A CHEMICAL BIOLOGY APPROACH TO DISCOVER THE BIOLOGICAL
TARGETS OF THE ANTIEPILEPTIC DRUG LACOSAMIDE

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ABSTRACT

PIERRE PHILIPPE MORIEUX: A Chemical Biology Approach to Discover the
Biological Targets of the Antiepileptic Drug Lacosamide
(Under the direction of Harold Kohn)

Lacosamide (Vimpat$^®$) is a potent antiepileptic drug that received market approval for the adjunctive treatment of partial-onset seizures in adults in Europe and the United States. The pharmacological studies document that lacosamide has a unique profile of activity that differentiates it from existing antiepileptic agents. Based on these findings, we hypothesized that lacosamide binds to different proteins, with low-to-modest affinity. This research project aims to identify the lacosamide biological targets that modulate function and toxicity. We propose a novel target identification approach where an affinity bait (AB) and a chemical reporter (CR) group are strategically placed within the lacosamide framework. These compounds are termed lacosamide AB&CR agents. The AB moiety leads to permanent capture of the binding protein while the bioorthogonal CR unit is used for either detection or isolation of the complex upon reaction with a probe. The understanding of lacosamide's mechanism(s) of action will help increase our understanding of epileptic disorders, and permit the rational development of new clinical agents.

In the first part of our study, we explored the structure-activity relationship (SAR) for the 3-oxy site in lacosamide. We showed that incorporation of non-bulky,

hydrophobic groups at this site provided lacosamide derivatives with excellent activities in animal seizure models. This information was used to design and stereospecifically synthesize a series of lacosamide AB&CR agents where either the AB or the CR group was installed at the 3-oxy site. Most lacosamide AB&CR agents were evaluated for anticonvulsant activity in animal models.

In the second part of our study, the lacosamide AB&CR agents were utilized to interrogate the rat brain soluble and membrane-bound proteome for potential binding partners of lacosamide. We used several subcellular fractionation methods to deconvolute the rat brain proteome. Within each subcellular fraction, different protein purification methods were employed to partition the lysate and aid the identification process. Several potential proteins were selectively targeted by the lacosamide AB&CR agents. Further analysis did not confirm that these proteins were directly linked to lacosamide function. These studies documented the strengths and limitations of the AB&CR strategy for receptor identification and are discussed.

DEDICATION

To my high school chemistry teacher Michel Schiano, for making me love chemistry and for developing my scientific curiosity.

To my niece Chloé Confais-Morieux, and my nephew Romain Morieux. I am looking forward to catch up with my uncle duties.

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PREFACE

"You do what you are"

I look at the work in my dissertation and see a reflection of myself. Who am I? A scientist passionate about what he does. A person who does not settle for mediocre and goes the extra step. An apprentice eager to learn, understand, and share new information. A human being who wants to help people not as lucky as he is. What am I? A hybrid, a reaction mixture, an alloy, a blend of different cultures, backgrounds, and trainings. What do I like to do? Synthesize compounds with a purpose. Design, craft and tailor molecular tools. As a reference to metalworkers, I like to look at myself like a carbonsmith.

I was born in December 1982 in Washington, D.C., from a French father, Christian Morieux, and a Japanese mother, Sumiko Morieux (née Ouchi), making me French/Japanese by blood, and French/American by citizenship. I speak French, English, fairly decent German, I can write, read, and speak Japanese at a more basic level, and these days I am trying to learn some Spanish. I am the youngest of three, with one sister, Christine, and one brother, Pascal. I always like to point out a unique feature of my family, which is that the five of us were each born in a different country, France and Japan for my parents, Beirut, Lebanon for my sister, and Algiers, Algeria for my brother. The origin of this diversity lies with my father's former

jobs as a French diplomat. Thanks to him, I have had the chance to be exposed to many different cultures and to grow in an open-minded familial environment. I am also the only scientist in my family. My father was a French teacher before being a diplomat, my mother was a journalist in Japan, and my brother and sister both went through a business/economy curriculum.

After my birth in the U.S., my family came back to live in France in 1985 for several years, and then moved to Rabat, Morocco, in 1989, where I spent 3 years going at a French elementary school. I learnt to read, speak and write Arabic as a child, but I unfortunately lost everything within years as we came back to France. After a couple more years in Paris, my father was nominated in 1995 as a cultural counselor at the French Embassy in Tokyo, Japan. We stayed there for 5 years, my high school years, where I attended the Lycée Franco-Japonais de Tokyo (LFJT), the French school in Tokyo. There, I met a person who played a very important role in my life, Michel Schiano, my physics and chemistry teacher. Michel had a passionate way to teach his classes and always tried to get his students exposed to more than the chemistry program requirements. He's the person who got me hooked on chemistry. He worked hard to have students from the French school in Tokyo participate in the French Chemistry National Olympiads. His efforts paid out and he made the LFJT the first French school in a foreign country to take part in the French National Chemistry Olympiads. Training under his supervision, I participated in the 16th Olympiads in 2000 where I ranked 10th, well beyond our expectations. Michel also taught us to look outside of science and got me interested in theater. He directed small theater plays with students, in which I was enrolled two consecutive

years. I then obtained my Baccalauréat in science in 2000, and me and my parents moved back to Paris.

The year following my return was not as bright. The next logical thing for me to do was to start my higher education in the classes préparatoires, the French traditional way to enter famous graduate schools Ecoles d'Ingénieurs. With chemistry on my mind, I set myself the goal to enter the Ecole Nationale Supérieure de Chimie de Paris (ENSCP), one of the most prestigious Ecole d'Ingénieurs in chemistry in France. However, the classe préparatoire work schedule proved to be as tough as advertised. With over 12 h of abstract mathematics, 10 h of physics, and only 2 h of chemistry per week, it did not take me long to start failing classes. Well before the end of the 1st year I knew I would not pass and looked for a reorientation. I joined the Institut Universitaire de Technologie of Orsay (IUT d'Orsay) in 2001, and followed a two-year training program as a chemistry technician. All the classes had a more practical aspect which was cruelly missing in the classes préparatoires. In this much more favorable, hands-on environment, I performed much better and learnt a lot, theoretically and practically, and I also continued playing theater. As part of my training, I completed my first internship as a chemistry technician in 2003 at BASF in Ludwigshafen am Rhein, Germany, for over a month. Ranking first of my class, I was able to apply for a parallel admission in the ENSCP where I was accepted in the fall of 2003 (class of 2006).

In the beginning of my second year, in the fall of 2004, a seminar was given by an English teacher at the Ecole Nationale Supérieure de Chimie de Rennes, Pierre Briend, a friend of Harold Kohn, who advertised for a Ph.D. exchange

program with the University of North Carolina at Chapel Hill. At this point in my studies, I was orienting myself towards research and organic synthesis, and decided to take advantage of this offer. I applied to the Division of Medicinal Chemistry and Natural Products and was accepted in 2005. I finished my second year at the ENSCP with a summer internship at Glaxosmithkline Stevenage's Medecines Research Center in Stevenage, U.K. There I worked, indirectly, for one of my future Ph.D. committee members, Dr. Stephen Frye. Within the four days following the end of my internship in Stevenage, in August 2005, I hopped back to Paris, grabbed my luggage and took off for Chapel Hill.

Exposed almost exclusively to chemistry in my training in France, I decided to learn something different and conducted my first laboratory rotation with Dr. Rihe Liu, learning basic biology techniques. I then moved on to work with Dr. Alexander Tropsha where I learnt more about computational chemistry and QSAR. In the summer of 2006, I joined Dr. Harold Kohn's laboratory and started working on my Ph.D. research project to identify the biological targets of lacosamide. Within the first year, Hal encouraged me to apply for a competitive NIH NRSA F31 fellowship, which he helped me obtain. I started working on the organic synthesis of molecular derivatives of lacosamide and later decided to learn how to use them myself in a biological environment. Several years down the road, and despite the fact that we did not find what we were looking for, I am more than happy with the overall result. With all this acquired knowledge I know have the ability to understand and speak both the language of chemistry and the language of biology. In the future I intend to perfect and take full advantage of this set of skills.

All these years as a graduate student, I have sadly witnessed some of my peers and even faculty members openly disregard, or look down on scientific fields other than their own. Biologists and pharmacologists looking at organic or computational chemistry with disdain. Organic chemists sneering at biology. I wish that my work serves as an invitation to other scientists, to reach out of their own field and learn about new areas of research. I believe it is a great approach to tackle the tough challenges we face as health scientists. It may, of course, feel scary to go to new, unknown places, but in my own experience it sure is a lot of fun!

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$(COCl)_2$	Oxalyl chloride
(*R*)	Rectus
(*S*)	Sinister
(v/v)	Volume per volume
(w/v)	Weight per volume
(w/w)	Weight per weight
$[\alpha]^{25}_D$	Optical rotation at 25 degrees Celsius using the D line of sodium
+ESI	Positive electrospray ionization
°C	Degree Celsius
β-DDM	β-Dodecylmaltoside
Δ	Heating
δ	Chemical shift
μA	Microampere
μM	Micromole per liter
2DG	2-Deoxy-D-glucose
AB	Affinity bait
AB&CR	Affinity bait and chemical reporter
ABPP	Activity-based protein profiling
Ac	Acetyl

Ac$_2$O	Acetic anhydride
ADP	Adenosine diphosphate
AED	Antiepileptic drug
Ag$_2$O	Silver (I) oxide
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMS	Ammonium sulfate
AP	Action potential
app	Apparent
Ar	Aryl; Argon
ASP	Anticonvulsant Screening Program
Asp, D	Aspartic acid
ATP	Adenosine triphosphate
BASP1	Brain acid soluble protein 1
BF$_3$•Et$_2$O	Boron trifluoride diethyl etherate
BME	β-Mercaptoethanol
Bn	Benzyl
Boc	*Tert*-butoxycarbonyl
Boc$_2$O	di-*tert*-butyl-dicarbonate
br	Broad

Br_2	Bromine
BSA	Bovine serum albumin
BuLi	*n*-Butyllithium
BVT	Brivaracetam
BZD	Benzodiazepine
c	Concentration in mg per 0.1 mL
C=O	Carbon-oxygen double bond
CA2	Carbonic anhydrase 2
Ca^{2+}	Calcium (II)
$CaCl_2$	Calcium (II) chloride
Calcd	Calculated
CaM	Calmodulin
CBZ	Carbamazepine
Cbz	Carboxybenzyloxy
CD_3OD	Perdeuterated methanol
$CDCl_3$	Deuterated chloroform
cDNA	Cloned deoxyribonucleic acid
C-H	Carbon-hydrogen bond
CH_2	Methylene
CH_2Cl_2	Methylene chloride
CH_3CN	Acetonitrile

CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
$CHCl_3$	Chloroform
CKB	Brain-type creatine kinase
CKB-/-	Brain-type creatine kinase double knockout
Cl^-	Chloride (-I)
CMC	Critical micellar concentration
CNS	Central nervous system
C-O	Carbon-oxygen single bond
COX IV	Cytochrome c oxidase subunit IV
Cr	Creatine
CR	Chemical Reporter
CRMP2	Collapsin response mediator protein 2
Cu(I)	Copper (+I)
Cu^{2+}	Copper (+II) ion
CuAAC	Cu(I)-catalyzed azide-alkyne cycloaddition
$CuSO_4 \bullet 5H_2O$	Copper (+II) sulfate pentahydrate
CV	Column volume
Cys, C	Cysteine
d	Day; doublet
D	Dextrorotatory
Da	Dalton

dB	Decibel
DBA/2	Dilute brown non-agouti
dd	Doublet of doublet
ddH$_2$O	Double distilled water
DEPC	Diethylpyrocarbonate
DIEA	Diisopropylethylamine
DITC	Di-(imidazolyl)thionocarbonate
DMAP	Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DMSO-d_6	Perdeuterated dimethylsulfoxide
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	Deoxyribonucleic acid
DNFB	2,4-Dinitrofluorobenzene
DPT	Di-(2-pyridyl)thionocarbonate
dt	Doublet of triplet
DTPP	Diethoxytriphenylphosphorane
DTPP-F$_6$	Di-(2,2,2-trifluoroethoxy)triphenylphosphorane
DTT	Dithiothreitol
E. coli	*Escherichia coli*
E_{Ca}	Equilibrium potential for calcium ions

E_{Cl}	Equilibrium potential for chloride ions
ED_{50}	Effective dose (50%)
EDTA	Ethylenediamine tetraacetate
EEG	Electroencephalogram
E_K	Equilibrium potential for potassium ions
E_{Na}	Equilibrium potential for sodium ions
equiv	Equivalent
ESM	Ethosuximide
Et, CH_2CH_3	Ethyl
Et_2O	Diethyl ether
Et_3N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol
F	Phenylalanine
FAA	Functionalized amino acid
FBM	Felbamate
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
g	Unit of gravity
G6PDH	Glucose-6-phosphate dehydrogenase
GABA	γ-Aminobutyric acid

GABAR	γ-Aminobutyric acid receptor
GABA-T	γ-Aminobutyric acid transaminase
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAT	γ-Aminobutyric acid transporter
GBP	Gabapentin
Glu, E	Glutamic acid
GPCR	G-protein coupled receptor
h	Hour
H_2	Hydrogen gas
H_2O	Water
HAT	Histone acetyltransferase
HCl	Hydrogen chloride
HDAC	Histone deactylase
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
His-tag	6-Histidine tag
HK	Hexokinase
HRMS	High resolution mass spectrometry
HSA	Human serum albumin
Hz	Hertz

I	Isoleucine
IBCF	Isobutylchloroformate
IC_{50}	Inhibitory concentration (50%)
iGluR	Ionotropic glutamate receptor
ip	Intraperitoneally
i-Pr	Isopropyl
i-PrOH	Isopropanol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Infrared
ITC	Isothermal titration calorimetry
IUPHAR	International union for basic and clinical pharmacology
iv	Intravenous
J	Coupling constant
K^+	Potassium (+I) ion
KA	Kainic acid
KAR	Kainic acid receptor
KCC2	Potassium and chloride cotransporter 2
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodalton
kg	Kilogram

L	Levorotatory
L	Liter
LB	Luria broth
LCM	Lacosamide
LGIC	Ligand-gated ion channel
LiAlH$_4$, LAH	Lithium aluminum hydride
LiCl	Lithium chloride
LiOH	Lithium hydroxide
LSAMP	Limbic system-associated protein
LTG	Lamotrigine
LVT	Levetiracetam
Lys, K	Lysine
m	Multiplet
M	Mole per liter
mA	milliampere
MASS1	Monogenic audiogenic seizure susceptibility 1
m-CPBA	*Meta*-chloroperbenzoic acid
Me, CH$_3$	Methyl
MeI	Methyl iodide
MeOH	Methanol
MES	Maximal electroshock seizure (epilepsy), Morpholineethanesulfonic acid (buffer)

Met, M	Methionine
mg	Milligram
$Mg(OAc)_2$	Magnesium (+II) acetate
$mg.kg^{-1}$	milligram per kilogram
Mg^{2+}	Magnesium (+II) ion
$MgCl_2$	Magnesium (+II) chloride
mGluR	Metabotropic glutamate receptor
min	Minute
mM	Millimole per liter
mmol	millimole
mp	Melting point
M_r, MW	Molecular weight
ms	Millisecond
Ms	Methanesulfonyl, mesyl
MsCl	Methanesulfonyl chloride
mV	millivolt
MWCO	Molecular weight cut-off
N_2	Nitrogen gas
N_3	Azide
Na Asc	Sodium ascorbate
Na^+	Sodium (+I) ion

Na_2SO_4	Anhydrous sodium sulfate
$NaBH_3CN$	Sodium cyanoborohydride
$NaBH_4$	Sodium borohydride
NaBr	Sodium bromide
NADP	β-Nicotinamide adenine dinucleotide phosphate, oxidated form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
NaH	Sodium hydride
$NaHCO_3$	Sodium hydrogenocarbonate
NaN_3	Sodium azide
NaOEt	Sodium ethoxide
nBu_4NF	Tetra-n-butylammonium fluoride
NCS	Isothiocyanate
NH_3	Ammonia
NINDS	National Institute of Neurological Disorders and Stroke
nM	Nanomole per liter
nm	Nanometer
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMM	N-methylmorpholine
NMR	Nuclear magnetic resonance
Nu	Nucleophile

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCr	Phosphocreatine
PCR	Polymerase chain reaction
Pd	Palladium
Pd/C	Palladium on charcoal
PDB	Protein Data Bank
PEG	Polyethylene glycol
Ph	Phenyl
pH	Potentiometric hydrogen ion concentration
photoAB	Photoaffinity bait
photoAB&CR	Photoaffinity bait and chemical reporter
PHT	Phenytoin
PI	Protective index
PILO	Pilocarpine
PKA	Protein Kinase A
PKC	Protein kinase C
PMSF	*Para*-methylsulfonylfluoride
PMT	Photomultiplier tube
PNS	Peripheral nervous system
po	Perorally

PPh$_3$	Triphenyphosphine
PPh$_3$(O)	Triphenylphosphine oxide
ppm	Part per million
Pr	Propyl
*p*TSA	*Para*-toluenesulfonic acid
*p*TsCl	*Para*-toluenesulfonyl chloride
PTZ	Pentylenetetrazole
R$_f$	Retention factor
RGB	Retigabine
RNA	Ribonucleic acid
rpm	Revolution per minute
Ru(II)	Ruthenium (+II)
s	Second (time); singlet (NMR)
SA	Serum albumin
SAR	Structure activity relationship
SBP	Sulfobathophenantroline
sc	Subcutaneous
SDS	Sodium dodecyl sulfate
SE	Status epilepticus
SiO$_2$	Silica
SNAP-25	Synaptosomal-associated protein 25

SRF	Sustained repetitive firing of neurons
STX	Saxitoxin
SV2A	Synaptic vesicle protein 2A
t	Triplet
T	Threonine
TAMRA	Tetramethylrhodamine
TBDMS	*Tert*-butyldimethylsilyl
TBST	Tris-buffered saline tween-20
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
t-Bu	*Tert*-butyl
t-BuOH	*Tert*-butanol
t-BuONO	*Tert*-butyl nitrite
TCEP HCl	Tris(2-carboxyethyl)phosphine hydrochloride
TD_{50}	Median toxic dose (50%)
TFA	Trifluoroacetic acid
TGB	Tiagabine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TLE	Temporal lobe epilepsy
TMS	Trimethylsilyl
$TMSN_3$	Trimethylsilyl azide

TPEN	*N*,*N*,*N'*,*N'*-Tetrakis(2-pyridylmethyl)ethylenediamine
TPM	Topiramate
Tris HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Trt	Triphenylmethyl
TTX	Tetrodotoxin
TX100	Triton X-100
U.mL-1	Unit per milliliter
UV	Ultraviolet
VGCC	Voltage-gated calcium channel
VGIC	Voltage-gated ion channel
VGKC	Voltage-gated potassium channel
VGSC	Voltage-gated sodium channel
VLGR1	Very large G-protein coupled receptor 1
VPA	Valproic acid
vs	Versus
Zn^{2+}	Zinc (+II) ion
Zw3-14	Zwittergent 3-14, 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate

CHAPTER 1

NEUROBIOLOGY OF EPILEPTIC DISORDERS

1.1. Basic neurobiology

1.1.1. Anatomy of neurons

Neurons are nerve cells found in the central nervous system (CNS) and the peripheral nervous system (PNS).[1] They are responsible for processing and transmitting sensory information.[2] Their discovery in the late part of the 19th century was made possible by the pioneering work of two scientists, Camillo Golgi[3] and Santiago Ramón y Cajal,[4,5] who shared the 1906 Nobel Prize for Physiology or Medicine.[6] A normal human brain contains approximately 100 billion neurons,[7] and the numerous types of stimuli we perceive come, in part, from the diversity of receptors and proteins localized within the brain.[1,2]

Like other cells, neurons are polarized by an electrochemical gradient generated by the differences between intracellular and extracellular ionic concentrations.[1,2] However, only a select set of cells (e.g., neurons) have the ability to electrically respond to a change in membrane potential. When the cell depolarizes and the potential reaches a certain threshold, it fires an electrical signal termed action potential (AP). In most cases, APs lead to neurotransmitter release from the presynaptic neuron, and the reception of the chemical signal is converted into

another electrical signal (excitatory or inhibitory) by the postsynaptic cell.[1,2] Neurons' morphologies and functions are intimately linked,[8] and they typically contain the following elements (Figure 1).
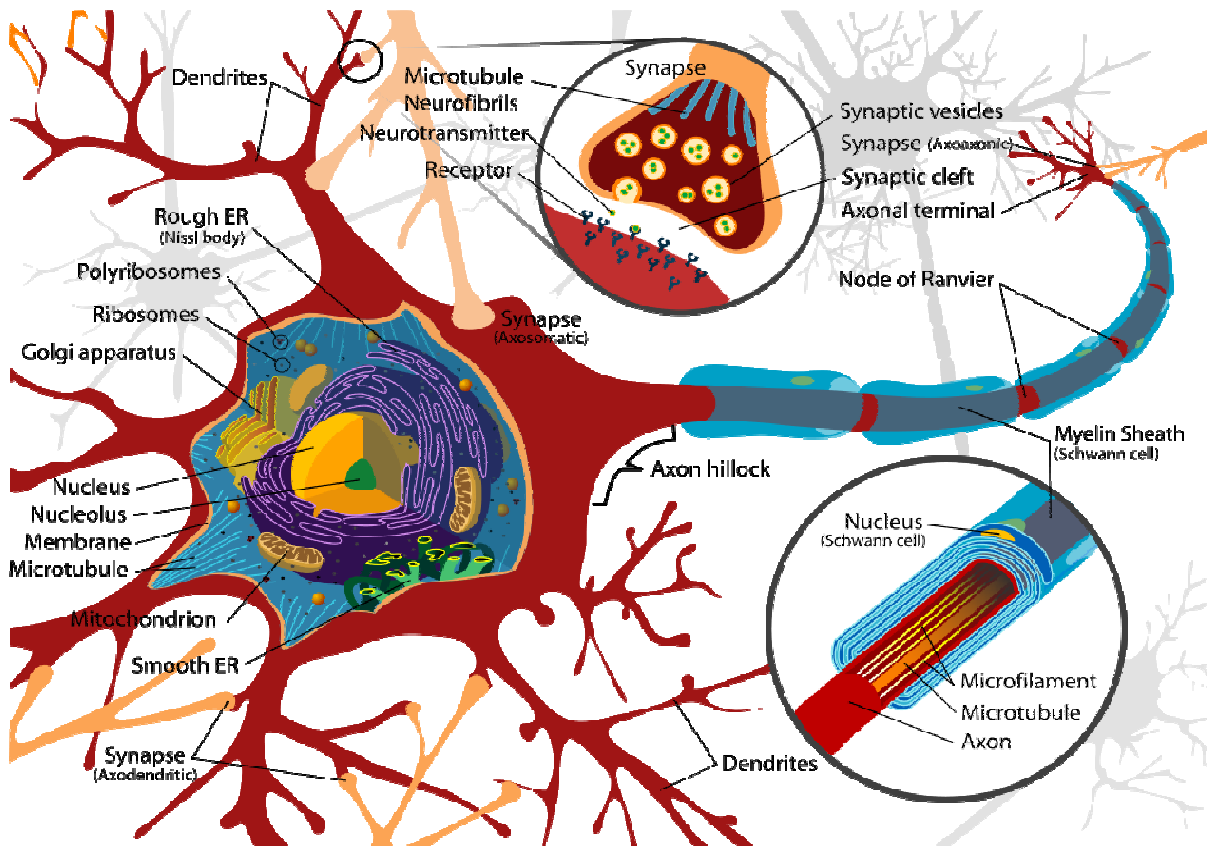
Figure 1. General morphology of a neuron (art by Mariana Ruiz Villareal)

• Dendrites, which are branched processes extending from the neuron, are designed to receive stimuli.

• The perikaryon or soma, core of the neuron, which contains the nuclear structure and organelles.

• The axon, a cellular wire-like projection towards other cells, acts as a transmitter of electrical information.

• Synapses, located at the end of the axon, are neuronal structures divided in three compartments: the presynaptic button, the synaptic cleft, and a post-synaptic density.

Upon reception of the AP, the depolarization of the presynaptic terminal releases vesicles filled with neurotransmitters into the synaptic cleft.[9] These neurochemicals, small molecules or peptides, interact with their cognate receptor to propagate or modulate signal transduction.[9]

1.1.2. Glial cells are crucial constituents of the brain

In a human brain, neurons represent approximately 10% of the brain tissue.[10] The remaining 90% are accounted for by non-neuronal cells called glia (greek for "glue").[10] Glial cells are present in both the CNS (astrocytes, oligodendrocytes, microglia) and the PNS (Schwann cells) where they play various roles.[1,2,11-14] Unlike neurons, glial cells are capable of undergoing mitosis and unable to generate action potentials.[10] They are also characterized by a lack of axon. One of their most basic roles is to provide a physical and energetic support to neurons and other brain cells. [1,2,10] Once thought of only fulfilling these "passive" tasks, glial cells in general have recently been shown to play crucial roles in neuronal signaling pathways.[11,13,14] In addition, overactivation of some types of glial cells is implicated in some neurological conditions.

Astrocytes are a type of cell indispensable to a neuron.[12,14] Briefly presented, their role ranges from tuning blood flow to different regions of the brain, clearing the synaptic cleft of excess neurotransmitter for recycling, to supplying neuronal cells

with nutrients and oxygen.[12,14] Microglia is the immune cell of the brain that is responsible for removing dead cells through phagocytosis.[10] Finally, oligodendrocytes and Schwann cells are a type of glia that forms a sheath of myelin, a lipid-rich membrane, which wraps around certain axons.[1,10,13] This electrical insulation increases the conduction speed of APs. Axonal regions exposed to the external milieu between two segments of myelin are called nodes of Ranvier. In these areas, the axonal membrane generally expresses high levels of ion channels that are essential for APs propagation.[1,2]

1.1.3. Neurons are polarized by differences in intracellular and extracellular ionic concentrations

Elaborate protein machinery allows neurons to maintain a membrane potential. In mammalian neuronal cells, the typical resting state membrane potential is approximately -70 mV and the intracellular part of the neuron is, therefore, more negatively charged. The concentration of K^+ ions is greater inside the cell while concentrations of Na^+, Ca^{2+}, and Cl^- are higher outside the cell (Figure 2). Highly selective membrane-spanning receptors called ion channels allow one specific type of ion to cross the membrane. For example, when a K^+-specific channel opens, K^+ ions tend to flow down the outwardly directed concentration gradient. As more and more positively charged ions leave the cell, the intracellular negative charge increases and creates an inwardly directed voltage gradient. This electrical force tends to attract K^+ ions inside the cell or slow the exiting ones. When the thermodynamic and electric forces cancel each other, the membrane is at its

equilibrium potential (E_K for potassium ions is equal to -102 mV), a value unique to each ionic species.
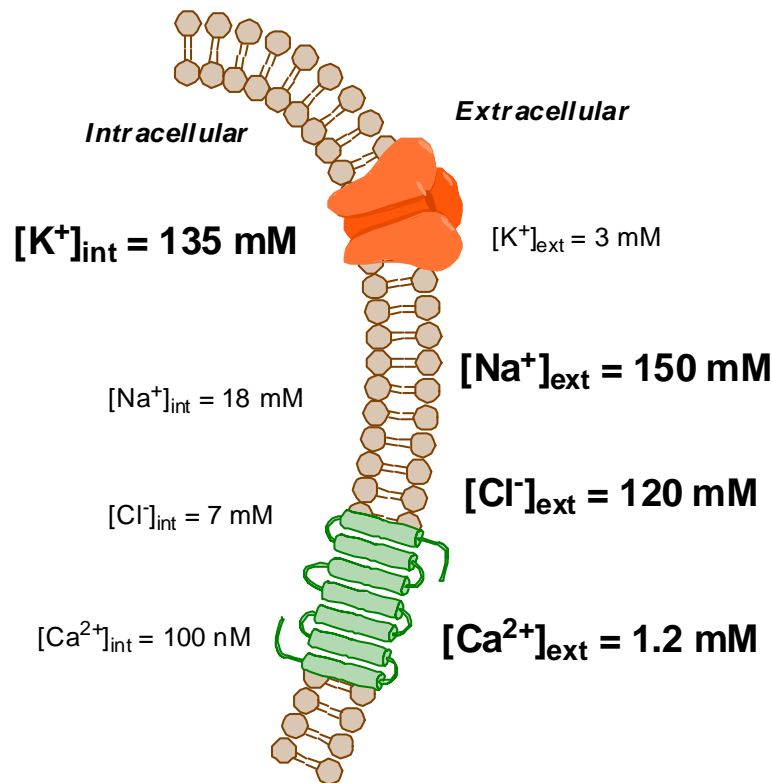
Figure 2. Typical intracellular and extracellular ionic concentrations for a mammalian neuron.

In addition to ion channels, a variety of ion pumps allow to maintain the unequal ionic distribution across the lipid bilayer. One of these, the Na^+/K^+-ATPase, maintains the Na^+ and K^+ gradients across the cell membrane by extruding three sodium ions and transporting two potassium ions inside the cell.

1.1.4. Mechanism of generation and propagation of action potentials

Different events are involved in the generation of an AP (Figure 3). First the membrane is at its resting potential (approx. -70 mV) (**1**) until a stimulus triggers the

opening of a small fraction of closed Na^+ channels on the membrane. Due to the concentration gradient, Na^+ ions start entering the cell leading to a membrane depolarization (**2**). If the initial depolarization reaches a threshold of approximately -55 mV, an AP is generated. If the threshold value is not met, no AP is produced. Upon reaching the threshold, a greater fraction of Na^+ channels open, leading to a rapid influx of Na^+ ions, which locally depolarizes the cell.
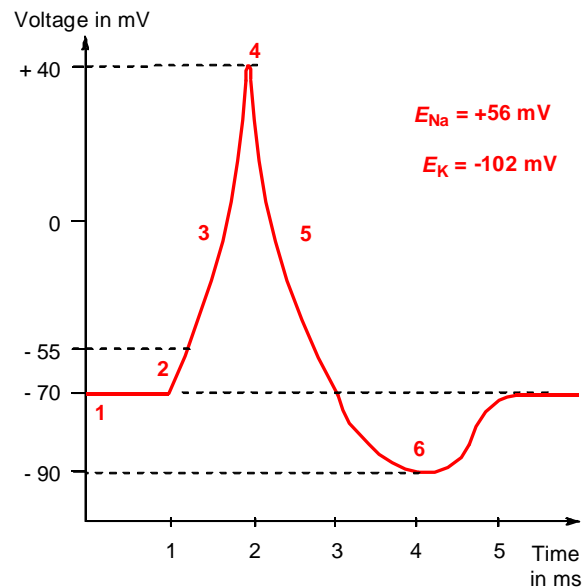
Figure 3. Schematic representation of the different phases occurring during the generation of an AP.

The membrane potential rapidly moves towards the equilibrium potential of Na^+ ions (E_{Na} = +56 mV) (**3**). Before E_{Na} can be reached, the membrane depolarization causes Na^+ channels to go into their inactive state (**4**), while nearby K^+ channels open and lead to an efflux of K^+ ions. As K^+ ions flow out, the membrane potential moves towards E_K (**5**). Before -102 mV can be reached, most K^+ channels go into their inactive state. During this refractory period (**6**), no AP can

6

occur. The initial membrane potential is then restored by action of specific K^+ channels.[15] After the resting concentrations are reached, another action potential may be fired. The AP generated travels unidirectionally down the axon by successive openings and closings of adjacent Na^+ channels expressed along the axonal membrane in a process termed "saltatory conduction" (Scheme 1).

Scheme 1. Simplified representation of the propagation of an action potential along the axon. Na^+ channels are represented in blue, the green signs indicate localized changes in membrane potential.
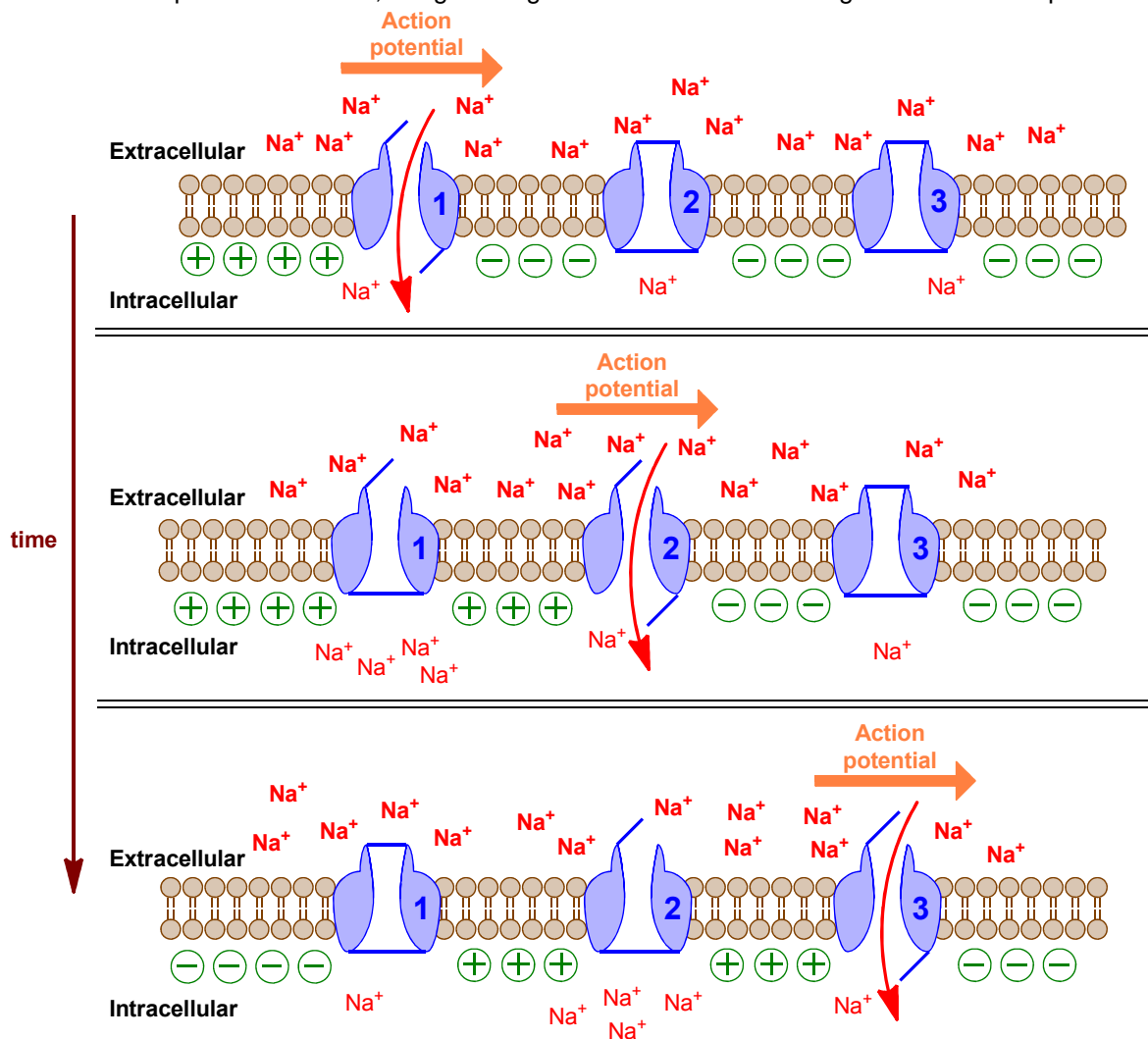
As its intracellular environment becomes more depolarized, Na$^+$ channel **1** opens and leads to a local influx of sodium ions (top drawing). This localized increase in Na$^+$ causes a membrane depolarization in the surroundings of Na$^+$ channel **2**, causing it to open, while **1** goes into its inactivated state (Scheme 1, middle drawing). Open **2** leads to Na$^+$ influx and membrane depolarization near to **3**, and its subsequent opening. As the AP travels down the axon towards the synapse, **1** goes from its inactivated to its closed state to allow transmission of another AP (Scheme 1, bottom drawing). This mechanism illustrates the physiological relevance of the refractory period, as it allows an AP to propagate in only one direction.

1.2. Epilepsy(ies): a multifaceted neurological disorder

1.2.1. A burden for people and economy

Epilepsy, also known as seizure disorder, is a chronic neurological condition that affects all populations[16] and is characterized by recurrent unprovoked epileptic seizures.[17-19] Such seizures are defined as discrete events resulting from simultaneous neuronal firing leading to an abnormal cellular behavior.[17,20] This disorder adversely affects the quality of life of approximately 50 million people worldwide,[21] that include 2 million people in the U.S. of which there are 340,000 children.[22-24] The U.S. annual cost of epilepsy is estimated at $15.5 billion in both medical costs and lost or reduced earnings and productivity.[25] Epilepsy is an extremely broad term that encompasses a large number of different seizure types.[20,26] While genetic mutations have been associated with some forms of epilepsy,[27] the etiology of epileptic seizures often remains unknown.[21,25]

1.2.2. Brief classification of epileptic seizures

Epileptic seizures can be classified according to the nature of the patient's electroencephalogram (EEG), brain scans, both of which are related to the seizure phenotype, and the level of consciousness of the patient.[20,26] So far over 40 different types of epilepsy have been reported.[28] Seizures confined to one region of one brain hemisphere are termed partial or focal, as opposed to generalized seizures that affect both hemispheres. The adjectives "complex" and "simple" respectively designate seizures that occur with and without loss of consciousness and "ictal" is the adjective that refers to a seizure.[20] Seizures can also evolve, for example from simple partial to complex generalized seizures and may or may not be convulsive.

Tonic-clonic, or grand mal seizures are the most common generalized seizures and they are typically (and misconceptually) associated with the word "epilepsy" itself.[26,29] They are characterized by a tonic phase, where the person violently arches in a strained position, followed by a clonic phase, during which the person experiences repetitive and uncontrolled jerks that may lead to injuries.[26,30]

Absence, or petit mal seizures are a type of generalized seizures characterized by a person's sudden interruption of activity, accompanied by a blank stare. This attack may or may not be accompanied with mild clonic, tonic or atonic components.[26,31] Atonic seizures are characterized by a sudden loss of muscle tone that may lead the person to abruptly fall on the ground, sometimes resulting in severe injury.[26]

Finally, status epilepticus (SE) is a potentially life-threatening condition where a generalized seizure, convulsive or non-convulsive, lasts for an extended period of time.[32-34] The precise duration after which a seizing person is considered to have SE is not clearly defined and ranges from 10 to 30 min during which the individual is unconscious.[33,35] Studies have shown that prolonged SE may lead to severe neuronal damage in epileptic patients.[36,37]

1.2.3. Small molecules to treat epilepsy: the need for antiepileptic drugs with new mechanisms of action

Epileptic seizures are typically treated with one or more antiepileptic drugs (AEDs). There are ~40 available AEDs on the market and they are classified into three categories: traditional, recent, and emerging.[38-41] Most traditional and recent anticonvulsants have been associated with well-defined pharmacological profiles. In many cases, they interact with either voltage-gated sodium channels,[42] calcium channels,[43] or with the benzodiazepine-binding site of the $GABA_A$ receptor.[44-46] Despite the availability of drugs, approximately *one third* of the patients diagnosed with epilepsy are refractory to treatment[47-50] and almost 40% of treated patients experience adverse side effects with current medications.[51] Current research on antiepileptic agents is hindered by the relatively few biological targets identified for epileptic seizures and by the need to conduct labor intensive animal behavioral tests for compound screening.

To respond to this health problem, the National Institute of Neurological Disorders and Stroke (NINDS) in 1975 created the Epilepsy Branch and the Epilepsy

Advisory Committee to expedite the development of more efficacious antiepileptic agents.[52] The Anticonvulsant Screening Program (ASP) is a division of the NINDS that allows research institutions to test small molecules in a variety of animal or *in vitro* pharmacological tests at no cost.[28] The requirement is the preparation of the compound on a 500–1000 mg scale which will be tested in mice and rats against a variety of seizure models.

1.2.4. Animal models used in the pharmacological evaluation of AEDs

Many animal models have been advanced to study epileptic syndromes.[53] Here, we briefly review the main pharmacological tests used at the ASP to identify new anticonvulsant molecules. The qualitative and quantitative assessments of a molecule are conducted at different time points ranging from 15 min to 4 h after administration. The anticonvulsant and toxicological profiles of compounds are mainly determined by intraperitoneal (ip) or peroral (po) administration to rodents. The dosage may range from 1 to 300 mg of compounds per kg of body weight, with several animals tested at each dosage. The dose at which half of the animals are protected is termed effective dose (50%) or ED_{50}. Similarly, the median toxic dose (50%) or TD_{50} is the dosage at which half of the animals display neurological impairment. The ratio of the TD_{50} over the ED_{50} is called the protective index (PI).

1.2.4.1. Maximal electroshock seizure test

The maximal electroshock seizure (MES) test consists in applying an electrical current to the animal via corneal electrodes and represents a model for generalized tonic-clonic seizures.[54] The electrical signal (60 Hz current, delivered for 0.2 sec) has a high frequency/short duration nature which elicits maximal seizures generally lasting no more than 30 sec.[55] A brief initial tonic flexion, and prolonged tonic extension period, followed by terminal clonus are characteristic of MES seizures.[55] Protection is defined as a failure of the animal to extend hindlimbs to an angle with the trunk greater than 90°.[56]

1.2.4.2. 6 Hertz test

The 6 Hz test is analogous to the MES test except for the nature of the electrical signal applied. A low frequency/long duration signal (6 Hz current, delivered for 3 sec, 32 or 44 mA intensity) triggers a different type of seizures in the animal that are representative of complex partial seizures.[57,58] Characteristics of 6 Hz-induced seizures are the immobility of the animal, accompanied by forelimb clonus, vibrissae (whiskers) twitching, and Straub-tail (elevated tail).[59] The absence of these signs upon electrical stimulation is defined as seizure protection.

1.2.4.3. Pentylenetetrazole administration

The subcutaneous pentylenetetrazole (scPTZ) test measures the ability of a compound to raise the seizure threshold produced by a chemoconvulsant (PTZ,

Scheme 5).[60] At a dose specific to the rodent model, PTZ induces clonic seizures and the test is considered a model for absence seizures.[52] A compound is considered protective if it abolishes the spasms triggered by the PTZ administration.[52] It is pharmacologically different from either the intravenous (iv)PTZ or the ipPTZ tests. In the iv route, the animal will start experiencing a series of myoclonic jerks (rapid contraction and relaxation of muscles) in a time-dependent manner.[61] These jerks will worsen and ultimately lead to loss of righting and clonic seizure. In the ip route, defined, subconvulsive doses of PTZ are administered at given time intervals. A gradual, reproducible response can then be observed, with initial jerks and full-blown seizure each starting after a specific number of injections.[62] In these tests, a molecule is considered protective if it can elevate the threshold PTZ dose necessary for convulsions and delay (iv) or increase the number of injections (ip) necessary for the onset of seizure.[61,62] Molecules with efficacy in the scPTZ test may have no effect in the ipPTZ or ivPTZ tests and vice-versa.[63]

1.2.4.4. Pilocarpine administration

In the lithium pilocarpine-induced status epilepticus (SE) model, animals are treated ip with a solution of LiCl 20 h prior to ip injection of pilocarpine (PILO), which results in the development of SE within 30 min after administration of PILO.[64] When administered alone, PILO induces secondarily generalized seizures that evolve into SE.[65] A variety of chemoconvulsants such as kainic acid,[66] picrotoxin,[67] and bicuculline[67] can be used to induce SE (Figure 4). After the SE period (8–24 h), animals will develop spontaneous recurrent seizures over a long-term period (up to 2

months).[65] These seizures are representative of complex partial seizures observed in humans.[20] Protection is defined by the suppression of recurring seizures.
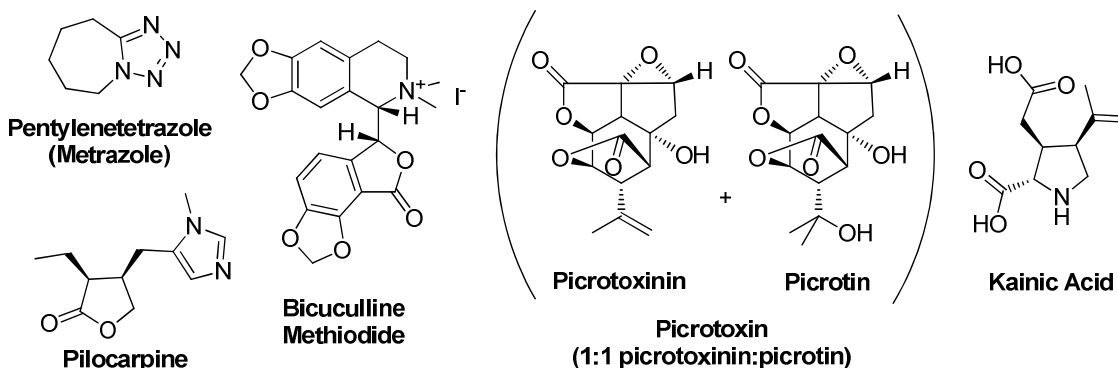
Figure 4. Chemical structures of various chemoconvulsants used in animal testing. Pentylenetetrazole, bicuculline and picrotoxin are GABA receptor antagonists. Kainic acid is a glutamate receptor agonist.

1.2.4.5. Audiogenic seizure animal models

The Frings mouse model is a model of generalized reflex epilepsy in which the seizure is triggered by a loud noise.[68] The epileptic phenotype is caused by a mutation in the *mass1* gene (monogenic audiogenic seizure susceptibility 1) which causes a premature termination of the membrane protein "very large G-protein coupled receptor 1" (VLGR1 or MASS1, 6300 amino acid residues).[69,70] The characteristic response of Frings mice to an intense stimulus (100 dB, 20 s) is wild running, followed by a loss of righting reflex that occurs with forelimb and hindlimb tonic extension.[52] Seizure protection is defined as the ability of a molecule to prevent tonic hindlimb extension.[52] The DBA/2 (dilute brown non-agouti) mouse is another genetic model used for audiogenic seizures.[71]

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1.2.4.6. Electrical kindling models

The rapid hippocampal kindling test is a model representative of focal seizures.[52,72] The term kindling refers to repetitive, infrequent stimulations that sensitize a specific region of the brain, progressively leading to a fully kindled state.[72] Once this stage is reached, stimulations that initially did not elicit a reaction will consistently trigger an enhanced response.[72] In the rat hippocampal kindling model, electrodes are surgically implanted in the hippocampus and kindled seizures are produced by a 10 s train of 1 ms biphasic 200 μA pulses (50 Hz) delivered every 30 min for 6 h on alternating days for a total of 60 stimulations (5 stimulus days).[52] Behavioral seizures are scored according to the Racine scale,[73] that ranges from 1 (whiskers twitching) to 5 (forelimb clonus, rearing and falling). Another brain region commonly used for the kindling procedure is the amygdala.[74] Compounds evaluated in this model are dosed based on their activity in other seizure tests. A molecule can be tested for its ability to stop kindled seizures (anticonvulsant activity), or its ability to delay the acquisition of the kindled state (antiepileptogenic effect).[52]

1.2.4.7. Neurological toxicity evaluation

The rotarod test is a measure of neurological toxicity, where a mouse is placed on a rotating rod (6 rpm).[75] Control mice have the ability to maintain themselves on the rod for an extended period of time.[75] Neurological impairment is defined as the inability of the mouse to remain on the rod for 1 min in three successive trials.[52] In rats, the behavioral toxicity of the compound is assessed. The positional stance test consists in lowering one hind leg over the edge of a table. A

neurologically impaired rat will fail to lift its leg back to a normal position.[52] The gait

and stance tests consists in observing symptoms of impairment in the animal, such

as zigzag gait, abnormal posture, and lack of exploratory behavior or catalepsy.[52]

1.3.   Pharmacology of traditional and recent AEDs

1.3.1. Ion channels are primary pharmacological targets to prevent seizures

Ion channels belong to a superfamily of transmembrane proteins that regulate

intracellular and extracellular concentrations of cations and anions essential for

triggering action potentials that propagate information or elicit biological responses.

[1,2] This superfamily of receptors can further be divided into two main classes, the

Voltage-Gated Ion Channels (VGIC) and the Ligand-Gated Ion Channels (LGIC).[1,2]

VGICs respond to changes in the membrane potential, while LGICs respond to

binding of an endogenous neurotransmitter or an exogenous molecule. Ion channels

play a crucial role in electrophysiological events pertaining to epilepsy and it is

therefore not surprising that they are targeted by numerous AEDs.[76-78]

1.3.1.1. Voltage-gated sodium channels

Voltage-gated sodium channels (VGSCs) are large Na^+-specific ion channels

that respond to changes in membrane potential. They are composed of one α

subunit (~260 kDa) consisting of 4 domains (I–IV) that form a central pore, each

containing 6 membrane-spanning α helical segments (S1–S6) (Figure 5), with

intracellular N- and C-termini.[76] The S4 segment of each domain contains one

positively charged amino acid every three residues.[79] These residues control the channel gating and respond to depolarization by moving across the membrane and initiating the activation of the receptor.[80] The ionic selectivity of the channel is determined by repetitive amino acid motifs in the membrane-reentrant loop between S5 and S6 of each domain, and a mutation of three residues in the S5S6 loop of each domain is enough to turn the Na^+ channel into a Ca^{2+} channel.[81]
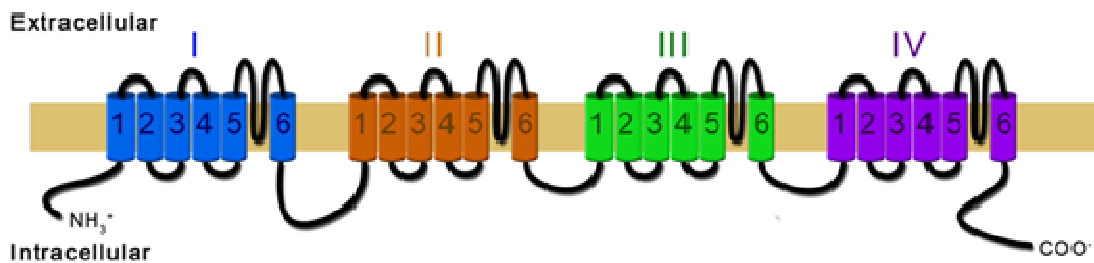
Figure 5. Schematic representation of a voltage-gated Na^+ channel α-subunit

Native VGSCs are associated with one or more β1, β2, or β3 subunits (~30 kDa) that can influence the gating properties of the channel, the kinetics of channel activation, as well as protein interactions with cell adhesion molecules.[82-84] In humans, 9 different types of voltage-gated Na^+ channels exist and they are designated $Na_v1.1$–$Na_v1.9$.[80] Among these, $Na_v1.1$–$Na_v1.3$ and $Na_v1.6$ are the main VGSCs found in the CNS.[80] Mutations in Scn1A, Scn2A and Scn1B, the genes that respectively code for the α subunits of $Na_v1.1$ and $Na_v1.2$, and the β1 subunit, are associated with severe forms of infant epilepsy.[85-87,27]

As described earlier, VGSCs can adopt different conformations depending on their state. When firing an AP, the channel goes from the closed (resting) state to the

open (activated) state to the inactivated state. Two distinct inactivation pathways exist, fast and slow, from which the Na$^+$ channel can recover. The fast inactivation pathway happens within a few milliseconds of channel opening, and involves a "hinged lid" mechanism.[76] The short intracellular region between domains III and IV physically obstructs the pore by docking a conserved hydrophobic peptide sequence isoleucine, phenylalanine, methionine, threonine (**IFMT**) into domain IV S6 segment (Scheme 2).[88] Recovery from the fast inactivated state depends on the membrane potential and time. This mechanism is responsible for the refractory period allowing APs to propagate in only one direction (Scheme 1).

Scheme 2. Schematic representation of the fast inactivation hinged lid mechanism of VGSCs.
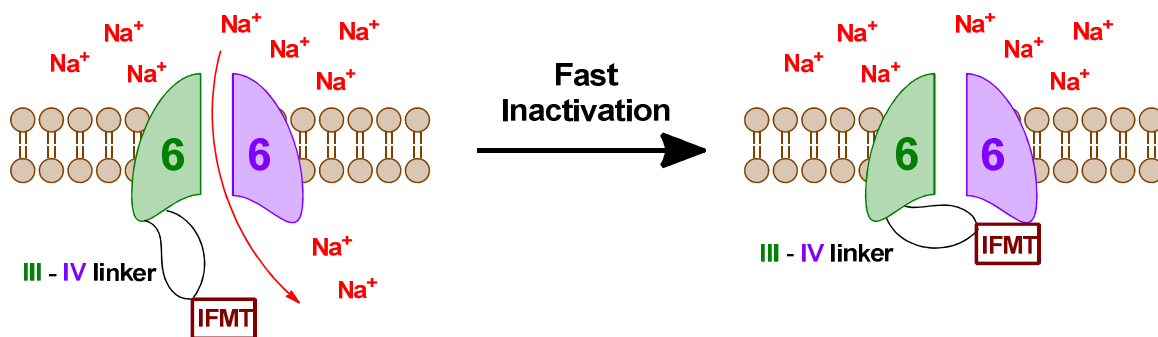
The VGSC slow inactivation pathway is a biological process independent of the fast inactivation.[89] Although its precise mechanism is subject to controversy, it is likely to involve a rearrangement of the pore, as well as specific domains of the channel.[89-92] The "slow" terminology refers to the prolonged time required for the channel to recover from its inactivated state, ranging from hundreds of milliseconds to a second.[76] This pathway is a cellular mechanism that only takes place under

conditions that may be relevant to an epileptic seizure such as high frequency depolarizations, or under sustained membrane depolarization (membrane potential at -60 or -55 mV). This type of inactivation acts as a protective way to lessen excessive neuronal excitability.[76]

1.3.1.2. Voltage-gated calcium channels

Voltage-gated Ca^{2+} channels (VGCCs), also termed voltage-dependent calcium channels (VDCCs) are transmembrane receptors that mediate calcium influx into the cell. Ten different types of VGCCs exist, $Ca_v1.1–1.4$, $Ca_v2.1–2.3$, and $Ca_v3.1–3.3$, that are comprised of four to five subunits, the largest and most important of which is the α_1 subunit (190–250 kDa).[93] Analogous to the VGSCs α subunit, the VGCC α_1 subunit is made of four domains (I–IV) each containing 6 α-helical membrane-spanning segments (S1–S6) where the S4 segment senses changes in membrane potential. Ion selectivity is controlled by the loop between S5 and S6 segments of domains I, III and IV.[93] VGCCs additionally possess an intracellular β subunit, as well as a disulfide-linked transmembrane δ subunit, and an extracellular α_2 subunit that forms a $\alpha_2\delta$ complex, and less frequently an intracellular γ subunit.[94] The pharmacological and electrophysiological properties of the Ca^{2+} channel are primarily determined by the different types of α_1 subunits whereas β, $\alpha_2\delta$ and γ subunits serve as modulators.[94] The alphabetical designation of VGCCs comes from the distinct types of currents they elicit and their sensitivity to established channel blockers: L-type for "long-lasting" ($Ca_v1.1–Ca_v1.4$), N-type for

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"neural" ($Ca_v2.2$), P/Q-type for "Purkinje neuron"[95] ($Ca_v2.1$), R-type for "resistant to the other blockers" ($Ca_v2.3$), and T-type for "transient" ($Ca_v3.1$–$Ca_v3.3$).[93]

The VGCC-mediated influx of Ca^{2+} ions has several consequences. Electrophysiologically, the increase of intracellular calcium leads to a depolarization of the neuron (E_{Ca} = +120 mV).[1] Pharmacologically, Ca^{2+} is an important second messenger signaling molecule that can start a cascade of cellular downstream events ranging from activation of protein kinase C (PKC), calmodulin (CaM) and Ca^{2+}-dependent proteases to triggering the transcription of pro-apoptotic factors.[96-101]

1.3.2. Targeting synaptic transmission helps control neuronal excitability

1.3.2.1. GABA$_A$ receptors
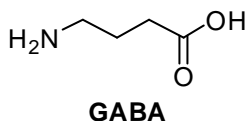
GABA

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. GABA receptors are a family of homo- or heteromeric transmembrane receptors that elicit their biological responses upon binding of GABA.[102] GABA$_A$ receptors (GABA$_A$Rs) are ligand-gated ion channels, or ionotropic receptors, while GABA$_B$ receptors (GABA$_B$Rs) are G-protein coupled receptors (GPCRs), or metabotropic receptors. GABA$_C$ receptors (GABA$_C$Rs) have recently been reclassified by the *International Union for basic and clinical Pharmacology* (IUPHAR)

as the ρ subfamily of GABA$_A$R based of their closely related sequence, structure and function.[103] That reclassification is, however, subject to controversy.[104]

There are 19 types of subunits, α1–6, β1–3, γ1–3, δ, ϵ, θ, π, and ρ1–3, that can assemble into a pentameric GABA$_A$Rs forming a central pore (Figure 6).[105] Each subunit possesses 4 transmembrane segments, with extracellular N- and C-termini. The extracellular N-terminal peptide chain contains a disulfide bridge that is characteristic of the so-called Cys-loop superfamily of receptors, to which other pentameric LGICs belong.[106]
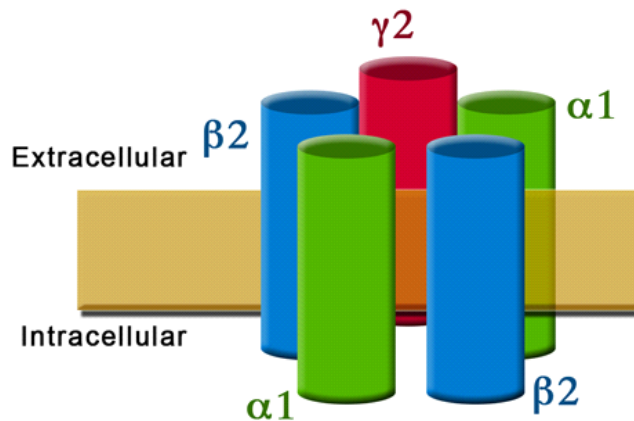
Figure 6. Simplified representation of a GABA$_A$R structure

GABA$_A$Rs containing ρ subunits (or GABA$_C$Rs) are primarily located in the retina.[103] The differences in subunit composition translate into different GABA$_A$Rs pharmacological and electrophysiological properties and reports indicate that receptors comprised of subunits α1β2γ2 (Figure 6) are the most abundant in the brain.[102] The binding site for GABA is located between an α and a β subunit, and up to two GABA molecules can, therefore, bind to the receptor.[107] Genetic forms of

juvenile epilepsies have been linked to mutations in the $GABA_ARs$ $\alpha 1$ and $\gamma 2$ subunits.[108,109]

GABAergic neurons are equipped with a machinery of enzymes and transporters that play an essential role in GABA-mediated signaling. GABA itself is biosynthetically derived from the excitatory amino acid L-glutamate through action of the glutamic acid decarboxylase enzyme (GAD).[110] GABA transporters (GAT) are present on the presynaptic membrane as well as on the membrane of astrocytes located near the synaptic cleft.[11,111-113] These transporters are responsible for excess GABA uptake into the glial cell or the presynaptic density, where GABA transaminase (GABA-T) further processes GABA into succinic semialdehyde.[114]

The biological effects of GABA are primarily mediated by $GABA_ARs$, which are GABA-activated chloride channels that are primarily localized on the post-synaptic membrane.[77] The higher extracellular concentration of Cl$^-$ ions leads to an inward flux upon channel opening. The electrophysiological basis of GABA-mediated inhibition can be explained by the equilibrium potential for chloride ions (E_{Cl} = -75 mV) which is slightly more negative than the average resting membrane potential (-70 mV). An influx of negatively charged chloride ions leads to a hyperpolarization of the post-synaptic neuron, thus reducing its probability of firing an AP.[1] It is important to note that the inhibitory effect of GABA is due to the average E_{Cl} in mature neurons. Changes in the intra- and extracellular concentrations of chloride ions can lead to an E_{Cl} value less negative than the resting membrane potential, thus rendering GABA excitatory.[115] Such conditions have been reported to occur in

developing cultured neurons,[116,117] as well as in the brain of patients suffering from temporal lobe epilepsy (TLE).[118,119]

1.3.2.2. Glutamate receptors

L-Glutamate is the main CNS excitatory neurotransmitter and acts at a variety of biological receptors. Mirroring the GABA$_A$Rs and GABA$_B$Rs, two different classes of glutamate receptors exist: the ionotropic glutamate receptors (iGluRs) that are LGICs permeable to several ionic species, and the metabotropic glutamate receptors (mGluRs) that are GPCRs.[120,121] While a recent investigation pointed out the potential anticonvulsant effects of mGluRs subtype-specific agonists and antagonists,[121] we will principally review the pharmacology of the ionotropic class.

L-glutamate   **AMPA**   **NMDA**   **Kainic Acid**
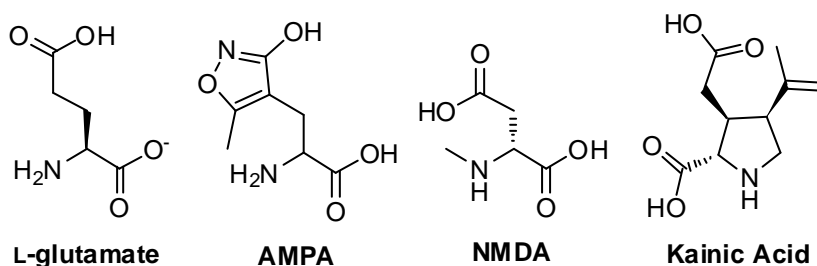
Figure 7. Chemical structures of cognate ligands of ionotropic glutamate receptors

iGluRs are divided into three major families, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs), the kainate (KA) receptors (KARs) and the *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) named after their selective small-molecule agonists (Figure 7).[122] Another less studied class called δ receptors are classified as orphan iGluRs.[123]

All classes of receptors have a similar heteromeric protein structure, comprised of four subunits forming the central pore.[120] Four different types of subunits exist for AMPARs (GluA1–GluA4), five types for KARs (GluK1–GluK5), seven for NMDARs (GluN1, GluN2A–D, GluN3A, GluN3B) and two for δ receptors (GluD1, GluD2).[106] The subunit nomenclature used above has recently been introduced (as of January 2009) by the nomenclature committee of IUPHAR (NC-IUPHAR) in order to bring a consistent nomenclature of LGICs.[106] iGluRs generally assemble into dimers of dimers: NMDARs comprise two sets of one mandatory GluN1 (former NR1) and one GluN2 or GluN3 (former NR2 and NR3) subunits,[124] and AMPARs contain a dimer of two GluA subunits that most often contains a GluA2.[125] The composition of the receptors varies with different brain regions and determines their electrophysiological and pharmacological properties.[124,125]
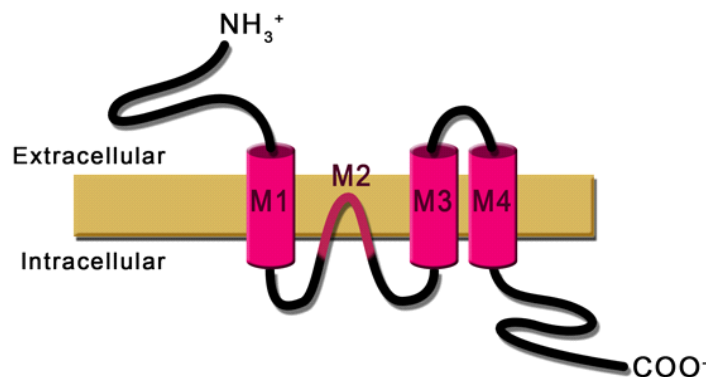
Figure 8. General topology of an ionotropic glutamate receptor subunit.

The subunits possess the same topology among AMPA, KA, and NMDA receptors: they are made of three transmembrane domains (M1, M3, M4) and one re-entrant membrane loop facing the intracellular side (M2), with extracellular N-

terminus and intracellular C-terminus (Figure 8).[122] The amino acid composition of the M2 loop determines the ion selectivity of the channel.[126,127]

While AMPA and kainate receptors are only permeable to Na^+, NMDA receptors are primarily permeable to Ca^{2+}.[125] In addition, the activation of NMDA receptors is unique in that it can occur through a ligand-gated mechanism involving glutamate and either glycine or D-serine, as well as through a voltage-dependent mechanism that can be modulated by Mg^{2+} ions.[124] The glutamate binding site is located on a GluN2 subunit, while either glycine or D-serine bind to a GluN1 subunit.

L-Glutamate and glutamate receptors mediate an important pathological process called excitotoxicity.[128] Excess glutamate may give rise to overactivation of iGluRs, such as NMDARs, leading to an increase of intracellular Ca^{2+}. In large excess, calcium cannot be adequately buffered and activates Ca^{2+}-dependent proteases.[129] In addition, excess Ca^{2+} may trigger swelling of mitochondria and release of proapoptotic molecules, leading to programmed death of the post-synaptic neuron.[130] Prolonged NMDARs overactivation and bioenergetic failure may ultimately drive the dying neuron to release excess glutamate towards other neurons, starting a neurodegenerative chain reaction.[131]

1.3.3. Pharmacology of traditional AEDs

In this section we briefly discuss leading marketed drugs with antiepileptic activity. AEDs that were identified as potential therapeutic agents in the early 1930s to the early 1970s are termed traditional. Despite their early year of discovery, traditional AEDs are still used for the treatment of epileptic seizures. As is the case

for many of these therapeutic agents, the complete mechanism of action of traditional AEDs still remains unknown.

1.3.3.1. Phenytoin
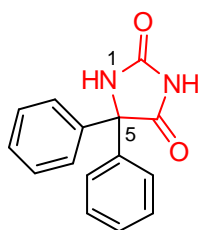
Phenytoin (PHT)

5,5-Diphenylhydantoin (phenytoin, PHT, Dilantin[®]) is an AED that was introduced in 1938 after its anticonvulsant effects were observed on electrically shocked laboratory animals.[132] It remains clinically used for the treatment of partial and tonic-clonic seizures but is ineffective in the treatment of absence seizures.[55] This pharmacological profile correlates with the high potency of the compound in the MES test, and its lack of activity in the scPTZ test.[55] Investigation of the structure activity relationship (SAR) of PHT analogs showed that the presence of the unmodified hydantoin ring (in red) was necessary for activity.[133] Replacing the 5-phenyl groups by alkyl groups leads to compounds with sedative effects, of which PHT is devoid.[134]

The primary mechanism of action of phenytoin has been established as the stabilization of the fast inactivated state of VGSCs, by binding to the intracellular part of the Na$^+$ channel (Scheme 7).[76,135] Indeed, *in vitro* studies showed PHT cannot prevent an initial action potential but blocks firing of a second one.[76] This blockade only affects epileptiform APs termed s̲ustained high-frequency r̲epetitive f̲iring of

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neurons (SRF),[136] without impairing spontaneous low frequency APs.[137,78] Importantly, the PHT block of VGSCs is highly voltage-dependent, with a 12-fold greater inhibitory effect at depolarized membrane potentials (IC_{50} = 10 μM at -60 mV) than at hyperpolarized potentials (IC_{50} = 120 μM at -85 mV).[138]

1.3.3.2. Carbamazepine

Carbamazepine
(CBZ)

5H-Dibenzo[b,f]azepine-5-carboxamide (carbamazepine, CBZ, Tegretol®) is an iminostilbene derivative (iminostilbene in red) with mood-stabilizing and anticonvulsant properties that was introduced as an AED in the 1970's.[139] Despite a lack of structural similarity, CBZ has an animal pharmacological profile closely related to that of PHT, being very potent in the MES test while having very limited efficacy in the scPTZ test.[55] Like phenytoin, this profile correlates with the use of CBZ for the treatment of tonic-clonic and partial seizures but not absence seizures.[136]

Based on their similar pharmacological profiles, it is not surprising that CBZ exerts its action through the fast inactivation of VGSCs.[135] Like phenytoin, carbamazepine is able to block SRF *in vitro* in a voltage-dependent fashion.[140,141] The main difference between PHT and CBZ resides in their binding kinetics with the VGSCs, with CBZ binding 5 times faster than PHT, but with 3-fold less affinity.[142]

This difference may account for the different therapeutic responses observed among subgroups of patients.[76,142] CBZ was also shown to have a weak inhibitory effect *in vitro* on NMDARs-mediated Ca^{2+} influx.[143] This effect was, however, highly potentiated in the presence of high extracellular K^+ concentration (50 mM KCl), a physiological concentration pertinent to epileptic seizures.[143]

1.3.3.3. Ethosuximide
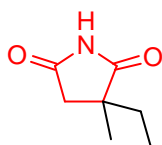
**Ethosuximide
(ESM)**

(*R*,*S*)-3-Ethyl-3-methyl-pyrrolidine-2,5-dione (ethosuximide, ESM, Zarontin®) is a member of the succinimide (succinimide core in red) family of anticonvulsants.[136] It was introduced in 1960 as an antiepileptic drug for the treatment of absence seizures in humans, and is ineffective against generalized tonic-clonic seizures. This profile correlates with the drug's animal pharmacology, ESM being able to prevent scPTZ- but not MES-induced seizures.[55]

The accepted mechanism of action responsible for suppressing absence seizures is the blockade of T-type calcium currents,[144,145] although different biological mechanisms are potentially implicated in the generation of absence seizures.[146] Unambiguous data concerning ESM reduction of Ca^{2+} currents was only recently published using cloned human T-type calcium channels, showing that T-type VGCCs block by ethosuximide is both voltage- and state-dependent.[147] ESM has a greater inhibitory effect on VGCCs at a depolarized membrane potential (IC_{50}

28

~2.5 mM at -75 mV, $Ca_v3.1$) than at a hyperpolarized potential (IC_{50} ~18 mM at -100 mV, $Ca_v3.1$),[147] a phenomenon reminiscent of the actions of PHT and CBZ on VGSCs.

1.3.3.4. Valproic acid

Sodium Valproate
(VPA)
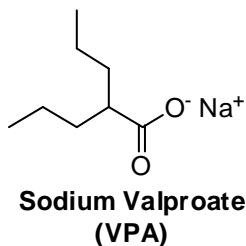
2-Propylvaleric acid (valproic acid, VPA, Depakene®) sodium salt is a branched fatty acid whose antiepileptic properties were fortuitously discovered in 1962, at which time it was used as a vehicle to evaluate new AEDs in animal models.[148] As positive results were obtained with any drug candidate at any dosage, the testing of valproate itself led to confirmation of its protective effects against seizures.[149] First marketed in 1967, it is still commonly used nowadays and has protective effects similar to ESM against absence seizures, and to CBZ and PHT against both tonic-clonic and partial seizures. Moreover, it is active in a variety of refractory epileptic disorders.[150] To date, VPA has one of the widest spectra of antiepileptic activity.[150]

The exact mechanism(s) of action of VPA is still subject to debate. VPA prevents MES-induced seizures with an efficacy comparable to PHT and CBZ, correlating with its use against generalized tonic-clonic and partial seizures.[55] Some studies indicate that valproate is able to reduce SRF in rat hippocampal cultured neurons,[151] probably through action on VGSCs. However, these results vary greatly

with preparation from different brain regions.[152,153] VPA does not seem to interact with the PHT binding site of VGSCs.[154] Similar to ESM, VPA's interaction with T-type VGCCs has been studied to explain its absence seizure protective effect.[155] T-Currents blockade by VPA proved to be specific to the type of neuron used for the *in vitro* culture.[156] In addition to its suggested interactions with VGICs, VPA has been shown to interact with GABA synthetic and degradative enzymes,[150] without directly interacting with GABA$_A$Rs.[157] Paralleling its effects on VGSCs and VGCCs, VPA displayed a brain regional specificity in its activating properties of glutamic acid decarboxylase (GAD).[157] In *ex vivo* and *in vitro* experiments, the valproate-induced increase in GAD activity matched the increase in GABA levels and its anticonvulsant effect.[158,159,157]

1.3.3.5. Benzodiazepines

Diazepam   **Midazolam**
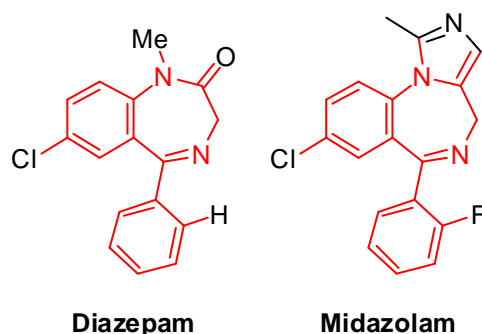
Diazepam (Valium®) belongs to the family of benzodiazepines (BZDs, benzodiazepine core in red), a class of drugs useful for treating a variety of neurological conditions, ranging from anxiety, agitation, insomnia, muscle spasms to seizures.[160] In 1963, diazepam, the second marketed BZD, was prescribed for its antidepressant properties. The anticonvulsant properties of BZDs were discovered

early in animal models. They are very potent in both the MES and scPTZ tests, but exhibit a pronounced neurotoxicity and short duration of action.[55] Benzodiazepines are unfortunately rendered useless for a chronic treatment of epilepsy due to the rapid onset of tolerance.[161] However, as an acute treatment, rectal administration of diazepam remains today the first-line emergency treatment against status epilepticus,[162] along with the more recent buccal formulation of midazolam[163] (Versed®).

The pharmacology of BZDs has been extensively studied, and have been shown to interact as positive allosteric modulators of $GABA_ARs$.[102,103,105,164,165] BZDs bind with at the interface of α and γ subunits and enhance hyperpolarizing chloride currents by increasing the frequency of channel opening.[166] This sole mechanism of action, however, can not explain all the pharmacological properties of benzodiazepines.[167] BZDs have been demonstrated to have an inhibitory effect on Ca^{2+} uptake through action on VGCCs,[168] and were shown *in vitro* to act on VGSCs by reducing SRF, which may correlate with the protective effects of the drug in animal seizure tests.[169]

1.3.3.6. Summary

Except for the family of benzodiazepines, most traditional AEDs mainly target VGICs. The prototypical drugs PHT, CBZ, VPA, ESM have proven to be invaluable tools for our understanding of biochemical mechanisms underlying epilepsy. Early research has led to the paradigm that blockades of Na^+ currents and transient Ca^{2+} currents, respectively, translate into protection against tonic-clonic and absence

seizures. The two respective animal models corresponding to these seizures are the MES and the scPTZ tests. In view of more recent studies, it has also become clearer that the neurotransmitter modulation through LGICs may account for part of the anticonvulsant properties of these AEDs.

1.3.4. Pharmacology of recent AEDs

One of the driving forces that led to the development of new AEDs was their undesirable side effects associated with long-term treatments.[170-172] The class of recent AEDs (mid-1970's to mid-1990's) has provided new chemical entities with diverse pharmacological profiles and mechanisms of actions. The compounds discussed in the following section are not exhaustive, but rather serve as an illustration of the variety of new mechanisms of action relevant to the treatment of epilepsy.

1.3.4.1. Lamotrigine

Lamotrigine (LTG)   **Folic acid (vitamin B9)**
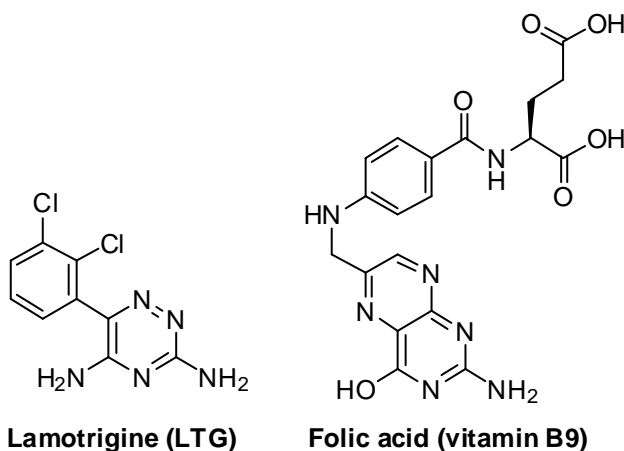
6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine (lamotrigine, LTG, Lamictal®) is an AED introduced in 1995 for the treatment of partial and generalized

tonic-clonic seizures. LTG was rationally designed based on the observation that folate derivatives possess proconvulsant activity in animals.[173,174] LTG, however, is only a weak folate antagonist, a property that has not been linked to its antiepileptic effects.[175] In animal models, the pharmacological profile of LTG was similar to that of PHT and CBZ.[176]

LTG blocked voltage-gated Na^+ channels by stabilizing the fast inactivated state of the channel.[177] In early studies, LTG was suggested to act at the slow inactivation pathway of VGSCs.[178] However, this pathway could not be distinguished from an alternative mechanism where the binding kinetics of LTG to the channel were slow.[76,179] In animal cultured neurons, lamotrigine demonstrated the ability to reduce SRF in a state- and voltage-dependent manner similar to that of PHT and CBZ.[180,181,177] Interestingly, evidence supports that LTG, although very different in structure from PHT and CBZ, binds, if not at the same fast-inactivation site, to a closely related receptor site.[182]

1.3.4.2. Topiramate
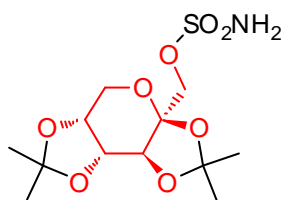
Topiramate (TPM)

2,3:4,5-Bis-*O*-(1-methylethylidene)-β-D-fructopyranose sulfamate (topiramate, TPM, Topamax®) is a recent AED with unusual structural features. The sulfamate derivative of D-fructose (in red) is used to treat simple and complex partial

seizures.[183] Originally synthesized in 1979 as a potential antidiabetic inhibitor of fructose 1,6-bisphosphatase, TPM was also tested the same year for anticonvulsant activity in mice where it displayed excellent anticonvulsant activity.[184,185] Following the same trend as its predecessors PHT, CBZ, and LTG, TPM displayed a marked protection in the MES test, and little effect in the scPTZ test.[186] A distinct feature of TPM was its extended duration of action (up to 16 h).[186]

In vitro experiments on neuronal cultures have identified three different potential mechanisms to explain the anticonvulsant activity of TPM. Similar to PHT, TPM reduced VGSC-dependent SRF (IC_{50} <100 μM) in rat hippocampal neurons.[187] In addition, TPM showed selective blockade of KARs (IC_{50} <5 μM) but not NMDARs,[188] and potentiated $GABA_A$R-mediated chloride currents (~150% increase in GABA-evoked Cl$^-$ currents at 10 μM) by binding to a site distinct from the BZD site.[189]

1.3.4.3. Felbamate
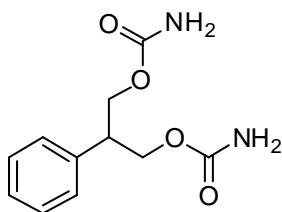
Felbamate (FBM)

2-Phenylpropane-1,3-diol dicarbamate (felbamate, FBM, Felbatol®) is a broad spectrum AED that was approved in 1993 for the treatment of partial and generalized seizures in adults and Lennox-Gastaut Syndrome (LGS) in children. At

non-toxic doses, FBM proved effective against both MES- and scPTZ- induced seizures in mice and rats.[190]

Like TPM, FBM possesses several distinct mechanisms of action that may account for its broad range of activities. In mice and rat cultured neurons it reduced VGSC-dependent SRF (IC$_{50}$ = 28 μM, rat striatal neurons),[191,192] and inhibited cloned human and rat VGSCs by stabilizing the fast-inactivated state of the channel.[193] At physiological concentrations (10–100 μM), it was shown to potentiate GABA-elicited currents through action on GABA$_A$Rs, in ways that differed with the neuronal *in vitro* preparations (e.g., mouse cortical neurons, rat hippocampal neurons).[194,195] In addition, FBM was shown to function as an antagonist on NMDA-evoked excitatory currents, albeit at high concentrations (IC$_{50}$ ~2 mM).[195] Knowing that administered anticonvulsant doses of FBM led to whole brain peak levels of 0.6 to 0.8 mM in rats,[196] it is likely that a modest inhibition of NMDARs may have occurred.

1.3.4.4. Tiagabine
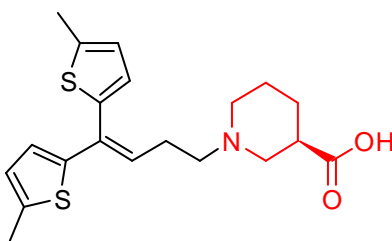
Tiagabine (TGB)

(*R*)-1-[4,4-Bis(3-methylthiophen-2-yl)but-3-enyl] piperidine-3-carboxylic acid (tiagabine, TGB, Gabitril®), is an antiepileptic drug advanced in the mid-1990's for the treatment of partial seizures.[197] TGB is another example of rationally designed molecule, based on the observation that nipecotic acid (in red) has inhibitory

properties on the GABA reuptake system.[198] Tiagabine is a more lipophilic analog of nipecotic acid with protective effects against scPTZ-induced, audiogenic, and amygdala kindled seizures, and moderate efficacy in the MES test.[197]

Unlike other traditional or recent AEDs, the mechanism of action of TGB is well-defined. Tiagabine has a potent inhibitory activity on GABA reuptake in glial (IC_{50} = 182 nM) and neuronal (IC_{50} = 446 nM) cultured cells, thereby prolonging the effects of GABA on its post synaptic cognate receptors.[199] The difference between the two IC_{50} values may be explained by different cellular levels of GAT-1, the GABA transporter selectively targeted by TGB.[200] Tiagabine was found to have little to no effect on a variety of neurotransmitter transporters, GPCRs, GABARs, Na^+ or Ca^{2+} VGICs.[201,197]

1.3.4.5. Summary

Recent AEDs are characterized by diversified, sometimes unusual, scaffolds compared with to traditional antiepileptics. An interesting feature of this class of drugs is the growing importance of rational design in the conception of the molecule. The rationally targeted pathway, however, turned out in some cases to be completely unrelated to the anticonvulsant properties of the molecule. AEDs such as TPM and FBM are unique in that they target both inhibitory and excitatory neurotransmitter receptors. TGB, in addition to modulating VGSCs, is the first antiepileptic rationally designed to effectively target one protein.

1.4. The emergence of new anticonvulsant entities and biological targets

1.4.1. What is new in antiepileptic drug development

The field of AEDs development is constantly evolving. The first decade of this millennium has seen the emergence of new therapeutic molecules acting on novel biological pathways. Following is a select list of such anticonvulsant molecules either marketed for the treatment of epilepsy or that are currently undergoing clinical trials.

1.4.1.1. Levetiracetam

Levetiracetam
(LVT)

Brivaracetam
(BVT)
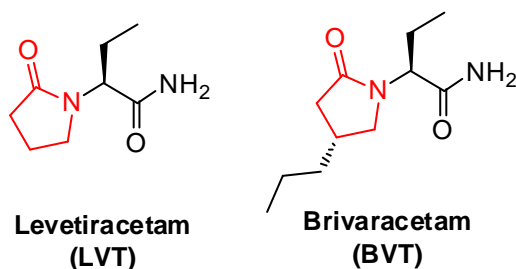
(*S*)-2-(2-Oxopyrrolidin-1-yl)butanamide   (Levetiracetam, LVT, Keppra[®]) is a recently approved AED (1999) for the treatment of partial onset seizures in humans and is structurally related to racetams, a class of pyrrolidine (in red) CNS-targeting agents.[202] LVT is unique in that it was not identified as active in the traditional MES and scPTZ animal models.[202,203] Instead, LVT proved very potent in the 6 Hz test and is one of the reasons why this pharmacological test was reintroduced as an important anticonvulsant screening method.[57] Levetiracetam has been proposed to have antiepileptogenic properties, in addition to its antiepileptic effects.[204] Such a profile may be linked to LVT's ability to prevent the acquisition of kindled seizures in

animals.[205] There are, however, some conflicting results about these properties of levetiracetam.[206]

LVT is also unique with respect to its mechanism of action. LVT has been shown to interact with the Synaptic Vesicle 2A (SV2A) protein, a macromolecule involved in the synaptic vesicle fusion process.[207-209] A correlation between interaction of SV2A and efficacy against partial and generalized seizures has been established.[210] Indeed, a tighter interaction with SV2A has been advanced as the primary mechanism of action of brivaracetam (BVT),[211] the second-generation analog of LVT currently undergoing Phase III clinical trials. Unlike its predecessor, BVT also appears to act on VGSCs, which may account for some of its anticonvulsant activity.[212]

1.4.1.2. Retigabine
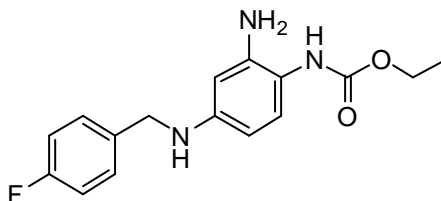
Retigabine (RGB)

Ethyl *N*-[2-amino-4-[(4-fluorophenyl)methylamino]phenyl]carbamate (Retigabine, RGB, D-23129) is a new anticonvulsant currently undergoing phase III clinical trials for the treatment of refractory partial-onset seizures.[212] RGB has demonstrated efficacy in many animal seizure tests like the MES and 6 Hz tests, in addition to several chemoconvulsant-induced seizure tests such as scPTZ and

picrotoxin.[213] Furthermore, it prevents sound-induced seizures in the Frings and DBA/2 mice models.[214]

The novel mechanism of action of RGB is the activation of K_v7.2–K_v7.5, a primarily neuronal subfamily of voltage-gated potassium channels (VGKCs).[215,216,207] Like VGCCs, these K^+ channels are characterized, in part, by the type of current they elicit.[217] Retigabine activates (1–10 μM) VGKC-mediated M-currents that are hyperpolarizing, with slow activation and deactivation kinetics.[218,219] RGB's effect is prevented by known, subtype-specific, VGKC blockers.[219]

1.4.1.3. 2-Deoxy-D-glucose

**2-Deoxy-D-glucose
(2DG)**
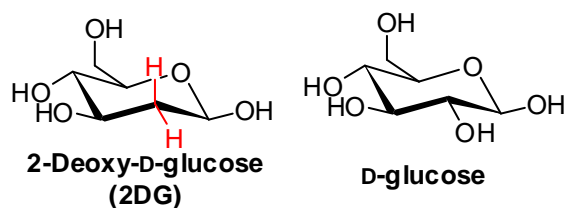
D-glucose

2-Deoxy-D-glucose (2DG) is a rather simple molecule with very promising anticonvulsant properties and is expected to undergo phase I clinical trial evaluation for the treatment of epilepsy in 2010.[212] Closely related to glucose, 2DG is taken up into cells by glucose transporters, but lacking the 2-hydroxyl group, the molecule cannot be metabolized and therefore act as an inhibitor of glycolysis.[220]

The ketogenic diet is a nutritional approach to the treatment of refractory epilepsy that consists in switching to a high-fat, low-carbohydrate food intake.[221] As a state of high energy demand, seizures rely on energetic pathways to "exist".[222] By switching from glucose to fat as the primary source of fuel, this diet is efficient at preventing epileptic seizures in patients.[222] These beneficial results can, however,

rapidly be lost by a small ingestion of excess carbohydrate.[223] Based on this observation, it was hypothesized that a glycolysis inhibitor may have protective effects against seizures and indeed, 2DG was found to have antiepileptic and antiepileptogenic effects in a variety of animal seizure models.[224]

1.4.2. Challenges for treating epilepsy

Most of the AEDs highlighted in this introductory chapter, from traditional drugs to recently discovered entities, have been presented in a favorable light, with their therapeutic mechanism of action as the focus. However, not mentioned are the sometimes life-threatening side-effects of some AEDs. As with most CNS-targeting agents, treatment with antiepileptics is often accompanied with a diverse set of side-effects, that can range from mild (drowsiness, nausea, sedation),[225] to severe (liver failure, aplastic anemia, teratogenicity).[226] Mostly for traditional and some recent AEDs, drug-drug interactions are numerous and multi-drug therapy, a standard practice in the treatment of epilepsy, requires caution by the practitioner.[227] In addition, antiepileptics are either classified by the Food and Drug Administration (FDA) as Schedule V substances because of an associated increased risk of suicidal thoughts, or as Schedule IV for AEDs with strong sedative effects (barbiturates and some benzodiazepines).

To tackle the problem of toxic side-effects often arising from a metabolite of the drug, one of the endeavors of AED research has been the development of prodrugs or metabolically stable analogs of already marketed molecules. This effort is exemplified by the modification of the traditional AED carbamazepine to the

second and third generation derivatives, oxcarbazepine and eslicarbazepine, respectively.[228,229] While these AEDs provide patients with safer treatment alternatives, they do not constitute a major breakthrough for the elucidation of new biological mechanisms underlying epilepsy.

1.4.3. Future hopes

Fortunately, change in AED development is likely to occur as scientists examine epilepsy in a new light. Although far from complete, our knowledge and understanding of epileptic disorders have greatly expanded since the early days of AED development. Importantly, researchers now realize the necessity to tackle the problem of epilepsy differently. Most marketed antiepileptics work as a prophylactic treatment, meaning preventing the symptoms rather than curing them. Research focus has begun to slowly shift towards the development of "disease-modifying" molecules and their related biology.

One interesting observation that began in the late 1980's, was the emergence of the word "antiepileptogenic" in epilepsy-related literature abstracts, a term that has gained greater prominence in the literature with each increasing year (Pubmed search). Antiepileptogenesis is a concept that designates the prevention or slowing of biological processes that eventually lead to an epileptic state. Recurring seizures have been shown to induce neuronal growth through a mechanism called mossy fiber sprouting.[230,231] The abnormal neural connections resulting from this process are thought to be essential for the initial development of seizures.[232] A better understanding of key macromolecules involved in neurogenesis will provide us with

new opportunities to help people with epilepsy. In addition, we can expect advances in seizure control using "disease"-representative models such as the kindled animal models, rather than tests that use healthy animals (e.g., MES, scPTZ, 6 Hz tests).

1.4.4. Functionalized Amino Acids (FAAs) are potent anticonvulsants with a broad spectrum of activity and a unique mechanism of action

1.4.4.1. Lacosamide
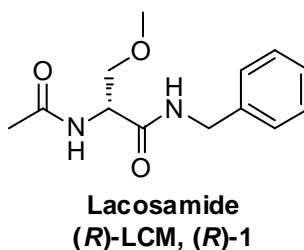
Lacosamide
(*R*)-LCM, (*R*)-1

(*R*)-*N*-Benzyl 2-acetamido-3-methoxypropionamide (lacosamide, (*R*)-LCM, Vimpat[®], (*R*)-**1**) is a functionalized amino acid (FAA) derived from D-serine that gained marketing approval for the treatment of partial-onset seizures in Europe (2008) and in the United States (2009).[203,233] FAAs were originally identified as potent anticonvulsant entities in the mid-1980s. Interestingly, the derivatives with the natural amino acid configuration displayed 10–20 fold less anticonvulsant activity.[234-241] (*R*)-LCM was extensively evaluated at the NINDS ASP and displayed excellent protection in the MES test, the Frings mouse model, the hippocampal kindled rat model and the 6 Hz test.[242] Compared with other standard AEDs, such as CBZ, PHT and LTG, the pharmacological profile of (*R*)-LCM was unique, leading to the speculation that (*R*)-LCM's mechanism of action was different from existing anti-epileptic agents.[233,243,244]

(*R*)-LCM function has been extensively studied using electrophysiology. Early studies showed that lacosamide had no effect on VGSCs (fast inactivation), VGCCs and VGKCs.[245,246] Radioligand displacement binding assays with (*R*)-LCM were conducted against a panel of GPCRs and LGICs, including GABA, adrenergic, dopamine, serotonin and muscarinic acetylcholine receptors.[233] (*R*)-LCM did not show any specific binding to these receptors at concentrations up to 10 μM. Because of its structural similarity to the unnatural amino acid D-serine, it was suggested that (*R*)-LCM might exert its action via the glycine binding site of NMDARs. However, binding studies showed no effect of (*R*)-LCM on this receptor at therapeutically relevant concentrations.[233]

1.4.4.2. Rationale and hypothesis to search and identify the biological targets of (*R*)-LCM

With little known about the mechanism of action of (*R*)-LCM, we initiated a chemical biology study to identify binding partners of (*R*)-LCM, by screening the rat brain proteome. Our rationale for this approach was based on the several observations. First, (*R*)-LCM and its FAA derivatives displayed very potent anticonvulsant activity in the rat. Second, AEDs, like many CNS agents, tend to have multiple targets for which they have a modest binding affinity. This phenomenon may be accounted for, in part, by their lower molecular weight and, in most cases, their relatively simple structures. The diminished structural size and complexity of most AEDs likely lessens the extent of protein-drug interactions (e.g., hydrogen bonds, hydrophobic, dipole-dipole, van der Waals) that foster binding. The average

molecular weight of the structurally diverse AEDs presented in this introductory chapter is ~220 g.mol^{-1} and (*R*)-LCM itself is 250 g.mol^{-1}. Third, the established or likely targets for anticonvulsant molecules are either soluble or membrane-bound proteins.

Shortly after the start of our investigation, (*R*)-LCM was reported to exert its function by enhancing the slow inactivation of VGSCs,[135,243] and by modulating the Collapsin Response Mediator Protein 2 (CRMP-2).[247] These target sites/pathways were novel for anticonvulsants and supported earlier assertions that (*R*)-LCM functioned via mechanisms *different* from existing antiepileptic agents. In light of the complex nature of AED function, we asked if there were additional (*R*)-LCM targets.

CHAPTER 2

ORGANIC SYNTHESIS OF LACOSAMIDE DERIVATIVES

2.1. Selecting the chemical parts to build molecular tools

2.1.1. Current methods for drug target identification

Many approaches exist for elucidating drug function.[248-259] Microarrays, RNA interference (RNA$_i$) and forward chemical genetics are three recent strategies. The first of these allows identification of genes or proteins whose expression profile is modified in the presence of the drug.[260] The second approach uses small RNAs to partially or completely silence or knock-down specific genes, and, therefore, proteins.[261] Though useful in rational drug discovery strategies, these methods do not identify the target's binding region for drug function. Forward chemical genetics is an attractive chemical biology approach that employs small molecules to elucidate biological pathways and protein targets by eliciting a certain phenotype.[250-255] Again, this method doesn't allow the identification of the target itself.

The most common approach for drug target identification is an affinity-based strategy where modified small molecules are immobilized on a matrix, and then incubated with purified proteins. Although protein targets have been successfully identified by this approach[262,263] the method does have limitations. The most

significant of these is that the structure of the ligand has been optimized. Subsequent introduction of a chemical bulky group (*i.e.,* biotin) often results in the disruption of key binding interactions, and with it, a loss of the drug's affinity for its target.

2.1.2. The use of small chemical groups for selective protein modification

Medicinal chemists and biochemists have traditionally appended reactive chemical groups on organic molecules to covalently label or inactivate macromolecules.[264-274] We term these small reactive moieties **Affinity Bait** (AB) groups. More recently the concept of bioorthogonal chemistry was advanced as a powerful approach to study or identify protein targets.[275-282] Alkynes and azides are two small, bioorthogonal groups: they are inert under biological conditions, yet quickly react with each other under Cu^I-catalyzed conditions ("Click Chemistry").[283,284] The alkyne and azide groups have been termed **Chemical Reporter** (CR) groups.

Our approach to target protein identification is based on the incorporation of both AB and CR groups on a low molecular weight compound. In this study, we focus on the mode of action of the novel AED lacosamide ((*R*)-**1**, LCM). Thus, we term these bi-functional derivatives as LCM AB&CR agents **2** (Scheme 3). Upon binding with the receptor (**3**), the AB group first creates a covalent linkage between the target protein and lacosamide (**4**, **5**). The CR group then reacts with a bioorthogonal Probe (**P**, **6**) containing a fluorophore or a biotin moiety to permit

complex **7**'s detection or purification, respectively. The following section provides a rationale for the choice of AB and CR groups used in our chemical biology study.

Scheme 3. Proposed strategy to identify the target proteins of (*R*)-LCM with AB&CR agents. The AB&CR analog **2** covalently modified receptor **3**. The covalent receptor/AB&CR complex **5** is then selectively tagged using a bioorthogonal Probe **6** for detection or purification (**7**).
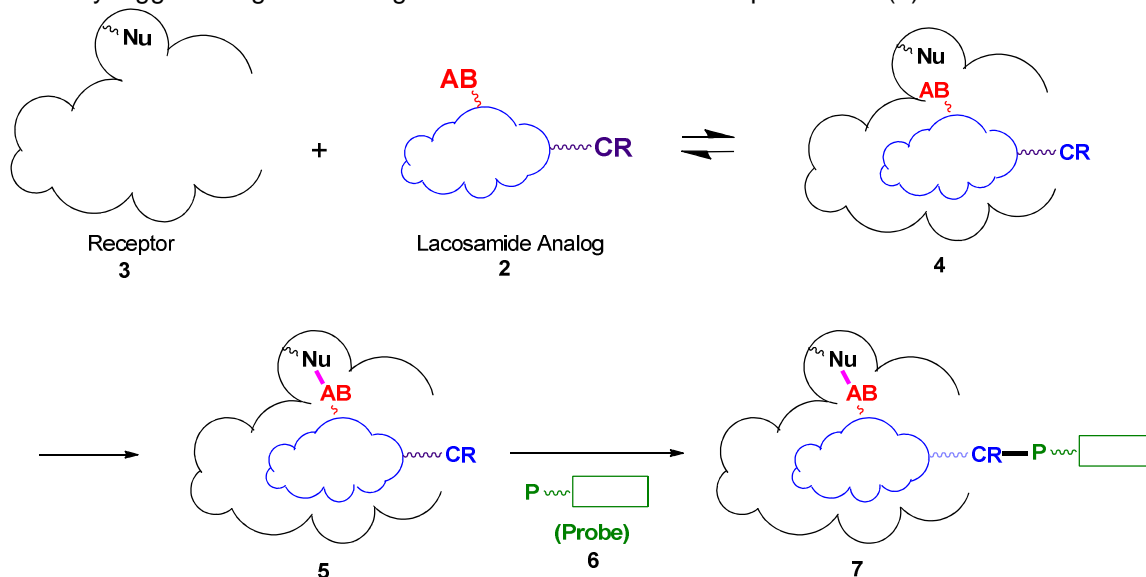
2.1.2.1. Electrophilic groups

One common application of the AB methodology is the development of selective enzyme inhibitors.[285-287] Typically, an electrophilic moiety is appended on the small molecule, peptide or peptidomimetic. The covalent bond formed between the active residue and the ligand leads to irreversible inhibition of the enzyme. Cravatt and coworkers have used an AB&CR methodology, Activity-Based Protein Profiling (ABPP), to characterize the reactivity profile of various AB groups within different classes of enzymes.[275,283,288] Interestingly, the nature of the electrophilic groups can be modulated to target a specific class of protease. For example

47

epoxide-containing peptides derivatives are commonly used to inactivate cysteine proteases,[269,274] while chloromethylketone groups are more prone to react with serine proteases.[289]

Almost any electrophile can, in principle, be used for protein modification. However, successful implementation of this approach requires the AB to display the adequate balance between chemical selectivity and reactivity. Employing very potent electrophilic moieties will inevitably lead to high levels of non-specific labeling.[290]

Covalent protein modification through small reactive groups is widely used in protein biology. Among the commonly utilized groups are the aromatic isothiocyanate,[291] the maleimide,[292] and the succinimidyl ester.[293] These electrophiles can react in a non-specific fashion since their adduction only requires an accessible lysine (isothiocyanate and succinimidyl ester) or cysteine (maleimide) residue. Fluorescein isothiocyanate (FITC),[291,294] and biotin succinimidyl esters,[293] are two examples of widely used low-molecular weight, non-specific protein modifiers.[295] Nonetheless, researchers have demonstrated that electrophilic groups can selectively target non-catalytic amino acid residues.[290,296,297]

2.1.2.1.1. Isothiocyanate

The aromatic isothiocyanate (NCS) may be considered a general amine-reactive chemical moiety with respect to proteins.[294] However, many studies document its utility as a selective, irreversible protein modifier. Indeed, brain G-protein coupled receptors (GPCRs) such as the δ-opioid,[264] cannabinoid,[265] *N*-methyl-D-aspartate (NMDA),[298] and α2-adrenergic receptors,[266] have been labeled *in*

vitro, and under certain conditions *in vivo*, by synthetic NCS-derivatives of their cognate ligand. Thus, this moiety can potentially be used as an AB group (**8**) for selective protein modification through a lysine residue within a binding pocket (Scheme 4).

Scheme 4. The NCS group reacts with a lysine residue to form a covalent thiourea linkage.
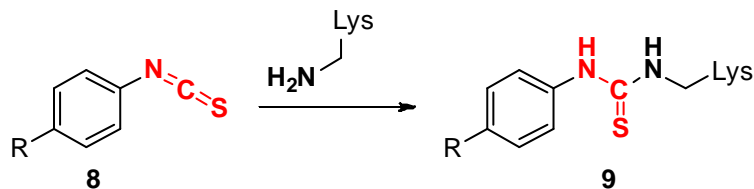
2.1.2.1.2. Aldehyde

Carbonyl compounds without an adjacent halomethyl group can also selectively label proteins.[299] Researchers have used aldehyde-containing small molecules to react with protein lysine residues (Scheme 5).[300]

Scheme 5. The aldehyde groups **10** and **13** react with an amine to give imine intermediates **11** and **14**. The transient species is then reduced to form a covalent amine bond using a hydride source.
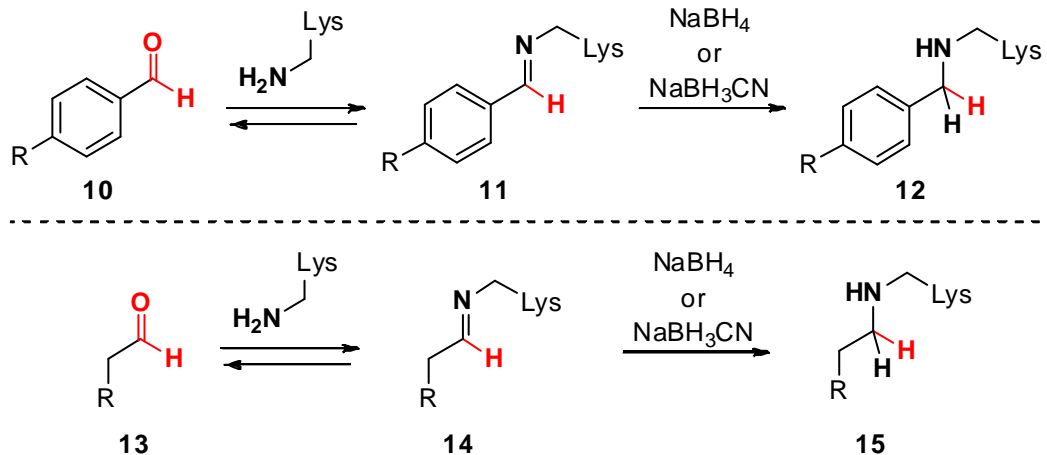
The transient imine (**11**, **14**) formed between the protein and the aldehyde (**10**, **13**) is reduced to a covalent amine bond using a hydride source. For instance, this approach led to the identification of the binding site of the antibiotic bicyclomycin with the *Escherichia coli* (*E. coli*) rho transcription terminator protein.[296,301,302] The aldehyde AB group can, therefore, be utilized to trap a small molecule/protein complex with the AB&CR methodology.

2.1.2.1.3. Epoxides

Epoxides are versatile electrophilic groups.[303-307] One of the driving forces of the reaction with electron-rich atoms is the release of the epoxide ring strain upon nucleophilic attack.[307] In addition to their use as cysteine protease irreversible inhibitors,[274,308,309] epoxide substrates (**16**, **19**) have been shown to react with the ε-amino group of lysines,[310] the carboxyl group of aspartates[311] and glutamates[312], and with the imidazole ring of histidines[290] to give the corresponding ring-opened adducts (Scheme 6).

Scheme 6. Epoxides undergo ring-opening with a variety of nucleophilic amino acids.
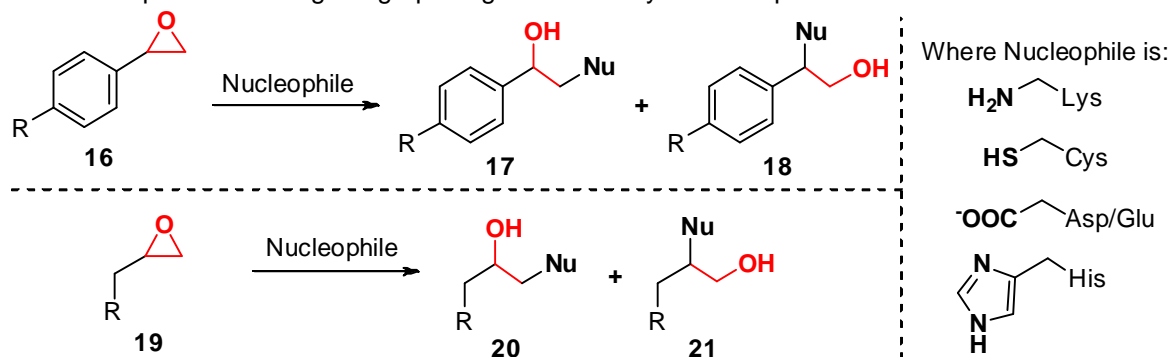
In a recent chemical biology study, Sames and coworkers examined at a panel of diverse electrophiles to construct a chemical probe to specifically target carbonic anhydrase 2 (CA2). Interestingly, the epoxide group was the only AB moiety that specifically targeted CA2.[290]

2.1.2.2. Photoreactive groups

A requirement for electrophilic AB groups is the need for a nearby protein nucleophilic moiety in the binding pocket. To overcome this limitation we included in our study several light-activated AB groups (**photoAB**). These reactive species can, upon binding to an active site, undergo less restrictive C–H bond insertion reactions to covalently label macromolecules.[313]

PhotoAB groups have been extensively used in protein modification studies.[314-316] Photophores become activated upon irradiation at a specific wavelength. Light triggers the formation of a reactive intermediate capable of undergoing carbon-hydrogen bond insertion or nucleophilic attack. A major advantage of this class of AB agents is their lack of reactivity under non-irradiative conditions. Photolabeling experiments may, however, suffer from the reactivity profile of some photoAB moieties, their low labeling efficiency, as well as the use of protein-damaging wavelengths.[313] Many studies have relied on the use of these light-activated groups for *in vitro* or *ex vivo* experiments.[263,268,316-320] Aromatic azides, diazirines and benzophenones are commonly used photoAB units for biological studies.

2.1.2.2.1. Aromatic azide

Aromatic azides are among the most widely used photoAB groups for protein labeling.[321,317,322,323,296] They can be readily obtained from the corresponding aromatic amine and nitro groups.[324,325] Irradiation of the Ar-N$_3$ group (**22**) at an energetic wavelength (254–360 nm) generates a short-lived (few nanoseconds)[326] singlet nitrene (**23**) that reacts with adjacent amino acids (**24**) (Scheme 7).

Scheme 7. Irradiation of **22** leads to the singlet nitrene **23**. The nitrene undergoes C-H bond insertion to give **24** or rearranges to the seven-membered ketinimine azepine electrophile **25**. The triplet nitrene is not shown
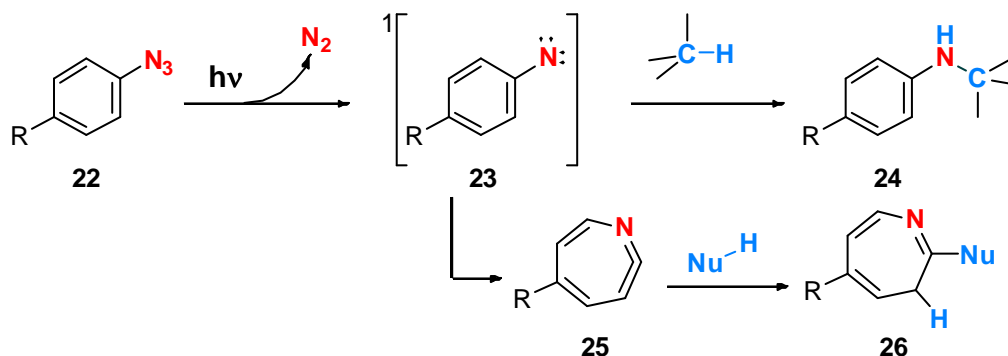
Two side reactions decrease the aryl azide photolabeling yield. The first one is the conversion of the singlet to the ground state triplet nitrene, a species with less value for photoaffinity labeling.[321] The second side reaction is the formation of a long-lived (few milliseconds[327]) reactive intermediate didehydroazepine **25**.[313,326,327] The ketenimine moiety in **25** reacts as an AB electrophile, and thus may lead to a less specific protein labeling through accessible nucleophilic residues.[313] The relative instability of aromatic azides to common reducing agents is also a problem.

Indeed, aryl azides are readily converted to the corresponding aniline upon exposure to dithiothreitol (DTT) and β-mercaptoethanol (BME).[317,328]

2.1.2.2.2. Diazirines

Diazirines were introduced more than 30 years ago as photoaffinity agents.[314] Upon irradiation (360 nm), they rearrange into diazo derivatives and/or carbenes (Scheme 8). Further excitation (312 nm) of the diazo intermediate leads to higher levels of the latter species.[313] The more pronounced carbene character of diazirines, as well as their stability to reducing, acidic and basic conditions[267,329] makes these reagents useful for protein modification studies.

Scheme 8. Irradiation of the diazirine moiety (**27**, **30**) generates the corresponding carbene (**28**, **31**) which undergoes nucleophilic attack or C-H bond insertion (**31**, **32**). Only the carbene intermediate is shown**.**
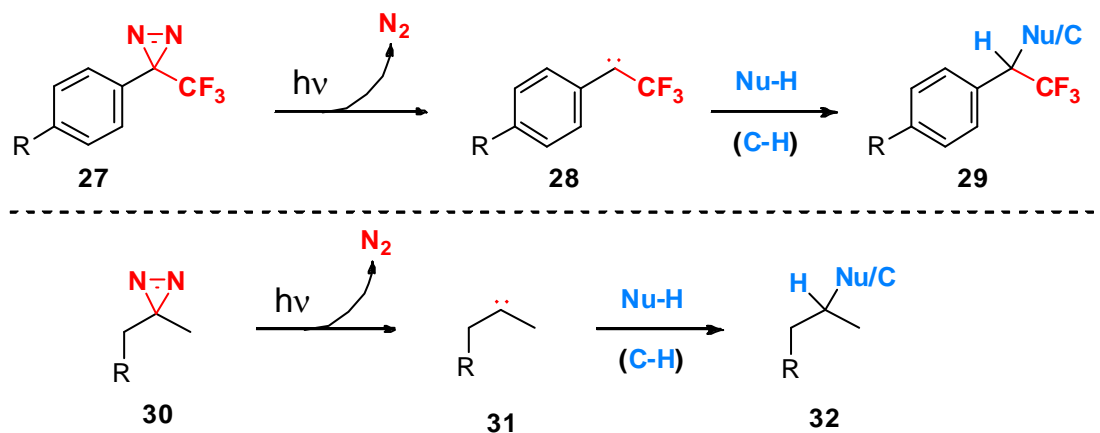
Two different types of diazirines are generally employed for photolabeling. The trifluoromethylaromatic diazirine (**27**) is the most common in biochemical studies.[318,330-334] At biologically relevant concentrations (1–1000 μM), Hatanaka and coworkers showed that irradiating this photoAB in buffered aqueous solution at 365

53

nm (10 min) and 312 nm (1 min) led to full conversion of the diazirine into its corresponding carbene **28**.[335] Used to a lesser extent is the alkyl diazirine photophore **30**.[268,336,337] The alkyl substituents flanking the diazirine moiety render the resulting carbene **31** less stable than its trifluoromethylaromatic counterpart, thereby reducing the efficiency of the irradiation step.[313]

Synthetic accessibility is one drawback of this photoAB moiety. While the alkyl diazirine is obtained in few steps,[338,339] the trifluoromethyldiazirine requires up to 10 synthetic transformations.[333,340,341] The reactive nature of carbenes is also a limitation. Indeed, a tight interaction is required for the short-lived intermediate to efficiently modify macromolecules.

2.1.2.2.3. Benzophenone

The benzophenone is another established photoAB for protein labeling (Scheme 9).[315,318,342,319,320,343-345] Unlike aryl azides and diazirines and their respective nitrene and carbene intermediates, the benzophenone undergoes protein C–H bond insertion via a radical pathway.[346] An important feature of this mechanism is that irradiation generates an excited, reactive intermediate (**34**) that returns to its ground state in the absence of a C–H bond substrate.[346] This non-destructive equilibrium between the ground and excited state,[347] as well as the required correct orientation of the $C^\bullet$–$O^\bullet$ diradical **34** with respect to the C–H bond[342] make the benzophenone a highly selective photoAB for biological studies.[313,346,347]

Scheme 9. Excitation of the C=O bond in **33** reversibly leads to an excited diradical **34** that undergoes C-H bond insertion reaction (**35**, **36**).

| **33** | **34** | **35** | **36** |
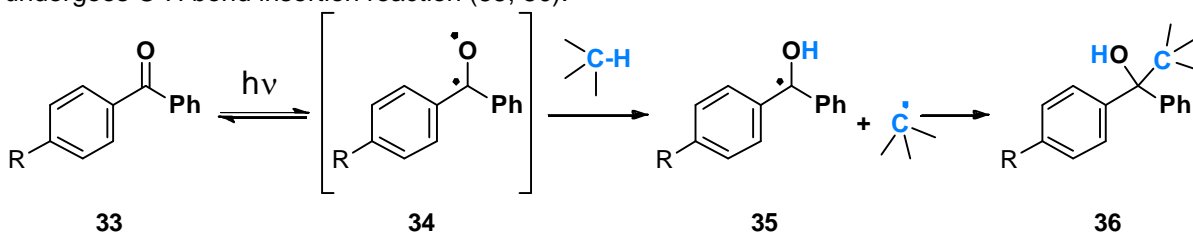
2.1.3. Bioorthogonal groups and click chemistry

1,3-Dipolar cycloadditions have found utility for the construction of a wide variety of heterocycles.[348] A copper-catalyzed version of these reactions, the 1,3 alkyne-azide cycloaddition (Scheme 10), has been extensively used in the field of chemical biology and polymer science over the past decade.[268,276,278,345,349-354]

Upon prolonged heating, alkynes and azides are known to form a mixture of 1,4- and 1,5-substituted triazoles.[355] However, under metal-catalyzed conditions, the reaction proceeds rapidly. Cu(I)-catalysis gives exclusively the 1,4-regioisomer (**39**), and Ru(II)-catalyzed conditions provide the 1,5-regioisomer (**40**).[356] Upon discovery of the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), Sharpless advanced the term of "Click Chemistry" as a concept to designate any organic reaction where reacting partners combine to give a single product and where the process is reliable, quantitative, selective, fast, clean, inexpensive, and environmentally friendly.[357] The CuAAC is the prototypical "Click Chemistry" reaction and is now commonly referred to by that name.[278,349-351]

Scheme 10. Terminal alkynes and azides lead to different triazole regioisomers under different conditions
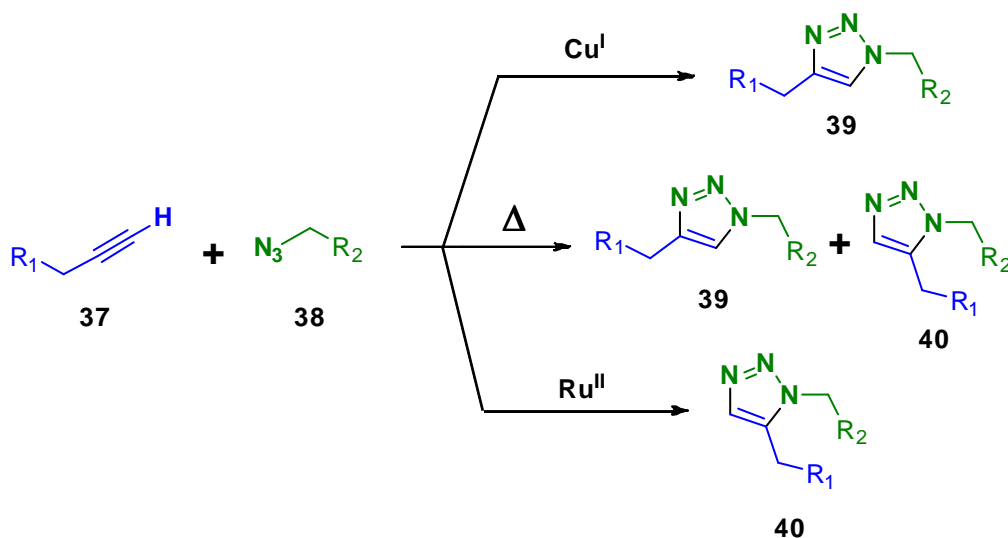
Along with others, Cravatt and coworkers pioneered the use of this transformation in *in vitro* biological studies and employed CuAAC to identify the target proteins for several small molecules.[275,345,354,358-360] However, this reaction cannot be extended to cells or living organisms due to the cytotoxicity of Cu(I) and Cu(II) species.[361] To circumvent this problem other types of copper-free click chemistry reagents have been advanced.

Scheme 11. Staudinger ligation reaction. The phosphorus atom reacts in **41** with an azide (**38**) to form an iminophosphorane intermediate that attacks the Me ester and forms an amide linkage and the phosphine oxide (**42**) upon hydrolysis
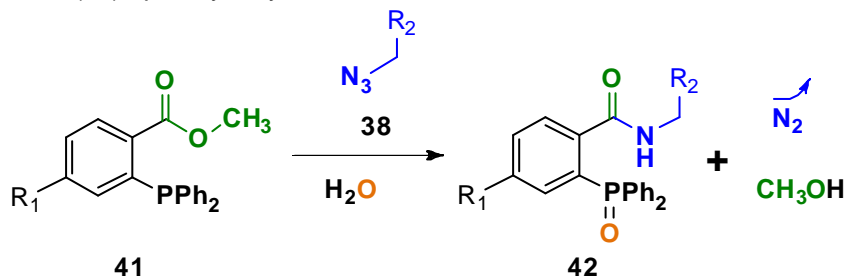
Bertozzi and coworkers have shown that both the Staudinger ligation (Scheme 11) and the strain-promoted azide-alkyne cycloaddition (Scheme 12) are

both suitable reactions to perform "Click Chemistry" in a biological milieu.[280-282,362-364]

Despite their excellent biocompatibility, both the phosphine and the first generation

cyclooctyne suffered from slow kinetics. Recently, progress has been made in the

development of more reactive cyclooctyne species.[352,365,366] Some of these copper-

free click reactions have been used to observe biological events in cells and living

organisms.[349,352]

Scheme 12. Two examples of strain-promoted cyclooctyne addition reactions. The strained ring as well as the electronic effects of neighboring groups improve the kinetics of the reaction. Only one of the two triazole regioisomers is drawn.
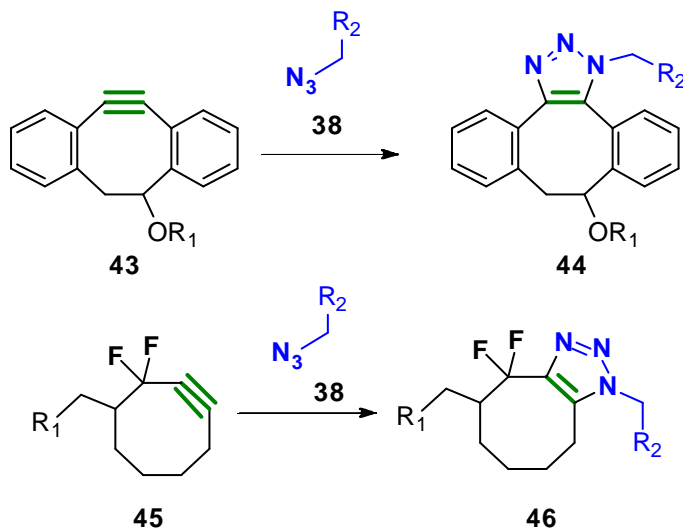
2.2. Structure-activity relationship of FAAs

2.2.1. FAAs

The potent anticonvulsant activity of FAAs (**47**) was discovered by the Kohn laboratory in 1985 and led to a focused SAR study.[234,367,241,368,237,369,238,370] More than 250 FAAs were prepared and then evaluated at the NINDS ASP and the Eli Lilly Laboratories. Compounds were tested for seizure protection in the MES and scPTZ tests and neurogical toxicity in the rotarod test (mice) and behavioral test (rats).

Scheme 13. Structure of FAAs. Different modification sites studied for the SAR of lacosamide are shown.
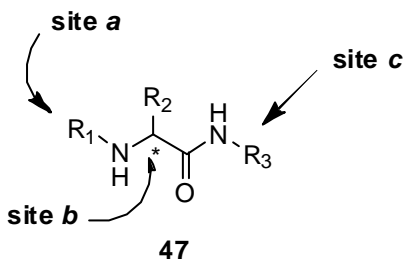
The SAR study of **47** focused on three different sites (*a*, *b* and *c*, Scheme 13). The optimal substituent for <u>site *a*</u> was the acetyl group (R_1 = C(O)CH$_3$). For <u>site *c*</u>, the requirements were also stringent. Excellent anticonvulsant activity was obtained for an unsubstituted benzyl group (*e.g.*, R_3 = CH$_2$Ph). Accordingly, most of the SAR study focused on modifications at <u>site *b*</u>. The Kohn laboratory showed that several compounds containing small aliphatic groups and heteroaromatic groups (*i.e.*, pyridyl, furanyl, oxazolyl) positioned at C(2) displayed significant anticonvulsant activity in the MES seizure test. When a heteroatom was introduced one atom removed from C(2), the seizure protection was comparable with or exceeded that of

phenytoin,[234,367,241,239] the prototypical antiepileptic agent in the MES-seizure model.[371] The highest anticonvulsant activities were obtained with oxygen as the heteroatom. The SAR study led to the discovery of lacosamide (R_2 = CH_2OCH_3). Most important, the SAR study demonstrated that one structural feature was recurrent: the anticonvulsant activity resided in the (R)-enantiomer (D-configuration) of the FAAs.

The initial SAR of FAAs concluded with lacosamide's invention. Efforts to explore the impact of the methoxy unit replacement in (R)-LCM were hampered by the unavailability of efficient synthetic pathways to the enantiomerically pure derivatives. At the start of our investigation, little was known about the structural tolerance at this site in lacosamide. The Kohn group reported the activities of the racemic O-ethyl (R,S)-**48** and O-allyl (R,S)-**49** analogs.[234] Both compounds exhibited significant activities but were 2–8-fold less active then (R,S)-**1** in mice (MES ED_{50} = 8.3 mg.kg^{-1} (0.5 h)).[234] Recently, a lacosamide analog was prepared where the OCH_3 moiety was replaced by a $OCHF_2$ (**50**).[372] This fluorinated derivative displayed excellent protection and prolonged duration of action (rat, po) in the MES test (ED_{50} = 3.0 mg.kg^{-1} (0.5 h), 4.2 mg.kg^{-1} (4 h)) when compared with (R)-LCM (ED_{50} = 1.8 mg.kg^{-1} (0.5 h), 8.3 mg.kg^{-1} (4 h)) under the same test conditions.[372]

48   **49**   **50**
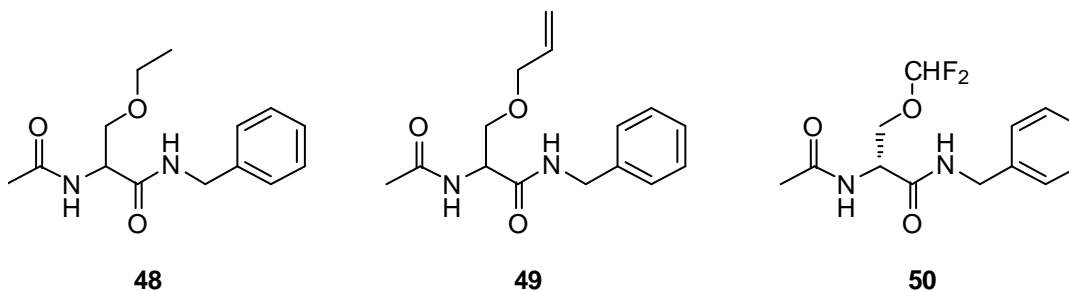
2.2.2. Synthetic approaches to side chain modification

2.2.2.1. Functionalization using *O*-alkylation

At the beginning of our studies two synthetic routes were available to synthesize enantiomerically pure LCM and LCM analogs. The shortest pathway (Scheme 14, **route 1**) to the AED started with commercial L- or D-serine (**51**). Acetylation with Ac$_2$O in acetic acid led to the *N*-acetylserine derivative **52** that directly reacted with benzylamine. Benzylamide **53** was then methylated with MeI under basic conditions to provide lacosamide enantiopure derivatives **55**. The need for the complete removal of excess AcOH prior to the amide coupling step was a major limitation of this pathway. Any acetic acid not removed resulted in the side-formation of *N*-acetylbenzylamide (**54**).

Scheme 14. Synthetic pathway to enantiomerically pure *O*-alkoxysubstituted derivatives of lacosamide: **route 1**
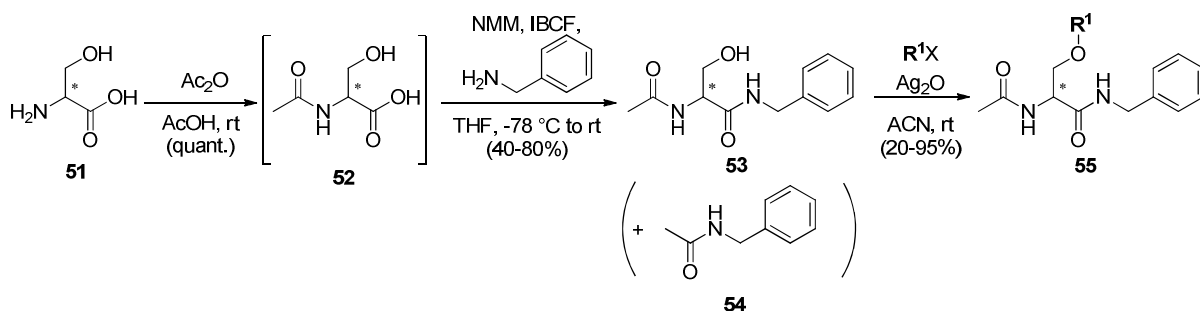
The second pathway (Scheme 15, **route 2**) was analogous to **route 1** except a protection/deprotection step was introduced. Enantiopure *N*-Cbz-serine (**56**) was either purchased or prepared from serine and Cbz-Cl. After amide coupling and methylation, the Cbz group was removed under catalytic hydrogenation conditions

and the free amine **59** acetylated with acetyl chloride or acetic anhydride. The enhanced solubility of *N*-protected serine derivatives in organic solvents made **route 2** more practical than **route 1** despite the increased number of steps. Nonetheless, a drawback to both pathways was the cost of Ag_2O (5 equiv required for the Williamson ether synthesis step). More important, only a narrow range of substituents could be installed at the 3-hydroxy site under alkylating conditions. Reaction yields rapidly dropped as the *O*-methyl group in LCM was increased to ethyl or allyl. Thus, **routes 1** and **2** were only useful to prepare either LCM AB or CR derivatives where the AB or CR moiety was introduced at the *N*-benzylamide position.

Scheme 15. Synthetic pathway to enantiomerically pure *O*-alkoxysubstituted derivatives of lacosamide: **route 2**
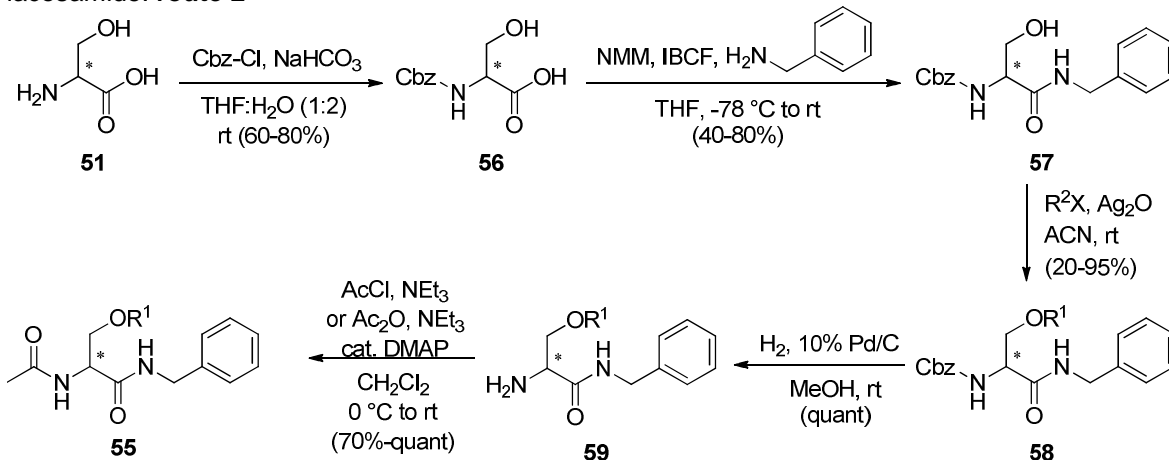
2.2.2.2. Functionalization using aziridine ring-opening, a general approach to LCM *O*-substituted derivatives

2.2.2.2.1. Synthesis via *N*-Trt aziridine carboxylate esters

N-Substituted aziridines carboxylate esters (**60**, R[1]= Ac, Cbz) are valuable intermediates in the synthesis of amino acid derivatives.[373-381] Under Lewis acid-catalyzed conditions, alcohols can add to the strained ring and produce an *O*-substituted serine analog **61** with the same C(2) stereochemistry as its precursor.[379]
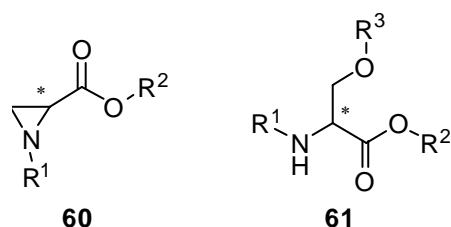
Scheme 16. Synthetic pathway to enantiomerically pure *O*-alkoxysubstituted derivatives of lacosamide: **route 3**
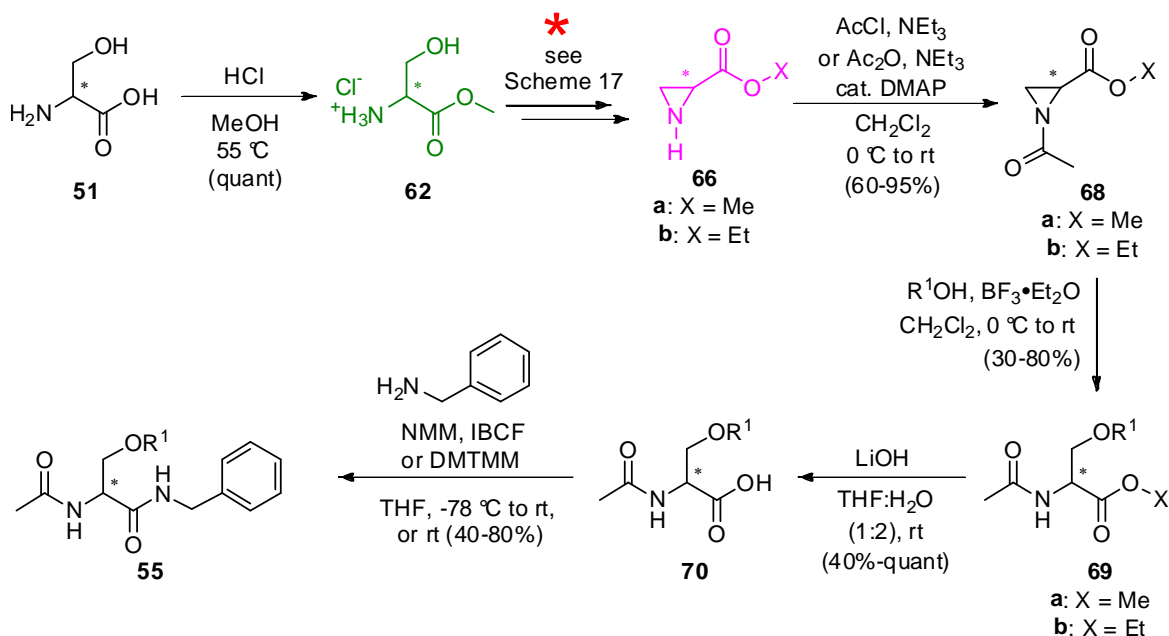
This methodology provided a third pathway (Scheme 16, **route 3**) for the preparation of LCM analogs and the desired AB&CR agents. L or D-Serine methyl

ester hydrochloride (**62**) was purchased or prepared from serine and HCl in MeOH.[382] The serine Me ester hydrochloride was successively *N*-tritylated, *O*-mesylated, and heated to reflux under basic conditions to form *N*-Trt-aziridinecarboxylate methyl ester (**65**, X = Me) (Scheme 17).[375,383] The trityl group was removed using TFA (Scheme 17) and the free aziridine **66a** acetylated with acetyl chloride to yield **68a** (Scheme 16). Ring-opening of **68a** with a variety of alcohols in the presence of BF$_3$•Et$_2$O gave **69a**.[378] The *O*-substituted *N*-acetyl serine esters (**69a**, and **69a,b**) were hydrolyzed with LiOH and coupled to benzylamine to provide LCM derivatives **55**.[384] The AB&CR agents were synthesized by coupling **70** with various substituted benzylamines. Depending on the nature of the *N*-benzylamide substituent, up to 3 additional steps were necessary to obtain the desired AB&CR compound.

Scheme 17. Different synthetic pathways used to access enantiomerically pure aziridine ester carboxylates

62

Trt-Cl, NEt$_3$
CH$_2$Cl$_2$
0 °C to rt
(75-90%)

63

MsCl, NEt$_3$
CH$_2$Cl$_2$
0 °C to rt
(90-95%)

64

NEt$_3$
THF, Δ
(60-80%)

65

NEt$_3$
ACN:EtOAc
(1:1)
0 °C, (80-90%)

CF$_3$COOH
CHCl$_3$:MeOH
(1:1)
0 °C to rt
(quant.)

67

PPh$_3$(OCH$_2$CH$_3$)$_2$ or PPh$_3$(OCH$_2$CF$_3$)$_2$
CH$_3$CN, rt
(30-70%)

66
a: X = Me
b: X = Et
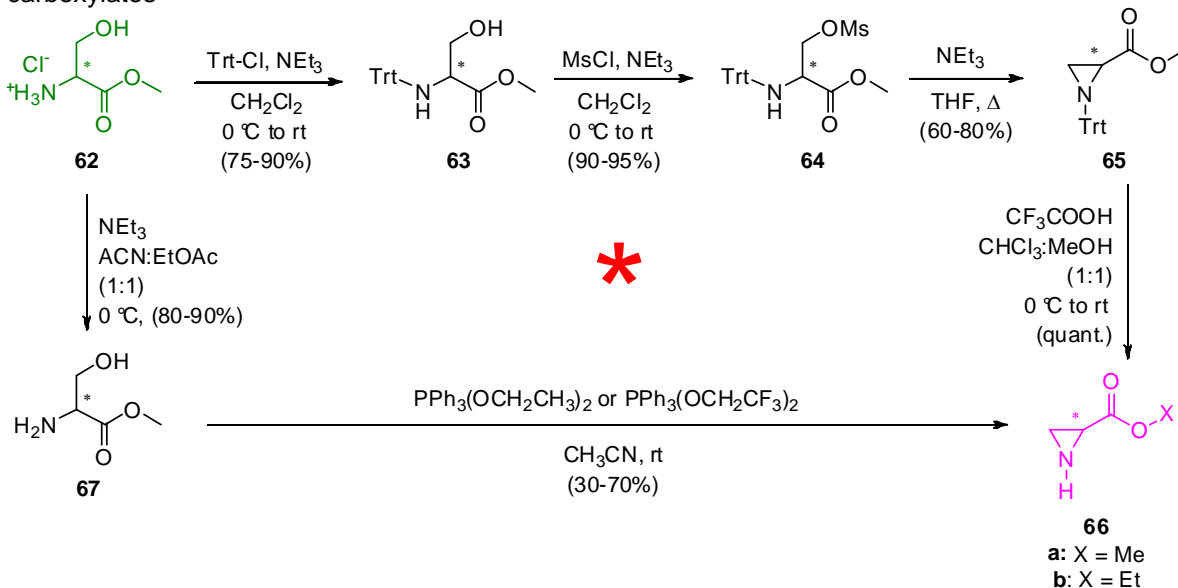
Despite the generality of this approach, two aspects in reaction Scheme 16 hindered our efficient synthesis of the lacosamide agents necessary for animal testing. The first was the generation of large quantities of **68**. The literature sequence[379,380,383] was time-consuming (4–5 d), labor intensive and the use of the Trt group as shown in Scheme 17 required its removal prior to *N*-acetylation, resulting in only moderate yields of **68a** (~40%). The second experimental roadblock involved the ring-opening of **68a** with select alcohols and the subsequent ester hydrolysis step. We found that the nature of the alcohol influenced the yield of the nucleophilic opening reaction. With 2-azidoethanol (**71**, 3–10 equiv), the reaction proceeded in a 30–40% yield while with 3-buten-1-ol (**72**, 3–5 equiv) it reached 65–70% yield. Furthermore, for water-soluble derivatives of **70**, we obtained poor to moderate recovery after ester hydrolysis and work-up.

71   **72**
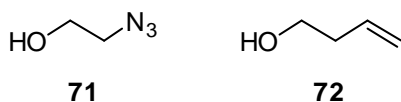
2.2.2.2.2. Synthesis using dialkoxyphosphoranes derivatives

Little could be done to resolve the second roadblock. Efforts to enhance the ring-opening step by increasing the number of equivalents of alcohol did not improve the yields of **69**. We addressed the first issue by employing a more efficient way to access **68**. Evans and coworkers demonstrated the versatility of dialkoxytriphenylphosphoranes (PPh$_3$(OR)$_2$) as cyclodehydration reagents to form heterocyclic systems.[385-389] Using an improved method,[387] we prepared

diethoxytriphenylphosphorane (PPh$_3$(OCH$_2$CH$_3$)$_2$, **DTPP**) from PPh$_3$, Br$_2$, and NaOEt (Scheme 18). Using serine methyl ester **67** and **DTPP** (1.2 equiv), we obtained aziridine **66a,b** as a mixture of methyl/ethyl esters. The reaction proceeded in 24 h, provided good yields (50–70%) of **66a,b** after bulb-to-bulb distillation, and was used to prepare up to 20 g of **68a,b** in a single experiment. We found that the reaction only proceeded if serine methyl ester **67** was first *isolated* as the free amine.[381] Acetylation of **66a,b** with acetic anhydride and catalytic DMAP afforded pure **68a,b** upon work-up. The remainder of the synthesis proceeded as previously described (Scheme 16).

Scheme 18. Synthetic route to dialkoxytriphenylphosphoranes **DTPP** and **DTPP-F$_6$**
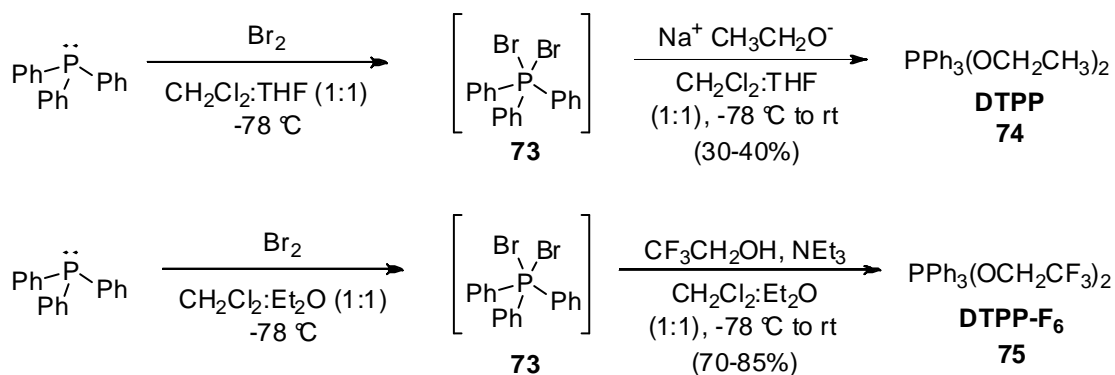
Although useful, **DTPP** proved to be a tedious reagent to prepare on large scale. The procedure required an initial centrifugation step (8000 rpm, 10 min) to remove finely divided NaBr, and 3–4 evaporation/filtration steps to remove PPh$_3$(O), a by-product of the reaction.[387] Accordingly, we prepared the hexafluoro derivative of **DTPP**, PPh$_3$(OCH$_2$CF$_3$)$_2$, **DTPP-F$_6$**,[390] by replacing NaOEt with a mixture of NEt$_3$ and CF$_3$CH$_2$OH (Scheme 18).[391] Phosphorane formation proceeded faster and required only filtration and evaporation to yield the pure compound.[391] Unfortunately,

DTPP-F$_6$ provided a decreased yield of **66** (25–30%) after bulb-to-bulb transfer compared with **DTPP** (40–70%).

We examined a more convergent synthesis of the desired *O*-substituted LCM analogs (Scheme 19, **route 4**). Derivative (*R*)-**55** was obtained by catalytic hydrogenation of (*R*)-**57** and reaction with either **DTPP** or **DTPP-F$_6$** to give aziridine *N*-benzylcarboxamide (*R*)-**77**. Following acetylation, the *N*-acetylaziridine benzylamide (*R*)-**78** was ring-opened with several alcohols (phenol, phenethyl alcohol and 3-butyn-1-ol) to yield the corresponding derivative of (*R*)-LCM. Paralleling the formation of **68-a,b**, greater yields of (*R*)-**77** were obtained with **DTPP** (50–60%) compared with **DTPP-F$_6$** (30–35%) (Scheme 19, **route 4**).

Scheme 19. Synthetic pathway to *O*-alkoxysubstituted derivatives of (*R*)-LCM: **route 4**
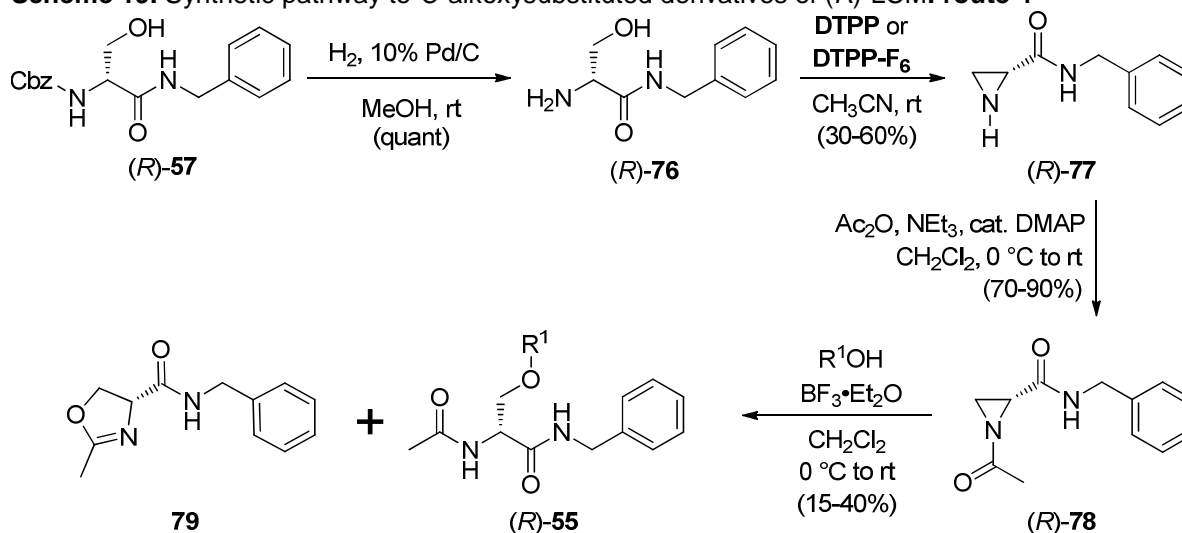
Several factors, however, made this synthetic route inconvenient. First, the low volatility of the aziridine benzylamide (*R*)-**77** prevented its bulb-to-bulb distillation, thus requiring its purification using silica gel flash chromatography. Second, 2-oxazoline **79** was generated as a by-product in the final step, presumably

through the rearrangement the *N*-acetylaziridine ring under Lewis-acid catalyzed conditions.[392] Interestingly, this rearrangement was not observed when using the *N*-acetylaziridine carboxylate ester (Scheme 16, **route 3**). Compound **79** was not readily separated from the final product and complicated the purification of (*R*)-**55** by flash column chromatography. The pure LCM derivatives were only obtained by recrystallization and we were not able to purify 2-oxazoline **79**. Compound **79** was tentatively identified by the characteristic ^{13}C resonance for the oxazoline CH$_3$ group (δ 13–14 ppm)[393] and by HRMS.

2.2.3. Structure activity relationship of the LCM 3-oxy-substituent

Large quantities of **68** were available using the **DTPP**-mediated aziridine synthesis. Using this new route we were able to explore the lacosamide SAR for the side chain oxy-substituent to identify potential structural constraints that would accompany either AB or CR placement at this site. We used different classes of alcohols to ring-open **68**, and in some instances **78**, and tested the new analogs of (*R*)-LCM at the NINDS ASP for anticonvulsant activity and neurotoxicity.[381]

2.2.3.1. Choice of compounds

With little knowledge of the structural tolerance at this site we designed, synthesized, and tested a series of *O*-substituted derivatives for anticonvulsant activity. We probed for several potential protein/drug interactions. We examined the effect of steric size by replacing the LCM methoxy unit ((*R*)-**1**) by progressively

larger alkoxy groups ((R)-**48**, (R)-**80**–(R)-**82**) (Table 1). We introduced unsaturated aliphatic and aromatic systems ((R,S)-**49**, (R)-**83**–(R)-**88**, (R)-**90**, (R)-**91**, (R)-**98**) to look for potential hydrophobic, π-π, and cationic-π interactions.[394] Within this set, we varied the length of the methylene spacer between several of these groups and the 3-oxy site in (R)-**55**. Not to limit ourselves to strictly lipophilic interactions, we prepared derivatives containing a polar side chain ((R)-**96**, (R)-**99**, (R)-**100**). These compounds could accept and/or donate a hydrogen bond(s) to a suitable amino acid residue within the putative drug binding pocket(s). Finally, we evaluated LCM oxy-substituted analogs that contained either an AB ((R)-**92**–(R)-**95**) or a CR group ((R)-**84**, (R)-**97**). In those cases where little or no anticonvulsant activity was observed for the AB agent, we prepared the corresponding isostere to see if metabolic factors, or structural and electrophysical constraints contributed to the loss of activity.[395-400]

Table 1. The Structure Activity Relationship of O-Substituted (R)-Lacosamide Derivatives

No.	R	mp (°C)	Mice (ip)[b]				Rat (po)[c]		
			MES,[d] ED_{50}	6 Hz, ED_{50}[e]	Tox,[f] TD_{50}	PI[g]	MES,[d] ED_{50}	Tox,[h] TD_{50}	PI[g]
1[i]	CH_3	142–143	4.5 [0.5] (3.7 – 5.5)		27 [0.25] (26 – 28)	6.0	3.9 [0.5] (2.6 – 6.2)	>500	>128
1-d_3	CD_3	142–143					5.2 [0.5] (4.3 – 5.7)	~200	~38
48	CH_2CH_3	129–130	7.9 [0.25] (5.3 – 10)		44 [0.25] (37 – 54)	5.6	5.6 [0.25] (2.5 – 16)	>500	>89
80	$CH(CH_3)_2$	151–153	23 [0.25] (20 – 26)	23 [0.25] (16 – 28)	77 [0.25] (66 – 96)	3.3	8.6 [0.25] (6.9 – 13)	>500	>58
81	$C(CH_3)_3$	126–127	30-100 [0.5]		~300 [0.5]				
82	C_6H_{11}	134–135	100-300 [0.5]		~300 [0.5]		~30 [0.25]	>30	
83	C_6H_5	169–170	100-300 [0.5]		>600 [0.5]				
49[i]	$CH_2CH=CH_2$ (R,S)	76–77	30–100		30–100				
84[j]	$CH_2C{\equiv}CH$	149	16 [0.25] (13 – 19)	29 [0.5] (21 – 40)	59 [0.25] (55 – 66)		7.9 [0.5] (4.7 – 11)	>500	>63
85[k]	$CH_2C{\equiv}CCH_3$	149–151	30-100 [0.5]		30-100 [0.5]				
86	$CH_2C_6H_{11}$	143–144	100-300 [0.5]		~300 [0.5]				

#	R	mp							
87	$CH_2C_6H_5$	145–146	64 [0.25] (56 – 76)		200 [0.25] (160 – 300)	3.2	>30	>30	
88	$CH_2CH_2CH=CH_2$	103–104	30-100 [0.5]		~100 [0.5]		17 [0.5] (13 – 21)	>500	>29
89	CH_2CH_2–cyclopropyl	97–99	46 [0.25] (42 – 50)		85 [0.25] (69 – 105)	1.9	>30	>30	
90	$CH_2CH_2C{\equiv}CH$	111–113	30–100 [0.5]		30–100 [0.5]				
91	$CH_2CH_2C_6H_5$	90–92	100-300 [0.5]		100-300 [0.5]		>30	>30	
92[l]	CH_2CH_2–diazirinyl		30-100 [0.5]		100-300 [0.5]		44 [1] (28–65)	>500	>11
93	$CH_2CH_2C(O)H$	120–121	>300 [0.5]		>300 [0.5]		>100		
94	CH_2CH_2–oxiranyl	104–110	>300 [0.5]		>300 [0.5]				
95[j]	CH_2CH_2NCS		>300 [0.5]		100-300 [0.5]		<30 [4]	>30 [4]	
96	$CH_2CH_2NHC(O)CH_3$	166–168	>300 [0.5]		>300 [0.5]		>30	>30	
97	$CH_2CH_2N_3$	111–113	100-300 [0.5]	44 [0.25] (32 – 65)			>40 [0.25] (po) 5.7 [0.25] (ip) (3.6 – 8.4)	78 [0.25] (ip) (72 – 83)	>13
98	CH_2CH_2–(4-CH_2OCH_3-triazol-1-yl)	127–129	>300 [0.5]	>100	>300 [0.5]				
99	$CH_2CH_2OCH_3$	109–110	30-100 [0.5]		~300 [0.5]				
100	$(CH_2CH_2O)_2CH_3$	48–52	>300 [0.5]	>100	>300 [0.5]		>30	>50	
phenytoin[m]			9.5 [2] (8.1–10)		66 [2] (53–72)	6.9	30 [4] (22–39)	[n]	> 100

phenobarbital [m]	22 [1] (15 – 23)	69 [0.5] (63 – 73)	3.2	9.1 [0.5] (7.6 – 12)	61 [0.5] (44 – 96)	6.7
valproate [m]	270 [0.25] (250 – 340)	430 [0.25] (370 – 450)	1.6	490 [0.5] (350 – 730)	280 [0.5] (190 – 350)	0.6

[a] All compounds tested corresponded to the (*R*)-enantiomer except **49**. The compounds were tested through the auspices of the NINDS ASP. [b] The compounds were administered intraperitoneally. ED_{50} and TD_{50} values are in milligrams per kilogram. [c] The compounds were administred orally unless otherwise indicated. ED_{50} and TD_{50} values are in mg/kg. [d] MES = maximal electroshock seizure test. [e] The 6 Hz test was carried out at 32 mA. [f] TD_{50} value determined from the rotorod test. [g] PI = protective index (TD_{50}/ED_{50}). [h] Tox = behavioral toxicity. [i] Ref.1. [j] Ref.23. [k] Work of Dr. Ki Duk Park. [l] Work of Dr. Christophe Salomé. [m] Porter, R. J.; Cereghino, J J.; Gladding, G. D.; Hessie, B. J.: Kupferberg, H. J.; Scoville, B.; White, B. G.; *Cleveland Clin. Q.* **1984**, *51*, 293-305. [n] No ataxia observed up to 3000 mg/kg.

2.2.3.2. Pharmacological results

Compounds (*R*)-**1**, (*R*)-**48**, (*R,S*)-**49**, (*R*)-**80**–(*R*)-**100** were tested for anticonvulsant activity at the NINDS ASP using the procedures described by Stables and Kupferberg.[401] The pharmacological data from the MES[401,402] and 6 Hz[57] tests are summarized in Table 1 along with clinical AEDs phenytoin,[403] valproate,[403] and phenobarbital.[403] All compounds were administered intraperitoneally (ip) to mice and orally (po) to rats unless otherwise indicated. The table lists the values that were determined to be protective in blocking hind limb extension induced in the MES seizure model from the rodent identification studies. For compounds that showed significant activity, we report the effective dose (50%) (ED_{50}) values obtained in quantitative screening evaluations. Also provided are the median doses for neurological impairment (50%) (TD_{50}) in mice, using the rotorod test,[75] and the behavioral toxicity effects observed in rats. TD_{50} values were determined for those compounds exhibiting significant activity in the MES test. The protective index (PI = TD_{50}/ED_{50}) for these analogs are also listed. Select compounds were evaluated in the psychomotor 6 Hz (32 mA) seizure models (mice, ip).[57] When the derivatives were evaluated in the scPTZ seizure model[401] none provided protection at 300 mg/kg doses at the times (0.5 and 4 h) tested (data not shown). The absence of seizure protection in this assay is a hallmark of FAA activity.[234,367,241,368,237,369,238,370]

In total, 23 new *O*-substituted LCM analogs were prepared and evaluated for anticonvulsant activity at the NINDS ASP. We observed a steady increase in activity in mice (ip, [0.25–0.5 h]) as the substituent steric size decreased from cyclohexyl ((*R*)-**82**, ED_{50} = 100–300 mg.kg^{-1}), phenyl ((*R*)-**83**, ED_{50} = 100–300 mg.kg^{-1}), and

tert-butyl ((R)-**81**, ED_{50} = 30–100 mg.kg^{-1}), to isopropyl ((R)-**80**, ED_{50} = 23 mg.kg^{-1}), to ethyl ((R)-**48**, ED_{50} = 7.9 mg.kg^{-1}) and to methyl ((R)-**1**, ED_{50} = 4.5 mg.kg^{-1}), indicating that non-bulky 3-oxy substituted groups in (R)-**55** analogs provided the highest anticonvulsant activity.[381] Interestingly, we observed a similar increase in activity as the size of the 3-oxy substituent decreased when the compounds were administered orally to rats, but the range of activities was narrower. For example, the *O*-cyclohexyl derivative (R)-**82** exhibited an ED_{50} of ~30 mg/kg while *O*-isopropyl (R)-**80** (ED_{50} = 5.6 mg/kg), *O*-ethyl (R)-**48** (ED_{50} = 5.2 mg/kg), and *O*-methyl (R)-**1** (ED_{50} = 3.9 mg/kg) all displayed excellent seizure protection. (R)-**48** and (R)-**80** showed no behavioral neurotoxicity in the rat at the highest dose (500 mg/kg) tested, leading to high PI values for both compounds ((R)-**48**: PI >89; (R)-**80**: PI >58). When we inserted a methylene group between the cyclohexyl group and 3-oxy site in (R)-**82** (ED_{50} = 100–300 mg/kg) to give (R)-**86** (ED_{50} = 100–300 mg/kg), we observed no improvement in anticonvulsant activity in mice (ip).

Next, we examined the effect of incorporating an unsaturated unit at the 3-oxy site in (R)-**55**. When the *O*-phenoxy (R)-**83** derivative was evaluated, we observed minimal protection in the MES test in mice (ip) (ED_{50} = 100–300 mg/kg).[381] Correspondingly, in mice we found pronounced anticonvulsant activity for other derivatives that contained an unsaturated 3-oxy substituent. The *O*-allyl[234] ((R,S)-**49**, ED_{50} = 30–100 mg/kg), *O*-propargyl ((R)-**84**, ED_{50} = 16 mg/kg),[384] and *O*-but-2-ynyl ((R)-**85**, ED_{50} = 30–100 mg/kg) (R)-**1** analogs all provided pronounced seizure protection. When the *O*-propargyl ((R)-**84**, ED_{50} = 7.9 mg/kg) and the *O*-but-2-ynyl ((R)-**85**, ED_{50} = 6.4 mg/kg) (R)-**55** derivatives were tested in the rat (po) we

observed excellent seizure protection, which was comparable with phenytoin (ED_{50} = 6.9 mg/kg),[403] and no behavioral neurological toxicity at 500 mg/kg. Significantly, the propargyl unit was shown to be a superior CR unit in bioorthogonal Cu(I)-mediated cycloaddition reactions.[384,276] Our finding that (*R*)-**84** and (*R*)-**85** exhibited excellent activity in the MES seizure model indicated that this CR unit in (*R*)-**1** AB&CR agents would not likely impact the unit's binding to its cognate receptor(s). Inserting a methylene group between the C(3)-oxy site and the phenyl group in *O*-phenoxy (*R*)-**83** (ED_{50} = 100-300 mg/kg) to give the *O*-benzyl derivative (*R*)-**87** (ED_{50} = 64 mg/kg) led to improved seizure protection in mice (ip). These collective findings suggest that beneficial protein interactions with the 3-oxy π-system in (*R*)-**55** existed at the drug binding site(s) responsible for MES-induced seizure protection. This interaction with the 3-oxy site in (*R*)-**55** analogs may offset adverse steric effects introduced when a larger, unsaturated group was included at this position.

We next explored the effect of adding a second methylene unit between the unsaturated unit and the (*R*)-**55** 3-oxy site to determine if the location of the unsaturated site affected anticonvulsant activity. When we added the second methylene group to *O*-propargyl (*R*)-**84** (ED_{50} = 16 mg/kg) and *O*-benzyl (*R*)-**87** (ED_{50} = 64 mg/kg) to give *O*-3-butynyl (*R*)-**90** (ED_{50} = 30–100 mg/kg) and *O*-2-phenylethyl (*R*)-**91** (ED_{50} = 100–300 mg/kg) (*R*)-**55** derivatives, we saw a decrease in anticonvulsant activity in mice (ip). Like the *O*-phenylethyl derivative (*R*)-**91** (ED_{50} = 100-300 mg/kg), the *O*-triazolylethyl (*R*)-**98** compound (ED_{50} = >300 mg/kg) did not display activity at the doses tested. Nonetheless, when tested orally in rats, the *O*-3-butynyl (*R*)-**90** (ED_{50} = <30 mg/kg) provided appreciable seizure protection.

These results indicated that the site of unsaturation at the 3-oxy site in (R)-**55** may be important for anticonvulsant activity. We observed one exception to this pattern, the O-allyl ((R,S)-**49**, ED_{50} = 30–100 mg/kg) and the O-3-butenyl ((R)-**88**, ED_{50} = 30–100 mg/kg) (R)-**55** derivatives showed similar anticonvulsant activity in mice (ip). Like O-3-butynyl (R)-**90**, the activity of the O-3-butenyl (R)-**88** derivative improved from mice (ip) (ED_{50} = 30–100 mg/kg) to rat (po) (ED_{50} = 17 mg/kg).

The effect of polar substituents at the C(2) site in (R)-**55** was assessed by incorporating either an ethylenoxy or an acetamidoethoxy unit two methylene units removed from the 3-oxy site. When we attached one ethylenoxy group to give methyl ether (R)-**99**, we observed noticeable seizure protection (ED_{50} = 30–100 mg/kg). Adding a second ethylenoxy spacer to (R)-**99** to give (R)-**100** produced no detectable activity under the test conditions (ED_{50} = >300 mg/kg). Similarly, the acetamidoethyl analog (R)-**96** (ED_{50} = >300 mg/kg) was inactive at the doses tested. The limited data do not allow us to speculate on the factors responsible for the loss of anticonvulsant activity of (R)-**96**, (R)-**99**, and (R)-**100**, and our findings indicated that introducing polar substituents at the 3-oxy site in (R)-**55** did not improve seizure protection.

We tested (R)-**55** derivatives, (R)-**93**–(R)-**95**, that contained AB groups at their 3-oxy sites. The electrophilic groups aldehyde (R)-**93** (ED_{50} = >300 mg/kg) and epoxide (R)-**94** (ED_{50} = >300 mg/kg) exhibited no anticonvulsant activity and no neurological toxicity at the doses tested. The lack of neurotoxicity may suggest that the compounds did not cross the blood brain barrier. The aliphatic isothiocyanate (R)-**95** was inactive in mice (ED_{50} = >300 mg/kg), active in rats (ED_{50} = <30 mg/kg),

but neurologically toxic (TD_{50} = 30–100 mg/kg, mice (ip); TD_{50} >30 mg/kg, rat (po)). We suspect that several of these AB groups may have metabolized under the test conditions. Indeed, aldehydes and epoxides are known substrates for a variety of enzymes (e.g., aldehyde reductases, epoxide hydrolases).[395-400] Since we planned to use these AB groups for in vitro experiments where metabolism would less likely be an issue, we evaluated, where possible, the corresponding AB isostere to determine if the steric size of the AB group precluded their use. We were gratified to find that the *O*-but-3-enyl isostere (*R*)-**88** (ED_{50} = 30–100 mg/kg, mice (ip); ED_{50} = 17 mg/kg, rat (po)) for aldehyde (*R*)-**93** and the *O*-cyclopropyl isostere (*R*)-**89** (ED_{50} = 46 mg/kg. mice (ip)) for epoxide (*R*)-**94** displayed pronounced-to-excellent anticonvulsant activity. Thus, we have not attributed the absence of activity for aldehyde (*R*)-**93** and epoxide (*R*)-**94** to steric factors. Similar to the *O*-cyclopropyl (*R*)-**89**, the photolabile methyldiazirine AB derivative (*R*)-**92** exhibited pronounced animal protection in the MES seizure test (ED_{50} = 30–100 mg/kg, mice (ip); ED_{50} = 44 mg/kg, rat (po)).

Of all the *O*-substituted LCM analogs prepared, the CR *O*-azidoethyl (*R*)-**97** was the most intringuing. Its anticonvulsant activity was dependent on the animal, the seizure test, and the route of administration. While (*R*)-**97** displayed poor MES seizure protection in mice (ip) (ED_{50} = 100–300 mg.kg^{-1}), it proved potent in the 6 Hz test when administered to mice by the same route of administration (ED_{50} = 44 mg.kg^{-1}, 32 mA stimulation). Furthermore, we observed that (*R*)-**97** exhibited exceptional activity in the MES test in the rat when administered ip (ED_{50} = 5.7

mg.kg^{-1}), but that activity dropped with oral administration (MES ED$_{50}$ >40 mg.kg^{-1}). The TD$_{50}$ for (R)-**97** was 78 mg.kg^{-1} (rat, po), providing a PI greater than 13.

2.2.3.3. Discussion

The composite SAR documented the stringent structural requirements for the 3-oxy site in (R)-**55**. In mice (ip), the O-substituent that afforded the best activity was methyl ((R)-**1,** ED$_{50}$ = 4.5 mg.kg^{-1}). Replacing the O-methyl group with ethyl ((R)-**48**, ED$_{50}$ = 7.9 mg/kg), isopropyl ((R)-**80**, ED$_{50}$ = 23 mg/kg), *tert*-butyl ((R)-**81**, ED$_{50}$ = 30-100 mg/kg), and cyclohexyl ((R)-**82**, ED$_{50}$ = 100-300 mg/kg) led to a steady drop in anticonvulsant activity in the MES seizure test. The difference in activity for (R)-**1**, (R)-**48** and (R)-**80** diminished in the rat (po) model where the MES ED$_{50}$ values were below 10 mg/kg. We concluded that including bulky alkyl substituents at the 3-oxy site in (R)-**55** adversely affected seizure protection in the MES seizure test.

Inserting one methylene (CH$_2$) group between the oxygen and the cyclohexyl ring in (R)-**82** (ED$_{50}$ = 100-300 mg/kg) to give (R)-**85** (ED$_{50}$ = 100–300 mg/kg) did not improve activity in mice (ip). However, we observed a significant increase in potency in mice going from O-phenyl (R)-**83** (ED$_{50}$ = 100–300 mg/kg) to O-benzyl (R)-**87** (ED$_{50}$ = 64 mg/kg). Pronounced-to-excellent activity was also observed for the O-allyl (R,S)-**49** (ED$_{50}$ = 30–100 mg/kg) and the O-propargyl (R)-**84** (ED$_{50}$ = 16 mg/kg) compounds. Inclusion of an ethylene (CH$_2$CH$_2$) spacer between the oxygen and the phenyl groups to provide (R)-**91** (ED$_{50}$ = 100–300 mg/kg) led to a loss of activity, compared with (R)-**87** that contained a methylene spacer. A similar activity loss in

mice was observed going from the *O*-propargyl compound (*R*)-**84** (ED_{50} = 16 mg/kg) to the *O*-3-butynyl derivative (*R*)-**90** (ED_{50} = 30-100 mg/kg). When several of these compounds were tested in the rat (po), we observed improved anticonvulsant activity (i.e., (*R*)-**84**, (*R*)-**88**, (*R*)-**90**). The pronounced-to-excellent activity in the *O*-benzyl (*R*)-**87** (ED_{50} = 64 mg/kg), *O*-propargyl (*R*)-**84** (ED_{50} = 16 mg/kg), *O*-but-2-ynyl (*R*)-**85** (ED_{50} = 30–100 mg/kg), and racemic *O*-allyl (*R,S*)-**49** (ED_{50} = 30–100 mg/kg) derivatives suggested that a favorable interaction with the 3-oxy π system in (*R*)-**55** at the target site(s) may foster binding, provided the group was not large and the unsaturated system was correctly positioned at the 3-oxy site.

Compounds (*R*)-**96**, (*R*)-**99**, and (*R*)-**100** were prepared to to help us determine if potential hydrogen bonding interactions at the 3-oxy site in (*R*)-**55** would affect anticonvulsant activity. Methyl ether (*R*)-**99** contained one ethylenoxy unit at the 3-oxy site and showed significant activity (ED_{50} = 30–100 mg/kg). Adding a second ethylenoxy unit to give (*R*)-**100** (ED_{50} = >300 mg/kg) led to a loss of anticonvulsant activity. Replacement of the ethylenoxy moiety in (*R*)-**99** with an acetamidoethoxy group provided (*R*)-**96** (ED_{50} = >300 mg/kg), which was inactive in the MES seizure test. When (*R*)-**99** was compared with two non-polar analogs, (*R*)-**88** (ED_{50} = 30–100 mg/kg) and (*R*)-**90** (ED_{50} = 30–100 mg/kg), we found that it displayed similar anticonvulsant activity. We concluded that introducing additional oxygen and nitrogen substituents at the (*R*)-**55** 3-oxy site did not improve activity. Several factors may be responsible for these whole animal pharmacological findings, such as (*R*)-**55** biodistribution in the brain, metabolism, and drug binding. Our data do not allow us to distinguish these factors but do provide valuable information for

the installation of both AB and CR units in the (R)-**1** framework and future drug design efforts.

As part of our (R)-**55** 3-oxy SAR study, we prepared several (R)-**55** AB agents ((R)-**92**–(R)-**95**) and (R)-**55** CR agents ((R)-**84**, (R)-**97**). The alkyne (R)-**84** and azide (R)-**97** CR compounds displayed excellent anticonvulsant activity (ED_{50} = <10 mg/kg) in the rat upon po and ip administration, respectively. These findings supported the use of these CR units in (R)-**1** proteomic target searches. Correspondingly, of the four (R)-**55** AB agents we prepared, only the diazirinyl (R)-**92** showed anticonvulsant activity, modest in mice at ED_{50} = 30–100 mg/kg and in rat at ED_{50} = 44 mg/kg. The three other (R)-**55** AB agents, (R)-**93**–(R)-**95** displayed no anticonvulsant activity in mice (ip) at the highest dose tested (300 mg/kg). When (R)-**95** was tested in the rat (po), we observed activity at 30 mg/kg. To test whether the structural size of the AB units in aldehyde (R)-**93** and epoxide (R)-**94** was responsible for anticonvulsant activity loss in the MES seizure model, we prepared the isosteres (R)-**88** and (R)-**89**, respectively. When (R)-**88** (ED_{50} = 30-100 mg/kg) and (R)-**89** (ED_{50} = 46 mg/kg) were evaluated in the MES seizure model in mice, we observed appreciable seizure protection for both compounds, suggesting that other factors, such as metabolism or drug biodistribution in the CNS, were responsible for the inactivity of these (R)-**55** AB agents in animals.

Finally, we asked whether the methyl-d_3 analog of (R)-**1**, (R)-**1**-d_3, would display enhanced anticonvulsant activity over (R)-**1**. Recently, pharmaceutical companies have prepared deuterated versions ("heavy drugs") of marketed medicinal agents.[404] The greater strength of C–D bonds compared with C–H bonds

is predicted to confer increased metabolic stability for the deuterated version of the drug.[404] Significantly, the major metabolite for (*R*)-**1** in humans is the desmethyl analog (*R*)-**53**.[405] The metabolic conversion of (*R*)-**1** to (*R*)-**53** has not been attributed to a specific metabolic enzyme.[406] Nonetheless, (*R*)-**53** accounts for ~30% of the (*R*)-**1** excreted in human urine,[203] and it exhibits little anticonvulsant activity (ED_{50} = 100–300 mg/kg).[234] Accordingly, we reasoned that replacing the 3-oxy methyl group in (*R*)-**1** with the perdeuterated methyl unit to give (*R*)-**1**-d_3 might reduce metabolic processes and thus lead to increased bioavailability of the AED. We prepared (*R*)-**1**-d_3 using a method we reported for (*R*)-**1**[48] and substituting CD_3I for CH_3I. The deuterated derivative (*R*)-**1**-d_3 (ED_{50} = 5.2 mg/kg) showed an anticonvulsant activity in the rat (po) comparable with (*R*)-**1** (ED_{50} = 3.9 mg/kg), without exceeding it, and exhibited greater neurological toxicity ((*R*)-**1**-d_3, TD_{50} = ~200 mg/kg; (*R*)-**1**, TD_{50} = >500 mg/kg).

(*R*)-**1**-d_3
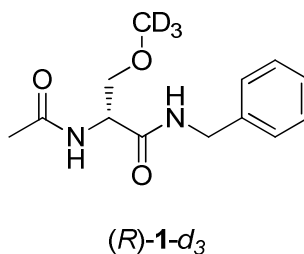
2.2.4. Experimental Section

General Methods

Melting points were determined in open capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra (IR) were run on an ATI Mattson Genesis FT-IR spectrometer. Absorption values are expressed in wavenumbers (cm^{-1}). Optical rotations were obtained on a Jasco P-1030 polarimeter at the sodium D line (589 nm) using a 1 dm path length cell and are given in units of deg cm^3 g^{-1} dm^{-1}. NMR spectra were obtained at 300 MHz (^1H) and 75 MHz (^{13}C) using TMS as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) from tetramethylsilane. Low-resolution mass spectra were obtained with a BioToF-II-Bruker Daltonics spectrometer by Drs Matt Crowe and S. Habibi at the University of North Carolina Department of Chemistry. The high-resolution mass spectrum was performed on a Bruker Apex-Q 12 Telsa FTICR spectrometer by Drs Matt Crowe and S. Habibi. Microanalyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Reactions were monitored by analytical thin-layer chromatography (TLC) plates (Aldrich, Cat # Z12272-6) and analyzed with 254 nm light. The reaction mixtures were purified by flash column chromatography using silica gel (Dynamic Adsorbents Inc., Cat #02826-25). All chemicals and solvents were reagent grade and used as obtained from commercial sources without further purification. THF was distilled from blue sodium benzophenone ketyl. Yields reported are for purified products and were not optimized. All compounds were checked by TLC, ^1H and ^{13}C

NMR, MS, and elemental analyses. The TLC, NMR and the analytical data

confirmed the purity of the products was ≥95%.

2.2.4.1. Synthetic procedures

2.2.4.1.1. General procedure for the aziridine ring-opening with alcohols. Method A

To a cooled CH_2Cl_2 solution (ice bath) of **68a,b** or **78** ([C] ~ 0.5–1 M) and the

appropriate alcohol (1–5 equiv) was added $BF_3 \bullet Et_2O$ (1 equiv) dropwise while

stirring. After addition, the mixture was warmed to room temperature and stirred (30

min), and then an equal volume of saturated aqueous $NaHCO_3$ was added. The

reaction was vigorously stirred (15 min) and the organic layer separated. The

aqueous layer was extracted with CH_2Cl_2 until no additional product could be

detected (TLC analysis). All the organic layers were then combined, dried (Na_2SO_4),

and concentrated in vacuo to yield a residue that was either or used directly for the

next step, purified by flash column chromatography, or recrystallized from EtOAc

and hexanes.

2.2.4.1.2. General procedure for the ester hydrolysis of *N*-acetylserine esters with LiOH. Method B

To a THF solution of serine methyl ester (2 volumes, [C] ~0.1 M) was added

an aqueous solution (1 volume) of LiOH (1 equiv). The homogeneous solution was

stirred at room temperature (60 min), after which time Et_2O (2 volumes) was added.

The aqueous layer was recovered, and washed with Et_2O. The remaining aqueous

layer was acidified (pH ~1) by the dropwise addition of aqueous concentrated HCl, saturated with NaCl, and extracted with EtOAc until no further product was detected (TLC analysis). The combined organic layers were combined, dried (Na_2SO_4), and evaporated to an oily residue that was used directly for the next step or recrystallized from EtOAc and hexanes to provide an analytical sample.

2.2.4.1.3. General procedure for the MAC reaction. Method C

To a cooled THF solution (–78 °C, dry ice/acetone bath) of acid ([C] ~0.1 M) were successively added NMM (1.0 equiv), stirred for 2 min, IBCF (1.0 equiv), stirred for 15 min, and then the desired benzylamine (1.0 equiv). Upon addition the reaction mixture was allowed to warm to room temperature and further stirred (2 h). The salts were filtered and rinsed with THF, and the filtrate was concentrated in vacuo. The residue obtained was purified by flash chromatography, followed by recrystallization from EtOAc and hexanes when necessary.

2.2.4.1.4. General procedure for the DMTMM coupling reaction. Method D

To a THF solution of acid ([C] ~0.1 M) at room temperature was added the desired benzylamine (1.2 equiv). The solution was stirred (5–10 min) until the benzylammonium carboxylate precipitated. With stirring, DMTMM (1.2 equiv) was added all at once, and the resulting suspension was stirred at room temperature (3–12 h). In those cases where a salt did not precipitate, DMTMM was added after 15 min to the solution. The salts were removed by filtration and washed with THF,

and the solvent was removed in vacuo. The residue obtained was purified by flash column chromatography to afford the benzylamide, and then recrystallized from EtOAc and hexanes.

2.2.4.1.5. General procedure for the Swern oxidation reaction. Method E

To a cooled solution (dry ice/acetone bath) of oxalyl chloride (1.3 equiv) in anhydrous CH_2Cl_2 ([C] ~0.5 M) was added dropwise a CH_2Cl_2 solution of DMSO (2.6 equiv) ([C] ~0.5 M). After stirring at -78 °C (15 min) a solution of alcohol (1.0 equiv) in CH_2Cl_2 or a mixture of CH_2Cl_2 and DMSO ([C] ~0.5 M) was added dropwise at -78 °C and the reaction was stirred at -78 °C (1 h). DIEA (5 equiv) was then added dropwise, stirred (20 min), warmed to room temperature and then stirred (30 min). Minimal amounts of aqueous 10% citric acid solution were added to the solution until the pH of the aqueous layer remained acidic (pH ~3). The CH_2Cl_2 layer was separated and the aqueous layer extracted with CH_2Cl_2 (~6 to 7 volumes) until no more UV visible product was detected (TLC analysis) in the organic layer. The combined organic layers were washed with brine (1 volume), dried (Na_2SO_4), and evaporated to give an oily residue. Recrystallization from EtOAc and hexanes afforded the pure aldehyde.

2.2.4.1.6. General procedure for the Corey-Chaykovsky epoxidation. Method F

Trimethylsulfoxonium iodide (1.2 equiv.) and NaH (60% dispersion in mineral oil, 1.2 equiv.) were suspended in DMSO or DMF ([C] ~0.1 M) and stirred under N_2

(1 h) until a clear solution was obtained (**solution A**). The aromatic aldehyde (1.0 equiv) was added at once as a solid and the reaction was stirred at room temperature (12 h). Brine was added (10 volumes) and the aqueous layer was extracted with CH_2Cl_2 until no additional product was detected in the aqueous layer. The combined organic layers were dried (Na_2SO_4), evaporated, and the residue was purified by flash chromatography and recrystallized from EtOAc and hexanes.

2.2.4.1.7. General procedure for the Williamson ether synthesis. Method G

A CH_3CN solution of alcohol ([C] ~0.05–0.5 M), Ag_2O (5 equiv) and MeI (10 equiv) was stirred at room temperature (2–3 d). The reaction was filtered through Celite®, and the solvent was evaporated in vacuo. The residue was purified by silica gel chromatography or used directly for the next step.

2.2.4.1.8. General procedure for the Pd-catalyzed hydrogenation reaction. Method H

A MeOH suspension of the starting material ([C] ~0.01–0.2 M) and 10% Pd/C (10% w/w) was vigorously stirred under an atmosphere of H_2 (balloon) at room temperature (16 h). The mixture was filtered through a bed of Celite®. The bed was washed with MeOH and CH_2Cl_2, the washings were collected and evaporated in vacuo. The amine was used without further purification.

2.2.4.1.9. General procedure for the aromatic aldehyde deprotection. Method I

To a THF solution of the protected aldehyde ([C] ~0.1–0.5 M, 2 volumes) was added 1 volume of aqueous 0.1 M HCl. The reaction was stirred at room temperature (15 h) and the THF was removed in vacuo. The remaining aqueous layer was extracted with CH_2Cl_2 until no compound was detectable (TLC analysis). The combined organic layers were washed with brine (1 volume), dried (Na_2SO_4) and evaporated to yield the aldehyde that was purified either by silica gel chromatography or recrystallized from EtOAc and hexanes.

2.2.4.1.10. General Procedure for the *m*-CPBA epoxidation. Method J

A CH_2Cl_2 ([C] ~0.1 M) solution of *m*-CPBA (1.0–1.2 equiv) was stirred in the presence of anhydrous Na_2SO_4 at room temperature (5 min). The starting material was then added at once as a solid (1.0 equiv) and the reaction was allowed to proceed at room temperature (15 h). The Na_2SO_4 was filtered, the solvent removed under vacuum, and the residue purified by silica gel chromatography.

2.2.4.2. Synthesis of *N*-acetylaziridine carboxylate esters and carboxamides

Methyl (2*R*)-1-Tritylaziridine-2-carboxylate ((*R*)-65).[383] D-Serine methyl ester hydrochloride (25.0 g, 161 mmol) was suspended in CH_2Cl_2 (200 mL) and cooled to 0 °C. While stirring, Et$_3$N (44.8 mL, 322 mmol) was added, followed by the portionwise addition of trityl chloride (44.8 g, 161 mmol). The reaction was stirred at

0 °C (24 h), filtered and the filtrate was successively washed with a 10% aqueous citric acid solution (200 mL) and brine (200 mL), dried (Na$_2$SO$_4$), and evaporated to yield 55.1 g of a white foam. The foam was dissolved in CH$_2$Cl$_2$ (400 mL), cooled (0 °C) and Et$_3$N (32 mL, 207 mmol) was added followed by the dropwise addition of mesyl chloride (13.2 mL, 167.6 mmol). The reaction was maintained at 0 °C (40 min), filtered, and the filtrate was washed with a 10% aqueous citric acid solution (400 mL), and brine (400 mL), dried (Na$_2$SO$_4$), and evaporated to give 65.0 g of a pale yellow foam. The foam was dissolved in 1,2-dimethoxyethane (110 mL) and Et$_3$N (44.8 mL, 322 mmol) was added. The solution was heated to reflux (24 h), cooled to room temperature and washed with 10% aqueous citric acid (2 x 250 mL), saturated aqueous sodium bicarbonate (2 x 250 mL), and brine (2 x 250 mL), dried (Na$_2$SO$_4$), and evaporated. The crude residue was recrystallized from EtOH to yield 32.2 g (58%) of (R)-**65** as a white solid. Pure (R)-**65** could also be obtained by SiO$_2$ chromatography (1/3 hexanes/CHCl$_3$): mp 124–126 °C (lit.[383] mp 123–125°C); [α]$^{25}_D$ +91.2°(c 1.0, EtOAc); R_f = 0.52 (1/3 hexanes/CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 1.41 (dd, J = 1.8, 7.0 Hz, NC**H**H'CH), 1.89 (dd, J = 3.0, 7.0 Hz, C**H**C(O)OCH$_3$), 2.55 (dd, J = 1.8, 3.0 Hz, NCH**H**'CH), 3.75 (s, C(O)OC**H$_3$**); ^{13}C NMR (CDCl$_3$) δ 28.7 (N**C**H$_2$CH), 31.8 (**C**HC(O)OCH$_3$), 52.2 (C(O)O**C**H$_3$), 74.5 (N**C**Ph$_3$), 127.1, 127.8, 129.4, 143.7 (3 **C$_6$**H$_5$), 172.0 (**C**(O)OCH$_3$).

Methyl (2S)-1-Tritylaziridine-2-carboxylate ((S)-65).[383] Following the preceding procedure, L-serine methyl ester hydrochloride (25.0 g, 161 mmol), Et$_3$N (44.8 mL, 322 mmol), trityl chloride (44.8 g, 161 mmol) in CH$_2$Cl$_2$ (200 mL), followed by Et$_3$N

(32 mL, 207 mmol), mesyl chloride (13.2 mL, 167.6 mmol) in CH_2Cl_2 (400 mL), and

Et_3N (44.8 mL, 322 mmol) in 1,2-dimethoxyethane (110 mL) gave 30.8 g (56%) of

(*S*)-**65** as a white solid upon work-up and recrystallization from EtOH: mp 124–126

°C (lit. [383] mp = 123–125 °C); $[\alpha]^{25}_D$ -90.9° (*c* 1.0, EtAc); R_f = 0.52 (1/3

hexanes/$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.41 (dd, *J* = 1.8, 7.0 Hz, NC**H**H'CH), 1.89 (dd,

J = 3.0, 7.0 Hz, C**H**C(O)OCH$_3$), 2.55 (dd, *J* = 1.8, 3.0 Hz, NCH**H'**CH), 3.75 (s,

C(O)OC**H$_3$**); ^{13}C NMR ($CDCl_3$) δ 28.7 (N**C**H$_2$CH), 31.8 (**C**HC(O)OCH$_3$), 52.2

(C(O)O**C**H$_3$), 74.5 (N**C**Ph$_3$), 127.1, 127.8, 129.4, 143.7 (3 **C$_6$**H$_5$), 172.0 (**C**(O)OCH$_3$).

(*R*)-Methyl 1-Acetylaziridine-2-carboxylate ((*R*)-68).[407] Compound (*R*)-**65** (26.0 g,

75.8 mmol) was dissolved in $CHCl_3$:MeOH (1:1) (200 mL) and cooled (ice bath).

While stirring, TFA (87 mL) was added dropwise and the reaction was stirred at 0 °C

(2 h). Solvents were removed in vacuo and the solid residue was dissolved in

CH_2Cl_2 (150 mL), cooled (0 °C) and then Et_3N (44.2 mL, 303 mmol) followed by

acetyl chloride (5.88 mL, 83.4 mmol) were added dropwise. The reaction was stirred

(2 h), filtered, and the filtrate was successively washed with 10% aqueous citric acid

(150 mL), saturated aqueous sodium bicarbonate (150 mL), and brine (150 mL). The

organic layer was dried (Na_2SO_4) and evaporated. The solid residue was purified

using silica gel chromatography (1/4 to 1/1 EtOAc/hexanes) to give 3.60 g (33%) of

(*R*)-**68** as a colorless residue: $[\alpha]^{25}_D$ +81.7° (*c* 1.3, $CHCl_3$); R_f = 0.41 (1/2

EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 2.15 (s, C**H$_3$**C(O)), 2.49 (dd, *J* = 1.8, 7.0 Hz,

NC**H**H'CH), 2.56 (dd, *J* = 3.0, 7.0 Hz, C**H**C(O)OCH$_3$), 3.14 (dd, *J* = 1.8, 3.0 Hz,

NCH**H'**CH), 3.78 (s, C(O)OC**H$_3$**); ^{13}C NMR ($CDCl_3$) δ 23.8 (**C**H$_3$C(O)), 31.0

(NCH$_2$CH), 34.5 (**C**HC(O)OCH$_3$), 53.0 (C(O)O**C**H$_3$), 169.0 (CH$_3$**C**(O)), 180.6 (**C**(O)OCH$_3$).

(*S*)-Methyl 1-Acetylaziridine-2-carboxylate ((*S*)-68).[407] Using the preceding procedure, (*S*)-**65** (17.4 g, 50.7 mmol) in CHCl$_3$:MeOH (1:1) (150 mL) was reacted with TFA (58 mL) at 0 °C (2 h). The solvents were rem oved and the residue was dissolved in CH$_2$Cl$_2$ (100 mL) and reacted with Et$_3$N (28.3 mL, 202.8 mmol) and acetyl chloride (3.96 mL, 55.8 mmol). Purification by SiO$_2$ flash chromatography (1/4 to 1/1 EtOAc/hexanes) afforded 2.50 g (34%) of (*S*)-**68** as a colorless residue: $[\alpha]^{25}_D$ -81.7° (*c* 1.3, CHCl$_3$); R_f = 0.41 (1/2 EtOAc/hexanes); ^{1}H NMR (CDCl$_3$) δ 2.15 (s, **CH$_3$**C(O)), 2.49 (dd, *J* = 1.8, 7.0 Hz, NC**H**H'CH), 2.56 (dd, *J* = 3.0, 7.0 Hz, **C**HC(O)OCH$_3$), 3.14 (dd, *J* = 1.8, 3.0 Hz, NCH**H'**CH), 3.78 (s, C(O)O**CH$_3$**); ^{13}C NMR (CDCl$_3$) δ 23.9 (**C**H$_3$C(O)), 31.1 (N**C**H$_2$CH), 34.6 (**C**HC(O)OCH$_3$), 53.1 (C(O)O**C**H$_3$), 169.1 (CH$_3$**C**(O)), 180.7 (**C**(O)OCH$_3$).

Serine Methyl Ester Free Amine (67).[381] L or D-Serine methyl ester hydrochloride (**62**, 1 equiv) was suspended in CH$_2$Cl$_2$ ([C] ~1 M) and Et$_3$N (1.5 equiv) was added. After stirring at room temperature (1 h), the salts were filtered and briefly rinsed with EtOAc, and an equal volume of EtOAc was then added. The mixture was stirred at 0 °C (10 min), the solids filtered, and the solvent redu ced in vacuo to one third in volume. The remaining reaction mixture was stirred at 0 °C (10 min), filtered, and the solvent removed. CH$_3$CN was then used to azeotropically remove excess Et$_3$N until

it could not be detected. Free amine **67** (80–90% yield) was immediately used in the next step.

(*R*)-Methyl *N*-Acetylaziridine-carboxylate ((*R*)-68a) and (*R*)-Ethyl *N*-Acetylaziridine-carboxylate ((*R*)-68b). To a solution of (*R*)-**67** (41.3 g, 347 mmol) in CH_3CN (500 mL) was added **DTPP** (86% by wt, 142.0 g, 346 mmol). The solution was stirred at room temperature (24 h). The solvent was removed in vacuo, the residue dissolved in minimal amount of CH_2Cl_2 (250 mL), and extracted with aqueous 0.1 M H_2SO_4 until the pH of the aqueous phase remained acidic (3 x 150 mL). The combined aqueous layers were washed with EtOAc (3 x 200 mL), basified (pH ~10) with solid Na_2CO_3, saturated with solid NaCl until the solution became cloudy, and extracted with EtOAc (6 x 200 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to give a crude yellow liquid. Bulb-to-bulb distillation of the liquid at 80 ℃ under vacuum (6 mm Hg) yielde d an approximately 9:1 molar mixture of (*R*)-**66a** and (*R*)-**66b** as a colorless liquid (24.60 g, 69%). The mixture was directly dissolved in CH_2Cl_2 (500 mL) and Et_3N (33.4 mL, 239 mmol) and DMAP (1.46 g, 12 mmol) were successively added. While stirring at room temperature (water bath), Ac_2O (22.6 mL, 239 mmol) was added dropwise (15 min) and the reaction was allowed to proceed at room temperature (45 min). The solution was successively washed with 10% aqueous citric acid (500 mL) and brine (500 mL), dried (Na_2SO_4), and the solvents were removed in vacuo to yield an approximately 9:1 molar mixture of a colorless residue (32.80 g, 94%) that did not require further purification: R_f = 0.38 ((*R*)-**68a**), 0.39 ((*R*)-**68b**) (2:1 hexanes/EtOAc). Spectral data

for (*R*)-**68a** (~90% mol. based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 2.16 (s, **CH$_3$**C(O)), 2.49 (dd, *J* = 1.8, 7.0 Hz, NC**H**H'CH), 2.58 (dd, *J* = 1.8, 3.0 Hz, NCH**H'**CH), 3.15 (dd, *J* = 3.0, 7.0 Hz, **CH**C(O)OCH$_3$), 3.80 (s, C(O)OC**H$_3$**); ^{13}C NMR (CDCl$_3$) δ 23.8 (**C**H$_3$C(O)), 31.0 (N**C**H$_2$CH), 34.5 (**C**HC(O)OCH$_3$), 52.9 (C(O)O**C**H$_3$), 168.9 (CH$_3$**C**(O)), 180.6 (**C**(O)OCH$_3$); (*R*)-**68a** was not detected by HRMS. Spectral data for (*R*)-**68b** (~10% mol. on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.31 (t, *J* = 6.9 Hz, C(O)OCH$_2$**CH$_3$**), 4.24 (q, *J* = 6.9 Hz, C(O)OC**H**H'CH$_3$), 4.25 (q, *J* = 6.9 Hz, C(O)OCH**H'**CH$_3$), the remaining signals were not detected and are believed to overlap with the ^{1}H signals for (*R*)-**68a**; ^{13}C NMR (CDCl$_3$) no ^{13}C signals were detected for (*R*)-**68b**; M_r (+ESI) 180.0631 [M+Na]$^+$ (calcd for C$_7$H$_{11}$NO$_3$Na$^+$ 180.0637).

(*R*)-*N*-Benzyl 2-*N*-(Benzyloxycarbonyl)amino-3-hydroxypropionamide ((*R*)-57).[235] Using Method C, Cbz-D-serine ((*R*)-**56**) (5.10 g, 21.3 mmol), NMM (2.3 mL, 21.3 mmol), IBCF (2.8 mL, 21.3 mmol), and benzylamine (2.3 mL, 21.3 mmol) gave (*R*)-**57** (5.33 g, 76%) as a white solid: mp 147–149 ℃ (lit.[235] mp 147–149 ℃); [α]$^{25}_D$ +4.5° (*c* 1.0, MeOH) (lit.[235] [α]$^{25}_D$ +4.6° (*c* 2.0, MeOH)); ^{1}H NMR (DMSO-*d$_6$*) δ 3.58 (d, *J* = 5.7 Hz, C**H$_2$**), 4.04–4.11 (m, C**H**CH$_2$), 4.27 (d, *J* = 6.0 Hz, C**H$_2$**N), 4.90–4.95 (m, CH$_2$O**H**), 5.02 (s, C**H$_2$**O), 7.20–7.38 (m, 10 Ar**H**, OC(O)N**H**), 8.32–8.37 (m, C(O)N**H**CH$_2$Ph).

(*R*)-*N*-Benzyl 2-Amino-3-hydroxypropionamide ((*R*)-76).[235] Using Method H, compound (*R*)-**57** (3.00 g, 9.14 mmol) and 10% Pd/C (300 mg) in MeOH (50 mL)

gave (*R*)-**76** (1.75 g, 98%) as a white solid: mp 89–91 °C (lit. [235] mp 88–90 °C); $[\alpha]^{25}_D$ -3.0° (*c* 1.0; MeOH) (lit.[235] $[\alpha]^{25}_D$ -3.2° (*c* 0.9; MeOH)); R_f = 0.15 (1/9 MeOH/CHCl$_3$); ^{1}H NMR (DMSO-*d*$_6$) δ 3.31–3.36 (m, **CH**CH$_2$OH), 3.40–3.60 (m, CHC**H$_2$**OH), 4.29 (d, *J* = 6.0 Hz, C(O)NHC**H$_2$**Ph), 4.78–4.98 (br s, CH$_2$O**H**), 7.20–7.32 (m, C$_6$**H$_5$**), 8.34–8.48 (br t, C(O)N**H**CH$_2$Ph); ^{13}C NMR (CDCl$_3$) δ 41.9 (NH**C**H$_2$Ph), 56.6 (**C**HCH$_2$O), 63.7 (CH**C**H$_2$OH), 126.7, 127.1, 128.2, 139.4 (**C$_6$**H$_5$), 172.4 (**C**(O)NHCH$_2$).

(*R*)-*N*-Benzyl 1-Acetylaziridine-2-carboxamide ((*R*)-78). Compound (*R*)-**76** (1.75 g, 9.1 mmol) was suspended in CH$_3$CN (20 mL) and while stirring **DTPP** (90% by wt, 3.60 g, 10.01 mmol) was added all at once. The solution became clear within minutes and was then stirred at room temperature (3 h). The solvent was removed in vacuo, and the residue was partitioned between aqueous 0.1 M H$_2$SO$_4$ (20 mL) and toluene (20 mL). The toluene layer was extracted with aqueous 0.1 M H$_2$SO$_4$ (20 mL). The combined aqueous layers were washed with toluene and basified (pH ~10) by addition of solid K$_2$CO$_3$, saturated with NaCl, and then extracted with EtOAc (3 x 40 mL). The combined EtOAc layers were dried (Na$_2$SO$_4$), and concentrated to yield 1.47 g (92%) of aziridine (*R*)-**77** as a crude yellow oil. Crude (*R*)-**77** was dissolved in CH$_2$Cl$_2$ (80 mL), cooled to 0 °C, and Et$_3$N (1.71 mL, 10.28 mmol) added. While stirring, AcCl (581 μL, 8.18 mmol) in CH$_2$Cl$_2$ (6 mL) was added dropwise. The reaction was stirred at 0 °C (1 h), and successively washe d with aqueous 10% citric acid (100 mL) and brine (100 mL). The organic layer was dried (Na$_2$SO$_4$) and evaporated. The residue was purified using flash chromatography (CH$_2$Cl$_2$/EtOAc 1:1) and further recrystallized from EtOAc and hexanes to yield 1.12 g of (*R*)-**78**

(62% for two steps): mp 82–84°C (lit. [384] mp 82–83 °C); $[\alpha]^{25}_D$ -106.6° (c 1.6; CHCl₃);

R_f = 0.44 (1/1 CH₂Cl₂/EtOAc); IR (nujol) 3230, 1660, 1558, 1459, 1390, 1154, 1092

cm⁻¹; ¹H NMR (CDCl₃) δ 2.17 (s, **CH₃**C(O)N), 2.37 (dd, J = 0.9, 3.0 Hz, NC**H**H'CH),

2.56 (dd, J = 0.9, 6.3 Hz, NCH**H'**CH), 3.08 (dd, J = 3.0, 6.3 Hz, NCHH'C**H**) 4.38–

4.50 (m, C(O)NHC**H₂**Ph), 6.46–6.62 (m, C(O)N**H**CH₂Ph), 7.20–7.40 (m, C₆**H₅**); ¹³C

NMR (CDCl₃) δ 23.7 (**C**H₃C(O)), 31.2 (N**C**H₂CH), 36.4 (NCH₂**C**H), 43.4 (NH**C**H₂Ph),

127.7, 127.8, 128.9, 137.8 (**C₆**H₅), 167.3 (**C**(O)NHCH₂), 181.5 (CH₃**C**(O)N); M_r

(+ESI) 219.1129 [M+H]⁺ (calcd for C₁₂H₁₄N₂O₂H⁺ 219.1134). Anal. Calcd for

C₁₂H₁₄N₂O₂: C, 66.04; H, 6.47; N, 12.84. Found: C, 66.06; H, 6.43; N, 12.73.

Alternate Procedure for (*R*)-*N*-Benzyl 1-Acetylaziridine-2-carboxamide ((*R*)-78).

PPh₃ (100 g, 377 mmol) was dissolved in CH₂Cl₂ (400 mL), cooled at -78 °C (dry

ice/acetone) under N₂ and Br₂ (19.3 mL, 380 mmol) was added quickly with a

syringe. After stirring at -78 °C (10 min), a solution of CF₃CH₂OH (54.2 mL, 754

mmol) and Et₃N (105 mL, 754 mmol) in Et₂O (300 mL) was added dropwise (15

min). Upon addition, the reaction was allowed to stir and slowly warm up to room

temperature (1 h), then quickly filtered using oven-dried glassware, and the solvents

were removed in vacuo at room temperature to give **DTPP-F₆** (138 g, 300 mmol,

79%) as a pale beige solid. **DTPP-F₆** was directly added to a suspension of (*R*)-**76**

(36.6 g, 189 mmol) in CH₃CN (500 mL) and the reaction was stirred at room

temperature (15 h). The solvent was removed under vacuum and the solid residue

was partitioned between EtOAc (500 mL) and aqueous 0.5 M H₂SO₄ (50 mL). The

aqueous layer was recovered and washed with EtOAc (3 x 200 mL), basified using

solid Na_2CO_3 (pH ~10), saturated with NaCl, and extracted with CH_2Cl_2 (5 x 200 mL). The EtOAc and CH_2Cl_2 layers were separately washed with brine (200 mL), combined, dried (Na_2SO_4), and concentrated. The residue was purified using flash chromatography (5/95 MeOH CH_2Cl_2) to give 9.70 g of a crude yellow oil (R_f = 0.43, 5/95 MeOH/CH_2Cl_2) that was dissolved in CH_2Cl_2 (500 mL). While stirring, Et_3N (7.7 mL, 55.0 mmol), DMAP (300 mg, 2.5 mmol) and Ac_2O (5.2 mL, 55.0 mmol) were successively added and the reaction was stirred at room temperature (1 h). The reaction was then washed with aqueous 0.1 M H_2SO_4 (300 mL), brine (200 mL), dried (Na_2SO_4), and the solvents were removed under vacuum. The residue was purified using flash chromatography (EtOAc/CH_2Cl_2 1/1 to 4/1) to yield (*R*)-**78** as a white solid (5.70 g, 15% overall yield for 2 steps): mp 82–84 ℃ (lit. [384] mp 82–83 ℃); $[\alpha]^{25}_D$ -106.0° (*c* 1.3, $CHCl_3$); R_f = 0.44 (1/1 EtOAc/CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 2.18 (s, **CH₃**C(O)), 2.37 (d, *J* = 3.3 Hz, C**H**H'(N)CH), 2.58 (d, *J* = 7.6 Hz, CH**H'**(N)CH), 3.09 (dd, *J* = 3.3, 7.6 Hz, CH_2(N)C**H**), 4.38–4.54 (m, NHC**H₂**C_6H_5), 6.42–6.54 (m, N**H**$CH_2C_6H_5$), 7.20–7.40 (m, CH_2C_6**H₅**); ^{13}C NMR ($CDCl_3$) δ 23.6 (**CH₃**C(O)), 31.4 (**C**H_2(N)CH), 36.4 (CH_2(N)**C**H), 43.4 (NH**C**H_2Ph), 127.8, 128.9, 137.6 (**C₆**H_5), 167.2 (**C**(O)NHCH_2), 181.3 (CH_3**C**(O)N), the remaining carbon signal was not detected and is believed to overlap with nearby peaks.

2.2.4.3. Synthesis of *O*-Substituted *N*-Acetylserine Esters

68a, X = Me
b, X = Et

69a, X = Me
b, X = Et
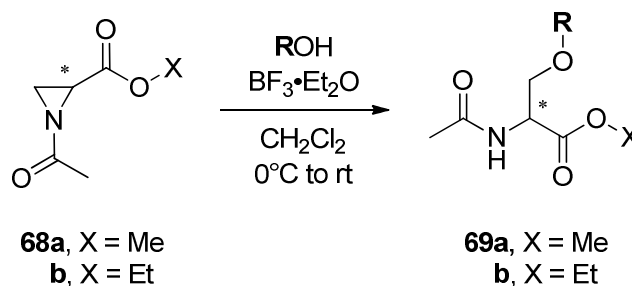
R =	Compound number (**a**, X = Me, **b**, X = Et)
Me	**101**
Et	**102**
i-Pr	**103**
t-Bu	**104**
C_6H_{11}	**105**
Ph	**106**
$CH_2C_6H_{11}$	**107**
$CH_2CH_2CH=CH_2$	**108**
CH_2CH_2◁	**109**
CH_2CH_2OBn	**110**
$CH_2CH_2CH_2OBn$	**111**
$CH_2CH_2N_3$	**112**
$CH_2CH_2OCH_3$	**113**
$(CH_2CH_2O)_2CH_3$	**114**

(*R*)-Methyl 2-Acetamido-3-methoxypropionate ((*R*)-101a) and (*R*)-Ethyl 2-Acetamido-3-methoxypropionate ((*R*)-101b). Using Method A, a ~9:1 mixture of (*R*)-**68a** and (*R*)-**68b** (5.46 g, 40.6 mmol) and $BF_3 \cdot Et_2O$ (5.1 mL, 40.6 mmol) in MeOH (50 mL) gave 3.95 g (56%) of (*R*)-**101a** and (*R*)-**101b** as a pale yellow residue that turned to a white solid upon concentration under high vacuum: R_f = 0.40 ((*R*)-**101a**), 0.42 ((*R*)-**101b**) (5/95 hexanes/EtOAc); IR (CH_2Cl_2 film) 3300, 3062,

2996, 2940, 1743, 1656, 1547, 1444, 1380, 1214, 1119 cm^{-1}. Spectral data for (*R*)-**101a** (approximately 90 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 2.06 (s, C**H$_3$**C(O)NH), 3.34 (s, OC**H$_3$**), 3.61 (dd, *J* = 4.0, 9.3 Hz, CHC**H**H'OCH$_3$), 3.77 (s, C(O)OC**H$_3$**), 3.81 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_3$), 4.75 (app dt, *J* = 4.0, 7.8 Hz, C**H**CH$_2$OCH$_3$), 6.74 (br d, *J* = 7.8 Hz, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 22.9 (**C**H$_3$C(O)), 52.4 (**C**HCH$_2$OCH$_3$ or C(O)O**C**H$_3$), 52.5 (C(O)O**C**H$_3$ or **C**HCH$_2$OCH$_3$), 59.1 (CH$_2$O**C**H$_3$), 72.2 (CH**C**H$_2$OCH$_3$), 170.1, 170.8 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$); *M*$_r$ (+ESI) 198.0740 [M+Na]$^+$ (calcd for C$_7$H$_{13}$NO$_4$Na$^+$ 198.0742).

Spectral data for (*R*)-**101b** (approximately 10 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.29 (t, *J* = 7.2 Hz, C(O)OCH$_2$C**H$_3$**) 1.99 (s, C**H$_3$**C(O)NH), 3.44 (s, OC**H$_3$**), 3.92 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_3$), 4.19–4.28 (m, C(O)OC**H$_2$**CH$_3$), 6.40–6.50 (br d, CH$_3$C(O)N**H**), the remaining signals were not detected and are believed to overlap with (*R*)-**101a** signals; ^{13}C NMR signals were not detected for (*R*)-**101b**; *M*$_r$ (+ESI) 212.0896 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_4$Na$^+$ 212.0899).

(*S*)-Methyl 2-Acetamido-3-(methoxy)propionate ((*S*)-101a) and (*S*)-Ethyl 2-Acetamido-3-(methoxy)propionate ((*S*)-101b). Using Method A, a ~9:1 mixture of (*S*)-**68a** and (*S*)-**68b** (1.50 g, 10 mmol) and BF$_3$•Et$_2$O (1.3 mL, 10 mmol) in MeOH (20 mL) gave upon work-up a mixture of (*S*)-**101a** and (*S*)-**101b** (1.43 g, 80%) as a pale yellow solid that was used without further purification: *R$_f$* = 0.40 ((*S*)-**101a**), 0.42 ((*S*)-**101b**) (5/95 hexanes/EtOAc); IR (neat) 3287, 3064, 2977, 2877, 1746, 1662, 1542, 1442, 1375, 1296, 1212 cm^{-1}. Spectral data for (*S*)-**101a** (~90% based on ^{1}H

NMR integrations): ^{1}H NMR (CDCl$_3$) δ 2.06 (s, **CH$_3$**C(O)), 3.35 (s, CH$_2$O**CH$_3$**), 3.61 (dd, J = 3.0, 9.0 Hz, C**H**H'OCH$_3$), 3.78 (s, C(O)O**CH$_3$**), 3.81 (dd, J = 3.0, 9.0 Hz, CH**H'**OCH$_3$), 4.73 (app. dt, J = 3.0, 7.2 Hz, C**H**CH$_2$O), 6.30–6.40 (br d, N**H**CHCH$_2$O); ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 52.8 (**C**HOCH$_2$ or C(O)O**C**H$_3$), 59.5 (CH$_2$O**C**H$_3$), 72.5 (CH**C**H$_2$OCH$_3$), 170.1, 171.0 (CH$_3$**C**(O), CH**C**(O)OCH$_3$), the remaining signal was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 198.0741 [M+Na]$^+$ (calcd for C$_7$H$_{13}$NO$_4$Na$^+$ 198.0742).

Spectral data for (S)-**101b** (~10% based on ^{1}H NMR integrations): ^{1}H NMR(CDCl$_3$) δ 1.28 (t, J = 7.2 Hz, OCH$_2$**CH$_3$**), 3.45 (s, CH$_2$O**CH$_3$**), 4.19–4.28 (m, O**CH$_2$**CH$_3$), the remaining signals were not detected and are believed to overlap with nearby signals or are too small to be detected; ^{13}C NMR signals were not detected for (S)-**14b**; M_r (+ESI) 212.0897 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_4$Na$^+$ 212.0899).

(R)-Methyl 2-Acetamido-3-ethoxypropionate ((R)-102a) and (R)-Ethyl 2-Acetamido-3-ethoxypropionate ((R)-102b). Using Method A, a ~9:1 mixture of (R)-**68a** and (R)-**68b** (1.88 g, 13.0 mmol) and BF$_3$•Et$_2$O (1.63 mL, 13.0 mmol) in EtOH (25 mL) gave 1.34 g (54%) of (R)-**102a** and (R)-**102b** as a pale yellow oil: R_f = 0.43 ((R)-**102a**), 0.45 ((R)-**102b**) (5/95 hexanes/EtOAc); IR (neat) 3287, 3064, 2977, 2876, 1745, 1661, 1542, 1442, 1375, 1212, 1119 cm^{-1}. Spectral data for (R)-**102a** (approximately 90 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.16 (t, J = 7.2 Hz, CH$_2$OCH$_2$**CH$_3$**), 2.06 (s, **CH$_3$**C(O)NH), 3.50 (q, J = 7.2 Hz, CH$_2$OC**H**H'CH$_3$), 3.51 (q, J = 7.2 Hz, CH$_2$OCH**H'**CH$_3$), 3.65 (dd, J = 4.0, 8.7 Hz, CHC**H**H'OCH$_2$CH$_3$), 3.76 (s, C(O)O**CH$_3$**), 3.84 (dd, J = 4.0, 8.7 Hz,

CHCH**H'**OCH$_2$CH$_3$), 4.75 (app dt, J = 4.0, 7.5 Hz, C**H**CH$_2$OCH$_2$CH$_3$), 6.54 (br d, J = 7.5 Hz, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 15.0 (OCH$_2$**C**H$_3$), 23.3 (**C**H$_3$C(O)), 52.7 (**C**HCH$_2$OCH$_3$ or C(O)O**C**H$_3$), 52.8 (C(O)O**C**H$_3$ or **C**HCH$_2$OCH$_2$H$_3$), 67.1 (CH**C**H$_2$OCH$_2$CH$_3$), 70.2 (CHCH$_2$O**C**H$_2$CH$_3$), 170.1, 171.1 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$); M_r (+ESI) 212.0897 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_4$Na$^+$ 212.0899).

Spectral data for (*R*)-**102b** (approximately 10 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.24 (t, J = 7.2 Hz, CH$_2$OCH$_2$C**H$_3$** or C(O)OCH$_2$C**H$_3$**), 1.29 (t, J = 7.2 Hz, C(O)OCH$_2$C**H$_3$** or CH$_2$OCH$_2$C**H$_3$**), 1.99 (s, C**H$_3$**C(O)NH), 3.96–4.04 (m, CH$_2$OC**H**H'CH$_3$), 4.19–4.30 (m, C(O)OC**H$_2$**CH$_3$), 6.10–6.22 (br d, J = 6.8 Hz, CH$_3$C(O)N**H**), the remaining signals were not detected and are believed to overlap with (*R*)-**102a** signals; ^{13}C NMR signals were not detected for (*R*)-**102b**; M_r (+ESI) 226.1054 [M+Na]$^+$ (calcd for C$_9$H$_{17}$NO$_4$Na$^+$ 226.1055).

(*R*)-Methyl 2-Acetamido-3-isopropoxypropionate ((*R*)-103a) and (*R*)-Ethyl 2-Acetamido-3-isopropoxypropionate ((*R*)-103b). Using Method A, a ~9:1 mixture of (*R*)-**68a** and (*R*)-**68b** (3.40 g, 23.5 mmol) and BF$_3$•Et$_2$O (2.95 mL, 23.5 mmol) in *i*-PrOH (30 mL) gave 2.98 g (62%) of (*R*)-**103a** and (*R*)-**103b** as a pale yellow oil: R_f = 0.46 ((*R*)-**103a**), 0.48 ((*R*)-**103b**) (5/95 hexanes/EtOAc); IR (neat) 3295, 3062, 2972, 2877, 1748, 1663, 1538, 1442, 1375, 1212, 1147 cm^{-1}. Spectral data for (*R*)-**103a** (approximately 90 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.10 (d, J = 6.0 Hz, CH$_2$OCHC**H$_3$**(C'H$_3$)), 1.12 (d, J = 6.0 Hz, CH$_2$OCHCH$_3$(C'**H$_3$**)), 2.06 (s, C**H$_3$**C(O)NH), 3.55 (hept, J = 6.0 Hz, CH$_2$OC**H**(CH$_3$)$_2$), 3.64 (dd, J = 3.7, 9.3 Hz, CHC**H**H'OCH(CH$_3$)$_2$), 3.76 (s, C(O)OC**H$_3$**), 3.84 (dd, J = 3.7, 9.3 Hz,

CHCH**H'**OCH(CH$_3$)$_2$), 4.72 (app dt, J = 3.7, 7.2 Hz, **C**HCH$_2$OCH(CH$_3$)$_2$), 6.41 (br d,

J = 7.2 Hz, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 21.5 (OCH**C**H$_3$(C'H$_3$)), 21.6

(OCHCH$_3$(**C'**H$_3$)), 22.9 (**C**H$_3$C(O)), 52.1 (**C**HCH$_2$OCH(CH$_3$)$_2$ or C(O)O**C**H$_3$), 52.6

(C(O)O**C**H$_3$ or **C**HCH$_2$OCH(CH$_3$)$_2$), 67.5 (CH**C**H$_2$OCH(CH$_3$)$_2$), 70.2

(CHCH$_2$O**C**H(CH$_3$)$_2$), 169.6, 170.4 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$); M_r (+ESI) 226.1054

[M+Na]$^+$ (calcd for C$_9$H$_{17}$NO$_4$Na$^+$ 226.1055).

Spectral data for (*R*)-**103b** (approximately 10% mol based on integrations): ^{1}H

NMR (CDCl$_3$) δ 1.16 (d, J = 6.0 Hz, CH$_2$OCHC**H$_3$**(C'H$_3$)), 1.21 (d, J = 6.0 Hz,

CH$_2$OCHCH$_3$(C'**H$_3$**)), 1.28 (t, J = 6.0 Hz, C(O)OCH$_2$C**H$_3$**), 1.99 (s, C**H$_3$**C(O)NH),

4.06–4.12 (m, CHC**H**H'OCH(CH$_3$)$_2$), 4.18-4.26 (m, C(O)OC**H$_2$**CH$_3$), 5.95-6.10 (br m,

CH$_3$C(O)N**H**), the remaining signals were not detected and are believed to overlap

with (*R*)-**103a** signals; ^{13}C NMR signals were not detected for (*R*)-**103b**; M_r (+ESI)

240.1211 [M+Na]$^+$ (calcd for C$_{10}$H$_{19}$NO$_4$Na$^+$ 240.1212).

(*R*)-Methyl 2-Acetamido-3-*tert*-butoxypropionate ((*R*)-104a) and (*R*)-Ethyl 2-Acetamido-3-*tert*-butoxypropionate ((*R*)-104b). Using Method A, a mixture of ~9:1

(*R*)-**68a** and (*R*)-**68b** (3.50 g, 24.2 mmol) and BF$_3$•Et$_2$O (3.05 mL, 24.2 mmol) in *t*-

BuOH (30 mL) gave 2.75 g (52%) of (*R*)-**104a** and (*R*)-**104b** as a pale yellow oil: R_f =

0.52 ((*R*)-**104a**), 0.54 ((*R*)-**104b**) (5/95 hexanes/EtOAc); IR (neat) 3298, 3062, 2974,

1749, 1663, 1537, 1370, 1204, 1098 cm^{-1}. Spectral data for (*R*)-**104a** (approximately

95 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.14 (s,

CH$_2$OC(C**H$_3$**)$_3$), 2.06 (s, C**H$_3$**C(O)NH), 3.56 (dd, J = 3.0, 9.0 Hz, CHC**H**H'OC(CH$_3$)$_3$),

3.76 (s, C(O)OC**H$_3$**), 3.81 (dd, J = 3.0, 9.0 Hz, CHCH**H'**OC(CH$_3$)$_3$), 4.72 (app dt, J =

3.0, 7.2 Hz, C**H**CH$_2$OC(CH$_3$)$_3$), 6.41 (br d, J = 7.2 Hz, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 22.9 (**C**H$_3$C(O)), 27.7 (OC(**C**H$_3$)$_3$), 52.1 (**C**HCH$_2$OC(CH$_3$)$_3$ or C(O)O**C**H$_3$), 53.3 (C(O)O**C**H$_3$ or **C**HCH$_2$OCH(CH$_3$)$_2$), 62.4 (CH**C**H$_2$OC(CH$_3$)$_3$), 73.8 (CHCH$_2$O**C**(CH$_3$)$_3$), 170.3, 171.5 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$); M_r (+ESI) 240.1211 [M+Na]$^+$ (calcd for C$_{10}$H$_{19}$NO$_4$Na$^+$ 240.1212).

Spectral data for (R)-**104b** (approximately 5% mol based on integrations): ^{1}H NMR (CDCl$_3$) δ 1.21 (s, CH$_2$OC(C**H$_3$**)$_3$), 1.99 (s, C**H$_3$**C(O)NH), 4.18–4.24 (m, C(O)OC**H$_2$**CH$_3$), 5.95–6.15 (m, CH$_3$C(O)N**H**), the remaining signals were not detected and are believed to overlap with (R)-**104a** signals or are too small to be detected; ^{13}C NMR signals were not detected for (R)-**104b**; M_r (+ESI) 254.1368 [M+Na]$^+$ (calcd for C$_{11}$H$_{21}$NO$_4$Na$^+$ 254.1368).

(R)-Methyl 2-Acetamido-3-(cyclohexyloxy)propionate ((R)-105a) and (R)-Ethyl 2-Acetamido-3-(cyclohexyloxy)propionate ((R)-105b). Using Method A, a ~1:1 mixture of (R)-**68a** and (R)-**68b** (2.70 g, 18.0 mmol), cyclohexanol (6.0 mL, 57 mmol) and BF$_3$•Et$_2$O (2.3 mL, 18.3 mmol) in CH$_2$Cl$_2$ (20 mL) gave a ~1:1 mixture of (R)-**105a** and (R)-**105b** (2.18 g, 48%) as a yellow oil after work-up and purification by flash chromatography (1/1 EtOAc/hexanes to EtOAc): R_f = 0.49 ((R)-**105a**), 0.51 ((R)-**105b**) (5/95 hexanes/EtOAc); IR (neat) 3308, 3061, 2926, 2861, 1747, 1662, 1537, 1447, 1372, 1206, 1106 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.18–1.35, 1.42–1.58, 1.62–1.86 (m, OCH(C**H$_2$**CH$_2$)$_2$C**H$_2$**, (R)-**105a,b**), 1.28 (t, J = 6.9 Hz, OCH$_2$C**H$_3$**, (R)-**105b**), 2.06 (s, C**H$_3$**C(O), (R)-**105a,b**), 3.18–3.26 (m, OC**H**(CH$_2$CH$_2$)$_2$CH$_2$, (R)-**105a,b**), 3.66 (dd, J = 3.0, 9.0 Hz, C**H**H'OCH(CH$_2$CH$_2$)$_2$CH$_2$, (R)-**105a,b**), 3.76 (s, C(O)OC**H$_3$**, (R)-

105a), 3.88 (dd, *J* = 3.0, 9.0 Hz, CH**H'**OCH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**105a,b**), 4.15–4.28 (m, C(O)OC**H$_2$**CH$_3$, (*R*)-**105b**), 4.66–4.78 (m, C**H**CH$_2$O, (*R*)-**105a,b**), 6.30–6.40 (br d, CH$_3$C(O)N**H**, (*R*)-**105a,b**); ^{13}C NMR (CDCl$_3$) δ 14.3 (C(O)OCH$_2$**C**H$_3$, (*R*)-**105b**), 23.4, 23.9, 24.0, 25.9 (**C**H$_3$C(O), OCH(CH$_2$**C**H$_2$)$_2$**C**H$_2$, (*R*)-**105a,b**), 31.9, 32.1 (OCH(**C**H$_2$CH$_2$)$_2$CH$_2$, (*R*)-**105a,b**), 52.7, 52.9, 53.0 (**C**HCH$_2$OCH$_2$, (*R*)-**105a,b**, C(O)O**C**H$_3$, (*R*)-**105a**), 61.7 (C(O)O**C**H$_2$CH$_3$, (*R*)-**105b**), 67.8, 67.9 (**C**H$_2$OCH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**105a,b**), 78.1 (O**C**H(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**105a,b**), 170.1, 170.7, 171.2 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$, (*R*)-**105a,b**), the remaining resonances were not detected and are believed to overlap with nearby signals; M_r (*R*)-**105a** (+ESI) 266.1368 [M+Na]$^+$ (calcd for C$_{12}$H$_{21}$NO$_4$Na$^+$ 266.1362), (*R*)-**105b** (+ESI) 280.1525 [M+Na]$^+$ (calcd for C$_{13}$H$_{23}$NO$_4$Na$^+$ 280.1524).

(*R*)-Methyl 2-Acetamido-3-phenoxypropionate ((*R*)-106a) and (*R*)-Ethyl 2-Acetamido-3-phenoxypropionate ((*R*)-106b). Using Method A, a ~3:7 mixture of (*R*)-**68a** and (*R*)-**68b** (2.00 g, 13.1 mmol), phenol (3.95 g, 42.0 mmol) and BF$_3$•Et$_2$O (1.6 mL, 13.1 mmol) in CH$_2$Cl$_2$ (20 mL) gave 1.4 g (43%) of (*R*)-**106a** and (*R*)-**106b** as a pale yellow residue: R_f = 0.50 ((*R*)-**106a**), 0.52 ((*R*)-**106b**) (5/95 hexanes/EtOAc); IR (neat) 3067, 2984, 1743, 1660, 1596, 1541, 1498, 1379, 1296, 1238, 1159 cm^{-1}; Spectral data for (*R*)-**106a** (approximately 30 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 2.06 (s, C**H$_3$**C(O)NH), 3.76 (s, C(O)OC**H$_3$**), 4.20–4.23 (m, CHC**H**H'OPh), 4.36–4.43 (m, CHCH**H'**OPh), 4.68–5.06 (m, C**H**CH$_2$OPh), 6.52 (br d, *J* = 7.2 Hz, CH$_3$C(O)N**H**), 6.84–6.90 (m, 2 ArH (*o*)), 6.95–7.00 (m, ArH (*p*)), 7.24–7.32 (m, 2 ArH (*m*)); ^{13}C NMR (CDCl$_3$) δ 23.1

($\mathbf{CH_3C(O)}$), 52.4 (**C**HCH$_2$OPh or C(O)OC**H$_3$**), 53.0 (C(O)OC**H$_3$** or **C**HCH$_2$OPh), 68.1 (CH**CH$_2$**OPh), 114.8, 121.7, 129.7, 158.4 (CH$_2$O**Ph**), 170.0, 170.1, 170.5 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$), additional peaks are believed to be part of (*R*)-**106b** but cannot be precisely attributed; M_r (+ESI) 268.0895 [M+Na]$^+$ (calcd for C$_{12}$H$_{15}$NO$_4$Na$^+$ 260.0899).

Spectral data for (*R*)-**106b** (approximately 70 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.25 (t, *J* = 7.2 Hz, C(O)OCH$_2$C**H$_3$**), 2.06 (s, C**H$_3$**C(O)NH), 4.20–4.23 (m, CHC**H**H'OPh), 4.24 (q, *J* = 7.2 Hz, C(O)OC**H$_2$**CH$_3$), 4.36–4.43 (m, CHCH**H**'OPh), 4.68–5.06 (m, C**H**CH$_2$OPh), 6.52 (br d, *J* = 7.2 Hz, CH$_3$C(O)N**H**), 6.84–6.90 (m, 2 ArH (*o*)), 6.95–7.00 (m, ArH (*p*)), 7.24–7.32 (m, 2 ArH (*m*)); ^{13}C NMR (CDCl$_3$) δ 14.3 (CH$_2$**C**H$_3$), 23.1 (**C**H$_3$C(O)), 52.5 (**C**HCH$_2$OPh), 68.2 (CH**CH$_2$**OPh), 114.8, 121.7, 129.7, 158.4 (CH$_2$O**Ph**), 170.0, 170.1, 170.5 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$), additional peaks are believed to be part of (*R*)-**106a** but cannot be precisely attributed; M_r (+ESI) 274.1052 [M+Na]$^+$ (calcd for C$_{13}$H$_{17}$NO$_4$Na$^+$ 274.1055).

(*R*)-Methyl 2-Acetamido-3-(cyclohexylmethoxy)propionate ((*R*)-107a) and (*R*)-Ethyl 2-Acetamido-3-(cyclohexylmethoxy)propionate ((*R*)-107b). Using Method A, a ~1:4 mixture of (*R*)-**68a** and (*R*)-**68b** (3.50 g, 22.7 mmol), cyclohexylmethanol (6.0 mL, 57 mmol) and BF$_3$•Et$_2$O (2.3 mL, 18.3 mmol) in CH$_2$Cl$_2$ (20 mL) gave a ~1:4 mixture of (*R*)-**107a** and (*R*)-**107b** (2.32 g, 38%) as a yellow oil after work-up and purification by flash chromatography (1/1 EtOAc/hexanes to EtOAc): R_f = 0.60 ((*R*)-**107a**), 0.62 ((*R*)-**107b**) (EtOAc); IR (neat) 3438, 3062, 2930, 2858, 1745, 1662,

1537, 1452, 1374, 1206, 1121 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 0.80–0.95, 1.05–1.38, 1.48–1.80 (m, CH$_2$**CH**(**CH$_2$CH$_2$**)$_2$**CH$_2$**, (*R*)-**107a,b**), 1.28 (t, *J* = 6.9 Hz, OCH$_2$**CH$_3$**, (*R*)-**107b**), 2.06 (s, **CH$_3$**C(O), (*R*)-**107a,b**), 3.12–3.30 (m, O**CH$_2$**CH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 3.66 (dd, *J* = 3.0, 9.6 Hz, **CH**H'OCH$_2$CH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 3.76 (s, C(O)O**CH$_3$**, (*R*)-**107a**), 3.88 (dd, *J* = 3.0, 9.0 Hz, CH**H'**OCH$_2$CH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 4.22 (q, *J* = 6.9 Hz, C(O)O**CH$_2$**CH$_3$, (*R*)-**107b**), 4.68–4.78 (m, **CH**CH$_2$O, (*R*)-**107a,b**), 6.28–6.40 (br d, CH$_3$C(O)N**H**, (*R*)-**107a,b**); ^{13}C NMR (CDCl$_3$) δ 14.4 (C(O)OCH$_2$**C**H$_3$, (*R*)-**107b**), 23.4, 26.0, 26.7 (**C**H$_3$C(O), OCH$_2$CH(**C**H$_2$**C**H$_2$)$_2$**C**H$_2$, (*R*)-**107a,b**), 30.0, 30.1 (OCH$_2$CH(**C**H$_2$**C**H$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 37.9 (OCH$_2$**C**H(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 52.7, 53.0 (C(O)O**C**H$_3$, (*R*)-**107a**, **C**HCH$_2$OCH$_2$, (*R*)-**107a,b**), 61.8 (C(O)O**C**H$_2$CH$_3$, (*R*)-**107b**), 67.8, 67.9 (**C**H$_2$OCH$_2$CH(CH$_2$CH$_2$)$_2$CH$_2$), (*R*)-**107a,b**), 77.5 (O**C**H$_2$CH(CH$_2$CH$_2$)$_2$CH$_2$, (*R*)-**107a,b**), 170.0, 170.6 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$, (*R*)-**107a,b**), the remaining resonances for (*R*)-**107a** were not detected and are believed to overlap with nearby signals or to be too small to be detected; M_r (*R*)-**107a** (+ESI) 280.1525 [M+Na]$^+$ (calcd for C$_{13}$H$_{23}$NO$_4$Na$^+$ 280.1524), (*R*)-**107b** (+ESI) 294.1682 [M+Na]$^+$ (calcd for C$_{14}$H$_{25}$NO$_4$Na$^+$ 294.1681).

(*R*)-Methyl 2-Acetamido-3-(but-3-enyloxy)propionate ((*R*)-108a) and (*R*)-Ethyl 2-Acetamido-3-(but-3-enyloxy)propionate ((*R*)-108b). Using Method A, a ~3:7 mixture of (*R*)-**68a** and (*R*)-**68b** (3.00 g, 20 mmol), 3-buten-1-ol (3.4 mL, 40 mmol) and BF$_3$•Et$_2$O (2.5 mL, 20 mmol) in CH$_2$Cl$_2$ (40 mL) gave a mixture of (*R*)-**108a** and (*R*)-**108b** (3.50 g, 77%) as a pale yellow oil that was used without further purification:

R_f = 0.48 ((R)-**108a**), 0.50 ((R)-**108b**) (5/95 hexanes/EtOAc); IR (neat) 3302, 3074, 2982, 2935, 2872, 1744, 1663, 1539, 1443, 1374, 1207 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.05 (s, C**H$_3$**C(O), (R)-**108a,b**), 2.24–2.35 (OCH$_2$C**H$_2$**CH=CH$_2$, (R)-**108a,b**), 3.40–3.58 (m, OC**H$_2$**CH$_2$CH=CH$_2$, (R)-**108a,b**), 3.62–3.70 (m, C**H**H'OCH$_2$CH$_2$, (R)-**108a,b**), 3.76 (s, C(O)OC**H$_3$**, (R)-**108a**), 3.72–3.80 (m, CH**H'**OCH$_2$CH$_2$, (R)-**108a,b**), 4.15–4.28 (m, OC**H$_2$**CH$_3$, (R)-**108b**), 4.58–4.68 (m, C**H**CH$_2$O, (R)-**108a,b**), 5.00–5.25 (m, CH$_2$CH=C**H$_2$**, (R)-**108a,b**), 5.69–5.85 (m, CH$_2$C**H**=CH$_2$, (R)-**108a,b**), 6.50–6.61 (br d, N**H**CHCH$_2$O, (R)-**108a,b**); ^{13}C NMR (CDCl$_3$) δ 14.1 (OCH$_2$**C**H$_3$, (R)-**108b**), 23.3 (**C**H$_3$C(O), (R)-**108a,b**), 33.7, 33.8 (CH$_2$**C**H$_2$CH=CH$_2$, (R)-**108a,b**), 52.5, 52.6, 52.7 (C(O)O**C**H$_3$, (R)-**108a**, **C**HOCH$_2$, (R)-**108a,b**), 61.5 (O**C**H$_2$CH$_3$, (R)-**108b**), 70.3, 70.4, 70.6, 70.7 (CH**C**H$_2$O**C**H$_2$, (R)-**108a,b**), 116.5 (CH$_2$CH$_2$CH=**C**H$_2$, (R)-**108a,b**), 134.9 (CH$_2$CH$_2$**C**H=CH$_2$, (R)-**108a,b**), 170.0, 170.4, 171.1 (CH$_3$**C**(O), CH**C**(O)O, (R)-**108a,b**), the remaining signal was not detected and is believed to overlap with nearby peaks. M_r (R)-**108a** (+ESI) 254.0794 [M+K]$^+$ (calcd for C$_{10}$H$_{17}$NO$_4$K$^+$ 254.0795), (R)-**108b** (+ESI) 252.1211 [M+Na]$^+$ (calcd for C$_{11}$H$_{19}$NO$_4$Na$^+$ 252.1212).

(S)-Methyl 2-Acetamido-3-(but-3-enyloxy)propionate ((S)-108a) and (S)-Ethyl 2-Acetamido-3-(but-3-enyloxy)propionate ((S)-108b). Using Method A, a ~9:1 mixture of (S)-**68a** and (S)-**68b** (1.50 g, 10 mmol), 3-buten-1-ol (1.3 mL, 17 mmol) and BF$_3$•Et$_2$O (1.3 mL, 23 mmol) in CH$_2$Cl$_2$ (20 mL) gave a mixture of (S)-**108a** and (S)-**108b** (550 mg, 25%) as a pale yellow oil after purification by flash chromatography (5/95 hexanes/EtOAc): R_f = 0.48 ((S)-**108a**), 0.50 ((S)-**108b**) (5/95 hexanes/EtOAc); IR (neat) 3300, 3071, 2944, 2870, 1747, 1662, 1537, 1441, 1372,

1211 cm^{-1}. Spectral data for (*S*)-**108a** (~90% based on ^{1}H NMR integrations): ^{1}H

NMR (CDCl$_3$) δ 2.05 (s, **CH$_3$**C(O)), 2.24–2.35 (m, OCH$_2$**CH$_2$**CH=CH$_2$), 3.42–3.56 (m,

O**CH$_2$**CH$_2$CH=CH$_2$), 3.62–3.70 (m, C**H**H'OCH$_2$CH$_2$), 3.76 (s, C(O)OC**H$_3$**), 3.72–3.80

(m, CH**H'**OCH$_2$CH$_2$), 4.68–4.73 (m, C**H**CH$_2$O), 5.00–5.12 (m, CH$_2$CH=C**H$_2$**), 5.69–

5.85 (m, CH$_2$C**H**=CH$_2$), 6.30–6.45 (br d, N**H**CHCH$_2$O); ^{13}C NMR (CDCl$_3$) δ 23.3

(**C**H$_3$C(O)), 33.9 (CH$_2$**C**H$_2$CH=CH$_2$), 52.7 (C(O)O**C**H$_3$ or **C**HOCH$_2$), 52.8 (**C**HOCH$_2$

or C(O)O**C**H$_3$), 70.4, 70.8 (CH**C**H$_2$O**C**H$_2$), 116.7 (CH$_2$CH$_2$CH=**C**H$_2$), 135.0

(CH$_2$CH$_2$**C**H=CH$_2$), 170.0, 171.0 (CH$_3$**C**(O), CH**C**(O)OCH$_3$); M_r no signal was

detected for (*S*)-**108a**.

Spectral data for (*S*)-**108b** (~10% based on ^{1}H NMR integrations): ^{1}H NMR

(CDCl$_3$) δ 1.23–1.35 (m, OCH$_2$C**H$_3$**), 4.18–4.25 (m, OC**H$_2$**CH$_3$), the remaining signals

were not detected and are believed to overlap with nearby signals or are too small to

be detected; ^{13}C NMR signals were not detected for (*S*)-**108b**; M_r no signal was

detected for (*S*)-**108b**.

(*R*)-Methyl 2-Acetamido-3-(2-cyclopropylethoxy)propionate ((*R*)-109a) and (*R*)-Ethyl 2-Acetamido-3-(2-cyclopropylethoxy)propionate ((*R*)-109b). Using Method

A, a ~1:1 mixture of (*R*)-**68a** and (*R*)-**68b** (2.75 g, 18.3 mmol), 2-cyclopropylethanol

(2.2 mL, 22 mmol) and BF$_3$•Et$_2$O (2.3 mL, 18.3 mmol) in CH$_2$Cl$_2$ (40 mL) gave a ~1:1

mixture of (*R*)-**109a** and (*R*)-**109b** (1.61 g, 37%) as a yellow oil after work-up and

purification by flash chromatography (1/1 EtOAc/hexanes to EtOAc): R_f = 0.48 ((*R*)-

109a), 0.50 ((*R*)-**109b**) (5/95 hexanes/EtOAc); IR (neat) 3264, 3071, 2995, 2926,

2869, 1744, 1662, 1537, 1442, 1373, 1210, 1119 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 0.01–0.08

(m, CH(C**H**H'C**H**H'), (*R*)-**109a,b**), 0.38–0.46 (m, CH(CH**H**'CH**H**'), (*R*)-**109a,b**), 0.60–0.68 (m, C**H**(CH$_2$CH$_2$), (*R*)-**109a,b**), 1.28 (t, *J* = 6.9 Hz, OCH$_2$C**H**$_3$, (*R*)-**109b**), 1.38–1.45 (m, C**H**$_2$CH(CH$_2$CH$_2$), (*R*)-**109a,b**), 2.06 (s, C**H**$_3$C(O), (*R*)-**109a,b**), 3.40–3.58 (m, OC**H**$_2$CH$_2$CH, (*R*)-**109a,b**), 3.54–3.60 (m, C**H**H'OCH$_2$CH$_2$, (*R*)-**109a,b**), 3.76 (s, C(O)OC**H**$_3$, (*R*)-**109a**), 3.82–3.90 (m, CH**H**'OCH$_2$CH$_2$, (*R*)-**109a,b**), 4.30–4.52 (m, C(O)OC**H**$_2$CH$_3$, (*R*)-**109b**), 4.70–4.78 (m, C**H**CH$_2$O, (*R*)-**109a,b**), 6.30–6.40 (br d, CH$_3$C(O)N**H**, (*R*)-**109a,b**); ^{13}C NMR (CDCl$_3$) δ 4.3 (CH(**C**H$_2$**C**H$_2$), (*R*)-**109a,b**), 7.9 (**C**H(CH$_2$CH$_2$), (*R*)-**109a,b**), 14.3 (C(O)OCH$_2$**C**H$_3$, (*R*)-**109b**), 23.1 (**C**H$_3$C(O), (*R*)-**109a,b**), 34.5, 34.6 (**C**H$_2$CH(CH$_2$CH$_2$), (*R*)-**109a,b**), 52.7, 52.9, 53.0 (**C**HCH$_2$OCH$_2$, (*R*)-**109a,b**, C(O)O**C**H$_3$, (*R*)-**109a**), 61.7 (C(O)O**C**H$_2$CH$_3$, (*R*)-**109b**), 70.6, 70.7, 71.9 (**C**H$_2$O**C**H$_2$CH$_2$, (*R*)-**109a,b**), 170.1, 170.6, 171.1 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$, (*R*)-**109a,b**), the remaining resonances were not detected and are believed to overlap with nearby signals; M_r (*R*)-**109a** (+ESI) 252.1212 [M+Na]$^+$ (calcd for C$_{11}$H$_{19}$NO$_4$Na$^+$ 252.1209), (*R*)-**109b** (+ESI) 266.1368 [M+Na]$^+$ (calcd for C$_{12}$H$_{21}$NO$_4$Na$^+$ 266.1368).

(*S*)-Methyl 2-Acetamido-3-(2-(benzyloxy)ethoxy)propionate ((*S*)-110). Using Method A compound (*S*)-**68a** (600 mg, 4.20 mmol), 2-benzyloxyethanol (775 μL, 5.45 mmol) and BF$_3$•Et$_2$O (527 μL, 4.20 mmol) in CH$_2$Cl$_2$ (20 mL) gave 600 mg (48%) of (*S*)-**110** as a pale yellow residue after purification by flash chromatography (5/95, cyclohexane/EtOAc): $[\alpha]^{25}_D$ +33.1° (*c* 1.0, CHCl$_3$); R_f = 0.43 (5/95 cyclohexane/EtOAc); IR (neat) 3286, 2869, 1744, 1663, 1537, 1446, 1369, 1207; 1111 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.97 (s, C**H**$_3$C(O)NH), 3.58–3.68 (m, OC**H**$_2$C**H**$_2$OBn), 3.71 (dd, *J* = 3.3, 8.7 Hz, CHC**H**H'OCH$_2$), 3.75 (s, OC**H**$_3$), 3.99 (dd, *J* = 3.3, 8.7 Hz,

CHCH**H'**OCH$_2$), 4.55 (s, CH$_2$CH$_2$OC**H$_2$**Ph), 4.74 (app. dt, J = 6.0, 8.7 Hz, C**H**CH$_2$O),

6.50 (br d, J = 6.0 Hz, N**H**CHCH$_2$O), 7.26–7.40 (m, C$_6$**H$_5$**); ^{13}C NMR (CDCl$_3$) δ 23.0

(**C**H$_3$C(O)), 52.5 (**C**HCH$_2$O or OC**H$_3$**), 52.8 (OC**H$_3$** or **C**HCH$_2$O), 69.4, 71.0

(OC**H$_2$**C**H$_2$**O), 71.1 (CHC**H$_2$**O), 73.2 (OC**H$_2$**Ph), 127.6, 127.7, 128.4, 138.0 (**C$_6$**H$_5$),

170.0, 170.8 (CH**C**(O)NH and **C**(O)OCH$_3$); M_r (+ESI) 318.1310 [M+Na]$^+$ (calcd for

C$_{15}$H$_{21}$NO$_5$Na$^+$ 318.1317). Anal. Calcd for C$_{15}$H$_{21}$NO$_5$•0.2H$_2$O: C, 60.27; H, 7.22; N,

4.69. Found: C, 60.14; H, 7.21; N, 4.65.

(*R*)-Methyl 2-Acetamido-3-(3-(benzyloxy)propoxy)propionate ((*R*)-111a) and (*R*)-Ethyl 2-Acetamido-3-(3-(benzyloxy)propoxy)propionate ((*R*)-111b). Using

Method A, a ~3:2 mixture of (*R*)-**68a** and (*R*)-**68b** (4.26 g, 28.4 mmol), 3-

benzyloxypropan-1-ol (12 mL, 75.5 mmol), and BF$_3$•Et$_2$O (3.57 mL, 28.4 mmol) in

CH$_2$Cl$_2$ (200 mL) gave 4.30 g (48%) of a mixture of (*R*)-**111a** and (*R*)-**111b** as a pale

yellow oil after purification by flash column chromatography (1/9 hexanes/EtOAc): R_f

= 0.53 ((*R*)-**111a**) and 0.55 ((*R*)-**111b**) (5/95 hexanes/EtOAc); IR (neat) 3306, 3063,

3033, 2941, 2869, 1744, 1665, 1535, 1449, 1372, 1210, 1109 cm^{-1}; ^{1}H NMR

(CDCl$_3$) δ 1.25 (t, J = 6.9 Hz, OCH$_2$C**H$_3$**, (*R*)-**111b**), 1.84 (quint, J = 6.3 Hz,

OCH$_2$C**H$_2$**CH$_2$O, (*R*)-**111a,b**), 1.98 (s, C**H$_3$**C(O), (*R*)-**111a,b**), 3.48–3.58 (m,

OC**H$_2$**CH$_2$CH$_2$O, (*R*)-**111a,b**), 3.60–3.66 (m, C**H**H'OCH$_2$CH$_2$CH$_2$, (*R*)-**111a,b**), 3.71

(s, C(O)OC**H$_3$**, (*R*)-**111a**), 3.86 (dd, J = 3.0, 9.6 Hz, CH**H'**OCH$_2$CH$_2$CH$_2$, (*R*)-

111a,b), 4.18 (q, J = 6.9 Hz, C(O)OC**H$_2$**CH$_3$, (*R*)-**111b**), 4.48 (s, OC**H$_2$**Ph, (*R*)-

111a,b), 4.68–4.74 (m, C**H**CH$_2$O, (*R*)-**111a,b**), 6.45–6.56 (m, CH$_3$C(O)N**H**, (*R*)-

111a,b), 7.24–7.38 (m, C$_6$**H$_5$**, (*R*)-**111a,b**); ^{13}C NMR (CDCl$_3$) δ 14.1 (C(O)OCH$_2$**C**H$_3$,

(*R*)-**111b**), 23.0, 23.1 (**C**H₃C(O), (*R*)-**111a,b**), 29.7, 29.8 (OCH₂**C**H₂CH₂O, (*R*)-**111a,b**), 52.4, 52.7, 52.8 (**C**HCH₂OCH₂, (*R*)-**111a,b**, C(O)O**C**H₃, (*R*)-**111a**), 61.5 (C(O)O**C**H₂, (*R*)-**111b**), 67.0, 68.5 (O**C**H₂CH₂**C**H₂O, (*R*)-**111a,b**), 70.4, 70.5 (CH**C**H₂O**C**H₂**C**H₂**C**H₂, (*R*)-**111a,b**), 73.0 (O**C**H₂Ph, (*R*)-**111a,b**), 127.6, 128.4, 138.4 (**C₆**H₅, (*R*)-**111a,b**), 170.0, 170.3, 170.9 (CH**C**(O)O, CH₃**C**(O)NH, (*R*)-**111a,b**), the remaining aromatic signal and additional signals corresponding to each ester were not detected and are believed to overlap with nearby signals; M_r (*R*)-**111a** (+ESI) 332.1467 [M+Na]⁺ (calcd for $C_{16}H_{23}NO_5Na^+$ 332.1474), (*R*)-**111b** (+ESI) 346.1624 [M+Na]⁺ (calcd for $C_{17}H_{25}NO_5Na^+$ 346.1630).

(*S*)-Methyl 2-Acetamido-3-(3-(benzyloxy)propoxy)propionate ((*S*)-111a). Using Method A, (*S*)-**68a** (1.00 g, 7.0 mmol), 3-benzyloxypropan-1-ol (1.44 mL, 9.11 mmol), and BF₃•Et₂O (878 μL, 7.0 mmol) in CH₂Cl₂ (3.5 mL) gave 816 mg (37%) of (*S*)-**111a** as a pale yellow oil after two purifications by flash column chromatography (5/95 hexanes/EtOAc) and (5/95 MeOH/CHCl₃): $[\alpha]^{25}_D$ +32.9° (*c* 0.5, CHCl₃); R_f = 0.53 (5/95 hexanes/EtOAc); IR (neat) 3294, 3032, 2931, 2867, 1746, 1665, 1535, 1445, 1371, 1212, 1109 cm⁻¹; ¹H NMR (CDCl₃) δ 1.85 (quint, *J* = 6.3 Hz, OCH₂**C**H₂CH₂O), 2.00 (s, **C**H₃C(O)), 3.48–3.59 (m, O**C**H₂CH₂**C**H₂O), 3.63 (dd, *J* = 3.0, 9.6 Hz, C**H**H'OCH₂CH₂CH₂), 3.73 (s, C(O)OC**H₃**), 3.86 (dd, *J* = 3.0, 9.6 Hz, CH**H'**OCH₂CH₂CH₂), 4.50 (s, OC**H₂**Ph), 4.73 (app dt, *J* = 3.0, 7.8 Hz, C**H**CH₂O), 6.33 (d, *J* = 7.8 Hz, CH₃C(O)N**H**), 7.24–7.38 (m, C₆**H₅**); ¹³C NMR (CDCl₃) δ 23.3 (**C**H₃C(O)), 29.9 (OCH₂**C**H₂CH₂O), 52.7 (**C**HCH₂OCH₂ or C(O)O**C**H₃), 52.9 (C(O)O**C**H₃ or **C**HCH₂OCH₂), 67.2, 68.8 (O**C**H₂CH₂**C**H₂O), 70.7

(CHCH$_2$OCH$_2$CH$_2$CH$_2$), 73.2 (OCH$_2$Ph), 127.7, 127.8, 128.6, 138.6 (C$_6$H$_5$), 170.1, 171.1 (CHC(O)OCH$_3$, CH$_3$C(O)NH); M_r (+ESI) 332.1469 [M+Na]$^+$ (calcd for C$_{15}$H$_{21}$NO$_5$Na$^+$ 332.1474). Anal. Calcd for C$_{16}$H$_{23}$NO$_5$•0.2H$_2$O: C, 61.40; H, 7.54; N, 4.48. Found: C, 61.38; H, 7.47; N, 4.48.

(*R*)-Methyl 2-Acetamido-3-(2-azidoethoxy)propionate ((*R*)-112). Using Method A, (*R*)-**68a** (2.35 g, 16.4 mmol), 2-azidoethanol (4.5 mL, 65.6 mmol) and BF$_3$•Et$_2$O (1 mL, 8.2 mmol) in CH$_2$Cl$_2$ (30 mL) gave 1.56 g (41%) of (*R*)-**112** after purification; [α]$^{25}_D$ +49.1° (*c* 1.0; EtOAc); R_f = 0.47 (EtOAc); IR (neat) 3302, 2948, 2107, 1746, 1668, 1534, 1443 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.06 (s, CH$_3$C(O)NH), 3.23–3.42 (m, CH$_2$N$_3$), 3.31–3.64 (m, OCH$_2$CH$_2$N$_3$, CHCHH'OCH$_2$), 3.78 (s, OCH$_3$), 3.96 (dd, *J* = 3.0, 9.1 Hz, CHCHH'O), 4.76–4.81 (m, CHCH$_2$O), 6.30–6.41 (br d, CH$_3$C(O)NH); ^{13}C NMR (CDCl$_3$) δ 23.2 (CH$_3$C(O)), 50.0 (OCH$_2$CH$_2$N$_3$), 52.6 (OCH$_3$ or CHCH$_2$O), 52.8 (CHCH$_2$O or OCH$_3$), 70.9 (CHCH$_2$O or OCH$_2$CH$_2$N$_3$), 71.1 (OCH$_2$CH$_2$N$_3$ or CHCH$_2$O), 170.1 (CH$_3$C(O)NH or C(O)OCH$_3$), 170.6 (C(O)OCH$_3$ or CH$_3$C(O)NH); M_r (+ESI) 253.0906 [M+Na]$^+$ (calcd for C$_8$H$_{14}$N$_4$O$_4$Na$^+$ 253.0913). No satisfactory elemental analysis was obtained.

(*S*)-Methyl 2-Acetamido-3-(2-azidoethoxy)propionate ((*S*)-112). Using Method A, (*S*)-**68a** (3.30 g, 23 mmol), 2-azidoethanol (6.3 mL, 92 mmol), and BF$_3$•Et$_2$O (1.4 mL, 11.5 mmol) in CH$_2$Cl$_2$ (115 mL) gave 1.67 g (31%) of (*S*)-**112** as a colorless residue after purification by silica gel chromatography (EtOAc): [α]$^{25}_D$ -48.0° (*c* 1.0; EtOAc); R_f = 0.47 (EtOAc); IR (neat) 3302, 2948, 2107, 1746, 1667, 1534, 1443 cm^{-1}; ^{1}H

NMR (CDCl$_3$) δ 2.06 (s, C**H$_3$**C(O)NH), 3.23–3.42 (m, C**H$_2$**N$_3$), 3.31–3.64 (m, OC**H$_2$**CH$_2$N$_3$, CHC**H**H'OCH$_2$), 3.78 (s, OC**H$_3$**), 3.96 (dd, J = 3.0, 9.1 Hz, CHCH**H'**O), 4.76–4.81 (m, C**H**CH$_2$O), 6.30–6.41 (br d, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 50.1 (OCH$_2$**C**H$_2$N$_3$), 52.7 (O**C**H$_3$ or CH**C**H$_2$O), 52.9 (**C**HCH$_2$O or O**C**H$_3$), 71.0 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 71.2 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 170.2, 170.7 (CH$_3$**C**(O)NH and **C**(O)OCH$_3$); M_r (+ESI) 253.0906 [M+Na]$^+$ (calcd for C$_8$H$_{14}$N$_4$O$_4$Na$^+$ 253.0913). No satisfactory elemental analysis was obtained.

(*R*)-Methyl 2-Acetamido-3-(2-methoxyethoxy)propionate ((*R*)-113a) and (*R*)-Ethyl 2-Acetamido-3-(2-methoxyethoxy)propionate ((*R*)-113b). Using Method A, a ~5:95 mixture of (*R*)-**68a** and (*R*)-**68b** (3.50 g, 23 mmol), ethylene glycol monomethyl ether (3.6 mL, 46 mmol) and BF$_3$•Et$_2$O (2.9 mL, 23 mmol) in CH$_2$Cl$_2$ (40 mL) gave upon work-up a ~5:95 mixture of (*R*)-**113a** and (*R*)-**113b** (4.24 g, 80%) as a pale yellow oil that did not require further purification: R_f = 0.41 ((*R*)-**113a**), 0.43 ((*R*)-**113b**) (EtOAc); IR (neat) 3303, 3061, 2930, 1743, 1668, 1535, 1451, 1374, 1204, 1113 cm^{-1}. Spectral data for (*R*)-**113a**: ^{1}H and ^{13}C NMR signals for (*R*)-**113a** were not detected and are believed to overlap with nearby signals or are too small to be detected; M_r (+ESI) 258.0744 [M+K]$^+$ (calcd for C$_9$H$_{17}$NO$_5$K$^+$ 258.0744).

Spectral data for (*R*)-**113b** (~95% based on ^{1}H NMR integrations): ^{1}H NMR (CDCl$_3$) δ 1.29 (t, J = 7.2 Hz, OCH$_2$C**H$_3$**), 2.05 (s, C**H$_3$**C(O)), 3.37 (s, CH$_2$OC**H$_3$**), 3.49–3.53 (m, CH$_2$OC**H$_2$**CH$_2$OCH$_3$ or CH$_2$OCH$_2$C**H$_2$**OCH$_3$), 3.59–3.64 (m, CH$_2$OCH$_2$C**H$_2$**OCH$_3$ or CH$_2$OC**H$_2$**CH$_2$OCH$_3$), 3.73 (dd, J = 3.0, 9.0 Hz, C**H**H'OCH$_2$CH$_2$), 3.94 (dd, J = 3.0, 9.0 Hz, CH**H'**OCH$_2$CH$_2$), 4.22 (d, J = 7.2 Hz,

OC**H₂**CH₃), 4.52 (app. dt, J = 3.0, 7.2 Hz, C**H**CH₂O), 6.48–6.60 (br d, N**H**CHCH₂O); ^{13}C NMR (CDCl₃) δ 14.3 (OCH₂**C**H₃), 23.3 (**C**H₃C(O)), 53.0 (**C**HC(O)OCH₂), 59.1 (CH₂O**C**H₃), 61.7 (C(O)O**C**H₂CH₃), 71.1, 71.3, 71.9 (CH**C**H₂O**C**H₂**C**H₂OCH₃), 170.1, 170.5 (CH₃**C**(O), CH**C**(O)OCH₂); M_r (+ESI) 272.0901 [M+K]⁺ (calcd for C₁₀H₁₉NO₅K⁺ 272.0900).

(*R*)-Methyl 2-Acetamido-3-(2-(2-methoxyethoxy)ethoxy)propionate ((*R*)-114a) and (*R*)-Ethyl 2-Acetamido-3-(2-(2-methoxyethoxy)ethoxy)propionate ((*R*)-114b). Using Method A, a ~1:1 mixture of (*R*)-**68a** and (*R*)-**68b** (4.5 g, 30.0 mmol), diethyleneglycol monomethyl ether (11.3 g, 94.5 mmol) and BF₃•Et₂O (3.8 mL, 30.0 mmol) in CH₂Cl₂ (30 mL) gave 2.84 g (35%) of (*R*)-**114a** and (*R*)-**114b** as a colorless viscous oil: R_f = 0.31 ((*R*)-**114a**), 0.33 ((*R*)-**114b**) (EtOAc); IR (neat) 3300, 3063, 2940, 2940, 1744, 1659, 1553, 1446, 1216, 1120 cm⁻¹. Spectral data for (*R*)-**114a** (approximately 50 mol percent based on ^{1}H NMR integrations): ^{1}H NMR (CDCl₃) δ 2.06 (s, C**H₃**C(O)NH), 3.39 (s, CH₂CH₂OC**H₃**), 3.52–3.58 (m, CH₂C**H₂**OCH₃), 3.60–3.64 (m, OC**H₂**CH₂OC**H₂**), 3.68–3.74 (m, CHC**H**H'OCH₂), 3.76 (s, C(O)OC**H₃**), 3.94 (app. t, J = 4.2, CHCH**H'**OCH₂), 4.68–4.76 (m, C**H**CH₂OCH₂), 6.52–6.70 (m, CH₃C(O)N**H**); ^{13}C NMR (CDCl₃) δ 23.1 (**C**H₃C(O)), 52.6 (**C**HCH₂OCH₂ or C(O)O**C**H₃), 52.9, 53.0 (C(O)O**C**H₃ or **C**HCH₂OCH₃, (*R*)-**114a**, **C**HCH₂OCH₃, (*R*)-**114b**)), 59.2 (CH₂CH₂O**C**H₃), 70.5, 70.6, 71.1, 71.2, 71.3 (O**C**H₂**C**H₂O**C**H₂**C**H₂OCH₃), 170.1, 170.4, 170.9 (CH₃**C**(O)NH, **C**(O)OCH₃), additional peaks were observed and are believed to be part of (*R*)-**114b** but cannot

be precisely attributed; Compound **114a** was only detected by HRMS as the acid. M_r

(+ESI) found 272.1107 $[M+Na]^+$ (calcd for $C_{10}H_{19}NO_6Na^+$ 272.1110).

Spectral data for (*R*)-**114b** (approximately 50 mol percent based on ^{1}H NMR

integrations): ^{1}H NMR (CDCl$_3$) δ 1.28 (t, *J* = 7.2 Hz, C(O)OCH$_2$**CH$_3$**), 2.06 (s,

CH$_3$C(O)NH), 3.39 (s, CH$_2$CH$_2$O**CH$_3$**), 3.52–3.58 (m, CH$_2$**CH$_2$**OCH$_3$), 3.60–3.64 (m,

O**CH$_2$**CH$_2$O**CH$_2$**), 3.68–3.74 (m, CHC**H**H'OCH$_2$), 3.97 (app. t, *J* = 4.2,

CHCH**H'**OCH$_2$), 4.22 (t, *J* = 7.2 Hz, C(O)OC**H$_2$**CH$_3$), 4.68–4.76 (m, C**H**CH$_2$OCH$_2$),

6.52–6.70 (m, CH$_3$C(O)N**H**); ^{13}C NMR (CDCl$_3$) δ 14.3 (C(O)OCH$_2$**C**H$_3$), 23.1

(**C**H$_3$C(O)), 52.6 (**C**HCH$_2$OCH$_2$), 52.9, 53.0 (C(O)O**C**H$_3$ or **C**HCH$_2$OCH$_3$, (*R*)-**114a**,

CHCH$_2$OCH$_3$ (*R*)-**114b**), 70.5, 70.6, 71.1, 71.2, 71.3 (O**C**H$_2$**C**H$_2$O**C**H$_2$**C**H$_2$O**C**H$_3$),

170.1, 170.4, 170.9 (CH$_3$**C**(O)NH, **C**(O)OCH$_3$), additional peaks were observed and

are believed to be part of (*R*)-**114a** but cannot be precisely attributed; M_r (+ESI)

found 300.1421 $[M+Na]^+$ (calcd for $C_{12}H_{23}NO_6Na^+$ 300.1423).

2.2.4.4. Synthesis of *O*-Substituted *N*-Acetylserine Derivatives.

69a, X = Me
b, X = Et

70
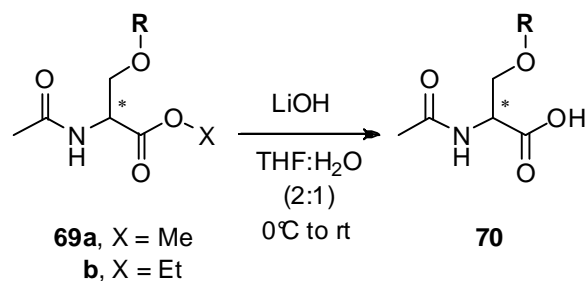
Scheme 20. Synthesis of acid **123** to prepare the side chain aldehyde AB group

122

123
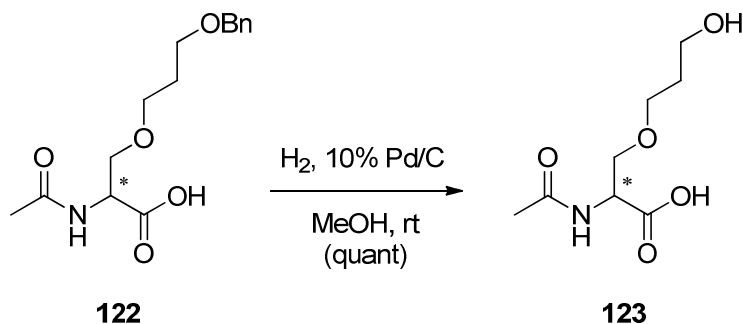
R =	Compound number
Me	**115**
Et	**116**
i-Pr	**117**
t-Bu	**118**
Ph	**119**
$CH_2CH_2CH=CH_2$	**120**
CH_2CH_2OBn	**121**
$CH_2CH_2CH_2OBn$	**122**
$CH_2CH_2CH_2OH$	**123**
$CH_2CH_2N_3$	**124**
$(CH_2CH_2O)_2CH_3$	**125**

(**R**)-2-Acetamido-3-methoxypropionic Acid ((**R**)-115). Using Method B, a mixture of (*R*)-**101a** and (*R*)-**101b** (3.79 g, 21.5 mmol) in THF (210 mL) and LiOH (515 mg, 21.5 mmol) in H_2O (100 mL) gave 1.31 g (38%) of (*R*)-**115** as a white solid after

work-up and recrystallization from EtOAc: mp 108–109°C; $[\alpha]^{25}_D$ -20.9° (c 0.7, MeOH) (lit.[235] $[\alpha]^{25}_D$ -16.9° (c 1.2; MeOH) for a partially racemized sample (~4:1, (R):(S))); R_f = 0–0.1 (EtOAc); IR (nujol) 3352, 3100–2200, 1746, 1631, 1549, 1459, 1375 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.86 (s, **CH₃**C(O)), 3.25 (s, CH₂OC**H₃**), 3.49 (dd, J = 3.9, 10.0 Hz, C**H**H'OCH₃), 3.63 (dd, J = 6.0, 10.0 Hz CH**H'**OCH₃), 4.36–4.45 (m, C**H**CH₂O), 8.20 (d, J = 7.2 Hz, CH₃C(O)N**H**), 12.7 (s, CO₂**H**); ^{13}C NMR (DMSO-d_6) δ 22.3 (**C**H₃C(O)), 52.1 (**C**HCH₂OCH₃), 58.3 (O**C**H₃), 71.8 (CH**C**H₂OCH₃), 169.4, 171.7 (CH**C**O₂H, CH₃**C**(O)NH). M_r (+ESI) 184.0582 [M+Na]$^+$ (calcd for C₆H₁₁NO₄Na$^+$ 184.0586). Anal. Calcd for C₆H₁₁NO₄: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.75; H, 6.82; N, 8.77.

(S)-2-Acetamido-3-(methoxy)propionic Acid ((S)-115). Using Method B, a ~9:1 mixture of (S)-**101a** and (S)-**101b** (1.43 g, 8 mmol) in THF (60 mL) and LiOH (192 mg, 8 mmol) in H₂O (30 mL) gave (S)-**115** (470 mg, 37%) upon work-up as a yellow oil that was recrystallized from EtOAc: mp 107–109°C; $[\alpha]^{25}_D$ +20.7° (c 0.5; MeOH); R_f = 0–0.10 (EtOAc); IR (nujol) 3351, 3300–2200 (br), 1746, 1631, 1549, 1459, 1375 cm^{-1}; ^{1}H NMR (CD₃OD) δ 2.01 (s, **CH₃**C(O)), 3.35 (s, CH₂OC**H₃**), 3.61 (dd, J = 3.3, 9.3 Hz, C**H**H'OCH₃), 3.81 (dd, J = 4.8, 9.3 Hz, CH**H'**OCH₃), 4.54–4.62 (m, C**H**CH₂O); ^{13}C NMR (CD₃OD) δ 22.5 (**C**H₃C(O)), 54.2 (**C**HOCH₂), 59.4 (CH₂O**C**H₃), 73.2 (CH**C**H₂OCH₃), 173.3, 173.5 (CH₃**C**(O), CH**C**(O)OH); M_r (+ESI) 184.0584 [M+Na]$^+$ (calcd for C₆H₁₁NO₄Na$^+$ 184.0586). Anal. Calcd for C₆H₁₁NO₄: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.47; H, 6.92; N, 8.46.

(*R*)-2-Acetamido-3-ethoxypropionic Acid ((*R*)-116). Using Method B, a mixture of (*R*)-**102a** and (*R*)-**102b** (2.48 g, 13.0 mmol) in THF (130 mL) and LiOH (312 mg, 13.0 mmol) in H_2O (65 mL) gave 1.57 g (69%) of (*R*)-**116** as a white solid after work-up and recrystallization from EtOAc: mp 149–151°C; $[\alpha]^{25}_D$ -31.5° (*c* 0.7, MeOH); R_f = 0–0.15 (5/95 hexanes/EtOAc); IR (nujol) 3355, 3300–2100 (br), 1951, 1747, 1630, 1545, 1457, 1374, 1204, 1107 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.09 (t, *J* = 6.9 Hz, OCH$_2$C**H$_3$**), 1.86 (s, **CH$_3$**C(O)), 3.39–3.49 (m, CH$_2$OC**H$_2$**CH$_3$), 3.53 (dd, *J* = 4.2, 10.0 Hz, C**H**H'OCH$_2$CH$_3$), 3.63 (dd, *J* = 6.0, 10.0 Hz, CH**H'**OCH$_2$CH$_3$), 4.36–4.42 (m, C**H**CH$_2$O), 8.15 (d, *J* = 7.2 Hz, CH$_3$C(O)N**H**), the carboxylic acid proton could not be detected; ^{13}C NMR (DMSO-d_6) δ 14.9 (OCH$_2$**C**H$_3$), 22.3 (**C**H$_3$C(O)), 52.4 (**C**HCH$_2$OCH$_3$), 65.8 (O**C**H$_2$CH$_3$), 69.6 (CH**C**H$_2$OCH$_2$CH$_3$), 169.4, 171.7 (CH**C**O$_2$H, CH$_3$**C**(O)NH); M_r (+ESI) 214.0480 [M+K]$^+$ (calcd for $C_7H_{13}NO_4K^+$ 214.0482). Anal. Calcd for $C_7H_{13}NO_4$: C, 47.99; H, 7.48; N, 8.00. Found: C, 48.21; H, 7.56; N, 7.95.

(*R*)-2-Acetamido-3-isopropoxypropionic Acid ((*R*)-117). Using Method B, a mixture of (*R*)-**103a** and (*R*)-**103b** (2.47 g, 12.0 mmol) in THF (120 mL) and LiOH (288 mg, 12.0 mmol) in H_2O (60 mL) gave 2.15 g (95%) of (*R*)-**117** as a white solid after work-up. Recrystallization from EtOAc and hexanes afforded an analytical sample: mp 128–130°C; $[\alpha]^{25}_D$ -36.5° (*c* 0.6, MeOH); R_f = 0.05–0.18 (5/95 hexanes/EtOAc); IR (nujol) 3366, 3300–2100 (br), 1751, 1636, 1548, 1457, 1376, 1328 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.06 (d, *J* = 6.0 Hz, OCHC**H$_3$**(C'H$_3$)), 1.07 (d, *J* = 6.0 Hz, OCHCH$_3$(C'**H$_3$**), 1.87 (s, **CH$_3$**C(O)), 3.49–3.50 (m, C**H**H'OCH(CH$_3$)$_2$), 3.64 (dd, *J* = 5.7, 9.9 Hz, CH**H'**OCH(CH$_3$)$_2$), 4.33–4.40 (m, C**H**CH$_2$O), 8.10 (d, *J* = 7.2 Hz,

CH$_3$C(O)N**H**), 12.70 (CO$_2$**H**); ^{13}C NMR (DMSO-d_6) δ 21.8 (OCH**C**H$_3$(C'H$_3$)), 21.9 (OCHCH$_3$(**C'**H$_3$), 22.4 (s, **C**H$_3$C(O)), 52.6 (**C**HCH$_2$OCH(CH$_3$)$_2$), 67.4 (**C**H$_2$OCH(CH$_3$)$_2$), 71.3 (CH$_2$O**C**H(CH$_3$)$_2$), 169.4, 171.8 (CH$_3$**C**(O)NH, **C**O$_2$H); M_r (+ESI) 212.0897 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_4$Na$^+$ 212.0899). Anal. Calcd for C$_8$H$_{15}$NO$_4$: C, 50.78; H, 7.99; N, 7.40. Found: C, 50.87; H, 8.02; N, 7.34.

(R)-2-Acetamido-3-*tert*-butoxypropionic Acid ((R)-118). Using Method B, a mixture of (R)-**104a** and (R)-**104b** (2.17 g, 9.90 mmol) in THF (100 mL) and LiOH (237 mg, 9.90 mmol) in H$_2$O (50 mL) gave 1.10 g (55%) of (R)-**118** as a white solid after work-up. Recrystallization from EtOAc and hexanes afforded an analytical sample: mp 154–156°C; [α]$^{25}_D$ -46.7° (*c* 0.8, MeOH); R_f = 0.48 (5/95 hexanes/EtOAc); IR (nujol) 3370, 3300–2100 (br), 1875, 1708, 1613, 1542, 1459, 1371, 1229, 1103 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.11 (s, OC(C**H**$_3$)$_3$), 1.87 (s, C**H$_3$**C(O)), 3.47 (dd, *J* = 4.2, 9.3 Hz, C**H**H'OC(CH$_3$)$_3$), 3.60 (dd, *J* = 5.1, 9.3 Hz, CH**H'**OC(CH$_3$)$_3$), 4.30–4.38 (m, C**H**CH$_2$O), 8.01 (d, *J* = 7.2 Hz, CH$_3$C(O)N**H**), 12.60 (CO$_2$**H**); ^{13}C NMR (DMSO-d_6) δ 22.4 (s, **C**H$_3$C(O)), 27.2 (OC(**C**H$_3$)$_3$), 52.8 (**C**HCH$_2$OC(CH$_3$)$_3$), 61.7 (**C**H$_2$OC(CH$_3$)$_3$), 72.8 (CHCH$_2$O**C**(CH$_3$)$_3$), 169.4, 171.9 (CH$_3$**C**(O)NH, **C**O$_2$H); M_r (+ESI) 242.0794 [M+K]$^+$ (calcd for C$_9$H$_{17}$NO$_4$K$^+$ 242.0795). Anal. Calcd for C$_9$H$_{17}$NO$_4$: C, 53.19; H, 8.43; N, 6.89; Found: C, 53.04; H, 8.49; N, 6.84.

(R)-2-Acetamido-3-phenoxypropionic Acid ((R)-119). Using Method B, a ~3:7 mixture of (R)-**106a** and (R)-**106b** (1.4 g, 5.7 mmol), LiOH (142 mg, 5.9 mmol) in

THF:H$_2$O (80 mL) gave 950 mg (74%) of (*R*)-**119** after recrystallization from EtOAc and hexanes: mp 168–170°C; [α]^{25}$_D$ -91.2° (*c* 0.5, MeOH); *R$_f$* = 0–0.15 (1/9 MeOH/CHCl$_3$); IR (nujol) 3362, 2300–2800 (br), 1950, 1746, 1607, 1551, 1461 cm^{-1}; ^{1}H NMR (DMSO-*d$_6$*) δ 1.90 (s, **CH$_3$**C(O)), 4.13 (dd, *J* = 3.9, 9.6 Hz, C**H**H'OPh), 4.37 (dd *J* = 5.1, 9.6 Hz, CH**H'**OPh), 4.60–4.68 (m, C**H**CH$_2$O), 6.88–6.98 (m, 3 Ar**H**), 7.24–7.32 (m, 2 Ar**H**), 8.42 (d, *J* = 7.5 Hz, N**H**CHCH$_2$), 12.50–13.00 (m, C(O)O**H**); ^{13}C NMR (DMSO-*d$_6$*) δ 22.3 (**C**H$_3$C(O)), 51.8 (**C**HCH$_2$OPh), 67.6 (**C**H$_2$OPh), 114.6, 121.0, 129.8, 158.1 (CH$_2$O**P**h), 169.5, 171.3 (CH$_3$**C**(O)NH, CH**C**(O)OH); *M$_r$* (+ESI) 246.0739 [M+Na]$^+$ (calcd for C$_{11}$H$_{13}$NO$_4$Na$^+$ 246.0742). Anal. Calcd for C$_{11}$H$_{13}$NO$_4$•0.25 H$_2$O: C, 58.02; H, 5.98; N, 6.15. Found: C, 58.00; H, 5.98; N, 5.91.

(*R*)-2-Acetamido-3-(but-3-enyloxy)propionic Acid ((*R*)-120). Using Method B, a ~3:7 mixture of (*R*)-**108a** and (*R*)-**108b** (3.28 g, 14.6 mmol) in THF (145 mL) and LiOH (350 mg, 14.6 mmol) in H$_2$O (70 mL) gave (*R*)-**120** (1.90 g, 65%) as a yellow oil that was used without further purification: [α]^{25}$_D$ -21.9° (*c* 0.9; MeOH); *R$_f$* = 0.10 (15/85 MeOH/CH$_2$Cl$_2$); IR (neat) 3300–2100 (br), 1957, 1735, 1635, 1544, 1432, 1376, 1228, 1118 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.09 (s, **CH$_3$**C(O)), 2.25–2.37 (m, OCH$_2$**CH$_2$**CH=CH$_2$), 3.46–3.51 (m, OC**H$_2$**CH$_2$CH=CH$_2$), 3.69 (dd, *J* = 4.0, 9.0 Hz, C**H**H'OCH$_2$), 3.93 (dd, *J* = 4.0, 9.0 Hz, CH**H'**OCH$_2$), 4.73 (app dt, *J* = 4.0, 7.7 Hz, C**H**CH$_2$O), 5.00–5.22 (m, CH$_2$CH=C**H$_2$**), 5.70–5.85 (m, CH$_2$C**H**=CH$_2$), 6.22 (d, *J* = 7.7 Hz, N**H**CHCH$_2$O), 9.80–10.00 (br s, C(O)O**H**); ^{13}C NMR (CDCl$_3$) δ 23.1 (**C**H$_3$C(O)), 33.9 (CH$_2$**C**H$_2$CH=CH$_2$), 52.9 (**C**HOCH$_2$), 70.0, 70.9 (CH**C**H$_2$O**C**H$_2$), 117.0 (CH$_2$CH$_2$CH=**C**H$_2$), 134.9 (CH$_2$CH$_2$**C**H=CH$_2$), 171.5, 173.4 (CH$_3$**C**(O),

CH**C**(O)OH); M_r (+ESI) 224.0897 [M+Na]$^+$ (calcd for $C_9H_{15}NO_4Na^+$ 224.0899). Anal.

Calcd for $C_9H_{15}NO_4$•0.30H_2O: C, 52.32; H, 7.61; N, 6.78; Found: C, 52.11; H, 7.37;

N, 6.77.

(*S*)-2-Acetamido-3-(but-3-enyloxy)propionic Acid ((*S*)-120). Using Method B, a

~9:1 mixture of (*S*)-**108a** and (*S*)-**108b** (550 mg, 2.5 mmol) in THF (25 mL) and LiOH

(61 mg, 2.5 mmol) in H_2O (12 mL) gave (*S*)-**120** (260 mg, 51%) as a yellow oil that

was used without further purification: $[\alpha]^{25}_D$ +22.2° (*c* 0.5; MeOH); R_f = 0.10 (15/85

MeOH/CH_2Cl_2); IR (neat) 3300–2200 (br), 1953, 1735, 1636, 1547, 1432, 1376,

1227, 1118 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.01 (s, **CH$_3$**C(O)), 2.28–2.36 (m,

OCH$_2$**CH$_2$**CH=CH$_2$), 3.49–3.52 (m, O**CH$_2$**CH$_2$CH=CH$_2$), 3.68 (dd, *J* = 4.0, 9.0 Hz,

C**H**H'OCH$_2$), 3.93 (dd, *J* = 4.0, 9.0 Hz, CH**H'**OCH$_2$), 4.73 (app dt, *J* = 4.0, 7.7 Hz,

C**H**CH$_2$O), 5.00–5.25 (m, CH$_2$CH=C**H$_2$**), 5.70–5.85 (m, CH$_2$C**H**=CH$_2$), 6.52 (d, *J* =

7.7 Hz, N**H**CHCH$_2$O), 9.10–9.60 (br s, C(O)O**H**); ^{13}C NMR (CDCl$_3$) δ 23.2

(**C**H$_3$C(O)), 33.9 (CH$_2$**C**H$_2$CH=CH$_2$), 52.8 (**C**HOCH$_2$), 69.9, 70.9 (CH**C**H$_2$O**C**H$_2$),

117.0 (CH$_2$CH$_2$CH=**C**H$_2$), 134.9 (CH$_2$CH$_2$**C**H=CH$_2$), 171.3, 173.5 (CH$_3$**C**(O),

CH**C**(O)OH); M_r (+ESI) 224.0901 [M+Na]$^+$ (calcd for $C_9H_{15}NO_4Na^+$ 224.0899). Anal.

Calcd for $C_9H_{15}NO_4$•0.30H_2O: C, 52.32; H, 7.61; N, 6.78; Found: C, 52.08; H, 7.47;

N, 6.88.

(*S*)-2-Acetamido-3-(2-(benzyloxy)ethoxy)propionic Acid ((*S*)-121). Using Method

B, (*S*)-**110a** (460 mg, 1.56 mmol) in THF (15 mL) and LiOH (37 mg, 1.56 mmol) in

H_2O (7 mL) gave 386 mg (87%) of (*S*)-**121** as a colorless residue that was used

directly in the next step: $[\alpha]^{25}_D$ +16.3° (*c* 1.0, CHCl$_3$); R_f = 0.05 (5/95 cyclohexane/EtOAc); IR (neat) 3400–2600 (br), 3322, 1736, 1631, 1541, 1449, 1372, 1214; 1121 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.97 (s, **CH$_3$**C(O)NH), 3.58–3.80 (m, O**CH$_2$CH$_2$**OBn, CHC**H**H'OCH$_2$), 3.95–4.04 (m, CHCH**H**'OCH$_2$), 4.54 (s, O**CH$_2$**Ph), 4.68–4.75 (m, C**H**CH$_2$OCH$_2$), 6.80 (d, *J* = 6.1 Hz, N**H**CHCH$_2$O), 7.22–7.40 (m, C$_6$**H$_5$**), 8.30–8.70 (br s, C(O)O**H**); ^{13}C NMR (CDCl$_3$) δ 23.0 (**C**H$_3$C(O)), 52.9 (**C**HCH$_2$O), 69.4, 70.7, 71.0 (O**C**H$_2$**C**H$_2$O and CH**C**H$_2$O), 73.2 (O**C**H$_2$Ph), 128.0, 128.6, 137.7 (**C$_6$**H$_5$), 171.3, 172.5 (CH$_3$**C**(O)NH and **C**(O)OH), the remaining aromatic resonance was not detected and is believed to overlap with nearby signals; M_r (+ESI) 304.1157 [M+Na]$^+$ (calcd for C$_{14}$H$_{19}$NO$_5$Na$^+$ 304.1161).

(*R*)-2-Acetamido-3-(3-(benzyloxy)propoxy)propionic Acid ((*R*)-122). Using Method B, a ~1:1 mixture of (*R*)-**111a** and (*R*)-**111b** (4.30 g, 13.6 mmol) in THF (150 mL) and LiOH (325 mg, 13.6 mmol) in H$_2$O (70 mL) gave 3.63 g (90%) of (*R*)-**122** as a viscous yellow oil that was used directly in the next step: $[\alpha]^{25}_D$ -17.7° (*c* 1.0, MeOH); R_f = 0.53 (1/9 MeOH/CHCl$_3$); IR (neat) 3500–2200 (br), 1959, 1735, 1630, 1540, 1449, 1373, 1213, 1107 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.85 (quint, *J* = 6.0 Hz, OCH$_2$**CH$_2$**CH$_2$O), 2.00 (s, **CH$_3$**C(O)), 3.54 (t, *J* = 6.0 Hz, O**CH$_2$**CH$_2$CH$_2$OBn or OCH$_2$CH$_2$**CH$_2$**OBn), 3.57 (t, *J* = 6.0 Hz, OCH$_2$CH$_2$**CH$_2$**OBn or O**CH$_2$**CH$_2$CH$_2$OBn), 3.64 (dd, *J* = 3.9, 9.7 Hz, C**H**H'OCH$_2$CH$_2$CH$_2$), 3.86 (dd, *J* = 3.3, 9.7 Hz, CH**H**'OCH$_2$CH$_2$CH$_2$), 4.49 (s, O**CH$_2$**Ph), 4.65–4.75 (m, C**H**CH$_2$O), 6.59 (d, *J* = 7.5 Hz, CH$_3$C(O)N**H**), 7.24–7.38 (m, C$_6$**H$_5$**), 9.10–9.50 (m, C(O)O**H**); ^{13}C NMR (CDCl$_3$) δ 23.1 (**C**H$_3$C(O)), 29.7 (OCH$_2$**C**H$_2$CH$_2$O), 52.9 (**C**HCH$_2$OCH$_2$), 67.2, 68.9

(O**C**H$_2$CH$_2$**C**H$_2$O), 70.2 (CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 73.1 (O**C**H$_2$Ph), 127.9, 128.0, 128.6,

138.4 (**C$_6$**H$_5$), 171.3, 173.1 (CH**C**(O)OH, CH$_3$**C**(O)NH); M_r (+ESI) 296.1492 [M+H]$^+$

(calcd for C$_{15}$H$_{21}$NO$_5$H$^+$ 296.1498). Anal. Calcd for C$_{15}$H$_{21}$NO$_5$•0.25H$_2$O: C, 60.09; H,

7.23; N, 4.67. Found: C, 59.93; H, 7.28; N, 4.67.

(*S*)-2-Acetamido-3-(3-(benzyloxy)propoxy)propionic Acid ((*S*)-122). Using

Method B, a ~9:1 mixture of (*S*)-**111a** and (*S*)-**111b** (8.30 g, 26.8 mmol) in THF (250

mL) and LiOH (643 mg, 26.8 mmol) in H$_2$O (125 mL) gave 5.78 g (73%) of (*S*)-**122**

as a viscous yellow oil that was used without further purification: [α]^{25}$_D$ +17.4° (c 1.0,

MeOH); R_f = 0.53 (1/9 MeOH/CHCl$_3$); IR (nujol) 3500–2200 (br), 1962, 1735, 1637,

1545, 1449, 1373, 1216, 1111 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.76 (quint, J = 6.9 Hz,

OCH$_2$**C**H$_2$CH$_2$O), 1.87 (s, **C**H$_3$C(O)), 3.38–3.52 (m, O**C**H$_2$CH$_2$**C**H$_2$O), 3.55 (dd, J =

4.2, 13.8 Hz, **C**HH'OCH$_2$CH$_2$CH$_2$), 3.86 (dd, J = 5.8, 13.8 Hz, CH**H**'OCH$_2$CH$_2$CH$_2$),

4.38–4.44 (m, O**C**H$_2$Ph, **C**HCH$_2$O), 7.24–7.38 (m, C$_6$**H$_5$**), 8.17 (d, J = 8.1 Hz,

CH$_3$C(O)N**H**), the carboxylic acid proton was not detected; ^{13}C NMR (DMSO-d_6) δ

22.3 (**C**H$_3$C(O)), 29.4 (OCH$_2$**C**H$_2$CH$_2$O), 52.4 (**C**HCH$_2$OCH$_2$), 66.6, 67.7

(O**C**H$_2$CH$_2$**C**H$_2$O), 70.0 (CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 71.9 (O**C**H$_2$Ph), 127.3, 127.4, 128.3,

138.7 (**C$_6$**H$_5$), 169.4, 171.8 (CH**C**(O)OH, CH$_3$**C**(O)NH); M_r (+ESI) 318.1318 [M+Na]$^+$

(calcd for C$_{15}$H$_{21}$NO$_5$Na$^+$ 318.1317). Anal. Calcd for C$_{15}$H$_{21}$NO$_5$: C, 61.00; H, 7.17; N,

4.74. Found: C, 60.75; H, 7.26; N, 4.86.

(*R*)-2-Acetamido-3-(3-hydroxypropoxy)propionic Acid ((*R*)-123). Using Method

H, compound (*R*)-**122** (1.80 g, 6.1 mmol) and 10% Pd/C (150 mg) in MeOH (20 mL)

gave 1.14 g (91%) of (*R*)-**123** as a pale yellow viscous oil that did not require further purification: $[\alpha]^{25}_D$ -18.2° (*c* 1.0, MeOH); R_f = 0.23 (1/4 MeOH/CHCl$_3$); IR (neat) 3500–2200 (br), 1958, 1734, 1655, 1547, 1427, 1377, 1232, 1127 cm^{-1}; ^{1}H NMR (DMSO-*d*$_6$) δ 1.62 (quint, *J* = 6.3 Hz, OCH$_2$**CH$_2$**CH$_2$O), 1.86 (s, **CH$_3$**C(O)), 3.36–3.50 (m, O**CH$_2$**CH$_2$CH$_2$O), 3.53 (dd, *J* = 4.2, 9.9 Hz, C**H**H'OCH$_2$CH$_2$CH$_2$), 3.64 (dd, *J* = 5.4, 9.9 Hz, CH**H'**OCH$_2$CH$_2$CH$_2$), 4.34–4.44 (m, C**H**CH$_2$O), 8.13 (d, *J* = 8.4 Hz, CH$_3$C(O)N**H**), the carboxylic acid proton was not detected; ^{13}C NMR (DMSO-*d*$_6$) δ 22.3 (**C**H$_3$C(O)), 32.4 (OCH$_2$**C**H$_2$CH$_2$O), 52.4 (**C**HCH$_2$OCH$_2$), 57.7 (OCH$_2$CH$_2$**C**H$_2$OH), 67.7 (CHCH$_2$O**C**H$_2$CH$_2$CH$_2$), 70.0 (CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 169.3, 171.7 (CH**C**(O)OH, CH$_3$**C**(O)NH); M_r (+ESI) 228.0842 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_5$Na$^+$ 228.0848). Anal. Calcd for C$_8$H$_{15}$NO$_5$•0.15H$_2$O: C, 46.62; H, 7.42; N, 6.74. Found: C, 46.26; H, 7.42; N, 6.55.

(*S*)-2-Acetamido-3-(3-hydroxypropoxy)propionic Acid ((*S*)-123). Using Method H, compound (*S*)-**122** (4.78 g, 16.2 mmol), 10% Pd/C (400 mg) in MeOH (50 mL) gave (*S*)-**123** as a pale yellow viscous oil that was used directly for next step: $[\alpha]^{25}_D$ +17.8° (*c* 1.2, MeOH); R_f = 0.23 (1/4 MeOH/CHCl$_3$); IR (neat) 3500–2200 (br), 1956, 1735, 1654, 1547, 1455, 1376, 1229, 1120 cm^{-1}; ^{1}H NMR (DMSO-*d*$_6$) δ 1.62 (quint, *J* = 6.6 Hz, OCH$_2$**CH$_2$**CH$_2$O), 1.87 (s, **CH$_3$**C(O)), 3.34–3.50 (m, O**CH$_2$**CH$_2$**CH$_2$**O), 3.55 (dd, *J* = 3.9, 9.9 Hz, C**H**H'OCH$_2$CH$_2$CH$_2$), 3.64 (dd, *J* = 5.7, 9.9 Hz, CH**H'**OCH$_2$CH$_2$CH$_2$), 4.34–4.44 (m, C**H**CH$_2$O), 8.12 (d, *J* = 8.1 Hz, CH$_3$C(O)N**H**), the carboxylic acid proton was not detected; ^{13}C NMR (DMSO-*d*$_6$) δ 22.3 (**C**H$_3$C(O)), 32.4 (OCH$_2$**C**H$_2$CH$_2$O), 52.3 (**C**HCH$_2$OCH$_2$), 57.7 (OCH$_2$CH$_2$**C**H$_2$OH), 67.8

(CHCH$_2$OCH$_2$CH$_2$CH$_2$), 69.9 (CHCH$_2$OCH$_2$CH$_2$CH$_2$), 169.3, 171.7 (CHC(O)OH,

CH$_3$C(O)NH); M_r (+ESI) 228.0845 [M+Na]$^+$ (calcd for C$_8$H$_{15}$NO$_5$Na$^+$ 228.0848). Anal.

Calcd for C$_8$H$_{15}$NO$_5$: C, 46.82; H, 7.37; N, 6.83. Found: C, 46.52; H, 7.42; N, 6.73.

(*R*)-2-Acetamido-3-(2-azidoethoxy)propionic Acid ((*R*)-124). Using Method B,

(*R*)-**112a** (1.56 g, 6.78 mmol) in THF (40 mL) and LiOH (195 mg, 8.14 mmol) in H$_2$O

(20 mL) gave 750 mg (51%) of (*R*)-**124** as a white solid upon work-up: mp 99–100

°C; [α]^{25}$_D$ -12.6° (*c* 1.8; MeOH); R_f = 0.21 (1/9 MeOH/CHCl$_3$); IR (nujol) 3356, 2119,

1735, 1625, 1547, 1457, 1267, 1232 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.07 (s, **CH$_3$**C(O)NH),

3.23–3.42 (m, **CH$_2$**N$_3$), 3.60–3.80 (m, O**CH$_2$**CH$_2$N$_3$, CHC**H'**HOCH$_2$), 4.01 (dd, *J* =

3.0, 9.1 Hz, CHC**H'**HO), 4.76–4.80 (m, **CH**CH$_2$O), 6.00–7.00 (m, CO$_2$**H**), 6.48 (d, *J* =

7.5 Hz, CH$_3$C(O)N**H**); ^{13}C NMR (DMSO-d_6) δ 22.3 (**CH$_3$**C(O)), 49.9 (OCH$_2$**CH$_2$**N$_3$),

52.2 (**CH**CH$_2$O), 69.4 (CH**CH$_2$**O or O**CH$_2$**CH$_2$N$_3$), 70.1 (O**CH$_2$**CH$_2$N$_3$ or CH**CH$_2$**O),

169.4, 171.5 (CH$_3$**C**(O)NH, **C**(O)OH); M_r (+ESI) 239.0750 [M+Na]$^+$ (calcd for

C$_7$H$_{12}$N$_4$O$_4$Na$^+$ 239.0756). Anal. Calcd for C$_7$H$_{12}$N$_4$O$_4$•0.07EtOAc: C, 39.30; H, 5.69;

N, 25.23. Found: C, 39.30; H, 5.73; N, 25.33.

(*S*)-2-Acetamido-3-(2-azidoethoxy)propionic Acid ((*S*)-124). Using Method B,

compound (*S*)-**112a** (1.67 g, 7.26 mmol) in THF (40 mL), and LiOH (209 mg, 8.71

mmol) in H$_2$O (20 mL) gave a white solid upon work-up and evaporation: mp 99–100

°C; [α]^{25}$_D$ +12.4° (*c* 1.3; MeOH); R_f = 0.21 (1/9 MeOH/CHCl$_3$); IR (nujol) 3356, 2119,

1735, 1624, 1547, 1458, 1267, 1233 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.86 (s,

CH$_3$C(O)NH), 3.30–3.45 (m, **CH$_2$**N$_3$), 3.50–3.78 (m, O**CH$_2$**CH$_2$N$_3$, CHC**H**H'OCH$_2$,

CHCH'HO), 4.39–4.48 (m, CHCH$_2$O), 8.13 (d, J = 8.1 Hz, CH$_3$C(O)NH); ^{13}C NMR

(DMSO-d_6) δ 22.3 (**CH$_3$**C(O)), 49.9 (OCH$_2$**CH$_2$**N$_3$), 52.2 (CH**CH$_2$**O), 69.4 (CH**CH$_2$**O

or O**CH$_2$**CH$_2$N$_3$), 70.1 (O**CH$_2$**CH$_2$N$_3$ or CH**CH$_2$**O), 169.4, 171.5 (CH$_3$**C**(O)NH,

C(O)OH); M_r (+ESI) 239.0750 [M+Na]$^+$ (calcd for C$_7$H$_{12}$N$_4$O$_4$Na$^+$ 239.0756). Anal.

Calcd for C$_7$H$_{12}$N$_4$O$_4$•0.07EtOAc: C, 39.30; H, 5.69; N, 25.23. Found: C, 39.28; H,

5.71; N, 25.37.

(*R*)-2-Acetamido-3-(2-(2-methoxyethoxy)ethoxy)propionic Acid ((*R*)-125). Using

Method B, a ~1:1 mixture of (*R*)-**114a** and (*R*)-**114b** (3.85 g, 14.2 mmol), LiOH (376

mg, 15.6 mmol) in THF:H$_2$O (160 mL) gave 2.44 g (68%) of (*R*)-**125** as a colorless

viscous oil after work-up: [α]$^{25}_D$ -38.5°(c 1.2, CHCl$_3$); R_f = 0–0.11 (1/9 MeOH/CHCl$_3$);

IR (neat) 3500-2500 (br), 1974, 1731, 1654, 1547, 1103 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ

2.08 (s, **CH$_3$**C(O)), 3.40 (s, OCH$_3$), 3.54–3.70 (m, O**CH$_2$CH$_2$**O**CH$_2$CH$_2$**OCH$_3$), 3.75

(dd, J = 3.3, 9.7 Hz, C**H**H'OCH$_2$), 3.98 (dd J = 3.3, 9.7 Hz, CH**H'**OCH$_2$), 4.68–4.78

(m, CHCH$_2$O), 6.98 (d, J = 7.8 Hz, C(O)NHCH), 9.10–9.50 (m, C(O)OH); ^{13}C NMR

(CDCl$_3$) δ 22.9 (**CH$_3$**C(O)), 53.0 (**C**HCH$_2$OCH$_2$CH$_2$), 59.0 (O**CH$_3$**), 70.3, 70.4, 70.7,

70.8, 72.1 (**CH$_2$**OCH$_2$CH$_2$OCH$_2$**CH$_2$**O), 171.4, 172.3 (**C**(O)OH, CH$_3$**C**(O)NH); M_r

(+ESI) 272.1107 [M+Na]$^+$ (calcd for C$_{18}$H$_{20}$N$_2$O$_3$Na$^+$ 272.1110). Anal. Calcd for

C$_{10}$H$_9$NO$_6$•0.33H$_2$O: C, 46.50; H, 7.81; N, 5.42. Found: C, 46.34; H, 7.74; N, 5.46.

2.2.4.5. Synthesis of *O*-substituted derivatives of lacosamide

R =	Compound number
Me	**1**
Et	**48**
i-Pr	**80**
t-Bu	**81**
C_6H_{11}	**82**
Ph	**83**
$CH_2C_6H_{11}$	**86**
CH_2Ph	**87**
$CH_2CH_2CH=CH_2$	**88**
CH_2CH_2 — (cyclopropyl)	**89**
$CH_2CH_2C{\equiv}CH$	**90**
CH_2CH_2Ph	**91**
CH_2CH_2OBn	**126**
CH_2CH_2OH	**127**
$CH_2CH_2CH_2OH$	**129**
$CH_2CH_2C(O)H$	**93**
CH_2CH_2 — (epoxide)	**94**
$CH_2CH_2NHC(O)CH_3$	**96**
$CH_2CH_2N_3$	**97**
CH_2CH_2—(triazole)—CH_2OCH_3	**98**
$CH_2CH_2OCH_3$	**99**
$(CH_2CH_2O)_2CH_3$	**100**
CD_3	**1**-*d*$_3$

Scheme 21. Different reactions used to obtain **55** derivatives after the amide coupling step (**route 3**).
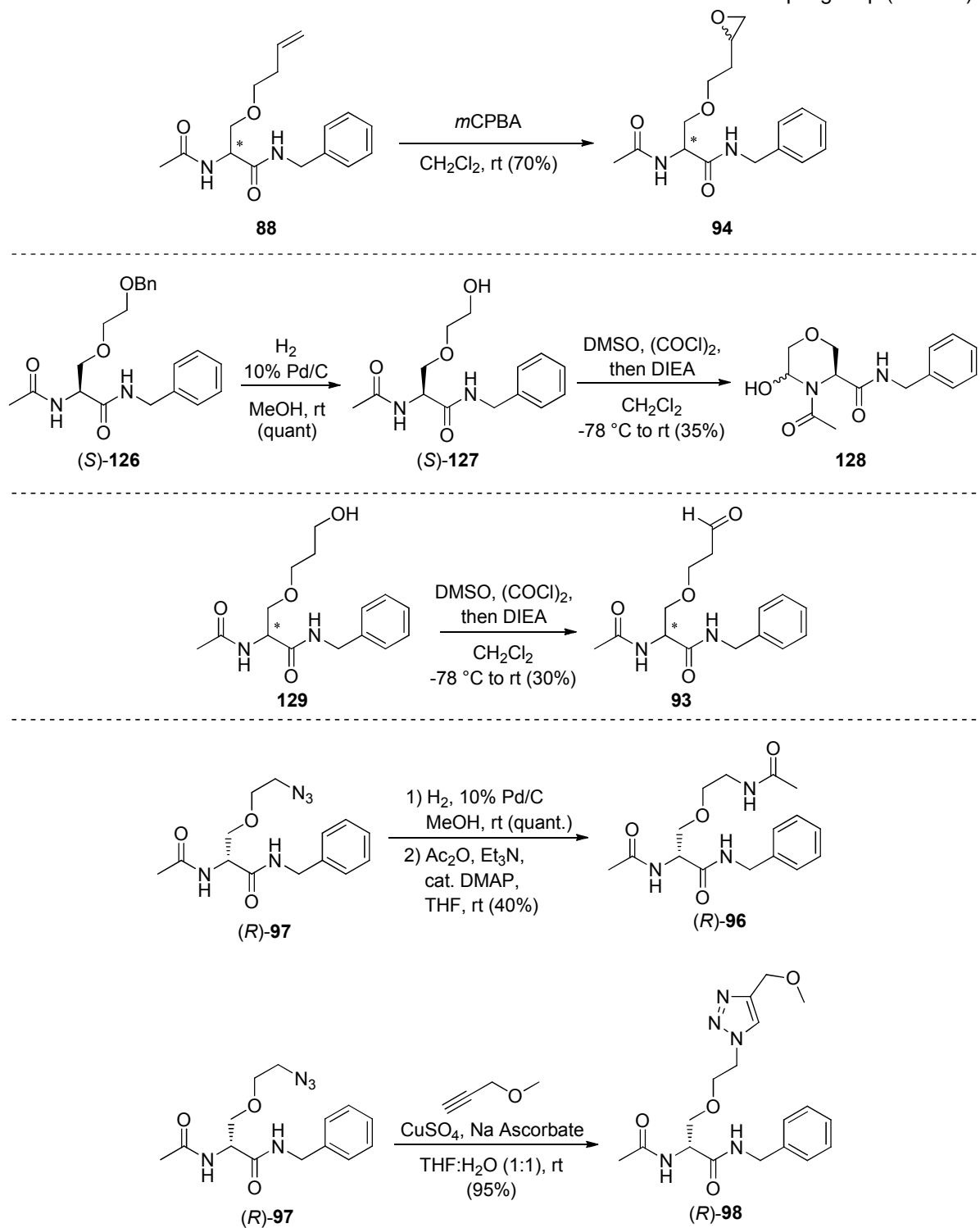
Scheme 22. Synthesis of the deuterated analog of (*R*)-lacosamide (*R*)-**1**-*d₃* (**route 1**)

(*R*)-**57**   (*R*)-**130**   (*R*)-**1**-*d₃*
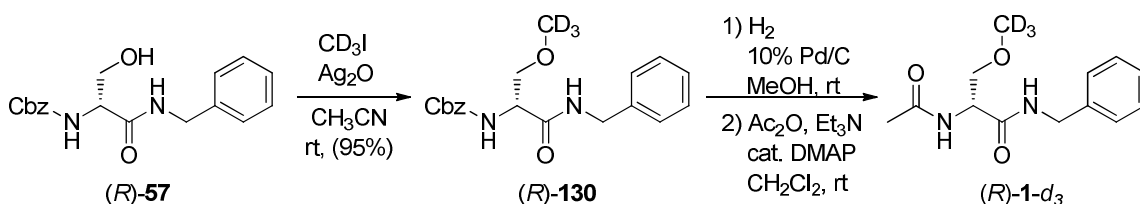
(*R*)-*N*-Benzyl 2-Acetamido-3-methoxypropionamide ((*R*)-1). Using Method D, (*R*)-**115** (100 mg, 0.62 mmol), benzylamine (81 μL, 0.74 mmol), and DMTMM (205 mg, 0.74 mmol) in anhydrous THF (10 mL) gave 95 mg (61%) of (*R*)-**1** after flash column chromatography (1/9 MeOH/CHCl₃) and recrystallization from EtOAc: mp 142–143 ℃ (lit.[235] mp 142–143℃); $[\alpha]^{25}_D$ +16.1° (*c* 0.9, MeOH) (lit.[235] $[\alpha]^{25}_D$ +16.0° (*c* 1.0; MeOH)); R_f = 0.47 (1/9 MeOH/CHCl₃); ¹H NMR (CDCl₃) δ 2.03 (s, **CH₃**C(O)), 3.37 (s, CH₂O**CH₃**), 3.46 (dd, *J* = 7.2, 8.4 Hz, C**H**H'OCH₃), 3.79 (dd *J* = 4.2, 8.4 Hz, CH**H'**OCH₃), 4.40–4.52 (m, NHC**H₂**C₆H₅), 4.52–4.60 (m, C**H**CH₂O), 6.40–6.60 (br m, CH₃C(O)N**H**), 6.78–6.92 (br m, C(O)N**H**CH₂Ph), 7.18–7.38 (m, NHCH₂C₆**H₅**), addition of excess (*R*)-(-)-mandelic acid to a CDCl₃ solution of (*R*)-**1** gave only one signal for the acetyl methyl protons and the methoxy protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl₃ solution of (*S*)-**1** and (*R*)-**1** (1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.023 (*S*) and 2.010 (*R*) (Δppm = 0.013)), and two signals for the methoxy protons (δ 3.311 (*S*) and 3.350 (*R*) (Δppm = 0.039)); ¹³C NMR (CDCl₃) δ 23.4 (**C**H₃C(O)), 43.7 (NH**C**H₂Ph), 52.6 (**C**HCH₂OCH₃), 59.3 (CH₂O**C**H₃), 71.9 (**C**H₂OCH₃), 127.6, 127.7, 138.1 (NHCH₂**C₆**H₅), 170.2, 170.5 (CH**C**(O)NH, CH₃**C**(O)NH), the remaining aromatic resonance was not detected and is believed to overlap with nearby signals

(*R*)-*N*-Benzyl 2-Acetamido-3-ethoxypropionamide ((*R*)-48). Using Method D, (*R*)-**116** (1.34 g, 7.7 mmol), benzylamine (1.00 mL, 9.2 mmol), and DMTMM (2.54 g, 9.2 mmol) in anhydrous THF (80 mL) gave 1.11 g (46%) of (*R*)-**48** as a white solid after flash column chromatography (8/92 MeOH/CHCl$_3$) and 2 recrystallizations from EtOAc: mp 129–130°C; [α]$^{25}_D$ -34.1° (*c* 0.6, CHCl$_3$); *R$_f$* = 0.35 (5/95 MeOH/CHCl$_3$); IR (nujol) 3283, 1634, 1555, 1456, 1375, 1114 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.15 (t, *J* = 7.2 Hz, OCH$_2$**CH$_3$**), 2.04 (s, **CH$_3$**C(O)), 3.44 (dd, *J* = 8.4, 9.3 Hz, C**H**H'OCH$_2$CH$_3$), 3.48–3.62 (m, OC**H$_2$**CH$_3$), 3.85 (dd *J* = 4.2, 9.3 Hz, CH**H'**OCH$_2$CH$_3$), 4.40–4.58 (m, C**H**CH$_2$OCH$_2$, NHC**H$_2$**C$_6$H$_5$), 6.40–6.50 (br d, CH$_3$C(O)N**H**), 6.78–6.90 (br t, C(O)N**H**CH$_2$Ph), 7.22–7.38 (m, NHCH$_2$C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**48** gave only one signal for the acetyl methyl protons (δ 2.017); ^{13}C NMR (CDCl$_3$) δ 15.1 (OCH$_2$**C**H$_3$), 23.3 (**C**H$_3$C(O)), 43.6 (NH**C**H$_2$Ph), 52.7 (**C**HCH$_2$OCH$_2$CH$_3$), 67.0 (CHCH$_2$O**C**H$_2$CH$_3$), 69.9 (CH**C**H$_2$OCH$_2$CH$_3$), 127.6, 127.7, 128.7, 138.1 (NHCH$_2$**C$_6$**H$_5$), 170.3, 170.5 (CH**C**(O)NH, CH$_3$**C**(O)NH); *M$_r$* (+ESI) 287.1374 [M+Na]$^+$ (calcd for C$_{14}$H$_{20}$N$_2$O$_3$Na$^+$ 287.1372). Anal. Calcd for C$_{14}$H$_{20}$N$_2$O$_3$: C, 63.62; H, 7.63; N, 10.60. Found: C, 63.62; H, 7.56; N, 10.47.

(*R*)-*N*-Benzyl 2-Acetamido-3-isopropoxypropionamide ((*R*)-80). Using Method D, (*R*)-**117** (1.90 g, 10.0 mmol), benzylamine (1.31 mL, 12.0 mmol), and DMTMM (3.32 g, 12.0 mmol) in anhydrous THF (100 mL) gave 2.01 g (72%) of (*R*)-**80** as a white solid after flash column chromatography (5/95 MeOH/CHCl$_3$) and recrystallization from EtOAc: mp 151–153°C; [α]$^{25}_D$ -23.4° (*c* 0.5, CHCl$_3$); *R$_f$* = 0.37 (5/95

MeOH/CHCl$_3$); IR (nujol) 3280, 3098, 1642, 1555, 1458, 1377, 1298, 1258, 1147

cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.09 (d, J = 6.0 Hz, OCHC**H$_3$**(C'H$_3$)), 1.13 (d, J = 6.0 Hz,

OCHCH$_3$(C'**H$_3$**)), 2.04 (s, **CH$_3$**C(O)), 3.40 (app t, J = 8.7 Hz, C**H**H'OCH(CH$_3$)$_2$), 3.63

(hept, J = 6.0 Hz, OC**H**(CH$_3$)$_2$), 3.84 (dd J = 3.9, 8.7 Hz, CH**H'**OCH(CH$_3$)$_2$), 4.38–

4.57 (m, C**H**CH$_2$OCH, NHC**H$_2$**C$_6$H$_5$), 6.42–6.50 (br d, CH$_3$C(O)N**H**), 6.82–6.94 (br t,

C(O)N**H**CH$_2$Ph), 7.24–7.38 (m, NHCH$_2$C$_6$**H$_5$**), addition of excess (R)-(-)-mandelic

acid to a CDCl$_3$ solution of (R)-**80** gave only one signal for the acetyl methyl protons

(δ 2.014); ^{13}C NMR (CDCl$_3$) δ 22.0 (OCH**C**H$_3$(C'H$_3$)), 22.2 (OCHCH$_3$(**C'**H$_3$)), 23.4

(**C**H$_3$C(O)), 43.7 (NH**C**H$_2$Ph), 52.9 (**C**HCH$_2$OCH(CH$_3$)$_2$), 67.5 (**C**H$_2$OCH(CH$_3$)$_2$), 72.7

(CH$_2$O**C**H(CH$_3$)$_2$), 127.7, 128.8, 138.1 (NHCH$_2$**C$_6$**H$_5$), 170.5 (CH**C**(O)NH or

CH$_3$**C**(O)NH), the remaining aromatic and C(O) peaks were not detected and are

believed to overlap with nearby signals; M_r (+ESI) 301.1530 [M+Na]$^+$ (calcd for

C$_{15}$H$_{22}$N$_2$O$_3$Na$^+$ 301.1528). Anal. Calcd for C$_{15}$H$_{22}$N$_2$O$_3$: C, 64.73; H, 7.97; N, 10.06.

Found: C, 64.56; H, 8.00; N, 10.12.

(R)-N-Benzyl 2-Acetamido-3-*tert*-butoxypropionamide ((R)-81). Using Method D,

(R)-**118** (1.10 g, 5.4 mmol), benzylamine (0.71 mL, 6.5 mmol), and DMTMM (1.80 g,

6.5 mmol) in anhydrous THF (50 mL) gave 730 mg (46%) of (R)-**81** as a white solid

after flash column chromatography (5/95 MeOH/CHCl$_3$) and recrystallization from

EtOAc: mp 126–127℃; [α]^{25}$_D$ -22.9° (c 0.9, CHCl$_3$); R_f = 0.39 (5/95 MeOH/CHCl$_3$);

IR (nujol) 3280, 3091, 1641, 1550, 1459, 1372, 1246, 1194, 1090 cm^{-1}; ^{1}H NMR

(CDCl$_3$) δ 1.14 (s, OC(C**H$_3$**)$_3$), 2.04 (s, **CH$_3$**C(O)), 3.40 (app t, J = 8.5 Hz,

C**H**H'OC(CH$_3$)$_3$), 3.84 (dd J = 4.2, 8.5 Hz, CH**H'**OC(CH$_3$)$_3$), 4.38–4.57 (m,

CHCH$_2$OC, NHC**H$_2$**C$_6$H$_5$), 6.40–6.50 (br d, CH$_3$C(O)N**H**), 6.80–6.92 (br t, C(O)N**H**CH$_2$Ph), 7.23–7.40 (m, NHCH$_2$C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**81** gave only one signal for the acetyl methyl protons (δ 2.009) and the *t*-Bu methyl protons (δ 1.112); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 27.6 (OC(**C**H$_3$)$_3$), 43.8 (NH**C**H$_2$Ph), 53.2 (**C**HCH$_2$OC(CH$_3$)$_3$), 61.7 (**C**H$_2$OC(CH$_3$)$_3$), 74.5 (CH$_2$O**C**(CH$_3$)$_3$), 127.7, 127.8, 128.8, 138.1 (NHCH$_2$**C$_6$**H$_5$), 170.3, 170.5 (CH**C**(O)NH, CH$_3$**C**(O)NH); M_r (+ESI) 315.1687 [M+Na]$^+$ (calcd for C$_{16}$H$_{24}$N$_2$O$_3$Na$^+$ 315.1685). Anal. Calcd for C$_{16}$H$_{24}$N$_2$O$_3$: C, 65.73; H, 8.27; N, 9.58. Found: C, 65.64; H, 8.08; N, 9.57.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-cyclohexyloxy)propionamide ((*R*)-82). Using Method B, a ~1:1 mixture of (*R*)-**105a** and (*R*)-**105b** (2.10 g, 8.4 mmol) in THF (80 mL) and LiOH (202 mg, 8.4 mmol) in H$_2$O (40 mL) gave upon work-up the corresponding acid (1.62 g, 7.1 mmol, 84%) as a yellow viscous oil (M_r (+ESI) 252.1212 [M+Na]$^+$ (calcd for C$_{11}$H$_{19}$NO$_4$Na$^+$ 252.1204)) that was directly dissolved in THF (70 mL). Using Method D, addition of benzylamine (930 μL, 8.5 mmol) and DMTMM (2.4 g, 8.5 mmol) gave (*R*)-**82** as a white solid (1.17 g, 65%) after purification by flash chromatography (1:2 hexanes/EtOAc to 1/9 MeOH/CH$_2$Cl$_2$) followed by recrystallization from EtOAc and hexanes: mp 134–135 °C; [α]$^{25}_D$ -33.5° (*c* 1.2; CHCl$_3$); R_f = 0.52 (EtOAc); IR (nujol) 3282, 2857, 1641, 1561, 1456, 1375, 1303, 1256 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.10–1.34, 1.42–1.88 (m, OCH(C**H$_2$**C**H$_2$**)$_2$C**H$_2$**), 2.03 (s, C**H$_3$**C(O)), 3.24–3.36 (m, OC**H**(CH$_2$CH$_2$)$_2$CH$_2$), 3.43 (app. t, *J* = 9.0 Hz, C**H**H'OCH$_2$CH), 3.86 (dd, *J* = 3.6, 9.0 Hz, CH**H'**OCH$_2$CH), 4.38–4.56 (m, NHC**H$_2$**Ph,

CHCH$_2$O), 6.48–6.58 (br d, CH$_3$C(O)N**H**), 6.92–7.02 (br t, N**H**CH$_2$Ph), 7.22–7.38 (m,

C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**82** gave

only one signal for the acetyl protons (δ 2.006); ^{13}C NMR (CDCl$_3$) δ 23.4, 23.9, 25.8

(**C**H$_3$C(O), OCH(CH$_2$**C**H$_2$)$_2$**C**H$_2$), 32.0, 32.2 (OCH(**C**H$_2$CH$_2$)$_2$CH$_2$), 43.8 (NH**C**H$_2$Ph),

52.9 (**C**HCH$_2$OCH), 67.3 (**C**H$_2$OCH(CH$_2$CH$_2$)$_2$CH$_2$), 78.4 (O**C**H(CH$_2$CH$_2$)$_2$CH$_2$),

127.7, 127.8, 128.9, 138.1 (**C$_6$**H$_5$), 170.4 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$), the remaining

resonance was not detected and is believed to overlap with a nearby signal; M_r

(+ESI) 341.3 [M+Na]$^+$ (calcd for C$_{18}$H$_{26}$N$_2$O$_3$Na$^+$ 341.3). Anal. Calcd for C$_{18}$H$_{26}$N$_2$O$_3$:

C, 67.90; H, 8.23; N, 8.80. Found: C, 67.86; H, 8.12; N, 8.74.

(*R*)-*N*-Benzyl 2-Acetamido-3-phenoxypropionamide (*R*)-83. Using Method A, (*R*)-

78 (2.66 g, 12.1 mmol), phenol (5.70 g, 60.6 mmol), and BF$_3$•Et$_2$O (1.52 mL, 12.1

mmol) in CH$_2$Cl$_2$ (50 mL) gave a crude residue that was recrystallized (3 x) from

EtOAc and hexanes to give (*R*)-**83** (340 mg, 8%) as a white solid. The mother

liquors from the recrystallization were concentrated and then purified using flash

chromatography (5/95 MeOH/CHCl$_3$). The obtained solid was recrystallized from

EtOAc and hexanes to yield an additional 100 mg (3%) of (*R*)-**83** (total yield: 440

mg, 11%): mp 169–170℃; [α]$^{25}_D$ -18.0° (*c* 0.4, MeOH); R_f = 0.52 (5/95

MeOH/CHCl$_3$); IR (nujol) 3288, 3073, 1687, 1551, 1458, 1375 cm^{-1}; ^{1}H NMR

(CDCl$_3$) δ 2.03 (s, **CH$_3$**C(O)), 4.05 (dd, *J* = 7.5, 9.6 Hz, C**H**H'OCH$_3$), 4.37 (dd *J* = 4.2,

9.6 Hz, CH**H'**OCH$_3$), 4.40–4.56 (m, NHC**H$_2$**C$_6$H$_5$), 4.78–4.86 (m, C**H**CH$_2$O), 6.66 (d,

J = 6.0 Hz, CH$_3$C(O)N**H**), 6.87 (d, *J* = 7.8 Hz, 3 Ar**H**) 6.98 (t, *J* = 7.8 Hz, 2 Ar**H**),

7.16–7.35 (m, CH$_2$C$_6$**H$_5$** and NHCH$_2$C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid

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to a CDCl$_3$ solution of (*R*)-**83** gave only one signal for the acetyl protons (δ 1.985); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.9 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.6 (**C**HCH$_2$OPh), 67.4 (**C**H$_2$OPh), 114.8, 121.9, 127.7, 127.8, 128.9, 129.8, 137.8, 157.9 (2 **C**$_6$H$_5$), 169.5, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)); M_r (+ESI) 335.1366 [M+Na]$^+$ (calcd for C$_{18}$H$_{20}$N$_2$O$_3$Na$^+$ 335.1372). Anal. Calcd for C$_{18}$H$_{20}$N$_2$O$_3$: C, 69.21; H, 6.45; N, 8.97; Found: C, 69.29; H, 6.52; N, 9.05.

(*R*)-*N*-Benzyl 2-Acetamido-3-phenoxypropionamide ((*R*)-83) (Alternate procedure). Using Method D, acid (*R*)-**119** (376 mg, 1.68 mmol), benzylamine (219 μL, 2.02 mmol), and DMTMM (557 mg, 2.02 mmol) in THF (20 mL) gave a (*R*)-**83** as a white solid (305 mg, 58%) after purification by flash chromatography (5/95 MeOH/CHCl$_3$) and further recrystallization from EtOAc: mp 169–170 ℃; [α]$^{25}_D$ -18.2° (*c* 1.0, MeOH); R_f = 0.52 (5/95 MeOH/CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 2.06 (s, **CH$_3$**C(O)), 4.03 (dd, *J* = 7.5, 9.6 Hz, C**H**H'OCH$_3$), 4.42 (dd *J* = 4.2, 9.6 Hz, CH**H'**OCH$_3$), 4.36–4.56 (m, NH**CH$_2$**C$_6$H$_5$), 4.78–4.84 (m, **C**HCH$_2$O), 6.53 (d, *J* = 6.0 Hz, CH$_3$C(O)N**H**), 6.61–6.71 (br t, C(O)N**H**CH$_2$Ph), 6.90 (d, *J* = 7.8 Hz, OC$_6$**H$_5$**), 7.00 (t, *J* = 7.8 Hz, OC$_6$**H$_5$**), 7.20–7.32 (m, CH$_2$C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**83** gave only one signal for the acetyl peak protons (δ 2.002).

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-cyclohexylmethoxy)propionamide ((*R*)-86). Using Method B, a ~1:4 mixture of (*R*)-**107a** and (*R*)-**107b** (2.32 g, 8.6 mmol) in THF (85 mL) and LiOH (207 mg, 8.6 mmol) in H$_2$O (40 mL) gave upon work-up the corresponding acid (1.88 g, 7.7 mmol, 90%) as a yellow viscous that was directly

dissolved in THF (75 mL). Using Method D, addition of benzylamine (1.0 mL, 9.2 mmol) and DMTMM (2.54 g, 9.2 mmol) gave (*R*)-**86** as a white solid (1.50 g, 59%) after purification by flash chromatography (1/2 hexanes/EtOAc to 1/9 MeOH/CH$_2$Cl$_2$) followed by recrystallization from EtOAc and hexanes: mp 143–144 °C; [α]$^{25}_D$ +6.7° (*c* 1.6; MeOH); R_f = 0.39 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3280, 3092, 2861, 1641, 1550, 1459, 1372, 1301, 1247 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 0.74–0.90, 1.00–1.26, 1.40–1.72 (m, OCH$_2$**CH**(CH$_2$CH$_2$)$_2$**CH$_2$**), 2.02 (s, **CH$_3$**C(O)), 3.16–3.32 (m, O**CH$_2$**CH(CH$_2$CH$_2$)$_2$CH$_2$), 3.42 (app. t, *J* = 9.0 Hz, C**H**H'OCH$_2$CH), 3.79 (dd, *J* = 3.6, 9.0 Hz, CH**H'**OCH$_2$), 4.38–4.50 (m, NH**CH$_2$**Ph), 4.51–4.60 (m, C**H**CH$_2$O), 6.53 (d, *J* = 6.3 Hz, CH$_3$C(O)N**H**), 6.88–7.00 (br t, **NH**CH$_2$Ph), 7.22–7.38 (m, C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**86** gave only one signal for the acetyl protons (δ 2.008); ^{13}C NMR (CDCl$_3$) δ 23.4 (**CH$_3$**C(O)), 25.9, 26.6, OCH$_2$CH(CH$_2$**CH$_2$**)$_2$**CH$_2$**), 30.0, 30.1 (OCH$_2$CH(**CH$_2$**CH$_2$)$_2$CH$_2$), 38.0 (OCH$_2$**CH**(CH$_2$CH$_2$)$_2$CH$_2$), 43.9 (NH**CH$_2$**Ph), 52.5 (**C**HCH$_2$OCH$_2$), 70.2 (**C**H$_2$OCH$_2$CH(CH$_2$CH$_2$)$_2$CH$_2$), 77.5 (O**CH$_2$**CH(CH$_2$CH$_2$)$_2$CH$_2$), 127.7, 127.8, 128.9, 138.0 (**C$_6$**H$_5$), 170.3, 170.4 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 355.1998 [M+Na]$^+$ (calcd for C$_{19}$H$_{28}$N$_2$O$_3$Na$^+$ 355.1997). Anal. Calcd for C$_{19}$H$_{28}$N$_2$O$_3$: C, 68.65; H, 8.49; N, 8.43. Found: C, 68.52; H, 8.43; N, 8.38.

(*R*)-*N*-Benzyl 2-Acetamido-3-(benzyloxy)propionamide (*R*)-87. To a stirred suspension of *O*-benzyl-D-serine (Astatech Inc.) (1.00 g, 5.13 mmol) in a 9:1 THF:H$_2$O mixture (50 mL) was added Ac$_2$O (1.45 mL, 15.3 mmol) at room temperature. The reaction became clear within 30 min and was further stirred at

room temperature (3 h). The solvents were removed in vacuo to yield a viscous clear residue (1.22 g, 5.13 mmol, quant.) that was directly dissolved in THF (60 mL). Using Method D, benzylamine (670 μL, 6.2 mmol) and DMTMM (1.71 g, 6.2 mmol) gave (R)-**87** as a white solid (1.55 g, 93% for 2 steps) upon purification by flash chromatography (EtOAc to 1/9 MeOH/CH$_2$Cl$_2$): mp 145–146 °C; $[\alpha]^{25}_D$ -28.7° (c 0.7; CHCl$_3$); R_f = 0.49 (EtOAc); IR (nujol) 3291, 2862, 1635, 1547, 1457, 1375, 1310, 1248 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.01 (s, **CH$_3$**C(O)), 3.51 (dd, J = 7.8, 9.0 Hz, C**H**H'OCH$_2$Ph), 3.90 (dd, J = 4.2, 9.0 Hz, CH**H'**OCH$_2$Ph), 4.38–4.64 (m, NHC**H$_2$**Ph, OC**H$_2$**Ph, C**H**CH$_2$O), 6.40–6.52 (br d, CH$_3$C(O)N**H**), 6.72–6.84 (br t, N**H**CH$_2$Ph), 7.18–7.38 (m, 2 C$_6$**H$_5$**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**87** gave only one signal for the acetyl protons (δ 1.996); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.9 (NH**C**H$_2$Ph), 52.5 (**C**HCH$_2$OCH$_2$), 69.6, 73.8 (**C**H$_2$O**C**H$_2$Ph), 127.7, 127.8, 128.1, 128.2, 128.6, 128.9, 137.4, 137.9 (2 **C$_6$**H$_5$), 170.1, 170.4 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 349.2 [M+Na]$^+$ (calcd for C$_{19}$H$_{22}$N$_2$O$_3$Na$^+$ 349.2). Anal. Calcd for C$_{19}$H$_{22}$N$_2$O$_3$: C, 69.92; H, 6.79; N, 8.58. Found: C, 70.30; H, 6.86; N, 8.64.

(R)-N-Benzyl 2-Acetamido-3-(but-3-enyloxy)propionamide ((R)-88). Using the Method D, (R)-**120** (1.32 g, 6.57 mmol), benzylamine (860 μL, 7.88 mmol), and DMTMM (2.18 g, 7.88 mmol) gave (R)-**88** (1.30 g, 67%) as a white solid after purification by flash chromatography (EtOAc) and further recrystallization from EtOAc and hexanes: mp 103–104 °C; $[\alpha]^{25}_D$ +51.3° (c 0.6; MeOH); R_f = 0.37 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3291, 3099, 1637, 1554, 1456, 1375, 1117 cm^{-1}; ^{1}H NMR

(CDCl$_3$) δ 2.03 (s, **CH$_3$**C(O)), 2.22–2.36 (m, CH$_2$**CH$_2$**CH=CH$_2$), 3.40–3.62 (m, CH$_2$OC**H$_2$**CH$_2$, C**H**H'OCH$_2$CH$_2$), 3.85 (dd, J = 4.2, 9.0 Hz, CH**H'**OCH$_2$CH$_2$), 4.45 (d, J = 7.2 Hz, NHC**H$_2$**Ph), 4.48–4.57 (m, C**H**CH$_2$O), 4.92–5.06 (m, CH$_2$CH=C**H$_2$**), 5.62–5.74 (m, CH$_2$C**H**=CH$_2$), 6.42–6.56 (m, N**H**CHCH$_2$O), 6.84–6.96 (br t, N**H**CH$_2$Ph), 7.20–7.36 (m, C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**88** gave only one signal for the acetyl protons (δ 2.011 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**88** and (*R*)-**88** in a ~1:2 ratio gave two signals with a relative ~1:2 intensity for the acetyl protons (δ 2.027 ppm (*S*)-**88**, δ 2.014 ppm (*R*)-**88**, (Δppm = 0.013)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**CH$_3$**C(O)), 34.2 (CH$_2$**CH$_2$**CH=CH$_2$), 43.8 (NH**CH$_2$**Ph), 52.5 (**C**HC(O)NH), 69.9, 70.6 (CH**CH$_2$**O**CH$_2$**), 117.0 (CH$_2$CH$_2$CH=**C**H$_2$), 127.7, 128.9, 135.3, 138.1 (**C$_6$**H$_5$, CH$_2$CH$_2$**C**H=CH$_2$), 170.2, 170.4 (CH$_3$**C**(O), CH**C**(O)NH), the remaining peak was not detected and is believed to overlap with nearby signals; M_r (+ESI) 313.1528 [M+Na]$^+$ (calcd for C$_{16}$H$_{22}$N$_2$O$_3$Na$^+$ 313.1528). Anal. Calcd for C$_{16}$H$_{22}$N$_2$O$_3$: C, 66.18; H, 7.64; N, 9.65. Found: C, 65.98; H, 7.55; N, 9.68.

(*S*)-*N*-Benzyl 2-Acetamido-3-(but-3-enyloxy)propionamide ((*S*)-88). Using Method D, (*S*)-**120** (164 mg, 0.87 µmol), benzylamine (113 μL, 1.04 mmol), and DMTMM (287 mg, 1.04 mmol) in THF (10 mL) gave (*S*)-**88** (150 mg, 60%) as a white solid after purification by flash chromatography (EtOAc) and further recrystallization from EtOAc and hexanes: mp 103–104 °C; [α]^{25}$_D$ -50.9° (c 0.6; MeOH); R_f = 0.37 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3298, 3093, 2861, 1637, 1553, 1455, 1375, 1117 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 2.22–2.36 (m, CH$_2$**CH$_2$**CH=CH$_2$), 3.40–

3.62 (m, CH$_2$OC**H$_2$**CH$_2$, C**H**H'OCH$_2$CH$_2$), 3.84 (dd, J = 4.2, 9.0 Hz, CH**H'**OCH$_2$CH$_2$),

4.45 (d, J = 7.2 Hz, NHC**H$_2$**Ph), 4.48–4.57 (m, C**H**CH$_2$O), 4.92–5.06 (m,

CH$_2$CH=C**H$_2$**), 5.62–5.74 (m, CH$_2$C**H**=CH$_2$), 6.48–6.60 (m, N**H**CHCH$_2$O), 6.88–7.00

(br t, N**H**CH$_2$Ph), 7.20–7.36 (m, C$_6$**H$_5$**), addition of excess (R)-(-)-mandelic acid to a

CDCl$_3$ solution of (S)-**88** gave only one signal for the acetyl protons (δ 2.026 ppm),

addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**88** and (R)-**88** in a

~1:2 ratio gave two signals with a relative ~1:2 intensity for the acetyl protons (δ

2.027 ppm (S)-**88**, δ 2.014 ppm (R)-**88**, (Δppm = 0.013)); ^{13}C NMR (CDCl$_3$) δ 23.2

(**C**H$_3$C(O)), 34.1 (CH$_2$**C**H$_2$CH=CH$_2$), 43.7 (NH**C**H$_2$Ph), 52.5 (**C**HC(O)NH), 69.9, 70.6

(CH**C**H$_2$O**C**H$_2$), 117.0 (CH$_2$CH$_2$CH=**C**H$_2$), 127.6, 127.7, 128.8, 134.9, 138.3 (**C$_6$**H$_5$,

CH$_2$CH$_2$**C**H=CH$_2$), 170.2, 170.4 (CH$_3$**C**(O), CH**C**(O)NH); M_r (+ESI) 313.1530

[M+Na]$^+$ (calcd for C$_{16}$H$_{22}$N$_2$O$_3$Na$^+$ 313.1528). Anal. Calcd for C$_{16}$H$_{22}$N$_2$O$_3$: C, 66.18;

H, 7.64; N, 9.65. Found: C, 66.27; H, 7.51; N, 9.51.

(R)-N-Benzyl 2-Acetamido-3-(2-cyclopropylethoxy)propionamide ((R)-89). Using

Method B, a ~1:1 mixture of (R)-**109a** and (R)-**109b** (1.60 g, 6.6 mmol) in THF (60

mL) and LiOH (158 mg, 6.6 mmol) in H$_2$O (30 mL) gave upon work-up the

corresponding acid (1.27 g, 5.9 mmol, 90%) as a yellow oil (M_r (+ESI) 238.1056

[M+Na]$^+$ (calcd for C$_{10}$H$_{17}$NO$_4$Na$^+$ 238.1054)) that was directly dissolved in THF (60

mL). Using Method D, addition of benzylamine (740 μL, 7.13 mmol) and DMTMM

(2.0 g, 7.13 mmol) gave (R)-**89** as a white solid (1.17 g, 65%) after purification by

flash chromatography (1:2 hexanes/EtOAc to 1/9 MeOH/CH$_2$Cl$_2$) followed by

recrystallization from EtOAc and hexanes: mp 97–99 ℃; [α]^{25}$_D$ -32.1° (c 1.5;

MeOH); R_f = 0.52 (EtOAc); IR (nujol) 3292, 2859, 1640, 1549, 1457, 1376, 1303, 1249 cm⁻¹; ¹H NMR (CDCl₃) δ -0.08–0.01 (m, CH(C**H**H'C**H**H')), 0.30–0.38 (m, CH(CH**H'**CH**H'**)), 0.50–0.64 (m, C**H**(CH₂CH₂)), 1.32–1.48 (m, **CH₂**CH(CH₂CH₂), 2.02 (s, **CH₃**C(O)), 3.40–3.62 (m, OC**H₂**CH₂CH(CH₂CH₂), C**H**H'OCH₂CH₂), 3.84 (dd, J = 3.9, 9.0 Hz, CH**H'**OCH₂CH₂), 4.38–4.58 (m, NHC**H₂**Ph, C**H**CH₂O), 6.48–6.58 (br d, CH₃C(O)N**H**), 6.90–7.00 (br t, N**H**CH₂Ph), 7.20–7.36 (m, C₆**H₅**), addition of excess (R)-(-)-mandelic acid to a CDCl₃ solution of (R)-**89** gave only one signal for the acetyl protons (δ 2.011); ¹³C NMR (CDCl₃) δ 4.3, 4.4 (CH(**CH₂CH₂**)), 8.0 (**C**H(CH₂CH₂)), 23.4 (**CH₃**C(O)), 34.7 (**C**H₂CH(CH₂CH₂)), 43.8 (NH**C**H₂Ph), 52.5 (**C**HCH₂OCH₂), 70.1, 71.8 (**C**H₂O**C**H₂CH₂), 127.7, 128.9, 138.0 (**C₆**H₅), 170.3, 170.4 (CH₃**C**(O)NH, **C**(O)NHCH₂), the remaining resonance was not detected and is believed to overlap with nearby signals; M_r (+ESI) 327.2 [M+Na]⁺ (calcd for C₁₇H₂₄N₂O₃Na⁺ 327.2). Anal. Calcd for C₁₇H₂₄N₂O₃: C, 67.08; H, 7.95; N, 9.20. Found: C, 67.06; H, 7.94; N, 9.27.

(R)-N-Benzyl   2-Acetamido-3-(but-3-ynyloxy)propionamide   ((R)-90). Using Method A, compound (R)-**78** (1.14 g, 5.22 mmol), homopropargyl alcohol (2.0 mL, 26.1 mmol), and BF₃•Et₂O (500 μL, 3.97 mmol) in CH₂Cl₂ (30 mL) gave (R)-**90** as a white solid (450 mg, 30%) upon work-up, purification by flash chromatography (2/1 EtOAc/CH₂Cl₂), and subsequent recrystallization from EtOAc and hexanes: mp 120–122 °C; $[\alpha]^{25}_D$ -49.1° (c 0.8, CHCl₃); R_f = 0.38 (2/1 EtOAc/CH₂Cl₂); IR (CHCl₃ film) 3088, 2932, 2867, 1746, 1640, 1548, 1449, 1377, 1303, 1243 cm⁻¹; ¹H NMR (CDCl₃) δ 1.80 (t, J = 2.7 Hz, CH₂C≡C**H**), 2.04 (s, **CH₃**C(O)), 2.38–2.46 (m, OCH₂**CH₂**), 3.46–3.74 (m,   CHH'OC**H₂**CH₂C≡CH), 3.90 (dd, J = 3.9, 9.0 Hz,

CHH'OCH$_2$CH$_2$C≡CH), 4.38–4.58 (m, NHCH$_2$C$_6$H$_5$, CHCH$_2$O), 6.48–6.56 (m, NHCHCH$_2$O), 6.92–7.06 (m, NHCH$_2$Ph), 7.20–7.38 (m, NHCH$_2$C$_6$H$_5$), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**90** gave only one signal for the acetyl protons (δ 2.006); ^{13}C NMR (CDCl$_3$) δ 20.0 (**C**H$_2$C≡CH), 23.4 (**C**H$_3$C(O)), 43.8 (NH$_2$**C**H$_2$Ph), 52.4 (**C**HCH$_2$OCH$_2$), 69.3, 69.8, 70.0 (**C**H$_2$O**C**H$_2$CH$_2$C≡CH), 81.6 (CH$_2$**C**≡CH), 127.7, 127.8, 128.9, 138.1 (**C$_6$**H$_5$), 170.1, 170.4 (CH**C**(O)NH, CH$_3$**C**(O)); M_r (+ESI) 421.1 [M+Cs]$^+$ (calcd for C$_{16}$H$_{20}$N$_2$O$_3$Cs$^+$ 421.1). Anal. Calcd for C$_{16}$H$_{20}$N$_2$O$_3$: C, 66.65; H, 6.99; N, 9.72. Found: C, 66.38; H, 6.90; N, 9.65.

(R)-N-Benzyl 2-Acetamido-3-phenethoxypropionamide ((R)-91). Using Method A, compound (R)-**78** (2.0 g, 9.2 mmol), phenethyl alcohol (6.0 mL, 49.2 mmol), and BF$_3$•Et$_2$O (1.0 mL, 8.0 mmol) in CH$_2$Cl$_2$ (30 mL) gave (R)-**91** as a white solid (392 mg, 10%) upon work-up and purification by flash chromatography (2/1 EtOAc/CH$_2$Cl$_2$) and recrystallization from EtOAc and hexanes: mp 90–92 °C; [α]^{25}$_D$ -29.2° (c 0.5, CHCl$_3$); R_f = 0.44 (3/1 EtOAc/CH$_2$Cl$_2$); IR (nujol) 3285, 3124, 1639, 1547, 1457, 1375, 1303, 1118 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.97 (s, CH$_3$C(O)), 2.84 (d, J = 6.3 Hz, OCH$_2$C**H$_2$**Ph), 3.40 (app d, J = 8.1 Hz, CHH'OCH$_2$CH$_2$Ph), 3.62–3.92 (m,CHH'OC**H$_2$**CH$_2$Ph), 4.20–4.38 (m, NHC**H$_2$**C$_6$H$_5$), 4.40–4.50 (m, CHCH$_2$O), 6.32–6.40 (m, NHCHCH$_2$O), 6.50–6.62 (m, NHCH$_2$Ph), 7.12–7.38 (m, CH$_2$CH$_2$C$_6$**H$_5$**, NHCH$_2$C$_6$**H$_5$**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**91** gave only one signal for the acetyl protons (δ 1.988); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 36.2 (CH$_2$**C**H$_2$Ph), 43.6 (NH$_2$**C**H$_2$Ph), 52.4 (**C**HCH$_2$OCH$_2$), 70.0, 72.1 (**C**H$_2$O**C**H$_2$CH$_2$), 126.6, 127.5, 127.6, 128.7, 128.8, 129.0, 138.2, 139.0 (2 **C$_6$**H$_5$),

170.2, 170.4 (CH**C**(O)NH, CH$_3$**C**(O)); M_r (+ESI) 363.2 [M+Na]$^+$ (calcd for C$_{20}$H$_{24}$N$_2$O$_3$Na$^+$ 363.2). Anal. Calcd C$_{20}$H$_{24}$N$_2$O$_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.40; H, 7.08; N, 8.15.

(*S*)-*N*-Benzyl 2-Acetamido-3-(2-(benzyloxy)ethoxy)propionamide ((*S*)-126). Using Method D, acid (*S*)-**121** (386 mg, 1.37 mmol), benzylamine (165 μL, 1.51 mmol) and DMTMM (418 mg, 1.51 mmol) in THF (14 mL) gave 365 mg (72%) of (*S*)-**126** as a white solid after purification by silica gel flash chromatography (5/95, MeOH/CHCl$_3$): mp 86–88 °C, [α]$^{25}_D$ +30.0° (*c* 1.0, CHCl$_3$); R_f = 0.56 (5/95 MeOH/CHCl$_3$); IR (nujol) 3284, 1637, 1560 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.86 (s, CH$_3$C(O)NH), 3.52–3.62 (m, OC**H$_2$**C**H$_2$**OBn, CHC**H$_2$**OCH$_2$), 4.20–4.38 (m, NHC**H$_2$**Ph), 4.46–4.54 (m, OC**H$_2$**Ph, CHC**H$_2$**OCH$_2$), 7.19–7.40 (m, 2 C$_6$**H$_5$**), 8.08 (d, J = 8.1 Hz, N**H**CHCH$_2$O), 8.51 (t, J = 6.0 Hz, N**H**CH$_2$Ph); ^{13}C NMR (DMSO-d_6) δ 22.5 (**C**H$_3$C(O)), 42.0 (NH**C**H$_2$Ph), 52.8 (**C**HCH$_2$O), 68.9, 69.8, 70.7 (O**C**H$_2$**C**H$_2$O, CH**C**H$_2$O), 72.0 (O**C**H$_2$Ph), 126.6, 126.9, 127.3, 127.4, 128.1, 128.2, 138.4, 139.2 (2 **C$_6$**H$_5$), 169.3, 169.7 (CH$_3$**C**(O)NH and **C**(O)OH); M_r (+ESI) 393.1807 [M+Na]$^+$ (calcd for C$_{21}$H$_{26}$N$_2$O$_4$Na$^+$ 393.1790). Anal. Calcd for C$_{21}$H$_{26}$N$_2$O$_4$•0.1H$_2$O: C, 67.76; H, 7.09; N, 7.53. Found: C, 67.57; H, 7.08; N, 7.90.

(*S*)-*N*-Benzyl 2-Acetamido-3-(2-hydroxyethoxy)propionamide ((*S*)-127). Using Method H, (*S*)-**126** (347 mg, 0.94 mmol), and 5% Pd/C (100 mg) in MeOH (12 mL) gave a residue that was purified using SiO$_2$ flash chromatography (5/95 MeOH/CHCl$_3$) to yield (*S*)-**127** as a colorless residue that slowly turned into a white

solid (193 mg, 73%): mp 100–102 °C, $[\alpha]^{25}_D$ -15.9° (c 0.2; MeOH); R_f = 0.28 (5/95 MeOH/CHCl$_3$); IR (neat) 3400–3000 (br), 2928, 1650, 1542, 1456, 1373 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.98 (s, **CH$_3$**C(O)NH), 3.20 (br s, CH$_2$O**H**), 3.52–3.78 (m, OC**H$_2$CH$_2$**OH, CHC**H**H'OCH$_2$), 3.82–3.88 (CHCH**H'**OCH$_2$), 4.38–4.50 (m, NHC**H$_2$**Ph), 4.60–4.68 (m, C**H**CH$_2$OCH$_2$), 6.84 (d, J = 7.2 Hz, N**H**CHCH$_2$O), 7.21–7.40 (m, C$_6$**H$_5$**, N**H**CH$_2$Ph); ^{13}C NMR (CDCl$_3$) δ 22.5 (**C**H$_3$C(O)), 42.0 (NH**C**H$_2$Ph), 52.9 (**C**HCH$_2$O), 61.7 (**C**H$_2$OH), 70.4 (O**C**H$_2$CH$_2$), 72.8 (CH**C**H$_2$O), 127.7, 128.9, 138.1 (**C$_6$**H$_5$), 170.3 (CH$_3$**C**(O)NH or **C**(O)OH), 170.9 (**C**(O)OH or CH$_3$**C**(O)NH), the remaining aromatic resonance was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 303.1314 [M+Na]$^+$ (calcd for C$_{14}$H$_{20}$N$_2$O$_4$Na$^+$ 303.1321). Anal. Calcd for C$_{14}$H$_{20}$N$_2$O$_4$•0.25H$_2$O: C, 59.04; H, 7.25; N, 9.84. Found: C, 58.80; H, 7.40; N, 9.99.

(3S)-N-Benzyl 4-Acetyl-5-hydroxymorpholine-3-carboxamide (S)-128. Using Method E, (COCl)$_2$ (63 μL, 732 μmol) in CH$_2$Cl$_2$ (1 mL), DMSO (105 μL, 1.46 mmol) in CH$_2$Cl$_2$ (1 mL) and (S)-**127** (189 mg, 665 μmol) in CH$_2$Cl$_2$ (10 mL) followed by DIEA (580 μL, 3.325 mmol) gave after work-up a residue that was purified using flash chromatography (6/94 MeOH/CHCl$_3$) to yield 53 mg (28%) of (S)-**128** a white solid : mp 146–148 °C, $[\alpha]^{25}_D$ -18.8° (c 0.9; MeOH); R_f = 0.26 (5/95 MeOH/CHCl$_3$); IR (CH$_2$Cl$_2$ film) 3400–3000 (br), 3283, 3102, 1646, 1575, 1460, 1403, 1133, 1056 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.17 (s, **CH$_3$**C(O)N), 3.53 (dd, J = 2.1, 12.0 Hz, CH(OH)CH$_2$OC**H**H'), 3.65 (dd, J = 4.3, 12.3 Hz CH(OH)C**H**H'), 3.80 (d, J = 12.0 Hz, CH(OH)CH**H'**), 4.07 (d, J = 12.3 Hz, CH(OH)CH$_2$OCH**H'**), 4.34 (app. d, J = 6.0 Hz,

NHC**H₂**Ph), 4.69 (d, *J* = 4.3 Hz, C**H**CH₂OCH₂), 5.14 (br d, *J* = 8.1 Hz, NC**H**(OH)CH₂O), 7.00 (d, *J* = 8.1 Hz, NCH(O**H**)CH₂O), 7.20–7.36 (m, C₆**H₅**), 8.98 (t, *J* = 6.0 Hz, N**H**CH₂Ph); ¹³C NMR (DMSO-*d₆*) δ 20.4 (**C**H₃C(O)), 42.5 (NH**C**H₂Ph), 53.1 (**C**HCH₂O), 68.1 (CH**C**H₂OCH₂), 71.7 (O**C**H₂CH(OH)N), 73.2 (OCH₂**C**H(OH)N), 126.9, 127.0, 128.4, 138.5 (**C**₆H₅), 172.1 (CH₃**C**(O)N or **C**(O)NHCH₂), 172.5 (**C**(O) NHCH₂ or CH₃**C**(O)N); *M*ᵣ (+ESI) 301.1157 [M+Na]⁺ (calcd for C₁₄H₁₈N₂O₄Na⁺ 301.1164). Anal. Calcd for C₁₄H₁₈N₂O₄: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.12; H, 6.55; N, 9.81.

(*R*)-*N*-Benzyl 2-Acetamido-3-(3-hydroxypropoxy)propionamide ((*R*)-129). Using Method D, acid (*R*)-**123** (1.07 g, 5.22 mmol), benzylamine (0.68 mL, 6.26 mmol), and DMTMM (1.73 g, 6.26 mmol) in anhydrous THF (50 mL) gave 1.34 g (87%) of (*R*)-**129** as a white solid after flash column chromatography (8/92 MeOH/CHCl₃): mp 90–92 ℃; [α]²⁵_D +8.0°(*c* 1.0, MeOH); *R*ₑ = 0.38 (8/92 MeOH/CHCl₃); IR (CH₂Cl₂ film) 3313, 3059, 2932, 2876, 1660, 1532, 1454, 1374, 1266, 1118 cm⁻¹; ¹H NMR (CDCl₃) δ 1.76 (quint, *J* = 6.0 Hz, OCH₂C**H₂**CH₂O), 1.98 (s, C**H₃**C(O)), 3.56 (dd, *J* = 6.6, 9.3 Hz, C**H**H'OCH₂CH₂CH₂), 3.58–3.68 (m, OC**H₂**CH₂C**H₂**O), 3.78 (dd, *J* = 3.9, 9.3 Hz, CH**H'**OCH₂CH₂CH₂), 4.36–4.52 (m, NHC**H₂**Ph), 4.60–4.65 (m, C**H**CH₂O), 6.76 (d, *J* = 7.2 Hz, CH₃C(O)N**H**), 7.20–7.38 (m, C₆**H₅**, C(O)N**H**CH₂); ¹³C NMR (CDCl₃) δ 23.1 (**C**H₃C(O)), 32.1 (OCH₂**C**H₂CH₂O), 43.6 (NH**C**H₂Ph), 52.7 (**C**HCH₂OCH₂), 60.3 (OCH₂CH₂**C**H₂OH), 69.3 (CHCH₂O**C**H₂CH₂CH₂), 70.6 (CH**C**H₂OCH₂CH₂CH₂), 127.5, 127.7, 128.8, 138.1 (**C**₆H₅), 170.3, 170.9 (CH**C**(O)NH, CH₃**C**(O)NH); *M*ᵣ (+ESI) 317.1477 [M+Na]⁺ (calcd for C₁₅H₂₂N₂O₄Na⁺

317.1477). Anal. Calcd for $C_{15}H_{22}N_2O_4 \bullet 0.15H_2O$: C, 60.65; H, 7.57; N, 9.43. Found: C, 60.40; H, 7.56; N, 9.67.

(*S*)-*N*-Benzyl 2-Acetamido-3-(3-hydroxypropoxy)propionamide ((*S*)-129). Using Method D, acid (*S*)-**123** (1.52 g, 7.41 mmol), benzylamine (0.97 mL, 8.9 mmol), and DMTMM (2.46 g, 8.9 mmol) in anhydrous THF (75 mL) gave 1.51 g (69%) of (*S*)-**129** as a white solid after flash column chromatography (8/92 MeOH/CHCl$_3$). The solid contained an unidentified impurity (~20 mol percent based on ^{1}H NMR integrations) that co-eluted using different solvent systems and could not be removed by recrystallization: mp 87–90 °C; $[\alpha]^{25}_D$ -7.5° (*c* 1.0, MeOH); R_f = 0.38 (8/92 MeOH/CHCl$_3$); IR (nujol) 3300–2600 (br), 3278, 1638, 1553, 1458, 1375, 1306, 1245, 1123 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.75 (quint, *J* = 5.7 Hz, OCH$_2$**CH$_2$**CH$_2$O), 1.98 (s, **CH$_3$**C(O)), 2.44-2.60 (br s, CH$_2$O**H**), 3.53 (dd, *J* = 6.9, 9.6 Hz, C**H**H'OCH$_2$CH$_2$CH$_2$), 3.36–3.50 (m, OC**H$_2$**CH$_2$C**H$_2$**O), 3.80 (dd, *J* = 4.2, 9.6 Hz, CH**H'**OCH$_2$CH$_2$CH$_2$), 4.35–4.51 (m, NHC**H$_2$**Ph), 4.58–4.66 (m, C**H**CH$_2$O), 6.74 (d, *J* = 6.9 Hz, CH$_3$C(O)N**H**), 7.20–7.40 (m, C$_6$**H$_5$**, C(O)N**H**CH$_2$); ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 32.1 (OCH$_2$**C**H$_2$CH$_2$O), 43.7 (NH**C**H$_2$Ph), 52.7 (**C**HCH$_2$OCH$_2$), 60.6 (OCH$_2$CH$_2$**C**H$_2$OH), 69.5 (CHCH$_2$O**C**H$_2$CH$_2$CH$_2$), 70.6 (CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 127.7, 127.8, 128.8, 138.0 (**C$_6$**H$_5$), 170.2, 170.7 (CH**C**(O)NH, CH$_3$**C**(O)NH); M_r (+ESI) 317.1478 [M+Na]$^+$ (calcd for $C_{15}H_{22}N_2O_4Na^+$ 317.1477).

(*R*)-*N*-Benzyl 2-Acetamido-3-(3-oxopropoxy)propionamide ((*R*)-93). Using Method E, alcohol (*R*)-**129** (1.20 g, 4.08 mmol) in CH$_2$Cl$_2$ (12 mL), (COCl)$_2$ (432 *µ*L,

4.92 mmol) in CH_2Cl_2 (12 mL), DMSO (720 μL, 9.84 mmol) in CH_2Cl_2 (24 mL), and DIEA (3.55 mL, 14.4 mmol) gave 506 mg (39%) of (R)-**93** as white needles after recrystallization from EtOAc: mp 120–121 °C; $[\alpha]^{25}_D$ -22.1° (c 0.9, $CHCl_3$); R_f = 0.34 (5/95 MeOH/CHCl₃); IR (CH_2Cl_2 film) 3306, 3057, 1724, 1662, 1523, 1374, 1266, 1119 cm⁻¹; ¹H NMR ($CDCl_3$) δ 2.04 (s, **CH₃**C(O)), 2.60–2.80 (m, **CH₂**C(O)H), 3.48 (dd, J = 7.5, 9.3 Hz, CH**CH**H'OCH₂CH₂), 3.70–3.78 (m, CH₂O**CH**H'CH₂C(O)H), 3.80–3.87 (m, CH₂OCH**H'**CH₂C(O)H), 3.90 (dd, J = 3.6, 9.3 Hz, CHCH**H'**OCH₂CH₂), 4.46 (d, J = 6.0 Hz, NH**CH₂**Ph), 4.51–4.58 (m, **CH**CH₂O), 6.61 (d, J = 6.3 Hz, CH₃C(O)N**H**), 6.85–7.04 (br t, C(O)N**H**CH₂), 7.22–7.38 (m, **C₆H₅**), 9.71 (t, J = 1.4 Hz, C(O)**H**), addition of excess (R)-(-)-mandelic acid to a $CDCl_3$ solution of (R)-**93** gave only one signal for the acetyl methyl protons and the aldehyde proton, addition of excess (R)-(-)-mandelic acid to a $CDCl_3$ solution of (S)-**93** and (R)-**93** (1:3 ratio) gave two signals for the acetyl methyl protons (δ 2.032 (S) and 2.018 (R) (Δppm = 0.014)), and two signals for the aldehyde protons (δ 9.658 (S) and 9.680 (R) (Δppm = 0.022)); ¹³C NMR ($CDCl_3$) δ 23.4 (**CH₃**C(O)), 43.8, 43.9 (OCH₂**CH₂**C(O)H, NH**CH₂**Ph), 52.4 (**CH**CH₂OCH₂), 64.5 (O**CH₂**CH₂C(O)H), 70.4 (CH**CH₂**OCH₂CH₂C(O)H), 127.6, 127.7, 128.8, 138.3 (**C₆**H₅), 170.0, 170.6 (CH**C**(O)NH, CH₃**C**(O)NH), 200.9 (**C**(O)H); M_r (+ESI) 315.1323 [M+Na]⁺ (calcd for $C_{15}H_{20}N_2O_4Na^+$ 315.1321). Anal. Calcd for $C_{15}H_{20}N_2O_4$: C, 61.63; H, 6.90; N, 9.58. Found: C, 61.34; H, 6.94; N, 9.42.

(S)-N-Benzyl 2-Acetamido-3-(3-oxopropoxy)propionamide ((S)-93). Using Method E, compound (S)-**129** (1.50 g, 5.10 mmol) in CH_2Cl_2 (10 mL), $(COCl)_2$ (580

μL, 6.63 mmol) in CH$_2$Cl$_2$ (20 mL), DMSO (941 μL, 6.63 mmol) in CH$_2$Cl$_2$ (10 mL),

and DIEA (4.44 mL, 25.5 mmol) gave 757 mg (49%) of (S)-**93** as white needles after

recrystallization from EtOAc: mp 120–121 °C; [α]$^{25}_D$ +22.0° (c 1.0, CHCl$_3$); R_f = 0.34

(5/95 MeOH/CHCl$_3$); IR (CHCl$_3$ film) 3306, 3057, 2984, 2922, 2875, 1724, 1662,

1523, 1373, 1265, 1119 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 2.60–2.80 (m,

CH$_2$C(O)H), 3.49 (dd, J = 7.2, 9.6 Hz, CHC**H**H'OCH$_2$CH$_2$), 3.69–3.78 (m,

CH$_2$OC**H**H'CH$_2$C(O)H), 3.78–3.85 (m, CH$_2$OCH**H'**CH$_2$C(O)H), 3.88 (dd, J = 3.9, 9.6

Hz, CHCH**H'**OCH$_2$CH$_2$), 4.45 (d, J = 5.7 Hz, NHC**H$_2$**Ph), 4.56 (app dt, J = 3.9, 7.2

Hz, **CH**CH$_2$O), 6.68 (d, J = 7.2 Hz, CH$_3$C(O)N**H**), 6.90–7.21 (br t, C(O)N**H**CH$_2$),

7.20–7.38 (m, C$_6$**H$_5$**), 9.70 (t, J = 1.4 Hz, C(O)**H**), addition of excess (R)-(-)-mandelic

acid to a CDCl$_3$ solution of (S)-**93** gave only one signal for the acetyl methyl protons

and the aldehyde proton, addition of excess (R)-(-)-mandelic acid to a CDCl$_3$

solution of (S)-**93** and (R)-**93** (~1:3 ratio) gave two signals for the acetyl methyl

protons (δ 2.032 (S) and 2.018 (R) (Δppm = 0.014)), and two signals for the

aldehyde protons (δ 9.658 (S) and 9.680 (R) (Δppm = 0.022)); ^{13}C NMR (CDCl$_3$) δ

23.3 (**CH$_3$**C(O)), 43.7, 43.8 (OCH$_2$**CH$_2$**C(O)H, NH**CH$_2$**Ph), 52.4 (**CH**CH$_2$OCH$_2$), 64.5

(O**CH$_2$**CH$_2$C(O)H), 70.4 (CH**CH$_2$**OCH$_2$CH$_2$C(O)H), 127.6, 127.7, 128.8, 138.2

(**C$_6$**H$_5$), 170.0, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)NH), 200.9 (**C**(O)H); M_r (+ESI) 315.1325

[M+Na]$^+$ (calcd for C$_{15}$H$_{20}$N$_2$O$_4$Na$^+$ 315.1321). Anal. Calcd for C$_{15}$H$_{20}$N$_2$O$_4$: C, 61.63;

H, 6.90; N, 9.58. Found: C, 61.71; H, 7.01; N, 9.52.

(2R)-N-Benzyl 2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide ((R)-94)

(mixture of diastereomers). Using Method J, (R)-**88** (320 mg, 1.10 mmol), Na$_2$SO$_4$

(50 mg) and *m*CPBA (77% wt, 420 mg, 1.88 mmol) in CH_2Cl_2 (3 mL) gave (*R*)-**94** (265 mg, 72%) as a ~1:1 mixture of diastereoisomers after purification by flash chromatography (15/85 acetone/EtOAc): mp 104–110 ℃; $[\alpha]^{25}_D$ -29.6° (*c* 0.3; $CHCl_3$); R_f = 0.31 (15/85 acetone/EtOAc); IR (nujol) 3292, 1637, 1545, 1457, 1376, 1127 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.52–1.64 (m, $CH_2C\mathbf{H}H'CH(O)CH_2$), 1.86–2.00 (m, $CH_2CH\mathbf{H}'CH(O)CH_2$), 2.03, 2.04 (s, $C\mathbf{H_3}C(O)$), 2.41–2.46 (m, $CH_2CH_2CH(O)C\mathbf{H}H'$), 2.66–2.71 (m, $CH_2CH_2CH(O)CH\mathbf{H}'$), 2.82–2.96 (m, $CH_2CH_2C\mathbf{H}(O)CH_2$), 3.44–3.52 (m, $C\mathbf{H}H'OCH_2CH_2$), 3.54–3.76 (m, $CH_2OC\mathbf{H_2}CH_2$), 3.84–3.93 (m, $CH\mathbf{H}'OCH_2CH_2$), 4.39–4.52 (m, $NHC\mathbf{H_2}Ph$), 4.50–4.58 (m, $C\mathbf{H}CH_2O$), 6.56–6.68 (m, $N\mathbf{H}CHCH_2O$), 6.88–6.98, 6.99–7.08 (m, $N\mathbf{H}CH_2Ph$), 7.22–7.36 (m, $C_6\mathbf{H_5}$), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*R*)-**94** gave only one set of signals for the acetyl protons (δ 2.015 and 2.022 ppm), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*S*)-**94** and (*R*)-**94** in a ~1:3 ratio gave two sets of signals with a relative ~1:3 intensity for the acetyl protons (δ 2.028 and 2.022 ppm (*S*)-**94**, δ 2.015 and 2.008 ppm (*R*)-**94**); ^{13}C NMR ($CDCl_3$) δ 23.4 ($\mathbf{C}H_3C(O)$), 32.4, 32.6 ($CH_2\mathbf{C}H_2CH(O)CH_2$), 43.8 ($NH\mathbf{C}H_2Ph$), 46.7 ($CH_2CH_2CH(O)\mathbf{C}H_2$), 50.3, 50.4, 52.6, 52.7 ($\mathbf{C}HC(O)NH$, $CH_2CH_2\mathbf{C}H(O)CH_2$), 68.5, 68.8, 69.9, 70.1 ($CH\mathbf{C}H_2O\mathbf{C}H_2$), 127.6, 127.7, 128.8, 128.9, 138.2, 138.3 ($\mathbf{C_6}H_5$), 170.2, 170.6 ($CH_3\mathbf{C}(O)$, $CH\mathbf{C}(O)NH$), the remaining peaks were not detected and are believed to overlap with nearby signals; M_r (+ESI) 329.1478 $[M+Na]^+$ (calcd for $C_{16}H_{22}N_2O_4Na^+$ 329.1477). Anal. Calcd for $C_{16}H_{22}N_2O_4$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.92; H, 7.37; N, 9.07.

(2*S*)-*N*-Benzyl 2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide ((*S*)-94) (mixture of diastereomers). Using Method J, (*S*)-**88** (100 mg, 0.35 mmol), Na_2SO_4 (25 mg) and *m*CPBA (77% wt, 131 mg, 0.59 mmol) in CH_2Cl_2 (3 mL) yielded (*S*)-**94** (76 mg, 72%) as a ~1:1 mixture of diastereoisomers: mp 104–109 °C; $[\alpha]^{25}_D$ +31.3° (*c* 1.4; $CHCl_3$); R_f = 0.31 (15/85 acetone/EtOAc); IR (nujol) 3281, 3062, 1638, 1547, 1457, 1376, 1127 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.52–1.64 (m, CH_2C**H**H'CH(O)CH$_2$), 1.84–1.96 (m, CH_2CH**H'**CH(O)CH$_2$), 2.00, 2.01 (s, **CH$_3$**C(O)), 2.40–2.46 (m, CH$_2$CH$_2$CH(O)C**H**H'), 2.65–2.71 (m, CH$_2$CH$_2$CH(O)CH**H'**), 2.82–2.96 (m, CH$_2$CH$_2$C**H**(O)CH$_2$), 3.44–3.52 (m, C**H**H'OCH$_2$CH$_2$), 3.54–3.72 (m, CH$_2$OC**H$_2$**CH$_2$), 3.81–3.90 (m, CH**H'**OCH$_2$CH$_2$), 4.36–4.52 (m, NHC**H$_2$**Ph), 4.53–4.61 (m, C**H**CH$_2$O), 6.70–6.80 (m, N**H**CHCH$_2$O), 7.04–7.11, 7.12–7.21 (2 br t, N**H**CH$_2$Ph), 7.22–7.36 (m, C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*S*)-**94** gave only one set of signals for the acetyl protons (δ 2.026 and 2.031 ppm), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*S*)-**94** and (*R*)-**94** in a ~1:3 ratio gave two sets of signals with a relative ~1:3 intensity for the acetyl protons (δ 2.028 and 2.022 ppm (*S*)-**94**, δ 2.015 and 2.008 ppm (*R*)-**94**); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H$_3$C(O)), 32.4, 32.6 (CH$_2$**C**H$_2$CH(O)CH$_2$), 43.7 (NH**C**H$_2$Ph), 46.7 (CH$_2$CH$_2$CH(O)**C**H$_2$), 50.3, 50.4, 52.6, 52.7 (**C**HC(O)NH, CH$_2$CH$_2$**C**H(O)CH$_2$), 68.4, 68.7, 70.0, 70.2 (CH**C**H$_2$O**C**H$_2$), 127.5, 127.6, 127.7, 128.7, 128.8, 138.3 (**C$_6$**H$_5$), 170.2, 170.5 (CH$_3$**C**(O), CH**C**(O)NH), the remaining peaks were not detected and are believed to overlap with nearby signals; M_r (+ESI) 329.1479 [M+Na]$^+$ (calcd for $C_{16}H_{22}N_2O_4Na^+$ 329.1477). Anal. Calcd for $C_{16}H_{22}N_2O_4$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.94; H, 7.34; N, 9.12.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-acetamidoethoxy)propionamide ((*R*)-96). Using Method H, (*R*)-**97** (1.14 g, 3.7 mmol) and 10% Pd/C (100 mg) in MeOH (25 mL) gave upon filtration and evaporation a residue that was dissolved in CH_2Cl_2 (50 mL). Et_3N (570 μL, 4.1 mmol), DMAP (1 mg, catalytic), and Ac_2O (390 μL, 4.1 mmol) were then successively added and the reaction was stirred at room temperature (30 min), filtered, and the solvents were removed under vacuum. The crude residue was purified by flash chromatography (1/9 MeOH/CH_2Cl_2) to give an oily residue that was dissolved in warm THF (25 mL). The white solid that precipitated upon cooling was filtered to give (*R*)-**96** (490 mg, 40% overall yield for two steps): mp 166–168 °C; $[\alpha]^{25}_D$ +11.7° (*c* 0.6, MeOH); R_f = 0.30 (5/95 MeOH/CH_2Cl_2); IR (nujol) 3494, 3089, 3288, 2861, 1637, 1554, 1456, 1372, 1290 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.79, 1.88 (s, 2 C**H$_3$**C(O)), 3.10–3.24 (m, OCH$_2$C**H$_2$**NHAc), 3.36–3.46 (m, OC**H$_2$**CH$_2$NHAc), 3.52–3.64 (m, CHC**H$_2$**OCH$_2$), 4.29 (d, *J* = 7.0 Hz, NHC**H$_2$**C$_6$H$_5$), 4.42–4.52 (m, C**H**CH$_2$O), 7.20–7.36 (m, CH$_2$C$_6$**H$_5$**), 7.80–7.88 (m, N**H**CH$_2$Ph or N**H**CH$_2$CH$_2$), 8.07 (d, *J* = 7.0 Hz, N**H**CHCH$_2$), 8.50 (t, *J* = 6.0 Hz, N**H**CH$_2$CH$_2$ or N**H**CH$_2$Ph); ^{13}C NMR (DMSO-d_6) δ 22.6 (2 **C**H$_3$C(O)), 38.3 (CH$_2$**C**H$_2$NHAc), 42.0 (NH$_2$**C**H$_2$Ph), 52.7 (**C**HCH$_2$OCH$_2$), 69.1, 70.3 (**C**H$_2$O**C**H$_2$CH$_2$), 126.6, 126.9, 128.2, 139.3 (**C$_6$**H$_5$), 169.3, 169.4, 169.7 (CH**C**(O)NH, 2 CH$_3$**C**(O)), the remaining signal was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 344.2 [M+Na]$^+$ (calcd for $C_{16}H_{23}N_3O_4Na^+$ 344.2). Anal. Calcd for $C_{16}H_{23}N_3O_4$: C, 59.80; H, 7.21; N, 13.08. Found: C, 59.63; H, 7.16; N, 12.94.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-97). Using

Method D, (*R*)-**124** (950 mg, 4.4 mmol), benzylamine (622 μL, 5.7 mmol), and

DMTMM (1.58 g, 5.7 mmol) in THF (50 mL) gave 715 mg (53%) of (*R*)-**97** as a white

solid after SiO$_2$ chromatography column (4/96 MeOH/CHCl$_3$) followed by

recrystallization from EtOAc: mp 111–113 ℃; [α]$^{25}_D$ +12.0°(*c* 1.0; MeOH); R_f = 0.51

(5/95 MeOH/CHCl$_3$); IR (nujol) 3139, 2107, 1635, 1547 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.98

(s, **CH$_3$**C(O)NH), 3.32–3.43 (m, OCH$_2$**CH$_2$**N$_3$), 3.50–3.56 (m, CHC**H**H'OCH$_2$), 3.58–

3.75 (m, O**CH$_2$**CH$_2$N$_3$), 3.88–3.96 (m, CHCH**H'**OCH$_2$), 4.39–4.51 (m, NHC**H$_2$**Ph),

4.52–4.58 (m, C**H**CH$_2$OCH$_2$), 6.55–6.65 (br d, N**H**CHCH$_2$O), 6.76–6.86 (m,

N**H**CH$_2$Ph), 7.21–7.35 (C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$

solution of (*R*)-**97** gave only one signal for the acetyl protons, addition of excess (*R*)-

(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**97** and (*R*)-**97** (1:2 ratio) gave two

signals for the acetyl protons (δ 2.006 (*S*) and 1.993 (*R*) (Δppm = 0.013)); ^{13}C NMR

(CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.9 (NH**C**H$_2$Ph), 50.9 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3

(O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 127.7, 128.9, 138.0

(**C$_6$**H$_5$), 169.8, 170.9 (CH$_3$**C**(O)NH and **C**(O)NHCH$_2$), the remaining aromatic

resonance was not detected and is believed to overlap with nearby signals; M_r

(+ESI) 328.1380 [M+Na]$^+$ (calcd for C$_{14}$H$_{19}$N$_5$O$_3$Na$^+$ 328.1386). Anal. Calcd for

C$_{14}$H$_{19}$N$_5$O$_3$: C, 55.07; H, 6.27; N, 22.94. Found: C, 54.85; H, 6.27; N, 22.94.

(*S*)-*N*-Benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-97). Using

Method D, acid (*S*)-**124** (1.08 g, 5 mmol), benzylamine (600 μL, 5.5 mmol) and

DMTMM (1.52 g, 5.5 mmol) in THF (50 mL) gave 854 mg (56%) of (*S*)-**97** as a white

solid after silica gel chromatography (4/96 MeOH/CHCl$_3$) followed by recrystallization from EtOAc and hexanes: mp 111–113 °C; [α]$^{25}_D$ -12.1° (*c* 1.0; MeOH); *R$_f$* = 0.51 (5/95 MeOH/CHCl$_3$); IR (nujol) 3139, 2107, 1635, 1547 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.98 (s, **CH$_3$**C(O)NH), 3.32–3.43 (m, OCH$_2$**CH$_2$**N$_3$), 3.50–3.56 (m, CH**CHH'**OCH$_2$), 3.58–3.75 (m, O**CH$_2$**CH$_2$N$_3$), 3.88–3.96 (m, CHCH**H'**OCH$_2$), 4.39–4.51 (m, NH**CH$_2$**Ph), 4.52–4.58 (m, **CH**CH$_2$OCH$_2$), 6.42–6.52 (br d, **NH**CHCH$_2$O), 6.74–6.84 (br t, **NH**CH$_2$Ph), 7.21–7.35 (C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**97** gave only one signal for the acetyl protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**97** and (*R*)-**97** (1:2 ratio) gave two signals for the acetyl protons (δ 2.006 (*S*) and 1.993 (*R*) (Δppm = 0.013)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.9 (NH**C**H$_2$Ph), 50.9 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 127.7, 128.9, 138.0 (**C$_6$**H$_5$), 169.8, 170.9 (CH$_3$**C**(O)NH and **C**(O)NHCH$_2$), the remaining aromatic resonance was not detected and is believed to overlap with nearby signals; *M$_r$* (+ESI) 328.1380 [M+Na]$^+$ (calcd for C$_{14}$H$_{19}$N$_5$O$_3$Na$^+$ 328.1386). Anal. Calcd for C$_{14}$H$_{19}$N$_5$O$_3$: C, 55.07; H, 6.27; N, 22.94. Found: C, 55.16; H, 6.28; N, 22.91.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-(4-(methoxymethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)propionamide ((*R*)-98). Compound (*R*)-**97** (400 mg, 1.3 mmol) was dissolved in a THF:H$_2$O (1:1, 50 mL) and while stirring, methyl propargyl ether (1.0 mL, 11.8 mmol), sodium ascorbate (25 mg, 0.13 mmol), and CuSO$_4$ (3 mg, 0.01 mmol) were successively added. The reaction was stirred at room temperature (24 h), and saturated aqueous NaHCO$_3$ (100 mL) was added. The aqueous layer was

extracted with CH_2Cl_2 (2 x 100 mL), the combined organic layers were washed with brine (100 mL) and filtered over a Celite® bed. Removal of solvents in vacuo gave an off-white solid that was purified by flash chromatography (1/9 $MeOH/CH_2Cl_2$) to yield (*R*)-**98** (470 mg, 96%) as a crystalline solid: mp 127–129 °C; $[\alpha]^{25}_D$ +1.8° (*c* 0.34; MeOH); R_f = 0.49 (1/9 $MeOH/CH_2Cl_2$); IR (nujol) 3296, 2861, 1641, 1545, 1457, 1377, 1143, 1095 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.00 (s, **CH₃**C(O)), 3.38 (s, CH_2OC**H₃**), 3.50 (dd, *J* = 6.5, 9.6 Hz, C**H**H'OCH_2CH_2), 3.76–3.85 (m, OC**H₂**CH_2N(N)CH), 3.92 (dd, *J* = 4.2, 9.6 Hz, CH**H'**OCH_2CH_2), 4.34–4.51 (m, NHC**H₂**Ph, OCH_2C**H₂**N(N)CH, C**H₂**OCH_3), 6.63 (d, *J* = 6.6 Hz, N**H**CHC(O)), 7.00–7.10 (br t, N**H**CH_2Ph), 7.18–7.35 (m, C_6**H₅**), 7.52 (s, NC**H**C(N)), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*R*)-**98** gave only one signal for the acetyl protons (δ 1.995); ^{13}C NMR ($CDCl_3$) δ 23.2 (**CH₃**C(O)), 43.7 (NH**CH₂**Ph), 50.0 (**CH₂**N(N)CH), 52.9 (**C**HC(O)NH), 58.6 (CH_2OC**H₃**), 66.0 (O**CH₂**CH_2N), 69.4, 70.5 (CH**CH₂**OCH_2, **CH₂**OCH_3), 123.8 (N**C**HC(N)), 127.6, 127.7, 128.8, 138.3 (**C₆**H_5), 145.3 (NCH**C**(N)), 169.7, 170.8 (CH_3**C**(O), CH**C**(O)NH); M_r (+ESI) 398.1806 [M+Na]$^+$ (calcd for $C_{18}H_{25}N_5O_4Na^+$ 398.1804). Anal. Calcd for $C_{18}H_{25}N_5O_4$: C, 57.59; H, 6.71; N, 18.65. Found: C, 57.34; H, 6.72; N, 18.49.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-methoxyethoxy)propionamide ((*R*)-99). Using Method B, a ~5:95 mixture of (*R*)-**113a** and (*R*)-**113b** (4.00 g, 17.4 mmol) in THF (170 mL) and LiOH (415 mg, 17.3 mmol) in H_2O (80 mL) gave 1.10 g (31%, 5.36 mmol) of a crude viscous yellow oil upon work-up (M_r (+ESI) 228.0848 [M+Na]$^+$ (calcd for $C_8H_{15}NO_5Na^+$ 228.0848)). Using Method D, the oil was directly dissolved

in THF (60 mL) and then benzylamine (732 μL, 6.7 mmol) followed by DMTMM (1.90 g, 6.7 mmol) were added. Purification by flash chromatography (1/9 hexanes/EtOAc to 1/9 MeOH/CH_2Cl_2) followed by recrystallization from EtOAc and hexanes afforded (*R*)-**99** (750 mg, 45%) as a white solid: mp 109–110 °C; $[\alpha]^{25}_D$ +10.3°(*c* 0.5; MeOH); R_f = 0.43 (1/9 MeOH/CH_2Cl_2); IR (CH_2Cl_2 film) 3427, 3306, 3058, 2998, 1741, 1664, 1528, 1454, 1372, 1267, 1206, 1107, 1028 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 3.18 (s, CH$_2$OC**H$_3$**), 3.43–3.53 (m, C**H**H'OCH$_2$, OC**H$_2$**CH$_2$OCH$_3$ or OCH$_2$C**H$_2$**OCH$_3$), 3.62–3.78 (m, OCH$_2$C**H$_2$**OCH$_3$ or OC**H$_2$**CH$_2$OCH$_3$), 3.85 (dd, *J* = 4.2, 9.6 Hz, CH**H'**OCH$_2$CH$_2$), 4.37–4.51 (m, NHC**H$_2$**Ph), 4.52–4.58 (m, C**H**CH$_2$O), 6.65 (d, *J* = 6.6 Hz, N**H**CHC(O)), 7.21–7.38 (m, N**H**CH$_2$C$_6$H$_5$, C$_6$**H$_5$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**99** gave only one signal for the acetyl protons (δ 1.997); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.8 (NH**C**H$_2$Ph), 51.9 (**C**HC(O)NH), 58.8 (CH$_2$CH$_2$O**C**H$_3$), 69.4, 70.5 (CH**C**H$_2$OCH$_2$, **C**H$_2$OCH$_3$), 127.6, 127.7, 128.8, 138.2 (**C$_6$**H$_5$), 170.2, 170.4 (CH$_3$**C**(O), CH**C**(O)NH), the remaining signal was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 317.1478 [M+Na]$^+$ (calcd for C$_{15}$H$_{22}$N$_2$O$_4$Na$^+$ 317.1477). Anal. Calcd for C$_{15}$H$_{22}$N$_2$O$_4$: C, 61.21; H, 7.53; N, 9.52; Found: C, 61.38; H, 7.36; N, 9.44.

(*R*)-*N*-Benzyl 2-Acetamido-3-(2-(2-methoxyethoxy)ethoxy)propionamide ((*R*)-100). Using Method D, (*R*)-**125** (1.67 g, 6.70 mmol), benzylamine (876 μL, 8.04 mmol) and DMTMM (2.22 g, 8.04 mmol) in THF (70 mL) gave a residue that was purified twice by flash chromatography (5/95 MeOH/CHCl$_3$) to yield (*R*)-**100** (1.20 g, 53%) as a yellow oil that progressively turned to an amorphous solid after 3 d under

vacuum: mp 48–52 °C; $[\alpha]^{25}_D$ +7.7° (c 1.2, MeOH); R_f = 0.51 (5/95 MeOH/CHCl$_3$); IR (neat) 3313, 3072, 2921, 2358, 2245, 1657, 1538, 1103 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 3.26 (s, O**CH$_3$**), 3.39–3.80 (m, CH**H'**O**CH$_2$CH$_2$**O**CH$_2$CH$_2$**OCH$_3$), 4.05 (dd, J = 3.9 Hz, 9.9 Hz, C**H**H'OCH$_2$CH$_2$O), 4.48 (d, J = 6.0 Hz, NHC**H$_2$**C$_6$H$_5$), 4.54–4.62 (m, C**H**CH$_2$O), 6.77 (d, J = 6.0 Hz, N**H**CH$_2$C$_6$H$_5$), 7.20–7.39 (m, C$_6$**H$_5$**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**100** gave only one signal for the acetyl peak protons (δ 1.998); ^{13}C NMR (CDCl$_3$) δ 23.4 (**CH$_3$**C(O)), 43.7 (NH$_2$**CH$_2$**C$_6$H$_4$), 52.5 (**C**HCH$_2$OCH$_2$CH$_2$), 59.0 (O**C**H$_3$), 70.4, 70.5, 70.6, 71.9 (O**CH$_2$CH$_2$**O**CH$_2$CH$_2$**O), 127.5, 127.7, 128.8, 137.8 (**C$_6$**H$_5$), 170.3, 170.4 (CH**C**(O)NH, CH$_3$**C**(O)), the remaining methylene signal was not detected and is believed to overlap with nearby signals; M_r (+ESI) 361.1743 [M+Na]$^+$ (calcd for C$_{17}$H$_{26}$N$_2$O$_5$Na$^+$ 361.1739). Anal. Calcd for C$_{17}$H$_{26}$N$_2$O$_5$•0.33 H$_2$O: C, 58.77; H, 7.83; N, 8.06. Found: C, 58.59; H, 7.88; N, 8.10.

(R)-N-Benzyl 2-N-(Benzyloxycarbonyl)amino-3-(methoxy-d_3)propionamide ((R)-130). Using Method G, compound (R)-**57** (1.40 g, 4.4 mmol), Ag$_2$O (5.00 g, 21.5 mmol) and CD$_3$I (2.7 mL, 43.5 mmol) in CH$_3$CN (40 mL) gave (R)-**130** as a white solid (1.50 g, 96%) upon purification by flash silica gel chromatography (2/98 MeOH/CH$_2$Cl$_2$): mp 130–131 °C; $[\alpha]^{25}_D$ -25.2° (c 0.6, CHCl$_3$); R_f = 0.52 (3/97 MeOH/CH$_2$Cl$_2$); IR (CHCl$_3$ film) 3021, 1719, 1675, 1501, 1326, 1217, 1131, 1073 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 3.48 (dd, J = 6.3, 9.0 Hz, C**H**H'OCD$_3$), 3.84 (dd, J = 3.9, 9.0 Hz, CH**H'**OCD$_3$), 4.30–4.40 (C**H**CH$_2$O), 4.46 (d, J = 5.7 Hz, NHC**H$_2$**C$_6$H$_5$), 5.10 (s, PhC**H$_2$**OC(O)), 5.64–5.78 (m, N**H**CHCH$_2$O), 6.70–6.80 (m, N**H**CH$_2$Ph), 7.20–7.38

(m, 2 C_6H_5); ^{13}C NMR (CDCl$_3$) δ 43.8 (NH$_2$**C**H$_2$Ph), 54.6 (**C**HCH$_2$OCH$_2$), 57.8–59.0

(m, O**C**D$_3$), 67.4 (Ph**C**H$_2$OC(O)), 72.1 (**C**H$_2$OCD$_3$), 127.7, 128.3, 128.4, 128.6, 128.8,

128.9, 136.3, 138.1 (2 **C**$_6$H$_5$), 156.3 (O**C**(O)NH), 170.1 (CH**C**(O)NH); M_r (+ESI)

[M+Na]$^+$ 368.2 (calcd for C$_{19}$H$_{19}$D$_3$N$_2$O$_4$Na$^+$ 368.2). Anal. Calcd for C$_{19}$H$_{19}$D$_3$N$_2$O$_3$: C,

66.07; H, 6.41; N, 8.11. Found: C, 66.05; H, 6.39; N, 8.04.

(R)-N-Benzyl 2-Acetamido-3-(methoxy-d_3)propionamide ((R)-1-d_3). Using Method

H, (R)-**130** (1.5 g, 4.25 mmol) and 10% Pd/C (200 mg) in MeOH (50 mL) gave after

evaporation an oily residue that was directly dissolved in CH$_2$Cl$_2$ (50 mL). While

stirring Et$_3$N (590 μL, 4.25 mmol), DMAP (25 mg, 212 μmol) and Ac$_2$O (400 μL, 4.25

mmol) were successively added and the reaction was stirred at room temperature (1

h). The organic layer was washed with aqueous 0.1 M H$_2$SO$_4$ (20 mL). The aqueous

layer was extracted with CH$_2$Cl$_2$ (4 x 20 mL). The organic layers were combined,

washed with brine (50 mL), dried (Na$_2$SO$_4$), and the solvents were removed under

vacuum. The obtained solid was recrystallized from EtOAc and hexanes to give (R)-

1-d_3 as a white solid (888 mg, 82% over 2 steps): mp 142–143 °C; $[\alpha]^{25}_D$ +16.0° (c

0.7, MeOH); R_f = 0.31 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3300, 3064, 2859, 1635,

1549, 1457, 1376, 1313, 1230, 1132 cm^{-1}; ^1H NMR (CDCl$_3$) δ 1.97 (s, **CH$_3$**C(O)),

3.47 (dd, J = 6.6, 9.0 Hz, C**H**H'OCD$_3$), 3.84 (dd, J = 4.0, 9.0 Hz, CH**H'**OCD$_3$), 4.34–

4.52 (m, NHC**H$_2$**C$_6$H$_5$), 4.62 (dt, J = 4.0, 6.6 Hz, C**H**CH$_2$O), 6.72 (d, J = 7.0 Hz,

N**H**CHCH$_2$O), 7.05–7.16 (m, N**H**CH$_2$Ph), 7.20–7.38 (m, C$_6$**H$_5$**), addition of excess

(R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**1**-d_3 gave only one signal for the

acetyl protons (δ 2.011); ^{13}C NMR (CDCl$_3$) δ 23.0 (**C**H$_3$C(O)), 43.4 (NH$_2$**C**H$_2$Ph), 52.5

($\mathbf{C}$HCH$_2$OCD$_3$), 58.1 (app quint, J = 21.6 Hz, O$\mathbf{C}$D$_3$), 71.8 ($\mathbf{C}$H$_2$OCD$_3$), 127.4, 128.6, 137.9 ($\mathbf{C_6}$H$_5$), 170.0, 170.3 (CH$_3\mathbf{C}$(O), CH$\mathbf{C}$(O)NH), the remaining signal was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 276.1 [M+Na]$^+$ (calcd for C$_{13}$H$_{15}$D$_3$N$_2$O$_3$Na$^+$ 276.1). Anal. Calcd for C$_{13}$H$_{15}$D$_3$N$_2$O$_3$: C, 61.64; H, 7.16; N, 11.06. Found: C, 61.60; H, 7.10; N, 10.99.

2.3. Synthesis of a molecular toolkit for use in chemical biology studies

2.3.1. Synthetic strategies to different AB, CR and AB&CR derivatives

To allow efficient synthesis of AB, CR and AB&CR derivatives we constructed a series of functionalized benzylamines (**134**, **137**, **141**, **144**, **146**, Scheme 23) using protection/deprotection strategies. Upon removal of the protecting group, the benzylamine was either stored in a cold, dry environment under Ar or directly coupled with select enantiomerically pure acids.

Scheme 23. Synthetic routes to *para*-substituted benzylamines used in the synthesis of AB, CR and AB&CR agents
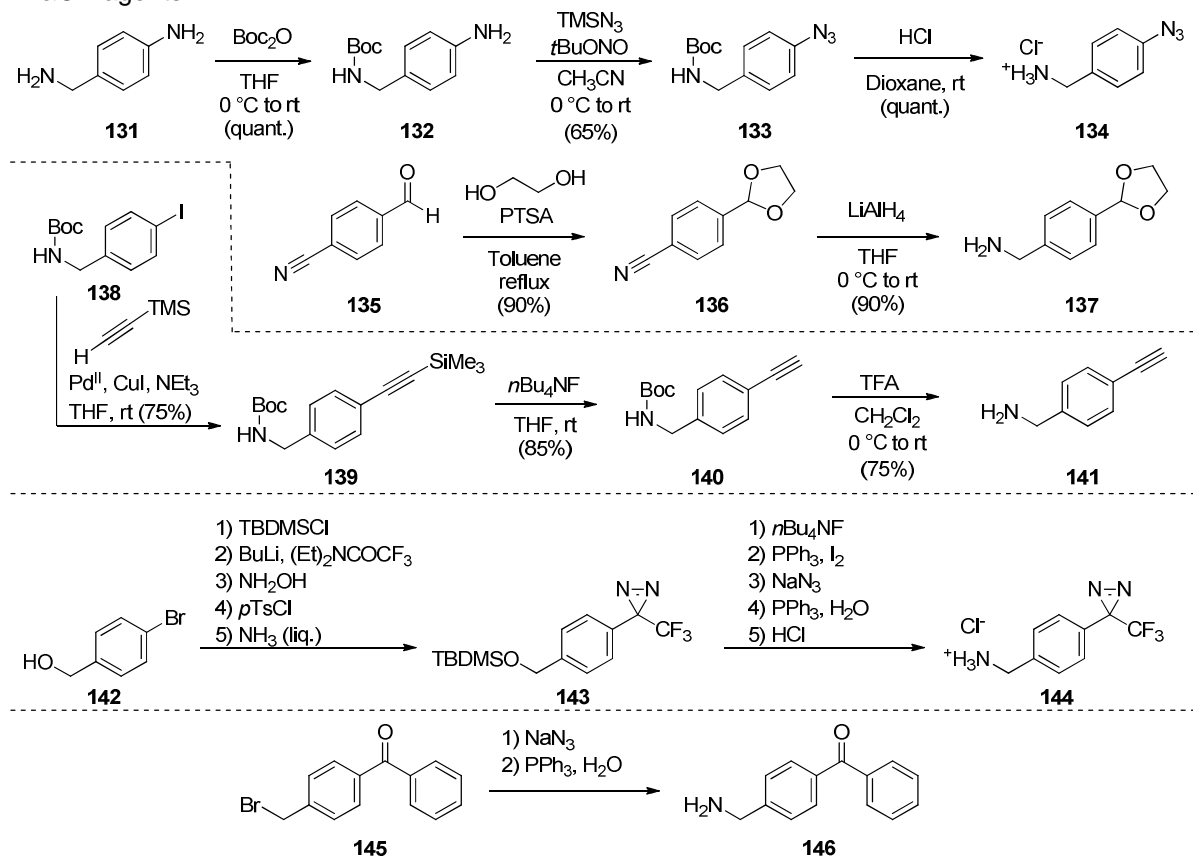
Commercially available 4-aminobenzylamine (**131**) was protected using di-*tert*-butyl dicarbonate to give **132**, and then the aniline moiety was converted to the azide using *tert*-butyl nitrite and trimethylsilylazide in a Sandmeyer-type reaction.[325] Deprotecting the Boc group with HCl yielded 4-azidobenzylammonium hydrochloride (**134**). To introduce the aromatic aldehyde and aromatic epoxide AB groups, commercial 4-cyanobenzaldehyde (**135**) was condensed with ethylene glycol and catalytic *p*TSA to protect the aldehyde moiety, and the cyano group was reduced with LiAlH$_4$ in THF to afford benzylamine **137**.[408] The aldehyde was deprotected after the amide coupling step.[409] The alkynyl benzylamine **141** was prepared from Boc-protected benzylamine **138** that was coupled with trimethylsilyl acetylene using a Sonogashira reaction (*work of Dr. Christophe Salomé*). The TMS protecting group in **139** was removed with tetrabutylammonium fluoride, and the Boc group deprotected with trifluoroacetic acid to yield benzylamine **141** as the trifluoroacetate salt. Benzylamine **141** degraded over time in a dry, refrigerated environment and was only stable for prolonged times (-20 °C, up to 6 months) as the Boc and TMS protected intermediate **139**. Finally, the benzylamine containing the trifluoromethyldiazirine was synthesized in 10 steps using the following sequence of reactions (*work of Dr. Christophe Salomé*): commercial 4-bromobenzyl alcohol (**142**) was protected with a *tert*-butyldimethylsilyl group. The *O*-TBDMS intermediate was reacted with *n*-BuLi and diethylamide trifluoroacetate to give the trifluoromethylaryl ketone. The ketone was condensed with hydroxylamine and the resulting oxime tosylated with *p*TsCl. Treatment with liquid ammonia generated the desired photoAB moiety (**143**). The TBDMS group was then removed by fluoridolysis and the hydroxyl

group converted to the iodo derivative under Mitsunobu conditions. Nucleophilic substitution with sodium azide followed by Staudinger reduction and treatment with HCl gave benzylammonium hydrochloride **144**. Finally, the 4-aminomethylbenzophenone was prepared by reacting commercial 4-bromomethylbenzophenone (**145**) with sodium azide and reducing the benzyl azide under Staudinger conditions to yield benzylamine **146** (*work of Dr. Christophe Salomé*).

2.3.1.1. Introduction of aldehyde AB groups

We initiated our study with compound **151**, that bore an C(O)H AB group at the *N*-benzylamide *para* position. The synthesis proceeded via **route 1** shown in Scheme 14.[408] *N*-Cbz-protected L- or D- serine (**56**) was coupled with **137** using the DMTMM method to give **147**.[234,236] The side chain hydroxyl group was alkylated using MeI and Ag$_2$O,[234,236] the Cbz-protecting group removed by hydrogenolysis (10% Pd/C), and the amine then acetylated using acetyl chloride.[234,236] Finally, removal of the dioxolanyl protecting group under aqueous acidic conditions[409] yielded enantiomerically pure compounds (*R*)-**151** and (*S*)-**151** (Scheme 24).

Scheme 24. Synthesis of the lacosamide AB derivative **151** (**route 1**).
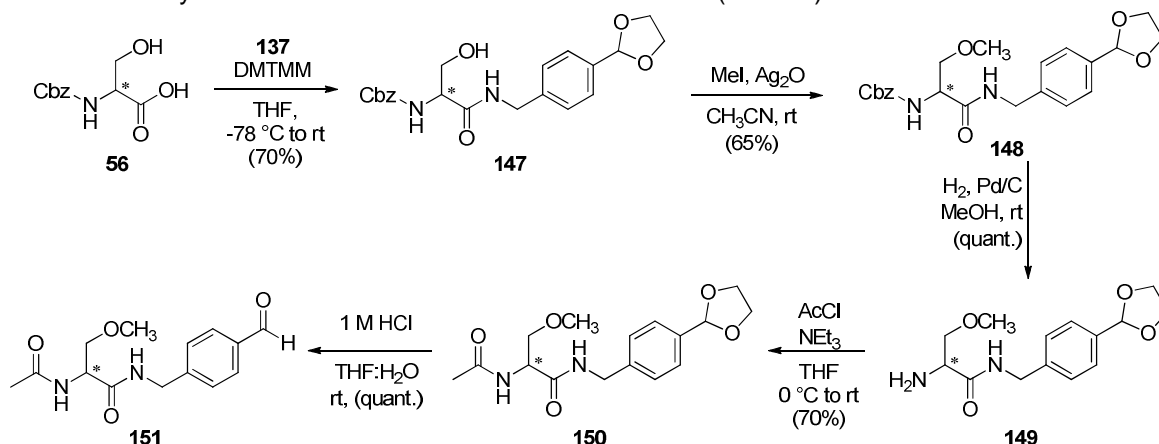
To introduce the aldehyde on the C(2) side chain we first ring-opened (*S*)-**68a** with commercial 2-benzyloxyethanol (Scheme 25). The ester was hydrolyzed, coupled with benzylamine and the *O*-benzyl protecting group was removed under hydrogenation conditions to give alcohol (*S*)-**127**. To our surprise, Swern oxidation (oxalyl chloride, DMSO, then DIEA)[410] of (*S*)-**127** gave the six-membered cyclized hemiaminal isomer **128** with no trace of the expected product.

Scheme 25. First attempt to synthesize the side chain aldehyde AB group (**route 3**).
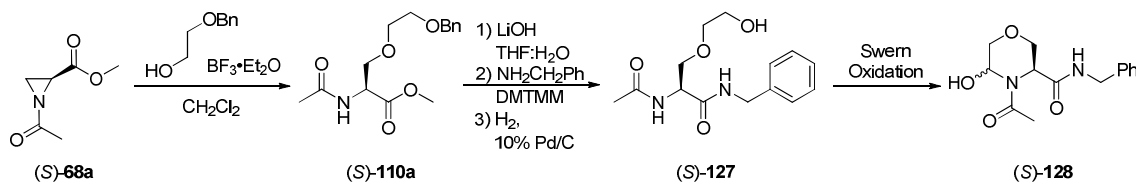
Accordingly, we increased by one the length of the carbon chain to entropically disfavor ring cyclization (6-membered vs. 7-membered rings).[411] Ring-opening of **68a,b** with commercial 3-benzyloxypropanol, followed by ester hydrolysis, removal of the benzyl protecting group, and coupling of the acid with the

157

desired benzylamine gave **152** (Scheme 26). Swern oxidation of the side chain hydroxyl group yielded the desired enantiomerically pure aldehyde **153**.

Scheme 26. Synthesis of the side chain aldehyde AB group (**route 3**)
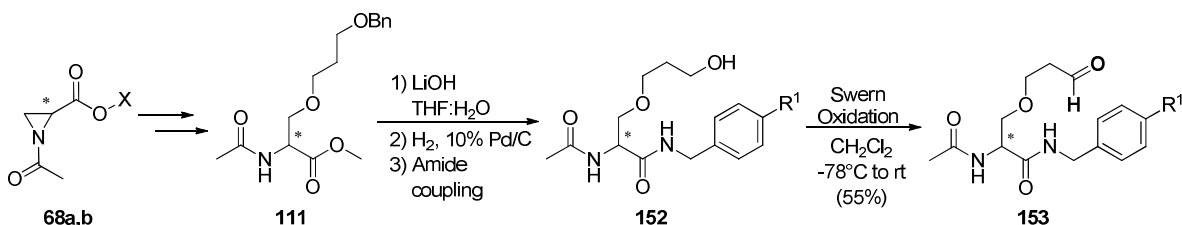
2.3.1.2. Introduction of isothiocyanate AB groups

Using **route 3**, we prepared the desired *O*-susbtituted *N*-acetylserine benzylamide derivative **155** containing an isothiocyanate moiety at the *N*-benzylamide *para* position from acid **70**. After amide coupling with 4-aminobenzylamine, the aniline was reacted with diimidazolylthionocarbonate (DITC) to form the aromatic isothiocyanate AB&CR analog **155** (Scheme 27).

Scheme 27. Synthesis of aromatic isothiocyanate AB&CR derivatives (**route 3**)
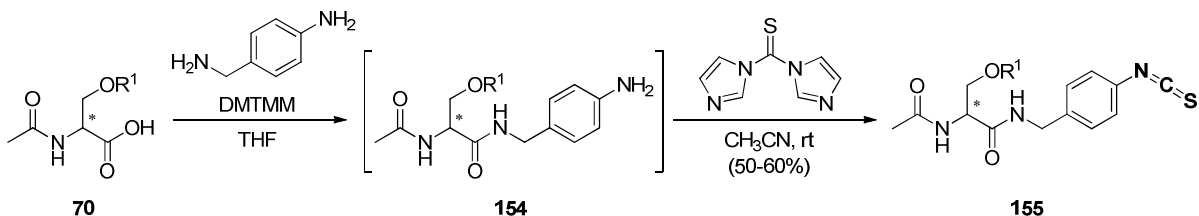
To introduce the NCS group on the C(2) side chain, **68a** was ring-opened with 2-azidoethanol under BF$_3$•Et$_2$O-catalyzed conditions, hydrolyzed, coupled with the

desired benzylamine and reacted with polymer-supported triphenylphosphine (Fluka, cat. # 93094). Reaction of the obtained amine with di-(2-pyridyl) thionocarbonate (DPT) gave the alkyl isothiocyanate derivative **157** (Scheme 28, *work of Dr. Christophe Salomé*).

Scheme 28. Synthesis of the side chain isothiocyanate AB&CR (**route 3**)
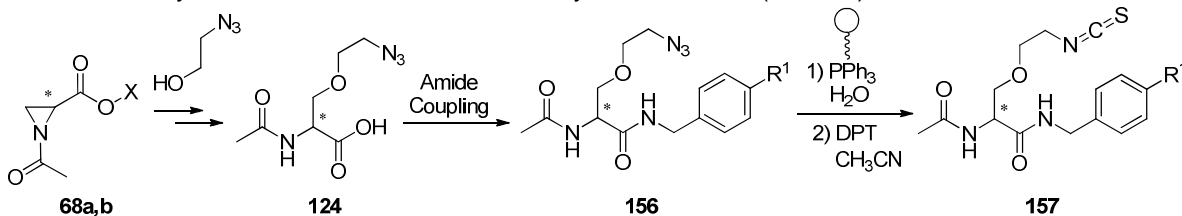
2.3.1.3. Introduction of epoxide AB groups

With aryl aldehyde **158** in hand, we used the conjugate base of trimethylsulfoxonium iodide (Corey-Chaykovsky reagent) to form the corresponding epoxides **159** in one step (Scheme 29).[412]

Scheme 29. Synthesis of aromatic epoxide AB&CR agents (**route 3**)
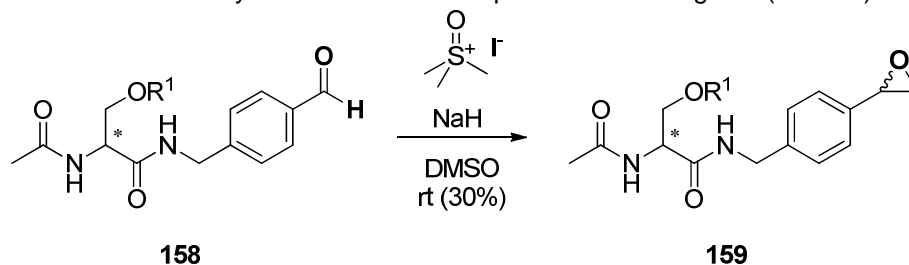
To introduce the epoxide on the C(2) side chain, we ring opened **68-a,b** with commercial 3-buten-1-ol. Saponification of the esters gave enantiopure acid **120**.

Subsequent coupling with the required benzylamine and epoxidation with *meta*-chloroperbenzoic acid (*m*CPBA) gave the diastereomeric epoxide **160** with the desired C(2) configuration (Scheme 30).[413]

Scheme 30. Synthesis of the side chain epoxide AB&CR agents (**route 3**)
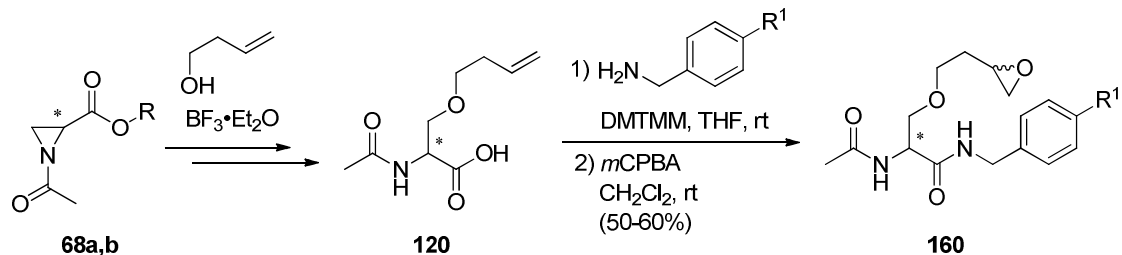
2.3.1.4. Introduction of azide CR groups

Benzylammonium hydrochloride **134** was coupled with various carboxylic acids to introduce the azide CR/photoAB on the phenyl ring (**162**, Scheme 31, bottom). To introduce the azide on the C(2) side chain, we prepared 2-azidoethanol (**71**) from 2-chloroethanol (**161**) and NaN$_3$[414] (Scheme 31, top) and used the established sequence to form the FAA **156** (see Scheme 28).

Scheme 31. Synthesis of 2-azidoethanol (top) and AB&CR agents bearing an aromatic azide (bottom) (**route 3**)
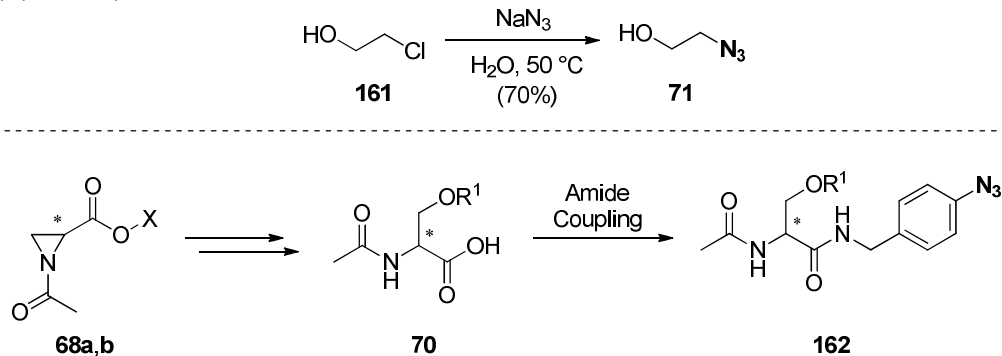
2.3.1.5. Introduction of alkyne CR groups

The alkyne CR was introduced on the lacosamide C(2) side-chain by ring-opening **68a,b** with propargyl alcohol and following **route 3** to benzylamide **164** (Scheme 32, top). For the CR agents bearing the *para*-substituted alkyne, carboxylic acid **70** was treated with benzylamine **141** using an amide coupling reaction (Scheme 32, bottom).

Scheme 32. Synthesis of the alkyne-containing AB&CR agents (**route 3**)
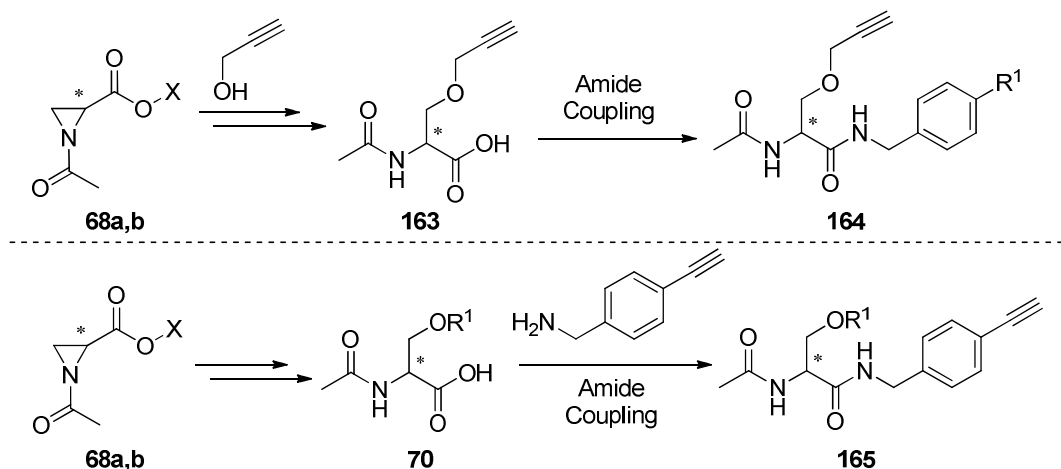
2.3.1.6. Introduction of diazirine and benzophenone photoAB groups (*work of Dr. Christophe Salomé*)

The trifluoromethylaromatic diazirine was introduced by coupling acid **163** with benzylamine **144**. For the C(2) side chain alkyl diazirine, 4-hydroxybutan-2-one (**166**) was condensed with hydroxylamine and the resulting oxime successively tosylated and reacted with liquid ammonia and iodine (Scheme 33, top). Methyldiazirinyl alcohol **167** was then reacted with **68** to give **168** after ester hydrolysis and amide coupling. The esters were hydrolyzed and coupled with

benzylamine **150** to provide the desired AB&CR derivative. Finally, 4-aminomethylbenzophenone **146** was coupled with acid **163** to provide AB&CR agent **170** (Scheme 33, bottom).

Scheme 33. Synthesis of 2-(3-methyl-3*H*-diazirin-3-yl)ethanol and the corresponding AB&CR agent (top), and photoAB&CR agents bearing an aromatic diazirine or benzophenone (bottom) (**route 3**)
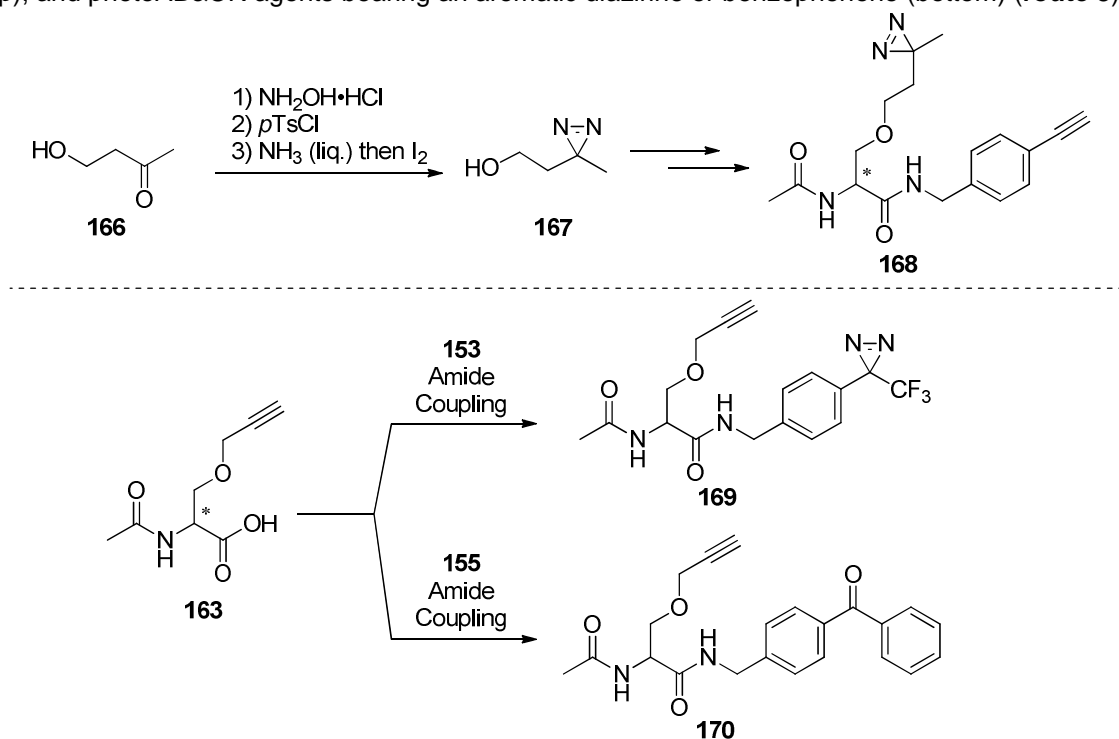
2.3.1.7. Summary

A comprehensive list of all the AB&CR agents synthesized and used for chemical biology studies is given in Figure 9. All molecules were prepared stereoisomerically pure at the C(2) following the previously described synthetic schemes. Compounds **172** and **181** were synthesized by Dr Ki Duk Park, and compounds **168**, **169**, **170**, and **177** were synthesized by Dr Christophe Salomé.

171; R^1 = CH$_2$CH$_2$N$_3$
172; R^1 = CH$_2$C≡CH

173; R^1 = CH$_2$CH$_2$N$_3$
174; R^1 = CH$_2$C≡CH

175; R^1 = CH$_2$CH$_2$N$_3$
176; R^1 = CH$_2$C≡CH

177

178

179; R^1 = N$_3$
180; R^1 = C≡CH

181

169

168

170
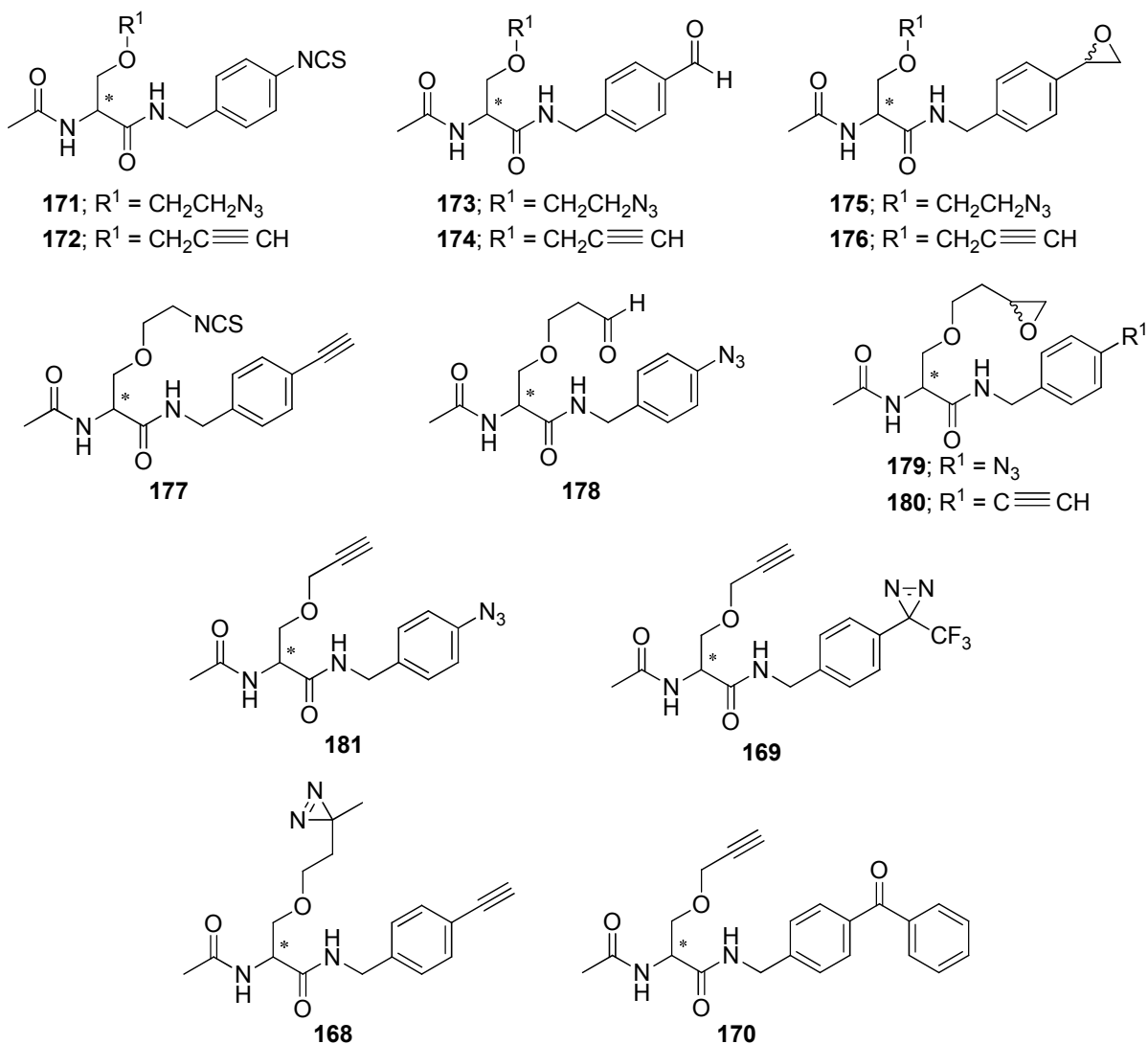
Figure 9. Structures of the different AB&CR agents synthesized for chemical biology studies.

2.3.2. Biotin and fluorescent alkyne and azide Probes

Two sets of Probes were constructed for chemical biology studies. The first set contained a biotin unit appended to the CR group. This set of Probes was used for isolating putative target proteins from the rat brain lysate. The biotinylated proteins were captured using Streptavidin beads, washed, eluted, resolved on SDS PAGE gel and analyzed by mass spectrometry. Although ideal for purifying

drug/protein complexes,[415,258,416] biotin/streptavidin-based purification is a time-consuming process. Therefore, we prepared the corresponding set of Probes containing a tetramethylrhodamine fluorophore (TAMRA). Incorporation of the TAMRA moiety readily permitted protein detection by in-gel fluorescence.

For both Probes we chose to include a polyethyleneglycol (PEG) spacer for two reasons. The first one was to improve the water solubility of the molecule and the second to minimize adverse protein (*i.e.* Streptavidin)/Probe interactions. To synthesize these, we first constructed the polyethylene glycol (PEG) moieties (Scheme 34). Commercial tetraethylene glycol (**182**) was reacted with MsCl (2 equiv) to form the di-mesylate intermediate **183**, and then treated with NaN$_3$ (2 equiv) to form diazido tetraethyleneglycol **184**.[417] Amine **185** was obtained by a mono-reduction of diazide **184** using a Staudinger reaction in a biphasic solvent system.[417] The PEG moiety of the alkyne Probe was constructed in a similar fashion. Tetraethylene glycol was reacted with NaH and propargyl bromide (1 equiv).[418,419] The hydroxyl group was mesylated, displaced with NaN$_3$, and reduced under Staudinger conditions to yield amine **189**.

Scheme 34. Synthetic pathway to azide and alkyne-containing PEG linkers
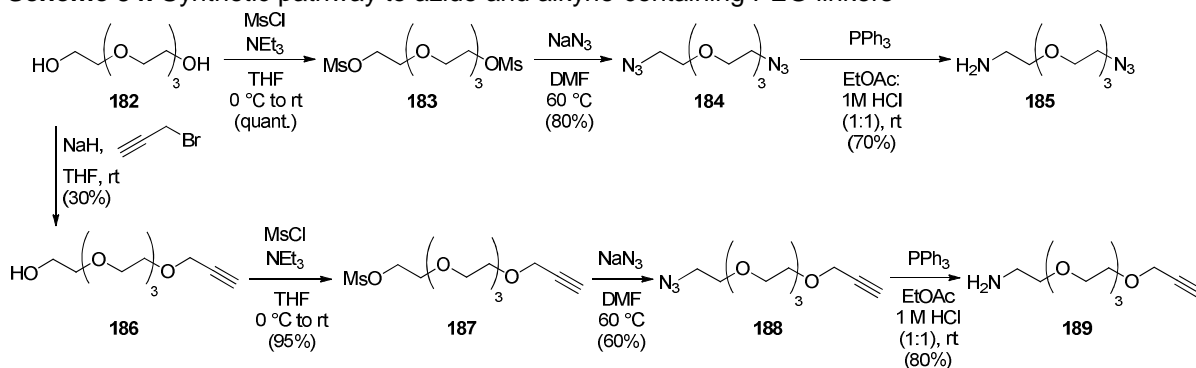
Biotin Probe **193** was prepared by linking commercial D-biotin (**190**) with **185** using an amide coupling reaction (DMTMM) to yield biotin-PEG-N$_3$ Probe **191**. The azide was reduced with PPh$_3$ and H$_2$O and the resulting amine **192** was coupled (DMTMM) with 4-pentynoic acid to yield biotin-PEG-alkyne Probe **193** (Scheme 35). Both amines **185** and **189** were readily coupled with 5,6-carboxytetramethylrhodamine to give fluorescent Probes TAMRA-PEG-N$_3$ **196** and TAMRA-PEG-alkyne **195**, respectively (Scheme 36).

Scheme 35. Synthesis of PEG-containing D-biotin Probes

Scheme 36. Synthetis of PEG-containing TAMRA fluorescent Probes
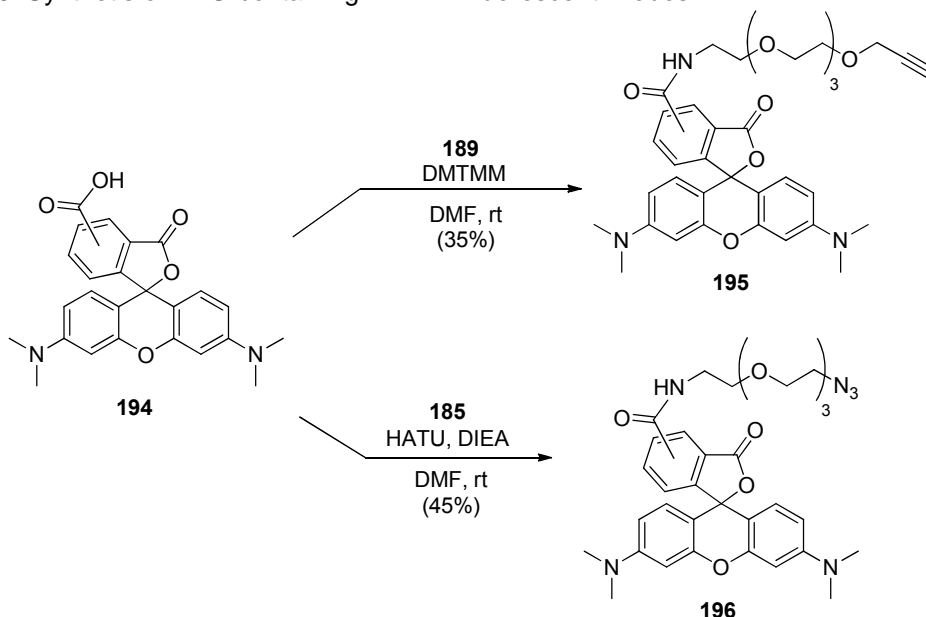
2.3.3. Experimental Section

2.3.3.1. Synthesis of *para*-substituted benzylamines

***Tert*-butyl 4-Azidobenzylcarbamate (133).**[324] To a cooled (ice bath) THF solution (40 mL) of 4-aminobenzylamine (1.00 g, 8.2 mmol) was slowly added a solution of Boc_2O (1.8 g, 8.2 mmol) in THF (10 mL). The reaction was stirred at room temperature (15 h) and the THF was evaporated to give **132** as a pale yellow solid (1.86 g, quant.) that was directly dissolved in CH_3CN (25 mL) and cooled (ice bath). While stirring, *t*-BuONO (1.5 mL, 12.6 mmol) and $TMSN_3$ (132 μL, 1 mmol) were added and the reaction was vigorously stirred at 0 ℃ u ntil bubbling was observed (10–20 min). More $TMSN_3$ (1.2 mL, 9 mmol) was then added dropwise at 0 ℃ and the reaction stirred at room temperature (14 h). The solvent was evaporated and the residue purified by flash chromatography (CH_2Cl_2) to give **133** as an orange oil (1.33 g, 65%) that slowly turned to yellow crystals: mp 58–60 ℃; R_f = 0.33 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.45 (s, C(**CH$_3$**)$_3$), 4.24–4.28 (m, NH**CH$_2$**C$_6$H$_4$), 4.90–5.10 (m, N**H**CH$_2$C$_6$H$_4$), 6.96 (d, *J* = 8.4 Hz, 2 Ar**H**), 7.25 (d, *J* = 8.4 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 28.5 (C(**CH$_3$**)$_3$), 44.2 (NH**CH$_2$**C$_6$H$_4$), 79.7 (**C**(CH$_3$)$_3$), 119.3, 129.0, 136.0, 139.1 (**C$_6$**H$_4$), 156.0 (O**C**(O)NH).

4-Azidobenzylammonium Hydrochloride (134). Compound **133** (7.17 g, 28.9 mmol) was dissolved in a 4 M HCl dioxane solution (25 mL, 100 mmol). The reaction was stirred at room temperature (12 h) and the resulting salt was filtered, and rinsed with Et_2O to give hydrochloride **134** (5.23 g, 98%) as a light beige solid: mp 80–82 ℃; R_f = 0–0.1 (1/9 MeOH/CH_2Cl_2); ^{1}H NMR (DMSO-*d$_6$*) δ 3.99 (d, *J* = 5.1 Hz,

$CH_2C_6H_4$), 7.14–7.18, 7.54–7.58 (m, $CH_2C_6H_4$), 8.25–8.80 (m, NH_3^+); ^{13}C NMR (DMSO-d_6) δ 41.5 ($CH_2C_6H_4$), 119.2, 130.9, 131.0, 139.5 (C_6H_4); **134** was not detected by mass spectrometry. Anal. Calcd for $C_7H_9N_4Cl$: C, 45.54; H, 4.91; N, 30.35; Cl, 19.20. Found: C, 45.78; H, 5.02; N, 30.18; Cl, 19.37.

4-(1,3-Dioxolan-2-yl)benzonitrile (136).[408] To a toluene solution (150 mL) of (4-cyano)benzaldehyde (**135**, 15.00 g, 108.8 mmol) was added ethylene glycol (23.9 mL, 435.1 mmol) and *p*TSA (21 mg, 0.11 mmol). The reaction solution was heated to reflux with a Dean-Stark apparatus until H_2O ceased forming (15 h), and then cooled to room temperature. The reaction washed with aqueous saturated $NaHCO_3$ (150 mL) and brine (150 mL). The organic layer was concentrated in vacuo to give a pale yellow residue that was recrystallized from Et_2O and hexanes to yield 17.20 g of **135** (90%) as white flakes: mp 44–45 °C (lit. [408] mp = 39–40 °C); R_f = 0.55 ($CHCl_3$); ^1H NMR ($CDCl_3$) δ 4.02–4.14 (m, OCH_2CH_2O), 5.84 (s, $OCHO$), 7.58 (d, J = 9.0 Hz, 2 Ar**H**), 7.66 (d, J = 9.0 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 65.6 (OCH_2CH_2O), 102.6 ($OCHO$), 113.0 (Ar**C**), 118.7 (**C**≡N), 127.3 (2 Ar**C**), 132.4 (2 Ar**C**), 143.3 (Ar**C**).

(4-(1,3-Dioxolan-2-yl)phenyl)methanamine (137).[408] A THF solution (60 mL) of **136** (23.12 g, 132 mmol) was added dropwise to a stirred THF solution of 1.0 M $LiAlH_4$ (400 mL, 400 mmol) at 0 °C. The reaction solution was stirred at 0 °C (15 min) and progressively turned yellow. It was further stirred at room temperature (15 h) and the excess $LiAlH_4$ was quenched by cooling the reaction (0 °C) and successively adding H_2O (12 mL), 15% aqueous NaOH (6 mL), and H_2O (12 mL)

dropwise. The mixture was stirred at room temperature (2 h) and filtered. The solid residue was rinsed CH_2Cl_2 and the combined organic layers were evaporated to give 21.80 g (92%) of **137** as a slightly yellow residue that did not require further purification: R_f = 0.20–0.44 (5/95 MeOH/CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 1.43 (s, N**H$_2$**), 3.85 (s, C**H$_2$**NH$_2$), 3.96–4.16 (m, OC**H$_2$**C**H$_2$**O), 5.79 (s, OC(**H**)O), 7.31 (d, J = 8.1 Hz, 2 Ar**H**), 7.43 (d, J = 8.1 Hz, 2 Ar**H**); ^{13}C NMR (CDCl$_3$) δ 46.4 (NH$_2$**C**H$_2$C$_6$H$_4$), 65.6 (O**C**H$_2$**C**H$_2$O), 103.7 (O**C**(H)O), 126.8, 127.3, 136.5, 144.6 (**C$_6$**H$_4$).

tert-Butyl 4-(Ethynyl)benzylcarbamate (140).[420] To a THF solution (130 mL) of compound **139** (4.10 g, 13.5 mmol) was added TBAF (1 M in THF, 28 mL, 28 mmol) in one portion and the reaction was stirred at room temperature (4 h). CH_2Cl_2 (150 mL) and 10% aqueous citric acid (100 mL) were added. The aqueous layer was extracted with CH_2Cl_2 (2 x 50 mL). All of the CH_2Cl_2 layers were washed with brine (200 mL), dried (Na$_2$SO$_4$), and evaporated to dryness. The dark brown residue obtained was purified by flash chromatography (15/85 EtOAc/hexanes) to yield **140** as a pale orange solid (2.70 g, 85%): mp 82–83 °C (lit.[420] mp 82 °C); R_f = 0.47 (15/85 acetone/EtOAc); ^{1}H NMR (CDCl$_3$) δ 1.46 (s, (C**H$_3$**)$_3$COC(O)NH), 3.06 (s, ArC≡C**H**), 4.31 (d, J = 6.0 Hz, C(O)NHC**H$_2$**), 4.80–4.92 (m, C(O)NHCH$_2$), 7.23 (d, J = 9.1 Hz, 2 Ar**H**), 7.45 (d, J = 9.1 Hz, 2 Ar**H**); ^{13}C NMR (CDCl$_3$) δ 28.6 ((**C**H$_3$)$_3$C(O)), 44.6 (C(O)NH**C**H$_2$), 77.4 (Ar**C**≡CH), 79.9 ((CH$_3$)$_3$**C**O), 83.6 (Ar**C**≡CH), 121.2, 127.5, 132.6, 140.0 (**C$_6$**H$_4$), 156.0 (O**C**(O)NH).

4-(Ethynyl)benzylamine (141).[420] Compound **140** (1.20 g, 5.2 mmol) was dissolved in CH_2Cl_2 (50 mL) and TFA (10 mL) was added. The dark brown solution was stirred at room temperature (1 h), after which time it was concentrated in vacuo. Saturated aqueous $NaHCO_3$ (100 mL) was added to the residue, and the emulsion was vigorously stirred until no more gas evolved. CH_2Cl_2 (50 mL) was added and the organic layer was separated. The aqueous layer was further extracted with CH_2Cl_2 (3 x 50 mL). All the organic layers were combined, washed with brine (100 mL), dried (Na_2SO_4), and evaporated to yield amine **140** as a dark brown oil (510 mg, 75%) that was used immediately in the next step.

Dimethoxytriazine *N*-Methylmorpholinium Hydrochloride (DMTMM).[421] Chlorodimethoxytriazine (CDMT) (25.00 g, 142 mmol) was suspended in THF (1 L) and while stirring NMM (17.3 mL, 157 mmol) was added. The suspension was stirred at room temperature (3 h) and filtered. The white solid was rinsed with THF and dried to yield 32.50 g (83%) of DMTMM that required no further purification: mp 121–123 °C (lit. [421] mp 116–117 °C); ^{1}H NMR (CD_3OD) δ 3.54 (s, N^+CH_3), 3.78–3.95, 4.02–4.10 , 4.51–4.58 ($N^+(CH_2CH_2)_2O$), 4.17 (s, 2 OCH_3); ^{13}C NMR ($CDCl_3$) δ 56.6 (N^+CH_3), 57.7 ($N^+(CH_2CH_2)_2O$), 61.5 (2 OCH_3), 63.4 (($N^+(CH_2CH_2)_2O$), 172.1, 175.6 (2 $COCH_3$).

2.3.3.2. Synthesis of lacosamide AB derivatives

Scheme 37. Synthesis of the AB derivative **197** bearing the epoxide at the benzylamide *para* position.

                    151   **197**
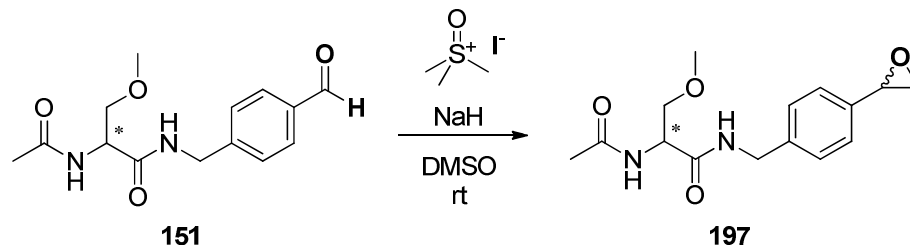
(*R*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl)   2-*N*-(Benzyloxycarbonyl)amino-3-

hydroxypropionamide ((*R*)-147). Using Method D, Cbz-D-serine (6.18 g, 25.85

mmol), **137** (5.09 g, 28.44 mmol) and DMTMM (7.86 g, 28.44 mmol) in THF (400

mL) gave 5.56 g (54%) of (*R*)-**147** as a white solid after recrystallization of the crude

material from CHCl$_3$. Purification of the mother liquors by silica gel chromatography

(6/93.5/0.5 MeOH/CHCl$_3$/NEt$_3$) yielded an additional 1.86 g (18%) (total yield: 7.42 g,

72%): mp 129–131 °C; $[\alpha]^{25}_D$ +2.9°(*c* 1.0, MeOH); R_f= 0.37 (5/95 MeOH/CHCl$_3$); IR

(nujol) 3288, 1689, 1642, 1540, 1459 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.53–3.59 (m,

CHC**H$_2$**O), 3.88–4.13 (m, OC**H$_2$CH$_2$**O, C**H**CH$_2$O), 4.30 (d, *J* = 5.7 Hz, NHC**H$_2$**C$_6$H$_4$),

4.90 (t, *J* = 5.4 Hz, CH$_2$O**H**), 5.04 (s, C$_6$H$_5$C**H$_2$**O), 5.69 (s, OC(**H**)O), 7.19–7.42 (m,

C$_6$**H$_5$**, C$_6$**H$_4$**), 8.44 (t, *J* = 5.7 Hz, N**H**CH$_2$C$_6$H$_4$), the carbamate NH was not detected;

^{13}C NMR (DMSO-d_6) δ 41.9 (NH**C**H$_2$C$_6$H$_4$), 57.4 (**C**HCH$_2$OH), 61.7 (C$_6$H$_5$**C**H$_2$O),

64.8 (O**C**H$_2$**C**H$_2$O), 65.5 (CH**C**H$_2$OH), 102.7 (O**C**(H)O), 126.2, 126.8, 127.7, 127.8,

128.3, 136.5, 137.0, 140.4, (**C$_6$**H$_5$, **C$_6$**H$_4$) 155.9 (O**C**(O)NH), 170.2 (CH**C**(O)NH); M_r

(+ESI) 423.1531 [M+Na]$^+$ (calcd for C$_{21}$H$_{24}$N$_2$O$_6$Na$^+$ 423.1532). Anal. Calcd for

C$_{21}$H$_{24}$N$_2$O$_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 63.00; H, 6.03; N, 6.97.

(*S*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl) 2-*N*-(Benzyloxycarbonyl)amino-3-hydroxypropionamide ((*S*)-147). Using Method D and following the preceding procedure, benzylamine **137** (5.09 g, 28.44 mmol), Cbz-L-serine (6.18 g, 25.85 mmol) and DMTMM (7.86 g, 28.44 mmol) in THF (500 mL) gave 5.66 g (55%) of (*S*)-**147** after recrystallization from $CHCl_3$ and an additional 1.78 g (17%) after purification of mother liquors (total yield: 7.44 g, 72%): mp 129–131°C; $[\alpha]^{25}_D$ -2.9° (*c* 1.0, MeOH); R_f = 0.37 (5/95 MeOH/$CHCl_3$); IR (nujol) 3288, 1689, 1642, 1540, 1459 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.53–3.59 (m, CHC**H$_2$**O), 3.88–4.13 (m, OC**H$_2$C**H$_2$O, CHC**H$_2$**O), 4.30 (d, *J* = 5.7 Hz, NHC**H$_2$**C$_6$H$_4$), 4.82–4.97 (br s, CH$_2$O**H**). 5.04 (s, C$_6$H$_5$C**H$_2$**O), 5.69 (s, OC(**H**)O), 7.19–7.42 (m, C$_6$**H$_5$**, C$_6$**H$_4$**), 8.44 (t, *J* = 5.7 Hz, N**H**CH$_2$C$_6$H$_4$), the carbamate NH could not be detected; ^{13}C NMR (CD$_3$OD) δ 44.0 (NH**C**H$_2$C$_6$H$_4$), 58.9 (**C**HCH$_2$OH), 63.4 (C$_6$H$_5$**C**H$_2$O), 66.4 (O**C**H$_2$CH$_2$O), 68.0 (CH**C**H$_2$OH), 104.9 (O**C**(H)O), 128.0, 128.4, 129.1, 129.2, 129.6, 138.2, 138.5, 141.0 (**C$_6$**H$_5$), 158.6 (O**C**(O)NH), 173.2 (CH**C**(O)NH); M_r (+ESI) 423.1525 [M+Na]$^+$ (calcd for $C_{21}H_{24}N_2O_6Na^+$ 423.1532). Anal. Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.86; H, 6.05; N, 7.06.

(*R*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl) 2-*N*-(Benzyloxycarbonyl)amino-3-methoxypropionamide ((*R*)-148). Using Method G, (*R*)-**147** (5.56 g 13.9 mmol), Ag$_2$O (16.19 g, 69.5 mmol) and MeI (8.66 mL, 139 mmol) gave a pale yellow residue after filtration and evaporation. Et$_2$O (50 mL) was added to the residue and (*R*)-**148** (3.95 g, 69%) was recovered as a white solid after filtration: mp 118–119°C; $[\alpha]^{25}_D$ +1.5° (*c* 1.0, MeOH); R_f = 0.57 (5/95 MeOH/$CHCl_3$); IR (nujol) 3298, 1690, 1645,

1542 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 3.34 (s, CH$_2$OC**H$_3$**), 3.47 (dd, J = 6.6, 9.3 Hz, C**H**H'OCH$_3$), 3.82 (dd J = 3.8, 9.3 Hz, CH**H'**OCH$_3$), 4.01–4.15 (m, OC**H$_2$**C**H$_2$**O), 4.08–4.18 (br m, C**H**CH'H), 4.45 (d, J = 5.7 Hz, NHC**H$_2$**C$_6$H$_4$), 5.10 (s, C$_6$H$_5$C**H$_2$**O), 5.73 (br d, J = 5.7 Hz, OC(O)N**H**), 5.78 (s, OC(**H**)O), 6.61–6.74 (m, N**H**CH$_2$C$_6$H$_4$), 7.26 (d, J = 7.8 Hz, 2 Ar**H**), 7.31–7.39 (m, C$_6$**H$_5$**), 7.42 (d, J = 7.8 Hz, 2 Ar**H**); ^{13}C NMR (CDCl$_3$) δ 43.4 (NH$_2$**C**H$_2$C$_6$H$_4$), 54.5 (**C**HCH$_2$O), 59.3 (CH$_2$O**C**H$_3$), 65.5 (O**C**H$_2$CH$_2$O), 67.4 (C$_6$H$_5$**C**H$_2$O), 72.2 (**C**H$_2$OCH$_3$), 103.6 (O**C**(H)O), 127.0, 127.7, 128.3, 128.4, 128.7, 136.2, 137.4, 139.1 (**C$_6$**H$_5$, **C$_6$**H$_4$), 156.3 (O**C**(O)NH), 170.0 (CH**C**(O)NH); M_r (+ESI) 437.1687 [M+Na]$^+$ (calcd for C$_{22}$H$_{26}$N$_2$O$_6$Na$^+$ 437.1689). Anal. Calcd for C$_{22}$H$_{26}$N$_2$O$_6$•0.25H$_2$O: C, 63.07; H, 6.38; N, 6.69. Found: C, 63.18; H, 6.38; N, 6.70.

(*S*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl)   2-*N*-(Benzyloxycarbonyl)amino-3-methoxypropionamide ((*S*)-148). Using Method G and following the preceding procedure, (*S*)-**147** (5.66 g, 14.2 mmol), Ag$_2$O (16.54 g, 71.0 mmol) and MeI (8.84 mL, 142.0 mmol) in CH$_3$CN (100 mL) gave 3.91 g (67%) of (*S*)-**148** as a white powder after precipitation of the residue with Et$_2$O (150 mL): mp 118–119°C; [α]^{25}$_D$ -1.5°(*c* 1.0, MeOH); R_f = 0.57 (5/95 MeOH/CHCl$_3$); IR (film) 3424, 3055, 2986, 1723, 1678 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 3.36 (s, CH$_2$OC**H$_3$**), 3.47 (dd, J = 6.6, 9.3 Hz, C**H**H'OCH$_3$), 3.87 (dd J = 3.8, 9.3 Hz, CH**H'**OCH$_3$), 4.01–4.15 (m, OC**H$_2$**C**H$_2$**O), 4.28–4.38 (m, C**H**CH'H), 4.48 (d, J = 5.7 Hz, NHC**H$_2$**C$_6$H$_4$), 5.12 (s, C$_6$H$_5$C**H$_2$**O), 5.61–5.70 (m, OC(O)N**H**), 5.80 (s, OC**H**O), 6.61–6.74 (m, N**H**CH$_2$C$_6$H$_4$), 7.22–7.48 (m, C$_6$**H$_5$**, C$_6$**H$_4$**), 8.44 (t, J = 5.7 Hz, N**H**CH$_2$C$_6$H$_4$); ^{13}C NMR (CDCl$_3$) δ 43.5

(NH$_2$**C**H$_2$C$_6$H$_4$), 54.5 (**C**HCH$_2$OCH$_3$), 59.3 (CH$_2$O**C**H$_3$), 65.5 (O**C**H$_2$**C**H$_2$O), 67.5

(C$_6$H$_5$**C**H$_2$O), 72.2 (**C**H$_2$OCH$_3$), 103.7 (O**C**(H)O), 127.1, 127.7, 128.4, 128.5, 128.8,

136.2, 137.5, 139.1 (**C$_6$**H$_5$, **C$_6$**H$_4$), 156.3 (O**C**(O)NH), 170.1 (CH**C**(O)NH); M_r (+ESI)

437.1681 [M+Na]$^+$ (calcd for C$_{22}$H$_{26}$N$_2$O$_6$Na$^+$ 437.1689). Anal. Calcd for

C$_{22}$H$_{26}$N$_2$O$_6$•0.25H$_2$O: C, 63.07; H, 6.38; N, 6.69. Found: C, 63.09; H, 6.37; N, 6.64.

(*R*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl) 2-Acetamido-3-methoxypropionamide ((*R*)-150). Using Method H, (*R*)-**148** (3.75 g, 9.1 mmol), and 10% Pd/C (700 mg) in

MeOH (50 mL) gave 2.54 g (100%) of a yellow oily residue that was directly

dissolved in THF (100 mL). After cooling (ice bath), Et$_3$N (1.26 mL, 9.07 mmol) and

AcCl (0.644 mL, 9.07 mmol) were successively added. The reaction was stirred at

room temperature (1 h) filtered and evaporated. Recrystallization of the residue from

EtOAc and hexanes gave 1.67 g (57%) of (*R*)-**150** as a pale beige solid: mp 138–

139°C; [α]^{25}$_D$ +13.0° (*c* 1.0, MeOH); R_f = 0.39 (5/95 MeOH/CHCl$_3$); IR (nujol) 3281,

3090, 1638, 1546, 1458 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.97 (s, **CH$_3$**C(O)), 3.35 (s,

CH$_2$O**CH$_3$**), 3.45 (dd, *J* = 6.9, 9.0 Hz, C**H**H'OCH$_3$), 3.73 (dd *J* = 4.2, 9.0 Hz,

CH**H'**OCH$_3$), 4.01–4.15 (m, OC**H$_2$**C**H$_2$**O), 4.20–4.55 (m, NHC**H$_2$**C$_6$H$_4$), 4.60 (app. dt,

J = 4.2, 6.9 Hz, C**H**CH$_2$O), 5.80 (s, OC(**H**)O), 6.76 (d, *J* = 6.9 Hz, CH$_3$C(O)N**H**), 7.14

(t, *J* = 5.7 Hz, N**H**CH$_2$C$_6$H$_4$), 7.25 (d, *J* = 7.8 Hz, 2 Ar**H**), 7.42 (d, *J* = 7.8 Hz, 2 Ar**H**),

addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**150** gave only one

signal for the acetyl methyl protons and the methoxy protons, addition of excess (*R*)-

(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**150** and (*R*)-**150** (~1:2 ratio) gave two

signals for the acetyl methyl protons (δ 2.021 (*S*) and 2.008 (*R*) (Δppm = 0.013) and

two signals for the methoxy protons (δ 3.317 (*S*) and 3.351 (*R*) (Δppm = 0.034)) in a

~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.2 (**C**H$_3$C(O)), 43.3 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.7 (**C**HCH$_2$O),

59.2 (CH$_2$O**C**H$_3$), 65.4 (O**C**H$_2$**C**H$_2$O), 72.0 (**C**H$_2$OCH$_3$), 103.6 (O**C**(H)O), 126.9,

127.6, 137.2, 139.2 (**C$_6$**H$_4$), 170.2 (CH**C**(O)NH or CH$_3$**C**(O)), 170.6 (CH$_3$**C**(O) or

CH**C**(O)NH): M_r (+ESI) 345.1424 [M+Na]$^+$ (calcd for C$_{16}$H$_{22}$N$_2$O$_5$Na$^+$ 345.1426).

Anal. Calcd for C$_{16}$H$_{22}$N$_2$O$_5$: C, 59.61; H, 6.88; N, 8.69. Found: C, 59.51; H, 6.90; N,

8.58.

(*S*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl) 2-Acetamido-3-methoxypropionamide ((*S*)-

150). Using Method H and following the preceding procedure compound (*S*)-**148**

(3.81 g, 9.2 mmol), 10% Pd/C (700 mg) in MeOH (50 mL) and Et$_3$N (1.28 mL, 9.18

mmol) and AcCl (650 μL, 9.18 mmol) in THF (100 mL) gave 1.70 g (57%) of (*S*)-**150**

as a light brown solid after recrystallization from EtOAc and hexanes: mp 138–139

℃; [α]^{25}$_D$ -13.0° (*c* 1.0, MeOH); R_f = 0.39 (5/95 MeOH/CHCl$_3$); IR (nujol) 3281,

3084, 1638, 1546, 1458 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.03 (s, **CH$_3$**C(O)), 3.38 (s,

CH$_2$O**CH$_3$**), 3.43 (dd, *J* = 7.5, 9.0 Hz, C**H**H'OCH$_3$), 3.80 (dd *J* = 3.7, 9.0 Hz,

CH**H'**OCH$_3$), 4.01–4.15 (m, O**CH$_2$CH$_2$**O), 4.40–4.50 (m, NH**CH$_2$**C$_6$H$_4$), 4.54 (app. dt,

J = 3.7, 7.5 Hz, C**H**CH$_2$), 5.80 (s, OC(**H**)O), 6.45 (d, *J* = 6.6 Hz, CH$_3$C(O)N**H**), 6.78–

6.82 (m, N(**H**)CH$_2$C$_6$H$_4$), 7.27 (d, *J* = 7.8 Hz, 2 Ar**H**), 7.45 (d, *J* = 7.8 Hz, 2 Ar**H**),

addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**150** gave only one

signal for the the acetyl peak protons and the methoxy protons, addition of excess

(*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**150** and (*R*)-**150** (~1:2 ratio) gave

two signals for the acetyl methyl protons (δ 2.021 (*S*) and 2.008 (*R*) (Δppm = 0.013)

and two signals for the methoxy protons (δ 3.317 (*S*) and 3.351 (*R*) (Δppm = 0.034)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.5 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.6 (**C**HCH$_2$O), 59.3 (CH$_2$O**C**H$_3$), 65.5 (O**C**H$_2$CH$_2$O), 71.8 (**C**H$_2$OCH$_3$), 103.7 (O**C**(H)O), 127.0, 127.7, 137.5, 139.1 (**C$_6$**H$_4$), 170.2, 170.5 (CH**C**(O)NH, CH$_3$**C**(O)); M_r (+ESI) 345.1421 [M+Na]$^+$ (calcd for C$_{16}$H$_{22}$N$_2$O$_5$Na$^+$ 345.1426). Anal. Calcd for C$_{16}$H$_{22}$N$_2$O$_5$: C, 59.61; H, 6.88; N, 8.69. Found: C, 59.50; H, 6.90; N, 8.56.

(*R*)-*N*-(4-Formylbenzyl) 2-Acetamido-3-methoxypropionamide ((*R*)-151). Using Method I, compound (*R*)-**150** (1.62 g, 5.03 mmol) and aqueous HCl in a THF:H$_2$O solution (2:1, 30 mL) gave 930 mg (66%) of (*R*)-**151** after work-up and recrystallization from EtOAc and hexanes. Mother liquors were purified by silica gel flash chromatography (MeOH/CHCl$_3$ 5/95) and yielded another 336 mg (24%) of the desired product (total yield: 1.27 g (90%)): mp 132–133°C; [α]^{25}$_D$ -10.4° (*c* 1.0, CHCl$_3$); R_f = 0.40 (5/95 MeOH/CHCl$_3$); IR (nujol) 3288, 3073, 1687, 1637, 1551, 1458, 1375 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.04 (s, **CH$_3$**C(O)), 3.40 (s, CH$_2$OC**H$_3$**), 3.46 (dd, *J* = 7.5 Hz, 9.6 Hz, C**H**H'OCH$_3$), 3.83 (dd *J* = 4.2 Hz, 9.6 Hz, CH**H'**OCH$_3$), 4.48–4.64 (m, NHC**H$_2$**C$_6$H$_4$, C**H**CH$_2$), 6.45 (d, *J* = 6.6 Hz, CH$_3$C(O)N**H**), 6.99–7.15 (m, N**H**CH$_2$C$_6$H$_4$), 7.42 (d, *J* = 8.1 Hz, 2 Ar**H**), 7.85 (d, *J* = 8.1 Hz, 2 Ar**H**), 9.99 (s, C(O)**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**151** gave only one signal for the the acetyl peak protons and the methoxy protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**151** and (*R*)-**151** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.037 (*S*) and 2.023 (*R*) (Δppm = 0.014) in a ~1:2 ratio, and two signals for the methoxy protons (δ 3.317 (*S*) and

3.351 (R) (Δppm = 0.034)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.4 (**CH$_3$**C(O)), 43.4 (NH$_2$**CH$_2$**C$_6$H$_4$), 52.7 (**C**HCH$_2$O), 59.4 (CH$_2$O**CH$_3$**), 71.7 (**CH$_2$**OCH$_3$), 128.0, 130.3, 135.9, 145.2 (**C$_6$**H$_4$), 170.5, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)), 192.0 (**C**(O)H); M_r (+ESI) 301.1161 [M+Na]$^+$ (calcd for C$_{14}$H$_{18}$N$_2$O$_4$Na$^+$ 301.1164). Anal. Calcd for C$_{14}$H$_{18}$N$_2$O$_4$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.13; H, 6.49; N, 9.91.

(R)-N-(4-Formylbenzyl) 2-Acetamido-3-methoxypropionamide ((R)-151) (alternate procedure). Using Method D, (R)-**115** (500 mg, 3.1 mmol), **137** (668 mg, 3.7 mmol) and DMTMM (1.030 g, 3.7 mmol) gave a pale yellow residue after filtration and evaporation. Using Method I, the crude benzylamide and aqueous HCl in a THF:H$_2$O solution (2:1, 30 mL) gave (R)-**151** (633 mg, 73%) as a white solid after work-up and silica gel chromatography: mp 131–133℃; [α]$^{25}_D$ -10.3° (c 1.0, CHCl$_3$); R_f = 0.40 (5/95 MeOH/CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 3.38 (s, CH$_2$O**CH$_3$**), 3.48 (app t, J = 9.0 Hz, **CH**H'OCH$_3$), 3.75 (dd J = 4.6, 9.0 Hz, CH**H'**OCH$_3$), 4.45–4.70 (m, NH**CH$_2$**C$_6$H$_4$, **CH**CH$_2$O), 6.24–6.40 (m, CH$_3$C(O)**NH**), 6.78–6.95 (m, **NH**CH$_2$C$_6$H$_4$), 7.42 (d, J = 8.3 Hz, 2 Ar**H**), 7.82 (d, J = 8.3 Hz, 2 Ar**H**), 9.92 (s, C(O)**H**); ^{13}C NMR (CDCl$_3$) δ 23.3 (**CH$_3$**C(O)), 43.3 (NH$_2$**CH$_2$**C$_6$H$_4$), 52.8 (**C**HCH$_2$OCH$_3$), 59.3 (CH$_2$O**CH$_3$**), 71.8 (**CH$_2$**OCH$_3$), 127.9, 130.3, 135.7, 145.2 (**C$_6$**H$_4$), 170.4, 170.5 (CH**C**(O)NH, CH$_3$**C**(O)), 192.1 (**C**(O)H).

(S)-N-(4-Formylbenzyl) 2-Acetamido-3-methoxypropionamide ((S)-151). Using Method I, compound (S)-**150** (1.65 g, 5.12 mmol) and aqueous HCl in THF:H$_2$O (2:1, 30 mL) gave 1.17 g of (S)-**151** as a white solid after recrystallization (EtOAc) and

silica gel chromatography (5/95 MeOH/CHCl$_3$): mp 132–133°C; $[\alpha]^{25}_D$ +10.4° (c 1.0, CHCl$_3$); R_f = 0.40 (5/95 MeOH/CHCl$_3$); IR (nujol) 3288, 1687, 1642, 1549, 1458, 1375 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.96 (s, **CH$_3$**C(O)), 3.36 (s, CH$_2$OC**H$_3$**), 3.53 (dd, J = 6.0, 9.3 Hz, C**H**H'OCH$_3$), 3.75 (dd J = 5.1, 9.3 Hz, CH**H'**OCH$_3$), 4.38–4.58 (m, NHC**H$_2$**C$_6$H$_4$), 4.71 (app. dt, J = 5.1, 6.0 Hz, C**H**CH$_2$O), 7.03 (d, J = 7.8 Hz, N**H**CH$_2$C$_6$H$_4$), 7.38 (d, J = 8.4 Hz, 2 Ar**H**), 7.68 (t, J = 5.4 Hz, CH$_3$C(O)N**H**), 7.77 (d, J = 8.4 Hz, 2 Ar**H**), 9.93 (s, C(O)**H**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**151** gave only one signal for the methoxy protons and the acetyl peak protons, addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**151** and (R)-**151** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.037 (S) and 2.023 (R) (Δppm = 0.014), in a ~1:2 ratio and two signals for the methoxy protons (δ 3.346 (S) and 3.377 (R) (Δppm = 0.031)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.0 (**C**H$_3$C(O)), 43.1 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.8 (**C**HCH$_2$O), 59.1 (CH$_2$OC**H$_3$**), 72.0 (**C**H$_2$OCH$_3$), 127.7, 130.0, 135.5, 145.3 (**C$_6$**H$_4$), 170.5, 170.7 (CH**C**(O)NH, CH$_3$**C**(O)), 192.0 (**C**(O)H); M_r (+ESI) 301.1158 [M+Na]$^+$ (calcd for C$_{14}$H$_{18}$N$_2$O$_4$Na$^+$ 301.1164). Anal. Calcd for C$_{14}$H$_{18}$N$_2$O$_4$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.40; H, 6.57; N, 9.90.

(S)-N-(4-Formylbenzyl)   2-Acetamido-3-methoxypropionamide   ((S)-151) (Alternate Procedure). Using Method D, (S)-**115** (200 mg, 1.24 mmol), **137** (267 mg, 1.49 mmol), and DMTMM (412 mg, 1.49 mmol) in THF (12 mL), followed by aqueous HCl in THF:H$_2$O (2:1, 20 mL) gave (S)-**151** (160 mg, 46%) as a white solid after work-up, purification by silica gel chromatography and recrystallization from

EtOAc: mp 132–133 ℃; $[\alpha]^{25}_D$ +10.2° (c 1.0, CHCl$_3$); R_f = 0.40 (5/95 MeOH/CHCl$_3$);

^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)), 3.38 (s, CH$_2$OC**H$_3$**), 3.48 (app t, J = 9.0 Hz,

CH**H'**OCH$_3$), 3.75 (dd J = 4.6, 9.0 Hz, C**H**H'OCH$_3$), 4.45–4.62 (m, NHC**H$_2$**C$_6$H$_4$),

4.60–4.68 (m, C**H**CH$_2$O), 6.62 (d, J = 8.1 Hz, CH$_3$C(O)N**H**), 7.15–7.25 (br t,

N**H**CH$_2$C$_6$H$_4$), 7.42 (d, J = 8.1 Hz, 2 Ar**H**), 7.82 (d, J = 8.4 Hz, 2 Ar**H**), 9.96 (s,

C(O)**H**); ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.3 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.8

(**C**HCH$_2$OCH$_3$), 59.3 (CH$_2$O**C**H$_3$), 71.9 (**C**H$_2$OCH$_3$), 127.9, 130.3, 135.7, 145.2

(**C$_6$**H$_4$), 170.5, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)), 192.0 (**C**(O)H).

(2R)-N-(4-(Oxiran-2-yl)benzyl) 2-Acetamido-3-methoxypropionamide ((R)-197)

(mixture of diastereomers). Using Method F, (R)-**151** (720 mg, 2.57 mmol) and

solution A (DMSO, 30.0 mL, [C]= 0.1 M, 3 mmol) gave (R)-**197** (312 mg, 42%) as a

white solid after work-up and purification by flash chromatography (15/85

acetone/EtOAc): mp 131–138 ℃; $[\alpha]^{25}_D$ -19.0° (c 0.6; CHCl$_3$); R_f = 0.36 (5/95

MeOH/CH$_2$Cl$_2$); IR (nujol) 3279, 1636, 1547, 1458, 1376, 1130 cm^{-1}; ^{1}H NMR

(CDCl$_3$) δ 2.01 (s, **CH$_3$**C(O)), 2.77 (dd, J = 2.4, 5.6 Hz, CH(O)CH**H'**), 3.14 (dd, J =

4.2, 5.6 Hz, CH(O)C**H**H'), 3.37 (s, CH$_2$OC**H$_3$**), 3.40–3.62 (dd, J = 7.4, 9.0 Hz,

CH**H'**OCH$_3$), 3.76 (dd, J = 4.2, 9.0 Hz, C**H**H'OCH$_3$), 3.84 (dd, J = 2.4, 4.2 Hz,

C**H**(O)CH$_2$), 4.38–4.52 (m, NHC**H$_2$**C$_6$H$_4$), 4.53–4.62 (m, C**H**CH$_2$O), 6.52 (d, J = 7.0

Hz, N**H**CHCH$_2$O), 6.86–6.92 (m, N**H**CH$_2$C$_6$H$_4$), 7.24 (s, C$_6$**H$_4$**), addition of excess

(R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**197** gave only one signal for the

acetyl protons (δ 2.008 ppm) and one signal for the methoxy protons (δ 3.356 ppm),

addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**197** and (R)-**197**

in a ~1:2 ratio gave two signals with a relative ~1:2 intensity for both the acetyl protons (δ 2.027 ppm (S)-**197**, δ 2.011 ppm (R)-**197**, (Δppm = 0.016)) and the methoxy protons (δ 3.331 ppm (S)-**197**, δ 3.357 ppm (R)-**197**, (Δppm = 0.026)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.4 (NH**C**H$_2$C$_6$H$_4$), 51.4, 52.3, 52.6 (**C**HC(O)NH, **C**H(O)CH$_2$), 59.3 (CH$_2$O**C**H$_3$), 71.9 (CH**C**H$_2$OCH$_3$), 126.0, 127.8, 137.1, 138.2 (**C$_6$**H$_4$), 170.2, 170.5 (CH$_3$**C**(O), CH**C**(O)NH); M_r (+ESI) 315.1323 [M+Na]$^+$ (calcd for C$_{15}$H$_{19}$N$_2$O$_4$Na$^+$ 315.1321). Anal. Calcd for C$_{15}$H$_{19}$N$_2$O$_4$: C, 61.63; H, 6.90; N, 9.58; Found: C, 61.53; H, 6.85; N, 9.44.

(2S)-N-(4-(Oxiran-2-yl)benzyl) 2-Acetamido-3-methoxypropionamide ((S)-197) (mixture of diastereomers). Using Method F, compound (S)-**151** (160 mg, 0.58 mmol) and solution A (DMSO, 6.9 mL, [C] = 0.1 M, 0.69 mmol) gave (S)-**197** (60 mg, 36%) as a white solid after work-up and purification by flash chromatography (15/85 acetone/EtOAc): mp 130–139 °C; [α]$^{25}_D$ +17.7° (c 1.4; CHCl$_3$); R_f = 0.36 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3281, 1636, 1547, 1458, 1376, 1130 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.00 (s, **CH$_3$**C(O)), 2.77 (dd, J = 2.4, 5.6 Hz, CH(O)C**H**H'), 3.14 (dd, J = 4.2, 5.6 Hz, CH(O)CH**H'**), 3.36 (s, CH$_2$OC**H$_3$**), 3.45 (dd, J = 7.4, 9.0 Hz, C**H**H'OCH$_3$), 3.76 (dd, J = 4.2, 9.0 Hz, CH**H'**OCH$_3$), 3.84 (dd, J = 2.4, 4.2 Hz, C**H**(O)CH$_2$), 4.34–4.52 (m, NH**CH$_2$**C$_6$H$_4$), 4.53–4.64 (m, C**H**CH$_2$O), 6.58–6.68 (br d, N**H**CHCH$_2$O), 6.98–7.08 (m, N**H**CH$_2$C$_6$H$_4$), 7.23 (s, C$_6$**H$_4$**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**197** gave only one signal for the acetyl protons (δ 2.030 ppm) and one signal for the methoxy protons (δ 3.328 ppm), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**197** and (R)-**197** in a ~1:2 ratio gave two

signals with a relative ~1:2 intensity for the acetyl protons (δ 2.027 ppm (S)-**197**, δ 2.011 ppm (R)-**197**, (Δppm = 0.016)) and the methoxy protons (δ 3.331 ppm (S)-**197**, δ 3.357 ppm (R)-**197**, (Δppm = 0.026)); ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.7 (NH**C**H$_2$C$_6$H$_4$), 51.3, 52.3, 52.7 (**C**HC(O)NH, **C**H(O)**C**H$_2$), 59.2 (CH$_2$O**C**H$_3$), 72.0 (CH**C**H$_2$OCH$_3$), 126.0, 127.7, 137.0, 138.2 (**C$_6$**H$_4$), 170.2, 170.6 (CH$_3$**C**(O), CH**C**(O)NH); M_r (+ESI) 315.1322 [M+Na]$^+$ (calcd for C$_{15}$H$_{19}$N$_2$O$_4$Na$^+$ 315.1321). Anal. Calcd for C$_{15}$H$_{19}$N$_2$O$_4$: C, 61.63; H, 6.90; N, 9.58. Found: C, 61.81; H, 7.01; N, 9.47.

2.3.3.3. Synthesis of lacosamide AB&CR derivatives

2.3.3.3.1. Derivatives bearing the CR group on the C(2) side chain

Scheme 38. Structure of the different intermediates and chemical reactions used in the synthesis of AB&CR agents **171**, **173**, **174**, **175** and **176**.
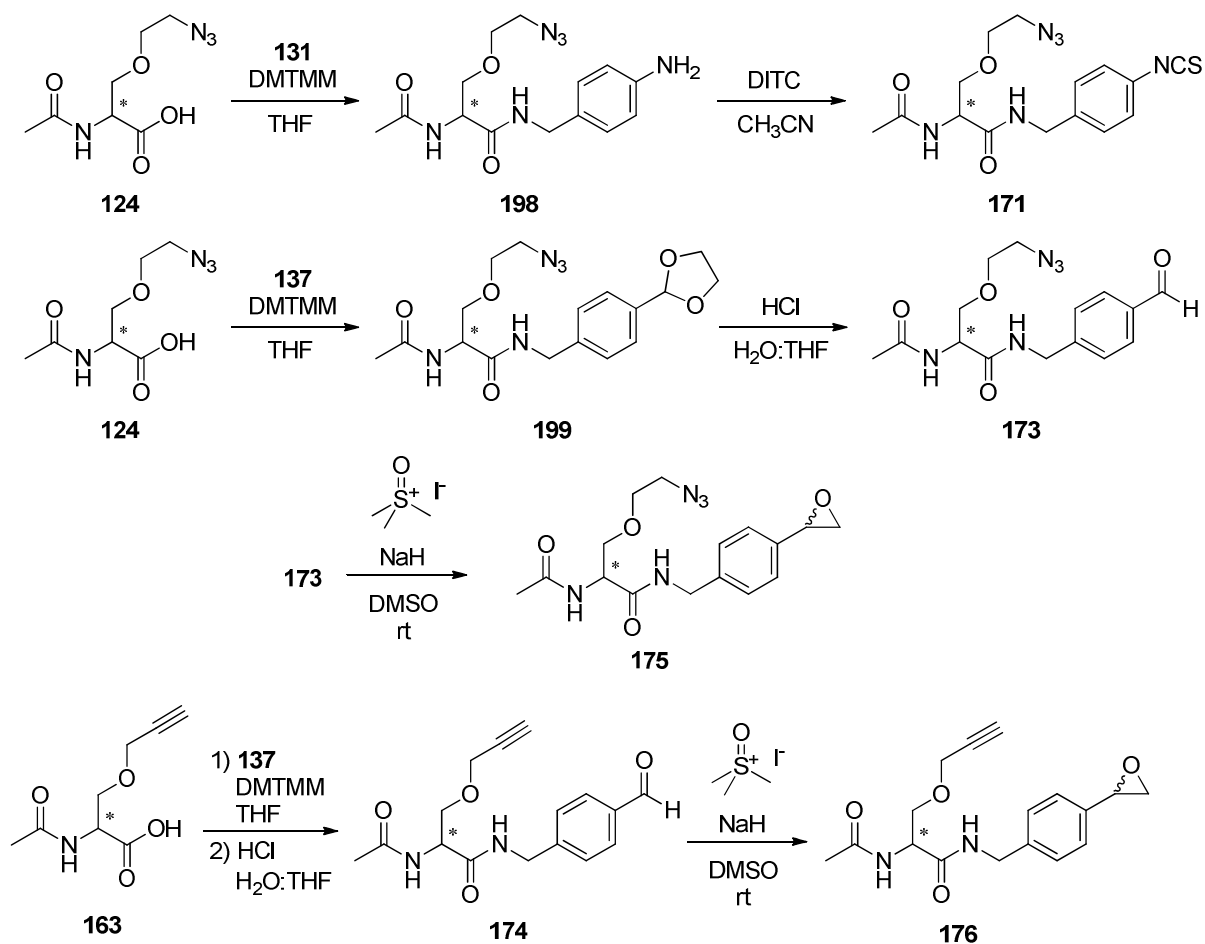
(*R*)-*N*-(4-Aminobenzyl) 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-198).

Using Method D, acid (*R*)-**124** (2.25 g, 10.4 mmol), 4-aminobenzylamine (1.52 g, 12.5 mmol) and DMTMM (3.46 g, 12.5 mmol) in THF (200 mL) gave 1.40 g (42%) of a dark brown residue *that was normally directly used in the next step.* Recrystallization from CHCl$_3$ and hexanes yielded a white solid that rapidly turned

brown when exposed to light or air: mp 98–99 °C; $[\alpha]^{25}_D$ +12.8° (c 0.5; MeOH); R_f = 0.36 (5/95 MeOH/CHCl$_3$); IR (nujol) 3314, 3205, 2107, 1628, 1522, 1457, 1374, 1285 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.85 (s, **CH$_3$**C(O)NH), 3.30–3.40 (m, **CH$_2$**N$_3$), 3.52–3.64 (m, O**CH$_2$**CH$_2$N$_3$, CHC**H$_2$**OCH$_2$), 4.08 (d, J = 6.0 Hz, C(O)NHC**H$_2$**Ph), 4.44–4.56 (m, C**H**CH$_2$O), 4.95 (s, C$_6$H$_4$N**H$_2$**), 6.48 (d, J = 8.7 Hz, 2 Ar**H**), 6.88 (d, J = 8.7 Hz, 2 Ar**H**), 8.02 (d, J = 8.4 Hz, CH$_3$C(O)N**H**), 8.29 (t, J = 6.0 Hz, C(O)N**H**CH$_2$Ph); ^{13}C NMR (DMSO-d_6) δ 22.5 (**C**H$_3$C(O)), 41.8 (NH**C**H$_2$Ph), 49.8 (**C**H$_2$N$_3$), 52.4 (**C**HCH$_2$O), 69.1 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 113.5, 125.9, 128.0, 147.4 (**C$_6$H$_4$**), 169.8, 169.2 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 321.1671 [M+H]$^+$ (calcd for C$_{14}$H$_{20}$N$_6$O$_3$H$^+$ 321.1675). Efforts to obtain satisfactory elemental analyses were unsuccessful.

(R)-N-(4-Isothiocyanatobenzyl)   2-Acetamido-3-(2-azidoethoxy)propionamide ((R)-171). Compound (R)-**198** (715 mg, 2.23 mmol) was dissolved in CH$_3$CN (40 mL) and DITC (90%, 510 mg, 2.57 mmol) was added all at once. The reaction solution was stirred at room temperature (1 h), the solvent was removed in vacuo, and the residue was purified using flash chromatography (5/95 MeOH/CHCl$_3$) and then recrystallized from EtOAc to yield 502 mg (62%) of an off-white solid: mp 142–143 °C; $[\alpha]^{25}_D$ -28.6° (c 0.8; CHCl$_3$); R_f = 0.45 (1/9 acetone/EtOAc); IR (nujol) 3276, 2181, 2114, 1631, 1547, 1459, 1374, 1285 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.05 (s, **CH$_3$**C(O)NH), 3.30–3.50 (m, **CH$_2$**N$_3$), 3.55 (dd, J = 7.5, 9.3 Hz, CHC**H**H'OCH$_2$), 3.62–3.80 (m, O**CH$_2$**CH$_2$N$_3$), 3.95 (dd, J = 4.0, 9.3 Hz, CHCH**H'**OCH$_2$), 4.46 (d, J = 6.0 Hz, C(O)NHC**H$_2$**Ph), 4.54–4.60 (m, C**H**CH$_2$O), 6.45 (br d, J = 6.2 Hz,

CH$_3$C(O)N**H**), 6.84–6.92 (br t, C(O)N**H**CH$_2$Ph), 7.18–7.22 (m, 2 Ar**H**), 7.23–7.29 (m, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**171** gave only one signal for the acetyl protons (δ 2.019), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**171** and (*R*)-**171** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.033 (*S*) and 2.020 (*R*) (Δppm = 0.013)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.2 (**C**H$_3$C(O)), 41.0 (NH**C**H$_2$Ph), 50.7 (**C**H$_2$N$_3$), 52.4 (**C**HCH$_2$O), 70.0 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.3 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 125.9, 128.6, 130.5 (3 Ar**C**), 135.6 (N**C**S), 137.4 (1 Ar**C**), 169.8, 169.2 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 363.1236 [M+H]$^+$ (calcd for C$_{15}$H$_{18}$N$_6$O$_3$SH$^+$ 363.1239). Anal. Calcd for C$_{15}$H$_{18}$N$_6$O$_3$S: C, 49.71; H, 5.01; N, 23.19; S, 8.85. Found: C, 49.55; H, 4.91; N, 23.13; S, 8.63.

(*S*)-*N*-(4-Isothiocyanatobenzyl)   2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-171). Using Method D, acid (*S*)-**124** (1.61 g, 7.52 mmol), 4-aminobenzylamine (1.02 mL, 9.03 mmol), and DMTMM (2.50 g, 9.03 mmol) in anhydrous THF (200 mL) gave after evaporation a brown residue to which EtOAc (100 mL) was added. After stirring (5 min) the insoluble materials were filtered, and rinsed with EtOAc. The filtrate was concentrated to yield a dark orange oily residue (2.38 g (crude)) (M_r (+ESI) 343.1496 [M+H]$^+$ (calcd for C$_{14}$H$_{20}$N$_6$O$_3$Na$^+$ 343.1495)). The residue was dissolved in CH$_3$CN (20 mL) and DITC (90%, 1.58 g, 7.90 mmol) was added at once. After stirring at room temperature (3 h), the reaction was concentrated in vacuo and the crude material was purified by flash column chromatography (5/95 MeOH/CHCl$_3$) and then recrystallized from EtOAc and hexanes to yield 706 mg (26% overall yield)

of (*S*)-**171** as an off-white solid: mp 142–143 °C; $[\alpha]^{25}_D$ +28.5° (*c* 1.0; CHCl$_3$); R_f = 0.45 (1/9 acetone/EtOAc); IR (nujol) 3280, 2177, 2108, 1637, 1548, 1452, 1374, 1293 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.01 (s, **CH$_3$**C(O)NH), 3.28–3.48 (m, **CH$_2$**N$_3$), 3.55 (dd, *J* = 7.2, 9.3 Hz, CHC**H**H'OCH$_2$), 3.62–3.78 (m, OC**H$_2$**CH$_2$N$_3$), 3.91 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_2$), 4.43 (d, *J* = 6.0 Hz, C(O)NHC**H$_2$**Ph), 4.56–4.62 (m, C**H**CH$_2$O), 6.56 (br d, *J* = 6.2 Hz, CH$_3$C(O)N**H**), 6.84–6.92 (br t, *J* = 6.0 Hz, C(O)N**H**CH$_2$Ph), 7.14–7.20 (m, 2 Ar**H**), 7.22–7.28 (m, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**171** gave only one signal for the acetyl protons (δ 2.032), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**171** and (*R*)-**171** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.033 (*S*) and 2.020 (*R*) (Δppm = 0.013)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.2 (NH**C**H$_2$Ph), 50.9 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 126.1, 128.8, 130.6 (3 Ar**C**), 135.8 (N**C**S), 137.6 (1 Ar**C**), 170.0, 170.7 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 363.1241 [M+H]$^+$ (calcd for C$_{15}$H$_{18}$N$_6$O$_3$SH$^+$ 363.1239). Anal. Calcd for C$_{15}$H$_{18}$N$_6$O$_3$S: C, 49.71; H, 5.01; N, 23.19; S, 8.85. Found: C, 49.86; H, 5.07; N, 23.10; S, 8.75.

(*R*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl) 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-199). Using Method D, acid (*R*)-**124** (400 mg, 1.87 mmol), benzylamine **137** (402 mg, 2.24 mmol), and DMTMM (620 mg, 2.24 mmol) in anhydrous THF (20 mL) gave 628 mg (89%) of (*R*)-**199** as a white solid after flash column chromatography (10/89.5/0.5 MeOH/CHCl$_3$/NEt$_3$). An analytical sample was obtained by recrystallization from EtOAc and hexanes: mp 112–113 °C; $[\alpha]^{25}_D$ -23.9°

(*c* 0.9; CHCl$_3$); R_f = 0.47 (10/89.5/0.5 MeOH/CHCl$_3$/NEt$_3$); IR (nujol) 3288, 3071, 2134, 1634, 1544, 1457, 1382, 1305, 1236, 1126 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)NH), 3.24–3.46 (m, **CH$_2$**N$_3$), 3.55 (dd, *J* = 7.2, 9.3 Hz, CHC**H**H'OCH$_2$), 3.60–3.74 (m, OC**H$_2$**CH$_2$N$_3$), 3.91 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_2$), 4.00–4.16 (m, OC**H$_2$CH$_2$**O), 4.40–4.54 (m, C(O)NHC**H$_2$**Ph), 4.56–4.62 (m, C**H**CH$_2$O), 5.79 (s, OC**H**(O)), 6.51 (d, *J* = 6.9 Hz, CH$_3$C(O)N**H**), 6.81–6.87 (br t, C(O)N**H**CH$_2$Ar), 7.28 (d, *J* = 8.1 Hz, 2 Ar**H**), 7.44 (d, *J* = 8.1 Hz, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**199** gave only one signal for the acetyl protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**199** and (*R*)-**199** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.028 (*S*) and 2.018 (*R*) (Δppm = 0.010)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.6 (NH**C**H$_2$Ph), 50.8 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 65.5 (O**C**H$_2$**C**H$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 103.6 (O**C**H(O)), 127.0, 127.7, 137.5, 139.1 (**C$_6$**H$_4$), 169.8, 170.6 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 400.1601 [M+Na]$^+$ (calcd for C$_{17}$H$_{23}$N$_5$O$_5$Na$^+$ 400.1597). Anal. Calcd for C$_{17}$H$_{23}$N$_5$O$_5$: 54.10; H, 6.14; N, 18.56. Found: C, 54.30; H, 6.09; N, 18.36.

(*S*)-*N*-(4-(1,3-Dioxolan-2-yl)benzyl)   2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-199). Using Method D, acid (*S*)-**124** (400 mg, 1.87 mmol), benzylamine **137** (402 mg, 2.24 mmol), and DMTMM (620 mg, 2.24 mmol) in anhydrous THF (20 mL) gave 518 mg (73%) of (*S*)-**199** as a white solid after flash column chromatography (10/89.5/0.5 MeOH/CHCl$_3$/NEt$_3$). An analytical sample was obtained by recrystallization from EtOAc and hexanes: mp 112–113 °C; [α]^{25}$_D$ +23.9°

(*c* 1.2; CHCl$_3$); R_f = 0.47 (10/89.5/0.5 MeOH/CHCl$_3$/NEt$_3$); IR (nujol) 3281, 3078, 2133, 1634, 1544, 1457, 1380, 1307, 1236, 1126 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.01 (s, **CH$_3$**C(O)NH), 3.24–3.44 (m, **CH$_2$**N$_3$), 3.55 (dd, *J* = 7.2, 9.0 Hz, CHC**H**H'OCH$_2$), 3.60–3.76 (m, O**CH$_2$**CH$_2$N$_3$), 3.91 (dd, *J* = 4.0, 9.0 Hz, CHCH**H'**OCH$_2$), 4.00–4.18 (m, O**CH$_2$**CH$_2$O), 4.40–4.54 (m, C(O)NH**CH$_2$**Ph), 4.54–4.60 (m, C**H**CH$_2$O), 5.79 (s, OC**H**(O)), 6.51 (d, *J* = 6.6 Hz, CH$_3$C(O)N**H**), 6.84–6.94 (br t, C(O)N**H**CH$_2$Ar), 7.28 (d, *J* = 8.1 Hz, 2 Ar**H**), 7.44 (d, *J* = 8.1 Hz, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**199** gave only one signal for the acetyl protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**199** and (*R*)-**199** (~1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.028 (*S*) and 2.018 (*R*) (Δppm = 0.010)) in a ~1:2 ratio; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.5 (NH**C**H$_2$Ph), 50.8 (**C**H$_2$N$_3$), 52.6 (**C**HCH$_2$O), 65.5 (O**C**H$_2$**C**H$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 103.6 (O**C**H(O)), 127.0, 127.7, 137.5, 139.1 (**C$_6$H$_4$**), 169.8, 170.6 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 400.1600 [M+Na]$^+$ (calcd for C$_{17}$H$_{23}$N$_5$O$_5$Na$^+$ 400.1597). Anal. Calcd for C$_{17}$H$_{23}$N$_5$O$_5$: 54.10; H, 6.14; N, 18.56. Found: C, 54.35; H, 6.20; N, 18.54.

(*R*)-*N*-(4-Formylbenzyl) 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-173).
Using Method I, (*R*)-**199** (280 mg, 0.74 mmol) and aqueous HCl in a THF:H$_2$O solution (2:1, 15 mL) gave 141 mg (57%) of (*R*)-**173** after work-up and recrystallization from EtOAc: mp 107–109 °C; [α]$^{25}_D$ -21.3° (*c* 1.1; CHCl$_3$); R_f = 0.45 (5/95 MeOH/CHCl$_3$); IR (nujol) 3277, 3080, 2860, 2106, 1699, 1638, 1550, 1456, 1378, 1304, 1131 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.06 (s, **CH$_3$**C(O)NH), 3.30–3.38 (m,

CH$_2$N$_3$), 3.59 (dd, J = 7.2, 9.0 Hz, CHC**H**H'OCH$_2$), 3.64–3.82 (m, OC**H$_2$**CH$_2$N$_3$), 3.97 (dd, J = 4.0, 9.0 Hz, CHCH**H'**OCH$_2$), 4.52–4.64 (m, C(O)NHC**H$_2$**Ph, C**H**CH$_2$O), 6.40–6.50 (br d, CH$_3$C(O)N**H**), 6.90–7.00 (br t, C(O)N**H**CH$_2$Ph), 7.43 (d, J = 8.1 Hz, 2 Ar**H**), 7.85 (d, J = 8.1 Hz, 2 Ar**H**), 10.01 (s, ArC(O)**H**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**173** gave only one signal for the acetyl protons, addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**173** and (R)-**173** (~2:1 ratio) gave two signals for the acetyl methyl protons (δ 2.043 (S) and 2.032 (R) (Δppm = 0.011)) in a ~2:1 ratio; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.4 (NH**C**H$_2$Ph), 50.8 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.5 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 128.0, 130.3, 135.8, 145.1 (**C$_6$**H$_4$), 170.2, 170.7 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$), 192.0 (Ar**C**(O)H); M_r (+ESI) 356.1341 [M+Na]$^+$ (calcd for C$_{15}$H$_{19}$N$_5$O$_4$Na$^+$ 356.1335). Anal. Calcd for C$_{15}$H$_{19}$N$_5$O$_4$: C, 54.05; H, 5.75; N, 21.01. Found: C, 54.30; H, 5.74; N, 20.73.

(S)-N-(4-Formylbenzyl) 2-Acetamido-3-(2-azidoethoxy)propionamide ((S)-173). Using Method I, (S)-**199** (256 mg, 0.68 mmol) and aqueous HCl in a THF:H$_2$O solution (2:1, 15 mL) yielded 110 mg (48%) of (S)-**173** after work-up and recrystallization from EtOAc: mp 107–109 °C; [α]$^{25}_D$ +21.3° (c 1.8; CHCl$_3$); R_f = 0.45 (5/95 MeOH/CHCl$_3$); IR (nujol) 3273, 3082, 2860, 2106, 1699, 1639, 1550, 1455, 1377, 1303, 1221 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.03 (s, C**H$_3$**C(O)NH), 3.28–3.44 (m, C**H$_2$**N$_3$), 3.60 (dd, J = 6.9, 9.0 Hz, CHC**H**H'OCH$_2$), 3.62–3.80 (m, OC**H$_2$**CH$_2$N$_3$), 3.92 (dd, J = 4.0, 9.0 Hz, CHCH**H'**OCH$_2$), 4.50–4.58 (m, C(O)NHC**H$_2$**Ar), 4.60–4.68 (m, C**H**CH$_2$O), 6.57 (d, J = 6.9 Hz, CH$_3$C(O)N**H**), 7.10–7.20 (br t, C(O)N**H**CH$_2$Ph), 7.43

(d, J = 8.1 Hz, 2 Ar**H**), 7.83 (d, J = 8.1 Hz, 2 Ar**H**), 9.98 (s, ArC(O)**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**173** gave only one signal for the acetyl protons, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**173** and (*R*)-**173** (~2:1 ratio) gave two signals for the acetyl methyl protons (δ 2.043 (*S*) and 2.032 (*R*) (Δppm = 0.011)) in a ~2:1 ratio; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.4 (NH**C**H$_2$Ph), 50.8 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.5 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 128.0, 130.3, 135.8, 145.1 (**C**$_6$H$_4$), 170.2, 170.7 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$), 192.0 (Ar**C**(O)H); M_r (+ESI) 356.1338 [M+Na]$^+$ (calcd for C$_{15}$H$_{19}$N$_5$O$_4$Na$^+$ 356.1335). Anal. Calcd for C$_{15}$H$_{19}$N$_5$O$_4$: C, 54.05; H, 5.75; N, 21.01. Found: C, 54.45; H, 5.81; N, 20.66.

(2*R*)-*N*-(4-(Oxiran-2-yl)benzyl)   2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-175) (mixture of diastereomers). Using Method F, solution A (DMF, 7.5 mL, [C]= 0.2 M, 1.5 mmol) and (*R*)-**173** (410 mg, 1.23 mmol) gave (*R*)-**175** (80 mg, 19%) as a white solid after purification by flash chromatography (15/85 acetone/EtOAc): mp 91–98°C; $[\alpha]^{25}_D$ -24.6° (*c* 0.4; CHCl$_3$); R_f = 0.38 (1/9 acetone/EtOAc); IR (CHCl$_3$ film) 3288, 3107, 2924, 2867, 2104, 1638, 1541, 1448, 1378, 1124 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.99 (s, **CH$_3$**C(O)), 2.76 (dd, J = 2.4, 5.6 Hz, CH(O)C**H**H'), 3.13 (dd, J = 4.0, 5.6 Hz, CH(O)CH**H'**), 3.24–3.44 (m, OCH$_2$C**H$_2$**N$_3$), 3.52–3.68 (m, O**CH$_2$**CH$_2$N$_3$, C**H**H'OCH$_2$), 3.83 (dd, J = 2.4, 4.0 Hz, C**H**(O)CHH'), 3.88 (dd, J = 4.5, 9.2 Hz, CH**H'**OCH$_2$), 4.43 (d, J = 7.2 Hz, NH**CH$_2$**C$_6$H$_4$), 4.58–4.64 (m, C**H**CH$_2$O), 6.65 (d, J = 7.2 Hz, N**H**CHCH$_2$O), 7.10–7.18 (br t, N**H**CH$_2$C$_6$H$_4$), 7.23 (s, C$_6$**H$_4$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*R*)-**175** gave only one signal for

the acetyl protons (δ 2.024 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**175** and (*R*)-**175** in a ~1:5 ratio gave two signals with a relative ~1:5 intensity for the acetyl protons (δ 2.030 ppm (*S*)-**175**, δ 2.020 ppm (*R*)-**175**, (Δppm = 0.010)), at 300 MHz, we were not able to detect individual peaks for the two diastereomers; ^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.5 (NH**C**H$_2$C$_6$H$_4$), 50.8, 51.3, 52.3, 52.6 (**C**HC(O)NH, **C**H(O)CH$_2$, **C**H$_2$N$_3$), 70.3, 70.4 (**C**H$_2$OCH$_2$CH$_2$N$_3$), 126.0, 127.9, 137.1, 138.1 (**C$_6$**H$_4$), 169.9, 170.6 (CH$_3$**C**(O), CH**C**(O)NH), at 75 MHz, we were not able to detect individual peaks for the two diastereomers; M_r (+ESI) 370.1491 [M+Na]$^+$ (calcd for C$_{16}$H$_{21}$N$_5$O$_4$Na$^+$ 370.1491). Anal. Calcd for C$_{16}$H$_{21}$N$_5$O$_4$: 55.32; H, 6.09; N, 20.16. Found: C, 55.36; H, 6.16; N, 20.00.

(2*S*)-*N*-(4-(Oxiran-2-yl)benzyl) 2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-175) (mixture of diastereomers). Using Method F, solution A (DMF, 7.5 mL, [C]= 0.2 M, 1.5 mmol) and (*S*)-**173** (334 mg, 1.00 mmol) gave (*S*)-**175** (71 mg, 20%) as a white solid after purification by flash chromatography (15/85 acetone/EtOAc): mp 92–98℃; [α]^{25}$_D$ +25.5° (*c* 1.0; CHCl$_3$); R_f = 0.38 (1/9 acetone/EtOAc); IR (CHCl$_3$ film) 3311, 3061, 2928, 2874, 2105, 1652, 1530, 1445, 1375, 1121 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.99 (s, **C**H$_3$C(O)), 2.76 (dd, *J* = 2.4, 5.6 Hz, CH(O)C**H**H'), 3.12 (dd, *J* = 4.0, 5.6 Hz, CH(O)CH**H'**), 3.22–3.44 (m, OCH$_2$**C**H$_2$N$_3$), 3.52–3.64 (m, C**H**H'OC**H$_2$**CH$_2$N$_3$), 3.82 (dd, *J* = 2.4, 4.0 Hz, **C**H(O)CHH'), 3.84 (dd, *J* = 4.5, 9.2 Hz, CH**H'**OCH$_2$CH$_2$N$_3$), 4.42 (d, *J* = 7.2 Hz, NHC**H$_2$**C$_6$H$_4$), 4.58–4.64 (m, C**H**CH$_2$O), 6.71 (d, *J* = 7.2 Hz, N**H**CHCH$_2$O), 7.10–7.18 (br t, N**H**CH$_2$C$_6$H$_4$), 7.23 (s, C$_6$**H$_4$**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**175** gave only one signal

for the acetyl protons (δ 2.030 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**175** and (*R*)-**175** in a ~1:5 ratio gave two signals with a relative ~1:5 intensity for the acetyl protons (δ 2.030 ppm (*S*)-**175**, δ 2.020 ppm (*R*)-**175**, (Δppm = 0.010)), at 300 MHz, we were not able to detect individual peaks for the two diastereomers; ^{13}C NMR (CDCl$_3$) δ 23.2 (**C**H$_3$C(O)), 43.4 (NH**C**H$_2$C$_6$H$_4$), 50.7, 51.3, 52.2, 52.6 (**C**HC(O)NH, **C**H(O)CH$_2$ **C**H$_2$N$_3$), 70.3, 70.4 (**C**H$_2$O**C**H$_2$CH$_2$N$_3$), 125.9, 127.8, 137.0, 138.1 (**C**$_6$H$_4$), 169.9, 170.6 (CH$_3$**C**(O), CH**C**(O)NH), at 75 MHz, we were not able to detect individual peaks for the two diastereomers; M_r (+ESI) 370.1491 [M+Na]$^+$ (calcd for C$_{16}$H$_{21}$N$_5$O$_4$Na$^+$ 370.1491). Anal. Calcd for C$_{16}$H$_{21}$N$_5$O$_4$: 55.32; H, 6.09; N, 20.16. Found: C, 55.63; H, 6.23; N, 19.98.

(*R*)-*N*-(4-Formylbenzyl) 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-174). (*initially prepared by Dr. Ki-Duk Park*) Using Method D, acid (*R*)-**163** (480 mg, 2.6 mmol), amine **137** (550 mg, 3.12 mmol) and DMTMM (862 mg, 3.12 mmol) in THF (30 mL) gave a crude yellow residue upon work-up. Using Method I, direct hydrolysis of the residue with HCl in a THF:H$_2$O solution (2:1, 20 mL) gave (*R*)-**174** as a white solid (382 mg, 49%, two steps) upon work-up and purification by flash chromatography: mp 142–144 ℃; [α]$^{25}_D$ -9.3° (*c* 0.3, CHCl$_3$); R_f = 0.51 (1/9 MeOH/CH$_2$Cl$_2$); ^{1}H NMR (CDCl$_3$) δ 2.04 (s, **CH$_3$**C(O)), 2.48 (t, *J* = 2.4 Hz, CH$_2$C≡C**H**), 3.67 (dd, *J* = 7.2, 9.0 Hz, C**H**H'OCH$_2$), 3.95 (dd *J* = 4.2, 9.0 Hz, CH**H'**OCH$_2$), 4.12–4.28 (m, OC**H$_2$**C≡CH), 4.47–4.68 (m, NHC**H$_2$**C$_6$H$_4$, C**H**CH$_2$O), 6.42 (d, *J* = 6.4 Hz, CH$_3$C(O)N**H**), 6.90–7.00 (m, N**H**CH$_2$C$_6$H$_4$), 7.43 (d, *J* = 8.3 Hz, 2 Ar**H**), 7.84 (d, *J* = 8.3 Hz, 2 Ar**H**), 10.00 (s, C(O)**H**), addition of excess (*R*)-(-)-

mandelic acid to a CDCl$_3$ solution of (*R*)-**174** gave only one signal for the acetyl protons (δ 2.029) and the propargyl proton (δ 2.610), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**174** and (*R*)-**174** in a ~2:1 ratio gave two signals with a relative ~2:1 intensity for the acetyl protons (δ 2.040 (*S*)-**174**, δ 2.030 (*R*)-**174**) and the propargyl protons (δ 2.469 (*S*), δ 2.434 (*R*)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.5 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.8 (**C**HCH$_2$OCH$_3$), 58.9 (CH$_2$O**C**H$_2$C≡CH), 69.2 (**C**H$_2$OCH$_2$C≡CH), 75.7 (CH$_2$C≡**C**H), 79.0 (CH$_2$**C**≡CH), 128.0, 130.3, 135.8, 145.1 (**C**$_6$H$_4$), 170.2, 170.7 (CH**C**(O)NH, CH$_3$**C**(O)), 192.0 (**C**(O)H).

(*S*)-*N*-(4-Formylbenzyl) 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*S*)-174).
Using Method D, acid (*S*)-**163** (480 mg, 2.6 mmol), amine **137** (550 mg, 3.12 mmol) and DMTMM (862 mg, 3.12 mmol) in THF (30 mL) gave a crude yellow residue upon work-up. Using Method I, the residue was reacted with aqueous HCl in THF:H$_2$O (2:1, 20 mL) to give (*S*)-**174** as a white solid (382 mg, 49%, two steps) upon work-up and silica gel flash chromatography (1/2 CH$_2$Cl$_2$/EtOAc to 1/9 MeOH/CH$_2$Cl$_2$): mp 142–144 °C; [α]$^{25}_D$ +9.1° (*c* 0.3, CHCl$_3$); R_f = 0.51 (1/9 MeOH/CH$_2$Cl$_2$); IR (nujol) 3442, 3372, 3288, 2856, 2111, 1687, 1635, 1554, 1457, 1375, 1108 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.05 (s, **CH$_3$**C(O)), 2.48 (t, *J* = 2.4 Hz, CH$_2$C≡**CH**), 3.67 (dd, *J* = 7.2 Hz, 9.0 Hz, C**H**H'OCH$_2$), 3.96 (dd, *J* = 4.2, 9.0 Hz, CH**H'**OCH$_2$), 4.14–4.30 (m, OC**H$_2$**C≡CH), 4.48-4.66 (m, NHC**H$_2$**C$_6$H$_4$, C**H**CH$_2$O), 6.42 (d, *J* = 6.4 Hz, CH$_3$C(O)N**H**), 6.82–6.92 (m, N**H**CH$_2$C$_6$H$_4$), 7.44 (d, *J* = 8.3 Hz, 2 Ar**H**), 7.85 (d, *J* = 8.3 Hz, 2 Ar**H**), 10.00 (s, C(O)**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**174** gave only one signal for the acetyl protons (δ 2.040) and the

propargyl proton (δ 2.469), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**174** and (*R*)-**174** in a ~2:1 ratio gave two signals with a relative ~2:1 intensity for the acetyl protons (δ 2.040 (*S*), δ 2.030 (*R*)) and the propargyl protons (δ 2.469 (*S*), δ 2.434 (*R*)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 43.4 (NH$_2$**C**H$_2$C$_6$H$_4$), 52.8 (**C**HCH$_2$OCH$_3$), 58.9 (CH$_2$O**C**H$_2$C≡CH), 69.2 (**C**H$_2$OCH$_2$C≡CH), 75.7 (CH$_2$C≡**C**H), 79.0 (CH$_2$**C**≡CH), 128.0, 130.3, 135.8, 145.1 (**C**$_6$H$_4$), 170.2, 170.7 (CH**C**(O)NH, CH$_3$**C**(O)), 192.0 (**C**(O)H); M_r (+ESI) 325.1 [M+Na]$^+$ (calcd for C$_{16}$H$_{18}$N$_2$O$_4$Na$^+$ 325.1). Anal. Calcd for C$_{16}$H$_{18}$N$_2$O$_4$: C, 63.56; H, 6.00; N, 9.27. Found: C, 63.34; H, 5.91; N, 9.20.

(2*R*)-*N*-(4-(Oxiran-2-yl)benzyl) 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-176) (mixture of diastereomers). Using Method F, solution A (DMSO, [C]= 0.1 M, 11.7 mL, 1.2 mmol) and (*R*)-**174** (410 mg, 1.23 mmol) gave (*R*)-**176** (80 mg, 19%) as a white solid after purification by flash chromatography (15/85 acetone/EtOAc): mp 131–136 °C; $[\alpha]^{25}_D$ +3.2° (*c* 0.5; MeOH); R_f = 0.30 (8/92 acetone/EtOAc); IR (nujol) 3283, 3061, 2109, 1634, 1544, 1458, 1375, 1306, 1096 cm^{-1}; ^{1}H NMR (CD$_3$CN) δ 1.94 (s, **CH$_3$**C(O)), 2.72–2.80 (m, CH$_2$C≡**CH**, CH(O)C**H**H'), 3.08 (dd, *J* = 4.0, 5.8 Hz, CH(O)CH**H'**), 3.63 (dd, *J* = 4.8, 9.0 Hz, C**H**H'OCH$_2$C≡CH), 3.80–3.88 (m, C**H**(O)CHH', CH**H'**OCH$_2$C≡CH), 4.09–4.22 (m, CH$_2$OC**H$_2$**C≡CH), 4.34 (d, *J* = 6.0 Hz, NHC**H$_2$**C$_6$H$_4$), 4.38–4.44 (m, C**H**CH$_2$O), 6.76–6.86 (br d, N**H**CHCH$_2$O), 7.18–7.28 (m, NHCH$_2$C$_6$**H$_4$**), at 300 MHz, we were not able to detect individual peaks for the two diastereomers; ^{13}C NMR (CD$_3$CN) δ 23.1 (**C**H$_3$C(O)), 43.2 (NH**C**H$_2$C$_6$H$_4$), 51.6, 52.6, 54.4, 59.1 (**C**HC(O)NH, **C**H(O)CH$_2$, O**C**H$_2$C≡CH),

70.5 (**C**H$_2$OCH$_2$C≡CH), 76.2 (CH$_2$OCH$_2$C≡**C**H), 80.5 (CH$_2$OCH$_2$**C**≡CH), 126.7, 128.3, 137.8, 140.3 (**C$_6$**H$_4$), 170.7, 171.2 (CH$_3$**C**(O), CH**C**(O)NH), at 75 MHz, we were not able to detect individual peaks for the two diastereomers; M_r (+ESI) 339.1 [M+Na]$^+$ (calcd for C$_{17}$H$_{20}$N$_2$O$_4$Na$^+$ 339.1). Anal. Calcd for C$_{17}$H$_{20}$N$_2$O$_4$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.38; H, 6.35; N, 8.65.

(2S)-N-(4-Oxiran-2-yl)benzyl)   2-Acetamido-3-(prop-2-ynyloxy)propionamide ((S)-176) (mixture of diastereomers). Using Method F, (S)-**174** and solution A (DMSO, 17.8 mL, [C] = 0.1 M, 1.78 mmol) gave (S)-**176** as a white solid (90 mg, 26%) after purification by flash chromatography (15/85 acetone/EtOAc): mp 132–138 ℃; [α]$^{25}_D$ -3.1° (c 0.3; MeOH); R_f = 0.30 (8/92 acetone/EtOAc); IR (nujol) 3412, 3277, 3138, 2874, 2109, 1633, 1544, 1458, 1375, 1306, 1096 cm^{-1}; ^{1}H NMR (CD$_3$CN) δ 1.94 (s, **CH$_3$**C(O)), 2.73–2.80 (m, CH$_2$C≡C**H**, CH(O)C**H**H'), 3.08 (dd, J = 4.0, 5.8 Hz, CH(O)CH**H'**), 3.63 (dd, J = 4.8, 9.0 Hz, C**H**H'OC**H$_2$**C≡CH), 3.80–3.88 (m, C**H**(O)CHH', CH**H'**OCH$_2$C≡CH), 4.08–4.21 (m, CH$_2$OC**H$_2$**C≡CH), 4.34 (d, J = 6.0 Hz, NHC**H$_2$**C$_6$H$_4$), 4.39–4.47 (m, C**H**CH$_2$O), 6.76–6.88 (br d, N**H**CHCH$_2$O), 7.17–7.28 (m, NHCH$_2$C$_6$**H$_4$**), at 300 MHz, we were not able to detect individual peaks for the two diastereomers; ^{13}C NMR (CD$_3$CN) δ 23.1 (**C**H$_3$C(O)), 43.2 (NH**C**H$_2$C$_6$H$_4$), 51.6, 52.7, 54.4, 59.1 (**C**HC(O)NH, **C**H(O)**C**H$_2$, O**C**H$_2$C≡CH), 70.5 (**C**H$_2$OCH$_2$C≡CH), 76.2 (CH$_2$OCH$_2$C≡**C**H), 80.5 (CH$_2$OCH$_2$**C**≡CH), 126.7, 128.3, 137.8, 140.3 (**C$_6$**H$_4$), 170.7, 171.2 (CH$_3$**C**(O), CH**C**(O)NH), at 75 MHz, we were not able to detect individual peaks for the two diastereomers; M_r (+ESI) 339.1 [M+Na]$^+$ (calcd for

$C_{17}H_{20}N_2O_4Na^+$ 339.1). Anal. Calcd for $C_{17}H_{20}N_2O_4$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.63; H, 6.40; N, 8.76.

2.3.3.3.2. Derivatives bearing the CR group on the aromatic ring

Scheme 39. Structure of the different intermediates and chemical reactions used in the synthesis of AB&CR agents **178**, **179**, and **180**.
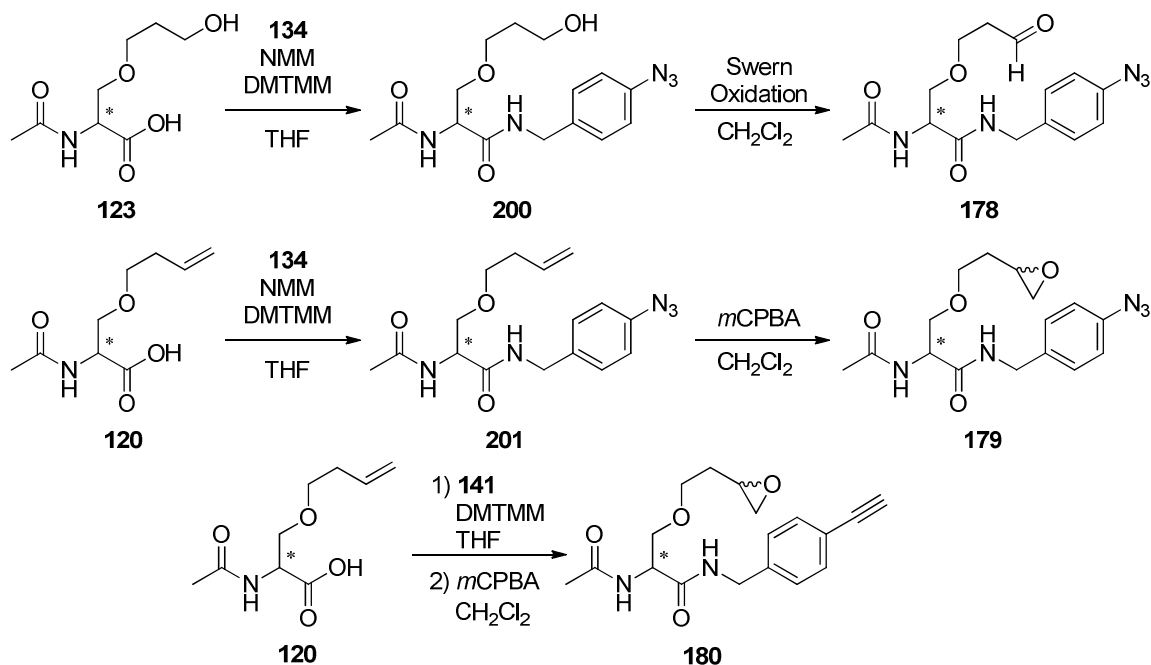
(*R*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(3-hydroxypropoxy)propionamide ((*R*)-200). Using Method D, acid (*R*)-**123** (1.00 g, 4.90 mmol), benzylamine hydrochloride **134** (1.07 g, 5.80 mmol), NMM (640 μL, 5.80 mmol), and DMTMM (1.60 g, 5.80 mmol) in anhydrous THF (100 mL) gave 1.20 g (73%) of (*R*)-**200** as a white solid after flash column chromatography (1/9 MeOH/CHCl$_3$). The solid slowly turned light yellow upon exposure to light: mp 127–130 ℃; $[\alpha]^{25}_D$ +46.3° (*c* 0.9, MeOH); R_f = 0.57 (1/9 MeOH/CHCl$_3$); IR (nujol) 3400–2700 (br), 3282, 3085, 2119, 1638, 1552,

1459, 1378, 1296, 1127 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.63 (quint, J = 6.3 Hz, OCH$_2$**CH$_2$**CH$_2$O), 1.87 (s, **CH$_3$**C(O)), 3.38–3.48 (m, OC**H$_2$**CH$_2$CH$_2$OH), 3.48–3.56 (m, **CH$_2$**OCH$_2$CH$_2$CH$_2$OH), 4.19–4.34 (m, NHC**H$_2$**Ph), 4.40 (t, J = 5.1 Hz, CH$_2$O**H**), 4.40–4.48 (m, C**H**CH$_2$O), 7.06 (d, J = 8.4 Hz, 2 Ar**H**), 7.28 (d, J = 8.4 Hz, 2 Ar**H**), 8.05 (d, J = 7.8 Hz, CH$_3$C(O)N**H**), 8.51 (t, J = 5.7 Hz, C(O)N**H**CH$_2$); ^{13}C NMR (DMSO-d_6) δ 22.5 (**C**H$_3$C(O)), 32.5 (OCH$_2$**C**H$_2$CH$_2$O), 41.4 (NH**C**H$_2$Ph), 52.8 (**C**HCH$_2$OCH$_2$), 57.7 (OCH$_2$CH$_2$**C**H$_2$OH), 67.6 (CHCH$_2$O**C**H$_2$CH$_2$CH$_2$), 70.3 (CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 118.8, 128.6, 136.4, 137.7 (**C$_6$**H$_4$), 169.4, 169.8 (CH**C**(O)NH, CH$_3$**C**(O)NH); M_r (+ESI) 358.1494 [M+Na]$^+$ (calcd for C$_{15}$H$_{21}$N$_5$O$_4$Na$^+$ 358.1491). Anal. Calcd for C$_{15}$H$_{21}$N$_5$O$_4$•0.43H$_2$O: C, 52.51; H, 6.42; N, 20.37. Found: C, 52.90; H, 6.30; N, 19.99.

(*S*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(3-hydroxypropoxy)propionamide ((*S*)-200). Using Method D, acid (*S*)-**123** (260 mg, 1.27 mmol), benzylamine hydrochloride **134** (281 mg, 1.52 mmol), NMM (167 μL, 1.52 mmol), and DMTMM (420 mg, 1.52 mmol) in anhydrous THF (13 mL) gave 327 mg (77%) of (*S*)-**200** as a white solid after flash column chromatography (1/9 MeOH/CHCl$_3$). The solid slowly turned light yellow upon exposure to light: mp 127–130 °C; [α]^{25}$_D$ -46.1° (*c* 1.1, MeOH); R_f = 0.57 (1/9 MeOH/CHCl$_3$); IR (nujol) 3400–2700 (br), 3282, 3084, 2119, 1638, 1552, 1459, 1377, 1296, 1127 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.63 (quint, J = 6.3 Hz, OCH$_2$**CH$_2$**CH$_2$O), 1.87 (s, **CH$_3$**C(O)), 3.38–3.48 (m, OC**H$_2$**CH$_2$CH$_2$OH), 3.48–3.56 (m, **CH$_2$**OCH$_2$CH$_2$CH$_2$OH), 4.19–4.34 (m, NHC**H$_2$**Ph), 4.40 (t, J = 5.1 Hz, CH$_2$O**H**), 4.40–4.48 (m, C**H**CH$_2$O), 7.06 (d, J = 8.7 Hz, 2 Ar**H**), 7.28 (d, J = 8.7 Hz, 2

ArH), 8.05 (d, J = 7.8 Hz, CH$_3$C(O)N**H**), 8.51 (t, J = 5.7 Hz, C(O)N**H**CH$_2$); ^{13}C NMR

(DMSO-d_6) δ 22.5 (**C**H$_3$C(O)), 32.5 (OCH$_2$**C**H$_2$CH$_2$O), 41.4 (NH**C**H$_2$Ph), 52.8

(**C**HCH$_2$OCH$_2$), 57.7 (OCH$_2$CH$_2$**C**H$_2$OH), 67.6 (CHCH$_2$O**C**H$_2$CH$_2$CH$_2$), 70.3

(CH**C**H$_2$OCH$_2$CH$_2$CH$_2$), 118.8, 128.6, 136.4, 137.7 (**C$_6$**H$_4$), 169.4, 169.8

(CH**C**(O)NH, CH$_3$**C**(O)NH); M_r (+ESI) 358.1494 [M+Na]$^+$ (calcd for C$_{15}$H$_{21}$N$_5$O$_4$Na$^+$

358.1491). Anal. Calcd for C$_{15}$H$_{21}$N$_5$O$_4$: C, 53.72; H, 6.31; N, 20.88. Found: C,

53.73; H, 6.32; N, 20.76.

(*R*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(3-oxopropoxy)propionamide ((*R*)-178).

Using Method E, alcohol (*R*)-**200** (1.20 g, 3.56 mmol) in a 2:1 CH$_2$Cl$_2$:DMSO mixture

(12 mL), (COCl)$_2$ (406 μL, 4.65 mmol) in CH$_2$Cl$_2$ (12 mL), DMSO (660 μL, 9.30

mmol) in CH$_2$Cl$_2$ (24 mL), and DIEA (3.1 mL, 17.8 mmol) gave 663 mg (56%) of (*R*)-

178 as a white solid after recrystallization from EtOAc: mp 120–122 °C; [α]^{25}$_D$ -18.3°

(*c* 2.4, CHCl$_3$); R_f = 0.68 (1/9 MeOH/CHCl$_3$); IR (CHCl$_3$ film) 3293, 3109, 2859, 2108,

1712, 1635, 1563, 1457, 1384, 1293 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.02 (s, **CH$_3$**C(O)),

2.60–2.80 (m, **CH$_2$**C(O)H), 3.48 (dd, J = 7.5, 9.6 Hz, CHC**H**H'OCH$_2$CH$_2$), 3.70–3.78

(m, CH$_2$OC**H**H'CH$_2$C(O)H), 3.78–3.90 (m, CH$_2$OCH**H'**CH$_2$C(O)H), 3.86 (dd, J = 3.9,

9.6 Hz, CHCH**H'**OCH$_2$CH$_2$), 4.40 (d, J = 6.0 Hz, NH**CH$_2$**Ar), 4.52–4.62 (m,

C**H**CH$_2$O), 6.73 (d, J = 6.6 Hz, CH$_3$C(O)N**H**), 6.84–7.02 (m, 2 Ar**H**), 7.15–7.20 (m,

C(O)N**H**CH$_2$, 2 Ar**H**), 9.72 (t, J = 1.4 Hz, C(O)**H**), addition of excess (*R*)-(-)-mandelic

acid to a CDCl$_3$ solution of (*R*)-**178** gave only one signal for the acetyl methyl

protons and the aldehyde proton, addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$

solution of (*S*)-**178** and (*R*)-**178** (~1:3 ratio) gave two signals for the acetyl methyl

protons (δ 2.027 (*S*) and 2.010 (*R*) (Δppm = 0.017)) in a ~1:3 ratio, and two signals

for the aldehyde protons (δ 9.671 (*S*) and 9.698 (*R*) (Δppm = 0.027)) in a ~1:3 ratio;

^{13}C NMR (CDCl$_3$) δ 23.3 (**C**H$_3$C(O)), 43.0, 43.7 (OCH$_2$**C**H$_2$C(O)H, NH**C**H$_2$Ar), 52.4

(**C**HCH$_2$OCH$_2$), 64.5 (O**C**H$_2$CH$_2$C(O)H), 70.4 (CH**C**H$_2$OCH$_2$CH$_2$C(O)H), 119.3,

129.2, 135.1, 139.3 (**C$_6$**H$_4$), 170.1, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)NH), 201.0 (**C**(O)H);

M_r (+ESI) 334.1518 [M+H]$^+$ (calcd for C$_{15}$H$_{19}$N$_5$O$_4$H$^+$ 334.1515). Anal. Calcd for

C$_{15}$H$_{19}$N$_5$O$_4$: C, 54.05; H, 5.75; N, 21.01. Found: C, 53.90; H, 5.77; N, 20.86.

(*S*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(3-oxopropoxy)propionamide ((*S*)-178).

Using Method E, alcohol (*S*)-**200** (327 mg, 0.98 mmol) in a 2:1 CH$_2$Cl$_2$:DMSO

mixture (5 mL), (COCl)$_2$ (127 μL, 1.46 mmol) in CH$_2$Cl$_2$ (5 mL), DMSO (207 μL, 2.92

mmol) in CH$_2$Cl$_2$ (10 mL), and DIEA (848 μL, 4.87 mmol) gave 188 mg (58%) of (*S*)-

178 as an off-white solid after work-up and recrystallization from EtOAc: mp 120–

122 °C; [α]$^{25}_D$ +18.1° (*c* 1.1, CHCl$_3$); R_f = 0.68 (1/9 MeOH/CHCl$_3$); IR (CHCl$_3$ film)

3293, 3108, 2859, 2108, 1712, 1635, 1552, 1457, 1384, 1293 cm^{-1}; ^{1}H NMR (CDCl$_3$)

δ 2.04 (s, **CH$_3$**C(O)), 2.62–2.82 (m, **CH$_2$**C(O)H), 3.48 (dd, *J* = 7.5, 9.3 Hz,

CHC**H**H'OCH$_2$CH$_2$), 3.70–3.78 (m, CH$_2$OC**H**H'CH$_2$C(O)H), 3.80–3.88 (m,

CH$_2$OCH**H'**CH$_2$C(O)H), 3.89 (dd, *J* = 3.9, 9.3 Hz, CHCH**H'**OCH$_2$CH$_2$), 4.42 (d, *J* =

6.0 Hz, NHC**H$_2$**Ar), 4.50–4.58 (m, C**H**CH$_2$O), 6.64 (d, *J* = 6.3 Hz, CH$_3$C(O)N**H**),

6.94–7.20 (m, 2 Ar**H**), 7.04–7.14 (m, C(O)N**H**CH$_2$), 7.22–7.30 (m, 2 Ar**H**), 9.73 (t, *J*

= 1.4 Hz, C(O)**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-

178 gave only one signal for the acetyl methyl protons and the aldehyde proton,

addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**178** and (*R*)-**178**

(~1:3 ratio) gave two signals for the acetyl methyl protons (δ 2.027 (*S*) and 2.010 (*R*)

(Δppm = 0.017)) in a ~1:3 ratio, and two signals for the aldehyde protons (δ 9.671

(*S*) and 9.698 (*R*) (Δppm = 0.027)) in a ~1:3 ratio; ^{13}C NMR (CDCl$_3$) δ 23.4

(**C**H$_3$C(O)), 43.1, 43.8 (OCH$_2$**C**H$_2$C(O)H, NH**C**H$_2$Ar), 52.3 (**C**HCH$_2$OCH$_2$), 64.5

(O**C**H$_2$CH$_2$C(O)H), 70.3 (CH**C**H$_2$OCH$_2$CH$_2$C(O)H), 119.4, 129.3, 135.1, 139.4

(**C$_6$**H$_4$), 170.0, 170.6 (CH**C**(O)NH, CH$_3$**C**(O)NH), 200.9 (**C**(O)H); M_r (+ESI) 334.1520

[M+H]$^+$ (calcd for C$_{15}$H$_{19}$N$_5$O$_4$H$^+$ 334.1515). Anal. Calcd for C$_{15}$H$_{19}$N$_5$O$_4$: C, 54.05; H,

5.75; N, 21.01. Found: C, 54.15; H, 5.80; N, 20.75.

(*R*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(but-3-enyloxy)propionamide ((*R*)-201).

Using Method D, (*R*)-**120** (300 mg, 1.50 mmol), benzylamine hydrochloride **134** (330

mg, 1.79 mmol), NMM (200 μL, 1.79 mmol), and DMTMM (495 mg, 1.79 mmol) in

THF (15 mL) gave (*R*)-**201** (280 mg, 56%) as a pale yellow solid after purification by

flash chromatography (EtOAc) and further recrystallization from EtOAc and hexanes:

mp 116–118 ℃; [α]$^{25}_D$ +42.4° (*c* 0.6; MeOH); R_f = 0.38 (5/95 MeOH/CH$_2$Cl$_2$); IR

(nujol) 3285, 3093, 2124, 1640, 1551, 1458, 1378, 1127 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ

2.03 (s, C**H$_3$**C(O)), 2.22–2.36 (m, CH$_2$C**H$_2$**CH=CH$_2$), 3.40–3.62 (m, CH$_2$OC**H$_2$**CH$_2$,

C**H**H'OCH$_2$CH$_2$), 3.85 (dd, *J* = 4.2, 9.0 Hz, CH**H'**OCH$_2$CH$_2$), 4.42 (d, *J* = 7.2 Hz,

NHC**H$_2$**Ph), 4.44–4.54 (m, C**H**CH$_2$O), 4.94–5.08 (m, CH$_2$CH=C**H$_2$**), 5.62–5.78 (m,

CH$_2$C**H**=CH$_2$), 6.45 (d, *J* = 7.2 Hz, N**H**CHCH$_2$O), 6.84–6.96 (m, N**H**CH$_2$Ph), 6.99 (d,

J = 8.7 Hz, 2 Ar**H**), 7.25 (d, *J* = 8.7 Hz, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic

acid to a CDCl$_3$ solution of (*R*)-**201** gave only one signal for the acetyl protons (δ

2.011 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**201**

and (*R*)-**201** in a ~3:1 ratio gave two signals with a relative ~3:1 intensity for the acetyl protons (δ 2.019 ppm (*S*), δ 2.011 ppm (*R*), (Δppm = 0.008)); ^{13}C NMR (CDCl$_3$) δ 23.5 (**C**H$_3$C(O)), 34.2 (CH$_2$**C**H$_2$CH=CH$_2$), 43.2 (NH**C**H$_2$Ph), 52.5 (**C**HC(O)NH), 69.8, 70.6 (CH**C**H$_2$O**C**H$_2$), 117.0 (CH$_2$CH$_2$CH=**C**H$_2$), 119.5, 129.2, 134.9, 135.3, 139.6 (**C$_6$**H$_4$, CH$_2$CH$_2$**C**H=CH$_2$), 170.3, 170.5 (CH$_3$**C**(O), CH**C**(O)NH); M_r (+ESI) 354.1542 [M+Na]$^+$ (calcd for C$_{16}$H$_{21}$N$_5$O$_3$Na$^+$ 354.1542). Anal. Calcd for C$_{16}$H$_{21}$N$_5$O$_3$: C, 57.99; H, 6.39; N, 21.13. Found: C, 58.08; H, 6.30; N, 21.00.

(*S*)-*N*-(4-Azidobenzyl) 2-Acetamido-3-(but-3-enyloxy)propionamide ((*S*)-201). Using Method D, (*S*)-**120** (164 mg, 0.87 μmol), benzylammonium hydrochloride **134** (192 mg, 1.04 mmol), NMM (114 μL, 1.04 mmol), and DMTMM (287 mg, 1.04 mmol) in THF (10 mL) gave (*S*)-**201** (150 mg, 60%) as an off-white solid after purification by flash chromatography (EtOAc) and further recrystallization from EtOAc and hexanes: mp 116–118 °C; $[\alpha]^{25}_D$ -42.1° (*c* 0.6; MeOH); R_f = 0.38 (5/95 MeOH/CH$_2$Cl$_2$); IR (nujol) 3278, 3091, 2123, 1640, 1551, 1458, 1378, 1127 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.03 (s, **CH$_3$**C(O)), 2.22–2.36 (m, CH$_2$**CH$_2$**CH=CH$_2$), 3.40–3.62 (m, CH$_2$OC**H$_2$**CH$_2$, C**H**H'OCH$_2$CH$_2$), 3.85 (dd, *J* = 4.2, 9.0 Hz, CH**H'**OCH$_2$CH$_2$), 4.42 (d, *J* = 7.2 Hz, NHC**H$_2$**Ph), 4.44–4.54 (m, C**H**CH$_2$O), 4.94–5.08 (m, CH$_2$CH=C**H$_2$**), 5.62–5.78 (m, CH$_2$C**H**=CH$_2$), 6.45 (d, *J* = 7.2 Hz, N**H**CHCH$_2$O), 6.84–6.96 (m, N**H**CH$_2$Ph), 6.99 (d, *J* = 8.7 Hz, 2 Ar**H**), 7.25 (d, *J* = 8.7 Hz, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**201** gave only one signal for the acetyl protons (δ 2.011 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**201** and (*R*)-**201** in a ~3:1 ratio gave two signals with a relative ~3:1 intensity for the

acetyl protons (δ 2.019 ppm (S), δ 2.011 ppm (R), (Δppm = 0.008)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 34.2 (CH$_2$**C**H$_2$CH=CH$_2$), 43.2 (NH**C**H$_2$Ph), 52.5 (**C**HC(O)NH), 69.8, 70.6 (CH**C**H$_2$O**C**H$_2$), 117.1 (CH$_2$CH$_2$CH=**C**H$_2$), 119.5, 129.2, 135.0, 135.3, 139.5 (**C$_6$**H$_4$, CH$_2$CH$_2$**C**H=CH$_2$), 170.3, 170.5 (CH$_3$**C**(O), CH**C**(O)NH); M_r (+ESI) 354.1536 [M+Na]$^+$ (calcd for C$_{16}$H$_{21}$N$_5$O$_3$Na$^+$ 354.1542). Anal. Calcd for C$_{16}$H$_{21}$N$_5$O$_3$: C, 57.99; H, 6.39; N, 21.13. Found: C, 58.09; H, 6.52; N, 20.85.

(2R)-N-(4-Azidobenzyl) 2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide ((R)-179) (mixture of diastereomers). Using Method J, (R)-**201** (80 mg, 0.24 mmol), Na$_2$SO$_4$ (15 mg) and mCPBA (77% wt, 176 mg, 0.41 mmol) in CH$_2$Cl$_2$ (3 mL) gave (R)-**179** (54 mg, 65%) as a mixture of diastereoisomers after purification by flash chromatography (15/85 acetone/EtOAc): mp 105–112 ℃; [α]$^{25}_D$ -15.3° (c 0.6; CHCl$_3$); R_f = 0.33 (15/85 acetone/EtOAc); IR (nujol) 3281, 2123, 1639, 1553, 1459, 1378, 1126 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.50–1.64 (m, CH$_2$C**H**H'CH(O)CH$_2$), 1.92–2.02 (m, CH$_2$CH**H'**CH(O)CH$_2$), 2.03, 2.04 (s, **CH$_3$**C(O)), 2.42–2.50 (m, CH$_2$CH$_2$CH(O)C**H**H'), 2.66–2.74 (m, CH$_2$CH$_2$CH(O)CH**H'**), 2.84–2.98 (m, CH$_2$CH$_2$C**H**(O)CHH'), 3.42–3.52 (m, C**H**H'OCH$_2$CH$_2$), 3.54–3.76 (m, CH$_2$OC**H$_2$**CH$_2$), 3.84–3.94 (m, CH**H'**OCH$_2$CH$_2$), 4.34–4.48 (m, NHC**H$_2$**C$_6$H$_4$), 4.48–4.58 (m, C**H**CH$_2$O), 6.58–6.70 (2 br d, N**H**CHCH$_2$O), 6.96 (m, 2 Ar**H**, ½ N**H**CH$_2$C$_6$H$_4$), 7.08–7.18 (m, ½ N**H**CH$_2$C$_6$H$_4$), 7.23 (d, J = 8.2 Hz, 2 Ar**H**), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (R)-**179** gave only one set of signals for the acetyl protons (δ 2.025 and 2.017 ppm), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$ solution of (S)-**179** and (R)-**179** in a ~2:3 ratio gave two sets of signals with a

relative ~2:3 intensity for the acetyl protons (δ 2.037 and 2.029 ppm (*S*), δ 2.030 and 2.019 ppm (*R*)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 32.4, 32.6 (CH$_2$**C**H$_2$CH(O)CH$_2$), 43.2 (NH**C**H$_2$C$_6$H$_4$), 46.7 (CH$_2$CH$_2$CH(O)**C**H$_2$), 50.5, 50.7, 52.6, 52.7 (**C**HC(O)NH, CH$_2$CH$_2$**C**H(O)CH$_2$), 68.6, 68.9, 69.8, 70.0 (CH**C**H$_2$O**C**H$_2$), 119.3, 119.4, 129.3, 135.1, 135.2 (**C$_6$**H$_4$), 170.3, 170.6 (CH$_3$**C**(O), CH**C**(O)NH), the remaining peaks were not detected and are believed to overlap with nearby signals; M_r (+ESI) 370.1491 [M+Na]$^+$ (calcd for C$_{16}$H$_{21}$N$_5$O$_4$Na$^+$ 370.1491). No satisfactory elemental analysis was obtained for (*R*)-**179**. The ^{1}H NMR indicated low levels (<10%) of an additional unidentified compound.

(2*S*)-*N*-(4-Azidobenzyl)   2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide ((*S*)-179) (mixture of diastereomers). Using Method J, (*S*)-**201** (100 mg, 0.30 mmol), Na$_2$SO$_4$ (25 mg) and *m*-CPBA (77% wt, 131 mg, 0.59 mmol) in CH$_2$Cl$_2$ (3 mL) gave (*S*)-**179** (85 mg, 81%) as a mixture of diastereoisomers after purification by flash chromatography (15/85 acetone/EtOAc): mp 106–112 ℃; [α]$^{25}_D$ +18.8° (*c* 0.6; CHCl$_3$); R_f = 0.33 (15/85 acetone/EtOAc); IR (nujol) 3279, 2122, 1640, 1550, 1459, 1378, 1127 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.50–1.64 (m, CH$_2$C**H**H'CH(O)CH$_2$), 1.84–1.96 (m, CH$_2$CH**H'**CH(O)CH$_2$), 2.02, 2.03 (s, **CH$_3$**C(O)), 2.42–2.48 (m, CH$_2$CH$_2$CH(O)C**H**H'), 2.66–2.74 (m, CH$_2$CH$_2$CH(O)CH**H'**), 2.82–2.98 (m, CH$_2$CH$_2$C**H**(O)CHH'), 3.44–3.52 (m, C**H**H'OCH$_2$CH$_2$), 3.54–3.72 (m, CH$_2$OC**H$_2$**CH$_2$), 3.82–3.90 (m, CH**H'**OCH$_2$CH$_2$), 4.34–4.50 (m, NHC**H$_2$**C$_6$H$_4$), 4.50–4.60 (m, C**H**CH$_2$O), 6.66–6.76 (m, N**H**CHCH$_2$O), 6.96 (d, *J* = 8.2 Hz, 2 Ar**H**), 7.06–7.14 (m, 0.5 N**H**CH$_2$C$_6$H$_4$), 7.18–7.28 (m, 2 Ar**H**, 0.5 N**H**CH$_2$C$_6$H$_4$), addition of excess (*R*)-(-)-

mandelic acid to a $CDCl_3$ solution of (*S*)-**179** gave only one set of signals for the acetyl protons (δ 2.040 and 2.032 ppm), addition of excess (*R*)-(-)-mandelic acid to a $CDCl_3$ solution of (*S*)-**179** and (*R*)-**179** in a ~2:3 ratio gave two sets of signals with a relative ~2:3 intensity for the acetyl protons (δ 2.037 and 2.029 ppm (*S*), δ 2.030 and 2.019 ppm (*R*)); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H_3C(O)), 32.4, 32.6 (CH_2**C**H_2CH(O)CH_2), 43.1 (NH**C**$H_2C_6H_4$), 46.6, 46.7 ($CH_2CH_2CH(O)$**C**H_2), 50.5, 50.6, 52.6, 52.7 (**C**HC(O)NH, CH_2CH_2**C**$H(O)CH_2$), 68.5, 68.8, 69.8, 70.1 (CH**C**H_2O**C**H_2), 119.3, 119.4, 129.2, 135.1, 135.2, 139.3 (**C$_6$**H_4), 170.2, 170.5, 170.6 (CH_3**C**(O), CH**C**(O)NH) the remaining peaks were not detected and are believed to overlap with nearby signals; M_r (+ESI) 370.1494 $[M+Na]^+$ (calcd for $C_{16}H_{21}N_5O_4Na^+$ 370.1491). No satisfactory elemental analysis was obtained for (*S*)-**179**. The ^1H NMR indicated low levels (<10%) of an additional unidentified compound.

(2*R*)-*N*-(4-Ethynylbenzyl) 2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide ((*R*)-180) (~1:1 mixture of diastereomers). Using Method D, (*R*)-**120** (350 mg, 1.74 mmol), 4-ethynylbenzylamine (272 mg, 2.08 mmol), DMTMM (575 mg, 2.08 mmol) in THF (40 mL) gave a residue that was directly used for the next step. Using Method J, Na_2SO_4 (500 mg) and *m*CPBA (70% by wt., 447 mg, 1.88 mmol) in CH_2Cl_2 (25 mL) were added to give (*R*)-**180** as a white solid (252 mg, 43% for two steps) after flash chromatography (1/9 to 3/7 acetone/EtOAc) and recrystallization from EtOAc and hexanes: mp 130–136 °C; $[\alpha]^{25}_D$ -19.2° (*c* 0.8; $CHCl_3$); R_f = 0.36 (15/85 acetone/EtOAc); IR (nujol) 3275, 3069, 1638, 1550, 1458, 1375, 1298, 1256, 1130 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.50–1.64 (m, CH_2**C**H**H**'CH(O)CH_2), 1.90–2.04 (m,

CH$_2$CHH'CH(O)CH$_2$), 2.03, 2.04 (s, **CH$_3$**C(O)), 2.42–2.48 (m, CH$_2$CH$_2$CH(O)C**H**H'),

2.66–2.74 (m, CH$_2$CH$_2$CH(O)CH**H'**), 2.82–2.98 (m, CH$_2$CH$_2$**C**H(O)CHH'), 3.07 (s,

ArC≡C**H**), 3.42–3.52 (m, C**H**H'OCH$_2$CH$_2$), 3.54–3.76 (m, CH$_2$OC**H$_2$**CH$_2$), 3.84–3.92

(m, CH**H'**OCH$_2$CH$_2$), 4.38–4.50 (m, NHC**H$_2$**C$_6$H$_4$), 4.51–4.58 (m, C**H**CH$_2$O), 6.60–

6.72 (m, N**H**CHCH$_2$O), 7.02–7.12 (m, 0.5 N**H**CH$_2$C$_6$H$_4$), 7.14–7.25 (m, 2 Ar**H**, 0.5

N**H**CH$_2$C$_6$H$_4$), 7.40–7.48 (d, J = 8.0 Hz, 2 Ar**H**), addition of excess (R)-(-)-mandelic

acid to a CDCl$_3$ solution of (R)-**180** gave only one set of signals for the acetyl

protons (δ 2.019 and 2.010 ppm), addition of excess (R)-(-)-mandelic acid to a CDCl$_3$

solution of (S)-**180** and (R)-**180** in a ~1:2 ratio gave two sets of two signals with a

relative ~1:2 intensity for the acetyl protons (δ 2.030 and 2.023 ppm (S); δ 2.017 and

2.009 ppm (R)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**CH$_3$**C(O)), 32.4, 32.6

(CH$_2$**CH$_2$**CH(O)CH$_2$), 43.4 (NH**CH$_2$**C$_6$H$_4$), 46.7 (CH$_2$CH$_2$CH(O)**CH$_2$**), 50.5, 50.6, 52.6,

52.7 (**C**HC(O)NH, CH$_2$CH$_2$**CH**(O)CH$_2$), 68.6, 68.9, 69.8, 70.1 (CH**CH$_2$**O**CH$_2$**), 77.5

(ArC≡**CH**), 83.5 (Ar**C**≡CH), 121.3, 121.4, 127.6, 132.5, 132.6, 139.1, 139.3 (**C$_6$**H$_4$),

170.3, 170.5, 170.6 (CH$_3$**C**(O), CH**C**(O)NH), the remaining signals were not detected

and are believed to overlap with nearby peaks; M_r (+ESI) 353.1 [M+Na]$^+$ (calcd for

C$_{18}$H$_{22}$N$_2$O$_4$Na$^+$ 353.1). Anal. Calcd for C$_{18}$H$_{22}$N$_2$O$_4$: C, 65.44; H, 6.71; N, 8.48.

Found: C, 64.76; H, 6.61; N, 8.19.

(2S)-N-(4-Ethynylbenzyl)   2-Acetamido-3-(2-(oxiran-2-yl)ethoxy)propionamide

((S)-180) (~1:1 mixture of diastereomers). Using Methods D and J, and following

the preceding procedure, (S)-**120** (348 mg, 1.73 mmol), amine **141** (272 mg, 2.08

mmol), and DMTMM (575 mg, 2.08 mmol) in THF (40 mL), followed by mCPBA

(70% by wt., 447 mg, 1.88 mmol), Na$_2$SO$_4$ (500 mg) in CH$_2$Cl$_2$ (25 mL) gave (*S*)-**180** as a white solid (152 mg, 26% for two steps) after work-up, purification by flash chromatography (1/9 to 3/7 acetone/EtOAc) and recrystallization from EtOAc and hexanes: mp 130–136 ℃; [α]$^{25}_{D}$ +18.7° (*c* 0.5; CHCl$_3$); R_f = 0.36 (15/85 acetone/EtOAc); IR (nujol) 3281, 3193, 3072, 2861, 1638, 1551, 1458, 1376, 1297, 1256, 1130 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.50–1.64 (m, CH$_2$C**H**H'CH(O)CH$_2$), 1.90–2.04 (m, CH$_2$CH**H'**CH(O)CH$_2$), 2.03, 2.04 (s, **CH$_3$**C(O)), 2.42–2.48 (m, CH$_2$CH$_2$CH(O)C**H**H'), 2.68–2.74 (m, CH$_2$CH$_2$CH(O)CH**H'**), 2.82–2.98 (m, CH$_2$CH$_2$**CH**(O)CHH'), 3.07 (s, ArC≡C**H**), 3.42–3.52 (m, C**H**H'OCH$_2$CH$_2$), 3.54–3.76 (m, CH$_2$OC**H$_2$**CH$_2$), 3.84–3.92 (m, CH**H'**OCH$_2$CH$_2$), 4.38–4.50 (m, NHC**H$_2$**C$_6$H$_4$), 4.51–4.58 (m, C**H**CH$_2$O), 6.58–6.72 (m, N**H**CHCH$_2$O), 7.02–7.12 (m, 0.5 N**H**CH$_2$C$_6$H$_4$), 7.14–7.25 (m, 2 Ar**H**, 0.5 N**H**CH$_2$C$_6$H$_4$), 7.40–7.48 (d, *J* = 8.0 Hz, 2 Ar**H**), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**180** gave only one set of signals for the acetyl protons (δ 2.030 and 2.023 ppm), addition of excess (*R*)-(-)-mandelic acid to a CDCl$_3$ solution of (*S*)-**180** and (*R*)-**180** in a ~1:2 ratio gave two sets of two signals with a relative ~1:2 intensity for the acetyl protons (δ 2.030 and 2.023 ppm (*S*); δ 2.017 and 2.009 ppm (*R*)); ^{13}C NMR (CDCl$_3$) δ 23.4 (**C**H$_3$C(O)), 32.4, 32.6 (CH$_2$**C**H$_2$CH(O)CH$_2$), 43.4 (NH**C**H$_2$C$_6$H$_4$), 46.7 (CH$_2$CH$_2$CH(O)**C**H$_2$), 50.5, 50.6, 52.6, 52.7 (**C**HC(O)NH, CH$_2$CH$_2$**C**H(O)CH$_2$), 68.6, 68.9, 69.8, 70.1 (CH**C**H$_2$O**C**H$_2$), 77.5 (ArC≡**C**H), 83.5 (Ar**C**≡CH), 121.4, 127.6, 132.5, 132.6, 139.1, 139.3 (**C$_6$**H$_4$), 170.3, 170.6 (CH$_3$**C**(O), CH**C**(O)NH), additional signals were not detected and are believed to overlap with nearby peaks; M_r (+ESI)

353.1 [M+Na]$^+$ (calcd for $C_{18}H_{22}N_2O_4Na^+$ 353.1). Anal. Calcd for $C_{18}H_{22}N_2O_4$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.17; H, 6.58; N, 8.28.

2.3.3.4. Synthesis of biotin and fluorescent Probes

1,11-Diazido-3,6,9-trioxaundecane (184).[417] To a cooled (ice bath) CH_2Cl_2 solution (250 mL) of tetraethylene glycol (10.30 g, 52.9 mmol) and Et_3N (16.5 mL, 60.8 mmol) was added MsCl (9.16 mL, 60.8 mmol) dropwise. The reaction was then stirred at room temperature (2 h), the salts filtered, and the CH_2Cl_2 layer washed with aqueous 10% citric acid (250 mL) and brine (250 mL). The organic layer was dried (Na_2SO_4) and evaporated to yield tetraethyleneglycol dimesylate (**183**) as a yellow liquid residue (18.70 g, quant.). Dimesylate **183** was dissolved in DMF (50 mL) and NaN_3 (8.65 g, 133 mmol) was added. The solution was stirred at 80 °C (12 h) and cooled to room temperature. H_2O (450 mL) was added to the DMF and the mixture was extracted with Et_2O (8 x 50 mL). The organic layers were combined, dried (Na_2SO_4), and evaporated to yield **184** (10.74 g, 83%) as a pale yellow liquid that was used directly in the next step: R_f = 0.23 (2/1 hexanes/EtOAc); ^{1}H NMR (CDCl$_3$) δ 3.39 (t, J = 5.1 Hz, 2 C**H$_2$**N$_3$), 3.65–3.70 (m, 6 OC**H$_2$**); ^{13}C NMR (CDCl$_3$) δ 50.9 (OCH$_2$**C**H$_2$N$_3$), 70.2 (O**C**H$_2$CH$_2$N$_3$), 70.9 (2 O**C**H$_2$**C**H$_2$O).

1-Amino-11-azido-3,6,9-trioxaundecane (185).[417] Compound **184** (1.52 g, 6.22 mmol) was dissolved in a mixture of EtOAc (50 mL) and aqueous 1 M HCl (10 mL). PPh$_3$ (1.94 g, 7.40 mmol) was added and the reaction was vigorously stirred (12 h). The aqueous layer was separated, extracted with EtOAc (4 x 50 mL), basified (pH

~11) with solid K_2CO_3, saturated with NaCl, and extracted with CH_2Cl_2 (6 x 30 mL) to yield **185** (950 mg, 70%) as a colorless residue that was used without further purification: R_f = 0.18 (1/9 MeOH/CHCl$_3$); ^{1}H NMR (DMSO-d_6) δ 2.74 (t, J = 5.4 Hz, C**H$_2$**NH$_2$), 3.36–3.46 (m, C**H$_2$**N$_3$, C**H$_2$**CH$_2$NH$_2$), 3.48–3.62 (m, 2 OC**H$_2$**C**H$_2$**O, C**H$_2$**CH$_2$N$_3$), 3.84–4.08 (m, CH$_2$N**H$_2$**); ^{13}C NMR (DMSO-d_6) δ 40.5 (**C**H$_2$NH$_2$), 50.0 (OCH$_2$**C**H$_2$N$_3$), 69.2, 69.5, 69.6, 69.7, 69.8, 71.3 (6 O**C**H$_2$).

3,6,9,12-Tetraoxapentadec-14-yn-1-ol (186).[418] To a cooled (ice bath) THF solution (50 mL) of tetraethylene glycol (2.00 g, 10.3 mmol) was added NaH (60% suspension in oil, 272 mg, 11.34 mmol). After stirring at 0 ℃ (15 min), propargyl bromide (80% wt in toluene, 1.68 mL, 11.34 mmol) was added dropwise at 0 ℃, the suspension warmed to room temperature and stirred (1 h). The salts were removed by filtration, the filtrate was concentrated in vacuo and purified by flash chromatography to yield **186** as a colorless oil (642 mg, 27%): R_f = 0.58 (1/9 MeOH/CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 2.44 (t, J = 2.2 Hz, OCH$_2$CC**H**), 2.68 (t, J = 6.3 Hz, CH$_2$O**H**), 3.58–3.64 (m, C**H$_2$**OH), 3.64–3.76 (m, 3 C**H$_2$**OC**H$_2$**, C**H$_2$**OCH$_2$CCH), 4.21 (d, J = 2.2 Hz, C**H$_2$**CCH); ^{13}C NMR (CDCl$_3$) δ 58.6 (**C**H$_2$CCH), 61.9 (**C**H$_2$OH), 69.3, 70.5, 70.6, 70.7, 70.8, 70.9, 72.7 (7 O**C**H$_2$), 74.7 (CH$_2$C**C**H), 79.8 (CH$_2$**C**CH).

1-Azido-3,6,9,12-tetraoxapentadec-14-yne (188). To a cooled (0 ℃) THF solution (25 mL) of **186** (624 mg, 2.69 mmol), Et$_3$N (450 μL, 3.22 mmol) was added mesyl chloride (250 μL, 3.22 mmol) dropwise. After stirring at room temperature (45 min), the salts were filtered, and the filtrate concentrated in vacuo. The residue was

dissolved in CH_2Cl_2 (25 mL), successively washed with aqueous 10% citric acid (25 mL) and brine (25 mL), dried (Na_2SO_4) and evaporated to dryness. The crude mesylated product **187** (788 mg, 2.54 mmol, 94%) was directly dissolved in DMF (10 mL). NaN_3 was added (214 mg, 3.3 mmol) and the reaction was heated at 60 °C (18 h). The reaction was cooled, H_2O (90 mL) was added and the mixture was extracted with Et_2O (3 x 100 mL). The combined organic layers were successively washed with H_2O (2 x 50 mL), brine (50 mL), and dried (Na_2SO_4). The solvents were removed under vacuum to yield **188** as pale yellow oil (400 mg, 61%) that was used without further purification: R_f = 0.46 (5/95 MeOH/CH_2Cl_2); IR (neat) 3251, 2869, 2106, 1454, 1531, 1293, 1107 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.44 (t, J = 2.2 Hz, OCH$_2$CC**H**), 3.41 (t, J = 5.1 Hz, C**H$_2$**N$_3$), 3.62–3.74 (m, 3 C**H$_2$**OC**H$_2$**, C**H$_2$**OCH$_2$CCH), 4.21 (d, J = 2.2 Hz, C**H$_2$**CCH); ^{13}C NMR (CDCl$_3$) δ 50.9 (**C**H$_2$N$_3$), 58.6 (**C**H$_2$CCH), 69.3, 70.2, 70.5, 70.6, 70.7, 70.8, 70.9 (7 O**C**H$_2$), 74.7 (CH$_2$C**C**H), 79.8 (CH$_2$**C**CH); M_r (+ESI) 296.1012 [M+K]$^+$ (calcd for $C_{11}H_{19}N_3O_4K^+$ 296.1013).

3,6,9,12-Tetraoxapentadec-14-yn-1-amine (189). Azide **188** (355 mg, 1.38 mmol) was dissolved in THF (20 mL) and PPh$_3$ (724 mg, 2.74 mmol) was added. After dissolution, H_2O (1 mL) was added and the reaction was stirred at room temperature (12 h). The solvents were removed in vacuo, the residue was dissolved in aqueous 0.1 M HCl (20 mL), and washed with CH_2Cl_2 (6 x 80 mL) and EtOAc (2 x 100 mL). The aqueous layer was evaporated to dryness and the remaining salts were suspended in CH_2Cl_2 (25 mL), vigorously stirred (5 min), and filtered. The cake was extensively rinsed with CH_2Cl_2, and the combined organic fractions were evaporated

to yield **189** (247 mg, 77%) as a hygroscopic pale yellow oil that did not require further purification: R_f = 0–0.10 (1/9 MeOH/CH$_2$Cl$_2$); IR (neat) 3367, 2870, 2112, 1661, 1596, 1457, 1353, 1294, 1250 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.38–1.56 (br s, CH$_2$N**H$_2$**), 2.44 (t, J = 2.2 Hz, OCH$_2$CC**H**), 2.87 (t, J = 5.4 Hz, C**H$_2$**NH$_2$), 3.51 (t, J = 5.4 Hz, C**H$_2$**CH$_2$NH$_2$), 3.60–3.75 (m, 2 C**H$_2$**OC**H$_2$**, C**H$_2$**OCH$_2$CCH, C**H$_2$**OCH$_2$CH$_2$NH$_2$), 4.21 (d, J = 2.2 Hz, C**H$_2$**CCH); ^{13}C NMR (CDCl$_3$) δ 42.0 (**C**H$_2$NH$_2$), 58.6 (**C**H$_2$CCH), 69.3, 70.5, 70.6, 70.7, 70.8, 73.7 (6 O**C**H$_2$), 74.7 (CH$_2$C**C**H), 79.8 (CH$_2$**C**CH), the remaining signal was not detected and is believed to overlap with nearby peaks; M_r (+ESI) 232.1547 [M+H]$^+$ (calcd for C$_{11}$H$_{21}$NO$_4$H$^+$ 232.1549). Anal. Calcd for C$_{11}$H$_{21}$NO$_4$•0.35H$_2$O: C, 55.60; H, 9.21; N, 5.89; Found: C, 55.34; H, 8.95; N, 6.26.
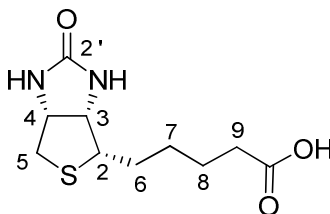
Biotin-PEG-azide (191).[418] Using Method D, amine **185** (415 mg, 1.9 mmol), D-biotin (464 mg, 1.9 mmol) and DMTMM (525 mg, 1.9 mmol) in DMF (20 mL) were stirred at room temperature (12 h). The DMF was removed in vacuo and the residue directly purified using flash chromatography (1/9 MeOH/CHCl$_3$) to yield biotin-PEG-azide **191** as an off-white solid (523 mg, 62%): mp 118–119 ℃; R_f = 0.40 (1/9 MeOH/CHCl$_3$); ^{1}H NMR (CD$_3$OD) δ 1.45 (q, J = 7.2 Hz, C(6)**H$_2$**), 1.50–1.80 (m, C(7)**H$_2$**, C(8)**H$_2$**), 2.22 (t, J = 7.2 Hz, C(9)**H$_2$**), 2.70 (d, J = 12.6 Hz, C(5)H**H**'), 2.92 (dd, J = 4.8, 12.6 Hz, C(5)**H**H'), 3.18–3.25 (m, C(2)**H**), 3.32–3.45 (m, NHC**H$_2$**CH$_2$,

CH$_2$N$_3$), 3.55 (t, J = 5.7 Hz, NHCH$_2$CH$_2$O), 3.59–3.71 (m, 2 OCH$_2$CH$_2$O, OCH$_2$CH$_2$N$_3$), 4.30 (dd, J = 4.8, 7.8 Hz, C(3)H), 4.49 (ddd, J = 0.9, 4.8, 7.8 Hz, C(4)H); ^{13}C NMR (CD$_3$OD) δ 27.0 (C(6)), 29.6, 29.9 (C(7), C(8)), 36.9 (C(9)), 40.6 (NHCH$_2$CH$_2$O), 41.2 (C(5)), 51.9 (OCH$_2$CH$_2$N$_3$), 57.1 (C(2)), 61.8 (C(4)), 63.5 (C(3)), 70.7 (NHCH$_2$CH$_2$O), 71.3, 71.4, 71.6, 71.7, 71.8 (2 OCH$_2$CH$_2$O, OCH$_2$CH$_2$N$_3$), 166.3 (C(2')), 176.3 (C(O)NHCH$_2$).

Biotin-PEG-amine (192).[422] Compound **191** (235 mg, 0.53 mmol) was suspended in THF (5 mL), and PPh$_3$ (208 mg, 0.79 mmol) was added. After 15 min stirring, H$_2$O (2 mL) was added and the reaction was stirred at room temperature (24 h). Aqueous 1 M HCl was added (5 mL) to the reaction and the mixture was washed with EtOAc (3 x 20 mL). The aqueous layer was basified (pH ~11) with K$_2$CO$_3$, saturated with NaCl, and then extracted with EtOAc (5 x 20 mL) and CH$_2$Cl$_2$ (5 x 20 mL). The aqueous layer was concentrated in vacuo and the residual salts triturated repeatedly with CH$_2$Cl$_2$ and filtered. The combined organic layers were evaporated to yield **192** (110 mg, 50%) as a colorless residue that did not require further purification: $[\alpha]^{25}_D$ +40.4° (c 1.6, CH$_2$Cl$_2$); R_f = 0.15 (1/4 MeOH/CHCl$_3$); ^{1}H NMR (CD$_3$OD) δ 1.58 (q, J = 7.2 Hz, C(6)H$_2$), 1.66–1.92 (m, C(7)H$_2$, C(8)H$_2$), 2.36 (t, J = 7.2 Hz, C(9)H$_2$), 2.84 (d, J = 12.6 Hz, C(5)HH'), 2.92 (br t, J = 5.1 Hz, OCH$_2$CH$_2$NH$_2$), 3.07 (dd, J = 4.8, 12.6 Hz, C(5)HH'), 3.30–3.39 (m, C(2)H), 3.50 (t, J = 5.1 Hz, C(O)NHCH$_2$), 3.62–3.71 (m, NHCH$_2$CH$_2$O, OCH$_2$CH$_2$NH$_2$), 3.72–3.84 (m, 2 OCH$_2$CH$_2$O, OCH$_2$CH$_2$N$_3$), 4.44 (dd, J = 4.8, 7.8 Hz, C(3)H), 4.49 (ddd, J = 0.6, 4.8, 7.8 Hz, C(4)H); ^{13}C NMR (CD$_3$OD) δ 26.8 (C(6)), 29.5, 29.7 (C(7), C(8)), 36.7 (C(9)), 40.6 (NHCH$_2$CH$_2$O), 41.2 (C(5)),

42.1 (OCH$_2$**CH$_2$**NH$_2$), 51.9 (OCH$_2$**CH$_2$**N$_3$), 57.1 (**C**(2)), 61.8 (**C**(4)), 63.5 (C(3)), 70.7 (NHCH$_2$**CH$_2$**O), 71.2, 71.5, 71.6 (3 O**CH$_2$**), 73.5 (O**CH$_2$**CH$_2$NH$_2$), 166.0 (**C**(2')), 176.0 (**C**(O)NHCH$_2$), the remaining methylene signal was not detected and is believed to overlap with a nearby peak; M_r (+ESI) 419.2324 [M+H]$^+$ (calcd for C$_{18}$H$_{34}$N$_4$O$_5$SH$^+$ 419.2328).

Biotin-PEG-alkyne (193).[423] Using Method D, amine **192** (96 mg, 229 μmol), 4-pentynoic acid (27 mg, 275 μmol) and DMTMM (76 mg, 275 μmol) in THF (5 mL) gave compound **193** (85 mg, 75%) as a white solid after silica gel flash chromatography (1/9 MeOH/CHCl$_3$): mp 108–110 °C; $[\alpha]^{25}_D$ +25.3°(c 1.1, CHCl$_3$); R_f = 0.47 (1/9 MeOH/CHCl$_3$); ^{1}H NMR (CD$_3$OD) δ 1.45 (q, J = 7.2 Hz, C(6)**H$_2$**), 1.50–1.80 (m, C(7)**H$_2$**, C(8)**H$_2$**), 2.22 (t, J = 7.2 Hz, C(9)**H$_2$**), 2.29 (t, J = 2.6 Hz, CH$_2$C≡C**H**), 2.35–2.50 (m, **CH$_2$CH$_2$**C≡CH), 2.70 (d, J = 12.6 Hz, C(5)H**H'**), 2.92 (dd, J = 4.8, 12.6 Hz, C(5)H**H'**), 3.18–3.25 (m, C(2)**H**), 3.32–3.42 (m, NH**CH$_2$**CH$_2$O, O**CH$_2$**CH$_2$NH), 3.55 (app. t, J = 5.1 Hz, NHCH$_2$**CH$_2$**O, O**CH$_2$**CH$_2$NH), 3.59–3.71 (m, O**CH$_2$CH$_2$**O), 4.30 (dd, J = 4.8, 7.8 Hz, C(3)**H**), 4.49 (ddd, J = 0.6, 4.8, 7.8 Hz, C(4)**H**); ^{13}C NMR (CD$_3$OD) δ 15.9 (**CH$_2$**CCH), 27.0 (**C**(6)), 29.6, 29.9 (**C**(7), **C**(8)), 36.1 (**CH$_2$**CH$_2$C≡CH) 36.9 (**C**(9)), 40.5, 40.6 (NH**CH$_2$**CH$_2$O, OCH$_2$**CH$_2$**NH), 41.2 (**C**(5)), 51.9 (OCH$_2$**CH$_2$**N$_3$), 57.1 (**C**(2)), 61.8 (**C**(4)), 63.5 (C(3)), 70.7 (NHCH$_2$**CH$_2$**O), 71.3, 71.4, 71.6, 71.7, 71.8 (2 O**CH$_2$CH$_2$**O, O**CH$_2$**CH$_2$N$_3$), 83.7 (**C**≡CH) 166.3 (**C**(2')), 176.3 (**C**(O)NHCH$_2$), the remaining alkyne carbon resonance was not detected. M_r (+ESI) 522.2407 [M+Na]$^+$ (calcd for C$_{23}$H$_{38}$N$_4$O$_6$SNa$^+$ 522.2410).
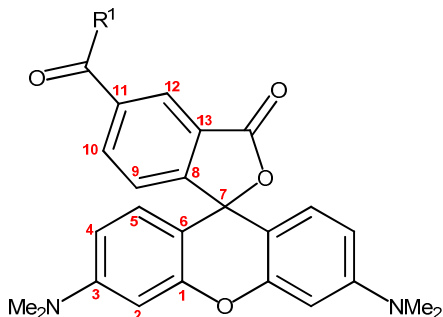
TAMRA-PEG-alkyne (195). Using Method D, 5-carboxytetramethylrhodamine (20 mg, 47 μmol), compound **189** (14 mg, 61 μmol), and DMTMM (17 mg, 61 μmol) in DMF (500 μL) gave **195** as a dark pink residue (10 mg, 33%) after purification by preparative TLC (5/95 to 15/85 MeOH/CH$_2$Cl$_2$): R_f = 0.25 (1/9 MeOH/CH$_2$Cl$_2$); ^{1}H NMR (CD$_3$OD) δ 2.85 (t, J = 2.4 Hz, OCH$_2$CC**H**), 3.30 (s, 2 N(C**H$_3$**)$_2$), 3.60–3.80 (m, 3 C**H$_2$**OC**H$_2$**, NHC**H$_2$**), 4.17 (d, J = 2.4 Hz, C**H$_2$**CCH), 6.92–7.08 (m, 2 C(4)**H**, 2 C(2)**H**), 7.13–7.28 (m, 2 C(5)**H**), 7.41 (d, J = 8.7 Hz, C(9)**H**), 8.09 (d, J = 8.7 Hz, C(10)**H**), 8.60–8.75 (br s, C(12)**H**); ^{13}C NMR (CD$_3$OD) δ 41.0 (2 N(**C**H$_3$)$_2$), 41.3 (C(O)NH**C**H$_2$), 59.2 (**C**H$_2$CCH), 70.2, 70.7, 71.4, 71.5, 71.6, 71.7, 71.8 (7 O**C**H$_2$), 76.1 (CH$_2$C**C**H), 80.8 (CH$_2$**C**CH), 97.5, 115.0, 115.2, 129.9, 131.0, 132.7, 137.3, 158.9, 159.2, 162.3, 169.4 (TAMRA), the remaining signals were not detected; M_r (+ESI) 644.2968 [M+H]$^+$ (calcd for C$_{36}$H$_{41}$N$_3$O$_8$H$^+$ 644.2972).

TAMRA-PEG-azide (196). 5,6-Carboxy-TAMRA (25 mg, 58 μmol) and amine **185** (12.6 mg, 70 μmol) were dissolved in DMF (300 μL) and EDCI (13 mg, 70 μmol), DMAP (1 mg, catalytic), DIEA (12 μL, 70 μmol) were successively added. After stirring at room temperature (1 d), the DMF was evaporated and the residue purified by silica gel chromatography (1/9 to 1/3 MeOH/CH$_2$Cl$_2$) to yield 16 mg (44%) of **196**

as a deep purple solid: R_f = 0.25 (1/9 MeOH/CH$_2$Cl$_2$); ^{1}H NMR (CD$_3$OD) δ 3.20–3.40 (m, C**H$_2$**N$_3$, 2 N(C**H$_3$**)$_2$), 3.50–3.80 (m, 3 C**H$_2$**OC**H$_2$**, NHC**H$_2$**), 6.92–7.08, 7.20–7.30, 7.38–7.42, 7.72–7.76, 8.05–8.20, 8.55–8.60 (m, 2 C(4)**H**, 2 C(2)**H**, 2 C(5)**H**, C(9)**H**, C(10)**H**, C(12)**H**); ^{13}C NMR (CD$_3$OD) δ 40.4, 41.0, 41.3 (2 N(**C**H$_3$)$_2$, C(O)NH**C**H$_2$), 51.9 (**C**H$_2$N$_3$), 70.7, 71.3, 71.5, 71.7, 71.8 (5 O**C**H$_2$), 97.4, 108.4, 115.1, 115.3, 130.2, 131.2, 132.7, 132.9, 137.3, 137.5, 159.0, 159.2, 163.1, 169.2 (TAMRA), the remaining signals were not detected; M_r (+ESI) 631.2876 [M+H]$^+$ (calcd for C$_{33}$H$_{38}$N$_6$O$_7$H$^+$ 631.2881).

CHAPTER 3

PROTEIN CHEMISTRY IN THE EYE OF THE CHEMIST

SAR studies demonstrated that placement of AB&CR moieties at the 3-oxy site and the 4'-benzylamide positions in (*R*)-LCM did not lead to significant loss of anticonvulsant activity in the MES test. In those cases where an electrophilic AB agent was inactive, the activity of the corresponding isostere suggested that the AB group could be used in *in vitro* experiments, where metabolism is less likely an issue. With these molecular tools at hand, we advanced to the biological questions of this project. In the following sections, general methods to prepare a protein lysate from animal tissues are presented, along with the protein purification methods used in these studies. These methods are detailed, discussed and explained from the perspective of an organic chemist learning how to manipulate large molecules of complexity. Considerations concerning click chemistry in a biological setting are also discussed.

3.1. General procedures for rat brain fractionation

Differential centrifugation is a widely used method to separate subcellular compartments from a given tissue.[424-427] It is usually preferred to use freshly prepared tissue samples, by sacrificing the animal and removing the organs

immediately before subcellular fractionation.[424] This ensures minimal subcellular cross-contamination that may arise from freeze/thaw cycles-induced compartment leakage. In our case, the purchased rat brains (6-7 weeks old, 200–300 g, male Sprague-Dawley rats, Rockland Immunochemicals or Pel-Freez Biologicals) were frozen in liquid nitrogen upon decapitation. The brains were shipped and received in dry ice, stored at -80 ℃ and thawed prior to fraction ation.

The first tissue preparation step involves homogenizing the tissue in an appropriate homogenization buffer at 4 ℃. A key comp onent in the buffer used is sucrose (280–320 mM), and serves as a molecular cushion to prevent the disruption of membranes (nucleus and organelles) by osmotic shock. Dounce homogenizers (glass/glass or glass/Teflon) are generally preferred to electric grinder or sonicators as they constitute a gentler way to disrupt tissues by shearing, leaving the bigger organelles intact. The clearance of the Dounce homogenizer is critical as a fitting too tight will lead to rupture of intracellular compartments and subcellular cross-contamination.

After grinding the tissue in a homogenization buffer the resulting crude homogenate can undergo several differential centrifugation steps to yield different subcellular compartments. The homogenization buffer typically includes a given buffer (5–50 mM Tris or HEPES [pH 7.4–8.0]), 320 mM sucrose, 5 mM $MgCl_2$ (improves stability of nuclei), and protease inhibitors. Generally, protease inhibitors were included in the different buffers to prevent proteolytic degradation that occurs as soon as the tissue is thawed. Accordingly, the following inhibitors were used for our studies. PMSF (paramethylsulfonyl fluoride) is an irreversible serine protease

inhibitor (working concentration 1 mM, 100 X stock solution in ethanol). PMSF hydrolyzes quickly in aqueous solutions, and should therefore be dissolved right before the homogenization step. E-64 (*N*-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide) is an epoxide containing tripeptide mimic that acts as an irreversible and selective inhibitor of cysteine proteases but does not inactivate proteins containing reactive cysteine groups (working concentration 10 μM, 100 X stock in water). Pepstatin-A is a peptide analog that acts as a reversible inhibitor of aspartyl proteases. Because of the reversible nature of the inhibitor, the lysate studied should be resupplemented with pepstatin-A after a dialysis step, and before storing the lysate at -80 ℃ (working concentration 1 μM, 100 X stock in DMSO). TPEN (*N,N,N',N'*-Tetrakis(2-pyridylmethyl)ethylenediamine) is a tight chelator of Zn^{2+} (pK_d = 15.2 at pH 7.6)[428] that prevents activation of zinc metalloproteases. As for Pepstatin A, this inhibitor should be resupplemented after a small molecule removing step such as dialysis (working concentration 1 μM, 100 X stock in ethanol).

Scheme 40. Subcellular fractionation using differential centrifugation
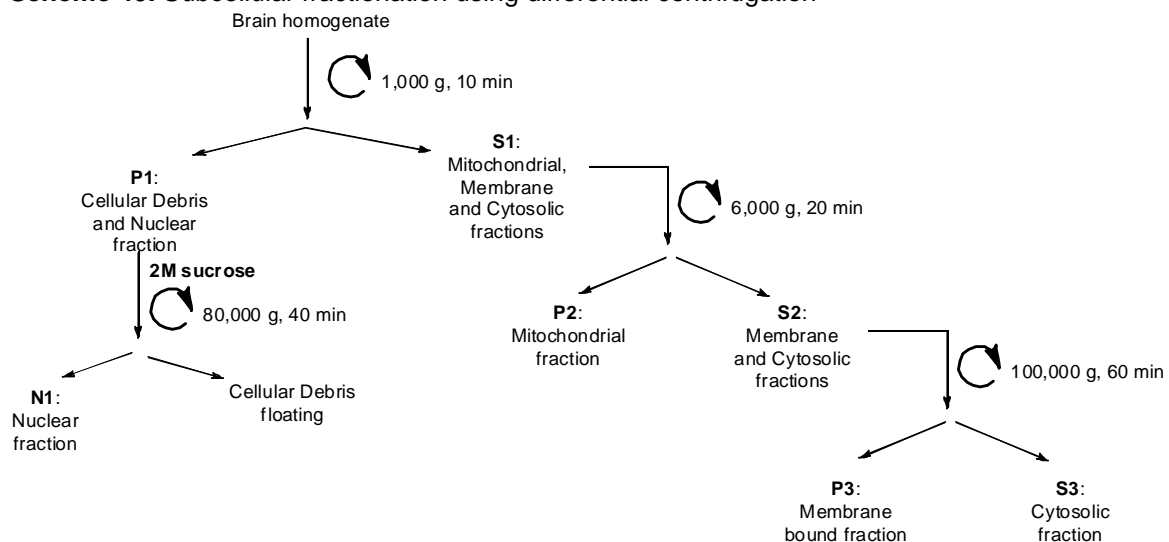
For our studies using rat brains, we utilized the following lysate preparation protocol (Scheme 40). All the following steps were carried out at 4 °C. The protocol was adapted from literature procedures.[424-426,429]

3.1.1. The nuclear fraction (N1)

N1 is obtained by centrifuging the crude homogenate at low speed (1,000 g) and comes with all the cellular debris. The supernatant (**S1**) is pulled for further separation. The pellet is rinsed 2 or 3 times with homogenization buffer and then resuspended in a high sucrose buffer (~4 to 5 times the approximate volume of the pellet), consisting of a given buffer (5–50 mM Tris or HEPES, pH 7.4), 2 M sucrose, and 5 mM $MgCl_2$. This suspension is then carefully and slowly layered on top of another high sucrose gradient (2 M) in an ultracentrifuge tube and spun at 80,000 g for 30–40 min. An optional step can be included at this stage that entails filtering the suspension through several layers of cheese cloth or gauze to remove most of the cellular debris, allowing the following step to be cleaner. While it is recommended that sucrose gradient separations be conducted with a swing bucket rotor, it was found that a fixed-angle rotor works fine for this procedure. Upon centrifugation (80,000 g, 35 min), the cellular debris (if not removed by filtration) is found floating at the surface of the tube due to the buoyancy of the solution, while pure nuclei, visible as a white fluffy cloud-like aggregate are pelletted at the bottom of the tube. Nuclei can be recovered by gently removing the supernatant with a pipette, and then the nuclear aggregate is rinsed one time with homogenization buffer (5 mL per rat brain nuclear fraction) to remove the sucrose.

Nuclei are generally lysed under hypertonic conditions (high salt) that cause the disruption of the nuclear membrane. A typical nuclear lysis buffer includes a given buffer (5-50 mM Tris or HEPES [pH 7.4–8.0]), 400–500 mM NaCl, 1.5 mM $MgCl_2$, 10–20% glycerol, and protease inhibitors. A high salt concentration and the presence of glycerol are critical to maintain proper folding of nuclear soluble proteins (mimics the high protein density inside the nucleus) as its removal by dialysis causes ~70% of nuclear proteins to precipitate. Nuclei are resuspended in the lysis buffer and can be sheared by 10 syringe passages through a 18 or 20 gauge needle. The suspension is gently rocked at 4 ℃ for 30 min. The DNA is then found floating at the surface as a white viscous material and removed with a pipette tip. Centrifugation at 14,000 rpm at 4 ℃ yields a soluble nuclear fraction as a supernatant and a nuclear membrane fraction as a pellet. The pellet is resuspended in a buffer typically consisting of a given buffer (5–50 mM, Tris or HEPES [pH 7.4–8.0]), 1.5 mM $MgCl_2$, 100 mM KCl, 10–20% glycerol and 0.5%–1% of a desired non-ionic detergent (Triton X-100, β-Dodecylmatoside, Zwittergent 3-10) and gently rocked at 4 ℃ for another 30 min. Centrifugation at 14,000 rpm for 10 min provides a solubilized membrane fraction. The fractions are stored at -80 ℃ and the pellet is discarded.

The soluble nuclear fraction should be resuspended in the smallest possible volume of nuclear lysis buffer and diluted with 4 volumes of lysis buffer depleted in NaCl to reduce the salt concentration to 100 mM NaCl to permit subsequent chemical biology experiments. Final protein concentration should be ideally 1~2 mg.mL^{-1}.

3.1.2. The mitochondrial fraction (P2)

P2 is obtained by centrifugation of the **S1** supernatant at 6,000 g. The supernatant **S2** is pulled for further purification. The mitochondrial fraction is comprised of mitochondria as well as the major cellular organelles (Golgi apparatus). The brain mitochondrial fraction is visible as a dark brown pellet and is lysed by osmotic shock with a hypotonic buffer (low salt). A typical mitochondrial lysis buffer includes a low concentration of buffer (5 mM Tris or 10 mM HEPES, pH 7.4) and protease inhibitors. The suspension is then gently rocked for 30 min at 4 ℃ and spun at 9,000 g for 30 min. The supernatant is then supplemented to a "normal" buffer concentration (5–50 mM Tris or HEPES, pH 7.4) and 50 mM NaCl. The pellet is then resuspended in a given buffer (5–50 mM Tris or HEPES, pH 7.4) containing 100 mM KCl and 0.5–1% of a desired detergent and gently rocked for 30 min at 4 ℃. Centrifugation at 9,000 g for 30 min provides a solubilized mitochondrial membrane fraction. The fractions are stored at -80 ℃ and the pellet is discarded.

3.1.3. The membrane fraction (P3)

P3 is obtained by centrifugation of the **S2** supernatant. Centrifugating at 100,000 g or more for 1 h provides the membrane fractions, also called microsomal fraction. Supernatant **S3** is pulled. The pellet obtained is resuspended in a given buffer (5–50 mM Tris or HEPES, pH 7.4), 100 mM KCl, 0.5%–1% of a desired detergent, and 15% glycerol and protease inhibitors, and then gently rocked for 30 min at 4 ℃ and centrifuged at 14,000 rpm (15 min) to provide a solubilized membrane fraction. The fraction is stored at -80 ℃ an d the pellet is discarded.

3.1.4. The cytoplasmic fraction (S3)

S3 contains the soluble, cytoplasmic proteins. Typically, approximately 40 mg of total cytoplasmic protein (Bradford Assay) are obtained per rat brain. The lysate (~20 mL per brain) is then dialyzed at 4 °C for 2 h against 3 L of a given buffer (5–50 mM Tris or HEPES, pH 7.4) containing 50 mM NaCl, changing the buffer after 1 h. Upon dialysis, the cytoplasmic fraction is resupplemented to 10 μM pepstatin A and 1 μM TPEN and stored at -80 °C.

3.2. Ammonium sulfate fractionation

Ammonium sulfate (AMS) fractionation is a widely used protein fractionation method[426,430-432] that uses the ability of high ionic concentrations to negate electrostatic repulsive charges on the solvent-exposed regions of a protein, leading to protein aggregation and precipitation. All proteins possess a specific AMS concentration range between which they progressively precipitate. That range is likely to change if the protein undergoes a conformational change, and therefore both pH and ligand binding can affect the needed AMS value for precipitation.

In contrast with protein denaturing precipitating conditions (trichloroacetic acid/acetone), AMS precipitation is non-denaturing and is very often the first step utilized in protein purification protocols or enrichment methods from a complex mixture. The protein pellet obtained from a given AMS fraction can be readily redissolved (1 to 10 min) by letting it stand in a desired buffer (5–50 mM HEPES or

Tris). If further protein purification is needed, it may be necessary to remove the excess AMS present in solution by dialysis, particularly if the following step involves ion exchange chromatography. A high amount of residual AMS present in a sample can be detrimental to S or Q Sepharose® fractionation as it will interact with the support's charged chemical groups and reduce their availability for proteins.

As a general trend, we observed that a slight majority of proteins that precipitated at low AMS saturation were of medium to high molecular weight (40–100 kDa) while a higher percentage lower molecular weight proteins (10–40 KDa) were found to precipitate in the higher AMS cut (>60–70%). Also, we did not observe proteins precipitating before ~15% AMS saturation. This information is important for the use of Phenyl Sepharose®. We also noticed an interesting pH-dependency in the AMS fractionation studies. At low levels (<30% AMS saturation), we found that as we lowered pH values from 8.5 to 7.5 to 6.5 we saw a general increase in the relative amounts of proteins that precipitate and it appeared that with decreasing pH we saw a buildup of large MW protein in the precipitate. At high AMS levels (>60–70%), as the pH of the buffer was decreased from 8.5 to 7.5 to 6.5 we observed the opposite trend. At high pH (8.5) we detected higher amounts of protein with pH 7.5 and 6.5.

AMS can be added either as a solution (aqueous AMS saturated solution, 4.1 M at room temperature) or as a solid. While it is easier to add a known precise volume (adding one volume of aqueous saturated AMS brings the solution to 50% saturation) this method becomes impractical at high AMS saturation (e.g., 9 volumes are required to bring a solution to 90% saturation) since it requires the use of larger

containers or multiple small containers. Moreover this method dilutes the proteins in solution and therefore increases the risk that the protein will not precipitate. Generally our experiments were carried out using AMS as a fractionation method employing liquid AMS up to 65-70% saturation (adding ~2 volumes of AMS_{sat}) and using solid AMS to saturate from 70 up to 95%. The amount of solid added was calculated using online software (http://www.encorbio.com/protocols/AM-SO4.htm) that takes into account the specific volume of AMS added to the solution.

A typical experiment involves the following protocol. A given volume of aqueous saturated AMS is added to the protein solution to bring the solution to the desired saturation, and the sample is gently mixed. The solution is allowed to stand at room temperature for 5 min and then centrifuged at 14,000 rpm for 4 min. The supernatant is transferred to another tube, and the process is repeated using the same procedure to obtain another protein AMS cut. Fractionation conducted in this way allowed the whole lysate to be "cut" in 5-6 different fractions within 90 min, including all the equilibration, centrifugation and supernatant transfer steps. The different cuts are hereon referred to as MXXYY, which designate the fraction that precipitate between XX% and YY% AMS saturation.

3.3. Ion exchange chromatography

Ion exchange resins are useful tools in protein purification and many different types have been developed. Protein ion exchange chromatography relies on the ability of proteins to form non-covalent electrostatic interactions between charged

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amino acids and complementarily charged chemical groups covalently attached to a solid matrix.[433]

In this method, the protein solution is incubated with the pre-equilibrated ion exchange resin under low salt conditions (equilibration buffer, typically 50 mM NaCl). Once proteins are adsorbed on the resin, the flow-through is either discarded or kept for further studies. The flow-through contains proteins that do not bind to the resin under these experimental conditions. The beads are then extensively washed with the equilibration buffer to remove unbound proteins present within the interstitial volume of the resin beads. The beads' interstitial volume is called the dead volume. Proteins are then eluted off the resin with increasing salt (typically NaCl) concentrations (elution buffers). The increasing ionic strength of the buffer (salt) progressively displaces macromolecules from the resin and leads to the gradual protein release from the beads. Other constituents can be included in the equilibration, washing and elution buffers in addition to the salt, such as organic type molecules (non-ionic or zwitterionic detergents) when fractionating membrane bound proteins, or inorganic species (Ca^{2+}, Mg^{2+}) to enhance stability of certain proteins.

A typical equilibration buffer contains 50 mM NaCl, and as a general trend for a cytoplasmic lysate, proteins generally start to elute off the column at ~150 mM NaCl, which is near the physiological concentration of NaCl.[434] By 350–500 mM NaCl, almost all the proteins have come off the beads employed in this study, Q-Sepharose® and S-Sepharose®.

Elution fractions can be assayed for protein concentration, and also resolved on a SDS PAGE gel, knowing though that high salt concentration may be

detrimental to proper electrophoretic migration. The sample may also be used directly for a reaction (*i.e.* click chemistry) or for further protein purification. Before the next step it may be necessary to remove or lower the salt concentration either by a desalting column or by dialysis.

In our studies, the ion exchange resins were Q-Sepharose® Fast-Flow (quaternary ammonium group) for anion exchange and S-Sepharose® Fast-Flow (sulphonate group) for cation exchange (GE Healthcare). For most proteins, the pI (isoelectric point) is below pH 7,[435] therefore a majority of proteins are negatively charged at physiological pH. Consequently, in a cytoplasmic lysate Q-Sepharose will bind a greater number of proteins than S-Sepharose at pH 7.4. In addition, since proteins are more positively charged at lower pH, and more negatively charged at higher pH, more proteins are anticipated to adsorb to S-Sepharose® and Q-Sepharose® under acidic and basic conditions, respectively.

Protein fractionation of the soluble cytoplasmic rat brain lysate was first carried out at two different pH values to identify differences in fractionation patterns. We found that S-Sepharose® chromatography was more sensitive to pH change (different fractionation patterns at pH 6.5 vs 8.5) than Q-Sepharose®. We also observed that the relative amount of protein that bind to S-Sepharose at pH 8.5 was low. These findings led us to combine these two types of ion-exchange chromatographies by adsorbing the lysate at pH 6.5 on S-Sepharose® and then adsorbing the unbound fraction on Q-Sepharose® without modifying the pH. Since more proteins typically bind to S-Sepharose® at pH 6.5 rather than 8.5, this provided an added benefit for the selective elution of proteins with various salt concentrations.

Therefore, we found it unnecessary to start the fractionation at pH 8.5 using S-Sepharose®.

While Sepharose® columns are mostly used for large scale protein purifications involving several different types of columns, they can also be used on small scale to purify or simply enrich a protein. A practical, small scale fractionation experiment with Sepharose® is to use Spin Filter tubes (650 µL capacity) with a polymer type filter to hold the resin bed. Ideally, experiments should be carried out at 4 ℃. Equilibration and elution buffers should be freshly prepared and chilled on ice prior to their use.

In general, in our experiments we used 1 mg of total protein (cytoplasmic lysate) for 100 μL (termed Column Volume, CV) of Fast-Flow® Sepharose (GE Healthcare, 50% slurry of beads in 20% EtOH). To preequilibrate the beads, the 20% EtOH is first removed by gravity or centrifugation at low speed (2,000 rpm, 5 sec when using Spin Filters) and the beads are washed with ice-chilled ddH$_2$O (2 x 10 CV) followed by the equilibration buffer (2 x 10 CV). On small scale, low speed centrifugation can be used to accelerate the process. It should be recognized that high speed (>5000 rpm, table-top centrifuge) can damage the beads' solid support. This general procedure was developed as a starting point for our studies of the whole lysate, and we expect that this protocol will be optimized when studying and purifying a specific protein.

On small scale experiments (20 μL to 200 μL beads, 50 μL to 2000 μL protein solution), the protein solution is tumbled with the beads (1–5 min). The flow-through is then recovered and the resin bed washed with 10 CV of equilibration buffer. To

elute the proteins, 5 CV of the desired elution buffer is added to the washed resin bed and gently swirled for 10–15 sec. The eluted fraction is then recovered by low speed centrifugation. 5–10 CV of the same elution buffer are then added to the beads to wash remaining bound proteins and centrifuged. The flow-through is discarded, and the next salt elution step can take place.

Sepharose type resins are useful in purifying or enriching a given pool of proteins based on electrostatic charges. Its other important role is to concentrate dilute samples, since the amount of resin used is proportional to the amount of protein that can be bound and not the protein concentration.

The main limitations of this method are the general setup, and the recovery of the different fractions. Also, when combining S and Q Sepharose together, while it is possible to adsorb and selectively elute approximately 60-70% of all the proteins present in the lysate, nearly 30-40% of the proteins remain unfractionated under various pH conditions (pH range from 6.5 to 8.5). Thus, it may be necessary to combine this method with other fractionation methods to obtain sufficiently pure material of select proteins.

Another type of Sepharose used in protein purification is the Phenyl Sepharose® Fast-Flow (GE Healthcare) type resin that allows proteins to be purified based on hydrophobic interactions. Chemical groups present on the beads of Phenyl Sepharose® include linear carbon chains ending with a phenyl group. In order to properly fractionate proteins by Phenyl Sepharose®, the protein mixture first needs to be adjusted to a certain AMS saturation (15% for a cytoplasmic lysate). This step will shield off the solvent accessible electronic charges of the protein, thus

maximizing interactions of the protein's hydrophobic residues with the Phenyl Sepharose. The purification methodology for Phenyl Sepharose® is similar to the one employed for S and Q Sepharose®, except the proteins are eluted off by progressively decreasing the AMS concentration of the buffer. This decrease frees the protein's electronic charges and releases the proteins from the hydrophobic matrix. The main disadvantage of this fractionation method is the necessity to dialyze the samples upon elution to remove the AMS.

3.4. Click chemistry

Click chemistry was performed using slightly modified conditions from those of Cravatt and co-workers.[276,345,359] Copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$) was used as the source of Cu^{2+}, and tris(2-carboxylethyl)phosphine hydrochloride (TCEP) was used as the reducing agent to generate the Cu(I) species. Earlier work by Sharpless and Finn have shown that the addition of an appropriate Cu(I) ligand in the click reaction increases the yield of this cycloaddition step by stabilizing the Cu(I) species in aqueous solutions and by catalyzing the click chemistry reaction.[279,284,436,437] When performing click chemistry between labeled proteins and a fluorescent- or a biotin-containing probe, the different ratios of compounds are critical to obtain a correct signal to noise ratio of product versus background labeling. Therefore, for one equivalent of alkyne or azide-containing Probe the following quantities of reagents were used.

25 equiv of $CuSO_4$: When the azide-alkyne cycloaddition is done under organic reaction conditions, the reaction only requires a catalytic amount of Cu(I) (5-

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10% mol vs the alkyne or azide). In our proteomics studies, the reaction was performed in aqueous buffered solutions and required the use of a high amount of $CuSO_4$ (25 equiv vs fluorescent probe). This is necessary if we are to detect a fluorescent signal within 1 h. Apart from the fluorescent or biotin probe, the $CuSO_4$ stock solution is the only component of the click chemistry reaction which does not need to be prepared freshly prior to using.

12.5 equiv of TCEP (50% mol vs Cu^{2+}). The theoretically optimal ratio should be 25 equiv since TCEP reacts stoichiometrically with $CuSO_4$. However, it was found that for a 1 h click chemistry reaction, there is no significant difference between 50% mol and 100% mol (25 equiv) of TCEP vs Cu^{2+}. Further increasing the TCEP vs Cu^{2+} ratio to 200% mol (50 equiv) resulted in an increase in background fluorescence. In experiments using sodium ascorbate (Na Asc) as the reducing agent, we did observe a slightly lower fluorescence signal compared with similar conditions using TCEP (data not shown). TCEP stock solution in water should be prepared prior to the experiment.

2.5 equiv (10% mol vs Cu^{2+}) of Cu(I)-chelating ligand: tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) has been advanced by Sharpless and coworkers as a ligand of choice to perform click chemistry reactions in biological systems. The main disadvantage of TBTA remains its poor solubility in water. Water-soluble Cu(I) chelators, such as sulfobathophenantroline (SBP), have recently been introduced as useful accelerating ligands for click chemistry.[438] Reports indicate that this ligand is more sensitive to oxidation than TBTA.[439] When comparing SBP with TBTA, we found that click chemistry with SBP gave rise to fluorescent signals in the no drug

control experiments that were as intense as the signal from the AB&CR-labeled proteins. This problem was not encountered when using TBTA as a ligand. We, therefore, opted to use TBTA. The theoretically optimal ratio of Cu(I) to ligand varied from 1:1 to 1:2 depending on the nature of the ligand. In the case of TBTA, 2 molecules of ligand are required to form the full complex with one Cu(I). We found that click chemistry performed with 200% mol of TBTA vs Cu^{2+} gave a higher fluorescence background when compared with only 10% mol TBTA vs Cu^{2+}. These findings were in agreement with published results from the Cravatt group.[276] As for TCEP, TBTA should also be prepared just prior to performing the click chemistry.

In initial experiments, the click chemistry was executed by adding given volumes of stock solutions in the following order: fluorescent probe, $CuSO_4$, TBTA, and TCEP. To combine 3 addition steps to one, we found it possible and more convenient to prepare a 10 x solution of premixed $CuSO_4$, TBTA and TCEP (termed 10X Cu(I) mix) and then add the appropriate volume to the protein solution containing the fluorescent probe. The 10X Cu(I) mix was prepared so that upon dilution the percentage (v/v) of organic solvents present in the reaction remained below 5%.

A typical click chemistry experiment of a lysate mixture modified with a lacosamide AB&CR agent involved the following (based on a 20 μM Probe final concentration). The fluorescent probe **195** or **196** (20 μM final concentration, stock solution in water) was first added to the modified protein solution. Next, a prepared 100 μL of 10X Cu(I) mix in a transparent tube was added. The Cu(I) mix was prepared by sequentially adding 10 μL of 50 mM aqueous $CuSO_4$, and then 40 μL of

625 μM TBTA (in a 1/4 DMSO/*t*BuOH mixture). The TBTA in the binary stock solution should be made fresh by first dissolving the solid in 1/5 of the final volume of DMSO, and adding 4/5 of the final volume of *t*BuOH. Solubilizing TBTA directly in a 1/4 DMSO/*t*BuOH mixture takes much longer. Upon mixing the Cu^{2+} and the TBTA stocks together, the solution turns cloudy quickly. At low $CuSO_4$ concentrations, the suspension looks white, while at higher concentrations it looks light blue (Cu^{2+} complex). To the suspension is then added 50 μL of freshly prepared 5 mM aqueous TCEP. Immediately upon mixing, the cloudy solution becomes clear and turns pale yellow, characteristic of a Cu(I)-complex. The 10X Cu(I) mix is diluted 10-fold by adding to the protein mixture and homogenized by vortexing. All the additions should be performed relatively quickly (5–10 min). A 10X Cu(I) mix should not be left at room temperature for more than 1 h. When many samples are involved, the use of a multi-channel pipettor is recommended. In our experiments, we found it unnecessary to rotate the tubes while performing click chemistry on soluble (cytoplasmic) proteins. Samples are then let to stand at room temperature for 1 h, and SDS loading buffer is added to stop the reaction and then boiled (75 °C for 4 min) and resolved on SDS PAGE gel.

CHAPTER 4

SCREENING THE RAT BRAIN CYTOPLASMIC FRACTION

4.1. The use of epoxide-based AB&CR molecules as selective labeling agents

4.1.1. Rationale

Early experiments with AB&CR agents **171** and **172** showed that the isothiocyanate (NCS) group led to appreciable non-specific protein labeling that gave complex in-gel fluorescence band patterns. The gel patterns made it difficult to identify unique targets associated with lacosamide function and almost impossible to identify potential tagets that are present in low abundance in the lysate. We hypothesized that by using a less reactive AB group, we would observe diminished protein adduction and an increased selectivity of target modification within the proteome. Recently, the epoxide AB was shown to selectively target certain classes of proteases in lysate experiments prepared from whole cells or organ tissue.[273,309,358] This apparent selectivity was, however, biased by the inherent affinity of protease cysteine residues for epoxide electrophiles. Interestingly, in a comparative study of various protein affinity labels, the aliphatic epoxide was able to selectively modify carbonic anhydrase 2 (CA2) without reacting with abundant or reactive enzymes (i.e., bovine serum albumin (BSA), protein kinase A (PKA),

glyceraldehyde-3-phosphate dehydrogenase (GAPDH)).[290] Optimal labeling conditions for epoxide adduction labeling were 20 h at room temperature.[290]

While aromatic epoxides differ from their aliphatic counterparts in terms of chemical reactivity,[307] they can also be used as protein labeling agents.[440,441] Accordingly, to increase our chances of target protein capture, we screened the proteome with AB&CR agents containing an epoxide moiety at two different positions, where the aliphatic and aromatic epoxide groups were located on the side chain of the molecule (**179**, **180**) and the benzylamide 4'-position (**175**, **176**), respectively.
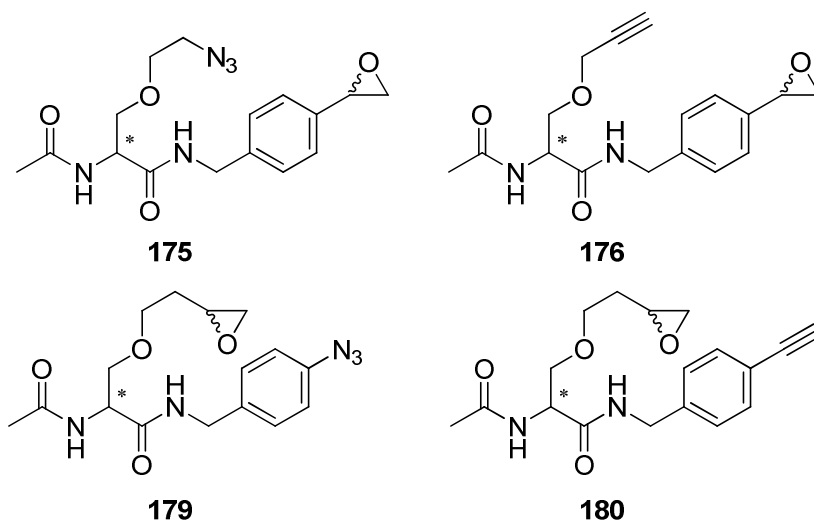
Figure 10. Structures of epoxide-based AB&CR agents used in the rat brain cytoplasmic fraction screening

4.1.2. Labeling and fractionation experiments of cytoplasmic proteins

Rat brain cytoplasmic lysate (**S3**) was prepared as previously described. Screening was initially performed using both enantiomers of **175**, **179**, and Probe **195** for visualization and later with (*R*) and (*S*) enantiomers of **176**, **180**, and Probe

196 (Figure 10, Figure 11). Starting with 200–300 μL of lysate (~2 mg.mL^{-1}) at the desired pH (6.5, 7.5, or 8.5), AB&CR compounds were allowed to react at room temperature (20 h),[290] and then the reaction mixture was fractionated with AMS. Recovered pellets were dissolved in 25 mM HEPES buffer (pH 7.5) and click chemistry (20 μM), CuSO$_4$ (25 equiv), TCEP (12.5 equiv), and TBTA[436,442,437] (200% vs. Cu^{2+}) was performed using compound **195**. The molar ratio of TBTA vs Cu^{2+} was reduced in later experiments (5% vs Cu^{2+}) since the higher molar ratio led to high fluorescence backgrounds, as observed by Cravatt and coworkers.[276,359] Samples were then resolved on SDS PAGE gel (8, 10, or 12.5%), scanned for fluorescence (532 nm excitation; 580 nm emission), and stained with Coomassie Brilliant Blue or silver stain for total protein quantification.
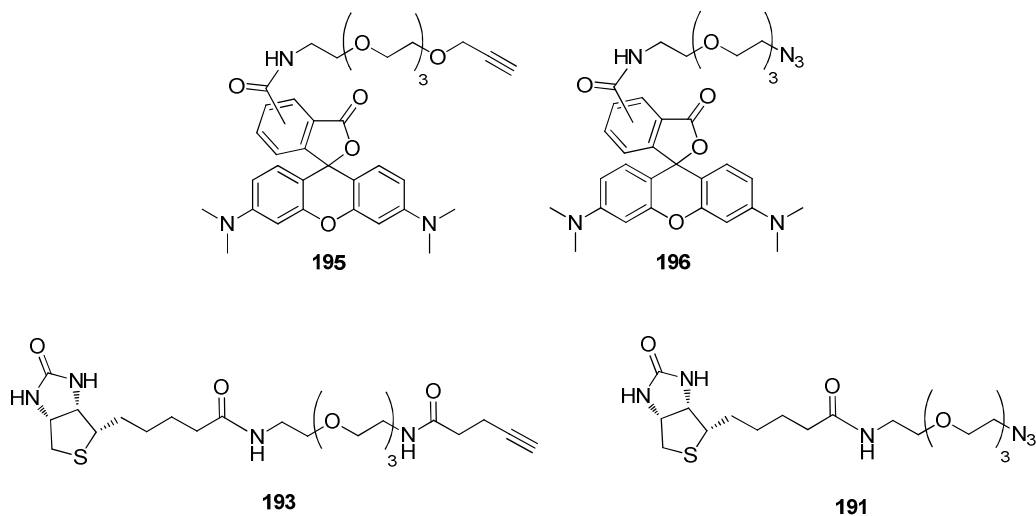
Figure 11. Structures of the different Probes used to react with the CR groups after protein labeling.

4.1.3. Identification of a protein of interest in the soluble fraction

The first experiment was carried out at two different concentrations of AB&CR agent (*R*)-**175** and (*R*)-**179** (50 μM and 5 μM), and fractions **M1030**, **M4050**, **M5058**, **M5865**, **M6590** were recovered then treated with **195** under Cu(I)-catalyzed conditions ("clicked"). Fluorescent labeling proved to be relatively high at 50 μM of AB&CR agent (*R*)-**179**, while only one protein in the **M4050** and the **M5058** fractions showed notable labeling at 5 μM. The protein was labeled by (*R*)-**179** and not by (*R*)-**175** (Figure 12). Analysis of the other AMS cuts (**M0040** and **M6590**) showed no bands that were selectively labeled by our AB&CR agents (data not shown).
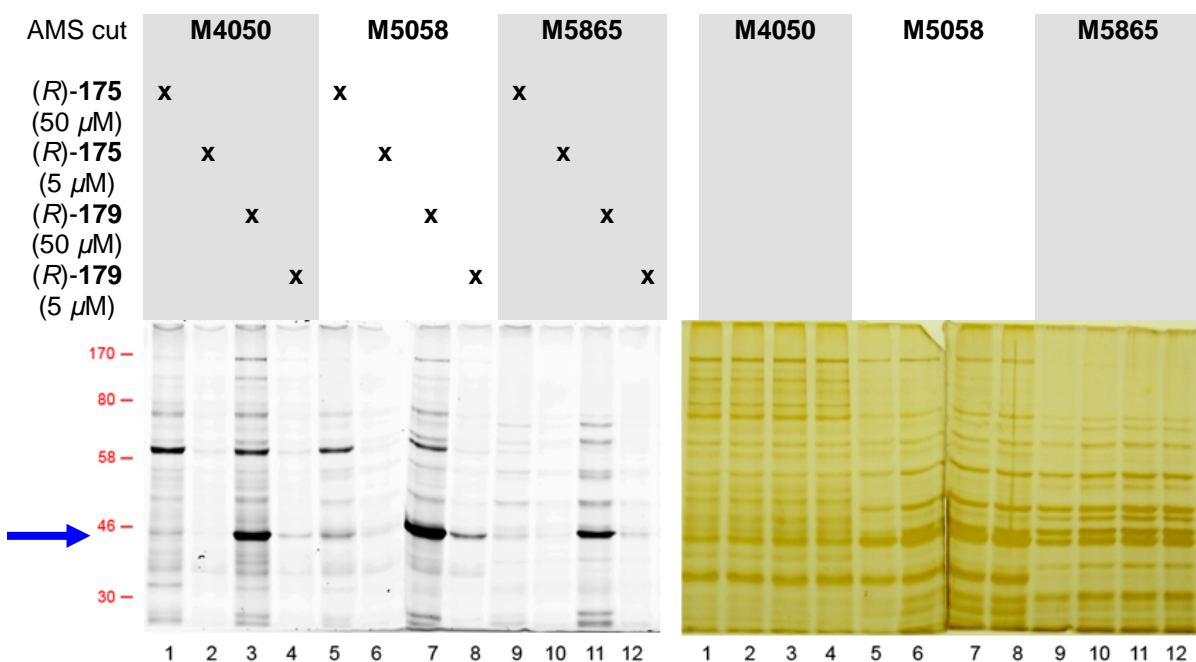
Figure 12. 6% SDS-PAGE gel of rat brain cytoplasmic AMS cuts containing a protein of interest labeled by (*R*)-**179** but not (*R*)-**175**. Cytoplasmic lysate samples (~300 μg total protein) were labeled with (*R*)-**179** and (*R*)-**175** at 5 or 50 μM at room temperature (20 h), fractionated with AMS, clicked with Probe **195**, and resolved. Left picture: in-gel fluorescence scan (ex.: 532 nm, em.: 580 nm); right picture: corresponding silver stain. Lanes 1–4: **M4050**; lanes 5–8: **M5058**; lanes 9–12: **M5865**. Lanes 1, 5, 9: (*R*)-**175** (50 μM); lanes 2, 6, 10: (*R*)-**175** (5 μM); lanes 3, 7, 11: (*R*)-**179** (50 μM); lanes 4, 8, 12: (*R*)-**179** (5 μM). Approximate molecular weight markers (kDa) are shown in red on the left.

A similar experiment with (R)-**179** at 50 *μ*M showed that the protein (~45 kDa) precipitated in the **M4065** fraction. However, it was possible to obtain a cleaner AMS cut containing the majority of the protein using the **M4555** cut. In a subsequent experiment, we found that this protein, enriched in **M4555**, could further be purified using Q-Sepharose® (HEPES, pH 8.0) upon elution between 200 and 300 mM NaCl at pH 8.0 (Figure 13). This experiment also demonstrated that (R)-**179** gave preferential labeling over (S)-**179**, while giving a solution highly enriched in the protein of interest. Utilizing this purification procedure, the protein was resolved on SDS PAGE gel (8%), Coomassie stained, excised, and sent for mass spectrometric (MS) analysis at the Michael Barber Centre for Mass Spectrometry (University of Manchester).
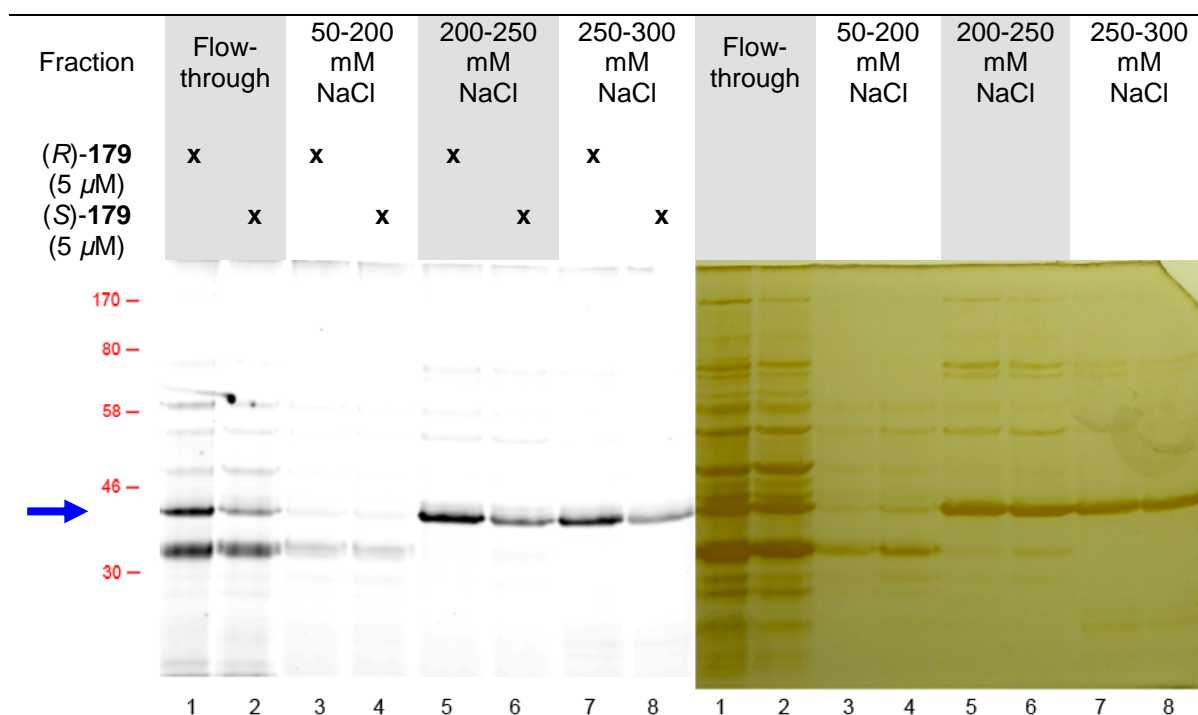
Figure 13. 8% SDS-PAGE gel of rat brain cytoplasmic preferentially labeled by (R)-**179** over (S)-**179**. Cytoplasmic lysate samples (~300 *μ*g total protein) were labeled with (R)-**179** and (S)-**179** at 5 *μ*M at room temperature (20 h), the **M4555** cut was recovered and further fractionated on Q-Sepharose® (pH 8.0). The flow-through, and elution fractions (50–200 mM NaCl, 200–250 mM NaCl, 250–300 mM NaCl) were recovered, clicked with Probe **195**, and resolved. Left picture: in-gel fluorescence scan

(ex.: 532 nm, em.: 580 nm), a small fraction of the labeled protein did not bind to the resin (lanes 1, 2); right picture: corresponding silver stain. Lanes 1,2: **M4555** Q-Sepharose[®] flow-through, lanes 3,4: 50–200 mM NaCl elution fraction, lanes 5,6: 200–250 mM NaCl elution fractions, lanes 7,8: 250–300 mM NaCl elution fractions. Lanes 1, 3, 5, 7: (*R*)-**179** (5 μM); lanes 2, 4, 6, 8: (*S*)-**179** (5 μM). Approximate molecular weight markers (kDa) are shown in red on the left.

4.2. Brain-type creatine kinase B (CKB)

The protein of interest was identified by mass spectrometry as brain-type creatine kinase (CKB). Mass spectral analysis of similarly cut gel bands from other experiments led to an approximate coverage of 60% of the enzyme sequence. These experiments did not reveal the modified residue on CKB. Table 2 provides representative tryptic fragments identified by MS.

Table 2. Representative list of the CKB tryptic digests identified from the fractionated cytoplasmic lysate

Creatine kinase B-type OS=Rattus norvegicus					
Observed	Mr(expt)	Mr(calc)	Miss	Score	Peptide
759.2673	758.26	758.3347	0	35	R.DWPDAR.G
439.7338	877.453	877.5021	1	43	K.FSEVLKR.L
524.175	1046.3354	1046.543	0	54	K.LLIEMEQR.L + Oxidation (M)
569.7446	1137.4746	1137.5601	1	43	K.GGNMKEVFTR.F
616.7395	1231.4644	1231.6085	0	49	K.DLFDPIIEDR.H
652.323	1302.6314	1302.7183	0	60	K.VLTPELYAELR.A
750.8008	1499.587	1499.7694	0	78	R.FCTGLTQIETLFK.S
801.8158	1601.617	1601.826	0	76	K.LAVEALSSLDGDLSGR.Y
836.3497	1670.6848	1670.8416	0	65	K.TFLVWINEEDHLR.V
841.8837	1681.7528	1681.8345	0	50	R.LEQGQPIDDLMPAQK.-
924.8934	1847.7722	1847.9703	0	110	R.LGFSEVELVQMVVDGVK.L
982.9081	1963.8016	1963.9236	0	75	R.GTGGVDTAAVGGVFDVSNADR.L
707.6211	2119.8415	2120.0247	1	77	K.RGTGGVDTAAVGGVFDVSNADR.L
729.2628	2184.7666	2184.9534	0	53	R.FPAEDEFPDLSSHNNHMAK.V
819.0059	2453.9959	2454.1386	1	34	K.LRFPAEDEFPDLSSHNNHMAK.V
839.9778	2516.9116	2517.1619	0	56	K.TDLNPDNLQGGDDLDPNYVLSSR.V

4.2.1. CKB is selectively labeled by epoxide-based AB&CR agents

Analogous experiments conducted using the AB&CR agents **176** and **180** (1–10 μM) containing the alkyne CR moiety and azide Probe **196** (10–50 μM) showed a

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reduced level of background fluorescence (Figure 15). We observed a similar reduced level of background protein labeling using azide Probe **196** compared with the alkyne Probe **195** in the *absence* of AB&CR agents. Finally, a slight reduction in background signal was observed between reactions where click chemistry was conducted with 5% TBTA vs Cu^{2+} compared with 200% TBTA vs Cu^{2+} (Figure 14).
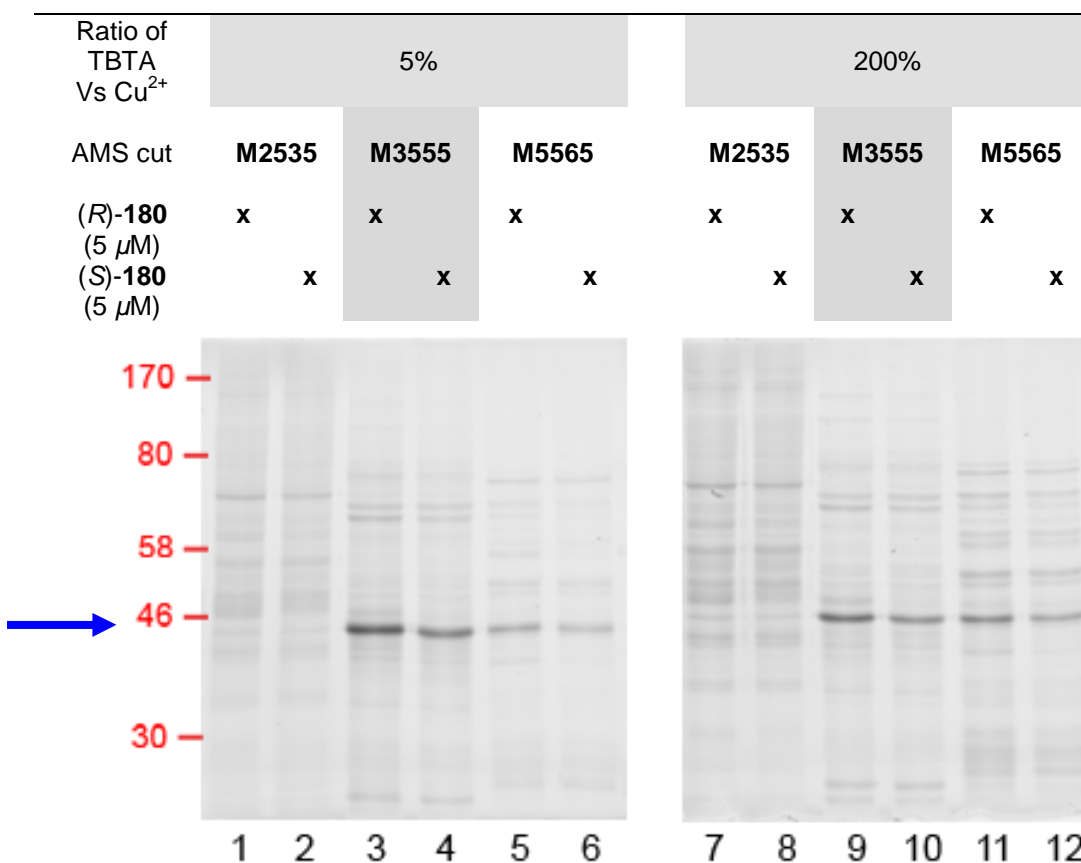
Figure 14. In-gel fluorescence scan of the brain cytoplasmic lysate (6% SDS-PAGE gel). Cytoplasmic lysate samples (~300 μg total protein) were labeled with (*R*)-**180** and (*S*)-**180** at 5 μM at room temperature (20 h), and fractionated using AMS. For each AMS cut recovered, the sample was split in half. The first half was clicked using a 5% TBTA vs Cu^{2+} ratio and the second half with 200% TBTA vs Cu^{2+} ratio. The samples were resolved, and scanned for fluorescence. Left gel (lanes 1–6): click chemistry was performed using a 5% TBTA vs Cu^{2+} ratio; right gel: (lanes 7–12): click chemistry was performed using a 200% TBTA vs Cu^{2+} ratio. Lanes 1, 3, 5, 7, 9, 11: (*R*)-**180** (5 μM); Lanes 2, 4, 6, 8, 10, 12: (*S*)-**180** (5 μM); Lanes 1, 2, 7, 8: **M2535**; lanes 3, 4, 9, 10: **M3555**; lanes 5, 6, 11, 12: **M5565**. CKB (blue arrow) is present in the **M3555** cut. Approximate molecular weight marker (kDa) are shown in red on the left

To confirm that CKB was specifically labeled by compound (*R*)-**179**, the cytoplasmic brain lysate (500 *μ*L, 1 mg total protein) was incubated with either DMSO (control) or 10 *μ*M (*R*)-**179** at room temperature (20 h). AMS fraction **M4555** was recovered, which was subsequently fractionated on Q-Sepharose® (pH 8.0) and the fraction eluting between 200 and 300 mM NaCl (HEPES, pH 8.0) was recovered. Click chemistry was performed using Probe **193** (20 *μ*M) at room temperature (1 h) and each reaction was then incubated with Streptavidin beads (50 *μ*L beads) that were pre-rinsed with 5 x 1 mL washing buffer (150 mM NaCl, 50 mM HEPES, pH 7.5) by tumbling at room temperature (1 h). The beads were centrifuged at 2000 rpm on a table-top centrifuge (30 sec), and the supernatant was removed. For each reaction the resin was then successively washed with 3 x 1 mL washing buffer, 3 x 1 mL washing buffer supplemented to 0.2% SDS, 3 x 1 mL aqueous 6 M urea, and 5 x 1 mL washing buffer. The beads were sent for MS analysis using off-the-beads trypsinization.[443] No proteins were found in the DMSO control sample, whereas only fragments of CKB were identified in the AB&CR sample, confirming that CKB was specifically labeled with (*R*)-**179** (Table 3)

Table 3. List of peptides identified after trypsin digestion of Streptavidin beads for the cytoplasmic sample treated with 10 *μ*M (*R*)-**179**. No peptides were identified after off-the-beads digestion from the DMSO treated sample.

Creatine kinase B-type OS=Rattus norvegicus					
Observed	Mr(expt)	Mr(calc)	Miss	Score	Peptide
516.23	1030.4454	1030.5481	0	38	K.LLIEMEQR.L
801.86	1601.7054	1601.826	0	84	K.LAVEALSSLDGDLSGR.Y
841.89	1681.7654	1681.8345	0	59	R.LEQGQPIDDLMPAQK.-
924.93	1847.8454	1847.9703	0	88	R.LGFSEVELVQMVVDGVK.L

4.2.2. Competition experiments with (*R*)-LCM and a known CKB inhibitor

Protein modification experiments using agent **180** reproducibly showed that CKB was preferentially labeled by (*R*)-**180** compared with (*S*)-**180** and that (*R*)-**176** did not modify the protein. However, (*R*)-**180** labeling experiments in the presence of excess (*R*)-**1** (100–5000 equiv) led, in most cases, to no reduction in the fluorescence intensity signal of the ~43 kDa band (Figure 15, blue arrow). In only one out of the 9 competition experiments did we see a reduction of the intensity of the fluorescent band labeled by (*R*)-**180** by (*R*)-LCM. This finding indicated (*R*)-LCM did not compete with (*R*)-**180** protein adduction. Nonetheless, other findings suggested a direct interaction between (*R*)-LCM and CKB (see Section 4.2.6).
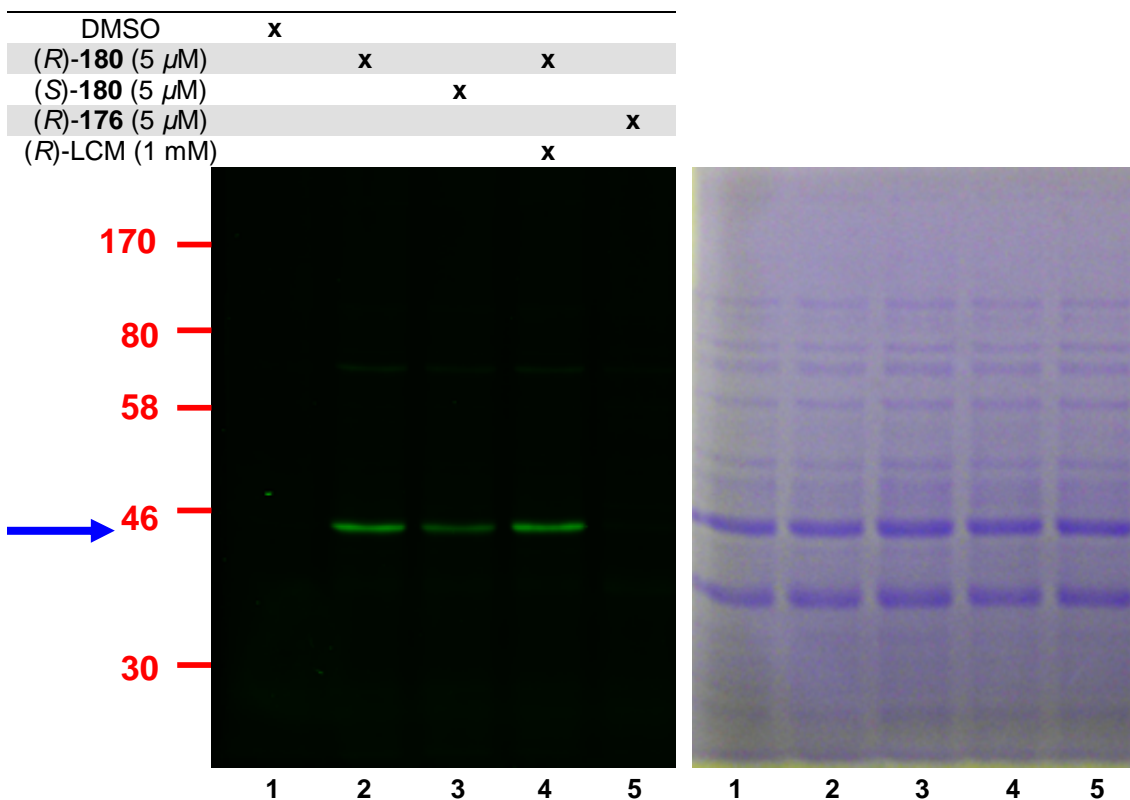
Figure 15. 8% SDS-PAGE gel of the cytoplasmic lysate **M4555** cut. Left picture: fluorescence scan; right picture: corresponding Coomassie stain. Samples (~200 μg total protein) were incubated at

room temperature (20 h) with the appropriate AB&CR agent or control, and fractionated with AMS. M4555 was recovered, click chemistry was performed using Probe **196**, and samples were resolved and scanned. Lane 1: DMSO; lane 2: (*R*)-**180** (5 μM); lane 3: (*S*)-**180** (5 μM); lane 4: (*R*)-**180** (5 μM) + (*R*)-LCM (1 mM); lane 5: (*R*)-**176** (5 μM). Approximate molecular weight markers (kDa) are shown in red on the left.

Next, we preincubated (5 min) the lysate with 2,4-dinitrofluorobenzene (DNFB, 10–20 μM), a known suicide inhibitor of CKB,[444-446] and then treated the lysate with (*R*)-**180** at room temperature (18 h). We observed a marked reduction (>50%, quantified with ImageJ®) in fluorescence intensity of the CKB containing band, after AMS fractionation (**M4555**) and click chemistry. By comparison, a more modest decrease in CKB-specific reduction (~10%) in the signal intensity was detected when DNFB was added <u>after</u> reaction of (*R*)-**180** with the lysate at room temperature (18 h). Interestingly, incubation with DNFB at room temperature (18 h) led to a shift in the AMS fraction of CKB, with a larger population of the protein now precipitating below 45% AMS saturation. This finding suggested that CKB underwent a protein conformational change upon DNFB modification. The DNFB findings were interesting and suggested that DNFB and (*R*)-**180** may interact at or near the same site. However, our observation that the protein likely underwent conformational change upon DNFB labeling raised several alternative explanations for the reduced (*R*)-**180** labeling with DNFB treatment. Among these, is that the CKB conformational change may have reduced either the efficiency of AB adduction or the extent of modification of the CR group of (*R*)-**180** with Probe **196** by click chemistry. Accordingly, the reduced fluorescence intensity could potentially be due to a combination of several factors. First, DNFB and (*R*)-**180** may label the same residue. Second, DNFB may have induced a conformational change of the protein resulting

in a reduction in (*R*)-**180** labeling. Third, a protein conformational change may have reduced the efficiency of the click chemistry reaction. Nonetheless, this set of experiments suggested the possibility that Cys283, the established residue modified by DNFB,[444-446] was covalently modified by (*R*)-**180**.

4.2.3. Possible significance of CKB as a target for epilepsy

CKB is a ~43 kDa cytoplasmic dimeric protein responsible for catalyzing the dephosphorylation of phosphocreatine to generate ATP, and is mainly localized in inhibitory neurons.[447,448] Importantly, this reaction represents the fastest way for a cell to generate ATP, using phosphocreatine as an energy buffer.[449] Many studies have shown the physiological importance of CKB in seizures and it has been established that the CKB reaction rate dramatically increases during a seizure, where brain levels of phosphocreatine decrease up to 50%.[449-452] This quick generation of ATP is required for fueling and maintaining the high-energy demand of the seizure.[450-452] Thus, it is possible that an inhibitor of CKB may act as a preventive agent by suppressing the seizure's energetic pathway. More recently, CKB has been shown to activate the K^+-Cl^--cotransporter 2 (KCC2) in GABAergic neurons.[453,444] KCC2 has been implicated in playing a crucial role in maintaining homeostasis of the neuron and dictating the inhibitory or excitatory role of GABA in developing neurons.[117] It has been recently found that some forms of epilepsy lead to dramatic changes in the expression profiles and the physiological properties of KCC2.[117,119,454,118] CKB knockout mice (CKB-/-) have been generated and are viable while displaying a mild phenotype, a gradual hearing loss, due to the abundance and

important role played by CKB in cochlear cells.[455] More interestingly, CKB-/- mice have been shown to have a higher seizure threshold in the ipPTZ seizure test when compared with normal mice.[456]

4.2.4. Creatine Kinase B activity assay

To further explore whether CKB was a potential target for (R)-**1** we determined if (R)-LCM, (S)-LCM and other derivatives could modulate the activity of the purified enzyme. Thus, we cloned, overexpressed and purified human His-tagged CKB in *E. coli* following literature procedures[457,458] and used the CKB activity assay to verify the functional integrity of the enzyme.
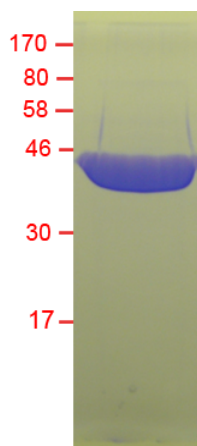
Figure 16. 12.5% SDS-PAGE gel Coomassie stain of overexpressed purified human CKB enzyme after His-tag, AMS, and Q-Sepharose® fractionation. Cloning and overexpression protocols can be found in the Experimental Section. Approximate molecular weight markers (kDa) are shown in red on the left.

To do so, we used a validated and widely used CKB coupled enzymatic assay.[446,459,460] The CKB activity assay was first performed using a commercial source of enzyme (human CKB, cat # 10-663-45059, GenWay Biotech, San Diego, CA), and later using purified overexpressed human CKB from *E. coli* (Figure 16, see

Sections 4.6.7 and 4.6.8 for experimental details). In addition we first used a commercial CK activity assay kit (BioAssay Systems, cat # ECPK-100, Hayward, CA) and later utilized an in house assay using commercially available reagents.[446,459,460] In this coupled assay, CKB catalyzes the transfer of a phosphate group from phosphocreatine to ADP, generating creatine and ATP (Scheme 41, top). The latter is used by hexokinase (HK) to convert D-glucose to glucose-6-phosphate, which is employed, in turn, by glucose-6-phosphate dehydrogenase (G6PDH), using NADP as a cofactor. NADPH, the reduced form of NADP, absorbs at 340 nm and 360 nm (Scheme 41, bottom).

Scheme 41. Chemical reactions used in the CKB activity assay. Top: CKB-catalyzed generation of ATP. Bottom: coupled enzymatic reactions used in the activity assay. NADPH is quantified by measuring the absorbance at either 340 or 360 nm. The amount of NADPH generated is proportional to the activity CKB.
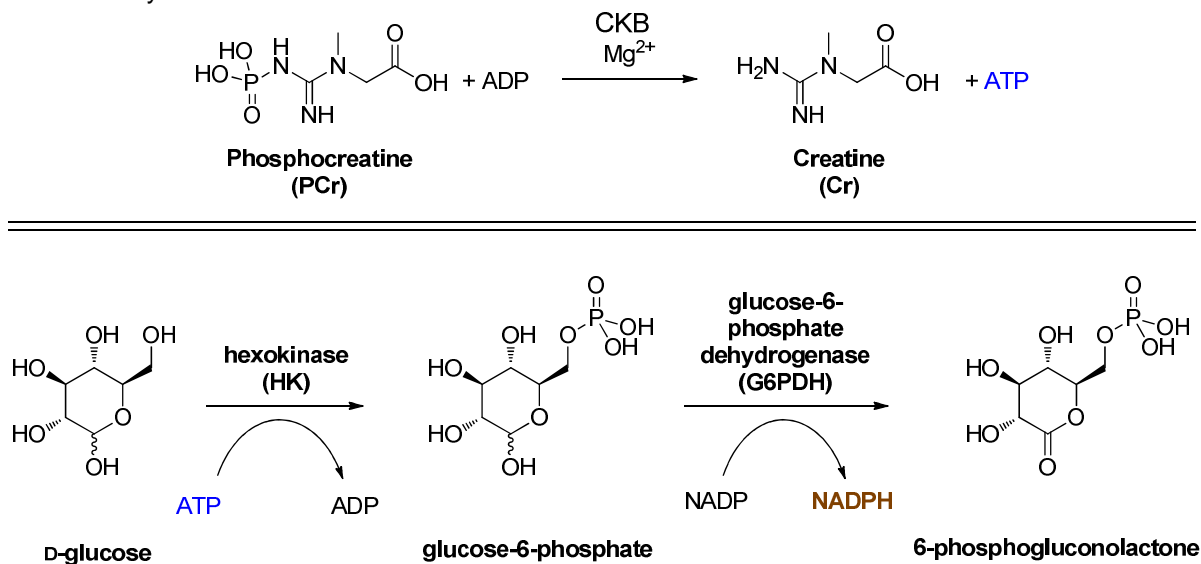
We compared the enzymatic activities of commercial CKB with the overexpressed purified CKB (Figure 17). The activities were virtually identical, and

both enzymes were completely inhibited by pre-incubation with 10 μM DNFB at room temperature (10 min). The amount of NADPH produced is directly proportional to the activity of CKB. In early experiments, equipment availability (HTS 7000 Plus Bio Assay Reader, Perkin Elmer) led us to monitor reactions by measuring the optical density (OD) at the sub-optimal 360 nm wavelength. The theoretically optimal 340 nm wavelength was used in later experiments using a different instrument (POLARstar OPTIMA, BMG Labtech).

CK-B NADP Assay (room temperature)

Legend:
- No Enz.
- 25 nM overexpressed CKB
- 25 nM overexpressed CKB + 50 μM DNFB
- 25 nM commercial CKB
- 25 nM commercial CKB + 50 μM DNFB
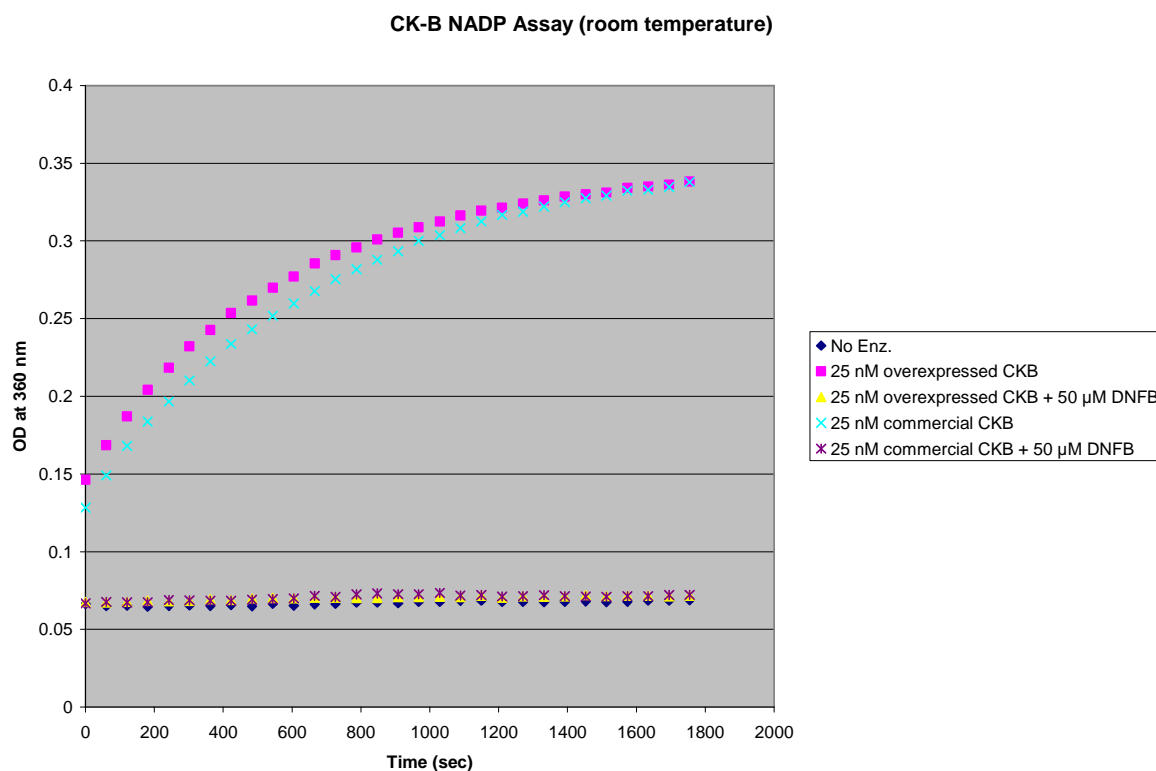
Figure 17. Comparison of enzymatic activities of human commercial CKB and overexpressed CKB. The coupled assay was conducted at room temperature and OD_{360} was plotted versus time. Legend is shown on the right.

4.2.5. Identification of modification sites of CKB

An (R)-**180** adduction experiment was conducted using overexpressed, purified human His-tagged CKB to determine which amino acid residue was modified by (R)-**180**. The buffer used for the labeling experiment was 100 mM MES (pH 6.5), containing 100 μM TCEP and a final concentration of 5% DMSO (v/v). One sample (0.5 mg) was incubated with no drug (control), and one with 500 μM of (R)-**180**. The samples were then resolved on 8% SDS-PAGE denaturing gel, the gel was Coomassie stained, and the band corresponding to CKB excised and sent for MS analysis.
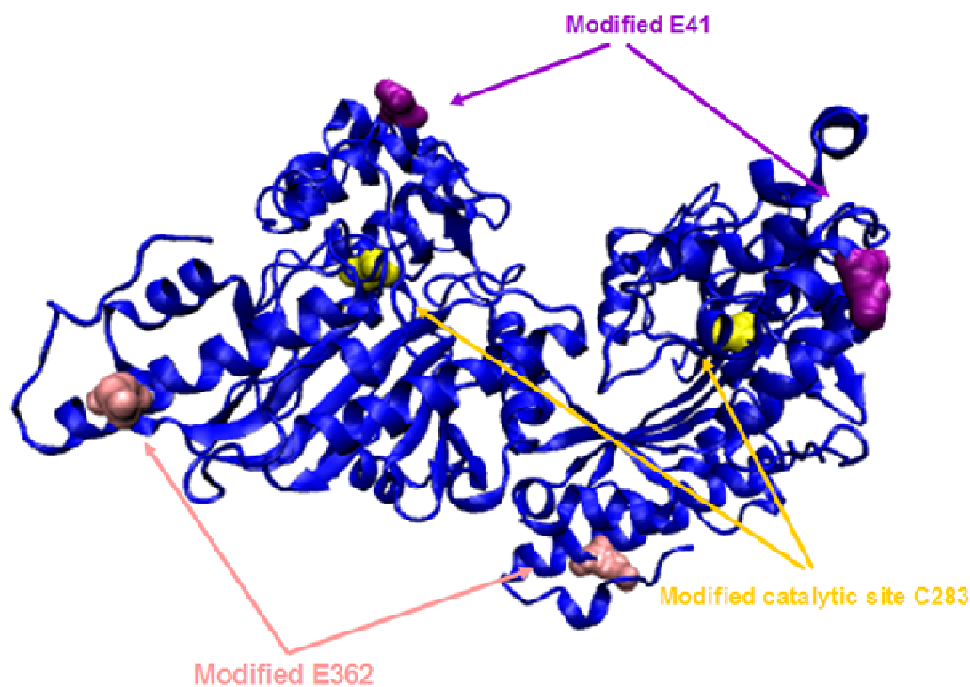
Figure 18. Crystal structure of human CKB (PDB ID: 3B6R). The protein dimer is represented in blue, and the modified residues identified from MS experiments are highlighted. Purified human CKB (~250 μg) was labeled with 500 μM (R)-**180** at room temperature (20 h), the protein was resolved on SDS-PAGE gel (8%), the gel was Coomassie stained and the ~43 kDa band corresponding to CKB was excised. Tryptic digests of the protein led to the identification of three residues. C283 (yellow) is a key amino acid for catalysis, while E362 (pink) and E41 (purple) are located on the protein surface. The figure was prepared by Ms. Onrapak Reamtong.

In the labeled enzyme sample, three residues were modified by the (*R*)-**180** epoxide AB group: C283, E41, and E362, but the extent of labeling of each residue could not be determined. The two glutamate residues are located on the surface of the enzyme, while C283 is a critical catalytic residue. D340, another key CKB catalytic residue,[461] was not modified under these conditions (Figure 18). The control reaction showed no evidence of amino acid modification.

A dose-dependent experiment was performed where a buffered solution of CKB (~200 μg per reaction) in 100 mM MES (pH 6.5), 5% DMSO, 100 μM TCEP was incubated at room temperature (20 h) with no drug, and 50, 500, 5000 μM of (*R*)-**180**. After labeling, an aliquot of each reaction was used in the CKB enzymatic assay (50 nM final concentration of enzyme). The remaining sample was equally split in two. One sample was dialyzed twice (1 h) against 3 L of double-distilled (dd) H_2O, lyophilized and sent on dry ice for MS analysis of the intact protein. The other sample was dialyzed twice (1 h) against 1.5 L of 50 mM MES (pH 6.5) and 100 μM TCEP. A 20 μL aliquot was used to perform click chemistry with Probe **196**. The remaining volume was snap-frozen (dry ice/acetone bath) and sent on dry ice for MS analysis.

Sample preparation proved to be critical for the detection of the intact protein. Only the samples that had undergone dialysis against ddH_2O and lyophilization gave a detectable signal. The CKB activity assay results showed an approximate correlation between the proportion of adducted protein and the inhibition of the enzyme. At 50 μM of (*R*)-**180**, the CKB enzymatic activity was virtually identical to

that of the unmodified enzyme (Figure 19) and no adduct could be detected by MS analysis (MW 44668 and MW 44710) (Figure 20). Interestingly, CKB appeared as a ~1:1 doublet at MW 44668 and MW 44710. The difference between the two signals (42 Da) likely corresponds to protein *N*-acetylation. We are unsure of the source and location of the acetyl residue, but have tentatively attributed this phenomenon to the CKB overexpression in *E. coli* where acetylation processes can occur.[462,463]

At 500 *µ*M, the enzymatic activity was reduced by ~60%, and approximately 33% of the enzyme, based on the MS peak intensities, corresponded to a (*R*)-**180** mono-adduct (330 Da modification: MW 44999 and MW 45040) with the rest matching the unmodified enzyme (MW of (*R*)-**180**: 330 Da).
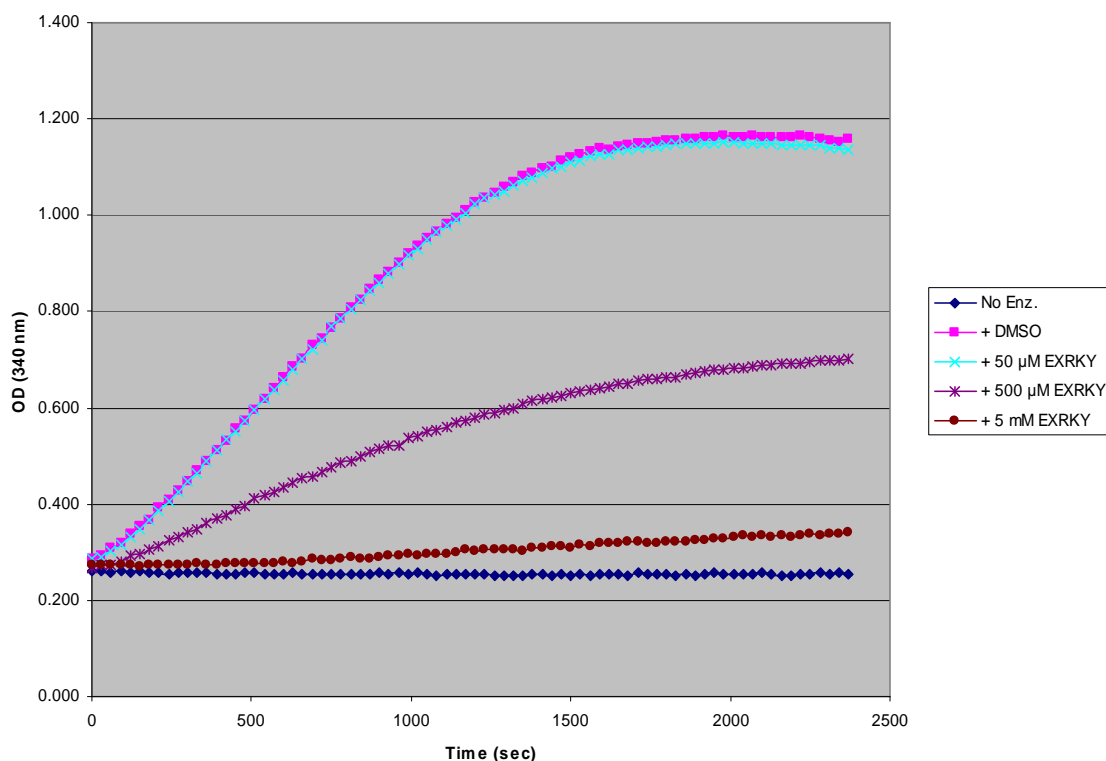
Figure 19. CKB enzymatic inhibition assay with (*R*)-**180** (abbreviated EXRKY). CKB samples were treated at room temperature (20 h) with DMSO, 50 *µ*M, 500 *µ*M, or 5 mM (*R*)-**180**. The assay was performed using an enzyme final concentration of 25 nM.

At 5 mM, the CKB activity was reduced ~95% and no MS peak characteristic of the unmodified enzyme could be observed. The major species in this sample was the (R)-**180** CKB mono-adduct (MW 45001 and MW 45042) and trace levels of a (R)-**180** di-adduct (MW 45330 and MW 45372). The evidence of a di-adduct may explain, in part, the three different modified residues observed in the tryptic digestion experiments.
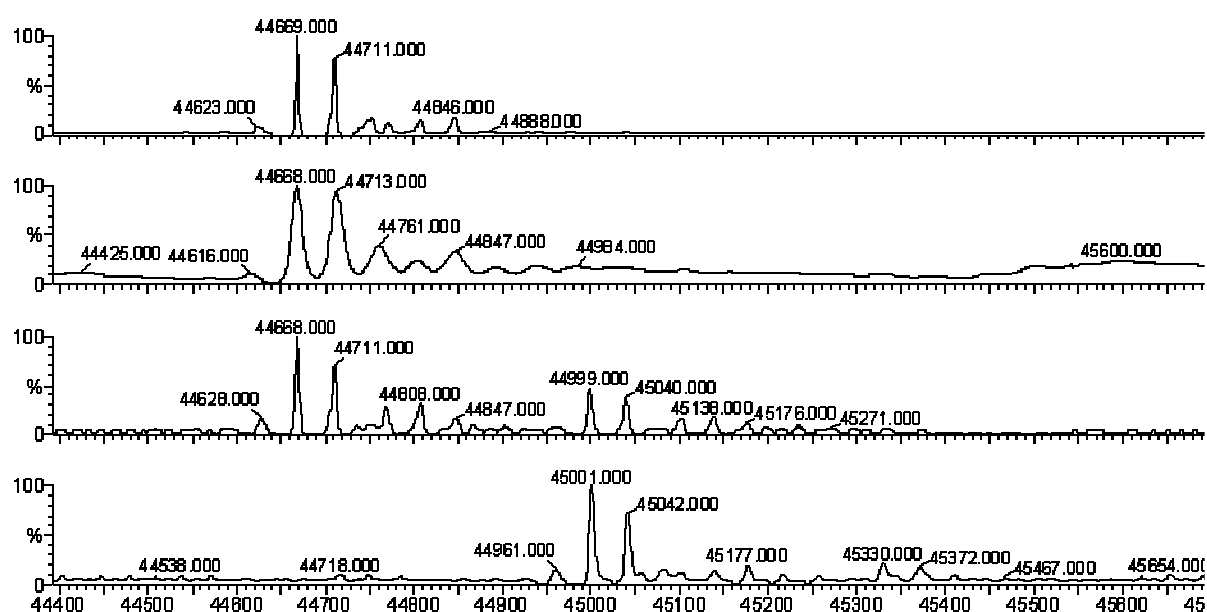
Figure 20. MS analysis of intact CKB (~50 μg) modified with (R)-**180** at different concentrations. Samples were incubated with DMSO or the AB&CR agent at room temperature (20 h), dialyzed against ddH$_2$O, lyophilized, and analyzed. CKB samples were treated with the following conditions, top to bottom: DMSO, 50 μM (R)-**180**, 500 μM (R)-**180**, 5 mM (R)-**180**.

The in-gel fluorescence experiment showed no adduction in the DMSO treated CKB sample, and a dose-dependent increase in the fluorescence intensity with samples modified with 50, 500 and 5000 μM of (R)-**180** (Figure 21, blue arrow). We reduced the sensitivity of the fluorescence scanner for this gel (PMT 300) in order to have non-saturating fluorescence levels.
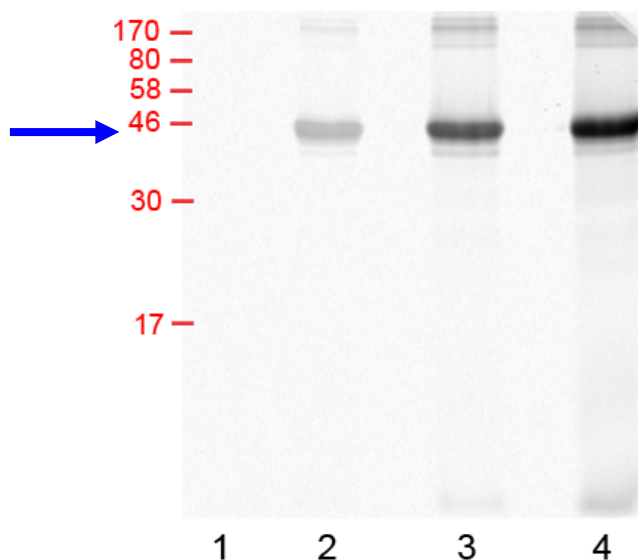
Figure 21. Fluorescence scan (very low sensitivity, PMT 300) of CKB samples treated with various concentrations of (*R*)-**180**. Samples were incubated with DMSO or (*R*)-**180** at room temperature (20 h), dialyzed against HEPES buffer (pH 7.5), clicked with Probe **196** and resolved on 15% SDS-PAGE gel. Lane 1: DMSO treated; lane 2: (*R*)-**180** (50 μM); lane 3: (*R*)-**180** (500 μM); lane 4: (*R*)-**180** (5 mM). The Coomassie stain of the gel showed equal amounts of protein per well (picture not shown). Approximate molecular weight markers are shown in red on the left.

The correlation of the MS data with the levels of CKB enzymatic inhibition was not exact. We found that the enzyme activity was reduced by ~60% in samples that showed ~33% of a (*R*)-**180** mono-adduct. Several factors may have accounted for this difference. One of these is the potentially different ionization properties of the CKB unmodified, (*R*)-**180** mono-adduct and (*R*)-**180** di-adduct samples in the mass spectrometer. Taken together, the results strongly suggest that the inhibitory activity of (*R*)-**180** is mediated by a covalent modification of Cys283. In addition, the strong in-gel fluorescent signal obtained at 50 μM of (*R*)-**180** combined with the absence of a detectable MS signal for the (*R*)-**180** mono-adduct (Figure 20) and no detectable loss of enzymatic activity (Figure 19) indicated that very low adduction levels (<1%) are sufficient to generate an intense signal in the in-gel experiments. These findings provide an insight into the sensitivity of this proteomic method.

4.2.6. Interaction of lacosamide with CKB

Most CKB labeling competition experiments using the rat cytoplasmic lysate or the purified enzyme with (*R*)-**180** (1–10 μM) in the presence of excess (*R*)- or (*S*)-LCM (100 to 5000 equiv) at room temperature (20 h) led to no reduction in fluorescence intensity of labeled CKB. These findings raised concerns since protein adduction by (*R*)-**180** should be competitively blocked by excess (*R*)-LCM. Accordingly, we asked whether CKB activity was inhibited by (*R*)-LCM.

Intriguingly, our first experiments conducted using purified CKB, showed that (*R*)-LCM inhibited the enzyme at therapeutically relevant concentrations (IC$_{50}$ ~20–50 μM), while (*S*)-LCM had no effect up to 500 μM. The IC$_{50}$ value of (*R*)-LCM, however, fluctuated with the amount of enzyme used per reaction. Furthermore, inhibition was only observed if the enzyme was pre-incubated with the drug for 10 min prior to starting the kinetic assay measurement. Noteworthy, incubation of (*R*)-LCM with a CKB sample containing 1 mM DTT led to no detectable inhibition of the enzyme.

The protective effect of DTT led us to hypothesize that the batch of (*R*)-LCM used was contaminated with trace amounts of a heavy metal and that this metal led to enzyme inactivation.[464,465] Significantly, the (*R*)-LCM sample used in these studies employed Ag$_2$O in the final synthetic step for (*R*)-**1** (**route 1**, see Section 2.2.2.1, Scheme 14). To confirm this hypothesis, we separately tested the effects of TPEN, a known heavy-metal chelator, on the enzyme. At 10 μM, TPEN did not have any inhibitory activity on CKB. When CKB (2 nM) was pre-incubated with 200 μM (*R*)-LCM, the enzyme was fully inhibited. However, in the presence of 10 μM TPEN, (*R*)-

LCM (200 and 500 μM) had no appreciable effect on the activity of the enzyme. Further supporting these results was the lack of activity of *O*-ethoxy (*R*)-**48**, which possesses anticonvulsant activity similar to (*R*)-LCM in the MES test,[466] and that was prepared using a heavy-metal-free synthetic route (**route 3**, see Section 2.2.2.2, Scheme 16).[466] Taking advantage of the large quantity of CKB that was available, we evaluated the binding affinity between CKB and (*R*)-LCM using isothermal titration calorimetry (ITC). In these experiments, highly concentrated stocks of CKB (5–10 mg.mL^{-1}, 115–230 μM) were dialyzed (2 x 2 h) against given equilibration buffers (phosphate buffer, pH 6.5 or 7.5, including TCEP or BME as reducing agent, in either the presence or absence of DMSO (5%)).
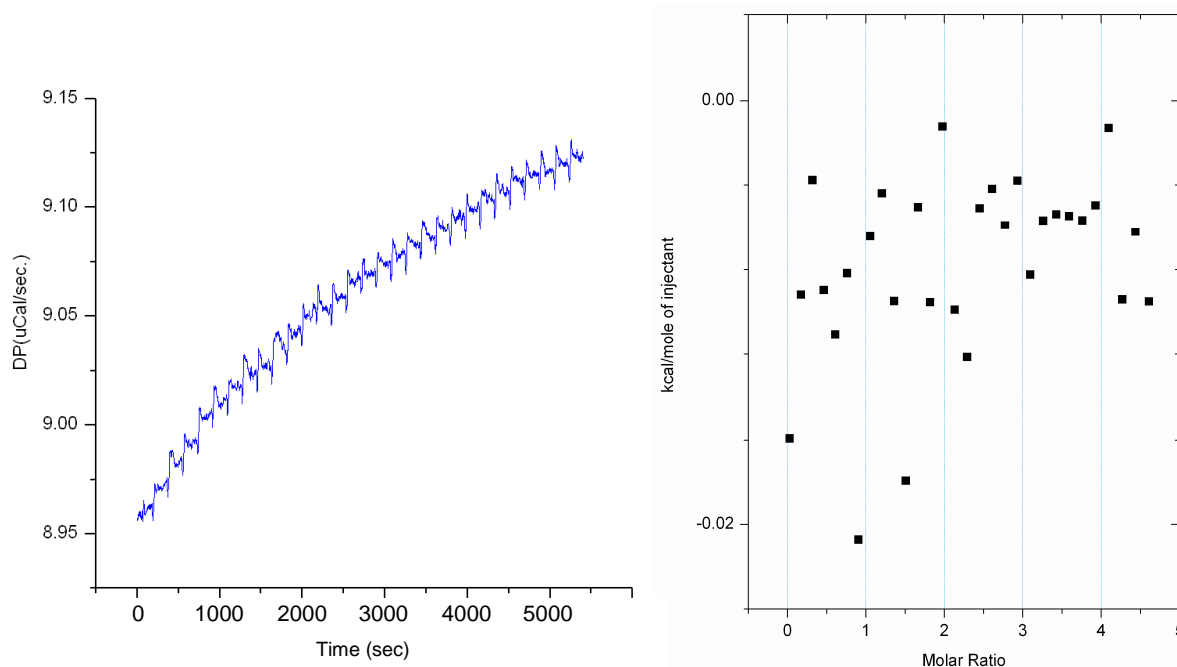
Figure 22. Left picture: ITC trace of the interaction between CKB and (*R*)-LCM. The purified enzyme (200 μM) was dialyzed (3 h) against 50 mM phosphate buffer (pH 7.4) containing 100 μM TCEP. The enzyme was then titrated with a 5 mM solution of (*R*)-LCM dissolved in the dialysis buffer. Right picture: corresponding integrated heat plot showing no correlation between the kcal.mol^{-1} of injectant and the molar ratio of the enzyme to the ligand. Machine settings are detailed in the Experiment Section.

The equilibration buffer of the second dialysis was then used to prepare the stock of (R)-LCM (20–25 times the protein concentration, 2.3–6.0 mM). Under no conditions was any significant binding affinity observed (Figure 22). The integrity of the enzyme was assessed by the CKB kinetic assay prior to beginning the ITC measurement. We concluded that CKB is selectively adducted by (R)-**180** in a complex protein lysate, but it is not a binding partner of (R)-**1**.

4.3. Photoaffinity labeling of the cytoplasmic lysate

Following our studies with epoxide-based AB&CR agents **175**, **179**, **176**, and **180**, other pairs of (R)- and (S)-AB&CR agents were employed to screen the rat brain soluble proteome (Figure 23).
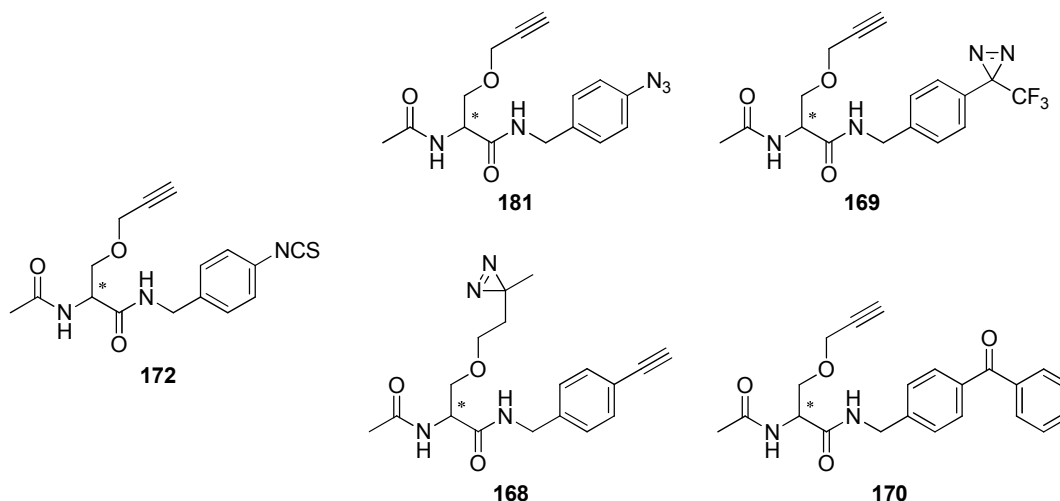
Figure 23. Structures of electrophilic AB&CR agents **172**, and photoAB&CR agents **181**, **169**, **168**, and **170** used in the screening of the rat brain cytoplasmic lysate.

One lesson learnt from the CKB experiments was that enantiospecific labeling of a protein should not be the sole criterion to identify potential targets. Instead, dose-

dependent and enantiospecific competition with (*R*)-LCM should serve as major experimental criteria for target identification. In addition, electrophilic AB groups can introduce a bias in the proteome search since they react with specific nucleophilic residues. Accordingly, the proteomic search was expanded to include a suite of photoAB&CR agents **181**, **169**, **168**, and **170** (Figure 23), and to rely primarily on competition experiments to select target(s) of interest. AB&CR agent **172** was used as a reference to compare the reactivity of the different photoAB&CR derivatives.

Following previously described protocols, we screened the rat brain cytoplasmic lysate with compounds **172**, **181**, **169**, **168**, and **170** using AMS to fractionate proteins. Each photoAB&CR agent was irradiated under specific conditions (see Experimental Section). After irradiation, the content of each well was recovered and AMS fractionation followed by click chemistry with fluorescent Probe **196** was performed. We screened the cytoplasmic lysate with AB&CR agent (*R*)-**172** (1 *μ*M, 15 min at rt, pH 7.4) and photoAB&CR agents **181**, **169**, **168**, and **170** (1 *μ*M) in the presence or absence of excess (*R*)-LCM (1 mM). Under these conditions, no protein of interest could be identified where the fluorescent labeling was diminished in the presence of excess (*R*)-LCM (1000 equiv) (Figure 24, Figure 25).

	1	2	3	4	5	6	7	8	9	10	11
DMSO	x										
(*R*)-**172**		x	x								
(*R*)-**181**				x	x						
(*R*)-**169**						x	x				
(*R*)-**168**								x	x		
(*R*)-**170**										x	x
(*R*)-LCM			x		x		x		x		x
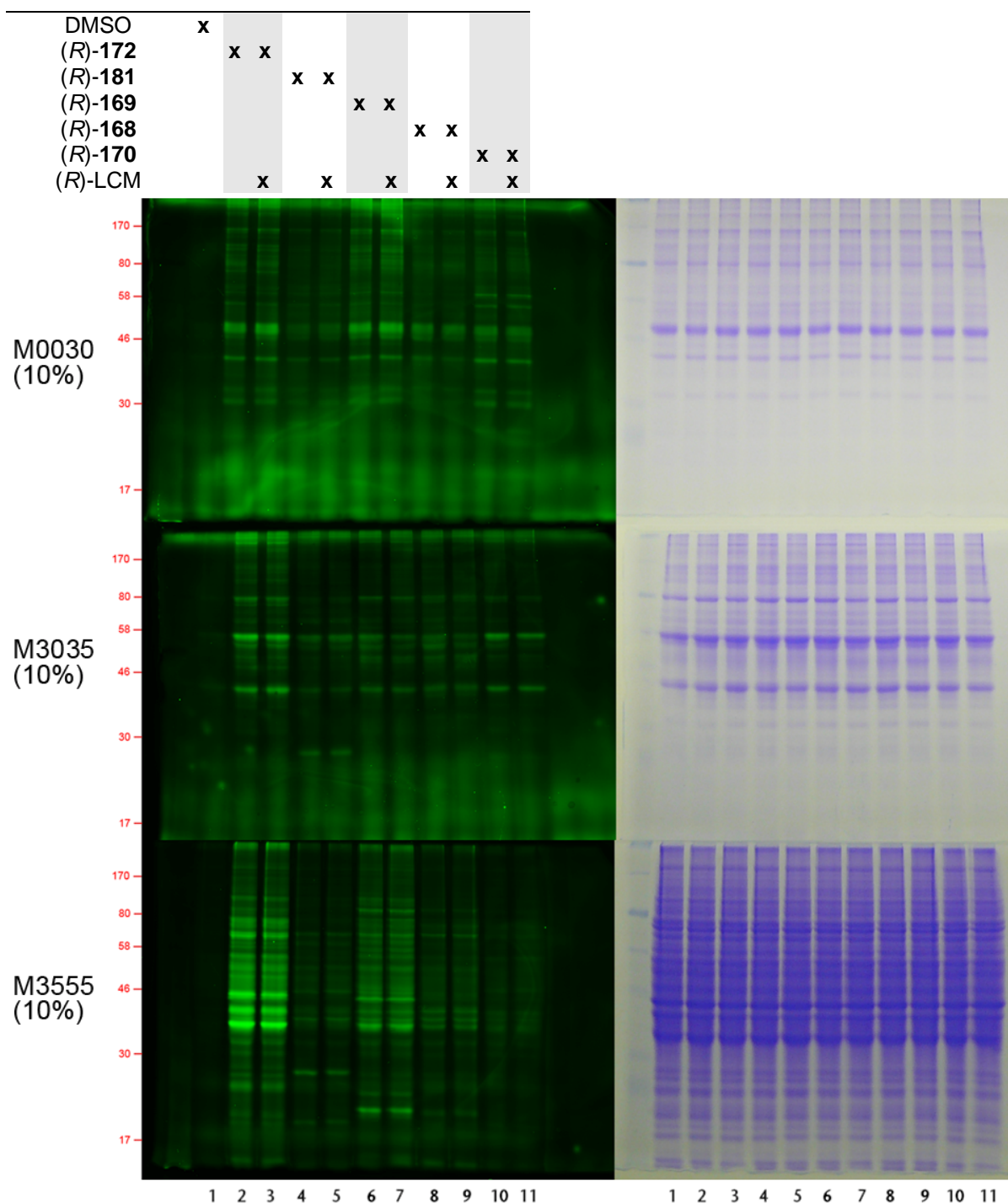
Figure 24. Left: in-gel fluorescence of different AMS cut from the cytoplasmic lysate after labeling with various AB&CR agents. Right: Corresponding Coomassie stains. Top to bottom: **M0030** (10% SDS-PAGE gel); **M3035** (10%); **M3555** (10%). Lane 1: DMSO; lane 2: (*R*)-**172** (1 µM); lane 3: (*R*)-**172** (1 µM) + (*R*)-LCM (1 mM); lane 4: (*R*)-**181** (1 µM); lane 5: (*R*)-**181** (1 µM) + (*R*)-LCM (1 mM); lane 6: (*R*)-**169** (1 µM); lane 7: (*R*)-**169** (1 µM) + (*R*)-LCM (1 mM); lane 8: (*R*)-**168** (1 µM); lane 9: (*R*)-**168** (1 µM) + (*R*)-LCM (1 mM); lane 10: (*R*)-**170** (1 µM); lane 11: (*R*)-**170** (1 µM) + (*R*)-LCM (1 mM). Approximate molecular weight marker (kDa) are shown in red on the left.
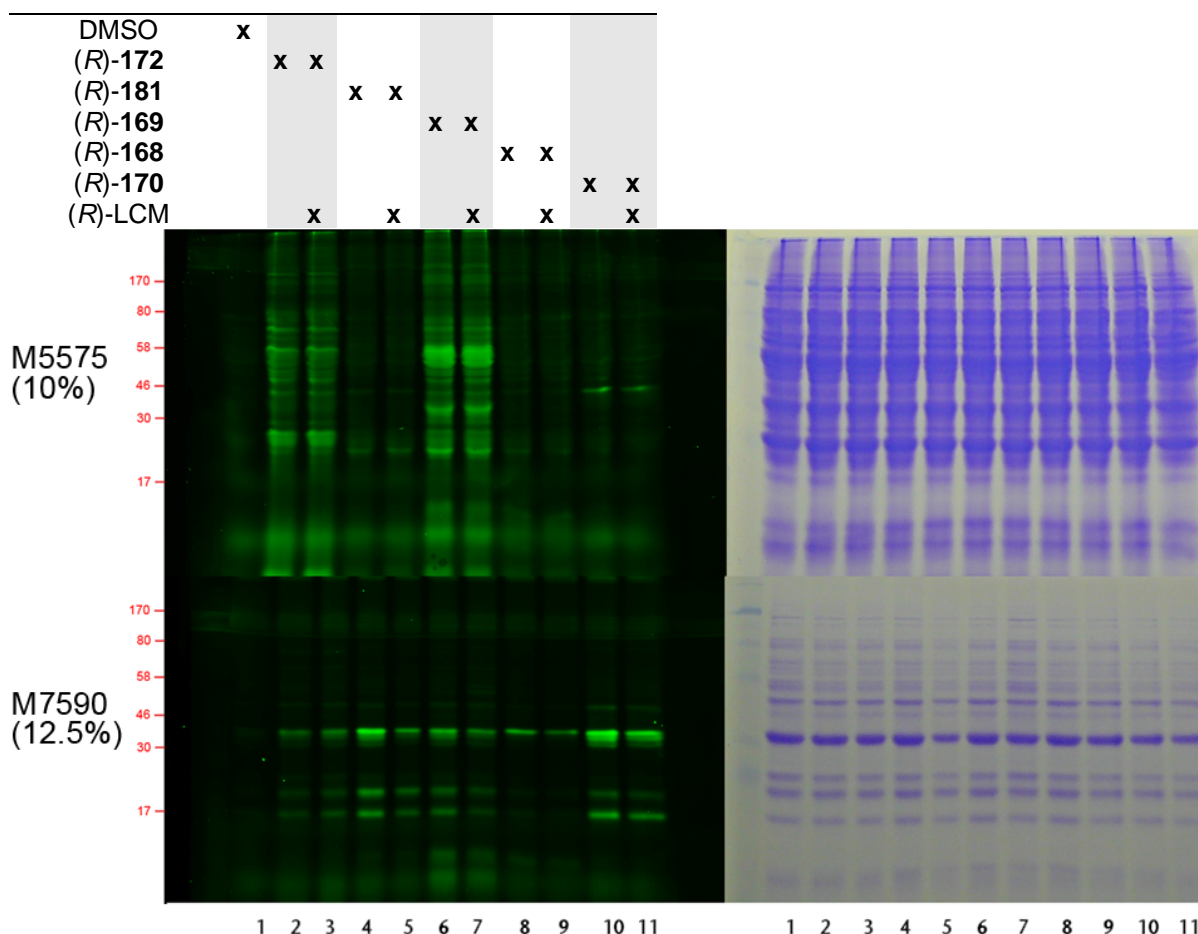
Figure 25. Left: in-gel fluorescence of different AMS cut from the cytoplasmic lysate after labeling with various AB&CR agents. Right: Corresponding Coomassie stains. Top to bottom: **M5570** (12.5%); **M7090** (12.5%). Lane 1: DMSO; lane 2: (*R*)-**172** (1 µM); lane 3: (*R*)-**172** (1 µM) + (*R*)-LCM (1 mM); lane 4: (*R*)-**181** (1 µM); lane 5: (*R*)-**181** (1 µM) + (*R*)-LCM (1 mM); lane 6: (*R*)-**169** (1 µM); lane 7: (*R*)-**169** (1 µM) + (*R*)-LCM (1 mM); lane 8: (*R*)-**168** (1 µM); lane 9: (*R*)-**168** (1 µM) + (*R*)-LCM (1 mM); lane 10: (*R*)-**170** (1 µM); lane 11: (*R*)-**170** (1 µM) + (*R*)-LCM (1 mM). Approximate molecular weight marker (kDa) are shown in red on the left.

4.3.1. A potential protein target for (*S*)-LCM

We did not identify a protein that was selectively competed with an excess of (*R*)-LCM using AB&CR agents (*R*)-**172**, (*R*)-**181**, (*R*)-**169**, (*R*)-**168**, and (*R*)-**170**. However, we found a ~25 kDa cytoplasmic protein that was selectively adducted by a (*S*)-AB&CR agent. The protein was first identified in two different AMS fractions (**M3045** and **M4555**) and solely labeled by (*S*)-**169** (Figure 26). Interestingly, [1]H

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NMR analysis of the (*S*)-**169** sample used showed that it was contaminated with an impurity. Nonetheless, despite the lowered concentration of (*S*)-**169** compared with (*R*)-**169** in this experiment, (*S*)-**169** gave a stronger fluorescence labeling while producing minimal non-specific protein labeling (Figure 26, blue arrow).
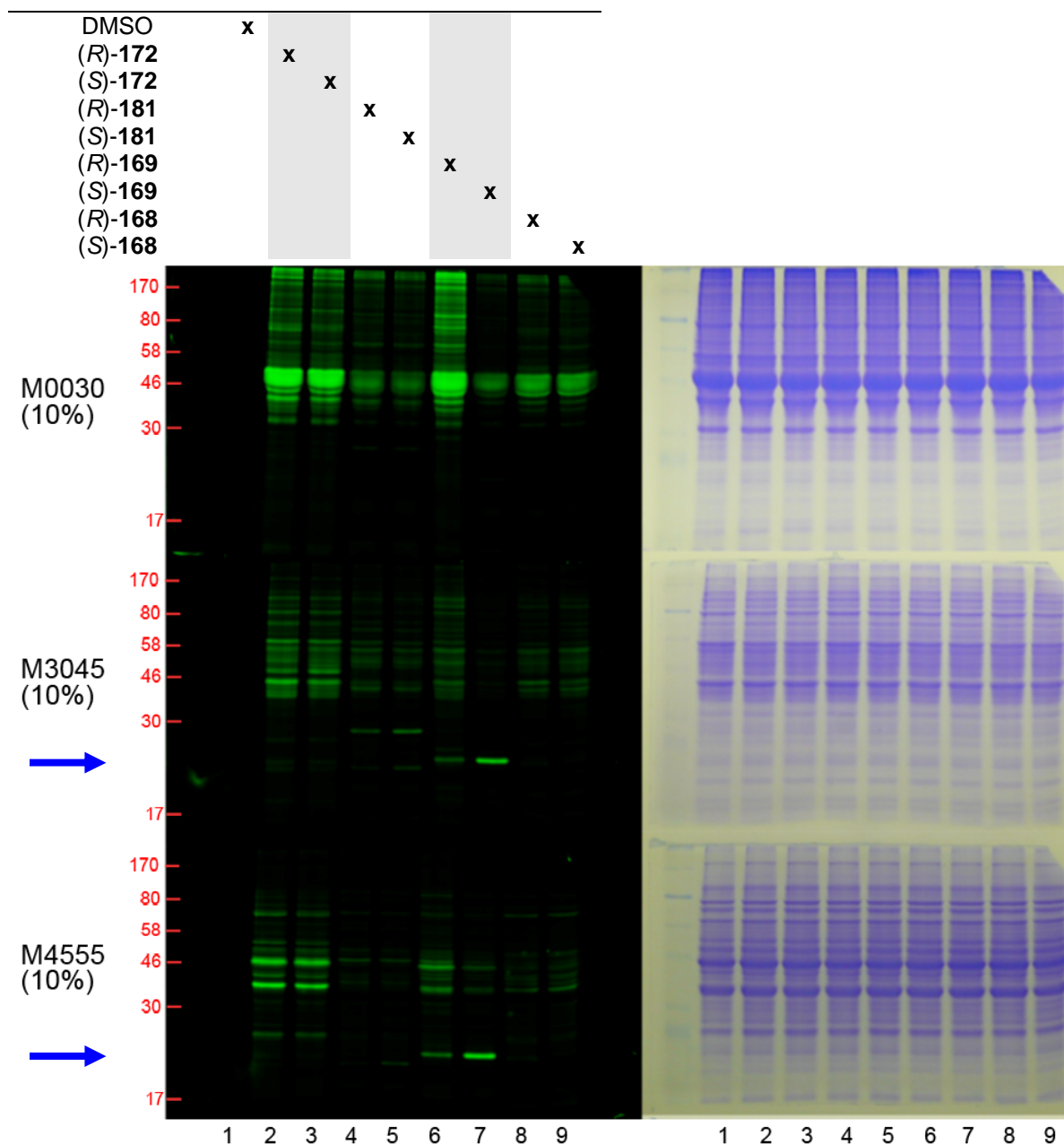
Figure 26. Left: in-gel fluorescence of different AMS cuts (**M0030**, **M3045**, **M4555**) from the cytoplasmic lysate after labeling with various AB&CR agents. Right: Corresponding Coomassie stains. The different AMS cuts corresponding to each gel are shown on the left, along with the percentage of SDS-PAGE gel between parentheses. Lane 1: DMSO; lane 2: (*R*)-**172** (5 *μ*M); lane 3:

(*S*)-**172** (5 *μ*M); lane 4: (*R*)-**181** (5 *μ*M); lane 5: (*S*)-**181** (5 *μ*M); lane 6: (*R*)-**169** (5 *μ*M); lane 7: (*S*)-**169** (5 *μ*M); lane 8: (*R*)-**168** (5 *μ*M); lane 9: (*S*)-**168** (5 *μ*M). An impurity was present in the (*S*)-**169** sample (lane 7), leading to a lower effective AB&CR concentration. Approximate molecular weight markers (kDa) are shown in red on the left.

Figure 27. Left: in-gel fluorescence of different AMS cuts (**M5565**, **M6590**) from the cytoplasmic lysate after labeling with various AB&CR agents. Right: Corresponding Coomassie stains. The different AMS cuts corresponding to each gel are shown on the left, along with the percentage of SDS-PAGE gel between parentheses. Lane 1: DMSO; lane 2: (*R*)-**172** (5 *μ*M); lane 3: (*S*)-**172** (5 *μ*M); lane 4: (*R*)-**181** (5 *μ*M); lane 5: (*S*)-**181** (5 *μ*M); lane 6: (*R*)-**169** (5 *μ*M); lane 7: (*S*)-**169** (5 *μ*M); lane 8: (*R*)-**168** (5 *μ*M); lane 9: (*S*)-**168** (5 *μ*M). An impurity was present in the (*S*)-**169** sample (lane 7), leading to a lower effective AB&CR concentration. Approximate molecular weight markers (kDa) are shown in red on the left.

We repeated the experiment with repurified (*S*)-**169**, and found that the protein of interest could be enriched in a refined AMS cut (**M3555**) and did not adsorb on S-Sepharose®, or Q-Sepharose® at pH 7.5. We saw a dose-dependent competition of the fluorescent signal with increasing concentrations of (*S*)-LCM

(100–1000 equiv). At 1000 equiv. of (*S*)-LCM, the (*S*)-**169** fluorescent signal

disappeared, while a 1000-fold excess of (*R*)-LCM had little to no effect (Figure 28).
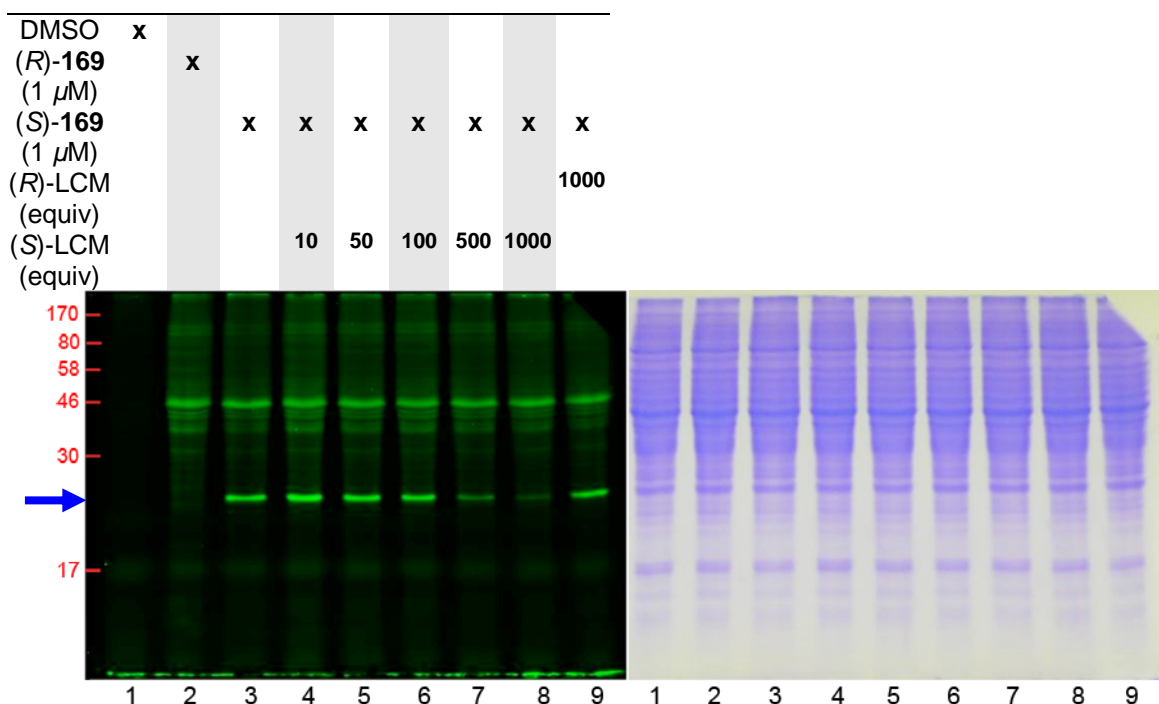
Figure 28. 12.5% SDS-PAGE gel of the rat cytoplasmic **M3555** cut. Left: in-gel fluorescence scan; right: corresponding Coomassie stain. Dose-dependant and enantioselective competition of the protein targeted by (*S*)-LCM. Rat cytoplasmic lysate was labeled with DMSO, (*R*)-**169**, and (*S*)-**169** in the presence or absence of (*R*)-LCM and (*S*)-LCM. Lane 1: DMSO; lane 2: (*R*)-**169** (1 *μ*M); lane 3: (*S*)-**169** (1 *μ*M); lane 4: (*S*)-**169** (1 *μ*M) + 10 μM (*S*)-LCM; lane 5: (*S*)-**169** (1 *μ*M) + (*S*)-LCM (50 *μ*M); lane 6: (*S*)-**169** (1 *μ*M) + (*S*)-LCM (100 *μ*M); lane 7: (*S*)-**169** (1 *μ*M) + (*S*)-LCM (500 *μ*M); lane 8: (*S*)-**169** (1 *μ*M) + (*S*)-LCM (1 mM); lane 9:(*S*)-**169** (1 *μ*M) + (*R*)-LCM (1 mM). Approximate molecular weight markers (kDa) are shown in red on the left.

Next, we tried to optimize conditions for biotin purification of the ~25 kDa

protein by conducting a dose-dependent labeling experiment. However, we

observed that while the extent of protein non-specific labeling increased with

increasing (*S*)-**169** concentrations, the intensity of the ~25 kDa fluorescent signal

rapidly reached a plateau between 5–10 *μ*M (Figure 29). This finding suggested that

the protein's extent of modification was maximal around 5 *μ*M of (*S*)-**169**, and led us

to hypothesize that, given the level of fluorescence intensity observed on the gels,

257

the ~25 kDa protein was present in low abundance in the lysate. In summary, a 1 μM concentration of (S)-**169** gave minimal non-specific protein labeling while providing a strong specific signal for the protein of interest.
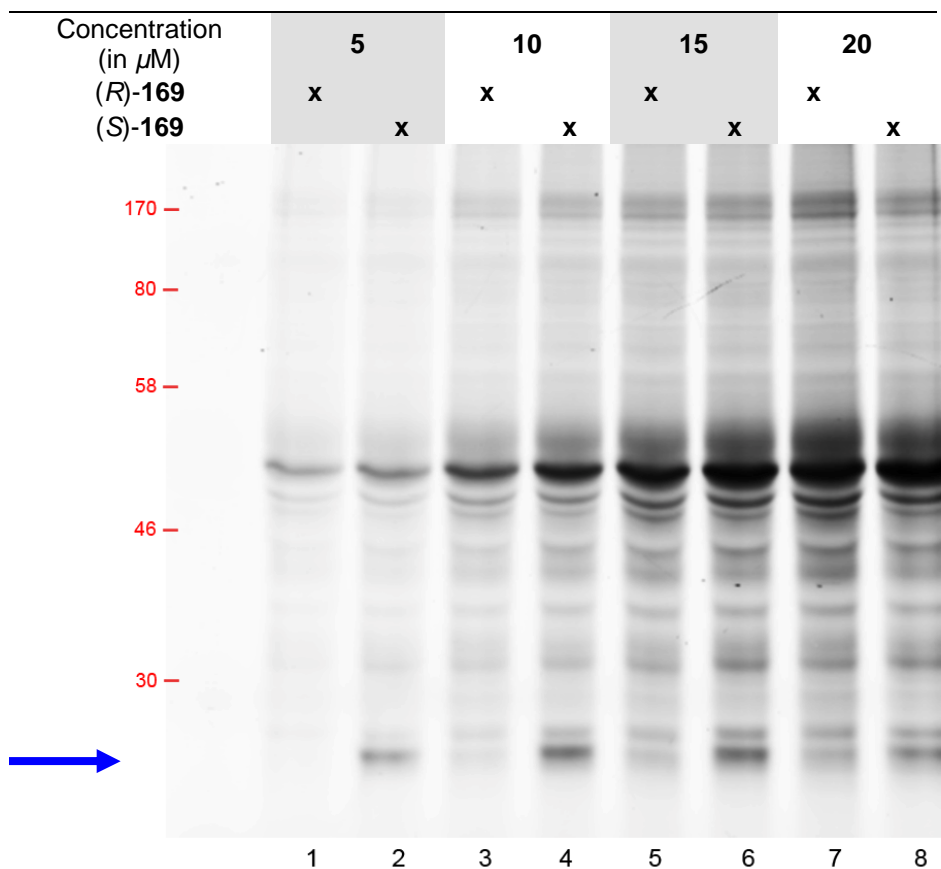
Figure 29. Fluorescence scan of the cytoplasmic AMS cut **M3555** after labeling with (R)-**169** or (S)-**169** at various concentrations (10% SDS-PAGE gel) and click chemistry with **196**. Lane 1: (R)-**169** (5 μM); lane 2: μM (S)-**169** (5 μM); lane 3: (R)-**169** (10 μM); lane 4: (S)-**169** (10 μM); lane 5: (R)-**169** (15 μM); lane 6: (S)-**169** (15 μM); lane 7: (R)-**169** (20 μM); lane 8: (S)-**169** (20 μM). The Coomassie stain (picture not shown) indicated equal amounts of protein loaded per well. Approximate molecular weight markers (kDa) are shown in red on the left.

4.3.2. Enrichment procedure for the ~25 kDa protein

Recognizing the low level of expression of the ~25 kDa protein, we implemented our protocol using two enrichment steps. First, we increased the molecular weight cut-off (MWCO) of the dialysis membrane used to dialyze the S3

supernatant in the lysate preparation. Using a MWCO of ~14 kDa instead of ~5–6 kDa led to the recovery of ~35 mg of cytoplasmic protein per rat brain instead of ~40 mg. Second, we included a pH-dependent protein precipitation step to further enrich the (*S*)-LCM target. Initially, we briefly (15 sec) lowered the pH of the lysate from 7.4 to either 5.5 (40 mM MES buffer), 5.0, or 4.5 (40 mM acetate buffer) <u>after</u> the photolabeling step. Denatured proteins were pelleted by centrifugation (14,000 rpm, 3 min), and the supernatant was readjusted to pH 7.4 by adding HEPES (pH 7.4) to 80 mM. AMS fractionation was subsequently performed, the **M3555** cut was clicked with Probe **196** and the gels were resolved and scanned for fluorescence. Gratifyingly, the fluorescence intensity levels corresponding to our protein of interest were not affected by the lysate acidification step (pH 4.5), while the Coomassie stain showed a strong reduction in the total amount of protein on the gel (data not shown). We further refined these conditions by precipitating proteins under even more acidic conditions. Using the same protocol, we precipitated proteins at pH 4.0, 3.5 (40 mM formate buffer) and pH 3.0 (40 mM citrate buffer). After AMS fractionation (pH 7.4) and click chemistry, we found that the protein was still unaffected after treatment at pH 4.0 and 3.5, while the Coomassie stain showed a more pronounced reduction in the total amount of protein. At pH 3.0, however, the fluorescent signal corresponding to the ~25 kDa protein disappeared (Figure 30). These combined steps (dialysis, acid precipitation, and AMS fractionation) led to the removal of ~90% of total cytoplasmic proteins (~10-fold enrichment, based on protein concentration measurement using the Bradford assay). We then endeavored to identify the ~25 kDa protein by performing a large scale biotin purification

experiment. The cytoplasmic S3 fraction from 3 rat brains was dialyzed through a 14

kDa MWCO membrane, and split into three equal volumes (9 mL, ~35 mg total

protein per reaction).
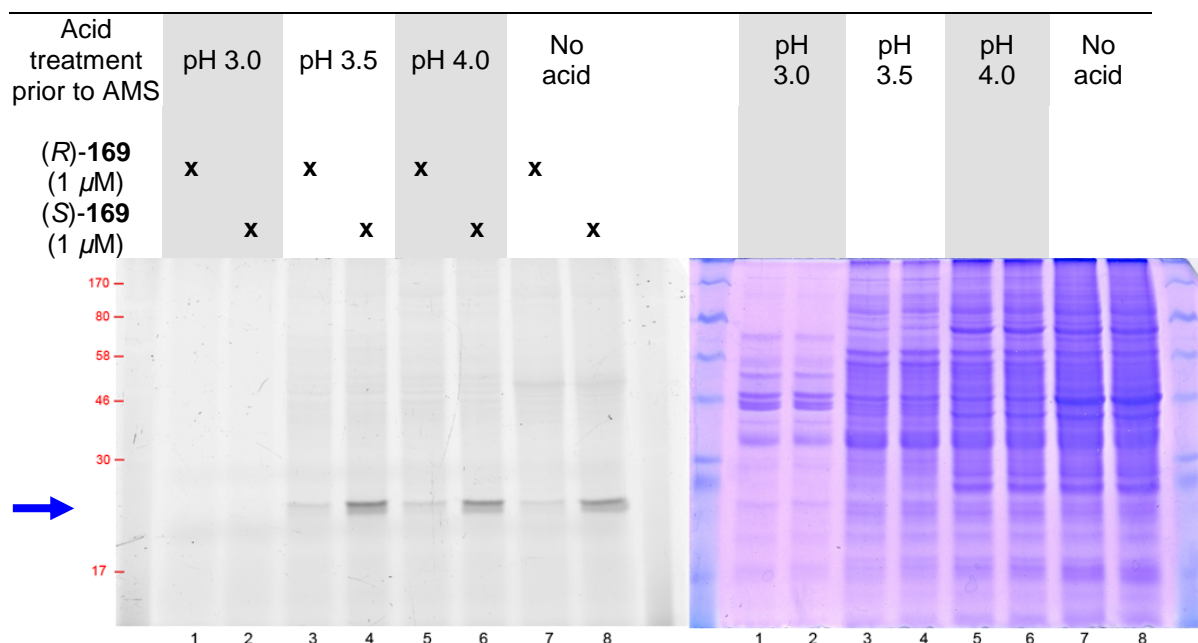
Figure 30. 10% SDS-PAGE gel of the rat brain cytoplasmic lysate **M3555** labeled with 1 μM (*R*)-**169** or (*S*)-**169**. Left picture: fluorescence scan; right picture: corresponding Coomassie stain. Lanes 1, 3, 5, 7: 1 μM (*R*)-**169**; lanes 2, 4, 6, 8: 1 μM (*S*)-**169**. Lanes 1,2: proteins were precipitated at pH 3.0 prior to AMS fractionation; lanes 3,4: at pH 3.5; lanes 5,6: at pH 4.0; lanes 7,8: no acid treatment was performed before AMS fractionation. Approximate molecular weight markers (kDa) are shown in red on the left.

Each reaction (DMSO control, (*R*)-**169** (1 μM), and (*S*)-**169** (1 μM)) was plated into a

6-well plate (1.5 mL per well) and irradiated at 4 °C (10 min at 365 nm, 1 min at 312

nm). The contents of each well was then recovered and brought to pH 3.5 by

addition of 1 M formate buffer (pH 3.5) to provide a 40 mM formate solution. The

reactions were mixed (10 sec) and centrifuged (5,500 g, 5 min). The supernatant

was transferred to a new tube (50 mL), the pH adjusted to pH 7.4 by addition of 1 M

HEPES buffer (pH 7.4) up to 80 mM, and the solution was brought to 35% AMS

saturation by addition of liquid saturated AMS (54% of solution volume). After mixing (5 min), the suspension was centrifuged (5,500 g, 5 min), the supernatant was transferred to a new tube and brought to 55% AMS with saturated aqueous AMS (64% of initial solution volume). After mixing (5 min), and centrifugation (5,500 g, 5 min) the supernatant was discarded, and the residual liquid on the side of the tube carefully wiped without touching the pellet. For each reaction, the pellet was resuspended in 3 mL of 50 mM NaCl, 25 mM HEPES (pH 7.4) and click chemistry was performed with 20 μM of biotin Probe **196** (1 h). Each reaction was supplemented to 0.1% SDS, dialyzed overnight against 50 mM NaCl, 25 mM HEPES buffer (pH 7.4), and tumbled with streptavidin beads for 30 min. Beads were then sequentially washed with 50 mM NaCl, 25 mM HEPES (pH 7.4) supplemented to 0.2% SDS (120 CV), 8 M urea (120 CV), and 50 mM NaCl, 25 mM HEPES (pH 7.4) (120 CV). The streptavidin beads were then boiled (95 °C, 15 min) in the presence of 2X SDS loading buffer, resolved on 10% SDS PAGE gel and silver stained. Traces of proteins were present in the DMSO control, while the lanes corresponding to (*R*)-**169** and (*S*)-**169** contained identical, low levels of protein despite using a extended silver stain developing time (>5 min, no picture available). The low recovery of the ~25 kDa band and our finding that the (*S*)-**169** reaction did not provide measurably enhanced levels of adduction compared with (*R*)-**169** did not warrant our MS analysis of this band, and we abandoned our efforts to identify this (*S*)-LCM interacting protein.

4.4. Discussion

Epoxide-containing AB&CR agent (*R*)-**180** and (*S*)-**180** proved highly selective at labeling CKB in a complex lysate. In addition we observed that the enzyme was preferentially labeled by (*R*)-**180** over (*S*)-**180**, and that the aromatic epoxide-containing AB&CR agent (*R*)-**176** did not adduct CKB. Mass spectrometric experiments strongly suggested that the principal CKB amino acid residue modified by (*R*)-**180** was Cys283, a catalytic residue crucial for enzymatic activity. However, no direct interaction between (*R*)-LCM and CKB was observed using fluorescence competition experiments, enzymatic inhibition assay, or ITC experiments. We have tentatively attributed the lack of correlation between the CKB selective, enantiospecific labeling by (*R*)-**180** and its binding with (*R*)-LCM to several reasons. First, epoxide electrophiles are prone to react with cysteine residues.[274,308,309]

180

Epoxycreatine 202

176
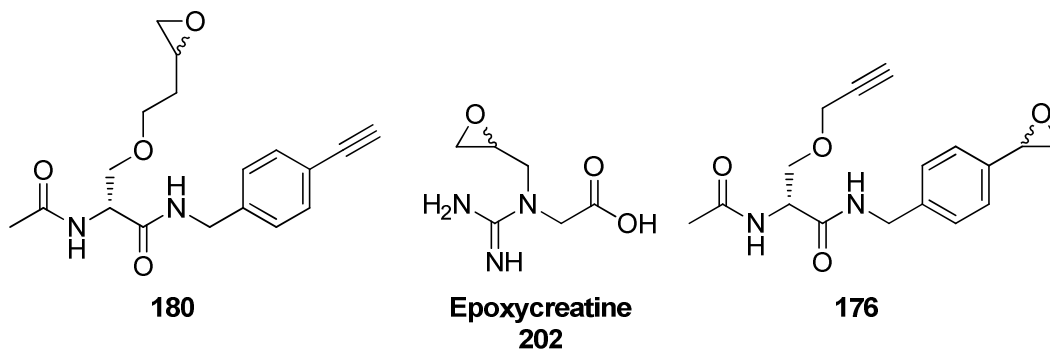
In this regard, the selective modification of CKB by aliphatic epoxide (*R*)-**180** compared with aromatic epoxide (*R*)-**176** may be rationalized by the report that epoxycreatine **202**, is a known rabbit muscle-type creatine kinase (CKM) epoxide inhibitor.[467] CKM shares an 80% identity with CKB and possesses the conserved catalytic site in humans, rats and rabbits that is seen in CKB. Epoxide **202** is known to modifiy Cys 282 of rabbit CKM, the CKB Cys283 homolog,[467] and (*R*)-**180** and

202 have structural features in common. They both possess an aliphatic epoxide moiety in proximity of an amino acid backbone. Among several other possibilities, one can hypothesize that this combination of pharmacophores in both (*R*)-**180** and epoxycreatine, but unmet in (*R*)-**176**, is required for the cysteine residue nucleophilic attack. Thus, only a small fragment of the lacosamide framework in (*R*)-**180** may be sufficient for binding and covalent modification of CKB, while the lack of an epoxide moiety in (*R*)-LCM results in a loss of interaction with CKB (Figure 22), and the absence of (*R*)-LCM competition for (*R*)-**180** modification of CKB in the in-gel fluorescence experiments (Figure 15). We have tentatively attributed compound **180**'s (*R*)- vs (*S*)- labeling selectivity to a greater binding affinity with the CKB **180** binding pocket.

Consistent with our hypothesis, epoxide-based AB&CR agents **175**, **179**, **176** and **180** all displayed high labeling selectivity that mirrored their expected low reactivity profiles. However, the extended reaction time (20 h) needed for epoxide adduction raises concerns that protein denaturation may have occurred during this reaction time. While a robust enzyme such as CKB remains functional over a long time, other proteins of interest in the lysate may denature quickly. This concern suggests that an ideal balance should be reached where the AB agent is sufficiently reactive over a moderate time period, but not too reactive leading to nonspecific adduction, to maximize chances of capturing potential targets.

We also screened the rat brain cytoplasmic fraction using the suite of photoAB&CR agents **181**, **169**, **168** and **170** and compared them with the NCS-containing agent **172**. We observed that the 4 photoAB moieties and the NCS AB all

displayed very different reactivity profiles at 1 μM (Figure 24, Figure 25) at 4 °C. The isothiocyanate group (15 min incubation, pH 7.4) and the trifluoromethylaromatic diazirine (10 min at 365 nm, 1 min at 312 nm) gave fluorescence signals with comparable intensities, yet with different labeling patterns. Aromatic azide (R)-**181** (10 min at 365 nm), methyldiazirine (R)-**168** (30 min at 365 nm, 1 min at 312 nm), and benzophenone (R)-**170** (1 h at 365 nm) all displayed lower adduction levels compared with (R)-**172** and (R)-**169**, and yet gave different protein labeling profiles. Unlike the three other photoAB&CR agents, we found that the protein labeling efficiency of the aromatic azide photoAB was highly temperature-dependent. At a 1 μM concentration, photoAB (R)-**181** went from a low fluorescence intensity (4 °C) comparable to that of benzophenone (R)-**170**, to a strong fluorescent signal (room temperature) that was greater than isothiocyanate (R)-**172** and aromatic diazirine (R)-**169** under the same conditions (data not shown). These findings emphasize the need to use the largest possible variety of affinity labels to maximize the chances of identifying a drug target in a complex protein environment. Despite the number of affinity labels (7) used in this proteomic search, we were not able to identify a selectively labeled protein whose fluorescent signal was competed by an excess amount of (R)-LCM.

Interestingly, a parallel study conducted in the laboratory using a mouse brain cytoplasmic lysate (*work of Dr Ki Duk Park*) showed that isothiocyanate-containing AB&CR **172** labeled CRMP2,[384] a ~62 kDa putative binding partner of (R)-LCM.[233] The extent of protein labeling was ~2-fold higher with (R)-**172** when compared with (S)-**172**.[384] Nonetheless, using our screening conditions, we did not observe any ~62

kDa protein that was preferentially labeled by (*R*)-**172** over (*S*)-**172** (Figure 24, Figure 25). Many possibilities may account for this difference. The animal used in the protocol should not, in principle, greatly influence the results given the fact that (*R*)-LCM is potent in both the mouse and the rat against MES-induced seizures. Perhaps more important, the amino acid sequences of the mouse and rat CRMP2 proteins are virtually identical. However, the two lysate preparations greatly differ and are likely the source of disparities observed. Significantly, the mouse brain cytoplasm was obtained by homogenizing the rat brain in plain HEPES buffer (50 mM, pH 7.4) that was not supplemented in sucrose, or protease inhibitors, and was used directly to conduct labeling experiments after centrifugation (100,000 g, 1 h). In contrast, the rat brain homogenate was prepared using literature established protocols,[424-427] using a buffer system (HEPES, 25 mM, pH 7.4) supplemented to 320 mM sucrose and 4 different protease inhibitors (see Section 3.1). Additionally, the rat cytosolic lysate obtained after centrifugation was extensively dialyzed (2 buffer changes) against 100 volumes HEPES buffer (25 mM, pH 7.4, supplemented to 50 mM NaCl). These three major differences may result in very different protein environments and therefore different screening conditions. Importantly, the absence of sucrose may result in some intracellular organelle leakage during homogenization, thus increasing the complexity of the lysate, and the lack of protease inhibitors may lead to some extent of protease degradation. In addition, the absence of a dialysis step will lead to the presence in the lysate of a variety of secondary messenger molecules[468-471] that might influence protein conformation.

We did, however, identify a soluble protein modified exclusively by (*S*)-**169**. This ~25 kDa soluble protein precipitated in the **M3555** AMS cut (pH 7.4) (Figure 28), and did not adsorb on S-Sepharose® (pH 4.5 or pH 7.5) or Q-Sepharose® (pH 7.5) (data not shown). Fluorescence labeling experiments showed a highly selective labeling by (*S*)-**169** over (*R*)-**169**, and the fluorescent signal of (*S*)-**169** disappeared in a dose-dependent manner with excess (*S*)-LCM, but not (*R*)-LCM (Figure 28). Use of a strongly acidic workup (~pH 3.5) did not cause the protein of interest to precipitate while ~50% of all the proteins present in the cytoplasmic lysate were denatured and precipitated. Dose-dependent labeling experiments with (*S*)-**169** showed that the relatively weak fluorescent signal of the ~25 kDa protein rapidly (5–10 μM) reached a plateau at 5–10 μM concentrations, while further increasing the photoAB&CR concentration (up to 20 μM) only resulted in a pronounced labeling of abundant proteins. This finding suggested that the protein was present in low abundance in the cytoplasmic protein lysate. Thus, we used a combination of protein dialysis (MWCO ~14 kDa), brief acidic treatment (pH 3.5), and AMS fractionation which resulted in a ~10-fold enrichment of the protein. The lysate was treated with DMSO, 1 μM (*R*)-**169**, and 1 μM (*S*)-**169**, irradiated, and each reaction was fractionated with AMS as described in the Experimental Section (see Sections 4.6.2 and 4.6.3). After click chemistry with biotin Probe **191**, dialysis, and streptavidin capture, the streptavidin-bound proteins were stringently washed, and eluted using SDS loading buffer. Traces of proteins were found in the DMSO control, while higher, yet minute amounts of proteins were present in samples treated with (*R*)-**169** and (*S*)-**169**. Both agents gave virtually identical protein profiles and did not mirror

the fluorescence scan previously observed (Figure 30). Even after *prolonged* developing, the silver stain did not show any ~25 kDa protein present in higher abundance in the (*S*)-**169** reaction compared with the (*R*)-**169** reaction. Thus, despite the use of acid and AMS fractionations and large amounts of protein per reaction (S3 fraction from one rat brain, ~35 mg per reaction, 3 rat brains), we were not able to isolate a protein band of interest after biotin purification. We concluded that the level of expression of this protein targeted (*S*)-LCM was too low for us to identify using the employed methodolgy.

4.5. Conclusions

Screening the rat brain cytoplasmic fraction with a large panel of AB&CR agents yielded a potential, low abundance protein targeted by (*S*)-LCM, but no protein selectively modified by our (*R*)-LCM AB&CR derivatives. While many factors may have been responsible for our lack of target identification in the cytoplasmic lysate, one likely explanation is the subcellular localization of the drug target. Indeed, membrane-bound proteins such as VGICs, LGICs, neurotransmitter transporters are key targets for many AEDs. Therefore, we advanced to the next step by interrogating the membrane-bound proteome.

4.6. Experimental Section

4.6.1. Preparation of the rat brain cytoplasmic lysate

Frozen male Sprague Dawley stripped rat brains (6–8 weeks old, Pel-Freez Biologicals, cat # 56999, or Rockland Immunochemicals, cat # RT-T081, 1.5–1.8 g per whole brain) were thawed on ice at 4 ℃ until sof t, finely minced with razor blades and homogenized (10 mL buffer per gram of wet tissue) using a glass/Teflon Dounce homogenizer in 320 mM sucrose 25 mM HEPES buffer (pH 7.4) (supplemented with 1 mM PMSF, 10 μM E-64, 10 μM Pepstatin A, and 1 μM TPEN) by 10–15 up-and-down strokes by hand, taking 20 sec to complete one stroke. The homogenate was centrifuged at 4 ℃ (100,000 g, 50 min), the supernatant was dialyzed twice (1 h) against 3 L of 25 mM HEPES (pH 7.4), 50 mM NaCl using a Spectra/Por$^{®}$ dialysis membrane (MWCO 3,500; Spectrum Laboratories Inc., cat # 132720) and stored at -80 ℃ until use for up to 2 mo nths.

4.6.2. Cytoplasmic protein labeling with AB&CR agents

The following AB&CR agents were incubated as follows prior to fractionation (AMS and/or ion exchange chromatography). Epoxide AB&CR agents **175**, **179**, **176** and **180** (1–10 μM, 2% (v/v) DMSO final concentration) were incubated with the cytoplasmic lysate (pH 7.4) at room temperature (20 h). The lysate then underwent fractionation and click chemistry. PhotoAB&CR molecules were irradiated as follows: **181**, 10 min at 365 nm; **169**, 10 min at 365 nm then 1 min at 312 nm; **168**, 30 min at 365 nm, then 1 min at 312 nm; **170**, 60 min at 365 nm. On small scale (100 μL

reactions), photolabeling was carried out at 4 ℃ in cle ar 96-well plates (BD Falcon®). Larger scale experiments were conducted using 6-well plates (1.0–1.5 mL per well). Two types of UV lamps were used to irradiate samples (365 nm; 312 nm: Spectroline, Longlife™ Filter), and were positioned 1 cm above the well. A maximum of 2 rows (2 x 12 wells) were irradiated at the same time when using 96-well plates and 1 row (1 x 3 wells) when performing large scale experiments.

Competition experiments were conducted by pre-incubating the lysate with an excess amount of (*R*)- or (*S*)-LCM for 5 min (500–1500 equiv) at room temperature prior to starting the labeling experiment.

4.6.3. General procedure for lysate fractionation

The rat brain lysate (100–200 μL reactions, 2–3 mg.mL^{-1}) was incubated with the desired AB&CR agents and AMS fractionation and ion exchange chromatography were performed as described in Section 3.2. Typical AMS cuts recovered were **M0030**, **M3045**, **M4555**, **M5565**, and **M6590**. The first 4 AMS cuts (0 to 65% saturation) were obtained by liquid addition of a saturated AMS solution at room temperature (65% ~2 initial volumes of sat. AMS added). After mixing, the solution was let to stand for 5 min and centrifuged (14,000 rpm, rt, 3 min). The last cut was obtained by addition of solid AMS. AMS pellets were redissolved in 25 mM HEPES (pH 7.4) and click chemistry was performed.

For ion exchange chromatography, the resin (S-Sepharose or Q-Sepharose, ~10 μL resin for 100 μg of total protein) was pre-equilibrated with the desired buffer, typically HEPES buffer supplemented with 50 mM NaCl, and the lysate was added

to the resin. After a short incubation (30 sec), the flow-through was recovered, and the resin rinsed with 10 CV of equilibration buffer. At pH 7.4, typical elution fractions recovered on both S-Sepharose® and Q-Sepharose® were 50–200 mM NaCl, 200–250 mM NaCl, 250–300 mM NaCl and 300–350 mM NaCl and click chemistry was performed on the eluates.

4.6.4. Click chemistry

Click chemistry was performed as described in Section 3.4. Fluorescent Probes **195** or **196**, and biotin Probes **193** or **191** were added to the fractionated lysate samples (20 μM), typically in PCR 8-tube strips (volume 250 μL). 10X Cu(I) mix was freshly prepared by sequentially adding (for 100 μL) 10 μL of 25 mM CuSO$_4$ (aqueous), 40 μL of 625 μM TBTA (in 1:4 DMSO:t-BuOH), and 50 μL of 5 mM TCEP HCl (aqueous), and added to each sample at a 1/10 dilution. Samples were gently mixed and let stand at room temperature (1 h). SDS-loading buffer was added to samples, and then the samples were heated at 75 °C (5 min) and resolved on SDS PAGE gels (6–12.5%).

4.6.5. ITC experiments

ITC experiments were conducted at 25 °C (Microcal^{TM}, VP-ITC microcalorimeter). The CKB enzyme (100–200 μM, ~2.5 mL) was dialyzed against the desired buffer (1.5 L) for at least 3 h, and a solution of (*R*)-LCM in the dialysis

buffer (2–5 mM) was prepared. In a typical ITC experiment, the enzyme was titrated with (*R*)-LCM, using the following conditions.

Experimental parameters: total number of injections: 30; cell temperature: 25 °C; reference power: 10 μCal.s^{-1}; initial delay: 60 s; stirring speed: 490 rpm.

Injection parameters: initial injection volume: 2 μL, duration: 4 s, spacing: 120 s, filter period: 2 s; injections: 10 μL, duration 20 s, spacing: 180 s.

4.6.6. Creatine Kinase B enzymatic assay

The components of the coupled enzymatic assay were as follows.

(A) CKB (GenWay Biotech cat # 10-663-45059): Commercial human CKB, tag-free. The overexpressed and purified human CKB was prepared according to literature procedures and contains a C-terminus His-tag (see Sections 4.6.7 and 4.6.8).[457]

(B) Hexokinase (HK) (Sigma cat # H4502): Catalyzes the transfer of a phosphate group from ATP to D-glucose to form glucose-6-phosphate (G6P)

(C) Glucose 6-Phosphate Dehydrogenase (G6PDH) from *leuconostoc mesenteroides* (Sigma cat # G5885): Catalyzes the oxidation of G6P to 6-phosphogluconolactone using NADP as a cofactor and generating NADPH. The absorbance of the solution is measured at 340 nm.

(D) Phosphocreatine (Sigma cat # P7936), ADP (Sigma cat # 01897), Mg(OAc)$_2$ (Sigma cat # M5661), D-glucose (Sigma cat # G7528), NADP (Sigma cat # N5755), DTT and MES. For assays where a complex lysate is used as a source of

creatine kinase, it may be necessary to include 10 mM AMP to stop myokinase activity.[446] AMP is not utilized in this assay.

The assay was conducted in 96-well plates using 150/200 μL total volume for each reaction either at room temperature (Plate reader: HTS 7000 Plus Bio Assay Reader, Perkin Elmer) or at 37 ℃ (Plate reader: POLARstar OPTIMA, BMG Labtech). HK was prepared as a 100 X stock in 100 mM MES buffer (pH 6.5) and stored at -20 ℃. HK can sustain many freeze/thaw cycles without losing activity. G6PDH was prepared as a 1000 X stock in 50 mM phosphate buffer (pH 7.2), 200 mM NaCl, and 1 mM EDTA, and stored at 4 ℃. Phospho creatine (PCr), ADP, D-glucose, Mg(OAc)$_2$, and DTT were prepared as 50 X stock solutions in 100 mM MES (pH 6.5) separately, and stored at -20 ℃. To prepar e a 10 X assay buffer mix, one volume of each of the 50 X stock solutions were mixed. When preparing 50 X stocks (concentrated solutions in MES buffer), the specific volumes of the solids were taken into account if the concentration of the stock solution was greater than 300 mM. NADP 10 X solutions were prepared prior to performing the assay and stored at -20 ℃. The NADP stock solutions did not undergo more than 2–3 freeze-thaw cycles. MES buffer (pH 6.5) was used at a 100 mM working concentration to dissolve all reagents and q.s. reactions to 150 or 200 μL.

For a typical CKB assay having a reaction volume of 200 μL reaction, CKB was used at 5–50 nM final concentration (MW = 43,466 Da, His-tagged CKB).

100 μL of 10 nM CKB (2 X)

4 μL of 500 mM DTT (50 X in 100 mM MES (pH 6.5))

4 μL of 1 M D-glucose (50 X in 100 mM MES (pH 6.5))

4 μL of 500 mM Mg(OAc)$_2$ (50 X in 100 mM MES (pH 6.5))

4 μL of 500 mM PCr (50 X in 100 mM MES (pH 6.5))

4 μL of 50 mM ADP (50 X in 100 mM MES (pH 6.5))

0.2 μL of 1000 U.mL^{-1} G6PDH (1000 X in 200 mM NaCl, 1 mM EDTA, 50 mM phosphate buffer (pH 7.2))

2 μL of 50 U.mL^{-1} HK (100 X in 100 mM MES (pH 6.5))

20 μL of 3 to 4 mM NADP.

53.8 μL of 100 mM MES (pH 6.5)

4.6.7. Cloning of CK-B

Cloning and overexpression of CKB was performed following literature procedures.[457,458] The CKB gene was first PCR-amplified from a human brain cDNA library (Liu lab) using the following conditions (Table 4) and established primers.[457,458]

5'-ATTGCC**CATATG**CCCTTCTCCAACAGC-3' (**Nde1 site**)

5'-ATACCG**CTCGAG**TCATTTCTGGGCAGG-3' (**Xho1 site**)

The following conditions were used for the PCR amplification. 1st denaturation step: 95 ℃ (5 min); 2nd denaturation step: 95 ℃ (20 sec); annealing step: 60 ℃ (15 sec); extension step: 72 ℃ (1 min); repeat from 2nd denaturation step: 30 cycles; final extension step: 72 ℃ (10 min).

Table 4. Components used for the PCR amplification of the CKB gene from a human cDNA library

Component	Volume	Final Concentration
10 X buffer for KOD DNA Polymerase	5 µL	1X
25 mM MgSO$_4$	3 µL	1.5 mM
dNTPs (2 mM each)	5 µL	0.2 mM (each)
DEPC-treated water	35.3 µL	
Sense (5') primer (50 µM)	0.4 µL	0.4 µM
Anti-Sense (3') primer (50 µM)	0.4 µL	0.4 µM
Template DNA	0.5 µL	
KOD DNA polymerase (2.5 U/µL)	0.4 µL	0.02 U/µL
Total reaction volume	**50 µL**	

The obtained CKB PCR product (23 ng.μL^{-1}) and pET28a vector were digested using Nde1 and Xho1 restriction enzymes at 37 °C (3.5 h). The enzymes were heat-inactivated at 65 °C (20 min) and the samples were resolved on a low melting point agarose gel (1%). Digested products were excised from the gel (visualization with UV irradiation) and extracted following QIAGEN Quick Spin DNA extraction protocol.

The digestion products were used for ligation using a T4 DNA ligase using either a DNA:vector ratio of 3:1 or a DNA:vector ratio of 1:1. Amounts were calculated using an online software (www.promega.com/biomath) based on concentrations of 9.0 ng.μL^{-1} of 1.1 kb DNA and 33 ng.μL^{-1} of 5.3 kb vector (Table 5).

Table 5. Components used for the ligation reaction between CKB and pET28a vector digested products

Conditions	3:1 DNA:vector	1:1 DNA:vector
Vector	5 µL	5 µL
CKB DNA	2.4 µL	0.8 µL
10 X buffer T4 ligase	1 µL	1 µL
DEPC H$_2$O	0.6 µL	2.2 µL
T4 DNA ligase	1 µL	1 µL

The reactions were incubated at 16 °C (18 h), ligati on reaction products (5 μL) were transformed into TOP10 competent cells, and colonies were grown overnight (37 °C) on LB agar plates supplemented with kanamycin (50 μg.mL^{-1}). Colony PCR was performed using T7 promoter (sequence: 5' TAA TAC GAC TCA CTA TAG GG 3') and terminator (5' TGC TAG TTA TTG CTC AGC GGT 3') primers to check for correct insertion of the gene of interest. Three colonies positive for insertion were grown on a 5-mL culture scale and the plasmid was purified using commercial plasmid purification kits (QIAGEN, cat # 27104). Each purified plasmid was transformed into the expression cell line Rosetta 21 (Novagen®, cat # 69450) (+ chloramphenicol, 34 μg.mL^{-1}), grown overnight on LB agar (+ kanamycin, 50 μg.mL^{-1}, + chloramphenicol, 34 μg.mL^{-1}) and a single colony of each was used to grow a 5-mL culture of bacteria. Each culture was supplemented to 25% glycerol and stored at -80 °C for future use. Sequencing (UNC-CH Genome Analysis Facility) showed one of the three CK-B plasmids contained a point mutation (K177R), while the other two plasmids were mutation-free. One of the mutation-free plasmids was used for protein overexpression.

4.6.8. Overexpression and purification of CK-B

A 1.5 L LB culture of CKB expressing *E. coli* was grown to OD$_{600}$ ~0.6 by shaking at 37 °C. The culture was then cooled to room te mperature, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and vigorously stirred with a magnetic stirring bar (1000 rpm) at room temperature (15 h). The cells were then harvested by centrifugation at 5000 rpm at 4 °C (10 m in), and the pellet

resuspended in lysis buffer (10 mL per gram of wet pellet, 300 mM NaCl, 50 mM HEPES (pH 7.5)) supplemented with 5% (w/v) glycerol, 1 mM PMSF, and 0.5% (w/v) lysozyme. After rotating at 4 ℃ (30 min), the cells we re disrupted by sonication on ice at 4 ℃ (7 x 1 min bursts, 30 sec pauses) and the suspen sion was centrifuged at 5000 rpm at 4 ℃ (15 min). The supernatant was adsorb ed on a cobalt-resin (TALON resin, BD Biosciences, cat # 635504) for His-tag purification. The resin was washed with the lysis buffer supplemented with 10 mM imidazole (10 CV) and eluted with lysis buffer supplemented with 250 mM imidazole (5 CV). The eluted fraction was purified by AMS fractionation and **M4070** was recovered. The AMS pellet was resuspended in 50 mM MES (pH 6.5), and 100 mM NaCl, dialyzed against the same buffer (1 h) and fractionated on Q-Sepharose® equilibrated with 100 mM NaCl 50 mM MES buffer (pH 6.5). The resin was washed with the equilibration buffer (10 CV) and a homogeneous solution of CK-B (~100 mg of protein) was obtained by eluting with 200 mM NaCl 50 mM MES buffer (pH 6.5). The enzyme was then precipitated with AMS (**M0070**) and stored (-20 ℃) either as an AMS pellet, or as a high-concentration stock in 100 mM MES buffer (pH 6.5) supplemented with 1 mM DTT and 20% (v/v) glycerol. Under these conditions, no detectable loss of activity was observed for up to 2 months.

CHAPTER 5

MEMBRANE-BOUND FRACTION SCREENING

We extensively screened the cytoplasmic fraction with different electrophilic and photoactivated AB&CR and did not identify a potential target protein displaying dose-dependent competition with (R)-LCM. Therefore, we turned to membrane-bound, subcellular fractions within the rat brain proteome to search for potential lacosamide targets. Membrane-associated proteins can be studied without solubilization as heterogeneous microsomes formed after tissue homogenization. Alternatively, they can be solubilized with a variety of commercially available detergents. Solubilized membranous proteins may then be enriched or purified by ion-exchange resin fractionation, provided that the equilibration and elution buffers are supplemented with the desired detergent.

5.1. Using non-denaturing detergents to solubilize membrane-bound proteins

Detergents, or surfactants, used in membrane protein biology are small molecules capable of forming micelles that serve as a water-soluble membrane-like environment for proteins. The specificity of these molecules lies in their amphiphilic character, and they are typically linear molecules with one hydrophilic head and one lipophilic tail. Other types of detergents exist, which are termed "facial amphiphiles"

and possess one polar and one non-polar surface area.[472,473] Surfactants come in several classes: cationic, anionic, neutral and zwitterionic. Ionic detergents (*i.e.*, with a net negative or positive charge) strongly interact with protein charged residues and can lead to a loss of protein structure. Anionic and cationic detergents are therefore considered denaturing. On the other hand, neutral and zwitterionic surfactants have been used as mild solubilizing agents to purify membrane-bound proteins while maintaining their activity.[429,474,475] Nonetheless, the use of a non-denaturing detergent does not guarantee the proper solubilization, folding, or stability of a protein.[476-478]
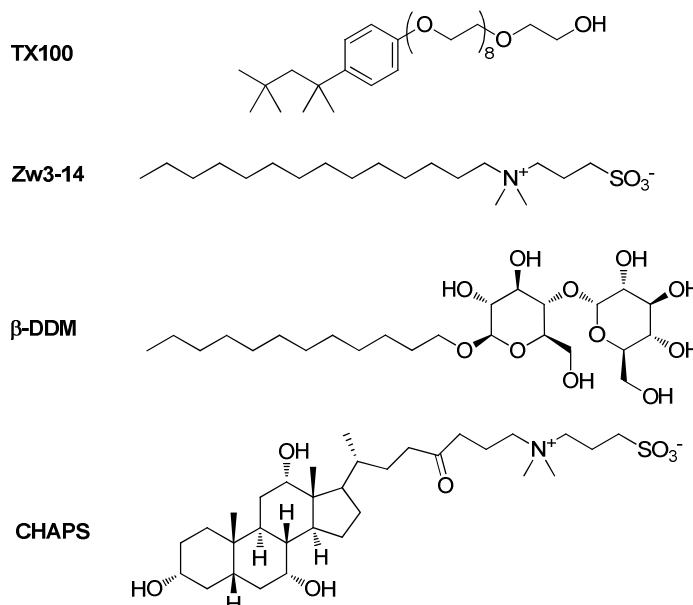
Figure 31. Structure of non-ionic and zwitterionic detergents used in our study of the membrane proteome.

One important physicochemical constant of a given detergent is the critical micellar concentration (CMC), which is the concentration at which it will start forming micelles. A given surfactant can, therefore, only solubilize membrane proteins at a

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concentration greater or equal to its CMC. For our studies, we used a small panel of structurally-diverse, non-denaturing detergents to solubilize a wide range of membrane-bound proteins. Triton X-100 (TX100), β-dodecylmaltoside (β-DDM), 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate (zwittergent 3-14, Zw3-14), and CHAPS (Figure 31) are commonly used surfactants for the solubilization and study of functional membrane proteins.[479-482]

5.1.1. Preparation of detergent-solubilized membrane extract from rat brain

In our initial approach, we recovered the heavy-membrane fraction (P2) and the light-membrane fraction (P3) after homogenization of the rat brain (Scheme 40). The organelles contained in the P2 fraction were hypo-osmotically lyzed, and the membrane fraction was recovered by centrifugation at 6,000 g (15 min) and the supernatant discarded. In other preparations, we directly centrifuged the S1 supernatant at 100,000 g (1 h) to obtain a combined P2+P3 pellet containing the whole membrane fraction of the brain. This combined pellet was also subjected to hypotonic lysis to discard the soluble content of intracellular organelles.

In a typical preparation, both membrane materials (P2+P3) was resuspended in isotonic buffer, such as 25 mM HEPES (pH 7.4) containing 150 mM NaCl, in separate tubes at 4 ℃ with a total protein concentrati on of ~5 mg.mL^{-1} (Bradford assay), and each suspension was then supplemented with a detergent at a specific concentration (*i.e.*, (w/v): 1% TX100, 0.5% β-DDM, 0.5% Zw3-14, 1% CHAPS). The suspensions were gently rocked for 15 min at 4 ℃ and cent rifuged at 100,000 g for 1 h. The pellet was discarded and the homogeneous supernatant containing

detergent-solubilized membrane proteins was used to perform labeling and click chemistry.

Membrane proteins fractionation was performed at pH 7.5 using sequential S and Q Sepharose® and by supplementing the equilibration and elution buffers with the following concentrations of surfactant: 1% for TX100, 0.5% for β-DDM, 0.1% for Zw3-14, and 1% for CHAPS. For solutions containing greater than 0.5% Zw3-14, the addition of SDS loading buffer to the high salt (>250 mM NaCl) eluted fraction led to a highly viscous solution that impeded proper electrophoretic resolution. Furthermore, the Zw3-14-solubilized protein solutions were not kept for an extended period of time at 0–4 ℃ since the surfactant is water- insoluble at this temperature. Failure to do so led to protein sample precipitation. Finally, the Zw3-14 samples could be frozen at -80 ℃ without any special procedure but were thawed quickly (*i.e.*, by immersion in a water bath at room temperature) to avoid loss of proteins. Following this procedure, we did not observe any significant precipitation in our samples.

5.1.2. Solubilized membrane fraction screening

Following the previously described protocol, we screened the rat, detergent-solubilized, membrane-bound proteome using both enantiomers of photoAB&CR agents **181**, **169**, **168**, **170** and where (*R*)-LCM and (*S*)-LCM served as competing agents. The irradiation conditions for the photolabeling step were identical to those described in Section 4.6.2. When Sepharose® fractionation was used after the labeling step, membrane fractions were resolved on SDS-PAGE gel (minigel size, ~8

cm high). Alternatively, when no fractionation was performed, samples were resolved using a larger (~20 cm high) SDS-PAGE gel system (Protean II XL cell, Bio Rad) to provide a better protein separation.
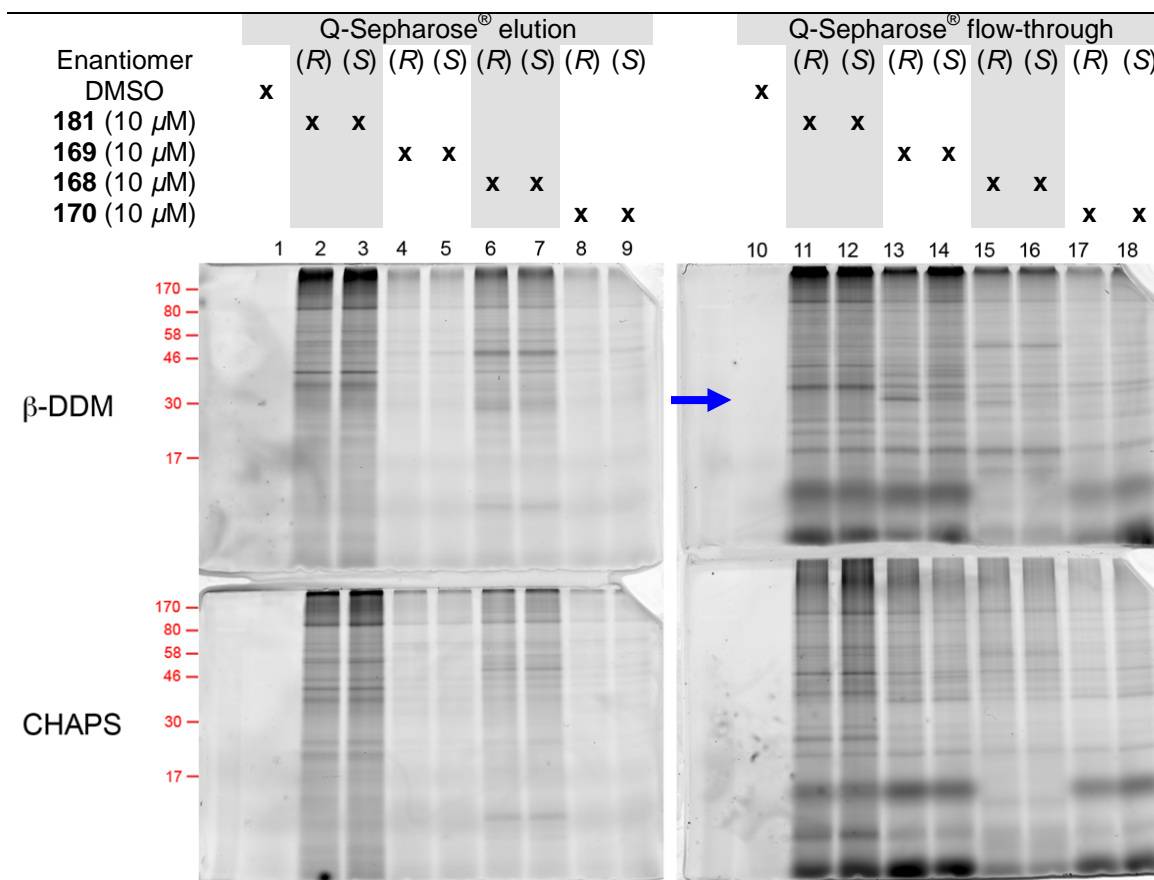
Figure 32. 12.5% SDS PAGE gel of the detergent-solubilized (β-DDM, or CHAPS) membrane fraction (P2+P3) labeled with photoAB&CR agents **181**, **169**, **168**, and **170**. The lysate (~200 μg total protein) was irradiated at 4 ℃ under the appropria te conditions and each reaction was fractionated using Q-Sepharose® (equilibrated with 25 mM HEPES (pH 7.4), 50 mM NaCl, supplemented with β-DDM or CHAPS). The flow-through (lanes 10–18) was recovered, the beads were rinsed with equilibration buffer, and eluted with 350 mM NaCl (lanes 1–9). Each fraction was then clicked with Probe **196**, resolved, and scanned for fluorescence. Lane 1, 10: DMSO control; lane 2, 11: (*R*)-**181** (10 μM); lane 3, 12: (*S*)-**181** (10 μM); lane 4, 13: (*R*)-**169** (10 μM); lane 5, 14: (*S*)-**169** (10 μM); lane 6, 15: (*R*)-**168** (10 μM); lane 7, 16: (*S*)-**168** (10 μM); lane 8, 17: (*R*)-**170** (10 μM); lane 9, 18: (*S*)-**170** (10 μM). The Coomassie stain (picture not shown) showed equal amounts of protein loaded in each gel. Approximate molecular weight markers (kDa) are shown in red on the left.

The reactivity profiles of the four photoAB&CR agents **181**, **169**, **168**, and **170** were varied, and were sometimes dissimilar within different Sepharose® fractions for a given photoAB&CR agent (Figure 32). The apparent order of reactivity of photoAB moieties was also different from the one observed in the cytoplasmic fraction screening.

Detergent used	Zw3-14				TX100				β-DDM				CHAPS			
(R)-**169** (10 μM)	x		x	x	x		x	x		x		x	x	x		x	x
(S)-**169** (10 μM)		x				x				x				x		
(R)-LCM (10 mM)		x				x				x				x	
(S)-LCM (10 mM)			x				x				x				x

Figure 33. 10% SDS-PAGE gel (minigel) of the membrane fraction (P2+P3) solubilized with a panel of detergents, flow-through of the S and Q Sepharose® (pH 7.4) fractionation steps. Top gels: fluorescence scan; bottom gels: corresponding silver stains. Black numbers: Zw3-14; green numbers:

TX100; blue numbers: β-DDM; red numbers: CHAPS. Solubilized proteins were labeled as follows. Lane 1: (R)-**169** (10 μM); lane 2: (S)-**169** (10 μM); lane 3: (R)-**169** (10 μM) + (R)-LCM (10 mM); lane 4: (R)-**169** (10 μM) + (S)-LCM (10 mM). After photolabeling (10 min 365 nm, 1 min 312 nm), each reaction was passed through pre-equilibrated S-Sepharose® (pH 7.4) and Q-Sepharose® (pH 7.4) supplemented with the appropriate detergent. The flow-through was then clicked with fluorescent Probe **203** and resolved. Approximate molecular weight markers (kDa) are shown in red on the left.

Using this approach, no potential lacosamide interacting proteins were identified that showed (R)- vs (S)-AB&CR specificity *and* displayed labeling dose-dependent competition with excess (R)-LCM. (Figure 33, Figure 34). Nonetheless, some bands were found to be specifically labeled by one photoAB group versus the other three. A few detergent-specific protein candidates were found to show near complete selectivity for the (R)-AB&CR versus the (S)-AB&CR agent (Figure 32, blue arrow), but failed to show any selective competition with a 1000–3000-fold excess of either (R)- or (S)-LCM. For example, we observed a ~30 kDa protein (Figure 32, blue arrow) that was selectively labeled by (R)-**169** over (S)-**169** at 10 μM. The protein was enriched in the β-DDM soluble fraction, and did not adsorb to S or Q Sepharose® (pH 7.4). This protein, however, did not display any fluorescence signal competition in the presence of excess (R)-LCM (1000 equiv, Figure 33). In addition, the overall fluorescence level of selectively labeled proteins was not intense, indicating either a low binding affinity with the AB&CR agent, a low expression level of the protein, a low level of recovered functional protein, a low efficiency for protein adduction or a combination of these factors.
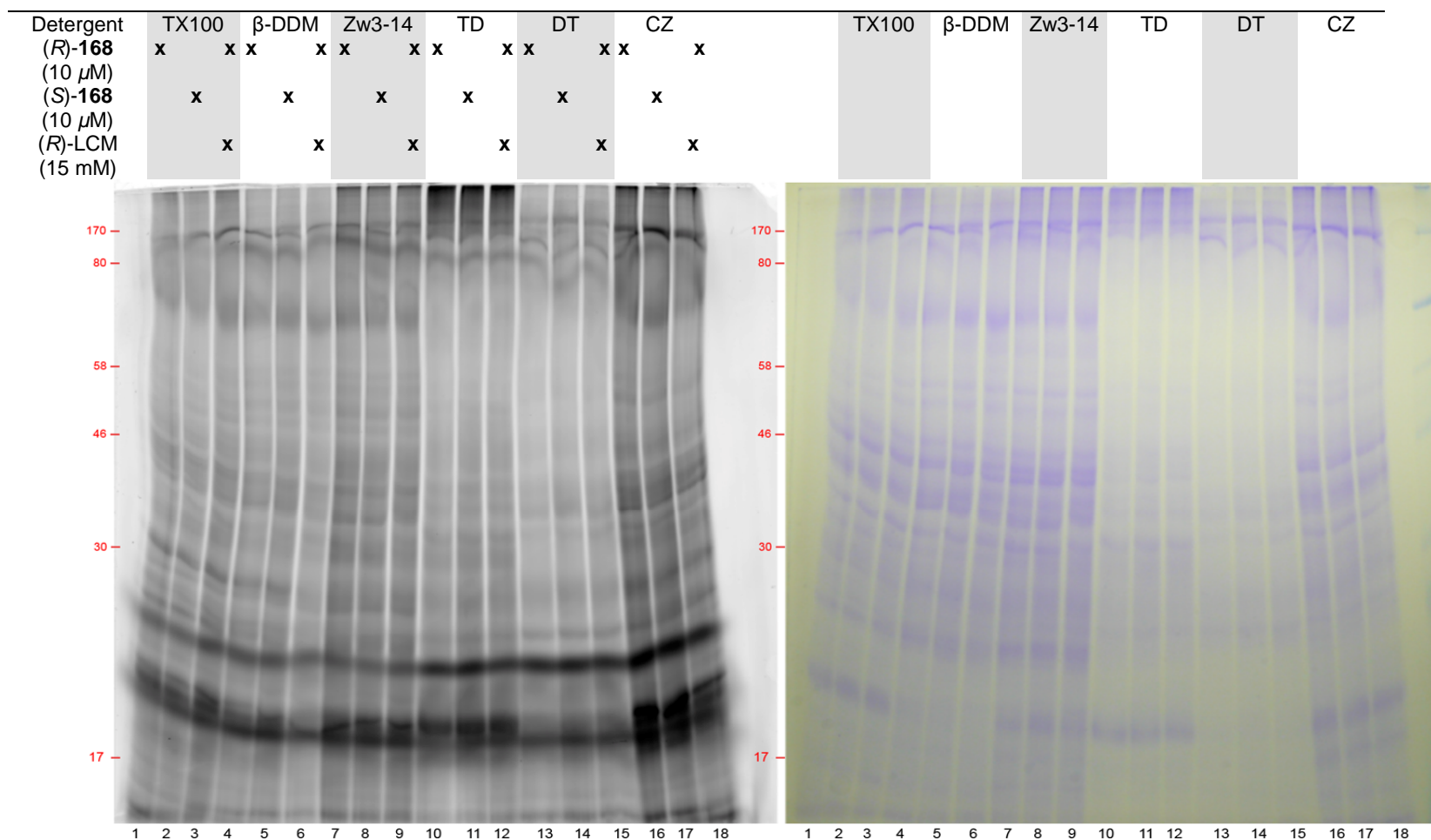
Figure 34. 10% SDS-PAGE gel (large size) of the membrane fraction (P2+P3) solubilized with a single detergent or sequentially-solubilized with two different surfactants. Left: fluorescence scan; right: corresponding Coomassie stain. Lanes 1–3: TX100 solubilized; lanes 4–6: β-DDM solubilized; lanes 7–9: Zw3-14 solubilized; lanes 10–12: the TX100 insoluble pellet was solubilized with β-DDM; lanes 13–15: the β-DDM insoluble pellet was solubilized with TX100; lanes 16–18: the CHAPS insoluble pellet was solubilized in Zw3-14. Lanes 1, 4, 7, 10, 13, 16: (*R*)-**168** (5 μM); lanes 2, 5, 8, 11, 14, 17: (*S*)-**168** (5 μM); lanes 3, 6, 9, 12, 15, 18: (*R*)-**168** (5 μM) + (*R*)-LCM (10 mM). Reactions were photolabeled (30 min 365 nm, 1 min 312 nm), clicked with Probe **196**, and resolved. Approximate molecular weight markers (kDa) are shown in red on the left.

Recognizing the complexity of the protein mixture, we also implemented another fractionation protocol using sequential detergent solubilization. The membrane material was first solubilized with a given surfactant, centrifuged, and the supernatant was discarded. The remaining pellet was solubilized in another detergent, centrifuged, and the supernatant was used for screening. The total protein stain (Coomassie blue or silver stain) showed diverse solubilization patterns for each detergent or combinations of detergents used (Figure 34). Three combinations were used and included β-DDM solubilization of the TX100 insoluble pellet (termed "TD"), the reverse combination (termed "DT"), and Zw3-14 solubilization of the CHAPS insoluble pellet (termed "CZ"). Unfortunately, no membrane lysate preparation yielded any potentially interesting protein band that met our selection criteria.

5.1.3. Interactions between fluorescent Probes and detergent molecules

Our studies showed direct interactions between the fluorescent Probes and detergent molecules. For example, click chemistry with PEG-containing Probe **196** proceeded smoothly in the presence of detergents lacking a PEG chain (β-DDM, Zw3-14, CHAPS), while the fluorescent labeling intensity was dramatically reduced in the presence of TX-100. This difference in click chemistry did not occur when the C3-azide Probe **203** was used for click chemistry. We suspect that this difference is due to the polyethylene glycol chain (containing 3 ethylenoxy units) in **196** that is likely solvated by the PEG chain in TX-100 (8–10 units), thus diminishing the Probe **196**'s ability to participate in the click chemistry reaction. We expect that these

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findings would also apply to biotin purification experiments, where a biotin Probe such as **191** would likely behave similarly with TX-100 molecules and provide lower product recovery yields.

203   **196**
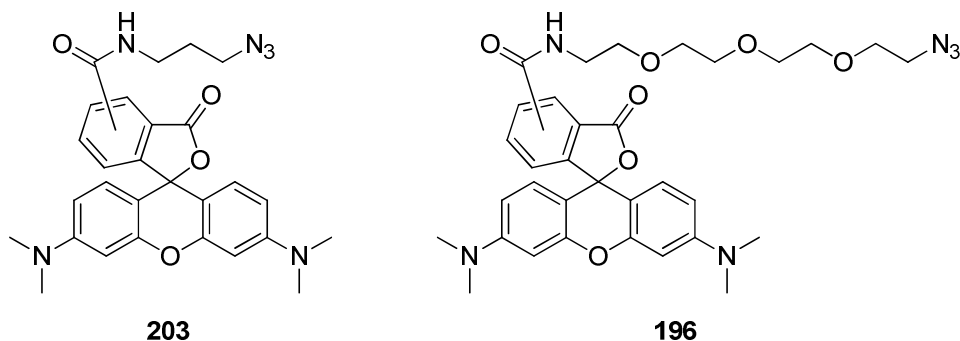
We observed one experimental complication using the C3 Probe **203** and all the detergents. We found an intense smear close to the protein migration front (Figure 33, green arrow) in the gel that was not washed away by several rinses with ddH$_2$O. Use of Probe **196** in place of Probe **203** eliminated this problem.

5.2. Screening whole membrane extracts for potential targets

The results using a detergent-based approach, suggested that treatment with surfactants may have been detrimental to the integrity of potential target proteins. Therefore, we decided to further pursue our interrogation of the proteome by using *unsolubilized* whole rat brain membrane extracts. We also tried to maintain a membrane environment as close as possible to native conditions and used a physiological-like buffer solution to conduct our photo-labeling experiments (Locke's buffer: 154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl$_2$, 1.0 mM MgCl$_2$, 3.8 mM NaHCO$_3$, 5 mM D-glucose, 5 mM HEPES (pH 7.2)).[483,484] Importantly, in our previous study, no special care was taken when freezing the material for storage. It has been

demonstrated that one single freeze-thaw cycle can lead to a near-complete loss of activity for some proteins.[485] Therefore, we also included a protocol that used a slow, cryopreserving method to freeze and store samples. After preparation, the samples were suspended to ~1 mg.mL^{-1} in Locke's buffer supplemented to 10 mg.mL^{-1} bovine serum albumin (BSA) and 10% DMSO (solution termed "cryobuffer").[485]

5.2.1. Rationale for the synaptosomal fraction screening

Homogenization of animal tissue leads to the shearing of different organelle structures. When brain tissue is used, pre-synaptic buttons are pinched at the end of the axon and close themselves to form pre-synaptic membrane vesicles.[486,487] These subcellular structures are termed synaptosomes, and are widely used as a model to study synaptic proteins or neurotransmission mechanisms.[488-491] Given the fact that several AEDs target proteins are located at the pre-synaptic level (TGB,[201] LVT,[210] gabapentin (GBP)[492]), we screened the synaptosomes for potential LCM targets.

Synaptosomes are usually recovered by centrifugation of the S1 supernatant at 15,000 g for 20 min (Scheme 40).[486,487,493,208,494] The pellet recovered at that speed also contains other organelles such as mitochondria and is, therefore, often referred to as the "crude synaptosomal fraction".[495] A typical enrichment procedure for synaptosomes involves further centrifugating the pellet through a sucrose density gradient.[485-487,208,209] Due to their specific buoyancy, synaptosomes are recovered at the interface of two specific sucrose layers. Such a procedure, though, requires a swing-bucket ultracentrifuge rotor,[485-487,208,209] which was unavailable for our studies.

We used an alternative approach based on phase partitioning, which takes advantage of the differences between the physicochemical properties of mitochondrial and synaptic membranes (Figure 35).[496,497] The method uses a biphasic system composed of two types of polymers: PEG-4000 (Sigma-Aldrich, cat # 81240), a polyethylene glycol polymer with an average molecular weight of 4000 g.mol^{-1}, and dextran 500 (Spectrum Chemicals, cat # D1004), a D-glucose glycopolymer with an average molecular weight of 500,000 g.mol^{-1}.
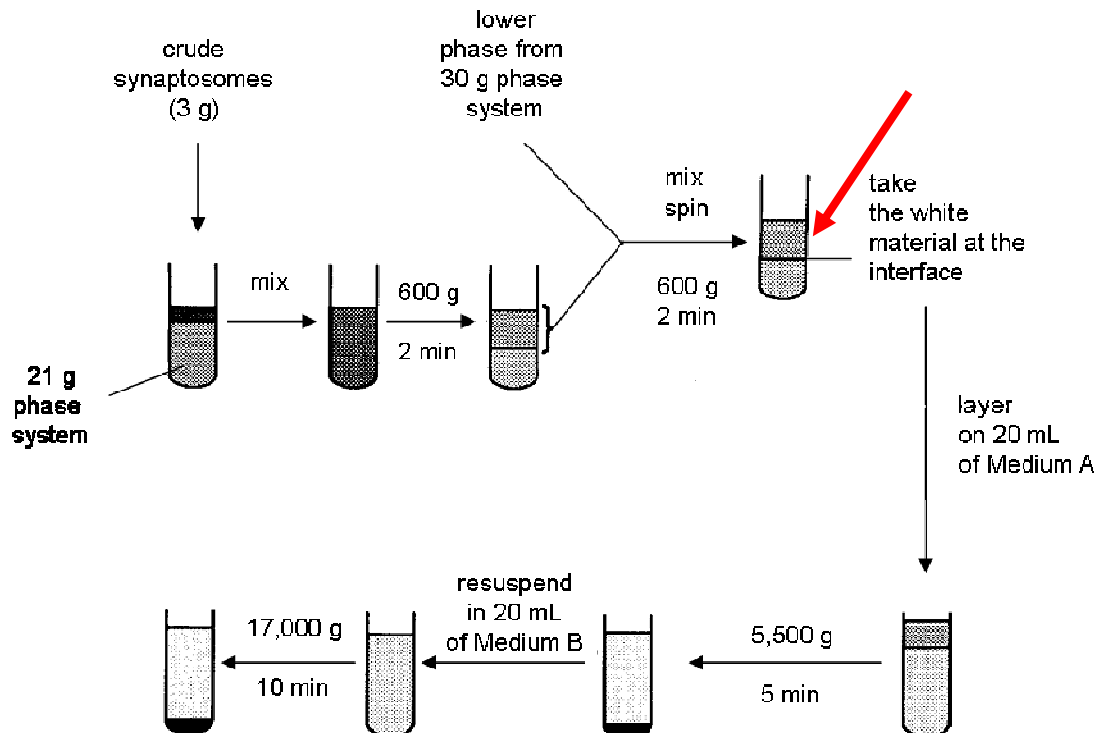
Figure 35. General approach to the purification of synaptosomes by phase partitioning. Experimental details are given in the Experimental Section. The red arrow indicates the procedure's step where differences were observed compared with the literature protocol. The figure was reproduced and modified from Ref. [496]

The two immiscible phases, a PEG-rich and a dextran-rich, are then mixed with the crude pellet and centrifuged at low speed to separate the layers. Synaptosomes are

recovered in the PEG-rich phase and then subjected to a similar, second round of purification to yield an enriched synaptosomal pellet. Utilizing this phase partitioning method, we observed one experimental difference between the synaptosomes preparation we used and the literature protocol (see Section 5.5.2).[496] After the second purification step, which uses a fresh lower phase from a 30-g phase system, the layer termed "final upper phase" did not contain any synaptosomal particles floating in the layer as described (Figure 35, red arrow).[496] Instead, a large amount of white material was found at the interface between the two layers. This material was collected with a pipette and directly pelleted at 17,000 g. The procedure yield was ~6 mg of synaptosomal protein per rat brain (~1.5 g wet tissue). To verify if the difference in the procedure was not detrimental to the preparation, we performed a Western blot of the enriched synaptosomes using an antibody raised against synaptosomal-associated protein 25 (SNAP-25), and using the crude rat brain homogenate as a positive control. In addition we also probed for the presence of mitochondrial proteins, using an antibody against the mitochondrial marker cytochrome c oxidase subunit IV (COX IV) (Figure 36). Mitochondria should have been removed during the preparation. Western blot analysis showed that, indeed, SNAP-25 was present in the membrane material recovered (Figure 36, left panel). The anti COX IV antibody was not functional and we could not, therefore, ascertain the absence of mitochondrial proteins (Figure 36, right panel). However, these results gave us confidence that the enrichment procedure worked correctly.
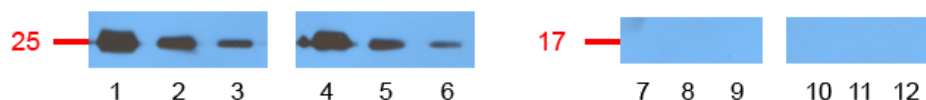
Figure 36. Western blot of the crude brain homogenate and synaptosomes. Proteins SNAP-25 (~25 kDa, synaptosomes) and COX IV (~17 kDa, mitochondria) antibodies were used as subcellular markers. Lanes 1–6: anti SNAP-25; lanes 7–14: anti COX IV. Lanes 1–3, 7–9: crude brain homogenate; lanes 4–6, 10–12: synaptosomes. Lanes 1, 7: ~10 μg of crude homogenate protein per lane; lanes 2, 8: ~1 μg; lanes 3, 9: ~0.1 μg; lanes 4, 10: ~2 μg of synaptosomal proteins per lane; lanes 5, 11: ~0.2 μg; lanes 6, 12: ~0.02 μg. Gels were transferred to nitrocellulose membranes and blocked with 5% milk in TBST. The rabbit polyclonal antibody against SNAP-25 was diluted 1:1000 in 5% milk in TBST and incubated at room temperature (1h). The rabbit polyclonal antibody against COX IV was diluted 1:750 in 5% milk in TBST and incubated at 4 ℃ (15 h). Both membranes were then probed with donkey anti rabbit IgG, horseradish peroxidase (HRP)-linked, diluted 1:1000 in 5% milk in TBST at room temperature (1 h) and developed (ECLplus). Developing time: SNAP-25: 1 min; COX IV: 15 min. Approximate molecular weight markers (kDa) are shown in red on the left.

5.2.2. Screening the synaptosomal and microsomal fractions

Following the previously established protocol, we screened the synaptosomal fraction using both enantiomers of compounds **181**, **169**, **168**, **170**, and (*R*)- and (*S*)-LCM as competing reagents. On small scale, unsolubilized proteins were dispensed in Locke's buffer (100 μL per well) after removing the cryobuffer and washing the pellet. PhotoAB&CR agents and competing reagents were added (5% (v/v) DMSO) and the suspension was incubated at 4 ℃ (10 min) prior to irradiation. After the photolabeling step, the content of each well was transferred to a tube, and centrifuged at 14,000 rpm at 4 ℃ (5 min). At this st age, several procedures were examined. Pelleted samples were either solubilized in non-denaturing detergents (β-DDM, Zw3-14, CHAPS) or in HEPES buffer (pH 7.4) supplemented to 1% SDS, and then "clicked" with Probe **196** (Method A). Alternatively, click chemistry was performed on the unsolubilized membrane pellet, after which the suspension was successively pelleted, rinsed with HEPES buffer (pH 7.4), spun down, solubilized

with a non-denaturing or denaturing detergent, centrifuged, and the supernatant was resolved on SDS-PAGE gel (Method B).

In the initial experiments, we used the set of photoAB&CR agents **181**, **169**, **168**, **170** to screen the unsolubilized membrane proteome as previously described (see Section 4.3). Using the synaptosomal preparation, we photolabeled proteins with 10 μM of the appropriate (R)-AB&CR and (S)-AB&CR agents. In addition, the synaptosomal preparation was irradiated with 10 μM (R)-AB&CR in the presence of 15 mM (R)-LCM.

Enantiomer		(R)	(S)	(R)	(R)	(S)	(R)	(R)	(S)	(R)	(R)	(S)	(R)
DMSO	x												
181 (5 μM)		x	x	x									
169 (5 μM)					x	x	x						
168 (5 μM)								x	x	x			
170 (5 μM)											x	x	x
(R)-LCM (15 mM)				x			x			x			x
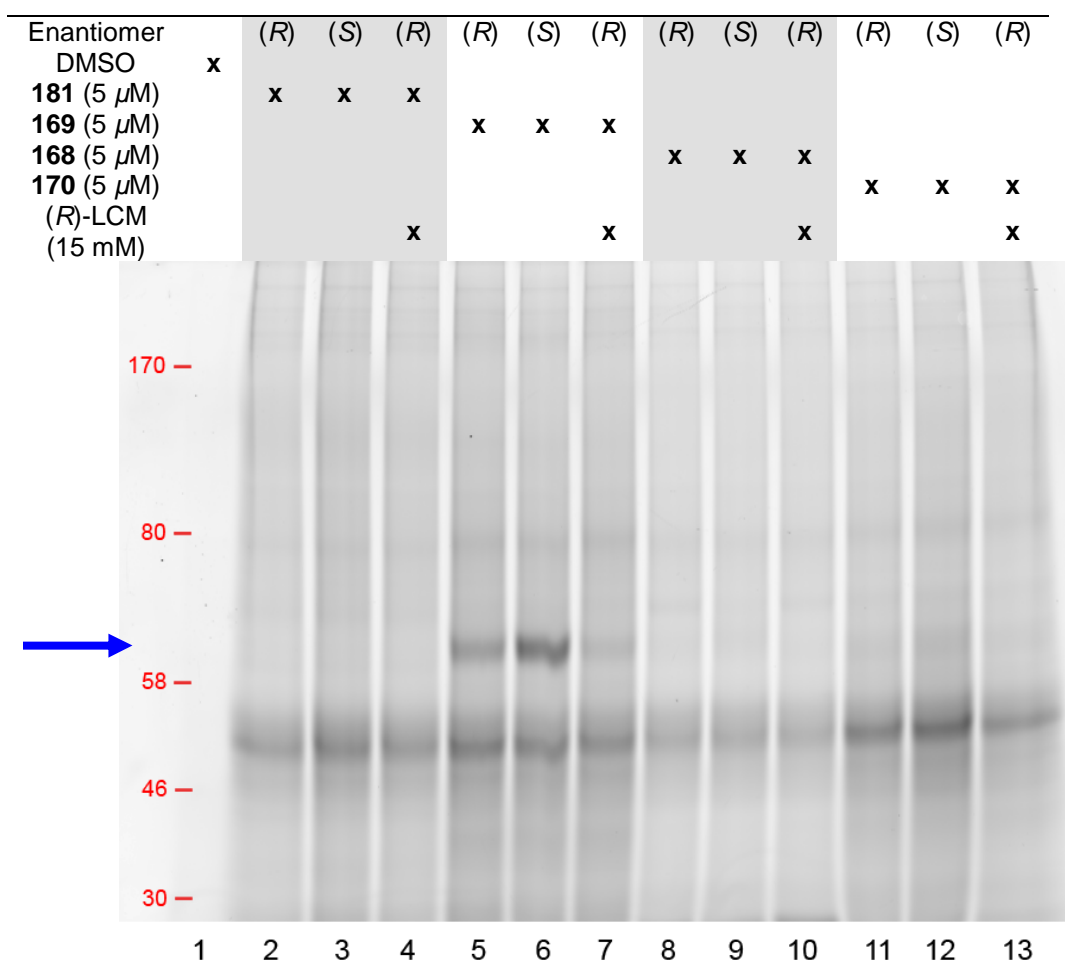
Figure 37. Fluorescence scan of the synaptosomal fraction screened with photoAB&CR agents **181**, **169**, **168**, and **180** (6%SDS PAGE gel). Whole synaptosomal extracts (~0.2 mg.mL^{-1} in Locke's

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buffer) were irradiated, the pellet was rinsed, dissolved in 25 mM HEPES (pH 7.4) supplemented with 1% SDS, clicked with Probe **196** and resolved. Lane 1: DMSO control; lane 2: (*R*)-**181** (5 *μ*M); lane 3: (*S*)-**181** (5 *μ*M); lane 4: (*R*)-**181** (5 *μ*M) + (*R*)-LCM (15 mM); lane 5: (*R*)-**169** (5 *μ*M); lane 6: (*S*)-**169** (5 *μ*M); lane 7: (*R*)-**169** (5 *μ*M) + (*R*)-LCM (15 mM); lane 8: (*R*)-**168** (5 *μ*M); lane 9: (*S*)-**168** (5 *μ*M); lane 10: (*R*)-**168** (5 *μ*M) + (*R*)-LCM (15 mM); lane 11: (*R*)-**170** (5 *μ*M); lane 12: (*S*)-**170** (5 *μ*M); lane 13: (*R*)-**170** (5 *μ*M) + (*R*)-LCM (15 mM). The Coomassie stain (picture not shown) showed equal amounts of protein loaded in each lane. Approximate molecular weight markers (kDa) are shown in red on the left.

Using Method A and SDS as a detergent, we observed a potential band of interest at ~60 kDa (Figure 37, blue arrow). This protein was exclusively labeled by (*R*)-**169** and (*S*)-**169**, with a pronounced preference for (*S*)-**169** and the presence of 1,500 equiv of (*R*)-LCM gave a pronounced reduction in the fluorescence intensity of the band.

We found that click chemistry in the presence of 1% SDS led to lower levels of fluorescence when compared with the three non-denaturing detergents β-DDM, Zw3-14, and CHAPS. All three detergents appeared to solubilize the protein of interest, as shown by the strong fluorescent band around 60 kDa on SDS-PAGE gels (Figure 38, blue arrow). Zw3-14 and β-DDM solubilized the protein equally well, while using CHAPS gave a slightly reduced fluorescent intensity. The amount of total protein solubilized with detergents decreased as follows: Zw3-14, β-DDM, CHAPS. We, therefore, chose to use β-DDM as it provided an adequate balance between its ability to provide a high recovery of the fluorescent signal and its solubilizing properties. The insoluble pellets corresponding to each detergent were then dissolved in 1% SDS (treatments termed "DS", "ZS", "CS", for β-DDM, Zw3-14, and CHAPS, respectively).

In later experiments, we also screened the microsomal fraction (see Section 3.1, Scheme 40) to refine the subcellular localization of the ~60 kDa protein. Thus, in

the same lysate preparation, we isolated rat brain synaptosomes and microsomes, and stored them under identical conditions using the cryobuffer. When screening the two membrane fractions under heterogeneous conditions, we found the ~60 kDa protein characteristic signal in both subcellular compartments (Figure 39). Using Method B, we found that the protein was preferentially labeled by (*S*)-**169** over (*R*)-**169**. However, a 3000-fold excess of (*R*)-LCM led to a strong reduction of fluorescence intensity when compared to (*S*)-LCM. Intriguingly, both signals from (*R*)-**169** and (*S*)-**169** were more affected by (*R*)-LCM than by (*S*)-LCM.

Several attempts were made to identify this protein by biotin purification. These efforts were unfruitful, while we observed some intriguing findings. First, when we used Method B to screen the microsomal fraction, the protein pellet after the click chemistry step could be solubilized in β-DDM. Fluorescence scans showed that the protein of interest was efficiently extracted in this detergent (data not shown). The corresponding Coomassie stain, however, showed that this protein was the predominant protein present in the entire gel, whereas all other proteins were only solubilized with denaturing conditions (1% SDS, Figure 39). These results contrasted with the high solubilization properties of β-DDM under native conditions (Method A, Figure 38). On large scale (~1 mg of membrane protein), the strong Coomassie stained band corresponding to ~60 kDa was excised and analyzed by MS (Figure 40). Serum albumin was identified as the likely major constituent in this band (Table 6)

293
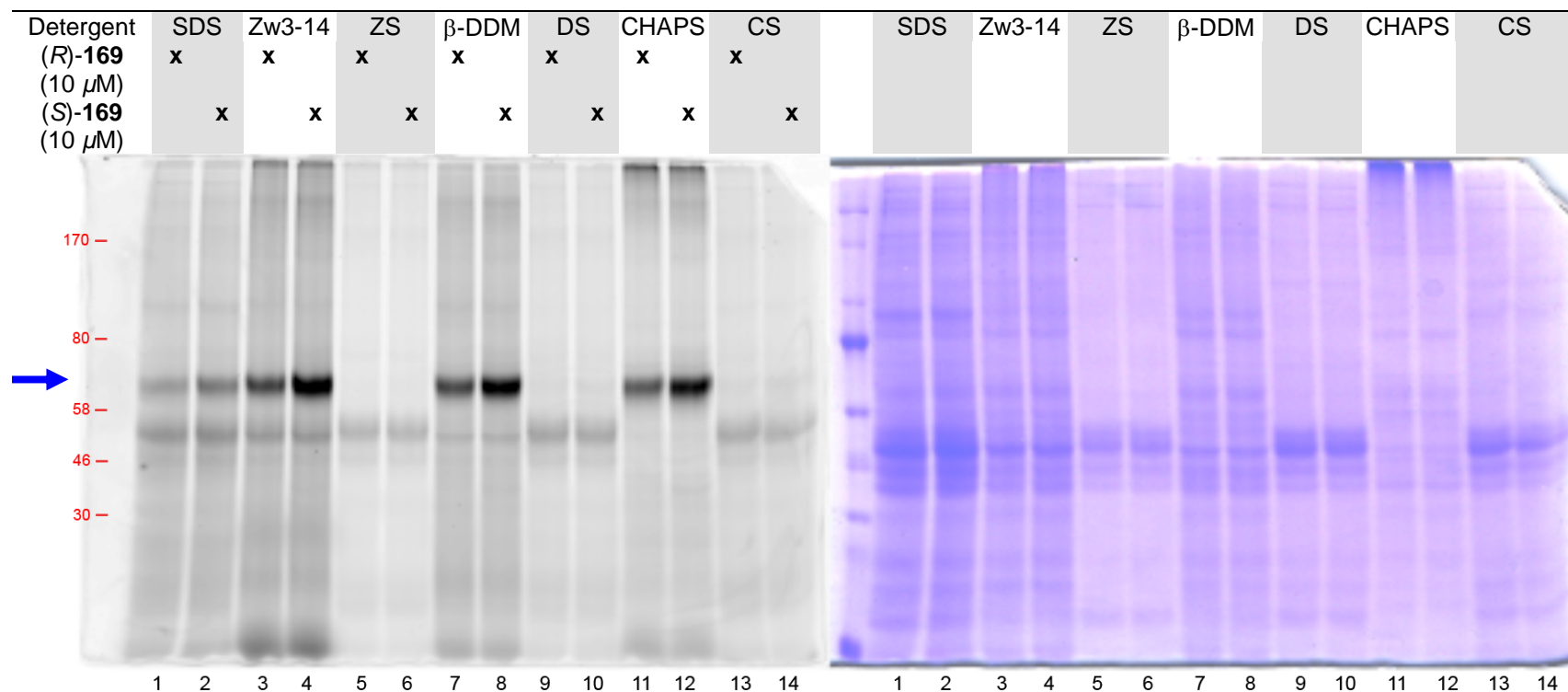
Figure 38. 10% SDS-PAGE gel of the synaptosomal fraction labeled with (*R*)-**169** and (*S*)-**169**. Left: fluorescence scan; right: corresponding Coomassie stain. Whole membrane extracts were irradiated in the presence of the photoAB&CR, solubilized with various detergents and the solubilized material was clicked with Probe **196** and resolved. Lanes 1, 3, 5, 7, 9, 11, 13: (*R*)-**169** (10 μM); lanes 2, 4, 6, 8, 10, 12, 14: (*S*)-**169** (10 μM). After photolabeling, membrane extracts were solubilized in detergent-supplemented HEPES buffer (pH 7.4) as follows. Lanes 1,2: 1% SDS; lanes 3, 4: 0.5% Zw3-14; lanes 5, 6: 0.5% Zw3-14 insoluble pellet solubilized in 1% SDS; lanes 7, 8: 0.5% β-DDM; lanes 9, 10: 0.5% β-DDM insoluble pellet solubilized in 1% SDS; lanes 11, 12: CHAPS; lanes 13, 14: CHAPS insoluble pellet solubilized in 1% SDS. Approximate molecular weight markers (kDa) are shown in red on the left.
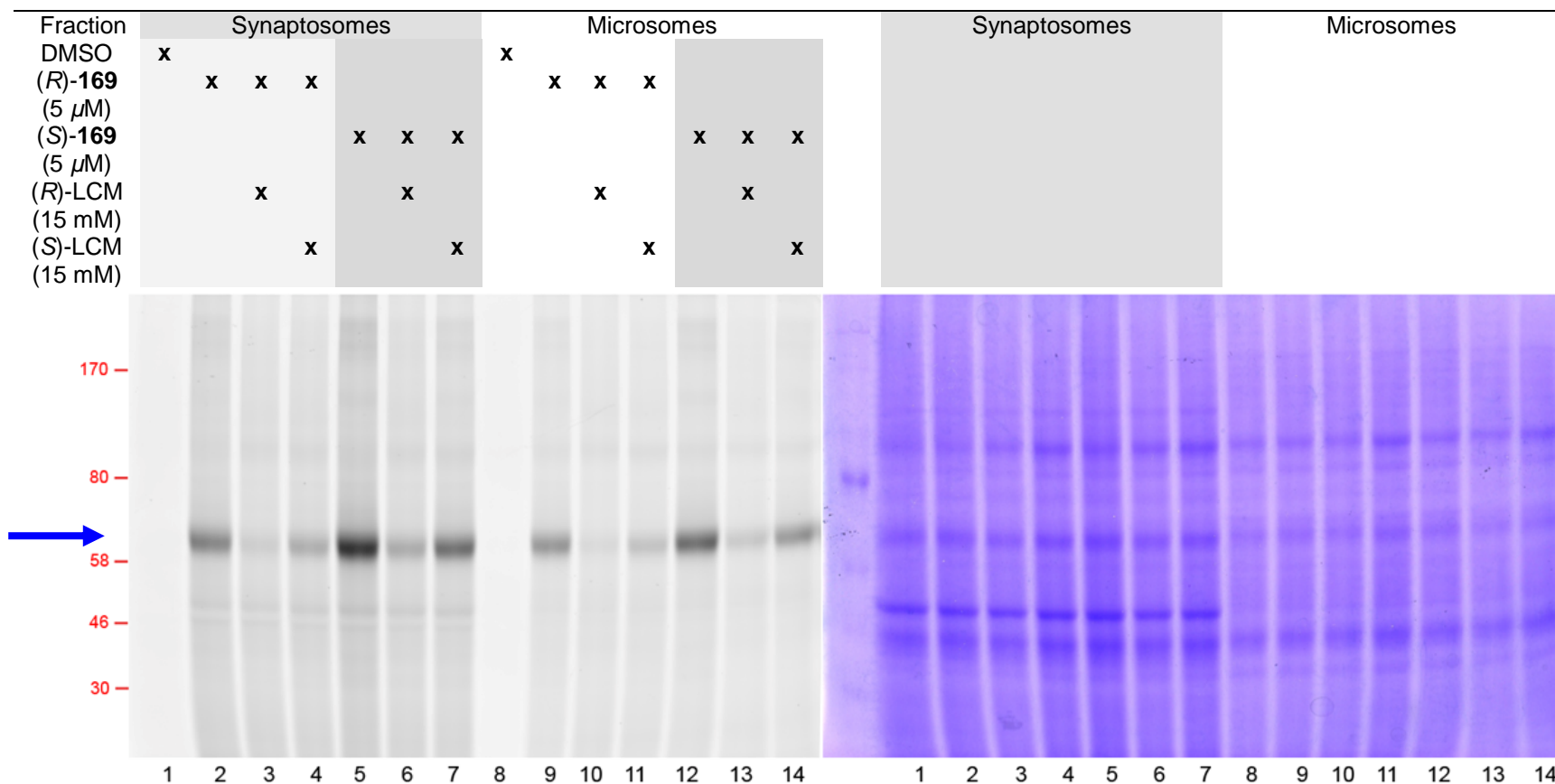
Figure 39. 8% SDS-PAGE gels of the intact membrane-bound fraction after photolabeling, click chemistry with **196** using Method B and 1% SDS 25 mM HEPES (pH 7.4). Left: fluorescence scan; right: corresponding Coomassie stain. Lanes 1–7: synaptosomal fraction; Lanes 8–14: microsomal fraction. Lanes 1, 8: DMSO; lanes 2, 9: (R)-**169** (5 µM); lanes 3, 10: (R)-**169** (5 µM) + (R)-LCM (15 mM); lanes 4, 11: (R)-**169** (5 µM) + (S)-LCM (15 mM); lanes 5, 12: (S)-**169** (5 µM); lanes 6, 13: (S)-**169** (5 µM) + (R)-LCM (15 mM); lanes 7, 14: (S)-**169** (5 µM) + (S)-LCM (15 mM). Approximate molecular markers (kDa) are shown in red on the left.
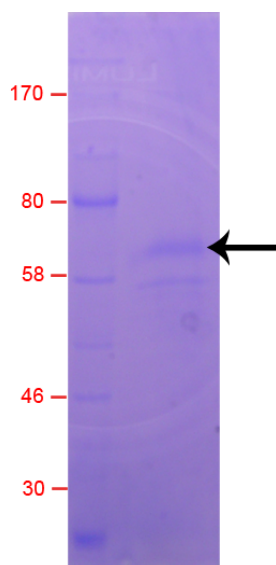
Figure 40. Coomassie stain of the enriched protein of interest (8% SDS-PAGE gel). Starting from 1.5 mg of total microsomal protein, the membrane lysate was irradiated in the presence of 10 *μ*M (*R*)-**169**, pelleted, rinsed, and clicked with biotin Probe **191** using Method B. After click chemistry, the membrane material was pelleted, rinsed with HEPES buffer and solubilized in 1% β-DDM 25 mM HEPES (pH 7.4). The suspension was centrifuged and the supernatant was resolved on a SDS-PAGE gel and Coomassie stained. The predominant band (black arrow) was excised and sent for MS analysis (see table below). Approximate molecular weight markers (kDa) are shown in red on the left.

Table 6. Representative list of tryptic fragments identified in the Coomassie stained band corresponding to ~60 kDa. Additional tryptic fragments corresponding to limbic system-associated protein (LSAMP) and brain acid soluble protein 1 (BASP1) were also identified.

Serum albumin			
Observed	Miss	Score	Peptide
486.7543	0	49	K.QTALAELVK.H
550.7948	1	47	K.KQTALAELVK.H
575.2831	0	37	K.LVQEVTDFAK.T
633.7795	0	64	R.FPNAEFAEITK.L
720.3389	0	59	K.APQVSTPTLVEAAR.N
733.339	0	72	K.LGEYGFQNAVLVR.Y

Second, the ~60 kDa protein's characteristic fluorescence signal was not observed when we used an aliquot of a freshly prepared microsomal fraction (Figure 41, blue arrow). However, when experiments were conducted on the cryopreserved material from the same preparation, the signal was again observed (data not shown). These findings indicated that BSA, the protein present at a high

concentration in the cryobuffer (10 mg.mL^{-1}) was likely targeted by (*S*)-**169** and to a lesser degree by (*R*)-**169**.
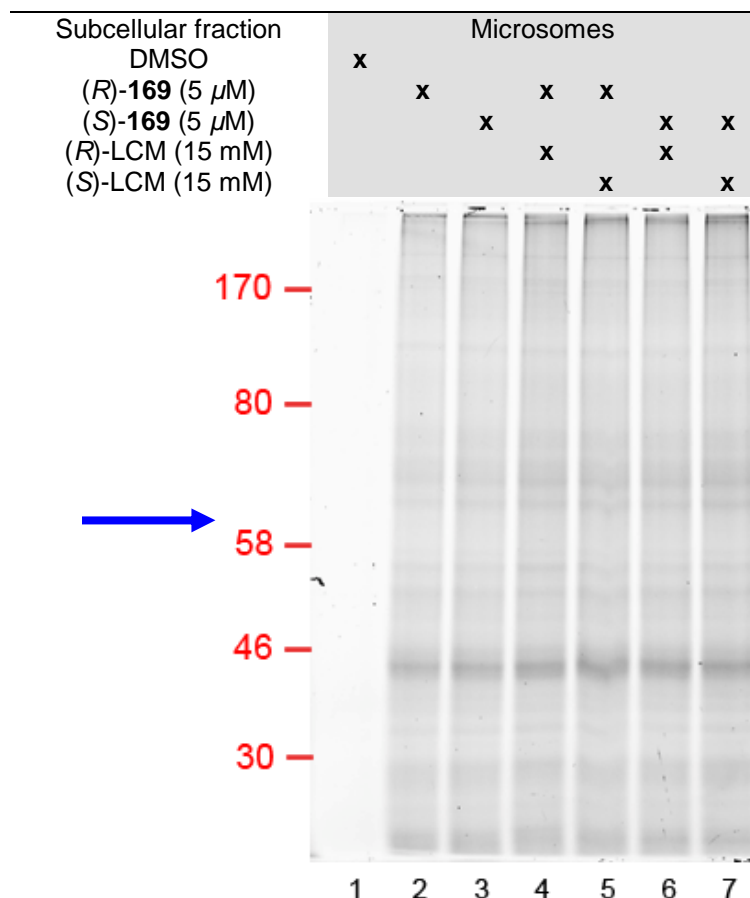
Figure 41. In-gel fluorescence scan of freshly prepared (non cryopreserved) microsomal fraction from the rat brain. Membrane extracts were incubate with photoAB&CR **169**, clicked with Probe **196** using Method B, and resolved (8% SDS PAGE gel). Lane 1: DMSO; lane 2: (*R*)-**169** (5 µM); lane 3: (*S*)-**169** (5 µM); lane 4: (*R*)-**169** (5 µM) + (*R*)-LCM (15 mM); lane 5: (*R*)-**169** (5 µM) + (*S*)-LCM (15 mM); lane 6: (*S*)-**169** (5 µM) + (*R*)-LCM (15 mM); lane 7: (*S*)-**169** (5 µM) + (*S*)-LCM (15 mM). No specific fluorescence signal was detected around 60 kDa under these conditions. The Coomassie stain (picture not shown) indicated equal amounts of protein loaded per lane. Approximate molecular weight markers (kDa) are shown in red on the left.

The serum albumin fragments identified in Table 6 could not be solely attributed to *Rattus norvegicus* and may have possibly originated from several other species, including *Bos taurus*. Photolabeling experiments conducted on pure BSA and human

serum albumin (HSA) was consistent with this hypothesis (Figure 42), although differences between (R)-**169** and (S)-**169** labeling were far less pronounced when using the *isolated* protein. Competition with (R)-LCM was also dramatically reduced from a fluorescent signal almost abolished with 3000-fold excess of (R)-LCM in the whole membrane lysate (Figure 39, lanes 2 and 3) to a barely detectable change with 5000-fold excess of (R)-LCM using the purified protein (Figure 42, lanes 2 and 4). Nonetheless, AB&CR **169** selectively labeled BSA and HSA while **181**, **168**, and **170** did not (Figure 43, blue arrow), thus mirroring the different photoAB labeling profiles observed in Figure 37. The differences observed in the two competition experiments (Figure 39, Figure 42) were surprising and have been tentatively attributed to a change of conformation due to the dissimilarity between the two protein environments.

Protein		Bovine Serum Albumin							Human Serum Albumin					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DMSO	x							x						
(R)-**169** (1 μM)		x		x	x				x		x	x		
(S)-**169** (1 μM)			x			x	x			x			x	x
(R)-LCM (5 mM)				x		x					x		x	
(S)-LCM (5 mM)					x		x					x		x
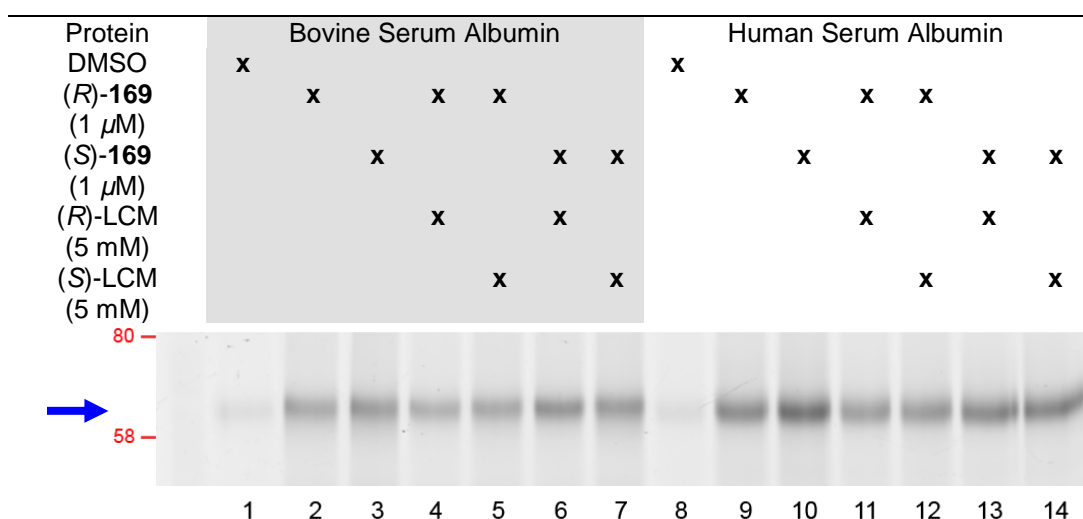
Figure 42. In-gel fluorescence scan of purified BSA and HSA labeled with photoAB&CR agent **169**. Purified proteins were solubilized (0.2 mg.mL^{-1}) in 25 mM HEPES (pH 7.4) containing 150 mM NaCl, photolabeled, clicked with Probe **196**, and resolved (~200 ng of protein were loaded per lane). Lanes 1–7: BSA; lanes 8–14: HSA. Lanes 1, 8: DMSO; lanes 2, 9: (R)-**169** (1 μM); lanes 3, 10: (S)-**169** (1 μM); lanes 4, 11: (R)-**169** (1 μM) + (R)-LCM (5 mM); lanes 5, 12: (R)-**169** (1 μM) + (S)-LCM (5 mM); lanes 6, 13: (S)-**169** (1 μM) + (R)-LCM (5 mM); lanes 7, 14: (S)-**169** (1 μM) + (S)-LCM (5 mM). The

Coomassie stain (picture not shown), showed equal protein loading in each lane. Approximate molecular weight marker (kDa) are shown in red on the left.
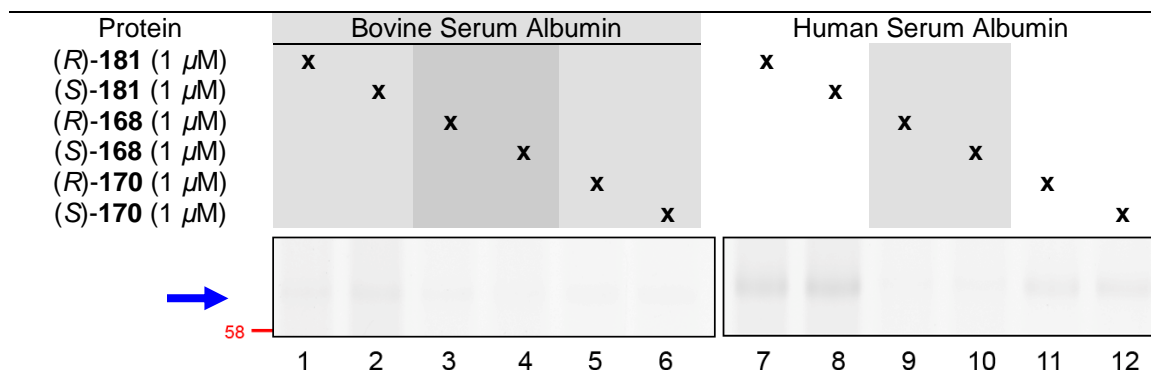
Figure 43. In-gel fluorescence scan of purified BSA and HSA labeled with photoAB&CR agent **181**, **168**, and **170**. Purified proteins were solubilized (0.2 mg.mL^{-1}) in 25 mM HEPES (pH 7.4) containing 150 mM NaCl, photolabeled, clicked with Probe **196**, and resolved (~200 ng of protein were loaded per lane). Lanes 1–6: BSA; lanes 7–12: HSA. Lanes 1, 7: (*R*)-**181** (1 µM); lanes 2, 8: (*S*)-**181** (1 µM); lanes 3, 9: (*R*)-**168** (1 µM); lanes 4, 10: (*S*)-**168** (1 µM); lanes 5, 11: (*R*)-**180** (1 µM); lanes 6, 12: (*S*)-**180** (1 µM). The Coomassie stain (picture not shown), showed equal protein loading in each lane. Approximate molecular weight marker (kDa) are shown in red on the left.

5.3. Discussion

The rat membrane-bound proteome was screened under a variety of conditions with the suite of photoAB&CR agents **181**, **169**, **168**, and **170**. Our first attempt at identifying potential LCM targets took advantage of the variety of commercially available non-denaturing detergents. Recognizing the complexity of the membrane proteome, we chose to use four structurally diverse surfactants to solubilize different sets membrane-bound proteins. Indeed, each detergent displayed pronounced differences in the solubilized protein patterns (Figure 33, Figure 34, Figure 38). For example, only CHAPS was found to efficiently solubilize very high molecular weight proteins (>300 kDa). Overall, TX100 (1%), β-DDM (0.5%), and Zw3-14 (0.5%) solubilized comparable amounts of protein (Bradford assay) that appeared greater than CHAPS (1%). We hypothesized that a wide range of

solubilization conditions would result in higher chances of identifying a potential target protein. Importantly, this non-denaturing solubilization protocol allowed us to use ion-exchange chromatography to deconvolute the membrane lysate. Another technique employed in this study was the use of a larger sized SDS PAGE electrophoresis system. Using longer SDS PAGE gels (~20 cm height) provided a greater separation of the different membrane proteins present in the lysate (15–250 kDa, Figure 34). We also gained in sensitivity by loading increased amounts of protein per well. The downside of the approach, however, was the prolonged gel migration times (from 50 min for mini-gels to 4 h for the larger gels) and the fact that some protein patterns were deformed or appeared smeared on the gel.

Using these screening conditions, we identified a few proteins displaying a strong (*R*) vs (*S*) selectivity for the photoAB&CR molecule by in-gel fluorescence. The overall order of reactivity for **181**, **169**, **168**, and **170** with the membrane fraction was slightly different than that observed for the cytoplasmic fraction screening. Under identical irradiation conditions (see Section 4.6.2), we observed for the membrane fraction that generally the aromatic azide **181** and alkyl diazirine **168** provided the most intense signals, followed by aromatic diazirine **169**, and benzophenone **170**. Nonetheless, these differences varied within different fractionation cuts (Q-Sepharose elution, left gels, and Q-Sepharose flow-through, right gels). By comparison, in the cytosolic fraction, **181** was more reactive, followed by **169**, **168** and **170**, and less disparities in reactivity were observed within different AMS cuts. Differences in the photoAB&CR reactivity profiles between the cytoplasmic and solubilized membrane fractions have been tentatively attributed, in

part, to differences in the inherent nature of proteins present in each subcellular fraction. The fluorescent signals in the membrane fraction were, however, not intense, and unaffected by an excess of competing (*R*)-LCM during the photolabeling step. In addition, the low-intensity fluorescent labeling observed for proteins specifically labeled suggested several potential problems. Among these are that (1) the protein labeled may have been present only at low levels in the lysate; (2) the photoAB&CR agent may only bind weakly to the protein; (3) the efficiency of the photolabeling step was low; (4) the detergent may have only solubilized a partial amount of membrane-bound protein; and (5) the detergent may have partially denatured the protein. A combination of these reasons may also explain the absence of protein targets that were strongly labeled in the membrane fractions. Indeed, some membrane-bound proteins cannot be easily solubilized or remain properly folded upon action of a detergent.[498-500]

Thus, we also screened the membrane-bound proteome under more native conditions. First, we changed from a simple HEPES-based buffer previously used to the more physiologically relevant Locke's buffer. Second, we took additional care in the handling of the freshly prepared membrane extracts by adding a slow cooling step in a cryobuffer prior to storage at -80 ℃. The cryobuffer used was Locke's buffer supplemented to 10% (v/v) DMSO and 1% (w/v) BSA and has been demonstrated to enhance the stability of sensitive membrane synaptosomal proteins.[485] Using this protocol, we prepared and screened intact synaptosomal and microsomal fractions with the photoAB&CR agents **181**, **169**, **168**, and **170**. We took advantage of a convenient phase-partitioning fractionation method[496] to quickly

enrich for synaptosomes from the P2 fraction of the rat brain. However, in our hands, we observed a difference in the synaptosome preparation. The literature method indicated that the synaptosomes would be present in the upper layer after the second purification step (Figure 35, red arrow).[496] Instead, we observed abundant white particles present at the interface between the two layers and assumed them to be the enriched synaptosomes. To check the integrity of the membrane extracts, we probed for the presence of a synaptosomal marker, SNAP-25, using an antibody and showed that the protein the material isolated after fractionation was enriched in SNAP-25 (Figure 36). We also probed the isolated synaptosomes for a mitochondrial marker (COX IV) to confirm that the protein isolated was not cross-contaminated with mitochondria, using the crude brain homogenate as a positive control. However, the antibody purchased was not functional and this secondary control was not pursued further. Recovery yields of synaptosomes (~6 mg per rat brain, 3–4 mg per gram of wet tissue) were in agreement with yields obtained using other literature procedures.[501,493,502]

Using this new screening condition and utilizing the same irradiating conditions, we observed another difference in the reactivity profile of photoAB&CR derivatives. In the intact membrane photolabeling, aromatic azide **181**, aromatic diazirine **169** and benzophenone **170** displayed a similar non-specific labeling profile whereas the photolabeled products from aliphatic diazirine **168** were less intense (Figure 37). However, we identified a ~60 kDa protein targeted in the synaptosomal fraction, which was exclusively labeled by aromatic diazirine **169**, and displayed a strong (*S*) vs (*R*) preference (Figure 37, Figure 38). Interestingly, both (*R*)-**169** and (*S*)-**169**

were competed by an excess of (*R*)-LCM, and to a lesser extent by (S)-LCM (Figure 39). The preference for (*S*)-**169** over (*R*)-**169** in the labeling step was intriguing, especially in view of the competition experiments. Indeed, the competition results suggested that (*R*)-LCM bound to that protein stronger than (*S*)-LCM. This (*S*)- vs (*R*)- preference for **169** has been tentatively attributed to an increased labeling efficiency of (*S*)-**169** due to a more ideally positioned amino acid residue, despite a potentially lower binding affinity when compared to (*R*)-**169**. We observed the presence of the same molecular weight protein in the microsomal fraction. The fluorescent signal observed for this photoadduction was intense (Figure 38, left panel) and did not correlate with the amount of total protein (Figure 38, right panel) present in the lane, thus suggesting a specific photolabeling. Attempts to purify this protein using a biotin purification experiment did not provide us with definitive identification of the protein but suggested that the putative interacting partner of (*R*)-LCM was likely serum albumin (Figure 40, Table 6). Supporting this hypothesis was the disappearance of the characteristic ~60 kDa fluorescent signal when we used a freshly prepared brain microsomal extract (*e.g.*, without BSA present) to perform the assay (Figure 41). The fluorescent signal returned when the same extract was stored using the slow cooling step in the presence of cryobuffer. A photolabeling experiment using pure BSA and HSA showed that serum albumin was indeed selectively labeled by photoAB&CR agent **169** (Figure 42), and not by **181**, **168**, and **170** (Figure 43). However, we observed notable differences when labeling was performed on the protein by itself. The difference in fluorescence intensity between (*R*)-**169** and (*S*)-**169** was greatly reduced, and competition with a large excess of

(*R*)-LCM (5000 equiv) had almost no effect on (*R*)-**169** or (*S*)-**169**'s fluorescent signals (Figure 42). We have hypothesized that the conformational states of serum albumin could be different in an isolated setting and in a complex hydrophobic environment, and that this difference could affect the AB&CR modification. Nonetheless, this finding indicates that lacosamide has an inherent binding affinity for serum albumin, a finding that has been previously observed for numerous marketed drugs.[503-506]

5.4. Conclusions

We screened the membrane-bound proteome with photoAB&CR agents using a variety of screening conditions. The search yielded one protein of interest for which the AB&CR **169**'s fluorescent labeling displayed enantiospecific competition with (*R*)-LCM over (*S*)-LCM. This protein has been tentatively identified as bovine serum albumin, a component of the cryobuffer for membrane extract storage. The labeling specificity was greatly reduced when either purified BSA or HSA was used for the experiment in buffer alone. While the therapeutic relevance of serum albumin for lacosamide overall function is unknown, we did not pursue this lead because the literature did not provide a rationale to link SA to epilepsy.

The identification of SA in our proteomic screening protocol suggested that the AB&CR methodology worked, in part, in that we were able to identify a medium-to-low affinity binding partner of (*R*)-LCM from a complex protein environment. Significantly, we do not assert that lacosamide function is intimately connected with SA binding. Rather, our finding suggests that lacosamide possesses an inherent,

modest binding affinity to serum albumin. If this is the case, SA sequestration of LCM may indirectly affect the AED's function by modulating the serum concentration of lacosamide and its biodistribution in the CNS.

5.5. Experimental Section

5.5.1. Preparation of the solubilized membrane lysate

The whole rat brain homogenate (320 mM sucrose, 25 mM HEPES (pH 7.4), supplemented with protease inhibitors 1 mM PMSF, 10 μM E-64, 10 μM pepstatin A, 1 μM TPEN) was centrifuged at 1,500 g (10 min). The supernatant (S1) was pulled and centrifuged at the desired speed. Centrifugation at 15,000 g (20 min) yielded the P2 fraction (heavy membrane), and a S2 supernatant that was centrifuged at 100,000 g (50 min) to yield a P3 fraction (light membrane). Alternatively, the S1 supernatant could also be centrifuged directly at 100,000 g to yield a combined P2+P3 membrane fraction. The pellet (P2, P3, or P2+P3) was hypoosmotically lysed by resuspension in 10 mM HEPES buffer (pH 7.4) on ice (20 min) and centrifuged at 15,000 g (20 min, P2) or 100,000 g (50 min, P3, P2+P3). The supernatant was discarded, and the pellets resuspended in 25 mM HEPES, 150 mM NaCl to a total protein concentration of 4–5 mg.mL^{-1}. The desired supernatant was added at the desired concentration (1% TX100, 0.5% β-DDM, 0.5% Zw3-14, 1% CHAPS), the suspension gently rocked at 4 ℃ (20 min) and centrifuged at 15,000 g (20 min, P2) or 100,000 g (50 min, P3, P2+P3). The supernatant was used as the detergent-solubilized membrane fraction for screening.

5.5.2. Protocol for the enrichment of synaptosomes by phase partitioning

Synaptosomes were prepared following a literature procedure.[496] One day prior to the experiment, the two phase systems (termed 21-g and 30-g (g for grams)) were prepared and stored at 4 °C. Because biomolecule s are very sensitive to small changes in polymer concentrations, all solutions were prepared by weighing the different reagents. The stock solution of PEG-4000 (40% w/w) was prepared by accurately weighing 40.0 g of PEG-4000 and made up to 100.0 g with ddH$_2$O. The dextran-500 was prepared at least 2 days in advance. For a 20.0% (w/w) stock, a ~35% (w/w) solution was prepared by layering 35 g of dextran over 65 g of water. The highly viscous solution was heated in a water bath (40–50 °C) while gently stirring until all the polymer was dissolved (~30 min). The solution was then dialyzed against ddH$_2$O (15 h) and transferred to a *weighed* container. The following was only carried out if no air bubbles were present in the viscous liquid. A known amount of dextran solution (~2 g) was precisely diluted to 10 mL with ddH$_2$O and the optical rotation was measured with a polarimeter. Using the specific rotation of dextran 500 ($[\alpha]_D^{25}$ = +199°.g.dm.mL^{-1}),[507] the exact concentration was determined and the stock solution was adjusted to 20.0% (w/w) by addition of ddH$_2$O. Care was taken to gently swirl the solution and avoid creating air bubbles.

The two stock solutions described were used as follows. The 21-g phase system was prepared in a 50 mL plastic centrifuge tube by weighing 7.680 g of 20.0% (w/w) dextran 500, 3.840 g of 40.0% PEG-4000, 6.720 g of 1 M sorbitol, 600 mg of 200 mM potassium phosphate (pH 7.4), 300 mg of 10 mM potassium EDTA,

and 1.860 g of ddH$_2$O. For convenience in later experiments, all volumes were increased by 20%. The 21-g solution was mixed and stored at 4 ℃.

The 30-g phase system was prepared by weighing 9.600 g of 20.0% dextran 500, 4.800 g of 40.0% PEG-4000, 9.600 g of 1 M sorbitol, 750 mg of 200 mM potassium phosphate (pH 7.4), 375 mg of 10 mM potassium EDTA and 4.875 g of ddH$_2$O. After mixing well, the phase system was allowed to settle at room temperature until two phases appeared. The process could be accelerated by low speed centrifugation (1,500 g, 3 min). The lower phase was collected without disturbing the interface and stored at 4 ℃ (fresh lowe r phase).

The following buffers were used in the preparation of rat brains. Homogenization was carried out using 320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris HCl (pH 7.4) (buffer A), supplemented with protease inhibitors (PMSF 1 mM, E-64 10 μM, Pepstatin A 10 μM, TPEN 1 μM). Resuspension of the synaptosomal pellet was carried out in 320 mM sorbitol, 0.1 mM potassium EDTA, and 5 mM potassium phosphate (pH 7.4) (buffer B).

Starting with 4 rat brains, homogenization was conducted as described in Section 3.1 using 15 mL buffer A per rat brain: The crude homogenate was centrifuged at 1,500 g (5 min), the supernatant (S1) was transferred to a new tube and the pellet was discarded.

The S1 fraction was centrifuged at 1,500 g (5 min), to give a cleaner supernatant (S1-clean) and the pellet was discarded.

The S1-clean fraction was centrifuged at 17,000 g (10 min), the supernatant (S2) was kept aside and later centrifuged at 100,000 g (50 min) to provide the microsomal fraction (P3) and the cytoplasmic fraction (S3).

The pellet (P2, crude synaptosomal pellet) was resuspended in 30 mL of buffer B and centrifuged at 12,000 g (10 min) and the supernatant was discarded.

The pellet was resuspended in a minimal amount of buffer B. Three grams of resuspension were precisely weighed in a conical tube and were added to one 21-g phase system, creating a 24-g phase system. Typically, one 21-g phase system was used for 2 rat brains.

The 24-g phase was mixed by 20 inversions and centrifuged at 600 g (2 min). The upper phase was carefully transferred to a tube containing 9 mL of fresh lower phase from the 30-g phase system. The new phase system was mixed by 20 inversions and centrifuged at 600 g (2 min). The final upper phase and the interface was layered on 20 mL of buffer A and centrifuged at 17,000 g (10 min). The pellet was rinsed with Locke's buffer, and then resuspended in cryobuffer for storage.

5.5.3. Photolabeling of the membrane fraction

The detergent-solubilized membrane lysate was photolabeled like the cytoplasmic lysate. Briefly, 100 μL (~200 μg total protein) aliquots of detergent-solubilized membrane lysate were dispensed in 96-well plates and irradiated under the appropriate conditions (see Section 4.6.2).

For the photolabeling of intact synaptosomes and microsomes, the extracts were rinsed from cryobuffer (see Section 5.5.5) and resuspended in Locke's buffer

(154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.8 mM $NaHCO_3$, 5 mM D-glucose, 5 mM HEPES (pH 7.2))[483,484] to a total protein concentration of 0.1–0.2 mg.mL^{-1}. Small scale experiments were conducted in 100 μL reaction volume in a 96-well plate. Photolabeling was performed at 4 °C with photoAB&CR **181**, **169**, **168**, and **170** as previously described.

5.5.4. Click chemistry on intact labeled membrane proteins (Methods A and B)

After photolabeling, the content of each well was transferred to an 1.5 mL Eppendorf tube, spun down (14,000 rpm, 10 min), resuspended in 200 μL 25 mM HEPES (pH 7.4), spun down again (14,000 rpm, 10 min), and the washing process was carried out one more time.

Using Method A, the pellet was resuspended in 30–50 μL of 25 mM HEPES buffer (pH 7.4) supplemented to the desired concentration of detergent (5 min) and centrifuged (14,000 rpm, 10 min). Click chemistry with the desired Probe was performed on the supernatant as previously described (see Section 4.6.4).

Using Method B, the pellet was resuspended in 30–50 μL of 25 mM HEPES buffer (pH 7.4), the desired Probe and click chemistry reagents were added, and the suspension was tumbled at room temperature (1 h). Each tube was spun down (14,000 rpm, 10 min) and the supernatant was discarded. The pellet in each tube was rinsed twice with 200 μL of 25 mM HEPES buffer (pH 7.4). The final pellet was resuspended in 30–50 μL 25 mM HEPES buffer (pH 7.4) supplemented to 1% SDS (5 min) and spun down (14,000 rpm, 10 min). SDS loading buffer was then added to the supernatant and the proteins resolved on SDS-PAGE gel.

5.5.5. Protocol for the cryopreservation step

The slow cooling step[485] was performed as follows. A thick-wall (~4–5 cm) Styrofoam box was filled halfway with loosely packed cheesecloth. The tubes were placed in the center of the box and more cheesecloth was added on top of the samples. The Styrofoam container was closed and allowed to cool in a dry ice chest at least 5 h before transferring the slowly frozen samples to a -80 ℃ freezer box. Before performing the labeling experiment, samples were quickly brought to room temperature using a water bath (37 ℃). The samples we re then spun down (14,000 rpm, 4 ℃, 5–10 min), the cryobuffer was discarded and the pellet resuspended in Locke's buffer. These steps were repeated once, and the final pellet was resuspended to a protein concentration of ~0.1–0.2 mg.mL^{-1} (10–20 μg in 100 μL) in Locke's buffer.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

In this research project, we have designed, synthesized, and evaluated a series of molecular tools to identify the biological targets of the antiepileptic drug lacosamide. Over the course of our study, we have extended the structure activity relationship of LCM at the 3-oxy site, and the *N*-benzylamide 4' position. Based on the SAR results, we have constructed a series of LCM AB&CR analogs to interrogate the rat brain proteome and identify potential binding partners of LCM.

Using up to 11 LCM AB&CR derivatives, we have screened the brain soluble and membrane-bound proteome. We took advantage of several established protein purification methods and subcellular fractionation protocols to reduce the complexity of the brain proteome. Following this approach, we were not able to identify a protein target with a therapeutic relevance to epilepsy. Nonetheless, our methodology allowed the identification of some proteins that were stereospecifically adducted by either (*R*)-AB&CR or (*S*)-AB&CR derivatives. In some instances, the protein displayed a labeling dose-dependent competition with excess (*R*)-LCM or (*S*)-LCM. Among these were creatine kinase B, serum albumin, and a ~25 kDa cytosolic protein in too low abundance for us to identify. These results have not allowed us to further define the mechanism of action of lacosamide.

Our overall findings exemplified two potential limitations in the use of the AB&CR methodology to identify protein targets of small molecules. First, the methodology may not allow the pull-down capture of proteins expressed at low levels, although the in-gel fluorescence approach is sensitive enough to allow their detection. Second, and perhaps most important, is that the method used can only yield an interacting partner, and no guarantee exists that the adducted protein is a biomolecule with therapeutic relevance to epilepsy. Following is a general discussion on this project's observations and learnt lessons, as well as potential shortcomings of our drug target identification method. Comparisons with the work of other groups are presented, where applicable. For each section, possible suggestions for future work are highlighted.

6.1. The importance of the lysate source

The handling of the animal tissue is a crucial step in the preparation of a lysate. In our study, we used a commercial source of rat brains that were frozen on dry ice prior to overnight shipment, and stored upon arrival at -80 °C. Ideally, the animal should have been sacrificed prior to brain homogenization, as potential proteins of interest may have been denatured by a single freeze-thaw step.[424] Lacosamide is able to prevent MES-induced seizures in healthy rodents and the protein targeted by this AED likely is present in the rat brain. Nonetheless, it is possible that the expression levels of this protein are too low to detect. Thus, it may be helpful to prepare a whole brain protein lysate from animals that have been undergone MES-seizure or 6 Hz treatment prior to sacrifice. Indeed, the protein(s)

targeted by (*R*)-LCM may be upregulated in the early events or during the course of a seizure.

Along the same line, screening the rat brain proteome of healthy animals with our AB&CR analogs would, if successful, only lead to identification of protein(s) involved in the development of artificially-induced seizures (*i.e.* implicated in *epileptic* pathways). In light of the unique pharmacological profile of (*R*)-LCM and notably its activity against kindled seizures, screening the brain proteome of kindled animals may lead to the identification of proteins implicated in *epileptogenic* pathways. These proteins and their biological pathways still remain to be discovered, and together this information would provide invaluable insights into the pathology of epileptic disorders. These could potentially translate into "cures" for epileptic patients. Similarly, using genetically modified animals (Frings and DBA/2 mouse models) as a source of brain tissue, would enhance the diversity of proteins screened, and thus the chances of identifying key macromolecules implicated in audiogenic or reflex epilepsy pathways.

6.2. Other subcellular fractions and proteins for screening

Our study focused on the cytosolic and membrane fractions. Other rat brain subcellular compartments yet to be screened include the mitochondria (soluble and membrane fraction) and the nucleus (nuclear membrane and nucleoplasm). Attempts to isolate nuclei from rat brain were unsuccessful, as the traditional sucrose gradient procedure requires a swing-bucket ultracentrifuge.[508-510,425] In addition, brain nuclei are reported to be more fragile than other organs' nuclei.[510]

Several findings support the importance of nuclear proteins in epileptic pathways. A recent publication indicates that drastic changes in the brain nucleus occur during a seizure, from increased protein activity to histone modifications.[511] It is interesting to note that curcumin, an established histone acetyltransferase (HAT) inhibitor,[512] has protective effects in a variety of animal seizure models including chemoconvulsant-induced kindled seizures.[66,513,514] In addition, the established AED VPA has been shown to have a direct inhibitory activity against histone deacetylases (HDAC).[515] However, the pharmacological relationship between HDAC inhibition and the antiepileptic or neuroprotective effects of VPA still remains to be validated.

Ion channels are extensively, and rightfully, studied for interactions with AEDs. Nonetheless, some currently emerging drugs (RGB, 2DG) do not interact with any epilepsy-related VGIC, LGIC, neurotransmitter transporter, or neurotransmitter degradative enzyme. These observations should encourage researchers to probe for different biological mechanisms and to approach epilepsy from a less electrophysiological perspective. The reemergence of the ketogenic diet as a front line treatment for refractory epilepsy[222,221] and the advancement of 2DG[224,212] in clinical trials suggest a trend to tackle epileptic syndromes from the energetic standpoint. In this regard, and despite its lack of interaction with (R)-LCM, it would be interesting to determine if small molecule CKB inhibitors have a protective effect on seizures. Indeed, the CKB-catalyzed phosphotransfer reaction is the fastest way for a cell to generate ATP.[449] Studies have shown the physiological importance of CKB in seizures and that the enzyme's reaction rate dramatically increases during a seizure, while brain levels of PCr decrease by up to 50%.[449-452] This quick

generation of ATP is required for fueling and maintaining the high-energy demand of the seizure.[450-452] Inhibitors of CKB may, therefore, act as preventive agents by suppressing the seizure's energetic pathway.

6.3. Drug target identification methods

Advantages and drawbacks of electrophilic and photo-activated affinity labels used in this study have already been highlighted. We took advantage of a variety of AB moieties with different reactivity profiles to cover a wide spectrum of potential amino acid modification sites. Experimental observations from our research project show distinct protein labeling patterns for each AB moiety and support the rationale for the use of a wide range of affinity labels. Significantly, all the "selectively modified" proteins we observed in our experiments (*e.g.*, proteins showing enantiospecific labeling but not necessarily displaying competition with either (*R*)-LCM or (*S*)-LCM) were only labeled by one AB moiety. For example, serum albumin (Figure 37) and the protein targeted by (*S*)-LCM (Figure 26) were only identified with the trifluoromethyl aromatic diazirine photoAB **169**. Other proteins in the membrane-bound fraction were exclusively labeled by the alkyl methyldiazirinyl photoprobe **168** (Figure 32, Figure 33). These findings support the idea that identification of unknown proteins using an affinity label/pulldown strategy is a delicate task that requires multiple approaches. In this regard, it is impossible to have a universal affinity label.

Whether or not click chemistry is involved, activity-based protein profiling (ABPP) has developed into a powerful proteomic method. ABPP involves the use of electrophilic moieties (*e.g.*, phenylsulfonates,[359] epoxides,[358,309] beta-lactones,[516] or

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fluorophosphonates[517]) to establish the reactivity profile of specific enzymes or identify new macromolecular entities that interact with specific AB moieties. In that sense, ABPP may be viewed as a drug target discovery method where the drug target is *a reactive enzyme* and possesses activated nucleophilic residues prone to react with the AB. Using this method, proteases[518] implicated in cancer and bacterial signaling pathways have been identified.[519-521,517] The ABPP ligands preferentially target small molecule binding sites or spatially related sites, because the enzyme's reactive residue is located within this pocket.

By comparison, in an epilepsy drug target discovery project such as ours, the biological target is more likely to be a membrane-bound or soluble protein where the AED binding site *does not possess reactive amino acids*. Rather than utilizing substrate-processing catalytic binding sites, AEDs seem to interact at sites modulating protein conformation. In these cases, photoactivated ligands may be more appropriate tools to covalently modify the target via a non-nucleophilic residue, and therefore be more suited for receptor binding site identification. For example, the use of the aromatic azide photoAB led to the identification of the binding site of LVT,[208] and photoreactive analogs of tetrodotoxin (TTX) and saxitoxin (STX) helped identify key regions for VGSCs function.[522]

Finally, we did not identify any specific labeling of VGSCs, an established binding partner for (*R*)-LCM. It is, however, possible that our lysate preparation did not yield functional Na^+ channel. Indeed, extensive purification studies by Catterrall and coworkers have demonstrated the extreme sensitivity of these proteins to detergent solubilization or proteases.[429,427] Thus, it would be interesting to use our

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array of photoAB&CR agents on oocytes overexpressing VGSCs to potentially identify the channel's binding region critical for slow inactivation.[135] In addition, it would also be interesting to use a complementary screening approach to the whole brain lysate. One could, for example, use a variety of readily available neuronal and glial cell lines to screen the AB&CR agents. These less complex protein sources may increase our chances of biological target capture.

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