DETECTING DNA DAMAGE: THE SYNTHESIS OF NEW ALDEHYDE REACTIVE PROBES FOR THE QUANTITATION OF APURINIC/APYRIMIDINIC SITES

John R. Ridpath

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering.

Chapel Hill
2006

Approved by

Advisor: Dr. Louise M. Ball

Reader: Dr. Avram Gold

Reader: Dr. Jun Nakamura

ABSTRACT

JOHN R. RIDPATH: Detecting DNA Damage: The Synthesis of New Aldehyde Reactive Probes for the Quantitation of Apurinic/Apyrimidinic Sites
(Under the direction of Dr. Louise M. Ball and Dr. Avram Gold)

Apurinic/apyrimidinic (abasic) sites are some of the most common lesions found in DNA. They are postulated to be intermediates of mutagenicity and carcinogenicity. Each human genome can have as many as 10^4 abasic sites per day. It is therefore important to be able to quantitate the number of these sites. Abasic sites are quantitated by an aldehyde reactive probe (ARP) assay highly specific for the aldehyde group of the ring-opened d-ribose. This assay is based on formation of a Schiff base adduct of a hydroxylamine on the probe end of the aldehyde.

The purpose of this project is to synthesize new ARPs that enhance reactivity without loss of specificity. A two-step synthetic scheme was devised to give a compound which replaces the hydroxylamine group with a hydrazino group expected to be more reactive. A long-chain ARP containing a polyethylene glycol moiety was also synthesized.

This thesis is dedicated to Dr. Ramiah Sangaiah – my mentor and my friend.

ACKNOWLEDGEMENTS

No endeavor of this sort can be successful, especially in the sciences, without the support, guidance and help of many people. First and foremost on this long list is my advisor, Dr. Louise Ball, who accepted me into the program, provided me with a project and the backing to bring the project to a close. Dr. Ball's knowledge, humor, common-sense and accessibility played no small role in making this project a success as well as enjoyable. Next, I wish to acknowledge my indebtedness to Dr. Avram Gold, my research advisor, for his continued attention to and interest in this project. His knowledge and willingness to impart that knowledge is extraordinary. I am also grateful for his accessibility and patience while I was making my way up the learning curve. I also am deeply indebted to the late Dr. Ramiah Sangaiah without whose mentoring I simply would not have been able to make my way in a chemistry laboratory. He was a kind, gentle, caring man who has been greatly missed. Thanks also to Dr. Karupiah Jayaraj who helped me through much of the tedium of the chemistry laboratory. Thanks to Dr. Jun Nakamura for conceiving this project along with having ideas for the new DNA probes and for being a reader of this thesis. Further thanks go to Yutai Li and Gunnar Boysen of the mass spectrometry laboratory. The project also would not have been possible without backing from the Super Fund Basic Research grant.

Last, and of course not least, I owe so much to my wife, Alice, that it could not possibly be repaid. I have made life much more difficult at a time of life when most people are hoping to wind down a bit.

TABLE OF CONTENTS

LIST OF FIGURES

I Introduction

The DNA code contains all of the information necessary to direct each cell of an organism to grow, perform its function as it relates to the organism, and reproduce itself. The code is manifested as a string of four chemical bases - adenine, cytosine, guanine, and thymine, which ultimately direct the production of proteins responsible for catalyzing most of the reactions within the cell. Any alteration (mutation) of the strict order of these DNA bases creates the risk that the protein coded for in that region will be changed in such a way so as to lose its functionality or have that functionality reduced to some degree. It is also possible that the protein could obtain a change in its function and become oncogenic. This loss or change of function can cause cell death (apoptosis), cause the inability of the cell to perform its proper function, or cause the cell to lose its normal reproductive controls and become tumorigenic or carcinogenic. Therefore, it is of utmost importance that mutations or any other damage to DNA be kept to a minimum so that stability of the genome can be maintained.

Due to its chemical nature, DNA is susceptible to damage by both exogenous and endogenous chemicals and ionizing radiation. There are an estimated $10^4 – 10^6$ DNA lesions per human cell per day (Sukhanova, et al., 2004.). The damage may take the form of chemically altered bases or sugars, chemically adducted bases, bases that are removed forming abasic sites (also known as apurinic/apyrimidinic, or AP sites), and single (SSB) and double strand breaks (Demple and Harrison, 1994). AP sites are a prevalent type of DNA

lesion. They arise from the cleavage of the glycosidic bond (between the DNA base and its corresponding deoxyribose), by the action of a DNA glycosylase (during base excision repair), by spontaneous depurination/depyrimidination of labile DNA adducts, by chemically induced depurination/depyrimidination or even by unmodified bases (Nakamura, 1998).

DNA repair mechanisms have evolved to remove damaged sites and restore the proper sequence while other mechanisms can bypass unrepaired lesions. Repair mechanisms include direct damage reversal, base excision repair (BER), nucleotide excision repair, various types of strand break repair such as nonhomologous end joining and homologous recombination, and mismatch repair (Paz-Elizur, et al., 2005).

BER is an essential repair mechanism of prokaryotic cells that acts continuously to mend single site lesions that are spontaneously occurring and also induced by hydrolysis, oxygen free radicals and alkylating agents (Wood, 1996). While BER is thought to repair damage caused mainly by endogenous pathways there is a long list of environmental agents that can cause the same type of damage (Nilson, et al., 2001). The initial event in BER is the removal from the deoxyribose-phosphate backbone of the damaged base by hydrolysis of the *N*-glycosidic bond utilizing one of many DNA glycosylases (Lindahl, 1993). This produces an AP site which can be mutagenic itself if the repair process is not continued with rapidity. Imbalances due to alterations to the enzymes in BER are known to cause mutations such as insertion of an incorrect base opposite an AP site. The next step in BER is the action of the enzyme, AP endonuclease, which creates a 5' nick or SSB in the DNA strand at the AP site. This is followed by the removal of the 5'-deoxyribose-phosphate completing the excision of the DNA repair intermediate. The repair is completed by Polymerase β (Pol β) and then

Ligase III (Lig III) that add the correct nucleotide and seal the resulting 3' nick respectively (Lindahl, 1993).

It has been shown that depurination to form AP sites is about 100 to 500 times greater than depyrimidination (Loeb and Preston, 1986). Although rare, it has also been shown that translesion synthesis of DNA can occur and that purines, especially adenine, are preferentially inserted opposite an AP site which leads to G:C to T:A and A:T to T:A transversion mutations (Schaaper, et al., 1983).

Since AP sites have been determined to be the most common lesion in DNA and therefore an important route to mutagenesis and carcinogenesis, researchers have found it beneficial to be able to quantitate them (Ide, et al., 1993). To this end many different assays have been developed over the last few decades. Most of these methods have not been proven to be wholly satisfactory as they are labor intensive, involve the use of radioactive materials or are not sensitive enough to detect low levels of AP sites. In 1992, Kubo, et al., developed an assay which makes use of a novel aldehyde reactive probe (ARP) which reacts with the aldehyde formed on the abasic deoxyribose. This biotinylated compound allows AP sites to be quantitated colorimetrically by an ELISA-like procedure using an avidin/biotin complex conjugated to horseradish peroxidase as an indicator system. This method is much simpler than previously described assays and Kubo reported comparable sensitivity. Ide, et al. (1993), characterized the ARP further and reported excellent specificity for the aldehyde on the deoxyribose. Five years later an improved method using ARP combined with a slot-blot assay was reported by Nakamura, et al. (1998).

A known problem with any AP site assay is that due to incubations at elevated temperature artifactual AP sites may be formed which will result in over-estimates of the lesions. This

research reports on the synthesis of novel ARPs in an attempt to increase reactivity and possibly sensitivity without losing specificity. If the reaction rate can be increased the reaction time can be reduced producing fewer artifacts. Three new ARP reagents have been synthesized; one which is similar to the original ARP but makes use of imino biotin rather than standard biotin (previously prepared in this laboratory), another which alters the aldehyde reactive end of the molecule from a hydroxylamine group to a hydrazino and a third reagent which uses a polyethylene glycol (PEG) chain to link the hydroxylamine to the biotin moiety.

II Literature Review

AP Site Formation

Once it was found that DNA contains all of the genetic information necessary to direct all of the activities of the cell – from growth to reproduction to even its death – it was assumed that the DNA molecule must be extremely stable in order to maintain the fidelity needed for a master blueprint. It thus was a surprise to learn that the DNA structure is actually dynamic and subject to constant change (damage and mutation) (Friedberg, 1995). The sources of damage to DNA can be exogenous (environmental) as well as endogenous (spontaneous). The damage may take the form of chemically altered bases or sugars, chemically adducted bases, bases that are removed forming AP sites, SSBs and double strand breaks (Demple and Harrison, 1994).

AP sites, some of the most prevalent lesions in DNA, are derived from the hydrolytic cleavage of the glycosidic bond either spontaneously or by one of many DNA glycosylases involved in DNA repair (Nakamura and Swenberg, 1999). AP sites can also be produced by chemical modification of bases by carcinogens and alkylating agents that destabilize the glycosidic bond and by ionizing radiation (Ide, et al., 1993). The formation of these sites is enhanced by the fact that the glycosidic bond is made more labile by the lack of the 2'-OH group in deoxyribose (which, however, greatly increases resistance of the phosphodiester bond) (Lindahl, 1993). It has been estimated that 10,000 AP sites occur in each human cell

per day (Lindahl and Nyberg, 1972). If these sites are not accurately and rapidly repaired they can lead to cell death or mutation of the DNA (Kunkel, 1983; Loeb and Preston, 1986).

Spontaneous Damage

Spontaneous damage to DNA is by definition the result of endogenous cellular processes. Spontaneous alterations and damage to DNA are caused by: mispairing of bases during replication, or mismatches; tautomeric shifts within bases; deamination of bases; loss of bases (depurination/depyrimidination); alkylation; and, attack by reactive oxygen species (Friedman, 1995). All appear to be able to cause AP site formation.

Tautomeric Shifts

All DNA bases can exist in relatively rare tautomeric forms (Langer and Doltsinis, 2003) (Figure II-1). A base tautomer occurs when one of the hydrogen atoms changes location such as when the N^6 amino group of adenine tautomerizes to the imino form or the N^6 keto group of guanine tautomerizes to the enol (Berg, et al., 2002). Tautomers are capable of mispairing as when the enol form of guanine pairs with the normal keto form of thymine or when the imino form of adenine pairs with the normal amino form of cytosine. The latter, for instance, can result in an A-T to G-C transition mutation upon replication.

Base Deamination

Of the four bases normally present in DNA, three have exocyclic amino groups. Deamination of these groups occurs spontaneously in certain conditions of pH and temperature and results in the conversion of cytosine, adenine, guanine and 5-methylcytosine to uracil, hypoxanthine, xanthine and thymine respectively (Anders and Dekant, 1994). Since some of these deamination products are miscoding lesions during DNA synthesis they can give rise to altered base pairs and mutations. For instance, if the cytosine to uracil

alteration were to be left unrepaired, it would result in a G-C to A-T base pair transition upon semiconservative DNA synthesis. The repair mechanism to remove uracil from DNA utilizes a repair enzyme, uracil glycosylase that removes the incorrect base, once again leaving an AP site (Figure II-2).

Loss of Bases

In a landmark study by Lindahl and Nyberg (1972) it was found that small but detectable amounts of purine bases are continuously released from DNA at elevated temperatures and physiological pH of 7.4. As it was difficult to directly determine the low rate of depurination at 37 ^{o}C and pH 7.4 with their methodology they extrapolated from data obtained at higher temperatures and estimated cells undergo 3 x 10^{-11} depurinations/nucleotide/second. This estimate was confirmed using DNA synthesized in vitro with labeled purines, as well as spectroscopically with unlabeled DNA (Loeb and Preston, 1986).

Oxidative Damage to DNA

Oxidative stress, or attack by reactive oxygen species (ROS) is a major source of spontaneous damage to DNA (as well as to proteins and lipids) (Pitot and Dragan, 1996). ROS appear as both oxygen radicals and nonradical oxygen derivatives. Examples of oxygen radicals are, the superoxide ion, the hydroxyl radical, the peroxyl radical, the alkoxyl radical and nitric oxide. Some nonradical oxygen derivatives are hydrogen peroxide, hypochlorous acid, ozone and peroxynitrite (Kohen and Nyska, 2002). Sources of ROS are both exogenous and endogenous. Exogenous sources include γ irradiation, UV radiation, food, drugs, pollutants, xenobiotics and toxins. Endogenous sources are cells (e.g., neutrophils), direct-producing ROS enzymes (e.g., NO synthase), indirect-producing ROS enzymes (e.g., xanthine oxidase), metabolism (e.g., mitochondria) and diseases (Kohen and Nyska, 2002).

The major intracellular source of oxygen radicals is most likely as byproducts of aerobic metabolism as the oxygen is being reduced to water during mitochondrial respiration (Riley, 1994). Most of the damage caused by ROS to DNA is attributable to the hydroxyl radical (Ward, 1988), a strong oxidizing agent that reacts with most organic and inorganic molecules in the cell (Kohen and Nyska, 2002). Cells have developed numerous defense mechanisms to protect against damage from reactive oxidative metabolites. Some of these include antioxidant defense (e.g., various enzymes, scavengers, chelating agents), physical defenses (e.g., stabilization of biological sites, steric interference) and, once again, DNA repair mechanisms which can produce AP sites (Kohen and Nyska, 2002)

Environmental Damage

Environmental, or exogenous, causes of AP site formation are due to either ionizing radiation or chemical induction.

Ionizing Radiation

Since the beginning of the evolution of life there has been exposure to and damage caused by ionizing radiation. Humans are exposed to low doses of radiation during air travel, from radon in homes, or in areas of low-level contamination including former sites of nuclear weapons production and can encounter much higher radiation doses in contaminated areas such as sites of nuclear accidents or radiotherapy (Sutherland, 2000). Ionizing radiation produces effects on DNA both through free radicals (indirectly) and direct action which include; SSBs, DSBs, damage to or loss of bases and cross-linking of DNA to itself or proteins (Montelone, 1998).

The radiolysis of water and generation of hydroxyl radicals along a track of ionizing radiation can yield clustered sites of base damage in both strands, and attempts to correct

such damage by standard BER can result in a DNA double-strand break (Lindahl, 1999). The high water content in cells suggests that the species formed from the radiolysis of water are the major source of indirect DNA damage. It has been estimated that more than 80% of the energy of ionizing radiation deposited in cells results from the abstraction of an electron from water which then leads to several other species (Friedman, 1995), including the most harmful hydroxyl radical.

Direct base damage is also caused by ionizing radiation in a way similar to the damage caused by the hydroxyl radical. It has been suggested that the direct action of ionizing radiation may lead to the ejection of an electron from the unsaturated C-5 or C-6 position (as in thymine) and the resulting cation radical may further react with a hydroxyl ion (Ward, 1988). Also, damage to DNA bases such as ring saturation can result in destabilization of the glycosidic bond and the formation of abasic deoxyribose residues (Teoule, 1987). Some of the bases damaged either indirectly or directly by ionizing radiation (such as 8-Hydroxy-guanine) are recognized and removed by glycosylases which are active in the BER pathway and thus form AP sites (Demple, 1991).

Chemical Agents

The earliest studies of the interactions of chemicals with DNA may have been in regard to how injurious and lethal chemical warfare agents act on the molecule (Brookes, 1990). Further advancement came from cancer chemotherapy which is based on the idea that damage to DNA can interfere with normal replication and can lead to the arrest of rapidly proliferating cell populations such as cancer cells (Friedberg, 1995). More recently, there has been a rapidly growing awareness of the effects of environmental genotoxic agents as evidenced by the tremendous amount of literature devoted to the biological actions of these

chemicals. The basic instability of the *N*-glycosydic bond can be enhanced by any chemical modification that changes the base into a better leaving group which would increase the rate of AP site formation (Loeb and Preston, 1986). DNA damaging chemicals come in many forms such as, alkylating agents, cross-linking agents, psoralens and chemicals that are metabolized to electrophilic reactants. Specifically, alkylating agents and metabolites of xenobiotics can cause AP sites.

Alkylating (methylating and ethylating) agents are electrophilic compounds with affinity for the nucleophilic centers in DNA (Friedberg, 1995). Many of these chemicals are proven or suspected mutagens. There are numerous reaction sites located on the DNA bases that could potentially be alkylated: in adenine, N^1, N^3, N^6, and N^7; in guanine, N^1, N^2, N^3, N^7, and O^6; in cytosine, N^3, N^4, and O^2; and in thymine, N^3, O^2, and O^4 (Roberts, 1978). Of these, the N^7 position of guanine and the N^3 position of adenine are the most reactive and alkylation at these sites causes the formation of an unstable quaternary ammonium ion intermediate that can be stabilized via resonance and glycosidic bond cleavage (Roberts, 1978). For instance, the formation of *N-7*-methylguanine is known to result in spontaneous depurination and AP site formation (Pieper, 1998.) Base modification by alkylation can increase the rate of depurination/depyrimidination by as much as six orders of magnitude (Wilson and Barsky, 2001).

All cells are continuously exposed to foreign chemicals (xenobiotics) which are both anthropogenic and naturally occurring and include drugs, pesticides, pollutants, pyrolysis products in cooked foods, alkaloids, secondary plant metabolites, and toxins produced by molds, plants and animals (Parkinson, 1996.). The cell reacts to the introduction of many hydrophobic xenobiotics by metabolizing these compounds to more polar forms that are

water-soluble (a process known as biotransformation) and thus easier to excrete. Many of these metabolized compounds are more reactive and, as in the case of alkylating agents, can interact with the nucleophilic centers in DNA bases (Anders and Dekant 1994.), eventually producing AP sites.

DNA Glycosylases

Although some types of DNA base damage can be repaired directly, most damage is repaired by removal of the damaged base or nucleotide. A repair mechanism known as base excision repair (BER) begins by the removal of the damaged or inappropriate base by making use of one of many glycosylases (Lindahl, 1976). These enzymes catalyze the hydrolysis of the glycosidic bond that links the base to its deoxyribose creating a free base and an AP site. An example of such catalysis is the removal of the inappropriate base, uracil, by uracil-*N*-glycosylase. BER, being a coordinated multistep process, then requires the action of several more repair enzymes, the first of which is an AP endonuclease 1 (APE1) that cleaves the DNA backbone on the 5' side of the AP site (Paz-Elizur, et al., 2005). Ape1 then recruits a DNA polymerase, POL β which releases the 5' remnant deoxyribose-phosphate and inserts the proper nucleotide. POL β then recruits a protein complex (XRCC1/LIG3) that seals the remaining nick in the DNA strand, completing the repair (Lindahl and Wood, 1999).

The Chemistry of AP Sites

An AP site exists as an equilibrium between three chemical species; an open-chain aldehyde, an open-chain hydrate and cyclic hemiacetals (α and β) (Wilde, et al., 1988)

Figure II-3). Manoharan, et al., (1988) characterized abasic sites to show that the two hemiacetal anomers are found in equal amounts and constitute the majority of species with open-chain aldehydes making up only about 1% (Wilde, et al., 1989). The aldehydic species is, however, predominant in terms of reactivity (Doetsch and Cunningham, 1990).

AP sites are susceptible to several different chemical attacks. A β-elimination reaction catalyzed by a nucleophile results in phosphodiester bond cleavage adjacent to the AP site creating an α,β-unsaturated aldehyde at the 3'-terminus of the nick and a C-5 phosphorylated deoxyribose at the 5'-terminus (Doetsch and Cunningham, 1990) (Figure II-4). The α,β-unsaturated aldehyde can then rearrange in alkaline conditions to form a 3'-2-oxocyclopent-1-enyl terminus (Jones, et al., 1968) or undergo a δ-elimination to yield a one-nucleotide gap with 3'-phosphoryl and 5'-phosphoryl DNA termini and a free 4-hydroxy-pent-2,4-dienal.

The α,β-unsaturated aldehyde remaining after a β-elimination is very reactive and nucleophiles can add to it readily (Doetsch and Cunningham, 1990). Manoharan, et al. (1988), reported the reaction of thiols with the aldehyde, and it has been shown that hydroxylamines will likewise react (Ide, et al., 1993), an interaction taken advantage of in the aldehyde reactive probe assay for AP sites to be discussed later. The reaction of thiols can compete with the δ-elimination reaction previously mentioned (Bailly and Verly, 1988).

Enzymatically, AP sites are cleaved on the 5' side by APE1, which leaves a deoxyribose 5'-phosphate and 3' hydroxyl residue that primes DNA repair synthesis (Wood, 1996). APE1 contains a conserved Asp-His pair that deprotonates a water molecule for nucleophilic attack on the DNA phosphate 5' of the abasic site (Mol, et al., 1999). The resulting hydroxide ion attacks the phosphate to form a pentacovalent intermediate after which the P-O-3' bond is

broken (Figure II-5). The putative mechanism involved in the cleavage 3' to the AP site by AP lyase begins with a nucleophilic attack on the aldehyde carbonyl by the amine of a lysine residue eventually forming an iminium ion product (Schiff base) (Figure II-6). This charged imine is susceptible to nucleophilic attack and a base-catalyzed proton abstraction at the α-carbon initiates a β-elimination resulting in the 5' cleavage. Hydrolysis then liberates the protein leaving the 4-hydroxy-2-pentenal at the 3' terminus (Doetsch and Cunningham, 1990).

Biological Consequences of AP Sites

AP sites are non-coding lesions and, as such, are both cytotoxic and mutagenic. They threaten the viability and integrity of the cell.

Cytotoxicity of AP Sites

Cytotoxicity due to AP sites may be caused by DNA polymerases pausing and dissociating on encountering the lesions. During replication this would lead to chromosomal strand breaks or, if during transcription, non-production of a viable protein product. If these events occur at high enough frequency the cell would be dysfunctional or not be able to survive (Wilson and Barsky, 2001). An AP sites' cytotoxicity may also be due to its ability to promote or inhibit the DNA cleavage activity of topoisomerases (Kingma and Osheroff, 1998).

Mutagenicity of AP Sites

All mutations consist of heritable changes in the sequence of bases in DNA, and may be spontaneous or induced by mutagens (Lewin, 2004). A point mutation changes a single base pair and can be caused by chemical alteration of a base or by an error during replication. The base pair change can be a transition where one purine is replaced by the other purine or when a pyrimidine is replaced by the other (G:C to A:T or vice-versa) or a transversion where a

purine is swapped for a pyrimidine or vice-versa (such as; G:C to T:A, et al.). Other types of mutation include insertions or deletions (frameshift mutations) of one or more base pairs and repeats, mainly of triplets (three base pair sequences that may repeat up to hundreds of times).

Mutations can have several different effects on the gene product. The effects can be either deleterious when they cause cancer or other genetic diseases or advantageous when they provide for genetic diversity. A *null* mutation completely eliminates the gene function and if that function is fundamental the mutation is lethal. A *loss-of-function* mutation inactivates a gene and is recessive. A *gain-of-function* mutation causes the gene product to take on a new function and is dominant. A *leaky* mutation can reduce the function of the protein but enough activity remains so that there is no phenotypic effect. A mutation can also be *silent* and have no effect whatsoever. This would occur if the amino acid is not changed or, if changed, has no effect on the protein function (Lewin, 2004).

By far the majority of AP sites are successfully repaired by the BER system. Mutagenesis can occur as a result of AP sites when BER is either overwhelmed by massive damage or there is an imbalance in BER activity. A recently reported example of such an imbalance in BER is in response to folate deficiency (Cabelof, et al., 2004). Uracil has been shown to accumulate in DNA in response to folate deficiency (Blount, et al., 1997). This inappropriate base (for DNA) is normally removed by BER. Cabelof, et al. (2004), found that while uracil glycosylase is up-regulated during folate deficiency (creating AP sites as uracil is removed), the rate-limiting enzyme in the BER process, Pol β, is not up-regulated. This results in the accumulation of AP sites which are not repaired or, at least, not repaired on a timely basis.

It has also been shown that DNA and RNA polymerases, when able to bypass an AP site, preferentially place an adenine opposite the site (the so-called 'A-Rule') (Boiteux and Laval, 1982) which may result in a transition or transversion mutation. Human DNA translesion synthesis polymerases Pol η, Pol κ and POLQ also preferentially incorporate adenine opposite AP sites in vitro, although inefficiently (Masutani, et al., 2000; Zhang, et al., 2000; Seki, et al., 2004). DNA polymerases can also bypass an abasic site resulting in a deletion (Wilson and Barsky, 2001). Lyons-Darden and Topal (1999) demonstrated that abasic sites can induce triplet repeat expansions which are associated with Fragile X syndrome and myotonic dystrophy. Johnson, et al. (2000), found that Pol ι and Pol ζ act sequentially with Pol ι inserting either guanine or thymine opposite on abasic site so that Pol ζ can bypass the damage.

Quantitation of AP Sites

AP sites are common and powerful cytotoxic and mutagenic lesions of DNA and therefore the ability to quantitate them is not only interesting but important. It is the best known case of a non-informational modification to DNA and, as such, is a benchmark against which other lesions can be measured (Lawrence, et al., 1990). It would be very valuable to be able to determine the base-line level of abasic sites so that further determinations of increases in the level due to toxicants or mutagens could be made. To this end much work on several types of assay has taken place in the last fifteen to twenty years.

Sometimes one needs to break something to study it. Car makers do this when they slam their vehicles into barriers to test for crash worthiness. In similar manner molecular biologists, geneticists, etc., search for or purposely generate mutations in genes involved in processes they wish to study. If that process happens to be one of the most important DNA

repair mechanisms like base excision repair, where AP sites are an intermediate, then the ability to quantitate the sites becomes essential. The folate deficiency study discussed above is an example of the use of AP site quantitation to aid in the determination of the deficiency's deleterious effect on BER and hence its influence on carcinogenesis.

Assays for detection of AP sites after first isolating the DNA can be fraught with inaccuracies as a result of several factors: (1) artifactual AP sites resulting from base loss and formation of AP sites in DNA by high temperature at neutral pH; (2) AP sites can be lost and levels may be underestimated as they are susceptible to β-elimination followed by δ-elimination at high temperatures, primary amines in histones, polyamines and thiols (Atamna, et al., 1999).

Alkali Treatment

The number of AP sites can be measured by subjecting the DNA to alkaline hydrolysis (Lindahl, 1981). Brent, et al. (1978), described such a method whereby the AP sites are converted into single strand breaks by incubation with NaOH. The number of strand breaks is then estimated by the proportion of nicked DNA by alkaline-CsCl sedimentation analysis (Teebor and Duker, 1975). A problem with this method is that it only detects intact AP sites. Any sites that were previously nicked will not be counted by this assay.

Chemical Modification

Another assay takes advantage of the fact that the aldehyde group present on the deoxyribose of an abasic site will react with (^{14}C)methoxyamine (Talpaert-Borle' and Liuzzi, 1983). The unreacted methoxyamine is removed by acid treatment leaving the acid-insoluble ^{14}C-labeled DNA. The radioactivity is then measured in a scintillation spectrometer to determine the number of AP sites.

Another chemical modification method has been reported much more recently in which Roberts, et al. (2006), describe a method in which AP site quantitation has been performed by prelabeling the sites with *O*-4-nitrobenzylhydroxylamine (NBHA) combined with a mass spectrometry technique. Once labeled with NBHA the DNA is enzymatically digested to monomeric subunits which are then isolated and detected with high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). The limit of detection for this assay is reported as 3 AP sites per 10^7 bases.

^{32}P-Postlabeling Assay for AP Sites

Weinberg, et al. (1990), have described a postlabeling method for measuring AP sites. In the beginning AP sites are reacted with methoxyamine to stabilize them for ensuing steps that entail long incubations at elevated temperature. This is done to prevent generation of AP sites during the incubations. The DNA is then digested to dinucleosides (designated d-NpM) and those with AP sites are separated. To generate labeled markers the dinucleotides are incubated with ^{32}P-labeled ATP using bacteriophage T4 polynucleotide kinase to get d-pNpM. Aliquots of each of the four d-pNpM markers are mixed and applied to a polyacrylamide/urea gel. The gel is run and an autoradiogram taken and the gel containing radioactive material is excised. The radioactive material is extracted from the gel and its activity counted and further resolved by HPLC. Advantages of this method are that it does not require pre-labeled DNA and it needs a very small amount of DNA for the assay. Disadvantages are that the method requires the use of radioactive materials and it is very labor intensive and time consuming compared to some of the other methods.

Aldehyde Reactive Probe Method

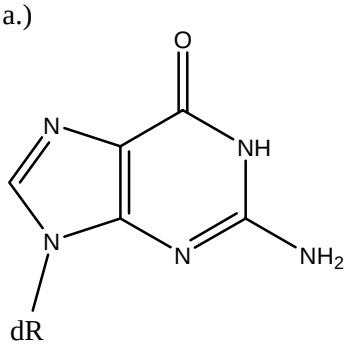
The aldehyde reactive probe (ARP) method was first described by Kubo, et al. (1992) and further improved by Ide, et al, (1993). The probe is a biotinylated reagent designed to react with the aldehydic carboxyl of the d-ribose in the AP site (Figure II-7). The reagent makes a nucleophilic attack by using a hydroxylamine group to form a stable Schiff base with the aldehyde (Figure II-8). The biotin group on the ARP-tagged AP sites is then reacted with avidin, a protein that has both strong affinity and high specificity for biotin. The avidin/biotin complex is conjugated to horseradish peroxidase. The number of AP sites is then determined colorimetrically in an ELISA-like assay using the avidin/biotin-horseradish peroxidase complex as an indicator. Kubo reported sensitivity (one AP site per 10^4 nucleotides) comparable to other methods. The ARP method has several advantages over others that have been reported. It requires no radioactive labeled DNA as in the alkali elution and DNA unwinding methods and is less cumbersome to set up than either. The ^{32}P-labeled method has no need for pre-labeled DNA, but is more difficult to set up than an ELISA. The monoclonal antibody assay to determine *O*-(nitrobenzyl)hydroxylamine tagged AP sites is comparable to the ARP method in terms of simplicity and sensitivity but the latter obviates the need for primary and secondary antibodies.

More recently, Nakamura, et al. (1998), developed an alternative assay, also making use of ARP, but combined with a slot-blot technique (ARP slot-blot assay, ASB). In this method the ARP-tagged DNA is immobilized on a BAS-85 NC membrane. The membrane is then incubated with strepavidin conjugated horseradish peroxidase. After incubation the membrane is rinsed and enzymatic activity is visualized with ECL reagents. The NC membrane was then photographed and analyzed using a densitometer. Quantitation was

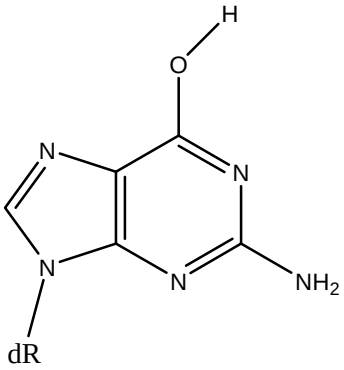
performed by comparison with an AP site standard. The ASB method was determined to have an AP site detection limit of 0.24 AP sites/10^6 nucleotides – an improvement over the previous method of one to two orders of magnitude.

In 1999 Atamna, et al., described a method for detecting abasic sites in living cells using the ARP. It was found that the ARP penetrates the plasma membrane and reacts with AP sites to form a stable ARP-DNA adduct. The DNA is then isolated and treated with avidin-horseradish peroxidase (HRP) forming a DNA-HRP complex at each AP site. The free HRP is then separated by selective precipitation with a DNA precipitating dye (DAPER). The number of AP sites can then be estimated by HRP activity toward chromogenic substrate in an ELISA.

a.)

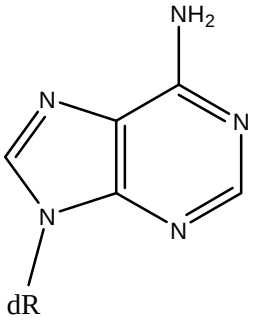


Normal Guanine

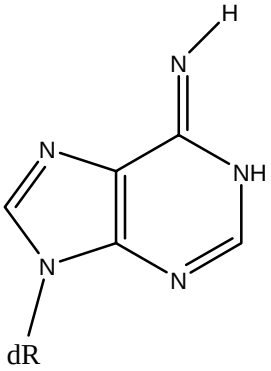


Enol Guanine Tautomer

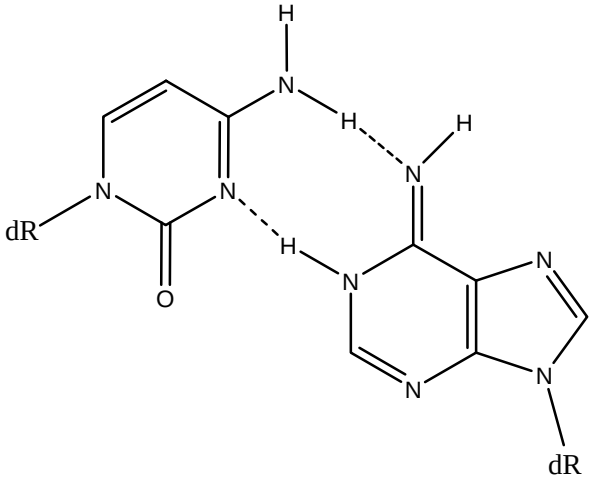
b.)



Normal Adenine



Imino Adenine Tautomer



Imino tautomer of adenine pairing with normal cytosine

Figure II-1 a.) Examples of DNA base tautomerization. b.) Example of abnormal base pairing due to tautomerization.

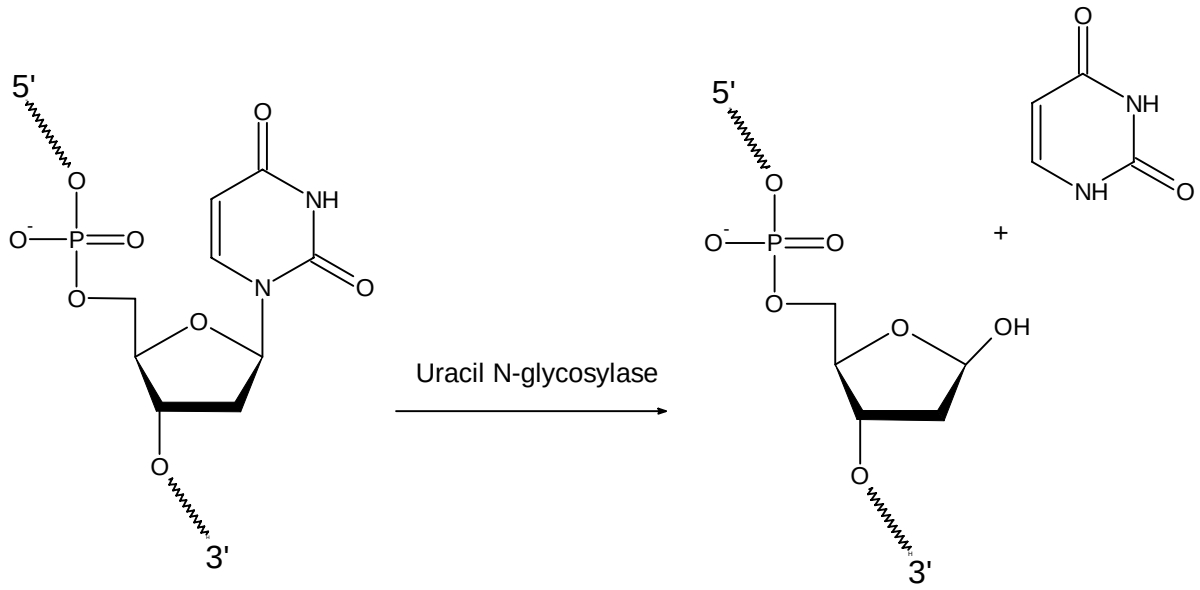


Figure II-2 Formation of an AP site in DNA by enzymatic removal of uracil.

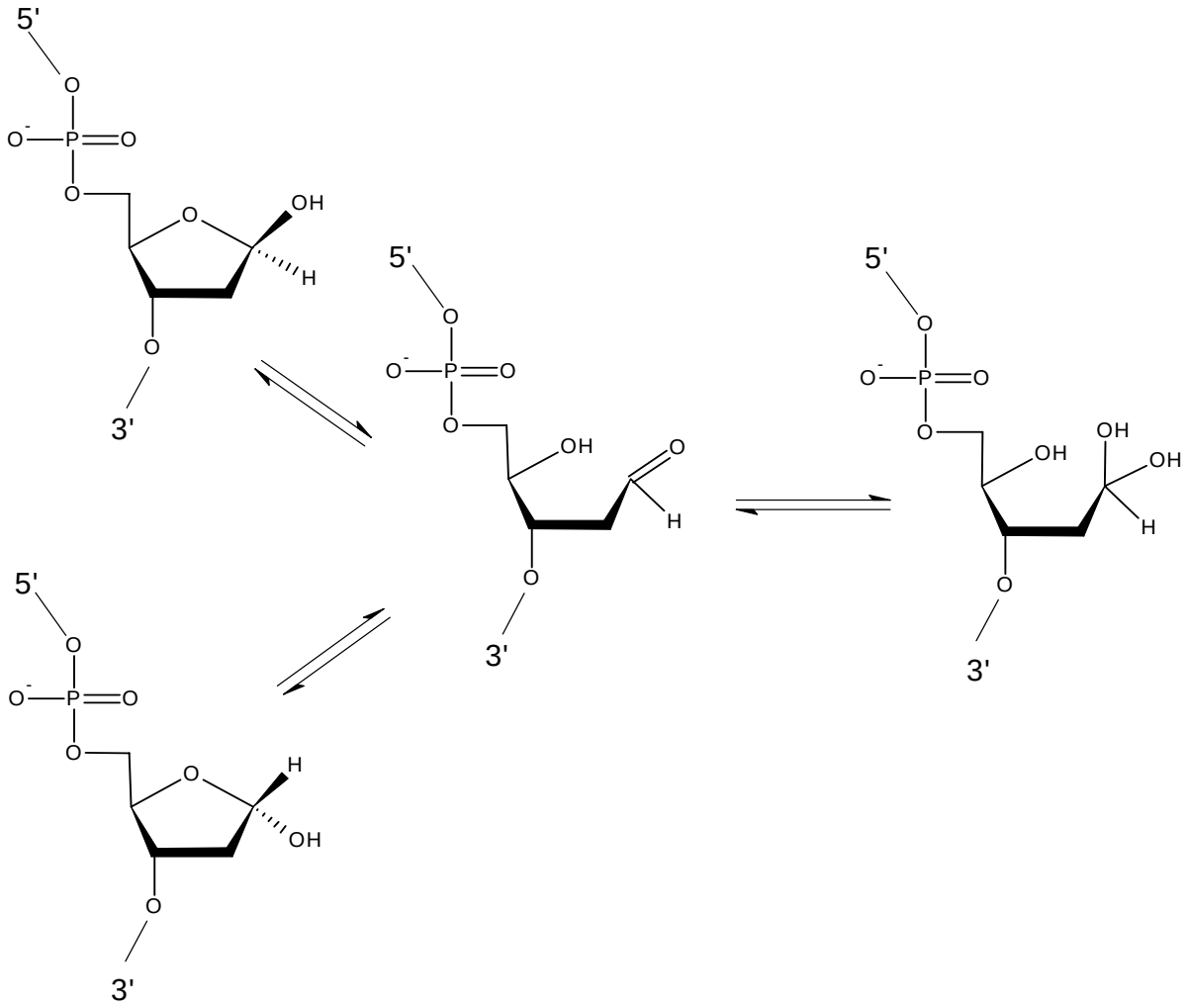


Figure II-3 Cleavage of the *N*-glycosidic bond results in the aldehydic form in equilibrium with several closed-ring forms of the 2'-deoxyribose.

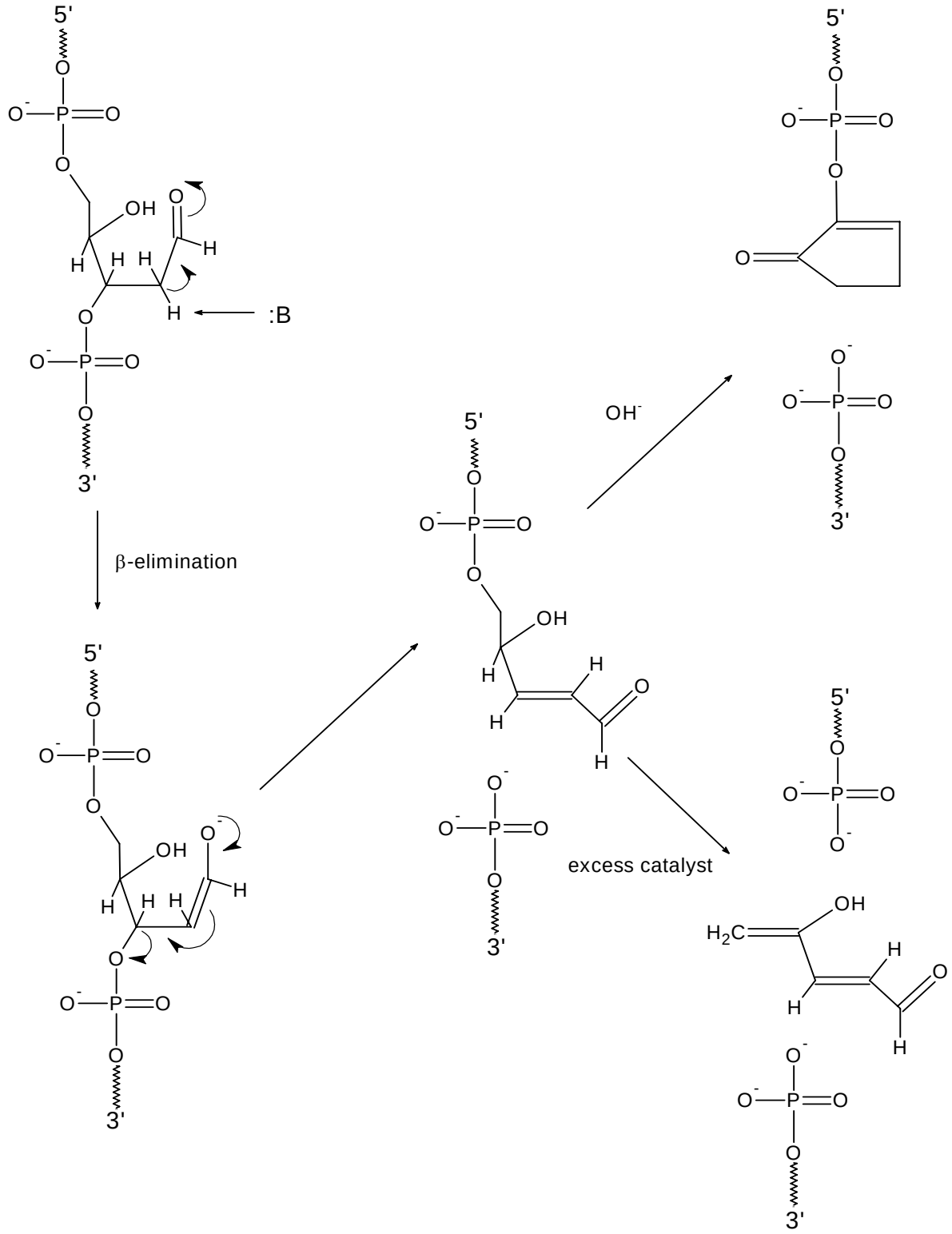


Figure II-4 AP site chemical reactions.

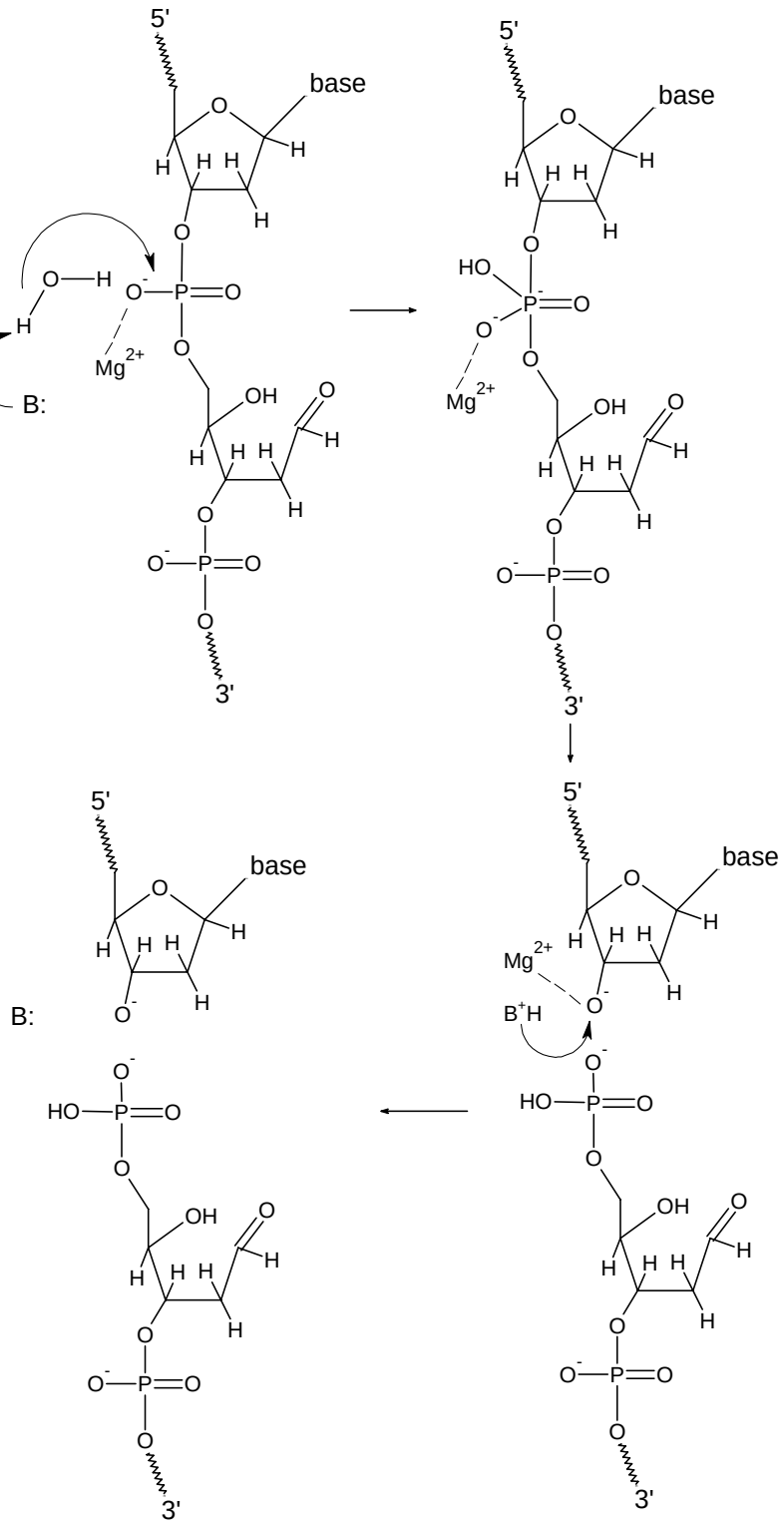


Figure II-5 5' cleavage of AP site by AP endonuclease 1 (adapted from Doetsch and Cunningham, 1990)

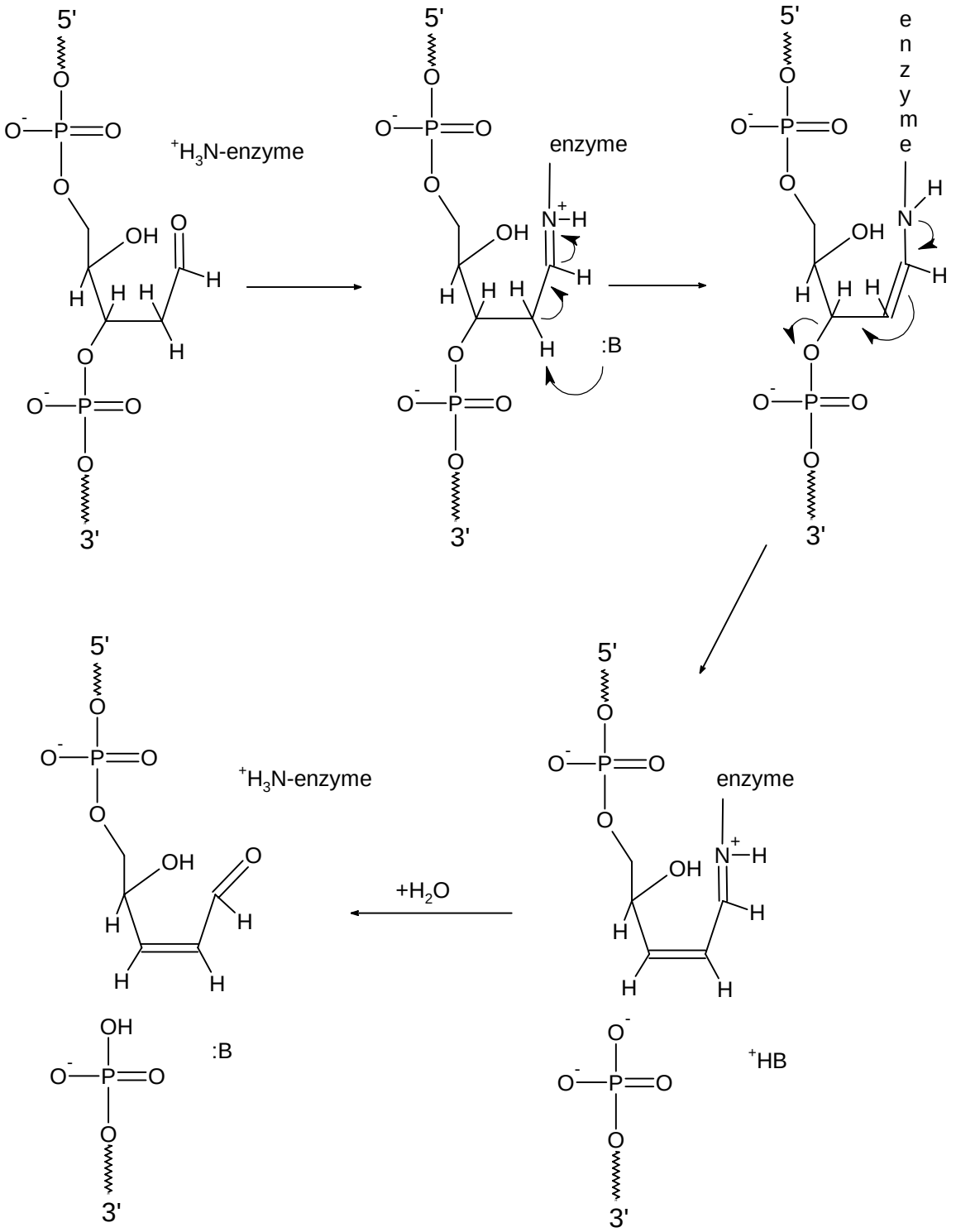
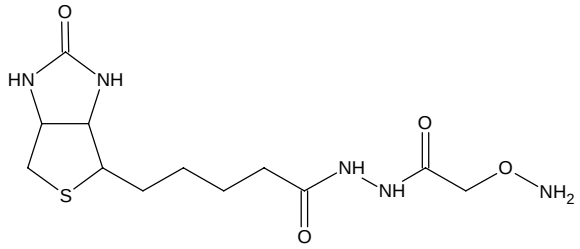
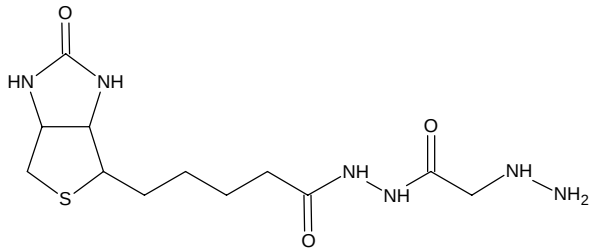


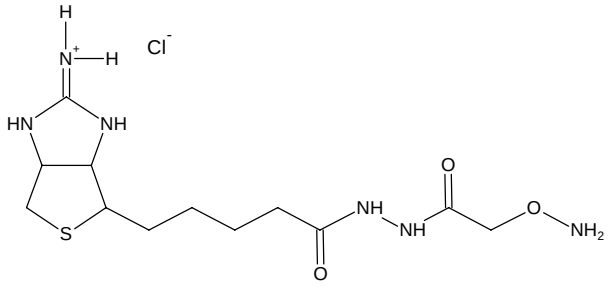
Figure II-6 Hydrolysis of an abasic site by β– and δ– elimination.



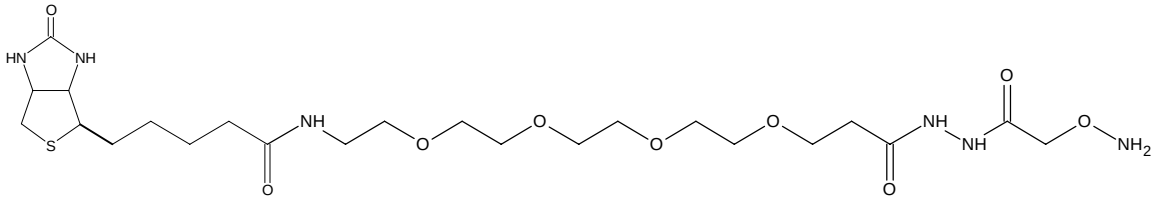
Aldehyde Reactive Probe



Hydrazino Aldehyde Reactive Probe



Iminobiotin Aldehyde Reactive Probe



Long Chain Aldehyde Reactive Probe

Figure II-7 DNA AP site probes.

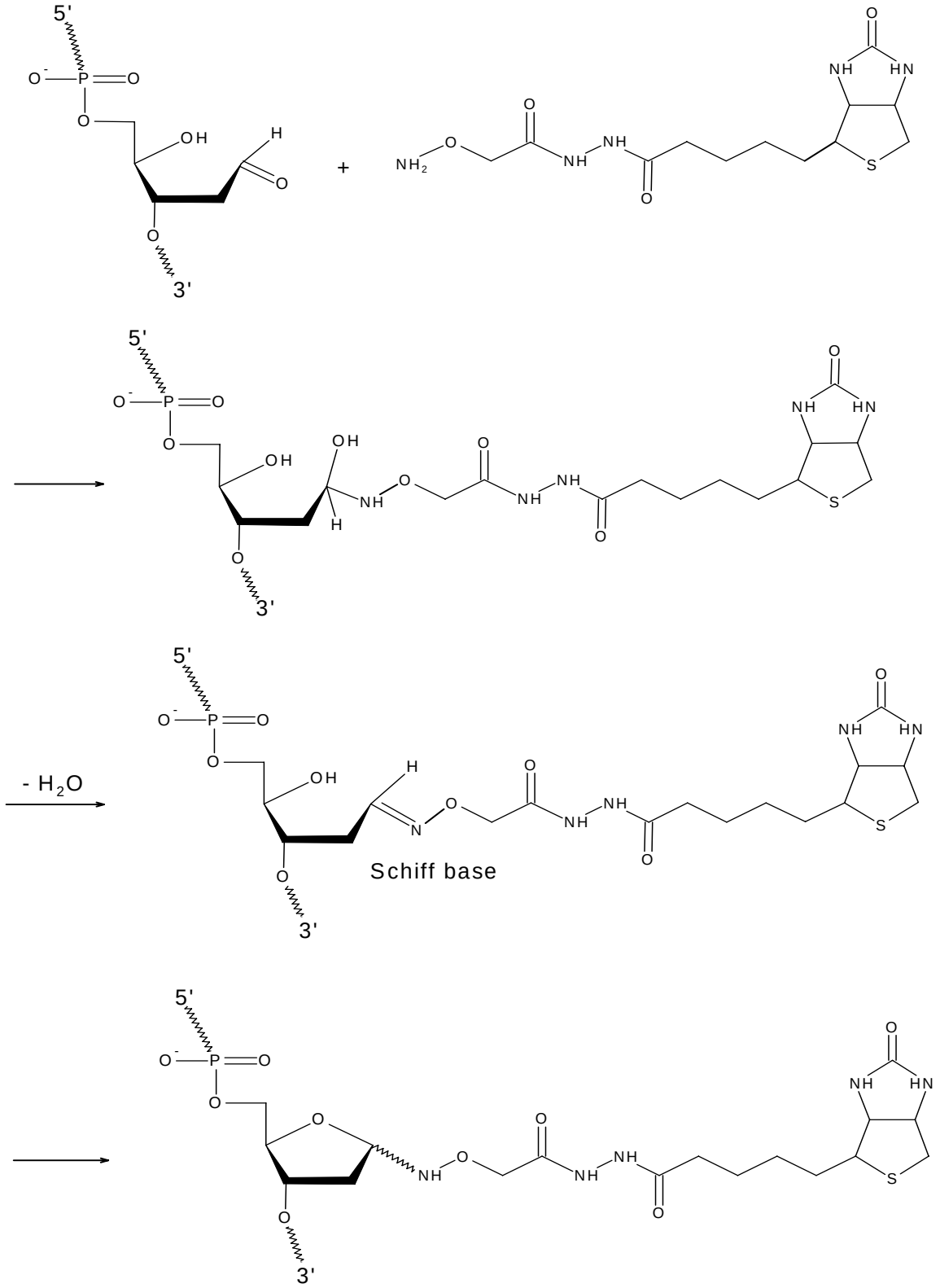


Figure II-8 Reaction of ARP with d-ribose of an AP site to form a stable ARP-DNA adduct.

III Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI) and used as received unless otherwise noted. Solvents were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ). Acetonitrile and dimethylformamide were dried by distillation from phosphorous pentoxide as required. Analytical thin layer chromatography (TLC) plates (Analtech Company; Newark, DE) were 5 x 10 cm with a gel thickness of 0.2 mm on aluminum backing. Preparative TLC was performed on 20 x 20 cm glass plates (Analtech) with silica gel GF of thickness 200, 500 or 1000 microns. For column chromatography, Sigma-Aldrich, Merck, grade 9385, 230-400 mesh, 60 Å silica gel was used. ^{1}H NMR solvents were from Aldrich except for $CDCl_3$ (99.8 % D) which was from Cambridge Isotope (Andover, MD). NMR solvents were generally 99.9 atom % D except for ^{1}H NMR spectra of final products when 100.0 atom % D was used.

Instrumentation

^{1}H NMR spectra were obtained on a Varian (Palo Alto, CA) Inova 500 spectrometer operated at 500 MHz. Chemical shifts were recorded in ppm relative to tetramethylsilane. NMR spectra were analyzed using MestRe-C, Mestrelab Research (Santiago de Compostela, A Coruña, Spain) NMR spectral software for Windows. The latest version used being 4.8.6. Full scan and tandem mass spectra were acquired on a Finnigan (Woburn, MA)

LCQ^{DECA} ion trap mass spectrometer in the electrospray ionization mode by direct loop injection. The spectrometer was operated in either positive or negative mode as needed. The mobile phase was typically 1% acetic acid in acetonitrile:water (1:1) with a flow rate of 100 μL/min.

Synthesis of Hydrazino ARP

Bromoacetyl biotin hydrazide (1)

Biotin hydrazide (1.940g, 7.51 mmol) was dissolved in 100 mL dry acetonitrile with stirring at room temperature. Bromoacetic acid (1.085 g, 7.81 mmol) was dissolved in 10 mL distilled acetonitrile and added to the biotin hydrazide solution. 1,3-Dicyclohexylcarbodiimide (DCC) (1.564 g, 7.58 mmol) was dissolved in 10mL dry acetonitrile and added to the reaction vessel. The reaction was allowed to stir at room temperature for 42 hours after which a white solid (dicyclohexylurea) was filtered and the filtrate was dried under vacuum to give a solid. The solid from the filtrate was dissolved in 50% v/v MeOH:$CHCl_3$ and was filtered off to remove remaining dicyclohexylurea. The product was then dried and redissolved in 5% HCl and filtered to remove unreacted biotin hydrazide. The reaction yielded 1.20 g (52%) of bromoacetyl biotin hydrazide (**1**) (Figure III-1).

^{1}H NMR (DMSO-d_6) δ ppm 1.23-1.67 (m, 6H, C9H_2, C10H_2, C11H_2); 2.13(t, 2H, $J_{11\text{-}12}$ = 7.38 Hz, C12H_2); 2.57(d, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, C7H_{7a}); 2.82(dd, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, $J_{7b\text{-}8}$ = 5.09 Hz, C7H_{7b}); 3.10(m, 1H, C5*H*); 3.90(s, 2H, C17H_2); 4.13(m, 1H, C4*H*); 4.30(m, 1H, C8*H*); 6.35(s, 1H, N1*H* or N3*H*); 6.41(s, 1H, N3*H* or N1*H*); 9.96(m, 1H, N14*H* or N15*H*); 10.27(m, 1H, N14*H* or N15*H*) (Figure III-2). ESI-MS: m/z 379, 381 (MH+). (Figure III-3).

Hydrazino ARP (2)

100mg (0.269 mmol) bromoacetyl biotin hydrazide was dissolved in 7.5 mL dry DMF. The solution was added drop-wise to 450 mL hydrazine with stirring. The reaction was stirred for 45 minutes at room temperature and then dried under argon and further by oil pump vacuum to obtain a sticky clear residue. The residue was dissolved in CH_2Cl_2 and dried by oil pump again to remove remaining DMF. A white powder was obtained which was dissolved in MeOH with 25 mg $NaHCO_3$. This solution was filtered to remove $H_2NNH_3^+Cl^-$ salt and then dried to obtain 58 mg (65%) hydrazino ARP (**2**).

^{1}H NMR (DMSO-d_6) δ ppm 1.23-1.66 (m, 6H, C9H_2, C10H_2, C11H_2); 2.13(t, 2H, $J_{11\text{-}12}$ = 7.35 Hz, C12H_2); 2.57(d, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, C7H_{7a}); 2.81(dd, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, $J_{7b\text{-}8}$ = 5.07 Hz, C7H_{7b}); 3.09(m, 1H, C5*H*); 3.49(s, 2H, C17H_2); 4.16(m, 1H, C4*H*); 4.30(m, 1H, C8*H*); 6.37(s, 1H, N1*H* or N3*H*); 6.40(s, 1H, N3*H* or N1*H*); 9.8(br s, 2H, N14*H*, N15*H*). (Figure III-4). FAB HRMS: calcd for C_{12} H_{22} O_3 N_6 S Na 353.1372, found 353.1370. ESI-MS: *m/z*, 353 $(M+Na)^+$, 331 $(M+H)^+$, 259 (biotin hydrazide + H)$^+$, 227 $(biotin)^+$ (Figure III-5).

Synthesis of Iminobiotin ARP

N-(tert-butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (3)

O-(carboxymethyl)hydroxylamine hemihydrochloride (2.36 g, 21.6 mmol) was dissolved in 15 mL DI water and 4.13 mL triethylamine. Di-*tert*-butyl dicarbonate (5.9 g, 27 mmol) was dissolved in 15 mL dioxane and added to the above solution. The reaction was allowed to stir at ambient temperature for 48 hours and then 50 mL DI water was added. The solution was washed with 70 mL ethyl acetate three times. The aqueous phase was cooled to 0 ^oC and

the pH adjusted to 2 using 5 M HCl. The aqueous phase was extracted with ethyl acetate (3 x 50 mL). The ethyl acetate phases were combined and washed with pre-cooled 5% HCl (3 x 40 mL) and then with brine (3 x 50 mL). The ethyl acetate fraction was filtered through sodium sulfate and evaporated to dryness, to yield white crystals. Recrystallization in ethyl acetate/hexane gave 3.19 g (77%) of white crystals (**3**) (Figure III-6).

^{1}H NMR (DMSO-d_6): δ ppm 1.40(s, 9H, *t*-BOC methyl); 4.26(s, 2H, CH_2); 10.10(br s, 1H, N*H*) (Figure III-7)

N-hydroxysuccinimide ester of N-(tert-butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (4)

N-(*tert*-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (**3**) (2.7 g, 14 mmol) and *N*-hydroxysuccinimide (1.9 g, 17 mmol) were dissolved in 25 mL dried acetonitrile. DCC (3.51 g, 17 mmol) was dissolved in 10 mL dried acetonitrile and added to the above solution with stirring at room temperature. The reaction was stirred for 24 hours and then filtered to remove dicyclohexylurea and evaporated to dryness. The residue was boiled in 150 mL CCl_4 and filtered hot. Recrystallization from CCl_4 yielded 0.47 g (12%) of white crystals (**4**).

^{1}H NMR (DMSO-d_6): δ ppm 1.42(s, 9H, *t*-BOC methyl); 2.84(s, 4H, succinyl CH_2CH_2); 4.82(s, 2H, acetyl CH_2); 10.36(s, 1H, N*H*) (Figure III-8)

2-Iminobiotin hydrazide-N-(tert-butoxycarbonyl)-O-(carboxymethyl) hydroxylamine conjugate (5)

Iminobiotin hydrazide hydrochloride (0.245 g, 0.85 mmol) was dissolved in 50 mL dry DMF. The solution was sonicated for one hour to aid dissolution. (**4**) (0.250 g, 0.85 mmol) was added with stirring at ambient temperature. The reaction stirred for four days and then evaporated to dryness on a rotary evaporator. The residue was extracted with boiling

isopropanol. After crystals formed the solution was centrifuged and the mother liquor was drawn off and reduced to one half volume for a second crop of crystals. The remaining mother liquor was dried and applied to silica gel preparative TLC plates for further purification. The product (**5**) was a fine gel and a total of 0.143 g (39%) was obtained.

^{1}H NMR (DMSO-d_6) δ ppm 1.4-1.7(m, 6H, C9H_2, C10H_2, C11H_2); 1.41(m, 9H, *t*-BOC methyls); 2.15(t, 2H, $J_{11\text{-}12}$ = 7.27 Hz, C12H_2); 2.82(d, 1H, $J_{7a\text{-}7b}$ = 12.9 Hz, C7H_{7a}); 2.92(dd, 1H, $J_{7a\text{-}7b}$ = 12.9 Hz, $J_{7b\text{-}8}$ = 4.87 Hz, C7H_{7b}); 3.25(m, 1H, C5*H*); 4.27(s, 2H, C17H_2); 4.47(m, 1H, C4*H*); 4.64(m, 1H, C8*H*); 7.72(s, 2H, N1*H* and N3*H*) (Figure III-9).

Iminobiotin ARP (6)

5 mL of pre-cooled trifluoroacetic acid (TFA) was added to (**5**) at 0 ^{o}C with stirring and stirred for an additional 25 minutes. The reaction was then allowed to stir at room temperature for 30 minutes. The TFA was evaporated under N_2 gas and dissolved in 15 mL DI water. Dowex 1x-4 resin (converted to OH^- form by washing with 5% aqueous NaOH three times) was added to pH 5. The resin was filtered and rinsed 2x with DI water. The water was removed by lyophilyzation and 0.023 g (30%) of white powder (**6**) was obtained.

^{1}H NMR (D_2O): δ ppm 1.5-1.9(m, 6H, C9H_2, C10H_2, C11H_2); 2.42(t, 2H, $J_{11\text{-}12}$ = 7.37 Hz, C12H_2); 2.96(d, 1H, $J_{7a\text{-}7b}$ = 13.4 Hz, C7H_{7a}); 3.12(dd, 1H, $J_{7a\text{-}7b}$ = 13.4 Hz, $J_{7b\text{-}8}$ = 4.89 Hz, C7H_{7b}); 3.49(m, 1H, C5*H*); 4.38(s, 2H, C17H_2); 4.71(m, 1H, C4*H*); 4.87(m, 1H, C8*H*) (Figure III-10).

Synthesis of Biotinylated Long-chain ARP

Biotin-PEG$_4$-hydrazide-acetylhydroxylamine-t-BOC (7)

115 mg (0.40 mmol) **4** was dissolved in 5 mL doubly distilled DMF. 200 mg (0.40 mmol) Biotin-dPEG$_4$-hydrazide (BPH) (Quanta BioDesign, Ltd., Powell, OH) was added with stirring at room temperature. The solution was sonicated 5 minutes until all BPH was dissolved. The reaction was stirred for 3 days at room temperature under argon. Product was purified by preparative thin layer chromatography (mobile phase; 20:80, MeOH:$CHCl_3$) to give 32 mg of an oil (**7**) (Figure III-11).

^{1}H NMR (DMSO-d_6) δ ppm 1.2-1.6(m, 6H, C9H_2, C10H_2, C11H_2); 1.40(m, 9H, *t*-BOC methyl); 2.06(t, 2H, $J_{11\text{-}12}$ = 7.4 Hz, C12H_2); 2.37(t, 2H, C15H_2); 2.57(d, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, C7H_{7a}); 2.81(dd, 1H, $J_{7a\text{-}7b}$ = 12.4 Hz, $J_{7b\text{-}8}$ = 5.0 Hz, C7H_{7b}); 3.09(m, 1H, C5*H*); 3.2(m, 4H, C16H_2, C27H_2); 3.40(t, 2H, C28H_2); 3.50(m, 12H, C18-C25H_{12}); 3.61(s, 2H, C33H_2); 4.13(m, 1H, C4*H*); 4.30(t, 1H, C8*H*); 6.35(s, 1H, N1*H* or N3*H*); 6.41(s, 1H, N3*H* or N1*H*); 7.84(m, 1H, N*H*); 7.93(s, 1H, N*H*); 8.3(s, 1H, N*H*) (Figure III-12). ESI-MS: *m/z*, 701 $(M+Na)^+$ (Figure III-13).

Biotin-PEG$_4$-hydrazide-acetylhydroxylamine (8)

22 mg (0.032 mmol) of **7** was pre-cooled to -10 ^oC and to this was added 2 mL TFA (also pre-cooled to -10 ^oC) with stirring. The reaction was stirred for 20 minutes at -10 ^oC and another 30 minutes at room temperature. The TFA was evaporated under argon and the resulting material was dissolved in 3 mL DI water. Dowex 1x4 Cl^- ion exchange beads (Sigma-Aldrich) were prepared by washing three times in 5% aqueous NaOH to convert the beads to the OH^- form. The Dowex beads were then added to the product solution until the pH reached 7. The beads were then filtered and washed in DI water. The beads were then

stirred in 5mL DI water for 3 hours and filtered again. The filtrates were collected and lyophilyzed to obtain 20 mg of a clear oil (**8**).

^{1}H NMR (CD_3OD) δ ppm 1.4-1.4(m, 6H, C9H_2, C10H_2, C11H_2); 2.22(t, 2H, $J_{11\text{-}12}$ = 7.4 Hz, C12H_2); 2.52(t, 2H, C15H_2); 2.70(d, 1H, $J_{7a\text{-}7b}$ = 12.5 Hz, C7H_{7a}); 2.92(dd, 1H, $J_{7a\text{-}7b}$ = 12.5 Hz, $J_{7b\text{-}8}$ = 5.0 Hz, C7H_{7b}); 3.20(m, 1H, C5*H*); 3.36(m, 2H, C16H_2); 3.54(m, 2H, C27H_2); 3.62(m, 14H, C18-C25H_{12}, C28H_2); 3.76(m, 2H, C33H_2); 4.30(m, 1H, C4*H*); 4.48(m, 1H, C8*H*) (Figure III-14). ESI-MS: *m/z*, 601 $(M+Na)^+$ (Figure III-15).

Synthesis of PEG_5 *bis*-hydroxylamine ARP

(Bis-[acetylhydroxylamino-tBOC])-1,19-diamino-4,7,10,13,16-pentaoxanonadecane (9)

1,19-Diamino-4,7,10,13,16-pentaoxanonadecane (Berry and Associates, Ann Arbor, MI.) (123 mg, 0.4 mmol) was dissolved in 3 mL DMF. **4** (300 mg, 1.04 mmol) was added with stirring at room temperature. The reaction was stirred at room temperature for five days at which time the DMF was evaporated under argon followed by oil pump. The product was separated by preparative thin layer chromatography (mobile phase: 85:15; $CHCl_3$:MeOH) and 203 mg (78%) of **9**, a clear oil, was obtained (Figure III-16).

^{1}H NMR (DMSO-d_6) δ ppm1.41(s, 18H, *t*-BOC methyl x2); 1.65(ψ−quintet, 4H, *J* = 6.6 Hz, $CH_2CH_2CH_2$, x2); 3.17(m, 4H, CH_2, x2); 3.40(m, 4H, CH_2, x2); 3.5(m, 16H, CH_2CH_2, x4); 4.13(s, 4H, CH_2, x2); 7.98(br s, 2H, N*H* x2) (Figure III-17).

(Bis-[acetylhydroxylamino])-1,19-diamino-4,7,10,13,16-

pentaoxanonadecane (10)

152 mg (0.23 mmol) of **9** was pre-cooled to -10 ^{o}C and to this was added 2 mL TFA (also pre-cooled to -10 ^{o}C) with stirring. The reaction was stirred for 20 minutes at -10 ^{o}C and another 30 minutes at room temperature. The TFA was then evaporated under argon and the resulting oil was dissolved in 5 mL DI water. Dowex 1x4 Cl^- ion exchange beads (Sigma-Aldrich) were prepared by washing three times in 5% NaOH to convert the beads to the OH^- form. The Dowex beads were then added to the product solution until the pH reached 7. The beads were then filtered and immersed in DI water overnight and filtered again. The filtrates were collected and lyophilyzed to obtain 133 mg of a clear oil (**10**).

^{1}H NMR (DMSO-d_6) δ ppm1.80(ψ–quintet, 4H, J = 6.31 Hz, $CH_2CH_2CH_2$, x2); 3.35(t, 4H, J = 6.61 Hz, CH_2, x2); 3.56(t, 4H, J = 5.97 Hz, CH_2, x2); 3.6(m, 4H, CH_2, x2); 3.63(m, 16H, CH_2CH_2, x4); 4.06(s, 4H, CH_2, x2) (Figure III-18)

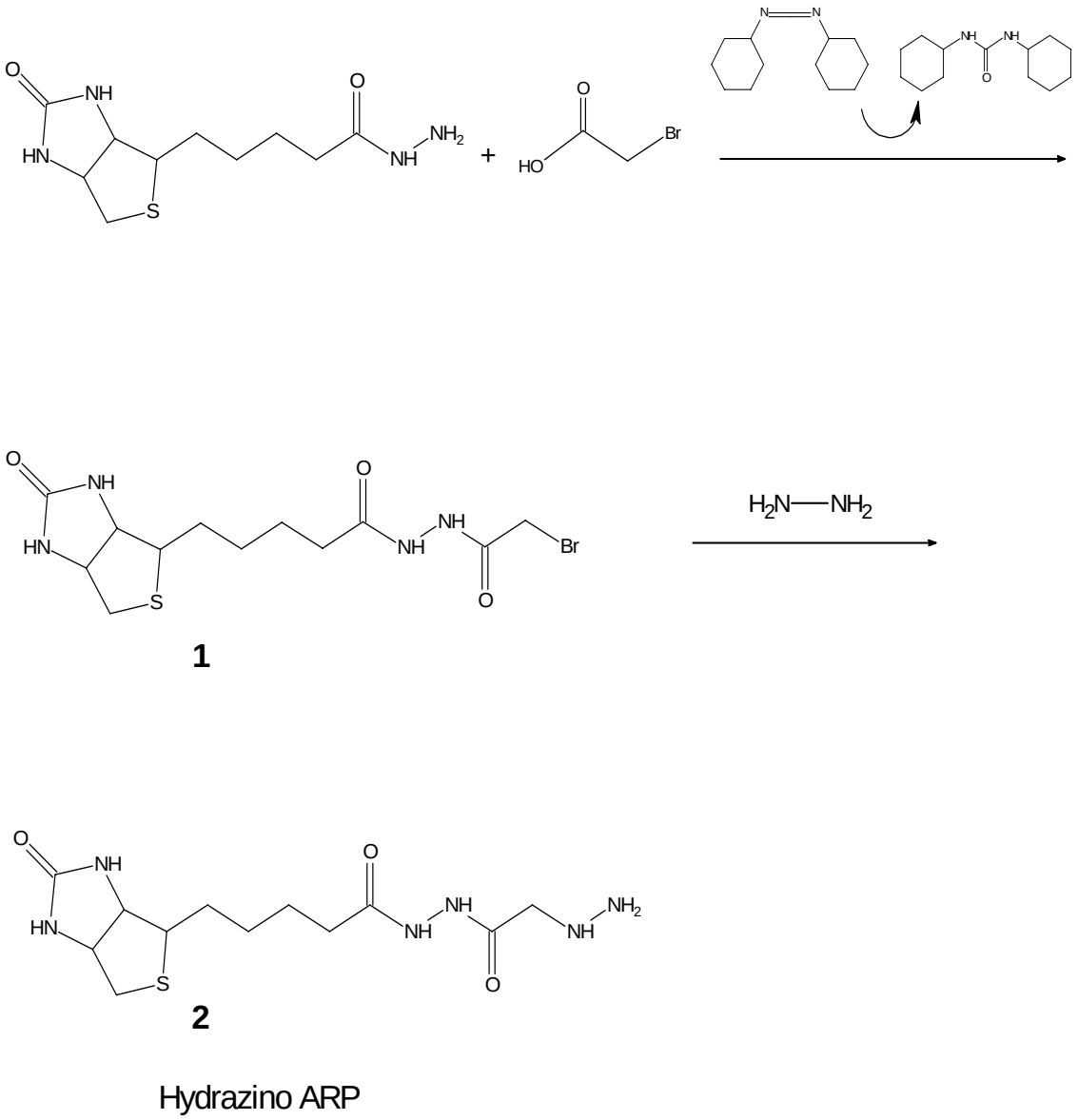


Figure III-1 Synthesis of hydrazino ARP.

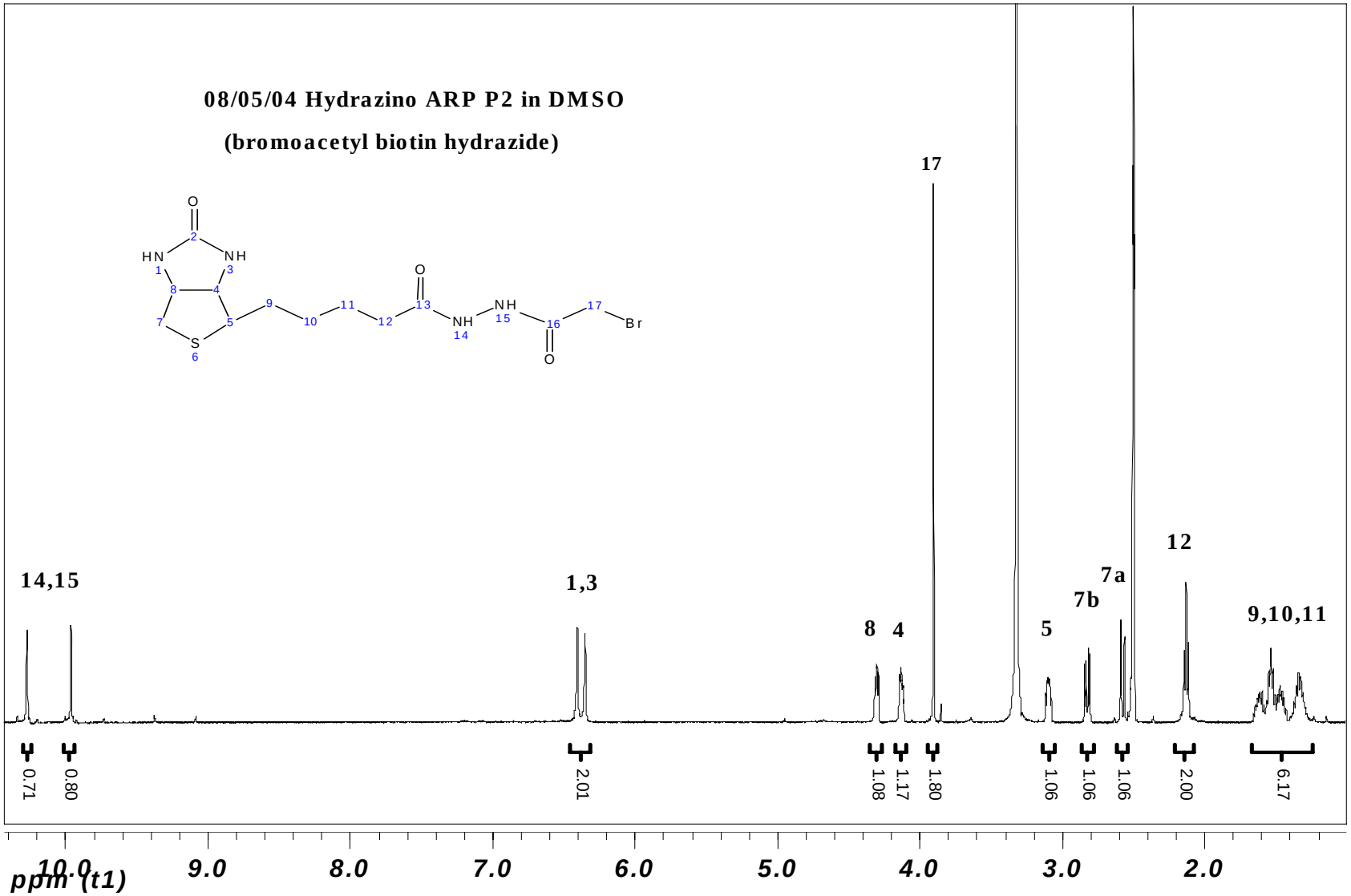


Figure III-2 ^{1}H NMR spectrum of bromoacetyl biotin hydrazide (1).

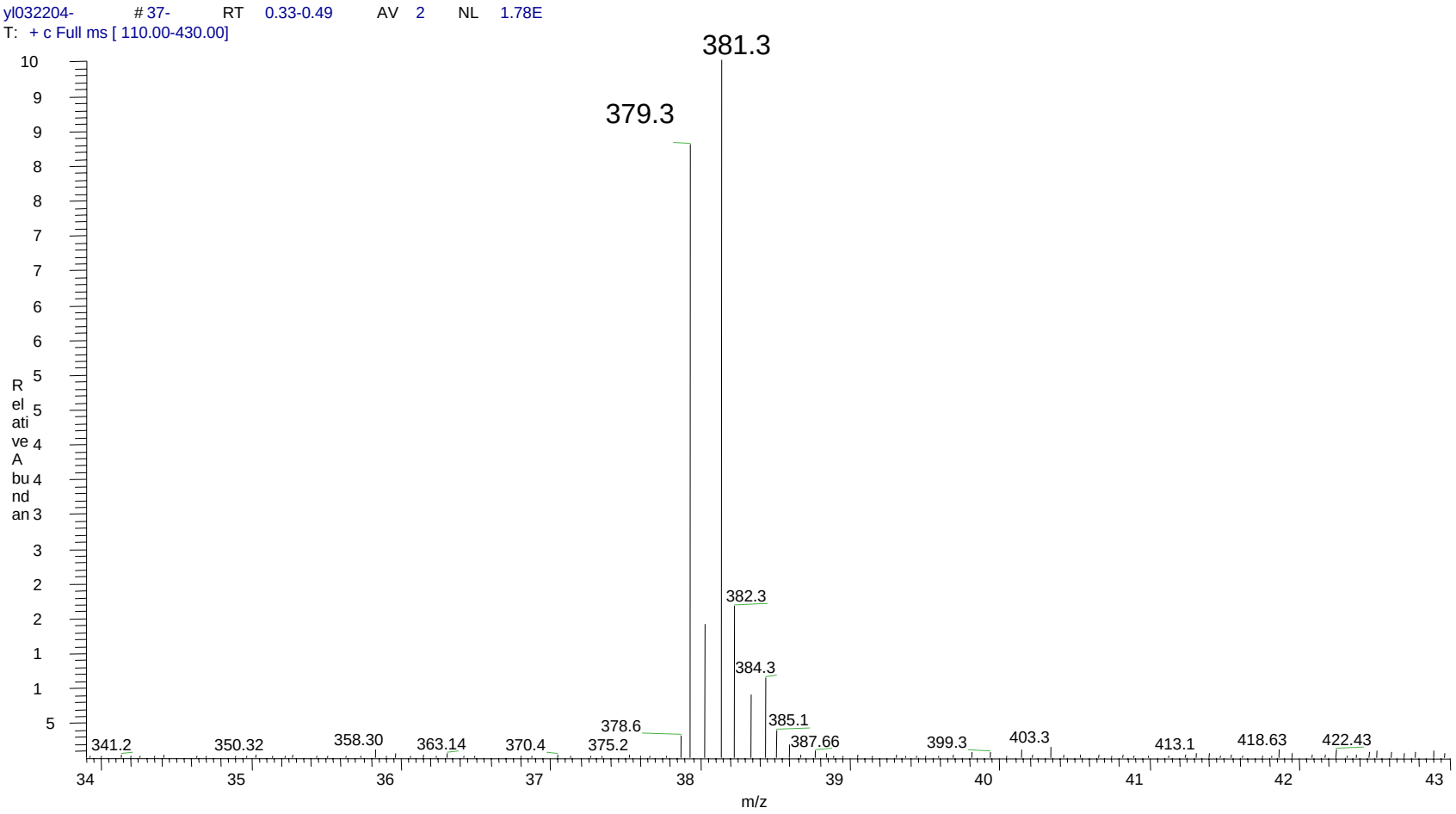


Figure III-3 Mass spectrum of bromoacetyl biotin hydrazide (1).

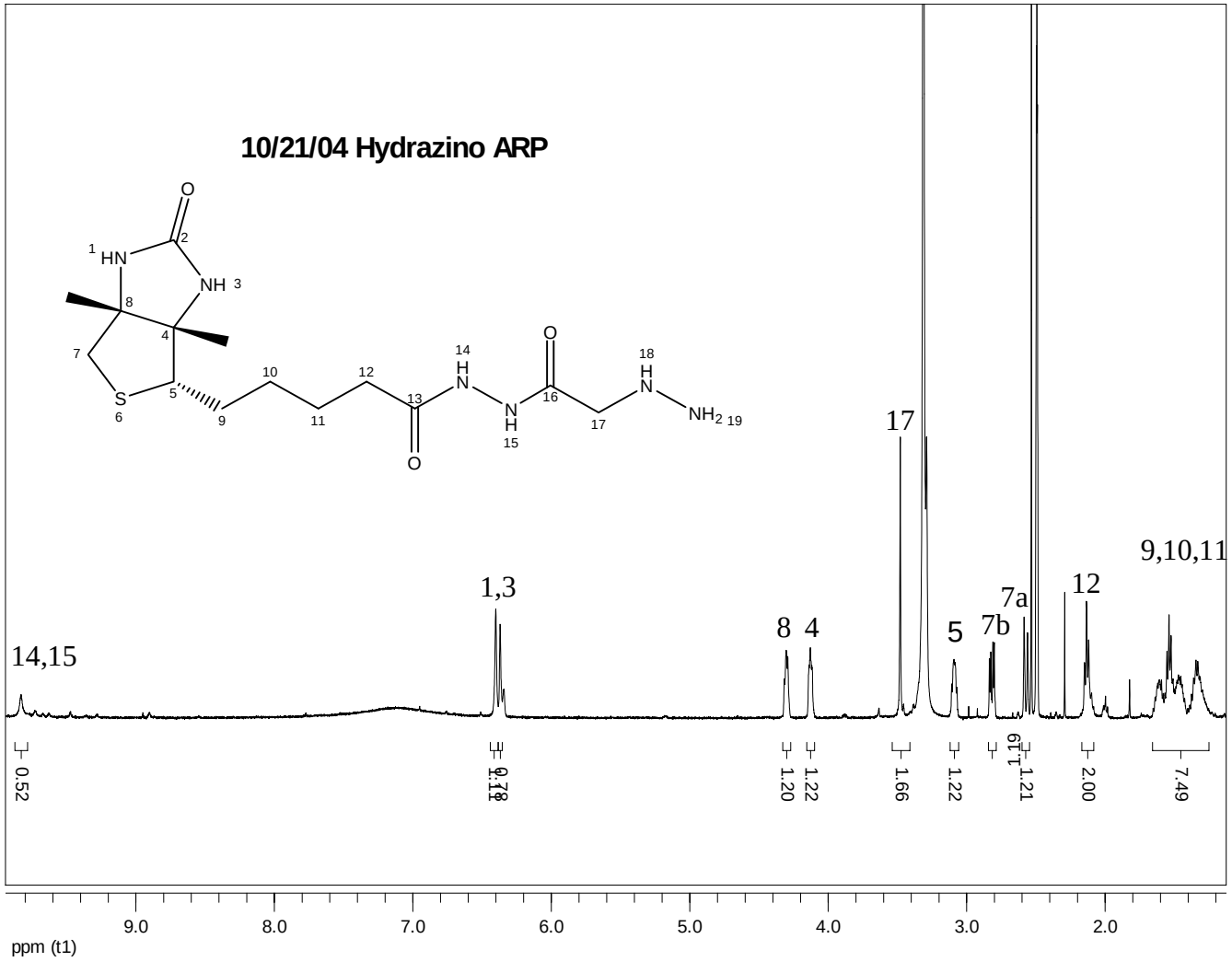


Figure III-4 ^{1}H NMR spectrum of hydrazino ARP (2).

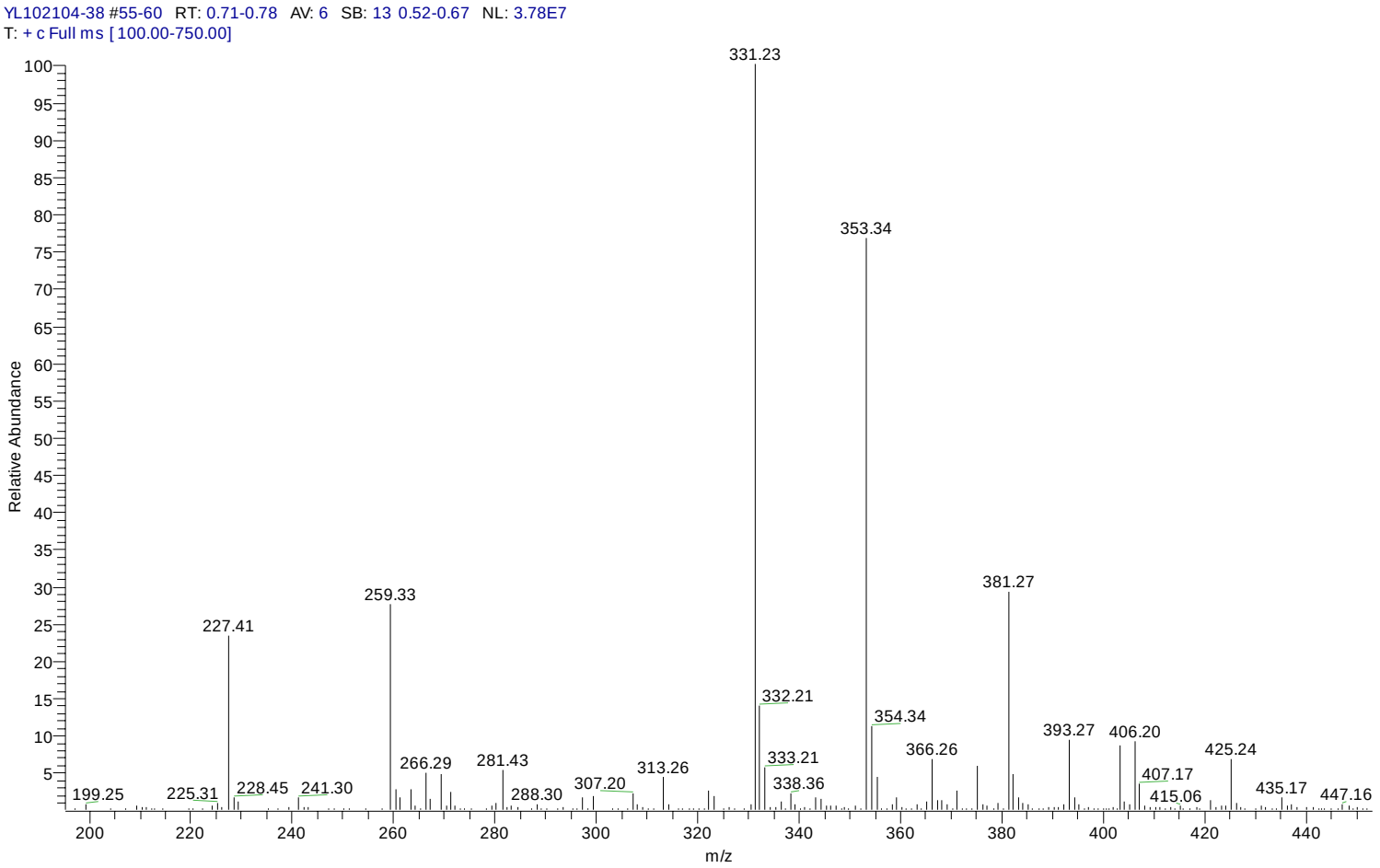


Figure III-5 Mass spectrum of hydrazino ARP (2).

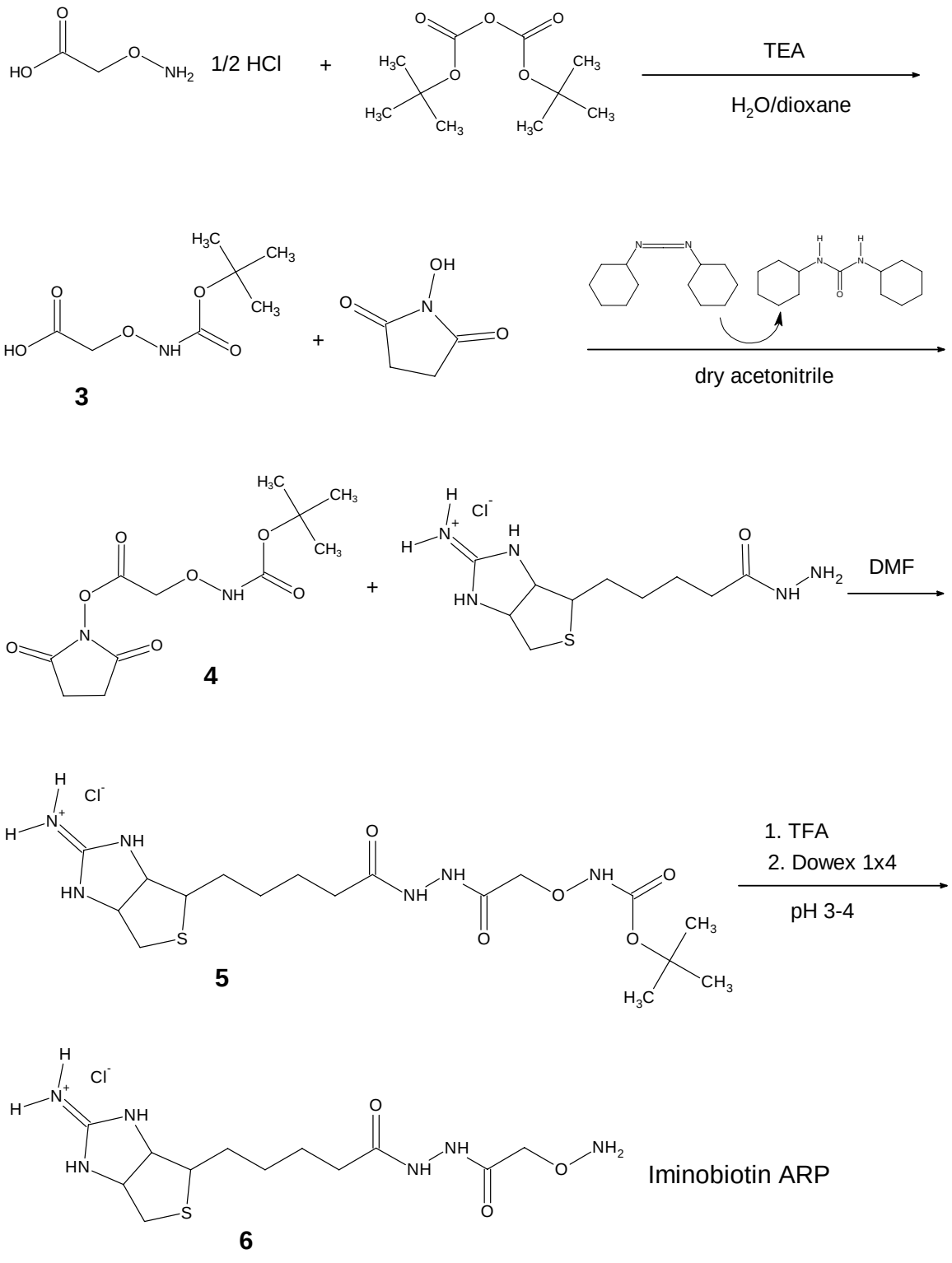


Figure III-6 Synthesis of iminobiotin ARP.

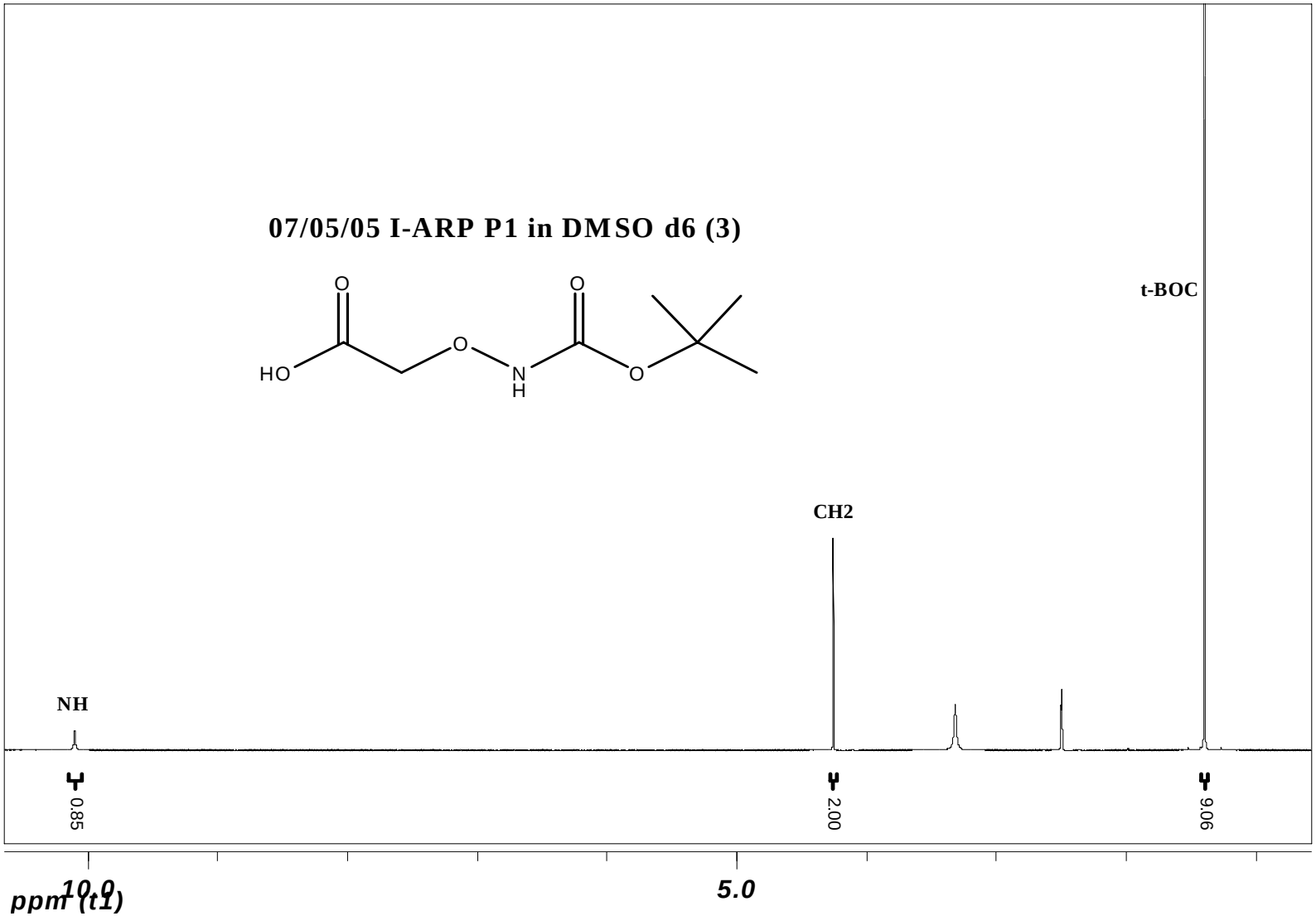


Figure III-7 ^{1}H NMR spectrum of *N*-(tert-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (3).

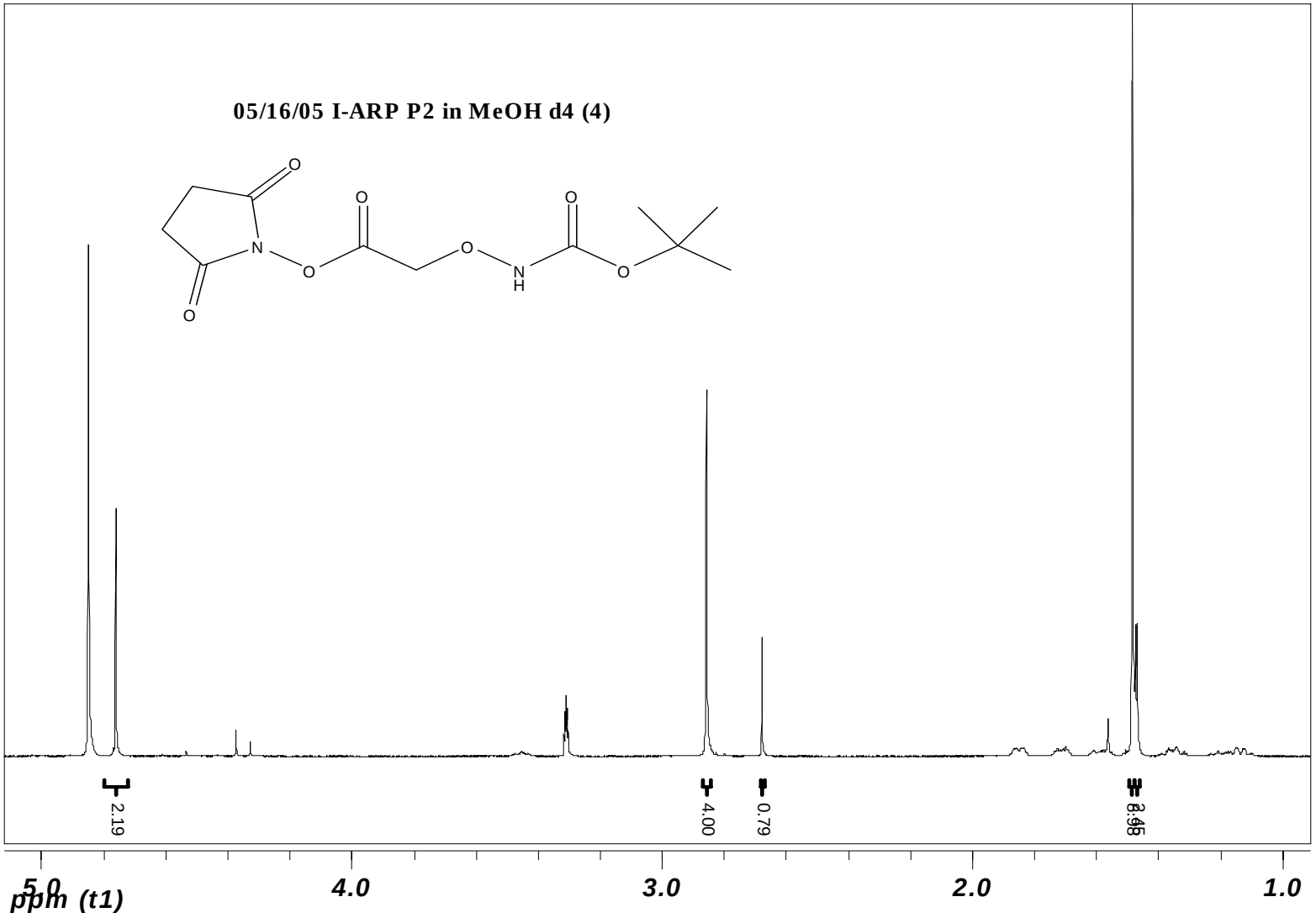


Figure III-8 ^{1}H NMR spectrum of *N*-hydroxysuccinimide ester of *N*-(tert-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (4).

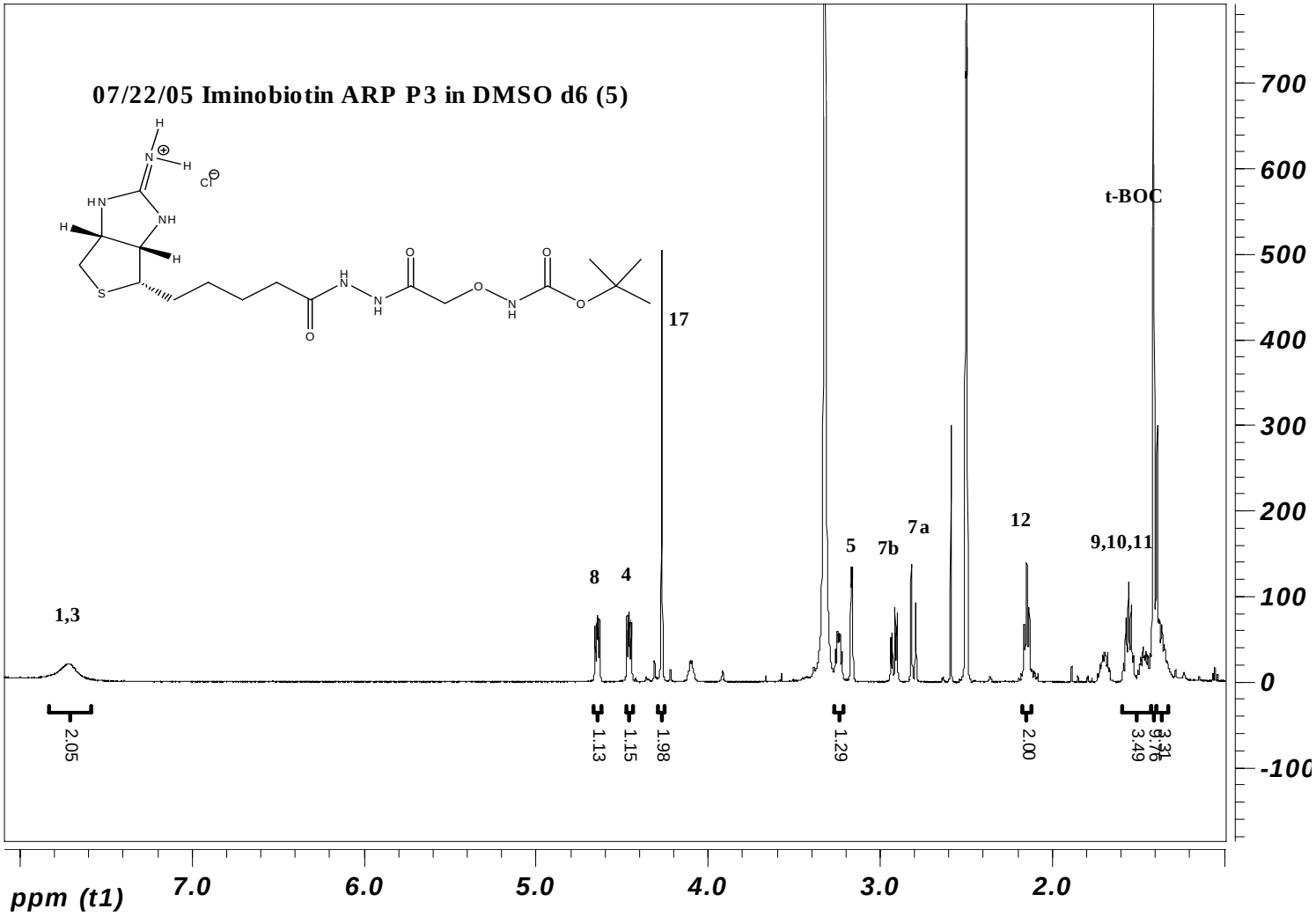


Figure III-9 ^{1}H NMR spectrum of *N*-(tert-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine ester of iminobiotin (5).

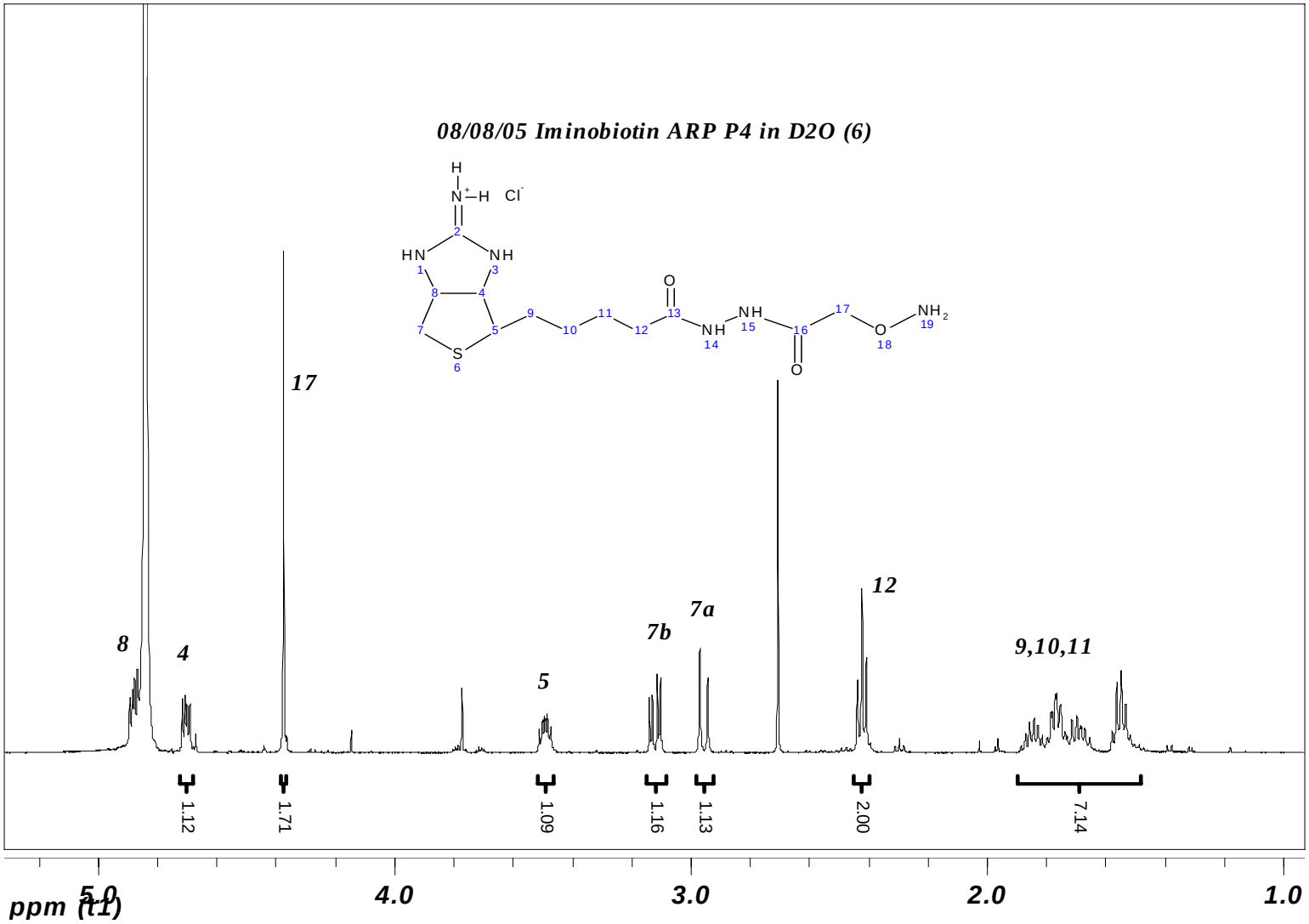


Figure III-10 ^{1}H NMR spectrum of iminobiotin ARP (6).

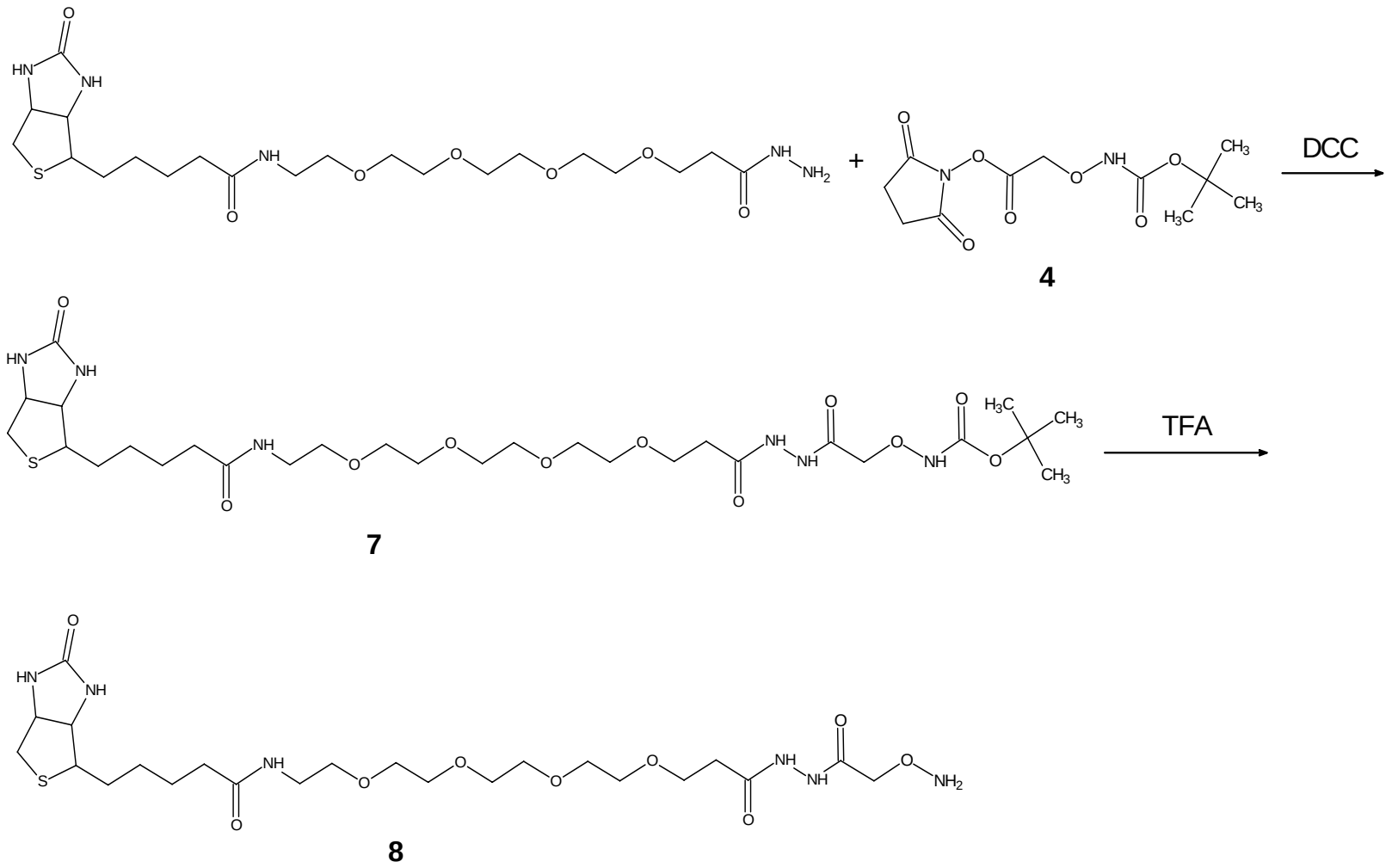


Figure III-11 Synthesis of biotin-PEG_4-hydrazide-acetylhydroxylamine (8) (LC-ARP).

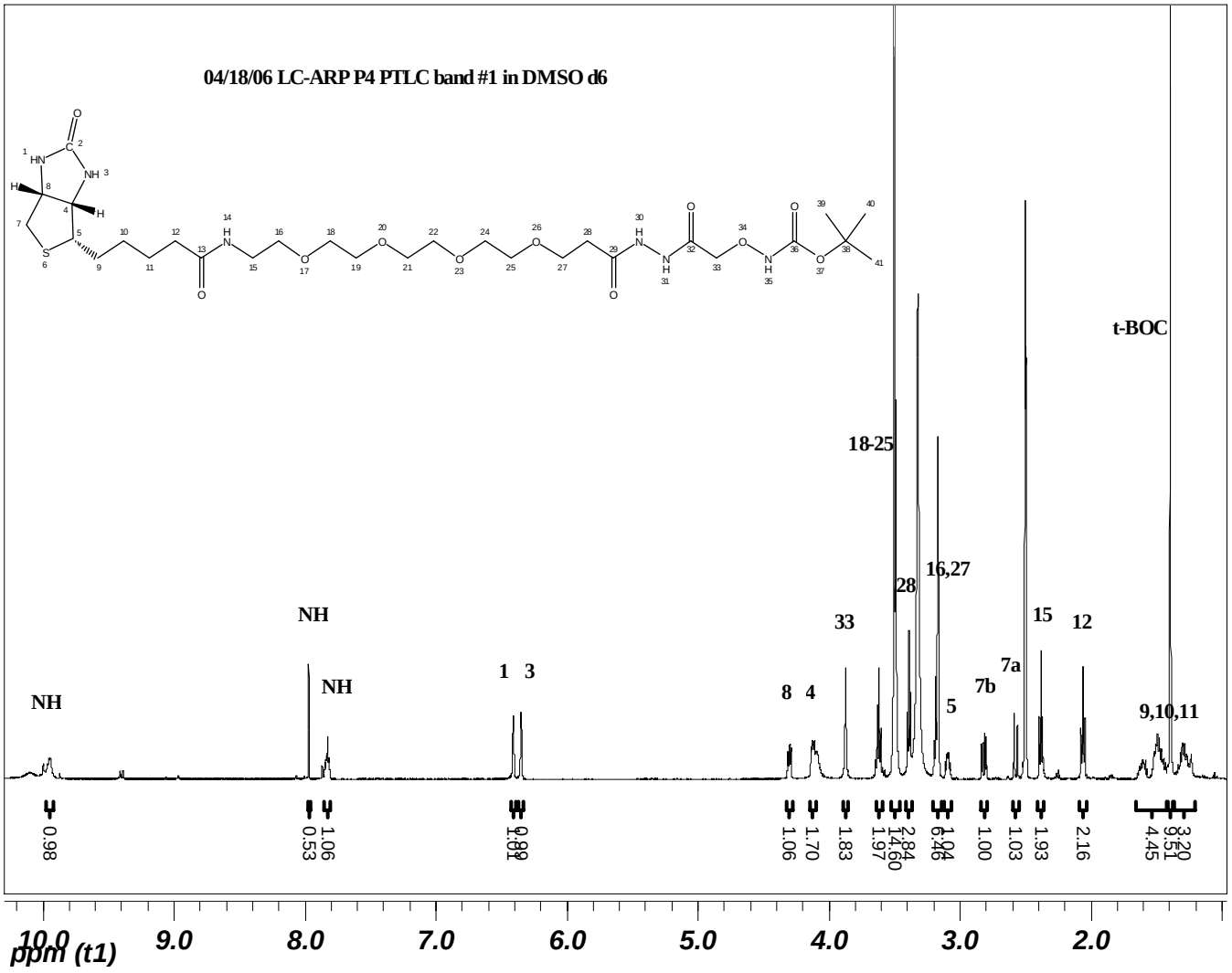


Figure III-12 ^{1}H NMR spectrum of biotin-PEG$_4$-hydrazide-acetylhydroxylamine-*t*-BOC (7).

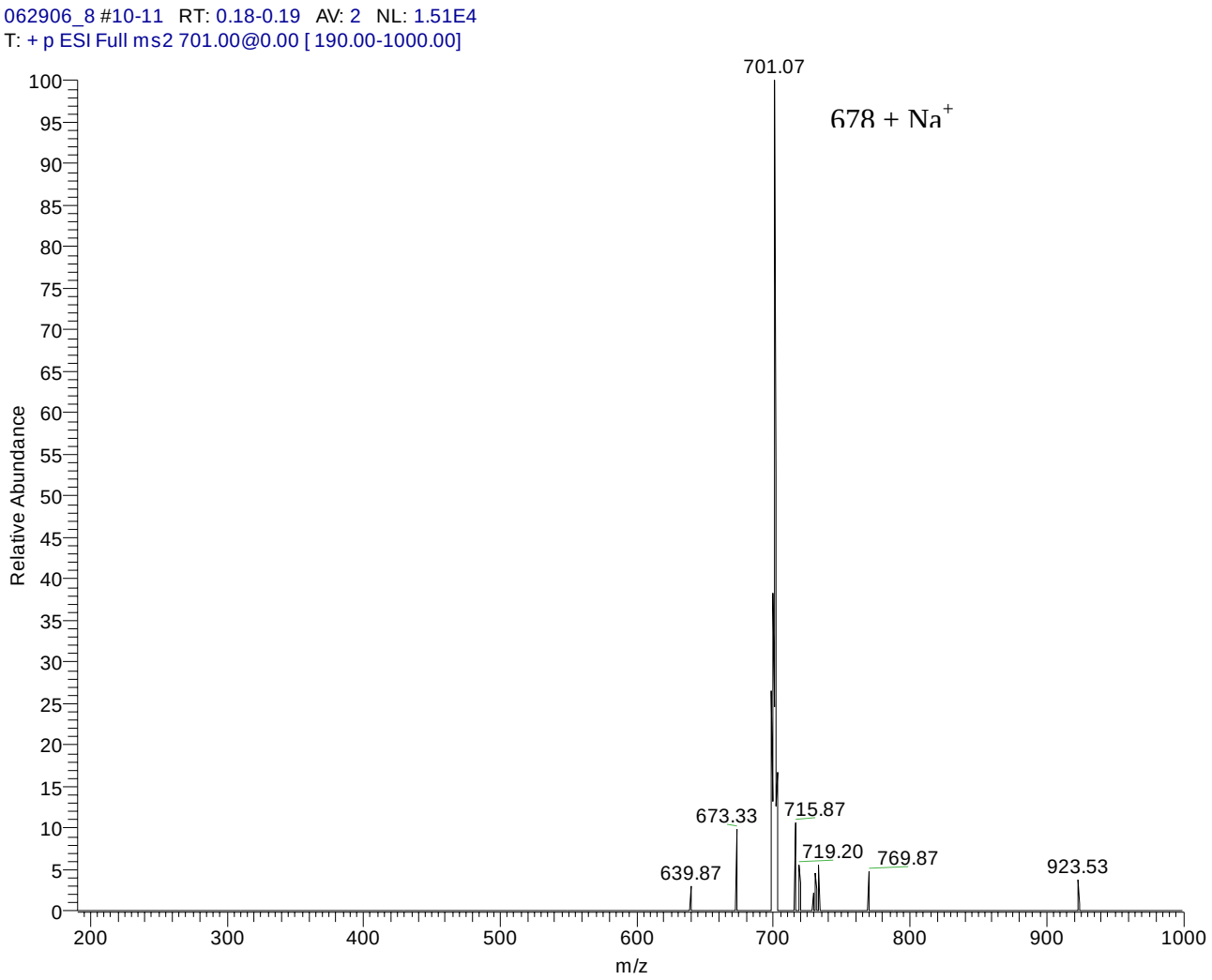


Figure III-13 Mass spectrum of biotin-PEG_4-hydrazide-acetylhydroxylamine-t-BOC (7).

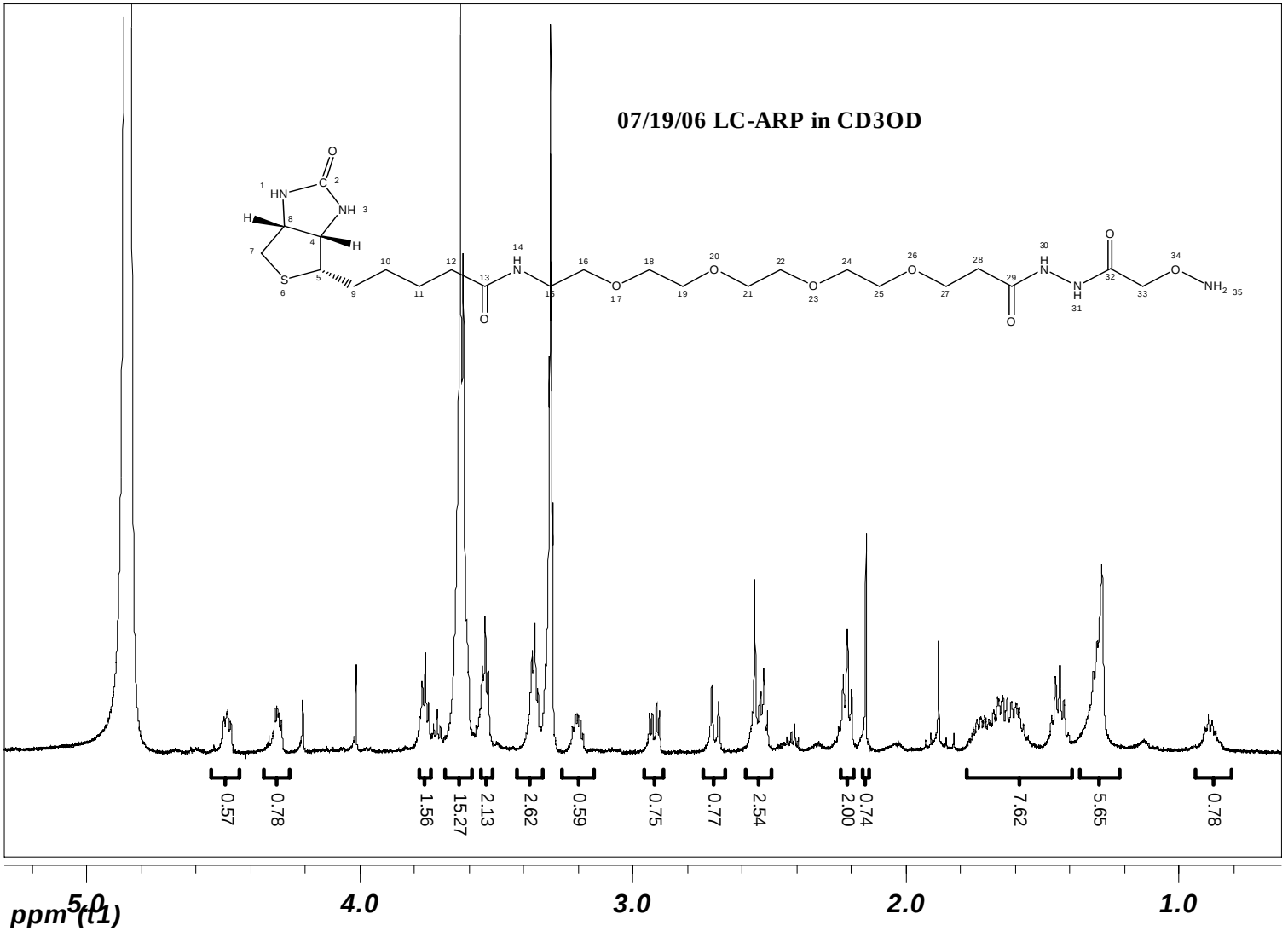


Figure III-14 ^{1}H NMR spectrum of biotin-PEG_4-hydrazide-acetylhydroxylamine (8) (LC-ARP).

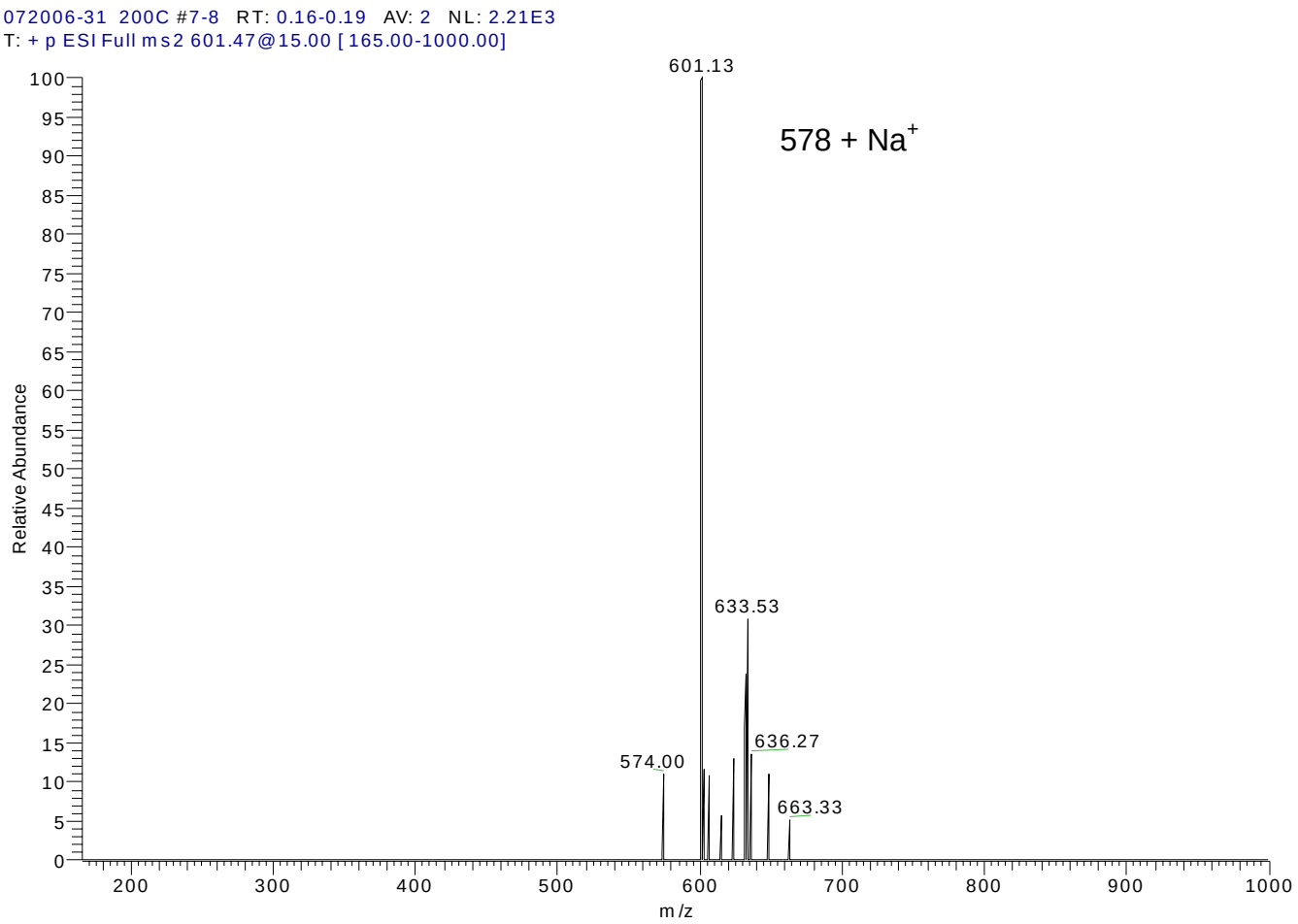


Figure III-15 Mass spectrum of biotin-PEG_4-hydrazide-acetylhydroxylamine (8) (LC-ARP).

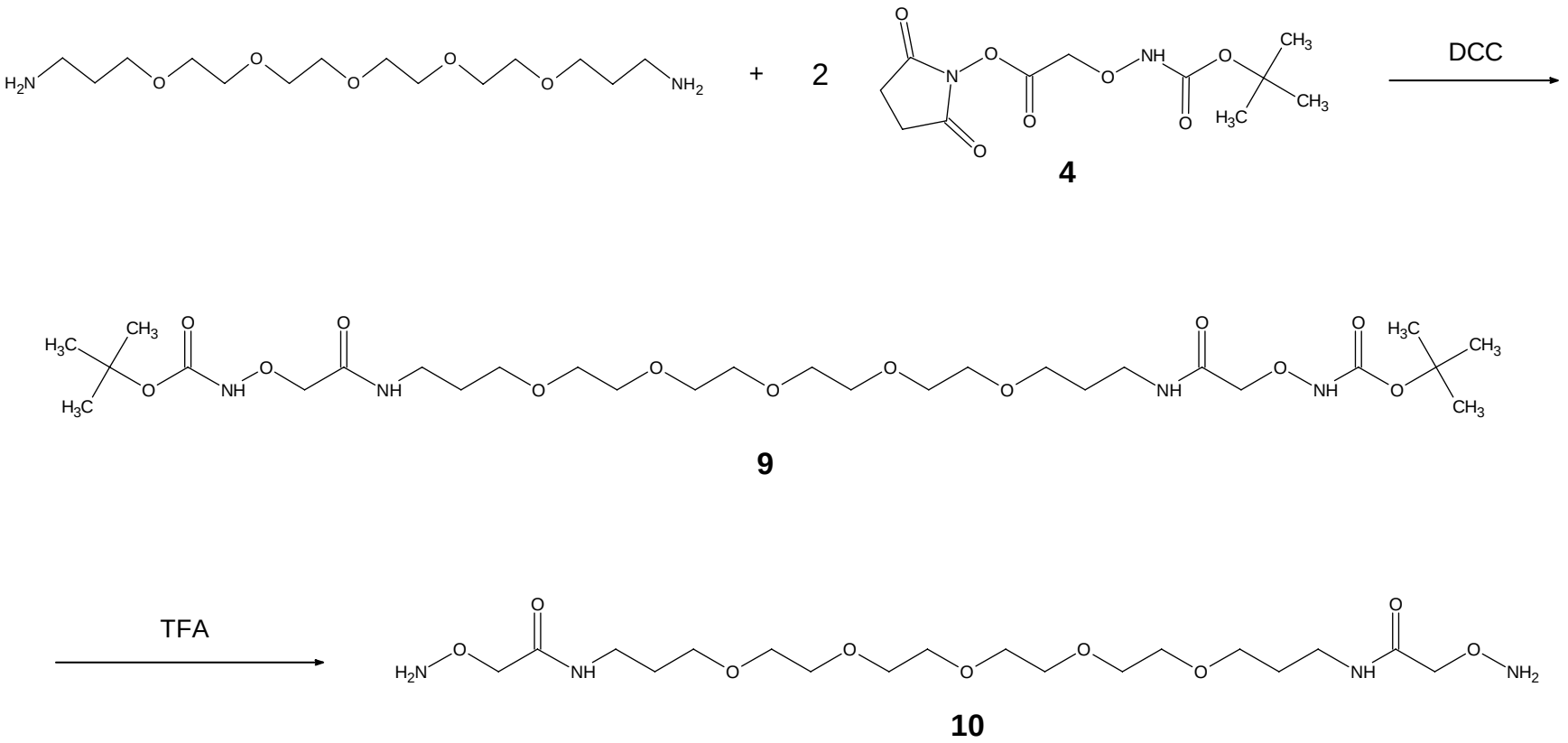


Figure III-16 Synthesis of (Bis-[acetylhydroxylamino]-1,19-Diamino-4,7,10,13,16-pentaoxanonadecane (10) (DE-ARP).

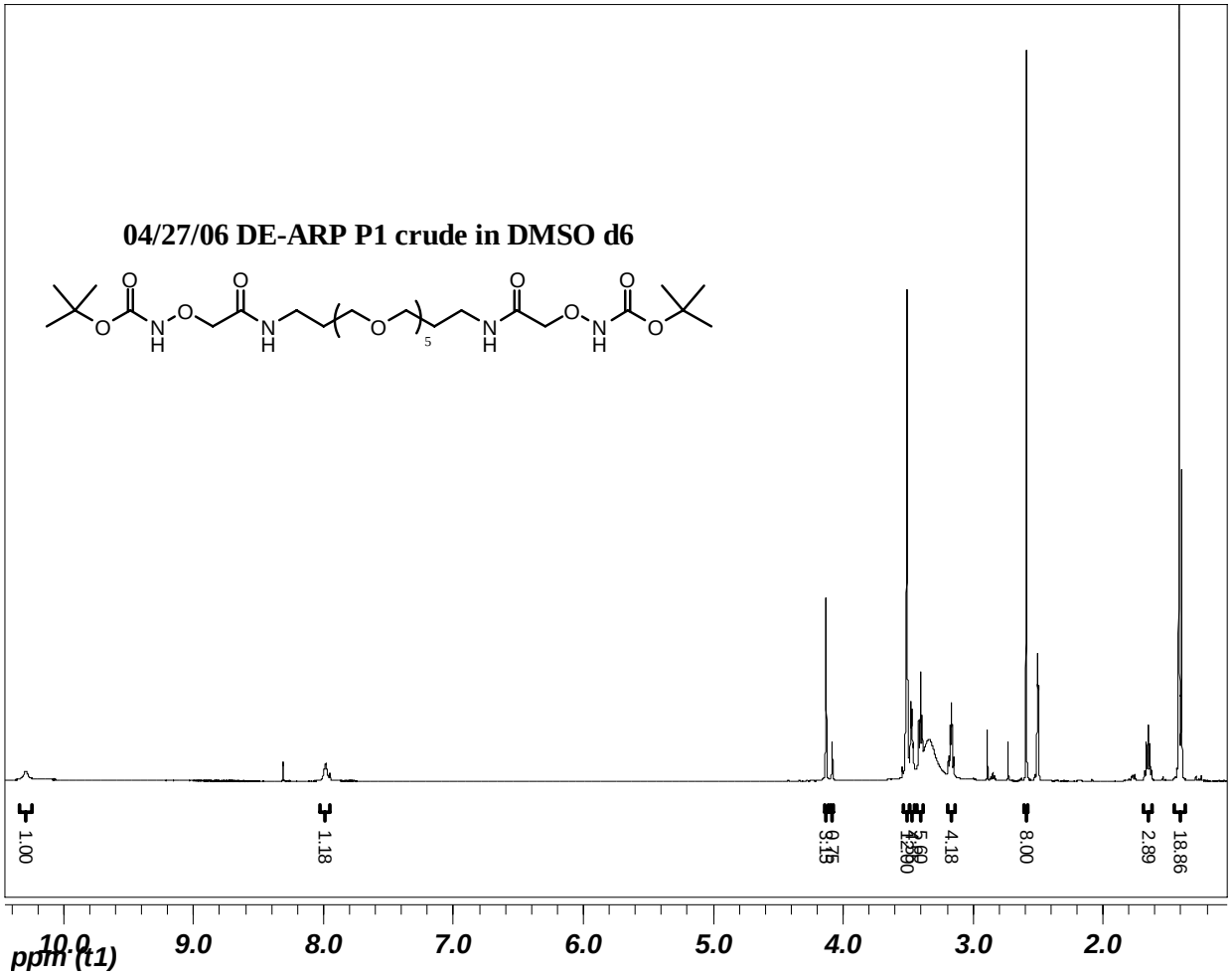


Figure III-17 ^{1}H NMR of (*Bis*-[acetylhydroxylamino-*t*-BOC]-1,19-Diamino-4,7,10,13,16-pentaoxanonadecane (9).

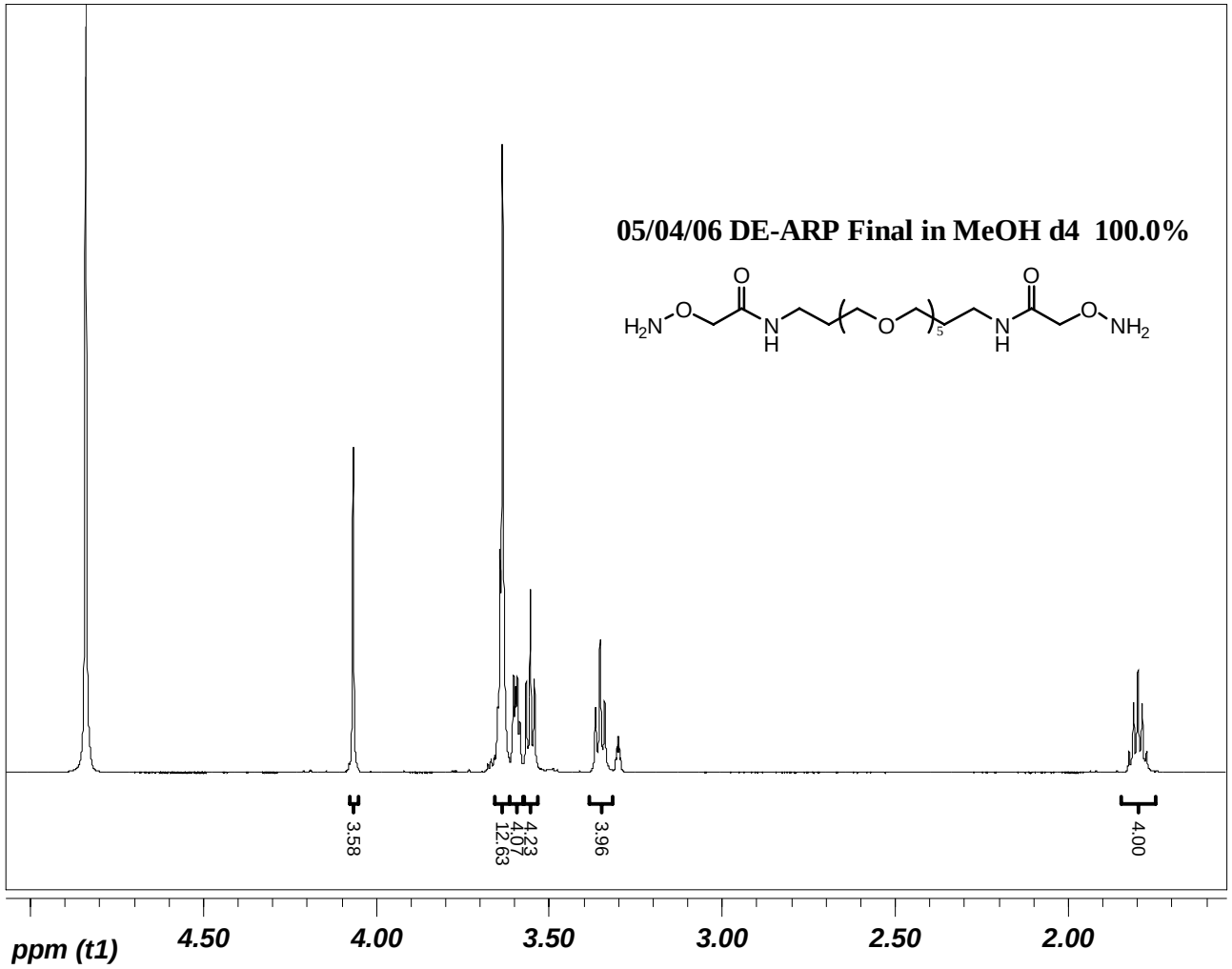


Figure III-18 ^{1}H NMR spectrum of DE-ARP (10).

IV Discussion

The purpose of this project was to synthesize new aldehyde reactive probes for use in the slot-blot analysis for quantitation of abasic sites in DNA. It was suspected the probe then being used, while having excellent specificity, might not be as reactive as possible and an underestimation of the number of AP sites could result. Alternatively, a less reactive probe necessitates a longer incubation period at elevated temperature during which more artifactual AP sites may be created resulting in an overestimation. In an attempt to improve upon this situation two new probes were synthesized; one that substitutes a hydrazino group for the hydroxylamine at the aldehyde reactive end of the molecule, and another that inserts a polyethylene glycol (PEG) into the molecule to create a long tether while retaining the hydroxylamine as the reactive moiety. The rationale for the substitution of the hydrazino group for the hydroxylamine was that the hydrazino group would be more basic and therefore more nucleophilic. If the compound is more reactive due to the increased nucleophilicity it is hoped that the ARP site assay incubation temperature could be reduced or the incubation time shortened, either of which should lessen the number of artifactual AP sites. The long-chain ARP may have two possible advantages: 1) it may be better able to react with double-stranded DNA, especially in vivo where the nucleosomal DNA is difficult to access; and, 2) after it has joined with the abasic site it may sterically hinder repair better than the shorter ARP.

During the course of the project it was discovered that another molecule, which is the result of a byproduct of an attempted long-chain ARP synthesis, may be of use, although not as a replacement probe for AP site quantitation. The compound is composed of a medium length PEG adducted at each end by a hydroxylamine group. This compound would be expected to create intra- and intermolecular cross-links between AP sites in DNA. Such cross-links could interfere with the action of RNA and DNA polymerases and thus prevent protein production and DNA replication resulting in cell death. Although it has been reported that as many as 10,000 AP sites may occur per mammalian genome per day, these sites are rapidly repaired and the toxicity of the compound to normal cells may be found to be low. If, however, such a compound were to be administered to cells immediately after or during irradiation (such as during cancer treatment) when AP site levels would be expected to be very high, toxicity of the compound may be expected to be commensurately high and increase lethality in these cells making the treatment more effective.

While the road to the final synthesis of the hydrazino ARP was relatively arduous, the successful synthesis was a simple two-step procedure. It should be noted that it was considered important to retain the acetyl methylene group to provide separation of the hydrazino group from the adjacent carbonyl since the carbonyl, being electron-withdrawing, would lessen the reactivity of the hydrazino group. Fortuitously, the methylene also provides an excellent marker for NMR characterization of the intermediate, as well as the final, product.

In the beginning a synthetic process similar to the one used by Ide, et al. (1993), for the hydroxylamine ARP was attempted (Figure IV-1). Standard DCC condensation chemistry was used to add *N*-hydroxysuccinimide to bromoacetic acid after which the bromine was

substituted with *tert*-butyl carbazate, the *tert*-butyl carbazate providing the terminal hydrazino group for the final product along with protection of the hydrazino group via the *tert*-butoxycarbonyl (*t*-BOC) group. The next step was to have been to react biotin hydrazide with the *N*-hydroxysuccinimidyl activated carbonyl to form the amide. A problem arose in the synthesis in which it appeared that, rather than acting as a leaving group, the succinimide ring opened and then inserted itself into the molecule. Although this result was not fully characterized, the mass spectrometry result indicated the correct mass for such a molecule. It was determined that the extra length caused by the open succinimide ring was acceptable and possibly even beneficial so the synthesis was continued with this compound. Standard deprotection of the *t*-BOC group using trifluoroacetic acid was attempted, but the result was not the desired product for an undetermined reason.

At this time it was decided to attempt an alternative synthetic path which involved first adding the bromoacetyl group to the biotin hydrazide followed by forming the terminal hydrazino group by substituting for the bromine by reaction with hydrazine (Figure IV-2). Bromoacetyl chloride was to have been reacted directly with biotin hydrazide in the first step of the pathway. However, even freshly synthesized bromoacetyl chloride proved to be too unstable under the conditions and this method was also abandoned.

The successful synthesis of hydrazino ARP once again depended on the substitution of the bromine on bromoacetic acid with hydrazine (see Figure III-I). In this case, DCC condensation was successfully used to react bromoacetic acid with biotin hydrazide. It should be noted here that biotin hydrazide has great difficulty dissolving in either dimethylformamide (DMF) or dimethyl sulfoxide (DMSO), the only two solvents which will dissolve the compound. DMF was chosen due to the difficulty of removing DMSO, but even

with DMF extended heating at 50 – 60 ^{o}C with stirring was necessary to dissolve the biotin hydrazide. A better alternative seemed to be to dissolve as much biotin hydrazide as possible without heating and start the reaction depending on the law of mass action to dissolve it further as it was consumed in the reaction. Also, since this is a condensation reaction, much care must be taken to exclude water from all reaction materials, including fresh distillation of the solvent. Since dicyclohexylurea (DCU) began to precipitate almost at once after addition of the DCC it was not possible to know when the biotin hydrazide was fully dissolved so the reaction was allowed to stir at room temperature for approximately two days when it was removed from stirring and the precipitate was filtered off and the filtrate dried. Based on NMR results and the weight of the filtered DCU it was determined that some DCU remained in the filtrate. The DCU was removed by dissolving the solid obtained from the filtrate in 50% MeOH:$CHCl_3$ which dissolves DCU but not the product. Also, if any excess biotin hydrazide was found to remain in the product it was removed with 5% HCl. The reaction gave a fair yield of 52% for the bromoacetyl conjugated biotin hydrazide.

The hydrazino ARP final product was synthesized by addition of the bromoacetyl conjugated biotin hydrazide to hydrazine monohydrate. In order to reduce the suspected possibility of producing the bis-adduct of the conjugated biotin hydrazide, the conjugate was introduced drop-wise slowly into excess hydrazine. Although a small amount of the bis-adduct was apparent from mass spectrometry results of the crude product, it appeared to be removed after the work-up to remove the hydrobromide salt by-product of the reaction. The yield for this reaction was good at 65%.

At this time only very preliminary results have been obtained as to the comparison of reactivity to AP sites of the hydroxylamine ARP to the hydrazino ARP. The result of the

single slot-blot assay performed indicated a stronger reaction with the hydroxylamine ARP. However, the slot-blot assay has been performed only at pH 7 and it is believed that the hydrazino group may be protonated ($-NHNH_3^+$) at pH 7 making it unavailable to react with the aldehyde. To obtain a true evaluation of the comparative reactivities of the two ARPs the hydrazino ARP will need to be used in a slot-blot assay performed at pH 8 or higher.

Several strategies were attempted for the synthesis of the long-chain (long-tether) ARP (LC-ARP) before finding a successful method. In all cases, PEGs with either 5 or 6 ethyl ethers were used to extend the length of the molecule. Both dihydroxy and diamino PEGs were used. All of the methods involved attaching various protection and activating groups to the ends of the PEG. *N*-hydroxyphthalimide was generally used as the protection group for one end of the PEG while several different synthetic methods were used to react biotin moieties to the other. These attempts all proved unsuccessful. The successful synthesis was performed after discovering a commercially available biotinylated PEG carboxyhydrazide that resembles very closely biotin hydrazide in terms of reactivity, hence by simply following the synthetic path described by Ide, et al., the desired LC-ARP could be obtained. To date, this synthesis has been performed successfully up to the penultimate product which still remains to be deprotected from the *t*-BOC.

During the attempted synthesis of the LC-ARP from PEG it was realized that what was a troublesome by-product may have value if further work was done with it. In some cases these syntheses were attempts to react with just one end of a symmetrical molecule and it is inevitable that some *bis*-adduct by-product will be made. This was taken advantage of by producing a PEG with hydroxylamine at both ends. In the attempted LC-ARP synthesis 1,19-diamino-4,7,10,13,16-pentaoxanonadecane was reacted with the *N*-hydroxysuccinimide

ester of *N*-(*tert*-butoxycarbonyl)-O-(carboxymethyl)hydroxylamine with the proper stoichiometry to react with just one end of the molecule. The *bis*-adduct by-product from this reaction was then deprotected with TFA to give the PEG with hydroxylamines at both ends (given the name "double-ended" or DE-ARP). Once this scheme was successful it was a simple matter to adjust the synthetic pathway to make this compound by adding two equivalents of the succinimidyl ester to the diamino PEG in the initial reaction followed by the deprotection. In the initial reaction of this scheme the crude product was worked up by separation on preparatory thin layer chromatography plates and it should be noted that the desired product was visible only after exposure to iodine vapor.

During the course of this project another ARP was synthesized. This compound, which had been previously synthesized in this laboratory, is identical to the original ARP but made use of iminobiotin hydrazide rather than biotin hydrazide. This compound was synthesized using the method described by Ide, et al., (1993).

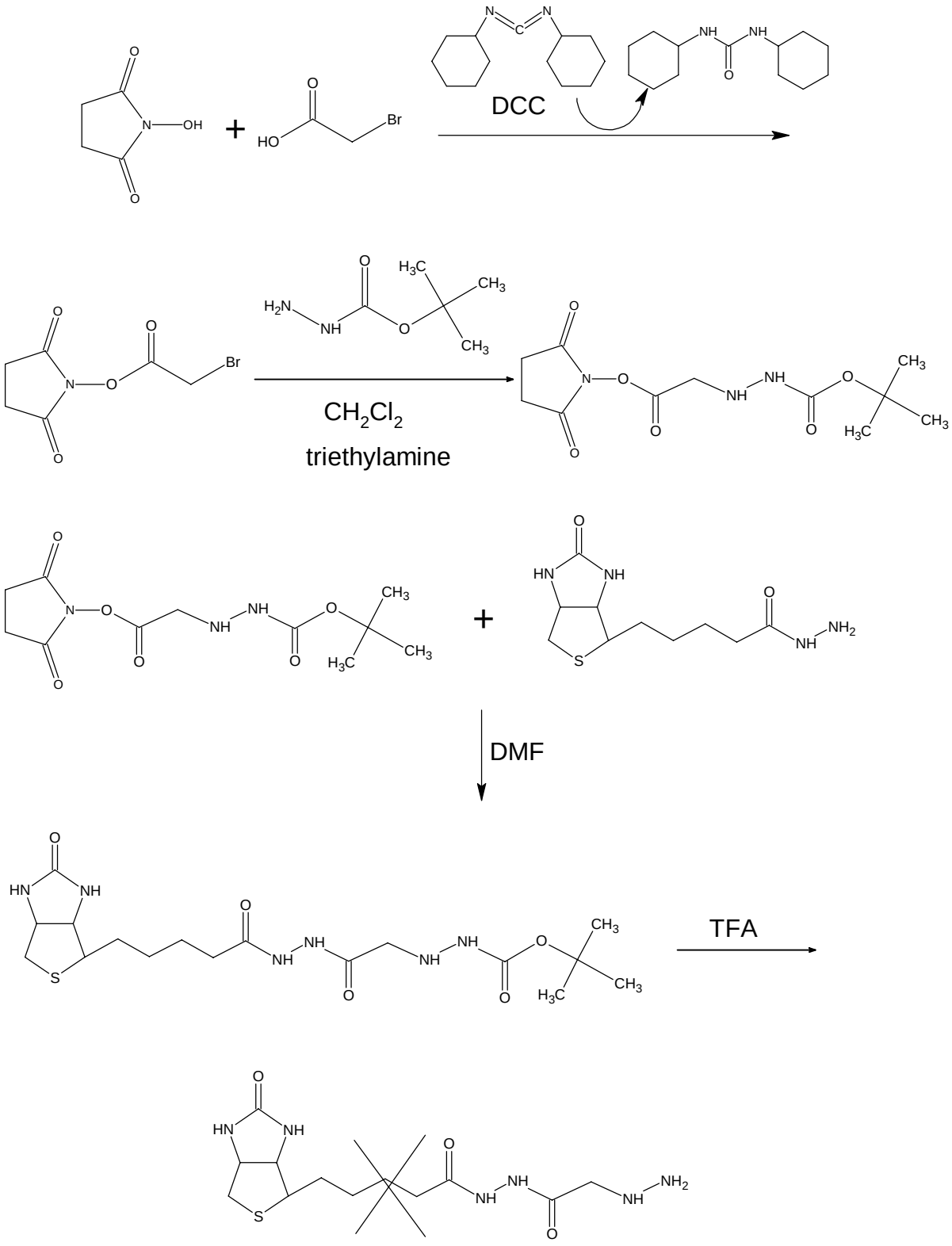


Figure IV-1 First attempted synthesis of hydrazino ARP.

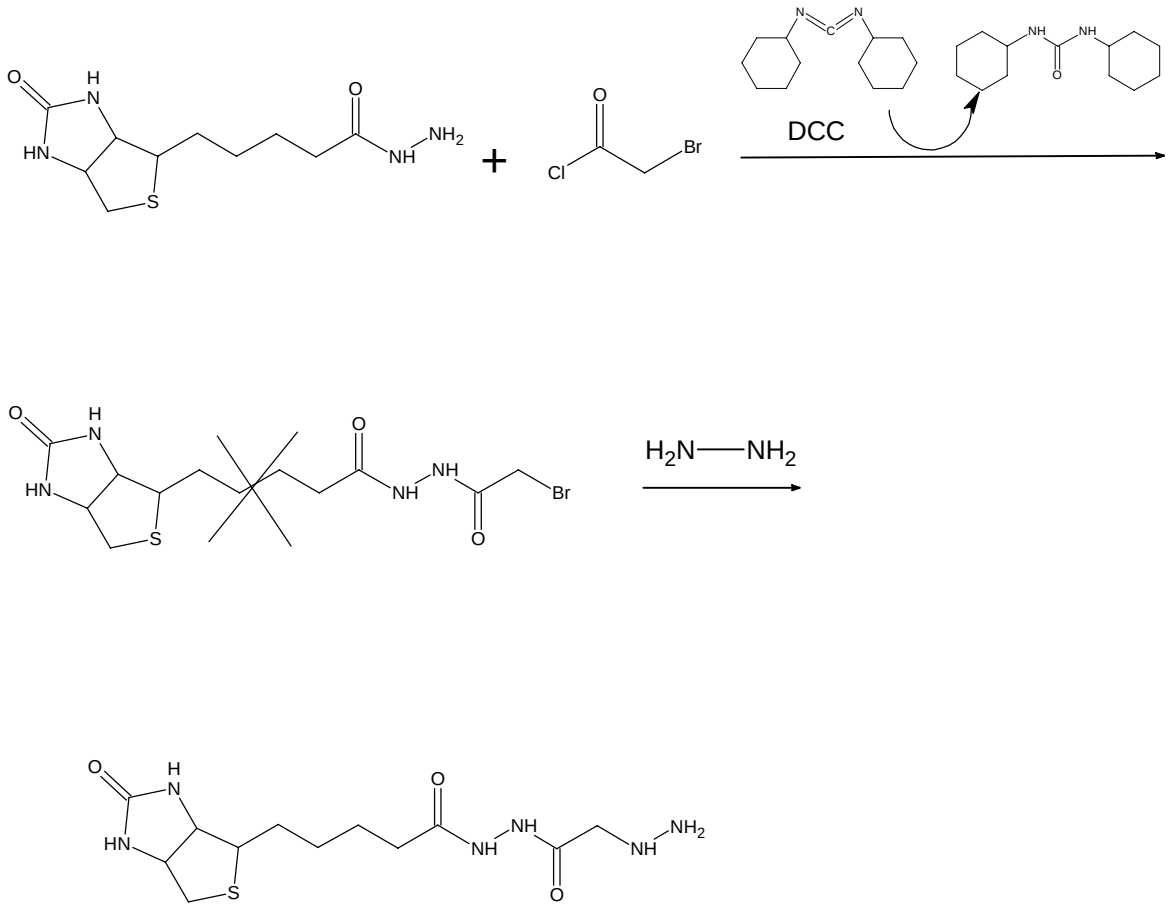


Figure IV-2 Second attempt to synthesize hydrazino ARP.

V. Conclusion

This project has resulted in the synthesis of two new aldehyde reactive probes that hopefully will contribute to the improvement of detection and quantitation of apurinic/apyrimidinic sites in DNA. A third compound has also been synthesized that may be more toxic to transformed cells than to normal ones.

While only one slot-blot assay has been performed with the new hydrazino ARP and that assay showed a lower reactivity than the original ARP, more work needs to be done with the new probe. A repeat of the assay should be performed at pH 7 (the normal pH for the assay) and, as it is probable that the hydrazino group is protonated at pH 7, the test should also be done at higher pH's in a systematic way. Synthesis of the hydrazino ARP uses inexpensive chemicals which are easily obtainable and is relatively easy.

The long-chain ARP also makes use of a straightforward synthetic pathway. However, the method, chosen for its expediency, makes use of a purchased starting material, the biotin PEG_4 hydrazide that is relatively expensive. Upon reflection, I believe this compound could be synthesized locally using low cost materials and possibly save some cost in the overall synthesis. Also, more work needs to be done to substantially increase the yield of the final product. This compound has not been tested in the slot-blot assay and, of course, this should be done to prove its efficacy in the test before work on improvements to the synthesis is contemplated.

The third new compound, the "double-ended" ARP, may be, after all, the most exciting. The synthesis is not difficult and inexpensive materials are used. As with the LC ARP, more

work should be done to improve yield. Preliminary in vivo tests done elsewhere show that the molecule has about the same toxicity as methoxyamine in normal cells. While this shows that the molecule survives and enters the cell, it will be interesting to see if it is making its way to the nucleus and reacting with abasic sites or if the toxicity is caused by some other reaction.

References

Anders, R. E., and Dekant, W., 1994. Conjugation-dependent carcinogenicity and toxicity of foreign compounds. *Adv. Pharmacol.* **27:**1-519.

Atamna, H., Cheung, I. and Ames, B. 1999. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. *PNAS*. Vol. 97, No. 2, 686-691.

Bailly, V. and Verly, W. G. 1988. Possible roles of β-elimination and δ-elimination in the repair of DNA containing AP (apurinic/apyrimidinic) sites in mammalian cells. *Biochem. J.*, 253, 553-559.

Berg, J. M., Tymoczko, J. L., and Stryer, L. *Biochemistry, 5th ed*.; W. H. Freeman and Company, 2002.

Blount, B. C., Mack, M. M., Wehr, D. M., MacGregor, J. T., Hiatt, R. A., Wang, G., Wickramasinghe, S. N., Everson, R. B. and Ames, B. N. 1997. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. *PNAS* 94: 3290-3295.

Boiteux, S. and Laval, J. 1982. Coding properties of poly(deoxycytidylic acid) templates containing uracil or apyrimidinic sites: In vitro modulation of mutagenesis by deoxyribonucleic acid repair enzymes. *Biochemistry* 21, 6746-6751.

Brookes, P. 1990. The early history of the biological alkylating agents. *Mutation Research.* **233:**3-14.

Cabelof, D. C., Raffoul, J. J., Nakamura, J., Kapoor, D., Abdalla, H. and Heydari, A. R. 2004. Imbalanced base excision repair in response to folate deficiency is accelerated by polymerase β haploinsufficiency. *The Journal of Biological Chemistry*. 279:35, 36504-36513.

Demple, B., and Harrison, L. 1994. Repair of Oxidative Damage to DNA: Enzymology and Biology. *Annual Revues in Biochemistry*. 63:915-948.

Demple, B. and Levin, J. D. 1991. Repair systems for radical damaged DNA, p. 119-154. In H. Sies (ed.), *Oxidative Stress: Oxidants and Antioxidants.* Academic Press, Ltd., London.

Doetsch, P. W. and Cunningham, R. P. 1990. The enzymology of apurinic/apurimidinic endonucleases. *Mutation Research*, 236,173-201.

Friedberg, E. C., Walker, G. C., and Siede, W. 1995. *DNA Repair and Mutagenesis.* American Society for Microbiology, Wash, D.C.

Ide, H., Akamatsu, K., Kimura, Y., Michiue, K., Makino, K., Asaeda, A., Takamori, Y. and Kubo, K. 1993. Synthesis and damage specificity of a novel probe for the detection of abasic sites in DNA. *Biochemistry,* 32, 8276-8283.

Johnson, R. E., Washington, M. T., Haracska, L., Prakash, S. and Prakash, L. 2000. Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions, *Nature* 406, 1015-1019.

Jones, A. S., Mian, A. M. and Walker, R. T. 1968. The alkaline degradation of deoxyribonucleic acid derivatives. *J. Chem. Soc.* (C), 2024-2044.

Kingma, P. S. and Osheroff, N. 1998. The response of eukaryotic topoisomerases to DNA damage. *Biochem. Biophys. Acta.* 1400, 223-232.

Kohen, R. and Nyska, A. 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxocologic Pathology.* Vol.30, No.6. pp. 620-650.

Kubo, K., Ide, H., Wallace, S. S. and Kow, Y. W. 1992. A novel, sensitive, and specific assay for abasic sites, the most commonly produced DNA lesion. *Biochemistry*, **31**, 3703-3708.

Kunkel, T. A., Schaaper, R. M. and Loeb, L. A. 1983. Depurination-induced infidelity of deoxyribonucleic acid synthesis with purified deoxyribonucleic acid replication proteins in vitro. *Biochemistry*. 22, 2378-2384.

Langer, H. and Doltsinis, N. L. 2003. Excited state tautomerism of the DNA base guanine: a restricted open-shell Kohn-Sham study. *Journal of Chemical Physics*, Vol. 118, No.12, pp. 5400-5407.

Lawrence, C. W., Borden, A., Banerjee, S. K. and LeClerc, J. E. 1990. Mutation frequency and spectrum resulting from a single abasic site in a single-stranded vector. *Nucleic Acids Research*. Vol. 18, No. 8., 2153-2157.

Lewin, B. 2004. *Genes XIII.* Pearson Prentice Hall, Upper Saddle River, N.J. p.10.

Lindahl, T. and Nyberg, B. 1972. Rate of depurination of native deoxyribonucleic acid. *Biochemistry*. Vol. 11, No. 19, pp. 3610-3618.

Lindahl, T. 1976. New class of enzymes acting on damaged DNA. *Nature* (London) **259:**64-66.

Lindahl, T. 1981. *DNA Repair: A Laboratory Manual of Research Procedures.* Friedberg, E. C. and Hanawalt, P. C., Eds. Marcel Dekker, Inc., New York. Vol. 1, Part A, pp. 213-216.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. *Nature.* Vol. 362, 22 April 709-715.

Lindahl, T. and Wood, R. D. 1999. Quality control by DNA repair. *Science*. Vol. 286, pp. 1897-1905.

Loeb, L. A. and Preston, B. D. 1986. Mutagenesis by apurinic/apyrimidinic sites. *Annual Revue of Genetics.* **20:**201-230.

Lyons-Darden, T. and Topal, M. D. 1999. Abasic sites induce triplet-repeat expansion during DNA replication in vitro. *Journal of Biological Chemistry.* 272, 25975-25978.

Manoharan, M., Ransom, S. C.., Mazumber, A. and Gerlt, J. A. 1988. The characterization of abasic sites in DNA heteroduplexes by site specific labeling with ^{13}C. *Journal of the American Chemical Society.* 110, 1620-1622.

Masutani, C., Kusumoto, R., Iwai, F. and Hanaika, F. 2000. Mechanisms of accurate translesion synthesis by human DNA polymerase η. *EMBO J.* 19, 3100-3109.

Mol, Clifford D., Parikh, Sudip S., Putnam, Christopher D., Lo, Terence P. and Tainer, John A. 1999. DNA Repair and Mechanisms for the Recognition and Removal of Damaged DNA Bases. *Annual Review of Biophysics and Biomolecular Structures.* 28:101-128.

Montelone, B. A. 1998. Mutation, Mutagens and DNA Repair. Supplement to BIOL400, Human Genetics. Division of Biology, Kansas State University.

Nakamura, J., Walker, V. E., Upton, P. B., Chiang, S., Kow, Y. W. and Swenberg, J. A. 1998. Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. *Cancer Research*, 58, 222-225.

Nakamura, J., Swenberg, J.A., 1999. Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. *Cancer Research*. 59, 2522-2526.

Nilson, H. and Krokan, H.E. 2001. Base excision repair in a network of defense and tolerance. *Carcinogenesis*. Vol. 22 No. 7 pp. 987-998.

Parkinson, A., In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th ed.; Klaassen, C. D. Ed. 1996. McGraw-Hill Medical Publishing: New York, p.113.

Paz-Elizur, T., Brenner, D. E. and Livneh, Z. 2005. Interrogating DNA repair in cancer risk assessment. *Cancer Epidemiology, Biomarkers and Prevention;* **14(7)**:1585-1587.

Pieper, R. O., In *DNA Damage and Repair, Volume II: DNA Repair in Higher Eukaryotes;* Nickoloff, J. A., Hoekstra, M. R., Eds. 1998. Humana Press Inc., Totowa, N.J.

Pitot, H. C., III, and Dragan, Y. P., In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th ed.; Klaassen, C. D. Ed. 1996. McGraw-Hill Medical Publishing: New York, p. 225.

Riley, P. A. 1994. Free radicals in biology: oxidative stress and the effects of ionizing radiation. *International Journal of Radiation Biology*. **65:**27-33.

Roberts, K. P., Sobrino, J. A., Payton, J. Mason, L. B. and Turesky, R. J. 2006. Determination of apurinic/apyrimidinic lesions in DNA with high-performance liquid chromatography and tandem mass spectrometry. *Chemical Research in Toxicology.* 19, 300-309.

Seki, M. Masutani, C., Yang, L. W., Schuffert, A., Iwai, S., Bahar, I. and Wood, R. D. 2004. High-efficiency bypass of DNA damage by human DNA polymerase Q. *The EMBO Journal.* **23,** 4484-4494.

Sukhanova, M.V., Larvik, O.I., Khodyreva, S.N. 2004. Poly(ADP-ribose)polymerase-1: A Regulator of Protein-Nucleic Acid Interactions in Processes Responding to Genotoxic Impact. *Molecular Biology*. Vol.38, No. 5, pp. 706-717.

Talpaert-Borle', M. and Liuzzi, M. 1983. Reaction of apurinic/apyrimidinic sites with (^{14}C)methoxyamine. A method for the quantitative assay of AP sites in DNA. *Biochim. Biophys. Acta.* **740**, 410-416.

Teebor, G. W. and Duker, N. J. 1975. *Nature*. 258, 544.

Teoule, R. 1987. Radiation-induced DNA damage and its repair. *Int. J. Radiat. Biol.* **51:**573-589.

Ward, J. F. 1988. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and reparability. *Prog. Nucleic Acid Res. Mol. Biol.* **35**: 95-125.

Weinfeld, M., Luizzi, M. and Paterson, M. C. 1990. Response of phage T4 polynucleotide kinase toward dinucleotides containing apurinic sites: design of a ^{32}P-postlabeling assay for apurinic sites in DNA. *Biochemistry*, **29,** 1737-1743.

Wilde, J. A., Bolton, P. H., Mazumber, A., Manoharan, M. and Gerlt, J. A. 1989. Characterization of the equilibrating forms of the aldehydic abasic site in duplex DNA by ^{17}O NMR. *Journal of the American Chemical Society.* 111, 1894-1896.

Wilson, D. and Barsky, D. 2001. Base excision repair intermediates as topoisomerase II poisons. *J. Biol. Chem.* 276: 46290-46296.

Wilson, D. M., III and Barsky, D. 2001. The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. *Mutation Research.* 485:283-307.

Wood, R. D. 1996. DNA Repair in Eukaryotes. *Annual Review of Biochemistry*. **65:**135-167.

Zhang, , Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J. S., Geacintov, N. E. and Wang, Z. 2000. Error-free and error-prone lesion bypass by human DNA polymerase kappa in vitro. *Nucleic Acids Research.* 28, 4138-4146.