**DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL NEO-TANSHINLACTONE ANALOGUES AS POTENT AND SELECTIVE ANTI-BREAST CANCER AGENTS**

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Pharmacy.

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## ABSTRACT

**YIZHOU DONG: Design, Synthesis, and Biological Evaluation of Neo-tanshinlactone**

**Analogues as Potent and Selective Anti-Breast Cancer Agents**

**(Under the direction of Kenan Professor Kuo-Hsiung Lee)**

The overall goals of this research are to design and synthesize novel neo-tanshinlactone analogues, to evaluate their antitumor activity and elucidate structure-activity relationships (SAR), to discover novel chemical entities, and to explore the mechanism(s) of action.

Neo-tanshinlactone (**90**) and its previously reported analogues are potent and selective *in vitro* anti-breast cancer agents. In a continuing study, a highly efficient synthesis of 4-ethyl neo-tanshinlactone **91** was accomplished with fewer steps and higher overall yield than those previously reported. This synthetic route was applied to develop new lead compounds and establish SAR of **90**. SAR studies on these compounds revealed the key molecular determinants of this family of anti-breast agents. Several analogues (**108-110** and **113**) displayed potent and selective anti-breast cancer activity.

We further explored how the individual rings in the molecule of **90** influence the anti-breast cancer activity. We designed and synthesized five new classes of compounds derived from neo-tanshinlactone. The results led to the discovery of two novel classes of anti-breast cancer agents, 2-(furan-2-yl)naphthalen-1-ol and 6-phenyl-4*H*-furo[3,2-*c*]pyran-4- one derivatives.

Preliminary SAR of 2-(furan-2-yl)naphthalen-1-ol derivatives was established.

Compounds **172**, **173**, and **181** were developed as new anti-breast cancer agents with better selectivity than **91**. Interestingly, **178** showed broad *in vitro* cytotoxicity against human cancer cell lines. Further development led to tetrahydronaphthalene-1-ol derivatives, a novel class of antitumor agents. **186** displayed potent activity against most tumor cell lines tested.

Novel 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives were synthesized and evaluated as novel anti-breast cancer agents. We explored the SAR and optimized the substituents. Promising lead compounds **198-201**, **211**, **213**, and **215** showed potent inhibition against the SK-BR-3 breast cancer cell line. Importantly, **213** and **215** showed the highest cancer cell line selectivity, being approximately 100- to 250-fold more potent against SK-BR-3 (0.08, and 0.14 µg/mL, respectively) compared with other cancer cell lines tested.

Enzyme assays suggested that **91** significantly suppressed CK2α1, ABL, and AKT1, and **178** showed higher inhibition activity against CDC42BPB, PKG1β, and SGK1. The results will guide us to further explore the mechanisms of action. In addition, we demonstrated that **91** is a potent and selective anti-breast cancer agent both *in vitro* and *in vivo.*

**ACKNOWLEDGEMENTS**

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AI | aromatase inhibitor |
| BRCA1 | breast cancer type 1 susceptibility gene |
| BRCA2 | breast cancer type 2 susceptibility gene |
| DASI | dual aromatase-steroid sulfatase inhibitors |
| DMAP | 4-Dimethylaminopyridine |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethyl sulfoxide |
| E1 | estrone |
| E2 | estrodiol |
| E1S | estrone sulfate |
| $EC_{50}$ | effective dose which causes 50% inhibition |
| EGFR | epidermal growth factor receptor |
| EMATE | estrone-3-methylthiophosphonate |
| ER | estrogen receptor |
| ERE | estrogen response element |
| FDA | Food and Drug Administration |
| Fox | forkhead box |
| 1H NMR | proton nuclear magnetic resonance |
| HER2 | Human epidermal growth factor receptor 2 |
| HDI | HER2 dimerization inhibitor |
| HIF1α | hypoxia-inducible factor-1α |
| HRT | hormone replacement therapy |

| | |
|---|---|
| *k*NN-QSAR | k nearest neighbor quantitative structure-activity relationship |
| MAbs | monoclonal antibodies |
| MAPK | mitogen-activated protein kinase |
| MeOH | methanol |
| mp | melting point (°C) |
| MS | mass spectrum |
| NSAIs | nonsteroidal aromatase inhibitor |
| NBS | *N*-bromosuccinimide |
| NF-κB | nuclear factor kappaB |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| SAR | structure-activity relationships |
| SERM | selective estrogen receptor modulators |
| SERD | selective estrogen receptor down regulators |
| STS | steroid sulfatase |
| TAM | tamoxifen |
| TCM | traditional Chinese medicine |
| TGD | tumor growth delay |
| TTE | time to endpoint |
| VEGF | vascular endothelial growth factor |
| μM | micromolar concentration |
| WHO | World Health Organization |

# CHAPTER 1

# BREAST CANCER AND TREATMENTS

## 1.1    Introduction

Breast cancer is the most common malignancy in women.[1] Worldwide, more than 1.2 million women are diagnosed with breast cancer every year, which accounts for one-tenth of all new cancers.[2] In the U.S., breast cancer accounts for more than one quarter of cancers diagnosed in women, according to the American Cancer Society. In 2007, the estimate for new cases of invasive breast cancer was 170,000, as well as an estimated 60,000 additional cases of in situ breast cancer.[3] Male breast cancer is significantly rarer than the female form of the disease, but still affects around 2,000 men yearly in the U.S., accounting for 1% of all breast cancer.[3, 4]

## 1.2    Risk Factors for Breast Cancer

A risk factor is anything that affects your chance of getting a disease, such as breast cancer. In 1940, the lifetime risk of a woman developing breast cancer was 5%. In 2008, the American Cancer Society increased that estimate to 12.3%.[3]

There are different kinds of risk factors. Some factors, such as a person's age or race, cannot be intervened. Others are related to cancer-causing factors in the environment. Still other risk factors are affected by personal behaviors, including smoking, drinking, and diet.[5]

**Increasing age**

Age is one of the most important risk factors for breast cancer.[3] Generally, breast

cancer incidence and death rates increase with age.[3] Based upon statistical data from the National Cancer Institute, women aged 20-24 years have the lowest breast cancer incidence rate, and women aged 75-79 years have the highest incidence rate, which is 464.8 cases per 100,000.[6] Age-specific incidence rates decrease in women aged 80 years and older.[6]

**Family history of breast cancer/genetic predisposition**

Women with a family history of breast cancer, especially in a first-degree relative (mother, sister, or daughter), have higher risk of developing breast cancer than those without a family history of breast cancer.[3, 7] Approximately 5% to 10% of breast cancer cases result from inherited mutations or alterations in the breast cancer susceptibility genes BRCA1 and BRCA2.[8] Based on population studies, BRCA1 mutations may cause a 65% risk for women to develop breast cancer by age 70; the corresponding risk for BRCA2 mutations is 45%. However, not all familial risk is due to a BRCA1 or BRCA2 mutation.[9]

**Hormonal factors**

Reproductive hormones can regulate cell proliferation and DNA damage, as well as promote cancer growth, and thus, influence breast cancer risk.[3] A woman's risk of breast cancer may be increased by factors related to the levels of reproductive hormones produced by her body, including early menarche (<12 years), older age at menopause (>55 years), older age at first full-term pregnancy (>30 years), and fewer numbers of pregnancies.[10]

In addition, recent studies demonstrated that the use of combination hormone replacement therapy (HRT), including estrogen and progestin, can be linked to increased breast cancer risk. A higher risk is associated with longer use.[11, 12]

**Clinical factors**

High breast tissue density is another strong independent risk factor for the development of breast cancer.[3] Women with the highest levels of breast density were found to have a four- to six-fold increased risk of breast cancer, compared with women with the least dense breasts.[3, 13-15]

2

Some benign breast conditions are closely related to breast cancer risk.[3, 16-18] There are three types of benign breast conditions based on the degree of risk: non-proliferative lesions, proliferative lesions without atypia, and proliferative lesions with atypia. Non-proliferative lesions do not show much effect on breast cancer risk, because they are not associated with any overgrowth of breast tissue. Proliferative lesions without atypia may increase a woman's risk of breast cancer slightly. Proliferative lesions with atypia (including atypical ductal hyperplasia and atypical lobular hyperplasia) have a stronger effect on breast cancer risk, increasing the risk four to five times higher than normal.[3]

## 1.3    Treatments for Breast Cancer

Many factors must be considered when choosing the optimal treatment for breast cancer, including the stage and biological characteristics of the cancer, the patient's age and preferences, and the risks and benefits associated with each treatment protocol.[19]

### Surgery

The primary goal of breast cancer surgery is to remove the cancer from the breast and to evaluate the stage of the disease.[3] Surgery is one of the primary treatments for most women with breast cancer. Only cancerous tissue plus a rim of normal tissue is removed in a lumpectomy. Total mastectomy includes removal of the entire breast.[3] However, breast cancer recurrence is still a concern after surgery.[1]

### Radiation therapy

Radiation aims to destroy cancer cells remaining in the breast, chest wall, or underarm area after surgery or to reduce the size of a tumor before surgery.[20] Radiation therapy is typically given for five to seven weeks, based on the patient's condition.[3] Radiation treatment is usually prescribed with other treatments such as chemotherapy.

### Hormone therapy

Estrogens play crucial roles in breast cancer development and growth, and estrogen-stimulated growth in tumor cells (as well as in normal cells) requires estrogen receptors (ERs).[21] About two-thirds of human breast tumors express higher levels of ERs than normal breast tissues.[22] If their breast cancers test positive for estrogen receptors, women can be given hormone therapy to block the effects of estrogen on the growth of breast cancer cells.[3] The most widely used therapy for antagonizing ER function is the antiestrogen tamoxifen (TAM), which binds to ERs and blocks downstream signaling.[22] Patients may stop taking tamoxifen because of drug resistance or adverse effects.

Another class of drugs known as aromatase inhibitors (AIs), which block estrogen production, has been approved for use in treating both early and advanced breast cancer. These drugs include letrozole, anastrozole, and exemestane.[3, 19] They can bind aromatase and then reduce the amounts of estrogen in postmenopausal women. Aromatase inhibitors are not effective in premenopausal women, because these women produce estrogen from the ovaries.[3] AIs are often preferred over tamoxifen as the first hormonal treatment for postmenopausal women, if their cancer is hormone receptor positive.[3] AIs have fewer side effect than tamoxifen; however, they can cause osteoporosis and bone fractures.[3]

**Biologic therapy**

An estimated 15% to 30% of breast cancers overproduce the protein HER2/neu (human epidermal growth factor receptor 2), which promotes tumor growth.[3] The American Cancer Society reported that "these tumors grow faster and are generally more likely to recur than tumors that do not overproduce HER2".[3] Herceptin is the first developed monoclonal antibody that directly targets the HER2 protein of breast tumors. Its use can increase the survival chance for some women with metastatic breast cancer.[23-25] Recently, herceptin also showed potent activity in treating early-stage breast cancer that over-expresses HER2.[26] As a result, many doctors suggest all invasive breast cancers should be tested for the HER2 protein in order to identify women who would benefit from this therapy.[27]

4

**Chemotherapy**

Chemotherapy refers to the use of chemicals that kill breast cancer cells after the tumor has been removed. Its purpose is to increase patient cure rates.[3] Combinations of drugs are more effective than just one drug alone for breast cancer treatment.[28] The drugs most commonly used in combination in early breast cancer are cyclophosphamide, methotrexate, fluorouracil, doxorubicin, epirubicin, paclitaxel, and docetaxel. Adjuvant chemotherapy is usually given for three to six months, based upon the combination of drugs. These and other chemotherapy drugs may also be used to reduce cancer size.[3] Although new cytotoxic agents with unique mechanisms of action have been developed continuously, many of them have not been therapeutically useful due to low tumor selectivity.[29] The toxic side effects, low tumor selectivity, and multidrug resistance with current cancer chemotherapy prompt us to develop novel potent anti-breast cancer agents.

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# CHAPTER 2

# ANTI-BREAST CANCER DRUGS (AGENTS) AND THEIR MECHANISMS OF ACTION

In the previous chapter, we discussed the risk factors and general treatments for breast cancer. Numerous studies have demonstrated that there are many mechanisms of action that could lead to breast cancer including over-expression of HER2, estrogen receptors, aromatases, sulfatases, and others. It is important to understand the mechanisms of action for the discovery and development of novel anti-breast cancer drugs. In this chapter, we will describe the anti-breast cancer drugs in use or under development based on their mechanisms of action.

## 2.1     HER2 Inhibitors

Receptor protein tyrosine kinases play an important role in signal transduction pathways that mediate cell division and differentiation.[1] The erbB receptor family contains four components: erbB-1/EGFR, erbB-2/Neu/HER2, erbB-3/HER3, and erbB-4/HER4.[1] Abnormal signaling in these pathways can have the following consequences: dysregulation of cell proliferation, evasion from apoptosis, angiogenesis, migration, and metastasis.[2-5] Over-expression of EGFR, as well as HER2, is a marker for poor prognosis in many human cancers.[3, 6, 7]

Figure 2-1 shows the main pathways activated by EGFR and HER2/*neu*, which can affect cell proliferation, angiogenesis, and other signaling transduction results.[8] Both the phosphoinositide 3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinase (MAPK) pathway can be activated by EGFR, HER2/*neu* heterodimerization, and tyrosine

phosphorylation.[9] AKT activation results in up-regulation of hypoxia-inducible factor-1α (HIF1α), which promotes vascular endothelial growth factor (VEGF) production and leads to angiogenesis. MAPK activation increases DNA synthesis to induce cell proliferation and tumor growth.[1, 8, 10]
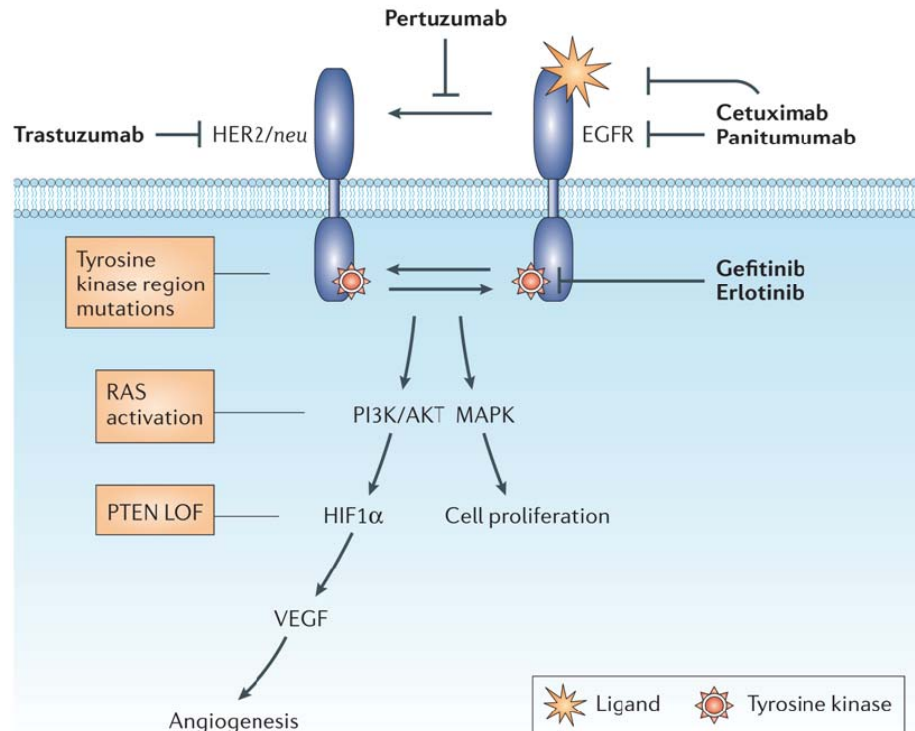


**Figure 2-1. Genetic variations and therapeutic targets: HER2/neu[8]**

As shown below, many approaches have been developed to target the erbB receptor family, especially HER2, because of its close link to breast cancer:[1]

(1) Monoclonal antibodies (MAbs) bind the extracellular domain of the receptor;

(2) Small molecules inhibit kinase activity by binding at the intracellular tyrosine kinase domain;

(3) Immunotoxin conjugates deliver toxins through erbB receptor targeting;

(4) Antisense oligonucleotides or RNAi reduce expression;

(5) Adaptor proteins block downstream signaling.

10

The first two approaches have been the most extensively explored. Therapeutic agents that act on the HER2/*neu* receptor through the first two approaches will be discussed in more detail below.

### 2.1.1 Monoclonal Antibodies

Trastuzumab (Herceptin) is a fully humanized monoclonal antibody jointly developed by Genentech, and was approved for treatment of breast cancers by the U.S. FDA in September 1998. It binds to the extracellular domain of HER2 and exerts anti-proliferative activity against breast cancers that over-express HER2 (Figure 2-1).[11, 12] Herceptin shows many effects, including antibody-mediated toxicity, prevention of downstream signaling, and promotion of receptor internalization.[13-15] However, herceptin cannot block heterodimerization of HER2, because the binding site of herceptin is not involved in dimerization.[1, 16]

Pertuzumab, another antibody, is the first in a new class of agents known as HER dimerization inhibitors (HDIs), being developed by Genentech. It also binds to the extracellular domain of HER2, but at a site distinct from that of trastuzumab.[17] This binding is believed to sterically block the region necessary for HER2 to collaborate with other erbB family members.[1, 18] Therefore, pertuzumab is active against tumors with low or high HER2 expression, because pertuzumab blocks ligand-induced HER2 heterodimerization.[1, 8, 17]

### 2.1.2 Small Molecular Inhibitors

#### 1. 4-Anilinoquinazolines

4-Anilinoquinazolines constitute the most widely studied class of small molecule inhibitors.[4] They are potent and selective reversible inhibitors of EGFR and HER2 tyrosine kinase, and act through competitive binding to the ATP site of the enzyme.[19, 20] Although Iressa (**1**, gefitinib) failed to be approved for clinical use, Tarceva (**2**, erlotinib) was approved by the FDA in 2005 for clinical use in cancers over-expressing EGFR (Figure 2-2).[21, 22] GW572016 (**3**, lapatinib), developed by GSK, is a potent dual inhibitor of EGFR and HER2

kinases.[19] Lapatinib is now in clinical use for the treatment of breast cancer. To obtain prolonged activity with selective kinase inhibitors, an irreversible inhibitor strategy has been explored by designing inhibitors to alkylate residues close to the ATP site.[23] Several irreversible inhibitors are under development, such as CI-1033 (**4**), EKB-569 (**5**), and HKI-272 (**6**).[24-26]



**1** Iressa (gefitinib)

**2** Tarceva (erlotinib)

**3** Lapatinib

**4** CI-1033

**5** EKB-569

**6** HKI-272
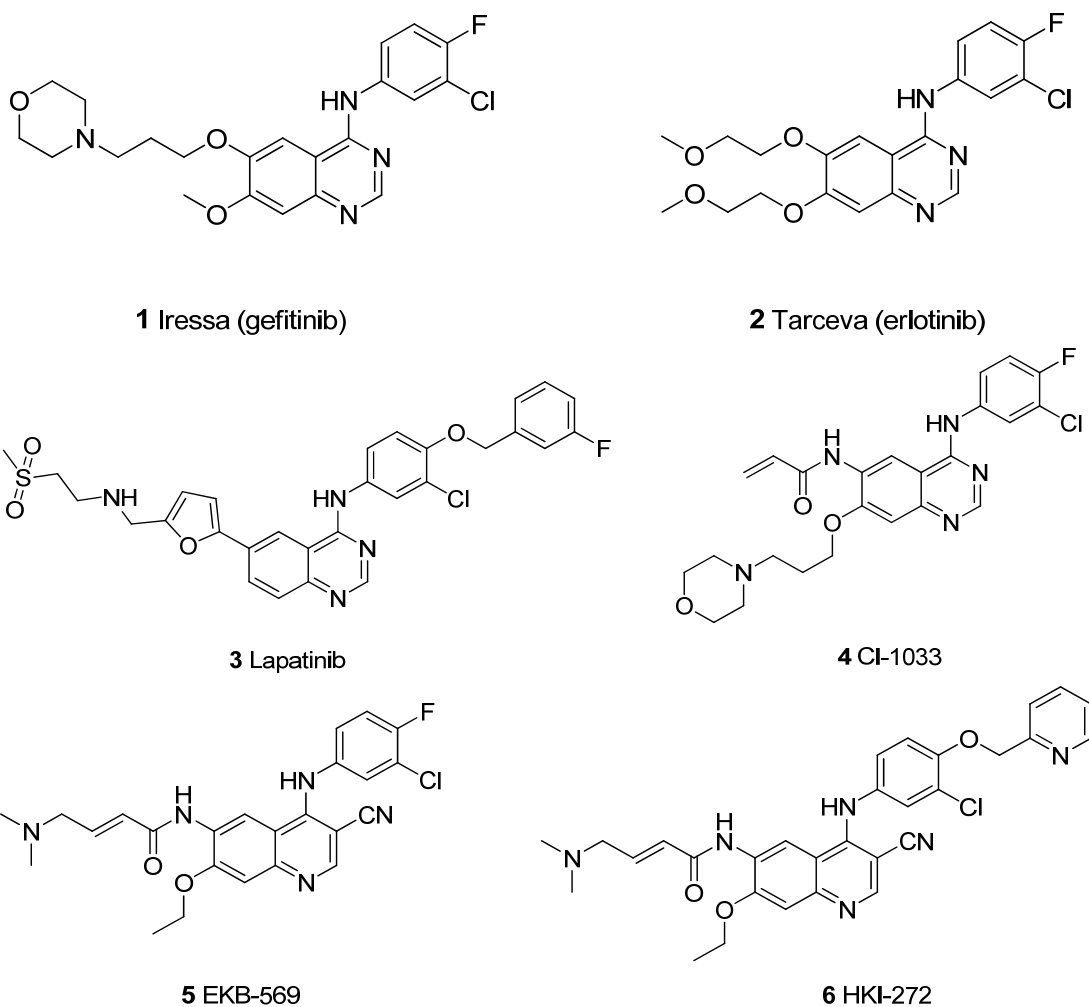
**Figure 2-2. Examples of 4-anilinoquinazoline inhibitors of HER2**

## 2. Tyrphostins

Tyrphostins could be classified as ATP competitive inhibitors, but also as mixed competitive inhibitors.[27] The characteristic feature of this class is a cis-cyano moiety, which

is important for HER2 inhibitory activity.[28, 29] Figure 2-3 shows some examples (**7-10**).
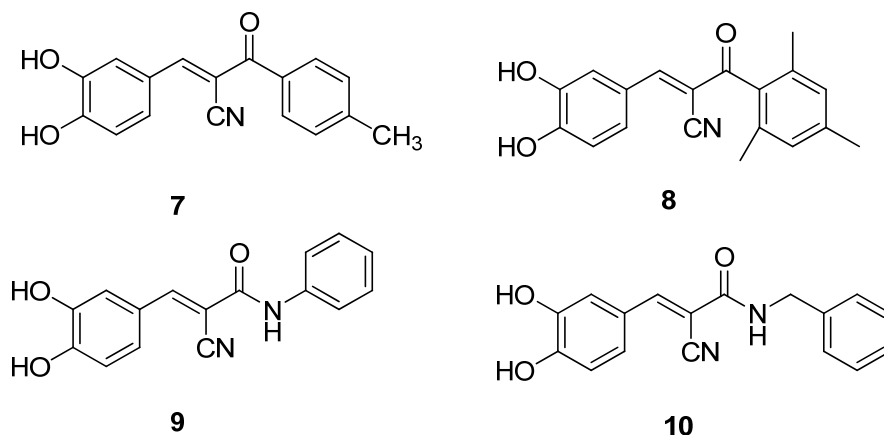


**Figure 2-3. Examples of tyrphostin inhibitors of HER2**


### 3. Pyrrolotriazines

The pyrrolo[2,1-*f*][1,2,4]triazine nucleus was developed from the quinazoline kinase inhibitor scaffold. Compounds **11**-**14** inhibited the kinase activity of EGFR, as well as inhibited proliferation of the human colon cancer cell line DiFi.[30] BMS-599626 (**15**, Figure 2-4), developed by BMS, is an effective inhibitor of both EGFR and HER2 tyrosine kinases. It shows superior efficacy to gefitinib in human tumor transplant models, and comparable activity to trastuzumab in mice with tumors over-expressing HER2.[1, 31] A Phase I clinical trial has been completed with BMS-599626.
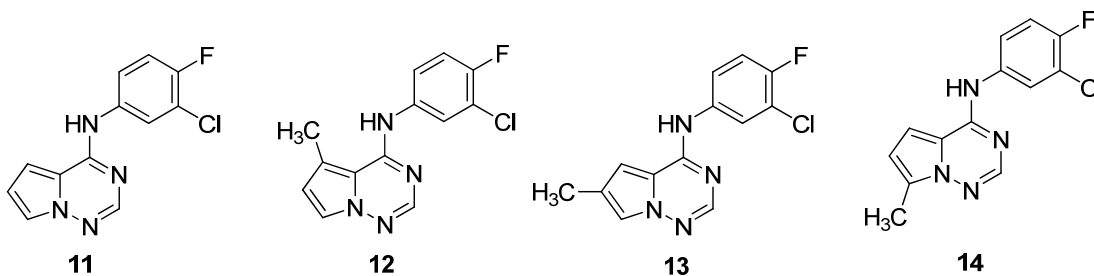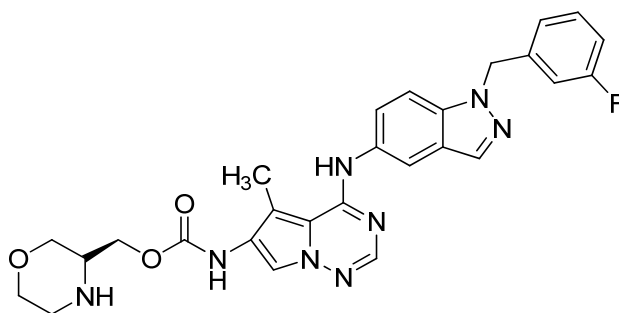
**15** BMS-599626

**Figure 2-4. Examples of pyrrolotriazine inhibitors of EGFR or HER2**

## 4. Pyrrolopyrimidines

AEE788 (**16**) and PKI166 (**17**) are dual EGFR/HER2 inhibitors (Figure 2-5), developed from 4-(phenylamino)pyrrolopyrimidines, and inhibit kinase activity in the low nanomolar range (Figure 2-5).[32-34] These compounds are under development in clinical trials by Novatis.
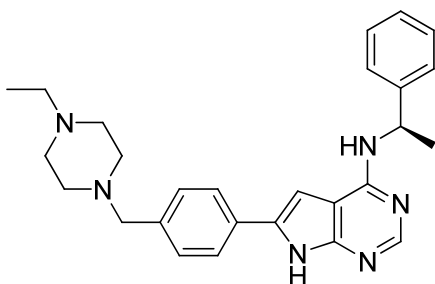


**16** AEE788
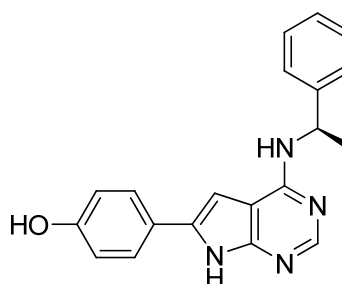
**17** PKI166

**Figure 2-5. Examples of pyrrolopyrimidines as dual EGFR/HER2 inhibitors**

## 5. 5,7-Diazaindolinones

5,7-Diazaindolinones were developed based on a combination of quinazoline and indolinone scaffolds (Figure 2-6). The combination of quinazoline and the five-membered lactam ring from the indolin-2-one core led to this class of compounds. Compounds **18**-**19** have been found to be dual EGFR/HER2 inhibitors.[35]

14

**18**

**19**
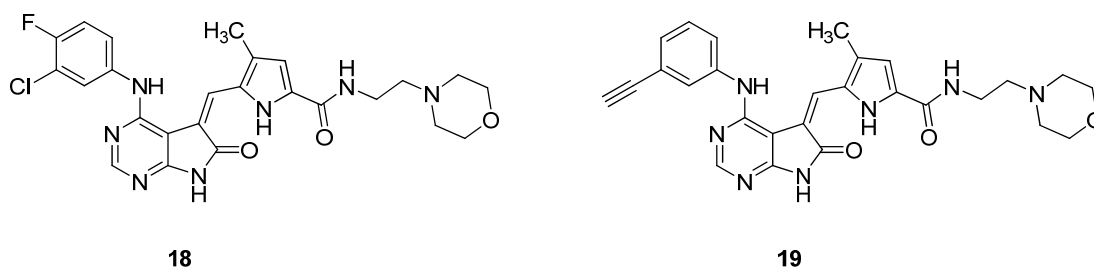
**Figure 2-6. Examples of 5,7-diazaindolinones as dual EGFR/HER2 inhibitors**

## 6. Others

There are many natural product "HER2-inhibitors" for which no real mechanism of action seems to have been established; some of which may actually inhibit the enzyme. Nakijiquinone C (**20**) is reported as a modestly potent erbB-2 inhibitor, with some selectivity over EGFR inhibition.[36] 11,11'-Dideoxyverticillin (**21**) is a dual EGFR/HER2 inhibitor (Figure 2-7).[37]



**20** Nakijiquinone C

**21** 11,11'-dideoxy-verticillin
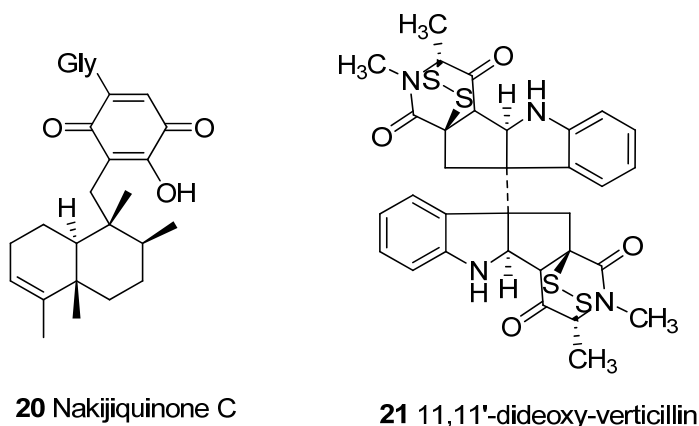
**Figure 2-7. Structures of nakijiquinone C and 11,11'-dideoxyverticillin**

## 2.2  Estrogen Receptor Inhibitors

Estrogens are a group of steroidal compounds, and play key roles in development and maintenance of normal sexual and reproductive functions, which regulate the growth, differentiation, and physiology of the reproductive process.[38, 39] Estrogens are also important

to the pathological processes of hormone-dependent cancers, such as breast, endometrial, prostate, ovarian, and thyroid cancers.[40, 41]

Estrogens regulate signal transduction through two main pathways, genomic and non-genomic (Figure 2-8).[41] In the genomic pathway, estrogens interact with two types of estrogen receptors, ERα and ERβ.[39] In general, an estrogen can cross the cell membrane and bind to its receptor. Estrogen receptors then dimerize, at which time they can undergo conformational changes. The dimeric receptor-ligand complex subsequently binds to estrogen response element (ERE) sequences in the promoter region of estrogen-responsive genes, resulting in the recruitment of co-regulatory proteins (co-activators or co-repressors) to the promoter, which leads to an increase or decrease in mRNA levels, the production of associated proteins, and finally a physiological response. The genomic pathway typically occurs over the course of hours. [42]

Recent studies demonstrated that ERα and ERβ can regulate transcription of some genes independent of ERE by interacting with other DNA-bound transcription factors, rather than binding directly to DNA.[40, 41] These factors include AP-1, SP-1, forkhead box (Fox), oct, nuclear factor kappaB (NF-κB), and GATA-3, which are some of the known non-ERE DNA-bound transcription factors that interact with ERs.[39, 43-45]
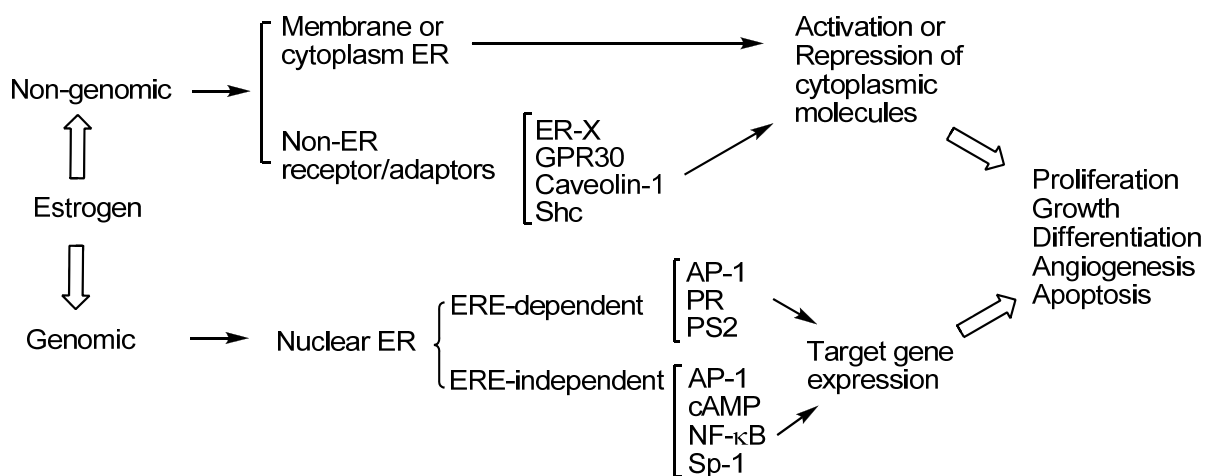


**Figure 2-8. Estrogen-mediated genomic and non-genomic pathways[39]**

In the non-genomic pathway, estrogens exert their biological effects either through the ER located in or adjacent to the plasma membrane or through other non-ER plasma membrane-associated estrogen-binding proteins (Figure 2-8).[39, 41, 46, 47] The non-genomic action of estrogens could regulate a vast range of cellular responses, such as increased levels of calcium or nitric oxide, and the activation of multiple intracellular kinase cascades, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase A (PKA), and protein kinase C (PKC).[39] The non-genomic mechanism is not as well understood as the genomic mechanism, but has been observed in many tissues.[38] The process involves many transcription factors that regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility, and apoptosis. The non-genomic effects occur within a few minutes.[48, 49]

Two types of compounds have been developed to regulate estrogen receptor function: selective estrogen receptor modulators (SERMs) and selective estrogen receptor down regulators (SERDs).[50] Baumann et al. summarized that "estrogens, SERMs, and SERDs interact with estrogen receptors in different ways: estrogens act only agonistically, SERMs act agonistically or antagonistically, and SERDs act only antagonistically. Estrogens and SERDs generally have a steroid structure, whereas SERMs lack the steroid structure of estrogens but have a tertiary structure that can bind to the estrogen receptors".[50]

## 2.2.1  Selective Estrogen Receptor Modulators (SERMs)

Tamoxifen (**22**) is a selective estrogen receptor modulator (SERM) and has been used for more than 30 years for the treatment of breast cancer.[51, 52] To improve the efficacy and reduce the toxicity of tamoxifen, new 'tamoxifen-like' analogues such as toremifene (**23**), droloxifene (**24**), and idoxifene (**25**) have been developed (Figure 2-9).[50] Toremifene citrate is FDA approved for use in advanced (metastatic) breast cancer. Droloxifene (**24**) and idoxifene (**25**) are still in clinical trials. Further development of tamoxifen also led to fixed-

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ring derivatives (Figure 2-9), including raloxifene (**26**), arzoxifene (**27**), acolbifene (**28**), and EM 800 (**29**).[50, 53] Raloxifene was approved by the FDA in 2007 for reducing the risk of invasive breast cancer in postmenopausal women with osteoporosis and in postmenopausal women at high risk for invasive breast cancer.



**22** Tamoxifen     **23** Toremifene     **24** Droloxifene     **25** Idoxifene

**26** Raloxifene     **27** Arzoxifene

**28** Acolbifene     **29** EM 800
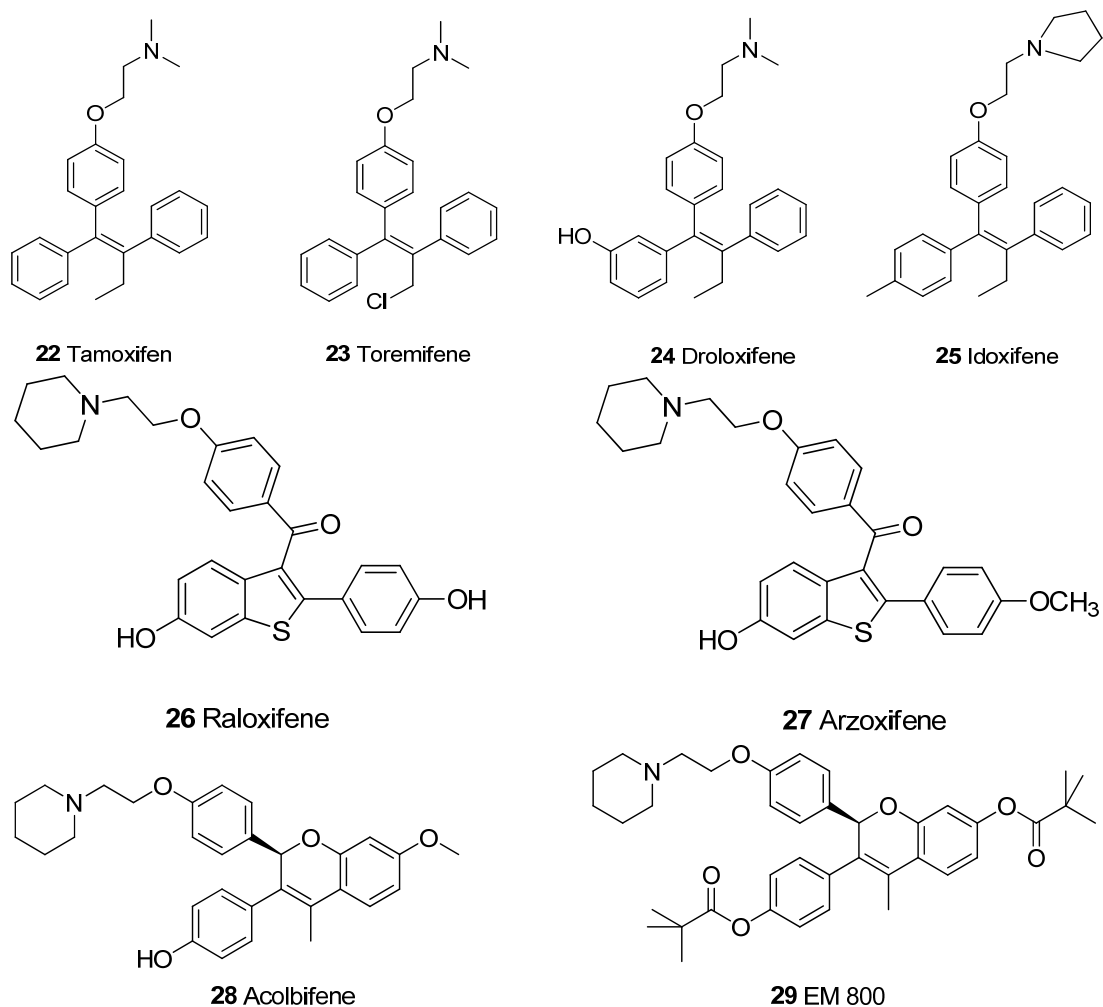
**Figure 2-9. Examples of selective estrogen receptor modulators**

## 2.2.2  Selective Estrogen Receptor Down Regulators (SERDs)

SERDs have different molecular structures and mechanism of actions compared with SERMs (Figure 2-10).[50] SERMs act agonistically or antagonistically, while SERDs act only antagonistically. Fulvestrant (**30**) is an analogue of estradiol with a 7α-alkyl sulfinyl chain. It

is used for the treatment of hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following antiestrogen therapy.[54] SR 16234 (**31**) is a steroidal compound developed by SRI International; it shows high affinity to the ERα.[55, 56] ZK 703 (**32**) and ZK 253 (**33**) display higher potency than tamoxifen and fulvestrant *in vivo*. Clinical studies with these two compounds are ongoing.[57]

**30** Fulvestrant

**31** SR-16234

**32** ZK 703

**33** ZK 253
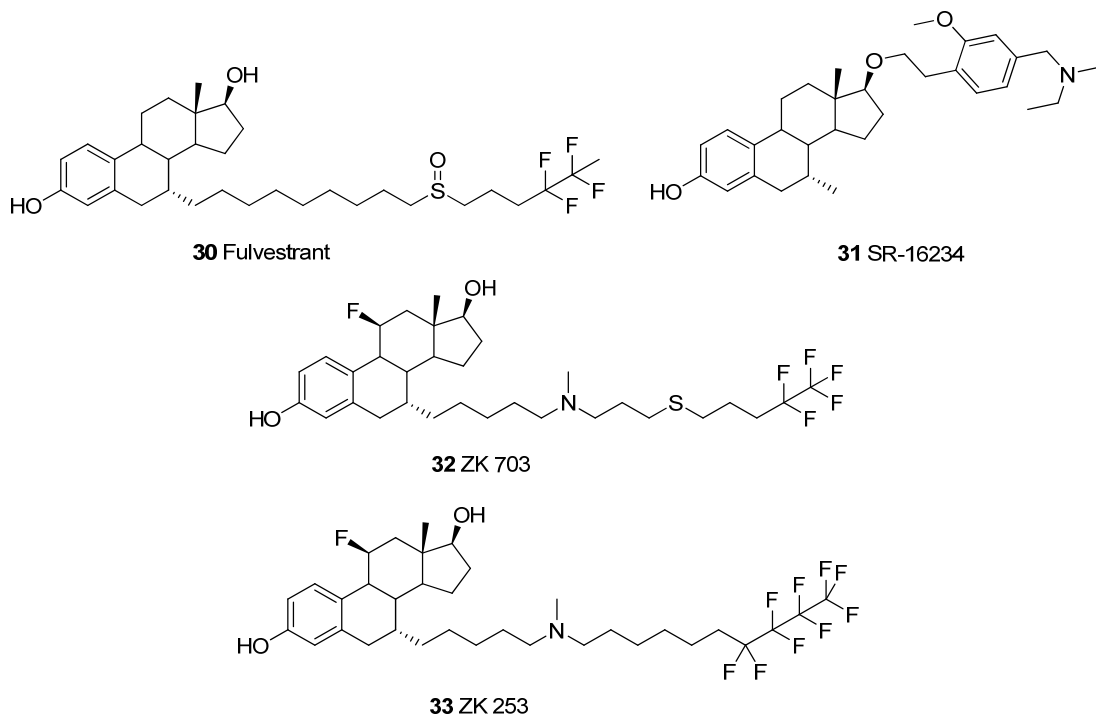
**Figure 2-10. Examples of selective estrogen receptor down regulators**

## 2.3    Aromatase Inhibitors

Another strategy to counteract ER function involves the reduction of estrogen levels by blocking a key enzyme required for their biosynthesis.[58] Although the different classes of steroidal hormones share a common biosynthetic pathway, a characteristic feature of estrogen biosynthesis is the formation of the aromatic A ring, which is the final and rate-limiting step.[58, 59] The enzyme aromatase regulates this step (Figure 2-11).[59] Aromatase belongs to the cytochrome P450 superfamily, located in the endoplasmic reticulum of cells. It is composed of a cytochrome P450 hemeprotein (P450 XIX, CYP19), which carries out the

19

aromatization reaction, and a NADPH-cytochrome P450 reductase, a flavoprotein required for the electron transfer from NADPH to the cytochrome P450 enzyme.[58, 60, 61] This enzyme catalyzes the synthesis of estrone (E1) and estrodiol (E2) via the aromatization of the A ring of androgen precursors, namely androstenedione and testosterone.[59]
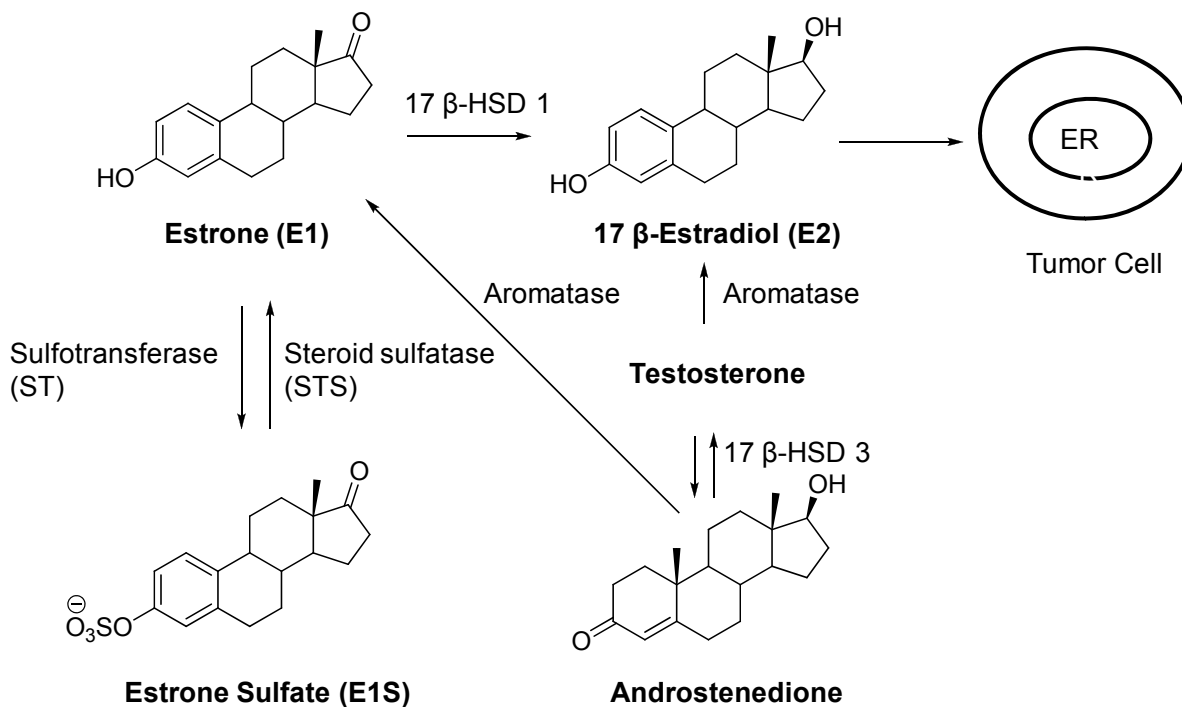


**Figure 2-11. The main pathways involved in production of estrogens by aromatase and steroid sulfatase complex[59]**

Inhibition of aromatase would selectively lead to estrogen deprivation, subsequently block hormone-mediated transcription, and finally result in loss of estrogenic effects, which cannot be achieved by the use of SERMs.[62] Aromatase has always been considered the most promising target for estrogen-related cancers, and considerable pharmacological and clinical data have been devoted to the study of this enzyme complex and to the development of potent and selective agents able to interfere with its action. Several classes of steroidal (Type I) and non-steroidal (Type II) aromatase inhibitors (AIs) have been developed, and potent and effective agents are now in clinical use. [63, 64]

### 2.3.1  Steroidal Inhibitors (Type I Inhibitors)

**1. Reversible inhibitors**

Reversible inhibitors are molecules that compete with the natural substrate for noncovalent binding to the active site of the enzyme. [63, 65] Steroidal inhibitors are built on the structure of androstenedione, with different substituents incorporated at various positions on the molecule.[65] These inhibitors interact with the aromatase cytochrome P450 enzyme in the same manner as the substrate androstenedione.[65] Some examples are shown in Figure 2-12. Compound **34** is also known as formestane, and is used clinically in some countries, but not in the U.S. [63]



**34**       **35**       **36**       **37**
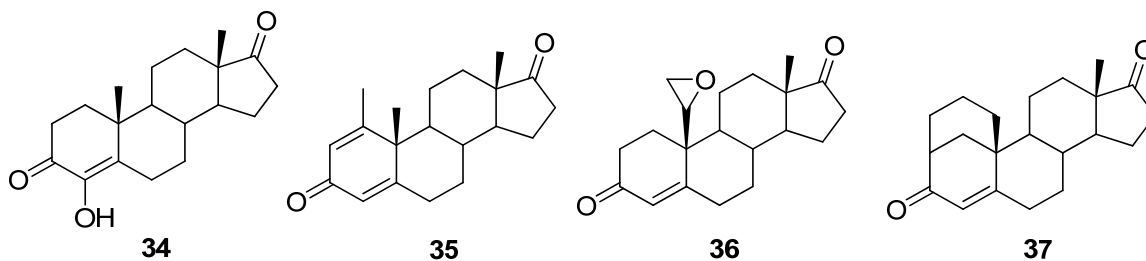
**Figure 2-12. Examples of reversible steroidal aromatase inhibitors**

**2. Irreversible inhibitors**

An irreversible inhibitor contains a reactive chemical moiety and reacts with the enzyme to produce a covalent bond between the inhibitor and the protein backbone of the enzyme.[66, 67] These inhibitors are also classified as affinity labeling inhibitors, alkylating agents, or active-site directed irreversible inhibitors.[65] Most irreversible aromatase inhibitors were designed with an alkylating moiety attached to the B-ring of androstenedione.[68, 69] Some examples are shown in Figure 2-13.

**38**, R= N(CH$_2$CH$_2$Cl)$_2$

**39**, R= HN—C(=O)—CH$_2$Br

**40**, R= N$_3$

**41**

**42**
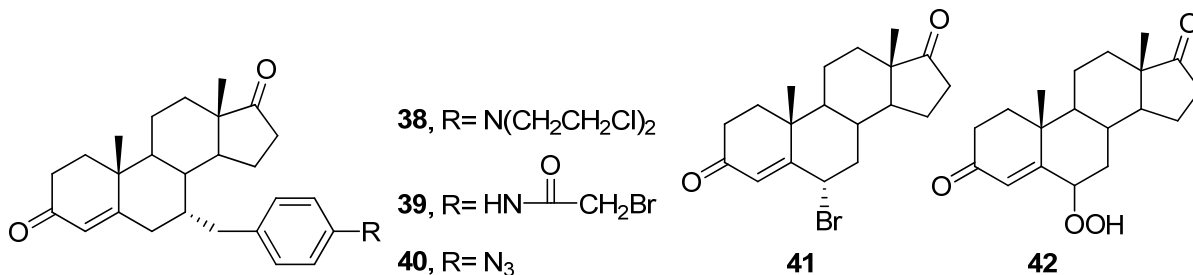
**Figure 2-13. Examples of irreversible steroidal aromatase inhibitors**

### 3. Mechanism-based inhibitors

A mechanism-based inhibitor, which mimics the substrate, is converted by the enzyme to a reactive intermediate and results in inactivation of the enzyme.[65] These inhibitors act on the enzyme during the normal catalytic process. They are also called enzyme-activated irreversible inhibitors and suicide inhibitors.[65] Mechanism-based inhibitors have distinct advantages in drug design because of they are highly enzyme specific and show prolonged inhibition and minimal toxicities.[65] Exemestane (**46)** is now in clinical use for the treatment of advanced breast cancer (Figure 2-14).[70, 71]



**43**  **44**  **45**  **46**
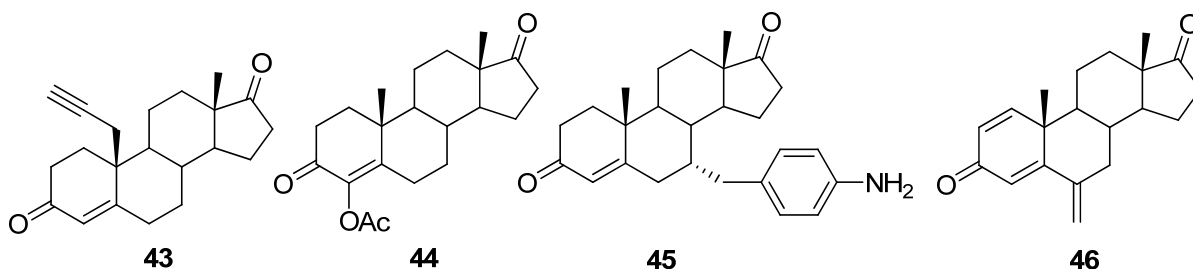
**Figure 2-14. Examples of mechanism-based steroidal aromatase inhibitors**

### 2.3.2 Nonsteroidal Inhibitors (Type II Inhibitors)

### 1. Imidazole and triazole derivatives

Aminoglutethimide (AG, **47**) is the pioneer nonsteroidal inhibitor used for the treatment of breast cancer. It opened the way to a different approach to the design and development of nonsteroidal AIs (NSAIs) inhibitors.[72] Many research teams began to

investigate compounds containing aza-heterocycles following the hypothesis that the AG mode of binding could involve interaction of a nitrogen atom with the heme iron of P450.[73] Second-generation compounds containing imidazole and triazole rings were subsequently developed by many groups; examples are shown in Figure 2-15.[72, 74] Third-generation agents such as letrozole (**51**) and anastrozole (**52**) are now used for the adjuvant treatment of hormonally-responsive breast cancer, and have proved to be more effective than tamoxifen in some clinical studies.[75, 76]



**47** Aminoglutethimide    **48** Fadrozole    **49** Liarozole

**50** Vorozole    **51** Letrozole    **52** Anastrozole
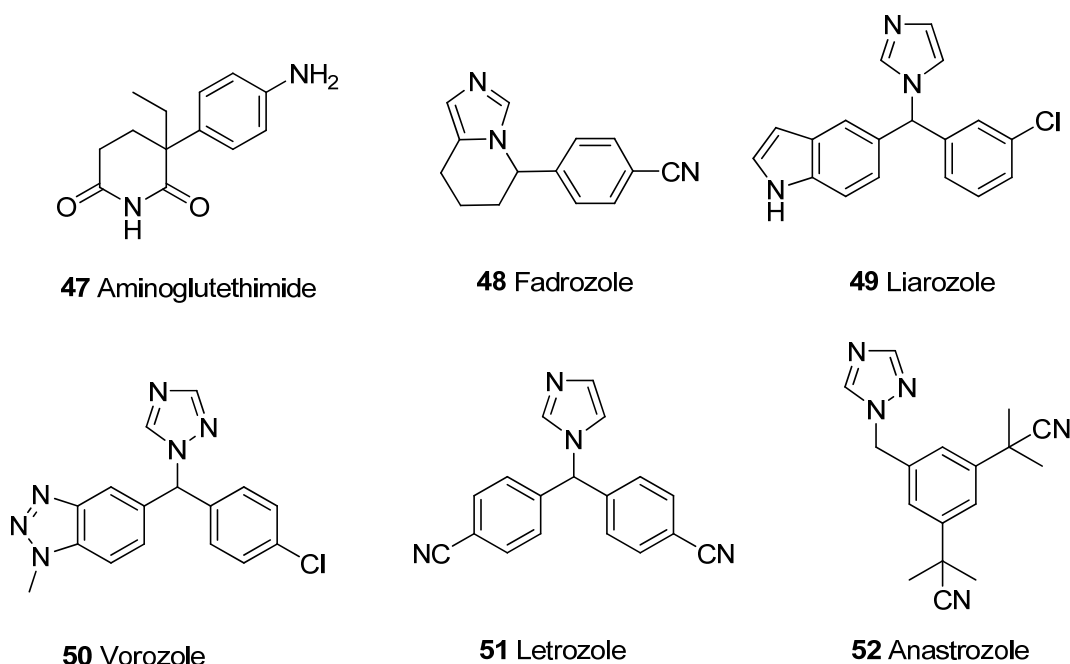
**Figure 2-15. Examples of NSAIs derived from imidazole and triazole**

## 2. Tetralone and tetraline derivatives

R.W. Hartmann et al. developed many tetrahydronaphthalene leads and obtained remarkable results with two major compounds classes, tetralone and tetraline derivatives.[77, 78] Compounds **53-56** showed high aromatase inhibitory potency at low micromolar concentrations (Figure 2-16).[79, 80]
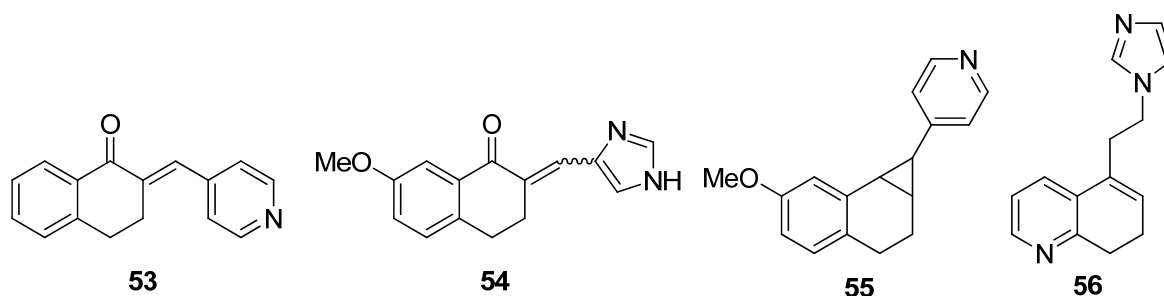
**Figure 2-16. Examples of NSAIs derived from tetralone and tetraline**

### 3. Benzopyranone derivatives

M. Recanatini used the method of comparative molecular analysis to develop a series of (di)benzopyranone derivatives.[81-83] The most interesting compounds were **57**-**60** (Figure 2-17), which showed strong inhibitory potency and selectivity with respect to P450.[84]
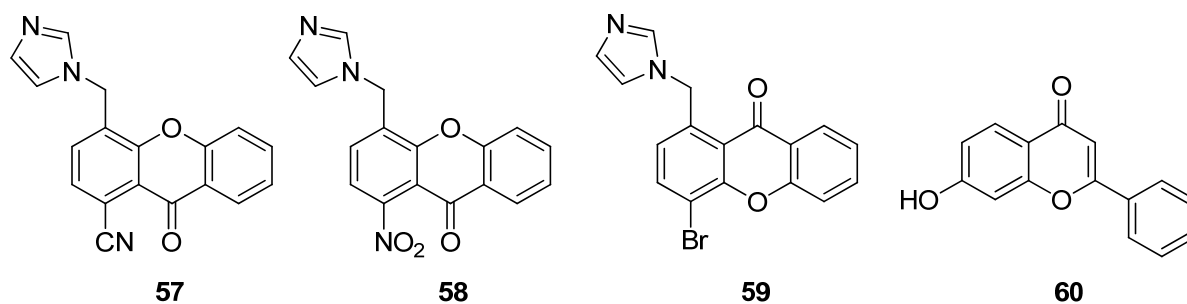


**Figure 2-17. Examples of NSAIs derived from benzopyranone**

## 2.4     Steroid Sulfatase Inhibitors

Sulfatases are enzymes of the esterase class, which catalyze the hydrolysis of sulfate monoester bonds in a wide variety of physiological substrates.[85] In human cells, 12 different sulfatases have been well characterized.[86-88] Steroid sulfatases (STS), a subset of sulfatases, are characterized by their ability to regulate the local production of estrogens and androgens from systemic precursors in several tissues.[85, 89] Many studies are ongoing to develop STS inhibitors as novel therapeutics for the treatment of breast cancer.

Estrone sulfate (E1S) is considered to be a major source of active estrogens in mammary tumors, especially in women after menopause (Figure 2-11).[85, 90] Although E1S

itself does not bind to estrogen receptors,[91] it is converted initially by STS to unconjugated E1 and then by a reducing enzyme (17$\beta$-hydroxysteroid dehydrogenase) to E2, which binds to the receptors with high affinity (Figure 2-11).[85, 92]

The current STS inhibitors have been designed based upon the natural substrates or have been discovered by screening compound libraries.[85] STS inhibitors can be classified as (i) steroidal inhibitors and (ii) non-steroidal inhibitors.[58, 65] An alternative classification is (i) reversible inhibitors and (ii) irreversible inhibitors.[85] In the following sections, the current status in the development of STS inhibitors will be reviewed.

### 2.4.1  Steroidal STS inhibitors

#### 1. Reversible STS inhibitors

Most of the reversible inhibitors feature a steroid skeleton derived from substrate or product. After the discovery of estrone-3-methylthiophosphonate (EMATE, **61**) in the early 1990s,[93] substantial efforts began to develop novel inhibitors.[85]

Many substrate-based inhibitors based upon EMATE were developed by installing different types of sulfate substituents, leading to E1S analogues (Figure 2-18),[92] such as **62**, which showed weak STS inhibitory activity.[85] In 1998, estradiol-based inhibitors were discovered by Poirier and Boivin.[94] Compounds **63**-**64** displayed STS inhibitory activity at nanomolar concentrations, and are still the most potent reversible inhibitors of STS known to date. Because compounds with a non-aromatic A-ring can be accepted by STS as substrates, Nussbaumer et al. investigated many steroidal non-aryl sulfamates.[95] However, compounds **65**-**66** showed weak STS inhibitory activity. In addition, investigations have shown that progestins, such as danazol (**67**), promegestone (**68**), tibolone, and medrogestone, inhibit STS.[96-99] Their inhibitory potency ranges from micromolar to nanomolar.
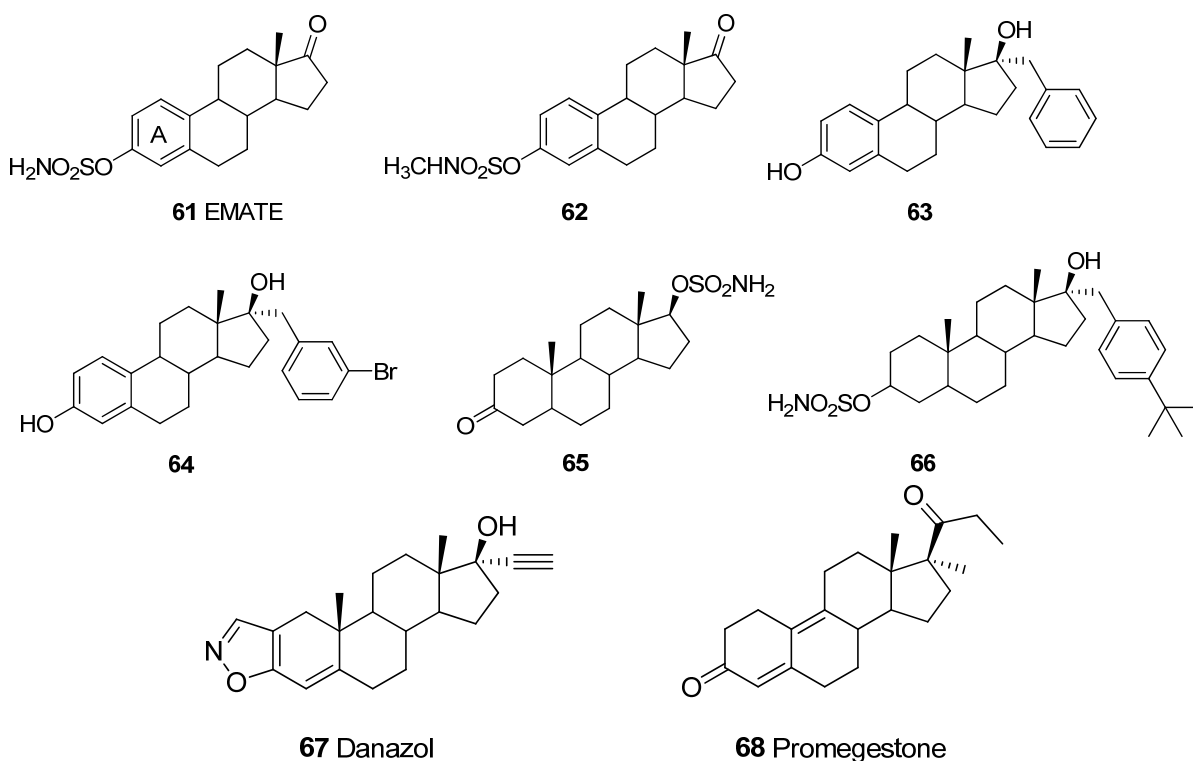
**Figure 2-18. Examples of steroidal reversible STS inhibitors**

## 2. Irreversible STS inhibitors

All irreversible inhibitors reported to date contain the arylsulfamate moiety [85, 92] EMATE, the first irreversible inhibitor, was reported by Howarth et al. in 1994. [78] However, due to its potent estrogenic activity, EMATE could not be used as a therapeutic agent.[100] Further development of novel derivatives was aimed at reducing estrogenic activity, while retaining STS inhibitory activity.[92] Structural modifications of EMATE focused on the A- and D-rings.[101-104] Compounds **69**-**72** showed high STS inhibitory activity and low estrogenic activity (Figure 2-19). The $IC_{50}$ of **70** was 1 nM, while that of EMATE was 18 nM.
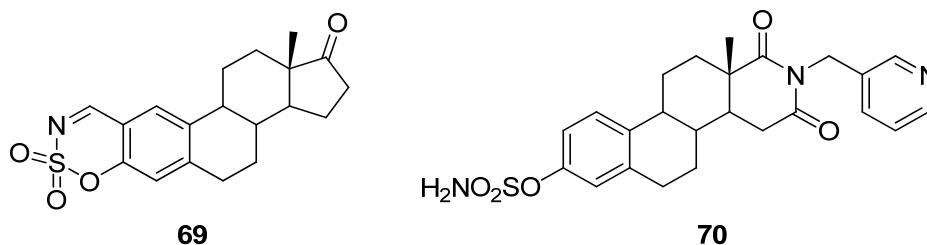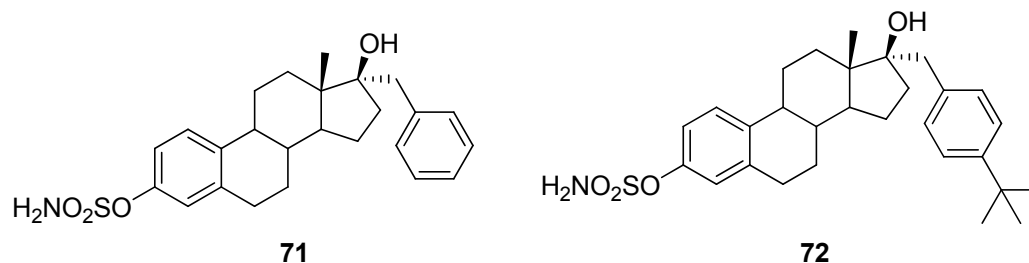
**Figure 2-19. Examples of steroidal irreversible inhibitors**

## 2.4.2 Nonsteroidal STS Inhibitors

### 1. Mono-, bi-, and tri-cyclic arylsulfamates

To mimic the A-, B-, C-rings of EMATE, mono-, bi-, and tri-cyclic arylsulfamates were designed by different groups (Figure 2-20).[105-107] Most monocyclic arylsufamates were less effective than EMATE, except compound **73**, which was three-fold superior to EMATE. Although most known bi- and tri-cyclic arylsulfamates, such as **75** and **76**, were less potent than EMATE, they merit further development based on their simplified scaffolds and increased selectivity.
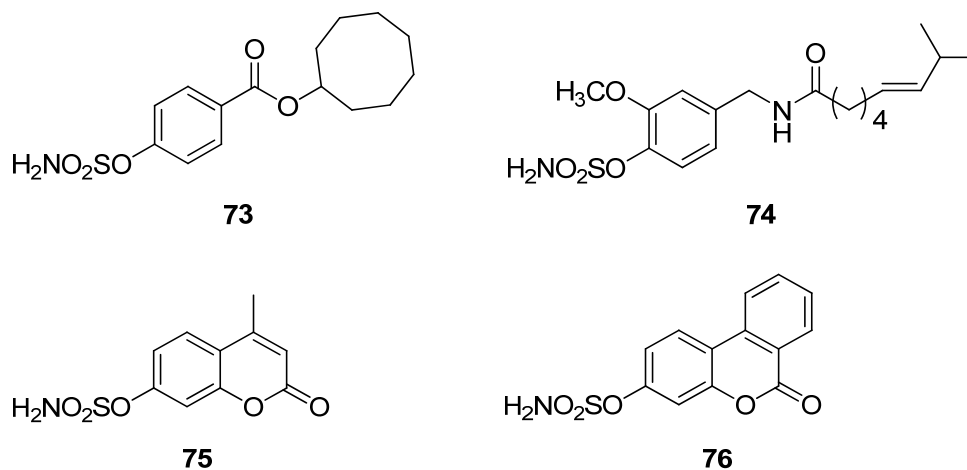


**Figure 2-20. Examples of nonsteroidal STS inhibitors**

### 2. STS inhibitors discovered from screening

Novel inhibitors with different scaffolds were discovered by screening different

compound libraries. Compounds **77**-**78** were found to have STS inhibitory activity (Figure 2-21). They were less potent than EMATE, but still provided new scaffolds without a sulfamate unit for possible development of novel inhibitors.[92, 108]
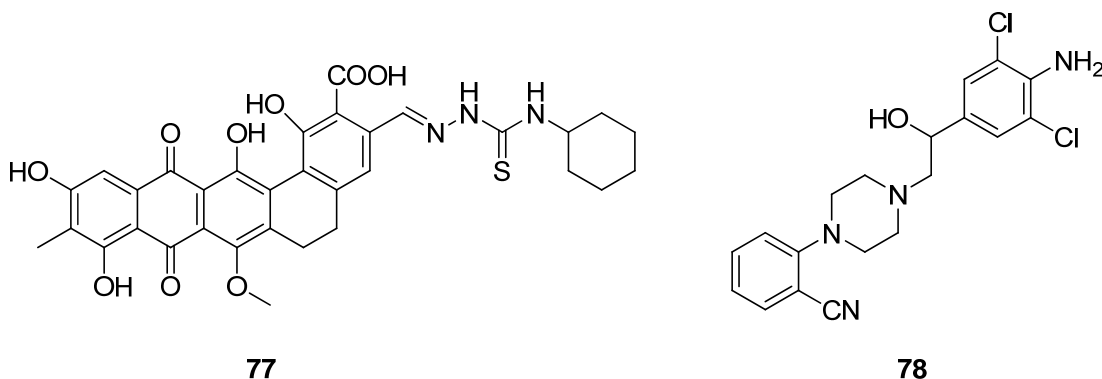


**Figure 2-21. Examples of nonsteroidal inhibitors from screening**

### 2.4.3  Dual Aromatase-Sulfatase Inhibitors

Recently, dual aromatase-steroid sulfatase inhibitors (DASIs), which combine features of STS inhibitors and AIs, were designed to enhance the response of hormone-dependent breast cancer both by providing more severe estrogen depletion and by reducing the synthesis of other steroids with estrogenic properties.[58, 109, 110] Initially, the sulfamate pharmacophore for STS inhibition was introduced into isoflavones, which are weak AIs, and the resulting compounds did inhibit STS both *in vitro* and *in vivo*, confirming the feasibility of the DASI concept.[101] Subsequent studies led to **79**, the first potent dual aromatase-steroid sulfatase inhibitor, which acted as a reversible AI and irreversible STS inhibitor (Figure 2-22).[111] Both **79** and **80** are promising DASIs. In compounds **81** and **82**, the sulfamate unit is incorporated into letrozole and anastrozole, respectively. Both compounds showed interesting inhibitory activity *in vitro* and *in vivo*.[59, 112]
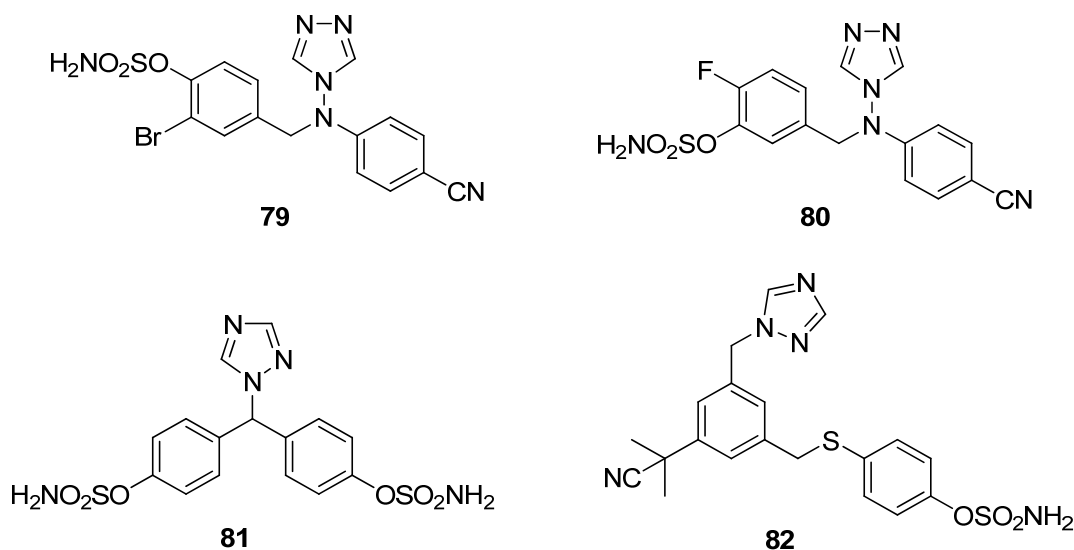
**Figure 2-22. Examples of dual aromatase-sulfatase inhibitors**

## 2.5    Chemotherapeutic Agents with Other Mechanisms of Action

Many classes of chemotherapeutic drugs are available for clinical use against breast cancer. Table 2-1 lists some of the commonly used drugs together with their mechanisms of action, and their structures are shown in Figure 2-23.

**Table 2-1. Classes of therapeutic agents for the treatment of breast cancer**

| Class | Mechanisms of Action | Examples in Clinical Use |
|---|---|---|
| Anthracyclines | Intercalate between base pairs of the DNA/RNA strand<br>Inhibit topoiosomerase II enzyme<br>Create iron-mediated free oxygen radicals | Daunorubicin<br>Doxorubicin<br>Epirubicin<br>Idarubicin<br>Valrubicin |
| Taxanes | Block microtubule assembly | Docetaxel, Paclitaxel |
| Pyrimidine analogues | DNA precursors/antimetabolites | Capecitabine and 5-Fluorouracil |
| Nucleosides | Inhibit DNA replication | Gemcitabine |
| Nitrogen mustard | Alkylating agent | Cyclophosphamide |
| Alkaloid | Block microtubule assembly | Vinorelbine |
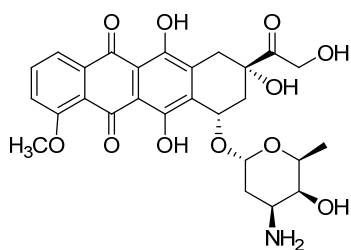
**83** Doxorubicin
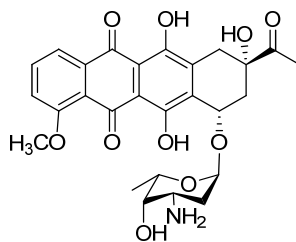
**84** Daunorubicin
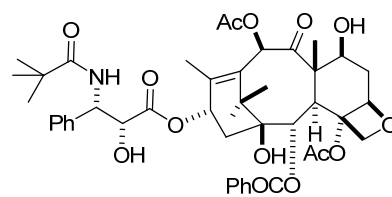
**85** Docetaxel
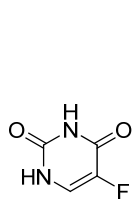
**86** 5-Fluorouracil
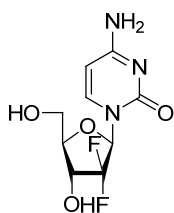
**87** Gemcitabine
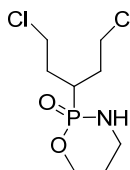
**88** Cyclophosphamide
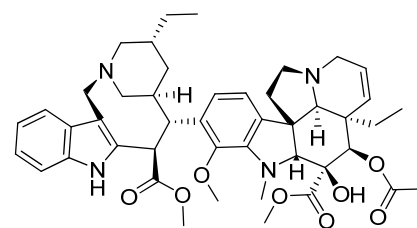
**89** Vinorelbine

**Figure 2-23. Examples of chemotherapeutic agents in clinical use**

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**CHAPTER 3**

**DESIGN, SYNTHESIS, AND STRUCTURE-ACTIVITY RELATIONSHIPS OF NOVEL NEO-**

**TANSHINLACTONE ANALOGUES**

**3.1      Introduction**

Historically, natural products have been an extremely significant source of drugs and drug leads, which have led to numerous clinically used medicines.[1-4] Accordingly, our group is interested in the discovery and development of novel anticancer drugs from natural plants. Drug discovery from medicinal plants has played an important role in the treatment of cancer, and about 74% of anticancer compounds are either natural products or natural product-derived.[5] Tamoxifen (TAM, Figure 1) is the most widely used selective estrogen receptor modulator (SERM) for the treatment of breast cancer.[6] However, it has shown some acute and long term toxicity, such as bone metastasis.[6] Other drugs, including cyclophosphamide, doxorubicin (adriamycin), and paclitaxel (Taxol), are also recommended to be used in combination in early breast cancer.[7] Although the death rate from breast cancer has declined significantly because of earlier detection and more effective treatments, toxic side effects, low tumor selectivity, and multidrug resistance with cancer chemotherapy still prompt the development of novel potent anti-breast cancer agents.[8]

Tanshen, the rhizome of *Salvia miltiorrhiza* Bunge, is used primarily in traditional Chinese medicine (TCM) for the treatment of coronary heart diseases, inflammatory diseases, and chronic hepatitis. Many biologically active constituents, including tanshinone I and tanshinone IIA, which have been studied extensively as anticancer agents, were first isolated from the roots of *Salvia miltiorrhiza*.[9] Recently, Dr. Xihong Wang in our group

reported that neo-tanshinlactone (**90**) (Figure 3-1), a minor component isolated from an EtOH extract of *S. miltiorrhiza*, showed significant selective *in vitro* anti-breast cancer activity as compared to TAM. Specifically, it was 10-fold more potent and 20-fold more selective than TAM against ER+ and HER2++ breast cancer cells.[10] Three series of analogues were synthesized by Dr. Wang to study the SAR (Figure 3-2).[11] The A series studied the C-4 and -6 positions, while the B and C series explored ring-D modification. In addition, Dr. Xihong Wang studied three series of furanocoumarin analogues, some of which showed antitumor promotion activity, but weak cytotoxic activity (Figure 3-2).[12] Compound **91** (Figure 3-1), a congener of **90**, was about twice as active as **90** against MCF-7 and SK-BR-3 cell lines.[11] Preliminary structure-activity relationships (SAR) showed that a methylated furan ring-D and the C-4 substituent in ring A are critical for anti-breast cancer activity.[11] These promising results encouraged us to continue the modification of this series to develop novel anticancer drug candidates. To increase the chemical availability, we also optimized the synthetic pathway. In this chapter, we describe further modifications of the A-, B-, C- and D-rings, as well as biological evaluation of newly synthesized analogues against several human cancer cell lines, including MCF-7 (estrogen receptor positive breast cancer), SK-BR-3 (estrogen receptor negative, HER2 over-expressing breast cancer), ZR-7-51 (estrogen receptor positive breast cancer), MDA-MB-231 (estrogen receptor negative breast cancer), A549 (human lung cancer), DU145 (prostate cancer), KB (nasopharyngeal carcinoma), and KB-vin (vincristine-resistant KB subline).



tamoxifen (TAM)          Tanshinone I          Tanshinone IIA

**90**: R = CH$_3$
**91**: R = C$_2$H$_5$
**92**: R = H
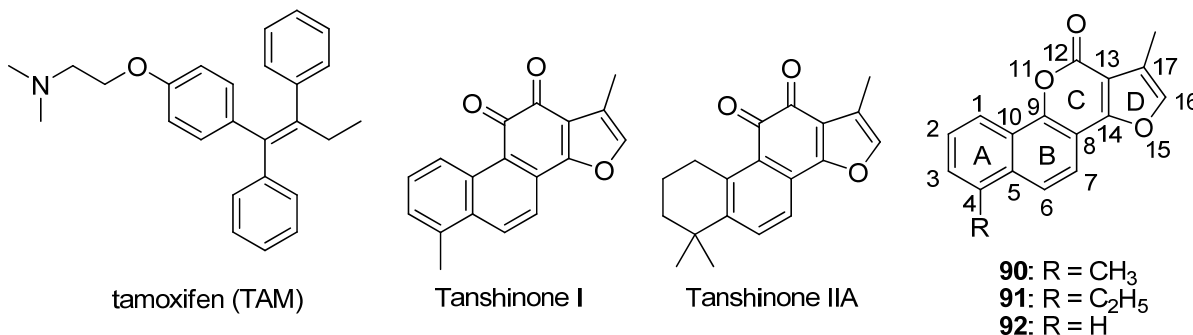
**Figure 3-1. Structures of tamoxifen, tanshinone I, tanshinone IIA, neo-tanshinlactone**

**(90) and its analogues 91-92**



A Series   B Series   C Series

$R_1$= H, $CH_3$, $CH_2CH_3$
$R_2$= H, Cl, $OCH_3$

A Series   B Series   C Series

R= 8-H, $CH_3$, $OCH_3$, F, Cl or
R= 7-$CH_3$, $OCH_3$
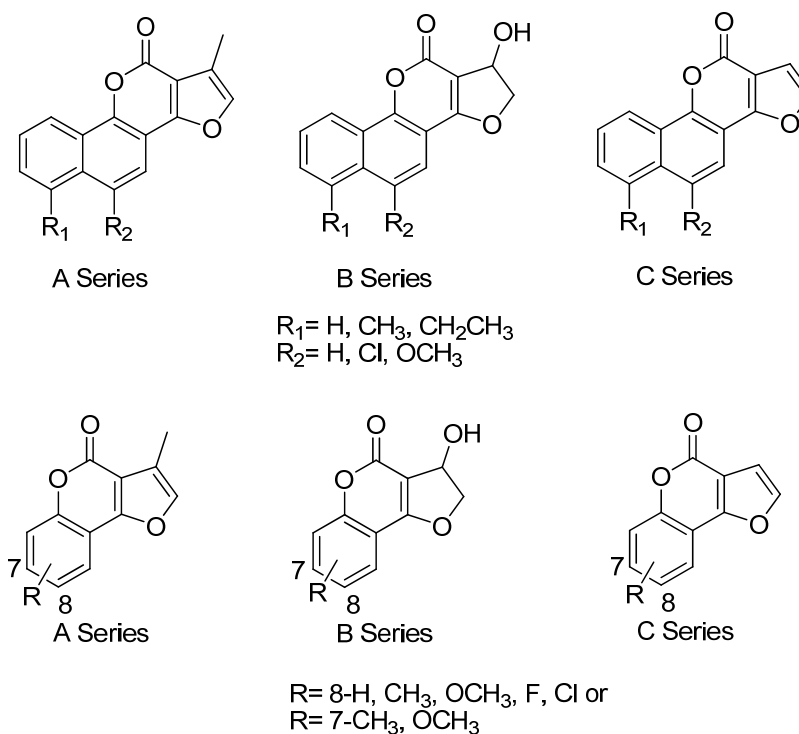
**Figure 3-2. Neo-tanshinlactone analogues and furanocoumarin analogues**

## 3.2 Design

Our general goals in drug design are to optimize the synthesis of active analogues, systematically explore SAR, and develop new lead compounds. Thus, our first goal in this study was to optimize the synthetic route to **91**. We aimed to reduce the number of steps and increase yields. The optimized synthetic route would then be applied to synthesize new analogues. Secondly, synthetic modifications of **90** were considered, because the resulting fundamental chemical and physical changes may affect molecular shapes, bond angles, and partition coefficients. Different substituents can have different hydrophobic interactions, sizes, and electrostatic effects that can influence interaction of a ligand with its target receptors. Structures of designed new neo-tanshinlactone analogues are shown in Figure 3-

3. For our **90**-analogues, we reported previously that a C-4 substituent in ring-A is critical for anti-breast cancer activity.[11] Thus, compounds **108-116** with substituents of different sizes and electrostatic properties were designed to find optimal groups at this position. In ring-B, we changed the phenyl ring to a pyridinone ring in **121** and **122** to explore a ring system effect. The strategy of bioisoteric replacement can be a useful tool in analogue design. Based on this concept, the oxygens in ring-C were changed to sulfur and nitrogen (**127-131**). Compounds with different substituents on the furan D-ring (**134-142**) were also designed. Moreover, the degree of saturation (number of double bonds) can change the orientation of a molecule and affect its *in vitro* activity and selectivity. Consequently, we designed **152-154** and **155-156** to have a more saturated non-aromatic ring-A and dihydrofuran ring-D, respectively. Finally, the furan D-ring was changed to a substituted phenyl ring in **159** to examine the ring system effect and interaction volume.

**108**: R= iPr
**109**: R= Pr
**110**: R= OMe
**111**: R= OEt
**112**: R= F
**113**: R= (structure with Br)
**114**: R= OH
**115**: R= OAc
**116**: R= (structure)

**121**: R= Me
**122**: R= Pr

**127**: R= H, $X_1$= S, $X_2$=O
**128**: R= H, $X_1$= NH, $X_2$= O
**129**: R= H, $X_1$= O, $X_2$= S
**130**: R= Et, $X_1$= O, $X_2$= S
**131**: R= Et, $X_1$= O, $X_2$= NOH

**134**: $R_1$= H, $R_2$= Et, $R_3$= H
**135**: $R_1$= Me, $R_2$= Et, $R_3$= H
**136**: $R_1$= Et, $R_2$= Et , $R_3$= H
**137**: $R_1$= H, $R_2$= Me, $R_3$= Me
**138**: $R_1$= Et, $R_2$= Me, $R_3$= Me
**139**: $R_1$= H, $R_2$= H, $R_3$= Me
**140**: $R_1$= Et, $R_2$= H, $R_3$= Me
**141**: $R_1$= H, $R_2$= 4-methoxyphenyl, $R_3$= H
**142**: $R_1$= Et, $R_2$= 4-methoxypheny, $R_3$= H

**152**: R= H, n= 0
**153**: R= H, n= 1
**154**: R= Me, n=1

**155**: R= H
**156**: R= Et

**159**

**Figure 3-3. Structures of designed neo-tanshinlactone analogues**

## 3.3    Chemistry

Synthesis of analogue **91** was achieved in five steps and an overall yield of 18%, compared with seven steps and 3% yield reported before[12,13] (Scheme 3-1). A Grignard reaction of **93** in the presence of zinc chloride gave **94** in an improved yield of 85%.[13] Addition of zinc chloride increased the yield more than 25%. Analogue **95** was obtained in one step by oxidation of **94** with Pd/C, rather than the prior two steps (hydrochloric acid & Pd/C).[11] Demethylation of **95** with boron tribromide gave naphthol **96**. Treatment of **96** with

polyphosphoric acid (PPA) in the presence of 85% $P_2O_5$ and malonic acid produced **102** in 53% yield. Phosphorus pentoxide can remove water from the reaction system and increase the yield and reproducibility. Finally, analogue **91** was obtained via a tandem alkylation /intramolecular Aldol reaction with an optimized procedure (70% yield).[14] This procedure can increase the yield around 20%. The optimized synthetic route can be applied to synthesize new analogues and produce **91** in large scale for animal test.

**Scheme 3-1. Optimized synthetic pathway to 91 and analogues 108-116**
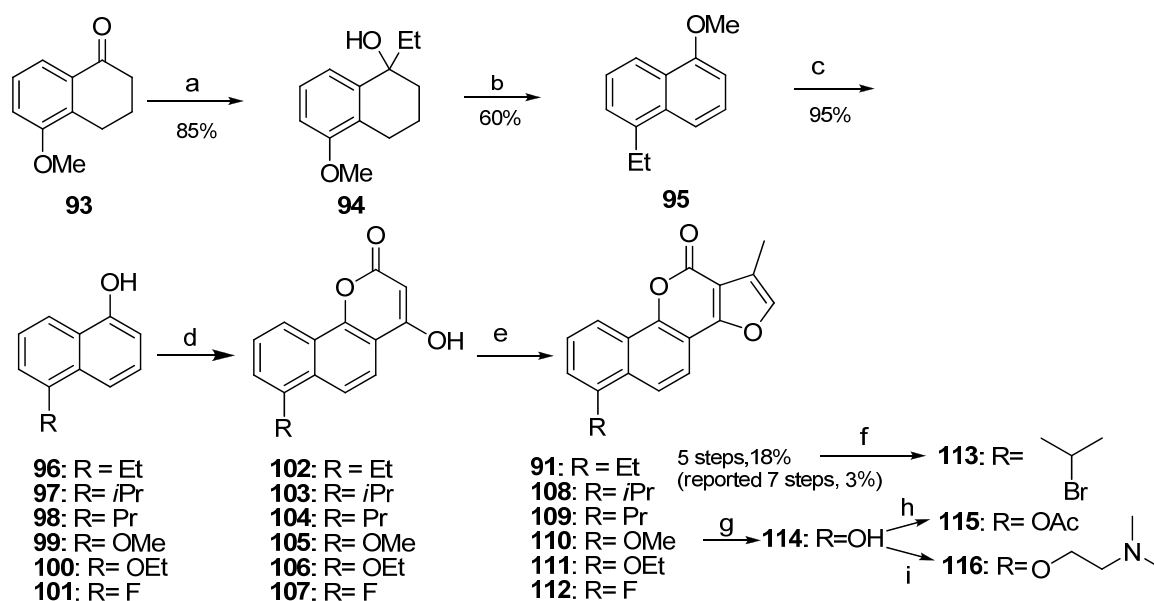


Reagents and conditions: (a) EtMgBr, ZnCl₂, THF, rt; (b) Pd/C, triglyme, reflux; (c) BBr₃, CH₂Cl₂; (d) malonic acid, PPA (85% P₂O₅), 75 °C, 3 h; (e) chloroacetone, HOAc/NH₄OAc, toluene/EtOH, reflux, 24 h; (f) NBS, dibenzoyl peroxide, toluene, reflux; (g) BBr₃, CH₂Cl₂, reflux, 3 h; (h) Ac₂O, Et₃N, 10 h; (i) 2-chloro-*N,N*-dimethylethanamine, K₂CO₃, acetone, 12h.

Using the optimized synthetic pathway, target compounds **108-112** were prepared from various substituted 1-naphthols (**97-101**), as shown in Scheme 3-1. Treating naphthols **97-101** with malonic acid in the presence of PPA (85% P₂O₅) provided benzochromenones **103-107**, which were converted to target compounds **108-112** under the same conditions as

synthesis of **91**.[14] Compound **113** was obtained by treatment of **91** with *N*-bromosuccinimide (NBS) and dibenzoyl peroxide.[15] Removal of the methyl group of **110** with boron tribromide afforded **114**, which was esterified with acetic anhydride, and alkylated with 2-chloro-*N,N*-dimethylethanamine under basic conditions to give **115** and **116**, respectively.

B-ring modification was achieved through a two-step reaction sequence. Commercially available substituted anilines **117** and **118** were reacted with diethyl malonate at 220 °C for 8 h to give intermediates **119** and **120** (Scheme 3-2).[16, 17] The desired compounds **121** and **122** were obtained through the tandem alkylation/intramolecular Aldol reaction described above and shown in Scheme 3-1.

**Scheme 3-2. Synthetic pathway to analogues 121-122**



**117**: R= Me  
**118**: R= Pr

**119**: R= Me  
**120**: R= Pr

**121**: R= Me  
**122**: R= Pr
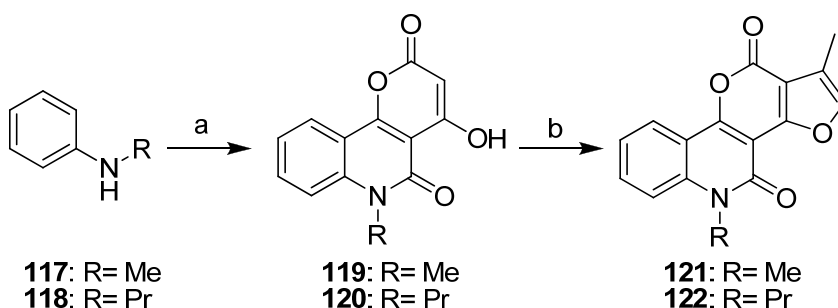
Reagents and conditions: (a) diethyl malonate, 220°C, 8h; (b) chloroacetone, HOAc, NH$_4$OAc, toluene/EtOH, reflux, 24 h.

Target compounds **127** and **128**, which are bio-isosteres of **92**, were obtained by using the same two synthetic steps shown in Scheme 1 for **91** from naphthol **96**, except that the starting materials were naphthalene-1-thiol **123** and naphthalen-1-amine **124** (Scheme 3-3). Compounds **92** and **91** were converted to thiolactones **129** and **130**, respectively, using Lawesson's reagent.[18] Compound **131** was obtained by treatment of **130** with sodium acetate and hydroxylamine hydrochloride.[19]

**Scheme 3-3. Synthetic pathway to analogues 127-131**
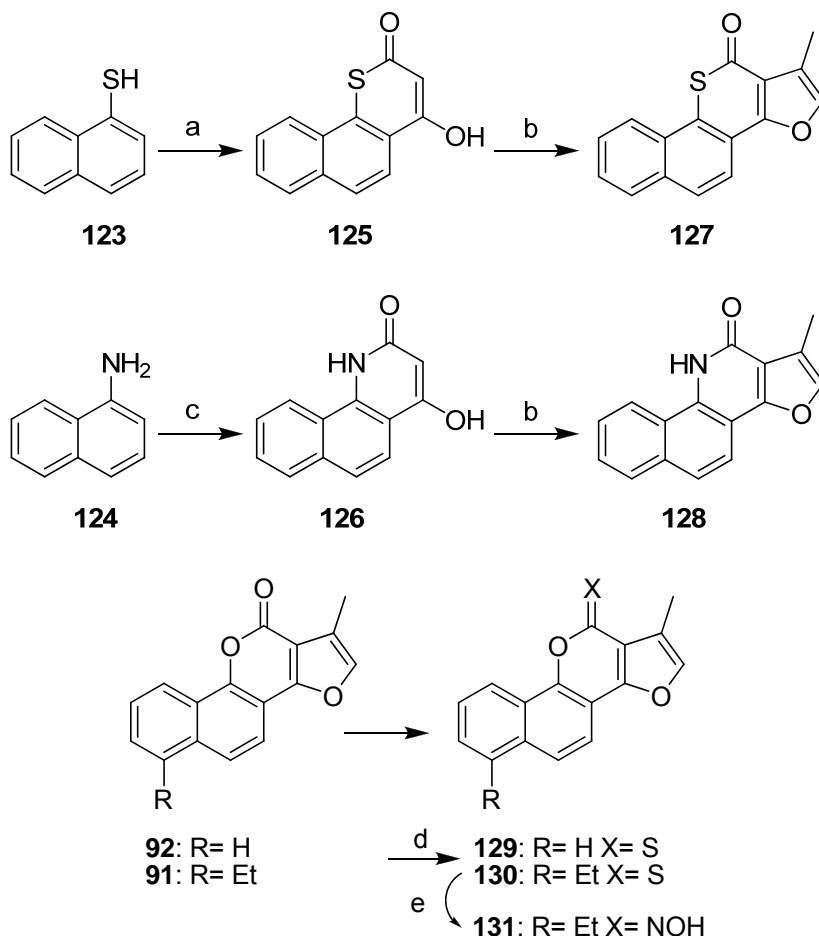


Reagents and conditions: (a) malonic acid, PPA (85% $P_2O_5$), 75 °C, 3 h; (b) chloroacetone, HOAc/NH$_4$OAc, toluene, EtOH, reflux, 24 h; (c) diethyl malonate, PPA (85% $P_2O_5$), 170 °C, 2h; (d) Lawesson's reagent, toluene, reflux, 5h; (e) NH$_2$OH HCl, NaOAc, MeOH, reflux, 12 h.

Target compounds **134-142** with various substituents on the D-ring were synthesized with the same tandem alkylation/intramolecular Aldol reaction using different bromoketones **148** (Scheme 3-4). Compound **144** was synthesized by Negishi cross-coupling reaction of compound **143** with 4-methylpent-3-enyl zinc(II) bromide in 96% yield.[20, 21] Treatment of **143** with AlCl$_3$ followed by demethylation by BBr$_3$ gave **145** in 84% yield.[21] Compounds **146** and **147**, with 5- and 6-membered A-rings, respectively, are commercially available. Compounds

**145-147** underwent the previously reported two-step ring closure reactions to afford **152-154** (Scheme 3-5). Reduction of **92** and **91** with palladium acetate, triethyl amine, and formic acid[22] afforded **155** and **156**, respectively (Scheme 3-6). Compound **159** was obtained from **157** by using esterification[23] and Heck reactions.[24]

**Scheme 3-4. Synthetic pathway to analogues 134-142**



**134**: R$_1$= H, R$_2$= Et, R$_3$= H
**135**: R$_1$= Me, R$_2$= Et, R$_3$= H
**136**: R$_1$= Et, R$_2$= Et , R$_3$= H
**137**: R$_1$= H, R$_2$= Me, R$_3$= Me
**138**: R$_1$= Et, R$_2$= Me, R$_3$= Me
**139**: R$_1$= H, R$_2$= H, R$_3$= Me
**140**: R$_1$= Et, R$_2$= H, R$_3$= Me
**141**: R$_1$= H, R$_2$= 4-methoxyphenyl, R$_3$= H
**142**: R$_1$= Et, R$_2$= 4-methoxyphenyl, R$_3$= H

**132**: R$_1$= H
**133**: R$_1$= Me
**102**: R$_1$= Et
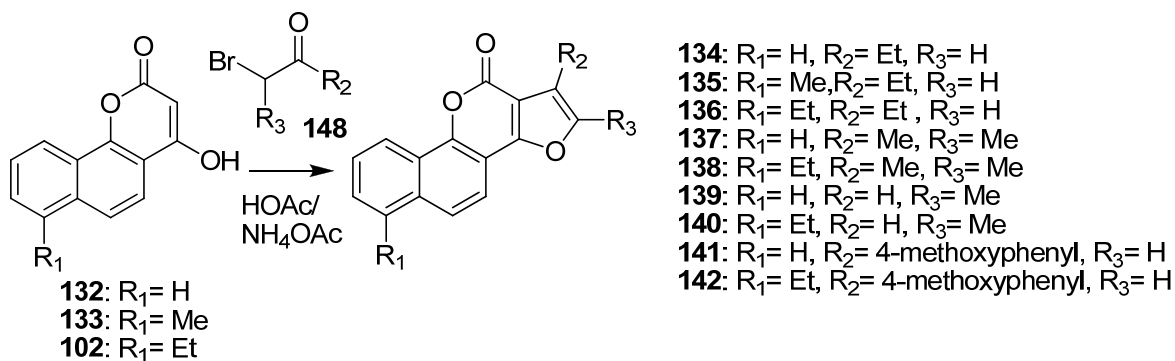
**Scheme 3-5. Synthetic pathway to analogues 152-154**



**143**          **144**          **145**

**146**: n=0, R=H    **149**: n=0, R=H    **152**: n=0, R=H
**147**: n=1, R=H    **150**: n=1, R=H    **153**: n=1, R=H
**145**: n=1, R= Me   **151**: n=1, R= Me   **154**: n=1, R= Me
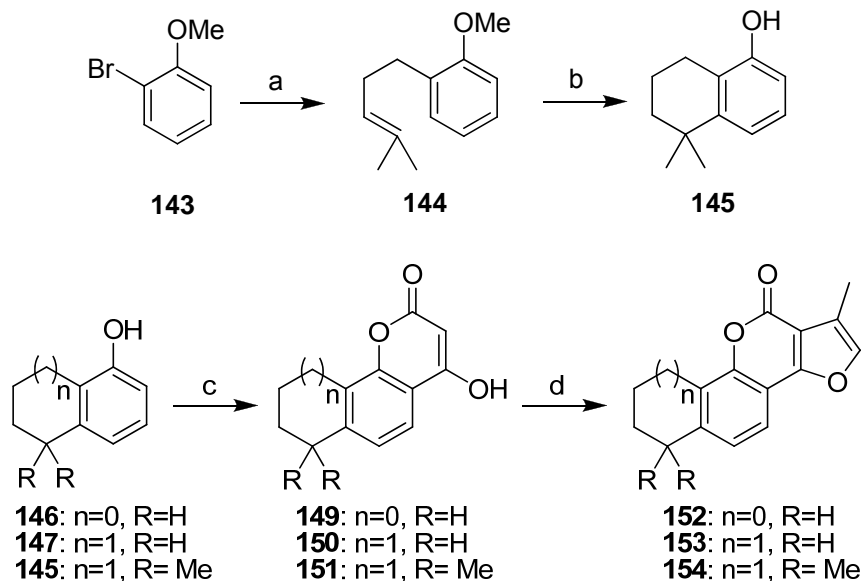
Reactions and conditions: (a) (4-methylpent-3-enyl)zinc(II) bromide, Pd(Cl$_2$)(dppf), THF, reflux, 1h; (b) (i) AlCl$_3$, DCM, 0 °C, 15 min; (ii) BBr$_3$, CH$_2$Cl$_2$; (c) malonic acid, PPA, 75 °C, 3 h; (d) chloroacetone, HOAc/NH$_4$OAc, toluene/EtOH, reflux, 24 h;

48

**Scheme 3-6. Synthetic pathway to analogues 155-156, and 159**



92: R= H
91: R= Et

155: R= H
156: R= Et

157

158

159
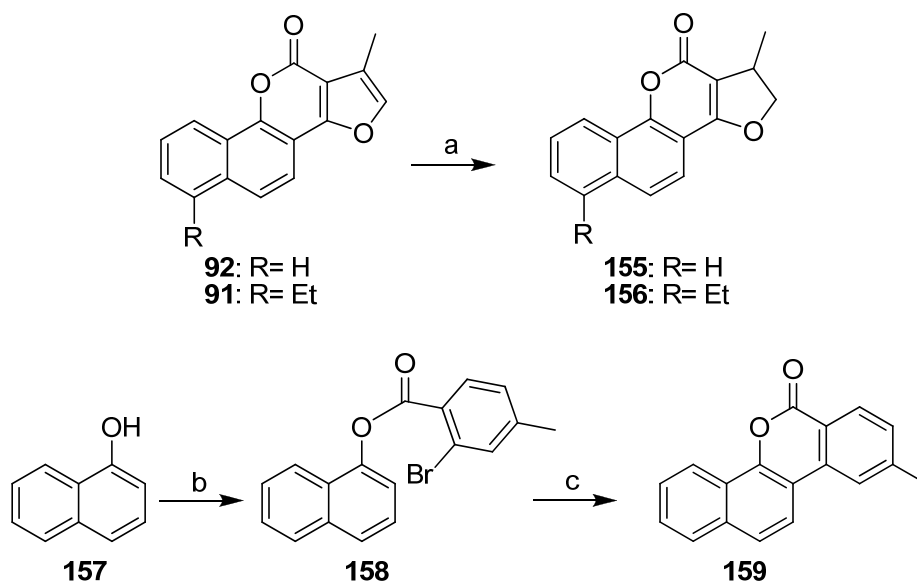
Reagents and conditions: (a) Et$_3$N, formic acid, Pd/C, acetone, 12 h; (b) 2-bromo-4-methylbenzoyl chloride, DMAP, DIEA, THF, rt, 12h; (c) Pd(OAc)$_2$, PPh$_3$, NaOAc, DMA, reflux, 3h.

## 3.4    Results and Discussion

Together with **90** and previously reported analogues **91** and **92**, the newly synthesized analogues (**108-116**, **121-122**, **127-131**, **134-142**, **152-156**, and **159**) were evaluated for *in vitro* anti-breast cancer activity against two human tumor cell lines: MCF-7 (ER+) and SK-BR-3 (HER2+). Compounds that had ED$_{50}$ values less than 4 µg/mL were also examined against ZR-75-1 (ER+, HER2+) and MDA-MB-231 (ER-) breast cancer cell lines (Table 3-1).

Initially, we investigated the effects of substitutions around the skeleton of **90** by comparing **90-92** with **108-116**. The compounds displayed different degrees of activity and selectivity toward the four breast cancer cell lines.

Against the MCF-7 cell line, small alkyl groups were favored relative to other groups at C-4 on ring-A. Analogue **91**, which has a C-4 ethyl group, was the most potent compound among those tested against MCF-7. Its $ED_{50}$ (0.2 µg/mL) was slightly better than that (0.6 µg/mL) of **90**, which has a C-4 methyl group. The rank order of potency for all C-4 substituted analogues was **91** (Et) > **90** (Me) > **109** (Pr) = **108** (*i*-Pr) > **110** (OMe) > **92** (H) > **112** (F) > **116** [2-(dimethylamino)ethoxy] > **115** (OAc) > **114** (OH) > **111** (OEt). The substituents on the furan (ring-D) double bond were also investigated. A methyl group (**91**) was better than either an ethyl (**136**) or 4-methoxyphenyl (**142**) group at the $R_2$ position. However, at the $R_3$ position, a methyl group was distinctly disfavored (**137-142**). These results indicated that the optimal combination on ring-D was methyl at $R_2$ and hydrogen at $R_3$.

Most compounds were equipotent or more potent against SK-BR-3 compared with MCF-7 cells. Compounds **91** and **90** were even more potent against SK-BR-3, with $ED_{50}$ values of 0.1 and 0.2 µg/mL, respectively.  However, **109** and **113** [4-(1-bromoethyl)] were also as potent as **91**, and **108** was equipotent to **90** against this cell line.  Compounds **92**, **112**, **108**, and **111** showed good but lower activity ($ED_{50}$ 1.0, 1.1, 2.0, 2.5 µg/mL, respectively), while **114**, **115**, and **116** were even less potent. These results indicate that the size, orientation, and electronic effect of groups at C-4 are important to the activity. Perhaps even more importantly, certain substituents could greatly affect the SK-BR-3/MCF-7 selectivity. Compounds **109-111** had approximately tenfold ratios of SK-BR-3/MCF-7 selectivity. For the D-ring analogues (**134-142**), most showed similar activity against SK-BR-3 and MCF-7. An exception was **140**, which showed moderate activity against SK-BR-3 ($ED_{50}$ 2.1 µg/mL), but was completely inactive against MCF-7.

To further explore the selectivity, compounds with $ED_{50}$ values less than 4 µg/mL, were further examined against two additional breast cancer cell lines, ZR-75-1 (ER+, HER2+) and MDA-MB-231 (ER-). Most compounds had similar potency against the SK-BR-3 and

ZR-75-1 cell lines. However, **108** had a tenfold ratio of ZR-75-1/SK-BR-3 selectivity, while **109** had a threefold ratio. Importantly, **110** showed a 23-fold ratio of ZR-75-1/MCF-7 selectivity. All compounds tested were not active against the MDA-MB-231 cell line, which confirmed that these novel analogues were highly selective.

As indicated by ZR-75-1/SK-BR-3 selectivity ratios, we observed that some compounds (e.g., **109** and **110**) were more sensitive to cell lines over-expressing only HER2 (SK-BR-3 and ZR-75-1), while others (e.g., **108**) were more sensitive to cell lines over-expressing both HER2 and ER (ZR-75-1). These results will facilitate our studies on the mechanism(s) of action. Because ring-A is critical to activity and selectivity, we will further explore C1-C4 positions in the future.

We also investigated analogues involving skeletal modifications in ring-B, -C, or –D. Compounds **121-122** contain a pyridinone rather than phenyl ring-B and were much less active than **92**. Bioisosteric modifications of either lactone oxygen to nitrogen or sulfur in ring-C led to dramatically decreased or abolished anti-breast cancer activity (**127-131**). The results demonstrated that the lactone ring is an important feature to the activity. Compounds **152**, **154** and **155-156** with a non-aromatic ring-A and dihydrofuran ring-D, respectively, showed decreased activity compared with **92** and **91**, respectively. Interestingly, **153** showed moderate activity against MCF-7, SK-BR-3, and ZR-75-1 cell lines. Compound **159** with a substituted phenyl rather than furan D-ring was inactive. These results, together with our previously reported data, indicated that an unsaturated furan is favored for ring-D.

**108-116, 127-131 134-142** | **121-122** | **152-154** | **155-156** | **159**
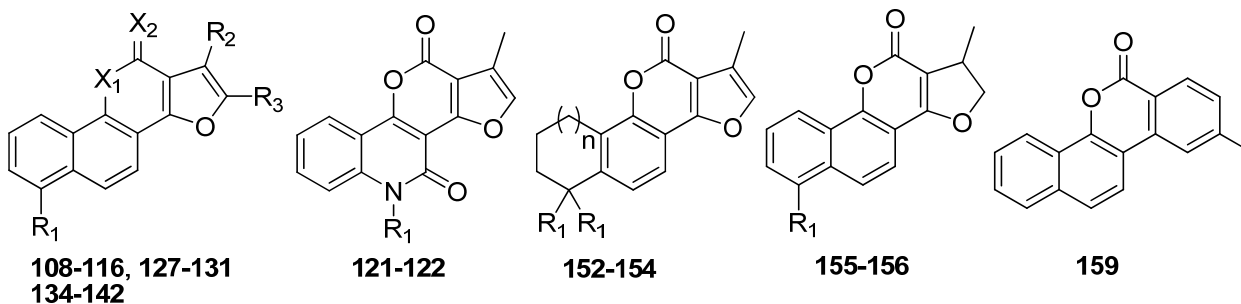
### Table 3-1. Cytotoxicity of compounds against tumor cell lines[a]

| Compd | $R_1$ | $R_2$ | $R_3$ | $X_1$ | $X_2$ | MCF-7 | SK-BR-3 | ZR-75-1 | MDA-MB-231 |
|---|---|---|---|---|---|---|---|---|---|
| 90 | Me | Me | H | O | O | 0.6 | 0.2 | 0.3 | 10.0 |
| 91 | Et | Me | H | O | O | 0.2 | 0.1 | 0.1 | 8.8 |
| 92 | H | Me | H | O | O | 4.0 | 1.0 | 4.0 | 10.3 |
| 108 | iPr | Me | H | O | O | 1.4 | 2.0 | 0.2 | >10 |
| 109 | Pr | Me | H | O | O | 1.2 | 0.1 | 0.3 | >10 |
| 110 | OMe | Me | H | O | O | 2.3 | 0.2 | 0.1 | 6.4 |
| 111 | OEt | Me | H | O | O | >20 | 2.5 | 1.9 | 9.8 |
| 112 | F | Me | H | O | O | 4.5 | 1.1 | 0.8 | >10 |
| 113 | BME[b] | Me | H | O | O | NT[c] | 0.1 | 0.1 | 14.1 |
| 114 | OH | Me | H | O | O | 15.0 | 5.0 | NT | NT |
| 115 | OAc | Me | H | O | O | 6.0 | 5.7 | NT | NT |
| 116 | DAE[d] | Me | H | O | O | 5.0 | 5.8 | NT | NT |
| 121 | Me | -- | -- | -- | -- | 11.0 | 12.1 | NT | NT |
| 122 | Pr | -- | -- | -- | -- | >20 | 15.1 | NT | NT |
| 127 | H | Me | H | S | O | >20 | 11.7 | NT | NT |
| 128 | H | Me | H | N | O | NT | 4.4 | NT | NT |
| 129 | H | Me | H | O | S | >20 | >20 | NT | NT |
| 130 | Et | Me | H | O | S | >20 | 16.3 | NT | NT |
| 131 | Et | Me | H | O | NOH | >20 | 18.8 | NT | NT |
| 134 | H | Et | H | O | O | 2.5 | 1.8 | 2.3 | >10 |
| 135 | Me | Et | H | O | O | 7.5 | 11.3 | NT | NT |
| 136 | Et | Et | H | O | O | 1.3 | 1.5 | 0.6 | >10 |
| 137 | H | Me | Me | O | O | >20 | 6.9 | NT | NT |
| 138 | Et | Me | Me | O | O | 8.0 | 9.8 | NT | NT |
| 139 | H | H | Me | O | O | >20 | 12.1 | NT | NT |
| 140 | Et | H | Me | O | O | >20 | 2.1 | 2.2 | 9.6 |
| 141 | H | PMP[e] | H | O | O | NT | 5.8 | NT | NT |
| 142 | Et | PMP | H | O | O | >20 | >20 | NT | NT |
| 152 | H | -- | -- | -- | -- | NT | >20 | NT | NT |
| 153 | H | -- | -- | -- | -- | 3.7 | 1.32 | 2.09 | 9.81 |
| 154 | Me | -- | -- | -- | -- | NT | 10.09 | NT | NT |
| 155 | H | -- | -- | -- | -- | NT | 13.7 | NT | NT |
| 156 | Et | -- | -- | -- | -- | NT | 5.1 | NT | NT |
| 159 | -- | -- | -- | -- | -- | NT | >20 | NT | NT |

a) Mean $ED_{50}$ (μg/mL), Standard error of independent determinations was less than 5%; b) BME: 1-bromoethyl; c) NT: not tested; d) PMP: 4-methoxyphenyl; e) DAE: 2-(dimethylamino)ethoxy.

To further investigate human tumor-tissue-type selectivity, compounds with $ED_{50}$ values less than 4 μg/mL against breast cancer cell lines were tested against four different human cancer cell lines, A549 (lung), DU145 (prostate), KB (nasopharnygeal), and KB-vin (its vincristine-resistant subline), using **91** as a positive control (Table 3-2). Except for **111** and **153**, none of the compounds were active against the four tumor cell lines tested. Compound **153** was active against A549 and KB-vin, but not active against DU145 and KB, which indicated that a cyclohexene ring-A could affect the tumor-tissue-type selectivity. In summary, these results demonstrated that our novel analogues were extremely selective for breast cancer cell lines.

**Table 3-2. Cytotoxicity of compounds against tumor cell lines[a]**

| Compd | A549 | DU145 | KB | KB-vin |
|:-----:|:----:|:-----:|:---:|:------:|
| **91** | 10.6 | 15.9 | 13.1 | 13.2 |
| **108** | 10.8 | 10.5 | 10.5 | 7.3 |
| **109** | 11.7 | 14.6 | 12.3 | 10.7 |
| **110** | 10.0 | 14.4 | 13.1 | 11.6 |
| **111** | 3.5 | 4.7 | 3.7 | 5.3 |
| **112** | 13.5 | 18.2 | 13.1 | 15.3 |
| **113** | >20 | 17.7 | 12.7 | >20 |
| **134** | 8.2 | 8.7 | 7.5 | 6.6 |
| **136** | 18.2 | 15.9 | 12.3 | 14.6 |
| **140** | 11.8 | 14.1 | 13.7 | 12.8 |
| **153** | 2.6 | 8.3 | 8.0 | 2.6 |

[a]See Table 3-1.

## 3.5    Conclusions

In summary, a highly efficient synthesis of **91** was accomplished with fewer steps and higher overall yield than those previously reported. This synthetic pathway was used to prepare new analogues. The SAR study led to the following observations. (1) C-4 position is critical for both potency and selectivity. The order of potency against SK-BR-3 was ethyl = 2-

bromoethyl = propyl > methyl = methoxy > fluoro = hydrogen > isopropyl > ethoxy > dimethylamino > acetate > hydroxyl. Analogues with 4-isopropyl, -propyl and -methoxy groups showed high selectivity against different breast cancer cell lines. (2) The order of potency at the C-17 position was methyl > ethyl > hydrogen, while the order of potency at the C-16 position was hydrogen > methyl. (3) Pyridinone ring is not favored for ring-B. (4) Lactone ring-C is essential for activity. (5) Ring-D is preferably an unsaturated furan ring. Based upon all results, a mechanism of action study is in progress. Due to their high selectivity and potency, **108-110** and **113** are novel promising anti-breast cancer candidates.

## 3.6    Experimental Section

### 3.6.1   Chemistry

**Materials and Methods**. Melting points were measured with a Fisher Johns melting apparatus without correction. ^{1}H NMR spectra were measured on a 300 MHz Varian Gemini 2000 spectrometer using TMS as internal standard. The solvent used was $CDCl_3$ unless indicated. Mass spectra were measured on a Shimadzu LC-MS2010 instrument. Thin-layer chromatography (TLC) and preparative TLC were performed on precoated silica gel GF plates purchased from Merck, Inc. Biotage Flash+ or Isco Companion systems were used for flash chromatography. Silica gel (200-400 mesh) from Aldrich, Inc., was used for column chromatography. All other chemicals were obtained from Aldrich, Inc, and Fisher, Inc. Intermediates **96-107** and **125-126** for target compounds **108-112** and **127-128** were prepared by the methods described in our previous paper.[11]

**Preparation of intermediates 119-120 for target compounds 121-122.** Methylaniline or *N*-propylaniline (9.942 mmol) and diethyl malonate (31.65 mmol) were refluxed at 250 °C for 7 h so that the EtOH generated escaped freely from the top of the air condenser. After cooling, the mixture, which had formed a red-brown crystalline paste, was

diluted with light petroleum and filtered. The solid was washed with MeOH to give the desired compound, **119** or **120**.

**4-Hydroxy-6-methyl-2*H*-pyrano[3,2-*c*]quinoline-2,5(6*H*)-dione  (119).** 28% yield; MS: *m/z* 244 (M+H$^+$); $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 3.80 (s, 3H, C*H$_3$*), 5.68 (s, 1H, COC*H*), 7.45-7.54 (m, 2H, aromatic), 7.78-7.83 (m, 1H, aromatic), 8.34 (d, *J* = 8.1 Hz, 1H, aromatic).

**4-Hydroxy-6-propyl-2*H*-pyrano[3,2-*c*]quinoline-2,5(6*H*)-dione  (120).** 28% yield; MS: *m/z* 272 (M+H$^+$); $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.08 (t, J = 7.5 Hz, 3H, C*H$_3$*), 1.82 (h, *J* = 7.5 Hz, 2H, C*H$_2$*CH$_3$), 4.31 (t, *J* = 7.8 Hz, 2H, C*H$_2$*CH$_2$CH$_3$), 5.67 (d, *J* = 0.9 Hz, 1H, COC*H*), 7.42-7.51 (m, 2H, aromatic), 7.75-7.81 (m, 1H, aromatic), 8.33-8.36 (m, 1H, aromatic).

**Synthesis of neo-tanshinlactone analogues 108-112, 121-122, 127-128, and 134-142.** To a solution of **102-107**, or **119-120**, or **125-126**, or **132-133** (0.20 mmol) in toluene (8 mL) was added a mixture of HOAc (59 mg, 1.0 mmol) and NH$_4$OAc (75 mg, 1.0 mmol) in EtOH (2 mL) and chloroacetone (90 mg, 1.0 mmol) or 3-bromobutan-2-one, 2-bromopropanal, 1-bromobutan-2-one, 2-bromo-1-(4-methoxyphenyl)ethanone. The mixture was stirred for 30 min at rt, and then heated to 60 °C for 30 min. Subsequently, it was refluxed for 24 h. After cooling, the mixture was diluted with H$_2$O and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The residue was purified by column chromatography (hexane/EtOAc) to give target compounds.

**6-Isopropyl-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one  (108).** 65% yield; mp 155-157 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.42 (t, *J* = 6.6 Hz, 6H, (C*H$_3$*)$_2$), 2.41 (d, *J* = 1.2 Hz, 3H, C*H$_3$*), 3.76 (h, *J* = 6.9 Hz, 1H, C*H*(CH$_3$)$_2$), 7.44 (d, *J* = 1.2 Hz, 1H, OC*H*), 7.56-7.64 (m, 2H, aromatic), 7.87 (d, *J* = 9.0 Hz, 1H, aromatic), 8.05 (d, *J* = 9.0 Hz, 1H, aromatic), 8.51 (d, *J* = 7.8 Hz, 1H, aromatic); HRMS Calcd for C$_{19}$H$_{17}$O$_3$ (M+H$^+$): 293.1178, found: 293.1168.

**1-Methyl-6-propyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (109).** 59% yield; mp 141-143 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.04 (t, *J* = 7.2 Hz, 3H, CH$_2$CH$_2$C*H*$_3$), 1.78 (m, 2H, CH$_2$C*H*$_2$CH$_3$), 2.40 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 3.06 (t, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_2$CH$_3$), 7.42-7.46 (m, 2H, aromatic & OC*H*), 7.55 (t, *J* = 8.4 Hz, 1H, aromatic), 7.83 (d, *J* = 9.0 Hz, 1H, aromatic), 7.94 (d, *J* = 9.0 Hz, 1H, aromatic), 8.49 (d, *J* = 8.4 Hz, 1H, aromatic); HRMS Calcd for C$_{19}$H$_{17}$O$_3$ (M+H$^+$): 293.1178, found: 293.1175.

**6-Methoxy-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (110).** 29% yield; mp 225-227 °C ; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.40 (s, 3H, C*H*$_3$), 4.02 (s, 3H, OC*H*$_3$), 6.95 (d, *J* = 7.8 Hz, 1H, aromatic), 7.42 (s, 1H, OC*H*), 7.54 (t, *J* = 7.8 Hz, 1H, aromatic), 7.78 (d, *J* = 9.0 Hz, 1H, aromatic), 8.15 (d, *J* = 9.0 Hz, 2H, aromatic); HRMS Calcd for C$_{17}$H$_{13}$O$_4$ (M+H$^+$): 281.0814, found: 281.0816.

**6-Ethoxy-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (111).** 28% yield; mp 201-203 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.56 (t, *J* = 7.2 Hz, 3H, CH$_2$C*H*$_3$), 2.39 (s, 3H, C*H*$_3$), 4.20 (q, *J* = 7.2 Hz, 2H, C*H*$_2$CH$_3$), 6.91 (d, *J* = 7.8 Hz, 1H, aromatic), 7.40 (s, 1H, OC*H*), 7.50 (t, *J* = 8.4 Hz, 1H, aromatic), 7.75 (d, *J* = 9.0 Hz, 1H, aromatic), 8.11 (d, *J* = 8.4 Hz, 1H, aromatic), 8.16 (d, *J* = 9.0  Hz, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{14}$O$_4$ (M+H$^+$): 295.0970, found: 295.0970.

**6-Fluoro-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (112).** 20% yield; mp 215-217 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.42 (d, *J* = 1.8 Hz, 3H, C*H*$_3$), 7.27-7.33 (m, 1H, aromatic), 7.48 (d, *J* = 1.2 Hz, 1H, OC*H*), 7.55-7.62 (m, 1H, aromatic), 7.93 (d, *J* = 9.0 Hz, 1H, aromatic), 8.03 (d, *J* = 9.0 Hz, 1H, aromatic), 8.40 (d, *J* = 8.7 Hz, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{10}$FO$_3$ (M+H$^+$): 269.0614, found: 269.0616.

**5-Aza-*N*-methyl-1-methyl-4*H*-benzo[*h*]furo[3,2,*c*]chromene-4,11-dione(121).** 50% yield; mp 265-267 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.38 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 3.82 (s, 3H, NC*H*$_3$), 7.39 (t, *J* = 8.1 Hz, 1H, aromatic), 7.46 (d, *J* = 8.4 Hz, 1H, aromatic), 7.54 (d,

$J$ = 1.5 Hz, 1H, OC$H$), 7.68-7.74 (m, 1H, aromatic), 8.36 (dd, $J$ = 1.2, 8.4 Hz, 1H, aromatic); HRMS Calcd for $C_{16}H_{12}NO_4$ (M+H$^+$): 282.0766, found: 282.0757.

**5-Aza-*N*-propyl-1-methyl-4*H*-benzo[*h*]furo[3,2,*c*]chromene-4,11-dione(122).** 55% yield; mp 238-240 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.08 (t, $J$ = 7.5 Hz, 3H, CH$_2$CH$_2$C$H_3$), 1.83 (h, $J$ = 7.5 Hz, 2H, CH$_2$C$H_2$CH$_3$), 2.38 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 4.35 (t, $J$ = 7.5 Hz, 2H, C$H_2$CH$_2$CH$_3$), 7.37 (t, $J$ = 7.8 Hz, 1H, aromatic), 7.43 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.53 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.66-7.72 (m, 1H, aromatic), 8.35 (dd, $J$ = 1.8, 7.8 Hz, 1H, aromatic); HRMS Calcd for $C_{18}H_{16}NO_4$ (M+H$^+$): 310.1079, found: 310.1068.

**1-Methyl-11*H*-benzo[*h*]furo[3,2-*c*]thiochromen-11-one (127).** 8% yield; mp 137-139 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.42 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 7.44 (q, $J$ = 1.5 Hz, 1H, OC$H$), 7.61-7.64 (m, 2H, aromatic), 7.85 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.88-7.92 (m, 1H, aromatic), 8.15 (d, $J$ = 8.7 Hz, 1H, aromatic), 8.21-8.22 (m ,1H, aromatic); HRMS Calcd for $C_{16}H_{11}O_2S$ (M+H$^+$): 267.0480, found: 267.0473.

**1-Methylbenzo[*h*]furo[3,2-*c*]quinolin-11(10*H*)-one (128).** 10% yield; mp 135-137 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.53 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 7.47 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.61-7.71 (m, 3H, aromatic), 7.93 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.98 (d, $J$ = 8.7 Hz, 1H, aromatic), 8.45 (d, $J$ = 8.1 Hz, 1H, aromatic); HRMS Calcd for $C_{16}H_{12}NO_2$ (M+H$^+$): 250.0868, found: 250.0855.

**1-Ethyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (134).** 64% yield; mp 151-153 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.32 (t, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 2.78 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 7.32 (s, 1H, OC$H$), 7.52-7.61 (m, 3H, aromatic), 7.66 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.76 (d, $J$ = 8.7 Hz, 1H, aromatic), 8.43 (d, $J$ = 7.5 Hz, 1H, aromatic); HRMS Calcd for $C_{17}H_{13}O_3$ (M+H$^+$): 265.0865, found: 265.0865.

**1-Ethyl-6-methyl-11H-benzo[*h*]furo[3,2-*c*]chromen-11-one (135).** 65% yield; mp 183-185 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.33 (q, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 2.65 (s,

3H, C*H*₃), 2.81 (q, *J* = 7.2 Hz, 2H, C*H*₂CH₃), 7.37 (s, 2H, aromatic & OC*H*), 7.45 (t, *J* = 8.1 Hz, 1H, aromatic), 7.71 (d, *J* = 8.7 Hz, 1H, aromatic), 7.78 (d, *J* = 9.0 Hz, 1H, aromatic), 8.36 (d, *J* = 8.4 Hz, 1H, aromatic); HRMS Calcd for $C_{18}H_{15}O_3$ (M+H⁺): 279.1021, found: 279.1017.

**1,6-Diethyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (136).** 75% yield; mp 101-103 °C; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.33 (q, *J* = 7.8 Hz, 6H, C*H*₃), 2.83 (q, *J* = 7.5 Hz, 2H, C*H*₂CH₃), 3.04 (q, *J* = 7.8 Hz, 2H, C*H*₂CH₃), 7.35-7.40 (m, 2H, aromatic & OC*H*), 7.48 (t, *J* = 7.5 Hz, 1H, aromatic), 7.70 (d, *J* = 9.3 Hz, 1H, aromatic), 7.83 (d, *J* = 8.7 Hz, 1H, aromatic), 8.37 (d, *J* = 8.1 Hz, 1H, aromatic); HRMS Calcd for $C_{19}H_{17}O_3$ (M+H⁺): 293.1178, found: 293.1169.

**1,2-Dimethyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (137).** 15% yield; mp 103-105 °C; ¹H NMR (300 MHz, CDCl₃, ppm): δ 2.32 (d, *J* = 0.9 Hz, 3H, OCC*H*₃), 2.42 (d, *J* = 0.9 Hz, 3H, C*H*₃), 7.59-7.65 (m, 2H, aromatic), 7.72 (d, *J* = 8.7 Hz, 1H, aromatic), 7.82 (d, *J* = 8.7 Hz, 1H, aromatic), 7.85-7.88 (m, 1H, aromatic), 8.58 (d, *J* = 8.7 Hz, 1H, aromatic); HRMS Calcd for $C_{17}H_{13}O_3$ (M+H⁺): 265.0865, found: 265.0860.

**6-Ethyl-1,2-dimethyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (138).** 29% yield; mp 141-143 °C; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.38 (t, *J* = 7.5 Hz, 3H, CH₂C*H*₃), 2.29 (s, 3H, OCC*H*₃), 2.39(s, 3H, C*H*₃), 3.09 (q, *J* = 7.5 Hz, 2H, C*H*₂CH₃), 7.42 (d, *J* = 6.9 Hz, 1H, aromatic), 7.50-7.55 (m, 1H, aromatic), 7.75-7.80 (m, 1H, aromatic), 7.88-7.91 (m, 1H, aromatic), 8.44 (d, *J* = 8.1 Hz, 1H, aromatic); HRMS Calcd for $C_{19}H_{17}O_3$ (M+H⁺): 293.1178, found: 293.1172.

**2-Methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (139).** 12% yield; mp 229-231 °C; ¹H NMR (300 MHz, CDCl₃, ppm): δ 2.53 (s, 3H, C*H*₃), 6.63 (s, 1H, OCC*H*), 7.61-7.65 (m, 2H, aromatic), 7.75 (d, *J* = 8.7 Hz, 1H, aromatic), 7.83-7.90 (m, 2H, aromatic), 8.59 (d, *J* = 7.5 Hz, 1H, aromatic); HRMS Calcd for $C_{16}H_{11}O_3$ (M+H⁺): 251.0708, found: 251.0703.

**6-Ethyl-2-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (140).** 2% yield; mp 175-177 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.41 (t, *J* = 7.5 Hz, 3H, CH$_2$C*H*$_3$), 2.54 (s, 3H, C*H*$_3$), 3.15 (q, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_3$), 6.64 (s, 1H, OCC*H*), 7.48 (d, *J* = 7.2 Hz, 1H, aromatic), 7.58 (t, *J* = 7.2 Hz, 1H, aromatic), 7.88 (d, *J* = 9.0 Hz, 1H, aromatic), 8.00 (d, *J* = 9.0 Hz, 1H, aromatic), 8.50 (d, *J* = 8.4 Hz, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{15}$O$_3$ (M+H$^+$): 279.1021, found: 279.1017.

**1-(4-Methoxyphenyl)-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (141).** 20% yield; mp 173-175 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 3.88 (s, 3H, C*H*$_3$), 7.02 (d, *J* = 9.0 Hz, 2H, aromatic), 7.64-7.68 (m, 2H, aromatic & OC*H*), 7.59-7.80 (m, 4H, aromatic), 7.90-7.94 (m, 2H, aromatic), 8.62-8.65 (m, 1H, aromatic); HRMS Calcd for C$_{22}$H$_{15}$O$_4$ (M+H$^+$): 343.0970, found: 343.0975.

**6-Ethyl-1-(4-methoxyphenyl)-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (142).** 34% yield; mp 185-187 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.39 (t, *J* = 7.5 Hz, 3H, CH$_2$C*H*$_3$), 3.11 (q, *J* = 7.2 Hz, 2H, C*H*$_2$CH$_3$), 3.86 (s, 3H, OC*H*$_3$), 6.85-7.01 (m, 2H, aromatic), 7.46 (d, *J* = 6.6 Hz, 1H, aromatic), 7.56 (t, *J* = 7.8 Hz, 1H, aromatic), 7.72-7.77 (m, 3H, aromatic & OC*H*), 7.86 (d, *J* = 8.7 Hz, 1H, aromatic), 7.95 (d, *J* = 8.7 Hz, 1H, aromatic), 8.47 (d, *J* = 8.1 Hz, 1H, aromatic); HRMS Calcd for C$_{24}$H$_{19}$O$_4$ (M+H$^+$): 371.1283, found: 371.1291.

**1-Methyl-7,8-dihydrocyclopenta[*h*]furo[3,2-*c*]chromen-10(6*H*)-one (152).** 20% yield; mp 210-212 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.19 (p, *J* = 7.5 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.36 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 3.04 (t, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 3.14 (t, *J* = 7.5 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 7.19 (d, *J* = 7.8 Hz, 1H, aromatic), 7.36 (q, *J* = 1.2 Hz, 1H, OC*H*), 7.63 (d, *J* = 8.1 Hz, 1H, aromatic); HRMS Calcd for C$_{15}$H$_{13}$O$_3$ (M+H$^+$): 241.0859, found: 241.0858.

**1-Methyl-8,9-dihydro-6*H*-benzo[*h*]furo[3,2-*c*]chromen-11(7*H*)-one (153).** 13% yield; mp 125-127 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.80-1.86 (m, 4H,

C$H_2$CH$_2$CH$_2$C$H_2$), 2.35 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 2.84 (t, $J$ = 5.7 Hz, 2H, CH$_2$C$H_2$CH$_2$CH$_2$), 2.94 (t, $J$ = 6.0 Hz, 2H, CH$_2$CH$_2$C$H_2$CH$_2$), 7.01 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.34 (d, $J$ = 0.9 Hz, 1H, OC$H$), 7.51 (d, $J$ = 8.1 Hz, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{15}$O$_3$ (M+H$^+$): 255.1016, found: 255.1012.

**1,6,6-Trimethyl-8,9-dihydro-6$H$-benzo[$h$]furo[3,2-$c$]chromen-11(7H)-one** **(154).** 38% yield; mp 101-103 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.33 (s, 6H, C(C$H_3$)$_2$), 1.67-1.71 (m, 2H, CC$H_2$CH$_2$CH$_2$), 1.84-1.88 (m, 2H, CCH$_2$C$H_2$CH$_2$), 2.35 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 2.97 (t, $J$ = 6.3 Hz, 2H, CCH$_2$CH$_2$C$H_2$), 7.32 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.35 (q, $J$ = 1.2 Hz, 1H, OC$H$), 7.61 (d, $J$ = 8.7 Hz, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{19}$O$_3$ (M+H$^+$): 283.1329, found: 283.1315.

**6-(1-Bromoethyl)-1-methyl-11$H$-benzo[$h$]furo[3,2-$c$]chromen-11-one (113).** To a solution of **91** (27 mg, 0.1 mmol) in CCl$_4$ (3 mL) was added $N$-bromosuccinimide (18 mg, 0.1 mmol) and dibenzoyl peroxide (2 mg). The reaction mixture was stirred and heated at reflux for 9 h. After the mixture was cooled in an ice bath, the solid was removed by filtration and washed with CCl$_4$. Concentration and silica gel flash column chromatography (hexane-EtOAc, 8:1) gave **113** (18 mg, 52%) as a white solid. mp 173-175 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.29 (d, $J$ = 6.9 Hz, 3H, CHBrC$H_3$), 2.42 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 5.97 (q, $J$ = 7.5 Hz, 1H, C$H$CH$_3$), 7.47 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.65 (t, $J$ = 7.8 Hz, 1H, aromatic), 7.88 (d, $J$ = 6.9 Hz, 1H, aromatic), 8.00 (d, $J$ = 9.0 Hz, 1H, aromatic), 8.15 (d, $J$ = 8.7 Hz, 1H, aromatic), 8.65 (d, $J$ = 8.7 Hz, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{14}$BrO$_3$ (M+H$^+$): 357.0126, found: 357.0120.

**6-Hydroxy-1-methyl-11$H$-benzo[$h$]furo[3,2-$c$]chromen-11-one (114).** To a solution of **110** (32 mg, 0.114 mmol) in DCM (3 mL) was added BBr$_3$ (1.12 mL, 1.12 mmol) dropwise at 0 °C. The reaction mixture was stirred and warmed to rt for 12 h. Water was added to quench the reaction. The solution was extracted with CHCl$_3$. The organic layer was

concentrated and purified with flash chromatography, eluting with DCM-MeOH, 15:1, to give **114**. 52% yield; ^{1}H NMR (300 MHz, DMSO, ppm): δ 2.30 (s, 3H, C*H₃*), 7.06 (d, *J* = 8.7 Hz, 1H, aromatic), 7.53 (t, *J* = 8.4 Hz, 1H, aromatic), 7.82-7.86 (m, 2H, aromatic), 7.97 (d, *J* = 1.2 Hz, 1H, OC*H*), 8.11 (d, *J* = 9.3 Hz, 1H, aromatic), 10.58 (s, 1H, O*H*); HRMS Calcd for $C_{16}H_{11}O_4$ (M+H⁺): 265.0501, found: 265.0505.

**1-Methyl-11-oxo-11*H*-benzo[*h*]furo[3,2-*c*]chromen-6-yl acetate (115).** Compound **114** (0.1 mmol) was dissolved in acetic anhydride under argon. Triethylamine (0.14 mL, 1.0 mmol) was added to the solution. After stirring overnight at 60 °C, the solution was washed with water and extracted with DCM, and dried (MgSO₄). Removal of solvent under reduced pressure yielded a residue, which was purified by column chromatography, eluting with EtOAc–hexane (1:4). 43% yield; ^{1}H NMR (300 MHz, CDCl₃, ppm): δ 2.41 (s, 3H, C*H₃*), 2.50 (s, 3H, COC*H₃*), 7.39 (d, *J* = 6.9 Hz, 1H, aromatic), 7.46 (s, 1H, OC*H*), 7.65 (t, *J* = 8.1 Hz, 1H, aromatic), 7.80 (d, *J* = 8.7 Hz, 1H, aromatic), 7.90 (d, *J* = 8.7 Hz, 1H, aromatic), 8.52 (d, *J* = 8.4 Hz, 1H, aromatic); HRMS Calcd for $C_{18}H_{13}O_5$ (M+H⁺): 309.0763, found: 309.0762.

**6-(2-(Dimethylamino)ethoxy)-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one (116).** Compound **114** (0.1 mmol) was dissolved in acetone under argon. K₂CO₃ (235 mg, 1.7 mmol) was added to the solution. After stirring for 10 min, 2-chloro-*N,N*-dimethylethylamine hydrochloride (30 mg, 0.2 mmol) was added to the mixture. After refluxing for 10 h, the mixture was filtrated and concentrated. The residue was purified by column chromatography, eluting with EtOAc–hexane (1:2). 9% yield; mp 209-211 °C; ^{1}H NMR (300 MHz, CDCl₃, ppm): δ 2.41 (s, 3H, C*H₃*), 2.44 (s, 6H, N(C*H₃*)₂), 2.96 (t, *J* = 5.1 Hz, 2H, OCH₂C*H₂*), 4.31 (t, *J* = 5.1 Hz, 2H, OC*H₂*CH₂), 6.98 (d, *J* = 7.5 Hz, 1H, aromatic), 7.45 (s, 1H, OC*H*), 7.55 (t, *J* = 8.1 Hz, 1H, aromatic), 7.83 (d, *J* = 8.7 Hz, 1H, aromatic), 8.20 (dd, *J* = 7.2, 8.7 Hz, 2H, aromatic); HRMS Calcd for $C_{20}H_{20}NO_4$ (M+H⁺): 338.1392, found: 338.1389.

Synthesis of neo-tanshinlactone analogues 129-130. A mixture of compound **92** or **91** (0.1 mmol) and Lawesson's reagent (81 mg, 0.2 mmol) in dry toluene (5 mL) was heated to reflux for 7 h. After cooling, toluene was removed in vacuo, and the red residue was dissolved in EtOAc and partitioned with $H_2O$. The organic phase was separated and dried over $MgSO_4$. Removal of solvent in vacuo afforded an oily residue, which was purified by column chromatography (EtOAc–hexane) to give a yellow solid.

**1-Methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromene-11-thione (129).** 90% yield; mp 267-269 °C; $^1$H NMR (300 MHz, $CDCl_3$, ppm): δ 2.53 (s, 3H, C*H*$_3$), 7.46 (s, 1H, OC*H*), 7.67-7.69 (m, 2H, aromatic), 7.79-7.92 (m, 3H, aromatic), 8.75 (d, *J* = 7.2 Hz, 1H, aromatic); HRMS Calcd for $C_{16}H_{11}O_2S$ (M+H$^+$): 267.0480, found: 267.0471.

**6-Ethyl-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromene-11-thione (130).** 84% yield; mp 189-191 °C; $^1$H NMR (300 MHz, $CDCl_3$, ppm): δ 1.38 (t, *J* = 7.5 Hz, 3H, CH$_2$C*H*$_3$), 2.49 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 3.09 (q, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_3$), 7.40 (d, *J* = 1.5 Hz, 1H, OC*H*), 7.46 (d, *J* = 7.2 Hz, 1H, aromatic), 7.56 (t, *J* = 7.2, 8.1 Hz, 1H, aromatic), 7.78 (d, *J* = 9.0 Hz, 1H, aromatic), 7.95 (d, *J* = 9.0 Hz, 1H, aromatic), 8.55 (d, *J* = 8.7 Hz, 1H, aromatic); HRMS Calcd for $C_{18}H_{15}O_2S$ (M+H$^+$): 295.0793, found: 295.0779.

**6-Ethyl-1-methyl-11*H*-benzo[*h*]furo[3,2-*c*]chromen-11-one oxime (131).** A mixture of **130** (22 mg. 0.075 mmol), hydroxylamine hydrochloride (10.4 mg, 0.15 mmol), sodium acetate (12 mg, 0.15 mmol), and MeOH (5 mL) was refluxed overnight and then filtered. The filtrate was concentrated under reduced pressure to give an oily residue. Purification by the column chromatography (EtOAc–hexane) gave **131** in 87% yield. mp 211-213 °C; $^1$H NMR (300 MHz, $CDCl_3$, ppm): δ 1.39 (t, *J* = 7.2 Hz, 3H, CH$_2$C*H*$_3$), 2.28 (d, *J* = 0.9 Hz, 3H, C*H*$_3$), 3.11 (q, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_3$), 7.18 (s, 1H, O*H*), 7.33 (d, *J* = 1.2 Hz, 1H, OC*H*), 7.40 (d, *J* = 6.9 Hz, 1H, aromatic), 7.52 (dd, *J* = 7.2, 8.4 Hz, 1H, aromatic), 7.71 (d, *J* = 9.0 Hz, 1H,

aromatic), 7.86 (d, $J$ = 9.0 Hz, 1H, aromatic), 8.40 (d, $J$ = 8.7 Hz, 1H, aromatic); HRMS Calcd for $C_{18}H_{16}NO_3$ (M+H$^+$): 294.1130, found: 294.1118.

**1-Methoxy-2-(4-methylpent-3-enyl)benzene (144).** Preparation of (4-methylpent-3-enyl)zinc(II) bromide: Zinc powder (0.98 g, 15 mmol) was stirred and heated to 70 °C under high vacuum for 30 minutes. *N,N'*-Dimethylimidazolidinone (DMI) (to give a total volume of 10 mL) and iodine (0.13 g, 0.50 mmol) were added under the protection of argon. After the red color of iodine had faded, the 5-bromo-2-methyl-2-pentene (10 mmol) was added. The colorless reaction mixture was stirred for 12 h at 70 °C, and then it was cooled to rt.

1-Bromo-2-methoxybenzene (1.0 mmol), Pd(Cl$_2$)(dppf) (0.1 mmol), and 10 mL THF were stirred in a 25 mL bottle. (4-Methylpent-3-enyl)zinc(II) bromide (2.0 mmol) was added. The mixture was refluxed for 1 h and cooled to rt. The reaction was quenched by addition of 4 N HCl (5 mL). The mixture was extracted with diethyl ether, and the organic layer dried over MgSO$_4$. Removal of solvent under reduced pressure yielded a light yellow liquid, which was purified by column chromatography, eluting with EtOAc–hexane (1:10). 96% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.57 (s, 3H, C$H_3$), 1.68 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 2.24 (q, $J$ = 7.5 Hz, CHC$H_2$CH$_2$), 2.62 (q, $J$ = 7.8 Hz, 2H, CHCH$_2$C$H_2$), 3.82, (s, 3H, OC$H_3$), 5.18-5.23 (m, 1H, C$H$C(CH$_3$)$_2$), 6.83-6.90 (m, 2H, aromatic), 7.11-7.20 (m, 2H, aromatic).

**5,5-Dimethyl-5,6,7,8-tetrahydronaphthalen-1-ol (145). 143** (8.66 g, 46 mmol) was dissolved in 250 mL of CH$_2$Cl$_2$ and cooled at 0 °C while AlCl$_3$ (6.13 g, 46 mmol) was added in one portion. The resulting orange solution was stirred at 0 °C for 15 min and then poured into 300 mL of ice-water. The aqueous phase was separated and extracted with two portions of Et$_2$O and dried to obtain pure 5-methoxy-1,1-dimethyl-1,2,3,4-tetrahydronaphthalene. [99% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.28 (s, 6H, (C$H_3$)$_2$), 1.61-1.65 (m, 2H, CC$H_2$), 1.76-1.80 (m, 2H, CC$H_2$CH$_2$), 2.65 (t, $J$ = 6.3 Hz, 2H, CCH$_2$CH$_2$C$H_2$), 3.81 (s, 3H,

OC*H*₃), 6.64 (d, *J* = 8.1 Hz, 1H, aromatic), 6.97 (d, *J* = 7.8 Hz, 1H, aromatic), 7.13 (t, *J* = 8.1 Hz, 1H, aromatic).]

To a solution of 5-methoxy-1,1-dimethyl-1,2,3,4-tetrahydronaphthalene (2 mmol) in DCM (3 ml) was added BBr₃ (6 mmol) dropwise at 0 °C. The reaction mixture was stirred and warmed to rt for 12 h. Water was added to quench the reaction. The solution was extracted with CHCl₃. The organic layer was concentrated and purified with flash chromatography, eluting with hexane: EtOAc=10:1, to give **145** as a solid. 85% yield; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.28 (s, 6H, (C*H*₃)₂), 1.62-1.66 (m, 2H, CC*H*₂), 1.79-1.88 (m, 2H, CCH₂C*H*₂), 2.63 (t, *J* = 6.6 Hz, 2H, CCH₂CH₂C*H*₂), 4.63 (s, 1H, O*H*), 6.59 (q, *J* = 6.6 Hz, 1H, aromatic), 6.95 (d, *J* = 7.8 Hz, 1H, aromatic), 7.04 (t, *J* = 7.8 Hz, 1H, aromatic).

**Synthesis of neo-tanshinlactone analogues 149-151.** A mixture of **146** (or **147, 145**) (1.0 g, 6.33 mmol), malonic acid (658 mg, 6.33 mmol), and PPA (85% P₂O₅, 10 g) was heated at 75 °C for 3 h. After cooling, ice-water was added to the black residue. The mixture was filtered, and the solid dissolved in MeOH. The organic layer was concentrated and purified with flash chromatography, eluting with DCM: MeOH=10:1, to yield **149** (or **150, 151**) as a yellow solid.

**4-Hydroxy-8,9-dihydrocyclopenta[*h*]chromen-2(7*H*)-one (149).** 35% yield; ¹H NMR (300 MHz, CD₃OD, ppm): δ 2.16-2.23 (m, 2H, CH₂C*H*₂CH₂), 3.01-3.08 (m, 4H, C*H*₂CH₂C*H*₂), 6.64 (d, *J* = 8.1 Hz, 1H, COC*H*), 7.20 (d, *J* = 8.1 Hz, 1H, aromatic), 7.68 (d, *J* = 8.1 Hz, 1H, aromatic); MS: *m/z* 201 (M-H⁺).

**4-Hydroxy-7,8,9,10-tetrahydro-2*H*-benzo[*h*]chromen-2-one (150).** 32% yield; ¹H NMR (300 MHz, CD₃OD, ppm): δ 2.64-2.73 (m, 4H, (C*H*₂)₄), 2.85 (t, *J* = 2.7 Hz, 4H, (C*H*₂)₄), 6.40 (s, 1H, COC*H*), 7.03 (d, *J* = 8.1 Hz, 1H, aromatic), 7.60 (d, *J* = 8.1 Hz, 1H, aromatic); MS: *m/z* 215 (M-H⁺).

**4-Hydroxy-7,7-dimethyl-7,8,9,10-tetrahydro-2*H*-benzo[*h*]chromen-2-one (151).** 42% yield; ¹H NMR (300 MHz, CD₃OD, ppm): δ 1.33 (s, 6H, (C*H*₃)₂), 1.69-1.73 (m, 2H,

CC*H*$_2$), 1.88-1.89 (m, 2H, CC*H*$_2$C*H*$_2$), 2.87 (t, *J* = 6.6 Hz, 2H, CC*H*$_2$CH$_2$C*H*$_2$), 7.38 (d, *J* = 8.4 Hz, 1H, aromatic), 7.68 (d, *J* = 8.4 Hz, 1H, aromatic); MS: *m/z* 243 (M-H$^+$).

**Synthesis of neo-tanshinlactone analogues 155-156.** Compound **92** or **91** (0.2 mmol) was dissolved in acetone at 40 °C under argon. Pd/C (81 mg, 10%), triethylamine (0.33 mL, 2.40 mmol) and formic acid (0.075 mL, 2.00 mmol) were added to the solution. After stirring overnight, TLC showed some substrate remained unreacted. The solution was filtered through Celite and solvent removed in vacuo to yield a dark oily residue. The residue was dissolved in DCM before washing with saturated aqueous sodium bicarbonate (5 mL), aqueous citric acid (5 mL, 10% v/v), water (5 mL) and brine (5 mL), and then dried (MgSO$_4$). Removal of solvent under reduced pressure yielded a white solid, which was purified by column chromatography, eluting with EtOAc–hexane (1:4).

**1-Methyl-1*H*-benzo[*h*]furo[3,2-*c*]chromen-11(2*H*)-one (155).** 30% yield (recovered); mp 143-145 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.46 (d, *J* = 6.9 Hz, 3H, CHC*H*$_3$), 3.68-3.77 (m, 1H, C*H*CH$_3$), 4.47 (q, *J* = 6.3 Hz, 1H, C*H*$_2$), 5.00 (t, *J* = 6.6 Hz, 1H, C*H*$_2$), 7.62-7.71 (m, 4H, aromatic), 7.87-7.90 (m, 1H, aromatic), 8.59-8.62 (m, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{13}$O$_3$ (M+H$^+$): 253.0865, found: 253.0856.

**6-Ethyl-1-methyl-1*H*-benzo[*h*]furo[3,2-*c*]chromen-11(2*H*)-one (156).** 56% yield (recovered); mp 83-85 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.39 (t, *J* = 7.8 Hz, 3H, CH$_2$C*H*$_3$), 1.46 (d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 3.13 (q, *J* = 7.5 Hz, 2H, C*H*$_2$CH$_3$), 3.66-3.78 (m, 1H, C*H*CH$_3$), 4.47 (q, *J* = 6.0 Hz, 1H, C*H*$_2$), 5.00 (t, *J* = 6.6 Hz, 1H, C*H*$_2$), 7.50-7.60 (m, 2H, aromatic), 7.64 (d, *J* = 9.3 Hz, 1H, aromatic), 7.91 (d, *J* = 9.3 Hz, 1H, aromatic), 8.49 (d, *J* = 8.7 Hz, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{19}$O$_3$ (M+H$^+$): 281.1178, found: 281.1163.

**Naphthalen-1-yl 2-bromo-4-methylbenzoate (158).** Thionyl chloride (0.17ml, 2.40 mmol) was added to 2-bromo-4-methylbenzoic acid (430 mg, 2 mmol) in DCM (3 mL) and DMF (0.1 mL), and the mixture was refluxed under nitrogen atmosphere for 1 h. After

cooling to rt, it was concentrated in vacuo to give the title compound as a pale yellow solid, which was used directly in the next step.

Naphthalen-1-ol (288 mg, 2.00 mmol) was dissolved in THF (5 mL), then DMAP (5 mg) and ethyldiisopropylamine (0.36 mL, 2.05 mmol) were added, and the mixture was cooled to 0 °C for 10 min. Freshly prepared 2-bromo-4-methylbenzoyl chloride in dry THF (10 mL) was added to the mixture via cannula, and the resulting mixture was stirred at 25 °C for 2 h, diluted with diethyl ether (150 mL), and quenched by the addition of water (15 mL). The organic layer was washed with HCl and NaHCO$_3$ and then dried (Na$_2$SO$_4$) and concentrated in vacuo. The residue was purified with flash chromatography, eluting with hexane: EtOAc=10:1, to give **158**. 96% yield; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.41 (s, 3H, C*H*$_3$), 7.27 (dd, *J* = 0.9, 8.1 Hz, 1H, aromatic), 7.40 (dd, *J* = 1.2, 7.5 Hz, 1H, aromatic), 7.48-7.52 (m, 3H, aromatic), 7.60 (d, *J* = 0.9 Hz, 1H, aromatic), 7.77 (d, *J* = 8.1 Hz, 1H, aromatic), 7.87-7.90 (m, 1H, aromatic), 7.96-7.99 (m, 1H, aromatic), 8.13 (d, *J* = 8.1 Hz, 1H, aromatic).

**9-Methyl-6*H*-dibenzo[*c,h*]chromen-6-one (159).** A mixture of **158** (68 mg, 0.2 mmol), Pd(OAc)$_2$ (4.5 mg, 0.02 mmol), PPh$_3$ (10.5 mg, 0.04 mmol), and NaOAc (32.8 mg, 0.4 mmol) was dissolved in dry dimethylacetamide (10 mL), and the solution was degassed and then heated to 150 °C for 3 h. On cooling to rt, the solution was diluted with diethyl ether (50 mL) and washed with HCl, and the organice extracts were dried over Na$_2$SO$_4$. The solution was filtered, the filtrate condensed in vacuo, and the resulting oil purified by flash chromatography (hexane:EtOAc 4:1) to give the title compound as a white solid.

65% yield; mp 193-195 °C; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.55 (s, 3H, C*H*$_3$), 7.35-7.38 (m, 1H, aromatic), 7.57-7.63 (m, 2H, aromatic), 7.71 (d, *J* = 9.0 Hz, 1H, aromatic), 7.82-7.85 (m, 1H, aromatic), 7.91 (s, 1H, aromatic), 8.00 (d, *J* = 9.3 Hz, 1H, aromatic), 8.30 (d, *J* = 7.8 Hz, 1H, aromatic), 8.53-8.56 (m, 1H, aromatic); HRMS Calcd for C$_{18}$H$_{13}$O$_2$ (M+H$^+$): 261.0916, found: 261.0909.

## 3.6.2   Biological Assay-Cell Growth Inhibition Assay.

All stock cultures are grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates with compounds added from DMSO-diluted stock. The plates were incubated for an additional 72 h after attachment and drug addition, and the assay was terminated by 10% TCA. Then, 0.4% SRB dye in 1% HOAc was added to stain the cells for 10 min. Unbound dye was removed by repeated washing with 1% HOAc and the plates were air dried. Bound stain was subsequently solved with 10 mM trizma base, and the absorbance read at 515 nm. Growth inhibition of 50% ($ED_{50}$) was calculated as the drug concentration that caused a 50% reduction in the net protein increase in control cells during the drug incubation. The mean $ED_{50}$ is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations. Variation between replicates was no more than 5% of the mean. The following human tumor cell lines were used in the assay: A549 (non-small cell lung cancer), MCF-7 (estrogen receptor positive breast cancer), MDA MB-231 (estrogen receptor negative breast cancer), SK-BR-3 (estrogen receptor negative, HER-2 over-expressing breast cancer), ZR-75-1 (estrogen receptor positive breast cancer, HER-2 over-expressing breast cancer), KB (nasopharyngeal carcinoma), KB-vin (vincristineresistant KB subline). All cell lines were obtained from the Lineberger Cancer Center (UNC-CH) or from ATCC (Rockville, MD). Cells were propagated in RPMI-1640 supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (1 μg/mL), and amphotericin B (0.25 μg/mL), and were cultured at 37 °C in a humidified atmosphere of 95% air/5% $CO_2$.

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# CHAPTER 4

# DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL 2-(FURAN-2-YL)

# NAPHTHALEN-1-OL AND TETRAHYDRONAPHTHALENE-1-OL DERIVATIVES

## 4.1    Introduction

Natural products continue to be an excellent source of new medicinal leads.[1-3] However, the structural complexity of natural products, including their intricate ring systems and numerous chiral centers, may hamper mechanism of action studies and clinical development.[4] For that reason, structural simplification of natural products is a powerful and highly productive tool for lead development and analogue design.[5] A well-known example is the simplification of morphine, which led to the clinically used medicines levophanol and meperidine.[6] Neo-tanshinlactone (**90**) is a steroid-like tetracyclic natural product originally isolated from the traditional Chinese medicine Tanshen (Figure 4-1). Structure-activity relationships of neo-tanshinlactone analogues were described in the previous chapter.[7, 8] However, several questions remained unanswered: how does the skeletal planarity affect activity and selectivity, how do each of the four individual rings contribute to activity, and how will the activity and selectivity change by simplification of the tetracyclic molecule of **90**. In this chapter, we will discuss our chemical and biological strategies to investigate structurally simplified neo-tanshinlactone (**90**) analogues to answer these questions.

## 4.2    Design

To study the individual contribution of the A-, C-, and D-rings of **90** to the selective activity against breast cancer cells, scaffolds 1-4 were designed as ring-opened model

compounds by breaking bonds 1-4 respectively (Figure 4-2). Different R groups were also incorporated to explore preliminary SAR of the four scaffolds. Designed analogues are shown in Figure 4-3 and Figure 4-4.
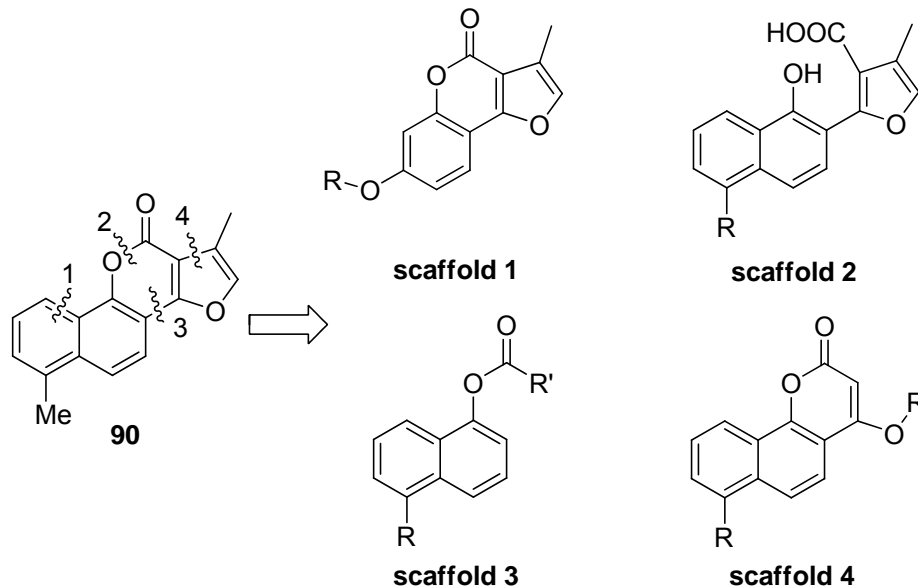


**Figure 4-1. Scaffolds 1-4 derived through structural simplification of 90**
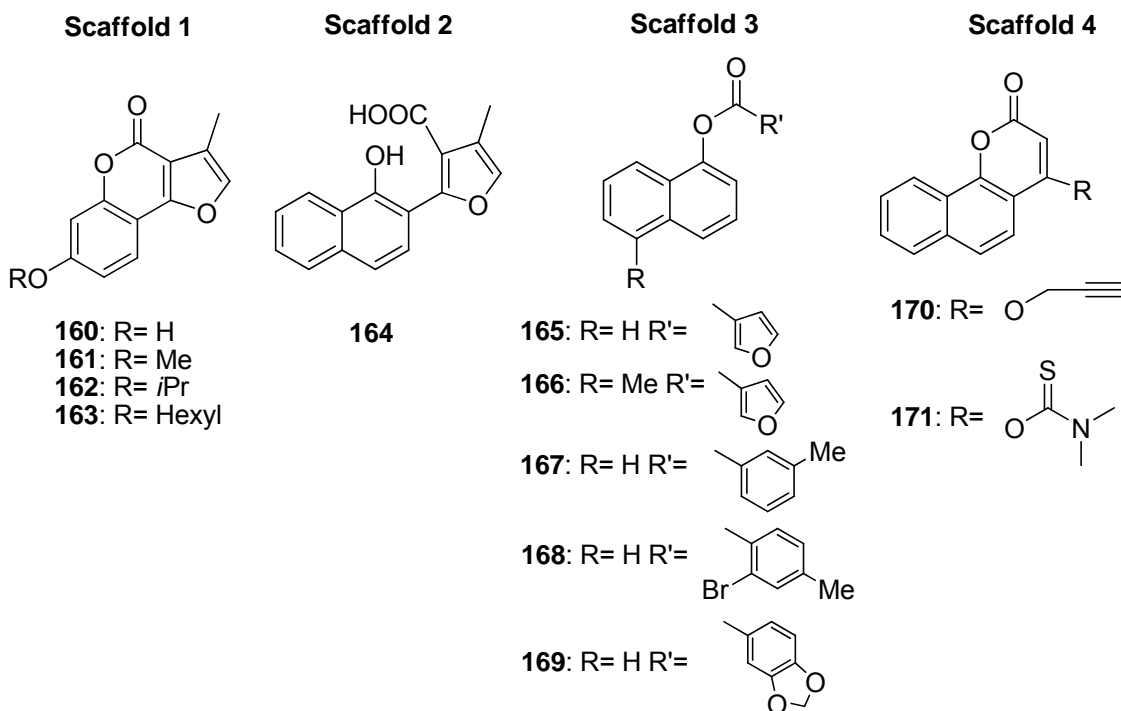


**Figure 4-2. Structures of designed target compounds 160-171 with scaffolds 1-4**

71

**172**: $R_1$= Et, $R_2$= H, $R_3$= H
**173**: $R_1$= OMe, $R_2$= H, $R_3$= H
**174**: $R_1$= OEt, $R_2$= H, $R_3$= H
**175**: $R_1$= H, $R_2$= Me, $R_3$= H
**176**: $R_1$= Et, $R_2$= Me, $R_3$= H
**177**: $R_1$= H, $R_2$= Me, $R_3$= Me
**178**: $R_1$= Et, $R_2$= Me, $R_3$= Me

**179**: $R_1$= H, $R_2$= H
**180**: $R_1$= Et, $R_2$= H
**181**: $R_1$= Et, $R_2$= Me

**182**: $R_1$= Me, $R_2$= Me
**183**: $R_1$= Et, $R_2$= H

**184**: R= H, n= 0
**185**: R= H, n= 1
**186**: R= Me, n= 1

**187**: $R_1$= Me, $R_2$= H
**188**: $R_1$= Me, $R_2$= Me

**189**: $R_1$= H, $R_2$= H
**190**: $R_1$= Me, $R_2$= H
**191**: $R_1$= Et, $R_2$= H
**192**: $R_1$= Me, $R_2$= Me
**193**: $R_1$= Et, $R_2$= Me
**194**: $R_1$= Me, $R_2$= Ac
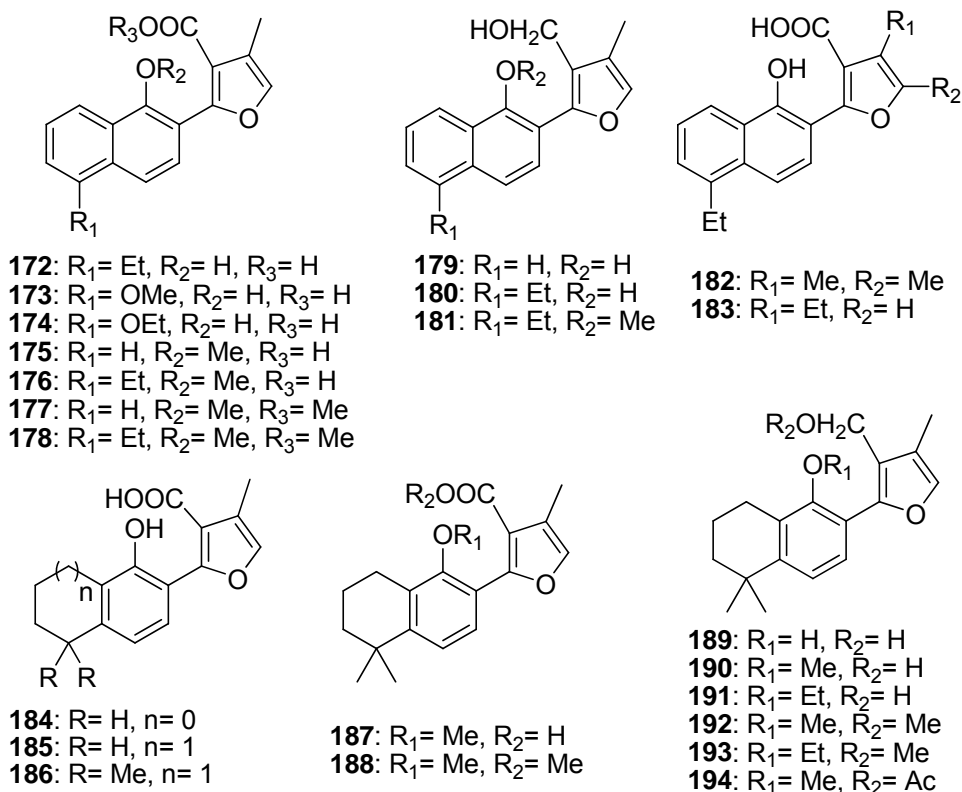
**Figure 4-3. Structures of designed target compounds 172-194 derived from scaffold 2**

## 4.3 Chemistry

### 4.3.1 Synthesis of compounds with scaffold 1

Intermediate **161** was obtained via a tandem alkylation/intramolecular Aldol reaction with commercially available **195** (Scheme 4-1).[9] Demethylation of **161** by BBr$_3$ generated **160**, which was converted to isopropyl and hexyl ethers, **162** and **163**, respectively.

**Scheme 4-1. Synthetic pathway to analogues 160-163 with scaffold 1**



**195**   **161**   **160**   **162**: R= *i*Pr
**163**: R= hexyl
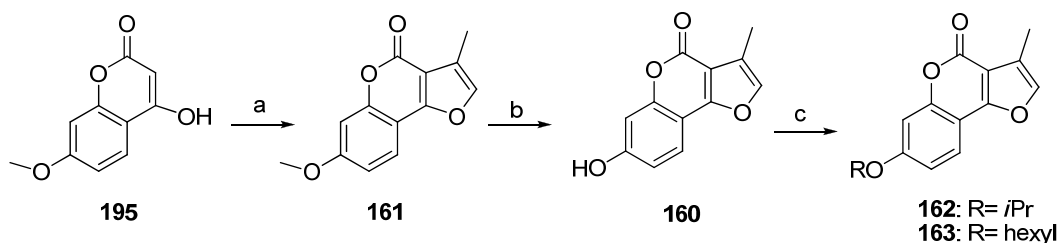
Reagents and conditions: (a) HOAc, NH$_4$OAc, chloroacetone, toluene, EtOH, reflux, 65%; (b) BBr$_3$, DCM, 50 °C, 42%; (c) 2-iodopropane for **162**, 1-bromohexane for **163**, Cs$_2$CO$_3$, DMF, acetone, 50 °C, 30% and 29%, respectively.

### 4.3.2  Synthesis of compounds with scaffold 2

Previously synthesized neo-tanshinlactone analogues, **91-92**, **110-111**, **136**, and **138**, were hydrolyzed to cleave the lactone ring and derive the related carboxylic acids, **164**, **172-174**, and **182-183**, respectively (Scheme 4-2).[10] After hydrolysis of **92** and **91**, MeI and 18-crown-6 ether were added directly to the crude mixture, without work up, to provide selective methylation of the hydroxy group of **164** and **172**.[11] The resulting methyl ethers **175-176** were converted to methyl esters (**177-178**) with thionyl chloride and methanol at room temperature.[12] Alcohols **179-180** were obtained by reduction of **91**-**92** with lithium aluminum hydride. Treatment of **180** with iodomethane in the presence of potassium carbonate gave **181**.

### Scheme 4-2. Synthetic pathway to analogues 164 and 172-183 with scaffold 2
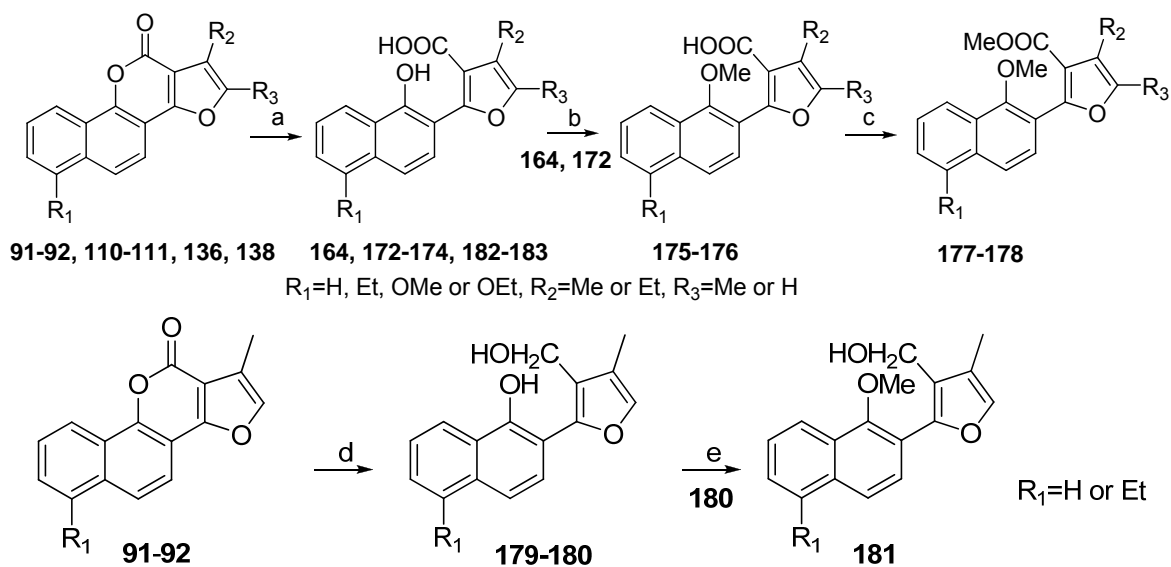


91-92, 110-111, 136, 138    164, 172-174, 182-183    175-176    177-178

R$_1$=H, Et, OMe or OEt, R$_2$=Me or Et, R$_3$=Me or H

91-92    179-180    180    181    R$_1$=H or Et

Reagents and conditions: (a) 5% NaOH (aq), reflux; (b) MeI, NaOH, 18-crown-6, CH$_3$CN, 90 °C; (c) SOCl$_2$, MeOH, rt; (d) LiAlH$_4$, THF; (e) MeI, K$_2$CO$_3$, acetone, rt.

### 4.3.3 Synthesis of compounds with scaffold 3

Compounds **157** and **196** were esterified with the appropriate acyl chlorides to give **165-169** (Scheme 4-3).

**Scheme 4-3. Synthetic pathway to analogues 165-169 with scaffold 3**
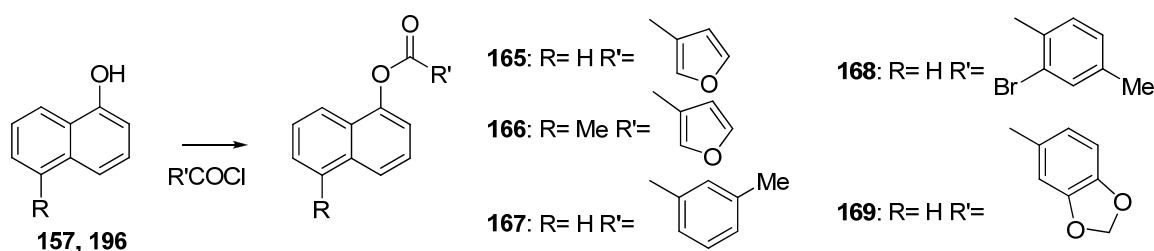


### 4.3.4 Synthesis of compounds with scaffold 4

Propargyl ether **170** and dimethylcarbamothioester **171** were synthesized from **132** by using 3-bromoprop-1-yne in the presence of K$_2$CO$_3$, and dimethylcarbamothioic chloride in the presence of NMP and DABCO, respectively (Scheme 4-4).[13, 14]

**Scheme 4-4. Synthetic pathway to analogues 170-171 with scaffold 4**
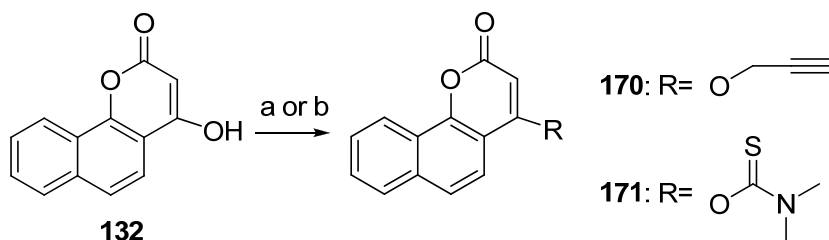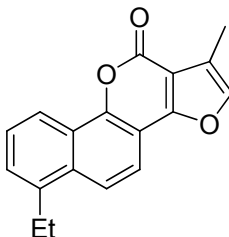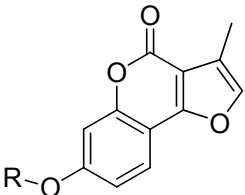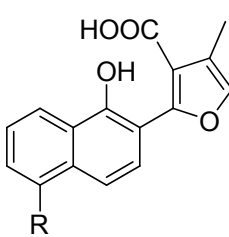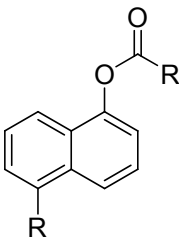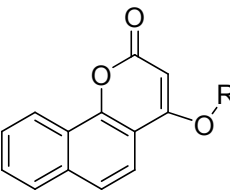


Reagents and conditions: (a) 3-bromoprop-1-yne for **170**, K$_2$CO$_3$, acetone, reflux, 40%; (b) dimethylcarbamothioic chloride for **171**, NMP, DABCO, 50 °C, 85%.

### 4.4 Results and Discussion

Initially, compounds **160-171** were tested for *in vitro* anticancer activity against two human breast cancer cell lines, MCF-7 (ER+) and SK-BR-3 (HER2+) (Table 4-1). Compounds with scaffolds 1 (**160-163**), 3 (**165-169**), and 4 (**170-171**) showed no activity against the two tested cancer cell lines. Compound **164** with scaffold 2 was less potent than **91**, but more potent than the remaining compounds. Thus, the results demonstrated that both the A-ring and D-ring are important in maintaining the biological activity of this compound type, while compounds with an opened ring-C could retain activity.

**Table 4-1. *In vitro* anticancer activity of 160-171 against MCF-7 and SK-BR-3 tumor cell lines[a]**



| 91 | scaffold 1 | scaffold 2 | scaffold 3 | scaffold 4 |

| Compd | scaffold | R | R' | MCF7 (ER+) | SK-BR-3 (HER2+) |
|---|---|---|---|---|---|
| 91 | | | | 0.2 | 0.1 |
| 160 | 1 | OH | --- | >20 | 7.8 |
| 161 | 1 | Me | --- | >20 | 10.5 |
| 162 | 1 | *i*Pr | --- | 12.0 | 10.8 |
| 163 | 1 | Hexyl | --- | >20 | 15.9 |
| 164 | 2 | H | --- | 6.0 | 7.0 |
| 165 | 3 | H | (furan) | >20 | >20 |
| 166 | 3 | Me | (furan) | >20 | >20 |
| 167 | 3 | H | (dimethylphenyl) | 11.0 | >20 |
| 168 | 3 | H | (Br, Me phenyl) | 15.5 | >20 |
| 169 | 3 | H | (methylenedioxyphenyl) | >20 | >20 |
| 170 | 4 | (butynyl) | --- | >20 | 17.9 |
| 171 | 4 | (thioamide) | --- | 15.5 | 10.9 |

a) Mean $ED_{50}$ (µg/mL), Standard error of independent determinations was less than 5%

In addition, we performed conformational analysis to study the molecular geometries of the four different scaffolds of **162**, **164**, **166**, and **170**. As seen in Figure 4-4, compounds **162**, **164**, **166**, and **170** possess most of the pharmacophore features present in the reference compound **91**, including an aromatic center, hydrophobic region, and hydrogen bond donor and acceptor groups. However, several key features are also missing in **162**, **164**, **166**, and **170** in comparison with **91**, i.e., one aromatic center in **162** and **170**, and a closed ring in **164** and **166**. The cytotoxicity results demonstrated that both the A- and D-ring are important in maintaining the molecule's biological activity. Interestingly, although **164** lacks a C-ring, an additional intramolecular hydrogen bond can form between –COOH and –OH groups in the lowest energy conformer of **164**. This hydrogen bond could 'lock' the structure into a conformation that is close to that of the tetracyclic scaffold. Thus, intramolecular hydrogen bonding in **164** may help the compound retain its biological potency.
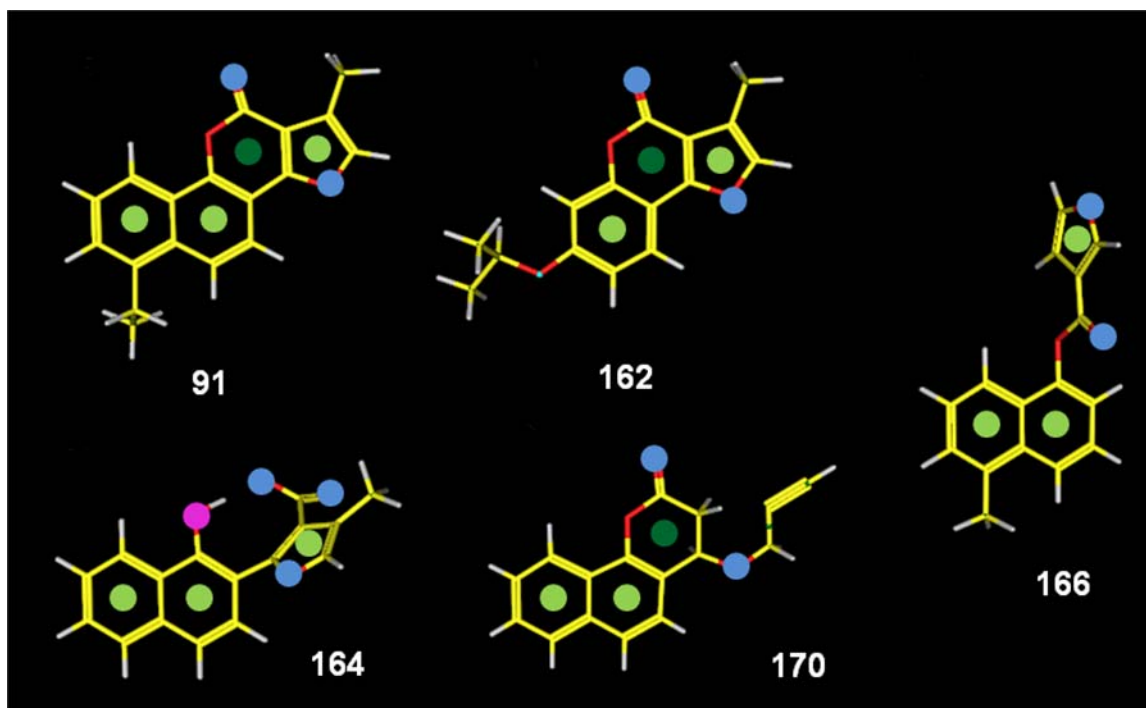


**Figure 4-4. Pharmacophore analysis of 162, 164, 166, and 170 with reference to 91 using the PCH annotation scheme.**

(Purple: H-bond donor; Blue: H-Bond acceptor; Light green: Aromatic ring center; Deep green: Hydrophobic region.) The structures of the global energy minima are shown by stick models.

Compound **164** was selected for further structure optimization in order to establish SAR correlations and to identify more active derivatives with the desired biological properties. Substituents on the **164**-scaffold will likely affect the molecule's overall three-dimensional structure and, thus, the compound's interaction with its target, which will translate into increased or decreased anti-breast cancer activity.

In order to test for a potential relationship between the intramolecular hydrogen bond (COOH- and OH-groups) and the selective *in vitro* anti-breast cancer activity, a specific target sub-set (**172**, **176** and **178**) was designed. Compound **172**, with OH at position $R_4$ and COOH at position $R_5$, can form an intramolecular hydrogen bond. However, in **176**, one hydrogen donor has been effectively removed by methylation of the OH in **172**, and in **178**, both hydrogen bond donors are blocked with methyl groups. Compounds **172-183** were designed to further study the SAR of various substituents on the molecule. The newly synthesized analogues (**172-183**) were tested initially for *in vitro* anticancer activity against two human breast cancer cell lines: MCF-7 (ER+) and SK-BR-3 cells (HER2+) (Table 4-2). Both **172** and **178** showed similar activity to TAM against MCF-7 ($ED_{50}$ values of 3.3 and 2.5 µg/mL, respectively), while **172** showed fivefold better activity than TAM against SK-BR-3 cells ($ED_{50}$ value of 1.0 µg/mL) and **178** showed about fourfold better activity than TAM against SK-BR-3 cells ($ED_{50}$ value of 1.2 µg/mL). Compound **176** displayed similar activity to TAM against both cell lines. From the $ED_{50}$ values of **164**, **172**, **173**, and **174**, the SAR study suggested that $R_1$ substituents influenced the *in vitro* anticancer activity and hydrophobic groups were favored at this position. At the $R_2$ position, a methyl group was preferable to ethyl (**172** vs **183**), and at the $R_3$ position, hydrogen was favored over methyl (**172** vs **182**).

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Proper combinations of $R_4$ and $R_5$ (hydroxy group, carboxylic acid, and alkoxy group) could

lead to potent analogues (**172**, **178**, and **181**).

**Table 4-2. Structure and cytotoxicity of analogues 172-183[a]**
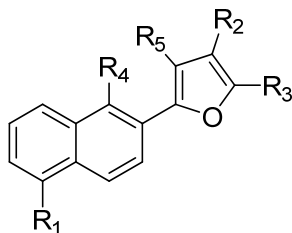


| Compd | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | MCF-7 | SK-BR-3 |
|-------|-------|-------|-------|-------|-------|--------|---------|
| TAM | | | | | | 5 | 5 |
| **164** | H | Me | H | OH | COOH | 6 | 7.0 |
| **172** | Et | Me | H | OH | COOH | 3.3 | 1.0 |
| **173** | OMe | Me | H | OH | COOH | 23 | 3.5 |
| **174** | OEt | Me | H | OH | COOH | 7.5 | 6.0 |
| **175** | H | Me | H | OMe | COOH | 18 | 16.7 |
| **176** | Et | Me | H | OMe | COOH | 4.3 | 8.5 |
| **177** | H | Me | H | OMe | COOMe | 8.5 | 6.5 |
| **178** | Et | Me | H | OMe | COOMe | 2.5 | 1.2 |
| **179** | H | Me | H | OH | $CH_2OH$ | 7 | 9.5 |
| **180** | Et | Me | H | OH | $CH_2OH$ | 12 | 12.8 |
| **181** | Et | Me | H | OMe | $CH_2OH$ | -- | 0.8 |
| **182** | Et | Me | Me | OH | COOH | 8.5 | 10.4 |
| **183** | Et | Et | H | OH | COOH | 5.1 | 5.4 |

[a]See Table 4-1.

To examine human tumor-tissue-type selectivity, active compounds **172**, **173**, **178**,

and **181** ($ED_{50}$ values >4.0 µg/mL were considered not active) were selected for testing

against a limited but diverse set of human cancer cell lines, using **91** as a positive control and "gold-standard" (Table 4-3). Compounds **172** and **173** were active only against certain breast cancer cell lines and not active against other tumor tissue cells tested, such as A549 lung cancer or DU145 prostate cancer cell lines. Thus, these two compounds had high tissue selectivity. More interestingly, **172** and **173** also showed high potency (ED$_{50}$ 0.3 and 0.6 µg/mL, respectively) and selectivity toward the ZR-7-51 (ER+, HER2+) cell line. Compound **172** was two times less potent against SK-BR-3 (HER2+) (ED$_{50}$ 0.9 µg/mL) and ten times less potent against MCF-7 (ER+) (ED$_{50}$ 3.3 µg/mL). Compound **173** was six times less potent against SK-BR-3 (HER2+) (ED$_{50}$ 3.5 µg/mL) and 33 times less potent against MCF-7 (ER+) (ED$_{50}$ >20 µg/mL). Meanwhile, compound **91** showed similar potency against ZR-7-51 and SK-BR-3 and was only three times less potent against MCF-7 than ZR-7-51. In summary, **172** and **173** were more potent against ZR-75-1 than cell lines over-expressing either ER or HER2 (MCF-7 or SK-BR-3), and much more potent than cell lines not over-expressing ER or HER2 (remaining cell lines in the panel). More importantly, **181** showed extremely high selectivity against SK-BR-3 cell line (ED$_{50}$ 0.8 µg/mL) compared with other cell lines tested (eight- to eleven-fold difference). This high selectivity could be applied for further analogue design and mechanism of action study. Unexpectedly, compound **178** was active against all cancer cell lines tested.

**Table 4-3. Cytotoxicity of compounds against tumor cell lines[a]**

|  | MCF-7 | SK-BR-3 | ZR-75-1 | MDA MB-231 | A549 | DU145 | KB | KBvin |
|---|---|---|---|---|---|---|---|---|
| **91** | 0.2 | 0.1 | 0.1 | >10 | 10.6 | 15.9 | 13.1 | 13.2 |
| **172** | 3.3 | 1.0 | 0.3 | >10 | 10.6 | 8.7 | 9.1 | 7.0 |
| **173** | >20 | 3.5 | 0.6 | >10 | 10.1 | 8.2 | 9.7 | 8.9 |
| **178** | 2.5 | 1.2 | 1.3 | 2.3 | 1.5 | 2.2 | 1.7 | 1.3 |
| **181** | -- | 0.8 | 6.5 | 9.1 | 9.6 | 7.2 | 7.0 | 6.6 |

[a]See Table 4-1.

To further explore the physicochemical basis for the different selectivity profiles between **172** and **178**, dihedral energy analyses between the naphthalene and furan rings were performed over 360° (Figure 4-5). Compared with **91**, compound **172** retains three aromatic centers and possesses one additional intramolecular hydrogen bond. This hydrogen bond helps to 'lock' the conformation close to that of **91**, which may explain why the activity pattern of **172** is similar to that of **91**, but with increased selectivity. The narrow shape of the potential energy well for the dihedral angle between the naphthalene and furan rings implies that it is difficult to vary the angle from the minimum of -135° (Figure 4-5). Thus, the compound's structure is fairly rigid, leading to a small probability for **172** to bind to a diverse set of targets. Compound **178** also retains three aromatic centers and hydrogen bond acceptors, in common with the tetracyclic compound **91**. However, the intramolecular hydrogen bond found in **172** cannot form in **178**. The dihedral angle between the naphthalene ring and the furan ring is more flexible as seen in Figure 4-5.[15] In comparison to **172**, the potential energy surface around the minimum is much flatter and there are fewer energy barriers (Figure 4-5). As a result, the increased structural flexibility in **178** could permit multi-target interactions and account for the compound's observed broader activity spectrum.
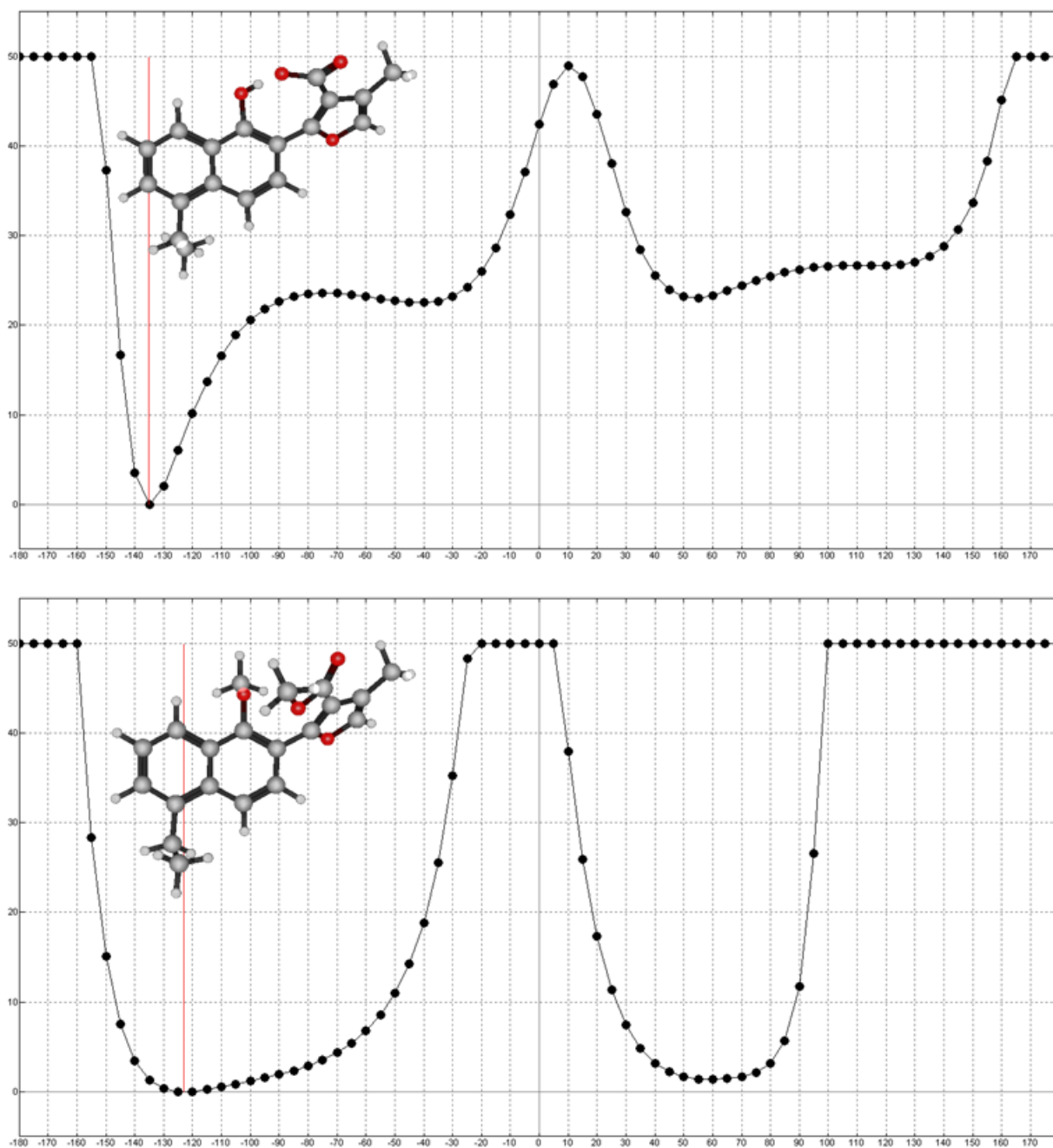
**Figure 4-5. Dihedral energy analyses of compounds between the naphthalene ring and the furan ring of 172 (Top panel) and 178 (Bottom panel). The structure of the global energy minimum is shown by a ball and stick model.**

## 4.5　Tetrahydronaphthalene-1-ol (TNO) Derivatives as Antitumor Agents

We further studied the effect of a non-aromatic ring-A in scaffold 2 on antitumor activity. Novel tetrahydronaphthalene-1-ol (TNO) derivatives were designed and eleven new analogues were synthesized as shown in Scheme 4-5. Compounds **152-154** were hydrolyzed by sodium hydroxide to give ring-opened compounds **184-186**. From a comparison of the activity data for **184-186** (Table 4-4), a *gem*-dimethyl group on ring-A seems to be important for activity. To study the functions of the hydroxyl and carboxylic acid groups of **186**, analogues **187-194** were designed and synthesized. Selective methylation of **186** generated methyl ether **187**, which was converted to methyl ester **188** through the same sequence as for the synthesis of **177** and **178**.[11] Meanwhile, reduction of **154** with lithium aluminum hydride afforded diol **189**, which was treated with iodomethane and iodoethane in the presence of $Cs_2CO_3$ to generate phenylethers **190** and **191**, respectively. Alkylation of the remaining primary alcohol was accomplished with iodomethane and iodoethane in the presence of NaH to give **192** and **193**, respectively. Acetate **194** was obtained by acetylation of **190** with $Ac_2O$.

**Scheme 4-5. Synthetic pathway to tetrahydronaphthalene-1-ol derivatives 184-194**
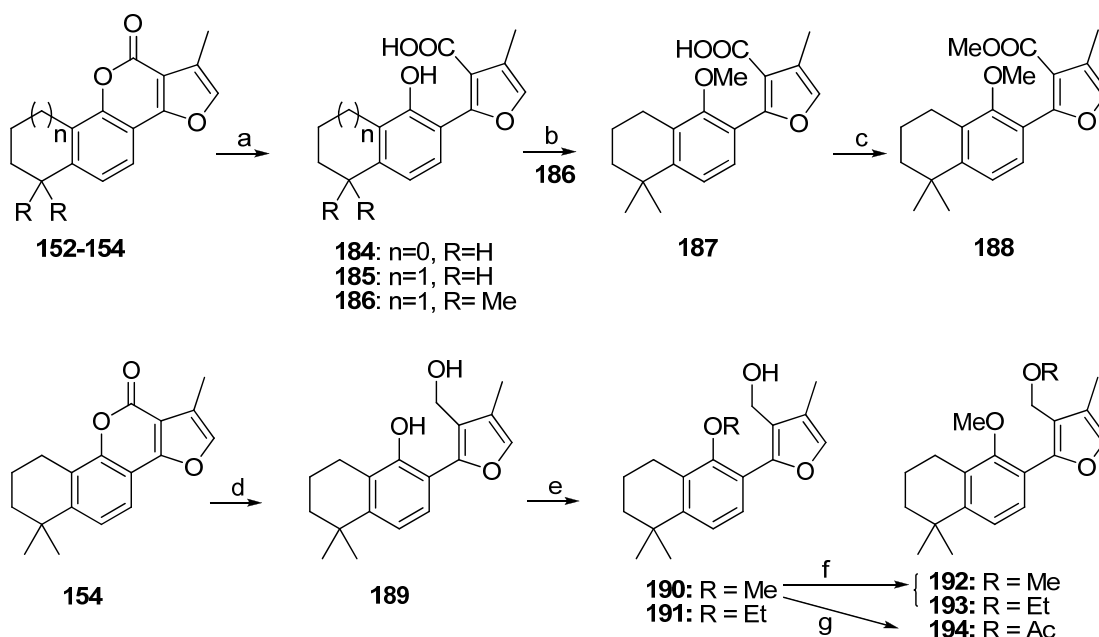
Reagents and conditions: (a) 5% NaOH (aq), reflux; (b) MeI, NaOH, 18-crown-6, $CH_3CN$, 90 °C; (c) $SOCl_2$, MeOH, rt; (d) $LiAlH_4$, THF; (e) MeI or EtI, $Cs_2CO_3$, acetone, 50 °C; (f) MeI or EtI, NaH, THF, rt; (g) $Ac_2O$, $Et_3N$, DMAP, $CH_2Cl_2$.

The newly synthesized analogues **184-194** were tested *in vitro* for their cytotoxic activity against a panel of human tumor cell lines: SK-BR-3 (estrogen receptor negative, HER2 over-expressing breast cancer), ZR-75-1 (estrogen receptor positive breast cancer), MDA-MB-231 (estrogen receptor negative breast cancer), A549 (non-small cell lung cancer), DU145 (prostate cancer cell line), KB (nasopharyngeal carcinoma), KB-vin (vincristine-resistant KB subline).

Tetrahydronaphthalene-1-ol (TNO) derivatives **184** and **185** displayed only weak antitumor activity, while **186** showed potent and broad antitumor activity against the tumor cell line panel ($ED_{50}$ 0.23 µg/mL against SK-BR-3 cell line; 0.49 µg/mL against ZR-75-1 cell line). The results demonstrated that analogues with a non-aromatic six-membered ring-A and *gem*-dimethyl substitution could retain significant activity. As to the tumor-tissue-type selectivity, **186** was highly active against all tumor cell lines tested ($ED_{50}$ 0.23–1.44 µg/mL), except MDA-MB-231 ($ED_{50}$ 7.42 µg/mL), while **91** and **172** were selectively active against certain breast cancer cell lines. These results demonstrated that introduction of a non-aromatic ring-A could greatly influence the selective antitumor activity, most likely by changing the molecular conformation and orientation. In our prior SAR studies of neo-tanshinlactone (**91**) and the ring-opened analogue **172**, the presence of two functional groups from the opened lactone ring-C was critical to the antitumor activity, which encouraged us to study additional derivatives of **186**. We synthesized a series of analogues with ether and ester substituents of various sizes. As seen in Table 4-4, **187-194** showed only weak to marginal activity against all tumor cell lines tested, which led to low selectivity ratios between MDA-MB-231 and other tumor cell lines. For example, **191** and **194** showed

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only four-fold higher potency against SK-BR-3 than MDA-MB-231, while **186** was 33-fold more potent against SK-BR-3 than MDA-MB-231. Thus, the current SAR study indicated that the optimal substituent combination is hydroxyl and carboxylic acid. The preliminary results indicated that the combination of *gem*-dimethylated ring-A, hydroxyl, and carboxylic acid is important to antitumor activity and selectivity. More analogues will be synthesized and evaluated to establish detailed SAR of this new compound series.

**Table 4-4. Cytotoxicity of compounds 184-194 against tumor cell lines[a]**

| Compd | SK-BR-3 | ZR-75-1 | MDA-MB-231 | A549 | DU145 | KB | KB-vin |
|-------|---------|---------|------------|------|-------|------|--------|
| **91** | 0.1 | 0.1 | >10 | 10.6 | 15.9 | 13.1 | 13.2 |
| **172** | 1.0 | 0.3 | >10 | 10.6 | 8.7 | 9.1 | 7.0 |
| **184** | 7.8 | 7.8 | 11.5 | 10.7 | 9.9 | 8.1 | 7.8 |
| **185** | 8.79 | 5.87 | 20 | 15.52 | 11.02 | 13.64 | 7.95 |
| **186** | **0.23** | **0.49** | 7.42 | **0.97** | **0.87** | **1.39** | **1.44** |
| **187** | 8.0 | 13.0 | >20 | 16.8 | 10.7 | 13.6 | 15 |
| **188** | 8.9 | 11.3 | 20 | 20 | 11 | 17.5 | 17.1 |
| **189** | 6.1 | 7.3 | 11.3 | 7 | 6.5 | 6.25 | 5.7 |
| **190** | 7.1 | 4.5 | 8.5 | 7 | 6.5 | 7.6 | 6.5 |
| **191** | 3.1 | 4.5 | 12.5 | 7.2 | 5.5 | 5.3 | 4.8 |
| **192** | 5.8 | 4.5 | 15.0 | 6.1 | 5.5 | 3.6 | 6.3 |
| **193** | 6.5 | 6.5 | 14.0 | 7.5 | 5.8 | 7.1 | 5.4 |
| **194** | 4.8 | 5.3 | 19.0 | 9.8 | 5.7 | 8.7 | 8.5 |

[a]See Table 4-1.

## 4.6    Conclusion

In summary, current data have led to new developments and insights about neo-tanshinlactone-based compounds that are active and selective against breast cancer cell

lines. We demonstrated that rings A and D were important for the activity. Importantly, we discovered that the lactone ring C could be opened through hydrolysis of the ester bond, while keeping the desired biological activity. A new class of active C-ring opened compounds, 2-(furan-2-yl) naphthalen-1-ol derivatives, was subsequently developed. Compounds **172-173** and **181** exhibited much higher selectivity against certain breast cancer cell lines than neo-tanshinlactone analogue **91**. In addition, compound **178** was active against all cell lines tested, suggesting a different mechanism of action from its structural derivatives. Conformational and dihedral energy analyses of **172** and **178** suggested that intramolecular hydrogen bonding was important to form a rigid conformation and improved the *in vitro* anticancer selectivity of **172**. We also discovered another novel class of antitumor agents, tetrahydronaphthalene-1-ol (TNO) derivatives. Compound **186** was the most potent analogue with $ED_{50}$ 0.23 µg/mL against SK-BR-3 cell line and showed broader antitumor activity compared with **91** and **172**. Overall, these results establish two new scaffolds as promising structures for the development of promising anti-breast cancer agents.

## 4.7    Experimental Section

### 4.7.1   Chemistry

**Materials and Methods**. Melting points were measured with a Fisher Johns melting apparatus without correction. ^{1}H NMR spectra were measured on a 300 MHz Varian Gemini 2000 spectrometer using TMS as internal standard. The solvent used was $CDCl_3$ unless indicated. Mass spectra were measured on a Shimadzu LC-MS2010 instrument. Thin-layer chromatography (TLC) and preparative TLC were performed on precoated silica gel GF plates purchased from Merck, Inc. Biotage Flash+ or Isco Companion systems were used for flash chromatography. Silica gel (200-400mesh) from Aldrich, Inc. was used for column chromatography. All other chemicals were obtained from Aldrich, Inc, and Fisher, Inc.

**7-Methoxy-3-methyl-4*H*-furo[3,2-*c*]chromen-4-one (161).** To a solution of **195** (199 mg, 1.04 mmol) in toluene (9 mL) was added a mixture of HOAc (0.30 mL, 5.20 mmol) and NH$_4$OAc (400 mg, 5.20 mmol) in EtOH (3 mL) and chloroacetone (0.42 mL, 5.20 mmol). The mixture was refluxed for 24 h. After cooling, the mixture was diluted with H$_2$O and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The residue was purified by column chromatography to give **161** as a white solid. 65% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, *J* = 1.2 Hz, 3H, C*H$_3$*), 3.88 (s, 3H, OC*H$_3$*), 6.88-6.93 (m, 2H, aromatic), 7.33 (d, *J* = 1.5 Hz, 1H, OC*H*), 7.71-7.74 (m, 1H, aromatic).

**7-Hydroxy-3-methyl-4*H*-furo[3,2-*c*]chromen-4-one (160).** To a solution of **161** (46 mg, 0.2 mmol) in DCM (3 mL) was added BBr$_3$ (0.6 mL, 0.6 mmol) dropwise at 0 °C. The reaction mixture was refluxed for 3 h. Water was added to quench the reaction. The solution was extracted with CHCl$_3$, concentrated, and purified with column chromatography. 42% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.88 (d, *J* = 1.2 Hz, 3H, C*H$_3$*), 6.81-6.88 (m, 2H, aromatic), 7.53 (d, *J* = 1.2 Hz, 1H, OC*H*), 7.72 (d, *J* = 8.7 Hz, 1H, aromatic).

**General procedure for synthesis of 162-163.** Compound **160** (0.2 mmol) was dissolved in DMF (1 mL) and acetone (3 mL). Cesium carbonate (195 mg, 0.6 mmol) and 2-iodopropane or 1-bromohexane (0.6 mmol) were added to the above solution. The reaction mixture was stirred at rt for 12 h.  After removal of solvent in vacuo, the residue was purified by column chromatography to give **162** or **163** as a white solid.

**7-Isopropoxy-3-methyl-4*H*-furo[3,2-*c*]chromen-4-one (162).** 30 % yield; mp 85-87°C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.38 (d, *J* = 6.0 Hz, 6H, CH(C*H$_3$*)$_2$), 2.34 (s, 3H, C*H$_3$*), 4.61 (h, *J* = 6.0 Hz, 1H, C*H*), 6.86-6.90 (m, 2H, aromatic), 7.33 (s, 1H, OC*H*), 7.71 (d, *J* = 8.4 Hz, 1H, aromatic); HRMS Calcd for C$_{15}$H$_{15}$O$_4$ (M+H$^+$): 259.0965, found: 259.0961.

**7-(Hexyloxy)-3-methyl-4*H*-furo[3,2-*c*]chromen-4-one (163).** 29% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 0.92 (t, *J* = 6.9 Hz, 3H, (CH$_2$)$_5$C*H$_3$*), 1.33-1.51 (m, 6H, (C*H$_2$*)$_3$), 1.81 (p, *J* = 6.6 Hz, 2H, OCH$_2$C*H$_2$*), 2.34 (d, *J* = 0.9 Hz, 3H, C*H$_3$*), 4.01 (t, *J* = 6.6 Hz, 2H,

OC$H_2$), 6.88-6.91 (m, 2H, aromatic), 7.28 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.71 (d, $J$ = 9.3 Hz, 1H, aromatic).

**General procedure for synthesis of 165-169.** Thionyl chloride (0.08 mL, 1.20 mmol) was added to the carboxylic acid (1.00 mmol) in CH$_2$Cl$_2$ (3 mL) and DMF (0.1 mL), and the mixture was refluxed under nitrogen atmosphere for 1 h. After cooling to rt, the mixture was concentrated in vacuo to give the corresponding acid chloride as a pale yellow solid, which was used directly in the next step. Naphthalen-1-ol or 5-methylnaphthalen-1-ol (1.00 mmol) was dissolved in THF (5 mL), then DMAP (5 mg) and ethyldiisopropylamine (0.18 mL, 1.02 mmol) were added, and the mixture was cooled to 0 °C for 10 min. Freshly prepared acid chloride in dry THF (10 mL) was added to the mixture via cannula, and the resulting mixture was stirred at 25 °C for 2 h, diluted with diethyl ether (150 mL), and quenched by the addition of water (15 mL). The organic layer was washed with HCl and NaHCO$_3$ and then dried (Na$_2$SO$_4$) and concentrated in vacuo. The residue was purified with flash chromatography eluting with hexane: EtOAc=10:1 to give **165-169**.

**Naphthalen-1-yl furan-3-carboxylate (165).** 87% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 6.96 (d, $J$ = 1.8 Hz, 1H, aromatic), 7.33 (dd, $J$ = 1.5, 7.5 Hz, 1H, aromatic), 7.46-7.55 (m, 4H, aromatic), 7.76 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.86-7.92 (m, 2H, aromatic), 8.32 (t, $J$ = 0.9 Hz, 1H, aromatic).

**5-Methylnaphthalen-1-yl furan-3-carboxylate (166).** 46% yield; mp 53-55°C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.72 (s, 3H, C$H_3$), 6.97 (dd, $J$ = 0.6, 5.2 Hz, 1H, aromatic), 7.33-7.42 (m, 3H, aromatic), 7.51-7.56 (m, 2H, aromatic), 7.79 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.93 (d, $J$ = 8.4 Hz,1H, aromatic), 8.32-8.33 (m, 1H, aromatic). HRMS Calcd for C$_{16}$H$_{13}$O$_3$ (M+H$^+$): 253.0859, found: 253.0869.

**Naphthalen-1-yl 3-methylbenzoate (167).** 67% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.49 (s, 3H, C$H_3$), 7.36 (dd, $J$ = 0.9, 8.1 Hz, 1H, aromatic), 7.4-7.55 (m, 5H,

aromatic), 7.79 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.89-7.95 (m, 2H, aromatic), 8.13-8.15 (m, 1H, aromatic).

**Naphthalen-1-yl 2-bromo-4-methylbenzoate (168).** 96% yield; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.41 (s, 3H, C$H_3$), 7.27 (dd, $J$ = 0.9, 8.1 Hz, 1H, aromatic), 7.40 (dd, $J$ = 1.2, 7.5 Hz, 1H, aromatic), 7.48-7.52 (m, 3H, aromatic), 7.60 (d, $J$ = 0.9 Hz, 1H, aromatic), 7.77 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.87-7.90 (m, 1H, aromatic), 7.96-7.99 (m, 1H, aromatic), 8.13 (d, $J$ = 8.1 Hz, 1H, aromatic).

**Naphthalen-1-yl benzo[*d*][1,3]dioxole-5-carboxylate (169).** 76% yield; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 6.11 (s, 2H, C$H_2$), 6.96 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.35 (dd, $J$ = 0.9, 7.8 Hz, 1H, aromatic), 7.48-7.54 (m, 3H, aromatic), 7.74 (d, $J$ = 1.5 Hz, 1H, aromatic), 7.78 (d, $J$ = 8.1 Hz, 1H, aromatic), 7.88-7.93 (m, 2H, aromatic), 7.96 (dd, $J$ = 1.5, 8.1 Hz, 1H, aromatic).

**4-(Prop-2-ynyloxy)-2*H*-benzo[*h*]chromen-2-one (170).** To a mixture of **132** (212 mg, 1.00 mmol mmol), K$_2$CO$_3$ (300 mg, 2.17 mmol) in acetone (8 mL) was added 3-bromoprop-1-yne (0.17 mL, 1.50 mmol). The mixture was refluxed 12 h. After cooling, the mixture was filtered, concentrated, diluted with H$_2$O and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The residue was purified by column chromatography to give **170** as a light yellow solid. 40% yield; mp 205-207 °C; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.70 (t, $J$ = 2.4 Hz, 1H, CC$H$), 4.92 (d, $J$ = 2.4 Hz, 2H, OC$H_2$), 5.93 (s, 1H, COC$H$), 7.62-7.71 (m, 3H, aromatic), 7.80-7.90 (m, 2H, aromatic), 8.54-8.58 (m, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{11}$O$_3$ (M+H$^+$): 251.0703, found: 251.0697.

***O*-2-oxo-2*H*-benzo[*h*]chromen-4-yl dimethylcarbamothioate (171).** 4-Hydroxy-2*H*-benzo[*h*]chromen-2-one (7.5 mmol) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (9.75 mmol, 1.3 equiv) were heated in *N*-methylpyrrolidone (NMP) (6 mL) to 50 °C to give a dark brown solution. Dimethylthiocarbamoyl chloride (8.25 mmol, 1.1 equiv) was dissolved in NMP (1.5 mL) and added dropwise to the previous solution over 18 min. Some fine precipitate formed

in the dark red solution during this addition. The reaction was monitored by LC and was complete within 90 min at 50 °C. Water was added over 15 min at 50 °C. The original solid dissolved readily, but a yellow precipitate formed later in the addition, which persisted to the end. The reaction mixture was cooled to 20 °C and the precipitate isolated by filtration. The product cake was washed twice with water and dried in vacuo at 50 °C to yield the title compound as a solid. 85% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 3.46 (s, 3H, N(C$H_3$)$_2$), 3.52 (s, 3H, N(C$H_3$)$_2$), 6.30 (s, 1H, COC$H$), 7.51 (d, $J$ = 9.0 Hz, 1H, aromatic), 7.64-7.70 (m, 3H, aromatic), 7.86-7.90 (m, 1H, aromatic), 8.56-8.59 (m, 1H, aromatic).

**General procedure for synthesis of 164, 172-174, 182-183, 184-186.** Lactone **91**-**92, 110-111, 136, 138, 152-154** (0.1 mmol) was refluxed in ethanolic aqueous potassium hydroxide (5%; 5 mL) for 3.5 h. Then the reaction mixture was cooled and quenched by pouring into ice, acidified with 6N HCl, and extracted with CHCl$_3$. Removal of solvent, drying (Na$_2$SO$_4$), and chromatographic purification (DCM-MeOH) gave the hydrolyzed product as a light yellow solid.

**2-(1-Hydroxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (164).** 93% yield; mp 194-196 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 2.27 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 7.51-7.55 (m, 5H, aromatic & OC$H$), 7.86-7.89 (m, 1H, aromatic), 8.40-8.44 (m, 1H, aromatic). HRMS Calcd for C$_{16}$H$_{11}$O$_4$ (M-H$^+$): 267.0657, found: 267.0663.

**2-(5-Ethyl-1-hydroxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (172).** 90% yield; mp 151-153 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 1.35 (t, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 2.25 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 3.08 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 7.35-7.37 (m, 2H, aromatic), 7.41 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.48 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.62 (d, $J$ = 9.0 Hz, 1H, aromatic), 8.24-8.28 (m, 1H, aromatic). HRMS Calcd for C$_{18}$H$_{15}$O$_4$ (M-H$^+$): 295.0976, found: 295.0972.

**2-(1-Hydroxy-5-methoxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (173).** 80% yield; mp 173-175 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 2.41 (s, 3H, C$H_3$), 4.00

(s, 3H, OC$H_3$), 6.98 (d, $J$ = 7.5 Hz, 1H, aromatic), 7.38 (t, $J$ = 8.1 Hz, 1H, aromatic), 7.51 (d, $J$ = 9.0 Hz, 1H, aromatic), 7.64 (s, 1H, OC$H$) 7.78 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.94 (d, $J$ = 8.7 Hz, 1H, aromatic). HRMS Calcd for $C_{17}H_{13}O_5$ (M-H$^+$): 297.0768, found: 297.0765.

**2-(5-Ethoxy-1-hydroxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (174).** 56% yield; mp 208-210 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 1.51 (t, $J$ = 7.8 Hz, 3H, CH$_2$C$H_3$), 2.33 (s, 3H, C$H_3$), 4.23 (q, $J$ = 6.9 Hz, 2H, C$H_2$CH$_3$), 6.97 (d, $J$ = 7.8 Hz, 1H, aromatic), 7.38 (t, $J$ = 8.7 Hz, 1H, aromatic), 7.48 (d, $J$ = 8.7 Hz, 1H), 7.59 (s, 1H, OC$H$), 7.82 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.82 (d, $J$ = 8.7 Hz, 1H, aromatic). HRMS Calcd for $C_{18}H_{15}O_5$ (M-H$^+$): 311.0919, found: 311.0940.

**2-(5-Ethyl-1-hydroxynaphthalen-2-yl)-4,5-dimethylfuran-3-carboxylic acid (182).** 66% yield; mp 158-160 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 1.35 (t, $J$ = 7.8 Hz, 3H, CH$_2$C$H_3$), 2.17 (s, 3H, C$H_3$), 2.26 (s, 3H, C$H_3$), 3.07 (q, $J$ = 7.8 Hz, 2H, C$H_2$CH$_3$), 7.32-7.38 (m, 2H, aromatic), 7.49 (d, $J$ = 9.0 Hz, 1H, aromatic), 7.60 (d, $J$ = 9.0 Hz, 1H, aromatic), 8.23-8.26 (m, 1H, aromatic). HRMS Calcd for $C_{19}H_{17}O_4$ (M-H$^+$): 309.1132, found: 309.1140.

**4-Ethyl-2-(5-ethyl-1-hydroxynaphthalen-2-yl)furan-3-carboxylic acid (183).** 90% yield; mp 153-155 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 1.24 (t, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 1.35 (t, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 2.78 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 3.07 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 7.34-7.36 (m, 2H, aromatic), 7.39 (s, 1H, OC$H$), 7.49 (d, $J$ = 9.3 Hz, 1H, aromatic), 7.60 (d, $J$ = 9.3 Hz, 1H, aromatic), 8.24-8.27 (m, 1H, aromatic). HRMS Calcd for $C_{19}H_{17}O_4$ (M-H$^+$): 309.1132, found: 309.1124.

**2-(4-Hydroxy-2,3-dihydro-1$H$-inden-5-yl)-4-methylfuran-3-carboxylic acid (184).** 90 % yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.06 (p, $J$ = 7.5 Hz, 2H, CH$_2$C$H_2$CH$_2$), 2.20 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 2.89 (q, $J$ = 7.5 Hz, 4H, C$H_2$CH$_2$C$H_2$), 4.94 (s, 1H, O$H$), 6.83 (d, $J$ = 7.8 Hz, 1H, aromatic), 7.14 (d, $J$ = 7.8 Hz, 1H, aromatic), 7.30 (d, $J$ = 0.9 Hz, 1H, OC$H$); MS: $m/z$ 257 (M-H$^+$).

**2-(1-Hydroxy-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3-carboxylic**

**acid (185).** 59% yield; mp 115-117 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 1.75-1.77

(m, 4H, C$H_2$), 2.20 (d, $J$ = 1.2 Hz, 3H, C$H_3$),  2.69-2.75 (m, 4H, C$H_2$) 6.67 (d, $J$ = 8.4 Hz, 1H,

aromatic), 7.10 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.43 (s, 1H, OC$H$); HRMS Calcd for C$_{16}$H$_{15}$O$_4$

(M-H$^+$): 271.0970, found: 271.0971.

**2-(1-Hydroxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3-**

**carboxylic acid (186).** 76% yield; mp 105-107 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 1.28

(s, 6H, C(C$H_3$)$_2$), 1.62-1.66 (m, 2H, C$H_2$), 1.78-1.82 (m, 2H, C$H_2$), 2.21(d, $J$ = 1.5 Hz, 3H,

C$H_3$),  2.70 (t, $J$ = 6.3 Hz, 2H, C$H_2$), 6.96 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.13 (d, $J$ = 8.4 Hz,

1H, aromatic), 7.33 (d, $J$ = 1.2 Hz, 1H, OC$H$); HRMS Calcd for C$_{18}$H$_{19}$O$_4$ (M-H$^+$): 301.1434,

found: 301.1425.

**General procedure for synthesis of methyl ethers.** Compound **91**, **92**, or **154** (0.1

mmol) was refluxed in ethanolic aqueous potassium hydroxide (5%; 5 mL) for 3.5 h. The

solution was washed with CHCl$_3$ and evaporated to give the dipotassium salt, which was

refluxed for 24 h with 18-crown-6 (4.35 mg, 0.0145 mmol) and methyl iodide (0.01 mL, 0.159

mmol) in acetonitrile (5 mL). The acetonitrile was removed in vacuo. After dilution with

CHCl$_3$, the mixture was washed with water (10 mL), dried, and concentrated to give an oily

residue. The residue was purified with flash chromatography, eluting with DCM:MeOH= 10:1,

to give the methyl ether derivative.

**2-(1-Methoxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (175).** 46% yield;

mp 118-120 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.25 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 3.74 (s,

3H, OC$H_3$), 7.35 (d, $J$ = 1.5 Hz, 1H, OC$H$), 7.47 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.51-7.54 (m,

2H, aromatic), 7.62 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.83-7.86 (m, 1H, aromatic), 8.18-8.22 (m,

1H, aromatic). HRMS Calcd for C$_{17}$H$_{13}$O$_4$ (M-H$^+$): 281.0814, found: 281.0827.

**2-(5-Ethyl-1-methoxynaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (176).** 39% yield; mp 128-130 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.37 (t, *J* = 7.8 Hz, 3H, CH$_2$C*H*$_3$), 2.46 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 3.09 (q, *J* = 7.8 Hz, 2H, C*H*$_2$CH$_3$), 3.73 (s, 3H, OC*H*$_3$), 7.35-7.52 (m, 4H, aromatic & OC*H*), 7.82 (d, *J* = 9.3 Hz, 1H, aromatic), 8.08 (d, *J* = 9.9 Hz, 1H, aromatic). HRMS Calcd for C$_{19}$H$_{17}$O$_4$ (M-H$^+$): 309.1127, found: 309.1139.

**2-(1-Methoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3-carboxylic acid (187).** 32 % yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.30 (s, 6H, (C*H*$_3$)$_2$), 1.63-1.67 (m, 2H, CCH$_2$C*H*$_2$CH$_2$), 1.77-1.83 (m, 2H, CC*H*$_2$CH$_2$CH$_2$), 2.36 (d, *J* = 0.9 Hz, 3H, C*H*$_3$), 2.76 (t, *J* = 6.3 Hz, 1H, CCH$_2$CH$_2$C*H*$_2$), 3.52 (s, 3H, OC*H*$_3$), 7.16 (d, *J* = 8.4 Hz, 1H, aromatic), 7.23 (d, *J* = 8.4 Hz, 1H, aromatic), 7.29 (d, *J* = 1.5 Hz, 1H, OC*H*); MS: *m/z* 315 (M+H$^+$).

**General procedure for synthesis of methyl esters.** Thionyl chloride (0.05 mL, 0.45 mmol) was added dropwise at 0 °C to a solution of **175**, **176**, or **187** (0.14 mmol) in MeOH (15 mL). The solution was then stirred at rt for 12 h. The solvent was evaporated, and the residue was dissolved in EtOAc and washed successively with saturated aqueous NaHCO$_3$ and brine. The organic phase was dried over Na$_2$SO$_4$ and evaporated under reduced pressure to give the methyl ester derivative.

**Methyl 2-(1-methoxynaphthalen-2-yl)-4-methylfuran-3-carboxylate (177).** 32% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.25 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 3.69 (s, 3H, OC*H*$_3$), 3.71 (s, 3H, COOC*H*$_3$), 7.35 (d, *J* = 1.5 Hz, 1H, OC*H*), 7.50-7.57 (m, 3H, aromatic), 7.64 (d, *J* = 8.4 Hz, 1H, d, *J* = 1.5 Hz, 1H, OC*H*), 7.84-7.88 (m, 1H, aromatic), 8.19-8.23 (m, 1H, aromatic). HRMS Calcd for C$_{18}$H$_{15}$O$_4$ (M-H$^+$): 297.1121, found: 297.1110.

**Methyl 2-(5-ethyl-1-methoxynaphthalen-2-yl)-4-methylfuran-3-carboxylate (178).** 82% yield; mp 121-123 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.39 (t, *J* = 7.8 Hz, 3H, CH$_2$C*H*$_3$), 2.52 (s, 3H, C*H*$_3$), 3.11 (q, *J* = 7.5 Hz, 1H, C*H*$_2$CH$_3$), 3.68 (s, 3H, OC*H*$_3$), 3.69 (s, 3H, COOC*H*$_3$), 7.34-7.49 (m, 3H, aromatic & OC*H*), 7.54 (d, *J* = 8.7 Hz, 1H, aromatic), 7.85

(d, *J* = 8.7 Hz, 1H, aromatic), 8.10 (d, *J* = 8.4 Hz, 1H, aromatic). HRMS Calcd for $C_{20}H_{19}O_4$ (M-H$^+$): 325.1434, found: 325.1430.

**Methyl 2-(1-methoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methyl furan-3-carboxylate (188).** 23 % yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.30 (s, 6H, (C*H*$_3$)$_2$), 1.63-1.67 (m, 2H, CC*H*$_2$CH$_2$CH$_2$), 1.77-1.83 (m, 2H, CCH$_2$C*H*$_2$CH$_2$), 2.20 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 2.75 (t, *J* = 6.3 Hz, 1H, CCH$_2$CH$_2$C*H*$_2$), 3.46 (s, 3H, OC*H*$_3$), 3.72 (s, 3H, COOC*H*$_3$), 7.14 (d, *J* = 8.1 Hz, 1H, aromatic), 7.23 (d, *J* = 8.1 Hz, 1H, aromatic),7.27 (d, *J* = 0.9 Hz, 1H, OC*H*); MS: *m/z* 329 (M+H$^+$).

**General procedure for synthesis of 179-180, and 189.** LiAlH$_4$ (60 mg, 16 mmol) was added at 0 °C to a solution of **91**, **92**, or **154** (0.1 mmol) in THF (5 mL). The solution was then refluxed for 5 h. Then the reaction mixture was cooled and quenched by pouring into ice, acidified with 2N HCl, and extracted with diethyl ether: DCM= 2:1 (3×5 mL). The solvent was evaporated after drying (Na$_2$SO$_4$). Chromatographic purification (hexane: EtOAc= 4:1) gave the product as a white solid.

**2-(3-(Hydroxymethyl)-4-methylfuran-2-yl)naphthalen-1-ol (179).** 87% yield; mp 95-97 °C; $^1$H NMR (300 MHz, CD$_3$COCD$_3$, ppm): δ 2.13 (s, 3H, C*H*$_3$), 4.58 (s, 2H, C*H*$_2$OH), 7.45-7.56 (m, 5H, aromatic & OC*H*), 7.83-7.87 (m, 1H, aromatic), 8.33-8.36 (m, 1H, aromatic); HRMS Calcd for $C_{16}H_{13}O_3$ (M-H$^+$): 253.0870, found: 253.0863.

**5-Ethyl-2-(3-(hydroxymethyl)-4-methylfuran-2-yl)naphthalen-1-ol  (180).** 95% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.38 (t, *J* = 7.5 Hz, 3H, CH$_2$C*H*$_3$), 2.14 (s, 3H, C*H*$_3$), 3.08 (q, *J* = 7.8 Hz, 2H, C*H*$_2$CH$_3$), 4.64 (s, 2H, C*H*$_2$OH), 7.35-7.44 (m, 3H, aromatic), 7.54 (d, *J* = 8.7 Hz, 1H, aromatic), 7.64 (d, *J* = 9.3 Hz, 1H, aromatic), 7.99 (br, 1H, OH), 8.25 (d, *J* = 8.1 Hz, 1H, aromatic). HRMS Calcd for $C_{18}H_{17}O_3$ (M-H$^+$): 281.1183, found: 281.1197.

**2-(3-(Hydroxymethyl)-4-methylfuran-2-yl)-5,5-dimethyl-5,6,7,8-tetrahydronaph thalene-1-ol (189).** 93% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.30 (s, 6H, (C*H*$_3$)$_2$), 1.63-1.67 (m, 2H, CC*H*$_2$CH$_2$CH$_2$), 1.80-1.84 (m, 2H, CCH$_2$C*H*$_2$CH$_2$), 2.11 (d, *J* = 0.9 Hz, 3H, C*H*$_3$),

2.71 (t, $J$ = 6.3 Hz, 2H, CC$H_2$CH$_2$C$H_2$), 4.58 (s, 1H, C$H_2$OH), 6.97 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.20 (d, $J$ = 8.4 Hz, 1H, aromatic), 7.28 (d, $J$ = 0.9 Hz, 1H, OC$H$); MS: $m/z$ 385 (M-H$^+$).

**General procedure for synthesis of 181, and 190-191.** Compound **180** or **189** (0.1 mmol) was dissolved in acetone. Methyl iodide or ethyl iodide (0.3 mmol) and potassium carbonate (for **180**) or cesium carbonate (for **189**) (1.0 mmol) were added to the above solution. The mixture was stirred overnight and purified with flash chromatography to obtain the ethers.

**(2-(5-Ethyl-1-methoxynaphthalen-2-yl)-4-methylfuran-3-yl)methanol (181).** 79% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.39 (t, $J$ = 7.5 Hz, 3H, CH$_2$C$H_3$), 2.17 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 2.75 (t, $J$ = 6.3 Hz, 1H, O$H$), 3.12 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 3.70 (s, 3H, OC$H_3$), 4.48 (d, $J$ = 6.0 Hz, 2H, C$H_2$OH), 7.37 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.40 (d, $J$ = 6.3 Hz, 1H, aromatic), 7.49 (dd, $J$ = 7.2, 8.1 Hz, 1H, aromatic), 7.55 (d, $J$ = 8.7 Hz, 1H, aromatic), 7.90 (d, $J$ = 9.0 Hz, 1H, aromatic), 8.09 (d, $J$ = 8.4 Hz, 1H, aromatic); HRMS Calcd for C$_{19}$H$_{21}$O$_3$ (M+H$^+$): 297.1485, found: 297.1470.

**(2-(1-methoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3-yl)methanol (190).** 100 % yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.30 (s, 6H, (C$H_3$)$_2$), 1.64-1.68 (m, 2H, CC$H_2$CH$_2$CH$_2$), 1.77-1.83 (m, 2H, CCH$_2$C$H_2$CH$_2$), 2.12 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 2.69 (t, $J$ = 6.3 Hz, 1H, CH$_2$O$H$), 2.77 (t, $J$ = 6.3 Hz, 2H, CCH$_2$CH$_2$C$H_2$), 3.46 (s, 3H, OC$H_3$), 4.41 (d, $J$ = 5.7 Hz, 2H, C$H_2$OH), 7.16-7.22 (m, 2H, aromatic), 7.27 (d, $J$ = 0.9 Hz, 1H, OC$H$); MS: $m/z$ 323 (M+Na$^+$).

**(2-(1-Ethoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3-yl)methanol (191).** 94 % yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.18 (t, $J$ = 7.2 Hz, 3H, CH$_2$C$H_3$), 1.30 (s, 6H, (C$H_3$)$_2$), 1.64-1.68 (m, 2H, CC$H_2$CH$_2$CH$_2$), 1.77-1.83 (m, 2H, CCH$_2$C$H_2$CH$_2$), 2.12 (d, $J$ = 0.9 Hz, 3H, C$H_3$), 2.76 (t, $J$ = 6.3 Hz, 1H, CCH$_2$CH$_2$C$H_2$), 2.87

(br, 1H, C*H*₂OH), 3.58 (q, *J* = 7.2 Hz, 2H, C*H*₂CH₃), 4.39 (s, 2H, C*H*₂OH), 7.18 (s, 2H, aromatic), 7.26 (d, *J* = 0.3 Hz, 1H, OC*H*); MS: *m/z* 313 (M-H⁺).

**General procedure for synthesis of 192-193.** Compound **190** or **191** (0.05 mmol) was dissolved in THF. Methyl iodide (0.5 mmol) and NaH (1.0 mmol) were added to the above solution. The mixture was stirred for 2 h and then poured into ice water. The solution was acidified and extracted with CH₂Cl₂. The organic phase was dried (Na₂SO₄) and concentrated. The residue was purified with flash chromatography eluting with hexane: EtOAc=10:1 to give **192-193**.

**2-(1-Methoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-3-(methoxymethyl) -4-methylfuran (192).** 44 % yield; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.30 (s, 6H, (C*H*₃)₂), 1.64-1.67 (m, 2H, CC*H*₂CH₂CH₂), 1.77-1.83 (m, 2H, CCH₂C*H*₂CH₂), 2.10 (d, *J* = 1.2 Hz, 3H, C*H*₃), 2.77 (t, *J* = 6.3 Hz, 1H, CCH₂CH₂C*H*₂), 3.33 (s, 3H, CH₂OC*H*₃), 3.49 (s, 3H, OC*H*₃), 4.32 (s, 2H, C*H*₂OCH₃), 7.17 (dd, *J* = 8.4 Hz, 2H, aromatic), 7.28 (d, *J* = 1.2 Hz, 1H, OC*H*); MS: *m/z* 315 (M+H⁺).

**2-(1-Ethoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-3-(methoxymethyl)- 4-methylfuran (193).** 61 % yield; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.19 (t, *J* = 7.2 Hz, 3H, CH₂C*H*₃), 1.30 (s, 6H, (C*H*₃)₂), 1.64-1.67 (m, 2H, CC*H*₂CH₂CH₂), 1.77-1.83 (m, 2H, CCH₂C*H*₂CH₂), 2.09 (d, *J* = 0.9 Hz, 3H, C*H*₃), 2.77 (t, *J* = 6.3 Hz, 1H, CCH₂CH₂C*H*₂), 3.32 (s, 3H, CH₂OC*H*₃), 3.59 (q, *J* = 6.9 Hz, 2H, C*H*₂CH₃), 4.32 (s, 2H, C*H*₂OCH₃), 7.16 (dd, *J* = 8.1 Hz, 2H, aromatic), 7.26 (d, *J* = 0.9 Hz, 1H, OC*H*); MS: *m/z* 329 (M+H⁺).

**(2-(1-Methoxy-5,5-dimethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-methylfuran-3- yl)methyl acetate (194).** Compound **190** (0.05 mmol) was dissolved in CH₂Cl₂. Acetic anhydride (0.50 mmol), Et₃N (0.65 mmol), and DMAP (5 mg) were added to the above solution. The mixture was stirred overnight and purified with flash chromatography to obtain the ester. 82 % yield; ¹H NMR (300 MHz, CDCl₃, ppm): δ 1.30 (s, 6H, (C*H*₃)₂), 1.63-1.67 (m, 2H, CC*H*₂CH₂CH₂), 1.77-1.83 (m, 2H, CCH₂C*H*₂CH₂), 2.06 (s, 3H, CH₂OCOC*H*₃), 2.06 (d, *J*

= 0.9 Hz, 3H, C$H_3$), 2.76 (t, $J$ = 6.3 Hz, 1H, CC$H_2$C$H_2$C$H_2$), 3.48 (s, 3H, OC$H_3$), 5.01 (s, 2H,

C$H_2$OCOC$H_3$), 7.13-7.19 (m, 2H, aromatic), 7.30 (d, $J$ = 1.2 Hz, 1H, OC$H$); MS: $m/z$ 365

(M+Na$^+$).

### 4.7.2 Biological Assay.

The assay methods and description of cell lines are described in Chapter 3.

### 4.7.3 Computational Methods

A conformational analysis was conducted to study the molecular geometries of compounds **162**, **164**, **166**, and **170** and their potential SAR relevance. The X-ray structure (CSD Refcode: VOBVIR) of 8-ethyl-4,10,12-trimethoxy-6*H*-benzo(*d*)naphtho(1,2-*b*)pyran-6-one was obtained by searching the Cambridge Structural Database version 5.29 and employed as the template by which compounds **162**, **164**, **166**, and **170** were aligned to obtain their starting conformations.[16] Systematic conformational searches were then carried out for compounds **162**, **164**, **166**, and **170**. The lowest energy conformers were further minimized by PM3 Hamiltonian. The final structures were rendered for pharmacophore elucidation and dihedral energy analysis. All calculations were conducted using the MOE 2007.09 package.

**4.8    References**

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# CHAPTER 5

# NOVEL SUBSTITUTUED 6-PHENYL-4*H*-FURO[3,2-*C*]PYRAN-4-ONE DERIVATIVES AS POTENT AND HIGHLY SELECTIVE ANTI-BREAST CANCER AGENTS

## 5.1    Introduction

In the previous chapter, we reported a study on how the individual A, C, and D rings of neo-tanshinlactone (**90**) analogues influence *in vitro* anti-breast cancer activity.[1] The results revealed that 2-(furan-2-yl)-naphthalen-1-ol derivatives (e.g., **172**),[1] in which ring-C of **90** is missing, are a new class of potent and selective anti-breast cancer agents. These results encouraged us to further simplify the scaffold of **90** and develop novel analogues as potential anti-breast cancer agents.
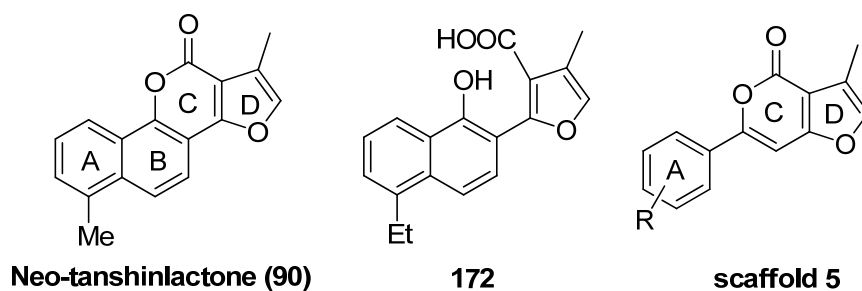


**Figure 5-1. Structures of neo-tanshinlactone (90), 2-(furan-2-yl)-naphthalen-1-ol derivative 172, and a newly designed scaffold 5**

## 5.2    Design

We designed scaffold 5 by removing ring-B of **90** to investigate how this ring affects the anti-breast cancer activity (Figure 5-1). Twenty-four new analogues were designed to explore the SAR and develop new leads. Firstly, new analogues with various substitutents

around ring-A were designed including mono-, di-, and tri-substituted analogues, as shown in Figure 5-2. Hydrogen, methyl, methoxy, ethoxy, hydroxy, and fluorine groups were incorporated at different positions (3' and 4' positions) of the phenyl ring (**197-206**) to study the effects of position, group size, halogen, etc. We also designed di-substituted analogues (2',3'; 3',4'; and 3',5'; **207-214**) and a tri-substituted analogue (3',4',5'; **215**). We further designed compounds **216-220**, which have a modified ring-C or -D, as shown in Figure 5-2. Insertion of an ethyl (**216**) or two methyl (**217**) groups rather than a single methyl group on the furan, as well as bioisosteric replacement of sulfur (thiolactone **218-219**) for oxygen in the lactone carbonyl, and reduction of the furan ring (**220**) were also investigated for their impact on anti-breast cancer activity.



**197**: R= H
**198**: R= Me
**199**: R= OMe
**200**: R= OEt
**201**: R= OH
**202**: R= F

**203**: R= F
**204**: R= Me
**205**: R= OMe
**206**: R= OH

**207**: $R_1$= $R_2$= OMe
**208**: $R_1$= $R_2$= OH
**209**: $R_2$= $R_3$= Me
**210**: $R_2$= $R_3$= OMe
**211**: $R_2$= $R_3$= OH
**212**: $R_2$= $R_4$= Me
**213**: $R_2$= $R_4$= OMe
**214**: $R_2$= $R_4$= OH
**215**: $R_2$= $R_3$= $R_4$= OMe

**216**: $R_1$= Et, $R_2$= H
**217**: $R_1$= $R_2$= Me

**218**: $R_2$= $R_4$= OMe
**219**: $R_2$= $R_3$= OMe

**220**
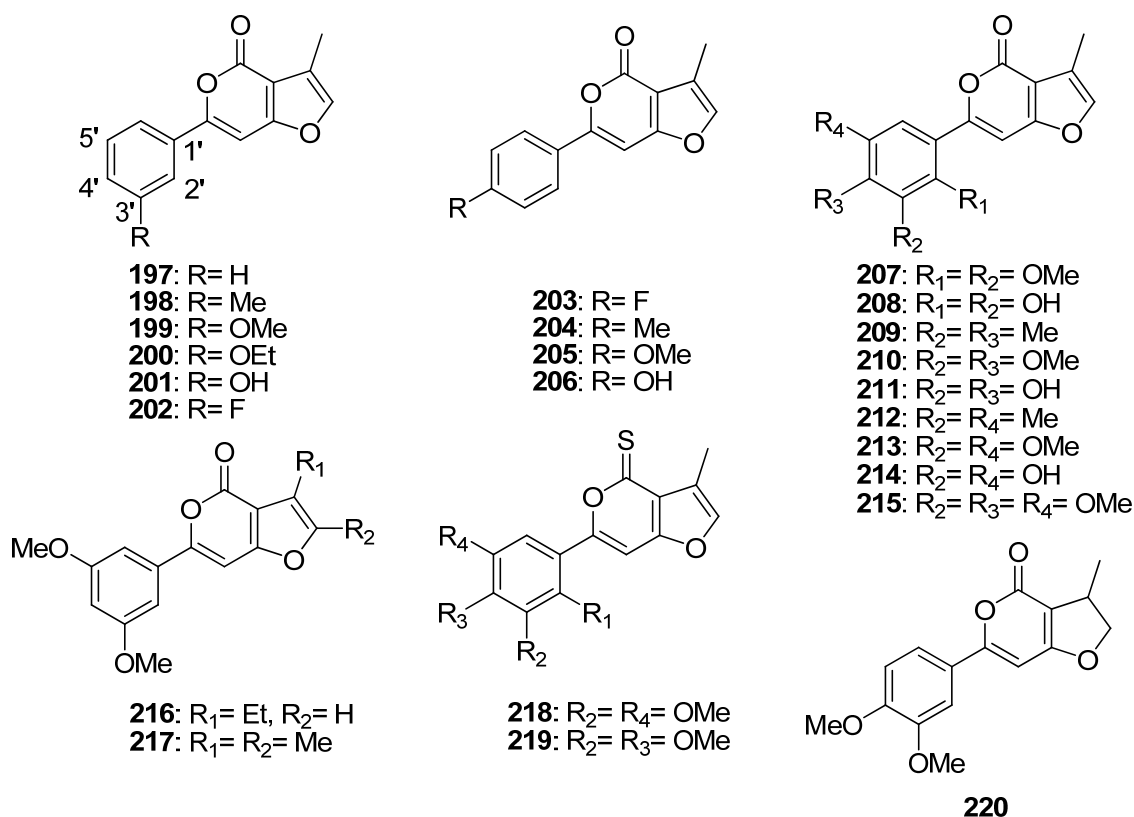
**Figure 5-2. Structures of designed 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives**

## 5.3    Chemistry

100

All target compounds (**197-220**) were synthesized through a three- to five-step sequence (Scheme 5-1). Various substituted esters **221** were reacted with a dianion intermediate generated from ethyl acetoacetate with LDA and TMEDA to give diketoesters **222** as tautomeric mixtures. Pyrones **223-235** were prepared by heating **222** at 170 °C under reduced pressure.[2] The resulting yellow solid was isolated by vacuum filtration, and the compound used directly in the next step.[2] Target compounds **197-199**, **202-205**, **207**, **209-210**, **212-213**, and **215-217** were obtained via a tandem alkylation/intramolecular Aldol reaction of **223-235**.[3, 4] Removal of the methyl group in **199**, **205**, **207**, **210**, and **213** by BBr$_3$ gave **201**, **206**, **208**, **211**, and **214**, respectively. Ethyl ether **200** was obtained by treatment of **201** with iodoethane under basic conditions (Scheme 5-1). Compounds **210** and **213** was reacted with Lawesson's reagent to afford **218** and **219**, respectively, [5] and **220** was synthesized by hydrogenation of **210** with formic acid.[6]

**Scheme 5-1 Synthetic pathway to analogues 197-220**



**Conditions**: a) Ethyl acetoacetate, LDA, TMDA, then HOAc; b) 170 °C, 5 mmHg; c) [structure], HOAc, NH$_4$OAc; d) BBr$_3$, CH$_2$Cl$_2$; e) EtI, K$_2$CO$_3$, acetone; f) Lawesson's reagent; g) Pd/C,Et$_3$N, HCOOH, acetone.
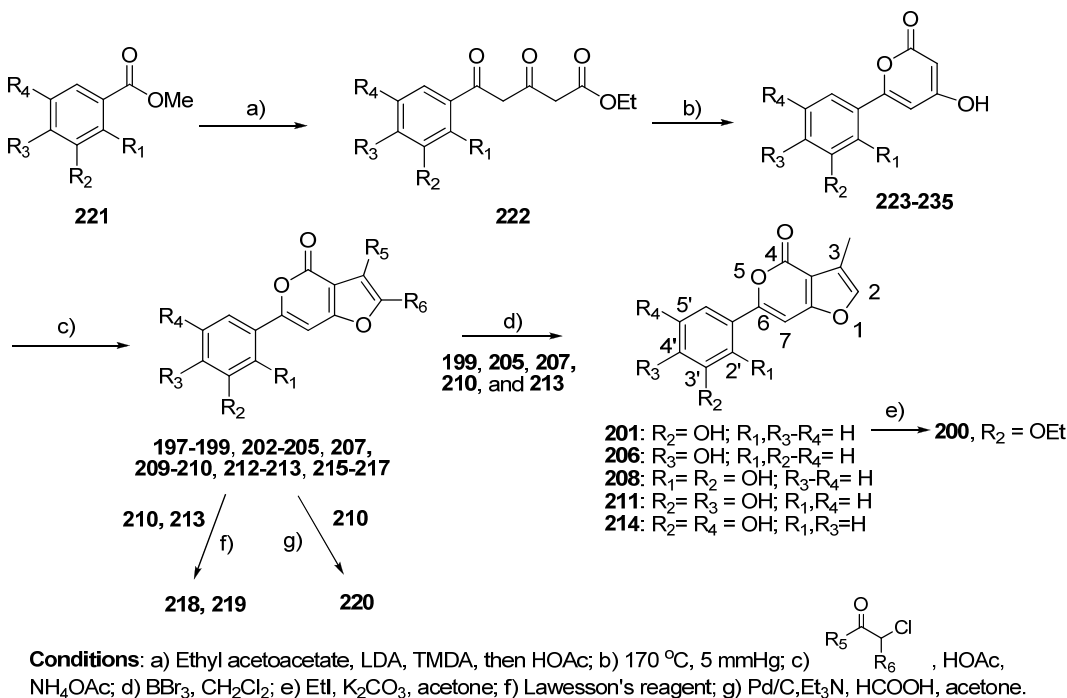
## 5.4    Results and Discussion

Together with **90,** the newly synthesized 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one analogues (**197-220**) were evaluated for *in vitro* anti-breast cancer activity against the SK-BR-3 human tumor cell line. Results from **197–215** (Table 5-1) showed that different substituents around the phenyl ring were critical to the potency and selectivity. Modifications in the furopyranone ring system were also explored with **216–220** (Table 5-2). Selected active compounds with $ED_{50}$ values less than 4 µg/mL against SK-BR-3 were further examined against ZR-7-51 (estrogen receptor positive breast cancer), MDA-MB-231 (estrogen receptor negative breast cancer), A549 (human lung cancer), DU145 (prostate cancer), KB (nasopharyngeal carcinoma), and KB-vin (vincristine-resistant KB subline) cancer cell lines (Table 5-3).
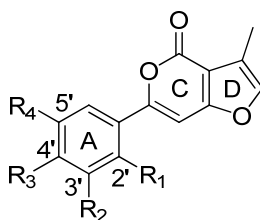


**Table 5-1. Cytotoxicity of 197-215 against SK-BR-3 tumor cell line[a]**

| Compd | 2' | 3' | 4' | 5' | SK-BR-3 |
|---|---|---|---|---|---|
| **90** | - | - | - | - | 0.25 |
| **197** | H | H | H | H | 3.5 |
| **198** | H | Me | H | H | 1.7 |
| **199** | H | OMe | H | H | 0.66 |
| **200** | H | OEt | H | H | 0.18 |
| **201** | H | OH | H | H | 0.39 |
| **202** | H | F | H | H | 5.0 |
| **203** | H | H | F | H | 4.7 |
| **204** | H | H | Me | H | >20 |
| **205** | H | H | OMe | H | >20 |
| **206** | H | H | OH | H | 8.7 |
| **207** | OMe | OMe | H | H | 18.6 |
| **208** | OH | OH | H | H | 8.8 |
| **209** | H | Me | Me | H | 3.7 |
| **210** | H | OMe | OMe | H | 14.8 |
| **211** | H | OH | OH | H | 0.12 |
| **212** | H | Me | H | Me | 5.7 |
| **213** | H | OMe | H | OMe | 0.08 |
| **214** | H | OH | H | OH | 9.9 |
| **215** | H | OMe | OMe | OMe | 0.14 |

Structurally, both **90** and **198** have a methyl substituent at corresponding positions on their phenyl rings. Thus, the two compounds are identical, except that **198** has no ring-B. Interestingly, although **198** showed potent activity against SK-BR-3 breast cancer cell line with an $ED_{50}$ value of 1.74 μg/mL, it was much less active than **90**. Addition of methyl (**198**), methoxy (**199**), ethoxy (**200**), and hydroxy (**201**) at the 3'-position of the phenyl ring increased activity against the SK-BR-3 cell line, compared with **197**. The rank order of potency of the five compounds was **200** > **201** > **199** > **198** > **197**. Especially, 3'-OEt analog **200** displayed slightly greater activity ($ED_{50}$ 0.18 μg/mL) than **90**. In contrast, fluorine at the 3'-position (**202**), as well as 4'-position (**203**), led to somewhat decreased potency compared with the unsubstituted analogue **197**. Addition of methyl, methoxy, or hydroxy at the phenyl 4'-position (**204-206**) reduced potency significantly. Compounds **207-214** and **215** are di- and tri-substituted derivatives, respectively, with one substituent always present at the phenyl 3'-position. Neither 2',3'-disubstituted compound (**207**, **208**) showed significant activity, leading us to speculate that a substituent in the 2'-position may have a steric effect on the orientation of the lactone ring and reduce the ligand-receptor interaction. Analogues with the same substituent at both the 3'- and 4'-positions showed increased potency relative to the corresponding 4'-monosubstituted analogue (**204** vs **209**, **205** vs **210**, **206** vs **211**). Thus, alkyl, alkoxy, and hydroxy groups are favored at the 3'-position, while they are not favored at the 4'-position. Comparison of **213** with **212** and **211** indicated that a methoxy group is favored at the 5'-position, while methyl and hydroxy groups are not. Furthermore, the 3',4',5'-trimethoxy (**215**) and 3',5'-dimethoxy (**213**) analogues showed dramatically enhanced potency compared with the 3'-methoxy compound (**199**), while the 3',4'-dimethoxy (**210**) and 2',3'-dimethoxy (**207**) analogues showed decreased potency. In fact, the 3',5'-dimethoxy analogue **213** ($ED_{50}$ 0.08 μg/mL) was the most active analogue among the 19

substituted phenyl A-ring analogues (**197–215**). It was also approximately three-fold more potent than **90**.

We also investigated the cytotoxic activity of **216-220**, which have a modified ring-C or -D, as shown in Table 5-2. Insertion of an ethyl (**216**) or two methyl (**217**) groups rather than a single methyl group on the furan, as well as bioisosteric replacement of sulfur (thiolactones **218-219**) for oxygen in the lactone carbonyl, and reduction of the furan ring-D (**220**) led to greatly reduced or no anti-breast cancer activity (Table 5-2).
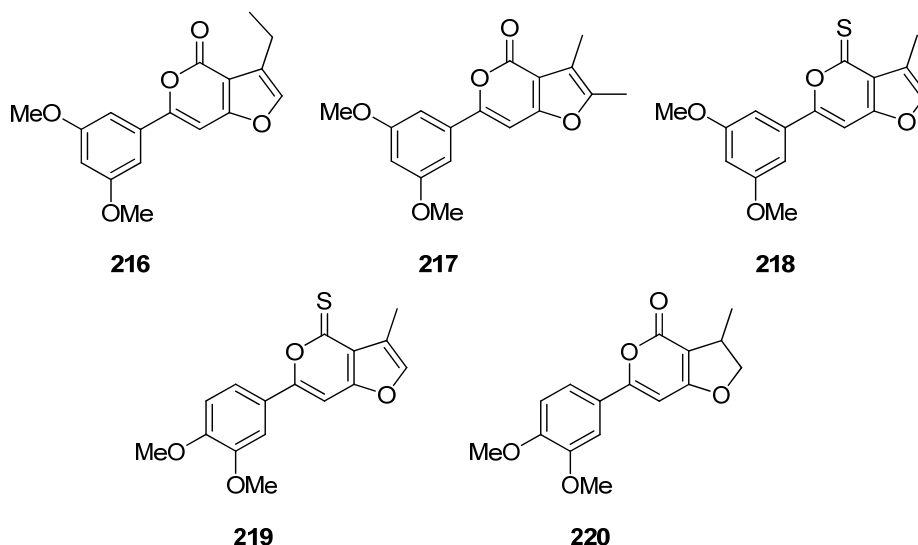


**216**        **217**        **218**

**219**        **220**

**Table 5-2. Cytotoxicity of 216-220 against SK-BR-3 cell line**[a]

| Compd | 216 | 217 | 218 | 219 | 220 |
|---|---|---|---|---|---|
| SK-BR-3 | 16.7 | >20 | >20 | 10.6 | 20 |

[a]See Table 5-1.

To examine the human tumor-tissue-type selectivity, active compounds **198-201**, **211**, **213**, and **215** were tested against a limited but diverse panel of human cancer cell lines, using **90** as a positive control (Table 5-3). Compounds **198-200** and **211** displayed similar and significant inhibition of the ZR-75-1 and SK-BR-3 cell lines. Interestingly, **213** and **215** showed only weak activity against ZR-75-1, although they were very active against SK-BR-3. Analogue **211** showed marginal inhibition, while the other six lead compounds had no

activity against MDA-MB-231 breast cancer or the remaining four non-breast cancer cell lines tested, which suggested high tumor-tissue-type selectivity. Importantly, **213** and **215** showed unique selectivity against the SK-BR-3 breast cancer cell line (HER2+), with approximately 100-250 fold differences compared with the other cancer cell lines tested. The unique selectivity of these novel lead compounds could be exploited to develop novel anti-breast cancer trials candidates and explore the mechanism(s) of action.

**Table 5-3. Cytotoxicity of selected compounds against human tumor cell lines[a]**

| Compd | ZR-75-1 | MDA-MB-231 | A549 | DU145 | KB | KBvin |
|:---:|:---:|:---:|:---:|:---:|:---:|:---:|
| **90** | 0.25 | 10.0 | 14.3 | 15.4 | >10 | >20 |
| **198** | 1.5 | >20 | 12.9 | 5.9 | 10.5 | 9.6 |
| **199** | 1.4 | >10 | 14.9 | >20 | >20 | 18.2 |
| **200** | 0.60 | >10 | 15.6 | 20 | 17.4 | >20 |
| **201** | 0.21 | >10 | 16.6 | 20 | 15.8 | 14.6 |
| **211** | 0.31 | 5.9 | 5.0 | 6.6 | 5.2 | 6.0 |
| **213** | 8.8 | >20 | >20 | 18.7 | >20 | >20 |
| **215** | 9.2 | >10 | 20 | >20 | >20 | >20 |

[a]See Table 5-1.

## 5.5    Conclusion

In conclusion, this study discovered a novel class of promising anti-breast cancer agents, substituted 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives. The $ED_{50}$ values of the two most potent analogues (**213** and **215**) against SK-BR-3 were 0.08 and 0.14 µg/mL, respectively. More importantly, **213** and **215** showed extremely high cancer cell line selectivity, being approximately 100- to 250-fold more potent against SK-BR-3 compared with six additional tested cancer cell lines. Preliminary SAR studies led to the following observations. (1) 3'-Methyl, methoxy, ethoxy, and hydroxy groups, but not a 3'-fluoro group,

could increase potency. (2) Among di-substituted phenyl compounds, 2'-, 4'-, or 5'-methyl groups, 2'- or 4'-methoxy groups, and 5'-hydroxy groups decreased potency; while a 4'-hydroxy or 5'-methoxy group increased potency. (3) Current modifications in ring-C and -D were not preferred. The SAR profile established from the current study is different from that with the neo-tanshinlactone series, which is a four-ring system. Thus, skeletal planarity is not indispensable for the entire molecule, though it may be important to some extent. Focused studies will continue to develop promising novel analogues as clinical trials candidates for anti-breast cancer treatment.

## 5.6    Experimental Section

### 5.6.1   Chemistry

**Materials and Methods**. Melting points were measured with a Fisher Johns melting apparatus without correction. ^{1}H NMR spectra were measured on a 300 MHz Varian Gemini 2000 spectrometer using TMS as internal standard. The solvent used was $CDCl_3$ unless indicated. Mass spectra were measured on a Shimadzu LC-MS2010 instrument. Thin-layer chromatography (TLC) and preparative TLC were performed on precoated silica gel GF plates purchased from Merck, Inc. Biotage Flash+ or Isco Companion systems were used for flash chromatography. Silica gel (200-400 mesh) from Aldrich, Inc. was used for column chromatography. All other chemicals were obtained from Aldrich, Inc. and Fisher, Inc. Preparation of intermediates **223-235** were reported by Douglas et al.[2]

**General procedure for preparation of 223-235.** To a solution of diisopropylamine (8.9 mL, 63.7 mmol, 2.5 eq) in 100 mL of THF at -10 °C (ice in acetone) was added n-BuLi (2.5 M in hexane) (25.5 mL, 63.7 mmol, 2.5 eq) via a syringe. This solution was stirred at -10 °C for 45 min. In a separate flask, a solution of ethyl acetoacetate (3.32 g, 25.5 mmol) in 50 mL of THF was cooled to -78 °C and the LDA solution was carefully transferred to the ethyl acetoacetate solution dropwise via a cannula. A yellow cloudy solution formed initially,

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but the mixture quickly turned homogeneous. After completing the addition of the LDA solution, freshly distilled TMEDA (3.84 mL, 25.5 mmol, 1.0 eq) was added in one aliquot via a syringe. The reaction mixture was then stirred for 3 h at -78 °C. The reaction mixture turned orange when a solution of **219** (25.5 mmol, commercially available) was added via cannulation, and a yellow precipitate was observed. The reaction mixture was warmed to rt and stirred for 48 h. When the reaction was completed, 4 mL of HOAc was added to the reaction mixture, which was subsequently concentrated under reduced pressure. The resulting crude residue was filtered through a small bed of silica gel eluted with ample $CH_2Cl_2$. After removal of the solvent in vacuo, the crude diketoester was purified using silica gel column chromatography eluting with a gradient of EtOAc in hexane to obtain **220**. In each case, numerous keto and enol tautomers could be found in many fractions. These tautomers also frequently equilibrated upon isolation. Hence, it was difficult to isolate one pure isomer and characterization was complicated. The mixtures of tautomers from different fractions were used directly for the pyrone formation without further characterization or purification. Compounds **220** were added to a flask equipped with an adaptor connected to a manifold and maintained under argon. The flask was then connected to a vacuum set at 5 mmHg pressure and heated over an oil bath at 170 °C. It was kept at this temperature for 0.5-1 h, and then cooled to rt. Diethyl ether was added to the reaction mixture, which was then filtered, and the solid residue was washed with diethyl ether. The solid was dried in vacuo and used in the next step.

**4-Hydroxy-6-phenyl-2*H*-pyran-2-one (223).** 58% yield; ^{1}H NMR (300 MHz, CD$_3$OD, ppm): δ 5.48 (d, *J* = 2.1 Hz, 1H, COC*H*), 6.70 (d, *J* = 1.8 Hz, 1H, OCC*H*), 7.48-7.51 (m, 3H, aromatic), 7.85-7.88 (m, 2H, aromatic); MS: *m/z* 187 (M-H^{+}).

**4-Hydroxy-6-m-tolyl-2*H*-pyran-2-one (224).** 26% yield; ^{1}H NMR (300 MHz, CD$_3$OD, ppm): δ 2.40 (s, 3H, C*H*$_3$), 5.47 (d, *J* = 2.4 Hz, 1H, COC*H*), 6.67 (s, 1H, OCC*H*), 7.30-7.39 (m, 2H, aromatic), 7.64-7.69 (m, 2H, aromatic); MS: *m/z* 201 (M-H^{+}).

**4-Hydroxy-6-(3-methoxyphenyl)-2*H*-pyran-2-one (225).** 45% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 3.85 (s, 3H, OC*H*$_3$), 5.46 (d, *J* = 1.8 Hz, 1H, COC*H*), 6.56 (s, 1H, OCC*H*), 7.01-7.05 (m, 1H, aromatic), 7.33-7.41 (m, 3H, aromatic); MS: *m/z* 217 (M-H$^+$).

**6-(3-Fluorophenyl)-4-hydroxy-2*H*-pyran-2-one (226).** 41% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 5.50 (d, *J* = 1.5 Hz, 1H, COC*H*), 6.75 (s, 1H, OCC*H*), 7.21-7.27 (m, 1H, aromatic), 7.48-7.71 (m, 3H, aromatic); MS: *m/z* 205 (M-H$^+$).

**6-(4-Fluorophenyl)-4-hydroxy-2*H*-pyran-2-one (227).** 46% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 5.46 (d, *J* = 1.8 Hz, 1H, COC*H*), 6.67 (s, 1H, OCC*H*), 7.20-7.26 (m, 2H, aromatic), 7.89-7.93 (m, 2H, aromatic); MS: *m/z* 207 (M+H$^+$).

**4-Hydroxy-6-p-tolyl-2*H*-pyran-2-one (228).** 52% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.39 (s, 3H, C*H*$_3$), 5.44 (d, *J* = 2.1 Hz, 1H, COC*H*), 6.63 (s, 1H, OCC*H*), 7.29 (d, *J* = 8.1 Hz, 2H, aromatic), 7.74 (d, *J* = 8.4 Hz, 2H, aromatic); MS: *m/z* 201 (M-H$^+$).

**4-Hydroxy-6-(4-methoxyphenyl)-2*H*-pyran-2-one (229).** 48% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 3.84 (s, 3H, OC*H*$_3$), 6.55 (s, 1H, OCC*H*), 7.01 (d, *J* = 8.7 Hz, 2H, aromatic), 7.79 (d, *J* = 8.7 Hz, 2H, aromatic); MS: *m/z* 217 (M-H$^+$).

**6-(2,3-Dimethoxyphenyl)-4-hydroxy-2*H*-pyran-2-one (230).** 14% yield; $^1$H NMR (300 MHz, DMSO, ppm): δ 3.77 (s, 3H, OC*H*$_3$), 3.85 (s, 3H, OC*H*$_3$), 5.38 (d, *J* = 1.8 Hz, 1H, COC*H*), 6.38 (d, *J* = 2.1 Hz, 1H, OCC*H*), 7.21-7.23 (m, 3H, aromatic), 11.82 (s, 1H, O*H*); MS: *m/z* 247 (M-H$^+$).

**6-(3,4-Dimethylphenyl)-4-hydroxy-2*H*-pyran-2-one (231).** 24% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.26 (s, 6H, C*H*$_3$), 5.40 (d, *J* = 1.5 Hz, 1H, COC*H*), 6.53 (s, 1H, OCC*H*), 7.16 (d, *J* = 7.8 Hz, 1H, aromatic), 7.48 (d, *J* = 7.8 Hz, 1H, aromatic), 7.54 (s, 1H, aromatic); MS: *m/z* 215 (M-H$^+$).

**6-(3,4-Dimethoxyphenyl)-4-hydroxy-2*H*-pyran-2-one (232).** 85% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 3.88 (s, 3H, OC*H*$_3$), 3.89 (s, 3H, OC*H*$_3$), 5.42 (d, *J* = 2.1 Hz, 1H, COC*H*), 6.62 (s, 1H, OCC*H*), 7.05 (d, *J* = 8.4 Hz, 1H, aromatic), 7.41-7.50 (m, 2H, aromatic);

MS: *m/z* 247 (M-H$^+$).

**6-(3,5-Dimethylphenyl)-4-hydroxy-2*H*-pyran-2-one (233).** 27% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.26 (s, 6H, C*H*$_3$), 5.03 (s, 2H, COC*H* & O*H*), 6.48 (s, 1H, OCC*H*), 7.01 (s, 1H, aromatic), 7.30 (s, 2H, aromatic); MS: *m/z* 215 (M-H$^+$).

**6-(3,5-Dimethoxyphenyl)-4-hydroxy-2*H*-pyran-2-one (234).** 27% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 3.83 (s, 6H, OC*H*$_3$), 5.47 (d, *J* = 2.1 Hz, 1H, COC*H*), 6.61 (t, *J* = 2.1 Hz, 1H, aromatic), 6.68 (s, 1H, OCC*H*), 6.99 (d, *J* = 2.1 Hz, 2H, aromatic); MS: *m/z* 247 (M-H$^+$).

**4-Hydroxy-6-(3,4,5-trimethoxyphenyl)-2*H*-pyran-2-one (235).** 50% yield; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 81 (s, 3H, OC*H*$_3$), 3.90 (s, 6H, OC*H*$_3$), 5.45 (d, *J* = 1.8 Hz, 1H, COC*H*), 6.70 (d, *J* = 1.8 Hz, 1H, OCC*H*), 7.17 (s, 2H, aromatic); MS: *m/z* 277 (M-H$^+$).

**General procedure of preparation of 197-199, 202-205, 207, 209-210, 212-213, 215.** To a solution of **223-235** (1.04 mmol) in toluene (9 mL) was added a mixture of HOAc (0.30 ml, 5.20 mmol) and NH$_4$OAc (400 mg, 5.20 mmol) in EtOH (3 mL) and chloroacetone (0.42 mL, 5.20 mmol; or 1-bromobutanone and 3-bromobutan-2-one for preparation of **216** and **217**, respectively). The mixture was stirred for 30 min at rt, and then heated to 60 °C for 30 min. Subsequently, it was refluxed for 24 h. After cooling, the mixture was diluted with H$_2$O and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography to give a white solid.

**3-Methyl-6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one (197).** 50% yield; mp 105-107 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 7.00 (s, 1H, OC*H*), 7.28-7.29 (m, 1H, C7-*H*), 7.43-7.46 (m, 3H, aromatic), 7.83-7.87 (m, 2H, aromatic); HRMS Calcd for C$_{14}$H$_{11}$O$_3$ (M+H$^+$): 227.0708, found: 227.0696.

**3-Methyl-6-*m*-tolyl-4*H*-furo[3,2-*c*]pyran-4-one (198).** 52% yield; mp 135-137 °C; $^1$H

NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 2.42 (s, 3H, C$H_3$), 6.99 (s, 1H, C7-$H$), 7.23-7.36 (m, 3H, aromatic), 7.62 (d, $J$ = 7.2 Hz, 1H, aromatic), 7.69 (d, $J$ = 1.5 Hz, 1H, OC$H$); HRMS Calcd for C$_{15}$H$_{13}$O$_3$ (M+H$^+$): 241.0865, found: 241.0851.

**6-(3-Methoxyphenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (199).** 44% yield; mp 119-121 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 3.87 (s, 3H, OC$H_3$), 6.96-7.00 (m, 2H), 7.29-7.44 (m, 4H); HRMS Calcd for C$_{15}$H$_{13}$O$_4$ (M+H$^+$): 257.0814, found: 257.0800.

**6-(3-Fluorophenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (202).** 34% yield; mp 158-160 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 7.01 (s, 1H, C7-$H$), 7.10-7.17 (m, 1H, aromatic), 7.31 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.39-7.46 (m, 1H, aromatic), 7.54-7.58 (m, 1H, aromatic), 7.61-7.65 (m, 1H, aromatic); HRMS Calcd for C$_{14}$H$_{10}$FO$_3$ (M+H$^+$): 245.0614, found: 245.0603.

**6-(4-Fluorophenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (203).** 52% yield; mp 175-177 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 6.94 (s, 1H, C7-$H$), 7.12-7.18 (m, 2H, aromatic), 7.29 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.82-7.87 (m, 2H, aromatic); HRMS Calcd for C$_{14}$H$_{10}$FO$_3$ (M+H$^+$): 245.0614, found: 245.0603.

**3-Methyl-6-$p$-tolyl-4$H$-furo[3,2-$c$]pyran-4-one (204).** 62% yield; mp 153-155 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, $J$ = 1.2 Hz, 3H, C$H_3$), 2.40 (s, 3H, C$H_3$), 6.95 (s, 1H, C7-$H$), 7.24-7.27 (m, 3H, aromatic & OC$H$), 7.74 (d, $J$ = 8.1 Hz, 2H, aromatic); HRMS Calcd for C$_{15}$H$_{13}$O$_3$ (M+H$^+$): 241.0865, found: 241.0848.

**6-(4-Methoxyphenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (205).** 60% yield; mp 146-148 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 3.87 (s, 3H, OC$H_3$), 6.88 (s, 1H, C7-$H$), 6.97 (d, $J$ = 9.0 Hz, 2H, aromatic), 7.26 (d, $J$ = 1.5 Hz, 1H, OC$H$), 7.80 (d, $J$ = 9.0 Hz, 2H, aromatic); HRMS Calcd for C$_{15}$H$_{13}$O$_4$ (M+H$^+$): 257.0808, found: 257.0816.

**6-(2,3-Dimethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (207).** 40% yield; mp 111-113 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 3.87 (s, 3H, OC*H*$_3$), 3.92 (s, 3H, OC*H*$_3$), 7.00 (dd, *J* = 1.2, 8.1 Hz, 1H, aromatic), 7.16 (t, *J* = 8.1 Hz, 1H, aromatic), 7.34 (q, *J* = 1.2 Hz, 1H, OC*H*), 7.48 (s, 1H, C7-*H*), 7.54 (dd, *J* = 1.2, 8.1 Hz, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{15}$O$_5$ (M+H$^+$): 287.0919, found: 287.0906.

**6-(3,4-Dimethylphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (209).** 67% yield; mp 182-184 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.30 (s, 3H, C*H*$_3$) 2.32 (s, 3H, C*H*$_3$), 2.33 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 6.95 (s, 1H, C7-*H*), 7.20 (d, *J* = 8.1 Hz, 1H, aromatic), 7.26 (d, *J* = 1.2 Hz, 1H, OC*H*), 7.57 (d, *J* = 8.1 Hz, 1H, aromatic), 7.64 (s, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{15}$O$_3$ (M+H$^+$): 255.1016, found: 255.1010.

**6-(3,4-Dimethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (210).** 83% yield; mp 154-156 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.98 (s, 3H, OC*H*$_3$), 6.90 (s, 1H, C7-*H*), 6.92 (d, *J* = 8.7 Hz, 1H, aromatic), 7.27 (t, *J* = 1.5 Hz, 1H, OC*H*), 7.34 (d, *J* = 2.1 Hz, 1H, aromatic), 7.43 (dd, *J* = 2.1, 8.4 Hz, 1H, aromatic); HRMS Calcd for C$_{16}$H$_{15}$O$_5$ (M+H$^+$): 287.0919, found: 287.0900.

**6-(3,5-Dimethylphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (212).** 30% yield; mp 171-173 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.33 (d, *J* = 0.9 Hz, 3H, C*H*$_3$),  2.37 (s, 6H, C*H*$_3$), 6.98 (s, 1H, C7-*H*), 7.07 (s, 1H, aromatic), 7.28 (d, *J* = 0.9 Hz, 1H, OC*H*), 7.48 (s, 2H, aromatic); HRMS Calcd for C$_{16}$H$_{15}$O$_3$ (M+H$^+$): 255.1021, found: 255.1010.

**6-(3,5-Dimethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (213).** 38%, yield; mp 153-155 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 3.86 (s, 6H, OC*H*$_3$), 6.54 (t, *J* = 2.1 Hz, 1H, C7-*H*), 6.98 (s, 1H, aromatic), 6.99 (d, *J* = 2.7 Hz, 2H, aromatic), 7.30 (d, *J* = 1.5 Hz, 1H, OC*H*); HRMS Calcd for C$_{16}$H$_{15}$O$_5$ (M+H$^+$): 287.0919, found: 287.0898.

**3-Methyl-6-(3,4,5-trimethoxyphenyl)-4*H*-furo[3,2-*c*]pyran-4-one (215).** 40% yield;

mp 201-203 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.34 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 3.91 (s, 3H, OC$H_3$), 3.95 (s, 6H, OC$H_3$), 6.94 (s, 1H, C7-$H$), 7.06 (s, 2H, aromatic), 7.29 (d, $J$ = 1.2 Hz, 1H, OC$H$); HRMS Calcd for C$_{17}$H$_{17}$O$_6$ (M+H$^+$): 317.1025, found: 317.1037.

**6-(3,5-Dimethoxyphenyl)-3-ethyl-4$H$-furo[3,2-$c$]pyran-4-one (216).** 51% yield; mp 131-133 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.31 (t, $J$ = 7.2 Hz, 3H, CH$_2$C$H_3$), 2.77 (q, $J$ = 7.5 Hz, 2H, C$H_2$CH$_3$), 3.86 (s, 6H, OC$H_3$), 6.54 (t, $J$ = 2.1 Hz, 1H, C7-$H$), 6.98 (d, $J$ = 2.1 Hz, 3H, aromatic), 7.30 (t, $J$ = 1.2 Hz, 1H, OC$H$); HRMS Calcd for C$_{17}$H$_{17}$O$_5$ (M+H$^+$): 301.1076, found: 301.1057.

**6-(3,5-Dimethoxyphenyl)-2,3-dimethyl-4$H$-furo[3,2-$c$]pyran-4-one (217).** 12% yield; mp 163-165 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 2.25 (d, $J$ = 0.6 Hz, 3H, C$H_3$), 2.33 (d, $J$ = 0.6 Hz, 3H, C$H_3$), 3.86 (s, 6H, OC$H_3$), 6.53 (t, $J$ = 2.1 Hz, 1H, aromatic), 6.93 (s, 1H, OC$H$), 6.97 (d, $J$ = 2.1 Hz, 2H, aromatic); HRMS Calcd for C$_{17}$H$_{17}$O$_5$ (M+H$^+$): 301.1071, found: 301.1067.

**General procedure of preparation of 201, 206, 208, 211, and 214.** To a solution of **199**, **205**, **207**, **210**, or **213** (0.2 mmol) in DCM (3 ml) was added BBr$_3$ (0.6 ml, 0.6 mmol) dropwise at 0 °C. The reaction mixture was stirred overnight. Water was added to quench the reaction. The solution was extracted with CHCl$_3$ and concentrated. The residue was purified by column chromatography to give a white solid.

**6-(3-Hydroxyphenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (201).** 78% yield; mp 225-227 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.29 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 6.86-6.90 (m, 1H, C7-$H$), 7.26-7.31 (m, 3H, aromatic & OC$H$), 7.35-7.39 (m, 1H, aromatic), 7.52 (dd, $J$ = 1.2, 2.7 Hz, 1H, aromatic); HRMS Calcd for C$_{14}$H$_{11}$O$_4$ (M+H$^+$): 243.0657, found: 243.0659.

**6-(4-Hydroxyphenyl)-3-methyl-4$H$-furo[3,2-$c$]pyran-4-one (206).** 80% yield; mp 258-260 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.27 (d, $J$ = 1.5 Hz, 3H, C$H_3$), 6.86 (d, $J$ = 9.0 Hz, 2H, aromatic) 7.18 (s, 1H, C7-$H$), 7.46 (d, $J$ = 1.2 Hz, 1H, OC$H$), 7.75 (d, $J$ = 9.3 Hz,

2H, aromatic); HRMS Calcd for $C_{14}H_{11}O_4$ (M+H$^+$): 243.0657, found: 243.0641.

**6-(2,3-Dihydroxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (208).** 66% yield; mp 239-241 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.28 (d, *J* = 0.9 Hz, 3H, C*H*$_3$), 6.76 (t, *J* = 1.5 Hz, 1H, aromatic), 6.86 (dd, *J* = 1.5, 1.8 Hz, 1H, aromatic), 7.38 (dd, *J* = 1.2, 1.5 Hz, 1H, aromatic), 7.48 (q, *J* = 1.2 Hz, 1H, OC*H*), 7.75 (s, 1H, C7-*H*); HRMS Calcd for $C_{14}H_{11}O_5$ (M+H$^+$): 259.0606, found: 259.0602.

**6-(3,4-Dihydroxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (211).** 60% yield; mp 259-261 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.69 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 6.83 (d, *J* = 7.8 Hz, 3H, C7-*H*), 7.12 (s, 1H, aromatic), 7.26-7.31 (m, 2H, aromatic), 7.46 (dd, J = 1.2 Hz, 1H, OCH); HRMS Calcd for $C_{14}H_{11}O_5$ (M-H$^+$): 257.0450, found: 257.0464.

**6-(3,5-Dihydroxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (214).** 70% yield; mp >300 °C; $^1$H NMR (300 MHz, CD$_3$OD, ppm): δ 2.86 (d, *J* = 1.2 Hz, 3H, C*H*$_3$), 6.35 (t, *J* = 2.1 Hz, 1H, aromatic), 6.80 (d, *J* = 2.1 Hz, 2H, aromatic), 7.21 (s, 1H, C7-*H*), 7.51 (d, *J* = 1.2 Hz, 1H, OC*H*); HRMS Calcd for $C_{14}H_{11}O_5$ (M+H$^+$): 259.0601, found: 259.0594.

**6-(3-Ethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-one (200).** To a mixture of **201** (212 mg, 1.00 mmol), K$_2$CO$_3$ (300 mg, 2.17 mmol) in actone (8 mL) was added iodoethane (0.4 mL, 5.00 mmol). The mixture was stirred for 12 h. The mixture was concentrated and diluted with H$_2$O and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The residue was purified by column chromatography to give a white solid. 35% yield; mp 128-130 °C; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.45 (t, *J* = 6.9 Hz, 3H, CH$_2$C*H*$_3$), 2.33 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 4.10 (d, *J* = 7.2 Hz, 2H, C*H*$_2$CH$_3$), 6.95-6.96 (m, 1H, aromatic), 6.98 (s, 1H, C7-*H*), 7.29 (d, *J* = 1.5 Hz, 1H, OC*H*), 7.32-7.43 (m, 3H, aromatic); HRMS Calcd for $C_{16}H_{13}O_4$ (M+H$^+$): 271.0965, found: 271.0962.

**General preparation of 218-219.** A mixture of **210** or **213** (0.1 mmol) and Lawesson's reagent (81 mg, 0.2 mmol) in dry toluene (5 mL) was heated to reflux for 12 h.

Toluene was removed and the red residue was dissolved in EtOAc and partitioned with $H_2O$. The organic phase was separated and dried over $Na_2SO_4$. Removal of solvent in vacuo afforded an oily residue, which was purified by column chromatography resulting in a yellow solid.

**6-(3,5-Dimethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-thione (218).** 60% yield; mp 147-149 °C; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.46 (d, *J* = 1.5 Hz, 3H, C*H*$_3$), 3.87 (s, 6H, OC*H*$_3$), 6.57 (t, *J* = 2.1 Hz, 1H, C7-*H*), 7.02 (s, 1H, aromatic), 7.03 (s, 1H, aromatic), 7.17 (s, 1H, aromatic), 7.32 (d, J = 1.5 Hz, 1H, OCH); HRMS Calcd for $C_{16}H_{14}O_4S$ (M+H$^+$): 303.0691, found: 303.0702.

**6-(3,4-Dimethoxyphenyl)-3-methyl-4*H*-furo[3,2-*c*]pyran-4-thione (219).** 50% yield; mp 157-159 °C; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 2.46 (s, 3H, C*H*$_3$),  3.96 (s, 3H, OC*H*$_3$), 3.96 (s, 3H, OC*H*$_3$), 6.95 (d, *J* = 8.7 Hz, 1H, aromatic), 7.11 (s, 1H, aromatic), 7.30 (s, 1H, OCCH), 7.40 (d, J = 2.1 Hz, 1H, OCH), 7.52 (m, 1H, aromatic); HRMS Calcd for $C_{16}H_{14}O_4S$ (M+H$^+$): 303.0691, found: 303.0681.

**6-(3,4-Dimethoxyphenyl)-3-methyl-2*H*-furo[3,2-*c*]pyran-4(3*H*)-one (220). 210** (57 mg, 0.2 mmol) was dissolved in acetone at 40 °C under argon. Pd/C (81 mg, 10%), triethylamine (0.33 mL, 2.4 mmol) and formic acid (0.075 mL, 2 mmol) were added to the solution. The solution was stirred overnight and filtered through celite and removal of solvent yielded a dark oily residue. The residue was dissolved in DCM before washing with saturated aqueous sodium bicarbonate and dried. Removal of solvent in vacuo afforded an oily residue, which was purified by column chromatography resulting in a white solid. 13% recovered yield; mp 95-97 °C; ^{1}H NMR (300 MHz, CDCl$_3$, ppm): δ 1.38 (t, *J* = 6.6 Hz, 3H, CHC*H*$_3$), 3.55 (m, 1H, C*H*CH$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.96 (s, 3H, OC*H*$_3$), 4.29 (dd, *J* = 6.0, 9.0 Hz, 1H, OCH*H*), 4.82 (t, *J* = 9.3 Hz, 1H, OC*H*H), 6.46 (s, 1H, OCC*H*), 6.91 (d, *J* = 8.7 Hz, 1H, aromatic), 7.33 (d, J = 2.1 Hz, 1H, aromatic), 7.41 (dd, J = 2.1, 8.7 Hz, 1H, aromatic);

HRMS Calcd for $C_{16}H_{17}O_5$ (M+H$^+$): 289.1076, found: 289.1079.

### 5.6.2   Biological Assay

The assay methods and description of cell lines are described in Chapter 3.

## 5.7    References

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# CHAPTER 6

# MECHANISM OF ACTION STUDY AND *IN VIVO* EVALUATION

**Mechanism of Action Study**

## 6.1    Cell Assays (MD Anderson Cancer Center)

## 6.1.1    Introduction

The MTT assay[1] was applied to examine the effect of the compounds on cell growth, cytotoxicity, and tumor-tissue-type selectivity. Twenty-nine tumor cell lines from different tissues and two normal cell lines treated with compound **91** were evaluated with this assay.

## 6.1.2    Methodology of MTT assay

The MTT assay was used to assess the killing effect of the drugs. Cells were seeded into 96-well plates at a density of 5000 cells in media per well. The drug was dissolved in DMSO. The drug was added into wells after overnight incubation. After 72 hours of incubation at 37 °C in 5% $CO_2$, 20 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent was added to each well and incubated for two hours. Then, the plates were read on a plate reader at OD 570 nM. The cell killing efficiency was determined by plotting the percent of survival cells with different concentrations of the compounds in the same family, which were tested side-by-side. The MTT reading derived from the cells treated with DMSO only was set as 100%. The following human cell lines were used in the assay. Breast cancer cell lines: MDA-MB-453, BT-483, MDA-MB-435, BT-549, MDA-MB-436, HBL100, Hs578t, BT20, BT474, MDA-MB-468, MCF7, Au565, ZR-75-1, T-47D, SK-BR-3; Prostate cancer cell lines: PC3, DU145, LnCAP; Ovarian cancer cell line: SKOV3ip1, Hey8, 2774; Esophagus cancer cell line: SKGF4, BE3, BIC1; Liver cancer cell line: Hep3B,

HepG2; Pancreatic cancer cell line: Panc1, Capan1; Lung cancer cell line: H1299; Pancreatic normal cell line: E6E7; Human lung fibroblast cell line: WI38.

### 6.1.3 Results and Discussion

As shown in Figure 6-1, **91** was effective in about 40% of human breast cancer cell lines (total 15 cell lines), was ineffective or weakly effective in ovarian, liver, pancreatic, lung, and esophagus cancer cell lines (total 11 cell lines), and promoted the cell growth in some prostate cancer cell lines (total 3 cell lines). Importantly, **91** was not active against E6E7, a pancreatic normal cell line and WI38, a human lung fibroblast cell line. The results demonstrated that **91** is highly selective against breast cancer cell lines.
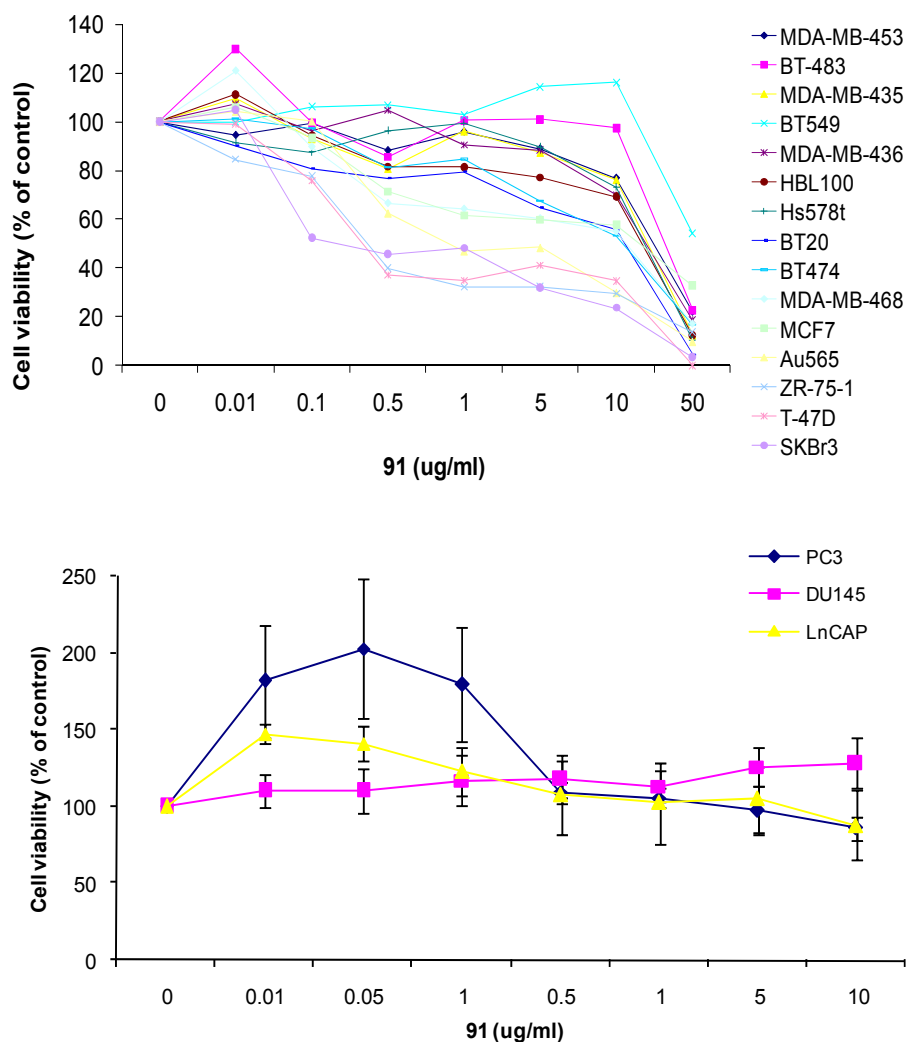


**Figure 6-1.** *In vitro* anticancer profiles of **91**

Compound **91** was effective in ER+/HER2+ positive cell lines, and also inhibited the ER-/HER2- MDA-MB-468 cell line, but did not affect ER+/HER2+ BT474 and MDA-MB-453 cell lines (Table 6-1). From the results in the cell-based assay, we postulated ER or HER2 is not the primary target of **91** in human breast cancer cell lines.

**Table 6-1. *In vitro* anticancer profiles of 91**

|  | MCF-7 | SK-BR-3 | ZR-75-1 | AU565 | T-47D | MDA-MB-468 | MDA-MB-453 | BT474 |
|---|---|---|---|---|---|---|---|---|
| ER | + | - | + | - | + | - | + | + |
| HER2 | - | ++ | - | ++ | + | - | ++ | ++ |
| Effective | + | + | + | + | + | + | - | - |

Compound **91** showed better killing effects compared to exemestane or tamoxifen in **91**-sensitive human breast cancer cell lines, but not in BT549, a human breast cancer cell line, and WI38, a human lung fibroblast cell line (Table 6-2).

**Table 6-2. *In vitro* anticancer profiles of tamoxifen, exemestane, and 91**

| Cell lines | Tamoxifen | Exemestane | **91** |
|---|---|---|---|
| MCF7 | ± | + | ++ |
| SK-BR-3 | ± | + | ++ |
| ZR-75-1 | ND | ± | ++ |
| Au565 | ND | + | ++ |
| T-47D | + | + | ++ |
| MDA-MB-468 | + | + | +± |
| BT549 | ND | ± | ± |
| WI38 | ND | ± | ± |

ND: not detected.

## 6.2    Enzyme Assays (MDS Pharma Services)

### 6.2.1    Introduction

With over 500 kinases in the human kinome and numerous drugs in phase 3 clinical trials and beyond, kinase inhibitors have become very attractive for drug design and development. To identify the targets of neo-tanshinlactone derivatives, compounds **91** and **173** were examined for inhibitory activity against 58 kinases. This investigation was performed by MDS Pharma Services.[2]

### 6.2.2    Methods

Methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Assays were performed under conditions described in the experimental section.

### 6.2.3    Results and Discussion

A summary of results meeting the significance criteria is presented in the following tables. Compound **178** showed higher inhibition activity against CDC42BPB, PKG1β, and SGK1 (Table 6-4), while **91** significantly suppressed CK2α1, ABL, and AKT1 (Table 6-3). The results suggested that these two compounds may have different mechanisms of action. More studies are focusing on these pathways to identify the possible targets.

**Table 6-3. Enzyme assay profiles of 91**

| Primary Biochemical Assay | Species | % INH |
| --- | --- | --- |
| Protein Serine/Threonine Kinase, AURKA (Aurora-A) | Human | 73 |
| Protein Serine/Threonine Kinase, CDC42BPB | Human | 82 |
| Protein Serine/Threonine Kinase, CSNK2A1 (CK2α1) | Human | 99 |
| Protein Serine/Threonine Kinase, MAPK8 (JNK1) | Human | 64 |
| Protein Serine/Threonine Kinase, MAPKAPK2 | Human | 50 |
| Protein Serine/Threonine Kinase, PDK1 | Human | 59 |
| Protein Serine/Threonine Kinase, PIM1 | Human | 71 |
| Protein Serine/Threonine Kinase, PRKG1 (PKG1β) | Human | 74 |
| Protein Serine/Threonine Kinase, SGK1 | Human | 82 |
| Protein Serine/Threonine Kinase, SGK2 | Human | 85 |
| Protein Serine/Threonine Kinase, TSSK2 (STK22B) | Human | 51 |
| Protein Tyrosine Kinase, ABL1 (ABL) | Mouse | 93 |
| Protein Tyrosine Kinase, ERBB2 (HER2) | Human | 69 |
| Protein Tyrosine Kinase, Insulin Receptor | Human | 83 |
| Protein Serine/Threonine Kinase, GSK3B | Human | 57 |
| Protein Serine/Threonine Kinase, AKT1 (PRKBA) | Human | 93 |

**Table 6-4. Enzyme assay profiles of 178**

| Primary Biochemical Assay | Species | % INH |
| --- | --- | --- |
| Protein Serine/Arginine-rich Kinase, SRPK1 | Human | 55 |
| Protein Serine/Threonine Kinase, CDC42BPB | Human | 90 |
| Protein Serine/Threonine Kinase, CSNK2A1 (CK2α1) | Human | 66 |
| Protein Serine/Threonine Kinase, MARK1 | Human | 68 |
| Protein Serine/Threonine Kinase, MAPKAPK2 | Human | 84 |
| Protein Serine/Threonine Kinase, PDK1 | Human | 86 |
| Protein Serine/Threonine Kinase, PIM1 | Human | 83 |
| Protein Serine/Threonine Kinase, PKD2 | Human | 63 |
| Protein Serine/Threonine Kinase, PRKG1 (PKG1β) | Human | 92 |
| Protein Serine/Threonine Kinase, SGK1 | Human | 96 |
| Protein Serine/Threonine Kinase, TSSK2 (STK22B) | Human | 60 |
| Protein Serine/Threonine Kinase, GSK3B | Human | 59 |

### 6.2.4 Experimental Section

**Protein Serine/Arginine-rich Kinase, CLK2.** Source: Human recombinant insect cells; Substrate: 50 µM S6 kinase/Rsk2 substrate peptide 2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 25 mM β-Glycerophosphate, 1 mM DTT, 5 mM EGTA, 20 mM $MgCl_2$, 1 mM $Na_3VO_4$; Quantitation Method: Quantitation of $[^{32}P]$S6 kinase/Rsk2 substrate peptide 2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Arginine-rich Kinase, SRPK1.** Source: Human recombinant E. Coli; Substrate: 5 µM SRPK1tide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 25 mM β-Glycerophosphate, 1 mM DTT, 5 mM EGTA, 20 mM $MgCl_2$, 1 mM $Na_3VO_4$; Quantitation Method: Quantitation of $[^{32}P]$SRPK1tide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, AKT1 (PRKBA).** Source: Human recombinant insect Sf21 cells; Substrate: 15 µg/mL Crosstide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 25 °C; Incubation Time/Temp: 60 minutes at 25 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM $Na_3VO_4$, 1 mM DTT; Quantitation Method: ELISA quantitation of Crosstide-P; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, AURKA (Aurora-A).** Source: Human recombinant insect Sf21 cells; Substrate: 100 µM Kemptide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 25 mM β-Glycerophosphate, 1 mM DTT, 5 mM

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EGTA, 20 mM MgCl$_2$, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P]S6 kinase/Rsk2 substrate peptide 2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, Ca$^{2+}$/Calmodulin-Dep. II.** Source: Rat brain; Substrate: 50 µM BB40; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 1 mM EGTA, 10 mM MgCl$_2$, 0.1 mM DTT, 1.5 mM CaCl$_2$, 0.02 mg/ml Calmodulin; Quantitation Method: Quantitation of [$^{32}$P]BB40; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, CAMK2D (KCC2D).** Source: Human recombinant insect cells; Substrate: 50 µM Autocamtide-2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM MgCl$_2$, 1.5 mM CaCl$_2$·2H$_2$O, 20 µg/mL Calmodulin; Quantitation Method: Quantitation of [$^{32}$P] Autocamtide-2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, CDC42BPB.** Source: Human recombinant insect Sf21 cells; Substrate: 50 µM S6 kinase/Rsk2 substrate peptide 2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P]S6 kinase/Rsk2 substrate peptide 2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, CHK2.** Source: Human recombinant E. Coli; Substrate: 10 µM CHKtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$;

Quantitation Method: Quantitation of [$^{32}$P] CHKtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, CSNK1A1 (CK1).** Source: Human recombinant E. Coli; Substrate: 9 µM CK1 substrate peptide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] CK1 substrate peptide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, CSNK2A1 (CK2α1).** Source: Human recombinant insect Sf21 cells; Substrate: 100 µM CK2 substrate peptide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] CK2 substrate peptide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, Dyrk1a.** Source: Rat recombinant E. Coli; Substrate: 50 µM Woodtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Woodtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, GSK3B.** Source: Human recombinant insect Sf21 cells; Substrate: 5 µM Phospho-glycogen synthase peptide-2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-

Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Phospho-glycogen synthase peptide-2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, IKK-1.** Source: Human recombinant insect Sf21 cells; Substrate: 20 µM IKKtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] IKKtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, IRAK4.** Source: Human recombinant insect Sf21 cells; Substrate: 50 µg/mL Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, LIMK1.** Source: Human recombinant insect Sf21 cells; Substrate: 0.7 µM Cofilin-1; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Cofilin-1; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MAP2K1 (MEK1).** Source: Human recombinant E. Coli; Substrate: 50 µg/mL Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-

Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MAP3K5 (ASK1).** Source: Human recombinant E. Coli; Substrate: 50 µg/mL Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MAPK8 (JNK1).** Source: Human recombinant insect Sf21 cells; Substrate: 1.5 µM ATF2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] ATF2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MAPKAPK2.** Source: Human recombinant E. Coli; Substrate: 50 µM MAPKAPK substrate peptide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MAPKAPK substrate peptide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MARK1.** Source: Human recombinant insect Sf21 cells; Substrate: 10 µM CHKtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM

Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] CHKtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, MYLK (MLCK).** Source: Human recombinant insect Sf21 cells; Substrate: 50 µM ZIPtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 4 hours at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 1.5 mM CaCl$_2$, 0.02 mg/ml Calmodulin, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] ZIPtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, NEK2.** Source: Human recombinant insect Sf21 cells; Substrate: 50 µg/ml Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PDK1.** Source: Human recombinant insect Sf21 cells; Substrate: 5.3 µM PDKtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] PDKtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PHKG2.** Source: Human recombinant insect Sf9 cells; Substrate: 50 µM ZIPtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$;

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Quantitation Method: Quantitation of [$^{32}$P] ZIPtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PIM1.** Source: Human recombinant E. Coli; Substrate: 50 µM S6 kinase/Rsk2 substrate peptide 2; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] S6 kinase/Rsk2 substrate peptide 2; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PKD2.** Source: Human recombinant insect Sf21 cells; Substrate: 50 µM MAPKAPK; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MAPKAPK; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PLK1.** Source: Human recombinant insect Sf9 cells; Substrate: 20 µG/ml Casein; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Casein; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PRKCG (PKCγ).** Source: Human recombinant insect Sf21 cells; Substrate: 250 µg/ml Histone; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 1 mM EGTA, 10 mM MgCl$_2$, 0.1 mM DTT, 50 µg/ml

Phosphatidylserine, 8 µg/mL Diacylglycerol; Quantitation Method: Quantitation of [$^{32}$P] Histone; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PRKCG (PKCη).** Source: Human recombinant insect Sf9 cells; Substrate: 250 µg/ml Histone; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 1 mM EGTA, 10 mM MgCl$_2$, 0.1 mM DTT, 50 µg/mL Phosphatidylserine, 8 µg/mL Diacylglycerol; Quantitation Method: Quantitation of [$^{32}$P] Histone; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, PRKG1 (PKG1β).** Source: Human recombinant insect Sf9 cells; Substrate: 50 µM BPDEtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$, 5 µM cGMP; Quantitation Method: Quantitation of [$^{32}$P] BPDEtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, RIPK2 (RIP2).** Source: Human recombinant insect Sf21 cells; Substrate: 50 µg/ml Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, RPS6KA2 (RSK3).** Source: Human recombinant insect Sf21 cells; Substrate: 50 µg/ml MAPKAPK substrate peptide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25

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mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MAPKAPK substrate peptide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, RPS6KA5 (MSK1).** Source: Human recombinant insect Sf21 cells; Substrate: 10 µM Crosstide KK; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Crosstide KK; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, SGK1.** Source: Human recombinant insect Sf21 cells; Substrate: 10 µM Crosstide KK; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Crosstide KK; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, SGK2.** Source: Human recombinant insect Sf21 cells; Substrate: 10 µM Crosstide KK; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Crosstide KK; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, STK17A (DRAK1).** Source: Human recombinant insect cells; Substrate: 50 µM ZIPtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM

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Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] ZIPtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, STK3 (MST2).** Source: Human recombinant insect Sf21 cells; Substrate: 50 µg/ml Myelin basic protein (MBP); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] MBP; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Serine/Threonine Kinase, TSSK2 (STK22B).** Source: Human recombinant insect Sf21 cells; Substrate: 10 µM CHKtide; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation Method: Quantitation of [$^{32}$P] CHKtide; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, ABL1 (ABL).** Source: Mouse recombinant E. coli; Substrate: 10 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 25 °C; Incubation Time/Temp: 60 minutes at 25 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT; Quantitation Method: ELISA quantitation of Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, ALK.** Source: Human recombinant insect cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 20 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 25 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, BTK.** Source: Human recombinant insect sf21 cells; Substrate: 200 μg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM $Na_3VO_4$, 1 mM DTT, 2 mM $MnCl_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, CSK.** Source: Human recombinant E. coli; Substrate: 200 μg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM $Na_3VO_4$, 1 mM DTT, 2mM $MnCl_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, EPHA4 (EphA4).** Source: Human recombinant insect sf21 cells; Substrate: 200 μg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM $Na_3VO_4$, 1 mM DTT, 2 mM $MnCl_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, ERBB2 (HER2).** Source: Human recombinant insect sf9 cells; Substrate: 200 μg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 25 °C; Incubation Time/Temp: 5 minutes at 25 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM $Na_3VO_4$, 1 mM DTT; Quantitation Method: ELISA quantitation of Poly(Glu:Tyr-P); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, FER (TYK3).** Source: Human recombinant insect sf21 cells; Substrate: 200 μg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM

HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, FGFR1.** Source: Human recombinant insect sf21 cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, FLT1 (VEGFR-1).** Source: Human recombinant insect sf21 cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, FLT3.** Source: Human recombinant insect sf21 cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, Insulin Receptor.** Source: Human recombinant insect sf9 cells; Substrate: 10 µg/mL Histone H1; Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 25 °C; Incubation Time/Temp: 60 minutes at 25 °C; Incubation Buffer: 50 mM

HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT; Quantitation Method: ELISA quantitation of Histone H1-P; Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, LYN A.** Source: Human recombinant insect cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

**Protein Tyrosine Kinase, MET (HGFR); Protein Tyrosine Kinase, NTRK1 (TRKA); Protein Tyrosine Kinase, PTK2B; Protein Tyrosine Kinase, RET; Protein Tyrosine Kinase, ROS1; Protein Tyrosine Kinase, TEK;Protein Tyrosine Kinase, TYRO3 (RSE);Protein Tyrosine Kinase, ZA70 (ZAP-70).** Source: Human recombinant insect sf21 cells; Substrate: 200 µg/mL Poly(Glu:Tyr); Vehicle: 1% DMSO; Pre-Incubation Time/Temp: 15 minutes at 37 °C; Incubation Time/Temp: 30 minutes at 37 °C; Incubation Buffer: 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, 2 mM MnCl$_2$; Quantitation Method: Quantitation of [$^{32}$P] Poly(Glu:Tyr); Significance Criteria: 50% of max stimulation or inhibition.

## 6.3    *In Vivo* Evaluation of Neo-tanshinlactone Analogue 91

### 6.3.1   Introduction

To examine the efficacy, tumor-tissue-type selectivity, and breast cancer subtype selectivity of **91**, it was tested for *in vivo* activity against human ZR-75-1 breast ductal carcinoma, PC-3 human prostate adenocarcinoma, and MDA-MB-231 breast ductal carcinoma xenografts, with paclitaxel as positive control. This work was performed in cooperation with Professor Che-Ming Teng at National Taiwan University.

### 6.3.2 Methodology[3]

**Mice**

Male (for PC-3) and female (for MDA-MB-231 and ZR-75-1) SCID mice (NTUH Animal Facility) were 5 weeks old, and had a body weight (BW) range of 20-24 g on D1 of the study. The animals were fed ad libitum water (reverse osmosis, 1 ppm Cl) and PicoLab Rodent Diet 20 Modified and Irradiated Lab Diet® consisting of 20.0% crude protein, 9.9% crude fat, and 4.7% crude fiber. The mice were housed on National Taiwan University Laboratory Animal Center, NTUMC, on a 12-hour light cycle at 21–23 °C and 60–85% humidity. Nude-athymic mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines.

**Tumor Cell Culture**

All human cancer cells were maintained in RPMI 1640 medium containing 100 units/mL penicillin G sodium, 100 $\mu$g/mL streptomycin sulfate, 0.25 $\mu$g/mL amphotericin B, and 25 $\mu$g/mL gentamicin. The medium was supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. The cells were cultured in tissue culture flasks in a humidified incubator at 37 °C, in an atmosphere of 5% $CO_2$ and 95% air.

***In Vivo* Implantation and Tumor Growth**

All human cancer cells used for implantation were harvested during log phase growth and resuspended in phosphate-buffered saline at $5 \times 10^7$ cells/mL. Each mouse was injected s.c. in the right flank with $1 \times 10^7$ cells (0.2 mL cell suspension). Tumors were monitored twice weekly and then daily as their volumes approached 80-150 mm$^3$. Tumor size, in mm$^3$, was calculated from:

$$\text{Tumor Volume} = w^2 \times l\,/2$$

where w = width and l = length in mm of the tumor. Tumor weight can be estimated with the assumption that 1 mg is equivalent to 1 mm3 of tumor volume.

**Time to Endpoint (TTE) and Tumor Growth Delay (TGD)**

Each animal was euthanized when the tumors reached the predetermined endpoint size. The time to endpoint (TTE) for each mouse was calculated by the following equation:

$$TTE = \frac{\log_{10} (\text{endpoint volume}) - b}{m}$$

where TTE is expressed in days, endpoint volume is in $mm^3$, b is the intercept, and m is the slope of the line obtained by linear regression of a log-transformed tumor growth data set. The data set was comprised of the first observation that exceeded the study endpoint volume and the three consecutive observations that immediately preceded the attainment of the endpoint volume. The calculated TTE was usually less than the day on which an animal is euthanized for tumor size. Animals that did not reach the endpoint were euthanized at the end of the study, and assigned a TTE value equal to the last day. An animal classified as having died from a treatment-related (TR) or non-treatment-related metastasis (NTRm) cause was assigned a TTE value equal to the day of death. An animal classified as having died from a non-treatment-related (NTR) cause was excluded from TTE calculations.

Treatment efficacy was determined from tumor growth delay (TGD), which is defined as the increase in the median TTE for a treatment group compared to the control group (expressed in days):

$$TGD = T - C,$$

, or as a percentage of the median TTE of the control group:

$$\%TGD = \frac{T - C}{C} * 100$$

where: T = median TTE for a treatment group, C = median TTE for control Group 1.

### 6.3.3 Results and Discussion

We have examined the anticancer activity of compound **91** in several xenograft models, such as PC-3 (androgen-independent human prostate carcinoma cells), MDA-MB-

231 (estrogen receptor negative breast cancer cells), and ZR-75-1 (estrogen receptor positive breast cancer cells). Compound **91** was administered intraperitoneally (i.p.) in a 4% benzyl alcohol/6% cremophor/90% D5W solution and was given at 10 mg/kg every other day to endpoint (qod to end). A positive reference group received paclitaxel i.p. at 20 mg/kg once daily every fourth day for 5 doses (q4d x 5). A control group received vehicle i.p. on a qod to end schedule. As shown in Figure 6-2, the treatment of SCID mice with compound **91** resulted in inhibition of estrogen-positive ZR-75-1 tumor xenograft growth. There was significant reduction in growth of estrogen-positive breast tumors in compound **91**-treated animals as compared with the control group. Treatment results were presented as percent tumor growth delay (%TGD), which is the percent increase in the mean time to endpoint (TTE) for drug-treated versus control mice. Logrank tests determine significance of the differences between TTE values for compound **91**-treated and control mice, at $P \leq 0.05$. In ZR-75-1 xenograft model, the mean TTE for the control group was 15.1 days. Paclitaxel produced a mean TTE of 35.0 days, corresponding to a %TGD of 132. Compound **91** at 10 mg/kg produced a mean TTE of 29.5 days, corresponding to a %TGD of 95 (p = 0.0067, logrank). However, compound **91** could not be evaluable statistically due to toxicity (data not shown). Of the xenografts studied, only estrogen-dependent breast cancer was suppressed by compound **91** treatment. With the PC-3 (androgen-independent human prostate carcinoma cells) and MDA-MB-231 (estrogen receptor negative breast cancer cells), **91** did not exert an effect.

In summary, **91** showed potent inhibition activity against the ZR-75-1 human breast ductal carcinoma xenograft, while it did not display any antitumor activity against PC-3 human prostate adenocarcinoma and MDA-MB-231 breast ductal carcinoma xenografts. The results proved that compound **91** is a selective anti-breast cancer agent *in vivo*. These findings suggest that compound **91** may be selectively used to inhibit the growth of

hormone-dependent breast cancers, particularly regrowth of residual tumor in postmenopausal breast cancer survivors receiving estrogen replacement therapy.
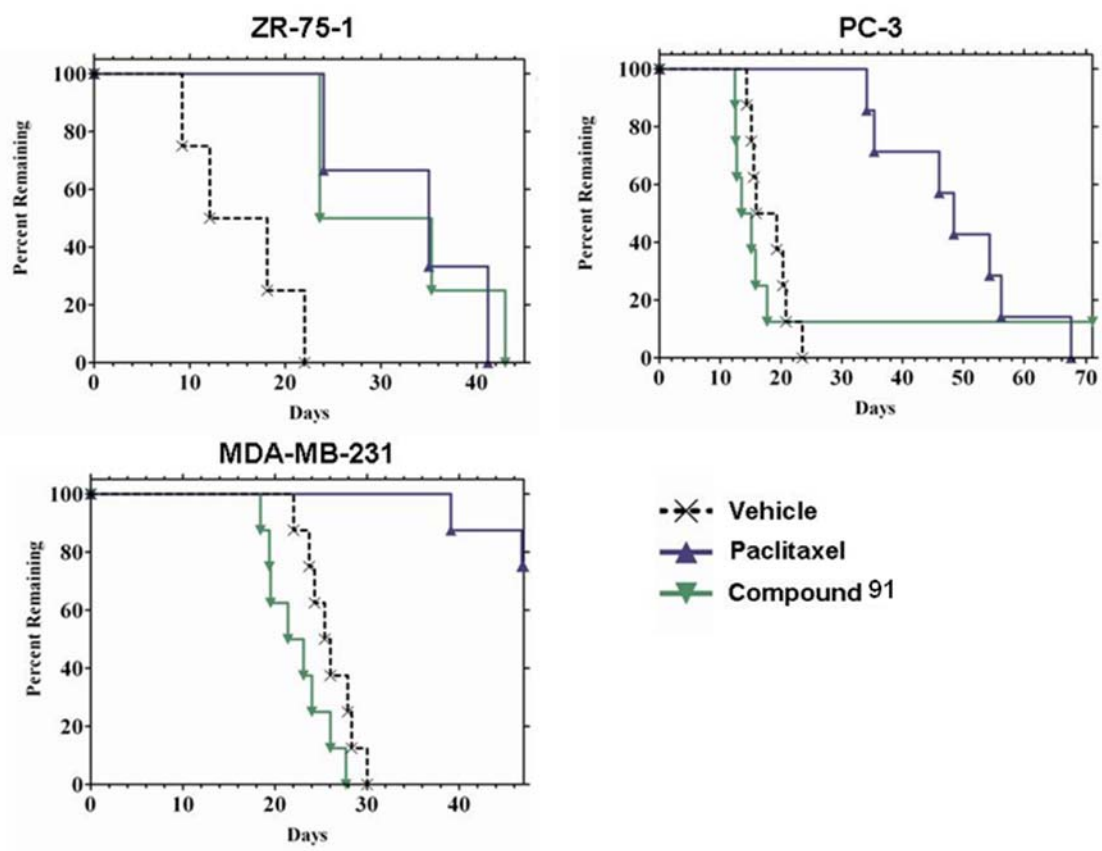


**Figure 6-2.** *In vivo* **anticancer activity of compound 91.**

## 6.4    References

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3.    Wei, L.; Shi, Q.; Bastow, K. F.; Brossi, A.; Morris-Natschke, S. L.; Nakagawa-Goto, K.; Wu, T.-S.; Pan, S.-L.; Teng, C.-M.; Lee, K.-H. Antitumor agents 253. Design, synthesis, and antitumor evaluation of novel 9-substituted phenanthrene-based tylophorine derivatives as potential anticancer agents. *J. Med. Chem.* **2007,** 50, 3674-3680.

# CHAPTER 7

# CONCLUDING REMARKS AND PERSPECTIVES FOR FUTURE STUDIES

## 7.1     Conclusions

### 7.1.1   Elucidation of SAR of New Neo-tanshinlactone Analogues

In this study, the synthetic pathway to 4-ethyl neo-tanshinlactone **91** was optimized by reducing the number of steps and increasing yields. Five series of new neo-tanshinlactone analogues were designed, synthesized, and evaluated in various biological assays as potential anti-breast cancer agents. Lead compounds **108-110** and **113** are shown in Figure 7-1. The SAR conclusions for neo-tanshinlactone analogues can be summarized as follows (Figure 7-1):

(1)     C-4 position is critical for both potency and selectivity. The order of potency with various substituents against SK-BR-3 was ethyl = 2-bromoethyl = propyl > methyl = methoxy > fluoro = hydrogen > isopropyl > ethoxy > hydroxyl, OAc, and 2-dimethylaminoethoxy. Analogues with isopropyl, propyl and methoxy groups showed high selectivity against different breast cancer cell lines.

(2)     Aromatic ring-A > non-aromatic ring-A

(3)    The order of potency for C-17 position was methyl > ethyl > hydrogen, while the order of potency for C-16 position was hydrogen > methyl.

(4)     Pyridinone ring is not favored for ring-B.

(5)     Lactone ring-C is essential for activity.

(6)     Ring-D is preferably an unsaturated furan ring.
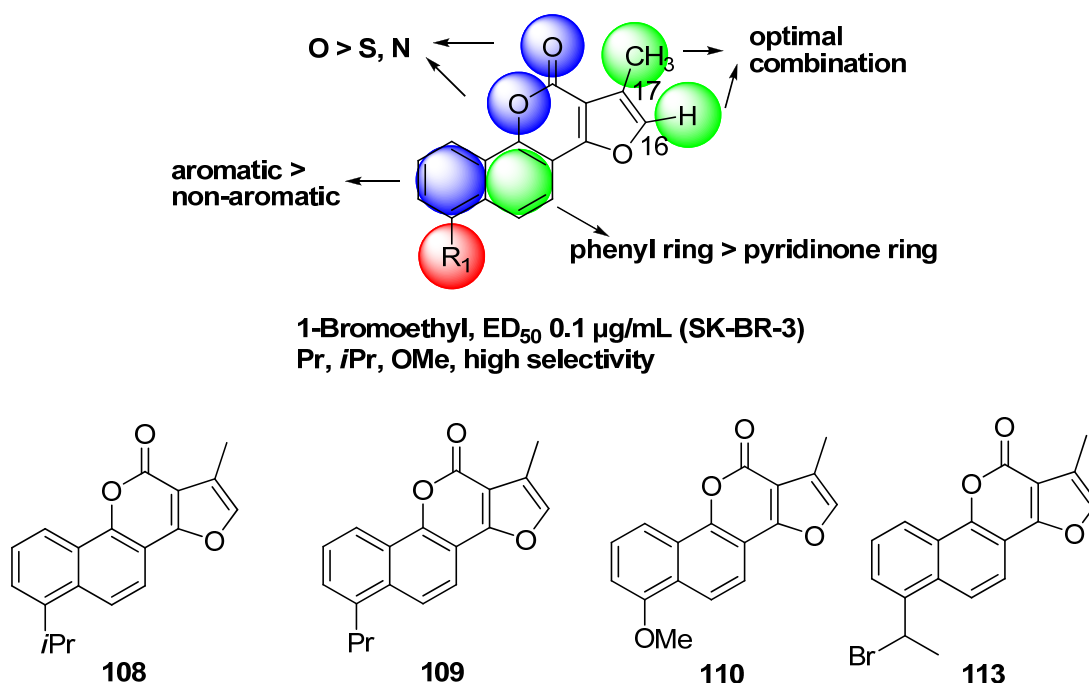
**Figure 7-1. SAR summary of neo-tanshinlactone analogues and lead compounds**

## 7.1.2 Discovery of Novel Compounds with New Scaffolds as Anti-Breast Cancer Agents

We studied the individual contribution of the A-, B-, C-, and D-rings of **90** to the selective activity against breast cancer cells. Scaffolds 1-5 were designed by breaking bonds 1-5 respectively (Figure 7-2). We demonstrated that aromatic rings A and D were important for the cytotoxic activity. Importantly, this study indicated that a simplified neo-tanshinlactone skeleton obtained by removal of ring-B still retained potent activity when the proper functional groups were present. Moreover, we discovered that ring-C could be opened through hydrolysis of the ester bond, without losing the desired biological activity. Based on this study, two novel chemical entities were discovered and developed as selective anti-breast cancer agents.
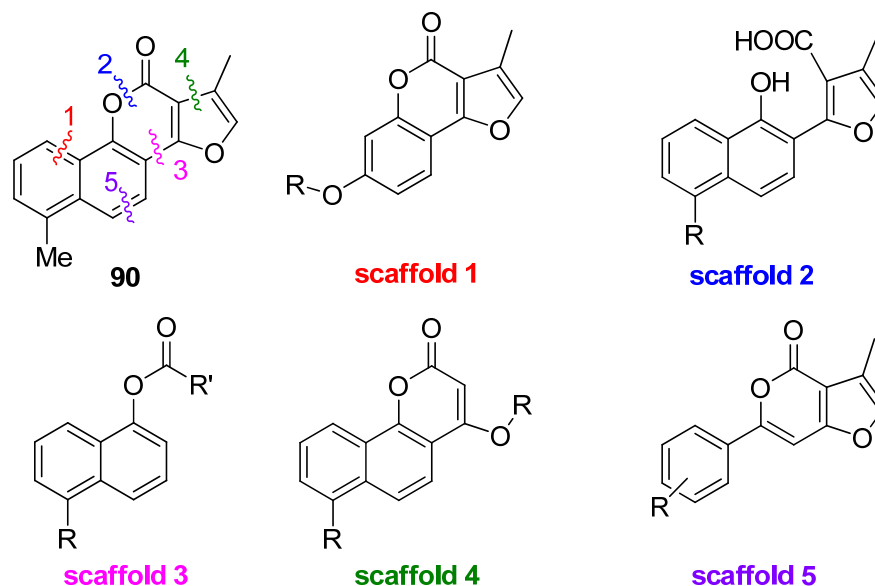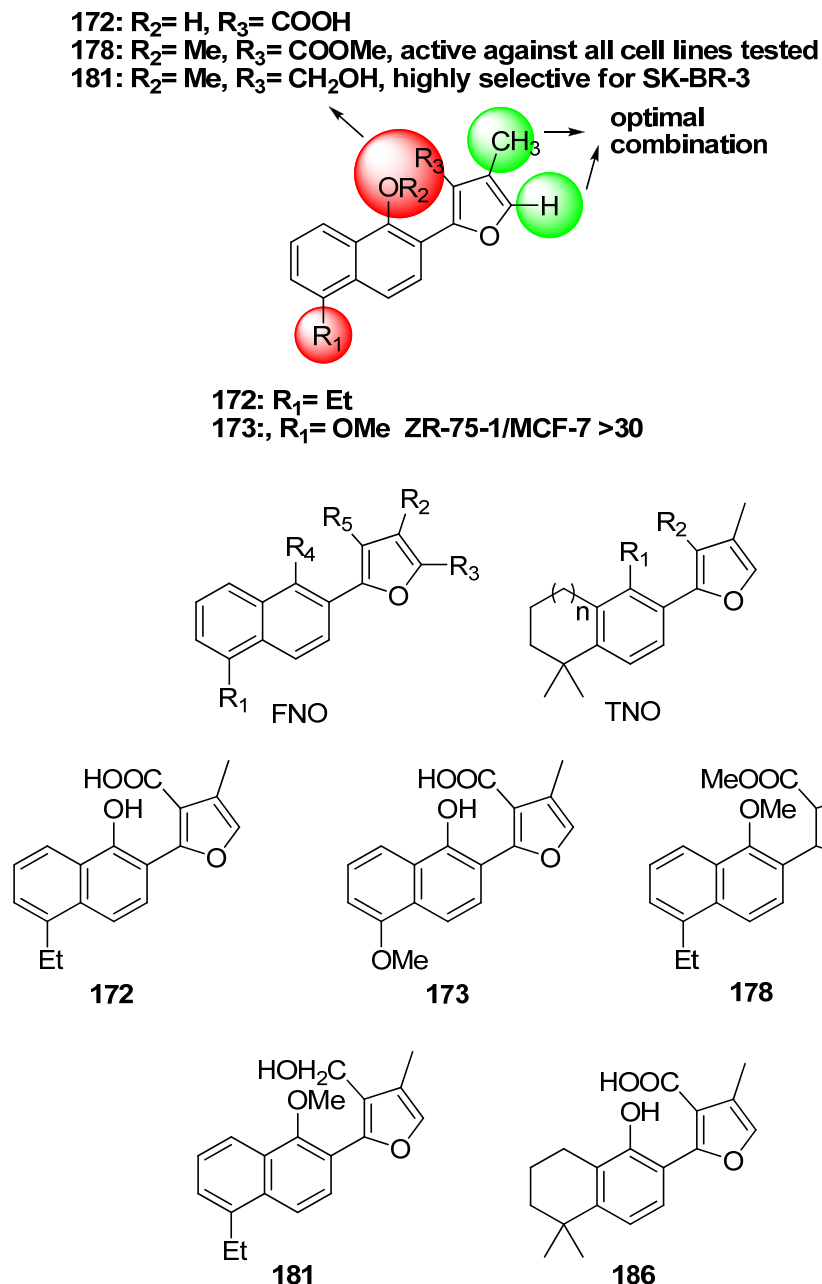
**Figure 7-2. Scaffolds 1-5 derived through structure simplification of neo-tanshinlactone (90)**

### 7.1.2.1 Design, Synthesis, and Biological Evaluation of Novel 2-(Furan-2-yl) naphthalen-1-ol and Tetrahydronaphthalene-1-ol Derivatives as Potent and Selective Anti-Breast Cancer Agents

We designed and developed 2-(furan-2-yl) naphthalen-1-ol (FNO) derivatives, a new class of anti-breast cancer agents. Further optimization led to **172**, **173**, and **181** as new lead compounds with better selectivity than neo-tanshinlactone analogue **91**. Interestingly, compound **178** showed broad *in vitro* cytotoxicity against human cancer cell lines tested, suggesting a different mechanism of action from its structural derivatives. Conformational searches and dihedral energy analyses of **172** and **178** suggested that intramolecular hydrogen bonding was important to form a rigid conformation and improved the *in vitro* anticancer selectivity of **172**. In addition, we further designed tetrahydronaphthalene-1-ol (TNO) derivatives and evaluated their antitumor activity. Compound **186** showed the highest potency with $ED_{50}$ values of 0.23 μg/mL against SK-BR-3 and 0.49 μg/mL against ZR-75-1. These results established two new scaffolds as promising structures for the development of

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investigational anti-breast cancer agents.

172: $R_2$= H, $R_3$= COOH
178: $R_2$= Me, $R_3$= COOMe, active against all cell lines tested
181: $R_2$= Me, $R_3$= CH$_2$OH, highly selective for SK-BR-3



optimal combination

172: $R_1$= Et
173:, $R_1$= OMe  ZR-75-1/MCF-7 >30



FNO

TNO

172

173

178

181

186

**Figure 7-3. SAR summary of 2-(furan-2-yl) naphthalen-1-ol & Tetrahydronaphthalene-**

**1-ol derivatives and lead compounds**

**7.1.2.2 Novel Substituted 6-Phenyl-4*H*-furo[3,2-*c*]pyran-4-one Derivatives as Potent**

**and Highly Selective Anti-Breast Cancer Agents**

Novel substituted 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives were designed and

synthesized as promising anti-breast cancer agents. Promising lead compounds **198-201**, **211**, **213**, and **215** showed potent inhibition against the SK-BR-3 breast cancer cell line. The $ED_{50}$ values of the two most potent analogues (**213** and **215**) against SK-BR-3 were 0.08 and 0.14 µg/mL, respectively. More importantly, **213** and **215** showed extremely high cancer cell line selectivity, being approximately 100- to 250-fold more potent against SK-BR-3 compared with six additional tested cancer cell lines. Preliminary SAR studies led to the following observations.

(1)    3'-Methyl, methoxy, ethoxy, and hydroxy groups, but not a 3'-fluoro group, could increase potency.

(2)    Among di-substituted phenyl compounds, 2'-methyl, 2'-methoxy, 4'-methyl, 4'-methoxy, 5'-methyl, and 5'-hydroxy groups decreased potency; while a 4'-hydroxy or 5'-methoxy group increased potency.

(3)    Current modifications in ring-C and -D were not preferred.
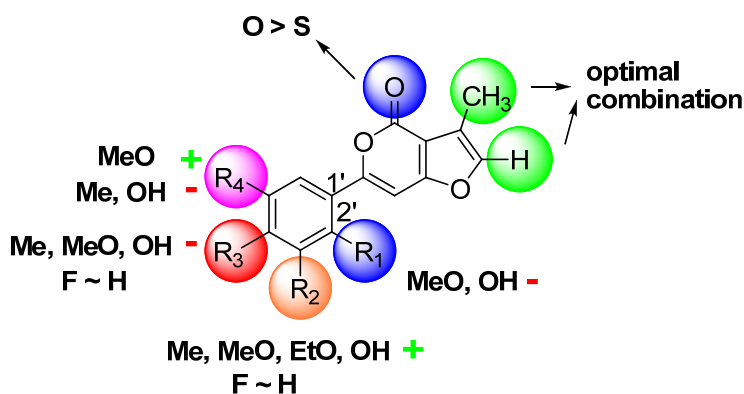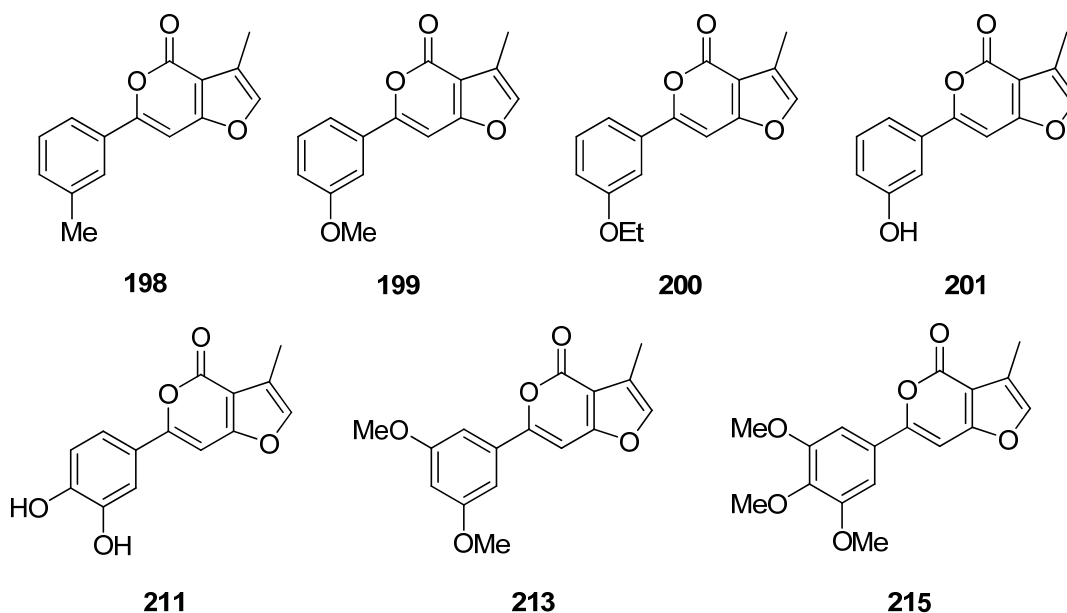
Figure 7-4.SAR summary of 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives and lead compounds

### 7.1.3 Mechanism of Action Studies and Preliminary *in vivo* Evaluation of 4-Ethyl Neo-tanshinlactone (91)

Enzyme assays suggested that **91** significantly suppressed CK2α1, ABL, and AKT1, while **178** showed higher inhibition activity against CDC42BPB, PKG1β, and SGK1. The results will guide our future explorations of the mechanisms of action. In addition, we demonstrated that 4-ethyl neo-tanshinlactone (analogue **91**) is a potent and selective anti-breast cancer agent *in vivo*. Compound **91** at 10 mg/kg showed significant antitumor activity against human ZR-75-1 breast ductal carcinoma xenograft, but was not active against PC-3 human prostate adenocarcinoma and MDA-MB-231 breast ductal carcinoma xenografts.

## 7.2 Perspectives for Future Studies

### 7.2.1 Further Exploration of SAR of Neo-tanshinlactone Analogues

Our further modification will focus on modifying ring-D of neo-tanshinlactone analogues **238-241** to study the SAR and develop new leads. Scheme 7-1 shows the

synthetic methodologies that we will use to achieve these goals. A similar synthetic route has been reported by Hsung's group.[1]

**Scheme 7-1. Synthetic pathway to designed neo-tanshinlactone analogues 238-241**
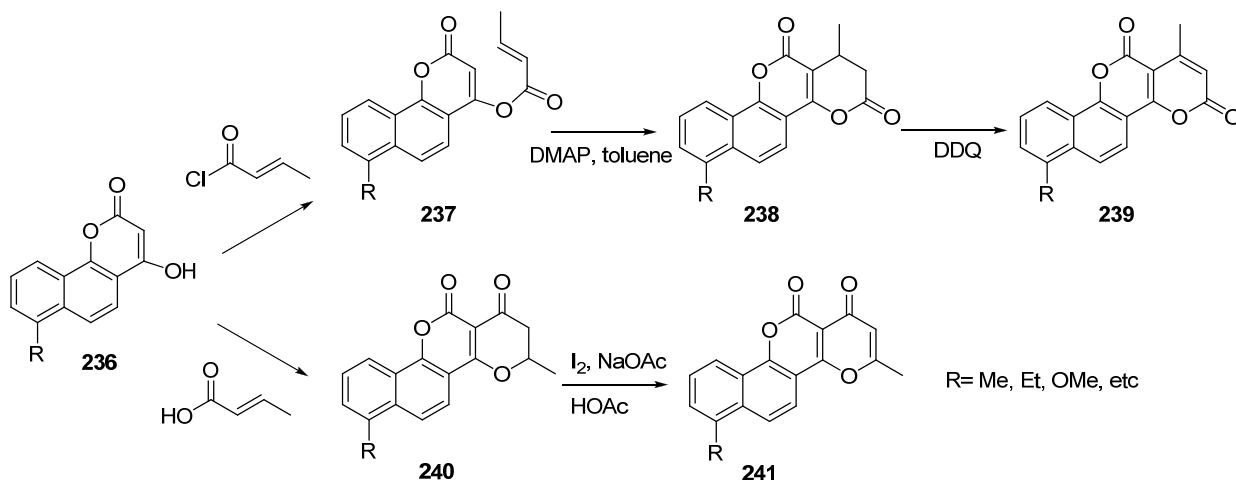


### 7.2.2 Design and Development of Novel 2-(Furan-2-yl) naphthalen-1-ol Derivatives as Anti-Breast Cancer Agents

Previous studies have indicated that the two functional groups (OH and COOH) resulting from an opened lactone ring-C are critical to anti-breast cancer activity and selectivity. We will explore different ether and ester substituents in new analogues **244-247** to find optimal lead compounds (Scheme 7-2).

**Scheme 7-2. Synthetic pathway to designed 2-(furan-2-yl) naphthalen-1-ol and tetrahydronaphthalene derivatives 244-247**

R₁= H, Me, Et, etc
R₂= Me, Et, OAc, etc

243          246          247
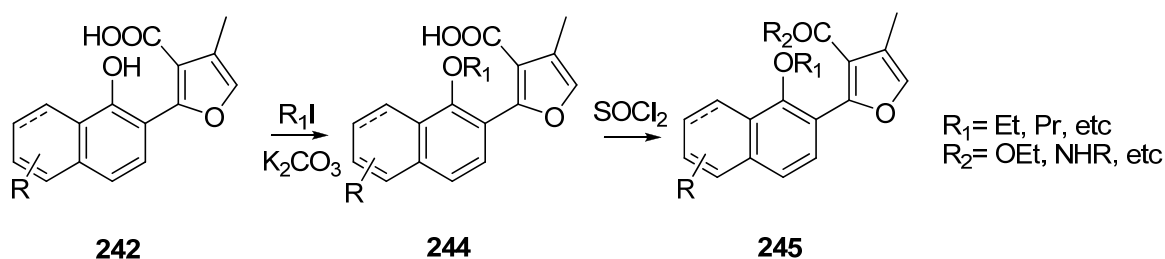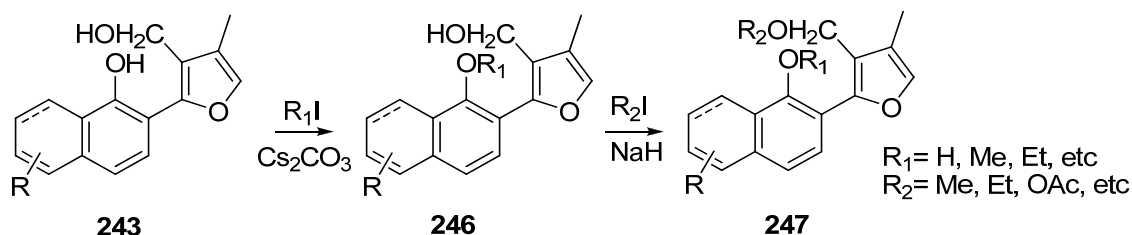
## 7.2.3 Design and Development of Novel Substituted 6-Phenyl-4*H*-furo[3,2-*c*]pyran-4-one Derivatives as Potent and Selective Anti-Breast Cancer Agents

(1) C-6 substituents play important roles in the anti-breast cancer activity of this compound class. To identify the SAR and find optimized substituents at this position, we will introduce different alkyl and heterocyclic groups, including methyl, ethyl, isopropyl, pyridine, and pyrimidine.[2] (2) Our study results also indicated that the orientation, ring size, and dihedral angle between rings could affect the anti-breast activity. We will insert a linker, such as a double bond, to find the best arrangement and combination of the rings. The syntheses of these new compounds are feasible based on prior studies (Scheme 7-3).[3]

**Scheme 7-3. Synthetic pathways to designed 4*H*-furo[3,2-*c*]pyran-4-one derivatives 250 and 255**



248          249          250

R= alkyl, heterocycle
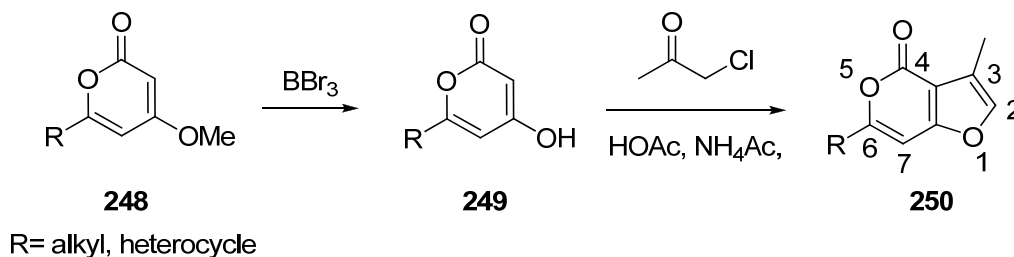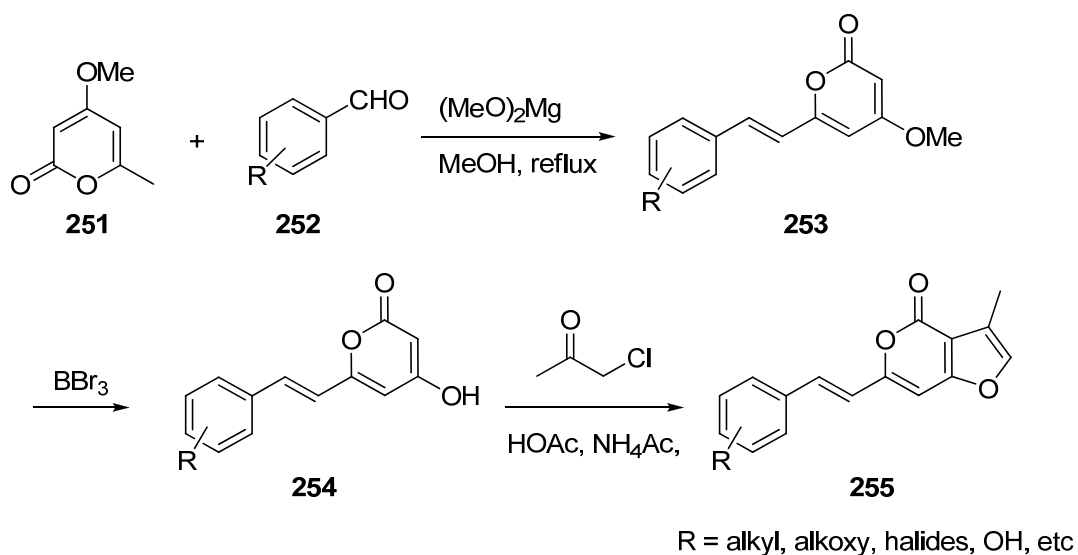
### 7.2.4 Design and Development of Novel Substituted 4-Amino-2*H*-benzo[*h*]chromen-2-one Derivatives as Anticancer Agents

In our most recent study, compounds **256-257** were designed, synthesized, and assayed against a panel of human tumor cell lines. As seen in Table 7-1, **256** showed potent antitumor activities against all cancer cell lines tested, while **257** was only moderately active. The results indicated that 4-amino-2*H*-benzo[*h*]chromen-2-one derivatives might have a different mechanism of action. In addition, Roma et al. reported that similar compounds displayed inhibition activity of DNA synthesis and cell growth in HeLa cell line.[4] More analogues will be synthesized as shown in Scheme 7-4 to discover new leads, establish the SAR, and explore the mechanism of action.[5]

**Table 7-1. Cytotoxicity of compounds 256-257 against tumor cell lines**



**256**, R= H
**257**, R = Et
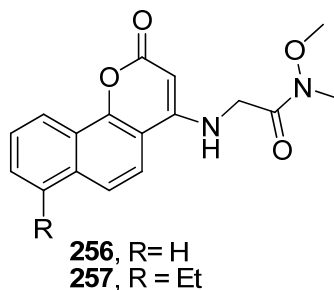
| | SK-BR-3 | ZR-75-1 | MDA MB-231 | A549 | DU145 | KB | KB-vin |
|---|---|---|---|---|---|---|---|
| **256** | 1.41 | 0.60 | 1.25 | 0.98 | 1.00 | 0.70 | 0.94 |
| **257** | 5.9 | 6.2 | >20 | 6.0 | 5.5 | 7.3 | 6.7 |

**Scheme 7-4. Synthetic pathways to designed 4-amino-2*H*-benzo[*h*]chromen-2-one derivatives 259**



R₁= H, Et; R₂, R₃= H, Me, OMe, etc
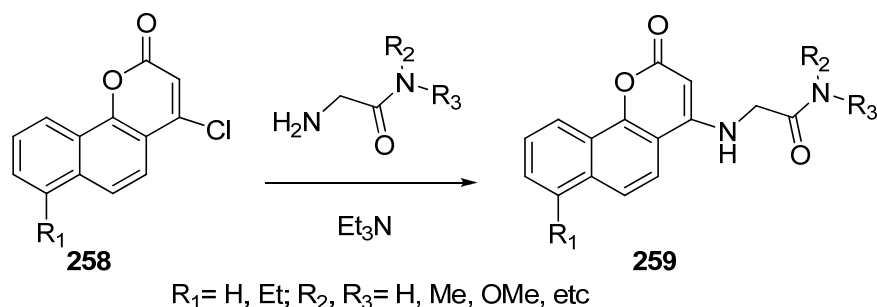
*N*-Methoxy-*N*-methyl-2-(2-oxo-2*H*-benzo[*h*]chromen-4-ylamino)acetamide  (256). 57% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 3.33 (s, 3H, NC$H_3$), 3.82 (s, 3H, OC$H_3$), 4.17 (d, *J* = 3.9 Hz, 1H, NC$H_2$), 5.36 (s, 1H, COC*H*), 7.27-7.72 (m, 4H, aromatic), 7.85-7.88 (m, 1H, aromatic), 8.57-8.61 (m, 1H, aromatic); MS for ([M+H]$^+$): 313.

2-(7-Ethyl-2-oxo-2*H*-benzo[*h*]chromen-4-ylamino)-*N*-methoxy-*N*-methylacetami de (257). 33% yield; $^1$H NMR (300 MHz, CDCl$_3$, ppm): δ 1.39 (t, *J* = 7.5 Hz, 3H, CH$_2$C$H_3$), 3.12 (q, *J* = 7.5 Hz, 2H, C$H_2$CH$_3$), 3.33 (s, 3H, NC$H_3$), 3.82 (s, 3H, OC$H_3$), 4.17 (d, *J* = 3.9 Hz, 2H, NC$H_2$), 5.35 (s, 1H, COC*H*), 6.46 (br, 1H, N*H*), 7.48-7.61 (m, 3H, aromatic), 7.90 (d, *J* = 9.0 Hz, 1H, aromatic), 8.48 (d, *J* = 8.1 Hz, 1H, aromatic); MS for ([M+H]$^+$): 341.

**7.2.5   Mechanism of Action Studies and *In Vivo* Evaluation of Lead compounds 91, 178, and 213 as Anti-Breast Cancer Agents**

Future mechanism of action studies will focus on **91** and **178**. In collaboration with Dr. Eva Lee's laboratory, **91** was tested on both BRCA1/p53 and wild-type mouse models.

Seven to ten days of daily injection of 0.1 mg of **91** dramatically reduced ductal branching of the mammary gland. BRCA1 is the first cloned breast cancer tumor susceptibility gene that is expressed in all cells. BRCA1 mutations mainly lead to breast and ovarian cancers.[6] Based on these results, we will extensively explore the interaction of **91** with progesterone, kinases, and aromatases. Because **178** showed higher inhibition activity against CDC42BPB, PKG1β, and SGK1, further studies will focus on the related pathways. In a collaborative study with Dr. Che-Ming Teng at National Taiwan University, preliminary *in vivo* data demonstrated that compound **91** is a potent and selective anti-breast cancer agent. Compounds **91**, **178**, and **213** have been synthesized on a gram scale, and will be examined *in vivo* for efficacy, toxicity, and dose effect. This study will also be performed in collaboration with Dr. Teng.

### 7.2.6   Ligand-based Drug Design and Development

In our research, we developed five different but related classes of anti-breast cancer agents: neo-tanshinlactone, 2-(furan-2-yl) naphthalen-1-ol (FNO), 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one (AFPO), tetrahydronaphthalene-1-ol (TNO), and 4-amino-2*H*-benzo[*h*] chromen-2-one derivatives (ABO). We established SAR with more than 100 analogues. To construct an informative SAR model, improve further drug design, and discover novel chemical entities with desired pharmacological properties, we will use molecular modeling studies to pursue quantitative structure-activity relationships (QSAR). QSAR is a well-known and useful method for drug discovery and development. Firstly, we will generate models with different QSAR methods, such as kNN,[7] and validate them with test sets. Secondly, the best models will be applied for virtual screening. Meanwhile, the generated QSAR models will be utilized for ligand-based drug design.

## 7.3    References

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