AD_____

Award Number: DAMD17-00-1-0238

TITLE: Development for Dual Acting Inhibitors for Breast Cancer

PRINCIPAL INVESTIGATOR: Pui-Kai Li, Ph.D.

CONTRACTING ORGANIZATION: The Ohio State University
Columbus, Ohio 43210-1239

REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual (3 Jul 00 - 2 Jul 01)

4. TITLE AND SUBTITLE
Development for Dual Acting Inhibitors for Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0238

6. AUTHOR(S)
Pui-Kai Li, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Ohio State University
Columbus, Ohio 43210-1239

E-Mail: li.27@osu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Purpose: To design dual acting inhibitors that can block the enzyme estrone sulfatase (an enzyme involves in the in situ formation of estrogen in breast cancer cells) and act as antiestrogens.

Scope: The design and synthesis of 30 dual inhibitors are proposed. The inhibitors contain 4 different structural core. The synthesized inhibitors will be tested on their ability to inhibit the enzyme estrone sulfatase and also their ability to inhibit the growth of breast cancer cells stimulated by estrone sulfate. In addition, selected inhibitors will be tested in vivo using NMU-induced mammary tumors in rats.

Major findings: More than 50 % (16 out of 30) of the proposed inhibitors have been synthesized. Six out of the 16 inhibitors have been tested for their ability to inhibit estrone sulfatase. activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM of substrate estrone sulfate. All the inhibitors tested so far are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity. It inhibits more than 95% of the sulfatase activity at 20 μM concentration. It is by far the most potent dual inhibitor we have ever obtained. It may serves as an important new lead in search of more potent and effective dual inhibitors for the treatment of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Breast cancer is the most common malignancy in the United States. It is estimated that approximately 30 - 40 % of all breast cancers are estrogen-dependent. Currently, the most common treatments use either antiestrogen or aromatase inhibitors. They are effective in 35-40 % of advanced postmenopausal breast cancer patients. In estrogen-dependent breast cancer patients, the estrogen levels in breast cancer cells are 5-10 times higher than in plasma. One of the possibilities to explain this observation is *in situ* production of estrogens from precursor substrates in the breast cancer cells. One of the pathways for the *in situ* production of estrogen is the conversion of androgens to estrogens by the enzyme aromatase (aromatase pathway). Another pathway for the *in situ* formation of estrogen is through the conversion of estrone sulfate to estrone by the enzyme estrone sulfatase (estrone sulfatase pathway). It has been pointed out that the estrone sulfatase pathway is significant and produce 10 times more estrogen than through the aromatase pathway in breast cancer cells. In addition, estrone sulfatase is also responsible for the conversion of dehydroepiandrosterone sulfate to androst-5-ene-3β,17β-diol, another estrogenic steroid in the body. Thus, potent estrone sulfatase inhibitors are potential agents for the treatment of estrogen-dependent breast cancer. Preliminary studies demonstrated that estrone sulfatase inhibitor can block the growth of NMU-induced tumor in rat stimulated by estrone sulfate. Thus the current approach is to design dual acting inhibitors that can not only block the estrone sulfatase pathway, but also act as antiestrogens. The proposed dual acting inhibitors will have advantage over the current drug treatments. The inhibitors will not only block the formation of estrogen, but also block the stimulatory effect of estrogen on cancer cells. This proposal will design and synthesize of dual acting inhibitors with sulfatase inhibitory and anti-estrogenic activity. The synthesized inhibitors will be tested using enzyme inhibition and cell culture assays. Finally, In vivo studies of dual acting inhibitors using NMU-induced mammary tumor in rats will be performed.

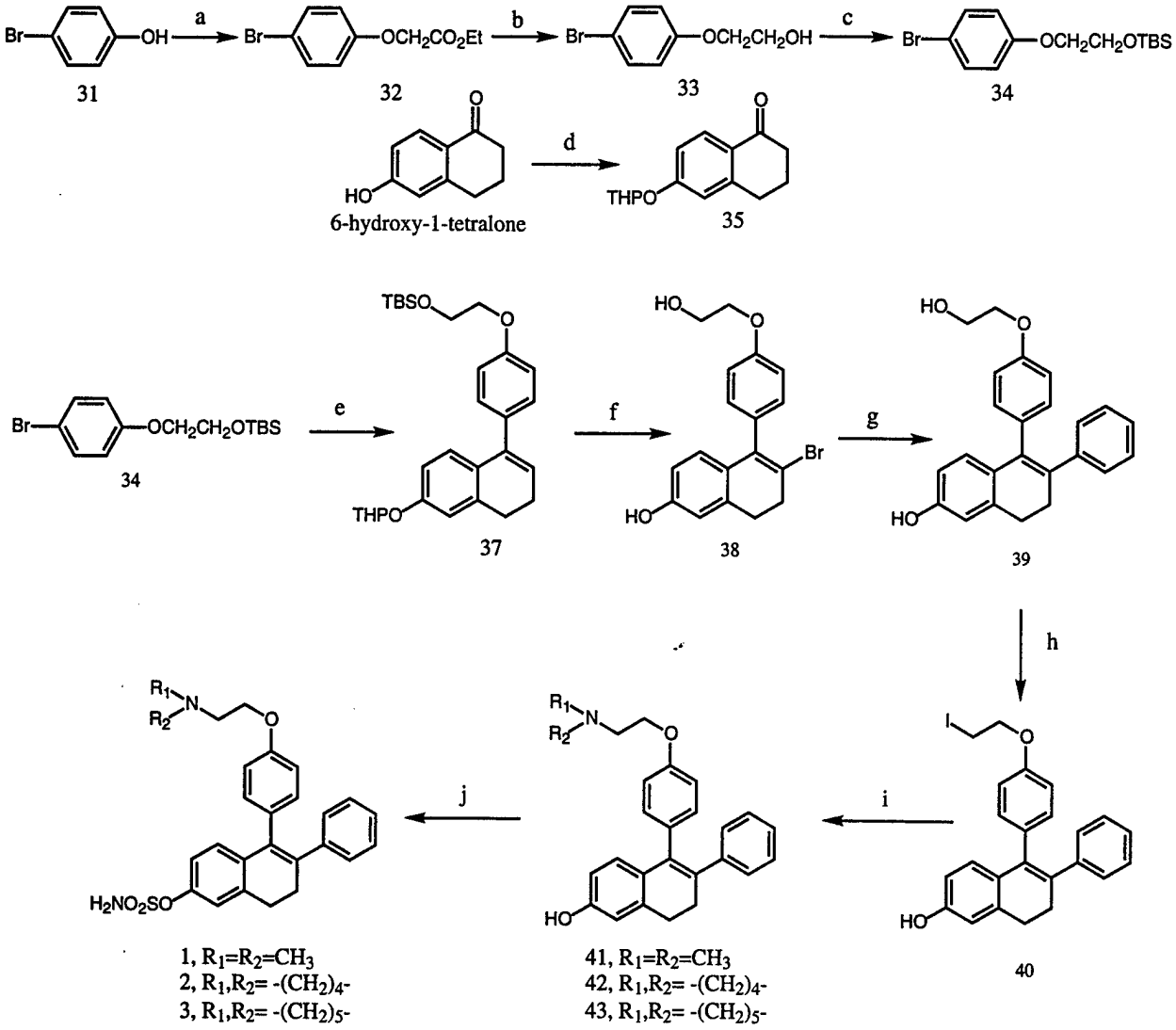
Body

As stated in the introduction, this proposal deals with the design, synthesis and biological testings of dual inhibitors with sulfatase inhibitory and anti-estrogenic activities. A total of 30 inhibitors were proposed. We have successfully synthesized 16 inhibitors and have also started the enzyme inhibitory studies of the synthesized inhibitors.

Synthesis of inhibitors 1-3

The synthesis of inhibitors **1-3** is shown in scheme 1.

Scheme 1. Synthesis of Inhibitors 1-3



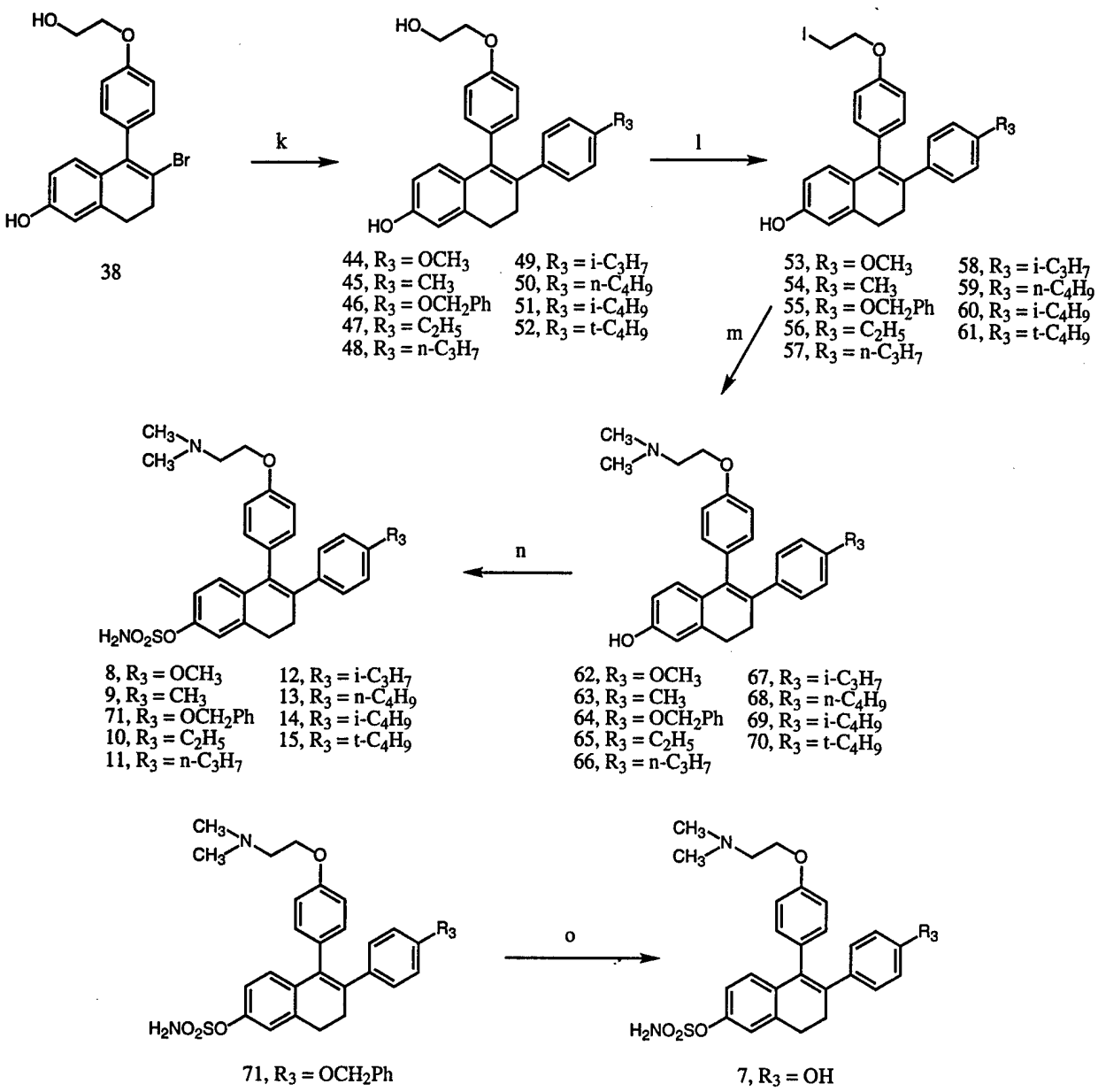
Reagents and Conditions: a. $BrCH_2CO_2Et$, K_2CO_3, acetone, reflux 2.5 h, 99.3 %; b. $LiAlH_4$, THF, r.t, 2 h; c. TBSCl, Imidazole, DMF, r.t, overnight, 96.4 % for 2 steps; d. Dihydropyran, PPTs, CH_2Cl_2, r.t, 2.5 h, 98 %; e. i) n-BuLi, THF, -78°C, 45 min; ii) **35**, -78°C to r.t, 3 h; iii) SiO_2, CH_2Cl_2, r.t, overnight, 65.7% based on **35**; f. i) $C_5H_5N.HBr_3$, CH_2Cl_2, 0°C, 1.5 h; ii) 2N HCl, THF, r.t, 1.5 h, 90.3 %; g. Ph-ZnCl, $Pd(PPh_3)_4$, THF, reflux, 2.5 h, 91 %; h. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 93 %; i. $(CH_3)_2NH$, pyrrolidine or piperidine, K_2CO_3, THF, r.t, 20 h, 88.3 - 91%; j. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t., 1 h, 91-94%.

The synthesis of inhibitors **1 - 3** is summarized in scheme 1. Reaction of 4-bromophenol **31** with ethyl bromoacetate gave ester **32** (99.3 %). Reduction of **32** with $LiAlH_4$ followed by protection of the resulting alcohol **33** as TBS ether yielded compound **34** (96.4 % for 2 steps). Treatment of **34** with n-butyllithium , then with ketone **35** which was prepared by tetrahydropyranylation of 6-hydroxy-1-tetralone (98%), followed by dehydration of the resulting tertiary alcohol with silica gel, afforded olefin **37** (65.7% based on **35**). Bromination of compound **37** with pyridinium tribromide followed by acidic hydrolysis furnished the vinyl bromide **38** (90.3%). Palladium catalyzed coupling (ref 1) of compound **38** with phenyl zinc chloride which was prepared by the treatment of the corresponding phenylbromide with n-butyllithium followed by zinc chloride, gave compound **39** (91%). Iodination of alcohols **39** with I_2/PPh_3/Imidazole yielded the iodide **40** (93%). Reaction of compound **40** with dimethylamine, pyrrolidine and piperidine gave the corresponding amines **41-43** respectively (88.3 –91%). Sulfamoylation (ref 2) of **41-43** with sulfamoyl chloride in the presence of hinder base: 2,6-di-tert-butyl-4-methyl pyridine, yielded the target compounds **1 - 3**.

Synthesis of inhibitors 7 – 15

The synthesis of inhibitors **7 – 15** is shown in scheme 2. Compound **38** in scheme 1 was used as the intermediate in the synthesis of inhibitors **7 – 15**. Palladium catalyzed coupling of compound **38** with various para-substituted phenyl zinc chlorides which were prepared by the treatment of the corresponding substituted phenylbromides with n-butyl lithium followed by zinc chloride, gave compounds 44 - 52 (91-94%). The remaining steps m and o (scheme 2) are similar to the steps i and j described in scheme 1 with high yields. Inhibitor **7** was obtained from sulfamate **71** through hydrogenation.

Scheme 2. Synthesis of inhibitors 7 - 15

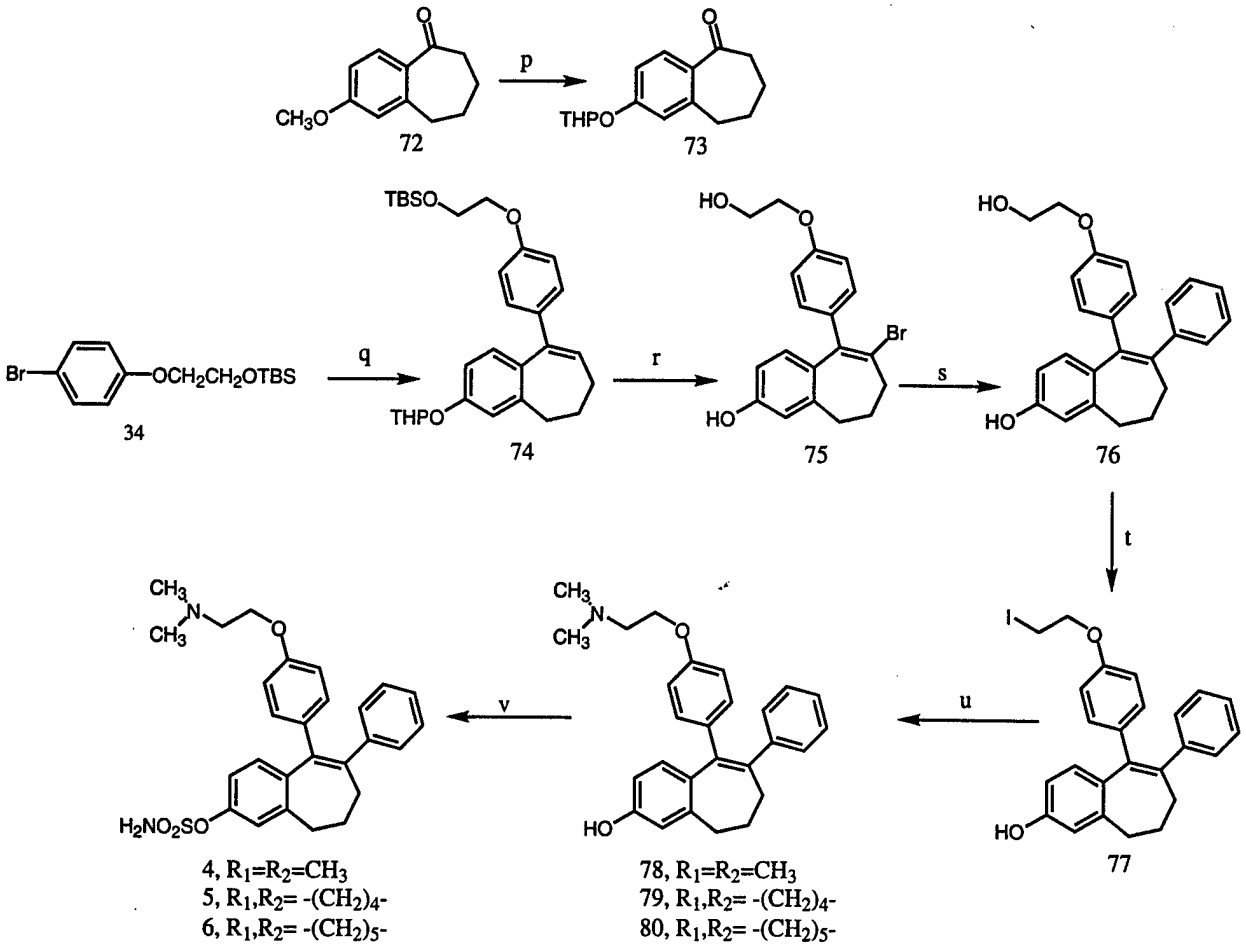


Reagents and Conditions: k. R-Ph-ZnCl (R = OCH_3, CH_3, OCH_2Ph, C_2H_5, n-C_3H_7, i-C_3H_7, n-C_4H_9, i-C_4H_9, t-C_4H_9), $Pd(PPh_3)_4$, THF, reflux, 2.5 h, 86 - 93 %; l. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 92 - 96 %; m. $(CH_3)_2NH$, K_2CO_3, THF, r.t, 20 h, 86-92%; n. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t, 1 h, 87-91%; O. H_2, 10% Pd/C, CH_2Cl_2-CH_3OH (3:1), r.t, 1 h, 77 %.

Synthesis of inhibitors 4 – 6

The synthesis of inhibitors **4 – 6** are shown in scheme 3. Inhibitor 4 – **6** are analogs of inhibitors **1 – 3** with a benzocycloheptene nucleus instead of a dihydronaphthalene. Compound **71** is one of the starting material which was prepared from m-anisaldehyde in 3 steps by the published procedure (ref 3). The methyl group in **72** was replaced by a tetrahydropyranyl group to form compound **73.** Treatment of **34** with n-butyllithium , then with ketone **73**), followed by dehydration of the resulting tertiary alcohol with silica gel, afforded olefin **74** (57.5 % based on **73**). Bromination of compound **74** with pyridinium tribromide followed by acidic hydrolysis furnished the vinyl bromide **75** (90.3%). The remaining steps s to v are similar to the steps g – j in scheme 1 with high yield.

Scheme 3. Synthesis of Inhibitors 4-6

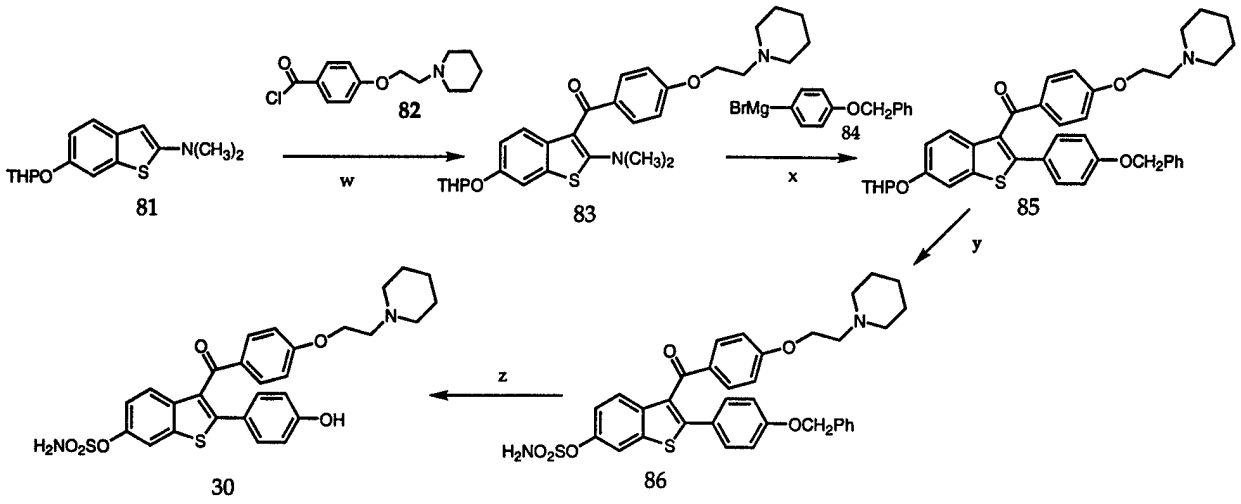


Reagents and Conditions p. i) BBr_3, CH_2Cl_2, r.t, 4 h; ii) Dihydropyran, PPTs, CH_2Cl_2, r.t, 2.5 h, 98 %; q. i) n-BuLi, THF, -78°C, 45 min; ii) **74**, -78°C to r.t, 3 h; iii) SiO_2, CH_2Cl_2, r.t, overnight, 57.5 % based on **74**; r. i) $C_5H_5N.HBr_3$, CH_2Cl_2, 0°C, 1.5 h; ii) 2N HCl, THF, r.t, 1.5 h, 92.6 %; s. Ph-ZnCl, $Pd(PPh_3)_4$, THF, reflux, 2.5 h, 87.1 %; t.. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 96 %; u. $(CH_3)_2NH$, pyrrolidine or piperidine, K_2CO_3, THF, r.t, 20 h, 82.2 – 90.3%; v. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t, 1 h, 78 – 89%.

Synthesis of inhibitor 30

The synthesis of inhibitor is shown in scheme 4. The synthesis begins with the coupling of compounds **81** and **82** in chlorobenzene through reflux yielded **83**. Compound **81** and **82** were synthesized according to the published procedure (ref 4). Reacting **83** with p-benzyloxyphenyl magnesium bromide **84** in THF obtained compound **85**. Deprotection followed by sulfamoylation afforded sulfamate **86**. The sulfamoylation procedure used in schemes 1 to 3 with chlorosulfonamide and hinder base in dichloromethane afforded very low yield (< 10%). It was probably due to low solubility of **85** in dichloromethane. A new sulfamoylation procedure reported by Okada et al. resulted in much improved yield 77%. Hydrogenation of **86** yielded inhibitor **30**.

Scheme 4 Synthesis of inhibitor 30

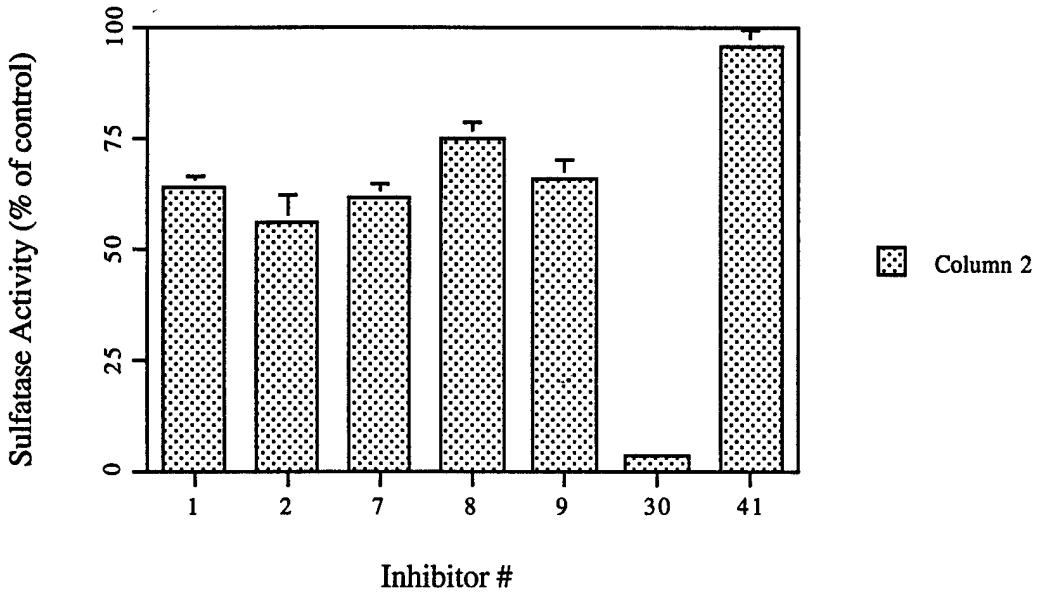


Reagents and Conditions: w. **82**, PhCl, reflux, 4 hr (73%); x. **84**, THF, 0°C to r.t , 2 h, 54%; y. i) 2N HCl, THF, r.t, 1.5 h; ii) $ClSO_2NH_2$, N,N-dimethylacetamide, r.t, 2 h, 77%; z. H_2, 10% Pd/C, CH_2Cl_2-CH_3OH (3:1), r.t, 1 h, 82 %.

Enzyme Inhibition studies of inhibitors

Of the 16 inhibitors (**1 – 15** and **30**) we have synthesized, inhibitors **1,2, 7 – 9** and **30** were tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. Table 1 shows the relative inhibition of estrone sulfatase by the inhibitors. All the inhibitors significantly inhibited estrone sulfatase activity. The sulfamate moiety is essential for sulfatase inhibition since compound **41**, the precursor of inhibitor **1**, did not show sulfatase inhibitory activity (Table 1). Varying the nature of the substituents in R_3 (H, CH_3, OCH_3, OH) has little effect on the sulfatase inhibitory activity. In addition, replacing the dimethylamino moiety in inhibitor **1** with a pyrrolidinyl group to form **2** also has no effect on sulfatase inhibitory activity. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity. It inhibits more than 95% of the sulfatase activity at 20 μM concentration. It is by far the most potent dual inhibitor we have ever obtained.

Table 1



Key Research Accomplishment

1. More than 50 % (16 out of 30 proposed inhibitors) of the proposed inhibitors have been synthesized

2. Six out of the 16 inhibitors have been tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. The inhibitors belong to the nafoxidine and raloxifene structural classes. Both classes of inhibitors showed significant inhibition of estrone sulfatase (Table 1). All the inhibitors tested so far are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity. It inhibits more than 95% of the sulfatase activity at 20 μM concentration. It is by far the most potent dual inhibitor we have ever obtained. It may serves as an important new lead in search of more potent and effective dual inhibitors for the treatment of breast cancer.

Reportable Outcomes

1. A manuscript is being prepared on the synthesis and sulfatase inhibitory activities of dual inhibitors with nafoxidine nucleus. In addition, an abstract will be submitted to the next national meeting of the American Chemical Society.

2. Currently a research associate is involved in the synthesis of and a second year graduate student on the biological testing of the inhibitors.

Conclusions:

Six out of the 16 synthesized inhibitors have been tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. The inhibitors belong to the nafoxidine and raloxifene structural classes. Both classes of inhibitors showed significant inhibition of estrone sulfatase (Table 1). All the inhibitors tested so far are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity. It inhibits more than 95% of the sulfatase activity at 20 μM concentration. It is by far the most potent dual inhibitor we have ever obtained. Since there is only one inhibitor (raloxifene sulfamate – inhibitor **30**)

Reference

1. R. McCague, G. Leclercq and C. Jordan. Nonisomerizable analogues of (Z)- and (E)-4-hydroxytamoxifen. Synthesis and endocrinological properties of substituted diphenylbenzocycloheptenes. J. Med. Chem. 31: 1285-1290 (1988).

2. P.K. Li, S. Milano, L. Kluth and M.E. Rhodes. Synthesis and Sulfatase Inhibitory Activities of Nonsteroidal Sulfatase Inhibitors. J Steroid Biochem. Molec. Biol. 59: No. 1, 41-48 (1996).

1. M.G. Hicks, G. Jones, H. Sheikh. The synthesis and photochemical behavior of some annelated tropones. J. Chem. Soc., Perkin Trans. 1, 10: 2297-304 (1984).

2. T. A. Grese L.D. Pennington, J.P. Sluka, M.D. Adrian, H.W. Cole, T.F. Fuson, D.E. Magee, D.L. Philips, E.R. Rowley, P.K. Shelter, L.L. Short, M. Venugopalan, N.N. Yang, M.Sato. A.L. Glasebrook and H.U. Bryant. Synthesis and Pharmacology of conformationally restricted raloxifene analogue: highly potent selective estrogen receptor modulators. J. Med. Chem. 41: 1272-1283 (1998).