AD ________________

Award Number: DAMD17-00-1-0238

TITLE: Development of 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 2002

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 2002	Annual (3 Jul 01 - 2 Jul 02)

4. TITLE AND SUBTITLE
Development of 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
breast cancer, dual inhibitors

15. NUMBER OF PAGES
15

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

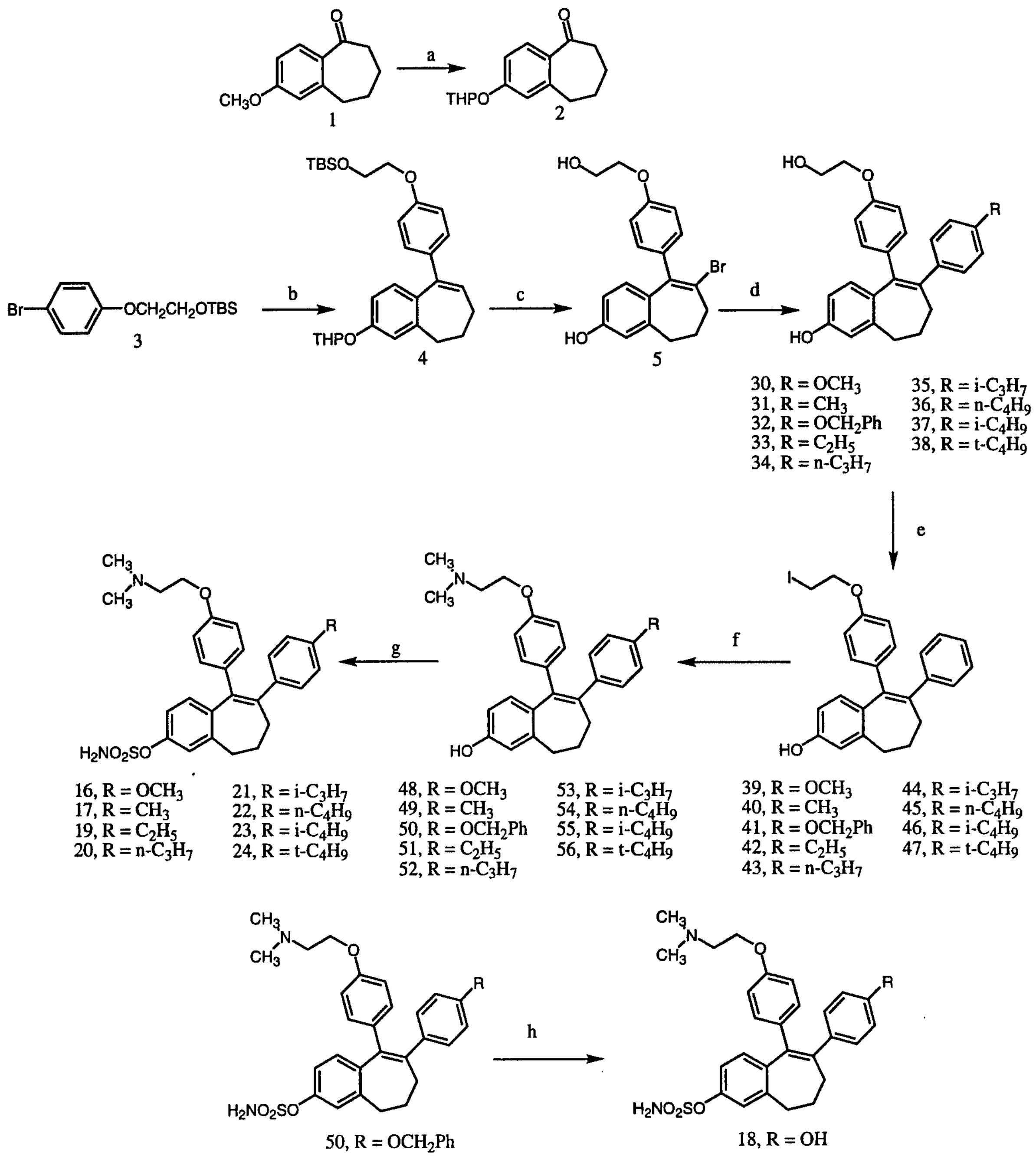
As stated in the introduction, this proposal deals with the design, synthesis and biological testings of dual inhibitors with sulfatase inhibitory and anti-estrogenic activities. A total of 30 inhibitors are proposed. In the first year we have synthesized 16 inhibitors (inhibitors **1-15** and **30** stated in the proposal). This year, we have completed the synthesis of inhibitors **16-24** proposed in the grant. We have completed 83 % of the proposed compounds. The synthesis of the remaining compounds (inhibitors **25-29**) should be completed in the near future.

The syntheses of inhibitors **16-24** are shown below (Fig. 1).

The syntheses of inhibitors **16-24** are shown in Fig. 1. Inhibitors **16-24** are analogs of inhibitors **7 – 15** with a benzocycloheptene nucleus instead of a dihydronaphthalene. Compound **1** is one of the starting material which was prepared from m-anisaldehyde in 3 steps by the published procedure (ref 1). The methyl group in **1** was replaced by a tetrahydropyranyl group to form compound **2.** Treatment of **3** with n-butyllithium , then with ketone **2**), followed by dehydration of the resulting tertiary alcohol with silica gel, afforded olefin **4** (57.5 % based on **4**). Bromination of compound **4** with pyridinium tribromide followed by acidic hydrolysis furnished the vinyl bromide **5** (92.6 %). Palladium catalyzed coupling of compound **5** 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 **30-38** (84-90%). Iodination of alcohols **30-38** with I_2/PPh_3/Imidazole yielded the iodides **39-47** (90-94%). Reaction of compound **39-47** with dimethylamine gave the corresponding amines **48-56** respectively (80-85 –91%). Sulfamoylation (ref 2) of **48-49, 51-56** with sulfamoyl chloride in the presence of hinder base: 2,6-di-tert-butyl-4-methyl pyridine, yielded the target compounds **16-17, 19-24**, respectively (81-87%). Hydrogenation of compound **50** yielded inhibitor **18** (87%).

The syntheses of inhibitors **16-24** are similar to the synthesis of inhibitors **7-15** and we did not come across any difficult.

Figure 1. Synthesis of Inhibitors 16-24



Reagents and Conditions a. i) BBr_3, CH_2Cl_2, r.t, 4 h; ii) Dihydropyran, PPTs, CH_2Cl_2, r.t, 2.5 h, 98 %; b. i) n-BuLi, THF, -78°C, 45 min; ii) **4**, -78°C to r.t, 3 h; iii) SiO_2, CH_2Cl_2, r.t, overnight, 57.5 % based on **4**; c. 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 %; d. 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, 84 - 90 %; e. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 90 - 94 %; f. $(CH_3)_2NH$, K_2CO_3, THF, r.t, 20 h, 80-85%; g. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t, 1 h, 81-87%; h. H_2, 10% Pd/C, CH_2Cl_2-CH_3OH (3:1), r.t, 1 h, 87 %.

Enzyme Inhibition studies of inhibitors

Inhibitors **16-24** were tested for their abilities to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. The sulfatase inhibitory activities of the inhibitors are similar to inhibitors **7-15**. At 20 μM inhibitor concentration, the % inhibiton of sulfatase activity activity range from 35- 55%. Raloxifene sulfamate (inhibitor **30**) is still the most potent inhibitor among all the inhibitors we synthesized (over 95% inhibition) at the same inhibitor concentration. We have synthesized 4 grams of the inhibitors for in vivo anti-tumor study.

Key Research Accomplishment

More than 83% (26 out of 30) of the proposed inhibitors have been synthesized 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. We have synthesized 4 grams of the inhibitors for in vivo antitumor study.

Reportable Outcomes

1. A manuscript has been prepared on the synthesis and sulfatase inhibitory activities of dual inhibitors with nafoxidine nucleus (please refer to the appendix)

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

Conclusions:

Twenty-five out of the 30 proposed inhibitors have been synthesized and 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, benzocycloheptene and raloxifene structural classes. All the inhibitors showed significant inhibition of estrone sulfatase and are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity and has been chosen to be one of the compounds for in vivo anti-tumor study. We have synthesized 4 grams of the compound.

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).

SYNTHESIS AND SULFATASE INHIBITORY ACTIVITIES OF CONFORMATIONAL RESTRICTED ANALOGS OF (E) -4-HYDROXYTAMOXIFEN SULFAMATE

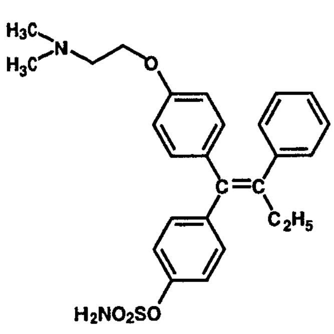
Guo-Hua Chu[+], Heidi Kabler, Kyle W. Selcer[#], Jiandong Shi, Zhigen Hu[+] and Pui-Kai Li[+*]

[+]*Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus OH, 43210*

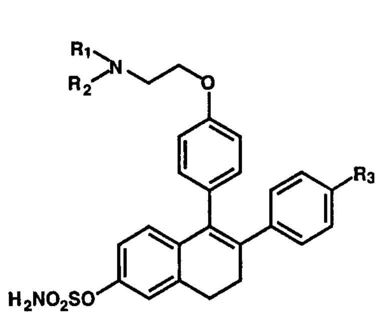
[#]*Department of Biology, Bayer School of Natural and Environmental Sciences, Duquesne University, Pittsburgh PA 15282*

Abstract: Eight conformational restricted analogs of (E)-4-hydroxytamoxifen sulfamate are synthesized as estrone sulfatase inhibitors. All the inhibitors significantly inhibited estrone sulfatase activity. Varying the nature of the substituents in R_3 (H, CH_3, OCH_3, OH) has little effect on the sulfatase inhibitory activity. However, inhibitors with pyrrolidinyl group consistently exhibit higher sulfatase inhibitory activities than the inhibitors with dimethylamino groups.

There is substantial evidence that breast tumors in post-menopausal women accumulate high concentration of estrogens[1,2] and possibly through conversion of estrone sulfate to estrone by the enzyme estrone sulfatase.[3,4] Several estrone sulfatase inhibitors (both steroidal and non-steroidal) have been developed as potential agents for the treatment of estrogen-dependent breast cancers.[5-26] Since the pharmacophore for sulfatase inactivation is a phenylsulfamoyl group, it occurs to us that a potent antiestrogen such as (Z) 4-hydroxytamoxifen can be easily converted to the respective sulfamate analog and becomes potential dual inhibitor (inhibitor with sulfatase inhibitor activity and antiestrogenic activity). Recently, we have synthesized (E) 4-hydroxytamoxifen sulfamate (Fig. 1), the sulfamoylated analog of the potent antiestrogen (Z) 4-hydroxytamoxifen, and demonstrated that it is four fold better than the substrate estrone sulfate in binding to estrone sulfatase.[27] However, the potent antiestrogen (Z)-4-hydroxytamoxifen has been shown in vitro to isomerize into a mixture of Z and E isomer.[28] (E)-4-Hydroxytamoxifen is estrogenic [101, 102].[29,30] Since the conjugation of the hydroxy group in (Z)-4-hydroxytamoxifen with the central double bond is responsible for the facile isomerization [103].[30], one method to fix the configuration of the double bond is incorporating it into a ring such as in nafoxidine. Thus, inhibitors **1 - 8** were synthesized as conformational restricted analogs of (E) 4-hydroxytamoxifen sulfamate.

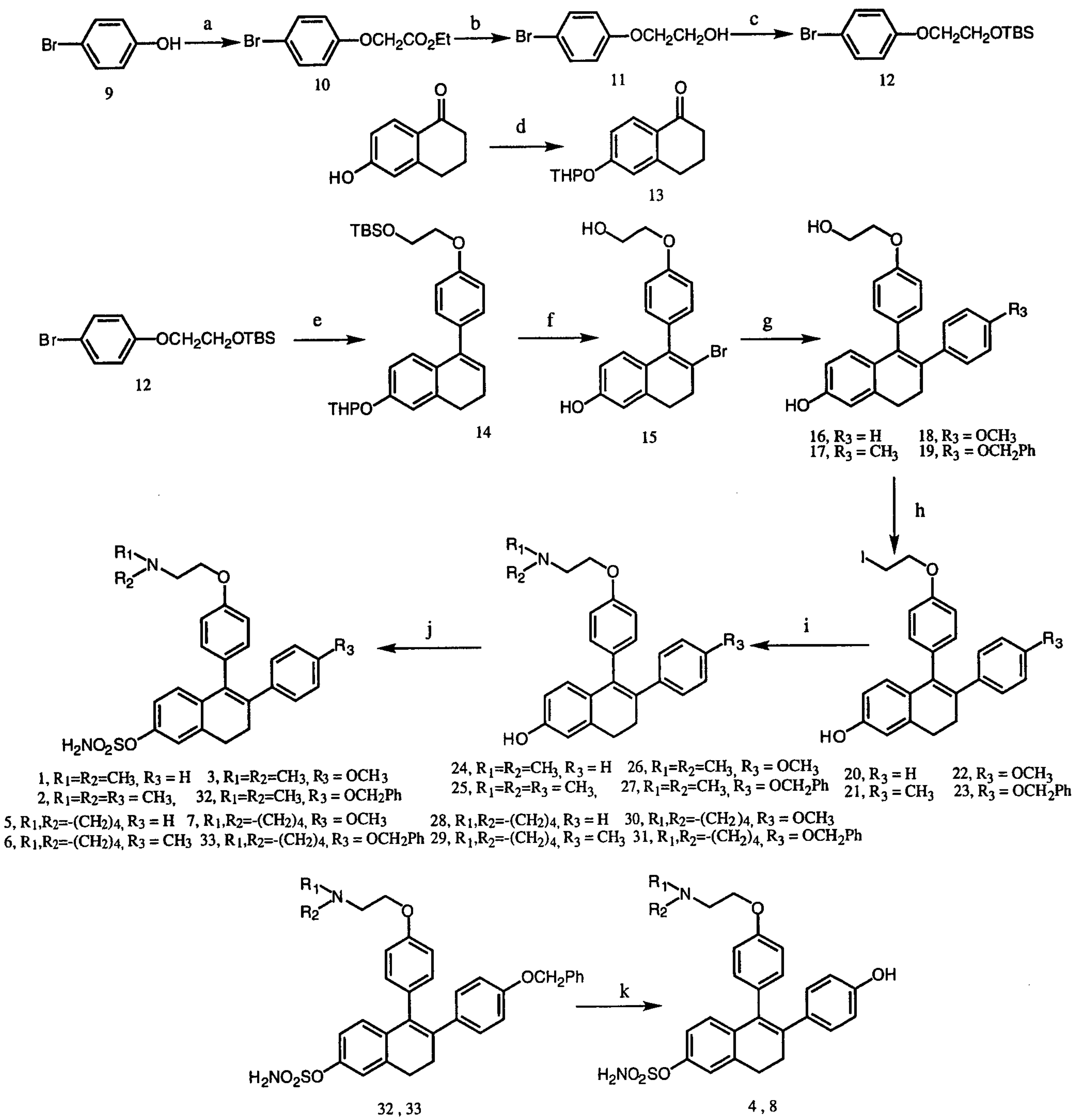


(E)-4-Hydroxytamoxifen sulfamate



Compound	R_1	R_2	R_3
1	CH_3	CH_3	H
2	CH_3	CH_3	CH_3
3	CH_3	CH_3	OCH_3
4	CH_3	CH_3	OH
5	- $(CH_2)_4$ -		H
6	- $(CH_2)_4$ -		CH_3
7	- $(CH_2)_4$ -		OCH_3
8	- $(CH_2)_4$ -		OH

Scheme 1

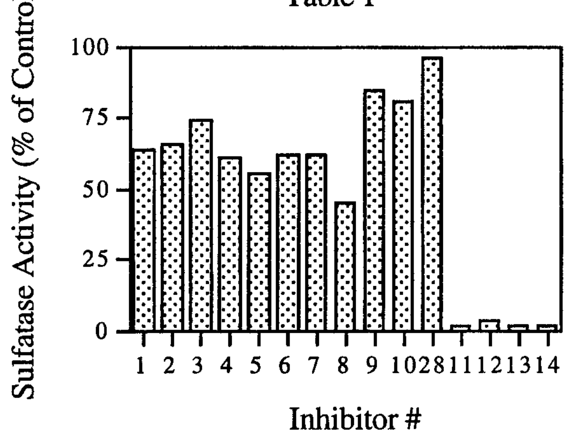


Reagents and Conditions: a. $BrCH_2CO_2Et$, K_2CO_3, acetone, reflux 2.5 h, 99.3 %; b. $LiAlH_4$, THF, r.t, 2 h; c. TBSCl, Imidazole, DMF, r.t, overnight, 96.4 % for 2 steps; d. Dihydropyran, PPTs, CH_2Cl_2, r.t, 2.5 h, 98 %; e. i) n-BuLi, THF, -78°C, 45 min; ii) **13**, -78°C to r.t, 3 h; iii) SiO_2, CH_2Cl_2, r.t, overnight, 65.7% based on **13**; 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. R-Ph-ZnCl (R = H, CH_3, OCH_3, OCH_2Ph), $Pd(PPh_3)_4$, THF, reflux, 2.5 h, 91 - 94 %; h. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 93 - 95 %; i. $(CH_3)_2NH$ or pyrrolidine, K_2CO_3, THF, r.t, 20 h, 88.3 - 94.1%; j. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t, 1 h, 91 - 94 %; k. H_2, 10% Pd/C, CH_2Cl_2-CH_3OH (3:1), r.t, 1 h, 79.2 % for **4**, 82 % for **8**.

The synthesis of compounds **1** - **8** is summarized in scheme 1. Reaction of 4-bromophenol **9** with ethyl bromoacetate gave ester **10** (99.3 %). Reduction of **10** with $LiAlH_4$ followed by protection of the resulting alcohol **11** as TBS ether yielded compound **12** (96.4 % for 2 steps). Treatment of **12** with n-butyl lithium , then with ketone **13** which was prepared by tetrahydropyranylayion of 6-hydroxy-1-tetralone (98%), followed by dehydration of the resulting tertiary alcohol with silica gel, afforded olefin **14** (65.7% based on **13**). Bromination of compound **14** with pyridinium tribromide followed by acidic hydrolysis furnished the vinyl bromide **15** (90.3%). Palladium catalyzed coupling[31] of compound **15** 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 **16** - **19** (91-94%). Iodination of alcohols **16** - **19** with I_2/PPh_3/Imidazole yielded the iodides **20** - **23** (93-95%). Reaction of compounds **20** - **23** with dimethylamine or pyrrolidine gave the corresponding amines **24** - **27** and **28** - **31** respectively (88.3 -94.1%). Sulfamoylation[32] of **24** -**26** and **28** - **30** with sulfamoyl chloride in the presence of hinder base: 2,6-di-tert-butyl-4-methyl pyridine, yielded the target compounds **1** - **3** and **5** - **7** respectively. Compound **4** was synthesized by sulfamoylation of compound **27** to form compound **32** followed by debenzylation through hydrogenation. The synthesis of inhibitor **8** was similar to **4** except compound **31** was sulfamoylated instead.

Inhibitors **1** - **8** can be divided into 2 series. Both series have the same modifications at the para position (R_3) of the 2-phenyl group. Series 1 (inhibitors **1** - **4**) contain the dimethylamino ethyl moiety while series 2 (inhibitor **5** - **8**) have the pyrrolidinyl ethyl moiety. Inhibitors **1** - **8** 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 **24**, 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. However, inhibitors with pyrrolidinyl group (inhibitors **5** - **8**) consistently exhibit higher sulfatase inhibitory activities than the inhibitors with dimethylamino groups (inhibitor **1** - **4**).

Table 1



References and Notes

1. Noel, C.T.; Reed, M.J.; Jacobs, H.S.; James, V.H.T. *J. Steroid Biochem.* **1981**, *14*, 1101.
2. Samojlik, E.; Santen, R.J.; Worgul, T.J. *Steroids* **1982**, *39*, 497.
3. Santner, S.J.; Feil, P.D.; Santen, R.J. *J. Clin. Endocrinol. Metab.* **1984**, *59*, 29.
4. Pasqualini, J.R.; Chetrite, G.; Nguyen, B.L.; Maloche, C.; Delalonde, L.; Talbi, M.; Feinstein, M.C.; Blacker, C.; Botella. J.; Paris, J. *J. Steroid Biochem. & Molec. Biol.* **1995**, *59*, 407.
5. Duncan, L.; Puroit, A.; Howarth, N.M.; Potter, B.V.L.; Reed, M.J. *Cancer Res.* **1993**, *53*, 298.
6. Howarth, N.M.; Cooper, G.; Purohit, A.; Duncan, L.; Reed, M.J.; Potter, B.V.L. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 313.
7. Li, P.K.; Pillai, R.; Young, B.L.; Bender, W.H.; Martino, D.M.; Lin, F.T. *Steroids* **1993**, *58*, 106.
8. Howarth, N.M.; Purohit, A.; Reed, M.J.; Potter, B.V.L. *J. Med. Chem.* **1994**, *37*, 219.
9.Dibbelt, L.; Li, P.K.; Pillai, R.; Knuppen, R. *J. Steroid Biochem. Molec. Biol.* **1994**, *50*, 261.
10. Dibbelt, L.; Li, P.K.; Pillai, R.; Knuppen, R. *J Steroid Biochem. & Molec. Biol.* **1995**, *52*, 281.
11. Purohit, A.; Williams, G.J.; Howarth, N.M.; Potter, B.V.L.; Reed, M.J. *Biochemistry* **1995**, *34*, 11508.
12. Purohit, A.; Williams, G.J.; Roberts, C.J.; Potter, B.V.L.; Reed, M.J. *Int. J. Cancer* **1995**, *63*, 106.
13. Li, P.K.; Pillai, R.; Dibbelt, L. *Steroids* **1995**, *60*, 299.
14. Li, P.K.; Milano, S.; Kluth, L.; Rhodes, M.E. *J. Steroid Biochem. & Molec. Biol.* **1996**, *59*, 41.
15. Selcer, K.W.; Jagannathan, S.; Rhodes, M.E.; Li, P.K. *J. Steroid Biochem. & Molec. Biol.* **1996**, *59*, 83.
16. Woo, L.W.; Lightowler, M.; Purohit, A.; Reed, M.J.; Potter, B.V.L. *J Steroid Biochem. & Mol. Biol.* **1996**, *57*, 79.
17. Woo, L.W.; Purohit, A.; Reed, M.J.; Potter, B.V.L. *J Med. Chem.* **1996**, *39*, 1349.
18. Selcer, K.W.; Hedge P.; Li, P.K.*Cancer Res.* **1997**, *57*, 702.
19. Chu, G.H.; Milano, S.; L. Kluth, Rhodes, M.E.; Johnson, D.A.; Li, P.K. *Steroids* **1997**, *62*, 530.
20. Li, P.K.; Chu, G.H.; Peters, A.; Selcer, K.W. *Steroids* **1998**, *63*, 425.
21. Poirier, D.; Boivin, R.P. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1891.
22. Purohit, A.; Vernon, K.A.; Hummelinck, A.E.; Woo, L.W.; Hejaz, H.A.; Potter, B.V.; Reed, M.J. *J Steroid Biochem. Mol. Biol.* **1998**, *64*, 269.
23. Woo, L.W.; Howarth, N.M.; Purohit, A.; Hejaz, H.A.; Reed, M.J.; Potter, B.V.L. *J Med. Chem.* **1998**, *41*, 1068.
24. Kolli, A.; Chu, G.H.; Rhodes, M.E.; Inoue, K.; Selcer, K.W.; Li, P.K. *J Steroid Biochem. Mol. Biol.* **1999**, *68*, 31.
25. Ciobanu, L.C.; Boivin, R.P.; Luu-The, V.; Labrie, F.; Poirier, D. *J Med. Chem.* **1999**, *42*, 2280.
26. Hejaz, H.A.; Purohit, A.; Mahon, M.F.; Reed, M.J.; Potter, B.V. *J Med. Chem.* **1999**, *42*, 3188.
27. Chu, G.H.; Peters, A.; Selcer, K.W.; Li, P.K. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 141.
28. Katzenellenbogen, J.A.; Carlson, K.E.; Katzenellenbogen, B.S. *J. Steroid Biochem.*, **1985**, *22*, 589.
29. Harper, M.J.; Walpole, A.L. *Nature* **1966**, *212*, 87.
30. Jordon, V.C.; Haldemann, B.; Allen, K.E. *Endocrinology* **1981**, *108*, 1353.
31. McCague, R.; Leclercq, G.; Jordan, V.C.; *J med. Chem.* **1988**, *31*, 1285.
32. Schwartz, S.; Thieme, I.; Richter, M.; Undeutsch, B.; Henkel, H.; Elger, W. *Steroids*, **1996**, *61*, 710.