Novel and Potent 17β-Hydroxysteroid Dehydrogenase Type 1 Inhibitors

Harshani R. Lawrence,[†] Nigel Vicker,[†] Gillian M. Allan,[†] Andrew Smith,[†] Mary F. Mahon,[†,§] Helena J. Tutill,[‡] Atul Purohit,[‡] Michael J. Reed,[‡] and Barry V. L. Potter*[,†]

Medicinal Chemistry, Department of Pharmacy and Pharmacology and Sterix Ltd., University of Bath, Claverton Down, Bath, BA2 7AY, U.K., Endocrinology and Metabolic Medicine and Sterix Ltd., Faculty of Medicine, Imperial College London, St. Mary's Hospital, London, W2 1NY, U.K., and Department of Chemistry, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

Received November 25, 2004

Abstract: Structure-based drug design using the crystal structure of human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) led to the discovery of novel, selective, and the most potent inhibitors of 17β-HSD1 reported to date. Compounds **1** and **2** contain a side chain with an *m*-pyridylmethylamide functionality extended from the 16β position of a steroid scaffold. A mode of binding is proposed for these inhibitors, and **2** is a steroid-based 17β-HSD1 inhibitor with the potential for further development.

Breast cancer is the most common cancer diagnosed among women, with an estimated global incidence in 2002 of ~1 150 000.[1] Approximately 60% of these are hormone-responsive breast cancer with circulating estrogens playing a vital role in promoting the growth and development of such tumors, and in this regard 17β-estradiol (E2) is crucial for tumor growth and development.[2] Elevated 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) activity has been found in many human tissues such as placenta, liver, ovary, endometrium and in breast cancers.[3] In human breast tissue, the principal activity of 17β-HSD1 is to convert weakly active estrone (E1) to active 17β-estradiol (E2), using nicotinamide adenine dinucleotide (NADH) or its phosphate derivative (NADPH) as the cofactor.[4,7] Occupation and activation of the estrogen receptor by estradiol are crucial for the stimulation of growth and development in hormone-dependent breast cancer.[5,6] Because E2 enhances breast cell proliferation in tumors,[7] suppression of the activity of 17β-HSD1 constitutes a way of reducing tumor estrogen levels and promoting tumor regression. Therefore, 17β-HSD1 inhibition is an attractive target for the design of potential antitumor drugs, but in vivo efficacy of an agent against this target has never been demonstrated.

Detailed knowledge of the biochemistry and molecular biology of 17β-HSD1 has grown rapidly in the recent years.[8] Crystallographic structural information on 17β-HSD1 with the enzyme in its native form, in complex with estradiol and NADP (PBD code 1FDT), with estradiol alone[9] and with equilin and NADP[10] provides information to design potential druglike inhibitors of 17β-HSD1. It has been suggested that the steroid and the NADPH binding sites can be independently targeted.[10] 17β-HSD1 is a 327 amino acid residue protein with a subunit mass of 35 kDa and exists as a homodimer. Several groups have reported selective inhibitors for 17β-HSD1,[11] many of which share common structural features such as a phenolic ring and hydrophobic scaffolds that interact with the hydrophobic amino acid residues in the active site that recognize the steroid substrate.

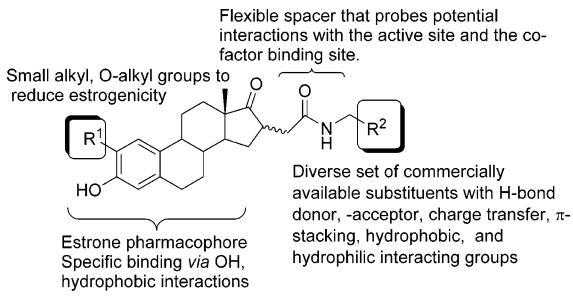


Figure 1. Estrone template modification for design of new inhibitors of 17β-HSD1.

Poirier et al. have reported estrone-based steroidal inhibitors of 17β-HSD1, with E2 derivatives bearing a short side chain at C17α and C16α positions of the estrone skeleton.[12] In an alternative approach, this group recently reported hybrid inhibitors of 17β-HSD1, the most potent being EM1745 with an IC_{50} of 52 nM, which had the estradiol template linked via an eight-methylene spacer to the adenosine moiety.[13] The crystal structure of 17β-HSD1 and EM1745 was resolved to 1.6 Å [14] and shows the substrate and cofactor binding sites occupied in the enzyme in a fashion similar to estradiol in the crystal structure of the ternary complex 17β-HSD1−E_2−$NADP^+$. This work highlights the size of the binding site and its accessibility to large flexible molecules.

As part of an ongoing program, we targeted the discovery of small-molecule inhibitors of 17β-HSD1 by incorporating a functionalized side chain at the 16 position of estrone (Figure 1). Structure-based drug design and parallel synthesis were used to optimize the interactions with the active site residues and to probe possible binding with the cofactor. From the therapeutic perspective of estrogen-dependent breast cancer, inhibitors of 17β-HSD1 should not display estrogenic effects. Attempts to generate dual action inhibitors that show antiestrogenic effects and inhibit 17β-HSD1 have been reported.[15] In our approach functionality is incorporated onto the steroid scaffold aimed at reducing estrogenicity. Here, we report the design, synthesis, and in vitro biological properties of compounds that represent a selective, potent, and novel class of 17β-HSD1 inhibitor (Table 1).

In the design of selective and potent inhibitors of 17β-HSD1, several structural requirements were taken into account (Figure 1). To obtain an inhibitor capable of exhibiting interactions with the substrate and the cofactor binding sites of 17β-HSD1, we designed mol-

* To whom correspondence should be addressed. Phone: +44 1225 386639. Fax: +44 1225 386114. E-mail: B.V.L.Potter@Bath.ac.uk.
[†] Department of Pharmacy and Pharmacology, University of Bath.
[§] Department of Chemistry, University of Bath.
[‡] Imperial College London.

10.1021/jm049045r CCC: $30.25
Published on Web 03/25/2005

Table 1. Inhibition of 17β-HSD1 and 17β-HSD2 in T47D and MDA-MB-231 Cell Lines, Respectively[a,22,23]

No.	R^1	R^2	IC_{50} (nM)	17β-HSD1 % inhibition[b]	17β-HSD2 % inhibition[b]
1	H		37	97	26
2	Et		27	99	-5.0
3	OMe		290	96	0

[a] Mean of at least two measurements typically ± 5% variation. [b] At 10 μM. The asterisk (*) indicates point of attachment.

ecules containing moieties with the ability to interact at each site with a linker of suitable length. The moieties selected to act as scaffolds and to bind to the substrate binding site were estrone and 2-ethylestrone; these were to be substituted at the 16 position with a distal carboxylic acid group that could be reacted with amines. The estrone skeleton is present in many inhibitors, and its binding mode is well demonstrated by X-ray crystallographic studies. As regards the spacer, we reasoned that a side chain bearing an amide function might favorably interact with amino acid residues in the active site of the enzyme while inclusion of an amide also aids the ease of synthesis for rapid optimization. Therefore, our strategy to incorporate carboxylamide and methylene carboxylamide linkers at the 16 position adds a point of diversity into the estrone pharmacophore, thus facilitating the probing of key interactions in the substrate binding site of the enzyme (Figure 1). The library design included different linkers and a diverse set of commercially available R^2 substituents. Full details of this work will be published elsewhere. The most promising side chain was a methylene carboxylamide moiety at the 16 position of the estrone (Figure 1), and molecular modeling studies guided the potential directions for synthetic lead optimization. To reduce the estrogenic activity, we focused on compounds with 2-ethyl and 2-methoxy groups on the A ring that are reported to display very low estrogenic activity,[16,17] and this strategy has previously been used successfully by our group to lower estrogenicity.[17]

The phenolic group on the A ring of estradiol undergoes bifurcated hydrogen bonding with His221 and Glu282 residues of the protein, and we found that the compounds with a hydroxyl group on the A ring showed high inhibition of 17β-HSD1. The amino acid residues in the catalytic region of the active site of 17β-HSD1 (Tyr 155, Ser 142, Glu 192) were targeted via hydrogen bonding with the 17-keto group on the estrone derivatives demonstrating high inhibition of 17β-HSD1. The R^2 substituents were chosen from commercially available amines that incorporated motifs of diverse characteristics.

Compounds **1** and **2** were identified as the most potent and selective inhibitors of 17β-HSD1 from our compound library with IC_{50} values of 37 and 27 nM, respectively. To understand how these compounds bind in accordance with our strategy, **1** and **2** were docked into 1FDT with estradiol removed using the flexible

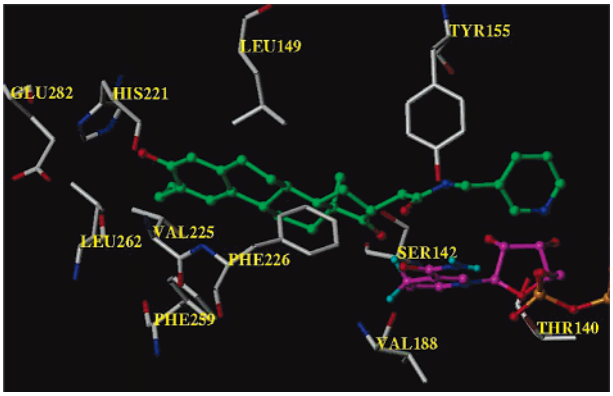


Figure 2. Compound **2** (green) docked into 1FDT with cofactor present (pink) and substrate removed.

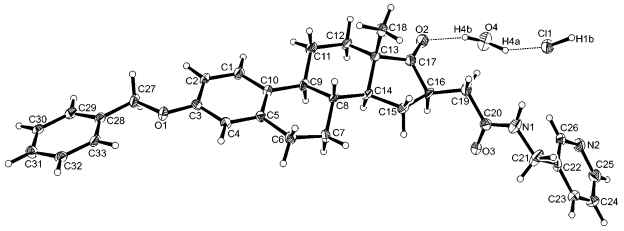


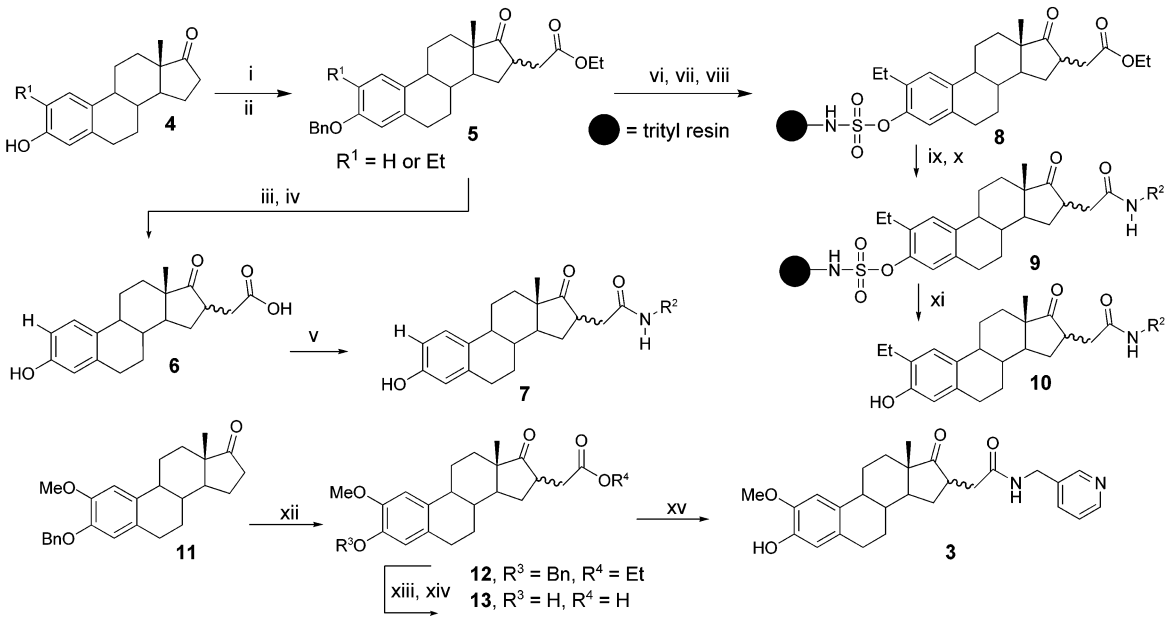
Figure 3. Crystal data and structure refinement of the hydrochloride salt of the major diastereoisomer of the 3-*O*-benzyl derivative of **1**. The asymmetric unit also contains one molecule of water. Ellipsoids are represented at the 30% probability level.

docking program GOLD.[18] The top-ranked conformations of **1** and **2** gave high fitness scores of 74.0 and 81.1, respectively. The steroid backbones of **1** and **2** are essentially overlaid when docked into 1FDT, forming the same hydrophobic interactions with the exception of the ethyl group in the 2 position of **2**, which is capable of forming an extra hydrophobic interaction with Leu262 and Phe259. The main loci of common hydrophobic interactions involving the steroid backbone of **1** and **2** are with Leu149, Val225, Phe226, Phe259; these hydrophobic interactions are also observed in the crystal structures reported wherein the steroid nucleus is cocrystallized.[8−10]

Figure 2 depicts **2** docked into 1FDT with key hydrophobic interactions with the steroid backbone and the phenolic oxygen at the 3 position and the carbonyl oxygen at the 17 position. In these potent inhibitors it was proposed that functionality in the 16β side chain (see Figure 3) might interact with the nicotinamide portion of the cofactor.

In 1FDT the nicotinamide carbonyl and the amide nitrogen form hydrogen bonds to the NH of Val188 and the oxygen of Thr140, respectively. The docked conformation of **2** shows that the carbonyl oxygen of the amide in the side chain is 3.16 Å from the closest nicotinamide NH. This observation indicates that there may be an interaction between the nicotinamide amide moiety and the amide carbonyl of the 16β side chain. In addition, a 16β side chain with sp^3 substitution may hinder the Pro(*S*) hydrogen being transferred to the 17 carbonyl 2.21 Å away because one of the hydrogen atoms in the methylene linker attached to the 16 position is only 2.63 Å from the Pro(*S*) hydrogen and the methine hydrogen at the 16 position is 1.81 Å away. The pyridyl nitrogen of the 16β side chain is 3.16 Å from a phosphate oxygen

Scheme 1. Synthetic Routes to Phenol Libraries **7** and **10** and 2-Methoxyestrone Analogue **3**[a]



[a] Reagents and conditions: (i) BnBr, K_2CO_3, DMF, 90−97%; (ii) (a) −10 °C, LDA, THF; (b) ethyl bromoacetate, −60 °C, 78−99%; (iii) H_2, Pd−C, EtOH, 94%; (iv) NaOH, MeOH/THF/H_2O (1:1:0.5), 80−90 °C, 90%; (v) EDCI, DMAP, Et_3N, 3-aminomethylpyridine (2 equiv), DCM, 48−70%; (vi) H_2, Pd−C, THF/MeOH (1:1), 97%; (vii) NH_2SO_2Cl, DMA, 98%; (viii) Cl-trityl-Cl resin, DIPEA, DCM, (loading of estrone is 0.86 mmol/g); (ix) 4 M NaOH, THF, room temp, 2 h; (x) 3-aminomethylpyridine, PyBroP, HOBt, DIPEA, DCM, 4 days; (xi) piperazine, THF, reflux, 10−45%; (xii) (a) −10 °C, LDA, THF; (b) ethyl bromoacetate, −60 °C, 95%; (xiii) H_2, Pd−C, THF/MeOH (1:1), 65%; (xiv) 4 M NaOH, THF, room temp, 2 h, 90%; (xv) PyBroP, HOBt, DIPEA, DCM, 50%.

and could form an interaction similar to that of Ile14 with an alternative phosphate oxygen at the same phosphorus center. These additional interactions of **1** and **2** with the cofactor may in part explain the high potency of these novel inhibitors.

The synthetic strategies for 17β-HSD1 inhibitors are shown in Scheme 1. The target compounds described here were prepared as part of a larger program to develop selective leads for 17β-HSD1. As a result, we utilized several different methods to obtain compounds. Here, we highlight the synthesis of the most potent compounds. The syntheses of the estrone derivatives with substitution at the 16 position were achieved using solid- and solution-phase parallel synthesis (Scheme 1). Estrone and 2-ethylestrone (R^1 = H and Et, respectively) were *O*-benzylated at the 3 position and then alkylated at the 16 position via enolization to give benzyl protected ethyl ester **5**.[19] The intermediate **5** was sequentially reacted with Pd−C/H_2 and NaOH to obtain the intermediate **6** in high yield. Acid **6** was subsequently reacted using parallel synthesis with commercially available amines using EDCI, DMAP, and Et_3N in DCM to obtain a focused library of amides **7** in good yields.

Additionally, as part of our combinatorial approach, a procedure reported by Poirier et al. was employed to produce the phenol library **10**.[20] This allowed rapid access to large numbers of diverse compounds. The 2-ethylestrone derivative **5** was debenzylated using Pd−C/H_2 to release the hydroxyl group, and the product reacted with sulfamoyl chloride to give the 3-*O*-sulfamate in excellent yield. This was attached to Cl-trityl-Cl resin to give the intermediate **8** with 0.86 mmol/g loading, using the standard conditions of DIPEA in DCM. The resin-bound sulfamate ester was then hydrolyzed and coupled with a series of amines using PyBroP and HOBt to provide resin-bound amides **9**.

Finally, the amides **9** were reacted with piperazine to give the deprotected phenol library **10** with good yields and high purities. Phenol libraries **7** and **10** were purified using SiO_2 chromatography and characterized by spectroscopic methods (^{1}H NMR, HPLC, LRMS, HRMS) to provide compounds with average purities of >90%. The synthesis of **3** is also outlined in Scheme 1, where 2-methoxybenzylestrone[16] **11** was reacted with ethyl bromoacetate via enolization to give **12**. The intermediate **12** was sequentially reacted with Pd−C/H_2 and NaOH to give the acid **13** in good yield. The final compound **3** was obtained in good yield as a mixture of diastereomers, using standard coupling conditions, i.e., PyBroP, HOBt, DIPEA, DCM. Focused libraries of amides displaying a variety of electronic and steric properties were generated to identify potential synthetic leads.

The major diastereomers (approximately 75% by ^{1}H NMR) of **1** and **2** were separated by SiO_2 chromatography and have a β orientation at the 16 position. The major diastereomers of **1** and **2** with >99% diastereomeric purity were used for biological studies. The X-ray crystal structure (Figure 3) of the major diastereomer of the 3-*O*-benzyl derivative of **1** confirmed that the side chain has a β orientation.[21]

The in vitro inhibitory activities of the **1**−**3** were evaluated against types 1 and 2 17β-HSDs using cell-based assays (Table 1). For this, T47D or MDA-MB-231 cells were used to assess the inhibitory potency of compounds in cells with 17β-HSD1 and 17β-HSD2 activities because T47-D and MDA-MB-231 cells have previously been shown to possess predominantly reductive and oxidative 17β-HSD activity, respectively.[22,23] Of those reported to date for 17β-HSD1, **1** and **2** were the most potent and selective inhibitors discovered by our approach.

The inhibitory activities shown by **1**−**3** suggest that the *m*-pyridyl unit is beneficial to the activity and selectivity in these compounds, and this supports our proposed novel mode of binding for these compounds. Small hydrophobic groups are tolerated at the steroid 2 position. Hence, **2** has a potency similar to that of **1** and may possess added hydrophobic interactions in the active site. Indeed, in addition to its potent activity and selectivity, **2** has built-in structural features proven to lower estrogenicity. Compound **3** containing a methoxy group at the 2 position is 10-fold less active than **2**. Because the methoxy group is less hydrophobic than an ethyl group and has some directional electronics, the hydrophobic interactions with Leu262 and Phe259 may be less favorable. In addition, the H-bonding interactions in the phenolic region may be lowered.

In conclusion, structure-based drug design using the crystal structure of human 17β-HSD1 has led to the discovery of novel potent inhibitors of 17β-HSD1. Compounds **1** and **2**, with IC_{50} values of 37 and 27 nM, respectively, and containing a side chain with an *m*-pyridylmethylamide functionality extended from the 16β position of a steroid scaffold, were identified from libraries using solid- and solution-phase parallel synthesis. A novel mode of binding is proposed for these new inhibitors. Moreover, **2** is a steroid-based 17β-HSD1 inhibitor with the potential for further development.

Acknowledgment. This work was supported by Sterix Ltd. as a member of the Ipsen Group. We thank Ms. A. C. Smith for technical assistance.

Supporting Information Available: Spectroscopic data for **1**−**3**, examples of R^2 substituents from commercially available amines used in the library construction as depicted in Scheme 1, details of biological assays, and Figure 2 enlarged. This material is available free of charge via the Internet at http://pubs.acs.org. Crystallographic data for the 3-*O*-benzyl derivative of **1** have been deposited with the Cambridge Crystallographic Data Centre as CCDC 253309. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. [fax(+44) 1223 336033, e-mail: deposit@ccdc.cam.ac.uk].

References

(1) Ferlay, J.; Bray, F.; Pisani, P.; Parkin, D. M. *Globocan 2002: Cancer Incidence, Mortality and Prevalence Worldwide*, version 2.0; IARC CancerBase No. 5; IARC Press: Lyon, France, 2004.
(2) Theobald, A. J. Management of advanced breast cancer with endocrine therapy: the role of primary healthcare team. *Int. J. Clin. Pract.* **2000**, *54*, 665−669.
(3) Martel, C.; Rhéaume, E.; Takahashi, M.; Trudel, C.; Couët, J.; Luu-The, V.; Simard, J.; Labrie, F. Distribution of 17β-hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues. *J. Steroid Biochem.* Mol. Biol. **1992**, *41*, 597−603.
(4) Gradishar, W. J.; Jordan, V. C. Hormonal therapy for breast cancer. An update. *Hematol. Oncol. Clin. North Am.* **1999**, *13*, 435−455.
(5) Adams, E. F.; Coldham, N. G.; James, V. H. Steroidal regulation of 17β-estradiol hydroxysteroid dehydrogenase activity of the human breast cancer cell line MCF-7. *J. Endocrinol.* **1988**, *118*, 149−154.
(6) McNeill, J. M.; Reed, M. J.; Beranek, P. A.; Bonney, R. C.; Ghilchik, M. W.; Robinson, D. J.; James, V. H. A comparison of the *in vivo* uptake and metabolism of 3*H*-estrone and 3*H*-estradiol by normal breast and breast tumour tissues in post menopausal women. *Int. J. Cancer* **1986**, *38*, 193−196.
(7) Penning, T. M. 17β-Hydroxysteroid dehydrogenase: Inhibitors and inhibitor design. *Endocr.*-Relat. Cancer **1996**, *3*, 41−56.
(8) (a) Luu-The, V. Analysis and characteristics of multiple types of human 17β-hydroxysteroid dehydrogenase. *J. Steroid. Biochem. Mol. Biol.* **2001**, *76*, 143−151. (b). Mindnich, R.; Moller, G.; Adamski, J. The role of 17 beta-hydroxysteroid dehydrogenase. *Mol. Cell. Endocrinol.* **2004**, *218*, 7−20.
(9) (a) Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. The structure of a complex human 17β-hydroysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* **1996**, *4*, 905−915. (b) Azzi, A.; Reshe, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S. X. Crystal structure of human estrogenic 17β-hydroysteroid dehydrogenase-1 complexed with 17β-estradiol. *Nat. Struct. Biol.* **1996**, *3*, 665−668.
(10) Sawicki, M. W.; Erman, M.; Puranen, T.; Vihko, P.; Ghosh, D. Structure of the ternary complex of human 17β-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and $NADP^+$. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 840−845.
(11) (a) Tremblay, M. R.; Poirier, D. Overview of a rational approach to design type 1 17β-hydroxysteroid dehydrogenase inhibitors without estrogenic activity: chemical synthesis and biological evaluation. *J. Steroid Biochem. Mol. Biol.* **1998**, *66*, 179−191. (b) Poirer, D. Inhibitors of 17β-hydroxysteroid dehydrogenase. *Curr. Med. Chem.* **2003**, *10*, 453−477.
(12) Sam, K.-M.; Boivin, R. P .; Tremblay, M. R.; Auger, S.; Poirier, D. C16 and C17 derivatives of estradiol as inhibitors of 17β-hydroysteroid dehydrogenase type 1: chemical synthesis and structure activity relationships. *Drug Des. Discovery* **1998**, *15*, 157−180.
(13) Poirier, D.; Boivin, R. P.; Bérubé, M.; Lin, S.-X. Synthesis of a first estradiol−adenosine hybrid compound. *Synth. Commun.* **2003**, *33*, 3183−3192.
(14) Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D; Lin, S.-X. A concerted, rational design of type 1 17β hydroxysteroid dehydrogenase inhibitors: estradiol−adenosine hybrids with high affinity. *FASEB J.* **2002**, *16*, 1829−1830.
(15) Tremblay, M. R.; Lin, S.-X.; Poirier, D. Chemical synthesis of 16β-propylaminoacyl derivatives of estradiol and their inhibitory potency on type1 17β-hydroxysteroid dehydrogenase and binding affinity on steroid receptors. *Steroids* **2001**, *66*, 821−831.
(16) Cushman, M.; He, H.-E.; Katzenellenbogen, J. A.; Lin, C. M.; Hamel, E. Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by binding to the colchicine binding site. *J. Med. Chem.* **1995**, *38*, 2041−2049.
(17) Leese, M. P.; Hejaz, H. A. M.; Mahon, M.; Newman, S. P.; Purohit, A.; Reed, M. J.; Potter, B. V. L. A-ring substituted estrogen-3-*O*-sulfamates: Potent multitargeted anticancer agents. *J. Med. Chem.*, submitted.
(18) Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **1997**, *267*, 727−748.
(19) Labaree, D. C.; Reynolds, T. Y.; Hochberg, R. B. Estradiol-16α-carboxylic acid esters as locally active estrogens. *J. Med. Chem.* **2001**, *44*, 1802−1814.
(20) (a) Liviu, C. C.; Poirier, D. Solid phase parallel synthesis of 17α-substituted estradiol sulfamate and phenol libraries using the multidetachable sulfamate linker. *J. Comb. Chem.* **2003**, *5*, 429−440. (b) Poirier, D.; Ciobanu, L. C.; Berube, M. A multidetachable sulfamate linker successfully used in a solid-phase strategy to generate libraries of sulfamate and phenol derivatives. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 2833−2838.
(21) Crystal data: $C_{33}H_{39}ClN_2O_4$, M = 563.11, λ = 0.710 73 Å, monoclinic, space group $P2_1$, a = 9.3190(8) Å,, b = 5.8060(6) Å,, c = 26.723(3) Å, β = 92.047(4)°, U = 1445.0(2) Å^3, Z = 2, D_c = 1.294 Mg/m^3, μ = 0.173 mm^{-1}, $F(000)$ = 600, crystal size 0.20 mm × 0.10 mm × 0.02 mm, unique reflections = 3707 [R_{int} = 0.0751], observed $I > 2\sigma(I)$ = 2057, data/restraints/paramaters = 3707/2/367, R1 = 0.0789, wR2 = 0.1724 (obsd data), R1 = 0.1625, wR2 = 0.2142 (all data), max peak/hole 0.360 and −0.271 e Å^{-3}.
(22) Details of these biological assays are in Supporting Information.
(23) Duncan, L. J.; Reed, M. J. The role and proposed mechanism by which oestradiol 17β-hydroxysteroid dehydrogenase regulates breast tumour oestrogen concentrations. *J. Steroid Biochem. Mol. Biol.* **1995**, *55*, 565−572.

JM049045R