

# In Vitro Inhibition of Estrogen Sulfoconjugation by Some 2- and 4-Substituted Estra-1,3,5(10)-trien-17 $\beta$ -ols<sup>1a</sup>

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Hormone-responsive rat and human mammary tumor, unlike normal epithelium, actively sulfoconjugates estrogens. The title compounds (9-11) were synthesized in search of specific inhibitors of estrogen sulfotransferase as a possible means of developing effective chemotherapeutic agents for treatment of hormone-dependent human mammary cancer. 4-Nitroestrone 3-triflate (7a) was converted to the corresponding estradiol derivative (8a) in 93% yield by reduction with NaBH<sub>4</sub> under phase-transfer conditions. Catalytic reduction (10% Pd/C) of the latter gave 4-aminoestra-1,3,5(10)-trien-17 $\beta$ -ol (9a) in 77% yield. These same reactions were applied consecutively to 4-nitroestrone 3-nonaflate (7b) to give 9a in 56% overall yield. The amino steroid (9a) was converted to 4-fluoroestra-1,3,5(10)-trien-17 $\beta$ -ol (10a) via a Balz-Schiemann reaction, in 17% overall yield. Successive NaBH<sub>4</sub> and (10% Pd/C) catalytic reductions of 4-fluoroestrone 3-O-(1-phenyl-1H-tetrazol-5-yl) ether (2b) provided a less satisfactory route to 10a. MCPBA oxidation of 9a gave 4-nitroestra-1,3,5(10)-trien-17 $\beta$ -ol (11a) in 56% yield. The same series of reactions were applied to 2-nitroestrone 3-triflate (7c) to give 2-amino- (9b), 2-fluoro- (10b), and 2-nitro- (11b) estra-1,3,5(10)-trien-17-ols in comparable yields. Substitution in the A ring results in improved inhibition of porcine endometrial sulfotransferase sulfoconjugation of estradiol relative to estra-1,3,5(10)-trien-17 $\beta$ -ol (4a). Moreover, electronegative substitution at C-4 of 4a is more effective than at C-2. In particular, the  $K_i$  (2.43  $\pm$  0.16  $\mu$ M) of 11a is sixfold smaller than that of the unsubstituted steroid (4a).

A distinguishing characteristic of both rat and human hormone-dependent mammary tumors, vis a vis the corresponding normal epithelial tissue, is the relatively high capacity to sulfoconjugate estrogens.<sup>2,3</sup> The mechanism of steroidal estrogen sulfurylation<sup>4</sup> by 3'-phosphoadenosine 5'-phosphosulfate (PAPS), as mediated by bovine adrenal estrogen sulfotransferase (EC 2.8.2.4), has been the subject of extensive study in this laboratory over the last several years.<sup>4-9</sup> From this work, there has emerged (inter alia) a hypothesis of an enzyme-bound transition state for estrogen sulfurylation that includes the concept of an association (stacking) of the adenine moiety of PAPS and the aromatic ring of the steroid.<sup>8</sup> More recently, we have isolated an estrogen sulfotransferase from porcine endometrium.<sup>10</sup> Although similar to the bovine adrenal enzyme ( $K_m$  = 3.77  $\mu$ M) in many of its properties and kinetics, this target tissue enzyme displayed an extremely low  $K_m$  (36 nM), as does the breast tumor sulfotransferase. The endometrial enzyme is induced by progesterone,<sup>11</sup> present only in secretory phase of the estrous<sup>12</sup> or menstrual<sup>13</sup> cycle.

Ring-A-substituted estrogens have proved to be effective inhibitors of estrogen 3-O-sulfurylation,<sup>5-8</sup> particularly if the substituent, e.g. a nitro group, is located at the 4-position of estrone.<sup>14</sup> Methyl ethers of 4-substituted estrones were prepared in search of competitive inhibitors that would not serve as substrates for the enzyme and, further, to prevent the final inhibitors from also being bound by estrogen receptor.<sup>15</sup> 4-Nitroestrone 3-O-methyl ether proved to be an efficient inhibitor of bovine adrenal<sup>8</sup> as well as porcine endometrial sulfotransferase,<sup>16</sup> with no affinity for estrogen receptor. The high level of sulfotransferase inhibition achieved with 4-nitroestrone 3-O-methyl ether was ascribed to a stabilization of the stack (vide supra) through hydrogen bonding between the 6-NH<sub>2</sub> group of adenine and the 4-nitro substituent.<sup>8</sup> Of considerable relevance and interest is the finding that 4-nitroestrone 3-O-methyl ether is, in addition, an effective inhibitor of the growth of the DMBA-induced (hormone-dependent) rat mammary tumor.<sup>17</sup> However, it was also observed that the in vivo fate of [6,7-<sup>3</sup>H]-4-nitroestrone 3-O-methyl ether included substantial (15%) O-deme-

**Table I.** Inhibition of Porcine Endometrial Estrogen Sulfotransferase by Some Estra-1,3,5(10)-trien-17 $\beta$ -ols and -17-ones<sup>a</sup>

substituent	structure no. <sup>b</sup>	app $K_i$ , $\mu$ M
Estra-1,3,5(10)-trien-17 $\beta$ -ol		
4-nitro	11a	2.43 $\pm$ 0.16
2-amino	9b	7.51 $\pm$ 1.63
2-nitro	11b	9.76 $\pm$ 1.18
4-fluoro	10a (4b)	10.2 $\pm$ 0.76
4-amino	9a	11.9 $\pm$ 0.56
2-fluoro	10b	13.6 $\pm$ 2.9
(unsubstituted)	4a	16.3 $\pm$ 3.9
Estra-1,3,5(10)-trien-17-one		
4-amino	12	10.51 $\pm$ 3.9
4-fluoro	6	14.8 $\pm$ 6.5
(unsubstituted)		25.1 $\pm$ 5.8

<sup>a</sup> Conditions for incubation are described in the Experimental Section. [6,7-<sup>3</sup>H]Estrone was added at 0.3  $\mu$ M and the inhibitor at 100 $\times$  the estrone concentrations. Apparent  $K_i$  values were calculated from the fractional inhibition data as described in previous work.<sup>8</sup> <sup>b</sup> See Schemes I and II for structures.

thylation.<sup>17</sup> The fact that 4-nitroestrone and 4-nitro-estradiol both bind to estrogen receptor,<sup>15</sup> albeit approx-

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of **2b** with NaBH<sub>4</sub> in ethanol provided 4-fluoroestradiol 3-*O*-(1-phenyl-1*H*-tetrazol-5-yl) ether (**3b**) cleanly in 74% yield.<sup>23</sup> Catalytic reduction of **3b** in ethanol over 10% Pd/C at 50 psi H<sub>2</sub> proceeded slowly to give **4b** in 49% yield.<sup>24</sup> The latter, which was obtained in the form of a foam, was further characterized by oxidation with Jones reagent to the corresponding 17-one (**6**), a crystalline solid isolated in 61% yield.

It seems unlikely that the slow replacement of the phenolic ether linkage in **3b** by hydrogen is a consequence of the adjacent electronegative substituent. Thus, the reduction of estradiol 3-*O*-(1-phenyl-1*H*-tetrazol-5-yl) ether (**3a**) under the same conditions was equally sluggish, though it provided **4a** in higher yield (78%). In this connection, it is worthy of note that the reduction of **3a** in the presence of Pd/BaCO<sub>3</sub> under a stream of deuterium gas, required 10 days to give 3-deuterioestra-1,3,5(10)-trien-17β-ol but in virtually quantitative yield.<sup>25</sup>

Attention was next focused on an appropriate synthesis of 4-amino-1,3,5(10)-trien-17β-ol (Scheme II, **9a**), which was to be utilized as an intermediate in an alternative route to **4b** as well as to other 4-substituted estra-1,3,5(10)-trien-17β-ols. Dannenberg and co-workers<sup>26</sup> achieved the synthesis of **9a**, in addition to its 2- and 3-positional isomers, by nitration of estra-1,3,5(10)-trien-17β-ol acetate to yield the precursory (2-, 3-, and 4-) nitro steroids. Reduction (N<sub>2</sub>H<sub>4</sub>-Raney Ni) of this mixture, followed by repeated chromatography, gave the individual amino derivatives.

It appeared that an extension of the reductive deoxygenation of an appropriate ether (vide supra) or ester derivative of the readily accessible 4-nitroestrone (**1c**)<sup>27</sup> would afford a more practical synthesis of **9a**. The requisite 4-nitroestrone 3-*O*-(1-phenyl-1*H*-tetrazol-5-yl) ether (**2c**) was prepared in good yield from **1c** and converted in the usual manner to the corresponding estradiol derivative (**3c**). However, attempts to effect concurrent reduction of the nitro substituent and hydrogenolysis of the heterocyclic ether function of **3c** with 10% Pd/C at hydrogen pressures in excess of 3 atm and for protracted periods of shaking were all unsuccessful. TLC showed the disappearance of **3c** with the formation of a complex reaction mixture from which no identifiable material could be isolated. In contrast, the 10% Pd/C catalyzed reduction of **3c** for 5 h with 1 atm of H<sub>2</sub> gave 4-aminoestradiol 3-*O*-(1-phenyl-1*H*-tetrazol-5-yl) ether (**5**) in 44% yield. However, the hydrogenolysis of **5** at elevated pressure (>3 atm H<sub>2</sub>) led once again to an intractable mixture of unidentified products. These findings point to a previously unrecognized limitation to the scope of the Musliner and Gates method of reduction of phenol ethers.<sup>22a,b</sup>

Catalytic hydrogenation techniques have been applied to aryl mesylates as a means of deoxygenating phenols.<sup>28</sup>

In this connection, Subramanian and co-workers<sup>29</sup> recently reported the facile conversion of phenolic nonafluorobutanesulfonic acid esters (nonaflates) to arenes by catalytic reduction with 10% Pd/C in methanol in an atmosphere of H<sub>2</sub>. Earlier, it had been reported that the catalytic reduction of *p*-nitrophenyl trifluoromethanesulfonate (triflate) with PtO<sub>2</sub> at 1 atm of H<sub>2</sub> gave anilinum triflate in 90% yield.<sup>30</sup> Under the same conditions, but with poorer yield, the triflate group was substituted by hydrogen in the reduction of phenyl triflate. It appeared then that catalytic reduction of an appropriate 4-nitroestrogen 3-perfluoroalkyl sulfonate ester would lead to **9a** (Scheme II). 4-Nitroestrone (**1c**), on treatment with triflyl chloride in acetone containing 1 equiv of triethylamine, gave the 3-triflate (Scheme II, **7a**) in excellent (96%) yield. The attempted NaBH<sub>4</sub> reduction in ethanol of the D-ring carbonyl group in **7a** provided 4-nitroestradiol as the predominant product due the apparent ease of solvolysis of the triflate ester under alkaline conditions. The desired reduction was effected by addition of a concentrated aqueous solution of NaBH<sub>4</sub> to a toluene solution of **7a** under phase-transfer conditions (ptc)<sup>31</sup> to give 4-nitroestradiol 3-triflate (**8a**) in 93% yield. Catalytic (10% Pd/C) reduction of the latter at 1 atm of H<sub>2</sub> in ethanol containing 1 equiv of triethylamine, which is reported to accelerate reductive cleavage,<sup>29</sup> provided **9a** in 70% yield.

4-Nitroestrone 3-nonaflate (**7b**), obtained in 77% yield from the esterification of **1c** with nonafllyl fluoride in CH<sub>2</sub>Cl<sub>2</sub>, was converted to **9a** in 56% yield on consecutive (ptc) NaBH<sub>4</sub> and 10% Pd/C catalyzed reductions. The nonaflate derivatives were not further explored since **7b** offered no preparative advantage over the 3-triflate (**7a**) in the preparation of **9a**.

The amino steroid **9a** was utilized to provide a more expeditious synthesis of the 4-fluoro derivative (**10a** = **4b**, Scheme I) and in a somewhat improved overall yield via a Balz-Schiemann reaction. Surprisingly, the intermediate diazonium tetrafluoroborate failed to separate from solution following either of two general methods of preparing these salts.<sup>32</sup> Apparently, the intermediate suffers spontaneous decomposition in solution. Thus, the crude, colorless product of diazotization in cold, aqueous fluoroboric acid showed no absorption in the vicinity of 2230 cm<sup>-1</sup>, which corresponds to -N≡N-stretch and is readily identifiable in the IR spectra of the diazonium fluoroborates derived from both 2- and 4-aminoestrone 3-*O*-methyl ethers.<sup>19</sup> Rather, the IR of the crude isolate revealed a band at 1260–1230 cm<sup>-1</sup> indicative of fluorine attached to an aromatic ring,<sup>33</sup> which was also noted in the IR spectrum of **4b** derived via the initial synthesis. Purification of **10a** was achieved by flash column chromatography to give crystalline material in 17% yield (based on **9a**), which was identical in every respect with **4b**.

The amino derivative (**9a**) also provided ready access to 4-nitroestra-1,3,5(10)-trien-17β-ol (**11a**), which was obtained in 56% yield by oxidation of the precursor with *m*-chloroperoxybenzoic acid.

Successive (ptc) NaBH<sub>4</sub> and (10% Pd/C) catalytic re-

- (23) Hydride reductions of D-ring keto steroids are known to proceed with selective hydride delivery to the α-face. See: Dryden, N. L., Jr. In *Organic Reactions in Steroid Chemistry*; Fried, J., Edwards, J. A., Eds.; Van Nostrand Reinhold: New York, 1972; Vol. 1, pp 1–60. The chemical shifts for 18-CH<sub>3</sub> and 17α-H in the <sup>1</sup>H NMR of **3b** are in accord with the expected selectivity of the carbonyl reduction.
- (24) The possibility of hydrogenolysis of the fluorine substituent leading to **4a** was considered, but the latter was not detected.
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ductions of 2-nitroestrone 3-triflate (**7c**), under the same conditions as those applied to the 4-nitro isomer (**7a**), provided 2-aminoestra-1,3,5(10)-trien-17-ol (**9b**) in comparable overall yield. Diazotization of the latter in aqueous fluoroboric acid led, in this case, to an isolable diazonium tetrafluoroborate. Decomposition of the latter in xylene produced the 2-fluoro steroid (**10b**) in 15–17% yield based on the diazonium salt.<sup>34</sup> 2-Nitroestra-1,3,5(10)-trien-17 $\beta$ -ol (**11b**) was obtained in 56% yield by oxidation of **9b** with *m*-chloroperbenzoic acid.

In the course of the present study, we examined the application of the same conditions of catalytic hydrogenation to the 3-deoxygenation of estrone 3-triflate (**7d**)<sup>35</sup> as a possible alternative route to estra-1,3,5(10)-trien-17-one. Surprisingly, **7d** was recovered unchanged after shaking with 1 atm H<sub>2</sub>–10% Pd/C either in the presence or absence of triethylamine. Estrone 3-nonaflate (**7e**) proved similarly resistant to reductive cleavage even after prolonged reaction periods (18 h) at 2 atm of H<sub>2</sub>. In contrast, 4-nitroestrone 3-triflate (**7a**), in accord with the behavior of the nitroestradiol 3-perfluoroalkyl sulfonate esters (**8a–c**), undergoes a facile conversion to 4-aminoestra-1,3,5(10)-trien-17-one (**12**) in 59% yield. The latter, on NaBH<sub>4</sub> reduction, provides **9a** in 80% yield and, in fact, this approach, i.e. via **7a**, developed as the method of choice for the preparation of **9a**.

There is, at present, no ready explanation of the failures encountered with the estrone 3-perfluoroalkyl sulfonates. Indeed, these findings stand in contrast with both the relatively high yields reported<sup>29</sup> for the deoxygenation of a number of phenol nonaflates and, as well, our own success with the nitroestrogen 3-perfluoroalkyl sulfonates (**7a**, **8a–c**). These observations are particularly puzzling in light of results with the 3-*O*-(1-phenyl-1*H*-tetrazol-5-yl) ethers (**3**) that show quite the opposite behavior. Thus, reductive cleavage failed in the case of the 4-aminoestradiol ether (**5**) but proved successful with the ethers of estradiol (**3a**) and its 4-fluoro derivative (**3b**). Apart from this presently inexplicable set of disparate findings, the two classes of estrogen derivatives do comprise useful and complementary synthetic routes to 17-oxygenated derivatives of estra-1,3,5(10)-triene.

**Biochemical Results and Discussion.** Apparent *K<sub>i</sub>* values for the inhibition of porcine endometrial estrogen sulfotransferase sulfoconjugation of estradiol (at C-3 OH) by the 2- and 4-amino- (**9**), 2- and 4-fluoro- (**10**), and nitroestra-1,3,5(10)-trien-17 $\beta$ -ols (**11**) are compared in Table I to the corresponding ring-A unsubstituted derivative **4a**. It is first of all noteworthy that, in the unsubstituted structure, a 17 $\beta$ -ol group (as in **4a**) leads to somewhat better inhibition (apparent *K<sub>i</sub>* 16.3  $\pm$  3.9  $\mu$ M) than the corresponding D-ring ketone (estra-1,3,5(10)-trien-17-one, apparent *K<sub>i</sub>* 25.1  $\pm$  5.8  $\mu$ M). Substitution results in improved inhibition of estrogen sulfurylation relative to estra-1,3,5(10)-trien-17 $\beta$ -ol (**4a**), and electronegative substitution at C-4 is more effective than at C-2. This generally modest, but measurable lowering of the apparent *K<sub>i</sub>* is, however, reversed in the case of the amino derivatives wherein the 2-substituted steroid (**9b**) was found to be a somewhat better inhibitor of esterification than its positional isomer (**9a**).

4-Nitroestra-1,3,5(10)-trien-17 $\beta$ -ol (**11a**) is clearly the most effective inhibitor, affording an apparent *K<sub>i</sub>* (2.43  $\pm$  0.16  $\mu$ M) that is sixfold smaller than that shown by the

unsubstituted steroid (**4a**). It is tempting to ascribe the significantly smaller apparent *K<sub>i</sub>* to enhanced stabilization provided by the 4-nitro substituent of **11a** to the stacking of the steroid with adenine (of PAPS) in the enzyme-bound transition state. The latter concept, as noted earlier, was proposed to explain the unusual substrate and inhibitory properties of, for example, 4-nitroestrone and its 3-*O*-methyl ether, respectively.<sup>8</sup> However, it is recognized that the extension of the concept of inhibition to the 4-substituted estra-1,3,5(10)-trien-17- $\beta$ -ols and **11a** in particular, which lack the phenolic methyl ether function, is somewhat tenuous. In this connection it is important to note that 4-nitroestrone 3-*O*-methyl ether is a significantly weaker inhibitor of estrogen sulfurylation, yielding an apparent *K<sub>i</sub>* (21.25  $\pm$  4.74  $\mu$ M, with the same enzyme system) that is approximately eightfold higher than that obtained for **11a**.

## Experimental Section

**Methods.** Infrared spectra were recorded on a Perkin-Elmer Model 1330 spectrophotometer. <sup>1</sup>H NMR spectra were obtained with JEOL FX 100 and Nicolet QE 300 FT spectrometers in CDCl<sub>3</sub> and are reported in parts per million downfield from internal (CH<sub>3</sub>)<sub>4</sub>Si. Electron-impact mass spectra were determined by direct-insertion probe with a Finnegan Model 4000 instrument.

Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. All solvent evaporations were carried out under reduced pressure in a Buchi rotoevaporator. Flash chromatography utilized E. Merck (40–63  $\mu$ m) silica gel. TLC was carried out with a precoated silica gel F-254 on aluminum foil in the following solvents: S<sub>1</sub>, CH<sub>2</sub>Cl<sub>2</sub>; S<sub>2</sub>, ethyl ether/CH<sub>2</sub>Cl<sub>2</sub>, 5/95; S<sub>3</sub>, EtOAc/toluene, 5/95. Nonafluorobutanesulfonyl fluoride was provided by Dr. L. R. Subramanian and was distilled (63–64 °C) prior to use.

**[(1-Phenyl-1*H*-tetrazol-5-yl)oxy]estra-1,3,5(10)-trien-17-one (**2a**).** This ether was prepared as described by Nambara et al.<sup>25</sup> from **1a** (1.35 g, 5 mmol), 5-chloro-1-phenyl-1*H*-tetrazole (910 mg 5 mmol), and anhydrous K<sub>2</sub>CO<sub>3</sub> (1.4 g, 10 mmol) in 150 mL of dry acetone to give 1.97 g (95% yield) of **1a**, which crystallized as colorless needles from EtOH: mp 196–198 °C (lit.<sup>25</sup> mp 204–206 °C). Anal. (C<sub>25</sub>H<sub>26</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

**4-Fluoro[(1-phenyl-1*H*-tetrazol-5-yl)oxy]estra-1,3,5(10)-trien-17-one (**2b**).** A solution of **1b** (210 mg, 0.73 mmol) in 30 mL of anhydrous acetone containing 5-chloro-1-phenyl-1*H*-tetrazole (130 mg, 0.73 mmol) and K<sub>2</sub>CO<sub>3</sub> (1.5 mmol) was maintained at reflux with careful exclusion of moisture for 24 h. The cooled reaction mixture was filtered through Celite, and filtrate was evaporated to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (30 mL), and the solution was washed first with (2  $\times$  5 mL) 10% NaOH and then water. The dried (Na<sub>2</sub>SO<sub>4</sub>) extract was evaporated to dryness and the product crystallized from EtOH in the form of colorless, fine needles: 250 mg (79% yield); mp 161–163 °C; <sup>1</sup>H NMR  $\delta$  0.93 (s, 3 H), 1.45–2.92 (m, 15 H), 7.19 (d, *J* = 5.56 Hz, 1 H), 7.39 (d, *J* = 5.36 Hz, 1 H), 7.53–7.89 (m, 5 H). Anal. (C<sub>25</sub>H<sub>25</sub>FN<sub>4</sub>O<sub>2</sub>) C, H, F, N.

**4-Nitro[(1-phenyl-1*H*-tetrazol-5-yl)oxy]estra-1,3,5(10)-trien-17-one (**2c**).** The reaction of **1c** (1.26 g, 4 mmol) in acetone (50 mL) containing (720 mg, 4 mmol) 5-chloro-1-phenyl-1*H*-tetrazole and (1.12 g, 8 mmol) K<sub>2</sub>CO<sub>3</sub> was carried out as described above to give a product that crystallized from EtOH as yellow needles: 1.36 g (74% yield); mp 169–171 °C; <sup>1</sup>H NMR  $\delta$  0.92 (s, 3 H), 1.22–2.86 (m, 15 H), 7.50–7.70 (m, 7 H). Anal. (C<sub>25</sub>H<sub>25</sub>N<sub>5</sub>O<sub>4</sub>) C, H, N.

**Estra-1,3,5(10)-trien-17 $\beta$ -ol (**4a**).** To a solution of **2a** (830 mg, 2 mmol), dissolved in 75 mL of hot MeOH and then carefully cooled to room temperature, was added, all at once, a solution of NaBH<sub>4</sub> (230 mg, 6 mmol) in a mixture of 2 mL of H<sub>2</sub>O and 6 mL of MeOH. The reaction mixture was stirred at room temperature for 0.75 h and then was evaporated to dryness. The residue was partitioned between H<sub>2</sub>O and CH<sub>2</sub>Cl<sub>2</sub> and the organic phase was washed with (2  $\times$  5 mL) 2% HCl and then twice with water. The dried (Na<sub>2</sub>SO<sub>4</sub>) extract was evaporated to give **3a** in the form of a colorless foam (660 mg, 72% yield) that appeared

(34) No attempt was made to synthesize **10b** from 2-fluorestrone (see ref 19).

(35) Kiesewetter, D. O.; Katzenellenbogen, J. A.; Kilbourn, M. R.; Welch, M. J. *J. Org. Chem.* 1984, 49, 4900.

as a single spot on TLC ( $S_1$ ). The product (**3a**) crystallized from EtOH as a colorless solid: mp 187–189 °C;  $^1\text{H}$  NMR  $\delta$  0.79 (s, 3 H), 1.25–2.95 (m, 16 H), 3.14 (t, 1 H), 7.09–7.86 (m, 8 H). Anal. ( $\text{C}_{25}\text{H}_{28}\text{N}_4\text{O}_2$ ) C, H, N.

To a solution of **3a** (250 mg, 0.6 mmol) in 100 mL of EtOH was added 10% Pd/C (50 mg), and the mixture was shaken for 9 h at room temperature in a Paar apparatus at 53 psi of  $\text{H}_2$ . TLC ( $S_1$ ) showed the presence of a significant amount of starting material and the reaction mixture, freshly charged with (50 mg) additional catalyst, was hydrogenated for another 9-h period at the same pressure. The catalyst was removed by filtration through Celite, and the clear solution was evaporated to dryness. The residue was dissolved in 50 mL of  $\text{CH}_2\text{Cl}_2$ , and the solution was washed first with (2  $\times$  10 mL) 10% NaOH and then water and dried ( $\text{Na}_2\text{SO}_4$ ). The residue, on evaporation of the solvent, showed three minor spots in addition to a major, slower moving spot on TLC ( $S_1$ ). The product, obtained following preparative TLC ( $S_1$ ), crystallized in the form of colorless needles (120 mg, 78% yield) from ether/petroleum ether (30–60 °C); mp 111–114 °C (lit.<sup>39</sup> mp 117–118.5 °C). This material was identical in all respects with a sample of **4a** prepared according to the method of Goldkamp et al.<sup>39</sup>

**4-Fluoroestra-1,3,5(10)-trien-17 $\beta$ -ol (4b).** (a) From **3b**. The reduction of **2b** (220 mg, 0.51 mmol) in MeOH (75 mL) with  $\text{NaBH}_4$  (120 mg, 1.5 mmol) dissolved in 25% aqueous MeOH (2.0 mL) was carried out as described above to give **3b** in the form of a colorless foam that was used directly.

Compound **3b** (160 mg, 0.37 mmol), dissolved in 50 mL of EtOH to which 10% Pd/C (50 mg) was added, was shaken under 50 psi of  $\text{H}_2$  in a Paar apparatus for two consecutive 9-h periods as described above for the preparation of **4a**. The product was isolated on preparative TLC ( $S_1$ ) as a colorless foam: 50 mg (49% yield);  $^1\text{H}$  NMR  $\delta$  0.78 (s, 3 H), 1.26–2.84 (m, 15 H), 3.73 (t, 1 H), 6.79–7.15 (m, 3 H); mass spectrum,  $m/z$  275 ( $M + 1$ )<sup>+</sup>. Anal. ( $\text{C}_{18}\text{H}_{23}\text{FO}$ ) C, H, F.

(b) From **4-Aminoestra-1,3,5(10)-trien-17 $\beta$ -ol (9a)**. A solution of **9a** (136 mg, 0.5 mmol) in absolute ethanol was cooled to 0 °C, and aqueous  $\text{HBF}_4$  (48%, 1.5 mL) followed by a cold solution of  $\text{NaNO}_2$  (38 mg, 0.55 mmol) in water (0.3 mL) was added. The reaction mixture was stirred at 0 °C for 1.5 h, after which the solution was diluted with ether (125 mL) and the resultant colorless precipitate was collected. Flash chromatography ( $\text{SiO}_2$ ,  $S_2$ ) gave a colorless solid: 23 mg (17% yield); mp 122–123 °C. The TLC ( $S_2$ ) and  $^1\text{H}$  NMR spectrum of this material (**10a**) were identical with those of the product (**4b**, foam) derived from **3b**: IR (KBr) 3300, 2930, 2860, 1580, 1460, 1350, 1250, 1060  $\text{cm}^{-1}$ .

**4-Fluoroestra-1,3,5(10)-trien-17-one (6).** To a solution of **4b** (100 mg, 36 mmol) in acetone (1.5 mL) at 0 °C was added, dropwise with stirring, a solution of 8 N  $\text{CrO}_3$  in 8 N  $\text{H}_2\text{SO}_4$  (95  $\mu\text{L}$ ). After approximately 5 min, the reaction mixture was poured into water and the precipitate was collected. The filter cake was stirred with MeOH, the inorganic salts were removed by filtration, and the clear filtrate was evaporated to give a tan solid. The latter crystallized from ether–petroleum ether (30–60 °C) as a colorless solid: 60 mg (61% yield); mp 139–141 °C; IR (KBr) 2850, 1724, 1610, 1565, 1460, 1255, 1230, 1080  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.92 (s, 3 H), 1.54–2.91 (m, 15 H), 6.81–7.21 (m, 3 H). Anal. ( $\text{C}_{18}\text{H}_{21}\text{FO}$ ) C, H, F.

**4-Amino-[(1-phenyl-1H-tetrazol-5-yl)oxy]estra-1,3,5(10)-trien-17 $\beta$ -ol (5).** Compound **2c** (920 mg, 2 mmol) in 100 mL of MeOH was reduced with  $\text{NaBH}_4$  (230 mg, 6 mmol) dissolved in a mixture of 2 mL of  $\text{H}_2\text{O}$  and 6 mL of MeOH as described above for the preparation of **3b**. The product (**3c**) was isolated as a yellow foam, 700 mg (76% yield).

To a solution of **3c** (462 mg, 1 mmol) in absolute EtOH (25 mL) was added 10% Pd/C (106 mg, 0.1 mol of Pd), and the mixture was shaken in a Paar apparatus at 16 psi  $\text{H}_2$  for 5 h. The catalyst was filtered, the filter cake was washed with  $\text{CH}_2\text{Cl}_2$  (25 mL), and the combined filtrates were evaporated to dryness. The residue was dissolved in  $\text{CH}_2\text{Cl}_2$  (25 mL) and the solution washed successively with (2  $\times$  15 mL) 10% aqueous NaOH and water. The dried ( $\text{Na}_2\text{SO}_4$ ) filtrate was evaporated, and the residue (380 mg) was flash chromatographed ( $\text{SiO}_2$ / $S_2$ ) to yield the product (**5**) that crystallized from EtOH in the form of a fine, colorless solid: 191 mg (44% yield); mp 216–217 °C; IR (KBr) 3450, 3370,

2930, 1540, 1510, 1490, 1450, 1300, 1210, 1070, 1030,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.77 (s, 3 H), 1.22–2.60 (m, 16 H), 3.68 (s, 2 H), 6.80 (d,  $J$  = 8.79 Hz, 1 H), 7.13, (d,  $J$  = 8.49 Hz, 1 H), 7.26–7.87 (m, 5 H). Anal. ( $\text{C}_{25}\text{H}_{29}\text{N}_5\text{O}_2$ ) C, H, N.

**4-Nitro-3-[(trifluoromethyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17-one (7a).** A well-stirred suspension of **1c** (3.15 g, 10 mmol) in dry acetone (100 mL) was cooled to 0 °C under a stream of argon. Triethylamine (2.1 mL, 15 mmol) followed by triflyl chloride (1.60 mL, 15 mmol) was added to the reaction mixture. The ice bath was removed and the mixture allowed to stir for 45 min. The reaction mixture was evaporated to dryness and the residue dissolved in  $\text{CH}_2\text{Cl}_2$  (50 mL). The solution was then washed with a saturated aqueous solution of  $\text{NaHCO}_3$  (2  $\times$  25 mL) and then water (2  $\times$  25 mL), and the dried ( $\text{Na}_2\text{SO}_4$ ) extract was concentrated to yield a bright yellow crude solid. Flash chromatography on  $\text{SiO}_2$  ( $S_3$ ) gave 4.2 g (96%) of an off-white solid: mp 194–195 °C (dec); IR (KBr) 1730, 1535, 1475, 1425, 1370, 1220, 1130, 1020,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.93 (s, 3 H), 1.47–2.98 (m), 7.33 (d,  $J$  = 9.08 Hz, 1 H), 7.58 (d,  $J$  = 8.79 Hz, 1 H). Anal. ( $\text{C}_{19}\text{H}_{20}\text{F}_3\text{NO}_6$ ) C, H, N.

**4-Nitro-3-[(nonafluorobutyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17-one (7b).** A solution of **1c** (157 mg, 0.5 mmol) in dry  $\text{CH}_2\text{Cl}_2$  containing triethylamine (77  $\mu\text{L}$ , 0.55 mmol) and freshly distilled nonafluorobutyl fluoride (166 mg, 0.55 mmol) was stirred at room temperature for 20 h with careful exclusion of moisture. The reaction mixture was washed successively with 5% NaOH (2  $\times$  5 mL) and water, and the dried ( $\text{Na}_2\text{SO}_4$ ) solution was evaporated to dryness. The residue crystallized from 2-propanol in the form of pale yellow needles: 230 mg (77% yield); mp 188–189 °C; IR (KBr) 1735, 1545, 1430, 1355, 1240, 1205, 1145, 1030  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.93 (s, 3 H), 1.18–2.97 (m, 15 H), 7.29 (d, 1 H), 7.54 (d, 1 H). Anal. ( $\text{C}_{22}\text{H}_{20}\text{F}_9\text{NO}_6$ ) C, H, F, N, S.

**2-Nitro-3-[(trifluoromethyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17-one (7c).** A well-stirred suspension of 2-nitroestrone<sup>26</sup> (3.15 g, 10 mmol) in dry acetone (100 mL) containing triethylamine (2.1 mL, 15 mmol) was treated with triflyl chloride (1.6 mL, 15 mmol) under argon as described for **7a**. The reaction product was flash chromatographed on  $\text{SiO}_2$  ( $S_3$ ) to give a pale yellow solid (4.2 g, 96% yield) that crystallized from 2-propanol in the form of pale yellow needles: mp 154–155 °C; IR (KBr) 1740, 1530, 1435, 1345, 1215, 1140, 1050, 1035  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.94 (s, 3 H), 1.18–3.11 (m, 15 H), 7.14 (s, 1 H), 8.10 (s, 1 H). Anal. ( $\text{C}_{19}\text{H}_{20}\text{F}_3\text{NO}_6$ ) C, H, F, N, S.

**3-[(Trifluoromethyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17-one (7d).** The reaction of **1a** (270 mg, 1.0 mmol) and triflyl chloride (0.14 mL, 1.25 mmol) in dry acetone (20 mL) containing triethylamine (0.18 mL, 1.25 mmol) was carried out as described above for **6a**. Estrone triflate (**6d**) was obtained as a crystalline solid (280 mg, 70% yield) following flash chromatography on  $\text{SiO}_2$  ( $S_2$ ). The analytical material crystallized in the form of colorless needles from EtOH; mp 99–101 °C (lit.<sup>35</sup> mp 87–88 °C); IR (KBr) 1735, 1483, 1418, 1242, 1225, 1210, 1135  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.92 (s, 3 H), 1.45–3.00 (m, 15 H), 6.99 (s, 1 H), 7.06 (d,  $J$  = 8.7 Hz, 1 H), 7.32 (d,  $J$  = 8.4 Hz, 1 H); mass spectrum,  $m/z$  402  $M^+$ . Anal. ( $\text{C}_{19}\text{H}_{21}\text{F}_3\text{O}_4\text{S}$ ) C, H, F, S.

**3-[(Nonafluorobutyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17-one (7e).** The reaction of estrone (12.7 g, 10 mmol) with nonafluorobutyl fluoride (3.3 mL, 15 mmol) in  $\text{CH}_2\text{Cl}_2$  (50 mL) containing triethylamine (1.54 mL, 15 mmol) was carried out as described above for **6b**. The crude product (mp 70–73 °C) was subjected to flash chromatography on  $\text{SiO}_2$  ( $S_1$ ) and the purified material, which showed a single spot on TLC ( $S_1$ ), crystallized as glistening plates: 3.30 g (60% yield); mp 74.5–75.5 °C; IR (KBr) 1723, 1482, 1438, 1415, 1349, 1285, 1235, 1180,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.92 (s, 3 H), 1.45–3.01 (m, 15 H), 7.00 (s, 1 H), 7.08 (d, 1 H); mass spectrum,  $m/z$  553 ( $M + 1$ )<sup>+</sup>. Anal. ( $\text{C}_{22}\text{H}_{21}\text{F}_9\text{SO}_4$ ) C, H, F, S.

**4-Nitro-3-[(trifluoromethyl)sulfonyl]oxy]estra-1,3,5(10)-trien-17 $\beta$ -ol (8a).** To a well-stirred solution of **7a** (4.0 g, 8.94 mmol) in toluene (75 mL) was added hexadecyltributylphosphonium bromide (0.46 g, 0.9 mmol), and the stirring was continued until all the phase-transfer agent dissolved. A solution of  $\text{NaBH}_4$  (1.02 g, 27 mmol) in water (10 mL) was carefully added to the clear pale yellow solution and stirred at ambient temperature for 1.5 h. The reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  (25 mL) and then washed with water (3  $\times$  15 mL). The organic phase was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to give a yellow solid.

Flash chromatography of the crude isolate on  $\text{SiO}_2$  ( $\text{S}_2$ ) yielded 3.67 g (91%) of a pale yellow solid: mp 132–133 °C; IR (KBr) 3400, 2940, 2870, 1535, 1475, 1430, 1360, 1220, 1135,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.79 (s, 3 H), 1.21–2.81 (m), 3.74 (1,  $J$  = 5.27 Hz, 1 H), 7.26 (d,  $J$  = 8.79 Hz, 1 H), 7.52 (d,  $J$  = 9.08 Hz, 1 H). Anal. ( $\text{C}_{19}\text{H}_{22}\text{F}_3\text{NO}_6\text{S}$ ) C, H, N.

**4-Nitro-3-[(nonafluorobutyl)sulfonyl]oxy]estra-1,3,5-(10)-trien-17 $\beta$ -ol (8b).** The reduction of **7b** (150 mg, 0.25 mmol) in toluene (1.0 mL), containing hexadecyltributylphosphonium bromide (13 mg, 0.025 mmol), with  $\text{NaBH}_4$  (30 mg, 0.75 mmol) in  $\text{H}_2\text{O}$  (100  $\mu\text{L}$ ) was carried out as described above for **7a**. The oily residue was isolated in the form of a foam (150 mg, 99% yield) upon evaporation from ether:  $^1\text{H}$  NMR  $\delta$  0.80 (s, 3 H), 1.26–2.81 (m), 3.72 (d, 1 H), 7.27 (d, 1 H), 7.40 (d, 1 H). Anal. ( $\text{C}_{22}\text{H}_{22}\text{F}_9\text{NO}_6\text{S}\cdot\text{C}_4\text{H}_{10}\text{O}$ ) C, H, N, S.<sup>37</sup>

**2-Nitro-3-[(trifluoromethyl)sulfonyl]oxy]estra-1,3,5-(10)-trien-17 $\beta$ -ol (8c).** Reduction of **7c** (4.47 g, 10 mmol) in toluene (75 mL) containing hexadecyltributylphosphonium bromide (508 mg, 1 mmol) with  $\text{NaBH}_4$  (1.13 g, 30 mmol) in  $\text{H}_2\text{O}$  (8 mL) was carried out as described for **7a**. The product, after flash chromatography on  $\text{SiO}_2$  ( $\text{S}_2$ ), was obtained in the form of a foam: 4.20 g (93% yield); IR (KBr) 3400, 2920, 1520, 1420, 1340, 1205, 1130  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.80 (s, 3 H), 1.17–3.03 (m), 3.76 (t,  $J$  = 6.15 Hz, 1 H), 7.10 (s, 1 H), 7.26 (s, 1 H). Anal. ( $\text{C}_{19}\text{H}_{22}\text{F}_3\text{NO}_6\text{S}\cdot\text{C}_4\text{H}_{10}\text{O}$ ) C, H, F, N, S.<sup>37</sup>

**4-Aminoestra-1,3,5(10)-trien-17 $\beta$ -ol (9a).** To a solution of **8a** (1.35 g, 3.0 mmol) in absolute ethanol (50 mL), containing triethylamine (0.418 mL, 3.0 mmol), was added 10% Pd/C (0.319 g, 0.3 mol of Pd), and the mixture was hydrogenated in a Parr apparatus at 16 psi of  $\text{H}_2$  for 5 h. The reaction mixture was filtered, and the residue was washed with  $\text{CH}_2\text{Cl}_2$  (25 mL). The combined filtrates were concentrated to dryness, and the residue was dissolved in  $\text{CH}_2\text{Cl}_2$  (25 mL). The solution was washed with saturated aqueous  $\text{NaHCO}_3$  ( $2 \times 15$  mL) and water ( $2 \times 25$  mL). The dried ( $\text{Na}_2\text{SO}_4$ ) extract, on evaporation, gave a solid that crystallized from methanol in the form of colorless needles: 0.567 g (70% yield); mp 191–192 °C; IR (KBr) 3440, 3340 (s), 2920, 2850, 1620, 1580, 1460, 1130  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.77 (s, 3 H), 1.12–2.58 (m), 3.72 (t,  $J$  = 6.0 Hz, 1 H), 6.56 (d,  $J$  = 7.73 Hz, 1 H), 6.80 (d,  $J$  = 7.81 Hz, 1 H), 7.00 (dd,  $J$  = 7.80 Hz, 1 H); mass spectrum,  $m/z$  271  $\text{M}^+$ . Anal. ( $\text{C}_{18}\text{H}_{25}\text{NO}$ ) C, H, N.

The same reduction of **8b** (1.944 g, 3.24 mmol) in EtOH (100 mL) containing triethylamine (0.53 mL, 3.8 mmol) and 10% Pd/C (404 mg, 0.38 mol of atom Pd) and at 16 psi of  $\text{H}_2$  for 5 h gave a tan product (0.87 g). The latter crystallized from MeOH as an off-white, crystalline solid: 0.496 g (56% yield); mp 188–190 °C. The NMR and IR spectral properties of the latter were identical with those of a sample of **9a** derived from **8a**.

**4-Aminoestra-1,3,5(10)-trien-17-one (12).** To a suspension of **7a** (1.166 g, 2.61 mmol) in absolute ethanol (100 mL) containing triethylamine (0.363 mL, 2.61 mmol) was added 10% Pd/C (277 mg, 0.26 mol of Pd), and the mixture was hydrogenated in a Parr apparatus at 16 psi of  $\text{H}_2$  for 7 h. The catalyst was filtered, the filtercake was washed well with  $\text{CH}_2\text{Cl}_2$  ( $\sim 100$  mL), and the combined filtrates were evaporated to dryness. The residue was dissolved in  $\text{CH}_2\text{Cl}_2$  (25 mL) and was washed with saturated aqueous  $\text{NaHCO}_3$  ( $2 \times 15$  mL) and water ( $2 \times 25$  mL) and dried ( $\text{Na}_2\text{SO}_4$ ). The extract was evaporated to yield 0.59 g of a crude solid that crystallized from absolute ethanol to give a fine, colorless crystalline solid: 353 mg (59% yield); mp 243–245 °C; IR (KBr) 3450, 3360, 2910, 1720, 1620, 1580, 1460, 1300, 1250,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.90 (s, 3 H), 1.42–2.63 (m, 15 H), 3.57 (s, 2 H), 6.57 (d,  $J$  = 7.62 Hz, 1 H), 6.78 (d,  $J$  = 7.61 Hz, 1 H), 7.06 (dd,  $J$  = 14.94, 7.91 Hz, 1 H). Anal. ( $\text{C}_{18}\text{H}_{23}\text{NO}$ ) C, H, N.

A solution of **12** (135 mg, 0.5 mmol) in THF (10 mL) was cooled to 0 °C and  $\text{NaBH}_4$  (0.113 g, 3 mmol) in  $\text{CH}_3\text{OH}$  (3 mL) was added. The ice bath was removed, and the reaction mixture was stirred for 45 min after which water (20 mL) was carefully added. The reaction mixture was then diluted with  $\text{CH}_2\text{Cl}_2$  (25 mL) and washed with saturated aqueous  $\text{NaHCO}_3$  ( $2 \times 20$  mL) and then water ( $2 \times 15$  mL). The dried ( $\text{Na}_2\text{SO}_4$ ) solution was filtered and concentrated to give 140 mg of a light brown solid. Flash chromatography ( $\text{SiO}_2$ ,  $\text{S}_2$ ) gave an off-white crystalline solid: 110 mg (81% yield); mp 183–185 °C. Its IR and  $^1\text{H}$  NMR spectral properties were superimposable with those of an authentic sample of **9a**.

**2-Aminoestra-1,3,5(10)-trien-17 $\beta$ -ol (9b).** Reduction of **8c** (3.48 g, 7.7 mmol) in EtOH (100 mL) containing triethylamine (1.1 mL, 7.7 mmol) and 10% Pd/C (0.848 g, 0.8 mol of Pd) and at 16 psi of  $\text{H}_2$  for 5 h was carried out as described above for **9a**. The reaction mixture gave a crude product (1.92 g) that crystallized from  $\text{CH}_2\text{Cl}_2$ –hexane as a colorless solid: 1.43 g (68% yield); mp 178–180 °C dec; IR (KBr) 3420, 3365, 2900, 1610, 1500, 1445, 1335, 1265, 1215, 1135, 1055  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.77 (s, 3 H), 1.06–2.81 (m), 3.72 (t,  $J$  = 8.2, 7.91 Hz, 1 H), 6.48 (d,  $J$  = 8.06 Hz, 1 H), 6.65 (s, 1 H), 6.87 (d,  $J$  = 7.91 Hz, 1 H). Anal. ( $\text{C}_{18}\text{H}_{25}\text{NO}$ ) C, H, N.

**2-Fluoroestra-1,3,5(10)-trien-17 $\beta$ -ol (10b).** To a well-stirred solution of **9b** (543 mg, 2 mmol) in a mixture of 48%  $\text{HBF}_4$  (3.0 mL, 16 mmol), THF (3 mL), and dioxane (0.5 mL) was added under the solution surface, via a syringe, a cold solution of  $\text{NaNO}_2$  (276 mg, 4 mmol) with external cooling. The temperature was then allowed to rise to –5 to 0 °C, and stirring was continued for 1 h. Cold water (20 mL) was added which led to the deposition of a yellow precipitate. Stirring was maintained for an additional 1 h at –5 to 0 °C after which the reaction mixture was extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 15$  mL). The organic layer was washed with  $\text{NaHCO}_3$  ( $3 \times 10$  mL), then dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated to yield 590 mg (80% yield) of a reddish solid: mp 63–70 °C dec; IR (KBr) 3420, 2920, 2860, 2260, 1630, 1050  $\text{cm}^{-1}$ .

A suspension of the diazonium salt (580 mg, 1.57 mmol) in xylene (50 mL) was refluxed for 18 h. The cooled, supernatant fraction was decanted, and the process was repeated with refluxing xylene ( $3 \times 50$  mL) carried out for 3-h periods. The combined xylene extracts were concentrated to an oil that TLC ( $\text{S}_1$ ) showed to be a mixture of products. The mixture was subjected to repeated (three) flash chromatographies ( $\text{SiO}_2$ ,  $\text{S}_1$ ) that gave a solid that was characterized as a single spot on TLC ( $\text{S}_1$ ). Two recrystallizations of this material from hexane gave an off-white product in the form of compact needles: 69 mg (16% yield); mp 124–126 °C; IR (KBr) 3300, 2940, 2910, 2850, 1610, 1580, 1390, 1260, 1130, 1050  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.78 (s, 3 H), 0.93–3.73 (m, 16 H), 6.80 (m, 3 H). Anal. ( $\text{C}_{18}\text{H}_{23}\text{FO}$ ) C, H, F.

**4-Nitroestra-1,3,5(10)-trien-17 $\beta$ -ol (11a).** To a stirred solution of MCPBA (518 mg, 3 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (10 mL) was slowly added, under gentle reflux and an atmosphere of argon, a solution of **9a** (136 mg, 0.5 mmol) in  $\text{CH}_2\text{Cl}_2$  (3 mL). After the addition of **9a**, the reaction mixture was cooled immediately to room temperature and was washed successively with 10% aqueous  $\text{Na}_2\text{SO}_3$  ( $2 \times 10$  mL), a saturated solution of  $\text{NaHCO}_3$  ( $2 \times 10$  mL), and water ( $2 \times 15$  mL). The dried ( $\text{Na}_2\text{SO}_4$ ) organic fraction was evaporated and the residue (140 mg) subjected to flash chromatography ( $\text{SiO}_2$ ,  $\text{S}_1$ ). The product, a yellow solid (85 mg, 56% yield) crystallized from ether–petroleum ether (30–60 °C) to provide the analytical sample: mp 151–152 °C; IR (KBr) 3400, 2920, 2850, 1515, 1340, 1130, 1070, 1045  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.82 (s, 3 H), 1.3–2.36 (m, 15 H), 3.74 (t, 1 H), 7.28 (d,  $J$  = 15.9 Hz, 1 H), 7.57 (d,  $J$  = 8.1 Hz, 1 H), 7.64 (d,  $J$  = 8.1 Hz, 1 H); mass spectrum,  $m/z$  301  $\text{M}^+$ . Anal. ( $\text{C}_{18}\text{H}_{23}\text{NO}_3$ ) C, H, N.

**2-Nitroestra-1,3,5(10)-trien-17 $\beta$ -ol (11b).** The oxidation of **9b** (136 mg, 0.5 mmol) in  $\text{CH}_2\text{Cl}_2$  (7.5 mL) with MCPBA (518 mg, 3 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 mL) was carried out as described above to yield a pale yellow foam, 110 mg. The latter, on flash chromatography ( $\text{SiO}_2$ ,  $\text{S}_2$ ) gave a pale yellow solid: 85 mg (56% yield); mp 165–167 °C; IR (KBr) 3350, 2900, 1520, 1350, 1060  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  0.79 (s, 3 H), 1.17–3.01 (m, 15 H), 3.76 (t,  $J$  = 7.62, 8.49 Hz, 1 H), 7.19 (d,  $J$  = 8.5 Hz, 1 H), 7.92 (d,  $J$  = 8.35 Hz, 1 H), 8.13 (s, 1 H); mass spectrum,  $m/z$  301 ( $\text{M}^+$ ). Anal. ( $\text{C}_{18}\text{H}_{23}\text{NO}_3$ ) C, H, N.

**Biochemical Evaluation. (a) Purification of Porcine Estrogen Sulfotransferase.** The enzyme was purified from porcine endometria according to the procedure of Freeman et al.<sup>36</sup> In general, this involves the neutral ammonium sulfate precipitation of proteins from the 100000g supernatant of a 0.02 M phosphate buffer (pH 7.4) homogenate. The fraction precipitating between 0.5 and 0.7 saturation is collected. The precipitate is

(36) Freeman, D. J.; Saidi, F.; Hopkirk, R. *J. Steroid Biochem.* 1983, 18, 23.

(37) The solvent incorporated in the foam could not be removed on drying the analytical sample at reduced (1 torr) pressure.



dissolved in the start buffer (10 nM monothioglycerol (MTG), 0.25 M sucrose, 25 mM imidazole, pH 7.0) for a chromatofocusing column (1 × 40 cm). This column yields a peak of estrogen sulfurylating activity that is stable for weeks at 0 °C and for months at -20 °C. The chromatofocusing column is eluted with Polybuffer-74 (pH 5.0) diluted 1 to 10 with 2× sucrose-MTG and H<sub>2</sub>O to give a final concentration equal to the starting buffer. This gives a pH range for the column of 5.0-7.0. The estrogen sulfotransferase is eluted at pH 6.1, and 1.0-mL aliquots are quick-frozen in liquid nitrogen and stored at -40 °C in 0.25 mM dithiothreitol. Although still a crude enzyme preparation, the peak of estrogen sulfotransferase activity from this chromatofocusing column has separated from the majority of the supernatant proteins and has apparently been freed from the deactivating factors in the tissue supernatant.

**(b) Standard Sulfotransferase Assay.** The enzyme assay is carried out as published previously.<sup>5,8</sup> Contained in a total volume of 0.2 mL are 0.1 mM PAPS, estrone (E<sub>1</sub>) 0.3 μM (60 pmol) in Me<sub>2</sub>SO-H<sub>2</sub>O (90:10), [6,7-<sup>3</sup>H]estrone (~1 × 10<sup>6</sup> dpm or 7 pmol), 12 mM magnesium acetate, 0.14 M Tris-HCl buffer, pH 7.8, and 12.7 μg of enzyme preparation. Incubation is carried out for 30 min at 37 °C and the reaction stopped by placing the tubes into boiling water for 4 min. The reaction products may then be extracted into ethyl acetate and the sulfurylated steroids resolved on instant thin-layer chromatography before the radioactivity in the two labels is measured by liquid scintillation counting with

absolute activity analysis.<sup>5,8</sup> This assay is also utilized in the kinetic, specificity, and inhibition studies in which case two (10 and 100 times the substrate concentration) concentrations of the compounds (inhibitors) are added to the incubation mixture in 5 mL of Me<sub>2</sub>SO-EtOH (90:10). The data are subjected to kinetic analysis, with Lineweaver-Burk plots yielding  $K_m$  and  $V_{max}$  values. computer analysis (NONLIN program) was employed to corroborate the  $K_m$  and  $V_{max}$  data derived by hand-drawn plots (Lineweaver-Burk). The inhibition values (apparent  $K_i$ ) were calculated from fractional inhibition data utilizing the equations described in ref 8. The apparent  $K_m$  of porcine endometrial estrogen sulfotransferase for reactions involving estrogen is 10<sup>-8</sup> M,<sup>16</sup> not unlike that reported for the enzyme from human endometria.<sup>38</sup>

**Acknowledgment.** This investigation was supported, in part, by a grant from the U.S. Public Health Service (Grant CA 37387) and an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

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## Conformational Energy Calculations and Electrostatic Potentials of Dihydrofolate Reductase Ligands: Relevance to Mode of Binding and Species Specificity

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Received April 15, 1985

Classical potential energy calculations are reported for a series of 11 structurally diverse substrates, products, and inhibitors of dihydrofolate reductase. In almost every case, the calculations reveal a range of potential biologically active conformations accessible to the molecule, and geometry optimization with molecular mechanics and molecular orbital calculations further expands the range of accessible conformations. The energy calculations are supplemented with electrostatic potential energy surfaces for the heterocyclic components of each molecule. These data are used in conjunction with the energy calculations and the crystallographically determined enzyme structures to compare two alternative proposed binding modes of folates known to bind with their pteridine rings inverted relative to that of methotrexate. It is shown that the conformational flexibility of the connecting chain between the benzoyl glutamate and pteridine moieties in the folates actually allows the pteridine ring to shift between these alternative binding modes, a combination of which may offer the best explanation for the observed activity. The electrostatic potentials and conformational energy data are also used in an attempt to account for the species specificity of inhibitors of mammalian, bacterial, and protozoal dihydrofolate reductases. The results show that while these techniques can be used to explain many of the observed results, others require recourse to the observed crystal structures to provide a satisfactory explanation.

Known inhibitors of dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate NADP<sup>+</sup> oxidoreductase, EC 1.5.1.3) vary widely in structure, and many of them display strong species specificity.<sup>1</sup> It is apparent that specificity for binding at the active sites of different DHFRs must eventually depend on the electronic structure and conformational properties of the individual DHFR inhibitors, but the size and conformational flexibility of the molecules is such that these data are generally not available.

In this paper we report complete conformational analyses and electrostatic potentials for a series of compounds representing the major structural classes of DHFR inhibitors (1-8), as well as the substrates and products of the enzyme (9-11). In each case we have used simple classical potential energy calculations, without geometry optimization, to identify all of the biologically accessible conformations. Where necessary, these calculations are sup-

**Table I.** Minimum Energy Conformations for the Amide and L-Glutamate Torsion Angles

torsion angle	conformational minima	conformation used for connecting chain calculations
ψ	30° → 140°, -140° → -40°	90°
φ	70° → 180°	160°
χ <sub>1</sub>	170° → 50°	-70°
χ <sub>2</sub>	60° → 60°	180°
ω <sub>1</sub>	free rotation	90°
ω <sub>2</sub>	free rotation	120°
amide bond	cis/trans	trans

plemented by molecular orbital and molecular mechanics calculations with geometry optimization; molecular orbital calculations have also been used to determine electron distributions and electrostatic potential surfaces. The results indicate that, while all of the ligands share some common electronic properties, virtually all of them can

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