Biochemical Pharmacology, Vol. 39, No. 11, pp. 1709–1713, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

SULFATE DERIVATIVES OF 2-PHENYLINDOLS AS NOVEL STEROID SULFATASE INHIBITORS

AN *IN VITRO* STUDY ON STRUCTURE–ACTIVITY-RELATIONSHIP

HERBERT BIRNBÖCK and ERWIN VON ANGERER*
Institut für Pharmazie, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany

(*Received* 14 *August* 1989; *accepted* 2 *January* 1990)

Abstract—The growth of hormone-dependent mammary tumor cells is stimulated by non-conjugated estrogens. One important source of these hormones in the tumors could be the enzymatic hydrolysis of circulating estrogen sulfates by steroid sulfatase (EC 3.1.6.2). Inhibition of this enzyme may result in reduced levels of endogenous estrogens and, consequently, in a reduced proliferation rate of estrogen-dependent tumors. This paper reports on a series of inhibitors of steroid sulfatase based on sulfated derivatives of 2-phenylindoles, a new class of mammary tumor inhibiting compounds. Starting from hydroxy-substituted 2-phenylindoles, a number of mono- and disulfates were synthesized and tested for steroid sulfatase inhibiting properties. The enzymatic test was based on the measurement of [^{3}H]estrone formed from [^{3}H]estrone sulfate in the presence of various amounts of inhibitor. The concentrations which result in a 50% reduction of the rate of hydrolysis (IC_{50}) were determined. Sulfates of hydroxylated 2-phenylindoles are substrates of steroid sulfatase. The most potent of these inhibitors show affinities which are comparable to the affinities of natural substrates. The test data further suggest that mono-sulfated compounds exhibit stronger enzyme-inhibiting properties than do disulfates.

Steroid sulfatase (EC 3.1.6.2) catalyses the hydrolysis of 3-hydroxy-steroid sulfates (cholesterolsulfate, E_1S,† DHAS). It is competitively inhibited by a number of natural steroids and steroid sulfates and may play a key role in intracellular steroid metabolism [1–4]. The enzyme is an integral glycoprotein of the mitochondrial membrane. An involvement of the carbohydrate residues in the native structure of the enzyme's catalytically active domain seems probable [5]. Steroid sulfatase mainly is localized in steroid-sensitive tissue, e.g. the placenta [4] and human breast tissue [6]. The enzyme is also present in high activity in breast cancer tissue and in MCF_7-cells, an estrogen receptor positive breast cancer cell line [7, 8]. It is discussed that steroid sulfatase—rather than aromatase—be responsible for estrone production in hormone-dependent tumors [9].

The enzyme activity seems to be correlated to the level of estrogen receptors in mammary tumors [10]. The inhibition of the enzyme resulting in a reduced intracellular production of estradiol might be a way to reduce proliferation of ER-positive tumor cells [11]. Carlström *et al.* [12] showed a marked reduction in the concentration of circulating steroids as a consequence of the therapeutic application of danazol which is a potent inhibitor of steroid sulfatase. The application of danazol in hormone-sensitive *in vitro* tumor models resulted in a strong obstruction of cell growth [13]. The therapy of the DMBA-induced mammary carcinoma of the rat also led to a significant reduction of tumor mass [14]. These observations clearly confirm the idea that the use of steroid sulfatase inhibitors could be advantageous in the therapy of hormone-dependent tumors.

Recently, von Angerer *et al.* [15–17] reported on a new class of mammary tumor inhibiting compounds, the 2-(hydroxyphenyl)indoles. One of the most promising new derivatives of this class, 5-acetoxy-2-(4-acetoxyphenyl)-1-ethyl-3-methylindole (zindoxifene), was shown to be metabolically converted to its sulfoconjugate to a substantial extent *in vivo* [18]. Therefore we intended to investigate whether sulfo-derivatives of 2-(hydroxyphenyl)indoles might act as inhibitors of steroid sulfatase. In this paper we report on a series of sulfo-derivatives and their sulfatase-inhibiting activity *in vitro*.

MATERIALS AND METHODS

4-[^{14}C]Estrone (sp. act. 57 mCi/mmol) and 6,7-[^{3}H]estrone sulfate (sp. act. 40 Ci/mmol) were purchased from New England Nuclear Division (Dreieich, F.R.G.). Radiochemical purity was checked prior to use by HPLC and on-line scintillation counting (Berthold LB506D). Non-labelled steroid sulfates and other biochemicals were purchased from the Sigma Chemical Co (Deisenhofen, F.R.G.).

Enzyme preparation and assay

The steroid sulfatase was prepared as a partially purified homogenate of calf uterus. Calf uterus tissue was homogenized (1 g of tissue/5 mL) in Tris–HCl (10 mM, pH 7.4) containing 1 mM EDTA and 0.5 mM dithioerythritol (TED-buffer). The supernatant of a low speed centrifugation (1000 *g*, 4°, 15 min) was used as steroid sulfatase preparation.

* Author to whom correspondence should be addressed: Prof. Dr Erwin von Angerer, Institut für Pharmazie, Universität Regensburg, Universitätsstraße 31, D-8400 Regensburg, F.R.G.

† Abbreviations used: BCA, bicinchoninic acid, DHAS, dehydroepiandrosterone-3-sulfate, DMBA, 7,12-dimethylbenz[*a*]anthracene, E_1, estrone, E_1S, estrone sulfate.

The enzymatic assay used to determine relative reaction rates was essentially based on the hydrolysis of [^{3}H]E_1S by steroid sulfatase in the presence of various concentrations of inhibitors [6]. All compounds tested as inhibitors were dissolved in TED-buffer. The assay solution contained various concentrations of inhibitor (100–4000 μM), 10 μM [^{3}H]E_1S and enzyme preparation (6 mg protein/mL) in TED-buffer in an assay volume of 1.0 mL. The concentration of [^{3}H]E_1S was chosen on the basis of previously determined K_m-values (9–12 μM) of the enzyme. The reaction was started by the addition of the enzyme preparation to the mixture of the components (all prewarmed to 37°). After 30 min incubation time, the reaction was stopped by the addition of 4 mM DHAS. [^{14}C]E_1 (*ca.* 1000 dpm) was added to each sample at the end of incubation as an internal standard to quantitate the amount of [^{3}H]E_1 formed. Estrone was extracted from the reaction mixture with 2 × 1 mL of toluene and counted for ^{3}H and ^{14}C-activities, respectively. Radioactivities were measured in a liquid scintillation counter (Beckman LS 1801) using Quickszint 212 (ZINSSER) as counting solution. Protein determinations were performed according to the BCA-assay procedure [19].

This assay was designed to determine relative inhibitory concentrations (IC_{50}) of the compounds to be studied. Since no K_i-values were determined the assay was not validated in all respects.

General procedures

Reagents used were reagent grade (Merck, Darmstadt, F.R.G.). Nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a 60 MHz Varian EM 360 L spectrometer. Chemical shift values are expressed as δ in ppm. Elemental analyses were performed at the Regensburg University Microanalytical Laboratory and are within ±0.5% for each element determined. HPLC was performed on LiChroSorb RP-18 columns (7 μm, 250 × 4.6 mm, Merck) with UV detection. MeOH/H_2O (60:40, v/v) was used as the mobile phase. Melting points are uncorrected.

Synthesis of the sulfates

Synthesis of the sulfates was performed according to literature procedures [20]. The hydroxy-2-phenylindoles [16] (1.0 mmole) were dissolved in 10 mL of anhydrous pyridine. To the solution was added twice the stoichiometric amount of pyridine–SO_3 complex. The reaction mixture was stirred overnight under anhydrous conditions.

Pyridine was removed *in vacuo* and the residue dissolved in anhydrous methanol. The solution was neutralized by the addition of 1 N NaOH in methanol. The precipitate was filtered off and the methanolic solution concentrated to 5–10 mL *in vacuo*.

Anhydrous ether (150–200 mL) was added dropwise with stirring to the solution. The precipitate formed was filtered and recrystallized from ethanol/ether to afford colorless or yellow crystals. The final product was dried at 50–60° *in vacuo*.

3-*Methyl*-5-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**1**). m.p. > 190° (dec.). ^{1}H-NMR (D_2O), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 6.9–7.5 (m, 8H, ArH). HPLC, t_R = 2.5 min. Elemental analysis for $C_{15}H_{11}NNa_2O_8S_2$.

1-*Ethyl*-3-*methyl*-5-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**2**). m.p. > 190° (dec.). ^{1}H-NMR (D_2O), δ 1.1 (t, J = 7 Hz, 3H, $CH_2C\underline{H_3}$), δ 2.2 (s, 3H, CC$\underline{H_3}$), δ 4.1 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 7.0–7.6 (m, 7H, ArH). HPLC, t_R = 1.7 min. Elemental analysis for $C_{17}H_{15}NNa_2O_8S_2 \cdot 2H_2O$.

3-*Methyl*-1-*propyl*-5-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**3**). m.p. > 240° (dec.). ^{1}H-NMR (DMSO-D_6), δ 0.6 (t, J = 7 Hz, 3H, $CH_2CH_2C\underline{H_3}$), δ 1.1–1.7 (m, J = 7 Hz, 2H, $CH_2C\underline{H_2}CH_3$), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 4.0 (t, J = 7 Hz, 2H, C$\underline{H_2}CH_2CH_3$), δ 6.9–7.5 (m, 7H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{18}H_{17}NNa_2O_8S_2 \cdot 2H_2O$.

1-*Ethyl*-6-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**4**). m.p. > 160° (dec.). ^{1}H-NMR (D_2O), δ 1.0 (t, J = 7 Hz, 3H, $CH_2C\underline{H_3}$), δ 4.0 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 6.3 (s, 1H, C$\underline{H}$), δ 6.7–7.5 (m, 7H, ArH). HPLC, t_R = 1.7 min. Elemental analysis for $C_{16}H_{13}NNa_2O_8S_2 \cdot 2H_2O$.

1-*Ethyl*-3-*methyl*-6-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**5**). m.p. > 155° (dec.). ^{1}H-NMR (DMSO-D_6), δ 1.1 (t, J = 7 Hz, 3H, $CH_2C\underline{H_3}$), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 4.0 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 6.7–7.5 (m, 7H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{17}H_{15}NNa_2O_8S_2 \cdot 2H_2O$.

3-*Methyl*-1-(4-*pyridylmethyl*)-6-*sulfooxy*-2-(4-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**6**). m.p. > 210° (dec.). HPLC, t_R = 1.5 min. Elemental analysis for $C_{21}H_{16}N_2Na_2O_8S_2$.

1-*Methyl*-6-*sulfooxy*-2-(3-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**7**). m.p. > 150° (dec.). ^{1}H-NMR (D_2O), δ 3.5 (s, 3H, NC$\underline{H_3}$), δ 6.3 (s, 1H, C$\underline{H}$), δ 6.7–7.5 (m, 7H, ArH). HPLC, t_R = 1.6 min. Elemental analysis for $C_{15}H_{11}NNa_2O_8S_2 \cdot 2H_2O$.

1-*Ethyl*-6-*sulfooxy*-2-(3-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**8**). m.p. > 170° (dec.). ^{1}H-NMR (D_2O), δ 1.1 (t, J = 7 Hz,3H, $CH_2C\underline{H_3}$), δ 4.1 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 6.3 (s, 1H, C$\underline{H}$), δ 6.6–7.5 (m, 7H, ArH). HPLC, t_R = 1.6 min. Elemental analysis for $C_{16}H_{13}NNa_2O_8S_2 \cdot 2H_2O$.

3-*Methyl*-1-*propyl*-6-*sulfooxy*-2-(3-*sulfooxyphenyl*)*indole*, *di-sodiumsalt* (**9**). m.p. > 140° (dec.). ^{1}H-NMR (DMSO-D_6), δ 0.6 (t, J = 7 Hz, 3H, $CH_2CH_2C\underline{H_3}$), δ 1.1–1.7 (m, J = 7 Hz, 2H, $CH_2C\underline{H_2}CH_3$), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 3.9 (t, J = 7 Hz, 2H, C$\underline{H_2}CH_2CH_3$), δ 6.5–7.5 (m, 7H, ArH). HPLC, t_R = 1.7 min. Elemental analysis for $C_{18}H_{17}NNa_2O_8S_2 \cdot H_2O$.

1-*Ethyl*-3-*methyl*-2-*phenyl*-5-*sulfooxyindole*, *mono-sodiumsalt* (**10**). m.p. > 140° (dec.). ^{1}H-NMR (DMSO-D_6), δ 1.1 (t, J = 7 Hz, 3H, $CH_2C\underline{H_3}$), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 4.1 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 6.9–7.6 (m, 8H, ArH). HPLC, t_R = 2.25 min. Elemental analysis for $C_{17}H_{16}NNaO_4S_2 \cdot H_2O$.

1-*Ethyl*-3-*methyl*-2-*phenyl*-6-*sulfooxyindole*, *mono-sodiumsalt* (**11**). m.p. > 150° (dec.). ^{1}H-NMR (DMSO-D_6), δ 1.1 (t, J = 7 Hz, 3H, $CH_2C\underline{H_3}$), δ 2.1 (s, 3H, CC$\underline{H_3}$), δ 4.1 (q, J = 7 Hz, 2H, C$\underline{H_2}CH_3$), δ 6.8–7.7 (m, 8H, ArH). HPLC, t_R = 1.6 min. Elemental analysis for $C_{17}H_{16}NNaO_4S_2 \cdot H_2O$.

Table 1. Inhibition of steroid sulfatase by natural substrates and simple phenyl derivatives

Substrate	IC_{50}* (μM)
Phenylsulfate	>8000
p-Nitrophenylsulfate	7000
17β-Estradiol-3-sulfate	35
DHAS	80

* Concentration of inhibitor required to reduce the rate of hydrolysis of [^{3}H]E_1S by 50%, mean value from two separate experiments, each performed in duplicate; the concentration of [^{3}H]E_1S was 10 μM.

5,6-*Disulfooxy*-1-*ethyl*-3-*methyl*-2-*phenylindole*, *di-sodiumsalt* (**12**). m.p. > 150° (dec.). ^{1}H-NMR (DMSO-D_6), δ 1.1 (t, J = 7 Hz, 3H, $CH_2\underline{CH_3}$), δ 2.1 (s, 3H, $C\underline{CH_3}$), δ 4.0 (q, J = 7 Hz, 2H, $C\underline{H_2}CH_3$), δ 7.2–7.7 (m, 7H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{17}H_{15}NNa_2O_8S_2 \cdot 2H_2O$.

1-*Ethyl*-3-*methyl*-2-(4-*sulfooxyphenyl*)*indole*, *mono-sodiumsalt* (**13**). m.p. > 180° (dec.). ^{1}H-NMR (D_6), δ 1.1 (t, J = 7 Hz, 3H, $CH_2\underline{CH_3}$), δ 2.2 (s, 3H, $C\underline{CH_3}$), δ 4.1 (q, J = 7 Hz, 2H, $C\underline{H_2}CH_3$), δ 6.9–7.7 (m, 8H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{17}H_{16}NNaO_4S_2 \cdot H_2O$.

5-*Ethyl*-7-*methyl*-6-(4-*sulfooxyphenyl*)-1,3-*dioxolo*[4,5-*f*]*indole*, *mono-sodiumsalt* (**14**). m.p. > 145° (dec.). ^{1}H-NMR (DMSO-D_6), δ 1.0 (t, J = 7 Hz, 3H, $CH_2\underline{CH_3}$), δ 2.1 (s, 3H, $C\underline{CH_3}$), δ 4.0 (q, J = 7 Hz, 2H, $C\underline{H_2}CH_3$), δ 6.0 (s, 2H, $O\underline{CH_2}O$), δ 7.0 (s, 1H, ArH), δ 7.1 (s, 1H, ArH), δ 7.3 (s, 4H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{18}H_{16}NNaO_6S \cdot 2H_2O$.

4-*Chlor*-2-(2,6-*dichlor*-4-*sulfooxyphenyl*)-1-*ethyl*-6-*sulfooxyindole*, *di-sodiumsalt* (**15**). m.p. > 175° (dec.). ^{1}H-NMR (D_2O), δ 1.1 (t, J = 7 Hz, 3H, $CH_2\underline{CH_3}$), δ 3.9 (q, J 7 Hz, 2H, $C\underline{H_2}CH_3$), δ 6.5 (s, 1H, $C\underline{H}$), δ 7.0–7.5 (m, 4H, ArH). HPLC, t_R = 1.7 min. Elemental analysis for $C_{16}H_{10}Cl_3NNa_2O_8S_2$.

3-*Methyl*-1-*pentafluorphenylmethyl*-6-*sulfooxy*-2-(4-*sulfooxyphenyl*)-4-*trifluormethylindole*, *di-sodiumsalt* (**16**). m.p. > 160° (dec.). ^{1}H-NMR (D_2O), δ 1.7 (s, 3H, $C\underline{H_3}$), δ 4.8 (s, 3H, $C\underline{H_3}$), δ 4.8 (s, 2H, $C\underline{H_2}$-Ar), δ 6.6–7.3 (m, 6H, ArH). HPLC, t_R = 2.3 min. Elemental analysis for $C_{23}H_{11}F_8NNa_2O_8S_2$.

RESULTS AND DISCUSSION

In order to confirm the selectivity of the steroid sulfatase preparation used, various compounds which should not show any inhibitory activities (phenylsulfate, *p*-nitrophenylsulfate) as well as compounds which are known to be substrates of the enzyme (E_2S, DHAS) were tested. E_2S and DHAS exhibit IC_{50} values of 35 and 80 μM, respectively, whereas *p*-nitrophenylsulfate as well as phenylsulfate show inhibitory effects only in very high excess (Table 1). These data clearly demonstrate the selectivity of the enzyme preparation used.

2-Phenylindole derivatives carrying two SO_4-groups in the positions 3 and 5 denoted in Table 2 (compounds **1**, **2** and **3**) show a clear correlation of their enzyme inhibiting potency with the chain length of the substituent R^1. With R^4 being $-CH_3$ the inhibitory activity decreases from IC_{50} = 500 μM to IC_{50} = 7000 μM with an increase in chain length from $R^1 = -H$ to $R^1 = -C_3H_7$. Compound **2** represents one of the main metabolites of zindoxifene *in vivo*. An IC_{50} value of 4000 μM indicates that it is only a very poor inhibitor of steroid sulfatase.

Compounds **4**, **5** and **6** carrying a SO_4-function at position 6 of the indole part generally show improved inhibitory activity which is not affected by the bulky 4-pyridyl-methyl-substituent at the indole nitrogen either. The comparison of **5** with its analogue **2** proves that shifting the sulfate group from position 5 to 6 is accompanied by a marked increase in enzyme inhibiting activity. This finding is confirmed by the results obtained with compounds **15** and **16** which carry sulfate groups at the denoted positions 6 and 4'. Furthermore the central regions of these molecules show hydrophobic character, and **16** carries a bulky substituent at the indole nitrogen. Compound **15** exhibits very good inhibiting properties (IC_{50} = 210 μM) whereas **16** is the most potent inhibitor tested (IC_{50} = 80 μM). Its affinity for steroid sulfatase is in the same order of magnitude as the affinities of the natural substrates E_1S and DHAS.

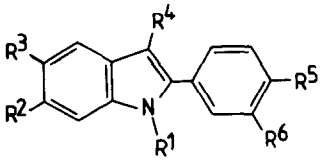
In the compounds **7**, **8** and **9** one SO_4-function was shifted from the *para*- to the *meta*-position of the phenyl ring while SO_4 at position 6 remained unchanged. This structural alteration slightly reduces the enzyme inhibiting activity.

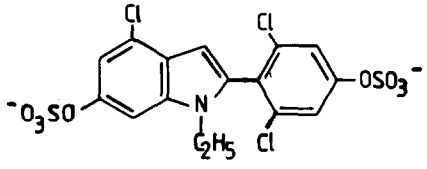
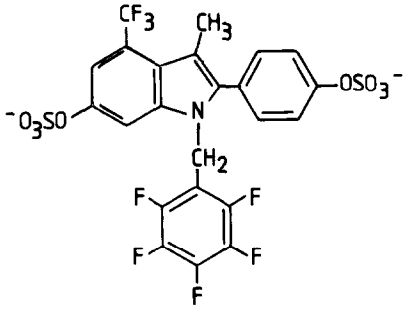
A further group of compounds is represented by those molecules which have only one SO_4-function. The monosulfate derivatives are related to the natural substrates of steroid sulfatase and therefore can be assumed to exert better inhibitory activities than do disulfates. A SO_4-group located in the indole part of the molecule leads to a rather poor enzyme inhibiting activity where it is irrelevant whether the SO_4-group is attached at position 5 or 6. A SO_4-function in the *para*-position of the phenyl part, however, further improves the inhibitory potency of this series of compounds by a factor of 3. Compound **14**, carrying a SO_4-group at position 4 of the phenyl part of the molecule and showing more polar characteristics due to the methylene-dioxy-function in the indole part, finally shows very strong inhibitory potency (IC_{50} = 120 μM). being only slightly weaker than the natural substrates tested.

In summary, we were able to demonstrate that starting from 2-(hydroxyphenyl)-indoles competitive inhibitors of steroid sulfatase can be developed by the introduction of sulfate groups at suitable positions of the molecule. Compounds with affinities to steroid sulfatase close to those of the natural substrates could be developed. When derivative **2** was incubated with the enzyme preparation, HPLC analysis [18] revealed its conversion to the free hydroxy compound (data not shown). Because of the structural similarity we assume that all of these 2-phenylindole sulfates are substrates of steroid sulfatase and are converted to their free hydroxy derivatives.

In the group of the disulfates tested, those carrying a SO_4-group in position 6 of the indole part proved to be the most potent inhibitors. The position of the

Table 2. Inhibition of steroid sulfatase by sulfate derivatives of 2-phenylindoles



No.	R^1	R^2	R^3	R^4	R^5	R^6	IC_{50}* (μM)
1	H	H	OSO_3^-	CH_3	OSO_3^-	H	500
2	C_2H_5	H	OSO_3^-	CH_3	OSO_3^-	H	4000
3	C_3H_7	H	OSO_3^-	CH_3	OSO_3^-	H	7000
4	C_2H_5	OSO_3^-	H	H	OSO_3^-	H	360
5	C_2H_5	OSO_3^-	H	CH_3	OSO_3^-	H	700
6	CH_2–C$_6$H$_5$	OSO_3^-	H	CH_3	OSO_3^-	H	450
7	CH_3	OSO_3^-	H	H	H	OSO_3^-	1600
8	C_2H_5	OSO_3^-	H	H	H	OSO_3^-	600
9	C_3H_7	OSO_3^-	H	CH_3	H	OSO_3^-	850
10	C_2H_5	H	OSO_3^-	CH_3	H	H	600
11	C_2H_5	OSO_3^-	H	CH_3	H	H	600
12	C_2H_5	OSO_3^-	OSO_3^-	CH_3	H	H	400
13	C_2H_5	H	H	CH_3	OSO_3^-	H	220
14	C_2H_5	O—CH_2—O		CH_3	OSO_3^-	H	120
15							210
16							80

* Concentration of inhibitor required to reduce the rate of hydrolysis of [^{3}H]E_1S by 50%, mean value from two separate experiments, each performed in duplicate; the concentration of [^{3}H]E_1S was 10 μM.

SO_4-group in the phenyl part, however, seems to be of minor importance. Large hydrophobic substituents at the indole nitrogen positively influence the inhibitory potential.

Sulfated zindoxifene-type analogues proved to be uneffective in inhibiting the enzyme as did sulfates of simple phenolic character. Among the monosulfates tested those carrying the SO_4-group in position 4 of the phenyl part rather than in the indole part are clearly preferred while the remaining part of the molecule has to exhibit hydrophobic properties.

In order to exert enzyme inhibition *in vivo* the compounds have to pass the cellular membrane. Natural steroid sulfates can be found in cells and act there as substrates of steroid sulfatase. Consequently, there has to be some transportation mechanism which facilitates the uptake of the ionic compounds. Due to structural analogies of the synthesized derivatives with natural steroid sulfates, intracellular uptake of the compounds can be expected. In order to test this hypothesis and to evaluate possible tumor inhibiting activities of the compounds, suitable experiments will be performed.

Acknowledgements—The authors wish to thank R. Ringshandl for technical assistance and the Deutsche Forschungsgemeinschaft (SFB 234) for financial support.

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