Thiosemicarbazones of Formyl Benzoic Acids as Novel Potent Inhibitors of Estrone Sulfatase†

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Thiosemicarbazones of the microbial metabolite madurahydroxylactone, a polysubstituted benzo[*a*]-naphthacenequinone, have been previously reported by us as potent nonsteroidal inhibitors of the enzyme estrone sulfatase (cyclohexylthiosemicarbazone **1**, IC_{50} 0.46 μM). The active pharmacophore of **1** has now been identified to be 2-formyl-6-hydroxybenzoic acid cyclohexylthiosemicarbazone (**25**, IC_{50} 4.2 μM). The active partial structure was derivatized in the search for novel agents against hormone-dependent breast cancer. Further substantial increases in activity were achieved by reversal of functional groups leading to the cyclohexylthiosemicarbazones of 5-formylsalicylic acid (**35**, IC_{50} 0.05 μM) and 3-formylsalicylic acid (**34**, IC_{50} 0.15 μM) as the most potent analogues identified to date. Both compounds were shown to be noncompetitive inhibitors of estrone sulfatase with K_i values of 0.13 μM and 0.12 μM, respectively. The compounds showed low acute toxicity in the hen's fertile egg screening test.

Introduction

Breast cancer is the leading site of new cancer cases in women, and the second leading cause of cancer death among women. In the countries of the European Union, one in twelve women will be at risk of having breast cancer during her life. Over one-third of all breast cancers require stimulation by estrogens for optimal growth. The estrogen 17β-estradiol has a pivotal role in the growth and maintenance of mamma carcinoma in postmenopausal women.[1] The source of estrogens in premenopausal women are predominantly the ovaries, but after the menopause, enzymatic synthesis of estradiol via sulfatase, aromatase, and 17β-hydroxysteroid dehydrogenase in the tumor itself contributes to the high estrogen levels measured in breast cancer tissue.

Much effort has been devoted to the development of inhibitors of the aromatase pathway which converts the androgen precursor androstenedione to estrone. However, estrone production is not completely abolished by aromatase inhibitors.[2] Furthermore, at physiological concentrations of estrone sulfate and androstenedione, productions of estrone from estrone sulfatase by hormon-dependent breast tumor is ten times faster than the rate of estrone production via the aromatase pathway.[3] Consequently, the control of estrone sulfatase inside the cancer cell with specific inhibitors may have considerable advantage over single therapy with aromatase inhibitors.[4−7]

Promising initial results in the blocking of this enzyme with steroidal inhibitors like estrone 3-*O*-sulfamate (EMATE[a]),[8] however, have been hampered by their strong estrogenic side effect.[9,10] There is currently increasing interest in developing a potent nonsteroidal inhibitor for treatment of endocrine-responsive breast cancer. A series of phenol sulfamate esters, like 4-methylcoumarin 7-*O*-sulfamate (COUMATE), proved to be inhibitors of the sulfatase,[11−29] thus demonstrating that the steroid nucleus as a whole is unnecessary for enzyme inhibition.[30,31]

We have recently reported a novel type of nonsteroidal sulfatase inhibitor. Thiosemicarbazone derivatives of the natural compound madurahydroxylactone (MHL), a polysubstituted benzo[*a*]naphthacenequinone, are highly potent but not estrogenic and show low acute toxicity.[32] Their structure−activity relationships have been disclosed, and the cyclohexylthiosemicarbazone (**1**, Figure 1) was shown to be the most potent inhibitor (IC_{50} 0.46 μM).

While we were pleased with our initial results, there were significant limitations and we wondered how further improvements could be achieved. Madurahydroxylactone is a highly complex benzo[*a*]naphthacenequinone. A laborious total synthesis was rejected, particularly as a promising route is far from being obvious. Moreover, because of their high affinity for protein binding and their molecular weight ($\approx$650 g mol^{-1}), it seemed doubtful whether the madurahydroxylactone thiosemicarbazones could be attractive as drug candidates themselves. Hence, we focused our further studies on the development of smaller molecules as inhibitors. Our approach was based on the hypothesis that A-ring analogues of **1** (see Figure 1) might be a promising lead structure. The present paper describes our studies into a series of thiosemicarbazones of formylbenzoic acids aiming at compounds with improved *in vitro* potency which might be expected to possess enhanced *in vivo* properties. Structure−activity relationships are discussed in detail.

Chemistry. The synthetic approaches to the target molecules are unexceptional and need no detailed description. All of the thiosemicarbazones and related semicarbazones in this study (Tables 1 and 2) were prepared by standard methods as outlined in Scheme 1 for compound **13**. With the exception of the di-ortho-substituted compounds **29** and **30**, no evidence for the formation of *E*−*Z* isomers mixture was noted, only the *E* isomers being obtained. The starting materials, for the synthesis of the thiosemicarbazones shown in Tables 1 and 2, were obtained from commercial sources, synthesized by literature methods (see Experimental Section), or were made as described below.

The tetrazole **40** was prepared readily by reaction of 3-cyanobenzaldehyde, protected as the diethyl acetal, with tributyltin azide (Scheme 1). The tributyltin group was removed employing sodium hydroxide, and the product was isolated most conveniently as the *N*-trityl derivative **39**. Subsequent deprotection

† This paper is dedicated to the memory of Prof. Dr. U. Gräfe who unexpectedly died during the course of this study.

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[a] Abbreviations: MHL, madurahydroxylacton; EMATE, estrone 3-*O*-sulfamate; COUMATE, 4-methylcoumarin 7-*O*-sulfamate.

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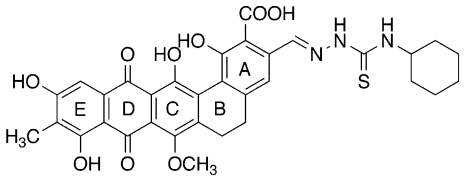
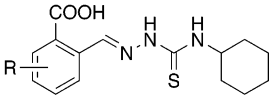


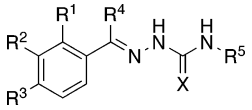
Madurahydroxylactone Cyclohexylthiosemicarbazone (**1**)



Partial Structure

Figure 1. MHL cyclohexylthiosemicarbazone (**1**) and its partial structure (A-ring analogue).

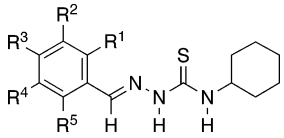
Table 1. Estrone Sulfatase Inhibition Data for **2**−**24**



compd	R^1	R^2	R^3	R^4	X	R^5	IC_{50} [μM]	pK_a
2	COOH	H	H	H	S	*c*-hexyl	4.0	6.4
3	H	COOH	H	H	S	*c*-hexyl	1.3	5.4
4	H	H	COOH	H	S	*c*-hexyl	24	
5	OH	H	H	H	S	*c*-hexyl	8.0	9.8
6	H	OH	H	H	S	*c*-hexyl	4.0	10.2
7	H	H	OH	H	S	*c*-hexyl	2.3	
8	H	COOH	H	H	O	*c*-hexyl	>100	
9	COOH	H	H	CH_3	S	*c*-hexyl	>100	
10	$COOCH_3$	H	H	H	S	*c*-hexyl	13	
11	SO_3Na	H	H	H	S	*c*-hexyl	4.0	-
12	$B(OH)_2$	H	H	H	S	*c*-hexyl	>100	7.7
13	H	tetrazol-5-yl	H	H	S	*c*-hexyl	0.81	5.2
14	COOH	H	H	H	S	*n*-butyl	NI[a]	
15	COOH	H	H	H	S	*n*-hexyl	NI[a]	
16	COOH	H	H	H	S	*c*-propyl	>100	
17	COOH	H	H	H	S	*c*-pentyl	43	
18	COOH	H	H	H	S	*c*-heptyl	13	
19	COOH	H	H	H	S	*c*-octyl	60	
20	COOH	H	H	H	S	phenyl	>100	
21	H	COOH	H	H	S	*n*-butyl	58	
22	H	COOH	H	H	S	*n*-hexyl	12	
23	H	COOH	H	H	S	*c*-pentyl	20	
24	H	COOH	H	H	S	*c*-heptyl	4.0	

[a] NI, no inhibition.

Table 2. Estrone Sulfatase Inhibition Data for Cyclohexylthiosemicarbazones **25**−**38** and Reference Compounds



compd	R^1	R^2	R^3	R^4	R^5	IC_{50} [μM]	pK_a
25	COOH	OH	H	H	H	4.2	
26	COOH	OCH_3	H	H	H	>100	
27	COOH	OH	CH_3	H	H	0.73	4.3
28	COOH	OCH_3	CH_3	H	H	>100	
29	COOH	OH	H	H	CH_3	2.5	
30	COOH	OCH_3	H	H	CH_3	35	
31	COOH	OH	OCH_3	H	H	20	
32	COOH	OH	OH	H	H	4.5	
33	COOH	OCH_3	OCH_3	H	H	>100	
34	OH	COOH	H	H	H	0.15	3.8
35	H	COOH	OH	H	H	0.05	4.2
36	OH	H	H	COOH	H	0.25	5.8
37	OH	H	H	COOEt	H	5.0	
38	OH	H	H	SO_3Na	H	4.6	
COUMATE	-	-	-	-	-	2.6	
EMATE	-	-	-	-	-	0.08	

effected by hydrochloric acid hydrolysis gave the target molecule **40**.

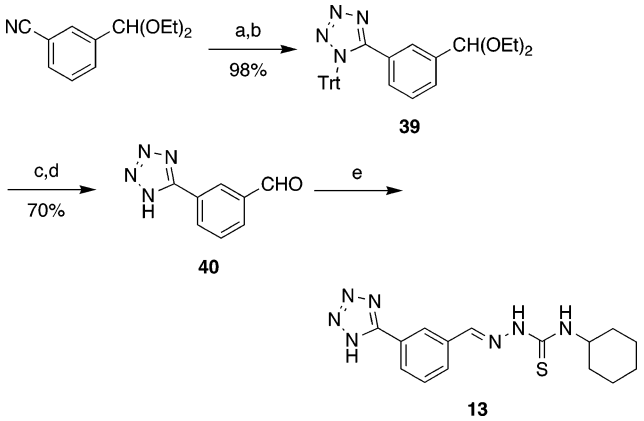
The phthalaldehydic acids **45**−**48**, which exist as an intramolecular hemiacetal, were prepared from the corresponding methyl ethers **(41**−**44**) by treatment with boron tribromide (Scheme 2). At −78 °C, boron tribromide regioselectively cleaved the methyl ether ortho to the carboxyl group. The catechol **49** was obtained from the guajacol **48** by dealkylation with aluminum trichloride. The details of these cleavage procedures are described in the Experimental Section.

Table 3. Kinetic Parameters and Acute Toxicity of **34**−**36** and **1**

compd	IC_{50} [μM]	K_m [μM]	K_i [μM]	acute tox. [mg/kg]
34	0.15	29	0.12	>50
35	0.05	31	0.13	>50
36	0.25	ND[a]	ND[a]	>100
1	0.46	27	0.46	>50

[a] ND, not determined.

Scheme 1[a]



[a] Reagents: (a) Bu_3SnN_3, toluene, reflux; (b) TrtCl, NaOH, H_2O; (c) HCl, H_2O, THF; (d) NaOH, H_2O; (e) cyclohexylthiosemicarbazide, EtOH, reflux.

Biological Testing. Enzyme activity was determined using a microsomal preparation from human placenta. The sulfatase activities in the presence of increasing amounts of inhibitor concentration were determined to evaluate the relative potency of the inhibitors. A negative control (absence of inhibitors) was run simultaneously. Some standard compounds (EMATE, COUMATE) were tested in our assay system for comparison with literature data. Our results were generally consistent with data obtained by other groups. The IC_{50} data are shown in Tables 1 and 2.

We performed kinetic analyses of **34** and **35**, some of the most active compounds. The analysis of the results following Lineweaver−Burk plot representations (data not shown) indicates that the compounds are noncompetitive inhibitors of human placental estrone sulfatase (Table 3). This behavior is in agreement with that of MHL cyclohexylthiosemicarbazone **1**.[32]

Results and Discussion

With the hypothesis in mind that A-ring analogues of **1** might be potent nonsteroidal estrone sulfatase inhibitors, we started a preliminary screening for a primitive definition of the minimal structural prerequisites. A set of ortho-, meta-, and para-substituted benzaldehyde cyclohexylthiosemicarbazones bearing methyl, fluorine, chlorine, bromine, hydroxy, methoxy, amino, acetamido, nitro, carboxy, and cyano groups were prepared and screened as inhibitors. The results obtained from these SAR studies are partly shown in Table 1 and can be summarized as follows: (1) Benzaldehyde cyclohexylthiosemicarbazone was a very poor inhibitor (IC_{50} >100 μM). Monosubstitution generally increased estrone sulfatase inhibition leading to compounds with moderate activity. (2) The introduction of certain substituents led to potent inhibitors (IC_{50}s in the range from 1.3 to 24 μM), the most potent compounds bearing a carboxy (**2**−**4**) or a hydroxy group (**5**−**7**). Both functional groups are found in the "natural" parent compound **1**! (3) The cyclohexylthiosemicarbazone of phthalaldehydic acid (**2**) and isophthalaldehydic acid (**3**) were about 10 times more active than the 4-isomer **4**. (4) The inhibitory activity of compounds **5**, **6**, and **7** was gradually increased as the position of the hydroxyl group was moved from ortho to para, with IC_{50} values of 8.0, 4.0, and 2.3 μM, respectively.

Having in this fashion identified the formylbenzoic acid thiosemicarbazones **2** and **3** as potent estrone sulfatase inhibitors, it was of interest to examine what structural features were required for their activity. Esterification of the carboxyl function caused a decrease in inhibitory activity. A similar result was obtained later in a second series of compounds (cf. **10** with **2**, Table 1, and **37** with **36**, Table 2). This suggests that the acid group plays an important role in its interaction with the enzyme.

Replacement of the sulfur in **3** by oxygen led to the corresponding semicarbazone **8**. Compound **8** showed a dramatical drop in activity (IC_{50} >100 μM). This agrees with our previously reported finding that the thiosemicarbazone is mandatory for potent inhibition of estrone sulfatase.[32] Substitution of the imine hydrogen with the more bulky methyl group produced the ketone derivative **9** which was inactive as an inhibitor at up to 100 μM. This argues for an interaction with the enzyme which is sensitive to steric effects.

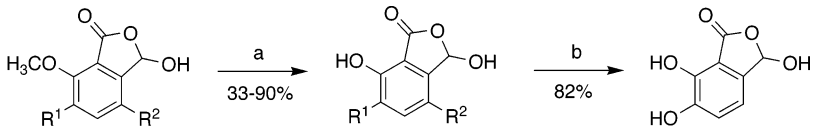
Having established the importance of the acid group and its proper position, we concluded that further study of this key substituent was well warranted. In an attempt to produce analogues of greater potency, the carboxylic acid groups of **2** and **3** were replaced by some isosteres. The acid mimics would allow us to vary pK_a values as well as the geometry and charge distribution about the acid center. pK_a values of selected compounds are listed in Tables 1 and 2. Since both carboxylic acids (compounds **2** and **3**) and phenols (**5**, **6**, and **7**) are inhibitors of estrone sulfatase, there is obviously no correlation between the acidity of the aromatic substituents and activity. It is, however, known that differences in charge distribution and geometry at the acid center can have profound effects.[33,34]

The most potent analogue arising from this effort was tetrazole **13**, which inhibited estrone sulfatase with an IC_{50} of 0.81 μM. This made the tetrazole the first compound with an IC_{50} below 1 μM. Tetrazoles are of particular interest to the medicinal chemist since they probably constitute the most commonly used bioisostere of the carboxyl group. The tetrazole is an "azole-type" carboxylic acid isostere and has a planar geometry. Tetrazole **13** has a pK_a value of 5.2 similar to that of the parent carboxylic acid **3** (pK_a 5.4).

The sulfonic acid **11** had an IC_{50} value of 4.0 μM which compared favorably with the parent carboxylic acid. On the other hand, in the hydroxyl-substituted series (Table 2), the sulfonic acid **38** reduced activity by a factor of 18 when compared to carboxylic acid **36**. These results indicate that, in certain circumstances, an acid with tetrahedral geometry, in which the charge of the anion is spread over three oxygen atoms, can be tolerated by the sulfatase enzyme. The boronic acid **12** was essentially inactive. Compound **12** was a weaker acid, but more important its acidity is based on hydroxyl ion acceptance rather than proton donation.

In the following compound series, the alkyl group in the thiosemicarbazono moiety was modified. The cycloalkylthiosemicarbazones (**2**, **3**, **16**−**19**, **23**−**24**) inhibited the sulfatase activity completely and concentration-dependent with IC_{50}s in the range of 1.3 (**3**) to 60 μM (**19**). These values were not as low as those reported for the madurahydroxylactone cycloalkylthiosemicarbazones[32] but the relative differences agree. Aliphatic chains (**14**, **15**, **21**, **22**) and aromatic substituents (**20**) gave rise to compounds of lower activity. This roughly parallels the madurahydroxylactone thiosemicarbazoness described previ-

Scheme 2[a]



41 $R^1 = R^2 = H$
42 R^1 = Me, $R^2 = H$
43 $R^1 = H$, R^2 = Me
44 R^1 = OMe, $R^2 = H$

45 $R^1 = R^2 = H$
46 R^1 = Me, $R^2 = H$
47 $R^1 = H$, R^2 = Me
48 R^1 = OMe, $R^2 = H$

49

[a] Reagents: (a) BBr_3, CH_2Cl_2, −78 °C; (b) $AlCl_3$, CH_2Cl_2.

ously.[32] Surprisingly, the butyl and hexyl derivatives **14** and **15** were essentially inactive. The data suggested a hydrophobic interaction in which a cycloalkyl of the proper size fits snugly. Therefore, the cyclohexylthiosemicarbazone was selected for all our investigations.

MHL cyclohexylthiosemicarbazone **1** (IC_{50} 0.46 μM), having at least one hydroxy group in the A-ring, was 8.7 times stronger than **2**. Therefore, a further study was subsequently undertaken to evaluate effects of introducing hydroxyl groups and other substituents in the compounds **2** and **3**. The results of this study are shown in Table 2. Salicylic acid derivative **25** represents a synthetic partial structure closer to **1** than **2**. It was disappointing that hydroxy analogue **25** was only equipotent with compound **2**. Increasing the hydrophobicity by introduction of a methyl group, compounds **27** and **29**, led to an increase in estrone sulfatase inhibition. The methyl group in 6-formyl-2-hydroxy-3-methylbenzoic acid cyclohexylthiosemicarbazone **27** (IC_{50} 0.73 μM) enhances activity more than threefold compared to the regioisomer **29**. Replacement of the methyl group in **27** by a methoxy subsitutent (**31**) reduced activity whereas the catechol **32** reestablished the inhibitory power. The IC_{50} values of the methyl ether derivatives **26**, **28**, **30**, and **33** were about 1 order of magnitude higher than those of the phenolic analogues (**25**, **27**, **29**, and **31**). Thus, the potency of all these compounds were clearly dependent on the presence of a free hydroxy group.

By analogy to the relationship between ortho-carboxylic acid **2** and the meta-compound **3**, we hypothesized that hydroxylated isophthalaldehydic acid cyclohexylthiosemicarbazones might well be more effective than **25** and **27**. Indeed, this was effectively the case. Salicylic acid derivative **34** enhanced the sulfatase inhibition by 1 order of magnitude (IC_{50} 0.15 μM). It is interesting to note that the position of the hydroxyl group plays an important role since the regioisomeric salicylate **35** (IC_{50} 0.05 μM) is three times more active than **34**. Moving the hydroxy group meta to both the carboxylic acid and the thiosemicarbazone (**36**) slightly reduces the potency. However, compound **36** still inhibits fairly well compared to **3**, which is much less active. The data clearly indicate the great importance of the hydroxy group in these compounds with respect to sulfatase inhibition. This represents a major difference in SAR between the isophthalaldehydic and phthalaldehydic acid series. Compounds **34**, **35**, and **36** all possess much greater inhibitory activity than COUMATE. 5-Formylsalicylic acid cyclohexylthiosemicarbazone **35** was the most potent inhibitor of all the compounds presented and inhibited estrone sulfatase even more stronger than EMATE.

In an attempt to rationalize the structure−activity data discussed above and presented in Tables 1 and 2, we assume that multiple hydrogen bonding and dipolar interactions of the aromatic acid unit and the alkylthiosemicarbazone region are important features for binding with the enzyme estrone sulfatase. The aldimine double bond and the restricted rotation about the C−N bonds in the thiourea moiety cause the thiosemicarbazones to be conformationally rather rigid molecules.[35] The ^{1}H NMR signals of the phthalaldehydic derivatives **25**, **27**, and **31**, as well as the isophthalaldehydic thiosemicarbazones **3**, **34**, **35**, and **36**, could be assigned unequivocally by a combination of COSY, HMBC, and HMQC experiments. We hoped that the ^{1}H−^{1}H-NOESY spectra might give some insight into conformation and configuration of these sulfatase inhibitors. While the NOESY spectra of **3** and **27** supported strong evidence that these molecules adopt an extended coplanar configuration, *E* relative to the aldimine bond and *Z*,*Z* regarding the thiourea, the other compounds, unfortunately, did not show all relevant cross-peaks. Further modular variations of the thiosemicarbazones completing the preliminary structure−activity study should form the basis of a molecular approach toward more potent nonsteroidal estrone sulfatase inhibitors.

The following studies were undertaken to investigate the pharmacological characteristics of the most potent compounds **34**, **35**, and **36** as new prototypic estrone sulfatase inhibitors. We expected that the compounds are not active-site directed inhibitors. In fact, *in vitro* kinetic studies revealed **34** and **35** to be noncompetitive inhibitors of estrone sulfatase. The kinetic parameters obtained from Lineweaver−Burk plots are outlined in Table 3. Thus, the mechanism of inhibition is different from that of the phenol sulfamates, which compete with estrone sulfate, but is similar to that of MHL cyclohexylthiosemicarbazone (**1**, see Table 3).[32]

The acute toxicities of **34** and **36** were studied in the hen's fertile egg screening test (HEST). Fifteen-day old chick embryos receive the test compound through the air cell and deaths were measured at 72−96 h after the treatment. The LD_{50} of HEST is strongly positively correlated to i.v. LD_{50} obtained in mice and rodents.[36] The acute toxicity of **34**−**36** determined in the hen's fertile egg screening test were >50 mg kg^{-1} and >100 mg kg^{-1}, respectively (Table 3). The compounds showed no antibacterial activity. They inhibited the growth of K-562 leukemia cells and HeLa cervix carcinoma cells with IC_{50} of >50 μg mL^{-1}.

The compounds **34**, **35**, and **36** are relatively small molecules capable of forming sodium salts of the carboxylic acid. The sodium salts obtained by careful treament with 1 equiv of sodium hydroxide are sufficiently water soluble (>0.1 g mL^{-1}) to permit easy formulation for *in vivo* tests of estrone sulfatase inhibition.

Conclusions

The active pharmacophore of the nonsteroidal sulfatase inhibitor MHL cyclohexylthiosemicarbazone (**1**) has now been identified to be 2-formylbenzoic acid cyclohexylthiosemicarbazone (**2**). The active partial structure was derivatized to improve the inhibitory power. Thiosemicarbazones of formylsalicylic acids exemplified by **25** and 5-formyl-2-hydroxybenzoic acid cyclohexylthiosemicarbazone (**35**) are a new class of

nonsteroidal estrone sulfatase inhibitors. The thiosemicarbazone **35** was characterized by an IC_{50} of 50 nM and possesses greater inhibitory activity than EMATE and COUMATE. Compounds **25**, **35**, and analogues such as **34** and **36** can be easily synthesized. Our studies revealed that the salicylic acid moiety, an isophthalaldehydic acid motif, and the thiosemicarbazone were all critical for high sulfatase inhibition. The carboxylic acid moiety can be replaced by selected acid mimics, for example, a tetrazol-5-yl, to give compounds of similar activity. The most potent inhibitors show low acute toxicity and are not cytotoxic. Compounds **34**, **35**, and **36** are therefore good lead compounds for the development of potent novel agents against hormone-dependent breast cancer.

Experimental Section

Chemical Compounds and Synthetic Procedures. General Information. The following substituted benzoic acids are available commercially: phthalaldehydic acid, isophthalaldehydic acid, 2-acetylbenzoic acid, 2-formylbenzenesulfonic acid, 2-formylboronic acid, 3-formylsalicylic acid, and 5-formylsalicylic acid, as well as the 2-, 3-, and 4-hydroxybenzaldehydes. Minor modifications of literature procedures furnished: 3-hydroxy-7-methoxy-3*H*-isobenzofuran-1-one (**41**),[37] 3-hydroxy-7-methoxy-6-methyl-3*H*-isobenzofuran-1-one (**42**),[38] 3-hydroxy-7-methoxy-4-methyl-3*H*-isobenzofuran-1-one (**43**),[38] 3-formyl-4-hydroxybenzoic acid,[39] and 3-formyl-4-hydroxybenzenesulfonic acid.[40] The thiosemicarbazides and semicarbazides are commercially available or were prepared by literature procedures.[41]

Analytical thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminum sheets silica gel 60 F_{254}). Products and starting material were detected by viewing under UV light (254 or 366 nm). Column chromatography was performed on silica gel 60 (0.063−0.1 mm; Merck). Nuclear magnetic resonance spectra (NMR) were obtained on a Bruker DPX 300 (^{1}H, 300 MHz; ^{13}C 75 MHz) and Bruker DRX 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) instruments; chemical shifts (δ) are expressed in parts per million (ppm) relative to the residual solvent peak. Electrospray mass spectra (ESMS) were obtained with a VG Quattro mass spectrometer. Melting points were taken on a Büchi B-540 melting point apparatus and are uncorrected. pK_a measurements are obtained from the titration curve applying the Kolthoff method. A 5−10 mg amount of the test compound was dissolved in 20% aqueous DMF. This was then titrated against 10 mM NaOH solution via a 5-mL burette. Elemental analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Solvents were dried according to the standard procedures. Chemical yields were not optimized.

General Procedure for the Preparation of Cyclohexylthiosemicarbazones 2−13 and 25−38. 4-Cyclohexylthiosemicarbazide (10 mmol) in one portion was added to a boiling solution of the aldehyde (10 mmol) in ethanol (3 mL). The reaction mixture was stirred under reflux until all the starting material was consumed (TLC). After cooling, the solid was filtered off and recrystallized from ethanol.

2-(4-Cyclohexylthiosemicarbazono)methylbenzoic Acid (2). mp 232−234 °C; ^{1}H NMR (DMSO-d_6) δ 1.13−1.93 (m, 10 H), 4.17 (m, 1 H), 7.44−7.51 (m, 1H), 7.54−7.62 (m, 1 H), 7.78−7.84 (m, 1 H), 8.13 (d, J = 7.4 Hz, 1 H), 7.94 (d, J = 8.5 Hz, 1 H), 8.74 (s, 1 H), 11.53 (s, 1 H). Anal. ($C_{15}H_{19}N_3O_2S$) C, H, N, S.

3-(4-Cyclohexylthiosemicarbazono)methylbenzoic Acid (3). mp 239−241 °C; ^{1}H NMR (DMSO-d_6) δ 1.05−1.52 (m, 5 H), 1.54−1.79 (m, 3 H), 1.80−1.94 (m, 2 H), 4.07−4.27 (m, 1 H), 7.55 (t, J = 8.0 Hz, 1 H, H-5), 7.94 (m, 1 H, H-6), 8.03−8.19 (m, 3 H, CH═N, H-2,4), 11.46 (s, 1 H, ═N−NH), 13.14 (br, 1 H). Anal. ($C_{15}H_{19}N_3O_2S$) C, H, N, S.

4-(4-Cyclohexylthiosemicarbazono)methylbenzoic Acid (4). mp 255−260 °C; ^{1}H NMR (DMSO-d_6) δ 1.11−1.35 (m, 3 H), 1.37−1.53 (m, 2 H), 1.55−1.65 (m, 1 H), 1.67−1.78 (m, 2 H), 1.81−1.92 (m, 2 H), 4.12−4.26 (m, 1 H), 7.87, 7.94 (2 d, J = 8.5 Hz, 2 H), 8.09 (m, J = 8.5 Hz, 1 H), 8.09 (s, 1 H), 11.50 (s, 1 H). Anal. ($C_{15}H_{19}N_3O_2S$) C, H, N, S.

3-(1*H*-Tetrazol-5-yl)-benzaldehyde Cyclohexylthiosemicarbazone (13). mp 228−229 °C; ^{1}H NMR (DMSO-d_6) δ 1.07−1.50 (m, 5 H), 1.60 (m, 1 H), 1.66−1.79 (m, 2 H), 1.82−1.95 (m, 2 H), 4.19 (m, 1 H), 7.65 (t, J = 7.8 Hz, 1 H), 7.99−8.07 (m, 2 H), 8.11 (d, J = 7.7 Hz, 1 H), 8.15 (s, 1 H, CH═N), 8.25 (br s, 1 H, H-2), 11.53 (s, 1 H, ═N−NH). Anal. ($C_{15}H_{19}N_7S$) C, H, N, S.

2-(4-Cyclohexylthiosemicarbazono)methyl-6-hydroxybenzoic Acid (25). mp 197−199 °C; ^{1}H NMR (DMSO-d_6) δ 1.05−1.47 (m, 5 H), 1.53−1.78 (m, 3 H), 1.82−1.96 (m, 2 H), 4.10 (m, 1 H), 6.91 (m, 1 H, H-5), 7.24−7.31 (m, 2 H, H-3,4), 7.74 (d, J = 8.4 Hz, 1 H, NH-cHex), 8.12 (s, 1 H, CH═N), 11.51 (s, 1 H, ═N−NH). Anal. ($C_{15}H_{19}N_3O_3S$) C, H, N, S.

6-(4-Cyclohexylthiosemicarbazono)methyl-2-hydroxy-3-methylbenzoic Acid (27). mp 197−198 °C; ^{1}H NMR (DMSO-d_6) δ 1.13 (m, 1 H), 1.27 (m, 2 H), 1.40 (m, 2 H), 1.59 (m, 1 H), 1.72 (m, 2 H), 1.87 (m, 2 H), 2.19 (s, 3 H, 3−CH_3), 4.15 (m, 1 H), 7.29 (d, J = 7.8 Hz, 1 H, H-4), 7.35 (d, J = 7.8 Hz, 1 H, H-5), 7.80 (d, J = 8.5 Hz, 1 H, NH-cHex), 8.45 (s, 1 H, CH═N), 11.46 (s, 1 H, ═N−NH). Anal. ($C_{16}H_{21}N_3O_3S$) C, H, N, S.

3-(4-Cyclohexylthiosemicarbazono)methyl-2-hydroxybenzoic Acid (34). mp 227−229 °C; ^{1}H NMR (DMSO-d_6) δ 1.02−1.52 (m, 5 H), 1.60 (m, 1 H), 1.65−1.79 (m, 2 H), 1.81−1.97 (m, 2 H), 4.18 (m, 1 H), 6.97 (t, J = 7.8 Hz, 1 H, H-5), 7.84 (dd, J = 7.8/1.7 Hz, 1 H, H-6), 8.00 (d, J = 8.4 Hz, 1 H, NH-cHex), 8.30 (dd, J = 7.7/1.7 Hz, 1 H, H-4), 8.42 (s, 1 H, CH═N), 11.47 (s, 1 H, ═N−NH). Anal. ($C_{15}H_{19}N_3O_3S$) C, H, N, S.

5-(4-Cyclohexylthiosemicarbazono)methyl-2-hydroxybenzoic Acid (35). mp 240−242 °C; ^{1}H NMR (DMSO-d_6) δ 1.14 (m, 1 H), 1.20−1.33 (m, 2 H), 1.34−1.48 (m, 2 H), 1.59 (m, 1 H), 1.65−1.76 (m, 2 H), 1.80−1.91 (m, 2 H), 4.17 (m, 1 H), 7.01 (d, J = 8.7 Hz, 1 H, H-3), 7.93 (d, J = 2.1 Hz, 1 H, H-6), 7.05 (d, J = 8.7 Hz, 1 H, HN−cHex), 8.01 (s, 1 H, CH═N), 8.12 (dd, J = 8.7/2.0 Hz, 1 H, H-4), 11.28 (s, 1 H, ═N−NH) Anal. ($C_{15}H_{19}N_3O_3S$) C, H, N, S.

3-(4-Cyclohexylthiosemicarbazono)methyl-4-hydroxybenzoic Acid (36). mp >350 °C; ^{1}H NMR (DMSO-d_6) δ 1.08−1.49 (m, 5 H), 1.51−1.76 (m, 3 H), 1.82−1.95 (m, 2 H), 4.16 (m, 1 H), 6.94 (d, J = 8.6 Hz, 1 H, H-5), 7.80 (dd, J = 8.6/2.2 Hz, 1 H, H-6), 7.94 (d, J = 8.4 Hz, 1 H, HN−cHex), 8.32 (d, J = 2.2 Hz, 1 H, H-2), 8.38 (s, 1 H, CH═N), 10.78 (br s, 1 H), 11.38 (s, 1 H, ═N−NH), 12.59 (br, 1 H). Anal. ($C_{15}H_{19}N_3O_3S$) C, H, N, S.

Biology. Materials. Estrone sulfate was purchased from Sigma Chemical Co. (Germany). [6,7-^{3}H]Estrone sulfate (specific activity, 53 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Radioactive samples were analyzed with a Packard Tri-Carb 1600 Liquid scintillation counter. Liquid scintillation cocktail was Ultima Gold from Packard (Meriden, CT).

Estrone Sulfatase Assay. ^{3}H-Estrone sulfate (500,000 dpm/tube) adjusted to 20 μM with unlabeled estrone sulfate in Tris-HCl buffer (0.2 M, pH 8.0, 0.1 mL) was added to a test tube. An inhibitor at various concentrations in Tris-HCl buffer (0.1 mL) was then added to each tube. The assay began by the addition of placental microsomes diluted with Tris-HCl buffer containing 2.5 mM dithiothreitol (0.3 mL). The protein concentration was measured using the Bradford method. The final volume of the assay was 0.5 mL. After 20 min of incubation at 37 °C, 1.5 mL of toluene was added to quench the assay. Control samples with no inhibitor were incubated simultaneously. The quenched samples were vortexed for 1 min, and 50 μL of toluene was removed from the organic phase and diluted with 3 mL of scintillation cocktail. The aliquots were counted for 3 min to determine the amount of product formation. All samples were run twice in duplicate. Product formation for samples containing an inhibitor was compared to that of the control sample.

Kinetic Analysis of the Inhibition of Estrone Sulfatase Activity. Estrone sulfatase assays were carried out as described above using various concentrations of the substrate (2−20 μM). The K_m, K_i, and type inhibition for 34 and 35 at pH 8.0 were determined by Lineweaver−Burk plot analysis and secondary

replots of the slopes of Lineweaver−Burk plot versus the corresponding inhibitor concentration (0, 0.5, 1, 2.5, 5 μM).

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Supporting Information Available: Full experimental details and analytical data for compounds **2**−**40** and **45**−**49**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pasqualini, J. R.; Schatz, B.; Varin, C.; Nguyen, B. L. Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 323−329.
(2) Purohit, A.; Dauvois, S.; Parker, M. G.; Potter, B. V. L.; Williams, G. J. et al. The hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate by human steroid sulfatase expressed in transfected COS-1 cells. *J. Steroid Biochem. Mol. Biol.* **1994**, *50*, 101−104.
(3) Pasqualini, J. R.; Cortesprieto, J.; Chetrite, G.; Talbi, M.; Ruiz, A. Concentrations of estrone, estradiol and their sulfates, and evaluation of sulfatase and aromatase activities in patients with breast fibroadenoma. *Int. J. Cancer.* **1997**, *70*, 639−643.
(4) Reed, M. J.; Purohit, A. Sulphatase inhibitors: the rationale for the development of a new endocrine therapy. *Rev. Endocr.-Rel. Cancer* **1993**, *45*, 51−62.
(5) Ahmed, S.; Owen, C. P.; James, K.; Sampson, L.; Patel, C. K. Review of estrone sulfatase and its inhibitors−an important new target against hormone dependent breast cancer. *Curr. Med. Chem.* **2002**, *9*, 263−273.
(6) Nussbaumer, P.; Billich, A. Steroid sulfatase inhibitors: their potential in the therapy of breast cancer. *Curr. Med. Chem. Anticancer Agents* **2005**, *5*, 507−528.
(7) Reed, M. J.; Purohit, A.; Woo, L. W.; Newman, S. P.; Potter, B. V. Steroid sulfatase: molecular biology, regulation, and inhibition. *Endocr. Rev.* **2005**, *26*, 171−202.
(8) Howarth, N. M.; Purohit, A.; Reed, M. J.; Potter, B. V. L. Estrone sulfamates: potent inhibitors of estron sulfatase with therapeutic potential. *J. Med. Chem.* **1994**, *37*, 219−221.
(9) Elger, W.; S. Schwarz, S.; Hedden, A.; Reddersen, G.; Schneider, B. Sulfamates of various estrogens are prodrugs with increased systemic and reduced hepatic estrogenicity at oral application. *J. Steroid Biochem. Mol. Biol.* **1995**, *66*, 395−403.
(10) Chander, S. K.; Purohit, A.; Woo, L. W.; Potter, B. V.; Reed, M. J. The role of steroid sulphatase in regulating the oestrogenicity of oestrogen sulphamates. *Biochem. Biophys. Res. Commun.* **2004**, *322*, 217−222.
(11) Purohit, A.; Woo, L. W. L.; Singh, A.; Winterborn, C. J.; Potter, B. V. L. et al. *In vivo* activity of 4-methylcoumarin-7-*O*-sulfamate, a nonsteroidal, nonestrogenic steroid sulfatase inhibitor. *Cancer Res.* **1996**, *56*, 4950−4955.
(12) Selcer, K. W.; Hedge, P. V.; Li, P.-K. Inhibition of estrone sulfatase and proliferation of human breast cancer cells by nonsteroidal (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines. *Cancer Res.* **1997**, *57*, 702−707.
(13) Woo, L. W.; Howarth, N. M.; Purohit, A.; Hejaz, H. A.; Reed, M. J. et al. Steroidal and nonsteroidal sulfamates as potent inhibitors of steroid sulfatase. *J. Med. Chem.* **1998**, *41*, 1068−1083.
(14) Kolli, A.; Chu, G. H.; Rhodes, M. E.; Inoue, K.; Selcer, K. W. et al. Development of (*p*-*O*-sulfamoyl)-*N*-alkanoyl-phenylalkyl amines as non-steroidal estrone sulfatase inhibitors. *J. Steroid Biochem. Mol. Biol.* **1999**, *68*, 31−40.
(15) Chu, G. H.; Peters, A.; Selcer, K. W.; Li, P. K. Synthesis and sulfatase inhibitory activities of (*E*)- and (*Z*)-4-hydroxytamoxifen sulfamates. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 141−144.
(16) Woo, L. L.; Purohit, A.; Malini, B.; Reed, M. J.; Potter, B. V. Potent active site-directed inhibition of steroid sulphatase by tricyclic coumarin-based sulphamates. *Chem. Biol.* **2000**, *7*, 773−791.
(17) Purohit, A.; Woo, L. W.; Barrow, D.; Hejaz, H. A.; Nicholson, R. I. et al. Non-steroidal and steroidal sulfamates: new drugs for cancer therapy. *Mol. Cell Endocrinol.* **2001**, *171*, 129−135.
(18) Billich, A.; Nussbaumer, P.; Lehr, P. Stimulation of MCF-7 breast cancer cell proliferation by estrone sulfate and dehydroepiandrosterone sulfate: inhibition by novel non-steroidal steroid sulfatase inhibitors. *J. Steroid Biochem. Mol. Biol.* **2000**, *73*, 225−235.
(19) Ahmed, S.; James, K.; Owen, C. P.; Patel, C. K. Synthesis and biochemical evaluation of novel and potent inhibitors of the enzyme oestrone sulphatase (ES). *J. Steroid Biochem. Mol. Biol.* **2002**, *80*, 419−427.
(20) Ahmed, S.; James, K.; Owen, C. P. The design, synthesis, and biochemical evaluation of derivatives of biphenyl sulfamate-based compounds as novel inhibitors of estrone sulfatase. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 180−183.
(21) Ahmed, S.; James, K.; Owen, C. Inhibition of estrone sulfatase (ES) by derivatives of 4-[(aminosulfonyl)oxy] benzoic acid. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2391−2394.
(22) Ciobanu, L. C.; Luu-The, V.; Poirier, D. Nonsteroidal compounds designed to mimic potent steroid sulfatase inhibitors. *J. Steroid Biochem. Mol. Biol.* **2002**, *80*, 339−353.
(23) Nussbaumer, P.; Bilban, M.; Billich, A. 4,4′-Benzophenone-O,O′-disulfamate: a potent inhibitor of steroid sulfatase. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2093−2095.
(24) Nussbaumer, P.; Lehr, P.; Billich, A. 2-Substituted 4-(thio)chromenone 6-O-sulfamates: potent inhibitors of human steroid sulfatase. *J. Med. Chem.* **2002**, *45*, 4310−4320.
(25) Walter, G.; Liebl, R.; von Angerer, E. 2-Phenylindole sulfamates: inhibitors of steroid sulfatase with antiproliferative activity in MCF-7 breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **2004**, *88*, 409−420.
(26) Billich, A.; Meingassner, J. G.; Nussbaumer, P.; Desrayaud, S.; Lam, C. et al. 6-[2-(adamantylidene)-hydroxybenzoxazole]-O-sulfamate, a steroid sulfatase inhibitor for the treatment of androgen- and estrogen-dependent diseases. *J. Steroid Biochem. Mol. Biol.* **2004**, *92*, 29−37.
(27) Hejaz, H. A.; Woo, L. W.; Purohit, A.; Reed, M. J.; Potter, B. V. Synthesis, in vitro and in vivo activity of benzophenone-based inhibitors of steroid sulfatase. *Bioorg. Med. Chem.* **2004**, *12*, 2759−2772.
(28) Patel, C. K.; Owen, C. P.; Ahmed, S. Inhibition of estrone sulfatase (ES) by alkyl and cycloalkyl ester derivatives of 4-[(aminosulfonyl)-oxy] benzoic acid. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 605−609.
(29) Saito, T.; Kinoshita, S.; Fujii, T.; Bandoh, K.; Fuse, S. et al. Development of novel steroid sulfatase inhibitors; II. TZS-8478 potently inhibits the growth of breast tumors in postmenopausal breast cancer model rats. *J. Steroid Biochem. Mol. Biol.* **2004**, *88*, 167−173.
(30) Ahmed, S.; James, K.; Sampson, L.; Mastri, C. Structure-activity relationship study of steroidal and nonsteroidal inhibitors of the enzyme estrone sulfatase. *Biochem. Biophys. Res. Commun.* **1999**, *254*, 811−815.
(31) Ahmed, S.; James, K. Derivation of a possible transition-state for the reaction catalysed by the enzyme estrone sulfatase (ES). *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1645−1650.
(32) Jütten, P.; Schumann, W.; Härtl, A.; Heinisch, L.; Gräfe, U. et al. A novel type of nonsteroidal estrone sulfatase inhibitors. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1339−1342.
(33) Carini, D. J.; Duncia, J. V.; Aldrich, P. A.; Chiu, A. T.; Johnson, A. L. et al. Nonpeptide angiotensin II receptor antagonists: The discovery of a series of *N*-(biphenylylmethyl)imidazoles as potent, orally active antihypertensives. *J. Med. Chem.* **1991**, *34*, 2525−2547.
(34) Drysdale, M. J.; Pritchard, M. C.; Horwell, D. C. Rationally designed "dipeptoid" analogues of CCK. Acid mimics of the potent and selective non peptide CCK-B receptor antagonist CI-988. *J. Med. Chem.* **1992**, *35*, 2573−2581.
(35) Walpole, C. S.; Wrigglesworth, R.; Bevan, S.; Campbell, E. A.; Dray, A. et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 2. The amide bond "B-region". *J. Med. Chem.* **1993**, *36*, 2373−2380.
(36) Nishigori, H.; Mizumura, M.; Iwatsuru, M. The hen's fertile egg screening test (HEST): a comparison between the acute toxicity for chick embryos and rodents of 20 drugs. *Cell. Biol. Toxicol.* **1992**, *8*, 255−265.
(37) Chenard, B. L.; Dolson, M. G.; Sercel, A. D.; Swenton, J. S. Annelation reactions of quinone monoketals. Studies directed at an efficient synthesis of anthracyclinones. *J. Org. Chem.* **1984**, *49*, 318−325.
(38) Hoffmann, B.; Lackner, H. Strukturmodifizierte Isokalafungine und Isonanaomycine. *Liebigs Ann.* **1995**, 87−94.
(39) Hullar, T. L.; Failla, D. L. Pyridoxyl phosphate. II. Benzene analogs. 2-Formylphenoxyacetic acids as potential antimetabolites of pyridoxyl phosphate. *J. Med. Chem.* **1969**, *12*, 420−424.
(40) Sunamoto, J.; Iwamoto, K.; Akutagawa, M.; Nagase, M.; Kondo, H. Rate control by restricting mobility of substrate in specific reaction field. Negative photochromism of water-soluble spiropyran in AOT reversed micelles. *J. Am. Chem. Soc.* **1982**, *104*, 4904−4907.
(41) Hagemann, H. Carbonic acid derivatives. In *Houben-Weyl*, 4th ed.; Georg Thieme: Stuttgart, 1983.

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