Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 639–656

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis of (bis)Sulfonic Acid, (bis)Benzamides as Follicle-Stimulating Hormone (FSH) Antagonists

Jay Wrobel,[a,*] Daniel Green,[a] James Jetter,[a] Wenling Kao,[a] John Rogers,[a] M. Claudia Pérez,[b] Jill Hardenburg,[b] Darlene C. Deecher,[b] Francisco J. López,[b,†] Brian J. Arey[b,‡] and Emily S. Shen[b]

[a]*Chemical Sciences, Wyeth Research, Inc, 500 Arcola Road, Collegeville, PA19426, USA*
[b]*Women's Health Research Institute, Wyeth Research, Inc, 500 Arcola Road, Collegeville, PA19426, USA*

Received 1 August 2001; accepted 4 September 2001

Abstract—Screening efforts identified (bis)sulfonic acid, (bis)benzamides (**1**–**3**) as compounds that interact with the follicle stimulating-hormone receptor (FSHR) and inhibit FSH-stimulated cAMP accumulation with IC_{50} values in the low micromolar range. Structure–activity relationship studies using novel analogues of **1**–**3** revealed that two phenylsulfonic acid moieties were necessary for activity and that the carbon–carbon double bond of the stilbene sub-series was the optimum spacer connecting these groups. Selected analogues (**2**, **14**, and **50**) were also able to block FSHR-dependent estradiol production in rat primary ovarian granulosa cells and progesterone secretion in a clonal mouse adrenal Y1 cell line. IC_{50} values for these compounds in these assays were in the low micromolar range. Optimization of the benzoic acid side chains of **1**–**3** led to gains in selectivity versus activity at the thyroid stimulating hormone (TSH) receptor (TSHR). For instance, while stilbene (bis)sulfonic acid congener **2** was only 10-fold selective for FSHR over TSHR, analogue **50** with an IC_{50} value of 0.9 μM in the FSHR-cAMP assay was essentially inactive at 30 μM in the TSHR-cAMP assay.

Introduction

Reproduction in women depends upon the dynamic interaction of several compartments of the female reproductive system. The hypothalamic–pituitary axis orchestrates a series of events affecting the ovaries and the uterine–endometrial compartment, which leads to the production of mature ova, ovulation, and ultimately appropriate conditions necessary for fertilization. Specifically, luteinizing hormone-releasing hormone (LHRH), released from the hypothalamus, initiates the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary. These hormones act directly on the ovary to promote the development of selected follicles by inducing granulosa and theca cell proliferation and differentiation. FSH stimulates aromatization of androgens to estrogens and increases the expression of LH receptors in the theca cells. Concentrations of estradiol and inhibin progressively increase during the follicular phase. Inhibin decreases FSH secretion from the pituitary gland, while estradiol acts on the hypothalamus and pituitary to induce the LH surge in mid-cycle, which results in ovulation. The ovulated follicle then forms the corpus luteum, which produces progesterone. Ovarian hormones, in turn, regulate the secretion of gonadotropins through a classical long-loop negative feedback mechanism. Identification of these control mechanisms has provided opportunities for the development of effective strategies to control fertility, including enhancement of both fertility and contraception.[1,2]

All current hormonal contraception methods are steroidal in nature (progestins and estrogens) and modulate long-loop feedback inhibition of gonadotropin secretion. The development of specific antagonists of FSHR would provide an alternative strategy for hormonal contraception. Such antagonists would block FSH-mediated follicular development, leading to disruption of ovulation and, thereby, produce the desired contraceptive effect. Support for the effectiveness of this strategy is provided by the mechanism that causes resistant ovary syndrome, which results in infertility in women. The infertility experienced by these women is the result of non-functional FSH receptors.[3] This approach to

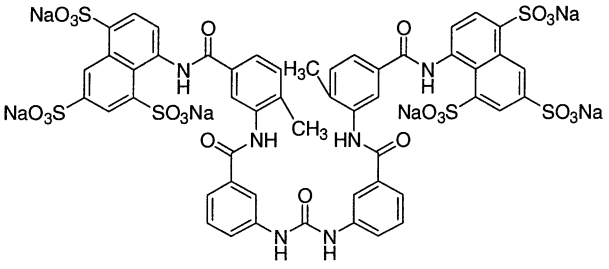
*Corresponding. Tel.: +1-484-685-2480; fax: +1-484-685-8228; e-mail: wrobelj@war.wyeth.com
†Current address: Ligand Pharmaceuticals Inc.,10275 Science Center Dr., San Diego, CA 92121, USA.
‡Current address: Metabolic and Cardiovascular Drug Discovery, Bristol-Myers Squibb, Inc., PO Box 4000, Lawrenceville, NJ 08543, USA.

0968-0896/02/$ - see front matter
PII: S0968-0896(01)00324-8

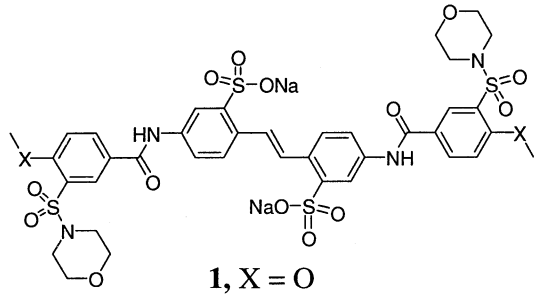
contraception may be applicable to men as well, because idiopathic male infertility seems to be related to a reduction in FSH binding sites. In addition, men with selective FSH deficiency are oligo-or azoospermic with normal testosterone levels and present with normal virilization.[4] Therefore, orally active FSH antagonists may provide a versatile method of contraception.

Follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH) constitute the glycoprotein hormone family. They are dimeric proteins that are composed of a common α-subunit (92–96 aa) and a hormone specific β-subunit (FSH: 111 aa). The receptors for these hormones belong to the super-family of G protein-coupled receptors (GPCRs), which possess seven transmembrane domains. Furthermore, upon hormone binding, the FSHR has been shown to associate with either Gs or Gi proteins to modulate the activity of adenylate cyclase and subsequent production of the second messenger cAMP.[5] Among GPCRs, the glycoprotein hormone receptors are distinguished by the presence of an extraordinarily long NH_2-terminal domain (~350 aa) containing several leucine-rich repeats. This domain of the receptor is known to be critical for hormone binding and activation of the signal transduction cascade.[6–9]

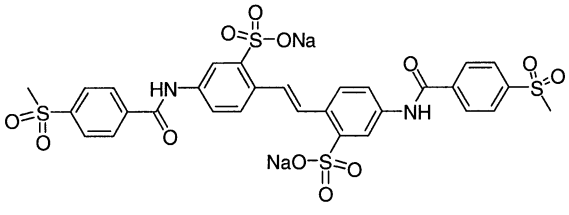
The mechanisms of action of these hormones have not been fully delineated and the paucity of pharmacological agents that modulate these receptors' activities has contributed to this situation. Recently suramin, a sulfonic acid anticancer agent with a wide variety of activities, was shown to inhibit FSH binding to its receptor.[10] Administration of suramin caused a decrease in testosterone production in rats and humans.[11] However, suramin is a nonselective agent that interacts with other glycoprotein hormone receptors as well as with proteins in other signal transduction pathways. We have recently reported other more selective sulfonic acid-based FSH receptor antagonists.[12]



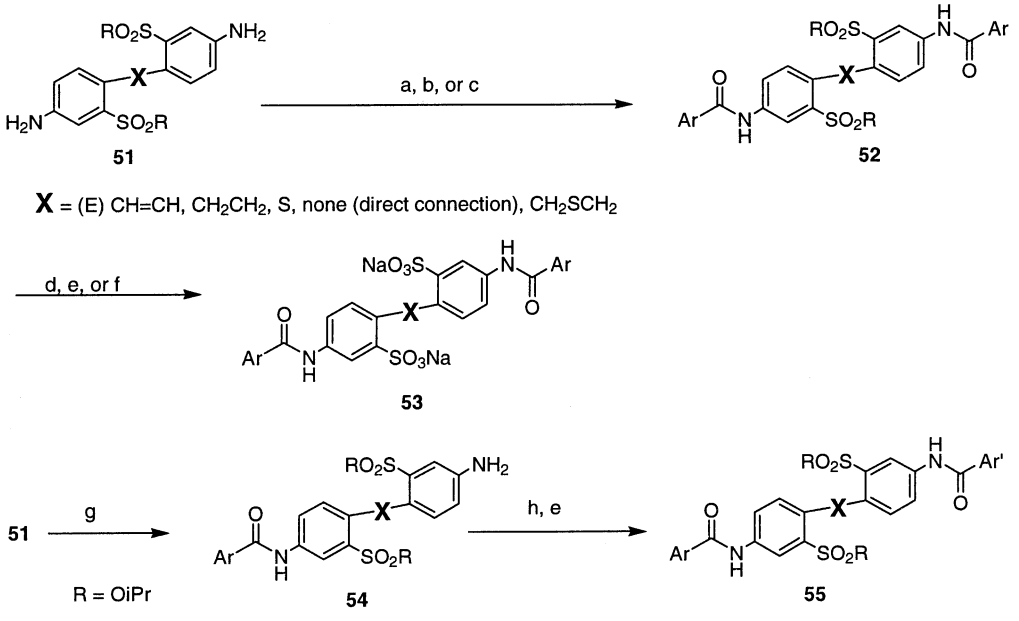
Suramin, Sodium



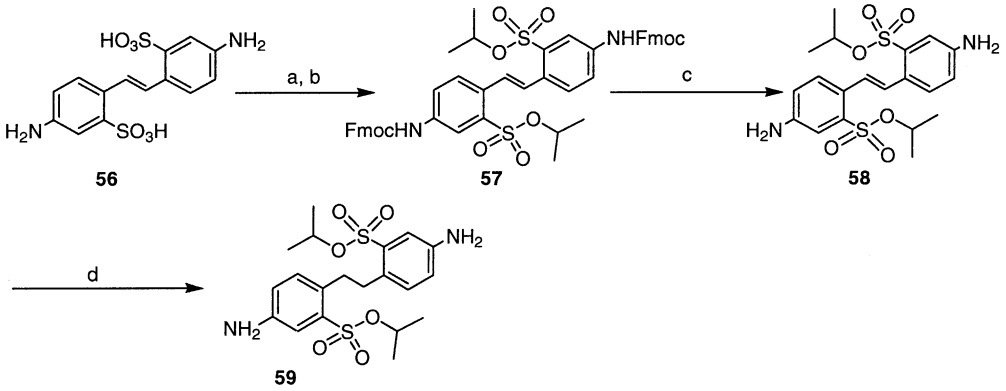
1, X = O
2, X = S



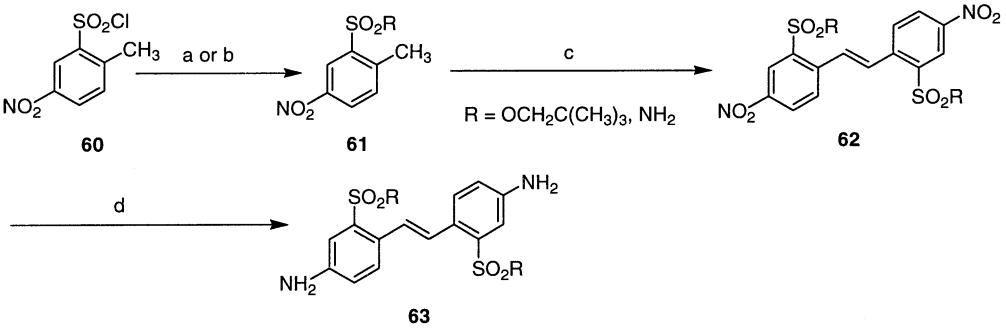
3



Scheme 1. Acylation of stilbene (bis) amines and congeners: (a) for R = OH, ONa: 2 ArCOCl, iPr$_2$EtN, DMA, 80 °C; (b) for R = ONBu$_4$: 2 ArCOCl, Et$_3$N, CH_2Cl_2; (c) for R = $OCH(CH_3)_2$, $OCH_2(CH_3)_3$, NH_2: 2 ArCOCl, K_2CO_3, THF; (d) for R = ONBu$_4$: Na^+ ion exchange, MeOH, H_2O; (e) for R = $OCH(CH_3)_2$: NaI, acetone, water, reflux; (f) for R = $OCH_2C(CH_3)_3$: Me$_4$NCl, DMF, 100 °C; (g) ArCOCl, K_2CO_3, THF; (h) Ar′COCl, K_2CO_3, THF.



Scheme 2. Preparation of stilbene and dihydrostilbene (bis)sulfonic acid, diisopropyl esters: (a) FMOC-Cl, Na_2CO_3, dioxane, water; (b) $HC(OiPr)_3$, dioxane, 110 °C, 82% (two steps); (c) piperidine, DMF, 0 °C, 80%; (d) H_2, Pd/C, THF, 85%.



Scheme 3. Preparation of stilbene (bis)sulfonic acid, dinepentyl esters and (bis)sulfonamides: (a) $R = OCH_2C(CH_3)_3$: $HOCH_2C(CH_3)_3$, pyridine, dioxane, reflux, 55%; (b) $R = NH_2$: aq NH_4OH, ether, 0 °C, 38%; (c) KO*t*Bu, DMF, air, 53% for $R = OCH_2C(CH_3)_3$, 21% for $R = NH_2$; (d) $SnCl_2$, ethyl acetate, 73% for $R = OCH_2C(CH_3)_3$, 31% for $R = NH_2$.

Herein, we report the identification of an additional class of sulfonic acid-based compounds we found from further compound screening. The stilbene (bis)sulfonic acids **1**–**3** competitively inhibited binding of [^{125}I]FSH to the FSHR. This prompted us to further explore the structure–activity relationship (SAR) of this class of compounds. The syntheses, SAR, radioligand binding activities, and cellular responses (inhibition of production of second messenger cAMP) for these compounds and analogues will be described.[13,14] Furthermore, the inhibition of FSHR-dependent estradiol production in rat primary ovarian granulosa cells, progesterone secretion in a clonal mouse adrenal Y1 cell line and selectivity against LH and TSH receptors will be discussed for selected analogues.

Chemical Syntheses

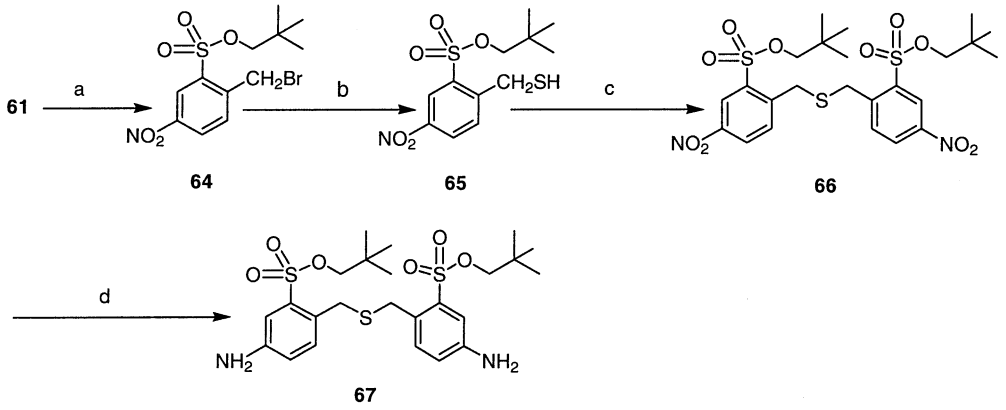
The low solubility of commercially available (bis)sulfonic acid, (bis)anilines (**51**, Scheme 1) in aqueous and organic solvents made (bis)acylation difficult. A method to increase their solubility utilizing dimethylacetamide as solvent at high temperatures (80 °C) was developed. However, this procedure was not satisfactory for most of the aryloyl chloride starting materials that were used due to frequent destruction of these starting materials. Additionally, a great deal of difficulty was encountered in removing inorganic salt and organic impurities from the products via chromatography, or by any other method of purification.

Derivatives of the (bis)sulfonic acid starting materials were examined as possible alternatives. For instance, the (bis)tetra-*N*-butylammonium salts of **51** were readily prepared and acylated and the product (bis) tetra-*N*-butylammonium salts (**52**) were purified via standard silica gel chromatography using dichloromethane solvent mixtures. Sodium ion exchange provided the (bis)-sodium salts (**53**).

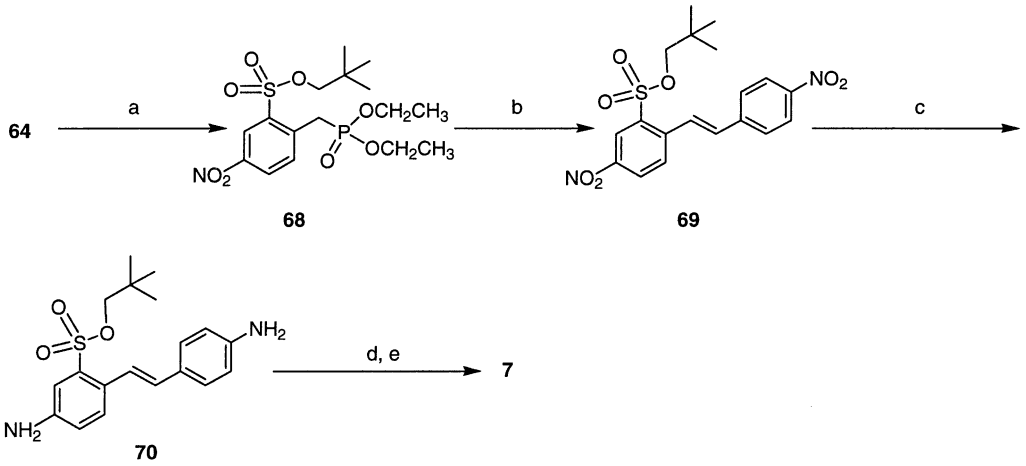
In another such method we developed, stilbene (bis)sulfonic acid, (bis) isopropyl esters of **51** were prepared (compounds **58**, see Scheme 2) using a three-step procedure involving (bis)FMOC protection of the aniline function, esterification with tri-isopropyl formate[15,16] and removal of the FMOC groups. In addition, dihydrostilbene analogue **59** could be prepared via reduction of the stilbene double bond using catalytic hydrogenation. Esters **58** and **59** were acylated under standard conditions (Scheme 1) and readily purified via silica gel chromatography. Facile deprotection with sodium iodide in acetone provided the desired (bis)sulfonic acid, (bis)sodium salts (**53**).

Unsymmetical analogues (**55**, Scheme 1) could also be efficiently prepared from the (bis) isopropyl ester of **51** ($R = OiPr$) via monoacylation with one equivalent of an aryloyl choride to afford **54**. A second acylation of compound **54** with a different aryloyl chloride, followed by deprotection with sodium iodide provided **55**.

Stilbene (bis)sulfonamide or (bis)sulfonic acid, (bis)-neopentyl esters (compounds **63** in Scheme 3) were pre-



Scheme 4. Preparation of thio(bis)methylene congeners: (a) NBS, $(PhCO_2)_2$, CCl_4, reflux, 55%; (b) CH_3CSNH_2, $CHCl_3$, reflux; (c) **64**, Et_3N, CH_2Cl_2, 36% (two steps); (d) $SnCl_2$, EtOH, reflux, 66%.



Scheme 5. Preparation of stilbene monosulfonic acid derivative: (a) $P(OEt)_3$, *o*-xylene, 100 °C, 60%; (b) *p*-nitrobenzaldehyde, NaH, THF, reflux, 91%; (c) $SnCl_2$, ethyl acetate, 80 °C, 33%; (d) ArCOCl, K_2CO_3, THF, 61%; (e) Me_4NCl, DMF, 100 °C, 40%.

pared via a base-induced oxidative dimerization of *o*-methyl-*m*-nitro sulfonic acid derivative **61** to stilbene **62**, followed by nitro group reduction to the (bis)aniline. Compounds **63** were acylated as in Scheme 1. (Bis)-neopentyl esters of **63** [$R = OCH_2C(CH_3)_3$] were much more stable than their (bis)isopropyl counterparts and were deprotected (Scheme 1) using more vigorous conditions (tetramethylammonium chloride in DMF at 100 °C).[17]

The thio(bis)methylene congener **11** (Table 2) was prepared as in Scheme 4. *o*-Methyl-*m*-nitro sulfonic acid derivative **61** was converted to benzyl bromide **64** using NBS and further converted to the mercaptan **65** using thioacetamide as the sulfur source.[18] Reaction of this mercaptan with another equivalent of bromide **64** resulted in the (bis)nitro deriviative **66** which was reduced to the (bis)aniline **67**. Standard acylation/neopentyl removal (Scheme 1) led to **11**. MCPBA oxidation of the neopenytyl ester of thioether **11**, followed by neopentyl deprotection provided (bis)methylene sulfone adduct **12** (Table 2).

The monosulfonic acid analogue **7** (Scheme 5, Table 1) was also prepared from benzyl bromide **64**. Arbuzov conversion to the phosphonate ester **68** followed by reaction with *p*-nitrobenzladyde under standard Horner–Emmons conditions led to (bis)nitro derivative **69**. Nitro group reduction, acylation and deprotection afforded **7**. Other standard transformations not shown in Schemes 1–5 include H_2/Pd-C reduction of (bis) nitro analogue **41** (Table 5) to (bis)aniline **42** (Table 5) and reaction of (bis)ester **38** (Table 5) with ethanolamine, to afford the (bis)amide derivative **39** (Table 5).

All carboxylic acid precursors were prepared from routes in Scheme 6 or were commercially available. These carboxylic acid precursors were converted to their corresponding acid chlorides for the acylation reactions in Scheme 1 using oxalyl chloride/DMF.

Sulfonamides **72** were obtained by reacting commercially available precursors **71** with chlorosulfonic acid, followed by amidation of the resulting sulfonyl chloride. *p*-Fluoro or bromo benzoic acid (**71**, X = F, Br) required considerably higher temperatures in the chlorosulfonation reaction (150 °C vs 65 °C) than the *p*-methoxy analogue (**71**, X = OMe). The thiomorpholine analogue **73** was also prepared by this method. Further oxidation of the thio moiety with hydrogen peroxide provided the sulfone analogue **74**.

The fluorosulfonamide **75** proved to be a versatile intermediate. Alcohols, thiols and amines readily displaced

the fluoro group via nucleophilic aromatic substitution reactions to provide the ethers **76**, thioethers **77**, and anilines **79**, respectively. Further oxidation of the thioether moiety of **77** with hydrogen peroxide provided sulfone **78**. Use of 2-hydroxyethylmethylsulfone as the nucleophile in the nucleophillic aromatic substitution reaction of **75** led to the phenol **76** (R = H) via beta-elimination of the intermediate methylsulfonylethylether of **76** ($R = CH_2CH_2SO_2CH_3$). This phenol was further elaborated to ethers **76** via standard Mitsunobu chemistry. Suzuki coupling of bromosulfonamide **80** with phenyl boronic acid led to biphenyl congener **81**. *p*-Sulfonylcarboxylic acids (**83**) were prepared via alkylation of 4-mercaptobenzoic acid (**82**) followed by oxidation of the resulting thioether with Oxone®.

High-Resolution Mass Spectroscopy data and HPLC purity analysis for all tested compounds is presented in Table 8.

Results and Discussion

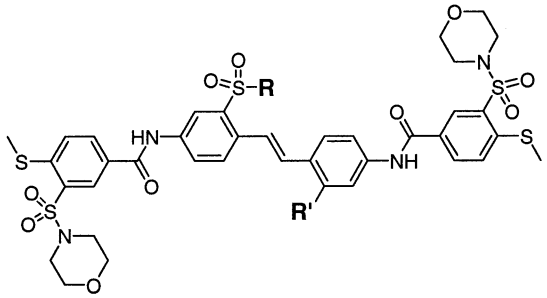
The compounds were first evaluated for their ability to inhibit the binding of [^{125}I]FSH to the human FSHR (hFSHR) in membranes from Chinese hamster ovary (CHO-K1) cells stably transfected with the hFSH gene (CHO-3D2 cell line).[9] The data are presented as 'hFSHR Binding IC_{50}' values in Tables 1–6. Functional activity of active compounds was further assessed by cAMP production in the CHO-3D2 cell line. In this assay, hFSH caused a dose–dependent 20- to 30-fold increase of cAMP levels over basal levels with $EC_{80} = 22.5$ ng/mL, $EC_{50} = 6.0$ ng/mL, and $EC_{20} = 1.85$ ng/mL. None of the stilbene (bis)sulfonic acids active in the radioligand binding assay were able to stimulate cAMP production over basal levels in this assay. However, many of these compounds did behave as antagonists in this system as measured by their ability to inhibit cAMP production induced by the EC_{20} concentration of hFSH. The data are presented as "hFSHR cAMP" IC_{50}'s values in Tables 2–6. Efficacy in this assay is a measure of the biological effectiveness of the compound to inhibit cAMP accumulation induced by an EC_{20} concentration of hFSH. Efficacy is calculated as a percent using the following equation:% Efficacy = [(Maximal response for ED_{20} of FSH− Maximal inhibitory response by test compound)/(Maximal response for ED_{20} of FSH− basal response)] ×100.

Our initial objective was to test the necessity of the arylsulfonic acid moieties of this series. Due to the low pK_a of the sulfonic acid group (generally < 2.5), it exists essentially in the unionized form under physiological conditions, and this results in greater partitioning into the aqueous phase rather than the lipid phase (i.e., logP < 0). Under these conditions, cell permeability via passive diffusion is greatly diminished.[19,20] Masking the sulfonic acid moiety via ester or amide formation, or replacement by a less acidic group, would provide more favorable permeability properties. However, as shown in Table 1, (bis)isopropyl and (bis)neopentyl sulfonic acid esters (**4** and **5**) and the (bis)sulfonamide (**6**) of compound **2** were inactive at the 30 μM concentration. In addition, both sulfonic acid moieties were necessary since the monosulfonic acid congener (**7**) showed substantially reduced activity.

We next examined (Table 2) whether the E-stilbene double bond of **1** was the optimal group between the two arylsulfonic acid moieties. In this regard, the dihydrostilbene analogue **8** was prepared. However, the increased flexibility imparted by the carbon–carbon single bond of **8** over the double bond of **1** had little effect on binding potency. The functional activity of **8** and **1** were also similar, although, in general, functional antagonist activity was slightly diminished for other dihydrostilbene analogues relative to their stilbene congeners.

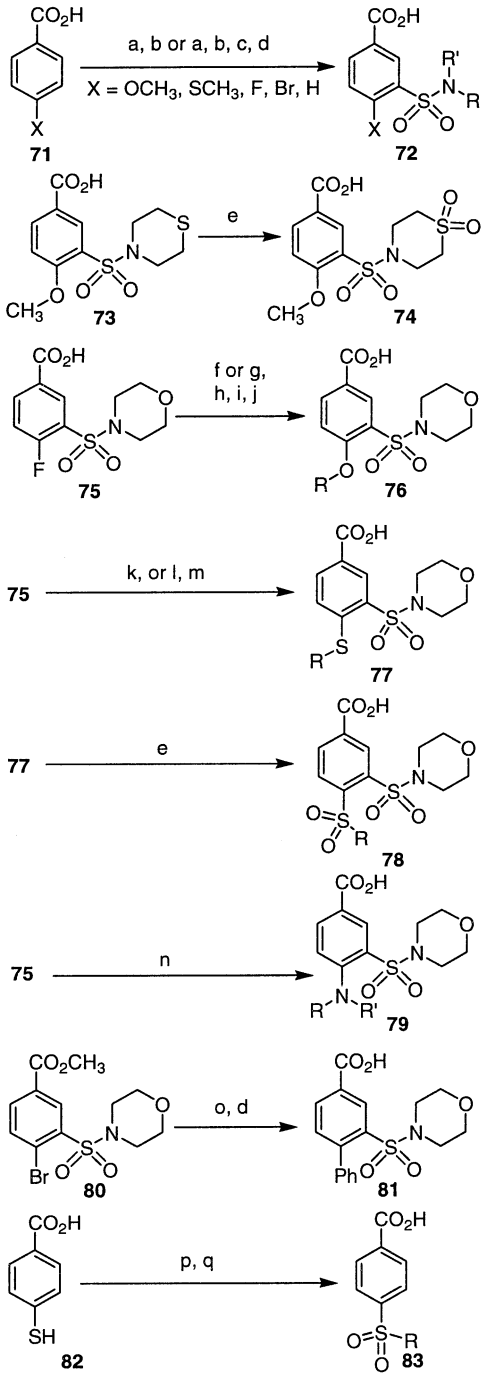
Reducing the number of atoms between the sulfonic acid containing aryl groups of **1**, whether by removing the stilbene double bond completely (biphenyl analogue

Table 1. Variation of sulfonic acid moiety of **2**



Compound	R	R′	hFSHR Binding IC_{50} (μM)
2	–ONa	$–SO_2ONa$	1.2 ± 0.06
4	$–OCH(CH_3)_2$	$–SO_2OCH(CH_3)_2$	—[a]
5	$–OCH_2C(CH_3)_3$	$–SO_2OCH_2C(CH_3)_3$	—[a]
6	$–NH_2$	$–SO_2NH_2$	—[a]
7	–ONa	–H	—[a]

Values represent the mean ± standard error of the mean. Compounds were evaluated in triplicate samples at each concentration tested.
[a]No inhibition observed at 30 μM, the highest concentration tested.



Scheme 6. Preparation of carboxylic acids precursors: (a) neat $ClSO_3H$; (b) HNRR′, CH_2Cl_2, ether; (c) for X = F, Br: CH_3I, K_2CO_3, DMF; (d) for X = F: LiOH, MeOH, H_2O; (e) H_2O_2, HOAc, 100 °C; (f) ROH, NaH, DMF, 30 °C; (g) $HOCH_2CH_2SO_2CH_3$, NaH, DMF; (h) $SOCl_2$, MeOH, 0 °C; (i) ROH, PPh_3, DEAD, toluene; (j) NaOH, MeOH; (k) RSH, NaH, DMF, 30 °C; (l) NaSH, DMF, 60 °C; RBr, KOH, EtOH, 80 °C; (n) RR′NH, K_2CO_3, DMF, reflux; (o) $PhB(OH)_2$, cat $(Ph_3P)_4Pd$, Na_2CO_3, toluene, EtOH, reflux, 38%; (p) RBr, Et_3N, CH_2Cl_2, 0 C; (q) oxone, MeOH, 0 °C.

10) or with a one-atom spacer (such as thioether **9**) decreased binding affinity by an order of magnitude. The cAMP activity of **9** was also decreased by a similar amount. Increasing the number of atoms between the sulfonic acid containing aryl groups of **1** by one atom, such as for compounds **11** and **12**, led to an even further drop in binding potency.

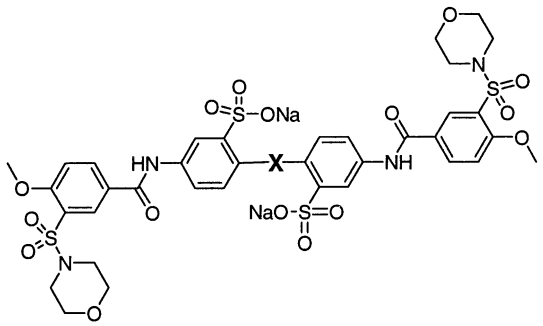
We found from screening a large number of stilbene (bis)sulfonic acids in our compound library that the nature and position of the substituents on the terminal benzoic acid amide groups of **1**–**3** had a major effect on the potency of the analogue. Furthermore, the 3-sulfonamide, 4-methoxy/thiomethyl groups of **1** and **2** and the 4-sulfonyl group of **3** were the substituents that provided the best binding affinity. In order to enhance potency further, we decided to explore the SAR of these groups in greater detail. The results are provided in Tables 3–5.

In this regard the morpholine group of **1** was replaced by a number of different cyclic and acylic amines (Table 3). No clear-cut trend was observed. Amines that contained polar, unionizable substituents provided compounds (**14**, **17**, and **18**) with the highest binding potency. Compound **14** also displayed the greatest functional activity in this sub-series. Compounds such as **13** that contained more lipophilic amines showed a several fold decrease in binding potency. Acyclic amine containing analogue **15** also showed a slight decrease in potency. The methyl piperidine congener **16** displayed a substantial drop in activity. The amine moiety in this compound would be protonated at physiological pH and presumably the positive charge was not tolerated in this position of the molecule. However, the *N*-ethylmorpholine analogue **19** should also be protonated at physiological pH and this compound showed only a slight decrease in binding affinity. Although the protonated amine group of **19** is of the same atomic distance as the same group in **16**, the aminoethylmorpholine side chain of **19** is considerably more flexible than the piperidine side chain of **16**. This increased flexibility may allow the positively charged nitrogen of **19** to avoid unfavorable interactions experienced by **16**, via adoption of a different side-chain conformation. Compound **20** illustrates another interesting observation in Table 3. This compound, despite the presence of two additional sulfonic acid groups, showed a three-fold reduction in activity relative to **1**.

The SAR of the methoxy substituent of **1** was also examined and the results are shown in Table 4. Removal of this substituent (**21**) resulted in a 2-fold reduction in binding potency. Small and/or polar unionizable substituents resulted in retention or slight improvement of binding and cAMP activity as seen with cyclic and acylic ether analogues **22**, and **26**, thiomethyl compound **2**, and methyl sulfone **28**. Increasing the lipophilicity with larger alkyl or aralkyl substituents resulted in a drastic reduction of activity. For example, compare the ether subseries **1** with **23**, **24**, and **25**, the thioether subseries **2** with **29** and **30**, and the dimethyl aniline **32** with the diethyl congener **33**. Also within this theme, replacement of the methoxy of **1** with a lipophilic phenyl substituent (**34**) led to a decrease in potency.

Next, we modified the methyl sulfonyl moiety of compound **3** (Table 5). One carbon homologation to the ethylsulfonyl congener **35** maintained in vitro potency, although two carbon extension to the propylsulfonyl

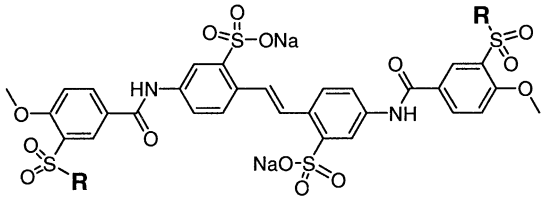
Table 2. Variation of Stilbene double bond of **1**

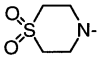
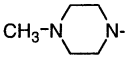
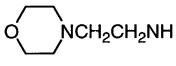


Compound	X	hFSHR Binding IC_{50} (μM)	hFSHR cAMP IC_{50} (μM)[a]
1	(E)–CH=CH–	3.0±0.29	7.0[b]±2.3
8	$–CH_2CH_2–$	2.5±0.11	11±0.4
9	–S–	23±0.9	67[b]±0.6
10	None (direct connection)	20±0.53	NT
11	$–CH_2–S–CH_2–$	34±0.9	NT
12	$–CH_2–S(O)_2–CH_2–$	41±1.24	NT
Suramin, sodium		43±2.8	33±12.5

Values represent the mean±standard error of the mean. Compounds were evaluated in either triplicate or quadruplicate samples at each concentration tested. NT = not tested.
[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 85% unless otherwise noted.
[b]Efficacy = 60–70%.

Table 3. Variation of terminal sulfonamide of **1**



Compound	R	hFSHR Binding IC_{50} (μM)	hFSHR cAMP IC_{50} (μM)[a]
1		3.0±0.29	7.0[b]±2.3
13		16±0.74	11±0.9
14		2.0±0.21	1.5±0.1
15	$-NEt_2$	6.2±0.35	4.5±0.6
16		48%±100 μM	NT
17	$(CH_3OCH_2CH_2)_2N–$	2.4±0.19	6.2±0.3
18	$CH_3OCH_2CH_2NH–$	2.2±0.09	5.8±0.4
19		5.9±0.26	5.5±0.3
20	-ONa	9.1±0.56	10±0.5

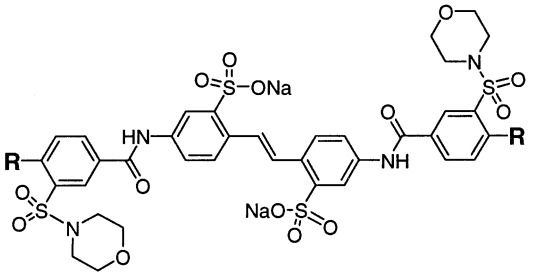
Values represent the mean±standard error of the mean. Compounds were evaluated in triplicate or quadruplicate samples at each concentration tested. NT = Not tested.
[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 89% unless otherwise noted.
[b]Efficacy = 68%.

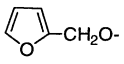
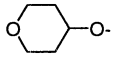
derivative **36** resulted in a loss in activity. Interestingly, the allyl derivative **37** was as potent as **3** and **35**. The methyl acetate analogue **38** was also active, albeit with slightly reduced potency. However, the corresponding hydroxyethylamide derivative **39** showed increased potency over ester **38** and **3** for both radioligand binding and cAMP activities. The sulfonamide analogue **40** showed a 10-fold drop in activity over **3**. Addition of an electron withdrawing substituent ortho to the methylsulfonyl group of **3**, such as a nitro (**41**) or sulfonamide (**28**) group, marginally increased potency. Replacement of the methylsulfonyl group of **3** with the sp^2 hybridized acetyl group of analogue **43** showed a substantial drop in potency as did ethyl analogue **44** which maintains the sp^3 hybridization of the sulfonyl moiety but loses the electron withdrawing capabilities.

Compounds **45**–**48** (Table 6) are modifications to the methylsulfonylphenyl group of **3**. Addition of a methylene spacer to afford *p*-methylsulfonylphenacyl derivative **45** resulted in a large loss of potency as did addition of a chlorine substituent meta to the methysulfonyl group (as for **46**). This latter change introduced a substituent *ortho* to the amide carbonyl moiety of **3**, which would force the amide carbonyl group out of its nearly coplanar relationship with the attached phenyl ring. The resulting change in phenyl ring conformation could thus result in this compound's reduction in potency. However, the phenyl ring of **3** could be replaced with a thiophene system, to afford compound **47**, without loss in binding or antagonist potency. Substitution of the methylsulfonyl group of **47** for a nitro group (**48**) did result in a large drop in potency, similar to what had been observed for the same change with phenyl derivative **3**.

Thus far, all of the compounds mentioned have been symmetrical [C2 axis perpendicular to the midpoint of the stilbene double bond for the stilbene (bis)sulfonic acids, for example]. Our synthetic methodology allowed us to prepare unsymmetrical stilbene (bis)sulfonic acids as well. Compounds **49** and **50** (Table 6) are examples utilizing substituted benzoic acid amide moieties found for some of the most potent symmetrical analogues

Table 4. Variation of terminal ether moiety of 1



Compound	R	hFSHR Binding IC_{50} (μM)	hFSHR cAMP IC_{50} (μM)[a]
1	$-OCH_3$	3.0 ± 0.29	7.0[d] ± 2.3
21	–H	6.9 ± 0.26	NT
22	$-OCH_2CH_2OCH_3$	1.3 ± 0.08	1.4 ± 0.07
23	$-OCH_2CH_2Ph$	—[b]	NT
24	$-OCH_2CH_2CH_2CH_3$	38% ± 10 μM	NT
25		53% ± 10 μM	NT
26		1.1 ± 0.04	1.1 ± 0.1
27	$-OCH_2CH_2N(CH_3)_2$	—[c]	NT
2	$-SCH_3$	1.2 ± 0.06	1.3 ± 0.1
28	$-S(O)_2CH_3$	1.5 ± 0.05	2.2 ± 0.08
29	$-SCH_2CH_3$	3.7 ± 0.71	4.9[d] ± 0.7
30	$-SCH(CH_3)_2$	28% ± 10 μM	NT
31	$-SCH_2CH_2OCH_3$	4.1 ± 0.29	NT
32	$-N(CH_3)_2$	4.8 ± 0.26	NT
33	$-N(CH_2CH_3)_2$	30% ± 10 μM	NT
34	–Ph	42% ± 10 μM	NT

Values represent the mean ± standard error of the mean. Compounds were evaluated in either triplicate or quadruplicate samples at each concentration tested. NT = Not tested.

[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 96% unless otherwise noted.

[b]No inhibition observed at 10 μM, the highest concentration tested.

[c]No inhibition observed at 30 μM, the highest concentration tested.

[d]Efficacy = 65–70%.

of **1**–**3** (in Tables 3–5). Each of the unsymmetrical analogues had a substituted benzoic acid group from the *p*-methylsulfonyl sub-series (**3**) and one from the *p*-alkoxy/alkylthio-*m*-sulfonamide sub-series (**1** and **2**). Both analogues **49** and **50** showed FSHR binding and inhibition of cAMP activity in the low micromolar region and were among the most potent compounds prepared.

In order to rule out cellular toxicity as a cause of the functional activity of these stilbene analogues, some of the more potent compounds, including **1**, **3**, **14**, **26**, **28**, **35**, **41**, **47**, and **50** were subjected to a cytotoxicity assay in CHO-3D2. None of these compounds were cytotoxic (details in Experimental section).

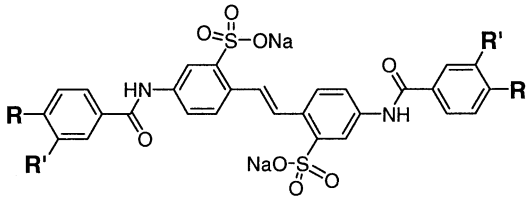
The more potent compounds were further evaluated in additional functional activity and receptor selectivity assays. The results of three representatives (**2**, **14**, and **50**) are presented in Table 7. All three compounds were able to inhibit hFSH-induced estradiol production in rat primary ovarian granulosa cells[2] with activities in the low micromolar range. In addition, these compounds could also block FSHR-dependent progesterone secretion with low micromolar IC_{50}s values in a clonal mouse adrenal Y1 cell line[21–24] that was engineered to stably express the hFSHR gene.

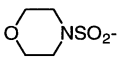
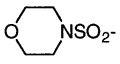
Luteinizing hormone (LH) and thyroid stimulating hormone (TSH) ligands and receptors show significant homology to FSH and FSHR,[7,8] and, therefore, are good candidates for selectivity assessment of our compounds. All three analogues (**2**, **14**, and **50**) demonstrated no binding interaction at concentrations up to 100 μM in the rat testis LHR radioligand binding assay. In contrast, nonselective FSHR antagonist, suramin, had similar binding interactions for both hFSHR and rLHR (Table 7).

These compounds showed a varying ability to block hTSH-stimulated cAMP production in CHO cells that stably express the hTSHR gene. Analogue **2** was the most potent, and, therefore, the least selective of the three in this assay, with about a 10-fold separation in activity between hFSHR and hTSHR. Compound **14** was more selective than **2** with approximately 47-fold difference in activity between the two receptors. For unsymmetrical analogue **50**, essentially no TSH antagonist activity could be detected at the 30 μM concentration.

As stated previously, these compounds were not expected to have good cell permeability properties, and because of this, oral absorption should be poor. The measured logP's (< -2.0) and CACO2 data (Amidon

Table 5. Variation of sulfone moiety of **3**



Compound	R	R′	hFSHR Binding IC_{50} (μM)	hFSHR cAMP IC_{50} (μM)[a]
3	$-SO_2CH_3$	–H	4.0±0.25	6.0±0.8
35	$-SO_2CH_2CH_3$	–H	2.9±0.09	2.3±0.5
36	$-SO_2CH_2CH_2CH_3$	–H	26% @ 10 μM	NT
37	$-SO_2CH_2CH=CH_2$	–H	3.5±0.17	2.3[b]±0.3
38	$-SO_2CH_2CO_2CH_3$	–H	6.9±0.65	NT
39	$-SO_2CH_2CONHCH_2CH_2OH$	–H	2.0±0.29	2.1±0.7
40		–H	68±11.2	NT
41	$-SO_2CH_3$	$-NO_2$	1.6±0.06	5.0±0.5
42	$-SO_2CH_3$	$-NH_2$	4.1±0.2	NT
28	$-SO_2CH_3$		1.5±0.05	2.2±0.08
43	$-COCH_3$	–H	38% @ 10 μM	NT
44	$-CH_2CH_3$	–H	17% @ 10 μM	NT

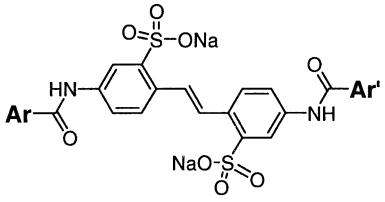
Values represent the mean±standard error of the mean. Compounds were evaluated in either triplicate or quadruplicate samples at each concentration tested. NT = Not tested.

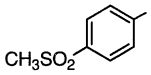
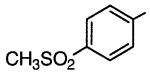
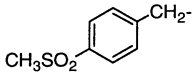
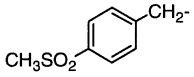
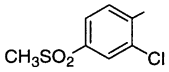
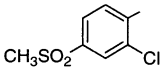
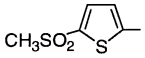
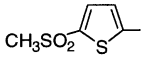
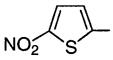
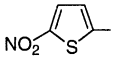
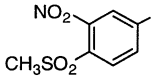
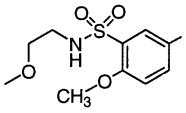
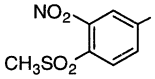
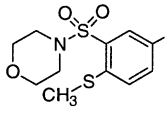
[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 94% unless otherwise noted.

[b]Efficacy = 83%.

Permiability rating of low, < 5%) for **2** and **50** were consistent with this supposition. Conversion of the sulfonic acid moieties to carboxylic acid groups would improve absorption properties; however, preliminary data suggest that such compounds lose most of their FSHR binding affinity. Despite these factors, compounds such as **14** and **50** are among the first potent and selective FSH antagonists, and should find use as tools to explore FSH-mediated processes. Further evaluation of several of the analogues is in progress.

Table 6. Variation of phenylsulfone moiety of **3**/unsymmetrical analogues



Compound	Ar	Ar′	hFSHR Binding IC_{50} (μM)	hFSHR cAMP IC_{50} (μM)[a]
3			4.0±0.25	6.0±0.8
45			15% @ 10 μM	NT
46			38% @ 10 μM	NT
47			3.2±0.2	2.6±0.3
48			[b]	NT
49			1.9±0.7	3.1±0.2
50			2.5±0.1	0.9±0.01

Values represent the mean±standard error of the mean. Compounds were evaluated in either triplicate or quadruplicate samples at each concentration tested. NT = Not tested.
[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 91%.
[b]No inhibition observed at 30 μM, the highest concentration tested.

Table 7. Additional functional activity and selectivity data for selected analogues

Compd	hFSHR Binding IC_{50} (μ)	hFSHR cAMP IC_{50} (μM)[a]	Granulosa (Aromatase) IC_{50} (μM)[b]	Y1 Adrenal IC_{50} (μM)[c]	rLHR Binding IC_{50} (μM)	hTSHR cAMP IC_{50} (μM)[e]
2	1.2±0.06	1.3±0.10	2.7±0.88	1.1±0.34	[d]	13±1.0[f]
14	2.0±0.21	1.5±0.10	3.1±0.26	NT	[d]	71±23[g]
50	2.5±0.10	0.9±0.01	1.7±0.06	3.6±1.09	[d]	13% @ 30 μM[h]
Suramin, Sodium	43±2.8	33±12.5	NT	NT	27±1.4	NT

Values represent the mean standard error of the mean. Compounds were evaluated in either triplicate or quadruplicate samples at each concentration tested. NT = Not tested.
[a]IC_{50} values determined in the presence of the EC_{20} of hFSH (1.85 ng/mL). Efficacy ≥ 85%.
[b]IC_{50} values determined in the presence of the EC_{50} of hFSH (0.5 ng/mL). Efficacy ≥ 89%.
[c]IC_{50} values determined in the presence of the EC_{50} of hFSH (25 ng/mL). Efficacy ≥ 100%.
[d]No inhibition at 100 μM, the highest concentration tested.
[e]IC_{50} values determined in the presence of the EC_{20} of hTSH (5 nM).
[f]Efficacy = 100%.
[g]Efficacy = 60%.
[h]Efficacy = 16%.

Table 8. Analytical data on tested compounds

Compd	Parent molecular formula	HRMS (ESI) [M−H]-calcd Found	HPLC Purity (%)
1	$C_{38}H_{40}N_4O_{16}S_4$	935.12493 935.12454	89
2	$C_{38}H_{40}N_4O_{14}S_6$	967.07925 967.07921	92
3	$C_{30}H_{26}N_2O_{12}S_4$	733.02958 733.02929	97
4	$C_{44}H_{52}N_4O_{14}S_6$	—[a]	CHN[b]
5	$C_{48}H_{60}N_4O_{14}S_6$	1109.25031 1109.2499	92
6	$C_{38}H_{42}N_6O_{12}S_6$	—[c]	CHN[d]
7	$C_{38}H_{40}N_4O_{11}S_5$	887.12243 887.12246	91
8	$C_{38}H_{42}N_4O_{16}S_4$	937.14058 937.1404	97
9	$C_{36}H_{38}N_4O_{16}S_5$	941.08135 941.08134	80
10	$C_{36}H_{38}N_4O_{16}S_5$	909.10928 909.10931	98
11	$C_{38}H_{42}N_4O_{16}S_5$	969.11265 969.11246	76
12	$C_{38}H_{42}N_4O_{18}S_5$	1001.10248 1001.10222	92
13	$C_{42}H_{48}N_4O_{14}S_4$	959.19771 959.19754	72
14	$C_{38}H_{40}N_4O_{18}S_6$	1031.05890 1031.05897	67
15	$C_{38}H_{44}N_4O_{14}S_6$	907.16640 907.16640	75
16	$C_{402}H_{46}N_6O_{14}S_4$	961.1882 961.18731	85
17	$C_{42}H_{52}N_4O_{18}S_4$	1027.20866 1027.20824	97
18	$C_{36}H_{40}N_4O_{16}S_4$	911.12493 911.1247	91
19	$C_{42}H_{50}N_6O_{16}S_4$	1021.20933 1021.20984	98
20	$C_{30}H_{26}N_2O_{16}S_4$	797.00924 797.00923	CHN[e]
21	$C_{36}H_{36}N_4O_{14}S_4$	875.10380 875.10328	95
22	$C_{42}H_{48}N_4O_{18}S_4$	1023.17736 1023.17722	91
23	$C_{52}H_{52}N_4O_{16}S_4$	1115.21883 1115.21886	93
24	$C_{44}H_{52}N_4O_{16}S_4$	1019.21883 1019.21912	98
25	$C_{46}H_{44}N_4O_{18}S_4$	1067.14606 1067.14619	91
26	$C_{46}H_{52}N_4O_{18}S_4$	1075.20866 1075.20889	96
27	$C_{44}H_{54}N_6O_{16}S_4$	1049.24063 1049.24070	84
28	$C_{38}H_{40}N_4O_{18}S_6$	1031.05890 1031.05836	86
29	$C_{40}H_{44}N_4O_{14}S_6$	995.11055 995.11023	98
30	$C_{42}H_{48}N_4O_{14}S_6$	1023.14185 1023.14181	97
31	$C_{42}H_{48}N_4O_{16}S_4$	1055.13168 1055.13147	99
32	$C_{40}H_{46}N_6O_{14}S_4$	961.18820 961.18801	93
33	$C_{44}H_{54}N_6O_{14}S_4$	1017.25080 1017.25086	96
34	$C_{48}H_{44}N_4O_{14}S_4$	1027.16640 1027.16653	91
35	$C_{32}H_{30}N_2O_{12}S_4$	761.06088 761.0671	98
36	$C_{34}H_{34}N_2O_{12}S_4$	789.09218 789.09256	83
37	$C_{34}H_{30}N_2O_{12}S_4$	785.06088 785.06089	95
38	$C_{34}H_{30}N_2O_{16}S_4$	849.04054 849.04055	85
39	$C_{36}H_{36}N_4O_{16}S_4$	907.09363 907.09280	75
40	$C_{36}H_{36}N_4O_{14}S_4$	875.10380 875.10348	92
41	$C_{30}H_{24}N_4O_{16}S_4$	822.99973 822.99981	95
42	$C_{30}H_{28}N_4O_{12}S_4$	763.05138 763.05130	81
43	$C_{32}H_{26}N_2O_{10}S_2$	661.09561 661.09549	95
44	$C_{32}H_{30}N_2O_8S_2$	663.13708 663.13686	99
45	$C_{32}H_{30}N_2O_{12}S_4$	761.06088 761.06118	83
46	$C_{30}H_{24}N_2O_{12}S_4$	800.95163 800.95172	99
47	$C_{26}H_{22}N_2O_{12}S_6$	744.94242 744.94242	89
48	$C_{24}H_{16}N_4O_{12}S_4$	678.95747 678.95731	96
49	$C_{33}H_{32}N_4O_{16}S_4$	867.06233 867.06206	91
50	$C_{34}H_{32}N_4O_{15}S_5$	895.03949 895.03907	98

[a]Ester unstable under MS conditions, −ESI: [M−H]- 967 observed, consistent with sulfonic acid **2**.
[b]calcd for $C_{44}H_{52}N_4O_{14}S_6\bullet 2H_2O$: C, 48.51; H, 5.18; N, 5.14. Found: C, 48.42; H, 5.29; N, 4.57.
[c]−ESI: [M-H]- 967, consistent with desired MF $C_{38}H_{42}N_6O_{12}S_6$.
[d]calcd for $C_{38}H_{42}N_6O_{12}S_6\bullet 4H_2O$: C, 43.92; H, 4.85; N, 8.09. Found: C, 43.79; H, 4.29; N, 8.04.
[e]calcd for $C_{30}H_{22}N_2O_{16}S_4Na_4\bullet 5H_2O$: C, 36.89; H, 3.30; N 2.87. Found: C, 36.86; H, 3.56; N 2.93.

Experimental

Proton magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz on a Bruker DPX300. Analytical HPLC were recorded on a HP1100 using a Xterra MS C18 column (3.5 micron, 4.6×50 mm) with a 5/95 to 80/20 acetonitrile/50 mM triethylammonium acetate (pH 7) gradient at a flow rate of 0.8 mL/min (5–10 μL injection of 0.2 mg/mL solution). Electrospray mass spectra were recorded using a Hewlett-Packard 5989B MS engine mass spectrometer. Electron Impact (EI, IE = 70 eV) and chemical ionization (CI, isobutane reagent gas) mass spectra were recorded on a Finnigan model 8230 spectrometer. Fast atom bombardment (FAB) were recorded on a Kratos MS50. High-resolution MS were recorded on a Bruker 9.4 T FTMS. Analyses (C, H, N) were carried out on a modified Perkin-Elmer model 240 CHN analyzer. Analytical results for elements were within ±0.4% of the theoretical values. Thin layer chromatography analyses were done on E. Merck Silica Gel 60 F-254 plates of 0.25 mm thickness. All commercial reagents and solvents were used as received unless otherwise noted. Suramin Sodium was purchased from Research Biochemicals International, Natick, MA, USA. All reactions were done under an inert, dry atmosphere unless otherwise noted.

2,2′-[(*E*)-1,2-Ethenediyl]bis[5-[3-(4-morpholinylsulfonyl) benzoyl]amino]benzene sulfonic acid] (21)

Commercially available 3-(chlorosulfonyl)benzoic acid (3 g, 13.5 mmol) was suspended in dichloromethane (50 mL). Diisopropylethylamine (5.19 mL, 30 mmol) and morpholine (1.67 mL, 21 mmol) were added slowly. After 2 h, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed three times with 3 N HCl. The ethyl acetate phase was dried ($MgSO_4$) and the solvent was removed, to provide 3-(morpholine-4-sulfonyl)benzoic acid (2.97 g, 81%, **72**, X = H, NRR″ = morpholine) as a white solid. ^{1}HNMR (DMSO-d_6) δ 8.28 (d, J = 8 Hz, 1H), 8.20 (s, 1H), 8.01 (d, 8 Hz, 1H), 7.83 (t, J = 8 Hz, 1H), 3.91(m, 4H), 3.12 (m, 4H). MS (EI-NEG): [M +] 270.

This compound (271 mg, 1 mmol) was suspended in dichloromethane (3 mL), and DMF (2 drops) was added. Oxalyl chloride (152 mg, 1.2 mmol) was added slowly, and the reaction mixture was allowed to stir for 2 h. The solvent was removed and the acid chloride was dissolved in dimethylacetamide (0.5 mL). This solution was added to a stirred solution of commercially available 4,4′-diaminostilbene-2,2′-disulfonic acid (110 mg, 0.33 mmol) and diisopropylethyamine (129 mg, 1 mmol) in dimethylacetamide (0.5 mL). The resulting solution was heated to 80 °C. After 12 h, the solvent was removed. The residue was added to aqueous sodium bicarbonate and stirred. The precipitate was filtered to provide the (bis)sodium salt of the title compound as a yellow solid (88 mg, 30%): ^{1}H NMR (DMSO-d_6) δ 0.41 (s, 2H), 8.43 (d, J = 3 Hz, 2H), 8.34 (m, 2H), 8.21 (d, J = 2 Hz, 2H), 8.08 (s, 2H), 7.94 (m, 2H), 7.80 (t, J = 8 Hz, 2H), 7.62 (d, 8 Hz, 2H), 3.72 (m, 4H), 3.10 (m, 4H); MS (ESI-NEG): [M−2H + Na]-897. Anal. calcd for $C_{36}H_{36}N_4O_{14}S_4$: C, 49.31; H, 4.14; N, 6.39. Found: C, 42.78; H, 3.83; N, 5.53.

4-Methylsulfanyl-3-(morpholino-4-sulfonyl)-benzoic acid (72, X = SCH$_3$, NRR″ = morpholine). Commercially available *p*-methylthiobenzoic acid (15 g, 90 mmol) was suspended in chlorosulfonic acid (40 mL), and it slowly went into solution. This solution was allowed to stir at room temperature for 5 h and then added slowly to crushed ice (about 300 mL). The resulting suspension was filtered. This solid sulfonyl chloride intermediate was suspended in ethyl acetate (150 mL), and morpholine (16 g, 184 mmol) was added. This suspension was allowed to stir overnight. It was extracted three times with 3 N HCl and dried ($MgSO_4$). The solvent was removed to provide the title compound (15 g, 53%) as a crystalline solid: ^{1}H NMR (DMSO-d_6) δ 13.2–12.8 (bs, 1H), 8.28 (d, J = 2 Hz, 1 H), 8.16 (dd, J = 9 Hz, 2 Hz, 1H), 7.62 (d, J = 9 Hz, 1H), 3.62 (m, 4H), 3.19 (m, 4H), 2.60 (s, 3H); MS (ESI-POS): [M + H] + = 318. Anal. calcd for $C_{12}H_{15}NO_5S_2$: C, 45.41; H, 4.76; N, 4.41. Found: C, 44.76; H, 4.57; N, 4.17.

3-(1,1-Dioxo-1-thiomorpholine-4-sulfonyl)-4-methoxy-benzoic acid (74). *p*-Anisic acid (10.0 g, 66 mmol) was added neat to stirring chlorosulfonic acid (30 mL). This reaction was heated to 65 °C for 4 h. The solution was added very slowly to crushed ice (500 mL), which was placed in a dry ice–acetone bath to keep it cool. The water suspension that formed was allowed to stir for 1 h, and it was then filtered. A portion of this crude sulfonyl chloride (3.0 g, 12 mmol) was dissolved in a 50:50 ether/dichloromethane (40 mL) solution and excess thiomorpholine (3.58 g, 35 mmol) was added. This solution was allowed to stir for 3 h. The solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate and 3 N HCl. The ethyl acetate was washed two additional times with 3 N HCl followed by a brine washing. The ethyl acetate was dried ($MgSO_4$) and the solvent was removed. The resulting residue was triturated in hexane, and filtered, to provide 4-methoxy-3-(thiomorpholine-4-sulfonyl)-benzoic acid (**73**, 1.7 g, 46%) as tan solid: (−ESI) [M−H]- = 316.

This compound (0.318 g, 1.00 mmol) was dissolved in 5 mL of glacial acetic acid and cooled to 0 °C in an ice-water bath. Hydrogen peroxide (0.41 mL, 4.00 mmol) was added dropwise, over a 10 min period, to the stirred solution. The ice bath was removed and the reaction mixture was heated to 100 °C in an oil bath. After stirring overnight, the solution was allowed to cool to room temperature and it was then concentrated and dried ($MgSO_4$) to afford 347 mg (99%) of the title compound as a white solid. ^{1}H NMR (DMSO-d_6) δ 8.31 (d, J = 2 Hz, 1H), 8.20 (dd, J = 9 Hz, 2 Hz, 1H), 7.39 (d, J = 9 Hz, 1H), 4.0 (s, 3H), 3.64 (m, 4H), 3.21 (m, 4H); MS (−ESI): [M−H]- = 348. Anal. calcd for $C_{12}H_{15}NO_7S_2$: C, 41.25; H, 4.33; N, 4.01. Found: C, 41.00; H, 4.01; N, 4.00.

2,2′-[(E)-1,2-Ethenediyl]bis[5-aminobenzenesulfonic acid], bis(1-methylethyl) ester (58). To a solution of commercially available 4,4′-diaminostilbene-2,2′-disulfonic acid (10 g, 27.0 mmol) in a mixture of 270 mL water and 30 mL dioxane was added 14.3 g (135 mmol) of sodium carbonate and the solution stirred until homogeneous. At this time 9-fluorenylmethyl chloroformate (14.7 g, 56.7 mmol) was added and the mixture was allowed to stir overnight. The solution was filtered and the solids were washed with water (200 mL). The collected solids were then suspended in 600 mL of water and 50 mL concd HCl was added. After 30 min, the solution was filtered and the solids dried in a vacuum oven overnight at 50 °C to provide 2,2′-[(*E*)-1,2-ethenediyl]bis(5-{[(9*H*-fluoren - 9 - ylmethoxy)carbonyl]amino}benzenesulfonic acid (21.8 g, 72%) as an orange-brown solid: MS (ES-NEG): [M–H] 813.

This compound (21.8 g, 26.75 mmol) was suspended in 270 mL dioxane and a reflux condenser was attached to the flask. Triisopropylorthoformate (17.88 mL, 80.26 mmol) was added and the mixture was heated to reflux. After complete conversion by TLC, the mixture was cooled to room temperature and the solids collected by filtration to provide 2,2′-[(*E*)-1,2-ethenediyl]bis(5-{[(9*H*)-fluorene - 9 - ylmethoxy)carbonyl]amino}benzene sulfonic acid), bis(1-methylethyl) ester (**57**, 18 g, 75%) as an off-white solid: ^{1}H NMR (DMSO-d_6) δ 10.25 (s, 2H), 8.28 (s, 2H), 7.92 (d, J = 7 Hz, 4H), 7.77 (d, J = 7

Hz, 8H), 7.60 (s, 2H), 7.44 (t, $J=7$ Hz, 4H), 7.37 (t, $J=7$ Hz, 4H), 4.5–4.7 (m, 6H), 4.34 (m, 2H), 1.18 (d, $J=6$ Hz, 12H); MS (ES-POS): [M + Na] 921. Anal. calcd for $C_{50}H_{46}N_2O_{10}S_2 0.5H_2O$: C, 66.13; H, 5.22; N, 3.08. Found: C, 66.00; H, 5.08; N, 3.04.

This compound (18.7 g, 20.89 mmol) was dissolved in 200 mL of dry DMF under a nitrogen atmosphere. The clear solution was cooled to 0 °C in an ice bath and piperidine (8.3 mL, 83.56 mmol) added in one portion. The solution was allowed to gradually warm to room temperature and after 3 h was diluted with 750 mL ethyl acetate. The mixture was washed with brine (3×). The aqueous layer was then back extracted with ethyl acetate (2×) and the organic layers were combined. The organic layer was dried ($MgSO_4$) and concentrated to a small volume. Hexane (200 mL) was added with stirring and the resulting precipitate was collected by filtration. The compound was further dried, under vacuum, overnight to afford the title compound (**58**, 7.3 g, 77%) as a dark yellow solid: ^{1}H NMR (DMSO-d_6) δ 7.48 (d, $J=8$ Hz, 2H), 7.38 (s, 2H), 7.20 (d, $J=2$ Hz, 2H), 6.90 (dd, $J=2$ Hz, 8 Hz, 2H), 5.90 (br s, 4H), 4.53 (m, 2H), 1.17 (d, $J=6$ Hz); MS (ES-NEG): [M−H] 453. Anal. calcd for $C_{20}H_{26}N_2O_6S_2.1.2H_2O$: C, 50.45; H, 6.01; N, 5.88. Found: C, 50.44; H, 5.83; N, 5.87.

2,2′-[(*E*)-1,2-Ethenediyl]bis[5-[4-(methylthio)-3-(4-morpholinyl sulfonyl) benzoyl] amino]benzenesulfonic acid] (2). To a solution of 4-methylsulfanyl-3-(morpholino-4-sulfonyl)-benzoic acid (**72**, X = SCH_3, NRR″ = morpholine, 2.96 g, 9.3 mmol) in 30 mL of dichloromethane under nitrogen was added dimethylformamide (50 μL) and the solution was cooled to 0 °C in an ice water bath. Oxalyl chloride (1.41 mL, 11 mmol) was added. After 2h the mixture was concentrated to dryness and then redissolved in 5 mL of dry tetrahydrofuran. This solution was added dropwise to a stirred suspension of bis(1-methylethyl) 2,2′-[(*E*)-1,2-ethenediyl]bis[5-aminobenzenesulfonate] (**58**, 2.04 g, 4.5 mmol) and potassium carbonate (2.57 g, 19 mmol) in 45 mL of dry THF. The resulting mixture was stirred for 18 h, concentrated to near dryness, and then partitioned between ethyl acetate and water. The organic fraction was washed three times with water followed by brine. The solvent was dried ($MgSO_4$) and concentrated. The residue was suspended in 4:1 ethyl acetate/hexane (25 mL), filtered and dried to afford 2,2-[(*E*)-1,2-ethenediyl]bis[5-[4-(methylthio)-3-(4-morpholinyl sulfonyl) benzoyl] amino] benzenesulfonic acid], bis(1-methylethyl) ester (**4**, 2.65 g, 55%) as a yellow solid: ^{1}H NMR (DMSO-d_6) δ 10.87 (s, 2H), 8.54 (s, 2H), 8.47 (s, 2H), 8.24 (m, 4H), 7.92 (d, $J=2$ Hz, 2H), 7.82 (s, 2H), 7.73 (d, $J=9$ Hz, 2H), 4.95 (m, 2H), 4.78 (m, 2H), 3.89 (m, 4H), 3.60 (m, 8H), 3.58 (m, 4H), 3.12 (m, 8H), 1.97 (m, 4H), 1.68 (m, 4H), 1.18 (d, $J=6$ Hz, 12H); CHN, calcd for $C_{44}H_{52}N_4O_{14}S_6 \cdot 2H_2O$: C, 48.51; H, 5.18; N, 5.14. Found: C, 48.42; H, 5.29; N, 4.57.

This compound (70 mg, 0.07 mmol) was dissolved in acetone (20 mL) and sodium iodide (120 mg, 0.80 mmol) was added. The mixture was stirred at 60 °C for 12 h. It was filtered, washed several times with acetone, and filtered again, to provide the bis(sodium) salt of the title compound (**2**, 50 mg, 66%):−ESI: [M−H]- 1066. ^{1}H NMR (DMSO-d_6) δ 10.55 (s, 2H), 8.21 (s, 2H), 8.14 (d, $J=8$ Hz, 2H), 8.07 (s, 2H), 8.03 (s, 2H), 7.95 (d, $J=8$ Hz, 2H), 7.61 (m, 4H), 3.31 (m, 8H), 3.06 (m, 8H), 2.58 (s, 6H).

2,2′-[(*E*)-1,2-Ethenediyl]bis[5-[3-[(1,1-dioxido-4-thiomorpholinyl)sulfonyl]-4-methoxybenzoyl]amino]benzenesulfonic acid] (14). Using the same procedure as for compound **2** above and starting from 3-(1,1-dioxo-1-thiomorpholine-4-sulfonyl)-4-methoxy-benzoic acid (**74**) the title compound was produced in 15% yield: ESI: [M−H]- 1031. ^{1}H NMR (DMSO-d_6) δ 10.50 (s, 2H), 8.46 (d, 2H, $J=2$ Hz), 8.35 (dd, 2H, $J=2$ Hz, 9 Hz), 8.16 (d, 2H, $J=2$ Hz), 8.07 (s, 2H), 7.89 (dd, 2H, $J=2$ Hz, 9 Hz), 7.62 (d, 2H, $J=9$ Hz), 7.43 (d, 2H, $J=9$ Hz), 4.04 (s, 6H), 3.67 (br s, 8H), 3.25 (br s, 8H).

4-Allylsulfonylbenzoic acid (83, R = allyl). A solution of commercially available 4-mercaptobenzoic acid (4 g, 26 mmol) in methylene chloride (200 mL) was treated with triethylamine (4 g, 40 mol) at room temperature. The resulting solution was cooled to 0 °C and treated with allyl bromide (10 g, 83 mmol) in methylene chloride (5 mL) under a nitrogen atmosphere. The mixture was stirred at 0 °C for 2 h and at room temperature for an additional 18 h. It was diluted with methylene chloride (500 mL), washed with water, dried, and evaporated. The crude product was recrystallized from methylene chloride/ether/hexane (1:5:20, 25 mL) to afford 4-allylthiobenzoic acid (3.5 g, 66% yield) as a pale solid: ^{1}H NMR (DMSO-d_6) δ 3.74 (d, $J=7$ Hz, 2H), 5.10 (d, $J=11$, 17 Hz, 1H), 5.30 (d, $J=11$, 17 Hz, 1H), 5.85 (m, 1H), 7.39 (d, $J=9$ Hz, 2H), 7.83 (d, $J=9$ Hz); MS (ES-Neg): $[M-H]^-$ 194. Anal. HPLC: 94% purity.

A solution of this acid (730 mg) in methanol (50 mL) was treated with Oxone® (5 g) in water (50 mL) at 0 °C. The resulting white suspension was stirred at room temperature for 48 h. The methanol was evaporated and the residue was diluted with water and extracted with methylene chloride. The methylene chloride extract was washed with water, dried and evaporated to give the title compound (**83**, R = allyl, 626 mg, 77% yield) as a white solid: ^{1}H NMR (DMSO-d_6) δ 4.20 (d, $J=7$ Hz, 2H), 5.20 (d, $J=17$, 11 Hz, 1H), 5.30 (d, $J=17$, 11 Hz, 1H), 5.68 (m, 1H), 7.97 (d, $J=9$ Hz), 8.10 (d, $J=9$ Hz). Anal. HPLC: 92.4% pure; MS (ES-Neg): $[M-H]^-$ 225.

2,2′-[(*E*)-1,2-Ethenediyl]bis[5-[4-(2-propenylsulfonyl)benzoyl]amino]benzenesulfonic acid (37). Commercially available 4,4′-diaminostilbene-2,2′-disulfonic acid (2.0 g, 5.4 mmol) was suspended in water (16 mL) and treated with 1 M tetra-*N*-butylammonium hydroxide solution (11.2 mL, 11.2 mmol). The resulting solution was stirred at rt for 1 h and then extracted with methylene chloride (3×50 mL). The methylene chloride extract was washed, dried and evaporated to dryness to afford 2,2′-[(*E*)-1,2-ethenediyl]bis[5-aminobenzenesulfonic acid, bis(tetra-*N*-butylammonium) salt (3.48 g, 75% yield) as an orange solid: ^{1}HNMR ($CDCl_3$) δ 0.90 (m, 24H), 1.25 (m, 16H), 1.45 (m, 16H), 3.00 (m, 16H), 6.57 (d, $J=8$ Hz, 2H),

7.40 (s, 2H), 7.65 (d, $J=8$ Hz, 2H), 8.07 (s, 2H); MS (ES-Neg): $[M-H]^-$ 369.

A solution of 4-allylsulfonyl-benzoic acid, (**83**, R = allyl, 225 mg, 1.0 mmol) in dichloromethane (1.5 mL) was treated with DMF (2 drops) and oxalyl chloride (1.5 mL) at 0 °C under a dry N_2 atmosphere. After 3 h, the solution was evaporated and the residue was dried. This residue was dissolved in dichloromethane (4.5 mL) and added to a 0 °C, stirred solution of 2,2′-[(*E*)-1,2-ethenediyl]bis[5-aminobenzenesulfonic acid, bis(tetra-*N*-butylammonium) salt (427 mg, 0.5 mmol) and triethylamine (141 mg, 1.4 mmol) in dichloromethane (25 mL). The resulting suspension was stirred for 2 h at 0 °C and at room temperature for an additional 18 h. It was diluted with 8% methanol in dichloromethane (200 mL), washed with water, dried and evaporated. Chromatography of the crude product on silica gel and elution with 7% methanol in dichloromethane afforded 55 mg (10% yield) of the bis(tetra-*N*-butylammonium) salt of (**37**) as a yellow solid: ^{1}HNMR (DMSO-d_6) δ 0.93 (m, 24H), 1.30 (m, 16H), 1.6 (m, 16H), 3.13 (m, 16H), 4.20 (d, $J=7$ Hz, 4H), 5.30 (m, 4H), 5.70 (m, 2H), 7.60–8.30 (m, 16H). Anal. HPLC = 97% pure; MS (ES Neg): $[M-H]^-$ 785. This bis(tetra-*N*-butylammonium) salt (50 mg) was dissolved in methanol/deionized water (5:3) (8 mL) and poured into an ion exchange resin column (3×20 cm, Toyopearl SP-650C, size range 100 micron). Elution with 10% methanol in deionized water (400 mL) and freeze-drying the eluant afforded 50 mg (100% yield) of the bis(sodium) salt of **37** as a white solid: ^{1}H NMR (DMSO-d_6) δ 4.21 (d, $J=7$ Hz, 4H), 5.28 (m, 4H), 5.71 (m, 2H), 7.60–8.25 (m, 16H). Anal. HPLC = 95.5% pure; MS (ES Neg): $[M-H]^-$ 785.

***N*,*N*-[(E)-1,2-Ethenediylbis[3-(aminosulfonyl)-4,1-phenylene]bis[4-(methylthio)-3-(4-morpholinylsulfonyl)benzamide] (6).** A solution of commercially available 2-methyl-5-nitrobenzenesulfonyl chloride (2.36 g, 10.0 mmol) and 75 mL dry ethyl ether under N_2 was cooled to 0 °C and concentrated ammonium hydroxide (10 mL) was added. After stirring overnight, the mixture was filtered and then diluted with dichloromethane (150 mL). The organic layer was separated and dried ($MgSO_4$). The solution was then concentrated to afford 2-methyl-5-nitrobenzenesulfonamide as a white solid (**61**, R = NH_2, 820, 38%). ^{1}H NMR (DMSO-d_6) δ 8.61 (d, $J=2.5$ Hz, 1H), 8.35 (dd, $J=8$ Hz, 2.5 Hz, 1H), 7.79 (br s, 2H), 7.70 (d, $J=8$ Hz, 1H), 2.71 (s, 3H).

To a solution of this compound (0.72 g, 3.33 mmol) in anhydrous dimethylformamide (30 mL) was added potassium *t*-butoxide (1.12 g, 9.99 mmol) and the resulting solution turned a deep red color. The mixture was allowed to stir for 16 h and was then poured over ice (50 g). The orange-brown mixture was diluted with ethyl acetate (150 mL), the organic layer was separated, and dried over $MgSO_4$. The material was then concentrated and dried under vacuum to afford 5-nitro-2-[(*E*)-2-(4-nitro-2-(aminosulfonyl)phenyl)ethenyl]benzene sulfonamide (**62**, R = NH_2, 300 mg, 21%) as an orange solid: ^{1}H NMR (DMSO-d_6) δ 8.70 (d, $J=2$ Hz, 2H), 8.55 (dd, $J=9$ Hz, 2 Hz, 2H), 8.20 (d, $J=9$ Hz, 2H), 8.09 (s, 2H), 8.06 (s, 4H); MS (FI-NEG): $[M-H]-$ 427.

To a solution of this compound (0.38 g, 0.887 mmol) in ethyl acetate (40 mL) was added tin chloride dihydrate (1.00 g, 4.44 mmol). The solution was heated in an oil bath to 80 °C and stirred for 16 h. After cooling to room temperature the solution was diluted with ethyl acetate (250 mL) and washed with saturated sodium bicarbonate. The mixture was filtered through Celite and the aqueous layer was discarded. The organics were then washed with brine, dried ($MgSO_4$) and concentrated. The crude material was then recrystallized from 10% methanol/dichloromethane and dried under vacuum to afford 5-amino-2-[(*E*)-2-(4-amino-2-(aminosulfonyl)-phenyl)ethenyl]benzenesulfonamide (**63**, R = NH_2, 100 mg, 31%) as a dark-yellow solid. ^{1}H NMR (DMSO-d_6) δ 7.58 (d, $J=8.5$ Hz, 2H), 7.49 (s, 2H), 7.31 (br s, 4H), 7.15 (d, $J=2$ Hz, 2H), 6.75 (dd, $J=8.5$ Hz, 2 Hz, 2H), 5.65 (br s, 4H).

To a solution of 4-methylsulfanyl-3-(morpholino-4-sulfonyl)-benzoic acid (**72**, X = SCH_3, NRR″ = morpholine, 0.198 g, 0.612 mmol) in 27 mL of dry dichloromethane under N_2 was added dimethylformamide (50 μL) and the solution was cooled to 0 °C in an ice-water bath. Oxalyl chloride (0.063 mL, 0.675 mmol) was added and the mixture stirred for 30 min and then allowed to warm to room temperature. After 3 h, the mixture was concentrated and then redissolved in 45 mL of dry THF. This solution was added to a suspension of 5-amino-2-[(*E*)-2-(4-amino-2-(aminosulfonyl)phenyl)ethenyl]benzenesulfonamide (**63**, R = NH_2, 0.09 g, 0.244 mmol) and potassium carbonate (0.135 g, 0.972 mmol) in 45 mL of dry THF under N_2 and the reaction mixture was stirred overnight. The reaction mixture was then diluted with ethyl acetate (300 mL) and washed with saturated sodium bicarbonate. A precipitate formed at the interface and was collected by filtration. After washing with ethyl acetate, this precipitate was dried under vacuum to afford the title compound (**6**) as a yellow solid (87 mg, 37%). ^{1}H NMR (DMSO-d_6) δ 10.80 (br s, 2H), 8.46 (s, 2H), 8.43 (s, 2H), 8.28 (d, $J=7$ Hz, 2H), 8.10 (d, $J=8$ Hz, 2H), 7.99 (d, $J=9$ Hz, 2H), 7.85 (s, 2H), 7.67 (d, $J=9$ Hz, 2H), 7.60 (br s, 4H), 3.62 (m, 8H), 3.15 (m, 8H), 2.61 (s, 6H); MS (ES-NEG): $[M-H]-$ 965. Anal. calcd for $C_{38}H_{42}N_6O_{12}S_6{\cdot}4H_2O$: C, 43.92; H, 4.89; N, 8.09. Found: C, 43.79; H, 4.29; N, 8.04.

5-[4-(Methylsulfonyl)-3-nitrobenzoyl]amino]-2-[(*E*)-2-[4-[4-(methylthio)-3-(4-morpholinylsulfonyl)benzoyl]amino]-2-sulfophenyl]ethenyl]benzenesulfonic acid] (50). To a solution of commercially available 4-methylsulfonyl-3-nitrobenzoic acid (0.27 g, 1.10 mmol) in 30 mL of dry dichloromethane under N_2 was added DMF (50 μL) and the solution cooled to 0 °C in an ice water bath. Oxalyl chloride (0.106 mL, 1.21 mmol) was added and the mixture was stirred for 30 min and then allowed to warm to room temperature. After 3 h, the mixture was concentrated and then redissolved in 50 mL of dry tetrahydrofuran. This THF solution was added dropwise, under N_2, over a 5 h period, to a stirred suspension of

2,2′-[(*E*)-1,2-ethenediyl]bis[5-aminobenzenesulfonic acid], bis(1-methylethyl) ester (**58**, 1.0 g, 2.20 mmol) and potassium carbonate (0.608 g, 4.40 mmol) in 170 mL of dry THF. The resulting reaction mixture was stirred overnight. The suspension was concentrated and then diluted with ethyl acetate (300 mL) and a small amount of water (20 mL). The mixture was washed with concentrated sodium bicarbonate solution followed by brine. The organic layer was dried ($MgSO_4$) and filtered. Concentration followed by flash column chromatography using a 5–20% ethyl acetate in dichloromethane gradient provided 5-[4-(methylsulfonyl)-3-nitrobenzoyl]amino] - 2 - [(*E*) - 2 - [4 - amino - 2 - sulfophenyl]-ethenyl]benzenesulfonic acid, bis(1-methylethyl) ester (**54**, 410 mg, 56%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 11.12 (s, 1H), 8.64 (d, J = 1 Hz, 1H), 8.55 (d, J = 2 Hz, 1H), 8.49 (dd, J = 8 Hz, 1 Hz, 1H), 8.32 (d, J = 8 Hz, 1H), 8.21 (dd, J = 9 Hz, 2 Hz, 1H), 7.85 (d, J = 9 Hz, 1H), 7.65 (d, J = 16 Hz, 1H), 7.57 (d, J = 9 Hz, 1H), 7.48 (d, J = 16 Hz, 1H), 7.25 (d, J = 2 Hz, 1H), 6.95 (dd, J = 8 Hz, 2 Hz, 1H), 6.10 (br s, 2H), 4.59 (m, 2H), 3.56 (s, 3H), 1.20 (m, 12H); MS (ES-NEG): [M−H] 680. Anal. calcd for $C_{28}H_{31}N_3O_{11}S_3$: C, 49.33; H, 4.58; N, 6.16. Found: C, 49.08; H, 4.35; N, 6.00.

To a solution of 4-methylsulfanyl-3-(morpholino-4-sulfonyl)-benzoic acid (**72**, X = SCH_3, NRR″ = morpholine, 0.105 g, 0.33 mmol) in 15 mL of dry dichloromethane under N_2 was added DMF (50 μL) and the solution cooled to 0 °C in an ice-water bath. Oxalyl chloride (0.030 mL, 0.345 mmol) was added and the mixture stirred for 30 min and then allowed to warm to room temperature. After 3 h the mixture was concentrated and then redissolved in 15 mL of dry tetrahydrofuran. This THF solution was added, under N_2, to a stirred suspension of 5-[4-(methylsulfonyl)-3-nitrobenzoyl]amino] - 2 - [(*E*) - 2 - [4 - amino - 2 - sulfophenyl]-ethenyl]benzenesulfonic acid, bis(1 - methylethyl) ester (**54**, 0.10 g, 0.15 mmol) and potassium carbonate (0.125 g, 0.90 mmol) in 15 mL of THF. The resulting reaction mixture was stirred overnight. The suspension was concentrated and then diluted with ethyl acetate (150 mL). The mixture was washed with saturated sodium bicarbonate, followed by brine. The organic layer was dried ($MgSO_4$) and filtered. Concentration, followed by flash column chromatography using 5% methanol in dichloromethane as an eluant provided 2-[(*E*)-2-[2-(isopropoxysulfonyl)-4-[4-(methylsulfonyl)-3-nitrobenzoyl]amino]phenyl] ethenyl]-5-[4-(methylthio)-3-(4 - morpholinylsulfonyl)benzoyl]amino]benzenesulfonic acid, (1 - methylethyl) ester (25 mg, 17%) as a yellow solid. ^{1}H NMR (DMSO - d_6) δ 11.18 (s, 1H), 10.91 (s, 1H), 8.65 (d, J = 1 Hz, 1H), 8.60 (t, 3 Hz, 2H), 8.51 (dd, J = 8 Hz, 1 Hz, 1H), 8.43 (d, J = 2 Hz, 1H), 8.33 (d, J = 8 Hz, 1H), 8.27 (m, 3H), 7.93 (t, J = 9 Hz, 2H), 7.73 (s, 2H), 7.68 (d, J = 9 Hz, 1H), 4.67 (m, 2H), 3.62 (m, 4H), 3.57 (s, 3H), 3.15 (m, 4H), 2.63 (s, 3H), 1.24 (m, 12H); MS (ESI): [M + NH_4] 998. Anal. RP-HPLC 90% pure, one major peak.

This compound (75 mg, 0.076 mmol) was suspended in 9:1 acetone/water (20 mL) and sodium iodide (46 mg, 0.31 mmol) was added. The mixture was stirred at 40 °C for 12 h. It was filtered, washed several times with acetone, and filtered again, to provide the bis(sodium) salt of the title compound (**50**, 36 mg, 44%): +ESI: [M + Na] + 919. ^{1}H NMR (DMSO-d_6) δ 10.83 (s, 1H), 10.62 (s, 1H), 8.65 (d, 1H, J = 1.4 Hz), 8.51 (dd, 1H, J = 1.4 Hz, 8 Hz), 8.42 (d, 1H, J = 2 Hz), 8.28 (dd, 2H, J = 3 Hz, 8 Hz), 8.18 (dd, 2H, J = 2 Hz, 0.8 Hz), 8.08 (s, 2H), 7.93 (d, 2H, J = 8 Hz), 7.63 (m, 3H), 3.62 (t, 4H, J = 6 Hz), 3.56 (s, 3H), 3.15 (t, 4H, J = 6 Hz), 2.60 (s, 3H).

Pharmacology

Cell preparations

Chinese hamster ovarian (CHO-K1) cells stably transfected with the ovarian hFSHR gene (CHO-3D2 cells) were provided by Dr. Kerry Koller, (Affymax, Inc., Palo Alto, CA) and grown in suspension culture in UltraCHO® medium (BioWhittaker, Inc., Walkersville, MD) containing 1% (v/v) fetal bovine serum (Life Technologies, Rockville, MD) and 200 μg/mL G418 (Life Technologies) for the FSHR radioligand membrane binding assay. The cells were collected by centrifugation and the resulting cell pellets were frozen and stored at −70 °C.

For the cAMP accumulation assays, CHO-3D2 cells were grown in growth medium consisting of DMEM/F12 (50:50) medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies), 2 mM GlutaMax®-I (Life Technologies), penicillin G sodium (100U/mL) and streptomycin sulfate (100 μg/mL). Cells were maintained at 37 °C in an atmosphere saturated with water and containing 95% air, 5% CO_2.

Chinese hamster ovarian cells that stably express the hTSHR gene (CHO-25) were kindly provided by Dr. Leonard D. Kohn, Metabolic Diseases Branch of the National Institute of Diabetes and Digestive and Kidney Diseases. CHO-25 cells were grown in F-12 Nutrient Mixture medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Life Technologies), 2 mM GlutaMax®-I (Life Technologies), penicillin G sodium (100 U/mL) and streptomycin sulfate (100 g/mL). Cells were maintained at 37 °C in an atmosphere saturated with water and containing 95% air, 5% CO_2.

For preparation of primary cultures of rat ovarian granulosa cells, 24 day-old immature female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used as donors for ovarian granulosa cells. The animals were treated by single daily injections of 100 mg diethylstilbestrol (DES; Sigma Chemical Co., St. Louis, MO, USA) per kg body weight in oil for three consecutive days prior to granulosa cell isolation. On the day following the last DES treatment, animals were sacrificed by carbon dioxide asphyxiation and the ovaries were removed. Ovaries were washed three times in 50 mL of sterile HEPES-buffered saline (HBS, pH 7.4).

Granulosa cells were harvested by incubating ovaries in a hypertonic medium consisting of serum-free McCoy's 5A medium (Life Technologies) supplemented with 5 mg/mL insulin, 5 mg/mL transferrin, 5 ng/mL sodium selenite (ITS, Sigma Chemical Co.), 146 mg/mL l-glutamine, 100 nM testosterone, 100 nM DES and 1X antibiotic-antimycotic solution (100 U/mL penicillin, 10 mg/mL streptomycin, 250 ng/mL amphotericin B; Life Technologies) containing 0.5 M sucrose and 0.1 mM EGTA (Sigma Chemical Co.). Ovaries were then incubated for 45 min at 37 °C in a humidified incubator gassed with 95% air/5% CO_2. They were washed three times with 10 mL isotonic medium (hypertonic medium without sucrose and EGTA) and incubated another 45 min. in isotonic medium at 37 °C. Granulosa cells were harvested by compressing the ovaries between two sterile glass microscope slides. Isolated granulosa cells were then placed in a 50 mL centrifuge tube and washed two times by the addition of 50 mL serum-free McCoy's 5A medium (growth medium) followed by centrifugation at 700*g* for 5 min. After the final spin, the cells were resuspended by gentle trituration in 25 mL growth medium. A portion of the cell suspension was counted in a hemocytometer and cell viability was estimated by trypan blue exclusion. Cells were plated into 24-well tissue culture plates (Nunc A/S; Roskilde, Denmark) at a density of 200,000 viable cells in 250 μL of growth medium/well.

Adrenocortical tumor Y1 cells (American Type Culture Collection; Rockville, MD, USA) were genetically engineered to stably produce the hFSHR (Y1/5E5/s3). The cells were plated two days prior to treatment into 24-well plates at a density of 200,000 cells/well in growth medium [F-10 Nutrient Mixture (Life Technologies), 2 mM GlutaMax®-I (Life Technologies), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate (Life Technologies), 12.5% (v/v) horse serum (heat-inactivated; BioWhittaker), 2.5% (v/v) fetal bovine serum (heat-inactivated; BioWhittaker), and 75 μg G418/mL (Life Technologies)]. On the day of treatment, each well of cells was washed twice with 1 mL prewarmed (37 °C) assay medium. After removing the last medium wash, an additional 1 mL of assay medium was added to each well and the cells incubated for 30 min at 37 °C in a humidified incubator with 5% CO_2/95% air.

FSH Receptor radioligand binding assay[9]

Receptor membrane preparation. Frozen cell pellets were weighed and resuspended in binding buffer (10 mM Trizma®-HCl (Sigma), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.025% (w/v) sodium azide, 0.1% (w/v) bovine serum albumin (BSA, fraction V; Sigma), 5 μg/mL aprotinin (Sigma), 5 μg/mL leupeptin (Sigma), 5 μg/mL pepstatin (Sigma), 5 μg/mL phenylmethylsulfonylfluoride (Sigma), 5 μg/mL phosphoramidon (Sigma), pH 7.2) to a final concentration of 30 mg wet weight/mL. Cell suspension for each pellet was homogenized using a Tempest homogenizer (VirTis, Gardiner, NY). Cell homogenates were pooled and 35 mL aliquots were transferred to polypropylene copolymer centrifuge tubes. Tubes were spun at 17,000×*g* for 12 min at 4 °C. Resulting supernatant fractions were discarded and pellets were stored at −70 °C until use.

Receptor binding assay. Cell membrane pellet was resuspended in 35 mL binding buffer and homogenized using a Tempest homogenizer. Membrane homogenate (100 μL) was added to each well of a 96-well microtiter plate. All samples were tested in triplicate. Test compound solutions (50 μL of 4× concentrated stock in binding buffer with 4% (v/v) DMSO) were added to the designated wells. Total bound counts were determined by adding 50 μL binding buffer containing 4% (v/v) DMSO to the designated wells. Non-specific binding was determined by adding 50 μL of hFSH solution [4 μM in 4% (v/v) DMSO; Reproductive Endocrine Unit, Massachusetts General Hospital, Charlestown, MA] to the designated wells. Plates were pre-incubated for 15 min at room temperature on a shaking platform. After pre-incubation [^{125}I]FSH (50 μL; specific activity: 50 pM final concentration, NEN Life Science Products, Inc., Boston, MA,) was added to each well and plates were incubated for 2 h at room temperature on shaking platform. The reaction was terminated by transfer of the membrane preparation to glass fiber filters (Skatron Instruments, Sterling, VA) that had been pretreated with 1% (w/v) BSA in wash buffer (50 mM Trizma®-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.2) for at least 30 min, but not longer than 1 h using a 96-well microtiter vacuum harvester (Skatron Instruments). The membranes collected on the filters were washed 5 times with ice-cold wash buffer (200 μL buffer/well/cycle) followed by three additional washes with wash buffer (100 μL buffer/well/cycle). The filters were dried by a 10 s aspiration. Disks corresponding to each well of the microtiter plate were punched out of the filter mat into 12×75 mm polypropylene tubes. The radioactivity present on each of the disks was measured using a gamma counter.

A hFSH dose response curve (0.001, 0.01, 0.1, 1, 10, and 100 nM) was generated for each binding assay to monitor assay to assay variability.

Rat LH receptor radioligand binding assay

Receptor membrane preparation. Rat testes were purchased from Pel-Freez Biologicals (Rogers, AR, USA). The tunica albuginea was removed from each testis before weighing. Two testes were minced in binding buffer (described above). Each pair of testes was homogenized using a Polytron homogenizer (model PT 1200C; Brinkmann Instruments, Inc, Westbury, NY, USA). The homogenates were spun at 500*g* for 10 min. The supernatant fractions were pooled and spun at 17,000*g* for 12 min. The supernatant fractions were discarded and the pellets were frozen and stored at −80 °C until use.

Receptor binding assay. Frozen pellets were weighed and resuspended in binding buffer to a concentration of 60 mg pellet weight/mL (approximately 0.73 mg protein/mL). Binding reactions were set up according the hFSHR binding assay described above with human LH

(50 μL, 4 μM solution, Reproductive Endocrine Unit, Massachusetts General Hospital) used instead of hFSH. The binding reactions were initiated by the addition to each well of 50 μL of [^{125}I]hLH (250 pM final concentration, Specific activity: 90–200 μCi/μg; NEN Life Science Products, Inc.). The plates were incubated for 2 h at room temperature on an orbital shaker. The reactions were terminated as described for the hFSHR binding assay.

cAMP accumulation assays

The day prior to the experiment, CHO-3D2 (hFSHR) cells were plated into 96-well tissue culture plates at a density of 30,000 cells/well in growth media and plates were incubated at 37 °C in an atmosphere saturated with water and containing 95% air, 5% CO_2. After overnight incubation cells were washed twice with 100 μL/well of Opti-MEM® I (Life Technologies), 0.1% (v/v) BSA, (Life Technologies). Medium was removed and 100 μL of Opti-MEM®-I, 0.1% (v/v) BSA was added to every well. Plates were incubated for an additional 30 min at 37 °C. Medium was removed and cells were challenged for 30 min at 37 °C in 50 μL of Opti-MEM I with 0.1% (v/v) BSA media containing vehicle or purified hFSH (>95% pure; Cortex Biochem, Inc., San Leandro, CA) in the presence or absence of test compounds. Experiments were terminated by the addition of 50 μL of 0.2 N HCL/well. All test compounds were evaluated in a dose–response paradigm ranging from 0.01 to 30 μM. Controls and test compounds were evaluated in quadruplicate. Cells were treated with vehicle, the EC_{20} of hFSH (1.85 ng/mL), and the compounds in the presence or absence of the EC_{20} of hFSH. The ability of the compounds to inhibit the cAMP-accumulation induced by hFSH was evaluated by radioimmunoassay.

cAMP accumulation assays using CHO-25 (hTSHR) cells were performed as described above for the CHO-3D2 cells with the following exceptions. CHO-25 cells were plated at a density of 20,000 cells/well. All test compounds were evaluated in a dose-response paradigm ranging from 0.01 to 30 μM. Controls and test compounds were evaluated in quadruplicate. Cells were treated with vehicle, the EC_{20} of hTSH (5nM; hTSH >98% pure, Cortex Biochem, Inc.), and the compounds in the presence or absence of the EC_{20} of hTSH. The ability of the compounds to inhibit the cAMP accumulation induced by hTSH was evaluated by radioimmunoassay.

In vitro bioassay of antagonists to the FSH receptor using primary cultures of rat granulosa[2]

Aromatase assay. Following plating of the cells, the plates were incubated at 37 °C for 2–4 h after which time the cells were treated with test compounds. Compounds were dissolved in DMSO (Sigma Chemical Co.) at a concentration of 0.1 M. The compounds were subsequently diluted in sterile isotonic medium and 0.2% (w/v) bovine serum albumin (Sigma Chemical Co) to 2× desired final concentration prior to use in the assay. The concentration of DMSO vehicle remained constant throughout all dilutions. Compounds were added to the wells (250 μL per well) and the cells were incubated at 37 °C for 72 h. Compounds were tested in quadruplicate wells. For antagonist mode, each compound was tested in a dose–response paradigm versus a constant level of purified hFSH (the EC_{50} dose of 0.5 ng/mL; Cortex Biochem, Inc.). Four different doses of compound were tested in the antagonist mode. In addition, vehicle alone was used to determine basal concentrations of estradiol and hFSH alone (EC_{50} dose) was used as a positive control. Along with the plates testing compounds, another plate was run in parallel using a dose–response of hFSH (0.01–100 ng/mL) as a positive control. At the end of the incubation period, the medium was removed from the wells and assayed for estradiol concentration by radioimmunoassay.

Evaluation of FSH receptor agonists and antagonists using mouse adrenocortical tumor (Y1) cells[21–24]

Stock compounds were solubilized in an appropriate vehicle, either phosphate-buffered saline (PBS, Life Technologies) or DMSO (Sigma Chemical Co.), at a concentration of 0.1 M. The compounds were subsequently diluted 1000-fold in sterile assay medium [α-MEM (Life Technologies) containing 15% horse serum (heat inactivated; BioWhittaker), 2.5% fetal bovine serum (heat inactivated; BioWhittaker), 2 mM GlutaMax® I (Life Technologies), penicillin G sodium (100 units/mL; Life Technologies), streptomycin sulfate (100 μg/mL, Life Technologies) and 0.1 mM MEM nonessential amino acids (Life Technologies). The concentration of the vehicle in each of the compound dilutions was the same.

Cells were treated with different concentrations of test compounds in 0.5 mL of assay medium containing 0.1% (v/v) DMSO or vehicle alone for 20–24 h at 37 °C in a humidified incubator with 5% CO_2/95% air. Each treatment condition was tested in quadruplicate. For antagonist mode, each compound was tested at four different concentrations (each concentration using four wells) in a dose–response paradigm versus a constant concentration of purified hFSH (EC_{50} of 25 ng/mL; Cortex Biochem). In addition, vehicle alone and hFSH alone (25 ng/mL final concentration) were tested as negative and positive controls, respectively. Along with the test compounds, purified hFSH was tested in a dose response (1, 10, 30, 300, and 1000 ng/mL) as an additional assay control. At the end of the incubation period, the medium was removed from each well and assayed for progesterone concentration by radioimmunoassay.

Radioimmunoassays. cAMP concentrations were measured using the cAMP BIOTRAK® radioimmunoassay kit purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA) in accordance with the manufacturer's instructions. Estradiol in the granulosa cell assay medium was measured by radioimmunoassay using the Coat-A-Count® Estradiol kit (Diagnostic Products Corporation; Los Angeles, CA, USA).

Estradiol concentrations in 50 μL samples were determined according to the manufacturer's recommendation. Progesterone concentration in the adrenal cell assay medium was determined using the Coat-A-Count® Progesterone kit (Diagnostic Products Corp.) according to the recommended specifications.

Analysis of results. Estradiol and progesterone concentrations were expressed as pg/mL. cAMP concentrations were expressed as fmol/mL. The ability of the compound to inhibit hFSH-induced estradiol or progesterone secretion was compared to the respective basal secretion and positive controls. The ability of compound to inhibit hFSH- or TSH-induced cAMP accumulation was compared to the respective basal accumulation and positive controls. Data were analyzed statistically by analysis of variance with Huber weighting of log-transformed data. Paired differences were determined using the least significant difference (LSD) test. The concentration at which compounds inhibited the hFSH- or hTSH-induced response by 50% (IC_{50}) was calculated using a four parameter logistic fit equation.

Cytotoxicity assay. Cellular toxicity resulting from compound treatment was assessed utilizing a CytoLux® kit (Perkin-Elmer Wallac, Inc., Gaithersburg, MD, USA). In this assay, CHO-3D2 cells, plated at 30,000/well, were pretreated with 3 or 30 μM of test compound for 30 min. Luciferase and luciferin were then added and the cellular ATP concentration, which is an indicator of cellular metabolic viability, was then measured using a luminometer according to the manufacturer's specifications.

Acknowledgements

We are grateful to the Discovery Analytical Chemistry Department of Wyeth-Ayerst for elemental analyses, 400-MHz ^{1}H NMR, mass spectroscopy and HPLC data, Hong Chen and William Gallaway for LogP data, Rob Ley for CACO2 data, and Gi-chung Chen, Jane Coyle-Morris, May Wu, and Jamin Chi for their technical assistance.

References and Notes

1. Fauser, B. C. J. M., Ed.: *FSH Action and Intraovarian Regulation, Studies in Profertility Series*; Parthenon Publishing Group, New York: 1997; Vol. 6.
2. Hsueh, A. J.; Bicsak, T.; Jia, X.-C.; Dahl, K. D.; Fauser, B. C. J. M.; Galway, A. B.; Czwkala, N.; Pavlou, S.; Pakoff, H.; Keene, J.; Boime, I. *Rec. Prog. Horm. Res.* **1989**, *45*, 209.
3. Aittomaki, K.; Lucena, J. L. D.; Pakarinen, P.; Sistonen, P.; Tapanainnen, J.; Gromoll, J.; Kaskikari, R.; Sankila, E.-M.; Lehvaslaiho, H.; Engel, A. R.; Nieschlag, E.; Huhtaniemi, I.; Chapelle, A. d. l. *Cell* **1995**, *82*, 959.
4. Lindstedt, G.; Nystrom, E.; Matthews, C.; Ernest, I.; Janson, P. O.; Chatterjee, K. *Clin. Chem. Lab. Med.* **1998**, *36*, 664.
5. Mukherjee, A.; Park-Sarge, O. K.; Mayo, K. E. *Endocrinology* **1996**, *137*, 3234.
6. Chappel, S.; Buckler, D.; Kelton, C.; Tayar, N. E. *Human Reproduction* **1998**, *13*, 18.
7. Tayar, N. E. *Mol. Cell Endocrinol.* **1996**, *125*, 65.
8. Jiang, X.; Dreano, M.; Buckler, D. R.; Cheng, S.; Ythier, A.; Wu, H.; Hendrickson, W. A.; Tayar, N. E. *Structure* **1995**, *3*, 1341.
9. Stevis, P. E.; Deecher, D. C.; Lopez, F. J.; Frail, D. E. *Endocrine*. **1999**, *10*, 153.
10. Daugherty, R. L.; Cockett, A. T. K.; Schoen, S. R.; Sluss, P. M. *J. Urol* **1992**, *147*, 727.
11. Danesi, R., Rocca, R. V. L.; Cooper, M. R.; Ricciardi, M. P.; Pellegrini, A.; Soldani, P.; Kragel, P. J.; Paparelli, A.; Tacca, M. D.; Myers, C. E. *J. Clin. Endocrinol. Metab.* **1996**, *81*, 2238
12. Arey, B. J.; Shen, E. S.; Deecher, D. C.; Perez, M. C.; Rogers, J. F.; Green, D. M.; Kao, W.-L.; Unwalla, R.; Wrobel, J.; Frail, D. E.; López, F. J. Identification and Characterization of a Selective, Non-peptide Follicle-Stimulating Hormone Receptor Antagonist, CL-272219, The Endocrine Society's 82nd Annual Meeting, Toronto, Canada, June 21–24, 2000.
13. Rogers, J.; Green, D.; Shen, E.; Hardenburg, J.; Perez, M.; Arey, B.; Wrobel, J. Synthesis of (bis)Sulfonic acid, (bis)-Benzamides as FSH Antagonists, 220th ACS National Meeting, Washington, DC, Aug 20–24, 2000.
14. Jetter, J.; Kao, W.; Shen, E.; Hardenburg, J.; Perez, M.; Arey, B.; Wrobel, J. Synthesis of (bis)Sulfonic acid, (bis)Benzamides as FSH Antagonists, 27th National Medicinal Chemistry Symposium, Kansas City, MI, June 13–17, 2000.
15. Padmapriya, A. A.; Just, G.; Lewis, N. G. *Synth. Commun.* **1985**, *15*, 1057.
16. Trujillo, J. I.; Gopalan, A. S. *Tetrahedron Lett.* **1993**, *34*, 7355.
17. Roberts, J. C.; Gao, H.; Gopalsamy, A.; Kongsjahju, A.; Patch, T. *J. Tetrahedron Lett.* **1997**, *38*, 355.
18. Takido, T.; Itabashi, K. *Synthesis* **1987**, 817.
19. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Deliv. Rev.* **1997**, *23*, 3.
20. Navia, M. A.; Chaturvedi, P. R. *Drug Disc. Today* **1996**, *1*, 179.
21. Yasumura, Y.; Buonassisi, V.; Sato, G. *Cancer Res.* **1996**, *26*, 529.
22. Schimmer, B. P. *Methods Enzymol.* **1979**, *58*, 570.
23. Kelton, C. A.; Cheng, S. V. Y.; Nugent, N. P.; Schweickhardt, R. L.; Rosenthal, J. L.; Overton, S. A.; Wands, G. D.; Kuzeja, J. B.; Luchette, C. A.; Chappel, S. C. *Mol. Cell. Endocrinol.* **1992**, *89*, 141.
24. Tilly, J. L.; Aihara, T.; Nishimori, K.; Jia, X.-C.; Billig, H.; Kowalski, K. I.; Perlas, E. A.; Hsueh, A. J. W. *Endocrinology* **1992**, *131*, 799.