Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane: Isolation and Structure Elucidation of a Novel Estrogen from Commercial Preparations of Phenol Red (Phenolsulfonphthalein)†

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Commercial preparations of phenolsulfonphthalein (Phenol Red), a pH indicator dye widely added to cell culture media, have weak estrogenic activity that can be accounted for by a minor lipophilic impurity (ca. 0.002%). We have isolated this impurity, determined its structure to be bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane, and synthesized it from phenolsulfonphthalein. This compound binds to the estrogen receptor with an affinity 50% that of estradiol; it stimulates the proliferation and increases the progesterone receptor content of estrogen-responsive breast cancer cells in vitro, and it stimulates uterine weight gain in rats in vivo, but shows a potency in these assays only 0.1–0.2% that of estradiol. We suggest how this novel estrogen may be generated during the preparation of phenolsulfonphthalein.

It has been a curious observation that, in the apparent absence of estrogenic stimulation, estrogen-responsive cells (such as MCF-7 human breast cancer cells) grow rapidly in culture at a rate that is often marginally, if at all, stimulated by estrogens, but is markedly suppressed by antiestrogens.[1] The possibility that estrogenic stimulatory substances might be present in the culture milieu has been examined, but rigorous efforts to identify residual levels of estrogens or estrogen sulfates in the serum, used to supplement the culture medium, have given negative results.[1a,2]

A while ago, we reported that commercial preparations of phenolsulfonphthalein (Phenol Red), a pH indicator dye commonly used in culture media to monitor acidity, had estrogenic activity.[3] After Phenol Red was removed from the culture medium, cell growth rate dropped to low levels, was largely unaffected by antiestrogens, and was stimulated to a large degree by added estrogens. This finding has been confirmed in a number of laboratories,[4] but some recent reports have suggested that it might be an impurity in the Phenol Red preparations, rather than the indicator phenolsulfonphthalein itself, that was responsible for the estrogenic stimulation.[5] In a chromatographic investigation of commercial Phenol Red preparations, we found that the indicator itself is devoid of estrogenic activity; most of the estrogen receptor binding activity of these dye preparations can be attributed to a more lipophilic impurity.[6] This impurity alone is capable of fully stimulating cell growth, and its removal from Phenol Red preparations eradicated their growth stimulatory properties.

In this paper, we describe the isolation of adequate quantities of this lipophilic impurity for the determination of its structure by spectroscopic means. We have synthesized this compound from phenolsulfonphthalein and confirmed the estrogenic activity of the synthetic material in uterotropic, cell proliferation, and progesterone receptor stimulation assays. In addition, the structure of an oxidized analogue of this impurity, which may be its precursor, is described.

Results and Discussion

Isolation. The purification of the novel estrogen binding component from commercial preparations of Phenol Red has involved solvent partitioning, followed by three steps of chromatographic purification. During each chromatography, fractions were collected and their estrogen receptor binding activity was assessed by measuring their competition with [^{3}H]estradiol toward preparations of estrogen receptor from rat uterus.[6,7] The profiles of competitive binding activity are indicated on the HPLC traces as the rectangularized line (histograms, Figure 1).

Extraction of a 300-g portion of Phenol Red monosodium salt (Sigma) with diethyl ether (20 L) provided 6 g of a dark brown oil. Reversed-phase HPLC analysis of this material (Figure 1A) showed a series of peaks, with the major estrogen receptor competing substance appearing in fractions 30 and 31; this corresponds to the descending portion of a small peak that follows the largest peak in the chromatogram. As indicated by the darkened horizontal lines, yellow material elutes in three regions of the chromatogram. Most of the colored material appears to be associated with the major UV-absorbing material (fractions 27–29); the yellow material that appears in the

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† In honor of Prof. E. J. Corey's 60th birthday.

(1) (a) Katzenellenbogen, B. S.; Norman, M. J.; Eckert, R. L.; Peltz, S. W.; Mangel, W. F. *Cancer Res.* **1984**, *44*, 1112–119. (b) Miller, M. A.; Katzenellenbogen, B. S. *Cancer Res.* **1983**, *43*, 3094–3100. (c) Sutherland, R. L.; Foo, M. S.; Greene, M. D.; Waybourne, A. M.; Krozowski, Z. S. *Nature (London)* **1980**, *288*, 273–275. (d) Sudo, K.; Monsma, F. J., Jr.; Katzenellenbogen, B. S. *Endocrinology* **1983**, *111*, 425–434. (e) Kon, O. L. *J. Biol. Chem.* **1983**, *258*, 3173–3177.

(2) (a) Vignon, F.; Terqui, M.; Westley, B.; Derocq, D.; Rochefort, H. *Endocrinology* **1980**, *106*, 1079–1086. (b) Darbre, P.; Yates, J.; Curtis, S.; King, R. J. B. *Cancer Res.* **1983**, *43*, 349–354.

(3) (a) Berthois, Y.; Katzenellenbogen, J. A.; Katzenellenbogen, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2496–2500. (b) The growth properties of MCF-7 cells are affected not only by the presence or absence of Phenol Red but by the duration of its presence or absence, as has been reported in detail elsewhere: Katzenellenbogen, B. S.; Kendra, K. L.; Norman, M. J.; Berthois, Y. *Cancer Res.* **1987**, *47*, 4355–4360.

(4) (a) Hubert, J. F.; Vincent, A.; Labrie, F. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 885–891. (b) Bronzert, D.; Silverman, S.; Lippman, M. *Cancer Res.* **1987**, *47*, 1234–1238. (c) Sheen, Y. Y.; Katzenellenbogen, B. S. *Endocrinology* **1987**, *120*, 1140–1151. (d) Klein-Hitpass, L.; Schorpp, M.; Wagner, U.; Ryffel, G. U. *Cell* **1986**, *46*, 1053–1061. (e) Fujii, D. K.; Lee, E. *Endocrinology* **1987**, *120* (supplement), page 127 (abstract 425). (f) Welshons, W. V.; Jordan, V. C. *Eur. J. Cancer Clin. Oncol.*, in press. (g) Nelson, J.; Clarke, R.; McFerran, N. V.; Murphy, R. F. *Biochem. Society Trans.* **1987**, *15*, 244.

(5) (a) Reese, C. C.; Warshaw, M. L.; Murai, J. T.; Siiteri, P. K. *Proceedings, 34th Annual Meeting, Society for Gynecologic Investigation* 1987, 104 (Abstract 153). (b) Warshaw, M. L.; Murai, J. T.; Reese, C. C.; Siiteri, P. K. *Endocrinology* **1987**, *120* (supplement), 71 (Abstract 201).

(6) Bindal, R. D.; Carlson, K. E.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. *J. Steroid Biochem.*, in press.

(7) Katzenellenbogen, J. A.; Johnson, H. J., Jr.; Myers, H. N. *Biochemistry* **1973**, *12*, 4085–4092.

0022-2623/88/1831-1978$01.50/0

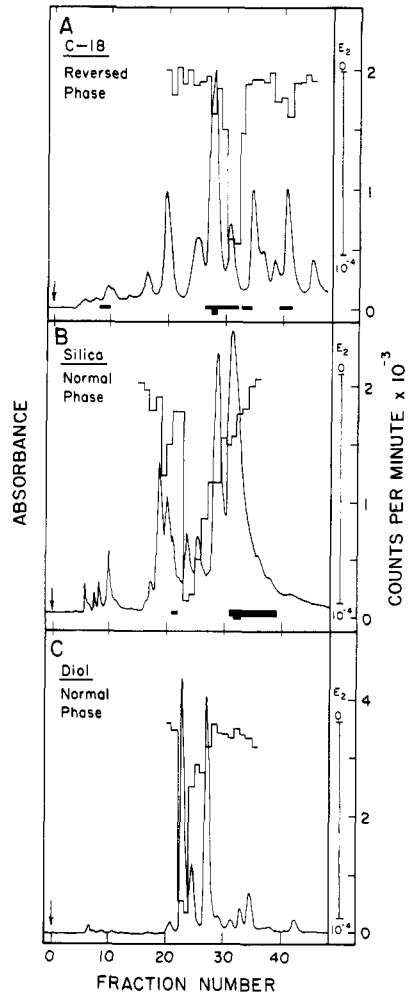


Figure 1. HPLC chromatographic analysis and estrogen receptor binding assay of Phenol Red samples at three stages during purification. Panel A: Reversed-phase analytical HPLC of the ether extract of Phenol Red sodium salt (C-18 silica gel column; 1 mL/min with a 0.1% aqueous trifluoroacetic acid/acetonitrile gradient from 60:40 to 30:70 over 25 min). Panel B: Normal-phase analytical HPLC of a sample of Phenol Red after partial purification by reversed-phase chromatography on a Waters Prep 500 C-18 column (silica gel; 1 mL/min with a hexane/20% 2-propanol in methylene chloride gradient from 90:10 to 50:50 over 20 min). Panel C: Analytical HPLC on a silica gel diol column of a sample of Phenol Red after both reversed-phase and normal-phase purification (silica gel diol; 1 mL/min with a hexane/20% 2-propanol in methylene chloride gradient from 60:40 to 40:60 over 20 min). In each case, the UV absorbance was monitored at 280 nm at 0.08 absorbance unit full scale (smooth curve at bottom). The dark horizontal lines in panels A and B indicate those fractions that have visible chromophores. Fractions, collected every 0.5 min, were lyophilized and redissolved in 60 μL of 1:1 dimethylformamide/buffer (10 mM Tris; 1.5 mM EDTA; 3 mM sodium azide; pH 7.2). For estrogen receptor competitive binding assay,[7] the fractions were analyzed either directly or after a 10-fold dilution (histogram). The vertical bars at the right-hand side indicate the count levels in the absence of competitive binding (0 added estradiol) and in the presence of 10^{-4} M estradiol (10^{-4}). The binding assay[7] is described more fully in the legend to Figure 4A.

receptor-binding fractions is due to the tail of this peak.

The initial reversed-phase LC purification of the 6 g of this ether extract was performed on a Waters Prep 500 system equipped with one C-18 cartridge, eluting with a gradient of acetonitrile and 0.1% aqueous trifluoroacetic acid (40:60 to 70:30). The effluent was monitored by UV absorbance, and fractions corresponding to the center of the chromatogram were collected and pooled. This material was concentrated and reinjected, with additional peak shaving to give a final recovery of 240 mg of a pale yellow oil.

Normal-phase HPLC analysis of this fraction on an analytical silica gel column is shown in Figure 1B. The receptor-competing material is found in fractions 24 and 25, again corresponding to a minor UV-absorbing peak. In this normal-phase system, the major colored impurities now elute after this peak (fractions 28–36).

Portions of this material were repeatedly injected onto a similar semipreparative silica gel column, and the material that corresponded to fractions 24 and 25 was collected and pooled to give 36 mg of a colorless oil. This material was analyzed by HPLC on a silica gel diol column under normal phase conditions (Figure 1C). The receptor-competing substance now appears to correspond to a single major peak in the chromatogram (fractions 23 and 24). A final purification on this column gave 5 mg of a colorless oil that appeared to be homogeneous upon reinjection on this column.

Structure Elucidation. Electron-impact (EI, 70 eV) and field-desorption (17 μA) mass spectrometry of the purified substance gave an intense molecular ion at m/z 432 (EI 432.101 977 0, calcd 432.102 064 0), indicating a composition of $C_{25}H_{21}O_5S$. Scanning high-resolution mass spectrometry (EI, 70 eV) and B/E linked scan of daughter ions from the first field-free region collision cell (Figure 2) showed losses of phenoxy radical (m/z 339), phenoxysulfonyl (m/z 274) and (phenoxysulfonyl)phenyl (m/z 199) groups. The last species (EI 199.074 203 5, calcd 199.074 373 5) indicated a composition of $C_{13}H_{11}O_2$, which could correspond to bis(hydroxyphenyl)methane cation and suggested a link with the (phenoxysulfonyl)phenyl fragment to give the molecular ion peak.

The 500-MHz J-correlated 2D ^1H NMR spectrum (inset Figure 3) established the presence of two 1,4-disubstituted aromatic four-spin systems (two doublets at δ 7.00 and 6.78, J = 8.5 Hz, four protons each). One 1,2-disubstituted aromatic four-spin system is indicated by the most downfield doublet at δ 7.86 (H_h, ortho to the sulfonate), ortho coupled (8.1 Hz) to the triplet centered at δ 7.44 (H_g). The doublet at δ 7.52 is assigned to the proton H_e meta to the sulfonate, which is coupled (J = 8 Hz) to the triplet at δ 7.74 (H_f); this latter triplet is also coupled to the triplet at δ 7.44 (H_g). The presence of five protons as two coupled sets of multiplets at δ 6.73 (2 protons) and δ 7.25 (3 protons) is consistent with the phenyl ester of the sulfonic acid.

The ^1H–^{13}C chemical shift correlation spectrum (Figure 3) showed the presence of a methine (δ (^{13}C) 50.4; (^1H) 6.64), indicating the point of attachment of the three four-spin systems. The signals for the five quarternary carbons are assigned following literature precedent,[8] the hydroxy-substituted ^{13}C being the most downfield at δ 157, followed by the ester carbon of phenyl sulfonate at δ 150.3. The carbon bearing the sulfonyl group is assigned at δ 146, followed by aryl carbons attached to the trityl carbon, at δ 131.3 (phenyl bearing ortho sulfonyl function) and δ 130.5 (phenyl bearing 4-hydroxyphenyl groups).

The corroboration of NMR spectroscopy and mass spectrometry led us to assign the structure of the estrogen

(8) (a) Pretsch, E.; Seibl, J.; Simon, W.; Clerc, T. In *Tables of Spectral Data for Structure Determination of Organic Compounds*; Boschke, F. L., Fresenius, W., Huber, J. F. K., Pungor, E., Rechnitz, G. A., Simon, W., West, T. S., Eds.; Springer-Verlag: Berlin, 1983; pp C120, C125. (b) Levy, G. C.; Lichter, R. L.; Nelson, G. L. In *Carbon-13 Nuclear Magnetic Resonance Spectroscopy*; Wiley-Interscience: New York, 1980; p 110.

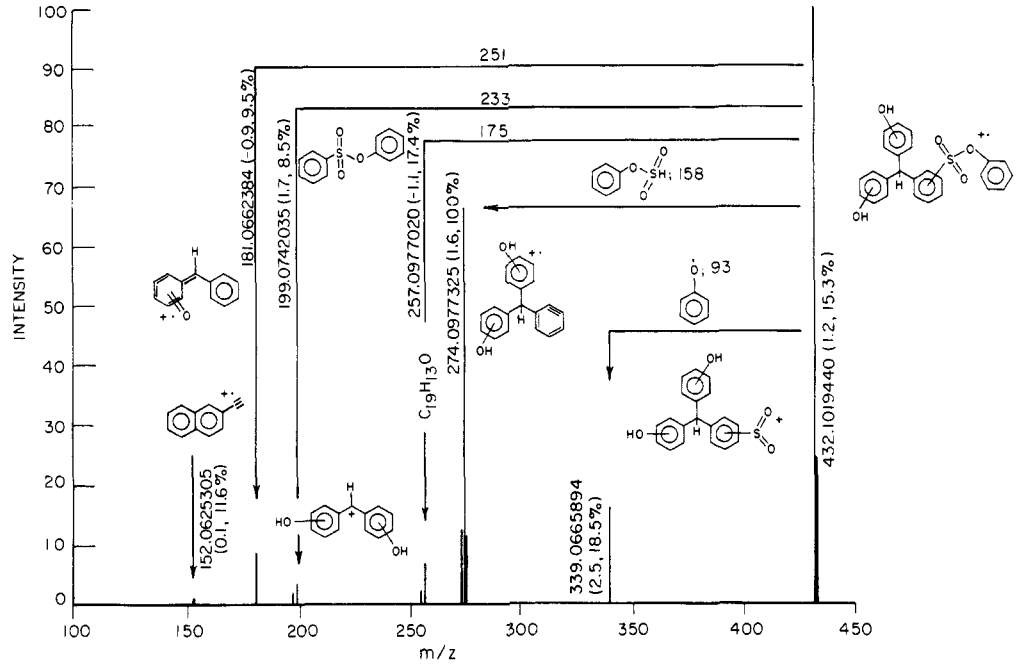
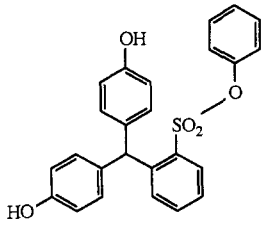


Figure 2. Scanning high-resolution mass spectroscopy (EI, 70 eV) and collision-activated daughter ion analysis of the molecular ion (m/z 432) of the bisphenolic phenyl sulfonate **1** on a VG ZAB-SE instrument. For each ion with intensity greater than 2% of the base peak (432) is listed the observe exact mass and in parentheses the deviation (in millimass units) from the calculated exact mass and the intensity as a percent of the base peak.

binding species to be bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane (**1**). The final proof of the structure came from the synthesis of **1**.



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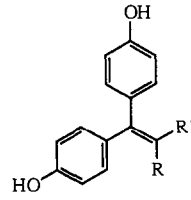
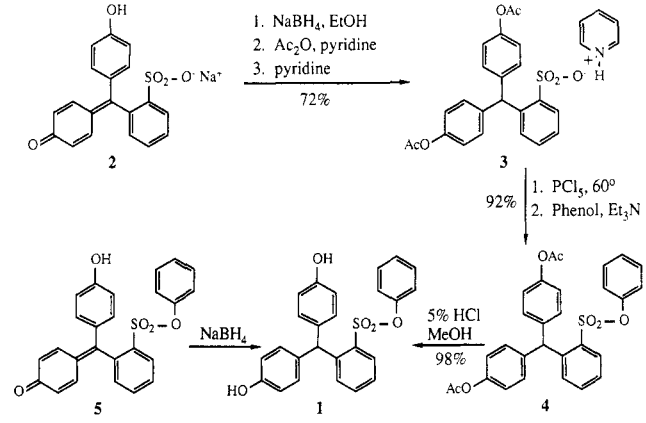


6 (R,R' = -$(CH_2)_5$-) (R = Et, R' = Ph)

Synthesis. The quinomethide system of Phenol Red monosodium salt (**2**, Scheme I) could be reduced with $NaBH_4$. Extraction of the reduced material was facilitated by the formation of the diacetate, which was converted to pyridinium salt **3** without further purification. The pyridinium sulfonate **3** was converted to the sulfonyl chloride with phosphorus pentachloride.[9] Treatment with phenol in the presence of triethylamine gave the sulfonate ester **4**. Selective hydrolysis of the acetates was achieved in methanol containing 5% hydrochloric acid. The synthetic material (**1**) was found to be identical with the material isolated from Phenol Red by ^1H NMR, mass spectrometry, and HPLC comparisons (silica gel diol column, as run in Figure 1C).

Determination of the Estrogen Receptor Binding Affinity, Cell Proliferation, and Progesterone Receptor Stimulating Activity and Uterotropic Activity of the Bis(phenol) Phenyl Sulfonate 1. The estrogenic activity of the synthetic preparation of the bisphenolic phenyl sulfonate **1** was evaluated in vitro by competitive receptor binding and cell proliferation assays and in vivo by uterine growth stimulation assays. As shown in the

Scheme I



cell-free, cytosolic estrogen receptor competitive binding assay (Figure 4A),[7] the bisphenolic phenyl sulfonate **1** binds to the rat uterine estrogen receptor with an affinity 50% that of estradiol.

Estrogens are known to stimulate the proliferation of certain estrogen receptor containing human breast cancer cell lines.[10] A quantitative MCF-7 cell proliferation assay,[11] shown in Figure 4B, indicates that the bisphenolic phenyl sulfonate is as effective as estradiol in achieving maximal stimulation. Its potency in this assay, however, is about 500-fold lower than estradiol, which is considerably

(9) Barco, A.; Benetti, S.; Pollini, G. P.; Taddia, R. *Synthesis* **1974**, 877–878.

(10) Sato, G. H.; Ross, R. *Hormones and Cell Culture*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1979; Books A and B. (b) Sirbasku, D. A.; Leland, F. E. In *Biochemical Actions of Hormones*; Litwack, G., Ed.; Academic: New York, 1982; Vol. 9, pp 115–140. (c) Katzenellenbogen, B. S.; Miller, M. A.; Mullick, A.; Sheen, Y. Y. *Breast Cancer Res. Treat.* **1985**, *5*, 231–245. (d) Aitken, S. C.; Lippman, M. E. *Cancer Res.* **1985**, *45*, 1611–1620.

(11) (a) Katzenellenbogen, B. S.; Norman, M. J.; Eckert, R. L.; Peltz, S. W.; Mangel, W. F. *Cancer Res.* **1984**, *44*, 112–119. (b) Eckert, R. L.; Katzenellenbogen, B. S. *Cancer Res.* **1982**, *42*, 139–144.

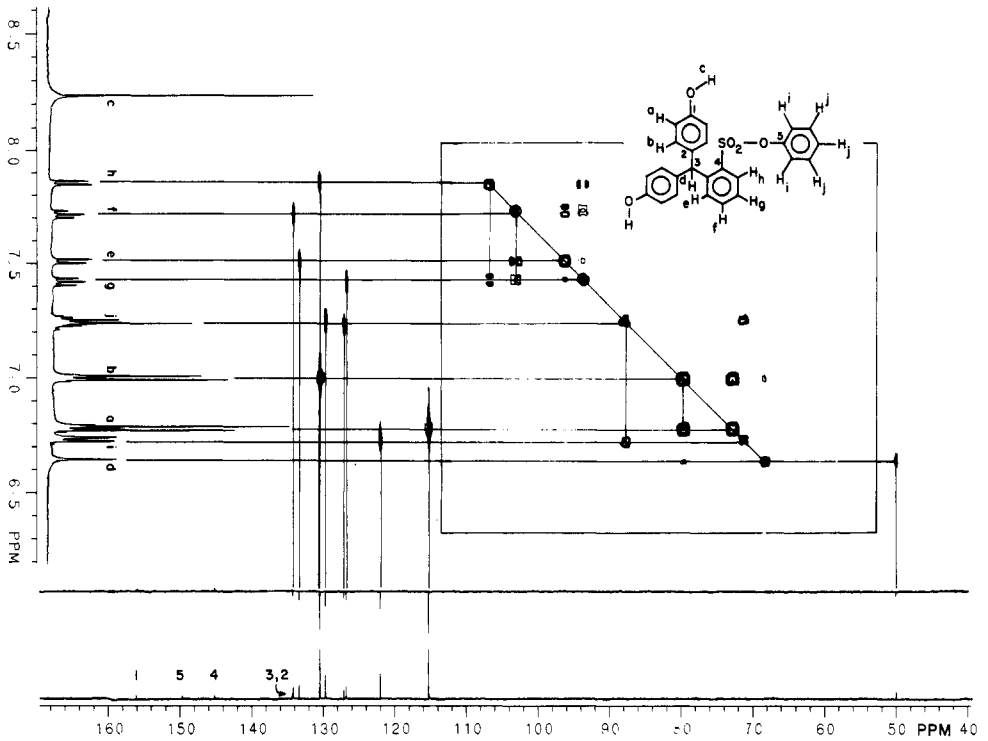


Figure 3. 500-MHz *J*-correlated 2D ^1H NMR (inset) and ^1H–^{13}C NMR chemical shift correlation spectrum of the bisphenolic phenyl sulfonate 1 in $CDCl_3$.

Table I. Induction of Progesterone Receptors by Estradiol and the Bisphenolic Phenyl Sulfonate in MCF-7 Cells[a]

	progesterone receptor levels, fmol/10^6 cells [% of control]	
concentration, M	E_2	bisphenolic phenyl sulfonate
0 (control)	5 [100]	5 [100]
10^{-11}	14 [280]	6 [120]
10^{-10}	70 [1400]	4 [80]
10^{-9}	76 [1520]	21 [420]
10^{-8}	81 [1620]	62 [1240]
10^{-7}	80 [1600]	83 [1660]
10^{-6}		84 [1680]

[a] MCF-7 cells, grown in the absence of Phenol Red for 1 week, were exposed to the indicated concentrations of estradiol or bisphenolic phenyl sulfonate for 5 days in media containing 5% charcoal dextran-treated calf serum. Fresh medium and compounds were added daily during this 5-day period. Cells were then harvested and assayed for progesterone receptor by a whole cell assay,[3b] utilizing 10 nM [^3H] R5020 in the absence and presence of a 100-fold excess of radioinert R5020. Values given are the mean of duplicate assays that are generally reproducible within 15%.

less than that expected on the basis of its receptor binding affinity. This difference may be accounted for by higher levels of nonspecific binding to serum or cell constituents or by as yet uncharacterized metabolic conversions.

The activity of estrogens in this same cell line can also be assessed by the stimulation of cellular progesterone receptor levels.[3b] Progesterone receptor levels are normally low in control cells grown in the absence of estrogens (Table I). The activity of the bisphenolic phenyl sulfonate is shown in Table I. Full induction of progesterone receptor levels (1500% of control, as with 10^{-9} M estradiol) is reached with 10^{-7} and 10^{-6} M of the bisphenolic phenyl sulfonate. The potency of the bisphenolic phenyl sulfonate in this assay (namely, ca. 0.1% that of estradiol), is similar to that observed in the cell proliferation assay.

A classic test for estrogenic activity in vivo is the uterotropic or uterine weight gain assay. As shown in Figure 4C, the bisphenolic phenyl sulfonate 1 is capable of full uterine weight gain stimulation. Its potency, however, is again much lower than estradiol (ca. 0.1% relative to estradiol). The reduced potency of compound 1 in this assay suggests that its metabolism or clearance is very rapid relative to estradiol. Estrogens that have a short duration of action are known to have a lower uterotropic potency than expected from their receptor binding affinity.[12]

The Formation of the Bisphenolic Phenyl Sulfonate Species 1 During the Synthesis of Phenol Red. Phenolsulfonphthalein is synthesized by heating *o*-sulfobenzoic acid with excess phenol at elevated temperatures.[13] The reaction can be accelerated by the addition of Lewis Acid catalysts, such as zinc chloride. However, under both conditions, a number of byproducts including phenyl sulfonate esters are generated.[12] Whether these phenyl sulfonates are true intermediates on the way to phenolsulfonphthalein or just in equilibrium with normal intermediates is not known.

The state of reduction of the bisphenolic methane 1 is also curious. The sulfobenzoic acid used in commercial syntheses might contain a trace of the corresponding aldehyde, which would give directly the appropriate level of reduction for 1. Alternatively, it is conceivable that at elevated temperatures, phenol might couple, generating a reducing species that could convert the quinone methide to a diarylmethane.

In this regard, we have isolated from the ether extract of Phenol Red, in addition to the bisphenolic phenyl sulfonate 1, the corresponding oxidized quinone methide, compound 5. This material appears to constitute around 20% of the ether extract of Phenol Red, and on C-18 reversed phase HPLC it elutes just ahead of the bis(phe-

(12) (a) Martin, L. *Steroids* **1969**, *13*, 1–10. (b) Terenius, L. *Acta Endocrinol. (Kbh.)* **1971**, *66*, 431–447. (c) Anderson, J. N.; Peck, E. J., Jr.; Clark, J. H. *Endocrinology* **1975**, *96*, 160–166. (d) Lan, N. C.; Katzenellenbogen, B. S. *Endocrinology* **1976**, *98*, 220–227.

(13) Orndorff, W. R.; Sherwood, F. W. *J. Am. Chem. Soc.* **1923**, *45*, 486–500.

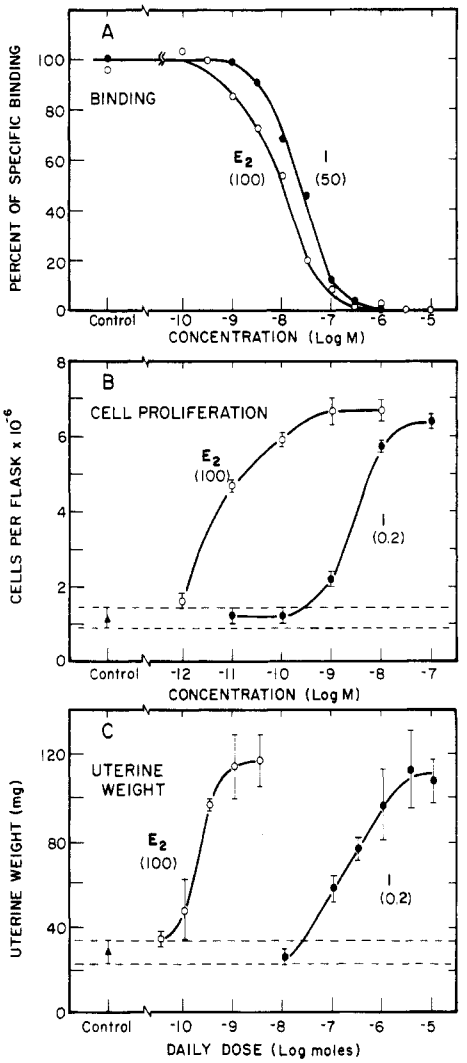


Figure 4. Characterization of the estrogenic activity of the bisphenolic phenyl sulfonate 1. Panel A: Competitive radiometric receptor binding assay. This assay was performed, as previously described.[7] A fixed concentration of [^{3}H]estradiol (10^{-8} M) and increasing concentrations (10^{-10}–10^{-5} M) of estradiol (○) or the sulfonate 1 (●) were incubated for 18 h at 0–4 °C with a cytosol preparation from immature rat uterus. Free ligand was adsorbed with dextran-treated charcoal and removed by centrifugation, and receptor-bound activity was determined by liquid scintillation counting. Points are the average of replicate determinations. The binding affinity (in parentheses) is expressed relative to that of estradiol (100). Panel B: Cell proliferation assay. This assay was performed as previously described.[11] Human breast cancer MCF-7 cells were grown in T-25 flasks in the presence of Phenol Red free improved minimal essential medium supplemented with 5% dextran-coated charcoal treated calf serum either alone (control) or in the presence of the indicated concentrations of estradiol (○) or sulfonate 1 (●). Media were changed every other day. On day 9, triplicate flasks of cells were counted; values represent the mean and stand and deviation of the three cell numbers for each group. Panel C: Assay of uterotropic activity. Immature female rats (20-day) were injected sc with the indicated dose of estradiol (○) or sulfonate 1 (●) in 0.5 mL of 4% ethanol in sunflower seed oil daily for three days. Twenty-four hours after the last injection, the animals were sacrificed and their uteri were excised and weighed. The assay was performed in quadruplicate, and the values represent the mean ± SD.

nol) 1, corresponding to the large yellow-colored peak in fractions 27–29 in Figure 1A. The identity of this compound is evident from its spectroscopic properties and from the fact that it is readily reduced to the bis(phenol) 1 with sodium borohydride.

2D J-correlated ^{1}H NMR spectroscopy of this substance showed the most downfield doublet at δ 8.01 (J = 7.9 Hz) ortho to the sulfonate, coupled to a triplet at δ 7.64 due to the meta proton. The other proton meta to the sulfonate appears as a doublet at δ 7.40 (J = 7.6 Hz), coupled to the proton para to the sulfonate, which appears as a triplet at δ 7.79, also coupled to the triplet at δ 7.64. The doublet at δ 6.65 for four protons are assigned to protons ortho to OH or α to the keto groups of the quinone methide system. The two-proton multiplet that appears at δ 6.78 is assigned to the two protons ortho to the phenyl of the sulfonate ester. The multiplet centered at δ 7.22 accounts for the other three protons of the phenyl sulfonate ester and the other four protons of the 1,4-disubstituted rings. The absence of a methine signal in ^{1}H NMR and ^{13}C NMR spectra and the yellow color of the sample are consistent with the quinone methide structure (5).

Conclusion

The estrogenic component in commercial preparations of Phenol Red has been isolated by solvent extraction and extensive chromatography and has been identified spectroscopically as bis(4-hydroxyphenyl)[(2-(phenoxysulfonyl)phenyl)]methane. It shows binding affinity for the estrogen receptor and is a stimulator of the proliferation and progesterone receptor content of estrogen responsive cells in culture and uterine weight gain in immature rats.

The binding affinity of the bisphenolic sulfonate for the estrogen receptor is remarkably high, 50% that of estradiol. While it is not apparent just in what way the sulfonate might resemble a steroidal ligand such as estradiol in terms of its orientation within the receptor binding site, it does bear structural relationship to other nonsteroidal estrogens,[14] particularly those of the 1,1-bis(4-hydroxyphenyl)ethylene class 6.[15,16] A large number of members of this class are known, and in general, their receptor binding affinity is increased by relatively bulky substituents (R,R′) at the ethylene 2,2-positions. However, despite this overall similarity with the 1,1-diarylethylenes, the bisphenolic sulfonate 1 is, in fact, a triaryl methane and has a different overall shape and conformational flexibility. So, further structural comparisons must await more extensive structure-binding/affinity studies and conformational modeling and analysis. Such studies are under way.

Experimental Section

General Procedures. Proton magnetic resonance spectra were recorded on a General Electric GN-500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR analyses). The spectra were recorded with an internal lock on the deuterium resonance of the solvent, with tetramethylsilane as the internal standard. Data are reported on the δ scale (downfield from TMS). Electron-impact (EI) mass spectrometry was obtained on a Finnigan 311A spectrometer; scanning, high-resolution (EI, 70 eV), and LINK scan of the molecular ion was done on a VG ZAB-SE instrument. Field-desorption mass spectroscopy was done on a Finnigan 731 instrument. High-performance liquid chromatography (reversed-phased water/acetonitrile, C-18 column; normal-phase hexane/methylene chloride/2-propanol, silica and diol columns) was performed on Spectra Physics ternary gradient liquid chromatograph (SP8700) with a Beckman UV detector (Model 153) at 280 nm. Reversed-phase preparative liquid chromatography

(14) Robertson, D. W.; Katzenellenbogen, J. A.; Long, D. J.; Rorke, E. A.; Katzenellenbogen, B. S. *Biochemistry* **1982**, *16*, 1–13.
(15) Raynaud, J.-P.; Ojasoo, T. In *Steroid Hormone Receptors: Structure and Function*; Eriksson, H., Gustafsson, J.-A., Eds.; Elsevier: Amsterdam, 1983; pp 141–170.
(16) Garg, S.; Bindal, R. D.; Durani, S.; Kapil, R. S. *J. Steroid Biochem.* **1983**, *18*, 89–95.

was done on Waters 500A instrument equipped with Preparative Gradient Generator, with a C-18 cartridge and Beckman UV detector (Model 153). A gradient of water/acetonitrile was used at a flow rate of 200 mL/min. Melting points were determined on a Thomas-Hoover capillary melting point apparatus, and temperatures reported are uncorrected. Elemental analyses were provided by the Microanalytical Laboratory of the University of Illinois.

Materials. Phenol Red monosodium salt (Lot No. 25F-36536) was obtained from Sigma, St. Louis, MO. Phosphorus pentachloride, triethylamine (Aldrich), reagent acetic anhydride (T. J. Baker), and pyridine (Fisher Scientific) were used without further purification. Reagent grade diethyl ether and analytical phenol were from Mallinckrodt. Sodium borohydride was an Alfa Product. Glass-distilled methylene chloride and hexane were dried over P_2O_5 prior to use.

The reversed-phase analytical column (0.46 × 30 cm, 10 μm C-18 silica gel) and the analytical normal-phase column (0.46 × 30 cm, 5 μm silica gel) were from Varian Associates; the preparative C-18 cartridge was from Waters Associates; the normal-phase semipreparative column (0.9 × 50 cm, 10 μm silica gel) was from Whatman; and the analytical diol column (0.46 × 25 cm, 10 μm diol silica gel) was from Alltech. Acetonitrile (American Burdick & Jackson) and water were filtered (Millipore 0.22 μm, (GS) and 0.2 μm (FG)) and degassed (He) prior to use in HPLC.

Pyridinium 2-[Bis(4-acetoxyphenyl)methyl]benzenesulfonate (3). A solution of phenolsulfonphthalein sodium salt (2) (1.88 g, 5 mmol) in 100 mL of 90% ethanol was stirred with sodium borohydride (567 mg, 15 mmol) at 25 °C for 48 h. Ten milliliters of 5% hydrochloric acid was then added dropwise, and the reaction mixture was lyophilized. The dried powder was dissolved in 50 mL of acetic anhydride and 10 mL of pyridine and stirred for 4 h at room temperature. Then, the reaction mixture was poured into a cold saturated solution of sodium bicarbonate with vigorous stirring. To this aqueous solution was added 300 mL of ethyl acetate, the pH of the aqueous layer was brought to 2 by the dropwise addition of 10% HCl, and the organic phase was separated. Two more extractions of the aqueous phase (100 mL each) were combined to the organic phase, washed (2 × 100 mL) with water, and then dried over anhydrous sodium sulfate. Solvent removal under reduced pressure furnished 1.76 g of an oil, which was dissolved in CH_2Cl_2 (100 mL) and stirred with 10 mL of pyridine. After 1 h the solution was diluted with hexane (50 mL), and the white precipitate that formed was collected by filtration on a sintered-glass funnel and washed with 1:1 hexane/CH_2Cl_2 to furnish 1.87 g (72%) of the pyridinium salt 3 (mp 213–215 °C): NMR (500 MHz, CD_3OD) δ 2.25 (s, 6 H, 2 $COCH_3$), 6.9 (s, 1 H, Ar_3CH), 6.96 (d, J = 8.6 Hz, 4H, 3,5-H of 2 4-$AcOC_6H_4$), 7.19 (d, J = 8.5 Hz, 4 H, 2,6-H of 2 4-$AcOC_6H_4$), 7.23 (d, J = 7.8 Hz, 1 H, 6-H of 2-SO_3^--C_6H_4), 7.26 (dd, J = 7.7 Hz, 1 H, 4-H of 2-SO_3^--C_6H_4), 7.36 (dd, J = 8.07, 6.51 Hz, 1 H, 5-H of 2-SO_3^--C_6H_4), 8.01 (dd, 2 H, 3,5-H of pyridinium), 8.03 (d, J = 7.7 Hz, 1 H, 3-H of 2-SO_3^--C_6H_4), 8.56 (dd, 1 H, 4-pyridinium), 8.75 (d, J = 5.6 Hz, 2 H, 2,6-H of pyridinium). Anal. ($C_{28}H_{25}NO_7S$) C, H, N, S.

Bis(4-acetoxyphenyl)[2-(phenoxysulfonyl)phenyl]methane (4). A solution of 3 (104 mg, 0.2 mmol) in 5 mL of anhydrous methylene chloride was refluxed with PCl_5 (42 mg, 0.2 mmol) for 1 h. The solvent was removed under reduced pressure, and the oil obtained was heated to 60–65 °C for 3 h at 0.1 Torr. The oil was redissolved in 5 mL of anhydrous methylene chloride, and phenol (18.8 mg, 0.2 mmol) and triethylamine (20.2 mg, 0.2 mmol) were added. After being stirred at 25 °C for 12 h, the reaction mixture was diluted with water (5 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic phase was washed with water (2 × 20 mL) and dried over anhydrous Na_2SO_4. Solvent removal under reduced pressure furnished the phenyl sulfonate diacetate 4 as a colorless oil (95 mg, 92%). Flash column chromatography[17] (silica, 5% ethyl acetate/benzene, R_f 0.23) furnished a colorless oil, which solidified when dried under high vacuum (mp 88–90 °C): NMR (500 MHz, $(CD_3)_2CO$) δ 2.24 (s, 6 H, 2 $COCH_3$), 6.74 (d, J = 7.6 Hz, 2 H, 2,6-H of phenoxy), 6.83 (s, 1 H, Ph_3CH), 7.10 (d, J = 8.5 Hz, 4 H, 3,5-H of 2 4-$AcOC_6H_4$), 7.22 (d, J = 8.5 Hz, 4 H, 2,6-H of 2 4-$AcOC_6H_4$), 7.27 (m, 3 H, 3,4,5-H of phenoxy), 7.51 (t, J = 7.1 Hz, 1 H, 4-H of 2-SO_3^--C_6H_4), 7.55 (d, J = 8.0 Hz, 1 H, 6-H of 2-SO_3^--C_6H_4), 7.79 (t, J = 7.8 Hz, 1 H, 5-H of 2-SO_3^--C_6H_4), 7.93 (d, J = 7.0 Hz, 1 H, 3-H of 2-SO_3^--C_6H_4). Anal. ($C_{29}H_{24}O_7S$) C, H, S.

Bis(4-hydroxyphenyl)[(2-phenoxysulfonyl)phenyl]methane (1). A solution of phenyl sulfonate diacetate (4; 52 mg, 0.1 mmol) in 2 mL of methanol containing 0.1 mL (5%) of concentrated HCl was stirred at room temperature for 20 h. Then solid sodium bicarbonate was added to bring the pH to neutrality, and the solvent was removed under reduced pressure. The colorless oil was chromatographed over silica gel (15% ethyl acetate/benzene, R_f 0.21) to furnish 1 (40 mg, 93%) as a colorless oil, which solidified to a white solid on drying under high vacuum. Anal. ($C_{25}H_{20}O_5S$) C, H, S. The mass spectrum and ^1H NMR and ^{13}C NMR spectra for this material was same as described in the text for the isolated 1.

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(17) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.