The Effect of Exchanging Various Substituents at the 2-Position of 2-Methoxyestradiol on Cytotoxicity in Human Cancer Cell Cultures and Inhibition of Tubulin Polymerization

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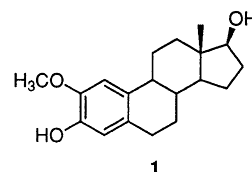
A new set of estradiol derivatives bearing various substituents at the 2-position were synthesized in order to further elucidate the structural parameters associated with the antitubulin activity and cytotoxicity of 2-substituted estradiols. The potencies of the new compounds as inhibitors of tubulin polymerization were determined, and the cytotoxicities of the analogues in human cancer cell cultures were investigated. The substituents introduced into the 2-position of estradiol included *E*-3′-hydroxy-1′-propenyl, 2′-hydroxyethoxy, 3-*N*,*N*-dimethylaminoethylideneamino, 2′-hydroxyethylineneamino, (*β*-3,4,5-trimethoxyphenyl)ethenyl, phenylethynyl, ethynly, 1′-propynyl, and cyano. The substituents conferring the ability to inhibit tubulin polymerization included *E*-3′-hydroxy-1′-propenyl, 2′-hydroxyethoxy, ethynyl, and 1′-propynyl. The remaining compounds were all inactive as inhibitors of tubulin polymerization when tested at concentrations of up to 40 μM. All of the compounds were cytotoxic in a panel of 55 human cancer cell cultures, and in general, the most cytotoxic compounds were also the most potent as inhibitors of tubulin polymerization. 2-(1′-Propynyl)estradiol displayed significant anticancer activity in the in vivo hollow fiber animal model.

Introduction

Inhibition of tumor growth,[1–4] metastasis,[3] and angiogenesis[1,2,5–11] in animal models in vivo by the endogenous human metabolite 2-methoxyestradiol (**1**) has stimulated a great deal of interest in its potential clinical utility as an anticancer agent.[11,12] 2-Methoxyestradiol (**1**) has very low affinity for the estrogen receptor[13] and is formed in mammals by hepatic hydroxylation of estradiol followed by 2-*O*-methylation.[14,15] The cytotoxic effects of 2-methoxyestradiol (**1**) in cancer cell cultures are associated with inhibition of DNA synthesis and mitosis, uneven chromosome distribution, faulty spindle formation, and an increase in the number of abnormal metaphases.[16,17] The available evidence suggests that these effects may result from inhibition of tubulin polymerization by 2-methoxyestradiol (**1**), which binds to the colchicine binding site.[18] However, a complete and correct picture of the mechanism of action of 2-methoxyestradiol (**1**) remains to be established, and a variety of other possibilities have also been suggested, including those involving p53,[19–24] nitric oxide synthase,[8] stress-activated protein kinase,[7] p34cdc2,[25] proliferating cell nuclear antigen (PCNA),[25] and DR5.[11,12] The low bioavailability of 2-methoxyestradiol (**1**) following oral administration in mice[26] has resulted in interest in the design and synthesis of analogues that might be more potent and have enhanced metabolic stability.[13,27–31] The present study was undertaken in order to further delineate the structure–activity relationships of 2-methoxyestradiol (**1**) and to maximize the potential clinical utility of the compounds in this series. More specifically, the effects of structural modification at the 2-position of 2-methoxyestradiol (**1**) on pharmacological activity have been investigated. The new compounds were tested as inhibitors of tubulin polymerization and for cytotoxic activity in human cancer cell cultures.



Chemistry

Treatment of 2-formylestradiol (**2**)[13] with excess *tert*-butyldimethylsilyl chloride and imidazole resulted in protection of both hydroxyl groups to afford the protected intermediate **3**, which reacted with (triphenylphosphoranylidene)acetaldehyde[32] to yield the *E*-3′-acrolenyl derivative **4** (Scheme 1). The trans geometry assigned to the double bond is consistent with the 18 Hz coupling constant observed for the olefinic protons in the ^{1}H NMR spectrum. Reduction of the aldehyde group of **4** with diisobutylaluminum hydride in toluene, followed by removal of the two *tert*-butyldimethylsilyl protecting groups, afforded the desired triol **5**.

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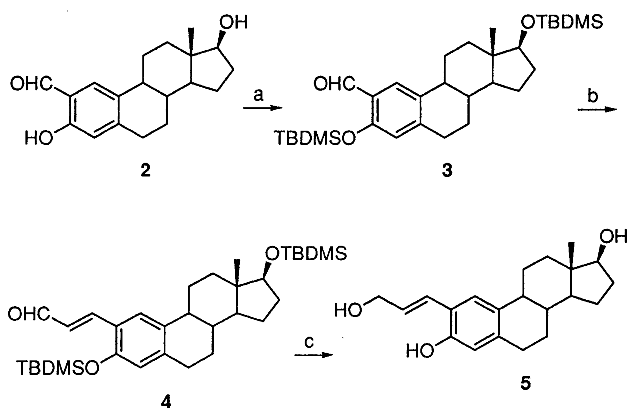
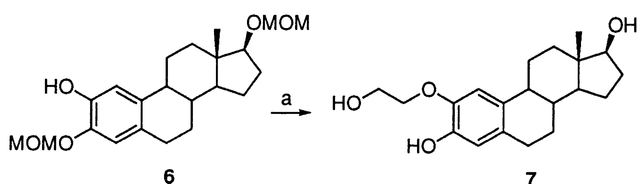
Scheme 1[a]



[a] Reagents and conditions: (a) TBDMSCl, imidazole, DMF, THF, 23 °C (12 h). (b) Ph_3P=CHCHO, THF, reflux (10 h). (c) (1) DIBAL, toluene, −40 °C (1 h); (2) TBAF, THF (12 h).

Scheme 2[a]



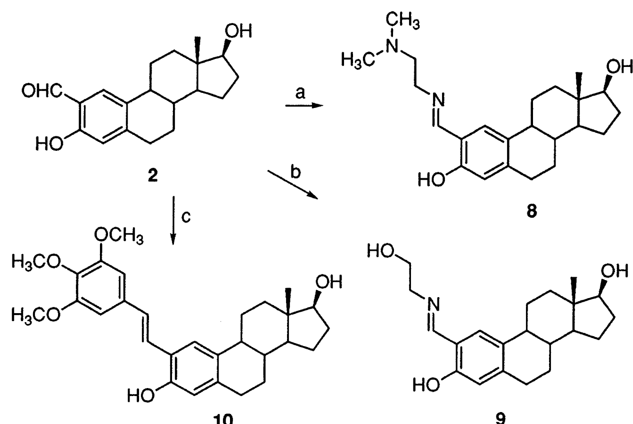
[a] Reagents and conditions: (a) (1) $BrCH_2COOEt$, K_2CO_3, CH_3CN, reflux (4 h); (2) LAH, THF, 23 °C (3 h); (3) 6 M HCl (10 h).

The effect of replacing the alkenyl moiety of **5** with an ether was investigated by synthesis of the estradiol derivative **7** (Scheme 2). Deprotonation of the bis(methoxymethyl) ether **6**[33] with potassium carbonate in acetonitrile, followed by nucleophilic displacement of the bromide from ethyl bromoacetate, afforded the expected ester derivative. Reduction of the ester moiety with lithium aluminum hydride in tetrahydrofuran (THF) and removal of the protecting groups in the presence of 6 M hydrochloric acid gave the target compound **7**.

Previously published work had demonstrated that a variety of substituted 2-vinylestradiols are cytotoxic in cancer cell cultures and also exhibit good activity as tubulin polymerization inhibitors.[13] The availability of the aldehyde **2** provided ready access to the corresponding imine derivatives, and the Schiff bases **8** and **9** were therefore synthesized by reaction of **2** with the corresponding primary amines (Scheme 3). The substituted trans stilbene-estradiol analogue **10** was also prepared easily from **2** using a Wittig reaction. The assignment of the trans stereochemistry to **10** is consistent with the 15 Hz coupling constant observed between the two vinyl protons in the ^{1}H NMR spectrum of **10**, and it is also in agreement with our prior observation that Wittig reactions involving ortho-substituted aromatic aldehydes give predominantly the trans alkene products.[34]

Both 2-methoxyestradiol (**1**) and 2-propynylestradiol (**19**)[27] (Scheme 4) have shown antitumor activity in an animal model for human neuroblastoma after oral administration.[4] Methods were therefore sought for improving the synthesis of **19** and for the synthesis of additional substituted alkyne derivatives. The Sonogashira coupling procedure of aryl halides with alkynes in the presence of amines and a catalytic amount of Pd(II) complex and CuI has been used extensively for the synthesis of aryl alkynes.[35−37] However, the Sonogashira

Scheme 3[a]



[a] Reagents and conditions: (a) $Me_2NCH_2CH_2NH_2$, reflux (6 h). (b) $HOCH_2CH_2NH_2$, 100 °C (6 h). (c) 3,4,5-Trimethoxybenzyl triphenylphosphonium chloride, LHMDS, THF, 0−23 °C (8 h).

coupling of the 2-iodoestradiol derivative **14** with propyne afforded only a 12% yield of the desired product **17**. An alternative procedure was therefore employed in which **14** was reacted with tetrakis(triphenylphosphine)palladium and 1-propynylzinc bromide (Negishi coupling), which afforded the alkyne **17** in 82% yield.[38] Deprotection of intermediate **17** with tetra-*n*-butylammonium fluoride provided 2-(1′-propynyl)estradiol (**19**). 2-Ethynylestradiol (**18**) was also prepared using this procedure. On the other hand, a Stille coupling of 2-iodoestradiol (**13**) with tri-*n*-butylphenylethynylstannane in the presence of tetrakis(triphenylphosphine)-palladium directly provided 2-phenylethynylestradiol (**15**) in 79% yield. In the present system, the Stille coupling has the advantage that it can be carried out effectively in good yield without installation and removal of any protecting groups.

Two syntheses of 2-cyanoestradiol (**21**) are outlined in Scheme 5. The first synthesis relies heavily on employment of 1-cyanobenzotriazole as a source of cyano cation (CN^+) equivalent.[39] Treatment of the bis(methoxymethyl (MOM)) ether **11**[40] of estradiol with *sec*-butyllithium in THF afforded the deprotonated, 2-lithio species, which was cyanated with 1-cyanobenzotriazole to yield the protected 2-cyanoestradiol intermediate **20**. The two MOM protecting groups were then removed under acidic conditions to provide 2-cyanoestradiol (**21**). Alternatively, the bis(MOM) ether **22**[40] of 2-formylestradiol was converted to the oxime with hydroxylamine hydrochloride in pyridine, and the oxime was then dehydrated with acetic anhydride to yield the desired nitrile **21**. Deprotection with hydrochloric acid provided 2-cyanoestradiol.

Biological Results and Discussion

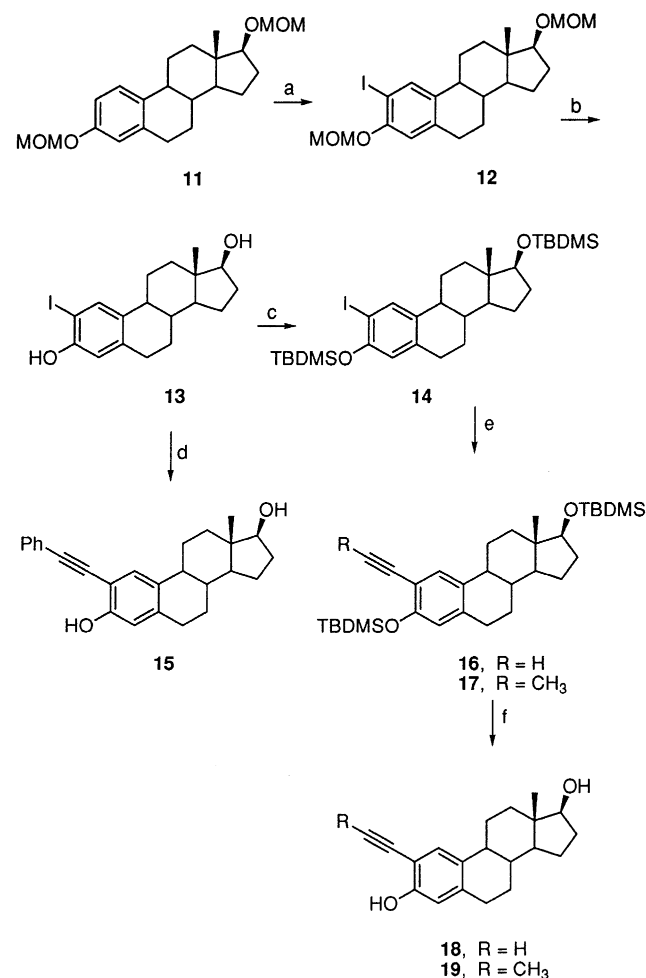
The new estradiol analogues were examined for antiproliferative activity against human cancer cell lines in the NCI screen, in which the activity of each compound was evaluated with approximately 55 different cell lines of diverse tumor origins. In addition, the new agents were examined for inhibitory effects on the polymerization of purified bovine brain tubulin under reaction conditions where 2-methoxyestradiol (**1**) had shown maximal inhibitory effect.[18] The activities against tubulin, the mean graph midpoints (MGMs) for 50%

Table 1. Cytotoxicities and Antitubulin Activities of 2-Methoxyestradiol and Analogues

	cytotoxicity (GI_{50} in μM)[a]									inhibition of tubulin polymerization IC_{50}[c]
no.	lung HOP-62	colon HCT-116	CNS SF-539	melanoma UACC-62	ovarian OVCAR-3	renal SN12C	prostate DU-145	breast MDA-MB-435	MGM[b]	(IC_{50}, μM ± SD)
1	0.70	0.47	0.32	0.36	0.21	0.95	1.8	0.080	1.3	4.7 ± 0.1[d]
5	5.5	0.47	0.25	0.47	5.3	6.2	0.90	0.052	1.1	6.3 ± 0.5
7	18	15	11	13	8.7	19	16	16	12	9.6 ± 0.4
8	19	15	22	17	21	22	22	19	16	>40
9	23	11	13	16	19	18	20	17	16	>40
10	3.6	3.2	2.0	2.7	3.2	2.4	2.8	4.4	3.4	>40
15	14	14	16	12	14	11	16	16	13.5	>40
18	6.2	3.9	2.3	4.8	3.8	6.4	10	2.2	5.5	13 ± 1
19	1.7	0.48	0.95	0.50	0.34	6.9	3.6	0.13	1.7	4.9 ± 0.8
21	56	>100	>100	6.1	>100	48	34	>100	20	>40

[a] The cytotoxicity GI_{50} values are the concentrations corresponding to 50% growth inhibition. [b] MGM for growth inhibition of all human cancer cell lines successfully tested. [c] Minimum of two independent determinations. Tubulin concentration was 1.2 mg/mL (12 μM). [d] Value for **1** obtained contemporaneously with values for the other active compounds.

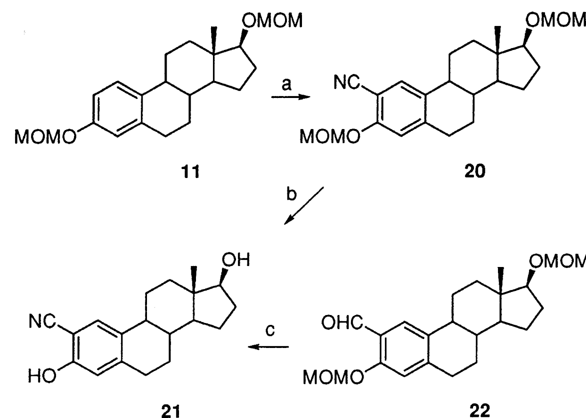
Scheme 4[a]



[a] Reagents and conditions: (a) (1) *sec*-BuLi, THF, −78 °C (2 h); (2) I_2, −78 °C to room temperature (12 h). (b) HCl, THF, 23 °C (10 h). (c) TBDMSCl, DMF, THF, imidazole, 23 °C (17 h). (d) PhC≡CSnBu$_3$, Pd(PPh$_3$)$_4$, THF, 60−65 °C (10 h). (e) (1) HC≡C-MgBr, ZnBr$_2$, THF, 23 °C (10 min); (2) Pd(PPh$_3$)$_4$, compound **14**, 23 °C (6 h). (f) TBAF, THF, 23 °C (10 h).

growth inhibition of all human cancer cell lines successfully tested, and the GI_{50} values obtained with selected cell lines are summarized in Table 1. The MGM is based on a calculation of the average GI_{50} for all of the cell lines tested (approximately 55) in which GI_{50} values below and above the test range (10^{-4} to 10^{-8} M) are taken as the minimum (10^{-8} M) and maximum (10^{-4} M) drug concentrations used in the screening test.[41]

Scheme 5[a]



[a] Reagents and conditions: (a) (1) *sec*-BuLi, THF, −78 °C (2 h); (2) 1-cyanobenzotriazole, −78 to 23 °C (4 h). (b) Aqueous HCl, THF, 23 °C. (c) (1) HONH$_2$·HCl, pyridine, 90−100 °C (1 h); (2) Ac$_2$O, 90−100 °C (2 h); (3) aqueous HCl, THF, 23 °C.

Table 1 includes published cell line data for 2-methoxyestradiol (**1**).[13]

Compounds **8**−**10**, **15**, and **21** were all inactive as inhibitors of tubulin polymerization. The inactivities of **8**−**10** and **15** are consistent with the previously expressed idea that there is a critical size restriction on the 2-substituent in estradiols that modulates the interaction of these substances with tubulin. For example, although 2-vinylestradiol, 2-((*E*)-1′-propenyl)-estradiol, and 2-((*E*)-1′-butenyl)estradiol are all active as inhibitors of tubulin polymerization, with maximal activity residing in the 2-((*E*)-1′-propenyl) analogue, 2-((*E*)-1′-pentenyl)estradiol is inactive as an inhibitor of tubulin polymerization.[13] Likewise, 2-(*n*-propyl)estradiol is more active than 1-ethylestradiol, but 2-(*n*-butyl)-estradiol and 2-(*n*-pentyl)estradiol are both inactive.[13] When viewed in the context of these results, the inactivities of **8**−**10** and **15** seem reasonable. On the other hand, the inactivity of 2-cyanoestradiol (**21**) vs tubulin polymerization is very surprising, especially in comparison with the activity of the 2-alkynyl compound **18**, which has a similar size and shape. The difference in activity seems to indicate that in addition to steric effects, electronic effects are also important, since nitriles are much more polar than alkynes, with a partial negative charge residing on the nitrile nitrogen and a partial positive charge located on the nitrile carbon.

Considering the compounds that are active vs tubulin polymerization, 2-(1′-propynyl)estradiol (**19**) (IC_{50} 4.9 μM) is the most potent, followed by the alkenyl alcohol **5** (IC_{50} 6.3 μM), the ether alcohol **7** (IC_{50} 9.5 μM), and 2-ethynylestradiol (**18**) (IC_{50} 13 μM). Only compound **19**, with its IC_{50} of 4.9 μM, had activity comparable to that of 2-methoxyestradiol (IC_{50} 4.7 μM). The range of potencies of these compounds vs tubulin polymerization is rather narrow, which limits the importance of the differences in activity among them. Overall, however, the IC_{50} values that vary by 20% or more in this assay have been reproducibly different from each other.

Concerning the cytotoxicities of the compounds in human cancer cell cultures, all of the compounds, including those that were inactive as inhibitors of tubulin polymerization, were cytotoxic to some extent. The four compounds **21** (MGM 20 μM), **8** (MGM 16 μM), **9** (MGM 16 μM), and **15** (MGM 13.5 μM) that displayed the lowest overall cytotoxicity were also all inactive as inhibitors of tubulin polymerization in the concentration range tested (up to 40 μM). On the other hand, the three most cytotoxic compounds **5** (MGM 1.1 μM), **1** (MGM 1.3 μM), and **19** (MGM 1.7 μM) were the best inhibitors of tubulin polymerization. In contrast, the stilbene analogue **10**, despite being one of the more cytotoxic compounds, was inactive as a tubulin polymerization inhibitor, and the ether alcohol **7** had relatively low cytotoxicity in comparison with the other tubulin polymerization inhibitors.

As mentioned above, 2-propynylestradiol (**19**), as well as 2-methoxyestradiol (**1**), have been shown to suppress neuroblastoma growth without signs of toxicity in nude mice after oral administration.[4] The antitumor activity resulted from both inhibition of tumor angiogenesis and from induction of tumor cell apoptosis.[4] Both compounds also resulted in chromaffin differentiation of neuroblastoma cells.[4] We therefore decided to further evaluate 2-propynylestradiol (**19**) in an in vivo animal model in which polyvinylidene fluoride hollow fibers containing various cancer cell cultures were implanted intraperitoneally (IP) and subcutaneously (SC) into mice, and the compound was administered by the IP route.[42] The compound was tested in the hollow fiber assay against a panel of 12 human tumor cell lines as described previously.[43] The effects of the compound on reduction of viable cancer cell mass as compared to those of controls was determined. The compound was solubilized in 10% dimethyl sulfoxide (DMSO) in saline/Tween-80 and administered intraperitoneally once daily for a total of four doses at each of two dose levels. The day after the last compound dose, the fibers were collected and assessed for viable cell mass. A score of 2 was assigned each time the compound produced a 50% or greater reduction in viable cell mass as compared to the vehicle-treated controls. The score was summed for the intraperitoneal fibers and the subcutaneous fibers, and the results indicated high in vivo anticancer activity for **19** in the hollow fiber animal model. The IP score for 2-(1′-propynyl)estradiol (**19**) was 20, and the SC score was 6, for a total score of 26. The estradiol derivative **19** also reproducibly reduced the viable cell mass of one or more of the cell lines to less than the original implant concentration (cell kill). These results strengthen the case for further preclinical evaluation of 2-(1′-propynyl)-estradiol (**19**) as an anticancer agent.

Experimental Section

General. Melting points were determined in capillary tubes on a Mel-Temp apparatus and are uncorrected. The proton nuclear magnetic resonance spectra (^{1}H NMR) were determined at 300 MHz. Microanalyses were performed at the Purdue Microanalysis Laboratory, and all values were within 0.4% of the calculated compositions. Silica gel used for column chromatography was 230−400 mesh.

3,17-Bis(*tert*-butyldimethylsilyl)estradiol-2-carboxaldehyde (3). A solution of aldehyde **2**[13] (3.3 g, 11 mmol), imidazole (10.4 g, 153 mmol), and *tert*-butyldimethylsilyl chloride (10 g, 66.3 mmol) in a mixture of dimethylformamide (DMF, 60 mL) and THF (60 mL) was stirred overnight under argon at room temperature. The reaction mixture was then poured into an ice-cold solution of 5% aqueous sodium bicarbonate (250 mL), and the crude product was extracted with ethyl acetate (2 × 100 mL). The organic layer was washed with water (100 mL) and brine (2 × 50 mL) and dried (Na_2SO_4). Removal of solvent followed by trituration of the crude product with methanol gave compound **3** (4.2 g, 72%); mp 182 °C. ^{1}H NMR ($CDCl_3$): δ 10.38 (s, 1 H), 7.73 (s, 1 H), 6.56 (s, 1 H), 3.66−3.61 (t, J = 9 Hz, 1 H), 2.86−2.82 (m, 2 H), 2.38−1.15 (m, 13 H), 1.01 (s, 9 H), 0.89 (s, 9 H), 0.73 (s, 3 H), 0.25 (s, 6 H), 0.04 (s, 3 H), 0.02 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 529 (MH^+, 100%).

2-(*E*-3′-Acrolenyl)-3,17β-bis(*tert*-butyldimethylsilyl)-estradiol (4). A solution of aldehyde **3** (0.5 g, 0.95 mmol) and (triphenylphosphoranylidene)acetaldehyde (0.432 g, 1.4 mmol) in dry THF (50 mL) was heated at reflux under argon for 10 h. The removal of solvent followed by column chromatographic purification (silica gel, 230−420 mesh, 10% ethyl acetate in hexane) gave compound **4** (0.32 g, 61%) as a pale yellow solid; mp 118 °C. IR (KBr): 2930, 2881, 1678, 1600 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 9.65−9.62 (d, J = 9 Hz, 1 H), 7.87−7.81 (d, J = 18 Hz, 1 H), 7.48 (s, 1 H), 6.70−6.62 (dd, J = 18 and 9 Hz, 1 H), 6.56 (s, 1 H), 3.67−3.62 (t, J = 9 Hz, 1 H), 2.84−2.80 (m, 2 H), 2.31−2.26 (m, 2 H), 1.93−1.88 (m, 3 H), 1.66−1.15 (m, 8 H), 1.03 (s, 9 H), 0.74 (s, 3 H), 0.24 (s, 6 H), 0.04 (s, 3 H), 0.03 (s, 3 H).

2-(*E*-3′-Hydroxy-1′-propenyl)estradiol (5). DIBAL in toluene (7.2 mL, 1 M) was slowly added to a solution of aldehyde **4** (0.5 g, 0.9 mmol) in dry toluene at −40 °C under argon. After the addition was completed, the reaction mixture was stirred at the same temperature for 1 h. The excess hydride was carefully quenched with methanol (2 mL), 10% sodium hydroxide (2 mL) was added, and the mixture was stirred at room temperature for 10 min. The organic layer was decanted, and the residue was washed with ethyl acetate (2 × 30 mL). The combined organic layer was dried (Na_2SO_4), and the solvent was removed in vacuo. The crude product was stirred with TBAF (10 mL, 1 M) in THF for 12 h. The reaction mixture was then poured into an ice-cold 5% aqueous solution (50 mL). The product was extracted into ethyl acetate (3 × 50 mL), washed with brine (50 mL), and dried (Na_2SO_4). The removal of solvent followed by column chromatographic purification (silica gel, 230−420 mesh, 1:1 ethyl acetate−hexane) gave compound **5** (0.25 g, 85%); mp 190 °C. ^{1}H NMR (methyl-d_3 alcohol-d): δ 7.26 (s, 1 H), 6.82−6.77 (d, J = 15 Hz, 1 H), 6.46 (s, 1 H), 6.33−6.29 (td, J = 15 and 6 Hz, 1 H), 4.20−4.18 (d, J = 6 Hz, 2 H), 3.64−3.61 (t, J = 9 Hz, 1 H), 2.72 (m, 2 H), 2.56−2.30 (m, 2 H), 2.14−1.14 (m, 11 H), 0.76 (s, 3 H). Anal. ($C_{21}H_{28}O_3$) C, H.

2-(2′-Hydroxyethoxy)estradiol (7). Anhydrous potassium carbonate (5.87 g, 42.5 mmol) and ethyl bromoacetate (2.35 mL, 21.2 mmol) were added to a solution of **6** (1.60 g, 4.25 mmol) in acetonitrile (50 mL), and the reaction mixture was heated at reflux for 4 h. The solvent was removed in vacuo, and the residue was extracted into ethyl acetate (3 × 30 mL), washed with water (50 mL), and dried (Na_2SO_4). The organic solvent was completely removed, and the crude product was dissolved in anhydrous THF (40 mL). LAH (0.5 g, 13.2 mmol)

was added, and the mixture was stirred at room temperature for 3 h. The excess LAH was carefully quenched with methanol (5 mL) and 10% NaOH (2 mL). The THF solution was decanted, and the residue was washed with THF (20 mL). The combined THF portion was stirred with 6 M HCl (50 mL) for 10 h. Evaporation of solvent from the organic layer, followed by trituration of the crude product with ethyl acetate, gave the pure compound **7** as a colorless solid (0.85 g, 62%); mp 214−216 °C. IR (KBr): 3328, 2923, 1511 cm^{-1}. ^1H NMR (DMSO-d_6): δ 8.4 (s, 1 H), 6.78 (s, 1 H), 6.45 (s, 1 H), 3.93−3.89 (t, J = 6 Hz, 2 H), 3.69−3.66 (t, J = 6 Hz, 2 H), 3.54−3.49 (t, J = 6 Hz, 1 H), 2.63 (m, 2 H), 2.26−1.07 (m, 13 H), 0.66 (s, 3 H). Anal. ($C_{20}H_{28}O_4{\cdot}0.2CH_3CO_2CH_2CH_3$) C, H.

2-(2′-*N,N*-Dimethylaminoethylimino)estradiol (8). 2-(Aminoethyl)dimethylamine (1.46 mL, 13.3 mmol) was added to the aldehyde **2** (0.4 g, 1.33 mmol), and the suspension was heated at reflux for 6 h. The excess amine was removed in vacuo, and the crude product was recrystallized from ethyl acetate−hexane (1:1) to give **8** (0.42 g, 85%) as a pale yellow solid; mp 138−140 °C. IR (KBr): 3206, 2946, 2864, 1630 cm^{-1}. ^1H NMR (DMSO-d_6): δ 13.17 (s, 1 H), 8.46 (s, 1 H), 7.29 (s, 1 H), 6.54 (s, 1 H), 4.50 (s, 1 H), 3.64−3.60 (t, J = 6 Hz, 2 H), 3.54−3.49 (t, J = 6 Hz, 1 H), 2.78−2.76 (m, 2 H), 2.52−2.47 (t, J = 6 Hz, 2 H), 2.30−1.12 (m, 19 H), 0.65 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 371 (MH^+, 100%). Anal. ($C_{23}H_{34}N_2O_2$) C, H, N.

2-(2′-Hydroxyethylimino)estradiol (9). Ethanolamine (0.4 mL, 6.66 mmol) was added to the aldehyde **2** (0.4 g, 1.33 mmol), and the suspension was heated at 100 °C for 6 h. Most of the excess ethanolamine was removed in vacuo, and the crude product was purified by passing it through a column of silica gel (230−420 mesh, ethyl acetate-methanol 2:8) to give compound **9** as a yellow solid (0.42 g, 92%); mp 190−192 °C. IR (KBr): 3357, 2923, 2867, 1636 cm^{-1}. ^1H NMR (DMSO-d_6): δ 13.17 (s, 1 H), 8.43 (s, 1 H), 7.31 (s, 1 H), 6.55 (s, 1 H), 4.74 (m, 1 H), 4.50−4.49 (d, J = 3 Hz, 1 H), 3.61 (s, 4 H), 3.55−3.48 (m, 1 H), 2.79−2.76 (m, 2 H), 2.34−1.11 (m, 13 H), 0.66 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 344 (MH^+, 100%). Anal. ($C_{21}H_{29}NO_3$) C, H, N.

2-(*β*-3,4,5-Trimethoxyphenyl)ethenylestradiol (10). LHMDS (1 M, 4.64 mL) was added to a suspension of 3,4,5-(trimethoxybenzyl)triphenylphosphonium chloride (1.59 g, 3.2 mmol) in dry THF (40 mL) at 0 °C, and the mixture was stirred under argon for 15 min. A clear orange color developed. Compound **2** (0.2 g, 0.66 mmol) in anhydrous THF (20 mL) was added dropwise under argon, and the reaction mixture was stirred for 8 h at room temperature. It was acidified to pH 5, extracted with ether (3 × 40 mL), washed with brine (2 × 50 mL), and dried (Na_2SO_4). Evaporation of the filtrate followed by column chromatographic purification (silica gel, 230−420 mesh; ethyl acetate−hexane, 1:1) gave compound **10** as a fluffy solid (0.24 g, 78%); mp 220−222 °C (ether). IR (KBr): 3430, 2931, 1580, 1508 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.43 (s, 1 H), 7.26−7.21(d, J = 15 Hz, 1 H), 7.03−6.98 (d, J = 15 Hz, 1 H), 6.74 (s, 2 H), 6.54 (s, 1 H), 5.2 (s, 1 H), 3.92 (s, 6 H), 3.85 (s, 3 H), 3.82−3.70 (m, 1 H), 2.80 (m, 2 H), 2.44−1.20 (m, 13 H), 0.80 (s, 3 H). PDMS m/z 464.4 (M^+). Anal. ($C_{29}H_{36}NO_2$) C, H, N.

2-Iodo-3,17-bis-*O*-(methoxymethyl)estradiol (12). A 1.3 M solution of *sec*-BuLi in cyclohexane (8.6 mL, 11.2 mmol) was added dropwise to a solution of 3,17-bis-*O*-(methoxymethyl)-estradiol (**11**)[40] (1 g, 2.8 mmol) in anhydrous THF (25 mL) at −78 °C, and the reaction mixture was stirred at the same temperature under argon for 2 h. A solution of iodine (2.8 g, 11.1 mmol) in dry THF (30 mL) was added to the pale yellow solution of the anion, and the reaction mixture was slowly raised to room temperature and stirred overnight. It was poured into ice water (100 mL). The crude product was extracted into ethyl acetate (3 × 30 mL), washed with a saturated solution of $Na_2S_2O_3$ (2 × 20 mL), and dried (Na_2SO_4). The removal of organic solvent followed by trituration of the crude product with methanol (30 mL) gave the pure iodo compound **12** as a colorless solid (1.1 g, 78%); mp 94 °C. ^1H NMR (300 MHz, $CDCl_3$): δ 7.65 (s, 1 H), 6.78 (s, 1 H), 5.18 (s, 2 H), 4.65 (s, 2 H), 3.60 (t, J = 7.5 Hz, 1 H), 3.51 (s, 3 H), 3.37 (s, 3 H), 2.82 (m, 2 H), 2.30−1.10 (m, 13 H), 0.80 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 486 (MH^+), 455, 425 (100%).

2-Iodoestradiol (13). Hydrochloric acid (6 M, 150 mL) was added to a solution of **12** (8 g, 16.4 mmol) in THF (150 mL), and the reaction mixture was stirred at room temperature for 10 h. It was poured into ice water (400 mL), extracted into ethyl acetate (3 × 50 mL), washed with brine (100 mL), and dried (Na_2SO_4). Removal of organic solvent gave 2-iodoestradiol (**13**) as a fluffy solid (6.4 g, 98%); mp 150−152 °C (lit.[44] mp 146−153 °C; lit.[13]mp 154−155 °C). ^1H NMR (300 MHz, $CDCl_3$): δ 7.52 (s, 1 H), 6.71 (s, 1 H), 3.76−3.70 (t, J = 7.5 Hz, 1 H), 2.80−2.76 (m, 2 H), 2.28−1.15 (m, 13 H), 0.81 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 399 (MH^+), 381 (100%).

3,17-Bis-*O*-(*tert*-butyldimethylsilyl)-2-iodo-estradiol (14). This compound was prepared according to the published procedure.[45]

2-(Phenylethynyl)estradiol (15). Tri-*n*-butylphenylethynylstannane (0.589 g, 1.51 mmol) was added to a solution of 2-iodoestradiol **13** (0.3 g, 0.75 mmol) in dry THF (30 mL), Pd(PPh_3)$_4$ (0.087 g, 0.075 mmol) was added, and the reaction mixture was maintained between 60 and 65 °C for 10 h under argon. The solvent was removed, and the crude product (0.22 g, 79%) was purified by passing it through a column of silica gel (230−420 mesh, 1:4 ethyl acetate−hexane); mp 156−158 °C. ^1H NMR (MeOH-d_4): δ 7.53−7.50 (dd, J = 6 and 3 Hz, 2 H), 7.36−7.29 (m, 3 H), 7.24 (s, 1 H), 6.55 (s, 1 H), 4.87 (s, 1 H, exchangeable with D_2O), 3.66−3.61 (t, J = 6 Hz, 1 H), 2.76 (m, 2 H), 2.29−2.25 (m, 1 H), 2.08−1.83 (m, 4 H), 1.68−1.25 (m, 8 H), 0.76 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 373 (MH^+, 100%), 355 (MH^+-H_2O). Anal. ($C_{26}H_{28}O_2{\cdot}0.3Et_2O{\cdot}0.5H_2O$) C, H.

3,17-Bis-*O*-(*tert*-butyldimethylsilyl)-2-(1-ethynyl)estradiol (16). A procedure similar to that for the preparation of compound **17** was employed, and the product was obtained as a pale yellow solid (0.321 g, 95%); mp 126−128 °C. IR (KBr): 3307, 2925, 2855, 2101, 1488, 1398 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.30 (s, 1 H), 6.49 (s, 1 H), 3.61 (t, J = 6 Hz, 1 H), 3.10 (s, 1 H), 2.77−2.75 (m, 15 H), 0.96 (s, 9 H), 0.87 (s, 9 H), 0.71 (s, 3 H), 0.19 (s, 6 H), 0.05 (s, 6 H). Anal. ($C_{32}H_{52}O_2Si_2$) C, H.

3,17-Bis-*O*-(*tert*-butyldimethylsilyl)-2-(1-propynyl)estradiol (17). Anhydrous $ZnBr_2$ (0.289 g, 1.28 mmol) was dissolved in dry THF (40 mL) under argon. A solution of 1-propynylmagnesium bromide (2.6 mL, 0.5 M) in THF was added, and stirring was continued under argon as the solution became turbid. After 10 min, Pd(PPh_3)$_4$ (0.074 g, 0.064 mmol) and iodo compound **14** (0.4 g, 0.64 mmol) were added, and stirring was continued for 6 h at room temperature. The reaction mixture was poured over ice water (100 mL), extracted into ethyl acetate (3 × 20 mL), washed with brine (50 mL), and dried (Na_2SO_4). Removal of organic solvent followed by column chromatographic purification (silica gel, 230−420 mesh, 5% ethyl acetate in hexane) gave 0.28 g (82%) of pure compound **17** as a colorless solid; mp 192−194 °C. IR (KBr): 2934, 2844, 1493, 1462, 1297, 1247, 1131, 1091 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.22 (s, 1 H), 6.48 (s, 1 H), 3.62 (t, J = 8.6 Hz, 1 H), 2.76 (m, 2 H), 2.25 (m, 1 H), 2.10 (m, 1 H), 2.04 (s, 3 H), 1.86 (m, 3 H), 1.70−1.10 (m, 8 H), 1.02 (s, 9 H), 0.89 (s, 9 H), 0.73 (s, 3 H), 0.20 (s, 6 H), 0.03 (s, 3 H), 0.02 (s, 3 H).

2-Ethynylestradiol (18). A solution of compound **16** (0.5 g, 0.95 mmol) in TBAF (1 M in THF, 9.6 mL) was stirred under argon at room temperature for 10 h. The reaction mixture was then poured into an ice-cold 5% aqueous sodium bicarbonate solution (50 mL). The crude product was extracted into ethyl acetate (2 × 50 mL), washed with brine (50 mL), and dried (Na_2SO_4). Removal of solvent followed by column chromatographic purification (silica gel, 230−420 mesh, 1:4 ethyl acetate−hexane) gave compound **18** (0.115 g, 41%); mp 102−104 °C. ^1H NMR ($CDCl_3$): δ 7.29 (s, 1 H), 6.67 (s, 1 H), 3.72 (m, 1 H), 3.40 (s, 1 H), 2.84−2.80 (m, 2 H), 2.32−1.15 (m, 13 H), 0.78 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 353

(MH-isobutane$^+$, 100%), 297 (MH$^+$), 279 (MH$^+$−H_2O). Anal. ($C_{20}H_{24}O_2$·0.4CH_3CO_2Et·0.4C_6H_{14}) C, H.

2-(1′-Propynyl)estradiol (19). Compound **19**[27] was prepared from **17** in 86% yield using the same procedure as that employed for the synthesis of **18**.

2-Cyano-3,17-bis-*O*-(methoxymethyl)estradiol (20). A 1.3 M solution of *sec*-BuLi in cyclohexane (8.6 mL, 11.2 mmol) was added to a solution of compound **11**[40] (1.05 g, 2.9 mmol) in anhydrous THF (30 mL) at −78 °C under argon, and the reaction mixture was stirred for 2 h. A solution of 1-cyanobenzotriazole (0.75 g, 5.2 mmol) was added to the reaction mixture, the temperature was slowly raised to room temperature, and the mixture was stirred for an additional 4 h. It was poured into ice water (100 mL), extracted into ethyl acetate (3 × 20 mL), and dried (Na_2SO_4). The crude product was passed through a column of silica gel (230−420 mesh, 1:4 ethyl acetate−hexane) to give **20** (0.22 g, 33%). IR (neat): 2928, 2224 (CN), 1609, 1498 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.46 (s, 1 H), 6.91 (s, 1 H), 5.24 (s, 2 H), 4.65 (m, 2 H), 3.61 (t, J = 8.1 Hz, 1 H), 3.50 (s, 3 H), 3.37 (s, 3 H), 2.9 (m, 2 H), 2.3−1.15 (m, 13 H), 0.80 (s, 3 H).

2-Cyanoestradiol (21). Hydrochloric acid (6 M, 30 mL) was added to a solution of **20** (0.2 g, 0.52 mmol) in THF (30 mL), and the mixture was stirred at room temperature for 8 h. The reaction mixture was poured into ice water (100 mL) and extracted into ethyl acetate (4 × 20 mL) and dried (Na_2SO_4). Removal of solvent followed by trituration of the crude product with methylene chloride gave pure compound **21** as a white solid (0.12 g, 78%); mp 320−324 °C. IR (KBr): 3385, 3144, 2925, 2231 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 10.41 (s, 1 H), 7.29 (s, 1 H), 6.63 (s, 1 H), 4.41 (d, J = 4.4 Hz, 1 H), 3.51 (q, J = 5.15 Hz, 2 H), 2.73 (m, 2 H), 2.20−1.05 (m, 13 H), 0.64 (s, 3 H). CIMS (isobutane) m/z (relative intensity) 298 (MH$^+$, 100%). Anal. ($C_{19}H_{23}NO_2$·0.4H_2O) C, H, N.

Preparation of 2-Cyanoestradiol 21 from Aldehyde 22. Hydroxylamine hydrochloride (1 g, 14 mmol) was added to a solution of aldehyde **22**[40] (0.5 g, 1.3 mmol) in pyridine (20 mL), and the mixture was heated at 90−100 °C with stirring for 1 h. Acetic anhydride (5 mL) was added, and stirring was continued at the same temperature for another 2 h. The pyridine was then removed, and the reaction mixture was poured onto crushed ice (100 mL). The crude product was extracted into ethyl acetate (4 × 30 mL), and the solution was dried (Na_2SO_4). Removal of solvent followed by cleavage of the methoxymethyl group (as described in the previous case) gave pure product (0.17 g, 44%). The IR and NMR of this product were identical in all aspects with those of the previous sample.

Tubulin Assays. Electrophoretically homogeneous tubulin was purified from bovine brain as described previously.[46] Determination of IC_{50} values for inhibition of polymerization of purified tubulin was performed as described in detail elsewhere,[18] except that Beckman DU7400/7500 spectrophotometers equipped with “high performance” temperature controllers were used. The temperature controllers were modified to include a water circuit for enhanced cooling of their electronic elements. The spectrophotometers were controlled by a program provided by MDB Analytical Associates, South Plainfield, NJ. In brief, tubulin was preincubated at 26 °C with varying compound concentrations, reaction mixtures were chilled on ice, GTP (required for the polymerization reaction) was added, and polymerization was followed at 26 °C by turbidimetry at 350 nm. The extent of polymerization after 20 min was determined. IC_{50} values were determined graphically. All compounds were examined in at least two independent assays.

Hollow Fiber Assay.[42,43] Polyvinylidene fluoride hollow fibers containing a standard panel of cancer cell lines were implanted IP or SC in mice, and compounds were injected IP using a qd × 4 treatment schedule. A total of 12 IP and 12 SC cell lines were tested in triplicate at two dosage levels, and each cell line with a 50% or greater reduction in viable cell mass was given a score of 2. The IP and SC scores were then totaled, and a net cell kill at one or more of the implant sites was noted.

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