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3-*O*-Phosphate ester conjugates of 17-β-*O*-{1-[2-carboxy-(2-hydroxy-4-methoxy-3-carboxamido) anilido]ethyl}1,3,5(10)-estratriene as novel bone-targeting agents

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ABSTRACT

A series of 3-*O*-phosphorylated analogs (**4**–**10**) of a novel bone-targeting estradiol analog (**3**) were synthesized after a thorough study of the reaction of **3** with a selection of phosphoryl chlorides under a variety of reaction conditions. Evaluation of these novel phosphate analogs for affinity for hydroxyapatite revealed that they bind with equal or higher affinity when compared to the bone tissue accumulator, tetracycline.

Osteoporosis is a condition that arises due to low mineral density of bone, and results in skeletal fragility with increased risk of bone fracture. At present, about ten million Americans suffer from osteoporosis with about eight million being women. Another thirty four million in the US population suffer from osteopenia, which is a condition where bone mineral density (BMD) is lower than normal, but not low enough to be classified as osteoporosis.[1,2] Clinical osteoporosis, which presents as fragility fractures, is a common condition in post-menopausal women; however, covert or subclinical osteoporosis that has not yet presented fractures is far more widespread, and is ultimately responsible for untold numbers of preventable bone fractures.[3] Numerous factors, including hyperthyroidism, menopause or ovariectomy, lead to conditions that cause increased osteoclast activity in proportion to bone growth.[4] Agents currently available for prophylaxis or treatment of osteoporosis include *bis*-phosphonates, calcitonin, selective estrogen receptor modulators (SERMs), selective androgen receptor modulators (SARMs), and parathyroid hormone (PTH). Estrogen replacement therapy is often a method of choice in the clinical treatment of osteoporosis that results from insufficient levels of estrogen.[5] The salubrious effect of estrogen on the resorption of bone tissue arises from its anti-apoptotic effect on osteoclasts. Unfortunately, direct treatment of osteoporosis and other related disorders with estrogens, such as estradiol (**1**), is hampered by the drug's non-specific effects, for example, its estrogenic properties, where increased concentrations of estradiol cause undesirable effects in males.[6]

Several years ago, we initiated a program aimed at restricting the effect of estrogens to bone tissue. Our strategy focused on the development of analogs of estradiol that would possess a tendency to localize predominantly in bone tissue. One of the drug design strategies we considered was the conjugation of an estradiol moiety to chemical entities that possessed high affinity for structural elements commonly contained in bone (i.e., osteotropic

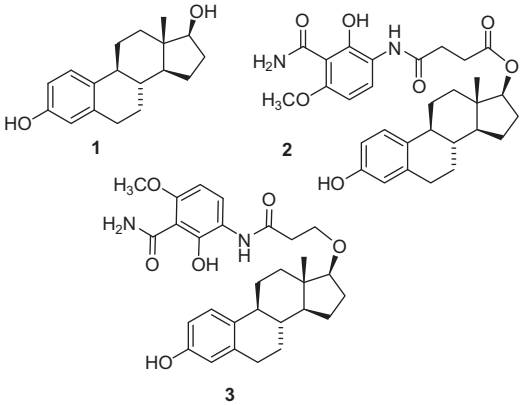

Figure 1. Estradiol (**1**) and bone-targeting analogs **2** and **3**.

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moieties).[6–8] We have recently reported on the design, synthesis and pharmacology of compounds in which Ca^{2+}-chelators have been conjugated to an estradiol moiety through succinoyl linker units (Fig. 1, compound **2**).[8] These compounds were subsequently found to possess poor hydrolytic stability in vivo, and were cleaved prior to their arrival at the site of action. To overcome this problem, we turned our attention to the design and synthesis of second generation analogs of **2** in which the succinoyl linker is replaced with a more stable carboxyethyl linker (Fig. 1, compound **3**).[9] Although this analog cannot be considered as an estradiol prodrug, due to its inability to be hydrolytically cleaved to estradiol, it did retain both the bone-targeting and anabolic properties of **2**.

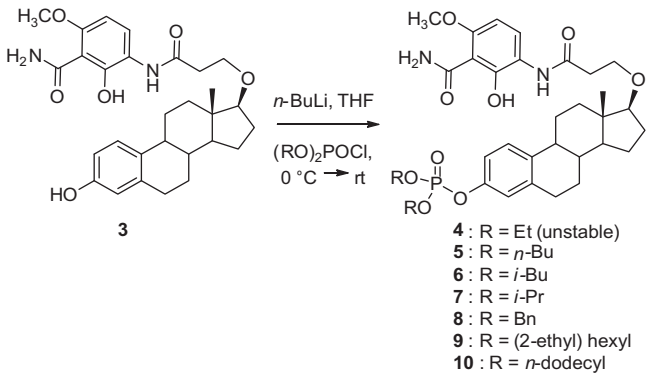
With regard to the current communication, it is well documented that phosphate groups have a strong affinity for Ca^{2+} and other divalent metal ions, as well as affording better hydrophilicity and water-solubility when incorporated into the structure of drug molecules.[10] To improve the bone-targeting capabilities of our second generation analogs, we have prepared a series of phosphate analogs of **3**. It has been reported that phosphate esters can be cleaved enzymatically, releasing phosphate in the body.[11] Thus, phosphorylated analogs of **3** would also serve as supplements to dietary phosphorus, which are frequently utilized in popular osteoporosis therapy regimens.

When considering the incorporation of a phosphate moiety into the structure of compound **3**, the potential sites of attachment of the phosphate ester are at the 3-*O*-position of the estradiol moiety and the phenolic oxygen of the aromatic Ca^{2+}-chelating moiety. The latter position was not considered to be a feasible strategy, since the free phenoxide of the aromatic moiety is necessary for its chelation with calcium ion. Thus, phosphate analogs of **3** were synthesized with the phosphate moiety conjugated at the 3-*O*-position of the estradiol moiety.

Since compound **3** has two potential phenolic sites for phosphorylation and several base-labile protons, we decided to attempt the use of bases of varied strengths and under a variety of reaction conditions. An attempt at the phosphorylation of **3** with diethyl phosphoryl iodide (formed in situ through the oxidation of triethylphosphite with iodine) in the presence of pyridine as a nucleophilic catalyst was unsuccessful, since unchanged **3** was isolated. It was noticed in the original literature report that this method was found to be successful with alcohols,[12] although one phenolic phosphorylation (with 4-hydroxy acetophenone, whose phenolic group is extremely labile to deprotonation by pyridine) was reported. This led us to suspect that the basicity of pyridine might be insufficient to deprotonate the phenolic 3-hydroxy function of **3**. Substitution of pyridine with triethylamine did not alter the reaction course, but this could have been due to the fact that phosphoryl iodides can react with triethylamine itself.[13] We next attempted the use of the less reactive diethylphosphoryl chloride in the presence of triethylamine. In this reaction, we observed traces of the desired product in the crude reaction mixture, and NMR spectral analysis of the reaction product showed a characteristic upfield shift of the C-3 signal of the estradiol moiety from 154 to 148 ppm, which strongly suggests the presence of the phosphoryl moiety. A downfield shift of the aromatic proton resonances of the 'A' ring of **3** at the 2 and 4-positions from 6.62 to 6.97 ppm was also observed. Unfortunately, this product **(4)** was found to be labile to silica gel chromatography. Attempts at purification of **4** through crystallization from various solvents led to degradation, and therefore **4** was not considered a viable phosphate analog for biological evaluation. However, when the same reaction was conducted with dibutylphosphoryl chloride, the product was found to be relatively stable, although the presence of numerous side-products in the reaction mixture prevented its isolation in a pure form. Since one of the reasons for the presence of side-products could be the reaction of triethylamine with dibutylphosphoryl chloride itself, as evidenced by a rapid darkening of an admixture of triethylamine and dibutylphosphoryl chloride, we sought to generate the required phenoxide anion by deprotonating **3** with an alkali metal base. Amongst various bases employed, (i.e., NaH, NaHMDS, KHMDS, LHMDS and *n*-BuLi, all in THF), *n*-BuLi was found to afford a clean white precipitate. The precise identity of this precipitate was not determined, however, quenching of this solution with silica gel led to the recovery of unchanged **3**, signifying that negligible degradation had occurred during the likely deprotonation step. Treatment of the suspension of this precipitate with dibutylphosphoryl chloride yielded product **5**, which exhibited NMR spectral characteristics consistent with the site of phosphorylation being at the 3-position of the estradiol moiety. This compound was stable to air, and could be isolated in a pure form after silica gel column chromatography.

Regarding the alkali metal amide bases that were utilized (i.e., LDA, LHMDS, KHMDS, etc.), the initial precipitate obtained had characteristics similar to that produced by *n*-BuLi and also afforded pure **3** when quenched, but the subsequent phosphorylation afforded very complicated reaction mixtures. This is likely due to the reaction of the conjugate acids (diisopropylamine and hexamethyl-disilazane, liberated during proton exchange) with dibutylphosphoryl chloride. *n*-BuLi on the other hand, produces butane gas as the conjugate acid, which possesses no nucleophilic character under the reaction conditions employed. In the optimization of the reaction conditions for the synthesis of phophorylated analogs of **3**, 1.05 equiv of *n*-BuLi were found to work best. The use of more than 1.05 equiv of phosphoryl chloride offered no advantage in the conversion ratio of **3** to phosphate esters **5**–**10**, which was generally around 70% in the best runs. Having optimized the conditions for this chemical transformation, a total of six phosphate esters, **5**–**10**, were synthesized (Scheme 1) and fully characterized.[14]

These phosphate esters were found to be stable during silica gel chromatographic purification. The conversion of **3** to analogs **5**–**10** was found to be clean, with estradiol (**1**) being the only significant side-product. Since **3** and its derivatives have previously been found to be highly susceptible to loss of the α-proton on the β-alkoxycarbonyl unit,[9] it is assumed that the formation of **1** in this reaction is caused by deprotonation of the α-proton of **3** by *n*-BuLi, followed by elimination to form an olefin (acrylamide). The short-chain aliphatic and benzyl phosphates **5**–**8** were isolated as solids, while the long-chain hydrocarbon phosphates **9** and **10** were found to be waxy semi-solids. All the phosphate esters were found to undergo slight degradation (about 10–20% conversion to estradiol over several weeks at ambient temperature in air). In contrast, compound **3**, upon storage at ambient temperature (in air), exhibited a significantly greater amount (40–50%) of hydrolytic degradation at the amidic linkage of the osteotropic aromatic moiety compared to its phosphoryl derivatives **5**–**10**.



Scheme 1. Phosphorylation conditions for the synthesis of compound **3**.

Table 1
Relative binding of 17-β-estradiol and analogs **5**–**10** to hydroxyapatite compared to tetracycline binding

Compound	Binding index (%) relative to tetracycline
17-β-Estradiol	0
Tetracycline	100
5	100
6	180
7	200
8	190
9	180
10	160

As a predictive measure of the ability of this class of compound to bind to bone tissue in vivo, the ability of compounds **5**–**10** to bind to crystalline hydroxyapatite in vitro was determined using UV–vis spectroscopy.[15] These studies were conducted in parallel with binding studies on tetracycline for comparison, as it is well established that tetracycline accumulates preferentially in bone-tissue because of a very strong affinity for hydroxyapatite.[16] The binding indices of the novel phosphate prodrugs are shown in Table 1. Superior binding to hydroxyapatite is clearly evident when compared to 17-β-estradiol alone, and is comparable to that of tetracycline. Within the phosphate prodrug series, increased lipophilicity or branching in the aliphatic substituents appears to improve hydroxyapatite binding, since analogs **6**–**10** are somewhat more potent than analog **5**.

In conclusion, we have developed a viable phosphorylation protocol for the regioselective synthesis of 3-*O*-phosphate analogs of the bone-targeting estradiol analog **3**. In vitro data indicate that these analogs have bone-targeting properties comparable to tetracycline, as indicated by their affinity for hydroxyapatite.

Acknowledgments

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14. *General procedure for the synthesis of 17-β-[[2-Carboxy(3-carboxamido-2-hydroxy-4-methoxy)anilido]ethoxy]estra-1,3,5-triene-3-dialkyl phosphates* (**5**–**10**). *n*-Butyllithium (0.8 mL, 2.5 M solution in hexane) was diluted to 10 times its volume with THF and the resulting mixture added drop-wise to a rapidly stirring solution of **3** in THF (0.6 M) that was immersed in a salt-ice bath (bath temperature −10 °C to −5 °C) under argon gas. The resulting white suspension was stirred for 5 min and then the appropriate dialkylphosphoryl chloride (1.0 equiv) was added to the reaction mixture in one portion. The suspension became a clear solution within 5–10 min and was stirred at ambient temperature for 18 h before being evaporated to dryness on a rotary evaporator (note: bath temperature not exceeding 30 °C). The resulting crude residue was purified by silica gel flash chromatography, utilizing mixtures of CH_2Cl_2 and CH_3OH as eluting solvent. The compounds were loaded onto the column either as solutions in benzene or as dispersions on silica gel (three times the weight of the compound). Dialkylphosphoryl chlorides were prepared in accordance with a previously reported method.[17]
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carbox-amido)anilido]ethyl}1,3,5-estratriene-3-dibutyl phosphate (**5**): White solid; R_f = 0.3 (CH_2Cl_2/MeOH, 20:1); mp = 78–80 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.4 (s, 1H), 8.72 (s, 1H), 8.46 (d, *J* = 9.0 Hz, 1H), 8.20 (br s, 1H), 7.24 (d, *J* = 8.2 Hz, 1H), 6.96 (app d, *J* = 11.3 Hz, 2H), 6.41 (d, *J* = 9.0 Hz, 1H), 5.76 (s, 1H), 4.13 (m, 4H), 3.95 (s, 3H), 3.82 (m, 2H), 3.50 (t, *J* = 8.1 Hz, 1H), 2.83 (m, 2H), 2.66 (m, 2H), 2.3–2.0 (m, 2H), 1.70–1.22 (m, 19H), 0.91 (t, *J* = 7.2 Hz, 6H), 0.84 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 170.2, 154.2, 148.6, 138.5, 137.1, 126.7, 124.3, 122.5, 120.0, 117.1, 102.6, 100.1, 89.9, 68.4, 66.1, 56.5, 50.6, 44.2, 43.6, 39.1, 38.5, 38.2, 32.5, 29.8, 28.2, 28.1, 27.2, 26.5, 23.3, 18.9, 13.9, 11.9 ppm; ESI-MS *m*/*z* 701 (M+H)$^+$. Anal. Calcd for $C_{37}H_{53}N_2O_9P$: C, 63.41; H, 7.62; N, 4.00. Found: C, 63.21; H, 7.49; N, 4.29.
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carboxamido)anilido]ethyl}1,3,5-estratriene-3-isobutyl phosphate (**6**): White solid; R_f = 0.35 (CH_2Cl_2/MeOH, 20:1), mp = 84–86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.4 (s, 1H), 8.68 (s, 1H), 8.43 (d, *J* = 9.0 Hz, 1H), 8.15 (br s, 1H), 7.19 (d, *J* = 7.8 Hz, 1H), 6.93 (app d, *J* = 11.4, 2H), 6.37 (d, *J* = 9.0 Hz, 1H), 5.86 (br s, 1H), 3.90 (s, 3H), 3.88–3.80 (m, 6H), 3.46 (t, *J* = 7.5 Hz, 1H), 2.80 (m, 2H), 2.65 (m, 2H), 2.25–1.16 (m, 16H), 0.94 (d, *J* = 6.6, 11H), 0.81 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 170.4, 154.3, 148.6, 138.6, 137.2, 126.7, 124.4, 122.5, 120.1, 117.1, 102.6, 100.1, 89.9, 74.4, 66.1, 56.4, 50.5, 44.1, 43.5, 39.0, 38.4, 38.5, 38.1, 29.7, 29.3, 29.2, 28.1, 27.2, 26.4, 23.3, 18.8, 11.8 ppm; ESI-MS *m*/*z* 701 (M+H)$^+$. Anal. Calcd for $C_{37}H_{53}N_2O_9P$: C, 63.41; H, 7.62; N, 4.00. Found: C, 63.32; H, 7.53; N, 4.28.
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carboxamido)anilido]ethyl}1,3,5-estratriene-3-isopropyl phosphate (**7**): White solid; R_f = 0.4 (CH_2Cl_2/MeOH, 15:1) mp = 104–106 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.4 (s, 1H), 8.68 (s, 1H), 8.46 (d, *J* = 9.0 Hz, 1H), 8.17 (br s, 1H), 7.20 (app. d, *J* = 9.0 Hz, 1H), 6.93 (m, 2H), 6.39 (d, *J* = 9.0 Hz, 1H), 5.81 (br s, 1H), 4.74 (m, 2H), 3.93 (s, 3H), 3.81 (m, 2H), 3.48 (t, *J* = 8.4 Hz, 1H), 2.81 (m, 2H), 2.62 (m, 2H), 2.27–0.84 (m, 19H), 1.32 (m, 6H), 0.82 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8, 170.4, 154.4, 149.0, 138.6, 137.1, 126.6, 124.5, 122.7, 121.7, 120.2, 117.3, 100.2, 90.0, 74.3, 73.5, 66.1, 56.4, 50.5, 44.2, 43.5, 39.1, 38.6, 38.2, 29.7, 28.1, 27.2, 26.4, 23.8, 23.7, 23.2, 11.9 ppm; ESI-MS *m*/*z* 673 (M+H)$^+$. Anal. Calcd for $C_{35}H_{49}N_2O_9P$: C, 62.49; H, 7.34; N, 4.16. Found: C, 62.21; H, 7.51; N, 4.34.
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carboxamido)anilido]ethyl}1,3,5-estratriene-3-dibenzyl phosphate (**8**): White solid; R_f = 0.5 (hexanes/CH_2Cl_2/MeOH, 2:10:3); mp = 140–142 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.41 (s, 1H), 8.73 (s, 1H), 8.48 (d, *J* = 9.0 Hz, 1H), 8.19 (br s, 1H), 7.32 (s, 10H), 7.17 (d, *J* = 8.7 Hz, 1H), 6.88 (dd, *J* = 8.7, 1.5 Hz, 2H), 6.41 (d, *J* = 9.0 Hz, 1H), 5.86 (br s, 1H), 5.13 (s, 2H), 5.10 (s, 2H), 3.94 (s, 3H), 3.79 (m, 2H), 3.48 (t, *J* = 8.1 Hz, 1H), 2.76 (m, 2H), 2.68 (m, 2H), 2.27–1.1 (m, 13H), 0.83 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 170.2, 154.2, 148.3, 138.6, 137.3, 135.7, 135.6, 128.6, 128.1, 126.7, 124.3, 122.5, 120.1, 117.2, 102.6, 100.2, 89.9, 70.1, 66.1, 56.5, 50.6, 44.2, 43.6, 39.1, 38.5, 38.2, 31.9, 29.8, 28.2, 27.3, 26.5, 23.4, 22.9, 14.4, 11.9 ppm; ESI-MS *m*/*z* 769 (M+H)$^+$. Anal. Calcd for $C_{43}H_{49}N_2O_9P$: C, 67.17; H, 6.42; N, 3.64. Found: C, 67.35; H, 6.61; N, 3.91.
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carboxamido)anilido]ethyl}1,3,5-estratriene-3-bis(2-ethylhexyl) phosphate (**9**): Yellow oil; R_f = 0.5 (pentane/CH_2Cl_2/MeOH, 2:10:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.42 (s, 1H), 8.72 (s, 1H), 8.46 (d, *J* = 9.0 Hz, 1H), 8.18 (br s, 1H), 7.21 (d, *J* = 8.4 Hz, 1H), 6.92 (br d, *J* = 8.2 Hz, 2H), 6.39 (d, *J* = 9.0 Hz 1H), 5.90 (br s, 1H), 4.03 (m, 4H), 3.93 (s, 3H), 3.81 (m, 2H), 3.48 (m, 2H), 2.83 (m, 2H), 2.63 (m, 2H), 2.26–2.02 (m, 4H), 1.90–0.86 (m, 38H), 0.81 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 170.2, 154.1, 148.6, 138.4, 137.0, 126.6, 124.3, 122.5, 120.0, 117.1, 102.5, 100.1, 89.9, 70.5, 66.1, 56.4, 50.5, 44.2, 43.6, 40.3, 40.2, 39.1, 38.5, 38.2, 30.1, 29.8, 29.1, 28.2, 27.3, 26.5, 23.5, 23.4, 23.2, 14.4, 11.9, 11.2 ppm; ESI-MS *m*/*z* 813 (M+H)$^+$. Anal. Calcd for $C_{45}H_{69}N_2O_9P$: C, 66.48; H, 8.55; N, 3.45. Found: C, 66.21; H, 8.82; N, 3.75.
17-β-O-{1-[2-Carboxy-(2-hydroxy-4-methoxy-3-carboxamido)anilido]ethyl}1,3,5-estratriene-3-dodecylphosphate (**10**): Yellow oil: R_f = 0.6 (hexanes/CH_2Cl_2/MeOH, 2:10:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.1 (s, 1H), 8.67 (s, 1H), 8.43 (d, *J* = 9.3 Hz, 1H), 8.14 (br s, 1H), 7.15 (d, *J* = 8.4 Hz, 1H), 6.93 (br d, 2H), 6.37 (d, *J* = 9.3 Hz, 1H), 5.87 (s, 1H), 4.10 (m, 4H), 3.90 (s, 3H), 3.79 (m, 2H), 3.46 (t, *J* = 8.1 Hz, 1H), 2.80 (m, 2H), 2.65 (m, 2H), 2.25–1.83 (m, 5H), 1.66 (m, 5H), 1.49–1.23 (m, 43H),0.86 (t, *J* = 7.2 Hz, 6H), 0.81 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 170.4, 154.3, 148.8, 138.6, 137.2, 126.7, 124.4, 122.6, 120.1, 117.1, 102.6, 100.1, 90.0, 68.7, 66.2, 56.4, 50.5, 44.2, 43.6, 39.0, 38.5, 38.1, 32.1, 30.5, 30.4, 29.9, 29.8, 29.7, 29.4, 28.2, 27.2, 26.5, 25.7, 23.4, 23.0, 14.4, 11.9 ppm; ESI-MS *m*/*z* 925 (M+H)$^+$. Anal. Calcd for $C_{53}H_{85}N_2O_9P$: C, 68.80; H, 9.26; N, 3.03. Found: C, 68.91; H, 9.47; N, 3.29.
15. *Hydroxyapatite binding assay*. A 1 mM solution of each analog was prepared in DMSO and diluted serially in 50 mM Tris–HCl buffer (pH 7, 1% DMSO), to attain a final concentration of 10 nM. Tetracycline was utilized as a reference analyte and ca. 50% bound to hydroxyapatite (HA) at a concentration of 10 nM. The HA slurry was prepared as an admixture of 50 mg HA in 100 mL in 50 mM Tris–HCl buffer containing 1% DMSO. For each analog, two samples were prepared. For one of the two samples, 1 mL of a 10 nM solution of the analog and 100 μL of the HA slurry was pipetted into a microcentrifuge tube. The mixture was agitated gently by inversion for 4 min and then centrifuged at 12,000g for 3 min to sediment the HA. The supernatant was decanted into another microcentrifuge tube. An electronic spectral scan (UV–vis) was recorded for

each analyte over the wavelength range 220–520 nm using a Varian Cary 300 Bio Scan spectrophotometer. The blank sample consisted of 50 mM Tris–HCl buffer containing 1% DMSO. The λ_{max} was determined and the extinction coefficient (e) was then calculated. The absorbance of each analog after incubation with HA was measured at the λ_{max}, and the molar concentration of the compound was then determined using the Beer–Lambert law and the previously calculated extinction coefficient. The fraction adsorbed by HA for each analog and for 17-β-estradiol was subsequently determined and compared to that obtained for tetracycline to afford a binding index relative to tetracycline binding.

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