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Design, synthesis, and biological evaluation of novel estradiol–bisphosphonate conjugates as bone-specific estrogens

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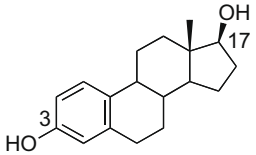
ABSTRACT

Bone deficiency causes osteoporosis and often decreases quality of life in patients with rheumatoid arthritis. Estrogens are known to protect elderly women from bone loss. Synthesis of new estradiol–bisphosphonate conjugates (E_2–BPs) was accomplished and their in vivo activity as bone-specific estrogens were examined. Among them, MCC-565 showed selective estrogenic activity in bones; but it showed little estrogenic activity in the uterus. We also found that the linker moiety in E_2–BPs was essential for the absorption and specificity of the conjugates.

1. Introduction

Bone deficiency causes osteoporosis and often decreases quality of life in patients with rheumatoid arthritis. Bones are always metabolized by osteoclasts and osteoblasts. Osteoclasts absorb bones and osteoblasts replace new bone materials. The increase of osteoclast activity or the decrease of osteoblast activity cause bone loss. One of the hormones that increases bone mass is estrogens including estradiol (E_2) having the steroid structure. Estrogen deficiency caused by menopause induces bone loss in about two-thirds of women. Therefore, it would be possible to treat osteoporosis by estrogen administration as a hormone replacement therapy, which was initiated about 10 years ago.[1,2] Although estrogen undoubtedly increases the bone mass, it often shows adverse effects such as abnormal uterine bleeding and generation of breast cancer and/or uterus cancer. Estrogen receptor agonists such as raloxifene are also employed to preserve bones in osteoporosis,[3–5] but its activity toward bone tissue is not prominent compared with that of estrogen therapy.[6] On the other hand, bisphosphonate compounds tend to be accumulated on the bone surface, increasing the bone mass; therefore, bisphosphonate-including compounds such as etidronate and alendronate were tested for use in osteoporosis therapy.[7–9] These compounds were clinically used; however, they showed toxicity toward the jawbone[10] and caused muscle pain.[11] Developing a drug delivery system targeting bones would be a unique approach for the therapy of postmenopausal bone loss, especially if the estrogens could be delivered selectively to the bone. For this purpose various estrogen conjugates have been prepared, and stable binding between E_2 and the bisphosphonate moiety have been mainly employed. Although several estrogen/bisphosphonate conjugates have been reported,[12–17] none of them has been used clinically for the treatment of osteoporosis.

As the previously prepared estrogen/bisphosphonate compounds showed stable binding between E_2 and the bisphosphonate moiety, the local release of E_2 from these compounds on the bone was found to be difficult. In 1996, Bauss et al. prepared substituted estradiols having a bisphosphonate moiety attached at the 17 position via a linker.[18] They suggested that the conjugate compounds might be hydrolyzed to release active estradiol into the bloodstream.



Estradiol (E_2)

Driven by the suggestion of Bauss et al., we sought to synthesize novel estradiol–bisphosphonate conjugates (E_2–BPs) in order to develop a useful anti-osteoporosis medication (Fig. 1).

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2. Results and discussion

2.1. Synthesis of conjugates

Conjugate **1** was synthesized as shown in Scheme 1. Monobenzyl esterification of Meldrum's acid **7** provided monobenzylester **8** in 54% yield. By use of this half-ester, the ester linkage was installed in the requisite C17 position of 3-methoxymethyl-estradiol **9** to give benzylmalonate **10** with the condensation agent dicyclohehxylcarbodiimide (DCC) in 98% yield. The benzyl group of **10** was reductively cleaved to yield the corresponding acid **11** under catalytic hydrogenation conditions. Coupling **10** with tetraethyl aminomethylenebisphosphonate **12**[19] using DCC in the presence of a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) afforded bisphosphonoamide **13** in 72% yield. Cleavage of the methoxymethyl (MOM) group at 3-position of estradiol and the tetra ethyl ester groups of phosphonate was successively accomplished by the treatment with trimethylsilyl iodide (TMSI) to provide conjugate compound **1** in 95% yield.

Conjugate **6** (MCC-565) was synthesized as shown in Scheme 2. Curtius rearrangement reaction of succinic acid monobenzyl ester **14** using diphenylphosphoryl azide (DPPA) followed by the addition of estrone to the intermediate isocyanate provided the carbamate, which was successively debenzylated by a catalytic hydrogenation to give the desired C-3 carbamate-rinked acid **15** in 28% overall yield. Then, condensation of **15** with **12** using

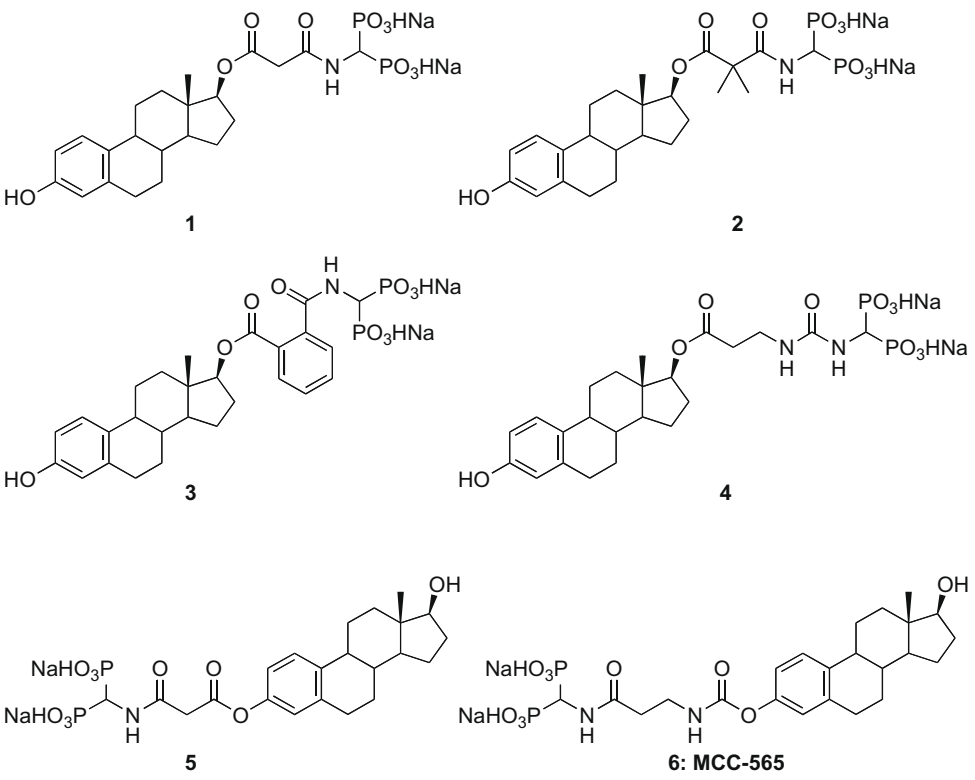
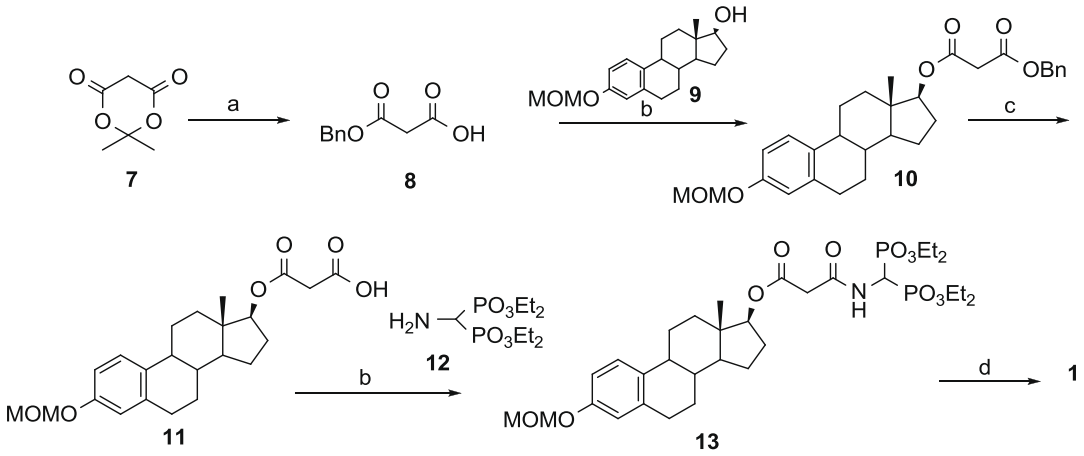
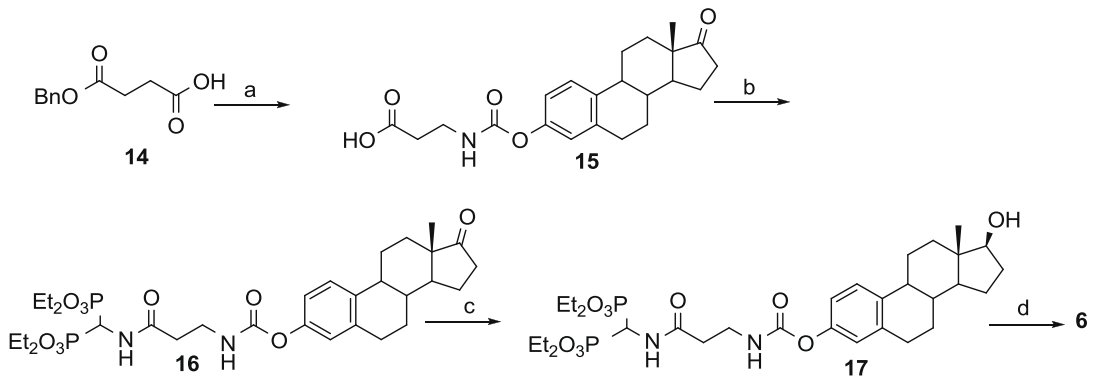


Figure 1. Structures of conjugate compounds.



Scheme 1. Reagents and conditions: (a) BnOH, toluene, reflux, 3 h; (b) DCC, DMAP, CH_2Cl_2, rt, 10 h; (c) 10% Pd-C, H_2, EtOH–THF, rt, 2 h; (d) TMSI, CH_2Cl_2, −20 °C, 0.5 h, then NaOAc, MeOH.



Scheme 2. Reagents and conditions: (a) (i) DPPA, TEA, toluene, then estorone, 100 °C, 1 h; (ii) Pd-C, H_2, EtOH–THF, rt, 1 h; (b) **12**, *N*-methylmorpholine, *iso*-butylthioroformate, −10 °C, 0.5 h; (c) $NaBH_4$, MeOH, 0 °C, 0.5 h; (d) TMSI, CH_3CN, −20 °C, 0.5 h, then NaOAc, MeOH.

isobutylthioroformate to give **16** followed by reduction with sodium borohydride afforded 17β-alcohol **17**. Finally, cleavage of the tetra ethyl ester groups of **16** was accomplished by the treatment with trimethylsilyl iodide (TMSI) to provide the conjugate compound **6** in 84% yield.

Conjugates **2–5** were synthesized by the same procedure used for conjugate **1** or **6**.

Conjugate **1** having malonic acid as a spacer between E_2 and the bisphosphonate was designed, supposing that malonic ester group might be more stable against enzymatic hydrolysis than the succinic or glutaric ester group due to steric hindrance. In fact, conjugate **1** could not be hydrolyzed by esterase to release E_2 after 8 h. Conjugate **2** was even more sterically hindered with the gem-dimethyl group, and it is considered to be more stable than conjugate **1**. Conjugate **3** contained phthalic acid as a spacer. Conjugate **4** showed higher water solubility than the other derivatives, since it contained two NH groups in the linkage. Conjugates **5** and **6** were equipped with activated ester and carbamate at the 3-position, respectively. Conjugate **6** (MCC-565) showed increased the solubility in water, since it also contained two NH groups in the linkage.

Esterase is known to have broad substrate specificity and therefore, is responsible for the hydrolysis of many exogenous compounds.[20] Cathepsin K is an essential protease for osteoporosis.[21,22] It is a cysteine-protease, but it may also hydrolyze esters and amides. Therefore, the free estrogen may be released from the bisphosphonate conjugates by esterase and/or proteases around the bone. In addition, labile E_2–BPs may also be cleaved under acidic conditions, which can be provided by the proton pump in the osteoclasts.[23] Thus, E_2–BPs are likely to be cleaved enzymatically or chemically around osseous tissue.

Conjugate **6** was not hydrolyzed by esterase (Sigma: E2884, positive control: phenylpropionic acid ethylester in PBS, 37 °C) or amidase (Sigma: acylamide amidohydrolase, positive control: benzoyl butylamide in PBS, 37 °C) within 8 h, nor was it hydrolyzed at pH 4 (buffer solution HPCE pH 4.0 solution) even after 1 month. However, E_2 was released from the conjugate into the rat bloodstream from 8 h, time dependently (TLC plate analyzed). Therefore, conjugate **6** is likely to be delivered to the bone, where it would slowly release E_2.

2.2. Bioactivity

The pharmacological effects of conjugates **1–6** were examined in ovariectomized rats. Synthesized E_2–BPs were given to SD female rats at 0.1 mg/kg, which is the optimized amount, by intravenous administration. After the treatment, the uterus weight and femur bone mass were scored. As shown in Figure 2, ovariectomized rats showed a dramatically reduced uterus weight and a slightly decreased bone weight. E_2 increased both the bone mass and uterus weight, indicating that it caused systemic hormonal effects without local selectivity. None of the synthesized compounds increased uterus weight. There was a significant difference of bone mass between estradiol and conjugate **6**.

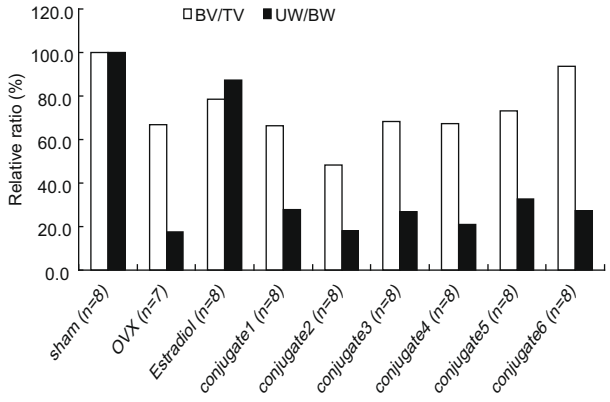


Figure 2. Estrogenic effects on bone and uterus of estradiol and each conjugate compounds in ovariectomized mice (Sham: ovary was exteriorized and interiorized again without ovariectomy. BV: bone volume, TV: tissue volume, UW: uterus weight, BW: body weight).

Conjugate **6** increased the bone mass even more effectively than E_2 without increasing the uterine weight. Bone loss was optimally prevented by conjugate **6** given at 0.1 mg/kg/4 weeks. Moreover, conjugates **6** showed no bisphosphonate activity in rats. Bisphosphonate activity was measured by the suppression of Ca^{2+} ion release from the bone by PTHrp treatment. Conjugate **6** did not suppress the release Ca^{2+} ion from the bone (data not shown). The estrogenic activity of conjugate **6** was studied by use of a competitive estrogen receptor binding assay employing estrogen receptors purified from rat uterus. Conjugate **6** exhibited 2000 times weaker binding activity toward the estrogen receptor than E_2 (Fig. 3).

Contamination of a very small amount of estradiol in conjugate **6** may be responsible for the effect at a high dose of the conjugate. Therefore we concluded that conjugate **6** could show selective estrogenic effects on bones.

Next, we studied whether the pharmacological effects on bones were due to the estrogenic activity of the conjugates. Fulvestrant is a known anti-estrogen.[24] As shown in Figure 4, administration of this chemical clearly suppressed the effect of conjugate **6** on bone. Considering that the uterus weight of the conjugate **6** treated rats did not increase (Fig. 2) Therefore, it is likely that conjugate **6** effectively released estradiol around the bones. It is possible that the 3-

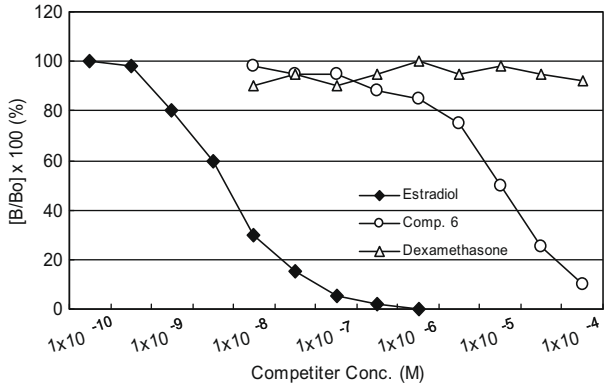


Figure 3. Comparison of estrogen receptor binding activity of conjugate **6** with that of estradiol by use of the competitive receptor binding assay. The total radioactivity of bound [3*H*]-estradiol (3 nM) was 4810.2 cpm. Non-specific binding was determined by radioactivity of bound [3*H*]-estradiol (3 nM) in the presence of excess non-radiolabeled estradiol (30 nM). The control value was 234.2 cpm. Percent binding of [3*H*]-estradiol in the presence of ligand was calculated by the following formula: Percent binding = [(B-NSB)/(Bo-NSB)] × 100 (%).

position is better than the 17-position to obtain more effective compounds. The linker structure of conjugate **6** is likely to be better than that of others, perhaps because compound **6,** having the carbamate structure at the 3-position of E_2, is more labile to biological stimuli.

In conclusion, we designed and synthesized novel E_2–BPs in which the linkers were sensitive to esterases, proteases, and acidic conditions on bones to act as bone-seeking E_2 prodrugs. Among them, conjugate **6** with an activated carbamate moiety was shown to be the most effective and specific for bones.

3. Experimental

^{1}H NMR spectra were recorded at ambient temperature on an AC-250 Brucker spectrometer in $CDCl_3$ or D_2O. ^{13}C NMR and ^{31}P-NMR spectra were recorded at ambient temperature on an Brucker ADBNCE 400 spectrometer in $CDCl_3$ or D_2O. ^{13}C NMR spectra of conjugate 1 was recorded at ambient temperature on an Brucker ADBNCE 600 spectrometer in D_2O. The chemical shifts were given in δ (ppm), and coupling constants were reported in Hz. Mass spectra were obtained with a HITACHI M2000A (positive-SIMS mode). Column chromatography was carried out on Silica Gel 60 (230–400 mesh ASTM; Merck). High Preparative Liquid Column chromatography (HPLC) was carried out on COSMOSIL (C-18 20 × 250 mm, Nakalai Tesque). Elemental analyses were performed with a PERKIN ELMER 240C Analyzer and were within ±0.4% of the theoretical values.

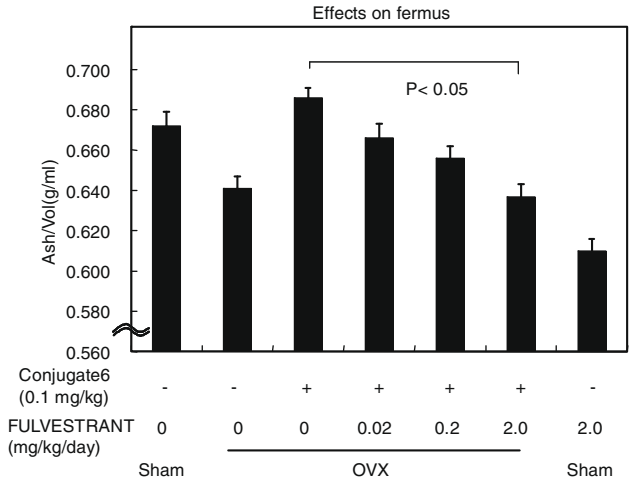


Figure 4. Estrogenic effect on bone of conjugate **6** was abolished by concurrent administration of pure anti-estrogen: FULVAESTRANT.

3.1. Malonic acid monobenzyl ester (8)

A solution of Meldrum's acid **7** (2.32 g) and benzyl alcohol (1.83 ml) in toluene (11.6 ml) was refluxed for 3 h. After removal of the solvent in vacuo, the residue was purified by chromatography over silica gel, with elution with $CHCl_3$–MeOH (10:1), to give **8** (1.7 g, yield: 54%): ^{1}H NMR ($CDCl_3$), δ 10.25 (br s, 1H), 7.37–7.34 (m, 5H), 5.20 (s, 2H), 3.48 (s, 2H). Elemental Anal. (%) Calcd for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 61.73; H, 5.28.

3.2. Malonic acid benzyl ester 3-methoxymethoxy-1,3,5-estratriene-17-yl ester (10)

To a solution of compound **9** (500 mg, 1.58 mmol) in CH_2Cl_2 (5.0 ml) were added DMAP (0.25 g, 2.05 mmol), DCC (0.42 g, 2.05 mmol), and **8** (0.31 g, 1.58 mmol). The resultant mixture was stirred at room temperature for 10 h, and the mixture was then diluted with EtOAc (30 ml). The organic layer was washed with H_2O and saturated brine, dried over anhydrous magnesium sulfate, and concentrated. The residue was purified by chromatography over silica gel with hexane–EtOAc (5:1) as the eluent, to give **10** (640 mg, yield: 98%): ^{1}H NMR ($CDCl_3$), δ 7.38–7.34 (m, 5H), 7.19 (d, 1H, *J* = 6.5 Hz), 6.83 (dd, 1H, *J* = 6.5 Hz, 2.0 Hz), 6.77 (d, 1H, *J* = 2.0 Hz), 5.18 (d, 2H, *J* = 2.3 Hz), 5.15 (s, 2H), 4.74 (t, 1H, *J* = 6.0 Hz), 3.47 (s, 3H), 3.43 (s, 2H), 2.90–2.80 (m, 2H), 2.30–2.10 (m, 3H), 1.90–1.65 (m, 3H), 1.60–1.20 (m, 7H), 0.73 (s, 3H). ^{13}C NMR (D_2O), δ 166.53, 166.43, 155.07, 137.97, 135.21, 133.72, 128.59, 128.52, 128.49, 126.38, 116.21, 113.77, 94.45, 83.85, 67.25, 55.90, 49.69, 43.78, 43.06, 41.85, 38.43, 36.71, 31.59, 29.71, 27.29, 27.16, 26.11, 23.21, 22.66, 14.13, 11.89. Elemental Anal. (%) Calcd for $C_{30}H_{36}O_6$: C, 73.15; H, 7.37. Found: C, 72.86; H, 7.37.

3.3. Malonic acid mono-(3-methoxymethoxy-1,3,5-estratriene-17-yl) ester (11)

A solution of **10** (640 mg, 1.58 mmol), 10% Pd-C (60 mg) in THF (6.0 ml), and EtOH (6.0 ml) was stirred under a H_2 atmosphere at room temperature for 2 h. The reaction mixture was then filtered through a Celite pad, and the filtrate was concentrated in vacuo to give **11** (648 mg, yield: 99%) as white crystals: ^{1}H NMR ($CDCl_3$), δ 7.19 (d, 1H, *J* = 6.5 Hz), 6.82 (dd, 1H, *J* = 6.5 and 1.9 Hz), 6.77 (d, 1H, *J* = 1.9 Hz), 5.14 (s, 2H), 4.78 (t-like, 1H), 3.47 (s, 3H), 3.44 (br s, 2H), 2.90–2.80 (m, 2H), 2.40–1.40 (m, 13H), 0.85 (s, 3H). ^{13}C NMR (D_2O), δ 170.42, 167.46, 155.06, 137.96, 133.65, 126.39, 116.23, 113.78, 94.44, 84.47, 55.91, 49.67, 43.78, 43.13, 40.56, 38.43, 36.78, 29.69, 27.34, 27.16, 26.09, 23.22, 11.99. Elemental Anal. (%) Calcd for $C_{23}H_{30}O_6 \cdot 0.2H_2O$: C, 68.03; H, 7.55. Found: C, 67.89; H, 7.60.

3.4. *N*-(Bis-(diethoxyphosphoryl)-methyl)-malonamic acid (3-methoxymethoxy-1,3,5-estratriene-17-yl) ester (13)

To a solution of **11** (620 mg, 1.54 mmol) in CH_2Cl_2 (7 ml) were added DMAP (245 mg, 2.0 mmol), DCC (635 mg, 2.0 mmol) and tetraethyl aminomethylenebisphosphonate **12** (467 mg, 1.54 mmol). After the resultant mixture had been stirred at room temperature for 10 h, the reaction mixture was diluted with EtOAc (30 ml).

The organic layer was washed with H_2O and saturated brine, dried over anhydrous magnesium sulfate, and concentrated in vacuo. The residue was then purified by chromatography over silica gel, with elution with $CHCl_3$–MeOH (10:1–1:1), to give **13** (504 mg, yield: 72%): ^{1}H NMR ($CDCl_3$), δ 7.77 (d, 1H, J = 7.5 Hz), 7.19 (d, 1H, J = 6.3 Hz), 6.83 (dd, 1H, J = 6.3 Hz, 2.0 Hz), 6.77 (d, 1H, J = 2.0 Hz), 5.15 (s, 2H), 5.06 (dt, 1H, J = 16.2 and 7.5 Hz), 4.76 (t, 1H, J = 6.0 Hz), 4.30–4.10 (m, 8H), 3.47 (s, 3H), 3.41 (s, 2H), 2.90–2.80 (m, 2H), 2.40–2.10 (m, 4H), 1.95–1.85 (m, 2H), 1.85–1.20 (m, 19H), 0.83 (s, 3H). HRMS (CI) calcd for $C_{32}H_{52}NO_{11}P_2$ $[M+H]^+$: 688.3016. Found: 688.3013.

3.5. *N*-(Bis-phosphono-methyl)-malonamic acid 3-hydroxy-1,3,5-estratrien-17-yl ester 2 sodium salt (1)

To a solution of **13** (252 mg, 0.366 mmol) in CH_3CN (10 ml) was added trimethylsilyl iodide (0.313 ml) at −20 °C, and the resultant mixture was stirred under cooling for 30 min. The reaction was then quenched by the addition of CH_2Cl_2 (3.0 ml) and H_2O (0.5 ml). After evaporation of the solvent in vacuo, the residue was diluted with MeOH (5.0 ml). To this reaction mixture was added a solution of 1 M-$CH_3COONa{\cdot}3H_2O$ in MeOH (5.0 ml), and the resultant precipitate was collected by filtration and washed with MeOH/H_2O (5:1, 20 ml) to give the title compound **1** (201 mg, yield: 95.3%) as a white powder: ^{1}H NMR (D_2O), δ 7.01 (d, 1H, J = 8.3 Hz), 6.48 (d, 1H, J = 8.3 Hz), 6.42 (s, 1H), 4.64 (t, 1H, J = 8.8 Hz), 3.38 (s, 2H), 2.85–2.60 (m, 2H), 2.32–1.09 (m, 13H), 0.76 (s, 3H). ^{13}C NMR (D_2O), δ 189.77, 188.63, 173.29, 158.52, 156.04, 141.92, 135.80, 129.84, 118.20, 115.84, 87.83, 52.19, 51.82, 51.35, 46.05, 45.74, 41.13, 39.21, 29.69, 29.44, 28.73, 25.59, 14.23. ^{31}P NMR (D_2O), δ 12.71, 12.59. IR(cm^{-1}), ν 3286, 2925, 1717, 1644, 1543, 1500, 1450, 1346, 1287, 1166, 1055, 1007, 964, 889, 817, 785. Elemental Anal. (%) Calcd for $C_{22}H_{31}NO_{10}P_2Na_2{\cdot}2.5H_2O$: C, 42.59; H, 5.52; N, 2.26. Found: C, 42.48; H, 5.26; N, 2.23. HRMS (CI) calcd for $C_{22}H_{31}NO_{10}P_2$ $[M+Na]^+$: 554.1319. Found: 554.1291.

3.6. 3-(17-oxo-1,3,5-estratrien-3-yloxycarbonylamino)-propionic acid (15)

To a solution of succinic acid monobenzyl ester **14** (571 mg, 2.74 mmol) and triethylamine (458 mg, 3.29 mmol) in toluene (5.7 ml) was added DPPA (0.62 ml, 2.88 mmol) at 0 °C, and the reaction mixture was then stirred at 100 °C for 30 min. After the gas evolution had ceased, the reaction mixture was cooled at 0 °C. To this reaction mixture was added estrone (740 mg, 2.74 mmol) in toluene (7.4 ml) dropwise, and the mixture was subsequently stirred at 100 °C for 1 h. Then the reaction mixture was allowed to cool to room temperature and diluted with chloroform (40 ml). The organic layer was washed with H_2O and saturated brine and dried over anhydrous magnesium sulfate. After removal of the solvent in vacuo, the residue was purified by chromatography over silica gel, with $CHCl_3$–MeOH (10:1) as the eluent, to give colorless crystals (343 mg, yield: 26%). A solution of this compound (343 mg), 10% Pd-C (34 mg) in EtOH (3.4 ml), and THF (3.4 ml) was stirred under a H_2 atmosphere for 1 h. The reaction mixture was then filtered through a Celite pad, and the filtrate was concentrated to give **15** (231 mg, yield: 83%) as a colorless crystal: ^{1}H NMR ($CDCl_3$): δ 7.66 (br s, 1H), 7.23 (d, 1H, J = 8.5 Hz), 6.90–6.80 (m, 3H), 5.62 (t, 1H, J = 6.2 Hz), 3.60–3.40 (m, 2H), 2.95–2.80 (m, 2H), 2.15–2.60 (m, 2H), 2.50–1.90 (m, 7H), 1.70–1.30 (m, 6H), 0.88 (s, 3H). ^{13}C NMR (D_2O), δ 177.10, 154.94, 148.74, 137.86, 136.98, 126.30, 121.67, 118.83, 50.41, 48.00, 44.12, 38.01, 36.48, 35.44, 34.03, 31.52, 29.40, 26.35, 25.76, 21.59, 13.83. IR(cm^{-1}), ν 3368, 2933, 1738, 1698, 1531, 1490, 1454, 1436, 1417, 1372, 1326, 1303, 1260, 1248, 1222, 1155, 1079, 1052, 1004, 965, 912, 896, 820, 792, 768. Elemental Anal. (%) Calcd for $C_{22}H_{27}NO_5$: C, 68.55; H, 7.06; N, 3.63. Found: C, 68.57; H, 7.03; N, 3.64.

3.7. ((3-(17-Oxo-1,3,5-estratrien-3-yloxycarbonylamino) propionylamino)methyl)-bisphosphonic acid tetraethyl ester (16)

A solution of **15** (231 mg, 0.6 mmol), *iso*-butylchloroformate (0.086 ml), and *N*-methylmorpholine (0.073 ml) in THF (10 ml) was stirred at −10 °C for 15 min. To this solution was added compound **12** (0.20 g, 0.66 mmol) in THF (2.0 ml) at −10 °C, and the reaction mixture was then stirred under cooling for 8 h. Next, the reaction mixture was diluted with $CHCl_3$ (40 ml), and washed with H_2O and saturated brine. After the organic layer had been dried over anhydrous magnesium sulfate and concentrated in vacuo, the residue was purified by chromatography over silica gel, with elution with $CHCl_3$–MeOH (10:1–1:1), to give **16** (440 mg, yield: 100%) as a light yellow syrup: ^{1}H NMR ($CDCl_3$), δ 7.49 (br d, 1H, J = 7.5 Hz), 7.24 (br d, 1H, J = 6.3 Hz), 6.85 (dd, 1H, J = 6.3 and 1.5 Hz), 6.83 (d, 1H, J = 1.5 Hz), 6.13 (t, 1H, J = 4.5 Hz), 5.26 (br s, 1H), 5.11 (dt, 1H, J = 16.5 and 7.5 Hz), 4.30–4.10 (m, 8H), 3.60–3.50 (m, 2H), 2.95–2.80 (m, 2H), 2.70–2.58 (m, 2H), 2.55–1.95 (m, 7H), 1.70–1.30 (m, 18H), 0.90 (s, 3H). HRMS (CI) calcd for $C_{31}H_{49}N_2O_{10}P_2$ $[M+H]^+$: 671.2862. Found: 671.2862.

3.8. ((3-(17-Hydroxy-1,3,5-estratrien-3-yloxycarbonylamino) propionylamino)methyl)-bisphosphonic acid tetraethyl ester (17)

To a solution of **16** (402 mg, 0.60 mmol) in MeOH (6.0 ml) was added $NaBH_4$ (34 mg, 0.90 mmol) at 0 °C under stirring, and the resultant mixture was stirred for 30 min. The reaction mixture was then poured into saturated aqueous NH_4Cl (50 ml) and extracted with $CHCl_3$ (30 ml × 2). The organic layer was concentrated in vacuo, after which the residue was purified by chromatography over silica gel with $CHCl_3$–MeOH (10:1–1:1) as the eluent, to give **17** (242 mg, yield: 60%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$): δ 7.21 (d, 1H, J = 8.4 Hz), 6.81 (dd, 1H, J = 2.0 and 10.4 Hz), 6.77 (d, 1H, J = 2.0 Hz), 6.02 (t, 1H, J = 6.2 Hz), 5.04 (dt, 1H, J = 16.5 and 7.5 Hz), 4.3–4.1 (m, 8H), 3.69 (t, 1H, J = 8.1 Hz), 3.62–3.50 (m, 2H), 2.85–2.70 (m, 2H), 2.60–2.50 (m, 2H), 2.35–1.10 (m, 13H), 1.31 (t, 12H), 0.73 (s, 3H). HRMS (CI) calcd for $C_{31}H_{51}N_2O_{10}P_2$ $[M+H\ ]^+$: 673.3019. Found: 673.3013.

3.9. ((3-(17-Hydroxy-1,3,5-estratrien-3-yloxycarbonylamino)-propionyl-amino)methyl)-bisphosphonic acid 2 sodium salts (6)

To a solution of **17** (242 mg, 0.359 mmol) in CH_3CN (2.3 ml) was added trimethylsilyl iodide (0.31 ml, 2.18 mmol) under stirring at −20 °C and the reaction mixture was then stirred for 30 min. Next, the reaction was quenched by the addition of CH_2Cl_2 (3.0 ml) and water (0.5 ml). After removal of the solvent in vacuo, the residue was dissolved with MeOH (3.0 ml). To this reaction mixture was added a solution of 1 M-$CH_3COONa{\cdot}3H_2O$ in MeOH (3.0 ml), and the resultant precipitate was collected by filtration and washed with MeOH/H_2O (5:1, 20 ml) to give the title compound **6** (164 mg, yield: 84%) as a white powder: ^{1}H NMR (D_2O): δ 7.18 (d, 1H, J = 7.6 Hz), 6.7–6.4 (m, 2H), 4.23 (t, 1H, J = 18.6 Hz), 3.52 (t, 1H, J = 7.8 Hz), 2.80–2.60 (m, 6H), 2.2–0.9 (m, 13H), 0.53 (s, 3H). ^{13}C NMR (D_2O), δ 173.54, 158.13, 149.06, 139.83, 139.37, 127.43, 122.61, 119.69, 82.28, 50.13, 48.87, 44.33, 43.54, 38.92, 37.01, 36.44, 29.71, 27.26, 26.60, 23.35, 11.44. ^{31}P NMR (D_2O), δ 12.93, 12.81. IR(cm^{-1}), ν 3370, 3321, 2932, 2872, 1737, 1699, 1532, 1490, 1453, 1436, 1417, 1372, 1327, 1303, 1260, 1248, 1211, 1156, 1079, 1052, 1006, 965, 911, 897, 884, 820, 792, 769.

Elemental Anal. (%) Calcd for $C_{23}H_{32}N_2O_{10}P_2Na_2{\cdot}2H_2O$: C, 43.13; H, 5.45; N, 4.32. Found: C, 43.11; H, 5.45; N, 4.32. HRMS (CI) calcd for $C_{23}H_{34}N_2O_{10}P_2$ $[M+Na]^+$: 583.1584. Found: 583.1614. Melting point: >300 °C, specific rotation: $[\alpha]_D^{25} = -1718$ (*c* 0.085, H_2O)

3.10. *N*-(Bis-phosphono-methyl)-2,2-dimethyl-malonamic acid 3-hydroxy-1,3,5-estratrien-17-yl ester 2 sodium salt (2)

Compound **2** was prepared from compound **10** as a white powder (yield: 52%) according to the similar procedure described for compound **1**: ^{1}H NMR (D_2O); δ 7.04 (d, 1H, J = 8.2 Hz), 6.48 (d, 1H, J = 8.2 Hz), 6.45 (s, 1H), 4.07 (t, 1H, J = 18.6 Hz), 2.70–2.50 (m, 2H), 2.10–1.00 (m, 13H), 1.34 (s, 3H), 1.32 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (D_2O), δ 176.07, 174.05, 153.09, 138.91, 132.79, 126.87, 115.24, 112.87, 84.72, 51.21, 48.91, 48.37, 43.09, 42.98, 38.14, 36.34, 28.95, 26.65, 26.51, 25.77, 22.68, 22.48, 22.40, 11.44. ^{31}P NMR (D_2O), δ 12.70, 12.58. IR(cm^{-1}), ν 3210, 2927, 1708, 1617, 1536, 1288, 1078, 872, 816. HRMS (CI) calcd for $C_{24}H_{35}NO_{10}P_2$ $[M+Na]^+$: 582.1632. Found: 582.1603.

3.11. *N*- (Bis-phosphono-methyl)-phthalamic acid 3-hydroxy-1,3,5-estratrien-17-yl ester 2 sodium salt (3)

Compound **3** was prepared from Compound **9** and phthalic anhydride as a white powder (yield: 11%) according to the similar procedure described for compound **1**: ^{1}H NMR (D_2O), δ 7.80–7.50 (m, 3H), 7.50–7.30 (m, 1H), 7.09 (d, 1H, J = 8.2 Hz), 6.60–6.40 (m, 2H), 4.73 (t, 1H, J = 7.4 Hz), 4.53 (t, 1H, J = 19.8 Hz), 2.70–2.50 (m, 2H), 2.30–1.10 (m, 13H), 0.71 (s, 3H). Elemental Anal. (%) Calcd for $C_{27}H_{31}N_2O_{10}P_2Na_2{\cdot}2.5H_2O$: C, 46.42; H, 4.93; N, 2.01. Found: C, 46.42; H, 4.71; N, 1.83. HRMS (CI) calcd for $C_{27}H_{33}NO_{10}P_2$ $[M+Na]^+$: 616.1475. Found: 616.1448.

3.12. 3-(3-(Bis-phosphono-methyl)-ureido)-propionic acid 3-hydroxy-1, 3, 5-estratrien-17-yl ester 2 sodium salt (4)

Compound **4** was prepared from compound **9** and succinic anhydride as a white powder (yield: 32%) according to the similar procedure described for compound **1**: ^{1}H NMR (D_2O); δ 7.18 (d, 1H, J = 7.6 Hz), 6.7–6.4 (m, 2H), 4.23 (t, 1H, J = 18.6 Hz), 3.52 (t, 1H, J = 7.8 Hz), 2.80–2.60 (m, 6H), 2.20–0.9 (m, 13H), 0.53 (s, 3H). ^{13}C NMR (D_2O), δ 153.06, 138.84, 135.44, 131.21, 129.47, 128.22, 126.81, 115.20, 112.82, 85.18, 48.84, 43.01, 38.17, 26.47, 25.79, 11.57. ^{31}P NMR (D_2O), δ 12.68, 12.55. IR(cm^{-1}), ν 3269, 2923, 2161, 1711, 1634, 1540, 1503, 1393, 1286, 1146, 1077, 889, 819, 785, 713. Elemental Anal. (%) Calcd for $C_{23}H_{32}N_2O_{10}P_2Na_2{\cdot}2H_2O$: C, 43.13; H, 5.67; N, 4.37. Found: C, 43.28; H, 5.39; N, 4.36. HRMS (CI) calcd for $C_{23}H_{34}N_2O_{10}P_2$ $[M+Na]^+$: 583.1584. Found: 583.1594.

3.13. *N*-(Bis-phosphono-methyl)-malonamic acid 17-hydroxy-1,3,5-estratrien-3-yl ester 2 sodium salt (5)

Compound **5** was prepared from estrone and compound **8** as a white powder (yield: 33%) according to the similar procedure described for compound **6**: ^{1}H NMR (D_2O), δ 7.26 (d, 1H, J = 8.4 Hz), 6.70–6.60 (m, 2H), 4.23 (t, 1H, J = 18.6 Hz), 3.51 (t, 1H, J = 7.5 Hz), 2.80–2.55 (m, 6H), 2.20–0.95 (m, 13H), 0.53 (s, 3H). Elemental Anal. (%) Calcd for $C_{22}H_{29}NO_{10}P_2Na_2{\cdot}2H_2O$: C, 43.22; H, 5.44; N, 2.29. Found: C, 43.27; H, 5.21; N, 2.31. HRMS (CI) calcd for $C_{22}H_{31}NO_{10}P_2$ $[M+Na]^+$: 554.1319. Found: 554.1318.

3.14. Animal experiments

Estradiol was dissolved with 95% of corn oil and 5% of benzyl alcohol. Conjugates **1–6** were dissolved in a mixture of 1% carboxymethyl cellulose and 1% citrate buffer. Female SD rats (7 weeks old) were purchased from Japan SLC Co. Ltd (Shizuoka, Japan) and were housed for 4 weeks in our laboratory. Before ovariectomy, each animal was allocated to one of the study groups so that mean body weight of each group would be equal. All animals except the sham-operated controls were bilaterally ovariectomized. On the next day, conjugates **1–6** were injected intravenously. The E_2 group was injected three times per week for 4 weeks on the first, second, and fifth days. The total amount of E_2 given was 560 mg/kg. The estrogenic effects on bone were analyzed by measuring the bone volume of the secondary spongiosa of the left tibia and by measuring ash weight of the right femurs.

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