Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 11, pp. 1375-1380, 1994

0960-894X/94 $7.00+0.00

0960-894X(94)00160-X

SYNTHESIS OF β-ESTRADIOL-3-BENZOATE-17-(SUCCINYL-12A-TETRACYCLINE): A POTENTIAL BONE-SEEKING ESTROGEN

Mark W. Orme* and Virender M. Labroo

ZymoGenetics, Inc.
A Subsidiary of Novo Nordisk A/S
4225 Roosevelt Wy NE
Seattle, WA 98105

Abstract: The title compound, designed as a bone-seeking estrogen prodrug, was synthesized, characterized and shown to bind to hydroxylapatite, the mineral constituent of bone tissue.

Introduction: Osteoporosis is the reduction of bone mass or the atrophy of skeletal tissue which can lead to debilitating hip fractures and vertebral compression fractures. It is one of the most common diseases of postmenopausal women, afflicting approximately 25% of this population[1]. It is generally accepted that a reduction in estrogen levels after menopause is the primary cause of postmenopausal osteoporosis[1,2]. Bone loss can be arrested by the administration of exogenous estrogens[3], and in fact this remains a preferred means to prevent this disease[4]. Unfortunately, there are a number of side effects associated with estrogen treatment, including a well-established correlation with endometrial cancer[5].

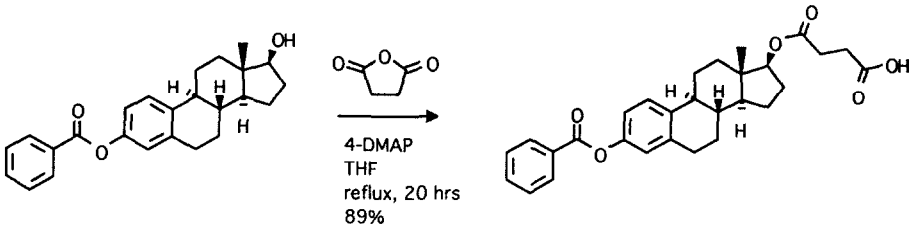
The prevention of postmenopausal bone loss by estrogen treatment may be attributed to a direct effect of estrogen on bone[6]. Consequently, the selective delivery of estrogen to bone tissue could result in a decrease in the side effects of estrogen therapy for the treatment of osteoporosis, both by limiting systemic estrogen levels, and by reducing the dosage required to achieve effective therapeutic results[7]. Therefore, we have attempted to prepare a prodrug of estrogen that will target bone.

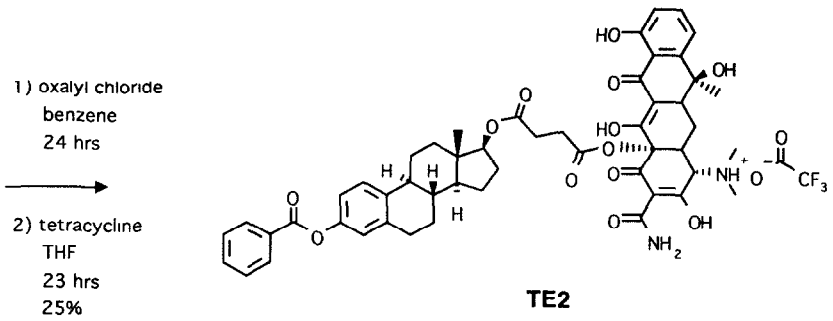
There are certain compounds which are known to exhibit bone affinity, that is, which bind to bone mineral, and tend to accumulate and become incorporated in mineralizing bone. The tetracyclines and bisphosphonates (such as (1-hydroxyethylidene)bisphosphonic acid or (1-aminohexylidene)bisphosphonic acid) are two such classes of compounds. For the purpose of being a bone-seeking moiety of an estrogen conjugate, tetracycline has the advantage of being relatively non-toxic and of possessing comparatively little biological activity in bone[8].

It has been claimed that the carbonic anhydrase inhibitor acetazolamide was linked to tetracycline and that this conjugate had significant affinity for bone mineral, however, no compound was actually purified and characterized[9]. Additionally, it has been reported that a number of hydroxy steroids have been conjugated to alkyl bisphosphonates[10]. There has also been a report of the linkage of several bioactive compounds to a putative bone seeking moiety based upon polymalonic acid, which is proposed to mimic the poly-α-carboxy-glutamic acid residues of the bone protein osteocalcin[11].

We have succeeded in the synthesis and characterization of a potential bone-seeking estrogen conjugate, TE2, by linking β-estradiol-3-benzoate with tetracycline via a succinate ester.

Synthetic Scheme





TE2

Chemistry: The preparation of TE2 is outlined in the scheme above. β-Estradiol-3-benzoate-17-hemisuccinate[12] was synthesized by the reaction of β-estradiol-3-benzoate with excess succinic anhydride and 4-DMAP in refluxing THF. After flash chromatography and recrystalization, pure material was obtained in 89% yield[13]. The acid chloride of this material (prepared by treatment with oxalyl chloride) was allowed to react with dry tetracycline (TC) in THF under anhydrous conditions[14]. Following the reaction by HPLC it was determined that an initial kinetic product formed, and slowly decomposed as a second thermodynamic product appeared. The key to the successful synthesis of this compound was the workup and purification. The reaction was filtered, and the filtrate concentrated to dryness on a rotary evaporator. This was then taken up in a small amount of THF and precipitated with ether. The solid was collected, rinsed, and the ether precipitation was repeated. Purification by preparative reverse phase HPLC, taking care to avoid excessively acidic conditions, gave the TFA salt of TE2 as a lyophilized powder in 25% yield[15].

The structure proposed for TE2 is based upon the evidence below. NMR assignments were made by comparison to those of TC[16]. The TC 3- and 12-enol and 10-phenol protons are clearly observed in the ^1H NMR spectrum of TE2. There is also a broad singlet at 5.09 ppm, which corresponds to the 4.9 ppm resonance in the ^1H NMR spectrum of TC. This 4.9 ppm peak has been previously assigned, although tentatively, as the 6-OH proton, with the 12a-OH resonance being hidden[16a]. We believe that this assignment is correct, and that TE2 is a 12a ester of TC, for the following reasons: (1) Anhydrotetracycline[16b] and minocycline[16c], that do not have a 6-OH group, have no peak in the 4.5-5.5 ppm region, and the 12a-OH is unobserved; (2) In 7-chlorotetracycline, the 4.9 ppm peak, as well as the 6-methyl resonance, are shifted downfield relative to those in TC[16a]; (3) In the ^{13}C NMR spectrum of TE2, the C-12a carbon is shifted downfield by 5.2 ppm relative to that in TC (the C-6 carbon is shifted upfield by 2.5 ppm), which is consistent with an alcohol to ester transformation[17]; (4) There is chemical precedence for the acylation of the 12a-OH and not the 6-OH under equilibrium conditions (5-hydroxytetracycline is bis-acetylated at the 5- and 12a- hydroxyls by acetic anhydride at rt, 14 days[18]); (5) TC can be silated to give tris(10-O,12a-O,2-carboxamido)trimethylsilyltetracycline without silation of the 6-OH[16b]; and (6) The inspection of 3D conformational models of TC clearly show that the 6-OH is more sterically hindered than the 12a-OH[16e,19].

Hydroxylapatite Binding: To test if the TE2 conjugate has the potential to retain the bone accumulation properties of tetracycline in vivo, it's binding to hydroxylapatite, the mineral constituent of bone, was measured[20]. 3.0 mM THF solutions of TE2 and controls were prepared. The concentration of each compound in solution was measured before and after a 1hr incubation with hydroxylapatite (100 mg/ml of solution) by integration of their HPLC chromatogram. The results summarized in the table below show that TE2 has significant affinity for bone mineral in organic solution.

Hydroxylapatite Binding

Compound	Percent Bound to Bone Mineral
Tetracycline	>99
β-Estradiol-3-benzoate	<1
β-Estradiol	<1
TE2	>99

Summary: A tetracycline-estrogen conjugate was prepared which retains the bone mineral affinity of tetracycline in vitro. This compound contains a potential biolabile linkage (ester) which may enable this compound to act as a bone-seeking prodrug of estrogen in vivo.

Acknowledgments: We wish to thank Prof. Stanley Raucher and the Dept. of Chemistry at the Univ. of Washington for the use of laboratory and instrument facilities. We also thank Dr. William Howald and Dr. Ross Lawrence for obtaining mass spectra, and Dr. Tom Pratum for help with NMR experiments. Finally, we thank Dr. Jim Piggott, Dr. Martin Edwards, and Dr. Steve Bain for their interest, discussions and support.

References and Notes

1. Komm, B. S.; Terpening, C. M..; Benz, D. J.; Graeme, K. A.; Gallegos, A.; Korc, M.; Greene, G. L.; O'Malley, B. W.; Haussler, M. R. *Science* **1988**, 241, 81.

2. (a) Richelson, L. S.; Heinze, H. W.; Melton III, L. J.; Riggs, B. L. *N. Engl. J. Med.* **1984**, 311, 1273.
(b) Stevenson, J. C.; Lees, B.; Devenport, M.; Cust, M. P.; Gangar, K. F. *Br. Med. J.* **1989**, 298, 924.

3. Thalassinos, N. C.; Gutteridge, D. H.; Joplin, G. F.; Fraser, T. R. *Clin. Sci.* **1982**, 62, 221.

4. (a) Nachtigall, L. E.; Nachtigall, R. H.; Nachtigall, R. D.; Beckman, E. M. *Obstet. Gynecol.* **1979**, 53, 277.
(b) Kreiger, N.; Kelsey, J. L.; Holford, T. R.; O'Connor, T. *Am. J. Epidemiol.* **1982**, 116, 141.

5. (a) Antunes, C. M.; Stolley, P. D.; Rosenshein, N. B.; Davies, J. L.; Tonascia, J. A.; Brown, C.; Burnett, L.; Rutledge, A.; Pokempner, M.; Garcia, R. *N. Engl. J. Med.* **1979**, 300, 9.
(b) Jick, H.; Watkins, R. N.; Hunter, J. R.; Dinan, B. J.; Madsen, S.; Rothman, K. J.; Walker, A. M. *N. Engl. J. Med.* **1979**, 300, 218.
(c) Vesey, M. P. *J. Roy. Soc. Med.* **1984**, 77, 542.

6. (a) Pilbeam, C. C.; Klein-Nulend, J.; Raisz, L. G. *Biochem. Biophys. Res. Commun.* **1989**, 163, 1319.
(b) Takano-Yamamoto, T.; Rodan, G. A. *Proc. Nat. Acad. Sci. USA*. **1990**, 87, 2172.

7. Peck, W. A.; Riggs, B. L.; Bell, N. H.; Wallace, R. B.; Johnston, C. C.; Gordon, S. L.; Shulman, L. E. *Am. J. Med*. **1988**, 84, 275.

8. (a) Likins, R. C.; Pakis, G. A. *Nature* **1964**, 203, 1069.
(b) Gruden, L. *Calc. Tiss. Res.* **1973**, 13, 41.

9. (a) Pierce Jr., W. M.; Waite, L. C. *Proc. Soc. Exp. Bio. Med.* **1987**, 186, 96.
(b) Pierce Jr., W. M.; Waite, L. C. **1986**, *EP Number* 0 201 057.

10. Saari, W. S.; Rodan, G. A.; Fisher, T. E.; Anderson, P. S. **1993**, *US Patent Number* 5,183,815.

11. Thompson, D. D.; Anderson, P. S.; Rodan, G. A. **1989**, *EP Number* 0 341 961 A2.

12. Blickenstaff, R. P.; Gerzon, K. *Cytotoxic Estrogens Horm. Recpt. Tumors, [Proc. Worshop]*; Raus, J.; Martens, H.; Leclercq, G., Eds.; Acedemic: London, Engl., **1980**; pp. 89-105.

13. Mp=138-139 °C, $[\alpha]^{24}_D$ +26.1° (c=2.16, dioxane). ^{1}H NMR (200 MHz, $CDCl_3$, ppm downfield from TMS) 8.20 (d, J=11.9 Hz, 2H, aromatics), 7.64-6.93 (m, 6H, aromatics), 4.73 (t, J=8.2 Hz, 1H, C_{17} methine), 2.90-1.10 (m, 19H, estradiol nucleus and succinyl methylenes), 0.84 (s, 3H, C_{18} methyl). ^{13}C NMR (50 MHz, $CDCl_3$, solvent as reference, ppm) 178.10, 172.07, 165.43, 148.65, 138.19, 137.84, 133.46, 130.11, 129.67, 128.49, 126.47, 121.59, 118.67, 83.15, 49.74, 43.96, 42.95, 38.19, 36.82, 29.50, 29.12, 29.01, 27.45, 27.00, 26.00, 23.29, 12.03. IR ($CHCl_3$, cm^{-1}) 3154, 3026-2865, 1732, 1711. UVmax (normalized, nm) 226, 272 (sh). MS (EI) *m/e* (relative intensity) 77 (18), 84 (62), 105 (100), 159 (16), 254 (12), 359 (16), 476 (M^+, 26). HRMS (EI) 476.2145 (476.2196 calcd.). Anal. Calcd. for $C_{29}H_{32}O_6$: C, 73.09: H, 6.77. Found: C, 72.66: H, 6.69.

14. Addition of pyridine or 4-DMAP resulted in the generation of many products, and the addition of TEA gave no improvement of purity, reaction rate, or yield.

15. 99+% purity by HPLC. $[\alpha]^{24}_D$ -72.6 (c=0.864, dioxane). ^{1}H NMR (500 MHz, DMSO-d6, ppm downfield from TMS) 16.53 (hump, 1H, tc 3-enol), 15.33 (br s, 1H, tc 12-enol), 11.60 (s, 1H, tc 10-phenol), 9.72 (br s, 1H, tc amide), 9.41 (br s, 1H, tc amide), 8.11 (d, J=7.4 Hz, 2H, E2 aromatics), 7.75 (t, J=7.4 Hz, 1H, E2 aromatic), 7.59 (m, 3H, E2 aromatics, tc 8-H), 7.32 (d, J=8.5 Hz, 1H, E2 aromatic), 7.12 (d, J=7.6 Hz, 1H, tc 7-H), 7.01 (d, J=8.3 Hz, 1H, tc 9-H), 6.95 (m, 2H, E2 aromatics), 5.09 (br s, 1H, tc 6-OH), 4.66 (t, J=8.4 Hz, 1H, E2 17-H), 4.58 (d, J=1.5 Hz, 1H, tc 4-H), 3.3-1.3 (m, 24H, E2 steroid nucleus, succinyl methylenes, tc 5a-H, 4a-H and 5-H_2) 2.50 (s, 6H, tc $N(CH_3)_2$), 1.50 (s, 3H, tc 6-methyl) and 0.81 (s, 3H, E2 18-methyl). ^{13}C NMR (125 MHz, DMSO-d6, ppm downfield relative to DMSO (39.50 ppm)) 192.52 (tc C-11), 187.50 (tc C-1, 3), 185.55 (tc C-12), 172.35, 172.31, 171.30 (tc $CONH_2$, succinyl carbonyls), 164.74 (benzoyl carbonyl), 161.33 (tc C-10), 158.12 (q, J=32 Hz, TFA), 148.39, 147.87, 137.86, 137.50, 136.80, 133.99, 129.71, 129.04, 128.98, 126.36, 121.58, 118.92, 117.24, 115.81, and 114.27 (aromatics), 107.20 (tc C-11a), 96.09 (tc C-2), 82.44 (E2 C-17), 79.36 (tc C-12a), 67.99 (tc C-4), 65.48 (tc C-6), 49.65 (E2 nucleus), 45.73 and 45.60 (br, $N(CH_3)_2$), 43.38, 42.65, 40.50, 37.80, 36.46, 34.66, 29.03, 28.98, 28.91, 27.15, 26.53, 25.67, 22.82, 22.26, 20.42, and 11.90 (tc C-5a, C-4a, 6-methyl, C-5, succinyl methylenes, and E2 nucleus carbons). IR (KBr, cm^{-1}) 3395 (br), 2930-2860, 1739, 1677, 1615, 1580, 1451, 1205, 1133, 800, 708. UVmax (nomalized, nm) 224, 268 (sh), 363. HRMS (FAB, M+H) 903.3682 (903.3704 calcd.).

16. (a) Williamson, D. E. and Everett Jr., G. W. *J. Am. Chem. Soc.* **1975**, 97, 2397.
(b) Hassall, C. H. and Thomas, G. J. *J. Chem. Soc. Sec. C.* **1970**, 636.
(c) Nilges, M.. J.; Enochs, W. S.; Swartz, H. M. *J. Org. Chem.* **1991**, 56, 5623.
(d) Drexel, R. E.; Olack, G.; Jones, C.; Chmurny, G. N.; Santini, R.; Morrison, H. *J. Org. Chem.* **1990**, 55, 2471.
(e) Celotti, M.; Fazakerley, G. V. *J. Chem. Soc. Perkin Trans. 2* **1977**, 2, 1319.
(f) Asleson, G. L.; Stoel, L. J.; Newman, E. C.; Frank, C. W. *J. Pharm. Sci.* **1974**, 63, 1144.

17. For example, the methine of t-butanol, which roughly matches the environment of C-12a, has a ^{13}C NMR resonance in $CDCl_3$ at 68.95 ppm. In t-butyl acetate, this peak is shifted to 79.90 ppm.

18. Hochstein, F. A.; Stephens, C. R.; Conover, L. H.; Regna, P. P.; Pasternack, R.; Gordon, P. N.; Pilgrim, F. J.; Brunings, K. J.; Woodward, R. B. *J. Am. Chem. Soc.* **1953**, 75, 5455.

19. (a) Palenik, G. J.; Mathew, M. *J. Am. Chem. Soc.* **1978**, 100, 4464.
(b) Molecular mechanics calculations, MM2/TINKER, Chem3D Plus 3.1.

20. (a) Misra, D. N. *Calcif. Tissue Int.* **1991**, 48, 362.
(b) Steendijk, R. *Acta Anat.* **1964**, 56, 368.

(*Received in USA* 5 *November* 1993; *accepted* 2 *May* 1994)