ELSEVIER

Synthesis and binding-analysis of 5E-[19-(2-bromoacetoxy)methyl]25-hydroxyvitamin D_3 and 5E-25-hydroxyvitamin D_3-19-methyl[(4-azido-2-nitro)phenyl]glycinate: Novel C_{19}-modified affinity and photoaffinity analogs of 25-hydroxyvitamin D_3

James K. Addo* and Rahul Ray†

*†Bioorganic Chemistry and Structural Biology Group, Vitamin D Laboratory, Departments of Medicine and Physiology, Boston University School of Medicine, Boston, Massachusetts 02218 USA and *Department of Chemistry, Boston University, Boston, Massachusetts, 02215 USA*

Synthesis of novel C_{19}-modified affinity and photoaffinity analogs of vitamin D_3 and 25-hydroxyvitamin D_3(25-OH-D_3) is described. A key step in the synthesis is a Horner–Emmons reaction between C_{19}-nor-cyclovitamin D_3-C_{19}-ketone or C_{19}-nor-25-hydroxy-cyclovitamin D_3-C_{19}-ketone and diethyl cyanomethylphosphonate. Competitive radioligand binding assays with human serum vitamin D-binding protein (DBP) and 5E-[19-(2-bromoacetoxy)methyl]25-hydroxyvitamin D_3 and 5E-25-hydroxyvitamin D_3-19-methyl[(4-azido-2-nitro)phenyl]-glycinate, 25-OH-D_3-analogs containing affinity and photoaffinity probes at C_{19}-position, demonstrated that these compounds displaced radiolabeled 25-OH-D_3 from the binding pocket of DBP in a dose-dependent manner. Thus, these affinity and photoaffinity analogs are potentially useful in determining the ligand binding site topographies of DBP and possibly the vitamin D receptor. (*Steroids* **63**:218–223, 1998) *© 1998 by Elsevier Science Inc.*

Keywords: affinity and photoaffinity analogs of vitamin D_3 and 25-hydroxyvitamin D_3; vitamin D-binding protein; vitamin D receptor; binding site mapping

Introduction

Research in the chemistry and biology of vitamin D_3 is currently being pursued vigorously by the revelations that $1\alpha,25(OH)_2D_3$ is involved in several cellular processes, including gene regulation, cell differentiation, immune regulation, and calcium homeostasis.[1] This has further been heightened by the observation that $1\alpha,25(OH)_2D_3$ and several of its synthetic analogs have the potential in the intervention and treatment of several disease states, including osteoporosis, psoriasis, renal osteodystrophy, leukemia, and cancer of various organs.[2,3] It is well understood that these properties of $1\alpha,25(OH)_2D_3$ are manifested by its highly specific interaction with a nuclear receptor (vitamin D_3 receptor, VDR) in the target cell.[4,5] Identification of the three-dimensional structure of this receptor, particularly its ligand-binding pocket, is a necessary step in the development of analogs useful for the rational design of more potent $1\alpha,25(OH)_2D_3$-based therapeutic agents.

The development of new affinity and photoaffinity analogs are of continuing importance in the vitamin D field because they are required for the characterization of the three-dimensional structure of the ligand-binding site of VDR, particularly in the absence of its crystal structure.[6] In the past, we and others have developed affinity and photoaffinity analogs of 25-hydroxyvitamin D_3 [25-OH-D_3, the biological precursor of $1\alpha,25(OH)_2D_3$], and successfully labeled the vitamin D_3 sterol-binding domains of vitamin D-binding protein, DBP, a serum receptor/transporter protein for vitamin D_3 and its metabolites,[7–14] and VDR.[15–21] These affinity and photoaffinity analogs contain labeling probes at C_3-position of the parent steroid,[7–21] except in

Address correspondence to Rahul Ray, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118. E-mail: bapi@bu.edu
Received July 9, 1997; accepted January 20, 1998.

655 Avenue of the Americas, New York, NY 10010

0039-128X/98/$19.00
PII S0039-128X(98)00009-9

1α,25-dihydroxyvitamin D_3-1-bromoacetate, where the affinity probe is attached at the C_1-position of the parent steroid.[14] In addition, we have recently developed a methodology for synthesizing affinity- and photoaffinity-labeling analogs of vitamin D_3 containing the affinity/photoaffinity probes at the C_6-position of vitamin D_3.[22]

In this paper, we report the synthesis of C_{19}-derivatized affinity and photoaffinity analogs of 25-OH-D_3 [(**17**) and (**18**), respectively] and results of the competitive binding analysis of these compounds with human serum DBP.[23] These compounds are potentially important in mapping the ligand-binding sites of DBP and VDR, the key proteins in the vitamin D endocrine system, which are responsible for the metabolic activation and physiological manifestations of 1α,25$(OH)_2D_3$, the vitamin D hormone.

Experimental

All the chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless mentioned otherwise. NMR spectra were taken in $CDCl_3$ using tetramethylsilane (TMS) as internal standard on either a 270 MHz (JEOL GSX 270, JEOL USA, Peabody, MA) or a 400 MHz (JEOL GSX 400) NMR spectrometer. Human DBP was from Calbiochem (San Diego, CA). 25-Hydroxy[26(27)-^{3}H]vitamin D_3 (^{3}H-25-OH-D_3), specific activity 20.6 Ci/mmol was purchased from New England Nuclear (Boston, MA). 25-OH-D_3 was a kind gift from Drs. Richard Gray and James Yager, Amoco Research Co., Naperville, IL. In general, all the operations involving compounds containing azido-nitrophenyl group were carried out in the dark.

6*R*-Methoxy-19-Nor-10-keto 3,5-cyclovitamin D_3 (5). This compound was synthesized by a modification of the published procedure.[24] A mixture of 6*R*-methoxy-3,5-cyclo-cholecalciferol (**3**), (500 mg, 1.25 mmol), 4-methylmorpholine-*N*-oxide, (415 mg, mmol, 0.7 eq.) and OsO_4 (150 mg, mmol, eq.) in tetrahydrofuran (THF) (10 mL), t-butanol (10 mL) and H_2O (5 mL) was stirred at room temperature for 2 h. Saturated aqueous $NaHSO_3$ (5 m L) was added, and the mixture extracted with EtOAC (3 $\times$ 10 mL). The organic phase was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness to give an oily residue. The oil (710 mg) was dissolved in methanol (15 mL) and H_2O (7 mL), and $NaIO_4$ (800 mg, 3.75 mmol) was added. The mixture was stirred at room temperature for 2 h and after evaporation to dryness, H_2O and EtOAC were added. The organic phase was separated from the aqueous phase and washed with brine. The organic phase was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness to give an oily residue. The residue was purified by column chromatography (5% EtOAc/hexane) to give (428 mg, 85% yield) of 19-nor-10-oxo-6*R*-methoxy-3,5-cyclovitamin D_3 (**5**). The corresponding 25-OH-D_3 compound (**6**) was also obtained by the same procedure in high yield. NMR spectra of (**5**)[24] and (**6**)[25] were consistent with the published ones.

10*E* and 10-*Z*-6*R*-methoxy-19-cyano-3,5-cyclovitamin D_3 (7a and 7b). To a stirred suspension of NaH (4.46 mg, 0.186 mmol, 3 eq.) in 5 mL of anhydrous 1,1-dimethoxyethane (DME) was added (30.1 μL, 0.186 mmol, 1 eq.) of diethylcyanomethylphosphonate, and the mixture stirred at room temperature for 15 min, followed by the addition of C_{19}-nor-3,5-cyclovitamin D_3-C_{19}-ketone (**5**) (25 mg, 0.06 mmol) in DME (5 mL). The reaction mixture was stirred at room temperature for 12 h followed by the addition of 5 mL of water and extraction with EtOAc (3 $\times$ 5 mL). The organic phase was washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by preparative thin-layer chromatrography (TLC) (5% EtOAc/Hexane) to give (19.27 mg) of the *E* isomer (**7a**) and (1.93 mg) of the *Z* isomer (**7b**) (80% combined yield) as an oil. *E*-isomer (**7a**): ^{1}H NMR ($CDCl_3$): δ 0.54 (3H, s, C_{18}-H), 0.868 and 0.870 (6H, d, $C_{26,27}$-H), 0.92 (3H, d, C_{21}-H), 1.06–2.88 (m), 3.25 (3H, s, OMe), 3.97 (1H, d, C_6-H), 4.96 (1H, d, C_7-H), 5.56 (1H, s, C_{19}-H). *Z*-isomer (**7b**): δ 0.49 (3H, s, C_{18}-H), 0.84 and 0.86 (6H, d, $C_{26,27}$-H), 0.91 (3H, d, C_{21}-H), 1.05–2.81 (m), 3.24 (3H, s, OMe), 3.98 (1H, d, C_6-H), 4.97 (1H, d, C_7-H), 5.58 (1H, s, C_{19}-H). The *E*, *Z* stereochemistry was proved by an NOE between C_7-H and C_{19}-H.

In a similar manner, 18.14 mg and 2.27 mg were respectively obtained for 10-*E* and 10-*Z*-25-hydroxy-19-cyano-6*R*-methoxy-3,5-cyclovitamin D_3 (**8a**) and (**8b**) in a combined yield of 75% using NaH (4.31 mg, 0.179 mmol, 3.0 eq.), diethyl cyanomethylphosphonate (28.97 μL, 0.179 mmol, 3 eq), and 19-nor-10-oxo-25-hydroxy-6*R*-methoxy-3,5-cyclovitamin D_3 (**6**) (25 mg, 0.0598 mmol). In general, NMR spectra of all the 25-OH-D_3-derivatives were very similar to those of the corresponding vitamin D_3 compounds, except in the aliphatic region, where vitamin D_3 compounds produced a 6H doublet at approximately δ0.9, and the corresponding 25-OH-D_3 compounds had two singlets (each 3H) centered at approximately δ0.95.

10*E*-19-formyl-6*R*-methoxy-3,5-cyclovitamin D_3 (9). To a solution of (**7a**) (61 mg, 0.143 mmol) in 1 mL of toluene at $-$60°C was added a 1 M solution of DIBAL in toluene (35 μL, 0.215 mmol, 1.5 eq.), and the reaction mixture was allowed to warm slowly to $-$20°C. After 30 min of stirring at $-$20°C, the mixture was quenched with 2 mL of 10% HCl. The product was extracted with EtOAC (3 $\times$ 5 mL), and the combined organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. The residue was purified by preparative TLC (5% EtOAC/hexane) to give 37.10 mg of (**9**) (60% yield). ^{1}H NMR: δ 0.56 (3H, s, C_{18}-H), 0.866 and 0.87 (6H, d, $C_{26,27}$-H), 0.93 (3H, d, C_{21}-H), 1.05–2.65 (m), 3.26 (3H, s, OMe), 4.25 (1H, d, C_6-H), 4.84 (1H, d, C_7-H), 6.25 (1H, d, C_{19}-H), 9.86 (1H, d, CHO). In a similar manner,10*E*-25-hydroxy-19-formyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**10**) (19.24 mg, 64% yield) was obtained from 10*E*-25-hydroxy-19-cyano-6*R*-methoxy-3,5-vitamin D_3 (**8a**) (30 mg).

10*E*-19-hydroxymethyl-6*R*-methoxy-3,5-cyclovitamin D_3 (11). A mixture of $NaBH_4$ (1.07 mg, 0.028 mmol, 1.2 eq.) in methanol (1 mL) at 0°C was treated dropwise with a solution of (**9**) (10 mg, 0.025 mmol) in methanol (1 mL). The resulting mixture was stirred at 0°C for 30 min, and 5 mL of saturated NH_4Cl was added. The reaction mixture was then extracted with EtOAC (3 $\times$ 5 mL). The combined organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. The residue was purified by preparative TLC (5% EtOAC/hexane) to give 5.53 mg. of (**11**) (55% yield). ^{1}H NMR: δ 0.49 (3H, s, C_{18}-H), 0.81(6H, d, $C_{26,27}$-H), 0 88 (3H, d, C_{21}-H), 1.04–2.01 (m), 3.19 (3H, s, OMe), 4.04 (1H, d, C_6-H), 4.19 (2H, d, OCH_2), 4.89 (1H, d, C_7-H), 5.71 (1H, t, C_{19}-H). In a similar manner, 10*E*-25-hydroxy-19-hydroxymethyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**12**) (5.02 mg, 50% yield) was obtained from 10*E*-25-hydroxy-19-formyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**10**) (10 mg, 0.023 mmol).

10*E*-19-[(2-bromoacetoxy)methyl]-6*R*-methoxy-3,5-cyclovitamin D_3 (13). A mixture of (**11**) (20 mg, 0.05 mmol), $BrCH_2CO_2H$ (19.50 mg, 0.14 mmol, 3 eq.), DCC (48 mg, 0.23 mmol, 5 eq.) and DMAP (8.53 mg, 0.07 mmol, 1.5 eq.) was stirred in 1 mL of anhydrous CH_2Cl_2 at room temperature for 15 min, after addition of 5 ml H_2O to quench the reaction, 10 mL of EtOAC was added. The organic layer was separated from the aqueous phase, dried with anhydrous $MgSO_4$, and concentrated in

vacuo. The residue was purified by preparative TLC (5% EtOAC/hexane) to give (18.45 mg, 72% yield) of 10*E*-19-(2-bromoacetoxy)methyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**13**). ^{1}H NMR: δ 0.50 (3H, s, C_{18}-H), 0.81 (6H, d, $C_{26,27}$-H), 0.89 (3H, d, C_{21}-H), 1.03–2.01 (m), 2.54 (m), 3.21 (3H, s, OMe), 3.82 (2H, s, CH_2Br), 4.18 (2H, d, C_6-H), 4.59–4.81 (2H, m, OCH_2), 4.95 (1H, d, C_7-H), 5.73 (1H, t, C_{19}-H). In a similar manner, 10*E*-25-hydroxy-19-(2-bromoacetoxy)methyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**14**). (7.12 mg, 70% yield) was obtained from 10*E*-25-hydroxy-19-(hydroxymethyl)-6*R*-methoxy-3,5-cyclovitamin D_3 (**12**) (8 mg, 0.02 mmol), $BrCH_2CO_2H$ (7.49 mg, 0.05 mmol, 3 eq.), DCC (18.56 mg, 0.09 mmol, 5 eq.), and DMAP (3.297 mg, 0.027 mmol, 1.5 eq.).

5*E*[19-(2-Bromoacetoxy)methyl]vitamin D_3 (16). A mixture of (**13**) (10 mg, 0.018 mmol) and p-TsOH.H_2O (1.38 mg, 0.07 mmol, 0.4 eq.) in 1 mL of a 3:1 mixture 1,4-dioxane:H_2O was heated for 15 min at 55°C. After cooling to room temperature, 5 mL of EtOAC was added and the mixture washed with 2 mL of saturated aqueous $NaHCO_3$ solution. The organic phase was separated and dried with anhydrous $MgSO_4$ and evaporated in vacuo. Preparative TLC (25% EtOAC/hexane) of the residue gave (4.59 mg, 48.70% yield) of compound (**16**).

In a similar manner, 5*E*-[19-(2-bromoacetoxy)methyl] 25-hydroxyvitamin D_3 (**17**) (2.13 mg, 45% yield) was obtained from 10*E*-25-hydroxy-19-(2-bromoacetoxy)methyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**14**) (5 mg, 0.008 mmol) and p-TsOH.H_2O (0.67 mg, 0.004 mmol, 0.40 eq.). ^{1}H NMR of (**17**) (400 MHz, in $CDCl_3$: δ 0.56 (3H, s, C_{18}-H), 0.95 and 0.96 (6H, two singlets, $C_{26,27}$-Hs), 1.01–2.82 (m), 3.81 (2H, s, CH_2Br), 3.99 (1H, m, C_3-H), 4.90 (2H, d, CH_2OCO), 5.43 (1H, t, C_{19}-H), 5.84 (1H, d, C_7-H), 6.22 (1H, d, C_6-H).

10*E*-6*R*-methoxy-25-hydroxy-3,5-cyclovitamin D_3-19-methyl-[(4-azido-2-nitro)phenyl]glycinate (15). A mixture of (**12**) (10 mg, 0.02 mmol), 4-azido-2-nitrophenylglycine[16] (15.93 mg, 0.067 mmol, 3 eq.), DCC (23 mg, 0.112 mmol, 5 eq.), and DMAP (4.09 mg, 0.033 mmol, 1.5 eq.) was stirred in 1 mL of anhydrous CH_2Cl_2 at room temperature for 30 min in the dark. The reaction was quenched with 5 mL of H_2O, and the mixture was evaporated with EtOAC (3 × 5 mL), filtered, dried with anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by preparative TLC (25% EtOAC/hexane) to give 6.5 mg (79% yield) of 10*E*-6*R*-methoxy-25-hydroxy-3,5-cyclovitamin D_3-19-methyl[(4-azido-2-nitro)phenyl]glycinate (**15**). ^{1}H NMR: δ 0.48 (3H, s, C_{18}-H), 0.91–0.92 (6H, two singlets, $C_{26,27}$-H), 0.88 (3H, d, C_{21}-H), 1.01–2.03 (m), 3.22 (3H, s, OMe), 3.81 (2H, s, CH_2Br), 4.16 (3H, m, C_6-H and -NCH_2), 4.61 and 4.62 (2H, dd, OCH_2), 4.95 (1H, d, C_7-H), 5.71 (1H, t, C_{19}-H), 6.71 (1H, d, C_5-phenyl), 7.12 (1H, d, C_6-phenyl), 7.89 (1H, C_3-phenyl), and 8.34 (1H, t, NH).

25-hydroxyvitamin D_3-19-methyl[(4-azido-2-nitro)phenyl]glycinate (18). To a solution of 10*E*-6*R*-methoxy-25-hydroxy-3,5-cyclovitamin D_3-19-methyl[(4-azido-2-nitro)phenyl]glycinate (**15**) (5 mg, 0.008 mmol) in 1 mL of 3:1 mixture of 1,4-dioxane-H_2O, was added p-TsOH.H_2O (0.57 mg, 0.003 mmol, 0.4 eq.), and the solution was stirred at room temperature for 30 min in the dark. After cooling to room temperature, EtOAc (3 × 5 mL) was added to extract the organic layer. The organic layer was separated from the aqueous layer, filtered, dried with anhydrous $MgSO_4$, and evaporated in vacuo. Preparative TLC (40% EtOAC/Hexane) of the crude extract gave 2.34 mg, 48% yield of (**18**). ^{1}H NMR: δ 0.51 (3H, s, C_{18}-H), 0.89 and 0.91 (6H, two singlets, $C_{26,27}$-H), 1.02–2.81 (m), 3.98 (1H, m, C_3-H), 4.07 (2H, d, CH_2O_2C), 4.84 (2H, d, $NHCH_2CO_2$), 5.45 (1H, t, C_{19}-H), 5.89 (1H, d, C_7-H), 6.23 (1H, d, C_6-H), 6.70 (1H, d, C_5-phenyl H), 7.14 (1H, d, C_6-phenyl H), 7.98 (1H, d, C_3-phenyl H), 8.34 (1H, t, NH).

Competitive radioligand binding assays of 25-OH-D_3, (17) and (18) with human serum DBP. These assays were carried out according to published procedure.[13] In general, samples of DBP (200 ng), ^{3}H-25-OH-D_3 (3,500 cpm in 10 μL of ethanol) and various concentrations of either 25-OH-D_3 or (**17**) or (**18**) (1 pmol–63.8 pmol) were incubated in a buffer (50 mM Tris.HCl, 150 mM NaCl, 1.5 mM EDTA, 0.1% Triton X-100, pH 8.3, total volume 0.5 mL) at 4°C for 20 h, followed by the incubation with of ice-cold slurry of Dextran-coated charcoal,[13] centrifugation and radioactive counting.

Results and discussion

Development of affinity/photoaffinity analogs require, as a general rule, synthesis of corresponding radiolabeled analog in a few short steps using a commercially available radioactive precursor of high specific activity.[26] Faced with these restrictions, synthesis of C_{19}-modified affinity/photoaffinity reagents by the procedure of Yamada et al., involving base-catalyzed functionalization of the sulfur-diooxide adduct of vitamin D_3,[27] deemed completely impractical.

In 1983, Paaren et al. reported the oxidation of C_{10-19} methylene of 25-hydroxy 3,5-cyclovitamin D_3 to its C_{19}-nor-C_{19}-keto derivative.[25] This information provided us with a starting point to elaborate the C_{19}-position of vitamin D molecule. We first set out to do model studies using vitamin D_3 due to the prohibitive cost of 25-OH-D_3. Cyclovitamin D_3 Ketone (**5**) was prepared in an improved yield of 85% from 6*R*-methoxy-3,5-cyclovitamin D_3 (**3**) by a modification of the of the published procedure.[23] We examined the reaction of this ketone using a variety of Wittig or Horner–Emmons reagents (Figure 1). In our hands, only diethylcyanomethylphosphonate [$(EtO)_2POCH_2CN$] gave a fruitful Horner–Emmons reaction with this ketone using DME as the solvent, to give a 10:1 mixture of the *E* and *Z*-isomers (**7a**) and (**7b**) separable by preparative TLC in a combined yield of 80%.[24] The following Wittig or Horner–Emmons reagents did not give any fruitful reaction with ketone (**5**): $(EtO)_2POCH_2COOEt$, $[Ph_3P^+$-CH_2CH_2CH-(OCH_2CH_2O) Br^-, Ph_3P^+-CH_2CH-(OCH_2CH_2O) Br^-, $(EtO)_2POCN$, $(EtO)_2POCH_2{=}{=}CH_2CO_2Et$. Also, the reaction with $(EtO)_2POCH_2CN$ does not occur in THF or toluene. The structure of the Horner–Emmons adducts were confirmed by their ^{1}H-NMR spectra showing a single C_{19}-H at δ5.6 for the *E*-isomer (**7a**) and δ5.8 for *Z*-isomer (**7b**). The C_{19}-H of (**7a**) was expected to be more shielded by the 7,8-olefinic electrons than that of (**7b**). An NOE experiment was conducted on each isomer to establish their stereochemistry. In the *E*-isomer (**7a**), a positive NOE was observed in the ^{1}H resonance signal corresponding to the C_{19}-H single proton when the C_7-H was irradiated. No NOE effect was observed in the ^{1}H resonance signal of the corresponding hydrogen of the *Z*-isomer (**7b**). Reduction of the major, *E*-isomer (**7a**) with DIBAL followed by acid treatment gave the aldehyde (**9**) (60% yield), which, on reduction with $NaBH_4$, gave the alcohol (**11**) (55% yield). The affinity label (**16**) was obtained by treatment of (**11**) with $BrCH_2COOH$ in the presence of DCC and DMAP (72%

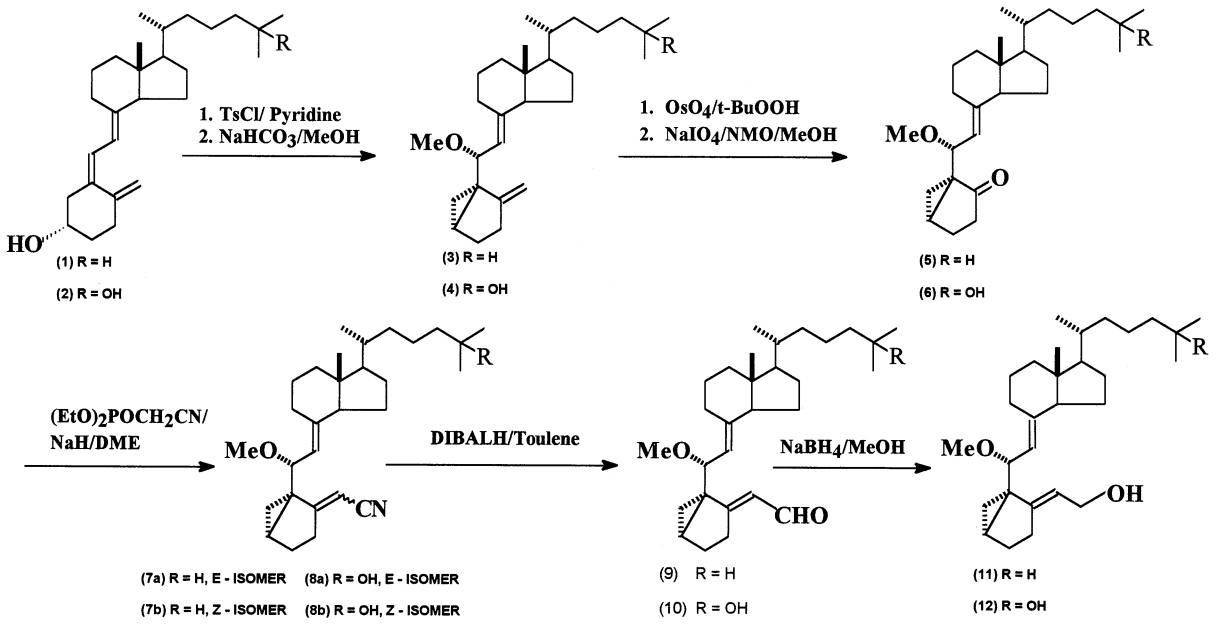

Figure 1 Scheme for the synthesis of 10*E*-19-hydroxymethyl-6*R*-methoxy-3,5-cyclovitamin D_3 and 10*E*-25-hydroxy-19-hydroxymethyl-6*R*-methoxy-3,5-cyclovitamin D_3 (**12**)

yield), followed by solvolysis with TsOH in 3:1 dioxane/H_2O) (48.7% yield).

Having successfully established the viability of the synthetic steps, we used 25-OH-D_3 as the starting material to synthesize the desired analogs by applying similar sequence of steps as above. The reaction of (**6**) with $(EtO)_2POCH_2CN$ proceeded smoothly to give 8:1 ratio of the *E* and *Z*-isomers (**8a**) and (**8b**) respectively in combined yield of 75%. DIBAL reduction of the major *E* isomer (**8a**) led to aldehyde (**10**) (64% yield), whose reduction with $NaBH_4$ gave alcohol (**12**) (50% yield). The affinity analog (**17**) was obtained by coupling $BrCH_2COOH$ with (**12**) in the pres-

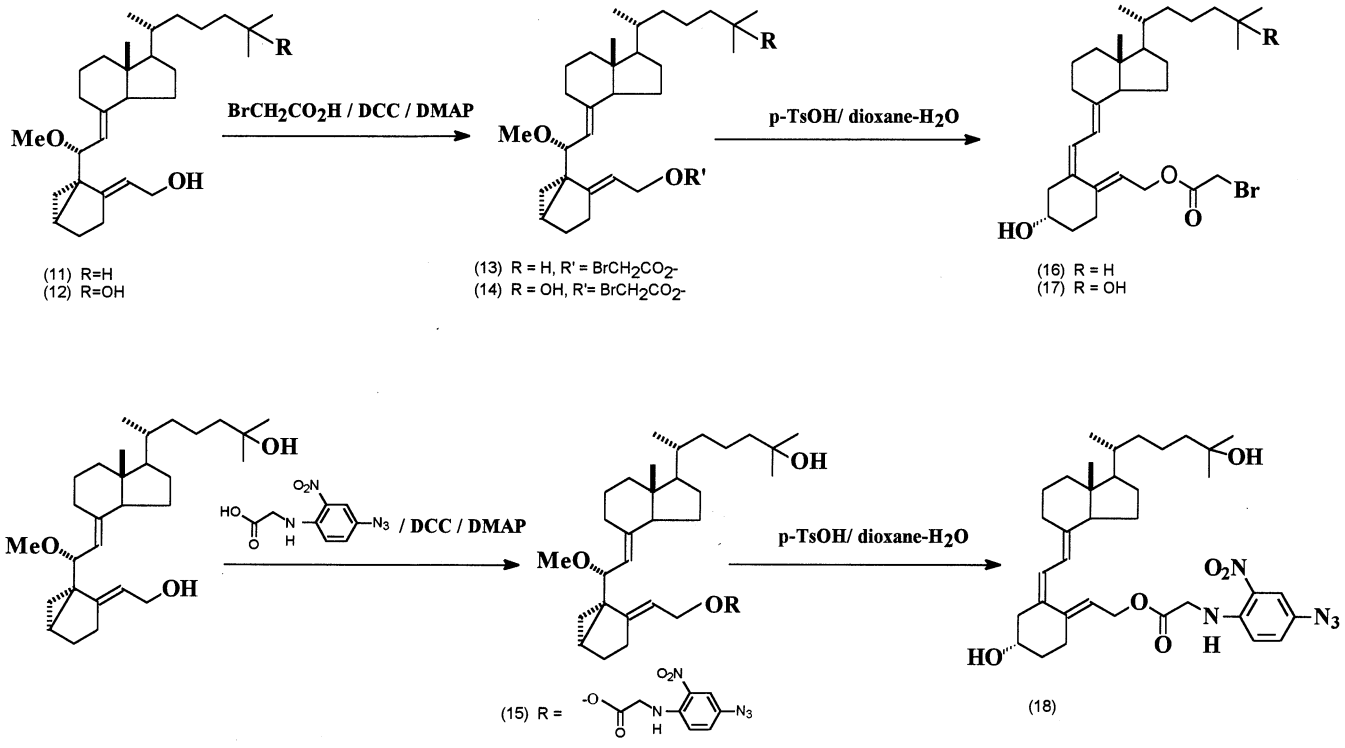

Figure 2 Scheme for the synthesis of C_{19}-modified affinity and photoaffinity labeling analogs of vitamin D_3 and 25-OH-D_3

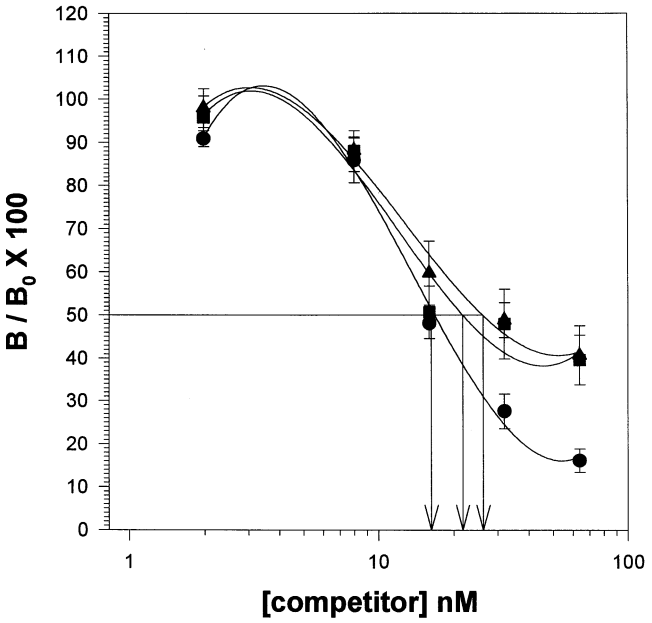

Figure 3 Competitive radioligand binding assays of 25-OH-D_3, compounds (**17**) and (**18**) with human serum DBP. Samples of DBP (200 ng), ^{3}H-25-OH-D_3 (3,500 cpm in 10 μL of ethanol) and various concentrations of either 25-OH-D_3 or (**17**) or (**18**) (1 pmol–63.8 pmol) were incubated in the dark in a buffer (50 mM Tris HCl, 150 mM NaCl, 1.5 mM EDTA, 0.1% Triton X-100, pH 8.3, total volume 0.5 mL) at 4°C for 20 h, followed by the incubation with an ice-cold slurry of Dextran-coated charcoal, centrifugation and radioactive counting.[13] Error bars represent standard deviation of three determinations. ●, 25-OH-D_3; ■, compound (**17**); ▲, compound (**18**).

ence of DCC and DMAP. (72% yield) followed by solvolysis with p-TsOH in a mixture of dioxane and H_2O (3:1) (45% yield). Additionally, the photoaffinity analog (**18**) was obtained from (**12**) by coupling with azidonitrophenylglycine[16] in the presence of DCC and DMAP (79% yield), and subsequent solvolysis (48% yield; Figure 2).

Although synthesis of C_{19}-alkanoic acid derivatives of vitamin D_3 was reported in 1983,[27] no biological/biochemical study has been reported to date, possibly due to the unavailability of the corresponding 25-OH-D_3 compounds. Thus, no information is currently available about the effect of C_{19}-modification of vitamin D_3 and its metabolites on DBP and VDR binding. Competitive binding assays of the affinity analog (**17**) and the photoaffinity analog (**18**) with DBP demonstrated that the binding affinities of these compounds are only 1.2- and 1.6-times less than that of 25-OH-D_3, the natural ligand (Figure 3). It is noteworthy that, although the photoaffinity probe has a much higher steric demand than the affinity probe, both (**17**) and (**18**) possessed almost equal binding affinity for DBP (Figure 3). These results strongly suggest that C_{19}-modification of 25-OH-D_3 is not significantly detrimental towards DBP-binding. Hence, the C_{19}-affinity and photoaffinity analogs of 25-OH-D_3, with the intact biologically relevant hydroxyl groups, imparting selectivity and specificity towards receptor-binding, could be valuable tools in determining the three-dimensional architecture of the ligand-binding pockets of these proteins.

Acknowledgments

The authors would like to thank Mr. Lincoln Scott (Chemistry Department, Boston University, Boston, MA) for many helpful discussions, and Dr. N. Swamy (Boston University School of Medicine) for assistance with the binding assays. This work was supported partly from grants from NIDDK (RO1 44337 and 47418) of the National Institute of Diabetes, Digestive and Kidney Diseases of the National Institutes of Health.

References

1. Holick MF (1989). Vitamin D: biosynthesis, metabolism and mode of action. In: DeGroot LJ, Besser GM et al (eds), *Endocrinology, vol. 2.* WB Saunders, Philadelphia, pp 902–926.
2. DeLuca HF (1988). The vitamin D story: a collaborative effort of basic science and clinical medicine. *Fed Proc Am Soc Ex Biol* **2**:224–236.
3. A review on vitamin D and cancer. Eisman JA (1994). New insight into vitamin D physiology and potential cancer therapy. In: Heersche JN, Kanis JA (eds) *J Bone Miner Res* Elsevier, Amsterdam, **8**:45–76.
4. Pike JW (1991). Vitamin D receptors: structure and function in transcription. *Ann Rev Nutr* **11**:189–21.
5. Haussler MR (1986). Vitamin D receptors: nature and function. *Ann Rev Nutr* **6**:527–562.
6. Sweet FW, Murdock GL (1987). Affinity labeling of hormone specific proteins. *Endocr Rev* **8**:154–184.
7. Ray R, Holick SA, Hanafin N, Holick MF (1986). Synthesis of 25-hydroxyvitamin D_3-3β-[3′-*N*-(4-azido-2-nitro)phenyl]glycinate: a photoaffinity analog of 25-hydroxyvitamin D_3 capable of cross-linking to the rat plasma vitamin D binding protein. *Biochemistry* **25**:4729–4733.
8. Ray R, Bouillon R, Van Baelen H, Holick MF (1991). Synthesis of 25-hydroxyvitamin D_3-3β-[3′-*N*-(4-azido-2-nitro)phenyl]amino-propylether, a second generation photoaffinity analog of 25-hydroxyvitamin D_3: photoaffinity labeling of rat serum vitamin D binding protein. *Biochemistry* **30**:4809–4813.
9. Ray R, Bouillon R, Van Baelen H, Holick MF (1991). Photoaffinity labeling of human serum vitamin D-binding protein, and chemical cleavages of the labeled protein: identification of a 11.5 KDa peptide, containing the putative 25-hydroxyvitamin D_3-binding site. *Biochemistry* **30**:7638–7642.
10. Haddad JG, Hu YZ, Kowalski MA, Laramore C, Ray K, Robzyk P, Cooke NE (1992). Identification of the sterol- and actin-binding domains of plasma vitamin D binding protein (Gc-globulin). *Biochemistry* **31**:7174–7181.
11. Swamy N, Ray R (1995). 25-Hydroxy[26,27-methyl-^{3}H]vitamin D_3-3β-(1,2-epoxypropyl)ether: an affinity labeling reagent for human vitamin D-binding protein. *Arch Biochem Biophys* **319**:504–507.
12. Link R, Kutner A, Schnoes HK, DeLuca HF (1987). Photoaffinity labelling of serum vitamin D binding protein by 3-deoxy-3-azido-25-hydroxyvitamin D_3. *Biochemistry* **26**:3957–3964.
13. Swamy N, Ray R (1996). Affinity labeling of rat serum vitamin D binding protein. *Arch Biochem Biophys* **333**:139–144.
14. Swamy N, Dutta A, Ray R (1997). Roles of structure and orientation of ligands and ligand mimicks inside the ligand-binding pocket of the vitamin D-binding protein. *Biochemistry* **36**:7432–7436.
15. Brown TA, DeLuca HF (1991). Photoaffinity labeling of the 1α,25-dihydroxyvitamin D_3 receptor. *Biochim Biophys Acta* **1073**:4729–4733.
16. Ray R, Holick SA, Holick MF (1985). Synthesis of a photoaffinity-labelled analogue of 1,25-dihydroxyvitamin D_3. *J Chem Soc Chem Comm* **11**:702–703.
17. Ray R, Rose SR, Holick SA, Holick MF (1985). Evaluation of a photolabile derivative of 1,25-dihydroxyvitamin D_3 as a photoaffinity probe for 1,25-dihydroxyvitamin D_3 receptor. *Biochem Biophys Res Commun* **132**:198–203.

18. Ray R, Holick MF (1988). The synthesis of a radiolabeled photoaffinity analog of 1,25-dihydroxyvitamin D_3. *Steroids* **51**:623–630.
19. Ray R, Ray S, Holick MF (1993). Photoaffinity labeling of chick intestinal 1α,25-dihydroxyvitamin D_3 receptor. *Steroids* **58**:462–465.
20. Ray R, Ray S, Holick MF (1994). 1α,25-dihydroxyvitamin D_3-3-deoxy-3β-bromoacetate, an affinity labeling analog of 1α,25-dihydroxyvitamin D_3. *Bioorg Chem* **22**:276–283.
21. Ray R, Swamy N, MacDonald PN, Ray S, Haussler MR, Holick MF (1996). Affinity labeling of 1α,25-dihydroxyvitamin D_3 receptor. *J Biol Chem* **271**:2012–2017.
22. Addo JK, Ray R. C-6-Functionalized analogs of vitamin D_3, 25-OH-D_3 and 1,25$(OH)_2D_3$: potential molecular probes for determining ligand binding site topographies of vitamin D-binding protein and vitamin D receptor. In: Norman AW, Bouillon R, Thomasset M (eds), Vitamin D chemistry, biology and clinical applications of the steroid hormone. Proceedings of the tenth workshop on vitamin D, Strasbourg, France, May 24–29, 1997, University of California, Riverside Press, pp 97–98.
23. Addo JK, Swamy N, Ray R. Synthesis and functional characterization of the C-19 affinity and photoaffinity analogs of 25-OH-D_3. Abstract presented at the Tenth workshop on vitamin D, Strasbourg, France, May 24–29, 1997.
24. Eguchi T, Kakinuma K, Ikegawa N (1991). Synthesis of 1α-[19-^{13}C]hydroxyvitamin D_3 and ^{13}C NMR analysis of the conformational equilibrium of the A-ring. *Bioorg Chem* **19**:327–332.
25. Paaren HE, Schnoes HK, DeLuca HF (1983). Electrophilic addition of OsO_4 25-hydroxycholecalciferol and its 3,5-cyclo derivative. J Org Chem **48**:3819–3820.
26. Bayley H, Knowles JR (1977). Photoaffinity labeling. *Methods Enzymol* **46**:69–114.
27. Yamada S, Suzuki T, Takayama H (1983). Stereoselective synthesis of (5E)- and (5Z)-vitamin D_3-alkanoic acids via vitamin D_3-sulfur dioxide adducts. *J Org Chem* **48**:3483–3488.