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The Synthesis of [26,27-^{11}C]Dihydroxyvitamin D_3, a Tracer for Positron Emission Tomography (PET)

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Abstract—1α,25-Dihydroxyvitamin D_3, an endogenous ligand with the highest affinity for the vitamin D receptor (VDR), was labeled with ^{11}C for use in biological experiments. The radionuclide was incorporated via the reaction of [^{11}C]methyllithium on a methyl ketone precursor in tetrahydrofuran at −10 °C. Deprotection of the labeled intermediate yielded 2.5–3 GBq [26,27-^{11}C]1α,25-dihydroxyvitamin D_3 [^{11}C-1,25$(OH)_2$ D_3] with specific radioactivity averaging 100 GBq/μmol at the end of synthesis and HPLC purification. The entire process took 48 min from the end of radionuclide production. In vitro binding experiments in rachitic chick purified VDR demonstrated the high affinity binding of this novel tracer. Thus; ^{11}C-1,25$(OH)_2$ D_3 is available for in vivo distribution studies and may be suitable for the positron emission tomography (PET) determination of VDR levels and occupancy in animals and humans.

Introduction

The hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3], a metabolite of vitamin D_3, maintains calcium homeostasis, inhibits proliferation, stimulates differentiation, and induces apoptosis in a wide range of normal and malignant cells by binding to specific intracellular receptors,[1,2] namely the vitamin D receptor (VDR), and is the most potent endogenous ligand for this receptor. Other, nongenomic, activities have been attributed to the binding of 1,25$(OH)_2$ D_3 to a membrane-bound receptor.[3] The actions of 1,25$(OH)_2$ D_3 and its analogues are thus of interest as pharmaceuticals in fields such as oncology,[4,5] endocrinology, immunology, and bone disease.[6–8]

Of potential interest to researchers and clinicians would be the ability to locate and quantify the VDR in tumors or other tissues in vivo in laboratory animals and humans. Of potential interest to those in VDR-related drug development would be the ability to measure the fraction of unoccupied VDR in vivo after administration of a pharmaceutical dose of a VDR-binding drug. Through the use of positron emission tomography (PET) and a tracer based on a VDR ligand, the goal of in vivo VDR measurements was our aim.

In this report, the ^{11}C ($t_{1/2} = 20$ min) labeling of 1,25$(OH)_2$ D_3 and its potential for VDR imaging were investigated. The choice for the incorporation of the positron-emitting radionuclide was at the 26,27-carbons. 26,27-Positional labeling of 1,25$(OH)_2$ D_3 has been reported incorporating tritium by reacting tritiated methylmagnesium bromide with 1α-hydroxy-26,27-dinorvitamin D_3-25-carboxylate.[9] In the current work, [^{11}C]methyllithium[10] was selected as the carbanion source, employing as precursor 1α and 3β hydroxyl *tert*-butyldimethylsilyl-protected 1α-hydroxy-25-keto-27-norvitamin D_3. Following rapid ^{11}C incorporation, protecting group removal would furnish the desired compound in a time frame compatible with the short half-life of this radionuclide.

The synthesis of [26,27-^{11}C]1α,25-dihydroxyvitamin D_3 (^{11}C-1,25$(OH)_2$ D_3) and the precursor required to make it, along with validation of radiotracer binding in vitro, are described herein.

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Results and Discussion

1,25(OH)$_2$ D$_3$ was radiolabeled with ^{11}C. The synthetic approach was one employing formation of a single carbon–carbon bond on an otherwise intact molecule, allowing completion of the synthesis and HPLC purification within the constraints of the 20 min half-life. In addition to standard chromatographic analysis for chemical and radiochemical purities, in vitro binding studies were performed using a commercially available solubilized rachitic chick purified VDR preparation to verify that the quality of the radiotracer was sufficient for further in vivo work.

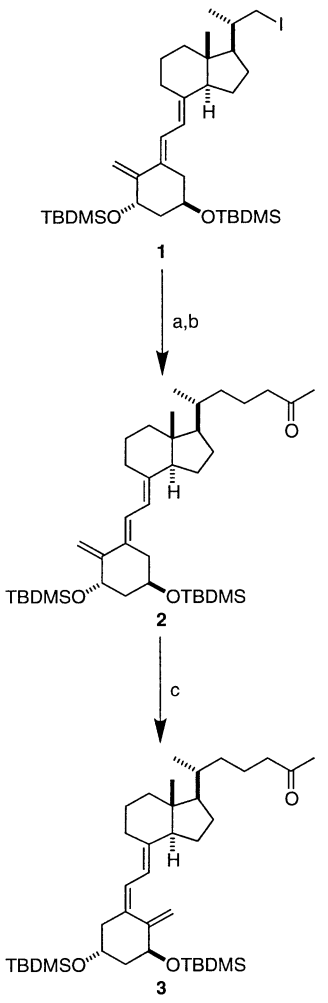
Chemistry and radiochemistry

Starting from the iodo compound **1**, the precursor for ^{11}C labeling **3** was synthesized in two steps[11] (Scheme 1). Synthesis of **4**, an HPLC standard for a ^{11}C-labeled intermediate, was achieved in one step[11] from 1,25(OH)$_2$ D$_3$ (Scheme 2).

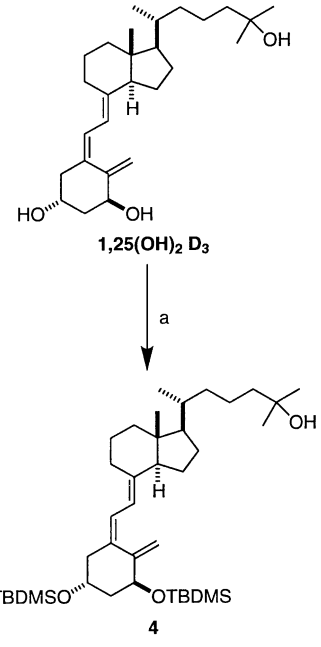
^{11}C-1,25(OH)$_2$ D$_3$ was synthesized rapidly (48 min average from the end of cyclotron irradiation) using a reliable method (more than 14 syntheses without failure) as outlined in Scheme 3. The first step, reaction of [^{11}C]methyllithium[10] with methyl ketone **3** was performed using a published method in which the reaction vial is charged with the precursor prior to trapping [^{11}C]methyl iodide.[12] The temperatures used in this work during trapping of methyl iodide (−70 °C) and reaction of [^{11}C]methyllithium (−10 °C) were significantly lower than those reported (−10 °C and room temperature, respectively). We chose the lower temperature because in our experience[13] higher yields of both [^{11}C]methyl iodide trapping and [^{11}C]methylithium reaction are achieved with fewer side reactions. The ^{11}C incorporation yield was estimated to be as high as 50%, although this value was difficult to measure due to loss of radioactivity as [^{11}C]methane and the very lipophilic nature of the labelled intermediate, [^{11}**C**]**4**. The second step, cleavage of two *tert*-butyldimethylsilyl (TBDMS) groups with tetrabutylammonium fluoride, was estimated to be quantitative.

The quantity of ^{11}C-1,25(OH)$_2$ D$_3$ produced, 2.5–3 GBq at end of synthesis, was more than adequate for both in vitro and in vivo studies (typically 0.4–0.8 GBq ^{11}C is used in a human PET examination). The specific radioactivity of ^{11}C-1,25(OH)$_2$ D$_3$ at the end of synthesis averaged 100 ± 39 GBq/μmol (n = 14), which is in the range of other tracers made from [^{11}C]methyl iodide synthesized at this laboratory.

Radiochemical purity was in all cases greater than 99%. Chemical purity, based on HPLC UV detection at λ = 265 nm, averaged 79%, and was generally higher in the later syntheses (91% average for the last four experiments) due to improved preparative HPLC separation and taking of a center cut fraction of the ^{11}C-1,25(OH)$_2$ D$_3$ eluent. The major mass impurity, thought to be fully *tert*-butyldimethylsilyl-deprotected starting material based on LC–MS (data not presented) and its



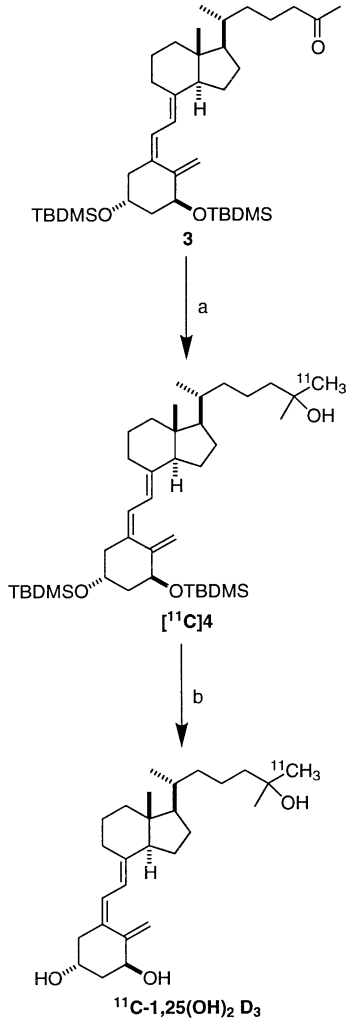
Scheme 1. (a) Prepare methyl vinyl ketone, $NiCl_2 \times 6H_2O$, Zn dust, pyridine, 65 °C, 30 min; (b) then **1**, pyridine, THF, rt, 2 h, dark; (c) 9-acetylanthracene, TEA, toluene, hν, 10 °C, 15 min. Overall yield 38%.



Scheme 2. (a) TBDMSCl, imidazole, DMF, rt, 1 h. Yield 80%.

λ_{max} of 265 nm, eluted prior to ^{11}C-1,25(OH)$_2$ D$_3$ on both preparative and analytical HPLC.[14] The suspected impurity, 1(*S*),3(*R*)-bis(hydroxy)-25-keto-9,10-seco-27-norcholesta-5(*Z*),7(*E*),10(19)-triene (1α-hydroxy-25-keto-27-norvitamin D$_3$), has been shown to exhibit vitamin D like activity, although, compared to 1,25(OH)$_2$ D$_3$, doses of this compound at least 40 and 2000 times higher were required, respectively, for similar effect on intestinal calcium transport and bone calcium mobilization.[15] Thus while the impurity can potentially bind to VDR, its influence on the effective specific radioactivity of the tracer would appear negligible due to its low relative potency in addition to its presence averaging about 9% (in the last four experiments) of the total VDR-active mass [the impurity plus 1,25(OH)$_2$ D$_3$]. Minimizing the VDR-binding mass is important due to its potential to bind the high-affinity, limited capacity receptor system, potentially blocking ^{11}C-1,25(OH)$_2$ uptake. In addition, prior to use of the tracer in humans, chemical impurities must be minimzed.

Curiously, considering the facts that butyllithium is in great excess over the mass associated with [^{11}C]methyllithium and the starting ketone is present at the end of [^{11}C]methyllithium reaction, formation of the butyl addition adduct was not observed. We propose that the low reaction temperatures employed (−70 to −10 °C) and short reaction time (10 min) allowed only the kinetically favored methyl anion to react with the ketone prior to the TBAF quench.



Scheme 3. (a) [^{11}C]methyl iodide, butyllithium, −70 to −10 °C, 10 min; (b) TBAF, THF, 105 °C, 3–5 min.

The position of the ^{11}C label was confirmed in a $^{11/13}$C-1,25(OH)$_2$ D$_3$ co-labeling experiment, followed by ^{13}C NMR analysis of fully radio-decayed product. The resonances found, 29.26 and 29.11 ppm, correspond within experimental error to the resonances assigned to the 26 and 27 carbons, respectively, 29.31 and 29.18 ppm.[16]

In vitro VDR binding

The VDR kit allowed determination of an equilibrium dissociation constant (K_d) of 61 pM and the maximum number of binding sites (B_{max}) of 22.3 fmol receptor per experiment as shown in Figure 1. These values were calculated from a non-linear regression curve fit of the specific binding data (Fig. 1, top) and the numbers were confirmed in a conventional Scatchard plot (Fig. 1, bottom). The K_d value measured in these studies is within the range of others reported for 1,25(OH)$_2$ D$_3$ binding to the VDR.[9] The calculated relative amount of

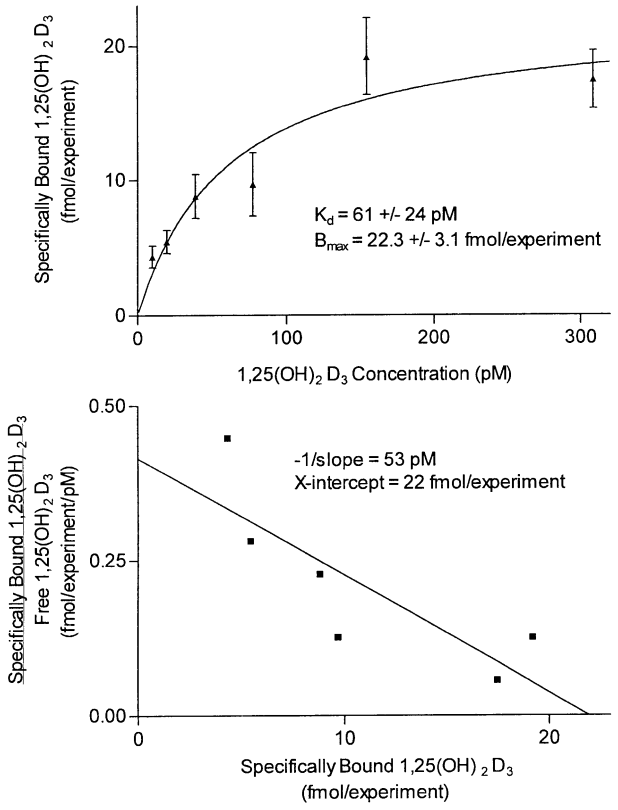


Figure 1. Top: Plot of specific ^{11}C-1,25(OH)$_2$ D$_3$ binding in rachitic chick purified VDR versus 1,25(OH)$_2$ D$_3$ concentration. The curve shown is a fit as described in the experimental section from which K_d and B_{max} were derived. Error bars represent the standard error of the mean (n = 3). Bottom: Scatchard plot of the ratio of specifically bound radiotracer to free radiotracer versus specifically bound radiotracer. The line shown is a linear regression fit. The values −1/slope and X-intercept are shown for comparison, respectively, to K_d and B_{max} obtained using non-linear regression.

specific binding[17] to total binding[18] at K_d and twice K_d, were 29 and 21%, respectively.

Conclusion

A reliable method for the production of [26,27-^{11}C]1α,25-dihydroxyvitamin D_3, a positron-emitting radiopharmaceutical for the vitamin D receptor, in adequate quantity, purity and specific radioactivity for PET studies has been developed. In vitro experiments demonstrated the high affinity binding of this novel PET tracer. However, further evaluation of this tracer in vitro in, for example, human tumor samples is warranted. ^{11}C-1,25(OH)$_2$ D_3 is thus available as a potential tool for in vivo assessment of the VDR in animals and humans using PET. Suggested applications are drug development, and cancer- and bone disease-related imaging.

Experimental

General

[^{11}C]Carbon dioxide production was performed by the ^{14}N(p,α)^{11}C nuclear reaction using a Scanditronix MC-17 cyclotron. A nitrogen (AGA, Nitrogen 6.0) gas target containing 0.05% oxygen (AGA, Oxygen 4.8) was used. Synthia, a robotic system developed in the laboratory,[19] was used for [^{11}C]methyl iodide production, HPLC injection and fraction collection. HPLC was performed on binary pump systems (126 Solvent Module, Beckman) using either, for semi-preparative, UV (265 nm, 166 Detector Module, Beckman) and radioactivity detectors, or, for analytical, diode array (190–300 nm, 168 Detector Module, Beckman) and radioactivity detectors. The HPLC columns used were an Ultrasphere ODS (5 μm, 10×250 mm, Beckman) for semi-preparative work and an Ultrasphere ODS (5 μm, 4.6×250 mm plus 4.6×45 mm guard column, Beckman) for analytical. NMR spectra were recorded using either a Varian XL 300 (Uppsala) or a Bruker AM300 (Ballerup) spectrometer and all chemical shifts are reported in ppm downfield from tetramethylsilane (δ scale). Mass spectra were recorded on a Micromass Autospec spectrometer. Tetrahydrofuran (THF) was distilled from Na-benzophenone ketyl. Lithium aluminum hydride, 0.05 M, was diluted with THF from Aldrich 1 M in THF.

Chemistry and radiochemistry

1(*S*),3(*R*)-Bis[(*tert*-butyldimethylsilyl)oxy]-25-keto-9,10-*seco* - 27 - norcholesta - 5(*E*),7(*E*),10(19) - triene (2). See Scheme 1. The method employed was adapted from a published procedure.[11] A suspension of methyl vinyl ketone (130 mg, 1.85 mmol), $NiCl_2\times6H_2O$ (180 mg, 0.76 mmol), and Zn dust (98 mg, 1.5 mmol) was stirred in pyridine (10 mL) under argon and slowly heated to 65 °C. After 30 min of heating at 65 °C, the mixture was cooled to room temperature. A solution of 1(*S*),3(*R*)-bis[(*tert*-butyldimethylsilyl)oxy]-20(*S*)-(iodomethyl)-9,10-*seco*-27-norcholesta-5(*E*),7(*E*),10(19)-triene **1**[11] (576 mg, 0.84 mmol) in pyridine–THF 1:2 (v/v) (6 mL) was added and the mixture was stirred in the dark for 2 h. The mixture was poured into ethyl acetate (75 mL) and filtered through Hyflo supercel. The Hyflo supercel was washed with ethyl acetate (2×75 mL). The combined organic filtrates were washed with 1 M hydrochloric acid (4×75 mL), a solution of EDTA disodium salt dihydrate (12 g) and $NaHCO_3$ (12 g) in water (150 mL) and brine (2×75 mL). The crude product obtained from drying ($MgSO_4$) and concentration in vacuo was chromatographed on Si gel (20 g) with 10% ether in hexane as eluent. Yield of **2**: 251 mg (0.40 mmol, 53%). ^{1}H NMR as described.[11] ^{13}C NMR δ 209.0, 153.4, 143.1, 135.1, 121.5, 116.2, 106.4, 70.0, 67.0, 56.2, 56.1, 45.7, 44.0, 43.8, 40.3, 36.4, 35.8, 35.2, 29.6, 28.7, 27.4, 25.7, 25.6, 23.3, 22.0, 20.2, 18.5, 18.0, 17.9, 11.8, −5.0, −5.1, −5.1.

1(*S*),3(*R*)-Bis[(*tert*-butyldimethylsilyl)oxy]-25-keto-9,10-*seco* - 27 - norcholesta - 5(*Z*),7(*E*),10(19) - triene (3). See Scheme 1. The method employed was adapted from a published procedure.[11] A mixture of **2** (251 mg, 0.40 mmol), 9-acetylanthracene (18.5 mg, 0.08 mmol) and triethylamine (290 μL) in toluene (32 mL) was irradiated under an argon atmosphere with a 500 W Hanau Z-2 UV lamp at 10 °C for 15 min. The reaction mixture was concentrated in vacuo and the residue was chromatographed on Si gel (80 g) with toluene as eluent to give **3** (178 mg, 0.28 mmol, 71%). ^{1}H NMR δ 6.22 (d, 1H), 5.99 (d, 1H), 5.16 (m, 1H), 4.84 (m, 1H), 4.35 (m, 1H), 4.17 (m, 1H), 2.80 (m, 1H), 2.11 (s, 3H), 0.92 (d, 3H), 0.86 (s, 18H), 2.49–0.79 (m, 22H), 0.51 (s, 3H), 0.04 (m, 12H); ^{13}C NMR δ 209.1, 148.1, 140.8, 134.8, 122.9, 117.7, 111.0, 71.8, 67.3, 56.1, 56.0, 45.8, 45.5, 44.6, 44.0, 40.4, 35.8, 35.2, 29.6, 28.6, 27.5, 25.6, 25.6, 23.3, 21.9, 20.2, 18.5, 18.0, 17.9, 11.7, −4.9, −5.0, −5.3.MS (EI+) calcd for $C_{38}H_{68}O_3Si_2$: 628.4707, found: 628.4694. Elemental analysis of **3** was not possible due to residual solvent. Compound **3** was stored at −25 °C in ethyl acetate (18 mg/mL) for more than 6 months with no apparent decomposition.

1,3-Bis[(*tert*-butyldimethylsilyl)oxy]-1α,25-dihydroxyvitamin D_3 (4). See Scheme 2. 1,25(OH)$_2$ D_3 (50 mg, 0.12 mmol), *tert*-butyldimethylsilyl chloride (54 mg, 0.36 mmol) and imidazole (49 mg, 0.72 mmol) were stirred in dimethylformamide (1.2 mL) at room temperature for 1 h. Calcium chloride (3 M, 3 mL) was added and the mixture was extracted with hexane (3×3 mL). The organic extracts were washed with water (3 mL) and brine (3 mL), dried with $MgSO_4$, concentrated in vacuo and chromatographed on Si gel (15 g) with pentane–dichloromethane–ethyl acetate 50:50:4 as eluent to give **4** (62 mg, 0.096 mmol, 80%). ^{1}H NMR as described.[11] ^{13}C NMR δ 148.1, 140.9, 134.7, 123.0, 117.7, 111.0, 71.9, 70.9, 67.3, 56.3, 56.1, 45.8, 45.6, 44.6, 44.2, 40.4, 36.2,35.9,29.2, 29.0, 28.7, 27.5, 25.7, 25.6,23.3, 21.9, 20.7, 18.6, 18.0, 18.0, 11.8, −4.9, −5.0, −5.3. Compound **4** was stored at −25 °C in ethyl acetate (17 mg/mL) for more than 6 months with no apparent decomposition.

[26,27-^{11}C]1α,25-Dihydroxyvitamin D_3 (^{11}C-1,25(OH)$_2$ D_3). ^{11}C-1,25(OH)$_2$ D_3 was synthesized according to

Scheme 3. Stock **3** in ethyl acetate (100 μL solution; 1.8 mg, 2.6 μmol) was added to an oven-dried 3 mL glass vial. The solvent was removed under a stream of nitrogen gas and the vial was placed under high vacuum for 5–10 min. The vial was sealed with a septum (PTFE-lined)-equipped screw cap and thoroughly flushed with nitrogen gas. THF was added, and **3** was dissolved via vigorous vortexing. [^{11}C]Methyl iodide (estimated 0.03–0.1 μmol, made from [^{11}C]CO_2, lithium aluminum hydride, and HI as described[13]) was distilled into the vial through a PTFE line and stainless steel needle over 3 min. The vial was cooled in a −70 °C bath (dry ice, ethanol) at the beginning of and throughout the [^{11}C]methyl iodide distillation.

At the end of distillation, the transfer and vent needles were removed, leaving a sealed vial, cooled to −70 °C, containing unreacted **3** and [^{11}C]methyl iodide in THF. To the vial was added butyllithium (Lancaster, 1.6 M in hexanes, 30 μL, 48 μmol). After 1 min the vial was moved to a −10 °C bath (ice, ethanol) for 10 min (contents turned yellow), providing ^{11}C-labeled intermediate **[^{11}C]4** in about 30–50% yield [calculated from a few early experiments, decay-corrected, from trapped [^{11}C]methyl iodide and based on analytical HPLC, acetonitrile–dichloromethane 1:1, 1 mL/min, t_R (**4** standard) = 5.28 min, λ_{max} = 267 nm, product not isolated].

The [^{11}C]methylation reaction was quenched by adding tetrabutylammonium fluoride (Lancaster, 1 M in THF, thought to contain about 5 wt% water, 200 μL, 200 μmol), and the vial was moved from −10 to 105 °C for protecting group cleavage. After 3–5 min, the vial was cooled to −10 °C and the reaction was quenched by adding 200 μL saturated aqueous sodium bicarbonate plus 2 mL ethanol–water 1:1. Semi-preparative HPLC [water–99.5% ethanol 9:13, 4 mL/min, t_R (1,25(OH)$_2$ D_3 standard) = 13.2 min] afforded 2.5–3 GBq ^{11}C-labeled 1,25(OH)$_2$ D_3.

The HPLC eluent fraction containing ^{11}C-1,25(OH)$_2$ D_3 was used directly in the biological experiments. Analysis of the fraction by HPLC [water–acetonitrile 1:9, 1 mL/min, t_R (1,25(OH)$_2$ D_3 standard) = 5.8 min, λ_{max} = 265 nm] was performed to determine radiochemical and chemical purity, and the concentration of 1,25(OH)$_2$ D_3 mass in the fraction for calculation of specific radioactivity.

[26,27-$^{11/13}$C]1α,25-Dihydroxyvitamin D_3 ($^{11/13}$C-1,25(OH)$_2$ D_3). The process for the $^{11/13}$C co-labeling synthesis was a slight modification of that described above for ^{11}C-1,25(OH)$_2$ D_3. At the end of [^{11}C]methyl iodide distillation, (^{13}C)methyl iodide (Larodan, Sweden, 20% v/v in octane, 10 μL, 30 μmol) was added to the cooled reaction vessel, proceeding as described above. The HPLC fraction containing $^{11/13}$C-1,25(OH)$_2$ D_3 was allowed to fully radio-decay at −25 °C, after which the solvent was removed in vacuo. The resulting residue was dissolved in CD_3OD (0.5 mL) and a ^{13}C NMR spectrum was recorded (ref CD_3OD = 49.00 ppm). ^{13}C NMR δ 29.26, 29.11.

Radiometric determination of radioligand binding in a solubilized rachitic chick purified VDR system. Three sequential binding experiments were performed using components from, and following the instructions in, the Amersham '1α,25-dihydroxyvitamin D [^{3}H]assay reagents system' (Amersham Pharmacia Biotech, Uppsala, Sweden, product code TRK 870), substituting ^{11}C-1,25(OH)$_2$ D_3 for the tritiated tracer supplied. Briefly, the purified VDR supplied by the manufacturer was incubated (30 min, 21 °C, with shaking) with different concentrations (9.6–308.7 pM) of ^{11}C-1,25(OH)$_2$ D_3 to give total binding. Parallel experiments with the same concentration of radiotracer plus 100 nM cold 1,25(OH)$_2$ D_3 were performed to give non-specific binding. Dextran-coated charcoal was added at the end of incubation to adsorb free radioligand. Centrifugation pelleted the dextran-coated charcoal, and radioactivity in the supernatant [containing receptor-bound ^{11}C-1,25(OH)$_2$ D_3] was determined using conventional gamma counting. The receptor supplied underwent a single additional freeze-thaw cycle between the first and second experiments. K_d (equilibrium dissociation constant) and B_{max} (maximum number of binding sites) were calculated using the software GraphPad Prism (GraphPad Software, Inc., San Diego, USA) via non-linear-regression analysis, fit to a single site binding model (hyperbola) on the specific binding data.

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