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Synthesis of 1α-[19-^{13}C]Hydroxyvitamin D_3 and ^{13}C NMR Analysis of the Conformational Equilibrium of the A-Ring

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1α-[19-^{13}C]Hydroxyvitamin D_3 (**6**) was synthesized and its conformational equilibrium was investigated by means of variable temperature ^{13}C NMR spectroscopy. The A-ring of **6** was found to exist in an equilibrium between two conformations in the ratio 52 : 48 at −100°C. The rate constants of the equilibrium were estimated by signal shape analysis according to the Gutowsky–Holm formulation. The thermodynamic parameters $\Delta G^{\ddagger}_{188\,K}$ (coalescence temperature), $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ were deduced from the exchange rate to be 8.0 kcal/mol, 6.3 kcal/mol, and −9.0 cal/mol · deg, respectively.

The study of active forms of biologically significant and conformationally flexible molecules is one of the more interesting subjects of biochemical and bioorganic research. Defining such conformations, however, presents a problem since flexible molecules per se have numerous possible conformations. The conformational mobility of vitamin D (*1*), especially in the A-ring, has been investigated by means of NMR spectroscopy (*2–13*). The A-ring of vitamin D derivatives has been shown to exist in two chair-like conformations, which are under dynamic equilibrium in solution. Even in the crystalline state, vitamin D_3 exists as a 1 : 1 mixture of two different chair conformations of the A-ring (*14*). We have recently discussed the equilibrium of the A-ring of 1α,25-dihydroxyvitamin D_3, the active form of vitamin D_3, by variable temperature ^{1}H and ^{13}C NMR techniques, and the free enthalpy of the equilibrium was estimated for the first time (*15*). However, further details of the equilibrium are necessary not only for mechanistic studies of the interconversion, but in particular for studies of the binding process with its receptor. We envisioned that dynamic ^{13}C NMR studies of the compound ^{13}C labeled at the A-ring would be worthwhile to investigate the kinetics of the equilibrium. Here we describe the synthesis of 1α-[19-^{13}C]hydroxyvitamin D_3 as the A-ring equivalent of the active hormone, 1α,25-dihydroxyvitamin D_3, and variable temperature ^{13}C NMR studies of it.

1α-[19-^{13}C]Hydroxyvitamin D_3 was synthesized from the known 3,5-cyclovitamin D (**1**; see Scheme 1) (*16*). Compound **1** was first treated with OsO_4 in the presence of *N*-methylmorpholine-*N*-oxide and then oxidized with $NaIO_4$ (*17*) to give **2** in a 55% yield. A specific ^{13}C label was introduced by the Wittig reaction of **2** with $^{13}CH_2{=}PPh_3$ to yield **3** in a 66% yield. 3,5-[19-^{13}C]Cyclovitamin D (**3**) was transformed into 1α-[19-^{13}C]hydroxyvitamin D_3 (**6**) in a 12% yield by a series of

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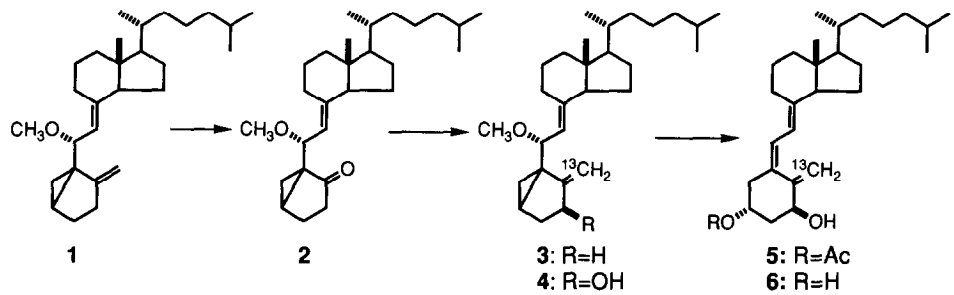

SCHEME 1

reactions developed by DeLuca *et al.* (*18*), i.e., selective 1α-hydroxylation with SeO_2, cycloreversion with acetic acid, and alkaline hydrolysis. The enrichment of ^{13}C at the 19-position of **6** was at least 95% on the basis of a mass spectrum as well as ^1H and ^{13}C NMR spectra.

^{13}C NMR spectra of **6**, which are shown in Fig. 1, were obtained at various temperatures ranging from $-100°C$ to 27°C. These experiments clearly indicated that the A-ring of **6** existed in two conformations, the abundances of which were determined to be 52 : 48 at $-100°C$ by integration of the peak area. These results agreed well with previous reports that suggested chair (or chair-like) conformations for both conformations, the major one having an axially oriented hydroxyl group at C-3 (*2–13, 15*). Computer-simulated ^{13}C NMR spectra of **6** are also presented in Fig. 1. The rate constants of the equilibria were deduced from computer-simulated signal shape analysis with the Gutowsky–Holm formulation (*19*). The resulting rate constants (k) were plotted as $\ln(k/T)$ vs $1/T$ as shown in Fig. 2. The plot demonstrated well-fitted linearity, and the $\Delta G^{\neq}_{188\,K}$ (coalescence temperature), $\Delta H^{\neq}$, and $\Delta S^{\neq}$ values were estimated to be 8.0 kcal/mol, 6.3 kcal/mol, and -9.0 cal/mol · deg, respectively.[1]

The actual conformation of 1α,25-dihydroxyvitamin D_3 binding to its receptor is clearly the one that should be further elucidated. Since the ^{13}C chemical shift difference between the two conformations was found to be relatively large at the C-19 position of the 1α-hydroxylated vitamin D_3, one way to look for the active conformation of the A-ring is to monitor the behavior of C-19 in the binding state.

EXPERIMENTAL

All reactions were carried out under an argon atmosphere. ^{13}C-labeled compounds were homogeneous on TLC and/or HPLC, showing behaviors identical to that of the corresponding nonlabeled compounds synthesized according to the

[1] We also synthesized [19-^{13}C]vitamin D_3 from **3** by the method of Mazur *et al.* (see Ref. (*16*). Mass spectrum (relative intensity) 385 (M^+, 19). ^{13}C NMR δ 112.39 (C-19). This compound showed no coalescence even at $-145°C$ in CD_3OD–$CFCl_3$ (1 : 1) under the same field conditions.

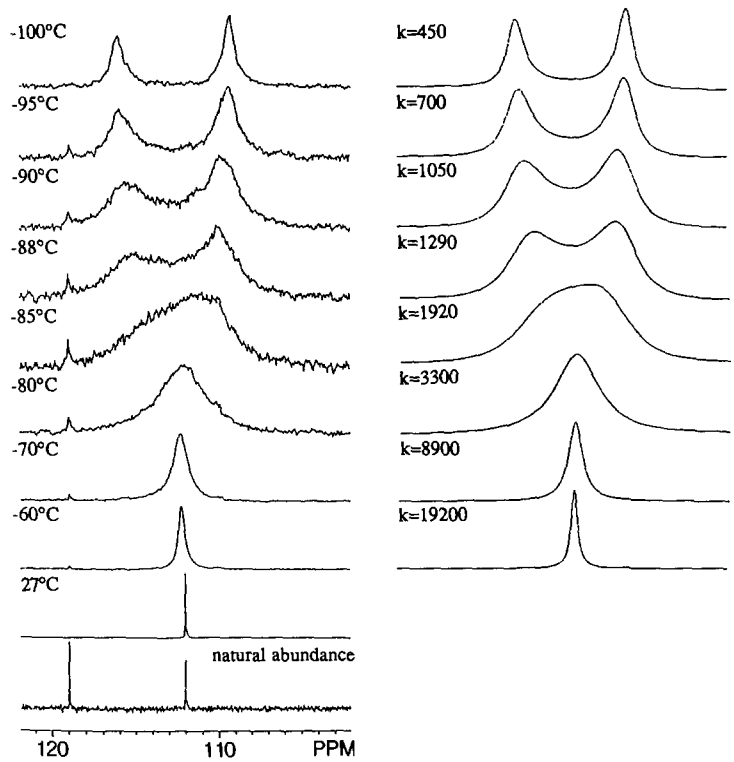

FIG. 1. Actual and computer-simulated ^{13}C NMR spectra of the C-19 signal of **6** at several temperatures (°C). The *k* values were obtained from computer-simulated signal shape analysis.

method of DeLuca *et al.* (*17*). Column chromatography was carried out with silica gel (Merck, silica gel 60, 70–230 mesh). ^1H and ^{13}C NMR spectra were taken on a Jeol GSX-270 spectrometer in $CDCl_3$ solution operating at 270 and 67.5 MHz, respectively. Variable temperature ^{13}C NMR spectra were recorded with a Jeol

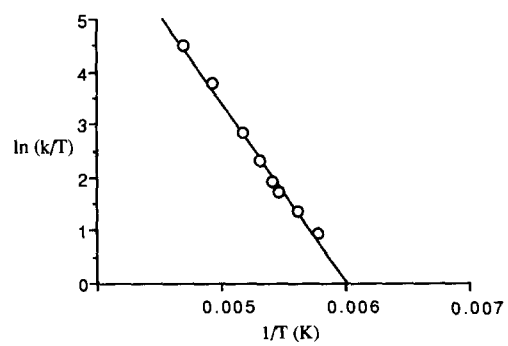

FIG. 2. Temperature dependence of the interconversion rate constants for **6**. ΔH^* and ΔS^* values of the interconversion were obtained from these plots.

GSX-500 spectrometer (125 MHz) at a concentration of 2.5 mg/ml in CD_3OD. An ir spectrum was measured with a Hitachi 285 infrared spectrometer. Ultraviolet spectra were recorded with a Shimadzu UV-200 double-beam spectrometer in ethanol solution. Electron ionization mass spectra were obtained with a Shimadzu DF-9020 spectrometer at 70 eV.

(6R)-3,5-Cyclo-6-methoxy-19-nor-10-oxo-9,10-secocholest-7-ene (**2**). A mixture of **1** (1.0 g), *N*-methylmorpholine-*N*-oxide (590 mg), and OsO_4 (100 mg) in THF (15 ml), *t*-butanol (15 ml), and water (5 ml) was stirred at room temperature for 1 day. Aqueous $NaHSO_3$ solution was added and the mixture was extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4, filtered, and concentrated to dryness to give an oily residue (1.3 g), which was dissolved in methanol (25 ml) and water (5 ml). Sodium periodate (800 mg) was added to this solution and the mixture was stirred overnight at room temperature. After most of the methanol was removed by evaporation, water and ethyl acetate were added. The organic phase was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography over silica gel with hexane–ethyl acetate (20 : 1–10 : 1) to give **2** (527 mg, 55%) as an oil: ^1H NMR δ 0.52 (3H, s, 18-H), 0.868 and 0.870 (6H, d, J = 6.6 Hz, 26- and 27-H), 0.92 (3H, d, J = 6.1 Hz, 21-H), 3.23 (3H, s, OMe), 4.56 (1H, d, J = 10.0 Hz, 6-H), 4.73 (1H, d, J = 10.0 Hz, 7-H). Infrared (neat) 1710 cm^{-1}. *Anal.* Calcd for $C_{27}H_{44}O_2$; C, 80.94; H, 11.07. Found: C, 80.91; H, 11.00.

(6R)-[19-^{13}C]-3,5-Cyclo-6-methoxy-9,10-secocholesta-7,10(19)-diene (**3**). Sodium hydride (60% in mineral oil) was washed three times with dry hexane and dried with a stream of argon, finally *in vacuo*. To this dried NaH (594 mg) was added DMSO (2 ml) and the mixture was heated at 80°C for 45 min. This solution (0.60 ml) was added to a solution of [*Me*-^{13}C]triphenylmethylphosphonium iodide (608 mg, 99.5% enrichment, Aldrich Chemical Co. Ltd.) in DMSO (2 ml) and the mixture was stirred at room temperature for 10 min. A solution of **2** (500 mg) in HMPA (5 ml) was added to the mixture, which was further stirred at room temperature for 4 h. Water was added and the mixture was extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was chromatographed over silica gel with hexane–ethyl acetate (20 : 1) to give **3** (330 mg, 66%) as an oil: ^1H NMR δ 0.54 (3H, s, 18-H), 0.72 (1H, m, 3-H), 0.868 and 0.870 (6H, d, J = 6.5 Hz, 26- and 27-H), 0.92 (3H, d, J = 6.7 Hz, 21-H), 3.25 (3H, s, OMe), 4.17 (1H, d, J = 9.5 Hz, 6-H), 4.93 (1H, d, $^1J_{C,H}$ = 156 Hz, 19*Z*-H), 5.00 (1H, d, J = 9.5 Hz, 7-H), 5.05 (1H, d, $^1J_{C,H}$ = 156 Hz, 19*E*-H). ^{13}C NMR δ 103.81 (C-19).

(6R)-[19-^{13}C]-3,5-Cyclo-1α-hydroxy-6-methoxy-9,10-secocholesta-7,10(19)-diene (**4**). A mixture of **3** (177 mg), SeO_2 (9.8 mg), and a 3 M solution of *t*-BuOOH in 2,2,4-trimethylpentane (0.30 ml) in CH_2Cl_2 (15 ml) was stirred at room temperature for 1.5 h. To the reaction mixture was added 10% aqueous NaOH solution (10 ml) and the stirring was continued for 10 min. The organic phase was separated, washed with 10% aqueous NaOH solution and brine, and dried over Na_2SO_4. After filtration and evaporation, the residual oil was chromatographed over silica gel with hexane–ethyl acetate (5 : 1) to give **4** (84.5 mg, 46%) as an oil: ^1H NMR δ 0.53 (3H, s, 18-H), 0.864 and 0.866 (6H, d, J = 6.6 Hz, 26- and 27-H), 0.92 (3H, d,

J = 6.8 Hz, 21-H), 3.24 (3H, s, OMe), 4.20 (2H, m, 6- and 1-H), 4.94 (1H, d, J = 9.0 Hz, 7-H), 5.16 (1H, d, $^1J_{C,H}$ = 157 Hz, 19*Z*-H), 5.24 (1H, d, $^1J_{C,H}$ = 157 Hz, 19*E*-H). ^{13}C NMR δ 104.87 (C-19).

[19-^{13}C]-3β-Acetoxy-1α-hydroxy-9,10-secocholesta-5,7,10(19)-triene (**5**). A mixture of **4** (96.0 mg) and acetic acid (1 ml) was heated with stirring at 55°C for 15 min. After the mixture had cooled to room temperature, ethyl acetate was added. The whole was washed with aqueous $NaHCO_3$ solution and brine, successively. After drying over Na_2SO_4, the solvent was removed and the residue was purified by chromatography over silica gel with hexane–ethyl acetate (10 : 1) to give **5** (44 mg, 43%): ^{1}H NMR δ 0.54 (3H, s, 18-H), 0.868 and 0.870 (6H, d, J = 6.6 Hz, 26- and 27-H), 0.92 (3H, d, J = 6.2 Hz, 21-H), 2.03 (3H, s, acetyl), 2.40 (1H, dd, J = 13.5 and 6.2 Hz, 4β-H), 2.59 (1H, dd, J = 13.5 and 3.3 Hz, 4α-H), 2.81 (1H, dd, J = 12.0 and 3.2 Hz, 9β-H), 4.40 (1H, m, 1-H), 5.02 (1H, d, $^1J_{C,H}$ = 159 Hz, 19*Z*-H), 5.21 (1H, tt, J = 6.8 and 3.3 Hz, 3-H), 5.35 (1H, d, $^1J_{C,H}$ = 159 Hz, 19*E*-H), 6.03 (1H, d, J = 11.2 Hz, 7-H), 6.34 (1H, d, J = 11.2 Hz, 6-H). ^{13}C NMR δ 111.85 (C-19). Ultraviolet λ_{max} 264 nm (ε 18,100). Further elution of the column with hexane–ethyl acetate (8 : 1) afforded the 5,6-*trans* isomer (33 mg, 32%).

1α-[19-^{13}C]Hydroxyvitamin D_3 (**6**). A solution of **5** (7.0 mg) and 5% KOH–methanol (0.1 ml) in ethanol (1 ml) was stirred at room temperature for 3 h. The mixture was diluted with ethyl acetate. The whole was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography over silica gel with hexane–ethyl acetate (3 : 1) to give amorphous **5** (5.2 mg, 81%): ^{1}H NMR δ 0.54 (3H, s, 18-H), 0.868 and 0.870 (6H, d, J = 6.6 Hz, 26- and 27-H), 0.92 (3H, d, J = 6.8 Hz, 21-H), 2.31 (1H, dd, J = 13.0 and 7.2 Hz, 4β-H), 2.59 (1H, dd, J = 13.0 and 3.2 Hz, 4α-H), 2.82 (1H, dd, J = 11.5 and 3.4 Hz, 9β-H), 4.22 (1H, tt, J = 6.8 and 3.3 Hz, 3-H), 4.42 (1H, br m, 1-H), 5.00 (1H, d, $^1J_{C,H}$ = 159 Hz, 19*Z*-H), 5.32 (1H, d, $^1J_{C,H}$ = 159 Hz, 19*E*-H), 6.02 (1H, d, J = 11.2 Hz, 7-H), 6.38 (1H, d, J = 11.2 Hz, 6-H). ^{13}C NMR δ 111.75 (C-19), 112.01 (C-19 in CD_3OD). Ultraviolet λ_{max} 264 nm (ε 17,800). Mass spectrum (relative intensity) 401 (M^+, 13), 383 (5), 153 (38), 135 (100).

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