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Isolation and Identification of 1α,25-Dihydroxy-24-oxovitamin D_3, 1α,25-Dihydroxyvitamin D_3 26,23-Lactone, and 1α,24(*S*),25-Trihydroxyvitamin D_3: In Vivo Metabolites of 1α,25-Dihydroxyvitamin D_3†

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ABSTRACT: Three new in vivo metabolites of 1α,25-dihydroxyvitamin D_3 were isolated from the serum of dogs given large doses (two doses of 1.5 mg/dog) of 1α,25-dihydroxyvitamin D_3. The metabolites were isolated and purified by methanol–chloroform extraction and a series of chromatographic procedures. By cochromatography on a high-performance liquid chromatograph, ultraviolet absorption spectrophotometry, mass spectrometry, Fourier-transform infrared spectrophotometry, and specific chemical reactions, the metabolites were identified as 1α,25-dihydroxy-24-oxovitamin D_3, 1α,25-dihydroxyvitamin D_3 26,23-lactone, and 1α,24(*S*),25-trihydroxyvitamin D_3. According to these procedures, the total amounts of the isolated metabolites were as follows: 1α,25-dihydroxyvitamin D_3, 23.6 μg; 1α,25-dihydroxy-24-oxovitamin D_3, 1.8 μg; 1α,25-dihydroxyvitamin D_3 26,23-lactone, 9.2 μg; 1α,24(*R*),25-trihydroxyvitamin D_3, 15.4 μg; 1α,24(*S*),25-trihydroxyvitamin D_3, 1.0 μg. With recovery corrections, the serum levels of each metabolite were approximately 49 ng/mL for 1α,25-dihydroxyvitamin D_3, 3.7 ng/mL for 1α,25-dihydroxy-24-oxovitamin D_3, 19 ng/mL for 1α,25-dihydroxyvitamin D_3 26,23-lactone, 32 ng/mL for 1α,24(*R*),25-trihydroxyvitamin D_3, and 2.1 ng/mL for 1α,24(*S*),25-trihydroxyvitamin D_3.

It has been well established that 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$][1] is a metabolite of vitamin D_3 active in intestinal calcium absorption and bone mineral mobilization (Norman, 1979). In recent studies, 1α,25$(OH)_2D_3$ may undergo further metabolism to several additional secosteroids, including (i) 1α,24,25-trihydroxyvitamin D_3 [1α,24,25$(OH)_3D_3$] (Kleiner-Bossaler & DeLuca, 1974; Tanaka et al., 1977; Ribovich & DeLuca, 1978; Reinhardt et al., 1982), (ii) side-chain oxidation and cleavage to yield CO_2 (Kumar et al., (1976) and a shortened side chain, C-23-COOH-containing secosteroid designated calcitroic acid (Esvelt et al., 1979), (iii) 1α,25,26-trihydroxyvitamin D_3 [1α,25,26$(OH)_3D_3$] (Reinhardt et al., 1981; Tanaka et al., 1981b), and (iv) 1α,25-dihydroxyvitamin D_3 26,23-lactone [1α,25$(OH)_2D_3$-26,23-lactone] (Ohnuma et al., 1980; Ishizuka et al., 1981). The stereochemical configurations of the biosynthesized 1α,24,25$(OH)_3D_3$ and 1α,25,26$(OH)_3D_3$ were determined to be 24(*R*) and 25(*S*), respectively (Tanaka et al., 1977; Reinhardt et al., 1981). Furthermore, the stereochemical configuration at the C-23 and C-25 positions of the 1α,25-$(OH)_2D_3$-26,23-lactone was determined to be (23*S*,25*R*)-1α,25$(OH)_2D_3$-26,23-lactone (Ishizuka et al., 1981).

During the course of the investigation of the further metabolism of 1α,25$(OH)_2D_3$, we became aware of three new metabolites of 1α,25$(OH)_2D_3$ that appeared in the serum of dogs given large doses of 1α,25$(OH)_2D_3$. It is the purpose of this paper to describe the isolation and to establish the structures of these new metabolites as 1α,25-dihydroxy-24-oxovitamin D_3 [1α,25$(OH)_2$-24-oxo-D_3], 1α,25$(OH)_2D_3$-26,23-lactone, and 1α,24(*S*),25-trihydroxyvitamin D_3 [1α,24(*S*),25$(OH)_3D_3$].

Materials and Methods

Compounds. The syntheses of 1α,25$(OH)_2D_3$ and the 24-isomers of 1α,24,25$(OH)_3D_3$ were carried out in our laboratory

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[1] Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 23(*S*),25-$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 24(*R*),25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 25(*S*),26$(OH)_2D_3$, 25(*S*),26-dihydroxyvitamin D_3; 25-OH-D_3-26,23-peroxylactone, 25-hydroxyvitamin D_3 26,23-(peroxylactone); 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,25$(OH)_2D_3$-26,23-lactone, 1α,25-dihydroxyvitamin D_3 26,23-lactone; 1α,25$(OH)_2D_3$-26,23-peroxylactone, 1α,25-dihydroxyvitamin D_3 26,23-(peroxylactone); 23(*S*),25(*R*),26$(OH)_3D_3$, 23(*S*),25(*R*),26-trihydroxyvitamin D_3; 1α,24(*R*),25$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; 1α,24(*S*),25$(OH)_3D_3$, 1α,24(*S*),25-trihydroxyvitamin D_3; 1α,25(*S*),26$(OH)_3D_3$, 1α,25(*S*),26-trihydroxyvitamin D_3; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; 1α-OH-25,26,27-trinor-24-CHO-D_3, 1α-hydroxy-25,26,27-trinor-24-oxovitamin D_3; 1α,23(*S*),25$(OH)_3D_3$, 1α,23(*S*),25(*R*)-trihydroxyvitamin D_3; 1α,23(*S*),25(*R*),26$(OH)_4D_3$, 1α,23(*S*),25(*R*),26-tetrahydroxyvitamin D_3; HPLC, high-performance liquid chromatography.

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as described previously (Morisaki et al., 1973; Ikekawa et al., 1975). The 1α,25(OH)$_2$-24-oxo-D$_3$ was synthesized in our laboratory as will be described elsewhere (S. Ishizuka et al., unpublished results). Natural 1α,25(OH)$_2$D$_3$-26,23-lactone was isolated as described previously (Ohnuma et al., 1980; Ishizuka et al., 1981). Nembutal was purchased from Abbott Laboratories (North Chicago, IL).

Procurement and Extraction of Dog Serum from Dogs Given Large Doses of 1α,25(OH)$_2$D$_3$. Three 12-month-old, male, beagle dogs weighing approximately 10 kg raised on a commercial diet (Nippon Clea Corp. DE-1; Ca, 1.0%, P, 0.8%, D$_3$, 1500 IU/kg) were each given orally 1.5 mg of 1α,25-(OH)$_2$D$_3$ in 20 mL of 5% ethanol–0.2% Triton X-100 solution by stomach tube. After 4 h, they were again dosed orally with 1.5 mg of 1α,25(OH)$_2$D$_3$. Four hours after this second dose, the dogs were anesthetized with Nembutal, and their blood was withdrawn from the carotid artery. The blood was immediately centrifuged, and 1000 mL of serum was obtained. Serum (800 mL) was diluted with 2 volumes of water and then extracted with 2 volumes of chloroform–methanol (1:1) for 30 min as described previously (Ishizuka et al., 1981). The chloroform phase was evaporated, and the residue was dried by ethanol azeotrope and chromatographed.

Chromatographic Purification of Peak I. The chloroform extract of 800 mL of serum chromatographed on a 1.5 × 25 cm Sephadex LH-20 column eluted with chloroform–*n*-hexane (65:35). Sixty 4-mL fractions were collected. The 1α,25-(OH)$_2$D$_3$ (tubes 27–45; 108–180 mL) fraction was well separated from the 25-OH-D$_3$ (tubes 9–15; 36–60 mL) and the 24,25(OH)$_2$D$_3$ (tubes 19–26; 76–103 mL). The 1α,25-(OH)$_2$D$_3$ fractions from the Sephadex LH-20 column were then subjected to high-performance liquid chromatography (HPLC) on a Hitachi Model 635A equipped with a 4.6 × 250 mm Zorbax Sil column and eluted with 20% 2-propanol in *n*-hexane at a flow rate of 1 mL/min. The eluate was continuously monitored by ultraviolet absorption at 264 nm, and the ultraviolet-absorbing peaks were separately collected. On this system, standard 1α,25(OH)$_2$D$_3$ and 1α,24(*R*),25(OH)$_3$D$_3$ eluted at 10.5 and 14.7 mL, respectively. The ultraviolet-absorbing peak I eluted between 1α,25(OH)$_2$D$_3$ and 1α,24-(*R*),25(OH)$_3$D$_3$ had ultraviolet absorption spectra with the typical vitamin D cis-triene absorbance maximum at 264 nm and the minimum at 228 nm. Peak I was rechromatographed in the same system. This new metabolite of 1α,25(OH)$_2$D$_3$ was further purified by HPLC on a Zorbax Sil column eluted with 3% methanol in dichloromethane at a flow rate of 1 mL/min. Peak I eluted before the elution position of 1α,25-(OH)$_2$D$_3$. The sole ultraviolet- (264-nm) absorbing peak, eluting from 11.0 to 12.5 mL, was pooled for structural identification.

Chromatographic Purification of Peaks II–IV. The chloroform extract from 800 mL of serum was chromatographed on a 1.5 × 25 cm Sephadex LH-20 column eluted first with 250 mL of chloroform–*n*-hexane (65:35) and then with 200 mL of chloroform–*n*-hexane–methanol (75:23:2). The 1α,25(OH)$_2$D$_3$ metabolites eluting in the position of authentic 1α,24,25(OH)$_3$D$_3$ (68–120 mL in the second solvent system) were collected and prepared for HPLC. Primary HPLC purification was performed on a 4.6 × 250 mm Zorbax Sil column, eluted with 3% methanol in dichloromethane at a flow rate of 1 mL/min. In this system, standard 1α,25(OH)$_2$D$_3$, 1α,24(*R*),25(OH)$_3$D$_3$, and 1α,24(*S*),25(OH)$_3$D$_3$ eluted at 16.4, 31.5, and 33.4 mL, respectively. The material eluting at 16.0–17.5 mL was collected for the isolation of peak II, while the materials eluting at 30.5–32.5 and 33.0–35.0 mL were collected for the isolation of peak III and peak IV, respectively. Peak II was then subjected to HPLC on a 4.6 × 250 mm Zorbax Sil column eluted with 20% 2-propanol in *n*-hexane. In this system, standard 1α,25(OH)$_2$D$_3$ and 1α,24(*R*),25(OH)$_3$D$_3$ eluted at 10.2 and 14.5 mL, respectively. Peak II was eluted at 17.5–19.5 mL after 1α,24(*R*),25-(OH)$_3$D$_3$. Final purification of peak II was performed on a 4.6 × 250 mm Zorbax Sil column eluted with 3% methanol in dichloromethane. The sole ultraviolet- (264-nm) absorbing peak, eluting from 16.0 to 17.5 mL, was pooled for structural identification.

Peak III and peak IV, eluting in the positions of authentic 1α,24(*R*),25(OH)$_3$D$_3$ and 1α,24(*S*),25(OH)$_3$D$_3$, respectively, on the first HPLC, were also purified by the same HPLC systems. Peak III eluted at 30.5–33.0 mL, and peak IV eluted at 32.5–35.0 mL.

Sodium Borohydride Reduction of Peak I. A total of 1 μg of peak I in 200 mL of ethanol was treated with 1 mg of $NaBH_4$. The reaction was allowed to proceed for 3 h at 25 °C at which time 2 mL of water was added and the product extracted 3 times with 2 mL of ethyl acetate. The ethyl acetate was removed under vacuum, and the product was chromatographed on a 4.6 × 250 mm Zorbax Sil column eluted with 3% methanol in dichloromethane. The purified product was collected and subjected to mass spectrometry.

Periodate Cleavage Oxidation. A total of 500 ng of the reduced product of peak I was dissolved in 100 μL of methanol, mixed with 10 μL of a 5% aqueous solution of $NaIO_4$, and incubated for 3 h at 25 °C. The reaction mixture was extracted with chloroform and evaporated to dryness. The residue of the chloroform extract was subjected to HPLC on a 4.6 × 250 mm Zorbax Sil column eluted with 3% methanol in dichloromethane. The periodate cleavage product was collected and subjected to mass spectrometry for structure identification.

Trimethylsilylation of Peak I. A total of 400 ng of the purified peak I was treated with 50 μL of (trimethylsilyl)-imidazole at 30 °C for 3 h. The trimethylsilyl ether product was extracted with ethyl acetate and purified on a 4.6 × 250 mm Zorbax Sil column eluted with dichloromethane. The major 264-nm absorbing material eluting at 6.5–7.5 mL was collected and used for mass spectrometry.

Spectroscopy. Ultraviolet spectra were recorded from ethanol solutions by using a Hitachi Model 200-10 spectrophotometer. Mass spectra were measured with a Shimadzu LKB Model 9000 mass spectrometer in the direct-probe inlet mode. Four-transform infrared (FT-IR) spectra were obtained by using a JEOL Model JIR-40X (Japan Electric Optical Laboratory, Ltd.). HPLC was carried out on a Hitachi Model 635A equipped with a double plunger type micropump, a Hitachi spectroflow UV monitor (Model 200-10 spectrophotometer) with a wavelength at 264 nm, and Zorbax Sil, a prepacked column (Du Pont, 25 cm × 4.6 mm i.d.), as the stationary phase (see paragraph at end of paper regarding supplementary material).

Results

During the course of our investigation of the further metabolism of 1α,25(OH)$_2$D$_3$, we became aware of three new metabolites of 1α,25(OH)$_2$D$_3$ that appeared in the HPLC chromatogram of lipid extracts obtained from the serum of dogs given large doses of 1α,25(OH)$_2$D$_3$. The chromatographic procedures used for purification of the metabolites are given under Materials and Methods. The highest peak from the Sephadex LH20 column was considered to be the substrate 1α,25(OH)$_2$D$_3$ since it migrated exactly as did authentic

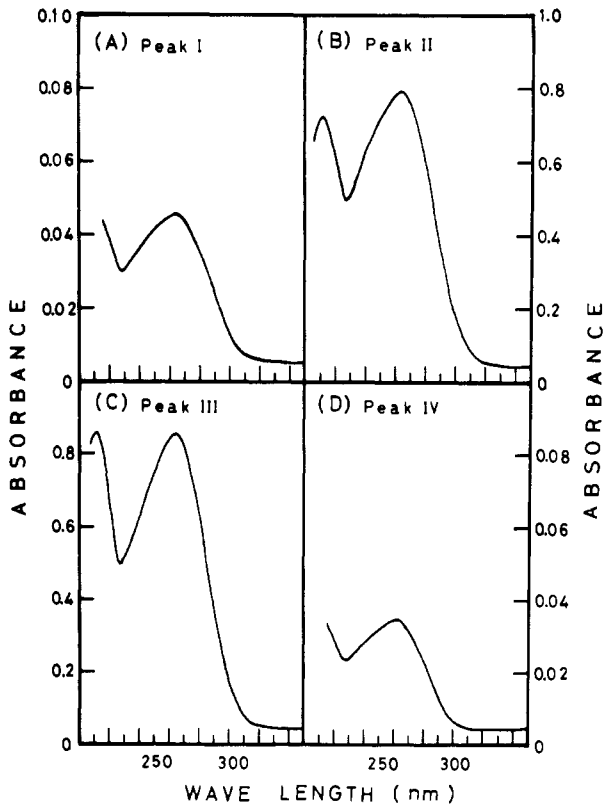

FIGURE 1: Ultraviolet spectra of isolated vitamin D_3 metabolites: (A) peak I [1α,25$(OH)_2$-24-oxo-D_3]; (B) peak II [1α,25$(OH)_2D_3$-26,23-lactone]; (C) peak III [1α,24(*R*),25$(OH)_3D_3$]; (D) peak IV [1α,24(*S*),25$(OH)_3D_3$]. All ultraviolet absorption measurements were made in ethanol.

1α,25$(OH)_2D_3$. The new metabolite of 1α,25$(OH)_2D_3$, peak I, was eluted immediately after authentic 1α,25$(OH)_2D_3$ when a Zorbax Sil column was used as an absorbent and developed with 20% 2-propanol in *n*-hexane. The peak I fraction was rechromatographed on the same column. However, when the same column was developed with 3% methanol in dichloromethane, peak I eluted before the elution of authentic 1α,25$(OH)_2D_3$. Peak I exactly comigrated with authentic 1α,25$(OH)_2$-24-oxo-D_3 in the two different solvent systems. Peak I was homogeneously purified by final HPLC.

The 1α,24,25$(OH)_3D_3$ fraction collected from the Sephadex LH-20 column was subjected to HPLC. The metabolite of 1α,25$(OH)_2D_3$, peak II, was eluted immediately after authentic 1α,25$(OH)_2D_3$, and peak III and peak IV eluted at the same positions as authentic 1α,24(*R*),25$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$, respectively, when a Zorbax Sil column was used as an absorbent and developed with 3% methanol in dichloromethane. Peak II was further purified by HPLC on a Zorbax Sil column eluted with 20% 2-propanol in *n*-hexane. Peak II was eluted at 17.5–19.5 mL after 1α,24-(*R*),25$(OH)_3D_3$. In the final purification, peak II was homogeneous and exactly comigrated with authentic 1α,25-$(OH)_2D_3$-26,23-lactone. Peaks III and IV were eluted in exactly identical positions with authentic 1α,24(*R*),25$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$, respectively. Thus, by two Sephadex LH-20 column chromatography and four successive high-performance liquid chromatographic procedures, four metabolites derived from 1α,25$(OH)_2D_3$ were isolated.

The ultraviolet spectra of all the metabolites of 1α,25-$(OH)_2D_3$ isolated from the serum display the typical vitamin D_3 cis-triene chromophore with λ_{max} = 264 nm and λ_{min} = 228 nm (Figure 1). From these spectra, the total amounts of isolated metabolites were calculated to be as follows: 1α,25-

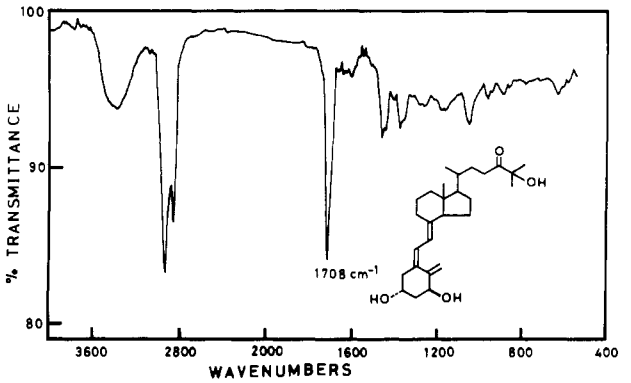

FIGURE 2: Fourier-transform infrared spectrum of isolated peak I, putative 1α,25$(OH)_2$-24-oxo-D_3, taken on crystalline potassium bromide.

$(OH)_2D_3$, 23.6 μg; peak I, 1.8 μg; peak II, 9.2 μg; peak III, 15.4 μg; peak IV, 1.0 μg. With recovery corrections, the serum levels of each metabolite were 49 ng/mL for 1α,25$(OH)_2D_3$, 3 ng/mL for peak I, 19 ng/mL for peak II, 32 ng/mL for peak III, and 2.08 ng/mL for peak IV.

When peak I was subjected to mass spectrometry, the diagnostic ions and structural assignments found were as follows: *m/e* 430, M^+; *m/e* 412, $M^+ - H_2O$; *m/e* 394, $M^+ - 2H_2O$; *m/e* 379, $M^+ - 2H_2O - CH_3$; *m/e* 343, $M^+ - C_4H_7O_2$; *m/e* 287, M^+ – side chain; *m/e* 269, M^+ – side chain – H_2O; *m/e* 251, M^+ – side chain – $2H_2O$; *m/e* 152, A ring + C-6 + C-7^+ [(A ring fragment)$^+$]; *m/e* 134, (A-ring fragment)$^+ - H_2O$ (data not shown). The molecular ion at *m/e* 430 suggests the incorporation of an additional oxygen function into 1α,25-$(OH)_2D_3$ concomitant with the introduction of one degree of unsaturation. The fragment ions at *m/e* 287, 269, 251, 152, and 134, characteristic of the mass spectrum of 1α,25$(OH)_2D_3$, indicate that the secosteroid nucleus of 1α,25$(OH)_2D_3$ has remained unchanged and that the metabolic alteration occurred on the side chain. The presence of the fragment ion at *m/e* 343 indicates that the molecule is unchanged to the C-23 position.

The Fourier-transform infrared spectrum of peak I demonstrated the presence of a carbonyl group showing strong absorption at 1708 cm^{-1} (Figure 2). For determination of the location of the oxygen and unsaturation functionalities, 400 ng of peak I was quantitatively silylated and used for mass spectrometry. Major ions and structural assignments are as follows: *m/e* 646, M^+; *m/e* 631, $M^+ - CH_3$; *m/e* 556, $M^+ - HOSi(CH_3)_3$; *m/e* 541, 556 – CH_3; *m/e* 515, $M^+ - C_3H_6OSi(CH_3)_3$; *m/e* 466, $M^+ - 2HOSi(CH_3)_3$; *m/e* 296, A ring + C-6 + C-7^+ [(A ring fragment)$^+$]; *m/e* 206, (A ring fragment)$^+ - HOSi(CH_3)_3$; *m/e* 131, $C^3H_6OSi(CH_3)_3$ (not shown). The apparent molecular ion at *m/e* 646 indicates the formation of a tris(trimethylsilyl) ether derivative. The presence of the fragment ion at *m/e* 131 demonstrated the C-26(H_3)–C-25(OH)–C-27(H_3) structure of the original 1α,25-$(OH)_2D_3$ was intact in the metabolite.

Treatment of 1 μg of peak I with $NaBH_4$ gave a major reduction product with chromatographic properties on HPLC very similar to those of authentic 1α,24,25$(OH)_3D_3$. Conclusive proof that the reduction product was 1α,24,25$(OH)_3D_3$ [a mixture of the 24(*R*) and 24(*S*) isomers] was provided by the mass spectrum of the reduction product, by comigration with authentic 1α,24,25$(OH)_3D_3$ on HPLC, and by periodate cleavage oxidation and HPLC analysis and mass spectrometry of the resulting aldehyde product. High-performance liquid chromatography of the reduction products of peak I and the authentic 24(*R*) and 24(*S*) isomers of 1α,24,25$(OH)_3D_3$ could

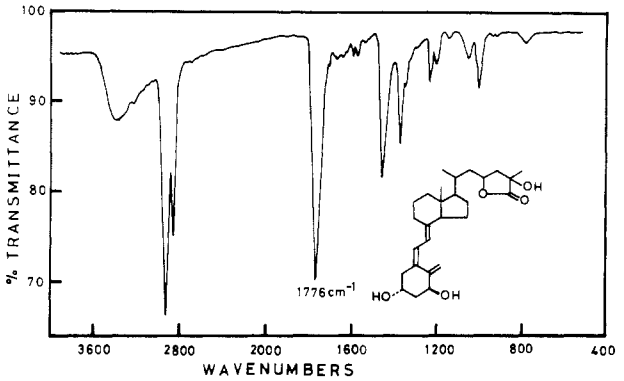

FIGURE 3: Fourier-transform infrared spectrum of isolated peak II, putative $1\alpha,25(OH)_2D_3$-26,23-lactone, taken on crystalline potassium bromide.

resolve the 24-isomers of $1\alpha,24,25(OH)_3D_3$. The elution volumes of the reduction products of peak I on HPLC were identical with those of the authentic $1\alpha,24(R),25(OH)_3D_3$ and $1\alpha,24(S),25(OH)_3D_3$, respectively. The mass spectrum of the reduction product exhibited the following pattern: *m/e* 432, M^+; *m/e* 414, $M^+ - H_2O$; *m/e* 396, $M^+ - 2H_2O$; *m/e* 381, $M^+ - 2H_2O - CH_3$; *m/e* 378, $M^+ - 3H_2O$; *m/e* 251, 269 – H_2O; *m/e* 152, A ring + C-6 + C-7 [(A ring fragment)$^+$]; *m/e* 134, (A ring fragment)$^+ - H_2O$. This spectrum is identical with that of $1\alpha,24,25(OH)_3D_3$.

When periodate cleavage product of reduced peak I was subjected to HPLC, the periodate cleavage product was eluted at 9–12 mL. The mass spectrum of the periodate cleavage product of reduced peak I showed major ions and structural assignments as follows: *m/e* 372, M^+; *m/e* 354, $M^+ - H_2O$; *m/e* 336, $M^+ - 2H_2O$; *m/e* 321, $M^+ - 2H_2O - CH_3$; *m/e* 287, M^+ – side chain; *m/e* 269, 287 – H_2O; *m/e* 251, 269 – H_2O; *m/e* 152, A ring + C-6 + C-7 [(A ring fragment)$^+$]; *m/e* 134, (A ring fragment)$^+ - H_2O$. This spectrum had a molecular ion at *m/e* 372 as would be expected for cleavage between the C-24 and C-25 positions of the reduced peak I. These results indicate that peak I is reduced by $NaBH_4$ to $1\alpha,24,25(OH)_3D_3$. Thus, peak I must be $1\alpha,3\beta,25$-trihydroxy-24-oxo-9,10-seco-5,7,10(19)-cholestatriene or $1\alpha,25$-dihydroxy-24-oxo-vitamin D_3 [$1\alpha,25(OH)_2$-24-oxo-D_3].

The Fourier-transform infrared spectrum of peak II is shown in Figure 3. It indicates the presence of an absorption at 1776 cm^{-1} due to a γ-lactone moiety. Thus, peak II was confirmed to have γ-lactone in the side chain.

The mass spectrum of peak II showed major ions and structural assignments as follows: *m/e* 444, M^+; *m/e* 426, $M^+ - H_2O$; *m/e* 408, $M^+ - 2H_2O$; *m/e* 393, $M^+ - 2H_2O - CH_3$; *m/e* 287, M^+ – side chain; *m/e* 269, 287 – H_2O; *m/e* 251, 269 – H_2O; *m/e* 152, A ring + C-6 + C-7$^+$ [(A ring fragment)$^+$]; *m/e* 134, (A ring fragment)$^+ - H_2O$. The anomalous chromatographic behavior of peak II is identical with that of the authentic $1\alpha,25(OH)_2D_3$-26,23-lactone. From these results, the structure of peak II was unequivocally determined to be $1\alpha,3\beta,25$-trihydroxy-9,10-seco-5,7,10(19)-cholestatriene 26,23-lactone or $1\alpha,25(OH)_2D_3$-26,23-lactone.

The structures of peak III and peak IV were determined by comigration with authentic $1\alpha,24,25(OH)_3D_3$ on HPL, by mass spectrometry, and by periodate cleavage oxidation and HPLC analysis and mass spectrometry of the resulting aldehyde product. Peak III and peak IV comigrated with authentic $1\alpha,24(R),25(OH)_3D_3$ and $1\alpha,24(S),25(OH)_3D_3$, respectively. The mass spectrum of peak III showed major ions and structural assignments as follows: *m/e* 432, M^+; *m/e* 414, $M^+ - H_2O$; *m/e* 396, $M^+ - 2H_2O$; *m/e* 381, $M^+ - 2H_2O - CH_3$; *m/e* 278, $M^+ - 3H_2O$; *m/e* 363, $M^+ - 3H_2O - CH_3$; *m/e* 387 M^+ – side chain; *m/e* 269, 287 – H_2O; *m/e* 251, 269 – H_2O; *m/e* 152, A ring + C-6 + C-7$^+$ [(A ring fragment)$^+$]; *m/e* 134, (A ring fragment)$^+ - H_2O$. In the case of peak IV, the fragmentation pattern is identical with that of peak III. The periodate cleavage products of peak III and peak IV were purified by HPLC and then were subjected to mass spectrometry. The mass spectra of the periodate cleavage products of peak III and peak IV gave *m/e* 372, 354, 336, 321, 287, 269, 251, 152, and 134. These spectra had a molecular ion *m/e* 372 as would be expected for cleavage between the C-24 and C-25 positions of peak III and peak IV. From these results, the structures of peak III and peak IV were unequivocally determined to be $1\alpha,24(R),25(OH)_3D_3$ and $1\alpha,24(S),25(OH)_3D_3$, respectively.

Discussion

This report demonstrates that $1\alpha,25(OH)_2$-24-oxo-D_3, $1\alpha,25(OH)_2D_3$-26,23-lactone, and $1\alpha,24(S),25(OH)_3D_3$ are metabolites of $1\alpha,25(OH)_2D_3$ in vivo. These metabolites as well as $1\alpha,24(R),25(OH)_3D_3$ were isolated in pure form from the serum of dogs given large doses of $1\alpha,25(OH)_2D_3$. The structural assignments are based on their chromatographic behavior, ultraviolet absorption spectrophotometry, Fourier-transform infrared spectrometry, mass spectrometry, and specific chemical reactions of materials isolated by successive chromatography on Sephadex LH-20 and HPLC columns of Zorbax Sil.

An essential feature of the HPLC Zorbax Sil chromatographic purification was the use of the different solvent systems 2-propanol–*n*-hexane (20:80 v/v) and methanol–dichloromethane (3:97 v/v). The subtle structural differences of many of the vitamin D metabolites are such that even the high resolving power of HPLC cannot achieve an unequivocal separation of all the vitamin D metabolites that may be present in the same sample with one solvent system. Thus, the $1\alpha,25(OH)_2D_3$ and $1\alpha,24,25(OH)_3D_3$ regions of the Sephadex LH-20 chromatogram were subsequently resolved by HPLC into two and three components, respectively.

The structural confirmation of $1\alpha,25(OH)_2$-24-oxo-D_3 was carried out as follows: (i) The isolated metabolite comigrates with the authentic $1\alpha,25(OH)_2$-24-oxo-D_3 on HPLC in two different solvent systems. (ii) The UV absorption spectrum of the metabolite displays the typical vitamin D_3 cis-triene chromophore with λ_{max} = 264 nm, λ_{min} = 228 nm, and OD_{264}/OD_{228} = 1.63 (Figure 1). (iii) The mass spectrum of the metabolite gives *m/e* 430, 412, 394, 379, 343, 287, 269, 251, 152, and 134. The fragment ions at *m/e* 287, 269, and 251 indicate that the secosteroid nucleus of $1\alpha,25(OH)_2D_3$ has remained unchanged and that all the metabolic alterations have taken place on the side chain. (iv) The Fourier-transform infrared spectrum of the metabolite indicates the very intense absorbance at 1709 cm^{-1}, indicative of a carbonyl group (Figure 2). (v) The reduction products of the metabolite with $NaBH_4$ comigrate with the synthetic, $1\alpha,24(R),25(OH)_3D_3$ and $1\alpha,24(S),25(OH)_3D_3$ on HPLC, respectively. (vi) The mass spectrum of the reduction product is consistent with the structural assignment of $1\alpha,24,25(OH)_3D_3$. (vii) The mass spectrum of the periodate cleavage product of reduced peak I is consistent with the structural assignment of 1α-hydroxy-25,26,27-trinor-24-oxovitamin D_3 (1α-OH-25,26,27-trinor-24-CHO-D_3). These results indicate the structure of peak I was unequivocally determined to be $1\alpha,25(OH)_2$-24-oxo-D_3 (Figure 4, I).

The structural confirmation of the $1\alpha,25(OH)_2D_3$-26,23-lactone was carried out as follows: (i) The chromatographic

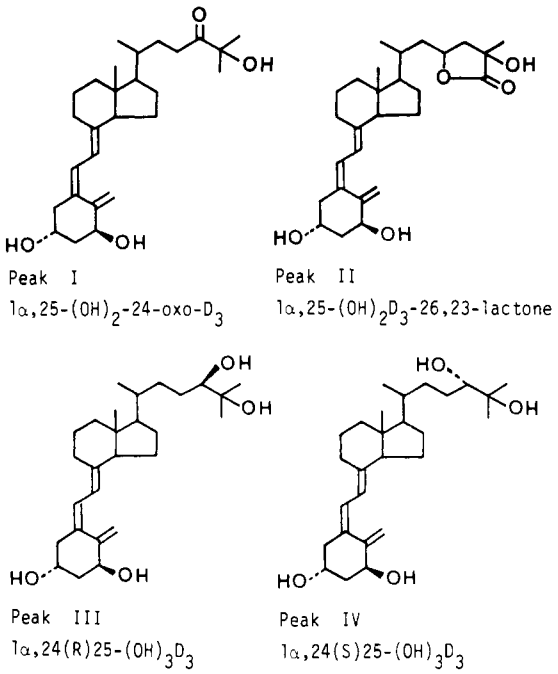

FIGURE 4: Proposed structures of the four isolated vitamin D_3 metabolites.

behavior of the metabolite is similar to that of the 1α,25-$(OH)_2D_3$-26,23-lactone (Ohnuma et al., 1980). (ii) The isolated metabolite comigrates with the authentic 1α,25-$(OH)_2D_3$-26,23-lactone on HPLC in two different solvent systems. (iii) The UV absorption spectrum of the metabolite displays the typical vitamin D_3 cis-triene chromophore with λ_{max} = 264 nm, λ_{min} = 228 nm, and OD_{264}/OD_{228} = 1.66 (Figure 1). (iv) The Fourier-transform infrared spectrum of the metabolite indicates the very intense absorbance at 1776 cm^{-1}, indicative of a γ-lactone moiety (Figure 3). (v) The mass spectrum of the metabolite is identical with that of 1α,25-$(OH)_2D_3$-26,23-lactone (Ohnuma et al., 1980; Ishizuka et al., 1981). From these results, the structure of the vitamin D_3 metabolite, peak II, was unequivocally determined to be 1α,25$(OH)_2D_3$-26,23-lactone (Figure 4, II).

The biochemical details of the metabolic pathway leading from 1α,25-$(OH)_2D_3$ to 1α,25-$(OH)_2D_3$-26,23-lactone are unknown. Two new metabolites of vitamin D_3 have been isolated and identified, from the serum of rats given large doses of vitamin D_3, 23(*S*),25-dihydroxyvitamin D_3 [23(*S*),25(OH)$_2$D$_3$] (Tanaka et al., 1981c; Ikekawa et al., 1981), and 23(*S*),25(*R*),26-trihydroxyvitamin D_3 [23(*S*),25(*R*),26-$(OH)_3D_3$] (Ishizuka et al., 1982a,b). Recently, Tanaka et al. reported that 23(*S*),25$(OH)_2D_3$ is an intermediate in the biosynthesis of 25-OH-D_3-26,23-lactone (Tanaka et al., 1981a). Thus, 23(*S*),25$(OH)_2D_3$ is a far better substrate for production of 25-OH-D_3-26,23-lactone than is 25,26$(OH)_2D_3$ (Tanaka et al., 1981a). We have previously reported that the production of 25-OH-D_3-26,23-lactone from 23(*S*),25-(*R*),26$(OH)_3D_3$ is 16.2 times more than that from 23(*S*),25-$(OH)_2D_3$ (Ishizuka et al., 1982b). Collectively, these results indicate that the 25-OH-D_3-26,23-lactone is biosynthesized from 25-OH-D_3 by way of 23(*S*),25$(OH)_2D_3$ to 23(*S*),25-(*R*),26$(OH)_3D_3$ (Ishizuka et al., 1982b). By analogy to the metabolic pathway leading from 25-OH-D_3 to 25-OH-D_3-26,23-lactone, it is reasonable to propose that 1α,25$(OH)_2D_3$ is first metabolized to 1α,23(*S*),25-trihydroxyvitamin D_3 [1α,23(*S*),25(*R*)$(OH)_3D_3$]. 1α,23(*S*),25$(OH)_3D_3$ is then metabolized to 1α,23(S),25(*R*),26-tetrahydroxyvitamin D_3 [1α,23(*S*),25(*R*),26$(OH)_4D_3$], although these metabolites have not yet been found, followed by a lactonization reaction to a metabolite having a γ-lactone ring, presumably 1α,25-$(OH)_2D_3$-26,23-lactone.

Very recently, we reported that 25-hydroxyvitamin D_3 26,23-(peroxylactone) (25-OH-D_3-26,23-peroxylactone) is a major metabolite of 25-OH-D_3 present in the serum of rats (Ishizuka et al., 1982c). The 25-OH-D_3-26,23-peroxylactone was converted upon storage at room temperature or −20 °C into the 25-OH-D_3-26,23-lactone. Since under the conditions of this isolation only the 25-OH-D_3-26,23-peroxylactone and no 25-OH-D_3-26,23-lactone was present in the rat serum, this suggests that the 25-OH-D_3-26,23-peroxylactone is the naturally occurring metabolite (Ishizuka et al., 1982c). If this is true, then it should be recognized that 25-OH-D_3-26,23-lactone (Ishizuka et al., 1981; Wichmann et al., 1979; Horst, 1979) and, by analogy, the 1α,25$(OH)_2D_3$-26,23-lactone (Ohnuma et al., 1980; Ishizuka et al., 1981) are not naturally occurring vitamin D_3 metabolites. We are conducting further experiments to more critically evaluate whether 1α,25$(OH)_2D_3$-26,23-peroxylactone exists in the serum or not.

The structural confirmation of 1α,24(*R*),25$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$ was carried out as follows: (i) The two isolated metabolites comigrate with authentic 1α,24(*R*),25-$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$ on HPLC, respectively. (ii) The UV absorption spectra of the metabolites display the typical vitamin D_3 cis-triene chromophore with λ_{max} = 264 nm, λ_{min} = 228 nm, OD_{264}/OD_{228} = 1.78, and OD_{264}/OD_{228} = 1.64, respectively (Figure 1). (iii) The mass spectra of the two metabolites as well as their periodate cleavage products are consistent with the structural assignment of 1α,24-(*R*),25$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$, respectively (Figure 4, III and IV).

In previous reports (Kleiner-Bossaler et al., 1974; Tanaka et al., 1977; Ribovich & DeLuca, 1978; Holick et al., 1973; Friedlander & Norman, 1975), evidence for the in vivo occurrence of 1α,24,25$(OH)_2D_3$ consisted of noting a radioactive peak in the appropriate elution volume on Sephadex LH-20 or HPLC columns after dosing with tritiated precursors. The putative 1α,24,25$(OH)_3D_3$ had never been isolated from in vivo sources to homogeneity and analyzed by UV absorption spectrum and mass spectrometry. 1α,24,25-$(OH)_3D_3$ was recently isolated from the plasma of cows injected intramuscularly with 375 mg of vitamin D_3 once a week for 4 weeks and identified by its comigration with synthetic 1α,24,25-$(OH)_3D_3$ and by mass spectrometry (Reinhardt et al., 1982). The isolated 1α,24,25$(OH)_3D_3$ comigrated with synthetic 1α,24(*R*),25$(OH)_3D_3$ and was homogeneous. Tanaka et al. previously reported that the 1α,24,25$(OH)_3D_3$ generated in vivo and in vitro has the 24-hydroxyl in the *R* configuration (Tanaka et al., 1977).

We describe in this paper that both 1α,24(*R*),25$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$ exist in the serum of dogs given large doses of 1α,25$(OH)_2D_3$. The metabolic pathway to 1α,24-(*S*),25$(OH)_3D_3$ from 1α,25$(OH)_2D_3$ in vivo is unknown. We found that 1α,25$(OH)_2$-24-oxo-D_3 was produced by an incubation of 1α,24(*R*),25$(OH)_3D_3$ or 1α,24(*S*),25$(OH)_3D_3$ with homogenates of intestinal mucosa or kidney obtained from vitamin D replete chicks (S. Ishizuka et al., unpublished results). Furthermore, we demonstrated that 1α,24(*R*),25-$(OH)_3D_3$ and 1α,24(*S*),25$(OH)_3D_3$ were isolated from the incubation of 1α,25$(OH)_2$-24-oxo-D_3 with homogenates of kidney from vitamin D deficient chicks or vitamin D replete chicks. Under these conditions, 1α,24(*S*),25$(OH)_3D_3$ was a more dominant metabolite than 1α,24(*R*),25$(OH)_3D_3$ from the 1α,25$(OH)_2$-24-oxo-D_3 (S. Ishizuka et al., unpublished

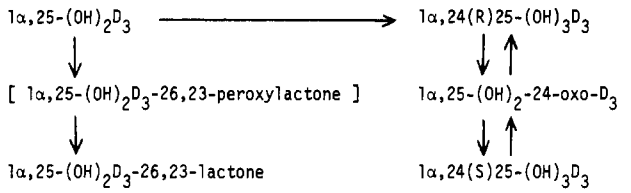

FIGURE 5: Pathways of metabolism of $1\alpha,25(OH)_2D_3$. The compound listed in brackets has not yet been chemically characterized; there is evidence for the existence of 25-OH-D_3-26,23-peroxylactone in serum (Ishizuka et al., 1982c).

results). Thus, it might be reasonable to consider that the $1\alpha,24(S),24(OH)_3D_3$ was biosynthesized from $1\alpha,25(OH)_2D_3$ by way of $1\alpha,24(R),25(OH)_3D_3$ to $1\alpha,25(OH)_2$-24-oxo-D_3.

From these considerations, it can be proposed that $1\alpha,25$-$(OH)_2D_3$ undergoes metabolism by two alternative pathways: one pathway includes C-23 and C-26 oxidations as the first steps and the second pathway includes C-24 oxidation as the first step. These relationships are summarized in Figure 5.

The pathways for the metabolism of vitamin D are becoming increasingly complex. To date, 28 vitamin D metabolites have been isolated and chemically characterized (Henry & Norman, 1984). With the exception of $1\alpha,25(OH)_2D_3$, 25-hydroxy-5,6-*trans*-vitamin D_3 (Kumar et al., 1981), and the 19-nor-10-oxo derivatives of vitamin D_3 and 25-OH-D_3 (Napoli et al., 1983), all the metabolites represent some form of structural modification of the 8-carbon side chain of vitamin D_3. The three new metabolites obtained from the serum of dogs given large doses of $1\alpha,25(OH)_2D_3$ represent structural modification of the side chain of vitamin D_3 as described in this report. The data presented here point out the need for additional research to determine the physiological function of these metabolites and their possible significance during $1\alpha,25(OH)_2D_3$ treatment and in diseases where $1\alpha,25(OH)_2D_3$ levels are endogenously elevated.

Supplementary Material Available

Additional data (HPLC separations, mass spectra, etc.) supporting the structure assignments for these four compounds (8 pages). Ordering information is given on any current masthead page.

Registry No. $1\alpha,25(OH)_2D_3$, 32222-06-3; $1\alpha,25(OH)_2$-24-oxo-D_3, 76338-50-6; $1\alpha,25(OH)_2D_3$-26,23-lactone, 81203-50-1; $1\alpha,24(S),25(OH)_3D_3$, 56142-95-1; $1\alpha,24(R),25(OH)_3D_3$, 56142-94-0.

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