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Chemistry

Synthesis and determination of configuration of natural 25-hydroxyvitamin D_3 26,23-lactone*

(vitamin D metabolite/iodolactonization/phenylselenolactonization)

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ABSTRACT **The four stereoisomers of 25-hydroxyvitamin D_3 26,23-lactone were synthesized by a stereoselective lactonization method. Natural 25-hydroxyvitamin D_3 26,23-lactone was produced from 25-hydroxy-[3α-^{3}H]vitamin D_3 by *in vitro* incubation of kidney homogenate prepared from vitamin D-supplemented chickens or was isolated from the serum of rats given 1,25-dihydroxyvitamin D_3 and 25-hydroxy-[3α-^{3}H]vitamin D_3. The four synthetic isomers and the naturally produced 25-hydroxyvitamin D_3 26,23-lactone were subjected to high-performance liquid chromatography in a system capable of separating the four isomers. The natural lactone comigrated with synthetic (23*S*,25*R*)-25-hydroxyvitamin D_3 26,23-lactone, establishing it as the natural vitamin D_3 metabolite.**

A major metabolite of vitamin D_3 was recently isolated in pure form and identified as 25-hydroxyvitamin D_3 26,23-lactone (25-OH-D_3 26,23-lactone) (1). Hollis *et al.* (2) claimed that 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] is a precursor in the biosynthesis of 25-OH-D_3 26,23-lactone but Pramanik *et al.* (3) reported that 25,26-$(OH)_2D_3$ may not be the intermediate in lactone biosynthesis. We recently isolated another major metabolite of vitamin D_3 and identified it as 23,25-$(OH)_2D_3$ (4). Chemical synthesis of the C-23 stereoisomers of 23,25-$(OH)_2D_3$ and configuration at C-23 of natural 23,25-$(OH)_2D_3$ was determined to be *S* (5). To determine the true precursor of the 26,23-lactone, four compounds—namely, (25*S*)-25,26-$(OH)_2D_3$, (25*R*)-25,26-$(OH)_2D_3$, (23*S*)-23,25-$(OH)_2D_3$, and (23R)-23,25-$(OH)_2D_3$—were incubated with kidney homogenate capable of producing lactone from 25-OH-D_3 (6). These results suggested that (23*S*)-23,25-$(OH)_2D_3$ is the likely precursor of 25-OH-D_3 26,23-lactone (7). This provided evidence that the C-23 configuration of the lactone is *S*. Recently, Morris *et al.* (8) synthesized four isomers of 25-OH-D_3 26,23-lactone and suggested that the natural lactone has a 23*R*,25*S* configuration on the basis that (25*S*)-25,26-$(OH)_2D_3$ is the precursor in lactone biosynthesis.

We have also synthesized four isomers of 25-OH-D_3 26,23-lactone. Only two isomers, (23*R*,25*S*)- and (23*S*,25*R*)-25-OH-D_3 26,23-lactone, give a C-23 proton resonance pattern similar to that of natural lactone, in agreement with data of Morris *et al.* (8). In addition, we demonstrate that biologically produced radioactive 25-OH-D_3 26,23-lactone comigrates only with (23*S*,25*R*)-25-OH-D_3 26,23-lactone.

MATERIALS AND METHODS

Synthesis of Stereoisomers of 25-OH-D_3 26,23-Lactone. The synthesis of one of the isomers, (23R)-25-OH-D_3 26,23-lactone has been reported (9). This compound was synthesized by using iodolactonization of 22,23-*trans*-26-acid **2b** which was prepared from the bisnorcholen-22-al **1** (Fig. 1) hydroxylation at C-25 with MoOPH (MoO_5·Py·), and then hydrolysis with HCl/MeOH to give the 25-OH 26,23-lactone (**4b**). Properties: mp 243–247°C; NMR 0.72(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 3.50(1H, *m*, 3-H), 4.42(1H, *m*, 6-H); IR 1765 cm^{-1}. The configuration at C-23 was determined by chemical transformation (9). The configuration at C-25 was clarified as *R* by transformation into 25,26-dihydroxycholesterol (18) (10).

The lactone **3b** (3-OH) was converted to the methoxyethoxymethyl ether **3c** (mp 125–127°C) with methoyethoxymethyl chloride and diisopropylethylamine in methylene chloride and then oxidized to the 25-ol **4c** (mp 175–176°C) with MoOPH. The 25-ol **4c** was reduced with $LiAlH_4$ in tetrahydrofuran at room temperature followed by acetonide formation to give the 23,-25,26-triol 25,26-acetonide **16**. Properties: mp 96–98°C; $[\alpha]_D^{20}$ −18.7° (c = 1.35). The 23-OH group was oxidized with pyridinium chlorochromate to afford the 23-oxo compound **17**. Properties: amorphous; $[\alpha]_D^{20}$ −20.7° (c = 1.09). The tosylhydrazone of **17** was reduced with $NaBH_4$ to give the 25,26-acetonide in 20% yield. Removal of the protecting groups with *p*-TsOH in MeOH and then $ZnBr_2$ in CH_2Cl_2 gave 25,26-dihydroxycholesterol (**18**). Identification of **18** with an authentic sample of (25*R*)-25,26-dihydroxycholesterol (10) was carried out by cochromatography as the (+)-methoxy trifluoromethylphenylacetic acid (MTPA) esters in a high-performance liquid chromatography (HPLC) system. HPLC separation was carried out with a column (15 cm × 4.6 mm) of Zorbax-SIL at 1.4 × 100 kg/cm^2 (13.7 mPa) with *n*-hexane/CH_2Cl_2 (5:1) as the solvent. The retention times were for 17.2 min for (25*R*)-25,26-dihydroxycholesterol di-MTPA ester and 19.1 min for the 25*S* isomers. Thus, it is evident that the oxidation of the lactone **3b** with MoOPH was performed by the less-hindered side attack.

(23*R*,25*S*)-25-OH 26,23-lactone (**7**) was synthesized by the following process. When the ester **2a** was oxidized with MoOPH, the 25-hydroxy 26-ester **5a** was obtained in 88% yield. Properties: mp 184–187°C; NMR 0.67(3H, *s*, 18-H_3), 1.01(3H, *s*, 19-H_3), 1.27(3H, *s*, 27-H_3), 5.30(3H, *m*, 22-, 23- and 6-H); IR 1720 cm^{-1}. Iodolactonization of the hydrolysis product **5b** (mp 196–199°C) afforded a nonseparable isomeric mixture **6** (1:1) at C-25 in 80% yield. Properties: mp 203–204°C; NMR(C^2HCl_3-$C^2H_3O^2H$) 0.77(3H, *s*, 18-H_3), 1.02(3H, *s*,

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Abbreviations: $(OH)_2D_3$, dihydroxycholesterol D_3; OH-D_3, hydroxyvitamin D_3; MoOPH, MoO_5·Py·hexamethylphosphoric triamide; HPLC, high-performance liquid chromatography; MTPA, methoxytrifluoromethylphenylacetic acid; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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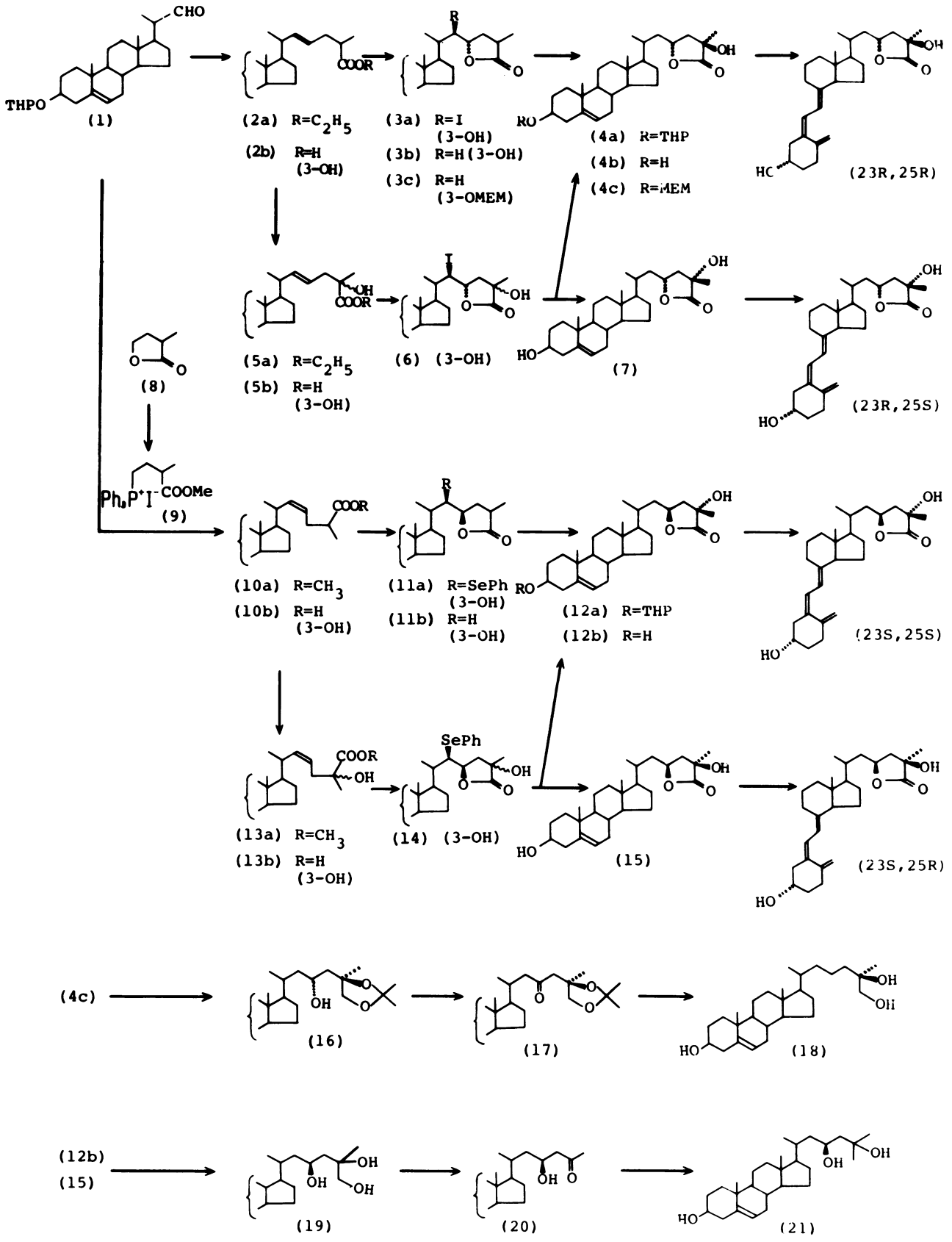

FIG. 1. Synthesis of 25-OH-D_3 26,23-lactone isomers.

19-H_3), 1.47(3H, *s*, 27-H_3), 5.32(1H, *m*, 6-H). After removal of iodine with *n*-Bu_3SnH, the mixture of 25-hydroxy lactone was separated by flash chromatography on Kieselgel 60 (230–400 mesh) with hexane/ethyl acetate (1:1) to give the more polar isomer 7 [properties: mp 252–254°C; NMR 0.72(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 1.49(3H, *s*, 27-H_3), 3.50(1H, *m*, 3-H),

4.42(1H, *m*, 23-H); IR 1765 cm^{-1}] and a less polar compound which was identical with the 25-hydroxy lactone **4b** obtained previously from **3a**. The conversion of **5b** to **6** may proceed via the same lactonization mechanism as in the case of **2b** to **3a** (11); thus, 25-hydroxy lactone **7** should have the 23*R*,25*S* configuration.

The synthesis of (23*S*)-25-OH 26,23-lactone **12b** and **15** were accomplished by cyclization of 22,23-*cis*-26-acid. α-Methylbutyrolactone (8) in conc. HI (1.2 equiv.) was refluxed for 20 min to give the iodo acid; this was converted to the methyl ester with diazomethane. The iodide was treated with triphenylphosphine (1.5 equiv.) in refluxing benzene for 1 day to afford phosphonium salt **9** in 50% yield from **8**. The solution of 22-aldehyde in hexamethylphosphoric triamide was added to the yield solution of **9** (2.4 equiv.) prepared by treatment with dimethyl sulfoxide and NaH (12) and then stirred for 18 hr at room temperature to give 22,23-*cis*-26-ester **10a**, exclusively, in 90% yield, as an oil. Properties: NMR 0.72(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 1.14(3H, *d*, 27-H_3), J=6.6 Hz), 3.67(3H, *s*, COOMe), 5.16(2H, *m*, 22- and 23-H), 5.32(1H, *m*, 6-H); IR 1720 cm^{-1}. Hydrolysis of **10a** with HCl/MeOH and then KOH/MeOH gave the acid **10b** (mp 163–164°C). Iodolactonization of **10b** with iodine failed but lactonization was achieved by using phenylselenyl chloride (13) in CH_2Cl_2 at −78°C to give **11a**, regio- and stereoselectively, in 70% yield. Properties: mp 214.5–217°C; NMR 0.72(3H, *s*, 18-H_3), 1.03(3H, *s*, 19-H_3), 4.60(1H, *m*, 23-H); IR 1765 cm^{-1}. The reduction with *n*-Bu_3SnH and azobisisobutyronitrile in refluxing toluene resulted in the removal of the phenylseleno group, giving rise to the lactone **11b** (mp 170–172°C) which was assumed to be an isomeric congener of 23*R* lactone **3b**. This assumption was confirmed by conversion of **12b** to 23,25-dihydroxycholesterol. The tetrahydropyranyl ether of **11b** was hydroxylated with MoOPH to give the 25-hydroxy lactone **12a**, which was converted to 3,25-dihydroxy lactone **12b**. Properties: mp 247.5–249.5°C; NMR 0.70(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 3.50(1H, *m*, 3-H), 4.72(1H, *m*, 23-H); IR 1765 cm^{-1}. From the evidence of less-hindered attack by MoOPH, the **12b** thus obtained should be the 23*S*,25*S* isomer.

Another 23*S* lactone was obtained by the following procedure. 25-Hydroxy ester **13a** [mp 163–165°C; NMR 0.72(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 1.23(3H, *s*, 27-H_3), 3.75(3H, *s*, COOMe), 5.30(3H, *m*, 22-, 23-, 6-H); IR 1725 cm^{-1}] was obtained from **10a** by the same procedure as **2a** to **5a**. Lactonization of the hydrolyzed product, **13b** (mp 208–210.5°C) afforded the isomeric mixture at C-25 of the phenylseleno lactone **14**. Properties: mp 203–206°C; NMR 0.72(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 4.50 and 4.85(1H, *m*, 23-H). The signal of 23-H revealed that **14** is a 1:1 mixture. After removal of the phenylseleno group, the mixture was separated by preparative thin layer chromatography with benzene/ethyl acetate (2:1) as a solvent (developed three times) to give the more-polar hydroxy lactone **15** [mp 248–249°C; NMR 0.71(3H, *s*, 18-H_3), 1.02(3H, *s*, 19-H_3), 3.50(1H, *m*, 3-H), 4.43(1H, *m*, 23-H); IR 1763 cm^{-1}] and the less-polar isomer **12b** which was identical with the 23*S*,25*S* lactone **12b**. Consequently, the fourth isomer **15** should have the configuration 23*S*,25*R*. The 23*S* configuration of **12b** and **15** was confirmed by transformation into 23,25-dihydroxycholesterol. For this purpose, **12b** or **15** was reduced with $LiAlH_4$ to the 23,25,26-triol **19**. After $NaIO_4$ oxidation of **19**, Grignard reaction with methyl magnesium bromide provided (23*S*)-23,25-dihydroxycholesterol (**21**) whose configuration was determined by direct comparison with the authentic sample (5).

The four isomers of the 25-hydroxy lactone thus synthesized had distinguishable NMR signals for C-23 proton depending on the stereochemical relationship between C-23 and C-25: 4.72 ppm for **4b** (23*R*,25*R*), 4.42 for **7** (23*R*,25*S*), 4.72 for **12b** (23*S*,25*S*), and 4.43 for **15** (23*S*,25*R*). This relationship also was mentioned by Morris *et al.* (8). The characteristic circular dichronism spectra of those isomers are shown in Fig. 2.

Four Δ^5 compounds (**4b**, **7**, **12b**, and **15**) were converted to the corresponding vitamin D compounds through the 5,7-dienes by the same method reported previously (9). Thus, by selective chemical reactions and conventional procedures, four isomers of 25-OH-D_3 26,23-lactone were obtained as the pure form showing identical ultraviolet and mass spectra as reported for the natural metabolite. The NMR chemical shifts of the C-23 proton of the vitamin D compounds were the same as those of corresponding Δ^5 compounds. (23*S*,25*R*)- and (23*R*,25*S*)-25-OH-D_3 26,23-lactone exhibited the same chemical shift of the C-23 proton as that of the natural metabolite.

Vitamin D Metabolites. Crystalline 1,25-$(OH)_2D_3$ and (24*R*)-24,25-$(OH)_2D_3$ were gifts from the Hoffmann–La Roche. 25-OH-D_3 was a gift from The Upjohn Company. (23*S*)-23,25-$(OH)_2D_3$ was synthesized by Ikekawa *et al.* (5); 25-OH-[3α-^{3}H]D_3 was synthesized by one of us (H.F.D.) and associates.

Animals. For *in vitro* production of 25-OH-D_3 26,23-lactone, 1-day-old White Leghorn chickens (Northern Hatcheries, Beaver Dam, WI) were fed a rachitogenic diet (14) for 2 weeks. They were given 2 μg each of 25-OH-D_3 and 1,25-$(OH)_2D_3$ intramuscularly daily for the last 2 days. For *in vivo* production of 25-OH-D_3 26,23-lactone, male weanling rats were purchased from Holtzman (Madison, WI) and fed a diet (15) adequate in calcium and phosphorus but deficient in vitamin D for 6 weeks. The rats were then given 4 μg 1,25-$(OH)_2D_3$ dissolved in 0.1 ml of 1,2-propanediol/ethanol mixture (95:5) subcutaneously daily for the last 2 days and 30 μCi (1 Ci = 3.7×10^{10} becque-

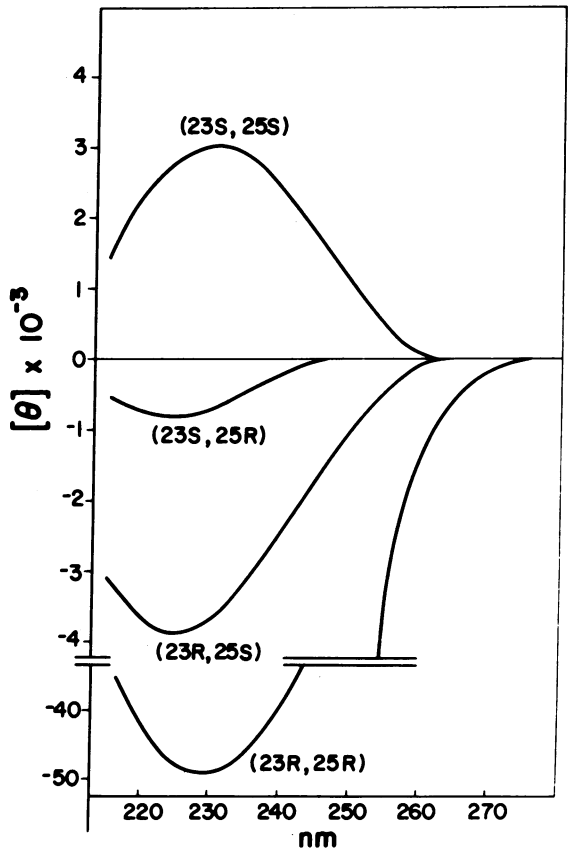

FIG. 2. Circular dichronism curves of four isomers of 25-OH 26,23-lactone. **12b** (23*S*,25*S*), $[\theta]_{230} = 3.04 \times 10^3$; **4b** (23*R*,25*R*), $[\theta]_{230} = 4.95 \times 10^4$; **15** (23*S*,25*R*), $[\theta]_{225} = -8.08 \times 10^2$; **7** (23*R*,25*S*), $[\theta]_{225} = -3.7 \times 10^3$.

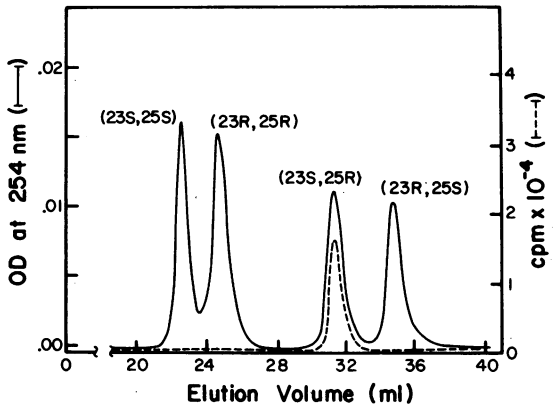

FIG. 3. Cochromatography of natural 25-OH-D_3 26,23-lactone produced *in vitro* (broken line) with synthetic isomers (solid line). Cochromatography was performed by HPLC on a Zorbax-SIL column with 5% 2-PrOH in hexane as solvent. Each 0.8-ml fraction was collected and assayed for radioactivity (broken line); the chromatographic profile of the isomers was obtained by UV detector (solid line).

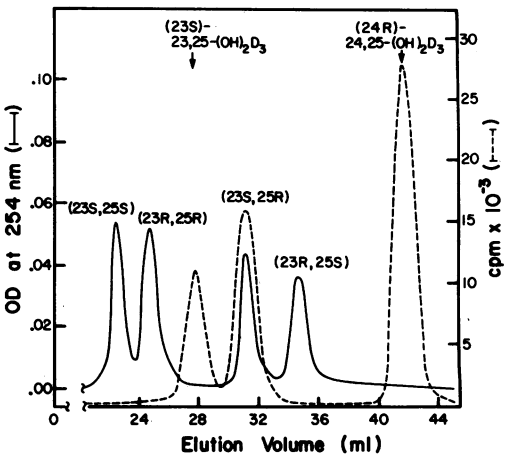

FIG. 4. Cochromatography of natural 25-OH-D_3 26,23-lactone produced *in vivo* with four synthetic isomers. Serum was prepared from 1,25-$(OH)_2D_3$-supplemented rats given 30 μCi of 25-OH-[3α-^{3}H]D_3 24 hr prior to sacrifice. The cochromatography was carried out as described in Fig. 3. Each 0.8-ml fraction was collected and assayed for radioactivity to obtain the chromatographic profile of metabolites biologically produced (broken line); the chromatographic profile of synthetic isomers was obtained by UV detector (solid line).

rels) of 25-OH-[3α-^{3}H]D_3 (28 Ci/mmol) dissolved in 0.1 ml of 95% ethanol intrajugularly 24 hr prior to sacrifice.

***In Vitro* Production of 25-OH-D_3 26,23-Lactone.** The *in vitro* production of 25-OH-[3α-^{3}H]D_3 26,23-lactone was carried out as described with 25-OH-[3α-^{3}H]D_3 as the substrate (6).

Extraction and Purification of 25-OH-D_3 26,23-Lactone from Rat Serum. Twenty-four hours after the administration of 25-OH-[3α-^{3}H]D_3, rats were killed and their blood was collected and centrifuged to obtain serum. The serum was diluted with 1 vol of water and then extracted with 10 vol of methylene chloride (16). The lipid extract was chromatographed as described (6).

Cochromatography of Natural 25-OH-D_3 26,23-Lactone with Synthetic Stereoisomers. 25-OH-[3α-^{3}H]D_3 26,23-lactone prepared by *in vitro* incubation of chicken kidney homogenate and purified through two consecutive HPLC systems as described (6) was mixed with the four isomers of synthetic 25-OH-D_3 26,23-lactone and analyzed by HPLC on the Zorbax-SIL column. HPLC was performed as described (6) except that 5% 2-PrOH/Hexane was used as solvent. The elution position of each isomer in this system had been previously determined. Each 0.8-ml fraction was collected, dried, and assayed for radioactivity in a toluene-based counting solution (17); the chromatographic profile of the synthetic isomers was obtained by an UV absorbance detector at 254 nm. The extract of rat serum purified through a Sephadex LH-20 column as described (6) was mixed with synthetic isomers and subjected to HPLC with 5% 2-PrOH/hexane. Elution positions of (23*S*)-23,25-$(OH)_2D_3$ and (24*R*)-24,25-$(OH)_2D_3$ were previously determined in addition to those of the 25-OH-D_3 26,23-lactone isomers.

Melting points were determined on a hot-stage microscope and are reported uncorrected. IR spectra and optical rotations were determined in chloroform solution. NMR spectra (δ, ppm) were taken in C^2HCl_3 solution. HPLC was carried out with a Waters Associates APC-GPC 203 or ALC/GPC 204 instrument and a Waters 440 fixed-wavelength detector. Radioactivity was determined with a liquid scintillation counter (Packard Instruments model 3255).

RESULTS

Fig. 3 shows that four stereoisomers of 25-OH-D_3 26,23-lactone could be resolved on the HPLC system (solid line) and that the 25-OH-[3α-^{3}H]D_3 26,23-lactone produced *in vitro* (broken line) exactly comigrated with one of the isomers, (23*S*,25*R*)-25-OH-D_3 26,23-lactone. Cochromatography of natural lactone produced *in vivo* with the synthetic isomers was performed on the same HPLC system. Fig. 4 shows that blood from 1,25-$(OH)_2D_3$-supplemented rats contained radioactive substances (broken line) that eluted exactly with (23*S*)-23,25-$(OH)_2D_3$, (24*R*)-24,25-$(OH)_2D_3$ (arrows), and synthetic (23*S*,25*R*)-25-OH-D_3 26,23-lactone. There was no trace of the 25-OH-D_3 26,23-lactone isomers other than 23*S*,25*R*. Thus, naturally produced 25-OH-D_3 26,23-lactone has the 23*S*,25*R* configuration.

DISCUSSION

Although it was clearly shown (18) that vitamin D is metabolized to its active form to express biological activity, the total metabolic pathway of the vitamin remains unclear. A significant number of metabolites of vitamin D_3 have been found in vitamin D-supplemented animals. Among those most recently identified are 25-OH-D_3 26,23-lactone and 23,25-$(OH)_2D_3$. Neither the biosynthetic pathway of 25-OH-D_3 26,23-lactone nor the configuration about C-23 and C-25 had been determined. However, successful synthesis of the stereoisomers of 23,25-$(OH)_2D_3$, determination of the C-23 configuration is the natural product as *S* (5), and the finding that (23*S*)-23,25-$(OH)_2D_3$ is a natural precursor of lactone (7) provided strong evidence that the C-23 configuration of the lactone is *S*. Because both isomers of 25,26-$(OH)_2D_3$ are poor substrates for lactone production (7), the configuration at C-25 remains unknown. After synthesis of four possible isomers of the lactone by Morris *et al.* (8) and by us, the isomer having the configuration 23*S*,25*S* was excluded as the natural lactone because the C-23 proton resonance of the isomer does not agree with that of natural lactone (δ 4.72 and δ 4.46, respectively). On the other hand, another possible isomer (23*S*,25*R*) does have a C-23 proton resonance at δ 4.43. Thus, the natural lactone must have the 23*S*,25*R* configuration. In contrast, Morris *et al.* (8) chose two isomers—namely, (23*S*,25*R*)- and (23*R*,25*S*)-25-OH-D_3 26,23-lactone that give NMR spectra (4.43 and 4.45, respectively) similar to those of the natural lactone. They concluded that the natural product has a 25*S* configuration based on the assumption that 25*S*,26-$(OH)_2D_3$ is the natural precursor to the lactone (2). We have now achieved direct cochromatography on HPLC of naturally pro-

duced lactone (both *in vitro* and *in vivo*) with the four synthetic isomers and the configurations of natural lactone are unambiguously determined as 23*S*,25*R*.

Involvement of 25,26-$(OH)_2D_3$ in lactone formation remains unclear. Absolute configuration at C-25 of natural 25,26-$(OH)_2D_3$ had been determined as *S* by cochromatography on HPLC with radioactive 25,26-$(OH)_2D_3$ produced *in vitro* with chicken kidney homogenate (19) or with nonradioactive 25,26-$(OH)_2D_3$ isolated from human plasma (20). Yet, (25*S*)-25,26-$(OH)_2D_3$ is a poorer substrate than the *R* isomer in production of the lactone (7). (23*S*)-23,25-$(OH)_2D_3$ is a far better precursor to the lactone than is either isomer of 25,26-$(OH)_2D_3$ (7). Although (25*R*)-25,26-$(OH)_2D_3$ cannot be totally excluded as a precursor in lactone formation, it is unlikely because (25*S*)-25,26-$(OH)_2D_3$, not (25*R*)-25,26-$(OH)_2D_3$, is the natural metabolite.

We purposely used two different sources of naturally produced lactone for cochromatography to examine the possibility that the C-23 and C-25 configurations of natural lactone may differ if it is produced *in vitro* or *in vivo* or if it is produced by avian or mammalian species. The natural lactones differently prepared, however, comigrated only with (23*S*,25*R*)-25-OH-D_3 26,23-lactone (Figs. 3 and 4). We also intentionally cochromatographed serum extract with the synthetic isomers without isolating the lactone from other metabolites in the serum to be sure that only one of the isomers of lactone was present. In good agreement with previous results (4), serum from vitamin D-supplemented rats contained 23,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, and 25-OH-D_3 26,23-lactone as major metabolites (Fig. 4, broken line). Because no radioactivity was found comigrating with the other three synthetic lactone isomers, it is clear that (23*S*,25*R*)-25-OH-D_3 26,23-lactone is the natural metabolite of vitamin D_3 and none of the other possible isomers is produced naturally.

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