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Natural 25,26-dihydroxyvitamin D_3 is an epimeric mixture

(vitamin D/vitamin D metabolites/steroid metabolism/vitamin D hydroxylations/calcium)

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ABSTRACT **Radiolabeled 25,26-dihydroxyvitamin D_3 was prepared *in vitro* by using chicken kidney homogenates and *in vivo* in rats from [23,24-^{3}H]-25-hydroxyvitamin D_3. These compounds were mixed with synthetic (25*S*)- and (25*R*)-25,26-dihydroxyvitamin D_3, converted to the corresponding (+)-α-methoxy-α-trifluoromethylphenylacetyl esters, and subjected to high-performance liquid chromatography that separates the derivatized epimers. The radiolabeled 25,26-dihydroxyvitamin D_3 derivatives were a 1:1 mixture of the 25*S* and 25*R* isomers. Similarly unlabeled 25,26-dihydroxyvitamin D_3 isolated from the plasma of rats given large amounts of vitamin D_3 was shown to be a 1:1 mixture of the *S* and *R* isomers. Therefore, naturally occurring 25,26-dihydroxyvitamin D_3 is a mixture of the 25*R* and 25*S* isomers and not just the *S* isomer reported previously.**

Previously we have determined the stereochemical configuration of side-chain-modified metabolites of 25-hydroxyvitamin D_3 (25-OH-D_3) by means of chromatographic comparison of the natural radiolabeled metabolites with synthetic isomers. Naturally produced 23,25-dihydroxyvitamin D_3 [23,25-$(OH)_2D_3$] (1), 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] (2), 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] (3), and 25-hydroxyvitamin D_3-26,23-lactone (25-OH-D_3-26,23-lactone) (4) have been shown to have a single configuration. In contrast to a base-line separation of isomers of 23,25-$(OH)_3D_3$ and 25-OH-D_3-26,23-lactone, epimers of 24,25-$(OH)_2D_3$ or 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] are not easily separated. In the case of 24,25-$(OH)_2D_3$, a sufficient chromatographic separation of the 24-epimers was possible only when their trimethylsilyl (Me_3Si) derivatives were prepared (2). However, separation of Me_3Si derivatives of the 25-epimers of 25,26-$(OH)_2D_3$ is only partial. Nevertheless, the stereochemical configuration of the naturally occurring 25,26-$(OH)_2D_3$ has been reported as *S* based on the partial separation of the Me_3Si derivatives (5–8).

We have now found that (+)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) ester (9) derivatives of the epimers of 25,26-$(OH)_2D_3$ can be more completely separated. Therefore, biologically produced 25,26-$(OH)_2D_3$ was derivatized as MTPA ester and cochromatographed with the MTPA derivatives of the synthetic isomers on HPLC. As a result, the natural 25,26-$(OH)_2D_3$ was found to be an epimeric mixture.

METHODS AND RESULTS

Isomers of 25,26-$(OH)_2D_3$ were synthesized and their stereochemistry determined as described elsewhere (10, 11). Biologically produced 25,26-$(OH)_2D_3$ was prepared in three different ways. (*i*) 25,26-$(OH)_2$-[^{3}H]D_3 was obtained by *in vitro* incubation of 100 μCi (1 Ci = 3.7×10^{10} Bq) of 25-OH-[23,24-^{3}H]-D_3 with kidney homogenate prepared from chickens given 6.5 nmol of vitamin D_3 48 hr prior to sacrifice, as described by Tanaka *et al.* (12). The 25,26-$(OH)_2D_3$ fraction was purified on a Sephadex LH-20 column and then by straight-phase HPLC by using a solvent mixture of 10% 2-propanol in hexane (12). In addition, the fraction was purified on a reversed-phase HPLC by using a solvent mixture of 20% H_2O in MeOH (13). The biologically produced 25,26-$(OH)_2$-[^{3}H]D_3 was then mixed with 20 μg of (25*R*)-25,26-$(OH)_2D_3$ and 50 μg of (25*S*)-25,26-$(OH)_2D_3$. The mixture was treated with MTPA chloride (30 mg) in pyridine (0.1 ml) at 50°C for 1 hr. Water (1 ml) was added to the mixture and reaction products were extracted with 1 ml of ethyl acetate. The organic phase was collected and washed with 2 M (aqueous solution) NaOH, 2 M (aqueous solution) HCl, saturated (aqueous solution) $NaHCO_3$, and 5% (aqueous solution) NaCl in order. The organic phase was then dried with $MgSO_4$ and the solvent was removed with a stream of argon gas. The resulting 3β,26-bis-MTPA ester was dissolved in a solvent mixture of 22% dichloromethane in hexane and injected into the HPLC (Shimadzu LC-4A), equipped with two Zorbax Sil columns (25 cm × 4.6 mm, DuPont), and eluted with the same solvent mixture. As shown in Fig. 1*A*, the 254-nm UV monitor was used to detect the derivatives of synthetic isomers of 25,26-$(OH)_2D_3$ (Fig. 1*A*, solid line), while 1.25 ml of each fraction was collected and its radioactivity (Packard Prias liquid scintillation counter) was determined to detect 25,26-$(OH)_2$-[^{3}H]D_3 derivatives (Fig. 1*A*, bars). Thus, it was unequivocally established that 25,26-$(OH)_2D_3$ produced *in vitro* by chicken renal homogenate has both *R* and *S* configurations. It is important to note that MTPA derivatization does not epimerize 25,26-$(OH)_2D_3$ because either (25*S*)-26-$(OH)_2D_3$ or (25*R*)-26-$(OH)_2D_3$ yields only one HPLC separable component under the above conditions. (*ii*) 25,26-$(OH)_2$-[^{3}H]D_3 was obtained from serum of rats given 4 μCi of 25-OH-[26,27-^{3}H]D_3 24 hr earlier. Purification of the 25,26-$(OH)_2$-[^{3}H]D_3 fraction, MTPA derivatization of both natural and synthetic 25,26-$(OH)_2D_3$, and HPLC cochromatography were carried out as described above. *In vivo*-produced 25,26-$(OH)_2$-[^{3}H]D_3 in rats was also shown to be roughly a 1:1

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Abbreviations: 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3-26,23-lactone; Me_3Si, trimethylsilyl; MTPA, (+)-α-methoxy-α-trifluoromethylphenylacetyl; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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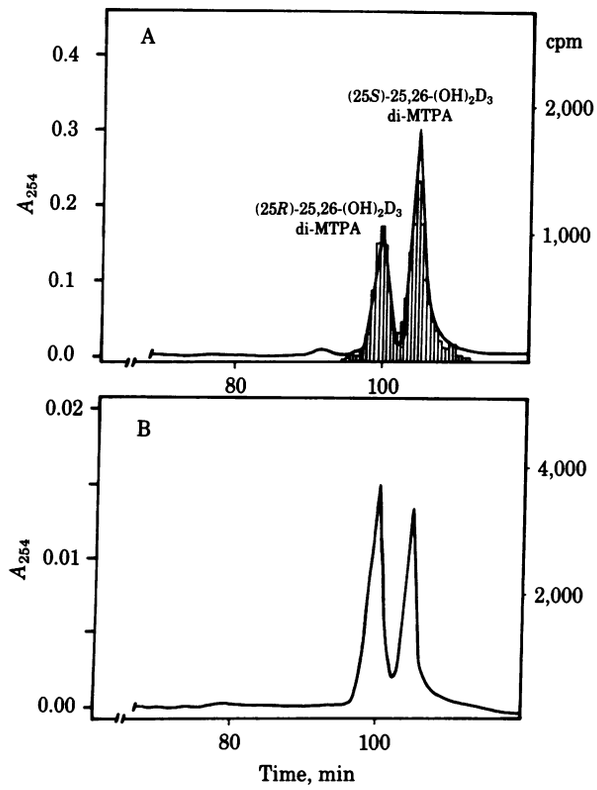

FIG. 1. (*A*) Cochromatography of MTPA derivatives of naturally produced 25,26-(OH)$_2$-[^{3}H]D$_3$ and synthetic isomers. The solid line represents UV absorbance of MTPA derivatives of synthetic isomers, whereas the bars represent radioactivity in the 1.25-ml fractions. (*B*) Chromatography of MTPA derivatives of 25,26-(OH)$_2$D$_3$ from serum of rats given a large dose of vitamin D$_3$. Chromatographic conditions are described in the text.

epimeric mixture (data not shown). (*iii*) Nonradioactive 25,26-(OH)$_2$D$_3$ was isolated from serum collected from 250 rats given 4×10^5 international units of vitamin D$_3$ per rat 6 days before followed by the same amount of vitamin D$_3$ 3 days before exsanguination. A total of 1 liter of the serum diluted with 1 liter of H_2O was extracted with 2 vol of MeOH/chloroform (1:1). The chloroform extract was purified on a Sephadex LH-20 column (1.5 × 25 cm) with chloroform/hexane (65:35). The 25,26-(OH)$_2$D$_3$ was further purified by HPLC by using a Zorbax Sil column (4.6 × 25 cm) eluted with 1.5% methanol in dichloromethane. The purified 25,26-(OH)$_2$D$_3$ from the rat serum (4 μg) was derivatized and chromatographed on HPLC as described above. As shown in Fig. 1*B*, the MTPA ester of natural 25,26-(OH)$_2$D$_3$ appeared as two peaks with retention times corresponding exactly to those of MTPA esters of synthetic (25*R*)-25,26-(OH)$_2$D$_3$ and (25*S*)-25,26-(OH)$_2$D$_3$ (solid lines in Fig. 1*A*). Each material was collected and subjected to mass spectrometry on a Shimadzu 6020. For each compound, a molecular ion of 848 was noted with fragments at mass-to-charge (*m/z*) of 614 (M^+-ROH), 380 (M^+-2ROH), 365 (M^+-2ROH-CH$_3$), and 189 (R-CO) (where R = MTPA). These spectra were identical to those of bis-MTPA esters of synthetic isomers.

DISCUSSION

This report presents clear evidence that both methyl groups of C-26 [the 25-pro-(*S*) methyl group]‖ and C-27 [the 25-pro-(*R*) methyl group]‖ are naturally hydroxylated. It is unknown whether or not the ratio of *R* and *S* isomers of natural 25,26-(OH)$_2$D$_3$ is different between species or is altered by physiological conditions. We intentionally prepared 25,26-(OH)$_2$D$_3$ by *in vitro* methods using a chicken preparation and by *in vivo* methods, from rats given a physiological dose and from rats given a large dose of vitamin D$_3$. In all of our trials *R* and *S* isomers appeared as a 1:1 ratio.

In general, it appears that nonstereospecific hydroxylation of the steroidal side chain occurs at the ends of the side chain. 26-Hydroxycholesterol isolated from human aortas was reported as a mixture of two epimers at C-25 (15). It has been established that 26-hydroxylation of cholesterol, as the initial step of bile acid biosynthesis in liver, is catalyzed by the microsomal hydroxylase at C-26, whereas the mitochondrial hydroxylase carries out hydroxylation at C-27 (16, 17), giving rise to an epimeric mixture. Fucosterol-epoxide, which is a key intermediate in cholesterol synthesis from sitosterol in insects, has been shown to be a (24*R*,28*R*)- and (24*S*,28*S*)-epoxide mixture (18, 19). Inokosterone (26-hydroxyecdysteroid), one of the common phytoecdysteroids, is a 1:2 mixture of the C-25 *R* and *S* epimers (20). In the case of 26-hydroxylation of 25-hydroxyvitamin D, it is possible that two 26-hydroxylase enzymes exist, one acting to produce the 25*S* configuration and another to produce the 25*R* configuration. This possibility would be consistent with the 25*R* configuration of the 25-OH-D$_3$-26,23-lactone (4) and with the idea that (23*S*,25*R*)-23,25,26-(OH)$_3$D$_3$ is an intermediate in the lactone biosynthesis (21). In any case, the physiologic importance of 26-hydroxylations to the function of vitamin D remains unknown.

‖ According to the nomenclature proposed by Popjak *et al.* (14), C-26 originating from C-2 of mevalonate is called the pro-(*R*) methyl group, whereas C-27 derived from C-3′ of mevalonate is called pro-(*S*) in the case of cholesterol. However, in the case of 25-OH-D$_3$ (assuming that the C-25 hydroxylation proceeds with retention of the configuration), the prochiralities of C-26 and C-27 should be reversed due to priority rule. Thus, when a hydroxyl is introduced into C-26, a newly created asymmetric C-25 holds the *R* configuration.

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1. Ikekawa, N., Taguchi, T., Hirano, Y., Tanaka, Y., DeLuca, H. F., Itai, A. & Iitaka, Y. (1981) *J. Chem. Soc. Chem. Commun.*, 1157–1159.
2. Tanaka, Y., DeLuca, H. F., Ikekawa, N., Morisaki, M. & Koizumi, N. (1975) *Arch. Biochem. Biophys.* **170**, 620–626.
3. Tanaka, Y., Castillo, L., DeLuca, H. F. & Ikekawa, N. (1977) *J. Biol. Chem.* **252**, 1421–1424.
4. Eguchi, T., Takatsuto, S., Ishiguro, M., Ikekawa, N., Tanaka, Y. & DeLuca, H. F. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6579–6583.
5. Cesario, M., Guilhem, J., Pascard, C. & Redel, J. (1978) *Tetrahedron Lett.*, 1097–1098.
6. Redel, J., Bazely, N., Tanaka, Y. & DeLuca, H. F. (1978) *FEBS Lett.* **94**, 228–230.
7. Redel, J., Bazely, N., Mawer, E. B., Hann, J. & Jones, F. S. (1979) *FEBS Lett.* **106**, 162–164.
8. Partridge, J. J., Shiuey, S. J., Chadaha, N. K., Baggiolini, E. G., Blount, J. F. & Uskokovic, M. R. (1981) *J. Am. Chem. Soc.* **103**, 1253–1255.
9. Chen, S. M. L., Nakanishi, K., Awata, N., Morisaki, M., Ikekawa, N. & Shimizu, Y. (1975) *J. Am. Chem. Soc.* **97**, 5297–5299.
10. Ishiguro, M., Koizumi, N., Yasuda, M. & Ikekawa, N. (1981) *J. Chem. Soc. Chem. Commun.*, 115–117.
11. Koizumi, N., Ishiguro, M., Yasuda, M. & Ikekawa, N. (1983) *J. Chem. Soc. Perkin Trans. 1*, in press.
12. Tanaka, Y., Shepard, R., DeLuca, H. F. & Schnoes, H. K. (1978) *Biochem. Biophys. Res. Commun.* **83**, 7–13.

13. Tanaka, Y. & DeLuca, H. F. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 196–199.
14. Popjak, G., Edmond, J., Anet, F. A. L. & Easton, N. R., Jr. (1977) *J. Am. Chem. Soc.* **2,** 931–935.
15. Redel, J. (1979) *J. Chromatogr.* **168,** 273–276.
16. Gustafsson, J. & Sjostedt, S. (1978) *J. Biol. Chem.* **253,** 199–201.
17. Atsuka, Y. & Okuda, K. (1981) *J. Biol. Chem.* **256,** 9144–9153.
18. Fujimoto, Y., Morisaki, M. & Ikekawa, N. (1980) *Biochemistry* **19,** 1065–1069.
19. Ikekawa, N., Fujimoto, Y., Takasu, A. & Morisaki, M. (1980) *J. Chem. Soc. Chem. Commun.,* 709–711.
20. Hikino, H., Mohri, K., Hikino, Y., Arihara, S. & Takemoto, T. (1976) *Tetrahedron* **32,** 3015–3021.
21. Ishizuka, S., Ishimoto, S. & Norman, W. (1982) *FEBS Lett.* **138,** 83–87.