# METABOLIC PATHWAY TO 25-HYDROXYVITAMIN $D_3$-26,23-LACTONE FROM 25-HYDROXYVITAMIN $D_3$

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## 1. Introduction

A new metabolite of vitamin $D_3$ has been isolated from the plasma of chickens, rats and pigs and identified as 25-hydroxyvitamin $D_3$-26,23-lactone (25-OH-$D_3$-26,23-lactone) [1–3]. The stereochemical configurations of natural 25-OH-$D_3$-26,23-lactone at the C-23 and C-25 positions were determined to be 23(*S*) and 25(*R*), respectively [4,5]. In [6] 25(*S*)26-dihydroxyvitamin $D_3$ (25(*S*)26-$(OH)_2D_3$) was reported as an intermediate in the biosynthesis of the 25-OH-$D_3$-26,23-lactone. But, in [7] 25(*S*)26-$(OH)_2D_3$ was shown not to be a precursor of the 25-OH-$D_3$-26,23-lactone [7]. The metabolic pathway to 25-OH-$D_3$-26,23-lactone from 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) was still unknown.

Two new metabolites of vitamin $D_3$ have been isolated and identified, from the serum of rats given large doses of vitamin $D_3$: 23,25-dihydroxyvitamin $D_3$ (23,25-$(OH)_2D_3$) [8] and 23,25,26-trihydroxyvitamin $D_3$ (23,25,26-$(OH)_3D_3$) [9]. The structure of the latter suggests a possible role as an intermediate in the biogenesis of the 25-OH-$D_3$-26,23-lactone.

The 25-OH-$D_3$-26,23-lactone is produced in the kidney [3,6,10]; therefore we designed experiments in an attempt to generate this metabolite from various vitamin $D_3$ metabolites in vitro by using chick kidney homogenates. Here we show that 25-OH-$D_3$-26,23-lactone is biosynthesized from 25-OH-$D_3$ by way of 23,25-$(OH)_2D_3$ to 23,25,26-$(OH)_3D_3$.

*Abbreviations:* 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 25-OH-$D_3$-26,23-lactone, 25-hydroxyvitamin $D_3$-26,23-lactone; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin $D_3$; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin $D_3$; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin $D_3$; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; 23,25,26-$(OH)_2D_3$, 23,25,26-trihydroxyvitamin $D_3$

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## 2. Materials and methods

### 2.1. *Compounds*

The synthesis of 25-OH-$D_3$, 24,25-dihydroxyvitamin $D_3$ (24,25-$(OH)_2D_3$), 1α,25-dihydroxyvitamin $D_3$ (1α,25-$(OH)_2D_3$) and 25,26-$(OH)_2D_3$ were done in our laboratory as in [11,12]. 25-OH-$D_3$-26,23-Lactone, 23,25-$(OH)_2D_3$ and 23,25,26-$(OH)_3D_3$ were isolated from the serum of rats given large doses of vitamin $D_3$ as in [5,8,9]. Four possible diastereoisomers of 25-OH-$D_3$-26,23-lactone were synthesized as in [5]. Vitamin $D_3$ was obtained from Sigma Chemical Co. (Chicago IL).

### 2.2. *In vitro incubation of chick kidney homogenate with vitamin $D_3$ metabolites*

Six-week-old White Leghorn cockerels were fed first with the rachitogenic diet [13] and then with a diet containing 2.0% strontium and no calcium. They were given orally 2 μg 1α,25-$(OH)_2D_3$ daily for 10 days. The chicks were sacrificed, their kidneys were taken and 10% tissue homogenates in 0.25 M sucrose were prepared with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. To 24 ml homogenate (13 mg protein/ml) in a 300 ml flask was added 56 ml reaction mixture containing 30 mM Tris–HCl (pH 7.4), 3.6 mM $MgCl_2$, 50 mM sucrose and 20 mM sodium succinate as in [9,14]. The incubations were initiated by addition of 4 μg 25-OH-$D_3$, 23,25-$(OH)_2D_3$, 23,25,26-$(OH)_3D_3$ or 25(*S*)26-$(OH)_2D_3$ in 0.5 ml ethanol. The incubations were carried out at 37°C for 60 min with shaking. Chloroform:methanol

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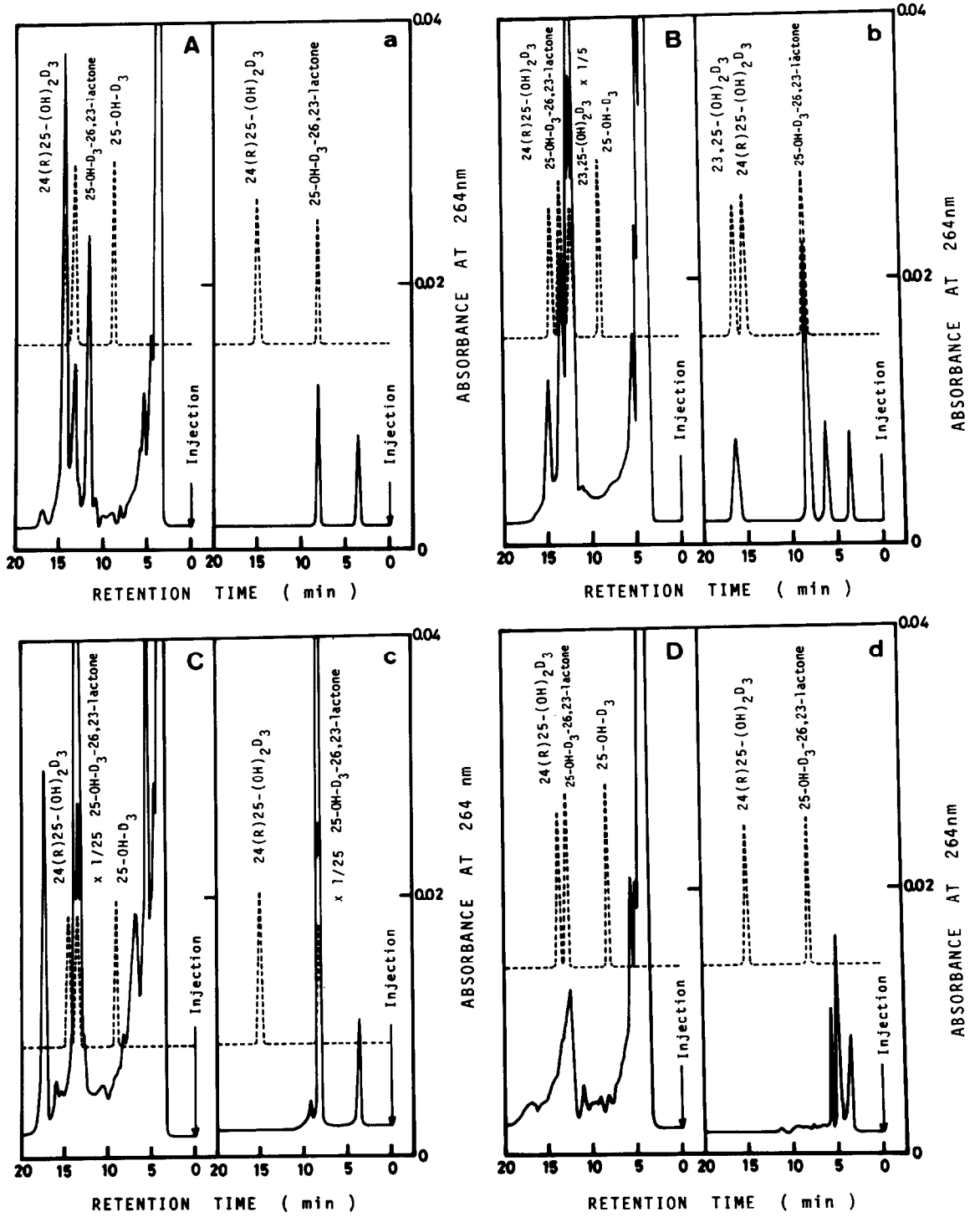


Fig.1. High-pressure liquid chromatographic profiles of the 24,25-$(OH)_2D_3$ fraction from Sephadex LH-20 column chromatography: (A) kidney homogenates incubated with 8 μg 25-OH-$D_3$; (B) kidney homogenates incubated with 8 μg 23,25-$(OH)_2D_3$; (C) kidney homogenates incubated with 8 μg 23,25,26-$(OH)_3D_3$; (D) kidney homogenates incubated with 8 μg 25(*S*)26-$(OH)_2D_3$. Each capital letter indicates that the 24,25-$(OH)_2D_3$ fraction from Sephadex LH-20 column was subjected to high-pressure liquid chromatography with a Zorbax Sil column eluted with 9% isopropanol in *n*-hexane. Each small letter indicates that the putative 25-OH-$D_3$-26,23-lactone in each capital letter was subjected to high-pressure liquid chromatography with a Zorbax Sil column eluted with 1.5% methanol in dichloromethane.

(1:1, 160 ml) was added to each flask to terminate the reaction. The 25-OH-$D_3$-26,23-lactone from incubation mixtures was separated and purified by Sephadex LH-20 column chromatography and high-pressure liquid chromatography using a Zorbax Sil column (4.6 × 250 mm).

2.3. *Preparation and purification of 25-OH-$D_3$-26,23-lactone by column chromatography*

The residue of chloroform extracts was dissolved in chloroform:*n*-hexane (65:35) and applied to a Sephadex LH-20 column (1.5 × 25 cm) packed and eluted with the same solvent. Sixty 4 ml fractions were collected. The 25-OH-$D_3$ (tubes 9–15), the 24,25-$(OH)_2D_3$ (tubes 19–28) and the 25,26-$(OH)_2D_3$ (tubes 34–42) from the column separately pooled and concentrated. The 24,25-$(OH)_2D_3$ fraction from the Sephadex LH-20 column was then subjected to high-pressure liquid chromatography on a Hitachi Model 635 apparatus equipped with a 4.6 × 250 mm Zorbax Sil column eluted with 9% isopropanol in *n*-hexane at 1 ml/min. Ultraviolet absorbance was monitored at 264 nm by a Hitachi Model 200-10 spectrophotometer. The putative 25-OH-$D_3$-26,23-lactone fraction was further purified by high-pressure liquid chromatography using a Zorbax Sil column (4.6 × 250 mm) eluted with 1.5% methanol in dichloromethane.

2.4. *Spectroscopy*

Ultraviolet absorption spectra were recorded with a Hitachi Model 200-10 spectrophotometer. Mass spectra were determined with a Simadzu-LKB mass spectrometer model 9000 in the direct probe inlet mode. The Fourier transform infrared (FT-IR) spectra were obtained by using a JEOL Model JIR-40X (Japan Electric Optical Laboratory Ltd.).

## 3. Results

The 25-OH-$D_3$-26,23-lactone was eluted from Sephadex LH-20 column in a fraction corresponding to 24,25-$(OH)_2D_3$ [1,2], was separated from various vitamin $D_3$ metabolites using high-pressure liquid chromatography with a Zorbax Sil column [1–3,10]. Kidney homogenates from 1α,25-$(OH)_2D_3$-supplemented chicks were incubated with 8 μg 25-OH-$D_3$, 23,25-$(OH)_2D_3$, 23,25,26-$(OH)_3D_3$ or 25(*S*)26-$(OH)_2D_3$. The chloroform extracts were dissolved in chloroform:*n*-hexane (65:35) and applied to a Sephadex LH-20 column and eluted with the same solvent. Fig.1 shows the high-pressure liquid chromatographic profiles of 24,25-$(OH)_2D_3$ fraction from Sephadex LH-20 column of extracts of kidney homogenates incubated with various vitamin $D_3$ metabolites. A small amount of 25-OH-$D_3$-26,23-lactone was produced from 25-OH-$D_3$ and 23,25-$(OH)_2D_3$ (fig.1A,B). In contrast, large amounts of 25-OH-$D_3$-26,23-lactone were synthesized from 23,25,26-$(OH)_3D_3$, while no detectable amount of 25-OH-$D_3$-26,23-lactone was found when 25(*S*)26-$(OH)_2D_3$ was added to the incubation mixture as the substrate (fig.1D). The 25-OH-$D_3$-26,23-lactone from 25-OH-$D_3$, 23,25-$(OH)_2D_3$ and 23,25,26-$(OH)_3D_3$ was identified by ultraviolet absorption, mass spectra and Fourier transform infrared spectra as in [5]. Table 1 shows that the production of 25-OH-$D_3$-26,23-lactone from chick kidney homogenates incubated with 8 μg various vitamin $D_3$ metabolites. This result indicated that the production of 25-OH-$D_3$-26,23-lactone from 23,25,26-$(OH)_3D_3$ was 35-times as much as that from 25-OH-$D_3$, whereas that from 23,25-$(OH)_2D_3$ was 2.2-times more than that from 25-OH-$D_3$. Fig.2A illustrates that the 4 synthetic diastereoisomers of 25-OH-$D_3$-26,23-lactone could be separated into separate peaks by high-pressure liquid chromatography. The generated 25-OH-$D_3$-26,23-lactone from 23,25,26-$(OH)_3D_3$ comigrated with the 23(*S*)25(*R*)-25-OH-$D_3$-26,23-lactone (fig.2B). Similarly, the 25-OH-$D_3$-26,23-lactone generated from 25-OH-$D_3$ and 23,25-$(OH)_2D_3$ comigrated with the 23(*S*)25(*R*)-25-OH-$D_3$-26,23-lactone.

Table 1
Production of 25-OH-$D_3$-26,23-lactone from various vitamin $D_3$ metabolites

| Substrate | 25-OH-$D_3$-26,23-lactone produced (ng . 4.8 g tissue$^{-1}$ . h$^{-1}$) |
|---|---|
| 25-OH-$D_3$ | 120 |
| 23,25-$(OH)_2D_3$ | 260 |
| 23,25,26-$(OH)_3D_3$ | 4200 |
| 25(*S*)26-$(OH)_2D_3$ | undetectable |

A 10% kidney homogenate from 1α,25-$(OH)_2D_3$-supplemented chicks was incubated with 8 μg 25-OH-$D_3$, 23,25-$(OH)_2D_3$, 23,25,26-$(OH)_3D_3$ or 25(*S*)26-$(OH)_2D_3$ as in section 2. 25-OH-$D_3$-26,23-lactone produced in vitro was isolated and purified by high-pressure liquid chromatography using 2 solvent systems. The amounts of 25-OH-$D_3$-26,23-lactone were quantitated by comparison with a standard curve made with authentic 25-OH-$D_3$-26,23-lactone

Thus, the stereochemistry of the biosynthesized 25-OH-$D_3$-26,23-lactone from various vitamin $D_3$ metabolites was definitely determined to be 23(*S*)25(*R*)-25-OH-$D_3$-26,23-lactone.

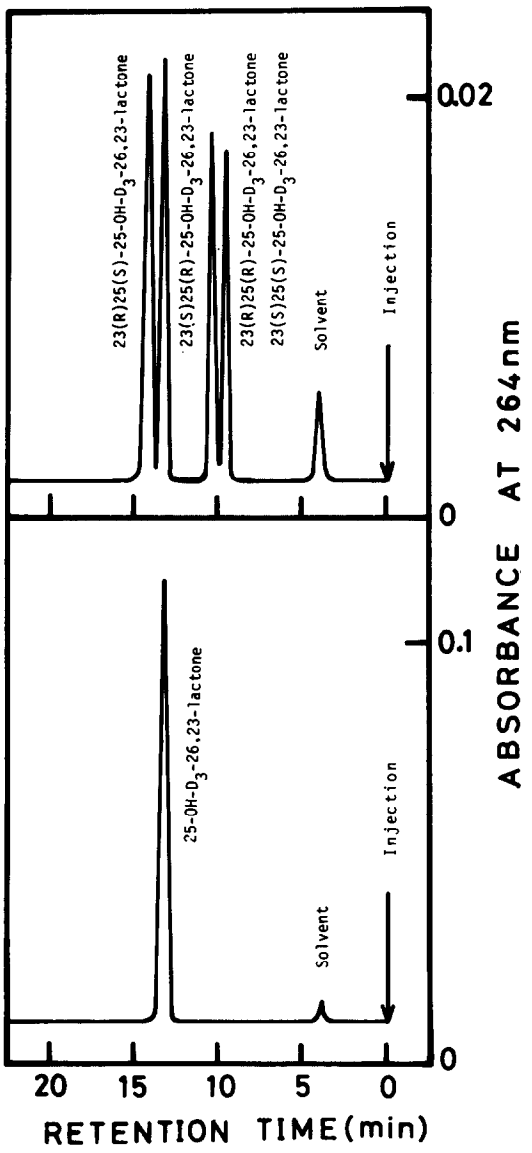


Fig.2. High-pressure liquid chromatographic profiles of diastereoisomers of synthetic 25-OH-$D_3$-26,23-lactone and biosynthetic 25-OH-$D_3$-26,23-lactone: (A) synthetic 25-OH-$D_3$-26,23-lactones; (B) biosynthetic 25-OH-$D_3$-26,23-lactone from 23,25,26-$(OH)_3D_3$. 25-OH-$D_3$-26,23-lactones were subjected to high-pressure liquid chromatography with a 4.6 × 250 mm Zorbax Sil column eluted with 9% isopropanol in *n*-hexane at 1 ml/min.

## 4. Discussion

This report demonstrates that the 25-OH-$D_3$-26,23-lactone was not produced from 25(*S*)26-$(OH)_2D_3$ but from 23,25-$(OH)_2D_3$ via 23,25,26-$(OH)_3D_3$ (fig.1). The stereochemical configuration of enzymically synthesized 25-OH-$D_3$-26,23-lactone from 23,25-$(OH)_2D_3$ and 23,25,26-$(OH)_3D_3$ was 23(*S*)25(*R*) at C-23 and C-25 positions (fig.2). Therefore, the stereochemistry of isolated 23,25-$(OH)_2D_3$ and 23,25,26-$(OH)_3D_3$ from the serum of rats was determined unequivocally to be 23(*S*)25-$(OH)_2D_3$ and 23(*S*)25(*R*)-26-$(OH)_3D_3$, respectively. Natural 25,26-$(OH)_2D_3$ was reported to possess the 23(*S*) absolute configuration [15–17]. With 25(*S*)26-$(OH)_2D_3$ as an intermediate in the biosynthesis of the lactone, the stereochemical inversion of hydroxy-group at C-25 position should occur during the course of lactone formation. The production of 25-OH-$D_3$-26,23-lactone from 23,25,26-$(OH)_3D_3$ was 16.2-times more than that from 23,25-$(OH)_2D_3$. In [18] 23(*S*)25-$(OH)_2D_3$ was reported as a far better substrate for production of 25-OH-$D_3$-26,23-lactone than is 25,26-$(OH)_2D_3$ [18]. Thus, it might be reasonable to consider that an oxidation reaction such as hydroxylation takes place initially on methylene group at C-23 position, followed by oxidation of methyl group at C-26 position. In short, the 25-OH-$D_3$-26,23-lactone was biosynthesized from 25-OH-$D_3$ by way of 23(*S*)25-$(OH)_2D_3$ to 23(*S*)-25(*R*)26-$(OH)_3D_3$.

The biological and physiological functions of the 25-OH-$D_3$-26,23-lactone are under investigation.

## References

[1] Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., Shepard, R. M. and Jorgensen, N. A. (1979) Biochemistry 18, 4775–4780.
[2] Horst, R. L. (1979) Biochem. Biophys. Res. Commun. 89, 286–293.
[3] Horst, R. L. and Littledike, T. (1980) Biochem. Biophys. Res. Commun. 93, 149–154.
[4] Yamada, S., Nakayama, K. and Takayama, H. (1981) Chem. Pharm. Bull. 29, 2393–2396.
[5] Ishizuka, S., Yamaguchi, H., Yamada, S., Nakayama, K. and Takayama, H. (1981) FEBS Lett. 134, 207–211.
[6] Hollis, B. W., Roos, B. A. and Lambert, P. W. (1980) Biochem. Biophys. Res. Commun. 95, 520–528.
[7] Pramanik, B. and Napoli, J. L. (1981) Fed. Proc. FASEB 40, 895 abstr.

[8] Tanaka, Y., Wichmann, J. K., Schnoes, H. K. and DeLuca, H. F. (1981) Biochemistry 20, 3875–3879.
[9] Ishizuka, S., Ishimoto, S. and Norman, A. W. (1982) submitted.
[10] Tanaka, Y., Wichmann, J. K., Paaren, H. E., Schnoes, H. K. and DeLuca, H. F. (1980) Proc. Natl. Acad. Sci. USA 77, 6411–6414.
[11] Ishizuka, S., Bannai, K., Naruchi, T. and Hashimoto, Y. (1981) Steroids 37, 33–43.
[12] Koizumi, N., Morisaki, M. and Ikekawa, N. (1978) Tetrahedron Lett. 2899–2902.
[13] Omdahl, J., Holick, M. F., Suda, T., Tanaka, Y. and DeLuca, H. F. (1971) Biochemistry 10, 2935–2940.
[14] Bannai, K., Ishizuka, S., Naruchi, T. and Hashimoto, Y. (1979) J. Steroid Biochem. 10, 411–418.
[15] Redel, J., Bazely, N., Tanaka, Y. and DeLuca, H. F. (1978) FEBS Lett. 94, 228–230; (1980) FEBS Lett. 113, 345.
[16] Cesario, M., Guilhem, J., Pascard, C. and Redel, J. (1978) Tetrahedron Lett. 1097–1098; (1980) Tetrahedron Lett. 1588.
[17] Partridge, J. J., Shiuey, S.-J., Chadha, N. K., Baggiolini, E. G., Blount, J. F. and Uskokovic, M. R. (1981) J. Am. Chem. Soc. 103, 1253–1255.
[18] Tanaka, Y., DeLuca, H. F., Schnoes, H. K., Ikekawa, N. and Eguchi, T. (1981) Proc. Natl. Acad. Sci. USA 78, 4805–4808.