
A BIOSYNTHETIC PRECURSOR OF (23S,25R)-25-HYDROXYVITAMIN D₃ 26,23-LACTONE*

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23S,25R,26-Trihydroxyvitamin D₃ and (23S,25R)-25-hydroxyvitamin D₃ 26,23-lactol were chemically synthesized, and the metabolism of the two compounds to (23S,25R)-25-hydroxyvitamin D₃ 26,23-lactone in chick kidney homogenates was studied. 23S,25R,26-trihydroxyvitamin D₃ was efficiently metabolized to the lactone in kidney homogenates from vitamin D-supplemented chicks, but not from vitamin D-deficient chicks. In contrast, the (23S,25R)-25-hydroxyvitamin D₃ 26,23-lactol was converted to the lactone in kidney homogenates regardless of the vitamin D status of the animals used.

A new metabolite was isolated in pure form from the incubation mixture of 23S,25R,26-trihydroxyvitamin D₃ with kidney homogenates prepared from vitamin D-supplemented chicks. The metabolite was identified as (23S,25R)-25-hydroxyvitamin D₃ 26,23-lactol by its ultraviolet and mass spectra and by derivatization. The structure was confirmed by direct comparison with an authentic sample on high pressure liquid chromatography. The evidence suggests that the stereochemistries of the isolated lactol at the 23- and 25-positions are S and R, respectively.

Since the outset of studies of vitamin D, hydroxylation at the 24- and 26-positions on the side chain has been known as the major metabolic pathways of 25(OH)D₃ in the kidney of vitamin D-supplemented animals (1). Recently, the existence of another important metabolic pathway in the kidney of vitamin D-supplemented animals, hydroxylation and further oxidation at the 23-position, has been disclosed, and nearly 10 metabolites prepared from vitamin D-deficient chicks, but not from vitamin D-deficient chicks. In contrast, the (23S,25R)-25-hydroxyvitamin D₃ 26,23-lactol was converted to the lactone in kidney homogenates regardless of the vitamin D status of the animals used.

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The abbreviations used are: 25(OH)D₃, 25-hydroxyvitamin D₃; 25(OH)₂D₃, 25,26-dihydroxyvitamin D₃; 25(OH)₃D₃, 25,26,23-trihydroxyvitamin D₃; 25(OH)₂D₃-26,23-lactone; 25(OH)₃D₃-26,23-lactone; 25(OH)₂D₃-26,23-peroxylactone; 25(OH)₃D₃-26,23-peroxylactone; MeSi, trimethylsilyl.
tamin D-supplemented chicks. While this study was progressing, isolation and identification of 23,25,26(OH)D₃ were reported (8, 26) which proved our prediction.

RESULTS AND DISCUSSION

According to our hypothesis, we have chemically synthesized the two postulated vitamin D metabolites, 23S,25(R)-26(OH)D₃ and (23S,25R)-25(OH)D₃-26,23-lactol, and lactol fraction from 25(OH)D₃.

The isolation and characterization of the two metabolites are shown in Fig. 2. The lactone fraction from the first HPLC column was purified on an HPLC column like the first one with 1% 2-propanol in n-hexane as the eluent. The 265 nm absorbing peak was collected and used for mass spectrometry. The UV spectrum of the purified lactone was taken in a flow cell of the UV detector attached to the HPLC instrument by stopping the flow during the second purification.

The lactol fractions from three runs on the first HPLC column were collected and subjected to purification on an HPLC column like the first one with 12% 2-propanol in n-hexane as the eluent. A portion of the metabolite isolated from the second HPLC column was subjected to trimethylsilylation, and the remainder was further purified on a reversed phase HPLC column (Finepak-C₁₈, JASCO, Hachioji, Tokyo; 0.46 × 25 cm) with 20% 2-propanol in n-hexane and the major 265 nm absorbing peak eluted between 8.0 and 9.5 ml was pooled and used for mass spectrometry. The UV spectrum of the metabolite was taken in a flow cell of the UV detector during the second HPLC purification.

The trihydroxyvitamin D fraction from the Sephadex LH-20 column was purified on an HPLC column (Finepak-SIL, JASCO; 0.46 × 25 cm). The HPLC column was eluted with 20% 2-propanol in n-hexane and the major 265 nm absorbing peak eluted between 8.0 and 9.5 ml was pooled and used for mass spectrometry. The UV spectrum of the metabolite was taken in a flow cell of the UV detector during the second HPLC purification.

The isolation of 25(OH)D₃-26,23-lactone—The compound isolated from the second HPLC column (about 20 ng) was dissolved in dichloromethane (10 μl). A large excess of trimethylsilylimidazole (10 μl) was added to the solution, and the mixture was stored at room temperature for 1 h. After evaporation of the solvent and the excess of the reagent, the hexane-soluble portion of the residue was purified on an HPLC column (Finepak-SIL, 0.46 × 25 cm). The major peak eluted between 6.3 and 6.8 ml was collected and used for mass spectrometry to identify the compound. The amounts of 23S,25R,26(OH)D₃ were quantified on the basis of the peak area on the first HPLC column.

Isolation of 25(OH)D₃-26,23-lactone and 25(OH)D₃-26,23-lactol—The incubation mixture was extracted as described by Gray et al. (28). The concentrated extracts were chromatographed on a column (1 × 30 cm) packed with 10 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was eluted with 65% chloroform in n-hexane. The fraction containing the lactone and lactol (30–200 ml) was collected separately from the one containing 23S,25R,26(OH)D₃ (200–450 ml). The lactone and lactol fraction from the Sephadex LH-20 column was evaporated in vacuo, and the residue was dissolved in 7% 2-propanol in n-hexane and subjected to HPLC (Zorbax-SIL, DuPont, Wilmington, DE; 0.46 × 25 cm). The HPLC column was eluted with the same solvent at a flow rate of 1 ml/min and monitored at 265 nm. The major 265 nm absorbing peaks which were collected at exactly the same retention volumes as those of the authentic (23S,25R)-25(OH)D₃-26,23-lactone (15.5–16.5 ml) and (23S,25R)-25(OH)D₃-26,23-lactol (19–21 ml) were collected separately. The amounts of the lactone and lactol shown in Table I were quantified at this stage by comparing the peak areas with the corresponding peaks in a standard curve made with the respective authentic samples.

Isolation of 25(OH)D₃-26,23-lactone from 25(OH)D₃—The lactone fraction from the first HPLC column was purified on an HPLC column like the first one with 1% 2-propanol in n-hexane as the eluent. The 265 nm absorbing peak was collected and used for mass spectrometry. The UV spectrum of the purified lactone was taken in a flow cell of the UV detector attached to the HPLC instrument by stopping the flow during the second purification.

The lactol fractions from three runs on the first HPLC column were collected and subjected to purification on an HPLC column like the first one with 12% 2-propanol in n-hexane as the eluent. A portion of the metabolite isolated from the second HPLC column was subjected to trimethylsilylation, and the remainder was further purified on a reversed phase HPLC column (Finepak-C₁₈, JASCO, Hachioji, Tokyo; 0.46 × 25 cm) with 20% 2-propanol in n-hexane and the major 265 nm absorbing peak eluted between 8.0 and 9.5 ml was pooled and used for mass spectrometry. The UV spectrum of the metabolite was taken in a flow cell of the UV detector during the second HPLC purification.

The trihydroxyvitamin D fraction from the Sephadex LH-20 column was purified on an HPLC column (Finepak-SIL, JASCO; 0.46 × 25 cm). The HPLC column was eluted with 20% 2-propanol in n-hexane and the major 265 nm absorbing peak eluted between 8.0 and 9.5 ml was pooled and used for mass spectrometry. The UV spectrum of the metabolite was taken in a flow cell of the UV detector during the second HPLC purification.

The isolation of 25(OH)D₃-26,23-lactone—The compound isolated from the second HPLC column (about 20 ng) was dissolved in dichloromethane (10 μl). A large excess of trimethylsilylimidazole (10 μl) was added to the solution, and the mixture was stored at room temperature for 1 h. After evaporation of the solvent and the excess of the reagent, the hexane-soluble portion of the residue was purified on an HPLC column (Finepak-SIL, 0.46 × 25 cm). The major peak eluted between 6.3 and 6.8 ml was collected and used for mass spectrometry to identify the compound. The amounts of 23S,25R,26(OH)D₃ were quantified on the basis of the peak area on the first HPLC column.

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The lactone fraction from the first HPLC column was purified on an HPLC column like the first one with 1% 2-propanol in n-hexane as the eluent. The 265 nm absorbing peak was collected and used for mass spectrometry. The UV spectrum of the purified lactone was taken in a flow cell of the UV detector attached to the HPLC instrument by stopping the flow during the second purification.

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(23S,25R)-25-Hydroxyvitamin D$_3$ 26,23-Lactol


volume and the UV and mass spectra. The metabolite showed exactly the same retention volume on the HPLC column as the authentic (23S,25R)-25(OH)D$_3$ 26,23-lactone. The UV spectra showed a $\lambda_{max}$ at 265 nm and a $\lambda_{max}$ at 228 nm. The mass spectra showed a molecular ion at $m/e$ 428 and diagnostic fragment ions at $m/e$ 410, 395, and 369.

Table I summarizes the results of the in vitro incubation. 23S,25R,26(OH)$_3$D$_3$ was converted to 25(OH)D$_3$ 26,23-lactone with high efficiency when the substrate was incubated with the kidney homogenates from vitamin D-supplemented chicks (Group I). Lactone production was completely inhibited by heating kidney homogenates prior to the incubation (Group II), indicating that the lactone is formed enzymatically. In contrast, 23S,25R,26(OH)$_3$D$_3$ was not converted at all to the lactone by the incubation with kidney homogenates from vitamin D-deficient chicks (Group III). These results indicate that 23S,25R,26(OH)$_3$D$_3$ with 23S,25R configuration is the true biosynthetic precursor of 25(OH)D$_3$ 26,23-lactone. When we completed this work, isolation and identification of 23S,25R,26(OH)$_3$D$_3$ from the incubation of 23S,25R,26(OH)$_3$D$_3$ with kidney homogenates of vitamin D-supplemented chicks and MeOH-CHC$_3$ extracts of the incubation were

No 25(OH)D$_3$ 26,23-lactol was detected in the kidney homogenates from either vitamin D-supplemented or -deficient chicks when kidney homogenates were incubated for 90 min with 23S,25R,26(OH)$_3$D$_3$. When 25(OH)D$_3$ 26,23-lactol was used as the substrate, however, it was converted to the lactone in good yields regardless of the vitamin D status of the animals used. The conversion of the lactol to the lactone was proved to be an enzymatic process, because the conversion was completely abolished by heating the homogenates before the incubation. In these experiments, yields of the lactone from 23S,25R,26(OH)$_3$D$_3$ were higher than those from the lactol. Recovery of the substrate after a 90-min incubation in Group I was about 30% with 23S,25R,26(OH)$_3$D$_3$ and 0% with the lactol. These results, therefore, suggest that in the described experiments the incubation period was too long so that all of the lactol formed was metabolized further to the lactone and some of the lactone formed was also metabolized further. So we next examined short term metabolism.

When 23S,25R,26(OH)$_3$D$_3$ was incubated for 15 min with kidney homogenates (20%) from vitamin D-supplemented chicks and MeOH-CHC$_3$ extracts of the incubation were
purified, a new metabolite, which migrated to the same retention volume as that of (23S,25R)-25(OH)D₃-26,23-lactol, was detected by HPLC (Fig. 3). The metabolite was purified by chromatography on Sephadex LH-20 followed by HPLC including straight and reversed phase columns to give a homogeneous metabolite. The metabolite migrated to the same retention volume as that of the authentic (23S,25R)-25(OH)D₃-26,23-lactol on three different HPLC columns as shown in Fig. 3. The purified metabolite showed a λₘₐₓ at 265 nm and a λₘᵢₙ at 228 nm in its UV spectrum. The mass spectrum of the metabolite was closely related to that of the synthetic 25(OH)D₃-26,23-lactol (Fig. 4), showing a molecular ion at m/e 430 and characteristic fragment ions at m/e 412 (M⁺ - H₂O), 342, 309 (342 - H₂O - CH₃), 136, and 118. The tris-Me₃Si ether of the metabolite was purified to the retention volume exactly the same as that of the tris-Me₃Si ether of the authentic (23S,25R)-25(OH)D₃-26,23-lactol. The mass spectrum (Fig. 5) of the Me₃Si ether of the metabolite was nearly identical with that of the authentic metabolite, showing a molecular ion at m/e 646 and fragment ions at m/e 556 (M⁺ - Me₃SiOH, 541 (556 - CH₃), 528 (556 - Me₃SiOH), 399 (414 - CH₃), 371 (399 - CO), 208, and 118 (208 - Me₃SiOH). The stereochemistry of the isolated lactol at the side chain is probably 23S,25R, since it was produced from 23S,25R,26(OH)D₃. Comparison of the HPLC retention volume of the lactol and its Me₃Si ether with those of the respective authentic samples provided supporting evidence.

Table I includes the results of the short term (15 min) incubation. As expected, (23S,25R)-25(OH)D₃-26,23-lactol was metabolized more rapidly than 23S,25R,26(OH)D₃. Therefore, to detect the lactol as the intermediate of the metabolism of 23S,25R,26(OH)D₃ to 25(OH)D₃-26,23-lactone, it is necessary to examine the incubation mixture at an early stage. 23S,25R,26(OH)D₃ produced 25(OH)D₃-26,23-lactol in about 15% yield together with the lactone (about 30% yield) after 15 min of incubation. The lactone formation was more efficient from the lactol than from 23S,25R,26(OH)D₃ in the short term incubation. All of the possible biosynthetic intermediates of 25(OH)D₃-26,23-lactone from 25(OH)D₃ were now isolated and identified, and the whole scheme of the biosynthesis of the lactone was established. As a result, biological oxidation of 25(OH)D₃ to 25(OH)D₃-26,23-lactone was proved to proceed in a stepwise manner as expected. Of all steps from 25(OH)D₃ to the lactone, the last step, conversion of the lactol to the lactone, was found to be out of the control of the vitamin D status of animals.

After we completed this manuscript, isolation and identification of 25-hydroxyvitamin D₃ 26,23-peroxylactone were reported by Ishizuka et al. (29). These authors reported that 25(OH)D₃-26,23-lactone is not the true metabolite of vitamin D₃ but is derived nonenzymatically from the labile peroxylactone. They also suggested a biosynthetic pathway of 25(OH)D₃-26,23-peroxylactone from 25(OH)D₃ where 25-hydroxy-23-hydroxyvitamin D₃ and 25,26-dihydroxy-23-hydroperoxylactone D₃ are postulated as intermediates. We could not detect a metabolite ascribed to 25(OH)D₃-26,23-peroxylactone in any significant amount during the purification of the metabolites of 23S,25R,26(OH)D₃ and 25(OH)D₃-26,23-lactol described above. Since it was conceivable that most of the peroxylactone formed had been converted to 25(OH)D₃-26,23-lactone during purification procedures prior to HPLC analysis (1 week), we re-examined carefully the 15-min incubation mixture of the lactol (Group I in
Fig. 4. Mass spectra of isolated (A) and synthetic (B) (23S, 25R)-25(OH)D₂-26,23-lactol.

Fig. 5. Mass spectra of tris(tri-methylsilyl) (TMS) ether of isolated (A) and synthetic (B) (23S, 25R)-25(OH)D₂-26,23-lactol.

Fig. 2) within the shortest possible period of time. However, even in this experiment, no significant amount of the peroxylactone was detected on HPLC, while the lactone was obtained in good yield (about 40%). These results indicate that the peroxylactone does not intervene in the lactone formation from the lactol.

Two possible metabolic routes to 25(OH)D₂-26,23-peroxylactone from 25(OH)D₂ can be considered, one via the known biosynthetic intermediates of 25(OH)D₂-26,23-lactone, 23S,25(OH)₂D₃, 23S,25R,26(OH)₃D₃, and (23S,25R)-25(OH)D₂-26,23-lactol, and the other via the corresponding 23-hydroperoxy derivatives suggested by Ishizuka et al. (29). The latter route via the 23-hydroperoxyvitamin D derivatives seems unlikely because (i) enzymatic hydroperoxidation at a nonactivated CH₂ group has not been known in well studied metabolism of steroid hormones, and (ii) none of the biosynthetic precursors of the peroxylactone with 23-hydroperoxy group, such as 25-hydroxy-23-hydroperoxyvitamin D₃, 25,26-dihydroxy-23-hydroperoxyvitamin D₃, etc., has been isolated, while all of their corresponding 23-hydroxy derivatives have been isolated. On the basis of oxidation stage, the peroxylactone is a further oxidation product of the lactone. Therefore, it appears more likely that the peroxyxylactone may be produced from the lactone.

To clarify the biosynthetic route to 25(OH)D₂-26,23-peroxylactone, detailed studies are progressing in our laboratory.

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