
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 the studies of vitamin D₃ 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 D₃-supplemented animals (1). Recently, the existence of another important metabolic pathway in the kidney of vitamin D₃-supplemented animals, hydroxylation and further oxidation at the 23-position, has been disclosed, and nearly 10 metabolites via that pathway have already been isolated and identified (2–12). Among these metabolites, 25(OH)D₃ 26,23-lactone has received special attention because of its high concentration in plasma of animals supplemented with large amounts of vitamin D₃ (13) and its characteristic physiological properties. The lactone was first isolated and identified from the blood plasma of chicks given maintenance level or large doses of vitamin D₃ (2) and later from the plasma of pigs and cows supplemented with toxic doses of vitamin D₃ (3). The structure of 25(OH)D₃ 26,23-lactone was confirmed by chemical synthesis (14–18), and the stereochemical configuration at the 23- and 25-positions has been determined to be S and R, respectively, by direct comparison of the natural metabolite with all of the possible synthetic stereoisomers with known stereochemistry (16, 18). The kidney was found to be the specific site for the production of the lactone from 25(OH)D₃ (13). 25(OH)D₃ 26,23-lactone has been reported to be five times more potent than 25(OH)D₃ for binding to vitamin D-binding protein in rat plasma (2, 3). A more striking feature of the lactone is that it suppresses the serum calcium level in competition with the action of 1α,25(oh)₂d₃ (19). 25S,26(OH)₂D₃ was first assumed to be the precursor of 25(OH)D₃ 26,23-lactone, and evidence to support this was reported (20). However, the assumption was shown to be incorrect by the unambiguous determination of the R stereochemical configuration of the lactone at carbon 23 (16, 18), and later 23S,25(OH)D₃ was proved to be the true natural precursor of the lactone (21, 22). Rigorous experiments done by Napoli and Horst (22) and Napoli et al. (23) have clearly ruled out the possibility that 25S,26(OH)₂D₃ is a biosynthetic precursor of the lactone.

We proposed a hypothetical process for the biosynthesis of 25(OH)D₃ 26,23-lactone (Fig. 1). If the metabolic oxidation of 23S,25(OH)₂D₃ to the lactone proceeds in a stepwise manner like that in the biosynthesis of cholic acids from cholesterol (24), 23S,25(OH)₂D₃ (I) would be first hydroxylated at the 26-position to yield 23S,25R,26(OH)₃D₃ (II), whose stereochemical configuration is the same as that of the natural lactone (IV). 23S,25R,26(OH)₃D₃ (II) would be converted to 25(OH)D₃ 26,23-lactol (III), the cyclized form of the aldehyde (IV'), by further oxidation at the 26-position, and the lactol (III) would be finally oxidized to 23S,25R-25(OH)D₃ 26,23-lactone (IV), the cyclized form of the carboxylic acid (IV'). To test our hypothesis, we have synthesized the two presumed metabolites, 23S,25R,26(OH)₂D₃ (II) and (23S,25R)-25(OH)D₃ 26,23-lactol (III), stereospecifically (25) and examined their in vitro metabolism using chick kidney homogenates. Both compounds were efficiently converted to 25(OH)D₃ 26,23-lactone. Furthermore, the lactol (III) was actually isolated and identified as the true biosynthetic precursor of the lactone which emanates from the incubation of 23S,25R,26(OH)₂D₃ with chick kidney homogenates from vi-
Vitamin D-supplemented chicks. While this study was progressing, isolation and identification of 23,25,26(OH)3 were reported (8, 26) which proved our prediction.

MATERIALS AND METHODS

General Procedures—Mass spectra were obtained using a JEOL D-300 mass spectrometer with an interfaced computer. HPLC was performed on a JASCO TWINCLE instrument equipped with a JASCO UVIDEC-610 spectrophotometer. UV spectra were recorded with a JASCO UVIDEC-610 spectrophotometer.


In Vitro Incubation of Chick Kidney Homogenates—One-day-old White Leghorn cockerel chicks were maintained for 4 weeks on a vitamin D-deficient diet containing 1% calcium and 0.45% phosphorus (27). They were then given orally 5 mg of 1α,25(OH)2D3/day or cotton seed oil as vehicle for 7 days. The chicks were killed by decapitation, and the kidneys were quickly removed, rinsed, minced with a garlic press, and homogenized in 4 volumes of incubation medium containing 0.2 M sucrose, 15 mM Tris-HCl (pH 7.4), 2 mM MgCl2, and 5 mM sodium succinate. The homogenates were incubated with either 23S,25R,26(OH)3D3 or (23S,25R)-25(OH)D3-26,23-lactol (0.83 μg/ml of the homogenates) at 37°C for either 15 or 90 min under 100% oxygen. The reaction was terminated by adding methanol/chloroform (2:1, v/v).

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 12% 2-propanol in n-hexane as the eluent. The 25(OH)D3-26,23-lactone peak eluted at the same retention volume as that of the authentic 23S,25R-25(OH)D3-26,23-lactol and the metabolite showed two 265 nm absorbing peaks between 6.5 and 9.0 ml, probably due to the two anomers at the 26-position (Fig. 3). Both peaks were pooled and subjected to further purification on a straight phase 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.

Trimethylsilylation of Isolated 25(OH)D3-26,23-lactol—The metabolite isolated from the second HPLC column (about 200 μg) 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 HPLC column was eluted with 3% ethyl acetate in n-hexane, and the major 265 nm absorbing peak eluted between 7.0 and 8.0 ml was pooled and used for mass spectrometry.

RESULTS AND DISCUSSION

According to our hypothesis, we have chemically synthesized the two postulated vitamin D metabolites, 23S,25R-, 26(OH)3D3 and (23S,25R)-25(OH)D3-26,23-lactol, in a stereospecific manner (25). Using these two chemically synthesized hypothetical metabolites with definite chemical and stereochemical structure, we studied biosynthesis of (23S,25R)-25(OH)D3-26,23-lactone. The metabolism was examined in three groups. In the first group (Group I) the substrates were incubated for 90 min with 20% kidney homogenates prepared from vitamin D-supplemented chicks. In the second group (Group II), kidney homogenates from vitamin D-supplemented chicks were heated at 100°C for 5 min and then incubated with the substrates. In the third group (Group III), the substrates were incubated with kidney homogenates (20%) from vitamin D-deficient chicks. In each of the three groups, the metabolism was examined in three separate runs. An outline of the procedure for the in vitro metabolism and purification of the metabolites is shown in Fig. 2.

25(OH)D3-26,23-lactone was the only major product observed on the HPLC column of the lactone and lactol fraction from the Sephadex LH-20 column of the 90-min incubation products. The structure of the isolated metabolite from each group was unequivocally determined to be (23S,25R)-25(OH)D3-26,23-lactone on the basis of the HPLC retention...
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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)D₃-26,23-lactone. The UV spectra showed a λ_max at 265 nm and a λ_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)₃D₃ was converted to 25(OH)D₃-26,23-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)₃D₃ 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,25,26(OH)₃D₃ with 23S,25R configuration is the true biosynthetic precursor of 25(OH)D₃-26,23-lactone. When we completed this work, isolation and identification of 23S,25,26(OH)₃D₃ from the incubation of 23S,25(OH)D₃ with kidney homogenates from vitamin D-deficient chicks were reported (8, 26). The 23S,25R configuration was suggested for the isolated 23S,25,26(OH)₃D₃ on the basis of the fact that it was metabolized to (23S,25R)-25(OH)D₃-26,23-lactone (8).

No 25(OH)D₃-26,23-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)₃D₃. When 25(OH)D₃-26,23-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)₃D₃ 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)₃D₃ 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)₃D₃ was incubated for 15 min with kidney homogenates (20%) from vitamin D-supplemented chicks and MeOH-CHCl₃ extracts of the incubation were
A 20% kidney homogenate was incubated with the substrates (0.83 
µg/ml of the homogenates) for either 90 or 15 min at 37°C. The 
metabolites produced were purified on a Sephadex LH-20 column 
followed by HPLC. Amounts of the metabolites were determined 
with the authentic compounds. Values show yield per cent of the 
substrate added. ND, undetectable.

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<th>Substrate</th>
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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 λ<sub>max</sub> at 265 
nm and a λ<sub>min</sub> 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, showing a molecular 
ion at m/e 646 and fragment ions at m/e 556 (M⁺ – Me₃SiOH,  
541 (556 – CH₃), 528, 438 (528 – 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 
biosynthesis 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 step-
wise 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-hydroperoxyvitamin D₃ and 25,26-dihydroxy-23-hydroperoxylactone D₃ are postulated as intermediates.

We could not detect a metabolite assigned 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 
procedure 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)D₃-26,23-lactol.

Fig. 5. Mass spectra of tris(tri-methylsilyl) (TMS) ether of isolated (A) and synthetic (B) (23S,25R)-25(OH)D₃-26,23-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)D₃-26,23-peroxylactone from 25(OH)D₃ can be considered, one via the known biosynthetic intermediates of 25(OH)D₃-26,23-lactone, 23S,25(OH)₂D₃, 23S,25R,26( OH)₃D₃, and (23S,25R)-25(OH)D₃-26,23-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 peroxylactone may be produced from the lactone.

To clarify the biosynthetic route to 25(OH)D₃-26,23-peroxylactone, detailed studies are progressing in our laboratory.

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


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