
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 stereochimical 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 stereochimy (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 stereochimical configuration of the lactone at carbon 25 (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 metabolite 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 stereochimical 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 aldehdyde (II′), 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)₃D₃ (II) and (23S,25R)-25(OH)₂D₃-26,23-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-
**RESULTS AND DISCUSSION**

According to our hypothesis, we have chemically synthesized the two postulated vitamin D metabolites, 23S,25R-25(OH)D$_3$, and 23S,25R-25(OH)D$_3$-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)D$_3$, and 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)D$_3$-26,23-lactone was the only major product observed from the Sephadex LH-20 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)D$_3$-26,23-lactone on the basis of the HPLC retention...
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 λₑₓ at 265 nm and a λₑₓ 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-supplemented 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 by comparison with the standard curves made with the authentic compounds. Values show yield per cent of the substrate added. ND, undetectable.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Group (D status)</th>
<th>Incubation time</th>
<th>Metabolites isolated</th>
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The mass spectrum of the metabolite was closely related to that of the synthetic (23S,25R)-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-Me2Si ether of the metabolite migrated to the retention volume exactly the same as that of the tris-Me2Si ether of the authentic (23S,25R)-25(OH)D3-26,23-lactol. The mass spectrum (Fig. 5) of the Me2Si ether of the metabolite was nearly identical with that of the authentic lactol, showing a molecular ion at m/e 646 and fragment ions at m/e 556 (M" - Me2SiOH), 541 (556 - CH3), 528, 438 (528 - MeSiOH), 399 (414 - CH3), 371 (399 - CD3), 208, and 118 (208 - MeSiOH). 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 time of the lactol and its Me2Si 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-peroxy lactone 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 peroxy lactone. They also suggested a biosynthetic pathway of 25(OH)D3-26,23-peroxy lactone from 25(OH)D3 where 25-hydroxy-23-hydroperoxyvitamin D3 and 25,26-dihydroxy-23-hydroperoxyvitamin D3 are postulated as intermediates.

We could not detect a metabolite ascribed to 25(OH)D3-26,23-peroxy lactone 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(trimethylsilyl) (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 hydroperoxydation 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.

REFERENCES
(23S,25R)-25-Hydroxyvitamin D₃ 26,23-Lactol

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