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THE SYNTHESIS AND BONE RESORBING ACTIVITY OF (25R)- AND (25S)-26-HYDROXYCHOLECALCIFEROL (26-OH-D_3)

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SUMMARY

The synthesis of C-25 epimers of 26-hydroxycholecalciferol (26-OH-D_3) (III)—the so far unknown 25-deoxy analogue of the metabolite 25,26$(OH)_2D_3$—is described.

(25R)- or (25S)-26-hydroxycholesterol 3,26-diacetate (I) was converted into the corresponding mixture of 5,7- and 4,6-diene, saponified and then directly irradiated with U.V. light. The obtained precholecalciferol analog was isomerized thermally to III and separated from the 4,6-diene on Sephadex LH 20.

Tested for bone resorption in organ cultures from mouse calvaria *in vitro*, (25R)-26-OH-D_3 is less effective than 25,26$(OH)_2D_3$, whereas (25S)-26-OH-D_3 is inactive. By the *in vivo/in vitro* method, (25R)- and (25S)-26-OH-D_3 are ineffective, while 25R,26- and 25S,26$(OH)_2D_3$ are moderately osteolytic.

INTRODUCTION

Among the dihydroxy-metabolites of vitamin D_3, the most ignored is undoubtedly 25,26-dihydroxycholecalciferol (25,26$(OH)_2D_3$). Although 25,26$(OH)_2D_3$ is a major dihydroxy-metabolite found in porcine [1] and human [2] plasma, its physiological function remains unknown as does its site of biosynthesis, and no data are available to indicate its configuration at carbon 25.

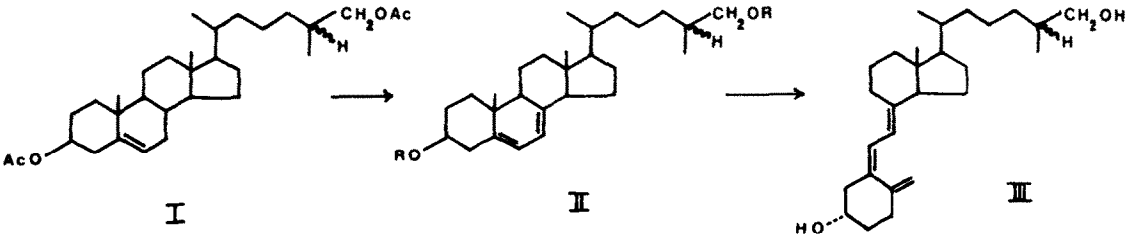
In our attempts to elucidate the stereochemistry of this metabolite of vitamin D_3, we have synthesized [3] the two epimers of 25,26$(OH)_2D_3$ but were unable at that moment to specify their chirality at C-25. One of these epimers must be identical with the natural 25,26$(OH)_2D_3$ of unknown configuration isolated from porcine plasma [1]. Recently, one of us has reported [4] the results of the analysis by X-ray diffraction, permitting us to assign absolute configuration to both epimers. In a biological study it was found [3] that 25S,26- and 25R,26$(OH)_2D_3$ have a selective activity on bone and, in particular, the former was superior to the latter in elevating serum phosphorus and in antirachitic activity.

These results assessing the importance of the configuration at C-25 prompted us to synthesize the unknown 26-hydroxy-derivatives of vitamin D_3, (25R)- and (25S)-26-OH-D_3, analogues of 25,26$(OH)_2D_3$ lacking the 25-hydroxyl, which may be helpful in defining more clearly the function of the 26-hydroxyl group.

The present report describes the chemical synthesis of two epimers of 26-OH-D_3 by a route outlined in Fig. 1, similar to that indicated [3] for 25R,26-$(OH)_2D_3$ and 25S,26$(OH)_2D_3$. Their activity on bone resorption is reported in a bone culture system [5], more likely to evaluate the effects at the level of the tissue receptors.

EXPERIMENTAL

Melting points were measured in open capillary tubes and are uncorrected. Ultraviolet spectra were determined with a Beckman Acta CIII spectrometer. Mass spectra were obtained with an AEI MS50 spectrometer using a direct probe. High pressure liquid chromatography was performed with a Waters Assoc. chromatograph equipped with a 6000A pump, a U6K injector and two columns (30 cm × 4 mm i.d.) packed with microporasil. The mobile phase was dichloromethane containing 1% (v/v) of methanol at a flow rate of 1 ml/min, and a pressure of 900 psi.



a : 25 R ; b : 25 S

Fig. 1. The synthesis of (25R)- and (25S)-26-hydroxycholecalciferol.

Optical rotations were measured for chloroform solutions.

Thin layer chromatography was performed on commercial 200 × 200 × 0.25 mm Merck plates of silica gel GF 254 using ethyl acetate-*n*-heptane (1:1, v/v) as solvent system. Spots were visualized by fluorescence and by spraying with 5% w/v phosphomobyldic acid in ethanol and heating.

(25R)-5-Cholestene-3β, 26-diol 3,26-diacetate (Ia) and (25S)-5-Cholestene-3β, 26-diol 3,26-diacetate (Ib). The two epimers were obtained by the resolution of a synthetic mixture of (25R)- and (25S)-5-cholestene-3β, 26-diol 3,26-diacetate by high pressure liquid chromatography, as recently described [6].

(25R)-5-cholestene-3β, 26-diol 3,26-diacetate: m.p. 128–129°C $[\alpha]_D^{20} = -37.0°C$ ($c = 0.9$).

(25S)-5-cholestene-3β, 26-diol 3,26-diacetate: m.p. 99–100°C $[\alpha]_D^{20} = -34.3°C$ ($c = 1.1$).

(25R)-5,7-Cholestadiene-3β, 26-diol 3,26-diacetate (IIa, R = Ac) (in admixture with the 4,6-diene). Ia (0.061 g) and 1,3-dibromo-5,5-dimethylhydantoin (0.024 g—25% excess) were suspended in light petroleum (b.p. 60–80°C, 3.6 ml) and heated under reflux, with stirring, for 30 min. After cooling to room temperature, the reaction mixture was filtered and the residue washed with light petroleum. Collidine (2 ml) was added to the filtrate, the low boiling solvent was evaporated *in vacuo* and the remaining solution heated at 150°C for 20 min, under nitrogen. After cooling ether was added and the precipitate filtered off and washed with ether. Work up of the ether extract in the usual manner gave a yellow residue (0.057 g) (mixture of 5,7 and 4,6-dienes). R_F 0.85.

(25S)-5,7-Cholestadiene-3β, 26-diol 3,26-diacetate (IIb, R = Ac) (in admixture with the 4,6-diene). Ib (0.071 mg) was treated as Ia and gave crude IIb (R = Ac) (0.069 g). R_F 0.85.

(25R)-5,7-Cholestadiene-3β, 26-diol (IIa, R = H) (in admixture with the 4,6-diene). IIa (R = Ac) (0.057 g) was dissolved in 5% methanolic potassium hydroxide (5 ml) and left at room temperature for 20 h. Work up in the usual manner gave 0.045 g of IIa (R = H) (mixture of 5,7 and 4,6-dienes). R_F 0.45.

(25S)-5,7-Cholestadiene-3β, 26-diol (IIb, R = H) (in admixture with the 4,6-diene). IIb (R = Ac) (0.069 g) was treated as IIa (R = Ac) and gave crude IIb (R = H) (0.057 g). R_F 0.45.

(25R)-26-Hydroxycholecalciferol (IIIa). IIa (R = H) (0.045 g) in diethyl ether–methanol (9:1, v/v, 100 ml) was irradiated with a medium-pressure mercury arc lamp for 25 min at 0°C. The solution was evaporated to dryness under reduced pressure at 5°C and the residue in a small volume of chloroform was applied to a silica gel plate (commercial Merck silica gel GF_{254} 200 × 200 × 0.5 mm) and developed by continuous elution for 22 h with ethyl acetate–*n*-heptane (1:5, v/v). Then the band containing the previtamin analogue and the isomeric 4,6-diene was scraped off and eluted with diethyl ether–ethanol (3:1, v/v, 100 ml). The eluate was evaporated to dryness and the residue refluxed in ethanol (25 ml) for 75 min to effect thermal isomerisation to IIIa. The product was applied to a 50 × 1.5 cm column of Sephadex LH-20 packed in chloroform–*n*-hexane (65:35, v/v). The column was eluted with the same solvent mixture, 2 ml fractions being collected. Each fraction was evaporated in a stream of nitrogen and redissolved in ethanol for measurement of ultraviolet absorbance. Fractions 48–54 with λ_{max} 265 nm were combined to give IIIa, whereas in fractions 57–58 the isomeric 4,6-diene, λ_{max} 240 nm was recovered. Final purification of IIIa was carried out by high pressue liquid chromatography. λ_{max} 265 nm and λ_{min} 228 nm. The yield was 2.5 mg (calculated assuming that $\epsilon 265 = 18.300$). R_F 0.45.

(25S)-26-Hydroxycholecalciferol (IIIb). IIb (R = H) (0.057 g) was treated as IIa (R = H) and after identical purification of the crude product gave IIIb (3.5 mg) of λ_{max} 265 nm and λ_{min} 228 nm.

The mass spectra are identical for IIIa and IIIb. Peaks at *m/e* (relative intensity): 400 (60; M^+), 382 (20; $M - H_2O$), 367 (52), 271 (30), 253 (40), 136 (84), 118 (100).

Biological activity

Methods and materials. The method of bone organ culture *in vitro* and *in vivo/in vitro* has been described in detail previously [5].

Mice were injected subcutaneously with ^{45}Ca (1 or 2 μCi) 1 or 2 days after birth. 4 days later the animals were either killed and paired calvaria explanted—one in control medium and the other in medium containing the test substances (*in vitro* method)—or injected with the test substance, sacrificed 18 h later and paired half calvaria, one intact and the other dead, prepared for organ culture (*in vivo/in vitro* method).

The ^{45}Ca released into the medium during a 48 h incubation was measured by liquid scintillation counting; the bones are counted after dissolving them in formic acid.

RESULTS

The synthesis of (25R)- and (25S)-26-OH-D_3 was achieved following the classical sequence outlined in Fig. 1. The known bromination and dehydrobromination reaction was applied to C-25 epimers of 5-Cholestene-3β, 26-diol 3,26-diacetate (I) and led to a mixture of isomeric 5,7-diene (II) and 4,6-diene. Our method, recently reported [7], consisted in making use of the latter mixture, without purification, for the irradiation stage and then separating the unchanged 4,6-diene from 26-hydroxycholecalciferol (III) by chromatography on Sephadex LH 20. Final purification was accomplished by high pressure liquid chromatography (h.p.l.c.).

The action of (25R)-26-OH-D_3 (IIIa) and (25S)-26-OH-D_3 (IIIb) on bone resorption in mouse calvaria is shown in Tables 1 and 2. The epimers are compared with 25R,26- and 25S,26$(OH)_2D_3$,

Table 1. Direct *in vitro* test of bone resorbing activity of the 26-hydroxy-derivatives of vitamin D_3

Compound	Concentration per ml (ng)	Increase in resorption (% ^{45}Ca release)	Significance
25-OH-D_3	100	12.5 ± 2.0 (7)	$P < 0.001$
(25R)-26-OH-D_3	200	2.60 ± 0.35 (13)	$P < 0.01$
(25S)-26-OH-D_3	200	−1.1 ± 0.5 (13)	N.S.
25R,26$(OH)_2D_3$	200	12.8 ± 2.2[a] (7)	$P < 0.001$
25S,26$(OH)_2D_3$	200	7.1 ± 1.9[a] (7)	$P < 0.001$

[a]Difference between 25R,26- and 25S,26$(OH)_2D_3$ was significant ($P < 0.001$).

The response is calculated as the increase in resorption expressed as the difference, treated half minus control half, in percentage release of total bone isotope into the medium during 48 h-culture. Number of pairs of explants in parentheses. Analysis of statistical significance was performed by Student's *t*-test.

Table 2. *In vivo/in vitro* test of bone resorption of 26-hydroxy-derivatives of vitamin D_3 injected 18 h before sacrifice

Compound	Dose per g body weight (ng)	Cell mediated resorption increase (% ^{45}Ca release)	Significance
25-OH-D_3	100	7.2 ± 1.5 (7)	$P < 0.001$
(25R)-26-OH-D_3	200	−0.7 ± 1 (12)	N.S.
(25S)-26-OH-D_3	200	−1.1 ± 0.7 (13)	N.S.
25R,26$(OH)_2D_3$	200	2.5 ± 1 (7)	$P < 0.01$
25S,26$(OH)_2D_3$	200	2.2 ± 1.2 (7)	$P < 0.05$

For each mouse the cell mediated resorption during the *in vitro* period was calculated as the difference, intact half minus dead half, in percentage of release of total bone isotope into the medium. The data are expressed as the increase in cell mediated resorption, treated mouse minus control paired mouse. Number of pairs of mice in parentheses. Analysis of statistical significance was performed by Student's *t*-test.

never tested before by *in vitro* methods. Table 1 illustrates the effect of 26-hydroxy-derivatives of vitamin D_3 when added to the medium. Among the C-25 epimers of 26-OH-D_3 only (25R)-26-OH-D_3 significantly stimulates bone resorption. 25R,26- and 25S,26$(OH)_2D_3$ both have pronounced osteolytic effects, the former compound being significantly ($P < 0.001$) more effective.

A comparison of the effects by the *in vivo/in vitro* method 18 h after injection is shown in Table 2. The C-25 epimers of 25,26$(OH)_2D_3$ have equivalent effects, whereas the C-25 epimers of 26-OH-D_3 are ineffective.

DISCUSSION

In organ cultures of mouse calvaria *in vitro* the C-25 epimers of 26-OH-D_3 are less effective in stimulating bone calcium mobilization than their 25-hydroxy-derivatives.

It is noteworthy that in the series of 24-hydroxy-derivatives of vitamin D_3, closely related to the metabolite 24,25-dihydroxycholecalciferol (24,25$(OH)_2D_3$), the 25-hydroxylation has only little effect on bone resorption *in vitro*. In an organ culture system from fetal rat bone [8] similar to ours, 24R-hydroxyvitamin D_3 (24-OH-D_3) and 24R,25-dihydroxyvitamin D_3 were equally potent [8], almost equivalent to 25-OH-D_3. The 24*S* epimers were less potent.

These results suggest that the 24-hydroxyl group, particularly in the 24R configuration, as in 24R-OH-D_3, is highly effective on bone resorption *in vitro*, whereas the 26-hydroxyl group, even in the more favoured 25R configuration, as in (25R)-26-OH-D_3, promotes only a weak activity. However, when hydroxylated at C-25, 24R-OH-D_3 and (25R)-26-OH-D_3 induce a very similar effect.

In the *in vivo/in vitro* experiments we were surprised to record the ineffectiveness of (25R)-26-OH-D_3, in spite of its activity *in vitro*. As it is known that *in vivo* a 25-hydroxylation by the calciferol-25-hydroxylase occurs, a decreased activity of the latter in vitamin D replete animals may be incriminated [9].

The bone resorption induced *in vivo/in vitro* by the C-25 epimers of 25,26$(OH)_2D_3$ is in agreement with their moderate activity on bone calcium mobilization in vitamin D and calcium deprived rats [3]. The latter effect was however, significant only with 25*S*,26$(OH)_2D_3$ [known in Ref. 3 as 25ξ^1,26$(OH)_2D_3$].

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