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ISOLATION AND IDENTIFICATION OF 25-HYDROXY-24-OXOCHOLECALCIFEROL: A METABOLITE OF 25-HYDROXYCHOLECALCIFEROL

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Summary: A metabolite of 25-hydroxycholecalciferol has been isolated in pure form from chicken kidney homogenates. It has been identified as 25-hydroxy-24-oxocholecalciferol by means of ultraviolet absorption spectrophotometry, mass spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and specific chemical reactions.

INTRODUCTION

It is well established that cholecalciferol (vitamin D_3) must be converted first in the liver to 25-hydroxycholecalciferol [25(OH)D_3], and then in the kidney to either 1α,25-dihydroxycholecalciferol [1α,25(OH)$_2D_3$] or 24,25-dihydroxycholecalciferol [24,25(OH)$_2D_3$](1). 1α,25(OH)$_2D_3$ has been thought to be an active form of vitamin D_3, whereas the biological significance of 24,25-(OH)$_2D_3$ is still controversial.

In the course of investigating renal 24-hydroxylase activity, we have found that kidney homogenates from chicks supplemented with vitamin D_3 metabolize in vitro 25(OH)[^{3}H]D_3 to 3 other radioactive metabolites (peaks A, C and E) besides 24,25(OH)$_2$[^{3}H]D_3 (2). The enzymes responsible for the production of peaks A and C appeared to be induced by 1α,25(OH)$_2D_3$ (3). Production of peak A increased in parallel with the increase of the amount of 25(OH)D_3

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Abbreviations used: 25(OH)D_3, 25-hydroxycholecalciferol; 1α,25(OH)$_2D_3$, 1α,25-dihydroxycholecalciferol; 24,25(OH)$_2D_3$, 24,25-dihydroxycholecalciferol.

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added as a substrate, while that of peak C was fairly constant irrespective of the increase of the substrate (3). Peak A was a slightly less polar metabolite than 25(OH)D_3 and it was eluted in the 25(OH)D_3 fraction on Sephadex LH-20 columns (2). Peak A has been isolated in pure form from chicken kidney incubations with 25(OH)D_3, and we now report the structure of the metabolite to be 25-hydroxy-24-oxocholecalciferol.

ISOLATION

One-day-old 1350 White Leghorn cockerel chicks were maintained for 10 days on a vitamin D-deficient diet containing 1.1% calcium and 0.6% phosphorus. The animals were orally dosed with 650 nmol of vitamin D_3 48 hr prior to sacrifice. The kidneys were quickly removed, rinsed, minced with a garlic press, and homogenized in 4 volumes of 0.2 M sucrose containing 15 mM Tris-Cl (pH 7.4), 2 mM $MgCl_2$ and 5 mM sodium succinate (2). A total of 4.6 l of the homogenate was obtained. Each 180 ml aliquot was incubated with 1 µCi (160 nmol) of 25-(OH)[26,27-^{3}H]D_3 (Radiochemical Centre, Amersham) in a 2 l Erlenmeyer flask at 37°C for 30 min. The reaction was terminated by the addition of 675 ml of methanol-chloroform (2:1, v/v). Extraction was performed as described by Gray et al.(4).

The concentrated extracts were divided into 7 equal parts, and each was chromatographed on a 2.2 x 55 cm column packed with 70 g of Sephadex LH-20. The column was eluted with 65% chloroform in n-hexane according to the method of Holick and DeLuca (5). Seventy 10 ml-fractions were collected and each radioactivity was counted. The 25(OH)D_3 (tubes 7-20) and the 24,25(OH)$_2D_3$ (tubes 28-50) fractions were separately pooled from each column run and concentrated. The pooled 25(OH)D_3 fraction was further chromatographed in 6 batches on a 4 x 20 cm column of Silica gel 60 (Merck, 100 g) using a solvent of n-hexane : ethyl acetate (2:1, v/v). Sixty 20 ml-fractions were collected. Each 25(OH)D_3 fraction (tubes 30-45) monitored by radioactivity was pooled and concentrated.

The 25(OH)D_3 fraction which, besides 25(OH)D_3, includes the metabolite referred to as peak A was applied to a high pressure liquid chromatograph, Waters HPLC Model 204, equipped with a semi-preparative μ-Porasil column (7.9 mm x 30 cm). The solvent system was 1% methanol in dichloromethane. A radioactive peak migrating prior to 25(OH)D_3 was collected and then chromatographed on a reverse-phase HPLC using μ-Bondapack C_{18} (4 mm x 30 cm) with 15% water in methanol. The major UV peak was collected and further purified on a straight-phase HPLC (Zorbax-Sil column, 4.6 mm x 15 cm, Du Pont) with 3.5% isopropanol in n-hexane. The major UV peak was pooled and rechromatographed on the same system. The sole UV peak obtained was used for structural identification. A total of 35.2 μg (on the basis of UV absorption at 265 nm) or 37.0 μg (on the basis of specific activity of tritium) of the purified metabolite was obtained. We also isolated 138 μg of purified 24,25(OH)$_2D_3$ from 1.6 mg of 25(OH)D_3. The amount of the new metabolite isolated was 2.3% of the substrate added and 26.8% of 24,25(OH)$_2D_3$ produced.

IDENTIFICATION

The ultraviolet absorption spectrum of the metabolite showed a λ_{max} at 265 nm and a λ_{min} at 228 nm, characteristic for the 5,6-cis-triene chromophore of the D vitamins. The mass spectrum of the metabolite exhibited a molecular ion at m/e 414 and fragment ions typical of vitamin D_3 at m/e 271 (M - side chain), 253 (271 - H_2O), 136 and 118 (see Fig. 1, I), indicating that the alteration of the structure by metabolism occurred at the side chain. The infrared spectrum of the metabolite showed an absorption due to a carbonyl group at 1710 cm^{-1}. The nuclear magnetic resonance spectrum of the metabolite showed an AB-type quartet centered at δ 6.17 (J = 11 Hz) for C-6 and C-7 protons, a pair of broad singlets at δ 4.84 and 5.08 for C-19 protons, a multiplet at δ 3.95 for C-3 proton, a three-proton singlet at δ 0.54 for 18-methyl group and six-proton singlet at δ 1.38 for 26- and 27-methyl groups. A downfield shift of the signal due to 26- and 27-methyl groups, from δ 1.20 to δ 1.38, was prominent when compared with the spectrum of the starting material,

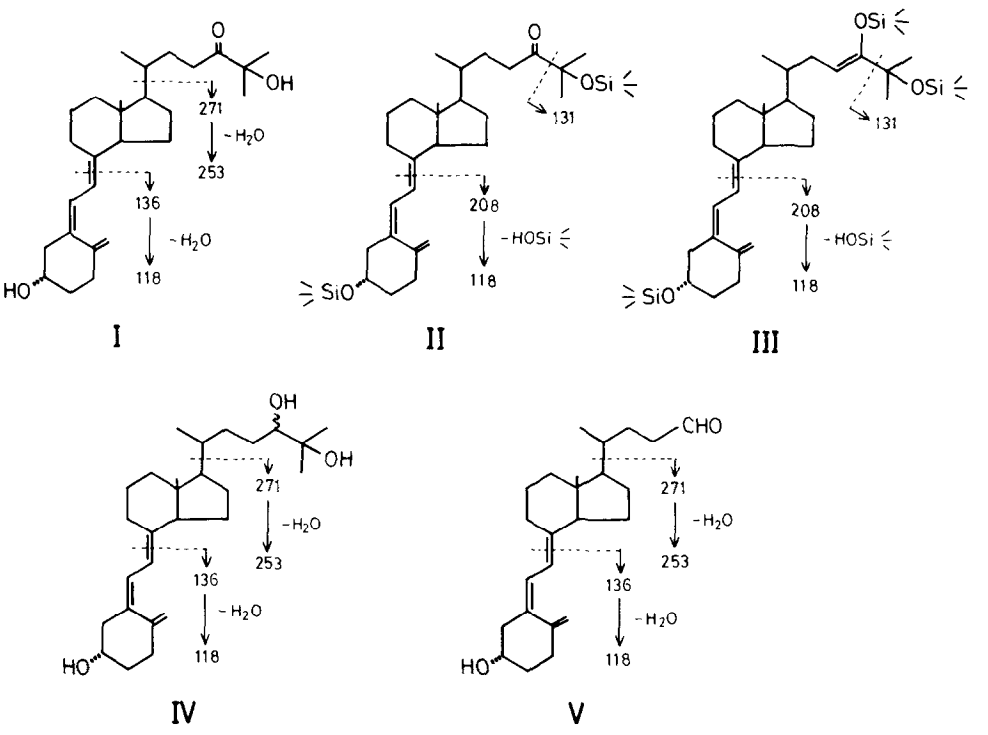

Fig. 1 Structures and schematic representation of bond ruptures in the mass spectrum of (I) 25-hydroxy-24-oxocholecalciferol, (II) its 3β,25-di(trimethylsilyl) ether, (III) its 3β,25-di(trimethylsilyl) ether-24-enol-trimethylsilyl ether, (IV) 24ξ,25(OH)$_2$D$_3$, and (V) 3β-hydroxy-9,10-seco-5,7,10(19)-cholatrien-24-al.

25(OH)D$_3$ (6). This, together with the data described above, strongly suggests the presence of a carbonyl function at C-24 and the metabolite to be 25-hydroxy-24-oxocholecalciferol (Fig. 1, I).

Further supporting evidence for the structure was provided by the following chemical transformations. Trimethylsilylation of the metabolite produced di(trimethylsilyl) ether (Fig. 1, II) together with a small amount of di(trimethylsilyl) ether-24-enol-trimethylsilyl ether (Fig. 1, III). The mass spectra of the both products verify the correctness of the assigned structure, showing significant peaks at m/e 558(M), 468 (M - HOSiMe$_3$), 208, 131 and 118 for II, and m/e 630(M), 540 (M - HOSiMe$_3$), 131 and 118 for III. Reduction of the metabolite with NaBH$_4$ gave a product (Fig. 1, IV) which was proved to be 24ξ,25(OH)$_2$D$_3$ based on the following reasons; i) the compound migrated to the

same retention volume as that of the authentic 24R,25(OH)$_2$D$_3$ on HPLC (column; μ-Porasil, eluted with 10% isopropanol in n-hexane), ii) the mass spectral fragmentation pattern of the compound (m/e 416(M), 271, 253, 136 and 118) was identical with that of 24R,25(OH)$_2$D$_3$ (7), iii) periodate oxidation of the compound produced a cleavage product (Fig. 1, V) which was proved to be identical with authentic 3β-hydroxy-9,10-seco-5,7,10(19)-cholatrien-24-al (7) both in HPLC retention volume (μ-Porasil, 5% isopropanol in n-hexane) and in the mass spectral fragmentation pattern (m/e 356(M), 271, 253, 136 and 118). Therefore, the new metabolite was established to be 25-hydroxy-9,10-seco-5,7,-10(19)-cholestatrien-24-one (25-hydroxy-24-oxocholecalciferol).

The biological activity of the metabolite is of considerable interest and is currently under investigation in our laboratories.

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