23,25,26-Trihydroxycholecalciferol

A 25-hydroxycholecalciferol-26,23-lactone precursor

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(Received 20 April 1982/Accepted 13 May 1982)

(23*S*)-23,25-Dihydroxycholecalciferol was converted into at least five metabolites in kidney homogenates prepared from 1,25-dihydroxycholecalciferol-treated chickens. One of these has been positively identified as 23,25,26-trihydroxycholecalciferol by u.v.-absorbance analysis, mass spectrometry and chemical formation of derivatives. 23,25,26-Trihydroxycholecalciferol produces 25-hydroxycholecalciferol-26,23-lactone when incubated in chick kidney homogenates.

The major circulating metabolite of cholecalciferol is 25(OH) D_3 (Hadded *et al.*, 1977; Horst *et al.*, 1981*a,b*). 25(OH) D_3 -lactone is a significant 25(OH) D_3 metabolite in animals receiving large doses of cholecalciferol (Wichmann *et al.*, 1979; Horst, 1979). This lactone has a 5-fold higher affinity for the vitamin D serum carrier protein than does 25(OH) D_3 (Horst & Littledike, 1980). Consequently, 25(OH) D_3 -lactone could be involved in vitamin D-toxicity; or perhaps the presence of high plasma 25(OH) D_3 -lactone concentrations could be used as a measure of vitamin D-toxicity.

25,26(OH) $_2D_3$, a kidney microsomal metabolite of 25(OH) D_3 (Napoli *et al.*, 1981), was initially reported as a 25(OH) D_3 -lactone precursor after preliminary experiments (Tanaka *et al.*, 1981). However, rigorous investigation excluded a role for (25*S*)-25,26(OH) $_2D_3$, the naturally occurring isomer (Partridge *et al.*, 1981), in 25(OH) D_3 -lactone biosynthesis (Pramanik *et al.*, 1981; Napoli & Horst, 1981). These results were subsequently verified (Ishizuka *et al.*, 1982) and therefore indicate that 25(OH) D_3 metabolism to 23,25(OH) $_2D_3$ is the first step in 25(OH) D_3 -lactone production. This report

establishes the identity of a new (23*S*)-23,25(OH) $_2D_3$ metabolite as 23,25,26(OH) $_3D_3$, and demonstrates that 23,25,26(OH) $_3D_3$ is an efficient precursor of 25(OH) D_3 -lactone.

Materials and methods

1,25(OH) $_2D_3$, (23*S*)-23,25(OH) $_2D_3$ and 1,24,25-(OH) $_3D_3$ were generously supplied by M. R. Uskokovic and J. J. Partridge, Chemical Research Department, Hoffmann-LaRoche, Nutley, NJ, U.S.A. 25(OH) D_3 was a gift from J. C. Babcock, The Upjohn Company, Kalamazoo, MI, U.S.A. Lactone standard was purified from cholecalciferol-toxic pig plasma (Horst, 1979). (23*S*)-23,25-(OH) $_2$ [3H] D_3 (sp. radioactivity 90 Ci/mmol) was made from 25OH[26,27- 3H] D_3 (0.5 mCi/15 ml of reaction mixture) by using homogenates prepared from 1,25(OH) $_2D_3$ -treated chick kidneys as described by Horst (1979). The structure of the natural product as (23*S*)- rather than (23*R*)-23,25(OH) $_2D_3$ was verified by h.p.l.c. analysis.

Mass spectra were obtained at 70 eV from the solids probe of a Finnigan model 4021 EI/CI GC/MS coupled with an INCOS Data System. To obtain spectra, the probe was heated from ambient temperature to 320°C over a 10 min period at a source temperature of 250°C. U.v.-absorbance spectra were taken in propan-2-ol with a Beckman model 25 recording spectrophotometer. H.p.l.c. was performed with Waters Associates model ALC/GPC 204 liquid-chromatographic equipment. H.p.l.c.

Abbreviations used: 25(OH) D_3 , 25-hydroxycholecalciferol; 1,25(OH) $_2D_3$, 1,25-dihydroxycholecalciferol; 24,25(OH) $_2D_3$, 24,25-dihydroxycholecalciferol; 23,25-(OH) $_2D_3$, 23,25-dihydroxycholecalciferol; 25(OH) D_3 -lactone, 25-hydroxycholecalciferol-25,23-lactone; 23,25,26(OH) $_3D_3$, 23,25,26-trihydroxycholecalciferol; 25,26-(OH) $_2D_3$, 25,26-dihydroxycholecalciferol; h.p.l.c. high-pressure liquid chromatography; ODS, octadecylsilane.

columns were purchased from Du Pont, unless noted otherwise.

White Leghorn cockerels (1 day old; Hy-line Indian River Co., Dallas Center, IA, U.S.A.) were fed vitamin D-deficient diets (1% calcium for 3 days and 3% calcium for 4 days) for 7 days. On days 4, 5 and 6, each chick received an intramuscular injection of cholecalciferol ($3.9\mu\text{mol}$) in propylene glycol ($100\mu\text{l}$). Each chick also received $1,25(\text{OH})_2\text{D}_3$ (600pmol) 24 h and 6 h before being killed. The animals were killed on day 7 and the kidneys were collected and rinsed in buffer A consisting of 15 mM-Tris/acetic acid/150 mM-sucrose/2 mM-magnesium acetate, pH 7.4. The kidneys were decapsulated and a 20% (w/v) homogenate was prepared in buffer A with a Polytron PT20 tissue disruptor. To the homogenate (6 ml) in each of nine Erlenmeyer flasks was added cofactor solution (3 ml) consisting of 7.5 mM-sodium succinate, 0.4 mM-NADP⁺, 160 mM-nicotinamide and 20 mM-ATP in buffer A. The reactions were initiated by the addition of (23*S*)-23,25(OH) $_2\text{D}_3$ ($0.12\mu\text{mol}$) in ethanol ($50\mu\text{l}$). Alternatively, a separate flask was prepared containing (23*S*)-23,25(OH) $_2$ [26,27- ^3H] D_3 ($25\mu\text{Ci}$, 0.31pmol). The mixtures were gassed with O_2 for 30 s, sealed and incubated with shaking for 1.5 h at 37°C . The reactions were quenched with 3.75 vol. of methanol/dichloromethane (2:1, v/v) and the lipids were extracted as previously described (Horst *et al.*, 1981b).

A portion of the extract from the flask containing the tritiated substrate was added to the extract of the flasks incubated with the unlabelled substrate. The solvents were evaporated and the residue was eluted with ethyl acetate through a column ($0.6\text{ cm} \times 10\text{ cm}$) of silicic acid and celite (1:1, v/v). The material obtained was applied to a silica-gel h.p.l.c. column ($0.95\text{ cm} \times 25\text{ cm}$) eluted with a concave gradient (curve 7 on Waters Associates model 660 Solvent Programmer) of 1%–14% propan-2-ol/hexane developed during 1 h at a flow rate of 5 ml/min. In this system, authentic $1,25(\text{OH})_2\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3$ elute at 288 and 335 ml respectively. The metabolite that eluted between 290 and 305 ml was applied to a reverse-phase h.p.l.c. column (ODS; $0.46\text{ cm} \times 25\text{ cm}$) developed with methanol/water (39:11, v/v) at 2 ml/min. The metabolite eluted in 19 ml, relative to elution volumes of 24, and 21 ml respectively, for $1,25(\text{OH})_2\text{D}_3$ and $2,24,25(\text{OH})_3\text{D}_3$. The recovered metabolite was applied to a silica-gel h.p.l.c. column ($0.46\text{ cm} \times 25\text{ cm}$) eluted with propan-2-ol/dichloromethane (7:93, v/v) at 2 ml/min. $1,25(\text{OH})_2\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3$ standards elute at 12 and 24 ml respectively in this system. The metabolite eluted at 23 ml. Finally, the new metabolite was re-applied to the silica-gel h.p.l.c. column ($0.46\text{ cm} \times 25\text{ cm}$) and eluted with propan-2-ol/hexane (11:89, v/v). The metabolite eluted in 24 ml

and was recycled on to the column for a total of two passes. The final elution volume was 52 ml.

$25(\text{OH})\text{D}_3$ -lactone was prepared from 23,25,26-(OH) $_3\text{D}_3$ by incubating the trihydroxy metabolite ($3.7\mu\text{g}$) with a 20% kidney homogenate (6 ml plus 3 ml of cofactor solution) prepared from $1,25(\text{OH})_2\text{D}_3$ -treated chickens as described previously (Horst, 1979). The $25(\text{OH})\text{D}_3$ -lactone was extracted with methanol/dichloromethane and eluted through a silicic acid/Celite column as described above. The residue was applied to a silica-gel h.p.l.c. column ($0.95\text{ cm} \times 25\text{ cm}$) eluted with the concave gradient described above, except that the development time was 40 min. In this system $23,25(\text{OH})_2\text{D}_3$ and $23,25,26(\text{OH})_3\text{D}_3$ standards elute at 155 and 215 ml respectively. The peak from the extract eluted at 173 ml and was placed on a silica-gel h.p.l.c. column ($0.46\text{ cm} \times 25\text{ cm}$) developed with dichloromethane/propan-2-ol (99:1, v/v). $25(\text{OH})\text{D}_3$ standard elutes at 24 ml in this system; the $23,25,26(\text{OH})_3\text{D}_3$ metabolite eluted at 34 ml. It was placed on a Zorbax-Amino h.p.l.c. column ($0.46\text{ cm} \times 25\text{ cm}$) and eluted with hexane/propan-2-ol (9:1, v/v) in the recycle mode for a total of two passes. The material recovered at 45 ml was placed on a reverse-phase h.p.l.c. column (ODS; $0.46\text{ cm} \times 25\text{ cm}$) developed with methanol/water (39:11, v/v). It eluted at 22 ml. It was re-applied to the Zorbax Amino h.p.l.c. column and again passed through the column twice.

Results and discussion

Incubation of (23*S*)-23,25(OH) $_2$ [^3H] D_3 (34 pM) with homogenates prepared from vitamin D-treated chick kidneys resulted in 83% substrate conversion into more polar compounds. At least five distinct products were observed. Included among these were $25(\text{OH})\text{D}_3$ -lactone (41% of total products), and 23-oxo-25-hydroxycholecalciferol (6% of total products). (R. L. Horst & J. L. Napoli, unpublished work). Preliminary studies showed that one of the unidentified tritiated peaks (7% of total products), upon re-introduction into the homogenate, produced a compound that co-migrated in three h.p.l.c. systems with $25(\text{OH})\text{D}_3$ -lactone. This new cholecalciferol metabolite was selected for isolation and identification. Thus the incubations were repeated with unlabelled (23*S*)-23,25(OH) $_2\text{D}_3$ as substrate.

A portion of the tritiated lactone precursor was added to the material extracted from the incubations done with unlabelled (23*S*)-23,25(OH) $_2\text{D}_3$. The new metabolite was freed from gross lipid contamination with a small silica-gel/Celite column and was purified to homogeneity through a series of four h.p.l.c. systems. In these h.p.l.c. procedures, the $23,25(\text{OH})_2\text{D}_3$ metabolite behaved significantly more polar than $1,25(\text{OH})_2\text{D}_3$ and slightly less polar than

1,24,25(OH)₃D₃. It also co-migrated with the tritiated lactone precursor.

Spectral analysis of the metabolite and its derivatives established its identity as 23,25,26(OH)₃D₃. A u.v.-absorbance spectrum had a λ_{max} at 265 nm and a λ_{min} at 229 nm with the ratio $\lambda_{\text{max}}/\lambda_{\text{min}}$ equal to 1.9. Therefore, the compound possessed a vitamin D-like *cis*-triene chromophore and was pure. The total amount of metabolite isolated was calculated to be 10 μg from the absorbance value, using a molar absorptivity (ϵ) of 18 200 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. The mass spectrum (Fig. 1a) had a molecular ion at m/z 432, which was consistent with addition of a hydroxy group to (23*S*)-23,25(OH)₂D₃. Peaks at m/z 414, 399, 396, 381 and 363 represented losses, from the molecular ion, of water, water and a methyl group, two molecules of water, two molecules of water and a methyl group and three molecules of water and a methyl group respectively. The peak at m/z 342 resulted from rupture of the C-23–C-24 bond, a unique characteristic of C-23-functionalized D₃ vitamins. Loss of water and a methyl group from

m/z 342 produced an ion with m/z 309. The peak at m/z 271 was produced by side-chain cleavage. Loss of water from the ion with m/z 271 gave an anion with m/z 253. The last three peaks demonstrated that the additional hydroxy group was added to the side chain. The peaks at m/z 383, 368 and 365 all resulted from loss of CH₂OH, accompanied by losses of water, water and a methyl group and two water molecules respectively. Loss of CH₂OH indicates the presence of a primary alcohol. Thus these peaks, considered with the peaks at m/z 271 and 253, suggested that the added hydroxy group was at C-26. The peaks at m/z 136 and 118 resulted from formal cleavage between C-7 and C-8, followed by loss of water from the initially formed fragment. These two peaks are diagnostic of a vitamin D triene system.

A portion of the metabolite (600 ng) was allowed to react with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (50 μl) at 90°C for 2 h to effect persilylation. The silylated product eluted at 20 ml from a reverse-phase h.p.l.c. column (Whatman ODS-3; 0.42 cm $\times$ 25 cm) with 0.1% dichloromethane in methanol. A mass spectrum had a molecular ion at m/z 720 (relative intensity, 0.5), which was consistent with a tetrasilylated cholecalciferol metabolite, i.e. a total of four hydroxy groups were present. Peaks (with relative intensities in parentheses) were observed at m/z 630 (0.5) and 540 (0.3), which represented a loss of one and two molecules of (CH₃)₃SiOH. A peak was observed at m/z 487 (5) caused by rupture of the C-23–C-24 bond. Peaks at m/z 397 (3) and 207 (10) arose from loss of one and two molecules of (CH₃)₃SiOH from m/z 487. Cleavage between C-24 and C-25 produced m/z 219 (20). The presence of a vitamin D triene system was indicated by the peaks at m/z 208 (15) and 118 (75) caused by formal cleavage between C-7 and C-8, followed by loss of water. The base peak was m/z 73 (100), corresponding to (CH₂)₃Si⁺. These data indicated beyond doubt that the compound was a hydroxylated (23*S*)-23,25(OH)₂D₃ metabolite.

To establish the location of the additional hydroxy group, the metabolite (1.5 μg) in methanol (50 μl) was allowed to react with aq. 5% NaIO₄ (135 μl) at room temperature for 2 h. A mass spectrum of the product (Fig. 1b) had a molecular ion at m/z 400, which indicated that the additional hydroxy group was vicinal to an existing side-chain hydroxy group. These data demonstrated positively that the added hydroxy group was on C-26. The peak at m/z 342 arose from cleavage between C-23 and C-24, which normally occurs in 23-hydroxylated cholecalciferol compounds. However, in this case, this cleavage could also result from a McLafferty rearrangement as indicated in Fig. 1. The peaks at m/z 324 and 309 indicated loss of water, and loss of water and a methyl group from m/z 342. The peak at m/z 382

Fig. 1. Mass spectra of 23,25,26(OH)₃D₃ (a) and its NaIO₄ cleavage product (b)

represented loss of water from the molecular ion. Peaks at m/z 136 and 118 indicated the presence of a vitamin D triene system.

To help verify its structure and to demonstrate that 23,25,26(OH)₃D₃ is a 25(OH)D₃-lactone precursor, the metabolite was incubated with a 1,25-(OH)₂D₃-treated chick kidney homogenate. It produced a material that migrated in the position of 25(OH)D₃-lactone standard in five separate h.p.l.c. procedures. The 23,25,26(OH)₃D₃ metabolite (approx. 386 ng) had an absorbance maximum at 265 nm and a minimum at 229 nm. The $\lambda_{\max}/\lambda_{\min}$ ratio was 1.7. The mass spectrum had a molecular ion (relative intensity in parentheses) at m/z 428 (5), and peaks at m/z 410 (3; $M^+ - H_2O$), 395 (9; $M^+ - H_2O - CH_3$), 253 (12; loss of the side chain and water), 136 (55) and 118 (100), i.e., it was similar to that of lactone standard (Horst, 1979). The u.v.-absorbance maximum, the mass-spectral data and the h.p.l.c. results leave no doubt that the 23,25,26-(OH)₃D₃ metabolite was 25(OH)D₃-lactone.

Since 23,25,26(OH)₃D₃ was synthesized from (23*S*)-23,25(OH)₂D₃ and produced 25(OH)D₃-lactone, which has the (23*S*,25*R*)-configuration (Morris *et al.*, 1981; Ishizuka *et al.*, 1981), it is highly probable that the configuration of 23,25,26-(OH)₃D₃ is 23*S*,25*R*.

We thank Bikash C. Pramanik for his expert assistance with the mass spectroscopy. This work was supported in part by Robert A. Welch Foundation grant I-797 and by N.I.H. grant AM 26535. The mass spectrometer

was purchased with N.I.G.M.S. shared Instrumentation grant GM27506.

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