23-Keto-25-hydroxyvitamin D3: a vitamin D3 metabolite with high affinity for the 1,25-dihydroxyvitamin D specific cytosol receptor

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Articles

23-Keto-25-hydroxyvitamin D_3: A Vitamin D_3 Metabolite with High Affinity for the 1,25-Dihydroxyvitamin D Specific Cytosol Receptor†

Ronald L. Horst,* Timothy A. Reinhardt, Bikash C. Pramanik, and Joseph L. Napoli

ABSTRACT: A new metabolite of 23,25-dihydroxyvitamin D_3 has been generated with kidney homogenates prepared from vitamin D treated chicks. The metabolite was purified with three high-performance liquid chromatographic steps and was identified as 23-keto-25-hydroxyvitamin D_3 by ultraviolet absorption spectroscopy, mass spectrometry, and chemical reactivity. The *R* stereoisomer of 23,25-dihydroxyvitamin D_3 was 10-fold more effective as an in vitro precursor to 23-keto-25-hydroxyvitamin D_3 than was the naturally occurring *S* stereoisomer. Approximately 500 ng of 23-keto-25-hydroxyvitamin D_3 was necessary to produce the same degree of intestinal-calcium transport as 25 ng of vitamin D_3—a difference of about 20-fold. 23-Keto-25-hydroxyvitamin D_3 was not active at stimulating bone calcium resorption at the doses and times tested. This new vitamin D_3 metabolite, however, had greater affinity than 25-hydroxyvitamin D_3 to both the rat plasma vitamin D binding protein and the 1,25-dihydroxyvitamin D specific cytosol receptor. Heretofore, only 1α-hydroxylated metabolites of 25-hydroxyvitamin D_3 or analogues possessing a pseudo 1α-hydroxy group were known to bind to the 1,25-dihydroxyvitamin D receptor with higher affinity than 25-hydroxyvitamin D_3. Ketone formation at the 23 position, therefore, is the first side-chain modification of 25-hydroxyvitamin D_3 that results in enhanced binding to the 1,25-dihydroxyvitamin D receptor binding protein.

The importance of the 25-hydroxylation and 1α-hydroxylation of vitamin D_2 and vitamin D_3 to the expression of biological activity is recognized (Haussler & McCain, 1977; Napoli & DeLuca, 1979; Norman, 1979). The resulting compounds 1,25-dihydroxyvitamin D_2 [1,25-$(OH)_2D_2$][1] and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] are biologically active forms produced primarily in the kidney cortex (Kodicek, 1974) during hypocalcemia, hypophosphatemia, or hypovitaminosis D. Recent evidence also suggests the presence of an extrarenal 1α-hydroxylase in bone (Howard et al., 1981), a target tissue for 1,25-$(OH)_2D$ (Stumpf et al., 1982). During normal vitamin D nutrition or vitamin D excess, there is enhancement of other enzymes responsible for the hydroxylation of 25-OHD_3 at C-23, C-24, and C-26 (Horst et al., 1981a,b; Napoli et al., 1981; Reinhardt et al., 1981a,b, 1982a,b; Tanaka et al., 1981b). Nephrectomy does not prevent the expression of these hydroxylases during vitamin D excess (Horst et al., 1981a; Napoli et al., 1982), but under normal vitamin D nutrition, nephrectomy abates hydroxylation of 25-OHD_3 at C-24 and C-23, whereas C-26 hydroxylation is unaffected (Horst et al., 1981a; Horst & Littledike, 1980; Taylor et al., 1982).

Recent reports have stressed the view that these side-chain modifications of 25-OHD and 1,25-$(OH)_2D$ are prerequisite

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[1] Abbreviations: 25-OHD, 25-hydroxyvitamin D; 25-OHD_3, 25-hydroxyvitamin D_3; 23-keto-25-OHD_3, 23-keto-25-hydroxyvitamin D_3; 24-keto-25-OHD_3, 24-keto-25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; lactone, 25-hydroxyvitamin D_3-26,23-lactone; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 1,25-$(OH)_2D$, 1,25-dihydroxyvitamin D; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 23,25,26-$(OH)_3D_3$, 23,25,26-trihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

to their deactivation (Tanaka et al., 1979). Contrasting views, however, suggest that 24(*R*),25-$(OH)_2D_3$, the major metabolite of 25-OHD_3 during normocalcemia and normal vitamin D status (Haddad et al., 1977; Horst et al., 1981a,b; Shepard et al., 1979), may have a biologic function, particularly in bone formation and embryonic development (Bordier et al., 1978; Endo et al., 1980; Henry & Norman, 1978). Specific side-chain modification(s), therefore, may result in metabolites with biologic activity.

Other side-chain modifications of 25-OHD_3 include the formation of 24-keto-25-OHD_3 (Takasaki et al., 1981) and lactone (Horst, 1979; Wichmann et al., 1979). These two metabolites possess a carbonyl group, which suggests the presence of heretofore uncharacterized dehydrogenase enzymes in the vitamin D metabolic scheme. Both compounds are generally formed during vitamin D_3 excess, but lactone is present in some species under normal vitamin D nutrition (Horst et al., 1981b). Hollis et al. (1980) and Tanaka et al. (1981a) reported that lactone biosynthesis could proceed from hydroxylation of 25-OHD_3 at C-26 to form 25(*S*),26-$(OH)_2D_3$. However, rigorous analysis has shown that 25(*S*),26-$(OH)_2D_3$, the naturally occurring isomer (Partridge et al., 1981), is not involved in lactone biosynthesis (Napoli & Horst, 1981; Pramanik et al., 1981); rather, the initial biosynthetic step is 23-hydroxylation of 25-OHD_3 to form 23(*S*),25-$(OH)_2D_3$ (Napoli et al., 1982).

In our studies of lactone biosynthesis (Napoli et al., 1982), we discovered that chick kidney homogenates, prepared from vitamin D_3 repleted chicks, metabolized 23(*S*),25-$(OH)_2D_3$ to five major metabolites (including lactone). One of these metabolites, 23-keto-25-OHD_3, is the subject of this paper. This metabolite was formed from either 23(*S*),25-$(OH)_2D_3$ or 23(*R*),25-$(OH)_2D_3$. The 23-keto-25-OHD_3 had greater affinity than any monohydroxylated vitamin D_3 metabolite to the plasma vitamin D binding protein and 1,25-$(OH)_2D$-specific cytosol receptor. The formation of 23-keto-25-OHD_3, therefore, represents the first reported side-chain modification of 25-OHD_3 that results in enhanced affinity to both the plasma vitamin D binding protein and 1,25-$(OH)_2D$ receptor.

Materials and Methods

High-performance liquid chromatography (HPLC) was performed on a Model LC-204 liquid chromatograph (Waters Associates, Milford, MA).[2] Mass spectra were obtained with the solids probe of a Finnigan Model 4021 automated EI/CI, GC/MS system coupled to an Incos data system. Electron-impact spectra were taken at 70 eV with an ionizer temperature of 250 °C while heating the probe from ambient to 300 °C. Methane was used as the reagent gas for chemical ionization mass spectra. Ultraviolet spectra were obtained in 2-propanol on a Beckman Model 25 recording spectrophotometer. A molar extinction coefficient of 18 200 was used for vitamin D_3 metabolites.

Sterols. Synthetic 23(*R*),25-$(OH)_2D_3$, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ were generously provided by Dr. Milan Uskokovič and Dr. John J. Partridge from Hoffmann-La Roche Inc. (Nutley, NJ). Synthetic 25-OHD_3 was generously provided by Dr. Marvin Ogilvie from Upjohn (Kalamazoo, MI). Lactone was prepared according to the procedure described by Horst (1979), and 24-keto-25-OHD_3 was prepared as previously described (Takasaki et al., 1981). Vitamin D_3 was purchased from Sigma Chemical Co. (St. Louis, MO). 23(*S*),25-$(OH)_2[26,27\text{-}^3H_2]D_3$ (90 Ci/mmol) was prepared from 25-$OH[26,27\text{-}^3H_2]D_3$ (90 Ci/mmol) according to the procedure of Horst et al. (1982a).

In Vitro Synthesis of 23-Keto-25-OHD_3. Thirty, 1-day-old leghorn cockerels (Hy-line Indian River Co., Dallas Center, IA) 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 3.9 μmol of vitamin D_3 in 100 μL of propylene glycol. In addition, each chick received 600 pmol of 1,25-$(OH)_2D_3$ at 24 h and once again 6 h before sacrifice. The animals were sacrificed on day 7, and the kidneys were collected and rinsed in a buffered solution containing 15 mM Tris–acetate, 150 mM sucrose, and 2 mM magnesium acetate, pH 7.4 (buffer A). The kidneys were cleaned of connective tissue, and a 20% broken cell suspension was prepared in buffer A with a polytron Pt-20 tissue disrupter at a setting of 7. Three milliliters of buffer A containing 7.5 mM sodium succinate, 0.4 mM $NADP^+$, 160 mM nicotinamide, and 20 mM ATP was added to the homogenate (6 mL) in 125-mL Erlenmeyer flasks. The reaction was initiated by the addition of 0.12 μmol of either 23(*S*),25-$(OH)_2D_3$, 23(*R*),25-$(OH)_2D_3$, or 25-OHD_3 in 50 μL of ethanol to each flask (three flasks/metabolite). A separate flask was prepared in the same manner containing 25 μCi (0.31 pmol) of 23(*S*),25-$(OH)_2[^3H]D_3$. The mixtures were flushed with a stream of O_2 for 30 s, sealed, and incubated for 1.5 h at 37 °C with shaking. The reactions were quenched with 3.75 volumes of methanol/methylene chloride (2:1) and lipid extracts prepared in the same manner previously described (Horst et al., 1979). A portion of the lipid extract from the incubation flask containing 23(*S*),25-$(OH)_2[^3H]D_3$ was added to the lipid extract of the flasks containing nonradioactive 23(*S*),25-$(OH)_2D_3$ to trace the elution of 23(*S*),25-$(OH)_2D_3$ metabolites. The products of the lipid extracts were dried and passed through a 0.6 × 10 cm column containing a 1:1 mixture of silicic acid (Bio-Rad, Richmond, CA) and Celite 503 (Baker, Phillipsburg, NJ) in 100% ethyl acetate. Twelve milliliters of eluent was collected and dried under N_2, and the residue was dissolved in 300 μL of 1:99 2-propanol/hexane.

The 23(*S*),25-$(OH)_2D_3$, 23(*R*),25-$(OH)_2D_3$, and 25-OHD_3 metabolites were separated by HPLC on a preparative (0.95 × 25 cm) silicic acid column (Zorbax Sil, Du Pont, Wilmington, DE) with a 1-h concave gradient (setting 7 on Water's gradient programmer) of 1–14% 2-propanol in hexane at a flow rate of 5 mL/min. On this system, 25-OHD_3 elutes at 165 mL, 23(*S*),25-$(OH)_2D_3$ at 210 mL, lactone at 235 mL, and 1,25-$(OH)_2D_3$ at 295 mL.

The fractions containing the 23-keto-25-OHD_3 (170–180 mL) were combined and purified further by HPLC on an analytical (0.45 × 25 cm) silicic acid column (Zorbax Sil) developed with 1% 2-propanol in methylene chloride. In this system, 25-OHD_3 elutes at 24 mL, and lactone elutes at 31 mL. The 23-keto-25-OHD_3 region (22–26 mL) was collected for final purification by HPLC on an analytical silicic acid column (Zorbax Sil) developed in 1:2:22 methanol/chloroform/hexane (Napoli et al., 1981). The 23-keto-25-OHD_3 region (14–18 mL) was recycled twice for a total of three passes to verify its homogeneity.

Sodium Borohydride Reduction. 23-Keto-25-OHD_3 (200 ng) in methanol (0.03 mL) was reduced with a 100-fold molar excess of sodium borohydride. After 6 min, the reaction was quenched with dilute HCl. The solvents were dried under a stream of nitrogen. The residue was extracted with chloro-

[2] Mention of a trade name, proprietary product, or vendor does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

Table I: Metabolites Isolated from Chick Kidney Homogenates Incubated with 23,25-$(OH)_2[^3H]D_3$[a]

peak	compound	% of total radioactivity	elution position (mL) from Zorbax Sil prep column (0.95 × 25 cm)[b]
I	23-keto-25-OHD_3	6.8	175
II	23,25-$(OH)_2D_3$	17.2	210
III	lactone	49.3	235
IV	?	8.1	245
V	23,25,26-$(OH)_3D_3$	8.5	300
VI	?	10.1	320

[a] The percent of total radioactivity present as the metabolites and their elution position on the HPLC chromatogram are given. [b] Refer to text for elution position of standards and solvent system.

form, and the products were analyzed by HPLC and mass spectroscopy.

Silylation. 23-Keto-25-OHD_3 (250 ng) and *N*-(trimethylsilyl)diethylamine (0.03 mL) were heated at 90 °C for 2 h. The excess reagent was removed under a stream of nitrogen. The residue was purified by HPLC with a Whatman ODS-3 column (0.42 × 25 cm) developed with 0.1% methylene chloride in methanol.

Radioligand Binding Assays. The comparison of the relative binding affinity of 23-keto-25-OHD_3 to other vitamin D_3 metabolites was accomplished with two sources of vitamin D binding proteins: (1) the rat plasma vitamin D binding protein diluted 1/5000 in 0.05 M potassium phosphate buffer containing 0.01% gelatin (Horst et al., 1981a) and (2) the 1,25-$(OH)_2D$-specific cytosol receptor with the isolation from bovine thymus and assay conditions repeated precisely as described by Reinhardt et al. (1982a,b).

Biological Evaluation. Weanling male rats were housed individually in overhanging wire cages. They were fed vitamin D deficient diets containing low calcium (0.005%) and normal phosphorus (0.3%) for 3 weeks prior to experimental use (Suda et al., 1970). At the end of 3 weeks, the rats were anesthetized with halothane and the test compounds given intrajugularly in 50 μL of a carrier solution containing ethanol/propylene glycol (3:7). Controls received the carrier solution alone. Twenty-four hours following injection of the test compounds, the rats were decapitated, and their duodena were used to measure intestinal calcium transport by the everted intestinal sac procedure (Martin & DeLuca, 1969). In addition, the blood was collected from the animals and centrifuged. The resulting serum was measured for calcium concentration by atomic absorption spectroscopy (Willis, 1960) to determine the degree of bone calcium resorption. Since the rats were on a diet essentially devoid of calcium, serum calcium increases reflect mobilization of calcium from bone and not intestinal absorption.

Results

In Vitro Production of 23-Keto-25-OHD_3 from 25-OHD_3, 23(R),25-$(OH)_2D_3$, and 23(S),25-$(OH)_2D_3$. The radioactive profile of the HPLC eluent established the presence of six major radioactive peaks (tabbed peaks I–VI) in the chick kidney incubations containing 23(*S*),25-$(OH)_2[^3H]D_3$. The elution positions and percent of total radioactivity present as the individual peaks are given in Table I. Peak II corresponds to the elution of the parent compound 23(*S*),25-$(OH)_2[^3H]D_3$. The major radioactive peak was peak III, which coelutes with lactone. Peak V has been recently identified as 23,25,26-$(OH)_3D_3$ (Napoli & Horst, 1982a).

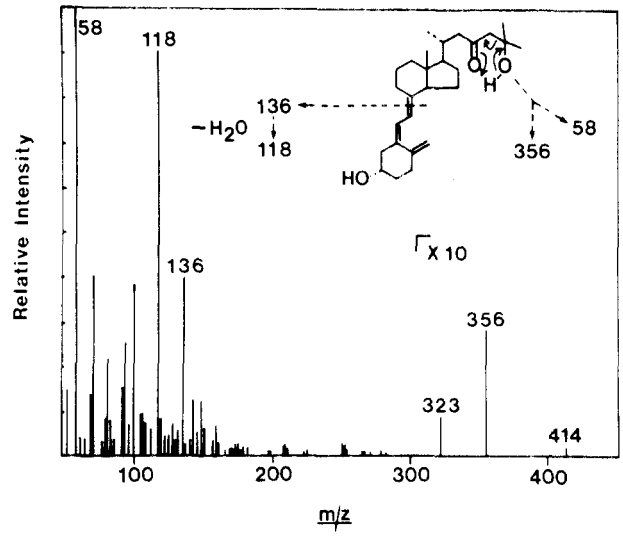

FIGURE 1: Electron-impact mass spectrum of 23-keto-25-OHD_3 produced by chick kidney homogenates from 23(*S*),25-$(OH)_2D_3$. The mass spectrum of 23-keto-25-OHD_3 produced from 23(*R*),25-$(OH)_2D_3$ was comparable.

The total amount of putative 23-keto-25-OHD_3 (peak I) isolated from kidney incubation containing the different precursors varied considerably. 23(*R*),25-Dihydroxyvitamin D_3, a compound not known to occur naturally, was the best in vitro substrate for 23-keto-25-OHD_3 synthesis. Approximately 2 μg of product was isolated from every 6 mL of 20% homogenate possessing 50 μg of the 23(*R*),25-$(OH)_2D_3$ substrate. Incubations with 23(*S*),25-$(OH)_2D_3$, the naturally occurring form, were about one-tenth as efficient with approximately 0.2 μg of 23-keto-25-OHD_3 being isolated for every 50 μg of substrate. The incubations containing 25-OHD_3 were least efficient and resulted in the isolation of approximately 0.005 μg of 23-keto-25-OHD_3 for every 50 μg of 25-OHD_3.

The UV absorbance spectrum of the new metabolite made from 23(*R*),25-$(OH)_2D_3$ or 23(*S*),25-$(OH)_2D_3$ had a λ_{max} at 264 nm and a λ_{min} at 228 nm. The ratio $\lambda_{max}/\lambda_{min}$ was 1.9. This indicates that the metabolite has a vitamin D like *cis*-triene chromophore, and that it was free of UV contaminants. The latter conclusion was also supported by inspection of the HPLC profiles generated during its purification (data not shown).

The structure of the metabolite as 23-keto-25-OHD_3 (Figure 1) was established by mass spectroscopy. An electron-impact mass spectrum showed a small molecular ion at *m/z* 414, which is consistent with dehydrogenation of the parent compound to form a ketovitamin D_3 derivative (Figure 1). The location of the ketone is firmly established by the mass spectrum. The base peak at *m/z* 58 and the rather significant peak at *m/z* 356 result from a McLafferty rearrangement between a 23-ketone and the proton on the 25-hydroxyl group giving rise to acetone (*m/z* 58) and 25,26,27-trinor-23-keto-vitamin D_3 (*m/z* 356). Given the starting material as 23,25-$(OH)_2D_3$, there is no feasible alternate explanation for these peaks. The peak at *m/z* 323 results from loss of a methyl group and a water molecule from *m/z* 356. The two peaks at *m/z* 136 and 118 result from formal cleavage between C-7 and C-8 and dehydration of the initially formed fragment, respectively. This process is peculiar to the vitamin D-triene system. These mass spectral data, considered with the UV absorbance spectrum, strongly support a structural assignment of 23-keto-25-OHD_3.

Further evidence for the assignment was provided by a chemical ionization mass spectrum (Figure 2). The quasi-

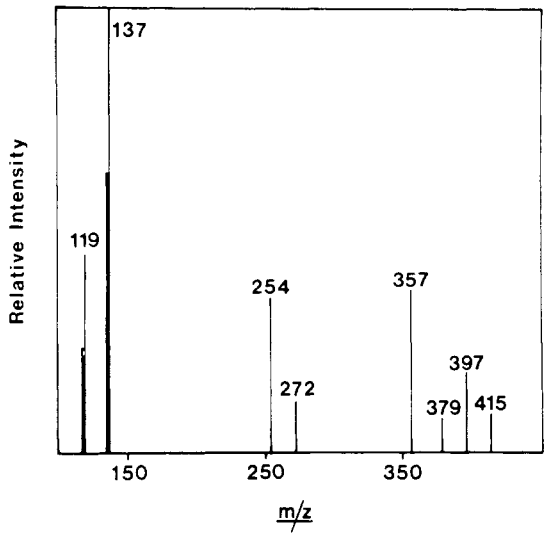

FIGURE 2: Chemical-ionization mass spectrum of 23-keto-25-OHD$_3$.

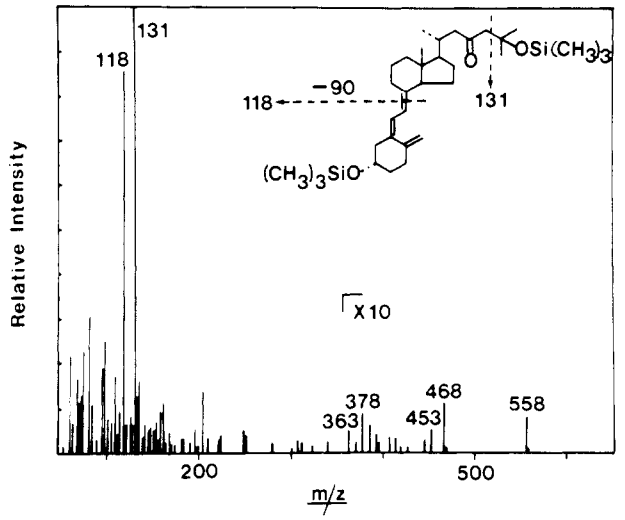

FIGURE 3: Electron-impact mass spectrum of disilylated 23-keto-25-OHD$_3$.

molecular ion at m/z 415 (MH^+) and the peaks at 397 ($MH^+ - H_2O$) and 379 ($MH^+ - 2H_2O$) confirm a molecular weight of 414 and the presence of two hydroxyl groups. The peaks at m/z 272 (MH^+ – side chain) and 254 (272 – H_2O) result from side-chain cleavage and dehydration of the remaining fragment, and provide independent confirmation that the group dehydrogenated was the 23-hydroxyl, not the 3-hydroxyl, function. The peak at m/z 357 arises from the McLafferty rearrangement discussed above. The base peak at m/z 137 is the protonated form of m/z 136 observed in the electron-impact mass spectrum. Likewise, m/z 119 is the protonated form of m/z 118.

An electron-impact mass spectrum of the silylated metabolite confirmed the presence of two hydroxyl groups (Figure 3). The molecular ion at m/z 558 is that expected of a disilylated 23-keto-25-OHD$_3$. Loss of $(CH_3)_3SiOH$ from m/z 558 produced m/z 468. Loss of a methyl group and $(CH_3)_3SiOH$, loss of two molecules of $(CH_3)_3SiOH$, and loss of two molecules of $(CH_3)_3SiOH$ plus a methyl group produced the peaks at m/z 453, 378, and 363, respectively. The base peak at m/z 131 represents cleavage between C-24 and C-25, demonstrating the presence of a silylated 25-hydroxyl group. This peak, along with the lack of a peak resulting from a McLafferty rearrangement in the silylated metabolite, is

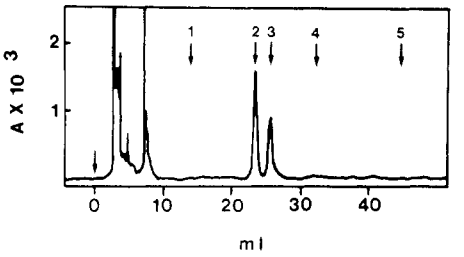

FIGURE 4: HPLC of 23-keto-25-OHD$_3$ after sodium borohydride reduction. A microparticulate silica gel column (0.42 × 25 cm) was eluted with hexane/methanol/chloroform (22:1:2) at a flow rate of 2 mL/min. Arrows point to the elution positions of synthetic standards: (1) 25-OHD$_3$ (14 mL); (2) 23(R),25-$(OH)_2D_3$ (24 mL); (3) 23(S),25-$(OH)_2D_3$ (26 mL); (4) 24(R),25-$(OH)_2D_3$ (33 mL); (5) 25(S),26-$(OH)_2D_3$ (46 mL). The two peaks that elute in the positions of 23(R),25-$(OH)_2D_3$ and 23(S),25-$(OH)_2D_3$ were collected for mass spectral analysis.

Table II: Ability of Vitamin D$_3$ Metabolites To Displace 25-OH[^{3}H]D$_3$ from the 4.2S Rat Plasma Vitamin D Binding Protein

metabolite	amount that produces 50% displacement of 25-OH[^{3}H]D$_3$ (ng)	competitive index relative to 25-OHD$_3$[a]
25(R)-OHD$_3$-26,23(S)-lactone	0.089	674
23-keto-25-OHD$_3$	0.22	272
25-OHD$_3$	0.60	100
24(R),25-$(OH)_2D_3$	0.60	100
24-keto-25-OHD$_3$	1.04	57.7
23(R),25-$(OH)_2D_3$	3.70	16.2
23(S),25-$(OH)_2D_3$	3.70	16.2
1,25-$(OH)_2D_3$	30.0	2.0

[a] The amount of 25-OHD$_3$ that will displace 50% of the 25-OH[^{3}H]D$_3$ divided by the amount of metabolite that will displace 50% of the 25-OH[^{3}H]D$_3$ times 100.

further evidence for the presence and participation of a 25-hydroxyl group in a McLafferty rearrangement. Finally as expected in a vitamin D metabolite, there was a major peak at m/z 118.

23-Keto-25-OHD$_3$ would produce upon sodium borohydride treatment a mixture of 23(R)- and 23(S),25-$(OH)_2D_3$. The new metabolite did produce, after reduction with sodium borohydride, two compounds that comigrated with synthetic 23(R)- and 23(S),25-$(OH)_2D_3$ in HPLC (Figure 4). An electron-impact mass spectrum of these peaks had the expected ions at m/z 416 (M^+, 5%), 398 ($M^+ - H_2O$, 2%), 380 ($M^+ - 2H_2O$, 4%), 136 (78%), and 118 (100%).

Relative Affinity for the Plasma Vitamin D Binding Protein and 1,25-$(OH)_2$D Cytosol Receptor. Table II presents the behavior of 23-keto-25-OHD$_3$ and several other vitamin D$_3$ metabolites in the diluted rat plasma radioassay used for the quantitation of vitamin D and vitamin D metabolites (Horst et al., 1981a). The 23-keto-25-OHD$_3$ was tested against other metabolites for its ability to displace 25-OH[^{3}H]D$_3$ from the 4.2S rat plasma vitamin D binding protein. About 2.7 times less 23-keto-25-OHD$_3$ than 25-OHD$_3$ was required to displace 50% of the 25-OH[^{3}H]D$_3$. Lactone was the only metabolite that bound with greater affinity. The reduced forms of 23-keto-25-OHD$_3$, namely, 23(R),25-$(OH)_2D_3$ or 23(S),25-$(OH)_2D_3$, were significantly less effective; a 6.2-fold excess of each relative to 25-OHD$_3$ was required to displace 50% of the 25-OH[^{3}H]D$_3$. Oxidation of 24,25-$(OH)_2D_3$ to 24-keto-25-OHD$_3$ did not provide any improvement in binding but rather resulted in a slight impairment. The compound

Table III: Ability of Vitamin D_3 Metabolites To Displace 1,25-$(OH)_2[^3H]D_3$ from the 3.7S 1,25-$(OH)_2D_3$ Cytosol Receptor

metabolite	amount that produces 50% displacement of 1,25-$(OH)_2$-$[^3H]D_3$ (ng)	competitive index relative to 1,25-$(OH)_2D_3$[a]
1,25-$(OH)_2D_3$	0.044	100.0
23-keto-25-OHD$_3$	1.74	2.5
25-OHD_3	7.59	0.58
24-keto-25-OHD_3	8.0	0.55
23(*R*),25-$(OH)_2D_3$	29.3	0.15
24(*R*),25-$(OH)_2D_3$	40.0	0.11
23(*S*),25-$(OH)_2D_3$	88.0	0.05
25(*R*)-OHD_3-26,23(*S*)-lactone	366	0.012
vitamin D_3	4681	0.00094

[a] The amount of 1,25-$(OH)_2D_3$ that will displace 50% of the 1,25-$(OH)_2[^3H]D_3$ divided by the amount of metabolite that will displace 50% of the 1,25-$(OH)_2[^3H]D_3$ times 100.

1,25-$(OH)_2D_3$ was the least potent metabolite in this system, requiring a 50-fold excess relative to 25-OHD_3 to displace 50% of the 25-$OH[^3H]D_3$.

Table III reports the potency of the 23-keto-25-OHD_3 in the 1,25-$(OH)_2D_3$ cytosol receptor assay. In this assay, metabolites were tested for their ability to displace 1,25-$(OH)_2[^3H]D_3$ from the 3.7S cytosol receptor protein. 23-Keto-25-OHD_3 was the most potent monohydroxylated vitamin D_3 metabolite. Compared to 1,25-$(OH)_2D_3$, it required a 43.3-fold excess to displace 50% of the 1,25-$(OH)_2[^3H]D_3$. The monohydroxylated metabolites 25-OHD_3 and 24-keto-25-OHD_3 were 4-fold less potent than 23-keto-25-OHD_3, requiring approximately a 170-fold excess relative to 1,25-$(OH)_2D_3$ to accomplish 50% displacement. The compounds 23(*S*),25-$(OH)_2D_3$ and 24(*R*),25-$(OH)_2D_3$, the reduced products of 23-keto-25-OHD_3 and 24-keto-25-OHD_3, were significantly less competitive than their oxidized products; a 670-fold excess and a 900-fold excess, respectively, were required for 50% displacement. In this assay, lactone and vitamin D_3 were the poorest competitors.

Biological Evaluation. The intestinal calcium transport responses of rats that received a single dose of test compound 24 h prior to experiment are recorded in Table IV. According to the data, about 500 ng of 23-keto-25-OHD_3 induced transport equivalent to the 25 ng of vitamin D_3. Therefore, about 20 times more 23-keto-25-OHD_3 than vitamin D_3 is necessary to produce the same calcium transport response. The 23-keto-25-OHD_3, however, was unable to mobilize bone calcium (increase serum calcium concentration) at the time and doses tested.

Discussion

The recent chemical synthesis of 23(*S*),25-$(OH)_2D_3$ and 23(*R*),25-$(OH)_2D_3$ (Partridge et al., 1982) has allowed us to demonstrate the in vitro metabolism of these two compounds to a new metabolite unequivocally identified as 23-keto-25-OHD_3. The assignment as 23-keto-25-OHD_3 was determined by spectral data and chemical reactivity. The new metabolite was shown to have a molecular weight of 414 by electron-impact mass spectroscopy, which is consistent with dehydrogenation of the parent compound 23,25-$(OH)_2D_3$ to form a ketovitamin D_3 derivative. The mass spectrum peaks at m/z 356, resulting from a McLafferty rearrangement between the 23-ketone and the proton on the 25-hydroxyl group to form 25,26,27-trinor-23-ketovitamin D_3, and the peak at m/z 58 to form acetone firmly establish the location of the ketone at C-23. The formation of 23(*S*),25-$(OH)_2D_3$ and 23(*R*),25-$(OH)_2D_3$ upon sodium borohydride treatment also adds credence to the structural assignment.

Table IV: Bone Calcium Mobilization and Intestinal-Calcium Transport in Rats Administered 23-Keto-25-OHD_3 and Vitamin D_3[a]

dose	serum calcium (mg/100 mL)	^{45}Ca serosal/^{45}Ca mucosa
control	4.4 ± 0.1	1.5 ± 0.3
25 ng of vitamin D_3	5.0 ± 0.3[b]	2.4 ± 0.3[b]
50 ng of vitamin D_3	5.6 ± 0.4[b]	3.3 ± 0.6[b]
100 ng of 23-keto-25-OHD_3	4.4 ± 0.2	1.5 ± 0.3
500 ng of 23-keto-25-OHD_3	4.4 ± 0.5	2.3 ± 0.3[b]

[a] Animals were maintained on a low calcium diet for 3 weeks. Under halothane anesthesia, the animals were dosed intrajugularly with the test compound in 50 μL of ethanol/propylene glycol (3:7). Twenty-four hours later, animals were decapitated for serum calcium and intestinal-calcium transport measurements. Data are expressed as mean ± SD of five rats per group. [b] Significantly different from control: $P < 0.05$.

The 23-keto-25-OHD_3 isolated from these in vitro incubations was shown to have higher affinity for the 4.2S vitamin D plasma binding protein and 3.7S 1,25-$(OH)_2D_3$ cytosol receptor than 25-OHD_3. This is the first example of side-chain metabolism of 25-OHD_3 that results in improved binding to both binding proteins. Side-chain metabolism of 25-OHD_3 generally results in unaltered (or enhanced, in the case of lactone) affinity for the 4.2S plasma protein and reduced binding to the 3.7S receptor protein (Tables II and III). Only the metabolism to 23,25-$(OH)_2D_3$ results in reduced affinity to both proteins. The simultaneous enhancement of affinity to both binding proteins suggests a binding mechanism for the side-chain common to both proteins that is sensitive to a carbonyl group at C-23 and unaffected by a carbonyl group at C-24. This structure modification will add insight to assessing the structural requirement for maximum binding to the 1,25-$(OH)_2D_3$ receptor and ultimate expression of biologic activity.

There is a high degree of correlation between the relative binding of analogues to the 1,25-$(OH)_2D_3$ receptor and their relative ability to stimulate intestinal calcium absorption (Eisman & DeLuca, 1977; Norman, 1979). The consistent nature of this relationship leads one to suspect that 1α-hydroxylation of 23-keto-25-OHD_3 could result in a metabolite with high affinity for the 1,25 receptor. The presence of this metabolite, i.e., 23-keto-1,25-$(OH)_2D_3$ or 23-keto-25-OHD_3 itself, could be a significant element of vitamin D_3 toxicity. They could either serve as agonists and promote toxicity or as antagonists and diminish toxicity. When tested by the classic in vivo bioassays, 23-keto-25-OHD_3 appeared to be about 20-fold less active than vitamin D_3 (Table IV). (The lack of necessary amounts of metabolite precluded adequate testing for antagonistic activity.) Therefore, when introduced systemically to vitamin D deficient rats, 23-keto-25-OHD_3 does not appear to have superior (relative to vitamin D_3) agonistic activity.

Although the in vivo presence of 23-keto-25-OHD_3 has yet to be demonstrated, there is recent evidence that vitamin D_3 metabolites, specifically 1,25-$(OH)_2D_3$, can be oxidized at C-23 by the intestine (Napoli & Horst, 1982b; Horst et al., 1982b). The appropriate enzymes are, therefore, present for the local production (in the intestine) of C-23 oxidized vitamin D_3 metabolites in a tissue that can respond to its presence and ultimately influence calcium metabolism. In the vitamin D repleted state, and more probably the vitamin D_3 toxic state,

23-keto-25-OHD_3 could be produced and initiate a biologic response locally without entering systemic circulation. Testing of this hypothesis will require in vitro evaluation with organ cultures that are responsive to calcinogenic activity.

This new metabolite was shown to be an in vitro product of 23(*R*),25-$(OH)_2D_3$, the nonnaturally occurring isomer and one of five major in vitro products of 23(*S*),25-$(OH)_2D_3$. Approximately 10-fold more 23-keto-25-OHD_3 was isolated from incubations containing 23(*R*),25-$(OH)_2D_3$ compared to incubations containing 23(*S*),25-$(OH)_2D_3$. The 23-hydroxy dehydrogenase responsible for 23-keto-25-OHD_3 formation is, therefore, not stereospecific. Presumably, part of the difference in the amount formed from the two diasteriomers could result from the ability of 23(*S*),25-$(OH)_2D_3$ to be converted to many compounds, with its major product being lactone. 23(*R*),25-$(OH)_2D_3$ is not a metabolic precursor to lactone (Napoli et al., 1982) and, therefore, may be more available to the 23-hydroxy dehydrogenase enzyme. Further, this firmly establishes that 23-keto-25-OHD_3 is not a metabolic precursor to lactone. However, in experiments where 23-keto-25-OHD_3 was reintroduced into kidney homogenates, it was metabolized to a compound that comigrates with lactone on silicic acid columns in two chemically different HPLC solvent systems (data not shown). More rigorous analysis, however, demonstrated that this metabolite was not lactone.

In summary, the in vitro metabolism of 23(*R*),25-$(OH)_2D_3$ and 23(*S*),25-$(OH)_2D_3$ to 23-keto-25-OHD_3 has been demonstrated. The 4.2S rat plasma vitamin D binding protein, as well as the 3.7S 1,25-$(OH)_2$D-specific cytosol receptor, demonstrated high affinities for this metabolite relative to 25-OHD_3. The biologic activity and further metabolism of this new metabolite are currently under investigation in our laboratory.

Registry No. 23,25-$(OH)_2D_3$, 77733-16-5; 23-keto-25-OHD_3, 83353-84-8; 23(*R*),25-$(OH)_2D_3$, 81738-09-2; 23(*S*),25-$(OH)_2D_3$, 79702-77-5.

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