1 α ,24(S)-Dihydroxyvitamin D₂: a biologically active product of 1 α -hydroxyvitamin D₂ made in the human hepatoma, Hep3B

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A major metabolite of the vitamin D analogue 1 α -hydroxyvitamin D₂ in human liver cells in culture has been identified as 1 α ,24(S)-dihydroxyvitamin D₂ [1 α ,24(S)-(OH)₂D₂]. 1 α -Hydroxyvitamin D₃ incubated with the same cells gives rise to predominantly 25- and 27-hydroxylated products. Our identification of 1 α ,24(S)-dihydroxyvitamin D₂ is based on comparisons of the liver cell metabolite with chemically synthesized 1 α ,24(S)-(OH)₂D₂ and 1 α ,24(R)-(OH)₂D₂ by using HPLC, GC and GC–MS techniques. The stereochemical orient-

ation of the 24-hydroxyl group was inferred after X-ray-crystallographic analysis of the 24(R)-OH epimer. 1 α ,24(S)-Dihydroxyvitamin D₂ binds strongly to the vitamin D receptor and is biologically active in growth hormone and chloramphenicol acetyltransferase reporter gene expression systems *in vitro*, but binds poorly to rat vitamin D-binding globulin, DBP. We suggest that this metabolite, 1 α ,24(S)-(OH)₂D₂, possesses the spectrum of biological properties to be useful as a drug in the treatment of psoriasis, metabolic bone disease and cancer.

INTRODUCTION

Vitamin D compounds are used throughout the world for the treatment of disorders involving disturbances of calcium homeostasis (e.g. those caused by vitamin D deficiency: rickets and osteomalacia) [1]. The compounds used most frequently are derived from vitamin D₃, the form of vitamin D that is synthesized in the skin of land-dwelling vertebrates. Vitamin D compounds, principally 1 α ,25-dihydroxyvitamin D₃ [1 α ,25-(OH)₂D₃] and 1 α -hydroxyvitamin D₃ [1 α -OH-D₃], have also been investigated for the long-term treatment of osteoporosis. Unfortunately, the toxic effects of these compounds, elevated urinary calcium and serum calcium, have contributed to the absence of approval of these compounds for osteoporosis in countries such as the United States [2].

A drug based on the vitamin D₂ form of the vitamin, 1 α -hydroxyvitamin D₂ (1 α -OH-D₂), first synthesized by Lam et al [3] in the 1970s, has been recently under development for the treatment of secondary hyperparathyroidism and metabolic bone disease. 1 α -OH-D₂ has been administered in clinical trials in the United States at levels of up to 10 μ g/day for 1 week, or up to 5 μ g/day for 2 years with minimal toxic side effects [4]. These tolerances to 1 α -OH-D₂ observed in human patients are analogous to the findings in rats of lower toxicity for vitamin D₂ analogues [5]. In a comparison of 1 α -OH-D₃ and 1 α -OH-D₂ in rats, these two compounds showed comparable effects on calcium transport and bone mobilization, but 1 α -OH-D₂ was significantly less toxic than was 1 α -OH-D₃ in terms of LD₅₀ and of nephrocalcinosis. One possible explanation for the lower toxicity may be differences in the metabolism of the two compounds.

During early studies of the metabolism of vitamin D₂ in the pig and rat, it was noted that, in addition to 25-hydroxyvitamin D₂ (25-OH-D₂), 24-hydroxyvitamin D₂ (24-OH-D₂) was a major circulating metabolite, its concentration exceeding that of the dihydroxylated vitamin D metabolites such as 24,25-dihydroxyvitamin D₂ [24,25-(OH)₂D₂] [6]. The site of formation of this metabolite was not immediately identified. Recent studies [7] have confirmed not only that 24-hydroxylation of vitamin D₂ occurs *in vivo*, but also that the product can be further 1 α -hydroxylated *in vivo* by the kidney, to a new metabolite, 1,24-dihydroxyvitamin D₂ [1,24-(OH)₂D₂]. Other work by Engstrom and Koszewski [8] suggested that the site of 24-hydroxylation of vitamin D₂ might be the liver, and they also postulated a mechanism of formation involving free radicals.

In recent studies from our own laboratory, we have established human hepatoma models for the study of the side-chain hydroxylation of vitamin D and have, using these models, also demonstrated the 24-oxidation/hydroxylation of a cyclopropane-ring containing analogue, MC969 [9,10], indicating the capacity of this system for hydroxylation at both C-24 and C-25.

We asked whether a difference in the pattern of side-chain hydroxylation between a vitamin D₂ and a vitamin D₃ analogue could be demonstrated in this system. In this report, we demonstrate that a difference in the metabolism of 1 α -OH-D₂ and 1 α -OH-D₃ does occur and is concentration-dependent. At concentrations up to 100 nM, the predominant metabolite of both compounds is 1 α ,25-(OH)₂D, whereas 24-hydroxylation of the vitamin D₂ analogue predominates at substrate concentrations above 100 nM, while for 1 α -OH-D₃ 25-hydroxylation

Abbreviations used: D₂ or D₃, vitamin D₂ or vitamin D₃; OH or (OH)₂, hydroxy or dihydroxy; CAT, chloramphenicol acetyltransferase; DBP, vitamin D serum binding protein; DPPD, 1,2-dianilinoethane; FCS, fetal-calf serum; hGH, human growth hormone; HIM, hexane/isopropanol/methanol; THF, tetrahydrofuran; TSIM, *N*-trimethylsilylimidazole; VDR, vitamin D receptor.

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continues to predominate. The work also describes the characterization of the stereochemistry of $1\alpha,24\text{-(OH)}_2\text{D}_2$ and demonstration of some of its biological properties *in vitro*.

A preliminary account of parts of this work was presented to the Eighth International Workshop on Vitamin D held in Paris, France, June 1991 [10a].

EXPERIMENTAL

Vitamin D compounds and cell lines used

25-OH-D_3 and $1\alpha,25\text{-(OH)}_2\text{D}_3$ were gifts from Milan Uskokovic, Hoffman-LaRoche. $1\alpha\text{-OH-D}_3$ was a gift from Martin Calverley, Leo Pharmaceuticals. $[6\text{-}^3\text{H}]1\alpha\text{-OH-D}_2$ (6 Ci/mmol) was a gift from Hector DeLuca (University of Wisconsin, Madison, WI, U.S.A.). $1\alpha\text{-OH-D}_2$ and $1\alpha,25\text{-(OH)}_2\text{D}_2$ were synthesized by the method of Paaren et al. [11].

The human hepatoma cell lines, Hep3B and HepG2, and the SV-40-transformed African Green Monkey kidney cell line, COS-1, were obtained from American Tissue Culture Collection. The two vectors used in transfection experiments, which were gifts from Dr. M. Haussler and Dr. K. K. Whitfield (University of Arizona, Tucson, AZ, U.S.A.), include: pSG5-hVDR1/3 plasmid [12], which has the hVDR1/3 DNA inserted into the *EcoRI* site of pSG5 vector, and (CT4)⁺TKGH plasmid, which has four copies of the CT4 synthetic rat osteocalcin VDRE ligated and annealed into pTKGH vector [13], which contains a thymidine promoter linked to the human GH (hGH) gene. Human GH radioimmunoassay kits were purchased from Nichols Diagnostics (San Juan Capistrano, CA, U.S.A.). The rat osteosarcoma (ROS17/2) stably transfected with the osteocalcin gene promoter coupled to a chloramphenicol acetyltransferase (CAT) reporter (ROSCO; [14]) was generously given by Dr. John Eisman, St. Vincent's Hospital, Sydney, Australia. ^{14}C -labelled chloramphenicol was purchased from Amersham Canada, Oakville, Ontario, Canada. Trypsin, penicillin G, gentamycin, fungizone and Earle's minimal essential medium was purchased from Gibco. *N*-Trimethylsilylimidazole (TSIM) was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Organic solvents of HPLC grade were obtained from Caledon (Georgetown, Ontario, Canada). Fetal-calf serum (FCS) and BSA were from ICN. DEAE-dextran was obtained from Pharmacia. Other reagents used for transfection: chloroquine, dimethyl sulphoxamine and the antioxidants 1,2-dianilinoethane (DPPD) and butylated hydroxytoluene (BHT) were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.

Hepatoma cell culture and incubation with vitamin D compounds

Hep3B and HepG2 cells were grown in Earle's medium supplemented with 10% (v/v) FCS and antibiotics (100 $\mu\text{g/ml}$ penicillin G, 5 $\mu\text{g/ml}$ gentamicin and 300 ng/ml fungizone). Cell stocks were cultured in 30 ml of supplemented medium in 150 mm tissue-culture dishes and subcultured when approaching confluence. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. For metabolism studies, cells were subcultured on 100 mm² plates at a density of 4×10^4 cells/cm² and grown to confluence in 10 ml of serum-supplemented medium. Before incubation, plates were washed with 3×2 ml of PBS and refed with 4 ml of medium containing 11% (w/v) BSA and antibiotics.

$1\alpha\text{-OH-D}_2$ and $1\alpha\text{-OH-D}_3$ were added to culture plates in 10 μl ethanol to give a range of final concentrations between 1 μM and 25 μM . Alternatively, $1\alpha\text{-OH-D}_2$ was mixed with $[6\text{-}^3\text{H}]1\alpha\text{-OH-D}_2$ in ethanol and added to culture plates to give a range of final concentrations between 1 nM and 100 nM. The antioxidant

DPPD was also added to cultured cells in 5 μl of ethanol to give a final concentration of 100 μM . Incubations were allowed to proceed for 20–40 h, after which the medium and cells were precipitated with methanol and extracted by the method of Bligh and Dyer [15]. The cells from representative plates were counted with a model ZM Coulter counter after trypsin treatment, pelleting by centrifugation and resuspension in PBS.

HPLC

Both straight-phase and reverse-phase HPLC was carried out with a model 590 pump, a U6K manual injector and a model 990 photodiode array detector (all from Waters Associates, Milford, MA, U.S.A.). This scanning spectrophotometer was used during metabolite purification to identify peaks possessing UV characteristics of the vitamin D *cis*-triene system ($\lambda_{\text{max.}} = 265$ nm, $\lambda_{\text{min.}} = 228$ nm, $\lambda_{\text{max.}}/\lambda_{\text{min.}} = 1.7$). Separations were achieved on Zorbax SIL (6.2 mm $\times$ 80 mm), Zorbax CN (4.6 mm $\times$ 250 mm) or Zorbax ODS (6.2 mm $\times$ 80 mm) columns (all from Dupont Instruments, Wilmington, DE, U.S.A.). Metabolites generated from $1\alpha\text{-OH-D}_2$ were initially chromatographed on Zorbax SIL using hexane/isopropanol/methanol (HIM) (91:7:2, by vol.), at flow rate of 1.0 or 1.5 ml/min. Further purification of metabolites was achieved by Zorbax CN chromatography using the solvent HIM (94:5:1, by vol.) at a flow rate of 1.5 ml/min. Finally, the purified peaks were compared by using one of three column packings under conditions specified in the Figure legends.

Mass spectrometry

Direct probe MS was carried out with a model HP5985 mass spectrometer (Hewlett Packard, Palo Alto, CA, U.S.A.) fitted with a direct insert probe. Samples (500 ng–1 μg) were dried in glass capillary tubes, placed in the probe, and the probe was heated from 20 °C to 200 °C over 20 min. Ionization voltage was 70 eV, and ions emerged 2–5 min after heating commenced.

Alternatively, mass spectra of the pertrimethylsilyl ethers of metabolites were obtained by GC-MS. Each purified metabolite (approx. 1 μg) was dried under N_2 and a derivative was prepared with 50 μl of TSIM, incubated for 1 h at 50 °C. The products were dissolved in hexane and passed through a small prewashed Lipidex column to remove excess TSIM. Samples were then dried down and redissolved in a small amount of hexane and transferred to small conical vials for injection on to the gas chromatograph. GC-MS was carried out on a Hewlett-Packard 5890 gas chromatograph using a HP-1 cross-linked methyl silicone gum (0.2 mm $\times$ 12 m, 0.33 mm film thickness) column with helium carrier gas at a flow rate of 1 ml/min and a Hewlett-Packard 5970 mass-selective detector in electron-impact (EI) mode. The linear temperature programme from 20 to 200 °C over 15 min was used, and ionization voltage was 70 eV. Positive-ion electron-impact spectra were recorded and stored on disk by using a Hewlett-Packard Series 21MX-E computer. Mass spectra were obtained by averaging each peak and subtracting the background from the trailing edge of the peak.

Chemical synthesis of $1\alpha,24\text{(R and S)-(OH)}_2\text{D}_2$

These compounds were synthesized by the scheme shown in Figure 1, starting from ergosterol (1), which was esterified to give ergosteryl acetate (2), converted into the β -ring diene protected Diels-Alder adduct (3) by using 4-phenyl-1,2,4-triazoline-3,5-dione, and ozonized to yield 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-23,24-dinor-6-cholesten-3 β -yl acetate (4). Condensation of this aldehyde with the anion of 3-methylbutan-2-one [lithium diisopropylamine (LDA)/tetrahydrofuran

Figure 1 Scheme for the synthesis of 1,24(*R* and *S*)-(OH)₂D₂ from ergosterol

(THF), at -79°C , then -20°C] followed by dehydration of the aldol product with 1.5 M HCl/THF (reflux, 1.5 h) yielded the C-24 ketone, namely (22*E*)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-cholesta-6,22-dien-24-on-3 β -yl acetate (**5**). Addition of CH₃MgBr to this compound yielded a mixture of the *R* and *S* allylic tertiary alcohols at C-24 (**6**). Deprotection of the protected β -ring diene was effected with LiAlH₄/THF to yield (22*E*)-5,7,22-ergostatriene-3 β ,24-diol (**7**). Irradiation of this compound in diethyl ether:benzene (4:1, v/v) (water-cooled quartz immersion well, by using a Hanovia medium-pressure UV lamp for 2 h) followed by thermal isomerization (ethanol, reflux overnight) yielded 24(*R* and *S*)-OH-D₂ (**8**). Conversion into the

C₁-hydroxylated derivative was accomplished via solvolysis of the derived tosylate (**9**) to yield the 3,5-cyclo-vitamin (**10**), which was hydroxylated by using SeO₂/t-butyl hydroperoxide to give (**11**). Cycloreversion using DMSO/acetic acid gave a mixture of the *cis*- and *trans*-vitamin (**12** and **13**), which were separated by using maleic anhydride and chromatography on silica gel. The *trans* C-24 stereoisomers (**12**) were separated by reverse-phase HPLC (Supelco C-8 column, 25 cm $\times$ 21.2 mm; acetonitrile/water, 3:2, v/v): 1 α ,24(*S*)-(OH)₂-D₂: m.p. = 160–162 $^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{24} = +53.3^{\circ}$ ($c = 0.75$, EtOH); IR (KBr) 3376 cm⁻¹ (OH); UV (EtOH) λ_{max} 265 nm (ϵ 18425); ¹H NMR (C²HCl₃) δ 0.57 (s, 3H, 18-CH₃), 0.88 and 0.9 (d, 6H, $J = 2.7$ Hz, 26-CH₃ and 27-CH₃),

1.05 (d, 3H, $J = 6.6$ Hz, 21-CH₃), 1.219 (s, 3H, 28-CH₃), 4.23 (m, 1H, 3-H), 4.43 (m, 1H, 1-H), 5.0 [m (sharp), 1H, 19(*E*)-H], 5.33 [m (sharp), 1H, 19(*Z*)-H], 5.45 (m, 2H, 22-H and 23-H), 6.0 (d, 1H, $J = 11.4$ Hz, 7-H), 6.4 (d, 1H, $J = 11$ Hz, 6-H); MS (CI) 429 ($M^+ + 1$, 3.8%), 411 ($M^+ + 1 - H_2O$, 100%), MS (EI) (M^+ , 7.8%); HRMS (EI) calc. 428.329, found 428.329: 1 α ,24(*R*)-(OH)₂-D₂: m.p. = 163–165 °C; $[\alpha]_D^{24} = +43.4^\circ$ ($c = 0.18$, EtOH), IR (KBr) 3374 cm⁻¹ (OH); UV (EtOH) λ_{max} 266 nm (ϵ 17785); ¹H NMR (C²HCl₃ δ 0.57 (s, 3H, 18-CH₃), 0.88 and 0.9 (d, 6H, $J = 2.7$ Hz, 26-CH₃ and 27-CH₃), 1.05 (d, 3H, $J = 6.6$ Hz, 21-CH₃), 1.214 (s, 3H, 28-CH₃), 4.23 (m, 1H, 3-H), 4.43 (m, 1H, 1-H), 5.0 [m (sharp), 1H, 19(*E*)-H], 5.33 [m (sharp), 1H, 19(*Z*)-H], 5.45 (m, 2H, 22-H and 23-H), 6.0 (d, 1H, $J = 11.4$ Hz, 7-H), 6.4 (d, 1H, $J = 11.2$ Hz, 6-H); MS (CI) 429 ($M^+ + 1$, 9.6%) 411 ($M^+ + 1 - H_2O$, 100%), MS (EI) 428 (M^+ , 9.5%); HRMS (EI) calc. 428.329, found 428.328.

Vitamin D receptor (VDR) binding assay

The binding affinity of 1 α ,24(*S*)-(OH)₂-D₂ for the calf thymus VDR was compared with that of 1,25-(OH)₂-D₃ by using a 1,25-(OH)₂-D₃ assay kit purchased from INCSTAR (Stillwater, MN, U.S.A.). Various concentrations of test compounds were incubated with receptor protein at 20 °C for 1 h. [³H]1,25-(OH)₂-D₃ was then added and the incubation continued for another 1 h. Charcoal/dextran was used to separate bound and free [³H]1,25-(OH)₂-D₃. After 25 min in an ice bath, the samples were centrifuged at 1800 *g* for 15 min at 4 °C and the supernatants containing the receptor-bound [³H]1,25-(OH)₂-D₃ were counted for radioactivity in a liquid-scintillation counter. The relative affinities of test compounds for the VDR were based on parallel displacement curves.

Vitamin D-binding protein (DBP) binding assay

The binding affinity of 1 α ,24(*S*)-(OH)₂-D₂ for the rat plasma DBP was compared with that of 25-OH-D₃ by using plasma obtained from vitamin D-depleted rats. Various concentrations of test compounds were incubated with 1:10000-diluted rat plasma (in a pH 8.6 Tris/HCl/phosphate buffer) and [³H]25-OH-D₃ (Amersham-Canada) (~20000 d.p.m.; 10 Ci/mmol) for 20 h at 4 °C. Charcoal/dextran was used to separate bound and free [³H]25-OH-D₃. After 1 h in an ice bath, the samples were centrifuged at 1800 *g* for 15 min at 4 °C and the supernatants containing the receptor-bound [³H]25-OH-D₃ were counted for radioactivity in a liquid-scintillation counter. The relative affinities of test compounds for the rat DBP were based on parallel displacement curves, in some cases requiring extrapolation of curves where these were incomplete.

Transient transfection and GH assays

The DEAE-dextran method of Sambrook et al. [16] was used for the co-transfection of the two vectors pSG5-hVDR1/3 and (CT4)⁺TKGH into COS-1 cells, which were about 80% confluent. Each plasmid, containing 5 μ g of total DNA, was added to a 150 mm culture plate of COS-1 cells. After a 30 min exposure to the DNA/DEAE-dextran mixture, cells were then treated with 80 μ M chloroquine for 4.5 h. Cells were subjected to DMSO shock for 2.5 min, then incubated overnight in Dulbecco's modified Eagle's medium containing 10% FCS. The cells were trypsin-treated and subcultured at a 1:54 pass ratio into 12-well plates in duplicate and incubated for 5 h. The cells were treated with vehicle (ethanol) or different concentrations of 1,25-(OH)₂-D₃ or 1 α ,24(*S*)-(OH)₂-D₂. After 48 h, 40 μ l samples of medium were removed from each well and transcriptional activity was

quantified by radioimmunoassay of hGH using the procedure supplied by the kit manufacturers.

Use of ROSCO cells and CAT assay

ROS cells containing the stably transfected osteocalcin promoter coupled to a CAT reporter gene were treated with various concentrations (10 pM–10 nM) of 1,25-(OH)₂-D₃ or 1 α ,24(*S*)-(OH)₂-D₂ in Ham's F12 medium containing 2% charcoal-stripped FCS for 24 h. After incubation, cells were harvested by trypsin treatment, pelleting and resuspension in a small volume of buffer. Cells were subjected to three freeze–thaw cycles to rupture cell membranes and release cytosolic CAT enzyme activity as described by Morrison and Eisman [14]. Subsequently, CAT activity was measured in samples of 12000 rev./min $\times$ 15 min supernatants by addition of [¹⁴C]chloramphenicol (100 μ M) and acetyl-CoA (4 mM) in a Tris/HCl buffer, pH 7.5. Products were extracted with 2 $\times$ 1 ml of ethyl acetate. Production of mono- and di-acetates of chloramphenicol was measured by liquid-scintillation counting of radioactivity of spots separated on TLC. Chloramphenicol 3-acetate was the principal product. Appropriate controls consisting of untreated ROSCO cells and no-cell incubations were also assayed.

RESULTS

Metabolism of 1 α -OH-D₂ in Hep3B and HepG2 cells

Incubation of [6-³H]1 α -OH-D₂ with Hep3B cells at concentrations ranging between 1 and 100 nM generated two radioactive products more polar than the radioactive substrate. These products, labelled Peaks A and B in Figure 2, had retention times of approx. 10 min and 11.5 min respectively. On the basis of co-migration with authentic standard, peak B was identified as 1 α ,25-(OH)₂-D₂; the former peak, A, remained unknown. As the

Figure 2 Representative radiochromatogram of an extract of Hep3B cells incubated with 1 nM [³H]1 α -OH-D₂

Trace (◆) represents the extract of 1 nM [³H]1 α -OH-D₂ incubated with cells; trace (■) represents the extract of 1 nM [³H]1 α -OH-D₂ incubated with medium alone in the absence of cells. Fractions were collected every 0.5 min. The peak in fraction 25, labelled Peak B, corresponded to the elution time of a synthetic 1 α ,25-(OH)₂-D₂ standard (11.5 min); the large peaks in fractions 6–8 and 11–13 correspond to esterified 1 α -OH-D₂ and 1 α -OH-D₂ respectively. [Note that there is a delay of ~2 fractions (1 min) between the UV detector and the fraction collection.] Conditions: Zorbax SIL column, 3 μ m particles (6.2 mm $\times$ 8 cm), HIM (91:7:2, by vol), 1.5 ml/min.

Table 1 Rates of 24- and 25-hydroxylation of 1 α -OH-D₂

'Substrate concn.' refers to use of 1 α -OH-D₂ as substrate. [6-³H]-1 α -OH-D₂ (sp. radioactivity 6 Ci/mmol) was mixed with non-radioactive 1 α -OH-D₂ to give the range of concentrations used. Units of activity based on Hep3B cells grown to confluence in 150 mm $\times$ 20 mm plates. A confluent plate contained approx. 4×10^7 cells; co-cultured plates of cells were trypsin-treated and counted at the end of the experiment to determine numbers accurately. Incubations were carried out in 10 ml of medium. ND, Not determined. Difficult to measure accurately; these two concentration points contained no radioactive substrate, so determination of metabolites was by quantification of UV absorbance on diode-array HPLC. Peaks that co-migrated with synthetic 1,25-(OH)₂D₂ were present at levels ≤ 5 ng.

Substrate concn. (nM)*	25-Hydroxylase activity (pmol/48 h per 10 ⁷ cells)	24-Hydroxylase activity (pmol/48 h per 10 ⁷ cells)
1	0.10	0.0275
10	0.318	0.275
100	3.82	4.5
1000	ND	39.0
10000	ND	468.0

substrate concentration was raised, the proportion of peak A to peak B changed such that, at concentrations ranging between 1 and 10 μ M, peak A was the major discernible product (Table 1). At 10 μ M substrate, by using diode-array UV detection, a peak possessing the vitamin D chromophore was observed at 9.79 min, corresponding to peak A (Figure 3a), and this was clearly resolved from 1 α -OH-D₂ substrate (5.6 min) and 1,25-(OH)₂D₂ standard (11.1 min). Under the same culture conditions the main product formed from 1 α -OH-D₃ (Figure 3b), even at a concentration of 10 μ M substrate, was 1 α ,25-(OH)₂D₃, accompanied by a small, but significant, quantity of 1,26(27)-(OH)₂D₃. Similar results were also obtained from HepG2 cells with 10 μ M 1 α -OH-D₂ and 10 μ M 1 α -OH-D₃ (results not shown). The unknown

peak A was collected, purified by HPLC on Zorbax CN and subjected to MS.

Identification of peak A as 1,24-(OH)₂D₂

The native mass spectrum of peak A obtained by direct-probe ms (results not shown) revealed a molecular ion, M^+ , at m/z 428, suggesting the insertion of an oxygen atom into the precursor (M^+ 412) and possibly representing an additional hydroxyl group. Two important fragments observed at m/z 367 and 349 were due to loss of a fragment of 43 mass units corresponding to C₃H₇, and possibly representing C-25,26,27 without additional hydroxylation and loss of one or two molecule(s) of water. The loss of such a C₃H₇ fragment was also observed in the mass spectra of 24-OH-D₂ and 24-OH-D₃ [6,17]. Other fragments found in the spectrum of peak A at m/z 287, 271, 253, 152 and 134 demonstrate retention of a 1 α -hydroxylated nucleus and are consistent with the extra oxygen atom being present in the side chain.

The trimethylsilyl derivative of peak A (Figure 4) gave a molecular ion at m/z 644, indicating the presence of three hydroxyl groups in the molecule. This confirms that the additional side-chain oxygen atom in peak A is in an hydroxyl group. As expected, the spectrum lacked a m/z -131 fragment produced from a derivative of a C-25 hydroxylated compound showing cleavage across the C-24–C-25 bond. The trimethylsilyl derivative of 1 α ,25-(OH)₂D₂ did show this fragment (results not shown; [18]). The spectrum of the trimethylsilyl derivative of peak A did, however, contain a major fragment at m/z 513 due to the loss of 131 mass units by cleavage through the A ring of the molecule. Such a cleavage has been observed in several 1 α -hydroxylated compounds. Other distinctive features of this mass spectrum were a series of fragments originally derived from loss of 43 mass units alone or in addition to the loss of one, two or three silanol

Figure 3 Products of 1 α -OH-D₂ and 1 α -OH-D₃ when incubated at a concentration of 10 μ M with Hep3B cells

(a) HPLC trace using diode-array detector at 265 nm of an extract of Hep3B cells incubated with 10 μ M 1 α -OH-D₂ for 36 h. Conditions: as in Figure 2. Peak at 5.56 min = 1 α -OH-D₂; Peak A [labelled putative 1 α ,24-(OH)₂D₂ and later identified as such] = 9.79 min. Note that, in the inset trace of authentic standards, 1 α -OH-D₂ has a retention time of 5.6 min and 1 α ,25-(OH)₂D₂ has a retention time of 11.1 min. Note that all labelled peaks possessed the vitamin D chromophore (results not shown). Subsequent analysis by GC-MS revealed that the peak at 11.22 min, which lacked a definitive vitamin D chromophore, does, however, contain a small, but detectable, amount of 1,25-(OH)₂D₂ as shown by M^+ of 644 and a major fragment at m/z = 131. (b) HPLC trace using diode-array detector at 265 nm of an extract of Hep3B cells incubated with 10 μ M 1 α -OH-D₃ for 36 h. Note that the peak at 12.04 min co-migrated with authentic 1 α ,25-(OH)₂D₃. The labelled peaks, including the peak at 13.6 min, possessed the vitamin D chromophore (results not shown) and the latter peak probably represents 1 α ,26(27)-(OH)₂D₃. The peak at 10.84 min is almost certainly a mixture of pre-1,25-(OH)₂D₃ (λ_{max} 260 nm, λ_{min} 235 nm) and 1 α ,24-(OH)₂D₃.

Figure 4 Mass spectrum of pertrimethylsilylated peak A obtained from GC-MS analysis

Abbreviation: TMSi, trimethylsilyl.

groups (90 mass units each). These correspond to fragments of m/z 601 ($M-43$), 511 ($M-43-90$), 421 ($M-43-90-90$) and 331 ($M-43-90-90-90$), which increase in relative intensity as the size of the fragment decreases. As with the native spectrum of peak A, we interpret this set of fragments to be derived from cleavage of the C-24-C-25 bond and loss of C_3H_7 . One other noticeable fragment is at m/z 143, which is presumably due to cleavage of the C-23-C-24 bond and subsequent loss of the C-28 methyl group from the terminal 24-*O*-trimethylsilyl-containing fragment. The spectra are thus consistent with a structure of $1\alpha,24-(OH)_2D_2$.

Synthesis of $1\alpha,24(S)-(OH)_2D_2$ and $1\alpha,24(R)-(OH)_2D_2$

The mass spectra, although indicating a structure of $1\alpha,24-(OH)_2D_2$, left in question the stereochemistry of the hydroxyl and methyl groups on C-24. To resolve this question, we chemically synthesized the two epimers shown in Figure 1 as an epimeric mixture. The two compounds were then separated and isolated by HPLC, and one of the compounds was crystallized and analysed by X-ray crystallography. It possessed the following properties: $C_{28}H_{44}O_3$, formula weight = 428.6, monoclinic space group C2, unit cell dimensions of crystal (a, b, c) $a = 25.017(6)$, $b = 6.878(2)$, $c = 17.264(4)$ Å (1 Å = 0.1 nm), unit cell angle $\beta = 118.85(2)^\circ$, vol. = $2608(1)$ Å³, molecules in unit cell, $Z = 4$, density(calc.) = $1.092 \text{ mg} \cdot \text{mm}^{-3}$, wavelength of X-ray source, $\lambda(\text{CuK}\alpha) = 1.54184$ Å, temperature, $T = 295$ °K.

A clear colourless $0.04 \text{ mm} \times 0.06 \text{ mm} \times 0.75 \text{ mm}$ crystal was used for data collection on an automated Siemens R3m/V diffractometer equipped with an incident beam monochromator. Lattice parameters were determined from 25 centred reflections within $32^\circ \leq 2\theta \leq 52^\circ$. The data-collection range of the Miller indices, hkl , was: $-4 \leq h \leq 27$, $-1 \leq k \leq 7$, $-18 \leq l \leq 16$, with $[(\sin \theta)/\lambda]_{\text{max.}} = 0.55$. Three standards, monitored after every 97 reflections, exhibited random variations, with deviations up to $\pm 2\%$ during the data collection. A set of 2089 reflections was collected in the $\theta/2\theta$ scan mode, with scan width $[\omega(K_{\alpha 1}) - 0.5]$ to $[\omega(K_{\alpha 2}) + 0.5]^\circ$ and ω scan rate (a function of count rate) from 4.0 to $10.2^\circ/\text{min}$. There were 1985 unique reflections, and 1389 were observed with $F_0 > 3\sigma(F_0)$. The structure was solved and refined with the aid of the SHELXTL system of programs [19]. The full-matrix least-squares refinement varied 282 parameters: scale factor, atom co-ordinates and anisotropic thermal parameters

Figure 5 Comparison of peak A with $1\alpha,24(R$ and $S)-(OH)_2D_2$ epimers on HPLC

Conditions: Zorbax ODS column, $3 \mu\text{m}$ particles ($6.2 \text{ mm} \times 8 \text{ cm}$); solvent acetonitrile/water (7:3, v/v), flow rate 1 ml/min; retention times: peak A = 14.3 min; $1\alpha,24(S)-(OH)_2D_2$ = 14.17 min; $1\alpha,24(R)-(OH)_2D_2$ = 15.83 min. Minor peaks in the $1\alpha,24(S)-(OH)_2D_2$ did not possess the vitamin D chromophore.

for the 31 non-H atoms, one common isotropic thermal parameter for all methyl hydrogen atoms, and another common isotropic thermal parameter for the non-methyl hydrogen atoms. H atoms visible in difference Fourier maps were included but not explicitly varied by using a riding model [co-ordinate shifts of C (or O) applied to attached H atoms]. No H-atom peaks were observed on hydroxyl atoms O-1 and O-3 in difference maps, which may indicate that they are randomly disordered or shifting among several alternative locations in the solid state. The X-ray-diffraction pattern established the stereochemistry of the $24(R)$ -OH epimer. Assignment of the $24(S)$ -OH stereochemistry to the other epimer was made by deduction. Co-migration of the biologically produced metabolite with the $24(S)$ -OH epimer established the identity of the biological metabolite.

Comparison of peak A with synthetic $1\alpha,24(R,S)-(OH)_2D_2$

Purified peak A was compared by reverse-phase HPLC with a synthetic mixture of the two $1\alpha,24(R,S)-(OH)_2D_2$ epimers and with authentic $1\alpha,24(R)-(OH)_2D_2$, the structure of which has been determined by X-ray crystallography (Figure 5). On this HPLC system, peak A co-migrated with $1\alpha,24(S)-(OH)_2D_2$ at 14.2 min, whereas the $1\alpha,24(R)-(OH)_2D_2$ epimer ran at 15.7 min. On other straight-phase HPLC systems the biological material co-migrated with the $24(S)$ -OH epimer, but the chromatographic resolution was found to be decreased. When the two synthetic $24(R$ and $S)$ -OH epimers were trimethylsilylated and subjected to GC-MS, we observed relative retention times for the $24(S)$ -OH epimer of 1.0165, for the $24(R)$ -OH epimer 1.0098, and for peak A 1.0163. Thus GC data are consistent with peak A being the $24(S)$ -OH epimer. The mass spectra of the two synthetic isomers were virtually identical with that of peak A, with $M^+ = 644$ and fragments at m/z 629 (M^+-15), 601 (M^+-43), 554 (M^+-90), 513 (M^+-131), 511 ($M^+-43-90$), 421 ($M^+-43-90-90$), 331 ($M^+-43-90-90-90$), 217 (A-ring cleavage) and 143 (side-chain fragment from C-23-C-24 cleavage and loss of methyl group). From HPLC, GC and MS data we conclude that peak A is $1\alpha,24(S)-(OH)_2D_2$.

Figure 6 Binding of 1 α ,24(S)-(OH)₂D₂ and 1 α ,24(R)-(OH)₂D₂ to bovine thymus VDR

Analogues are compared with 1 α ,25-(OH)₂D₃, which is the natural ligand for the VDR. Graphs represent means of triplicate determinations and are representative of several replicate experiments.

Table 2 Affinity of mammalian DBP for 1 α ,24(S)-(OH)₂D₂ compared with other analogues of vitamin D

B₅₀ is the concentration of ligand which causes 50% displacement of [³H]25-OH-D₃ from a curve of B/B₀ versus log dose, with the following concentrations of ligand: 100 pg/tube to 10 ng/tube for 25-OH-D₃; 15 ng/tube to 1 μ g/tube for 1,25-(OH)₂D₃; 100 ng/tube to 10 μ g/tube for 1 α ,24(S)-(OH)₂D₂; values are averages of four independent sets of curves.

Analogue	B ₅₀ of analogue	Affinity relative to 25-OH-D ₃	Affinity relative to 1,25-(OH) ₂ D ₃
25-OH-D ₃	653 pg/tube	1	0.0026
1,25-(OH) ₂ D ₃	250 ng/tube	383	1
1 α ,24(S)-(OH) ₂ D ₂	3.4 μ g/tube	5207	13.6

Biological activity of 1 α ,24(S)-(OH)₂D₂: VDR binding studies

As shown in Figure 6, 1 α ,24(S)-(OH)₂D₂ was found to have a 1.3-fold lower affinity for the bovine thymus VDR than 1 α ,25-(OH)₂D₃. On the other hand, the epimer 1 α ,24(R)-(OH)₂D₂ showed a 30-fold lower affinity for the VDR than the natural hormone.

DBP studies

The binding affinity of 1 α ,24(S)-(OH)₂D₂ for the rat DBP was found to be 1000 times weaker than that for 25-OH-D₃ or approx. 10 times weaker than that of 1 α ,25-(OH)₂D₃ (Table 2). With the same DBP preparation, binding of the hormone 1 α ,25-(OH)₂D₃ is approx. 300 times weaker than of 25-OH-D₃ in this system.

hGH gene-expression studies

Table 3 shows the results of using 25-OH-D₃, 1 α ,25-(OH)₂D₃, 1 α ,24(S)-(OH)₂D₂ or 1 α ,24(R)-(OH)₂D₂ to induce hGH production in a transiently transfected COS-1 cell. Similarly to the VDR studies, the 24(S)-OH epimer was about as effective as 1 α ,25-(OH)₂D₃ in this expression system, with the 24(R)-OH epimer 10-fold less effective. The control vitamin D compound, 25-OH-D₃, required ~1000-fold higher concentrations to give similar gene expression.

Table 3 Vitamin D-inducible GH production in transfected COS-1 cells

Inducer	Concn.	Vitamin D-inducible GH production	
		Total GH production* (ng/ml)	Net vitamin D-inducible GH production (ng/ml)
Ethanol		43.8	0
25-OH-D ₃	0.1 μ M	245	201
	1 μ M	1100	1056
	10 μ M	775	731
1 α ,25-(OH) ₂ D ₃	0.1 nM	73.8	30
	1 nM	925	881
	10 nM	1475	1441
1 α ,24(S)-(OH) ₂ D ₂	0.5 nM	425	381
	5 nM	1350	1306
	50 nM	1182	1138
1 α ,24(R)-(OH) ₂ D ₂	1 nM	80	36
	10 nM	1100	1056
	100 nM	1300	1256

* Average of duplicate determinations.

CAT reporter gene-expression studies

ROSCO cells proved to be extremely sensitive to exogenously supplied 1 α ,25-(OH)₂D₃, showing significant inducible-gene activation with as little as 10 pM and maximal activity with 100 pM (Table 4). 1 α ,24(S)-(OH)₂D₂ showed significant activity at a concentration of 10 pM, with less than maximal induction at 100 pM and maximal activity at 1 nM. Thus it appears that 1 α ,24(S)-(OH)₂D₂ is ~2–5-fold less effective than 1 α ,25-(OH)₂D₃ in this model system.

DISCUSSION

In this paper we have shown that 1 α -OH-D₂ is converted by human hepatoma cells into a biologically active metabolite, 1 α ,24(S)-(OH)₂D₂, as well as the expected 1 α ,25-(OH)₂D₃ identified previously [18]. In the hepatoma cell Hep3B, exposed to concentrations of 1 α -OH-D₂ above 100 nM, 1 α ,24(S)-(OH)₂D₂ is the main product, its concentration exceeding that of 1 α ,25-(OH)₂D₃. In contrast, cells incubated with 1 α -OH-D₃ at the same concentrations produced 1 α ,25-(OH)₂D₃ as the principal product. The results of the assays *in vitro* for VDR binding affinity, for enhancing activity of a heterologous promoter, or for induction of a vitamin D-responsive promoter indicate that the metabolite, 1 α ,24(S)-(OH)₂D₂, has significant activity associated with active vitamin D compounds. In contrast, the binding affinity of 1 α ,24(S)-(OH)₂D₂ for the vitamin D-binding protein indicates that, as with many 'non-calcaemic' vitamin D analogues, the pharmacodynamics of this compound may differ from that of 1 α ,25-(OH)₂D₃.

Although 1,24-(OH)₂D₂ has been isolated and identified previously, it was in a different context, being *in vivo* and as a result of renal 1 α -hydroxylation of 24-OH-D₂ in rats given large doses of vitamin D₂ [7]. In the present study, we used human hepatoma cells, showing that they too possess the enzymes necessary to produce 1 α ,24-(OH)₂D₂. It seems reasonable to postulate that human beings therapeutically dosed with large amount of vitamin D₂ produce 1 α ,24-(OH)₂D₂ as another active metabolite of vitamin D₂. In this study, we also have established unequivocally the stereochemistry of the 1 α ,24-(OH)₂D₂ in the 24-position,

Table 4 CAT activity in 1,25-(OH)₂D₃- and 1,24(S)-(OH)₂D₂-induced ROSCO cells

ROSCO cells were incubated with vitamin D analogues as described in the text; cytosolic CAT activity was harvested and assayed by using 5 µl of extract in the presence of 100 µM chloramphenicol. Results are expressed as percentage of radioactivity recovered in acetylated product spots on TLC compared with that in the substrate remaining. Abbreviation: ND, not detectable. Values are means of duplicate determinations. Total acetylated products = (sum of 1-acetate + 3-acetate + 1,3-diacetate).

Inducer concn. (M)	Percentage of radioactivity recovered as†				Total acetylated products
	Chloramphenicol (substrate)	Chloramphenicol 1-acetate	Chloramphenicol 3-acetate	Chloramphenicol 1,3-diacetate	
10 nM 1,25-(OH) ₂ D ₃	60.8	3.8	35.4	0.11	39.3
1 nM 1,25-(OH) ₂ D ₃	51.8	4.5	43.6	0.11	48.2
0.1 nM 1,25-(OH) ₂ D ₃	46.5	4.8	48.5	0.09	53.4
10 pM 1,25-(OH) ₂ D ₃	84.4	1.5	14.1	0.06	15.7
Non-induced control	91.5	0.8	7.7	0.04	8.5
No-cell control	100	ND	ND	ND	0
10 nM 1,24(S)-(OH) ₂ D ₂	50.6	4.6	44.7	0.13	49.4
1 nM 1,24(S)-(OH) ₂ D ₂	54.2	4.9	41.9	0.08	45.8
0.1 nM 1,24(S)-(OH) ₂ D ₂	74.2	2.5	23.4	0.06	25.9
10 pM 1,24(S)-(OH) ₂ D ₂	89.8	1.1	9.1	0.06	10.2
Non-induced control	94.4	0.6	5.0	0.06	5.6
No-cell control	100	ND	ND	ND	0

with the hydroxyl group in the 24(S) orientation. This involves retention of the configuration of the 24-methyl group of the parent vitamin after hydroxylation. We used X-ray-crystallographic techniques to establish the structure of the 24(R)-OH epimer, and we were then able to show that the peak from Hep3B and G2 cells was exclusively composed of the other (presumed) 24(S)-OH epimer. This is in contrast with the results of Engstrom and Koszewski [8], who observed an epimeric mixture of 24(R)- and 24(S)-OH forms in a liver homogenate broken-cell system and thus ascribed 24-hydroxylation to a free-radical-mediated process. In the studies described here we used whole cells, added DPPD to media and observed no product formation in no-cell or heat-denatured-cell controls. Furthermore, product formation is blocked by ketoconazole treatment, suggesting involvement of a cytochrome *P*-450. We therefore believe that the 24(S)-hydroxylation observed here is not the result of a free-radical-mediated process, but is instead the result of a stereospecific reaction mediated by a cytochrome *P*-450 isoform, CYP-27, and have recently published data to support this hypothesis [20].

In studies of the biological activity of the new metabolites reported here, we have shown that 1α,24(S)-(OH)₂D₂ is 1.3–5-fold less active than 1α,25-(OH)₂D₃ *in vitro*. We used VDR binding, vitamin D-inducible GH expression and vitamin D-inducible CAT expression, but all gave approximately the same results. The other epimer, 1α,24(R)-(OH)₂D₂, was 10–30-fold less active than 1α,25-(OH)₂D₃. We also showed that the DBP-binding affinity of 1α,24(S)-(OH)₂D₂ is even poorer than 1α,25-(OH)₂D₃ [and presumably 1α,25-(OH)₂D₂], suggesting that it might be more readily cleared from the bloodstream if administered *in vivo*. However, preliminary pharmacokinetic findings in rats suggest that, although the level of 1α,24-(OH)₂D₂ circulating after administration of 1α,24-(OH)₂D₂ is less than the plasma level of 1α,25-(OH)₂D₂ or 1α,25-(OH)₂D₃ observed after a similar dose of 1α,25-(OH)₂D₂ or 1α,25-(OH)₂D₃ respectively, the half-life in plasma of all three compounds is similar [21]. The route of metabolism of 1α,24(S)-(OH)₂D₂ remains unknown, although the C-22–C23 double bond and the C-28 methyl group of the vitamin D₂ side chain would seem to restrict side-chain oxidation at C-23 and C-24, as is seen for 1α,25-(OH)₂D₃ [22].

The results presented in this paper offer a possible explanation for the previous report on the equivalent activities, yet differing toxicities, of 1α-OH-D₂ and 1α-OH-D₃ [5]. Our results suggest that, as the substrate concentration of 1α-OH-D₂ in the liver of an animal increases, the route of metabolism of this drug is altered from 25-hydroxylation to 24-hydroxylation. The resulting product, 1α,24(S)-(OH)₂D₂, has similar biological potency to 1α,25-(OH)₂D₃ but may have lower toxicity by virtue of altered pharmacodynamics (maximal blood level, distribution).

The above-mentioned characteristics of 1α,24(S)-(OH)₂D₂ also suggest that this compound may be especially useful as a drug for treatment of conditions such as psoriasis, metabolic bone disease and cancer, although it remains to be established if its unique spectrum of properties offer advantages in any of these conditions *in vivo*.

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REFERENCES

- Jones, G. and Calverley, M. J. (1993) Trends Endocrinol. Metab. **4**, 297–303
- Gallagher, J. C. and Goldgar, D. (1990) Ann. Intern. Med. **113**, 649–655
- Lam, H. Y., Schnoes, H. K. and DeLuca, H. F. (1974) Science **186**, 1038–1040
- Gallagher, J. C., Bishop, C. W., Knutson, J. C., Mazess, R. B. and DeLuca, H. F. (1994) J. Bone Miner. Res. **9**, 607–614
- Sjoden, G., Smith, C., Lindgren, V. and DeLuca, H. F. (1985) Proc. Soc. Exp. Biol. Med. **178**, 432–436
- Jones, G., Schnoes, H. K., LeVan, L. and DeLuca, H. F. (1980) Arch. Biochem. Biophys. **202**, 450–457
- Horst, R. L., Koszewski, N. J. and Reinhardt, T. A. (1990) Biochemistry **29**, 578–582
- Engstrom, G. W. and Koszewski, N. J. (1989) Arch. Biochem. Biophys. **270**, 432–440
- Tam, T., Strugnell, S., Deeley, R. and Jones, G. (1988) J. Lipid Res. **29**, 1637–1642
- Strugnell, S., Bishop, C. W., Knutson, J. C. and Jones, G. (1991) in Vitamin D: Gene Regulation, Structure–Function Analysis and Clinical Application (Norman, A. W., Bouillon, R. and Thomasset, M., eds.), pp. 267–268, De Gruyter, Berlin and New York
- Strugnell, S., Calverley, M. J. and Jones, G. (1990) Biochem. Pharmacol. **40**, 333–341

-
- 11 Paaren, H., DeLuca, H. F. and Schnoes, H. K. (1980) *J. Org. Chem.* **45**, 3253–3258
- 12 Hsieh, J. C., Jurutka, P., Galligan, M. A., Terpening, C. M., Haussler, C. A., Samuels, D. S., Shimizu, Y., Shimizu, N. and Haussler, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9315–9319
- 13 Terpening, C. M., Haussler, C. A., Jurutka, P. W., Galligan, M. A., Komm, B. S. and Haussler, M. R. (1991) *Mol. Endocrinol.* **5**, 373–385
- 14 Morrison, N. A. and Eisman, J. A. (1991) *J. Bone Miner. Res.* **6**, 893–899
- 15 Bligh, E. G. and Dyer, W. G. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- 16 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., pp. 16.42–16.44, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 17 Wichmann, J. K., Schnoes, H. K. and DeLuca, H. F. (1981) *Biochemistry* **20**, 2350–2353
- 18 Jones, G., Schnoes, H. K. and DeLuca, H. F. (1975) *Biochemistry* **14**, 1250–1256
- 19 Sheldrick, G. M. (1989). SHELXTL-Plus, Version 4.2 (computer software and manuals), Siemens Analytical X-Ray Instruments, Inc., Madison, WI
- 20 Guo, Y. D., Strugnell, S., Back, D. W. and Jones, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8668–8672
- 21 LeVan, L. W., Knutson, J. C. and Bishop, C. W. (1993) *J. Bone Miner. Res.* **8**, S224, abstr. 432
- 22 Makin, G., Lohnes, D., Byford, V., Ray, R. and Jones, G. (1989) *Biochem. J.* **262**, 173–180
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