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METABOLISM OF A CYCLOPROPANE-RING-CONTAINING ANALOG OF 1α-HYDROXYVITAMIN D_3 IN A HEPATOCYTE CELL MODEL

IDENTIFICATION OF 24-OXIDIZED METABOLITES

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Abstract—MC 969 is an analog of the calcemic drug 1α-hydroxyvitamin D_3 (1α-OH-D_3) in which carbons 25, 26, and 27 in the side chain are incorporated into a cyclopropane ring. Metabolites of MC 969 were generated in an *in vitro* human hepatocyte cell model, Hep 3B. The identity of the metabolites was established by comigration on HPLC with authentic standards, and by mass spectrometry of native and chemically modified metabolites. Unequivocal identification of the 24-keto- and the two epimeric 24-alcohol metabolites is provided. No 25-hydroxylated metabolites were detected. In competition studies, MC 969 was able to inhibit 25-hydroxylation of tritiated vitamin D_3 more effectively than 1α-OH-D_3 itself, indicating that the vitamin D_3-25-hydroxylase may be responsible for generation of one or more of the metabolites observed.

Analogs of hydroxylated forms of vitamin D in which the carbon atoms at the 25, 26 and 27 positions are incorporated into a cyclopropane ring (MC 969, MC 1015; Fig. 1) have been synthesized recently [1] in an attempt to modify the receptor-mediated responses and the processes of metabolism and catabolism of the parent forms. Both cyclopropane ring-analogs were found to possess potent calcemic effects *in vivo* in rats.§ It is reasonable to propose that metabolism of MC 969 will be similar to that of 1α-OH-D_3‖ and will start with a 25-hydroxylation step in the liver [2] to give MC 1015, an analog of 1,25-$(OH)_2D_3$ as the active form. Further metabolism might be expected to introduce a second hydroxyl group at the 24 or 26 position by analogy with 1,25-$(OH)_2D_3$ [3]. The liver vitamin D_3-25-hydroxylase has been shown to possess quite broad substrate specificity and will metabolize molecules with differing steroid nuclei. Mitochondrial and microsomal versions of the enzyme [4, 5] have been shown collectively to catalyse the 25-hydroxylation of 1α-OH-D_3, the 26-hydroxylation of some bile acid precursors [6, 7], and possibly the 16α-hydroxylation of testosterone [8], as well as some other hydroxylation reactions with lower efficiency. The microsomal vitamin D_3-25-hydroxylase has been purified sufficiently to obtain some N-terminal sequence [9] and it is concluded that it may be closely related to or identical with cytochrome P-450h [10].¶ Anticipating 25-hydroxylation of MC 969 to MC 1015, we examined the metabolism of MC 969 in a new *in vitro* hepatocyte cell model, Hep 3B, which we recently used to study the 25-hydroxylation of vitamin D_3 [12]. Much to our surprise, we found instead three metabolites representing C-24-oxidation products of MC 969 in the Hep 3B cell system. We also describe preliminary competition studies of the enzyme vitamin D_3-25-hydroxylase using the substrate [^{3}H]vitamin D_3, designed to further probe the possibility that this enzyme may be involved in the observed metabolism.

MATERIALS AND METHODS

Materials. Hep 3B cells were obtained from the American Type Culture Collection. Trypsin, penicillin G, gentamycin, fungizone, and Earle's Minimum Essential Medium (MEM) were obtained from Gibco. Fetal calf serum was from Flow Laboratories. Radiolabelled [1α,2α(n)-^{3}H]vitamin D_3 was from Amersham. The analogs, MC 969 and MC 1015, were synthesized as outlined [1]. Their systematic names are: 1(*S*),3(*R*)-dihydroxy-20(*R*)-(3′-cyclopropylpropyl) - 9,10 - secopregna - 5(*Z*),7(*E*),10(19)-triene(MC 969); and 1(*S*),2(*R*)-dihydroxy-20(*R*)-[3′-(1″- hydroxy- cyclopropyl)propyl]- 9,10- secopregna-5(*Z*),7(*E*),10(19)-triene (MC 1015). MC 1080 [1(*S*),3(*R*)- dihydroxy-20(*R*)-(3′-cyclopropyl-3′-oxopropyl) - 9,10 - secopregna - 5(*Z*),7(*E*),10(19)triene]

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§ L. Binderup, Biology Dept., Leo Pharmaceutical Products, unpublished results (cited with permission). As commented in Ref. 1, MC 1015 is equipotent to 1,25-$(OH)_2D_3$ and MC 969 is about ten times less active than 1α-OH-D_3 in causing hypercalcemia in normal rats.

‖ Abbreviations: 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; HIM, hexane/isopropyl alcohol/methanol; BHT, butylated hydroxytoluene; DPPD, *N*,*N*′-diphenylethylenediamine; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 1,24*R*-$(OH)_2D_3$, 1,24*R*-dihydroxyvitamin D_3.

¶ For nomenclature used, consult Nebert *et al.* [11] and Ref. 9.

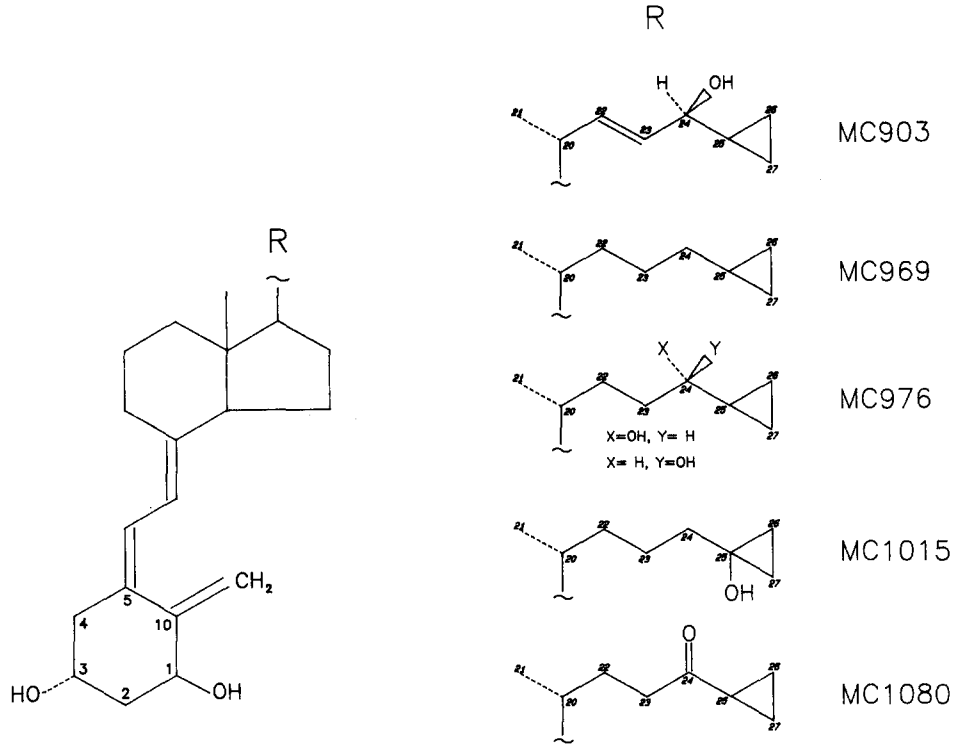

Fig. 1. Structures of cyclopropane-ring-containing analogs of vitamin D. The 1α-OH-D_3 nucleus common to all analogs in this study is shown on the left. The differing side chains and the names assigned to each analog are shown on the right.

was synthesized as described [13]. Solvents were of HPLC grade and were obtained from Caledon (Georgetown, Ontario).

Cell culture. Hep 3B cells were seeded at an initial density of 4×10^4 cells/cm^2 in 25- or 75-cm^2 flasks and cultured in 10 or 20 mL of Earle's MEM containing 10% fetal calf serum and antibiotics (100 μg/mL penicillin G, 5 μg/mL gentamycin, and 300 ng/mL fungizone). Cultures were maintained at 37° in a humidified atmosphere of 5% carbon dioxide in air. Cells were subcultured at late log phase.

Competition studies with MC 969. Cells used in concentration–response assays were removed from 75-cm^2 flasks by treatment with trypsin/EDTA solution (5 min, 10 mL of 0.01% trypsin/5 mM EDTA). Cells were pooled and pelleted by centrifugation (5 min, 500 *g*). The cell pellet was resuspended in medium and used to inoculate 25-cm^2 flasks, which were grown to late log phase. Prior to incubation, the flasks were washed with 3×5 mL of Earle's MEM. Earle's MEM (2 mL) supplemented with antibiotics was then pipetted into each flask. Substrate [1α,2α(n)-triated vitamin D_3 ± MC 969 or 1α-OH-D_3 (0–500 nM)] was added in 10 μL of ethanol, and the cells were incubated for a further 48 hr.

Generation of large quantities of MC 969 *metabolites*. Hep 3B cells were removed from 75-cm^2 flasks as described above and used to inoculate 150-cm^2 tissue culture dishes. The cells were grown to late log phase. Prior to incubation, the plates were washed with 3×10 mL Earle's MEM. Earle's MEM (10 mL) supplemented with antibiotics was added to each plate. MC 969 was then added, in absolute ethanol, to a final concentration of 5 μg/mL of 10 μg/mL. Ethanol concentrations did not exceed 0.1% (v/v). For determination of cell number, medium was removed from the tissue culture dishes, and the cells were washed with 10 mL of Dulbecco's modified salt solution (containing 5.4 mM KCl, 1.4 mM KH_2PO_4, 137 mM NaCl, and 8 mM $Na_2HPO_4 \cdot 7H_2O$ at pH 7.4) containing 1% bovine serum albumin (w/v). The medium and wash from each plate were combined for extraction. Cells were removed from dishes using trypsin/EDTA solution, pelleted and resuspended in salt solution, and aliquots were counted in a model ZM automatic cell counter (Coulter Electronics, Luton, England).

Lipid extraction. For concentration–response studies, cells and medium were extracted in 20-mL test tubes using a modified version of the method of Bligh and Dyer [14], in which methylene chloride was substituted for chloroform. Pooled medium and phosphate-buffered saline (PBS) wash from tissue culture plates were extracted in separatory funnels using the same technique. The methylene chloride layer was evaporated to dryness and the residue redissolved in hexane/isopropyl alcohol/methanol (HIM) in the proportions (by vol.) 91/7/2, or 94/5/1, or 96/3/1 depending on the subsequent chromatography.

High pressure liquid chromatography. Analytical HPLC of the MC 969 extracts was performed on a

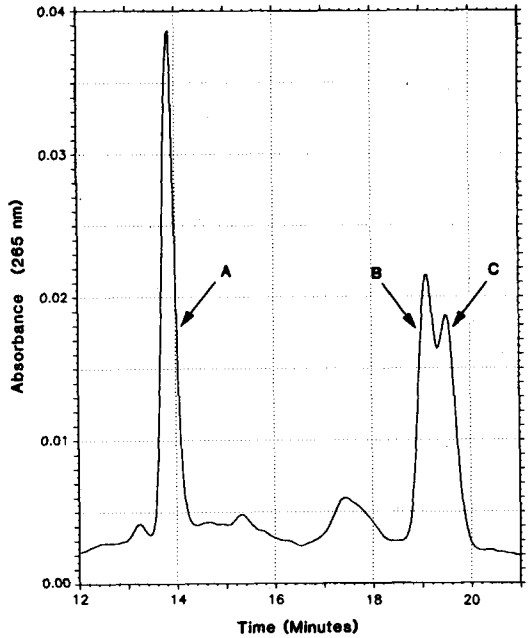

Fig. 2. Chromatogram of extract of Hep 3B cells incubated with MC 969 depicting metabolites A, B and C. Metabolite peaks were recognized by possession of the vitamin D chromophore: UV_{max} = 265 nm UV_{min} = 228 nm. HPLC conditions: Zorbax-SIL, HIM 91/7/2, flow rate 2 mL/min. Only the 12–21 min region of the chromatogram is shown. Peak A = 24-ketone; peaks B and C = 24 (*R*,*S*) alcohols of MC 969.

modular system comprised of a Waters model 590 pump, a Waters U6K manual injector, a Waters model 440 fixed wavelength detector (254 nm), and a Waters 990 photodiode array detector. Separations were performed with a Zorbax-SIL column (6.2 mm × 25 cm, Du Pont) using HIM, 91/7/2, at a flow rate of 2 mL/min [15] (Fig. 2). Effluent was collected in glass scintillation vials, and the following fractions containing metabolites were pooled: (a) the effluent emerging between 13 and 15 min was pooled for peak A; (b) the effluent emerging between 18.5 and 20.5 min was pooled for a combined peak B + C fraction. Peaks were identified as metabolites when they possessed the UV characteristics of the vitamin D *cis*-triene system (UV_{max} = 265 nm, UV_{min} = 228 nm, UV_{max}/UV_{min} = 1.8–2.0).

Peak A was purified further on the same HPLC system using Zorbax-SIL, 3 μm (6.2 mm × 8 cm, Du Pont) with HIM, 94/5/1, at a flow rate of 3 mL/min, the peak of interest emerging between 7.2 and 8.5 min. This step was repeated once more in order to generate pure peak A.

The combined B and C fraction was rechromatographed on Zorbax-Sil, 3 μm (6.2 mm × 8 cm) using the solvent HIM, 96/3/1, with a flow rate of 3 mL/min (see Fig. 4). The two peaks obtained were resolved by selecting early cuts (22.2 to 23.2 min) for peak B; late cuts (24 to 25.3 min) for peak C; and recycling the central portion of the merged peaks (23.2 to 24.0 min) through four successive steps of identical HPLC. In some cases, crude mixtures of peaks B and C from the Zorbax-SIL, 3 μm 22.2- to 25.3-min fraction were used for subsequent steps of the identification procedure.

For analysis of extracts from competition studies, automatic analytical HPLC was performed using a Waters model 510 pump, a WISP automatic sample injector, and a Waters 490 programmable multiwavelength detector. The extracts were run on Zorbax-SIL using HIM, 94/5/1, at a flow rate of 2 mL/min. Effluent was collected in 1-min fractions using a programmable fraction collector (Superrac, Pharmacia, Dorval, Quebec) and allowed to evaporate at room temperature in a fume hood. Scintillation counting was performed using an organic based scintillant (Scintiverse, Fisher) in an automatic counter (model LS 7500 Beckman Instruments).

Mass spectrometry. Metabolites which possessed the vitamin D chromophore and had been purified to homogeneity were subjected to electron impact–mass spectrometry using model HP5985 mass spectometer (Hewlett–Packard) fitted with a direct insert probe. Samples (500 ng to 1 μg) were dried in glass capillary tubes, placed in the probe, and the probe heated from 20 to 200° over 20 min. Ionization voltage was 70 eV, and ions emerged 2–5 min after heating commenced.

Sodium borodeuteride reduction. The putative 24-keto-containing metabolite of MC 969 (Peak A) was subjected to reduction with $NaBD_4$. Briefly, 2.4 nmol of putative metabolite was evaporated to dryness under oxygen-free nitrogen. The metabolite was redissolved in 200 μL methanol and 10 μL water, a large molar excess of $NaBD_4$ was added, and the reaction was allowed to proceed for 1 hr at 0°. At the end of the incubation period, the reactants were extracted twice with 2.0 mL ethyl acetate. The ethyl acetate was then evaporated to dryness and the residue redissolved in 150 μL HIM 91/7/2. The sample was then subjected to HPLC on Zorbax-SIL at 2.0 mL/min, and the eluting peaks were collected.

Trimethylsilylation. Putative metabolites (2.4 nmol of each) were dried under N_2 and redissolved in 50 μL of Tri-Sil "Z" (*N*-trimethylsilyl-imidazole in pyridine, Pierce Chemical Co.), and the solution was heated to 50° for 1 hr. The reaction mixture was applied to a 1-cm column of Lipidex (hydroxyalkoxypropyl-dextran Type IX, Sigma) previously washed with 10 mL hexane, and derivatized metabolites were eluted with 2 mL of hexane [16]. The hexane was then evaporated under N_2 and the sample redissolved in a small volume of hexane and subjected to mass spectrometry as described previously. Alternatively, the reaction mixture was dried under N_2 and redissolved in 100 μL of hexane/ethyl acetate (75/25), followed by purification on Zorbax-SIL at a flow rate of 1 mL/min. The putative 24-ketone eluted in a single peak at approximately 6 min.

Chemical synthesis of MC 976 [1(S),3(R)-*dihydroxy*- 20(R)- 3′-*cyclopropyl*- 3′ζ-*hydroxypropyl*)- 9,10 - *secopregna* - 5(Z),7(E),10(19) - *triene*]. Authentic MC 976 as an approximately equal mixture of 24-epimers was synthesized from the described [17] bis-*tert*-butyldimethylsilyl ether of the corresponding (5*E*)-vitamin D analog. The standard sequence of triplet-sensitized photoisomerization

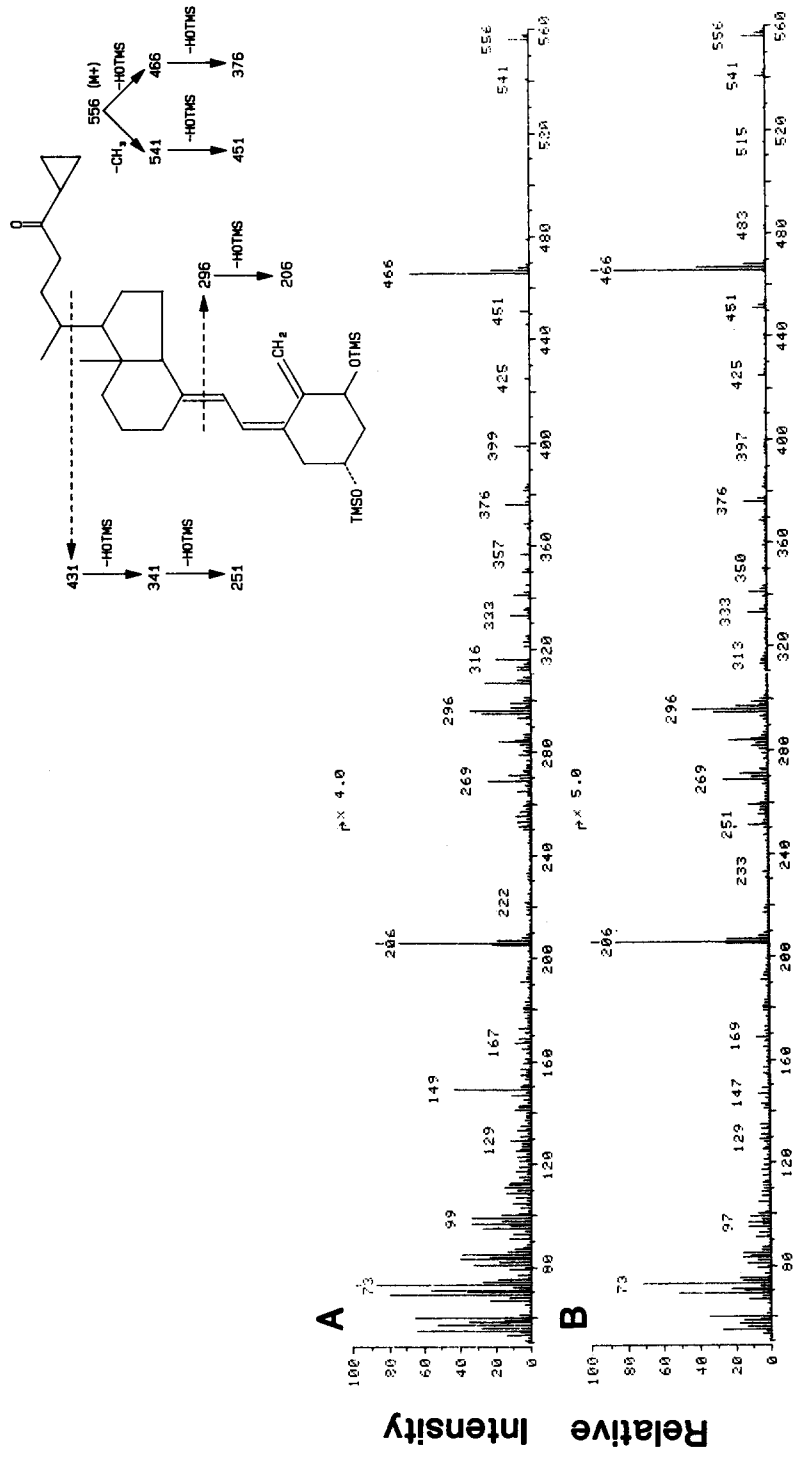

Fig. 3. Comparative mass spectra of (A) TMS-derivative peak A (500 ng) and (B) TMS-derivatized MC 1080 (1 μg). Structure of the derivatized compound and fragmentation pattern are inserted at the top.

Table 1. Effects of antioxidants on rate of metabolite production in Hep 3B cells *in vitro**

	Metabolite production† (ng/10^6 cells/day)	
	Peak A	Peaks B + C
Control Hep 3B cells	8.2 ± 1.0	9.0 ± 1.8
BHT‡-treated Hep 3B cells	6.5 ± 1.7	6.8 ± 1.2
DPPD‡-treated Hep 3B cells	10.5 ± 1.0§	11.2 ± 1.3

* Cultured in 150 × 25 mm dishes.
† Results are means ± SD of triplicate determinations.
‡ Added to culture in ethanol at time of addition of substrate to achieve a final concentration of 50 μM.
§ Significantly different from control, $P < 0.05$ (Student's *t*-test).

into the (5*Z*) series followed by desilylation with tetrabutylammonium fluoride under the conditions described in Ref. 17 (including chromatographic purification to homogeneity on silica gel with ethyl acetate as eluant) gave MC 976 as an oil, which was stored in ethanol solution. The data refer to solvent-free material: UV (EtOH): λ_{max}265 nm (ε_{265nm} 17,500 dm^3/mol cm); ^{1}H-NMR (300 MHz, $CDCl_3$): 0.17–0.30 (2H, m) 0.44–0.57 [5H, m, including 0.55 (3H, s)], 0.88 (1H, m), 0.94 and 0.95 (3H, two doublets of equal height, $J = 6.3$ Hz), 2.31 (1H, dd, $J = 7$ and 13 Hz), 2.60 (1H, dd $J = 3$ and 13 Hz), 2.77–2.87 (2H, m), 4.23 and 4.43 (each 1H, m), 5.00 and 5.33 (each 1H, bs), 6.02 and 6.38 (each 1H, d, $J = 11$ Hz) ppm.

RESULTS

Extracts of Hep 3B cells incubated with MC 969 contained three metabolites, termed peaks A, B and C. When Hep 3B cells were omitted and MC 969 incubated with medium alone, no metabolites were formed. Note that peaks B and C were difficult to resolve in Fig. 2. Metabolites were selected through possession of the distinctive vitamin D chromophore. It should be noted that no metabolite which comigrated with authentic MC 1015 on this HPLC system was detected. When incubations were carried out in 75-cm^2 flasks at two concentrations of MC 969, 5 and 10 μg/mL, production of peak A was approximately doubled at the higher concentration, from 12.1 ± 1.8 ng/10^6 cells/day to 23.7 ± 2.9 ng/10^6 cells/day. Production of peaks B and C was also doubled, from 10.4 ± 1.1 ng/10^6 cells/day to 24.3 ± 3.7 ng/10^6 cells/day. In another series of incubations, using MC 969 at a concentration of 5 μg/mL and Hep 3B cells grown on 150 × 25 mm plates, we studied the effects of two antioxidants, butylated hydroxytoluene (BHT) and *N,N'*-diphenylethylenediamine (DPPD), on the rate of metabolite synthesis. Production rates, though slightly lower than in initial experiments presumably due to differences in the cell/medium ratio, were affected only marginally by the addition of antioxidants. BHT treatment caused no change in either peak A or peak B + C production, but DPPD treatment resulted in a slight increase in production of peak A and no change in the synthesis of peak B + C (Table 1).

Table 2. Co-chromatography of synthetic standards of metabolite peaks on HPLC

Compound	Retention time* (min)
Peak A	7.68
MC 1080	7.68
Peak B†	13.09
MC 976 Isomer 1‡	13.03
Reduced MC 1080 Isomer 1§	13.06
Peak C†	13.50
MC 976 Isomer 2‡	13.46
Reduced MC 1080 Isomer 2§	13.48
MC 1015	12.66

* HPLC conditions: Zorbax-SIL, 3 μm; HIM, 94/5/1; flow rate, 3 mL/min.
† Area ratio of peak B:peak C = 1.198:1.
‡ Area ratio of MC 976 Isomer 1:Isomer 2 = 1.110:1.
§ Area ratio of reduced MC 1080 Isomer 1:Isomer 2 = 1.092:1.

In another control experiment, we incubated the hepatoma cell line Hep G2, which possesses low 25-hydroxylase activity, with 5 μg/mL MC 969. No detectable metabolites were observed in this cell line.

Peak A. The least polar metabolite, peak A, comigrated exactly with authentic 24-ketone (MC 1080) on Zorbax-SIL (Table 2). Purification of Peak A followed by mass spectrometry identified a molecular ion with $m/z = 412$, suggesting addition of oxygen to the molecule (data not shown). Fragments at m/z 287 (side chain cleavage), 269 (287 − H_2O), 251 (269 − H_2O), 152 (*cis*-triene cleavage) and 134 (152 − H_2O) are those expected for the 1α-OH-D_3 nucleus [18] and indicate that the additional oxygen is in the side chain. The same molecular ion and fragments were obtained from mass spectrometry of authentic 24-ketone (data not shown).

Borodeuteride reduction of peak A produced a roughly equimolar mixture of two peaks which comigrated on Zorbax-SIL with peaks B and C, and with authentic 24-(*R,S*) alcohol MC 976 (Table 2). Mass spectrometry of the reduced metabolite revealed a molecular ion with $m/z = 415$, consistent with the

introduction of a deuterium atom and a hydrogen atom into the molecule (data not shown). Fragments which could be ascribed to the derivative were found at m/z 397 (molecular ion $-$ H_2O); 379 (397 $-$ H_2O), 364 (379 $-$ CH_3), 287 (loss of side chain), 269 (287 $-$ H_2O), 251 (269 $-$ H_2O), 152 (*cis*-triene cleavage) and 134 (152 $-$ H_2O). In contrast, the synthetic alcohol mixture MC 976 showed a molecular ion of m/z = 414 and fragments at m/z 396 (molecular ion $-$ H_2O), 378 (396 $-$ H_2O), 363 (378 $-$ CH_3), 287 (loss of side chain), 269 (287 $-$ H_2O), 251 (269 $-$ H_2O), 152 (*cis*-triene cleavage) and 134 (152 $-$ H_2O). Both the borodeuteride reduced peak A and MC 976 contained prominent fragments at m/z 285 and 167 which were not identified. Thus, both spectra were identical except for the ions above m/z 320 where the single deuterium atom increased m/z by 1 mass unit. The fact that ions m/z below 320 were identical is evidence that the deuterium atom is in the side chain. In summary, the mass spectral and chromatographic evidence is consistent with interpretation that borodeuteride reduction of peak A produces an epimeric mixture of C_{24}-alcohols.

TMS-derivatization of peak A produced a compound with a molecular ion of m/z = 556, consistent with the addition of TMS groups to two hydroxyl groups (Fig. 3A). Fragments at m/z 541 (556 $-$ CH_3), 466 (566 $-$ HOTMS), 451 (466 $-$ CH_3), 376 (466 $-$ HOTMS), and 296 (*cis*-triene cleavage) are all consistent with the derivatized 1α-OH-D_3 nucleus. Fragments at m/z 341 (side-chain cleavage) are consistent with an underivatized side chain. The TMS-derivatized synthetic 24-ketone produced an identical mass spectrum (Fig. 3B).

Peaks B and C. The second and third metabolites, peaks B and C, were resolved only with difficulty on Zorbax-SIL (Fig. 4). The retention times of the two metabolites, which were present in roughly equal amounts (see Table 2), proved to be identical with the retention times of the two epimers of authentic 24-alcohol (MC 976) (Table 2). Mass spectrometry revealed for both peak B and peak C a molecular ion with m/z = 414, consistent with the introduction of a hydroxyl function into the molecule (data not shown). Additionally, fragments with m/z 396 (414 $-$ H_2O), 378 (396 $-$ H_2O), 363 (378 $-$ CH_3), 285, 267 (285 $-$ H_2O), and 251 were present in the mass spectra of both peaks B and C and in the mass spectra of the synthetic 24-alcohol (data not shown).

Mass spectrometry of the TMS-derivatized peaks B and C showed molecular ions with m/z = 630, consistent with attachment of TMS groups to three hydroxyl groups in the molecule (Fig. 5A). Fragments with m/z 540 (630 $-$ HOTMS), 450 (540 $-$ HOTMS), 341 (630 $-$ side chain $-$ HOTMS), 296 (*cis*-triene cleavage) and 206 (296 $-$ HOTMS) are consistent with retention of the 1α-OH-hydroxylated vitamin D_3 nucleus and with the presence of a hydroxyl group in the side chain. TMS-derivatization of synthetic 24-alcohol yielded a mass spectrum with identical molecular ions and fragmentation pattern (Fig. 5B).

Studies of possible metabolic sequence. Incubations of Hep 3B cells with MC 1080 gave rise to biosynthesis of mainly peak B (MC 976 Isomer 1) with little or no discernible peak C (MC 976 Isomer 2). When we separated synthetic isomers of MC 976 by extensive recycle chromatography, we were able to obtain sufficient pure material to try each as a substrate with Hep 3B cells. Both MC 976 Isomer 2 and MC 976 Isomer 1 acted as precursors to peak A (MC 1080). From these results we tentatively propose the following pathway:

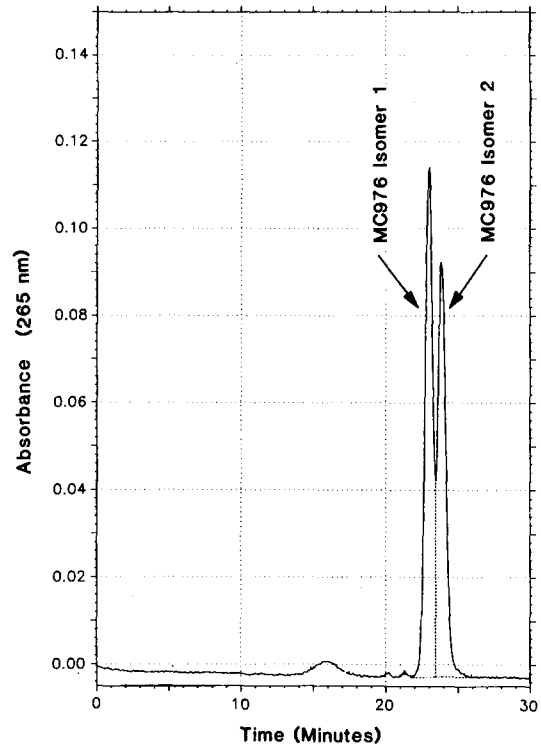

Fig. 4. HPLC chromatogram of MC 976 showing similar retention times of two isomers. The chromatogram represents the UV trace at 265 nm. HPLC conditions: Zorbax-SIL, 3 μm, HIM 96/3/1, flow rate 3 mL/min.

MC 969 $\rightarrow$ MC 976 Isomer 2 (Peak C) $\rightarrow$

MC 1080 (Peak A) $\rightleftharpoons$ MC 976 Isomer 1 (Peak B)

Competition studies. We studied the abilities of MC 969 and 1α-OH-D_3 at concentrations of 0–100 nM to inhibit the 25-hydroxylation of tritiated vitamin D_3 in Hep 3B cells. 1α-OH-D_3, in concentrations as low as 25 nM, decreased the formation of [^{3}H]25-OH-D_3 by 86% (Fig. 6). The identification of metabolites of MC 969 which were 24-hydroxylated rather than 25-hydroxylated prompted us to examine whether MC 969 would also block the formation of [^{3}H]25-OH-D_3 in this system. The data in Fig. 6 indicate that MC 969 was even more effective than 1α-OH-D_3 over the same concentration range.

DISCUSSION

We report here the identification of 24-substituted

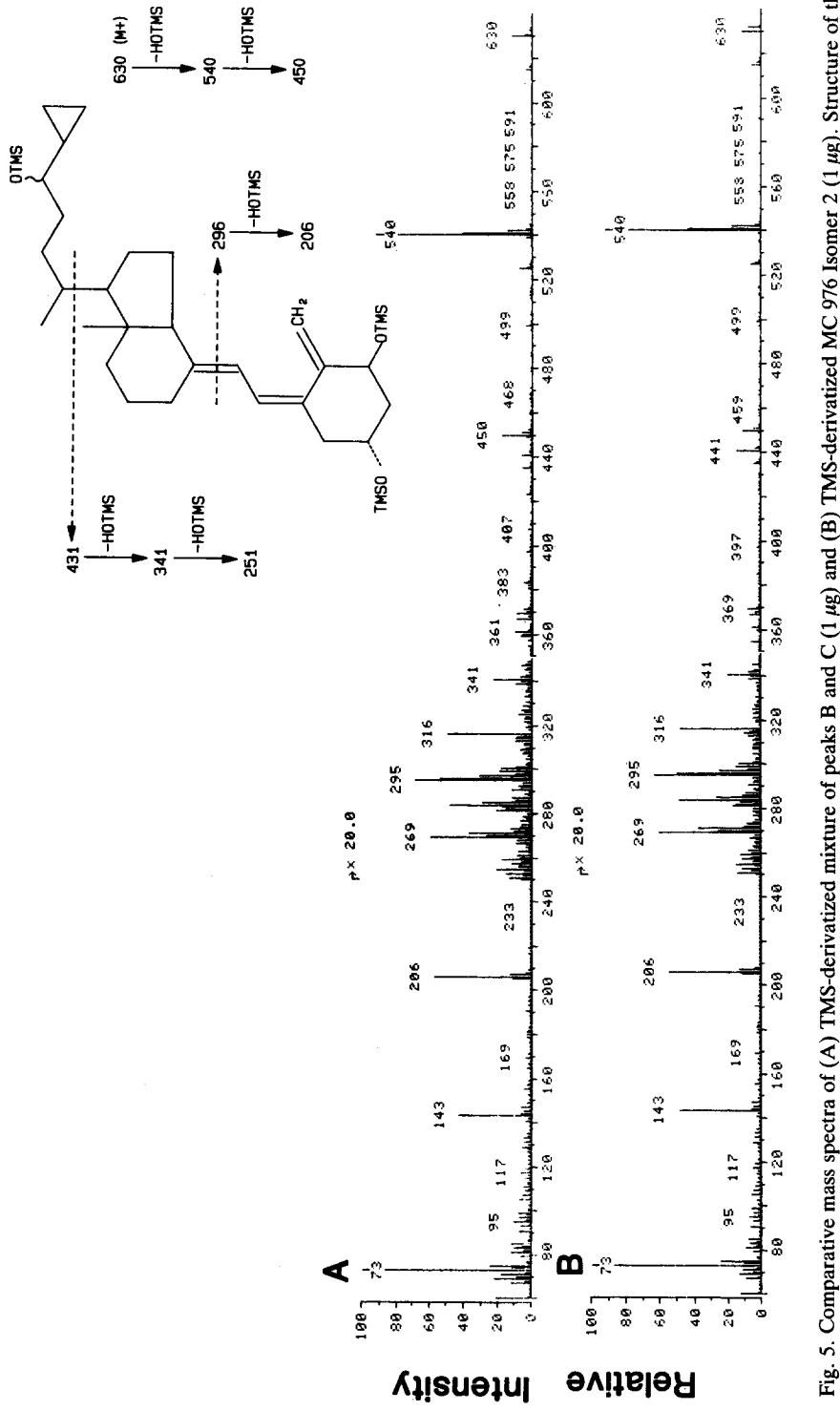

Fig. 5. Comparative mass spectra of (A) TMS-derivatized mixture of peaks B and C (1 μg) and (B) TMS-derivatized MC 976 Isomer 2 (1 μg). Structure of the derivatized compound and fragmentation pattern are inserted at the top.

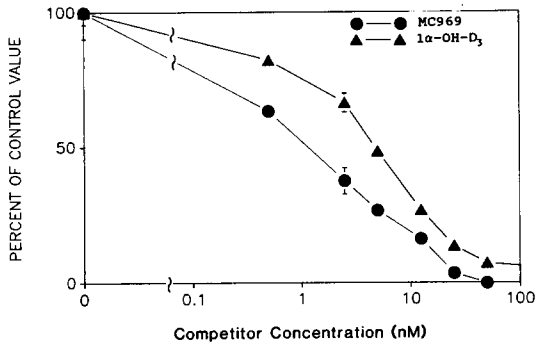

Fig. 6. Inhibition of [^{3}H]25-OH-D_3 production from [^{3}H]vitamin D_3 by increasing concentrations of 1α-OH-D_3 or MC 969. Incubation conditions are described in Materials and Methods. The rate of [^{3}H]25-OH-D_3 formation with no added competitor was assigned a value of 100% in each assay. For the incubation with MC 969, this rate was 0.127 pmol/10^6 cells/day; the rate for the incubation with 1α-OH-D_3 was similar. Each point in the figure is the mean ± SE of three flasks. SE values for most points were around 1% and could not be depicted in the figure due to the size of the symbols.

metabolites of MC 969, a synthetic cyclopropane-ring-containing analog of 1α-OH-D_3. The metabolites, a 24-keto- and the two epimeric 24-hydroxy derivatives, were generated enzymatically in a new *in vitro* hepatocyte cell model, Hep 3B. Chemical reduction of the 24-ketone gave the same mixture of 24-alcohols. Identity of the native metabolites was established by comigration on HPLC, with chemically synthesized standards and by comparative mass spectrometry. Though these metabolites were synthesized *in vitro*, it is not yet proven that MC 969 is subjected to the same metabolism *in vivo*. However, preliminary evidence from *in vivo* animal studies of another cyclopropane analog, MC 903 (Fig. 1), suggests that it too is converted to a 24-ketone [13]. Thus, the Hep 3B cell model, which has so far provided an accurate *in vitro* reflection of *in vivo* metabolism of vitamin D and its analogs (e.g. for the study of 25-hydroxylation of vitamin D_3, dihydrotachysterol [19] and 1α-OH-D_3), appears to be a reliable model for studying metabolism of the cyclopropane-ring-containing analogs as well.

The formation of 24-oxidized rather than 25-oxidized metabolites of the cyclopropane analog was unexpected. There is evidence in the literature [20, 21] that two other molecules, vitamin D_2 and 1α-hydroxy-25-fluorovitamin D_3, both undergo 24-hydroxylation without 25-hydroxylation *in vivo* but there is no evidence that the 24-hydroxylase is hepatic. Incubation of 1α-OH-D_3 with Hep 3B cells at the same concentration used in these experiments for MC 969 resulted in the production of 1,25-$(OH)_2D_3$, as verified by comigration on HPLC and mass spectrometry (Strugnell S and Jones G, unpublished results). Further metabolism of peaks A, B and C in the 25-position was ruled out by the absence of peaks in the chromatogram in the region where polar metabolites such as 1,24,25-$(OH)_2D_3$ migrate. It seemed possible to us that the 24-substituted metabolites could be formed by the same enzyme performing the 25-hydroxylation of vitamin D_3. With this in mind, a competition assay was employed in which Hep 3B cells were incubated with tritiated vitamin D_3 in the presence of increasing concentrations of MC 969. As our results indicated (Fig. 6), MC 969 was more effective than 1α-OH-D_3 at inhibiting vitamin D_3-25-hydroxylase activity. One possible explanation for these data is that MC 969, 1α-OH-D_3 and vitamin D_3 are all side-chain hydroxylated by the same enzyme. In this model, incorporation of a cyclopropane ring into the side chain of the vitamin D_3 analog directs the 25-hydroxylase to introduce a hydroxyl group into the 24-position of the side chain. Consistent with this idea was the finding that another hepatoma Hep G2 known to be low in the 25-hydroxylase activity is unable to metabolize MC 969 to peaks A, B and C. However, the many other enzymes present in the cell make it impossible to state unequivocally that it is solely the vitamin D_3-25-hydroxylase which generates the metabolites. This question can only be addressed by isolation and expression of the cDNA encoding the cytochrome P-450 for this enzyme.

The cyclopropane-ring-containing analogs employed in this work were originally synthesized in an attempt to probe the mechanism of action of MC 903, a 24(*S*)-hydroxylated, Δ^{22}-analog [17], in which the cell-differentiating activity of the vitamin D compound has been separated from the calcemic effects [22] and which is currently undergoing clinical trials for the treatment of psoriasis. The data presented here indicate that MC 969 can undergo biological 24-hydroxylation. The findings that MC 969 *in vitro* and MC 903 *in vivo* both undergo 24-oxidation to the 24-ketone, MC 1080, could be considered to be a step towards activation or catabolism. Since MC 903 is itself inactive as a calcemic factor, one would assume that its metabolite MC 1080 must also lack calcemic activity. Preliminary biological testing shows that this metabolite, MC 1080, also possesses a considerably reduced activity compared to MC 903 in cell differentiation and proliferation assays [13].

Preliminary studies reported here using each metabolite as a substrate for Hep 3B cells have revealed a possible metabolic sequence. This is:

$$\text{MC 969} \rightarrow \underset{\substack{\text{(24-hydroxy)}\\ \textit{Isomer 2}}}{\text{Peak C}} \rightarrow \underset{\text{(24-ketone)}}{\text{Peak A}} \rightleftharpoons \underset{\substack{\text{(24-hydroxy)}\\ \text{Isomer 1}}}{\text{Peak B}}$$

By analogy with 1,24*R*-$(OH)_2D_3$, a known calcemic factor [23], one would presume that the calcemic effect of MC 969 may be through the formation of one (or both) of these 24-hydroxy compounds. We are currently engaged in the stereoselective synthesis of these isomers for biological testing in order to resolve this question.

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