19-Nor-10-ketovitamin D Derivatives: Unique Metabolites of Vitamin D_3, Vitamin D_2, and 25-Hydroxyvitamin D_3†

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ABSTRACT: Three metabolites were isolated after incubation of vitamin D_3, vitamin D_2, or 25-hydroxyvitamin D_3 with bovine rumen microbes. They are identified as 5(*E*)-19-nor-10-ketovitamin D_3, 5(*E*)-19-nor-10-ketovitamin D_2, and 5(*E*)-19-nor-10-keto-25-hydroxyvitamin D_3, respectively. The identifications were based on ultraviolet absorbance, mass spectroscopy, and chemical reactivity. All 5(*E*)-19-nor-10-ketovitamin D derivatives examined had an absorbance maximum at 312 nm and a characteristic fragment in their mass spectra corresponding to loss of 43 amu from their molecular ions. The vitamin D_3 metabolite was identical in all essential spectral and chromatographic aspects with authentic synthetic 5(*E*)-19-nor-10-ketovitamin D_3. These metabolites represent a unique pathway of vitamin D metabolism and the first characterized products of microbial vitamin D metabolism. The conversion of vitamin D and its metabolites to their 19-nor-10-keto forms likely represents a detoxification mechanism.

Cows are especially sensitive to peritoneally administered pharmacological doses of vitamin D (Littledike & Horst, 1982) but are comparatively insensitive to oral pharmacological doses (Hibbs & Pounden, 1955). A cause of this difference could be metabolic inactivation of vitamin D by bovine rumen microbes. To examine this question, we studied vitamin D metabolism by bovine rumen microbes and demonstrated that these microbes convert [^{3}H]vitamin D into several more polar metabolites (Sommerfeldt et al., 1979, 1982). This paper will report the structural characterization of one group of these metabolites as 19-nor-10-ketovitamin D derivatives. These are the first examples of a metabolic steroidal A-ring modification in D vitamins, with the exception of 1α-hydroxylation (Lawson, 1980). These compounds also represent the first structurally characterized microbial vitamin D metabolites.

Experimental Procedures

General. HPLC[1] was performed with Waters Associates equipment (Model ALC/GPC 204). All solvents used for HPLC were glass distilled and filtered through a 0.45-μM filter. The normal-phase column used was a Du Pont Zorbax-Sil 5-μM particle column (0.42 × 25 cm), unless otherwise noted. UV spectra were obtained in ethanol with a Beckman Model 25 recording spectrophotometer. High-resolution mass spectra were taken with a Kratos MS50 magnetic sector mass spectrometer at the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Nebraska. Low-resolution mass spectra were taken with a Finnigan Model 4021 quadrupole GC/MS. NMR spectra were taken in $CDCl_3$ with a JEOL 200-MHz FT NMR spectrometer.

Compounds. [3α-^{3}H]Vitamin D_2 and [3α-^{3}H]vitamin D_3 (1.2 Ci/mmol) were prepared by the sodium borotritide reduction of the α-tricarbonyliron complex of 3-ketovitamin D (Barton & Patin, 1976). Radiochemical purity was established by analysis on a normal-phase HPLC column developed with 2-propanol/hexane (1/99). Both compounds were diluted to a specific activity of 33000 dpm/μg. 25-OH[26,27-^{3}H]D_3 (90 Ci/mmol) was synthesized by allowing [^{3}H]methylmagnesium bromide to react with 27-nor-25-ketovitamin D_3. Radiochemical purity was established by analysis on a normal-phase HPLC column eluted with 2-propanol/hexane (5/95). This substrate was also diluted to a specific radioactivity of 33000 dpm/μg. Synthetic 5(*E*)-19-nor-10-ketovitamin D_3 was prepared by the procedure of Inhoffen et al. (1958, 1959) and had the following characteristics: colorless solid, mp 140–141 °C; λ_{max} (ethanol) 312 nm (ϵ = 27000); $[\alpha]_D$ +194° (*c* 1.03, benzene); NMR ($CDCl_3$) δ 0.53 (s, 18-CH_3), 0.85 [d, *J* = 6 Hz, 26,27-(CH_3)$_2$], 0.92 (d, *J* = 6 Hz, 21-CH_3), 4.2 (m, 3α-H), 5.9 and 7.6 (2 d, *J* = 12 Hz, 6- and 7-H); mass spectrum, *m*/*z* (relative intensity) 386 (M^+, 0.82), 271 (0.14), 343 (0.64), 273 (0.5), 179 (0.42), 175 (0.57), 135 (0.93), 133 (1.00).

Generation of Vitamin D Metabolites. A steer with an abdominal fistula, fed a maintenance diet of alfalfa hay and cracked corn, was used as the source of rumen contents for the in vitro incubations. Samples of the rumen contents were obtained from the rumen cannula 4–6 h after feeding. Rumen contents were strained immediately through cheese cloth, kept at 38 °C, and purged with CO_2 gas. Strained rumen fluid (20 mL) was transferred into 14 Erlenmeyer flasks (50 mL) while being gassed with CO_2. Sterilized controls were obtained by autoclaving the contents of the flasks at 121 °C and 15 psi for 15 min. [^{3}H]Vitamin D (30 μg) was added to each flask under CO_2. Flasks were stoppered with Bunsen valves and incubated for 24 h at 38 °C with constant shaking. The initial pH was 6.6; the medium gradually reached a pH of 6.2 during incubation.

Isolation of Vitamin D Metabolites. The contents of the Erlenmeyer flasks were extracted by the procedure of Bligh & Dyer (1959). The organic layer was concentrated under reduced pressure, and the solvent was evaporated from the

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[1] Abbreviations: HPLC, high-performance liquid chromatography; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; UV, ultraviolet absorbance; NMR, nuclear magnetic resonance; FT, Fourier transform; NMR, nuclear magnetic resonance.

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concentrate under a stream of nitrogen. The residue was dissolved in 1 mL of hexane/chloroform (9/1) and applied to a Lipidex 5000 column (system 1, 0.9 × 58 cm). The column was eluted with 100 mL of hexane/chloroform (9/1), followed by 100 mL of hexane/chloroform (1/1). Aliquots of fractions (5 mL) were measured for radioactivity. Peak Q (130–150 mL) was collected. The recovered materials were applied to a heat-activated (110 °C, 16 h) Celite/silicic acid (1/1, w/w) column (system 2, 0.9 × 58 mL) and eluted with 50 mL of hexane/ethyl acetate (9/1), followed by 300 mL of hexane/ethyl acetate (4/1). The material eluting between 150 and 250 mL was collected and applied to a Sephadex LH-20 column (system 3, 0.9 × 58 cm) developed with hexane/chloroform/methanol (79/20/1). The material eluting between 40 and 80 mL was recovered and applied to a normal-phase HPLC column (system 4) developed with 2-propanol/hexane (4/96). Two major radioactive peaks were observed at 28 and 33 mL, respectively. The total material between 27 and 35 mL was collected and applied to the same HPLC column (system 5) and eluted with 2-propanol/dichloromethane (1/99). This HPLC column also resolved peak Q into two peaks, Q_1 (29 mL) and Q_2 (48 mL). The recovered materials (50 μg each) were used for spectral analyses.

Generation and Isolation of the 25-OH-D_3 Metabolites. The 25-OH-D_3 metabolite was produced from rumen fluid as described above (10 flasks, 30 μg of [^{3}H]25-OH-D_3/flask). The material recovered after a Bligh and Dyer extraction was filtered through a Celite/silicic acid column (1.8 × 25 cm) with ethyl acetate. The first 50 mL were collected and evaporated. The residue was applied to a semipreparative normal-phase HPLC column (0.94 × 25 cm, Zorbax-Sil) eluted with 2-propanol/hexane (1/9). The peak eluting between 65 and 75 mL was collected. This peak was placed on an analytical normal-phase column and eluted with 2-propanol/dichloromethane (2.5/77.5). The metabolite eluted between 17 and 19 mL. In this system, 1,25-$(OH)_2D_3$ eluted at 37 mL. The metabolite was placed on the same column and was eluted in 27 mL with 2-propanol/hexane (1/9). In this system, 1,25-$(OH)_2D_3$ eluted at 28 mL. The last column procedure was repeated to yield 40 μg of a homogeneous metabolite.

Silylation of Metabolites. About 400 ng of each metabolite was heated at 50 °C for 80 min in the presence of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (40 μL). The reaction was cooled, and the volatile materials were removed under a stream of nitrogen. The residue was analyzed by mass spectroscopy.

Sodium Borohydride Reduction. About 900 ng of metabolite was treated with sodium borohydride (40 μL, 10 mg/100 mL of ethanol) for 20 min. Acetone (100 μL) was added to quench the reaction. The solvents were removed under a stream of nitrogen, and the product was extracted with chloroform. The material obtained after evaporating the chloroform was analyzed by mass spectroscopy.

Irradiations. Compounds were irradiated in ethanol (0.5 mL) for 10 min by placing them 8 in. from a GE sunlamp fitted with a 275-W bulb, which emitted light between 280 and 340 nm. The solvent was evaporated under a stream of nitrogen, and the compounds were immediately analyzed by HPLC.

Results

Rumen fluid microbes converted 75% of either [^{3}H]vitamin D_2 or [^{3}H]vitamin D_3 to more polar metabolites during 24 h of incubation. The main metabolite peaks of each were designated Q, R_1, and R_2. The conversions to Q, R_1, and R_2 were

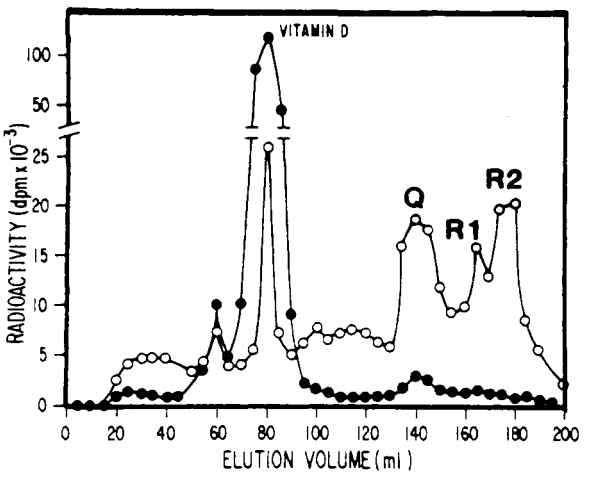

FIGURE 1: Profile of [^{3}H]vitamin D_3 and its metabolites recovered from a rumen fluid microbial incubation. Materials extracted after incubation with sterilized (●) or intact (○) rumen microbes were eluted from a Lipidex 5000 column with hexane/chloroform (system 1) as described under Experimental Procedures. Similar results were obtained with [^{3}H]vitamin D_2.

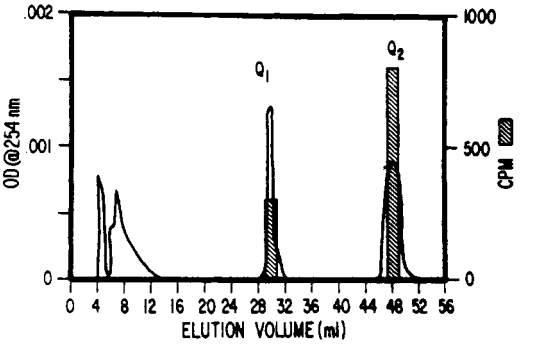

FIGURE 2: HPLC profile of Q_1D_3 and Q_2D_3 isolated from a rumen fluid microbial incubation of [^{3}H]vitamin D_3 (system 5).

not catalyzed by sterilized rumen fluid. The small amount of materials that migrated with Q on the first column (system 1), illustrated in Figure 1, did not migrate with the radioactive material in the Q area, obtained from unsterilized rumen, on a second column (system 2). Peaks Q from each of vitamin D_2 and vitamin D_3 were individually purified with a total of five distinct chromatographic procedures (see Experimental Procedures). System 4, based on a HPLC column, resolved each peak Q into two peaks, Q_1 and Q_2, but they were collected as a unit. The Q peaks from vitamin D_2 and vitamin D_3 were separately applied to the final HPLC column (system 5, Figure 2), which also resolved them. This time, Q_1 and Q_2 from each parent compound were collected individually. Thus, a total of four compounds were obtained, two each from vitamin D_2 and vitamin D_3 designated, respectively, Q_1D_2, Q_2D_2, Q_1D_3, and Q_2D_3.

The UV spectrum of Q_2D_3 displayed an absorbance maximum at 312 nm (Figure 3). The UV spectrum of Q_2D_2 was identical with that of Q_2D_3. The spectra of Q_1D_3 and Q_1D_2 differed from those of Q_2, but were identical with each other, with absorbance maxima at 308 nm. These spectra were different from the typical spectra produced by the 5(*Z*),7(*E*),10(19)-triene or (5*E*),7(*E*),10(19)-triene chromophores of *cis*- and *trans*-vitamin D, which have absorbance maxima at 265 and 273 nm, respectively. In fact, the spectra of the Q metabolites are different from those of all known triene isomers of vitamin D. These data show that a triene chromophore is not present in the Q compounds but extended conjugation is and suggest that Q_1 is isomeric with Q_2.

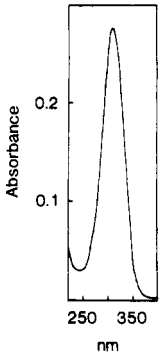

FIGURE 3: Ultraviolet absorbance spectrum of metabolite Q_2D_3. The absorbance maximum is 312 nm. This spectrum is identical with that of Q_2D_2 and the metabolite generated from 25-OH-D_3 and differs from those of the Q_1 compounds only in absorbance maximum. The maxima of the Q_1 compounds were 308 nm.

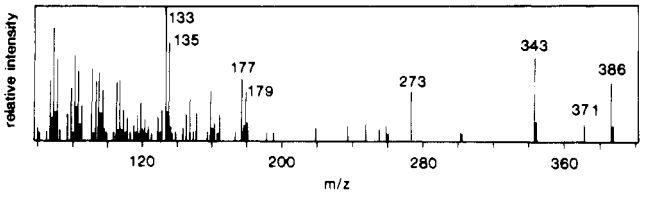

FIGURE 4: High-resolution electron-impact mass spectrum of metabolite Q_2D_3. This spectrum was virtually identical with that of Q_1D_3.

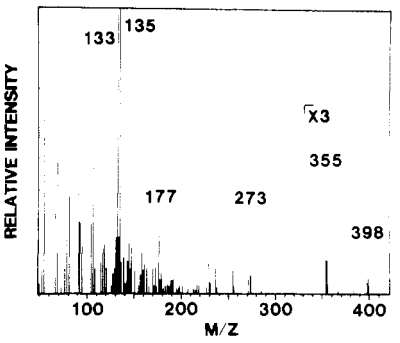

FIGURE 5: Low-resolution electron-impact mass spectrum of metabolite Q_2D_2.

High-resolution mass spectra were obtained of Q_1D_3 and Q_2D_3. The spectrum of Q_2D_3 (Figure 4) had a molecular ion (M^+) at m/z 386.3189, indicating an elemental composition of $C_{26}H_{42}O_2$ (calculated value 386.3185). The peak at 371 ($M^+ - 15$) was caused by loss of a methyl group from the molecular ion. The peak at 343.2646 ($M^+ - 43$) had an elemental composition of $C_{23}H_{35}O_2$ (calculated value 343.2637), representing loss of C_3H_7 (not C_2H_3O) from the molecular ion. The peak at 273 arises from cleavage between C(17) and C(20) and represents loss of the steroid side chain. Major peaks were also observed at 179, 177, 133, and 135. The mass spectrum of Q_1D_3 was virtually identical. Low-resolution mass spectra were taken of Q_1D_2 and Q_2D_2. The spectrum of Q_2D_2 (Figure 5) had a molecular ion at m/z 398 and had a large peak at m/z 355 ($M^+ - 43$). Loss of the side chain was indicated by the peak at m/z 273. Major peaks were also observed at m/z 177, 135, and 133. This spectrum was identical with that of Q_1D_2, within experimental limits. These data indicate that Q_1 and Q_2 differ from vitamin D by substitution of a CH_2 group by an oxygen atom. The substitution is not in the side chain but probably is in the A ring. The mass spectral data are also consistent with the conclusion drawn from the UV results, that Q_1 and Q_2 are isomers. We had also noticed the appearance of Q_1 (or Q_2) in pure samples

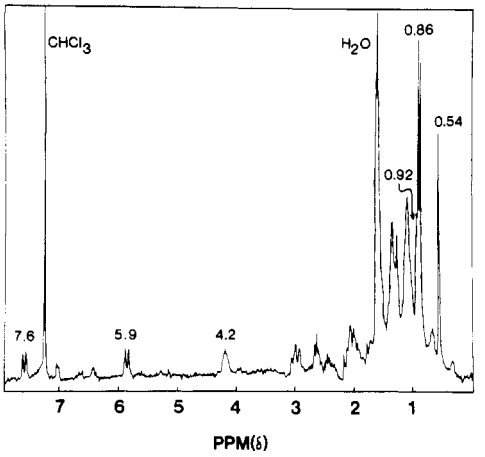

FIGURE 6: High-resolution NMR spectrum of metabolite Q_2D_3. The sample concentration was 100 μg/100 μL of $CDCl_3$.

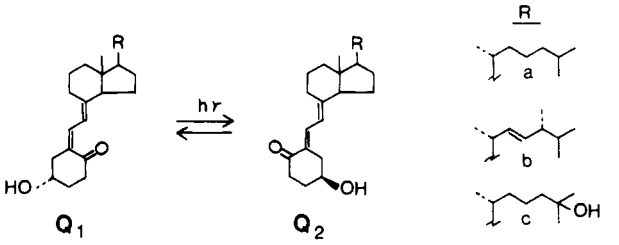

FIGURE 7: Structures of Q_1 [5(Z)-19-nor-10-ketovitamin D] and Q_2 [5(E)-19-nor-10-ketovitamin D] metabolites. Two compounds each were initially obtained from vitamin D_3, Q_1D_3 and Q_2D_3 (R = a), and from vitamin D_2, Q_1D_2 and Q_2D_2 (R = b). One metabolite was generated from 25-OH-D_3, Q_2 (R = c).

of Q_2 (or Q_1) upon prolonged handling.

All four compounds were silylated, and the silyl derivatives were analyzed by mass spectroscopy. The mass spectra of silylated Q_1 did not differ from those of silylated Q_2. Addition of one trimethylsilyl group to the parent compound was observed with all four compounds. The results with Q_2D_3 are illustrative. A molecular ion at m/z 458 (0.25 relative intensity) was consistent with the presence of a single hydroxyl group in the molecule. A peak at m/z 443 (0.05) indicated loss of a methyl group. A peak at m/z 415 (0.2) indicated loss of 43 amu from the molecular ion and was consistent with the high-resolution data, which demonstrated that the $M^+ - 43$ peak arose from loss of C_3H_7, not the isobaric C_2H_3O. A peak at m/z 368 (0.35) resulted from loss of $(CH_3)_3SiOH$. A peak at m/z 329 (0.35), not observed in the spectra of underivatized Q_1, most likely represents loss of $(CH_3)_3SiO$-C_3H_4 from the molecular ion. Metabolite Q_2D_3 was treated with sodium borohydride. Mass spectral analysis showed that the product had a molecular ion of m/z 388. These derivatization data indicate that one of the oxygen atoms in Q is in a hydroxyl group and the other is in a ketone.

A 200-MHz proton NMR spectrum was taken of Q_2D_3 (Figure 6). Signals at δ 0.54 (s), 0.86 (d, J = 6 Hz), and 0.92 (d, J = 6 Hz) in the spectrum confirmed that the steroidal side chain is intact and are similar to those observed in vitamin D_3. The multiplet at δ 4.2 represents the 3α proton and confirms the presence of a 3β-hydroxyl group. Notably, no signals representing the C(19)-methylene protons of vitamin D were observed (δ 4.8 and 5.4). The olefinic protons on C(6) and C(7) are present but have chemical shifts different from those on vitamin D. The C(7) proton at δ 5.9 (d, J = 12 Hz) is shielded relative to that of vitamin D [C(7)-H, δ 6.0]; and the C(6) proton at δ 7.6 (d, J = 12 Hz) is deshielded relative to that of vitamin D [C(6)-H, δ 6.2]. These data are consistent

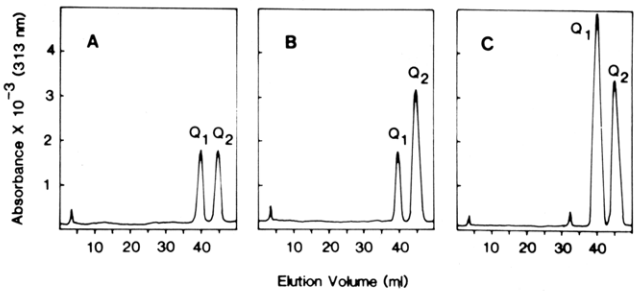

FIGURE 8: HPLC analyses of Q compounds. All samples were eluted from a normal-phase column with 2-propanol/hexane (3/97). (A) A mixture of Q_1D_3 and Q_2D_3 (100 ng each) was eluted from the column. (B) A mixture of Q_1D_3 and Q_2D_3 (100 ng each) to which synthetic 5(*E*)-19-nor-10-ketovitamin D_3 had been added (100 ng) was eluted. (C) A mixture of Q_2D_3 and synthetic 5(*E*)-19-nor-10-ketovitamin D_3, irradiated with ultraviolet light, was eluted. Individual irradiations of Q_1D_3, Q_2D_3, or synthetic 5(*E*)-19-nor-10-ketovitamin D_3 produced similar chromatograms.

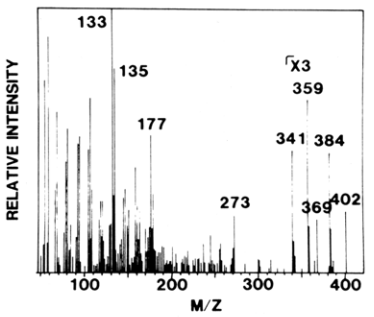

FIGURE 9: Low-resolution mass spectrum of a rumen microbial metabolite generated from 25-OH-D_3.

with the substitution of a keto function for the C(19)-methylene group. Thus, the UV, mass spectral, and NMR data indicate that Q_1 and Q_2 are the 5(*Z*) and 5(*E*) isomers of 19-nor-10-ketovitamin D_3 (Figure 7).

At this point we compared the compounds obtained from rumen fluid incubations with authentic synthetic 5(*E*)-19-nor-10-ketovitamin D_3 (Inhoffen et al., 1958, 1959). The UV, mass spectra, and NMR characteristics of synthetic 5(*E*)-19-nor-10-ketovitamin D_3 (Experimental Procedures) were cospecific with those of Q_2D_3. The synthetic compound and the natural product were also identical in their chromatographic behavior and their sensitivity to light, as indicated by the following experiments. An equimolar mixture of Q_1D_3 and Q_2D_3 was applied to HPLC. Q_1D_3 eluted at 40 mL and Q_2D_3 eluted at 45 mL (Figure 8A). A second injection was made with the addition of synthetic 5(*E*)-19-nor-10-ketovitamin D_3 to the mixture of the natural products. The synthetic compound coeluted with Q_2D_3 (Figure 8B). Since we had observed isomerization between Q_1 and Q_2 and 19-nor-10-ketovitamin D compounds are known to be light sensitive (Inhoffen et al., 1958), we tested the sensitivity to UV irradiation of Q_1D_3, Q_2D_3, and the synthetic compound. All three compounds, when irradiated separately, isomerized to mixtures of both isomers (chromatograms not shown). A mixture of Q_2D_3 and synthetic 5(*E*)-19-nor-10-ketovitamin D_3 was also irradiated. HPLC analysis showed that this combination gave rise to a mixture of isomers, corresponding to Q_1D_3 and Q_2D_3, upon exposure to UV light (Figure 8C).

We therefore conclude that the structures of Q_1D_3, Q_2D_3, Q_1D_2, and Q_2D_2 are 5(*Z*)-19-nor-10-ketovitamin D_3, 5(*E*)-19-nor-10-ketovitamin D_3, 5(*Z*)-19-nor-10-ketovitamin D_2, and 5(*E*)-19-nor-10-ketovitamin D_2. A subsequent isolation was conducted with the knowledge that we were manipulating light-sensitive compounds. *When care was taken to minimize exposure to light during isolation, only the 5(E) metabolites were observed as products of rumen incubations.*

[^{3}H]25-OH-D_3 was also incubated with rumen fluid microbes. A metabolite was isolated that had an UV absorbance spectrum with a maximum at 312 nm, i.e., consistent with a 5(*E*)-19-nor-10-keto chromophore. A low-resolution mass spectrum of the metabolite (Figure 9) had a molecular ion at *m/z* 402 (M^+) and major peaks at *m/z* 384, 369 ($M^+ - H_2O - CH_3$), 359 ($M^+ - C_3H_7$), 341 ($M^+ - C_3H_7 - H_2O$), 273 (M^+ – side chain), 179, 177, 135, and 133. These data, especially the unique fragments at *m/z* 359 and 341, indicate a structure of 5(*E*)-19-nor-10-keto-25-OH-D_3. Notably, this compound migrated very close to 1,25-$(OH)_2D_3$ in a hexane-based, normal-phase HPLC system but not in one based on dichloromethane.

Discussion

Polar microbial metabolites of vitamin D_3, vitamin D_2, and 25-OH-D_3 have been purified from incubations in bovine rumen fluid. The metabolites were identified as 5(*E*)-19-nor-10-ketovitamin D_3, 5(*E*)-19-nor-10-ketovitamin D_2, and 5-(*E*)-19-nor-10-keto-25-OH-D_3, respectively, by spectral and chemical means. Low-resolution mass spectra and ultraviolet absorbance spectra pointed out changes in the usual 5-(*Z*),7,10(19)-triene chromophore, and its replacement with a heretofore unknown metabolite chromophore, in all three of these metabolites. The vitamin D_3 metabolite had an elemental composition of $C_{26}H_{44}O_2$ according to high-resolution mass spectrometry, suggesting substitution of an oxygen atom for a methylene group. This notion was supported further by the metabolite's chemical reactivity, which indicated the presence of a single hydroxyl group and a ketone function. An NMR spectrum confirmed the absence of the C(19)-methylene group and indicated its replacement by an oxygen atom. These data provide inescapable evidence that each vitamin D metabolite had the C(19)-methylene group replaced by a 10-keto group and indicate that the 25-OH-D_3 metabolite was similarly derivatized.

Confirmation of the structural assignment, and specification of the C(5)-alkene stereochemistry as 5(*E*), rather than 5(*Z*), was achieved by comparing the natural product, generated from vitamin D_3, to authentic synthetic 5(*E*)-19-nor-10-ketovitamin D_3. The synthetic compound was identical, within experimental limits, with the natural product. The UV absorbance spectra had the same maxima, the mass spectral fragmentation patterns were the same, and the characteristic NMR signals were the same. Furthermore, the synthetic compound and the natural product comigrated on HPLC. They had also reacted similarly to ultraviolet irradiation. A mixture of the 5(*E*) and 5(*Z*) isomers, however, will be obtained if the samples are not protected adequately from ultraviolet light during isolation. It is possible that the 5(*Z*) isomer is formed directly from vitamin D. Alternatively, the 5(*E*) isomer could be formed first and undergo acid-catalyzed isomerization in rumen fluid to the isolated 5(*Z*) isomer. In fact, this type of dienone has been shown to undergo acid-catalyzed *E* to *Z* isomerization (Harrison & Lythgoe, 1958).

Fortunately, the 19-nor-10-keto-5(*E*),7-diene functionality has a distinctive chromophore with a λ_{max} at 312 nm. A distinctive mass spectral fragmentation pattern, different from that of the 5,7,10(19)-triene of vitamin D is helpful in identifying these compounds. Namely, the mass spectrum of vitamin D does not have a peak at $M^+ - 43$. On the other hand, it has major peaks (90–100% relative intensity) at 136 and 118 resulting from C(7)/C(8) bond cleavage and subsequent

dehydration (Okamura et al., 1976). The spectral characteristics of 19-nor-10-keto derivatives will be useful in determining whether this class of vitamin D metabolites occurs systematically in ruminants and nonruminants. In this regard, we have observed 5(*E*)-19-nor-10-ketovitamin D_3, but not the 5(*Z*) isomer, in bovine blood, indicating that it is absorbed.

In preliminary assays of activity, 5-μg doses of 19-nor-10-ketovitamin D_3 did not stimulate bone calcium mobilization in rats. The same dose did stimulate intestinal calcium absorption, but the effect was less than that produced by 50 ng of vitamin D_3.[2] It therefore appears that these metabolites are in a pathway of vitamin D metabolic deactivation. The generation of these metabolites in rumen would appear to explain bovine tolerance to oral vitamin D.

This paper has reported the observation and structural characterization of a unique class of vitamin D metabolites and has reported the first example of microbial vitamin D metabolism. It appears that these metabolites are significant to ruminant detoxification of vitamin D. Work is under way to determine whether nonruminant mammals have 19-nor-10-ketovitamin D derivatives in circulation.

Registry No. Vitamin D_3, 67-97-0; vitamin D_2, 50-14-6; 25-hydroxyvitamin D_3, 19356-17-3; 27-nor-25-ketovitamin D_3, 77531-55-6; 5(*E*)-19-nor-10-ketovitamin D_3, 62743-72-0; 5(*E*)-19-nor-10-ketovitamin D_2, 85925-89-9; 5(*E*)-19-nor-10-keto-25-hydroxyvitamin D_3, 85925-90-2.

[2] J. L. Sommerfeldt, J. L. Napoli, R. Gardner, D. C. Beitz, E. T. Littledike, and R. L. Horst, unpublished results.

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Spectral Properties of Three Quaternary Arrangements of *Pseudomonas* Pilin†

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ABSTRACT: *Pseudomonas aeruginosa* possess multisubunit, filamentous appendages called pili which are involved in adhesion, twitching motility, and bacteriophage adsorption. The spectral properties of three forms of pili have been compared. These are native pili, pilin dimers in octyl glycoside, and an in vitro assembled form of pilin which we call reassembled pilin filaments. Alkaline pH titrations, solvent perturbation, quenching of tryptophan fluorescence with acrylamide, and circular dichroism were used to demonstrate that tyrosines-24 and -27 are at a dimer/dimer interface in both native pili and in the reassembled pilin filaments. Dissociation of pili by octyl glucoside results in exposure of the two tyrosines and in partial exposure of a least one tryptophan in pilin.

Pili are filamentous, multisubunit appendages distinct from flagella found on the surfaces of bacteria. *Pseudomonas aeruginosa* pili are involved in such processes as adhesion of the bacteria to host mucosal surfaces (Woods et al., 1980), twitching motility (Bradley, 1980), and bacteriophage adsorption (Bradley & Pitt, 1974). The pili appear to be able to retract into the bacterial cell, thereby bringing attached bacteriophage in contact with the cell surface (Bradley, 1974). To explain the mechanism of assembly and disassembly of pili, it is important to understand the interactions at the regions of subunit contact. To do this we have compared the spectral properties of three different arrangements of pilin subunit. As will be seen below, these are native pili, pilin dimers in octyl glucoside (Watts et al., 1982a), and reassembled pilin filaments (Watts et al., 1982b).

Native pili consist of a single subunit, pilin, of M_r 15 000, according to its sequence (Sastry et al., 1983). Previous publications (Frost & Paranchych, 1977; Paranchych et al., 1979) had suggested a molecular weight of 18 000 based on migration of pilin in sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels and upon its amino acid composition. This led Folkhard et al. (1981) to interpret from X-ray fiber diffraction data that native pili consist of 4.06–4.08 subunits in

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