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Structures of Fecapentaenes, the Mutagens of Bacterial Origin Isolated from Human Feces†

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ABSTRACT: The structures of two fecapentaenes isolated by a combination of chromatographic procedures have been determined as 1-(1-glycero)tetradeca-1,3,5,7,9-pentaene (I) and 1-(1-glycero)dodeca-1,3,5,7,9-pentaene (II). Two additional fecapentaenes are tentatively assigned structures of geometric isomers of II.

The importance of exogenous factors, like chemicals, viruses, radiation, etc., in the etiology of cancer cannot be overemphasized. Epidemiological evidence points to the role that diet may play in the causation of many cancers, cancer of the large bowel being one of the most common varieties of the disease in the Western world (Doll & Peto, 1981). It therefore seemed logical to search for chemicals that might be responsible for colon cancer among those substances with which the intestine wall is in constant contact, i.e., in feces. The cancer-causing substances would be detected easily in most cases by a bacterial assay of mutagenicity (Ames et al., 1975). In fact, a purified fraction from feces of approximately 40% of the Caucasian population has been shown to be strongly mutagenic by using the *Salmonella* test strain TA-100 without activation (Bruce et al., 1977; Dion & Bruce, 1982; Reddy et al., 1980; Wilkins et al., 1980). It has also been shown that this mutagenic fraction is a product of bacterial action on feces within the colon rather than constituents of the bacteria residing in the large bowel itself (Wilkins et al., 1981).

The first attempts in our laboratory as well as those of Wilkins et al. to isolate the compound(s) from feces and characterize it (them) have shown that the mutagenic fraction is very labile (Bruce et al., 1981; Kingston et al., 1981). Also, it was shown that a certain UV spectrum (327, 340, 358 nm) accompanies the mutagenicity (Bruce et al., 1981, 1982) and that the instability of the mutagenic fraction is associated with exposure to light, oxygen, and acidity (Bruce et al., 1981; Kingston et al., 1981). The recognition of the chromophore as being characteristic of a conjugated pentaene (Bruce et al., 1981; Kingston et al., 1981; Oroshnik & Mebane, 1963; Hamilton-Miller, 1973; Weedon, 1969) exhibiting very high extinction coefficients ($\epsilon \sim 10^6$) suggested that very small quantities are involved (Bruce et al., 1981; Gupta et al., 1982). In order to isolate pure material in sufficient amounts to take ^{1}H NMR[1] spectra and mass spectra, as well as to study some of the elementary reactivity of the compounds, we have designed a procedure described under Methods. The analysis of these data led directly to the structures, as is discussed under Results.

Experimental Procedures

Materials

The feces were collected as described by Bruce et al. (1977, 1981) and anaerobically incubated according to Lederman et al. (1980) at 37 °C. The cooled batch (~2 kg) was thoroughly mixed with anhydrous Na_2SO_4 (~4 kg), anhydrous Na_2CO_3 (~300 g), and purified cellulose powder (~1 kg), and the resulting powder was stored until it was used as described under Methods.

Florisil (100–200 mesh, Fisher Scientific) and *silica gel* (60–200 mesh, J. T. Baker Chemical Co.) were thoroughly

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[1] Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; EI, electron ionization; CI, chemical ionization; Me_2CO, acetone; DMF-d_7, perdeuterated dimethylformamide.

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washed with 10% aqueous Na_2CO_3, H_2O, EtOH, and Et_2O, dried on air, and activated in a vacuum oven at 120 °C and 20 mmHg for 20 h. *Cellulose* (Whatman CF 11 medium fiber, ashless powder) was treated as florisil but activated at 100 °C, while *Sephadex LH-20* (25–100 μm, Pharmacia, Uppsala, Sweden) was used as such.

Solvents. All solvents were distilled immediately before use with 3% (w/w) anhydrous K_2CO_3 except tetrahydrofuran [distilled from 2% (w/w) CaH_2] and MeOH (distilled from Mg turnings). The solvents for HPLC were filtered through glass fiber filter paper.

Glassware was rinsed thoroughly with 10% aqueous Na_2CO_3 or K_2CO_3, water, and ethanol and dried in an oven. NMR tubes (5 mm) or cylindrical microcells (Wilmad Glass Co., Inc.) were treated with saturated aqueous Na_2CO_3 overnight, rinsed with H_2O, dried at 120 °C, treated with 99.7% D_2O overnight, dried at 120 °C, cooled under Ar, and kept in a desiccator until use.

Silica gel G_{254} was used for TLC; for analytical HPLC, *μPorasil* (30 cm × 3.9 mm, Waters) and *Zorbax ODS* columns (25 cm × 4.6 mm, Du Pont) were used. For preparative HPLC, a *Magnum 40* column (50 cm × 48 mm) dry packed with silica gel (53 μm, *Partisil* Prep 40, Whatman) and a cartridge (*PrepPak*-500/C_{18}, Waters) were used.

Methods

All operations were done in a darkened room (only desk lamps allowed far away from benches; the glassware was either red or wrapped in an aluminum foil), usually in N_2 or Ar atmosphere.

Chromatography: (*1*) *Extractive Chromatography on a Florisil Column.* Feces mix (1.8 kg, cf. Materials) was placed on top of a Florisil (1.5-kg) column prepared in C_6H_6 and washed with C_6H_6 (3 L), CH_2Cl_2 (2.5 L), 20% Me_2CO in CH_2Cl_2 (4 L), and Me_2CO (2 L). Mutagenic substances were usually eluted in the penultimate fraction as monitored by TLC in 10% MeOH in $CHCl_3$ visualized by long-wave UV lamps (Mineralight) as a green fluorescent spot. To the fractions containing mutagenic substances was added 0.01% antioxidant BHT (butylated hydroxytoluene, Sigma); this amount of BHT was added after every chromatographic step.

(*2*) *Separation on Silica Gel.* The combined fractions containing mutagens from ten Florisil columns were placed on a silica gel column (750–1000 g) prepared in hexane and eluted sequentially with hexane, CH_2Cl_2, a gradient of Me_2CO in CH_2Cl_2, Me_2CO, and MeOH. The TLC (as above) monitoring showed which Me_2CO fractions contained mutagens.

(*3*) *Separation on Sephadex LH-20.* The combined fractions containing mutagens from the silica gel columns were slowly fractionated on a Sephadex LH-20 column (300 g) prepared and run in acetone (flow rate 1 mL/min). The presence of mutagens was monitored by TLC (as above) and HPLC on a μPorasil column with 5% 2-propanol in CH_2Cl_2 and UV detection at 340 nm (Beckman, Model 324).

(*4*) *Preparative HPLC on Silica Gel.* Combined fractions containing mutagens from two Sephadex LH-20 columns were fractionated on a Magnum 40 Partisil column with a preparative HPLC system (Prep LC/system 500A, Waters) modified to allow UV detection at 340 nm (Du Pont UV spectrophotometer) according to J. Baptista, I. Gupta, and J. J. Krepinsky (unpublished results). The column was first flushed with 5% 2-propanol in CH_2Cl_2 containing 1% $EtNH_2$; the separation was done without the base (flow rate 100 mL/min).

(*5*) *Preparative reversed-phase HPLC* utilized two columns (PrepPak-500/C_{18}) in series, and the optimal mobile phase composition was determined by using the solvent selectivity triangle method on a Du Pont Series 8800 instrument and a Zorbax ODS (25 cm × 4.6 mm) column (Lehrer, 1981; Freeman, 1982; Glajch et al., 1980) as follows: acetonitrile–water–methanol–tetrahydrofuran, 36.2:32:25.4:6.4. The combined fractions from separation step 4 (cf. Figure 1) were injected into acetonitrile–MeOH (1:1) solution and eluted with the above quaternary solvent mixture (flow rate 100 mL/min, detection at 340 and 230 nm). The resulting fractions were monitored by HPLC on a Zorbax ODS (25 cm × 4.6 mm) column (no antioxidant was added to these fractions). The compound corresponding to band 4 (Figure 1) was isolated in pure form, while several fractions containing compounds **1**–**3** in various proportions were obtained, one of them being 90% **3** and 10% **2** (the last named fraction will be called compound **3** in the following discussions).

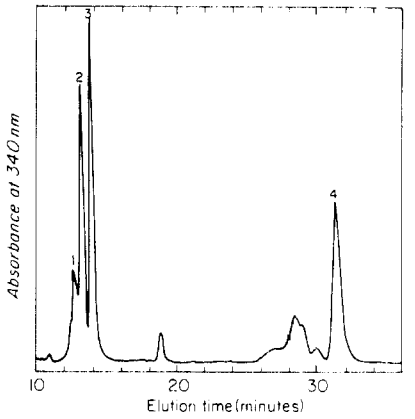


FIGURE 1: HPLC separation profile of fecapentaenes (after chromatography step 4) using C_{18} reversed phase and quaternary solvent mixture (cf. Methods); UV monitoring at 340 nm was employed.

Mutagenicity Assay. Fractions (cf. Figure 1) containing compounds **4** and combined compounds **1**–**3** as well as the fractions immediately before **1**, immediately after **4**, and those in between **4** and **1**–**3** as a group were examined by the *Salmonella* test (Ames et al., 1975) using TA-100 and TA-98 strains as described before (Bruce et al., 1977, 1981). Compound **4** as well as the mixture **1**–**3** showed the typical mutagenicity (approximately 5000 revertants/1 OD unit at 340 nm) while the other fractions were negative, including those between **1**–**3** and **4**.

Nuclear Magnetic Resonance. The samples dried at room temperature at 10^{-3} mmHg for at least 3 h were dissolved in either dimethylformamide-d_7 (99.96% isotopic purity, Merck Sharp & Dohme, Montreal) or benzene-d_6 (99.999% isotopic purity, Merck Sharp & Dohme, Montreal), and depending on the amounts of material available, the final volume was either 100 μL in a cylindrical microcell or 400 μL in a 5-mm NMR tube. Chemical shifts are quoted relative to $(CH_3)_4Si$ with residual ^1H solvent signals as an internal reference. All spectra were collected, and decoupling experiments performed, in the Fourier transform mode on a Nicolet 360-MHz instrument. In general, the spectrometer conditions were 4-kHz sweep width, 4-s cycle time, 16K data points, and 512–1024 (or more if required) scans.

Mass Spectrometry. The direct-inlet EI mass spectrometry using various conditions was not successful. HPLC interfaced with a mass spectrometer (LC/MS Hewlett-Packard, Model HP5985, interface for nonvolatiles) using the Zorbax ODS column (25 cm × 4.6 mm), and the quaternary solvent system (cf. Figure 1) was utilized to record good quality mass spectra (CI type) of compound **4**, and **1**–**3**.

Derivatization. It was anticipated that the purification of mutagenic substances might be assisted by derivatization of

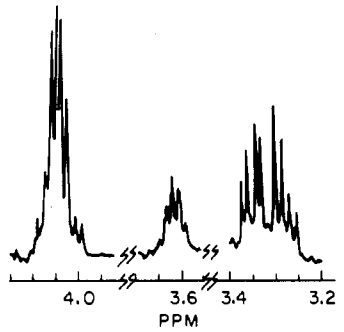


FIGURE 2: Typical NMR signals of the glycerol portion of fecapentaenes in benzene-d_6.

fractions containing mutagens **1–4** after chromatography step 3 or 4.

(*A*) *Acetylation and Benzoylation.* Mutagenic fractions (~5–10 mg) were allowed to stand overnight with 0.1 mL each of dry pyridine and acetic anhydride or benzoic anhydride. The reagents were distilled off at room temperature at 10^{-4} mmHg, and TLC (10% MeOH in $CHCl_3$) as well as HPLC (μPorasil, 5% 2-propanol in CH_2Cl_2) revealed the absence of the starting material and appearance of new less polar substances showing the same UV characteristics as the starting material. The derivatized fractions were hydrolyzed in 10% NaOH in aqueous EtOH (50%; 0.4 mL) for 4 h at 50 °C, giving materials corresponding to those before the acylations.

(*B*) *Attempted Methylation.* Mutagenic fractions (~5–10 mg) dissolved in 0.5 mL of Et_2O were treated with an excess of a solution of CH_2N_2 in Et_2O for 30 min at room temperature. No discoloration was observed, and the starting material exhibiting the characteristic UV absorptions was recovered after evaporation of CH_2N_2 and Et_2O.

(*C*) *Hydrogenation.* Compounds **3** and **4** were separately hydrogenated in MeOH with Pd/C (10%) as a catalyst for 42 h. The catalyst was filtered off, the solution was diluted with H_2O, and the products were taken up into pentane and examined by gas chromatography using Varian gas chromatography system Vista 44 connected with chromatography data system Vista 401 (column 10% AT-1000 on Chromosorb WAW 80–100 mesh, 5 ft × $^1/_8$ in., 180 °C, N_2 flow rate 25 mL/min).

Results

Fecapentaene-14 (*I*). The structure of compound **4** was essentially deduced from its NMR spectra [cf. Serdarevich & Carroll (1966), Hermetter & Paltauf (1982), and Kresge & Chen (1972)] (cf. Figures 2 and 3): NMR (DMF-d_7) δ 6.84 (1 H, d, J = 12 Hz, -OCH=), 6.10–6.35 (7 H, m, -CH=CH-), 5.65–5.75 (2 H, m, OCH=C*H*-[2] and =C*H*-CH_2), 4.10 (2 H, dd, J = 6 and 12 Hz, $-OCH_2-$), 3.80 (1 H, m, C*H*OH), 3.65 (2 H, dd, J = 4.5 and 12 Hz, $-CH_2OH$), 2.09 (2 H, m, $=CHCH_2-$), 1.23 [s, $(CH_2CH_2)_n$], and 0.87 (3 H, t, J = 8 Hz, CH_2CH_3); NMR (C_6D_6) δ 6.35 (1 H, d, J = 12 Hz), 5.60–3.70 (2 H, m, -OCH=C*H*- and =C*H*-CH_2), 5.40–5.57 (7 H, m, -CH=CH-), 4.05 (2 H, dd, J = 4.5 and 12 Hz, $-CH_2O-$), 3.62 (1 H, m, -C*H*OH), 3.30 (2 H, dd, J = 6 and 12 Hz, $-CH_2OH$), 2.1 (2 H, m, $=CHCH_2$), 1.235 [s, $(CH_2CH_2)_n$], and 0.93 (3 H, t, J = 8 Hz, CH_2CH_3).

The above assignments were confirmed by the decoupling experiments. The presence of a small amount of impurities in the samples made it difficult to estimate how many CH_2 groups (1.235 ppm) were present in the molecule. The trans geometry of the double bond OCH=CH- follows from the coupling constant observed on H-1 (12 Hz). The stereochemistry of the other double bonds could not be determined at this stage. The uncertainty about the length of the aliphatic chain was solved by mass spectrometry: the mass spectrum exhibited an M + 1 ion at m/e 279 corresponding to a C_{14} unsaturated chain as it is portrayed in formula I. This chain length was further corroborated by the presence in the spectrum of fragments at m/e 205 for $(C_{14}H_{21}O)^+$ and at m/e 187 for $(C_{14}H_{19})^+$. This formula also explains the unreactivity of I toward CH_2N_2, the stability toward bases, and the esterification reactions.

We utilized the instability of vinyl ethers in acidic media in hydrogenation of I (fecapentaene-14) using acidic catalyst Pd/C. Tetradeca-2,4,6,8-tetraen-1-al is formed first (Scheme I); it gives on hydrogenation either tetradecanal, tetradecanol [X, R = $(CH_2)_3CH_3$], or tetradecane [XI, R = $(CH_2)_3CH_3$], depending on the course of hydrogenation. In fact, gas chromatographic comparison with appropriate standards with

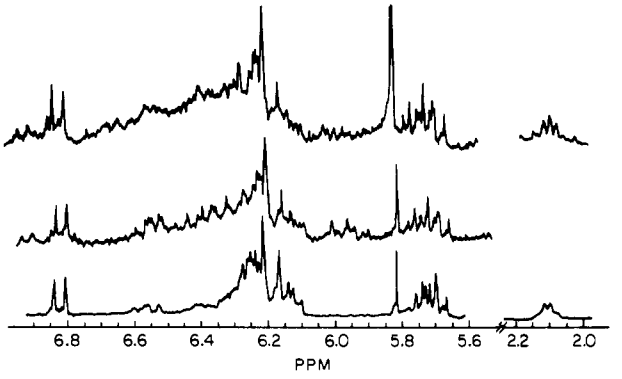
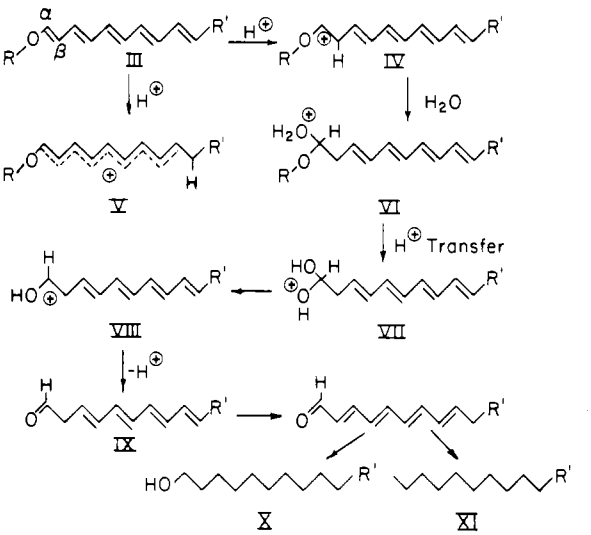
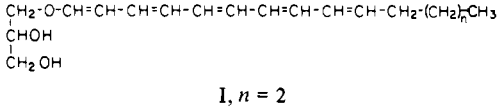


FIGURE 3: NMR signals of the fecapentaenes related to the unsaturated system in DMF-d_7: bottom tracings, fecapentaene-14; central tracing, fecapentaene-12 (compound **3**); top tracings, compounds **1–3** (cf. text and Figure 1). The signal at δ 5.82 is assigned to residual CH_2Cl_2.

Scheme I: Reaction Pathway Leading to Formation of Saturated Aliphatic Compounds from Fecapentaenes



[2] In our previous paper (Bruce et al., 1982), to this proton was erroneously assigned a signal at δ 5.14 (dd, J = 12 and 6 Hz).



I, n = 2
II, n = 0

chain lengths over C_{10} showed that only tetradecanol was formed [X, R = $(CH_2)_3CH_3$].

Having thus established the gross structure of I, we hoped that the chromophore would interact with the chiral C-2 carbon of the glycerol moiety to such an extent that it would yield a measurable Cotton effect in circular dichroism. However, the compound showed no Cotton effect in CH_2Cl_2 solution.

Fecapentaene-12 (II). The structure of compound **3** was deduced similarly to the case of fecapentaene-14. Its NMR spectrum showed the pattern of signals between δ 3.2 and 4.1 (the intensities of the multiplets centered at δ 4.05, 3.62, 3.31 were again in the ratio 2:1:2) as in the previous case, and the spectra of both compounds were virtually identical in this region (cf. Figure 2). The differences became noticeable in the olefinic region (cf. Figure 3, the middle tracing) and in the splitting pattern of the allylic protons at δ 2.11. While the olefinic proton located on the ethereal carbon maintains its characteristic shape and position at δ 6.84, the rest of the signals show a markedly changed pattern. The mass spectrum obtained under the same conditions as in the case of fecapentaene-14 showed the presence of an $(M + 1)^+$ ion at m/e 251, suggesting strongly that the chain in the case of compound **3** is shorter by two methylene units, thus containing 12 carbons.[3,4] Again, we corroborated this finding by gas chromatographic analysis of the hydrolysis–hydrogenation products as in the case of fecapentaene-14 and found as the only aliphatic alcohol, dodecanol, $C_{12}H_{25}OH$ (X, R = CH_2CH_3). It is possible to explain the differences in the olefinic regions in the NMR spectra of fecapentaene-14 and -12 by the presence of various geometrical isomers of the double bonds in fecapentaene-12. This possibility is further supported by the following facts: The NMR spectrum in the olefinic region of a mixture of compounds **1–3** (ratio approximately 15:50:35) shown as the upper tracings in Figure 3 is more complex than the tracing below it. This suggests that even greater variety of the double bonds is present in this sample. The mass spectra taken at various points between compounds **1**, **2**, and **3** (cf. Figure 1) show consistently only the same spectrum, i.e., M + 1 ion at m/e 251. Consequently, we conclude that compounds **1–3** are various cis,trans isomers of the same general structure of fecapentaene-12 (II).

Discussion

Fecapentaene-12 and -14 may not be the only chain lengths present in feces. We have observed some minor constituents in our not completely purified samples that according to their chromatographic properties might contain longer chains than 14. It is, however, unlikely that we could isolate these compounds because of the instability of fecapentaenes. Even by taking all the precautions described under Methods (dark room, inert atmosphere, antioxidant additions, extensive "basification"), fecapentaenes decomposed to a certain degree. In fact, the purer the samples, the faster was the decomposition (Bruce et al., 1982). The explanation of this phenomenon in all likelihood lies in an association of fecapentaenes with other lipids in micelles thus protecting the most sensitive part of the molecule. When the other lipids are removed, fecapentaenes may not form micelles themselves, and consequently are exposed to acidity and oxidizing agents.

Another difficulty connected with the isolation of fecapentaenes was the remarkable variability of feces. No two batches were quite identical and sometimes were very different, depending apparently on the variation in diet and overall status of the donor. We added another factor of variability by pooling the feces of different donors. Thus very often we observed during our isolation procedure the appearance of unusual accompanying compounds in some batches. The structures of fecapentaenes, however, are now known in sufficient detail to prepare the pure compounds in larger quantities by synthesis, which would make it possible to study their chemical and biological properties in detail.

The mutagenicity of the fecapentaenes is one of the highest among the mutagens (5000 revertants/1 OD unit; 1 OD $\sim$ 1 μg). This is probably due to the ease with which a highly stabilized (by charge delocalization) carbocation can be formed. While the significance of the fecapentaenes with relation to the origin of colon cancer remains to be determined, it seems likely that those compounds represent some hazard for human health since virtually all direct-acting mutagens are carcinogenic to some degree (McCann & Ames, 1976). These compounds are likely to represent only a fraction of the mutagen exposure faced by colonic cells in the human body (Venitt, 1982). Nevertheless, detailed structural information should lead to the development of more rational methods to reduce the level of the exposure and the risk of malignancy in the colon (Dion et al., 1982).

Acknowledgments

Our special thanks are due to many anonymous donors and collectors of fecal samples without whose cooperation this work could never have been undertaken and completed. We are also indebted to Dr. A. G. Harrison, Chemistry Department, University of Toronto, and Dr. B. H. Khouw, Chemistry Department, York University, Toronto, for their patient help with EI mass spectrometry.

Registry No. I, 84000-58-8; II, 84000-59-9; tetradeca-2,4,6,8-tetraen-1-al, 84000-60-2; tetradecanal, 124-25-4; 1-tetradecanol, 112-72-1; tetradecane, 629-59-4; 1-dodecanol, 112-53-8.

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[3] This fact suggests that fecapentaene-12 is most likely identical with the "potent mutagen from feces" whose structure was reported recently (Kingston, 1982).

[4] Added in Proof: The communication of Hirai and co-workers has just appeared; cf. Hirai et al. (1982).

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Articles

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

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

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

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

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