DeKruijff, B., Gerritsen, W. J., Oerlemans, A., Van Dijck, P. W. M., Demel, R. A., & Van Deenen, L. L. M. (1974b) *Biochim. Biophys. Acta 339*, 44–56.
Feingold, D. S. (1965) *Biochem. Biophys. Res. Commun. 19*, 261–267.
Heider, J. G., & Boyett, R. L. (1978) *J. Lipid Res. 19*, 514–518.
Holland, J. F., Teets, R. E., & Timnick, A. (1973) *Anal. Chem. 45*, 145–153.
Huang, C. (1969) *Biochemistry 8*, 344–352.
Kinsky, S. C. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1049–1056.
Kinsky, S. C. (1963a) *Arch. Biochem. Biophys. 102*, 180–188.
Kinsky, S. C. (1963b) *Antimicrob. Agents Chemother. (1962)*, 387–394.
Kinsky, S. C., Avruch, J., Permutt, M., Rogers, H. B., & Schonder, A. A. (1962) *Biochem. Biophys. Res. Commun. 9*, 503–507.
Kinsky, S. C., Luse, S. A., & Van Deenen, L. L. M. (1966) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 25*, 1503–1510.
Kinsky, S. C., Demel, R. A., & Van Deenen, L. L. M. (1967a) *Biochim. Biophys. Acta 135*, 835–843.
Kinsky, S. C., Luse, S. A., Zopf, D., Van Deenen, L. L. M., & Haxby, J. (1967b) *Biochim. Biophys. Acta 135*, 844–861.
Lampen, J. O., Arnow, P. M., Borowska, Z., & Laskin, A. I. (1962) *J. Bacteriol. 84*, 1152–1160.
Norman, A. W., Demel, R. A., DeKruijff, B., & Van Deenen, L. L. M. (1972a) *J. Biol. Chem. 247*, 1918–1929.
Norman, A. W., Demel, R. A., DeKruijff, B., Geurts Van Kessel, W. S. M., & Van Deenen, L. L. M. (1972b) *Biochim. Biophys. Acta 290*, 1–14.
Norman, A. W., Spielvogel, A. M., & Wong, R. G. (1976) *Adv. Lipid Res. 14*, 127–170.
Patterson, J. M., Olinger, M. R., Holland, J. F., & Bieber, L. L. (1979) *J. Antibiot.* (in press).
Rose, H. G., & Oklander, M. (1965) *J. Lipid Res. 6*, 428–434.
Schroeder, F., & Bieber, L. L. (1975) *Insect Biochem. 5*, 201–221.
Schroeder, F., Holland, J. F., & Bieber, L. L. (1972) *Biochemistry 11*, 3105–3111.
Schroeder, F., Holland, J. F., & Bieber, L. L. (1973) *Biochemistry 12*, 4785–4789.
Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245–281.
Tillack, T. W., & Kinsky, S. C. (1973) *Biochim. Biophys. Acta 323*, 43–54.
Van Hoogevest, P., & DeKruijff, B. (1978) *Biochim. Biophys. Acta 511*, 397–407.
Verkleij, A. J., DeKruijff, B., Gerritsen, W. F., Demel, R. A., Van Deenen, L. L. M., & Ververgaert, P. H. J. (1973) *Biochim. Biophys. Acta 291*, 577–581.
Weber, M. M., & Kinsky, S. C. (1965) *J. Bacteriol. 89*, 306–312.

25-Hydroxyvitamin D_3 26,23-Lactone: A New in Vivo Metabolite of Vitamin D†

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ABSTRACT: A major vitamin D metabolite was isolated in pure form from the blood plasma of chicks given either maintenance levels or large doses of vitamin D_3. The isolation involved methanol–chloroform extraction and five column chromatographic procedures. The metabolite purification and elution position on these columns were followed by a competitive protein binding assay. The metabolite was identified, using high- and low-resolution mass spectrometry, 270-MHz proton nuclear magnetic resonance spectrometry, ultraviolet absorption spectrophotometry, Fourier transform infrared spectrophotometry, and specific chemical reactions, as 3β,-25-dihydroxy-9,10-seco-5,7,10(19)-cholestatrieno-26,23-lactone. The trivial names 25-hydroxyvitamin D_3 26,23-lactone or calcidiol 26,23-lactone are suggested for this compound.

The metabolism of vitamin D_3 to physiologically active forms has been well established (DeLuca & Schnoes, 1976; Kodicek, 1974; DeLuca, 1974). Vitamin D_3 is first hydroxylated in liver to 25-hydroxyvitamin D_3 (25-OH-D_3).[1] 25-OH-D_3 is then hydroxylated in kidney to form either 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the most active form of the vitamin, or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. Other known metabolites include 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] and 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$]. These compounds, however, do not represent all vitamin D_3 metabolites. Other metabolites have been detected, several of which appear to have their circulating levels influenced by dietary factors such as calcium and phosphorus (Ribovich & DeLuca, 1978a–c).

During the course of developing an assay procedure for determining plasma levels of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$, a previously unidentified compound was detected in chick plasma by using the competitive protein binding radioassay technique (Horst et al., 1979; Shepard et al., 1979). We wish to report here the isolation of this vitamin D_3 metabolite from plasma of chicks receiving maintenance and

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,-24,25-trihydroxyvitamin D_3; MLP, plasma from chicks on maintenance levels of vitamin D_3; HDP, plasma from chicks given high doses of vitamin D_3; LC, high-pressure liquid chromatography; FT-IR, Fourier transform infrared spectroscopy; Me_3Si, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

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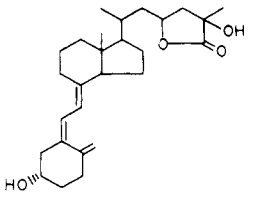

Calcidiol lactone
25 hydroxy vitamin D_3-26,23-lactone
3β,25 dihydroxy-9,10-seco-5,7,10(19) cholestatrieno-26,23 lactone

FIGURE 1: Structure of calcidiol lactone or 25-hydroxyvitamin D_3 26,23-lactone or 3β,25-dihydroxy-9,10-seco-5,7,10(19)-cholestatrieno-26,23-lactone.

elevated levels of vitamin D_3 and its identification as 25-hydroxyvitamin D_3 26,23-lactone (Figure 1).

Materials and Methods

Procurement and Extraction of Chick Plasma from Chicks Given Maintenance Levels of Vitamin D_3. A total of 24 L of blood was collected by decapitation of 8-week-old fryers (A-G coop; Arcadia, WI). These chicks had been fed a diet containing a maintenance level of vitamin D_3 (1000 IU/lb of feed). Blood was collected in containers having a sufficient amount of 0.1 M sodium oxalate, pH 7.0, to give a final volume/volume concentration of 10% and stored at 0 °C until used (not more than 4 h). The blood was separated in a continuous flow DeLaval blood separator and yielded 16 L of plasma.

Plasma was then heated with stirring at 70 °C for 1 h in 1 L batches. After cooling to 4 °C, we centrifuged the denatured plasma at 13000*g* for 1 h in a Beckman J-21C centrifuge equipped with a JA-14 rotor. This facilitated handling large volumes of plasma and gave the same results as when plasma was extracted directly with methanol–chloroform. The recovered pellet was resuspended in 400 mL of distilled water and extracted at 4 °C for 1 h with 2:1 methanol–chloroform by using the procedure of Blunt et al. (1968).

The combined chloroform phases from all extractions were concentrated and partitioned at 4 °C in 500 mL of hexane and 500 mL of 10% water in methanol for 1 h with shaking. The phases were separated, and the methanol–water phase was extracted 2 times with 500 mL of chloroform. Ethanol was added to the chloroform extract, and solvents were evaporated under vacuum. The residue taken up in dry chloroform was then used for chromatography.

Procurement and Extraction of Plasma from Chicks Receiving High Levels of Vitamin D_3. Seventy-one, 1-day-old white Leghorn Cockerals (Northern Hatcheries, Beaver Dam, WI) were maintained on a purified 22% protein diet containing 3% calcium for 10 weeks. The chicks were then dosed intramuscularly with 10^5 IU of vitamin D_3 (Aldrich Chemicals, Milwaukee, WI) in 50 μL of ethanol daily for 3 days. On the fourth day they were dosed intramuscularly with 10^7 IU of vitamin D_3 in four doses in 125 μL of ethanol. Five days after this dose, blood was collected by cardiac puncture using a small amount of heparin to avoid clotting. The blood was immediately centrifuged and 1.1 L of plasma was obtained. The plasma was extracted at 4 °C for 1 h by using 2:1 methanol–chloroform as described by Blunt et al. (1968). The chloroform phase was evaporated, and the residue was dried by ethanol azeotrope and used for chromatography.

Chromatographic Purification Procedures. The chloroform extracts of both plasma samples were first chromatographed on a 3 × 30 cm Sephadex LH-20 column eluted with 9:1:1 hexane–chloroform–methanol. The plasma extract obtained from chickens given maintenance levels (MLP) of vitamin D_3 was chromatographed in three equal batches while the plasma extract from chicks given large doses of vitamin D_3 (HDP) was chromatographed in one run. A total of 110 fractions (9 mL) were collected from each column run. Fractions were assayed by using the competitive protein binding method of Haddad et al. (1977). For MLP runs, an aliquot equivalent to 10 mL of plasma was used for assay purposes while the HDP run was assayed by using 1 mL of plasma-equivalent aliquots. The competitive binding peak eluting from 618 to 806 mL was pooled and concentrated from the MLP sample. A larger binding peak eluting from 608 to 913 mL was pooled and concentrated from the HDP sample. These binding peaks were then chromatographed individually on a 2 × 55 cm Sephadex LH-20 column eluted with 70:30 chloroform–hexane. The MLP sample was chromatographed in three equal batches while the HDP sample was chromatographed in one run. A total of 110 fractions (6 mL) were collected and assayed as described above. The binding peak eluting from 240 to 258 mL was pooled and concentrated from the HDP sample, while the binding peak eluting from 240 to 318 mL was pooled and concentrated from the MLP sample. The peak from the MLP sample was further chromatographed on a 1 × 58 cm Sephadex LH-20 column eluted with 9:1:1 hexane–chloroform–methanol. Seventy fractions (3.7 mL) were collected and assayed as described above. The binding peak eluting from 133 to 162 mL was pooled and concentrated.

The MLP peak from the 1 × 58 cm Sephadex LH-20 column and the HDP sample from the 2 × 55 cm Sephadex LH-20 column were then subjected to high-pressure liquid chromatography (LC) on a Waters Model ALP/6PC 204 instrument equipped with a Model 440 absorbance detector (Waters Associates, Milford, MA). The samples were chromatographed on a 0.46 × 25 cm Partisil ODS (Whatman Inc., Clifton, NJ) column eluted with 25% v/v water in methanol at a flow rate of 1.5 mL/min. A total of 40 1-mL fractions were collected in both cases and assayed as described above. The binding peak eluting from 25.5 to 28.5 mL was pooled from both runs. Both MLP and HDP samples were rechromatographed by using the same system, and the sole binding peak and major ultraviolet (254-nm) absorbing peak eluting from 25.5 to 28.5 mL were pooled and concentrated. These samples were further purified by LC using a 0.46 × 25 cm Zorbax-SIL (Du Pont, Inc.) column eluted with 8% v/v 2-propanol in hexane at a flow rate of 2 mL/min. A total of 40 1-mL fractions were collected and assayed as described above. The sole binding peak and major 254-nm absorbing peak eluting from 14.5 to 16.5 mL were pooled for both samples. These samples were then rechromatographed on the same system, and the sole binding peak and sole ultraviolet 254-nm absorbing peak eluting from 14.5 to 16.5 mL were pooled for each sample and used for structural identification.

Spectroscopy. Ultraviolet spectra were recorded from ethanol solutions by using a Beckman Model 24 spectrophotometer.

Low-resolution and high-resolution mass spectra were obtained by using an AEI 902 mass spectrometer (Associated Electrial Industries, Ltd., Manchester, England) interfaced with a DS-50 data system (Data General Corporation, Southboro, MA). All spectra were run at 70 eV, with a source temperature between 100 and 130 °C above ambient temperature.

The Fourier transform infrared (FT-IR) spectrum was obtained from a chloroform solution by using a Nicolet 7199 FT-IR instrument (Nicolet Instrument Corp., Madison, WI).

CHROMATOGRAPHY OF PLASMA EXTRACTS

column No. HDP	MLP	
1	1	3 x 30 cm LH-20 9:1:1 hexane:$CHCl_3$:MeOH pool 25,26-$(OH)_2D_3$ region
2	2	2 x 55 cm LH-20 70:30 $CHCl_3$:hexane pool 24,25-$(OH)_2D_3$ region
	3	1 x 58 cm LH-20 9:1:1 hexane:$CHCl_3$:MeOH pool 25,26-$(OH)_2D_3$ region
3,4	4,5	HPLC on .45 x 25 cm Zorbax ODS 25% H_2O in MeOH pool binding peak eluting before 24,25-$(OH)_2D_3$
5,6	6,7	HPLC on .45 x 25 cm Zorbax-SIL 8% iPrOH in Hexane pool binding peak eluting before 24,25-$(OH)_2D_3$

HDP = High dosed D_3 plasma extract

MLP = Maintenance level D_3 plasma extract

FIGURE 2: Flow sheet of the isolation of calcidiol lactone from both MLP and HDP samples.

Proton nuclear magnetic resonance spectra were taken in $CDCl_3$ solution (56 μg of compound in 190 μL) on a Brucker 270-MHz instrument (Bruker Instruments, Billerica, MA) at 4 and 21 °C.

Preparation of Derivatives. The trimethylsilyl (Me_3Si) derivative was prepared by reacting 1 μg of compound in 30 μL of pyridine with 25 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride at 55 °C for 40 min. The reaction mixture was purified by LC on a 0.46 × 25 cm Zorbax-SIL column eluted with 5% v/v ethyl acetate in hexane. The major 254-nm absorbing peak eluting between 8.5 and 9.5 mL was collected and used for mass spectrometry and further derivatization.

The methyl lactol of the Me_3Si derivative was prepared by reacting 1 μg of $(Me_3Si)_2$ compound (prepared as above) with 25 μL of 0.01 M methyllithium in diethyl ether at room temperature for 1 h. The reaction was quenched with 50 μL of water and extracted twice with 200 μL of methylene chloride. The methylene chloride extract was purified by LC on a 0.46 × 25 cm Zorbax-SIL column eluted with 5% ethyl acetate in hexane. The major 254-nm absorbing peak eluting between 30 and 42 mL was collected and concentrated for mass spectrometry and further derivatization.

The $(Me_3Si)_3$ derivative of the methyl lactol was prepared by reacting 1 μg of $(Me_3Si)_2$ methyl lactol (prepared as described above) in 30 μL of pyridine with 25 μL of BSTFA as described above for the preparation of the $(Me_3Si)_2$ derivative. The reaction mixture was used without further purification for mass spectrometry.

Results and Discussion

A flow sheet of the procedures used in the isolation of the metabolite is presented in Figure 2. To conserve space, the actual column profiles are not presented except for the final LC profile demonstrating homogeneity of the isolated substance from the HDP sample (Figure 3). To follow the purification and elution position of the metabolite, we used the binding assay of Haddad et al. (1977) as modified by Horst et al. (1979). Following our identification of the lactone, we determined that it is 5 times more effectively bound to the rat plasma transport protein than is 25-hydroxyvitamin D_3. It is interesting that the lactone shows the highest affinity among the vitamin D metabolites for the serum vitamin D transport protein.

The ultraviolet spectrum of the compound isolated from the HDP sample is presented in Figure 4. The spectrum displays the typical vitamin D_3 triene chromophore with λ_{max} = 264 nm, λ_{min} = 228 nm, and OD_{264}/OD_{228} = 1.51. The ultraviolet spectrum from the MLP sample (not shown) gave an essentially identical spectrum with λ_{max} = 264 nm, λ_{min} = 229 nm, and OD_{264}/OD_{229} = 1.40. A total recovery of 8 μg was calculated for the MLP sample, assuming an ε of 18 600 M^{-1} (DeLuca, 1971), and a total recovery of 56.3 μg was calculated for the HDP sample, assuming the same ε.

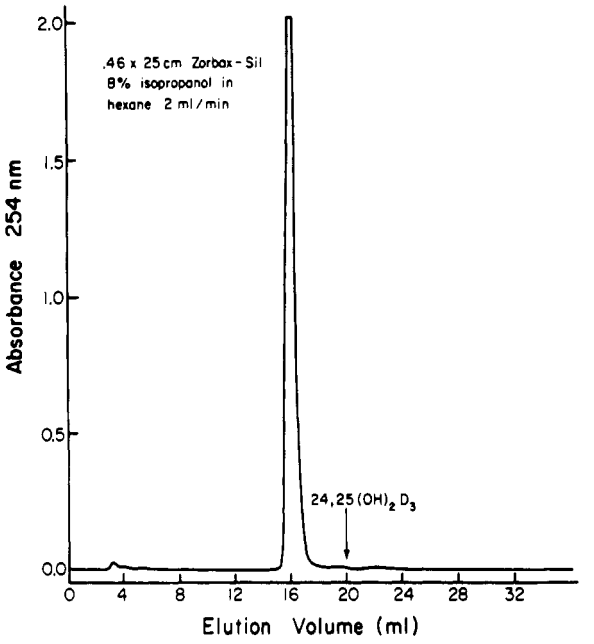

FIGURE 3: Column profile of the final LC purification step from the HDP sample. The arrow indicates the elution volume of 24,25-$(OH)_2D_3$.

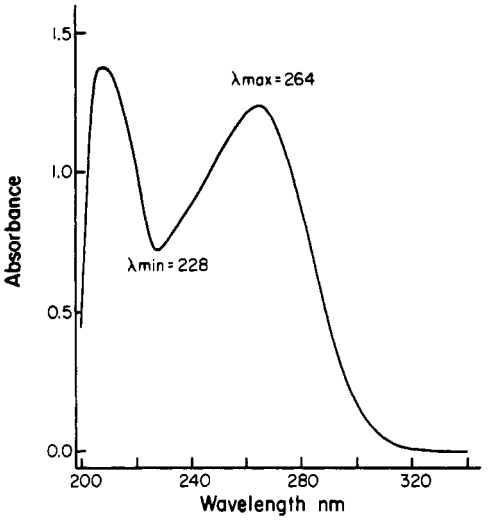

FIGURE 4: Ultraviolet absorption spectrum of the isolated calcidiol lactone taken in ethanol.

Identical low-resolution mass spectra were obtained from the compound isolated from both sources. Figure 5 shows the mass spectrum of the compound isolated from the HDP sample. The diagnostic ions, relative intensities, and structural assignments are as follows: *m/e* 428, 27.6%, M^+; *m/e* 410, 4.1%, $M^+ - H_2O$; *m/e* 395, 12.0%, $M^+ - H_2O - CH_3$; *m/e* 271, 4.5%, M^+ – side chain; *m/e* 253, 9.3%, M^+ – side chain – H_2O; *m/e* 136, 100%, A ring + C-6 + C-7$^+$ (A-ring fragment$^+$); *m/e* 118, 94%, A-ring fragment$^+ - H_2O$. This spectrum displays the diagnostic ions (*m/e* 271, 253, 136, and 118) of vitamin D_3 derivatives containing only the 3β-hydroxyl group on the A ring (DeLuca, 1971). It is thus assumed that

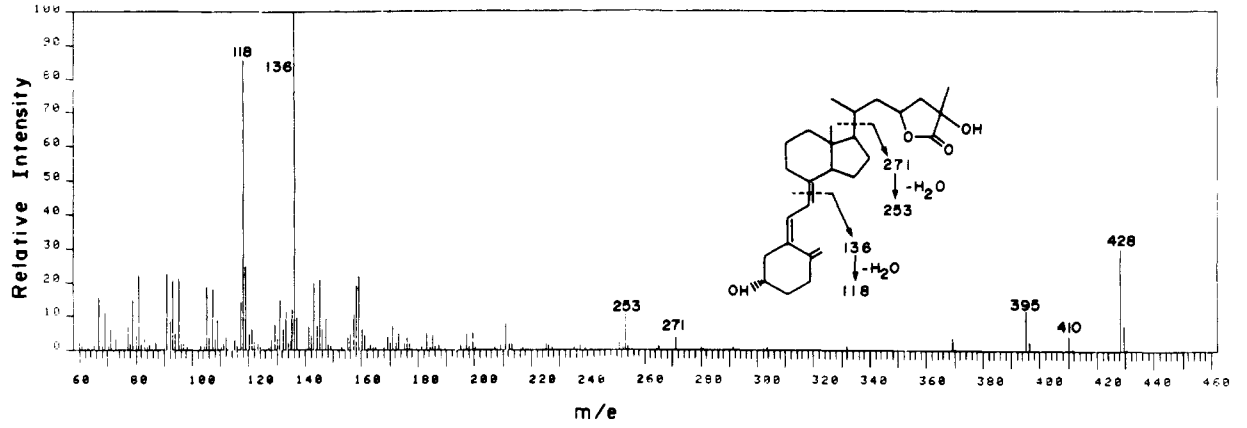

FIGURE 5: Mass spectrum of calcidiol lactone.

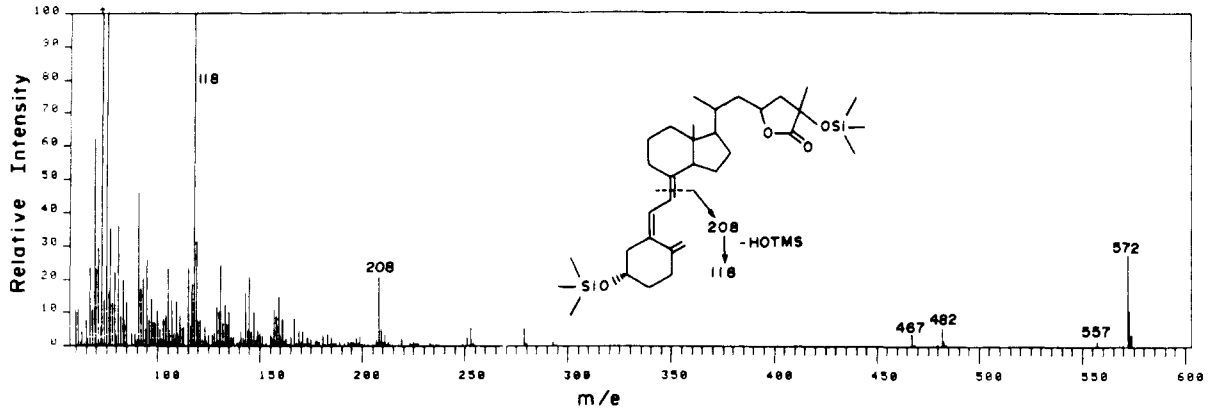

FIGURE 6: Mass spectrum of the $(Me_3Si)_2$ derivative of calcidiol lactone.

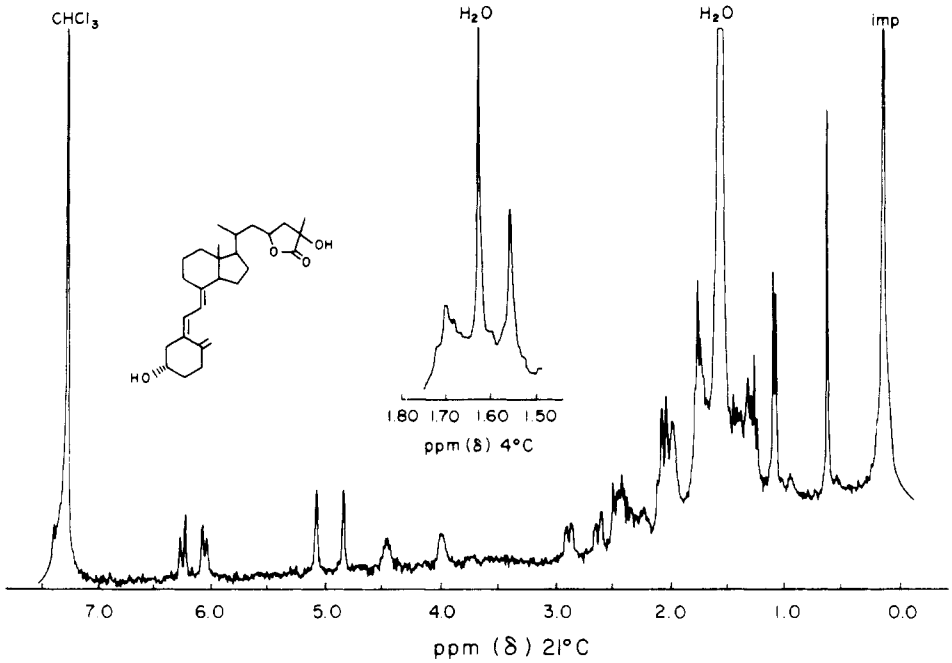

FIGURE 7: Proton nuclear magnetic resonance spectrum of calcidiol lactone taken in $CDCl_3$ at 21 °C. Insert shows the δ 1.5–1.8 region at 4 °C.

the isolated compound has the monohydroxylated (3β-OH) A ring of vitamin D_3. The fragments at *m/e* 271 and 253 further illustrate that the secosteroid nucleus of vitamin D has remained unchanged and that all the metabolic alterations have taken place on the side chain.

High-resolution mass spectrometry of the compound yielded a molecular weight of 428.2901 for the MLP sample and 428.2905 for the HDP sample which requires the molecular formula $C_{27}H_{40}O_4$ (calcd M_r of M^+ = 428.2926). The high-resolution mass spectra also confirmed the peak assignments given for the low-resolution mass spectra. Thus, the compound is a derivative of vitamin D_3 and not a dihydroxylated derivative of vitamin D_2 ($C_{28}H_{44}O_3$; calcd M_r of M^+ = 428.3290). The ions of *m/e* 271 ($C_{19}H_{27}O$) and 253 ($C_{19}H_{25}$) which represent the steroid nucleus establish the presence of the other three oxygen atoms on the side chain.

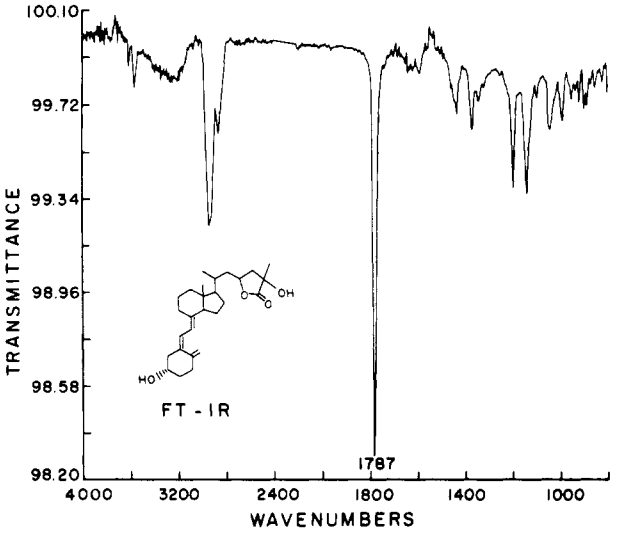

FIGURE 8: Fourier transform infrared spectrum of calcidiol lactone taken in chloroform.

Identical mass spectra were also obtained from the Me_3Si derivative of the compound isolated from both sources. Figure 6 presents the mass spectrum of the $(Me_3Si)_2$ derivative of the compound. Diagnostic ions, their relative intensities, and structural assignments are as follows: *m/e* 572, 20%, M^+; *m/e* 482, 15%, $M^+ - HOSiMe_3$; *m/e* 467, 10%, $M^+ - HOSiMe_3CH_3$; *m/e* 208, 34%, A-ring fragment$^+$; *m/e* 118, 100%, A-ring fragment$^+ - HOSiMe_3$. The formation of only a $(Me_3Si)_2$ derivative establishes that only two of the four oxygen atoms of the compound are present as free hydroxyl groups, one of which must be on the side chain. The ions of *m/e* 208 and 118 indicate only one hydroxyl group on the A ring, probably at the 3β position. The location of the hydroxyl group on the side chain cannot be deduced from the mass spectral data. The two nonhydroxyl oxygen atoms must then be present as carbonyl functions and/or as ethers or esters.

The 270-MHz proton NMR spectrum of the metabolite is presented in Figure 7. The spectrum shows the typical resonances for the olefinic protons of the vitamin D 5,6-*cis*-triene chromophore, including the C-6 and C-7 protons (AB quartet centered at δ 6.14) and the C-19 protons at δ 4.82 and 5.05. The multiplet resonance at δ 3.97, which represents the 3α proton, confirms the presence of a 3β-hydroxy group (in the spectrum of vitamin D_3 this signal occurs at δ 3.95). The sharp three-proton singlet at δ 0.63 and the three-proton doublet at δ 1.09 can be assigned to the C-18 and C-21 methyl groups, respectively. Both resonances are shifted slightly downfield from their position in the spectrum of vitamin D_3. The doublet resonance (J = 6.3 Hz) for the C-21 protons indicates that C-20 is not functionalized. The key signals in the spectrum of the metabolite are the one-proton multiplet at δ 4.46, which must represent a carbinyl proton of an ether or ester, and the three-proton singlet at δ 1.56 (see 4 °C inset in Figure 7) which can be assigned to a terminal methyl group (C-27) of the side chain. The singlet resonance for this methyl group requires that C-25 be substituted, and the absence of any signal attributable to another methyl group implies that C-26 is fully substituted or oxidized. Since the side chain must contain one hydroxy group, the postulate of a C-25 hydroxy and a C-26 carbonyl function is consistent with these observations and also explains the downfield chemical shift of the C-26 methyl group (δ 1.56). Assignment of the third side-chain oxygen as a part of a lactone then accounts for the carbinyl proton signal at δ 4.46 and satisfies the elemental composition determined for the metabolite. The only logical

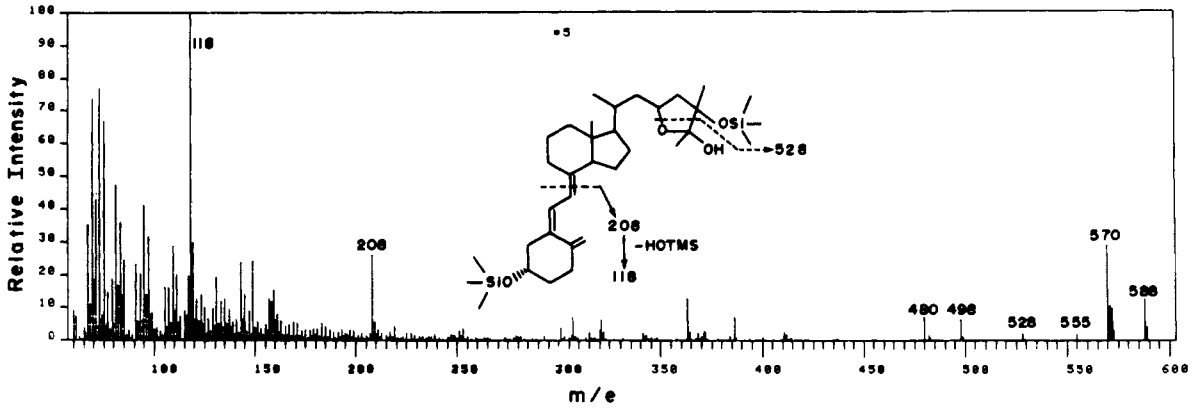

FIGURE 9: Mass spectrum of the methyl lactol of the $(Me_3Si)_2$ derivative of calcidiol lactone. Intensities of all ions > *m/e* 300 are multiplied by 5.

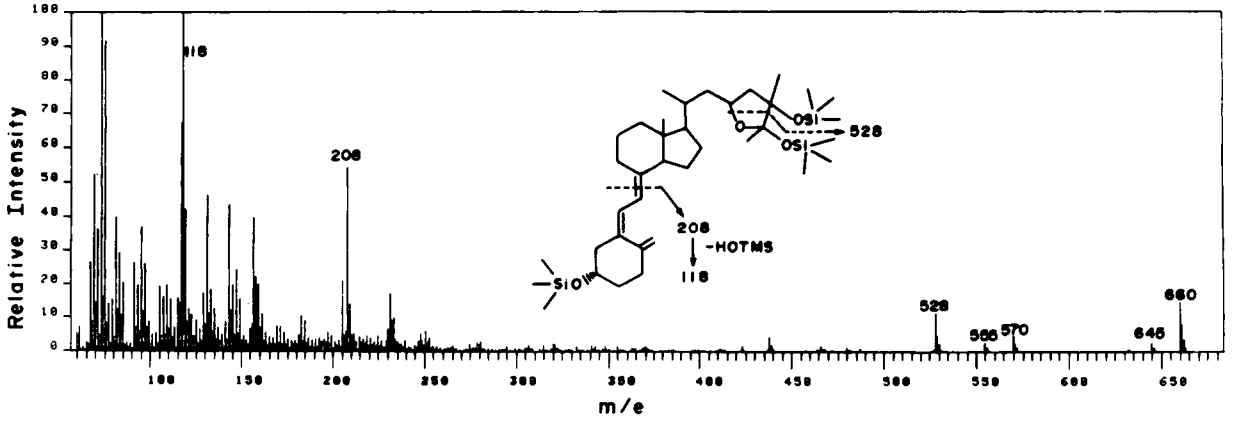

FIGURE 10: Mass spectrum of the $(Me_3Si)_3$ methyl lactol derivative of calcidiol lactone.

interpretation of these results is a 25-hydroxy 26,*X*-lactone derivative of vitamin D_3. The lactone ring size cannot be determined from the NMR and mass spectral data.

The FT-IR spectrum of this compound (in chloroform) is presented in Figure 8. The salient feature that differentiates the spectrum from that of a typical vitamin D_3 derivative is the very intense absorbance at 1787 cm^{-1}, indicative of a γ-lactone. The structure of the compound must therefore be 3β,25-dihydroxy-9,10-seco-5,7,10(19)-cholestatrieno-26,23-lactone (25-hydroxyvitamin D_3 26,23-lactone).

Further evidence for this structure is presented in Figures 9 and 10. Figure 9 is the mass spectrum of the methyl lactol of the $(Me_3Si)_2$ derivative of the lactone. Diagnostic ions, their relative intensities, and fragment assignments are as follows: *m/e* 588, 4.4%, M^+; *m/e* 570, 7.3%, $M^+ - H_2O$; *m/e* 528, 1.5%, $M^+ - HOAc$; *m/e* 498, 2%, $M^+ - HOSiMe_3$; *m/e* 208, 33%, A-ring fragment$^+$; *m/e* 118, 100%, A-ring fragment$^+ - HOSiMe_3$. The molecular ion of *m/e* 588 indicates the addition of a methyl group and conversion of lactone to lactol. Loss of H_2O from the lactol group leads to the ion of *m/e* 570; *m/e* 528 arises by elimination of the elements $C_2H_4O_2$, as shown schematically in Figure 9. Figure 10 shows the mass spectrum of the $(Me_3Si)_3$ derivative of the methyl lactol. Diagnostic ions, their relative intensities, and fragment structures are as follows: *m/e* 660, 12.7%, M^+; *m/e* 645, 1.8%, $M^+ - CH_3$; *m/e* 570, 4%, $M^+ - HOSiMe_3$; *m/e* 555, 2%, $M^+ - HOSiMe_3CH_3$; *m/e* 528, 10%, $M^+ - AcOSiMe_3$; *m/e* 208, 54%, A-ring fragment$^+$; *m/e* 118, 100%, A-ring fragment$^+ - HOSiMe_3$. The molecular ion at *m/e* 660 confirms the formation of a tris(trimethylsilyl ether) derivative. The elimination of $C_2H_3O_2SiMe_3$ by fragmentation through the lactol ring accounts for the fairly prominent peak at *m/e* 528.

It is interesting to note that the preparation of the methyl lactol discussed above was only successful when the alkylation reaction was performed on the $(Me_3Si)_2$ derivative of the lactone. Treatment of the underivatized lactone with methyllithium gave no recoverable product. Similarly no product could be recovered upon attempted hydride reduction of the lactone using a variety of reagents [$LiAlH_4$, $LiB(Et)_3H$, and $LiAl(C_4H_9)_3H$] and conditions that proved quite successful with other vitamin D model compounds. Hydrolysis in either aqueous or anhydrous methanolic base gave either starting lactone or no recoverable product. Effective chelation of the products to be expected from these reactions with the reagent, or with trace metal in the reagent, appears to be a reasonable explanation of these results. All reactions were performed on microgram quantities of sample, and, at such low levels, the formation of a chelated complex could well preclude recovery of the desired derivative.

The identification of 25-hydroxyvitamin D_3 26,23-lactone represents the second vitamin D_3 metabolite isolated to date with the 23-carbon functionalized. This laboratory recently isolated 1α,3β-dihydroxy-24-nor-5,7,10(19)-cholatrien-23-oic acid from livers and intestines of rats dosed with 1,25-$(OH)_2D_3$ (Esvelt et al., 1979). Thus, the functionalization of the 23-carbon of vitamin D_3 compounds represents a new area of study in the metabolism of vitamin D_3. The biological activity and isolation of intermediates in the formation of 25-hydroxyvitamin D_3 26,23-lactone are currently under investigation in this laboratory.

As pointed out in a previous publication, this compound comigrates with 24,25-$(OH)_2D_3$ on Sephadex LH-20 and silica LC (Horst et al., 1979). Thus, it represents a potential error in both metabolic studies and plasma measurements concerned with 24,25-$(OH)_2D_3$. Its potential presence must therefore now be taken into account in future investigations.

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References

Blunt, J. W., DeLuca, H. F., & Schnoes, H. K. (1968) *Biochemistry 7*, 3313.

DeLuca, H. F. (1971) in *The Vitamins* (Sebrell, W. H., Jr., & Harris, R. S., Eds.) 2nd ed., Vol. III, Chapter 7, pp 230–239, Academic Press, New York.

DeLuca, H. F. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 2211.

DeLuca, H. F., & Schnoes, H. K. (1976) *Annu. Rev. Biochem. 45*, 631.

Esvelt, R. P., Schnoes, H. K., & DeLuca, H. F. (1979) *Biochemistry 18*, 3977–3983.

Haddad, J. G., Jr., Min, C., Mendelsohn, M., Slatopolsky, E., & Hahn, T. J. (1977) *Arch. Biochem. Biophys. 182*, 390.

Horst, R. L., Shepard, R. M., Jorgensen, N. A., & DeLuca, H. F. (1979) *J. Lab. Clin. Med. 93*, 277.

Kodicek, E. (1974) *Lancet 1*, 325.

Ribovich, M. L., & DeLuca, H. F. (1978a) *Arch. Biochem. Biophys. 188*, 145.

Ribovich, M. L., & DeLuca, H. F. (1978b) *Arch. Biochem. Biophys. 188*, 157.

Ribovich, M. L., & DeLuca, H. F. (1978c) *Arch. Biochem. Biophys. 188*, 164.

Shepard, R. M., Horst, R. L., Hamstra, A. J., & DeLuca, H. F. (1979) *Biochem. J. 182*, 55.