Isolation and Identification of 25-Hydroxyvitamin D\(_3\)-26,23-peroxylactone

A NEW VIVO METABOLITE OF VITAMIN D\(_3\)*

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A new vitamin D\(_3\) metabolite was isolated in pure form (18.2 µg) from the serum of rats given large doses (two doses of 26 µmol/rat) of vitamin D\(_3\). The new metabolite has been unequivocally identified as 3β,25-dihydroxy-9,10-seco-5,7,10(19)-cholestatrino-26,23-peroxylactone by ultraviolet absorption spectrophotometry, Fourier transform infrared spectrophotometry, mass spectrometry, field desorption mass spectrometry, and specific chemical reaction with triphenylphosphine. The stereochemical configuration at the C-23 and C-25 positions of the 25-hydroxyvitamin D\(_3\)-26,23-peroxylactone was definitely determined to be the 23(S)25(R)-25-hydroxyvitamin D\(_3\)-26,23-peroxylactone. The trivial name 25-hydroxyvitamin D\(_3\)-26,23-peroxylactone is suggested for this metabolite. The isolation involved chloroform-methanol extraction and four column chromatographic procedures. The metabolite purification and elution position on these columns were followed by UV measurement at 264 nm. This metabolite was ultimately resolved from the previously known 25-hydroxyvitamin D\(_3\)-26,23-lactone by high pressure liquid chromatography using a Zorbax Sili column. The 25-hydroxyvitamin D\(_3\)-26,23-peroxylactone was converted upon storage at room temperature or -20 °C into the 25-hydroxyvitamin D\(_3\)-26,23-lactone. Since under the conditions of this isolation only the 26,23-peroxylactone and no 26,23-lactone of 25-hydroxyvitamin D\(_3\) was present in the rat serum, this suggests that the 25-hydroxyvitamin D\(_3\)-26,23-peroxylactone is the naturally occurring metabolite.

It is widely accepted that vitamin D\(_3\) must undergo metabolism in vivo to express its physiological activities (1, 2). Since the discovery that 25(OH)D\(_3\) is the major circulating form of vitamin D\(_3\) (3), the further metabolism of vitamin D\(_3\) has been the subject of intensive investigation. Some of the metabolites have been structurally identified and their physiological functions are well defined, but others either remain unidentified or their physiological functions are unknown. The two dihydrorylated metabolites, 1,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_3\), are believed to be responsible for the spectrum of biological responses attributable to the parent vitamin D\(_3\) (4-6).

To date, 20 vitamin D metabolites have been isolated and chemically characterized (7). With the exception of 1,25(OH)\(_2\)D\(_3\) (8, 9) and 25(OH)5,6-trans-vitamin D\(_3\) (10), all the metabolites represent some form of structural modification of the 5-carbon side chain of vitamin D\(_3\). Of the many known side chain modifications of vitamin D\(_3\), the lactone ring structure of 25(OH)D\(_3\)-26,23-lactone (11-13) and 1,25(OH)\(_2\)D\(_3\)-26,23-lactone (14) is the most complex. The 25(OH)D\(_3\)-26,23-lactone was isolated from the plasma of chickens, rats, and pigs given large doses of vitamin D\(_3\). Four possible diastereomers of 25(OH)D\(_3\)-26,23-lactone have been synthesized (15-20) and they have been directly compared to the natural 25(OH)D\(_3\)-26,23-lactone by high pressure liquid chromatography. The stereochemical configurations of the natural 25(OH)D\(_3\)-26,23-lactone at the C-23 and C-25 positions were determined to be 23(S) and 25(R), respectively (13, 20). Also, we have reported that the 25(OH)D\(_3\)-26,23-lactone was not produced from 25(S)26(OH)\(_3\)D\(_3\) but from 23(S)25(OH)\(_3\)D\(_3\) via 23(S)25(R)26(OH)\(_3\)D\(_3\) (21).

During the course of the investigation of the stereochemical configuration of the natural 25(OH)D\(_3\)-26,23-lactone, we became aware of a new metabolite of vitamin D\(_3\) that appeared in the serum of rats given large doses of vitamin D\(_3\). It is the purpose of this report to describe the isolation and to establish the structure of this new metabolite as 25(OH)D\(_3\)-26,23-peroxylactone.

**MATERIALS AND METHODS**

**RESULTS**

During the course of our investigation of the stereochemical configuration of the 25(OH)D\(_3\)-26,23-lactone, we became aware of a new metabolite of vitamin D\(_3\) that appeared in the serum of rats given large doses of vitamin D\(_3\). It is the purpose of this report to describe the isolation and to establish the structure of this new metabolite as 25(OH)D\(_3\)-26,23-peroxylactone.

**REFERENCES**

Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 5-12) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda MD 20814. Request Document No. 82M-1519, cite authors, and include a check for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
aware of a new metabolite of 25(OH)D$_3$ that appeared in the HPLC chromatogram of lipid extracts obtained from rats given vitamin D$_3$. The proposed structure of the new metabolite, namely 25(OH)D$_3$-26,23-peroxylactone, is shown in Fig. 1. A flow sheet of the procedures used in the isolation of the metabolite is presented in Table I. To conserve space, the initial Sephadex LH-20 column chromatographic profile is not presented. Fig. 2 shows the high pressure liquid chromatographic profiles of the 24,25(OH)$_2$D$_3$ fraction collected from the Sephadex LH-20 column. The new metabolite of 25(OH)D$_3$, the 25(OH)D$_3$-26,23-peroxylactone, is eluted just before the elution of the authentic 25(OH)D$_3$-26,23-lactone when a Zorbax Sil column was used as an absorbent and developed with isopropyl alcohol:n-hexane, 9:91 (Fig. 2A). However, when the same column was used with methanol:dichloromethane, 1.5:98.5, the 25(OH)D$_3$-26,23-peroxylactone was eluted immediately after the elution of the authentic 25(OH)D$_3$-26,23-lactone (Fig. 2B). This anomalous chromatographic behavior of the 25(OH)D$_3$-26,23-peroxylactone is similar to the results presented in previous reports of the 25(OH)D$_3$-26,23-lactone (11-13). The 25(OH)D$_3$-26,23-peroxylactone was purified to homogeneity by HPLC. According to these procedures, approximately 18.2 µg of the new metabolite, 25(OH)D$_3$-26,23-peroxylactone, were isolated in pure form from the serum of 300 male rats assuming an ε of 18,000 M$^{-1}$ cm$^{-1}$.

The UV spectrum of the 25(OH)D$_3$-26,23-peroxylactone isolated from the serum displays the typical vitamin D$_3$ cis-triene chromophore with λ$_{max}$ = 264 nm, λ$_{max}$ = 228 nm, and A$_{254}$/A$_{325}$ = 1.76 (Fig. 3). To conserve space, the bulk of the experimental data on the isolation and chemical characterization of the new metabolite is presented in the Miniprint.

**DISCUSSION**

This report demonstrates that the 25(OH)D$_3$-26,23-peroxylactone is a major metabolite of 25(OH)D$_3$ present in the serum of rats under the conditions of the isolation procedure employed. The structure assignment is based on UV absorption spectrometry, Fourier transform infrared spectrophotometry, mass spectrometry, field desorption mass spectrometry, as well as specific chemical reaction of the peroxylactone-secosteroid with triphenyl phosphate. The new metabolite was isolated in pure form by successive chromatography on Sephadex LH-20 and HPLC columns of Zorbax Sil.

An essential feature of the HPLC Zorbax Sil chromatographic purification was the use of the two different solvent systems, isopropyl alcohol:n-hexane (9:91) and methanol:dichloromethane (1.5:98.5). The subtle structural differences of many of the vitamin D metabolites are such that even the high resolving power of HPLC cannot achieve with one solvent system an unequivocal separation of all the vitamin D metabolites that may be present in the same sample. Thus, the
24,25(OH)2D3, region of the Sephadex LH-20 chromatogram was subsequently resolved by HPLC into two or three components. The clear separation of the 25(OH)D3-26,23-peroxylactone from 25(OH)D3-26,23-lactone or 24R(25(OH)D3) required two different HPLC solvent systems. The anomalous chromatographic behavior of the 25(OH)D3-26,23-peroxylactone on HPLC using two different solvent systems is similar to the results presented in previous reports on 25(OH)D3-26,23-lactone (11-13) as well for 1,25(OH)2D3-26,23-lactone (14).

The structural confirmation of the 25(OH)D3-26,23-peroxylactone was carried out as follows: (i) the anomalous chromatographic behavior of the metabolite is similar to that of the 25(OH)D3-26,23-lactone (11-14) (Fig. 2); (ii) the UV absorption spectrum of the metabolite displays the typical vitamin D3 cis-triene chromophore with \( \lambda_{\text{max}} = 264 \text{ nm} \), \( \lambda_{\text{min}} = 228 \text{ nm} \), and \( A_{264}/A_{228} = 1.76 \) (Fig. 3); (iii) the field desorption mass spectrum of the metabolite gives a parent ion \( m/z = 444 \) (Fig. 5); (iv) the mass spectrum of the metabolite gives \( m/z = 412, 400, 372, 354, 399, 313, 271, 253, 136 \), and 116 (Fig. 6). The fragments \( m/z = 271 \) and 253 indicate that the secosteroid nucleus of vitamin \( D_3 \) has remained unchanged and that all the metabolite alterations have taken place on the side chain; (v) the Fourier transform infrared spectrum of the metabolite indicates the very intense absorbance at 1733 cm\(^{-1} \), indicative of a \( \delta \)-lactone moiety (Fig. 7); (vi) the metabolite easily converts nonenzymatically to the 25(OH)D3-26,23-lactone at room temperature or \(-20^\circ \text{C} \), because it is extremely unstable (Figs. 8, 9, and 10). It is well known that the peroxylactone derivatives such as \( \gamma \)-methyl-\( \gamma \)-peroxyvalerolactone and \( \gamma \)-peroxy-\( \gamma \)-butyrolactone are extremely unstable (24, 25); (vii) the reaction of the metabolite with triphenylphosphine results in stoichiometric amounts of 25(OH)D3-26,23-lactone and triphenyl phosphine oxide (Fig. 11). A similar reaction was reported by Adam and Szendry (24). The reaction of the \( \gamma \)-methyl-\( \gamma \)-peroxyvalerolactone with triphenylphosphine resulted in a stoichiometric amount of \( \gamma \)-methyl-\( \gamma \)-valerolactone and triphenyl phosphine oxide; and (viii) the stereochemistry of the generated 25(OH)D3-26,23-lactone from the metabolite is definitely determined to be 23S(25R)(25(OH)D3-26,23-lactone (Fig. 12). From these results, the structure of the new vitamin D metabolite was unequivocally determined to be 23S(25R)(25(OH)D3-26,23-peroxylactone.

Our observation that the "freshly" isolated lactone metabolite has in reality the peroxy-\( \gamma \)-lactone ring structure (Fig. 6), coupled with our observation (Fig. 7) that on storage the \( \gamma \)-peroxylactone disproportionates into the \( \gamma \)-lactone ring structure of 25(OH)D3-26,23-peroxylactone, raises the interesting possibility that in reality the \( \gamma \)-lactone ring is a nonenzymatic artifact of storage. If this is true, then it should be recognized that 25(OH)D3-26,23-lactone (11-13), and by analogy 1,25(OH)2D3-26,23-lactone (14), are not naturally occurring vitamin \( D_3 \) metabolites. We are conducting further experiments to evaluate more critically these possibilities.

The biochemical details of the metabolic pathway leading from 25(OH)D3 to 25(OH)D3-26,23-peroxylactone are unknown. Recently, Tanaka et al. (26) reported that 23S(25)(25(OH)D3 is an intermediate in the biosynthesis of the 25(OH)D3-26,23-lactone. Thus, 23S(25)(25(OH)D3 is a far better substrate for production of 25(OH)D3-26,23-lactone than is 25,26(OH)2D3 (26). We have previously reported that the production of 25(OH)D3-26,23-lactone from 23S(25R)(25(OH)D3 is 16.2 times more extensive than that from 24S(25)(25(OH)D3 (21). Collectively, these results indicate that the 25(OH)D3-26,23-lactone is biosynthesized from 25(OH)D3 by way of 23S(25)(25(OH)D3 to 23S(25R)(25(OH)D3 (21). Since the 25(OH)D3-26,23-lactone is apparently generated nonenzymatically from 25(OH)D3-26,23-peroxylactone, the metabolic pathway leading from 25(OH)D3-26,23-peroxylactone is proposed as shown in Fig. 4. First, 25(OH)D3-23-hydroxyperoxide is made from 25(OH)D3. This is followed by the oxidative introduction of the hydroxyl group at the C-26 position to yield 25,26(OH)2D3-26,23-hydroxyperoxide which is then subjected to peroxylactonization between the C-26 and C-23 positions to yield 25(OH)D3-26,23-peroxylactone. Also, kidney homogenates prepared from vitamin D supplemented rats (21, 27), as well as chickens (28), are known under in vitro conditions to be capable of producing the 25(OH)D3-26,23-lactone. It is generally accepted that the 25(OH)D3-26,23-lactone is not produced in nephrectomized animals. This suggests, therefore, that the metabolism of 25(OH)D3 to the 25(OH)D3-26,23-peroxylactone occurs mainly in the kidney.

It is also possible that the 1α-hydroxylated version of 25(OH)D3-23-hydroxyperoxide is the immediate precursor of the recently chemically characterized 1,25(26,23)-dihydroxyvitamin D3 (29) and 25,26(OH)2-23-oxo-D3 (29). Thus, the step of introduction of the hydroxyperoxide on carbon-23 could create a branch point in further metabolism and lead either to the production of 25(OH)D3-26,23-peroxylactone (by generation of a carboxyl group on C-26) or to 1,25,26(OH)3-23-oxo-D3 (by generation of a C-23 oxo and a C-26 hydroxyl) on the 1α-hydroxylated secosteroid (30).

Gross changes in the plasma concentrations of the known vitamin \( D_3 \) metabolites in rats have been shown to occur on administration of large amounts of vitamin \( D_3 \) and 25(OH)D3 (28). Use of this fact permitted us to identify the 25(OH)D3-26,23-peroxylactone (this report). However, the metabolite is present in the serum at <1 ng/ml under physiological conditions, but under circumstances of elevated vitamin \( D_3 \) levels, it becomes one of the major circulating metabolites. A total recovery of 30.3 µg of 25(OH)D3-26,23-peroxylactone was calculated for 1200 ml of the serum sample, assuming an \( E_260 = 18,000 \text{ M}^{-1} \text{cm}^{-1} \) and \( M = 444 \). This corresponds to a serum level of approximately 25.3 ng/ml serum. The biological and physiological functions of the new 25(OH)D3-26,23-peroxylactone are currently under evaluation in our laboratories.

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REFERENCES

1. Norman, A. W. (1979) *Vitamin D: the Calcium Homeostatic Steroid Hormone*, 490 pp., Academic Press, New York
2. DeLuca, H. F. (1979) *Nutr. Rev.* 37, 161-193
3. Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968) *Biochemistry* 7, 3317-3322
4. Norman, A. W., Henry, H. L., and Malluche, H. H. (1980) *Life Sci.* 27, 229-237
5. Henry, H. L., and Norman, A. W. (1978) *Science (Wash. D. C.)* 201, 835-837
6. Norman, A. W., Schaefer, K., Herrath, D. v., Grigoleit, H.-G., Coburn, J. W., DeLuca, H. F., Mawer, E. B., and Suda, T. (eds) (1979) *Vitamin D: Basic Research and Its Clinical Application, Proceedings of the Fourth Workshop on Vitamin D, Berlin, West Germany, February 1979*, 1318 pp. Walter de Gruyter, Berlin
7. Mayer, E., Ohnma, N., Wanamaker, G., and Norman, A. W., (1982) in (Norman, A. W., Schaefer, K., Grigoleit, H. G., and von Herrath, D., eds) *Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism, Proceedings of the Fifth Workshop on Vitamin D, Williamsburg, Virginia, February 1982*, pp. 7-12, Walter de Gruyter, Berlin
8. Norman, A. W., Myrtle, J. F., Midgett, R. J., Nowicki, H. G., Williams, V., and Popjak, G. (1971) *Science (Wash. D. C.)* 173, 51-54
9. Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R., and Williams, D. H. (1971) *Nature (Lond.)* 230, 228-230
10. Kumar, R., Nagubandi, S., Jardine, L., Londowski, J. M., and Bollman, S. (1981) *J. Biol. Chem.* 256, 9369-9392
11. Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., Shepard, R. M., and Jorgensen, N. A. (1979) *Biochemistry* 18, 4770-4780
12. Horst, R. L. (1979) *Biochem. Biophys. Res. Commun.* 89, 286-295
13. Ishizuka, S., Yamaguchi, H., Yamada, S., Nakayama, K., and Takayama, H. (1981) *FEBS Lett.* 134, 207-211
14. Ohnuma, N., Bannai, K., Yamaguchi, H., Hashimoto, Y., and Norman, A. W. (1980) *Arch. Biochem. Biophys.* 204, 387-391
15. Wichmann, J. K., Paaren, H. E., Fivizzani, M. A., Schnoes, H. K., and DeLuca, H. F. (1980) *Tetrahedron Lett.* 21, 4667-4670
16. Ikekawa, N., Hirano, Y., Ishiguro, M., Oshida, J., Eguchi, T., and Miyasaka, S. (1980) *Chem. Pharm. Bull. (Tokyo)* 28, 2852-2854
17. Morris, D. S., Williams, D. H., and Norris, A. F. (1981) *J. Chem. Soc. Commun.* 424-425
18. Morris, D. S., Williams, D. H., and Norris, A. F. (1981) *J. Org. Chem.* 46, 3422-3426
19. Yamada, S., Nakayama, K., and Takayama, H. (1981) *Tetrahedron Lett.* 22, 2591-2594
20. Yamada, S., Nakayama, K., and Takayama, H. (1981) *Chem. Pharm. Bull. (Tokyo)* 29, 2393-2396
21. Ishizuka, S., Ishimoto, S., and Norman, A. W. (1982) *FEBS Lett.* 138, 83-87
22. Ishizuka, S., Bannai, K., Naruchi, T., and Hashimoto, Y. (1981) *Steroids* 37, 33-43
23. Norman, A. W. (1972) *J. Nutr.* 102, 1243-1246
24. Adam, W., and Szendry, L. (1971) *Chem. Commun.* 1299-1300
25. Gibson, D. H., and Joseph, J. T. (1978) *J. Am. Chem. Soc.* 100, 3641-3642
26. Tanaka, Y., DeLuca, H. F., Schnoes, H. K., Ikekawa, N., and Eguchi, T. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 4805-4808
27. Tanaka, Y., Wichmann, J. K., Paaren, H. E., Schnoes, H. K., and DeLuca, H. F. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 6411-6414
28. Shepard, R. M., and DeLuca, H. F. (1980) *Arch. Biochem. Biophys.* 202, 43-53
29. Ohnuma, N., Kruse, J., Popjak, G., and Norman, A. W. (1982) *J. Biol. Chem.* 257, 5087-5102
30. Ohnuma, N., and Norman, A. W. (1982) *J. Biol. Chem.* 257, 8261-8271
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**Results**

Figure 7 provides the mass spectrum of the isolated 25(OH)D₂-26,23-peroxylactone. The molecular ion at m/z 644 was not observed by the electron impact mass spectrometer. The diagnostic ions and structural assignments are as follows: m/z 466, M⁻, m/z 412, M⁻- 34, m/z 382, M⁻- 37, M⁻- 32, m/z 364, m/z 288, m/z 320, m/z 232, m/z 210, m/z 200, m/z 192, m/z 176, m/z 136, A-ring x 2 + C-F (A-ring fragment) x 1, m/z 118, A-ring fragment⁻ (F⁻). The fragment ions at m/z 434 and 416 (432) (414) were assigned to be 25(OH)D₂-26,23-lactone by comparison of the 25(OH)D₂-26,23-peroxylactone during electron impact ionization process. The presence of the additional oxygen and hydroxyl functions on the side chain could be detected from the peaks at m/z 271 and 253 (251). This indicates that the cis double bond located intact and that the additional oxygen function was not on the A-ring were confirmed by the characteristic ions at m/z 135 and 118 (116).-

The structure of the new metabolite was therefore, 25(OH)D₂-26,23-peroxylactone [25(OH)D₂-26,23-peroxylactone].

**Figure 1.** Fourier transform infrared spectra of the isolated 25(OH)D₂-26,23-peroxylactone taken on crystalline potassium bromide.

**Figure 2.** High pressure liquid chromatographic profile of the isolated 25(OH)D₂-26,23-peroxylactone after it had been stored in ethanol at -20°C for one week. The elution position of the "steroid" 25(OH)D₂-26,23-peroxylactone from an HPLC Zorbax S1 column differed from that of freshly isolated 25(OH)D₂-26,23-peroxylactone. The "steroid" 25(OH)D₂-26,23-peroxylactone migrated on the same position as authentic, chemically synthesized 25(OH)D₂-26,23-lactone. A similar change in the HPLC chromatographic behavior of the recently isolated 25(OH)D₂-26,23-peroxylactone was observed after it had been stored for one week at -20°C in isopropanol, in ether, or in ether.

**Figure 3.** Fourier transform infrared spectra of the isolated 25(OH)D₂-26,23-peroxylactone taken on crystalline potassium bromide.
Figure 9 and 10 illustrate the mass spectra and the Fourier transform infrared spectrum of the product generated from the 25(OH)D3-26,23-peroxylactone in Figure 8. The mass spectrum of the product generated from the 25(OH)D3-26,23-peroxylactone indicated the presence of an absorption of 170 cm\(^{-1}\) due to a 2-lactone moiety (Figure 10). These results demonstrated that the 3-peroxylactone moiety of the "freshly" isolated peroxylactone was converted on standing to the 2-lactone of 25(OH)D3-26,23-lactone. Accordingly, the "stoichiometric" lactone was unequivocally identified as 25(OH)D3-26,23-lactone.

Figure 10. Fourier transform infrared spectrum of the product generated from the 25(OH)D3-26,23-peroxylactone in Figure 8.

Figure 11. High pressure liquid chromatographic profile of the reaction mixture of the 25(OH)D3-26,23-peroxylactone reacted with triphenylphosphine. The solid line indicated that 2 \(\mu\)l of the 25(OH)D3-26,23-peroxylactone reacted with 200 \(\mu\)l of triphenylphosphine dissolved in 100 \(\mu\)l of n-hexane at 0°C for 60 min. The dotted line showed that 200 \(\mu\)l of triphenylphosphine reacted with 100 \(\mu\)l of n-hexane at 0°C for 60 min. The dotted line illustrated that 1 \(\mu\)l of the 25(OH)D3-26,23-peroxylactone stood in n-hexane at 0°C for 60 min. The reaction mixture was chromatographed by HPLC using a 4.6 x 250 mm Zorbax 511 column eluted with isopropanol: n-hexane, 9:1, at a flow rate of 1 ml/min.

Figure 12. High pressure liquid chromatographic profile of the reaction mixture of the 25(OH)D3-26,23-lactone generated from the 25(OH)D3-26,23-peroxylactone. The four synthetic stereneisomers of 25(OH)D3-26,23-lactone could be separated into resolved peaks by HPLC (Figure 12c). The generated 25(OH)D3-26,23-lactone from the 25(OH)D3-26,23-peroxylactone was definitely determined to be 25(OH)D3-26,23-lactone. The reaction course of 25(OH)D3-26,23-peroxylactone to 25(OH)D3-26,23-lactone the stereochemical configurations at C-22 and C-23 positions of 25(OH)D3-26,23-peroxylactone should not be capable of inversion by known mechanisms. So, the stereochemical configuration of 25(OH)D3-26,23-peroxylactone was unequivocally determined to be 25(OH)D3-26,23-peroxylactone.