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ISOLATION AND IDENTIFICATION OF 25-HYDROXYDIHYDROTACHYSTEROL$_2$ 1α,25-DIHYDROXYDIHYDROTACHYSTEROL$_2$ AND 1β,25-DIHYDROXYDIHYDROTACHYSTEROL$_2$

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Summary—Three metabolites of orally administered dihydrotachysterol$_2$ have been isolated in impure form from serum of rats. These metabolites have been identified as 25-hydroxydihydrotachysterol$_2$ and two epimers of formula 1-ambo,25-dihydroxydihydrotachysterol$_2$ by means of gas chromatography–mass spectrometry and ultraviolet absorption spectrometry. For the first time this provides evidence for 9,10-seco steroid hydroxylation at pseudo C_3. The stereochemistry of the 1-hydroxyl group of the two epimers could be established tentatively by quantitative comparison of the mass spectra of their respective trimethylsilyl derivatives. Since purity requirements were not achieved, biological activities could not be determined.

INTRODUCTION

Dihydrotachysterol$_2$ (DHT$_2$)[1] is successfully used in the treatment of hypoparathyroidism and renal osteodystrophy [2]. However, in contrast with dihydrotachysterol$_3$—the vitamin D_3 analogue of DHT$_2$—little is known concerning its metabolism and action. Because synthetic 25-hydroxydihydrotachysterol$_3$ is more effective than dihydrotachysterol$_3$ in increasing intestinal calcium transport and bone mobilization [3], it is generally assumed that DHT$_2$ requires 25-hydroxylation before it can be effective. It has been shown that reconstituted liver mitochondrial cytochrome P-450 can carry out the 25-hydroxylation of DHT$_2$ when combined with ferredoxin and ferredoxin reductase [4]. On the other hand 1-hydroxylation of DHT$_2$ is assumed to be most unlikely because insertion of a 1-hydroxyl group into [4-^{14}C, 1,2-^{3}H]dihydrotachysterol$_3$ could not be established under *in vivo* conditions in rats [5]. The purpose of this study was to check these assumptions.

In a previous paper [6] we reported the occurrence of 9 distinct radioactive substances in serum of rats after an oral dose of tritiated DHT$_2$ [7]. The present report describes the isolation and identification of 3 of these substances. These substances, preliminarily designated "b", "c2" and "e", cochromatographed with DHT$_2$, 25-hydroxydihydrotachysterol$_3$ and 1α,25-dihydroxyvitamin D_3 respectively [7].

EXPERIMENTAL

Materials

Crystalline DHT$_2$ was a gift from Duphar b.v., Weesp, The Netherlands. 25-Hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 were gifts from Hoffmann–LaRoche, Basel, Switzerland; their respective tritium labelled analogues were obtained from Radiochemical Centre, Amersham, England. Ethanol and solvents for HPLC came from Rathburn Chemicals Ltd, Walkerburn, Scotland. Vitamin D_3, *bis*(trimethylsilyl)trifluoracetamide and trimethylchlorosilane were purchased from Merck, Darms-

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The trivial names for the sterols used are: dihydrotachysterol$_2$, (5E,7E,22E)-(3S,10S)-9,10-seco-5,7,22-ergostatrien-3-ol; 25-hydroxydihydrotachysterol$_2$, (5E,7E,22E)-(3S,10S)-9,10-seco-5,7,22-ergostatrien-3,25-diol; 1β,25-dihydroxydihydrotachysterol$_2$, (5E,7E,22E)-(1S,3R,10R)-9,10-seco-5,7,22-ergostatrien-1,3,25-triol; 1α,25-dihydroxydihydrotachysterol$_2$, (5E,7E,22E)-(1R,3R,10R)-9,10-seco-5,7,22-ergostatrien-1,3,25-triol; vitamin D_3, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3-ol; 25-hydroxyvitamin D_3, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3,25-diol; 1α,25-dihydroxyvitamin D_3, (5Z,7E)-(1S,3R)-9,10-seco-5,7,10(19)-cholestatrien-1,3,25-triol; vitamin D_2, (5Z,7E,22E)-(3S)-9,10-seco-5,7,10(19),22-ergostatetraen-3-ol; dihydrotachysterol$_3$, (5E,7E)-(3S,10S)-9,10-seco-5,7-cholestadien-3-ol; 25-hydroxydihydrotachysterol$_3$, (5E,7E)-(3S,10S)-9,10-seco-5,7-cholestadien-3,25-diol; 3-deoxy-(1)-hydroxyvitamin D_3, (5Z,7E)-(1S)-9,10-seco-5,7,10(19)-cholestatrien-1-ol; dihydrovitamin D_2-II, (5Z,7E,22E)-(3S,10S)-9,10-seco-5,7,22-ergostatrien-3-ol; 25-hydroxydihydrovitamin D_2-II, (5Z,7E,22E)-(3S,10S)-9,10-seco-5,7,22-ergostatrien-3,25-diol; 1α,25-dihydroxydihydrovitamin D_2-II, (5Z,7E,22E)-(1S,3R,10R)-9,10-seco-5,7,22-ergostatrien-1,3,25-diol; 5,6-*trans* vitamin D_3, (5E,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3-ol.

Abbreviations used are: DHT$_2$, dihydrotachysterol$_2$; 25(OH)D_3, 25-hydroxyvitamin D_3; HPLC, high performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; TMSOH, $(CH_3)_3SiOH$.

tadt, Western Germany. Tritium labelled "c2" and "e" were obtained in a previous study [6].

General procedures

HPLC was performed with a Model 6000 A solvent delivery system and a Model WISP 710 A automatic sample processor, at a flow rate of 1 ml/min; runs were recorded at 254 nm with a Model 440 absorbance detector (Waters Associates). In developing chromatographic procedures for the purification of "c2" and "e" the objective was to achieve adequate resolution between respectively "c2" and 25-hydroxyvitamin D_3, and "e" and 1α,25-dihydroxyvitamin D_3. Using the radioactive analogues of these compounds, several types of columns at a variety of liquid combinations were examined to find these qualities. A chromatographic system developed to separate DHT_2 and vitamin D_3 [8] appeared to be suitable for the purification of "b". Drying of chromatographic fractions (0.5 ml) was performed with a stream of nitrogen at 40°C. Radioactive measurements were made in a Packard Tri-Carb scintillation counter, Model 3380. Ultraviolet spectra were recorded with a Pye Unicam P 8800 UV/VIS spectrophotometer. Gas chromatographic–mass spectrometric determinations were carried out with a Kratos MS 80 GC/MS instrument using on-column injection; source temperature 225°C, electron energy 50 eV, ionizing current 100 μA, flow rate of carrier gas 50 ml/min.

RESULTS

Isolation of "b", "c2" and "e"

Male Wistar rats (Central Institute for Nutrition Research, Zeist, The Netherlands) were fed a diet adequate in Ca, P and Vitamin D_3. Thirty rats (275–308 g) were each given 1.84 μmol of DHT_2 in 50 μl of ethanol (96%) by gastric tube. Ten hours later, blood was drawn by cardiac puncture yielding 128 ml of serum which was extracted as previously described [6]. The dried lipid extract and redissolved in 2600 μl of *n*-hexane–propanol-2–water (85:15:0.05, by vol). This was applied in 150 μl batches to a Zorbax Sil 850 HPLC column (250 × 6.2 mm; Dupont de Nemours, Den Bosch, The Netherlands) and eluted with the solvent. As described previously [6, 7], crude "b" appeared in fractions 12–16, crude "c2" in 17–22 and crude "e" in 37–48. After drying the crude samples were stored under dry nitrogen at −70°C.

Crude "b" was redissolved in 470μl of *n*-hexane–propanol-2 (98.2:1.8, v/v) and applied in three 150 μl batches to a Zorbax Sil 850 HPLC column (250 × 6.2 mm) which was eluted with the solvent. "b" appeared in fractions 30–34. Rechromatography (same procedures) gave single peaks which migrated at the position of authentic DHT_2. After drying, the combined fractions were redissolved in 1.5 ml of ethanol. The u.v. absorption spectrum of this solution corresponded of DHT_2 showing peaks at 260.5, 251.2 (λ_{max}) and 242.8 nm and troughs at 256.5, 246.0 and 220.5 nm. Assuming an extinction coefficient of 40.200 at 251.2 nm [9] as for DHT_2 the yield was 5.05 μg.

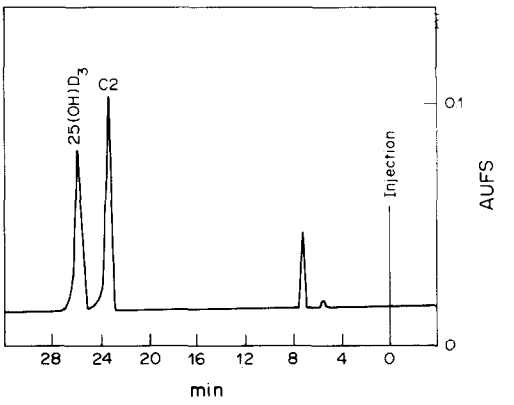

Fig. 1. HPLC profile of 25-hydroxydihydrotachysterol$_2$ ("c2") and 25-hydroxyvitamin D_3 ($25(OH)D_3$) on a 0.62 × 25 cm Lichosorb Si-60-5 column developed in a system of *n*-hexane–propanol–water (95:5:0.5, by vol). For details: see text. Ordinate: u.v. absorption at 254 nm in AUFS; abcissa: elution time in min.

Crude "c2" was redissolved in 470 μl of *n*-hexane–propanol-2–water (95:5:0.05, by vol) and applied in three 150 μl batches to a LichoSorb Si-60-5 HPLC column (250 × 6.2 mm; Chrompack, Middelburg, The Netherlands) which was developed with the solvent. "c2" occurred in fractions 46–51 and migrated sufficiently different from 25-hydroxyvitamin D_3, as is shown in Fig. 1. After rechromatography fractions 46–51 were combined, dried and redissolved in 1.5 ml of ethanol. Unfortunately, when ethanol was used as the reference the u.v. spectrum of this solution showed a massive absorbance band without fine structure at 200–270 nm. However, when the solution was recorded against an ethanolic solution (1.5 ml) of the combined fractions 40–45 (second chromatography) the spectrum obtained was almost identical with that of authentic DHT_2. The only difference concerned the relative height of the absorbancy at 242.8 nm. The yield was estimated at 1.3 μg assuming an extinction coefficient at 40.200 at 251.2 nm. This preparation was used for mass spectrometric determinations.

Our efforts at resolving "e" and 1α,25-dihydroxyvitamin D_3 failed. Chromatographic systems tested were: Zorbax Sil 850, methylene chloride–propanol-2; Lichosorb Si-60-5, *n*-hexane–propanol-2 and methylene chloride–propanol-2; Hypersil ODS, methanol–water. Partial separation could be achieved by the system Zorbax Sil 850/*n*-hexane–propanol-2–water (85:15:0.05, by vol). In this system 1α,25-dihydroxyvitamin D_3 migrated in the trailing edge of peak "e".

Crude "e" was redissolved in 470 μl of the eluent and applied in three 150 μl batches to the column. The fractions 39–42 were collected and re-

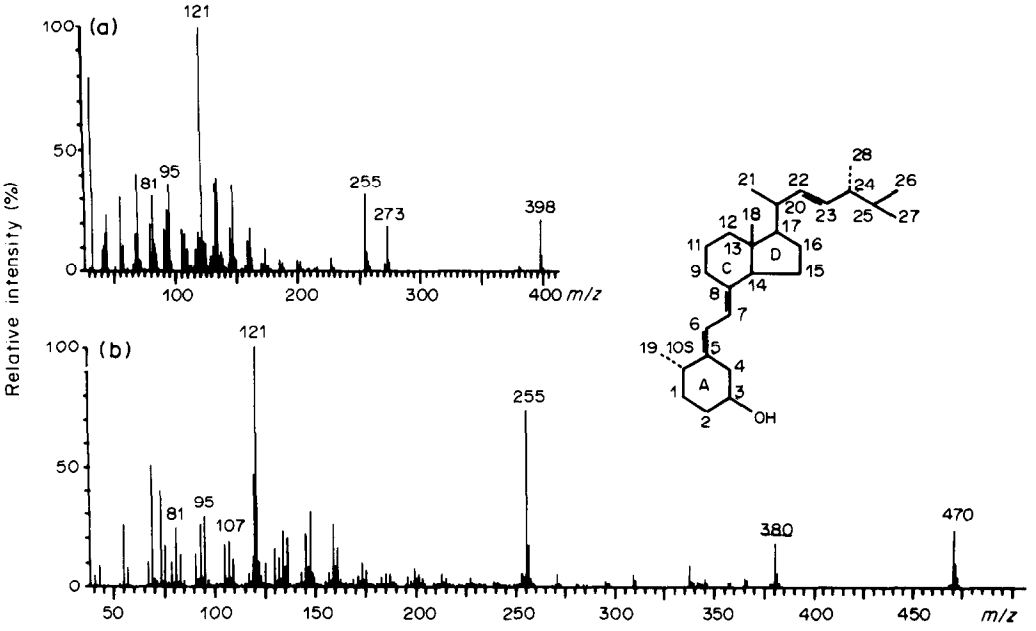

Fig. 2. Mass spectra of dihydrotachysterol$_2$ (A) and the trimethylsilyl ether of dihydrotachysterol$_2$ (isolated from serum of rats) (B). Inserted: the chemical structure and numbering of carbon atoms of dihydrotachysterol$_2$. For details: see text. Ordinate: relative intensity to m/z 121; abcissa: m/z.

chromatographed in the same system. After drying the residue was redissolved in 1.5 ml of ethanol. When recorded against ethanol, however, the u.v. spectrum of this solution showed a similar distortion as that of an ethanolic soution of "c2". When an ethanolic solution (1.5 ml) of the fractions 35–38 was used as the reference the shape of the u.v. spectrum was identical to that of DHT_2. 1.3 μg of "e" was obtained assuming a molar absorptivity of 40.200 at 251 nm.

Trimethylsilylation of "b", "c2" and "e"

Considering the limited amounts of "c2" and "e" available and their degree of purity it was decided to perform their mass spectrometric analyses by GC–MS using the on-column injection technique. Furthermore, for these analyses it appeared to be desirable to increase the volatility of the samples which was attained by trimethylsilylation. The ethanolic solutions of "b", "c2" and "e" were transferred to conical glass tubes and evaporated to dryness by rotary evaporation. The samples were each redissolved in 5 μl of pyridine and reacted with 5 μl of *bis*(trimethylsilyl)trifluoroacetamide containing 20% trimethylchlorosilane for 30 min at 65°C.

Identification of "b" as DHT_2

2.5 μl of sample "b" was applied to a 3% OV-101 glass packed tubular column (1 m, 2.5 mm in dia.; oven temperature: programmed for 280 to 300°C at 2°C/min). The total ion current chromatogram (not shown) showed a single peak demonstrating a high degree of purity for the sample. Its mass spectrum (Fig. 2B) was identical with that of the trimethylsilyl ether of DHT_2. The u.v. absorption spectrum, the chromatographic data and the mass spectrum unequivocally demonstrated that DHT_2 is present in the circulation after oral administration.

Apart from the peaks at m/z 273 [$C_{19}H_{29}O^+$; 398–125(C_9H_{17}, side chain) and 380 ($C_{28}H_{44}^{+\cdot}$; 470–90(TMSOH)] the mass spectra of authentic DHT_2 (M = 398; $C_{28}H_{46}O^{+\cdot}$) [Fig. 2A; 200 ng, direct probe inlet] and its trimethylsilyl ether (M = 470; $C_{31}H_{54}OSi^{+\cdot}$) show analogous fragmentation patterns. The fragment ion at m/z 255 arises by loss of the side chain through C_{17}–C_{20} cleavage; [(398–125(C_9H_{17})–18(H_2O)]$^+$ and [470–125(C_9H_{17})–90(TMSOH)$^+$]. The prominent ion at m/z 121 (base peaks) which is also present in the mass spectrum of dihydrotachysterol$_3$ [3] may be interpreted to result from C_7–C_8 bond cleavage and a concomitant H shift; [398–260($C_{19}H_{32}$)–18(H_2O)]$^+$ and [470–260($C_{19}H_{32}$)–90(TMSOH)$^+$]. It is worth noting that C_7–C_8 cleavage is the main fragmentation reaction in D vitamins [10].

Identification of "c2" as 25-hydroxydihydrotachysterol$_2$

2.5 μl of sample "c2" was applied to the OV-101 column as described before. The total ion current chromatogram (not shown) demonstrated several distinct peaks. The mass spectrum (Fig. 3A) of one of the components showed a peak at m/z 121 (base peak), characteristic in the mass spectra of DHT_2 and its trimethylsilyl ether. The presence of a molecular ion at m/z 486 is consistent with a monotrimethylsilyl ether of a monohydroxylated DHT_2 derivative ($C_{31}H_{54}O_2Si^{+\cdot}$). The fragments at m/z 468

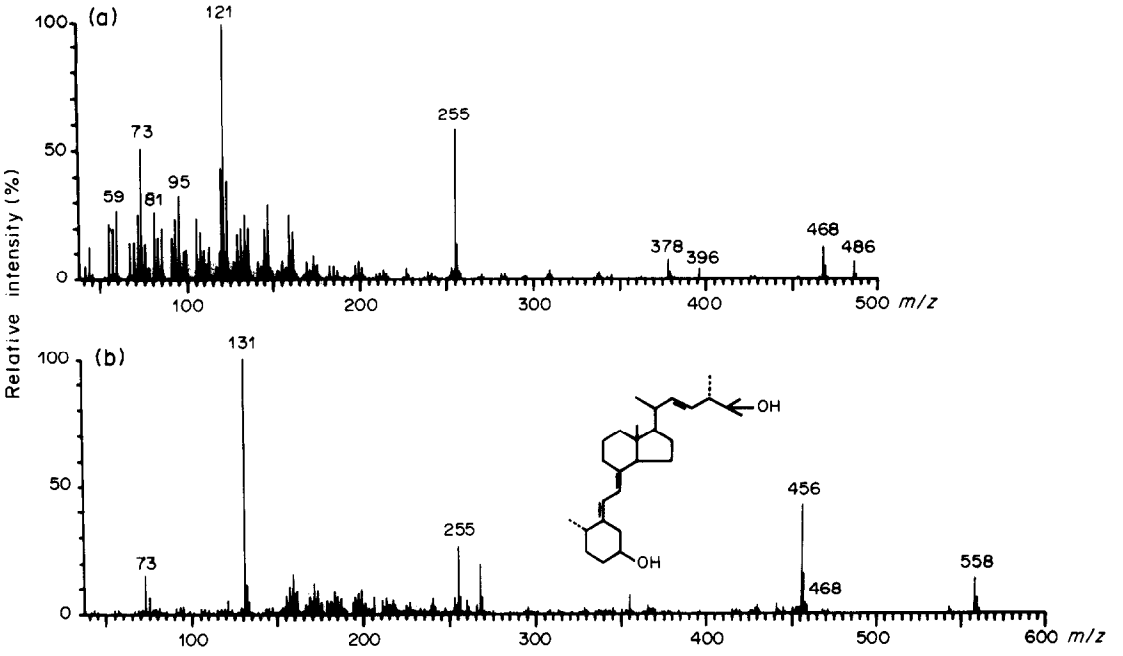

Fig. 3. Mass spectra of the monotrimethylsilyl ether (with the silyl group attached to C_1) (A) and the ditrimethylsilyl ether (B) of 25-hydroxydihydrotachysterol$_2$. Inserted: the chemical structure of the proposed parent compound. The peaks after m/z 150 in spectrum B have been amplified 10-fold. The peak at m/z 456 in spectrum B derived from an unknown contaminant. For details: see text. Ordinate: intensity relative to m/z 121 (A) and 131 (B); abcissa: m/z.

$[M^{+\cdot}-18(H_2O)]$, 396 $[M^{+\cdot}-90(TMSOH)]$ and 378 $[M^{+\cdot}-90(TMSOH)-18(H_2O)]$ confirm the presence of a sylilated hydroxyl and a free hydroxyl function in the compound. The peak at m/z 255 $[M^{+\cdot}-141(C_9H_{17}O)-90(TMSOH)$; C_{17}–C_{20} cleavage] indicates that the free, additional hydroxyl group is associated with the side chain.

Additional evidence for structural assignment was obtained after prolonged silylation. Again 2.5 μl of the reaction mixture was applied to the OV-101 column as described. The total ion current chromatogram (not shown) showed 4 distinct peaks. The mass spectrum (Fig. 3B) of the principal component indicated a molecular ion of m/z 558. This corresponds to the molecular ion of a ditrimethylsilyl ether of DHT$_2$ ($C_{34}H_{62}O_2Si_2^{+\cdot}$) and therefore confirms that an additional hydroxyl function has been incorporated into DHT$_2$. The presence of a peak at m/z 255 which is also observed in the mass spectra of DHT$_2$ and its monotrimethylsilyl ether indicates the location of this hydroxyl group on the side chain since it arises by cleavage of the C_{17}–C_{20} bond $[M^{+\cdot}-213(C_9H_{16}OSi(CH_3)_3$; side chain)–90(TMSOH)]. Further, the intense peak at m/z 131 {base peak; $(CH_3)_2C{=}O^+$–$Si(CH_3)_3$) [11]} which is also present in the spectra of the trimethylsilyl ethers of 25-hydroxylated metabolites of vitamin D_3 and vitamin D_2 [12, 13, 14], but not in the spectra of DHT$_2$ and its monotrimethylsilyl ether, can only arise by cleavage of the bonds between C_{24} and C_{25}, with the hydroxyl group attached to C_{25}. The peak at m/z 131 corresponds to m/z 59 ($C_3H_7O^+$) in the spectrum of monotrimethylsilylated "c2" (Fig. 3A).

Assuming that a hydroxyl group is present at C_3 these mass spectral data, together with the data of u.v. absorption spectral analysis and chromatography, establishes the structure of "c2" as 25-hydroxydihydrotachysterol$_2$.

The peak at m/z 456 results from some contaminant. The presence of this contaminant could be ascertained by recording the change in the spectra with the retention time, the ratio contaminant/"c2" becoming a maximum at the trailing edge of the GC peak.

Identification of "e" as 1α,25-dihydroxydihydrotachysterol$_2$ and 1β,25-dihydroxydihydrotachysterol$_2$

2.5 μl of sample "e" was applied to the OV-101 column as described before. The total ion current chromatogram (Fig. 4) showed 4 peaks. Two components, preliminarily designated "e1" and "e2", exhibited essentially identical mass spectra (Fig. 5 "e1" and "e2"). Therefore, the following interpretation of the mass spectral data applies to both compounds. The presence of a molecular ion at m/z 646 suggests the incorporation of 2 additional hydroxyl functions into DHT$_2$; it corresponds to the molecular ion of a tritrimethylsilyl ether of DHT$_2$ ($C_{37}H_{79}O_3Si_3^{+\cdot}$). The fragments at m/z 343 $M^{+\cdot}-213(C_{12}H_{25}OSi)-90(TMSOH)$ and m/z 253 $[343^{+}-90(TMSOH)]$ which arise by loss of the side chain (C_{17}–C_{20} cleavage) indicate that only one hydroxyl is present in the side chain. The peak at m/z

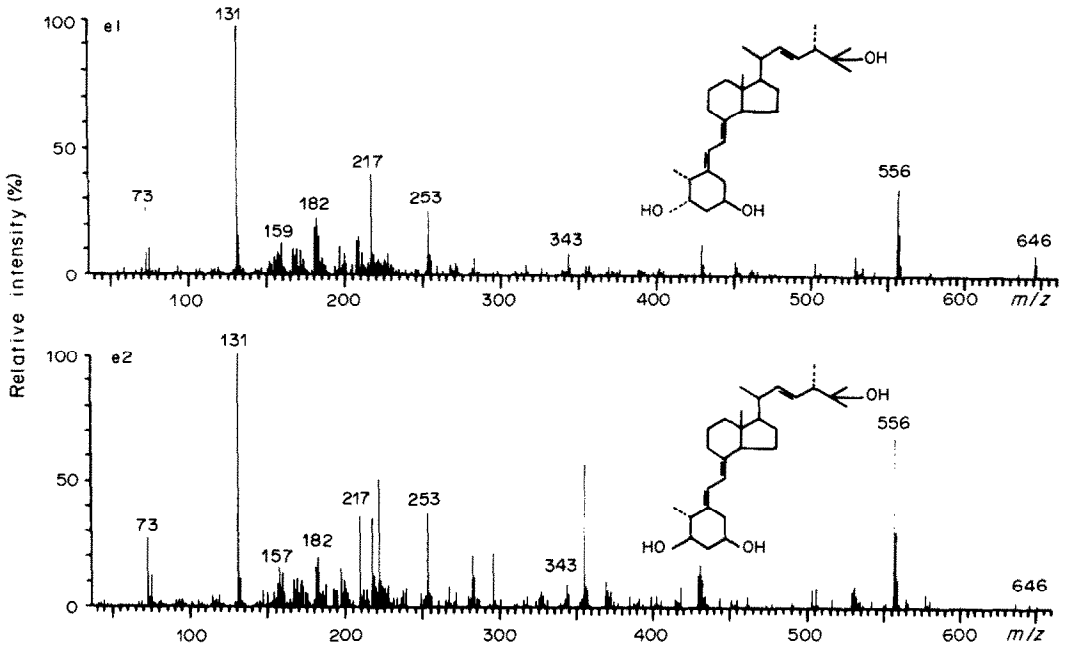

Fig. 5. Mass spectra of the trimethylsilyl ethers of 1β,25-dihydroxydihydrotachysterol$_2$ ("e1") and 1α,25-dihydroxydihydrotachysterol$_2$ ("e2"). Inserted: the chemical structure of the proposed parent compounds. The peaks after m/z 150 have been amplified 10-fold. For details: see text. Ordinate: intensity relative to m/z 131; abcissa: m/z.

131 [base peak; $(CH_3)_2C{=}O^+Si(CH_3)_3$] requires C_{25} as its position. It is further assumed that the 3α-hydroxyl of DHT_2 is still intact, thus leaving one hydroxyl group unaccounted for. The u.v. absorption spectrum rules out C_6 and C_7 as possible positions. A hydroxyl group attached to the 5,7-diene structure could conceivably cause a small bathochromic or hypochromic shift. The peak at m/z 182 could result from the cleavage of the C_5–C_6 bonds

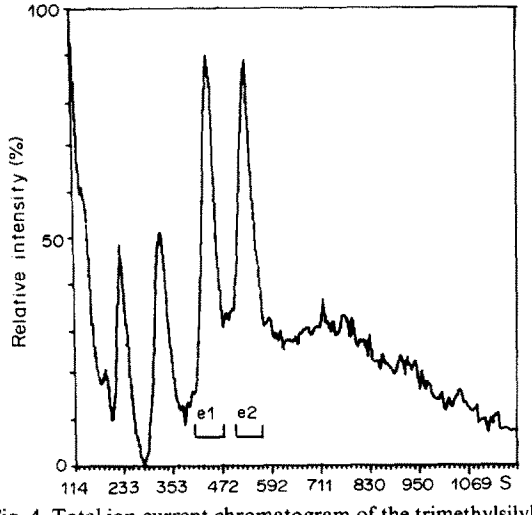

Fig. 4. Total ion current chromatogram of the trimethylsilyl ethers of 1β,25-dihydroxydihydrotachysterol$_2$ ("e1") and 1α,25-dihydroxydihydrotachysterol$_2$ ("e2"). Chromatographic conditions: 3% OV-101 glass packed tubular column (1 m, 2.5 mm in diam) oven temperature: programmed for 280 to 300°C at 2°C min. For details: see text. Ordinate: relative intensity; abcissa: elution time in s.

[$M^{+\cdot}$–213($C_{12}H_{25}OSi$; side chain)–135($C_{10}H_{15}$; C and D rings)–26(C_2H_2; C_6 and C_7)–90(TMSOH)]. This suggests that the second additional hydroxyl function is in ring A. The ion at m/z 217 which is also observed in the mass spectrum of the cyclised tritrimethylsilyl ether of 1α,25-dihydroxyvitamin D_3 [14] confirms this interpretation since it can only be attributed to the fragment $(CH_3)_3SiO{-}CH{=}CH{-}CH{=}O^+Si(CH_3)_3$ formed by cleavage of the C_1–C_{10} and C_3–C_4 bonds, with the second hydroxyl group attached to C_1. These data establishes that the structure of the epimeric parent compounds is 1-ambo, 25-dihydroxydihydrotachysterol$_2$. The tentative stereochemistry of the 1-hydroxyl function can be deduced from the relative intensities of the peaks at m/z 646 and m/z 556 [$M^{+\cdot}$–90($HOSi(CH_3)_3$)] [see also Ref. 15 for an analogues case]. It is seen that the molecular ions of the tritrimethylsilyl ethers of the epimers differ in the rate of loss of TMSOH. An axial trimethylsilyl ether function at C_1 introduces non-bonded interactions into the A-ring and these increase the reactivity of the molecular ion. However, due to A-ring chair–chair interconversion [16] a trimethylsilyl ether group at C_1 can take up an axial position in both the 1α- and 1β-conformer. Yet, crowding of the hydrogens of the trimethylsilyl ether functions at C_1 and C_3 becomes maximal when both are axial. As a result the molecular ion of the diaxial conformer will have a greater tendency to lose TMSOH compared to its equatorial(C_1)–axial(C_3) counterpart. Thus spectrum "e2" is tentatively assigned to be that of the tritrimethylsilyl ether of 1α,25-dihydroxydihydrotachysterol$_2$. Consequently, spectrum "e1" is that of

the tritrimethylsilyl ether of 1β,25-dihydroxydihydrotachysterol$_2$.

DISCUSSION

For the first time the present report provides evidence for the putative chemical structure of 3 metabolites of dihydrotachysterol$_2$. It is obvious that complete structural verification awaits the synthesis of these metabolites. As suspected, *in vivo* DHT$_2$ is readily [6] hydroxylated at C_{25} to yield 25-hydroxydihydrotachysterol$_2$. However, it came as a total surprise that this metabolite undergoes [6] a second hydroxylation at C_1 to give both 1α,25-dihydroxydihydrotachysterol$_2$ and 1β,25-dihydroxydihydrotachysterol$_2$. The A-ring of DHT$_2$ is inverted 180° with respect to the A-ring of the vitamins D. Consequently the 1α- and 1β-hydroxy groups of the 1,25-dihydroxy derivatives of DHT$_2$ are displaced, occupying respectively a pseudo 3β- and pseudo 3α-position. Hitherto there were no reports of the existence of 9,10-seco steroid 3α- and 3β-hydroxylases in higher animals. Furthermore, the findings indicate that generally accepted concepts on the functional role of hydroxy groups at C_3 in determining the expression of vitamin D activity may have to be reconsidered. As a first consequence the statements on the biological activity of 3-deoxy-1α-hydroxyvitamin D$_3$ [17, 18] should be viewed with circumspection.

The application of HPLC methods did not result in complete purification of the metabolites of DHT$_2$. Both the preparations "c2" and "e" showed massive contamination by ultraviolet spectrometry. Since this extraneous material was also present in the corresponding HPLC fractions used as a reference, whereas it was absent in preparation "b", it is not unlikely that the appearance of this material is connected with the water-containing solvent system used. It cannot be excluded that the problems of purification might be reduced by using gel chromatography prior to HPLC, by using extracts of other body tissues, by raising the metabolites in a different species or by changing the chromatographic properties of the material through derivatization [19]. Since purity requirements were not achieved, biological activities could not be determined.

Similarly, the structural assignment of the metabolites of DHT$_2$ was complicated by the fact that purity requirements were not achieved. In fact, all conclusions drawn from the mass spectral data were based on the assumption that the DHT$_2$ carbon skeleton and the 3α-hydroxy function remain intact during metabolism. This assumption was supported partly by the u.v. absorption spectral data. It was further strengthened by taking into account some similarities between the features of the fragmentation patterns of the trimethylsilyl ethers of DHT$_2$ and its metabolites. Evidence in support of the existence of the additional hydroxyl groups came from the liquid-chromatographic data and the nominal increases of the weights of molecular ions and fragments. Furthermore, it was a matter of elegance to conclusively prove the presence of DHT$_2$ in serum after oral administration.

Since *in vivo* hydroxylation of vitamin D sterols at positions equivalent to 3α and 3β is without precedent, another possible explanation of the results should be considered as well. Orally administered vitamin D$_3$ is converted to a small extent into 5,6-*trans* vitamin D$_3$ [20]. It is conceivable that in a similar way DHT$_2$ is converted into 25-hydroxydihydrovitamin D$_2$-II. Dihydrovitamin D$_2$-II is the 5,6-*trans* isomer of DHT$_2$. Unfortunately, the ultraviolet spectra of DHT$_2$ and dihydrovitamin D$_2$-II are identical [9] and it is most likely that this also applies to their corresponding hydroxylated forms. This implies that it cannot be excluded that "c2" represents 25-hydroxydihydrovitamin D$_2$-II. If that is the case, it is not unlikely that the latter may be hydroxylated at C_1 forming 1α,25-dihydroxydihydrovitamin D$_2$-II which would explain the occurence of one 1,25-dihydroxy form after DHT$_2$ administration. The appearance of two 1,25-dihydroxy forms following gas chromatography could be the result of partial, thermal isomerization of "e". Current studies should resolve these questions.

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