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POLAR METABOLITES OF DIHYDROTACHYSTEROL$_3$ IN THE RAT

COMPARISON WITH *IN VITRO* METABOLITES OF 1α,25-DIHYDROXYDIHYDROTACHYSTEROL$_3$

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Abstract—The metabolism of 25-hydroxydihydrotachysterol$_3$ (25-OH-DHT$_3$) to more polar metabolites was investigated *in vivo* in the rat and compared with the *in vitro* metabolism of 1α,25-dihydroxy-DHT$_3$ (1α,25-(OH)$_2$DHT$_3$) in the osteosarcoma cell line UMR 106. Rats were given 2 mg of DHT$_3$ in divided doses at 0 and 6 hr. Plasma was collected 24 hr after the initial dose, extracted, separated, and polar metabolites purified by HPLC. A number of polar metabolites were formed *in vivo* with mass spectrometric characteristics which suggested that they were derived from a previously isolated metabolite of 25-OH-DHT$_3$, T3/H. Of these, four were isolated and identified as 24-oxo-T3/H, 24-hydroxy-T3/H, 26-hydroxy-T3/H and the 26,23-lactone of T3/H. In view of the identification of T3/H as a mixture of 1α- and 1β-hydroxylated 25-OH-DHT$_3$, osteosarcoma cells (UMR 106) were incubated with chemically synthesized 1α,25-(OH)$_2$DHT$_3$ in an attempt to determine from which component of the T3/H mixture these metabolites were derived. Again, more polar metabolites were formed and five of these were isolated by lipid extraction, purified by HPLC and identified as 24-oxo-1α,25-(OH)$_2$DHT$_3$, 1α,23,25-(OH)$_3$DHT$_3$, 24-oxo-1α,23,25-(OH)$_3$DHT$_3$, 1α,24,25-(OH)$_3$DHT$_3$ and 1α,25,26-(OH)$_3$DHT$_3$. Three of the *in vitro* metabolites were similar to those found in rat plasma but only two of these metabolites were available in sufficient amounts to allow comparison. The chromatographic characteristics, using HPLC and gas chromatography, of these two pairs of metabolites (24-oxo and 24-hydroxy) were examined and it was demonstrated that they were not the same. It is therefore suggested that the polar metabolites formed *in vivo* are in fact metabolites of the T3/Hb component (1β,25-(OH)$_2$DHT$_3$) rather than the T3/Ha component (1α,25-(OH)$_2$DHT$_3$). Supporting evidence for this suggestion was obtained when a small quantity of 1β,25-(OH)$_2$DHT$_3$, obtained from chemically synthesized 1β-OH-DHT$_3$ by incubation with Hep 3B cells, was further incubated in the osteosarcoma UMR 106 system. Preliminary studies indicated that the putative 24-oxo and 24-hydroxy metabolites formed from 1β,25-(OH)$_2$DHT$_3$ had chromatographic and mass spectral properties almost indistinguishable from those of corresponding metabolites of T3/H formed *in vivo*. All the metabolites formed *in vivo* and *in vitro* are components of two metabolic pathways described previously for 25-hydroxyvitamin D$_3$ and also for 25-OH-DHT$_3$.

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§ *Trivial names and abbreviations:* DHT$_2$, dihydrotachysterol$_2$, 10*S*-9,10-seco-ergosta-5(*E*),7(*E*),22(*E*)-trien-3β-ol; DHT$_3$, dihydrotachysterol$_3$, 10*S*-9,10-seco-cholesta-5(*E*),7(*E*)-dien-3β-ol. The product of DHT$_3$ is also illustrated in Fig. 9. Where it is not necessary or desired to distinguish between DHT$_2$ and DHT$_3$, the term DHT is used. Hydroxylated metabolites are referred to in the text using the shorthand illustrated here for 1α,25-dihydroxy-DHT (1α,25(OH)$_2$DHT). Similar considerations apply to vitamins D$_2$ (D$_2$) and D$_3$ (D$_3$). T3/H refers to the metabolite of 25-OH-DHT$_3$ (and T2/H from 25-OH-DHT$_2$) whose two components (T3/Ha and T3/Hb) have not been separated. T3/Ha has now been shown to be 1α,25(OH)$_2$DHT$_3$ and T3/Hb to be 1β,25(OH)$_2$DHT$_3$. Similar shorthand is used for the components of T2/H. GC-MS, gas chromatography–mass spectrometry; TSIM, *N*-trimethylsilylimidazole; TMSi, trimethylsilyl; nBBA, *n*-butylboronate derivative; Me, methyl; EI(+), electron impact, monitoring positive ions; AUFS, absorbance units full scale; HIM, hexane–isopropanol–methanol.

Dihydrotachysterol (DHT§), like vitamin D, exists in two forms with differing side-chains, of which only DHT$_2$ is licensed for administration to human patients and has been used as a calcaemic agent for the last 50 years. We have described recently the metabolism of 25-OH-DHT$_3$ in the isolated perfused rat kidney [1] and demonstrated the existence of two pathways, analogous to the 24-oxidation and lactone pathways of 25-OHD$_3$ [2, 3]. We have also demonstrated that similar metabolic pathways are seen *in vivo*, although all the metabolic intermediates have not yet been isolated. We originally isolated, but did not identify, non-renal metabolites of DHT$_3$ and DHT$_2$ (T3/H and T2/H) formed *in vivo*, of which the latter bore a striking resemblance to a previously reported metabolite of DHT$_2$ [4]. During the course of our *in vivo* studies in the rat, more polar metabolites of DHT$_3$ were observed. Preliminary studies [5] of these metabolites suggested that they are derived from one or both components of T3/H, which has been shown to be a mixture (T3/Ha and T3/Hb). T3/Ha has now been

identified as $1\alpha,25(OH)_2DHT_3$ and T3/Hb as $1\beta,25(OH)_2DHT_3$) [6]. This paper describes the isolation and identification of some of these polar metabolites and also compares their chromatographic characteristics using HPLC and GC with those metabolites generated *in vitro* by incubation of chemically synthesized $1\alpha,25(OH)_2DHT_3$ in a rat osteosarcoma cell line (UMR106). This cell line, a known target cell for $1,25(OH)_2D_3$, has been shown previously to metabolize 25-OH-DHT_3 along the same two pathways already demonstrated in the perfused rat kidney [7] and also to catabolize $1\alpha,25(OH)_2D_3$ [8].

MATERIALS AND METHODS

Materials

DHT_3 was a generous gift from Dr L. Downey, Duphar Pharmaceuticals Ltd (Southampton, U.K.). DHT_2, methoxyamine hydrochloride and polyethylene glycol were obtained from the Sigma (Poole, U.K.). *N*-trimethylsilylimidazole (TSIM) was purchased from Pierce and Warriner (Chester, U.K.). Sodium hydroxide, sodium periodate, sodium borohydride and solvents which were of an analytical grade were obtained from BDH Chemicals Ltd (Poole, U.K.). Solvents used for HPLC were purchased from either Rathburn Chemical Ltd (Wakeburn, U.K.) or Caledon (Georgetown, Canada). Trypsin, penicillin G, gentamycin, fungizone and McCoy's medium were purchased from Gibco (Grand Island, NY, U.S.A.). Foetal calf serum was supplied by Flow Laboratories (Costa Mesa, DA, U.S.A.). Sep-Pak cartridges were obtained from Milliport U.K. Ltd (Watford, U.K.).

Equipment

HPLC was performed on a system comprising of a WISP 712 sample injector, Model 590 pump and Model 990 photodiode array detector all supplied by Millipore U.K. Ltd (Watford, U.K.). HPLC columns employed were Hibar LiChrospher Si60 (119 × 4 mm i.d.) and Hibar Superspher RP-18 (119 × 4 mm i.d.) from E. Merck (Darmstadt, Germany), and Zorbax-CN 5μ (250 × 4.6 mm i.d.) from Jones Chromatography (Mid Glamorgan, U.K.). Gas chromatography-mass spectrometry (GC-MS) was performed on an HP (Hewlett–Packard, Palo Alto, CA, U.S.A.) 5890 GC using a HP-1 cross linked methyl silicone gum (12 m × 0.2 mm × 0.33 μm film thickness) column with helium carrier gas at a flow rate of 1 mL/min with an HP 5970 Series Mass Selective Detector. Direct probe MS was carried out on a model HP 5985 mass spectrometer.

Chemical synthesis

$1\alpha,25(OH)_2DHT_3$ was synthesized from the 1-*O*, 3-*O*-bis-tertiary butyldimethylsilyl ether obtained by silyation of $1\alpha,25(OH)_2$-(5E)-D_3 as described elsewhere*.

* Qaw F, Calverley MJ, Schroeder NJ, Trafford DJH, Makin HLJ and Jones G, 25-hydroxylation of synthetic 1-hydroxydihydrotachysterols: comparison with *in vivo* metabolites in the rat. Unpublished work.

In vivo *experiments*

Adult male and female Wistar and Sprague–Dawley rats bred in house and fed on a standard rodent diet *ad lib.* were used. Each rat weighing between 200 and 300 g was given two 1 mg injections of DHT_3 in 500 μL of 6% v/v ethanol in polyethylene glycol. The second injection was given 6 hr after the initial dose and 18 hr after the last dose the rats were anaesthetized with CO_2 and exsanguinated by cardiac puncture. Blood was collected into lithium heparin tubes and the plasma recovered by centrifugation.

Pooled plasma (from 40 rats approximately 100 mL) was extracted and DHT metabolites recovered using previously published methods [9]. Briefly, the extraction procedure consisted of addition of an equal volume of acetonitrile to plasma, whirlmixing for 5 min and centrifuging. Solid phase extraction was performed by application of the acetonitrile supernatant after adjustment to 60% aqueous with 0.4 M phosphate buffer (pH 10.5) to a column (height 4 × 4.5 cm diameter) packed with C18 material obtained from Sep-Pak C18 cartridges. The column was washed with water and water : methanol (50 : 50, v/v) and DHT metabolites eluted from the column with methanol. The methanol extract was then applied to a column (height 2 × 3 cm diameter), packed with SIL material obtained from Sep-Pak SIL cartridges and separated into three fractions which were eluted with 1, 3 and 30% isopropanol in hexane. The 30% (v/v) isopropanol–hexane fraction from this separation was dried down in a vacuum oven at 30° and redissolved in 200 μL of HPLC mobile phase. Metabolites were purified using three different chromatography systems prior to chemical modification and/or mass spectrometric characterization. Initial separation was performed on a Hibar LiChrospher Si60 column using a mobile phase hexane–2-propanol–methanol (94 : 3 : 3, v/v/v) at a flow rate of 1.5 mL/min. Peaks separated on this column, identified as metabolites of DHT by their characteristic UV spectra, were collected manually. Where sufficient material was recovered, the isolated peaks were then purified on a Zorbax-CN column using a mobile phase hexane–2-propanol–methanol (90/6/4, v/v/v). Final purification was achieved on a Hibar Superspher RP-18 column using a mobile phase methanol–water–acetonitrile (60 : 25 : 15, v/v/v).

In vitro *osteosarcoma cell line incubations*

Osteoblast-like UMR 106 cells (1.5×10^7) [10] obtained from J. N. M. Heersche, University of Toronto, Canada were grown in 75-cm^2 culture flasks containing 20 mL of McCoy's modified 5A medium supplemented with 10% fetal calf serum, penicillin G (100 μg/mL), gentamycin (5 μg/mL) and fungizone (300 ng/mL). Cultures were maintained at 37° in humidified atmosphere of 5% CO_2 in air where medium was changed every 3 days. At late log phase of growth the medium was discarded and cells in air-tight culture flasks were incubated further in 6 mL/flask fetal calf serum-supplemented medium, in the presence of 27 μg of $1\alpha,25(OH)_2DHT_3$ (10 μM), for 36 hr at 37° room temperature on 60 cycles/min shaker. Cells and medium were extracted

using a modification of the method of Bligh and Dyer [11], substituting dichloromethane for chloroform. Lipid extracts were dried down under a stream of nitrogen or *in vacuo*. The residues were dissolved in hexane–isopropanol–methanol (HIM) (88:10.2, v/v/v) prior to purification on HPLC using 5μ-Zorbax SIL eluted with HIM (88:10:2, v/v/v) at a flow rate of 2 mL/min. Peaks with the characteristic chromophore of dihydrotachysterol compounds were collected, redissolved in HIM (88:10:2, v/v/v) and chromatographed on Zorbax-CN eluting with HIM (88:10:2, v/v/v) at a flow rate of 1.3 mL/min.

Characterization of metabolites

Chemical modifications and/or GC-MS were carried out as follows.

Borohydride reduction. Borohydride reduction was carried out to confirm the presence of oxo groups by reduction to the corresponding hydroxyl. Material (100–250 mg) was dissolved in a small volume of ethanol and diluted with 10 mL 0.01 M phosphate buffer (pH 6). Two millilitres of a 10% (w/v) solution of sodium borohydride in 0.01 M sodium hydroxide was added and incubated for 15 min at 50°. Excess borohydride was removed by addition of 2 mL 10% (v/v) acetic acid and further incubation at 50° for 15 min. Reaction products were extracted with dichloromethane or ethyl acetate and purified by straight-phase HPLC.

Periodate oxidation. Periodate oxidation was carried out to confirm the presence of vicinal hydroxyl groups. Approximately 30 ng of material was incubated with 50 μL of a 10% (w/v) sodium periodate in 0.01 M sodium hydroxide solution at 50° for 15 min. Any products were then extracted into 1 mL of dichloromethane or ethyl acetate and purified by straight-phase HPLC.

Methyloxime formation. Approximately 200 ng of material were treated with 10% (w/v) solution of methoxyamine hydrochloride in pyridine. The sample was incubated for 1 hr at 50°, the pyridine solvent removed under nitrogen and the products extracted into 2 mL of toluene. The toluene was

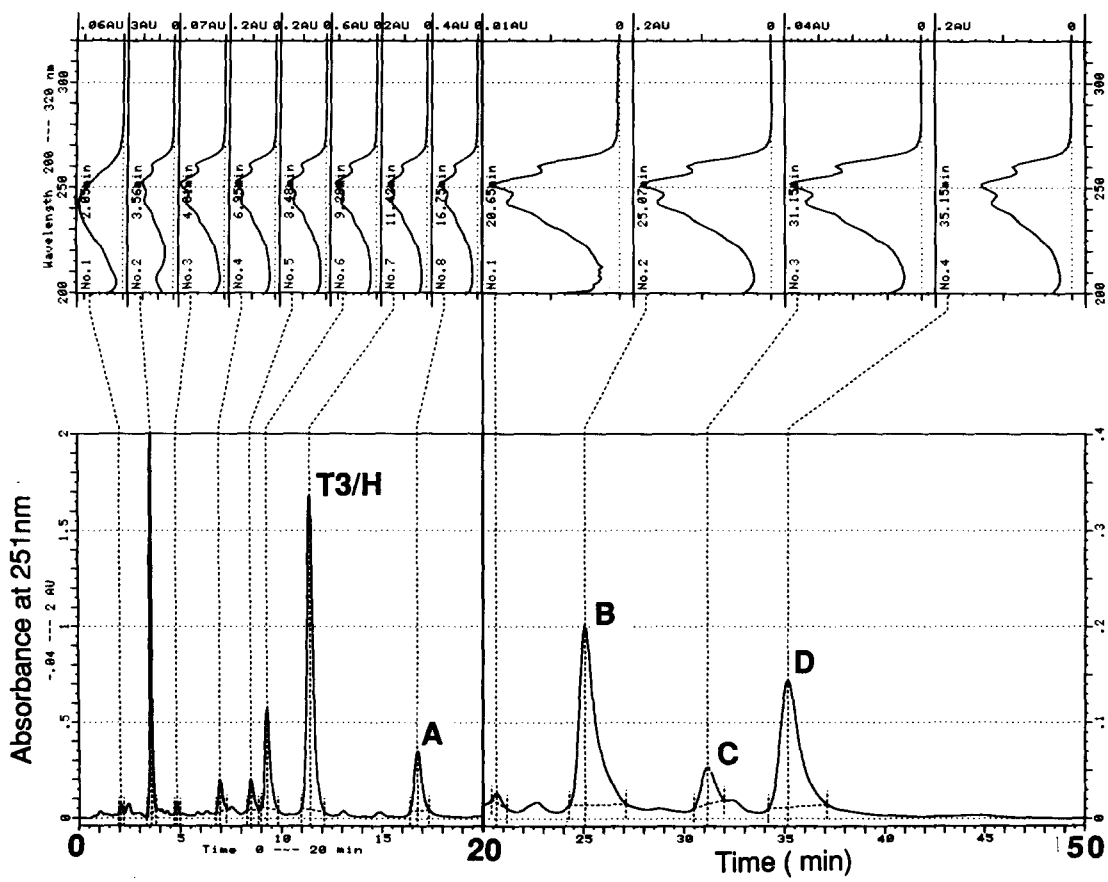

Fig. 1. Straight-phase HPLC of the 30% isopropanol in hexane fraction of *in vivo* DHT$_3$ metabolites isolated from rat plasma. The peak corresponding to T3/H is indicated. More polar metabolites eluting after T3/H are (A) 24-oxo-T3/H, (B) 24-hydroxy T3/H, (C) 26-hydroxy T3/H, (D) 26,23-lactone T3/H. These structures are assigned on the basis of evidence described in the text. The upper panels show the UV spectrum (200–320 nm) of each peak and all the labelled metabolites can be seen to possess the UV spectrum characteristic of DHT compounds. The absorbance axis after 20 min has been reduced by a factor of 5 (from 2 AUFS to 0.4 AUFS) thus amplifying the signal from the polar peaks under investigation.

evaporated under a nitrogen steam, and the products purified by straight-phase HPLC.

MS. Direct probe MS was carried out on some of the *in vitro* metabolites. Samples (400–800 ng) were dissolved in hexane or methanol and transferred to clean dry capillary tubes which were then introduced into the mass spectrometer on a direct insertion probe which was heated from a starting temperature of between 25 and 40° at a programme rate of 15°/min. Ionization voltage was 70 eV. EI(+) spectra were recorded and stored on disk with a Hewlett–Packard Series 21MX-E computer.

EI(+) mass spectra of the per-trimethylsilyl ethers of metabolites from both *in vitro* and *in vivo* studies were obtained by GC-MS. Each purified metabolite was derivatized with 50 μL of TSIM, incubated for 1 hr at 50°. The products were dissolved in hexane and passed through a small prewashed Lipidex column to remove excess TSIM. Samples were then dried down and transferred to small conical vials for injection onto the GC. Mass spectra were obtained by averaging each peak and subtracting the background. Cyclic *n*-butyl boronate derivatives were formed by addition of 50 μL of *n*-butylboronic acid to approximately 100 ng of the metabolite of interest. This was incubated for 15 min at room temperature then evaporated to dryness using a nitrogen stream prior to treatment with TSIM as described above.

RESULTS

In vivo *metabolism of* DHT_3

In vivo injection of DHT_3 into rats gave rise to a large number of metabolites which were identified by their characteristic UV spectrum. Figure 1 shows the HPLC separation of an extract of rat plasma and indicates the presence of at least 11 metabolites. The two most abundant metabolites are 25-OH-DHT_3 eluting at 3.56 min and the non-renal metabolite T3/H at 11.42 min. Seven DHT_3 metabolites having longer retention times than T3/H are present, although not all are indicated. Of these seven only four (designated A, B, C and D) have been isolated in sufficient quantities to allow identification. The retention times of these metabolites on two of the HPLC columns used are listed in Table 1. Figure 2 shows the EI(+) mass spectra of the two components of T3/H (T3/Ha, $1\alpha,25(OH)_2DHT_3$ and T3/Hb, 1β-$25(OH)_2DHT_3$). The mass spectra of these two compounds contain two significant fragments, *m/z* 253 and *m/z* 217. These fragments can arise: (a) by loss of 2 silanol from the steroid nucleus after C17–20 cleavage (i.e. 433-90-90), indicating the presence of two hydroxyls in the nucleus, and (b) by cleavage of the A-ring between C1 and 10 and C3 and 4 giving a fragment Me_3SiO–C(1)H=C(2)H=C(3)H=O^+SiMe_3 suggesting that the two hydroxylations are on C1 and 3 [4]. The mass spectra of all the *in vivo* metabolites examined here contain these two fragments and this leads to the postulation that these more polar compounds are metabolites of T3/H. Each of these four metabolites has been isolated and identified as follows.

A. 24-oxo-T3/H. This peak had a retention time of 16.75 min on Lichrospher Si60 and was the least polar of the four metabolites studied. It had a UV spectrum which identified it as a DHT metabolite. It was retained on Zorbax-CN which suggested the presence of an oxo group. GC-MS of the perTMSi ether derivative showed a molecular ion of *m/z* 648 indicating the addition of 14 amu (i.e. addition of an oxygen and loss of two hydrogen atoms) to the tri-TMSi ether of T3/H ($M^+ = 634$). Reaction of peak A with methoxyamine hydrochloride produced two less polar products on straight phase HPLC with mass spectra consistent with the formation of *syn*- and *anti*-methyloximes. Peak A was unaffected by treatment with periodate. Further fragments in the mass spectrum of the perTMSi ether were *m/z* $(M-90)^+$, *m/z* 131 (indicating the presence of a 25-hydroxyl group), *m/z* 217 and 253 (fragments seen in the perTMSi ether of T3/H). It is suggested that peak A is the 24-oxo metabolite of T3/H.

B. 24-hydroxy T3/H. This peak had a retention time of 25.07 min on Lichrospher Si60 and the UV spectrum again indicated that it was a DHT metabolite. Periodate oxidation gave rise to a less polar product on straight-phase HPLC suggesting the presence of vicinal hydroxyls. The mass spectrum of the perTMSi ether showed the presence of the fragments *m/z* 217 and 253. The molecular ion *m/z* 722 was at very low abundance and was difficult to see. However, the presence of the fragment at *m/z* 707 $(M-15)^+$ confirmed that the M_r was indeed 722, indicating the presence of four TMSi groups. Other fragments at *m/z* $(M-90)^+$, *m/z* 501 $(M-90-131)^+$ and 411 $(M-90-90-131)^+$ are also seen, the loss of 131 in the latter fragment being due to cleavage of the steroid A ring with loss of $C(2)H_2$-C(3)H($OSiMe_3$)–$C(4)H_2 + H$ (=131). (The frag-

Table 1. Retention times of *in vivo* metabolites in two different chromatography systems

Peak*	Lichrospher Si60 (94/3/3 HIM) (1.5 mL/min)	Zorbax-CN 5μ (90/6/4 HIM) (1.2 mL/min)
A (24-oxo-T3/H)	16.75	16.21
B (24-hydroxy-T3/H)	25.07	16.58
C (26-hydroxy-T3/H)	31.15	18.31
D (23,26-lactone of T3/H)	35.15	33.29
T/3H	11.42	10.49
$1\alpha,25(OH)_2D_3$	13.07	11.34

* Suggested identities (in parentheses) are based on mass spectral and other evidence presented in the text and are included here for convenience.

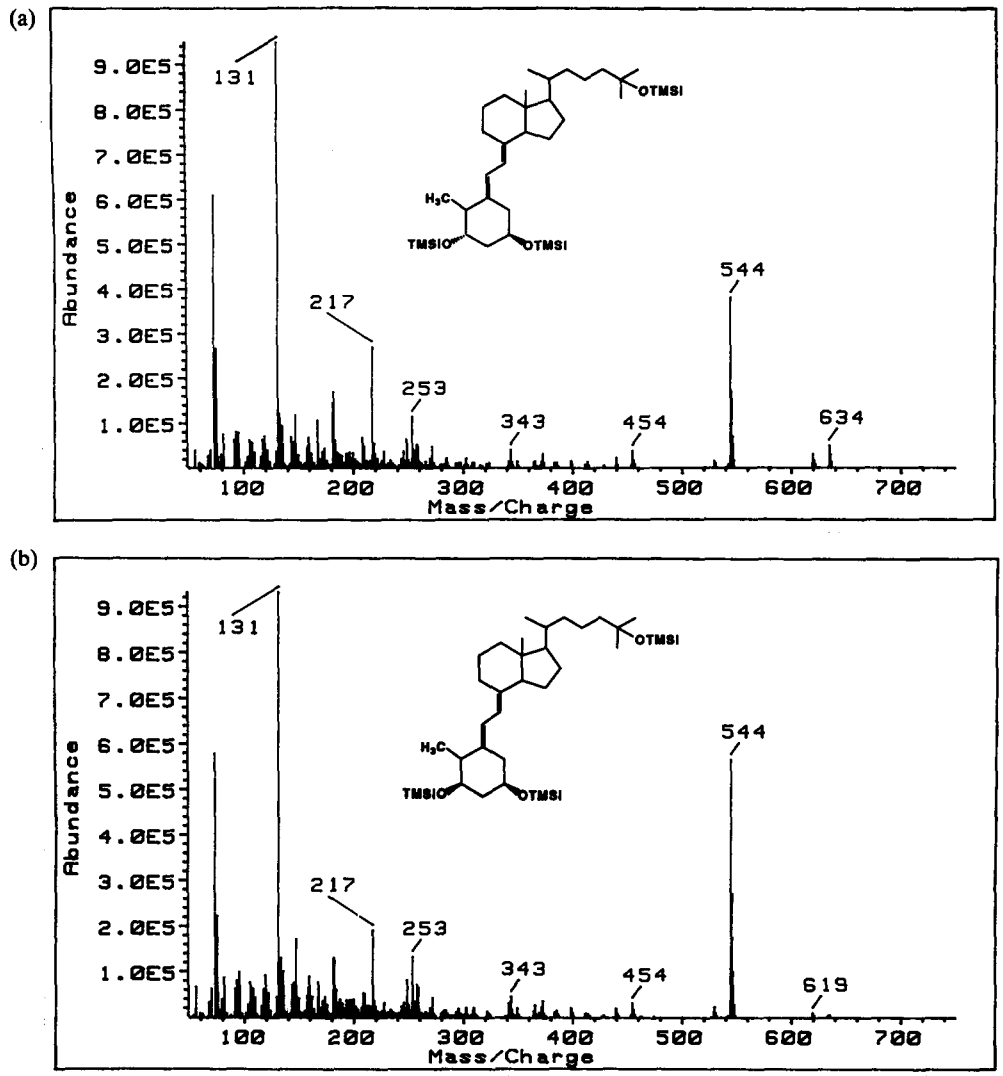

Fig. 2. EI(+) mass spectra of the TMSi ether derivatives of the two components of T3/H, identified as (a) 1α,25(OH)$_2$DHT$_3$ (T3H/a) and (b) 1β,25(OH)$_2$DHT$_3$ (T3/Hb), possessing the molecular ion m/z 634.

ment m/z 131 in high abundance on the other hand is from C24–25 cleavage giving C(26)H$_3$–C(25)(OSiMe$_3$)–C(27)H$_3$). This compound formed an *n*-butyl boronate-TMSi ether derivative giving a prominent ion at m/z 644, again confirming the presence of vicinal hydroxyl groups. It is suggested that peak A is the 24-hydroxy metabolite of T3/H.

C. 26-hydroxy T3/H. This peak had a retention time of 31.15 min on Lichrospher Si60 and once again had a UV spectrum indicating that it was a DHT metabolite. Of the four metabolites studied here, this was in the lowest concentration. Periodate oxidation gave rise to a less polar product on straight-phase HPLC. The mass spectrum of the perTMSi ether gives a very low abundance of the molecular ion at m/z 722 but as with peak B the presence of the ion at m/z 707 (M-15)$^+$ confirmed that the M_r was indeed 722. Ions were also seen at m/z 632 (M-90)$^+$ and m/z 619 (M-103)$^+$. The 103 fragment is suggested to be due to the loss of C(26)H$_2$-OSiMe$_3$. m/z 529 is obtained by loss from the molecular ion of a silanol molecule and the 103 fragment (M-90-103)$^+$. Thus m/z 439, also present, represents (M-90-90-103)$^+$. Another characteristic fragment can be seen at m/z 219 which corresponds to a cleavage between C24 and 25 giving Me$_3$SiO–C(26)H$_2$-C(25)(OSiMe$_3$)–C(27)H$_3$. Insufficient material was available for attempted formation of *n*-butyl boronate-TMSi derivative and subsequent examination by GC-MS. It is suggested that peak C is a 26-hydroxy metabolite of T3/H.

D. 26,23-lactone of T3/H. This peak had a retention time of 35.15 min on Lichrospher Si60 possessing the UV spectrum of DHT and was retarded on Zorbax-CN indicating the presence of an oxo group in the molecule. However no methyloxime could be formed on this molecule and periodate had no effect. The mass spectrum (Fig. 3) of the perTMSi ether of this metabolite gave a

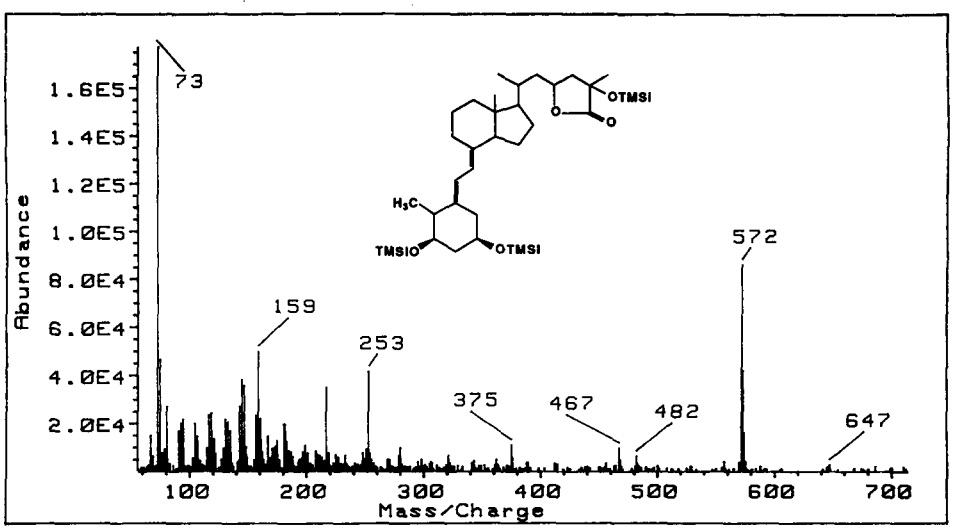

Fig 3. EI(+) mass spectrum of the TMSi ether derivative of the *in vivo* metabolite 26,23-lactone-T3/H, isolated and purified from rat plasma.

molecular ion of very low abundance but the presence of the fragment at m/z 647 $(M\text{-}15)^+$ suggested that the M_r was 662 (i.e. three TMSi groups). Fragments at m/z 217 and 253 were again observed, although the fragment m/z 217 has not been assigned in the Figure, as were fragments at m/z 572 $(M\text{-}90)^+$ and m/z 482 $(M\text{-}90\text{-}90)^+$. m/z 467 represents $(M\text{-}90\text{-}90\text{-}15)^+$. If this metabolite is derived from T3/H, a M_r of 662 indicates the addition of 28 amu and this represents the addition of two oxygen and loss of four hydrogen atoms. Despite the addition of two oxygen functions no further TMSi groups have been added indicating that the extra oxygens are not hydroxyls. The evidence available suggests that Peak D is the 26,23-lactone of T3/H.

In vitro *metabolism of* $1\alpha,25(OH)_2DHT_3$

Similar experiments were carried out using the rat osteosarcoma cell line, incubating with chemically synthesized $1\alpha,25(OH)_2DHT_3$. Figure 4 shows the HPLC separation of an extract of the cell line incubation medium, again identifying metabolites by their UV spectrum. Retention time data of these metabolites on HPLC are given in Table 2. A number of these metabolites, designated E, F, G, H and I, are indicated and these have been isolated and identified as follows.

E. 24-*oxo*-$1\alpha,25(OH)_2DHT_3$. This peak had a retention time of 12.46 min on Zorbax-SIL being the least polar of all metabolites in this study. It had a characteristic DHT UV spectrum. Retention on Zorbax-CN indicated the presence of an oxo group in the molecule. GC-MS of the perTMSi ether derivative showed a molecular ion at m/z 648, indicating the addition of 14 amu to the tri-TMSi ether of $1\alpha,25(OH)_2DHT_3$ ($M^+ = 634$) which represents the addition of an oxygen atom and the loss of two hydrogens. Direct probe MS of the underivatized peak gave a molecular ion of m/z 432 which again indicated an increment of 14 amu to the substrate $1\alpha,25(OH)_2DHT_3$ ($M^+ = 418$). The site of the additional oxygen atom was suggested from the ion at m/z 373 $(M\text{-}59)^+$, which it is suggested arises from C24–25 cleavage. Periodate treatment had no effect on peak E while sodium borohydride reduction gave a peak with a similar retention time to $1\alpha,24,25\text{-}(OH)_3DHT_3$ shown below. Further fragments in the mass spectrum of the perTMSi ether were m/z 558 $(M\text{-}90)^+$, m/z 131 (indicating the presence of a 25-hydroxyl group), m/z 217 and 253 (fragments seen in the perTMSi ether of $1\alpha,25(OH)_2DHT_3$). Peak E is suggested to be the 24-oxo metabolite of $1\alpha,25(OH)_2DHT_3$.

F. $1\alpha,23,25(OH)_3DHT_3$. This peak had a retention time of 14.55 min on Zorbax-SIL and its UV spectrum indicated that it was a DHT metabolite. The molecular ion of m/z 434 in the direct probe MS of the underivatized compound suggested the incorporation of an extra oxygen atom to $1\alpha,25(OH)_2DHT_3$. Fragments due to C23–24 cleavage can be seen at m/z 360 $(M\text{-}C(24)H_2\text{-}C(25)(OH)(Me)_2)^+$ and m/z 342 $(360\text{-}H_2O)^+$. GC-MS of the perTMSi ether derivative showed a molecular ion m/z 722 indicating the incorporation of a fourth hydroxyl group into the substrate (Fig. 5). Peak F was unaffected by treatment with periodate. The fragment at m/z 577 $(M\text{-}145)^+$ in the mass spectrum of the perTMSi ether was due to the C23–24 cleavage and the loss of $C(24)H_2C(25)(OSiMe_3)(Me)_2$. Other fragments at m/z 487 $(M\text{-}145\text{-}90)^+$ and m/z 397 $(M\text{-}145\text{-}90\text{-}90)^+$ in addition to m/z 217 and 253 are also seen. It is suggested that peak F is a 23-hydroxy metabolite of $1\alpha,25(OH)_2DHT_3$.

G. 24-*oxo*-$1\alpha,23,25(OH)_3DHT_3$. This peak had a retention time of 15.98 min on Zorbax-SIL, with a UV spectrum indicating that it was a DHT metabolite. It was retained on Zorbax-CN behind peak H which suggested the presence of an oxo group in the molecule. GC-MS of the perTMSi ether derivative showed a molecular ion of m/z 736

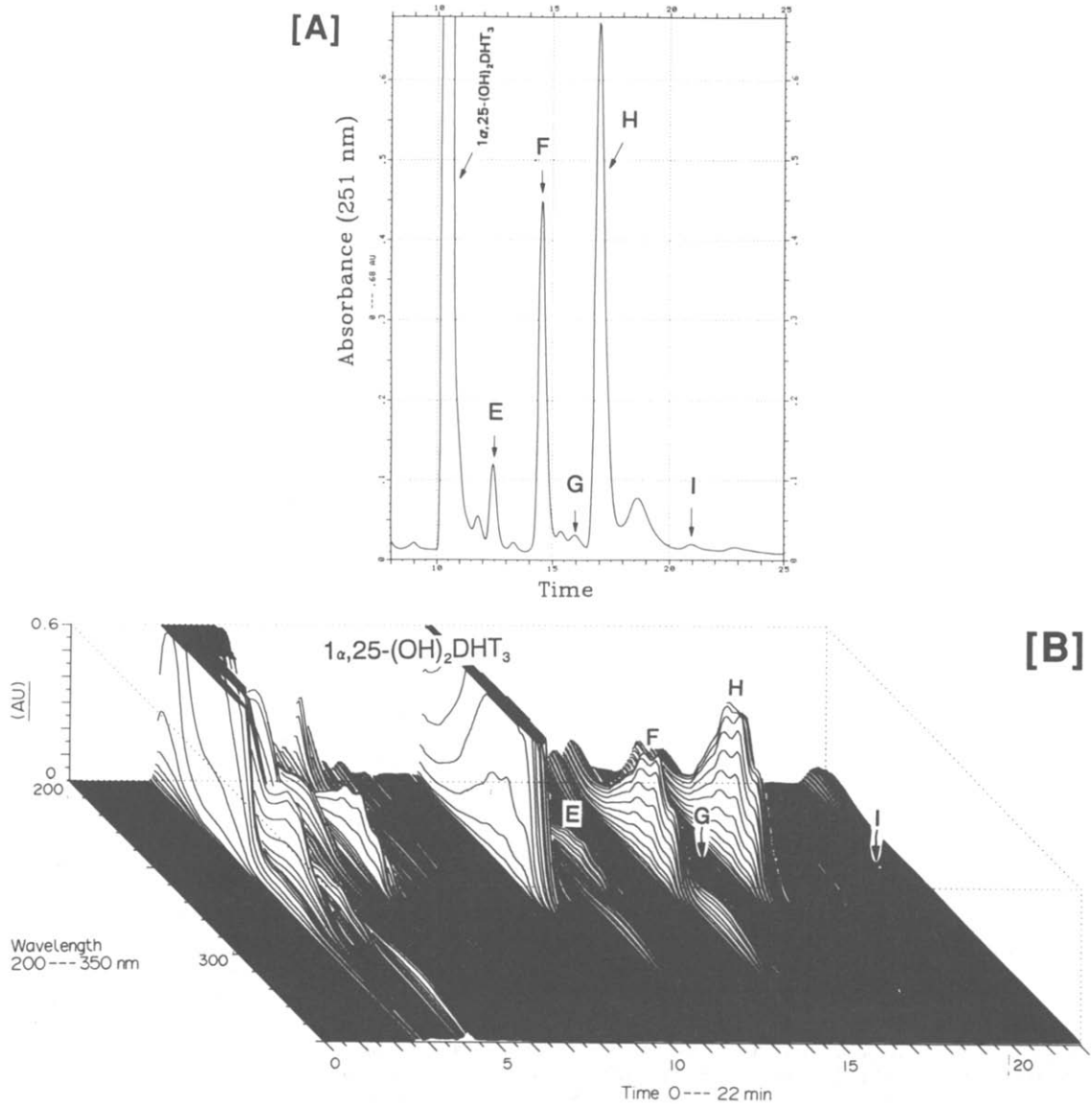

Fig. 4. Straight-phase HPLC of the lipid extract of *in vitro* DHT_3 metabolites isolated from osteosarcoma cells after incubation with synthetic $1\alpha,25(OH)_2DHT_3$. The peak $1\alpha,25(OH)_2DHT_3$ is labelled and the more polar metabolites identified as (E) 24-oxo-$1\alpha,25(OH)_2DHT_3$, (F) $1\alpha,23,25(OH)_3DHT_3$, (G) 24-oxo-$1\alpha,23,25(OH)_3DHT_3$, (H) $1\alpha,24,25(OH)_3DHT_3$, (I) $1\alpha,25,26(OH)_3DHT_3$. These structures are assigned on the basis of evidence described in the text. (A) Shows the chromatogram obtained monitoring at 251 nm and (B) shows the 3-D chromatograph which enables peaks with the characteristic DHT UV spectra to be identified.

Table 2. Retention times (in min) of *in vitro* metabolites of chemically synthesized $1\alpha,25(OH)_2DHT_3$ in different chromatography systems

Peak*	Zorbax-SIL (88/10/2 HIM) (2 mL/min)	Zorbax-CN (88/10/2 HIM) (1.3 mL/min)
E (24-oxo-$1\alpha,25(OH)_2DHT_3$)	12.46	12.48
F (23-hydroxy-$1\alpha,25(OH)_2DHT_3$)	14.55	13.03
G (24-oxo-23-hydroxy-$1\alpha,25(OH)_2DHT_3$)	15.98	16.01
H (24-hydroxy-$1\alpha,25(OH)_2DHT_3$)	17.01	13.29
I (26-hydroxy-$1\alpha,25(OH)_2DHT_3$)	20.93	15.09
$1\alpha,25(OH)_2DHT_3$	10.46	8.56
$1\alpha,25(OH)_2D_3$	10.93	9.17

* Suggested identities (in parentheses) are based on mass spectral and other evidence presented in the text and are included here for convenience.

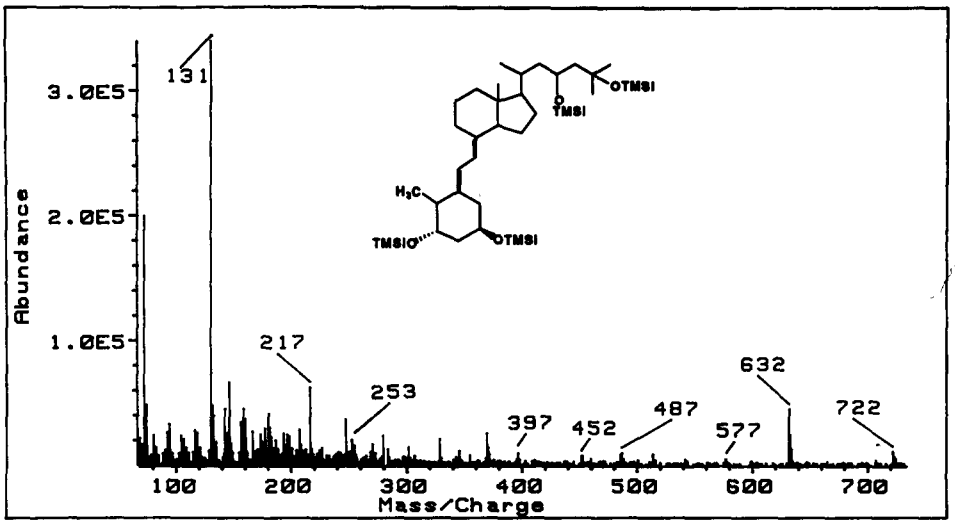

Fig. 5. EI(+) mass spectrum of the TMSi ether derivative of the *in vitro* metabolite 1α,23,25(OH$_3$)DHT$_3$, isolated and purified from UMR 106 osteosarcoma cells.

indicating the addition of 14 amu (i.e. addition of an oxygen and loss of two hydrogen atoms) to the perTMSi ether of peak F ($M^+ = 722$). Major fragments at m/z 646 represent $(M\text{-}90)^+$, m/z 487 $(M\text{-}159\text{-}90)^+$, m/z 397 $(M\text{-}159\text{-}90\text{-}90)^+$ and at a lower abundance m/z 577 $(M\text{-}159)^+$ is also seen. The loss of 159 in those fragments being due to the C23–24 cleavage and the loss of C24,25,26,27-OTMSi with the oxo group on C24. Other fragments at m/z 131, m/z 253 and m/z 217 are also present. The evidence available suggests that peak G is the 24-oxo-23-hydroxy metabolite of 1α,25(OH)$_2$DHT$_3$.

H. 1α,24,25(OH)$_3$*DHT*$_3$. This peak had a retention time of 17.01 min on Zorbax-SIL and had a UV spectrum which identified it as a DHT metabolite. Periodate oxidation gave rise to a less polar product on straight-phase HPLC. Direct probe MS of the underivatized compound gave a molecular ion with m/z 434, an increase of 16 amu, suggesting the incorporation of an oxygen atom to the substrate. The mass spectrum (Fig. 6c) of the perTMSi ether showed a molecular ion on m/z 722 indicating the addition of a fourth TMSi group to the tri-TMSi ether of 1α,25(OH)$_2$DHT$_3$. Further fragments in the mass spectrum of the perTMSi ether were m/z 632 (M-90), m/z 131, m/z 592 $(M\text{-}131)^+$ and m/z 501 $(M\text{-}90\text{-}131)^+$ as seen in 24-hydroxy T3/H. A cyclic *n*-butyl boronate ester-TMSi ether derivative could be formed giving a mass spectrum with a molecular ion at m/z 644 while the ion at m/z 131 disappeared. This indicated the incorporation of *n*-butyl boronate besides the addition of two TMSi groups to C1 and 3 which were sequentially lost as indicated from the fragments at m/z 554 $(M\text{-}90)^+$ and m/z 464 $(M\text{-}90\text{-}90)^+$. All evidence suggests that peak H is a 24-hydroxy metabolite of 1α,25(OH)$_2$DHT$_3$.

I. 1α,25,26(OH)$_3$*DHT*$_3$. This peak had a retention time of 20.93 min on Zorbax-SIL and the UV spectrum again indicated that it was a DHT metabolite. Besides peak G, this metabolite was found in the lowest concentration and no periodate treatment was carried out on it. As in 26-hydroxy T3/H, the mass spectra of the perTMSi ether of this metabolite gave a very low abundance of the molecular ion at m/z 722 but the presence of the ion at m/z 632 $(M\text{-}90)^+$ suggested that the M_r was 722. An important ion at m/z 619 $(M\text{-}103)^+$ is obtained by loss from the molecular ion of the fragment 103 which represents C26-OTMSi. m/z 529 represents $(M\text{-}90\text{-}103)^+$ and m/z 439 represents $(M\text{-}90\text{-}90\text{-}103)^+$. Other fragments seen are m/z 253, 217 and m/z 219 from C25,26,27-(OTMSi)$_2$. The formation of cyclic *n*-butyl-boronate ester-TMSi ether derivative gave a mass spectrum with a molecular ion at m/z 644 and no ion at m/z 131 could be seen which indicated again the presence of only two TMSi groups at C1 and 3. The loss of silanol groups was indicated from fragments at m/z 554 $(M\text{-}90)^+$ and m/z 464 $(M\text{-}90\text{-}90)^+$. It is suggested that peak I is the 26-hydroxy metabolite of 1α,25(OH)$_2$DHT$_3$.

Comparison of in vivo *and* in vitro *metabolites*

Three of the *in vivo* generated metabolites are similar to three of the 1α-hydroxylated metabolites generated in the osteosarcoma cell line. Figure 6 shows (*inter alia*) a comparison of the mass spectra of the *in vivo* generated 24-OH T3H (a) and the *in vitro* generated 1α,24,25(OH)$_2$DHT$_3$ (c). The mass spectra demonstrate the similarity between these two metabolites as fragmentation is almost identical, although there are differences in the relative ion intensities. Similarly, *in vivo* 24-oxo T3H together with 26-OH T3H have mass spectra qualitatively, but not quantitatively, identical to their *in vitro* counterparts, 24-oxo-1α,25(OH)$_2$DHT$_3$ and 1α,25,26(OH)$_3$DHT$_3$, respectively. A comparison of the HPLC retention time data, for two of the *in vivo* and *in vitro* 1α-hydroxylated, generated metabolites, are illustrated in Table 3, whilst Fig. 7 shows the separation of the TMSi ether derivatives of these four metabolites on GC from which it can

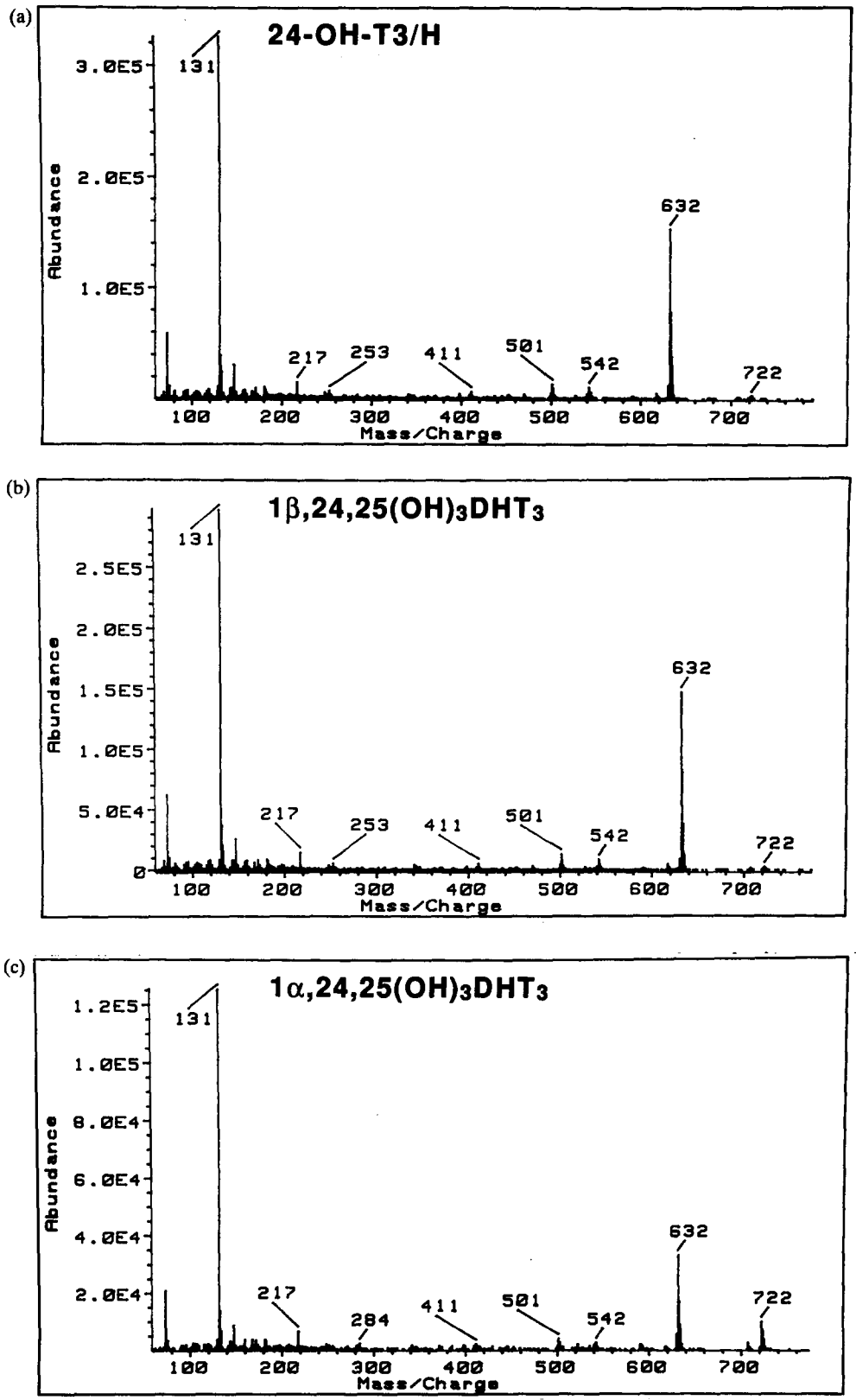

Fig. 6. EI(+) mass spectra of the TMSi ether derivatives of the 24-hydroxylated metabolites from *in vitro* and *in vivo* experiments demonstrating that 24-OH-T3/H (a) and 1α,24,25(OH)$_3$DHT$_3$ (c) undergo similar fragmentation, although differences in the relative intensities of the ions are observed, but that the mass spectra of (a) 24-OH-T3/H and (b) 1β,24,25(OH)$_3$DHT$_3$ are identical.

Table 3. Comparison of the HPLC and GC retention characteristics of *in vivo* and *in vitro* polar metabolites of T3/H, 1α- and 1β-hydroxylated 25-OH-DHT_3

	HPLC relative retention times*	GC relative retention tunes†
Comparison with 1α-OH metabolites‡		
In vivo 24-oxo-T3/H	1.466 ± 0.002	1.469
In vitro 24-oxo-1α,25$(OH)_2DHT_3$	1.292 ± 0.003	ND
In vivo 24-OH T3/H	1.878 ± 0.004	1.573
In vitro 1α,24,25$(OH)_3DHT_3$	1.915 ± 0.001	1.394
Comparison with 1β-OH metabolites‡		
In vivo 24-oxo-T3/H	1.497 ± 0.005	1.469
In vitro 24-oxo-1β,25$(OH)_2DHT_3$	1.492 ± 0.002	1.467
In vivo 24-OH T3/H	2.004 ± 0.004	1.573
In vitro 1β,24,25$(OH)_3DHT_3$	2.006 ± 0.009	1.578

* Retention times are relative to that of chemically synthesized 1α,25$(OH)_2DHT_3$ which was included in each run. Mean values ± SD. (σ_N is recorded (N = 3).

† Retention times of the perTMSi derivatives were relative to that of tri-TMSi ether of 25-OH DHT_3. Only a single value for each is recorded since very limited quantities of the 1β-hydroxylated metabolites were available.

‡ HPLC chromatographic comparisons were carried out on two different occasions on different LiChrospher Si60 columns using mobile phase HIM (91/7/2) at a flow rate of 1 mL/min. Slightly different relative retention times for the same compounds are observed in the two different chromatographic runs.

ND, Not determined.

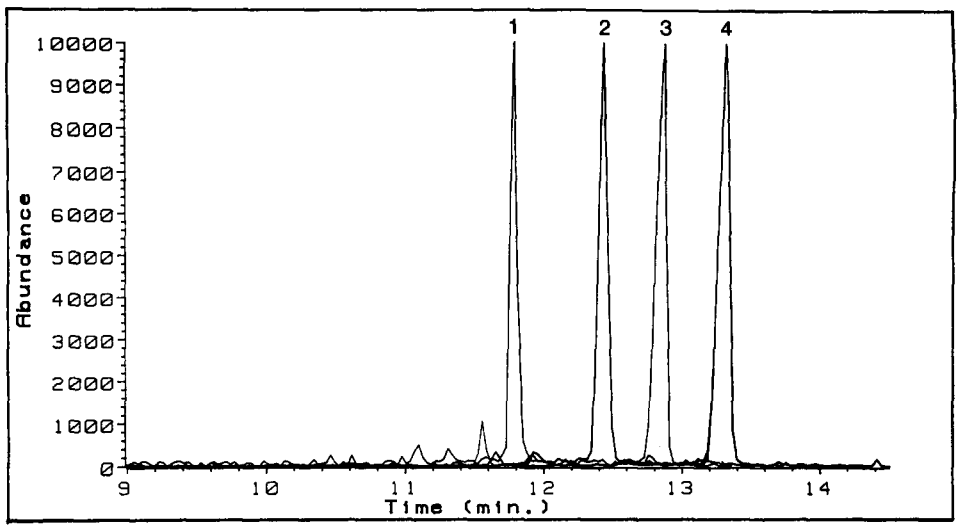

Fig. 7. Single ion chromatographs of the TMSi ether derivatives of (1) 24-oxo-1α,25$(OH)_2DHT_3$, (2) 1α,24,25$(OH)_3DHT_3$, (3) 24-oxo-T3/H and (4) 24-OH-T3/H, monitoring the $(M-90)^+$ ion, indicating the GC retention times of these metabolites. The data handling facilities of the mass spectrometer allowed the normalization and overlaying of each peak to enable visualization at the same time.

be seen that in no case are *in vivo* and *in vitro* metabolites the same. Insufficient material did not allow similar comparison of the 26-hydroxylated metabolites.

This data demonstrated that the polar metabolites of 1α,25$(OH)_2DHT_3$ formed in the osteosarcoma cell line were similar but not identical to those metabolites formed *in vivo* and were thus likely to be metabolites of 1β,25$(OH)_2DHT_3$, a chemically synthesized standard of which was not available to us. 1β-hydroxy DHT_3, whose chemical synthesis and characterization are described elsewhere*, was therefore incubated in the Hep 3B cell line as described previously [12]. Small quantities of 1β,25$(OH)_2DHT_3$ were isolated from the medium, identified as such by HPLC retention and mass

* Qaw F, Calverley MJ, Schroeder NJ, Trafford DJH, Makin HLJ and Jones G, 25-hydroxylation of synthetic 1-hydroxydihydrotachysterols: comparison with *in vivo* metabolites in the rat. Unpublished work.

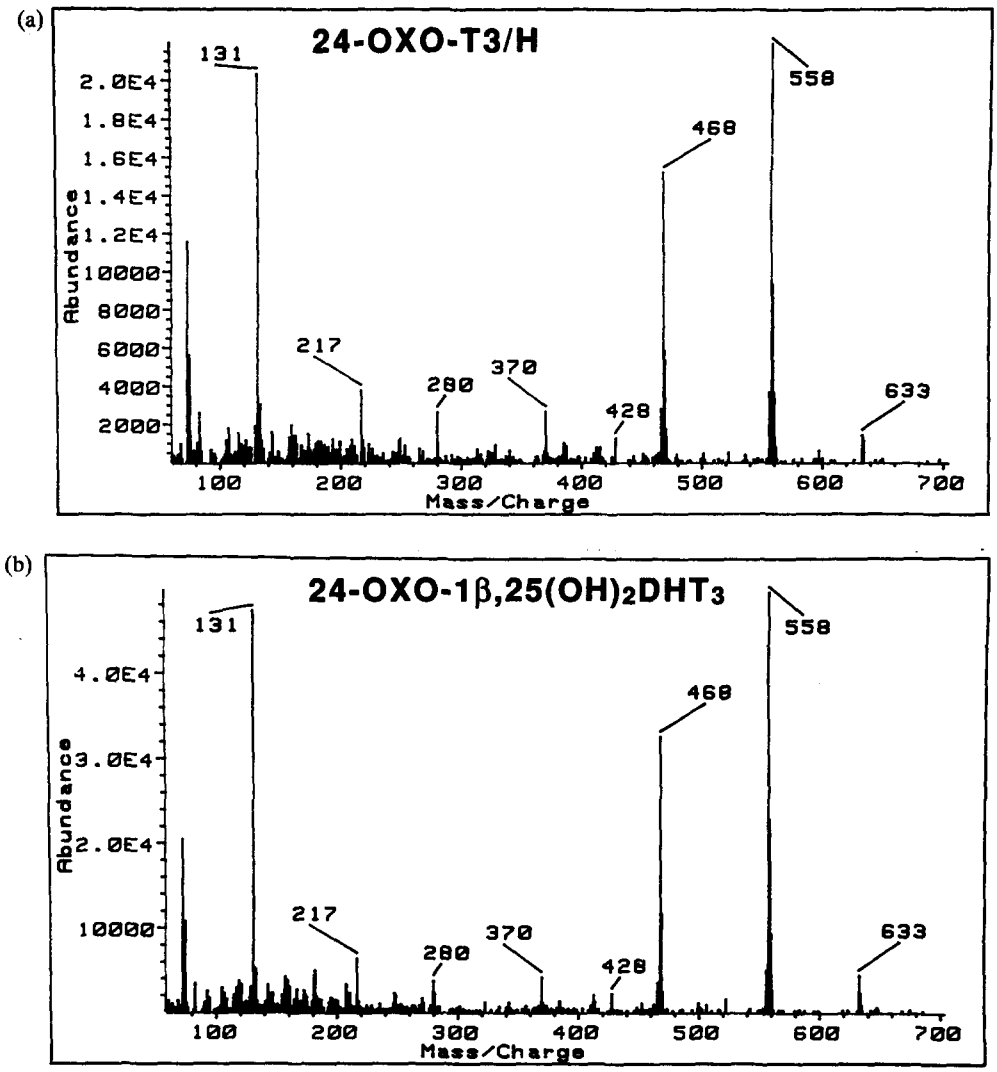

Fig. 8. EI(+) mass spectra of the perTMSi ether derivatives of (a) the 24-oxo metabolite of T3/H and (b) 24-oxo-1β,25(OH)$_2$DHT$_3$. These two spectra are identical.

spectral characteristics. 1β,25(OH)$_2$DHT$_3$ was then incubated with the osteosarcoma cell line as described for 1α,25(OH)$_2$DHT$_3$ and the putative 24-oxo and 24-hydroxy metabolites isolated. HPLC and GC retention times (Table 3) and mass spectral (Figs 6 and 8) characteristics of these metabolites were identical to those of the corresponding *in vivo* metabolites of T3/H. Although this approach allowed us to identify 24-oxo-1β,25-(OH)$_2$DHT$_3$ and 1β,24,25-(OH)$_3$DHT$_3$ as two of the metabolites of T3/H formed *in vivo*, insufficient quantities of other metabolites of 1β,25-(OH)$_2$DHT$_3$ were formed preventing extensive characterization of these metabolites. Furthermore, recent incubations of 1β,25-(OH)$_2$DHT$_3$ with UMR106 cells have allowed the isolation of the 26,23-lactone of 1β,25-(OH)$_2$DHT$_3$, the TMSi ether of which had a very similar mass spectrum to that illustrated in Fig. 3.

DISCUSSION

This paper reports the isolation and identification of four previously unidentified polar metabolites of DHT$_3$ formed *in vivo* in the rat and five *in vitro* metabolites of 1α,25(OH)$_2$DHT$_3$ generated by rat osteosarcoma cells (UMR106). The mass spectral characteristics of the *in vivo* metabolites suggested strongly that they were derived from the previously described non-renal metabolite of 25-OH-DHT$_3$, T3/H [1]. However when T3/H is examined by reverse-phase HPLC, it is resolved into two components, a very small early eluting peak (designated T3/Ha) and a later peak (T3/Hb). In the DHT$_3$ series, a $\ll$ b but in the DHT$_2$ series, a is approximately = 0.3 b. We have shown elsewhere [6] that there is strong evidence to suggest that peak a is 1α,25(OH)$_2$DHT and peak b is 1β,25(OH)$_2$DHT.

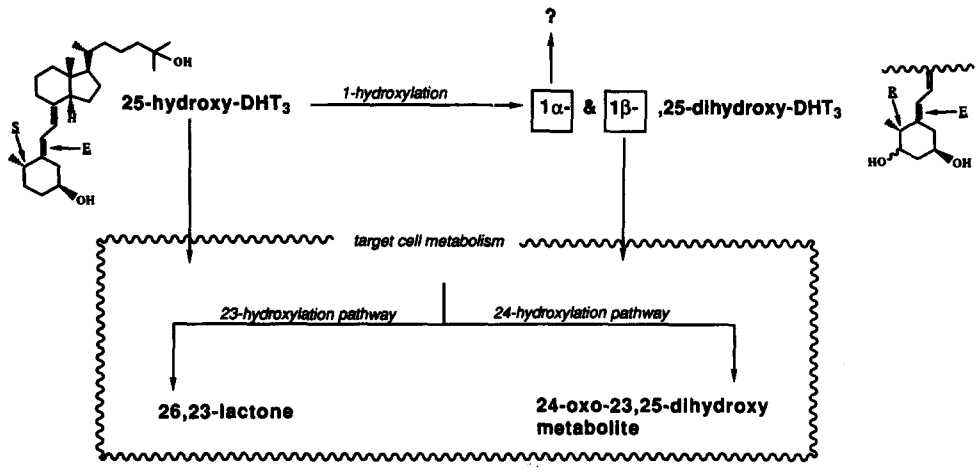

Fig. 9. Suggested scheme for the *in vivo* metabolism of 25-OH-DHT_3. The evidence at present available to us suggests that 25-OH-DHT_2 is metabolized in a similar fashion except that structural differences in the side chain will prevent formation of many of the intermediates produced from 25-OH-DHT_3. The structure of 25-OH-DHT_3 and the A-ring of the 1-hydroxylated metabolites are also illustrated.

The *in vivo* polar metabolites which have been described in the present paper are therefore generated by metabolism of T3/Ha or T3/Hb or both. The synthesis of 1α,25$(OH)_2DHT_3$ has enabled the origin of the *in vivo* metabolites to be identified. Unfortunately, confirmation of our conclusions by incubations with chemically synthesized 1β,25$(OH)_2$DHT cannot be carried out as this material is not yet available to us. However preliminary studies using *in vitro* generated 1β,25$(OH)_2DHT_3$ have proved support for our suggestions. Generation of polar metabolites of 1α,25$(OH)_2DHT_3$ in an osteosarcoma cell line has allowed the comparison of some of the *in vitro* generated metabolites with those formed *in vivo* from T3/H. The demonstration that the 1α-hydroxylated metabolites are not the same and the demonstration that metabolites of 1β,25$(OH)_2DHT_3$ are very similar to the *in vivo* metabolites, suggest that *in vivo* the polar metabolites which have been studied are not derived from T3/Ha but are rather target cell metabolites of T3/Hb. Since T3/Ha is present in such small concentrations in the plasma of rats *in vivo*, it is still possible that these side-chain modified metabolites are also produced from T3/Ha but that these are present at concentrations beyond the detection limit of our techniques. What is clear is that the polar metabolites of T3/Hb (i.e. 1β,25-$(OH)_2DHT_3$) appear to represent the dominant if not exclusive compounds found in plasma *in vivo*.

We therefore suggest that DHT_3, after 25-hydroxylation in the liver, is metabolized *in vivo* in the kidney and other target cells along the two metabolic pathways described previously in the isolated perfused rat kidney [1] and the osteosarcoma cell line [7, 8]. In addition, we suggest that 25-OH-DHT is 1-hydroxylated in a non-renal tissue to give rise to the metabolite T/H, which is composed of a mixture of 1α- and 1β-hydroxylated compounds, and that these 1-hydroxylated compounds are then further metabolized along the same two 23- and 24-oxidation pathways already demonstrated for 25-OH-DHT_3. We have attempted to summarize our present state of knowledge of the metabolism of 25-OH-DHT_3 in Fig. 9. The isolation of 26-hydroxylated metabolites both *in vivo* and *in vitro* may indicate a further pathway of metabolism. A similar metabolite of D_3, 1α,25,26 trihydroxyvitamin D_3, has also been isolated previously [13]. Studies reported elsewhere [14] have indicated that 1α,25$(OH)_2$DHT is probably the physiologically active calcaemic metabolite of DHT, since it has the highest affinity for the vitamin D receptor. If this is so, the role of the quantitatively more significant 1β-hydroxylated compound, which has a low affinity for the vitamin D receptor, and the fact that it appears *in vivo* to be metabolized preferentially in vitamin D target tissues require explanation. The low concentration of 1α,25$(OH)_2$DHT found in rat plasma after administration of DHT may indicate either a reduced rate of formation, a short half-life due to rapid removal by target cells or lower affinity of the rat vitamin D transport protein or other plasma proteins for this metabolite. A combination of the latter two explanations is quite attractive since it might be assumed that high affinity for the VDR and low affinity for plasma transporters would imply rapid sequestration inside the target cell and thus increased availability for metabolism. The site and mechanism of formation of these two interesting 1-hydroxylated metabolites of 25-OH-DHT_3 are still not known and investigations continue.

The results presented in this paper confirm that the 23- and 24-oxidation pathways already described for 25-OH-D_3 and 1α,25$(OH)_2D_3$ will also metabolize the same hydroxylated metabolites of DHT_3. In addition, the evidence presented here suggests that the enzymes involved in these pathways will also metabolize 1β,25(OH)DHT in addition to 1α,25(OH)DHT, although it is not clear whether one or other of these compounds may be a preferential substrate. This possibility may be the explanation for the very low concentrations of the

1α-hydroxy metabolite found in rat and human [15] plasma. Our investigations described here and elsewhere have indicated that DHT_3 is extensively metabolized in many of the same tissues as vitamin D but also at an as yet unknown non-renal 1-hydroxylation site or sites to produce a multiplicity of products whose possible functions are unknown but are worthy of investigation. Further metabolites of DHT have been observed but await isolation and characterization. Previous theories of the mechanism of the calcaemic effect of DHT now need re-appraisal.

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