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Identification of di- and tri-substituted hydroxy and ketone metabolites of Δ^1-tetrahydrocannabinol in mouse liver

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In vivo liver metabolites of Δ^1-tetrahydrocannabinol (Δ^1-THC) were examined with a gas chromatograph—mass spectrometer—computer system as trimethylsilyl (TMS), [$^2H_9$]TMS and methyloxime-TMS derivatives. In addition to the reported monohydroxy, acid, and hydroxyacid metabolites, the following multiply substituted metabolites were identified: 2″,7-, 3″,7-, and 6β,7-dihydroxy-Δ^1-THC; 2″,6α,7-, and 3″,6α,7-trihydroxy-Δ^1-THC; 2″-, 3″-, and 7-hydroxy-6-oxo-Δ^1-THC, and 2″,7- and 3″,7-dihydroxy-6-oxo-Δ^1-THC. The ketones and hydroxyacids were reduced to common alcohols with lithium aluminium deuteride and the number of deuterium atoms in the product was used to distinguish the metabolic alcohols from those produced by reduction.

Previous studies on the metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC) in the mouse have shown that 6α- and 7-hydroxy-Δ^1-THC are the main monohydroxylated metabolites (Ben-Zvi, Burstein & Zikopoulos, 1974; Jones, Widman & others, 1974). More recently, Δ^1-THC-7-oic acid and several of its mono- and di-hydroxy derivatives have also been characterized as major metabolites *in vivo* (Harvey & Paton, 1976a, b). The presence of the latter compounds suggested that other polysubstituted metabolites might be produced but, to date, only the 6α,7-diol appears to have been reported (Jones & others, 1974). In addition, the presence of Δ^1-THC-6-one as an *in vitro* liver metabolite (Jones & others, 1974) suggested that hydroxy-ketone metabolites may also be formed. This paper reports the presence in mouse liver of three monohydroxy and two dihydroxy ketone metabolites together with several di- and trihydroxy-Δ^1-THC's.

MATERIALS AND METHODS

Δ^1-THC (98% pure as determined by g.l.c., remaining 2% was cannabinol) was suspended in Tween-80 and isotonic saline and was administered intraperitoneally at 100 mg kg^{-1} to male Charles River CDl mice at 26 h and 2 h before stunning and decapitation. The livers were homogenized in isotonic saline (10 ml), and the metabolites, together with unchanged Δ^1-THC were extracted with 3 portions (10 ml) of redistilled ethyl acetate. The solution was dried over magnesium sulphate, evaporated to dryness under reduced pressure and the residue from each liver was chromatographed on Sephadex LH-20 in chloroform to remove endogenous lipids as described previously (Harvey & Paton, 1976a, b). Fractions were taken as outlined in Table 1a. Subfractionation of the main metabolite fraction (fraction 5) was made by increasing the concentration of methanol in 2% stages and eluting 10 ml at each concentration as shown in Table 1b. Aliquots of these fractions, equivalent to 0·5 g of liver, were converted into derivatives for combined g.c.-ms as described below.

Table 1. *Fractionation of the liver extracts on Sephadex LH-20.*

a. Whole liver extract

Fraction	Solvent	Vol. (ml)	Contents
1	$CHCl_3$	0–18	triglycerides, cholesterol
2	$CHCl_3$	18–26	Δ^1-THC
3	$CHCl_3$	26–35	Fatty acids
4	$CHCl_3$	35–70	comps 1–7 (Table 2)
5	10% MeOH/$CHCl_3$	70–120	polar metabolites

b. Subfractionation of fraction 5

5a	2% MeOH/$CHCl_3$	70–80	—
5b	4% MeOH/$CHCl_3$	80–90	comps 8, 9
5c	6% MeOH/$CHCl_3$	90–100	comps 8, 9, 10, 11, 13
5d	8% MeOH/$CHCl_3$	100–110	comps 9, 12, 14, 15, 16, 19
5e	10% MeOH/$CHCl_3$	110–120	comps 17, 21
5f	20% MeOH/$CHCl_3$	120–150	—

Preparation of derivatives

(a) *Trimethylsilyl (TMS) derivatives.* 5 μl of a solution of *N,O*-bistrimethylsilyltrifluoroacetamide (BSTFA, 2 parts), trimethylchlorosilane (TMCS, 1 part) and acetonitrile (2 parts) was added to the dried (N_2 stream) metabolite sample which was then heated at 60° for 10 min. The total sample was injected into the gas chromatograph.

* Correspondence.

(b) [$^2H_9$] *TMS derivatives.* These were prepared by the addition of [$^2H_{18}$] bistrimethylsilylacetamide ([$^2H_{18}$]BSA, 3 μl), acetonitrile (2 μl) and TMCS (trace) to the sample which was then treated as above.

(c) *Methyl ester-TMS derivatives.* A solution of diazomethane in ether (prepared from *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine as described by Fales, Jaouni & Babashak, 1973) was added to a solution of the metabolites in methanol (20 μl). After 3 min the remaining diazomethane and solvents were removed with a nitrogen stream and the residue was converted into TMS derivatives as described above.

(d) *Methyloxime-TMS derivatives.* The metabolites were dissolved in pyridine (20 μl) and an excess of methoxyamine hydrochloride was added. The mixture was then heated at 60° for 1 h, and the residue was converted into TMS derivatives.

(e) *Lithium aluminium deuteride reduction.* The metabolites, dissolved in dry ether, were refluxed with an excess of $LiAlD_4$ for 1 h. The reduced metabolites were recovered using a standard work-up procedure and converted into TMS derivatives.

G.c.-ms

Low resolution mass spectra were recorded at 25 eV with a V.G. Micromass 12B mass spectrometer. This was interfaced via a glass jet separator to a Varian 2400 gas chromatograph fitted with a single 2 m × 2 mm glass column packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa. U.S.A.). The column oven was temperature programmed from 170–280° at 2° min^{-1} with the injector block and transfer line temperatures maintained at 270° and 230° respectively. Helium at 30 ml min^{-1} was used as the carrier gas. The mass spectrometer was operated with an accelerating voltage of 2·5 kV and an ion source temperature of 260°. Spectra were recorded with a V.G. data system type 2040 which was set to aquire spectra repetitively throughout the elution of the chromatogram. A scan speed of 3 s $decade^{-1}$ with an inter-scan delay of 2 s was employed and data acquisition was started when the g.c. column reached 190°.

RESULTS AND DISCUSSION

Figs 1 and 2 show the g.l.c. profiles of the TMS derivatives of the metabolites present in fractions 4 and 5 from the Sephadex LH-20 column. They were produced by computer enhancement of the total ion chromatograms as described by Powers, Wallington & others (1975). Only ions above *m/e* 300 were used,

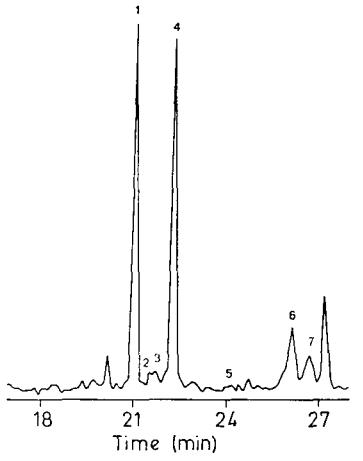


FIG. 1. Computer reconstructed chromatogram (*m/e* 300–700) of the Δ^1-THC metabolites (TMS derivatives) present in fraction 4 from the Sephadex LH-20 column. The gas chromatographic conditions are given in the 'experimental' section and the peak numbers are listed in Table 2.

to minimize interference by endogenous tissue constituents (Harvey & Paton, 1976a).

The major metabolites in fraction 4 (peaks 1 and 4) were identified as 6α- and 7-hydroxy-Δ^1-THC (base peaks at $[M-90]^{+\cdot}$ and $[M-103]^+$) respectively by comparison of their g.c.-ms characteristics with those of authentic samples. Peaks 2 and 3 were also monohydroxy metabolites (18 a.m.u. shift of $M^{+\cdot}$ in the spectra of the [$^2H_9$] TMS derivatives, McCloskey, Stillwell & Lawson, 1968) and were identified as 3″- and 6β-hydroxy-Δ^1-THC respectively by their mass spectra (Wall & Brine, 1976). Fraction 5

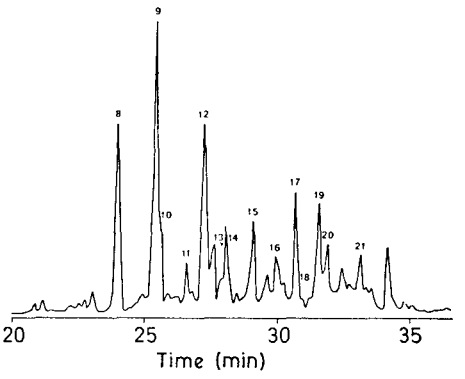
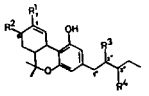


FIG. 2. Computer reconstructed chromatogram (*m/e* 300–700) of the Δ^1-THC metabolites (TMS derivatives) present in fraction 5 from the Sephadex LH-20 column. Separation conditions and peak identification are given in the experimental section and Table 2 respectively.

contained the following previously reported compounds: 6α,7-dihydroxy-Δ^1-THC (Jones & others, 1974; Harvey & Paton, 1976a; Wall & Brine, 1976) (peak 8, Fig. 2), Δ^1-THC-7-oic acid (Harvey & Paton, 1976a,b; Wall & Brine, 1976) (peak 9, Fig. 2), 2″-, 3″-, and 6α-hydroxy-Δ^1-THC-oic acid (peaks 15, 19, 12), 2″, 6α-and 3″,6α-dihydroxy-Δ^1-THC-oic acid (Harvey & Paton, 1976a,b) (peaks 17, 21, Table 2).

Table 2. *Structures of the metabolites of Δ^1-THC found in mouse liver.*



Peak no.[a]	Compound	R^1	R^2	R^3	R^4
1	6α-OH-Δ^1-THC	H	α-OH	H	H
2	3″-OH-Δ^1-THC	H	H	H	OH
3	6β-OH-Δ^1-THC	H	β-OH	H	H
4	7-OH-Δ^1-THC	OH	H	H	H
5	2″-OH-6-oxo-Δ^1-THC	H	=O	OH	H
6	7-OH-6-oxo-Δ^1-THC	OH	=O	H	H
7	3″-OH-6-oxo-Δ^1-THC	H	=O	H	OH
8	6α,7-Di-OH-Δ^1-THC	OH	α-OH	H	H
9	Δ^1-THC-7-oic acid	COOH	H	H	H
10	2″,7-Di-OH-Δ^1-THC	OH	H	OH	H
11	6β,7-Di-OH-Δ^1-THC	OH	β-OH	H	H
12	6α-OH-Δ^1-THC-7-oic acid	COOH	α-OH	H	H
13	3″,7-Di-OH-Δ^1-THC	OH	H	H	OH
14	2″,6α,7-Tri-OH-Δ^1-THC	OH	α-OH	OH	H
15	2″-OH-Δ^1-THC-7-oic acid	COOH	H	OH	H
16	3″,6α,7-Tri-OH-Δ^1-THC	OH	α-OH	H	OH
17	2″,6α-Di-OH-Δ^1-THC-7-oic acid	COOH	α-OH	OH	H
18	2″,7-Di-OH-6-oxo-Δ^1-THC	OH	=O	OH	H
19	3″-OH-Δ^1-THC-7-oic acid	COOH	H	H	OH
20	3″,7-Di-OH-6-oxo-Δ^1-THC	OH	=O	H	OH
21	3″,6α-Di-OH-Δ^1-THC-7-oic acid	COOH	α-OH	H	OH

a In order of elution. Compounds 1–7, Fraction 4; 8–21 Fraction 5.

*Diols**

In addition to 6α,7-dihydroxy-Δ^1-THC, three other diol metabolites were identified in fraction 5 (peaks 10, 11 and 13). All of these eluted in fractions 5b and 5c when the 10% methanol–chloroform fraction was subfractionated as described in the experimental section. None of these compounds reacted with diazomethane or methoxyamine hydrochloride indicating the absence of acid or ketone groups. Peak 11 was associated with an abundant molecular ion at *m/e* 562 (27 a.m.u. shift in the [$^2H_9$] TMS spectrum indicated three OTMS groups) and abundant fragment ions at *m/e* 459 ([M-CH_2OTMS]$^+$), *m/e* 472 ([M-TMSOH]$^{+\cdot}$) and *m/e* 369 ([M-TMSOH-CH_2OTMS]$^+$, Table 3). This, together with its relative retention time enabled the compound to be identified as 6β,7-dihydroxy-Δ^1-THC by comparison with the g.c.-ms data reported by Wall & Brine (1976). This diol has not previously been reported as an *in vivo* metabolite of Δ^1-THC in the mouse.

* For clarity, the term 'diol' refers only to hydroxy groups introduced metabolically. The phenol group is ignored. Other metabolites are named similarly.

Table 3. *G.c.-ms data for the diol and triol metabolites of Δ^1-THC (TMS derivatives).*

Compound	No.	Rt (min)	Diagnostic ions M$^{+\cdot}$	[M-90]$^{+\cdot}$	[M-103]$^+$	Side chain	Other
6α,7-Di-OH-Δ^1-THC	8	24·2	562 (0)	472[a] (100) *18*	459 (8) *18*	—	—
2″,7-Di-OH-Δ^1-THC	10	26·3	562 (4) *27*	—	459 (76) *18*	145 (100) *9*	—
6β,7-Di-OH-Δ^1-THC	11	27·3	562 (35) *27*	472 (28) *18*	459 (80) *18*	—	369[b] (100) *9*
3″,7-Di-OH-Δ^1-THC	13	28·2	562 (—) *27*[c]	—	459 (100) *18*	418 (60) *18*	—
2″,6α,7-Tri-OH-Δ^1-THC	14	28·4	650 (0) (0)	560 (57) *27*	547 (8) *27*	145 (100) *9*	635[d] (3)
3″,6α,7-Tri-OH-Δ^1-THC	16	30·8	650 (1)	560 (100) *27*	547 (42) *27*	416[e] (39) *27*	635 (2)[d]

(a) Relative intensity in parentheses; shift in [$^2H_9$]TMS spectra in italics. (b) [M-90-103]$^+$. (c) Mixed spectrum. (d) [M-15]$^+$ (e) [M-90-144]$^{+\cdot}$.

Peaks 10 and 13 (Fig. 2) were both associated with abundant ions at *m/e* 459 ([M-CH_2OTMS]$^+$) indicating 7-hydroxylation. Abundant ions were also present at *m/e* 145 and 418 ([M-144]$^{+\cdot}$) for peaks 10 and 13 respectively (Table 3) and indicated hydroxylation at positions 2″ and 3″ of the pentyl side-chain (Binder, Augrell & others, 1974; Binder, 1976; Wall & Brine, 1976). The retention increments observed between 6α,7-, 2″,7- and 3″,7-dihydroxy-Δ^1-THC were similar to those for the corresponding 7-acids (Harvey & Paton, 1976a, b) and to those found by Wall & Brine (1976) for the same metabolites isolated from rhesus monkey liver homogenates.

Further evidence for these structures was obtained by reduction of the metabolite fraction with lithium aluminium deuteride. All acid metabolites in this fraction were reduced to primary alcohols with the incorporation of 2 deuterium atoms and ketones were reduced to secondary alcohols with the incorporation of one deuterium atom at the site of reduction. Alcohols produced by reduction could thus be distinguished from those produced metabolically by the shift in the ions containing deuterium. By this means the three monohydroxyacids, 2″-, 3″-, and 6α-hydroxy-Δ^1-THC-7-oic acids (15, 19, 12) were

reduced to primary alcohols having the same retention times and mass spectra (with two deuterium atoms at C_7) as the 2″,7-, 3″,7- and 6α,7-dihydroxy-Δ^1-THC's respectively.

Single ion chromatograms of ions characteristic of other diol metabolites such as the series containing a 6α-hydroxy group together with side-chain hydroxylation suggested the presence of these compounds; however, the spectra were too weak for confirmation of these additional diols.

Triols

Two triols (peaks 14 and 16) with the hydroxy substituents in positions corresponding to the positions substituted in the two dihydroxy acids (17 and 21, Harvey & Paton, 1976a, b) were also identified in fraction 5. These compounds eluted from the Sephadex column after the diols (fractions 5b and 5c) but before the dihydroxy acids (fraction 5e). Their retention times on SE-30 were related to those of the diols in the same way as the retention times of the dihydroxy acids were related to those of the monohydroxy acids thus indicating a similar structure. No reaction with diazomethane or methoxyamine hydrochloride was observed. The molecular ion was absent from the spectrum of peak 14 (TMS derivative) but the spectrum of the second triol (peak 16) contained a weak molecular ion at *m/e* 650. The $[M-15]^+$ ions were present in low abundance in both spectra (Table 3) and deuterium labelling ($[^2H_9]$ TMS) confirmed the presence of four OTMS groups. Major fragment ions (Table 3) were present at *m/e* 560 ($[M-TMSOH]^{+\cdot}$) in both spectra indicating 6α-substitution and at *m/e* 145 and 416 ($[M-90-144]^{+\cdot}$, (see Harvey & Paton, 1976a, b for analagous fragmentations of 2″-, and 3″-,6α-dihydroxy-Δ^1-THC-7-oic acids) in the spectra of compounds 14 and 16 respectively, confirming the presence of hydroxyl groups in the 2″- and 3″-positions. In addition, both spectra contained ions produced by loss of the primary alcohol at C_7 ($[M-CH_2OTMS]^+$, *m/e* 547). The shifts of these ions in the $[^2H_9]$ TMS spectra were consistent with these fragmentations. Reaction of the metabolic fraction with lithium aluminium deuteride reduced the dihydroxy acids 17 and 21 to triols having the same g.c.-ms properties but with the incorporation of two deuterium atoms as the triols 14 and 16 and the latter compounds were thus identified as 2″,6α,7- and 3″,6α,7-trihydroxy-Δ^1-THC respectively. As with the diols, single ion chromatograms of diagnostic ions suggested the presence of two additional triols, possibly of the 6β-series. Although their retention times differed from those of the 6α-compounds definitive mass spectra could not be obtained.

Monohydroxyketones

The mass spectra of the three compounds (5, 6 and 7) eluting after the monohydroxy metabolites in fraction 4 (Fig. 1) contained molecular ions at *m/e* 488 which shifted by 18 a.m.u. in the $[^2H_9]$ TMS spectra indicating the presence of two hydroxyl groups. The difference in molecular weights between these compounds and the monohydroxy metabolites ($M^{+\cdot}$ at *m/e* 474) suggested oxidation of a methylene or methyl group to a carbonyl group. That this was not incorporated into a carboxylic acid was demonstrated by the inability of these compounds to form methyl esters by reaction with diazomethane. The presence of the carbonyl group was subsequently confirmed by the preparation of the methyloxime (MO) derivative of each metabolite. The presence of the ions at *m/e* 145 and $[M-144]^{+\cdot}$ (Table 4) in the spectra of both the TMS and MO-TMS derivatives of compounds 5 and 7 located the hydroxyl group in the 2″- and 3″-positions (side-chain). The spectra of the derivatives of compound 6 contained abundant ions at $[M-CH_2OTMS]^+$ indicating 7-hydroxylation. None of these spectra, however, gave any further

Table 4. *G.c.-ms data for the ketol metabolites of* Δ^1*-THC extracted from mouse liver.*

Compound	No.	Derivative	Rt (min)	$M^{+\cdot}$	Base	Other ions	
2″-OH-6-oxo-Δ^1-THC	5	TMS	23·8	488[a] (19) *18*	145[b] *9*	473 (18)	
		MO-TMS	25·3	517 (16) *18*	145[c] *9*		
7-OH-6-oxo-Δ^1-THC	6	TMS	26·1	488 (57) *18*	385[c] *9*		
		MO-TMS	26·9	517 (5) *18*	414[c] *9*	427 (41)	
3″-OH-6-oxo-Δ^1-THC	7	TMS	26·6	488 (49) *18*	344[d] *9*		
		MO-TMS	27·8	517 (27) *18*	373[d] *9*		
2″,7-Di-OH-6-oxo-Δ^1-THC	18	TMS	30·2	576 (e) *27*	145[b] *9*		
		MO-TMS	31·5	605 (4)	145[b] *9*		
3″,7-Di-OH-6-oxo-Δ^1-THC	20	TMS	e	576 (e) *27*	432[d] *18*		
		MO-TMS	33·6	605 (14)	502[c] *18*	461[d] (35)	371 (39)

(a) Relative intensity in parentheses, shift in $[^2H_9]$TMS derivative in italics. (b) $C_3H_7\overset{+}{C}H_2$ = $\overset{+}{O}$TMS. (c) $[M-CH_2OTMS]^+$. (d) $[M-144]^{+\cdot}$. (e) Not separated from the 7-acid metabolite.

indication on the location of the carbonyl group, although position 6 was favoured by analogy with the other metabolites and the report by Jones & others (1974) of 6-oxo-Δ^1-THC as an *in vitro* metabolite of THC in the mouse. In addition Martin, Agurell & others (1976a) have recently reported 3″-, and 4″-hydroxy-6-oxo-cannabidiol as metabolites of cannabidiol in the rat.

Confirmation that the carbonyl group was located at C_6 was obtained by reduction of the three hydroxyketones with lithium aluminium deuteride. Compound 6 was reduced to a diol with g.c.-ms properties identical with those of 6α,7-dihydroxy-Δ^1-THC but with the incorporation of one deuterium atom at C_6 (the mass spectrum showed loss of 103 amu from C_7). The other two ketols were reduced to compounds whose retention times and mass spectra were consistent with those expected for 2″,6α- and 3″,6α-dihydroxy-Δ^1-THC. The three ketols were thus 2″-, 3″-, and 7-hydroxy-6-oxo-Δ^1-THC.

Dihydroxyketones

Two other new ketol metabolites, 2″,7- and 3″,7-dihydroxy-6-oxo-Δ^1-THC (peaks 18 and 20, Fig. 2) were identified in fraction 5 in an analogous manner. Both compounds formed methyloximes and the spectra of the TMS, [$^2H_9$] TMS, and MO-TMS derivatives contained ions indicative of hydroxyl substitution in the 2″-, 3″-, and 7-positions (Table 4). Reduction with lithium aluminium deuteride gave the corresponding triols (14 and 16) which were distinguished from the metabolic triols and triols produced by reduction of the dihydroxy acids (17 and 21) by the incorporation of only one deuterium at the site of reduction.

The metabolites were not quantitated because reference compounds were not available for the calculation of g.l.c. response factors. However, the relative peak heights in Figs 1 and 2 give an indication of the relative amounts of each compound. In general the concentrations of all the diol, triol and ketol metabolites were lower than those of the acid and substituted acids.

Thus, four main sites of biotransformation have so far been identified in the mouse: the allylic positions 6 and 7 on the terpene moiety and positions 2″ and 3″ on the side-chain. Position 7 was the most readily substituted; most of the metabolites reported to date have contained substitution at this position. Dihydroxy metabolites with one hydroxyl group at C_7 and the second group at one of the other preferred positions, 6α, 2″, or 3″ (6β substitution is regarded as a minor biotransformation step in this strain of mouse), were abundant as were the two possible triols (14 and 16). In addition to the 7-hydroxy series, the corresponding series containing the fully oxidized 7-group (7-COOH) and the oxidized 6-group (6-oxo) were also present, the former in high abundance. The observation that the positions substituted are the same in each series parallels results obtained with other cannabinoids (Martin, Agurell & others, 1976a; Martin, Nordqvist & others, 1976b). Of the many possible permutations among these substitutions, the principal absentees are double substitution and further oxidation of the side chain, and ketoacids.

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