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Dihydroxylated metabolites of cannabinol formed by rat liver *in vitro*

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Cannabinol (CBN) was metabolized *in vitro* by a 10 000 *g* supernatant from rat liver. After removal of unchanged CBN and its monohydroxylated metabolites four dihydroxylated metabolites were isolated. By nuclear magnetic resonance and mass spectrometry the compounds were identified as 1″,7-dihydroxy-CBN, 2″,7-dihydroxy-CBN, 3″,7-dihydroxy-CBN and 4″,7-dihydroxy-CBN. Side chain hydroxylation occurred predominantly at C–4″ and C–3″.

Cannabinol (CBN) which is one of the major constituents of cannabis has received very little attention. This could be due to the fact that CBN has been regarded to be without psychotomimetic activity (Mechoulam, 1973) compared to Δ^1-tetrahydrocannabinol (Δ^1-THC). However, Perez-Reyes, Timmons & others (1973) have found that high doses of CBN administered to man exhibit a mild psychoactive effect. Ben-Zvi, Bergen & Burstein (1974), after their discovery of urinary CBN metabolites arising from Δ^1-THC in rhesus monkey, have suggested that CBN is formed as a transient metabolite of Δ^1-THC and may play a role in the pharmacology of cannabis. Hence a further examination of the metabolism of CBN would seem to be worthwhile.

Previous metabolic studies have shown that CBN is converted mainly to 7-hydroxy-CBN by rat (Widman, Nilsson & others, 1971; Wall, 1971) and rabbit (Widman, Dahmén & others, 1975a) liver *in vitro*. Metabolites hydroxylated in the pentyl side chain have also been found, in the rat 2″-, 3″-, 4″-, and 5″-hydroxy-CBN were formed as minor metabolites while in the rabbit 4″-hydroxy-CBN was shown to be a major product together with small amounts of 3″- and 5″-hydroxy-CBN (Widman & others, 1975a).

We now report the identification of four dihydroxylated metabolites of CBN formed by an enriched rat liver microsomal preparation.

MATERIALS AND METHODS

In vitro *metabolism of CBN*

1″-^{3}H-CBN was synthesized as described earlier (Widman & others 1971; Agurell, Gustafsson & others, 1973). The radiochemical purity was determined to be over 97% by gas chromatography (g.c.) and thin-layer chromatography (t.l.c.) The final specific activity was adjusted to 0·14 mCi mmol^{-1} by the addition of nonlabelled CBN.

Ten male rats (Sprague-Dawley) were pretreated with phenobarbitone and then decapitated. The livers (105 g) were removed, homogenized in ice cold potassium chloride solution (1·15%) and subsequently centrifuged at 10 000 *g*. The supernatant was enriched with appropriate cofactors before the addition of 45 mg of tritiated CBN (Widman & others, 1975a). The incubation was at 37° for 3 h.

Isolation of metabolites

The incubate was extracted with three portions (280 ml each) of light petroleum (b.p. 40–60°) followed by another three portions (280 ml each) of diethyl ether (ether). The combined ether extract was chromatographed on Florisil (50 g) with the following eluents of 250 ml:—20% v/v ether–light petroleum; 50% ether–light petroleum; 100% ether; 5% methanol–ether; 20% methanol–ether and 100% methanol.

The metabolites in the 5% methanol–ether fraction were further purified and separated by t.l.c. Precoated Silica Gel F plates (Merck, 0·25 mm thickness, 5 × 10 cm) that had been prewashed with benzene–methanol (1 : 1) were used. The plates were developed twice in 0·5% methanol–ether, and the metabolites were visualized with 0·2% Fast Blue B salt (Merck) in 2N aqueous sodium hydroxide.

Instrumental methods

Radioactivity was assayed in a Packard Tricarb model 3375 spectrometer with external standardization.

Gas chromatographic data were obtained from a Varian Aerograph Model 2100, using a 2% SE-30

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column (ultraphase/Gas-chrom Q, 125–150 mesh) at 250°.

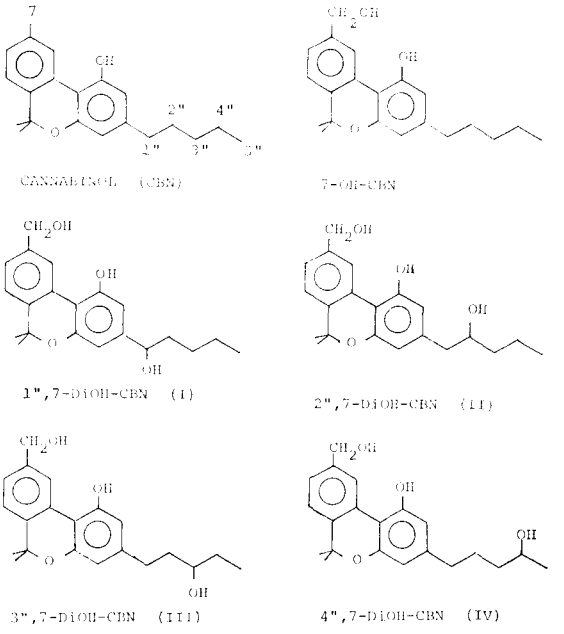
For mass spectrometry (m.s.), a LKB 9000 gas chromatograph-mass spectrometer (LKB, Bromma, Sweden) at 20 eV with a 2% SE-30 column (ultraphase/Gas-chrom Q, 100–120 mesh) at 200° was used. Before g.c. and m.s. analysis, all samples were silylated with *N,O*-bis-(trimethylsilyl)-acetamide in dry acetonitrile.

^{1}H-nuclear magnetic resonance (^{1}H-nmr) spectra were recorded on a Varian 100 MHz instrument ($CDCl_3$, Fourier Transform).

RESULTS AND DISCUSSION

CBN was converted to several metabolites by rat liver (80% conversion). Unchanged CBN was predominantly extracted by light petroleum whereas the metabolites as well as any residual CBN were extracted by ether. The ether extract was chromatographed on a Florisil column, and the first eluate contained unchanged CBN. The second and third eluates were composed of monohydroxylated metabolites as described earlier (Widman & others, 1975a). From the 5% methanol–ether eluate (about 10% of total activity) 7-hydroxy-CBN and four more polar metabolites (I–IV) were isolated and separated by t.l.c. The metabolites were identified by nmr and by comparison of their m.s. with those of mono-oxygenated CBN metabolites (Widman & others, 1975a).

The nmr spectra of the metabolites (I–IV) were similar to that of CBN (Petrzilka, Haefliger & Sikemeier, 1969) with a few modifications. They all lacked the C-7 methyl signal at 2·38 which is present in the spectrum of CBN. Instead a two proton singlet was present around $\delta = 4{\cdot}7$ showing the metabolites were hydroxylated at C-7 position.



(Widman & others, 1971). Furthermore all the spectra showed the presence of the aromatic protons of the olivetol moiety around $\delta = 6{\cdot}3$ and $\delta = 6{\cdot}4$ as well as the protons from the other aromatic nucleus at $\delta = 7{\cdot}2$ (C-5 and C-6) and $\delta = 8{\cdot}4$ (C-2). Metabolites I, II and III showed a triplet signal around $\delta = 0{\cdot}9$ corresponding to protons at C-5″. This triplet was absent in metabolite IV. Instead a doublet at $\delta = 1{\cdot}21$ was present showing 4″-hydroxylation.

In Table 1 the m.s., g.c. and t.l.c. data are presented for the metabolites (I, II, III and IV). The m.s. of each silylated metabolite showed a molecular ion at *m/e* 558 which is consistent with silylation of a dihydroxylated CBN metabolite. The fragment

Table 1. *Dihydroxy metabolites of CBN.*

Metabolite	Quantity μg	M.s.[a] % at *m/e* 558 (M$^+$)	543	528	501	414	145	131	117	T.l.c.[b] (R_F)	G.c.[c] Rt (min)
1″,7-dihydroxy-CBN (I)	120[d]	51	100	7	79	1	—	—	—	0·72	7·9
2″,7-dihydroxy-CBN (II)	126	7	8	1	—	1	100	—	—	0·63	8·3
3″,7-dihydroxy-CBN (III)	200	39	73	5	—	100	—	3	2	0·51	9·7
4″,7-dihydroxy-CBN (IV)	220	31	100	7	—	7	—	—	5	0·41	10·5

a. M.s. of the silylated derivative at 20 eV.
b. Developed in 0·5% methanol–ether.
c. Retention time of the silylated derivative on 2% SE-30 at 250°.
d. Calculated by g.c.

m/e 543 (M^+-CH_3), as well as the metastable ion *m/e* 528·4, was present in all metabolites. All the m.s. indicated 7-hydroxylation by the presence (1–3%) of either *m/e* 455 (loss of -$CH_2OSi(CH_3)_3$) and/or *m/e* 103 ($CH_2\overset{+}{O}Si(CH_3)_3$). The m.s. also established that the compounds were side chain hydroxylated. The exact postion of the hydroxyl group was determined according to Binder, Agurell & others (1974).

Metabolite I showed a prominent *m/e* 501 which is consistent with the loss of -C_4H_9, a characteristic fragment of silylated 1″-hydroxy cannabinoids. The nmr also supported 1″-hydroxylation since the benzylic protons at $\delta = 2{\cdot}5$ disappeared to give a broad poorly resolved triplet in the $\delta = 5$ region. Thus metabolite I was assigned the structure of 1″, 7-dihydroxy-CBN.

Metabolite II with a base peak at *m/e* 145 ($C_4H_8\overset{+}{O}Si(CH_3)_3$) which is diagnostic for 2″-hydroxylated cannabinoids was determined to be 2″,7-dihydroxy-CBN.

Metabolite III was assigned the structure 3″,7-dihydroxy-CBN. It exhibited a base peak at *m/e* 414 (benzylic cleavage with proton transfer) and *m/e* 131 (α-cleavage between C-2″ and C-3″).

Metabolite IV was identified as 4″, 7-dihydroxy-CBN. Hydroxylation at C-4″ was indicated by the fragment *m/e* 117 ($C_2H_4\overset{+}{O}Si(CH_3)_3$) and confirmed by nmr as discussed earlier.

Previously, Wall (1971) has tentatively identified 2″,7-dihydroxy-CBN as a metabolite from rat liver. Burstein & Varanelli (1975) have isolated two metabolites from mouse which seemed to be dihydroxy-CBN derivatives. These compounds were suggested to be precursors for metabolites with an acidic function at C-7 and a hydroxyl group somewhere in the side chain. Our results lend support to this proposed pathway for the transformation of CBN *in vivo*.

Regarding dihydroxy metabolites of cannabinoids other than CBN 6,7-dihydroxy-Δ^6-THC (Wall, Brine & others, 1970), 5α,7- and 5β, 7-dihydroxy-Δ^6THC (Wall, 1971) have been reported. Recently several dihydroxy-cannabidiol (CBD) metabolites have been identified by Martin, Agurell & others (1976). They found that CBD, like CBN, was hydroxylated predominantly at C-4″ and to a lesser degree at C-3″. Trace amounts of metabolites were hydroxylated at C-1″, -2″ or 5″. The abundance of isolated dihydroxy metabolites of both CBD and CBN seems to reflect the quantity of the monohydroxy metabolites (cf. Agurell, Binder & others. 1976). Also when Δ^1-THC is side chain hydroxylated, the formation of 3″- and 4″-hydroxy metabolites (isolated from lung and liver of dog) is favoured (Widman, Nordqvist & others, 1975b). It also appears that those side chain hydroxylated metabolites which are major metabolites are also the pharmacologically most potent compounds (Agurell & others, 1976).

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