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BIOSYNTHESIS OF PROPYL CANNABINOID ACID AND ITS BIOSYNTHETIC RELATIONSHIP WITH PENTYL AND METHYL CANNABINOID ACIDS*

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Key Word Index—*Cannabis sativa*; Moraceae; *in vitro* incubation, propyl cannabinoid acid; biosynthesis.

Abstract—Biosynthesis of propyl cannabinoid acid has been determined by *in vitro* incubation with a crude enzyme solution from three strains of *Cannabis sativa* using ^{14}C-labelled cannabinoid acid. Biosynthetic relationships between methyl, propyl and pentyl cannabinoid acids have been demonstrated.

INTRODUCTION

A number of neutral propyl cannabinoids have been isolated [1–6] and other methyl cannabinoids have been detected [7]. Four new propyl cannabinoid acids have been isolated recently: tetrahydrocannabivarinic acid (**1**), cannabidivarinic acid (**2**), cannabichromevarinic acid (**3**) and cannabigerovarinic acid (**4**); also pentyl cannabinoid acid has been obtained from the Thai cannabis, 'Meao strain' and biogenetic pathways have been proposed [8]. The biosynthetic pathway to propyl cannabinoid acid, and the biosynthetic relationships between propyl-, pentyl- and methyl cannabinoid acids are described in this paper.

RESULTS AND DISCUSSION

Cannabis sativa *strains*

For this investigation, three strains of *Cannabis sativa* were employed: the Mexican strain, the Meao variant-1 and Meao variant-2. The Mexican strain contains tetrahydrocannabinolic acid (**5**) as the major and cannabichromenic acid (**6**) as a minor cannabinoid acid. Compound **6** has previously been described as the only cannabinoid acid present in the 7-day-old seedlings [9]. The variant-1 strain of *C. sativa* (1-month-old) contains **1** as the major acid, **3** as a second major acid and trace amounts of **5** and **6** [8]. The variant-2 strain (6-month-old) contains **2** and cannabidiolic acid (**7**) as major acids and **3** and **6** as minor acids; variant-2 strain lacks **1** and **5** [8].

Preparation of the crude enzyme solution and determination of radiochemical purity

Previous investigations based on *in vivo* feeding produced little incorporation of labelled precursors into the corresponding cannabinoid acid [10], due to the fact that cannabinoid acids are very sensitive to high temperature [11], light changes [12] and an acidic medium [13]. Therefore, in this investigation a facile and more convenient *in vitro* procedure was employed; fresh leaves from different variants of *C. sativa* were utilized and the products were isolated as described in Experimental.

Since almost all cannabinoid acids are non-crystalline, the determination of radiochemical purity of the cannabinoid acids obtained in *in vitro* incubation was confirmed by repeated TLC with different solvents (see Experimental) and by methylation with diazomethane which caused no decrease in activity. The position of labelling in the individual cannabinoid acids was determined by decarboxylation which indicated the intact incorporation of precursor.

Although artificial transformation of labelled precursor during the incubation was feared, the non-enzymatic transformation of cannabigerolic acid-carboxyl-^{14}C (**8**-carboxyl-^{14}C) [14] to **6** is negligible in the presence of boiled enzyme. The conversion of **8**-carboxyl-^{14}C to **6** using the enzyme solution from the Mexican strain was complete within 24 hr at 25° (Fig. 1) and thus 24 hr incubation was used routinely.

Biosynthesis of propyl cannabinoid acid

When **4**-carboxyl-^{14}C [14] was incubated with the variant-1 enzyme solution, a high incorporation of ^{14}C into **1** was observed as well as into **3** (Exp. 1, Table 1). The incubation of **4**-carboxyl-^{14}C with the variant-2 enzyme solution was tested. Its transformation into **2** and **3** was sought, but none of the radioactivity was incorporated into the pentyl cannabinoid acids. This agrees with the finding of Kajima and Piranx [15] who found that propyl cannabinoids are not formed from pentyl cannabinoids by a shortening of the side chain.

2-carboxyl-^{14}C [14] was incubated with the variant-1 enzyme solution, resulting in ^{14}C incorporation into **1** and none into **3** (Exp. 3). When **2**-carboxyl-^{14}C was incubated with the variant-2 enzyme solution (Exp. 4) no ^{14}C-labelled cannabinoid acid resulted, suggesting that the variant-2 does not possess an enzyme which transforms **2** to **3**. Thus **4** is a key intermediate for propyl cannabinoid

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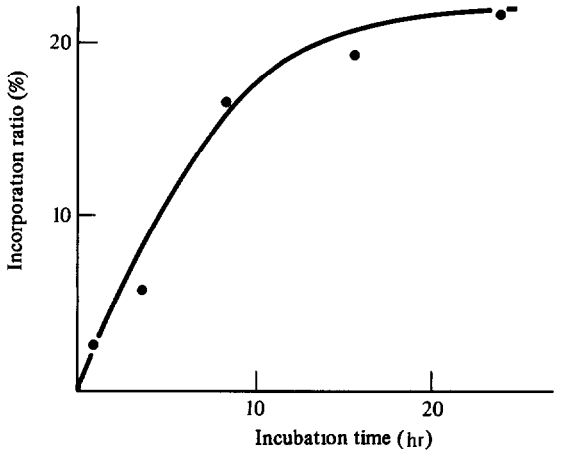

Fig 1. Time course for the conversion of cannabigerolic-carboxyl-^{14}C acid to cannabichromenic acid by *in vitro* incubation.

acids just as **8** is for pentyl cannabinoid acids [10].

Thus, in the biosynthesis of cannabinoid acids (Fig. 2) the major pathway is that of **4** to **1** via **2**: conversion of **4** to **2** is dominant over conversion of **4** to **3**. The fact that the cannabis plant of the variant-2 strain does not have the ability to change **2** to **1** could be due to the lack of transferase activity.

The relationship of propyl-, pentyl- and methyl cannabinoid acids

The biosynthetic relationship of pentyl-, propyl- and methyl cannabinoid acid remains unclear (Fig. 2). The variation in cannabinoid components displayed in the various cannabis strains may be due to: (a) the lack of a chain elongation enzyme which causes the build-up of polyketide (C-10) and C-2 units; (b) the enzyme which condenses the polyketide and C-10 unit (geraniol or nerol) is specific for the chain length of the polyketide; or (c) the enzymes which transform each type of cannabinoid acid are specific for the alkyl side chains. The first two possibilities can be rejected: (a) spiro compounds biosynthesized from a C-14 polyketide [16–18] have been found in all cannabis plants [16] together with the corresponding cannabinoid acids; (b) the second possibility appears implausible due to the difficulty of synthesizing the appropriate polyketides. Thus, the remaining possibility (c) was investigated.

In order to determine whether there is a single enzyme or multiple enzyme systems responsible for pentyl-, propyl- and methyl cannabinoid acid transformations, the crude enzyme fraction from seedlings of the Mexican strain were used. Compounds **6**, **3** and cannabichromeorcinic acid (**9**) were used as standards. Individual radioactive precursors shown in Table 2 (0.02 μmole) were incubated and a radiochromatograph was produced in each case. The first radiochromatogram indicated that the conversion rate of **8**-carboxyl-^{14}C to **6** is 25%. When **4**-carboxyl-^{14}C was used **3** was labelled indicating that the incorporation ratio is almost the same as **8**-carboxyl-^{14}C to **6**. The incubation of cannabigerorcinic acid-carboxyl-^{14}C (**10**-carboxyl-^{14}C) resulted in the conversion to two products. Though the peak near the origin has not been identified, the incorporation of **10**-carboxyl-^{14}C into **9** is 2.5% (Table 2).

In spite of the different transformation ratios of pentyl-, propyl- and methyl cannabinoid acids, it is clear that the seedlings of the Mexican strain possess the enzymes necessary for the transformation of methyl and propyl cannabinoid acids. Thus, possibility (c) must be rejected. In other words, the variation in cannabinoid acid content found in different strains of cannabis is a function of the enzyme specificity of the particular strain at the level of condensation of polyketide with geraniol or nerol.

EXPERIMENTAL

General. All mps are uncorr UV: MeOH, IR: $CHCl_3$, ^{1}H NMR: 100 MHz, $CDCl_3$, TMS as int. standard; EIMS: 70 eV and elevated temp. TLC (silica gel F_{254}, Merck) were developed in hexane–EtOAc (1.2), $CHCl_3$–MeOH–H_2O (30:10·1) and C_6H_6–MeOH–AcOH (45:8·4) (for cannabinoid acids), C_6H_6 (for neutral cannabinoids) and hexane–$CHCl_3$–EtOAc (40:2:1) (for cannabinoid acid methyl esters). Radiochromatography was detected by using a TLC scanner (Aloka Model TRM-1B) Radioactivity was measured on a liquid scintillation spectrometer (Packard-TRI-Carb Model 3375) using the scintillator mixture (POP 4 g, diethyl POPOP 0.1 g, EtOH 50 ml and toluene 950 ml).

Synthesis of cannabichromeorcinic acid (**9**). Compound **10** [14] (197 mg) in 12 ml dioxane containing 402 mg DDQ was stirred at 30° for 3 hr in the dark. The solvent was evaporated *in vacuo* and the residue was redissolved in $CHCl_3$–MeOH (2:1) (10 ml) After washing by a modification of Folch's method [19], which produced a colourless $CHCl_3$ layer, the crude product was purified

Table 1. Tracer experiments with various strains of *Cannabis sativa*

Expt. no.	Strain	Precursor fed ($\times 10^5$ dpm)	Cannabinoid acid isolated	Incorporation (%)
1	Variant-1	4-carboxyl-^{14}C (2.79)	**1**	7 04
			3	0 91
2	Variant-2	4-carboxyl-^{14}C (2.79)	**2**	0 88
			3	0.12
			7	0
			6	0
3	Variant-1	2-carboxyl-^{14}C (3.40)	**1**	3 53
			3	0
4	Variant-2	2-carboxyl-^{14}C (3.40)	**1**	0
			3	0

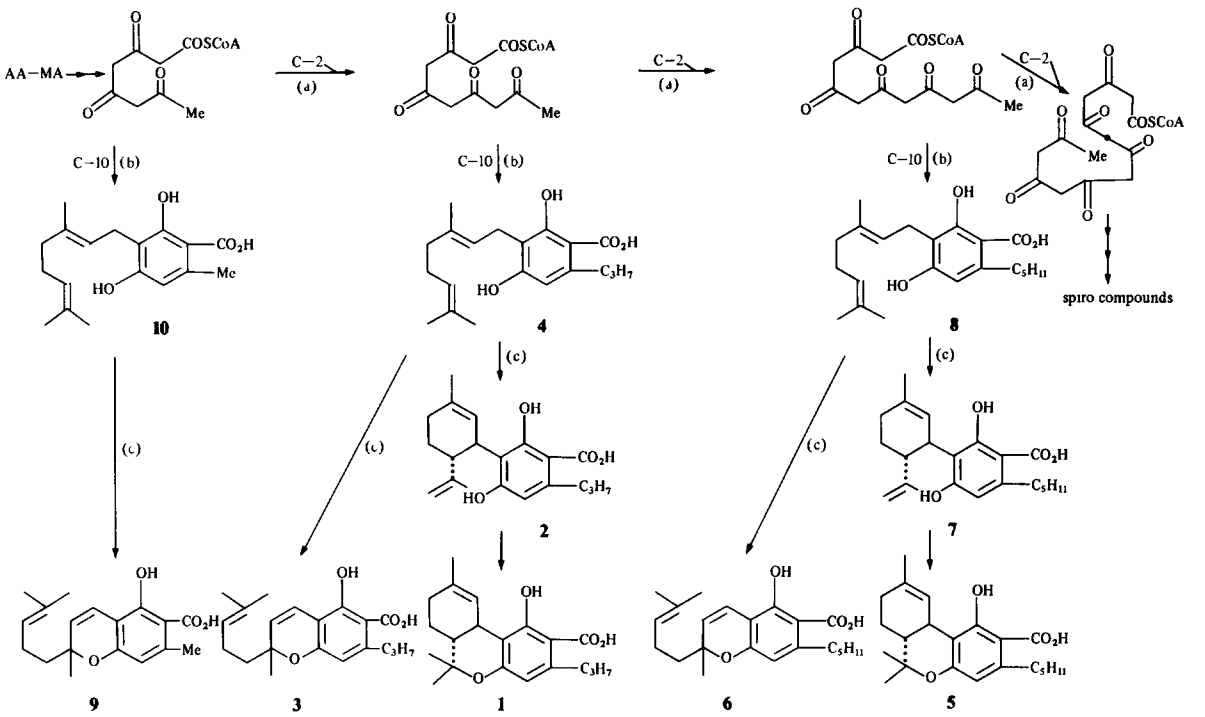

Fig. 2. Biosynthesis of propyl cannabinoid acids and biogenetic relation of methyl-, propyl- and pentyl cannabinoid acids

Table 2 *In vitro* conversion of cannabinoid acids by seedlings of the Mexican strain

Precursor fed (dpm)	Cannabinoid acid isolated	Incorporation (%)
8-carboxyl-^{14}C (82 800)	**6**	25.40
4-carboxyl-^{14}C (94 650)	**3**	24 96
10-carboxyl-^{14}C (90 080)	**9**	2 50

by CC over silica gel (75 g of Merck silica gel 60) using $CHCl_3$–MeOH–H_2O (30.10:1) as a solvent Compound **9** was crystallized from Et_2O–hexane as colourless needles (148 mg), mp 92–94°; UV λ_{max}^{MeOH} nm (log ε): 247 (4.20), 255 (4.14), 290 (3.55), 3.27 (3.28) IR $\nu_{max}^{CHCl_3}$ cm^{-1}. 3520 (OH), 1705, 1655 (C=O), 1620, 1590 (C=C). ^{1}H NMR: δ1.36 (3H, *s*), 1.58, 1.67 (3H × 2), 1 90, 2.30 (4H, *m*), 2.34 (3H, *s*), 5 10 (1H, *t*), 5.40 (1H, *d*, *J* = 8 Hz), 6.10 (1H, *s*), 6.64 (1H, *d*, *J* = 8 Hz) MS *m/z* 302 [M]$^+$ 258, 243, 201, 176, 175 (100%); Calc for $C_{18}H_{22}O_4$ 302 1581. Found. 302.1516.

Cannabichromeorcinic acid methyl ester. Crystallized from hexane–Et_2O as colourless needles, mp 64–66°. UV λ_{max}^{MeOH} nm (log ε): 225 (4.39), 261 (4.40), 283 (3.43). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600–3100 (OH), 1725, 1650 (C=O), 1620, 1570 (C=C). ^{1}H NMR: δ1.40, 1.56, 1.60 (3H × 3, each *s*), 2.60 (2H, *t*), 2.48 (3H, *s*), 3.92 (3H, *s*), 5.08 (1H, *t*), 5.46 (1H, *d*, *J* = 10 Hz), 6.18 (1H, *s*), 6.72 (1H, *d*, *J* = 10 Hz), 11.94 (OH). MS *m/z*: 316 [M]$^+$, 301, 269, 234, 233, 201 (100%); Calc for $C_{19}H_{24}O_4$: 316.1675. Found: 316.1670.

Cannabichromeorcin This was obtained by decarboxylation of **9** after heating at 150° for 10 min A colourless syrup was obtained, UV λ_{max}^{MeOH} nm (log ε): 230 (4.10), 281 (3.56), 291 (sh. 3 48) IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3240 (OH), 1630, 1580 (C=C). ^{1}H NMR: δ1 36, 1.58, 1 66 (3H × 3, each *s*), 2.20 (3H, *s*), 5.08 (1H, *t*, *J* = 8 Hz), 5.47 (1H, *d*, *J* = 10 Hz), 6 10, 6.22 (1H × 2, *d*, *J* = 1 Hz), 6 58 (1H, *d*, *J* = 10 Hz). MS *m/z* 258 [M]$^+$, 243, 176, 175 (100%).

Radioactive cannabinoid acids [14]. **10**-carboxy-^{14}C: mp 128–130°, 2.55 mCi/mmol, **4**-carboxyl-^{14}C: mp 66–68°, 2 54 mCi/mmol, **8**-carboxyl-^{14}C: mp 106–110°, 2.69 mCi/mmol.

Preparation of crude enzyme solution. Five grams of 7-day-old seedlings of *C sativa* (Mexican strain) were homogenized with 0.1 M K_2HPO_4 (10 ml, pH 6.7) at 4° and centrifuged (2000 rpm, 10 min) The supernatant was used for the crude enzyme soln. The Meao variant-1 (1-month-old; 2 g) and the Meao variant-2 were also homogenized in the same way.

Incorporation of precursors and extraction. Radioactive precursor (1 2 × 10^5 dpm – 1 1 × 10^5 dpm), 0.1 M $MgCl_2$ (0 1 ml) and 1 mg of Tween 80 were added to 1 ml of the crude enzyme soln. After sonication, the mixture was incubated at 25° in the dark for 24 hr. Incubation was terminated by the addition of 3 ml of $CHCl_3$–MeOH (2·1). The H_2O layer was extracted with 1 ml of $CHCl_3$–MeOH (2:1) × 3. The pooled $CHCl_3$ layer was washed by Folch's Method [19] and the $CHCl_3$ was evaporated in N_2. After the addition of carrier standard (10 mg), the material was purified by prep. TLC. The plates underwent radiochromatography, and the radioactive zones were scraped, extracted with MeOH (10 ml) and counted A control assay was performed by using a denatured crude enzyme soln, obtained by boiling a portion of crude enzyme soln for 5 min.

Confirmation of radiochemical purity for cannabinoid acid obtained by in vitro incubation. Purified cannabinoid acid in Et_2O was methylated by treatment with CH_2N_2 for 5 min. After evapn of the solvent, the product was purified by prep. TLC in hexane–$CHCl_3$–EtOAc (40:2:1); the corresponding band was scraped from the TLC plate, extracted with MeOH and counted.

Determination of incorporation by decarboxylation Individual pure cannabinoid acids obtained by *in vitro* incubation were decarboxylated by heating. The liberated neutral cannabinoids were purified by prep. TLC in C_6H_6. The corresponding zone was scraped off, extracted and counted.

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