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BIOSYNTHESIS OF CANNABINOID ACIDS*

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Abstract—Malonic acid, mevalonic acid, geraniol and nerol were incorporated into tetrahydrocannabinolic acid and cannabichromenic acid in *Cannabis sativa*. The pathway from cannabigerolic acid to tetrahydrocannabinolic acid via cannabidiolic acid was established by feeding labelled cannabinoid acids. Cannabichromenic acid was shown to be formed on a side pathway from cannabigerolic acid.

INTRODUCTION

Mechoulam and Gaoni have postulated the two parallel routes for the biogenesis of the cannabinoids [1], the first involves the condensation of geraniol with olivetol to give the neutral cannabinoids and the second, the condensation of geraniol with olivetolic acid to give the cannabinoid acids. In the course of our investigation on *Cannabis* [2], the amounts of the neutral cannabinoids were observed to increase in stored material, whereas several cannabinoid acids, including cannabigerolic acid (**1**), cannabidiolic acid (**2**), cannabichromenic acid (**3**) and tetrahydrocannabinolic acid (**4**) (Fig. 1), have been isolated from fresh plants. Previously, we noted that the neutral cannabinoids as well as cannabinolic acid are artifacts formed during harvesting and the storage, and proposed a biogenetic pathway of the cannabinoid acids [3].

This paper deals with feeding studies with *Cannabis* plants using ^{14}C-labelled malonate and mevalonate, ^{3}H-labelled geraniol and nerol, and with labelled cannabigerolic and cannabidiolic acids in order to throw light on the overall biosynthetic pathway.

RESULTS AND DISCUSSION

Strains of Cannabis sativa

For the feeding experiments, four strains of *Cannabis sativa* L., regarded as genetic types, were used (Table 1). Three of them, Mexican, Minamioshihara-1 (M-1) and Domestic-1 (D-1), have tetrahydrocannabinolic acid (**4**) as the major cannabinoid acid. M-1 also contains cannabigerolic acid monomethyl ether (**5**) as the second major constituent, Domestic-2 (D-2) contains cannabidiolic acid (**2**), but almost no **4**, and thus seems to lack the capacity for production of the latter acid.

Tracer compounds

The tracers employed in this study are shown in Table 2. Geraniol-[1-^{3}H] and nerol-[1-^{3}H] were prepared by $NaB^{3}H_4$ reduction of the corresponding aldehydes. The labelled cannabinoid acids [U-^{14}C] **1** and **2** were obtained from *Cannabis* plants cultivated with $^{14}CO_2$ [4]. Cannabinoid acids **1** and **2**, labelled at the carboxyl carbon, were synthesized by the reaction of the neutral cannabinoids with ^{14}C-labelled methyl mag-

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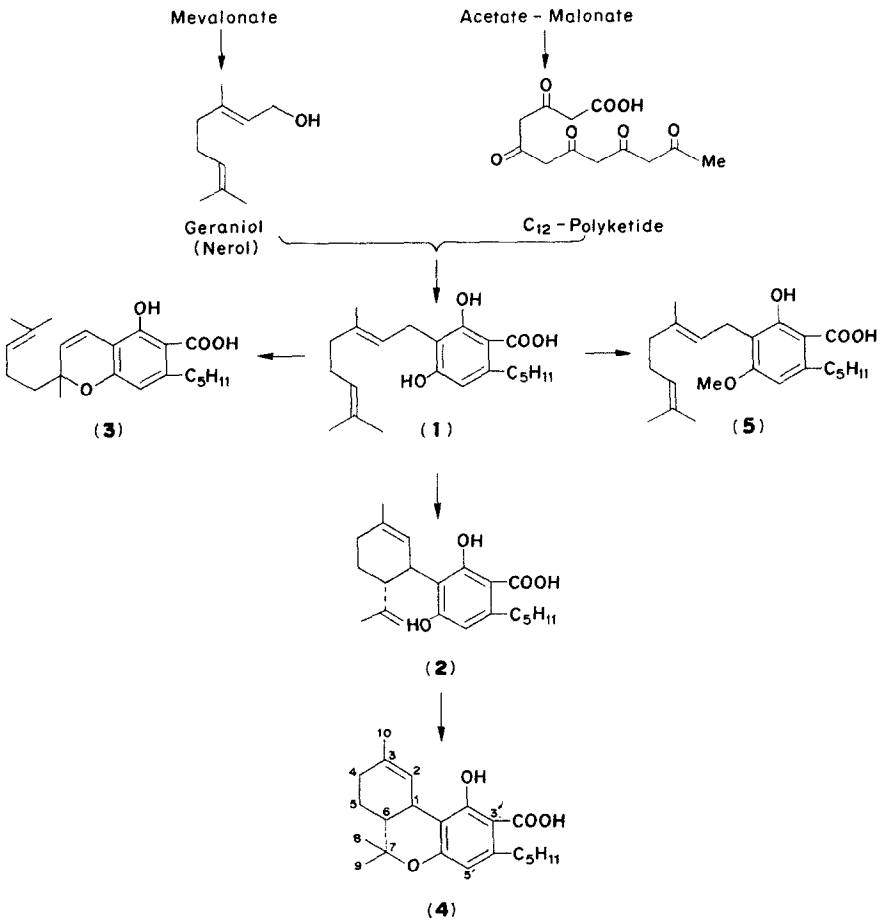

Fig. 1. Biosynthetic pathway of cannabinoid acids.

nesium carbonate [5]. Cannabigerolic acid-[U-^{3}H] was prepared according to Wilzbach's procedure. The labelled substrates were fed to the plants either by using Bennett and Heftmann's procedure [6] (geraniol and nerol) or by means of cotton wicks. The final identification of the labelled cannabinoids isolated from plants is given in the Experimental.

Incorporation of primary precursors

The biosynthesis of the cannabinoid acids from acetate-malonate (MA) and mevalonate (MVA) was shown by the incorporation of MA-[2-^{14}C] and MVA-[2-^{14}C] into **3** and **4** (Table 2). The incorporation ratio of both precursors, however, was quite low. Geraniol-[1-^{3}H] and nerol-[1-^{3}H] were administered, and the radioactive cannabinoid acids (**3** and **4**) were isolated and decarboxylated to the corresponding neutral compounds (tetrahydrocannabinol and cannabichromene) in a chromatographycally pure form. The production of radioinactive cannabinol from the active tetrahydrocannabinol by the removal of C-1-^{3}H [7], originally from geraniol- and nerol-1-^{3}H, indicates the intact incorporation of both monoterpenes into **4**. Since **1** is formed from geraniol and C_{12}-polyketide, some difference might have been

Table 1. Cannabinoid content of various strains of *Cannabis sativa*

Strain	Cannabinoid Present (%)* 2	3	4	5
Mexican	neg	0·58	2·10	neg
Minamioshihara No. 1 (M-1)	neg	0·17	0·76	0·18
Domestic No. 1†, (D-1)	neg	0·17	0·26	trace
Domestic No. 2 (D-2)	0·51	0·03	neg	trace

* Contained in fresh dried leaves.
† 50-day-old plant.

Table 2. Tracer experiments with various strains of *C. sativa*

Expt. no	Strain	Precursor Fed.	Incor. (%)*	Sp. act. (dpm/M)
1	Mexican	Malonic acid-[2-^{14}C]-Ca (30 μCi)	**4*** 0·012 **3*** 0·007	
2	Mexican	(±)-Mevalonic acid-[2-^{14}C]-Na (20 μCi)	**4*** 0·013 **3*** 0·003	
3	Mexican	Geraniol-[1-^{3}H] ($1·67 \times 10^{9}$ cpm)	**4*** 0·419 **3*** 0·044	11·1† 9·78†
4	Mexican	Nerol-[1-^{3}H] ($3·66 \times 10^{8}$ cpm)	**4*** 0·284 **3*** 0·019	1·09† 0·748†
5	(D-2)	**1**-[carboxyl-^{14}C] ($3·15 \times 10^{6}$ dpm)	**2** 3·16	1·32
6	Mexican	**1**-[carboxyl-^{14}C] ($3·15 \times 10^{6}$ dpm)	**4** 2·94 **3** 0·12	0·826 0·249
7	(D-1)	**1**-[carboxyl-^{14}C] ($3·12 \times 10^{6}$ dpm)	**4** 0·68 **3** 0·22	0·173 0·134
8	(M-1)	**1**-[U-^{3}H] ($1·22 \times 10^{9}$ dpm)	**4*** 0·039 **3*** 0·023 **5*** 0·004	91·3 307·0 7·33
9	Mexican	**2**-[U-^{14}C] ($9·87 \times 10^{5}$ dpm)	**4*** 0·42 **3*** 0	0·062 0
10	Mexican	**2**-[carboxyl-^{14}C] ($1·18 \times 10^{6}$ dpm)	**4** 2·80	0·776

* Compounds marked thus, isolated after decarboxylation as corresponding neutral compounds.
† Sp. act. marked thus, indicated by cpm/mM.

expected between the incorporation ratios of geraniol and of nerol into **3** and **4**, if they are biosynthesized through **1**. However, no remarkable difference between the ratios was observed (Table 2, experiments 3 and 4), probably due to the equilibration of the terpenes before condensation with the olivetolic acid moiety. Similar results have been reported for some indole alkaloids [8].

Incorporation of cannabigerolic and cannabidiolic acids

When labelled **1** was fed, the label in the resulting metabolites varied according to the *Cannabis* variety to which it was applied. High incorporation into **2** was observed in variety D-2 (Expt. 5 Table 2), while **3** and **4** were labelled in Mexican and D-1 (Expts. 6 and 7). Upon application to M-1, **5** was observed to be labelled as well as **3** and **4** (Expt. 8). The decarboxylation of the acids furnished the corresponding neutral cannabinoids and radioactive CO_2, indicating the direct transformation of **1** to **3** and **4**. Cannabigerolic acid (**1**) was, therefore regarded as the first intermediate produced (Fig. 1).

Predominate incorporation of **1** in **3** as compared with that in **4** was observed mainly in young plants. These results are in good accordance with the evidence previously reported by us [9] that **3** is present exclusively in young plants and is gradually replaced by **4** as the plants produce flowering shoots.

Upon administration of either cannabidiolic acid-[U-^{14}C] or -[carboxyl-^{14}C], to Mexican strain in which **3** was detected as a second major cannabinoid acid besides **4**, the incorporation of **2** into the former substance was not observed and only the latter was labelled (Table 2 Expt. 9). When the carboxyl-^{14}C acid was applied, the location of the radioactivity in the carboxyl moiety of the acids was determined in the same way as with cannabigerolic acid-[carboxyl-^{14}C].

According to these feeding experiments, therefore, the biosynthetic pathway of the cannabinoid acids appears to be as shown in Fig. 1.

EXPERIMENTAL

Identification of neutral cannabinoids and cannabinoid acids. The following TLC [on Si gel G (Merck)] solvent systems were used: C_6H_6 or hexane–C_6H_6–Et_2NH (20:10:1) [10] (for neutral cannabinoids); hexane–EtOAc (1:1) and C_6H_6–MeOH–HOAc (45:8:4) (for cannabinoid acids). Spots were detected with UV-light or with diazotized benzidine. Isolation of the labelled cannabinoids from the plants was effected using authentic crystalline cannabigerolic acid [**1**: mp 106–110°, UV_{max}^{MeOH} 223 (20400), 267 (7400), 306 (2900). $IR_{max}^{CHCl_3}$ 3320–3400

(OH), 1640 (C=O)] and the derivatives, tetrahydrocannabinol-3,5-dinitrophenylurethane [11] (mp 115–116°) and cannabidiolic acid diacetate [12] (mp 118–125°) in order to dilute labelled compounds and crystallize them to constant specific activity. Purity of uncrystallized cannabinoids (**3**, **4** and cannabichromene) were determined radiochromatographically.

Preparation of the labelled compounds. Geraniol-[1-^{3}H] and nerol-[1-^{3}H]: To a soln of geranial or neral in MeOH, NaB^3H_4 (50 μCi) was added and the soln allowed to stand for 1 hr, dil with H_2O, and extracted with Et_2O. The extract was purified by preparative TLC to give radiochemically pure geraniol- and nerol-[1-^{3}H] ($2{\cdot}01 \times 10^{10}$ cpm/mM and $4{\cdot}55 \times 10^9$ cpm/mM, respectively). **1**-[carboxyl-^{14}C] and **2**-[carboxyl-^{14}C]: Cannabigerol and cannabidiol respectively were treated with ^{14}C-labelled methyl magnesium carbonate (produced from 3 mCi $Ba^{14}CO_3$) giving the cannabinoid acids which were identified by mmp, TLC, and GLC with authentic samples [**1**-(carboxyl-^{14}C)], mp 106–110°, $2{\cdot}08 \times 10^9$ dpm/mM, **2**-[carboxyl-^{14}C], mp of diacetate 118–125°, $1{\cdot}83 \times 10^9$ dpm/mM). **1**-[U-^{3}H] was prepared according to Wilzbach's procedure, mp 106–110°, $5{\cdot}15 \times 10^{12}$ dpm/mM. **2**-[U-^{14}C]: Cuttings of variety D-2 (15 cm high, 20 leaves) were cultivated with $^{14}CO_2$ (10 mCi) for 6 days. Leaves were extracted with MeOH and the MeOH extractives were fractionated by Si gel (column and preparative TLC with hexane–EtOAc). **2**-[U-^{14}C], mp of the diacetate 118–125°, $5{\cdot}07 \times 10^8$ dpm/mM.

Feeding experiments. Female plants were used. The aq soln of MA-[2-^{14}C]-Ca(30 μCi) and (±)-MVA-[2-^{14}C]-Na(20 μCi) were fed to cuttings of Mexican variety (20 cm high, with 10 leaves) by means of cotton wicks. Geraniol-[1-^{3}H] ($1{\cdot}67 \times 10^9$ cpm) and nerol-[1-^{3}H] ($3{\cdot}66 \times 10^8$ cpm) were dissolved in Me_2CO and applied on the leaves of Mexican variety (100 cm high, with 30 leaves) for 6 days according to the procedure of Bennett and Heftmann [6]. The labelled cannabinoid acids (10^5–10^6 dpm) were emulsified in H_2O with the aid of Tween 85 and applied to the cuttings of the various strains shown in Table 2.

Isolation of the cannabinoids. The leaves were extracted with MeOH and the MeOH extractives were fractionated on a Si gel column (hexane–EtOAc) and preparative TLC (same solvent and C_6H_6–MeOH–HOAc). The cannabinoid acids isolated were then decarboxylated to neutral cannabinoids, which were also obtained by direct decarboxylation of the total MeOH extractives, followed by the purification by preparative TLC.

Dehydrogenation. Tetrahydrocannabinol (19·4 mg, 7770 cpm; from geraniol-[1-^{3}H] and 18·8 mg, 7740 cpm; from nerol-[1-^{3}H]) was refluxed in toluene containing chloranil for 8 hr and purified by preparative TLC to give non-radioactive cannabinol.

Decarboxylation. Radioactive **3** ($3{\cdot}10 \times 10^3$ dpm, from Expt. 7) was decarboxylated by heating at 160° for 10 min to liberate radioactive CO_2 ($2{\cdot}95 \times 10^3$ dpm) which was trapped in the mixture of 2-methoxyethanol and ethanolamine (2:1) [13]. Radioactive **4** ($8{\cdot}24 \times 10^4$ dpm, from Expt. 10) and **2** ($7{\cdot}99 \times 10^3$ dpm, from Expt. 5) were decarboxylated in the same way to liberate radioactive CO_2 ($6{\cdot}18 \times 10^4$ dpm and $5{\cdot}91 \times 10^3$ dpm, respectively).

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