First Direct Evidence for the Mechanism of Δ^1-Tetrahydrocannabinolic Acid Biosynthesis

Futoshi Taura, Satoshi Morimoto,* and Yukihiro Shoyama

Faculty of Pharmaceutical Sciences, Kyushu University Maidashi 3-1-1, Fukuoka 812, Japan

Raphael Mechoulam

Department of Natural Products, Hebrew University Medical Faculty, Jerusalem 91120, Israel

Received May 8, 1995

Numerous plausible hypotheses have been advanced regarding the biogenesis of Δ^1-tetrahydrocannabinol (Δ^1-THC, **1a**), the psychoactive constituent of marihuana (*Cannabis sativa* L.);[1] however, they all lack experimental support. Thus all hypothetical biogenetic schemes assume that Δ^1-tetrahydrocannabinolic acid (Δ^1-THCA, **1b**), the precursor of Δ^1-THC, is formed by cyclization from cannabidiolic acid (**2**).[2] We now present experimental evidence that establishes that **1b** is actually formed from cannabigerolic acid (**3b**) through oxidocyclization by a new enzyme, Δ^1-THCA synthase, and that **2**, a plausible intermediate, is not a substrate for this enzymatic reaction.

Since the enzymes that catalyze the formation of **1b** have not been purified and studied, we first attempted to identify an enzyme in *C. sativa* that could cyclize **2** into **1b**. Using various extraction and assay conditions we were unable to obtain such an enzyme preparation; however, by contrast, when **3b** was used as a substrate,[3] the crude enzyme preparation extracted with 1 M $CaCl_2$ exhibited a potent Δ^1-THCA-producing activity.[4] We assume therefore that **1b** is derived biosynthetically by the oxidative cyclization of **3b** rather than by the ring closure of **2**.

In order to characterize the oxidocyclase (Δ^1-THCA synthase), we attempted to purify this new enzyme. Leaf buds of the Mexican *Cannabis* strain were used for the extraction of the enzyme,[5] as they showed a much higher enzyme activity than any other parts of this plant. The Δ^1-THCA synthase was purified to homogeneity by chromatography on (diethylaminoethyl)cellulose, phenyl Sepharose CL-4B, and hydroxylapatite.[6] The purified enzyme migrated on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as a single band with a molecular mass of 75 kD (Figure 1). The native molecular mass determined by gel filtration using Sephadex G-75 was 74 kDa, indicating that Δ^1-THCA synthase is a monomeric enzyme. The isoelectric point for this enzyme was determined as 6.4 by isoelectric focusing. The purified enzyme was sequenced by Edman degradation, and the following NH_2-terminal sequence was identified: Asn-Pro-Arg-Glu-Asn-Phe-Leu-Lys-x-Phe-Ser-Lys-His-Ile-Pro-Asn.[7] The Δ^1-THCA synthase is the first enzyme involved in cannabinoid biogenesis to be identified and purified. The Δ^1-THCA (**1b**) produced by the enzymatic reaction[8] was found to be identical with **1b** isolated from the plant by comparison of their CD spectra as well as their FAB-MS. Therefore, the purified Δ^1-THCA synthase was confirmed to catalyze the stereoselective conversion of **3b** to **1b**.

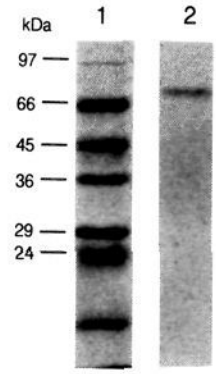

Figure 1. SDS−PAGE (12.5% gel) of purified Δ^1-THCA synthase. The gel was stained with Coomassie Blue. Lane 1: molecular mass standards. Lane 2: purified Δ^1-THCA synthase (1 μg). Numbers at left indicate positions of molecular mass standards in kilodaltons.

In order to evaluate the kinetic properties of Δ^1-THCA synthase, the enzyme activity was measured under various conditions (Table 1). Since the Δ^1-THCA synthase activity was highest between pH 5.5 and 6.0, an assay system with 200 μM **3b** at pH 6.0 was chosen as a standard.[9] Ions such as Mg^{2+} and Mn^{2+}, which are known to be cofactors for monoterpene cyclase,[10] had little influence on the Δ^1-THCA synthase activity, whereas Hg^{2+} strongly inhibited the production of **1b**. It is noteworthy that Δ^1-THCA synthase is not inhibited by the absence of molecular oxygen, contrary to oxygenases and oxidases which absolutely require molecular oxygen for the

(1) Gaoni, R.; Mechoulam, R. *J. Am. Chem. Soc.* **1964**, *86*, 1646.

(2) (a) Mechoulam, R. *Science* **1970**, *168*, 1159−1166. (b) Shoyama, Y.; Yagi, M.; Nishioka, I.; Yamauchi, T. *Phytochemistry* **1975**, *14*, 2189−2192.

(3) Owing to the low CBGA content in *C. sativa*, **3b** was chemically synthesized by coupling of olivetol (Sigma) and geraniol (Aldrich),[13a] followed by carboxylation with methylmagnesium carbonate.[13b]

(4) The enzyme activity was determined by HPLC. HPLC was carried out under essentially the same conditions as previously described,[14] but 85% aqueous CH_3CN containing 50 mM phosphoric acid was used for this study as a solvent system.

(5) Extraction was carried out at 4 °C. Leaf buds (66 g) of *C. sativa* (Mexican strain) were homogenized with a Waring blender at high speed together with 800 mL of 1 M $CaCl_2$ containing 10 mM mercaptoethanol. The homogenate was centrifuged at 20000 *g* for 15 min. The supernatant fluid was concentrated by ultrafiltration and dialyzed against 10 mM aqueous mercaptoethanol. During dialysis, most of the Δ^1-THCA synthase activity precipitated. The resulting precipitates were collected by centrifugation at 49000*g* for 60 min and resuspended in 10 mM sodium phosphate buffer (pH 7.0) containing 3 mM mercaptoethanol. The insoluble material was removed by centrifugation at 49000*g* for 60 min, and the supernatant (crude enzyme extracts) was used for the purification of the Δ^1-THCA synthase.

(6) All purification procedures were conducted at 4 °C. The following buffers were used for the purification of the Δ^1-THCA synthase: buffer A, 10 mM sodium phosphate buffer (pH 7.0) containing 3 mM mercaptoethanol; buffer B, buffer A containing 0.8 M ammonium sulfate; buffer C, 250 mM sodium phosphate buffer (pH 7.0) containing 3 mM mercaptoethanol. The crude enzyme extracts as described in footnote 5 were applied to a Whatman DE-52 chromatographic column (2.5 × 24 cm) using buffer A (flow rate: 1.7 mL/min). The enzyme was eluted soon after the void peak (void volume: 65 mL). This step gave a 3-fold purification, while approximately 45% of loaded activity was recovered by this procedure. The Δ^1-THCA synthase active fractions were concentrated by ultrafiltration. After ammonium sulfate was added to the concentrated sample to raise the concentration to 0.8 M, the sample was loaded onto a phenyl Sepharose CL-4B column (Pharmacia; 1.5 × 14 cm) previously equilibrated with buffer B. Δ^1-THCA synthase was eluted by a 500-mL linear gradient of buffer B to buffer A (flow rate: 1.5 mL/min), resulting in an enrichment of ca. 152-fold. The Δ^1-THCA synthase active fractions eluted at about 0.3 M ammonium sulfate were concentrated by ultrafiltration and dialyzed against buffer A. A final purification was achieved by hydroxylapatite chromatography (Nacalai Tesque, Japan; 1.0 × 8 cm) pre-equilibrated with buffer A. A 300-mL linear gradient of buffer A to buffer C followed at a flow rate of 1.2 mL/min. The enzyme was eluted at about 230 mM phosphate buffer. The overall yield of Δ^1-THCA synthase from this purification scheme was 3.3%. The purified enzyme (250 μg) was stored at 4 °C in buffer A.

(7) The letter "x" indicates that the identity of the residue is ambiguous.

(8) In the presence of the purified enzyme (50 μg), the substrate buffer (500 mL), which was composed of 100 mM sodium phosphate buffer (pH 6.0), 200 μM **3b**, and 0.1% (w/v) Triton X-100, was incubated for 6 h at 30 °C. The reaction mixture was partitioned with AcOEt. The AcOEt layer was subjected to HPLC[4] to yield **1b** (0.5 mg).

(9) Standard assay consisted of the substrate buffer[8] (500 μL) and enzyme solution (100 μL). After the sample was incubated for 2 h at 30 °C, the reaction was stopped by the addition of 600 μL of MeOH.

(10) (a) Croteau, R. *Chem. Rev.* **1987**, *87*, 929−954. (b) Rajaonarivony, J. I. M.; Gershenzon, J.; Croteau, R. *Arch. Biochem. Biophys.* **1992**, *296*, 49−57. (c) Colby, S. M.; Alonso, W. R.; Katahira, E. J.; McGarvey, D. J.; Croteau, R. *J. Biol. Chem.* **1993**, *268*, 23016−23024.

0002-7863/95/1517-9766$09.00/0

Table 1. Δ^1-THCA Synthase Activity under Various Conditions

conditns	rel act.[a]	conditns	rel act.[a]
standard	100	1 mM NAD	101
2 mM $MgCl_2$	104	1 mM NADP	101
2 mM $MnCl_2$	103	1 mM FAD	64
2 mM $HgCl_2$	4	1 mM FMN	70
2 mM $CaCl_2$	103	1 mM H_2O_2	78
2 mM $FeCl_3$	91	N_2[b]	99

[a] Data were obtained means of three replicated assays. [b] To remove molecular oxygen the standard assay solution was saturated with N_2 gas.

oxidation of substrates. Furthermore, addition of hydrogen peroxide did not stimulate the enzyme, indicating that Δ^1-THCA synthase is not peroxidase. Therefore, Δ^1-THCA synthase should be a dehydrogenase, although coenzymes such as NAD, NADP, FAD, and FMN did not stimulate the enzyme activity. Inhibition by both flavins was shown to be due to nonenzymatical degradation of **3b**. Purified Δ^1-THCA synthase displayed a high activity (V_{max}: 2.68 nkat/(mg of enzyme)) and a high affinity (K_m: 134 μM) for **3b**.[11] Cannabinerolic acid (**4b**),[12] which is a *Z* isomer of **3b**, is also converted to **1b** by Δ^1-THCA synthase, although lower specificity (V_{max}: 0.37 nkat/(mg of enzyme), K_m: 254 μM) for **4b** was found as compared to **3b**. The C-1/C-2 double bond of **4b** has the same configuration as that of **1b**, suggesting that **4b** could be an intermediate in the oxidocyclization of **3b** into **1b**. However, the lower activity for **4b** shows that the enzymatic reaction does not proceed from **3b** through **4b** to **1b**. We propose that **1b** is formed via a common intermediate in the reactions of both **3b** and **4b** (Figure 2). Recently, kinetic properties similar to those reported above were found for a monoterpene cyclase (limonene synthase), which catalyzes limonene formation with higher V_{max} and lower K_m values for geranyl pyrophosphate as compared to neryl pyrophosphate.[10b] The limonene synthase, whose full amino acid sequence was revealed,[10c] did not possess a region similar to the NH_2-terminal sequence of Δ^1-THCA synthase. Various monoterpene cyclases including limonene synthase have been purified and characterized; however, cyclization by such enzymes was not accompanied by an oxidative reaction.[10] The Δ^1-THCA synthase described here is apparently a unique cyclase. Δ^1-THCA synthase did not convert the neutral cannabinoids cannabigerol (**3a**) and cannabinerol (**4a**) to **1a**, indicating that the presence of the carboxyl group in the substrate is essential for enzymatic cyclization of the terpene moieties.

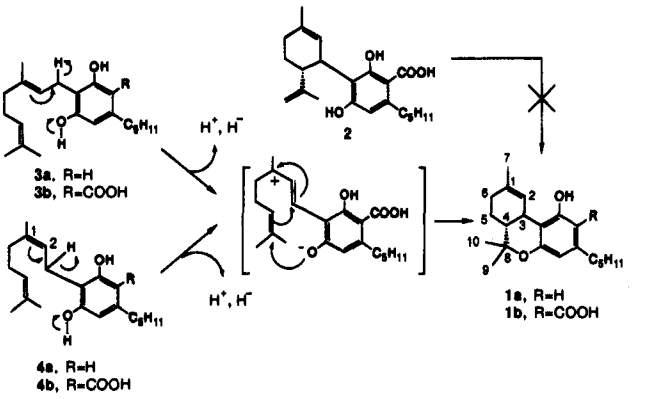

Figure 2. Mechanism of Δ^1-THCA (**1b**) biosynthesis by Δ^1-THCA synthase.

In conclusion, we have identified *C. sativa* a new oxidocyclase, which converts **3b** and **4b** to **1b**. This reaction does not proceed through the generally postulated precursor **2**. These findings cast serious doubt on the hypothesis that **2** is a biosynthetic precursor of **1b**.

Acknowledgment. The research reported in this paper was supported in part by a Grant-in-Aid (No.05671830 for S.M.) from the Ministry of Education, Science and Culture of Japan.

JA951490B

(11) V_{max} and K_m values were determined by Lineweaver–Burk double-reciprocal plots of the velocity curves of the THCA-producing reaction with concentration of substrates. The assays were carried out with 0.34 μg of the purified enzyme.

(12) Taura, F.; Morimoto, S.; Shoyama, Y. *Phytochemistry* **1995**, *39*, 457–458.

(13) (a) Mechoulam, R.; Yagen, Y. *Tetrahedron Lett.* **1969**, 5349–5352. (b) Mechoulam, R.; Ben-Zvi, Z. *J. Chem. Soc. D* **1969**, 343–344.

(14) Morimoto, S.; Goto, Y.; Shoyama, Y. *J. Natl. Prod.* **1994**, *57*, 817–823.