Chem. Pharm. Bull.
25(8)1941—1946(1977)

UDC 547.914.04 : 581.192 : 542.98

Biotransformation of Cannabinoid Precursors and Related Alcohols by Suspension Cultures of Callus induced from *Cannabis sativa* L.

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(Received December 3, 1976)

In the suspension cultures using callus induced from *Cannabis sativa* L., geraniol, nerol, olivetol and ethyl olivetolate which are cannabinoid precursors were not biotransformed to cannabinoids. We found that primary and secondary allylic alcohols such as geraniol, nerol, *trans*-cinnamyl alcohol, isophorol and *trans*-verbenol were biotransformed to the corresponding aldehydes. On the other hand, acyclic secondary allylic alcohols such as α-ionol, β-ionol and 4-phenyl-3-buten-2-ol were not biotransformed. Further, we clarified that the enzyme which catalyzed the oxidation of allylic alcohols was an alcohol oxidase.

Keywords——*Cannabis sativa* L.; Moraceae; callus; suspension cultures; cell-free system; bio-oxidation of allylic alcohols; alcohol oxidase

In a previous paper,[2] we reported that tetrahydrocannabinol, the other cannabinoids, and essential oil which observed in the extract of original plant were not detected in the callus induced from *Cannabis sativa* L. (Moraceae) under various conditions.

In the present paper, we report on the biotransformation of cannabinoid precursors and related alcohols using Cannabis callus.

It has been assumed that cannabinoids are biosynthesized by the condensation of geraniol (or nerol) with olivetol or olivetolic acid.[3] But Nishioka, *et al.* have postulated that the routes involved the condensation of geraniol with C_{12}-polyketide formed from Acetate-Malonate pathway.[4] We achieved the biotransformation of cannabinoid precursors using Cannabis callus based on the above postulate.

In the combination of geraniol or nerol with olivetol or ethyl oilvetolate,[5] the biotransformation was carried out by suspension cultures using Cannabis callus cultured on Murashige and Skoog agar medium for about one month. After one week, the callus and the medium were investigated chemically, but compounds related to cannabinoids were not observed in the suspension cultures. On the other hand, in the biotransformation of geraniol, a new spot giving a negative Fast blue B color reagent test was detected by thin-layer chromatography (TLC). Furthermore, two new peaks were also detected with gas-liquid chromatography (GLC) using 25% PEG-6000 packing. The peaks were identified as citral and nerol by comparison of retention time (t_R) and mass fragment patterns with the authentic samples. Since citral has citral-a (*trans*) and citral-b (*cis*), the biotransformed citral was isolated chromatographically and its proton magnetic resonance (PMR) was measured. We found that the citral consisted of a mixture of citral-a and citral-b according to PMR data, $CDCl_3$(δ) ppm, citral-a:[6] 2.20 (d, J=1.5 Hz, CH_3–C=C), 9.98 (d, J=8.0 Hz, CHO), citral-b: 2.00 (d, J=1.5 Hz, CH_3–C=C), 9.90 (d, J=8.0 Hz, CHO). The t_R values of citral-a and citral-b

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prepared by oxidation (MnO_2)[7] of geraniol and nerol agreed with the t_R values of the obtained aldehydes by GLC using 15% silicon DC-200 packing. In the biotransformation of nerol, the result also was similar to that described above. Consequently, it was clear that geraniol was biotransformed to citral-a, citral-b and nerol, and nerol to citral-a, citral-b and geraniol in the suspension cultures of Cannabis callus. But the biotransformation of geraniol to nerol or nerol to geraniol was observed only occasionally and was not found in a cell-free system of Cannabis callus.

On the basis of the fact that geraniol and nerol which are cannabinoid precursors were biotransformed to citral, we attempted to biotransform other alcohols such as *trans*-cinnamyl alcohol, 3-phenyl-1-propanol, citronellol, borneol, *l*-menthol, isophorol (3,5,5-trimethyl-2-cyclohexen-1-ol), *trans*-verbenol, *cis*-verbenol, α-ionol, β-ionol, 4-phenyl-3-buten-2-ol and linallol using Cannabis callus. The suspension cultures, originating from Cannabis callus cultured on Murashige and Skoog agar medium containing 1-naphthaleneacetic acid (NAA) and kinetin, were grown for a week in a 1 l Erlenmeyer flask containing the medium described above but without agar and the alcohols dissolved in a very small amount of ethanol. After a week, the cultured material was divided into the portions of cells and medium solution, and the biotransformed compounds were extracted with *n*-hexane from each portion. The products were obtained mostly from the medium solution. The products were identified by comparison of infrared (IR), PMR, MS and t_R in GLC with authentic samples. The results are shown in Table I. As can be seen from the table, the oxidation ability of alcohols using Cannabis callus was characterized by the oxidative activity for primary and secondary allylic alcohols, and *trans*-cinnamyl alcohol of many alcohols was biotransformed efficiently. But acyclic secondary allylic alcohols such as α-ionol, β-ionol and 4-phenyl-3-buten-2-ol were not biotransformed. On the other hand, in the biotransformation of geraniol and nerol, the

TABLE I. The Biotransformation of Alcohols by Suspension Cultures

		Sample	Recovery (%)	Product	Ratio of coversion (%)
Primary alcohol	Normal type	Citronellol	91±5	—	—
		3-Phenyl-1-propanol	96±5	3-Phenyl-1-propanal	+
	Allylic type	Geraniol	78±5	Citral	35.2
				Nerol	14.8
		Nerol	81±5	Citral	46.8
				Geraniol	8.2
		trans-Cinnamyl alcohol	98±5	Cinnamaldehyde	90.5
				Cinnamic acid[1)]	+
Secondary alcohol	Normal type	Borneol	90±5	—	—
		l-Menthol	93±5	—	—
	Cyclic allylic type	*trans*-Verbenol	86±5	Verbenone	55.9
		cis-Verbenol	93±5	Verbenone	61.7
				trans-Verbenol[a)]	26.5
		Isophorol	95±5	Isophorone	60.2
	Acyclic allylic type	α-Ionol	95±5	—	—
		β-Ionol	93±5	—	—
		4-Phenyl-3-buten-2-ol	98±5	—	—
Tertiary alcohol		Linallol	96±5	—	—

a) The respective compounds produced as artifacts in suspension cultures.

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recoveries were less than those in the biotransformation of other allylic alcohols. Although *cis*-verbenol was biotransformed to *trans*-verbenol and verbenone in the suspension cultures, the biotransformation of *cis*-verbenol to *trans*-verbenol was also observed in the control experiments. In the cell-free system (homogenized with Cannabis callus and Tris-HCl buffer: 0.2 M, pH 7.2), the biotransformation of *cis*-verbenol to *trans*-verbenol and verbenone was hardly observed. Consequently, it was evident that the biotransformation of *cis*-verbenol to verbenone was due to *trans*-verbenol which arose as an artifact of *cis*-verbenol in the suspension cultures.

This oxidation also has been possible to carry out in the cell-free system prepared by homogenization of Cannabis callus. As shown in Fig. 1, the oxidative activity was most strongly exhibited in a cell-free system prepared by the homogenization of the same amounts of Cannabis callus and McIlvaine type buffer solution[8] of a pH range of about 7. In addition, it was evident that the biotransformation rate of *trans*-cinnamyl alcohol was about 2—3 times as high as that of geraniol and that geraniol (*trans*) was biotransformed more efficiently to citral than its geometrical isomer, nerol (*cis*). This enzyme activity was unaffected by addition of 10 mM of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleo tide phosphate (NADP), and as shown in Table II, the amounts of cinnamaldehyde and hydrogen peroxide produced in the reaction of *trans*-cinnamyl alcohol and the cell-free solution were approximately equivalent after 60 min. On basis of the facts described above, the enzyme which catalyzed the oxidation of *trans*-cinnamyl alcohol and the other allylic alcohols was determined to be an alcohol oxidase.[9]

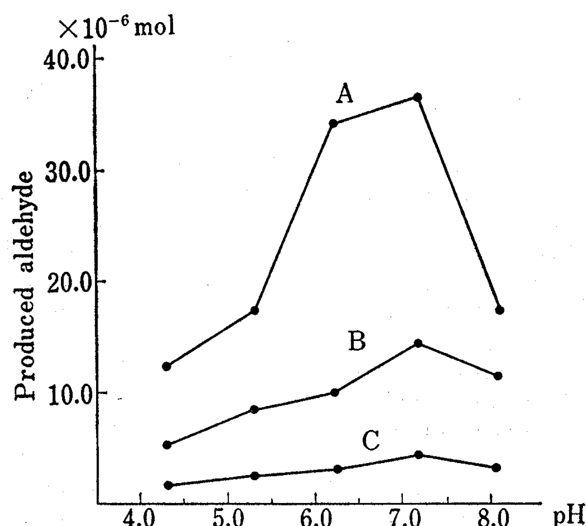

Fig. 1. Effect of pH for the Activity in Cell-free System

The cell-free solution was prepared by the homogenization of 20 g of callus and 20 ml of buffer solution.
The activity was measured by GLC after 30 min.
Buffer solution: McIlvaine type buffer solution.
A, *trans*-cinnamyl alcohol; B, geraniol; C, nerol.

TABLE II

	Cell-free solution	Hydrogen peroxide	Cinnamaldehyde
Experiment I	1.0 ml	16.5×10^{-9} mol	13.6×10^{-9} mol
Experiment II	1.0 ml	35.2×10^{-9} mol	31.4×10^{-9} mol

The reaction was carried pout in the cell-free solution at 25° for 60 min. Cannabis callus was added with Tris-HCl buffer (pH 7.2, 0.2 M) and was homogenized using blender. The cell and debris were removed by centrifugation at $1000\times g$ for 10 min, and the supernatant solution was used as the cell-free solution.
Experiment I: the cell-free solution was prepared from 10 g of callus and 40 ml of the buffer solution.
Experiment II: the cell-free solution was prepared from 20 g of callus and 40 ml of the buffer solution.
The determination of products was performed as described in experimental section.

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Discussion

The investigation of enzymes which catalyze the oxidation of alcohols in plants has been reported[10] and recently, the participation of alcohol dehydrogenase and alcohol oxidase has been discussed in the formation of leaf aldehyde in several plants.[9g,11] Banthorpe, *et al.*[9g] have speculated that alcohol oxidase may have significance for biosynthesis of terpenoids. We agree with the speculation on the basis of our biotransformation experiment. Investigations related to *trans-cis* isomerization of primary allylic alcohols have also been reported recently.[12] Although this *trans-cis* isomerization may be due to a redox system involving the corresponding aldehydes, alcohol oxidase also is thought to relate to the isomerization in a complex reaction system from the fact that this enzyme activity is characterized by the oxidation for allylic alcohols.

Experimental

Materials——Purified horseradish peroxidase, NAD and NADP were purchased from Sigma Chemical Co., U.S.A. Other chemicals were obtained from commercial sources and were used after further purification, when necessary. The aldehydes corresponding to allylic alcohols were prepared by oxidation using MnO_2.[7] (±)-Isophorol, (±)-α-ionol and (±)-β-ionol were prepared by reduction of the corresponding ketones using $LiAlH_4$. (±)-4-Phenyl-3-buten-2-ol was prepared from the ketone according to the method of Meerwein-Ponndorf-Verley Reduction.[13] *trans*-Verbenol, verbenone and *cis*-verbenol were prepared from α-pinene, $[\alpha]_D$ +14.59° (no solvent), according to the method of Whitham.[14] Olivetol and ethyl olivetolate were prepared from β-ketoester and diketene according to the method of Kato.[5] The respective materials proved to be more than 95% pure by GLC.

Tissue Cultures——Callus tissues were induced from seedlings of *Cannabis sativa* L. (Moraceae) on Murashige and Skoog basal medium.[15] The basal medium was supplied with the concentration of kinetin from 0.01 to 0.1 ppm, 1.0 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA, 3% of sucrose and 0.7% of agar. Callus tissues were subcultured in the same medium for callus induction at 3—5 week intervals, at 26° in the dark and have been subcultured for about 5 years.

Biotransformation of Cannabinoid Precursors and Related Alcohols——The biotransformation of cannabinoid precursors and related alcohols was carried out with suspension cultures. 250 ml of Murashige and Skoog basal medium containing 0.05 ppm of kinetin, 0.5 ppm of NAA and 3% of sucrose was added to each 1 l Erlenmeyer flask. The pH of the medium was adjusted to 5.6 and sterilized in an autoclave (2 atmospheric pressure, 15 min). Cannabinoid precursors or 0.5×10^{-3} mol of alcohols dissolved in 0.3 ml of ethanol (70%) and about 100 g of Cannabis callus were transferred to the basal medium under sterile conditions. The flask was incubated for a week at 26° in the dark. After incubation, the cultured material was divided into the cells and medium solution using a Büchner funnel. From the respective portions, the biotransformed principles were extracted with *n*-hexane or chloroform. The controls were carried out under the same conditions except for the use of Cannabis callus deactivated by autoclaving (2 atm., 5 min). The cell-free system was prepared by homogenization of the same amounts of Cannabis callus and Tris-HCl buffer (pH 7.2, 0.2 M) in an ice bath for 1—2 min using blender, and biotransformation of alcohol was carried out by incubation of the mixture of alcohol and cell-free solution at 26° for 15—20 hr. After incubation, the biotransformed products were isolated from the *n*-hexane extract by column chromatography on alumina.

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(I) Biotransformation of Cannabinoid Precursors: 0.25 or 0.5×10^{-3} mol of olivetol or ethyl olivetolate and the same amounts of geraniol or nerol were used as cannabinoid precursors. The detection of cannabinoids was carried out using GLC and TLC. The conditions of GLC were as follows; column: 1.5% SE-30, 1.5 m, carrier gas: N_2, 55 ml/min, column temp.: 205°. The conditions of TLC were as follows; support: silica gel G. solvent: benzene, color reagent: Fast blue B[16] or I_2.

(II) Biotransformation of Geraniol: In GLC of the extract, 3 peaks (peak I: 8.0 min, peak II: 10.6 min, peak III: 12.2 min) were observed under the following conditions; column: 25% PEG-6000, 2 m, carrier gas: N_2, 30 ml/min, column temp.: 175°. The compounds were identified as follows; peak I: citral (*m/e* 152 (M^+), 69 (base peak)), peak II: nerol (*m/e* 154 (M^+), 69 (base peak)), peak III: geraniol (*m/e* 154 (M^+), 69 (base peak)) by comparing with GLC or GLC–MS of the authentic samples. Furthermore, citral was isolated by silica gel column chromatography and the PMR spectra were measured, $CDCl_3$ (δ) ppm, citral-a: 1.63, 1.70 (s, respectively, $\genfrac{}{}{0pt}{}{CH_3}{CH_3}\!>$C=C), 2.20 (d, $J=1.5$ Hz, CH_3–C=C), 5.07 (br, m, $\genfrac{}{}{0pt}{}{Me}{Me}\!>$C=C–H), 5.86 (d, $J=8.0$ Hz, C=C$<\genfrac{}{}{0pt}{}{CHO}{H}$), 9.98 (d, $J=8.0$ Hz, CHO), citral-b: 1.61, 1.70 (s, respectively, $\genfrac{}{}{0pt}{}{CH_3}{CH_3}\!>$C=C), 2.00 (d, $J=1.5$ Hz, CH_3–C=C), 5.11 (br, m, $\genfrac{}{}{0pt}{}{Me}{Me}\!>$C=C–H), 5.87 (d, $J=8.0$ Hz, C=C$<\genfrac{}{}{0pt}{}{H}{CHO}$), 9.90 (d, $J=8.0$ Hz, CHO). Citral-a and citral-b were separated and identified under the conditions of GLC as follows; column: 15% Silicon DC-200, 1.5 m, carrier gas: N_2, 35 ml/min, column temp: 140°, t_R: 8.3 min (citral-a), 9.5 min (citral-b).

(III) Biotransformation of Nerol: The data of the extract were consistent with those in the biotransformation of geraniol.

(IV) Biotransformation of *trans*-Cinnamyl Alcohol: The extract provided a new peak (11.8 min) under the conditions of GLC as follows; column: 25% PEG-6000, 2 m, carrier gas: N_2, 30 ml/min, column temp.: 200°. The compound was identified as *trans*-cinnamaldehyde (*m/e* 132 (+), 131 (Mbase peak)) by comparing with GLC and GLC–MS of authentic sample. But the presence of 5—15% of *cis*-cinnamaldehyde was pointed out by the PMR spectrum. The PMR spectra ($CDCl_3$ (δ) ppm) of obtained aldehydes were as follows; *trans*-cinnamaldehyde: 6.63 (dd, $J=15.5$, 7.5 Hz, C=C$<\genfrac{}{}{0pt}{}{CHO}{H}$), 7.42 (d, $J=15.5$ Hz, $\genfrac{}{}{0pt}{}{Ph}{H}\!>$C=C), 7.43 (aromatic protons), 9.63 (d, $J=7.5$ Hz, CHO), *cis*-cinnamaldehyde: 6.12 (dd, $J=12.0$, 8.0 Hz, C=C$<\genfrac{}{}{0pt}{}{H}{CHO}$), 7.35 (d, $J=12.0$ Hz, $\genfrac{}{}{0pt}{}{H}{Ph}\!>$C=C), 9.88 (d, $J=8.0$ Hz, CHO).

(V) Biotransformation of *trans*-Verbenol: The extract provided two peaks (peak I: 7.6 min, peak II: 9.2 min) under the conditions of GLC as follows; column: 25% PEG-6000, 2 m, carrier gas: N_2, 40 ml/min, column temp.: 160°. The respective compounds were identified as *trans*-verbenol (peak I: *m/e* 152 (M^+), 109 (base peak)) and verbenone (peak II: *m/e* 150 (M^+), 135, 107, 91) by comparing with GLC and GLC–MS of authentic samples.

(VI) Biotransformation of *cis*-Verbenol: The extract provided two new peaks in GLC. The data of extract were consistent with those in the biotransformation of *trans*-verbenol.

(VII) Biotransformation of Isophorol: The extract provided a new peak (6.6 min) under the conditions of GLC as follows; column: 25% PEG-6000, 2 m, carrier gas: N_2, 30 ml/min, column temp.: 170°. The compound was identified as isophorone (*m/e* 138 (M^+)) by comparing with GLC and GLC–MS of authentic sample.

(VIII) Biotransformation of Other Alcohols: The new compounds which were biotransformed from citronellol, 3-phenyl-1-propanol, *l*-menthol, α-ionol, β-ionol or 4-phenyl-3-buten-2-ol, were not detected by GLC and TLC.

(IX) Quantitative Analysis of the Products: Under the conditions of GLC described above, the amounts of products were determined using *l*-menthol as an internal standard in the biotransformation of geraniol, nerol, *trans*-verbenol and *cis*-verbenol, thymol in the case of *trans*-cinnamyl alcohol, and naphthalene in the case of isophorol.

Quantitative Analysis of Cinnamaldehyde and Hydrogen Peroxide produced in Cell-free Solution——All spectrophotometric measurements were made with a Shimadzu UV-210. After the homogenization of a proper amount of Cannabis callus and Tris-HCl buffer (pH 7.2, 0.2 M), the cell and debris were removed by centrifugation at $1000 \times g$ for 10 min, and the supernatant solution was used as the cell-free solution. Cinnamaldehyde was determined by spectrophotometric measurement of the complex formed between cinnamaldehyde and 2,4-dinitrophenylhydrazine according to the method of Ariga.[17] The cell-free solution (1.0 ml) was added to *trans*-cinnamyl alcohol (10 μmol—0.5 ml, small amounts of Tween 80 were added as the solubilizer) and after incubation (60 min), 2,4-dinitrophenylhydrazine (5 μmol—1.0 ml, dissolved in 4 N HCl) was added to the reactant and after 30 min, 5.0 ml of NaOH solution (96 g/l) was added and the color developed was measured at 500 nm after 5—10 min. Hydrogen peroxide was analysed spectrophotometrical-

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ly by the ordinary method using peroxidase and *o*-dianisidine.[9c] The cell-free solution (1.0 ml) was added to horseradish peroxidase (100 μg), *o*-dianisidine (100 μg) and *trans*-cinnamyl alcohol (10 μmol) in Tris-HCl buffer (pH 7.2, 0.2 M, 2.0 ml). After incubation (60 min), the reaction was stopped by addition of HCl (4 N, 0.2 ml) and the color developed was measured at 400 nm.

Acknowledgement The authors are grateful to Dr. M. Akasu for his valuable advice.

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