[Chem. Pharm. Bull. 25(8)1947—1951(1977)]

UDC 547.361.04 : 542.98 : 581.192

Stereochemistry in Oxidation of Allylic Alcohols by Cell-free System of Callus induced from *Cannabis sativa* L.

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(Received December 3, 1976)

In the cell-free system of callus induced from *Cannabis sativa* L. (Moraceae), the pro-R hydrogen from C-1 methylene of primary allylic alcohols such as *trans*-cinnamyl alcohol and geraniol was abstracted. In an example of secondary allylic alcohols biotransformations, S-(−)-isophorol of the racemate was biotransformed to isophorone, and (+)-*trans*-verbenol of four isomers, such as (+)- and (−)-*trans*-verbenol, and (+)- and (−)-*cis*-verbenol, was preferentially biotransformed to (+)-verbenone. We reported previously that the enzyme which catalyzed the oxidation of these allylic alcohols in the cell-free system of Cannabis callus was an alcohol oxidase.

Keywords——*Cannabis sativa* L.; Moraceae; callus; cell-free system; bio-oxidation of allylic alcohols; stereochemistry; pro-R; enantiomer differentiating reaction; alcohol oxidase

In a previous paper,[2] we reported that primary and secondary allylic alcohols such as geraniol, nerol, *trans*-cinnamyl alcohol, isophorol, and *trans*-verbenol were biotransformed to the corresponding aldehydes in suspension cultures using the callus[3] induced from *Cannabis sativa* L. (Moraceae). Furthermore, we clarified that the enzyme which catalyzed the oxidation of allylic alcohols in the cell-free system of Cannabis callus was an alcohol oxidase.[2] Although the stereochemistry in connection with oxidation-reduction of alcohols using alcohol dehydrogenase has been widely investigated,[4] the stereochemistry in oxidation of allylic alcohols using alcohol oxidase still has not been reported. Therefore, we investigated the stereochemistry in oxidation of allylic alcohols using Cannabis callus.

We carried out experiments on stereochemistry to distinguish C-1 enantiomeric hydrogens of primary allylic alcohols such as *trans*-cinnamyl alcohol (I) and geraniol (II).[5] (*S*)-*trans*-cinnamyl alcohol-1-D and (*S*)-geraniol-1-D were prepared by reduction of the corresponding aldehydes with isobornyloxymagnesium-2-D bromide according to the method of Streitwieser.[6] The prepared (*S*)-alcohols-1-D were incubated with the cell-free system of Cannabis callus for 4 hr at 26°. After incubation, the biotransformed aldehydes were isolated chromatographically from the *n*-hexane extract of incubated filtrate. We observed that in the resulting aldehydes, the aldehydes containing deuterium were found to be in higher amounts than the normal aldehydes by the proton magnetic resonance (PMR) spectra. Consequently, as shown in Fig. 1, it was proved that the pro-R hydrogen from C-1 methylene of primary allylic alcohols such as *trans*-cinnamyl alcohol and geraniol is abstracted in the cell-free system of Cannabis callus.

Next, we carried out experiments on the stereochemistry of oxidation products of secondary allylic alcohols such as isophorol (3,5,5-trimethyl-2-cyclohexen-1-ol) and verbenols (VI, VIII) using Cannabis callus. (±)-Isophorol was incubated with a cell-free system of Cannabis

1) Location: *1432-1, Horinouchi, Hachioji, Tokyo.*
2) H. Itokawa, K. Takeya, and S. Mihashi, *Chem. Pharm. Bull.* (Tokyo), **25**, 1941 (1977).
3) H. Itokawa, K. Takeya, and M. Akasu, *Shoyakugaku Zasshi*, **29**, 106 (1975).
4) R. Bentley, "Molecular Asymmetar in Biolgy," Voll. II, Academic Press, New York, 1970.
5) H. Itokawa, K. Takeya, and M. Akasu, *Chem. Pharm. Bull.* (Tokyo), **24**, 1681 (1976).
6) A. Streitwieser Jr., J.R. Wolfe Jr., and W.D. Shaeffer, *Tetrahedron*, **6**, 338 (1959).

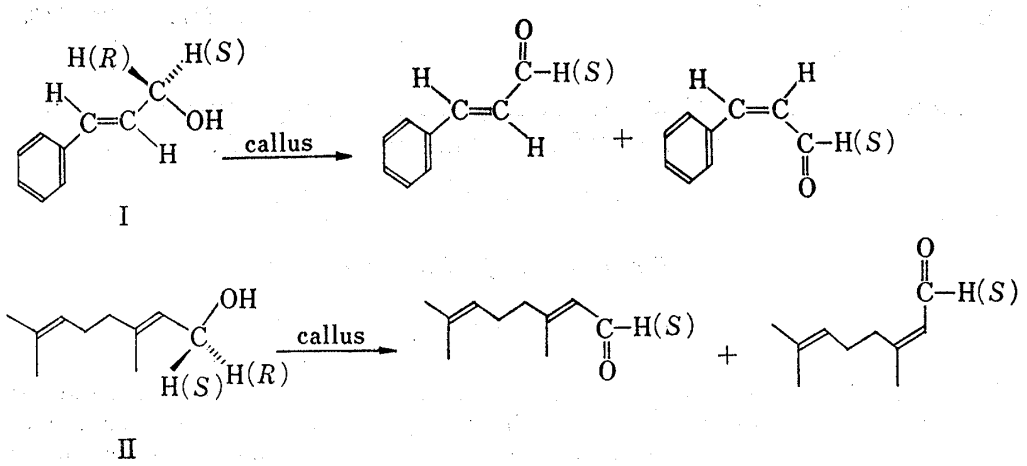

Fig. 1. The Stereochemistry of Primary Allylic Alcohols Oxidized by a Cell-free System of Callus induced from *Cannabis sativa* L.

callus for 15 hr at 26°. After incubation, the residual isophorol and biotransformed isophorone were isolated chromatographically from *n*-hexane extract of incubated filtrate. The specific rotation of residual isophorol showed $[\alpha]_D+14°$. Since the optical purity of residual isophorol was not determined satisfactorily by the PMR spectra of diastereomer-ester of isophorol and ketopinic acid[7] or chiral shift reagent[8] of tris[3-(trifluoromethylhydroxymethylene)-*d*-camphorate]europium (III),[9] the specific rotation was calculated from amounts of optically pure (+)-isophorol which was calculated from the produced isophorone/the residual isophorol ratio[10] on the assumption that the enantiomer differentiating reaction[11] proceeded completely. This isophorol was derived to benzoate (III) in the usual way. We applied the

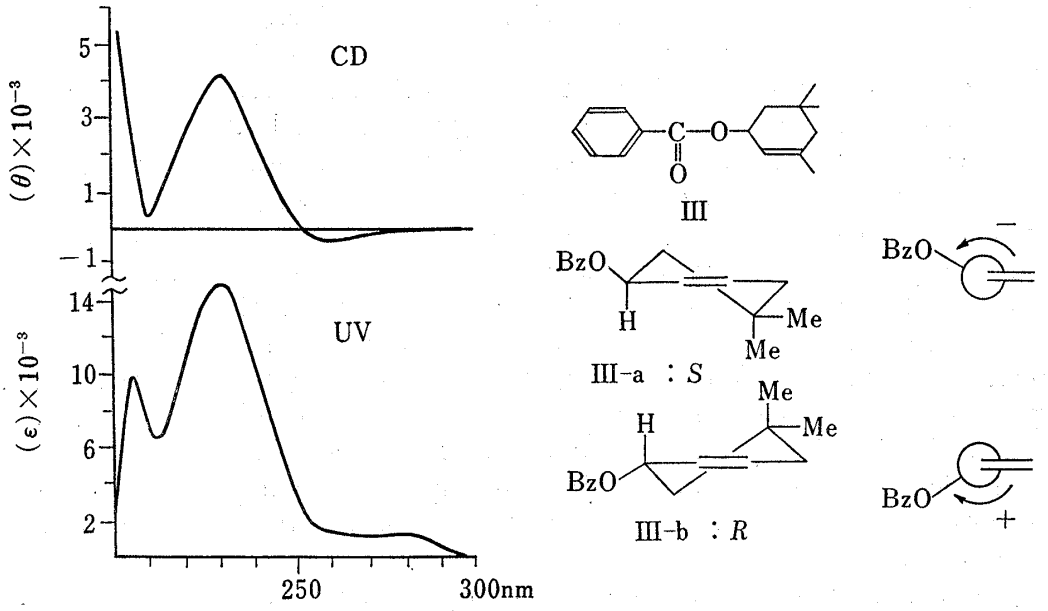

Fig. 2. The CD and UV Spectra of Isophorol-benzoate

7) P.D. Bartlett and L.H. Knox, "Org. Syntheses," Coll. Vol. **5**, 689, (1973).
8) R.E. Siever, "Nuclear Magnetic Resonance Shift Reagents," Academic Press, New York, 1973, p. 87.
9) H.L. Goering, J.N. Eikenberry, and G.S. Koermer, *J. Am. Chem. Soc.*, **93**, 5913 (1971).
10) The ratio was determined from the relative peak areas under the conditions of GLC as follows; column: 25% PEG-6000, 2 m, carrier gas: N_2, 30 ml/min, column temp.: 170°.
11) Y. Izumi, *Angew. Chem. Int. Ed. Engl.*, **10**, 871 (1971). The Chemical Society of Japan, "Kagaku Sosetsu," No. 4, University of Tokyo Press, Tokyo, 1974.

exciton chirality method[12] for determining the absolute configuration of (+)-isophorol. As shown in Fig. 2, the circular dichroism (CD) spectrum of benzoate shows a split type Cotton effect due to interaction between the benzoate and ene chromophore. The positive first Cotton effect, $[\theta]^{30}$ (nm): +4100 (232), indicates a positive chirality (III-b) between two axes of electric transition moments. The absolute configuration of (+)-isophorol is hence an R configuration. Consequently, it was evident that *S*-(−)-isophorol of the racemate was biotransformed to isophorone in the cell-free system of Cannabis callus.

Four stereoisomers of verbenols were also investigated as examples of secondary allylic alcohols for stereoisomer differentiating reaction. Verbenols were prepared chemically from commercial α-pinene (V), $[\alpha]_D$ +14.59°, or β-pinene (IV), $[\alpha]_D$ −21.14°, according to the method of Whitham[13] and the specific rotation is shown in Fig. 3. It was calculated from the

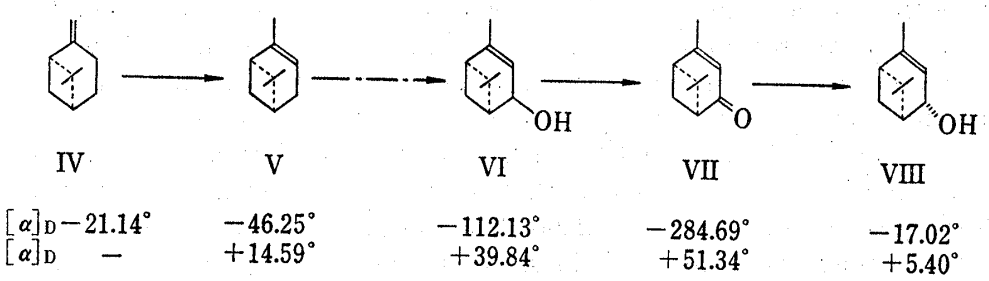

Fig. 3. The Specific Rotation of Pinene-derivatives prepared chemically

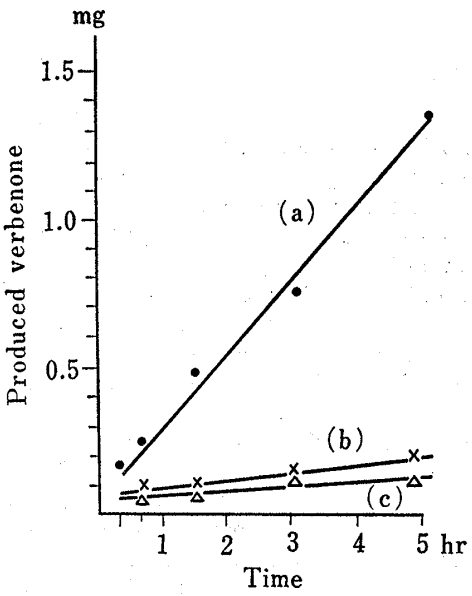

Fig. 4. The Biotransformation of Verbenols by Cannabis callus

(a) *trans*-verbenol prepared from (+)-α-pinene.
(b) *cis*-verbenol prepared from (+)-α-pinene.
(c) *trans*-verbenol prepared from (−)-β-pinene.
Sample 9 mg/Cannabis callus 30 g.

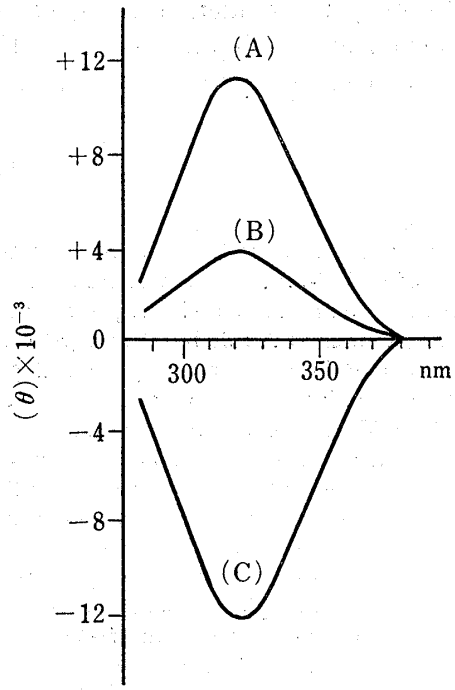

Fig. 5. The CD Spectra of Verbenones prepared biochemically and chemically

(A) the CD curve converted (+)-VI into VII by oxidation with callus.
(B) the CD curve converted (+)-VI into VII by chemical oxidation.
(C) the CD curve converted (−)-VI into VII by chemical oxidation.

12) N. Harada and K. Nakanishi, *Accounts Chem. Res.*, **5**, 257 (1972).
13) G.H. Whitham, *J. Chem. Soc.*, **1961**, 2232; *cf*) M.A. Cooper, J.R. Salmon, D. Whittaker, and U. Scheidegger, *J. Chem. Soc.* (*B*), **1967**, 1259.

specific rotation of optically pure α-pinene[14] and β-pinene[15] that the α-pinene consisted of 64% of (+)- and 36% of (−)-compound, and the β-pinene of (+)-compound 3% and (−)-compound 97%. Further, it has been reported[16] that (−)-β-pinene has the configuration 1*S*: 5*S*. The biotransformation of prepared verbenols was carried out in the cell-free system of Cannabis callus for 15 hr at 26°. As can be seen from Fig. 4, (+)-*trans*-verbenol (VI) prepared from α-pinene was rapidly biotransformed to verbenone (VII) by Cannabis callus, but (−)-*trans*-verbenol prepared from β-pinene, and (+)-*cis*-verbenol (VIII) prepared from α-pinene, were hardly biotransformed. From the fact that almost optically pure (−)-*trans*-verbenol was hardly biotransformed and (+)-*trans*-verbenol consisted of 64% of (+)- and 36% of (−)-compound was biotransformed, it can be presumed that only (+)-*trans*-verbenol of the racemate will be biotransformed. On the other hand, both (+)- and (−)-*cis*-verbenol cannot be biotransformed, since (+)-*cis*-verbenol consisted of 64% of (+)- and 36% of (−)-compound was not biotransformed. On basis of the matter described above, the CD curve of verbenone biotransformed from (+)-*trans*-verbenol by Cannabis callus was compared with that of verbenone prepared chemically[17] from (+)-*trans*-verbenol used above and (−)-*trans*-verbenol. As shown in Fig. 5, the CD curve of verbenone biotransformed shows $[\theta]^{25}$ (nm): +11000 (319) (positive maximum) and that of verbenone prepared chemically, $[\theta]^{25}$ (nm): +4100 (319) (positive max.) and −12500 (319) (negative max.). The individual samples were measured under the same conditions. Consequently, in view of the fact that *trans*-verbenol consisted of 64% of (+)- and 36% of (−)-compound was rapidly biotransformed to (+)-verbenone of good optical purity by Cannabis callus, though *cis*-verbenol was hardly biotransformed, it is evident that (+)-*trans*-verbenol of four isomers such as (+)- and (−)-*trans*-verbenol, and (+)- and (−)-*cis*-verbenol, is preferentially biotransformed to (+)-verbenone in the cell-free system of Cannabis callus.

Experimental

PMR spectra were recorded on a Varian S-60T or JEOL JNM-PS-100. UV measurements were made with a Shimadzu UV-210. CD measurements were performed on a Jasco model J-20 spectropolarimeter. Optical rotation was measured with a Jasco model DIP-SL automatic polarimeter.

(*S*)-*trans*-Cinnamyl Alcohol-1-D and (*S*)-Geraniol-1-D[6]——Reduction of 13 g of (+)-D-camphor with 1 g of lithium aluminum deuteride gave 12.5 g of (−)-deuterioisoborneol (90% in GLC). This material (3 g) was converted into the alkoxymagnesium salt by addition to the Grignard reagent prepared from 2.8 g of *n*-butyl bromide and 0.48 g of magnesium. Benzene (100 ml) was added and the ether was distilled off. After cooling, 5.4 g of *trans*-cinnamaldehyde was added and stirred overnight at room temperature. Next, water was added and extracted with ether. 2.2 g of (*S*)-*trans*-cinnamyl alcohol-1-D as crude product was isolated from the ether extract by column chromatography on alumina. In a similar manner, 2.3 g of (*S*)-geraniol-1-D was prepared by reduction of 4 g of citral-a with 19.4×10^{-3} mol of isobornyloxymagnesium-2-D bromide. The deuterium contents of prepared alcohols were respectively over 98% from the PMR spectra.

Isophorol——(±)-Isophorol was prepared from isophorone by reduction using lithium aluminum hydride.

***trans*-Verbenol, Verbenone and *cis*-Verbenol**——These were prepared from β-pinene, $[\alpha]_D$ −21.14° (no solvent), and α-pinene, $[\alpha]_D$ +14.59° (no solvent). β-Pinene (20 g) was isomerized by stirring with 5% palladised charcoal (3.5 g) under hydrogen until 250 ml had been absorbed.[18] Fractional distillation gave α-pinene (18.2 g). *trans*-Verbenol (3.2 g) was prepared from α-pinene (5.5 g) by oxidation of lead tetra-acetate (18 g), isomerization in glacial acetic acid (25 ml) for 30 min at room temperature and hydrolysis with 10% of KOH solution (H_2O–MeOH, 1: 1).[13] The prepared *trans*-verbenol contained 5% of *cis*-verbenol by GLC. Verbenone (380 mg) was prepared by oxidation[17] of *trans*-verbenol (500 mg). *cis*-Verbenol (850 mg) was prepared by reduction of verbenone (1 g) using sodium borohydride (0.3 g). The prepared *cis*-verbenol contained 10% of *trans*-verbenol by GLC. The specific rotation of products is shown in Fig. 3.

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15) G. Dupont, *Ann. Chim.*, [X] **1**, 184 (1924).
16) D.V. Banthorpe and D. Whittaker, *Chem. Rev.*, **66**, 643 (1966).
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Tissue Cultures——Cannabis callus was induced from seedling of *Cannabis sativa* L. (Moraceae) on Murashige and Skoog medium containing 0.7% of agar, 3.0% of sucrose, 0.1 ppm of kinetin and 1.0 ppm of 2,4-dichlorophenoxyacetic acid.[3] Cannabis callus has been subcultured at 3—5 week intervals for about 5 years.

Biotransformation of Allylic Alcohols——Biotransformation of alcohol was carried out at 26° by incubation of the mixture of alcohol and cell-free solution prepared by homogenization of the same amounts of Cannabis callus and Tris-HCl buffer (pH 7.2, 0.2 M) at 0° for 1—2 min by a blender. After incubation, the biotransformed products were isolated from the *n*-hexane extract by column chromatography on alumina.

Biotransformation of (*S*)-*trans*-cinnamyl alcohol-1-D (470 mg) was carried out for 4 hr by 600 g of Cannabis callus. The biotransformed cinnamaldehyde was obtained in a 45% yield. These data were reported previously.[5]

Biotransformation of (*S*)-geraniol-1-D (200 mg) was carried out for 4 hr by 720 g of Cannabis callus. The biotransformed citral was obtained in a 65% yield. These data were reported previously.[5]

Biotransformation of (+)-isophorol (400 mg) was carried out for 15 hr by 720 g of Cannabis callus. The biotransformed isophorone was obtained in a 45% yield. The specific rotation of residual isophorol showed $[\alpha]_D$ +14.35° (c=1.2, *n*-hexane) when the optically pure isophorol was calculated from the yield. The benzoate (III) was prepared from the residual isophorol (300 mg) by esterification using benzoyl chloride (450 mg) in a solution consisting of pyridine (5 mg) and chloroform (5 ml). The CD curve of benzoate showed $[\theta]^{30}$ (nm): +4100 (232) (c=0.12, methanol) (positive max.) and +80 (213) (negative max.).

Biotransformation of *trans*-verbenol (222 mg), $[\alpha]_D$ +39.84° (c=1.23, chloroform), prepared from α-pinene was carried out for 15 hr by 600 g of Cannabis callus. The biotransformed verbenone was obtained in a 38% yield. The specific rotation of biotransformed verbenone showed $[\alpha]_D$ +226.14° (c=0.48, ethanol) and that of residual *trans*-verbenol, $[\alpha]_D$ −23.19° (c=0.35, chloroform). The CD curve of biotransformed verbenone showed $[\theta]^{25}$ (nm): +11000 (319) (c=0.48, ethanol) (positive max.) and that of verbenone prepared from α-pinene chemically, $[\theta]^{25}$ (nm): +4100 (319) (c=0.45, ethanol) (positive max.) and that of verbenone prepared from β-pinene chemically, $[\theta]^{25}$ (nm): −12500 (319) (c=0.42, ethanol) (negative max.). The detection of biotransformed product was carried out under the conditions of GLC as follows; column: 25% PEG-6000, 2 m, carrier gas: N_2, 40 ml/min, column temp.: 160°.

Acknowledgement We thank Professor T. Furuya, School of Pharmaceutical Science, Kitasato University, Japan, for CD spectral measurements.