

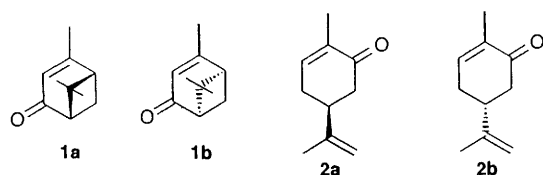
Novel reductase participation in the *syn*-addition of hydrogen to the C=C bond of enones in the cultured cells of *Nicotiana tabacum*

Kei Shimoda, Diana I. Ito, Shunsuke Izumi and Toshifumi Hirata *

Department of Chemistry, Faculty of Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739, Japan

A reductase isolated from cultured cells of *Nicotiana tabacum* has been characterized and used in the reduction of a C=C bond adjacent to a carbonyl group. The stereochemistry of the latter reaction has been investigated by ²H NMR and mass spectroscopy. It was found that the reductase reduces stereospecifically the C=C bond of verbenone and carvone by *syn* addition of hydrogen from the *re* face at the β-position and the *re* face at the α-position to the carbonyl group; the hydrogen atoms participating in the enzymatic reduction at the α- and β-positions originate from the medium (H₂O) and the *pro*-4S hydrogen of NADPH, respectively.

Although much work has been carried out on the enzymatic reduction of the C=C bond of α,β-unsaturated ketones with reductases from microorganisms¹⁻³ and animals,^{4,5} there have been few investigations of the isolation and characterization of enone reductases from plant cells. In studying the biotransformation of exogenous substrates by the *Nicotiana tabacum* cell cultures, we found that the cells are able to reduce the C=C bond



adjacent to the carbonyl group of enones such as verbenone **1** and carvone **2**.⁶⁻⁸ A reductase responsible for the stereochemical reduction of carvone **2** and isolated from the cultured cells of *N. tabacum*⁹ was reported to reduce the C=C bond of carvone **2**, but not that of verbenone **1**.¹⁰ We have reported in preliminary form¹¹ that the reductase responsible for the reduction of the C=C bond of verbenone **1** is different from many other enone reductases,^{9,12-14} and operates in a manner having unique stereochemical features. We now demonstrate in detail the isolation and characterization of this reductase and the stereochemistry in the reduction of the C=C bond of enones.

Results and discussion

Isolation and characterization of the verbenone reductase

A soluble cell-free extract was obtained from the *N. tabacum* cell cultures and then treated with ammonium sulfate (40–80% sat'd). The crude enzyme preparation was subjected to chromatography on a Sephadex G-25 column and a Diethylaminoethyl (DEAE) Toyopearl column (see Fig. 1). The activity of the enzyme was assayed at pH 7.2 using verbenone **1** as the substrate in the presence of NADPH. The active fraction for the reduction of verbenone **1** was different from that for the reduction of carvone **2**.^{9,10} in the elution pattern on the DEAE-Toyopearl column. The active fraction was further subjected to a Red Toyopearl column to give a pure enzyme preparation (see Table 1). The purified enzyme preparation was homogeneous as judged by the presence of a single protein band on the SDS gel electrophoresis.

The molecular weight (*M_r*) of the enzyme was estimated as *ca.*

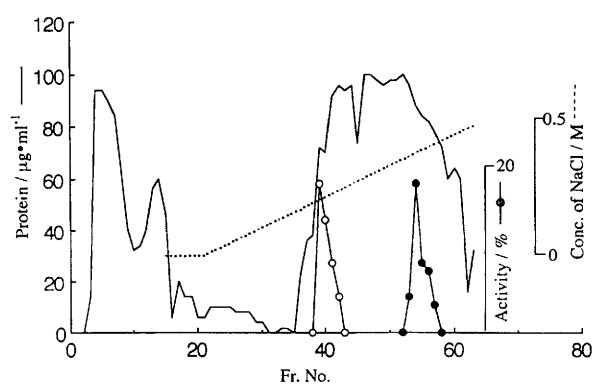


Fig. 1 Purification of the reductases from the cultured cells of *N. tabacum* by DEAE-Toyopearl column chromatography, ●—: activity for verbenone reductase, ○—: activity for carvone reductase.^{9,10}

80–90 kDa by gel filtration on a Sephadex G-150 column. The TOF mass spectrum of the enzyme preparation showed signals at *m/z* 44 500 and 89 000. The SDS gel electrophoresis of the enzyme preparation showed a single band at *ca.* 45 kDa. These observations indicate that the enzyme is composed of two identical subunits. The enzyme had a pH optimum at 7.2 with half maximal activity at 6.5 and 8.0 in 3-morpholinopropane-sulfonic acid–NaOH buffer. The enzyme required NADH and NADPH as coenzymes, but NADPH was a better donor of hydride ion by a factor of 6.7 as compared to NADH. FAD and FMN were ineffective for the enzyme activity.

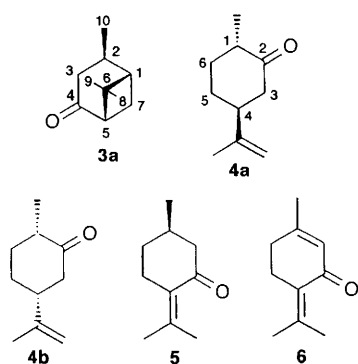
The substrate specificities of the enzyme were investigated by use of several enones as the substrates (see Table 2). Although the enzyme reduced enantioselectively the C=C bond of (1*S*,5*S*)-verbenone **1a** to give (1*S*,2*R*,5*S*)-*cis*-verbanone **3a**, that of (1*R*,5*R*)-verbenone **1b** was not reduced. Similarly, whilst reduction of cyclohex-2-enone to give cyclohexanone was highly effective, that of 3-methylcyclohex-2-enone and 2-methylcyclohex-2-enone was not. With carvone **2** as the substrate, the *S*-enantiomer **2a** was reduced enantioselectively to yield (1*S*,4*S*)-dihydrocarvone **4a**, although the conversion was lower than that of verbenone **1a**. These observations indicate that the enzyme is clearly different from the carvone reductase isolated from the cultured cells of *N. tabacum*.^{9,10} The reductase isolated here may be different from the enone reductase from the leaves of *M. piperita*,¹⁵ because the latter reduces 3-oxo *p*-menthane derivatives such as piperitenone **6** but not 2-oxo derivatives such as carvone **2**.¹⁵

Table 1 Purification of the reductase from the cultured cells of *N. tabacum*

	Total protein (mg)	Total reductase (units × 10 ³)	Sp. act. (units/mg pro.)	Fold	Yield (%)
Crude extract	280	64	0.23	1	100
DEAE-Toyopearl	17	57	3.25	14	89
AF-Red Toyopearl	0.064	6	94	409	9

Table 2 Activities of the reductase towards various enones

Substrate	Conversion (%)	Product
(1 <i>S</i> ,5 <i>S</i>)-Verbenone 1a	25	(1 <i>S</i> ,2 <i>R</i> ,5 <i>S</i>)-Verbanone 3a
(1 <i>R</i> ,5 <i>R</i>)-Verbenone 1b	0	—
(<i>S</i>)-Carvone 2a	12	Dihydrocarvone 4a
(<i>R</i>)-Carvone 2b	2	Isodihydrocarvone 4b
(<i>R</i>)-Pulegone 5	0	—
Cyclohex-2-enone	68	Cyclohexanone
2-Methylcyclohex-2-enone	2	2-Methylcyclohexanone
3-Methylcyclohex-2-enone	2	3-Methylcyclohexanone



Stereochemistry in the reduction of the C=C bond of enones

That (1*S*,2*R*,5*S*)-*cis*-verbanone **3a** was produced by the enzymatic reduction of (1*S*,5*S*)-verbenone **1a** indicates that the hydrogen attack to the conjugated C=C double bond takes place stereospecifically from the *re*-face at C-2. However, the stereochemistry of the hydrogen attack at C-3 of **1a** has yet to be elucidated. To complete the stereochemical studies on the reduction of the C=C double bond with the enzyme preparation, we investigated the stereochemistry of the hydrogen attack on **1a** by performing the following four experiments, and examined the orientation of the deuterium in the resultant product. (1*S*,5*S*)-Verbenone **1a** was incubated with the enzyme preparation in the presence of: (a) NADPH in H₂O; (b) NADPH in ²H₂O; (c) (4*S*)-[4-²H]-NADPH in H₂O and (d) (4*R*)-[4-²H]-NADPH in H₂O. The labelling pattern of deuterium and the deuterium contents in the resulting *cis*-verbanone **3a** were determined by NMR and mass spectroscopy. *cis*-Verbanone **3a** produced in the incubation in the presence of NADPH in ²H₂O [experiment (b)] showed a peak at *m/z* 153 in the mass spectrum, *i.e.* one mass unit higher than the molecular ion peak (*m/z* 152) of *cis*-verbanone **3a** produced in the control experiment (a). A fragment ion peak at *m/z* 138 due to expulsion of a methyl group was also one mass unit higher compared with the fragment peak observed for verbanone in the control experiment (a). This indicates that the deuterium atom originating from ²H₂O is incorporated into *cis*-verbanone. On the other hand, the mass spectrum of *cis*-verbanone **3a** produced when (4*S*)-[4-²H]-NADPH was included in the incubation mixture [experiment (c)] showed a peak at *m/z* 153 [M + 1]⁺. However, when the (4*S*)-[4-²H]-NADPH was replaced by (4*R*)-[4-²H]-NADPH [experiment (d)], no deuteriation of *cis*-verbanone **3a** was observed. These observations indicate that only the *pro*-4*S* hydrogen of

NADPH is incorporated into *cis*-verbanone **3a** during the enzymatic reduction of the double bond. The labelled sites in the deuterium-labelled *cis*-verbanone were determined from their ¹H and ²H NMR spectra. The ¹H NMR spectrum of *cis*-verbanone produced in experiment (b) had no signal at δ 2.87 for 3-H *cis*-orientated to the 2-H as seen in the spectrum of standard *cis*-verbanone. The deuteriated site of the *cis*-verbanone was confirmed by ²H {¹H} NMR spectroscopy. The spectrum of *cis*-verbanone produced in experiment (b) showed only a signal at δ 2.87 (99% enrichment) due to the C(3)-²H with a *cis*-orientation to the C(2)-H. On the other hand, verbanone produced in experiment (c) exhibited a signal at δ 2.38 due to C(2)-H in the ²H NMR spectrum; the ²H-enrichment factor at the labelled sites was 99%. These observations indicate that the deuterium atoms at C-2 and C-3 of the deuteriated *cis*-verbanone originate from (4*S*)-[4-²H]-NADPH and ²H₂O, respectively, and that the reduction occurs stereospecifically by *syn*-addition of hydrogen atoms to the *re-re* face of the C=C bond.

However, the occurrence of such a *syn*-addition might be due to the bulkiness of the gem-dimethyl bridge of verbenone **1**. To confirm the stereochemistry of the enzymatic reduction, therefore, similar experiments to those described above were performed using (*S*)-carvone **2a** as substrate. Dihydrocarvone **4a** produced in the incubation in the presence of NADPH in ²H₂O showed a peak at *m/z* 153 in the mass spectrum, *i.e.* one mass unit higher than the molecular ion peak (*m/z* 152) of dihydrocarvone **4a** produced in the control experiment. This indicates that the deuterium atom originating from ²H₂O is incorporated into dihydrocarvone. On the other hand, the mass spectrum of dihydrocarvone produced when (4*S*)-[4-²H]-NADPH was present in the incubation mixture showed a peak at *m/z* 153 [M + 1]⁺. However, when (4*R*)-[4-²H]-NADPH was used, no deuteriation of dihydrocarvone was observed. The deuteriation sites of dihydrocarvone **4a** produced in the enzymatic reductions were determined from ²H NMR spectroscopy. The spectrum of dihydrocarvone **4a** produced in the presence of NADPH in ²H₂O showed only a signal at δ 2.37 (99% enrichment) due to the ²H at C-1. However, dihydrocarvone produced in the presence of (4*S*)-[4-²H]-NADPH exhibited a signal at δ 2.13 (96% enrichment) due to the equatorial ²H at C-6. These observations indicate that reduction of carvone **2** with the reductase also involves the *syn*-addition of hydrogen atoms to the C=C bond.

Thus, it was concluded that the reduction of the C=C bond of enones with the verbenone reductase from *N. tabacum* occurs enantio- and stereo-specifically by the *syn*-addition of hydrogen atoms from the *re-re* face of the C=C bond. The hydrogen atoms

participating in the reduction at the α - and β -positions of the carbonyl group originate from the medium and the *pro*-4S hydrogen of NADPH, respectively. All of the previous reports on the stereochemistry in the enzymatic reduction of enones involve reductions occurring by the *anti*-addition of hydrogen atoms to the C=C bond. The verbenone reductase isolated shows unique behaviour in terms of the stereochemistry of the reduction which it induces.

Experimental

^1H NMR spectra were measured on a JEOL GSX-270 spectrometer with solutions in $^2\text{H}_2\text{O}$ and CDCl_3 , containing Me_4Si as internal standard; J values are given in Hz. Mass spectra were recorded on a Hewlett Packard 5890SII spectrometer at 70 eV in EI mode. Time-of-Flight (TOF) mass spectrum was recorded on a Bruker TOF mass spectrometer.

Substrates

(1*S*,5*S*)-(-)-Verbenone, $[\alpha]_{\text{D}}^{25} -196$ (c 0.49, EtOH) and (1*R*,5*R*)-(+)-verbenone, $[\alpha]_{\text{D}}^{25} +211$ (c 1.5, CHCl_3) were prepared from (-)- and (+)- α -pinenes, respectively, by oxidation with *tert*-butyl chromate.¹⁶ (*R*)-(-)-Carvone, $[\alpha]_{\text{D}}^{25} -60.1$ (neat) was purchased from Aldrich Chemical Co. Ltd. (*S*)-(+)-Carvone, $[\alpha]_{\text{D}}^{25} +57.1$ (neat) and (*R*)-(+)-pulegone, $[\alpha]_{\text{D}}^{25} +22.3$ (c 4.6, EtOH) were donated from Takasago Perfumery Co. Ltd. 2-Methylcyclohex-2-enone was prepared from 2-methylcyclohexanone by chlorination with sulfur chloride followed by dehydrochlorination with collidine according to the reported method.¹⁷ Cyclohex-2-enone and 3-methylcyclohex-2-enone were commercial products. NADH, NADP^+ , NADPH, FAD and FMN were purchased from the Oriental Yeast Co. Ltd.; Sephadex G-25 was from Pharmacia; DEAE Toyopearl and AF-Red Toyopearl 650ML were from TOSOH Co. Ltd.; $^2\text{H}_2\text{O}$ (99.9% atom D) was from Aldrich Chemical Co. Ltd.

Preparation of (4*R*)-[4- ^2H]-NADPH

Following the reported method,¹⁸ (4*R*)-[4- ^2H]-NADPH was prepared by reduction of β - NADP^+ (125 mg) with $[\text{H}_6]$ -EtOH (99% enrichment; 400 mg) and yeast alcohol dehydrogenase (27 units, 10 mg). The crude product was subjected to chromatography on a DEAE-Toyopearl column to give (4*R*)-[4- ^2H]-NADPH (89% ^2H -enrichment; 59 mg); $\delta_{\text{H}}(\text{D}_2\text{O})$ 2.66 (1 H, br s, 4-H), 6.18 (2 H, d, OCH_2) and 6.90 (1 H, s, 2-H).

Preparation of (4*S*)-[4- ^2H]-NADPH

Following the reported method,¹⁹ (4*S*)-[4- ^2H]-NADPH was prepared by enzymatic reduction of [4- ^2H]- NADP^+ (120 mg) with sodium isocitrate and isocitrate dehydrogenase. The reaction mixture was chromatographed on a DEAE-Toyopearl column to give (4*S*)-[4- ^2H]-NADPH (99% ^2H -enrichment; 31 mg), $\delta_{\text{H}}(\text{D}_2\text{O})$ 2.81 (1 H, br s, 4-H), 6.19 (2 H, d, OCH_2) and 6.92 (1 H, s, 2-H).

Purification of enzyme

A suspension of *N. tabacum* cells was prepared as described in ref. 6; cells cultured for 3 weeks were used in the present work. All purification procedures were carried out at 4 °C. Cultured cells (50 g) were frozen with liq. N_2 , ground and homogenized with Na-Pi buffer (100 mol dm^{-3} , pH 6.8; 100 cm^3) containing sucrose (0.25 mol dm^{-3}), glycerol (10%), dithiothreitol (5 mmol dm^{-3}) and $\text{Na}_2\text{S}_2\text{O}_5$ (5 mmol dm^{-3}) in a Waring blender. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 10 000 g for 10 min. The supernatant was collected and treated with ammonium sulfate to 40% saturation. The supernatant solution was collected and brought to 80% ammonium sulfate saturation. The residue was collected by centrifugation, redissolved in a minimal volume of 0.025 mol

dm^{-3} Tris buffer (pH 7.2) containing $\text{Na}_2\text{S}_2\text{O}_5$ (1 mmol dm^{-3}) and dithiothreitol (1 mmol dm^{-3}). The $(\text{NH}_4)_2\text{SO}_4$ fraction was desalted through a Sephadex G-25 column previously equilibrated with the standard buffer. The protein fraction was subsequently loaded onto a DEAE-Toyopearl column (3.1 \times 23 cm) equilibrated with the standard buffer. After being washed with the buffer soln, the DEAE-Toyopearl column was eluted with a linear gradient of aq. NaCl (0 ~ 0.5 mol dm^{-3} in the standard buffer). The active enzyme fractions were dialysed against the standard buffer. The enzyme solution was subsequently subjected to further purification on an AF-Red Toyopearl 650ML column (1.2 \times 9 cm) equilibrated with the standard buffer. After washing with the buffer soln, the enzymes were eluted with a linear NADPH gradient (0 ~ 4 mmol dm^{-3} in the standard buffer). Effluent fractions containing enzyme activity were pooled and used for experiments.

Molecular weight

The M_r values of the reductase was estimated by gel filtration through a Sephadex G-150 column (1.5 \times 75 cm) by using aldolase, bovine serum albumin, ovalbumin and ribonuclease A as protein markers. The enzyme prep. was dialysed to distilled water and then its mass spectrum was recorded on a TOF mass spectrometer.

According to Laemmli's method,²⁰ 0.4% SDS-15% PAGE of the enzyme was performed. The molecular weight of enzyme was determined by reference to the mobilities of proteins of known molecular mass (LMW electrophoresis calibration kit from Pharmacia Co. Ltd.).

Optimum pH

The reaction mixture was composed of MOPS buffer (100 mmol dm^{-3} ; 900 mm^3) with pH adjusted from 6.5 to 8.0, (-)-verbenone (3 μmol), NADPH (6 μmol), 0.1 Triton X-100 (1%; 1 cm^3) and enzyme prepn (0.1 cm^3). The enzyme activity was analysed according to the same method as the standard assay.

Enzyme assay

The standard assay mixture was composed of enzyme prepn. (1 cm^3) in Na-Pi buffer (25 mmol dm^{-3}) with pH adjusted to 7.2, (-)-verbenone (3 μmol), NADPH (6 μmol) and Triton X-100 (1%; 0.1 cm^3). The mixture was incubated for 12 h at 36 °C and then extracted with Et_2O and subjected to GLC and GC-MS analyses. The enzyme activity was expressed as the amount of *cis*-verbanone produced.

Incubation of enones with the enzyme preparation

Following the standard assay method, enzymatic reduction of several enones was performed. The enzymatic reduction products of **1a** and **2a** were identified as (1*S*,2*R*,5*S*)-*cis*-verbanone **3a** and (1*S*,4*S*)-dihydrocarvone **4a**, respectively, by direct comparison of GLC, TLC, GC-MS and NMR characteristics with those of authentic samples.^{6,7} (1*S*,2*R*,5*S*)-*cis*-Verbanone **3a**: m/z 152 (M^+ , 11%), 137 (12), 95 (50) and 83 (100); $\delta_{\text{H}}(\text{CDCl}_3)$ 1.01 (3 H, s, 9-Me), 1.17 (3 H, d, J 7.3, 10-Me), 1.34 (3 H, s, 8-Me), 2.16 (1 H, dd, J 20.0 and 4.9, 3-H *trans*-orientated to the 2-H), 2.38 (1 H, m, 2-H), 2.87 (1 H, dd, J 20.0 and 10.7, 3-H *cis*-orientated to the 2-H). Assignments of ^1H NMR signals of *cis*-verbanone **3a** were made by evaluation of their H,H coupling constants on a 270 MHz NMR spectrum; the assignments were confirmed by a 2D ^1H , ^1H shift correlation NMR spectrum of authentic (1*S*,2*R*,5*S*)-verbanone prepared by hydrogenation of (-)-verbenone **1a** in the presence of Pd-C.^{7,21} In addition, assignment of the ^1H NMR signals due to two protons at C-3 was justified by the fact that the proton signal at δ 2.16 showed NOEs for the signals at δ 1.01 due to the C(9)-3 H and at δ 1.17 due to the C(10)-3 H in the difference NOE spectrum measurements. (1*S*,4*S*)-dihydrocarvone **4a**: m/z (rel. int.) 152 (M^+ , 20%), 137 (14), 109 (30), 95 (80) and 67 (100); $\delta_{\text{H}}(\text{CDCl}_3)$ 1.02 (3 H, d, J 6.5, 1-Me), 1.37 (1 H,

qd, J 12.8 and 3.8, 6-H *trans*-orientated to the 1-H), 1.74 (3 H, s, 8-Me), 2.13 (1 H, dq, J 12.8 and 2.8, 6-H *cis*-orientated to the 1-H) and 4.77 (2 H, br s, C=CH₂). Assignments of ¹H NMR signals of dihydrocarvone **4a** were made by evaluation of their H,H coupling constants on a 270 MHz NMR spectrum; the assignments were confirmed by 2D ¹H,¹H and ¹H,¹³C shift correlation NMR spectra of authentic (1*S*,4*S*)-dihydrocarvone prepared by reduction of (+)-carvone **2a** with zinc powder in 25% potassium hydroxide–EtOH.^{6,22}

Incubation of (1*S*,5*S*)-verbenone **1a with the enzyme preparation in the presence of (4*S*)-[4-²H]-NADPH.** To the enzyme preparation (5 cm³; pH 7.2) in a glass stoppered tube, a soln. of (1*S*,5*S*)-verbenone (30 μmol) and the labelled NADPH (60 μmol) in Na-Pi buffer (25 mmol dm⁻³; 100 mm³) containing Triton X-100 (1%) was added. The mixture was incubated for 20 h at 30 °C after which it was extracted with Et₂O. The extract was purified by prep. GLC to give *cis*-verbanone: m/z 153 (M + 1⁺, 5%), 138 (7), 95 (18) and 83 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ 2.38 (s, 2-²H).

Incubation of (1*S*,5*S*)-verbenone **1a with the enzyme preparation in the presence of (4*R*)-[4-²H]-NADPH.** In the presence of (4*R*)-[4-²H]-NADPH, (1*S*,5*S*)-verbenone was converted into *cis*-verbanone: m/z 152 (M⁺, 3%), 137 (4), 95 (36) and 83 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ no ²H-signal was detected.

Incubation of (1*S*,5*S*)-verbenone **1a with the enzyme preparation in the presence of ²H₂O.** The enzyme preparation (5 cm³) was lyophilised and then re-suspended in Na-Pi buffer (50 mmol dm⁻³, pH 7.2; 3 cm³) prepared with ²H₂O. To the suspension, (1*S*,5*S*)-verbenone (30 μmol) and NADPH (60 μmol) dissolved in Na-Pi buffer (50 mmol dm⁻³; 100 mm³) containing Triton X-100 (1%) was added. The mixture was incubated for 20 h at 30 °C after which it was extracted with Et₂O. The extract was purified by prep. GLC to give ²H-labelled *cis*-verbanone: m/z 153 (M + 1⁺, 6%), 138 (7), 95 (35) and 83 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ 2.87 (s, 3-²H *cis*-orientated to the 2-H).

Incubation of (S)-carvone **2a with the enzyme preparation in the presence of (4*S*)-[4-²H]-NADPH.** The enzymatic reduction of (S)-carvone (30 μmol) with the labelled NADPH (60 μmol) was performed in the same way as above to give ²H-labelled dihydrocarvone: m/z 153 (M + 1⁺, 13%), 138 (6), 110 (20), 95 (81) and 67 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ 2.13 (s, 6-²H *cis*-orientated to the 1-H).

Incubation of (S)-carvone **2a with the enzyme preparation in the presence of (4*R*)-[4-²H]-NADPH.** In the presence of (4*R*)-[4-²H]-NADPH, (S)-carvone was converted into dihydrocarvone: m/z 152 (M⁺, 19%), 137 (9), 109 (14), 95 (67) and 67 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ no ²H-signal was detected.

Incubation of (S)-carvone **2a with the enzyme preparation in the presence of ²H₂O.** (S)-Carvone (30 μmol) was incubated with the enzyme fraction in the buffer prepared with ²H₂O to give ²H-labelled dihydrocarvone: m/z 153 (M + 1⁺, 15%), 138 (7), 110 (25), 95 (63) and 67 (100); $\delta_{\text{H}}(\text{CHCl}_3)$ 2.37 (s, 1-²H).

Acknowledgements

The authors thank Dr S. Ohta, Instrument Center for Chemical Analysis of Hiroshima University, for the measurements of ¹H (270 MHz) and ²H (41.5 MHz) NMR, Professor Tohru Kojima and Dr Mikio Yashiki, School of Medicine of Hiroshima University, for the measurement of GC–MS, Professor Katsutoshi Yoshizato, Faculty of Science of Hiroshima University, for the measurement of TOF mass spectrum and Miss Masae Hayashi for helpful assistance with parts of the experiments.

References

- 1 S. J. Davidson, *Methods Enzymol.*, 1969, **15**, 656.
- 2 T. L. Miller and E. J. Hessler, *Biochim. Biophys. Acta*, 1970, **202**, 354.
- 3 T. Oda and Y. Sato, *Chem. Pharm. Bull.*, 1986, **34**, 4635.
- 4 O. Berseus, H. Danielsson and A. Kallner, *J. Biol. Chem.*, 1965, **240**, 2396.
- 5 O. Berseus and I. Bjorkhem, *Eur. J. Biochem.*, 1967, **2**, 503.
- 6 T. Hirata, H. Hamada, T. Aoki and T. Suga, *Phytochemistry*, 1982, **21**, 2209.
- 7 T. Suga, H. Hamada and T. Hirata, *Chem. Lett.*, 1987, 471.
- 8 T. Suga, T. Hirata and H. Hamada, *Bull. Chem. Soc. Jpn.*, 1986, **59**, 2865.
- 9 T. Hirata, Y. Tang, K. Okano and T. Suga, *Phytochemistry*, 1989, **28**, 3331.
- 10 Y. Tang and T. Suga, *Phytochemistry*, 1992, **31**, 2599.
- 11 T. Hirata, S. Izumi, K. Shimoda and M. Hayashi, *J. Chem. Soc., Chem. Commun.*, 1993, 1426.
- 12 M. Desrut, A. Kergomard, M. F. Renard and H. Veshambre, *Biochem. Biophys. Res. Commun.*, 1983, **110**, 908.
- 13 H. L. Holland, W. Xu and D. W. Hughes, *J. Chem. Soc., Chem. Commun.*, 1989, 1760.
- 14 D. Schlieper, K. Tiemann and W. Barz, *Phytochemistry*, 1990, **29**, 1519.
- 15 A. J. Burbott and W. D. Loomis, *Plant Physiol. (Suppl.)*, 1980, **65**, 96.
- 16 T. Matsuura and K. Fujita, *J. Sci. Hiroshima Univ., Ser. A*, 1952, **16**, 173.
- 17 E. W. Warnhoff, D. G. Martinx and W. S. Johnson, *Org. Synth., Coll. Vol.*, 1963, **4**, 162.
- 18 J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Pobjak, G. Ryback and G. J. Schroepfer, *Proc. R. Soc. London*, 1965, **163**, 436.
- 19 S. P. Colowick and N. O. Kaplan, *Methods Enzymol.*, 1957, **4**, 840.
- 20 U. K. Laemmli, *Nature (London)*, 1970, **227**, 680.
- 21 H. Hamada, *Bull. Chem. Soc. Jpn.*, 1988, **61**, 869.
- 22 Y. Noma and C. Tatsumi, *Nippon Noeikagaku Kaishi*, 1973, **47**, 705.

Paper 5/04706D

Received 18th July 1995

Accepted 22nd September 1995