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STEREOCHEMISTRY OF REDUCTION OF THE ENDOCYCLIC DOUBLE BOND OF (−)-CARVONE WITH THE ENZYME PREPARATION FROM CULTURED CELLS OF *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; cultured cells; enzymatic reduction; stereochemistry; carvone.

Abstract—The stereochemistry of the reduction of the endocyclic C–C double bond of (4*R*)-(−)-carvone with an enzyme preparation from cultured cells of *Nicotiana tabacum* was investigated by ^{2}H NMR and mass spectroscopy. It was found that: (i) the enzyme preparation regioselectively reduces only the endocyclic double bond; (ii) the reduction occurs stereospecifically by *anti* addition of hydrogen from the *si* face at C-1 and the *re* face at C-6 of carvone, resulting in the formation of (1*R*, 4*R*)-(+)-dihydrocarvone; (iii) the hydrogen atoms participating in the enzymatic reduction at C-1 and C-6 originate from the medium and the *pro*-4*R* hydrogen of NADH, respectively.

INTRODUCTION

Several studies on the reduction of α,β-unsaturated ketones with plant cell cultures have been reported [1–5]. In recent studies [3, 6], we have shown the stereospecific reduction of the C–C double bond adjacent to the carbonyl group of carvone (**1**) by cultured cells of *Nicotiana tabacum* L. In connection with these studies, we have now investigated the stereochemistry of the reduction of carvone (**1**) by incubation of an enzyme preparation from cultured cells of *N. tabacum* in the presence of either stereospecifically ^{2}H-labelled NADH or ^2H_2O.

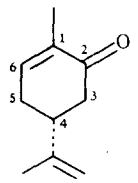
RESULTS AND DISCUSSION

An enzyme responsible for the reduction of the C–C double bond of carvone (**1**) was prepared from cultured suspension cells of *N. tabacum*. Incubation of (*R*)-(−)-carvone (**1**) with the enzyme preparation gave only (1*R*, 4*R*)-(+)-dihydrocarvone (**2**) in 20–60% yield, although the biotransformation of carvone (**1**) with the cultured cells was always accompanied by the formation of dihydrocarveol, formed by further reduction of dihydrocarvone as reported in our previous paper [3]. The formation of only (+)-dihydrocarvone (**2**) by this enzymatic reaction indicates that the enzyme preparation catalyses the regio- and stereoselective reduction of the endocyclic double bond of carvone (**1**).

In order to investigate the stereochemistry of the enzymatic reduction of the C–C double bond of carvone, four experiments were performed: carvone (**1**) was incubated with the enzyme preparation in the presence of (a) NADH in H_2O, (b) (4*R*)-[4-^{2}H]NADH in H_2O, (c) (4*S*)-[4-^{2}H]NADH in H_2O and (d) NADH in ^2H_2O. The labelling pattern of deuterium and the deuterium contents in the resulting dihydrocarvones were determined by mass and NMR spectroscopy.

The dihydrocarvone (**2**) produced in the incubation performed in the presence of (4*R*)-[4-^{2}H]NADH [experiment (b)] shows a peak at *m/z* 153, i.e. one mass unit higher than the molecular ion peak (*m/z* 152) of dihydrocarvone (**2**) produced in the control experiment (a) (Fig. 1a and b). A fragment ion peak at *m/z* 110 due to expulsion of the 1-methylethenyl group together with one hydrogen atom [7] is also one mass unit higher compared to the fragment peak observed for dihydrocarvone in the control experiment (a). This indicates that deuteration is restricted to the cyclohexene part of carvone (**1**). When the (4*R*)-[4-^{2}H]NADH is replaced by (4*S*)-[4-^{2}H]-NADH, no deuteration of dihydrocarvone is observed (Fig. 1c). Thus, the deuterium atom originating from (4*R*)-[4-^{2}H]NADH is incorporated into dihydrocarvone on enzymatic reduction of carvone (**1**). On the other hand, the mass spectrum of dihydrocarvone produced when ^2H_2O is included in the incubation mixture shows peaks at *m/z* 153 $[M+1]^+$ and 154 $[M+2]^+$ (Fig. 1d). This observation indicates that deuterium originating from ^2H_2O was incorporated into two sites of dihydrocarvone (**2**).

The labelled sites in the deuterium-labelled dihydrocarvone on enzymatic reduction of carvone (**1**). On the ^{2}H NMR spectra with those of un-labelled dihydrocarvone (**2**). Complete assignments of ^{1}H NMR signals of dihydrocarvone (**2**) were made on a 500 MHz NMR spectrum. The assignments were confirmed by a 2D



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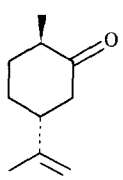

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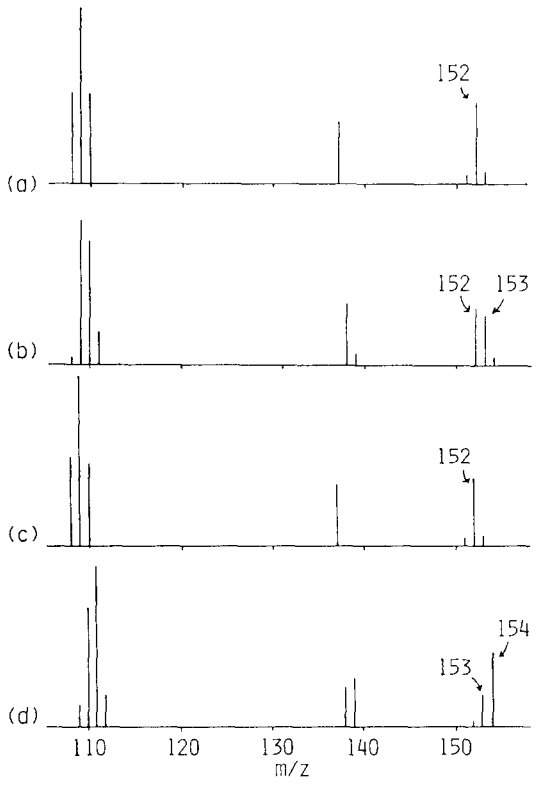

Fig. 1. Mass spectra of dihydrocarvone (**2**) obtained by enzymatic reduction of (−)-carvone (**1**): (a) incubation with NADH in H_2O, (b) incubation with (4*R*)-[4-^{2}H]NADH in H_2O, (c) incubation with (4*S*)-[4-^{2}H]NADH in H_2O and (d) incubation with NADH in ^2H_2O.

^{1}H–^{1}H shift correlation NMR spectrum of the unlabelled dihydrocarvone (**2**). The ^{1}H NMR spectrum of dihydrocarvone produced in experiment (b) revealed a decrease in the intensity of the signal at δ1.37 due to the C-6 axial proton. The ^{1}H NMR spectrum of dihydrocarvone produced in experiment (d) showed the disappearance of the signal at δ2.37 due to the C-1 proton and a decrease in the signal at δ1.37 due to the C-6 axial proton. However, the intensities of other signals, especially the signal due to the 1-methylethenyl group, were unchanged. These observations indicate deuteration at C-6 of dihydrocarvone produced in experiment (b) and at C-1 and C-6 of dihydrocarvone produced in experiment (d). The deuteration sites of dihydrocarvones produced in the enzymatic reductions were thus confirmed by ^{2}H{^{1}H} NMR spectroscopy. The spectrum of dihydrocarvone produced in experiment (b) showed only a signal at δ1.37 due to the axial ^{2}H at C-6. The ^{2}H-enrichment factors at the labelled site was determined from the intensity of the corresponding ^{2}H peak on the basis of the peak intensity of natural abundant ^{2}H in $CHCl_3$ used as the solvent and was 20%. On the other hand, dihydrocarvone produced in experiment (d) exhibited two signals at δ1.37 and 2.37; these signals are due to the axial ^{2}H at C-6 and the ^{2}H at C-1 of dihydrocarvone, respectively. The ^{2}H-enrichment factors at the labelled sites were 95% for C-1 and 41% for C-6. It was thus established that the deuterium atoms at C-1 and C-6 of the deuterated dihydrocarvone originate from ^2H_2O and (4*R*)-[4-^{2}H]NADH, respectively. In addition, the *pro*-4*R* hydrogen from NADH tends to be exchanged in part with ^{2}H of ^2H_2O in the process of the enzymatic reduction.

It was concluded that (a) the enzyme preparation from the cultured cells of *N. tabacum* regioselectively reduces only the endocyclic double bond of carvone (**1**); (b) the reduction occurs stereospecifically by *anti* addition of hydrogens from the *si* face at C-1 and the *re* face at C-6; (c) the hydrogen atoms participating in the enzymatic reduction at C-1 and C-6 originate from the medium and the *pro*-4*R* hydrogen of NADH, respectively.

EXPERIMENTAL

Analytical and prep. TLC: 0.25 mm and 0.5 mm silica gel (Merck silica gel 60; GF_{254}). Prep. HPLC: Fine Pak-SIL column with pentane -Et_2O (19:1); GC: FID and a glass column (3 mm × 2 m) packed with 15% DEGS and 2% OV-17 on Chromosorb W (AW-DMCS; 80–100 mesh) at 100 and 90–200° (3°/min), respectively. GC-MS: 15% DEGS column (3 mm × 2 m) by EI mode at 70 eV; ^{1}H and ^{13}C NMR: 500 and 125 MHz, respectively, in $CDCl_3$ with TMS as int. standard; ^{2}H NMR: 76.7 MHz in $CHCl_3$ by use of the solvent peak as an internal standard. The enrichment factor (%) at a labelled site in deuterated dihydrocarvone was calculated from the integral of the deuterium signals and the deviations were within ±3%.

Substrate. (4*R*)-(−)-Carvone, donated from Takasago Perfumery Co. Ltd, was rectified by distillation to give a sample with bp 93–94°/1067 Pa, $[\alpha]_D^{25}$ −60.1° (neat), n_D^{25} 1.5005, and d_4^{25} 0.9610 (lit. [8] $[\alpha]_D$ −59.7°, n_D^{20} 1.4988, and d_4^{15} 0.9652).

Preparation of (4R)-[4-^{2}H]*NADH.* Following the reported method [9], (4*R*)-[4-^{2}H]NADH was prepared by reduction of β-NAD^+ (125 mg) with EtOH-d_6 (98% enrichment; 400 mg) and horse-liver alcohol dehydrogenase (27 units; 10 mg). The crude product was subjected to chromatography on a DEAE Toyopearl gel column to give (4*R*)-[4-^{2}H]NADH (59 mg): UV $\lambda_{max}^{H_2O}$ nm (log ε): 339 (3.79), 258 (4.11) and 234 (3.80); ^{1}H NMR (90 MHz, D_2O): δ2.66 (1H, *br s*, 4*S*-H), 5.98 (2H, *d*, –O–CH_2–), 6.13 (2H, *d*, –O–CH_2–) and 6.94 (1H, *s*, 2-H).

Preparation of (4S)-[4-^{2}*H*]*NADH.* Following the reported method [9], (4*S*)-[4-^{2}H]NADH was prepared by enzymatic reduction of [4-^{2}H]NAD^+ (120 mg) with EtOH (170 mg) and horse-liver alcohol dehydrogenase (27 units; 10 mg). The reaction mixture was chromatographed on a DEAE Toyopearl gel column to give (4*S*)-[4-^{2}H]NADH (31 mg): UV $\lambda_{max}^{H_2O}$ nm (log ε): 340 (3.79), 259 (4.12) and 233 (3.80); ^{1}H NMR (90 MHz, D_2O): δ2.76 (1H, *br s*, 4*R*-H), 5.97 (2H, *d*, –O–CH_2–), 6.12 (2H, *d*, –O–CH_2–) and 6.92 (1H, *s*, 2-H).

Enzyme preparation of the cultured cells of N. tabacum. The fresh tissue (30 g) was frozen with liq. N_2, ground in a mortar and suspended in 50 mM K-Pi buffer (pH 6.8, 120 ml). The resulting paste was filtered through two layers of cheesecloth. The filtrate was centrifuged at 10000 g for 20 min. The supernatant was subjected to gel-filtration on a Sephadex G-25 column to give an enzyme fraction (37 ml; 0.61 mg protein/ml). All of the operations were carried out at 4°.

Large scale incubation of carvone (**1**) *with the enzyme preparation.* To the enzyme preparation (20 ml; pH 6.8) (−)-carvone (5 mg) dissolved in 1% Triton X-100 (0.1 ml) was added. The mixture was incubated for 20 hr at 30°. A control experiment was carried out in parallel in which the enzyme preparation was deactivated by heating at 85° for 10 min. After incubation, the reaction mixture was extracted with Et_2O. The Et_2O extract was subjected to GC and GC-MS; only one peak, except for the peak

of unchanged carvone, was observed. It was attributed to dihydrocarvone by direct comparison of GC and GC-MS with those of an authentic dihydrocarvone. However, no product was found in the control experiment. The extract in the incubation with the active enzyme preparation, after removal of the solvent, was subjected to prep . TLC with EtOAc–hexane (1:4) to give (+)-dihydrocarvone (**2**) (1.4 mg); $[\alpha]_D^{25}$ +12.5° (EtOH, *c* 0.1) [lit. [10] +13.1° (EtOH; *c* 6); IR ν_{max}^{film} cm^{-1}: 1710 (C=O) and 1637 (C=C); ^{1}H NMR (60 MHz): δ1.02 (3H, *d*, *J* = 6 Hz, 1-Me), 1.75 (3H, *s*, 8-Me) and 4.79 (2H, *br s*, C=CH_2); ^{13}C NMR (125 MHz): δ_c 212.6 (C-2), 147.6 (C-8), 109.6 (C-9), 47.0 (C-4), 46.9 (C-3), 44.7 (C-1), 34.9 (C-6), 30.8 (C-5), 20.5 (C-10) and 14.3 (C-7); MS *m/z* (rel. int.): 152 $[M]^+$ (20), 137 (14), 109 (30), 95 (80) and 67 (100).

Incubation of carvone (**1**) *with the enzyme preparation in the presence of* (4R)- *and* (4S)-[4-^{2}H]*NADH*. To the enzyme preparation (3 ml; pH 6.8) in a glass stoppered tube, a soln of (−)-carvone (2 μmol) and the ^{2}H-labelled NADH (6 μmol) in 50 mM K-Pi buffer (100 μl) containing 1% Triton X-100 was added. The mixture was incubated for 20 hr at 30° and then the reaction mixture was extracted with Et_2O. The Et_2O extract, after removal of the solvent, was subjected to GC and GC-MS. The extract was purified by prep. HPLC to give dihydrocarvone (**2**). MS: see Fig. 1; ^{2}H NMR: δ1.37 (*s*, 6β-^{2}H); the intensities of the signal at δ1.37 (*qd*, *J* = 13.2 and 3.7 Hz, 6β-H) in the ^{1}H NMR spectra of the dihydrocarvones showed 0.8H for the dihydrocarvone produced in the incubation in the presence of (4*R*)-[4-^{2}H]NADH and 1H for that in the presence of (4*S*)-[4-^{2}H]NADH.

Incubation of carvone (**1**) *with the enzyme preparation in the presence of* ^2H_2O. The enzyme preparation (3 ml) was lyophilized and then resuspended in 50 mM K-Pi buffer (3 ml; pH 6.8) prepared with ^2H_2O. To the suspension, (−)-carvone (2 μmol) and NADH (6 μmol) dissolved in 50 mM K-Pi buffer (100 μl) containing 1% Triton X-100 was added. The mixture was incubated for 20 hr at 30° and then the reaction mixture was extracted with Et_2O. The Et_2O extract was subjected to GC and GC-MS and then purified by prep. HPLC to give ^{2}H-labelled dihydrocarvone (**2**). MS: see Fig. 1; ^{1}H NMR (500 MHz): δ1.03 (1H, *s*, 1-Me), 1.37 (0.6H, *br t*, *J* = 13.2 Hz, 6β-H), 1.64 (1H, *br t*, *J* = 13.2 Hz, 5α-H), 1.94 (1H, *br d*, *J* = 13.2 Hz, 5β-H), 2.10 (0.4H, *br s*, 6α-H) and 2.12 (0.6H, *dt*, *J* = 13.2 and 3.3 Hz, 6α-H), 2.28 (1H, *t*, *J* = 12.3 Hz, 4-H), 2.31 (1H, *t*, *J* = 12.3 Hz, 3α-H), 2.45 (1H, *dt*, *J* = 12.3 and 1.8 Hz, 3β-H), 4.73 (1H, *dq*, *J* = 1.3 and 1.1 Hz, 9*Z*-H) and 4.76 (1H, *d*, *J* = 1.3 Hz, 9*E*-H); ^{2}H NMR: δ1.37 (*s*, 6β-^{2}H) and 2.37 (*s*, 1-^{2}H).

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