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STEREOCHEMISTRY IN THE REDUCTION OF ENONES BY THE REDUCTASE FROM *EUGLENA GRACILIS* Z

KEI SHIMODA, TOSHIFUMI HIRATA* and YOSHIAKI NOMA†

Department of Chemistry, Faculty of Science, Hiroshima University,1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan; † Faculty of Domestic Science, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan

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Key Word Index—*Euglena gracilis* Z; Euglenaceae; stereochemistry; enone reductase; carvone; verbenone; pulegone; piperitenone.

Abstract—A reductase, which catalyses the NADH-dependent reduction of the C═C bond adjacent to the carbonyl group, was characterized with regard to the stereochemistry of the hydrogen transfer into the substrate. The reductase was isolated from *Euglena gracilis* Z and was found to reduce stereospecifically the C═C bond of carvone by *anti*-addition of hydrogen from the *si* face at α-position to the carbonyl group and the *re* face at β-position. The hydrogen atoms participating in the enzymatic reduction at α- and β-position to the carbonyl group originate from the medium and the *pro*-4*R* hydrogen of NADH, respectively. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Hitherto known biochemical reduction of the C═C bond of α,β-unsaturated ketones were most investigated with microorganisms [1–3] and animals [4, 5]. Recently, three kinds of enone reductases were isolated from the cultured cells of *Nicotiana tabacum* and the stereochemical studies in the reduction of the C═C bond by these reductases have been reported [6–8]. On the other hand, Noma *et al*. reported that the cultured cells of *Euglena gracilis* Z classified in Phytomastigophoria also reduce the C═C bond of carvone (**1**) with high stereospecificity [9, 10]. In connection with the studies on the enone reductase from plant cells, we now investigate the isolation and characterization of the enone reductase from *E. gracilis* and the stereochemistry in the reduction of the C═C bond of enones with the reductase.

RESULTS AND DISCUSSION

A microsomal enzyme was obtained from the *E. gracilis* cell cultures by extraction with Triton X-100 and then subjected to chromatography on a Sephadex G-25 column and a diethylaminoethyl (DEAE) Toyopearl column. The activity of the enzyme was assayed at pH 7.4 using (*R*)-carvone (**1a**) as the substrate in the presence of NADH. The active fraction was further subjected to a Blue Toyopearl column to give a pure enzyme preparation (Table 1). The purified enzyme preparation was homogeneous as judged by the presence of a single protein band on the SDS gel electrophoresis, as shown in Fig. 1.

The *Mr* of the enzyme was estimated as about 55 k by gel filtration on a Sephadex G-150 column. The SDS gel electrophoresis of the enzyme preparation showed a single band at about 55 k (Fig.1). These observations indicate that the native enzyme is monomer of 55 k protein. The enzyme shows a pH optimum at 7.4 with half maximal activity at 6.3 and 8.1 in 3-morpholinopropanesulfonic acid-NaOH buffer. The enzyme required NADH and NADPH as coenzymes, and the activity in presence of NADH was twice as high as that in presence of NADPH. 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, as shown in Table 2. The data show a rather limited substrate specificity; only the C═C bond having a hydrogen atom at the position β to the carbonyl group can be reduced. When carvone (**1**) was used as the substrate, the (*R*)-enantiomer (**1a**) was reduced enantioselectively to yield (1*R*, 4*R*)-dihydrocarvone (**3**), and the conversion was higher than that of (*S*)-enantiomer (**1b**), yielding (1*R*, 4*S*)-isodihydrocarvone (**4**). No reduction of the enones such as 3-methylcyclohexenone, (1*S*, 5*S*)-verbenone (**2a**), (1*R*, 5*R*)-verbenone (**2b**), (*R*)-pulegone (**5**) and piperitenone (**6**) was observed. These observations show that the enzyme is similar to the carvone reductase isolated

* Author to whom correspondence should be addressed. E-mail: thirata@sci.hiroshima-u.ac.jp

Table 1. Purification of the reductase from *E. gracilis* Z

	Total protein mg	Total activity unit $\times 10^4$	Sp. act. units per g protein	Fold
Crude extract	125	2.2	1.7	1
DEAE-Toyopearl	7	1.5	21	12
AF-Blue Toyopearl	0.1	0.03	30	18

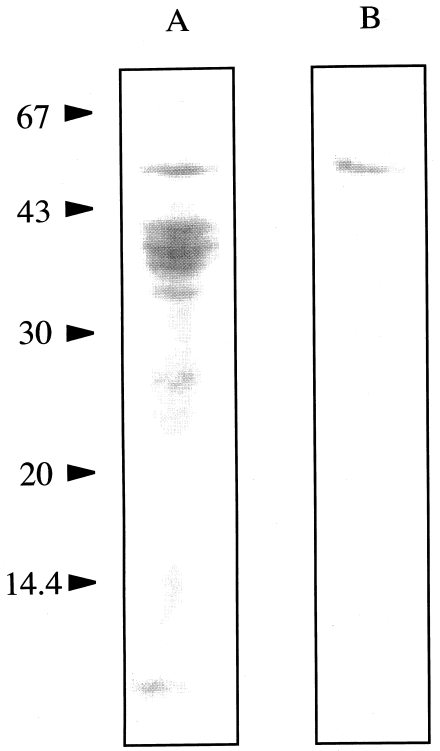
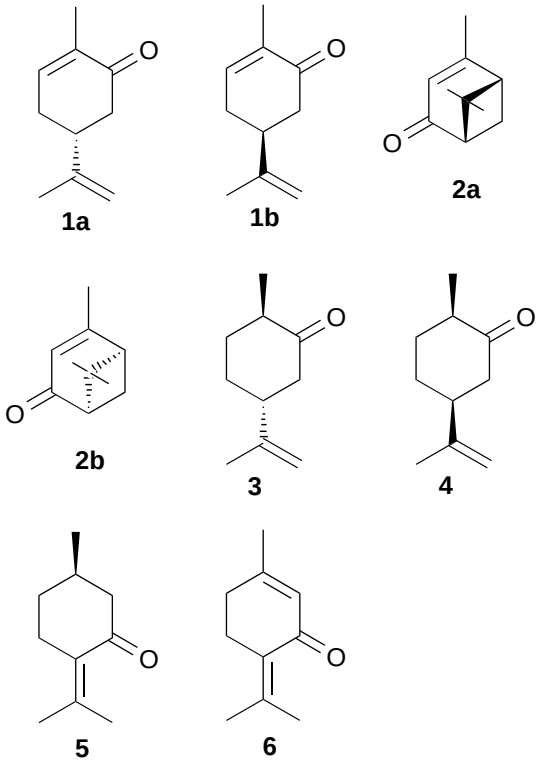


Fig. 1. SDS-gel electrophoretic analyses of the reductase from *E. gracilis* cell cultures. (a) DEAE Toyopearl fraction, (b) Blue Toyopearl fraction.



from the cultured cells of *N. tabacum* [6, 7] in respect of substrate specificity.

The fact that (1*R*, 4*R*)-dihydrocarvone (**3**) and (1*R*, 4*S*)-isodihydrocarvone (**4**) were produced by the enzymatic reduction of (*R*)- and (*S*)-carvone (**1a** and **1b**), respectively, indicates that the hydrogen attack at the conjugated C=C bond takes place stereospecifically from the *si* face at C-1. However, the stereochemistry of the hydrogen attack at C-6 of carvone (**1**) was unknown. To complete the stereochemical studies on the reduction of the C=C bond with the enzyme preparation, we investigated the stereochemistry of the hydrogen attack to **1a** by performing the following four experiments. (*R*)-Carvone (**1a**) was incubated with the enzyme preparation in the presence of: (a) NADH in H_2O; (b) NADH in ^2H_2O; (c) (4*R*)-[4$-^2$H]NADH in H_2O and (d) (4*S*)-[4$-^2$H]NADH in H_2O. Through these experiments, the orientation of deuterium in the resultant deuterated product was examined. The labelling pattern of deuterium and the deuterium contents in the resulting dihydrocarvone (**3**) were determined by NMR and mass spectroscopy. Dihydrocarvone (**3**) produced in the incubation in the presence of NADH in ^2H_2O [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 dihydrocarvone (**3**) produced in the control experiment (a). This indicates that the deuterium atom originating from ^2H_2O is incorporated into dihydrocarvone. On the other hand, the mass spectrum of dihydrocarvone (**3**), produced when (4*R*)-[4$-^2$H]NADH was included in the incubation mixture [experiment (c)], showed a peak at *m*/*z* 153 $[M+1]^+$. However, when the (4*R*)-[4$-^2$H]NADH was replaced by (4*S*)-[4$-^2$H]NADH [experiment (d)], no deuteration of dihydrocarvone (**3**) was observed. These observations indicate that only the *pro*-4*R* hydrogen of NADH is incorporated into dihy-

Table 2. Substrate specificity in the reduction of various enones with the reductase from *E. gracilis* Z

Substrate	Conversion/%[a]	Product
(*R*)-Carvone (**1a**)	23	(1*R*,4*R*)-Dihydrocarvone (**3**)
(*S*)-Carvone (**1b**)	6	(1*R*,4*S*)-Isodihydrocarvone (**4**)
(1*S*,5*S*)-Verbenone (**2a**)	0	—
(1*R*,5*R*)-Verbenone (**2b**)	0	—
(*R*)-Pulegone (**5**)	0	—
Cyclohex-2-en-l-one	45	Cyclohexanone
2-Methylcyclohex-2-en-l-one	5	(*R*)-2-Methylcyclohexan-l-one
3-Methylcyclohex-2-en-l-one	0	—
Piperitenone (**6**)	0	—

(a) Conversions are expressed as the percentage of the products obtained in the reduction of summarized substrates under the assay conditions.

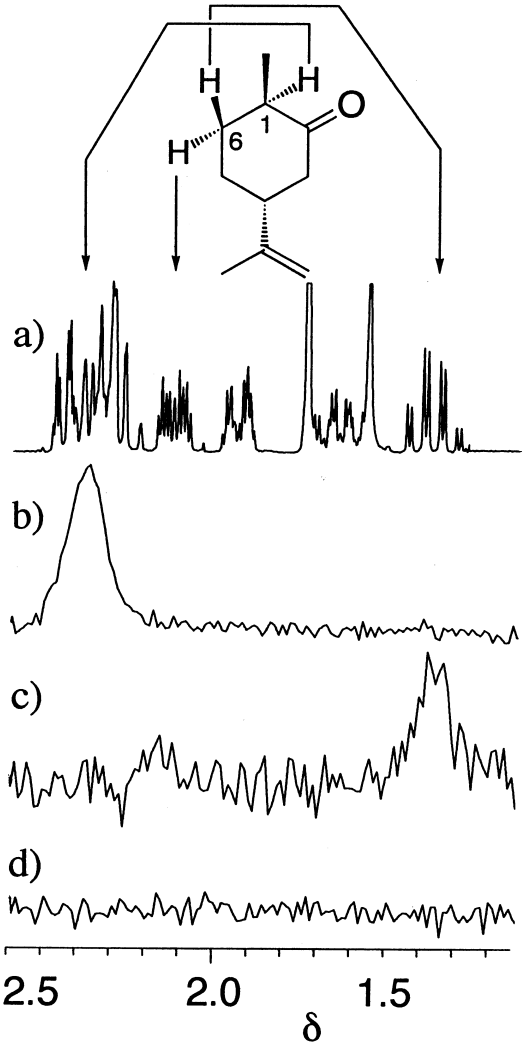


Fig. 2. NMR spectra of dihydrocarvone (**3**) obtained by the enzymatic reduction of (*R*)-carvone (**1a**): (a) ^{1}H NMR spectrum for the product by the incubation with NADH in H_2O; (b) ^{2}H NMR spectra for the products by the incubation with NADH in ^2H_2O; (c) the incubation with (4*R*)-[4−^{2}H]NADH in H_2O; and (d) incubation with (4*S*)-[4−^{2}H]NADH in H_2O.

drocarvone (**3**) during the enzymatic reduction of the double bond. The labelled sites in the deuterium-labelled dihydrocarvone were determined from ^{2}H NMR spectroscopy. The spectrum of dihydrocarvone (**3**) produced in the presence of NADH in ^2H_2O showed only a signal at δ 2.37 due to the ^{2}H at C-1. 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 ^{2}H in $CHCl_3$ used as the solvent and was 99%. However, dihydrocarvone produced in the presence of (4*R*)-[4−^{2}H]NADH exhibited a signal at δ 1.37 (99% enrichment) due to the ^{2}H at C-6 *trans*-oriented to the hydrogen atom at C-1. These observations indicate that the deuterium atoms at C-6 and C-1 of the deuterated dihydrocarvone originate from (4*R*)-[4−^{2}H]NADH and ^2H_2O, respectively.

Thus, it was concluded that the reduction of the C=C bond of enones with the reductase from *E. gracilis* occurs stereospecifically by the *anti*-addition of hydrogen atoms from the *si* face at C-1 and the *re* face at C-6 of the C=C bond of carvone, as shown in Fig.3. The hydrogen atoms participating in the reduction at α- and β- positions of the carbonyl group originate from the medium and the *pro*-4*R* hydrogen of NADH, respectively. The reductase isolated from the cultured cells of *E. gracilis* is similar to the carvone reductase from *N. tabacum* in respect of stereochemistry of the hydrogen transfer, but composed of different *Mr* from the carvone reductase, i.e., although the reductase from *E. gracilis* is a monomer of 55 k protein, the carvone reductase from *N. tabacum* is composed of two identical subunits of a 22 k protein; although the carvone reductase had been reported to be a tetramer of 22 k protein by Tang *et al.* [7], our recent investigation showed the reductase to be dimer of 22 k protein.

EXPERIMENTAL

Analytical and preparation

TLC: 0.25 mm silica gel (Merck silica gel 60; GF_{254}). GC: FID and a glass column (3 mm × 2 m) packed

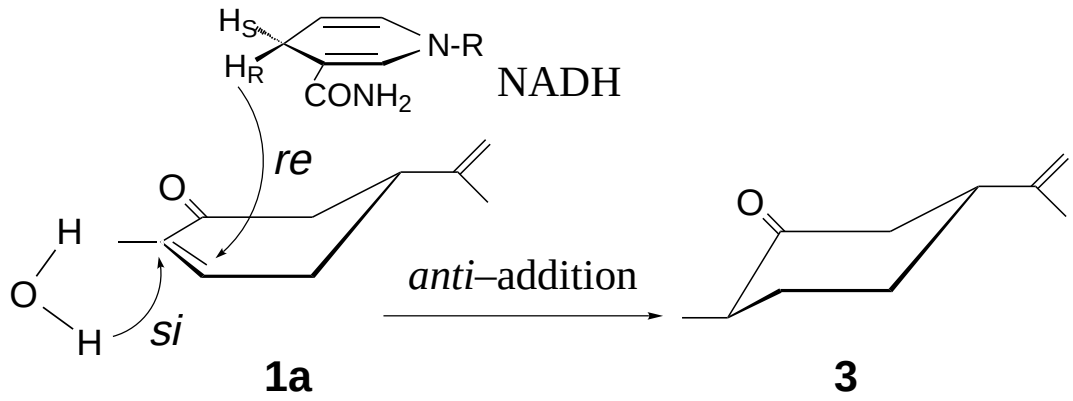


Fig. 3. Stereochemistry in the reduction of (*R*)-carvone by the reductase from *Euglena gracilis* Z.

with 15% DEGS at 100° or a capillary column (0.25 mm × 25 m) coated with 0.25 μm CP-cyclodextrin β 236M-19. GC-MS: a GCMS-QP 2000A spectrometer by El mode at 70 eV. ^{1}H NMR: 270 MHz in ^2H_2O and $CDCl_3$ with Me_4Si as internal standard. ^{2}H NMR: 41.5 MHz in $CHCl_3$ by use of the solvent peak as an internal standard.

Substrate

(1*S*,5*S*)-(−)-Verbenone, $[\alpha]_D^{25}$ −196 (*c* 0.49, EtOH) and (1*R*,5*R*)-(+)-verbenone, $[\alpha]_D^{25}$ +211 (*c* 1.5, $CHCl_3$) were prepared from (−)-α-pinene and (+)-α-pinene, respectively, by oxidation with *t*-butyl chromate [11]. (*R*)-(−)-Carvone, $[\alpha]_D^{25}$ −60.1 (neat) was purchased from Aldrich. (*S*)-(+)-Carvone, $[\alpha]_D^{25}$ +57.1 (neat) and (*R*)-(+)-pulegone, $[\alpha]_D^{25}$ +22.3 (*c* 4.6, EtOH) were donated by Takasago Perfumery Co. Ltd. 2-Metylcyclohex-2-en-l-one was prepared from 2-methylcyclohexan-l-one by chlorination with sulfuryl chloride followed by dehydrochlorination with collidine according to the reported method [12]. Cyclohex-2-en-l-one and 3-methylcyclohex-2-en-l-one were commercial products. NADH, $NADP^+$, NADPH, FAD and FMN were purchased from Oriental Yeast Co. Ltd.; Sephadex G-25 was from Pharmacia; DEAE Toyopearl and AF-Blue Toyopearl 650ML were from TOSOH Co. Ltd.; ^2H_2O (99.9% atom D) was from Aldrich.

*Preparation of (4*R*)-[4−^{2}H]NADH*

Following the reported method [13], (4*R*)-[4−^{2}H]NADH was prepared by reduction of β-NAD^+ (120 mg) with EtOH-d_6 (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−^{2}H]NADH (60 mg, 99% ^{2}H-enrichment), ^{1}H NMR (D_2O) δ 2.66 (1H, *brs*, 4*S*-H), 6.18 (1H, *d*, OCH_2) and 6.90 (1H, *s*, 2-H).

*Preparation of (4*S*)-[4−^{2}H]NADH*

Following the reported method [13], (4*S*)-[4−^{2}H]NADH was prepared by enzymatic reduction of [4−^{2}H]NAD^+ (120 mg) with EtOH (400 mg) and yeast alcohol dehydrogenase (27 units; 10 mg). The reaction mixture was chromatographed on a DEAE-Toyopearl column to give (4*S*)-[4−^{2}H]NADH (30 mg, 99% ^{2}H-enrichment), ^{1}H NMR (D_2O) δ 2.81 (1H, *brs*, 4*R*-H), 6.19 (1H, *d*, OCH_2) and 6.92 (1H, *s*, 2-H).

Purification of enzyme

Cultured cells of *E. gracilis* Z were prepared as described in Ref. [14]; cells cultured for 3 weeks were used in the present work. All purification procedures were carried out at 4°. Cultured cells (5 g) were frozen with liq. N_2, ground and homogenized with 100 mM Na-Pi buffer (pH 6.8, 10 ml) containing 0.25 M sucrose, 10% glycerol, 5 mM dithiothreitol, 5 mM $Na_2S_2O_5$ and 0.5% Triton X-100 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 desalted through a Sephadex G-25 column previously equilibrated with Tris buffer (ph 8.0) containing 1 mM $Na_2S_2O_5$ and 1 mM dithiothreitol. The protein fraction was subsequently loaded onto a DEAE-Toyopearl column (3.1 × 23 cm) equilibrated with the standard buffer. After washing with the buffer soln, the enzymes were eluted with a liner gradient of NaCl (0 ~ 0.5 M in the standard buffer). The active enzyme fractions were dialysed against the standard buffer. The enzyme soln was subsequently subjected to further purification on an AF-Blue Toyopearl column (1.2 × 9 cm) equilibrated with the standard buffer. After washing with the buffer soln, the enzymes were eluted with a linear NaCl gradient (0 ~ 2 M in the standard buffer). Effluent fractions containing the active enzyme were pooled and used for experiments.

The *Mr* values of the reductase were estimated by gel filtration through a Sephadex G-150 column

(1.5 × 75 cm), using aldolase, bovine serum albumin, ovalbumin and ribonuclease A as protein markers. According to method of Ref. [15], 0.4% SDS-15% PAGE of the enzyme was preformed. The *Mr* of enzyme was determined by reference to the mobilities of proteins of known *Mr* (LMW electrophoresis calibration kit from Pharmacia).

Optimum pH

The reaction mixture was composed of 900 μl 100 mM 3-(*N*-morpholino)propane sulfonic acid buffer with pH adjusted from 6.0 to 8.5, 3 μmol (*R*)-carvone, 6 μmol NADH, 0.1 ml 1% Triton X-100 and 0.1 ml enzyme prepn. The enzyme activity was determined according to the same method as the standard assay.

The standard enzyme assay mixture contained 1 ml enzyme prepn in 25 mM Na-Pi buffer with pH adjusted to 7.4, 3 μmol (*R*)-carvone, 6 μmol NADH and 0.1 ml 1% Triton X-100. The mixture was incubated for 12 h at 36° and then extracted with Et_2O and subjected to GC and GC-MS analyses. The enzyme activity was expressed as the amount of dihydrocarvone produced.

Incubation of enones with the enzyme preparation

Following the standard assay method, enzymatic reduction of several enones was preformed. The reduction products were identified with NMR, GC-MS and CD spectroscopy. Enantiomeric purity was determined from the peak area of GLC with CP cyclodextrin β 236M-19 column. In the case of (*R*)-carvone as substrate, the product was (1*R*, 4*R*)-dihydrocarvone : *m*/*z* (rel. int.) 152 (M^+, 16%), 137 (10), 109 (11), 95 (76) and 67 (100); ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, *d*, *J* = 6.5 Hz, 1-Me), 1.37 (1H, *qd*, *J* = 12.8 and 3.8 Hz, 6-H *trans*-oriented to the 1-H), 1.74 (3H, *s*, 8-Me), 2.13 (1H, *dq*, *J* = 12.8 and 2.8 Hz, 6-H *cis*-oriented to the 1-H) respectively. Incubation of 2-methylcylohexen-l-one with the reductase produced (*R*)-2-methylcyclohexan-l-one (> 99% enantiomeric excess): CD $[\theta]_{max}$ −990 at 289 nm (*c* 0.02, MeOH) [Ref. [16]: $[\theta]_{max}$ −987 at 288 nm]

*Incubation of (*R*)-carvone (**1a**) with the enzyme preparation in the presence of (4*R*)-[4−^{2}H]NADH*

The enzymatic reduction of (*R*)-carvone (30 μmol) with the labelled NADH (60 μmol) was performed in the same procedure as above to give ^{2}H-labelled dihydrocarvone: *m*/*z* (rel. int.) 153 ($M+1^+$, 4), 138 (4), 110 (17), 95 (45) and 67 (100); ^{2}H NMR ($CHCl_3$) δ 1.37 (*s*, 6-^{2}H *trans*-oriented to the 1-H).

*Incubation of (*R*)-carvone (**1a**) with the enzyme preparation in the presence of (4*S*)-[4−^{2}H]NADH*

In the presence of (4*S*)-[4−^{2}H]NADH, (*R*-carvone was converted to dihydrocarvone: *m*/*z* (rel. int.) 152 (M^+, 9), 137 (9), 109 (22), 95 (51) and 67 (100); ^{2}H NMR ($CHCl_3$) no ^{2}H-signal was detected.

*Incubation of (*R*)-carvone (**1a**) with the enzyme preparation in the presence of ^2H_2O*

(*R*)-carvone (30 μmol) was incubated with the enzyme fraction in the buffer prepared with ^2H_2O to give ^{2}H-labelled dihydrocarvone: *m*/*z* (rel. int.) 153 ($M+1^+$, 5), 138 (5), 110 (16), 95 (34) and 67 (100); ^{2}H NMR ($CHCl_3$) δ 2.37 (*s*, 1−^{2}H).

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