Stereochemistry in the Reduction of the C=C Bond of Verbenone with a Reductase from the Cultured Cells of *Nicotiana tabacum*

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Reduction of the C=C bond of verbenone by a reductase from the cultured cells of *Nicotiana tabacum* involves *syn*-addition of the hydrogen atom to the *re-re* face of the double bond; the hydrogen atoms participating in the reduction at C-2 and C-3 of verbenone originate from the *pro*-4*S* hydrogen of NADPH and the medium, respectively.

Many studies have been reported on the enzymatic reduction of the C=C bond of α,β-unsaturated ketones with enone-reductases from microorganisms[1–3] and animals.[4–6] In these cases the reduction occurs stereospecifically by the *anti*-addition of hydrogen atoms to the C=C bond. However, in connection with our studies on the biotransformation of the α,β-unsaturated ketones such as carvone **1** and verbenone **2** with plant cell cultures,[7–9] we have now found that reduction of verbenone with an enzyme preparation from cultured cells of *Nicotiana tabacum* involves the *syn*-addition of hydrogen atoms to the C=C bond.

An enzyme responsible for the reduction of the C=C bond of verbenone **2** was prepared from cultured suspension cells of *N. tabacum*.† Incubation of (1*S*,5*S*)-verbenone **2a** {$[\alpha]_D^{25}$ −208° (neat)} with the verbenone reductase gave (1*S*,2*R*,5*S*)-*cis*-verbanone **3** in 20–60% yield,‡ while incubation of (1*R*,5*R*)-verbenone **2b** {$[\alpha]_D^{25}$ +210° (neat)} did not give any reduction products. These results indicate that the enzyme catalyses the reduction of the C=C bond of verbenone in both an enantio- and stereo-specific manner.

To clarify the stereochemistry of the enzymatic reduction, four experiments were performed. (1*S*,5*S*)-Verbenone **2a** was incubated with the enzyme preparation in the presence of: (*a*) NADPH in H_2O; (*b*) NADPH in ^2H_2O; (*c*) (4*S*)-[4-^{2}H]NADPH in H_2O and (*d*) (4*R*)-[4-^{2}H]NADPH in H_2O.§ The deuterium contents and labelling patterns in the resulting *cis*-verbanones were determined by NMR and mass spectroscopy.

cis-Verbanone **3** produced in the incubation in the presence of NADPH 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 *cis*-verbanone 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

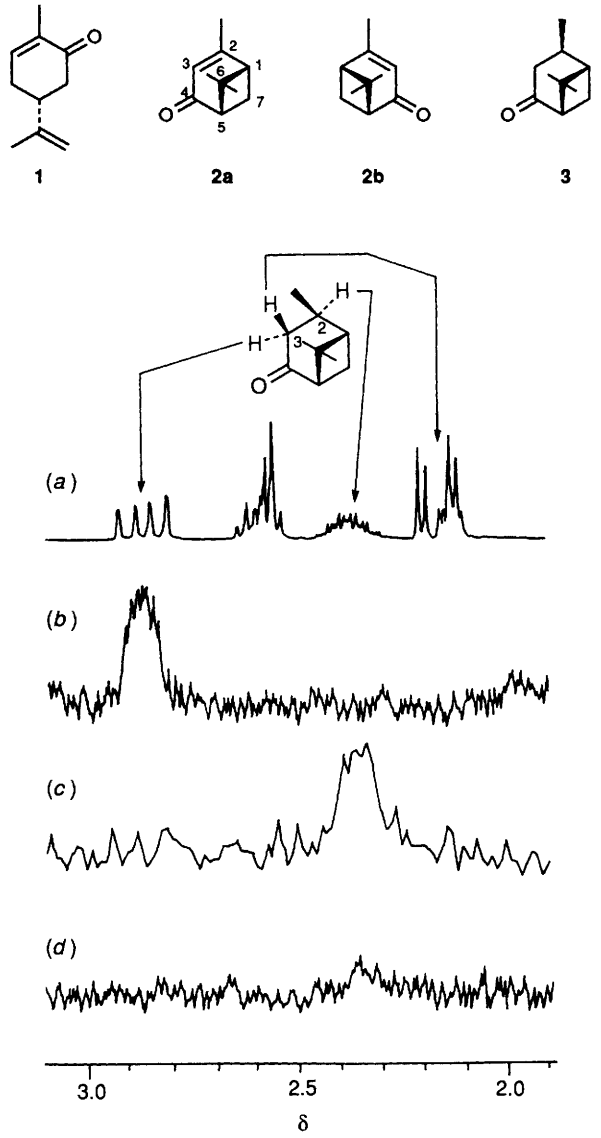

Fig. 1 NMR spectra of *cis*-verbanone **3** obtained by the enzymatic reduction of (1*S*,5*S*)-verbenone **2a**: (*a*) ^{1}H NMR spectrum for the product by the incubation with NADPH in H_2O, (*b*) ^{2}H NMR spectra for the products by the incubation with NADPH in ^2H_2O, (*c*) the incubation with (4*S*)-[4-^{2}H]NADPH in H_2O and (*d*) incubation with (4*R*)-[4-^{2}H]NADPH in H_2O

† Homogenates of the cultured cells in 50 mmol dm^{-3} phosphate buffer (pH 6.8) were centrifuged at 10 000 g to give a cell-free extract, which was treated with ammonium sulfate (40–80% sat.) to give a crude enzyme preparation. The preparation was subjected to chromatography on a Sephadex G-25 column, a diethylaminoethyl-Toyopearl column, and then a Red Toyopearl column to give a verbenone reductase: M_r *ca.* 37 000 by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, NADPH dependent and optimum pH 8.0.

‡ Verbenone (2.0 mg) and NADPH (20 mg) in 1% Triton X-100 (2 ml) was added to the enzyme preparation (8 ml, 100 μg protein; pH 7.7). The mixture was incubated for 24 h at 35 °C. After incubation, the reaction mixture was extracted with diethyl ether and the extract was analysed by GC and GC-MS. The extract was also subjected to prep. GC to give *cis*-verbanone **3** { 0.6 mg; *m/z* (rel. int.) 152 (M^+, 11), 137 (12), 95 (50) and 83 (100); ^{1}H NMR ($CDCl_3$) δ 1.01 [3H, s, C(9)Me], 1.17 [3H, d, *J* 7.3 Hz, C(10)Me], 1.34 [3H, s, C(8)Me], 2.16 [dd, *J* 20.0 and 4.9 Hz, C(3)-H *trans*-oriented to the C(2)-H], 2.38 [m, C(2)-H] and 2.87 [dd, *J* 20.0 and 10.7 Hz, C(3)-H *cis*-oriented to the C(2)-H]}. Assignments of ^{1}H NMR signals of *cis*-verbanone **3** were made by evaluation of their H/H coupling constants[10] on a 500 MHz NMR spectrum; the assignments were confirmed by a 2D ^{1}H–^{1}H shift correlation NMR spectrum of an authentic verbanone. In addition, assignment of the signals due to two protons at C-3 was jusified by the fact that the proton signal at δ 2.16 showed NOEs to the signals at δ 1.01 due to the C(9)-3H and at δ 1.17 due to the C(10)-3H.

§ (4*S*)-[4-^{2}H]NADPH [99% ^{2}H-enrichment; ^{1}H NMR (D_2O): δ 2.81 (bs, 4*R*-H), 5.95 (d, –O–CH_2–), 6.19 (d, –O–CH_2–) and 6.92 (s, 2-H)] was prepared by the enzymatic reduction of [4-^{2}H]NADP$^+$ with sodium isocitrate and isocitrate dehydrogenase following the reported method.[11,12] (4*R*)-[4-^{2}H]NADPH [89% ^{2}H-enrichment; ^{1}H NMR (D_2O) δ 2.66 (b s, 4S-H), 5.94 (d, –O–CH_2–), 6.18 (d, –O–CH_2–) and 6.90 (s, 2–H)] was prepared by the reduction of β-NADP$^+$ with [$^2H_6$]EtOH (99% ^{2}H-enrichment) and yeast alcohol dehydrogenase.

the control experiment (*a*). This indicates that the deuterium atom originating from ^2H_2O is incorporated into *cis*-verbanone **3**. On the other hand, the mass spectrum of *cis*-verbanone produced when (4*S*)-[4-^{2}H]NADPH was included in the incubation mixture [experiment (*c*)] showed a peak at *m/z* 153 $[M + 1]^+$. However, when the (4*S*)-[4-^{2}H]NADPH was replaced by (4*R*)-[4-^{2}H]NADPH [experiment (*d*)], no deuteriation of *cis*-verbanone was observed. These observations indicate that only the *pro*-4*S* hydrogen of NADPH is incorporated into *cis*-verbanone **3** during the enzymatic reduction of the double bond.

The labelled sites in the deuterium-labelled *cis*-verbanone were determined from their ^{1}H and ^{2}H NMR spectra. The ^{1}H NMR spectrum of *cis*-verbanone produced in experiment (*b*) revealed the absence of the signal at δ 2.87 due to the proton at C-3 *cis*-oriented to the C(2)-H which was seen in the spectrum of standard of *cis*-verbanone. The deuteriated site of the *cis*-verbanone was confirmed by ^{2}H{^{1}H} NMR spectroscopy, as shown in Fig. 1. The spectrum of *cis*-verbanone produced in experiment (*b*) showed only a signal at δ 2.87 (95% enrichment)¶ due to the C(3)-^{2}H with a *cis*-orientation to the C(2)-H. However, verbanone produced in experiment (*c*) exhibited a signal at δ 2.38 due to the C(2)-H; the ^{2}H-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-^{2}H]NADPH and ^2H_2O, respectively.

It was thus established that the reduction of the C=C bond of verbenone with the reductase from *N. tabacum* occurs enantio- and stereo-specifically by the *syn*-addition of hydrogen atoms to the *re-re* face of the C=C bond and the hydrogen atoms participating in the reduction at C-2 and C-3 originate from the *pro*-4*S* hydrogen of NADPH and the medium, respectively.

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¶ The ^{2}H-enrichment factor at the labelled sites 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.

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