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Hydroxylation of Acetoxy-*p*-menthenes in the Cultured Cells of *Nicotiana tabacum*. Epoxidation of the Carbon-Carbon Double Bond

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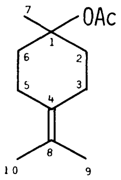
The process of the formation of glycols in the biotransformation of 1-acetoxy-*p*-menth-4(8)-ene and 8-acetoxy-*p*-menth-1-ene in the cultured cells of *Nicotiana tabacum* was examined. The formation of the glycols was found to result from epoxidation of the C–C double bond, followed by hydrolysis of the resulting epoxides.

Recent studies on the biotransformation of acetoxy-*p*-menthenes in the cultured cells of *Nicotiana tabacum* showed that the cells have the ability to hydroxylate regio- and stereoselectively the C–C double bond and its allylic position to yield the corresponding glycols and allyl alcohols.[1—3)] Also the hydroxylation of 8-acetoxy-*p*-menth-1-ene was enantioselective;[3,4)] the C–C double bond of the 4*S* enantiomer was hydroxylated in preference to the 4*R* one, whereas the hydroxylation at the allylic position was in favor of the 4*R* isomer. Such a difference in enantioselectivity in two types of the hydroxylation remarked us that the mechanism may differ from one another. We have investigated the process of hydroxylation of the C–C double bond in the cultured cells of *N. tabacum.*

Results and Discussion

Formation of Epoxides in the Biotransformation of Acetoxy-*p*-menthenes. In order to find intermediates in the hydroxylation of the C–C double bond, each of 1-acetoxy-*p*-menth-4(8)-ene (**1**) and (±)-8-acetoxy-*p*-menth-1-ene (**2**) was incubated for 2 d with a suspension of cultured cells of *N. tabacum* similarly to the manner described in our previous paper,[1)] and careful TLC and GLC analyses indicated that the products were composed of unidentified products (**3**—**6**), in addition to the previously described hydroxylated products, such as *t*-1-acetoxy-*p*-menthane-*r*-4,8-diol (**7**) and 8-acetoxy-*c*-4-*p*-menthane-*r*-1,*t*-2-diol (**8**).[1,3)] The unidentified products **3**—**6** were proved to be the corresponding epoxides of the acetoxy-*p*-menthenes by comparison of TLC, GLC, and GLC-MS with those of synthetic specimens described in the next section. Incubation of **1** gave *r*-1-acetoxy-*t*-4,8-epoxy-*p*-menthane (**3**) and *r*-1-acetoxy-*c*-4,8-epoxy-*p*-menthane (**4**) in 3.8 and 1.0% yields, respectively. On the other hand, incubation of **2** gave 8-acetoxy-*r*-1,*c*-2-epoxy-*c*-4-*p*-menthane (**5**) and 8-acetoxy-*r*-1,*c*-2-epoxy-*t*-4-*p*-menthane (**6**) in 2.0 and 0.1% yields, respectively.



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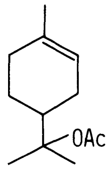

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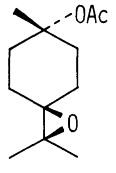

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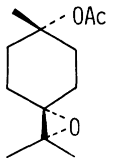

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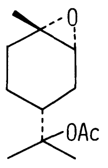

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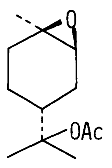

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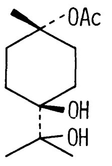

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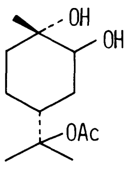

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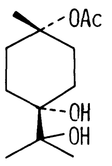

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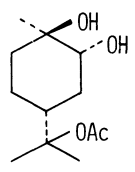

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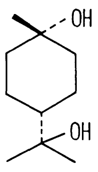

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The time-courses in the biotransformation of the acetoxy-*p*-menthenes **1** and **2** in a suspension of the cultured cells were followed. In the case of **1**, the formation of the epoxides **3** and **4** was observed at an earlier period of incubation, but their content gradually decreased with the lapse of time after incubation for 4 d, as shown in Fig. 1a. Similar phenomenon was also observed in the case of 8-acetoxy-*p*-menth-1-ene (**2**) (Fig. 1b). Such a decrease in the amount of the epoxides is probably due to further transformation to other products such as glycols, which had been the main products in the biotransformation of the acetoxy-*p*-menthenes.1,3)

To elucidate the conversion of the epoxides to the glycols, each of the epoxides **3**—**6** was incubated under the same conditions as described above. The remarkable formation of the corresponding glycols was observed as shown in Fig. 2. The epoxides **3** and **6** were transformed to a single product, *t*-1-acetoxy-*p*-menthane-*r*-4,8-diol (**7**) and 8-acetoxy-*c*-4-*p*-menthane-*r*-1,*t*-2-diol (**8**), respectively, whereas the epoxides **4** and **5** gave a mixture of glycols, the glycol (**7**) and *c*-1-acetoxy-*p*-menthane-*r*-4,8-diol (**9**) in a ratio of 1:6 from **4** and the glycol **8** and 8-acetoxy-*t*-4-*p*-menthane-*r*-1,*t*-2-diol (**10**) in a ratio of 4:1 from **5**. Exclusive formation of the glycol **8** in the conversion of the epoxide **6** indicates occurrence of the cleavage of the epoxy ring at only C-1 and not at C-2. Such an exclusive conversion is probably due to the difference in the stability of intermediate cations in the transition state, as indicated by the MO calculation5) that the total energy of the cation having a positive charge at C-1 is lower by 26 kcal† mol^{-1} than that of the cation at C-2. The transformation of the epoxides to the glycols was also found when the epoxides were treated with Murashige and Skoog's solution6) used as culture medium. These findings clearly indicate that the epoxides are chemically converted to the corresponding glycols with this solution. The rate of the transformation of the epoxide **6** to the glycol **8** was very fast, as shown in Fig. 2d; this may be responsible for a little accumulation of the epoxide **6** in the biotransformation of **2**.

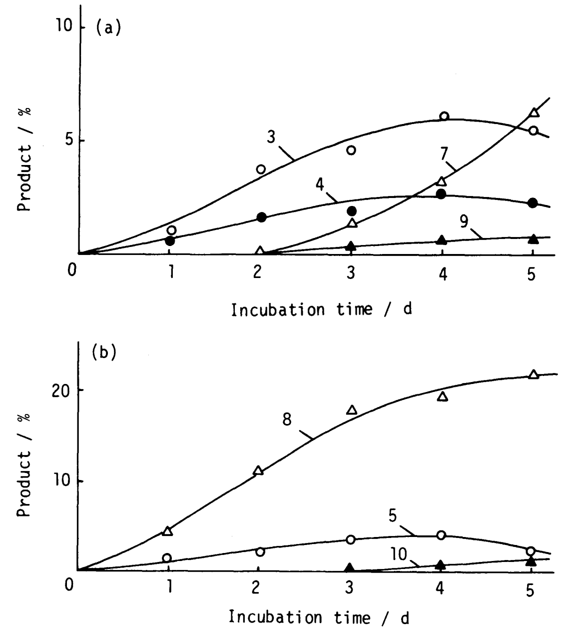

Fig. 1. Formation of the epoxides **3**—**5** and the glycols **7**—**10** in the biotransformation of (a) 1-acetoxy-*p*-meth-4(8)-ene (**1**) and (b) 8-acetoxy-*p*-menth-1-ene (**2**) in the cultured cells of *N. tabacum.*

The transformation of the acetoxy-*p*-menthenes to glycols in the cultured cells of *N. tabacum* was thus found to involve epoxidation of the double bond by a biological process, followed by hydrolysis of the resulting epoxides by a chemical one.

Structure Elucidation of the Synthetic Epoxides. For identification of the stereochemical structures of the epoxides produced in the biotransformation of the

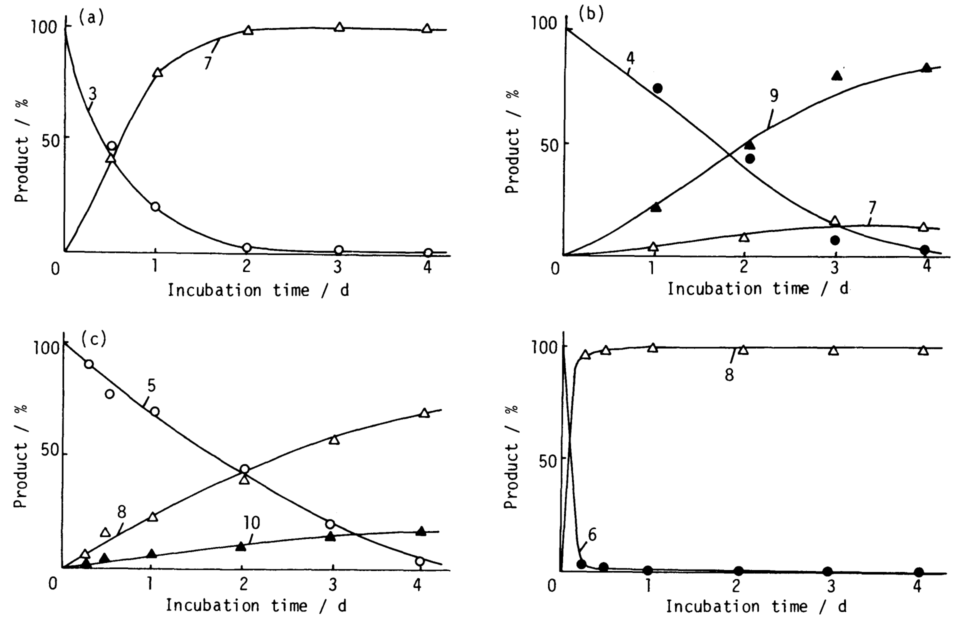

Fig. 2. The time-courses in the biotransformation of (a) *r*-1-acetoxy-*t*-4,8-epoxy-*p*-menthane (**3**), (b) *r*-1-acetoxy-*c*-4,8-epoxy-*p*-menthane (**4**), (c) 8-acetoxy-*r*-1,*c*-2-epoxy-*c*-4-*p*-menthane (**5**) and 8-acetoxy-*r*-1,*c*-2-epoxy-*t*-4-*p*-menthane (**6**) in the cultured cells of *N. tabacum.*

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† 1 cal=4.184 J.

acetoxy-*p*-menthenes in the cultured cells, epoxides were prepared from **1** and **2** by epoxidation with *m*-chloroperbenzoic acid to determine their relative spatial arrangements between the epoxy ring and the 1-acetoxyl and 1-acetoxy-1-methylethyl groups, as described below. Epoxidation of **1** gave a 3:7 mixture of epoxides A and B which were identical with the products **4** and **3** in the biotransformation of **1**, respectively, in all respects of their spectra and chromatographic behavior. The fragmentation pattern in the mass spectrum of the epoxide A was quite similar to that of the epoxide B; this implies the epoxide A to be a geometrical isomer of the epoxide B. The epoxides A and B exhibited the ^{1}H NMR signals at δ 1.36 and 1.33, respectively, due to a tertiary *gem*-dimethyl group, instead of the signal (δ 1.67) due to the methyl groups attached to the 4(8)-double bond of **1**. Accordingly, the epoxides A and B were suggested to be 4,8-epoxides of **1**. However, these spectral data did not give any reliable informations for determining the relative orientation between the epoxy ring and the 1-acetoxyl group in the epoxides. The structure of the epoxide A was finally determined by X-ray crystallography. An ORTEP drawing[7] of the epoxide A is given in Fig. 3; the epoxy ring was found to be cis to the acetoxyl group. The epoxide A was thus established to be *r*-1-acetoxy-*c*-4,8-epoxy-*p*-menthane (**4**). Therefore, the epoxide B should be *r*-1-acetoxy-*t*-4,8-epoxy-*p*-menthane (**3**).

Epoxidation of **2** gave a 9:11 mixture of epoxides C and D which were identical with the epoxides **5** and **6** in the biotransformation of **2**, respectively. The relative orientation between the epoxy ring and the 1-acetoxy-1-methylethyl group in these epoxides was evidenced by ^{1}H NMR spectral analysis as described below. On the basis of Karplus's equation[8] and the ^{1}H NMR data reported for limonene 1,2-oxide,[9] ^{1}H–^{1}H coupling patterns for the C-2 proton of both the epoxides were predicted to be a doublet (J=4—7 Hz) for a *cis*-epoxide and a broad singlet or a triplet (J=0—2 Hz) for a *trans*-epoxide. The observed signals were a doublet (J=4.6 Hz) at δ 2.81 for the epoxide C and a broad singlet at δ 2.87 for the epoxide D; this observation suggests the relative orientation between the epoxy ring and the 1-acetoxy-1-methylethyl group in the epoxide C to be cis and that of the epoxide D to be trans. The suggested structure for the epoxide C was supported by the formation of *c*-4-*p*-menthane-*r*-1,8-diol (**11**)[10] on reduction with lithium aluminum hydride. The epoxides C and D were thus established to be 8-acetoxy-*r*-1,*c*-2-epoxy-*c*-4-*p*-menthane (**5**) and 8-acetoxy-*r*-1,*c*-2-epoxy-*t*-4-*p*-menthane (**6**), respectively. All the epoxides prepared above were used as authentic samples for the purpose of identification.

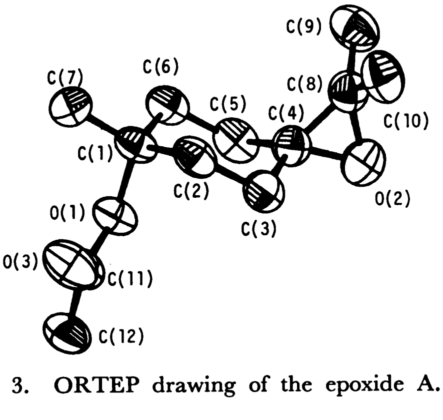

Fig. 3. ORTEP drawing of the epoxide A.

Experimental

Analytical TLC and preparative TLC were carried out on 0.25 mm and 0.75 mm thick silica-gel plates (Merck silica gel 60, GF_{254}), respectively. GLC analyses were performed on a Shimadzu GC-6A gas chromatograph equipped with an 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) by programming the column temperature at 100—200 °C with a rate of 3 °C min^{-1} for DEGS and at 90—250 °C with a rate of 2 °C min^{-1} for OV-17. GLC-MS were recorded on a Shimadzu QP-1000 mass spectrometer which was equipped with an EI ion source operating at 70 eV and installed with a gas chromatograph equipped with an OV-17 column operating at 80—200 °C with a rate of 3 °C min^{-1}.

Substrate. 1-Acetoxy-*p*-menth-4(8)-ene (**1**) [IR ν_{max} (film) 1740 cm^{-1} (ester C=O); ^{1}H NMR ($CDCl_3$) δ=1.49 (3H, s, 7-Me), 1.67 (6H, bs, 9- and 10-Me), and 1.98 (3H, s, OAc); *m/z* (rel intensity) 136 (M^+—AcOH, 80%), 121 (100), and 93 (65)] was prepared from limonene (Ogawa Perfumery Co. Ltd.), according to the procedure described in our previous paper.[1]

(±)-8-Acetoxy-*p*-menth-1-ene (**2**) [IR ν_{max} (film) 1730 cm^{-1} (ester C=O); ^{1}H NMR ($CDCl_3$) δ=1.43 (6H, s, 9- and 10-Me), 1.64 (3H, bs, 7-Me), 1.96 (3H, s, OAc), and 5.32 (1H, bs, >C=CH–); *m/z* (rel intensity) 136 (M^+—AcOH, 85%), 121 (100), and 93 (63)] was prepared from (±)-*p*-menth-1-en-8-ol (α-terpineol) (Takasago Perfumery Co. Ltd.) by acetylation, followed by chromatography on a silica-gel column with hexane-ethyl acetate (97:3, v/v). Both the substrates used were >99.5% pure on GLC.

Incubation of Acetoxy-*p*-menthenes 1 and 2 in a Suspension of the Cultured Cells. A suspension of the cultured cells of *N. tabacum* "Bright Yellow" were prepared as described in our previous paper;[1] the cells were cultured in each 300-ml conical flask containing 100 ml of Murashige and Skoog's solution.[6] To the flask containing a suspension of the cultured cells, 10 mg of the substrate, such as 1-acetoxy-*p*-menth-4(8)-ene (**1**) and (±)-8-acetoxy-*p*-menth-1-ene (**2**), were administered; the total amount of the substrate used was 100 mg. The mixture was incubated at 25 °C for 2 d on a rotary shaker (70 rpm) in the dark. The cultured mixture was worked up in a manner similar to that described in our previous paper.[1] An incubation product obtained was subjected to the repeated TLC with hexane–ethyl acetate (7:3 and 1:1, v/v). Constituents of the product were identified by comparing their TLC, GLC, and GLC-MS with those of authentic specimens.

Time-Courses in the Biotransformation of Acetoxy-*p*-menthenes 1 and 2 and Epoxides 3—6. Each of the substrates was administered to a suspension of the precultured cells in each flask, as described above. A part of the incubation mixture (10 ml) was pipetted out and extracted with ether at a regular time interval. Each ether extract was dissolved in ethyl acetate and then subjected to GLC. The products were identified by comparison (co-GLC and GLC-MS) with authentic samples.[1,3] Yields of the products were determined on the basis of the peak areas on GLC and are expressed as the relative percent for the total amount of a whole reaction product extracted. The time-courses in the biotransformation are given in Figs. 1 and 2.

Treatment of Acetoxy-*p*-menthenes 1 and 2 and Epoxides 3—6 with Murashige and Skoog's Solution. Each of the substrates (10 mg) were shaken with 100 ml of Murashige and Skoog's solution (pH 5.8) at 25 °C for 4 d in the dark. A reaction mixture was extracted with ether to give an oily substance, which was subjected to TLC, GLC, and GLC-MS. The oily substance was composed of only the unchanged acetoxy-*p*-menthene in both the cases of the acetoxy-*p*-menthenes **1** and **2**.

The epoxides **3**—**6** were completely converted to the corresponding glycols. These glycols were identified by comparison of their TLC, GLC, and GLC-MS with those of authentic samples.[1,3] Each of the oily substances (9 and 10 mg) obtained in the reactions of **3** and **6** with Murashige and Skoog's solution was composed of only *t*-1-acetoxy-*p*-menthane-*r*-4,8-diol (**7**) and 8-acetoxy-*c*-4-*p*-menthane-*r*-1,*t*-2-diol (**8**), respectively, by GLC and GLC-MS analyses. In the case of the epoxide **4**, the oily substance (10 mg) was composed of the glycol **7** and *c*-1-acetoxy-*p*-menthane-*r*-4,8-diol (**9**) in a ratio of 1 : 6 on the basis of the peak areas on GLC. On the other hand, the oily substance (9 mg) from the epoxide **5** was composed of the glycol **8** and 8-acetoxy-*t*-4-*p*-menthane-*r*-1,*t*-2-diol (**10**) in a ratio of 4 : 1. The time-courses of the conversion of the epoxides **3**—**6** to the corresponding glycols in Murashige and Skoog's solution were quite similar to those in the case of the transformation of the epoxides in a suspension of the cultured cells (Fig. 2).

Table 1. Final Atomic Coordinates and Thermal Parameters of the Epoxide **4** with Standard Deviations in Parentheses

Atom	$10^4 x$	$10^4 y$	$10^4 z$	B_{eq}[a)]/Å^2
C(1)	6117 (7)	4790 (9)	1682 (4)	4.2
C(2)	6791 (7)	4292 (9)	2700 (4)	4.5
C(3)	8269 (7)	4490 (9)	2998 (5)	4.5
C(4)	8594 (7)	6330 (9)	2806 (5)	4.3
C(5)	8041 (7)	6765 (9)	1775 (5)	5.1
C(6)	6565 (7)	6601 (8)	1480 (5)	4.5
C(7)	4636 (7)	4690 (10)	1414 (5)	5.3
C(8)	8895 (7)	7690 (10)	3532 (5)	4.7
C(9)	8697 (8)	9598 (10)	3268 (6)	6.3
C(10)	8909 (8)	7307 (11)	4524 (5)	6.2
C(11)	6386 (7)	1990 (9)	971 (5)	4.8
C(12)	6924 (9)	1199 (10)	271 (5)	6.2
O(1)	6586 (5)	3690 (5)	1045 (3)	4.6
O(2)	9933 (5)	6843 (7)	3261 (4)	5.9
O(3)	5819 (5)	1188 (7)	1419 (4)	7.0

a) $B_{eq}=(B_{11}+B_{22}+B_{33})/3$.

Preparation of Authentic Samples. i) *r*-1-Acetoxy-*t*-4,8-epoxy-*p*-menthane (**3**) and *r*-1-acetoxy-*c*-4,8-epoxy-*p*-menthane (**4**). Following the procedure described in the reference,[11] 1-acetoxy-*p*-menth-4(8)-ene (**1**) (66 mg) dissolved in dichloromethane (4 ml) was epoxidized with *m*-chloroperbenzoic acid (75 mg) at 0 °C for 4 h. The reaction mixture, after working-up as usual, was subjected to preparative TLC with hexane–ethyl acetate (9 : 1, v/v) to afford the epoxide **3** [35 mg; IR ν_{max} (film) 1730 cm^{-1} (ester C=O); ^{1}H NMR ($CDCl_3$) δ=1.33 (6H, s, 9- and 10-Me), 1.54 (3H, s, 7-Me), and 2.02 (3H, s, OAc); *m/z* (rel intensity) 152 (M^+—AcOH, 6), 110 (45), and 79 (100)] and the epoxide **4** [15 mg; mp 86.5—87.0 °C, IR ν_{max} (film) 1735 cm^{-1} (ester C=O); ^{1}H NMR ($CDCl_3$) δ=1.36 (6H, s, 9- and 10-Me), 1.54 (3H, s, 7-Me), and 2.04 (3H, s, OAc); *m/z* (rel. intensity) 152 (M^+—AcOH, 2), 110 (44), and 79 (100)].

ii) 8-Acetoxy-*r*-1,*c*-2-epoxy-*c*-4-*p*-menthane (**5**) and 8-acetoxy-*r*-1,*c*-2-epoxy-*t*-4-*p*-menthane (**6**). Epoxidation of 8-acetoxy-*p*-menth-1-ene (**2**) (1.02 g) with *m*-chloroperbenzoic acid (1.2 g) in dichloromethane (25 ml) at 5 °C for 4 h, followed by working-up in the same manner as above, gave the epoxide **5** [269 mg; IR ν_{max} (film) 1729 (ester C=O); ^{1}H NMR ($CDCl_3$) δ=1.23 (3H, s, 9-Me), 1.29 (3H, s, 9-Me), 1.31 (3H, s, 10-Me), 1.88 (3H, s, OAc), and 2.81 (1H, d, *J*=4.6 Hz, 2-H); *m/z* (rel intensity) 152 (M^+—AcOH, 8), 137 (7), 126 (21), and 43 (100)] and the epoxide **6** [335 mg; IR ν_{max} (film) 1725 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ=1.26 (3H, s, 7-Me), 1.38 (6H, s, 9- and 10-Me), 1.88 (3H, s, OAc), and 2.87 (1H, bs, 2-H); *m/z* (rel intensity) 152 (M^+—AcOH, 5), 137 (7), 126 (25), and 43 (100)].

Reduction of the Epoxide 5. The epoxide **5** (10 mg) dissolved in ether (2 ml) was refluxed with a suspension of $LiAlH_4$ (10 mg) in ether (2 ml) for 30 min. The reaction mixture, after working-up as usual, gave a crude product (6 mg). This crude product, after PTLC with hexane–ethyl acetate (7 : 3, v/v), gave *c*-4-*p*-menthane-*r*-1,8-diol (**11**):[10] 6 mg; mp and mmp 105—106 °C (lit,[12] mp 104—105 °C); IR ν_{max} (film) 3450 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$) δ=1.18 (6H, s, 9- and 10-Me) and 1.21 (3H, s, 7-Me); *m/z* (rel intensity) 136 (M^+—$2H_2O$, 1), 96 (23), and 81 (100).

X-Ray Crystallographic Analysis of the Epoxide 4. The X-ray crystallographic analysis was performed on a Syntex R3 diffractometer using graphite-monochromated Mo *Kα* radiation. The crystal analyzed was 0.62 mm×0.35 mm×0.87 mm in size. Crystal data: $C_{12}H_{20}O_3$, monoclinic, a=10.891 (5), b=7.747 (7), c=15.112 (8) Å, β=108.47 (4)°, U=1209.4 Å^3, Z=4, D_c=1.17 g cm^{-3}, D_m=1.20 g cm^{-3}, μ(Mo *Kα*)=0.9 cm^{-1}, space group $P2_1/c$. A total of 1316 reflections were collected by ω-scan ($2\theta_{max}$=50.0) and used in the structure determination. The phases of 204 reflections with $|E|>1.30$ were determined by MULTAN.[13] An E map for the best solution yielded positions for all non-hydrogen atoms. Anisotropic refinement for carbon and oxygen atoms and isotropic refinement for hydrogen atom by full-matrix least-squares calculation reduced the R index finally to 0.042. Final atomic coordinates are shown in Table 1. The lists of anisotropic thermal parameters and final structure factors have been deposited as Document No. 8718 at the Office of the Editor of the Chemical Society of Japan.

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