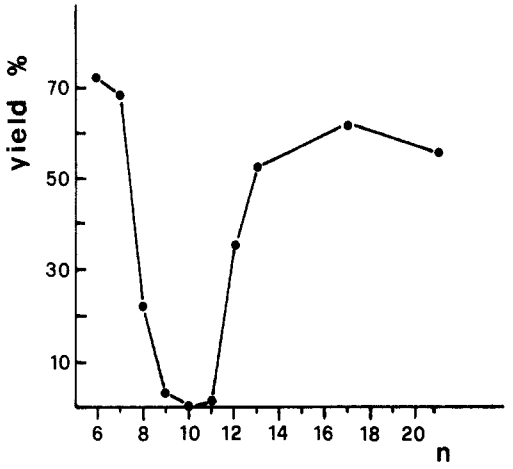

Figure 1. Plot of yields vs. ring size n for the cyclization of diethyl ω-bromoalkylmalonates in Me_2SO at 80 °C.

24 h. This finding and the failure to obtain quantitative yield of cyclic product even in the common ring region suggests the reaction might involve side reactions other than polymerization.[5] Keeping in mind the difficulties one has to overcome to generate a carbanion and to have it survive long enough to give the desired reaction, we suspect that better results than those reported here can probably not be obtained in the formation of wholly saturated carbocycles via intramolecular nucleophilic substitution.

Yield data from the present work may be compared with those reported for other cyclization reactions via C–C bond formation.[6] A plot of yields vs. ring size for the cyclization of ω-bromoalkylmalonates (Figure 1) has the familiar shape of the yield profiles for classical cyclization reactions, such as the Ruzicka, Thorpe–Ziegler, and Dieckmann reactions.[6] It is worth noting that the minimum is located at ring sizes 9 to 11, which exactly corresponds to the maximum strain energy of cycloalkanes.[6] Since the same rate of feed was used throughout the series, the yield data very likely reflect the relative ring-closure tendencies, thus providing additional evidence as to the importance of strain on the ease of cyclization of bifunctional chain molecules.

Experimental Section

The diethyl ω-bromoalkylmalonates were prepared and purified as previously reported.[2b,7] The preparation of $Br(CH_2)_5CH(CO_2Et)_2$ also gave 1,1-bis(ethoxycarbonyl)cyclohexane as a by-product, bp 100–104 °C (3 mmHg) [lit.[8] bp 105–106 °C (5mmHg)]. A.R. grade Me_2SO (ERBA RPE) was purged with argon before use. 18-Crown-6 was from Aldrich.

A Sage Instrument syringe pump Model 355 was used. ^1H NMR spectra were taken from CCl_4 solutions on a JEOL JNM-C60 HL spectrometer. Mass spectra were obtained on a AEI MS 12 spectrometer. For GLC–MS analyses the latter instrument was matched to a Varian Aerograph Series 1400 gas chromatograph. GLC analyses were performed by the internal standard method on a Hewlett-Packard Model 5830 A instrument, fitted with a 1.8-m OV-17 column operated in the range of 100 to 220 °C, depending on molecular weight.

Cyclization Procedure. All operations were carried out under pure nitrogen. The reaction was run in a 250-mL three-necked flask containing a well-stirred solution of 18-crown-6 (2.2 mmol) in Me_2SO (200 mL) heated at 80 °C. The reagents were added separately and simultaneously over a 3.5-h period by means to two identical motor-driven syringes. One syringe contained 2 mmol of the proper ω-bromoalkylmalonic ester in 30 mL of Me_2SO and the other 2 mmol of KOH in 30 mL of Me_2SO–EtOH (93:7, v/v). After being stirred at 80 °C for an additional 15 min, the mixture was cooled to room temperature, poured into ice–water containing $Ba(NO_3)_2$, and worked up with ether. A small portion of the ether solution was used for GLC analysis. The remaining part was evaporated and the residue was eluted with benzene/CCl_4 (1:1) on aluminum oxide 90 (Merck). The dimeric cycles were obtained upon further elution with benzene.

All the isolated monomeric rings were further purified by sublimation or microdistillation in vacuo and found to be at least 99.5% pure (GLC). They were characterized by elemental analysis data, and ^1H NMR and mass spectra. Along with the expected molecular ion, all the compounds showed a strong peak at m/e 173, possibly due to the fragment $[CH_2CH(CO_2Et)_2]^+$. The 9-, 10-, and 11-membered rings were detected and characterized by GLC–MS analysis. The dimeric rings showed ^1H NMR spectra very similar to those of the monomeric isomers and mass spectra consistent with the assigned structures.

Registry No. 1,1-Bis(ethoxycarbonyl)cyclohexane, 1139-13-5; 1,1-bis(ethoxycarbonyl)cycloheptane, 6557-83-1; 1,1-bis(ethoxycarbonyl)cyclooctane, 76999-11-6; 1,1-bis(ethoxycarbonyl)cyclononane, 76999-12-7; 1,1-bis(ethoxycarbonyl)cyclodecane, 76999-13-8; 1,1-bis(ethoxycarbonyl)cycloundecane, 76999-14-9; 1,1-bis(ethoxycarbonyl)cyclododecane, 76999-15-0; 1,1-bis(ethoxycarbonyl)cyclotridecane, 37689-04-6; 1,1-bis(ethoxycarbonyl)cycloheptadecane, 76999-16-1; 1,1-bis(ethoxycarbonyl)cycloheneicosane, 76999-17-2; 1,1,9,9-tetrakis(ethoxycarbonyl)cyclohexadecane, 76999-18-3; 1,1,10,10-tetrakis(ethoxycarbonyl)cyclooctadecane, 76999-19-4; 1,1,11,11-tetrakis(ethoxycarbonyl)cycloeicosane, 76999-20-7; 1,1,12,12-tetrakis(ethoxycarbonyl)cyclodocosane, 37689-02-4; 1,1,13,13-tetrakis(ethoxycarbonyl)cyclotetracosane, 76999-21-8; 1,1,14,14-tetrakis(ethoxycarbonyl)cyclohexacosane, 37689-00-2; $Br(CH_2)_5CH(CO_2Et)_2$, 1906-95-2; $Br(CH_2)_6CH(CO_2Et)_2$, 6557-85-3; $Br(CH_2)_7CH(CO_2Et)_2$, 76999-22-9; $Br(CH_2)_8CH(CO_2Et)_2$, 77011-21-3; $Br(CH_2)_9CH(CO_2Et)_2$, 76999-23-0; $Br(CH_2)_{10}CH(CO_2Et)_2$, 76999-24-1; $Br(CH_2)_{11}CH(CO_2Et)_2$, 76999-25-2; $Br(CH_2)_{12}CH(CO_2Et)_2$, 35295-45-5; $Br(CH_2)_{16}CH(CO_2Et)_2$, 76999-26-3; $Br(CH_2)_{20}CH(CO_2Et)_2$, 63099-18-3.

(5) Diethyl butylmalonate can be spectrophotometrically titrated with EtOK in Me_2SO owing to the strong absorption of its conjugate base (λ_{max} 274 nm; ϵ_{max} 2.4×10^4 M^{-1} (cm^{-1}). The absorption is not stable, but disappears with a half-life time of ~16 min at room temperature. The fate of the carbanion is unknown.

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Practical Approach to High-Yield Enzymatic Stereospecific Organic Synthesis in Multiphase Systems

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The stereoselective synthesis of epoxides is of current interest because of the large number of stereospecific reactions undergone by epoxides.[1] There have been many attempts to prepare optically active epoxides via asymmetric oxidation using chiral peracids,[2-4] chiral phase-transfer catalysts,[5,6] and organometallic complexes with chiral ligands.[7-9] Some of these procedures have been quite successful. Using chiral titanium complexes, Sharpless has epoxidized allyl alcohols in optical yields above 90%.[9] Epoxidation of α,β-unsaturated ketones with chiral catalysts has, however, not exceeded 55% enantiomeric excess (ee).[6] The preparation of nonfunctionalized chiral epoxides of high enantiomeric purity remains a

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formidable task,[2,3] usually requiring tedious resolution of appropriate precursors.[10,11] Only one case of direct resolution of a simple racemic epoxide has been recorded.[12]

Microbial systems, on the other hand, exhibit a considerable degree of stereoselectivity due to the stereospecificity of the enzymes involved. Thus, *Corynebacterium equi* oxidizes 1-hexadecene to the corresponding optically pure (*R*)-epoxide.[13] Similarly, the conversion of 1,7-octadiene into (*R*)-7,8-epoxy-1-octene by the "ω-hydroxylation" system of *Pseudomonas oleovorans* proceeds with an optical yield greater than 80%.[14]

Nevertheless, while efficient conversion has sometimes been observed,[15] the total yield of epoxide per liter of culture has been very low, because the culture never contains more than 1% (v/v) alkene and because the epoxide formed enzymatically is isolated in very low yields or not at all.[14,15] Such microbial systems have therefore not yet proven practical in the synthesis of optically pure epoxides.

Since it is our aim to produce large amounts of chiral epoxide, we have made considerable efforts to optimize the conversion of 1-octene to 1,2-epoxyoctane by *P. oleovorans*, as described elsewhere.[16] This was achieved by epoxidation of 1-octene by *P. oleovorans* in a two-phase system in which the substrate 1-octene itself was present in sufficient amounts (20–50% (v/v)) to serve as the second phase. In addition, in optimizing the *P. oleovorans* biotransformation system, we found that the bulk apolar phase causes considerable damage to the cell membranes, while the product (epoxide) reacts with cellular enzymes, both of which factors reduce and eventually abolish all enzymatic activity.[16] To circumvent these problems, it is necessary to remove the damaged cells from the fermentation, and to add fresh cells to the apolar phase at regular intervals. In principle, this system, which shows some resemblance to the mixed two-phase system of Schwartz and McCoy,[15] allows the practical production of chiral epoxides because first, substrate is never limiting, second, there are no side products which derive from the bulk phase (e.g., cyclohexanol may be formed in the experiments of Schwartz and McCoy[15,17]), third, enzyme and cell damage are circumvented by cell renewal, and fourth, purification of the product requires the separation of epoxide from only one component, namely, 1-octene.

In this paper we describe the isolation of (*R*)-1,2-epoxyoctane produced in large amounts by *P. oleovorans* cells and show that this product has an enantiomeric purity of 70%.

Experimental Section

General Methods. Hydrocarbon substrates were purchased from various sources and were of the highest grades commercially available.

Infrared spectra were recorded on a Unicam SP-200 infrared spectrophotometer. A Varian XL-100 was used for the 100-MHz ^{1}H NMR spectra. Chemical shift values were expressed as δ (parts per million) values relative to a tetramethylsilane internal standard.

Optical rotations were determined with a Perkin-Elmer 241 polarimeter. *P. oleovorans* TF4-1L (ATCC 29347) was used throughout the experiments. This strain was a gift from Dr. C. J. McCoy (Corporate Research Laboratories, Exxon Research and Engineering). *P. oleovorans* was grown in minimal P-1 medium as described previously.[18] The carbon source was *n*-octane or 1-octene as indicated.

Epoxide assay. For quantitation of 1,2-epoxyoctane in the organic phase, a 20-μL sample was diluted 50-fold in *n*-hexane which contained 2-octanol (0.3 mg/mL) as an internal standard; this dilution was analyzed with gas chromatography as described by May and Abbott.[19]

A stainless-steel column was used (12 ft × $^1/_8$ in., packed with 10% Carbowax 20M on 80/100 Chromosorb W). The column temperature was maintained isothermally at 130 °C, and the carrier gas flow was 30 mL of helium/min. All determinations were performed in duplicate.

Syntheses. 1,2-Epoxyoctane was synthesized from *m*-chloroperbenzoic acid and 1-octene as described previously.[19]

Enzymatic Synthesis of 1,2-Epoxyoctane (2). Two 5-L flasks, each containing 1000 mL of P-1 medium[18] and 250 mL of 1-octene were inoculated with octane-grown cells of *P. oleovorans* and were incubated at 30 °C on a rotary shaker at 250 rpm. After 70 h the cultures were centrifuged (10000*g*, 30 min) at 0 °C, and the octene layers were separated from the water layers. The octene layers were transferred to two fresh cultures of 1000 mL inoculated with octane-grown cells. After being shaken for 70 h at 30 °C, the cultures were treated in the same way as mentioned above. The organic layers were collected and centrifuged again (18000*g*, 30 min) to remove remaining water. During growth the cells produced a white polymeric substance, which was responsible for the fact that a lot of water (about 200 mL) remained "solvated" in the organic phase.

The octene layer was dried (Na_2SO_4), and the 1-octene was evaporated under diminished pressure. The distillate, which contained 1-octene and 1,2-epoxyoctane was saved to be used in later syntheses.

The crude product was purified by distillation under diminished pressure to give 5.6 g (1.4%) of 1,2-epoxyoctane, bp 60–61 °C (15 mmHg).

1,2-Epoxyoctane was further purified by preparative gas chromatography using the same column and conditions as described under Epoxide Assay. The product showed $[\alpha]^{21}_D$ +9.3° (*c* 3.1, EtOH).

IR and ^{1}H NMR data were identical with those of chemically synthesized 1,2-epoxyoctane.

1-Methoxy-2-octanol. A small amount of sodium was added to 120 mL of dry methanol. After the reaction of the sodium was complete 4.8 g (38 mmol) of 1,2-epoxyoctane was added, and the mixture was refluxed for 7 h. After evaporation of the methanol the residue was filtered to remove the sodium methoxide; the filtrand was rinsed with n-hexane. The *n*-hexane was evaporated and the crude product was purified by distillation under diminished pressure, yielding 5.5 g (92%) of 1-methoxy-2-octanol; bp 97–98 °C (17 mmHg).

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IR and ^1H NMR data were identical with those reported in the literature.[20]

(*R*)-1-Methoxy-2-octanol (3). The same procedure as above was applied, except that enzymatically produced 1,2-epoxyoctane was used to give (*R*)-1-methoxy-2-octanol: bp 124.5–126.5 °C (36–40 mmHg); $[\alpha]^{20}_D$ +5.8° (neat).

Tris[3-(trifluoroacetyl)-*d*-camphorato]europium(III). This compound was prepared from 3-bromo-*d*-camphor and ethyl trifluoroacetate as described previously.[21,22]

Determination of the Enantiomeric Excess of 1,2-Epoxyoctane. Optical purity determinations were carried out by using the chiral shift reagent tris[3-(trifluoroacetyl)-*d*-camphorato]europium(III) as described previously.[13] At a concentration ratio of 0.3 mol of the shift reagent/mol of racemic 1-methoxy-2-octanol in CCl_4 the ^1H NMR peak due to the methoxygroup was split into two peaks at δ 5.09 and 5.00.

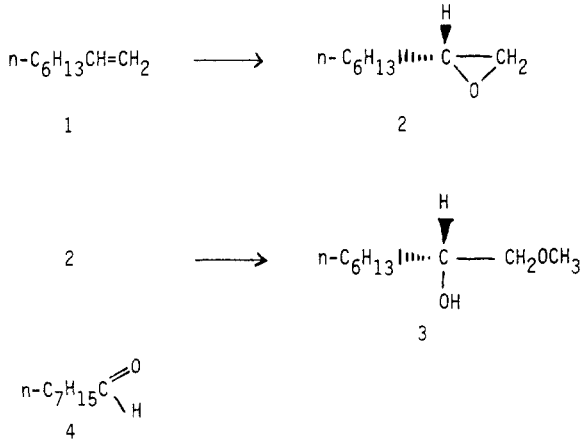
All determinations were performed in triplicate.

Results

Enzymatic Synthesis and Isolation of 1,2-Epoxyoctane. Because preliminary experiments[16] showed that *P. oleovorans* produces 1,2-epoxyoctane in high yields when it is grown on high concentrations of 1-octene, this microorganism was grown in a culture which contained 20% (v/v) 1-octene. After incubation for 70 h at 30 °C the octene layer contained 15 mg/mL of 1,2-epoxyoctane. To raise the epoxide concentration, we transferred the organic layer to a fresh culture of *P. oleovorans*, which resulted in an epoxide concentration of 32 mg/mL in this phase and in a total yield of 12.4 g of 1,2-epoxyoctane. After isolation and purification, 5.6 g of nearly chemically pure of (95%, GLC) epoxide was obtained.

The remaining 1,2-epoxyoctane was lost during evaporation of the 1-octene, probably as a consequence of azeotropy; the distillate, which contained a low concentration of the epoxide might be used in a next enzymatic synthesis, because no other compounds could be detected by GLC. No effort was made to optimize the distillation procedure.

For accurate optical rotation measurements the product was further purified by preparative gas chromatography. However, under the conditions indicated in the Experimental Section it appeared that the purified 1,2-epoxyoctane contained about 10% *n*-octanal (4) as shown by



NMR and by comparison of the GLC retention time with that of an authentic specimen. Lowering[23] the temperature of both the injection port and the detector by 30 °C resulted in a decrease of the amount of *n*-octanal to about 2%.

Determination of the Optical Purity. The purified epoxide showed $[\alpha]^{21}_D$ +9.3° (*c* 3.1, EtOH). By comparison, Hill[11] has reported an $[\alpha]^{21}_D$ value of +14.5° (*c* 3.6, EtOH) for chemically synthesized (*R*)-1,2-epoxyoctane, the optical rotation and absolute configuration of which have been correlated.[11,24] Thus, *P. oleovorans* stereoselectivity epoxidizes 1-octene to give preferentially (*R*)-1,2-epoxyoctane.

The optical purity of the enzymatic product was determined by NMR with the chiral shift reagent tris[3-(trifluoroacetyl)-*d*-camphorato]europium(III). In order to accurately integrate the NMR signals corresponding to the two enantiomeric forms of 1,2-epoxyoctane, the epoxide was converted into 1-methoxy-2-octanol.[25]

In the case of the chemically synthesized epoxide the ^1H NMR peak due to the methoxy group of 1-methoxy-2-octanol was split into two peaks of equal intensity at δ 5.09 and 5.00, whereas in the case of the enzymatic product the ratio of the integrated intensity of the peak at δ 5.00 to that of the δ 5.09 peak was 8.50:1.50.

On the basis of three such experiments the optical purity of the 1,2-epoxyoctane produced by *P. oleovorans* was found to be 70 ± 2%.

This result is in accord with the finding of May et al.[26] that the epoxidation of 1,7-octadiene by *P. oleovorans* resulted preferentially in (*R*)-7,8-epoxy-1-octene with an optical purity of 84%.

Control Experiments for Epoxide Product Isomerization or Stereospecific Degradation by Enzymes Other Than the "ω-Hydroxylation" System. First, it might be postulated that the "ω-hydroxylation" system of *P. oleovorans* produces optically pure (*R*)-1,2-epoxyoctane but that the occurrence of enzymatic or nonenzymatic isomerization processes is responsible for the 70% enantiomeric excess of (*R*)-1,2-epoxyoctane.

If this were the case, the specific rotation of the enzymatic product might not be constant during the epoxidation of 1-octene by *P. oleovorans*.

The time course of the specific rotation of 1,2-epoxyoctane produced by *P. oleovorans* was followed during growth in a culture which contained 20% (v/v) 1-octene. The results (Figure 1) clearly show that the specific rotation of the enzymatic product is constant during epoxidation of 1-octene.

Second, the possibility may exist that the "ω-hydroxylation" system is nonstereospecific and produces both enantiomers of 1,2-epoxyoctane from 1-octene but that other unrecognized enzymes are present, which stereoselectively degrade or isomerize one enantiomer.

In order to exclude this hypothetical situation, racemic 1,2-epoxyoctane, obtained by peracid epoxidation of 1-octene, was incubated for 30–48 h at 30 °C with octane-grown *P. oleovorans* cells in a culture which contained 30% (v/v) *n*-octane or *n*-hexadecane.

The presence of a second phase which harbors the epoxide decreases the toxicity of this compound, because in its absence the cells are inactivated, and no conversion of 1,2-epoxyoctane can be observed (results not shown).

The results in the Table I show that when *n*-octane is the second phase there is little degradation of 1,2-epoxy-

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(25) To our knowledge, optically active 1-methoxy-2-octanol has not been made previously. 1-Methoxy-2-octanol (3) prepared from the enzymatic product showed $[\alpha]^{20}_D$ +5.8° (neat).
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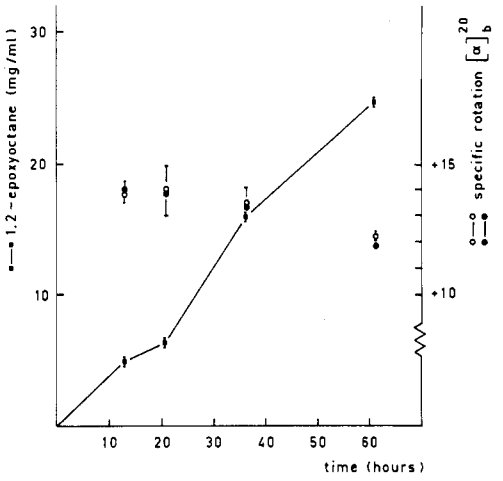

Figure 1. Time course of the specific rotation of 1,2-epoxyoctane produced by *P. oleovorans* in a 20% (v/v) 1-octene culture. A culture with 20% (v/v) 1-octene was incubated at 30 °C. At different times samples were taken from the culture. The octene layer was separated from the water layer by centrifugation; the optical rotation of the octene layer was determined, and the amount of 1,2-epoxyoctane was assayed as described in the Experimental Section: ○, specific rotation of 1,2-epoxyoctane in the octene layer; ●, corresponding values from a standard curve, for which enzymatically produced 1,2-epoxyoctane was used, and which represent the relation between the epoxide concentration and the specific rotation.

octane (5%), and a small negative optical rotation could be detected.

It is likely that this is a consequence of the higher affinity of the "ω-hydroxylation" system for *n*-octane than for the epoxide. In contrast, the epoxide concentration decreases when *n*-hexadecane, which is not metabolized by the cells, is used. At the same time, the specific rotation increases.

Three principal conclusions emerge from these results: (1) racemic 1,2-epoxyoctane is converted by the "ω-hydroxylation" system of *P. oleovorans*; (2) the *R* enantiomer is degraded faster than the *S* enantiomer to give an enrichment in (*S*)-1,2-epoxyoctane; (3) no other enzymes are present, which eventually degrade or isomerize the product of the 1-octene epoxidation.

Finally, this experiment nicely demonstrates that it is possible to degrade racemic 1,2-epoxyoctane stereospecifically with *P. oleovorans*.

Discussion

Our previous results[16] have established that large amounts of 1,2-epoxyoctane are produced by *P. oleovorans* in a two-phase fermentation with 1-octene as the second phase. The present study extends these findings and shows that the product is optically active with an enantiomeric purity of 70% (*R*)-1,2-epoxyoctane. This specific example may be expected to be generally applicable. First, numerous studies show that the "ω-hydroxylation" system can be used to oxidize a variety of aliphatic compounds.[17,27] Second, we have recently carried out a similar conversion of 1-decene to 1,2-epoxydecane with results comparable to those described in this paper for 1-octene.

Our control experiments (Figure 1) show that the specific activity of the epoxide remains constant during growth of *P. oleovorans* on 1-octene. Furthermore, when racemic 1,2-epoxyoctane was not degraded by *P. oleovorans*, there was no significant change in optical rotation (Table I).

Table I. Enzymatic Degradation of Racemic 1,2-Epoxyoctane by *P. oleovorans*[a]

incubation time, h	1,2-epoxyoctane[b] in organic phase, mg/mL	solvent	$[\alpha]^{20}_{365}$ of epoxide	% ee
0	20	*n*-octane	0	0
30.5	19	*n*-octane	−0.8	2
0	20	*n*-hexadecane	0	0
30.5	14	*n*-hexadecane	−5.4	12
48[c]	5	*n*-hexadecane	−16.0	33
0	11	*n*-hexadecane	0	0
36.5	7	*n*-hexadecane	−3.9	8
46.5[c]	3	*n*-hexadecane	−19.4	40

[a] Chemically synthesized 1,2-epoxyoctane, dissolved in *n*-octane or *n*-hexadecane, was added to a 5% (v/v) *n*-octane culture of growing *P. oleovorans* cells. The final concentration of the organic phase was 30% (v/v). Cultures were incubated at 30 °C, after which the organic phase was separated from the water phase by centrifugation. [b] The quantitation of 1,2-epoxyoctane was determined as described in the Experimental Section. No compounds other than 1,2-epoxyoctane were present in the organic phase, as shown by GLC. [c] The organic phase was transferred one time to a fresh culture of octane-grown cells.

These results indicate that there is no isomerization after product release, which is in agreement with the findings of May et al.,[26,28] who showed that the stereochemical configuration of preformed 7,8-epoxy-1-octene does not undergo significant isomerization during diepoxidation of *P. oleovorans*. When racemic 1,2-epoxyoctane was degraded by *P. oleovorans*, this degradation occurred stereoselectively, the *R* enantiomer being hydroxylated and subsequently oxidized faster than the *S* enantiomer.

It is interesting to note that the 1,2-epoxyoctane produced by *P. oleovorans* consists of 85% of the *R* form and 15% of the *S* form. May et al.[26] found that the monoepoxide produced by *P. oleovorans* from 1,7-octadiene consisted of 92% of the *R* form and 8% of the *S* form. It appears, therefore, that the "ω-hydroxylation" system is more stereospecific when 1,7-octadiene is the substrate than when 1-octene is the substrate, and we are therefore exploring the influence of structural variations in the substrate on the stereospecificity of the "ω-hydroxylation" system of *P. oleovorans*.

Finally, although the 1,2-epoxyoctane synthesized by *P. oleovorans* and described here is not optically pure, the enantiomeric excess exceeds that ever reached by chemical routes.

Since, in addition, the chemical yields exceed those normally found with other microbial systems by one or two orders of magnitude, this process may find application in the preparation of chiral compounds.

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Registry No. 1, 111-66-0; (*R*)-2, 77495-66-0; (±)-2, 77549-73-6; (*R*)-3, 77495-67-1.

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