Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 46 (2007) 14–19
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Conversion of α-methyltropate to optically active α-phenylpropionate by tropate-degrading *Rhodococcus* sp. KU1314

Kenji Miyamoto, Shinji Hirokawa, Hiromichi Ohta *

Department of Biosciences and Informatics, Center for Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan

Received 5 October 2006; received in revised form 30 January 2007; accepted 7 February 2007
Available online 15 February 2007

Abstract

Microorganisms which can assimilate tropate were screened from soil. Among them, we found a microorganism which has an ability to convert α-methyltropate to optically active α-phenylpropionate, and it was identified as *Rhodococcus* sp. KU1314. Substrate specificity of the microorganism has been studied. When the aryl group was phenyl, 4-methoxyphenyl and 2-naphthyl, the substrate gave optically active α-propionate in good yields. To estimate the reaction mechanism, some compounds considered to be the intermediates were subjected to the reaction. Both enantiomers of α-methyltropate were converted to (*R*)-α-phenylpropionate with almost the same enantiomeric excess (68 and 72% from *R*-and *S*-enantiomers, respectively) and yield (605 and 48% from *R*-and *S*-enantiomers, respectively).

Keywords: *Rhodococcus*; Enzymatic oxidation; Tropate; Screening; α-Phenylpropionate

1. Introduction

Atropine (**1**) is one of the tropane alkaloids synthesized by solanaceous plants. Its degradation by microorganisms has been reported and the mechanism has also been studied [1–7]. The initial step is the hydrolysis to give alcohol **2**, which has been shown to be oxidized to phenylacetate by the intact cells of *P. putida* PMBL-1 and mutant [5]. It was reported that an NAD^+-linked dehydrogenase was responsible for this oxidation and the enzyme was active toward both enantiomers of tropate. Also, two dehydrogenases (tropate and phenylacetaldehyde dehydrogenases) were detected in the cell-free extracts of the strain AT3 [8]. Thus, the metabolism of tropate to phenylacetate has been shown to proceed *via* phenylacetaldehyde (**5**) via the estimated intermediata, phenylmalonic semialdehyde (**4**) Scheme 1.

We supposed that if this metabolic path is effective to synthetic compounds, optically active products might be obtained starting from racemic compounds in a quantitative yield. However, no such investigation including the one on the substrate specificity of the dehydrogenases has been reported. In this paper, we report the screening of tropate-degrading microorganisms and conversion of α-methyltropate to optically active α-phenylpropionate using the selected microorganism, *Rhodococcus* sp. KU1314.

2. Experimental

2.1. Materials

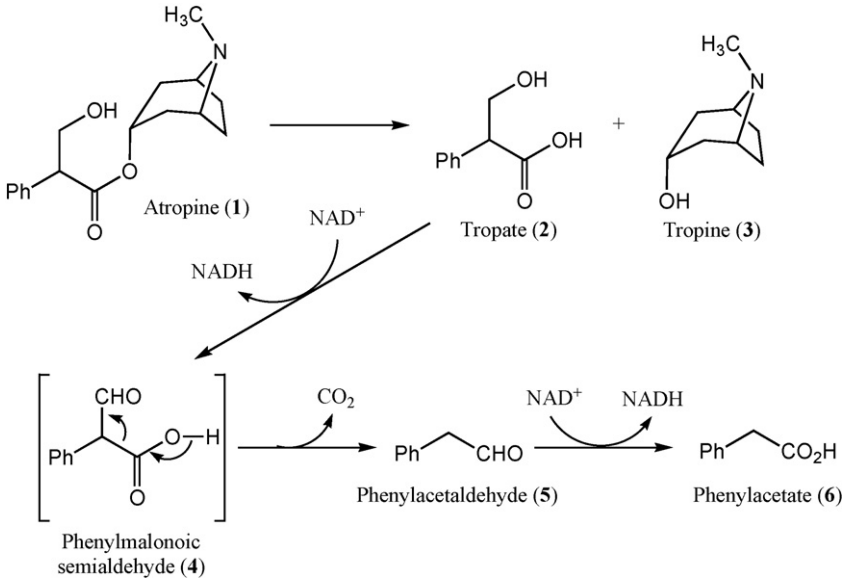
Tropic acid, methyl phenylacetate, 4-methoxyphenylacetic acid, 4-chlorophenylacetatic acid, 2-phenylbutyric acid and 2-napththylacetatic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. (Japan) or Wako Pure Chemicals (Osaka, Japan). Methyl arylacetates were synthesized by esterification of the corresponding carboxylic acids. Methyl α-arylpropionates were prepared by alkylation of the corresponding methyl arylacetates with a base and methyl iodide. All other chemicals were purchased from commercial source and used without further purification.

2.2. Preparation of the substrates

As shown in Scheme 2, various tropate derivatives (**7a–e**) were synthesized by the benzyloxymethylation of methyl

* Corresponding author. Fax: +81 45 566 1551.
E-mail address: hohta@bio.keio.ac.jp (H. Ohta).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.02.004



Scheme 1. The structure and metabolic pathway of atropine.

α-arylalkanoates (**11a–e**) followed by deprotection of the esters (**12a–e**) according to the reported methods [9].

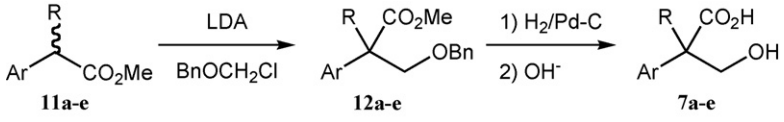
α-Hydroxymethyl-α-phenylpropionate (α-methyltropic acid) (**7a**): mp 89–91 °C ^{1}H NMR($CDCl_3$) δ(ppm): 1.69 (s, 3H), 3.67 (d, J = 11.6 Hz, 1H), 4.11 (d, J = 11.6 Hz, 1H), 7.25–7.64 (m, 5H); IR ν (cm^{-1}): 3400, 2988, 1706, 1235, 1024, 963, 760, 732.

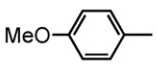
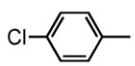
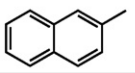
α-Hydroxymethyl-α-phenylbutylate (**7b**): mp 94–95 °C ^{1}H NMR($CDCl_3$) δ(ppm): 0.93 (t, J = 7.5 Hz, 3H), 2.09–2.22 (m, 2H), 4.02 (d, J = 11.6 Hz, 1H), 4.13 (d, J = 11.6 Hz, 1H), 7.25–7.40 (m, 5H); IR ν (cm^{-1}): 3407, 2974, 1706, 1241, 1029, 742, 727, 700, 664.

α-Hydroxymethyl-α-(4-methoxyphenyl)propionate (**7c**): mp 95–96 °C ^{1}H NMR($CDCl_3$) δ(ppm): 1.67 (s, 3H), 3.65 (d, J = 11.6 Hz, 1H), 3.80 (s, 3H), 4.09 (d, J = 11.6 Hz, 1H), 6.88–6.94 (m, 2H), 7.26–7.32 (m, 2H); IR ν (cm^{-1}): 3410, 3267, 1713, 1514, 1256, 1024, 829, 798, 730.

α-Hydroxymethyl-α-(4-chlorophenyl)propionate (**7d**): mp 109–110 °C ^{1}H NMR($CDCl_3$) δ(ppm): 1.66 (s, 3H), 3.69 (d, J = 11.6 Hz, 1H), 4.06 (d, J = 11.6 Hz, 1H), 7.25–7.36 (m, 4H); IR ν (cm^{-1}): 3395, 2941, 1711, 1295, 1037, 1011, 924, 822, 751, 719.

α- Hydroxymethyl-α-(2-naphthyl)propionate (**7e**): mp 160–161 °C ^{1}H NMR($CDCl_3$) δ(ppm): 1.80 (s, 3H), 3.80 (d, J = 11.4 Hz, 1H), 4.22 (d, J = 11.4 Hz, 1H), 7.46–7.52 (m, 3H), 7.82–7.87 (m, 4H); IR ν (cm^{-1}): 3365, 3050, 2985, 1666, 1596, 1132, 1033, 754.



Compound	Ar	R
a		Me
b		Et
c		Me
d		Me
e		Me

Scheme 2. Preparation of tropate derivatives.

2.3. Screening of tropate-degrading microorganisms

The enrichment culture technique [10] was used to screen for various tropate-degrading microorganisms. A soil sample was added to an inorganic medium (10 ml) [$(NH_4)_2HPO_4$, 10 g; K_2HPO_4, 2 g; $MgSO_4{\cdot}7H_2O$, 300 mg; $FeSO_4{\cdot}7H_2O$, 10 mg; $ZnSO_4{\cdot}7H_2O$, 8 mg; $MnSO_4{\cdot}4H_2O$, 8 mg, and yeast extract, 200 mg; in 1000 ml of distilled water, adjusted pH 7.0] containing 0.2% of tropate (**2**), and the mixture was incubated with shaking for some days at 30 °C. After repetition of this operation for several times, a loopful of grown cells was streaked onto an agar plate containing the same medium. The microorganisms which degraded tropate were obtained in this way. The strain KU1314 was identified to be *Rhodococcus* sp. by NCIMB Japan Co. Ltd. (Shimizu, Japan).

2.4. General experimental procedure of microbial oxidation by Rhodococcus sp. KU1314

Rhodococcus sp. KU 1314 was subcultured in an inorganic medium (10 ml) containing 0.2% of tropate for 24 h at 30 °C. The subculture broth (1 ml) was added into a 500-ml Sakaguchi flask containing 50 ml of the same medium. Incubation was carried out for 3 days at 30 °C. The cells were harvested from the culture broth (30 ml) by centrifugation (5000 × *g*, 10 min) and were suspended in 0.1 M glycine–NaOH buffer (pH 9, 5 ml). Substrate (final concentration 10 mM) was added to this suspension, and the mixture was shaken at 30 °C for several days. To this suspension, was added 2 M HCl (1 ml) and the products were extracted with diisopropyl ether three times. The combined diisopropyl ether extract was washed with brine, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under vacuum. The residue was treated with an excess amount of methanol and trimethylsilyldiazomethane. The crude products were purified by preparative TLC and identified by comparing the NMR spectra and the retention times of HPLC with dose of racemic authentic specimen.

Methyl (*R*)-α-phenylpropionate (**11a**): yield: 61% based on α-hydroxymethy-α-phenylpropionate (**7a**) ^{1}H NMR($CDCl_3$) δ(ppm): 1.50 (d, *J* = 7.0 Hz, 3H), 3.60 (s, 3H), 3.72 (q, *J* = 7.0 Hz, 1H), 7.20–7.50 (m, 5H); enantiomeric excess: 68% (determined by HPLC analysis): column, CHIRALCEL OJ (4.6 mm × 250 mm); mobile phase, hexane/iPrOH = 9/1; flow rate, 0.5 ml/min; retention time, (*R*) 21.1 min, (*S*) 18.8 min.

Methyl (*R*)-α-phenylbutyrate (**11b**): yield: 25% based on α-hydroxymethy-α-phenylbutyrate (**7b**) ^{1}H NMR($CDCl_3$) δ(ppm): 0.88 (t, *J* = 7.5 Hz, 3H), 1.75–1.85 (m, 1H), 2.03–2.15 (m, 1H), 3.45 (t, *J* = 7.7 Hz, 1H), 3.65 (s, 3H), 7.22–7.35 (m, 5H); enantiomeric excess: 85% (determined by HPLC analysis); column, CHIRALCEL OD-H (4.6 mm × 250 mm); mobile phase, hexane/iPrOH = 200/1; flow rate, 0.5 ml/min; retention time, (*R*) 14.4 min, (*S*) 17.9 min.

Methyl (*R*)-α-(4-methoxyphenyl)propionate (**11c**): yield: 61% based on α-hydroxymethy-α-(4-methoxyphenyl)propionate (**7c**) ^{1}H NMR($CDCl_3$) δ(ppm): 1.45 (d, *J* = 7.0 Hz, 3H), 3.62 (s, 3H), 3.63 (q, *J* = 7.0 Hz, 1H), 3.79 (s, 3H), 6.28 (d, *J* = 8.7 Hz, 2H), 7.21 (d, *J* = 8.7 Hz, 2H); enantiomeric excess: 74% (determined by HPLC analysis); column, CHIRALCEL OJ (4.6 mm × 250 mm); mobile phase, hexane/iPrOH = 50/1; flow rate, 0.5 ml/min; retention time, (*R*) 25.3 min, (*S*) 23.6 min.

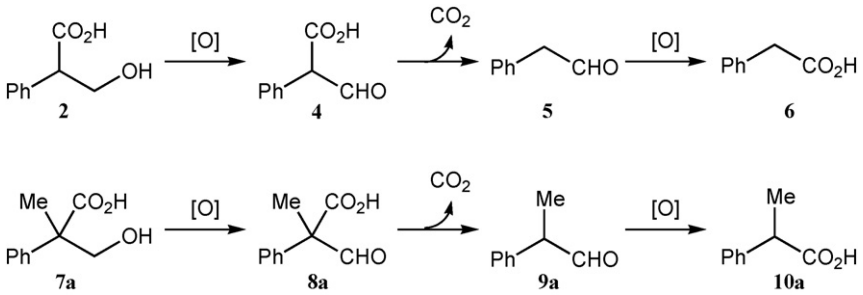
Methyl (*R*)-α-(4-chlorophenyl)propionate (**11d**): yield: 75% based on α-hydroxymethy-α-(4-chlorophenyl)propionate (**7d**) ^{1}H NMR($CDCl_3$) δ(ppm): 1.97 (d, *J* = 6.6 Hz, 3H), 3.63 (s, 3H), 3.67 (q, *J* = 6.6 Hz, 1H), 7.11–7.41 (m, 4H); enantiomeric excess: 0% (determined by HPLC analysis); column, CHIRALCEL OJ (4.6 mm × 250 mm); mobile phase, hexane/iPrOH = 50/1; flow rate, 0.5 ml/min; retention time, (*R*) 21.8 min, (*S*) 23.8 min.

Methyl (*R*)-α-(2-naphthyl)propionate (**11e**): yield: 60% based on α-hydroxymethy-α-(2-naphthyl)propionate (**7e**) ^{1}H NMR($CDCl_3$) δ(ppm): 1.58 (d, *J* = 7.1 Hz, 3H), 3.65 (s, 3H), 3.88 (q, *J* = 7.1 Hz, 1H), 7.40–7.81 (m, 7H); enantiomeric excess: 48% (determined by HPLC analysis); column, CHIRALCEL OD-H (4.6 mm × 250 mm); mobile phase, hexane/iPrOH = 200/1; flow rate, 0.5 ml/min; retention time, (*R*) 30.2 min, (*S*) 32.6 min.

3. Results and discussion

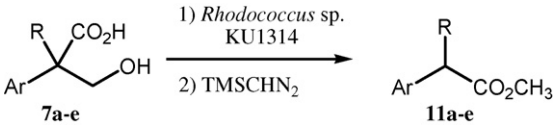
3.1. Screening of microorganisms

First, we screened microorganisms which could assimilate tropate (**2**) as a sole source of carbon (Scheme 3). It was supposed that the first step of the metabolic path is oxidation followed by spontaneous decarboxylation of phenylmalonic semialdehyde (**4**) to give α-phenylpropanal (**5**), which is oxidized to phenylacetate (**6**). The acid will be further metabolized, for example, *via* α-oxidation or hydroxylation of the benzene ring. Of various microorganisms that were isolated after growing on tropate (**2**)



Scheme 3. Screening of tropate-degrading microorganisms.

Table 1
Substrate specificity



7a-e 11a-e

Entry	Substrate	Reaction time (day)	Product (11a–e)		
			Yield (%)	e.e. (%)	Configuration
1	**7a**	2	61	68	*R*
2	**7b**	7	25	85	*R*
3	**7c**	2	61	74	*R*
4	**7d**	5	75	0	–
5	**7e**	6	60	48	*R*

from about 100 soil samples, a strain assigned as *Rhodococcus sp.* KU1314 was found to be the most active to convert α-methyltropate (**7a**) to optically active α-phenylpropionate (**10a**).

3.2. Identification of the strain KU 1314

The bacterium is Gram-positive rods (0.7–0.8 × 1.0–2.0 μm). It is non-spore-forming and non-motile without flagella. No acid and gas is produced from glucose. Type of 16S rDNA shows that tropate-utilizing strain KU1314 is closely related to *Rhodococcus* sp. 871-AN040 (100%), *Rhodococcus wratislaviensis* (99.8%) and *Rhodococcus opacus* (99.8%). Based on these results, the strain KU1314 was identified as *Rhodoccus* sp.

3.3. Optimization of the cultivation conditions and reaction conditions

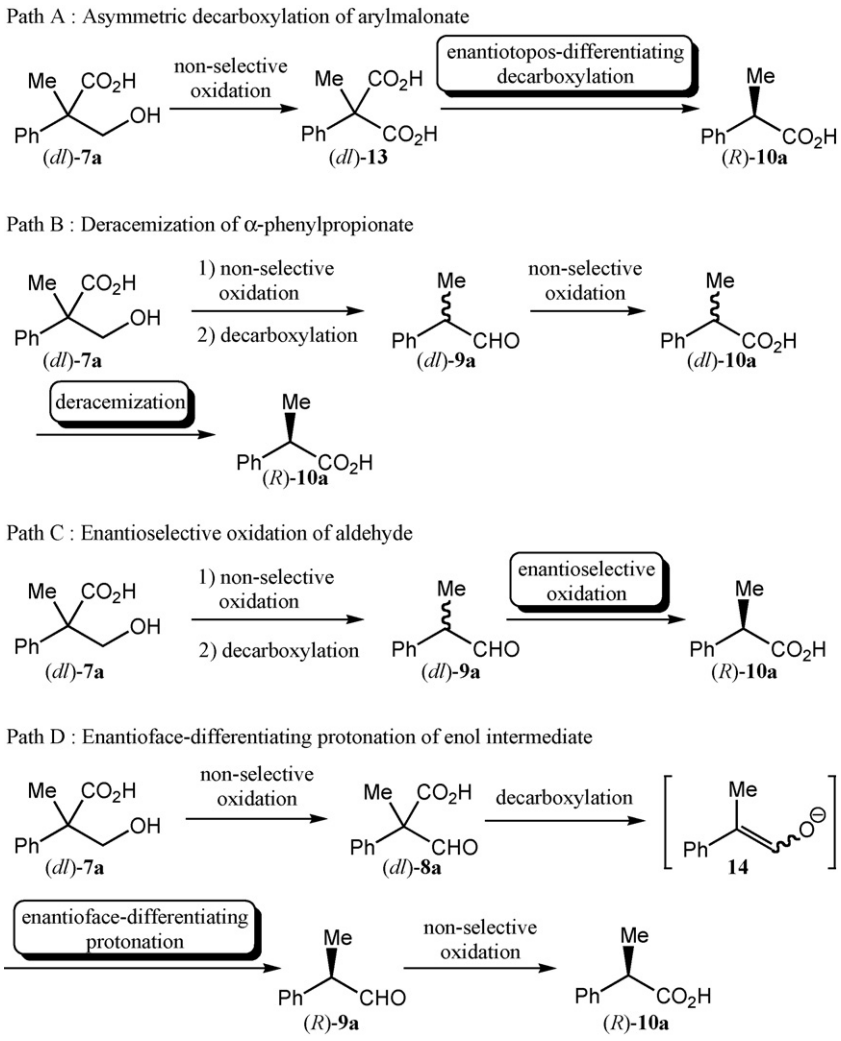
The cultivation conditions suitable to induce a potent oxidizing activity in intact cells were examined. The oxidation activity was inductively expressed in the cells grown in the presence of tropate (**2**). Consistent activity for tropate oxidation was obtained by using the inorganic medium containing tropate. The optimum concentration of tropate and the adequate cultivation period were found to be 0.2% and 3 days, respectively. A higher concentration (>0.5%) of tropate inhibited cell growth (data not shown). Next, the conditions for the α-phenylpropionate (**10a**) production from α-methyltropate (**7a**) were examined. The strain exhibited the highest productivity at around pH 9. When the concentration of α-methyltropate (**7a**) was below 10 mM, the oxidation reaction proceeded smoothly, and the corresponding carboxylic acid (**10a**) was obtained in a good yield. The oxidation activity decreased when the concentration of α-methyltropate was higher than 20 mM. It is probably because the regeneration system of co-factor (eg. $NAD(P)^+$) is not acting well enough to convert the higher concentration of the substrate. Therefore, the enzymatic reaction was performed with 10 mM of the substrate concentration.

3.4. Substrate specificity

Under the optimum condition, present microbial oxidation was extended to other tropate derivatives. As shown in Table 1, when the R group was changed to ethyl (Entry 2), the yield of the product (**11b**) decreased compared to the case of methyl group (Entry 1). This must be due to the difference of the steric bulkiness of ethyl and methyl groups. Next, the effect of variation of the aromatic part was examined. When Ar group was 4-methoxyphenyl (**7c**), 4-chlorophenyl (**7d**) and 2-naphthyl (**7e**), the oxidation reaction proceeded and the corresponding esters (**11c–e**) were obtained in good yields, although a long reaction time was required for the conversion of **7e**. The products were identified after esterification of the primary product with TMS diazomethane. In the case of 4-chlorophenyl, the desired product (**11d**) was a racemate. It is probably because the acidity of α-proton of the supposed intermediate aldehyde **9a** (Scheme 4, Path D) is stronger than those of other compounds due to the electron-withdrawing effect of the *p*-chloro substitution and accordingly **9a** racemizes before it is oxidized to the corresponding carboxylic acid. It seems difficult at present to consistently interpret the effect of the structure of Ar and R on the reactivity. However, it is certain that electron-donating substituents are favorable for this enzyme system.

3.5. Reaction mechanism

To obtain a better understanding of the reaction mechanism, some compounds that are considered to be intermediates were subjected the reaction. Various reaction courses can be considered as illustrated in Scheme 4. Path A: α-methyltropate (**7a**) is oxidized to α-phenyl-α-methylmalonate (**13**). Then, the malonate (**13**) is converted to optically active α-phenylpropionate (**10a**) by arylmalonate decarboxylase [11–14]. In order to confirm this assumption, incubation of the malonate **13** with *Rhodococcus* sp. was carried out. The result obtained was the total recovery of the substrate, indicating that no decarboxylase is present in the bacterium. Path B: α-methyltropate is converted to racemic α-phenylpropionate (**10a**), which is deracemized to optically active propionate [15,16]. In order to confirm the possibility, racemic α-phenylpropionate was subjected to the reaction to find that the deracemization did not occur at all. Path C: α-methytropate is converted to racemic 2-phenylpropanal (**9a**). If **9a** can racemize under the reaction conditions and (*R*)-enantiomer is preferentially oxidized to (*R*)-phenylpropionate, the final product can be optically active. Actually, racemic α-



Scheme 4. Estimated pathway for the conversion of α-methyltropate to optically active α-phenylpropionate.

phenylpropanal gave almost racemic α-phenylpropionate. Thus, it is clear that intermediate aldehyde **9a** should be optically active. In this way, the most possible reaction path is the following. Path D: both enantiomers of α-methyltropate are oxidized to racemic malonic semialdehyde (**8a**), which is converted by spontaneous decarboxylation to enol-type intermediate (**14**) in the active site of the enzyme. The enolate is immediately protonated in an enantioface-selectively manner to result in optically active aldehyde (**9a**). The optically active aldehyde is oxidized to α-phenylpropionate without losing its enantiomeric excess. In this way, the (*R*)-enantiomer of the final product would be obtained utilizing both enantiomer of α-methytropate.

Each enantiomer of α-methyltropate (**7a**) was resolved with the aid of quinine and obtained in 98% e.e. The enzymatic reaction was performed using optically active α-methyltropate at 30 °C for 24 h. As shown in Table 2, both enantiomers were converted to (*R*)-α-phenylpropionate (**10a**) with almost the same enantioselectivity. Also there was no significant difference in the rate of reaction between the two enantiomers. Thus, it can be concluded that both enantiomers of α-methyltropate are non-selectively oxidized to the corresponding malonic semialdehyde (**8a**). The semialdehyde is converted by decarboxylation to an enol-type intermediate. Because the final product is optically active, the protonation to this intermediate should be enantioface-selective to give (*R*)-aldehyde (Scheme 4, Path D). Similarly tandem reactions occurred in the conversion of isocitrate into 2-oxoglutarate by the NAD^+-linked dehydrogenase [17] and L-malate in to pyruvate by the malate dehydrogenase [18]. Stereochemistry of isocitrate dehydrogenase-catalyzed decarboxylation of isocitrate had been reported [19–21]. Thus, it is thought that the mechanism which we presumed is also sufficiently possible.

Table 2
Effect of the configuration of the starting compound

Entry	Substrate	Reaction time (h)	Product (11 a)		
			Yield (%)	e.e. (%)	Configuration
1	*rac*-**7a**	24	53	65	*R*
2	(*R*)-**7a**	24	60	68	*R*
3	(*S*)-**7a**	24	48	72	*R*

In summary, a new type of conversion of α-methyltropate has been realized by incubation with *Rhodococcus* sp. KU1314 resulting in the formation of optically active α-phenylpropionate, although the transformations take a long time in some cases. To raise the efficiency of the reaction, it is desirable to increase the amount of the key enzyme(s). For this purpose and to clarify the detailed reaction mechanism, the purification of the enzyme and cloning of the gene are problems to be challenged.

References

[1] J. Kackowski, Acta Soc. Bot. Polon. 208 (1959) 677–694.
[2] J. Kackowski, M. Mazejko-Toczko, Acta Microb. Polon. 9 (1960) 173–179.
[3] H. Niemer, H. Bucherer, A. Kohler, Z. Physiol. Chem. 317 (1959) 238–242.
[4] A. Rörsch, F. Berends, H.C. Bartlema, W.F. Stevens, Proc. Kon. Ned. Akad. Wetensch. Ser. C Biol. Med. Sci. 74 (1971) 132–147.
[5] H.O. Michel, E. Hackley, D.N. Kramer, Anal. Biochem. 36 (1970) 294–302.
[6] M.T. Long, D.J. Hopper, P.W. Trudgill, FEMS Microbiol. Lett. 106 (1993) 111–116.
[7] B.A. Bartholomew, M.J. Smith, P.W. Trudgill, D.J. Hopper, Appl. Environ. Microbiol. 62 (1966) 3245–3250.
[8] M.T. Long, B.A. Bartholomew, M.J. Smith, P.W. Trudgill, D.J. Hopper, J. Bacteriol. 179 (1997) 1044–1050.
[9] K. Miyamoto, S. Tsuchiya, H. Ohta, J. Am. Chem. Soc. 114 (1992) 6256–6257.
[10] Y. Asano, J. Biotech. 94 (2002) 65–72.
[11] K. Miyamoto, H. Ohta, J. Am. Chem. Soc. 112 (1990) 4077–4078.
[12] K. Miyamoto, H. Ohta, Biocata1ysis 5 (1991) 49–60.
[13] K. Miyamoto, H. Ohta, Eur. J. Biochem. 210 (1992) 475–481.
[14] K. Miyamoto, H. Ohta, Appl. Microbiol. Biotechnol. 38 (1992) 234–238.
[15] D. Kato, S. Mitsuda, H. Ohta, J. Org. Chem. 68 (2003) 7234–7242.
[16] D. Kato, K. Miyamoto, H. Ohta, Tetrahedron Asymmetry 15 (2004) 2965–2973.
[17] J.A. Hathaway, D.E. Atkinson, J. Biol. Chem. 238 (1963) 2875–2883.
[18] S. Korkes, A. Del Campillo, S. Ochoa, J. Biol. Chem. 187 (1950) 891–905.
[19] S. Englard, I. Listowsky, Biochem. Biophys. Res. Commun. 12 (1963) 356–360.
[20] Z.B. Rose, J. Biol. Chem. 235 (1960) 928–933.
[21] G.E. Lienhard, I.A. Rose, Biochem. 3 (1964) 185–190.