JOC Article

Microbial Deracemization of α-Substituted Carboxylic Acids: Substrate Specificity and Mechanistic Investigation

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A new enzymatic method for the preparation of optically active α-substituted carboxylic acids is reported. This technique is called deracemization reaction, which provides us with a route to obtain the enantiomerically pure compounds, theoretically in 100% yield starting from the racemic mixture. This means that the synthesis of a racemate is almost equal to the synthesis of the optically active compound, and this concept is entirely different from the commonly accepted one in the asymmetric synthesis. Using the growing cell system of *Nocardia diaphanozonaria* JCM3208, racemates of 2-aryl- and 2-aryloxypropanoic acid are deracemized smoothly and (*R*)-form-enriched products are recovered in high chemical yield (>50%). In addition, using optically active starting compounds and deuterated derivatives as well as inhibitors, we have disclosed the fact that a new type of enzyme takes part in this biotransformation, and that the reaction proceeds probably via the same mechanism as that in rat liver.

Introduction

Biological activity of a compound often depends on the absolute configuration of chiral centers in the molecule, because the receptor to which the molecule binds is made of an enantiomerically pure protein and its active site is chiral. For example, (*S*)-enantiomers of 2-arylpropanoic acids are widely used as nonsteroidal antiinflammatory drugs (NSAIDs), while the (*R*)-antipodes are either inactive or only weakly active in vivo.[1] Furthermore, the (*R*)-enantiomers of 2-aryloxypropanoic acids are utilized as herbicide, while the (*S*)-antipodes are inactive.[2] In addition, (*R*)-2-(4-chlorophenoxy)propanoic acid lowers the level of serum cholesterol and prevents platelet aggregation. As the (*S*)-antipode of this compound inhibits the chloride channel in muscles, it causes side effects, such as muscle irritability and spasms. Thus the preparation of optically pure enantiomers is extremely important, and many kinds of approaches have been tried so far.

The methods of obtaining the enantiomerically enriched compounds are classified into two broad categories: optical resolution of racemates and asymmetrization of prochiral or meso compounds. Biocatalysts are widely utilized in both cases.[3] When the starting material is a racemic mixture, the kinetic resolution process is a standard procedure for the preparation of optically active compounds (Scheme 1). There have been many reports concerning this method and much effort has been devoted to increase the enantioselectivity through the various modifications of the reaction conditions and/or biocatalysts.[2,4] Although kinetic resolution has already been a well-established method for the preparation of optically active compounds, this has a disadvantage in that the maximum yield of the desired enantiomer is theoretically limited to 50%. Also the separation of the product and the recovered starting material is inevitable, which sometimes is tedious.

To overcome this limitation, dynamic kinetic resolution (DKR), which is a method of the combination of enzymatic kinetic resolution and in situ racemization of the substrate, is attracting considerable attention.[5] Theoretically, DKR is capable of giving the desired enantiomer in 100% yield, which is a marked advantage for industrial application. In this case, however, it is essential that the starting material racemizes under the reaction conditions, while the product does not. Accordingly, combinations of sophisticated design of the substrate and incubation conditions are the key to the successful procedure.

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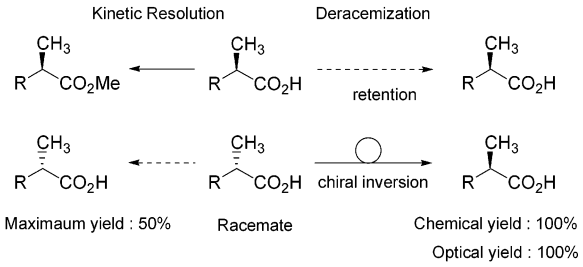
SCHEME 1. Comparison of the Concept of Two Techniques for the Preparation of Optically Active Compounds

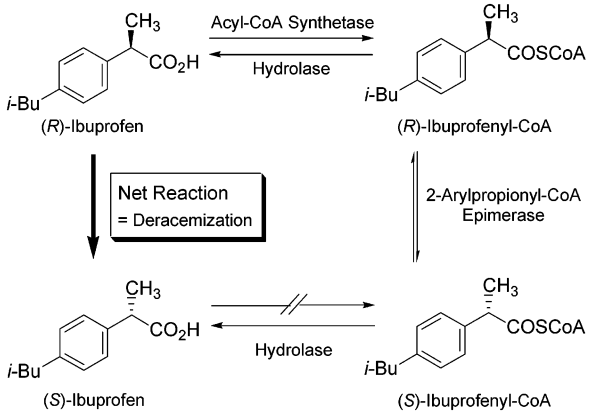


Because of its structural limitation of the substrate, the DKR method cannot be widely applied at present despite its potential. In both resolution techniques, one enantiomer of the racemic compound is converted to a different compound, such as the esterification of a carboxylic acid (Scheme 1).

On the other hand, if there is a method by which we can convert a racemic compound to the corresponding optically active one without changing its chemical structure, this will be the third option for obtaining an optically active compound from a racemic mixture. In such a case, we will be free from the fear of racemization of the "product" and a tedious separation in the workup procedure. We would like to call such a process "deracemization", distinguishing it from DKR as well as kinetic resolution. In this deracemization process, the chirality of either enantiomer of a racemate is converted to the other antipode via single or plural reactions, resulting in an optically active compound starting from a racemic mixture as a whole (Scheme 1). In total, successful application of this process means that the synthesis of a racemate is almost equal to the synthesis of the optically active compound. This concept is entirely different from the commonly accepted one in the asymmetric synthesis. Most of the enzymatic deracemization reactions so far reported utilize the oxidation−reduction process. A representative example is the deracemization of secondary alcohols, the key being the combination of two enzymes that catalyze the oxidation of one enantiomer followed by enantioselective reduction to the antipode.[5c] Quite recently, Turner and co-workers reported the deracemization of α-amino acids[6] and α-methylbenzylamine[7] combining the enantioselective enzyme-catalyzed oxidation of the amine to imine and the nonenantioselective chemical reduction. Here we present an enzymatic deracemization reaction of a quite different type of compounds, 2-arylpropanoic acid and 2-aryloxypropanoic acid. Not only are these important, physiologically active compounds, but also it is interesting from the standpoint of the reaction mechanism. That is because these compounds are difficult to subject to the oxidation−reduction process by either chemical or enzymatic reaction.

There are only a few reports on the enzymatic deracemization of α-substituted carboxylic acids with biocatalysts, i.e., the use of rat liver[8,9] and two fungi, *Cordyceps militaris*[1,10] and *Verticillium lecanii*,[11] which

SCHEME 2. Proposed Mechanism of Deracemization Reaction in Rat Liver



are capable of inverting the chirality of the (*R*)-enantiomer of 2-arylpropanoic acids to the (*S*)-antipode. There is also an actinomycete, *Rhodococcus* sp.,[12] which inverts the configuration of 2-aryloxypropanoic acids from *S* to *R*. In the case of rat liver, the reaction mechanism was proposed based on various studies with enantiomerically pure compounds and deuterated derivatives in which three enzymes take part in this biotransformation system (Scheme 2).[7−9] The initial step of this biotransformation is considered to be the enantioselective formation of the Coenzyme A (CoA) thioester of (*R*)-acid. The thioester is subsequently racemized by the aid of an epimerase and cleaved by a hydrorase to release the free (*S*)-acid. Among these three enzymes, only the acyl-CoA synthetase is enantioselective. Thus, the enantiomeric ratio in the reaction mixture shifts to the (*S*)-form with the repetion of the reactions. These enzymes as well as genes were purified and identified. In comparison to the rat liver enzymes, the others have been little studied and many reports on these biocatalysts concern only the pharmacological aspect of the system and not their synthetic potentials. In addition, all of the previously reported enzymes are only capable of inverting the chirality of 2-arylpropanoic acids from (*R*)- to (*S*)-configuration. Thus, from the standpoint of synthetic chemistry, it is very important to find new enzymes possessing the deracemization activity for a wide range of compounds and to invert the chirality of 2-arylpropanoic acid from (*S*)- to (*R*)-configuration. Recently, we found a new biocatalyst, *Nocardia diaphanozonaria* JCM3208, a kind of actinomycetes, affording the (*R*)-form of 2-aryl- and 2-aryloxypropanoic acid from their racemates.[13] In addition, Mitsukura et al. also have reported the efficient preparative

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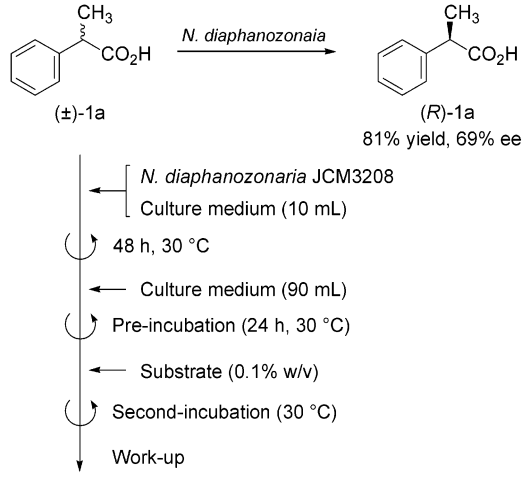
(13) Kato, D.-i.; Mitsuda, S.; Ohta, H. *Org. Lett.* **2002**, *4*, 371−373.

SCHEME 3. Optimization of the Reaction Conditions with Use of the Growing Cell System of *N. diaphanozonaria*

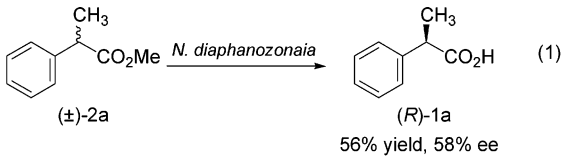


method of (*R*)-2-phenylpropanoic acid using the resting cells of this bacterium.[14] In the present paper, we would like to report the reaction mechanisms as well as the substrate specificities using the whole cell system of *N. diaphanozonaria*.

Results and Discussion

Substrate Specificity. (*S*)-2-Phenylpropanoic acid ((*S*)-**1a**) was converted to the (*R*)-form (Scheme 3) by incubation with the growing cells of *N. diaphanozonaria*. To the best of our knowledge, this bacterium is the first example that exhibits the deracemization activity toward (*S*)-**1a**. Thus, 0.1% (w/v) of **1a** was added to the suspension of 24-h incubated cells, and the mixture was shaken at 30 °C (second incubation). When the reaction was stopped after 48 h, enantiomerically enriched **1a** was recovered as the sole product, the yield and enantiomeric excess (ee) of the product being 81% and 69% (*R*), respectively. The period of the second incubation was optimized based on two parameters, the yield and the ee of the product. Although these two values consistently increased over 48 h, the remarkable decrease was observed thereafter. This is probably because of the oxidative degradation of the substrate.

The methyl ester of 2-phenylpropanoic acid (**2a**) was also deracemized to the (*R*)-enantiomer although the yield and ee of the product were lower (eq 1). In this case,



the primary product was revealed to be free acid. Thus the actual substrate would also be the free acid formed in situ by the aid of hydrolase of *N. diaphanozonaria*. On the other hand, 2-phenylpropanamide (**3**) could not be recognized by either the deracemization enzymes or the hydrolase and was recovered intact (eq 2). These results suggest that the free acid moiety is essential to this enzyme system.

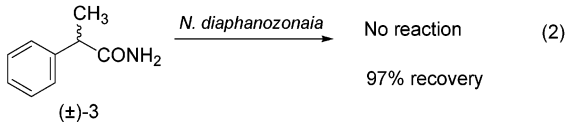
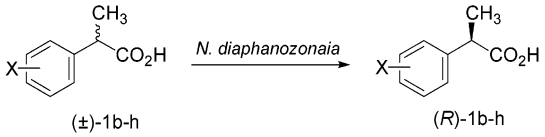


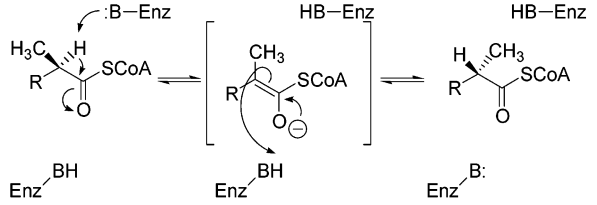
TABLE 1. Microbial Deracemization of 2-Arylpropanoic Acid[a]



entry	sub.	X	time (h)	yield[b] (%)	ee[c] (%)
1	**1b**	4-isobutyl	48	85	9
2	**1c**	3-fluoro-4-phenyl	48	99	6
3	**1d**	4-methoxy	48	60	4
4	**1e**	2-chloro	48	59	9
5	**1f**	3-chloro	48	62	4
6	**1g**	4-chloro	48	61	11
7	**1h**	4-fluoro	6	83	racemic
8	**1h**	4-fluoro	16	58	6
9	**1h**	4-fluoro	48	35	10

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**2b**—**h**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

SCHEME 4. Enolate-Type Intermediate: Proposed Intermediate during the Chiral Inversion Process



To expand the substrate specificity of the deracemization reaction, several 2-phenylpropanoic acid derivatives which have a substituent on the aromatic ring were subjected to the reaction (Table 1). In the case of 2-(4-isobutylphenyl)propanoic acid (ibuprofen, **1b**) and 2-[(3-fluoro-4-phenyl)phenyl]propanoic acid (flurbiprofen, **1c**), *N. diaphanozonaria* did not show the deracemization activity in contrast to the fact that both of these compounds were deracemized by *C. militalis*.[1,10] In addition, 2-(4-methoxyphenyl)propanoic acid (**1d**) was not accepted by the enzyme system of *N. diaphanozonaria* (entry 3).

If the reaction proceeds via an enolate type intermediate as proposed by Shieh et al.[9a] (Scheme 4), the introduction of an electron-withdrawing substituent on the aromatic ring will stabilize the transition state and accelerate the reaction. Unfortunately, however, 2-(chlorophenyl)propanoic acid derivatives possessing a chlorine atom on the ortho-, meta-, or para-position (**1e**—**g**) did not undergo the deracemization reaction (entries 4—6). Furthermore, the introduction of a fluorine atom on the para-position (**1h**) did not enhance the apparent chiral

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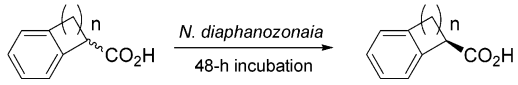
TABLE 2. Microbial Deracemization of Cyclic Analogues of 2-Phenylpropanoic Acid[a]



(±)-4a-c (*R*)-4a-c

entry	sub.	n	yield[b] (%)	ee[c] (%)
1	**4a**	1	65	racemic
2	**4b**	2	70	12
3	**4c**	3	58	9

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**5a**−**c**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

inversion to any significant extent regardless of the small size and the electronegativity of the fluorine atom (entries 7−9). In addition, after 48 h of incubation, the yield of the product significantly decreased. This result was estimated to be caused by the increase of the rate of metabolic degradation by the introduction of a fluorine atom on the aromatic ring. As expected, shortening the incubation period raised the yield, although the ee of the product was not improved (entry 7).

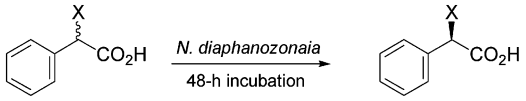
If the reaction proceeds via an enolate-type intermediate, which will be stabilized by the resonance effect of the adjacent phenyl ring (Scheme 4). In this situation, the orbital of the anion and the π electrons of the phenyl ring should be parallel, and consequently the conformation of the intermediate in the active site of the enzyme would be restricted. Accordingly, if the racemization step is rate-determing, fixing the conformation of the substrate in advance to the one that is favorable to the delocalization of the carbanion will be expected to accelerate the reaction because of the lowering of the activation entropy. Thus we prepared some compounds with a ring system (Table 2). However this attempt was unsuccessful. The substrates with a four-, five-, or six-membered ring (**4a**−**c**) were recovered as almost racemates.

It is also interesting how the α-substituent affects the reactivity. α-Fluorophenylacetic acid (**6a**), which has a fluorine atom instead of a methyl group, was accepted by the deracemization enzymes to give the (*R*)-form in a moderate optical yield (Table 3, entry 1). On the other hand, the enzyme system did not exhibit the deracemization activity with 2-phenylbutanoic acid (**6b**), α-methoxyphenylacetic acid (**6c**), and 3-hydroxy-2-phenylpropanoic acid (**6d**), probably because of the steric bulkiness of the α-substituents (entries 2−4).

Mandelic acid derivatives (**8a**−**c**), which have a hydroxyl group on the α-carbon, exhibited entirely different reactivity, i.e., they underwent the enantioselective degradation reaction (Table 4). Only the (*S*)-form was converted to the corresponding benzoic acid derivatives and (*R*)-mandelic acid derivatives were recovered in the optically pure form.

2-Aryloxypropanoic acid was selected as the next substrate. These compounds have an oxygen atom between the aromatic ring and the asymmetric center. At first, we presumed that these compounds would be unfavorable for the deracemization reaction because the

TABLE 3. Microbial Deracemization of α-Substituted Phenylacetic Acid[a]

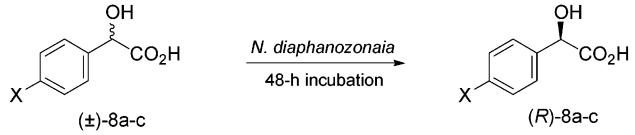


(±)-6a-d (*R*)-6a-d

entry	sub.	X	yield[b] (%)	ee[c] (%)
1	**6a**	F	74	55
2	**6b**	C_2H_5	77	racemic
3	**6c**	OMe	85	racemic
4	**6d**	CH_2OH	76	racemic

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**7a**−**d**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

TABLE 4. Microbial Deracemization of Mandelic Acid Derivatives[a]



(±)-8a-c (*R*)-8a-c

			starting compd	product		
entry	sub.	X	ee (%)	yield[b] (%)	ee[c] (%)	metabolic product
1	**8a**	H	racemic	45	>99	benzoic acid
2	**8a**	H	*R*, >99	84	>99	
3	**8a**	H	*S*, 83	34	*S*, 23	benzoic acid
4	**8b**	Cl	racemic	43	>99	4-chlorobenzoic acid
5	**8c**	OMe	racemic	38	>99	4-methoxybenzoic acid

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**9a**−**c**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

insertion of an oxygen atom will lower the acidity of the α-methyne proton. To our surprise, however, the deracemization reaction of 2-phenoxypropanoic acid (**10a**) cleanly proceeded under the same reaction conditions as those for 2-phenylpropanoic acid (**1a**), both the yield and the ee of the product being 75% after 48 h (Table 5, entry 2). Elongation of the second incubation period to 72 h resulted in the improvement of the ee of the product, being as high as 97% (entry 4). However, similar to the case of **1a**, incubation over 72 h had a negative effect on the yield and the ee of the product (entry 5). Surprisingly enough, the absolute configuration of the product was revealed to be *R* judging from the sign of optical rotation.[15] This means that the spatial arrangement of the ligands around the asymmetric center was opposite that

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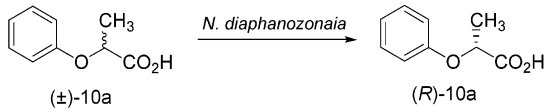
(20) Bach, T.; Körber, C. *J. Org. Chem*. **2000**, *65*, 2358−2367.

TABLE 5. Microbial Deracemization of 2-Phenoxypropanoic Acid[a]

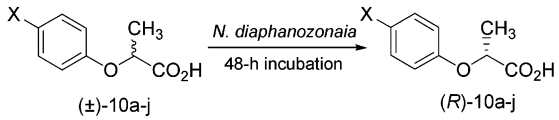


(±)-10a (*R*)-10a

entry	reaction time (h)	yield[b] (%)	ee[c] (%)	$[\alpha]_D$[d]
1	24	68	50	
2	48	75	75	+41.0 (EtOH)
3	60	66	91	
4	72	71	97	+41.2 ($CHCl_3$)
5	84	86	86	

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**11a**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester. [d] Optical rotation was measured after conversion to the corresponding methyl ester.

TABLE 6. Microbial Deracemization of Para-Substituted 2-Phenoxypropanoic Acid[a]



(±)-10a-j (*R*)-10a-j

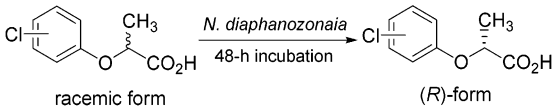
entry	sub.	X	yield[b] (%)	ee[c] (%)	$[\alpha]_D$(EtOH)[d]
1	**10a**	H	75	75	+41.0
2	**10b**	F	64	96	+52.0
3	**10c**	Cl	95	97	+44.7
4	**10d**	Br	83	99	+47.5
5	**10e**	I	76	97	+43.4
6	**10f**	CF_3	64	99	+43.3
7	**10g**	CH_3	69	90	+53.3
8	**10h**	OMe	83	44	+34.0
9	**10i**	COMe	59	racemic	
10	**10j**	OH	79	racemic	

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**11a**−**j**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester. [d] Optical rotation was measured after conversion to the corresponding methyl ester.

of **1a**. To the best of our knowledge, this is the first example of the inversion of the enantioselectivity between these two substrates as we described in the previous communication.[13] It is very interesting that the insertion of only one atom brought about a dramatic change in enantioselectivity.

As seen from the results of compounds **10b**−**j**, introduction of a substituent on the para-position of the aromatic ring had no negative effect on the deracemization reaction in contrast to the case of **1a** (Table 6). In particular, this enzyme system was shown to be active to a series of compounds that have an electronegative substituent on the para-position. For example, 2-(4-chlorophenoxy)propanoic acid (**10c**) was deracemized smoothly, with the yield and the ee of the product being as high as 95% and 97% (*R*) after 48 h of incubation (entry 3). Moreover, the compounds which have an electron-donating substituent also underwent a deracemization reaction, with a slight decrease of the ee of the product after 48 h of incubation (entries 7 and 8). Unfortunately, however, in the case of *p*-acetyl- or

TABLE 7. Microbial Deracemization of 2-(Chlorophenoxy)propanoic Acid[a]

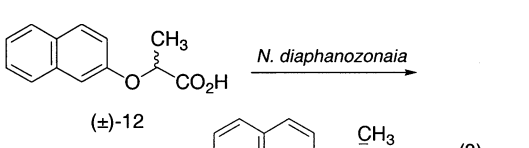


racemic form (*R*)-form

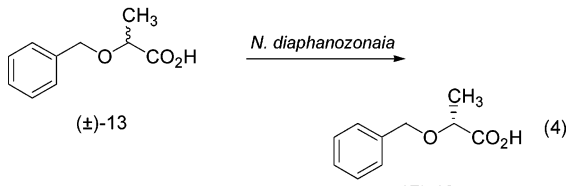
entry	sub.	position	yield[b] (%)	ee[c] (%)
1	**10*l***	2-Cl	61	17(*S*)
2	**10m**	3-Cl	73	21(*R*)
3	**10c**	4-Cl	95	97(*R*)

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**11c**, **l**, and **m**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

p-hydroxyl-substituted compounds, we observed only the recovery of the racemate (entries 9 and 10). When the aromatic ring was converted to the more bulky naphthyl group, the deracemization reaction hardly proceeded (eq 3). Furthermore, while the enzyme system of *N. diaphanozonaria* could accept the *p*-chloro derivative, it could not recognize the other chloro-substituted compounds, such as 2-(2-chlorophenoxy)- and 2-(3-chlorophenoxy)-propanoic acid (**10*l*,m**, Table 7, entries 1 and 2). The introduction of one methylene spacer in 2-phenoxypropanoic acid between the oxygen atom and the aromatic ring had considerable negative effect on the deracemization reaction, as can be seen from the remarkable decrease of the enantioselectivity for this type of compound (eq 4).



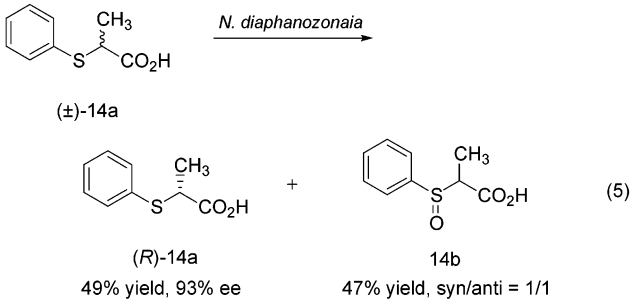
(±)-12 (*R*)-12
78% yield, 19% ee



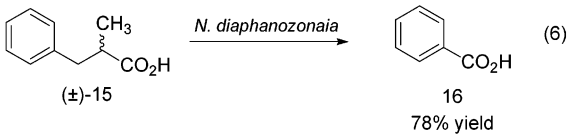
(±)-13 (*R*)-13
77% yield, 21% ee

Then we focused our attention on the effect of the "spacer" atom between the α-carbon and the aromatic ring. Thus we selected two compounds, 2-phenylthiopropanoic acid (**14a**) and 2-methyl-3-phenylpropanoic acid (**15**).

By the incubation with the growing cells of *N. diaphanozonaria*, however, degradation reactions proceeded preferentially for these compounds, although the enzymes concerning the oxidation of **14a** and **15** will be different, judging from the products. In the case of **14a**, the (*S*)-enantiomer was oxidized on the sulfur atom to give 2-phenylsulfinylpropanoic acid (**14b**, eq 5). The relative configuration of this product could be determined by ^1H



NMR analysis, and equal amounts of syn and anti isomers were detected. On the other hand, the (*R*)-enantiomer was recovered intact in high optical yield. In the case of **15**, complete degradation proceeded without enantioselectivity to give benzoic acid (**16**, eq 6).



If the enzyme system of *N. diaphanozonaria* also works via the same reaction mechanism as that established for the rat liver system (Scheme 2), the key intermediate will be acyl-CoA, which is also the intermediate of the *β*-oxidation process of carboxylic acids. Thus it is not surprising that the degradation reaction took precedence over the deracemization reaction for these compounds under an aerobic condition (Scheme 5). Even if the oxidative degradation process is a minor pass, the substrate will be gradually metabolized because this process is irreversible. In fact, 2-alkylpropanoic acid and the analogous compounds, such as 2-methyldecanoic acid, 2-heptyloxypropanoic acid, and 2-chlorooctanoic acid, were completely metabolized under the same conditions by the growing cells of *N. diaphanozonaria*, and no reaction product could be detected.

If the hypothesis that the *β*-oxidation process is competing with the deracemization reaction is correct, then it might be possible to obtain the optically active form of **15** and related carboxylic acids by suppressing the oxidation step by designing the reaction conditions and/or by some additives. Thus we studied the reaction mechanism aiming at obtaining more information.

Investigation of the Reaction Mechanism. In general, there are two possible reaction paths for the formation of the optically active compounds starting from the racemates. One is the deracemization of the substrate via some mechanism and the other is the enantioselective degradation. In case of the above-mentioned conversion by the aid of *N. diaphanozonaria*, the former process is considered to be the major path based on the yield of the product although the degradation path is not negligible. For the study of the strict reaction mechanism, the isolation of the enzyme is essential. However, for the purpose of designing the reaction conditions to obtain better results, information that is available by using the whole cell system is sometimes useful. To obtain such information, we carried out some experiments. First, optically active 2-phenylpropanoic acid (**1a**) was subjected to the reaction (Table 8). It was revealed that the configuration of the product was *R* (entries 2 and 3). It is clear that the configuration of (*S*)-**1a** was inverted to the other antipode based on a calculation with the values of the yield and ee of the product. It is impossible to explain the result shown in Table 8 by supposing only the enantioselective degradation via the *β*-oxidation process. It is also interesting that the ee of (*R*)-**1a** was decreased from 88% to 65% after 48 h of incubation (entry 2). These results suggest that the deracemization process by this bacterium consists of the combination of equilibrium reactions and that a set of enzymes are participating.

To explain each reaction, the following experiments were performed. When racemic 2-deuterio-2-phenylpropanoic acid (**1i**, D content 98%) was incubated with the whole cells of *N. diaphanozonaria*, the D content of the product decreased to 20% (entry 4). Because no decrease of the D content was observed in the absence of the microorganism, D−H exchange should be catalyzed by enzymes and the reaction path should include C−D bond fission. There are at least two possible paths that will cause the loss of the α-methyne proton of **1a** (Scheme 6). One is the path via an enolate-type intermediate and the other is an oxidation−reduction process via the exo methylene-type intermediate; the former is proposed by Shieh et al.[9a] for rat liver and the latter is the analogous process for the deracemization of secondary alcohols. To clarify which path was working, racemic 3,3,3-trideuterio-2-phenylpropanoic acid (**1j**, D content >99%) was subjected to the reaction. The substrate was recovered as its optically active form without losing any deuterium atom (entry 5). This fact strongly suggests that no C−D bond fission occurred on the methyl group, although one cannot totally neglect the possibility that the same hydrogen atom comes back to the methyl group during the oxidation−reduction process. However, considering such a mechanism, it is still difficult to explain the fact that only the hydrogen on C-2 exchanges, while the one on C-3 does not. Thus, the most probable mechanism at present is the path via an enolate-type intermediate similar to the case of rat liver (Scheme 4).

In case of rat liver, it is clarified that acyl-CoA synthetase plays an important role in the recognition of the configuration of the asymmetric center.[8,9] Several studies have confirmed that only the (*R*)-enantiomer of 2-arylpropanoic acid can yield acyl-CoA thioester. Formation of acyl-CoA may be essential not only in the chiral inversion process but also in the *β*-oxidation of fatty acid. It is of interest whether acyl-CoA synthetase also takes part in the deracemization reaction in the case of *N. diaphanozonaria*. Thus the deracemization procedure by the growing cell system was carried out in the presence of the typical substrate of acyl-CoA synthetase, i.e., benzoic acid and palmitic acid. These are known to be the substrates of medium-chain acyl-CoA synthetase and long-chain acyl-CoA synthetase, respectively. The influence of these carboxylic acids on the chiral inversion of 2-phenylpropanoic acid (**1a**) and 2-(4-chlorophenoxy)-propanoic acid (**10c**) was examined. The results are summarized in Table 9. Racemic substrates were incubated for 12 h with growing cells of *N. diaphanozonaria* in the presence of 5 equiv of benzoic acid or palmitic acid. When benzoic acid was added to the reaction mixture, the deracemization reaction was completely inhibited. On the other hand, palmitic acid had no effect on the chiral inversion process. These experiments show that the

SCHEME 5. Two Different Kinds of Reaction Paths in the Metabolic Reaction of 2-Methyl-3-phenylpropanoic Acid (15)

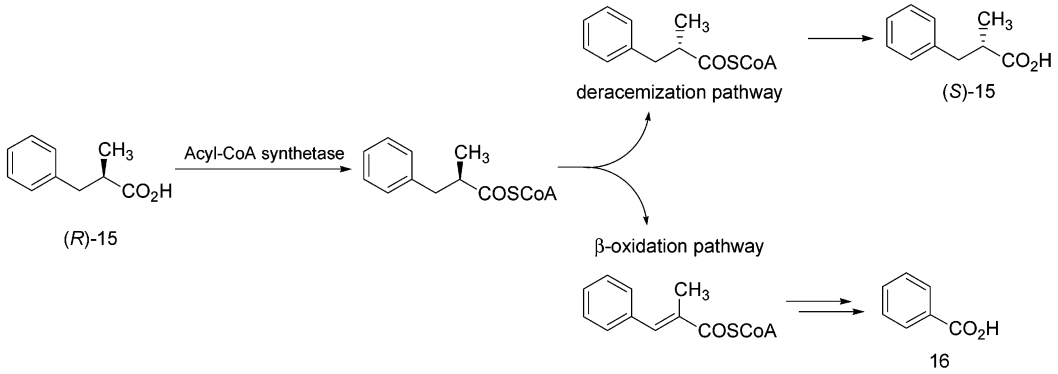
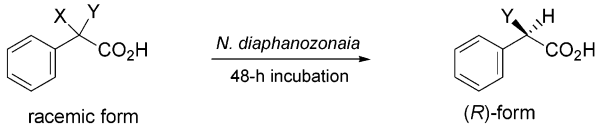


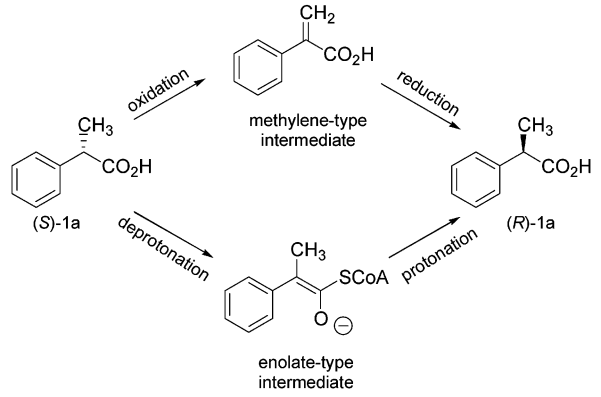
TABLE 8. Mechanistic Investigation of Deracemization Reaction with 2-Phenylpropanoic Acid Derivatives[a]



				starting compd		product		
entry	sub.	X	Y	ee (%)	D content (%)	yield[b] (%)	ee[c] (%)	D content[d] (%)
1	**8a**	H	CH_3	racemic		81	69	
2	**1a**	H	CH_3	R, 88		79	65	
3	**1a**	H	CH_3	S, 93		59	50	
4	**1i**	D	CH_3	racemic	98	58	72	20
5	**1j**	H	CD_3	racemic	>99	61	71	>99

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* at 30 °C. [b] Isolated yield after conversion to the corresponding methyl ester (**2a**,**i**,**j**). [c] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester. [d] D content was calculated from ^{1}H NMR analysis.

SCHEME 6. Two Types of Intermediate during the Deracemization Reaction of 2-Phenylpropanoic Acid (1a)



medium-chain acyl-CoA synthetase plays an important role in the present deracemization reaction. This is in marked contrast with the case of rat liver, in which the chiral inversion process was inhibited by palmitic acid, a typical substrate of long-chain acyl-CoA synthetase.[8c] In addition, it is very interesting that the same compound inhibited the deracemization of both **1a** and **10c**, which means that the same acyl-CoA synthetase is involved in both processes despite the fact that the enantioselectivity between **1a** and **10c** was opposite (Scheme 3, Table 6).

Figure 1 shows the comparison of the inhibitory activity of benzoic acid toward **1a** and **10c**. In this figure, the relative activity is expressed by the ratio of ee values of the product after 12 h of incubation in the presence and absence of benzoic acid. Apparently, the effect of benzoic acid on **1a** is more serious compared to that on **10c**. These results show that the affinity of **10c** to acyl-CoA synthetase is stronger than that of **1a**, which is reflected in the higher ee of **10c** compared to that of **1a**.

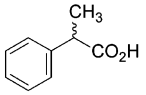
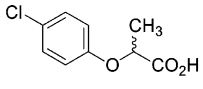
To obtain more information on the inhibitory activity on the deracemization enzymes, the reaction of **10c** was carried out in the presence of 5 equiv of *n*-alkanoic acids with various chain lengths (Table 10). It is known that medium-chain acyl-CoA synthetase recognizes C4−C12 lengths of *n*-alkanoic acids and that long-chain acyl-CoA synthetase is capable of catalyzing the reaction of C14−C22 *n*-alkanoic acids.[8c] When *n*-alkanoic acids with medium chain lengths (C4−C13) were present in the reaction mixture, the chiral inversion process was completely inhibited (entries 2−9). On the other hand, long-chain *n*-alkanoic acids, such as myristic acid, palmitic acid, and stearic acid, affected the deracemization reaction only a little and optically active **10c** was obtained (entries 10−12). Thus it was also shown that medium-chain acyl-CoA synthetase takes part in the chiral inversion process in *N. diaphanozonaria*.

Conclusion

We found a novel enzymatic method for the preparation of optically active α-substituted carboxylic acids. This process can be performed under mild reaction conditions via an extremely simple procedure. It gives optically active products in good to high yields as well as enantiomeric excess. This deracemization reaction is interesting, in that a small modification of the substrate brings about a drastic change in the enantioselectivity.

Though the detail of the reaction mechanism of this biotransformation is not clear at present, plural enzymes are likely to be related. The inhibition studies suggested that the formation of acyl-CoA will be the key step as reported in the case of rat liver, although the enantioselectivity and reactivity are quite different. Low yields of the product in some cases will be due to the oxidation of these intermediates. Further investigation on the reac-

TABLE 9. Relation of Acyl-CoA Synthetase to the Deracemization Reaction[a]

entry	substrate	benzoic acid	palmitic acid	control
1	 (±)-1a	racemic N.D.	24% ee 43% yield	24% ee 65% yield
2	 (±)-10c	racemic 61% yield	91% ee 76% yield	92% ee 75% yield

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* for 12 h at 30 °C. Inhibitor was added (5 equiv) to substrate **1a** or **10c**. Isolated yield was determined after conversion to the corresponding methyl ester (**2a** and **11c**). Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester. Benzoic acid and palmitic acid are typical substrates of medium-chain acyl-CoA synthetase and long-chain acyl-CoA synthetase, respectively.

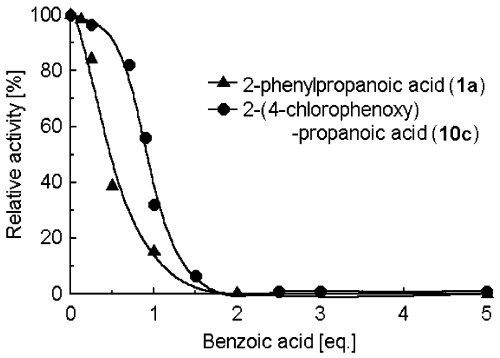


FIGURE 1. Comparison of the inhibitory activity of benzoic acid toward 2-phenylpropanoic acid (**1a**) and 2-(4-chlorophenoxy)propanoic acid (**10c**).

TABLE 10. The Influence of Various Chain Lengths of *n*-Alkanoic Acid on the Deracemization Process in *N. Diaphanozonaria*[a]

entry	inhibitor[b]	chain length	ee[d] (%)	yield[c] (%)
1	none		92	75
2	*n*-butanoic acid	4	racemic	76
3	*n*-valeric acid	5	racemic	99
4	*n*-hexanoic acid	6	racemic	87
5	*n*-heptanoic acid	7	racemic	76
6	*n*-octanoic acid	8	racemic	82
7	*n*-decanoic acid	10	racemic	68
8	*n*-dodecanoic acid	12	racemic	90
9	*n*-tridecanoic acid	13	racemic	65
10	myristic acid	14	52	62
11	palmitic acid	16	91	76
12	stearic acid	18	52	76

[a] The starting compounds were incubated with growing cells of *N. diaphanozonaria* for 12 h at 30 °C. [b] The inhibitor was added (5 equiv) to substrate **10c**. [c] Isolated yield after conversion to the corresponding methyl ester (**11c**). [d] Ee of the product was determined by HPLC analysis after conversion to the corresponding methyl ester.

tion mechanism as well as the isolation of the enzymes is now underway.

Experimental Section

Microorganism. The strain, *Nocardia diaphanozonaria* JCM3208, used in this experiment is available from the Japan Collection of Microorganism: The Institute of Physical and Chemical Research (Riken), 2-1 Hirosawa, Wako 351-0106, Japan.

2-Phenylpropionamide (3). To a solution of 2-phenylpropionitrile (0.52 g, 3.4 mmol) in MeOH (5 mL) was added a 35% hydrogen peroxide solution (0.4 mL). The pH was adjusted at 8.0 by adding 2 M sodium hydroxide. After 1 h, 35% hydrogen peroxide solution (0.4 mL) was added to the reaction. The reaction mixture was stirred for an additional 1 h. The solvent was removed in vacuo, and the residue was extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc/EtOH 6/2/1) to give 2-phenylpropionamide (0.50 g, 95% yield) as a colorless needle: mp 87−88 °C; IR (KBr disk) 3361, 3185, 2983, 2801, 1657, 1451, 1405, 1287, 1264, 1134, 1114, 696, 656 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.33 (m, 5H), 5.27 (br s, 2H), 3.60 (q, J = 7.3 Hz, 1H), 1.55 (t, J = 7.3 Hz, 3H).

Methyl 2-(4-Fluorophenyl)propanoate (2h). To a solution of diisopropylamine (0.5 mL, 3.6 mmol) in tetrahydrofuran (4 mL) was added *n*-butyllithium (1.52 M in hexane, 2.4 mL) at −78 °C under Ar, and the mixture was stirred for 30 min. Then a solution of methyl 4-fluorophenylacetate (0.50 g, 3.0 mmol) in tetrahydrofuran (5 mL) was added dropwise, and the mixture was stirred for 20 min at the same temperature. The mixture was then allowed to warm to 0 °C. Methyl iodide (0.3 mL, 4.8 mmol) was added and the mixture was stirred for 30 min. The reaction mixture was acidified by 2 M hydrochloric acid and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 19/1) to give methyl 2-(4-fluorophenyl)propanoate (0.48 g, 89% yield) as a colorless oil; IR (film) 2983, 2953, 1739, 1604, 1510, 1456, 1436, 1224, 1170, 1070, 838, 732, 551 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.19 (dt, J = 2.0, 8.8 Hz, 2H), 6.94 (tt, J = 2.0, 8.8 Hz, 2H), 3.64 (q, J = 6.8 Hz, 1H), 3.59 (s, 3H), 1.41 (d, J = 6.8 Hz, 3H).

4-Fluoropnenylpropanoic Acid (1h). A solution of **2h** (0.25 g, 1.4 mmol) in EtOH (3 mL) was added to an aqueous solution (3 mL) of potassium hydroxide (0.5 g, 9.0 mmol) at 0 °C. After being stirred for 8 h, the reaction mixture was acidified by 2 M hydrochloric acid, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrated in vacuo to give 2-(4-fluorophenyl)propaoic acid (0.23 g, quant) as a colorless oil: IR (film) 2985, 2626, 1705, 1603, 1506, 1457, 1410, 1225, 1161, 838, 722, 559 cm^{-1}.

Compounds **1d**−**g** were prepared according to a similar procedure.

Indan-1-carboxylic Acid (4b). A solution of indan-1-one (5.0 g, 37.8 mmol) in freshly distilled ethyl chloroacetate (6.9 g, 56.3 mmol) was added dropwise over 1 h to a solution of potassium *tert*-butoxide (6.4 g, 57.0 mmol) in dry *tert*-butyl alcohol (50 mL) at 0 °C. After completion of the addition, the reaction mixture was allowed to warm to room temperature and stirred for further 1.5 h. The excess *tert*-butoxide was decomposed by passing CO_2 through the reaction mixture and the solvent was removed in vacuo at 35 °C. Ether (20 mL) was added to the residue and the mixture was filtered through a pad of Celite. The Celite was washed with ether and the washing was combined with the filtrate and concentrated in vacuo to give a dark-brownish residue. To a solution of potassium hydroxide (3.5 g, 46.0 mmol) in ethanol (5 mL) was added the solution of this residue in ethanol (5 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for an additional 3 h. The precipitate was filtered and washed with ice-cold ethanol and ether. A vigorous evolution of gas occurred when acetic acid (20 mL) was added to this precipitate at 0 °C. After being stirred at room temperature for 15 min, the reaction mixture was heated at 130 °C for 1 h. The reaction mixture was then cooled to room temperature, diluted with water (10 mL), and extracted with

ether. The organic layer was washed with saturated sodium hydrogen carbonate and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo to give a brownish oil. To a solution of this oil in acetone (10 mL) was added dropwise Jones' reagent (12 mL) over 20 min at 0 °C and the reaction mixture was stirred for an additional 1 h. The excess Jones' reagent was quenched by the addition of MeOH, and the reaction mixture was stirred for 15 min before additing 10% NaCl solution. The reaction mixture was extracted with ether. The organic layer was washed with brine and saturated sodium hydrogen carbonate solution, dried over anhydrous sodium sulfate, and concentrated in vacuo to give crude indan-1-carboxylic acid (3.9 g, 64% yield) as a brownish oil. The crude product was recrystallized from hexane/EtOAc to give indan-1-carboxylic acid (2.0 g, 32% yield (4 steps starting from indan-1-one)) as a yellowish crystal: IR (KBr disk) 2942, 2730, 1710, 1418, 1318, 1282, 1228, 940, 738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.42 (m, 1H), 7.20 (m, 3H), 4.08 (dd, J = 6.3, 8.1 Hz, 1H), 3.12 (m, 1H), 2.93 (m, 1H), 2.40 (m, 2H).

Ethyl 2-(4-Fluorophenoxy)propanoate (17b). Sodium hydride (60% in paraffin, 80 mg, 2.0 mmol) was stirred for 5 min in hexane (5 mL) under Ar, then the solvent was removed by a syringe followed by evaporation with a vacuum pump. After the same operation was repeated three times, the residue was suspended in tetrahydrofuran (5 mL) and the mixture was cooled to 0 °C. To this mixture was added a solution of 4-fluorophenol (0.21 g, 1.9 mmol) in tetrahydrofuran (3 mL) dropwise over 5 min, and the mixture was stirred for 5 min at the same temperature. Then the mixture was allowed to warm to room temperature and stirred for an additional 15 min. A solution of ethyl 2-bromopropanoate (0.7 g, 3.9 mmol) in tetrahydrofurane (2 mL) was added and the mixture was stirred for 14 h. The reaction mixture was acidified by 2 M hydrochloric acid and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 9/1) to give ethyl 2-(4-fluorophenoxy)propanoate (0.34 g, 86% yield) as a colorless oil: IR (film) 2988, 2940, 1753, 1505, 1447, 1377, 1274, 1203, 1134, 1052, 829, 748 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.96 (m, 2H), 6.83 (m, 2H), 4.67 (q, J = 6.8 Hz, 1H), 4.22 (q, J = 6.8 Hz, 2H), 1.60 (d, J = 6.8 Hz, 2H), 1.25 (t, J = 6.8 Hz, 3H).

2-(4-Fluorophenoxy)propanoic Acid (10b). A solution of **17b** (0.21 g, 1.0 mmol) in EtOH (3 mL) was added to an aqueous solution (3 mL) of potassium hydroxide (0.62 g, 9.5 mmol) at 0 °C. After being stirred for 4 h, the reaction mixture was acidified by 2 M hydrochloric acid and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo to give 2-(4-fluorophenoxy)propanoic acid (0.18 g, quant) as a colorless solid: IR (KBr disk) 2999, 2645, 1717, 1505, 1291, 1226, 1139, 922, 831, 757, 685, 516 cm^{-1}.

Compounds **10d−j, 12, 13, 14a**, and **15** were prepared according to a similar procedure. The spectral data of these compounds are available as Supporting Information.

2-Deuterio-2-phenylpropanoic Acid (1i). To a solution of methyl 2-phenylpropanoate (**1a**, 2.0 g, 13.6 mmol) in tetrahydrofuran (200 mL) was added dropwise *n*-butyllithium (1.52 M in hexane, 8.8 mL) at −78 °C under Ar. After 1 h, *n*-butyllithium (1.52 M in hexane, 17.5 mL) was added dropwise. During this stage the colorless solution turned yellow. The reaction mixture was stirred for an additional 100 min at −78 °C. Then the reaction mixture was allowed to warm to 0 °C and deuterium chloride solution (30% in D_2O, 5 mL) was added. The yellow color immediately disappeared. After 60 min of stirring at room temperature, the solvent was removed under reduced pressure and the residue was extracted with ether. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 3/1) to give 2-deuterio-2-phenylpropanoic acid (2.0 g, 98% yield) as a colorless oil: IR (film) 2982, 2655, 2548, 1704, 1498, 1449, 1409, 1294, 1231, 1142, 943, 726, 697 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.27 (m, 5H), 1.45 (s, 3H).

Methyl 3,3,3-Trideuterio-2-phenylpropanoate (2j). Methyl 3,3,3-trideuterio-2-phenylpropanoate (0.47 g, 96% yield) was obtained as a colorless oil starting from methyl 2-phenylacetate (0.44 g, 3.0 mmol) according to the same procedure as described above for the synthesis of **2h** except with trideuterio methyl iodide instead of methyl iodide: IR (films) 3030, 2952, 2233, 1739, 1602, 1495, 1454, 1326, 1246, 1202, 1169, 1022, 943, 806, 768, 730, 698 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.22 (m, 5H), 3.66 (s, 1H), 3.61 (s, 3H).

3,3,3-Trideuterio-2-phenylpropanoic acid (1j). 3,3,3-Trideuterio-2-phenylpropanoic acid (0.20 g, 97% yield) was obtained as a colorless oil starting from **2j** (0.22 g, 1.3 mmol) according to the same procedure as described above for the synthesis of **1h**: IR (film) 3032, 2929, 2722, 2233, 1705, 1601, 1497, 1455, 1416, 1290, 1226, 1186, 939, 731, 696 cm^{-1}.

General Procedure for the Deracemization Reaction of α-Substituted Carboxylic Acids with the Aid of *N. diaphanozonaria* JCM3208. The ingredients of the medium were as follows: glycerol (10 g/L), peptone (2 g/L), beef extract (3 g/L), yeast extract (3 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), pH 7.0. To 90 mL of a nutrient medium was added a suspension of 48-h incubated cells of *N. diaphanozonaria* in 10 mL of the broth and the incubation was carried out at 30 °C for 24 h (first incubation). Then, 100 mg of (±)-α-substituted carboxylic acid was added to the suspension, and the mixture was shaken for the appropriate time indicated in the tables depending on the substrates (second incubation). The reaction mixture was filtered through a pad of Celite to remove the cells. The Celite was washed with EtOAc and the washing was combined with the filtrate. After being acidified by 2 M hydrochloric acid, the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was treated with diazomethane and purified by pTLC (hexane/EtOAc 9/1) to give the methyl ester of the starting acid as a colorless oil. The incubation time, yield, and spectral data of the esters are available as Supporting Information.

Inhibition Study of Acyl-CoA Synthetase. To 90 mL of a nutrient medium was added a suspension of 48-h incubated cells of *N. diaphanozonaria* in 10 mL of the broth and the incubation was carried out at 30 °C for 24 h (first incubation). Then, the substrate (**1a** or **10c**, 0.1 g) and appropriate amount of the inhibitor (benzoic acid, palmitic acid, or *n*-alkanoic acid) were added to the suspension, and the mixture was shaken for 12 h (second incubation). The reaction mixture was filtered through a pad of Celite to remove the cells. The Celite was washed with EtOAc and the washing was combined with the filtrate. After being acidified by 2 M hydrochloric acid, the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was treated with diazomethane and purified by pTLC (hexane/EtOAc 9/1) to give the methyl ester of the starting acid as a colorless oil. The ee of the product was determined by HPLC with a Daicel Chiralcel OJ column (9/1 hexane/2-propanol; 0.5 mL/min; 254 nm).

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Supporting Information Available: The spectral data for **10d−j, 12, 13, 14a**, and **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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