Bull. Chem. Soc. Jpn., 70, 2895—2911 (1997)

Accounts

Enzyme-Mediated Enantioselective Protonation to Enolates in an Aqueous Medium

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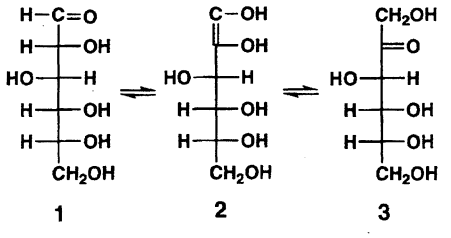
(Received July 23, 1997)

An enzyme sometimes provides a entirely hydrophobic chiral reaction field to a synthetic substrate. This is one of the most characteristic features of an enzyme compared to ordinary chemical catalyst. Taking advantage of being able to carry out a reaction of a prochiral compound in this special pocket, it is possible to prepare an optically active product via asymmetric protonation in an aqueous medium. Two such examples are demonstrated in this article, i.e., asymmetric hydrolysis of enol esters, and asymmetric decarboxylation of α-aryl-α-methylmalonic acid to give optically active ketones and α-arylpropionic acids, respectively. Some related reactions are described as well.

Biocatalysts (enzymes, microbial whole cells, plant cultures, and catalytic antibodies) are now widely used in organic synthesis.[1—4] In almost all cases, enantio-, regio-, and chemoselectivities of the reactions, and mild conditions under which the reactions proceed are emphasized as the characteristics of biocatalysts. Of course, the authors are correct, but there is another important feature that we should not overlook: That biocatalysts provide unique hydrophobic reaction fields in an aqueous medium. The most representative well-known example will be the isomerism between glucose (**1**) and fructose (**3**). The isomerization proceeds via the common enolate form **2** (Eq. 1).

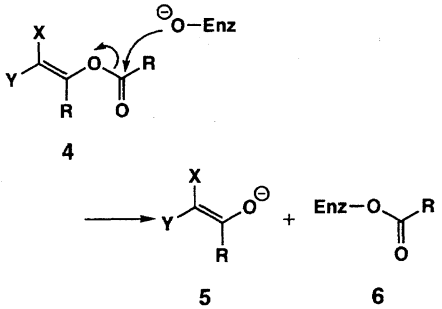


1 2 3 (1)

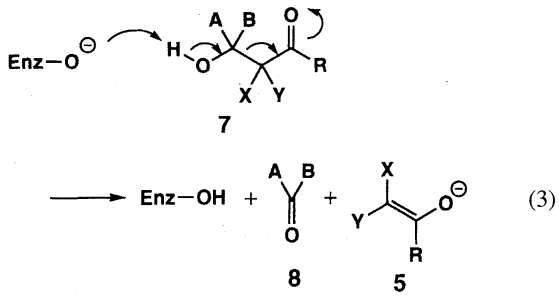
One observes no epimerization of glucose through the rearrangement, which means that the protonation on C-2 of enol **2** is completely enantioselective (or more accurately, diastereoselective). If a free water molecule comes in contact with this reaction, the stereoselectivity of protonation could not be perfect and this would cause some epimerization of glucose. In this way, this reaction demonstrates that in some cases the active site of an enzyme completely excludes free water molecules or, even if they do participate in the reaction, they are entirely under the control of the enzyme, even though the reaction is performed in an aqueous medium.

Applying such type of asymmetric protonation to a synthetic enolate, it would be possible to develop a new route to an optically active compound which has its chiral center adjacent to a carbonyl group. However, it is practically impossible to use an enol or enolate as a substrate of enzymatic reaction in an aqueous medium because of its unstability. The substrate enolate will react with water to give a racemic product before it binds to the active site of the enzyme. Thus the enolate should be generated in situ in the enzyme pocket. We might suppose two possible routes to form an enolate in the enzyme pocket, using a water-stable precursor.

The first route is realized when an enzyme reacts as a nucleophile, which is very common with esterases and lipases. An esterase attacks the acyl carbon of an ester to form an enolate and an acyl enzyme complex (**6**) (Eq. 2).

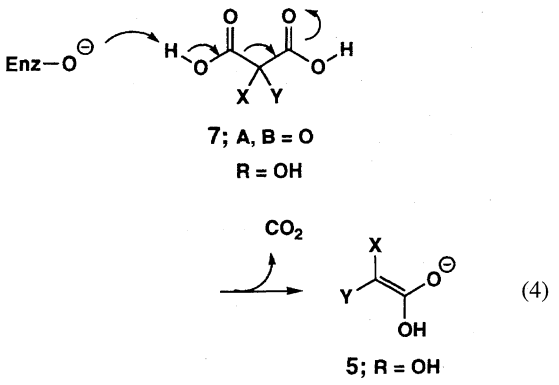


4 → 5 + 6 (2)



(3)

Thus, when we use an enol ester of type **4** as the substrate, the expected enolate (**5**) will be generated in the active site of the enzyme. Enantioface differentiating protonation on the C=C double bond of **5** will give an optically active compound. The second route will be possible when an enzyme works as a base. Although it will be difficult for a weak base such as an enzyme to abstract a proton from a carbon, even if it is α to a carbonyl group, we can think of a retroaldol-type reaction shown in Eq. 3. Aldol reaction and retroaldol reaction are the most general paths for the biosynthesis and degradation of sugars in living cells. Thus the conversion of an aldol type compound (**7**) according to Eq. 3 will be expected to be the most promising way of generating an enolate from a carbonyl compound. In addition, if we want to prepare an optically active compound starting from a prochiral one rather than a racemate, the ligands A and B of **7** must be a carbonyl oxygen and R should be a hydroxy group. As an inevitable consequence, the substrate compound **7** should be a disubstituted malonic acid and the target enzyme will be a decarboxylase (Eq. 4).

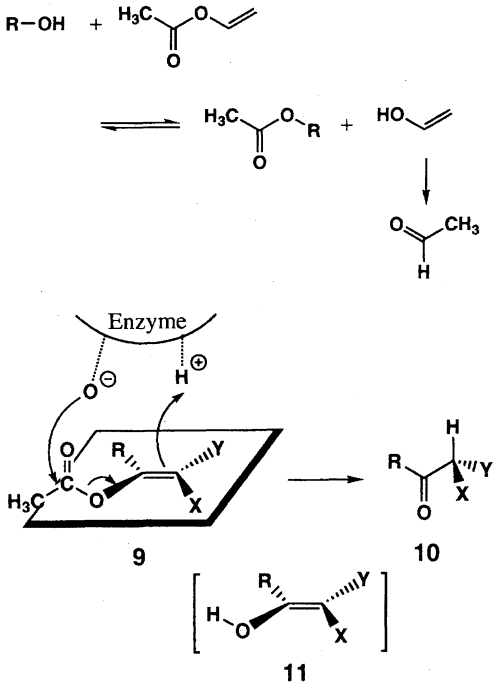


(4)

The results of these two types of reactions as well as of some related ones are presented in this article.

1. Asymmetric Protonation to Enol Esters

1-1. Asymmetric Hydrolysis of Cyclic Enol Acetates. Enzymatic hydrolysis of esters has been established as a useful method for the production of a variety of optically active alcohols, carboxylic acids, and esters. All such syntheses are based on the ability of enzymes to distinguish the chiral or prochiral centers. On the other hand, vinyl or propenyl acetate is extensively used as an active acylating agent in transesterification reactions.[5,6] In these cases, the product from the alcohol part of the starting ester is a carbonyl compound, but not an alcohol! Because of this reason, vinyl acetate makes the equilibrium of transesterification reaction practically irreversible (Eq. 5). However, there had been no example until our first report that paid any attention to the potential possibility of this reaction as a tool for providing a new route to optically active ketones and aldehydes. Equation 6 illustrates the guiding principle of our design. In the course of a hydrolysis of an enol ester (**9**), if a protonation occurs on the β-carbon with differentiation of the prochiral face of the enol ester, concomitantly with nucleophilic attack of the enzyme residue, and thus the ketone (**10**) forms without intermediary formation of enol (**11**), then the resulting ketone will be optically active. Even when the protonation occurs on the oxygen atom and forms enol **11**, there still remains some possibility of resulting in the formation of an optically active product if the enol form isomerizes to keto form in the chiral reaction field of the enzyme.

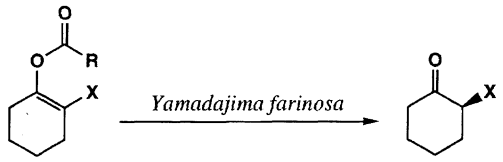


(5)

(6)

First, we have chosen 1-acetoxy-2-methylcyclohexene (**12**) as the reference substrate for screening of enzymes and microorganisms, because it can readily be prepared from racemic 2-methylcyclohexanone by the reaction with acetic anhydride and perchloric acid without contamination of the regio- and stereo-isomers. Many kinds of commercially available enzymes and microorganisms were examined to learn that most of them did hydrolyze **12a**, but to afford racemic ketones. Among them, *Yamadazyma farinosa* (formally classified as *Pichia miso*, then *Pichia farinosa*) IFO 10896 gave the best results.[7,8] Enol acetate (**12a**) (0.1 mL) was added to 50 mL of a suspension of *Y. farinosa* (collected from 400 mL of broth) in phosphate buffer (pH 6.5) and incubated for 3 h (Eq. 7). Extraction of the broth with an organic solvent and purification of the product with preparative TLC afforded 2-methylcyclohexanone. The sign of optical rotation indicates its configuration to be (*S*). Enantiomeric excess of the product was determined as follows. Ketone

13a was reduced with super hydride ($LiBEt_3H$) and derived to the corresponding MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) ester, which was analyzed by capillary GLC. Propionyl ester gave the best result (92%e.e.). The results for other esters are summarized in Table 1. Although benzoyl ester decreased the enantioselectivity, use of propionyl esters allowed some variations in the structure of the substituents. Not only esters which have *n*-alkyl side chains but also the ones with unsaturated substituents and benzyl group were hydrolyzed with good to high enantioselectivity. Because non-enzymatic hydrolysis is negligible under the reaction conditions, the enantiomeric excess of the products can be regarded as reflecting the enantioselectivity of the enzyme. The special arrangements of the ligands around the chiral center of the products were the same for all compounds, although the *R*, *S* definitions are different because of the reversal of the priority rule.

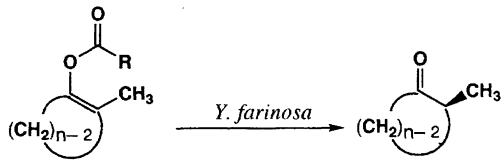


(7)

12 **13**

a R = CH_3, X = CH_3
b R = C_2H_5, X = CH_3
c R = C_2H_5, X = n-C_3H_7
d R = CH_3, X = n-C_7H_{15}
e R = C_2H_5, X = CH_2=CH-CH_2-
f R = CH_3, X = $PhCH_2$

This unique biocatalyst-mediated hydrolysis can be extended to medium-sized cycloalkanone enol esters (Eq. 8).



(8)

14 **15**

a n = 8, R = CH_3
b n = 10, R = C_2H_5
c n = 10, R = C_6H_5
d n = 12, R = CH_3
e n = 15, R = CH_3

As seen from Table 2, optically active 2-methylcycloalkanones of eight-, ten-, and twelve-membered rings were smoothly obtained from the corresponding enol esters, whereas larger ring compounds, such as fifteen-membered ring compound (**14e**) showed only poor reactivity and selectivity. It is noteworthy that 2-methylcyclododecanone (**14d**) had extremely high optical purity and the opposite absolute configuration (*R*)[9,10] to that of other cycloalkanones (*S*). Two explanations are possible for the inversion of configuration. The configuration of the starting material could be reversed as the carbon numbers of the ring are large enough to take the *trans* configuration or the substrate could interact in a different manner with the enzyme. An NOE experiment indicated that the configuration of **14d** was consistent with the others. Thus, it is believed that the mode of interaction between the enzyme and the twelve-membered ring compound is somewhat different from the other cases of smaller rings, although the details are not clear.

Table 1. Asymmetric Hydrolysis of α-Substituted Cyclohexanone Enol Esters

R	X	[Sub]	Time	Yield	e.e.	Config.
		%	h	%	%	
CH_3	CH_3	0.2	3	79	90	*S*
C_2H_5	CH_3	0.2	3	78	92	*S*
C_2H_5	n-C_3H_7	0.2	3	78	86	*S*
CH_3	n-C_7H_{15}	0.2	3	77	87	*S*
C_2H_5	CH_2=CH-CH_2	1.0	24	92	77	*R*
CH_3	$PhCH_2$	0.2	3	75	84	*R*

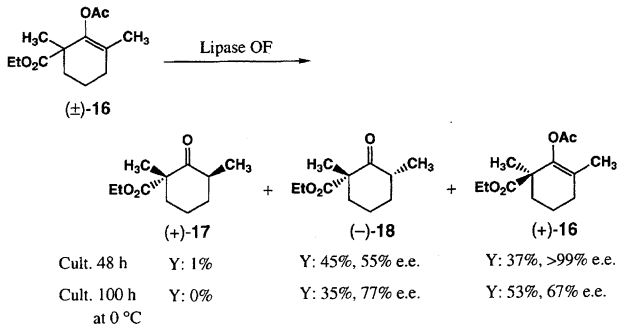
Table 2. Asymmetric Hydrolysis of α-Substituted Cycloalkanone Enol Esters

n	R	Time	Yield	e.e.	Config.
		h	%	%	
8	CH_3	3	71	67	*S*
10	C_2H_5	3	83	89	*S*
10	C_6H_5	24	84	86	*S*
12	CH_3	24	67	96	*R*
15	CH_3	24	38	<2	

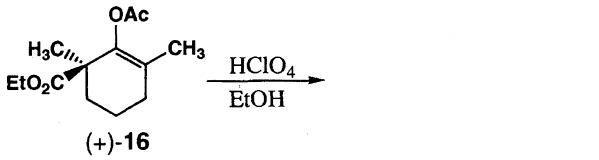
1-2. Asymmetric Hydrolysis of Cyclic Enol Acetates with a Chiral or Prochiral Center. The above described enantioface differentiating hydrolysis was applied to a compound which already has a chiral center near the double bond, expecting to prepare an optically active building block with two chiral centers. Because six-membered ring enol esters gave a pretty good result, we tried a kinetic resolution via enantioface differentiating hydrolysis of racemic enol acetate **16**. However, to our disappointment, *Y. farinosa* showed little enantioselectivity to give diastereomeric mixture of hydrolysis product (**18**). Thus, commercially available hydrolyzing enzymes were tested to find that lipase OF (originated from *Cadida rugosa*, purchased form Meito) gave agreeable results.[11]

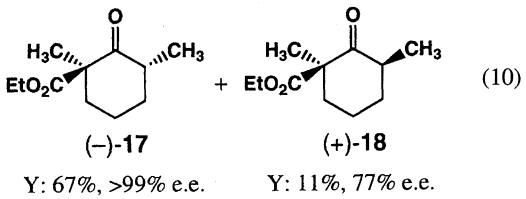
Cultivation of racemic **16** with the lipase gave optically active **18** (yield 45%) and unreacted starting material of high enantiomeric excess (yield 37%, >99%e.e.), as shown in Eq. 9. Although the enantiomeric excess of the hydrolyzed product is not high enough to be used as a chiral building block for an optically active useful compounds, recovered starting material exhibited a high e.e. Accordingly if (+)-**16** can be hydrolyzed in a diastereoselective manner, to give the corresponding ketone, then the product would serve as an optically active intermediate for the synthesis of diterpenes. Fortunately, ethanolysis with a catalytic amount of perchloric acid resulted in the formation of optically active ketone (**17**) in a good yield (Eq. 10). Nucleophilic addition of phen-

ylacetylene to the carbonyl carbon of (−)-**17** followed by reduction gave **19**. Treatment of **19** with an acid resulted in cyclization in a completely diastereoselective manner, and following esterification gave deoxypodocarpic acid methyl ester (**20**) (Eq. 11), which is a useful intermediate for the synthesis of various natural products.[12]

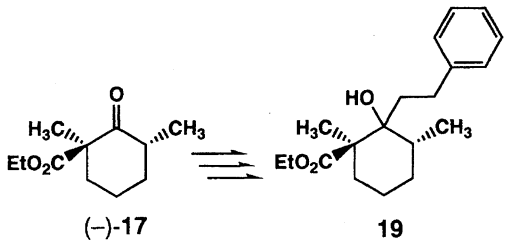


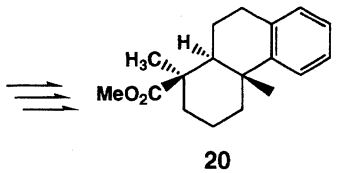
(9)





(10)

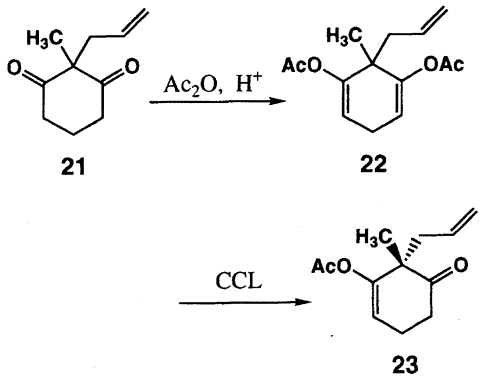




(11)

Total Y: 48%

Hydrolysis of enol esters can be utilized to prepare an optically active ketone starting from a compound with a prochiral center,[13] via an enantio-topos differentiating reaction but not via enantioface differentiating one. A French group treated dienol diacetate (**22**), which is readily prepared from the corresponding diketone (**21**) via acetylation with acetic anhydride or propenyl acetate in the presence of an acid. Although hog liver esterase and *Pseudomonas fluorescens* lipase gave only disappointing results from the standpoint of enantioselectivity, CCL (*Candida cylindracea* lipase) nicely hydrolyzed **22** to give optically active monoketone (**23**) with e.e. over 98% (Eq. 12). Since carbon chain elongation reactions are possible to both sides of the cyclohexane ring utilizing the carbonyl and masked carbonyl groups, **23** is expected to be a useful starting material of optically active compounds.

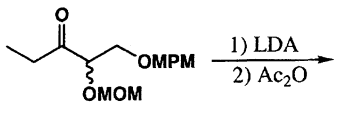


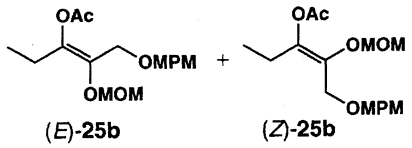
(12)

Y: 80%, >98% e.e.

1-3. Asymmetric Hydrolysis of Acyclic Enol Acetates. Enantioface differentiating hydrolysis of enol esters is also applicable to acyclic compounds. In these cases, it is important to prepare a stereochemically pure substrate because in all cases there will be a possibility to form both (*E*)- and (*Z*)-forms of enol esters starting from a racemic α,α-disubstituted ketones.

Among a variety of chiral ketones, optically active glycerol derivatives are considered to be versatile building blocks. For example, protected dihydroxy ketone of type **24** has been demonstrated to be one of the key intermediates in the synthesis of mycinolyde IV by Suzuki and coworkers.[14] We tried the enol ester method in the preparation of optically active **24**. Treatment of racemic **24** with LDA and subsequent acetylation with acetic anhydride gave a mixture of (*E*)- and (*Z*)-isomers of enol acetate **25b**, (*E*)-isomer being the major product (Eq. 13). Thus a screening of enzymes and microorganisms which show efficient enantioselectivity in the hydrolysis was performed using (*E*)-**25b** as the substrate.





(13)

MPM = -CH_2-C_6H_4-OCH_3 (*p*) MOM = -CH_2OCH_3

Curiously enough, again in this case, *Yamadazyma farinosa* IFO 10896, which was used in the hydrolysis of cyclic enol esters, was found to be the best biocatalyst to hydrolyze the substrate in an enantioselective manner. The product was

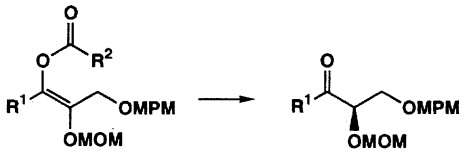
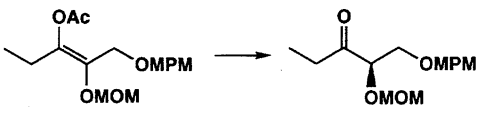


Table 3. Asymmetric Hydrolysis of Acyclic Enol Esters (*E*)-**25**

	R^1	R^2	Yield %	e.e. %
a	C_2H_5	CH_3	83	85
b	C_2H_5	C_2H_5	78	82
c	C_2H_5	CH_3O	82	81
d	*n*-C_3H_7	CH_3	86	87
e	*n*-C_4H_9	CH_3	54	90
f	3-Butenyl	CH_3	84	86

revealed to be (*R*)-ketone **24b**, of which the yield and the e.e. were 83 and 85%, respectively (Eq. 14).[15] The yield and e.e. of the product after 24 h was little affected by the initial concentration of the substrate up to 1%. When the concentration of the substrate was 0.1%, the reaction was completed in 3 h after the addition of the substrate to a suspension of the cells of *Y. farinosa* grown in an nutrient broth. This enantioface differentiating hydrolysis was successfully applied to other structurally related compounds, as shown in Table 3. The alkyl chain (R^1) was not limited to ethyl, but longer ones and ones contain an unsaturated bond also gave good results. As for the acid part, not only carboxylic moieties but also methyl carbonate was accepted as the substrate. In this case, because the resulting acid decomposes to carbon dioxide and methanol, instead of forming carboxylic acid, the control of pH of the reaction medium became easier.



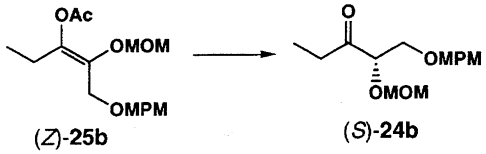
(14)

Y: 83%, 85% e.e.

Reaction conditions;

Y. farinosa, 24 h, [sub] = 0.1%, pH 6.8

In relation to the configuration (*E* and *Z*) of the substrate enol ester, the mode by which the enzyme recognizes the substrates proposes an important and interesting problem. If the enzyme recognizes only acyl and alkyl chain parts of the substrate (left half of **25**) and delivers a proton from the same prochiral face concerning the carbon binding to the ester moiety, to both (*E*)- and (*Z*)-isomers, then the configuration of the products would be reversed depending on the configuration of the starting material. On the other hand, if the enzyme recognizes the stereochemistry of the right half of the starting esters, and a proton approaches from the same prochiral face concerning the carbon which will be the asymmetric center, then the configuration of the resulting ketone would be the same regardless of (*E*)- or (*Z*)-stereochemistry of **25**. The actual result was the worst, being the middle of these two extremes. When (*Z*)-**25b** was subjected to the microbial hydrolysis, the reaction did proceed and gave (*S*)-ketone, but the e.e. of the product was only 39% (Eq. 15). Moreover, when the acyl part of the substrate was substituted with propionyl, the reaction itself was inhibited. The shape of the active site of the enzyme is estimated not to be capable of interacting smoothly with the (*Z*)-isomer because the enzyme had been originally screened using the (*E*)-isomer as the substrate.



(15)

Y: 80%, 39% e.e.

Reaction conditions;

Y. farinosa, 24 h, [sub] = 0.1%, pH 6.8

The above described difference of reactivity between (*E*)- and (*Z*)-**25b** convinces us the fact that the same microorganism which has high enantioselectivity to cyclic enol esters (**12**, **14**) exhibited also high selectivity to acyclic enol esters of (*E*)-stereochemistry rather than to (*Z*)-isomer, when the conformation of three types of compounds are depicted as in Fig. 1. The aryl moiety of **14** and (*E*)-**25b** can occupy the right part of the acyl group and the ring methylenes of **14** and methoxymethyl group of (*E*)-**25b** can be drawn in a similar manner. On the contrary, if the acyl group and the double bond of (*Z*)-**25b** are drawn in the same manner, then the methoxymethyl and aryl parts of the molecule occupy the opposite direction to the ones of the former two substrates. Consequently, **14** and (*E*)-**25b** can be good substrates for the same enzyme, while (*Z*)-**25b** can not. In the case of cyclic enol esters, the α-substituent was not restricted to benzyl and some alkyl groups were allowed to bind on this position. Accordingly, the presence of MOM group on the right hand side of acetyl group in the case of (*Z*)-**25b** may not be serious enough to inhibit the normal binding of the substrate to the active site, but the enzyme would allow an aryl group to locate only on the upper right hand site of the ester moiety.

1-4. How Can a Yeast Catalyze Enantioselective Pro-

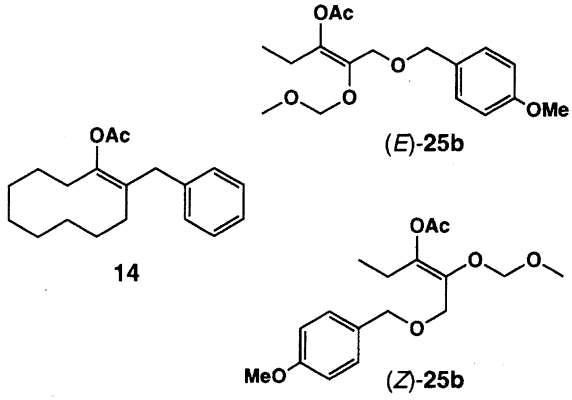


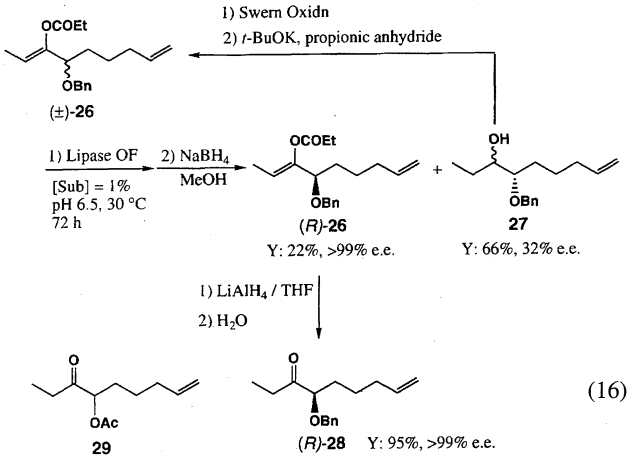
Fig. 1. Supposed conformations of enol esters.

tonation? K. Matsumoto and co-workers tried to isolate the enzyme which is responsible for this enantioselective hydrolysis of enol esters and found an interesting protein.[16] *Y. farinosa* was disrupted by a French press and the hydrolysis of 2-benzylcyclohexanone enol acetate (**12f**) was tried using the cell free extract. Although some decrease of activity was observed, the enantioselective reaction proceeded in a similar manner to give (*R*)-2-benzylcyclohexanone (**13f**). Then, a curious phenomenon was observed when this cell free extract was subjected to ultracentrifugation. Although the clear supernatant kept its hydrolyzing ability, the resulting ketone was revealed to be racemic. On the other hand, the suspension of precipitate obtained by centrifugation showed no hydrolyzing activity.

Surprisingly, when the suspension of this precipitate was added to a solution of the supernatant, the resulting mixture showed hydrolyzing activity to give an optically active product. The enantiomeric excess of ketone **13f** increased according to the amount of added precipitate. Thus it can be concluded that this precipitate is essential to the enantioselectivity of the reaction. The novel effect of this "enantioselectivity-promoting factor" was further confirmed when it was used in combination with PLE (pig liver esterase) and lipase OF (from *Candida rugosa*), both of which hydrolyzed the substrate **12f**, but in a nonenantioselective manner. Addition of a small amount of the unknown factor to a solution of the above enzymes resulted in the formation of ketone **13f** with 23 and 16%e.e., respectively. Although e.e.'s are not enough from the synthetic standpoint, this was the first example to demonstrate the existence of "enantioselectivity promoting factor." This enantioselectivity-promoting effect was not affected when the precipitate was treated with DNase I or RNase A, but decreased after the treatment with trypsin or α-chymotrypsin, indicating that this factor is an insoluble protein.

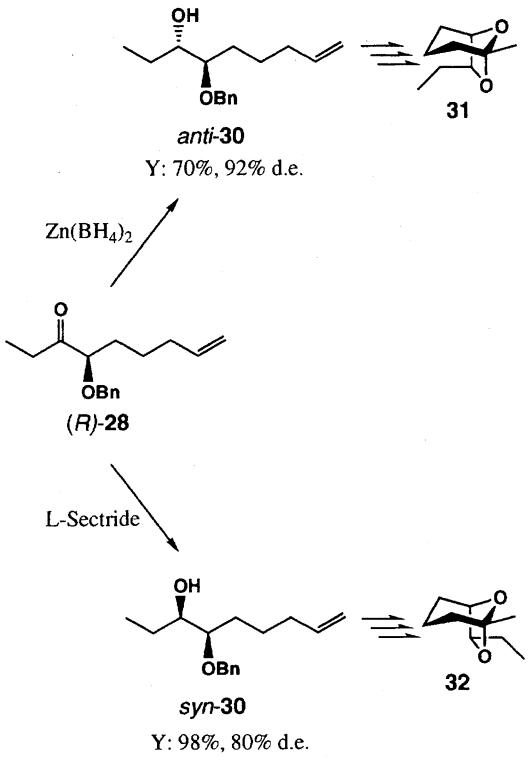
A similar "enantioselectivity-promoting factor" was found in commercially available lipase AP, which catalyzes enantioselective hydrolysis of enol ester **12f**. While PLE promotes the hydrolysis of the ester, the resulting ketone is racemic as described above. However, it was endowed with enantioselectivity in the hydrolysis of **12f** as the model enol ester, by adding a small amount of unknown protein obtained from lipase AP. Starting from 10.2 g/420 mL protein (from 70 g of lipase AP), the enantioselectivity-promoting factor was purified to 120 mg/8 mL. A solution of PLE (1.5 μL, 1850 units/mL) and 100 μL of the above solution of "enantioselectivity-promoting factor" was mixed and diluted to 1 mL. The hydrolysis reaction was carried out using 1 mg of **12f** at 30 °C for 15 min. The e.e. of resulting ketone **13f** was as high as 92%.[17] Although the precise mechanism is not clear, a protein originally contained in lipase AP evidently enhanced the enantioselectivity of the hydrolysis, and the role of this unique factor is the first example in asymmetric reactions catalyzed by enzymes.

1-5. Optical Resolution of Racemic Ketones via Enantioselective Hydrolysis. In general, enzymatic hydrolyses are applied to prepare optically active alcohols, amines, carboxylic acids, esters, and amides. Different from these usual compounds, hydrolysis of enol esters can be utilized in optical resolution of ketones.[18] One such typical example is illustrated in Eq. 16. Optically active ketone **28** is a straightforward common intermediate for the synthesis of (+)-*endo*- and (+)-*exo*-brevicomins (**31** and **32**, respectively). In this synthesis, the key step is asymmetric induction via diastereoselective reduction of the adjacent carbonyl group based on the chirality of an already existing asymmetric center. Accordingly, the protecting group of the hydroxy group of **28** should be large and stable to acid and base, i.e., benzyl rather than an ester group. Thus, if one plans to obtain such type of molecule via a biocatalyst mediated reaction, the general method, i.e., hydrolysis of the corresponding ester (for example, acetate **29**), or an acylation of the hydroxy group is a rather indirect method. In such a case, the smartest way would be to transform the carbonyl group to the one which can serve as the substrate of enzymatic reactions. In this way, we applied the present "enol esterification and subsequent hydrolysis" method to racemic benzyloxy ketone **28**.



(16)

Unfortunately, *Y. farinosa* didn't work well to enol propionate (**26**). Among some commercially available lipases and esterases screened, lipase OF showed relatively high enantioselectivity, although it was not excellent.[19] Optically pure (*R*)-enol propionate was recovered in 22% yield after a rather prolonged reaction time (72 h). Since the separation of resulting ketone (*S*-**28**) and optically active starting material (*R*-**26**) was very difficult, the reaction mixture was immediately treated with sodium borohydride. Reduction of ketone to diastereomeric diol monobenzyl ether (**27**) facilitated the isolation of remaining enol ester (*R*)-**26**. Swern oxidation of **27** followed by treatment with potassium *t*-butoxide and propionic anhydride afforded the racemic starting material which can be recycled again. Optically pure enol propionate was reduced with LAH to form lithium enolate, which in turn readily converted to optically pure (*R*)-ketone (**28**) by the treatment with acidic water.



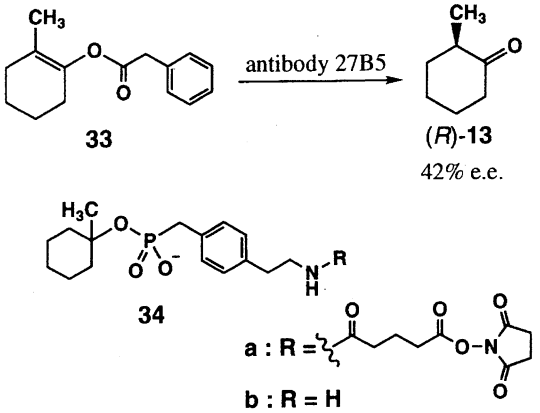
(17)

Equation 17 illustrates the further transformations. Reduction of the carbonyl group of (*R*)-**28** with zinc borohydride gave *anti*-diol monobenzyl ether (*anti*-**30**) in a highly diastereoselective manner. This was converted to (+)-*endo*-brevicomin (**31**). On the other hand, treatment of (*R*)-**28** with L-selectride afforded *syn*-**30**, which served as the key intermediate for the synthesis of *exo*-brevicomin (**32**).[20]

1-6. Asymmetric Hydrolysis of Enol Esters and Vinyl Ethers by the Aid of Catalytic Antibodies. Catalytic antibodies are now well established to be a powerful tool as tailor-made biocatalysts in the synthesis of chiral compounds.[21,22] The first successful example in which a catalytic antibody was applied to the enantioface-differentiating hydrolysis of enol esters was reported by I. Fujii, R. A. Lerner, and K. D. Janda.[23] Hapten **34a** was utilized as the antigen for induction of an antibody which is capable of promoting enantioselective hydrolysis of 1-methyl-2-(phenylacetoxy)cyclohexene (**33**) (Eq. 18). From 26 monoclonal antibodies screened, three were found to accelerate the hydrolysis of **33**. All these three exhibited saturation kinetics and could be completely inhibited by the addition of free hapten **34b**. The maximum enantiomeric excess determined by GLC was 42%, which was less than that achieved by natural enzymes. However this was the first demonstration of an antibody which influenced chirality via enantiofacial protonation and opened the door to further antibody-aided biotransformations of analogous compounds.

Hydrolysis of vinyl ether (**35**) is considered to be a reaction of the same category with the hydrolysis of enol ester, because the protonation on carbon is the key step. Thus, antibodies against hapten **40** were assayed for the hydrolysis of **35** in order to produce optically active aldehyde **37** (Eq. 19). They reasoned that the carboxyl groups expected in the binding sites of antibodies raised against the cationic center of hapten **40** will be in an optimal position to assist carbon protonation of vinyl ether **35**. Antibody 14D9 showed a remarkable activity for the expected cleavage of (*Z*)-**35**. The enantiomeric purity of resulting aldehyde **37** was revealed to be as high as 96% by ^{1}H NMR. When stereoisomeric substrate (*E*)-**35** was used as a substrate, it was smoothly hydrolyzed to give the same (*S*)-enantiomer of aldehyde **37**, in 93% e.e. (Eq. 20).[24—26]

Catalytic antibody 14D9 was also found to be particularly effective for the hydrolysis of the cyclic vinyl ether **38** to produce enantiomerically pure ketone **39** (Eq. 21) at a rate enhancement k_{cat}/k_{uncat} of 10^3.[25,27] Usually, one of the drawbacks of antibody catalyzed reactions is the difficulty in carrying out the reaction on a large scale. In the case of transformation of vinyl ether **38**, the authors succeeded to carry out the reaction on gram scale.[27] Substrate **38** (500 mg, 1.83 mmol) was added to a solution of antibody (57 mL, 7 mg mL^{-1}, 400 mg) in tris buffer, and magnetically stirred at room temperature for 5 h. The reaction proceeded to 80% conversion. The reaction mixture was transferred to a dialysis bag and dialyzed to 1 L of the same buffer at pH 7. The pure ketone **39** was isolated by recrystallisation in 60—65% yield with an optical purity of 86% e.e. The antibody in the dialysis bag could be used repeatedly without significant loss of the activity. Recycling of this procedure for 5 times produced 1.5 g (62% based on investigated substrate) of (−)-**39** with 86% e.e.[27] In addition, the use of crude antibody preparation provides significant cost and labor advantages. In this way, this example demonstrated that antibody catalyzed reactions are useful for the preparative organic chemistry.



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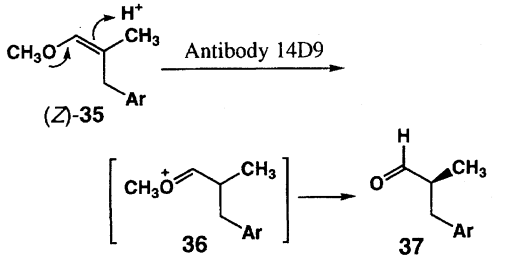

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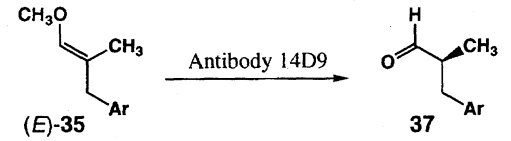

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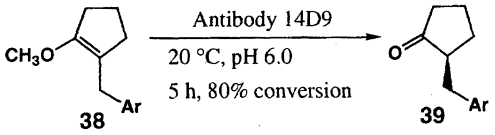

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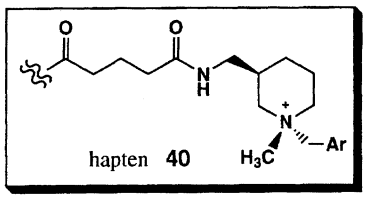
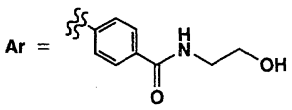
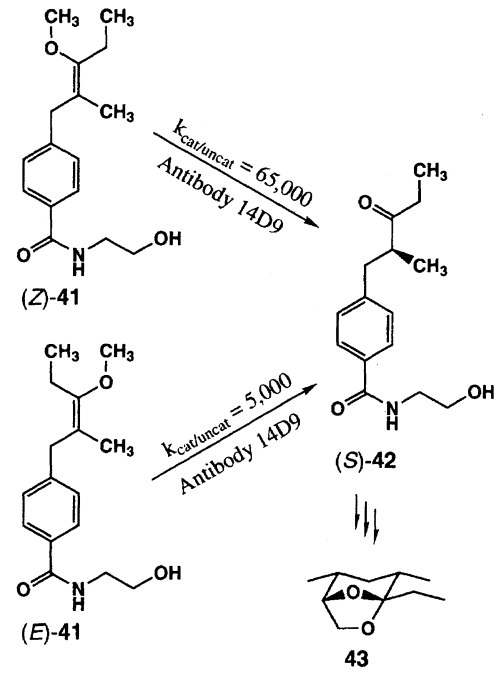




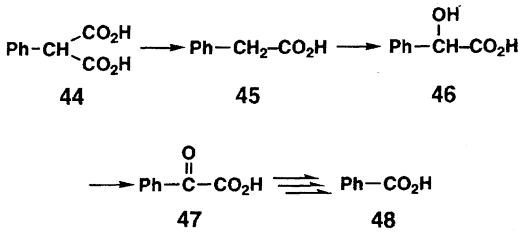
The first example of application of a catalytic antibody-promoted reaction to a natural product synthesis was demonstrated by Keinan and his co-worker.[28] Vinyl methyl ether (*Z*-**41**) was hydrolyzed by antibody 14D9 to produce (*S*)-ketone (**42**) in a high yield (Eq. 22). It will be worthy to mention that (*E*)-isomer of **41** was also hydrolyzed smoothly to give the same enantiomer, although the rate enhancement was not so high as with the (*Z*)-isomer. Kinetic studies revealed that a smaller k_{cat} value for (*E*)-**41** rather than K_m was responsible for the lower reactivity. Optically active ketone (*S*)-**42** was derived via some steps to naturally occurring (−)-α-multistriatin (**43**).



(22)

2. Asymmetric Decarboxylation of α-Aryl-α-methylmalonic Acid

2-1. Screening and Substrate Specificity. As supposed in Eqs. 3 and 4, decarboxylation of disubstituted malonic acid might be expected to be another route to generate an enolate anion in the active site of an enzyme. Thus we decided to screen a microorganism which has some decarboxylation activity to disubstituted malonic acid derivatives. The method of screening was simple. We screened a microorganism having an ability to grow on a medium which contains phenylmalonic acid (**44**) as the sole source of carbon. The first step of the metabolism of phenylmalonic acid (**44**) was supposed to be decarboxylation to give phenylacetic acid (**45**) (Eq. 23), which would be further degraded probably via oxidation of α-carbon.



(23)

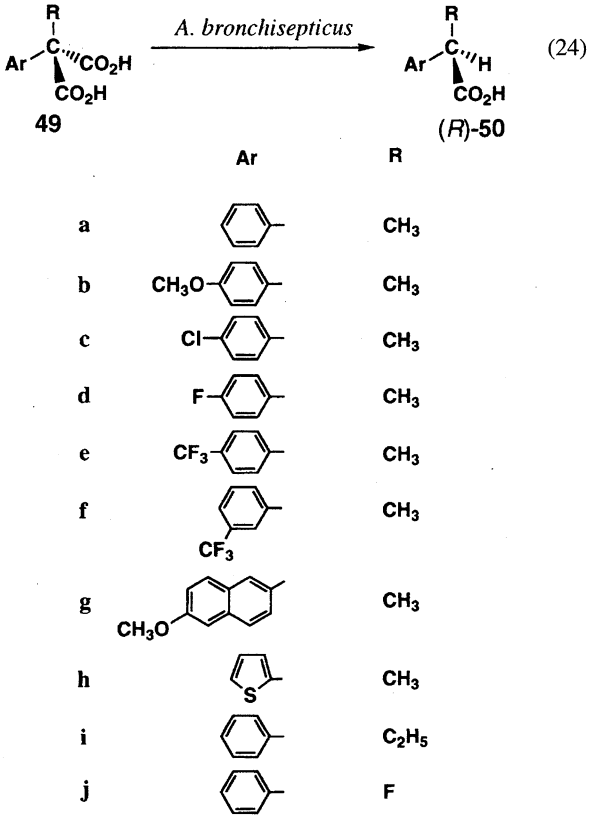
Actually, this estimation was confirmed to be really operating by identification of some of the supposed intermediates (**45**, **46**, and **47**), at least for a microorganism described.[30]

After screening of many soil samples and type cultures, we finally found that a microorganism isolated from a soil sample picked up from the road side along the beach of Izu–Ohshima Island has the strongest activity to metabolize and grow on the screening medium. Fortunately, the biocatalysis decarboxylated α-methyl-α-phenylmalonic acid (**49a**) to give α-phenylpropionic acid (Eq. 24).[31] The present microorganism was identified as a kind of bacterium, *Alcaligenes bronchisepticus* KU1201. This is the first example of asymmetric decarboxylation of malonic acid derivatives, and thanks to mother nature, the enantioselectivity was extremely high (96% e.e. for **49a**). Thus we examined the applicability of this new type of reaction. The enzyme accepted a wide variety of α-aryl substituents with an electron-donating substituents as well as electron-withdrawing ones. Electron-withdrawing substituents are favorable to the reaction. Both naphthyl and thienyl groups were allowed to be incorporated to the active site of the enzyme.[32] On the other hand, as for the alkyl part on the α-position, the enzyme showed rather strict and narrow specificity, i.e., only hydrogen, methyl, and fluorine atoms[33] were allowed as the α-substituents. The representative results are summarized in Table 4. Because aryl malonic acids are generally good substrates, we tentatively named the enzyme as arylmalonate decarboxylase, AMDase in short. The absolute configuration of the products were *R*, as determined by the specific rotation and derivatization to known compounds in some cases. Although the absolute configuration was opposite to that of *anti*-inflammatory agent, we became strongly interested in the reaction mecha-

Table 4. Asymmetric Decarboxylation of Disubstituted Malonic Acid (**49**)

Substrate	Substrate concn/%	Yield %	e.e. %
a	0.4	93	96
b	0.1	48	99
c	0.5	85	97
d	0.5	54	97
e	0.5	91	95
f	0.5	99	>95
g	0.5	96	95
h	0.3	98	95
i	0.1	0	—
j	0.1	64	95

nism and decided to study further to clarify if this reaction is enantiotopos differentiating or enantioface differentiating as we supposed at the starting point of the investigation.



2-2. Isolation of Enzyme and Cloning of the Gene. To promote studies on an enzymatic reaction mechanism, the first work to be done is isolation of the enzyme. To this end, *A. bronchisepticus* was cultivated aerobically at 30 °C for 72 h in an inorganic medium [$(NH_4)_2HPO_4$, K_2HPO_4, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $MnSO_4$, and yeast extract containing polypeptone and phenylmalonic acid, pH 7.2. In the course of the study, this enzyme was revealed to be an inducible enzyme and consequently the addition of an inducer such as phenylmalonic acid was inevitable to produce the enzyme.

All the procedures for the purification of the enzyme were performed under 5 °C. Potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA and 5 mM 2-mercaptoethanol (1 M=1 mol dm^{-3}) was used throughout the purification. About 200 g of wet cells from 16 L culture broth were suspended in 2 L of the above solution. The cell suspension was homogenized by a Dyno-mill homogeniser and centrifuged at 10000 g. To the cell-free extract was added 5% protamine sulfate to remove nucleic acids as precipitates. After centrifugation, the supernatant fluid was heat treated at 55 °C for 2 min, then chilled quickly in an ice-water bath. The coagulated protein was removed by centrifugation. The supernatant was fractionated with ammonium sulfate (0—60%), and active precipitate was collected and dialyzed. The resulting enzyme solution was applied to a DEAE-Toyopearl, butyl-Toyopearl, and QAE-Toyopearl columns. The enzyme was purified to homogeneity by SDS/PAGE and 377 fold to the cell-free extract. The yield was about 15%, as summarized in Table 5.

The molecular mass of the native AMDase was estimated to be 22 kDa by gel filtration on HPLC. Determination of the molecular mass of denatured protein by SDS/PAGE gave a value of 24 kDa. These results indicate that AMDase is a monomeric enzyme.

To investigate the cofactor requirements of the enzyme, we examined the effects of additives using phenylmalonic acid as the representative substrate. The addition of ATP or ADP to enzyme reaction mixtures, with or without coenzyme A, did not enhance the rate of reaction. These results clearly show that AMDase requires no coenzyme A, which is inevitable for analogous enzymes, such as acyl-CoA carboxylases,[35] methylmalonyl-CoA decarboxylases,[36,37] and transcarboxylases.[35,38]

Then, effects of various compounds and inhibitors on the enzyme activity were investigated. Some divalent metal cations, metal chelating reagents, such as EDTA, 8-quinolinol, 2,2′-bipyridyl, 1,10-phenanthroline, serine inhibitors, such as phenylmethanesulfonyl fluoride, and carbonyl reagents, such as sodium azide, hydroxylamine, potasium cyanide had little or no effects. On the other hand, sulfhydryl reagents, such as mercuric chloride, silver nitrate, iodoacetate, and *p*-chloromercuribenzoate (PCMB) totally inhibited the reaction, indicating that AMDase contains at least one free cysteine residue in the active site. The most characteristic feature of the present enzyme is that it is not in-

Table 5. Summary of Purification of AMDase from *A. bronchisepticus*

Purification step	Total protein mg	Total activity U	Specific activity U/mg protein	Yield %
Cell-free extract	8630	10950	1.26	100
Heat fractionation	4280	9540	2.22	87
Ammonium sulfate	2840	10350	3.64	95
DEAE-Toyopearl	244	5868	24.1	54
Butyl-Toyopearl	14.7	3391	231	31
QAE-Toyopearl	4.32	1627	377	15

hibited by avidin. Avidin is a kind of protein which strongly binds to biotin and inhibits biotin-containing enzyme. Ordinary decarboxylases require biotin as an essential cofactor. In the present case, as avidin has no effect, AMDase catalyzes decarboxylation reaction without the aid of biotin nor, as already described, coenzyme A and ATP, which are usually required by ordinary decarboxylases and transcarboxylases.

In fact, just mixing a solution of the enzyme and α-aryl-α-methylmalonic acid caused a smooth decarboxylation reaction, resulting in the formation of the corresponding α-arylpropionic acid in quantitative chemical and optical yields as shown in Table 6.

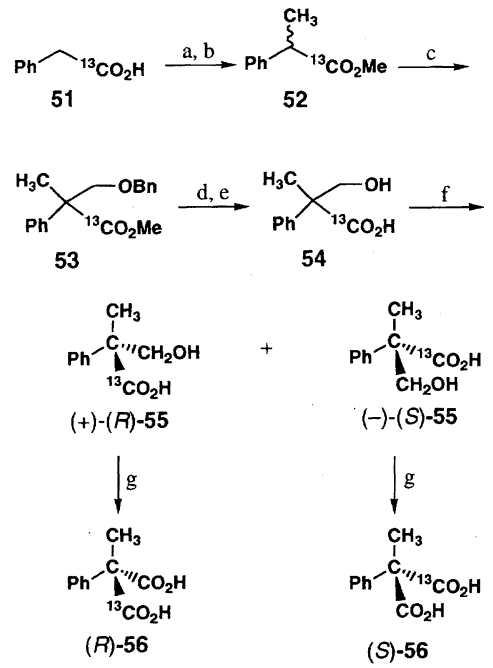
The gene for AMDase from *A. bronchisepticus* was isolated from genomic DNA, cloned, and expressed in a mutant of *E. coli*.[39] The genomic DNA of *A. bronchisepticus* was digested by a restriction enzyme *Pst*I and cloned in commercially available plasmid pUC 19. An *E. coli* mutant was transformed by this plasmid. AMDase activity was screened on a plate by the development of the blue color of bromothymol blue (BTB) due to the pH change. The transition interval of BTB is pH 6.0 (yellow) to pH 7.6 (blue). The basis of the selection is the change of pH by AMDase-catalyzed decarboxylation of phenylmalonic acid to phenylacetic acid. Formation of monobasic acid from dibasic acid causes some increase of pH around the colony. The decarboxylase activity was easily detected as a blue halo around the colony. One of approximately 700 transformants exhibited AMDase activity. The plasmid (pAMD 100) contained an insert of about 2.8 kb. The insert DNA fragment from the plasmid designated as pAMD 100 was digested with *Pst*I and *Hind*III. The *Pst*I–*Hind*III fragment (1.2 kb) was subcloned in pUC 19 to generate pAMD101. The *E. coli* transformed by this plasmid also exhibited AMDase activity. The procedures of purification of the enzyme from *E. coli* was more simple compared to that from the original bacterium, and the yield of AMDase increased by about two-fold.

Various deletion mutants of the 2.8-kb insert were prepared and sequenced by Sanger's method. An open reading frame encoding 240 amino acids showed the same NH_2-terminal amino acids sequence as that obtained from *A. bronchisepticus*. The molecular weight of the encoded protein was determined as 24734 from the deduced amino acid sequence and is in good agreement with that of the enzyme purified from the bacterium determined by SDS/PAGE. Based on the effects of some additives, we had estimated that free cysteine residue of the enzyme played an important role for its activity. DNA sequencing revealed that four cysteine residues are included in this enzyme at the positions of 101, 148, 171, and 188 from amino terminal.

The DNA sequence encoding AMDase and deduced amino acid sequence was compared to the data base using DNASIS (Hitachi). No significant similarities were observed with any of the sequences searched.

2-3. Stereochemistry and Electronic Effect. The absolute configuration of the product from α-methyl-α-phenylmalonic acid is (*R*), as unambiguously determined based on the sign of specific rotation. Then, which carboxyl group remains in the propionic acid and which releases carbon dioxide? To solve this problem we have to distinguish two prochiral carboxyl groups, and the most effective way would be to prepare both enantiomers of chiral α-methyl-α-phenylmalonic acid (**56**) of known configuration containing ^{13}C on either one of the two carboxyl groups.[40]

Preparation of both enantiomers of **56** was carried out according to Scheme 1, starting from ^{13}C containing phenylacetic acid (**51**). Methylation followed by benzyloxymethylation of methyl ester of **51** afforded **53** in a high yield. Deprotection of **53** gave free hydroxy acid, which was resolved via formation of a salt with quinine and subsequent recrystarization from acetone. Both enantiomers were isolated in 25—30% yield. Since the optical rotations of both enantiomers of **55** are known, the absolute configurations of (+)- and (−)-enantiomers were unambiguously determined. Jones' oxidation gave the desired chiral malonic acid (**56**),



(a) MeOH, TsOH; (b) LDA, MeI; (c) LDA, $ClCH_2OBn$

(d) H_2/Pd-C; (e) KOH/EtOH; (f) quinine resolution;

(g) Jones' oxidation

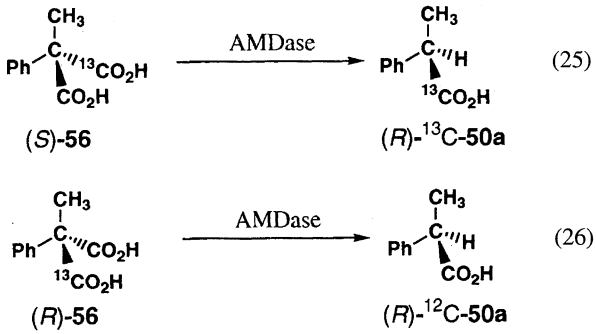
Scheme 1. Synthesis of both enantiomers of ^{13}C-α-methyl-α-phenylmalonic acid.

Table 6. Decarboxylation Reaction Catalyzed by Purified AMDase

	Arylpropionate	Reaction time	Yield	e.e.	Config.
		h	%	%	
49a	Phenyl	20	100	>99	*R*
49b	*p*-Methoxyphenyl	20	99	>99	*R*
49h	Thienyl	1	97	>99	*S*

although they could not be distinguished by optical rotation.

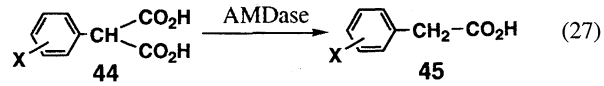
The enzymatic reaction was performed at 30 °C for 2 h in a volume of 1 mL of 250 mM phosphate buffer (pH 6.5) containing 50 mM of KOH, 32 U/mL of the enzyme, and [1-^{13}C]-**56**. The product was isolated as the methyl ester. When (*S*)-**56** was employed as the substrate, ^{13}C remained completely in propionate **50a**, as confirmed by ^{13}C NMR and HRMS (Eq. 25). In addition, spin–spin coupling between ^{1}H and ^{13}C was observed in the product, and the frequency of the C–O bond stretching vibration shifted lower to 1690 cm^{-1} (cf. 1740 cm^{-1} for ^{12}C–O). On the contrary, reaction of (*R*)-**56** resulted in the formation of (*R*)-**50a** containing ^{13}C only within natural abundance (Eq. 26). These results clearly indicate that the *pro-R* carboxyl group of malonic acid is eliminated to form (*R*)-**50a** with inversion of configuration.[40] This is in sharp contrast to the known decarboxylation reaction by malonyl CoA decarboxylase[41] and serine hydroxymethyl transferase,[42] which proceeds with retention of configuration.



(25)

(26)

Via what kind of intermediate or transition state does the reaction proceed? To elucidate this problem, we carried out a kinetic study. Initial reaction rates of some substituted phenylmalonic acids were measured at several substrate concentrations, and the k_{cat} and K_m values of each compound were determined by Lineweaver–Burk plots. As illustrated in Fig. 2, the logarithm of relative values of k_{cat} for non-substituted compound cleanly correlated in a linear fashion with Hammett's σ-value, the ρ-value being +1.9.[34] The fact that the Hammett ρ-value is positive clearly shows that the transition state is negatively charged. The structure of the transition state can not be elucidated from these data alone, but the enolate form (Eq. 4) which is initially postulated would be, at least one of the possible structure. If this is the case, the chirality of the final product can be said to be determined by the enantioface differentiating ability of the enzyme, and the key step of the reaction as a whole is an asymmetric protonation to an enolate.



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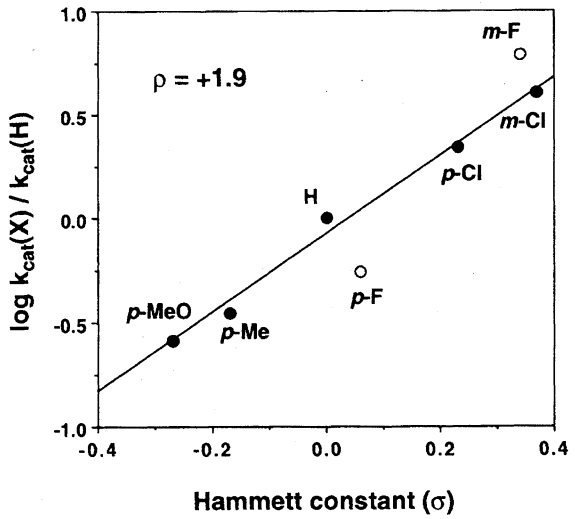


Fig. 2. Hammett plot of k_{cat} (relative to X=H) for the AMDase catalyzed reaction of substituted phenylmalonic acids.

2-4. Conformation of the Substrate in the Active Site of the Enzyme. Next, let's see what conformation is required for a smooth reaction. It is a common understanding that the spatial arrangements of substituents of a molecules have an essential effect on whether an enzyme can accept the compound as a substrate. Evaluation of the effects of configuration on the difference of reactivities of enantiomers may be examined, as the two enantiomers can be separated and treated as individual starting materials and products. In fact, some beautiful models of enzyme-substrate interactions have been proposed by J. B. Jones and co-workers, and permit successful interpretation of the difference of reactivities between given pair of enantiomers.[43,44] On the other hand, analysis of the reactivity of the conformational isomers of a substrate is rather difficult, because conformers are readily interconvertible under ordinary enzymatic reaction conditions. In the present case, there are relatively few C–C single bonds of which rotations are considered to affect the shape of the molecule. Accordingly this reaction is expected to be a good model that enables the evaluations of the effect of conformation on the enzyme reactivity.[45]

First, we examined the kinetic parameters (K_m and k_{cat}) of some *ortho*-substituted compounds, as well as a control substrate. The results are shown in Fig. 3. The K_m and k_{cat} values of a standard substrate (**44**) are 13.9 mM and 353 s^{-1}, respectively. Introduction of a chlorine atom on the *ortho*-position of the benzene ring (**57**) accelerates the rate of reaction obviously because of its electron-withdrawing property. The steric effect of this substituent is considered to be small, as the K_m value is nearly the same as that of non-substituted phenylmalonic acid (**44**). On the other hand, substitution of the α-hydrogen with a methyl group (**49a**) decreases the k_{cat} value less than one tenth (30 s^{-1}). This can be accounted for by the direct binding of an electron-donating group to the anionic center of the developing enolate. Again in this case, the steric effect of a methyl group is not so large judging from the K_m values of **44** and **49a**. Taking into consideration of all these results, substitution of the *ortho* position of α-methyl compound with a chlorine atom is reasonably expected to bring about some rate enhancement due

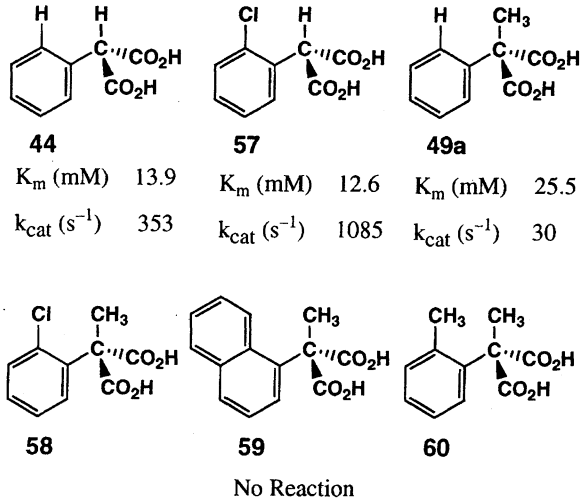


Fig. 3. Kinetic parameters of *ortho*-substituted phenylmalonic acids.

to its electron-withdrawing effect. However, the reality was entirely different; α-(*o*-chlorophenyl)-α-methyl derivative (**58**) does not undergo decarboxylation at all. The starting material is recovered intact after incubation with AMDase. It should be emphasized that the corresponding *p*-chlorophenylmalonic acid smoothly decarboxylated to give the expected monocarboxylic acid. As is clear from the case of *o*-chlorophenylmalonic acid (**57**), chlorine atom alone is not bulky enough to inhibit the reaction, so the inactiveness of **58** is concluded to come from the presence of two substituents on *ortho* and α-positions. Further evidence that the steric repulsion between *ortho*- and α-substituents is the crucial factor inhibiting the enzymatic reaction is also demonstrated by the *o*-methyl derivative (**60**), which is not affected by the enzyme.

The most probable interpretation of the above results is that the conformation disfavored by steric repulsion between *ortho*- and α-substituents is the same conformation that is required for the substrate to be bound in the active site of the enzyme. Undoubtedly it is the conformation (A) (*syn*-periplanar concerning the *ortho*- and α-substituents) illustrated in Fig. 4. If the substrate could occupy the another planar conformation (B) in the active site of the enzyme, it is free from steric repulsion between the two substituents, and

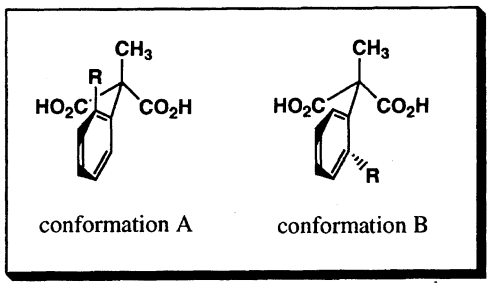


Fig. 4. Possible planar conformations of α-methyl-α-(*o*-substituted phenyl)malonic acids. Substituent R and α-methyl group occupy *syn*-relation in conformation A and *anti*-relation in confomation B.

$$E + S \underset{}{\overset{K^{\ddagger}_{ES}}{\rightleftharpoons}} [ES] \xrightarrow{k_{cat}} E + P$$

$$\Delta G^{\ddagger}_{ES} = \Delta H^{\ddagger}_{ES} - T\Delta S^{\ddagger}_{ES}$$

$$\ln K^{\ddagger}_{ES} = -\Delta G^{\ddagger}_{ES}/RT$$

(where $R = 1.986$ cal K^{-1} mol^{-1}, $T = 298$ K)

$$K^{\ddagger}_{ES} = {K_m}^{-1}$$

Scheme 2. Binding energy between substrates and enzymes.

would give the expected product. The actual inactivity of the two compounds (**58** and **60**) suggests that this conformation in the pocket of the enzyme is disfavored by some reasons not clarified at present. Then, how much is the energy difference between the two conformers A and B? Apparently *syn*-periplanar conformer A will be the less favored one compared to B. However, if binding energy with the enzyme overcomes the difference in potential energy between free A and B, and the enzyme forces the substrate to take the conformation A, the decarboxylation of the substrate will be allowed to proceed. Thus whether a compound can undergo smooth reaction or not would depend on the difference between enzyme-substrate binding energy and the potential energy of the "reactive conformer". The free energy of the formation of enzyme-substrate complex is easily calculated based on K_m value, according to Scheme 2.

Of course the K_m value of **58** is not available because of its inactivity. It would not be so curious if we suppose that the K_m value of **58** is not much different from that of *o*-chlorophenylmalonic acid (**57**) (12.6 mM), then the free energy of formation of enzyme-substrate complex can be calculated as -2.6 kcal mol^{-1} at 25 °C. In addition, supposing that the values of $\Delta S^{\ddagger}$ for **57** and **58** are not so much different because the ligands around the prochiral centers are similar, then the difference of ΔH between the favored and unfavored conformation will be the key to interpret the different reactivity of **57** and **58**. To find the potential energy surfaces for two types of arylmalonic acid **57** and **58**, we have employed ab initio molecular orbital method on the internal rotation of the benzene ring. The theoretical calculations were carried out by using Gaussian 92 program system.[46] The molecular structures for various rotational angles were optimized by using 3-21G* basis set with Hartree–Fock method.[47] The results are shown in Figs. 5 and 6.

The potential energy diagram for *o*-chlorophenylmalonic acid (**57**) is shown in Fig. 5. We have obtained two stable structures which correspond to the *syn*- and *anti*-periplanar conformer A and B in Fig. 4, respectively. The energy difference between these two conformers is calculated to be 0.8 kcal mol^{-1}. This small energy difference clearly indicates that the steric repulsion between α-C–H and chlorine atom is extremely small.

The rotational energy diagram for α-(*o*-chlorophenyl)-α-methylmalonic acid (**58**) is shown in Fig. 6. In contrast to a simple potential curve for **57**, we have obtained four energy minima for **58** at the dihedral angles 24.6°, 73.0°, 178.2°,

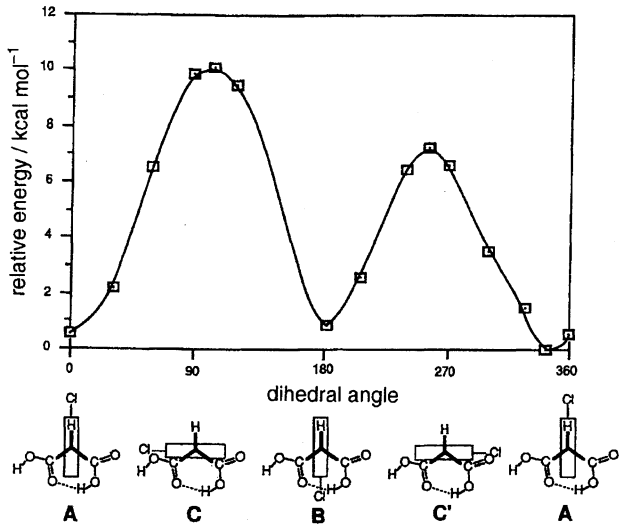


Fig. 5. The potential energy diagram on the C–C bond rotation for α-(*o*-chlorophenyl)malonic acid (**57**) calculated with HF/3-21G* method.

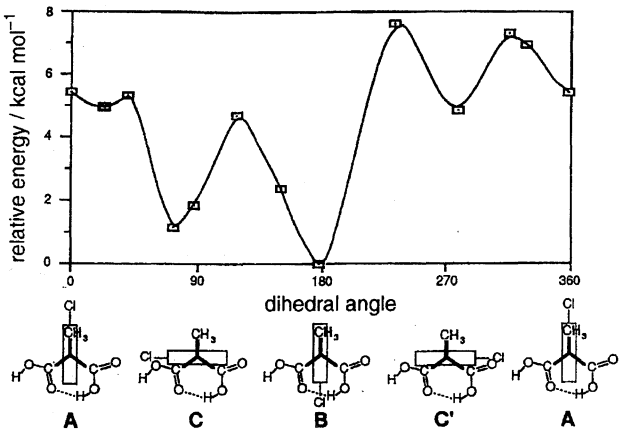
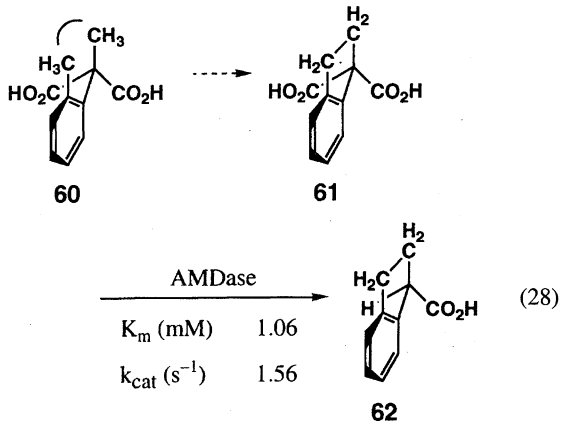


Fig. 6. The potential energy diagram on the C–C bond rotation for α-(*o*-chlorophenyl)-α-(methylmalonic acid (**58**) calculated with HF/3-21G* method.

and 278.8°. The most stable conformer takes 178° of dihedral angle, which corresponds to *anti*-periplanar conformer B. On the other hand, the potential energy of *syn*-periplanar conformation is about 5.5 kcal mol^{-1} higher than that of the most stable one. This potential surface curve clearly indicates that the structure corresponding to *syn*-periplanar conformation is unstable due to the steric repulsion between the chlorine atom and the α-methyl group. Accordingly, chlorophenyl derivative **57** can be incorporated in the active site of the enzyme in *syn*-periplanar form, whereas α-(*o*-chlorophenyl)-α-methylmalonic acid is unable to overcome the energy loss for occupying *syn*-conformation by binding with the enzyme.

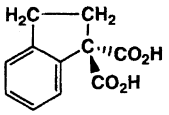
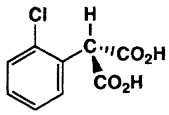
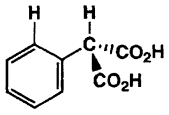
Further, the essential importance of *syn*-periplanar conformation A was demonstrated by designing and subjecting a substrate to the reaction, which mimics the *syn*-periplanar conformation of an unreactive compound. As described earlier, α-(*o*-methylphenyl)-α-methylmalonic acid (**60**) is entirely inactive to the enzyme, and the reason is now proposed, analogous to the *o*-chloro- derivative, that this compound cannot occupy the *syn*-periplanar conformation because of steric repulsion of two methyl groups. Accordingly if the conformation of this compound could be fixed to *syn*-periplanar, it would be decarboxylated smoothly. But, how can the conformation be fixed as an unstable one? The only way to realize this making compensation for a loss of potential energy is to make a covalent bond between two methyl groups. In this way, 1,1-indanedicarboxylic acid (**61**) was prepared and incubated with the enzyme. As expected, this cyclic substrate afforded the corresponding (*R*)-indane-1-carboxylic acid (**62**) in high yield (Eq. 28). The k_{cat} value is smaller than that of phenylmalonic acid (**44**), because of the electron-donating property of two methylene groups on *ortho*- and α-positions. It is worth noting that the K_m value of this substrate is also smaller by one order than those of acyclic compounds. Evidently, it is due to its conformation already being arranged in the form that fits to the binding site of the enzyme, or in other words probably because of the decrease of activation entropy. If the $\Delta H^{\ddagger}$ values for α-methyl-α-phenylmalonic acid (**49a**) and indanedicarboxylic acid (**61**) are assumed to be the same, the difference in $\Delta S^{\ddagger}$ between two compounds is calculated to be 6.3 cal K^{-1} mol^{-1}.



(28)

We could actually confirm this estimation to be true by kinetic studies.[48] Three kinds of substrates were subjected to the reaction. Phenylmalonic acid (**44**) as the standard compound, *ortho*-chloro derivative (**57**) as a substrate with an electron-withdrawing group, and indanedicarboxylic acid (**61**) as a conformationally restricted compound. The initial rates of the enzymatic decarboxylation reaction of three compounds were measured at several substrate concentrations at 15, 25, and 35 °C. The k_{cat} and K_m values at each temperature were obtained by a Lineweaver–Burk plot, and an Arrhenius plot was made based on these data. We can calculate the activation enthalpy and entropy for each compound, these are summarized in Table 7. Clearly, the activation entropy of indanedicarboxylic acid (**61**) is smaller than the others by 9 to 11 cal per degree per mol. The following conclusion can then be drawn, i.e., the benzene ring and the other α-substituent of the substrate should occupy coplanar conformation in the enzyme pocket, and when there is a substituents at the *ortho*-position of the phenyl ring, it must take the *syn* position with

Table 7. Activation Parameters fro AMDase-Catalyzed Decarboxylation

Substrate			
	61	**57**	**44**
k_{cat}/s^{-1}	1.9	934	250
K_m/mM	0.92	12.9	11.9
$\Delta S^{\ddagger}$/cal mol^{-1} K^{-1}	−27.6	−36.8	−38.5
$\Delta H^{\ddagger}$/kcal mol^{-1}	8.9	2.4	2.7
$\Delta G^{\ddagger}$/kcal mol^{-1}	17.1	13.3	14.1

the α-hydrogen to undergo a smooth reaction.

This planar conformation will be also favorable to form a enolate type intermediate or transition state as estimated from the Hammett plot, since the π-electron orbitals of the phenyl ring are already arranged in the best positions so as to be able to readily conjugate with the growing orbital of the enolate.

2-5. Active Site Directed Inhibitor. To clarify the mode of interaction of the cysteine residue of the enzyme with the substrates, we screened an active-site directed inhibitor. After trying some compounds structurally resembling the substrate, we found that α-bromophenylacetic acid (BPA, **63**) is a potent inhibitor. The Lineweaver–Burk plot (Fig. 7) indicated that the mode of this inhibitor is competitive and reversible.[49]

First, we tested how many molecules bind to the enzyme. The result is shown in Fig. 8, demonstrating only one cysteine is binding with this inhibitor. When a thiol-specific reagent, *para*-chloromercuribenzoate (PCMB), was added to a solution of AMDase, an increase of UV absorption was observed due to the binding of PCMB and the enzyme, according to curve A. On the other hand, when PCMB was added to the enzyme solution after a short-time incubation of the enzyme with α-bromophenylacetic acid (**63**), the increase of UV absorption was about three-fourth (Curve B) compared to that of without no pre-treatment with the bromo acid. This result clearly indicates that one of four cysteines was blocked by α-bromophenylacetic acid, and can not bind with PCMB. Naturally, the blocked one will be the one which is in the active site. In other words, α-bromophenylacetic acid is proved to

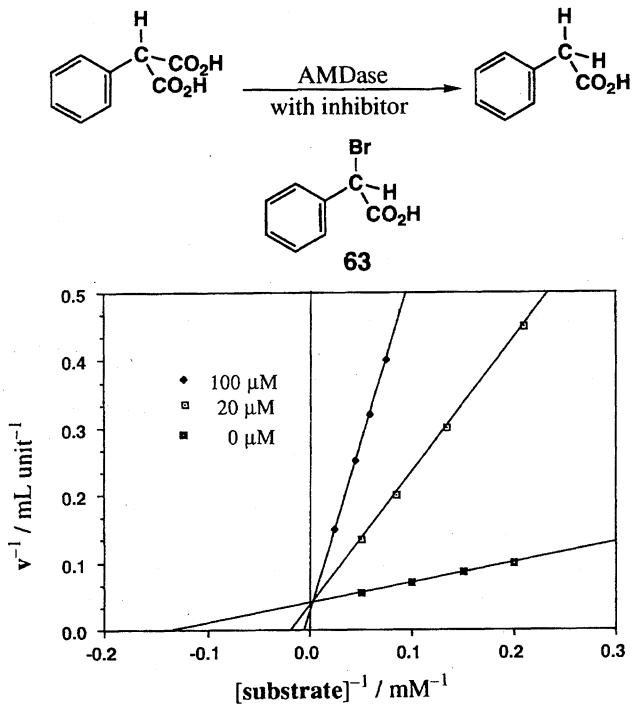


Fig. 7. Lineweaver–Burk plot of decarboxylation of phenylmalonic acid in the presence of α-bromophenylacetic acid.

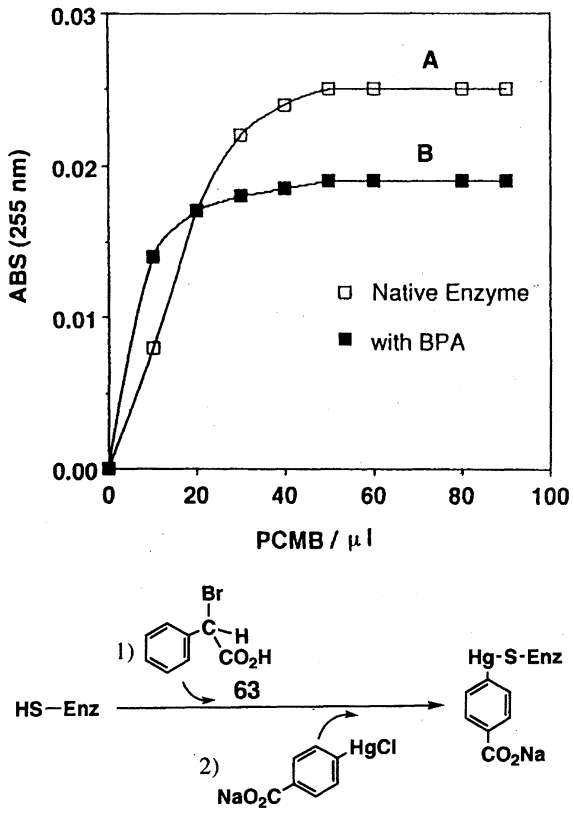
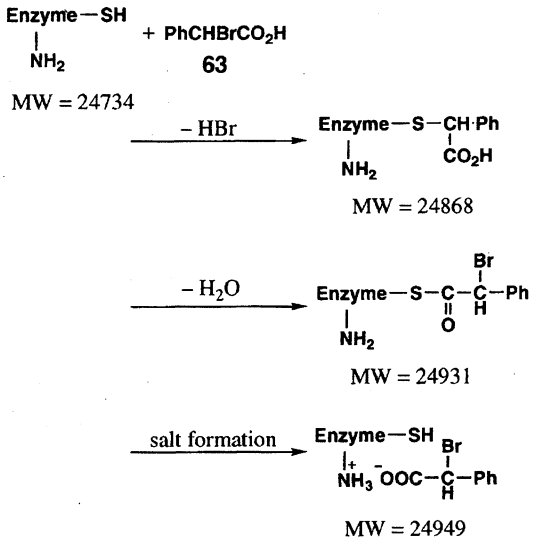


Fig. 8. Titration of cysteine residue of AMDase with PCMB. Curve A shows the increase of absorption at 255 nm on addition of PCMB in the absence of α-bromophenylmalonic acid (BPA). Curve B shows the increase of absorption when PCMB was added after a short incubation with BPA.

react with only one cysteine located in the active site.[50]

If the mode of binding of the bromo acid inhibitor **63** becomes apparent, then we will be able to estimate analogously how the substrate binds to the active site. To this end, the molecular weight of the enzyme and enzyme-inhibitor complex was measured with matrix-assisted laser desorption ionization—time of flight mass spectrometry. The mass number of the native AMDase was observed as 24766, which is in good accordance with the calculated value, 24734. An aqueous solution of α-bromophenylacetic acid (**63**) was added to the solution of AMDase. Its mass spectrum was measured after 10 min. The peak is observed at mass number 24967. Three possibilities are considered as the mode of binding between the enzyme and inhibitor **63** as illustrated in Scheme 3. If the inhibitor and the enzyme bind with the formation of a sulfide bond and elimination of HBr, the mass number should be 24868, which is smaller by about one hundred units than the observed value. On the other hand, if the binding mode is by the formation of a thiol ester or a formation of only a salt, the mass number is expected to be 24931 and 24949, respectively. Accordingly, sulfide formation is very unlikely, although it is difficult to distinguish the formation of a thiol ester or a salt from this mass spectroscopy alone.[49]

From the kinetics and mass measurement results, BPA (**63**) was found to bind with AMDase in a reversible manner, and the resulting bond was estimated to be a thiol ester or a simple salt. If the former is true, some thiol compounds would attack the carbonyl group to liberate the free enzyme resulting in the recovery of AMDase activity, while these thiol compounds would have no effect on the dissociation of the carboxylate-enzyme complex. In this way, two possibilities would be distinguished, and this prediction turned out to be true as indicated in Table 8. The activity of the enzyme gradually increased when a large excess of 2-mercaptoethanol (ME) was added to the enzyme-inhibitor complex, until the activity finally recovered to 100%. This result clearly shows that BPA was released from the active site of AMDase when ME



Scheme 3. Binding of α-bromophenylacetic acid and AMDase.

Table 8. Conversion of α-Phenylmalonic Acid in the Presence of 2-Mercaptoethanol by the Inhibited Enzyme and Native Enzyme

Reaction time	Yield of phenylacetic acid/%	
h	Native enzyme	Inhibited enzyme[a]
10	70	53
23	100	100

a) The enzyme was once treated with BPA. Then, the reaction was performed in the presence of excess 2-mercaptpethanol.

was added to the AMDase–BPA complex. Thus, it can be concluded that the potent inhibitory effect of α-bromophenylacetic acid is very likely to come from the formation of a thiol ester with a cysteine residue which is present in the active site of the enzyme. In addition to mass spectroscopic studies, we have succeeded to observe a peak which can be assigned to deformation vibration of a C–S bond (1103 cm^{-1}) by FT-IR spectroscopy.[51]

The remarkably small K_i value can be accounted for by a strong electron-withdrawing effect of the bromine atom which causes the high reactivity of the carbonyl group in the nucleophilic attack of a thiol. Further, as this inhibitor is competitive with the substrate, the first event which would occur between the substrate and the active site of the enzyme would be an interaction similar to that between the inhibitor and the enzyme. Thus the first step of the activation of the substrate by the enzyme is considered to be the attack of cysteine on the *pro*-(*S*) carboxyl group of the substrate. The electron-withdrawing effect of the thiol group would lower the potential energy of the negatively charged transition state and facilitate the cleavage of the C–C bond to release carbon dioxide from the free carboxyl group. In this way, the enzyme itself plays the role of coenzyme A, which is required in ordinary decarboxylation reactions of malonate.

2-6. Site-Directed Mutagenesis. As is clear by the studies so far described, the asymmetric decarboxylation reaction of disubstituted malonic acids will be initiated with a nucleophilic attack of a cysteine residue to a free carboxyl group of the substrate, and the electron-withdrawing effect of a thiol ester group will facilitate the formation of intermediate enolate. Then, how can the Cys responsible for the reaction be specified? Site-directed mutagenesis is one of the most powerful techniques for studying mechanisms of enzyme-catalyzed reactions. Since this technique provides a method of replacing a specific amino acid residue of an enzyme instead of an arbitrary one, it is especially useful to specify the catalytic amino acid residue(s) of an enzyme.[52–54]

In the case of AMDase, one of four cysteine residues was supposed to be involved in the catalytic site. Preparation and kinetic studies of four mutant enzymes in which one of four cysteines is replaced by some amino acid are expected to be most informative. Which amino acid should be introduced in place of cysteine? As described earlier, the transition state has a negative charge as is clear from the sign of Hammett ρ value being positive. Taking into consideration the fact that in ordinary enzymatic decarboxylation reaction, coenzyme

A forms a thiol ester with the substrate, the rate-determining step is supposed to be either a nucleophilic attack of cysteine to one of the two carboxyl groups or decarboxylation to form an enolate, because in both cases, the transition state will have negative charge. This supposition leads to an estimation that in either case, substitution of cysteine in the active site for serine would greatly decrease the reaction rate because of relatively small nucleophilicity and anion-stabilizing effect of a hydroxy group compared to a thiolate functionality.

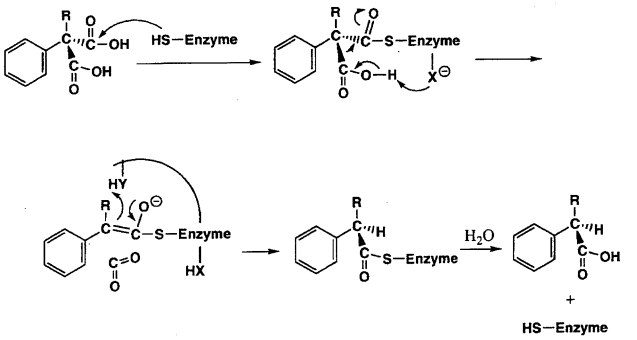
In this way, if the mutant enzyme partially retains its catalytic activity, even when the essential cysteine in the active site is replaced by serine, the k_{cat} value would greatly decrease while the K_m value would not be seriously affected. On the other hand, if the cysteine residue other than the catalytic one is replaced by serine, the effect on reactivity will be moderate, because the steric bulkiness of serine resembles that of cysteine and it will more or less keep the hydrogen bonding(s) of the wild enzyme. Thus, we prepared four mutant genes in which one of four codons corresponding to cysteine was replaced by that of serine, via site-directed mutagenesis according to the Kunkel protocol.[55] Four AMDase mutants expressed in *E. coli* were purified to homogeneity and used in kinetic studies of decarboxylation of phenylmalonic acid (**44**).

Enzyme activity was measured and kinetic parameters were determined by Lineweaver–Burk plots (Table 9). Among four mutants, C188S showed a drastic decrease in the activity (k_{cat}/K_m). This low activity was due to decrease in catalytic turnover number (k_{cat}) rather than affinity to the substrate (K_m). CD spectrum of C188S mutant was almost the same as that of wild enzyme. This shows that there is no significant change in the tertiary structure of C188S mutant. The fact that the k_{cat} value of this mutant is extremely small despite a little change in conformation clearly indicates that Cys188 is located in the active site.

2-7. Conclusion. We suppose that the active site pocket is very hydrophobic, and some basic amino acid assists cysteine to form a thiolate anion and attacks the *pro*-(*S*) carboxyl group of the substrate. Then, the electron-withdrawing effect of the thiol group lowers the potential energy of the negatively charged transition state or maybe of an intermediate, and facilitates the cleavage of the C–C bond of another carboxyl group. Enantioface differentiating protonation followed by hydrolysis will give the observed product (Eq. 29).

Table 9. Relative Activities and Kinetic Parameters of the Wild Type and Four Mutant Enzymes

	Relative activity	K_m	k_{cat}	k_{cat}/K_m
	U/mg	mM	s^{-1}	
Wild type	406.4	13.3	365.9	27.5
C101S	547.6	4.3	247.6	57.6
C148S	166.5	11.5	100.1	8.7
C171S	118.0	9.1	62.3	6.8
C188S	1.3	4.9	0.62	0.13



(29)

In this way, the enzyme itself activates the substrate in place of coenzyme A without the aid of ATP.

It is a great pleasure to thank all those who collaborated on enol ester project and decarboxylation project for their enthusiastic endeavors. The names of all co-workers and colleagues are mentioned in the references. These works were financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture.

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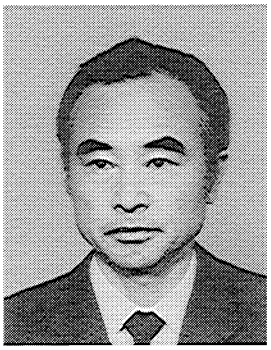

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