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Effect of Conformation of the Substrate on Enzymatic Decarboxylation of α-Arylmalonic Acid

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Abstract—The configuration and conformation of a compound are critical factors that determine whether it can be accepted by an enzyme as a substrate or not. We have examined enzyme-catalyzed decarboxylation of some α-substituted malonic acids and proposed that the *syn*-periplanar conformation is required for the substrates to be bound to the active site of the enzyme. Theoretical calculation of potential energy surfaces also supports the conclusion from experimental results.

Introduction

It is a common understanding that the spatial arrangements of substituents of a molecule 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 are configurational isomers, which can be physically separated and treated as individual starting materials or products. By these means, some models of enzyme–substrate interactions permit successful interpretation of the difference between reactivities of the given pair of substrate enantiomers on the basis of their configurational isomerism, namely their chirality.[1] On the other hand, analysis of the reactivity of the conformational isomers of a substrate is rather difficult, because in general, conformers are readily interconvertible under ordinary enzymatic reaction conditions. We wish to report here an example which clearly shows that a specific conformation is required for the substrate to be incorporated in smooth enzymatic transformation. The key for a successful demonstration of a conformational effect consists of a synthesis of a conformationally restricted substrate and a theoretical rationalization of the stability of conformers in the problem.

Results and Discussion

Characteristics of AMDase-catalyzed decarboxylation

We have already reported an enzyme-catalyzed enantioselective decarboxylation of α-arylmalonic acid and its α-methyl[2] and α-fluoro[3] derivatives (Scheme I).

This reaction is very characteristic compared with ordinary enzyme-catalyzed decarboxylation and transcarboxylation reactions for two reasons. Firstly, the present enzyme, AMDase,[4] originally purified from *Alcaligenes bronchisepticus*,[5] requires no additional co-factors such as biotin, coenzyme A or ATP for its full activity. Secondly, the structural allowance for two α-substituents is rather strict, i.e. one must be an aromatic ring directly attached to the α-carbon and only a small group such as H, F or CH_3 (**1a–c**) is allowed as the other substituent. For example, α-methyl-α-benzylmalonic acid (**3a**) or its heteroatom derivatives (**3b, c**) and α-ethyl-α-phenylmalonic acid (**1d**) are recovered intact on incubation with the isolated

1 2

	X	R
a	H	H
b	H	CH_3
c	H	F
d	H	C_2H_5
e	Cl	H
f	Cl	CH_3
g	CH_3	CH_3
h	$-CH_2CH_2-$	

3

a X = CH_2
b X = O
c X = S

Scheme I. Decarboxylation reaction by the aid of arylmalonate decarboxylase (AMDase).

Dedicated to Professor John Bryan Jones of the University of Toronto on the occasion of his 60th birthday.

enzyme.[2b] Accordingly, this system is expected to be a good example for stereochemical studies on enzyme–substrate interactions. In addition to these facts, the electronic effect of the substituents of the aromatic ring upon the rate of reaction clearly correlates to Hammett's σ value in a linear fashion as shown in Figure 1, ρ value for substituted **1a** being +1.9.[5a] This is also very important to the stereochemical studies of enzyme reaction, because (1) the quantitative evaluation of electronic effect allows us to isolate only the stereochemical one from the apparent total effect of a substituent; (2) based on comparison of the ρ value with that of already established enzyme-catalyzed racemization of mandelic acid,[6] the present reaction is estimated to proceed via an enolate-type transition state (**4**) as depicted in Scheme II (as for X of compound **4**, we have no definite evidence, that it can be a hydroxyl group or covalently bound enzyme via Cys).

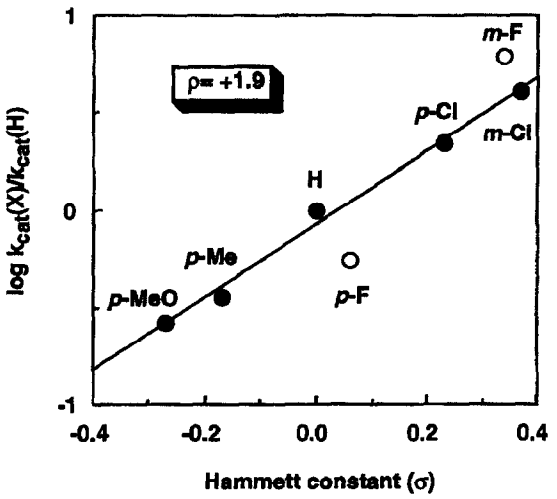
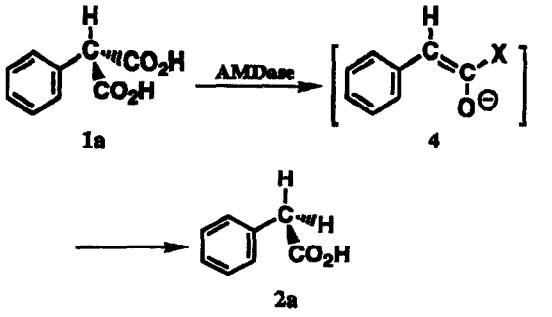


Figure 1. Hammett plot for the enzymatic decarboxylation of substituted α-phenylmalonic acids.



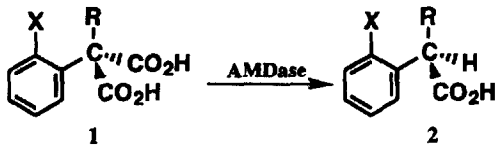
Scheme II. Reaction path of AMDase catalyzed decarboxylation of phenylmalonic acid.

This argument suggests that the conformation of the substrate in the transition state will be arranged so that the π-electrons of the aromatic ring and enolate double bond are parallel with each other for efficient interaction. As a result, the aromatic ring and α-substituent are expected to be coplanar to one another. Then, in what stage of the reaction is the substrate required to be arranged in this conformation? Studies on this problem are expected to clarify the conformation required for the substrate to be incorporated in the active site of the enzyme.

Kinetics of substituted α-arylmalonic acids

First, kinetic parameters of some compounds were examined and these are summarized in Table 1. The effect of substitution on the aromatic ring and α-carbon is clear when Michaelis constant (K_m) and k_{cat} of each compound are compared with those of **1a**. When a chlorine atom is introduced to the *ortho* position of the benzene ring (**1e**), the rate of reaction is accelerated obviously because of its electron-withdrawing property. The steric effect of this substituent is presumed to be small as K_m is nearly the same as that of the original compound **1a**. On the other

Table 1. Kinetic parameters of enzymatic decarboxylation of α-arylmalonic acid



Substrate	X	R	K_m (mM)	K_{cat} (s^{-1})
1a	H	H	13.9	353
1e	Cl	H	12.6	1085
1b	H	CH_3	25.5	30
1f	Cl	CH_3	No reaction	
1g	CH_3	CH_3	No reaction	
1h	$-CH_2CH_2-$		1.06	1.56

hand, substitution of α-hydrogen with a methyl group (**1b**) decreased the k_{cat} value to less than one-tenth. This can be accounted for by the direct binding of an electron-donating group to the center of developing enolate. Taking into consideration the above results, substitution of *ortho*-position of α-methyl-α-phenylmalonic acid (**1b**) with chlorine atom was expected to produce some rate enhancement compared with **1b**. However, the reality was entirely different, α-(*o*-chlorophenyl)-α-methylmalonic acid (**1f**) did not undergo decarboxylation at all. It must be emphasized that previous work showed the *para*-chloro derivative of **1b** was smoothly decarboxylated to give the expected monocarboxylic acid.[2b] As is obvious from the case of **1e**, the *o*-chlorophenyl group alone is not so bulky as to inhibit the reaction, the inactiveness of **1f** is concluded to come from the presence of two substituents on *ortho* and α-positions.

Effect of conformation on the reactivity

The most probable interpretation for 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 *syn*-periplanar conformation as illustrated in Figure 2. Further evidence that the steric repulsion between *ortho* and α-substituents is the crucial factor inhibiting the enzymatic decarboxylation reaction was also demonstrated by the *o*-methyl derivative **1g**, which was not affected by the enzyme. There are two possible planar conformations as illustrated in Figure 2, A and B. If the substrates could occupy the conformation B in the active site of the enzyme, they are free from the steric repulsion between *ortho* and α-substituents and would give the expected product. The actual inactivity of **1f** and **1g** suggests that conformation B in the pocket of the enzyme is disfavored. Then, how much is the energy difference between the two conformers A and B? Apparently conformer A will be the unfavored one compared with B.

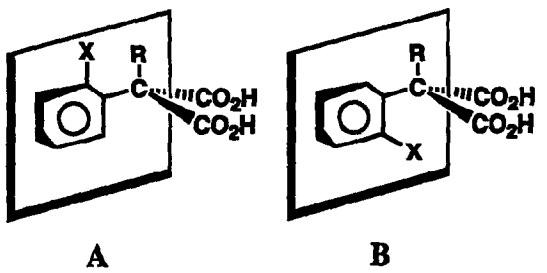


Figure 2. Possible planar conformations. (A) Benzene ring and α-substituent R are arranged in *syn*-periplanar; (B) Benzene ring and α-substituent R are arranged in *anti*-periplanar.

However, binding with the enzyme forced the substrate to take the conformer A, thus overcoming the difference in potential energy between free A and B, and be incorporated in the active site occupying conformation A, allowing decarboxylation to proceed. The free energy of formation of enzyme–substrate complex is easily calculated based on K_m values shown in Table 1. For compound **1e**, the value of $\Delta G^{\ddagger}$ can be calculated to be –2.6 kcal/mol from equations (1)–(4). If we suppose the values of $\Delta S^{\ddagger}$ for **1e** and **1f** are not so different as the ligands around the prochiral center are similar, then the energy difference between the favored and unfavored conformations for each compound will be the key to interpret the different reactivity of two compounds. If the difference of energy gap between the most stable conformer and *syn*-periplanar one for **1e** and **1f** is far more than 2.6 kcal/mol, the inactivity of **1f** can be attributed to the instability of conformer A.

$$E + S \underset{k_{-1}}{\overset{k_1 \quad K^{\ddagger}_{ES}}{\rightleftharpoons}} [ES] \xrightarrow{k_{cat}} P + E \qquad (1)$$

$$\Delta G^{\ddagger}_{ES} = \Delta H^{\ddagger}_{ES} - T\Delta S^{\ddagger}_{ES} \qquad (2)$$

$$\ln K^{\ddagger}_{ES} = -\Delta G^{\ddagger}/RT \qquad (3)$$

where R = 1.986 cal/°K·mol; T = 298 °K

$$K^{\ddagger}_{ES} = K_m^{-1} \qquad (4)$$

The potential energies for two conformers of **1e** and **1f** were obtained by computer modeling. The energy diagram rotating the benzene ring is illustrated in Figures 3 and 5, and discussed below.

Computational procedure

In order to obtain the potential energy surfaces for two types of α-arylmalonic acids **1e** and **1f** theoretically, we have employed *ab initio* molecular orbital method on the internal rotation of the benzene ring. The theoretical calculations were carried out by using the Gaussian 92 program system.[7] The molecular structures for various rotational angles were optimized by using 3-21G* basis set with Hartree–Fock (HF) method.[8] We have evaluated the analytical second derivatives with respect to nuclear coordinates in order to confirm the calculated structures to be at stable conformations or transition states. We have corrected the relative energies by using the zero-point vibrational energies obtained with the analytical second derivative method. The zero-pointed corrected energies were, however, found to be almost identical to the values without corrections within 0.5 kcal/mol. Since the energy barrier calculated with HF method tends to be overestimated, we have reevaluated the relative energies with the second-order Møller–Plesset perturbation (MP2) method[8] at the optimized structures obtained by HF method in order to include the electron correlation effect. The values shown in the text are the ones obtained from the MP2 method with 3-21G* basis set, and the values described in parentheses are from the HF method.

Theoretical results

In the preliminary calculation with STO-3G basis set, we have roughly estimated the energy difference between two

conformers A and B. The energies calculated for *o*-chlorophenylmalonic acid (**1e**) are almost identical for conformers A and B. The energy of conformer A for α-(*o*-chlorophenyl)-α-methylmalonic acid (**1f**) is calculated to be more than 6 kcal/mol higher than that of conformer B. This energy difference seems to agree well with the experimental results. The rotational barrier for **1e** is estimated to be 10 kcal/mol and this energy is *ca* 2 kcal/mol higher than that of **1f**. Since the rotational barrier obtained with STO-3G basis set tends to be overestimated, we have calculated the entire potential energy surface on the C–C bond rotation of α-phenylmalonic acid with the 3-21G* basis set.

The potential energy diagram for *o*-chlorophenylmalonic acid (**1e**) is shown in Figure 3. We have obtained two stable structures for *o*-chlorophenylmalonic acid which correspond to the conformers A and B. The most stable structure whose dihedral angle is –17 ° is illustrated in Figure 4. The α-C–H bond is not strictly coplanar to the phenyl ring, but this structure corresponds to the conformer A. The second energy minimum is found to be at the dihedral angle 183.8 °. The energy difference between these two structures is calculated to be 0.8 (0.9) kcal/mol. This small energy difference clearly indicates that the steric repulsion between α-C–H bond and Cl atom is extremely small. It is interesting that the hydrogen atom on the α-carbon prefers the conformation which takes the same side with the chlorine atom of the phenyl ring, although the energy difference is small.

There are two potential energy barriers on the C–C bond rotation for *o*-chlorophenylmalonic acid (**1e**). The transition states are calculated to be at 101 ° and 257 ° of the dihedral angles, which correspond to the conformations C and C' shown in Figure 3, respectively. The potential barrier at the conformation C is 8.1 (10.1) kcal/mol, and the one of conformation C' is 5.4 (7.3) kcal/mol. These relatively high energy barriers are mainly due to the steric repulsion between chlorine atom and carboxyl groups.

The rotational energy diagram for α-(*o*-chlorophenyl)-α-methylmalonate (**1f**) is shown in Figure 5. Since the malonic acid forms the internal hydrogen bond, the rotational energy curve is not symmetric for 0–180 ° and 180–360 ° of the C–C bond rotational angle due to the chirality. While the rotational barrier of **1e** is mainly produced by the repulsive force between carboxyl groups and a phenyl group, the steric repulsion due to the three groups, i.e. two carboxyl groups and methyl group toward the phenyl group in **1f** leads to the complicated potential energy surface for **1f**. This evidence is clearly depicted in the case of α-(*o*-chlorophenyl)-α-methylmalonic acid (**1f**) compared with the case of *o*-chlorophenylmalonic acid (**1e**).

In contrast to the simple potential curve for **1e**, we have obtained four energy minima for **1f** at the dihedral angles 24.6 °, 73.0 °, 178.2 ° and 278.8 °. While the most stable structure B takes 178 ° of dihedral angle, the second most stable conformation is 73 ° of dihedral angle which corresponds to perpendicular form C. The energy difference between these two conformations is calculated to be -0.2 (1.2) kcal/mol. The structures at 25 ° and 278 ° are shown to be located to shallow potential minima. The relative energies of these two local minima are *ca* 4 (*ca* 5) kcal/mol higher than the energy of the most stable conformation B. The potential energy curve for **1f** clearly indicates that the structure corresponding to the conformation A is unstable due to the steric repulsion between the chlorine atom and methyl group. The optimized structure at the dihedral angle 24.5 ° has the large angle 126 ° from normal sp^2 hybrid-

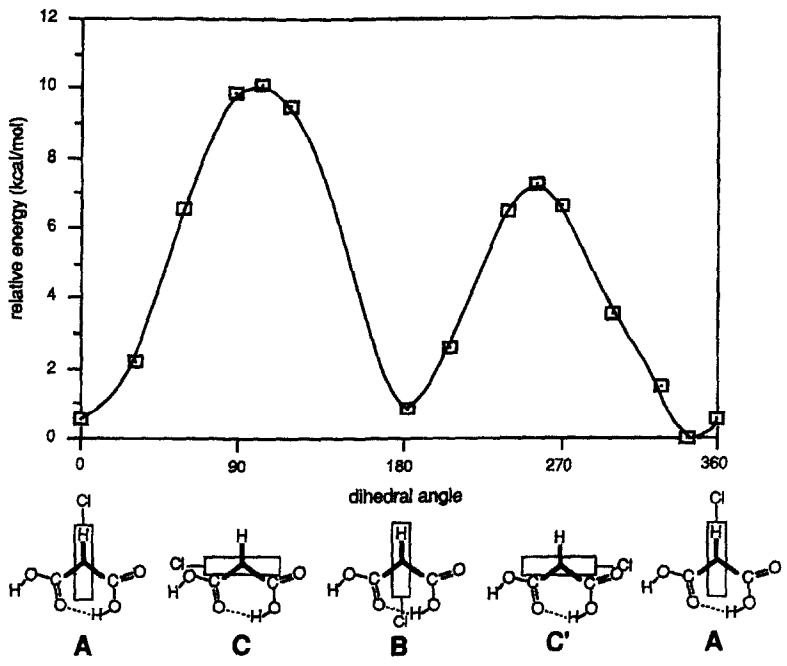


Figure 3. The potential energy diagram on the C–C bond rotation for *o*-chlorophenylmalonic acid (**1e**) calculated with HF/3-21G* method.

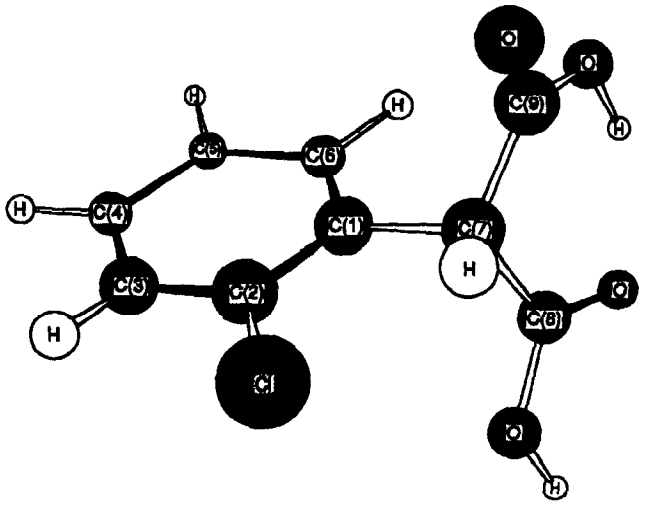


Figure 4. The most stable optimized structure for *o*-chlorophenylmalonic acid (**1e**).

ization for C(4)–C(1)–C(7). Since the relative energies obtained by the MP2 method show that the structure at dihedral angle 24.5 ° is less stable than the transition state at 40.6 °, the conformation A would not be local minimum structure when we calculate with the higher level of theory. This result concludes that the conformation A for **1f** would hardly occupy and mostly take the conformation B and perpendicular form C.

Concerning the fact that both rotational barriers for **1e** and **1f** are estimated to *ca* 6 (*ca* 7) kcal/mol, the most probable conformations at thermally equilibrated environment are concluded to be the conformations A and B for *o*-chlorophenylmalonic acid (**1e**), and B and C for α-(*o*-chlorophenyl)-α-methylmalonic acid (**1f**). Accordingly, **1e** can be incorporated in the active site in form A, whereas **1f** is unable to overcome the energy loss for occupying conformation A by binding with the enzyme.

Thus it is concluded that the results of theoretical calculations are in support of the importance of conformer A for smooth reaction.

Preparation of and reaction of a conformationally restricted substrate

Finally, the essential importance of conformation A was clearly demonstrated by designing and subjecting a substrate to the reaction, which mimics the conformer A of unreactive compound **1g**. As mentioned earlier, dimethyl derivative **1g** is entirely inactive to the enzyme and the reason is now proposed that **1g** cannot occupy the *syn*-periplanar conformation A because of steric repulsion between two methyl groups. Accordingly, if the conformation of **1g** could be fixed to *syn*-periplanar, it would be decarboxylated. The only way to fix the conformation of **1g** to A making compensation for a loss of potential energy is to make a covalent bond between two methyl groups. In this way, indane-1,1- dicarboxylic acid (**1h**) was prepared and incubated with the enzyme. As expected, **1h** afforded the corresponding (*R*)-indane-1-carboxylic acid (**2h**) in high yield. It is worth noting that the K_m value of this substrate is smaller by one order than those of acyclic compounds. Evidently, this is due to its conformation already being arranged to the one that fits to the binding site of the enzyme, or in other words probably because of decrease of activation entropy. If the $\Delta H^{\ddagger}$ value for **1g** and **1b** are assumed to be the same, the difference in $\Delta S^{\ddagger}$ between two compounds is calculated to be 6.3 cal/°K/mol. The fact that **1h** is a substrate in contrast to the inactivity of **1g** strongly suggests that the conformation of the substrates in the active site of the enzyme is very similar to **1h**.

In conclusion, the active conformation of arylmalonic acid for a smooth reaction has been proposed to be *syn*-periplanar based on both kinetic parameters of model compounds and theoretical calculation of energy diagrams of α-(*o*-chlorophenyl)- and α-(*o*-chlorophenyl)-α-methylmalonic acid as a function of the dihedral angle between the benzene ring and the α-substituent.

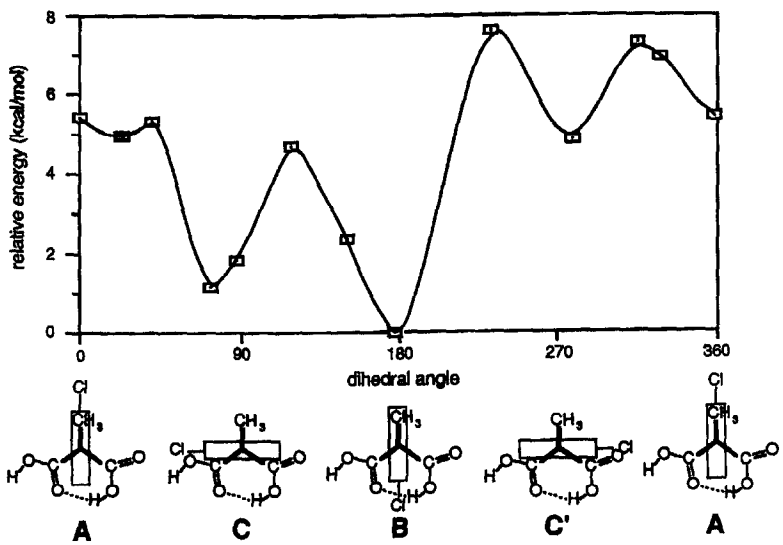


Figure 5. The potential energy diagram on the C–C bond rotation for α-(*o*-chlorophenyl)-α-methylmalonic acid (**1f**) calculated with HF/3-21G* method.

Experimental

All bps were uncorrected. IR spectra were measured as films on a Jasco IRA-202 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ with TMS as the internal standard at 90 MHz on a JEOL JNM FX-90 spectrometer. Optical rotations were recorded on a Jasco DIP 360 polarimeter. Mass spectra were recorded on a Hitachi M-80 spectrometer at 70 eV. GC analyses were performed on a Shimadzu GC-14A gas chromatograph, equipped with a Silicon OV-1 column (3 mm x 2 m, GL Science Co., Japan). HPLC analysis for the determination of enantiomeric excess of resulting methyl indane-1-carboxylate was done by using CHIRALCEL OJ (Daicel Chem. Ind. Ltd, Japan, 4.6 mm x 250 mm) and a mixture of hexane and isopropanol (9:1, v/v) as solvent.

Preparation of arylmalonate derivatives

α-Arylmalonic and α-aryl-α-methylmalonic acids were prepared according to the procedure described in ref. 5a, except for indane-1,1-dicarboxylic acid (**1h**).

*Preparation of indane-1,1-dicarboxylic acid (**1h**)*

Indane-1-carboxylic acid (**2h**) was prepared according to known procedures starting from 1-indanone.[9] The carboxylic acid was esterified with benzyl alcohol to give benzyl indane-1-carboxylate in 54 % total yield from 1-indanone: ^{1}H NMR ($CDCl_3$, 90 MHz) δ 2.17–2. 60 (m, 2H), 3.02 (dt, 2H, J_1 = 7.0 Hz, J_2 = 7.0 Hz), 4.10 (t, 1H, J = 7.0 Hz), 5.17 (s, 2H), 7.00–7.50 (m, 9H); IR (film) ν 2900, 1720, 1440, 1205, 1155, 1015, 740, 690 cm^{-1}.

A solution of *n*-butyllithium in hexane (1.58 M, 9.0 mL, 14.2 mmol) was added to a solution of diisopropylamine (1.54 g, 15.5 mmol) in 30 mL of THF and the mixture was stirred at 0 °C for 15 min, then cooled to –78 °C. Benzyl indane-1-carboxylate (3.00 g, 11.9 mmol) was added with stirring and the stirring was continued for 30 min. Benzyl chloroformate (4.06 g, 23.8 mmol) was slowly added to the mixture and the temperature was gradually raised to room temperature. The reaction mixture was quenched with phosphate buffer (pH 6) and extracted with ethyl acetate. After evaporation of the solvent, resulting oil was purified by column chromatography on silica gel (eluent, hexane/ethyl acetate = 9/1) to afford dibenzyl indane-1,1-dicarboxylate (4.52 g, 98%) as an oil; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 2.60–2.88 (m, 2H), 2.92–3.18 (m, 2H), 5.14 (s, 4H), 6.93–7.70 (m, 14H); IR (film): ν 2900, 1720, 1680, 1440, 1215, 1140, 1050, 735, 690 cm^{-1}.

A solution of dibenzyl 1,1-indanedicarboxylate (1.50 g, 3.88 mmol) in 50 mL of ethanol was vigorously stirred over a catalytic amount of 10 % Pd–C under an atmosphere of hydrogen for 2 h. Filtration of the catalyst followed by removal of solvent afforded indane-1,1-dicarboxylic acid (**1h**), which was recrystallized from a mixture of ether and hexane: mp 151–152 °C (dec.); ^{1}H NMR ($CDCl_3$/acetone-d_6 = 9/1) δ 2.63–2.94 (m, 2H), 2.94–3.24 (m, 2H), 7.10–7.78 (m, 4H), 8.70–9.35 (m, 2H); IR (film): ν 2980, 1685, 1400, 1280, 1245, 915, 730 cm^{-1}.

*Enzymatic reaction of indane-1,1-dicarboxylic acid (**1h**)*

To a 20 mL tube containing 5 mL of 1 M phosphate buffer (pH 6.5) were added 1 mL of 1 N aqueous solution of NaOH, 500 μL of 50 mM aqueous solution of mercaptoethanol, 500 μL of 1 mM aqueous solution of EDTA, 2 mL of H_2O and 226 u/mL of AMDase[5a] in 1 mL of Tris buffer (pH 8.5) and 206 mg of substrate (1h, 1 mmol). The mixture was incubated at 30 °C for 20 h. The reaction mixture was acidified with 2 N HCl and extracted with ether. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residual oil was purified by preparative TLC on silica gel developed with a mixture of hexane–ethyl acetate–acetic acid (70:30:1) to give indane-1-carboxylic acid (**2h**, 161 mg, 99.8 %): $[\alpha]_D^{21}$ +41.5 ° (*c* 1.2, benzene)[lit.[10] $[\alpha]_D$ +43.3 ° (benzene)], configuration *R*; ^{1}H NMR δ 2.20–2.70 (m, 2H), 2.85–3.40 (m, 2H), 3.06 (t, 1H, J = 7.5 Hz), 7.10–7.80 (m, 4H); IR (film) ν 2930, 1700, 1470, 1410, 1280, 1225, 905, 750, 730 cm^{-1}.

Kinetic measurement: general procedure

To a 2 mL tube, were added 25 μL of aqueous solution of a substrate, 25 μL of 1 M Tris buffer (pH 8.5) and diluted with 400 μL of water, and kept at 25 °C. The enzymatic reaction was started by addition of 50 μL of aqueous solution of the enzyme. The reaction was quenched by adding 2 N HCl. Resulting monocarboxylic acid was extracted with 500 μL of diisopropyl ether containing methyl phenoxyacetate (3 mg/mL) as the internal standard for gas chromatographic determination. The organic layer (*ca* 400 μL) was transferred to a new tube. Excess amount of trimethylsilyldiazomethane and 100 μL of methanol were added. The resulting methyl ester of the primary product was determined by GC based on a calibration curve. The measurement was done within a period in which the yield of the product increased proportionally to the reaction time. To make a Lineweaver–Burk plot, kinetic measurement was carried out at five different concentrations of the substrate.

Purification of AMDase

The decarboxylation enzyme (AMDase) was purified from *Alcaligenes bronchisepticus* KU 1201 according to the procedure already reported.[5a]

Acknowledgement

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