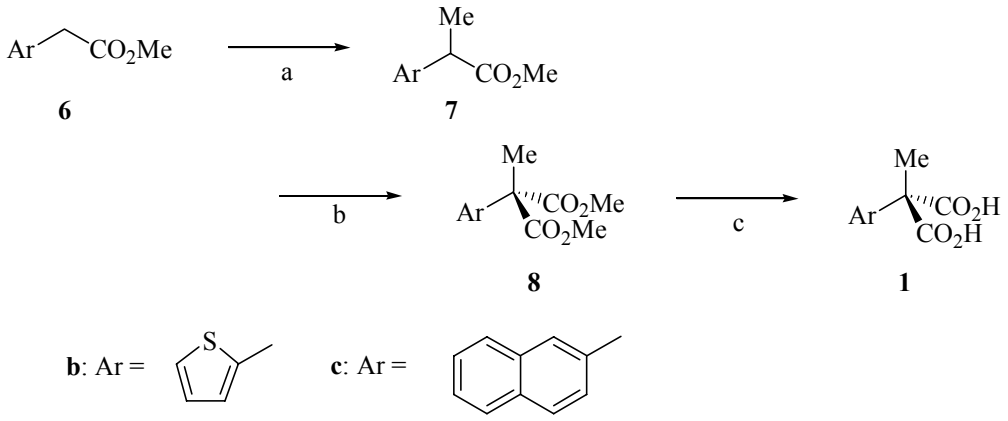
Experimental

Preparation of the Substrates



Reagents: a; LDA, MeI, THF b; LDA, $ClCO_2Me$, THF c; KOH, EtOH

1. Methyl α-(2-thienyl)propionate (7b)

To a solution of diisopropylamine (2.53 mL, 18 mmol) in dry THF (20 mL) was added 1.56 M solution of *n*-BuLi in hexane (9.87 mL, 18 mmol) with stirring at –78 °C over a period of 30 min. Methyl 2-thienylacetate (**6b**) (2.34 g, 12 mmol) in THF (20 mL) was added at –78 °C and the mixture was stirred for 30 min. Then methyl iodide (1.40 mL, 18 mmol) was added and the mixture was allowed to warm to room temperature with stirring for 1 h. The mixture was quenched with 2 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane / ethyl acetate = 5 / 1) to give 2.19 g of **7b** as colorless oil (86 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$)δ: 1.59 (3H, d, J=7.2 Hz), 3.71 (3H, s), 4.02 (1H, q, J=7.1 Hz), 6.95~7.23 (3H, m); IR ν_{max}: 3108, 2982, 2952, 1739, 1532, 1435, 1377, 1327, 1200, 1059, 853, 702 cm^{-1}

2. Dimethyl α-methyl-α-(2-thienyl)malonate (8b)

To a solution of diisopropylamine (1.05 mL, 7.5 mmol) in dry THF (10 mL) was added 1.56 M solution of *n*-BuLi in hexane (4.11 mL, 7.5 mmol) with stirring at –78 °C over a period of 30 min. Methyl α-(2-thenyl)propionate (**7b**) (0.85 g, 5 mmol) in THF (10 mL) was added at –78 °C and the mixture was stirred for 30 min. Then methyl chloroformate (0.58 mL, 7.5 mmol) was added and the mixture was allowed to warm to room temperature with stirring for 1 h. The mixture was quenched with 2 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, and dried over anhydrous

Na_2SO_4. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane / ethyl acetate = 9 / 1) to give 1.10 g of **8b** as colorless oil (97 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$)δ: 1.94 (3H, s), 3.78 (6H, s), 6.96~7.31 (3H, m); IR ν_{max}: 3478, 3108, 3002, 2953, 1731, 1434, 1377, 1276, 1119, 983, 890, 709 cm^{-1}

3. α-Methyl-α-(2-thienyl)malonic acid (1b)

A solution of dimethyl α-methyl-α-(2-thienyl)malonate (**8b**) (0.99 g, 4.3 mmol) in ethanol (5 mL) was added to a solution of potassium hydroxide (3.18 g, 43 mmol) in ethanol (20 mL). The mixture was stirred at 0 °C for 1 h. After the mixture was acidified with 6 M HCl, ethanol was evaporated and the residue was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by recrystallization from ethyl acetate and hexane to give 0.63 g of **1b** as colorless crystals (72 % yield). Mp : 157~159 °C ;

^{1}H-NMR (400MHz, CD_3CD)δ: 1.90 (3H, s), 6.96~7.41 (3H, m) ; IR ν_{max} : 3000, 2650, 1700, 1455, 1410, 1350, 1280, 1200, 1130, 1100, 1050, 1020, 910, 850 , 785, 745, 700 cm^{-1}

4. Methyl α-(2-naphtyhl)propionate (7c)

To a solution of diisopropylamine (2.10 mL, 15 mmol) in dry THF (20 mL) was added 1.57 M solution of *n*-BuLi in hexane (8.22 mL, 15 mmol) with stirring at –78 °C over a period of 30 min. Methyl 2-naphthylacetate (**6c**) (2.00 g, 10 mmol) in THF (20 mL) was added at –78 °C and the mixture was stirred for 30 min. Then methyl iodide (0.93 mL, 15 mmol) was added and the mixture was allowed to warm to room temperature with stirring for 1 h. The mixture was quenched with 2 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane / ethyl acetate = 4 / 1) to give 2.08 g of **7c** as colorless oil (97 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$)δ: 1.60 (3H, d, J=7.3 Hz), 3.67 (3H, s), 3.90 (1H, q, J=7.1 Hz), 7.42~7.83 (7H, m); IR ν_{max}: 3449, 3056, 2979, 2951, 1919, 1732, 1601, 1508, 1455, 1434, 1378, 1331, 1198, 1093, 1066, 949, 859, 822, 795, 748, 709 cm^{-1}

5. Dimethyl α-methyl-α-(2-naphthyl)malonate (8c)

To a solution of diisopropylamine (1.47 mL, 11.5 mmol) in dry THF (10 mL) was added 1.57 M solution of *n*-BuLi in hexane (5.76 mL, 11.5 mmol) with stirring at –78 °C for 30 min. Methyl α-(2-naphthyl)propionate (**7c**) (1.50 g, 7 mmol) in THF (10 mL) was

added at –78 °C and the mixture was stirred for 30 min. Then methyl chloroformate (0.90 mL, 11.5 mmol) was added and the mixture was allowed to warm to room temperature with stirring for 1 h. The mixture was quenched with 2 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexane / ethyl acetate = 4 / 1) to give 1.73 g of **8c** as colorless oil (95 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$)δ: 1.99 (3H, s), 3.79 (6H, s), 7.46~7.83 (7H, m); IR ν_{max}: 3458, 3000, 2952, 1733, 1434, 1378, 1255, 1113, 820, 750 cm^{-1}

6. α-Methyl-α-(2-naphthyl)malonic acid (1c)

A solution of dimethyl α-methyl-α-(2-naphthyl)malonate (**8c**) (0.67 g, 2.5 mmol) in ethanol (5 mL) was added to a solution of potassium hydroxide (2.24 g, 25 mmol) in ethanol (20 mL). The mixture was stirred at 0 °C for 1 h. After the mixture was acidified with 6 M HCl, ethanol was evaporated and the residue was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by recrystallization from ethyl acetate / hexane to give 0.48 g of **1c** as colorless crystals (80 % yield). Mp : 170~ 172 °C ; ^{1}H-NMR (400MHz, $CDCl_3$)δ: 1.97 (3H, s), 7.48~7.97 (7H, m) ; IR ν_{max} : 3529, 3060, 2629, 1715, 1455, 1287, 1129, 815, 751, 707 cm^{-1}

Site-directed Mutagenesis of AMDase

A pUC19-based plasmid pAMD101 and pAMD101-C188S plasmid were used as the templates for G74C single mutant and for G74C/C188S double mutant, respectively for PCR-based site-directed mutagenesis. Both template plasmids were previously prepared in our laboratory (ref 6 and 7 in the article). To introduce mutation, partially overlapping PCR products were generated spanning the 5' and 3' end of the gene. The synthetic oligonucleotides used for the preparation of the 5' fragment were the forward primer which corresponds to the anti-codon of the multi-cloning site sequence of the vector (P5: 5'-CAGGAAACAGCTATGACC-3', 18 mer), and the reverse primer containing the mutation site to change the amino acid residue of 74th glycine to cysteine (G74Creverse: 5'-GCTGAGCGAGGTGCACATCAGCGAAACCAC-3', 30 mer). The forward primer, which contained mutation site (G74Cfowrawd: 5'-GTTTCGCTGATGTGCACCTCGCTCAGCTTC-3', 30 mer), and the reverse primer corresponding to the vector sequence (P1: 5'-GTAAAACGACGGCCAGTG-3', 18 mer) were used to prepare the 3' fragment.

After preparation of the 5' and 3' PCR fragments for mutation, they were combined by

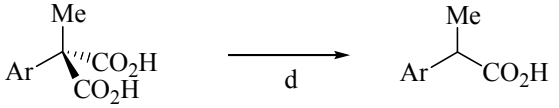
mixing and second PCR amplification with the same primers, P5 and P1. The final PCR product was digested with the restriction enzymes, HindIII and Pst I. This mutated gene was connected with pUC19 which was digested with the same restriction enzymes. Then *E. coli* JM109 was transformed by the mutant plasmid of pAMD101 or pAMD101-C188S. The transformant harboring the mutant plasmid was cultivated in 1.5 L of LB-broth (pH 7.0, containing 150 mg/L of ampicillin) at 30 °C with shaking (200 rpm). After cultivation for 2 h, starting from 1/100 volume of over-night preculture inoculum, IPTG (isopropyl-β-D-galactopyranoside) was added at 0.1 mM. The cultivation was continued for additional 16 h.

Purification of AMDase

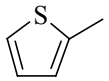
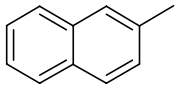
The potassium phosphate buffer of various concentrations containing 0.5 mM EDTA and 5 mM β-mercaptoethanol was used throughout the purification. All the procedures for the purification of the enzyme were performed below 4 °C. The *E. coli* cells containing AMDase were collected by centrifugation at 6000 g for 20 min. The cells were suspended in 200 mL of 100 mM buffer (pH 7.0) and homogenized by French press (1500 kg/cm^2). This suspension was centrifuged (12,000 g, 20 min) to remove the insoluble precipitates. To the resulting solution, was added 1% volume of aqueous solution of protamine sulfate (conc. 2%), and the mixture was stirred for 30 min. The resulting precipitate was removed by centrifugation (12,000 g, 20 min). To the obtained enzyme solution, was added ammonium sulfate to the concentration of 60% saturation and stirred for 1 h. The precipitated protein was isolated by centrifugation (12,000 g, 20 min) and dissolved in 10 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer. The enzyme solution was charged to a DEAE-Toyopearl column, which had been equilibrated with 10 mM Tris–HCl buffer (pH 8.0) and eluted with aqueous solution of NaCl with a linear gradient of 10–50 mM. The active fractions were collected and concentrated to 30 mL by ultrafiltration. Ammonium sulfate was added to this solution to 25% saturation and this mixture was applied to a butyl-toyopearl column which had been equilibrated with 25% ammonium sulfate solution of 10 mM Tris–HCl buffer (pH 8.0). The enzyme was eluted with an aqueous solution of ammonium sulfate with a linear gradient of 25–15%. The active fractions were combined and dialyzed against 10 mM Tris–HCl buffer.

The concentration of protein was determined by Bio-Rad protein Assay kit or from the absorbance at 280 nm. As to the mutant enzymes, the protein which was identical with AMDase on SDS-PAGE was recovered after the purification by column chromatography.

Enzymatic Decarboxylation of α-Methyl-α-Arylmalonic Acid (1)



1 **2**

b: Ar =  **c**: Ar = 

d: 10 mM Tris-HCl buffer, pH 8.5, AMDase

1. Decarboxylation of α-Methyl-α-(2-Thienyl)malonic Acid (1b)

α-Methyl-α-(2-thienyl)malonic acid (**1b**, 400 mg, 2 mmol) was dissolved in water (ca. 8 mL) and the pH of the mixture was adjusted to 8.0 with 2 M HCl and 2 M NaOH. Final volume was adjusted to 10 mL by adding water. To 20 mL round-bottomed flask was added 1 mL of 10 mM Tris-HCl buffer (pH = 8.5), 2 mL of the substrate solution (80.0 mg, 0.4 mmol) and 1 mL of AMDase solution (Wild Type, 0.87 mg; C188S mutant, 0.77 mg; G74C mutant, 1.07 mg; G74C/C188S mutant, 0.52 mg). The mixture was stirred at 30 °C for 1~72 h. The mixture was quenched with 1 mL of 2 M HCl and filtrated with cerite. The filtrate was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by preparative TLC (hexane/ethyl acetate/acetic acid =33/66/1) to give **2b** as colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.61 (3H, d, J=7.3 Hz), 4.04 (1H, q, J=7.3 Hz), 6.95~7.23 (3H, m) ; IR ν_{max}: 2982, 1715, 1457, 1417, 1236, 699 cm^{-1}

After esterification with TMS-diazomethane, the enantiomeric excess of the product was determined by HPLC : Chiralcel OJ (Daicel Chemical Industries Ltd.); eluent : hexane / 2-propanol = 50 / 1; flow rate : 0.5 mL/min

Retention time : (R)-form 19 min, (S)-form 32 min

Wild Type: 62.4 mg, 99% yield, 94% ee (S)-form
C188S mutant: 10.6 mg, 17% yield, 50% ee (R)-form
G74C mutant: 23.1 mg, 37% yield, 0% ee (R/S)-form
G74C/C188S mutant: 37.5 mg, 60% yield, 84% ee (R)-form

Enzymatic Decarboxylation of α-Methyl-α-(2-naphthyl)malonic Acid (1c)

α-Methyl-α-(2-naphthyl)malonic acid (**1c**, 488 mg, 2 mmol) was dissolved in water (ca. 8 mL) and the pH of the mixture was adjusted to 8.0 with 2 M HCl and 2 M NaOH. Final volume was adjusted to 10 mL by adding water. To 20 mL round-bottomed flask was

added 1 mL of the 10 mM Tris-HCl buffer (pH = 8.5), 2 mL of the substrate solution (97.7 mg, 0.4 mmol) and 1 mL of AMDase solution (Wild Type, 0.87 mg; C188S mutant, 0.77 mg; G74C mutant, 1.07 mg; G74C/C188S mutant, 0.52 mg). The mixture was stirred at 30 °C for 1~72 h. The mixture was quenched with 1 mL of 2 M HCl and filtrated with cerite. The filtrate was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by preparative TLC (hexane/ethyl acetate/acetic acid =50/50/1) to give **2c** as colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.61 (3H, d, J=7.1 Hz), 3.93 (1H, q, J=6.9 Hz), 7.44~7.83 (7H, m) ; IR ν_{max}: 2980, 1698, 1419, 1273, 1225, 963, 747 cm^{-1}
After esterification with TMS-diazomethane, the enantiomeric excess of the product was determined by HPLC : Chiralcel OD (Daicel Chemical Industries Ltd.); eluent : hexane / 2-propanol = 100 / 1; flow rate : 0.5 mL/min
Retention time : (R)-form 20 min, (S)-form 22 min

Wild Type: 76.8 mg, 96% yield, 92% ee (R)-form
C188S mutant: 4.8 mg, 6% yield, 70% ee (S)-form
G74C mutant: 10.4 mg, 13% yield, 6% ee (R)-form
G74C/C188S mutant: 13.6 mg, 17% yield, 96% ee (S)-form