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Substrate specificity analysis and inhibitor design of homoisocitrate dehydrogenase

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Abstract—Homoisocitrate dehydrogenase is involved in the α-aminoadipate pathway of biosynthesis of L-lysine in fungi, yeast, some prokaryotic bacteria, and archaea. This enzyme catalyzes the oxidative decarboxylation of (2*R*,3*S*)-homoisocitrate into 2-oxoadipate using NAD^+ as a coenzyme. Substrate specificity of two homoisocitrate dehydrogenases derived from *Deinococcus radiodurans* and *Saccharomyces cerevisiae* was analyzed using a series of synthetic substrate analogs, which indicated a relatively broad substrate specificity of these enzymes. Based on the substrate specificity, 3-hydroxyalkylidene- and 3-carboxyalkylidenemalate derivatives were designed as a specific inhibitor for homoisocitrate dehydrogenase. The synthetic inhibitors showed a moderate competitive inhibitory activity and (*R*,*Z*)-3-carboxypropylidenemalate was the most inhibitory among the synthesized inhibitors. Therefore, homoisocitrate dehydrogenase appeared to recognize preferentially an extended conformation of homoisocitrate.

1. Introduction

Mechanistic enzymology is among major interests in biochemistry. Ultimate goals of this field include precise understanding of the molecular interactions in enzyme reaction and development of potential chemicals for medicinal as well as agricultural use.

The α-aminoadipate pathway for biosynthesis of lysine is present in fungi, yeast, some prokaryotic bacteria, and archaea.[1] Therefore, this unique metabolic pathway is now considered to be a new target for treatment of fungal pathogens.[2,3] Homoisocitrate dehydrogenase (HICDH, EC: 1.1.1.115) is involved in the third step of the α-aminoadipate pathway and catalyzes the oxidative decarboxylation of (2*R*,3*S*)-homoisocitrate **1** into 2-oxoadipate using NAD^+ as a coenzyme as shown in Figure 1.[1]

HICDH is categorized into the β-decarboxylating oxidation enzyme family including isocitrate dehydrogenase (ICDH) in TCA cycle, 3-isopropylmalate dehydrogenase (IPMDH) in leucine biosynthesis, and other related enzymes. Recently, the crystal structure of HICDH from thermophilic bacteria *Thermus thermophilus* was analyzed by X-ray crystallography and the polypeptide regions responsible for the binding of cofactor and substrate were discussed by comparison with the crystal structures of IPMDH and ICDH.[4] However, detailed features of HICDH–substrate interaction have not yet been clarified. In this paper, we describe the substrate specificities of HICDHs from *Deinococcus radiodurans* (DraHICDH) and *Saccharomyces cerevisiae* (ScHICDH) using substrate analogs. Also described are inhibitor design for these HICDHs and evaluation of the synthesized inhibitors.

2. Results and discussion

2.1. Substrate specificity for HICDH

In order to design a specific inhibitor of HICDH, we first analyzed the substrate specificity using a series of synthesized substrate analogs having various side chains. The structures of the tested compounds **1**–**19** are shown in Figure 2. Homoisocitrate **1**[5] and their homoanalogs **3**[6]

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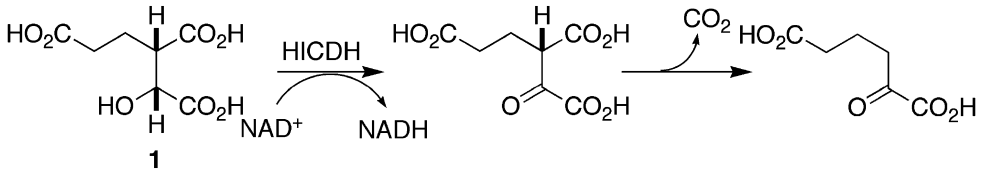


Figure 1. The enzyme reaction of HICDH.

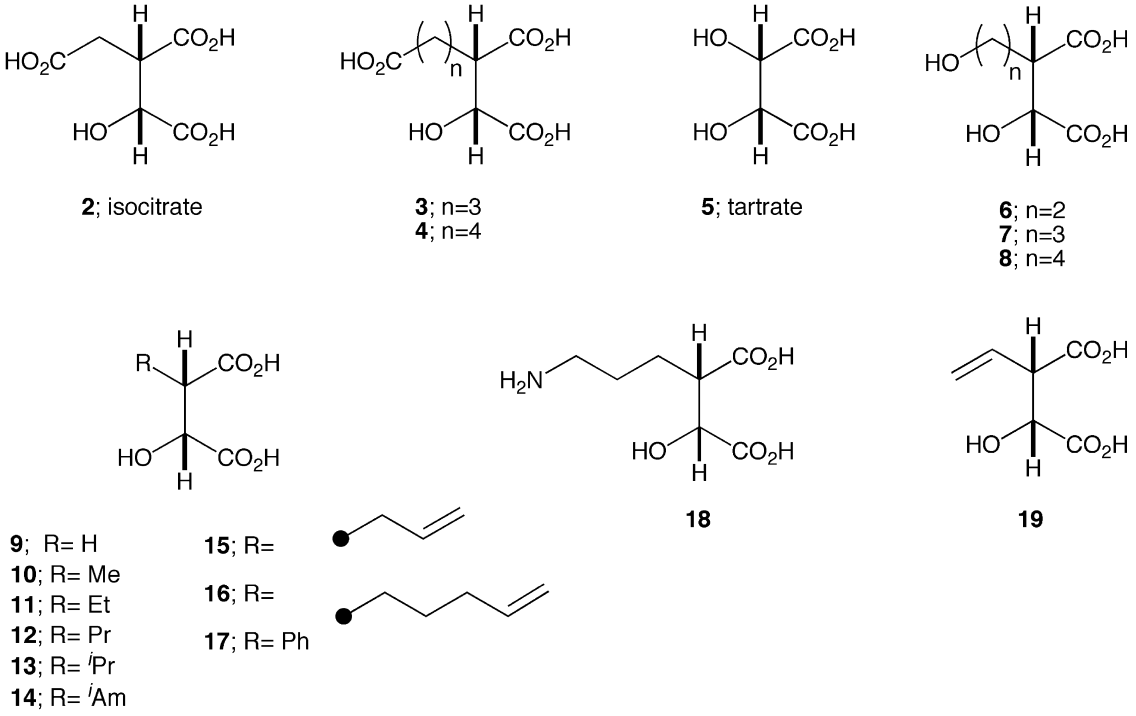
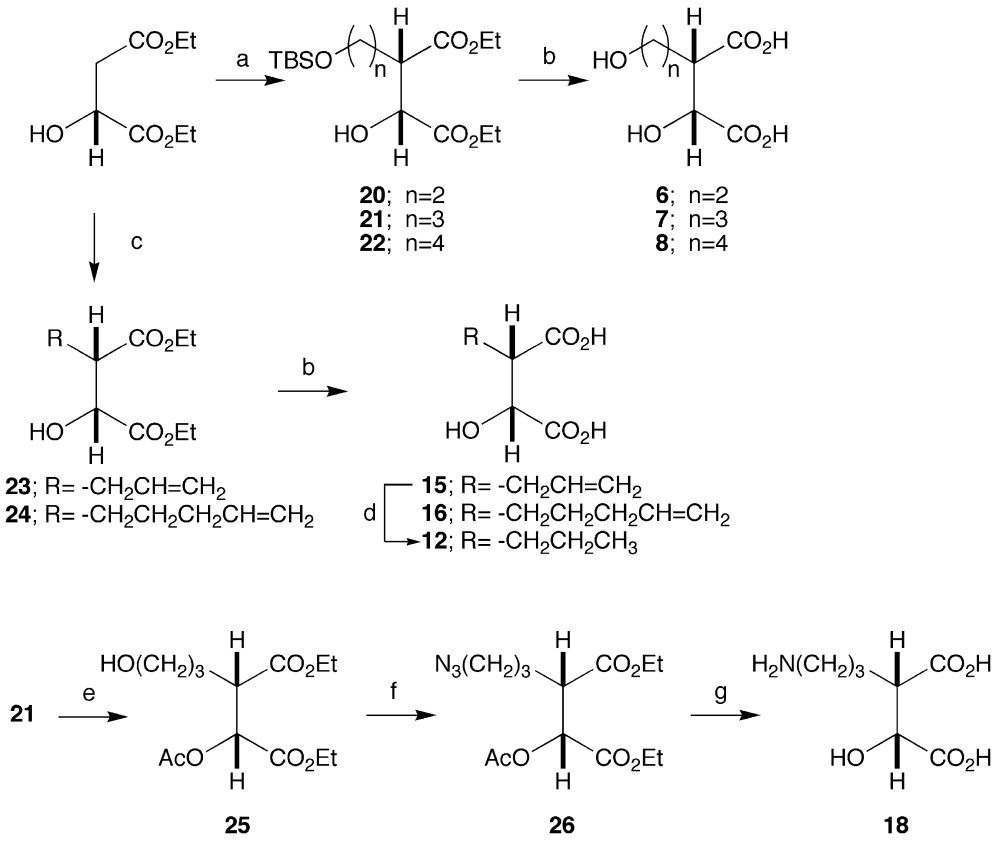


Figure 2. Structures of substrate analogs.

and **4**[6] were synthesized according to the reported methods. 3-Alkylmalates[7] **10**, **11**, **13**, **14**, **17**, and 3-vinylmalate **19**[8] were previously synthesized in our laboratory for investigation of substrate specificity and inhibitory analysis of IPMDH. Other 3-alkylmalates **12**, **15**, **16**, and 3-(ω-hydroxyalkyl)malates **6**–**8** were prepared in this study via alkylation[9] of diethyl D-malate as shown in Scheme 1. Thus, diethyl D-malate was treated with two equivalents of LDA at −78 °C to form ester enolate, which was then alkylated with 1-(*t*-butyldimethylsiloxy)-2-iodoethane at −20 °C to give stereoselectively 3-alkylated malate in 8:1 ratio. After separation of diastereomers, the desired isomer **20** was hydrolyzed with NaOH, followed by deprotection of TBS group under acidic conditions to yield **6**. Compounds **7**, **8**, **12**, **15**, and **16** were similarly prepared from diethyl D-malate. Further, in order to see the effect of electrostatic interaction between substrate and enzyme, we synthesized 3-(3-aminopropyl)malate **18**, which has a positively charged ammonium group in the side chain instead of a negatively charged carboxylate group of the substrate under the physiological conditions. As also shown in Scheme 1, compound **21** was converted by a conventional manner to desired **18**.

The prepared analogs were subjected to the enzyme reaction to test their potentials to be incorporated into the active site of HICDH. The reactions were monitored by measuring the formation of NADH from NAD^+ and the reaction kinetics were analyzed by double reciprocal plot. In this study, we used two HICDHs, DraHICDH[10,11] and ScHICDH, derived from *D. radiodurans* and *S. cerevisiae*, respectively. DraHICDH and ScHICDH were used as examples of HICDH from prokaryote and eukaryote, respectively. Although the native ScHICDH was purified and characterized from yeast,[12] cloning, expression, and purification of ScHICDH was carried out in this study.

The kinetic parameters are listed in Table 1. As for DraHICDH, this enzyme recognized most of the analogs as a substrate. It is known that prokaryotic and archaeal HICDHs including DraHICDH have a relatively broad substrate specificity.[10,11,13,14] Most HICDHs are able to recognize not only homoisocitrate but also isocitrate as a substrate. Furthermore, DraHICDH can recognize isopropylmalate as a substrate, suggesting the unusually broad specificity of the enzyme. In addition to the previous results, the present study clearly demonstrated that DraHICDH possessed a large pocket in the substrate-binding site.[11] Among the tested, ω-carboxylalkylmalate analogs (**1**–**4**) were the most potent substrates and the modest substrate activity was



Scheme 1. Reagents and conditions; (a) 2 equiv LDA, $TBSO(CH_2)_nI$, THF-HMPA, 0 °C; (b) NaOH, H_2O–THF, and then H_3O^+; (c) 2 equiv LDA, allyl bromide or 5-iodo-1-pentene, THF–HMPA, 0 °C; (d) H_2–Pd/C, ethanol; (e)—1 Ac_2O, DMAP, Et_3N, CH_2Cl_2, —2 PPTS, ethanol; (f)—1 TsCl, Et_3N, CH_2Cl_2, —2 NaN_3, DMF; (g) —1 H_2–Pd/C, ethanol, —2 NaOH, H_2O–THF.

Table 1. Kinetic parameters of substrate analogs

Compound	DraHICDH			ScHICDH		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/ μM)	K_m(μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/μM)
1	211[a]	46[a]	0.22[a]	18	17	0.94
2	291[a]	43[a]	0.15[a]	ND	ND	ND
3	200	40	0.20	26	1.6	0.062
4	140	40	0.29	170	3.1	0.018
5	ND	ND	ND	ND	ND	ND
6	590	25	0.042	2100	0.78	0.00037
7	510	29	0.057	580	4.8	0.0083
8	470	28	0.060	440	2.1	0.0048
9	ND	ND	ND	ND	ND	ND
10	3600	19	0.0053	5700	0.32	0.000056
11	1000	43	0.043	600	0.61	0.0010
12	780	53	0.068	290	0.70	0.0024
13	1330[a]	0.37[a]	0.00028[a]	ND	ND	ND
14	3200	3.7	0.0012	7200	0.11	0.000015
15	2800	37	0.013	410	0.34	0.00083
16	2700	3.0	0.0011	9400	0.14	0.000015
17	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND
19	83	0.77	0.0093	3400	0.50	0.00015

[a] Data from Ref. 11. ND, substrate activity was not detected at a high concentration of the substrate (>10 mM).

observed in ω-hydroxyalkylmalates (**6**–**8**) compared to 3-alkylmalates (**10**–**17**). Further, aminoalkylmalate analog (**18**) showed no activity at all. Therefore, these results indicated that a polar hydroxyl group and a negatively charged carboxyl group in side chain might contribute to the substrate recognition of DraHICDH.

HICDHs from *Saccharomyces* species are highly homologous to fungal HICDHs in the amino acid level. These

enzymes were reported to recognize specifically homoisocitrate, and isocitrate and isopropylmalate are not accepted at all.[13,15] Actually, isocitrate **2** and isopropylmalate **13** did not show any substrate activity, however, most of the analogs acted as a substrate as shown in Table 1. ω-Carboxyalkylmalate derivatives were also the most potent and the tendency of substrate recognition was found to be similar to DraHICDH. Although the substrate recognition in ScHICDH seemed to be more strict than DraHICDH, ScHICDH also turned out to have a broad substrate specificity.

Interesting phenomenon was observed in the case of vinylmalate **19**, which was designed as a potent and mechanism-based inhibitor for IPMDH.[8] Vinylmalate (K_m, 83 μM) appeared to be tightly recognized by DraHICDH rather than the original substrate, homoisocitrate (K_m, 211 μM).[11] However, ScHICDH showed a weak affinity for vinylmalate (K_m, 3400 μM) compared to homoisocitrate (K_m, 18 μM). Since vinylmalate showed a high-affinity and a relative low catalytic efficiency to DraHICDH, we tested the inhibitory activity of vinylmalate against HICDHs. The inhibitory activity was analyzed against the natural homoisocitrate substrate employing a standard protocol of HICDH assay method as described above. While ScHICDH was not inhibited, vinylmalate appeared to competitively inhibit the DraHICDH reaction and the inhibition constant, K_i, was estimated to be as strong as 88 μM as shown in Table 2. Because the inhibition mechanism of vinylmalate appeared to be competitive and the time-dependent inactivation was not observed (data not shown), it seems likely that the strong inhibition activity of vinylmalate is due to its high affinity to DraHICDH. Considering the binding affinities of structurally similar analogs such as ethylmalate (K_m, 1000 μM) and allylmalate (K_m, 2800 μM) for DraHICDH, the presence and position of the double-bond of vinylmalate seem to be important for the strong binding, which may be caused by the π–π interaction between vinylmalate and a amino acid residue of HICDH or a nicotinamide ring of NAD^+. It is intriguing that significant difference was observed between two HICDHs and further analysis is underway in our laboratory.

Table 2. Competitive inhibitory constants of the designed inhibitors

Inhibitor	K_i (μM)	
	DraHICDH	ScHICDH
19	88	ND
27a	5200	1400
27b	15,300	510
28a	3100	790
28b	260	72

ND, inhibitory activity was not detected.

2.2. Design and evaluation of inhibitors

Based on the two-step mechanism of the HICDH reaction, an inhibitor which cannot be susceptible for the second decarboxylation reaction may reside in the active site. Thus, when alkylidenemalate derivative is recognized by the enzyme and once oxidized in the first oxidation step, the resulting alkylidenoxaloacetate should not be decarboxylated, and hence, may stay in the active site as shown in Figure 3. Alternatively, a neighboring nucleophilic residue in the active site may attack the electron-deficient double bond to form a covalent bond. Actually, we successfully demonstrated that 3-isopropylidenemalic acid[16] and 3-ethylidenemalic acid[8] behave as a mechanism-based inhibitor for IPMDH. Along this line and according to the result of substrate recognition experiments, 3-hydroxypropylidenemalate (**27ab**) and 3-carboxypropylidenemalate (**28ab**) were designed as a specific inhibitor of HICDH.

The syntheses of **27ab** and **28ab** were performed as shown in Scheme 2. Compound **21** was treated with 2 equiv of LDA and the resulting enolate was reacted with phenyl benzenethiosulfate, after acetylation of hydroxyl group, to give phenylthiolated product **29**. The oxidative elimination of **29** was accomplished by treatment with *m*-CPBA, followed by thermal elimination of the resulting sulfoxide in refluxing toluene to give **30a** and **30b** in a ratio of 5:3. After separation of isomers, the geometry of the double bond was determined by NOE experiments. Finally, acidic and basic hydrolysis of **30ab** afforded desired **27ab**, respectively. 3-Carboxypropylidenemalates **28ab** were similarly synthesized as shown in

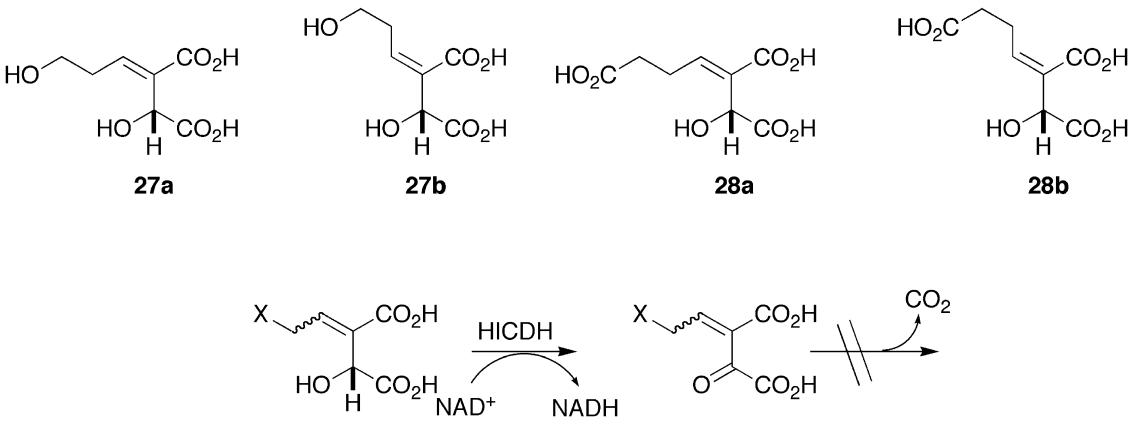
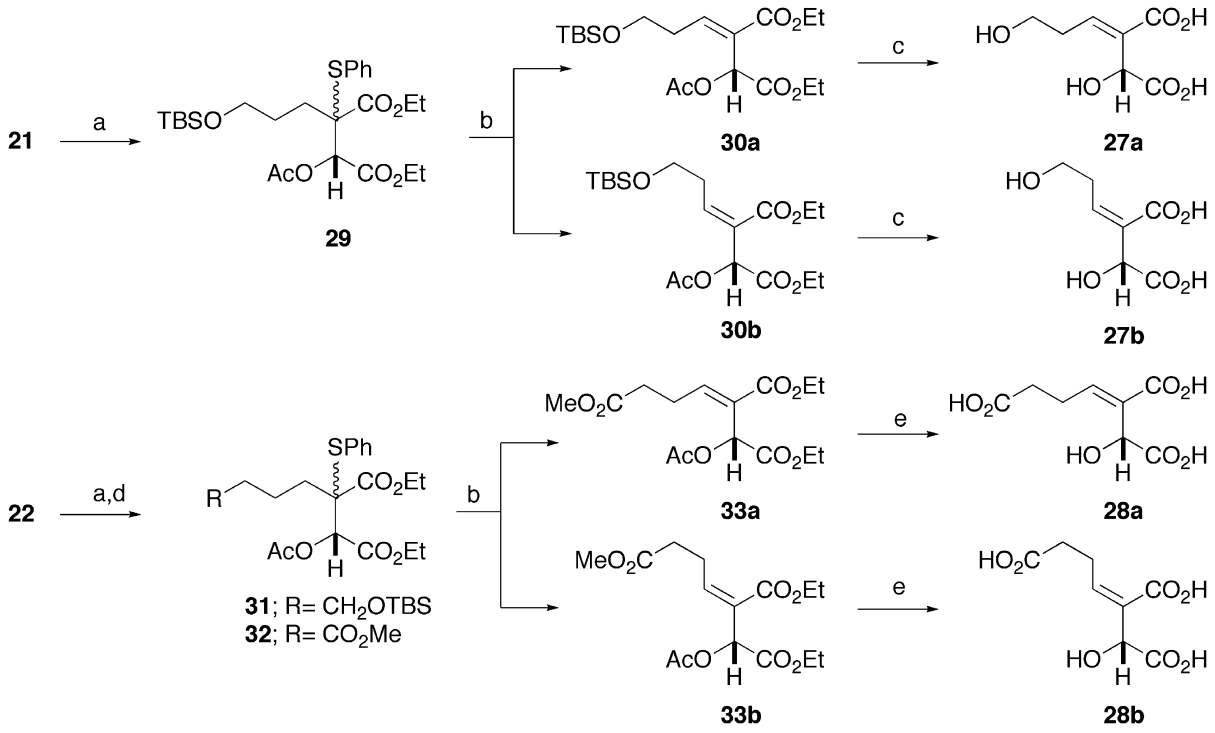


Figure 3. Structures of designed inhibitors and the expected inhibitory mechanism.



Scheme 2. Reagents and conditions; (a)—1 2 equiv LDA, $PhSO_2SPh$, THF, −20 °C, —2 Ac_2O, DMAP, Et_3N, CH_2Cl_2; (b)—1 *m*-CPBA, CH_2Cl_2, —2 toluene, reflux; (c)—1 PPTS, ethanol, —2 LiOH, H_2O–THF; (d)—1 PPTS, ethanol, —2 PDC, DMF, —3 $TMSCHN_2$, methanol; (e) LiOH, H_2O–THF.

Scheme 2. After phenyl thiolation of **22** as described above, deprotection of silyl group of the obtained **31** and conversion of the resulting hydroxyl group into carboxylic group by PDC, followed by methyl esterification with $TMSCHN_2$, gave triester **32**. Oxidation of **32**, followed by thermal elimination and hydrolysis as described above afforded desired **28ab**.

The synthesized inhibitors were subjected to the enzyme reaction and their inhibitory activities were studied. The results are also summarized in Table 2. All of the designed inhibitors appeared to behave as a competitive inhibitor. Although 3-hydroxypropylidenemalates were not so potent compared to the corresponding 3-carboxypropylidenemalates, these inhibitors showed moderate inhibitory activities in comparison with K_m value of homoisocitrate. Since the mechanism of inhibition for these inhibitors appeared to be competitive and these inhibitors did not show the time-dependent inactivation for these enzymes, therefore, these inhibitors may be reversibly incorporated into the active site of enzyme and reside without further oxidation unexpectedly. Further, it should be noted that significant difference in the inhibitory activity for HICDH was observed between the geometric isomers, especially 3-carboxypropylidenemalates. In both enzymes, *Z*-isomers showed more potent inhibitory activity except for 3-hydroxypropylidenemalates in ScHICDH. Therefore, it is conceivable that the *Z*-configuration of inhibitors mimics the active conformation of homoisocitrate, i.e., HICDH preferentially recognizes an extended conformation of homoisocitrate.

In conclusion, we demonstrated the substrate specificities of HICDHs from *D. radiodurans* and *S. cerevisiae* using substrate analogs, which indicated a relatively broad substrate specificity of these enzymes. Based on the substrate specificity, 3-hydroxyalkylidene and 3-carboxyalkylidenemalate derivatives were designed as a specific inhibitor for HICDH. The synthetic inhibitors showed a moderate competitive inhibitory activity and (*R*,*Z*)-3-carboxypropylidenemalate was the most inhibitory among the synthesized inhibitors. Therefore, homoisocitrate dehydrogenase appeared to recognize preferentially an extended conformation of homoisocitrate. This is the first approach to in vitro inhibition experiments for the enzyme involved in the α-aminoadipate pathway. The present results appear to be significant in elucidating the interaction of HICDH with the substrate and cofactors. The development of a highly potent inhibitor such as 3-carboxypropylidenemalate may allow us to obtain crystals of HICDH–inhibitor–NAD^+ complex appropriate for crystallographic analysis and efforts are being directed toward this goal.

3. Experimental

3.1. General

^1H and ^{13}C NMR spectra were recorded on a JEOL AL-400 or a JEOL Lamda-400 spectrometer. IR spectra were recorded on a Horiba FT-710 Fourier-transform infrared spectrometer. Elemental analyses were performed with a Perkin-Elmer 2400 apparatus. Column

chromatography was carried out with Merck Kieselgel 60 (70–230 mesh or 230–400 mesh, Merck) or Merck Kieselgel 60 silanisiert (70–230 mesh, Merck).

Escherichia coli DH5α (TaKaRa) was used for subcloning of DNA fragments. Restriction enzymes were purchased from TaKaRa. *Escherichia coli* was grown at 37 °C in Luria–Bertani (LB) broth or on LB agar supplemented, when necessary, with appropriate antibiotics. Ampicillin (100 μg/ml) or kanamycin (30 μg/ml) was added for selection of plasmid-containing *E. coli* cells. Recombinant protein concentration was determined by using Lowry–Folin method with 2.0 mg/ml BSA as a standard. Enzyme reaction was monitored by measuring the NADH absorption at 340 nm on a Shimadzu UV-160A UV–Vis recording spectrometer. DNA sequencing was carried out with a LONG READIR 4200 (Li-Cor) according to the manufacturer's protocol. All other reagents were of the highest grade commercially available.

3.2. Synthesis

3.2.1. Diethyl (2*R*,3*S*)-3-{2-(*t*-butyldimethylsiloxy)ethyl}malate (20). *n*-BuLi (61.3 ml, 94.6 mmol, 1.58 M in hexane) was added dropwise to a stirred solution of diisopropylamine (15.9 ml, 113 mmol) in 120 ml of anhydrous THF at −78 °C. After 30 min, a solution of diethyl D-malate (9.00 g, 47.3 mol) in THF (10 ml) was added dropwise at −78 °C. The mixture was warmed to −20 °C and stirred for 40 min, and then HMPA (40 ml) was added to the solution. After cooling to −78 °C, 1-(*t*-butyldimethylsiloxy)-2-iodoethane (16.2 g, 56.8 mmol) was added, and the reaction mixture was stirred at −20 °C for 15 min. Acetic acid (11 ml) was added at −78 °C. After warmed to room temperature, the reaction mixture was poured into aqueous NH_4Cl (100 ml) and the mixture was extracted with ether (four times). The combined organic layer was washed with saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. After evaporation of the solvent, a crude product was purified by flash silica-gel chromatography with hexane–ethyl acetate (80/20) to afford **20** (5.27 g, 32%) as an oil: IR (neat): 3521, 2979, 2931, 1739 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.30 (dd, 1H, *J* = 3.2, 6.8 Hz), 4.24 (d q, 1H, *J* = 12.2, 7.2 Hz), 4.22 (d q, 1H, *J* = 12.2, 7.2 Hz), 4.13 (d q, 1H, *J* = 11.5, 7.2 Hz), 4.09 (d q, 1H, *J* = 11.5, 7.2 Hz), 3.75 (ddd, 1H, *J* = 5.2, 6.8, 10.5 Hz), 3.68 (ddd, 1H, *J* = 5.2, 6.8, 10.5 Hz), 3.33 (d, 1H, *J* = 6.8 Hz), 3.15 (d t, 1H, *J* = 3.2, 7.1 Hz), 2.08 (dd t, 1H, *J* = 13.9, 5.2, 7.1 Hz), 1.85 (dd t, 1H, *J* = 13.9, 5.2, 7.1 Hz), 1.28 (t, 3H, *J* = 7.1 Hz), 1.21 (t, 3H, *J* = 7.1 Hz), 0.87 (s, 9H), 0.02 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 173.4, 172.4, 70.9, 61.6, 60.7, 60.4, 45.0, 30.7, 25.8, 14.0, −5.5, −5.5. Anal. Calcd for $C_{16}H_{32}O_6Si$: C, 55.14; H, 9.25. Found: C, 54.98; H, 9.02.

3.2.2. Diethyl (2*R*,3*S*)-3-{3-(*t*-butyldimethylsiloxy)propyl}malate (21). Compound **21** was synthesized by the similar procedure described for the preparation of **20** (45%). IR (neat): 3521, 2931, 1739, 1463, 836 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.25 (m, 3H), 4.14 (d q, 1H, *J* = 11.5, 7.2 Hz), 4.13 (d q, 1H, *J* = 11.5, 7.2 Hz), 3.63 (t, 2H, *J* = 6.5 Hz), 3.18 (d, 1H, *J* = 5.3 Hz), 2.90 (ddd, 1H, *J* = 2.5, 4.8, 5.7 Hz), 1.95–1.85 (m, 1H), 1.77–1.66 (m, 1H), 1.65–1.56 (m, 1H), 1.29 (t, 3H, *J* = 7.1 Hz), 1.23 (t, 3H, *J* = 7.1 Hz), 0.87 (s, 9H), 0.02 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 173.3, 172.5, 71.1, 62.8, 61.7, 60.7, 48.3, 30.4, 25.9, 24.7, 14.2, 14.1, −5.3. Anal. Calcd for $C_{17}H_{34}O_6Si$: C, 56.32; H, 9.45. Found: C, 56.07; H, 9.17.

3.2.3. Diethyl (2*R*,3*S*)-3-{3-(*t*-butyldimethylsiloxy)butyl}malate (22). Compound **22** was synthesized by the similar procedure described for the preparation of **20** (58%). IR (neat): 2929, 1743, 1473, 836 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.19–4.30 (m, 1H), 4.24 (d q, 1H, *J* = 12.2, 7.2 Hz), 4.22 (d q, 1H, *J* = 12.2, 7.2 Hz), 4.13 (d q, 1H, *J* = 11.5, 7.2 Hz), 4.09 (d q, 1H, *J* = 11.5, 7.2 Hz), 3.61 (t, 2H, *J* = 6.2 Hz), 3.17 (d, 1H, *J* = 7.7 Hz), 2.85 (m, 1H), 1.73–1.81 (m, 1H), 1.61–1.73 (m, 1H), 1.38–1.57 (m, 4H), 1.30 (t, 3H, *J* = 7.1 Hz), 1.23 (t, 3H, *J* = 7.1 Hz), 0.88 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 173.0, 172.3, 70.6, 62.4, 61.3, 60.7, 48.1, 32.1, 27.4, 25.5, 23.3, 17.9, 13.6, −5.8. Anal. Calcd for $C_{18}H_{36}O_6Si$: C, 57.41; H, 9.64. Found: C, 57.21; H, 9.64.

3.2.4. Diethyl (2*R*,3*S*)-3-(4-pentenyl)malate (24). Compound **24** was synthesized by the similar procedure described for the preparation of **20** (42%). IR (neat): 2981, 1739 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.78 (dd t, 1H, *J* = 10.0, 16.8, 6.8 Hz), 4.9–5.0 (m, 2H), 4.10–4.25 (m, 5H), 3.20 (d, 1H, *J* = 7.5 Hz), 2.83 (m, 1H), 2.08 (q, 2H, *J* = 7.0 Hz), 1.87–1.51 (m, 2H), 1.41 (m, 2H), 1.28 (t, 2H, *J* = 7.2 Hz), 1.22 (t, 2H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$): δ 173.3, 172.6, 138.1, 114.8, 71.2, 61.7, 60.7, 48.4, 33.4, 27.5, 26.6, 14.1. Anal. Calcd for $C_{13}H_{22}O_5$: C, 60.45; H, 8.58. Found: C, 60.23; H, 8.40.

3.2.5. (2*R*,3*S*)-3-(2-hydroxyethyl)malate (6). To a solution of **20** (420 mg, 1.20 mmol) in THF (5 ml) was added 10% aqueous sodium hydroxide (10.4 ml) and the mixture was stirred for 10 h at room temperature. The solution was evaporated to remove THF, and then Dowex 50W-X8 (H^+ form) was added to acidify until pH 3. The mixture was filtered and evaporated. The residue was chromatographed over silica gel (Merck silicagel 60-silanized, water). After evaporation of the residue, sodium hydroxide (82 mg, 2.05 mmol) was added to the residue and the solution was evaporated to give **6** as sodium salt (64 mg, 21%): IR (KBr): 3332, 1606 cm^{-1}; ^1H NMR (D_2O): δ 3.12 (d, 1H, *J* = 7.2 Hz), 3.49–3.39 (m, 2H), 2.38 (m, 1H), 1.70–1.53 (m, 2H); ^{13}C NMR (D_2O): δ 181.2, 180.0, 74.7, 60.0, 49.5, 31.7. Anal. Calcd for $C_6H_8O_6Na_2$: C, 32.45; H, 3.63. Found: C, 32.49; H, 3.81.

3.2.6. (2*R*,3*S*)-3-(3-hydroxypropyl)malate (7). Compound **7** was synthesized by the similar procedure described for the preparation of **6** (63%). IR (KBr): 3403, 1593 cm^{-1}; ^1H NMR (D_2O): δ 4.25 (d, 1H, *J* = 4.7 Hz), 3.44 (t, 2H, *J* = 6.4 Hz), 2.78 (m, 1H), 1.68–1.40 (m, 4H); ^{13}C NMR (D_2O): δ 181.4, 180.1, 74.6, 61.5, 52.2, 29.7, 25.4. Anal. Calcd for $C_7H_{10}O_6Na_2$: C, 35.61; H, 4.27. Found: C, 35.84; H, 4.51.

3.2.7. (2*R*,3*S*)-3-(4-Hydroxybutyl)malate (8). Compound **8** was synthesized by the similar procedure described for the preparation of **6** (76%). IR (KBr): 3386, 1602 cm^{-1}; ^1H NMR (D_2O): δ 3.86 (d, 1H, J = 7.2 Hz), 3.46 (t, 2H, J = 6.4 Hz), 2.37 (m, 1H), 1.30–1.50 (m, 4H), 1.21 (q, 2H, J = 7.2 Hz); ^{13}C NMR (D_2O): δ 182.0, 180.3, 75.0, 61.5, 52.9, 31.3, 28.9, 23.3. Anal. Calcd for $C_8H_{12}O_6Na_2$: C, 38.41; H, 4.84. Found: C, 38.16; H, 4.62.

3.2.8. (2*R*,3*S*)-3-Allylmalic acid (15). Compound **15** was synthesized from **23**[5] by the similar procedure described for the preparation of **6** (63%). IR (KBr): 2981, 1727 cm^{-1}; ^1H NMR (D_2O): δ 5.69 (dd t, 1H, J = 10.8, 14.4, 7.6 Hz), 5.02 (d q, 1H, J = 14.4, 1.0 Hz), 4.95 (d t, 1H, J = 14.4, 1.0 Hz), 4.18 (d, 1H, J = 4.4 Hz), 2.84 (d t, 1H, J = 4.4, 7.9 Hz), 2.31–2.36 (m, 1H), 2.17–2.29 (m, 1H); ^{13}C NMR (D_2O): δ 177.2, 176.5, 135.0, 117.3, 70.7, 49.0, 31.9. Anal. Calcd for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C, 47.99; H, 5.80.

3.2.9. (2*R*,3*S*)-3-(4-Pentenyl)malic acid (16). Compound **16** was synthesized by the similar procedure described for the preparation of **6** (79%). IR (KBr): 3417, 1589 cm^{-1}; ^1H NMR (D_2O): δ 5.75 (dd t, 1H, J = 10.0, 16.8, 6.4 Hz), 4.95–4.84 (m, 2H), 4.27 (d, 1H, J = 4.8 Hz), 2.79 (d t, 1H, J = 4.8, 7.2 Hz), 1.95 (q, 2H J = 6.8 Hz), 1.57–1.63 (m, 1H), 1.42–1.51 (m, 1H), 1.34 (q, 2H, J = 8.0 Hz); ^{13}C NMR (D_2O): δ 176.4, 176.1, 138.9, 114.6, 70.7, 59.8, 48.6, 39.1, 32.7, 26.8, 25.7. Anal. Calcd for $C_9H_{14}O_5$: C, 53.46; H, 6.98. Found: C, 53.23; H, 7.16.

3.2.10. (2*R*,3*S*)-3-Propylmalic acid (12). To a solution of **15** (44 mg, 0.25 mmol) in ethanol (0.5 ml) and H_2O (0.5 ml) was added Pd/C (10 mg), and the mixture was stirred for 6 hr at room temperature under a hydrogen atmosphere. The mixture was filtered and the filtrate was evaporated. The residue was chromatographed over silica gel (Merck silicagel 60 silanized) with water to afford **12** (40 mg, 89%). (KBr): 3399, 1655 cm^{-1}; ^1H NMR (D_2O): δ 4.09 (d, 1H, J = 5.6 Hz), 2.65 (d t, 1H, J = 5.6, 9.5 Hz), 1.37-1.56 (m, 2H), 1.28-1.18 (m, 2H), 0.78 (t, 3H, J = 7.4 Hz); ^{13}C NMR (D_2O): δ 178.6, 178.2, 72.2, 50.0, 30.0, 20.1, 13.2. $C_7H_{12}O_5$: C, 47.72; H, 6.81. Found: C, 47.99; H, 6.80.

3.2.11. Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-(3-hydroxypropyl)malate (25). To a solution of **21** (2.25 g, 5.98 mmol), 4-(dimethylamino)pyridine (146 mg, 1.20 mmol), and triethylamine (1.68 ml, 12.0 mmol) in CH_2Cl_2 (20 ml) was added acetic anhydride (0.84 ml, 8.96 mmol) at 0 °C. After 30 min, the reaction was quenched by addition of water at 0 °C. The mixture was extracted with ethyl acetate (four times) and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. The crude residue was chromatographed over silica gel with hexane–ethyl acetate (95/5) to afford acetylated product (2.22 g, 91%). To a solution of the product (1.10 g, 2.62 mmol) in ethanol (13 ml) was added pyridinium *p*-toluenesulfonate (394 mg, 1.57 mmol) at room temperature. After 10 h, the reaction was quenched by addition of $NaHCO_3$ aq at 0 °C. The mixture was extracted with ethyl acetate (four times) and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. The crude residue was chromatographed over silica gel with hexane–ethyl acetate (70/30) to afford **25** (794 mg, 96 %). IR (neat): 2938, 1754, 1733 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.20 (d, 1H, J = 5.7 Hz), 4.25–4.09 (m, 4H), 3.61 (t, 2H, J = 6.4 Hz), 2.91 (d t, 1H, J = 5.3, 8.6 Hz), 2.10 (s, 3H), 1.79–1.36 (m, 7H), 1.26 (t, 3H, J = 7.3 Hz), 1.23 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$): δ 171.2, 170.1, 168.5, 72.3, 62.2, 61.5, 60.9, 46.5, 32.2, 27.1, 23.3, 14.0, 14.0. Anal. Calcd for $C_{13}H_{22}O_7$: C, 55.25; H, 7.95; S, 7.95. Found: C, 55.19; H, 8.16.

3.2.12. Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-(3-azidopropyl)malate (26). To a solution of **25** (210 mg, 0.69 mmol), 4-(dimethylamino)pyridine (17 mg, 0.14 mmol), and triethylamine (210 μl, 2.10 mmol) in CH_2Cl_2 (2.3 ml) was added *p*-toluenesulfonyl chloride (197 mg, 1.03 mmol) at 0 °C. After 30 min at room temperature, the reaction was quenched by addition of water at 0 °C. The mixture was extracted with ethyl acetate (four times), and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. To a solution of the residue in DMF (2.3 ml) was added sodium azide (179 mg, 2.76 mmol) at room temperature. After stirring for 30 min at 40 °C, the mixture was filtered and the filtrate was evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (90/10) to afford **26** (203 mg, 89%): IR (neat): 2098, 1743 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.23 (d, 1H, J = 5.6 Hz), 4.28–4.12 (m, 4H), 3.28 (t, 2H, J = 6.8 Hz), 2.97–2.93 (m, 1H), 2.13 (3H, s) 1.65–1.57 (m, 1H), 1.54–1.37 (m, 3H), 1.29 (t, 3H, J = 7.1 Hz), 1.26 (t, 3H, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$): δ 170.8, 169.9, 168.2, 72.3, 61.6, 61.0, 51.0, 46.5, 28.7, 27.0, 24.5, 14.1, 14.1. Anal. Calcd for $C_{11}H_{19}N_3O_5$: C, 50.16; H, 7.37; N, 14.63. Found: C, 50.40; H, 7.12; N, 14.39.

3.2.13. (2*R*,3*S*)-3-(3-Aminopropyl)malate (18). To a solution of **26** (203 mg, 0.62 mmol) in ethanol (3 ml) was added Pd/C (10 mg). The mixture was stirred for 2 h at room temperature under hydrogen atmosphere. The mixture was filtered and the filtrate was evaporated. Ten percent aqueous sodium hydroxide (10.4 ml) was added to the residue and the mixture was stirred for 10 h at room temperature. After evaporation of the solvent, the residue was purified by silica-gel chromatography (Merck silicagel 60-silanized, water) and gel-filtration (CG-50, water) to afford **18** as sodium salt (80 mg, 61%): IR (KBr): 3301, 1611 cm^{-1}; ^1H NMR (D_2O): δ 3.82 (d, 1H, J = 6.0 Hz), 2.84 (t, 2H, J = 7.2 Hz), 2.33 (m, 1H), 1.15–1.58 (m, 4H); ^{13}C NMR (D_2O): δ 181.4, 180.0, 74.7, 52.4, 39.2, 28.3, 26.6. Anal. Calcd for $C_9H_{12}NO_5Na$: C, 39.44; H, 5.67; N, 6.57. Found: C, 39.61; H, 5.80; N, 6.79.

3.2.14. Diethyl (2*R*)-2-*O*-acetyl-3-{3-(*t*-butyldimethylsiloxy)propyl}-3-(phenylthio)malate (29). *n*-BuLi (30.1 ml, 46.4 mmol, 1.56 M in hexane) was added dropwise to a stirred solution of diisopropylamine (7.23 ml, 53.0 mmol) in 55 ml of anhydrous THF at −78 °C. After 30 min, a solution of **21** (8.01 g, 22.1 mol) in THF (5 ml) was added dropwise at −78 °C. The mixture was warmed

to −20 °C and stirred for 30 min. After cooling to −78 °C, phenyl benzenethiosulfonate (7.18 g, 28.7 mmol) was added, and the reaction mixture was warmed to 0 °C. After 5 min, acetic acid (6.0 ml) was added. After warmed to room temperature, the reaction mixture was poured into aqueous NH_4Cl (70 ml) and the mixture was extracted with diethyl ether (four times). The combined organic layer was washed with saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by flash column chromatography with hexane–ethyl acetate (92/8) to give a mixture (1:1) of two diastereoisomers (3.25 g, 31%). To a solution of the product (3.20 g, 6.80 mmol), 4-(dimethylamino)pyridine (166 mg, 1.36 mmol), and triethylamine (1.91 ml, 13.6 mmol) in CH_2Cl_2 (6.8 ml) was added acetic anhydride (0.95 ml, 10.2 mmol) at 0 °C. After 30 min at room temperature, the reaction was quenched by addition of water. The mixture was extracted with ethyl acetate (four times), and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. The crude was chromatographed over silica gel with hexane–ethyl acetate (95/5) to afford a mixture of two diastereoisomers of **29** (3.18 g, 91%): IR (neat): 2983, 1747, 1643 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.66–7.62 (m, 1H), 7.66–7.62 (m, 1H), 7.54–7.51 (m, 1H), 7.54–7.51 (m, 1H), 7.37–7.25 (m, 3H), 7.37–7.25 (m, 3H), 5.74 (s, 1H), 5.62 (s, 1H), 4.33–3.95 (m, 4H), 4.33–3.95 (m, 4H), 3.62–3.58 (m, 2H), 3.62–3.58 (m, 2H), 2.14 (s, 3H), 2.03–1.78 (m, 2H), 2.03–1.78 (m, 2H), 1.86 (s, 3H), 1.32 (t, 3H, J = 7.1 Hz), 1.24 (t, 3H, J = 7.1 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.13 (t, 3H, J = 7.1 Hz), 0.89 (s, 9H), 0.88 (s, 9H), 0.05 (s, 6H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 173.1, 173.0, 171.4, 170.9, 137.2, 137.2, 130.5, 130.3, 130.1, 129.9, 128.8, 128.5, 77.4, 77.3, 65.4, 64.3, 62.9, 62.7, 62.3, 62.3, 61.1, 61.0, 45.4, 46.1 30.0, 28.7, 27.5, 27.5, 20.2, 20.2, 18.4, 18.4, 14.0, 13.9, 13.7, 13.7, −5.5, −5.5. Anal. Calcd for $C_{25}H_{40}O_7SSi$: C, 58.56; H, 7.86; S, 6.25. Found: C, 58.53; H, 7.61; S, 6.51.

3.2.15. Diethyl (*R*,*E* and *R*,*Z*)-2-*O*-acetyl-3-{3-(*t*-butyldimethylsiloxy)propylidene}malate (30a and 30b). To a solution of **29** (396 mg, 0.770 mmol) in CH_2Cl_2 (3.1 ml) was added *m*-CPBA (214 mg, 0.71 mmol) at 0 °C. After stirring for 30 min, the reaction was quenched with aq $Na_2S_2O_3$. The mixture was neutralized by addition of aq $NaHCO_3$ and then the layers were separated. The aqueous layer was extracted with ethyl acetate (four times), and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was dissolved in toluene (30 ml) and the solution was warmed to 80 °C. After 5 h, the solution was evaporated and the residue was purified by flash silica gel chromatography with hexane–ethyl acetate (80/20) to afford **30a** (170 mg, 55%) and **30b** (114 mg, 37%): **30a**: IR (neat): 2970, 1747 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.21 (t, 1H, J = 7.4 Hz), 6.21 (s, 1H), 4.27–4.16 (m, 4H), 3.74 (t, 2H, J = 6.4 Hz), 2.66–2.50 (m, 2H), 1.29 (t, 3H, J = 7.1 Hz), 1.25 (t, 3H, J = 7.1 Hz), 0.89 (s, 9H), 0.05 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 170.0, 168.6, 143.3, 131.7, 62.2, 62.0, 61.8, 54.9, 32.0, 25.1, 14.1, −5.0. Anal. Calcd for $C_{19}H_{34}O_7Si$: C, 56.91; H, 8.51, Found: C, 56.40; H, 8.22. **30b**: IR (neat): 2998, 1744 cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.53 (t, 1H, J = 6.6 Hz), 5.87 (s, 1H), 4.24 (q, 2H, J = 7.1 Hz), 4.20 (q, 2H, J = 7.2 Hz), 3.73 (t, 2H, J = 6.3 Hz), 2.81 (q, 2H, J = 6.3 Hz), 1.30 (t, 3H, J = 7.1 Hz), 1.26 (t, 3H, J = 7.1 Hz), 0.89 (s, 9H), 0.05 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 172.8, 165.8, 144.1, 131.3, 66.7, 61.9, 61.5, 60.9, 32.0, 25.9, 14.1, −5.4. Anal. Calcd for $C_{19}H_{34}O_7Si$: C, 56.40; H, 8.51. Found: C, 56.40; H, 8.22.

3.2.16. (*R*,*E*)-(3-Hydroxypropylidene)malic Acid (27a). A mixture of **30a** (112 mg, 0.299 mmol) and 2 M HCl (1.2 ml) was stirred for 20 h at 50 °C. The solvent was evaporated and the crude product was chromatographed over silica gel (Merck silica gel 60-silanized, water) to give **27a** (51 mg, 90%): IR (KBr): 3570, 1798 cm^{-1}; ^1H NMR (D_2O): δ 7.00 (t, 1H, J = 7.6 Hz), 4.90 (s, 1H), 3.55 (t, 2H, J = 6.4 Hz), 2.41 (q, 2H, J = 6.4 Hz); ^{13}C NMR (D_2O): δ 174.4, 170.4, 144.2, 135.2, 66.3, 60.1, 38.9. Anal. Calcd for $C_7H_{10}O_6$: C, 44.21; H, 5.30. Found: C, 43.97; H, 5.56.

3.2.17. (*R*,*Z*)-(3-Hydroxypropylidene)malate (27b). Compound **27b** was synthesized by the similar procedure described for the preparation of **27a** (60% yield): IR (KBr): 3476, 1772 cm^{-1}; ^1H NMR (D_2O): δ 6.31 (t, 1H, J = 7.6 Hz), 4.78 (s, 1H), 3.55 (t, 2H, J = 6.4 Hz), 2.59 (q, 2H, J = 6.4 Hz); ^{13}C NMR (D_2O): δ 176.9, 169.5, 147.2, 132.2, 66.3, 60.6, 31.8. Anal. Calcd for $C_7H_8Li_2O_6$: C, 41.62; H, 3.99. Found: C, 41.20; H, 4.28.

3.2.18. Diethyl (2*R*)-2-*O*-acetyl-3-{3-(*t*-butyldimethylsiloxy)butyl}-3- (phenylthio)malate (31). Compound **31** was synthesized by the similar procedure described for the preparation of **29** (37% yield). IR (neat): 2980, 1760 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.64–7.62 (m, 1H), 7.64–7.62 (m, 1H), 7.53–7.51 (m, 1H), 7.53–7.51 (m, 1H), 7.37–7.25 (m, 3H), 7.37–7.25 (m, 3H), 5.71 (s, 1H), 5.61 (s, 1H), 4.33–4.09 (m, 4H), 4.33–4.09 (m, 4H), 3.63–3.58 (m, 2H), 3.63–3.58 (m, 2H), 2.13 (s, 3H), 1.98–1.78 (m, 4H), 1.98–1.78 (m, 4H), 1.86 (s, 3H), 1.51–1.48 (2H, m), 1.51–1.48 (2H, m) 1.32 (t, 3H, J = 7.1 Hz), 1.24 (t, 3H, J = 7.1 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.14 (t, 3H, J = 7.1 Hz), 0.89 (s, 9H), 0.88 (s, 9H), 0.05 (s, 6H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 169.6, 169.4, 169.4, 169.3, 130.1, 129.8, 129.6, 129.5, 128.5, 128.3, 76.6, 74.2, 62.8, 62.0, 61.9, 61.9, 61.8, 61.5, 60.8, 33.9, 33.5, 33.4, 26.0, 26.0, 21.3, 21.0, 20.6, 20.3, 14.3, 14.2, 14.1, 14.1, −5.2, −5.2. Anal. Calcd for $C_{26}H_{42}O_7SSi$: C, 59.28; H, 8.04; S, 6.09. Found: C, 59.48; H, 8.19; S, 6.30.

3.2.19. Diethyl (2*R*)-2-*O*-acetyl-3-{3-methoxycarbonylpropyl}-3-(phenylthio)malate (32). To a solution of **31** (1.26 g, 2.52 mmol) in ethanol (25 ml) was added pyridinium *p*-toluenesulfonate (256 mg, 1.00 mmol) at room temperature. After 10 h at room temperature, the reaction was quenched by addition of $NaCO_3$ aq at 0 °C. The mixture was extracted with ethyl acetate (four times). The combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (70/30) to afford a mixture (1:1) of

thiolated compounds (931 mg, 96%). To a solution of the product (920 mg, 2.39 mmol) in DMF (20 ml) was added pyridinium dichromate (3.14 g, 8.37 mmol). After 18 h at room temperature, the mixture was diluted with diethyl ether and Celite was added. The mixture was filtered and the filtrate was extracted with ethyl acetate (three times). The combined organic layer was dried over $MgSO_4$, filtered, and evaporated. The residue was dissolved in methanol (16 ml) and a solution of trimethylsilyldiazomethane (2.0 M in diethyl ether, 6.0 ml) was added at −78 °C. After 30 min at room temperature, the mixture was quenched by addition of acetic acid and concentrated. The residue was chromatographed over silica gel with hexane–ethyl acetate (80/20) to afford **32** (420 mg, 40%): IR (neat): 2983, 1747, 1643 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.64 (d, 1H, J = 6.8 Hz), 7.64 (d, 1H, J = 6.8 Hz), 7.52 (d, 1H, J = 6.8 Hz), 7.52 (d, 1H, J = 6.8 Hz), 7.40–7.30 (m, 3H), 7.40–7.30 (m, 3H), 5.75 (s, 1H), 5.58 (s, 1H), 4.32–3.97 (m, 4H), 4.32–3.97 (m, 4H), 3.67 (s, 3H), 3.67 (s, 3H), 2.33–2.29 (m, 2H), 2.33–2.29 (m, 2H), 2.17 (s, 3H), 2.10–2.00 (m, 2H), 2.10–2.00 (m, 2H), 1.95–1.84 (m, 2H), 1.95–1.84 (m, 2H), 1.90 (s, 3H), 1.34 (t, 3H, J = 7.1 Hz), 1.25 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz), 1.13 (t, 3H, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$): δ 173.0, 172.9, 169.2, 169.0, 168.8, 168.6, 166.8, 166.7, 137.6, 137.0, 129.5, 129.4, 129.3, 128.4, 128.2, 76.2, 73.8, 61.7, 61.7, 61.6, 61.3, 61.0, 60.3, 60.0, 51.3, 51.2, 34.1, 33.8, 33.2, 32.5, 20.8, 20.3, 20.0, 19.9, 14.0, 13.9, 13.7, 13.6. Anal. Calcd for $C_{21}H_{28}O_8S$: C, 57.26; H, 6.41; S, 7.28. Found: C, 57.39; H, 6.33; S, 7.09.

3.2.20. Diethyl (*R*,*E* and *R*,*Z*)-2-*O*-acetyl-3-{3-(methoxycarbonyl)propylidene}malate (33a and 33b). To a solution of **32** (396 mg, 0.770 mmol) in CH_2Cl_2 (3.1 ml) was added *m*-CPBA (214 mg, 0.71 mmol) at 0 °C. After 30 min, the reaction was quenched with aq $Na_2S_2O_3$. The mixture was neutralized by addition of aq $NaHCO_3$, and then the layers were separated. The aqueous layer was extracted with ethyl acetate (four times), and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was dissolved in toluene (30 ml) and the solution was warmed to 60 °C. After 5 h, the solution was evaporated and the residue was purified by flash silica-gel chromatography with hexane–ethyl acetate (80/20) to afford **33a** (170 mg, 55%) and **33b** (114 mg, 37%): **33a**: IR (neat): 2970, 1747 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.06 (t, 1H, J = 7.5 Hz), 6.40 (s, 1H), 4.27–4.18 (m, 4H), 3.70 (s, 3H), 2.79–2.60 (m, 2H), 2.51 (t, 2H, J = 7.2 Hz), 2.16 (s, 3H), 1.30 (t, 3H, J = 7.2 Hz), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$): δ 172.2, 169.7, 167.8, 164.9, 147.1, 128.1, 66.2, 61.5, 61.0, 51.6, 32.3, 23.8, 20.5, 13.9, 13.8. Anal. Calcd for $C_{15}H_{22}O_8$: C, 54.54; H, 6.71. Found: C, 54.36; H, 6.75. Compound **33b**: IR (neat): 2998, 1744 cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.40 (t, 1H, J = 7.3 Hz), 5.84 (s, 1H), 4.33-4.09 (m, 4H), 3.69 (s, 3H), 2.69 (m, 2H), 2.51 (t, 2H, J = 7.3 Hz), 2.18 (s, 3H), 1.31 (t, 3H, J = 7.2 Hz), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$): δ 172.7, 169.7, 168.0, 164.6, 147.0, 127.7, 72.5, 61.6, 60.9, 51.6, 32.8, 24.8, 20.6, 13.9, 13.9. Anal. Calcd for $C_{15}H_{22}O_8$: C, 54.54; H, 6.71. Found: C, 54.33; H, 6.42.

3.2.21. (*R*,*E*)-(3-Carboxypropylidene)malic acid (28a). To a solution of **33a** (16.0 mg, 0.056 mmol) in THF (0.3 ml) was added 10% aqueous solution of lithium hydroxide (0.6 ml), and the mixture was stirred for 18 h at room temperature. The solution was evaporated and the residue was purified by gel-filtration (CG-50, H^+ form) to give **28a** (10.5 mg, 88%): IR (KBr): 3476, 1772 cm^{-1}; ^1H NMR (D_2O): δ 6.94 (t, 1H, J = 7.2 Hz), 5.08 (s, 1H), 2.47–2.50 (m, 4H); ^{13}C NMR (D_2O): δ 176.8, 176.1, 168.8, 147.7, 130.6, 65.5, 32.3, 23.5. Anal. Calcd for $C_8H_{10}O_7$: C, 44.04; H, 4.62. Found: C, 44.15; H, 4.84.

3.2.22. (*R*,*Z*)-(3-Carboxypropylidene)malic acid (28b). Compound **28b** was synthesized by the similar procedure described for the preparation of **28a** (80% yield): IR (KBr): 3476, 1772 cm^{-1}; ^1H NMR (D_2O) δ 6.30 (t, 1H, J = 7.2 Hz), 4.73 (s, 1H), 2.66 (q, 2H, J = 7.2 Hz), 2.44 (t, 2H, J = 7.2 Hz); ^{13}C NMR (D_2O) δ 177.2, 175.5, 169.0, 146.1, 131.0, 72.6, 66.8, 32.7, 24.6. Anal. Calcd for $C_8H_{10}O_7$: C, 44.04; H, 4.62. Found: C, 43.80; H, 4.83.

3.3. Molecular cloning of ScHICDH encoding gene

The HICDH gene (*lys12*) was amplified by PCR from genome of *S. cerevisiae* with primer *lys12*-Nterm (5′-AAACATATGTTTAGATCTGTTGC-3′) and *lys12*-Cterm (3′-CCTATTATGAGCTCTATAATCTCG-5′). PCR conditions: 1 cycle at 94 °C for 7 min followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 68 °C for 1 min using KOD-plus DNA polymerase (TaKaRa). The introduced *Nde*I site (solid underline) and *Sac*I site (dashed underline) are underlined, respectively. The amplified fragments were digested with *Eco*RV and *Sac*I, and were cloned into LITMUS28 (New England Biolabs) to give LITMUS28-*lys12*. After the nucleotide sequence was verified, the *Nde*I–*Sac*I fragment of the resulting plasmid was subcloned into the corresponding site of pET30b(+) (Novagen) to give pET-*lys12*. The pET-*lys12* was transformed into *E. coli* Rosetta (TaKaRa) for overexpression.

3.4. Expression and purification of the recombinant ScHICDH

The *E. coli* Rosetta cells having pET-*lys12* were grown in LB medium supplemented with 30 μg/ml kanamycin and 100 μg/ml chloramphenicol. Culture was grown at 37 °C until OD_{600} reached 0.6, 0.3 mM isopropyl β-D-thiogalactoside was added, and the culture was continued at 32 °C for additional 5 h. The harvested wet cells (3.5 g) were suspended in 30 mM Tris–HCl (pH 8.5) and disrupted by sonication (5 min, five times), and debris was removed by centrifugation (15,000 rpm, 20 min). The supernatant fractions were applied onto an anion exchange column, DEAE F.F. (Amersham). The column was washed with 2.0 M NaCl, pre-equilibrated with 30 mM Tris–HCl (pH 8.5), and eluted with the buffer containing 1.0 M NaCl. The eluted fractions were concentrated by centrifugation with VIVASPIN concentrator (Ieda Trading Corporation, Tokyo) and applied onto a Hi Load 26/60 Superdex200 prep grade column

(Pharmacia Biotech) pre-equilibrated with 20 mM Tris–HCl (pH 8.0) containing 150 mM NaCl. Purity of the recombinant enzyme was verified by SDS–PAGE.

3.5. Assay of DraHICDH and ScHICDH

HICDH reaction was monitored by measuring the NADH absorption at 340 nm on a Shimadzu UV-160A UV–Vis spectrometer. Kinetic measurements were performed at 28 °C (for DraHICDH) or 36 °C (for ScHICDH) in an assay mixture (total 700 μl) containing enzyme, 0.2 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD^+, and homoisocitrate or substrate analog in 50 mM Hepes–NaOH (pH 7.8). In the assay of DraHICDH, the reaction was started by addition of the enzyme (0.1 μg) to the assay mixture. In the assay of ScHICDH, the reaction mixture with all required components except for NAD^+ was preincubated for about 3 min and the reaction was started by addition of NAD^+ to the mixture. The formation of NADH was measured for 30 s. Data were graphically analyzed by Lineweaver–Burk double reciprocal plots.

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