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# Thiahomoisocitrate: A highly potent inhibitor of homoisocitrate dehydrogenase involved in the α-aminoadipate pathway

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**Abstract**—Homoisocitrate dehydrogenase is involved in the α-aminoadipate pathway of L-lysine biosynthesis in higher fungi such as yeast and human pathogenic fungi. This enzyme catalyzes the oxidative decarboxylation of (2*R*,3*S*)-homoisocitrate into 2-ketoadipate using $NAD^+$ as a coenzyme. A series of aza-, oxa-, and thia-analogues of homoisocitrate was designed and synthesized as an inhibitor for homoisocitrate dehydrogenase. Among them, thia-analogue showed strong competitive inhibitory activity as $K_i$ = 97 nM toward homoisocitrate dehydrogenase derived from *Saccharomyces cerevisiae*. Kinetic studies suggested that the formation of the enolate intermediate played an important role in inhibition.


## 1. Introduction

In some of bacteria and higher fungi such as yeast and human pathogenic fungi as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, and plant pathogens like *Magnaporthe grisea*, L-lysine is biosynthesized via the α-aminoadipate pathway.[1] Since this pathway is unique for these organisms, enzymes involved in this pathway are considered to be a potential target for new antifungal drugs.[2] Homoisocitrate dehydrogenase (HICDH, EC 1.1.1.87) is involved in the fourth step of the α-aminoadipate pathway and catalyzes the conversion of homoisocitrate (HIC) to 2-ketoadipate through the oxidation of a hydroxy group by $NAD^+$, the subsequent decarboxylation, and the final protonation of enolate as shown in Figure 1.[1] We have been involved in designing substrate analogues and inhibitors for HICDH.[3] In this paper, we describe the first successful example of design and synthesis of a highly potent inhibitor for HICDH. Evaluation of the synthesized inhibitor is also described.

## 2. Results and discussion

### 2.1. Inhibitor design and synthesis

It is well known that substitution of a heteroatom such as sulfur and oxygen at the α-position of a ketone enhances the acidity of α-carbon and increases the stability of its enolate form.[4,5] We hence designed a potential inhibitor for homoisocitrate dehydrogenase by introduction of a heteroatom into the 3-position of HIC as shown in Figure 2. When such a compound is once accepted by HICDH and the enzyme reaction proceeds, the stability of the intermediary enolate (or enol) in the HICDH reaction would be increased by heteroatom substitution and the enolate intermediate might reside in the active site. Therefore, these compounds may act as an inhibitor of HICDH. Based on this idea, we designed and synthesized a series of aza-, oxa-, and thia-analogues **1**–**3** as a potential inhibitor for HICDH.

The preparation of **1**–**3** was straightforward and is depicted in Scheme 1. Aza-analogue **1** was obtained in 82% yield by treatment of glycine with epoxyfumarate under basic conditions. Synthesis of oxa-analogue **2** was achieved by treatment of diethyl *meso*-tartrate with methyl iodoacetate in the presence of silver (I) oxide, followed by alkaline hydrolysis to give **2** in 72% yield (2 steps). Thia-analogue **3** was prepared by the same procedure for synthesis of **1** using thioglycolate instead of glycine in 82% yield.

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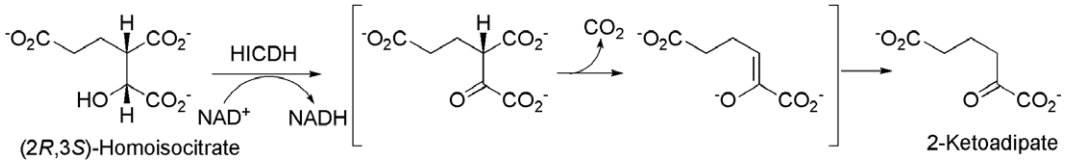



**Figure 1.** Reaction catalyzed by HICDH.

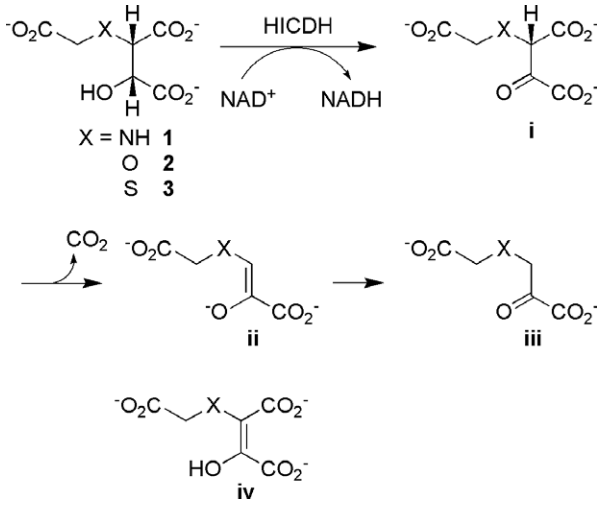
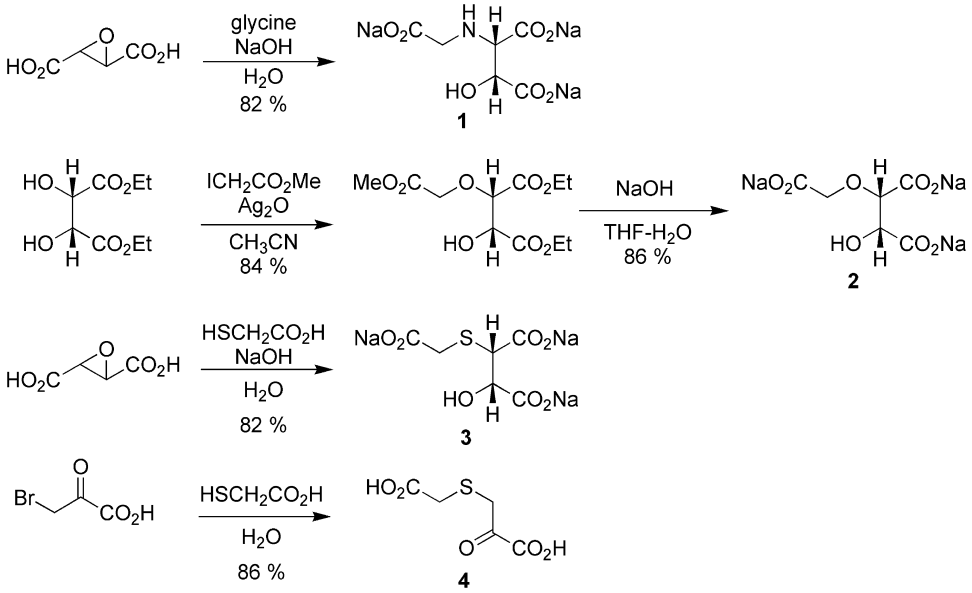



**Figure 2.** Heteroatom substituted analogues.

## 2.2. Inhibitory activity

The synthesized racemic analogues **1**–**3** were subjected to reaction with HICDH derived from *Saccharomyces cerevisiae* (ScHICDH).[3] HICDHs from *Saccharomyces* species are known to be highly homologous to fungal HICDHs in the amino acid level. The reactions were monitored by measuring the formation of NADH from $NAD^+$ as described previously,[3] and the reaction kinetics were analyzed by double reciprocal plots. As shown in Table 1, while aza-analogue **1** showed moderate substrate activity ($K_m$ = 150 μM, $k_{cat}$ = 2.0 $s^{-1}$), oxa- and thia-analogues (**2** and **3**) revealed very low substrate activity. Furthermore, oxa- and thia-analogues (**2** and **3**) were found to act as a strong competitive inhibitor against HIC in ScHICDH reaction, which indicates that they bind to the homoisocitrate binding site. Notably, thia-analogue **3** appeared to significantly inhibit the ScHICDH reaction ($K_i$ = 97 nM). This is the first successful example of design and synthesis of a highly potent inhibitor for ScHICDH. Since substitution by sulfur is known to be the most effective for stabilization of enolate rather than oxygen and nitrogen,[5] the inhibitory activities of **1**–**3** were in good agreement to ability for the stabilization effect as we anticipated. Similar inhibitors by heteroatom substitution were previously reported as 3-mercapto-2-ketoglutarate and 3-methylmercapto-2-ketoglutarate toward isocitrate dehydrogenase,[6] although details of the inhibition mechanism have not been discussed.

Since thia-analogue strongly inhibited the enzyme reaction, our attention was thus focused to which step of the ScHICDH reaction was slowed by this analogue. At first, the pre-steady state kinetic analysis of thia-analogue was performed. The enzyme reactions with thia-analogue were carried out in the presence of different concentrations of thia-analogue and a fixed concentration of ScHICDH and $NAD^+$ by a stopped-flow instrument. The two-phase reaction was observed under various concentrations of thia-analogues as shown in Figure 3. The rate of the NADH formation in the first




**Scheme 1.** Synthesis of compounds **1**–**4**.

**Table 1.** Kinetic data of synthetic compounds **1**–**4** and HIC

| | $K_i$ (μM) | $K_M$ (μM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| **1** | — | 150 | 2.0 |
| **2** | 10 | 20 | 0.59 |
| **3** | 0.097 | nd | nd |
| **4** | 12[a] | — | — |
| HIC | | 18[b] | 17[b] |

nd: these values were not determined under these conditions because of its low substrate activity.
[a] Uncompetitive inhibitor.
[b] Data from Ref. 3.

faster phase was found to be concentration dependent, while the rate of second slower phase was apparently independent of the concentration of thia-analogue. The formation of NADH in all reactions finally reached almost plateau at the same level. These results clearly indicated that the oxidation of thia-analogue by $NAD^+$ proceeded in a concentration dependent manner as usual and that inhibition of ScHICDH reaction by thia-analogue was caused by basically slow down after the oxidation by $NAD^+$, in which decarboxylation and protonation are involved.

When the enolate intermediate **ii** in Figure 2 plays an important role in the inhibition mechanism, the enzyme reaction product of thia-analogue would also inhibit HICDH reaction after keto–enol tautomerization. Thus, we examined inhibition activity of the plausible enzyme reaction product **4** as a product inhibitor. Compound **4** was synthesized by adding thioglycolate to a slightly excess of bromopyruvate as shown in Scheme 1,[8] and the reaction product was purified by ion-exchange chromatography. In a polar solvent such as acetonitrile and water, compound **4** exists as a mixture of enol- and keto-form in a 1:2 ratio in acetonitrile, which indicates that sulfur-substitution actually stabilizes its enol form. In the reaction of ScHICDH, compound **4** appeared to be a strong uncompetitive inhibitor ($K_i$ = 12 μM) at dif-

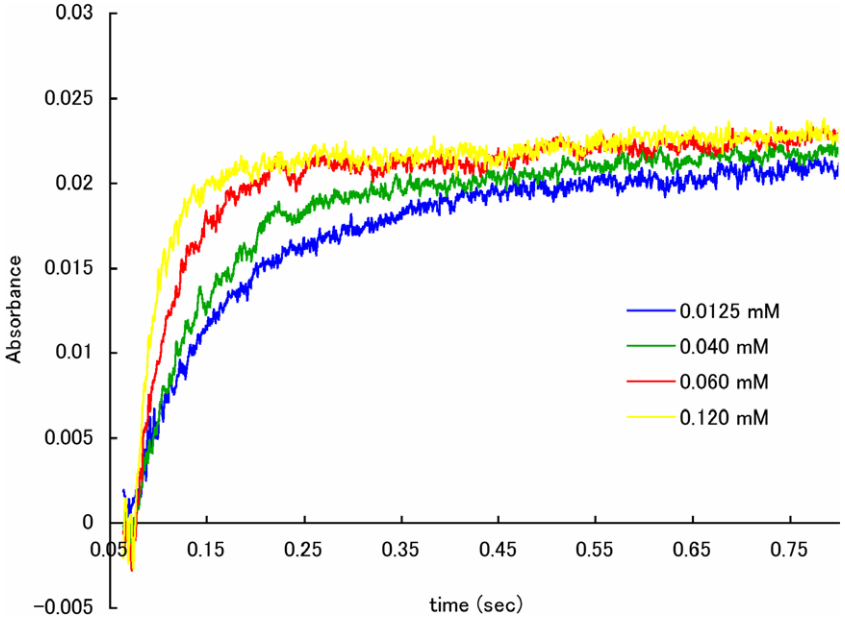



**Figure 3.** Time-course of the NADH formation in ScHICDH reaction; thia-analogue 12.5–120 μM, NAD 5.0 mM, ScHICDH 3.0 μM, 10 °C.

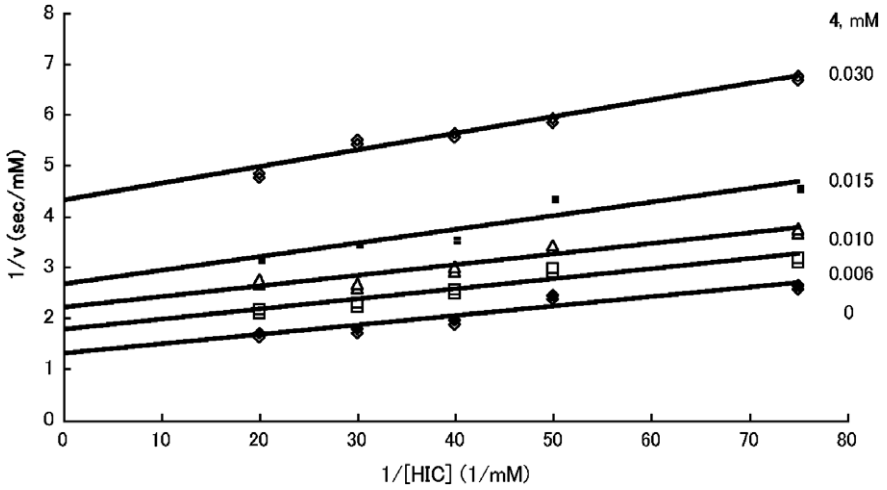



**Figure 4.** Lineweaver–Burk plot of the effect of compound **4** on ScHICDH reaction.

ferent concentrations of HIC and a fixed concentration of $NAD^+$ as shown in Figure 4. The same uncompetitive inhibition was observed at different concentrations of $NAD^+$ and a fixed concentration of HIC (data not shown). It was reported that 2-ketoadipate, the product of the original reaction, was shown to be an uncompetitive inhibitor for ScHICDH ($K_i$ = 5.8 mM),[7] so that 2-ketoadipate was suggested to be released from ScHICDH before NADH. Therefore, since compound **4** was found to be an uncompetitive inhibitor as 2-ketoadipate, the binding of the intermediary enolate **ii** to ScHICDH could participate in inhibition by thia-analogue. In the case that the binding of the enolate intermediate to ScHICDH is a predominant factor for inhibition of thia-analogue, the inhibition constant of thia-analogue is likely to be of a similar order of magnitude to that of compound **4**, however it was not the case. It seems less likely that the decarboxylation step is slowed in this case. With regard to another possible inhibition mechanism of thia-analogue, if the enol **iv** in Figure 2 is spontaneously formed from the intermediate **i** by the effect of the introduced heteroatom, this compound cannot be decarboxylated, thereby residing in the active site. Therefore, the possibility of spontaneous formation of the enol **iv** cannot be ruled out for the inhibition mechanism at the moment. In a preliminary test, thia-analogue did not affect growth of *S. cerevisiae* probably due to its low permeability into cells. Further derivatization and mechanistic analysis of thia-analogue is underway in our laboratory.

In conclusion, a series of aza-, oxa-, and thia-analogues of homoisocitrate was designed and synthesized as a potential inhibitor for HICDH. The synthesized thia-analogue showed strong competitive inhibitory activity toward homoisocitrate dehydrogenase. This is the first successful example of design and synthesis of a highly potent inhibitor for HICDH. The development of a highly potent inhibitor such as thia-homoisocitrate may provide a way to understand more detailed enzyme reaction mechanism of this class of enzymes.

## 3. Experimental

### 3.1. General

$^1H$ and $^{13}C$ NMR spectra were recorded on a JEOL LA-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, Merck). Enzyme reactions were monitored by measuring the NADH absorption at 340 nm on a Shimadzu UV-160 A UV–vis recording spectrometer. Pre-steady state kinetics was monitored on a Photal stopped-flow apparatus (Otsuka Electronics).

### 3.2. Inhibitory activity

Kinetic measurements were performed at 36 °C in an assay mixture (total 700 μl) containing 50 mM Hepes–NaOH (pH 7.8), 0.2 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM $NAD^+$. A reaction mixture including ScHICDH (0.1 μg) and HIC (5–50 μM) or the alternative analogues was pre-incubated for ca. 3 min and the reaction was started by addition of $NAD^+$ to the reaction mixture. The formation of NADH was measured at 340 nm for 5 s. Data were graphically analyzed by Lineweaver–Burk double reciprocal plots, and the kinetic parameters were estimated by Hanes plots or Dixon plots. Pre-steady state kinetic measurements were performed at 10 °C by mixing A and B components; component A, ScHICDH (3.0 μM) and thia-analogue **3** (12.5–120 μM), component B, 5.0 mM $NAD^+$.

### 3.3. Synthesis

**3.3.1. Aza-analogue 1.** To a solution of epoxyfumaric acid (20.1 mg, 0.152 mmol) in distilled water (1.0 ml) was added 0.2 ml of 3.0 M aqueous sodium hydroxide, followed by glycine (11.4 mg, 0.152 mmol). The mixture was stirred for 10 h at 80 °C. The solution was evaporated and the residue was chromatographed by gel-filtration (Sephadex G-10, water). Evaporation of the solvent gave sodium salt of **1** (31 mg, 82%): IR (KBr): 3469, 1616, 1396 $cm^{-1}$; $^1H$ NMR ($D_2O$) $\delta$ 3.08 (d, $J$ = 15.8 Hz, 1H), 3.15 (d, $J$ = 15.8 Hz, 1H), 3.33 (d, $J$ = 3.6 Hz, 1H), 4.05 (d, $J$ = 3.6 Hz, 1H); $^{13}C$ NMR ($D_2O$) $\delta$ 178.2, 178.1, 176.9, 73.1, 65.6, 50.7. Anal. Calcd for $C_6H_7NNa_2O_7{\cdot}3H_2O$: C, 23.62; H, 4.29; N, 4.59. Found: C, 23.83; H, 4.20; N, 4.48.

**3.3.2. Oxa-analogue 2.** To a solution of diethyl *meso*-tartrate (1.30 g, 6.30 mmol) and silver (I) oxide (4.38 g, 18.9 mmol) in dry $CH_3CN$ (2.6 ml) was added methyl iodoacetate (1.39 g, 6.94 mmol). The mixture was stirred for 20 h at 40 °C. The mixture was filtered and the filtrate was concentrated. The residue was chromatographed over silica gel (hexane/ethyl acetate = 3:7) to afford triester of **1** (1.60 g, 84%): IR (NaCl): 3094, 1754 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 1.24 (t, $J$ = 7.2 Hz, 3H), 1.26 (t, $J$ = 7.2 Hz, 3H), 3.72 (s, 3H), 3.84 (d, $J$ = 8.0 Hz, 1H), 4.13–4.16 (m, 5H), 4.36 (d, $J$ = 2.4 Hz, 1H), 4.43 (d, $J$ = 17.2 Hz, 1H), 4.64 (dd, $J$ = 2.4, 8.0 Hz, 1H); $^{13}C$ NMR ($CDCl_3$) $\delta$ 170.7, 170.3, 168.0, 81.1, 72.1, 67.7, 61.9, 61.5, 52.0, 14.0, 14.0. Anal. Calcd for $C_{11}H_{18}O_8$: C, 47.48; H, 6.52. Found: C, 47.73; H, 6.65.

To a solution of the obtained triester (600 mg, 2.16 mmol) in THF (5.0 ml) and water (7.0 ml) was added aqueous sodium hydroxide (3.0 M, 2.5 ml). The mixture was stirred for 3 h. The solution was evaporated to remove THF, and the residue was chromatographed by gel-filtration (Sephadex G-10, water). Evaporation of the solvent gave sodium salt of **1** (401 mg, 86%): IR (KBr): 3421, 1602, 1417 $cm^{-1}$; $^1H$ NMR ($D_2O$) $\delta$ 3.73 (d, $J$ = 15.2 Hz, 1H), 3.91 (d, $J$ = 2.8 Hz, 1H), 3.93 (d, $J$ = 15.2 Hz, 1H), 4.18 (d, $J$ = 2.8 Hz, 1H); $^{13}C$ NMR ($D_2O$) $\delta$ 177.8, 177.2, 176.0, 83.4, 73.5, 69.1. Anal. Calcd for $C_6H_5Na_3O_8{\cdot}3H_2O$: C, 21.96; H, 3.38. Found: C, 22.19; H, 3.44.

**3.3.3. Thia-analogue 3.** To a solution of epoxyfumaric acid (78.2 mg, 0.592 mmol) in distilled water (2.0 ml) was added 3.0 M of aqueous sodium hydroxide until

pH 12. Then, thioglycolic acid (54.5 mg, 0.592 mmol) was added. The mixture was stirred for 3 h at 80 °C. The solution was evaporated and the residue was chromatographed by gel-filtration (Sephadex G-10, 5 mM NaOH aq). Evaporation of the solvent gave sodium salt of **3** (140 mg, 82%): IR (KBr): 3442, 1596, 1396 $cm^{-1}$; $^1H$ NMR ($D_2O$) $\delta$ 3.22 (d, $J = 14.8$ Hz, 1H), 3.26 (d, $J = 14.8$ Hz, 1H), 3.43 (d, $J = 6.0$ Hz, 1H), 4.00 (d, $J = 6.0$ Hz, 1H); $^{13}C$ NMR ($D_2O$) $\delta$ 178.6, 177.4, 177.2, 73.9, 53.6, 37.1. Anal. Calcd for $C_6H_5Na_3O_7S{\cdot}3H_2O$: C, 20.94; H, 3.22; S, 9.32. Found: C, 20.93; H, 3.42; S, 9.06.

**3.3.4. Enzyme reaction product 4.** To a solution of 3-bromopyruvic acid (600 mg, 3.59 mmol) in acetone (7.0 ml) was added thioglycolic acid (191.9 ml, 2.76 mmol) at −78 °C. After the mixture was warmed to 0 °C, triethylamine (2.33 ml, 16.6 mmol) was added. The mixture was evaporated and the residue was chromatographed over ion-exchanged resin (DEAE Sephadex A-20, 0–2.0 M formic acid) to give **4** (460 mg, 86%): IR (KBr): 3093, 2979, 1691 $cm^{-1}$; $^1H$ NMR ($CD_3CN$) $\delta$ 6.42 (s, 1H), 3.78 (s, 2H), 3.51 (s, 2H), 3.25 (s, 2H); $^{13}C$ NMR ($CD_3CN$) $\delta$ 188.7, 171.1, 171.0, 163.6, 161.3, 138.7, 112.8, 37.6, 35.2, 33.8. Anal. Calcd for $C_5H_6O_5S$: C, 33.71; H, 3.39; S, 18.00. Found: C, 33.68; H, 3.68; S, 18.28.

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