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Synthesis of Conformationally Restricted Substrate Analogs and Their Interaction with 3-Isopropylmalate Dehydrogenase Derived from *Thermus thermophilus*

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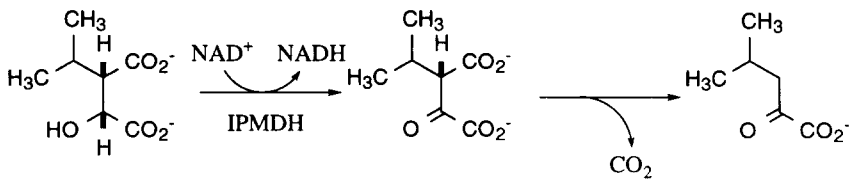
Abstract: The finding that 2-*O*-methyl-3-isopropylmalate was an uncompetitive inhibitor of 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85), involved in the rate-determining step in the biosynthetic pathway of the essential amino acid L-leucine, prompted to design conformationally restricted substrate analogs, in which the hydroxy oxygen is intramolecularly bound to an isopropyl carbon to form a ring structure. The oxirane **2** was the most inhibitory among those synthesized. IPMDH appeared to recognize preferentially the *anti*-conformation of the butanedioic acid structure.

Introduction

Mechanistic enzymology is among the major interests in chemical biology. We have been involved in the mechanistic and molecular recognition studies on *threo*-Ds-3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85), derived from the extremely thermophilic bacteria *Thermus thermophilus* HB8. IPMDH catalyzes an oxidation and decarboxylation reaction of (2*R*, 3*S*)-3-isopropylmalate (IPM) into 2-oxoisocaproate with the aid of NAD^+ in the penultimate step of the biosynthetic pathway of L-leucine (Scheme 1).[1-4]

The cryptic stereochemistry of the IPMDH reaction was already elucidated; *i. e.*, the hydride transfer from the C-2 position of IPM to NAD^+ is *pro-R* (A-site) specific, and the decarboxylation proceeds with retention of configuration at C-3 of the substrate.[5,6] These stereochemical features of IPMDH demonstrate significant similarity to those of *threo*-Ds-isocitrate dehydrogenase (ICDH, EC 1.1.1.42) functioning in the tricarboxylate cycle. Genetic comparison of IPMDH and ICDH also suggested an evolutionary relationship between the two enzymes.[7,8]



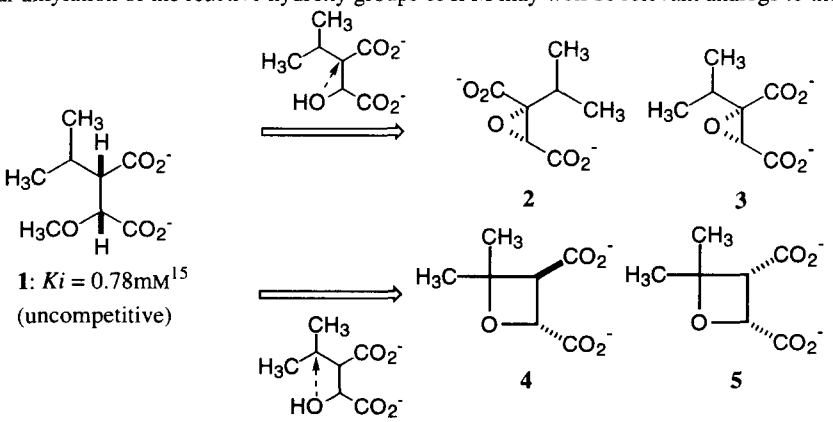
(2*R*, 3*S*)-IPM 2-oxoisocaproate

Scheme 1

X-ray analyses of IPMDH have been carried out for the crystals of various compositions. Recently, the crystals of the enzyme containing the NAD^+ cofactor and the ones containing the IPM substrate have been analyzed separately, and the NAD^+- and IPM-recognized regions of IPMDH have been elucidated by Hurley *et al.*, and Tanaka *et al.*, respectively.[8-10] However, even at this stage, precise features of the IPMDH-substrate interaction have not yet been clarified, because i) the conformation of the nicotinamide nucleoside was not fixed in the crystals of IPMDH with NAD^+, and ii) when the IPMDH crystals were subjected to soaking with IPM, the resulting complexed crystals with the substrate were reported to be formed only at low substrate concentration. It was also reported that, at high substrate concentrations, the enzyme crystals were destroyed during the soaking. Tanaka *et al.* argued with these observations that the enzyme conformation must be altered rather significantly upon substrate binding, and the crystal structure deduced with the soaked specimen at low substrate concentration may not represent the features of the native IPMDH-substrate complex. It is well-established that most of the enzymes utilizing a nicotinamide coenzyme bind the coenzyme first and the substrate binding is followed. Accordingly, in the complexation of IPMDH with the cofactor and the substrate, when the enzyme molecule captures the cofactor, significant conformational changes are induced to make the resulting enzyme-cofactor complex prone to accept the IPM substrate. At any rate, the structure of the ternary complex of IPM has yet to be elucidated.

Among other approaches to this end is to analyze the enzyme reaction and the complexed structure with highly potential inhibitors and/or with conformationally restricted substrate analogs. By these approaches, one may obtain appropriate crystals of enzyme-cofactor-inhibitor complex and may predict actual conformations of the substrate in the active site of enzyme. In this paper, we describe the latter approach.

We have been involved for quite some time in designing the substrate analogs and inhibitors for IPM.[13-16] One of the designed analogs, 2-*O*-methyl IPM (**1**), in which electrostatic interactions between the enzyme and the reactive hydroxy group of the substrate was reduced by methylation, turned out to be an uncompetitive inhibitor (Ki = 0.78 mM).[15] Actually, IPMDH is a homodimer (monomer, 36 Kd) having two active sites and only one site is active for catalysis. Apparently, the conformation of IPMDH is changed in the enzyme-substrate complex by binding one IPM at the active site of enzyme. 2-*O*-methyl IPM (**1**) may subsequently be bound to the other altered active site of the enzyme-substrate complex, and turns out to be an uncompetitive inhibitor. By extending this observation, the molecules containing a structure formed by intramolecular alkylation of the reactive hydroxy groups of IPM may well be relevant analogs to the substrate.



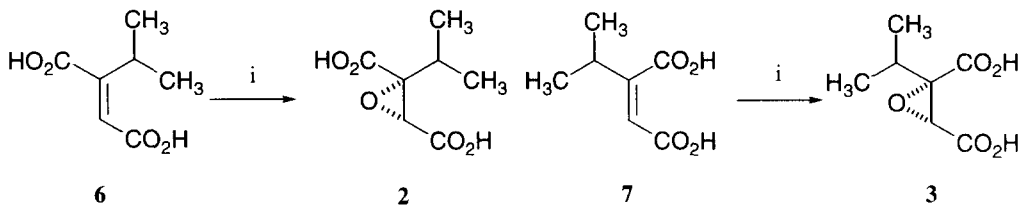
Scheme 2

The newly formed O-C bond can also play a key role in restricting the conformation of the analogs so that the crucial functional groups may be located in rather fixed orientation. Thus, the inhibitory activity of these analogs is an important clue to the structural features of the substrate in the active site.

Our design is illustrated in Scheme 2, in which oxirane (**2** and **3**) and oxetane (**4** and **5**) rings are introduced by making a bond from the oxygen at C-2 to an appropriate carbon. Our minor expectation with these ring systems was that a suitably-oriented functional group of the enzyme active site might react with these strained cyclic structures to hopefully form a covalent bond by nucleophilic ring opening.

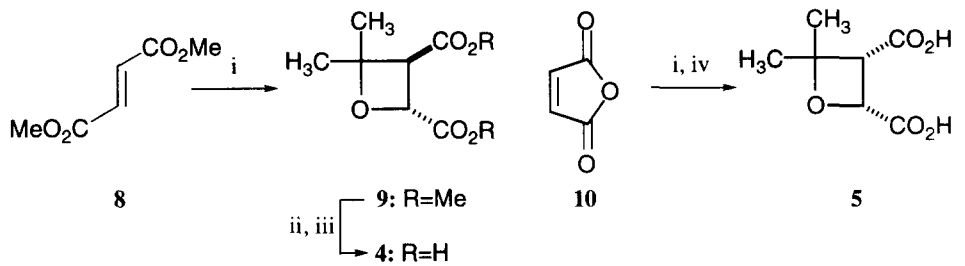
Results and Discussion

Since the early observation suggested that the enantiomer of IPM is neither a substrate nor inhibitor, the designed analogs were synthesized in racemic forms by well-established chemistry. The oxirane derivatives, **2** and **3**, were prepared from isopropylfumaric acid (**6**) and isopropylmaleic acid (**7**), respectively, by epoxidation, which involved a treatment of each diacid with aqueous H_2O_2 in the presence of 0.1 equivalent of sodium tungstate at pH 4-5.5. Heating at 90°C was required for completion of the reaction in both cases (Scheme 3).[17] The oxetanes, **4** and **5**, were prepared principally by [2+2] photocycloaddition (Scheme 4).[18] The intermediary ester **9**, prepared from acetone and dimethyl fumarate (**8**) by illuminating with a mercury lamp, was hydrolyzed with LiOH and the product was purified by ion-exchange resin chromatography to yield the *trans*-dicarboxylate **4**. The *cis*-counterpart **5** was prepared alternatively by photocycloaddition of acetone with maleic anhydride (**10**), followed by hydrolysis.



6 **2** **7** **3**

Scheme 3. Reagents: i, H_2O_2, $Na_2WO_4 \cdot 2H_2O$, NaOH



8 **10** **5**

Scheme 4. Reagents: i, hυ, acetone ; ii, LiOH; iii, Dowex 50W-X2 (H^+); iv, H_2O

These conformationally restricted cyclic substrate analogs were subjected to the enzyme reaction to test the potentials of these substrate analogs to be incorporated into the active site of IPM. Actually, their inhibitory

activities were studied against the natural IPM substrate employing a standard protocol of the IPMDH bioassay.[4] The reaction kinetics were analyzed by Lineweaver-Burk plot and the results are summarized in Table 1. In every case, inhibitory effect was observed against the formation of NADH. Both oxiranes **2** and **3** appeared to behave as competitive inhibitors, and the inhibition constant, *Ki*, were estimated to be 3.2 mM for **2** (Fig. 1) and 17 mM for **3.** Apparently, the inhibitory activity of these oxiranes depended on the orientation of the two carboxylate groups. Conversely, the oxetanes **4** and **5** appeared to behave as uncompetitive inhibitors. The *Ki* value for **4** was estimated to be 60 mM, and *Ki* for **5** was estimated to be 70 mM. Although the inhibitory activity of the oxetanes was not so potent as the oxiranes, the inhibition seemed to be independent on the orientation of the two carboxylates. Although the substrate analogs were inhibitors of moderate activity in comparison with *Km* for IPM (1.8 μM), significant difference was observed between the analogs in the inhibitory activity for IPMDH.

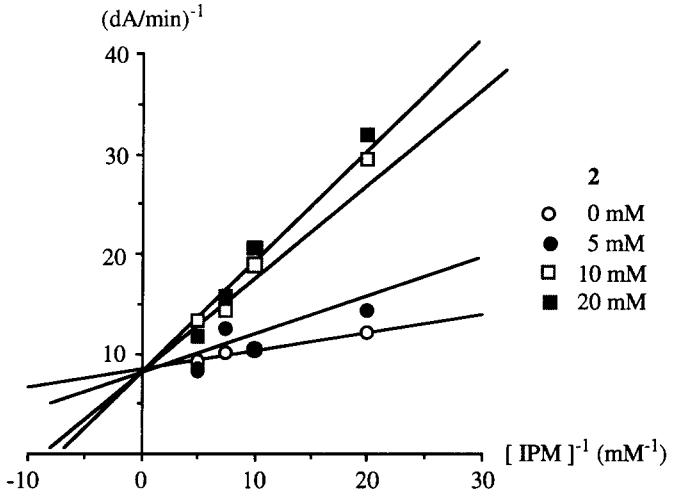
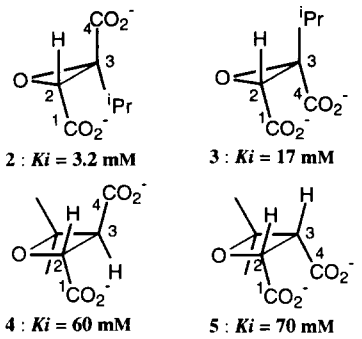


Fig. 1. Lineweaver-Burk plot of the effect of the oxirane **2** on IPMDH reaction.

To rationalize these observations and to elucidate the actual conformation of substrate in IPMDH, possible conformations of these four conformationally restricted substrate analogs were estimated by the molecular mechanics calculation with the CHARMm force field. The resulting dihedral angles between the two carboxylates of each analog are also shown in Table 1. The dihedral angle of the two carboxylates in the oxirane **2**, the most inhibitory among those studied, was estimated to be -172°, which is essentially identical with the dihedral angle (-165°) of the corresponding two carboxylates of isocitrate in the ICDH - isocitrate complex as determined by X-ray crystallographic analysis.[12] Therefore, it is conceivable that the configuration of the oxirane **2** mimics the conformation of IPM in the active site of IPMDH: *i. e.*, IPMDH preferentially recognizes an *anti*-conformation between the two carboxylate groups. Further, we have suggested recently that a conformation of isopropyloxalacetate that is the immediate product of the first oxidation step of the IPMDH reaction is closely similar to the preferred conformation in the enzyme active site for the subsequent decarboxylation reaction; that is, the bond between C-3 and C-4 is almost perpendicular to the plane of the C-2 carbonyl group.[16] The corresponding dihedral angle (O2-C2-C3-C4) of the oxirane **2** was estimated to be almost equal to 90° (Table 1). Therefore, the configuration of the oxirane **2** may also mimic the conformation of the isopropyloxalacetate intermediate in the enzyme active site.



	C1-C2-C3-C4	O2-C2-C3-C4
2	-172°	96°
3	-2°	-95°
4	-114°	125°
5	-6°	-129°

Table 1. The dihedral angles of C1-C2-C3-C4 and O2-C2-C3-C4 in the conformationally restricted substrate analogs were calculated by the molecular mechanics, CHARMm force field.

Although the oxirane analogs were not highly potent inhibitors, the mechanism of inhibition appeared to be competitive (Fig. 1). Therefore, the oxiranes may be incorporated properly into the active site of IPMDH. On the other hand, since the less potent oxetanes are uncompetitive inhibitors, the mode of interaction between the enzyme and the oxetanes is somewhat different. Further, the observation that the inhibition of the oxiranes was reversible may suggest either that the epoxide structure of **2** is not reactive enough to accept a nucleophilic group near by or that no suitable nucleophilic group is located in the active site. The result that the oxirane **2** was less inhibitory than **1** could be due to an inappropriate location of the oxygen atom of **2**, resulting in weak contact with the corresponding enzyme residues.

In conclusion, the oxirane **2** showed significant inhibitory activity against IPMDH among the conformationally restricted substrate analogs so far synthesized. By combining the results of molecular mechanics calculations, IPMDH is suggested to preferentially recognize the *anti*-conformation of the dicarboxylate system of the substrate. These results appear to be significant in elucidating the electrostatic interaction of IPMDH with IPM, metal ion, and the NAD^+ cofactor.

Experimental

Mps were measured with a Yanagimoto BY-1 micromelting point apparatus and are uncorrected. IR spectra were taken on a Hitachi 285 infrared spectrometer and/or a Horiba FT-710 fourier transform infrared spectrometer. ^1H and ^{13}C NMR were recorded on JEOL GSX-270, EX-270, LA-300, and/or JEOL EX-400 spectrometers. Deuteriochloroform (99.8% atom enriched, Kanto Chemicals or Merck), deuterium oxide (99.9% atom enriched, Kanto Chemicals or Merck), or dimethyl sulfoxide-d_6 (99.9% atom enriched, Kanto Chemicals) was used for the NMR solvent. ^1H and ^{13}C NMR chemical shifts were reported in δ-values based on internal $SiMe_4$ ($\delta_H = 0$) or dioxane ($\delta_C = 67.4$), or solvent signal ($CDCl_3$: $\delta_C = 77.0$; HOD: $\delta_H = 4.65$; DMSO-d_6: $\delta_H = 2.49$, $\delta_C = 39.5$) as reference. Column chromatography was carried out with Kieselgel 60 (70-230 mesh, Merck) or Lobar Fertigsaule RP-18 (40-63 mm, Merck). All reactions were carried out in an inert (Ar or N_2) atmosphere. Acetone was distilled from calcium sulfate.

(*2R, *3R**)-2,3-Epoxy-2-isopropylbutane-1,4-dioic acid (2)**

To a solution of isopropylfumaric acid[19] (3.38 g, 21.3 mmol) in water (30 ml), 15M aq. NaOH (2.1 ml) was added at 0 °C. $Na_2WO_4 \cdot 2H_2O$ (710 mg, 2.15 mmol) and 31% aq. H_2O_2 (2.3 ml, 23.3 mmol) were added to the mixture, which was then stirred at room temperature. After 30 min, the mixture was heated at 90 °C and was stirred for 18 h at the same temperature. The mixture was cooled to 0°C, and was acidified to pH 1 with 6M HCl. The reaction mixture was filtered and the filtrate was extracted three times with diethyl ether (total 500 ml). The combined extract was evaporated. The residue was recrystallized from water to give *title compound* **2** (1.07 g). The mother liquor was evaporated and the residue was chromatographed over Lobar RP-18 column with methanol-water (1:10) to yield additional *title compound* **2** (1.41 g) (total 2.48 g, 67%), mp 58-59°C; υ_{max}(KBr)/cm^{-1} 3520, 3050, 1740; δ_H(D_2O) 0.82 (d, J =7 Hz, 3H), 0.85 (d, J =7 Hz, 3H), 1.52 (septet, J =7 Hz, 1H) and 3.62 (s, 1H); δ_C (D_2O) 17.8, 18.4, 29.7, 58.5, 66.6, 170.9 and 171.8. *Anal.* Calcd. for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C. 47.99; H, 5.74.

(*2S, *3R**)-2,3-Epoxy-2-isopropylbutane-1,4-dioic acid (3)**

To a stirred solution of the crude isopropylmaleic acid[20] (1.72 g, 70% purity, containing 30% of isopropylfumaric acid) in water (10 ml), 15M aq. NaOH (1.0 ml) was added at 0 °C. $Na_2WO_4 \cdot 2H_2O$ (337 mg, 1.02 mmol) and 31% aq. H_2O_2 (1.0 ml, 10.1 mmol) were added and the mixture was stirred for 5.5 h at room temperature. The mixture was concentrated under reduced pressure and acetone was added to the residue. The resulting precipitate was collected to give solid (1.41 g). The solid (1.31 g) was dissolved in 15M aq. NaOH (0.25 ml) and acetone was added to the solution. The resulting precipitate was collected to yield the sodium salt of *title compound* **3** (1.52 g, 67%). A part of the sodium salt (285 mg) was dissolved in water. The solution was acidified to pH 1 with 6M HCl and was extracted three times with diethyl ether. The extract was dried over Na_2SO_4 and evaporated to give *title compound* **3** (137 mg), υ_{max}(neat)/cm^{-1} 2980, 1750; δ_H(D_2O) 0.74 (d, J =7 Hz, 3H), 0.81 (d, J =7 Hz, 3H), 1.85 (septet, J =7 Hz, 1H) and 3.56 (s, 1H); δ_C(D_2O) 17.2, 18.1, 31.5, 57.3, 69.6 and 171.4 (2C). *Anal.* Calcd. for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C. 48.02; H, 6.03.

(*3R, *4R**)-2,2-Dimethyl-3,4-dimethoxycarbonyloxetane (9)**

A solution of dimethyl fumarate (5.67 g, 39.3 mmol) in acetone (170 ml) was deoxygenated by refluxing and cooling in a stream of nitrogen, and was transferred to the irradiation apparatus under nitrogen. The solution was irradiated for 19 h using a 200 W Hanovia medium-pressure mercury arc at 0 °C. The solution was evaporated. The residue was chromatographed over silica gel with benzene-ethyl acetate (15:1-10:1) to give *title compound* **9** (880 mg, 11%), υ_{max}($CHCl_3$)/cm^{-1} 3000, 1740 ; δ_H($CDCl_3$) 1.39 (s, 3H), 1.58 (s, 3H), 3.58 (d, J =7 Hz, 1H), 3.77 (s, 3H), 3.81 (s, 3H) and 5.25 (d, J =7 Hz, 2H); δ_C($CDCl_3$) 24.5, 30.3, 51.4, 52.2, 52.4, 71.5, 83.8, 169.5 and 171.7.

(*3R, *4R**) -2,2-Dimethyl-3,4-dicarboxyoxetane (4)**

Lithium hydroxide monohydrate (43.5 mg, 1.04 mmol) was added to a stirred solution of compound **9** (92.5 mg, 0.46 mmol) in THF (1 ml) / water (0.5 ml), and the mixture was stirred for 10 min. The solution was evaporated to remove THF. Then, Dowex 50W-X2 (H^+-form) was added (to pH 3 by pH paper) and the mixture was filtered. The filtrate was evaporated to give *title compound* **4** (77.0 mg, 96%), υ_{max}(KBr)/cm^{-1} 3470, 3000, 1720; δ_H(D_2O) 1.20 (s, 3H), 1.32 (s, 3H), 3.44 (d, J =7 Hz, 1H) and 4.94 (d, J =7 Hz, 1H);

$\delta_C(D_2O)$ 24.5, 30.2, 52.8, 72.9, 85.9, 174.0 and 176.9. *Anal.* Calcd. for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C. 48.40; H, 5.98.

(*3S*, 4R) -2,2-Dimethyl-3,4-dicarboxyoxetane (5)**

A solution of maleic anhydride (3.50 g, 35.7 mmol) in acetone (175 ml) was deoxygenated by refluxing and cooling in a stream of nitrogen, and was transferred to the irradiation apparatus under nitrogen. The solution was irradiated for 19 h using a 200 W Hanovia medium-pressure mercury arc with water-cooling. The solution was evaporated to give a crude product (4.56 g). The residue (3.27 g) was chromatographed over Lobar RP-18 column with water and water-methanol (10:1) to yield *title compound* **5** (1.39 g, 22%), mp 73-75 °C; υ_{max}(KBr)/cm^{-1} 3480, 3000, 1720; $\delta_H(D_2O)$ 1.13 (s, 3H), 1.35 (s, 3H), 3.68 (d, *J*= 9 Hz, 1H) and 4.94 (d, *J* =9 Hz, 1H); $\delta_C(D_2O)$ 25.0, 29.5, 52.2, 71.9, 86.0, 172.9 and 175.9. *Anal.* Calcd. for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C. 48.24; H, 6.09.

Enzyme assay for the analogs

The thermophilic IPMDH derived from *T. thermophilus* HB-8 was prepared and purified as described previously.[4] IPMDH reaction was monitored by measuring the NADH absorption at 340 nm on a Shimadzu UV-160A UV-Visible recording spectrophotometer. Kinetic measurements were performed at 60 °C in an assay mixture (total volume 700 µl) containing 50 mM HEPES buffer (pH 7.8), 5 mM NAD^+, 5 mM $MgCl_2$ and 100 mM KCl. The reaction was started by addition of the enzyme (0.1 mg/ml, 7 µl) to the reaction mixture with all required components including IPM (50-200 µM) and the inhibitor (5-150 mM). The formation of NADH was measured for 1 min as described above. Data were graphically analyzed by Lineweaver-Burk double reciprocal plots.

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References

1. Oshima, T.; Imahori, K. *J. Syst. Bacteriol.*, **1974**, *24*, 102.
2. Tanaka, T.; Kawano, N.; Oshima, T. *J. Biochem. (Tokyo)*, **1981**, *89*, 677.
3. Kagawa, Y.; Nojima, H.; Nukiwa, N.; Ishizuka, M.; Nakajima, T.; Yasuhara, T.; Tanaka, T.; Oshima, T. *J. Biol. Chem.*, **1984**, *259*, 2956.
4. Yamada, T.; Akutsu, N.; Miyazaki, K.; Kakinuma, K.; Yoshida, M.; Oshima, T. *J. Biochem. (Tokyo)*, **1990**, *108*, 449.
5. (a) Yamada, T.; Kakinuma, K.; Oshima, T. *Chem. Lett.*, **1987**, 1745. (b) Yamada, T.; Kakinuma, K.; Endo, T.; Oshima, T. *Chem. Lett.*, **1987**, 1749.
6. Kakinuma, K.; Ozawa, K.; Fujimoto, Y.; Akutsu, N.; Oshima, T. *J. Chem. Soc., Chem. Commum.*, **1989**, *17*, 1190.
7. (a) Nakamoto, T.; Vennesland, B. *J. Biol. Chem.*, **1960**, *235*, 202. (b) England, S.; Listowsky, I. *Biochem. Biophys. Res. Commun.*, **1963**, *12*, 356.

8. Imada, K.; Sato, M.; Tanaka, N.; Katsube, Y.; Matsuura, Y.; Oshima, T. *J. Mol. Biol.*, **1991**, *222*, 725.
9. Hurley, J. H.; Dean, A. M. *Structure*, **1994**, *2*, 1007.
10. Kadono, S.; Sakurai, H.; Moriyama, H.; Sato, M.; Hayashi, Y.; Oshima, T.; Tanaka, N. *J. Biochem. (Tokyo)*, **1995**, *118*, 745.
11. Hurley, J. H.; Dean, A. M.; Sohl, J. L.; Koshland, Jr.,D. E.; Stroud, R. M. *Science*, **1990**, *249*, 1012.
12. (a) Hurley, J. H.; Dean, A. M.; Koshland, Jr., D. E.; Stroud, R. M. *Biochemistry*, **1991**, *30*, 8671. (b) Stoddard, B. L.; Dean, A. M.; Koshland, Jr., D. E. *Biochemistry*, **1993**, *32*, 9310.
13. Kakinuma, K.; Terasawa, H.; Li, H.-Y.; Miyazaki, K.; Oshima, T. *Biosci. Biotech. Biochem.*, **1993**, *57*, 1916.
14. Miyazaki, K.; Kakinuma, K.; Terasawa, H.; Oshima, T. *FEBS Lett.*, **1993**, *332*, 35.
15. Terasawa, H.; Miyazaki, K.; Oshima, T.; Eguchi, T.; Kakinuma, K. *Biosci. Biotech. Biochem.*, **1994**, *58*, 870.
16. Aoyama, T.; Eguchi, T.; Oshima, T.; Kakinuma, K. *J. Chem. Soc., Perkin Trans. 1*, **1995**, 1905.
17. Payne, G. B.; Williams, P. H. *J. Org. Chem.*, **1959**, *24*, 54.
18. Albone, E. S. *J. Am. Chem. Soc.*, **1968**, *90*, 4663.
19. Rydon, H. N. *J. Chem. Soc.*, **1936**, 829.
20. Eck, R.; Simon, H. *Tetrahedron*, **1994**, *50*, 13631.

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