

## Synthesis and reactivities of a novel flavoenzyme model, 5-deazaflavin with $C_2$ -symmetry

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**Abstract:** The title model compound represents a redox property of the active site of flavoenzymes and an environmental effect of the apoproteins. Its stereostructure was elucidated by <sup>1</sup>H-NMR spectra and energy minimum calculations. The stereoselectivity of this model was observed during the reaction with Me<sub>2</sub>PNPH, as NAD(P)H model.  
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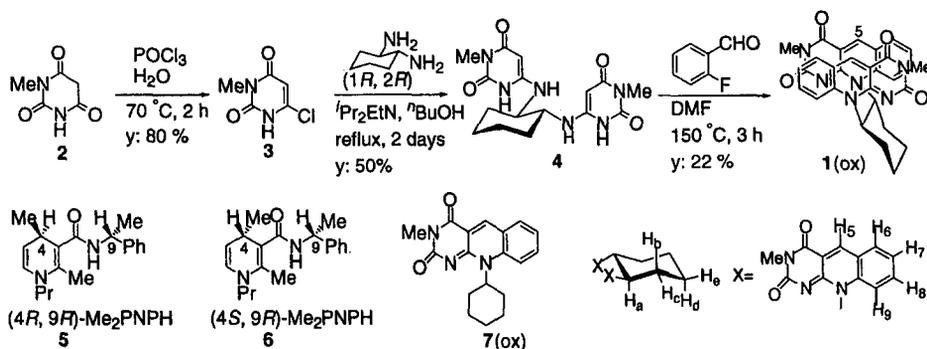
The flavin and 5-deazaflavin coenzymes play essential roles as redox agents in a wide variety of flavoenzymes.<sup>1</sup> These flavin coenzymes are covalently bound or tightly held to apoproteins at the active sites of flavoenzymes. The apoproteins have been accepted to enhance markedly the reactivity of flavin coenzymes and the stereoselectivity in the reactions with substrates by blocking one face of the flavin moiety to allow only the other face to interact with them. In order to clarify the above concept, we have synthesized several chiral 5-deazaflavins possessing the functions of apoproteins and investigated the redox reactivities as flavoenzyme models.<sup>2</sup> Now we have designed 3,3'-dimethyl-10,10'-[(1*R*,2*R*)-1,2-cyclohexylene]bis(pyrimido[4,5-*b*]quinoline-2,4(3*H*,10*H*)-dione) **1**(ox) with  $C_2$ -symmetry as a novel flavoenzyme model compound, in which two 5-deazaflavin (5-dFl) moieties are situated equivalently with each other because of the  $C_2$ -symmetry. It is predicted that one 5-dFl in **1**(ox) is blocked by the other 5-dFl and that an electron withdrawing effect of one 5-dFl would affect the reactivity of the other 5-dFl like the reactive face activation by apoproteins. We report here the stereoselectivity observed during the reaction of **1**(ox) with Me<sub>2</sub>PNPH as NAD(P)H model and the increased reactivity of one 5-dFl in **1**(ox) brought by the other 5-dFl.

Scheme 1 shows the synthetic route to **1**(ox). 1-Methylbarbituric acid **2** was treated with phosphorus trichloride and a small amount of hydrogen oxide to give 6-chloro-3-methyluracil **3**<sup>3</sup> in 80% yield. It was then treated with (1*R*,2*R*)-(-)-1,2-diaminocyclohexane in the presence of *N,N*-diisopropylethylamine in <sup>n</sup>BuOH under reflux to give compound **4** in 50% yield. The condensation reaction<sup>4</sup> of **4** and 2-fluorobenzaldehyde in DMF gave **1**(ox) in 22% yield.

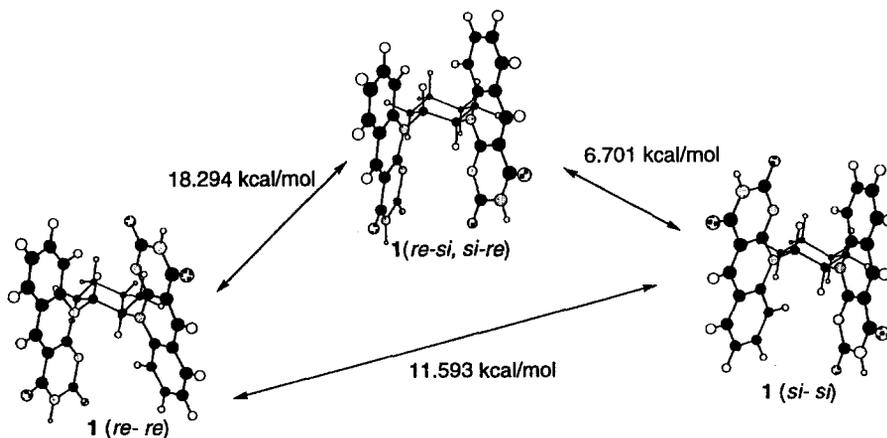
The structure of **1**(ox) was elucidated by NMR spectroscopic analysis and *ab initio* calculations. The <sup>1</sup>H-NMR spectrum of **1**(ox) is very simple, though it is a complex compound. It shows that **1**(ox) has a  $C_2$ -symmetrical structure because the starting material ((1*R*,2*R*)-(-)-1,2-diaminocyclohexane) has a  $C_2$ -symmetrical structure. The boat-type conformation of cyclohexane ring is excluded because it requires the plane of symmetry. The <sup>1</sup>H-NMR spectrum was fully assigned by correlation spectroscopy (H–H COSY) and nuclear Overhauser effect spectroscopy (H–H NOESY) (Scheme 1 and Experimental). The proton ( $\delta$  8.18) was elucidated to be H<sub>9</sub> of 5-dFl from the NOE between H<sub>a</sub> proton ( $\delta$  7.16, the root one of 5-dFl on cyclohexane ring) on NOESY. The vicinal coupling constant of H<sub>a</sub> and H<sub>b</sub> (the neighbor axial proton of H<sub>a</sub>) is  $J_{ab}$ =9.7 Hz. It shows that the relation between H<sub>a</sub> and H<sub>b</sub> is vicinal diaxial and then two 5-dFl groups in **1**(ox) occupy equatorial position on cyclohexane ring.

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Scheme 1.

Figure 1. Relative energies between configurations. They are shown in  $\Delta E(\text{RHF}/3\text{-}21\text{g}/\text{RHF}/\text{PM}3)$ .

There can be three possible stereoisomers for **1(ox)** because of rotational hindrance of two big 5-dFls. That is, the combinations of the open faces at the C(5) positions of 5-dFls in **1(ox)** are *re*- and *re*-faces (*re-re*), *si*- and *si*-faces (*si-si*), and *re*- and *si*-faces (*re-si*) (*si-re* is the same as *re-si*) (Figure 1). Because **1(ox)** has a  $C_2$ -symmetrical structure, it has *re-re* or *si-si* faces. No temperature dependence of the  $^1\text{H-NMR}$  spectra was observed ( $-50$ – $+60^\circ\text{C}$ ). The stereostructure of **1(ox)** was analyzed by molecular modeling. If **1(ox)** has *re-re* faces, the distance between  $\text{H}_9$  and  $\text{H}_a$  is about 1.5 Å. If **1(ox)** has *si-si* faces, the distances between  $\text{H}_9$  and  $\text{H}_a$  or  $\text{H}_9$  and  $\text{H}_b$  are both about 1.4 Å. The NOE between  $\text{H}_9$  and  $\text{H}_a$  was observed clearly on NOESY but that between  $\text{H}_9$  and  $\text{H}_b$  was not observed. It implies that **1(ox)** has *re-re* faces.

The energy minimum calculations supported that **1(ox)** had *re-re* faces. This configuration was predicted to have approximately 11.6 and 18.3 kcal mol $^{-1}$  lower in energy than *si-si* and *re-si* face configuration of **1(ox)**. They were located by full optimizations with RHF/PM3 method, followed by a single-point energy calculations at the RHF/3-21G level $^5$  of theory (Figure 1 and Table 1).

The reactivity of **1(ox)** was compared with that of 10-cyclohexyl-3-methyl-5-deazaflavin **7(ox)** during the (net) hydride transfer reactions with chiral NAD(P)H models, (4*R*,9*R*)-Me $_2$ PNPH **5** and (4*S*,9*R*)-Me $_2$ PNPH **6**. $^6$  The reaction of **1(ox)** or **7(ox)** (0.01 mmol) with **5** (0.01 mmol) and **6** (0.01 mmol) in  $\text{CDCl}_3$  (0.6 ml) under an argon atmosphere was followed by  $^1\text{H-NMR}$  (Figure 2). Figure 2 shows that **1(ox-red)** (only one of two 5-dFl groups was reduced) was produced more rapidly than **7(red)**. It means that two 5-dFl groups in **1(ox)** are interacted with each other and the oxidation ability of one 5-dFl is increased by the other 5-dFl. The consumption of **5** and **6** was analyzed by  $^1\text{H-NMR}$ .

Table 1. Calculated relative energies of configurations<sup>a</sup>

dFl <b>1</b> (ox)	RHF/3-21g//RHF/PM3 <sup>b</sup>	
	Calculated energy <sup>c</sup>	Relative energy <sup>d</sup>
<b>1</b> ( <i>re-re</i> )	-1 689.8688	0.000
<b>1</b> ( <i>si-si</i> )	-1 689.8503	11.593
<b>1</b> ( <i>si-re, re-si</i> )	-1 689.8397	18.294

<sup>a</sup>The character of all energy minima was confirmed with calculation of force constant analyses at the Hartree-Fock level.

<sup>b</sup>Computed at the RHF/3-21g//RHF/PM3 optimized geometries. <sup>c</sup>Hartree. <sup>d</sup>kcal mol<sup>-1</sup>.

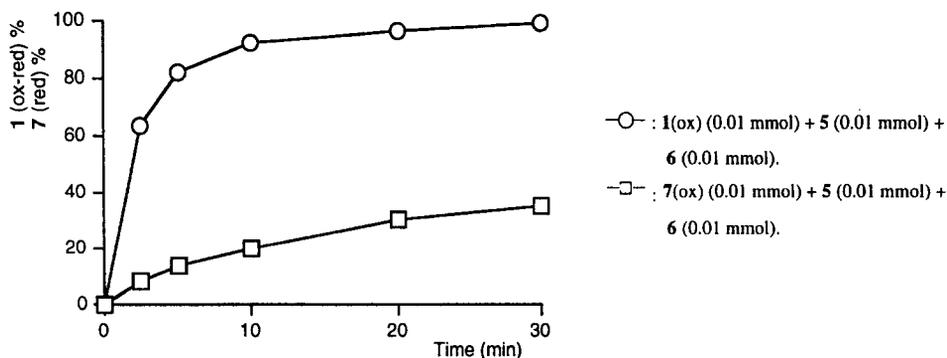


Figure 2. The reaction of **1**(ox) or **7**(ox) with NAD(P)H models, **5** and **6**.

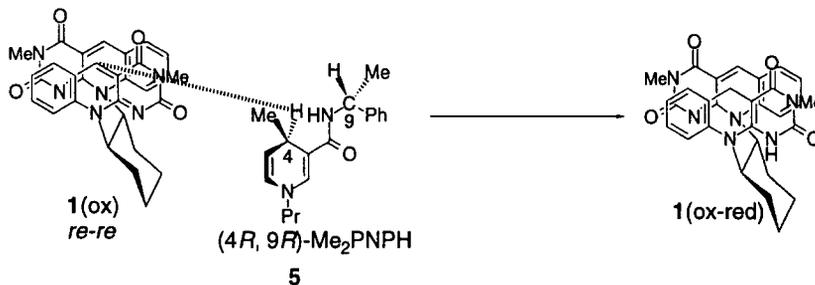


Figure 3.

The methyl doublets at C(4)-Me of **5** and **6** were used for this purpose. When **1**(ox) was used, **5** and **6** remained in **7** and 93% after 30 min respectively. When **7**(ox) was used, **5** and **6** remained in 51 and 49%. These show that one face of the C(5) reaction center of 5-dFl is actually blocked by the other 5-dFl as for the apoprotein and that the *re* faces of **1**(ox) recognize predominantly **5**.<sup>7</sup> It has been found in our laboratory that the pyrimidine moiety of 5-dFl interacts with the carbonyl group of NAD(P)H model in the transition state of the (net) hydride transfer reaction.<sup>8</sup> A transition state of the reaction of **1**(ox) and **5** has been proposed as depicted in Figure 3. It should be noted that the transition state is similar to the stacking mode between NADPH and FAD at the active site of glutathione reductase determined by X-ray crystallography.<sup>9</sup>

In conclusion, C<sub>2</sub>-symmetrical **1**(ox) was synthesized as a novel flavoenzyme model.<sup>10</sup> It has *re-re* faces at the C(5) active center of 5-dFls and oxidized (4*R*,9*R*)-Me<sub>2</sub>PNPH **5** selectively and the reaction rate of **1**(ox) was faster than 5-dFl monomer **7**(ox). Compound **1**(ox) is thought to be an advanced flavoenzyme model because one 5-dFl in **1**(ox) would work just as a 'wall' in the transition state to shield one side of the other 5-dFl and an electron withdrawing effect of one 5-dFl would affect the reactivity of the other 5-dFl similar to the environmental effect of the apoproteins.

## Experimental

Melting points were determined on YANAGIMOTO micromelting point apparatus and are uncorrected. The NMR spectra were recorded on a GE NMR OMEGA 600 instrument at 600 MHz, JEOL 270 and 200 MHz using tetramethylsilane as an internal reference. High- and low-resolution mass spectra were recorded on a JEOL JMS-01SG-2 or JMS-HX/HX 110A mass spectrometer. Optical rotations were measured in CHCl<sub>3</sub> with a JASCO DIP-360 digital polarimeter. The UV spectra were obtained with SHIMADZU UV-2100 spectrophotometer.

### *3,3'-Dimethyl-6,6'-[(1R,2R)-1,2-cyclohexylenediamino]dipyrimidine-2,4(1H,3H)-dione 4*

6-Chloro-3-methyluracil **3** was synthesized by a modification of the general procedure reported by Bruce and co-workers.<sup>3</sup> Hydrogen oxide (0.57 g, 31.67 mmol) was added dropwise to 3-methylbarbituric acid **2** (3 g, 21.13 mmol) and phosphorus trichloride (32 g, 208.74 mmol) at 0°C. The mixture was heated at 80°C for 4 h. After cooling, the mixture was evaporated to remove phosphorus trichloride, and then the residue was washed with a small amount of methanol. Recrystallization of the residue from methanol gave **3** as white powder (2.72 g, 80%). The mixture of **3** (8.4 g, 52 mmol), (1R,2R)-(-)-1,2-diaminocyclohexane (3 g, 26 mmol), and *N,N'*-diisopropylethylamine (10 g, 78 mmol) in *n*BuOH (20 ml) was refluxed for 2 days. After cooling, the solid of the reaction mixture was filtered, and then it was washed with methanol. Recrystallization from methanol gave **4** as pale yellow needles (4.71 g, 50% yield). m.p. >300°C. <sup>1</sup>H-NMR (200 MHz, *d*<sub>6</sub>-DMSO) δ: 1.37 (m, 4H), 1.67 (m, 2H), 1.96 (m, 2H), 3.04 (s, 6H, 2×Me), 3.20 (m, 2H), 4.73 (s, 2H, 2×C<sub>5</sub>H), 5.98 (br. s, 2H, 2×NH), 10.10 (br. s, 2H, 2×NH). MS (EI) *m/z*: 362 (M<sup>+</sup>).

### *3,3'-Dimethyl-10,10'-[(1R,2R)-1,2-cyclohexylene]bis(pyrimido[4,5-b]quinoline-2,4(3H,10H)-dione) 1(ox)*

A mixture of **4** (2g, 5.52 mmol) and 2-fluorobenzaldehyde (1.53 g, 12.34 mmol) in *N,N*-dimethylformamide (DMF) (8 ml) was refluxed for 3 h and then cooled. The reaction mixture was evaporated to remove DMF, then the residue was washed with ethanol. Recrystallization of the residue from ethanol afforded pale yellow needles **1(ox)** (657 mg, 22% yield). m. p. 285–286°C. Anal. Calcd for C<sub>30</sub>H<sub>26</sub>N<sub>6</sub>O<sub>4</sub>: C, 67.41; H, 4.90; N, 15.72. Found: C, 67.42; H, 4.71; N, 15.89. <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ: 1.94 (m, 2H, 2×H<sub>d</sub>), 2.09 (m, 2H, 2×H<sub>e</sub>), 2.16 (m, 2H, 2×H<sub>c</sub>), 3.12 (s, 6H, 2×Me), 3.23 (m, 2H, 2×H<sub>b</sub>), 7.16 (d, *J*<sub>ab</sub>=9.7 Hz, 2H, 2×H<sub>a</sub>), 7.36 (dd, *J*<sub>6,7</sub>=8.1, *J*<sub>7,8</sub>=8.9 Hz, 2H, 2×H<sub>7</sub>), 7.66 (dd, *J*<sub>6,7</sub>=8.1, *J*<sub>6,8</sub>=1.6 Hz, 2H, 2×H<sub>6</sub>), 7.88 (ddd, *J*<sub>7,8</sub>=8.9, *J*<sub>8,9</sub>=9.3, *J*<sub>6,8</sub>=1.6 Hz, 2H, 2×H<sub>8</sub>), 8.18 (d, *J*<sub>8,9</sub>=9.3 Hz, 2H, 2×H<sub>9</sub>), 8.49 (s, 2H, 2×H<sub>5</sub>). UV(CHCl<sub>3</sub>) λ<sub>max</sub> (ε): 264 (57600), 324 (17200), 402 (15600), 425 (11100) nm. MS *m/z*: 534.2013 (Calcd for C<sub>30</sub>H<sub>26</sub>N<sub>6</sub>O<sub>4</sub>: 534.2018). [α]<sub>D</sub><sup>25</sup>=−676 (*c*=1.00, CHCl<sub>3</sub>). The enantiomeric excess (ee) was determined by using HPLC column (Chiralcel OD, Daicel, ethanol) and found to be >99%.

### *10-Cyclohexyl-3-methylpyrimido[4,5-b]quinoline-2,4(3H,10H)-dione 7(ox)*

Compound **7(ox)** was synthesized newly by a modification of the general procedure by Yoneda and co-workers.<sup>11</sup> A mixture of **3** (1 g, 6.30 mmol) and cyclohexylamine (3 ml) was heated at 140°C for 1 h and then cooled. The solidified melt was triturated in methanol and the crude material was collected by filtration. Recrystallization from methanol gave 6-(cyclohexylamino)-3-methyluracil (1 g, 72% yield). The mixture of 6-(cyclohexylamino)-3-methyluracil (2 g, 9.01 mmol) and 2-fluorobenzaldehyde (1.4 ml) was heated at 130°C for 18 h and then cooled. The solidified melt was triturated in methanol and the crude material was collected by filtration. Recrystallization from methanol gave **7(ox)** as pale yellow needles (1.75 g, 63% yield). m. p. 260–262°C. Anal. Calcd for C<sub>18</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub>: C, 69.88; H, 6.19; N, 13.58. Found: C, 69.70; H, 6.29; N, 13.38. <sup>1</sup>H-NMR (270 MHz, CDCl<sub>3</sub>) δ: 1.55–2.05 (m, 10H), 3.48 (s, 3H, Me), 6.35–6.70 (m, 1H), 7.45 (t, *J*=7.9 Hz, 1H), 7.81 (t, *J*=7.9 Hz, 1H), 7.90 (dd, *J*=7.9 and 1.7 Hz, 1H), 8.15 (d, *J*=7.9 Hz, 1H), 8.87 (s, 1H). UV(CHCl<sub>3</sub>) λ<sub>max</sub> (ε): 267 (42400), 322 (8200), 405 (10100), 426 (8200) nm. MS *m/z*: 309.1479 (Calcd for C<sub>18</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub>: 309.1477).

*The reaction of 5-dFl, 1(ox) or 7(ox) with NAD(P)H models, 5 and 6*

The mixture of **1(ox)** or **7(ox)** (0.01 mmol) with **5** and **6** (each 0.01 mmol) in CDCl<sub>3</sub> (0.6 ml) was put into NMR tube. The reaction was carried out at 27°C under argon. It was followed by <sup>1</sup>H-NMR (270 MHz). The yields of **1(ox-red)** were measured by the <sup>1</sup>H-NMR integration of H<sub>5</sub> of **1(ox-red)**. This proton (δ 8.49) of **1(ox-ox)** was gradually decreased and the proton (δ 8.60) of **1(ox-red)** was increased. The yields of **7(red)** were measured by the <sup>1</sup>H-NMR integration of N-Me proton. This proton (δ 3.48) of **7(ox)** was gradually decreased and the proton (δ 3.56) of **7(red)** was increased.

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