Racemization Catalyst for Amino Acid. IV* Enantiomer Differentiating Racemization with (*S*)-2′-Nitro-5-nitroso-6,6′-dimethylbiphenyl-2-ol

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The racemizations of both enantiomers of alanine were carried out with (*S*)-2′-nitro-5-nitroso-6,6′-dimethylbiphenyl-2-ol (**1**) in the presence of cupric ion. In time course studies, the apparent half-life of D-alanine is longer than that of L-alanine. Such an enantiomer differentiating ability is explained by a mechanism involving a reactive product formed from the preferred conformation of the diastereomeric cupric complexes, consisting of **1** and D- or L-alanine, respectively. Synthesis of **1** *via* the resolution of 2′-nitro-6,6′-dimethylbiphenyl-2-amine is also presented.

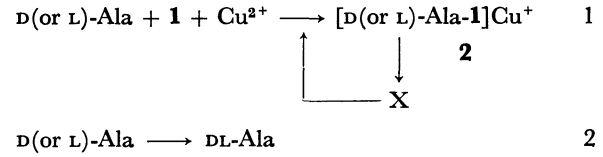
Amino acid racemases are known to catalyze the racemization of both D- and L-amino acids.[1] Studies of the enantiomer diferentiating ability of the racemases are particularly interesting, for they could provide significant stereochemical information regarding the formation of enzyme-substrate complex.

Lambert *et al.*[2] investigated the rate of racemization of alanine with racemase and found that the initial racemization rate for L-alanine is faster than that for D-alanine and that the kinetic parameters of the reaction, which would reflect the stereochemical properties of the enzyme-substrate complex, differ significantly between D- and L-alanine, regardless of the invariability of thermodynamic parameters.

Apart from the enzymatic studies, we have attempted to prepare a synthetic racemization catalyst with a chiral structure, assuming that if there are stereochemical interactions between substrate and catalyst during the reaction process, analogous to the enzyme-substrate complex in enzymatic system, the chiral catalyst should exhibit an enantiomer differentiation against the chiral substrate.

Thus, (*S*)-2′-nitro-5-nitroso-6,6′-dimethylbiphenyl-2-ol (**1**), which contains 4-nitrosophenol structure in the hindered biphenyl, has been synthesized and its enantiomer differentiating ability toward D- and L-alanine has been confirmed.[3]

The mechanism of racemization of alanine with 4-nitrosophenol derivatives has also been presented on the basis of kinetic and stereochemical studies.[4] The proposed reaction pathways are as follows:



1

D(or L)-Ala → DL-Ala

2

Ala: Alanine. X: Unidentified compound.

That is, 4-nitrosophenol derivatives are decomposed into an unidentified compound (X) *via* the cupric complex (**2**) consisting of 4-nitrosophenol and alanine, the resulting X then catalyzes both reactions 1 and 2. The induced period was regarded as the time necessary to accumulate sufficient concentration of X through the decomposition of **2**, according to the reaction mechanism shown in reaction 1.

Thus, the time course of the reaction, which shows a sigmoid curve and also the effect of steric bulk of substituents surrounding the hydroxyl group of the catalyst on the induced period of the reaction, is explained by this mechanism. In the present paper, the origin of an enantiomer differentiation in the racemization process with **1** is discussed from the view point that the racemization proceeds *via* the mechanism shown in reactions 1 and 2.

Results and Discussion

Racemizations of D- and L-alanine were carried out in the presence of **1** and cupric ion. The resulting time

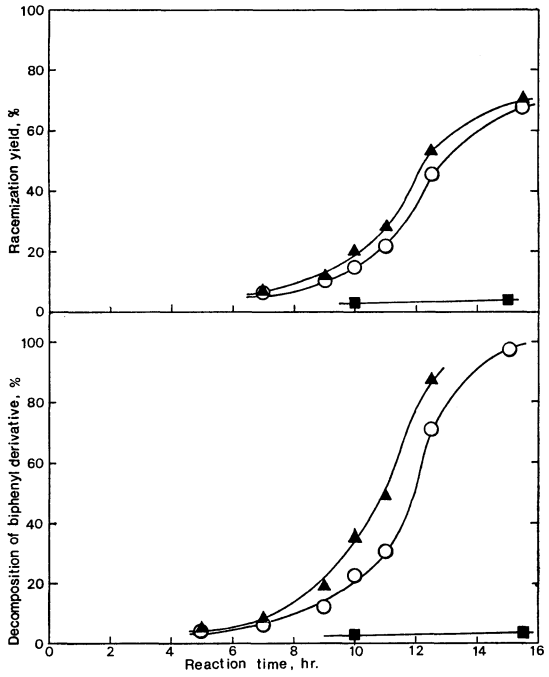


Fig. 1. The reaction mixture (0.5 ml) containing 0.5 mmol D- or L-alanine, 0.05 mmol $CuSO_4 \cdot 5H_2O$, and 0.0125 mmol the above biphenyl derivative in 0.025 M borate buffer (pH, 10.4 at 50 °C).
○: Reaction with D-alanine, ▲: Reaction with L-alanine, ■: Reaction with D-alanine in the absence of the biphenyl.

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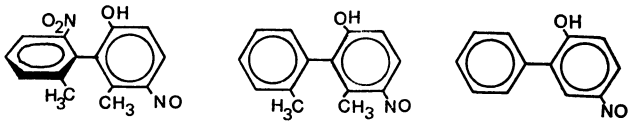
courses of racemization and those of the decomposition of catalysts used exhibit sigmoid curves consisting of the induced period, the acceleration and maximum rate periods, and final stage, as shown in Fig. 1.

Actually the apparent racemization half-life of D-alanine is found to be longer than that of L-alanine as the result of the enantiomer differentiating ability of **1**. However the difference of half-life does not correspond to the difference in rate constants of reaction as was found in the enzymatic reaction, but solely depends on the difference in the induced period.



1 **3** **4**

In order to make clear the relationship between the apparent half-life of racemization and the structure of the catalyst, the racemization of alanine and decomposition of the catalyst used were further studied with two representative catalysts (**3** and **4**) whose structures closely resemble that of **1** (Fig. 2). In all cases, the time courses of the reactions show similar sigmoid curves, differing however, in the length of the induced period. The apparent half-lives of racemization, and as well the lengths of the induced period, are in the order; **1**>**3**≫**4**.

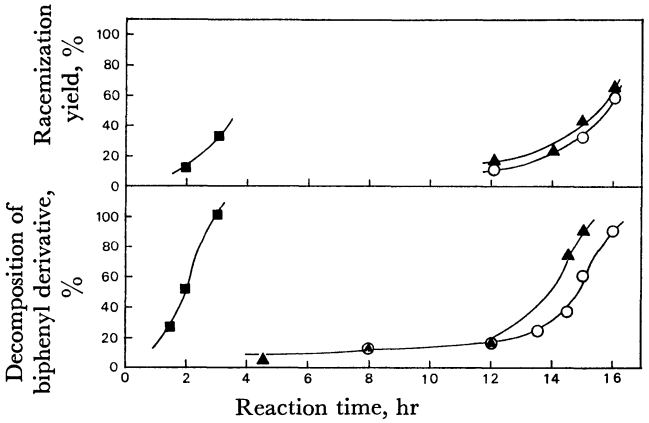


Fig. 2. The reaction mixture (0.5 ml) containing 0.5 mmol D-alanine, 0.05 mmol $CuSO_4 \cdot 5H_2O$ and 0.0125 mmol biphenyl derivatives in 0.025 M borate buffer (pH, 10.4 at 50 °C).
■: with **4**. ▲: with **3**. ○: with **1**.

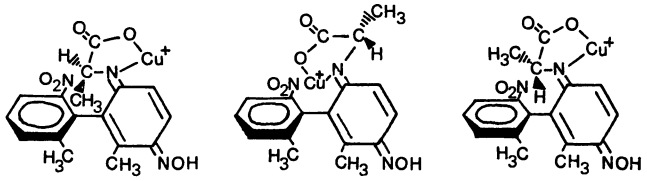
The good correlation of time course between racemizations of alanine and decompositions of 4-nitrosophenol derivatives implies that both racemization and decomposition proceed though the same intermediate consisting of 4-nitrosophenol derivatives, alanine and cupric ion as shown in **2**.

Taking into account the steric effects on the induced period, it is reasonable that the rate of formation of X is directly related to the equilibrium concentration of **2** which reflects steric effects of substituents of 4-nitrosophenol derivatives in the reaction system. Thus, the direct correlation between the induced period and the structure of **2** was made clear.[4]

The same concept can be used to explain the enantiomer differentiating ability of **1**. The different effects of D-alanine and L-alanine in the decomposition of **1** could be ascribed to the equilibrium concentrations of D-alanine-**1**-cupric complex (D-**1**-Cu) and L-alanine-**1**-cupric complex (L-**1**-Cu) since this complex is an intermediate in the decomposition of **1**. As D-**1**-Cu and L-**1**-Cu are diastereomers, the concentration of D-**1**-Cu should be different from that of L-**1**-Cu.

The apparent half-life of D-alanine is longer than that of L-alanine is also explained by the stereochemical aspects of intermediate, **2**. Actually the results are found to correspond well with predictions using the stereo model as follows: Two types of stereo models, **5** and **6**, can be proposed for L-**1**-Cu. Structure **6**, however, has much less stereo interaction between the chiral center of alanine and the biphenyl ring of **1** than structure **5**. Accordingly structure **5** must make a larger contribution to the enantiomer differentiation of **1**. Structure **7** is the corresponding version of structure **5** for D-**1**-Cu. The steric hindrance between the α-methyl group of alanine and the 2′-nitro group of **1** in **5** is less than in **7**. Hence the concentration of X would be higher for L-**1**-Cu rather than for D-**1**-Cu, resulting in shorter half-life of racemization for L-alanine than for D-alanine.

Although it is not possible to speculate on the mechanism of enantiomer differentiating ability of alanine racemase with the results obtained from **1** because of the decomposition of **1** during the reaction, the enantiomer differentiating process in the formation of enzyme-substrate complex was considered to be well simulated by the complexes **5** and **7**.



5 **6** **7**

The simulation of enantiomer differentiating racemization in the enzymatic systems with synthetic catalysts will be more feasible when the stable catalysts with a chiral structure are available. Efforts along this line are continuing.

Experimental

Melting points are uncorrected. IR spectra were determined on a Shimadzu 27-G spectrometer. NMR spectra were obtained on a Hitachi R-24 spectrometer with TMS as an internal standard.

2′-Nitro-6,6′-dimethylbiphenyl-2-amine (8). 2,2′-Dinitro-6,6′-dimethylbiphenyl[5] was reduced with sodium sulfide nonahydrate to get 2′-nitro-6,6′-dimethylbiphenyl-2-amine according to Sako's procedure.[6]

(S)-2′-nitro-6,6′-dimethylbiphenyl-2-amine (9). L-Cysteic acid monohydrate[7] (10 g) was dissolved in water (70 ml) with warming and then the solution (75 °C) was added dropwise to

a solution of **8** (12 g) in ethanol (550 ml) previously heated to the same temperature. The mixture was allowed to stand at room temperature for three hours to precipitate a white salt and chilled with ice-water for an hour. This salt was filtered by suction, washed with 90% ethanol (50 ml) and dried at 60 °C to give a white powder (11 g). It was boiled with 90% ethanol (1100 ml) and the excess L-cysteic acid which remained insoluble was filtered off. After standing at room temperature for several hours, the salt began to precipitate as a white powder and then the solution was kept in the refrigerator overnight. The precipitated salt was collected on a filter, washed with 90% ethanol and dried at 60 °C. In the successive recrystallizations, the following procedure was used in order to minimize the total volume of solvent; the salt was again dissolved in the minimum amount of boiling ethanol (80 ml) and then boiling absolute ethanol was added until the final concentration of ethanol was 90%. The yellow solution was allowed to stand overnight in the refrigerator. The white precipitate was filtered by suction and dried at 60 °C. The above recrystallization procedure was repeated four or five times. The color of the solution, during this repeated recrystallization, changed from reddish yellow to pale yellow and also the crystalline state of the salt changed from powder to white needles. Although the course of the recrystallization could be roughly monitored by observations of the above phenomena, it was accurately** followed by decomposition of the salt and measureing the optical rotation value of **9**. The salt was decomposed with 2 M sodium hydroxide to give yellow crystals, which were extracted with ether. The ether solution was washed with water and dried over sodium sulfate. After the evaporation of the ether, the residue started to crystallize almost immediately on cooling. Its specific rotation was measured on the dried sample, and the recrystallization was discontinued when there was essentially no further changes in rotation. After the resolution was complete, **9** liberated from the L-cysteic acid was recrystallized from petroleum ether to afford lustrous yellow crystals (1.8 g) in 30% yield: mp 108—109 °C (lit,[8] 108.3—109.0 °C); $[\alpha]_D$ −74° (*c* 1.0, ethanol), $[\alpha]_D$ −185° (*c* 1.0, 0.2 M HCl) (lit,[8] $[\alpha]_D$ −74.6° (*c* 1.0, 95% ethanol), $[\alpha]_D$ −187° (*c* 0.92, 0.2 M HCl)).

(S)-2-nitro-6,6′-dimethylbiphenyl-2-ol (10). **10** was prepared by the method of Angelletti,[9] but with a greatly improved yield. To a solution of 25% sulfuric acid (70 ml) was added the fine powder of **9** (1.20 g) with vigorous stirring and the diazotization performed at 0—5 °C with a solution of sodium nitrite (0.38 g) in water (1.0 ml). The resulting yellow solution was poured dropwise into continuously boiling water (20 ml) in over ten minutes. During this time, reaction took place with vigorous foaming of nitrogen and brown pitch-like material separated. After cooling, the mixture was made alkaline with 2 M sodium hydroxide and the insoluble brown materials was filtered off by suction. The filtrate was acidified with dilute sulfuric acid solution and the liberated oil was extracted with ethyl acetate. The extract was washed with water, dried over anhydrous sodium sulfate and the solvent was evaporated to give a dark colored syrupy oil containing **10**. The purification was carried out *via* acetylation as follows: The above syrupy oil was gently refluxed with acetic anhydride (5.0 ml) for forty minutes. After removal of the excess acetic anhydride under reduced pressure, the residue was dissolved in ether and the insoluble matter was filtered off. The filtrate was then washed with water, dried with anhydrous sodium sulfate, and the ether was distilled off. The brown residue was dissolved in hot ethanol (20 ml) and decolorized with charcoal.

After removal of the solvent, the residue was recrystallized from 50% ethanol to afford acetylated **10**: mp 77—78 °C, $[\alpha]_D$ +270° (*c* 1.0, ethanol), Found: C, 67.70; H, 5.04; N, 4.85%. Calcd for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91%. IR(KBr): 1763 cm^{-1} (C=O) and 1530 cm^{-1} (NO_2).

The acetylated **10** (400 mg) was stirred rapidly with 2 M sodium hydroxide (30 ml) at 70—80 °C for an hour. After acidification of the reaction mixture with 2 M hydrochloric acid, the liberated oily matter was extracted with ethyl acetate (10 ml). After washing with water of the solvent, the mixture was dried over anhydrous sodium sulfate and was evaporated. The crystalline residue was recrystallized from cyclohexane to afford white crystals (0.5 g) in 41% yield from **9**: mp 109—110 °C $[\alpha]_D$ +60.0° (*c* 1.0, ethanol), Found: C, 68.55; H, 5.39; N, 5.63%. Calcd for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.76; N, 5.39%. IR (KBr): 3450 cm^{-1} (OH) and 1523 cm^{-1} (NO_2).

(S)-2′-Nitro-5-nitroso-6,6′-dimethylbiphenyl-2-ol (1). **1** was prepared by the nitrosation of **10** according to the procedure described in a previous paper.[4] mp 205—208 °C, $[\alpha]_D$ −12.0 ° (*c* 1.0, ethanol), Found: C, 61.65; H, 4.22; N, 10.04%. Calcd for $C_{14}H_{12}N_2O_4$: C, 61.76; H, 4.44; N, 10.29%. IR ($CHCl_3$): 2550 cm^{-1} (OH). NMR (CD_3COCD_3): 12.5 ppm (OH), 1.96 and 2.22 ppm (CH_3).

5-Nitroso-6,6′-dimethylbiphenyl-2-ol (3). 2-Amino-6,6′-dimethylbiphenyl (4.0 g)[8] which was prepared from **8** was added to 20% sulfuric acid (50 ml) and diazotized by adding sodium nitrite (1.6 g) in water (10 ml) at 0—5 °C. The mixture was poured into boiling water (200 ml) and the resulting pitch-like material was separated by decantation of the solvent. It was stirred with 2 M sodium hydroxide (100 ml) and the insoluble material was filtered. After acidification of the filtrate with 4 M hydrochloric acid, the mixture was extracted with ether. The extract was washed with water, dried over anhydrous sodium sulfate and the solvent evaporated to afford 6,6′-dimethylbiphenyl-2-ol. The crude product was dissolved in 95% acetic acid and a solution of sodium nitrite (1.0 g) in water (30 ml) was added dropwise with stirring at 0—5 °C. The resulting brown precipitate was collected on a filter, washed with 60% acetic acid and recrystallized from 50% ethanol to afford yellow crystals (1.2 g) in 25% yield: mp 199—200 °C, Found: C, 74.47; H, 5.66; N, 6.01%. Calcd for $C_{14}H_{13}NO_2$: C, 73.99; H, 5.77; N, 6.16%.

5-Nitrosobiphenyl-2-ol (4). Biphenyl-2-ol was treated with sodium nitrite in acetic acid by the method described in the preparation of **1**. The precipitate from the reaction mixture was recrystallized from 30% ethanol to afford yellow crystals in 16% yield: mp 169—171 °C (lit,[10] mp 170—172 °C) Found: C, 72.63; H, 4.49; N, 6.84%. Calcd for $C_{12}H_9NO_2$: C, 72.35; H, 4.55; N, 7.03%.

Racemization Reaction. An aqueous solution of D- or L-alanine (0.5 mmol), cupric sulfate pentahydrate (0.05 mmol), and one of the biphenyl derivatives (0.0125 mmol) was made. The pH was adjusted to 10.4 (at 50 °C) with 1 M sodium hydroxide and diluted to a total volume of 0.25 ml. After dilution with 0.05 M borate buffer (0.25 ml, pH 10.4 at 50 °C), the mixture was shaken at 50 °C in a sealed tube. At specific time intervals the reaction was stopped by the addition of 6 M hydrochloric acid (0.25 ml).

Determination of the Ratio of D- *and* L-*Alanine and the Recovery of Biphenyl Derivatives.* The ratio of D- and L-alanine in the reaction mixture was determined by gas chromatography after conversion to *l*-menthyl D- and L-trifluoroacetylalaninates by the method described previously.[4] Careful measurements, compared with the calibrated curve, indicate an estimated

** Following the optical rotation of the salt itself could not be used as a criterion of purity due to a constant contamination of the salt with the L-cysteic acid during the procedure.

error of $\pm 0.5\%$. Biphenyl derivatives were determined spectrophotometrically after separation from reaction mixtures by thin layer chromatography according to the previously outlined procedure.[4]

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