Racemization Catalyst for Amino Acids. III.* Racemization of Alanine Induced by 4-Nitrosophenol

Kazuhiro HIROTA, Hisanori KOIZUMI,** Yoshio HIRONAKA, and Yoshiharu IZUMI

Division of Organic Chemistry, Institute for Protein Research, Osaka University, Suita, Osaka 565

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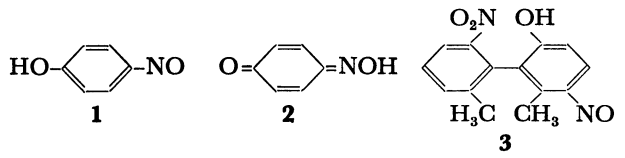
The racemization of D-alanine was carried out with several derivatives of 4-nitrosophenol in the presence of cupric ion. Their racemization activities have been found to decrease with an increase in the bulk of alkyl group in 4-nitrosophenol. The racemization is accompanied with the decomposition of 4-nitrosophenol, and has been found to be catalyzed by an unidentified product generated in the reaction mixture. The proposed mechanism involves an autoaccelerating reaction.

In the course of studies on the racemization of amino acids, derivatives of 2- or 4-nitrosophenol[1,2] were found to be good catalyst for the racemization of alanine. The catalytic function of these compounds in the reaction was first expected to be the same as that of pyridoxal, since they require similar reaction conditions to those of the pyridoxal-catalyzed racemization.[3] Further they are able to form a Schiff's base with alanine, as pyridoxal does in a step of the racemization reaction. However rate studies of the racemization with 4-nitrosophenol revealed that these compounds are not the actual catalyst but they induce the formation of catalysts for the racemization whose mechanism differs from that of pyridoxal.

In this paper the functions of 4-nitrosophenols in the racemization reaction of D-alanine are explained.

Results and Discussion

Effect of the Substituent on 2,6 or 3,5 Positions in 4-Nitrosophenol on the Racemization. The racemization of D-alanine was performed with several derivatives of 4-nitrosophenol in the presence of cupric ion. The racemization yields and the recoveries of 4-nitrosophenols are shown in Figs. 1 and 2 (Fig. 2 includes some data used in Fig. 1). The time course is sigmoid, consisting of an induced stage and a subsequent steep rise, suggesting an autoaccelerating reaction. The induced period of the racemizations with 2-alkyl substituted 4-nitrosophenols, as seen in Fig. 1, are in the oder: 4-nitrosophenol < 2-methyl-4-nitrosophenol ≃ 2-ethyl-4-nitrosophenol < 2-isopropyl-4-nitrosophenol < 2-*tert*-butyl-4-nitrosophenol. Thus the apparent catalytic activity increases with decreasing steric bulk of the groups in the position *ortho* to the hydroxyl group of the 4-nitrosophenol derivatives. This suggests the contribution of the hydroxyl group of 4-nitrosophenol (**1**) to the initial stage of reaction. With this regard, the Schiff's base formed with alanine and the carbony



1 **2** **3**

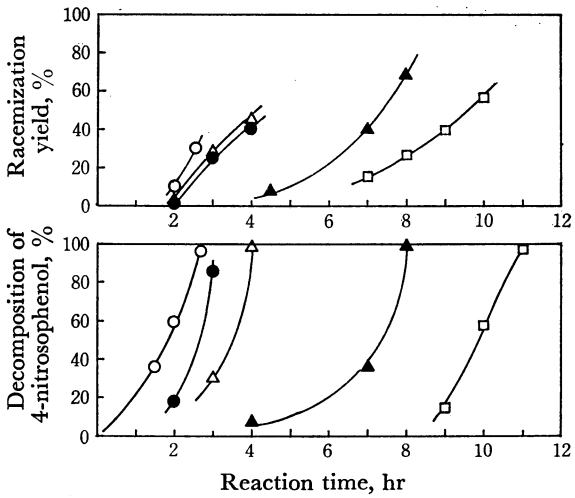


Fig. 1. Effect of 2-alkyl substituted 4-nitrosophenol on the racemization. Reaction mixture (0.5 ml, pH 10.4) containing 0.5 mmol D-alanine, 0.05 mmol $CuSO_4 \cdot 5H_2O$ and 0.0125 mmol one of the 4-nitrosophenol derivatives was shaken at 50 °C.
○: 4-Nitrosophenol, ●: 2-methyl-4-nitrosophenol, △: 2-ethyl-4-nitrosophenol, ▲: 2-isopropyl-4-nitrosophenol, □: 2-*tert*-butyl-4-nitrosophenol.

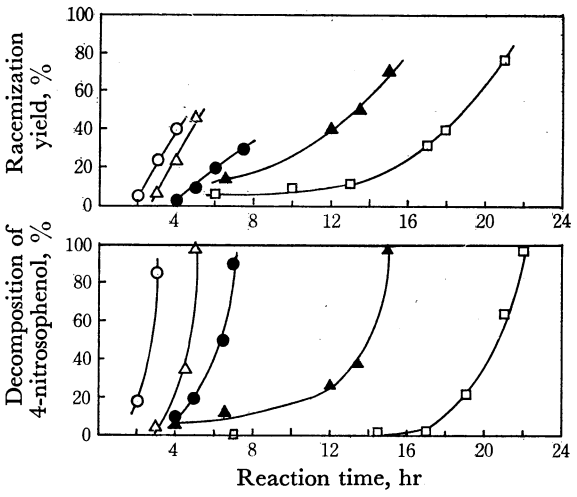


Fig. 2. Effect of 3-alkyl substituted 4-nitrosophenol on the racemization. The reaction condition of each experiment is same as that shown in Fig. 1.
○: 2-Methyl-4-nitrosophenol, ●: 3-methyl-4-nitrosophenol, △: 2,6-dimethyl-4-nitrosophenol, ▲: 3,5-dimethyl-4-nitrosophenol, □: 2-isopropyl-5-methyl-4-nitrosophenol.

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** Present adress: Nippon Senka Chemical Ind. Ltd. 1-83 Naka Hanatencho, Jyotoku, Osaka.

group of the quinone form (**2**) of 4-nitrosophenol would play an important role in the initial stage of the reaction.

On the other hand, a function of the nitroso group in the phenol form, **1** in the racemization was suggested by the experiment shown in Fig. 2. The apparent catalytic activity of 3,5-dimethyl-4-nitrosophenol, in which the nitroso group is protected by steric hindrance of the two methyl groups, is lower than that of 3-methyl-4-nitrosophenol. These results indicate that the catalytic activity of the 4-nitrosophenols depends not only on the carbonyl group in **2**, but also on the nitroso group in **1**. Consistent with this is the fact that the lowest activity of racemization among the derivatives of 4-nitrosophenol employed is observed with 2-isopropyl-5-methyl-4-nitrosophenol, in which both of the functional groups are sterically hindered by alkyl groups.

The relative reactivities of the two functional groups, were determined by comparing racemization activities of two structural isomers. The apparent catalytic activities, as seen in Fig. 2, are in the order: 2-methyl-4-nitrosophenol>3-methyl-4-nitrosophenol and 2,6-dimethyl-4-nitrosophenol > 3,5-dimethyl-4-nitrosophenol, respectively. These orders show that the participation of the nitroso group in **1** in the racemization is greater than that of the carbonyl group in **2**.

Relationship between the Racemization of D-*Alanine and the Decomposition of 4-Nitrosophenol.* The racemizations with 4-nitrosophenol, as seen in Figs. 1 and 2, are accompanied by the decomposition of 4-nitrosophenol.

A more detailed rate experiment was performed with 2′-nitro-2-hydroxy-5-nitroso-6,6′-dimethylbiphenyl (**3**). The result, comparing the rates of racemization and the decomposition of **3**, is shown in Fig. 3. The induction period of the racemization corresponds to that of the decomposition of **3** and increases when the concentration of **3** is decreased as seen in comparing A and B of Fig. 3. This indicates that the racemization of D-alanine is actually catalyzed by an unidentified product (X) generated in the reaction mixture, and suggests that the initiation of racemization activity might be a response to the appearance of this product, which is the actual catalyst.

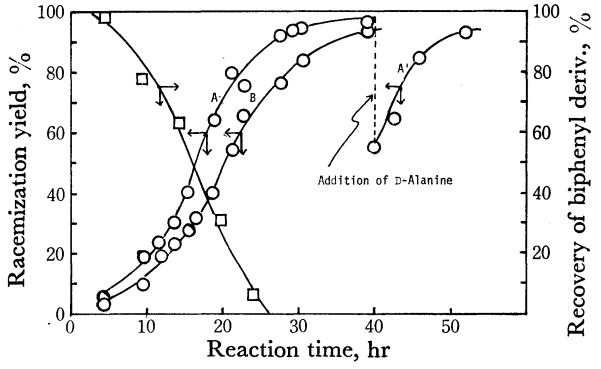

Fig. 3. Time courses of racemization yield and recovery of 2′-nitro-2-hydroxy-5-nitroso-6,6′-dimethylbiphenyl (**3**).
○: Racemization yield of alanine,
A: The reaction mixture of which initial composition consists of D-alanine 445 mg, $CuSO_4 \cdot 5H_2O$ 125 mg, **3** 3.4 mg, and borate buffer (0.05 M, pH 10.4 at 50 °C) was incubated at 50 °C.
A′: To the reaction mixture of A at the final stage of racemization, D-alanine 445 mg and $CuSO_4 \cdot 5H_2O$ 125 mg were added. In this manner, the initial racemization yield of the reaction mixture becomes 50%. The mixture was reincuvated another 10 hr at 50 °C.
B: The reaction condition and the initial composition of reaction mixture are same as A, except the amount of **3** (14 mg).
□: Recovery of **3** in the system of A.

The above conclusion is also supported by the experiment shown in A′ of Fig. 3; D-alanine and cupric sulfate pentahydrate were added again to the reaction mixture at the final stage of the reaction A in which **3** had already disappeared, and the racemization time course was followed. Additional D-alanine is racemized immediately without the induced period seen in curves A and its initial racemization rate corresponds to the previous maximum observed in curve A. This indicates that the relatively stable compound, X, has accumulated in the reaction mixture and that additional D-alanine can undergo racemization catalyzed by X already present.

Proposed Mechanism of the Racemization. Because the decomposition of 4-nitrosophenol in the reaction condition requires the presence of both D-alanine and cupric ions, the decomposition should proceed *via* a cupric complex DN–Cu containing D-alanine and 4-nitrosophenol. The resulting decomposition product, X, catalyzes both the racemization of D-alanine and the decomposition of DN–Cu in an autoaccelerating reaction. The proposed mechanism of racemization for D-alanine is shown in Eq. 1.

$$D + N \overset{Cu^{2+}}{\rightleftharpoons} DN\text{–}Cu \longrightarrow X \qquad (1)$$

$$D \overset{X}{\rightleftharpoons} L$$

$$D + N \overset{Cu^{2+}}{\rightleftharpoons} DN\text{–}Cu \rightleftharpoons LN\text{–}Cu \overset{Cu^{2+}}{\rightleftharpoons} L + N \qquad (2)$$

D: D-alanine, L: L-alanine, N: 4-nitrosophenol, X: an unidentified compound.

Since the initial rate of racemization is negligible, the chief role of DN–Cu may be as a promoter to produce X rather than an intermediate in the racemization of D-alanine *via* reaction shown in Eq. (2). The racemization mechanism with 4-nitrosophenol, in this respect, is quite different from that with pyridoxal[3] which forms the metal-amino acid complex to catalyze the racemization.

We have no information about the composition or the structure of X, but we are currently working to confirm them.

Experimental

All melting points are uncorrected. The IR spectra were taken with a Shimadzu 27-G spectrometer. The NMR spectra were recorded by a Hitachi R-24 spectrometer using TMS as the internal standard. Thin layer chromatography was performed on precoated plates of silica gel (E. Merck). The glc analyses were carried on a Shimadzu 4APF apparatus, using neopentylglycolsuccinate (1.5%)-Chromosorb W (300 cm × 0.5 cm).

Materials. 4-Nitrosophenol,[4] 2-methyl-4-nitrosophe-

nol,[5] 3-methyl-4-nitrosophenol,[5] 2,6-dimethyl-4-nitrosophenol,[6] 3,5-dimethyl-4-nitrosophenol,[7] and 2-isopropyl-5-methyl-4-nitrosophenol[8] were prepared from substituted phenols and sodium nitrite according to the methods described previously.

2-Ethyl-4-nitrosophenol. To a solution of sodium nitrite (2.5 g) in water (2000 ml), 2-ethylphenol (6.0 g) was dissolved and 1.5% sulfuric acid (200 ml) was added dropwise while stirring at 0—5 °C for 2 hr. A brown yellow precipitate was collected and recrystallized from benzene with a small amount of charcoal. Yield, 3.0 g (40%); mp 101—102 °C. Found: C, 63.85; H, 6.06; N, 9.13%. Calcd for $C_8H_9N_1O_2$: C, 63.57; H, 6.00; N, 9.27%.

2-Isopropyl-4-nitrosophenol. Concentrated hydrochloric acid (30 ml) was added to a solution of 2-isopropylphenol (13.6 g) dissolved in ethanol (40 ml). The second solution of sodium nitrite (7.0 g) dissolved in water (10 ml) was poured with continuous stirring into the 2-isopropylphenol solution at 0—5 °C. The reaction mixture was allowed to stand for 2 hr at this temperature. After the addition of a solution of urea (0.5 g) in water (2.0 ml), it was evaporated to dryness. The residue was extracted with ether (50 ml). The extract was washed with water and dried over anhydrous sodium sulfate. The ether solution was chromatographed on a column of alumina and eluted with ether to separate an orange yellow fraction from the pitch-like material. Further purification was carried out with this layer chromatography on silica gel in a solvent system of ether–hexane (1: 1 v/v). A yellow band which gave a positive Liebermann test was scraped off the plate, and extracted with ethyl acetate. After removing the solvent, the residue was recrystallized from 30% ethanol. Yield, 1.0 g (6%); mp 106—107 °C. Found: C, 65.25; H, 6.77; N, 8.61%. Calcd for $C_9H_{11}N_1O_2$: C, 65.44; H, 6.71; N, 8.48%.

2-tert-Butyl-4-nitrosophenol. 2-*tert*-Butylphenol (4.0 g) and sodium nitrite (4.0 g) were dissolved in 0.3% sodium hydroxide (1000 ml). To this solution 6% sulfuric acid (100 ml) was added dropwise with stirring over one hour at 0—3 °C. After standing for 2 hr., a red brown material precipitated. On cooling more of the precipitate formed. The precipitate was recrystallized twice from 25% ethanol to give yellow crystals. Yield, 1.0 g (21%); mp 136—137 °C. Found: C, 67.34; H, 7.35; N, 7.70%. Calcd for $C_{10}H_{13}N_1O_2$: C, 67.02; H, 7.31; N, 7.82%.

2′-Nitro-2-hydroxy-5-nitroso-6,6′-dimethylbiphenyl. 2-Hydroxy-2′-nitro-6,6′-dimethylbiphenyl (243 mg)[9] was dissolved in glacial acetic acid (1.0 ml) and then water (0.2 ml) was added. A solution of sodium nitrite (83 mg) in water (0.2 ml) was added dropwise with stirring at 0—3 °C. The mixture was allowed to stand overnight at the same temperature. The reddish yellow precipitate was collected on a filter and washed twice with 10% ethanol (1.5 ml). The crystals were recrystallized from 30% ethanol with a small amount of charcoal. Yield, 90 mg (33%); mp 200—202 °C. Found: C, 61.10: H, 4.26; N, 10.00%. 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): δ 12.5 (OH), 1.96 and 2.22 (CH_3).

Racemization Reaction. An aqueous solution of D-alanine (0.5 mmol), cupric sulfate pentahydrate (0.05 mmol), and one of the 4-nitrosophenol 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 diluting 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 0.25 ml of 6 M hydrochloric acid.

Determination of the Racemization Yield. The reaction mixture (0.2 ml) was dried *in vacuo*. The residue was heated with *l*-menthol (1.0 g) 110—120 °C and bubbled with dry hydrogen chloride for 1.5 hr to prepare the *l*-menthyl ester of alanine. Excess *l*-menthol was removed by sublimation at 110—120 °C under reduced pressure. Trifluoroacetic anhydride (0.05 ml) was added and allowed to stand overnight at room temperature to obtain *l*-menthyl L- and D-trifluoroacetylalaninate. After diluting with tetrahydrofuran (0.5 ml), the diastereomers were gas chromatographed at 170 °C. From the ratio of the diastereomers, the racemization yield of D-alanine was calculated.

Determination of the Recovery of 4-Nitrosophenol. The reaction mixture (0.2 ml) was extracted with ethyl acetate (2.0 ml) and the extract (120 μl) was chromatographed on a silica gel with ether–hexane (1: 1 v/v) as the developing solvent. A yellow band containing 4-nitrosophenols was scraped off the plate and extracted with 0.5 M sodium hydroxide (5.0 ml). The absorption of the extract was measured at 403 nm and the concentration was calculated.

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