Micellar and Metal Ion Catalysis in the Pyridoxal-Promoted α,β-Elimination of *S*-Phenylcysteine*

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The effects of metal ions and detergents on the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine have been investigated in 0.01 M borate buffer at 50 °C (the copper ion catalysis at 40 °C in the presence of CTAB micelles). Both copper(II) and nickel(II) ions markedly accelerated the elimination reaction of *S*-phenylcysteine. Since the catalytic efficiency of nickel(II) seems to be obtained due to the poor coordination ability of the leaving group in the present reaction, the previous working hypothesis which was provided for the elucidation of catalytic roles of metal ions in the α,β-elimination of *O*-phosphothreonine has now been approved. Among cationic, anionic, and non-ionic detergents employed in this work as apoenzyme models, only CTAB of cationic nature showed a considerable rate acceleration even in the absence of metal ions. The micellar binding constant was evaluated from the saturation-type correlation between reaction rate and CTAB concentration. The surfactant micelles demonstrated some enzyme-like features of catalysis in the pyridoxal-promoted α,β-elimination reaction, where the reaction is controlled by the entropy term. The CTAB catalysis was greatly inhibited by organic and inorganic anions due to the electrostatic nature of the micellar surface. The catalytic mechanisms for the micellar reactions and their significance as an enzyme model system are discussed.

Although the apoenzyme moieties of pyridoxal-dependent enzymes have not been fully characterized up to the present time, their specific roles may be counted tentatively as follows: (1) they provide the hydrophobic binding site inside the enzymes where pyridoxal phosphate is buried in its vicinity as the coenzyme function, and then (2) catalyze the specified reactions by their various functional groups such as the imidazole group of histidine residue and the ε-amino group of lysine. In our previous work, the catalytic effect of some organic bases of different base-strength was examined in the pyridoxal-catalyzed α,β-elimination reaction of *S*-(*p*-substituted phenyl)cysteines in connection with the catalytic nature of base moieties which are planted in an apoenzyme skeleton.[1] Our special concern has also been placed on the correlation between hydrophobic medium effect and reaction kinetics in these reactions by adopting acetonitrile-water mixed solvent system.[1]

In spite of the substrate and reaction specificity as well as the acceleration effect due to the vitamin B_6 apoenzymes, only a limited information has been obtained on their catalytic and structural nature. Micellar surfactants have often been granted to behave as an enzyme or apoenzyme model as reviewed by several workers.[2,3] In the present study, we have investigated the effects of these detergents as an apoenzyme model for the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine. As for the catalytic roles of metal ions in the elimination, *S*-phenylcysteine would render the metal ion effects different from those for *O*-phosphothreonine.[4] A metal ion of strong hexa-coordination tendency has been suggested to bind the leaving phosphate group in the Schiff base derived from pyridoxal and the latter amino acid, thus inactivating the amino acid toward elimination reaction. On the contrary, such a metal ion would not demonstrate any significant coordination ability toward thioether-sulfur in the present amino acid. We have studied from these viewpoints the effects of copper(II) and nickel(II) ions on the pyridoxal-catalyzed α,β-elimination of *S*-phenylcysteine.

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Experimental

Materials. *S*-Phenylcysteine was prepared by the reaction of thiophenol with α-acetoamidoacrylic acid and the subsequent acid hydrolysis.[5] Pyridoxal hydrochloride was obtained from Mann Research Laboratories, Inc., New York, U.S.A. Cetyltrimethylammonium bromide (CTAB, Katayama Chemical Co.), sodium lauryl sulfate (NaLS, Eastman Kodak Co.), and polyoxyethylene(23) dodecanol (Brij 35, Nakarai Chemical Co.) were obtained from commercial sources.

Kinetic Measurements. For the micellar catalysis, the reaction was carried out in 0.01 M borate buffer (pH 9.0) at 50.0±0.1 °C without inorganic salt as being a supporting electrolyte. Fifty ml of a reaction mixture containing specified amounts of substrate, detergent, and borate buffer was placed in a jacketed reaction vessel. When the constant temperature was attained, 5 ml of the pyridoxal solution was added. The initial concentrations of *S*-phenylcysteine and pyridoxal were 7.31×10^{-4} and 9.09×10^{-4} M, respectively and the detergent concentration was varied. Aliquot samples were withdrawn from the reaction mixture at appropriate time intervals after the thermal equilibrium was attained (5 min after the pyridoxal solution was added). The reaction rate was determined by measuring the yielded pyruvate as its 2,4-dinitrophenylhydrazone. The analytical procedures were essentially the same as those described previously,[1] but with slight modifications. In the presence of detergents the 2,4-dinitrophenylhydrazone of pyridoxal did not precipitate and the extraction of an excess hydrazine from the resulting filtrate with toluene was not possible due to foam formation. In order to overcome these difficulties 5 ml of acetonitrile was added to the sample solution before treating it with the potassium phosphate—sodium hydroxide buffer to allow the precipitation of pyridoxal hydrazone. Secondly, the extraction of an excess hydrazine with toluene was omitted since this modification did not cause any serious analytical error.

For the metal ion catalysis, the reaction was carried out in borate buffer at either 40 or 50 °C with or without 0.91×10^{-4} M CTAB. The reaction was started by the addition of 5 ml of cupric nitrate or nickel nitrate solution prewarmed to the reaction temperature to 50 ml of a metal-free reaction mixture. The initial concentrations of substrate, pyridoxal, and borate buffer were the same as those for the micellar catalysis, and the initial metal ion concentration was adjusted at either 1.81×10^{-4} or 4.54×10^{-4} M. The reaction rate was measured by the above-mentioned procedures.

Results

Effects of Micellar Surfactants. The kinetic runs for the pyridoxal-catalyzed α,β-elimination reaction were carried out at 50 °C in 0.01 M borate buffer in the presence of three kinds of detergents, cetyltrimethylammonium bromide (CTAB), sodium lauryl sulfate (NaLS), and polyoxyethylene(23) dodecanol (Brij 35). Each reaction rate followed apparent first-order kinetics with respect to the total concentration of unreacted substrate. While the anionic and non-ionic micelles showed little catalytic effect or rather retarding, the cationic micelle promoted the elimination rate (Table 1). The correlation between the apparent first-order rate constant and the CTAB concentration is illustrated in Fig. 1. The rate is markedly accelerated above 1×10^{-3} M CTAB, and tends to level off beyond approximately 3×10^{-3} M of the detergent concentration. The rate acceleration at this saturation level is about 18-fold of the rate without the cationic detergent. On the other hand, CTAB did not show any significant catalytic effect in the absence of pyridoxal under the similar conditions.

The micellar catalysis by CTAB was effectively inhibited by inorganic and organic salts as shown in Table 2. Potassium nitrate is the more effective inhibitor than potassium chloride in a manner as observed for the solvolysis reactions of carboxylic esters,[6] while both of the organic salts, sodium benzoate and sodium benzenesulfonate, demonstrated the larger inhibition effect than the inorganic salts.

Activation parameters for the micellar reaction are listed in Table 3 together with those for the reaction without detergent. The micellar reaction becomes enthalpically unfavorable, and the catalysis is apparently controlled by the entropy term.

Table 1. The effects of anionic and non-ionic surfactants on the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine in 0.01 M borate buffer (pH 9.0) at 50 °C[a]

Surfactant	concentration M	$k_{obs}\times10^5$ s^{-1}	Relative rate
—	—	1.38[b]	1[b]
NaLS	2.90×10^{-3}	1.69	1.23
Brij 35	3.10×10^{-4}	0.84	0.61

a) Initial concentrations: substrate, 7.31×10^{-4} M; pyridoxal, 9.09×10^{-4} M. b) Micellar surfactant absent.

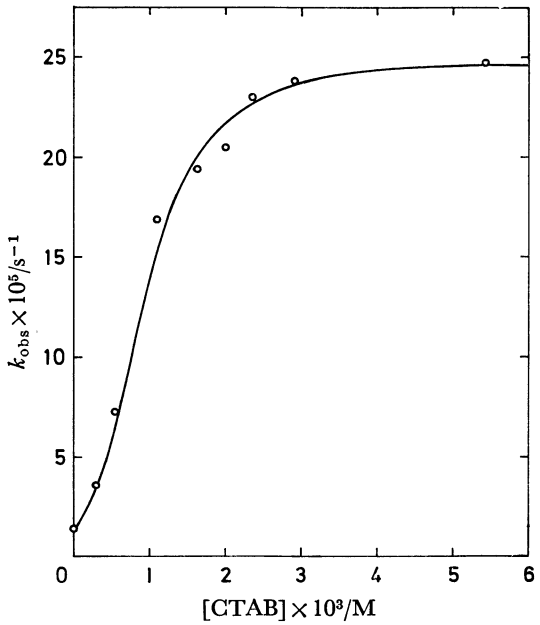


Fig. 1. Correlation between rate and CTAB concentration in the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine at 50 °C in 0.01 M aqueous borate buffer with the following initial concentrations: substrate, 7.31×10^{-4} M; pyridoxal, 9.09×10^{-4} M.

Table 2. Inhibition of CTAB catalysis in the pyridoxal-promoted α,β-elimination reaction of *S*-phenylcysteine in 0.01 M borate buffer at 50 °C[a]

Salt	Concentration M	$k_{obs}\times10^5$ s^{-1}	Relative rate
—	—	1.38[b]	1[b]
—	—	23.8	17.1
KNO_3	0.05	4.22	3.04
KNO_3	0.10	2.68	1.93
KCl	0.10	5.71	4.13
Na Benzoate	0.040	2.27	1.64
Na Benzenesulfonate	0.040	2.67	1.92

a) Initial concentrations: substrate, 7.31×10^{-4} M; pyridoxal, 9.09×10^{-4} M, CTAB, 2.91×10^{-3} M. b) Both CTAB and salt absent.

Table 3. Activation parameters for the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine in the presence and absence of CTAB at 50 °C[a]

[CTAB] M	$\Delta H^{\neq}$ kcal mol^{-1}	$\Delta S^{\neq}$ e. u.	$\Delta G^{\neq}$ kcal mol^{-1}
2.91×10^{-3}	16.4	−25	24.5
0	12.5	−41	25.7

a) Initial concentrations: substrate, 7.31×10^{-4} M; pyridoxal, 9.09×10^{-4} M.

Effects of Metal Ions. When the copper(II) and nickel(II) solutions were added to the reaction mixture containing substrate, pyridoxal, and borate buffer, precipitates were formed. The reduction of metal ion concentration and/or the increase in the acidity of the medium did not improve the situation. Thus, the reaction was carried out in a heterogeneous system.

The reaction followed a fairly good apparent first-order kinetics up to 70% conversion of the substrate in the presence of nickel(II); the initial concentration of the metal ion is 4.54×10^{-4} M. The rate constant of 2.84×10^{-4} s^{-1} corresponds to the 20.6-fold acceleration relative to the non-metallic system. On the other hand, any detectable amount of pyruvate was not liberated in about 30 min of the reaction time upon addition of copper(II); the initial concentration of the metal ion is 4.54×10^{-4} M. This apparently means that copper(II) inhibited the α,β-elimination of S-phenylcysteine completely through the complex formation. The precipitates were recovered for IR measurements (Fig. 2). The precipitates formed in the reaction of these metal ions with the substrate in the absence of pyridoxal were also subjected to IR measurements (Fig. 2). A broad band at 2800 cm^{-1} due to the ammonium group of S-phenylcysteine disappeared in the spectra of these metal complexes. The 1640 cm^{-1} band attributable to the carbonyl stretching mode shifted to the lower frequency region upon coordination. From these evidences the precipitates formed during the reaction can be identified as bis(S-phenylcysteinato)copper(II) and bis(S-phenylcysteinato)nickel(II).

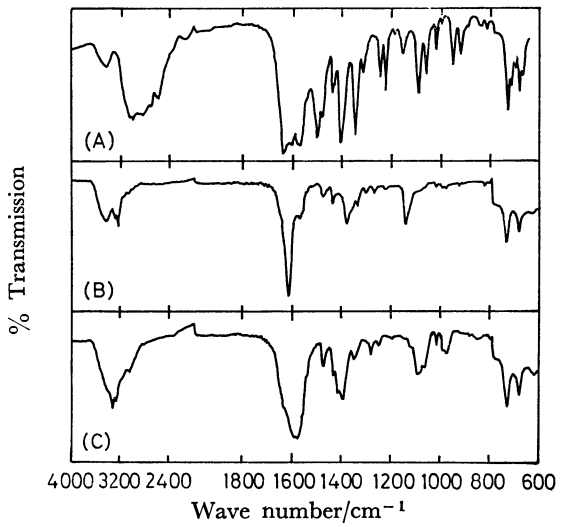


Fig. 2. Infrared spectra (KBr disc method): (A), S-phenylcysteine; (B), the copper(II) complex of S-phenylcysteine; (C), the nickel(II) complex of S-phenylcysteine. S-phenylcysteine underwent reaction with the metal ions in the absence of pyridoxal at the same respective concentrations as employed for the kinetic runs. The precipitates obtained by raising pH up to 9.0 with sodium hydroxide were used for IR measurements. The respective precipitates recovered from the reaction mixtures for kinetic runs gave the completely identical spectra.

The addition of CTAB prevented the precipitation of the metal complexes and consequently the reaction was carried out in a homogeneous system. But the situation is rather complicated in this system (0.91×10^{-3} M CTAB present) as follows. At the outset of the reaction the "burst" of pyruvate formation was detected, followed by the rate deceleration. The overall reaction, therefore, did not follow the first-order kinetics. The rate constants estimated from the initial "burst" part (~2 min period after the addition of metal ions), where the true thermal equilibrium was not still attained, are listed in Table 4. This table also gives the rate data for the reaction period following the "burst" part (a 2—10 min period after the metal addition). The reaction rate was so fast at 50 °C in the presence of copper(II) that the reaction temperature needs to be lowered to 40 °C and the metal concentration to two-fifths of the nickel(II) ion. The rate constants obtained for the "burst" part are greater by one order than those for the following period. Approximately a 60% portion of the substrate has been converted to the products in the "burst" part under these experimental conditions.

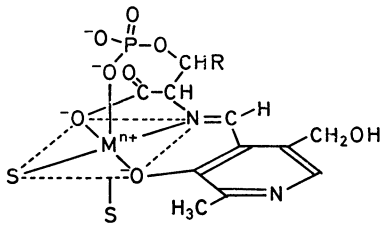
Discussion

Metal Ion Catalysis. In our previous investigations on the pyridoxal-catalyzed α,β-elimination of O-phosphothreonine,[4] the examined six metal ions were classified into two categories from the catalytic aspect: the active group includes copper(II) and vanadyl(IV) while the inactive group consists of nickel(II), iron(III), aluminum(III), and thorium(IV). We pointed out that the differences in catalytic activity of metal ions could not be explained merely in terms of the thermodynamic stability of the metal complexes. But the structural feature of these metal complexes was suggested to be responsible for the catalytic activity. The axial coordination site is available for metal ions of the latter category to bind the leaving phosphate group as shown by **1**. The lack of catalysis by these metal ions was thus attributed to this coordination effect. Taking for example nickel(II) ion of the latter category, this metal ion does not demonstrate any significant coordination ability toward the thioether group as investigated for S-methylcysteine and methionine.[7] The leaving group of S-phenylcysteine, therefore, must be free from coordination with nickel(II) ion. Consequently, nickel ion can be

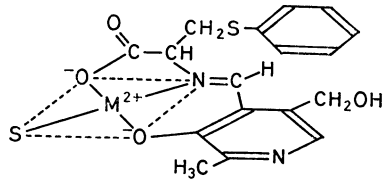
Table 4. Metal ion catalysis in the pyridoxal-promoted α,β-elimination reaction of S-phenylcysteine in the presence of 0.91×10^{-3} M CTAB

Metal ion	concentration, M	Temp. °C	After "burst" part		Initial "burst" part	
			k_{obs}, s^{-1}	k_{rel}[a]	k_{obs}, s^{-1}	k_{rel}[a]
Ni^{2+}	4.54×10^{-4}	50	5.7×10^{-4}	4.1	3×10^{-3}	21
Cu^{2+}	1.82×10^{-4}	40	5.7×10^{-4}	9.5	5×10^{-3}	83

a) Relative to the corresponding rate constant in the absence of metal ions: $k_{obs}(Ni^{2+})$, 1.40×10^{-4} s^{-1}; $k_{obs}(Cu^{2+})$, 6.00×10^{-6} s^{-1}.



1 S : solvent



2 M^{2+} : Cu^{2+}, Ni^{2+}

expected to exert an effective catalytic activity in the present elimination reaction through formation of a metal complex as shown by **2**. In fact, a profound rate acceleration was observed in the presence of nickel(II) under the present experimental conditions, even though a minor part of the substrate and metal ion was out of the reaction mixture due to the complex formation. The apparent complete inertness of copper(II) is undoubtedly attributed to the high thermodynamic stability of bis(*S*-phenylcysteinato)copper(II) which resulted in precipitation in the aqueous system. These behaviors of metal ions are consistent with the reaction sequence given in Scheme 1.

When the reaction was carried out in a homogeneous micellar system, the rate acceleration at the "burst" part reaches nearly a hundred-fold in the copper(II) catalysis (Table 4). In our previous work,[4] the copper(II) catalysis in the α,β-elimination of *O*-phosphothreonine was so enormous that the reaction proceeded smoothly at 45 °C, although the reaction did not occur in the absence of the metal ion even at 80 °C. In conformity with the previous work, both nickel(II) and copper(II) form the corresponding metal complexes at a 1 : 1 ratio of metal to Schiff base ligand of structure **2** in the electrostatic layer of surfactant micelles as the preequilibrium stage. In addition, the metal complexes would be more favorably incorporated into the micellar phase than the metal-free Schiff base since the anionic character of the Schiff base ligand can be neutralized through metal-coordination. The rate-determining α,β-elimination then takes place by the attack of hydroxide ion which has been accumulated in the electrostatic layer due to the micellar effect. It is quite reasonable that copper(II) showed a larger catalytic activity than nickel(II) in homogeneous systems on the basis of the relative stability constants of their Schiff base complexes.[8]

In conclusion, it is interesting to note that copper and nickel did show the catalytic effect on the pyridoxal-catalyzed α,β-elimination reaction of *S*-phenylcysteine, although a part of the catalytic activity was exerted by the micellar effect.

Micellar Catalysis. Only the cationic detergent showed a considerable effect on the pyridoxal-catalyzed α,β-elimination of *S*-phenylcysteine. This micellar catalysis resulted in the 18-fold rate acceleration at the level-off region as shown in Fig. 1. Since the conventional micellar catalysis demonstrates rate enhancement in a 10- to 100-fold range, the present value can be referred to as the indication of moderate catalytic efficiency.[2,3]

One of the prominent features of the CTAB-catalyzed reaction is the saturation behavior of the reaction rate with respect to the detergent concentration as shown in Fig. 1. The rate-saturation is characteristic of the enzymic and related model reactions, and the following formula is consistent with the present scheme.

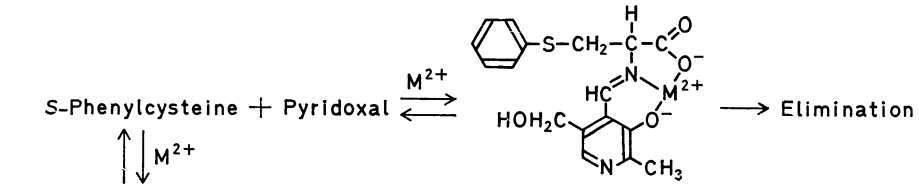
$$\begin{array}{l} S + M \overset{K}{\rightleftharpoons} SM \xrightarrow{k_m} P + M \\ \quad\downarrow k_0 \\ \quad P \end{array}$$

where S, M, and P respectively represent substrate (a Schiff base derived from *S*-phenylcysteine and pyridoxal), micelle, and product, and SM is the substrate-micelle complex. Upon defining C_D, N, and K as detergent concentration, aggregation number (assumed to be 61 in this case), and binding constant, respectively, the above scheme provides the correlation among observed (k_{obs}), spontaneous (k_0), and micellar (k_m) rate constants by Eq. (1).

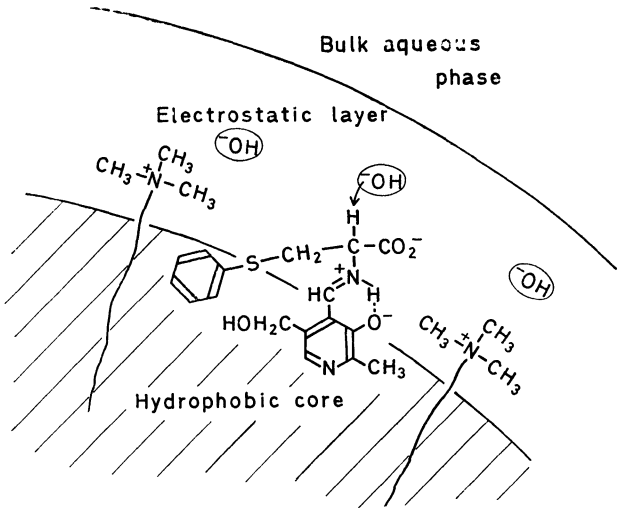
$$\frac{1}{k_{obs}-k_0} = \frac{1}{k_m-k_0} - \left(\frac{1}{k_m-k_0}\right)\left(\frac{N}{K(C_D-CMC)}\right) \quad (1)$$

An analysis of th present data (Fig. 1) gave fairly good linear relationship: $K \simeq 10^5$ and $CMC \simeq 5\times10^{-4}$ M.

A question arises as to the binding and reaction sites in the micellar phase. It has been shown that charged and polar organic compounds are bound to the electrostatic layer of micelle where the charged head groups of surfactant molecules are located, while apolar



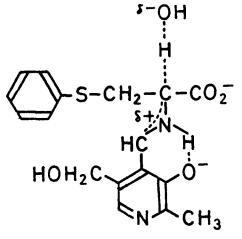
Scheme 1.



Scheme 2.

hydrocarbons are incorporated into the hydrophobic interior of the micelle.[9] Thus, a reactive Schiff base molecule of the present study is most likely incorporated into the micellar phase where the apolar pyridyl ring moiety is inserted into the hydrophobic core and the polar part composed of the Schiff base bond is located on the micellar surface (Stern and electric double layers). This state of affairs is schematically shown in Scheme 2.

Activation parameters obtained at the level of rate saturation for the micellar reaction of *S*-phenylcysteine are listed in Table 2. Although the increase in activation enthalpy has been observed by Bunton and Robinson for the micellar reaction of *p*-nitrophenyldiphenyl phosphate with hydroxide and fluoride ions,[10] it would seem rather unexpected. They ascribed the larger activation enthalpy for the micellar reaction to the high solubility of the neutral substrate in the micellar phase. In the present study, however, another explanation is needed for the high activation enthalpy since the Schiff base formed between *S*-phenylcysteine and pyridoxal possesses a much polar structure relative to the above neutral substrate. In conformity with the reaction scheme given for the non-micellar system,[1] the reaction center of the Schiff base may become less polar in the transition state **3** relative to the initial one. The electrostatic micellar surface in which the reactive center is incorporated destabilizes the transition state



3 (transition state)

and may consequently provide a larger activation enthalpy.

The hydroxide ion is presumably more concentrated on the micellar surface than in the bulk aqueous phase, and the included Schiff base may be readily subject to the attack of hydroxide ion. This state of affairs acts in favor of the activation entropy. As a whole, the CTAB micellar catalysis is exclusively controlled by the entropy term.

When inorganic salts are added to the micellar reaction system, hydroxide ion on the micellar surface is presumably substituted with the chloride or nitrate ion to some extent depending upon the relative concentrations of these salts. Since these inorganic anions are much poorer nucleophiles than the hydroxide ion, the substitution may result in the effective inhibition of the CTAB catalysis. The organic anions may exclude not only the hydroxide ion from the micellar surface but also the Schiff base by occupying the binding site around the charged head groups of surfactant molecules. Thus, these organic salts exerted an inhibition effect much greater than the inorganic salts.

In conclusion, the surfactant micelles demonstrated some enzyme-like features of catalysis in the pyridoxal-promoted α,β-elimination reaction of *S*-phenylcysteine, where the reaction is controlled by the entropy term. This seems to be the first effective organic catalyst ever provided for the pyridoxal-concerned reactions. The catalytic effectiveness is rather small at present relative to the enzyme system, but the modification of the detergent structure by introducing various functional groups into the skeleton will lead to the development of novel and eminent enzyme models of higher activity.

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