ELSEVIER

Journal of Molecular Catalysis A: Chemical 112 (1996) 1–7

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

Hydrolysis catalyzed with a resin containing histidine groups

Wei-Hsiu Hung, Cho-Chun Hu, Chuen-Ying Liu *

Department of Chemistry, National Taiwan University, Taipei, Taiwan

Received 8 September 1995; revised 8 January 1996; accepted 11 January 1996

Abstract

A histidine-containing polymer was synthesized in which the amino group of the histidine was attached chemically via an azide coupling method to the carboxylic acid of Amberlite IRC-50. The resultant polymer was applied as a catalyst for hydrolysis of *p*-nitrophenyl acetate (PNPA). PNPA in aqueous solution was hydrolyzed at 25°C with a phosphate buffer (pH 7.8). The observed kinetics obey those of Michaelis–Menten. The reaction rates at various temperatures were measured. The activation parameters, pre-exponential factor (A) and activation energy (E_a), were 6.64×10^{-4} min^{-1} and 37.5 kJ mol^{-1} respectively. At a pH of the medium greater than 7.8, the reaction rate remained almost constant ($k_{obs} = 0.024$ min^{-1}) and seemed to be controlled by the rate of diffusion of PNPA from the bulk solution into the catalytically active site at the resin channel surface. For catalysed hydrolysis, the effect of ionic strength in solution demonstrated that bifunctional cooperation between adjacent histidine groups existed through the nucleophilicity of nitrogen. The effects of metal ions and aspartic acid or serine on hydrolysis were also investigated.

Keywords: Histidine-containing resin; Hydrolysis; *p*-Nitrophenyl acetate

1. Introduction

Enzyme models are not proteins; they have the advantage of being more stable and can thus be prepared by synthetic methods. Because the mechanism of action of hydrolytic enzymes such as α-chymotrypsin was the first to be elucidated in detail, tests of enzyme models mostly involved the hydrolysis of phenyl esters. The imidazole group of the histidyl residue is involved in the catalytic action of many hydrolytic enzymes, and imidazole itself can act as a basic catalyst in various reactions. Therefore, in a typical catalytic-group approach, imidazole compounds are used widely as a hydrolytic enzyme model [1,2].

Many reactions in aqueous surfactant solutions occur at the interface between micellar hydrocarbon and water. The adsorption of reactants at these micellar interfaces and their subsequent reactions resemble reactions controlled by enzymes. Large rate enhancements are observed because reactants are concentrated in the micellar pseudophase. Gitler and Solano used a nucleophile that contained the bulky alkyl group *N*-myristyl-L-histidine for the catalytic reaction of *p*-nitrophenyl esters [3]. Bunton and Ihara tested the catalytic activity of *N*-decanoylhistidine in the presence of co-micellization with CTABr [4]. Ihara and Hosako investigated stereoselective reactions of enantiomers of *N*-

* Corresponding author.

PII S1381-1169(96)00031-3

acylamino acid *p*-nitrophenyl esters with *N*-decanoyl-(D or L)-histidine in an optically active surfactant derived from (L)-ephedrine [5]. Chiral *p*-nitrophenyl esters derived from phenylalanine were cleaved with histidine-containing dipeptides at a micellar interface [6]. Ohkubo et al. found a unique enantioselective hydrolysis of *N*-acyl amino acid esters with L (or D)-histidine-attached β-cyclodextrins [7] and in bilayer vesicular systems containing the tripeptide-type histidine derivative Z-L-Leu–L-His–L-Leu [8].

We previously synthesized a histidine-containing resin to extract metal ions [9]. In the present work, we report the catalytic effects of histidine-containing polymers on the hydrolysis of *p*-nitrophenyl acetate. Catalytic activities of polymer-supported catalysts are essentially smaller than those of the corresponding catalysts of small molar mass, but insoluble polymer catalysts are easily separated by filtration from the reactant or product at the end of a reaction and can be reused for further runs.

2. Experimental

2.1. Apparatus

Elemental analysis was performed on an elemental analyzer (Perkin Elmer 240C). The IR spectra of the resin in KBr pellets were recorded on an infrared spectrophotometer (Perkin Elmer 983). A UV–Vis spectrophotometer (Hitachi U-3200) was used for absorbance measurements. A pH meter (Radiometer, model PHM 61, Copenhagen, Denmark) was used to measure the pH of solutions.

2.2. Chemicals

Most chemicals were of analytical reagent grade (E. Merck, Darmstadt, Germany). *p*-Nitrophenyl acetate (PNPA) (Aldrich, Milwaukee, WI, USA) was purified from ethyl acetate. Pure water (18 MΩ cm) from a water purification system (Milli-Q Ultrapure Water System, Millipore, USA) was used to prepare all solutions. The stock solutions (ca. 0.1 M) of metal ions were prepared by dissolving an appropriate amount of the metal salt in nitric acid (0.1 M), diluting with pure water (to 250 ml), adjusting to pH 1.6, and storing in polyethylene bottles. The concentration of metal ion was verified on EDTA complexometric titration.

2.3. Synthesis of histidine-containing resin

The histidine-containing resin was prepared as described previously [9]. To the pretreated Amberlite IRC-50 resin (6 g) placed in a flask containing methanol (600 ml), sulfuric acid (18 M, 1.5 ml) as catalyst was added dropwise. The resultant mixture was refluxed at 70°C for 20 h. The product was cooled to room temperature and collected by filtration under suction and washed sequentially with water and methanol until no sulfate ion appeared in the filtrate. The procedures were repeated to obtain a higher yield. Then hydrazine (3 ml) was added dropwise to product **1** and reacted at room temperature for 30 h. The product was washed with water until no hydrazine appeared in the filtrate. To product **2** was added hydrochloric acid (0.5 M, 50 ml). While the mixture was constantly agitated in a freezing mixture of salt and ice, a solution of sodium nitrite (0.2 M, 10 ml) was added dropwise. After the addition was complete, the mixture was stirred at 0°C for 30 min, and histidine (4 g) was added. In this process, triethylamine (0°C, 50 ml) was added to adjust the pH to 8.0–8.5. The reaction mixture was stirred and kept at 0°C for 48 h. Then ammonia–ammonium chloride (1 M, 5 ml) was added to remove the unreacted azide. The final product was collected on suction filtration and washed sequentially with hydrochloric acid (0.1 M), water and methanol to remove unreacted starting material.

2.4. Potentiometric titrations

2.4.1. Acid dissociation constant

In this investigation, at least 14 samples of the resin (0.2 g) were accurately weighed and placed respectively in each PE bottle (100 ml). Aqueous solutions containing various amounts of sodium hydroxide (0.1 M) were made to ionic strength (0.1 M) with potassium chloride and brought to a total volume (25 ml). The solutions were added to a PE bottle containing the resin. The mixture was stirred for 4 h at (25 ± 0.1)°C. After equilibration, the pH was measured.

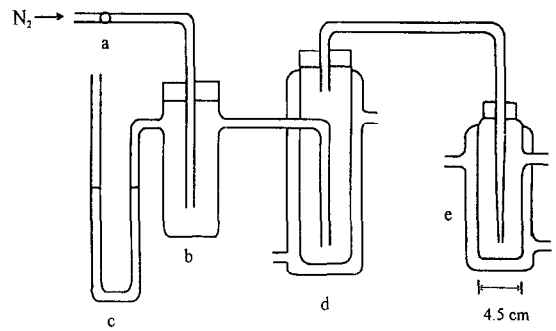
2.5. Kinetic of hydrolysis of p-nitrophenyl acetate

The reactions were carried out by measuring the absorbance of *p*-nitrophenol with a spectrophotometer, a thermostated cell compartment and a thermostated cell holder. The reaction medium, potassium chloride (0.068 M, 145 ml) and phosphate buffer (0.014 M) of varied pH in dioxane aqueous solution (2:3, v/v) was placed in an apparatus under nitrogen and thermostated at (25 ± 0.1)°C for 30 min. The reaction was initiated on the addition of a stock solution (2 ml, 5.35×10^{-3} M) of the ester in acetonitrile.

3. Results and discussion

The histidine-containing resin (PHis) was prepared and characterized in accordance with the previously reported method [9]. The pK_a value of the protonated imidazolium group of the resin determined according to potentiometric titration is 5.80. Bjerrum's method was applied to the system according to a detailed procedure [10]. Scheme 1 shows the apparatus for the catalytic system. We chose *p*-nitrophenyl acetate (PNPA) as the model compound.

In the presence of a catalytic species, the coefficient of the rate of reaction is, $k_{obs} = k_w + k_{cat}$ in which k_w is the kinetic coefficient for the spontaneous reaction and k_{cat} for the catalytic reaction. By varying the amount of resin, the rate coefficients, k_{obs}, were obtained by plotting $\ln A_\infty/(A_\infty - A_t)$ vs. time (Fig. 1). Only under the condition $\ln A_\infty/(A_\infty - A_t) < 0.3$ did a linear relationship exist, such that k_{obs} is constant; at greater values of $\ln A_\infty/(A_\infty - A_t)$, a curvature appeared. The initial rate of production of *p*-nitrophenol increases with increasing temperature. Catalysis is demonstrated when the rate of hydrolysis is greater in the presence of the potential catalyst than in its absence. Observed coefficients for the rate of



Scheme 1. Apparatus for catalytic experiments. a. Pressure gauge; b. trap; c. manometer; d. thermostat flask; e. reactor.

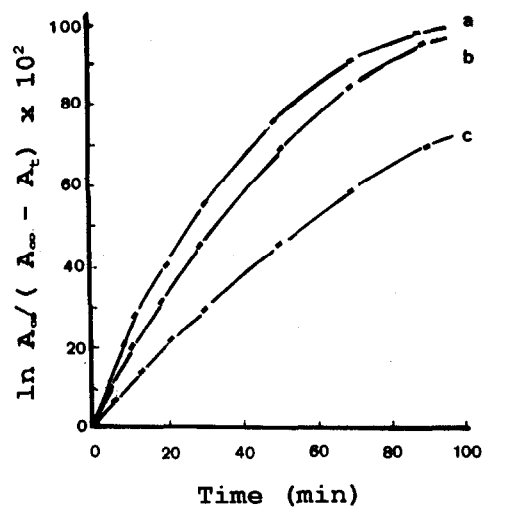


Fig. 1. Plot of $\ln A_\infty/(A_\infty - A_t)$ vs. time for deacylation of PNPA. Phosphate buffer (0.014 M, pH 7.5)–KCl (0.068 M) mixture in dioxane aqueous solution (2:3, v/v), at 25°C. Weight of PHis: a. 0.15 g; b. 0.10 g; c. 0.05 g. Volume of solution: 147 ml.

Table 1
Rates of cleavage of PNPA with various catalysts

Catalyst	Concentration	k_{obs} [a]/10^{-4} min^{-1}
spontaneous hydrolysis		4.3
acetamide	5.0×10^{-3} M	4.3
Amberlite IRC-50	0.1 g/147 ml	3.9
histidine (His)	3.2×10^{-3} M	95
histidine resin (PHis) [b]	0.1 g/147 ml	220

[a] Phosphate buffer (pH 7.5, 0.014 M)–KCl (0.068 M) mixture in dioxane aqueous solution (2:3, v/v), at 25°C.
[b] Bead size: 60–100 mesh; water regain: 49 mmol g^{-1}; nitrogen content: 1.59 mmol g^{-1}; hydrogen capacity: 1.42 mmol g^{-1}; pK_a of imidazolium group: 5.80.

hydrolysis increased from 4.3×10^{-4} min^{-1} with no catalyst to a maximum 2.2×10^{-2} min^{-1} at 0.1 g of PHis per 147 ml of PNPA (Table 1). Acetamide or Amberlite IRC-50 resin gave no significant enhancement of the hydrolysis. Only histidine and histidine resin catalysed the hydrolysis. The rate was enhanced most for the histidine-containing resin among all materials tested. At a constant PNPA concentration, the rate increased linearly with the weight of resin, whereas at a constant amount of resin, the rate is proportional to the amount of PNPA at a concentration less than 3×10^{-4} M until it levels to a plateau at a concentration greater than 3×10^{-4} M (Fig. 2). These phenomena indicate saturated binding of catalyst to substrate and that the mechanism obeys the Michaelis–Menten equation, in which V_s, the maximum reaction velocity, is 4.02×10^{-4} M min^{-1}, and k_s, the Michaelis parameter, is 1.68×10^{-2} M. At this stage, the catalyst is completely acylated and the rate is independent of the substrate concentration. Hydrolysis of PNPA by both histidine (His) and histidine-containing resin (PHis) shows a significant dependence of rate on pH (Fig. 3). At low pH, the reaction with PHis was lower than that with His, but as the media became more basic, a sharply increased reaction rate and gradual saturation at pH greater than 7.8 for PHis were observed. Although neutral and anionic forms of imidazole are present in greater proportions in both

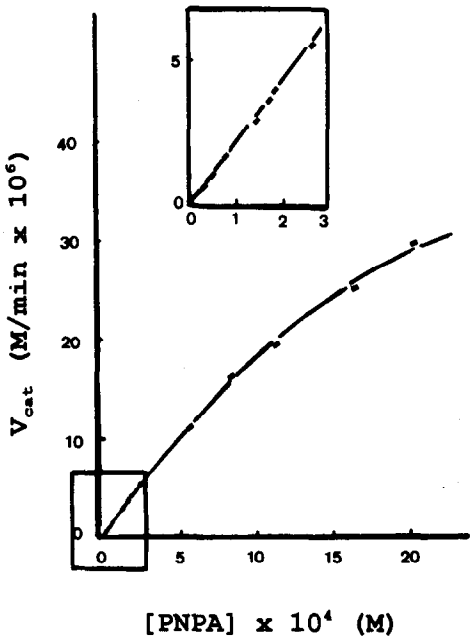


Fig. 2. Dependence of k_{obs} on PNPA concentration. Phosphate buffer (0.014 M, pH 7.5)–KCl (0.068 M) mixture, at 25°C. Amount of PHis: 0.10 g. Volume of solution: 147 ml.

His and PHis at higher pH, which act as the catalytic sites, Phis might exhibit another driving force for the above-mentioned property. The

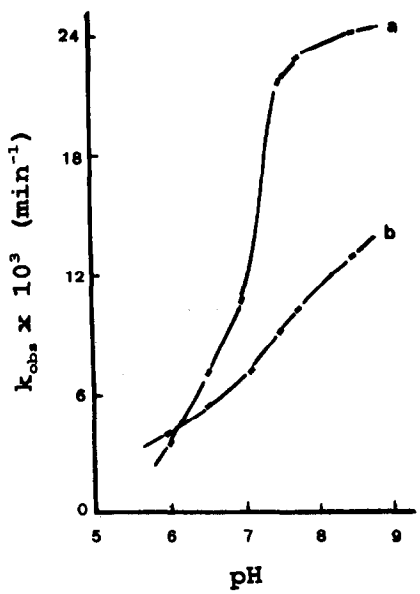


Fig. 3. Rate vs. pH for deacylation of PNPA. Conditions as for Fig. 1. a. Amount of PHis: 0.1 g; b. [His] = 3.24×10^{-3} M.

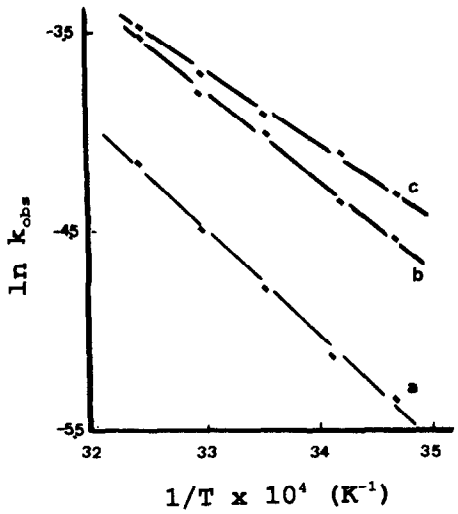


Fig. 4. Effect of temperature on deacylation of PNPA. Conditions as for Fig. 1, except c. at pH 8.0. a. [His]: 3.24×10^{-3} M; b, c. Amount of PHis: 0.1 g.

effect of temperature on the catalytic activity of His and PHis at varied pH toward PNPA is shown in Fig. 4. Below pH 7.8, the rate-controlling step for PHis with activation energy E_a = 37.5 kJ mol^{-1} and pre-exponential factor A = 8.64×10^4 min^{-1}, might be attributed to a pseudo-homogeneous system like that for the monomeric histidine catalyst. The E_a for the monomeric histidine is 45.2 kJ mol^{-1} and A is 7.67×10^5 min^{-1}. At pH above 7.8, that E_a for PHis is 34.7 kJ mol^{-1} might be attributed to diffusion control by the resin matrix [1]. That the activation energy for the histidine-containing resin is smaller than for monomeric histidine indicates that bifunctional groups interact in the resin to offer an easier mechanism for the catalysis [11]. Additionally, this distinction may indicate that deacylation is assisted by the binding force of the polymer chain. That the pre-exponential factor for the resin is smaller than that of monomeric histidine might be due to steric hindrance of the resin matrix. In considering the salt effect upon hydrolysis of PNPA, we find the catalytic behavior of histidine to be similar to that commonly found in catalytic reactions [12], but different from that of histidine-containing resin (Table 2). Lee and Ford found a similar phenomenon in that added chloride ion depressed the rate of hydrolysis by displacing *o*-iodosobenzoate from latex [13]. For reactions in various solvents, the less polar the solvent is, the slower the reaction (Table 2). As there is some variation in the solvent regain, the catalytic conditions chosen are under pH 8.5, for which the rate of reaction is predominantly controlled by diffusion. The rate decreases in the order $H_2O > CH_3OH > C_2H_5OH >$ 1,4-dioxane. The results also indicate that His is less affected by the polarity of the solvent than PHis (Fig. 5). According to these properties, bifunctional cooperation between adjacent histidine groups for the resin might exist through the nucleophilicity of nitrogen in a more polar solvent. Bifunctional cooperation becomes less important in the less polar solvent. By comparing the catalytic activity of the monomeric functional group with the polymer-supported catalyst with the same functional group, we can improve the explanation of the mechanism.

The presence of metal ion in the catalytic system was also tested (Table 3). Although zinc seems to be required for the catalysis, at higher concentrations it inhibited the hydrolysis. Copper and nickel ions are not effective reactiva-

Table 2
Solvent and salt effects for hydrolysis of PNPA [a]

Solvent	Solvent regain/ ml g^{-1}	$k_{obs}/10^{-3}$ min^{-1}	
		His	PHis
H_2O	0.878	9	25
H_2O [b]		10	22
H_2O [c]		10	19
H_2O [d]		11	14
H_2O [e]			24
CH_3OH/H_2O [e,f]	0.856	4	12
C_2H_5OH/H_2O [e,f]	0.791		10
Dioxane/H_2O [e,f]	0.505		5

[a] Phosphate buffer (pH 7.5, 0.014 M)–KCl (0.034 M), at 25°C [His]: 3.24×10^{-3} M; amount of resin: 0.1 g; volume of solution: 147 ml.
[b] Conditions as in a, except [KCl]: 0.068 M.
[c] Conditions as in a, except [KCl]: 0.170 M.
[d] Conditions as in a, except [KCl]: 0.340 M.
[e] Conditions as in b, except phosphate buffer at pH 8.5.
[f] Solvent ratio: 2/3 by v/v.

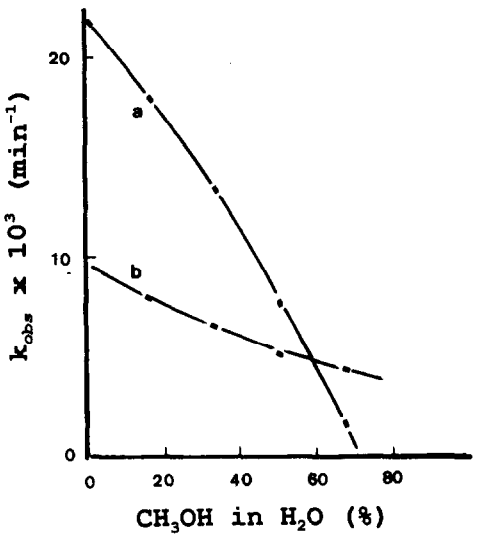


Fig. 5. Variation of reaction rate with composition of solvent. Conditions as for Fig. 1. a. Amount of PHis: 0.1 g; b. [His]: 3.24×10^{-3} M.

tors, perhaps because the chelate formation toward the histidine functional groups might be followed by a conformational change or blockage of the active site of the catalyst. Carboxypeptidase A, among the first metalloenzymes to be discovered, has been extensively investigated by kinetic, structural and spectral methods. There is no direct coordination of the carbonyl oxygen to Zn^{2+} before addition of water. The role of Zn^{2+} is therefore to stabilize negatively charged intermediates formed during hydrolysis rather than to polarize a bound carbonyl [14,15]. In view of the catalytic properties of zinc ion in the metalloenzyme, the above results seem rational. In order to assess the deacylation of PNPA, we also examined the effect of aspartic acid and serine (Table 4), as histidine, serine and aspartic acid make up the active site of the hydrolytic enzyme. They insignificantly affected the reaction rate. These phenomena indicate a less favorable fit of the substrate and the catalytic environment in the transition structure.

Table 3
Effect of metal ions on hydrolysis of PNPA

Metal ion concentration/ 10^{-5} M	k_{obs} [a]	
	His [b] $/10^{-3}$ min^{-1}	PHis [c] $/10^{-2}$ min^{-1}
control	9.51	2.18
Cu(II)		
1.53	9.02	1.93
2.96	8.25	1.70
6.62	5.94	1.05
Ni(II)		
1.61	8.59	2.28
3.12	6.70	2.18
6.61	5.33	1.99
Zn(II)		
1.73	9.13	2.53
3.34	8.79	1.90
7.09	8.13	1.55

[a] Phosphate buffer (pH 7.5, 0.014 M)–KCl (0.068 M) mixture in dioxane aqueous solution (2:3, v/v), at 25°C.
[b] [His]: 3.24×10^{-3} M.
[c] Amount of resin: 0.1 g; volume of solution: 147 ml.

Table 4
Effect of aspartic acid and serine upon deacylation

	k_{obs} [a] $/10^{-3}$ min^{-1}	
	Control	PHis (0.1 g/147 ml)
control	0.43	21.8
aspartic acid and serine [b]	0.40	19.1

[a] Phosphate buffer (pH 7.5, 0.014 M)–KCl (0.068 M) mixture in dioxane aqueous solution (2:3, v/v), at 25°C.
[b] [aspartic acid] = [serine] = 8.9×10^{-4} M.

4. Conclusion

In dioxane–aqueous solution the histidine moiety in the resin is an active site in the hydrolysis of *p*-nitrophenyl acetate. Upon increasing the substrate concentration, typical Michaelis–Menten kinetics are observed. The polymer matrix provides a hydrophobic force for catalytic binding, and adjacent functional groups in the resin play an important role of bifunctional interaction in the catalytic process. Zinc ion at the optimum concentration stabilizes the transition structure.

Although it is impossible to prove an enzyme mechanism based on simple model experiments, detailed mechanistic tests on simple enzyme

models can provide some insight into how enzymes work. In addition, the catalyst will be highly promising in organic synthesis, pharmaceutical use and food chemistry.

Acknowledgements

We thank the National Science Council of the Republic of China for financial support.

References

[1] T. Kunitake, in P. Hodge and D.C. Sherrington (Eds.), Polymer-Supported Reactions in Organic Synthesis, Wiley, Chichester, 1980.
[2] J. Chin, M. Banaszczyk, V. Jubian, J.H. Kim and K. Mrejen, in H. Dugas (Ed.), Bioorganic Chemistry Frontiers, Vol. 2, Springer-Verlag, Berlin, 1991.
[3] C. Gitler and A. Ochoa-Solano, J. Am. Chem. Soc., 90 (1968) 5004.
[4] C.A. Bunton and Y. Ihara, J. Org. Chem, 42 (1977) 2865.
[5] Y. Ihara and R. Hosako, Bull. Chem. Soc. Jpn., 55 (1982) 1979.
[6] M.C. Cleij, W. Drenth and R.J.M. Nolte, J. Org. Chem., 56 (1991) 3883.
[7] K. Ohkubo, H. Ishida, K. Yamaki and M. Kawata, Chem. Lett., (1991) 1723.
[8] K. Ohkubo, Y. Nakano and H. Nagamura, J. Mol. Catal., 28 (1985) 1.
[9] C.Y. Liu, Anal. Chim. Acta, 192 (1987) 85.
[10] C.Y. Liu, M.J. Chen, N.M. Lee, H.C. Hwang, S.T. Jou and J.C. Hsu, Polyhedron, 11 (1992) 551.
[11] R. Breslow, Pure Appl. Chem., 66 (1994) 1573.
[12] M.L. Bender and B.W. Turnquest, J. Am. Chem. Soc., 79 (1957) 1052.
[13] J.J. Lee and W.T. Ford, J. Am. Chem. Soc., 116 (1994) 3753.
[14] E.T. Kaiser and B.L. Kaiser, Acc. Chem. Res., 5, (1992) 219.
[15] D.W. Christianson and W.N. Lipscomb, Acc. Chem. Res. 22, (1989) 62.