Crown Ethers as Regulators of Enzymatic Reactions: Enhanced Reaction Rate and Enantioselectivity in Lipase-Catalyzed Hydrolysis of 2-Cyano-1-methylethyl Acetate

Toshiyuki Itoh,*[,†] Yumiko Takagi,[‡] Takako Murakami,[†] Yuji Hiyama,[†] and Hiroshi Tsukube*[,§]

Department of Chemistry, Faculty of Education, and Department of Chemistry, Faculty of Science, Okayama University, Okayama 700, Japan

Received September 1, 1995 (Revised Manuscript Received December 15, 1995[⊗])

Both reaction rate and enantioselectivity in lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate were significantly changed by addition of some crown ethers. Although various crown ethers accelerated the reaction rate, the enantioselectivity greatly depended on the nature of each one. Among 27 crown ethers and 5 polyethers tested, benzo-crown, armed azacrown, and thiacrown ethers offered high enantioselectivity of the lipase-catalyzed hydrolysis. Hydrophobicity and chirality of the crown ether did not affect the hydrolysis behavior, but its concentration greatly influenced enantioselectivity. Two hundred fifty or more times the crown ether, molarity based on the enzyme, was required to attain satisfactorily high reaction rate and enantioselectivity.

Introduction

The synthetic value of lipases has been well recognized because their reactions proceed efficiently and selectively under mild conditions.[2] Since only a limited number of lipase-catalyzed reactions are applicable for practical optical resolution, several methods were recommended to improve their reaction performance: optimization of reaction conditions, modification of substrate,[3] selection of nonaqueous media,[4] and use of an additive that regulates lipase reactivity.[5] Of these, the additive method is the most advantageous. It is simple to use, but only a few compounds have been reported to enhance enantioselectivity of the lipase reaction.[5] Sih et al. first reported that dextromethorphan and levomethorphan worked as enantioselective inhibitors in *Candida cylindracea* lipase-catalyzed hydrolysis of (±)-arylpropionic acids and (±)-(aryloxy)propionic esters.[5a] We found that (*S*)-2-amino-4-(methylthio)-1-butanol similarly enhanced enantioselectivity in *Pseudomonas cepacia* lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate (**1**).[5b] Each of these remarkably improved enantioselectivity but rarely increased reaction rate. We recently demonstrated that some crown ethers had potential to enhance not only enantioselectivity but also reaction rate in the lipase-catalyzed hydrolysis of acetate **1**.[1]

We examine here a total of 32 crown ethers and their derivatives as additives in this lipase reaction and explain our search to optimize the utility of the crown ethers. Although nitrile **1** was not an appropriate substrate for the lipase PS employed, addition of some crown ethers surprisingly enhanced both enantioselectivity and reaction efficiency. The reaction profiles are investigated in detail to discuss the mechanism behind the crown ether modification of the lipase performance. This is the first systematic study of regulation of an enzymatic reaction by crown ether-type additives, though they are known as complexing agents for several proteins.[6]

Results and Discussion

Actions of a Crown Ether Additive. We chose 2-cyano-1-methylethyl acetate (**1**) and lipase PS as substrate and enzyme in this study, because nitrile **1** is a useful chiral building block and lipase PS is applicable to various substrates.[2c,d] This purified lipase showed a single band in a SDS disk gel electrophoresis experiment and has a molecular weight of 32000.[1] It effectively catalyzed hydrolysis of many unnatural ester substrates but often exhibited modest enantioselectivity.[5b] Although nitrile **1** was not a favorable substrate for lipase PS, some crown ethers were found to significantly enhance both enantioselectivity and reaction efficiency in the hydrolysis.

† Department of Chemistry, Faculty of Education, Okayama University.

‡ Department of Chemistry, Faculty of Education, Okayama University, Research Fellow of the Japan Society for the Promotion of Science. Present address: Department of Chemistry, Faculty of Teacher Education, Hyogo University of Education, Yashiro-cho, Hyogo 673−14, Japan.

§ Department of Chemistry, Faculty of Science, Okayama University. Present address: Department of Chemistry, Faculty of Science, Osaka City University, Osaka 558, Japan.

⊗ Abstract published in *Advance ACS Abstracts*, February 15, 1996.

(1) A preliminary account has appeared: Itoh, T.; Hiyama, Y.; Betchaku, A.; Tsukube, H. *Tetrahedron Lett.* **1993**, *34*, 2617.

(2) (a) Whiteside, G. M.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 617. (b) Jones, J. B. *Tetrahedron* **1986**, *42*, 3351. (c) Chen, C.-S.; Sin, C. J. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 695. (d) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114. (e) Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev.* **1992**, *92*, 1071 and references cited therein.

(3) For example, see: Itoh, T.; Takagi, Y.; Nishiyama, S. *J. Org. Chem.* **1991**, *56*, 1521 and references cited therein.

(4) (a) Sakurai, T.; Margolin, A. L.; Russell, A. J.; Klivanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 7236. (b) Parida, S.; Dordick, J. S. *J. Am. Chem. Soc.* **1991**, *113*, 2253. (c) Parida, S.; Dordick, J. S. *J. Org. Chem.* **1993**, *58*, 3238. (d) Kamat, S. *Biotechnol. Bioeng.* **1992**, *40*, 158. (e) Arroyo, M.; Sinisterra, J. V. *J. Org. Chem.* **1994**, *59*, 4410. Control of enzyme enantioselectivity by pressure change has recently been reported. (f) Kamat, S. V.; Beckman, E. J.; Russell, A. J. *J. Am. Chem. Soc.* **1993**, *115*, 8845 and references cited therein.

(5) Dextrometrophan,[5a] (*S*)-2-amino-4-(methylthio)-1-butanol,[5b] sodium chloride,[5c,f] calcium chloride,[5d] potassium chloride,[5e] and Triton X-100[5g] were reported as effective additives. (a) Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* **1989**, *111*, 6839. (b) Itoh, T.; Ohira, E.; Takagi, Y.; Nishiyama, S.; Nakamura, K. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 624. (c) Tsukube, H.; Betchaku, A.; Hiyama, Y.; Itoh, T. *J. Chem. Soc., Chem. Commun.* **1992**, 1751. (d) Holmberg, E.; Holmquist, M.; Hedenstrom, E.; Berglund, P.; Norin, T.; Hogberg, H.-E.; Hult, K. *Appl. Microbiol. Biotechnol.* **1992**, *35*, 572. (e) Khmelnitsky, Y. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647. (f) Tsukube, H.; Hiyama, Y.; Betchaku, A.; Itoh, T. *J. Org. Chem.* **1994**, 59, 7014. (g) Bhaskar, Rao A.; Rehman, H.; Krishnakumari, B.; Yadav, J. S. *Tetrahedron Lett.* **1994**, *35*, 2611.

(6) (a) Odell, B.; Earlam, G. *J. Chem. Soc., Chem. Commun.* **1985**, 359. (b) Reinhoudt, D. N.; Eondebak, A. M.; Nijenhuis, W. F.; Verboom, W.; Kloosterman, M.; Schoemaker, H. E. *Ibid.* **1989**, 399.

0022-3263/96/1961-2158$12.00/0

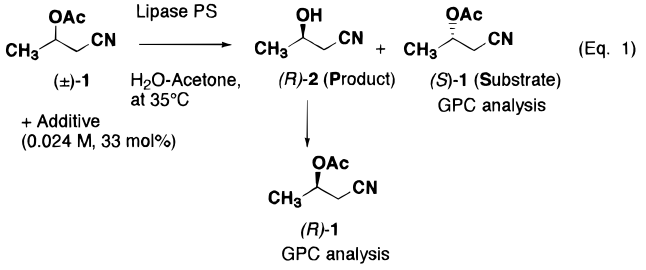


Figure 1.

The hydrolysis of **1** was generally carried out in a nonbuffered aqueous solution to exclude an effect of complexation between crown ether and metal cation (eq 1). Typically, 5.0 mL of an aqueous lipase solution[7] was added to a solution of (±)-**1** (65.0 mg, 0.511 mmol), together with a crown ether additive (33 mol % toward the substrate) in 0.5 mL of acetone. The resulting mixture was stirred at 35 °C, and the reaction was stopped when spots of ester **1** and alcohol **2** became the same size on a silica-gel TLC plate. The alcohol (*R*)-**2** produced and remaining ester (*S*)-**1** were extracted with ethyl acetate and separated by silica-gel TLC (hexane/ethyl acetate = 2:1). The enantiomeric excess of the remaining acetate **3** was determined by capillary GPC analysis using chiral stationary phase (Chiraldex G-Ta). The enantiomeric excess of alcohol (*R*)-**2** was also determined by GPC analysis of the corresponding acetate (Figure 1). Typical results obtained for reaction at 35°C are summarized in Table 1.

We examined ten crown ethers **3**−**12**, one cryptand **13**, eight heteromacrocycles **15**, **16**, **20**−**24**, **26**, eight armed macrocycles **14**, **17**−**19**, **25**, **27**−**29**, and five acyclic analogs **30**−**34** as additives (Figure 2). They vary widely in structures and guest binding properties.

The effect of these crown ethers on the enantioselectivity of the lipase-catalyzed hydrolysis was evaluated by *E* value,[8] while the relative rate was calculated from percentage conversion per reaction time compared with that in the absence of additive.[9] It is noteworthy that the reaction rate was enhanced in the presence of most additives employed, but the enantioselectivity depended strongly on the nature of the additive (see Table 1). Among 32 additives, benzocrown ether (entry 10), armed azacrown ethers (entries 21−26), and thiacrown ethers (entries 28−33) apparently enhanced both enantioselectivity and reaction rate. The highest *E* value recorded was 37 when the hydrolysis was carried out in the presence of 1,4,8,11-tetrathiacyclotetradecane (**23**) (entry 30). Other thiacrown ethers also offered satisfactorily large *E* values: 34 for **24**; 34 for **25**; and 27 for **22**.

(7) The enzyme content in lipase PS employed here was less than 10% by weight, and the remainder was mostly amorphous inorganic compounds (Celite). The content of the enzyme molecule is thus estimated as less than 3.1×10^{-3} mmol/g of lipase PS.

(8) Chen, C. -S.; Fujimoto, Y.; Girdauskas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *102*, 7294. Chen, C-S.; Wu, S-H.; Girdauskas, G.; Sih, C. J. *Ibid*. **1987**, *109*, 2812. The enantiomeric ratio was calculated from $E = \ln[(1 - c)(1 - \text{ee}(S)\text{-}\mathbf{1})]/\ln[(1 - c)(1 + \text{ee}(S)\text{-}\mathbf{1})]$, where *c* means conversion ratio and $c = \text{ee}(S)\text{-}\mathbf{1}/(\text{ee}(S)\text{-}\mathbf{1} + \text{ee}(R)\text{-}\mathbf{2})$.

(9) We have reported the *E* value of this control experiment as 7−8 based on results of ^{19}F NMR analysis of (+)-α-methoxy-α-phenyl-α-(trifluoromethlyl)acetate (MTPA ester method).[1] GPC analysis of all experiments gave slightly higher E values than those obtained using MTPA ester method.[10] In the MTPA ester method, the remaining acetate was first converted to alcohol by LAH reduction and then to the corresponding MTPA ester.[5b] Since kinetic resolution may occur during the MTPA formation process, capillary GPC analysis provided us highly reliable results.

(10) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1972**, *95*, 512.

Table 1. Effect of Crown Ether Additives on Lipase-Catalyzed Hydrolysis

entry	additive	time (h)	*c* (% conv)	relative rate (*c*/h)	% ee of (*R*)-**2** (% yield)	% ee of *S*-**1** (% yield)	*E*
1	none	14	18	1.3	77 (18)	17 (48)	9
2	none	60	38	0.6	80 (3.5)	16 (40)	16
3	none	96	41	0.4	68 (41)	8 (51)	8
4	none[a]	3	8	2.7	75 (18)	6 (62)	8
5	none[a]	22	37	1.7	81 (20)	48 (43)	17
6	none[a]	33	46	1.4	79 (32)	68 (51)	17
7	3	14	18	1.3	80 (15)	18 (61)	11
8	4	14	25	1.8	87 (13)	29 (56)	19
9	5	14	11	0.8	72 (11)	9 (60)	7
10	6	13	52	3.7	80 (48)	89 (32)	28
11	7	14	16	1.1	84 (16)	16 (58)	13
12	8	14	45	3.5	79 (38)	65 (37)	17
13	9	14	34	2.4	75 (30)	40 (38)	11
14	10	14	30	2.1	72 (23)	30 (41)	8
15	11	14	46	3.3	76 (39)	64 (34)	14
16	12	14	52	3.7	70 (42)	77 (36)	13
17	13	14	62	4.4	56 (52)	91 (28)	10
18	14	14	50	3.6	73 (43)	72 (32)	14
19	15	14	41	2.2	69 (37)	48 (29)	9
20	16	14	67	4.8	47 (53)	95 (29)	9
21	17(*RS*)	14	54	3.9	77 (54)	91 (44)	24
22	18(*RS*)	14	44	3.1	87 (40)	68 (39)	28
23	18(*R*)	19	46	2.4	81 (40)	68 (26)	20
24	18(*S*)	11	42	3.8	83 (42)	60 (30)	20
25	18(*RS*)[a]	6.5	35	5.4	83 (32)	46 (43)	18
26	19(*RS*)	14	46	3.3	87 (34)	72 (42)	30
27	20	14	50	3.6	74 (40)	75 (32)	15
28	21	13	41	3.2	84 (24)	59 (32)	22
29	22	14	60	4.3	65 (39)	99 (33)	27
30	23	14	59	4.2	71 (39)	99 (40)	37
31	24	14	57	4.1	74 (40)	99 (21)	34
32	25	14	57	3.1	79 (26)	99 (21)	34
33	26	13	37	2.8	84 (24)	50 (38)	20
34	27	13	42	3.2	84 (7)	61 (55)	21
35	28(*RS*)	15	32	2.1	85 (18)	41 (47)	18
36	28(*S*)	24	46	1.9	80 (45)	67 (44)	18
37	29	15	23	1.5	84 (23)	25 (57)	15
38	30	14	46	3.3	56 (38)	48 (42)	7
39	31	14	52	3.7	74 (34)	80 (43)	15
40	32	14	22	1.6	85 (19)	24 (57)	15
41	33	14	18	1.3	70 (18)	18 (55)	9
42	34	14	52	3.7	79 (30)	84 (33)	22

[a] Reaction was carried out at pH 7.2 in 0.1 M phosphate buffer.

Reaction Profile of Lipase-Catalyzed Hydrolysis in the Presence of Crown Ether. The *E* value is the most convenient factor by which to evaluate the enantioselectivity in the hydrolysis of racemic substrates. This is known to be constant when the reaction possesses a small K_m,[8] but the *E* value varied greatly in the reaction conversion in this hydrolysis (see entries 1−6 in Table 1). This seems due to large K_m of the enzymatic reaction between the lipase and substrate **1**.[8] We determined *E* values in three different reaction media: in pure water, in a buffer solution, and in an aqueous solution of crown ether additive (Figure 3). *E* values obtained were extremely reduced at over 40% conversion when the reaction was carried out in the absence of buffer or additive. This must be a result of reverse reaction.

The reaction in the buffer solution (pH 7.2) also offered a constantly high *E* value at 30−50%, while a lower E value was observed at lower conversion.[9] It was noteworthy that thiacrown **23** maintained satisfactorily high enantioselectivity even at 60% conversion. Since the hydrolysis in the presence of thiacrown **23** provided a much higher *E* value than those in the buffer solution, this was confirmed as a useful additive that improves this enzymatic reaction.

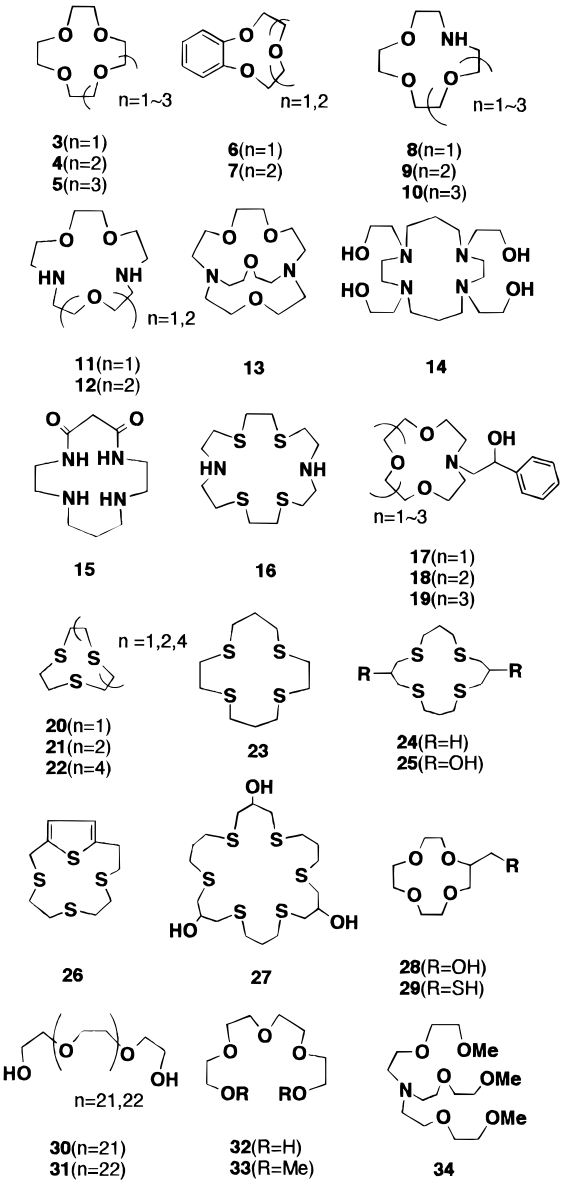
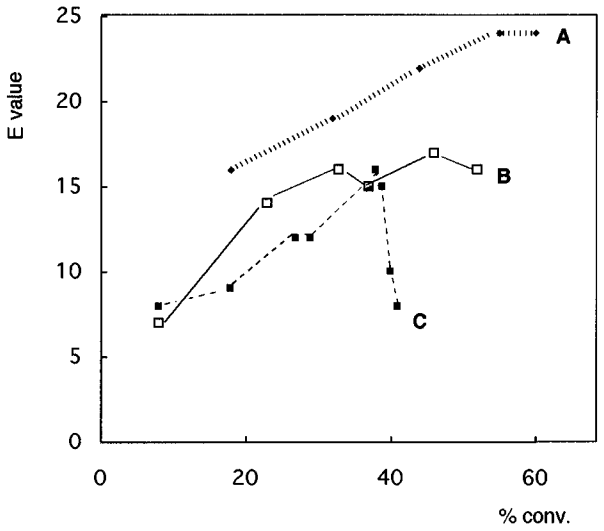


Figure 2. List of tested compounds.

Chirality of crown ether did not affect either enantioselectivity or reaction efficiency of the hydrolysis (entries 22−25 and 35−36 in Table 1). We examined two kinds of chiral crown ethers: crowns **17-19** have asymmetric carbons on their sidearms; crown **28** has an asymmetric carbon in the parent crown ring. The similar *E* values and reaction rates were typically recorded when (*R*)-**28** and (*S*)-**28** were employed as additives (entries 35 and 36 in Table 1).[11] The chirality of the sidearm of crown **18** also slightly affected the reactivity (entries 22−25 in Table 1). Since no significant difference was observed between enantiomers of chiral crown ethers, the stereochemistry of the crown ether additives had no effect on hydrolysis rate or enantioselectivity.

Figure 4, parts a and b, illustrate the effect of additive concentration on the *E* value and reaction rate. There are very interesting contrasts between the additive activities of crown ethers **23**, **28**, and **15**. The relative rate increased similarly with increased additive concentration for the three cases but changes in the enantioselectivity differed. Addition of 5−33 mol % of thiacrown **23** increased the *E* value from 16 to 29. Because the *E* value showed a decrease in the presence of larger amounts of **23**, 250 or 1650 equiv of thiacrown ether **23**, based on the lipase, were optimum.[7] Crown ether **28** and polyamine **15** acted as regulators in different fashions: the former changed the *E* value only slightly, and the latter decreased it more noticeably. The nature of crown ether additive clearly had an impact on the reaction behavior.



A: Crown **23**, 33mol%. **B**: Buffer at pH=7.2.
C: No buffer and no additive.

Figure 3. *E* value dependence of the lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate (**1**) upon reaction conversion.

Why Crown Ethers Modify Lipase Performance. Crown ethers bind metal cations with varying strength depending on structural variations. The lipase solution employed was confirmed to contain the following alkali and alkaline earth metal cations: Na^+, 6.5×10^{-4} mol/L; K^+, 4.6×10^{-4} mol/L; Mg^{2+}, 6×10^{-4} mol/L; Ca^{2+}, 6.3×10^{-4} mol/L. These metal cations are good guests for crown ethers **17**−**19** but not for thiacrown ethers **21**−**27**, though both types of additive enhanced the *E* value and reaction rate of the hydrolysis. Some metal salts were recently reported to influence the catalytic activities of enzymes,[5c−f] but crown ether−metal cation complexation may not be involved in this.

The reactivity of an enzyme is known to be significantly influenced by the nature of a solvent.[4] Since polyethylene glycols and related polyethers provided unique microenvironments for proteins, we compared the additive activity of the crown ether with its hydrophobicity. The hydrophobicity is a good indicator by which to evaluate solvent character and is generally characterized by log *P*.[12] Since the log *P* values of the employed crown ethers have not been reported, we estimated "relative hydrophobicity" from R_f value of the additive recorded on reversed phase thin layer chromatography (see Experimental Section). Figure 5 shows plots of E value and relative hydrophobicity, $1/R_f$, for various additives. Although the additives **12**, **15**, and **25** having polar amino, amide, and hydroxyl groups exhibited similarly higher hydrophobicity values, thiacrown ether **25** offered a much

(11) (*S*)-**28** was derived from (*S*)-isopropylideneglycerol by a modification of the published method: Miyazaki, T.; Yanagita, S.; Itoh, A.; Okahara, M. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2005. For the method of preparation, see reference 5f.

(12) Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81.

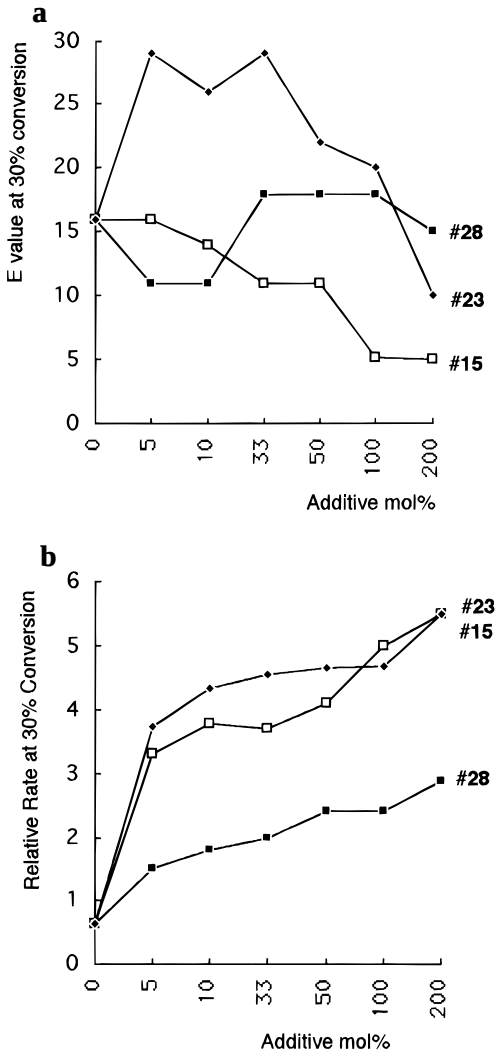


Figure 4. (a) Effect of concentration of additive on the *E* value of lipase-catalyzed hydrolysis. (b) Effect of concentration of additive on rate of lipase-catalyzed hydrolysis.

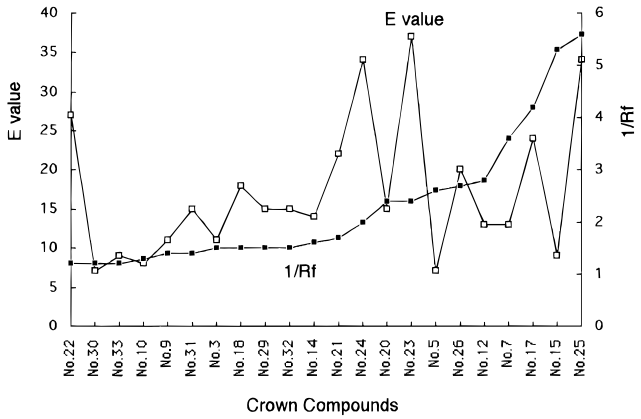


Figure 5. Relationship of relative hydrophobicity of additive ($1/R_f$) with the *E* value in hydrolysis.

higher *E* value than additives **12** and **15**. All other additives showed lower "relative hydrophobicity" values but their *E* values varied broadly. Thus, there is no significant correlation between hydrophobicity and the *E* value. Since the reaction rate was also independent of the relative hydrophobicity, the lipase employed was believed not to accommodate these additives site-specifically near the active site or allosteric site.

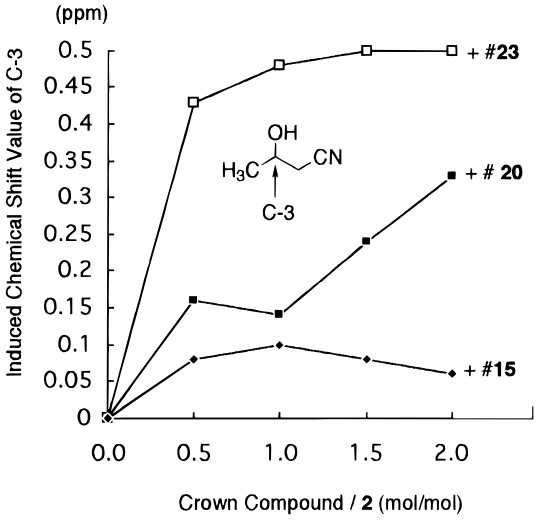


Figure 6. Induced chemical shift values of ^{13}C NMR of 3-hydroxybutyronitrile **2** by thiacrown **20**, **23**, and azacrown **15**.

Crown ethers bind neutral organic molecules in the crystal states and nonpolar media.[13] Since our substrate **1** has a nitrile group, and product **2** has a further hydroxyl moiety, crown ether may trap such neutral molecules having acidic moieties in the course of the reaction. We conducted ^{13}C NMR binding experiments in $CDCl_3$ solution using polyamine **15** and thiacrowns **20** and **23**. These three additives accelerated the hydrolysis, but only thiacrown **23** enhanced its enantioselectivity. Figure 6 illustrates typical spectral changes of product **2** in the presence of crown ether additive. Large induced ^{13}C NMR spectral changes were observed at C-3 position by addition of these crown ethers, while no significant change was found in the case of substrate **1**. It is suspected that they may bind the product and depress the reverse reaction. Thiacrown **23** was suggested to bind product **2** particularly strongly; this may explain the high additive activity of thiacrown **23**. Since the lipase itself preferentially hydrolyzes (*R*)-enantiomer of **1**,[5b] (*R*)-alcohol **2** should be preferentially acylated by the lipase. However, it is impossible to run the reverse reaction with 0.1 M acetate as the acetylating agent because the concentration of water in a 10% aqueous acetone solution is in excess of 45 M under the experimental conditions. Both reaction rate and enantioselectivity can be enhanced if the reverse reaction occurs inside the enzyme near or at the entrance of the active site and is inhibited by trapping of the product with the crown ether. Large induced shifts of carbon signals for **2** were similarly observed with other additives, **6**, **20**, **23**, **24**, and **26**. Since these additives mediated highly enantioselective hydrolysis (see Table 1), the induced chemical shift provides a good guideline for selection of a potential additive.

Molecular Basis for Enhanced Enantioselectivity. There are three possibilities in additive-enhanced enantioselectivity of the lipase hydrolysis. (1) The first is the enantioselective inhibition,[5a] in which the crown ether additive may be specifically located near the active site of the lipase and modify its reactivity. Since the lipase should provide a chiral cavity, chirality and hydrophobicity of the crown ether additive should reflect

(13) Goldberg, I. In Crown Ethers and Analogs; Patai, S., Rappoport, Z., Eds.; John Wiley & Sons: Chichester, 1989; p 399.

on the reactivity. Because the lipase hydrolysis did not depend on the chirality and hydrophobicity of the additive, this possibility can be ruled out. (2) The second is inhibition of the reverse reaction, which may occur inside in the enzyme close to or at the entrance to the active site, by the presence of crown ethers. The interaction between the crown ether and product alcohol (*R*)-**2** seems adequate to inhibit the reverse reaction, and it explains both accelerations of the hydrolysis rate and enhancement of the enantioselectivity. However, it should be noted that large amounts of crown ether decreased the enantioselectivity, although it increased the rate; thus, another mechanism must be cooperatively involved. (3) The last possibility is modification of the lipase local conformation. Enzyme reactivity was sometimes modified by addition of lipid[14] or salt.[5d] The employed crown ethers may interact with certain sites of the lipase as proposed in some proteins, thereby activating the lipase and changing its enantioselectivity. Previously it was reported that covalent modification of the arginine side chain in the lid of lipases from *Humicola lanuginosa* and from *Rhizomucor miehei* decreased their enantioselectivity toward esters of 2-methyldecanoic acid.[15] The crystal structure of *Candida rugosa* lipase has shown two different conformations.[16] In one, called "closed", the helical surface loop partially covers the hydrophobic crevice containing the active site, while in the other conformation, called "open", the surface loop moves and the crevice is uncovered.[17] It was recently reported by Kazlauskas and his colleagues[18] that 2-propanol treatment of *Candida rugosa* lipase caused modification of enantioselectivity. 2-Propanol treatment was proposed to convert the closed form of this lipase to the open form, thereby enhancing the enantioselectivity.[18] Although *Pseudomonas cepacia* lipase has not been characterized in detail, such conformational changes may be induced by crown ether additive.

While we have not ruled out other possibilities, both the second and third points mentioned explain our observations in Figure 4, parts a and b. When crown ethers remain at the entrance of the lipase, they may modify local conformation of the lipase, trap the product alcohol, and accelerate diffusion of the alcohol into the bulk water phase (Figure 7).

The present study demonstrated that the crown ether derivatives have the potential to enhance both reaction rate and enantioselectivity of the lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate. These crown compounds cannot change the original enantioselectivity of enzyme, but enhance its potential ability to a level at which the reaction can be used practically.[19] This work represents not only a significant advance in improvement of lipase-catalyzed organic synthesis but also provides an interesting combined use of crown ether with enzyme. The approach is therefore recommended as a new technique by which to regulate enzymatic reactions by chemical reagents.

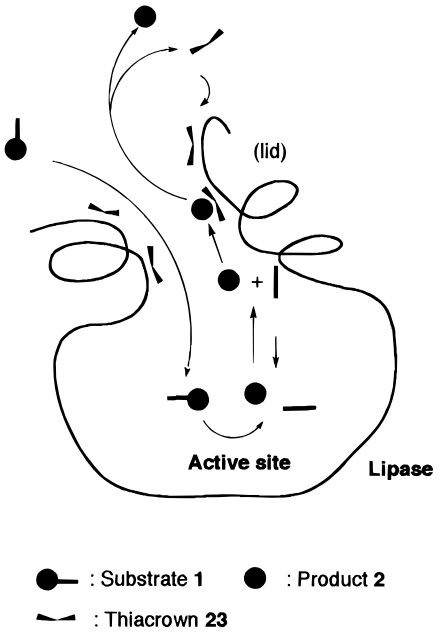


Figure 7. Plausible mechanism of acceleration of the lipase-catalyzed reaction by thiacrown **23**.

Experimental Section

Materials. Crown ethers were purchased from Aldrich or Jensen and used without further purification. Azacrown ethers **17**−**19** were prepared by reaction of corresponding unsubstituted azacrown ethers (Aldrich) and (1,2-epoxyethyl)-benzene. *Pseudomonas cepacia* lipase was provided by Amano Pharmaceutical Co., Ltd. (Japan).

Enzyme-Catalyzed hydrolysis. A typical example is described below: To an acetone solution (0.5 mL) of ester **1** (65.0 mg, 0.511 mmol) and 1,4,8,11-tetrathiacyclotetradecane (38.4 mg, 0.168 mmol) was added the enzyme solution (5.0 mL), and the resulting mixture was incubated at 35 °C. The reaction was stopped by addition of small pieces of ice and then extracted with ethyl acetate, dried over $MgSO_4$, and evaporated to dryness. The product (*R*)-**2** and remaining substrate (*S*)-**1** were separated by silica-gel TLC (hexane:ethyl acetate = 2:1). The enzyme solution was prepared in the following way: A boltex shaking suspension of lipase PS (50 mg) in deionized water (7.7 mL) was centrifuged at 3000 rpm for 5 min at room temperature, and then 5.0 mL of the supernatant was immediately used as the enzyme solution. GPC analysis for determination of % ee of 2-cyano-1-methylethyl acetate was carried out using a capillary column on chiral phase; Chiraldex G-Ta, ∅ 0.25 mm × 20 m; carrier gas: He 40 mL/min; temp (°C): 100; inlet pressure: 1.35 kg/cm^2; amount 400 ng; detection, FID; (*R*)-2-cyano-1-methylethyl acetate: t_{R^1} 5.2; K'_1 1.1, (*S*)-2-cyano-1-methylethyl acetate: t_{R^2} 9.4; K'_2 2.8, K_2/K_1 = 2.5.

Relative Hydrophobicity of Crown Compounds. Relative hydrophobicity is defined by $1/R_f$ value recorded on reversed phase TLC of Merck-LKC18 using a mixed solvent of acetone and water (4:1). Triplicate TLC experiments were conducted, and R_f values shown are the average. Crowns **3**: R_f 0.65, **5**: R_f 0.38, **7**: R_f 0.28. Azacrowns **9**: R_f 0.69, **10**: R_f 0.79, **12**: R_f 0.36. Armed macrocycles **14**: R_f 0.63, **17**: R_f 0.24, **18**: R_f 0.67, **28**: R_f 0.18, **29**: R_f 0.67. Heteromacrocycle **15**:

(14) Okahata, Y.; Ijiro, K. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2411; Hatano, A.; Ijiro, K.; Okahata, Y. The 6th Conference of Synthetic Organic Chemistry Using Biocatalysts, Seto Japan, January 1994, Abst No. 2017

(15) Holmquist, M.; Martinelle, M.; Berglund, P.; Clausen, I. G.; Patkar, S.; Svendsen, A.; Hult, K. J. *J. Protein Chem.* **1993**, *12*, 749.

(16) (a) Rubin, B.; Jamison, P.; Harrison, D. *Lapases: Structure, Mechanism and Genetic Engineering*; Alberghina, L.; Schmid, R. D.; Verger, R., Eds.; VCH: New York, 1991; pp 63−66. (b) Grochulski, P.; Li, Y.; Schrag, J. D.; Bouthillier, F.; Smith, P.; Harrison, D.; Rubin, B.; Cygler, M. *J. Biol. Chem.* **1993**, *268*, 12843.

(17) Grochulski, P.; Li, Y.; Schrag, J. D.; Cygler, M. *Protein Sci.* **1994**, *3*, 82.

(18) Colton, I. J.; Sharmin, N. A.; Kazlauskas, R. J. *J. Org. Chem.* **1995**, *60*, 212.

(19) We employed thiacrown **23** as an additive in hydrolysis of 2-substituted-2-propenyl acetates or 1-alkyl-3-cyanopropyl acetates. Hydrolysis rate of all substrates was accelerated by addition of 5 mol % of thiacrown **23**, but enantioselectivity strongly depended on the nature of the substrate. Significant enhancement of enantioselectivity was observed when the original *E* values were from 7 to 15.

Table 2. ^{13}C NMR Spectra of Acetate 1 in the Presence of Crown Ethers (in $CDCl_3$)

additive (equiv)	C-6(Ac), ppm	C-4, ppm	C-2, ppm	C-3, ppm	C-1(CN), ppm	C-5(CO), ppm	shift at C-3, ppm	shift at C-1, ppm	shift at C-5, ppm
none	19.08	20.83	24.27	65.43	116.17	169.92	reference	reference	reference
5 (2.0)	18.90	20.65	24.07	65.27	116.03	169.69	−0.16	−0.15	−0.24
6 (2.0)	19.17	20.90	24.36	65.51	116.13	170.04	0.08	−0.05	0.12
7 (2.0)	19.05	20.78	24.23	65.40	116.09	169.87	−0.03	−0.08	−0.06
9 (2.0)	19.03	20.76	24.19	65.37	116.07	169.70	−0.06	−0.10	−0.22
15 (2.0)	19.21	20.99	24.41	65.51	116.07	170.10	0.08	−0.10	0.18
18 (2.0)	19.21	20.98	24.39	65.50	116.23	170.09	0.06	0.06	0.17
20 (2.0)	19.08	20.82	24.27	65.38	116.07	169.86	−0.05	−0.10	−0.07
23 (2.0)	19.10	20.88	24.29	65.38	116.14	169.92	−0.05	−0.04	0.00
24 (2.0)	19.16	20.91	24.34	65.45	116.16	169.91	0.02	−0.01	−0.01
25 (2.0)	19.01	20.78	24.19	65.34	116.08	169.88	−0.09	−0.10	−0.05
32 (2.0)	19.09	20.84	24.25	65.47	116.21	169.96	0.04	0.04	0.04
33 (2.0)	19.06	20.78	24.20	65.43	116.16	169.84	0.00	−0.01	−0.09

R_f 0.19. Thiacrowns **20**: R_f 0.41, **21**: R_f 0.58, **22**: R_f 0.82, **23**: R_f 0.41, **24**: R_f 0.49, **25**: R_f 0.18, **26**: R_f 0.37. Acyclic polyethers **30**: R_f 0.83, **31**: R_f 0.73, **32**: R_f 0.67, **33**: R_f 0.83.

Synthesis of (*R*)-4,7,10,13-Tetraoxa-*N*-(2-phenyl-2-hydroxyethyl)azacyclopentadecane (18). To a solution of 1-aza-15-crown-5 (**9**) (97%, 468 mg, 2.07 mmol) and (*R*)-(1,2-epoxyethyl)benzene (249 mg, 2.07 mmol) in dry ether (10 mL) was added of activated alumina powder[20] (1.0 g, activity 1). The resulting mixture was stirred for 23 days at room temperature and then filtered through a glass sintered filter and evaporated to dryness. Silica-gel flash column chromatography (hexane:ethyl acetate:methanol, 4:4:1) gave (*R*)-**18** (530 mg, 1.56 mmol) in 75% yield as colorless oil: $[\alpha]^{23}_D$ −34.5° (*c* 0.96, $CHCl_3$), ^1H NMR (200 MHz, δ, $CDCl_3$, *J* = Hz) 2.47 (1 H, dd, *J* = 13.1, 12.9), 2.66 (1 H, dd, *J* = 6.1, 5.4), 2.73 (1 H, OH, s), 2.73 (1 H, t, *J* = 4.8), 2.75−2.85 (2 H, m), 3.55−3.73 (16 H, m), 4.61 (1 H, dd, *J* = 10.4, 3.1), 7.1−7.4 (5 H, m); ^{13}C NMR (50 MHz, $CDCl_3$, ppm) 55.75, 65.26, 69.91, 70.11, 70.41, 70.54, 70.78, 125.92, 127.13, 128.12, 142.33; IR (neat, cm^{-1}) 3420, 2900, 1460, 1360, 1120, 940, 710. Anal. Calcd for $C_{18}H_{29}NO_5$: C, 63.69; H, 8.61; N, 4.13. Found: C, 63.66; H, 8.60; N, 4.11.

(*S*)-**18** was prepared from (S)-(1,2-epoxyethyl)benzene by the procedure described above. (*S*)-**18** ; $[\alpha]^{23}_D$ +35.4° (*c* 1.01, $CHCl_3$). The spectroscopic data were exactly the same as (*R*)-**18**.

4,7,10,13-Trioxa-*N*-(2-phenyl-2-hydroxyethyl)azacyclododecane (17) was prepared from crown **8** and (1,2-epoxyethyl)benzene (oil, 77%): ^1H NMR (200 MHz, δ, $CDCl_3$, *J* = Hz) 2.44 (1 H, dd, *J* = 12.9, 10.7), 2.58 (1 H, dd, *J* = 4.3, 4.0), 2.66 (1 H, dd, *J* = 7.1, 4.1), 2.69 (1 H, dd, *J* = 13.8, 3.4), 2.83 (1 H, dd, *J* = 14.1, 3.4), 2.87 (1 H, dd, *J* = 14.2, 3.4), 3.54−3.72 (12 H+OH, m), 4.63 (1 H, dd, *J* = 10.6, 3.3), 7.14−7.3 (2 H, m), 7.3−7.4 (3 H, m); ^{13}C NMR (50 MHz, $CDCl_3$, ppm) 55.68, 64.50, 69.44, 70.44, 70.63, 70.83, 125.86, 127.04, 128.07, 142.32; IR (neat, cm^{-1}) 3430, 3030, 2850, 1450, 1360, 1090, 1030, 910, 700. Anal. Calcd for $C_{16}H_{25}NO_4$: C, 65.06; H, 8.53; N, 4.74. Found: C, 65.09; H, 8.60; N, 4.70.

4,7,10,13,16-Pentaoxa-*N*-(2-phenyl-2-hydroxyethyl)azacyclooctadecane (19) was prepared from crown **10** and (1,2-epoxyethyl)benzene (oil, 56%): ^1H NMR (200 MHz, δ, $CDCl_3$, *J* = Hz) 2.50 (1 H, dd, *J* = 12.5, 10.5) 2.63 (1 H, t, *J* = 3.0) 2.70 (1 H, t, *J* = 4.8) 2.86 (1 H, dd, *J* = 11.9, 4.9) 2.90 (1 H, dd, *J* = 13.6, 4.9) 3.5−3.7 (20 H+OH, m) 4.61 (1 H, dd, *J* = 10.1, 3.0) 7.1−7.4 (5 H, m); ^{13}C NMR (50 MHz, $CDCl_3$, ppm) 53.40, 55.04, 64.52, 69.78, 69.91, 70.29, 70.50, 70.56, 70.64, 70.78, 125.91, 127.16, 128.16, 142.46; IR (neat, cm^{-1}) 3400, 2850, 1440, 1340, 1240, 1100, 930, and 750. Anal. Calcd for $C_{20}H_{33}NO_6$: C, 62.64; H, 8.67; N, 3.65. Found: C, 62.33; H,8.72; N,3.68.

NMR Binding Experiments. ^{13}C NMR studies were carried out with a Varian VXR-200 spectrometer (SC-NMR Laboratory of Okayama University). Crown ethers were dissolved in $CDCl_3$ at a concentration of 0.05−0.06 mol/L. Results are summarized in Tables 2 and 3.

Table 3. ^{13}C NMR spectra of alcohol 2 in the presence of crown ethers (in $CDCl_3$)

additive (equiv)	C-4, ppm	C-2, ppm	C-3, ppm	C-1(CN), ppm	shift at C-3, ppm	shift at C-1, ppm
none	22.41	27.24	63.65	117.80	reference	reference
5 (2.0)	22.38	27.19	63.38	117.78	−0.27	−0.02
6 (2.0)	22.63	27.39	64.00	117.54	0.36	−0.26
7 (2.0)	22.48	27.23	63.70	118.04	0.05	0.24
9 (2.0)	22.63	27.36	63.07	117.88	−0.58	0.08
15 (0.5)	22.83	27.59	63.73	117.89	0.08	0.09
15 (1.0)	22.87	27.62	63.75	117.89	0.10	0.09
15 (1.5)	22.88	27.64	63.73	117.91	0.08	0.11
15 (2.0)	22.88	27.63	63.71	117.92	0.06	0.12
18 (2.0)	22.74	27.47	64.02	117.53	0.37	−0.27
20 (0.5)	22.54	27.33	63.81	117.59	0.16	−0.21
20 (1.0)	22.50	27.33	63.79	117.54	0.14	−0.26
20 (1.5)	22.49	27.27	63.88	117.49	0.24	−0.31
20 (2.0)	22.67	27.42	63.98	117.41	0.33	−0.39
21 (2.0)	22.66	27.44	63.99	117.57	0.34	−0.23
22 (2.0)	22.63	27.38	63.90	117.47	0.25	−0.33
23 (0.5)	22.73	27.48	64.08	117.49	0.44	−0.31
23 (1.0)	22.76	27.49	64.13	117.42	0.48	−0.38
23 (1.5)	22.77	27.49	64.14	117.43	0.50	−0.37
23 (2.0)	22.78	27.50	64.15	117.40	0.50	−0.40
24 (2.0)	22.61	27.37	63.86	117.44	0.21	−0.36
25 (2.0)	22.56	27.33	63.73	117.55	0.08	−0.25
26 (2.0)	22.53	27.32	63.77	117.56	0.12	−0.24
27 (2.0)	22.53	27.30	63.67	117.60	0.02	−0.20
32 (2.0)	22.45	27.21	63.53	117.65	−0.12	−0.15
33 (2.0)	22.42	27.16	63.46	117.57	−0.18	−0.23

Acknowledgment. This research was supported in part by a Grant-in-Aid to Y. T. as a JSPS Research Fellow from the Ministry of Education, Science and Culture of Japan. It was also supported in part by the Ryobi-Teien Fund for Bioscience and the Nishida Research Fund for Fundamental Organic Chemistry. The authors are grateful to Dr. Yoshihiko Hirose of Amano Pharmaceutical Co. for providing information on lipase PS. They also thank the SC-NMR Laboratory of Okayama University for the ^{13}C NMR experiment. The provision of lipases by Amano Pharmaceutical Co. is also appreciated.

Supporting Information Available: Tables of experimental results detailing the data of Figures 3 and 4, parts a and b, ^1H and ^{13}C NMR and IR spectra for compounds **17**−**19** (12 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO951598+

(20) Yang, L.; Weber, A. E.; Greenlee, W. J.; Patchett, A. A. *Tetrahedron Lett.* **1993**, *34*, 7035.