Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 493–501
Carbohydrate RESEARCH

Preparation of gemini-type amphiphiles bearing cyclitol head groups and their application as high-performance modifiers for lipases

Yurie Mine,[a] Kimitoshi Fukunaga,[a,*] Ken-ichi Samejima,[a] Makoto Yoshimoto,[a] Katsumi Nakao[a] and Yoshiaki Sugimura[b]

[a]*Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, Yamaguchi University, 2-16-1 Tokiwadai, Ube 755-8611, Japan*
[b]*General Education Division, Oshima National College of Maritime Technology, Komatsu, Oshima-cho, Yamaguchi 742-2193, Japan*

Received 14 June 2003; accepted 8 December 2003

Abstract—Five gemini-type amphiphiles bearing cyclitol head groups, which have abundance of axial hydroxy groups, are newly synthesized. The syntheses are based on a common mixed anhydride method utilizing *N*,*N*′-[iminobis(trimethylene)]bisquinamide, prepared from iminobispropylamine and quino-1,5-lactone, and dialkyl *N*-(3-carboxypropanoyl)-L-glutamates as polar and hydrophobic components, respectively. *Candida rugosa* lipase (CRL) and *Pseudomonas cepacia* lipase (PCL) are co-lyophilized with these synthesized gemini-type amphiphiles, and their transesterification activities in organic solvents are evaluated. The modified PCL and CRL prepared by using each amphiphile showed highly enhanced and moderately enhanced enzyme activity, respectively. These results are discussed in terms of the increased preferential exclusion of the hydrophilic heads of the amphiphile and of the topological view of the amphiphile.

Keywords: Amphiphile; Axial hydroxy group; Modified lipase; Quinic acid

1. Introduction

In recent years, enzymatic catalysis in non-aqueous media has gained considerable interest as an efficient preparative approach to biosynthesis, because the development of enzymes in non-aqueous media has numerous advantages compared to their use in conventional aqueous media. Although numerous enzymes are catalytically active in organic media, the activity of native enzymes in anhydrous organic media is much lower than that in water. Hence, many methods have been introduced leading to improved enzyme activity in organic media.[1] One of the most practical and simple methods of enzyme modification among them is co-lyophilization with lyoprotectants such as carbohydrates.[2,3] For lipase activation, however, the co-lyophilizates[4] or co-precipitates[5,6] with amphiphiles that have sugar alcohol as a hydrophilic moiety were more active than those with lactose, sucrose, or D-glucitol (sorbitol).[4] It might be suggested that the poorer activating abilities of carbohydrates seem consistent with the difficulty to open the lid of the lipase and to crystallize it in the activated form, because the amphiphiles, which contain a hydrophobic tail and a hydrophilic head group, have a high efficiency in making a lipid–water interface, and in turn in crystallizing the enzyme in an open lid form upon co-lyophilization.[4] More recently, we have prepared the gemini or dimeric amphiphiles that have two alkyl chains and two D-arabinitol groups connected with a spacer.[7,8] These amphiphiles were about 11-fold more efficient for activating lipase than conventional single-head and single-tail amphiphiles. This high enzyme activity could be explained in terms of steric exclusion of polyolic heads of the amphiphiles from the non-polar

* Corresponding author. Tel.: +88-836-85-9272; fax: +88-836-85-9201; e-mail: fukun@yamaguchi-u.ac.jp

0008-6215/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2003.12.006

region of protein surface in the aqueous system prior to freeze-drying.[9] The steric exclusion of the sugar is enhanced as the number of equatorial OH (eq OH) groups per sugar molecule decreases.[10] In fact, it is known that *myo*-inositol, one of a typical cyclic polyol, is strongly repelled from the non-polar region of the protein surface and displays a very extensive preferential hydration of the protein different from other linear polyols.[11,12] Since quinic acid is the most readily available cyclic polyol at present, a chemical synthesis of the gemini amphiphile containing quinic acid as the heads was carried out. The preparative details of the synthesis and the activation behavior of the resulted gemini amphiphiles toward lipases in non-aqueous media are reported herein.

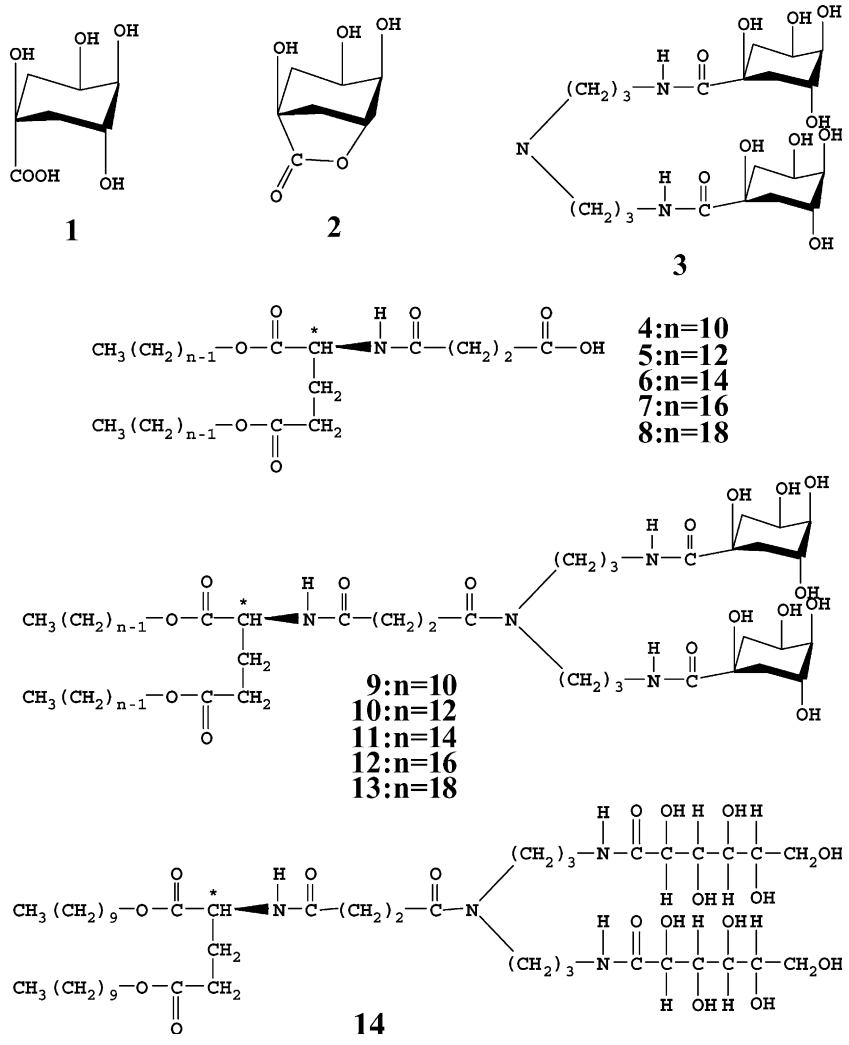
2. Results and discussion

2.1. Synthesis of the gemini amphiphiles

The gemini-type amphiphiles, 2-[3-(bis{3-[(1,3,4,5-tetrahydroxycyclohexanecarbonyl)amino]propyl}carbam-oyl)-propionylamino]pentanedioic acid dialkyl esters (**9**: didecyl ester; **10**: didodecyl ester; **11**: ditetradecyl ester; **12**: dihexadecyl ester; **13**: dioctadecyl ester), were prepared using the previously reported mixed anhydride method,[7,8] substituting *N*,*N*′-[iminobis(trimethylene)]-bis-D-gluconamide for *N*,*N*′-[iminobis(trimethylene)]-bisquiamide (**3**) as a polar component, in moderate yields. Lactonization of D-(−)-quinic acid (**1**) was carried out by azeotropic dehydration,[6] since the reported reaction is known to proceed under severe conditions in low yield.[13] The gemini-type amphiphile bearing linear polyols as heads, 2-(3-{bis[3-(2,3,4,5,6-pentahydroxyhexanoylamino)propyl]carbamoyl}propionyl-amino]pentanedioic acid didecyl ester (**14**)[14] was also prepared for comparison. Compounds **9**–**13** and compound **14** were purified by a combination of column and gel-filtration chromatography and by repeated recrystallization from 2-propanol, respectively, and their structures were confirmed by ^{13}C NMR spectroscopy. Scheme 1 shows the molecular structures of the compounds **1**–**14**.

2.2. Enzyme activity of the gemini amphiphile–lipase complexes in organic solvent

For this study *Pseudomonas cepacia* lipase (PCL) and *Candida rugosa* lipase (CRL) were selected as lipases, because serious differences in the enzyme activity between the PCL- and CRL-co-lyophilizate with poly(oxyethylene) detergent, crown ethers, or cyclodextrins were previously observed.[14,15] Figure 1 shows representatively the time courses of the transacetylation by the reaction of racemic 6-methyl-5-hepten-2-ol (racemic sulcatol) with isopropenyl acetate catalyzed by PCL co-lyophilized with each amphiphile, **9**–**13**, or native powder (control) PCL in diisopropyl ether at 30 °C. From most of the data points in Figure 1, the yield of (*R*)-(−)-acetyloxy sulcatol in the reaction catalyzed by the PCL-co-lyophilizate with the amphiphile was approximately 100% after only about 150 min, whereas the native powder PCL showed little catalytic activity. Initial reaction rates up to ca. 20% conversion were used to evaluate the specific activity: mmol h^{-1} (g of lipase)$^{-1}$, and the enantioselectivity was expressed as the enantiomeric excess (ee) of the enantiopreferred product at ca. 30% conversion. Table 1 summarizes enzyme activities and enantioselectivities for the transacetylations by using the PCL co-lyophilized with each amphiphile at various amphiphile concentrations. The primary difference of the molecular structure among the amphiphiles used in this investigation is in the hydrophile–lipophile balance (HLB). In Table 2, we list the calculated HLB values of the amphiphiles, **9**–**14**, from so-called group numbers assigned to each group on the basis of the equation:[16] $\mathrm{HLB} = \sum(\text{hydrophilic group numbers}) + \sum(\text{lipophilic group numbers}) + 7$, and the solubilities of the amphiphiles in water. Tables 1 and 2 illustrate that the HLB value correlated with the effectiveness of the amphiphiles as well as the results in our previous paper.[8] The PCL co-lyophilized with **9** (HLB 10.7) from the aqueous amphiphile solution of 32–65 mM (the molar ratio of amphiphile/PCL is 100–200, assuming PCL is 100% pure) showed the largest enzyme activity for the transacetylation between racemic sulcatol and isopropenyl acetate in diisopropyl ether, and the enzyme activity of the PCL-**10** (HLB 8.8) or **11**-co-lyophilizate at the aqueous amphiphile solution of 48–65 mM (the molar ratio is 150–200) was comparable to those of the gemini amphiphiles in our previous works.[7,8] Use of the amphiphile of HLB below 5 was less effective. It has been clearly established that lid displacement, which uncovers the catalytic site, is involved in the mechanism of interfacial activation of lipases,[4,17] and it might be suggested that the extent of the activation of lipases in non-aqueous media is directly proportional to the performance of an amphiphile to open its lid in an aqueous solution prior to co-lyophilization. Thus, this great enhancement of the PCL activity induced by the co-lyophilization with amphiphiles of HLB 7–11, can be accounted for by its HLB value close to 10, at which the hydrophilic–lipolytic property at the interface just balances. In turn the performance as an oil–water interface is maximized. However, the enzyme activity of the PCL-co-lyophilizate with **9** (HLB 10.7) was 2-fold over that of its counterpart amphiphile, **14** (HLB 11.3),[8] even though their HLB values are very close [107 vs 53 mmol h^{-1} (g PCL)$^{-1}$ at a 1:100 molar ratio as shown in Table 1]. The critical micelle concentration (cmc) of



Scheme 1. The structure of compounds **1**–**14**.

the novel non-ionic gemini amphiphiles has not been measured yet. However, it has been known that ionic gemini amphiphiles are more likely than conventional surfactants to adsorb at the aqueous solution surface rather than to form micelles.[18] Thus, it could be presumed that when the gemini amphiphile was mixed with lipase in aqueous buffer solution before co-lyophilization, it interacts monomerically with enzyme protein. Furthermore, this estimate was borne out by the dependence of the amphiphile-derived activation of lipase in non-aqueous medium on the aqueous concentration of the amphiphile as shown in Table 1: if the amphiphiles are organized as micelles, a sudden increase in the activation should be expected at a certain concentrations.[4] Therefore, the factor governing the enzyme activity is attributable to the abundance of axial hydroxy groups, which enhance the natural-like characteristics of PCL due to the preferential hydration of the enzyme protein, by the hydrophilic heads of **14**

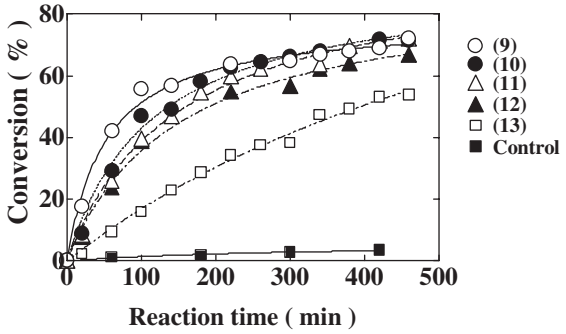


Figure 1. Comparison of the time course of the PCL-co-lyophilizate and non-co-lyophilizate with an amphiphile-mediated transesterification between racemic sulcatol (2 mmol) and isopropenyl acetate (2 mmol) in anhyd diisopropyl ether (4 mL) at 30 °C. Enzymes were obtained by lyophilization of 10 mg PCL from 10 mL of aq phosphate buffer (10 mM, pH 7) containing 32 μmol of the corresponding amphiphile (○: **9**; ●:**10**; △: **11**; ▲: **12**; □: **13**) or no additive (i.e., control PCL powder: ■), and enzyme concentration as native PCL in diisopropyl ether was 2.5 mg mL^{-1}.

Table 1. Enzyme activities and enantioselectivities of the PCL co-lyophilized with an amphiphile and of the native PCL

Co-lyophilized amphiphile	Molar quantities[a] (μmol)	Molar ratio of amphiphile/PCL	Enzyme activity (mmol h^{-1} (g-PCL)$^{-1}$)	ee_R[b] (%)
9	16	50	61	92
9	32	100	107	88
9	48	150	118	88
9	65	200	124	90
10	16	50	47	85
10	32	100	59	85
10	48	150	75	84
10	65	200	77	83
11	16	50	42	88
11	32	100	52	84
11	48	150	55	83
11	65	200	60	82
12	16	50	29	88
12	32	100	48	88
12	48	150	48	88
12	65	200	47	85
13	16	50	26	90
13	32	100	20	87
13	48	150	17	85
13	65	200	20	86
14	32	100	53	87
None[c]	—	—	1	86

[a] PCL (10 mg) was co-lyophilized with the amphiphile of different molar quantities from 10 mL of aq phosphate buffer.
[b] Enantiomeric excess (ee) of the (*R*)-(−)-acetyloxy sulcatol determined by the enantiomeric purity of the product at ca. 30% conversion.
[c] Control PCL powder.

Table 2. The calculated HLB values and solubilities in water of the amphiphiles

Amphiphile	HLB value	Solubilities in water[a]
9	10.7	++
10	8.8	+
11	6.9	+
12	5.0	−
13	3.1	−
14	11.3	++

[a] The extent of solubility of 30 mg of amphiphile in 10 mL of water at 25 °C was graded from + (fairly soluble) to ++ (easily soluble); −; insoluble.

compared to that of **9**.[11,12] Regarding enantioselectivity, enantiomeric specificity of every activated PCL was unaltered (Table 1). Next, we investigated the dependence of the enzyme activity of the CRL co-lyophilized with each amphiphile, **9**, **10**, **12**–**14**, or native powder CRL. The reaction used for CRL was transbutyrylation between racemic solketal and vinyl butyrate in anhydrous cyclohexane at 30 °C. As shown in Table 3, moderately enhanced enzyme activities and slightly enhanced enantioselectivities were observed in this case. In our previous paper, it was found that the enzyme activity of the co-lyophilized CRL with L-2-(3-{bis[3-(2,3,4,5,6-pentahydroxyhexanoylamino)propyl]carbamoyl}propionylamino)pentanedioic acid didodecyl ester (L-BIG2C_{12}CA: HLB 9.4) from the aqueous amphiphile solution of 62 mM (the molar ratio is 200) was greatly enhanced as high as 78-fold, whereas, there was only a 5-fold increase for the co-lyophilized CRL with its diastereomeric amphiphile, D-BIG2C_{12}CA (HLB 9.4) from the same aqueous concentration.[19] The lack of enhancement for the CRL co-lyophilizate with D-BIG2C_{12}CA was correlated to the inhibited contact of carbonyl groups of the amphiphile with Tyr69 in the lid of the CRL by the steric hindrance between potential oligosaccharide chains attached at Asn351 of the CRL and polyol chains of the D-amphiphile, when two hydrophobic tails of the amphiphile bind to the strands of the small and large β-sheet situated in N-terminal end side of the CRL,[19,20] as are illustrated in Figure 2. Thus, it was suggested that by comparing the enhanced enzyme activity of the CRL co-lyophilizate with **9** (HLB 10.7) or **10** (HLB 8.8) from the aqueous amphiphile solution of 62 mM (21- or 29-fold, respectively) to that of the co-lyophilizate with **14** (HLB 11.3) from the same aqueous concentration (84-fold) (Table 3), the steric hindrance between potential oligosaccharide chains, attached at Asn351, of the CRL and cyclic polyol chains of the present amphiphiles are intermediate between those of D-BIG2C_{12}CA and L-BIG2C_{12}CA. The respective low-energy conformer of three amphiphiles L-BIG2C_{12}CA, D-BIG2C_{12}CA, and **10**, shown in Figure 3, supports this reasoning. The geometry of these molecules was optimized by using MM2 in Chem. 3D of the Chemical Structure Markup Language package (Cambridge Software Co.).

Table 3. Enzyme activities and enantioselectivities of the CRL co-lyophilized with an amphiphile and of the native CRL

Co-lyophilized amphiphile	Molar quantities[a] (μmol)	Molar ratio of amphiphile/CRL	Enzyme activity (mmol h^{-1} (g-CRL)$^{-1}$)	ee_S[b] (%)
9	7.7	50	8	34
9	15	100	11	28
9	31	200	21	39
9	62	400	16	31
10	7.7	50	10	34
10	15	100	12	29
10	31	200	29	36
10	62	400	15	35
11	7.7	50	9	34
11	15	100	29	32
11	31	200	26	34
11	62	400	34	36
12	7.7	50	9	36
12	15	100	3	35
12	31	200	23	37
12	62	400	21	33
13	7.7	50	3	34
13	15	100	5	32
13	31	200	6	31
13	62	400	12	34
14	31	200	84	38
None[c]	—	—	3	29

[a]CRL (10 mg) was co-lyophilized with the amphiphile of different molar quantities from 10 mL of aq phosphate buffer.
[b]Enantiomeric excess (ee) of the (*S*)-(+)-butyryloxy solketal determined by the enantiomeric purity of the product at ca. 30% conversion.
[c]Control CRL powder.

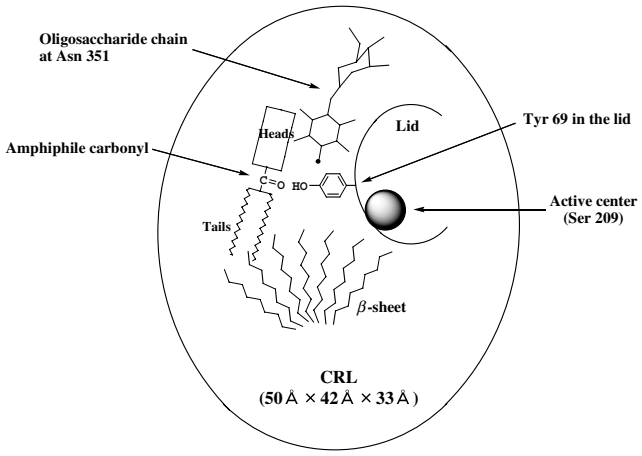


Figure 2. Schematic representation of the interfacial activation of CRL with an amphiphile.

3. Experimental

3.1. Materials

Two lipases [EC3.1.1.3] from *C. rugosa* (CRL: 64.6 kD; Meito Sangyo Co., Nagoya) and *P. cepacia* (PCL: 31 kD; Amano Enzyme Co., Nagoya) were used. CRL was employed without further purification. PCL was employed after the removal of the diatomaceous earth stabilizer according to procedures described in a previous paper.[14] Racemic sulcatol (6-methyl-5-heptene-2-ol), racemic solketal (2,2-dimethyl-1,3-dioxolone-4-methanol) and isopropenyl acetate and vinyl butyrate were purchased from Aldrich Chemical Co. (Milw, WI, USA), Tokyo Kasei Kogyo Co. (Tokyo), and Japan VAM & POVAL (Osaka), respectively. Diisopropyl ether was purchased from Wako Pure Chemical Co. (Osaka). All other organic and inorganic reagents were of the purest grade available from commercial sources.

3.2. Preparation of (−)-quinide (quino-1,5-lactone) (2)

Quinic acid (**1**) (25 g, 130 mmol) and 100 mL of 2-methoxyethanol were added to a 300-mL round flask. The reaction mixture was heated under stirring at 100–115 °C. Then the solution was diluted with 50-mL of abs toluene, and again heated under reflux for 3 h, followed by concentration by azeotropic distillation until the boiling point of the distillate reached 122 °C. Addition of 2-methoxyethanol and toluene, followed by azeotropic distillation, was repeated two more times. The residue was dissolved in 150 mL of boiling EtOH, and after cooling to room temperature, the solution was left to stand overnight in a refrigerator. After removing unreacted needles of **1**, the filtrate was concentrated in vacuo to give a colorless crystalline product, **2**, which was collected by filtration and air dried. Yield: 7.6 g (34%); mp 185–187 °C, lit. 175–195 °C[13]; IR (KBr): 3300 (OH), 2900 (CH), and 1780 cm^{-1} (lactone).

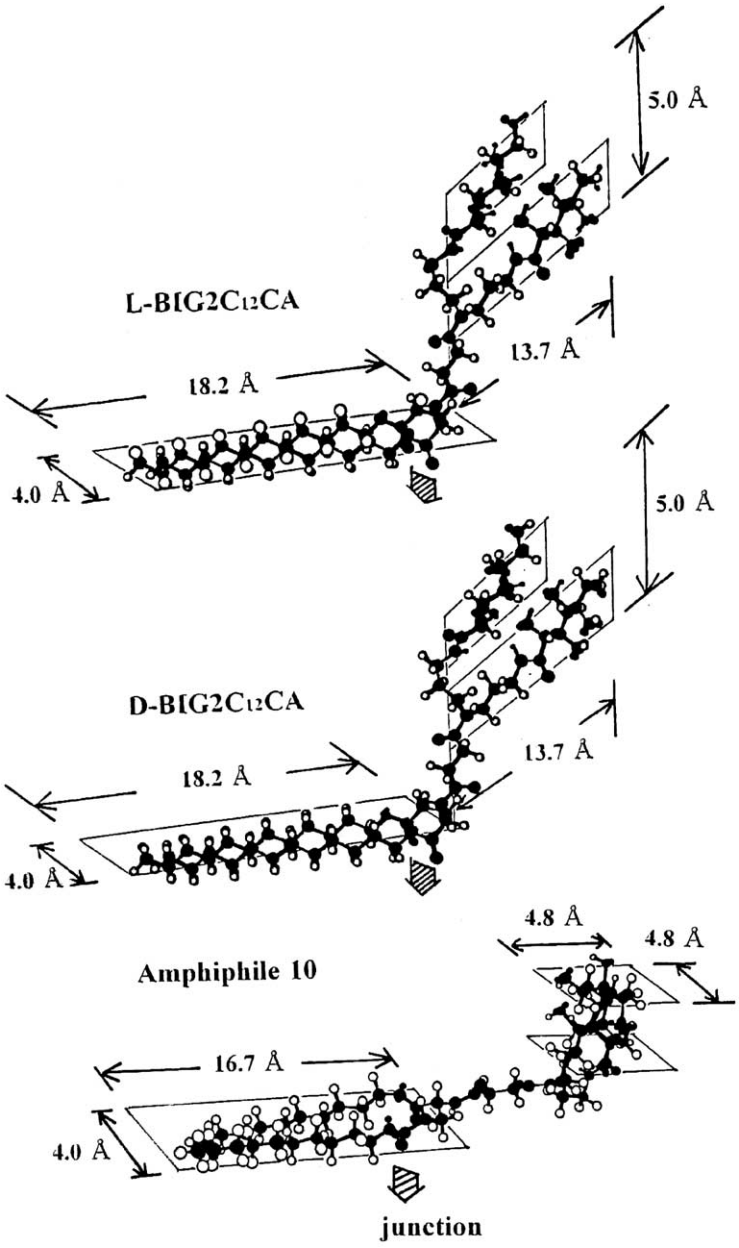


Figure 3. Stereoview of three amphiphiles. The geometry of these molecules was optimized by using MM2 in Chem. 3D of Chemical Structure Markup Language package (Cambridge Software Co.).

3.3. Preparation of *N*,*N*′-[iminobis(trimethylene)]-bis[quinamide] (3)

To a solution of 113 mg (8.6 mmol) of iminobispropylamine in 100 mL of refluxing abs MeOH was added 3.0 g (17.2 mmol) of **2**. After refluxing for 6 h, the mixture was allowed to warm up to room temperature, evaporated by a rotary evaporator under vacuum, and the viscous residue was triturated with 100 mL of $CHCl_3$. The crude product **3** was collected by vacuum filtration, washed with cold abs THF, and used without further purification.

3.4. Preparation of the gemini amphiphile (9)

To a solution of 4.3 mmol (251 mg) of didodecyl *N*-(3-carboxypropanoyl)-L-glutamate (**4**)[21] dissolved in 50 mL of dry DMF, was added 4.3 mmol (0.6 mL) of Et_3N. The resulting solution was cooled to 0 °C in an ice–salt bath, after which 4.3 mmol (0.59 g) of isobutyl chloroformate was added all at once. After mixing for 30 min, the mixture was allowed to stand in a refrigerator for 2 h. The mixed anhydride thus formed was then filtered into the solution of 4.3 mmol (206 mg) of **3** in 30 mL of dry DMF, which was warmed to 50 °C (leaving the

$Et_3N{\cdot}HCl$ on the funnel). The mixture was further stirred at 50 °C for 2 days, and then the solvent was evaporated under high vacuum, by maintaining the temperature of the bath at 50 °C. Approximately 80 mL of MeOH were added to the residue. The mixture was heated until all the material had dissolved. The solution was allowed to cool slowly to room temperature. The precipitated unreacted materials were filtered off, and concentration and cooling of the mother liquor yielded the crystalline, crude compound. The crude product was chromatographed on a column of silica gel (Wako Gel C-300) with MeOH as the eluent. Gel-filtration chromatography of the product on a column of Sephadex LH-20 with MeOH methanol as eluent gave further purified **9** (234 mg, 55%) as pale-yellow needles: mp 90–92 °C; IR (KBr): *ν* 3220 (–OH), 1720 (–C=O) and 1620 (–CONH–); ^{13}C NMR data ($CDCl_3$): δ 14.0 (CH_3–×2), 23.1 (CH_3CH_2–×2), 26.5 (–$CH_2CH_2CH_2$–O–CO–×2), 26.6 (–O–CO–CH_2CH_2CH<), 27.8 (>CH–CONH–CH_2CH_2–CO–N<), 27.9 (–O–CO–CH_2CH_2CH<), 29.9 (–CH_2CH_2–O–CO–×2), 30.0 ($CH_3(CH_2)_2CH_2(CH_2)_2CH_2$–×2), 30.3 ($CH_3(CH_2)_3CH_2CH_2$–×2, >CH–CONH–$CH_2CH_2$–CO–N<), 30.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 32.5 ($CH_3CH_2CH_2$–×2), 37.8 (>C(OH)$CH_2CH$(OH)-(quinic)×2), 41.1 (>N–$CH_2CH_2CH_2$–CONH–×2), 44.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 54.7 (–O–CO–CH_2CH_2CH<), 58.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 61.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 66.7 (–CH_2–O–CO–×2), 67.8 (>C(OH)CH_2CH(OH)-(quinic)×2), 79.2 (–CH(OH)CH(OH)-CH(OH)-(quinic)×2), 172.0 (–CH_2–O–CO–×2), 173.6 (>CH–CONH–CH_2CH_2–CO–N), 175.4 (>CH–CONH–CH_2CH_2–CO–N<), 178.5 (>N–$CH_2CH_2CH_2$–CONH–×2). Anal. Calcd for $C_{49}H_{88}N_4O_{16}$: C, 59.49; H, 8.97; N, 5.66. Found: C, 59.31; H, 9.05; N, 5.70.

3.5. Preparation of the gemini amphiphile (10)

Didodecyl *N*-(3-carboxypropanoyl)-L-glutamate (**5**) (4.3 mmol)[21] was treated in an analogous method to that described above for **9**. A combination of column and gel-filtration chromatography gave **10** as pale-yellow needles (243 mg, 54%): mp 119–123 °C; ^{13}C NMR data ($CDCl_3$): δ 14.0 (CH_3–×2), 23.1 (CH_3CH_2–×2), 26.5 (–$CH_2CH_2CH_2$–O–CO–×2), 26.6 (–O–CO–CH_2CH_2CH<), 27.8 (>CH–CONH–CH_2CH_2–CO–N<), 27.9 (–O–CO–CH_2CH_2CH<), 29.9 (–CH_2CH_2–O–CO–×2), 30.0 ($CH_3(CH_2)_2CH_2(CH_2)_4CH_2$–×2), 30.3 (–$CH_2$–×8, >CH–CONH–$CH_2CH_2$–CO–N<), 30.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 32.5 ($CH_3CH_2CH_2$–×2), 37.8 (>C(OH)$CH_2CH$(OH)-(quinic)×2), 41.1 (>N–$CH_2CH_2CH_2$–CONH–×2), 44.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 54.7 (–O–CO–CH_2CH_2CH<), 58.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 61.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 66.7 (–CH_2–O–CO–×2), 67.8 (>C(OH)CH_2 CH(OH)-(quinic)×2), 79.2 (–CH(OH)-CH(OH)-(quinic)×2), 172.0 (–CH_2–O–CO–×2), 173.6 (>CH–CONH–CH_2CH_2–CO–N<), 175.4 (>CH–CONH–CH_2CH_2–CO–N<), 178.5 (>N–$CH_2CH_2CH_2$–CONH–×2). Anal. Calcd for $C_{53}H_{96}N_4O_{16}$: C, 60.90; H, 9.26; N, 5.36. Found: C, 60.72; H, 9.34; N, 5.40.

3.6. Preparation of the gemini amphiphile (11)

Ditetradecanoyl *N*-(3-carboxypropanoyl)-L-glutamate (**6**) (4.3 mmol)[21] was treated in an analogous method to that described above for **9**. A combination of column and gel-filtration chromatography gave **11** as pale-yellow needles (303 mg, 64%): mp 122–125 °C; ^{13}C NMR data ($CDCl_3$): δ 14.0 (CH_3-2), 23.1 (CH_3CH_2–×2), 26.5 (–$CH_2CH_2CH_2$–O–CO–×2), 26.6 (–O–CO–CH_2CH_2CH<), 27.8 (>CH–CONH–CH_2CH_2–CO–N<), 27.9 (–O–CO–CH_2CH_2CH<), 29.9 (–CH_2CH_2–O–CO–×2), 30.0 ($CH_3(CH_2)_2CH_2(CH_2)_6CH_2$–×2), 30.3 (–$CH_2$–×12, >CH–CONH–$CH_2CH_2$–CO–N<), 30.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 32.5 ($CH_3CH_2CH_2$–×2), 37.8 (>C(OH)$CH_2CH$(OH)-(quinic)×2), 41.1 (>N–$CH_2CH_2CH_2$–CONH–×2), 44.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 54.7 (–O–CO–CH_2CH_2CH<), 58.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 61.4 (>C(OH)CH_2 CH(OH)-(quinic)×2), 66.7 (–CH_2–O–CO–×2), 67.8 (>C(OH)CH_2CH(OH)-(quinic)×2), 79.2 (–CH(OH)-CH(OH)CH(OH)-(quinic)×2), 172.0 (–CH_2–O–CO–×2), 173.6 (>CH–CONH–CH_2CH_2–CO–N<), 175.4

3.7. Preparation of the gemini amphiphile (12)

Dihexadecanoyl *N*-(3-carboxypropanoyl)-L-glutamate (**7**) (4.3 mmol)[21] was treated in an analogous method to that described above for **9**. A combination of column and gel-filtration chromatography gave **12** as pale-yellow needles (344 mg, 69%): mp 135–140 °C; ^{13}C NMR data ($CDCl_3$): δ 14.0 (CH_3–×2), 23.1 (CH_3CH_2–×2), 26.5 (–$CH_2CH_2CH_2$–O–CO–×2), 26.6 (–O–CO–CH_2CH_2CH<), 27.8 (>CH–CONH–CH_2CH_2–CO–N<), 27.9 (–O–CO–CH_2CH_2CH<), 29.9 (–CH_2CH_2–O–CO–×2), 30.0 ($CH_3(CH_2)_2CH_2(CH_2)_8CH_2$–×2), 30.3 (–$CH_2$–×16, >CH–CONH–$CH_2CH_2$–CO–N<), 30.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 32.5 ($CH_3CH_2CH_2$–×2), 37.8 (>C(OH)$CH_2CH$(OH)-(quinic)×2), 41.1 (>N–$CH_2CH_2CH_2$–CONH–×2), 44.9 (>N–$CH_2CH_2CH_2$–CONH–×2), 54.7 (–O–CO–CH_2CH_2CH<), 58.4 (>C(OH)CH_2CH(OH)-(quinic)×2), 61.4 (>C(OH)CH_2 CH(OH)-(quinic)×2), 66.7 (–CH_2–O–CO–×2), 67.8 (>C(OH)CH_2CH(OH)-(quinic)×2), 79.2 (–CH(OH)-CH(OH)CH(OH)-(quinic)×2), 172.0 (–CH_2–O–CO–×2), 173.6 (>CH–CONH–CH_2CH_2–CO–N<), 175.4

(>CH–*C*ONH–CH_2CH_2–CO–N<), 178.5 (>N–$CH_2CH_2CH_2$–*C*ONH–×2). Anal. Calcd for $C_{61}H_{112}N_4O_{16}$: C, 63.29; H, 9.75; N, 4.84. Found: C, 63.26; H, 9.78; N, 4.86.

3.8. Preparation of the gemini amphiphile (13)

Dioctadecanoyl *N*-(3-carboxypropanoyl)-L-glutamate (**8**) (4.3 mmol)[21] was treated in an analogous method to that described above for **9**. A combination of column and gel-filtration chromatography gave **13** as pale-yellow needles (402 mg, 77%); mp 142–145 °C; ^{13}C NMR data ($CDCl_3$): δ 14.0 (*C*H_3–×2), 23.1 (CH_3*C*H_2–×2), 26.5 (–*C*$H_2CH_2CH_2$–O–CO–×2), 26.6 (–O–CO–CH_2*C*H_2CH<), 27.8 (>CH–CONH–CH_2*C*H_2–CO–N<), 27.9 (–O–CO–*C*H_2CH_2CH<), 29.9 (–*C*H_2CH_2–O–CO–×2), 30.0 ($CH_3(CH_2)_2$*C*$H_2(CH_2)_{10}$*C*H_2–×2), 30.3 (–*C*H_2–×20, >CH–CONH–*C*H_2CH_2–CO–N<), 30.9 (>N–CH_2*C*H_2CH_2–CONH–×2), 32.5 (CH_3CH_2*C*H_2–×2), 37.8 (>C(OH)*C*H_2*C*H(OH)-(quinic)×2), 41.1 (>N–CH_2CH_2*C*H_2–CONH–×2), 44.9 (>N–*C*$H_2CH_2CH_2$–CONH–×2), 54.7 (–O–CO–CH_2CH_2*C*H<), 58.4 (>C(OH)CH_2*C*H(OH)-(quinic)×2), 61.4 (>C(OH)*C*H_2CH(OH)-(quinic)×2), 66.7 (–*C*H_2–O–CO–×2), 67.8 (>*C*(OH)CH_2CH(OH)-(quinic)×2), 79.2 (–CH(OH)*C*H(OH)CH(OH)-(quinic)×2), 172.0 (–CH_2–O–*C*O–×2), 173.6 (>CH–CONH–CH_2CH_2–*C*O–N<), 175.4 (>CH–*C*ONH–CH_2CH_2–CO–N<), 178.5 (>N–$CH_2CH_2CH_2$–*C*ONH–×2). Anal. Calcd for $C_{65}H_{120}N_4O_{16}$: C, 64.33; H, 9.97; N, 4.62. Found: C, 64.30; H, 10.0; N, 4.65.

3.9. Modified enzyme preparation

Modification by the co-lyophilization of the lipase, CRL or PCL, was performed as follows: Lipase was dissolved (1 g L^{-1}) in 10 mL of an aqueous phosphate buffer solution (10 mM, pH 7.0) in a round-bottom flask (25 mL). After the desired amount of the gemini amphiphile was added to the lipase solution, mixing was carried out with a homogenizer (Ultra-Turrax T8.01; IKA, Staufen) at 20,000 rpm for 2 min in an ice bath, followed by rapid freezing in liquid nitrogen, and lyophilization in a freeze dryer (Model VD-16; TAITEC Co., Saitama). The light-brown or white solid thus obtained was employed as the modified lipase. As a control enzyme powder, lyophilized native lipase was obtained by lyophilization from the same aqueous buffer solution in the absence of modifier.

3.10. Lipase assay

The reactions used to determine enzyme activity of the lipase preparations were transacetylation between racemic slucatol and isopropenyl acetate catalyzed by PCL in diisopropyl ether, and transbutyrylation between racemic solketal and vinyl butyrate catalyzed by CRL in cyclohexane, respectively. A general run was as follows: after organic solvent (4 mL) had been added to the co-lyophilized enzyme powder, racemic alcohol substrate (2 mmol) was added to the resulting suspension with magnetic stirring at 30 °C, and then acyl source substrate (2 mmol) was added to start the reaction. At 30-min intervals, aliquots were withdrawn from the reaction mixture, and the extent of conversion, and the enantiomeric purity of the product were determined by capillary gas chromatography using an FID detector and a chiral capillary column (Supelco β-DEX 120, 60 m) under the analytical conditions described previously.[7,14] From the GC data, initial reaction rates up to ca. 20% conversion were used to evaluate the specific activity: mmol h^{-1} (g of lipase)$^{-1}$. Conversion was defined as the percentage of the alcohol substrate which had reacted to form (*R*)- and (*S*)-acyloxy products. The enantioselectivity was expressed as the enantiomeric excess (ee) of the enantio-preferred product at ca. 30% conversion.

Acknowledgements

The authors are grateful to Amano Enzyme Co., Ltd. (Nagoya) and Meito Sangyo Co., Ltd. (Nagoya) for the provision of PCL and CRL, respectively.

References

1. Adlerkreutz, P. Modes of Using Enzyme in Organic Media. In *Enzymatic Reactions in Organic Media*; Koskinen, A. M. P., Klibanov, A. M., Eds.; Blackie Academic & Professional: Glasgow, 1996; pp 9–42.
2. Dabulis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **1993**, *41*, 566–571.
3. Triantafyllou, A. Ö.; Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1995**, *45*, 406–414.
4. Mingarro, I.; Abad, C.; Braco, L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3308–3312.
5. Okahata, Y.; Fujimoto, Y.; Ijiro, K. *J. Org. Chem.* **1995**, *60*, 2244–2250.
6. Zhang, Z.; Fukunaga, K.; Sugimura, Y.; Nakao, K.; Shimizu, T. *Carbohydr. Res.* **1996**, *292*, 47–59.
7. Fukunaga, K.; Maruoka, N.; Sugimura, Y.; Nakao, K.; Shimizu, T. *Biotechnol. Lett.* **1998**, *20*, 1161–1165.
8. Mine, Y.; Fukunaga, K.; Maruoka, N.; Nakao, K.; Sugimura, Y. *J. Biosci. Bioeng.* **2000**, *90*, 631–636.
9. Bhat, B.; Timasheff, S. N. *Protein Sci.* **1992**, *1*, 1133–1143.
10. Uedaira, H.; Uedaira, H. *Bull. Chem. Soc. Jpn.* **1980**, *53*, 2451–2455.
11. Gekko, K.; Morikawa, T. *J. Biochem.* **1981**, *90*, 39–50.
12. Carpenter, J. F.; Crowe, J. H. *Biochemistry* **1989**, *28*, 3916–3922.
13. Wolinksy, J.; Novak, R.; Vasileff, R. *J. Org. Chem.* **1964**, *29*, 3596–3598.
14. Mine, Y.; Fukunaga, K.; Yoshimoto, M.; Nakao, K.; Sugimura, Y. *J. Biosci. Bioeng.* **2001**, *92*, 539–543.

15. Mine, Y.; Fukunaga, K.; Itoh, K.; Yoshimoto, M.; Nakao, K.; Sugimura, Y. *J. Biosci. Bioeng.* **2003**, *95*, 441–447.
16. Davies, J. T. In *Interfacial Phenomena*; Davies, J. T., Rideal, E. K., Eds.; Academic: New York, 1961; p 374.
17. Verger, R. *TIBTECH* **1997**, *15*, 32–38.
18. Rosen, M. J.; Zhu, Z. H.; Hua, X. Y. *J. Am. Oil Chem. Soc.* **1992**, *69*, 30.
19. Fukunaga, K.; Mine, Y.; Samejima, K.; Yoshimoto, M.; Nakao, K.; Sugimura, Y. *Biotechnol. Lett.* **2002**, *24*, 1157–1160.
20. Mine, Y.; Fukunaga, K.; Yoshimoto, M.; Nakao, K.; Sugimura, Y. *Biotechnol. Lett.* **2003**, *25*, 1863–1867.
21. Zhang, Z.; Fukunaga, K.; Sugimura, Y.; Nakao, K.; Shimizu, T. *Carbohydr. Res.* **1996**, *290*, 225–232.