A Lipid–Lipase Aggregate with Ether Linkage as a New Type of Immobilized Enzyme for Enantioselective Hydrolysis in Organic Solvents[1)]

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For the purpose of carrying out smoothly enzymatic reaction of water-insoluble substrates in organic solvents, a new type of immobilized enzyme, a lipid–lipase aggregate, was developed. In order to prepare various kinds of lipid–lipase aggregates, 27 kinds of dialkyl ether-type phospholipid analogues were newly synthesized and used for the preparation of aggregates with lipase. Thus obtained lipid–lipase aggregates were found to catalyze the enantioselective hydrolysis of the (±)-α-acyloxy ester 2 much more efficiently than lipase immobilized with synthetic prepolymer (ENTP-4000) in water-saturated isopropyl ether. Namely, the reaction time became much shorter (2 to 3 d for completion as compared with 21 d) and the chemical and optical yields of the reaction products were found to be high.

Keywords enantioselective hydrolysis; water-insoluble substrate; α-acyloxy ester; lipid–lipase aggregate; diltiazem hydrochloride

In order to achieve enantioselective hydrolysis of water-insoluble substrates by enzymes, the use of immobilized enzymes in organic solvents has been extensively employed.[2)] An important chiral intermediate, (2*S*,3*S*)-**2**, for synthesis of the drug diltiazem hydrochloride **1** was obtained with high yield (48%) and high optical purity (94% ee) when the (±)-α-acyloxy ester **2** was exposed to lipases immobilized with synthetic prepolymer (ENTP-4000) in a water-saturated organic solvent.[2a)] However, the reaction proceeded extremely slowly, requiring a long time (21 d) for completion, and polar solvents could not be used as reaction media.

We now report a new type of immobilized enzyme, a lipid–lipase aggregate, which can catalyze the same enantioselective hydrolysis of (±)-**2** much more efficiently than lipase immobilized with ENTP-4000 in water-saturated isopropyl ether.

Generally, phospholipids are considered to form unilamellar vesicles (liposomes) A under certain conditions and such vesicles are regarded as a model of biomembranes. If the reverse micelle B in which the outer layer is lacking, were obtained and the complex C containing hydrophilic enzyme in the water pool of B were formed, then intact C would show affinity for an external organic solvent and be stable without losing enzymatic activity, because the complex C is expected to be covered by the lipophilic part of lipids.

As regards studies aimed at developing the synthetic application of reverse micelles containing enzymes in lipophilic organic solvents, several reports have appeared: the synthesis of tripeptide by using reverse micelles formed by the anionic surfactant **4**,[3)] the synthesis of triacylglycerol by lipase in a phosphatidylcholine **5** reverse micelle system[4)] and enantioselective esterification by using lipase/sugar-lipid **6** complex.[5)] Since our interest is in the enantioselective hydrolysis of esters, it is desirable to avoid amphiphiles having ester linkage in their molecules. Thus, the ether-type amphiphiles **7** having a hexadecyl group instead of a palmitoyl group were synthesized and used for the preparation of aggregates with lipase.

Synthesis of Phospholipid Analogues (±)-1,2-Di-*O*-hexadecyl-glycerol (**8**) was obtained from commercially available (±)-2,3-dimethyl-1,3-dioxolane-1-methanol in 49% overall yield (4 steps) by applying the reported method.[6)] For the synthesis of all types of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy alkylamine (inner salt), six kinds of improved methods (A—F) were used, because the

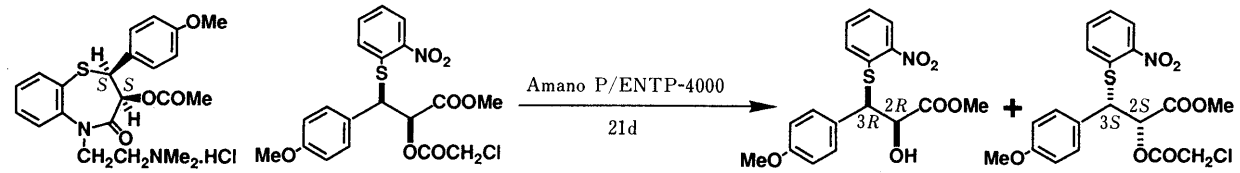


Chart 1

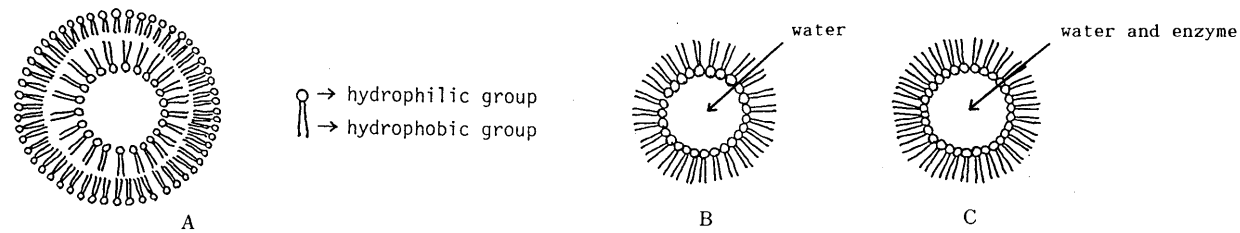


Chart 2

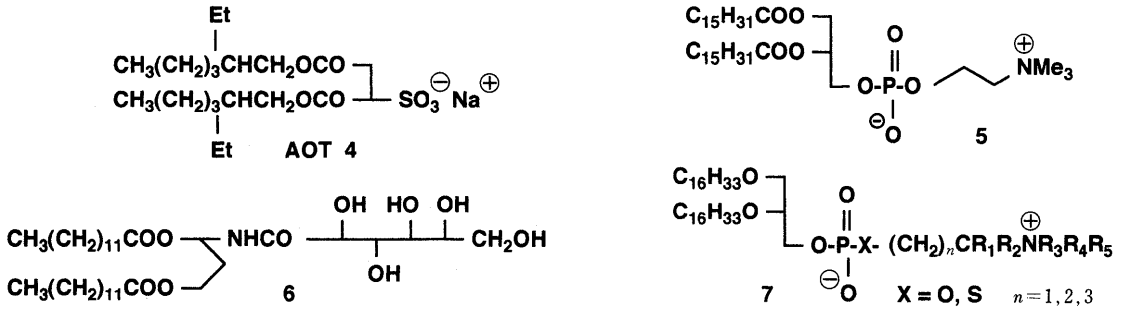


Chart 3

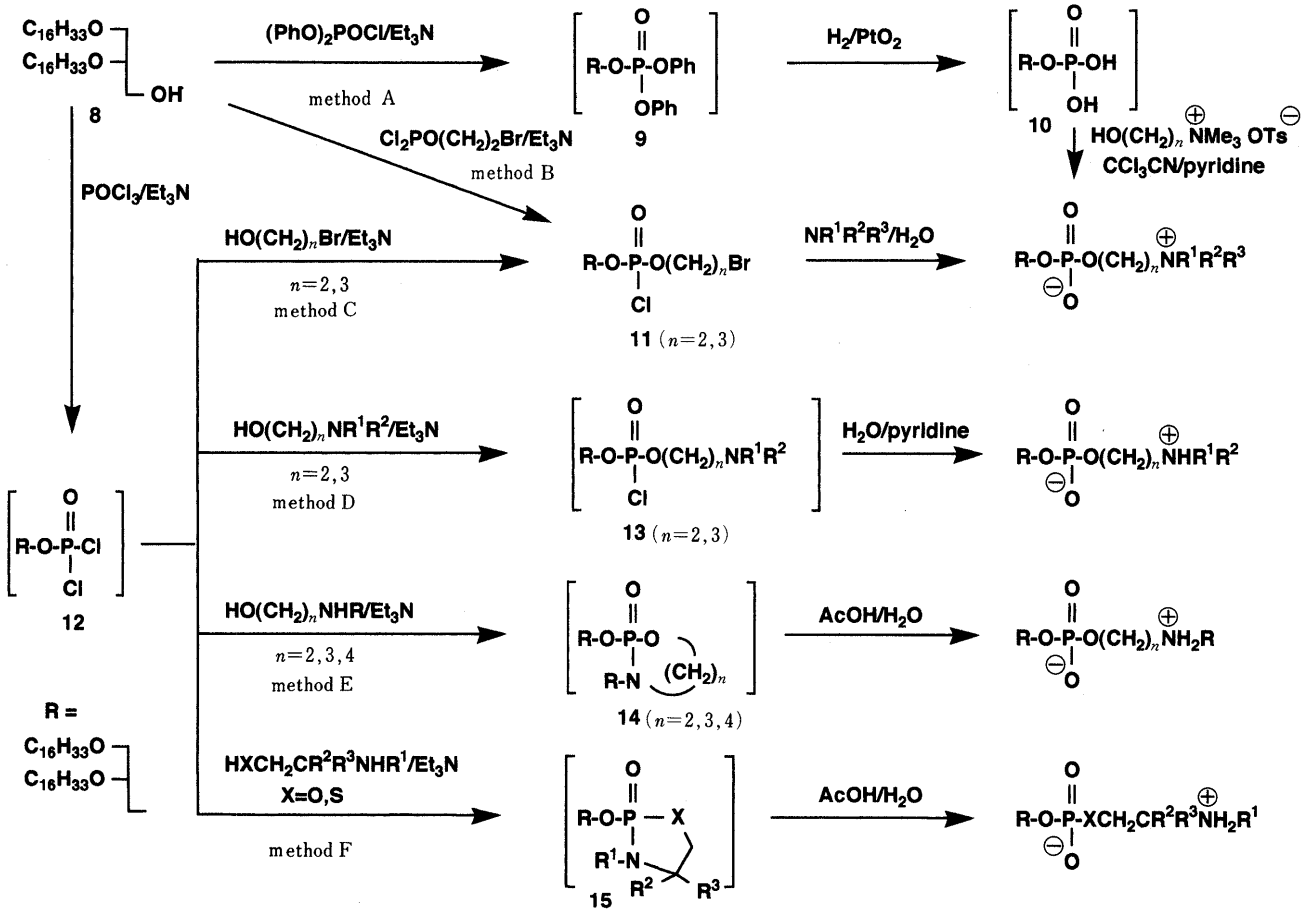


Chart 4

synthetic yield of ether-type phospholipid analogues by the known procedure[7] was poor. The general synthetic scheme of phospholipid analogues by our methods (A—F) is shown in Chart 4.

Method A: 1,2-Di-*O*-hexadecyl-*rac*-glycerol phosphoric acid (**10**) was prepared from **8** by applying the known procedure.[8] Treatment of **10** with *N*,*N*,*N*-trimethylamino-1-ethanol tosylate (choline *p*-toluenesulfonate salt)[9] gave 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonocholine (P2NM3)[10] in 78% overall yield from **8**.

Method B: A mixture of **8**, 2-bromoethyl phosphoryl dichloride[11] and Et_3N was stirred to give an intermediate **11** ($n=2$), which was treated with 30% aqueous NMe_3 to provide P2NM3 in 77% overall yield. In method B, crude **11** ($n=2$) was reacted with *N*-methylpiperidine or *N*-methylpyrrolidine to give 1,2-di-*O*-hexadecyl-*rac*-3-phosphonoxy ethyl *N*-methyl piperidium (inner salt; P2NMPIP) or 1,2-di-*O*-hexadecyl-*rac*-3-phosphonoxy ethyl *N*-methyl pyrrolidium (inner salt; P2NMPYRR) in 48% overall yield or 61% overall yield from **8**, respectively.

Method C: Treatment of **8** with $POCl_3$ gave a crude 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphoric acid dichloride (**12**), which was treated with ω-bromoalkanol to afford an intermediate **11** ($n=2,3$). Treatment of **11** with pyridine, thiazole, *N*-methylmorpholine and 30% aqueous NMe_3 afforded the corresponding 1,2-di-*O*-hexadecyl-*rac*-glycero-phosphonoxy ethyl pyridium (inner salt; P2PY; 52% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy propyl pyridium (inner salt; P3PY; 33% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl thiazolium (inner salt; P2TAZ; 20% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl *N*-methyl morpholium (inner salt; P2NMMO; 27% overall yield from **8**), P2NM3 (77%

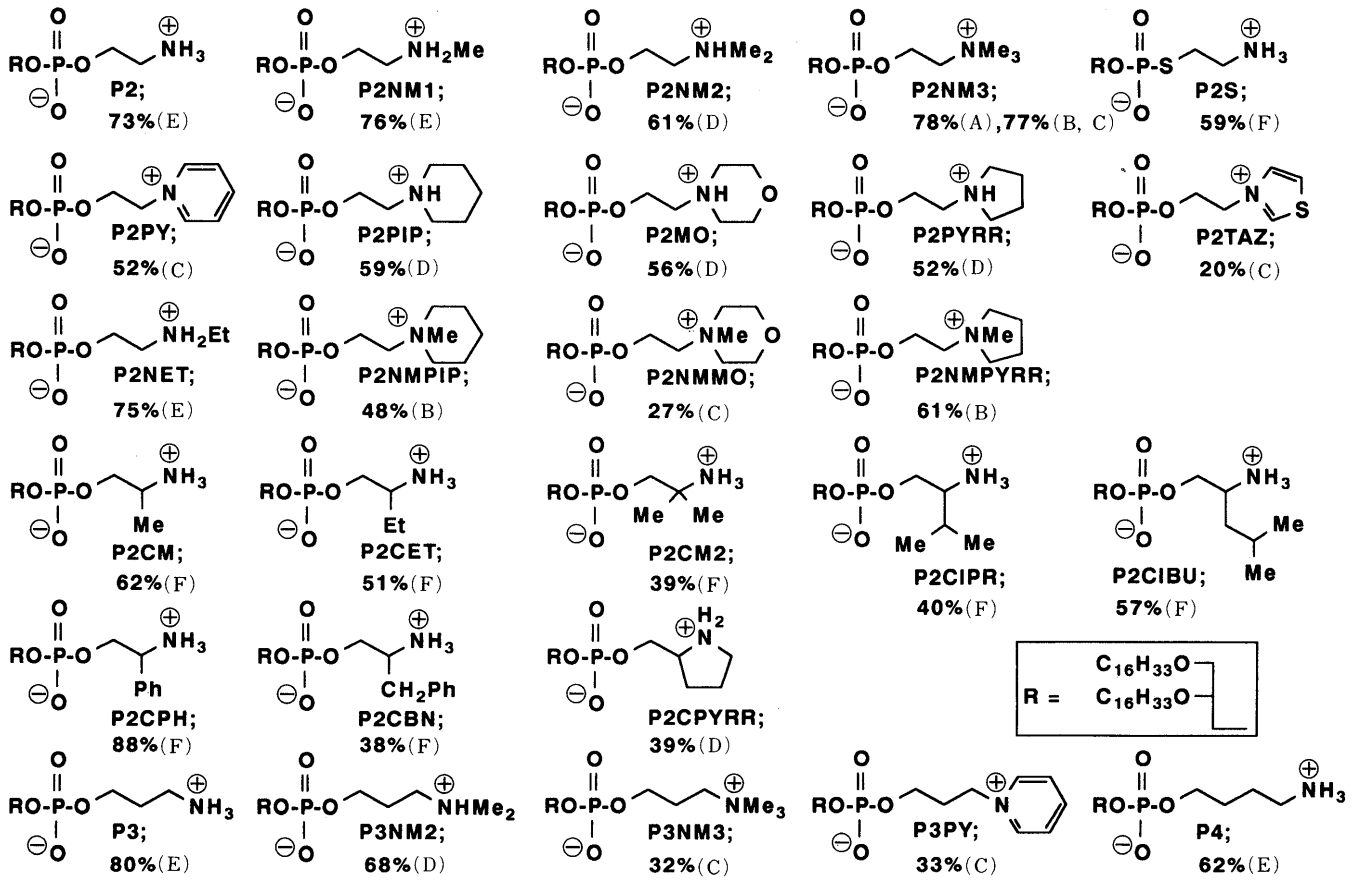
overall yield from **8**), and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy propyl trimethylamine (inner salt; P3NM3; 32% overall yield from **8**).

Method D: Crude **12** was treated with *N*,*N*-disubstituted amino alkanol to provide an intermediate **13** ($n=2$, 3), which was hydrolyzed with aqueous pyridine to produce 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phsophonoxy alkyl amine (inner salt). By using this method, 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl dimethylamine (inner salt;. P2NM2; 61% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl piperidium (inner salt; P2PIP; 59% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-3-phosphonoxy ethyl morpholium (inner salt; P2MO; 36% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl pyrrolidium (inner salt; P2PYRR; 52% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy pyrrolidine methanol (inner salt; P2CPYRR; 39% overall yield from **8**), and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy propyl dimethylamine (inner salt; P3NM2; 68% overall yield from **8**) were obtained.

Method E: Crude **12** was treated with alkanolamine or *N*-monosubstituted aminoethanol to give an intermediate **14** ($n=2,3,4$), which was hydrolyzed with aqueous AcOH to produce 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy alkylamine (inner salt). By using this method, 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl amine (inner salt; P2; 73% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl methylamine (inner salt; P2NM1; 76% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl ethylamine (inner salt; P2NET; 75% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy propylamine (inner salt; P3; 80% overall yield from **8**), and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy butylamine (inner salt; P4; 62% overall yield from **8**) were obtained.

Method F: Crude **12** was treated with 2-aminoethanethiol or many amino alcohols to provide an intermediate **15** (X=S, O), which was hydrolyzed with aqueous AcOH to produce 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy thio-ethylamine (inner salt) or 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-substituted ethylamine (inner salt).

By using this method, 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy thio-ethylamine (inner salt; P2S; 59% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-methylethylamine (inner salt; P2CM; 62% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-ethylethylamine (inner salt; P2CET; 51% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′,2′-dimethylethylamine (inner salt; P2CM2; 39% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-isopropylethylamine (inner salt; P2-CIPR; 40% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-isobutylethylamine (inner salt; P2CIBU; 57% overall yield from **8**), 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-phenylethylamine (inner salt; P2CPH; 88% overall yield from **8**), and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy 2′-benzylethylamine (inner salt; P2CBN; 38% overall yield from **8**) were ob-



(A)method A; (B) method B; (C) method C; (D) method D; (E)method E; (F) method F

Chart 5

TABLE I. Yields of Various Kinds of "Dry Aggregates"

Amano P/P2	(63 mg)	Amano P/P2NM1	(110 mg)	Amano P/P2NM2	(74 mg)
Amano P/P2NM3	(56 mg)	Amano P/P2S	(96 mg)	Amano P/P2PY	(55 mg)
Amano P/P2PIP	(61 mg)	Amano P/P2MO	(43 mg)	Amano P/P2PYRR	(27 mg)
Amano P/P2TAZ	(97 mg)	Amano P/P2NET	(77 mg)	Amano P/P2NMPIP	(17 mg)
Amano P/P2NMMO	(48 mg)	Amano P/P2NMPYRR	(40 mg)	Amano P/P2CM	(57 mg)
Amano P/P2CET	(77 mg)	Amano P/P2CM2	(60 mg)	Amano P/P2CIPR	(85 mg)
Amano P/P2CIBU	(21 mg)	Amano P/P2CPH	(29 mg)	Amano P/P2CBN	(61 mg)
Amano P/P2CPYRR	(71 mg)	Amano P/P3	(63 mg)	Amano P/P3NM2	(44 mg)
Amano P/P3NM3	(44 mg)	Amano P/P3PY	(50 mg)	Amano P/P4	(101 mg)

TABLE II. Screening Experiment for Finding a Suitable Reaction Solvent

Entry	Solvent	Product (2*R*,3*R*)-**3** Yield (%)	Optical purity (% ee)	(2*S*,3*S*)-**2** Yield (%)	Optical purity (% ee)
1	CH_2Cl_2	2.4	78	80.5	4
2	$CHCl_3$	3.0	81	92.4	5
3	CCl_4	7.9	95	90.0	9
4	CH_3CCl_3	7.5	92	91.1	9
5	$CHCl=CCl_2$	0.8	9	96.0	1
6	Benzene	4.5	88	94.2	5
7	Toluene	3.1	88	94.6	4
8	Ether	9.1	88	88.7	10
9	Isopropyl ether	33.3	98	63.8	53
10	CH_3CN	27.5	95	70.7	38

tained.

The structures of the present synthesized phospholipid analogues were confirmed by elemental analysis, fast atom bombardment mass spectroscopy (FAB-MS) and nuclear magnetic resonance (NMR) analysis.

Preparation of Lipid–Lipase Aggregates The known lipid–lipase complex[5] is prepared in aqueous solution because the sugar-lipid **6** is soluble in aqueous media. This complex is freely soluble in organic solvents. On the other hand, we prepared lipid–lipase aggregates by a different method because the present synthesized dialkyl phospholipid analogues are insoluble in aqueous media. A mixture of 100 mg of lipase Amano P from *Pseudomonas* sp. in water (5 ml) and 50 mg of dialkyl phospholipid analogues in benzene (40 ml) was sonicated for 30 min at 0 °C. The resulting precipitate was centrifuged at $3000 \times g$ and the solvent was decanted off. The residual precipitate was dried under reduced pressure to provide a "dry aggregate" (Table I). The amount of the dry aggregate depended on the dialkyl ether-type phospholipid analogues used.

Enantioselective Hydrolysis by Using Lipid–Lipase Aggregates In a preliminary experiment, it was found that a 1 : 1 mixture of two racemates ((±)-**2** and (±)-**3**) was well separated by high-performance liquid chromatographic (HPLC) analysis with a chiral column (CHIRALCEL OD (4.6 × 250 mm)). The best reaction solvent was found to be isopropyl ether, as shown in Table II, when (±)-**2** was exposed to the enzymatic reaction using lipase Amano P itself in various kinds of water-saturated organic solvents.

Then the catalytic activity of lipid–lipase aggregates was investigated in the enantioselective hydrolysis of (±)-**2** in water-saturated isopropyl ether. Selected data from the enzymatic reaction are given in Table III.

TABLE III. The Result of Enzymatic Reaction with 10 mg of (±)-**2**

Entry	Aggregate	Time (d)	Product (2*R*,3*R*)-**3** Yield (%)	Optical purity (% ee)	(2*S*,3*S*)-**2** Yield (%)	Optical purity (% ee)
1	Amano P/P2	2	48.5	>99	49.5	94
2	Amano P/P2	3	44.7	>99	45.9	99
3	Amano P/P2NM1	3	4.4	>99	93.5	5
4	Amano P/P2NM2	3	27.4	>99	71.5	39
5	Amano P/P2NM3	3	44.7	>99	52.5	87
6	Amano P/P2S	3	49.9	97	49.7	98
7	Amano P/P2PY	3	46.4	99	51.3	92
8	Amano P/P2PIP	2	46.8	>99	51.8	92
9	Amano P/P2PIP	3	47.6	99	48.7	99
10	Amano P/P2MO	2	43.3	>99	47.1	94
11	Amano P/P2MO	3	48.9	97	50.2	96
12	Amano P/P2PYRR	2	46.5	98	47.3	98
13	Amano P/P2PYRR	3	48.1	97	47.3	>99
14	Amano P/P2TAZ	3	41.8	98	46.5	89
15	Amano P/P2NET	3	46.4	97	53.0	84
16	Amano P/P2NMPIP	2	48.9	98	50.9	94
17	Amano P/P2NMPIP	3	50.5	97	48.6	99
18	Amano P/P2NMMO	2	50.0	98	49.7	>99
19	Amano P/P2NMMO	3	50.6	96	49.2	>99
20	Amano P/P2NMPYRR	2	50.0	98	48.9	98
21	Amano P/P2NMPYRR	3	49.3	97	46.6	>99
22	Amano P/P2CM	2	47.7	98	51.8	91
23	Amano P/P2CM	3	49.4	98	50.0	98
24	Amano P/P2CET	2	48.5	>99	49.8	>99
25	Amano P/P2CET	3	49.2	>99	50.0	>99
26	Amano P/P2CM2	2	47.5	99	52.3	90
27	Amano P/P2CM2	3	49.0	97	50.2	96
28	Amano P/P2CIPR	3	47.6	98	51.5	92
29	Amano P/P2CIBU	3	46.9	97	52.4	88
30	Amano P/P2CPH	2	49.7	98	50.0	98
31	Amano P/P2CPH	3	49.1	98	47.3	99
32	Amano P/P2CBN	2	48.6	>99	49.4	95
33	Amano P/P2CBN	3	49.6	98	49.7	99
34	Amano P/P2CPYRR	3	47.0	>99	51.4	93
35	Amano P/P3	2	45.8	>99	49.9	94
36	Amano P/P3	3	47.9	>99	51.0	95
37	Amano P/P3NM2	2	48.9	97	50.5	95
38	Amano P/P3NM2	3	49.4	96	49.7	97
39	Amano P/P3NM3	2	48.1	98	51.3	91
40	Amano P/P3NM3	3	50.2	98	49.1	99
41	Amano P/P3PY	2	48.0	99	49.5	97
42	Amano P/P3PY	3	50.1	96	49.2	99
43	Amano P/P4	2	48.6	97	51.3	92
44	Amano P/P4	3	50.5	97	48.9	99

It was found that the reaction time became particularly short, with only 2 to 3 d for being required completion as compared with the previous case (21 d).[2a] In the case of entry 18, the chemical yield (49.7%) and optical purity (>99% ee) of the desired (2*S*,3*S*)-**2** were extremely high and those (chemical yield; 50%, and optical purity; 98% ee) of the hydrolyzed product (2*R*,3*R*)-**3** were also found to be high. Even if a large amount (100 mg) of substrate was used, the chemical and optical yields were reproducible

TABLE IV. The Result of Enzymatic Reaction with 100 mg of (±)-2

Entry	Aggregate	Time (d)	Product (2R,3R)-3 Yield (%)	(2R,3R)-3 Optical purity (% ee)	(2S,3S)-2 Yield (%)	(2S,3S)-2 Optical purity (% ee)
1	Amano P/P2	3	50	90	49	93
2	Amano P/P2NM3	2	44	94	53	76
3	Amano P/P2PY	3	51	95	42	91
4	Amano P/P2PIP	2	47	96	50	87
5	Amaon P/P2MO	3	49	96	50	90
6	Amano P/P2CPYRR	3	47	97	50	94

and generally similar to those of a small-scale experiment (10 mg), as shown in Table IV. A repeat experiment using the recovered aggregate has not been carried out yet.

The relation between the structure of the amphiphile and catalytic activity of the aggregate remains to be fully elucidated. Further exploration of amphiphiles having different types of hydrophilic portion as well as a long-chain ether linkage might lead to still more effective aggregates. Investigation along this line and application of the present immobilization method to a wide variety of enzymes are in progress. The structural analysis of the lipid–lipase aggregate by means of physical methods is also being undertaken.

Experimental

All melting points were measured on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. NMR spectra were measured on a JEOL GX-400 spectrometer and spectra were taken as 5—10% (w/v) solutions in $CDCl_3$ with Me_4Si as an internal reference. FAB-MS were obtained with a JEOL JMS-HS 100 instrument. HPLC system was composed of two SSC instruments (ultraviolet (UV) detector 3000B and flow system 3100). All the reactions were carried out in an atmosphere of argon. All evaporations were performed under reduced pressure.

The Synthesis of Phospholipid Analogues. Method A A mixture of 1,2-di-*O*-hexadecyl-glycerol (**8**, 1.08 g), diphenyl chlorophosphate (1.08 g) and Et_3N (4 ml) in pyridine (4 ml) was stirred for 20 h at room temperature. The reaction mixture was diluted with H_2O and extracted with AcOEt. The organic layer was washed with 10% aqueous HCl, saturated aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$. Removal of the solvent gave an oily product **9**, which was subjected to hydrogenation (H_2 atm; 4 kg/cm^2) in the presence of PtO_2 (200 mg) in AcOEt to give a crude phosphoric acid **10**. This crude acid **10** was used immediately in the next step without further purification. A mixture of the crude **10**, choline *p*-toluenesulfonate salt[9] (7.77 g) and CCl_3CN (7.5 ml) in pyridine (30 ml) was stirred at 0 °C for 5 min and then at 50 °C for 2 d. The reaction mixture was evaporated to give a brown residue, which was extracted with a mixed solvent system ($CHCl_3$–MeOH–H_2O = 15 : 20 : 16, v/v). The extract was dried with $MgSO_4$, then the solvent was removed to afford an oily residue, which was chromatographed on silica gel (100 g) to provide crude P2NM3 from the $CHCl_3$–MeOH–H_2O (65 : 35 : 5, v/v) eluate. The crude P2NM3 was re-chromatographed on Sephadex LH-20 (750 ml) and the $CHCl_3$–MeOH (2 : 3, v/v) eluate gave P2NM3 as a colorless amorphous powder (78% overall yield from **8**). P2NM3: mp 198—204 °C. *Anal.* Calcd for $C_{40}H_{84}NO_6P \cdot 3H_2O$: C, 63.20; H, 11.94; N, 1.84. Found: C, 63.43; H, 11.69; N, 1.78. FAB-MS *m/z*: 706 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14} \times 2$), 3.33 (9H, s, NMe_3).

Method B A solution of 2-bromoethyl phosphoryl dichloride[11] (5.32 g) in toluene (50 ml) was added to a mixture of **8** (8.18 g) and Et_3N (3.17 ml) in toluene (100 ml) with stirring at 0 °C and the reaction mixture was stirred for 2 d at room temperature, then filtered. The filtrate was concentrated to provide a residue, which was chromatographed on silica gel (200 g) to give **11** (n = 2, 9.38 g; 83% yield) as a colorless crystals from the $CHCl_3$–MeOH (3 : 1, v/v) eluate. **11**: NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14} \times 2$).

A mixture of **11** (490 mg), 30% aqueous NMe_3 (0.5 ml), CH_3CN (4 ml) and iso-PrOH (4 ml) was stirred at 60 °C for 12 h in a screw-top pressure tube. The reaction mixture was worked up in the same way as described under method A to give P2NM3 (428 mg, 77% overall from **8**).

A solution of **11** (2 g) and *N*-methylpiperidine (3 g) in H_2O (1.25 ml) was refluxed for 12 h. After cooling, the reaction mixture was diluted with toluene and evaporated to provide a residue, which was chromatographed on silica gel (100 g) to give crude P2NMPIP from the $CHCl_3$–MeOH–H_2O (65 : 35 : 5, v/v) eluate. The crude P2NMPIP was re-chromatographed on Sephadex LH-20 (750 ml) to give P2NMPIP (1.16 g, 48% overall yield from **8**) as a colorless amorphous powder. In the same way as described for the synthesis of P2NMPIP, P2NMPYRR (61% overall yield from **8**) was obtained as a colorless amorphous powder. P2NMPIP: mp 160—164 °C. *Anal.* Calcd for $C_{43}H_{88}NO_6P \cdot 3/2H_2O$: C, 66.80; H, 11.86; N, 1.81. Found: C, 66.93; H, 11.82; N, 1.80. FAB-MS *m/z*: 746 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14} \times 2$), 3.33 (3H, s, NMe). P2NMPYRR: mp 215—216 °C. *Anal.* Calcd for $C_{42}H_{86}NO_6P \cdot 5/2H_2O$: C, 64.91; H, 11.80; N, 1.80. Found: C, 65.10; H, 11.46; N, 1.81. FAB-MS *m/z*: 732 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14} \times 2$), 3.26 (3H, s, NMe).

Method C 1) A mixture of **8** (4.82 g) and Et_3N (1.74 ml) in dry Et_2O (50 ml) was added to a solution of $POCl_3$ (1.92 g) in dry Et_2O (50 ml) with stirring at 0 °C. Stirring was continued for 2 h at room temperature, then the precipitated salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated. A mixture of the resulting residue and Et_3N (2.75 ml) in toluene (10 ml) was added to a solution of 2-bromoethanol (1.33 g) in toluene (20 ml) with stirring at 0 °C. Stirring was continued for 2 h at room temperature, then the precipitated salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated to give a crude reaction mixture, which was chromatographed in the same way as described under method B to give **11** (n = 2, 5.81 g, 87% yield from **8**) as a colorless solid.

2) A solution of **8** (4.32 g) in a mixed solvent (dry Et_2O–CH_2Cl_2 = 2 : 1, v/v, 50 ml) was added to a mixture of $POCl_3$ (1.72 g) and Et_3N (1.26 g) in a mixed solvent (dry Et_2O–CH_2Cl_2 = 2 : 1, v/v, 5 ml) over 30 min with stirring at 0 °C. Stirring was continued for 1 h at room temperature, then the precipitated salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated. A mixture of the resulting residue and Et_3N (3.6 g) in dry Et_2O (20 ml) was added to a solution of 3-bromopropanol (1.33 g) in dry Et_2O (20 ml) over 30 min with stirring at 0 °C. Stirring was continued for 1 h at room temperature, then the precipitated salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated to give a crude product, which was chromatographed on silica gel (100 g) to provide homogeneous crystals from the $CHCl_3$–MeOH (10 : 1, v/v) eluate. The product was crystallized from MeOH to afford **11** (n = 3, 3.53 g, 58% yield from **8**) as a colorless solid.

3) A mixture of **11** (n = 2, 1.30 g), pyridine (10 ml) and H_2O (5 ml) was refluxed for 12 h. The reaction mixture was diluted with toluene (20 ml) and concentrated to give a crude product, which was chromatographed on silica gel (150 g). The $CHCl_3$–MeOH–H_2O (65 : 35 : 5, v/v) eluate gave crude P2PY. The crude P2PY was re-chromatographed on Sephadex LH-20 (750 ml) and evaporation of the $CHCl_3$–MeOH (2 : 3, v/v) eluate gave P2PY as a colorless amorphous powder. In the same way as described for the synthesis of P2PY, P2TAZ and P2NMMO were obtained from **11**. P2PY: 52% overall yield from **8**. mp 196.5—197.5 °C *Anal.* Calcd for $C_{42}H_{80}NO_6P \cdot 3/2H_2O$: C, 66.98; H, 11.11; N, 1.86. Found: C, 66.81; H, 10.94; N, 1.83. FAB-MS *m/z*: 726 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 7 Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14} \times 2$), 8.03—9.40 (5H, m, aromatic proton). P2TAZ: colorless amorphous powder. 20% overall yield from **8**. mp 192—193 °C. *Anal.* Calcd for $C_{40}H_{78}NO_6PS \cdot 3/2H_2O$: C, 63.29; H, 10.76; N, 1.85; S, 4.22. Found: C, 63.13; H, 10.82; N, 1.87; S, 4.38. FAB-MS *m/z*: 732 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14} \times 2$). P2NMMO: colorless amorphous powder. 27% overall yield from **8**. mp 195—196 °C. *Anal.* Calcd for $C_{42}H_{86}NO_7P \cdot 2H_2O$: C, 64.33; H, 11.57; N, 1.79. Found: C, 64.51; H, 11.40; N, 1.82. FAB-MS *m/z*: 748 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 6.8 Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14} \times 2$), 3.48 (3H, s, NMe).

4) A mixture of **11** (n = 3, 1.20 g) and 30% aqueous NMe_3 (12.5 ml) in a mixed solvent of $CHCl_3$ (10 ml), CH_3CN (10 ml) and iso-PrOH (10 ml) was kept at 60 °C for 12 h in a screw-top pressure tube. The reaction mixture was worked up in the same way as described under method B (P2NM3) to afford P3NM3 (625 mg, 32% overall yield from **8**) as a colorless amorphous powder. P3NM3: mp 211—212 °C. *Anal.* Calcd for $C_{41}H_{86}NO_6P \cdot 3/2H_2O$: C, 65.92; H, 12.01; N, 1.87. Found: C, 66.03; H, 11.95; N, 1.79. FAB-MS *m/z*: 720 ($M^+ + 1$). NMR δ: 0.88 (6H, t, J = 7 Hz,

ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$), 3.24 (9H, s, NMe_3).

5) A mixture of **11** ($n=3$, 1.20 g), pyridine (5 ml) and H_2O (2 ml) was refluxed for 2 h. The reaction mixture was worked up in the same way as described for the synthesis of P2PY to afford P3PY (654 mg, 33% overall yield from **8**) as a colorless amorphous powder. P3PY: mp 198—199 °C. *Anal.* Calcd for $C_{43}H_{82}NO_6P\cdot 9/2H_2O$: C, 62.89; H, 11.17; N, 1.71. Found: C, 62.86; H, 10.46; N, 1.66. FAB-MS *m/z*: 740 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$), 8.08—9.40 (5H, m, aromatic proton).

Method D A solution of *N,N*-dimethylaminoethanol (0.25 ml) in toluene (10 ml) was added to a mixture of the crude **12** obtained by method C, and Et_3N (0.8 ml) in toluene (10 ml) at 0 °C, and the reaction mixture was stirred for 1 h at room temperature. The resulting salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated to give a crude product, which was refluxed for 1 h in H_2O (1 ml) and pyridine (2 ml). The reaction mixture was diluted with a mixed solvent (PhH–MeOH = 5 : 1, v/v) and evaporated to give a residue, which was chromatographed on silica gel (100 g). The $CHCl_3$–MeOH–H_2O (45 : 15 : 1, v/v) eluate afforded crude P2NM2. The crude P2NM2 was re-chromatographed on Sephadex LH-20 (750 ml) and the $CHCl_3$–MeOH (2 : 3, v/v) eluate gave P2NM2 (940 mg, 61% overall yield from **8**) as a colorless amorphous powder. In the same way as described for the synthesis of P2NM2, P2PIP, P2MO, P2PYRR and P3NM2 were obtained from **8**. P2NM2: mp 135—136 °C. *Anal.* Calcd for $C_{39}H_{82}NO_6P\cdot 2H_2O$: C, 64.33; H, 11.91; N, 1.92. Found: C, 64.41; H, 11.46; N, 1.87. FAB-MS *m/z*: 692 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=7$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$), 2.86 (6H, s, NMe_2). P2PIP: colorless amorphous powder. 59% overall yield from **8**. mp 100—102 °C. *Anal.* Calcd for $C_{42}H_{86}NO_6P$: C, 68.90; H, 11.84; N, 1.91. Found: C, 68.62; H, 11.87; N, 1.91. FAB-MS *m/z*: 732 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2MO: colorless amorphous powder. 56% overall yield from **8**. mp 159.5—162 °C. *Anal.* Calcd for $C_{41}H_{84}NO_7P$: C, 65.47; H, 11.53; N, 1.86. Found: C, 65.37; H, 11.24; N, 1.84. FAB-MS *m/z*: 734 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2PYRR: colorless amorphous powder. 52% overall yield from **8**. mp 101—102.5 °C. *Anal.* Calcd for $C_{41}H_{84}NO_6P\cdot H_2O$: C, 66.90; H, 11.78; N, 1.90. Found: C, 66.60; H, 11.75; N, 1.92. FAB-MS *m/z*: 718 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2CPYRR: pale yellow amorphous powder. 39% overall yield from **8**. mp 88.5—89 °C. *Anal.* Calcd for $C_{40}H_{82}NO_6P$: C, 68.24; H, 11.74; N, 1.99. Found: C, 67.90; H, 11.76; N, 1.96. FAB-MS *m/z*: 704 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P3NM2: colorless amorphous powder. 68% overall yield from **8**. mp 128—130 °C. *Anal.* Calcd for $C_{40}H_{84}NO_6P\cdot 1/2H_2O$: C, 67.18; H, 11.98; N, 1.96. Found: C, 67.22; H, 11.96; N, 1.98. FAB-MS *m/z*: 706 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$), 2.75 (6H, s, NMe_2).

Method E A solution of *N*-ethylaminoethanol (0.48 g) in toluene (10 ml) was added to a mixture of the crude **12** obtained from **8** (2.41 g) by method C, and Et_3N (1.38 ml) in toluene (20 ml) at 0 °C, and the reaction mixture was stirred for 1 h at room temperature. The resulting salt was filtered off and washed with toluene. The filtrate and washing were combined and evaporated to give a crude product, which was stirred for 12 h at room temperature after addition of a mixed solvent (iso-PrOH (30 ml), AcOH (2 ml) and H_2O (8 ml)). The reaction mixture was diluted with H_2O (150 ml) and the resulting precipitate was collected. The crude precipitate was chromatographed on silica gel (120 g) and the $CHCl_3$–MeOH–H_2O (45 : 15 : 1, v/v) eluate afforded crude P2NET. The crude P2NET was re-chromatographed on Sephadex LH-20 (750 ml), and the $CHCl_3$–MeOH (2 : 3, v/v) eluate provided P2NET (2.31 g, 75% overall yield from **8**) as an amorphous powder. In the same way as described for the synthesis of P2NET, P2, P2NM1, P3 and P4 were obtained from **8**. P2NET: mp 106—107 °C. *Anal.* Calcd for $C_{39}H_{82}NO_6P$: C, 67.68; H, 11.94; N, 2.02. Found: C, 67.55; H, 12.04; N, 2.00. FAB-MS *m/z*: 692 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$), 1.38 (3H, t, $J=7.3$ Hz, -$CH_2\underline{CH_3}$). P2: colorless amorphous powder. 73% overall yield from **8**. mp 170.5—172 °C. *Anal.* Calcd for $C_{37}H_{78}NO_6P\cdot 1/2H_2O$: C, 66.03; H, 11.83; N, 2.08. Found: C, 66.35; H, 11.84; N, 2.03. FAB-MS *m/z*: 664 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$). P2NM1: colorless amorphous powder. 76% overall yield from **8**. mp 162.5—163 °C. *Anal.* Calcd for $C_{38}H_{80}NO_6P\cdot 5/2H_2O$: C, 63.12; H, 11.85; N, 1.94. Found: C, 62.83; H, 11.41; N, 1.97. FAB-MS *m/z*: 678 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=7$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$), 2.67 (3H, s, NMe). P3: colorless amorphous powder. 80% overall yield from **8**. mp 180.5—181.5 °C. *Anal.* Calcd for $C_{38}H_{80}NO_6P$: C, 67.31; H, 11.89; N, 2.07. Found: C, 67.47; H, 12.03; N, 1.88. FAB-MS *m/z*: 679 ($M^+ +2$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P4: colorless amorphous powder. 62% overall yield from **8**. mp 164.5—165.5 °C. *Anal.* Calcd for $C_{39}H_{82}NO_6P$: C, 67.78; H, 11.94; N, 2.02. Found: C, 67.40; H, 12.01; N, 1.99. FAB-MS *m/z*: 692 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$).

Method F 1) Et_3N (1.38 ml) and 2-aminoethanethiol (413 mg) were added to a solution of the crude **12** obtained from **8** (2.41 g) by method C in dry Et_2O (15 ml) at 0 °C, and the reaction mixture was stirred for 1 h at room temperature. The mixture was worked up in the same way as described under method E to give a precipitate, which was recrystallized from MeOH to provide crude P2S. The crude P2S was chromatographed on Sephadex LH-20 (750 ml) and the $CHCl_3$–MeOH (2 : 3, v/v) eluate afforded P2S (1.79 g, 59% overall yield from **8**) as a colorless amorphous powder. In the same way as described for the synthesis of P2S, crude P2CM2 was obtained from **8** and purified by means of silica gel column chromatography followed by Sephadex LH-20 column chromatography. P2S: mp 138—140 °C. *Anal.* Calcd for $C_{37}H_{78}NO_5PS$: C, 65.34; H, 11.56; N, 2.06; S, 4.72. Found: C, 65.21; H, 11.64; N, 1.83; S, 4.29. FAB-MS *m/z*: 680 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2CM2: colorless amorphous powder. 39% overall yield from **8**. mp 113—114 °C. *Anal.* Calcd for $C_{39}H_{82}NO_6P\cdot 3/2H_2O$: C, 65.14; H, 11.92; N, 1.95. Found: C, 65.32; H, 11.62; N, 1.93. FAB-MS *m/z*: 692 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.7$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$).

2) Et_3N (1.38 ml) and 2-phenylglycinol (0.74 g) were added to a mixture of the crude **12** obtained from **8** (2.41 g) by method C in toluene (40 ml) at 0 °C, and the reaction mixture was stirred for 12 h at room temperature. The resulting salt was filtered off and washed with toluene (20 ml). The filtrate and washing were combined and evaporated to give a residue, which was diluted with a mixed solvent system of iso-PrOH (30 ml), AcOH (2 ml) and H_2O (8 ml). After being stirred for 12 h at room temperature, the reaction mixture was treated with H_2O (150 ml) and the resulting precipitate was collected. The crude precipitate was purified by silica gel column chromatography followed by Sephadex LH-20 column chromatography to provide P2CPH (88% overall yield from **8**) as a colorless amorphous powder. P2CPH: mp 163—164 °C. *Anal.* Calcd for $C_{43}H_{82}NO_6P\cdot H_2O$: C, 68.12; H, 11.17; N, 1.85. Found: C, 67.80; H, 10.79; N, 1.90. FAB-MS *m/z*: 740 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=7$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$), 7.26 (5H, br s, aromatic proton).

3) Et_3N (1.38 ml) and a solution of alaninol (0.41 g) in toluene (10 ml) were added to a mixture of the crude **12** obtained from **8** (2.41 g) by method C in toluene (40 ml) at 0 °C, and the reaction mixture was stirred for 1 h at room temperature. The mixture was worked up in the same way as described for the preparation of P2CPH to yield a crystalline compound. This product was washed with MeOH to afford P2CM (62% overall yield from **8**) as a colorless amorphous powder. In the same way as described for the preparation of P2CM, P2CET, P2CIPR, P2CIBU and P2CBN were obtained from **8**. P2CM: mp 160—161 °C. *Anal.* Calcd for $C_{38}H_{80}NO_6P\cdot 1/2H_2O$: C, 66.43; H, 11.88; N, 2.04. Found: C, 66.57; H, 11.76; N, 2.04. FAB-MS *m/z*: 678 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2CET: colorless amorphous powder. 51% overall yield from **8**. mp 115—116 °C. *Anal.* Calcd for $C_{39}H_{82}NO_6P\cdot 1/2H_2O$: C, 66.8l; H, 11.93; N, 2.00. Found: C, 67.01; H, 11.88; N, 2.00. FAB-MS *m/z*: 692 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2CIPR: colorless amorphous powder. 40% overall yield from **8**. mp 79—80 °C. *Anal.* Calcd for $C_{40}H_{84}NO_6P\cdot 1/2H_2O$: C, 67.18; H, 11.98; N, 1.96. Found: C, 66.86; H, 11.93; N, 1.94. FAB-MS *m/z*: 706 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.00, 1.08 (each 3H, d, $J=6.7$ Hz, iso-Pr), 1.26 (56H, br s, $(CH_2)_{14}\times 2$). P2CIBU: colorless amorphous powder. 57% overall yield from **8**. mp 90—91 °C. *Anal.* Calcd for $C_{41}H_{86}NO_6P\cdot 1/2H_2O$: C, 67.54; N, 12.03; N, 1.92. Found: C, 67.28; H, 11.96; N, 1.87. FAB-MS *m/z*: 720 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=7$ Hz, ω-Me), 0.92, 0.94 (each 3H, d, $J=6.3$ Hz, iso-Pr), 1.25 (56H, br s, $(CH_2)_{14}\times 2$). P2CBN: colorless amorphous powder. 38% overall yield from **8**. mp 149.5—151 °C. *Anal.* Calcd for $C_{44}H_{84}NO_6P\cdot H_2O$: C, 68.44; H, 11.23; N, 1.81. Found: C, 68.66; H, 11.13; N, 1.81. FAB-MS *m/z*: 754 ($M^+ +1$). NMR δ: 0.88 (6H, t, $J=6.8$ Hz, ω-Me), 1.26 (56H, br s, $(CH_2)_{14}\times 2$).

Preparation of Lipid–Lipase Aggregate The procedure was described in the test.

Screening Experiment for Finding a Suitable Reaction Solvent A mixture of (±)-**2** (10 mg) and lipase Amano P (5 mg) from *Pseudomonas* sp. in a water-saturated organic solvent (2 ml) was shaken at 33 °C for 3 d. The

reaction mixture was dried over $MgSO_4$ and evaporated to give a crude product, which was analyzed by HPLC. The results are shown in Table II.

HPLC Analysis of Two Racemates ((±)-2 and (±)-3) by Using a Chiral Column A 1:1 mixture of two racemates ((±)-**2** and (±)-**3**) gave four well separated peaks ((±)-**2**; 6.70 and 7.60 min, (±)-**3**; 9.03 and 13.78 min) corresponding to each enantiomer under the following analytical conditions (eluent, hexane–EtOH (85:15)+AcOH (0.1%); detection, UV at 254 nm; flow rate, 1.5 ml/min). The assignment of these peaks was achieved by comparing them with those of authentic sample[2a] ((2*S*,3*S*)-**2** and (2*R*,3*R*)-**3**). Namely, the peak with shorter retention time (t_R = 7.60 min) was found to correspond to that of the (2*S*,3*S*)-**2** enantiomer and the peak with longer retention time (t_R = 9.03 min) to that of the (2*R*,3*R*)-**3** enantiomer.

General Procedure of Enantioselective Hydrolysis. 1) Using 10 mg of Substrate (±)-2 A mixture of (±)-**2** (10 mg) and lipid–lipase aggregate (5 mg) in water-saturated (iso-Pr)$_2$O (2 ml) was shaken at 33 °C for a selected time. The reaction mixture was dried over $MgSO_4$ and evaporated to prodive a crude product, which was analyzed by HPLC. The results are shown in Table III.

2) Using 100 mg of Substrate (±)-2 A mixture of (±)-**2** (100 mg) and lipid–lipase aggregate (50 mg) in water-saturated (iso-Pr)$_2$O (20 ml) was shaken at 33 °C for 3 d. The reaction mixture was dried over $MgSO_4$ and evaporated to afford a crude product, which was subjected to silica gel (40 g) column chromatography. The fraction with 10% AcOEt in hexane (v/v) gave (2*S*,3*S*)-**2**. The second fraction eluted with 25% AcOEt in hexane (v/v) provided (2*R*,3*R*)-**3**. Both fractions were analyzed by HPLC and the results are shown in Table IV.

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