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Enzymatically Enantioselective Hydrolysis of Prochiral 1,3-Diacyloxyglycerol Derivatives

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An enzymatically enantioselective ester hydrolysis of prochiral 1,3-diacyloxy-2-substituted-2-propanol to chiral 1-acyloxy-2,3-propanediol was studied. The (*R*)-monoester was prepared by selection of a suitable lipase and alkyl chain length of the substrate diester. Lipase D from *Rhizopus delemer* gave (*R*)-1-isobutyryloxy-2-(2,4-difluorophenyl)-2,3-propanediol with 97%ee and 87% yield at 15°C and pH 5.5. The (*R*)-monoester is a key intermediate of azole antifungal agents.

Key words: enantioselective hydrolysis; lipase; glycerol derivative

The importance of optically active compounds is being increasingly recognized in pharmaceutical fields.[1] The enzymatic kinetic resolution by a lipase is a general method used organic chemistry for synthesizing optically active alcohols,[2-4] but its theoretical maximum yield is 50%. In contrast, the theoretical yield from the enzymatically stereoselective transformation of a prochiral substrate is 100%. Stereoselective reduction is an effective method for synthesizing optically active alcohols,[5] but it is necessary to regenerate a coenzyme that is an expensive substrate.[5,6] Enzymatically enantioselective hydrolysis or esterification, however, does not require such a special material, and the racemic alcohol is a cheap and widely available substrate.

Optically active derivatives of glycerol 2-substituted homologues are versatile chiral building blocks.[7-11] Monoacyloxyesters (**1**) can be induced to an important intermediate (**2**) for synthesizing azole antifungal agents (Fig. 1).[7-9] Two strategies were considered for preparating an optically active monoacyloxyester (**1**) by the enzymatic reaction from a prochiral substrate: one was enantioselective esterification of a triol (**3**), and the other was enantioselective hydrolysis of a diacyloxyester. We have reported that a prochiral substrate, a diacyloxyester (**4**), could be easily prepared by condensing 1,3-diacyloxyacetone (**5**) and using a 1-bromo-2,4-difluorobenzene Grignard reagent.[7] By this method, the enantioselective hydrolysis of **4** becomes a more advantageous strategy for the synthesis of **1** (Fig. 2). In the present study, we screened enzymes and substrates for possible use in the hydrolysis of **4** to **1** and then attempted to optimize the reaction conditions.

Experimental

General. All chemicals used in this study were of reagent grade or better. 1,3-Diisobutyryloxyacetone was prepared as previously described.[7] Lipase AP-6 (60,000 units/g), MAP-10 (10,000 units/g), D

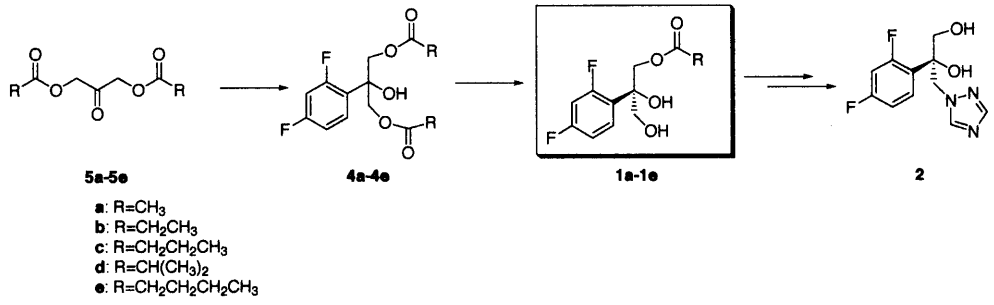

Fig. 1. Synthesis of the Intermediate of an Azole Antifungal Agent.

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Abbreviation: ee, enantiomeric excess

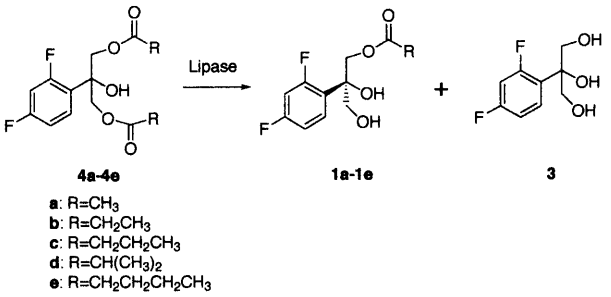

Fig. 2. Enzymatic Hydrolysis of a Diacyloxyester.

(370,000 units/g), FAP-15 (150,000 units/g), PS (30,00 units/g), and GC (50,000 units/g) were purchased from Amano Enzyme, Japan. Lipase MY (6,000 units/g) was purchased from Maito Sangyo, Japan. Lipase SP523 (4,000 units/g), SP524 (2,500 units/g), SP525 (150 units/g), and SP539 (250 units/g) were purchased from Novo Nordisk, Denmark. One unit of the lipase is defined as the amount of enzyme to liberate 1 μmole per minute of a carboxylic acid from an ester under a set of standard conditions determined by the enzyme manufacturer. Reactions were monitore on Kieselgel $60F_{254}$ TLC plates purchased from Merck, Germany. Detection was achieved by UV inspection (254 nm), and by spraying with a phosphomolybdic acid solution (5 g in 100 ml of ethanol) with subsequent heating at approximately 120°C. Column chromatography was carried out with a glass column on Kieselgel 60 (230–400 mesh, Merck, Germany).

*Preparation of diisobutyryloxyester (**4d**).* 1,3-Diisobutyryloxy-2-(2,4-difluorophenyl)-2-propanol (**4d**) was prepared from 1-bromo-2,4-difluorobromobenzene and 1,3-diisobutyryloxyacetone as described previously.[7] A solution of 1-bromo-2,4-difluorobenzene (**5d**; 96.5 g, 0.5 mol) in dry THF (450 ml) was added to magnesium (12.8 g, 0.53 mol) at under 18°C over a 3-hour period. The reaction mixture was stirred at 5°C for 1 hour to produce a Grignard reagent, which was then slowly poured into the solution of **5d** (103 g, 0.45 mol) in THF (150 ml) at below 15°C over a 1-hour period. After stirring at 5°C for 1 hour, the reaction was stopped by the addition of a 1.2 M HCl solution (500 ml) at below 5°C, and the mixture was extracted twice with ethyl acetate (500 ml). The organic layer was washed with brine and evaporated to dryness to give **4d** as an oil. Crude **4d** was purified by distillation to give pure **4d** (146 g, 85% yield for bromobenzene), bp 114–119°C at 0.5–0.6 mm Hg. ^{1}H-NMR δ (ppm): 7.73–7.65 (1H, m), 6.95–6.88 (1H, t), 6.85–6.77 (1H, m), 4.54–4.45 (4H, q), 3.90 (1H, s), 2.55–2.47 (2H, m), 1.10–1.03 (12H, m). IR ν_{max} (film) cm^{-1}: 3468, 1720, 1618, 1500, 968, 850. Anal. Found: C, 59.1; H, 6.5%. Calcd. for $C_{17}H_{22}F_2O_5$: C, 59.3; H, 6.4%.

*Preparation of the triol (**3**).* **4d** (100 g, 0.3 mol) in toluene (300 ml) was added to a 30% aqueous KOH solution (300 ml). The resulting mixture was stirred at room temperature for 24 hours. The toluene and aqueous layers were then separated, and crude 2-(2,4-difluorophenyl)-1,2,3-propanetriol (**3**) was extracted twice from the aqueous layer with ethyl acetate (500 ml). The organic layer was washed with brine and then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified by column chromatography on silica gel to give the pure desired compound (50 g, 80% yield), mp 58–59°C. ^{1}H-NMR δ (ppm): 7.73–7.68 (1H, m), 6.92 (1H, t), 6.79 (1H, t), 4.13 (2H, d), 3.80 (3H, t). IR ν_{max} (KBr) cm^{-1}: 3382, 1622, 1503, 1123, 1071, 994, 968, 851. Anal. Found: C, 52.9; H, 4.9%. Calcd. for $C_9H_{10}F_2O_3$: C, 59.2; H, 4.9%.

*Preparation of the diacyloxyesters (**4a**, **4b**, **4c**, **4e**).* In general, an acid anhydride (0.1 mol) was added dropwise to **3** (8.4 g, 0.04 mol) in pyridine (80 ml) at room temperature. The resulting mixture was then stirred at room temperature for 1 hour. Next, ethyl acetate (40 ml) was added to the reaction mixture, and the organic layer was separated, washed with 1 M aqueous HCl, and further washed with brine. The organic layer was then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified by column chromatography on silica gel to give **4**. **4a**: 90% yield. ^{1}H-NMR δ (ppm): 7.70–7.67 (1H, m), 6.93 (1H, t), 6.81 (1H, t), 4.52 (2H, d), 4.44 (2H, d), 3.65 (1H, s), 2.03 (6H, s). IR ν_{max} (KBr) cm^{-1}: 3403, 1744, 1240, 1042, 849. Anal. Found: C, 54.1; H, 4.9%. Calcd. for $C_{13}H_{14}F_2O_5$: C, 54.2; H, 4.9%. **4b**: 85% yield. ^{1}H-NMR δ (ppm): 7.71–7.67 (1H, m), 6.94–6.89 (1H, m), 6.83–6.78 (1H, m), 4.54–4.43 (4H, dd), 3.77 (1H, s), 2.32–2.26 (2H, q), 1.08–1.04 (3H, t). **4c**: 85% yield. ^{1}H-NMR δ (ppm): 7.72–7.65 (1H, m), 6.95–6.88 (1H, t), 6.85–6.76 (1H, m), 4.55–4.44 (4H, dd), 3.82 (1H, s), 2.28–2.23 (4H, m), 1.60–1.50 (4H, m), 0.88–0.83 (6H, m). **4e**: 83% yield. ^{1}H-NMR δ (ppm): 7.72–7.64 (1H, m), 6.95–6.88 (1H, t), 6.85–6.78 (1H, m), 4.52–4.44 (4H, dd), 2.40–2.35 (2H, m), 2.30–2.25 (2H, m), 1.66–1.58 (2H, m), 1.55–1.45 (2H, m), 1.43–1.33 (2H, m), 1.30–1.18 (2H, m), 0.96–0.90 (3H, m), 0.88–0.82 (3H, m).

*Preparation of the racemic monoacyloxyesters (**1a**–**1e**).* The racemic monoacyloxyesters as standards for the analysis were synthesized from diacyloxyesters. In general, a 1 M sodium hydroxide aqueous solution (5 ml) was added dropwise to **4** (5 mmol) in water (5 ml) and THF (5 ml) in a water-ice bath. The resulting mixture was stirred for 1 hour, and the pH of the reaction mixture was adjusted to 6.5 with acetic acid. The organic solvent was then removed *in vacuo*, and the aqueous layer was extracted

with ethyl acetate (10 ml). The organic layer was washed with brine. The solvent was removed *in vacuo*, and the residue was purified by column chromatography on silica gel to give racemic **1**. 1-Acetyloxy-2-(2,4-difluorophenyl)-2,3-propanediol (**1a**): 90% yield. ^{1}H-NMR δ (ppm): 7.69–7.65 (1H, m), 6.92 (1H, t), 6.80 (1H, t), 4.52 (2H, s), 3.97 (1H, d), 3.91 (1H, s), 3.80 (1H, d).2.00 (3H, s). IR ν_{max} (KBr) cm^{-1}: 3420, 1711, 1501, 1240, 1055, 970, 847. Anal. Found: C, 53.6; H, 4.9%. Calcd. for $C_{11}H_{12}F_2O_4$: C, 53.7; H, 4.9%. **1b**: 92% yield. ^{1}H-NMR δ (ppm): 7.69–7.63 (1H, m), 6.93–6.89 (1H, m), 6.83–6.77 (1H, m), 4.57–4.50 (2H, q), 3.99–3.96 (2H, d), 3.80–3.77 (1H, d), 2.38 (1H, s), 2.29–2.22 (2H, m), 1.04–1.00 83H, t). **1c**: 90% yield. ^{1}H-NMR δ (ppm): 7.67–7.64 (1H, m), 6.92–6.89 (1H, m), 6.82–6.77 (1H, m), 4.58–4.51 (2H, q), 4.02 (1H, s), 3.98–3.95 (1H, d), 3.79–3.76 (1H, d), 2.36 (1H, s), 2.23–2.19 (2H, m), 1.54–1.48 (2H, q), 0.84–0.81 (3H, t). **1d**: 90% yield. ^{1}H-NMR δ (ppm): 7.69–7.66 (1H, m), 6.91–6.87 (1H, m), 6.78–6.77 (1H, m), 4.54–4.45 (3H, m), 3.97–3.78 (2H, dd), 2.49–2.42 (1H, m), 1.01–0.99 (6H, m). IR ν_{max}(film) cm^{-1}: 3400, 1738, 1600, 1500, 968, 850. Anal. Found: C, 56.8; H, 5.9%. Calcd. for $C_{13}H_{16}F_2O_4$: C, 56.9; H, 5.9%. **1e**: 90% yield. ^{1}H-NMR δ (ppm): 7.67–7.64 (1H, m), 6.93–6.89 (1H, m), 6.83–6.77 (1H, m), 4.58–4.51 (2H, q), 4.05 (1H, s), 3.98–3.95 (1H, d), 3.77–3.76 (1H, d), 2.35 (1H, s), 2.25–2.21 (2H, t), 1.47–1.43 (2H, t), 1.23–1.19 (2H, q), 0.85–0.82 (3H, t).

Enzymatic hydrolysis of the diacyloxyesters. In general, a mixture of **4**, an enzyme, and an appropriate buffer was stirred, the other reaction conditions being as described for each result. The reaction mixture was extracted with ethyl acetate, and the organic solvent was removed *in vacuo*. The residue was dissolved in methanol, and an HPLC analysis was carried out as described next.

Analyses. ^{1}H-NMR spectra were recorded in $CDCl_3$ with JM-400 FT-NMR spectrometer (400 MHz; Jeol, Japan). Chemical shifts are expressed in parts/million (ppm), with tetramethylsilane as the internal standard. IR spectra were recorded with an 8100 M FTIR spectrometer (Shimadzu, Japan). Optical rotation was determined with a digital polarimeter (SEPA-200, Horiba, Japan). The reaction products were analyzed by HPLC with a Chiralpak AD (4.6 mmϕ × 250 mm) column (Daicel Chemicals, Japan). The HPLC conditions included the use of *n*-hexane:ethanol = 9:1 (v/v) as the mobile phase, a flow rate of 1.2 ml/min, an ambient column temperature, and detection at 254 nm. As examples, the retention times of **4a**, (*S*)-**1a**, (*R*)-**1a**, and **3** were 10.1, 14.9, 16.5, and 22.0 min, respectively. The absolute configurations of **1** were determined by converting to compound **4** whose configuration is known, as described previously.[7-9]

*Preparative-scale reactions and preparation of optically active (R)-***1d**. **4d** (5 g), cyclohexane (5 ml), lipase D (50 mg), and water (45 ml) were stirred at the desired temperature and pH value. The pH value of the reaction mixture was kept constant with 2 M NaOH. After the reaction, the reaction mixture was extracted twice with ethyl acetate (50 ml), and the organic layer was washed with brine. The solvent was removed *in vacuo*, and the residue was purified by column chromatography on silica gel to give **1d** (3.2 g, 80% yield, 96.7%ee), $[\alpha]_D^{25}$ −7.04° (c,1.0, CH_3OH).

Results and Discussion

Synthesis of the diacyloxyesters

4 was prepared from **5** with the 1-bromo-2,4-difluorobenzene Grignard reagent. The derivatives of **4** having other substituent groups on the aromatic ring instead of the 2,4-difluoro group were also synthesized by this procedure (data are not shown). In addition, **4** was prepared from **3** by a general method using an acid anhydride under basic conditions. In this case, the tertiary hydroxy group of **3** was not acylated by using a large excess of the acylating reagent. The by-product (**3**) was acylated to **4** and reused as the substrate for the enzymatic reaction.

Screening of the lipase hydrolyzing diacyloxyesters

Eleven commercially available lipases were tested for their ability to hydrolyze two diacyloxyesters (**4a** and **4d**) to optically active monoacyloxyesters (**1a** and **1d**; Table 1). Ten lipases, excepting lipase GC, hydrolyzed **4a** to **1a**, but their enantioselectivity was not sufficiently high. The enantioselectivity of lipase D, FAP-15, PS, GC, SP524, and SP525 markedly varied, although that of lipase AP-6, MAP-10, MY, and SP539 did not vary according to the chain length of the substrate. Industrially available lipase D was selected for enzymatic hydrolysis of the diacyloxyester, because it gave the desired (*R*)-form product with high yield and enantioselectivity from **4d**.

Selection of the substrate for lipase hydrolysis

Five kinds of dialkyloxyesters were examined for their enantioselective hydrolysis to monoacyloxyesters by lipase D (Table 2). Monoacyloxyesters were obtained with over 90%ee except when using the acetyloxyester (**4a**) as the substrate. The low yield and low enantioselectivity of **1a** may have been caused by a probable 1,3-rearrangement of the acetoxy group, or possible non-enzymatic hydrolysis to **3**. The yield of **3** from **4e** was the highest with these five substrates. As the substrates with long alkyl chains are similar to triacylglycerides, which are the original substrate of lipase, they are easily hydro-

Table 1. Enzymatic Hydrolysis of 1,3-Diacetyloxy-2-(2,4-difluorophenyl)-2-propanol (**4a**) and 1,3-Diisobutyryloxy-2-(2,4-difluorophenyl)-2-propanol (**4d**)[a]

Lipase	Origin	Substrate **4a**				Substrate **4d**			
		1a			**3**	**1d**			**3**
		Yield (%)		%ee[b]	Yield (%)	Yield (%)		%ee	Yield (%)
AP-6	*Aspergillus niger*	47	*S*	10	30	28	*R*	35	4
MAP-10	*Mucor javanicus*	13	*R*	80	n.d.	90	*R*	94	1
MY	*Candida cylindracea*	47	*R*	56	36	33	*R*	48	67
D	*Rhizopus delemer*	50	*R*	11	10	73	*R*	98	1
FAP-15	*Rhizopus javanicus*	56	*S*	18	21	97	*R*	96	3
PS	*Pseudomonas fluorescens*	37	*S*	51	2	13	*R*	80	1
GC	*Geotrichum candidum*	3	*S*	46	n.d.	3	*S*	6	n.d.
SP523	*Humicola* sp.	76	*S*	54	8	n.d.	—	—	100
SP524	*Humicola meihei*	51	*R*	46	11	39	*R*	99	60
SP525	*Candida antarctica*	32	*R*	4	42	9	*S*	26	1
SP539	*Bacillus* sp.	20	*S*	60	1	17	*S*	62	2

[a] Each reaction mixture, composed of 10 mg of **4** and 10 mg of lipase in 1 ml of a 50 mM acetate buffer (pH 5.0), was stirred at 30°C for 18 hours.
[b] The yield and enantiomeric excess of **1** were measured as described in the text. n.d.: not detected

Table 2. Hydrolysis of Diacyloxyesters **4a-4e** by Lipase D[a]

Substrate	Product			
	1			**3**
	Yield (%)		%ee[b]	Yield (%)
4a	50	*R*	11	10
4b	70	*R*	91	2
4c	81	*R*	97	5
4d	73	*R*	98	1
4e	71	*R*	97	17

[a] Each reaction mixture, composed of 10 mg of **4** and 10 mg of lipase D in 1 ml of a 50 mM acetate buffer (pH 5.0), was stirred at 30°C for 18 hours.
[b] The yield and enantiomeric excess of **1** were measured as described in the text.

lyzed to **3**. We selected **4d** as the most suitable substrate.

Optically active 1-monoacyloxyglycerol derivatives can be easily racemized by 1,3-rearrangement.[12] In addition, their synthesis has been achieved by means of enantioselective esterification of 1,3-diols in an organic solvent.[13-15] However, enantioselective hydrolysis of the diacyloxyester was found to be possible by selecting a suitable acyl group.

Effect of organic solvents

The effects of organic solvents on hydrolysis or esterification by a lipase have been reported.[16-20] It has also been reported that enzymatic kinetic resolution by a lipase can be efficiently carried out; that is, the *E* value can be changed by adding an organic solvent such as acetone or hexane.[16,18] We examined the efficacy of various organic solvents for the lipase hydrolysis of **4d** (Table 3). The yield of **1d** was higher with the addition of a hydrocarbon when compared with the absence of a solvent. A water-soluble solvent, defined by a partition coefficient (log *P*) less than 2,[20] or a solvent containing a halogen atom or an aromatic ring, damaged the enzyme activity. The yield of **1d** was influenced by the presence of an organic solvent, but the enantioselectivity and yield of **3** were not affected. An organic solvent was found to affect only the reactivity of a lipase. The formation of an emulsion was observed when cyclohexane was added to the reaction mixture in the gram-scale reaction. The solubility of **4d** and **1d** in hydrocarbons was

Table 3. Effect of Organic Solvents on the Enzymatic Hydrolysis of 1,3-Diisobutyryloxy-2-(2,4-difluorophenyl)-2-propanol (**4d**)[a]

Organic solvent	Product		
	1d		**3**
	Yield (%)	%ee[b]	Yield (%)
None	44	97.1	n.d.
Methanol	n.d.	—	n.d.
Acetone	n.d.	—	n.d.
1,4-Dioxane	n.d.	—	n.d.
Acetonitrile	n.d.	—	n.d.
Isopropyl ether	51	97.6	n.d.
1,2-Dimethoxyethane	3	n.d.	1
Hexane	52	97.4	1
Cyclohexane	68	97.6	1
Heptane	52	97.0	1
Octane	52	96.7	1
Decane	51	96.4	1
Petroleum ether	57	97.0	1
Benzene	36	96.8	n.d.
Toluene	30	96.8	n.d.
p-Xylene	20	94.3	n.d.
Dichloromethane	6	90.7	n.d.
Chloroform	13	96.3	n.d.
N,*N*-Dimethylformamide	n.d.	—	n.d.

[a] Each reaction mixture, composed of 100 mg of **4**, 10 mg of lipase D and 0.5 ml of an organic solvent in 0.5 ml of a 50 mM acetate buffer (pH 5.0), was stirred at 30°C for 18 hours.
[b] The yield and enantiomeric excess of **1** were measured as described in the text. n.d.: not detected

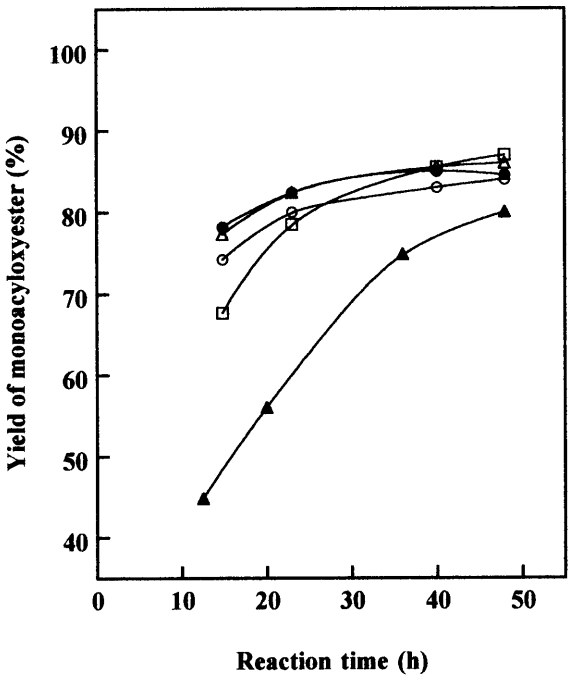

Fig. 3. Effect of Temperature and pH on the Hydrolysis of 1,3-Diisobutyryloxy-2-(2,4-difluorophenyl)-2-propanol.

Time-course plots are shown of the enzymatic hydrolysis of 1,3-diisobutyryloxy-2-(2,4-difluorophenyl)-2-propanol (**4d**) by lipase D under various conditions. The yield of the 1-isobutyryloxy-2-(2,4-difluorophenyl)-2,3-propanediol (**1d**) is plotted. ○, 30°C, pH 4.5; ●, 30°C, pH 5.0; △, 30°C, pH 5.5; ▲, 6°C, pH 5.5; □, 15°C, pH 5.5.

low, as hydrocarbons can affect both the stability of a lipase and the interaction between the substrate and the lipase through the formation of a three-phase reaction system.

Preparative-scale reactions

Based on the foregoing results, gram-scale reactions were carried out under several conditions (Fig. 3). The yield of **1d** at pH 5.5 and 30°C was higher than that at pH 4.5 and 30°C. Although the enantiomeric excess of **1d** after a 48-hour reaction at pH 4.5 was 97.0%ee, that at pH 5.5 was 96.1%ee. The racemization and non-enzymatic hydrolysis of **1d** were depressed at lower temperatures and pH values. The hydrolysis of **1d** by lipase D was therefore carried out at a relatively low temperature such as 6°C or 15°C. After a reaction time of 48 hours, the yield of **1d** at 6°C and pH 5.5 was the lowest, but that at 15°C and pH 5.5 was as high as that at 30°C and pH 5.5. The enantiomeric excess of **1d** in the reaction carried out at 15°C was 97.3%ee, although that at 30°C and pH 5.5 was 96.1%ee. Under the optimized conditions, 15°C and pH 5.5, the yield and enantiomeric excess of **1d** were 87% and 97.3%ee, respectively. After the reaction, the product (**1d**) was extracted from the reaction mixture with ethyl acetate, and the crude product after evaporation could be used for the synthesis of **2**. Purer product could be obtained by a purification procedure such as flash column chromatography.

In conclusion, we established a method for synthesizing optically active 2-aryl-1-monoacyloxyglycerol derivatives by enantioselective enzymatic hydrolysis of prochiral substrates.

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