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Highly sensitive high-performance liquid chromatographic method for the determination of the absolute configuration and the optical purity of di-O-acylglycerols using a chiral derivatizing agent, (*S*)-(+)-2-*tert.*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid

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Abstract

A general method was developed to determine the optical purity of 1,2-(or 2,3-)di-O-acyl-*sn*-glycerol, which involves the chemical transformation to the key compound 3-(or 1-)O-*tert.*-butyldimethylsilyldi-O-(+)-TBMB-*sn*-glycerol (**4a** or **4b**). [TBMB = (*S*)-(+)-2-*tert.*-butyl-2-methyl-1,3-benzodioxole]. The chiral di-O-acylglycerols were first silylated and the acyl groups were removed by Grignard degradation to 3-(or 1-)O-*tert.*-butyldimethylsilyl-*sn*-glycerol. Subsequent fluorescent labelling by (+)-TBMB-COOH gave the key compound **4a** or **4b** without arising acyl migration. The diastereoisomers were separated by silica gel TLC or normal-phase silica column HPLC with fluorescence detection. This is an extremely sensitive method to determine the stereoselectivities of lipase reactions.

1. Introduction

Glycerol has a symmetrical plane centre at C-2, being achiral and optically inactive itself. 1,2-Di-O-acyl-*sn*-glycerol is, however, chiral with the enantiomer of 2,3-di-O-acyl-*sn*-glycerol. Although they may be generated by lipase-catalysed hydrolysis of triacylglycerols or esterification of monoacylglycerols, the stereoselectivities of these lipase reactions have not been well established because of the difficulty of determining the optical purities of di-O-acylglycerols. The facile acyl migration may be another reason for the poor stereoselectivities observed in the previous lipase reaction.

Several methods have been reported to determine the optical purity of 1,2-(or 2,3-)di-O-acylglycerol [1–6]. Recently, Takagi and coworkers [3,4] and Sempore and Bezard [5] reported the enantiomeric separation of di-O-acylglycerols using either normal- or reversed-

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phase chiral columns after derivatization to the 3,5-dinitrophenylurethane (3,5-DNPU) derivatives. Another HPLC separation was also reported by Rogalska et al. [6] using diastereoisomeric carbamate derivatives on a normal-phase column. However, all the mentioned methods were less general and authentic samples with known configurations were necessary in each acyl group to determine the absolute configuration.

More recently, we have reported two methods for the determination of the optical purity and absolute configuration of 1,2-(or 2,3-)di-O-acylglycerol via the key compound 1,2-di-O-benzoyl-3-O-*tert.*-butyl-dimethylsilyl-*sn*-glycerol or its enantiomer either by a circular dichroism (CD) method or by chiral column HPLC using UV detection [7,8]. However, the former method needs CD equipment and the latter needs supplementary GC data for correction for the contaminated 1,3-isomer which overlaps the peak of the dibenzoyl derivative of 1,2-di-O-acyl-*sn*-glycerol in HPLC. Therefore, a simpler and more sensitive method is required to determine the absolute configuration and the opital purity of di-O-acylglycerols in order, for example, to determine the stereoselectivity of lipase hydrolysis in the early stages.

We have also developed (*S*)-(+)-2-*tert.*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [(*S*)-(+)-TBMB-COOH] as a fluorescent chiral derivatizing agent [9–11]. This reagent was designed to determine the absolute configuration and opital purity of amines and alcohols using HPLC, NMR and CD.

In this paper, we describe enantiomer separations of di-O-acylglycerols using this reagent. Di-O-acyl-*sn*-glycerols (**1a** and **1b**) were derivatized to *tert.*-butyldimethylsilylated 1,2-(and 2,3-)di-O-(+)-TBMB-*sn*-glycerols (**4a** and **4b**), and the diastereomers thus derived were well separated by normal-phase silica column HPLC. The sensitivity was greatly improved by using fluorescent detection, and possible all isomers of *sn*-1,2-, *sn*-2,3- and 1,3-di-O-acylglycerols were completely separated within 30 min.

2. Experimental

2.1. Chemicals

1,2-Di-O-palmitoyl-*sn*-glycerol [**1a**, *ca.* 100% enantiomeric excess (e.e.) as determined by HPLC of the (+)-TBMB derivative] and 1,3-di-O-palmitoyl-*sn*-glycerol (**1c**) were purchased from Sigma (St. Louis, MO, USA). D,L-1,2-Dipalmitin (**1**, racemate) was obtained from Nacalai Tesque (Kyoto, Japan). Triolein, substrate of lipase-catalysed hydrolysis, was also purchased from Sigma. Lipase (Amano AP, EC 3.1.1.3) from *Pseudomonas* sp. was purchased from Amano Pharmaceutical (Nagoya, Japan). (+)-TBMB-COOH (100% e.e. as determined by ^{1}H NMR of the (−)-cinchonidine salt) was synthesized according to our method [9].

2.2. Derivatizations of di-O-acyl glycerols

Preparation of 1,2-di-O-(+)-TBMB-3-O-tert.-butyldimethylsilyl-rac-glycerol (4) from 1,2-di-O-palmitoyl-rac-glycerol (1)

Silylation of 1,2-di-O-acyl-rac-glycerol. 1,2-Di-O-palmitoyl-*rac*-glycerol (**1**, 18.0 mg, 0.03 m*M*) in methylene chloride (10 ml) was added to *tert.*-butyldimethylsilyl chloride (TBDMS-Cl, 300 mg, 1.99 m*M*) in dry pyridine (10 ml) at room temperature. After 2 h, the reaction mixture was diluted with CH_2Cl_2 (20 ml) and washed with saturated $NaHCO_3$ solution (2×10 ml) and water (20 ml) to neutrality. The methylene chloride solution was dried over $MgSO_4$, the latter was removed by filtration and the solvent was evaporated in vacuo at 40°C to afford the monosilyl compound **2**, which was purified by column chromatography on silica gel [toluene–ethyl acetate (20:1, v/v)] before use in the next reaction (20.0 mg, yield 93%) [7].

Replacement of acyl groups of 2 with (+)-TBMB groups. The acyl groups were removed a Grignard reaction as described previously [7]. Methylmagnesium bromide in diethyl ether (0.1

ml, 0.3 m*M*) was added to the solution of **2** (20.0 mg, 0.03 m*M*) in dry diethyl ether (10 ml) under a nitrogen atmosphere. The mixture was stirred for a few minutes and saturated NH_4Cl solution (20 ml) was added carefully to decompose the excess of the Grignard reagent at 0°C. After 1 h, the usual work-up in a similar manner to that described previously [7] using ethyl acetate (3 × 20 ml) gave the deacylated derivative **3** (yield *ca.* 100%).

On the other hand, (+)-TBMB-COOH (71 mg, 100% e.e., 0.3 m*M*) in dry benzene (10 ml) was added into $SOCl_2$ (450 mg, 3.77 m*M*) and the mixture was kept at 60°C. After 10 min, excess of $SOCl_2$ and benzene were removed in vacuo to give (+)-TBMB-COCl. Then, dry pyridine (10 ml), 4-dimethylaminopyridine (DMAP, *ca.* 10 mg) and (+)-TBMB-COCl were added to the solution of **3** in dry CH_2Cl_2 (10 ml) at room temperature. After 4 h, the usual work-up gave optically active 1,2-di-O-(+)-TBMB-3-O-*tert.*-butyldimethylsilyl-*rac*-glycerol (**4**), which was purified by preparative TLC [*n*-hexane–ethyl acetate (20:1, v/v)] (18.2 mg, yield 97%).

1,2 - Di - O - (+) - TBMB - 3 - O - TBDMS - *rac* - glycerol (**4**): $[\alpha]_D^{22}$ + 19.5° (*c* = 0.58, MeOH); high-resolution electron impact (EI) MS, found 642.3199, calculated for $C_{35}H_{50}O_9Si$ $[M^+]$ 642.3221; ^1H NMR (400 MHz, $CDCl_3$), δ 0.032–0.038 [12H, s × 2, $(Si–Me_2) \times 2$], 0.869–0.870 [18H, s × 2, (Si–*tert.* - Bu) × 2], 0.999–1.046 [36H, s × 3, (TBMB–*tert.* - Bu) × 4], 1.485–1.568 [12H, s × 3, (TBMB–Me) × 4], 3.940–5.401 [10H, m, glycerol (*sn*-1, *sn*-2, *sn*-3) × 2], 6.707–7.348 [12H, m, (TBMB-aromatic 3H) × 4].

Preparation of 1,2-di-O-(+)-TBMB-3-O-tert.-butyldimethylsilyl-sn-glycerol (4a) from 1,2-di-O-palmitoyl-sn-glycerol (1a)

Silylation and subsequent di-O-(+)-TBMB derivatization of 1,2-di-O-palmitoyl-*sn*-glycerol [**1a**, *ca.* 100% e.e. as determined by HPLC of the (+)-TBMB derivative], conducted in the same manner as described above, gave optically active 1,2-di-O-(+)-TBMB-3-O-*tert.*-butyldimethylsilyl-*sn*-glycerol (**4a**, *ca.* 100% e.e., yield 92%). The HPLC analysis indicated that **4a** is optically pure.

1,2 - Di - O - (+) - TBMB - 3 - O - TBDMS - *sn* - glycerol (**4a**, standard sample): $[\alpha]_D^{22}$ + 7.7° (*c* = 1.08, MeOH); high-resolution EI-MS, found 642.3222, calculated for $C_{35}H_{50}O_9Si$ $[M^+]$ 642.3221; ^1H NMR (400 MHz, $CDCl_3$), δ 0.032–0.038 (6H, s × 2, $Si–Me_2$), 0.869 (9H, s, Si–*tert.*-Bu), 1.046 [18H, s, (TBMB–*tert.*-Bu) × 2], 1.485 ([6H, s, (TBMB–Me) × 2], 3.913–3.992 [2H, dd × 2, glycerol (*sn*-3)], 4.553–4.622 [2H, dd × 2, glycerol (*sn*-1)], 5.378–5.401 [1H, m, glycerol (*sn*-2)], 6.722–7.335 [6H, m, (TBMB–aromatic 3H) × 2].

Preparation of 2,3-di-O-(+)-TBMB-1-O-tert.-butyldimethylsilyl-sn-glycerol (4b)

2,3 - Di - O - (+) - TBMB - 1 - O - *tert.* - butyl - dimethylsilyl - *sn* - glycerol [**4b**, R_F = 0.26 on TLC, *n*-hexane–ethyl acetate (20:1, v/v)] was separated from 1,2-di-O-(+)-TBMB-3-O-*tert.*-butyl-dimethylsilyl-*rac*-glycerol (**4**) on a preparative TLC plate [*n*-hexane–ethyl acetate (20:1, v/v), developed five times]. The lower band on the preparative TLC plate corresponding to **4b** was carefully scraped off and dissolved in CH_2Cl_2 (10 ml). Then, silica gel was removed by filtration (CH_2Cl_2, 3 × 10 ml) and the filtrate was evaporated to dryness at 40°C under reduced pressure to give **4b** (diastereomer of **4a**).

1,2 - Di - O - (+) - TBMB - 3 - O - *tert.* - butyl - dimethylsilyl - *sn* - glycerol (**4a**, R_F = 0.28) was also separated on the preparative TLC plate from 1,2-di-O-(+)-TBMB-3-O-*tert.*-butyl-dimethylsilyl-*rac*-glycerol (**4**) in the same manner as described above.

2,3 - Di - O - (+) - TBMB - 1 - O - TBDMS-*sn*-glycerol (**4b**): $[\alpha]_D^{22}$ + 32.3° (*c* = 0.67, MeOH); high-resolution EI-MS, found 642.3223, calculated for $C_{35}H_{50}O_9Si$ $[M^+]$ 642.3221; ^1H NMR (400 MHz, $CDCl_3$), δ 0.037 (6H, s, $Si–Me_2$), 0.870 (9H, s, Si–*tert.*-Bu), 0.999–1.011 [18H, s × 2, (TBMB–*tert.*-Bu) × 2], 1.562–1.568 [6H, s × 2,(TBMB–Me) × 2], 3.932–3.995 [2H, dd × 2, glycerol (*sn*-1)], 4.525–4.631 [2H, dd × 2, glycerol (*sn*-3)], 5.375–5.401

[1H, m, glycerol (*sn*-2)], 6.719–7.347 [6H, m, (TBMB–aromatic 3H) × 2].

1,2 - Di - O - (+) - TBMB - 3 - O - TBDMS-*sn*-glycerol (**4a**, scraped off from **4** on TLC plate): $[\alpha]_D^{22} + 7.7°$ ($c = 0.94$, MeOH); high-resolution EI - MS, found: 642.3222, calculated for $C_{35}H_{50}O_9Si$ [M^+] 642.3221; ^1H NMR (400 MHz, $CDCl_3$), δ 0.032–0.038 (6H, s × 2, Si-Me_2), 0.869 (9H, s, Si–*tert.*-Bu), 1.046 [18H, s, (TBMB–*tert.*-Bu) × 2], 1.485 [6H, s, (TBMB–Me) × 2], 3.913–3.992 [2H, dd × 2, glycerol (*sn*-3)], 4.553–4.622 [2H, dd × 2, glycerol (*sn*-1)], 5.378–5.401 [1H, m, glycerol (*sn*-2)], 6.722–7.335 [6H, m, TBMB–aromatic 3H) × 2].

*Preparation of 1,3-di-O-(+)-TBMB-2-O-tert.-butyldimethylsilyl-sn-glycerol (**4c**) from 1,3-di-O-palmitoyl-sn-glycerol (**1c**)*

Silylation and subsequent di-O-(+)-TBMB derivatization of 1,3-di-O-palmitoyl-*sn*-glycerol (**1c**, achiral) were also conducted by the above method to give 1,3-di-O-(+)-TBMB-2-O-*tert.*- butyldimethylsilyl-*sn*-glycerol (**4c**, yield 89%).

1,3 - Di - O-(+) - TBMB - 2 - O - TBDMS - *sn*-glycerol (**4c**): $R_F = 0.28$ on TLC [*n*-hexane–ethyl acetate 20:1, (v/v)]; high-resolution EI-MS, found 642.3197, calculated for $C_{35}H_{50}O_9Si$ [M^+] 642.3221; ^1H NMR (400 MHz, $CDCl_3$), δ 0.087–0.093 (6H, s × 2, Si–Me_2), 0.860 (9H, s, Si-*tert.*-Bu), 1.063–1.066 [18H, s × 2, (TBMB–*tert.*-Bu) × 2], 1.587–1.600 [6H, s × 2, (TBMB–Me) × 2], 4.264–4.291 [1H, m, glycerol (*sn*-2)], 4.390–4.500 [4H, m, glycerol (*sn*-1, *sn*-3)], 6.745–7.346 [6H, m, (TBMB–aromatic 3H) × 2].

2.3. Fluorescence spectra and HPLC separations

For measurement of the excitation and emission spectra of each derivative (**4a** or **4b**), a JASCO (Tokyo, Japan) Model FP-550A spectrofluorimeter with a 1-cm quartz cell was employed without spectral correction. Excitation and emission spectra of 6.86 μM of each derivative (**4a** or **4b**) were measured in the same solvent as the HPLC mobile phase [*n*-hexane–*n*-butanol (750:1, w/w)]. The fluorescence intensities of the derivatives were determined at the maximum excitation and emission wavelengths.

HPLC separations were conducted with a Tosoh (Tokyo, Japan) CCPM instrument connected to an FS-8010 fluorescent detector monitoring at $\lambda_{ex} = 310$ nm and $\lambda_{em} = 370$ nm. Separations were achieved on a Deverosil 60-3 silica gel column (Nomura Chemical) (stainless steel, 5 cm × 4.6 mm I.D.). The analyses were carried out isocratically using a mixture of HPLC-grade *n*-hexane and *n*-butanol (750:1, w/w) at a flow-rate of 0.6 ml/min as the mobile phase at room temperature. For quantitative determination, peak areas were calculated using an 807-IT integrator (JASCO).

2.4. Applications to the determination of stereochemistry of lipase-catalysed reaction

Lipase-catalysed hydrolysis of triolein in the initial stages

A suspension of triolein (100 mg) in 4 ml of 1 *M* Tris–HCl buffer (pH 7.5) containing the enzyme (Amano AP, 25 mg) was incubated at 40°C with vigorous shaking. After the reaction had proceeded to the extent of *ca.* 5–10% (10 min), monitored by silica gel TLC [toluene–ethyl acetate (20:1, v/v)], the enzymatic hydrolysate was extracted with diethyl ether [7]. Silylation and subsequent di-O-(+)-TBMB derivatization were then conducted according to the above method.

Lipase-catalysed esterification of 2-O-benzoyl-glycerol

The substrate of lipase-catalysed esterification, 2-O-benzoylglycerol, was prepared according to the literature [12]. 2-O-Benzoyl-1,3-di-O-benzylglycerol (120 mg, 0.32 m*M*) in ethyl acetate (10 ml) was debenzylated with Pd–C under a hydrogen atmosphere at room temperature (yield *ca.* 100%). At the end of the reaction, the mixture was filtered off and concentrated under reduced pressure at 40°C.

2-O-Benzoylglycerol (60 mg) in diisopropyl

ether (5 ml) was mixed with palmitic acid (660 mg, 2.56 m*M*) and lipase (Amano AP, 15 mg), and the mixture was incubated at 40°C. After 20 h, the reaction mixture was filtered off, washed with ethyl acetate (3 × 10 ml) and concentrated under reduced pressure at 40°C. Silylation and subsequent di-O-(+)-TBMB derivatization were then conducted according to the above method.

3. Results and discussion

By di-O-TBMB derivatization, the chiral di-O-acylglycerols were subjected to the introduction of two new chiral centres, which led to the generation of eight kinds of diastereomers. Hence it is essential for the determination of the original optical purity of 1,2-(or 2,3)di-O-acyl-*sn*-glycerol (**1a** or **1b**) to use (+)-TBMB-COOH acid of 100% optical purity for the derivatization. (+)-TBMB-COOH [100% e.e. as determined by ^{1}H NMR of the (−)-cinchonidine salt] used in this study was synthesized according to our method, and its optical purity was confirmed in our previous studies [9–11]. Consequently, only two diastereomers can be derived from a mixture of 1,2- and 2,3-di-O-acylglycerols, as can be seen in Fig. 3.

The TBMB derivatization shown in Fig. 1 was detailed in the Experimental section. It proceeded to an overall yield of more than 90% and no acyl migration was observed during the reactions. The TBMB carboxylation of **3** (or **3a** or **3c**) was performed under the usual conditions using pyridine in CH_2Cl_2. The use of more than a fourfold excess of (+)-TBMB-COOH was necessary for the completion of the derivatization. The other reaction conditions from **1** to **2** and then to **3** had been optimized previously [7,8].

Fig. 1 represents all reactions starting from di-O-acylglycerol (racemate **1** or optically active **1a** or achiral **1c**) to obtain the corresponding TBMB derivative (**4** or **4a** or **4c**). Compound **4a** could be also obtained from **4** by the TLC separation. Compound **4b** (diastereomer of **4a**) was also obtainable in this way.

Fig. 2 shows a typical HPLC trace of the key compounds (**4**) derived from di-O-acylglycerols. HPLC gave the 1,2-di-O-(+)-TBMB-3-O-TBDMS-*sn*-glycerol (**4a**) and 2,3-di-O-(+)-TBMB-1-O-TBDMS-*sn*-glycerol (**4b**, diastereomer of **4a**) as two symmetrical peaks in less than 30 min after injection with a separation factor (α) of 1.35. The *sn*-1,2-isomer (**4a**) was eluted first, followed by the *sn*-2,3-isomer (**4b**). In addition, the 1,3-di-O-(+)-TBMB-2-O-TBDMS-glycerol (**4c**) derivatized from 1,3-di-O-acylglycerol (**1c**, achiral), which may be contaminated in the lipase hydrolysate, was eluted in front of the *sn*-1,2-isomer (**4a**). Hence, all three regioisomers of di-O-acylglycerols were well separated using TBMB derivatization.

For the fluorescence spectra, the maximum excitation and emission wavelengths of 1,2-di-O-(+)-TBMB-3-O-TBDMS-*sn*-glycerol (**4a**) were approximately 315–317 and 358–361 nm [in *n*-hexane–*n*-butanol (750:1, w/w)], respectively. The same results were obtained in the fluorescence spectra of 2,3-di-O-(+)-TBMB-1-O-TBDMS-*sn*-glycerol (**4b**). Both of the excitation and emission fluorescence intensities were identical for **4a** and **4b**, confirming that the two diastereomers can be monitored quantitatively in HPLC analysis using a fixed single wavelength for fluorescence excitation and detection. The HPLC fluorescence detector was therefore, fixed at 310 and 370 nm for excitation and detection, respectively.

In order to confirm the reproducibility and the efficiency of the present method, the derivatization was carried out using di-O-acylglycerols of known optical purities to give the results summarized in Table 1 and Fig. 3. By using a series of standard solutions containing a mixture of di-O-acylglycerols (**1** and **1a**) with a known ratio, excellent agreement was obtained for the optical purities before and after the derivatization of di-O-acylglycerols within the usual limits of variation (S.D. = 0.91, $n = 4$).

Fig. 3 shows HPLC traces of di-O-TBMB-glycerol derivatives derived from four standard di-O-acylglycerols represented in Table 1. It should be noted that the chromatogram (iv) of the corresponding 1,2-di-O-(+)-TBMB-3-O-TBDMS-*sn*-glycerol derivative from commercial-

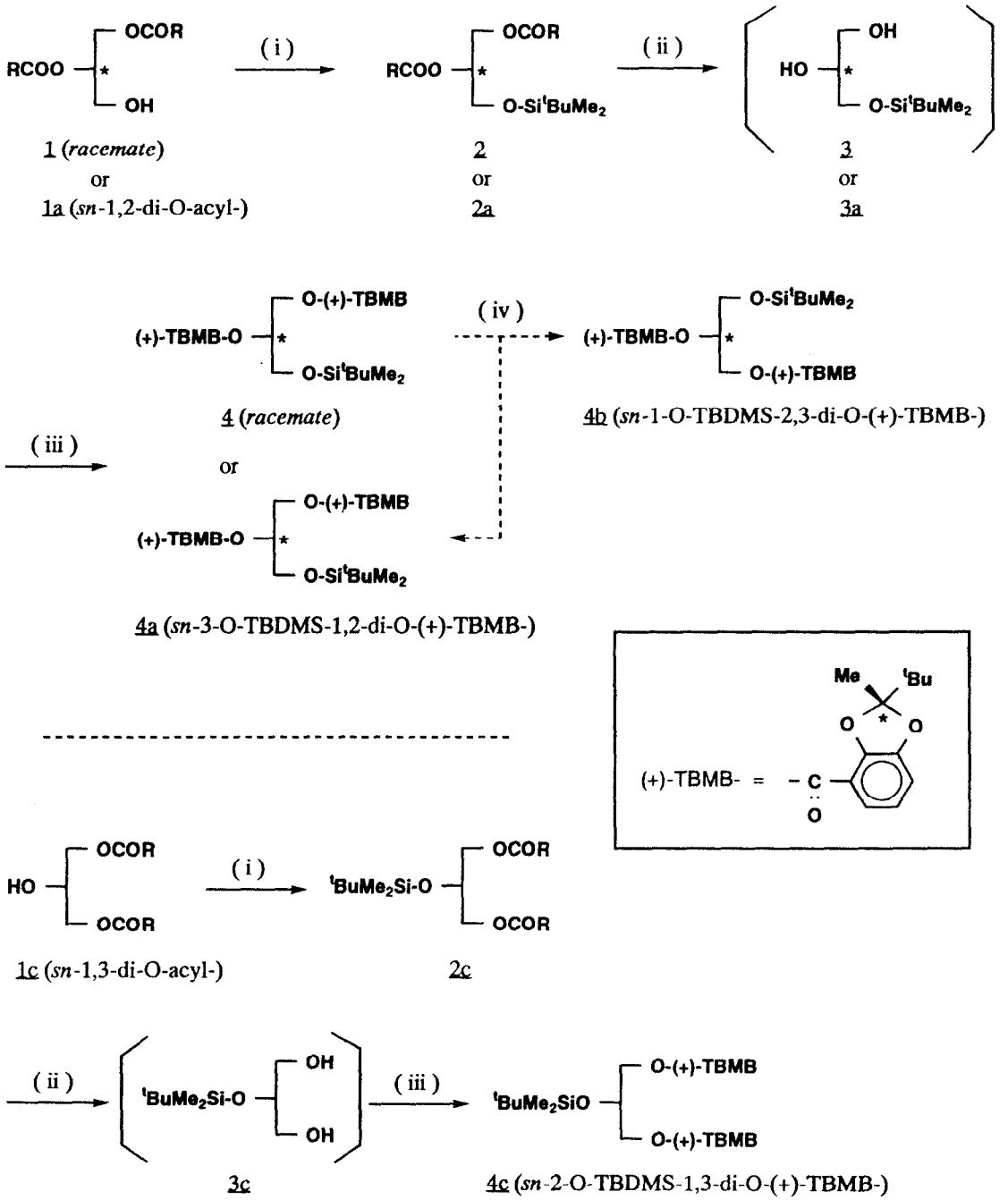

Fig. 1. Scheme for the derivatization to the di-O-(+)-TBMB-glycerol derivatives from di-O-acylglycerols. 1 = D,L-1,2-dipalmitin (racemate); **1a** = 1,2-di-O-palmitoyl-*sn*-glycerol (*sn*-1,2-enantiomer); 1c = 1,3-di-O-palmitoyl-*sn*-glycerol, R = $-(CH_2)_{14}CH_3$. (i) Pyridine, *tert.*-butyl-dimethylsilyl chloride (TBDMS-Cl), r.t.; (ii) methylmagnesium bromide, diethyl ether, r.t.; (iii) (+)-TBMB-COCl, pyridine, 4-dimethylaminopyridine (DMAP), r.t.; (iv) separation by TLC [*n*-hexane–ethyl acetate (20:1, v/v), developed five times] (r.t. = room temperature).

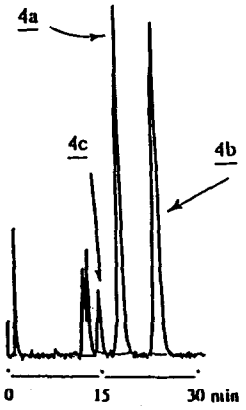

Fig. 2. Chromatogram of di-O-acylglycerols labelled with (+)-TBMB-COOH. **4a** = *sn*-3-O-TBDMS-1,2-di-O-(+)-TBMB-glycerol; **4b** = *sn*-1-O-TBDMS-2,3-di-O-(+)-TBMB-glycerol; **4c** = *sn*-2-O-TBDMS-1,3-di-O-(+)-TBMB-glycerol. HPLC conditions: silica gel column (Deverosil 60-3, 5 cm × 4.6 mm I.D.); λ_{ex} = 310 nm, λ_{em} = 370 nm; eluent, *n*-hexane–*n*-butanol (750:1, w/w); flow-rate 0.6 ml/min; temperature, 22–24°C.

ly available 1,2-di-O-palmitoyl-*sn*-glycerol (99% purity, Sigma) gave no peak of 2,3-di-O-(+)-TBMB-1-O-TBDMS-*sn*-glycerol (**4b**) or the 1,3-isomer (**4c**). This means that the 1,2-di-O-palmitoyl-*sn*-glycerol (**1a**) used is optically pure (*ca.* 100% e.e.). This, together with the other results in Fig. 3 and Table 1, indicates that during the derivatization procedures (silylation, Grignard deacylation and TBMB derivatization) no migration of acyl and silyl groups occurs, which might affect the quantification in the present method. The use of di-O-palmitoyl-*rac*-glycerol (**1**) gave two peaks corresponding to **4a** and **4b** with identical intensities. This also reveals that there is no chiral discrimination by (+)-TBMB-COOH in the derivatization.

Therefore, the peak areas of **4a** and **4b** under the present HPLC conditions could be used directly to determine the optical purity of the mixture of di-O-acyl glycerols without a calibration process. The detection limit of 1,2-di-O-palmitoyl-*sn*-glycerol (**1a**) was about 0.3 pmol on-column (signal-to-noise ratio = 3).

We applied this method to determine the stereoselectivities both in lipase-catalysed hydrolysis and esterification using triolein and 2-O-benzoylglycerol, respectively (Table 2). The lipase hydrolysate and the esterificate were both derivatized to the key compound di-O-(+)-TBMB-glycerol, which was subjected to the HPLC analysis to determine the optical purity. HPLC showed that the optical purity was 42.0% e.e. (S.D. = 0.84, n = 5) with *sn*-1 preference in the hydrolysis and 7.9% e.e. (S.D. = 0.40, n = 4) with *sn*-3 preference in the esterification. Although the di-O-acylglycerols obtained by these lipase reactions were extremely small amounts

Table 1
Comparison of optical purities before and after the derivatization of standard di-O-acylglycerols

Standard di-O-acylglycerol mixture before derivatization[a]			After derivatization to di-O-(+)-TBMB-glycerol derivatives[b]	
D,L-1,2-Dipalmitin (**1**, racemate) (mg)	1,2-Di-O-palmitoyl-*sn*-glycerol (**1a**) (mg)	Calculated optical purity (% e.e.)	Average observed optical purity (% e.e.)	S.D.
27	Free	0	0.3 (*sn*-1,2-) (n = 4)	0.91
18.22	9.04	33.2	32.9 (n = 2)	–
7.25	14.75	67.1	68.3 (n = 2)	–
Free	18	100	99.6 (n = 5)	0.83

[a] Each standard solution was prepared by mixing **1** (racemate) and **1a** (*ca.* 100% e.e.) in the required and their optical purity was calculated from the ratio of the **1** and **1a** contents [% e.e. before the derivatization = **1a**/(**1a** + **1**) · 100].

[b] The HPLC peak areas of di-O-(+)-TBMB glycerol derivatives (**4a** and **4b**) derived from each standard di-O-acylglycerol mixture were used directly to determine the optical purity of the mixture of di-O-acylglycerols without correction [% e.e. after the derivatization = (peak area of **4a** − peak area of **4b**)/(peak area of **4a** + peak area of **4b**) · 100].

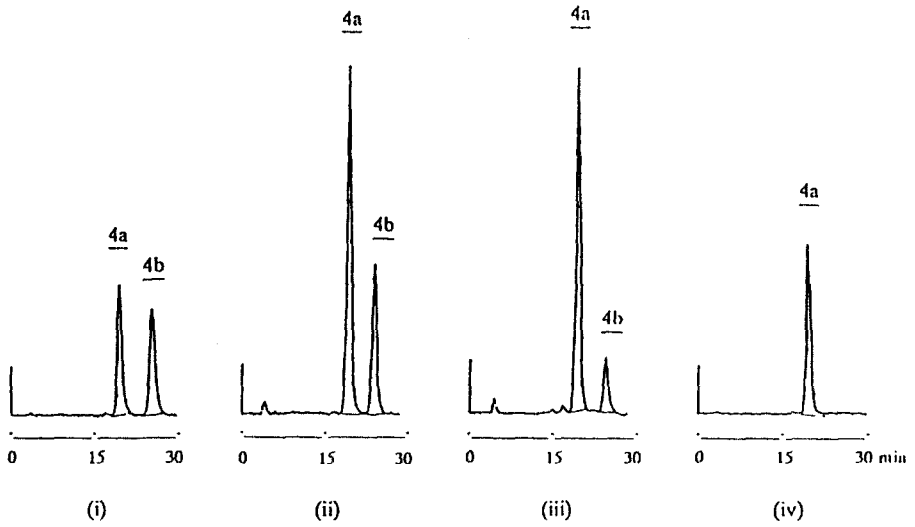

Fig. 3. Chromatograms of di-O-(+)-TBMB-glycerol derivatives derived from each standard mixture of di-O-acylglycerols (**1** and **1a**) with a known ratio (see Table 1). (i) Racemate, (ii) 33.2%, (iii) 67.1% and (iv) 100% optical purity of di-O-acylglycerols. HPLC conditions as in Fig. 2.

(*ca.* 5 mg, <10% yield), their corresponding di-O-TBMB derivatives were analysed with good reproducibility.

For comparison of these results, the same lots of the lipase hydrolysate and the esterificate were also subjected to our previous method based on chiral column HPLC [8]. The two methods indicated the well matched optical

Table 2
Optical purities of di-O-(+)-TBMB-glycerols derived from di-O-acylglycerols obtained by lipase-catalysed hydrolysis and esterification

Substrate —(lipase, diisopropyl ether)→ di-O-acylglycerol → **4**	Optical purity (% e.e.)		Preference of lipase reaction
	Average	S.D.	
Hydrolysis:			
$RCOO\left\{\begin{matrix}OCOR\\OCOR\end{matrix}\right. \rightarrow RCOO\left.\right\}* \begin{matrix}OH\\OCOR\end{matrix} \rightarrow$ **4**	42.0 ($n = 5$)[a]	0.84	*sn*-1
$R = -(CH_2)_7CH=CH(CH_2)_7CH_3$			
Esterification:			
$BzO\left\{\begin{matrix}OH\\OH\end{matrix}\right. \rightarrow BzO\left.\right\}* \begin{matrix}OH\\OCOR'\end{matrix} \rightarrow$ **4**	7.9 ($n = 4$)[a]	0.40	*sn*-3
$R' = -(CH_2)_{14}CH_3$			

[a] For the comparison of the results for the present di-O-(+)-TBMB derivative method with those for our previous method [8], the same lots of the lipase hydrolysate and the esterificate were also subjected to our previous method based on chiral column HPLC. The optical purities of the former and the latter were 43.5% e.e. with *sn*-1 preference and 5.6% e.e. with *sn*-3 preference, respectively.

purities and absolute configurations of the di-O-acylglycerols. However, it should be noted that the previous method was less sensitive and needed an expensive chiral HPLC column and GC equipment to separate the 1,3-di-O-benzoylated isomer from the *sn*-1,2-di-O-benzoylated glycerol overlapping in the HPLC separation.

Using the present method, *sn*-1,2-, *sn*-2,3- and 1,3-di-O-(+)-TBMB isomers obtained from their corresponding di-O-acylglycerols could be completely separated within 30 min by silica gel column HPLC and detected with higher sensitivity (>100-fold) using fluorescence detection than using our previous dibenzoate method with UV detection.

In conclusion, a new approach was developed to determine the optical purity of 1,2-(or 2,3-)di-O-acylglycerol, which has been one of the most difficult targets in enantiomeric separations. This method needs no special analytical equipment and allows the optical purity to be determined at the picomole level taking advantage of the strong fluorescence of (+)-TBMB-COOH.

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