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Simple and highly sensitive high-performance liquid chromatographic method for separating enantiomeric diacylglycerols by direct derivatization with a fluorescent chiral agent, (*S*)-(+)-2-*tert*.-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid

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Abstract

A simple and highly sensitive HPLC method was developed for the determination of the absolute configuration and the optical purity of diacylglycerols. The method involves direct fluorescent labelling of diacylglycerols with (*S*)-TBMB-carbonyl chloride in pyridine and HPLC separation of the derived diastereomeric (*S*)-TBMB-carboxylated diacylglycerol derivatives [(*S*)-TBMB = (*S*)-(+)-2-*tert*.-butyl-2-methyl-1,3-benzodioxole]. Complete separation of the diastereomeric (*S*)-TBMB-carbonyl-diacylglycerol derivatives and thus indirectly of the enantiomers of the parent diacylglycerols and of the *sn*-1,3-regioisomer was achieved using normal-phase silica gel HPLC within 30 min for every saturated single acid ($R_1 = R_2$) diacylglycerol ($C_{12:0}$–$C_{22:0}$) examined.

1. Introduction

During the last decade, several methods have been proposed for separating chiral diacylglycerols, in which a combination of 3,5-dinitrophenylurethane (3,5-DNPU) derivatization and chiral-phase HPLC was applied by Takagi and co-workers [1–3] and Sempore and Bezard [4]. Alternatively, a combination of chiral urethane derivatization and a normal-phase silica gel column was also employed by Michelsen et al. [5]. Rogalska et al. [6] and Laakso and Christie [7]. However, these methods have limited sensitivities with UV detection and needed relatively long elution times and long columns. Another approach was reported by Kruger et al. [8] to increase the sensitivity by using a fluorescent chiral agent, but chiral separation of diacylglycerols by HPLC was not satisfactory.

In previous papers, we have reported general methods for determining the optical purity and the absolute configuration of diacylglycerols via derivatization into a key compound, 1,2- (or 2,3-) di-O-benzoyl-3-O-*tert*.-butyldimethylsilyl-*sn*-glycerol, which was determined either by circular dichroism (CD) or chiral column HPLC with UV detection [9,10]. More recently, we have proposed a different approach using a

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chiral derivatizing agent, (*S*)-TBMB-carboxylic acid [(*S*)-TBMB = (*S*)-(+)-2-*tert.*-butyl-2-methyl-1,3-benzodioxole] [11], in order to increase the sensitivity due to the strong fluorescence activity of the reagent [12]. Our previous methods are convenient because they need no authentic sample with a known configuration to determine the absolute configuration and the optical purity of diacylglycerols. However, the derivatizing procedures may be laborious, and acyl moieties of diacylglycerol cannot be characterized, as acylglycerols are deacylated leading to a mono-O-*tert.*-butyldimethylsilyl di-O-benzoyl or di-O-(*S*)-TBMB-carbonylated derivative. In order to solve these problems, we attempted a direct derivatization of diacylglycerols with (*S*)-TBMB-carboxylic acid and the separation of the diastereoisomers by HPLC with fluorescence detection.

In this paper, we report that all possible isomers of *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerols can be completely separated within 30 min and detected in a highly sensitive manner based on the fluorescence of the (*S*)-TBMB reagent. We applied this method to determine the optical purity of sub-microgram levels of diacylglycerols produced by the lipase-catalysed hydrolysis of triacylglycerol.

2. Experimental

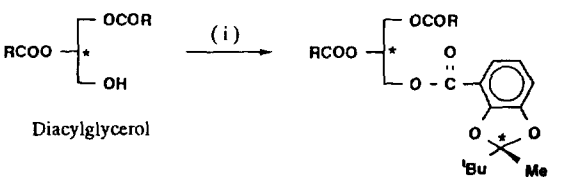
2.1. Chemicals

Optically active 1,2-dipalmitoyl-*sn*-glycerol {ca. 100% enantiomeric excess (e.e.) as determined with our previous method [12] by HPLC of the (*S*)-TBMB derivative} and 1,3-dipalmitoyl-*sn*-glycerol were purchased from Sigma (St. Louis, MO, USA) and its racemate, 1,2-dipalmitoyl-*rac*-glycerol, was obtained from Nacalai Tesque (Kyoto, Japan). (*S*)-(+)-2,2-Dimethyl-1,3-dioxolane-4-methanol and its racemate (solketal) were purchased from Tokyo Kasei (Tokyo, Japan) and used for the synthesis of (*S*)-TBMB derivatives of the saturated single-acid ($C_{12:0}$–$C_{22:0}$) diacylglycerols as described below. Lipase AP (Amano PS, EC 3.1.1.3) from *Pseudomonas* sp. was purchased from Amano Pharmaceutical (Nagoya, Japan) and PPL (EC 3.1.1.3, type II) from porcine pancreas from Sigma. (*S*)-TBMB-COOH [100% e.e. as determined by ^{1}H NMR of the (−)-cinchonidine salt] was synthesized according to a previously described method [11].

2.2. (S)-TBMB derivatization of diacylglycerols

Diacylglycerols were directly derivatized to the (*S*)-TBMB-carbonylated derivatives according to the scheme in Fig. 1. To a solution of (*S*)-TBMB-COOH (33 mg, 100% e.e., 0.14 m*M*) in dry benzene (5 ml) was added $SOCl_2$ (370 mg, 3.11 m*M*), and the mixture was kept at 60°C. After 10 min, excess $SOCl_2$ and benzene were removed in vacuo to give (*S*)-TBMB-COCl [12]. A dry pyridine solution (0.5 ml) of 10% of 4-dimethylaminopyridine (DMAP) and (*S*)-TBMB-COCl (24 mg, 0.1 m*M*) was added to the solution of dipalmitoyl-*rac*-glycerol (17 mg, 0.03 m*M*) in dry CH_2Cl_2 (5 ml) with stirring at room temperature. After 2 h, the reaction mixture was diluted with CH_2Cl_2 (5 ml) and washed with saturated $NaHCO_3$ solution (3 × 10 ml) and water (20 ml). 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 (*S*)-TBMB-carbonyl-dipalmitoyl-*rac*-glycerol, which was purified by preparative TLC [*n*-hexane–ethyl acetate (10:1, v/v)] (20 mg, yield 85%).

1-(*S*)-TBMB-carbonyl-2,3-dipalmitoyl-*rac*-glycerol: high-resolution electron-impact (EI) MS, found 786.6033, calculated for $C_{48}H_{82}O_8$,



Diacylglycerol

Fig. 1. Scheme for the direct derivatization of diacylglycerols with (*S*)-TBMB-COCl forming diastereomeric derivatives. (i) (*S*)-TBMB-COCl, pyridine, 4-dimethylaminopyridine (DMAP), room temperature.

[M^+] 786.6005; ^{1}H NMR (400 MHz, C^2HCl_3), δ0.862–0.896 [12H, m, (dipalmitoyl-Me) × 2], 1.077 and 1.083 [9H × 2, s × 2, (TBMB-*tert.*-Bu) × 2], 1.255 [96H, m, (dipalmitoyl-$(CH_2)_{12}$–) × 2], 1.513–1.606 [8H, m, (dipalmitoyl-β-CH_2–) × 2], 1.603 and 1.606 [3H × 2, s × 2, (TBMB-Me) × 2], 2.278–2.325 [8H, m, (dipalmitoyl-α-CH_2–) × 2], 4.276–5.392 [10H, m, glycerol (*sn*-1, *sn*-2, *sn*-3) 5H × 2], 6.749–7.327 [6H, m, (TBMB-aromatic 3H) × 2].

(*S*)-TBMB derivatization of 1,2-dipalmitoyl-*sn*-glycerol and optically inactive 1,3-dipalmitoyl-*sn*-glycerol were also conducted in the same manner as described above.

3-(*S*)-TBMB-carbonyl-1,2-dipalmitoyl-*sn*-glycerol: high-resolution EI-MS, found 786.5948, calculated for $C_{48}H_{82}O_8$, [M^+] 786.6005; ^{1}H NMR (400 MHz, C^2HCl_3), δ0.862–0.896 (6H, m, dipalmitoyl-Me), 1.079 (9H, s, TBMB-*tert.*-Bu), 1.250 [48H, m, dipalmitoyl-$(CH_2)_{12}$–], 1.554 (4H, m, dipalmitoyl-β-CH_2–), 1.603 (3H, s, TBMB-Me), 2.287–2.334 (4H, m, dipalmitoyl-α-CH_2–), 4.263–5.379 [5H, m, glycerol (*sn*-1, *sn*-2, *sn*-3)], 6.756–7.326 (3H, m, TBMB-aromatic).

2-(*S*)-TBMB-carbonyl-1,3-dipalmitoyl-*sn*-glycerol: high-resolution EI-MS, found 786.6027, calculated for $C_{48}H_{82}O_8$, [M^+] 786.6005); ^{1}H NMR (400 MHz, C^2HCl_3), δ0.862–0.897 (6H, m, dipalmitoyl-Me), 1.078 (9H, s, TBMB-*tert.*-Bu), 1.235–1.255 [48H, m, dipalmitoyl-$(CH_2)_{12}$–], 1.518–1.633 (4H, m, dipalmitoyl-β-CH_2–), 1.595 (3H, s, TBMB-Me), 2.279–2.336 (4H, m, dipalmitoyl-α-CH_2–), 4.275–5.508 [5H, m, glycerol (*sn*-1, *sn*-2, *sn*-3)], 6.749–7.317 (3H, m, TBMB-aromatic).

2.3. Preparation of standard samples of diastereoisomeric (S)-TBMB-carbonylated homologeous single-acid diacylglycerol derivatives for the HPLC separations

1-(S)-TBMB-carbonyl-2,3-diacyl-rac-glycerols

(*S*)-TBMB-carbonylated single-acid diacyl-*rac*-glycerols ($C_{12:0}$–$C_{22:0}$) were prepared from solketal (*rac*-2,2-dimethyl-1,3-dioxolane-4-methanol) as follows. Solketal (**1**, 40 mg, 0.3 m*M*) and dry pyridine (1 ml) containing 10% of DMAP were added to a solution of (*S*)-TBMB-COCl (24 mg, 0.1 m*M*) in dry CH_2Cl_2 (5 ml) with stirring at room temperature. After 6 h, the reaction mixture was diluted with CH_2Cl_2 (10 ml) and washed with saturated $NaHCO_3$ solution (3 × 10 ml) and water (20 ml). 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 1-(*S*)-TBMB-carbonyl-2,3-O-isopropylidene-*rac*-glycerol (**2**). This mixture of diastereomers was purified by column chromatography on silica gel [*n*-hexane–ethyl acetate (20:1, v/v)] (31 mg, yield 89%).

1-(*S*)-TBMB-carbonyl-2,3-O-isopropylidene-*rac*-glycerol (**2**): high-resolution EI-MS, found 350.1682, calculated for $C_{19}H_{26}O_6$, [M^+] 350.1728); ^{1}H NMR (400 MHz, C^2HCl_3), δ1.085 and 1.086 [9H × 2, s × 2, (TBMB-*tert.*-Bu) × 2], 1.382 and 1.441 [6H × 2, s × 2, (isopropylidene-2Me) × 2], 1.599 [6H, s, (TBMB-Me) × 2], 3.897–4.454 [10H, m, glycerol (*sn*-1, *sn*-2, *sn*-3) 5H × 2], 6.753–7.357 [6H, m, (TBMB-aromatic 3H) × 2].

A solution of **2** in 75% acetic acid solution (5 ml) was stirred for 4 h at room temperature. The solvent was evaporated in vacuo at 50°C with toluene (10 ml) to give crude 1-(*S*)-TBMB-carbonyl-*rac*-glycerol derivative (**3**, ca. 100% yield). Compound **3** (mixture of diastereomers) was diacylated with the corresponding acyl anhydride or acyl halide in the following manner using lauroyl chloride as a typical procedure.

Commercially available lauroyl chloride (290 mg, 1.32 m*M*) and dry pyridine (1 ml) containing 10% of DMAP were added to a methylene chloride solution (5 ml) of **3** (18 mg, 0.06 m*M*) with stirring at room temperature. After stirring overnight, the reaction mixture was worked up in the same manner as described above for the preparation of **2** to give a diastereomeric mixture of 1-(*S*)-TBMB-carbonyl-2,3-dilauroyl-*rac*-glycerol (**4**), which was purified by preparative TLC [*n*-hexane–ethyl acetate (20:1, v/v)] (31 mg, yield 77%).

1-(*S*)-TBMB-carbonyl-*rac*-glycerol (**3**): high-resolution EI-MS, found 310.1451, calculated for

$C_{16}H_{22}O_6$, [M^+] 310.1415); ^{1}H NMR (400 MHz, C^2HCl_3), δ1.089 and 1.091 [9H × 2, s × 2, (TBMB-*tert.*-Bu) × 2], 1.616 and 1.619 [3H × 2, s × 2, (TBMB-Me) × 2], 3.710–4.541 [10H, m, glycerol (*sn*-1, *sn*-2, *sn*-3) 5H × 2], 6.786–7.375 [6H, m, (TBMB-aromatic 3H) × 2].

1-(*S*)-TBMB-carbonyl-2,3-dilauroyl-*rac*-glycerol (**4**): high-resolution EI-MS, found 674.4764, calculated for $C_{40}H_{66}O_8$, [M^+] 674.4754); ^{1}H NMR (400 MHz, C^2HCl_3), δ0.861–0.895 [12H, m, (dilauroyl-Me) × 2], 1.079 and 1.084 [9H × 2, s × 2, (TBMB-*tert.*-Bu) × 2], 1.251–1.262 [64H, m, (dilauroyl-$(CH_2)_8$–) × 2], 1.568–1.666 [8H, m, (dilauroyl-β-CH_2–) × 2], 1.604 and 1.607 [3H × 2, s × 2, (TBMB-Me) × 2], 2.288–2.335 [8H, m, (dilauroyl-α-CH_2–) × 2], 4.264–5.405 [10H, m, glycerol (*sn*-1, *sn*-2, *sn*-3) 5H × 2], 6.756–7.327 [6H, m, (TBMB-aromatic 3H) × 2].

3-(S)-TBM-carbonyl-1,2-diacyl-sn-glycerols

3-(*S*)-TBMB-carbonyl-1,2-diacyl-*sn*-glycerols ($C_{12:0}$–$C_{22:0}$) were prepared from optically active (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol in a similar manner to that described for the preparation of the racemates.

3-(*S*)-TBMB-carbonyl-1,2-O-isopropylidene-*sn*-glycerol (**2a**): high resolution EI-MS, found 350.1698, calculated for $C_{19}H_{26}O_6$, [M^+] 350.1728); ^{1}H NMR (400 MHz, C^2HCl_3), δ1.086 (9H, s, TBMB-*tert.*-Bu), 1.384 and 1.441 (3H × 2, s × 2, isopropylidene-2Me), 1.600 (3H, s, TBMB-Me), 3.936–4.469 [5H, m, glycerol (*sn*-1, *sn*-2, *sn*-3)], 6.745–7.365 (3H, m, TBMB-aromatic).

3-(*S*)-TBMB-carbonyl-*sn*-glycerol (**3a**): high-resolution EI-MS, found 310.1387, calculated for $C_{16}H_{22}O_6$, [M^+] 310.1415); ^{1}H NMR (400 MHz, C^2HCl_3), δ1.088 (9H, s, TBMB-*tert.*-Bu), 1.617 (3H, s, TBMB-Me), 3.769–4.534 [5H, m, glycerol (*sn*-1, *sn*-2, *sn*-3)], 6.783–7.369 (3H, m, TBMB-aromatic).

3-(*S*)-TBMB-carbonyl-1,2-dilauroyl-*sn*-glycerol (**4a**): high-resolution EI-MS, found 674.4783, calculated for $C_{40}H_{66}O_8$, [M^+] 674.4754); ^{1}H NMR (400 MHz, C^2HCl_3), δ0.861–0.895 (6H, m, dilauroyl-Me), 1.079 (9H, s, TBMB-*tert.*-Bu), 1.252 [32H, m, dilauroyl-$(CH_2)_8$–], 1.568–1.666 (4H, m, dilauroyl-β-CH_2–), 1.604 (3H, s, TBMB-Me), 2.289–2.348 (4H, m, dilauroyl-α-CH_2–), 4.266–5.394 [5H, m, glycerol (*sn*-1, *sn*-2, *sn*-3)], 6.757–7.327 (3H, m, TBMB-aromatic).

The other homologous (*S*)-TBMB-carbonylated single-acid diacylglycerol derivatives ($C_{14:0}$–$C_{22:0}$) showed ^{1}H NMR data almost identical with those of **4** or **4a**, except for the integration of –(CH_2)– signals (ca. 1.25 ppm). On the basis of the TBMB-Me (ca. 1.6 ppm), TBMB-*tert.*-Bu (ca. 1.0 ppm) and the other ^{1}H NMR signals and EI-MS data, their structures were confirmed.

2.4. HPLC separations

Prior to the HPLC analysis, the crude (*S*)-TBMB derivatives were preliminarily purified by silica gel TLC [*n*-hexane–ethyl acetate (10:1, v/v)]. The TLC bands of the derivatives were cut out from the TLC sheet and extracted with the HPLC solvents.

HPLC separations were conducted with a Jasco (Tokyo, Japan) Model 880-PU instrument connected to a Tosoh Model FS-8010 fluorescent detector monitoring at $\lambda_{ex.}$ 310 nm and $\lambda_{em.}$ 370 nm. Separations were performed on a Develosil 60-3 (Nomura Chemical) silica gel column (stainless steel, 25 cm × 4.6 mm I.D.). The analyses were carried out isocratically using a mixture of HPLC-grade *n*-hexane and *n*-butanol (300:1, w/w; flow-rate 0.6 ml/min) as the mobile phase at ambient temperature. For quantitative determinations, peak areas were calculated with a Model 807-IT integrator (JASCO).

2.5. Applications to the determination of stereochemistry of lipase-catalysed hydrolysis of tripalmitin

Tripalmitin obtained from Sigma was purified by chromatography on silica gel using *n*-hexane–ethyl acetate (20:1, v/v) as the mobile phase. A suspension of tripalmitin (20 mg) in 4 ml of 1 *M* Tris–HCl buffer (pH 7.5) containing the enzyme Amano AP (25 mg) or PPL (20 mg) was incubated at 40°C with vigorous shaking. After the

reaction had proceeded up to ca. 5–10% (1 h), monitored by silica gel TLC [toluene–ethyl acetate (20:1, v/v)], the enzymatic hydrolysate was extracted with diethyl ether [11]. Then, subsequent direct (*S*)-TBMB derivatization of diacylglycerols was conducted as described above.

3. Results and discussion

3.1. Direct (S)-TBMB acylation of diacylglycerols and HPLC separation of derivatives

The (*S*)-TBMB-COOH used is optically pure as determined by ^{1}H NMR analysis of the (−)-cinchonidine salt [11]. (*S*)-TBMB acylations of diacylglycerols as detailed under Experimental (Fig. 1) were performed with yields of >80%. A methylene chloride solution of (*S*)-TBMB-COCl, easily available from (*S*)-TBMB-COOH, was treated with diacylglycerols under mild conditions in the presence of pyridine containing 10% DMAP at room temperature. After ca. 2 h the reaction mixture was worked up and placed on a silica gel TLC sheet (5 cm × 5 cm) and eluted with *n*-hexane–ethyl acetate (10:1, v/v). The TLC spots ($R_F = 0.4$) corresponding to fluorescent (*S*)-TBMB-carbonylated diacylglycerol derivatives, owing to the visible fluorescence of the (*S*)-TBMB group, were cut out from the TLC sheet and extracted with the HPLC solvents (*n*-hexane–*n*-butanol) for direct HPLC injection.

Fig. 2i shows HPLC profiles of dipalmitoylglycerols derivatized with (*S*)-TBMB-COCl. Under normal-phase conditions using a Develosil 60-3 silica column (25 cm × 4.6 mm I.D.) and *n*-hexane–*n*-butanol (300:1, w/w) as the mobile phase, a complete separation of *sn*-1,2-, *sn*-2,3- and 1,3-dipalmitoylglycerols was achieved within 30 min; the *sn*-1,2-isomer (a) was eluted first, followed by the *sn*-2,3-isomer (b), and then the *sn*-1,3-isomer (c), derived from optically inactive 1,3-dipalmitoyl-*sn*-glycerol.

3.2. Reproducibility and quantification

In order to confirm the reproducibility and the quantification of the proposed method, (*S*)-TBMB derivatization was carried out using dipalmitoylglycerols of known optical purities (0,

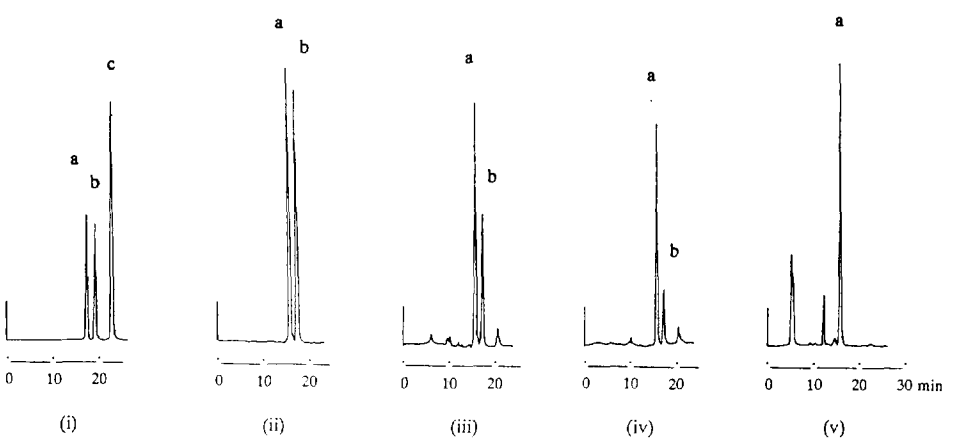


Fig. 2. Typical HPLC separation of isomeric dipalmitoylglycerols labelled with (*S*)-TBMB-COOH and chromatograms of (*S*)-TBMB glycerol derivatives derived from each standard mixture of dipalmitoylglycerols (racemate and *sn*-1,2-) with known ratio (see Table 1). (i) Mixture of 1,3- and 1,2-dipalmitoyl-*rac*-glycerols as their (*S*)-TBMB derivatives (Peaks: a = *sn*-1,2-; b = *sn*-2,3-; c = *sn*-1,3-), (ii) racemate; (iii) 28.5%, (iv) 63.6% and (v) 100% optical purity of dipalmitoylglycerols. HPLC conditions: Develosil 60-3 silica gel column (25 cm × 4.6 mm I.D.): λ_{ex} 310 nm, λ_{em} 370 nm; eluent, *n*-hexane–*n*-butanol (300:1, w/w); flow-rate, 0.6 ml/min; temperature, 22–24°C.

Table 1
Comparison of optical purities before and after derivatization of standard diacylglycerols

Standard diacylglycerol mixture[a]					
Before derivatization			After derivatization with (*S*)-TBMB-COCL[b]		
Dipalmitoyl-*rac*-glycerol (racemate) (mg)	1,2-Dipalmitoyl-*sn*-glycerol (*sn*-1,2) (mg)	Calculated optical purity (% e.e.)	Average of observed optical purity (% e.e.)	*n*	S.D.
10	0	0	0.3 (*sn*-1,2)	7	0.72
2.53	1.01	28.5	29.1	2	–
2.89	5.05	63.6	61.2	2	–
0	5	100	98.9–ca. 100	4	0.55

[a] Each standard solution was prepared by mixing the racemate and *sn*-1,2-diacylglycerol (ca. 100% e.e.) in the appropriate ratio, and their optical purity was calculated from the ratio of the racemate and *sn*-1,2-diacylglycerol contents [% e.e. before derivatization = *sn*-1,2/(racemate + *sn*-1,2) × 100].

[b] The HPLC peak areas of (*S*)-TBMB-diacylglycerol derivatives (*sn*-1,2 and *sn*-2,3) derived from each standard diacylglycerol mixture were used directly to determine the optical purity of the mixture of diacylglycerols without correction [% e.e. after derivatization = (peak area of *sn*-1,2 − peak area of *sn*-2,3)/(peak area of *sn*-1,2 + peak area of *sn*-2,3) × 100].

28.5, 63.6 and 100% e.e.) to give the results summarized in Table 1. Fig. 2ii–v show the HPLC traces for (*S*)-TBMB-glycerol derivatives derived from these standard dipalmitoylglycerols.

As shown in Fig. 2iii and iv, in addition to the peaks of *sn*-1,2- and *sn*-2,3-dipalmitoylglycerols, a small peak of 1,3-dipalmitoylglycerol appeared. This isomer might be generated by acyl migration from *sn*-2 to *sn*-3 or *sn*-1 during the derivatization procedures or the storage of the diacylglycerols, but has no effect on the determination of the optical purities of *sn*-1,2- and *sn*-2,3-diacylglycerols, as all of these three isomers were separated completely. Moreover, excellent agreement was obtained for the optical purities before and after the (*S*)-TBMB derivatization within the usual limits of variation (S.D. = 0.72, $n = 7$), as summarized in Table 1. Fig. 2ii and v show 100:100 and 100:0 peak area ratios of *sn*-1,2- and *sn*-2,3- isomers in the respective HPLC traces for the racemate and optically pure *sn*-1,2-dipalmitoylglycerol.

These results indicate that the peak areas of (*S*)-TBMB-diacylglycerols can be used to determine the absolute configuration and the optical purity without a calibration process. Owing to the strong fluorescence of the (*S*)-TBMB group, these isomers could be determined in less than picomolar concentrations on the HPLC column; the detection limit of 3-(*S*)-TBMB-carbonyl-1,2-dipalmitoyl-*sn*-glycerol could be lowered to about 0.3 pmol on-column (signal-to-noise ratio = 3).

3.3. Indirect HPLC separation of enantiomeric single-acid diacylglycerols after derivatization with (S)-TBMB-COOH

Similar indirect enantiomer separations were also achieved for other homologeous single-acid diacylglycerols, as shown in Fig. 3. These chromatograms represent the diacyl-*rac*-glycerol derivatives prepared from solketal as detailed under Experimental. In every case, the *sn*-1,2-isomer (a) was eluted faster than the *sn*-2,3-isomer (b), and these two peaks also appeared with equal integration to each other. Identification of the diastereomers (and thus of the parent enantiomers) of each (*S*)-TBMB derivative was carried out by co-injection of *sn*-1,2-diacyl derivatives synthesized from (*S*)-(+)-solketal. Another peak (ca. 2% peak area except for ca. 23% in chromatogram i for the dibehenoyl

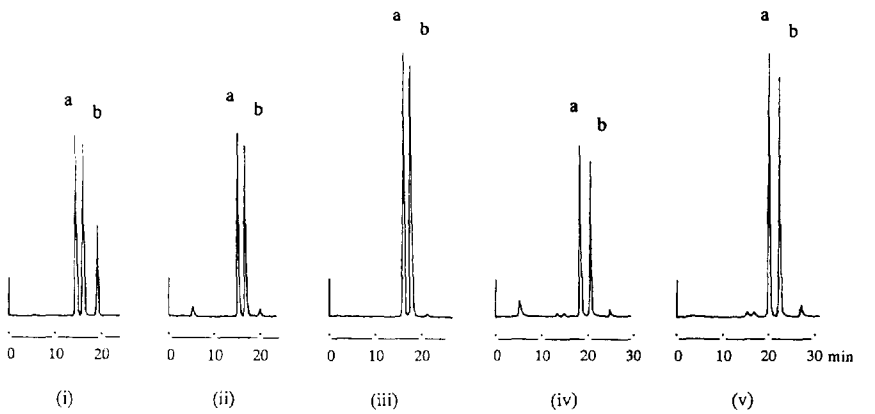


Fig. 3. HPLC separation of each (*S*)-TBMB derivatized homologous saturated single-acid diacyl-*rac*-glycerol ($C_{12:0}$–$C_{22:0}$) synthesized from solketal. (i) Dibehenoyl-; (ii) diarachidoyl-; (iii) distearoyl-; (iv) dimyristoyl-; (v) dilauroyl-. (Peaks: a = *sn*-1,2- and b = *sn*-2,3- in each chromatogram.) HPLC conditions as in Fig. 2.

derivative) appeared after the *sn*-2,3-isomer (b) in each chromatogram, and this peak is highly likely to be a trace amount of their 1,3-isomer derived from acyl migration during the synthetic procedures in the same manner as for dipalmitoylglycerol, judging from the relative retention volumes, which agreed well with the values expected from that of dipalmitoylglycerol.

The HPLC data summarized in Table 2 clearly show that all the single-acid diacylglycerols ($C_{12:0}$–$C_{22:0}$) examined here could be determined by the present method. High separation coefficients ($\alpha = 1.16$) and peak resolutions ($R_s = 2.3$) were obtained for all the isomeric (*S*)-TBMB derivatized diacylglycerols.

Moreover, it is obvious from the retention times that the longer-chain diacylglycerols are eluted faster than the shorter compounds. At-

Table 2
Chromatographic data for homologous single-acid diacylglycerols as their (*S*)-TBMB derivatives

Acyl group	Position	V_r(ml)	k'	α	R_s
Dilauroyl	*sn*-1,2	8.24	2.71	1.16	3.5
	sn-2,3	9.55	3.14		
Dimyristoyl	*sn*-1,2	7.35	2.42	1.16	3.3
	sn-2,3	8.53	2.81		
Dipalmitoyl	*sn*-1,2	6.69	2.20	1.16	3.1
	sn-2,3	7.79	2.57		
	sn-1,3	9.96	3.28	1.28	5.5
Distearoyl	*sn*-1,2	6.01	1.98	1.16	2.9
	sn-2,3	7.00	2.30		
Diarachidoyl	*sn*-1,2	5.44	1.79	1.17	2.7
	sn-2,3	6.34	2.09		
Dibehenoyl	*sn*-1,2	4.81	1.59	1.17	2.3
	sn-2,3	5.62	1.85		

V_r = Retention volume corrected by column void volume (3.04 ml); k' = capacity factor; α = separation coefficient; R_s = peak resolution.

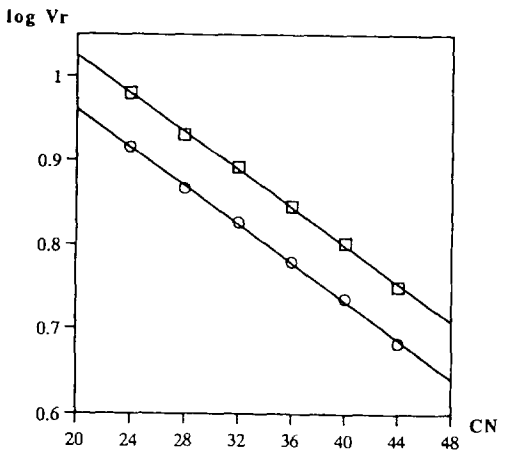


Fig. 4. Relationships of log V_r (retention volume) versus *CN* (total number of acyl carbon atoms) for homologous and isomeric diacylglycerols as their (*S*)-TBMB derivatives separated by HPLC on a silica gel column (Develosil 60-3). ○, Log V_r (*sn*-1,2-); □, log V_r (*sn*-2,3-).

tempts to plot log V_r (retention volume) versus *CN* (total number of acyl carbon atoms) revealed a linear relationship, as can be seen in Fig. 4, and a similar relationship using 3,5-DNPU derivatives was reported by Takagi and co-workers [1–3]. The two straight lines for *sn*-1,2- and *sn*-2,3-diacyl derivatives in Fig. 4 are parallel and the relationships could be expressed by following equations: log V_r (*sn*-1,2-) = $-0.011\ CN + 1.191$; log V_r (*sn*-2,3-) = $-0.011\ CN + 1.251$; E = log V_r (*sn*-2,3-) − log V_r (*sn*-1,2-) = 0.06 where E is the diastereomer separation factor. The above rules will be useful not only for determining indirectly the enantiomer but also the acyl group composition of diacylglycerols with the same acyl moieties.

3.4. Applications to the determination of stereochemistry of lipase-catalysed reactions

We applied the present method to re-examine [6,12] the stereoselectivity of the lipase-catalysed hydrolysis of tripalmitin (Table 3). PPL is known to have poor stereoselectivity for triglycerides [6], and our results with the present method also showed low selectivity (less than 10% e.e.) with a weak *sn*-3 preference. On the other hand, Amano-AP showed higher stereoselectivity (43.1% e.e.) with an *sn*-1 preference, which accorded well with our previous results [12]. In order to confirm these results, we tested a lipase-free reaction using optically pure 1,2-dipalmitoyl-*sn*-glycerol in the reaction buffer. Although the 1,3-isomer appeared at levels up to ca. 3%, no *sn*-2,3-isomer by acyl migration appeared for at least 4 h under the present lipase reactions conditions or during (*S*)-TBMB derivatization procedures, which otherwise would affect the quantification by this method.

In conclusion, we have developed a facile and highly sensitive method for determining the optical purity of 1,2- (or 2,3-) diacyl-*sn*-glycerol which involves derivatization with (*S*)-TBMB-carboxylic acid under mild conditions. The diastereomeric derivatives, including the symmetric 1,3-isomer, were completely separated by HPLC on a normal-phase silica column (Develosil 60-3) within 30 min and detected in less than a 1 p*M*

Table 3
Optical purities of (*S*)-TBMB-dipalmitoylglycerols derived from dipalmitoylglycerols obtained by lipase-catalysed hydrolysis of tripalmitin and reference (lipase-free) with 1,2-dipalmitoyl-*sn*-glycerol (ca. 100% e.e.) in 1.0 *M* Tris–HCl buffer

Substrate	Lipase reaction (40°C)		Optical purity (% e.e.)		Preference of lipase reaction
	Lipase	Time (h)	Average (n = 4)	S.D.	
Reference; 1,2-dipalmitoyl-*sn*-glycerol	–	1	97.4	0.84	–
	–	2	97.6	0.56	–
	–	4	99.2	0.74	–
Hydrolysis; tripalmitin	PPL	1	8.2	0.51	*sn*-3
	AP	1	43.1	0.47	*sn*-1

amount of diacylglycerols, taking advantage of the strong fluorescence of the (*S*)-TBMB group. The study showed also that 3-(*S*)-TBMB-carbonyl-1,2-diacyl-*sn*-glycerol derivatives were always eluted faster than the *sn*-2,3-isomers. This rule is empirically useful for assigning the absolute configurations of chiral diacylglycerols (and for evaluating the optical purity), although the mechanism has still not been clarified sufficiently well to be able to rationalize these phenomena.

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