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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF GLYCEROL DERIVATIVES ON A CHIRAL STATIONARY PHASE

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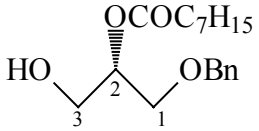
SUMMARY

We report herein the separation of enantiomers of glycerol derivatives on a cellulose-modified chiral column (Chiracel OD) in normal-phase mode. This method is suitable for monitoring the progress of the reaction and for determination of the enantiomeric purity of products. The lipase Novozyme 435-catalysed enantioselective hydrolysis of 1-*O*-benzyl-2,3-di-*O*-octanoyl-*rac*-glycerol has been shown to be an efficient method for preparation of 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol with high enantiomeric excess (*ee* > 95%).

INTRODUCTION

The synthesis of pseudoglycerides, in which one or two fatty acid moieties have been replaced by a drug, is a feasible way of modification of well known drugs [1,2]. Literature studies show that there is no universal way of preparing enantiomerically pure glycerides. The main difficulty is enantioselective construction of a hydroxyl group at C-2 of the glycerol moiety. The most effective methods involve transformation in the presence of enzymes. In this work we studied the use of lipases for synthesis of enantiomerically pure 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol, **4S.**



4S

The crucial task was establishing an analytical method to monitor the progress of the reaction and for determination of optical purity. Separation of a racemate into enantiomers and analytical monitoring of the process is a common problem in stereochemistry research and in the preparation of biologically active compounds, in particular drugs. Enantioselective separations can be achieved by all separation techniques, including chromatography and electromigration methods [3]. Among many chromatographic techniques, gas chromatography (GC), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and supercritical-fluid chromatography (SFC) using chiral selectors are extensively used for separation and quantification of the enantiomers. Because of its wide range of application, in this work we used HPLC analysis to monitor the progress of the reaction and to determine enantiomeric excess [4–8].

EXPERIMENTAL

General Methods

^{1}H NMR spectra were recorded for solutions in $CDCl_3$ (internal standard TMS) with a Varian spectrometer at a frequency of 300 MHz. Optical rotation was measured with a Perkin-Elmer 141 polarimeter using a sodium lamp (589 nm) at room temperature. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. IR spectra were recorded on a Specord M-80 Carl Zeiss Jena instrument. Column chromatography was performed on silica gel 60 (70–230 mesh, Merck) with hexane–ethyl acetate, 10:1 (*v*/*v*), as mobile phase. Reactions were monitored by TLC on precoated silica gel G plates (Merck) with hexane–acetone, 5:2 (*v*/*v*), as mobile phase. Components were detected by spraying the plates with 1% ethanolic bromophenol blue (detection of diesters and acids) or 5% ethanolic molybdatophosphoric acid (detection of monoesters and diols), then heating. All evaporation was performed under reduced pressure at 50°C.

Reagents

1,2-*O*-Isopropylideneglycerol, benzyl bromide, octanoic acid, and octanoyl chloride were purchased from Sigma–Aldrich. 2-Propanol and hexane used as solvents were of HPLC grade from Merck. 1-*O*-benzylglycerol [9] and 1-*O*-benzyl-2,3-di-*O*-octanoylglycerol [10] were prepared according to published procedures. The enzymes used were Novozyme 435 (10 U mg^{-1}; Novo Nordisk), Lipozym IM (30 U mg^{-1}; Amano), PPL type

II (30 U mg^{-1}; Sigma), and lipases from *Mucor racemosus* (MRA37-12) and *Mucor javanicus* (MJT45-2), which were kindly provided by Dr Hab. Tadeusz Antczak from the Technical University of Lodz.

HPLC

HPLC analysis was performed with a Hewlett–Packard 1050 Series pump and a Hewlett Packard 1050 Series variable wavelength detector. Normal-phase HPLC separations were performed with a Chiracel OD column (250 mm × 4.6 mm, particle size 10 μm, Daicel) at ambient temperature. UV detection was performed at 257 nm. Hexane and 2-propanol were used as components of the mobile phase at a flow rate of 0.5 mL min^{-1}. Sample concentrations were 1% (*w*/*v*) in mobile phase as solvent and injection volumes were 20 μL.

Procedure for Enzymatic Esterification

Synthesis of 1-O-benzyl-3-O-octanoyl-rac-glycerol **3**

Octanoic acid (0.288 g, 2 mmol) and 1-*O*-benzylglycerol **1** (0.364 g, 2 mmol) were dissolved in 95 mL hexane and sodium sulphate (1 g) was added. The mixture was stirred magnetically at room temperature for 10 min. Lipase (MJT45-2, MRA37-22, or Novozyme 435, 50 mg) was then added. The course of the reaction was followed by TLC analysis. The reaction was interrupted after 4 h by removal of the enzyme by filtration. The filtrate was extracted with ethyl acetate and the extract was dried over magnesium sulphate and evaporated under reduce pressure. Products were

Table I

Lipase-catalysed esterification and hydrolysis

	Entry	Substrate	Lipase	Time	Conversion/yield*	3R:3S	4S:4R	3:4
Esterification	1	**1**	Novozyme 435	4 h	53/37	1:1.06	–	–
	2	**1**	MJT45-2	4 h	36/26	1:1.16	–	–
	3	**1**	MRA37-22	4 h	27/20	1:1.17	–	–
Hydrolysis	4	**2**	Novozyme 435	1 h	31/17**	1:0	1:0.009	1:10
	5	**2**	MRA37-22	1 h	28/12**	1:0.7	1:1.5	1:1.8
	6	**2**	PPL	1 h	39/18**	1:0.8	1:1.3	1:3.8
	7	**2**	Lipozym IM	1 h	25/11**	1:1.5	1:1.7	1:21

*Calculated for products isolated by column chromatography
Summary of yield of products **3 and **4**

purified on a column of silica gel to give **3** as a colourless oil. Yields are summarised in Table I (entries 1-3).

TLC: R_F (octanoic acid) = 0.63; R_F (**1**) = 0.14; R_F (**3**) = 0.49.

HPLC: mobile phase hexane–2-propanol, 90:10, t_R (**3S**) = 19.78, t_R (**3R**) = 22.26.

^{1}H NMR of **3**: δ (ppm) 0.85–0.90 (m, 3H, CH_3 in $C_7H_{15}CO$), 1.20–1.35 (m, 8H, CH_2 in $C_7H_{15}CO$), 1.55–1.70 (m, 2H, CH_2 in $C_7H_{15}CO$), 2.35 (t, 2H, J = 7.5 Hz, CH_2 in $C_7H_{15}CO$), 3.50 (dd, 1H, $J_{1a,b}$ = 9.8 Hz, $J_{1a,2}$ = 6.0 Hz, H-1a), 3.56 (dd, 1H, $J_{1b,a}$ = 9.8 Hz, $J_{1b,2}$ = 4.3 Hz, H-1b), 4.04 (dddd, 1H $J_{2,3a}$ = 6.3 Hz, $J_{2,3b}$ = 4.8 Hz, $J_{2,1a}$ = 6.0 Hz, $J_{2,1b}$ = 4.3 Hz, H-2), 4.14 (dd, 1H, $J_{3a,b}$ = 11.4 Hz, $J_{3a,2}$ = 6.3 Hz, H-3a), 4.20 (dd, 1H, $J_{3b,a}$ = 11.4 Hz, $J_{3b,2}$ = 4.8 Hz, H-3b), 4.57 (s, 2H, CH_2Ph), 7.28-7.37 (m, 5H, Ph).

IR (CH_2Cl_2, cm^{-1}): 3540–3630 (br, OH), 3020 (Ph), 2960, 2920, 2855 (aliphatic), 1730 (C=O), 1215 (C–O). Anal. calc. for $C_{18}H_{28}O_4$: C, 70.10; H, 9.15. Found: C, 70.56; H 9.29.

Procedure for Enzymatic Hydrolysis

Synthesis of 1-O-benzyl-2-O-octanoyl-sn-glycerol **4S**

1-*O*-benzyl-2,3-di-*O*-octanoylglycerol **2** (0.250 g, 0.58 mmol) was dissolved in a solution of sodium hydroxide (pH 8, 2.5 mL) and lipase (Novozym 345, MRA37-22, PPL, or Lipozym IM, 50 mg) was added. The mixture was stirred magnetically at room temperature and the course of the reaction was followed by TLC analysis. The reaction was interrupted after 1 h by removal of the enzyme by filtration. The filtrate was extracted with ethyl acetate and the extract was dried over magnesium sulphate and evaporated under reduce pressure. Products were purified on a silica gel column. Summarized yields of products **4** and **3**, which were inseparable by column chromatography, are listed in Table I (entries 4–7). Physicochemical measurements were performed only for products of the reaction in the presence of Novozyme 435 (Table I, entry 4)**,** which contained 91% product **4S**.

TLC: R_F (**2**) = 0.70; R_F (**1**) = 0.14; R_F (products) = 0.50.

HPLC: mobile phase hexane–2-propanol, 90:10, t_R (**3S**) = 19.78, t_R (**3R**) = 22.26, t_R (**4S**) = 14.79, t_R (**4R**) = 16.25.

Optical rotation **4S** $[\alpha]_D^{20} = -4.93°$ ($CHCl_3$, c = 5.0).

^{1}H NMR of **4**: δ (ppm) 0.85–0.90 (m, 3H, CH_3 in $C_7H_{15}CO$), 1.20–1.35 (m, 8H, CH_2 in $C_7H_{15}CO$), 1.55–1.70 (m, 2H, CH_2 in $C_7H_{15}CO$), 2.35 (t, 2H, J = 7.5 Hz, CH_2 in $C_7H_{15}CO$), 3.64 (dd, 1H, $J_{3a,b}$ = 10.4 Hz, $J_{3a,2}$ = 4.8 Hz, H-3a), 3.68 (dd, 1H, $J_{3b,a}$ = 10.4 Hz, $J_{3b,2}$ = 4.9 Hz, H-3b), 3.82 (d 2H, $J_{1,2}$ = 4.4 Hz, H-1a,b), 4.53 and 4.57 (qAB, 2H, J = 12.0 Hz, CH_2Ph), 5,04 (ddt, 1H $J_{2,3a}$ = 4.8 Hz, $J_{2,3b}$ = 4.9 Hz, $J_{2,1}$ = 4.4 Hz, H-2), 7.28–7.37 (m, 5H, Ph).

RESULTS AND DISCUSSION

HPLC Method Development – Separation of Enantiomers of Glycerol Derivatives

In recent years high-performance liquid chromatography (HPLC) has been studied and refined as an efficient procedure for resolution of racemic mixtures of monoacyl [11], diacyl [12–16] and triacylglycerol derivatives [17]. One approach used to separate glycerol enantiomers, indirect enantiomeric resolution, involves coupling of the enantiomers with an auxiliary chiral reagent to form diastereoisomers and then separation by HPLC on a chiral phase [11–15]. One feasible method involves use of ultraviolet sensitive derivatives of diacylglycerol, for example 3,5-dinitrophenylurethanes [14,15]. Another method is derivatization of the glycerol component with a fluorescent chiral agent, for example (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [11,12] or 2-anthrylurethanes [13]. These methods are, however, laborious.

Yet another approach involves direct separation methods in which the enantiomers are placed in an appropriate chiral environment. A variety of chiral stationary phases for HPLC column have been developed and used to separate chiral compounds into their optical isomers [18]. Among these, polysaccharide-based columns play an important role [19]. For example the chiral stationary phase Chiracel OB has been used to separate benzyl monoglycerides [16] and Chiracel OD or OF has been used to resolve asymmetric triacylglycerides [17].

In the work discussed in this report, a commercially available chiral stationary phase (Chiracel OD) was used to separate enantiomers of a selectively protected glycerol and to estimate the optical purity of 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol **4S**, without derivatisation. Experiments were conducted to find a mobile phase enabling optimum separation and sensitivity. According to Diacel, manufacturer of Chiracel OD, hexane, 2-propanol, and ethanol in any compositions are the solvents of choice for use with this column. To perform normal-phase HPLC analysis hexane and 2-propanol were chosen as solvents. Mobile phase composition was optimised by varying the proportion of the constituents at ambient temperature. The mobile phases finally used were hexane–2-propanol, 99:1 (*v*/*v*), for diacyl-substituted derivatives **2R** and **2S** and hexane–2-propanol, 90:10 (*v*/*v*) for monoacyl-substituted derivatives **1R**, **1S**, **3R**, **3S**, **4R**, and **4S**. Spectrophotometric detection was performed at 257 nm. Chromatographic results obtained from separation of glycerol derivatives on a Chiracel OD

column are summarized in Table II. The method is capable of separating the two enantiomers with separation factors of 1.06–1.25. Typical chromatograms are shown in Fig. 1.

Table II

Chromatographic properties of glycerol derivatives on Chiracel OD

Mobile phase	Compound	t_R (min)	α
Hexane–2-propanol, 90:10 (*v*/*v*)	1S	29.20	1.25
	1R	36.50	
	3S	19.78	1.11
	3R	22.26	
	4S	14.79	1.13
	4R	16.25	
Hexane–2-propanol, 99:1 (*v*/*v*)	2S	20.70	1.06
	2R	21.93	

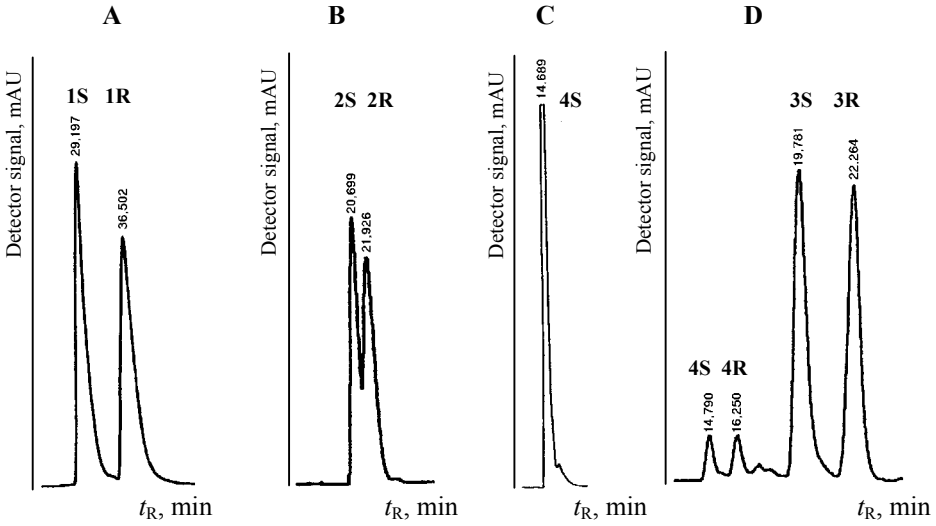


Fig. 1

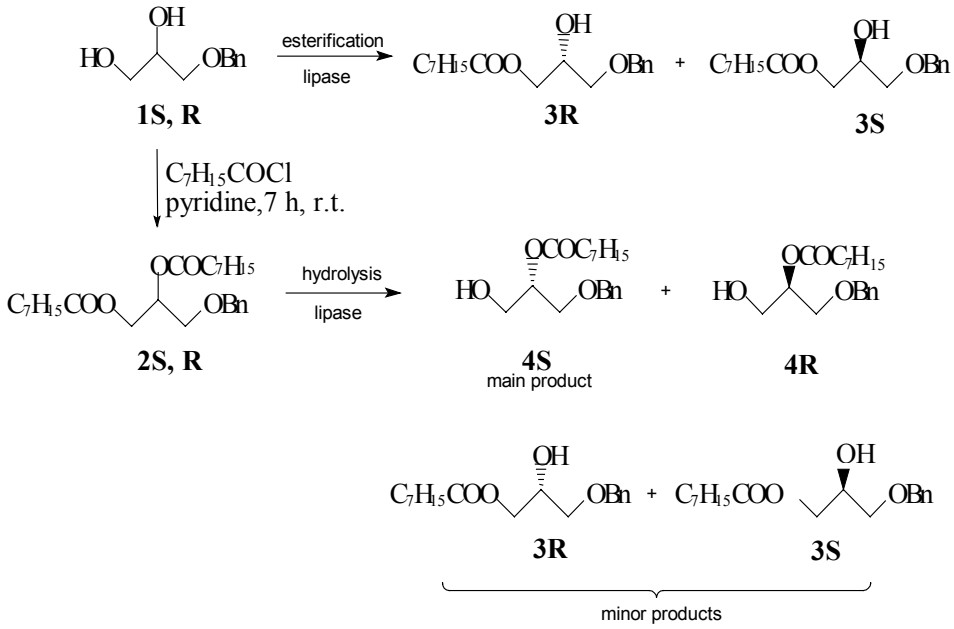
Analytical HPLC separation of glycerol derivatives. A. 1-*O*-benzyl-*rac*-glycerol **1S, R**; B. 1-*O*-benzyl-2,3-di-*O*-octanoyl-*rac*-glycerol **2S, R**; C. 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol **4S**; D. 1-*O*-benzyl-3-*O*-octanoyl-*sn*-glycerol **3S**; 3-*O*-benzyl-1-*O*-octanoyl-*sn*-glycerol **3R**; 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol **4S**; 3-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol **4R**. Analysis was performed on a 250 mm × 4.6 mm Chiracel OD column, UV detection was at 257 nm. The mobile phase was hexane–2-propanol, the flow rate 0.5 mL min^{-1}

Because of a lack of standards of mono and diacyl derivatives of glycerol, assignment of peaks was achieved indirectly as follows. The lipase Novozyme 435-catalysed enantioselective hydrolysis of 1-*O*-benzyl-2,3-di-*O*-octanoyl-*rac*-glycerol **2** afforded predominantly one product **4S** with high enantioselectivity (*ee* > 95%). Close examination of ^{1}H NMR spectra of **4S** indicated that the product had one free, primary hydroxyl group. For 1-*O*-benzyl-2-*O*-octanoylglycerol the H-2 proton occurred at lower field than for the 1-*O*-benzyl-3-*O*-octanoylglycerol derivative with a free secondary hydroxyl group (multiplets at δ = 5.05 ppm and 4.04 ppm, respectively). The absolute configuration of the prevailing enantiomer **4S** was assigned by measuring the optical rotation of unreacted substrate **2** enriched in one enantiomer and comparison with a literature value. Thus unreacted diester **2** was isolated by column chromatography and its optical rotation measured at 589 nm, $[\alpha]_D^{20} = +2.13°$ ($CHCl_3$, $c = 4.3$), was compared with the literature value [20], $[\alpha]_D^{20} = +9.7^o$ ($CHCl_3$, $c = 9.0$), for the (*S*)-(+) enantiomer. This result clearly indicates that separated unreacted substrate was enantiomerically enriched in the (*S*)-(+) enantiomer. So we have implied that the (*R*)-(−) enantiomer of **2** is involved in the hydrolysis reaction and afforded 1-*O*-benzyl-2-*O*-octanoyl-*sn*-glycerol **4S**. The retention time for this compound is 14.7 min as shown in Fig. 1. Stereochemical assignment of the other glycerol derivatives of unknown configuration was achieved by analogy with these assignments. The order of elution of the *S* and *R* enantiomers of the analyte is dictated by the absolute configuration of the chiral stationary phase which is constant for the column.

Application – HPLC Analysis of Lipase-Catalysed Reactions

Lipases are used to catalyse a wide variety of chemo-, regio-, and stereoselective transformations [21,22]. Resolution of racemic mixtures by lipases is one of the most useful means of preparing enantiomerically pure compounds [23]. Examples of their use include resolution of racemic glycerol derivatives by enantioselective esterification [24], hydrolysis [25] or interesterification [17]. These methods are, however, far from universal.

To synthesize enantiomerically pure glycerol derivative **4S** we have explored two synthetic approaches (Scheme 1). The first involved the lipase-catalysed esterification of 1-*O*-benzyl-*rac*-glycerol **1** in the presence of octanoic acid, the second was lipase-catalysed hydrolysis of 1-*O*-benzyl-2,3-di-*O*-octanoyl-*rac*-glycerol **2**.



Scheme 1

Enzymatic esterification and hydrolysis

Esterification of **1** with octanoic acid was regioselective and afforded products **3R** and **3S** with free, secondary hydroxyl groups. Unfortunately the reaction was not enantioselective, a mixture of products was only slightly enriched in the *S* enantiomer, irrespective of the lipase employed (Table I, entries 1–3). The best result, approximately 17% predominance of the *S* enantiomer, was obtained by use of lipase MRA37-22 (Table I, entry 3). Only traces of the desired product **4S** were observed in reaction mixture. We therefore turned our attention to the lipase-catalyzed hydrolysis of **2**. Reactions were conducted in the presence of two native lipases, MRA37-22 and PPL, and two immobilized lipases, Novozyme 435 and Lipozym IM. The highest regioselectivity was achieved with Lipozym IM and the worst with MRA37-22 (Table I, entries 7 and 5). High enantioselectivity and 31% conversion were achieved with Novozyme 435 (Table I, entry 4). The reactions were monitored up to about 50% conversion, because a significant decrease in *ee* was observed at higher conversions. Because of these successful results, lipase Novozyme 435 was chosen as catalyst for further experiments. The best regio- and stereoselectivity were obtained in the hydrolysis reaction after 1 h and with an enzyme-to-substrate ratio of

0.26. Varying the concentration of the substrate in the range C_S = 0.2–0.9 M had almost no effect on the stereoselectivity of the reaction. We decided that the isolated, unreacted diester could be employed in the next cycle. Unfortunately a significant decrease of enantioselectivity was observed.

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