ELSEVIER

Journal of Chromatography A, 724 (1996) 367–372

JOURNAL OF CHROMATOGRAPHY A

Short communication

Preparative separation of the diastereomers of methyl branched-chain phosphatidylcholines

Frank Bringezu, Bodo Dobner, Runald Stritzel, Barbara Elsner, Peter Nuhn*

Institut für Pharmazeutische Chemie, Martin-Luther-Universität Halle, Weinbergweg 15, D-06110 Halle, Germany

First received 18 July 1995; revised manuscript received 14 September 1995; accepted 14 September 1995

Abstract

Phosphatidylcholines with methyl branched-chain fatty acids linked to the *sn*-2-position of the glycerol backbone were prepared. Diastereomers were isolated if the methyl branching was at the 2-position of the fatty acids. The separation of the diastereomers was connected with the distance between the chiral centres, which was varied systematically. An increasing distance resulted in a decrease in the separation factors calculated from HPLC data. Starting the synthesis from chiral glycerol educts after separation, optically pure diastereomers of α-methyl branched chain phospholipids were obtained.

Keywords: Diastereomer separation; Preparative chromatography; Enantiomer separation; Phosphatidylcholines; Phospholipids; Glycerols; Glycerophosphocholines; Fatty acids

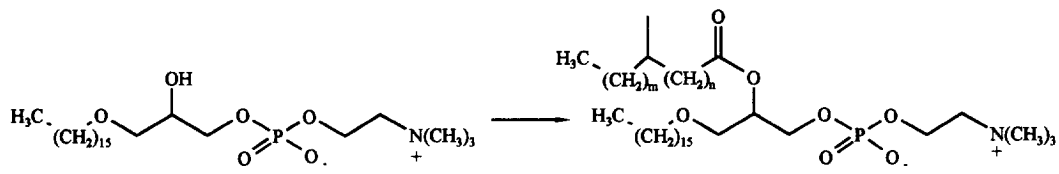
1. Introduction

Methyl branched-chain fatty acids are widely distributed in nature, especially as components of acylated glycerols [1]. The synthesis and characterization of branched-chain phospholipids have been investigated owing to their importance as model systems for biological membranes and thin films [2,3]. Wide investigations of the influence of chirality at the phosphorus atom have been carried out [4]. Both the synthesis and physicochemical characterization of pure enantiomers of non-branched-chain phospholipids have been described previously [5], whereas the studies of the phase behaviour of phospholipids substituted with methyl branched-chain fatty acids having the branching close to the carboxyl function have dealt in all cases with racemic mixtures of the diastereomers of these compounds [3,6]. Nevertheless, the use of pure branched-chain phospholipid diastereomers as model systems for biomembranes is of great significance, because the physico-chemical parameters might exhibit differences.

Continuing our investigations on glycerophosphocholines (GPCs) containing branched-chain fatty acids [6,7], 1-O-alkyl-2-acyl-GPCs which are racemic both at the glycerol and at the position of the branching in the acid chain were synthesized (Fig. 1).

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01014-9



1 2-7

	2	3	4	5	6	7*)
n	0	0	0	1	2	0
m	10	12	14	14	15	12

*) chiral sn-glycero-1-phosphocholine

Fig. 1. Phospholipids prepared.

2. Experimental

^{1}H NMR spectra (in deuterochloroform) were recorded on a Bruker WP 200 instrument at 20°C. Fast atom bombardment (FAB) mass spectra were measured on an AMD 402 mass spectrometer at 70 eV. Thioglycol was used as the matrix.

2.1. Synthesis

2-Methyl branched-chain fatty acids were prepared using previously described procedures [6]. The synthesis of the 3-methylstearic acid has been described [8]. 4-Methylicosanoic acid was prepared following the method of Weitzel and Wojahn [9,10] by chain elongation of 2-methylstearic acid. The identity of the products was checked using mass spectrometry (MS) an gas chromatography of the methyl esters. The melting points were found to be in agreement with the literature [6,8–10].

The acylation of *rac*-1-O-hexadecyl-*glycero*-3-phosphocholine (**1**) with the anhydride of the branched-chain fatty acid in presence of 4-DMAP, as described previously [7], is the final step in the synthesis of **2–6**. For purification, column chromatography was used. The column was packed with 100 g of silica gel per gram of the crude product and first eluted with 100 ml of chloroform per gram of the crude product followed by 100 ml of chloroform–methanol–25% ammonia solution (90:10:1), 100 ml (80:20:2), 100 ml (70:30:3) and 100–400 ml (65:35:5) until the eluation was complete. For characterization FAB-MS microanalysis and ^{1}H NMR spectrometry were applied (see Tables 1 and 2).

2.2. Capillary gas chromatography

The enantiomer separations of the fatty acid methyl esters (FAME) were accomplished with a Shimadzu GC14A gas chromatograph equipped with a flame ionization detector (FID). The experiments were performed on a 25 m × 0.25 mm I.D. FS-Hydrodex-β-3P column (Machery–Nagel, Düren, Germany) containing heptakis(2,6-di-O-methyl-3-pentyl)-β-cyclodextrin in OV-1701. Both the injector and detector were maintained at 250°C and split injection was set at 100:1. Hydrogen was used as the carrier gas at a flow-rate of 1.3 ml/min. A Shimadzu C-R6 Chromatopac was used to record and process the chromatographic data. The chiral column was equipped with a 2 m deactivated fused-silica

Table 1
Characterization of the 1-O-hexadecyl-2-acyl-GPCs **2–7**

Compound	Phosphorus micro analysts (calc./found)	Yield (%)	Mol. mass		t_{Ra} [a] (s)	t_{Rb} [a] (s)	A_a (%)	A_b (%)	α [d]
			Calc.	Found					
2	4.35/4.48	75	692.0	692.0 $(M+H)^+$	21.46	24.15	98.2[b]	98.5[b]	1.125
3	4.23/4.30	73	720.1	720.7 $(M+H)^+$	21.05	23.85	98.3[b]	98.6[b]	1.133
4	4.15/4.14	70	748.1	748.7 $(M+H)^+$	23.30	26.00	99.8[b]	99.4[b]	1.116
5	3.99/4.06	65	762.1	762.6 $(M+H)^+$	18.85	19.74	48.21[c]	50.74[c]	1:047
6	3.81/3.92	73	790.2	790.6 $(M+H)^+$	18.72	18.72	98.97[c]		1
7	4.23/4.28	71	720.1	720.7 $(M+H)^+$	21.07	23.83	90.3[b]	100.0[b]	1.131

[a] t_{Ra} and t_{Rb} are the retention times for the diastereomers **a** and **b**, respectively.
[b] Integrated areas for preparatively isolated diastereomers.
[c] Integrated areas for not preparatively isolated diastereomers.
[d] Separation factor.

retention gap with same I.D. The retention gap was necessary for the splitless mode.

2.3. High-performance liquid chromatography

The HPLC separations of the diastereomers of **2–7** were performed with a JASCO chromatograph fitted with a 250 × 4.6 mm I.D. Kromasil Si 100 column (100–5 μm) (JASCO) equipped with an ELSD IIA evaporative light-scattering detector. Chloroform–methanol–water (60:40:4) was used as the mobile phase. The flow-rate was 0.5 ml/min for 10 min and then 1 ml/min until the end of the analysis. The column temperature was maintained at 20°C. The lipids were diluted in sufficient volumes of a standard mixture of chloroform–methanol (80:20) and 20 μl of the solution were injected. Chromatograms and inte-

Table 2
^{1}H NMR chemical shift of selected phospholipids

Compound	^{1}H NMR chemical shift (ppm)[a]
3a	0.83 (t, 2 CH_3, 6.9 Hz, 6H), 1.10 [d, $-CH(CH_3)-$, 7.0 Hz, 3H], 1.21 [s, alkyl $-(CH_2)_{13}-$, acyl $-(CH_2)_{11}-$, 50H], 1.47–1.49 (m, $-CH_2CH_2O-$, 2H), 2.36–2.41 (m, $-CHCOO-$, 1H), 3.16 [s, $-N(CH_3)_3^+$, 9H], 3.39–3.42 (m, $-CH_2CH_2O-$, $-CH_2N^+$, 4H), 3.51–3.59 (m, alkyl–O–CH_2-, 2H), 3.9–3.93 (m, $-CHCH_2-O-PO_3-$, 2H), 4.18 (m, $-CH_2-CH_2-N^+$, 2H), 5.07–5.09 (m, $-CH_2CHCH_2-$, 1H)
3b	0.82 (t, 2 CH_3, 6.7 Hz, 6H), 1.07 [d, $-CH(CH_3)-$, 7.0 Hz, 3H], 1.20 [s, alkyl $-(CH_2)_{13}-$, acyl $(CH_2)_{11}-$, 50H], 1.46–1.49 (m, $-CH_2CH_2O-$, 2H), 2.38–2.42 (m, $-CHCOO-$, 1H), 3.15 [s, $-N(CH_3)_3^+$, 9H], 3.39–3.42 (m, $-CH_2CH_2O-$, $-CH_2N^-$, 4H), 3.5–3.57 (m, alkyl–O–CH_2-, 2H), 3.88–4.0 (m, $-CHCH_2-O-PO_3-$, 2H), 4.17 (m $-CH_2-CH_2-N^+$, 2h), 5.07–5.09 (m, $-CH_2CHCH_2-$, 1H)
7a	0.83 (t, 2 CH_3, 6.9 Hz, 6H), 1.11 [d, $-CH(CH_3)-$, 7.0 Hz, 3H], 1.22 (s, alkyl $-(CH_2)_{13}-$, acyl $-(CH_2)_{11}-$, 50H], 1.47–1.49 (m, $-CH_2CH_2O-$, 2H), 2.37–2.41 (m, $-CHCOO-$, 1H), 3.18 [s, $-N(CH_3)_3^+$, 9H], 3.38–3.43 (m, $-CH_2CH_2O-$, $-CH_2N^+$, 4H), 3.51–3.59 (m, alkyl–O–CH_2-, 2H), 3.91–3.93 (m, $-CHCH_2-O-PO_3-$, 2H), 4.20 (m, $-CH_2-CH_2-N^+$, 2H), 5.07–5.09 (m, $-CH_2CHCH_2-$, 1H)
7b	0.83 (t, 2 CH_3, 6.7 Hz, 6H), 1.08 [d, $-CH(CH_3)-$, 7.0 Hz, 3H], 1.21 [s, alkyl $-(CH_2)_{13}-$, acyl $-(CH_2)_{11}-$, 50 H], 1.46–1.50 (m, $-CH_2CH_2O-$, 2H), 2.40–2.42 (m, $-CHCOO-$, 1H), 3.16 [s, $-N(CH_3)_3^+$, 9H], 3.37–3.40 (m, $-CH_2CH_2O-$, $-CH_2N^+$, 4H), 3.52–3.57 (m, alkyl–C–CH_2, 2H), 3.91–4.0 (m, $-CHCH_2-O-PO_3-$, 2H), 4.19 (m, $-CH_2-CH_2-N^+$, 2H), 5.08–5.10 (m, $-CH_2CHCH_2-$, 1H)

[a] 500 MHz; solvent $CDCl_3$.

grated data were recorded with a JASCO Chromatopac. Averaged values were used for all calculations.

3. Results and discussion

Owing to the two chiral centres in the lipid molecules, the products obtained are mixtures of diastereomers. In the case of GPCs containing 2-branched fatty acids (**2–4**) we achieved the preparative separation of two fractions (**a** and **b**) possessing identical FAB mass spectra. The ^{1}H NMR spectra of the isolated fractions were measured to confirm the absence of 1,3-isomers. For such isomers, the chemical shift for the proton at the C-2 position in the glycerol differs [11]. However, in all cases a multiplet at 5.07–5.09 ppm for this proton was obtained. Further analysis showed that the comparable protons in the glycerol have the same chemical shift. As a result, the isolated lipid fractions correspond to the 1-O-alkyl-2-acyl-*glycero*-3-phosphocholine structure. The association of the ^{1}H signals was obtained from ^{1}H-COSY spectra and the analytical data from Ref. [11].

Comparing the retention time (t_R) values from the HPLC measurements of the diastereomers of **2** and **3**, it was found that with increasing acyl chain length the t_R values decrease slightly. On going from **3** to **4** a jump to a higher retention was observed.

In **4**, the distance between the chiral carbon atoms is equivalent to one C–C single bond, one ester bond and one O–C single bond. Varying this distance should have an effect on the capability to separate diastereomers. Therefore, derivatives were synthesized using 3- and 4-methyl branched-chain fatty acids. On going to larger distances, the intramolecular stereochemical interaction between the asymmetric substituted carbon atoms should disappear.

Inserting 3-methylstearic acid leads to **5** and inserting 4-methylicosanoic acid yields **6**. In **5** this distance has been elongated by one C–C single bond and in **6** by two C–C single bonds.

For **5**, the presence of the diastereomers was detected by analytical HPLC, although no preparative separation was obtained. Comparing the retention times of **5a** and **b** with those of **4a** and **b**, it is seen that with increase in the distance the values decrease owing to the differences in the acyl chain length between these substances. Compound **6** shows no separation of diastereomers and the t_R of **6** is smaller than that of **4**, which corresponds to an increase in the acyl chain length. Nevertheless, the Δt_R between **4a** and **5a** is significantly higher than that between **5a** and **6**.

With increasing distance between the chiral carbon atoms, the calculated separation factors α decrease. The HPLC traces of **4–6** are shown in Fig. 2.

For further investigation, the synthesis was started using the chiral glycerol derivative 3-O-hexadecyl-*sn*-glycerol, obtained from commercially available 1,2-isopropylidene-*sn*-glycerol using the procedure described in Ref. [12]. After conversion according to Ref. [7], 3-O-hexadecyl-*sn*-glycero-1-phosphocholine was obtained. Acylation led to **7**, from which **7a** and **b** were isolated. Both are optically pure diastereomers of methyl-branched glycerophospholipids with optical rotations of $[\alpha]_D + 1.7°$ for **7a** and $+5.2°$ for **7b** ($CHCl_3$, $c = 0.01$ mol/l, 20°C).

After removing the acyl residues from the lipids **7a** and **b** using a mineral acid in methanol, the corresponding FAMEs were obtained. In Fig. 3 the gas chromatograms of the FAME removed from **7a** and the corresponding racemate are shown. The measurements were made at a pressure near the optimum due to the van Deemter curve [13].

For the FAMEs from **7a** and **b**, only one peak was observed in each case, whereas the racemate resolved into two peaks, one for each enantiomer. In Ref. [13] the elution order for higher methyl branched-chain FAMEs was determined after GC analysis of pure methyl (*R*)-2-methylhexadecanoate prepared according to Ref. [14]. In agreement with the data from Ref. [15], it could be shown that the (*R*)-2-methyl-FAME elutes first, which means that the FAME removed from **7a** has the (*R*)-2-methyl configuration.

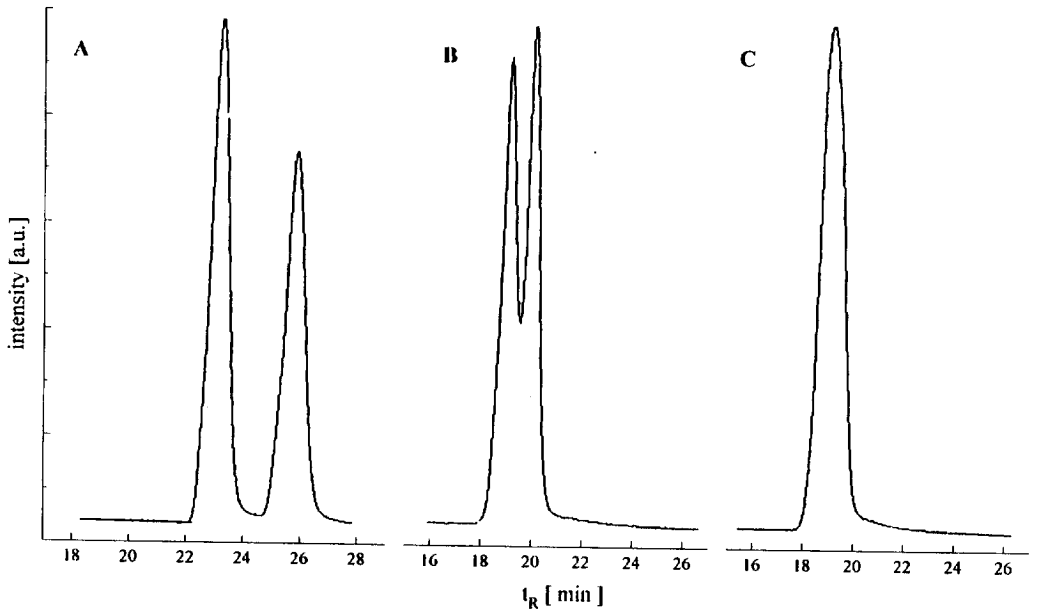


Fig. 2. Resolution of the diastereomers. HPLC traces for (a) **4**, (B) **5** and (C) **b**.

4. Conclusion

The present experiments have demonstrated that if the fatty acid in 1-O-alkyl-2-acyl-*glycero*-phosphocholines has α-methyl branching, it is possible to isolate diastereomers preparatively in high purity of >98% (see Table 1).

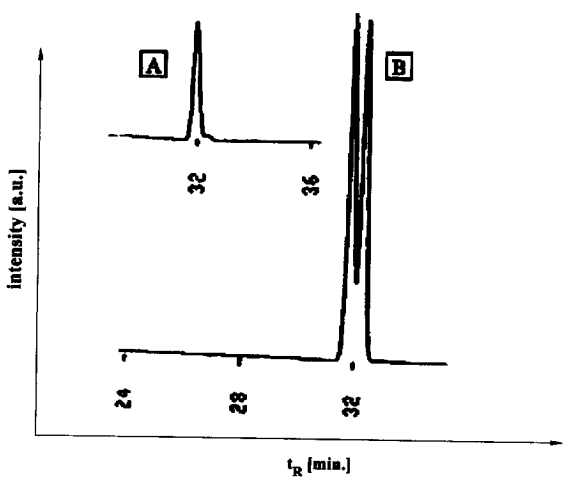


Fig. 3. GC chromatograms of the FAME removed from (A) **7a** and (B) *rac*-α-methylpentadecanoate at 130°C.

By GC analysis of the FAMEs removed from **7a** and **b**, it was shown that these lipids are optically pure diastereomers. The separation of **2–4** into two fractions gives the diastereomeric pairs which are racemic both at the *sn*-2-position of the glycerol and at C-2-position of the fatty acid. The absolute configuration of these products could be determined if one considers that according to Ref. [13] the FAME removed from **7a** has the *R*-configuration. In this case the lipid consists of the 3-O-hexadecyl-*sn-glycero*-1-PC substituted at the *sn*-2-position with the (*R*)-2-methyl acyl residue. The lipid **7a** elutes before **7b**. From this, one can conclude that the separation of the diastereomers of **2–4** first gives the elution of the racemic mixture of 1-O-hexadecyl-2-[(*S*)-2-methylacyl[-*sn*-*glycero*-3-phosphocholine and 3-O-hexadecyl-2-[(*R*)-2-methylacyl]-*sn*-*glycero*-1-phosphocholine. The fractions **2–4b** consist of a racemic mixture of 1-O-hexadecyl-2-[(*R*)-2-methylacyl]-*sn*-*glycero*-3-phosphocholine and 3-O-hexadecyl-2-[(*S*)-2-methylacyl]-*sn*-*glycero*-1-phosphocholine.

The GC analysis results indicate that it is possible to separate enantiomers of α-methyl branched-chain fatty acids by separation of dia-

stereomers of GPCs if the acid is in the *sn*-2-position of the glycerol backbone.

Since it is possible to separate the phospholipid diastereomers, one could see differences in the physico-chemical parameters of these products. Therefore, such pure diastereomers are suitable model compounds for studying the phase behaviour of mono- and bilayers of such compounds, especially for investigations concerning the influence of chirality on phase transition parameters of lipid–water systems.

The dependence of the chemical structure, i.e., the substitution pattern at the glycerol or the influence of the head group, on relevant parameters of the separation of the diastereomers of these lipids is currently being studied.

Acknowledgements

The authors thank Dr. D. Ströhl (FB Chemie, Universität Halle) for measuring the ^{1}H NMR spectra and helpful discussions and Mrs. Horn (Institut für Pflanzenbiochemie, Halle) for preparing the FAB mass spectra. This work was supported financially by the Deutsche Forschungsgemeinschaft (DFG).

References

[1] P. Nuhn, M. Gutheil and B. Dobner, Fette Seifen Anstrichm., 87 (1985) 135.

[2] F.M. Menger, M.G. Wood, Q.Z. Zhou, H.P. Hopkins and J. Fumero, J. Am. Chem. Soc., 110 (1988) 6804.

[3] R.N.A.H. Lewis, R.N. McElhaney, P.E. Harper, D.C. Turner and S.M. Gruner, Biophys. J., 66 (1994) 1088.

[4] D.A. Wisner, T. Rosario-Jansen and M.D. Tsai, J. Am. Chem. Soc., 108F (1986) 8064.

[5] P.N. Guivisdalsky and R. Bittman, Tetrahedron Lett., 29 (1988) 4393.

[6] P. Nuhn, G.Brezesinksi, B. Dobner, G. Förster, M. Gutheil and H.-D. Dörfler, Chem. Phys. Lipids, 39 (1986) 221.

[7] B. Rattay, G. Brezesinski, B. Dobner, G. Förster and P. Nuhn, Chem. Phys. Lipids, 75 (1995) 81.

[8] B. Dobner, P. Nuhn, A. Büge and U. Burkhardt, Pharmazie, 39 (1984) 21.

[9] G. Weitzel and J. Wojahn, Z. Physiol. Chem., 287 (1951) 65.

[10] G. Weitzel and J. Wojahn, Z. Physiol. Chem., 287 (1951) 296.

[11] J.G. Lammers, T.J. Liefkens, J. Bus and J. van der Meer, Chem. Phys. Lipids, 22 (1978) 293.

[12] F. Paltauf and A. Hermetter, Methods Enzymol., 197 (1991) 134.

[13] R.Stritzel, P. Nuhn, B. Dobner and F. Bringezu, J. High Resolut. Chromatogr., in press.

[14] G. Helmchen, G. Nill, D. Flockerzi and M.S.K. Youssef, Angew, Chem., Int. Ed. Engl., 18 (1979) 63.

[15] S. Hartmann, G.S. Besra, J.L. Fraser, W.A. König, D.E. Minnikin and M. Ridell, Biochim. Biophys. Acta, Gen. Subj., 1201 (1994) 339.