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ORIGINAL PAPER

Discrimination of Chain Positions in Mixed Short/Long-Chain Glycerophosphocholines by NMR Chemical Shift Variations

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Abstract The synthesis of a series of (1,2-) mixed short/long-chain glycerophosphocholines has been performed. Starting from glycerophosphorylcholine (GPC), and using regioselective acylation in the presence of dibutyltin oxide, a set of high-purity isomeric mixed-chain phospholipids was obtained. This has allowed the development of a simple NMR method for the structural determination of the isomeric 1(2)-short-2(1)-long-diacylglycerophosphocholines. The method is based on the observation that selected protons in the two series of isomeric phospholipids undergo systematic chemical shift variations $\Delta\delta$ that can be ascribed to the acyl substituents on the glycerol backbone. The observed patterns can be exploited as a simple method for the discrimination of regioisomeric unsymmetrical 1,2-diacylglycerophosphocholines.

Keywords Regioselective acylation · Diacylglycerophosphocholine · Isomeric phospholipids · Mixed chains · NMR

Introduction

Recently, research has increasingly been focused on biologically active phospholipids (PLs), and in particular on lysophospholipids (LPLs), due to their active role in many biological processes [1, 2]. LPLs are widely distributed in nature in both animals and plants, even though they represent only a small fraction of the cellular lipid component. Although lysophospholipids are well-known PL metabolites, their role as intercellular signaling molecules is being studied in more and more depth [3, 4]. Lysophospholipids and their receptors have been found in a great range of tissues and cell types, and are involved in many processes such as reproduction, angiogenesis, and nervous system regulation [5, 6].

2-Acyl-1-lyso-*sn*-glycerophosphocholines are very interesting biologically active compounds because they are potential in vivo delivery systems for chains situated at position *sn*-2. It has recently been shown [7] that 2-docosahexaneoyl-1-lysophosphatidylcholine is preferentially transferred through the in vitro blood–brain barrier over unesterified docosahexanenoic acid. Nevertheless, these compounds are not stable in vivo due to spontaneous acyl chain migration from position *sn*-2 to the more stable *sn*-1 [8].

It has been stated [9] that the presence of a small acyl chain in position *sn*-1 causes the PL to display the same biochemical behavior as 1-lysoPL, because the *sn*-1 position is occupied and not free for spontaneous acyl migration from the less stable *sn*-2 position. We have also recently reported [10] a practical synthesis of mixed short/long 1,2-diacyl-chain phosphatidylcholines starting from glycerophosphorylcholine (GPC). Figure 1 shows the general formulae and atom numbering of the newly synthesized compounds.

During the preparation of mixed-chain PLs from GPC, we had the opportunity to compare different sets of 1,2-phosphatidylcholine (PC) isomers, as formally obtained by swapping the same pairs of substituents (e.g., C2 and C16 fatty acid residues) from position 1 to position 2 of the glycerol backbone and vice versa.

The determination of fatty acid composition in phospholipids is usually obtained either by selective enzymatic

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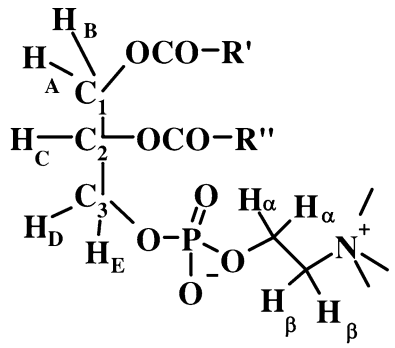

1: R'= CH_3; R''= $C_{15}H_{31}$
2: R'= $C_{15}H_{31}$; R''= CH_3
3: R'= C_2H_5; R''= $C_{15}H_{31}$
4: R'= $C_{15}H_{31}$; R''= C_2H_5
5: R'= C_3H_7; R''= $C_{15}H_{31}$
6: R'= $C_{15}H_{31}$; R''= C_3H_7
7: R'= C_5H_{11}; R''= $C_{15}H_{31}$
8: R'= $C_{15}H_{31}$; R''= C_5H_{11}
9: R'= C_8H_{17}; R''= $C_{15}H_{31}$
10: R'= $C_{15}H_{31}$; R''= C_8H_{17}
11: R'= C_9H_{19}; R''= $C_{15}H_{31}$
12: R'= $C_{15}H_{31}$; R''= C_9H_{19}

Fig. 1 Molecular formulae and atom numbering of compounds **1–12**

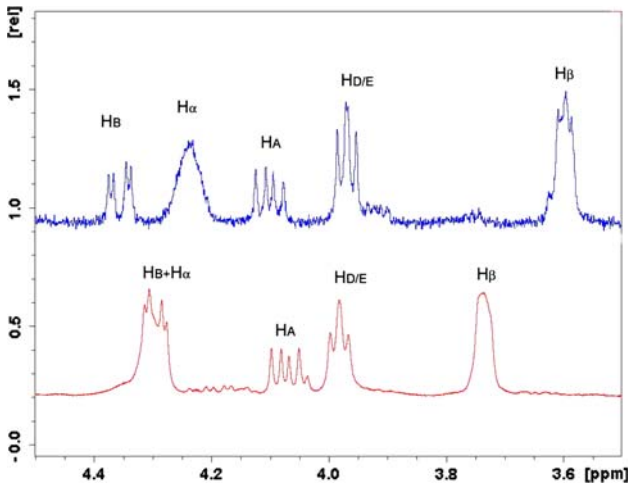

Fig. 2 Expansions of the ^{1}H NMR spectra of compounds **11** (*top trace*) and **12** (*bottom trace*)

hydrolysis in positions *sn*-1 and *sn*-2 with some lipases [11] and phospholipase A_2 [12, 13], respectively, and the subsequent analysis of released fatty acid residue [14], or by using liquid chromatography/electrospray ionization/mass spectrometry [15]. In all cases the analyzed sample cannot recovered.

In the present work, the long- and short-chain components of the PLs were palmitic acid and short-chain saturated linear acid residues ranging from C2 to C10, respectively. The analysis and discrimination of regioisomers is hampered by the fact that the homologous pairs exhibit similar retentions in both HPLC and TLC analysis. Nevertheless, the analysis of ^{1}H NMR spectra showed the existence of diagnostic signals for each isomeric pair, such as those labeled as H_A-H_E and H_α and H_β in Fig. 2. The observed chemical shift turned out to be dependent upon the chain length and the position of the acyl substituent on the glycerol backbone. Based on these observations, we propose a simple nondestructive method for the easy discrimination of regioisomers based on chemical shift data for selected protons without any chemical manipulations.

Experimental Procedures

Materials and Methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents were of analytical grade.

The ion exchange resin is an H^+-form cation exchange resin (Fluka 44443, Buchs, Switzerland), 20–50 mesh.

^{1}H NMR spectra were recorded on Bruker (Rheinstetten, Germany) ARX 400 and AV 500 instruments operating at ^{1}H resonance frequencies of 400 and 500 MHz, respectively. Chemical shifts (δ, ppm) are reported relative to tetramethylsilane (TMS) used as internal standard. All spectra were recorded in $CDCl_3/CD_3OD$ (see "Synthesis") at 305 K. The assignment of proton spectra was supported by standard 2D correlation experiments (COSY and TOCSY) obtained by using library pulse sequences. Nuclear Overhauser enhancement correlation experiments (NOESY) were carried out by using standard pulses in the literature. The spectra were acquired by using magnetic field gradients for coherence pathway selection. The data were collected with three different mixing times, 300, 600, and 900 ms.

Mass spectra were recorded on an ESI/MS (Bruker Esquire 3000) by direct infusion of methanol solutions of compounds **1–12**.

Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) were used for analytical TLC; mixtures of $CHCl_3/CH_3OH/NH_3$ in different ratios were used as eluant. Detection was achieved with UV light followed by I_2 staining.

HPLC analysis were performed on an Agilent (Palo Alto, CA, USA) 1100 series apparatus fitted with a LiChrospher (Merck) 100 diol 5 µm column, length/internal diameter = 125/4, and an evaporative light scattering detector (ELSD) (Sedex model 75, Sedere, Lawrenceville, NJ, USA, 41 °C). The operating temperature and pressure were 55 °C and 3.5 bar. A binary solvent system of solvent A: hexane/isopropanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v) and solvent B: isopropanol/water/acetic acid/triethylamine (837/140/15/0.8, v/v/v/v) was used in a gradient mode starting from 60% A, 40% B and ramping to 100% B in 16 min. The 20 µl samples of a 1 mg/ml CH_2Cl_2/CH_3OH solution were injected into the column at a mobile phase flow rate of 1.5 ml/min.

Synthesis

The synthesis of the isomers reported in Fig. 1 was carried out with the same procedure. We report here as an example the detailed synthesis of the isomeric compounds **1** and **2** and analytical data for all compounds.

1-O-Acetyl -2-O- hexadecanoyl-sn-glycero-3-phosphocholine (1)

GPC (250 mg, 0.97 mmol) and dibutyltin oxide (DBTO;275 mg, 1.1 mmol) were suspended in 2-propanol (10 ml) and refluxed for 1 h. The mixture was cooled to 25 °C. Then dimethylamino pyridine (DMAP; 147 mg, 1.2 mmol) and acetyl chloride (83.2 µl, 1.2 mmol) were added. After 48 h, the suspension was filtered and the solution was concentrated under vacuo without heating. The residue was suspended in dry dichloromethane (10 ml) and treated with DMAP (0.335 g, 2.74 mmol) and palmitic anhydride (1.36 g, 2.74 mmol). The reaction mixture was stirred for 24 h at r.t., then concentrated under vacuo. The crude product was then dissolved in heptane (10 ml) and water (10 ml). After centrifugation the organic phases were evaporated to give a residue which was dissolved in dichloromethane (2 ml) and precipitated in acetone (30 ml) at −20 °C. Solid **1** (160 mg, 30 %) was recovered by filtration and dried under vacuo.

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.15 (1H, m, H_C), 4.33 (1H, m, H_B), 4.18 (2H, m, H_α), 4.07 (1H, m, H_A), 3.93 (2H, m, $H_{D/E}$), 3.52 (2H, m, H_β), 3.11 (9H, s, $N(CH_3)_3$), 2.22 (2H, m, $COCH_2$), 2.03 (3H, s, $COCH_3$), 1.54 (2H, m, $COCH_2CH_2$), 1.18 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 0.82 (3H, m, $CO(CH_2)_{14}CH_3$). ESI/MS: $[M + H]^+ = 538.2$; $[M + Na]^+ = 560.2$. t_R (HPLC) = 5.178 min.

1-O-Hexadecanoyl-sn-glycero-3-phosphocholine (1-O-PalmPC)

GPC (1 g, 3.9 mmol) and DBTO (1.1 g, 3.9 mmol) were suspended in 2-propanol (40 ml) and refluxed for 1 h. The mixture was cooled to 25 °C and treated with triethylamine (TEA; 0.65 ml, 4.7 mmol) and palmitoyl chloride (1.28 g, 4.7 mmol). The formation of the lysoPC was followed by HPLC. After 15 min the reaction mixture contained 97% of 1-palmitoyl-2-*lyso*-phosphatidylcholine and 3% of GPC as the only phospholipids. The solution was then treated with water (40 ml) and extracted with heptane (40 ml). The water–alcohol solution was extracted three times with heptane (3 × 40 ml) and evaporated in vacuo; the crude residue was dissolved in ethanol (10 ml) and precipitated with acetone (40 ml) at −10 °C to give 1.5 g of 1-*O*-Palm-PC as a white powder (80%). The analytical samples were obtained after crystallization from ethanol (yield 40%).

The ^{1}H NMR spectrum has been already reported in the literature [11]. ESI/MS: $[M + H]^+ = 496.3$; $[M + Na]^+ = 518.2$. t_R (HPLC) = 6.24 min.

1-O-Hexadecanoyl-2-O-acetyl-sn-glycero-3-phosphocholine (2)

To a solution of 1-*O*-hexadecanoyl-*sn*-glycero-3-phosphocholine (200 mg, 0.404 mmol) in dry dichloromethane (8 ml), DMAP (148 mg, 1.21 mmol) and acetic anhydride (0.112 ml, 1.21 mmol) were added. The reaction mixture was stirred for 20 h at r.t., then vacuum-distilled. The crude product was then dissolved in dichloromethane and stirred at room temperature for 1 h with Dowex 50 × 8 cation exchange resin (H^+-form). The suspension was then filtered and concentrated to give a residue which was dissolved in dichloromethane (2 ml) and precipitated in acetone (30 ml) at −20 °C. Solid **2** (141 mg, 65% yield) was recovered by filtration and dried under vacuo.

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.20 (1H, m, H_C), 4.36 (3H, m, $H_B + H_\alpha$), 4.15 (1H, m, H_A), 3.99 (2H, m, $H_{D/E}$), 3.80 (2H, m, H_β), 3.34 (9H, s, $N(CH_3)_3$), 2.27 (2H, m, $COCH_2$), 2.05 (3H, s, $COCH_3$), 1.58 (2H, m, $COCH_2CH_2$), 1.26 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 0.88 (3H, m, $CO(CH_2)_{14}CH_3$). ESI/MS: $[M + H]^+ = 538.4$; $[M + Na]^+ = 560.4$. t_R (HPLC) = 5.305 min.

1-O-Propanoyl -2-O- hexadecanoyl-sn-glycero-3-phosphocholine (3)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.16 (1H, m, H_C), 4.33 (1H, m, H_B), 4.22 (2H, m, H_α), 4.09 (1H, m, H_A), 3.95 (2H, m, $H_{D/E}$), 3.60 (2H, m, H_β), 3.25 (9H, s, $N(CH_3)_3$), 2.32 (4H, m, $COCH_2$), 1.61 (2H, m, $COCH_2CH_2$), 1.25 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 0.98 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+ = 552.4$; $[M + Na]^+ = 574.4$. t_R (HPLC) = 4.682 min.

1-O-Hexadecanoyl-2-O-propanoyl-sn-glycero-3-phosphocholine (4)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.21 (1H, m, H_C), 4.36 (3H, m, $H_B + H_\alpha$), 4.14 (1H, m, H_A), 3.99 (2H, m, $H_{D/E}$), 3.85 (2H, m, H_β), 3.38 (9H, s, $N(CH_3)_3$), 2.28 (4H, m, $COCH_2$), 1.38 (2H, m, $COCH_2CH_2$), 1.25 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 1.15 (3H, m, $COCH_2CH_3$), 0.88 (3H, m, $CO(CH_2)_{14}CH_3$). ESI/MS: $[M + H]^+ = 552.2$; $[M + Na]^+ = 574.2$. t_R (HPLC) = 4.582 min.

1-O-Butanoyl -2-O-hexadecanoyl-sn-glycero-3-phosphocholine (5)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.20 (1H, m, H_C), 4.37 (1H, m, H_B), 4.29 (2H, m, H_α), 4.11 (1H, m, H_A), 3.97 (2H, m,

$H_{D/E}$), 3.70 (2H, m, H_β), 3.22 (9H, s, $N(CH_3)_3$), 2.31 (4H, m, $COCH_2$), 1.61 (4H, m, $COCH_2CH_2$), 1.23 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 0.87 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + Na]^+$ = 588.3. t_R (HPLC) = 4.354 min.

1-O-Hexadecanoyl-2-O-butanoyl-sn-glycero-3-phosphocholine (6)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.19 (1H, m, H_C), 4.34 (1H, m, H_B), 4.28 (2H, m, H_α), 4.11 (1H, m, H_A), 3.98 (2H, m, $H_{D/E}$), 3.68 (2H, m, H_β), 3.25 (9H, s, $N(CH_3)_3$), 2.26 (4H, m, $COCH_2$), 1.63 (4H, m, $COCH_2CH_2$), 1.22 (24H, m, $COCH_2CH_2(CH_2)_{12}CH_3$), 0.93 (3H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 566.3; $[M + Na]^+$ = 588.3. t_R (HPLC) = 4.434 min.

1-O-Hexanoyl-2-O-hexadecanoyl-sn-glycero-3-phosphocholine (7)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.12 (1H, m, H_C), 4.30 (1H, m, H_B), 4.20 (2H, m, H_α), 4.04 (1H, m, H_A), 3.90 (2H, m, $H_{D/E}$), 3.58 (2H, m, H_β), 3.18 (9H, s, $N(CH_3)_3$), 2.23 (4H, m, $COCH_2$), 1.64 (4H, m, $COCH_2CH_2$), 1.16 (28H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.81 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 594.4; $[M + Na]^+$ = 616.4. t_R (HPLC) = 3.941 min.

1-O-Hexadecanoyl-2-O-hexanoyl-sn-glycero-3-phosphocholine (8)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.23 (1H, m, H_C), 4.38 (3H, m, $H_B + H_\alpha$), 4.14 (1H, m, H_A), 4.02 (2H, m, $H_{D/E}$), 3.94 (2H, m, H_β), 3.34 (9H, s, $N(CH_3)_3$), 2.26 (4H, m, $COCH_2$), 1.58 (4H, m, $COCH_2CH_2$), 1.26 (28H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.88 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 594.3; $[M + Na]^+$ = 616.3. t_R (HPLC) = 4.195 min.

1-O-Nonanoyl-2-O-hexadecanoyl-sn-glycero-3-phosphocholine (9)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.14 (1H, m, H_C), 4.32 (1H, m, H_B), 4.19 (2H, m, H_α), 4.06 (1H, m, H_A), 3.92 (2H, m, $H_{D/E}$), 3.53 (2H, m, H_β), 3.25 (9H, s, $N(CH_3)_3$), 2.28 (4H, m, $COCH_2$), 1.60 (4H, m, $COCH_2CH_2$), 1.23 (34H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.85 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 636.5; $[M + Na]^+$ = 658.5. t_R (HPLC) = 3.51 min.

1-O-Hexadecanoyl-2-O-nonanoyl-sn-glycero-3-phosphocholine (10)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.16 (1H, m, H_C), 4.30 (3H, m, $H_B + H_\alpha$), 4.07 (1H, m, H_A), 3.95 (2H, m, $H_{D/E}$), 3.76 (2H, m, H_β), 3.28 (9H, s, $N(CH_3)_3$), 2.23 (4H, m, $COCH_2$), 1.53 (4H, m, $COCH_2CH_2$), 1.20 (34H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.81 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 636.5; $[M + Na]^+$ = 658.5. t_R (HPLC) = 3.609 min.

1-O-Decanoyl-2-O-hexadecanoyl-sn-glycero-3-phosphocholine (11)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.13 (1H, m, H_C), 4.30 (1H, m, H_B), 4.18 (2H, m, H_α), 4.05 (1H, m, H_A), 3.91 (2H, m, $H_{D/E}$), 3.55 (2H, m, H_β), 3.15 (9H, s, $N(CH_3)_3$), 2.23 (4H, m, $COCH_2$), 1.55 (4H, m, $COCH_2CH_2$), 1.17 (36H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.80 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 650; $[M + Na]^+$ = 672. t_R (HPLC) = 3.65 min.

1-O-Hexadecanoyl-2-O-decanoyl-sn-glycero-3-phosphocholine (12)

^{1}H NMR ($CDCl_3/CD_3OD$): δ 5.15 (1H, m, H_C), 4.30 (3H, m, $H_B + H_\alpha$), 4.07 (1H, m, H_A), 3.98 (2H, m, $H_{D/E}$), 3.74 (2H, m, H_β), 3.24 (9H, s, $N(CH_3)_3$), 2.23 (4H, m, $COCH_2$), 1.52 (4H, m, $COCH_2CH_2$), 1.18 (36H, m, $COCH_2CH_2(CH_2)_nCH_3$), 0.80 (6H, m, $CO(CH_2)_nCH_3$). ESI/MS: $[M + H]^+$ = 650; $[M + Na]^+$ = 672.4. t_R (HPLC) = 3.71 min.

Results and Discussion

We have recently described [10] a new procedure for the synthesis of mixed short/long-chain glycerophosphocholines which exploits the tin-mediated synthesis of lysophospholipids [16] and subsequent selective acylation in the *sn*-2 position of the glycerol backbone.

In this procedure, glycerophosphocholine (GPC) is transformed into the cyclic stannylene derivative by treatment in 2-propanol with dibutyltin oxide (DBTO) at reflux. The cyclic tin ketal is then acylated with the desired acid chloride and dimethylaminopyridine (DMAP) as a base. The solvent was then removed at room temperature and replaced with dry dichloromethane. The second acylation is carried out with the acid anhydride in the presence of fresh DMAP. After different purification steps, yields from GPC were in the range of 20 to 69% for compounds **1**–**12**. This tin-catalyzed synthesis of phospholipids always leads to a single regioisomer. The purities of the compounds were investigated by HPLC and their structures were determined by ^{1}H NMR.

For the sake of clarity, compounds **1**–**12** can be divided into two families of isomers: odd-numbered compounds correspond to PCs carrying a variable length acyl substituent at position 1 of GPC and a palmitoyl moiety at position 2, whilst even-numbered compounds correspond

to their regioisomeric counterparts, namely those PCs bearing the palmitoyl chain at position 1 and the same acyl group as before at position 2. Accordingly, short indicative names for the isomeric pairs can be used, such as C2-PalmPC and Palm-C2PC for compounds **1** and **2**, respectively.

The NMR spectra of compounds **1–12** showed the presence of isolated signals in the chemical shift range 5.40–3.60 ppm, along with the envelope due to nonassignable protons of the hydrocarbon chains, the terminal methyl groups and the singlet of the three equivalent CH_3 groups of the choline moiety. Expansions of the ^{1}H-NMR spectra for the homologous pair **11–12** are reported in Fig. 2.

The protons belonging to the glycerol frame (see Fig. 1) are expected to give rise to an ABCMX spin system. As a matter of fact, the protons $H_{D/E}$ attached to C3 of GPC are isochronous and thus the observed spin system is $ABCM_2$. Any attempt to remove their chemical shift degeneracy by changing solvent and/or temperature failed. The NMR signal of the latter protons then appears as a deceptively simple triplet. In a similar way, the CH_2 groups of the choline fragment gave rise to a triplet ($CH_2\alpha$) and to a broad singlet ($CH_2\beta$).

The chemical shifts of H_A-H_E, H_α and H_β were collected and tabulated for all of the examined compounds. The results are summarized in Table 1.

Some systematic trends could be better highlighted by calculating the difference in the chemical shift for the same proton between the homologous isomeric pairs. The results are reported in the $\Delta\delta$ columns of Table 1.

For example, the column $\Delta\delta H_C$ reports the difference in the chemical shift of H_C between the Palm-C*n*PC compounds and the corresponding C*n*-PalmPC (C*n* = C2, C3, C4, C6, C9, C10). After studying the $\Delta\delta$ values, it is clear that: (1) the chemical shift variations can be related to the length of the acyl substituent and to the position of the latter in the GPC backbone; (2) the $\Delta\delta$ values are always positive, with the exception of the pair **5–6** (C4-PalmPC and Palm-C4PC); and (3) the values for the choline CH_2 protons are one order of magnitude larger than all of the others. A graphical summary is reported in Fig. 3.

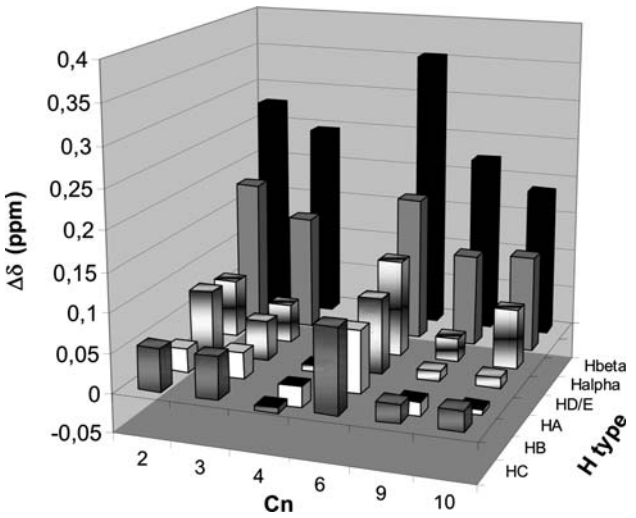

Fig. 3 Reported differences in chemical shift ($\Delta\delta$, ppm) for selected protons in compounds **1–12** vs the number C*n* of carbon atoms in the variable length acyl chain. $\Delta\delta$ for a given proton is defined as δ(H in Palm-C*nPC*)$-\delta$(H in C*n*-PalmPC) for all of the examined C*n*

The chemical shift data clearly indicate that both the glycerol protons and the choline methylene groups are sensitive probes of regioisomeric substitution and useful probes for recognizing the acyl substitution pattern at the C1–C2 frame of GPC. The large $\Delta\delta$ observed for the choline methylene groups are far beyond the experimental uncertainty and suggest that this quantity could be used as an unbiased descriptor for discriminating the regioisomeric substitution of mixed short/long-chain glycerophosphocholines.

Table 1 Chemical shifts (δ, ppm) and the differences in chemical shift ($\Delta\delta$, ppm) for selected protons from compounds **1–12**

Compd.	R′	R″	Short name	H_C	$\Delta\delta H_C$	H_B	$\Delta\delta H_B$	H_A	$\Delta\delta H_A$	$H_{D/E}$	$\Delta\delta H_{D/E}$	H_α	$\Delta\delta H_\alpha$	H_β	$\Delta\delta H_\beta$
1	C2	C16	C2-PalmPC	5.150	–	4.330	–	4.069	–	3.925	–	4.177	–	3.524	–
2	C16	C2	Palm-C2PC	5.205	0.055	4.360	0.03	4.150	0.081	3.999	0.074	4.360	0.183	3.802	0.278
3	C3	C16	C3-PalmPC	5.160	–	4.330	–	4.090	–	3.950	–	4.220	–	3.600	–
4	C16	C3	Palm-C3PC	5.214	0.054	4.364	0.034	4.140	0.05	3.999	0.049	4.364	0.144	3.845	0.245
5	C4	C16	C4-PalmPC	5.200	–	4.370	–	4.114	–	3.974	–	4.294	–	3.698	–
6	C16	C4	Palm-C4PC	5.193	−0.007	4.343	−0.027	4.106	–0.008	3.977	0.003	4.275	−0.019	3.683	−0.015
7	C6	C16	C6-PalmPC	5.120	–	4.303	–	4.040	–	3.901	–	4.201	–	3.582	–
8	C16	C6	Palm-C6PC	5.227	0.107	4.383	0.08	4.136	0.096	4.022	0.121	4.383	0.182	3.936	0.354
9	C9	C16	C9-PalmPC	5.136	–	4.316	–	4.061	–	3.920	–	4.185	–	3.532	–
10	C16	C9	Palm-C9PC	5.159	0.023	4.298	−0.018	4.073	0.012	3.950	0.03	4.300	0.115	3.755	0.223
11	C10	C16	C10-PalmPC	5.126	–	4.303	–	4.053	–	3.906	–	4.175	–	3.549	–
12	C16	C10	Palm-C10PC	5.152	0.026	4.296	−0.007	4.067	0.014	3.981	0.075	4.296	0.121	3.736	0.187

In order to provide a possible rationale for the empirical criterium reported above, a conformational investigation of compounds **1–12** was undertaken. The conformational preferences of the examined compounds can be described in terms of two dihedral angles, θ and φ, corresponding to the torsions around the C1–C2 and C2–C3 chemical bonds of GPC, respectively. In turn, θ and φ drive the values of vicinal proton spin–spin coupling constants via the Karplus–Altona equation [17]. It should be noted that, in the present case, the tremendous overlap of the signals from the acyl chains R′ and R″ (see Fig. 1) severely hampers the use of inter-chain NOE for conformational analysis. Therefore, the conformational preferences of the title compounds can only be spotted via the analysis of J couplings. In detail, the experimental values of J_{AC} and J_{BC} are related to θ, while those of J_{CD} and J_{CE} are related to φ. In the case of different conformations that rapidly interconvert on the time-scale of NMR spectroscopy, the observed vicinal coupling constants are the average of the coupling constants of pure conformations weighted by the fractional population of the conformers. In our case, the values of J_{CD} and J_{CE} could not be experimentally determined due to the degeneracy of the D and E protons, as described above. Thus, our data allow us to discuss the conformational preferences of **1–12** concerning the torsion around the C1–C2 bond of GPC only. A sketch of the rotamers that are appreciably populated is reported in Fig. 4.

For the conformational analysis, we took advantage of the work published by Martin and Pitzer [18] on symmetrically substituted 1,2-dipropanoyl-, 1,2-dibutanoyl- and 1,2-dihexanoylphosphatidylcholines (C3PC, C4PC, and C6PC, respectively). Those substrates gave high-resolution NMR spectra in D_2O solution without any chemical shift degeneracy, and thus allowed the authors to measure all of the vicinal coupling constants related to both θ and φ. The authors found that the conformation about the C1–C2 fragment was mainly dictated by conformers A and B, with the third conformation C providing only a minor contribution to the overall conformer distribution. The C2–C3 fragment of the GPC moiety was more flexible, with the three conformations D, E and F being nearly equally populated. The observed vicinal coupling constants of **1–12** are summarized in Table 2.

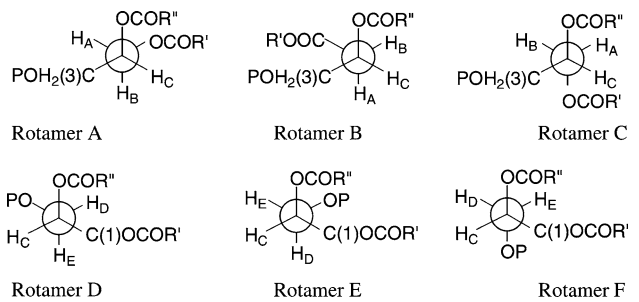

Fig. 4 Sketch of populated rotamers resulting from torsion around the C1–C2 bond of GPC. Rotamer nomenclature are as given in [18]

Table 2 Values (Hz) of the vicinal (^3J) coupling constants for the examined compounds

Compound	R′	R″	Short name	J_{BC}	J_{AC}	$R_J = J_{AC}/J_{BC}$
1	C2	C16	C2-PalmPC	3.3	6.9	2.1
2	C16	C2	Palm-C2PC	3.2	6.8	2.1
3	C3	C16	C3-PalmPC	3.3	7.0	2.1
4	C16	C3	Palm-C3PC	3.0	6.8	2.3
5	C4	C16	C4-PalmPC	3.3	7.1	2.2
6	C16	C4	Palm-C4PC	3.5	6.7	1.9
7	C6	C16	C6-PalmPC	3.0	7.1	2.4
8	C16	C6	Palm-C6PC	3.4	6.9	2.0
9	C9	C16	C9-PalmPC	3.3	7.0	2.1
10	C16	C9	Palm-C9PC	3.3	6.7	2.0
11	C10	C16	C10-PalmPC	3.3	7.0	2.1
12	C16	C10	Palm-C10PC	3.5	6.8	1.9
C3PC[a]	C3	C3		3.5	6.6	1.9
C4PC[a]	C4	C4		3.4	6.7	2.0
C6PC[a]	C6	C6		3.2	7.0	2.2

[a] From [18]

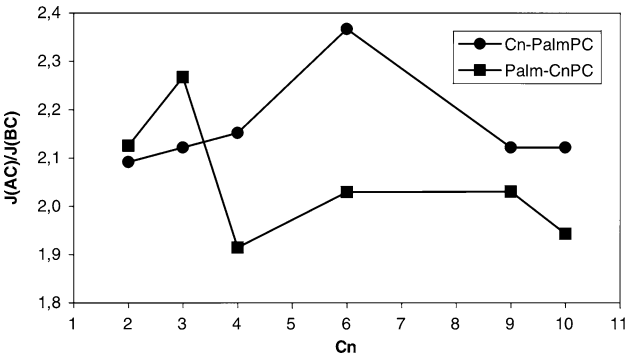

Fig. 5 Plot of $R_J = J_{AC}/J_{BC}$ versus the number C*n* of carbon atoms in the variable-length acyl chain

The literature values for the references C3PC, C4PC and C6PC are also reported for the sake of comparison. The ratio $R_J = J_{AC}/J_{BC}$ can be used as suitable empirical descriptor to compare the conformations about C1–C2 in corresponding pairs of regioisomers. A plot of R_J values vs the number C*n* of carbon atoms in the acyl chains is shown in Fig. 5.

Starting from the crossover point corresponding to $n = 4$, the data show that the R_J values of the odd-numbered terms (C*n*-PalmPC) are systematically greater than the R_J values of the even-numbered isomers (Palm-C*n*PC). This fact, in turn, corresponds to a higher contribution of the A conformation with respect to the C conformation in the C*n*-PalmPC series compared to the Palm-C*n*PC.

Therefore, significant chemical shift differences $\Delta\delta$ observed for the choline CH_2 groups in unsymmetrical 1,2-diacyl glycerophosphocholines can be related to both the substitution patterns at the *sn*-1 and *sn*-2 positions of the GPC and the length of the hydrocarbon chain of the acyl moiety. The observed patterns can be exploited as a simple, quick and nondestructive method for discriminating regioisomeric unsymmetrical 1,2-diacyl glycerophosphocholines. However, these chemical shift variations need to be theoretically investigated further in order to find a general model that can explain their behavior. Nevertheless, significant correlations between the observed $\Delta\delta$ values and the vicinal coupling constants across the GPC backbone suggest that the observed trends can be ascribed to different conformational preferences of the target molecules.

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