Synthesis of Acetyl,docosahexaenoyl-Glycerophosphocholine and Its Characterization Using Nuclear Magnetic Resonance

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ABSTRACT: Docosahexaenoic acid (DHA) circulates in mammals in lipoproteins and bound to serum albumin as a nonesterified fatty acid as well as esterified in lysophosphatidylcholine (lysoPC). 1-Lyso,2-DHA-glycerophosphocholine (GPC) is an unstable isomer because of a primary alcohol at the *sn*-1 position. To keep DHA at the *sn*-2 position of lysoPC, its usual position for the corresponding lysoPC to be acylated into PC in tissues, we synthesized 1-acetyl,2-DHA-GPC and confirmed its structure by use of nuclear magnetic resonance (NMR) spectroscopy in comparison with its positional isomer, 1-DHA,2-acetyl-GPC. 1-Lyso,2-DHA-GPC was prepared from 1-stearoyl,2-DHA-GPC by enzymatic hydrolysis and purified by high-performance liquid chromatography. The isomerization of 1-lyso,2-DHA-GPC into 1-DHA,2-lyso-GPC was obtained by keeping the former overnight at room temperature under nitrogen. Both lysoPC isomers were acetylated by acetic anhydride into 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC, respectively, and the resulting phospholipids were fully characterized by NMR. In particular, the 1,2 substitution pattern of the acetyl and DHA chains could be easily detected by 2D heteronuclear multibond correlation. We conclude that 1-acetyl,2-DHA-GPC might be considered as a stable form of 1-lyso,2-DHA-GPC for its delivery to tissues, if the latter exhibits acetyl hydrolase activity.

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Since the pioneering epidemiological studies of Bang *et al.* (1), there has been a considerable interest in the potential health benefits of marine polyunsaturated fatty acids, namely eicosapentaenoic and docosahexaenoic (DHA) acids. In addition to the beneficial effects of those fatty acids in the circulatory cardiovascular system (2), DHA is of particular interest for the cerebrovascular system, especially the retina, in which it represents almost the only polyunsaturated fatty acid (3,4), and in brain grey matter, where it may be more prominent than arachidonic acid, depending on the area (5). Indeed, DHA is assumed to be required for proper visual functions and brain development (6,7). *In vivo*, DHA circulates in the bloodstream under various chemical forms attached to either albumin or lipoproteins (8). Previous studies in which ^{13}C-labeled DHA esterified in triglycerides or phosphatidylcholine (PC) was ingested as a single dose by human volunteers showed that nonesterified DHA and DHA esterified in lysophosphatidylcholine (lysoPC-DHA) are two chemical forms bound to albumin that could specifically bring DHA to platelets and erythrocytes, respectively (9,10). There is evidence that the incorporation of DHA into erythrocytes may serve as an indicator of DHA incorporation into the brain (11), and we consider this observation to be in agreement with the preferential brain uptake of DHA in the rat when DHA is injected as lysoPC-DHA compared to nonesterified DHA, both chemical forms being bound to albumin (12). Evidence for the preferential uptake of DHA has recently been provided using a reconstituted *in vitro* blood brain barrier with co-cultures of capillary endothelial cells and astrocytes (13). However, the metabolic fate of DHA in the brain is to accumulate at the *sn*-2 position of phospholipids. Although previous experiments have been done with 1-lyso,2-docosahexaenoyl-glycerophosphocholine (1-lyso,2-DHA-GPC), it is expected that part (if not the bulk) of this chemical form would have been isomerized into 1-DHA,2-lyso-GPC because of the primary alcohol reactivity at the *sn*-1 position.

The objective of the present study was to prevent such an isomerization by esterification of the *sn*-1 position with an acetyl group, keeping the molecule at a polarity closer to that of lysoPC-DHA. A stable molecule of this kind might be useful as a vehicle for delivering DHA through lysoPC-DHA, provided that the target tissues exhibit acetyl hydrolase activity.

EXPERIMENTAL PROCEDURES

Preparation of lysoPC-DHA. 1-Lyso,2-DHA-GPC was prepared by enzymatic hydrolysis from 1-stearoyl,2-DHA-GPC, which was provided by Avanti Polar Lipids (Alabaster, AL). The enzyme used was a lipase (EC 3.1.1.3) from *Rhizopus arrhizus* which exhibits phospholipase A_1 activity (14). The enzyme was furnished by Sigma (L'Isle d'Abeau, France). Two milligrams of substrate (PC) was dissolved in 1 mL of diethylether and sonicated for 4 min in a sonication bath with 1 mL of phosphoboric buffer (pH 6) containing 200,000 U of

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Abbreviations: COSY, correlation spectroscopy; DHA, docosahexaenoic acid; GPC, glycerophosphocholine; HPLC, high-performance liquid chromatography; HMBC, heteronuclear multibond correlation; HSQC, heteronuclear single quantum coherence; lysoPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; TLC, thin-layer chromatography.

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enzyme. This mixture was further incubated with vigorous shaking for 1 h at room temperature under nitrogen. The reaction was stopped in ice and total lipids were extracted according to Bligh and Dyer (15) at pH 3 and 4°C to minimize lysoPC isomerization.

The lysoPC formed was separated from the remaining PC substrate and the fatty acid was released by the lipase treatment using thin-layer chromatography (TLC) on silica gel 60G (Merck, Darmstadt, Germany) with the eluent mixture chloroform/methanol/water (65:25:4, by vol) at 4°C (16). The R_f of the compounds were 0.2, 0.47, and 0.91, respectively. The purity of 1-lyso,2-DHA-GPC obtained was checked by high-performance liquid chromatography (HPLC) as shown in Figure 1. The isomerization of 1-lyso,2-DHA-GPC into 1-DHA,2-lyso-GPC was induced by keeping the former overnight at room temperature under nitrogen. Under these conditions, further isomerization of 1-DHA,2-lyso-GPC into 1-DHA,3-lyso-GPC is very unlikely because of the lower reactivity of the *sn*-2 secondary alcohol. The positional isomers were analyzed by HPLC with a 5-µm Superspher C_{18} column (4.6 × 250 mm, i.d.; Hewlett-Packard, Les Ulis, France) isocratically eluted with meth-anol/water/acetonitrile (90:25:2.5, by vol) containing 0.35% choline chloride at a flow rate of 1.2 mL/min. Each lysoPC was detected at 210 nm. Figure 1 gives an example of chromatograms. If 1-DHA,3-lyso-GPC was formed subsequent to isomerization of 1-lyso,2-DHA-GPC, it would presumably have migrated in peak 1 of Figure 1B together with 1-lyso,2-DHA-GPC, the sum representing at most 12% of the two isomers. In addition, the nuclear magnetic resonance (NMR) spectrum of the acetylated derivative (2) appeared at least 95% pure, and no signal could be detected for a putative 1-acetyl,3-DHA-GPC.

Each lysoPC isomer was quantified on the basis of its DHA content by gas–liquid chromatography (9). LysoPC was treated with 5% H_2SO_4 in methanol for 90 min at 100°C in the presence of an appropriate amount of 1-heptadecanoyl,2-lyso-GPC as an internal standard. The mixture of DHA and heptadecanoic acid methylesters were separated and measured as previously described by gas chromatography, using an SP2380 capillary column (30 m × 0.32 mm; Supelco, Bellefonte, PA) (9).

Acetylation of lysoPC isomers. Both lysoPC isomers (1-lyso,2-DHA-GPC and 1-DHA,2-lyso-GPC) stored in chloroform were dried under reduced pressure at low temperature in the dark. Each lysoPC isomer was dissolved in 0.55 mL of a 0.135 M chloroformic solution of 4-pyrrolidinopyridine and 0.8 mL of a 0.138 M chloroformic solution of acetic anhydride and was continuously agitated for 16 h at 4°C to ensure complete reaction. Added to this were 1.7 mL of chloroform and 1.5 mL of water. After stirring, the mixture was centrifuged for 10 min at 800 × *g*. The chloroformic phase was separated and evaporated under reduced pressure. The 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC were separated from the remaining 1-lyso,2-DHA-GPC and 1-DHA,2-lyso-GPC, respectively, by TLC on silica gel 60G with the chloroform/methanol/water (65:25:4, by vol) mixture. The expected 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC at

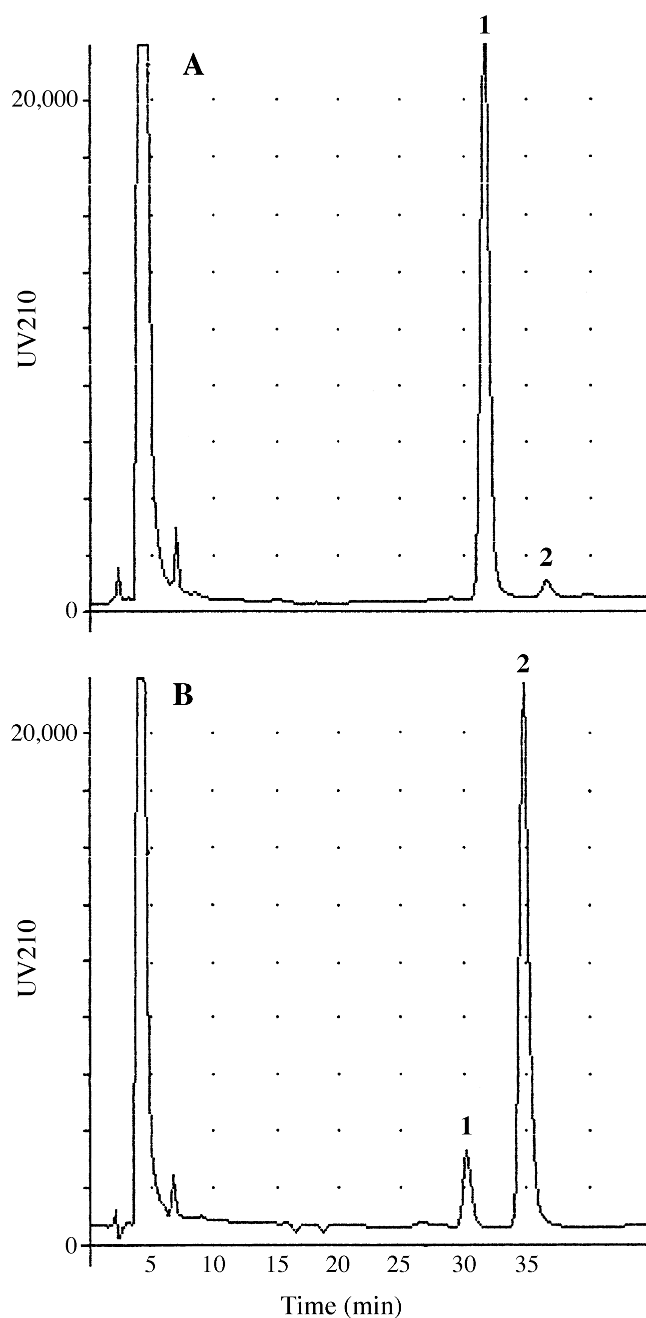
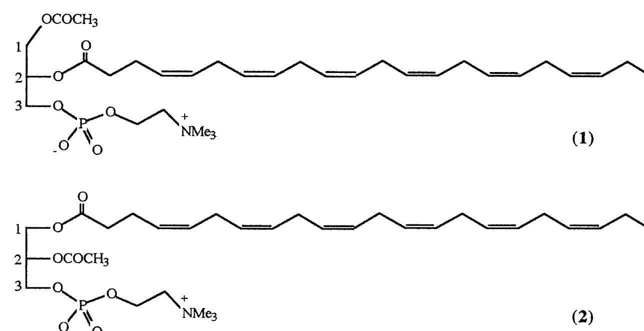


FIG. 1. Separation of 1-lyso,2-docosahexaenoyl-glycerophosphocholine (1-lyso,2-DHA-GPC) (peak 1) from 1-DHA,2-lyso-GPC (peak 2) by reversed-phase high-performance liquid chromatography. (A) Lysophosphatidylcholine (lysoPC) isomers obtained by enzymatic cleavage of 1-stearoyl,2-DHA-GPC were injected onto a 5-µm Superspher column (250 × 4.6 mm), and eluted at 1.2 mL/min by methanol/water/acetonitrile (90:25:2.5, by vol) containing 0.35% choline chloride. They were detected by ultraviolet light at 210 nm. (B) LysoPC isomers obtained by enzymatic cleavage were maintained overnight at room temperature. They were separated as indicated in A.

R_f 0.18 were scraped off the plate by extraction with chloroform/methanol/water (10:20:3, by vol) and the extract was dried under nitrogen before being dissolved in 0.4 mL of $CDCl_3$ for further NMR analysis.

NMR analysis. Nuclear magnetic spectra were recorded in $CDCl_3$ with a Bruker (Wissembourg, France) DRX-500 (500/125 MHz) spectrometer. Chemical shifts are given in ppm and are referenced to $CDCl_3$ resonances (7.26 and 77.0 ppm). Splitting pattern abbreviations are *s*, singlet; *d*, doublet; *t*, triplet; and *m*, multiplet. Resonance attributions were made according to the following experiments: Standard ^{1}H NMR, 2D H,H-correlation spectrocopy (COSY), 2D heteronuclear single quantum coherence (HSQC), and 2D gradient-selected heteronuclear multibond correlation (HMBC) with a mixing period of 60 ms.



SCHEME 1

RESULTS AND DISCUSSION

LysoPC isomers. The hydrolysis of 1-stearoyl,2-DHA-GPC by the *Rhizopus arrhizus* lipase led to 1-lyso,2-DHA-GPC with a yield recovery of about 93%, as based on ultraviolet quantitation. The lysoPC produced was extracted in acidic conditions to minimize its isomerization into 1-DHA,2-lyso-GPC. A typical HPLC profile of the lipase hydrolysis product is given in Figure 1. From this HPLC profile, it can be calculated that the isomerization was limited to around 4%. To prevent the 1-lyso,2-DHA-GPC isomer from its isomerization into 1-DHA,2-lyso-GPC, it was kept at pH 4 at 4°C. Leaving the 1-lyso,2-DHA-GPC isomer at room temperture overnight under neutral conditions was enough to provoke more than 85% isomerization into the 1-DHA,2-lyso-GPC isomer (Fig. 1). Both isomers have been taken into consideration in the present study as we found them bearing polyunsaturated fatty acids bound to human albumin, both forms being found in similar proportions (17). These lysoPC appear to be the main lysophospholipids released from the liver (18) and are considered to be the main form to provide polyunsaturated fatty acids in the brain (12,16). An important issue in this context is the biological relevance of 1-lyso,2-DHA-GPC, expected to result from liver phospholipase A_1 activity, compared to that of 1-DHA,2-lyso-GPC, which is unlikely to be acylated to yield PC, as that PC would contain DHA at the *sn*-1 position. In contrast, we may reasonably expect that 1-lyso,2-DHA-GPC could be easily acylated into physiological PC. In order to keep DHA stable at the *sn*-2 position, we aimed to chemically prepare 1-acetyl,2-DHA-GPC. This form could be then the precursor of 1-lyso,2-DHA-GPC, provided that it was first cleaved by an acetyl hydrolase.

NMR characterization of acetyl-DHA-GPC. 1-Acetyl,2-DHA-GPC **(1)** and its positional isomer, 1-DHA,2-acetyl-GPC **(2)** were obtained by acetylation of the corresponding DHA-lysoPC with acetic anhydride, and their structures (Scheme 1) were characterized by NMR spectroscopy, as described in the Experimental Procedures section.

As expected, the ^{1}H NMR spectra of compounds **1** and **2** were very similar and no distinction between them could be made on this basis. The 2D HMBC experiment, which is related to $^2J_{CH}$ and $^3J_{CH}$ connectivities (carbon–hydrogen connectivity through two or three bonds), appeared very useful to connect directly the carbon-1 of the glycerol backbone with the acetyl group in compound **1** or the DHA chain in compound **2**, thus revealing the 1,2 substitution pattern. In the 2D HMBC experiment with compound **1** (Fig. 2), cross peaks were observed between the carbon resonance at δ 171.13 ($^1CH_2$–O–$\underline{C}$O–CH_3) and proton resonances at δ 2.06 ($^1CH_2$–O–CO–$C\underline{H}_3$) and δ 4.17 ($^1C\underline{H}_2$–O–CO–CH_3); a weak cross peak also was observed between the carbon resonance at

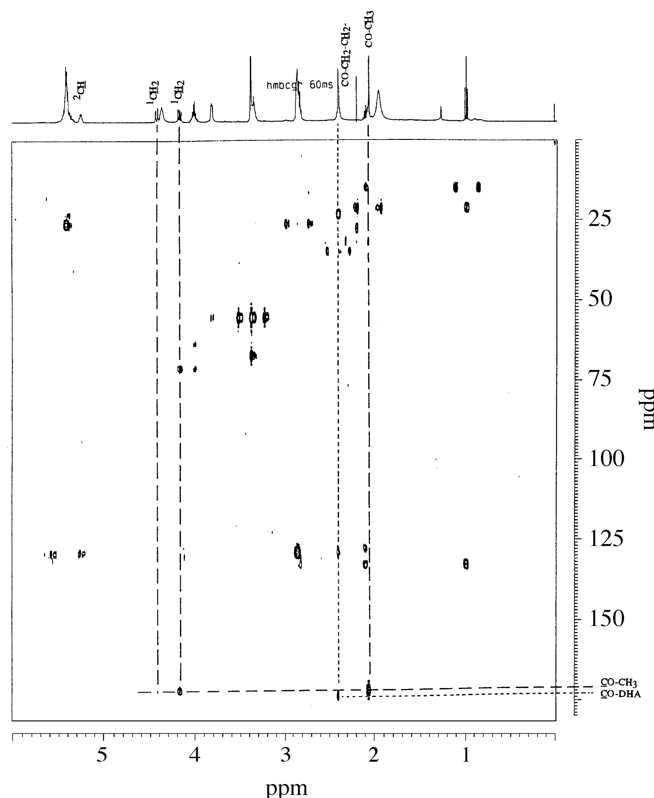


FIG. 2. 2D gradient-selected heteronuclear multibond correlation nuclear magnetic resonance spectrum of 1-acetyl,2-DHA-GPC (**1**). The presence of cross peaks on the $COCH_3$ line (horizontal) demonstrates the attachment of the acetyl group to C-1 of the glycerol backbone. The spectrum was obtained without filtering out the $^1J_{CH}$ coupling interaction since the expected information occurs in the carbonyl region where such a coupling is absent. Acquisition parameters: NS, 32; TD2, 2048; SW2, 6.008 ppm; relaxation delay, 1.5 s; mixing time, 60 ms; TDI, 128; SW1, 179.998 ppm. Processing parameters: F2 SI, 1024; F1 SI, 512. The time domain data were multiplied by a sine-bell function prior to Fourier transformation.

δ 171.13 and the other $^{1}CH_2$ proton resonance at δ 4.41 ($^{1}C\underline{H}_2–O–CO–CH_3$). Thus, the connection between the $^{1}CH_2$ and $O–CO–CH_3$ parts of the molecule was unambiguously established. A similar result was obtained with compound **2** since cross peaks were observed between the carbon resonance at δ 173.43 ($^{1}CH_2–O–\underline{C}O–CH_2–CH_2$) and proton resonances at δ 2.42–2.37 ($^{1}CH_2–O–CO–C\underline{H}_2–C\underline{H}_2$) and δ 4.18 ($^{1}C\underline{H}_2–O–CO–CH_2–CH_2$), establishing the connection between the $^{1}CH_2$ and O–DHA parts of the molecule (in this case, no cross peak was observed between the carbon resonance at δ 173.43 and the other $^{1}CH_2$ proton at 4.40). With chains bonded to carbon-2, DHA in **1** (Fig. 2), or acetyl in **2**, a cross peak was observed between the carbonyl carbon and the methylene or methyl protons of the chains, but we were unable to connect the chains with carbon-2 since the expected cross peaks between the carbonyl carbons and $^{2}C\underline{H}$ proton resonances at ~5.25 could not be observed. This missing observation is presumably related to the splitting of the $^{2}C\underline{H}$ proton resonances and to the small amount of product in the NMR sample. The following proton and carbon resonances were attributed to each compound from the different NMR experiments: 1-acetyl,2-DHA-GPC (**1**): ^{1}H NMR: δ 5.44–5.34 (*m*, 12H, $C\underline{H}=C\underline{H}$); 5.26–5.23 (*m*, 1H, $^{2}C\underline{H}$); 4.41 (*dd*, 1H, *J* = 12.0 and 3.2 Hz, $^{1}C\underline{H}_2$); 4.40–4.30 (*m*, 2H, $O–C\underline{H}_2–CH_2–N$); 4.17 (*dd*, 1H, *J* = 12.0 and 7.0 Hz, $^{1}C\underline{H}_2$); 4.04–3.96 (*m*, 2H, $^{3}C\underline{H}_2$); 3.84–3.79 (*m*, 2H, $O–CH_2–C\underline{H}_2–N$); 3.38 [*s*, 9H, $N(C\underline{H}_3)_3$]; 2.89–2.81 (*m*, 10H, $=CH–C\underline{H}_2–CH=$); 2.43–2.38 (*m*, 4H, $O–CO–C\underline{H}_2–C\underline{H}_2–CH=$); 2.12–2.03 (*m*, 2H, $=CH–C\underline{H}_2–CH_3$); 2.06 (*s*, 3H, $CO–C\underline{H}_3$); 1.00 (*t*, 3H, *J* = 7.5 Hz, $=CH–CH_2–C\underline{H}_3$). ^{13}C NMR: δ 172.81 ($^{2}CH–O–\underline{C}O–CH_2–CH_2$); 171.13 ($^{1}CH_2–O–\underline{C}O–CH_3$); 133.0–127.4 ($\underline{C}H=\underline{C}H$); 71.00 ($^{2}\underline{C}H$); 66.84 ($O–CH_2–\underline{C}H_2–N$); 63.55 ($^{3}\underline{C}H_2$); 63.37 ($^{1}\underline{C}H_2$); 59.40 ($O–\underline{C}H_2–CH_2–N$); 54.75 [$N(\underline{C}H_3)_3$]; 26.3–25.6 ($=CH–\underline{C}H_2–CH=$); 34.36 ($^{1}CH_2–O–CO–\underline{C}H_2–CH_2$); 22.86 ($^{2}CH–O–CO–CH_2–\underline{C}H_2$); 21.11 ($^{1}CH_2–O–CO–\underline{C}H_3$); 20.97 ($=CH–\underline{C}H_2–CH_3$); 14.47 ($=CH–CH_2–\underline{C}H_3$).

1-DHA,2-acetyl-GPC (**2**): ^{1}H NMR: δ 5.44–5.32 (*m*, 12H, $C\underline{H}=C\underline{H}$); 5.25–5.20 (*m*, 1H, $^{2}C\underline{H}$); 4.40 (*dd*, 1H, *J* = 12.0 and 3.2 Hz, $^{1}C\underline{H}_2$); 4.40–4.25 (*m*, 2H, $O–C\underline{H}_2–CH_2–N$); 4.18 (*dd*, 1H, *J* = 12.0 and 7.0 Hz, $^{1}C\underline{H}_2$); 4.04–3.98 (*m*, 2H, $^{3}C\underline{H}_2$); 3.80–3.74 (*m*, 2H, $O–CH_2–C\underline{H}_2–N$); 3.35 [*s*, 9H, $N(C\underline{H}_3)_3$]; 2.89–2.81 (*m*, 10H, $=CH–C\underline{H}_2–CH=$); 2.42–2.37 (*m*, 4H, $O–CO–C\underline{H}_2–C\underline{H}_2–CH=$); 2.13–2.02 (*m*, 2H, $=CH–C\underline{H}_2–CH_3$); 2.09 (*s*, 3H, $CO–C\underline{H}_3$); 1.00 (*t*, 3H, *J* = 7.5, $=CH–CH_2–C\underline{H}_3$). ^{13}C NMR: δ 173.43 ($^{1}CH_2–O–\underline{C}O–CH_2–CH_2$); 170.78 ($^{2}CH–O–\underline{C}O–CH_3$); 133.0–127.4 ($\underline{C}H=\underline{C}H$); 70.89 ($^{2}\underline{C}H$); 66.78 ($O–CH_2–\underline{C}H_2–N$); 63.79 ($^{3}\underline{C}H_2$); 63.25 ($^{1}\underline{C}H_2$); 59.45 ($O–\underline{C}H_2–CH_2–N$); 54.75 [$N(\underline{C}H_3)_3$]; 26.3–25.6 ($=CH–\underline{C}H_2–CH=$); 34.30 ($^{1}CH_2–O–CO–\underline{C}H_2$); 22.81 ($^{1}CH_2–O–CO–CH_2–\underline{C}H_2$); 21.34 ($O–CO–\underline{C}H_3$); 21.07 ($=CH–\underline{C}H_2–CH_3$); 14.48 ($=CH–CH_2–\underline{C}H_3$).

The carbonyl carbon chemical shifts deserve some comments. The carbonyl resonance of the C-1 DHA chain in **2** (173.43) appears at a higher chemical shift than its C-2 DHA chain counterpart in **1** (172.81), the same relationship being observed with the acetyl group (171.13 in **1** > 170.78 in **2**). This cross comparison may be related to the observed carbonyl chemical shifts in saturated or unsaturated α and β chains of triglycerides, the carbonyl of the α chain having the higher chemical shift (19–21).

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