Synthesis of Phosphatidylcholine Having a Very Long Chain Polyunsaturated Fatty Acid

Syed Shabbir Haider, Masatoshi Tanaka,† Md Khorshed Alam, Shuhei Nakajima,† Naomichi Baba,*† and Sakayu Shimizu††
Graduate School of Natural Science and Technology, Okayama University, Tsushimanaka, Okayama 700
†*Faculty of Agriculture, Okayama University, Tsushimanaka, Okayama 700*
††*Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606*

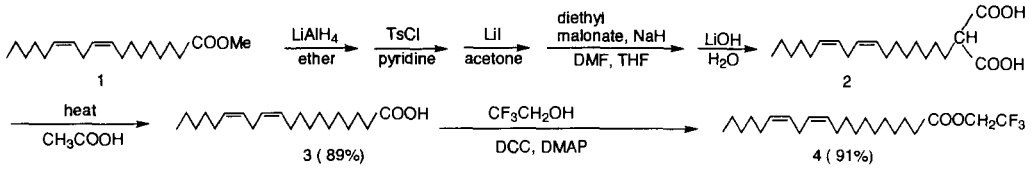
(Received October 6, 1997; CL-970773)

A phosphatidylcholine **9** bearing icosadienoyl and docosahexaenoyl groups at the 1- and 2-positions respectively was synthesized. The synthetic route involves carbon chain elongation of linoleic acid via malonic ester synthesis, preparation of lyso-phosphatidylcholine via lipase-catalyzed mono-acylation of 2-*O*-methoxyethoxymethylglycerol and phosphodiester synthesis, and finally DCC-mediated esterification.

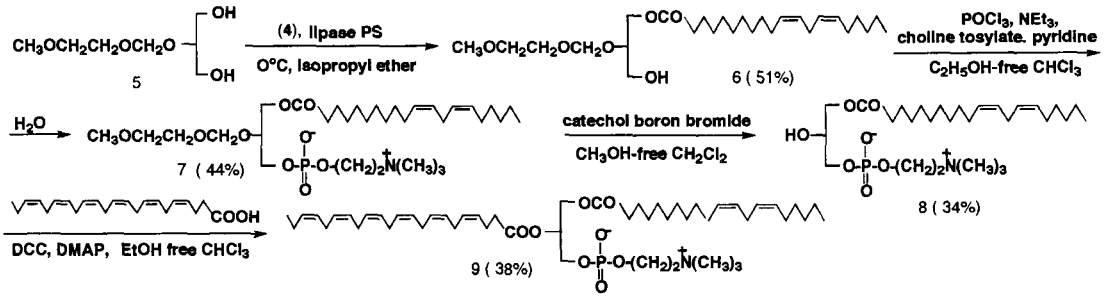
In recent years, polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and icosapentaenoic acid (EPA) have attracted wide interest because of their diverse but prominent biological functions. These fatty acids usually have carbon numbers of 18-22. On the other hand, a number of less common unsaturated fatty acids have been discovered whose carbon chain length are longer by 2-34 or more carbon atoms. These fatty acids are called very long chain fatty acids (VLCFA) and are found mostly as glycerophospholipids and sphingolipids in mature supermatozoa of a number of mammalian species, retina, human brain etc.[1] Also, VLCFA of n-6 series were reported to occur in rat seminiferous tubules.[2] Studies on their biological roles have just started and thus it is essential to develop synthetic methods for preparing these biomolecules since it is not easy to isolate them in a pure state and in a large amount from the complex mixtures of biological materials. This communication describes a convenient route to synthesize a model phosphatidylcholine, 1-*O*-icosadienoyl-2-*O*-docosahexaenoyl-*sn*-glycero-3-phosphocholine **9** having an elongated fatty acid, prepared from linoleic acid methyl ester, at 1-position and docosahexaenoyl group at 2-position.

Since such VLCFA as icosadienoic acid **3** is not available, we synthesized it for the first time as follows (Scheme 1). Methyl linoleate **1** was converted to a malonic acid derivative **2** via reduction, tosylation, iodination, conversion to a diethyl malonate derivative and hydrolysis with lithium hydroxide. The diacid **2** was subjected to decarboxylation without purification. The decarboxylation reaction was conducted in acetic acid medium under nitrogen atmosphere in the presence of butylated hydroxytoluene (BHT) since the usual acid catalyzed decarboxylation by heating might cause decomposition of the non-conjugated *cis*, *cis*-olefinic structure. After neutralization, the reaction mixture was extracted with ethyl acetate. The residue, obtained after drying and evaporation of the organic phase, was chromatographed on a silica gel column (hexane/EtOAc, 8:2) to afford the carboxylic acid **3** (yield 89%). A part of this product was esterified with diazomethane and analyzed by EIMS[3] and NMR[3] which confirmed the structural integrity. The methyl ester of **3** had a retention time (t_R) of 29.8 min in a gas chromatograph (column DB-1, 0.25 mm ϕ x 30 m, temp. 50-250 °C, prog. 10 °C / min), where as t_R of methyl linoleate was found to be 25.3 min under the same conditions. The acid **3** was converted to a trifluoroethyl ester **4** to activate the carboxylic acid group for lipase-catalyzed acylation.

In the next step, enzymatic mono-acylation of 2-*O*-methoxyethoxymethylglycerol **5**, which was prepared by the reaction of glycerol-1,3-diacetate with methoxyethoxymethyl chloride, was conducted using the ester **4** and lipase PS (Amano) in dry isopropyl ether (51% yield) (Scheme 2). In our previous study for the synthesis of phosphatidylcholine hydroperoxides,[4] we employed 2-*O*-benzylglycerol as a substrate and conducted mono-acylation with vinyl stearate. In the present case, however, the benzyl group could not be applied for protection since



Scheme 1.



Scheme 2.

removal of this group requires catalytic hydrogenolysis which will saturate the olefinic bonds of the fatty acyl moiety at 1-position. Thus, we introduced methoxyethoxymethyl (MEM) group as a protective function for 2-position instead of the benzyl group.

Phosphodiester synthesis of the monoacyl-MEM-glycerol **6** was conducted by a reported method [5] and purified by silica gel column chromatography ($CHCl_3/CH_3OH/28\%$ aq.NH_3, 65:35:5) to give the phosphatidylcholine **7** (yield 44%). To prepare the lysophosphatidylcholine **8** from **7**, MEM group should be removed, and Lewis acids such as titanium tetrachloride,[6] zinc bromide[6] and catechol boron bromide complex[7], which can be prepared by the reaction of boron tribromide and catechol in dry methylene chloride, were examined. Although the removal of the MEM group with the former two reagents afforded a complex mixture containing undesirable products, the last one was found to remove the group successfully as viewed by silica gel TLC ($CHCl_3$ / CH_3OH / 28% aq.NH_3, 65:35:10). After silica gel column purification ($CHCl_3$ / CH_3OH / 28% aq.NH_3, 65:35:10) the lyso-compound **8** was obtained in 34% yield. The structural integrity and purity were confirmed by ion spray mass spectrometry and ^{1}H-, ^{13}C- and ^{31}P-NMR data.[8]

As a final step, esterification of **8** was conducted with DHA activated by DCC with dimethylaminopyridine (10% of DCC) in ethanol-free chloroform at room temperature for 48 hours. After filtration and evaporation of the solvent, the residue was chromatographed on a silica gel column eluted with a mixture of $CHCl_3/CH_3OH/$ 28% aq.NH_3, 65:35:10 to afford the product **9** in 38% yield. The structural integrity was confirmed by ion spray mass spectrometry as well as ^{1}H-, ^{13}C- and ^{31}P-NMR data.[9]

The possibility of acyl migration from *sn*-1 to *sn*-2 position of the lyso-form **8** was ruled out by conducting enzymatic hydrolysis of **9** with phospholipase A_2 (from hog pancreas) in tris-maleate buffer of pH 7.4 following a reported procedure.[10] This 2-position specific enzyme liberated only DHA from the phospholipid **9** which was identified as its methyl ester by GC analysis.

For determination of optical purity and absolute configuration, the monoacylated glycerol **6** was converted to *sn*-2,3-di-*O*-MEM-glycerol via introduction of a second MEM group at 3-position by reacting with methoxyethoxymethyl chloride in CH_2Cl_2 and reductive cleavage of the ester group at 1-position with lithium aluminum hydride. The 2,3-di-*O*-MEM-glycerol thus obtained was found to have $[\alpha]_D$ =+0.78° in $CHCl_3$. The same compound derived from 1-*O*-linoleoyl-2-*O*-MEM-*sn*-glycerol following the same reaction sequences, however, showed $[\alpha]_D$ = +0.66° in $CHCl_3$ and an absolute configuration of *S*.[11] By a correlation using these data, the enatiomeric excess of **6** was found to be 98% with a stereochemical configuration of *S*. Since the chemical reactions from **6** to **9** are considered to be accompanied with no racemization, the final product **9** was assumed to have the same optical purity as that of **6** and *R*-configuration.

It is generally known that unconjugated all-*cis*-polyene structures are unstable and their first chemical change is radical-initiated autoxidation accompanied with conjugation of the double bonds as well as simultaneous *cis/trans* isomerization. In the present study, however, the signal pattern of the olefinic moiety in the ^{1}H- and ^{13}C-NMR spectrum of **9** [9] suggested that no such chemical change occurred during the course of synthesis as all the reactions were carried out under nitrogen atmosphere in the presence of BHT. In brief, a convenient synthetic route for the preparation of phosphatidylcholines containing polyunsaturated VLCFA at the 1-position has been developed in the present study. Based on this route, any type of polyunsaturated fatty acid can be incorporated at *sn*-1 or -2 positions to prepare optically active ester-type phospholipids.This is a superior point of our study over other reported methods.[12] Construction of such phospholipid homologs having natural occurrence is now in progress in our laboratory which may inspire an intense research on their biological functions.

We are grateful to Venture Business Laboratory and SC-NMR Laboratory of Okayama University for ^{1}H, ^{13}C and ^{31}P-NMR measurements.

References and Notes

1 A. Poulos, *Lipids* , **30**, 1 (1995).

2 M. I. Aveldano, B. S. Robinson, D. W. Johnson, and A. Poulos, *J. Biol. Chem.*, **268**, 11663 (1993).

3 Methyl ester of **3** : TLC (SiO_2), R_f = 0.76 (hexane / EtOAc = 8:2). ^{1}H-NMR ($CDCl_3$, 200 MHz, TMS) δ : 0.89 (3H, t, *J* = 6.9Hz, ω-CH_3), 1.20-1.40 (18H, m, C4, C5, C6, C7, C8, C9, C17, C18, C19-CH_2 x 9), 1.62 (2H, m, β-CH_2), 2.05 (4H, m, C10, C16-CH_2 x 2), 2.30 (2H, t, *J* =7.1 Hz, α-CH_2), 2.78 (2H, t, *J* = 6.6Hz, C13-CH_2), 3.67 (3H, s, -$COOCH_3$), 5.35 (4H, m, C11, C12, C14, C15-CH x 4). EIMS *m/z* : 322 (M^+), 291 (M^+- OCH_3).

4 N. Baba, K. Yoneda, S. Tahara, J. Iwasa, T. Kaneko, and M. Matsuo, *Chem. Soc., Chem. Commun.*, 1281 (1990).

5 M. Witzke and R. Bittman, *J. Lipid Res.*, **27**, 344 (1986); P. Guirisdalsky and R. Bittman, *Tetrahedron Lett.*, **29**, 4393 (1988).

6 J. Corey, J. Gras, and P. Ulrich, *Tetrahedron Lett.*, **17**, 809 (1976).

7 K. Boeckman and J. C. Potenza, *Tetrahedron Lett.*, **26**, 1411 (1985).

8 TLC (SiO_2), R_f = 0.19 ($CHCl_3$ / CH_3OH / 28% aq. NH_3, 65:35:10), Mo-blue positive. $[\alpha]_D$: -15.0° (c 0.38, $CHCl_3$, 25°C). ^{1}H-NMR ($CDCl_3$, 200 MHz, TMS) δ : 0.88 (3H, t, *J* = 7.1Hz, ω-CH_3), 1.20-1.38 (18H, m, 9 x CH_2), 1.55 (2H, m, CH_2CH_2COO), 2.05 (4H, m, 2 x $CH=CHCH_2$), 2.28 (2H, t, *J* = 8.0Hz, CH_2CH_2COO), 2.75 (2H, t, *J* = 6.2Hz, $CH=CHCH_2CH=CH$), 3.29 (9H, s, $N(CH_3)_3$), 3.75 (2H, br, $POCH_2CH_2N$), 3.88 (1H, br, CH(OH)), 3.93 (2H, br, CH_2O-icosadienoyl), 4.05 (2H, br, CH_2OP), 4.30 (2H, br, $POCH_2CH_2N$), 5.35 (4H, m, 2 x CH=CH). ^{13}C-NMR ($CDCl_3$, 50.3 MHz, TMS) δ : 13.9, 21.0, 22.4, 24.7, 25.4, 27.2, 29.2, 30.1, 31.3, 34.0, 54.1, 59.3, 64.9, 65.9, 67.0, 68.5, 125.3, 127.8, 128.0, 129.9, 173.7. ^{31}P-NMR ($CDCl_3$, 81.0 MHz, 85% H_3PO_4 as an ext. std.) δ : 0.32. Ion Spray MS *m/z* : 548.4 (MH^+).

9 TLC (SiO_2), R_f = 0.45 ($CHCl_3$ / CH_3OH / 28% aq.NH_3, 65:35:10), Mo-blue positive. $[\alpha]_D$: -0.59° (c 1.70, $CHCl_3$, 25°C). ^{1}H-NMR ($CDCl_3$, 500 MHz, TMS) δ : 0.87 (3H, t, *J* = 5.2Hz, ω-CH_3 of icosadienoyl), 0.96 (3H, t, *J* = 5.2Hz, ω-CH_3 of docosahexaenoyl), 1.20-1.40 (18H, m, 9 x CH_2), 1.56 (2H, m, $CH_2CH_2CH_2COO$), 2.05 (8H, m, 4 x $CH=CHCH_2$), 2.27 (2H, m, $CH_2CH_2CH_2COO$), 2.32 (2H, m, $CHCH_2CH_2COO$), 2.80 (12H, m, 6 x $CH=CHCH_2CH=CH$), 3.32 (9H, s, $N(CH_3)_3$), 3.78 (2H, br, $POCH_2CH_2N$), 3.93 (2H, m, CH_2O-icosadienoyl), 4.10 (1H, m, CH_2OP), 4.32 (2H, br, $POCH_2CH_2N$), 4.36 (1H, m, CH_2OP), 5.19 (1H, br, OCH_2CH(O-docosahexaenoyl)CH_2OP). 5.35 (16H, m, 8 x CH=CH). ^{13}C-NMR ($CDCl_3$, 50.3 MHz, TMS) δ : 14.1, 14.3, 20.5, 22.4, 24.7, 25.6, 27.2, 29.3, 29.5, 29.7, 30.3, 31.5, 33.9, 54.5, 59.3, 62.9, 63.5, 66.4, 70.5, 125.5, 127.0, 128.0, 128.3, 128.6, 129.3, 130.2, 132.0, 172.6, 173.6. ^{31}P-NMR ($CDCl_3$, 81.0 MHz, 85% H_3PO_4 as ext. std.) δ : 0.80. Ion Spray MS *m/z* : 858.6 (MH^+).

10 N. Baba, Y. Mikami, Y. Shigeta, S. Nakajima, T. Kaneko, and M. Matsuo, *Biosci. Biotech. Biochem.*, **57**, 2200 (1993).

11 N. Baba, T. Akiyama, S. Tahara, and S. Nakajima, *Biosci. Biotech. Biochem.*, **59**, 353 (1995).

12 E. Baer, *J. Amer. Oil Chem. Soc.*, **42**, 257 (1965) and references sited there in; F.Paltauf, *Chem. Phys. Lipids*, **11**, 270 (1973) and references sited there in; R. G. Jensen and D. T. Gordon, *Lipids* , **7**, 611 (1972); D. W. Hopper, J. G. Catalano, and T. L. Macdonald, *Tetrahedron Lett.*, **37**, 7871 (1996).