PERKIN 1 COMMUNICATION

A first synthesis of a phosphatidylcholine bearing docosahexaenoic and tetracosahexaenoic acids

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A phosphatidylcholine bearing tetracosahexaenoic [24:6 (*n*-3)] and docosahexaenoic acid [22:6 (*n*-3), DHA] at the 1- and 2-positions, respectively, was synthesized for the first time by a new method.

A series of glycerophospholipids in which docosahexaenoic acid (DHA) and a very long chain polyunsaturated fatty acid are linked to the glycerol moiety has been discovered from bovine retina by Aveldano and co-workers.[1] These lipids are associated with rhodopsin which is an essential protein for visual functions, and the lipids themselves are also very important for these functions. The mechanism of the physiological function remains unclear and ample quantity of such phospholipids for biological studies should be provided by chemical synthesis rather than isolation from complex mixtures of biological materials. Very long chain polyunsaturated fatty acids in these phospholipids constitute a series of longer chain homologs of *n*-3 hexaenoic acid whose carbon numbers range from 24 to 36. The shortest fatty acid among them is therefore longer than DHA by two carbons. Thus, the structure of the simplest phosphatidylcholine (PC) is **11** in Scheme 1.

In general, synthesis of glycerophospholipids having different polyunsaturated fatty acyl groups at the 1- and 2-positions of the glycerol moiety is difficult and only a very few reports on this have been published.[2] In particular, enantiomers having different stereochemical configuration as well as different polyunsaturated fatty acyl groups at the *sn*-1- and *sn*-2-position are more difficult to prepare. According to Scheme 1, the present method constitutes two parts, *i.e.*, two-carbon elongation of DHA ethyl ester **1** to give an acid **5** and synthesis of lysophosphatidylcholine **10** starting from lipase-catalyzed mono-acylation of 2-*O*-TBDMS-glycerol **7** followed by the introduction of choline phosphate and removal of the silyl group. In the usual route to lysophosphatidylcholine, the hydroxy group at the 2-position of the glycerol moiety is protected as a benzyl ether for regioselective introduction of an acyl group at the 1-position. The benzyl group can be removed only by catalytic hydrogenolysis. In the present case, however, hydrogenolysis cannot be applied after acylation at the 1-position with unsaturated fatty acyl group because of the inevitable saturation of the olefinic bonds. Therefore, the protective group should be one that can be removed under non-hydrogenolytic conditions. In addition, the conditions of cleavage should be so mild that the unstable all-*cis* nonconjugated polyolefinic structure remains intact without autoxidation, partial conjugation or *cis*/*trans* isomerization. To overcome this problem, we previously employed 2-*O*-MEM-glycerol as a substrate for lipase-catalyzed mono-acylation instead of 2-*O*-benzylglycerol for the synthesis of 2-*O*-docosahexaenoyl-1-*O*-eicosadienoylphosphatidylcholine in which eicosadienoic acid [22:2 (*n*-6)] was derived from linoleic acid.[2b] However, this approach proved to be inappropriate when the longer chain polyunsaturated fatty acid was derived from DHA because the MEM group was considerably hydrophilic and its derivative was so difficult to free from moisture that column chromatography and drying took a long time rendering partial decomposition of the unstable non-conjugated all *cis*-olefinic bond structure in the DHA. Moisture and impurities remaining in the sample sometimes prevented the recording of clear spectral data for the molecule. As a result, synthesis of our desired target compound, 2-*O*-DHA-1-*O*-tetracosahexaenoylphosphatidylcholine (**11**), in which the long chain fatty acid [24:6 (*n*-3)] was derived from DHA, was unsuccessful. Therefore, in the present study, we employed the *tert*-butyldimethylsilyl group as a much more non-polar candidate instead of the MEM group. Thus, 2-*O*-TBDMS-glycerol **7** was prepared according to a reported method[3] and submitted to the mono-acylation using the trifluoroethyl ester of **5** in the presence of lipase PS (Amano) in diisopropyl ether. The reaction was stopped simply by filtering out the enzyme before 1,3-diacyl product formation.[4] All the reactions involving the polyunsaturated moiety in the present synthesis were conducted in the presence of a trace amount of butylated hydroxytoluene (BHT) as an antioxidant and in a nitrogen atmosphere in the dark to prevent autoxidation of the olefins.

Carbon chain elongation of DHA was performed according to our reported method.[2b] Briefly, DHA ethyl ester was converted to an iodide **2** *via* reduction with $LiAlH_4$, conversion to a tosylate, and iodination with LiI. Diethyl malonate was then introduced to the iodide and, after hydrolysis of the diester with LiOH, decarboxylation was conducted in a mixture of distilled THF and CH_3COOH with concentrated HCl (0.0001%) at 90 °C for 48 h. After evaporation of the solvent under reduced pressure and silica gel column purification, the two-carbon elongated tetracosahexaenoic acid (**5**) was obtained and converted to a trifluoroethyl ester **6**.[5] The trifluoroethyl group activates the carbonyl group in the ester for nucleophilic attack by the electron withdrawing nature of the fluorine atom. This activated carbonyl group may produce an activated ester with a functional group at the active site of the lipase PS rendering effective nucleophilic attack by one of the hydroxy groups of 2-*O*-TBDMS-glycerol **7**. Thus, the lipase PS-catalyzed reaction of **6** and **7** in diisopropyl ether afforded **8**.[6]

The monoacyl glycerol **8** was submitted to phosphodiester synthesis under the usual conditions using $POCl_3$ to afford **9**.[7] Usual work-up of the reaction mixture involving extraction of the product with chloroform is very often accompanied by emulsification which makes separation difficult. However, this problem was solved by using a mixture of chloroform–methanol (2:1). Thus, no emulsification occurred and easy

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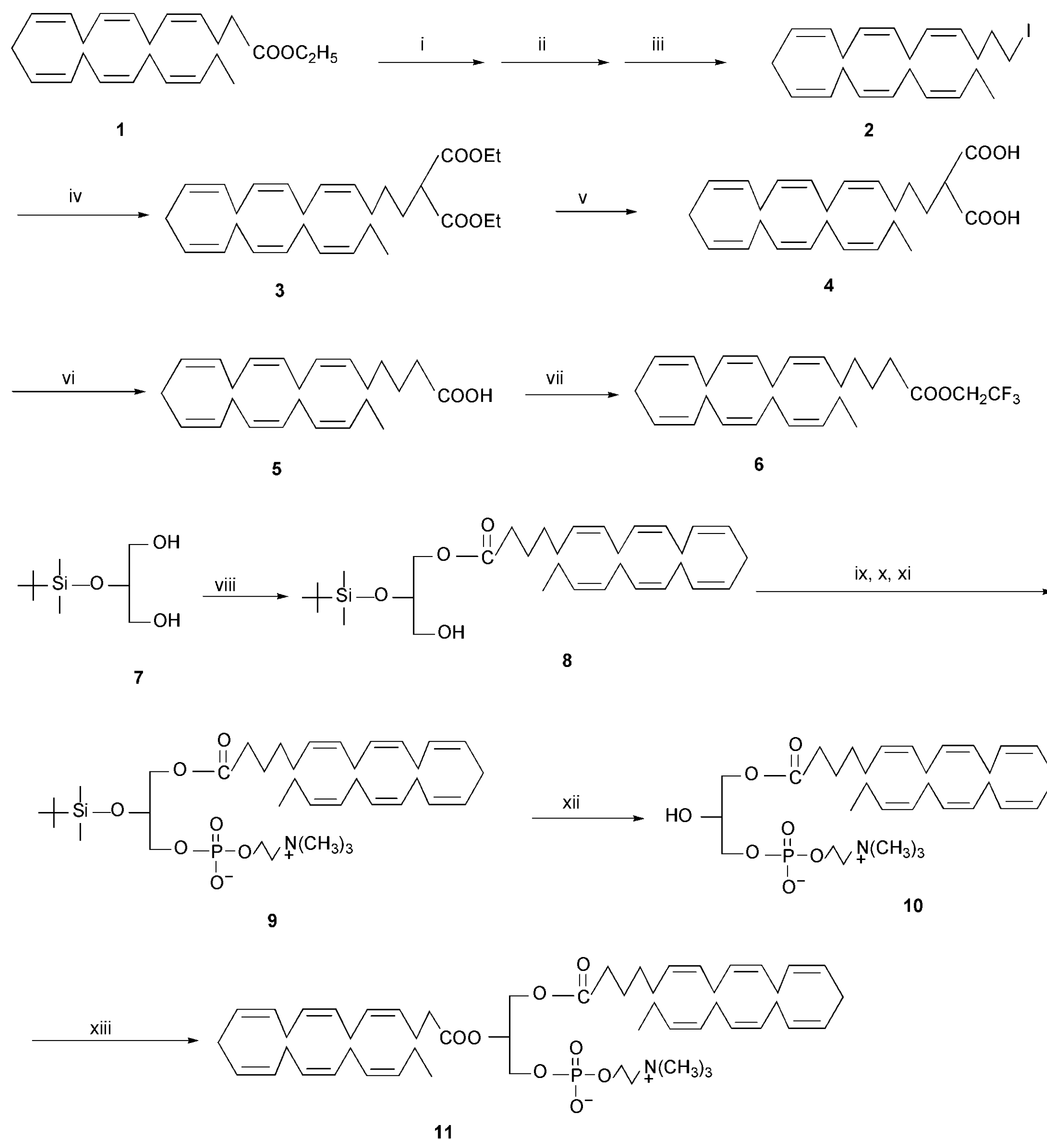

Scheme 1 *Reagents*: i. $LiAlH_4$; ii. A toluene-*p*-sulfonyl chloride; iii. LiI; iv. diethyl malonate, NaH in DMF–THF (1 : 1); v. 2 M LiOH (aq.); vi. 0.0001% HCl in AcOH, 90 °C, 74 h; vii. CF_3COOH, DCC, DMAP; viii. **6**, lipase PS (Amano), 0 °C; ix. $POCl_3$, Et_3N; x. choline tosylate, pyridine; xi. H_2O; xii. CH_3COOH–THF–H_2O; xiii. DHA, DCC, DMAP in EtOH-free $CHCl_3$.

separation of the aqueous and organic phase was possible. After evaporation of the extraction solvent, the residue was chromatographed on silica gel eluted with CH_3OH–$CHCl_3$–28% aq. NH_3 (30 : 60 : 5), giving **9**. Treatment of **9** with a solution of CH_3COOH in THF, followed by silica gel chromatographic isolation of the product afforded the lysophosphatidylcholine **10**.[8] As a final step, the lyso-form was esterified with DHA activated with DCC–DMAP in ethanol-free chloroform to afford the final compound **11**[9] in 36% yield from **10**. Proton NMR and electrospray mass spectral analyses confirmed the structural integrity. The ^{1}H NMR spectrum in the olefinic region showed that no autoxidation occurred and the non-conjugated all *cis*-olefins are completely intact. This product showed optical activity of $[a]_D^{rt}$ −0.5 (*c* 2, $CHCl_3$). This chirality was derived from **8** that was given by the stereoselective lipase-catalyzed mono-acylation of the diol **7**, which afforded the optically active monoester **8** {$[a]_D^{rt}$ −2.7 (*c* 22.5, $CHCl_3$)}. Although the stereochemistry of the asymmetric centre, including the configuration and optical purity, have not been determined yet, it was presumed to be *S* since it has been recognized that lipases including, lipase PS (Amano) produced by *Pseudomonas* species[4,10] exhibit *S*-enantioselectivity almost unexceptionally regardless of the structural differences in the the 2-*O*-substituent, or differences in the acyl group to be esterified. On the other hand, the configuration and optical purity of the natural product **11** obtained from the retina have not been reported. Therefore, we have to wait for the determination of the stereochemistry of the natural product or synthesize both enantiomers separately and test their activity for the visual function of retina in combination with rhodopsin. The mono-acyl form **8** with opposite configuration may be obtained *via* lipase-catalyzed hydrolytic mode of the 1,3-diester of the TBDMS-glycerol **7** with tetracosahexaenoic acid (**5**).[10b,11]

The synthetic route in the present study may serve as a potential source of phosphatidylcholines bearing DHA [22 : 6 (*n*-3)] and tetracosahexaenoic acid [24 : 6 (*n*-3)] for physiological studies. The present method may also be applicable to the synthesis of phosphatidylcholines having other longer homologs also identified in bovine retina.

Experimental

Lipase-catalyzed acylation of 2-*O*-TBDMS-glycerol 7

To a solution of **6** (450 mg, 1.03 mmol) and **7** (335 mg, 1.62 mmol) in diisopropyl ether (10.6 ml) was added lipase PS (450 mg, 13500 units, a gift from Amano Pharmaceutical Co., Ltd. Nagoya, Japan), and the solution was stirred at 0 °C for 3 h under N_2 in the presence of trace BHT. After filtering the enzyme through Celite, the filtrate was concentrated and the residue was chromatographed on silica gel, eluted with hexane–

ethyl acetate (9:1→8:2), giving **8** (225 mg, yield 40%). The characterization data are given in ref. 6.

Introduction of choline phosphate into 8

To phosphoric trichloride (26.3 ml) in nitrogen atmosphere was added a solution of **8** (100 mg, 0.18 mmol) and Et_3N (33.0 ml) in ethanol-free distilled $CHCl_3$ (2.0 ml) at 0 °C in the presence of a trace of BHT and the solution was stirred for 0.5 h at this temperature, then, at room temperature for an additional 1 h. Dry choline tosylate (100 mg) and freshly distilled pyridine (75 ml) were added successively, and the reaction mixture was stirred at room temperature for 48 h. The reaction mixture was poured into a mixture of $CHCl_3$–methanol (2:1) to extract the product into the organic phase. The extract was concentrated and the residue was chromatographed on silica gel eluted with CH_3OH–$CHCl_3$–28% aq. NH_3 (30:60:5) giving **9**. The characterization data are given in ref. 7.

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5 *Tetracosahexaenoate* (**5**): ^1H NMR (500 MHz, $CDCl_3$): δ 0.95 (t, $J = 7.8$, 3H, C24-CH_3), 1.26 (m, 2H, C4-CH_2), 1.65 (dd, $J = 7.1$, 7.1, 2H, C3-CH_2), 2.05 (m, 4H, C5, C23-CH_2), 2.35 (m, 2H, C2-CH_2), 2.83 (m, 10H, C8, C11, C14, C17, C20-CH_2), 5.35 (m, 12H, all olefin protons).

6 *2-O-tert-Butyldimethylsilyl-1-tetracosa (all Z)-6,9,12,15,18,21-hexaenoyl-sn-glycerol* (**8**): ^1H NMR (500 MHz, $CDCl_3$): δ 0.08 (s, 6H, $Si(CH_3)_2$), 0.85 (s, 9H, $C(CH_3)_3$), 0.95 (t, $J = 7.8$, 3H, C24-CH_3), 1.40 (m, 2H, C4-CH_2), 1.63 (dd, $J = 7.1$, 7.1, 2H, C3-CH_2), 2.05 (m, 4H, C5, C23-CH_2), 2.33 (m, 2H, C2-CH_2), 2.83 (m, 10H, C8, C11, C14, C17, C20-CH_2), 3.58–4.10 (m, 5H, protons on glycerol structure), 5.35 (m, 12H, all olefin protons). Found (ES) *m*/*z*: (M + H^+) 545.4; $C_{33}H_{56}O_4Si$ requires: 544.9. $[\alpha]_D^{rt}$ −2.7 (*c* 22.5, $CHCl_3$). Yield 25%.

7 *2-O-tert-Butyldimethylsilyl-1-tetracosa (all Z)-6,9,12,15,18,21-hexaenoyl-sn-glycerophosphocholine* (**9**): ^1H NMR (500 MHz, $CDCl_3$): δ 0.08 (s, 6H, $Si(CH_3)_2$), 0.85 (s, 9H, $N(CH_3)_3$), 0.95 (t, $J = 7.8$, 3H, C24-CH_3), 1.40 (m, 2H, C4-CH_2), 1.62 (dd, $J = 7.1$, 7.1, 2H, C3-CH_2), 2.05 (m, 4H, C5, C23-CH_2), 2.30 (m, 2H, C2-CH_2), 2.85 (m, 10H, C8, C11, C14, C17, C20-CH_2), 3.35 (s, 9H, $N(CH_3)_3$), 3.78 (br, 2H, OCH_2CH_2N), 3.80–4.10 (m, 5H, protons on glycerol structure), 4.30 (br, 2H, OCH_2CH_2N), 5.25 (m, 12H, all olefin protons). Found (ES) *m*/*z*: (M + H^+) 711.1; $C_{38}H_{68}NO_7PSi$ requires: 710.1. $[\alpha]_D^{rt}$ −0.12 (*c* 6.0, $CHCl_3$). Yield 46%.

8 *1-Tetracosa (all Z)-6,9,12,15,18,21-hexaenoyl-sn-lysophosphocholine* (**10**): ^1H NMR (500 MHz, $CDCl_3$): δ 0.95 (t, $J = 7.8$, 3H, C24-CH_3), 1.40 (m, 2H, C4-CH_2), 1.60 (dd, $J = 7.1$, 7.1, 2H, C3-CH_2), 2.05 (m, 4H, C5, C23-CH_2), 2.30 (m, 2H, C2 and C2′-CH_2), 2.85 (m, 10H, C8, C11, C14, C17, C20, C6′, C9′, C12′, C15′, C18′-CH_2), 3.30 (s, 9H, $N(CH_3)_3$), 3.75 (br, 2H, O CH_2CH_2N), 3.80–4.40 (m, 5H, protons on glycerol structure), 4.25 (br, 2H, OCH_2CH_2N), 5.30 (m, 12H, all olefin protons). Found (ES) *m*/*z*: (M + H^+): 596.4; $C_{32}H_{54}NO_7P$ requires: 595.4. $[\alpha]_D^{rt}$ −0.05 (*c* 4.5, $CHCl_3$). Yield 92%.

9 *2-Docosahexaenoyl-1-tetracosa (all Z)-6,9,12,15,18,21-hexaenoyl-sn-glycerophosphocholine*: (**11**): ^1H NMR (500 MHz, $CDCl_3$): δ 0.90 (t, $J = 7.8$, 6H, C24-CH_3 and C22′-CH_3), 1.35 (m, 2H, C4-CH_2), 1.55 (dd, $J = 7.1$, 7.1, 2H, C3-CH_2), 2.00 (m, 8H, C3-CH_2), 2.25 (m, 4H, C2 and C2′-CH_2), 2.75 (m, 10H, C5, C11, C14, C17, C20, C6′, C9′, C12′, C15′, C18′-CH_2), 3.30 (s, 9H, $N(CH_3)_3$), 3.75 (br, 2H, OCH_2CH_2N), 3.80–4.40 (m, 5H, protons on glycerol structure), 4.25 (br, 2H, OCH_2CH_2N), 5.30 (m, 24H, all olefin protons). Found (ES) *m*/*z*: (M + H^+) 906.6; $C_{54}H_{84}NO_8P$ requires: 905.6. $[\alpha]_D^{rt}$ −0.5 (*c* 2, $CHCl_3$). Yield 36%.

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