A new approach to phospholipid synthesis using tetrahydropyranyl glycerol: rapid access to phosphatidic acid and phosphatidylcholine, including mixed-chain glycerophospholipid derivatives

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Received 17th March 2006, Accepted 8th May 2006
First published as an Advance Article on the web 17th May 2006
DOI: 10.1039/b603788g

A new synthesis of phosphatidic acid and phosphatidylcholine is reported, relying on the preparation of 3-tetrahydropyranyl-*sn*-glycerol as the key intermediate for sequential introduction of the primary and secondary acyl functions to produce chiral diglycerides that are phosphorylated to obtain the target phospholipid compounds.

Development of new synthetic methods for the preparation of biologically active phospholipid compounds is one of the most important problems of membrane-chemistry and biochemistry today.[1] Specifically, while phospholipids have long been known as constituents of membrane bilayers,[2] they were recently recognized as essential molecules for regulation of cell functions by hormones, neurotransmitters, growth factors and inflammatory cytokines.[3] Membrane glycerophospholipids are precursors of lipid metabolites with second messenger functions, serving as substrates for phospholipases, lipid kinases and phosphatases that generate signaling lipid molecules.[3,4] Elucidation of the mechanistic details involved in the enzymological, cell-biological, and membrane-biophysical roles of phospholipids remains to be accomplished, and it depends on the availability of efficient synthetic methods for the preparation of structurally variable phospholipid compounds.[3–5] The synthetic compounds are required for the establishment of structure–activity relationships with respect to phospholipid–phospholipid and phospholipid–protein interactions,[6] as well as for mechanistic studies of phospholipid metabolizing enzymes.[7]

Despite their apparent structural simplicity the synthesis of mixed-chain 1,2-diacyl-3-phosphoglycerols has been a challenging task that involves: 1) regioselective incorporation of three different substituents at the three glycerol positions that normally requires the use of multiple protecting groups, and 2) development of conditions that allow deprotection of the hydroxyl groups and subsequent acylation/phosphorylation while preventing acyl migration in the course of introducing the desired substituents.[8] We now report an efficient general route to phosphatidylcholine[2] and phosphatidic acid,[9] that should be applicable to the preparation of a wide range of structurally related glycerophospholipid derivatives as well. The key features of the sequence rely on tetrahydropyranylation of the incipient *sn*-3-glycerol position that allows regioselective monoacylation at the *sn*-1-primary hydroxyl group, followed by introduction of the secondary ester substituent. Subsequent acid-catalyzed deprotection of the *sn*-3-glycerol function is achieved using dilute hydrochloric acid in a mixture of chloroform–methanol solution under experimental conditions where *acyl migration is prevented.*[10] Finally, elaboration of the phospholipid headgroup is accomplished using phosphorylation methods suitable for the preparation of phosphomonoester and phosphodiester functions, respectively. The usefulness of the method is illustrated by the preparation of a double-labeled mixed-chain phosphatidylcholine, carrying chain-terminal fluorescent reporter groups applicable for spectroscopic (FRET) studies of lipolytic enzymes.[11]

Significantly, the sequence became possible because we discovered, that isopropylidene protection of glycerol can be selectively cleaved using bismuth(III) triflate[12] as catalyst in aqueous tetrahydrofuran, while under the same reaction conditions the other acid-labile tetrahydropyranyl protecting group remains essentially unaffected. Thus, we were able to prepare the key intermediate of the sequence **3**, from commercially available 1,2-*O*-isopropylidene-3-*sn*-glycerol **1** rapidly, in two steps (Scheme 1).

Reaction of D-α,β-*O*-isopropylidene glycerol **1** with twofold excess of dihydropyran in CH_2Cl_2 in the presence of pyridinium *p*-toluenesulfonate as catalyst at room temperature for 2 h yielded compound **2**, which was isolated by extraction from a mixture of benzene–water as a colorless oil (92%). The product **2** was treated with 0.3 equiv. bismuth(III) triflate tetrahydrate in THF–H_2O (4 : 1) at room temperature for 1 h to give the tetrahydropyranyl glycerol **3**. It was purified by silica gel chromatography with $CHCl_3$–EtOAc (1 : 1), freeze-dried from benzene and obtained as a colorless oil (in 76% yield).

This intermediate **3** was used for the synthesis of both symmetric- and mixed-chain phospholipids including phosphatidic acid and phosphatidylcholine. Thus, selective acylation of compound **3** at the primary *sn*-1-glycerol position was achieved using a threefold excess of diol **3** in reaction with 10-(7′-mercapto-4′-methylcoumarin)decanoic acid[8d]–DCC in the presence of a catalytic amount of DMAP in chloroform at room temperature for 20 h. The resulting fluorescent *sn*-1-ester **4** was chromatographed on a silica gel column with $CHCl_3$–EtOAc (4 : 1) as eluant, then freeze-dried from benzene to give a white solid (79%). The regioisomeric purity of compound **4** could readily be ascertained by high-field ^{1}H NMR spectroscopy. Specifically, the ^{1}H NMR spectrum of the product **4** shows baseline resonance in the δ 5.00–5.09 range indicating that there is no *sn*-2-ester group in the molecule,[13] such that acylation occurred at the *sn*-1-position with complete regioselectivity. In the next step the incipient *sn*-2-substituent of the mixed-chain phospholipid was introduced by treatment of compound **4** with 1.2 equiv. 12-(2′-naphthylacetyl)aminododecanoic acid–DCC in chloroform in the presence of catalytic amount of DMAP at room temperature

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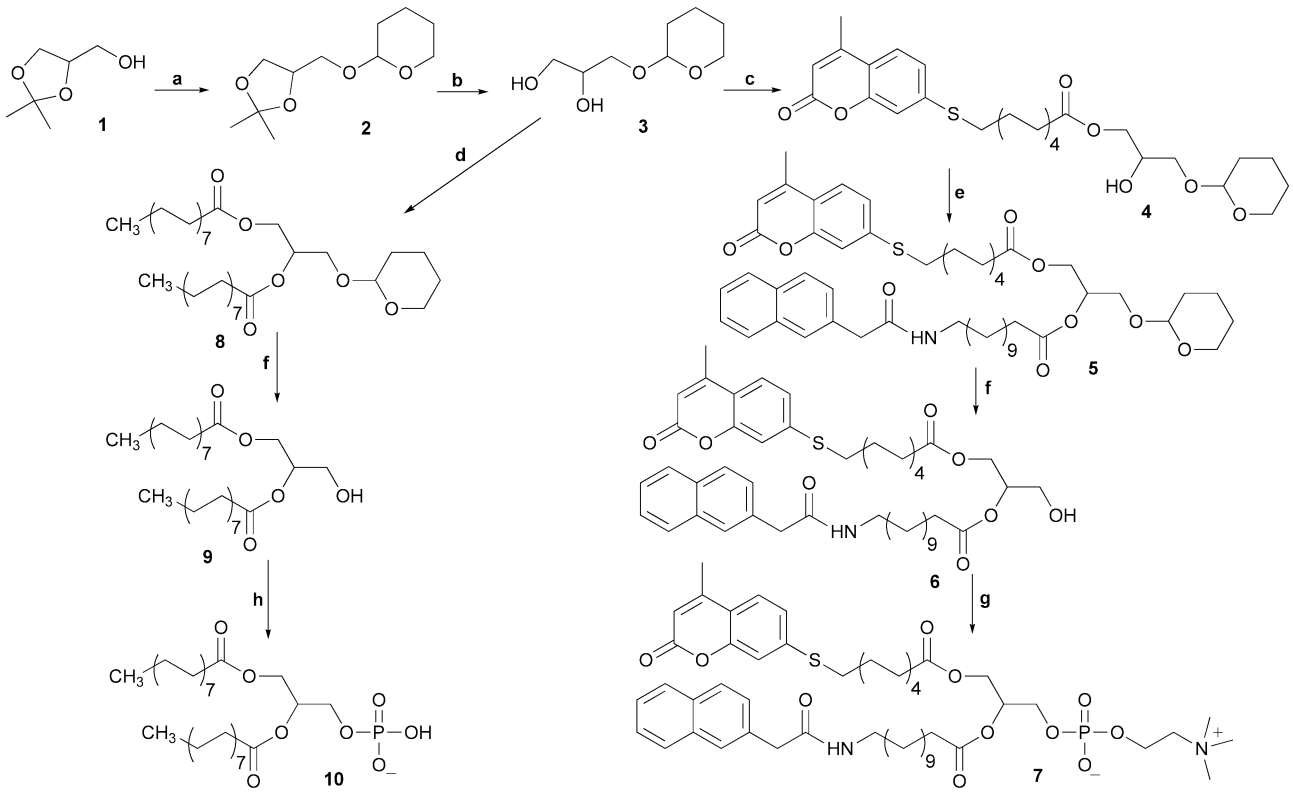

Scheme 1 *Reagents and conditions*: (a) DHP, PPTS, CH_2Cl_2; (b) 0.3 equiv. $Bi(OTf)_3$, THF–H_2O (4 : 1), rt, 1 h; (c) 10-(4′-methyl-7′-mercapto-coumarin)dodecanoic acid–DCC–DMAP, $CHCl_3$; (d) 2.2 equiv. palmitic acid–DCC–DMAP, $CHCl_3$; (e) 12-(2′-naphthylacetyl)aminododecanoic acid–DCC–DMAP, $CHCl_3$; (f) 0.15 M HCl, $CHCl_3$–MeOH, 2 h; (g) (i) ethylene chlorophospate, Et_3N, benzene, (ii) $(CH_3)_3N$, MeCN, 65 °C; (h) (i) $iPr_2P(OCH_2CH_2CN)_2$, tetrazole, $CHCl_3$–MeCN (3 : 1), (ii) 30% aq. H_2O_2–CH_2Cl_2, (iii) 0.2 M DBU, toluene, 110 °C, 16 h.

for 72 h. The diacyl compound **5** was purified by silica gel chromatography using $CHCl_3$–EtOAc (4 : 1), freeze-dried from benzene and isolated as a white powder in 85% yield. Deprotection of the *sn*-3-hydroxyl group was achieved in 0.15 M HCl in chloroform-methanol (1 : 1, 83% yield).[10]

For elaboration of the phosphodiester headgroup, compound **6** was allowed to react with 2-chloro-2-oxo-1,3,2-dioxaphospholane–triethylamine in anhydrous benzene, followed by treatment of the phosphorylated intermediate with trimethylamine in acetonitrile at 65 °C (in a pressure bottle) for 48 h. The product was purified by silica gel chromatography ($CHCl_3$–MeOH–H_2O 65 : 25 : 4) and freeze-dried from benzene to give the target phospholipid **7** as a yellow solid (54%).[14–16]

Preparation of symmetric-chain phospholipids from *sn*-3-tetrahydropyranyl glycerol **3** is illustrated by synthesis of 1,2-dipalmitoylphosphatidic acid **10**. Thus, compound **3** was treated with 2.2 equiv. palmitic acid–DCC and 20 mol% DMAP in chloroform at room temperature for 12 h. The dipalmitoyl product **8** was chromatographed on silica gel with $CHCl_3$ as eluant, and isolated as an analytically pure white solid in 79% yield. Acid-catalyzed deprotection of the *sn*-3-alcohol function in 0.15 M HCl in chloroform–methanol (1 : 1) as above, gave the product **9** in 93% yield.

Compound **9** was phosphorylated by treatment with bis(β-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite in the presence of tetrazole in $CHCl_3$–MeCN (3 : 1), at room temperature for 48 h (51%), followed by oxidation of the phosphite intermediate in a biphasic mixture of 30% aq. H_2O_2–CH_2Cl_2 in 85% yield. Finally, base catalyzed elimination of the cyanoethyl protecting groups with 2.5 equiv. DBU in refluxing toluene overnight gave the phosphomonoester **10** in 70% yield.[17]

In conclusion, the synthesis here reported provides a facile and efficient method for the preparation of a wide range of diacylglycerols and phospholipids, including phosphatidic acid, symmetric- and mixed-chain phosphatidylcholines. The strength of the method is in its flexibility with respect to the substituent groups that can be introduced, and in its applicability to the development of new phospholipid analogues with desired target structures for biochemical and membrane-biophysical studies. Work along these lines is under way in our laboratory.

Acknowledgements

We are grateful to the National Institutes of Health, grant S06 GM/HD48680, for financial support. J.H. is pleased to acknowledge support by the College of Science and Mathematics, and the Research and Grants Committee/ORSP of California State University, Northridge.

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16 The two fluorophores incorporated at the chain-terminals of compound **7** comprise a donor–acceptor pair suitable for fluorescence resonance energy transfer (FRET) studies, including real time monitoring of phospholipase A_2 activity,[11] since the emission peak of the 2-naphthylacetyl group (λ_{max} = 340 nm) shows substantial overlap with the excitation spectrum of 7-mercapto-4-methylcoumarin group (λ_{max} = 335 nm).
17 All new compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, HRMS and elemental analysis. *Selected data*: Compound **3**: IR ($CHCl_3$): 3330 br, 2857 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.49–1.92 (br m, 6H, 3C**H$_2$**), 3.27 (m, 1H, CH_2C**H**H(OCH)), 3.53 (m, 3H, CHC**H$_2$**O and CH_2CH**H**(OCH)), 3.82 (m, 1H, CH_2C**H**(CH_2)OH), 3.99 (m, 2H, C**H$_2$**OH), 4.87 (br m, 1H, OC**H**$(OCH_2)CH_2$). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 19.15 (CH_2**C**H_2CH_2), 26.74 (CH_2**C**H_2CH_2), 37.35 (CH**C**H_2CH_2), 62.07 (O**C**H_2CH_2), 67.06 (O**C**H_2CH), 73.46 (CH**C**H_2O), 87.42 (CH_2**C**H(O)CH_2), 111.66 (O**C**H(O)CH_2). R_f ($CHCl_3$–EtOAc, 1 : 1) 0.23. Anal. Calcd for $C_8H_{16}O_4$: C, 54.53; H, 9.15; Found: C, 54.43; H, 9.49%; MS MH$^+$ $C_8H_{16}O_4H$ Calcd: 177.1127, Found: 177.1121. Compound **4**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.28 (m, 10H, 5C**H$_2$**), 1.42–1.72 (br m, 10H, 5C**H$_2$**), 2.29–2.47 (br m, 5H, CH_2C**H$_2$**C=O and C**H$_3$**), 2.82 (br m, 1H, C**H**H), 2.96 (t, 2H, J = 6Hz, SC**H$_2$**), 3.55 (m, 2H, C**H$_2$**O), 3.66 (d, 2H, J = 5 Hz, C**H$_2$**CH), 3.94 (m, 1H, CH**H**), 4.14 (d, 2H, J = 5 Hz, OC**H$_2$**CH), 4.55 (m, 1H, CH_2C**H**CH_2), 6.18 (s, 1H, C**H**), 7.10–7.14 (m, 2H, C**H**-aromatic), 7.44 (d, 1H, J = 8.8 Hz, C**H**-aromatic). R_f ($CHCl_3$–EtOAc 4 : 1) 0.41. Anal. Calcd for $C_{28}H_{40}O_7S$: C, 64.59; H, 7.74; Found: C, 64.26; H, 7.58%; MS MH$^+$ $C_{28}H_{40}O_7SH$ Calcd: 521.2573, Found: 521.2595. Compound **5**: Anal. Calcd for $C_{52}H_{71}NO_9S{\cdot}H_2O$: C, 69.07; H, 8.14; Found: C, 68.99; H, 8.79%; MS MNa$^+$ $C_{52}H_{71}NO_9SNa$ Calcd: 908.4742, Found: 908.4789. Compound **6**: IR ($CHCl_3$): 3330, 1737 br, 1685 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.25 (m, 24H, 12C**H$_2$**), 1.61 (m, 8H, 4C**H$_2$**), 2.37 (m, 7H, 2C**H$_2$**C(O) and C**H$_3$**), 2.97 (t, 2H, J = 6.8 Hz, SC**H$_2$**), 3.20 (m, 2H, NHC**H$_2$**), 3.73 (s, 2H, CC**H$_2$**CO), 4.15 (m, 2H, OC**H$_2$**CH), 5.07 (m, 2H, OC**H$_2$**CH), 5.60 (m, 1H, CH_2C**H**(O)CH_2), 6.19 (s, 1H, C**H**), 7.12–7.15 (m, 2H, C**H**-aromatic), 7.21–7.85 (m, 8H, C**H**-aromatic). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 18.26 (**C**H$_3$), 23.21 (**C**H$_2$), 26.32 (**C**H$_2$), 27.75 (**C**H$_2$), 28.89 (**C**H$_2$), 29.14 (**C**H$_2$), 29.24 (**C**H$_2$), 29.34 (**C**H$_2$), 29.65 (**C**H$_2$), 29.99 (**C**H$_2$), 32.38 (**C**H$_2$), 33.87 (**C**H$_2$), 39.53 (**S**CH$_2$), 42.37 (NH**C**H$_2$), 62.07 (CH**C**H$_2$OH), 63.79 (O**C**H$_2$CH), 74.75 (CH_2**C**H(O)CH_2), 110.75 (C**C**HC), 113.09 (**C**-aromatic), 122.56 (**C**-aromatic), 124.54 (**C**-aromatic), 125.89 (**C**-aromatic), 126.51 (**C**-aromatic), 127.32 (**C**-aromatic), 127.47 (**C**-aromatic), 128.02 (**C**H-aromatic), 128.34 (**C**-aromatic), 128.57 (**C**-aromatic), 143.73 (**C**-aromatic), 152.41 (**C**-aromatic), 154.75 (**C**-aromatic), 160.41 (O**C**=O), 169.71 (NH**C**=O), 173.04 (O**C**=O), 173.37 (O**C**=O). R_f ($CHCl_3$–EtOAc 4 : 1) 0.54. Anal. Calcd for $C_{47}H_{63}NO_8S{\cdot}\frac{1}{2}H_2O$: C, 69.60; H, 7.95; N, 1.73; Found: C, 69.83; H, 8.22; N, 2.17%. MS MNa$^+$ $C_{47}H_{63}NO_8SNa$ Calcd: 824.4167, Found: 824.4187. $[\alpha]_D^{20}$ +9.2 (*c* 1.14, $CHCl_3$–MeOH 3 : 2). Compound **7**: R_f ($CHCl_3$–MeOH–H_2O 65 : 25 : 4) 0.42. Anal. Calcd for $C_{52}H_{75}N_2O_{11}PS{\cdot}3H_2O$: C, 61.16; H, 7.99; N, 2.74; Found: C, 61.28; H, 7.47; N, 2.42%. MS MH$^+$ $C_{52}H_{75}N_2O_{11}PSH$ Calcd: 967.4902, Found: 967.4928. $[\alpha]_D^{20}$ +5.7 (*c* 1.14, $CHCl_3$–MeOH 4 : 1). Compound **8**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.87 (br t, 6H, 2C**H$_3$**), 1.25 (m, 44H, 22C**H$_2$**), 1.61 (m, 14H, 7C**H$_2$**), 2.31 (br t, 4H, $2CH_2CH_2$C**H$_2$**C=O), 3.52 (m, 2H, OC**H$_2$**CH_2), 3.77 (m, 2H, CHC**H$_2$**O), 4.18 (m, 1H, OC**H**HCH), 4.35 (m, 1H, OCH**H**CH), 4.61 (m, 1H, OC**H**$(OCH_2)CH_2$), 5.22 (m, 1H, CH_2C**H**$(OC{=}O)CH_2$). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 14.05 (**C**H$_3$), 18.94 (**C**H$_3$), 22.64 (**C**H$_2$),24.85 (**C**H$_2$), 24.93 (**C**H$_2$), 25.31 (**C**H$_2$), 29.04 (**C**H$_2$), 29.08 (**C**H$_2$), 29.25 (**C**H$_2$), 29.31 (**C**H$_2$), 29.44 (**C**H$_2$), 29.61 (**C**H$_2$), 29.65 (**C**H$_2$), 30.2 (**C**H$_2$), 30.25 (**C**H$_2$), 31.87 (**C**H$_2$), 34.08 (CH_2**C**H_2C=O), 34.29 (CH_2**C**H_2C=O), 61.75 (O**C**H_2CH_2), 62.68 (CH**C**H_2O), 65.30 (O**C**H_2CH), 70.11 (CH_2**C**H(OC=O)CH_2), 98.71 (CH_2O**C**H$CH_2(OCH_2)$, 173.00 (CH_2**C**=O), 173.37 (CH_2**C**=O). R_f ($CHCl_3$) 0.47. Anal. Calcd for $C_{40}H_{76}O_6$: C, 73.57; H, 11.73; Found: C, 73.18; H, 11.72%; MS MNa$^+$ $C_{40}H_{76}O_6Na$ Calcd: 675.5534, Found: 675.5550.

