Pergamon

Tetrahedron Letters, Vol. 38, No. 8, pp. 1369-1370, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00082-8

Enzymatic Introduction of N-Heterocyclic and As-Containing Head Groups into Glycerophospholipids

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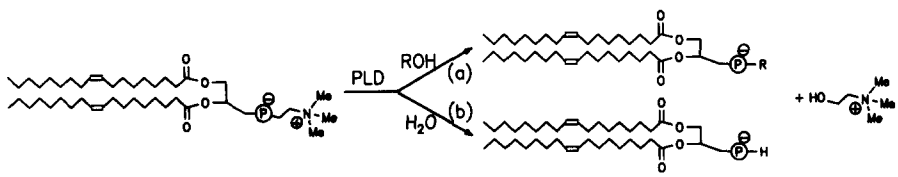
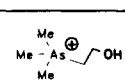
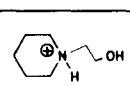
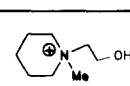
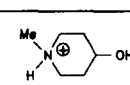
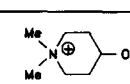
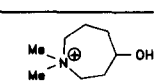
Abstract: The synthesis of six new glycerophospholipids with choline-analogous head groups by enzymatic transphosphatidylation with phospholipase D is described. Starting from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, primary or secondary N-heterocyclic alcohols and (2-hydroxyethyl) trimethylarsonium were substituted for choline in two-phase systems.

The great interest in natural and artificial phospholipids results from their importance as surfactants and as building units for biological membranes and liposomes as well as from their biological action in cell signalling. A large variety of strategies and experimental procedures for the synthesis of phospholipids have been elaborated.[1,2] One method is the transformation of phospholipids in the polar head group region via enzymatic transphosphatidylation (route (a)) by phospholipase D (PLD)[1,3-5] as shown in the scheme for 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. Generally, this reaction competes with the hydrolysis (route (b)) of the phosphatidylcholine (PC) to phosphatidic acid (PA).



Since the resulting product of transphosphatidylation is potentially also a substrate for the PLD, the product formation is kinetically controlled. In the present work we used PLD from cabbage prepared as described elsewhere[6] and from *Streptomyces* sp. (PLD Type VII, Sigma, Germany) to synthesize six new glycerophospholipids bearing the As-analogue of choline or N-heterocyclic alcohols as head groups. In 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DioPC), choline was replaced by (2-hydroxyethyl) trimethylarsonium **(1)**, N-(2-hydroxyethyl)-piperidine **(2)**, N-(2-hydroxyethyl)-N-methylpiperidine **(3)**, 4-hydroxy-N-methylpiperidine **(4)**, 4-hydroxy-N,N-dimethylpiperidine **(5)**, and 4-hydroxy-N,N-dimethylazepane **(6)** (**Table 1**).

Table 1. New 1,2-dioleoyl-*sn*-glycero-3-phospholipids

Acceptor alcohol (ROH)						
Product abbreviation	DioP(**1**)	DioP(**2**)	DioP(**3**)	DioP(**4**)	DioP(**5**)	DioP(**6**)
Product yield (%)[x]	15 [a] / 78 [d]	37 [c] / 87 [c]	46 [d] / 87 [d]	11 [b] / 51 [e]	37 [b] / 73 [e]	18 [b] / 18 [e]

[x] by use of PLD from cabbage / PLD from *Streptomyces* sp.
The optimum reaction time was: [a] 1.25, [b] 2.7, [c] 4, [d] 7, and [e] 20 hours.

The reactions were performed in a diethyl ether/buffer emulsion system. The composition of a typical reaction mixture was 6200 µl diethyl ether, 30 µmol DioPC (98.0 %, Lipoid KG, Germany), 300 µmol of the

respective acceptor alcohol (all were gifts from ASTA Medica, Germany) diluted from an aqueous stock solution (pH 5.6), 0.33 U PLD from cabbage or 0.013 U PLD from *Streptomyces* sp., and 800 µl 100 mM acetate buffer, 40 mM $CaCl_2$, pH 5.6. The activity of the enzymes was determined as described elsewhere.[6] The reaction mixtures were shaken at 300 min^{-1} at 25°C. After phase separation the organic layer was evaporated to dryness on a centrifugal evaporator (Jouan, France). The course of reaction was followed by thin layer chromatography on HPTLC plates (silica gel 60, Merck, Germany).[6] The plates were developed by (a) chloroform/methanol/water (65:25:2, v/v) or (b) the same solvent system under the addititon of 50 µl glacial acetic acid per ml in a horizontal chamber (Camag, Switzerland)[6] at 50-52 % relative humidity (R_f-values in the ***Appendix***). The product yields in the reaction mixtures are shown in **Table 1**.

For purification 30-50 mg of the dried reaction components were dissolved in 1 ml of chloroform and applied to a HPLC column Eurospher Si 100, 10 µm, 0.8 x 25 cm^2 (Knauer, Germany).[6] The products were obtained after the evaporation of the collected peak fractions and were homogenous in HPTLC (>95 %). The structure of the products was evaluated by ^{1}H NMR and MALDI-mass spectrometry (MALDI-MS). The ^{1}H NMR measurements were performed in $CDCl_3$ on a Bruker ARX 500 spectrometer working at 500.13 MHz for protons (***Appendix***). For MALDI-MS a reflectron-type time-of-flight mass spectrometer Reflex™ (Bruker-Franzen, Germany) was used applying a saturated solution of α-cyano-4-hydroxy cinnamic acid in acetone as matrix. The maximum difference between the calculated and the measured values was 0.24 $g \cdot mol^{-1}$.

The described syntheses show that the synthetic potential of PLD from cabbage is larger as generally assumed. Even secondary alcohols proved to be reactive in the enzymatic reaction, whereas hitherto only examples for transphosphatidylations with primary alcohols have been reported.[3, 5, 7] In our experiments the microbial PLD, however, mostly resulted in higher yields of the transphosphatidylation products than the cabbage enzyme. The synthesized compounds represent a series of analogues of DioPC with certain differences in the head group structure and polarity. Therefore, they should be suitable for systematic investigation on the phase behaviour and on the biological action of phospholipids.

The financial support by ASTA Medica AG, Frankfurt, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Bonn, and the Studienstiftung des deutschen Volkes is gratefully acknowledged.

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Appendix

DioP(1): ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm = 0.88 (t, 6 H, $C\underline{H}_3$-$C_{17}H_{32}$-); 1.22-1.36 (m, 40 H, -$(C\underline{H}_2)_n$-); 1.58 (m, 4 H, -$C\underline{H}_2$- acyl C-3); 2.0 (m, 8 H, -$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-); 2.09 (s, 9 H, $(C\underline{H}_3)_3As^+$-); 2.28 (2 t, 4 H, -$C\underline{H}_2$- acyl C-2); 2.89 (t, 2H, -$C\underline{H}_2$-As-); 3.95 (m, 2 H, CH_2OR-CHOR-$C\underline{H}_2$-O-PO_2-); 4.13 (dd, 1 H, $H^1C\underline{H}^2$OR-CHOR-CH_2-O-PO_2-); 4.28 (m, 2 H, -PO_2-O-$C\underline{H}_2$-); 4.39 (dd, 1 H, $H^1C\underline{H}^2$OR-CHOR-CH_2-O-PO_2-); 5.21 (m, 1H, CH_2OR-$C\underline{H}$OR-CH_2-O-PO_2-); 5.34 (m, 4 H, -$C\underline{H}$=$C\underline{H}$-), R_{fa} = 0.16, R_{fb} = 0.21

DioP(2), DioP(3), DioP(4), DioP(5), DioP(6) ^{1}H NMR (500 MHz, $CDCl_3$), δ/ppm, head group protons only

DioP(2): 1.21-1.36 (m, 40+2 H, -$(C\underline{H}_2)_n$-, 2 rp); 1.7 (m, 2 H, rp); 1.85 (b, 1 H, rp); 1.9-2.1 (m, 8+1 H, -$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-, 1 rp); 2.6 (m, 2 H, rp, or); 3.11 (m, 2 H, rp, or); 3.6 (m, 2 H, -PO_2-O-$CH_2C\underline{H}_2$-); 4.2 (m, 2 H, -PO_2-O-$C\underline{H}_2$-), R_{fa} = 0.63, R_{fb} = 0.69
DioP(3): 1.72 (m, 2 H, rp); 1.93 (m, 4 H, rp); 3.36 (s, 3 H, $C\underline{H}_3$-N^+); 3.52 (m, 2 H, rp, or); 3.68 (m, 2 H, rp, or); 3.83 (m, 2 H, -PO_2-O-$CH_2C\underline{H}_2$-); 4.36 (m, 2 H, -PO_2-O-$C\underline{H}_2$-), R_{fa} = 0.12, R_{fb} = 0.19
DioP(4): 1.63 (b, 4 H, rp, me); 2.72 (s, 3 H, $C\underline{H}_3$-N^+); 3.2 (b, 2 H, rp, or); 3.5 (b, 1 H, rp, or); 4.28 (m, 1 H, rp, or); 4.5 (b, 1 H, rp, pa), R_{fa} = 0.16, R_{fb} = 0.21
DioP(5): 2.19 (b, 4 H, rp, me); 3.23+3.28 (2 s, 6 H, $(C\underline{H}_3)_2$-N^+-); 3.55 (b, 2 H, rp, or); 3.68 (b, 2 H, rp, or); 4.5 (b, 1 H, rp), R_{fa} = 0.06, R_{fb} = 0.08
DioP(6): 1.85 (m, 2 H, rp); 2.08 (m, 2 H, rp); 2.2 (b, 1 H, rp); 2.47 (b, 1 H, rp); 3.29+3.3 (2 s, 6 H, $(C\underline{H}_3)_2$-N^+-); 3.38 (m, 1 H, rp); 3.55 (m, 1 H, rp); 3.9 (m, 1 H, rp); 4.0 (m, 1 H, rp); 4.53 (b, 1 H, rp, pa), R_{fa} = 0.05, R_{fb} = 0.07

s - singlet, d - doublet, t - triplet, m - multiplet, b - broad signal, or - ortho, me - meta, pa - para, rp - ring proton(s)

DioPC: R_{fa} = 0.09, R_{fb} = 0.14 **DioPA:** R_{fa} = 0.19, R_{fb} = 0.62

(*Received in Germany* 15 *November* 1996; *revised* 13 *January* 1997; *accepted* 14 *January* 1997)