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The chemical synthesis and cytotoxicity of new sulfur analogues of 2-methoxy-lysophosphatidylcholine

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ABSTRACT

The chemical synthesis of phosphorothioate/phosphorodithioate analogues of 2-methoxy-lysophosphatidylcholine has been described. For the preparation of new sulfur derivatives of lysophosphatidylcholine both oxathiaphospholane and dithiaphospholane approaches have been employed. Each lysophospholipid analogue was synthesized as a series of five compounds, bearing different fatty acid residues both saturated (12:0, 14:0, 16:0, 18:0) and unsaturated (18:1). The methylation of glycerol 2-hydroxyl function was applied in order to increase the stability of prepared analogues by preventing 1→2 acyl migration. The cellular toxicity of newly synthesized 2-methoxy-lysophosphatidylcholine derivatives was measured using MTT viability assay and lactate dehydrogenase release method.

For many years lysophospholipids (LPL) have been considered mainly as 'structural and storage components without informational functions'. However, more recent studies have demonstrated that some lysophospholipids are intercellular lipid mediators acting as hormone-like signaling molecules. They can activate specific membrane receptors and/or nuclear receptors and in this way regulate many important physiological and pathophysiological processes.[1] The LPL have been shown to be involved in many cellular processes and diseases such as diabetes, obesity, atherosclerosis and cancer. Several lysophospholipids were identified under in vivo conditions including lysophosphatidic acid (LPA), cyclic phosphatidic acid (cPA), lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS) and lysophosphatidylthreonine (LPT).[1] There is a growing evidence that various lysophospholipids are ligands activating membrane receptors coupled to G proteins (GPCRs). However, the LPL receptors and corresponding signaling pathways have not been recognized so far. Lysophospholipids need to be further characterized with regard to diversity of their structures and specific biological activities. These studies will require the use of LPL analogs with well-defined fatty acid residues and various polar head groups.

One of the most important natural lysophospholipid is 1-*O*-acyl-*sn*-glycerol-3-*O*-phosphocholine (lysophosphatidylcholine, LPC, **1**).[2–5] LPC has a zwitterionic structure (see Fig. 1) and is the most abundant lysophospholipid in animals and plants.

Natural LPC exists in optically active form (R enantiomer), as a mixture of several compounds, possessing fatty acids of different length and saturation status (saturated, monounsaturated, polyunsaturated). Although LPC is only a minor phospholipid present in cell membranes (<3%), it is the most abundant LPL with relatively high (around 150 μM) concentration in human blood.[5] Most of the circulating LPC molecules are associated with albumin. LPC is also a major phospholipid component of oxidized low-density lipoproteins. LPC present in plasma is derived from phosphatidylcholine by lecithin–cholesterol acyltransferase that catalyzes the transacylation of the *sn*-2 fatty acid residue of lecithin to the free cholesterol, resulting in a formation of cholesterol ester and LPC formation.[6] LPC is generated by membrane phosphatidylcholine digestion with phospholipases A_2 (PLA_2) and phospholipases A_1 (PLA_1) that are able to cleave the *sn*-1 and *sn*-2 ester bond, respectively.[7,8] Appreciable amounts of LPC are also formed in plasma by the action of endothelial lipase.[5]

LPC is a proinflammatory, proatherogenic substance and plays an important role in the initiation, progression and destabilization of atherosclerotic plaques. It induces differentiation and morphological changes of various cells and plays important role in tumor progression. Recent reports have shown that LPC induces insulin secretion from pancreatic β-cells.[2] It has been also found that LPC activates glucose uptake and effectively lowers blood glucose levels in mouse models of type 1 and 2 diabetes.[3,4] However, to date the precise mechanism of this phenomenon is obscure. On the other hand, oleoyl-lysophosphatidylcholine (OLPC) was reported to bind to one of membrane receptors known as GPR119 that causes intracellular cAMP accumulation and an

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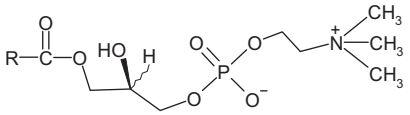


Figure 1. The structure of natural R-lysophosphatidylcholine (LPC, **1**). Substituents R–C(O)– refer to various fatty acid residues present in lysophospholipids.

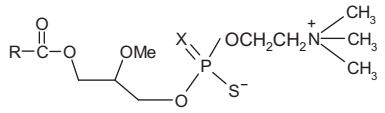
increase of glucose stimulated insulin secretion in NIT-1 insulinoma cells.[4] Nevertheless, the nature of the endogenous ligands of GPR119 and its physiological role in direct regulation of insulin secretion by the pancreatic β cells still have to be explained.[5]

In our previous publication we have described the chemical synthesis of sulfur analogues of LPA and cPA, in which either one or two nonbridging phosphate oxygen atoms are substituted by sulfur to give phosphorothioate or phosphorodithioate analogues, respectively.[9] Intriguing biological properties of natural LPC prompted us to undertake studies on the synthesis of its sulfur analogues modified in the same manner. On the basis of our earlier studies performed on the synthesis of phosphorothioate and phosphorodithioate derivatives of nucleotides and oligonucleotides[10] we expected that lysophospholipid sulfur analogues should have similar physicochemical properties as natural lysophospholipids, yet should be more resistant towards hydrolytic enzymes.[11] In order to prevent possible 1→2 acyl migration in LPC analogues, the oxygen atom in position 2 of glycerol was protected by methylation.[9,12–14] The conditions of chemical synthesis enabled us also to obtain LPC sulfur analogues as homogenic compounds, each containing only one fatty acid residue. Thus, LPC sulfur analogues, both phosphorothioates (**2a**–**e**) and phosphorodithioates (**3a**–**e**), were prepared as a series of five different compounds, bearing the residues of the following fatty acid: (a) lauric (12:0), (b) myristic (14:0), (c) palmitic (16:0), (d) stearic (18:0), (e) oleic (18:1) (see Fig. 2).

The diversity of biological functions and activity of lysophosphatidylcholine allowed us to assume that phosphorothioate/phosphorodithioate derivatives **2** and **3** may also have interesting biological or even therapeutic properties. For preliminary biological studies the phosphorothioates (**2a**–**e**) and phosphorodithioates (**3a**–**e**) were synthesized from racemic glycidol.

The chemical synthesis of 1-acyl-2-methoxyglycerols (**4a**–**e**), that were crucial building blocks for the preparation of **2a**–**e** and **3a**–**e**, was performed exactly as described in our previous paper (see Scheme 1).[9]

Thus, all five aforementioned fatty acids were reacted with commercially available racemic glycidol (**5**) in the presence of catalytic amounts of *n*-tributylamine into 1-acylglycerols (**6a**–**e**) according to the procedure described by Lok et al.[15] In the following step 1-acylglycerols were regioselectively silylated with *t*-butyldimethylsilyl chloride in the position 3 of glycerol (primary hydroxyl group).[16–18] The silyl ethers (**7a**–**e**) were then methylated at the central oxygen of glycerol with trimethylsilyldiazomethane in the presence of 40% fluoroboric acid.[19] The 2-methoxy ethers



LPC sulfur analogues **2,3**

2a-e X = O phosphorothioates **3a-e** X = S phosphorodithioates

a - lauroyl **b** - myristoyl **c** - palmitoyl **d** - stearoyl **e** - oleoyl

Figure 2. The structure of LPC 2-methoxy-sulfur analogues (phosphorothioates **2a**–**e** and phosphorodithioates **3a**-**e**).

(**8a**–**e**) were desilylated with tetra-*n*-butylammoniumfluoride in tetrahydrofuran, yielding desired glyceride building blocks **4a**–**e**.[16–18] All aforementioned products were isolated and purified by silica gel flash column chromatography and characterized by spectroscopic methods as described earlier.[9]

The introduction of one or two nonbridging sulfur atoms into lysophosphatitydylcholine molecule was performed using oxathiaphospholane or dithiaphospholane approach, respectively. These methods were successfully employed for the synthesis of phosphorothioate/phosphorodithioate nucleotide and oligonucleotide analogues of phosphodiester type (see Scheme 2 and the Supplementary data).[10]

The application of aforementioned approach to the synthesis of sulfur analogues of 2-methoxy-lysophosphatidylcholine **2** and **3** is shown on Scheme 3.

For the synthesis of phosphorothioate analogues racemic 1-acyl-2-methoxyglycerols **4a**–**e** were reacted in anh. dichloromethane solution with equimolar amount of 2-*N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane in the presence of *S*-ethylthiotetrazole, and then with elemental sulfur, to give 3-*O*-(2-thio-1,3,2-oxathiaphospholane) lipid derivatives **9a**–**e** (X = O), each showing in ^{31}P NMR two signals at δ ca. 105 ppm. Such chemical shifts were earlier described as characteristic for various 2-alkoxy-2-thio-1,3,2-oxathiaphospholane derivatives.[10a] Compounds **9a**–**e** were isolated by silica gel flash column chromatography as pale-yellow oils in 54–78% yield, and characterized by spectroscopic methods. Due to appearance of two signals in ^{31}P NMR spectra it was concluded that each of **9a**–**e** is a mixture of two stereoisomers not separable by chromatography (TLC).

Each of oxathiaphospholane derivatives **9a**–**e** was then treated in anh. dichloromethane with 3 mol equiv of choline *p*-toluenesulfonate[20] and 2 mol equiv of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The ring opening condensation was accompanied by spontaneous elimination of episulfide.

Crude products were purified by silica gel flash column chromatography to give 2-methoxy-LPC phosphorothioates (**2a**–**e**) as white solids in 50–60% yield. The products were obtained as a mixture of stereoisomers (two signals in ^{31}P NMR at δ ca. 59 ppm), which could not be separated by chromatography (column or TLC). Such chemical shifts are characteristic for dialkyl phosphorothioates.[10a] The purified products **2a**–**e** were characterized by spectroscopic methods (^{1}H and ^{31}P NMR, MALDI TOF MS). They were soluble in chloroform, methanol and water, and did not decompose when stored at −20 °C. The details are given in Supplementary data.

The presence of two signals in ^{31}P NMR spectra of each **2a**–**e** (and also **9a**–**e**) can be attributed to the presence of two centers of asymmetry (at C2 and at phosphorus), leading to the formation of two pairs of diastereoisomers. Unfortunately, unlike the previously phosphorothioate cPA analogues,[9] these stereoisomers could not be separated by either column chromatography or thin layer chromatography.

For the synthesis of phosphorothioate analogues racemic 1-acyl-2-methoxyglycerols **4a**–**e** containing one of the aforementioned fatty acid residues were reacted in anh. dichloromethane solution with 2-*N*,*N*-diisopropylamino-1,3,2-dithiaphospholane in the presence of *S*-ethylthiotetrazole, and then with elemental sulfur, to give 3-*O*-(2-thio-1,3,2- dithiaphospholane) lipid derivatives **10a**–**e** (X = S), showing in ^{31}P NMR single signals at δ ca. 123 ppm). Such chemical shifts were earlier described as characteristic for various 2-alkoxy-2-thio-1,3,2-dithiaphospholane derivatives.[10b,g] Pure compounds **10a**–**e** were isolated by silica gel flash column chromatography as pale-yellow oils in 39–50% yield, and characterized by spectroscopic methods.

Each of dithiaphospholane derivatives **10a**–**e** was then treated in anh. dichloromethane with 3 mol equiv of choline