Enantioselective Responses to a Phosphorothioate Analogue of Lysophosphatidic Acid with LPA_3 Receptor-Selective Agonist Activity

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Abstract: The metabolically stabilized LPA analogue, 1-oleoyl-2-*O*-methyl-*rac*-glycerophosphothioate (OMPT), is a potent agonist for the LPA_3 G-protein-coupled receptor. A new enantiospecific synthesis of both (2*R*)-OMPT and (2*S*)-OMPT is described. Calcium release assays in both LPA_3-transfected insect Sf9 and rat hepatoma Rh7777 cells showed that (2*S*)-OMPT was 5- to 20-fold more active than (2*R*)-OMPT. Similar results were found for calcium release, MAPK and Akt activation, and IL-6 release in human OVCAR3 ovarian cancer cells.

Introduction. Lysophosphatidic acid (LPA, 1- or 2-*O*-acyl-*sn*-glycero-3-phosphate) is a deceptively simple intercellular signaling phospholipid with a wide variety of biologic effects.[1,2] LPA induces cell proliferation and morphological changes and has been shown to be involved in many physiological and pathological processes including neurogenesis,[3] myelination, angiogenesis,[4] wound healing,[5] ischemia-reperfusion,[6] and cancer progression.[7−9] As a result, the attention has turned to the development of modulators of LPA signaling as potential new therapeutic agents.[10]

Although four mammalian genes (LPA_1/Edg-2, LPA_2/Edg-4, LPA_3/Edg-7, and LPA_4/p29y/G*P*23) encoding high-affinity LPA receptors have been cloned and characterized,[11,12] the function of particular receptors in the mammalian system and the molecular mechanism of LPA actions are still subjects of intensive investigation.[13] Until recently, the relative paucity of molecular tools, e.g., metabolically stable and selective agonists and antagonists for LPA receptors, has constrained progress.[14] Recently, two highly potent agonists for the LPA_3 receptor have been identified: [1-fluoro-3-(*S*)-hydroxyl-4-(oleoyloxy)butyl]phosphonate (XY-17), an α-fluoromethelyene phosphonate analogue,[15] and OMPT, an *O*-methylated thiophosphate analogue[16] (Figure 1).

Racemic OMPT at concentrations below 100 nM triggered cellular responses through the LPA_3, but not the LPA_1 or LPA_2 receptors.[16] Since the LPA_3 receptor may play a role in feedback inhibition of activity at LPA_1 and LPA_2 receptors,[9] LPA_3 selective agonists could be used to selectively decrease LPA_1 and LPA_2 mediated responses, thus preventing the abnormal cell growth and differentiation that occurs in cancer.

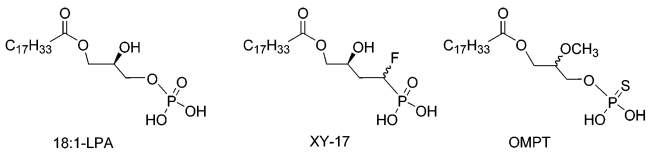

Figure 1. Structures of naturally occurring LPA and *rac*-OMPT.

Ligand recognition by G-protein-coupled receptors (GPCR), as well as substrate recognition by enzymes, generally shows a preference for the naturally occurring enantiomer over the unnatural one. However, recognition of LPA by its receptors can be viewed as an exception, as both the natural L (*R*) and unnatural D (*S*) stereoisomers of LPA are equally active in some bioassays.[17] In contrast to the enantiomers of natural LPA, however, the activities of LPA analogues based on nonglycerol backbones show strong enantioselectivity. For example, analogues of *N*-acyl-serine phosphoric acid (NASPA), *N*-acyl-ethanolamine phosphoric acid (NAEPA), and *N*-acyl-2-benzyl-4-oxybenzyl-ethanolamine phosphoric acids (D-VPC12204) and L-VPC12249), which contain a serine or an ethanolamine backbone in place of glycerol, are recognized in a stereoselective manner.[18−20] This duality of responses has been recently reviewed.[17,21] Since the stereochemically restricted interaction appears to be distinct between the $LPA_{1/2/3}$ receptors, it may lead to the development of receptor subtype-selective antagonists.

The first synthesis of OMPT involved selective protection of *sn*-1 and *sn*-3 hydroxyl groups, introduction of *sn*-2 *O*-methyl under strong basic conditions, and finally construction of the LPA backbone by selective deprotection, phosphorylation, and esterification.[16] Herein we describe a streamlined route that provides the first enantioselective synthesis of both OMPT enantiomers, and we illustrate enantioselective biological responses in LPA-receptor-transfected insect and mammalian cell lines as well as in human ovarian cancer cells.

Results and Discussion. The synthesis of (2*R*)-OMPT began with the phosphorylation of (2*S*)-solketal (**1**) using phosphoramidite methodology (Scheme 1).[22] The resulting phosphite triester was oxidized with elemental sulfur to yield the corresponding phosphorothioate triester (**2**). Under acidic conditions, the acetonide was removed to provide the diol intermediate **3**. Initial attempts to selectively acylate the primary alcohol using DCC-promoted esterification at 4 °C gave disappointing yields. However, selective acylation was readily accomplished with oleoyl chloride in dichloromethane in the presence of 2,6-lutidine at −78 °C[23] (2 h, 45% yield); substituting 2,4,6-collidine as the base further increased the yield to 87% with no loss of regioselectivity. This approach avoids the cumbersome (and acyl-migration susceptible) selective protection/deprotection methods to achieve *sn*-1 *O*-acylation. Next,

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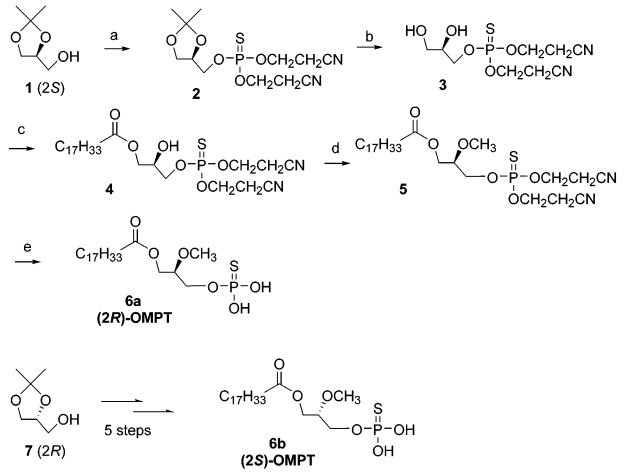
Scheme 1. Synthesis of OMPT Enantiomers[a]



[a] Reagents: (a) $(CNCH_2CH_2O)_2PN(^iPr)_2$, 1*H*-tetrazole, S, CS_2/pyridine, 86%; (b) *p*-TsOH, MeOH, 67%; (c) oleoyl chloride, 2,4,6-collidine, -78 °C, 87%; (d) $TMSCHN_2$, HBF_4, 58%; (e) *t*-$BuNH_2$, BTMSA, 84%.

the *O*-methylation of the secondary *sn*-2 hydroxyl in **4** was achieved using trimethylsilyldiazomethane ($TMSCHN_2$) in dichloromethane in the presence of 42% aqueous fluoroboric acid (HBF_4)[24] to give the corresponding methyl ether (**5**) in good yield.[25] The acidic conditions used in this reaction were compatible with the presence of the acyl chain and the phosphorothioate group.[24] Next, the cyanoethyl ester groups were removed under basic aprotic conditions to yield the desired (2*R*)-OMPT (**6a**). The enantiomer (2*S*)-OMPT (**6b**) was prepared using the analogous procedures starting from (2*R*)-solketal (**7**). All the compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR spectroscopy and MS. (2*R*)-OMPT and (2*S*)-OMPT are over 95% pure as determined by high-resolution MS.

First, we evaluated the effect of 18:1-LPA and stereoisomers of OMPT for agonist-induced Ca^{2+} release in both insect (Sf9) cells and mammalian (Rh7777) cells expressing the human LPA_3 receptor.[13,16] Cells were loaded with a membrane-permeant calcium-sensitive dye, Fura-2, and agonist-induced Fura-2-Ca^{2+} complex formation was monitored at 500 nm (excitation 340, 380 nm). The calcium response is a common read-out for activation of the transfected GPCR and occurs downstream of PLC cleavage of PtdIns(4,5)P_2 to give Ins(1,4,5)P_3, followed by Ins(1,4,5)P_3-receptor-mediated calcium mobilization. Figure 2 illustrates the dose–response curves for 18:1 LPA, racemic OMPT, (2*R*)-OMPT (**6a**), and (2*S*)-OMPT (**6b**). As expected, the response to racemic OMPT was intermediate to the responses to the two enantiomers, indicative of a stronger response to one stereoisomer. In both mammalian (Figure 2, panel A) and insect cells (Figure 2, panel B), 5- to 20-fold lower EC_{50} values, respectively, were observed for the (2*S*)-OMPT having the unnatural configuration.

Second, we evaluated Ca^{2+} release in two human cancer cell lines using the calcium sensing dye Indo 1 (Figure 3).[16] The OVCAR3 ovarian cancer cells naturally express $LPA_3 \gg LPA_2 > LPA_1$ as determined by RT-PCR,[26] while the HT-29 colon cancer cells express almost exclusively LPA_2.[27] In the OVCAR3 cells, (2*S*)-OMPT and (2*R*)-OMPT showed EC_{50} values of 9.0 and

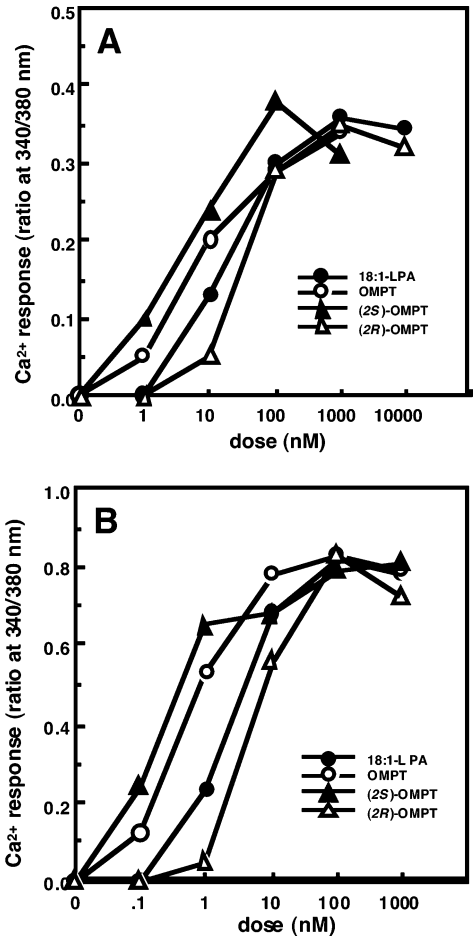


Figure 2. Panel A: Ca^{2+} release (peak concentration) by Rh7777 cells-expressing human LPA_3 receptor; Panel B: Ca^{2+} responses (peak concentration) of Sf9 cells-expressing human LPA_3 receptor.

46.5 nM, respectively. In HT-29 cells, EC_{50} values exceeded 1 mM for both enantiomers, confirming that the previous receptor specificity defined for the racemic mixture applies to both enantiomers.[16]

Third, we determined the phosphorylation of Thr-473 of PKB/Akt and of MAPK in the signaling pathways further downstream in LPA-activated GPCR signaling.[9,28] Figure 4a shows Western blots illustrating the dose-dependent phosphorylation of these two protein kinases in OVCAR3 cells in response to 18:1-LPA, and to racemic OMPT and the separate enantiomers. Note that in Figure 4a, 10-fold higher concentrations were employed for LPA because of its lower activity relative to OMPT. Once again the (2*S*)-OMPT analogue demonstrated increased activity with activation detected at concentrations as low as 0.01 μM. The peak activity in these assays was not as high for OMPT or the OMPT enantiomers likely due to interactions between LPA receptor isoforms at concentrations higher than 100 nM.[16] Nevertheless, the (2*S*)-OMPT analogue was more active than the (2*R*)-OMPT analogue, with the racemic mixture showing intermediary activity at 0.01 μM. At higher concentration, the responses appeared saturated in these experiments.

Finally, we measured IL-6 production in the OVCAR3 cells.[29] IL-6 is an important inducer of neovascularization and of white blood cells, potentially contributing to ovarian tumorigenesis. As illustrated in Figure 4b,

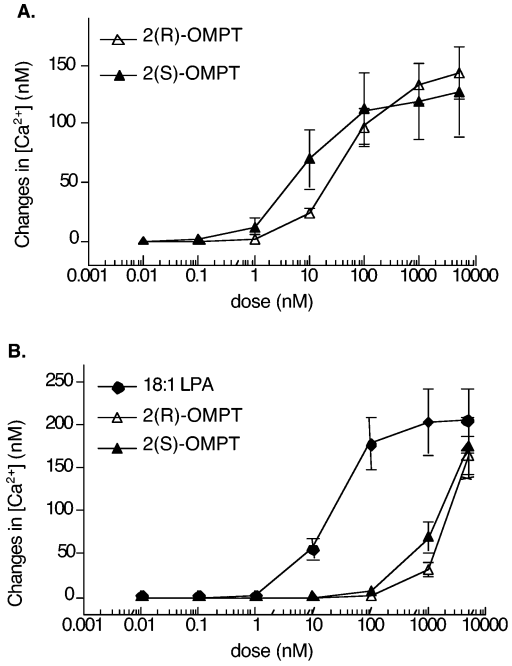


Figure 3. Dose-response curves for Ca^{2+} release by two human ovarian cancer cell lines. Panel A: OVCAR3, which express receptors in the relative abundance $LPA_3 \gg LPA_1 > LPA_2$; Panel B: HT-29, which express LPA_2 receptor only.

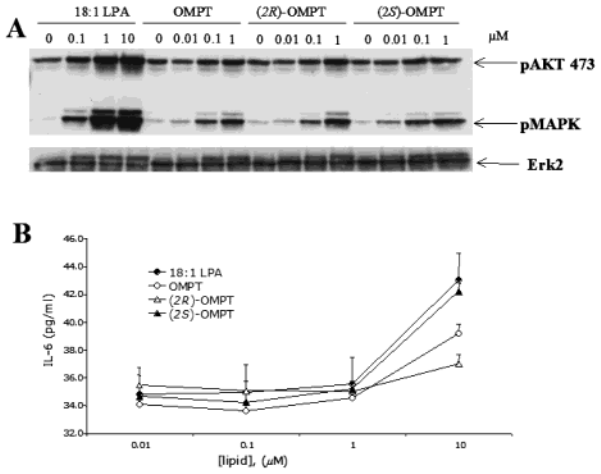


Figure 4. Panel A: Phosphorylation of MAPK and Akt in OVCAR3 cells treated with 18:1-LPA, racemic OMPT, (2*S*)-OMPT, and (2*R*)-OMPT. Panel B: Release of IL-6 from OVCAR3 cells activated by treatment with 18:1 LPA, racemic OMPT (2*S*)-OMPT, and (2*R*)-OMPT.

(2*S*)-OMPT was equipotent with 18:1-LPA in eliciting IL-6 production in response to extracellular stimulation. LPA_2 is more efficient than LPA_3 at inducing IL-6 production, potentially explaining the relatively high activity of 18:1-LPA in this functional assay.[29] As with the other assays, the response to racemic OMPT was intermediate to the responses to the (2*S*) and (2*R*) enantiomers.

Taken together, the (2*S*)-OMPT enantiomer was a highly effective probe for the function of LPA_3 in vitro. It is more active than either racemic OMPT or (2*R*)-OMPT in activating cells as indicated by increases in cytosolic calcium, activation of downstream signaling molecules and production of the potent neovascularizing factor, IL-6. The resistance of (2*S*)-OMPT to degradation suggests that it will prove a useful probe to determine the functions of LPA_3 in intact animals.[16]

In conclusion, we have described an efficient enantioselective synthesis of *O*-methylatd LPA analogues bearing unsaturated acyl chains and metabolically stabilized phosphate surrogates. Biological responses in four cell-based assay systems comparing LPA, racemic OMPT, and its (2*R*) and (2*S*)-enantiomers consistently demonstrated that the unnatural (2*S*)-isomer was more effective at activating LPA_3 and concomitant downstream signaling events than either (2*R*)-OMPT or the racemic mixture. This result suggests that, relative to the native hydroxyl group, the methoxy group is less well accommodated in the receptor binding site. Thus, the natural (2*R*)-enantiomer suffers a steric interference avoided by the (2*S*)-enantiomer, leading to the apparent "inverse" enantioselectivity.

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Supporting Information Available: Synthesis procedure and ^1H and ^{13}C NMR and MS data of intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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