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Phosphorothioate Analogues of Alkyl Lysophosphatidic Acid as LPA_3 Receptor-Selective Agonists

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*The metabolically stabilized LPA analogue 1-oleoyl-2-O-methyl-rac-glycerophosphorothioate (OMPT) was recently shown to be a potent subtype-selective agonist for LPA_3, a G-protein-coupled receptor (GPCR) in the endothelial differentiation gene (EDG) family. Further stabilization was achieved by replacing the sn-1 O-acyl group with an O-alkyl ether. A new synthetic route for the enantiospecific synthesis of the resulting alkyl LPA phosphorothioate analogues is described. The pharmacological properties of the alkyl OMPT analogues were characterized for subtype-specific agonist activity using Ca^{2+}-mobilization assays in RH7777 cells expressing the individual EDG family LPA receptors. Alkyl OMPT analogues induced cell migration in cancer cells mediated through LPA_1. Alkyl OMPT analogues also activated Ca^{2+} release through LPA_2 activation but with less potency than sn-1-oleoyl LPA. In contrast, alkyl OMPT analogues were potent LPA_3 agonists. The alkyl OMPTs **1** and **3** induced cell proliferation at submicromolar concentrations in 10T 1/2 fibroblasts. Interestingly, the absolute configuration of the sn-2 methoxy group of the alkyl OMPT analogues was not recognized by any of the LPA receptors in the EDG family. By using a reporter gene assay for the LPA-activated nuclear transcription factor PPARγ, we demonstrated that phosphorothioate diesters have agonist activity that is independent of their ligand properties at the LPA-activated GPCRs. The availability of new alkyl LPA analogues expands the scope of structure–activity studies and will further refine the molecular nature of ligand–receptor interactions for this class of GPCRs.*

Introduction

Lysophosphatidic acid (LPA, 1-radyl-*sn*-glycerol-3-phosphate) has been shown to elicit growth-factor-like effects, including cell proliferation, cell survival, Ca^{2+} mobilization, and changes in cell shape and motility in a variety of cell types.[1–4] Based on animal experiments, LPA is implicated in complex physiological responses that include immunological competence, brain development, wound healing, coagulation, and regulation of blood pressure.[5,6] The physiological functions of LPA suggest that LPA could contribute to a number of pathophysiological states including cancer, autoimmunity, immunodeficiency, atherosclerosis, and ischemia reperfusion injury.[7] LPA elicits its effects by binding to G-protein-coupled receptors (GPCRs) with subsequent activation of multiple heterotrimeric G-protein-linked downstream events.[7–9] Four different LPA GPCR types on mammalian cell surfaces have been characterized so far, including three members of the Edg family (LPA_1, LPA_2, and LPA_3, previously known as Edg 2, Edg 4, and Edg 7, respectively). A purinergic receptor family GPCR (GPR23/P2Y9) was recently identified as a putative LPA receptor.[10] LPA also activates the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ). Through activation of these GPCRs and PPARγ, LPA induces multiple physiological and pathological responses. Our understanding of these complex responses at the present time is limited by the lack of specific probes for the receptor types and subtypes.

Under normal circumstances, the production and degradation of LPA is tightly regulated to maintain a concentration range of 50–100 nM for LPA bound to carrier proteins. LPA levels are elevated during blood clotting, wound healing, and

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in a number of biological fluids such as saliva and ovarian cyst fluid.[7] Although the underlying mechanisms remain elusive, aberrations in the production and degradation of LPA have been identified in cancer cells as well as in cancer patients.[7] Markedly elevated levels of LPA have been found in the ascitic fluid of ovarian cancer patients and in autocrine activation loops in ovarian and prostate cancer.[3] In combination with alterations in LPA receptor expression and potentially receptor function, LPA appears to be an important mediator in the pathophysiology of cancer.[6,7,11] Therefore, LPA receptors have emerged as highly attractive targets for therapeutic intervention.

As with many other GPCRs, LPA receptors should be amenable to the development of highly specific and potent agonists or antagonists that have favorable pharmacokinetic, bioavailability, and metabolic characteristics. Currently available compounds represent a promising start to the development of useful chemical tools, although none can be considered definitive in determining receptor selectivity or biological functions, especially for studies in vivo. Several groups have reported the characterization of LPA agonists and antagonists. A partial list of compounds with reported LPA_1 receptor selectivity includes the ethanolamide phosphate and phosphorothioate derivatives,[12,13] 3-(4-[4-([1-(2-chlorophenyl)-ethoxy]carbonylamino)-3-methyl-5-isoxazolyl] benzylsulfanyl)propanoic acid (Ki16425),[14] and additional ethanolamide derivatives.[13,15] Compounds with LPA_2 agonist activity are less common but include the decyl and dodecyl fatty alcohol phosphates (FAP-10 and FAP-12).[16] Most common are the LPA_3 agonists, including OMPT, a phosphorothioate analogue of LPA,[17] a monofluorophosphonate analogue of LPA,[18] phosphates and phosphorothioates based on a carbohydrate scaffold,[19] and several ethanolamide derivatives.[15] Appropriately validated compounds are essential to advance in vivo studies, particularly in view of potential off-target effects. The development of more selective, more stable, more potent, and more druglike agonists and antagonists is eagerly awaited.

The metabolically stabilized LPA analogue 1-oleoyl-2-*O*-methylglycerophosphorothioate (OMPT) is a potent agonist for the LPA_3 receptor that shows enantioselective action.[17,20] Subsequently, we showed that alkyl LPA analogues were equipotent to natural acyl LPAs as LPA receptor agonists for each receptor isoform (Figure 1).[21] Catabolism of LPA proceeds by three principal pathways.[7] First, phosphate hydrolysis to form monoacylglycerol is catalyzed by phosphatase (LPP) or phosphohydrolase enzymes. Second, conversion into PA is mediated by LPA acyltransferases (LPAATs). Third, LPA-specific lysophospholipases hydrolyze the *sn*-1 acyl chain to form the corresponding glycerol phosphate. Alkyl substitution may introduce unexpected biological activities, as ether analogues are selectively phospholipase-resistant[22] and might be strong activators of PPARγ as a result of their unique properties.

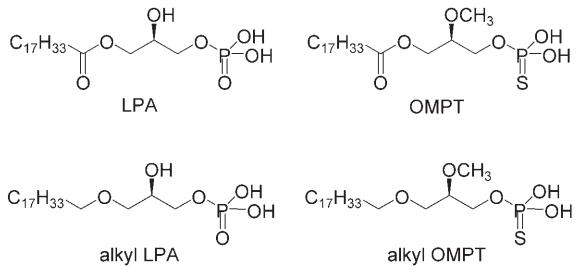


Figure 1. Structures of LPA, OMPT, and the corresponding *O*-alkyl analogues.

The alkyl OMPT analogues described herein are threefold metabolically stabilized with a phospholipase-resistant *sn*-1 ether chain, a migration and acyltransferase-resistant *sn*-2 *O*-methoxy group, and the less-readily hydrolyzed phosphorothioate head group.[20] To further elucidate the structural features of OMPT that determine agonist activity, we designed compounds **1**–**7** (Figure 2). Each analogue contains an *sn*-1 alkyl hydrophobic chain, an *sn*-2 methoxy group, and an anionic phosphorothioate group. To investigate the importance of absolute configuration of the *sn*-2 *O*-methoxy group for GPCR and PPARγ activity, we designed two pairs of enantiomers: **1** and **3**, plus **2** and **4**. Herein, we describe a convenient enantioselective synthetic approach to make these LPA analogues, and we describe cell-based assays for structure–activity relationships (SARs) for transfected human LPA receptors in the context of cell proliferation and migration, and for the activation of PPARγ.

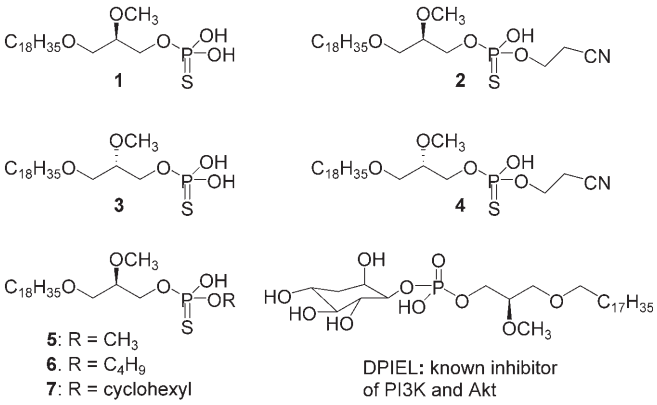
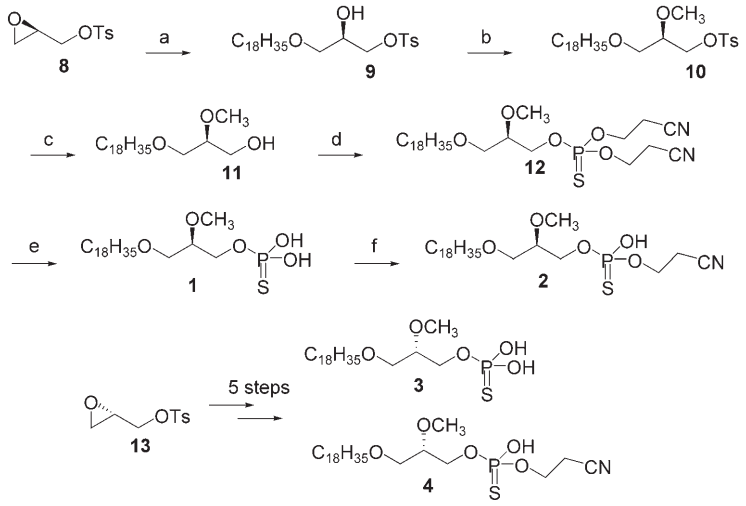


Figure 2. LPA analogues designed as LPA receptor agonists and antagonists.

Results

Chemistry

The enantioselective synthesis of OMPT involved the use of 2-cyanoethyl (CE) phosphate protecting groups, which can be removed under mild basic conditions.[17] For the synthesis of phosphorothioate analogues **1**–**7**, we used a common synthetic approach. Scheme 1 shows the concise conversion of the *p*-toluenesulfonyl (Ts) derivative of (*R*)-glycidol **8** to both enantiomerically pure phosphorothioates **1** and **2**. The key carbon–oxygen bond-forming step was a regio- and stereospecific nucleophilic opening of (*R*)-glycidyl tosylate **8** with a long-chain alcohol using BF_3 etherate as a catalyst.[23,24] As reported by

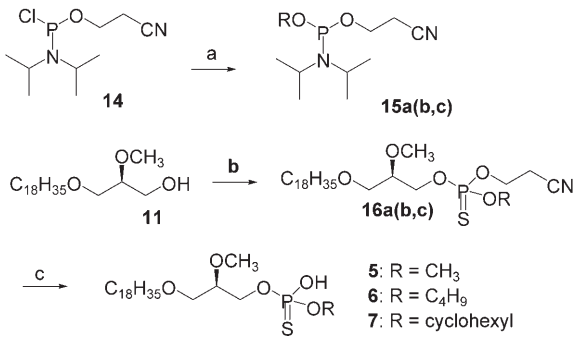


Scheme 1. Reagents and conditions for the synthesis of analogues **1**–**4**: a) (*Z*)-9-octadecen-1-ol, BF_3-$(C_2H_5)_2O$, CH_2Cl_2, 95%; b) CH_3OTf, 2,6-di-*t*Bu-4-MePy, CH_2Cl_2, 73%; c) Mg, CH_3OH, 91%; d) di-(2-cyanoethyl)-*N*,*N*-diisopropylphosphodiamidite, 1*H*-tetrazole; S, CS_2, Py, 86%; e) *t*$BuNH_2$, BTMSA, 72 h, 84%; f) *t*$BuNH_2$, BTMSA, 10 h, 84%. BTMSA = bis(trimethylsilyl)acetamide, Tf = trifluoromethanesulfonyl (triflate).

Guivisdalsky and Bittman, epoxide opening of (*R*)- or (*S*)-glycidol toluenesulfonate with oleyl alcohol occurred exclusively at the C3 position, stereospecifically affording the ring-opened product in very high enantiomeric excess (97–99% *ee*) as determined by HPLC on a chiral stationary phase.[25,26] Therefore, there is no loss of optical purity during the synthesis of the phosphorothioate LPA analogues. Mild conditions were required for the conversion of ring-opened product **9** into *O*-methyl compound **10** to avoid epoxide formation by intramolecular nucleophilic displacement. Methylation of the *sn*-2 hydroxy group was carried out by using methyl triflate in the presence of the hindered 2,6-di-*tert*-butyl-4-methylpyridine in dichloromethane at reflux, to generate **10** in good yield.[25] It was important to use the bulky and non-nucleophilic base 2,6-di-*tert*-butyl-4-methylpyridine to avoid the unwanted methylation of the base. Tosylate **10** was converted into alcohol **11** in high yield by using magnesium[27] in methanol instead of potassium superoxide with 18-crown-6.[25] Alcohol **11** was then phosphorylated with phosphoramidite methodology. The resulting phosphoramidite triester was oxidized with elemental sulfur to yield the corresponding phosphorothioate triester **12**.[17] Finally, one cyanoethyl ester group was removed under aprotic basic conditions (*tert*-butyl amine in acetonitrile) for 10 h to yield the desired phosphorothioate diester **2**. After 72 h, both cyanoethyl groups were removed to give phosphorothioate monoester **1**. The analogues **3** and **4** were prepared by using the analogous procedures starting from (*S*)-glycidyl tosylate **13** (Supporting Information). All compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR spectroscopy and by MS.

To investigate the steric effect of the ester group of the phosphorothioate diester analogues, we synthesized analogues **5**–**7**, which are similar to the phosphorothioates **2** and **4**. The butyl side chain in **6** approximates the physical size of the cyanoethyl group, whereas the methyl group in **5** is smaller, and the cyclohexyl group in **7** is bulkier. These structures were selected based on the observed antagonist effect of DPIEL[28] toward LPA receptor activation (Y. Hasegawa, G. Mills, unpublished results), and allowed us to probe the relationship between the bulk of the phosphorothioate ester side chain and its biological effect in receptor activation or antagonism.

Thus, three phosphoramidite reagents **15a**, **15b**, and **15c** were prepared by the reaction of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite with methanol, butanol, or cyclohexanol, respectively, with diethylisopropylamine (DIPEA) as the base (Scheme 2).[29] The air-sensitive phosphoramidites were purified by rapid flash chromatography with a basic solvent system. The phosphoramidites were then condensed with primary alcohol **11** in the presence of 1*H*-tetrazole to yield the phosphoramidite intermediates. Sulfurization with S_8 in CS_2/pyridine (1:1 *v*/*v*) gave the corresponding phosphorothioate diesters **16a**–**c**. The final reaction included deprotection of the cyanoethyl group on the phosphorothioate by the addition of triethylamine (TEA), combined with bis(trifluoromethylsilyl)acetamide (BTFSA). BTFSA was added to prevent the phosphorothioate anion from undergoing re-alkylation. Compounds **2** and **4**–**7** all contain a chiral phosphorothioate phosphorus atom; the ^{31}P NMR of these compounds indicates that each analogue is, as anticipated, a 1:1 mixture of the resulting diastereomers.



Scheme 2. Reagents and conditions for the synthesis of analogues **5**–**7**: a) ROH, DIPEA, 43–64%; b) phosphodiamidite **15**, 1*H*-tetrazole; S, CS_2, Py, 60–68%; c) triethylamine, BTFSA, 72 h, 59–65%.

Biology

The ligand properties of the compounds were first evaluated on the activation of LPA_1, LPA_2, and LPA_3 by the mobilization of Ca^{2+} in Sf9 or RH7777 cells expressing LPA_1, LPA_2, and LPA_3 receptors, with a FlexStation II automated fluorometer (Molecular Devices, Sunnyvale, CA).[16] In a biological context, the activation of LPA_1 was evaluated with a cell-migration assay with

203G murine glioma cells, MDA-MD-231 human breast cancer cell lines, and PC-3 prostate cancer cells.[30]

The enantiomers of alkyl OMPTs **1** and **3** induced cell migration, but with lower potency than 1-oleoyl LPA (Supporting Information Fig. 1). As LPA-induced cell migration in various cell types is LPA_1-dependent, the cell migration assay was found to be more sensitive than the system using LPA_1–LPA_2 chimeric receptor calcium assay, and was thus employed for comparing the biological activities of the synthetic analogues toward the LPA_1 receptor.[21] Therefore, **1** and **3** were weak agonists for the LPA_1 receptor.

Supporting Information Fig. 1 also illustrates calcium responses elicited through the activation of human LPA_1, LPA_2, and LPA_3 receptors.[16,31] In these experiments, RH7777 cells, which are intrinsically unresponsive to LPA,[16,31] were transfected with human LPA_1, LPA_2, and LPA_3, respectively. Both **1** and **3** are weak LPA_2 receptor agonists. Importantly, both **1** and **3** are potent agonists for LPA_3 subtypes, with higher potency than 1-oleoyl-LPA. The phosphorothioate diesters **2** and **4** (Supporting Information Fig. 2) showed the same activities as **1** and **3**. Similar to the activity of **1** and **3**, analogues **2** and **4** are LPA_3-selective agonists. However, the phosphorothioate diesters **5**–**7** did not show any activity toward LPA receptors, with the exception that **5** showed weak LPA_3 agonist activity (Supporting Information Fig. 3).

Alkyl OMPT **1** and **3** are LPA_3-selective agonists in one assay system using RH7777 cells expressing the human LPA_3 receptor. These independent assay results with RH7777 cells confirm that alkyl OMPT analogues are LPA_3 agonists and that the monoester alkyl OMPT analogue **2** is a weak LPA_3 agonist (Table 1). Interestingly, among the monoester alkyl OMPTs **5**–**7**, only the methyl phosphodiester **5** showed weak agonist activity, whereas the butyl phosphodiester **6** and the cyclohexyl phosphodiester **7** slightly inhibited LPA activation in this assay system.

Table 1. Effects of LPA analogues on RH7777 cells transfected with LPA_1 and LPA_3.[a]

	LPA_1		LPA_3	
Compd	E_{max} [%]	EC_{50} [nM]	E_{max} [%]	EC_{50} [nM]
1	79.7	790	130	62
2	123	3880	123	817
3	74.2	571	ND[c]	ND[c]
4	116	2720	124	207
5	73.9	13500	100	4840
6	NE[b]	ND[c]	51[d]	inhibited
7	NE[b]	ND[c]	68[d]	inhibited
18:1 LPA	100	17	100	263

[a] Intracellular Ca^{2+} transients were measured in response to the application of increasing concentrations of LPA analogues and compared with transients elicited by 1-oleoyl (18:1) LPA; results represent the average of four measurements. [b] NE = no effect. [c] ND = not determined. [d] Inhibition of 200 nM LPA response.

LPA is a member of the phospholipid growth factor family and exerts pleiotropic effects that include enhancement of cell survival and cell proliferation. In vitro, LPA has been shown to function as a growth and survival factor for renal proximal tubular cells, inhibiting apoptosis induced by growth factor deprivation.[7] To further examine the potential biological activities of the analogues, we used an MTT assay to analyze effects on cell proliferation. We found that starting at a concentration of 100 nM, compounds **1** and **3** stimulated the growth of 10T 1/2 cells in a dose-dependent manner with a robust induction shown by **3** at 10 μM (Figure 3). No significant difference was

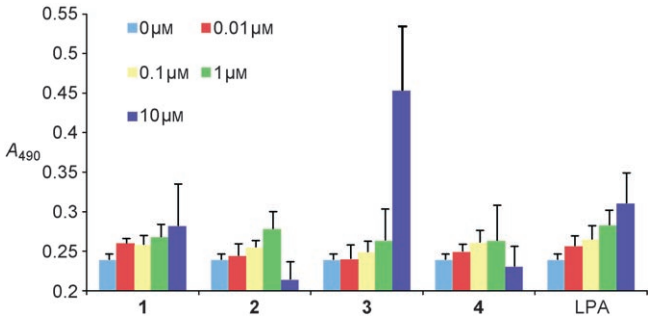


Figure 3. Stimulation of DNA synthesis in 10T 1/2 cells by LPA and analogues **1**–**4** as determined by MTT assay.

observed between compounds **1**, **3** and natural LPA within the concentration range of 0.01–1 μM. At the higher concentrations, the 2*S* enantiomer **3** was more effective than either the 2*R* enantiomer **1** or natural LPA toward the proliferation of 10T 1/2 cells. This result is consistent with previous reports of the enantioselectivity of OMPT, in which (2*S*)-OMPT was threefold to eightfold more active than (2*R*)-OMPT in activating cytosolic calcium release, downstream kinase activation, and production of the potent neovascularizing factor IL-6.[17] In the present case, the enantioselectivity of compounds **1** and **3** was revealed at a much higher concentration (10 μM) than was that of the enantiomers of OMPT (0.1 μM).

In addition to LPA plasma membrane receptors, LPA was found to activate the nuclear transcription factor PPARγ.[32] Many agents have been reported to be agonists of the nuclear transcription factor PPARγ including members of the thiazolidinedione family represented by rosiglitazone (Rosi) as well as oxidized phospholipids, fatty acids, eicosanoids, and oxidized LDL. LPA and the alkyl ether analogue of LPA directly bind to the ligand-binding domain of PPARγ. The activation of PPARγ is direct, and is enhanced when LPA entry into cells is facilitated by carrier amine sulfonamides capable of increasing the transmembrane movement of LPA.[32] Compounds **1**, **3**, **5**, **6**, and **7** were tested for PPARγ activation in CV1 cells that had been transfected with an acyl-coenzyme A oxidase–luciferase (PPRE-Acox-Rluc) reporter gene construct as previously reported.[33] The results are illustrated in Figure 4. Interestingly, the phosphorothioate diesters **5**–**7**, which are inactive or have low activity toward LPA receptors, can nonetheless activate the PPRE-Acox-Rluc reporter more robustly than can arachidonoyl LPA and alkyl OMPT **1** and **3**. These results are consistent with our previously reported observations, in that LPA analogues

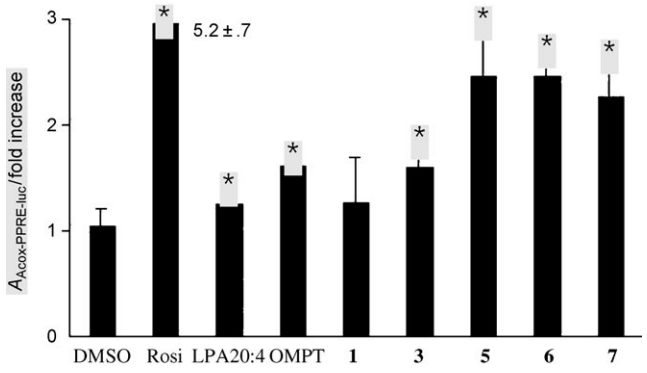


Figure 4. Results of in vitro activation of PPARγ by selected compounds in CV1 cells transfected with PPARγ and the PPRE-Acox-Rluc reporter gene. Rosiglitazone, a known PPARγ agonist, was used as the positive control. CV1 cells were treated with DMSO (1%) or test compound (10 μM in DMSO) for 20 h. Luciferase and β-galactosidase activities (reported as mean ± SE) were measured in the cell lysate ($n = 4$); $^*p < 0.05$, significant differences over vehicle control.

that lack the ability to activate GPCRs for LPA receptors may still be potent activators of PPARγ.

Discussion

Ligand recognition by GPCRs generally shows a preference for the naturally occurring enantiomer. 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.[34] The lack of stereoselectivity of the LPA GPCRs distinguishes them from PPARγ, which shows enantioselective activation.[33] In contrast to the enantiomers of acyl and alkyl LPA, however, the activities of LPA analogues based on non-glycerol backbones show strong enantioselectivity. For example, analogues of *N*-acylethanolamine phosphoric acid (NAEPA) derivatives[13] (which contain a serine or an ethanolamine backbone in place of glycerol), analogues of *N*-palmitoyl-2-methylenehydroxyethanolamide phosphoric acid ((*R*)-MHEPA and (*S*)-MHEPA),[35] and analogues of carbohydrate scaffolds[19] are recognized in a stereoselective manner, in which the 2*R* enantiomers (natural configuration) are more potent than the 2*S* enantiomers (unnatural configuration). This duality of responses has been recently reviewed.[34,36] It appears that OMPT and its alkyl analogues further reinforce this duality of enantioselective recognition. At different concentration ranges, both (2*S*)-acyl OMPT and (2*S*)-alkyl OMPT (the unnatural configuration) show higher activity than their 2*R* enantiomer (natural configuration) counterparts in bioassays. This result further confirms that relative to other kinds of LPA analogues, OMPT and its analogues may interact with LPA receptors through a different orientation than does natural LPA. Furthermore, enantioselective degradation by lipid phosphatases and other metabolic enzymes might also be responsible for differences observed in cellular assays.

As expected, 1-oleoyl LPA stimulated cell survival in 10T 1/2 cells at all concentrations tested (Figure 3). Compounds **1** and **3** stimulated cell viability in a dose-dependent manner, with a robust induction exhibited by **3** at 10 μM. Phosphorothioate diesters **2** and **4** increased cell viability at low concentrations (0.01–1 μM), but functioned as inhibitors at 10 μM, with **2** showing a more prominent effect. These data prompted us to further assess whether compounds **2** and **4** are potential antagonists for LPA signaling. For this purpose, we evaluated these compounds on LPA receptors. However, we could not confirm antagonist activity after the evaluation of all of these compounds. It is thus possible that the inhibitory effect on cell viability arose from the cytotoxicity of cyanoethanol resulting from phosphodiester hydrolysis.

To investigate the function of the ester group on the alkyl OMPT analogues, we prepared phosphorothioate diester compounds **5**–**7**, each with the natural 2*R* configuration at the *sn*-2 position. Among these three analogues, only methyl phosphodiester **5** had agonist activity toward the LPA_3 receptor after biological evaluation. The butyl and cyclohexyl phosphodiesters failed to activate the LPA receptors. Although analogues **2**, **4**, and **5** have similar steric sizes, their activities are quite different. It appears that the polar cyanoethyl group may explain this biological activity.

LPA and its structural analogues all have a polar head group, a linker, and a hydrophobic tail. As reported, of these three motifs, modifications to the polar head group may be the least tolerated. Only modifications of the phosphate head group that retain a negative charge under physiological conditions have been found to retain receptor activation. So far, only two classes of phosphate mimics are good LPA agonists. One class are the phosphonate derivatives, such as α-hydroxymethylene phosphonate, α-ketomethylene phosphonate[35] and α-fluoromethylene phosphonate.[18] The second class includes the phosphorothioate analogues such as OMPT. Therefore, comparison of analogues **1**–**4** with **4**–**6**, switching phosphorothioate monoester to phosphorothioate diester decreases and even deletes agonistic activity. Recently, different substituents at the *sn*-2 position of the *N*-acylethanolamide phosphate backbone were explored.[13] Compounds with smaller substituents at the *sn*-2 position were more potent and efficacious agonists; indeed, in some cases, these compounds are more potent and efficacious than LPA. The fact that efficacy decreased sharply with the bulk of hydrophobic substituents at position 2 led to the discovery of a dual LPA_1/LPA_3 competitive antagonist, VPC12249.[13] This observation further demonstrates that agonists and antagonists might have similar molecular structures, and that small changes can convert an LPA agonist to an antagonist. An excellent LPA agonist can potentially be used as a good lead compound for design of antagonists, because agonists and antagonists may share common features for binding with LPA receptors.

McIntyre and colleagues provided the first evidence that the transcription factor PPARγ can function as an intracellular LPA receptor.[32] The authors showed that oleoyl LPA, palmitoyl LPA, and hexadecyl glycerol phosphate competitively displaced rosiglitazone and hexadecyl azelaic phosphatidylcholine, previously identified ligands of PPARγ. The authors provided conclusive evidence that LPA activates the transcription of genes that contain the peroxisomal proliferator responsive element (PPRE)

including the scavenger receptor CD36. Subsequently, the unsaturated and alkyl ether analogues of LPA were found to induce neointima formation, an early step leading to the development of atherogenic plaques, through PPARγ activation. The SAR of neointima formation by LPA analogues in vivo was identical to PPARγ activation in vitro and was clearly different from the SAR for LPA GPCRs.[33] In the PPARγ activation assays reported herein, both alkyl OMPT (**1** and **3**) and phosphorothioate diesters **5**–**7** activated PPARγ.[33] In contrast to their activities on LPA receptors, the phosphorothioate diesters were more potent than alkyl OMPT and natural LPA. This result strengthens the point that the SAR for PPARγ activation is different from that for GPCR activation.

In summary, an initial SAR analysis of alkyl-chain-substituted OMPT analogues was described, and these alkyl OMPTs were found to be selective agonists of LPA_3. We also found that switching the phosphorothioate monoester to a phosphorothioate diester changed their activities sharply. This study has resulted in the discovery of a new series of agonists for the LPA receptors through the use of an in vitro cell viability assay. The discovery of LPA receptor-specific agonists and antagonists that have favorable properties will facilitate the understanding of the physiological and pathophysiological roles played by single LPA receptor subtypes, which is a major current challenge for LPA biology. Effective LPA subtype-specific agonists will be important for understanding the fundamental biology of LPA receptors and for the development of human therapies based on targeting LPA receptors and components of their signaling pathways. The findings presented herein provide a useful platform for further optimization of OMPT-type analogues, both for agonists and antagonists.

Experimental Section

Chemicals were purchased from Aldrich and Acros Chemical Corporation and were used without prior purification. Solvents were reagent grade and distilled before use; CH_2Cl_2 was distilled from CaH_2 and THF was distilled from sodium wire. TLC: precoated silica gel aluminum sheets (EM SCIENCE silica gel $60F_{254}$). Flash Chromatography (FC): Silica gel Whatman 230–400 mesh ASTM. NMR spectra were recorded on a Varian INOVA 400 at 400 MHz (^{1}H), 101 MHz (^{13}C), or 162 MHz (^{31}P) at 25 °C. Chemical shifts are given in ppm with TMS as internal standard ($\delta = 0.00$); ^{31}P, 85% H_3PO_4 ($\delta = 0.00$). Low- and high-resolution mass spectra were obtained on HP5971A MSD and Finnigan MAT95 double focusing mass spectrometer instruments, respectively.

1-*O*-(9*Z*-octadecen-1-yl)-2(*R*)-*sn*-glycerol-3-*O*-*p*-toluenesulfonate (9): Freshly distilled BF_3 etherate (50 μL) was added to (2*R*)-glycidyl tosylate (200 mg, 0.88 mmol) and 9*Z*-octadecen-1-ol (330 mg, 1.23 mmol) in 4 mL anhydrous CH_2Cl_2. The mixture was stirred at room temperature for 24 h under argon. The solvent was removed under decreased pressure to give a residue. FC (EtOAc/hexane 1:5 *v/v*, $R_f = 0.36$) gave **9** (320 mg) as a colorless oil (0.642 mmol, 73%). ^{1}H NMR ($CDCl_3$): $\delta = 0.88$ (t, $J = 8.0$ Hz, 3H), 1.26 (br s, 24H), 1.51 (t, $J = 6.8$ Hz, 2H), 2.01 (m, 4H), 2.04 (s, 1H), 2.45 (s, 3H), 3.37–3.48 (m, 4H), 3.97 (m, 1H), 4.01–4.15 (m, 2H), 5.35 (m, 2H), 7.35 (d, $J = 8.4$ Hz, 2H), 7.80 ppm (d, $J = 8.8$ Hz, 2H); ^{13}C NMR: $\delta = 14.11$, 21.65, 22.68, 26.00, 27.18, 29.24, 29.31, 29.41, 29.47, 29.51, 29.69, 29.75, 31.89, 68.28, 70.39, 70.54, 71.74, 127.99, 129.79, 129.89, 129.97, 132.63, 144.99 ppm; MS (CI) *m/z* 497.3 [M^++H−OH]; HRMS [M^++H−OH] calcd for $C_{28}H_{49}SO_5$: 497.3301, found: 497.3344.

3-*O*-(9*Z*-octadecen-1-yl)-2(*S*)-*O*-methyl-*sn*-glycerol-1-*O*-*p*-toluenesulfonate (10): A solution of **9** (2.74 g, 5.50 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (7.76 g, 37.8 mmol) in dry dichloromethane (50 mL) was treated with methyl triflate (4.28 mL, 37.8 mmol). After the mixture was heated at reflux for 16 h under nitrogen, the solvents were evaporated. EtOAc (500 mL) and 2 N HCl (100 mL) were added to the residue. The organic phase was isolated and washed again with 2 N HCl (30 mL). To recover the excess hindered pyridine, the combined aqueous phase was neutralized with 20% aqueous NaOH, and 2,6-di-*tert*-butyl-4-methylpyridine was extracted into dichloromethane. The EtOAc phase was washed with water, saturated $NaHCO_3$ and water, and then dried (Na_2SO_4). Removal of the solvents gave a residue that was purified by FC (hexane/EtOAc 9:1 *v/v*, $R_f = 0.18$) to give 2.10 g of **10** as a colorless oil (3.99 mmol, 73%). ^{1}H NMR ($CDCl_3$): $\delta = 0.85$ (t, $J = 7$ Hz, 3H), 1.28 (br s, 22H), 1.45 (t, $J = 6.4$ Hz, 2H), 1.97 (m, 3H), 2.42 (s, 3H), 3.32 (m, 5H), 3.37 (m, 2H), 3.50 (m, 1H), 5.32 (m, 2H), 7.28 (d, $J = 8.0$ Hz, 2H), 7.77 ppm (d, $J = 8.0$ Hz, 2H); ^{13}C NMR: $\delta = 14.11$, 21.64, 22.67, 25.99, 27.20, 29.25, 29.31, 29.42, 29.48, 29.51, 29.69, 29.75, 31.89, 58.11, 68.92, 69.15, 71.82, 127.99, 129.80, 129.95 ppm; MS (CI) *m/z* 527.3 [M^++H]; HRMS [M^++H] calcd for $C_{29}H_{51}SO_6$: 527.3406, found: 527.3272.

1-*O*-(9*Z*-octadecen-1-yl)-l-2(*S*)-*O*-methyl-*sn*-glycerol (11): Dry methanol (15 mL) was added to **10** (1.4 g, 2.66 mmol) and magnesium (650 mg, 26.6 mmol) in a flask fitted with a condenser and a calcium chloride guard tube. The solution was stirred at room temperature, with the flask kept in a water bath overnight. When the reaction was complete, the reaction mixture was neutralized with cold 1 N HCl and extracted with diethyl ether (3×20 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was purified by chromatography (hexane/EtOAc 3:1 *v/v*) to afford 860 mg of **11** as a colorless oil (2.41 mmol, 91%). ^{1}H NMR ($CDCl_3$): $\delta = 0.88$ (t, $J = 7.2$ Hz, 3H), 1.26 (br s, 24H), 1.56 (m, 2H), 2.01 (m, 4H), 3.44 (m, 3H), 3.46 (s, 3H), 3.54 (m, 2H), 3.62–3.77 (m, 2H), 5.34 ppm (m, 2H); ^{13}C NMR: $\delta = 14.09$, 22.67, 26.05, 27.18, 27.19, 29.23, 29.31, 29.41, 29.46, 29.51, 29.56, 29.64, 29.68, 29.75, 31.89, 57.76, 62.67, 70.61, 71.90, 79.81, 129.81, 129.93 ppm; MS (CI) *m/z* 357.3 [M^++H]. HRMS [M^++H] calcd for $C_{22}H_{45}O_3$: 357.3369, found: 357.3369.

3-*O*-bis-(2-cyanoethoxy)thiophosphoryl-1-*O*-(9*Z*-octadecen-1-yl)-2(*R*)-*O*-methyl-*sn*-glycerol (12): Di(2-cyanoethyl) diisopropylphosphorodiamidite (32 mg, 0.12 mmol) was added under an argon atmosphere to a solution of **11** (14 mg, 0.04 mmol) and 1H-tetrazole (8.4 mg, 0.12 mmol) in 1 mL dry CH_2Cl_2. After stirring for 1 h, sulfur (10 mg, 0.2 mmol) and CS_2/pyridine (10 μL, 1:1 *v/v*) were added. After stirring at room temperature for 2 h, the reaction mixture was filtered and the filtrate was washed with brine, dried over Na_2SO_4, and concentrated. FC (EtOAc/hexane/CH_2Cl_2 1:2:2 *v/v/v*) gave 20 mg of **12** as a colorless oil (0.109 mmol, 91%). ^{1}H NMR ($CDCl_3$): $\delta = 0.87$ (t, $J = 7.2$ Hz, 3H), 1.28 (br s, 24H), 1.56 (m, 2H), 2.00 (m, 4H), 2.76 (m, 4H), 3.44 (m, 2H), 3.46 (s, 3H), 3.50 (m, 2H), 3.55 (m, 1H), 4.14 (m, 2H), 4.25–4.33 (m, 5H), 5.34 ppm (m, 2H); ^{13}C NMR: $\delta = 14.09$, 19.36, 19.43, 22.57, 22.54, 22.64, 26.03, 27.17, 29.09, 29.23, 29.28, 29.41, 29.47, 29.54, 29.61, 29.66, 29.73, 31.86, 58.01, 62.43 (t, $J = 4.6$ Hz), 67.95 (d, $J = 6.2$ Hz), 68.99, 71.87, 78.44 (d, $J = 7.6$ Hz), 116.34, 116.38, 129.79, 129.92 ppm; ^{31}P NMR: $\delta = 68.91$ ppm (s); MS (CI) *m/z* 559.3 [M^++H]; HRMS [M^++H] calcd for $C_{28}H_{52}PSN_2O_5$: 559.3334, found: 559.3365.

1-*O*-(9*Z*-octadecen-1-yl)-2(*R*)-*O*-methyl-*sn*-glycero-3-phosphothionate (1): *tert*-Butylamine (1 mL) was added to a solution of compound **12** (28 mg, 0.05 mmol) in CH_3CN (1 mL) under N_2 followed by the addition of bistrimethylsilylacetamide (43 μL, 0.175 mmol). After 72 h, the reaction mixture was concentrated and the residue was purified by chromatography (EtOAc/MeOH 8:1→1:1 *v/v*) to afford 18 mg of **1** as a light yellow oil (0.041 mmol, 84%). ^{1}H NMR (CD_3OD): $\delta = 0.78$ (t, $J = 7.2$ Hz, 3H), 1.23 (m, 24H), 1.45 (m, 2H), 1.91 (m, 4H), 3.35 (m, 3H), 3.50 (m, 3H), 3.85 (m, 2H), 5.22 ppm (m, 2H); ^{13}C NMR: $\delta = 13.42$, 15.52, 22.60, 25.13, 25.29, 26.07, 26.99, 27.02, 29.19, 29.31, 29.47, 29.59, 31.91, 37.48, 57.20, 58.12, 65.08, 65.14, 71.85, 72.56, 80.78, 80.86, 118.07, 130.73 ppm; ^{31}P NMR: $\delta =$ 52.18 ppm (s); MS (CI) *m/z* 453.3 [M^++H]; HRMS [M^++H] calcd for $C_{22}H_{46}PSO_5$: 453.2804, found: 453.2822.

3-*O*-mono-(2-cyanoethoxy)thiophosphoryl-1-*O*-(9*Z*-octadecen-1-yl)-2(*R*)-*O*-methyl-*sn*-glycerol (2): *tert*-Butylamine (1 mL) was added to a solution of compound **12** (28 mg, 0.05 mmol) in CH_3CN (1 mL) under N_2 followed by the addition of bistrimethylsilylacetamide (43 μL, 0.175 mmol). After 10 h, the reaction mixture was concentrated and the residue was purified by chromatography (EtOAc/MeOH 8:1 *v/v*) to afford 21 mg of **2** as a light yellow oil (0.042 mmol, 84%). ^{1}H NMR (CD_3OD): $\delta = 0.78$ (t, $J = 7.2$ Hz, 3H), 1.23 (m, 24H), 1.45 (m, 2H), 1.91 (m, 4H), 2.69 (t, $J = 6.4$ Hz, 2H), 3.46 (m, 6H), 3.60 (m, 2H), 3.95 (m, 2H), 4.12 (m, 2H), 5.22 ppm (m, 2H); ^{13}C NMR: $\delta = 13.39$, 15.49, 22.57, 25.06, 25.26, 26.04, 26.96, 26.98, 29.16, 29.28, 29.44, 29.56, 31.88, 37.45, 54.76 (m), 57.17, 58.09, 62.07 (m), 65.05, 65.11, 71.82, 72.53, 80.75, 80.83, 116.34 (s), 118.04, 129.73 (s), 129.83 (s),130.70 ppm; ^{31}P NMR: $\delta = 58.64$ (s), 58.57 ppm (s); MS (CI) *m/z* 506.3 [M^++H]; HRMS [M^++H] calcd for $C_{25}H_{49}NO_5PS$: 506.3069, found: 506.3081.

Ca^{2+} mobilization assay for receptor activation: The assay for mobilization of intracellular Ca^{2+} was performed as described.[16,30,31] Briefly, RH7777 cells stably expressing human LPA_1, LPA_2, or LPA_3 were loaded with Fura-2 AM. Changes in the intracellular Ca^{2+} concentration were monitored by determining the ratio of emitted light intensities at $\lambda = 520$ nm in response to excitation at $\lambda = 340$ and 380 nm. Responses were monitored for 80–120 s. Ca^{2+} transients were quantified automatically by calculating the difference between maximum and baseline ratio values for each sample. To determine antagonist properties, varying concentrations of the compounds were mixed with LPA (200 nM, 18:1) and responses were recorded. Each test was performed in quadruplicate.

Chemotaxis assay: Cancer cells (203G, MDA-MD-231, and PC-3) that each exhibit LPA_1-dependent cellular migration were used. Cells were cultured in RPMI 1640 (Sigma) with 5% fetal bovine serum (FBS) plus streptomycin (100 μg mL^{-1}) and penicillin (100 U mL^{-1}). Cell migration was measured in a modified Boyden chamber as described previously.[30] The number of cells that migrated to the underside was determined by measuring optical densities at $\lambda = 590$ nm using a 96-well microplate reader (Nalge Nunc International).

MTT assay for cell viability: 10T 1/2 cells were plated into 96-well plates at a density of 5000 cells per 100 μL^{-1}well^{-1} in Basal Medium Eagle Medium containing 10% FBS for one day. Cells were starved in serum-free medium for 24 h prior to stimulation with different compounds for 48 h. At this time, 20 μL well^{-1} of MTS/PMS solution was added to the cells and incubated for an additional 1–4 h at 37 °C. The amount of soluble formazan produced by cellular reduction of MTS was measured at $\lambda = 490$ nm by using a plate reader. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

PPARγ activation assay: PPARγ activation using CV1 cells transfected with an acyl-coenzyme A oxidase–luciferase (PPRE-Acox-Rluc) reporter gene construct was performed as previously reported. Briefly, CV-1 cells were plated in 96-well plates at a density of 5×10^3 cells well^{-1} in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The next day, the cells were transiently transfected with 125 ng pGL3-PPRE-Acox-Rluc, 62.5 ng pcDNAI-PPARγ, and 12.5 ng pSV-β-galactosidase (Promega, Madison, WI, USA) by using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with OptiMEMI (Invitrogen) supplemented with 1% FBS, containing DMSO or 10 μM test compound dissolved in DMSO for 20 h. Luciferase and β-galactosidase activities were measured with Steady-Glo Luciferase Assay system (Promega) and the Galacto-Light Plus system (Applied Biosystems, Foster City, CA, USA), respectively. Samples were run in quadruplicate and the mean ± SE were calculated. Data are representative of at least two independent transfections.

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