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Alkyl lysophosphatidic acid and fluoromethylene phosphonate analogs as metabolically-stabilized agonists for LPA receptors

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Abstract—We describe an efficient method for the synthesis of alkyl lysophosphatidic acid (LPA) analogs as well as alkyl LPA mono- and difluoromethylene phosphonate analogs. Each alkyl LPA analog was evaluated for subtype-specific LPA receptor agonist activity using a cell migration assay for LPA_1 activation in cancer cells and an intracellular calcium mobilization assay for LPA_2 and LPA_3 activation. Alkyl LPAs induced pronounced cell migration activity with equivalent or higher potency than *sn*-1-oleoyl LPA, while the alkyl LPA fluoromethylene phosphonates proved to be less potent agonists in this assay. However, each alkyl LPA analog activated Ca^{2+} release by activation of LPA_2 and LPA_3 receptors. Interestingly, the absolute configuration of the *sn*-2 hydroxyl group of the alkyl LPA analogs was not recognized by any of the three LPA receptors. The use of alkyl LPA analogs further expands the scope of structure–activity studies, which will better define LPA–LPA receptor interactions.

Lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycerol 3-phosphate) is a naturally occurring phospholipid that shows a variety of cell-specific biological activities,[1] and modulation of LPA signaling has emerged as an important area in development of new therapeutics.[2] For example, LPA signaling is under intense scrutiny for development of new cancer therapeutics via inhibitors of LPA biosynthesis and antagonism of specific LPA receptors.[3,4] A primary route of LPA production results from the action the secreted enzyme lysophospholipase D, previously known as autotaxin,[5] on lysophosphatidylcholine. LPA activates three related G protein-coupled receptors (GPCRs) designated as LPA_1, LPA_2, and LPA_3 (formerly Edg-2, Edg-4, Edg-7),[6,7] as well as p2y9/GPR23, an orphan GPCR,[8] and PPARγ.[9]

The molecular diversity of LPA is well-recognized; it comprises acyl-, alkyl-, and alkenyl-LPAs (Fig. 1) that contain one hydrophobic chain that can vary in length.[10] In addition, the acyl subclass includes two positional isomers having the same fatty acyl group: *sn*-1-acyl- and *sn*-2-acyl-LPAs. Acyl LPAs are widely distributed in mammalian tissues.[11] Alkyl LPAs have been identified only in rat brain,[12] human platelets,[13] and human ascitic fluid.[14] Alkenyl LPAs have been identified in human ascitic fluid[14] and rabbit aqueous humour.[15] Alkyl LPA acts via LPA receptors, albeit with less potency than the corresponding acyl LPA, at least in A431 cells.[16] In human platelet aggregation assays, on the other hand, alkyl LPAs appear to be the most potent species.[13,17] Taken together, alkyl LPAs appear to be bona fide receptor agonists and are physiologically relevant lipid mediators.

Although LPA action is most often associated with cell proliferation and morphological changes, less attention has been paid to the effects of LPA on cell motility and migration. Cell migration is fundamental to many normal processes and plays a central role in embryonic development. Cell migration is also important in the pathophysiology of cancer, and is dysregulated during the progression of tumor cells from a non-invasive to an invasive and metastatic phenotype. Thus, LPA signaling exacerbates the invasion of tumor cells across a monolayer of normal cells,[18] but also promotes wound

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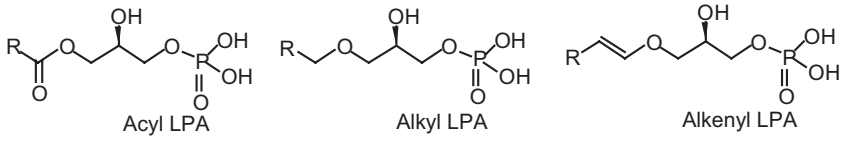


Figure 1.

healing both in vitro and in vivo.[19] However, it is not well understood how LPA receptors signal cell migration. In general, cell migration is driven by signaling pathways controlled by the three Rho GTPases—RhoA, Rac1, and Cdc42—acting in a coordinated fashion.[20] Rac1 regulates lamellipodia protrusion and forward movement; Cdc42 establishes cell polarity, and RhoA mediates actomyosin-driven cytoskeletal contraction and stress fiber formation as well as the detachment of the rear end of migrating cells.[20] In contrast, very little is yet known about the effect of alkyl LPA on cell migration and in most studies on LPA-driven cell migration only acyl LPAs have been used. To facilitate examination of the cell migration potential of alkyl LPA, we have developed a methodology for the synthesis of alkyl LPAs and metabolically-stabilized alkyl LPA fluoromethylene phosphonate analogs. Moreover, we report subtype-specific receptor activation of LPA_1 in cancer cell migration assays, and LPA_2 and LPA_3 activation in insect cells expressing human receptors as well as LPA_2-expressing HT29 colon cancer cells.

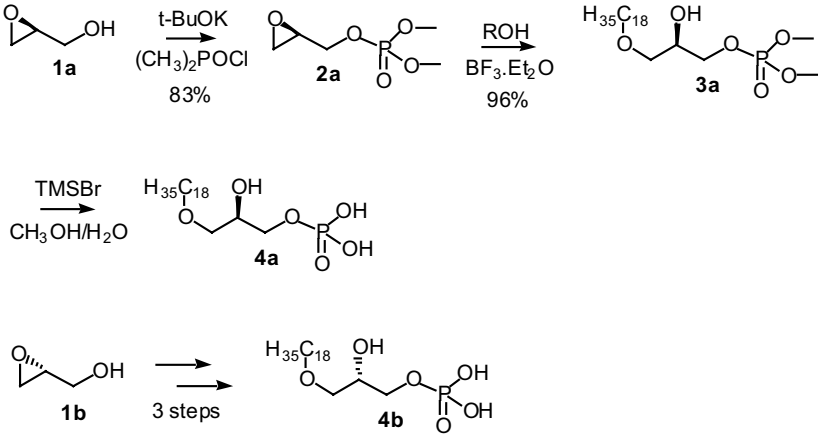
1. Chemistry

The synthetic strategies of the target alkyl LPA, 1-seracyl-*sn*-glycerol-3-phosphate were based on three design considerations. First, we wanted to employ an epoxide ring-opening reaction with a phosphorylated substrate to minimize protecting group manipulations. Moreover, epoxides are readily prepared in optically pure form. Second, we desired access to both the natural 2*R*- and unnatural 2*S*-enantiomers of alkyl LPA and related analogs in order to examine the enantiospecificity of the biological responses. Third, for ease of synthetic manipulations, the deprotection of the penultimate dimethyl phosphate with trimethylsilane bromide[21,22] was selected to permit incorporation of an unsaturated alkyl chain as well as to reveal the charged phosphate in neutral condition at the final step of the synthesis. It was the first report that uses the dimethyl protecting group for the synthesis of alkyl LPA.

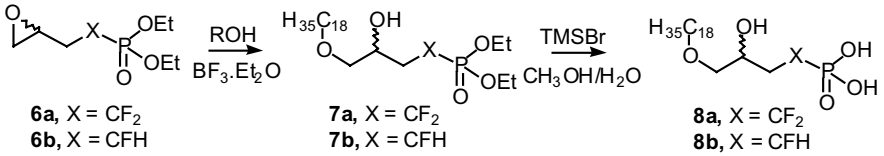
The primary alcohol (2*R*)-glycidol was phosphorylated with dimethylphosphoryl chloride in the presence of *t*-BuOK to give good yield of the protected glycidol 1-phosphate. Previously, Lewis acid-catalyzed epoxide opening of the glycidol derivatives, using hexadecanol and $BF_3{\cdot}Et_2O$ has been shown to proceed regio- and stereospecifically.[23,24] Thus, opening of the epoxide ring using catalytic $BF_3{\cdot}Et_2O$ (0.13 equiv) and serachyl alcohol (Z9-octadecenol) proceeded with complete regiospecificity to produce the dimethyl-protected lysoalkyl phosphatidic acid in excellent yield. Deprotection of protected phosphate **3a** gave target compound **4a** in an overall yield of 77% from (2*R*)-glycidol. The enantiomer **4b** was synthesized similarly from (2*S*)-glycidol (Scheme 1).

To determine the enantiomeric excess (ee) of the ring-opened products **3a** and **3b**, we prepared their (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (*R*)-(+)-MTPA esters.[25] The diastereomeric ratio of the resulting mixture was analyzed by ^1H and ^{19}F NMR. The NMR spectra indicated that the ee value of ring-opened products **3a**(**b**) was 97.8–98.6%. Considering the optical purity of the starting compounds, glycidol (99%, ee), the ring opening of the epoxide by serachyl alcohol proceeded without any detectable loss of chiral purity.

The isoelectronic and isosteric replacement of oxygen by difluoromethylene or monofluoromethylene in phosphate analogs confers metabolic stability and imparts



Scheme 1.



Scheme 2.

important features for receptor binding.[26,27] Recently, we designed and synthesized fluorinated LPA and PA analogues, which have been evaluated as LPA receptor agonists and lipid phosphate phosphatase inhibitors.[22,28–30] Since the phosphate moiety is readily hydrolyzed by the action of lipid phosphate phosphatases, the strategic substitution of oxygen by difluoromethylene or monofluoromethylene in alkyl LPA would be expected to prolong biological activity by altering pharmacokinetics, metabolism, and ligand binding. We planned to employ the ring-opening of terminal epoxide, diethyl 1-monofluoro-3,4-epoxy-butylphosphonate, and diethyl 1,1-difluoro-3,4-epoxy-butylphosphonate,[22,29] to obtain access to α-fluorinated methylene phosphate analogs of the alkyl LPA. Since the preparation of enantiomerically pure 1-fluorinated-3,4-epoxy-butylphosphonates has not been reported, we first attempted hydrolytic kinetic resolution (HKR) of racemic fluorinated epoxy to obtain enantiomeric pure epoxides. However, this method gave less than 89% ee for monofluorinated epoxides.[22] Thus, to access to α-fluorinated methylene phosphate analogs of alkyl LPA for initial biological evaluation, we employed racemic 1-fluorinated-3,4-epoxy-butylphosphonates and the corresponding racemic α-fluorinated analogs. Given the lack of enantioselective biological response to **4a** and **4b** as described below, this appeared to be an appropriate and expeditious strategy.

Thus, the ring-opening reaction of epoxides **3** by seryachyl alcohol catalyzed by $BF_3{\cdot}Et_2O$ gave the corresponding diethyl-protected lysoalkyl phosphatidic acid **7** in 64–69% yield. Initial attempts to open the epoxide by using catalytic $BF_3{\cdot}Et_2O$ only afforded low yield (20%) of product. However, treatment of the fluorinated epoxides **6a** and **6b** with excess $BF_3{\cdot}Et_2O$ (4.0 equiv) gave good yields of product. It is possible that coordination of BF_3 with the fluorine(s) in the fluoromethylene-containing epoxides competes with binding between BF_3 and the epoxide. After dealkylation of phosphonic acid diethyl esters with excess bromotrimethylsilane (10.0 equiv) for 8 h at rt, α-fluorinated alkyl LPA analogs **8a** and **8b** were obtained in essentially quantitative yield (Scheme 2).

2. Biological evaluation

We previously tried to detect LPA_1 activation using a calcium release assay in receptor-transfected Sf9 insect cells. However, binding of LPA to LPA_1 receptor did not result in the mobilization of intracellular calcium since LPA_1 is not coupled to $G_{i/q}$ type G-protein in Sf9 insect cells.[31] Actually, the LPA_1–LPA_2 chimeric receptor, in which seven-transmembrane domain of LPA_1 is fused with carboxy terminus of LPA_2, does mediates a small calcium mobilization.[32] However, the sensitivity is too low for evaluation of LPA_1 activation. However, we recently showed that LPA-induced cell migration in various cell types is LPA_1-dependent.[33] This assay was found to be more sensitive than the system using LPA_1–LPA_2 chimeric receptor calcium assay, and was thus employed herein for comparing biological activities of the synthetic analogs. Thus, in this study we utilized the cell migration using the LPA_1-expressing three cell lines (203G, PC-3, MDA-MD-231).

Cell migration was measured in a Boyden-chamber (pore size 8 μm) as described.[33] Filters were pretreated with phosphate-buffered saline (PBS) containing bovine plasma fibronectin overnight at 4 °C, rinsed once with PBS, and placed on the lower chamber containing serum-free DMEM supplemented with agonists. Cells suspended in serum-free DMEM containing 0.1% fatty acid-free bovine serum albumin were added to the upper chamber (1×10^5 cells/well). Cells were allowed to migrate for 4 h at 37 °C. Non-migratory cells were removed from the top filter surface using a cotton swab. Migrated cells, attached to the bottom surface, were fixed in 3% formaldehyde/PBS, permeabilized in methanol, stained with crystal violet, and evaluated by measuring optical densities at 590 nm.

The enantiomeric alkyl LPA analogs **4a** and **4b**, the racemic difluoromethylene phosphonate **8a** and the monofluoromethylenephosphonate **8b** were evaluated for stimulation of cell migration activity in 203G murine glioma cells, PC-3 human prostate cancer, and MDA-MB-231 human breast cancer cell lines. LPA_1 is responsible for LPA-induced cell migration in each of these cell lines,[33] which predominantly express the LPA_1 receptor. Figure 2 shows that the enantiomeric alkyl LPAs **4a** and **4b** both induced cell migration with the same potency as natural acyl LPA (18:1). Since LPA_3 receptor is not expressed at all in PC-3, 203G or MDA-MB-231cells, we can conclude that LPA-induced cell migration in these cells is LPA_1-dependent. The LPA_1 receptor-specific effect can be abolished by addition of an LPA_1 antagonist-Ki16425.[33,34] Therefore, **4a** and **4b** are potent agonists for the LPA_1 receptor.

Figure 3 illustrates the mobilization of calcium release via activation of human LPA_2 and LPA_3 receptors. In these experiments, Sf9 insect cells, which are totally irresponsive to LPA, were infected with baculovirus encoding human LPA_2 and LPA_3, respectively.[31] Of importance, both **4a** and **4b** were agonists for both receptor subtypes, with almost equivalent potency to acyl LPA (18:1).[42] In the bottom panel of Figure 3,

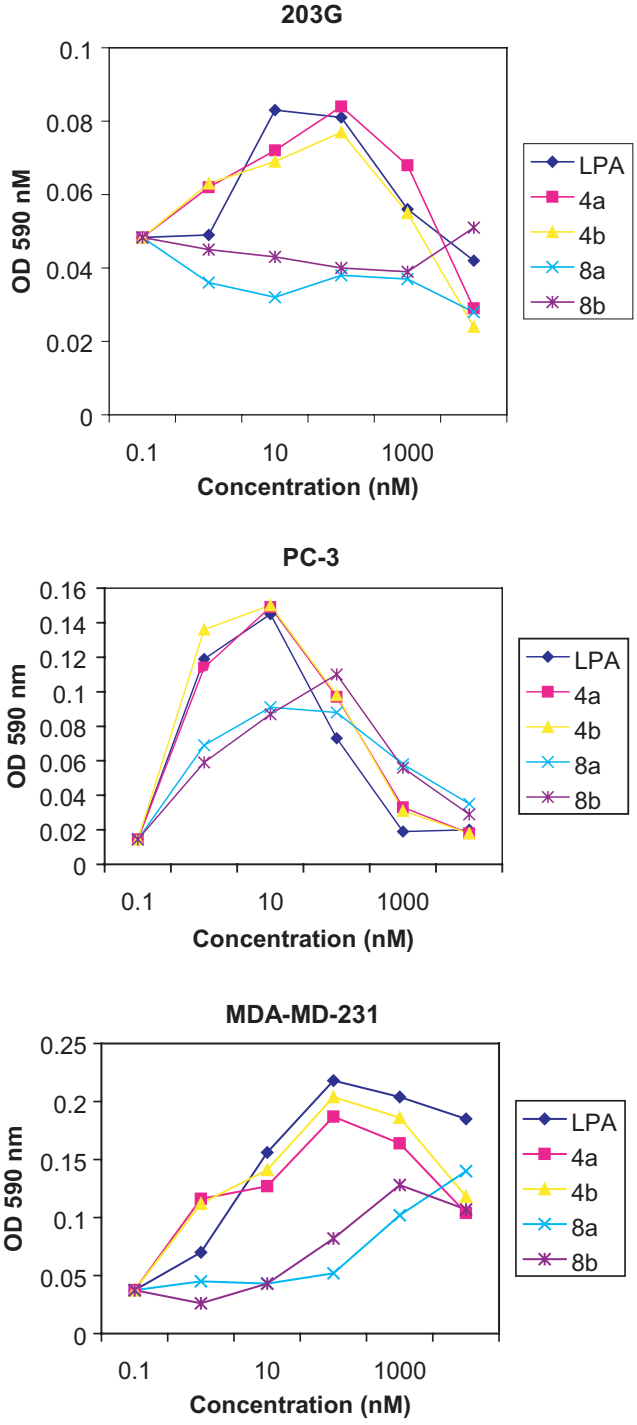


Figure 2. Alkyl LPAs and their analogs induced cell migration.

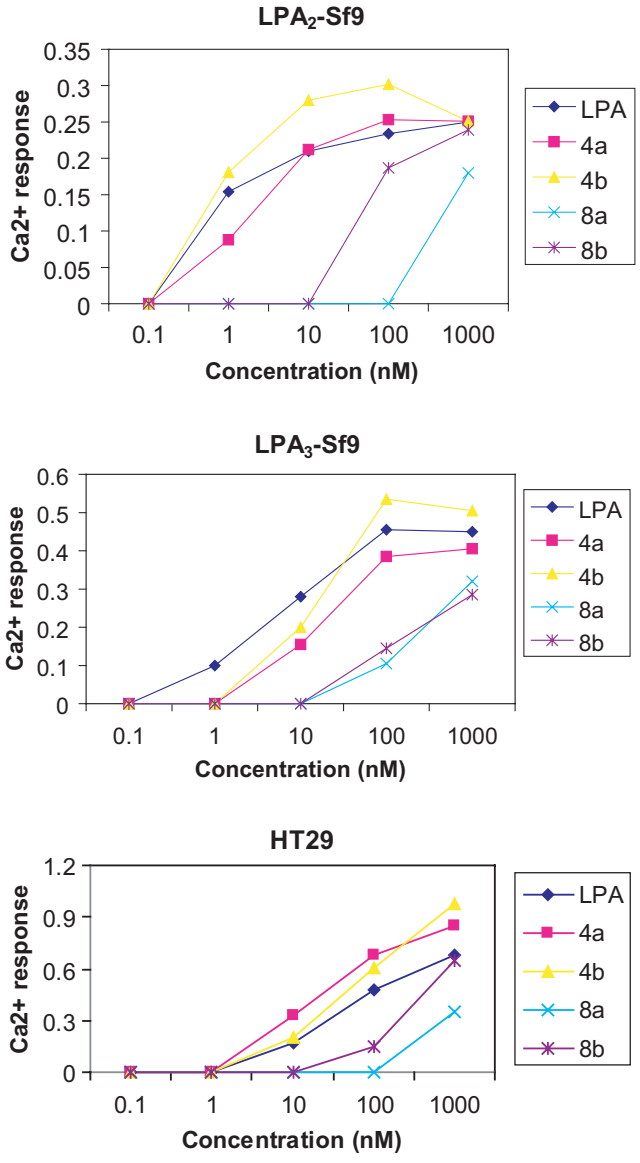


Figure 3. Ca^{2+} release (peak concentration) by Sf9 cells-expressing human $LPA_{2/3}$ receptors and HT29 cells.

HT29 cells, which predominantly expressed the LPA_2 receptor,[33] showed greater responses to **4a** and **4b** than to 18:1 acyl LPA. Thus, taken together, **4a** and **4b** were non-selective, non-enantioselective, agonists for each of the three LPA receptor subtypes. An earlier enzymatic preparation of alkyl LPA from PAF (platelet-activating factor). using PLD followed by PAF acetylhydrolase[32] gave incomplete deacylation. Thus it is possible that we underestimated the activity of alkyl LPA in this previous work.[32] Using the totally synthetic alky LPA analogs, we now clearly show that alkyl LPA is equipotent to natural LPA toward LPA_{1-3}.

We recently showed that monofluorinated form of 1-oleoyl-LPA is a potent and selective agonist for LPA_3.[30] Indeed, replacement of *sn*-3 oxygen of natural LPA by monofluoromethylene resulted in an LPA_3-selective agonist with over 100 times the potency of natural LPA. However, the fluoromethylene phosphonate analogs of alkyl LPA **8a** and **8b** were found to be poor agonists for $LPA_{1/2/3}$ receptors. In effect, the fluoromethylene phosphonates with *sn*-1 alkyl rather than acyl chains were not be recognized by LPA receptors, in contrast to the similarly modified fluoromethylene phosphonates analogs of acyl LPA and PA.[30,35] In a preliminary experiment, we examined the stability of these LPA analogs. We applied the LPA analogs to the cell culture, and after several hours we measured the activity remains

by either bioassay or mass spectrometry. We found **8a** and **8b** are much more stable than **4a** and **4b**. However, the biological activities showed that **4a** and **4b** were more potent than **8a** and **8b** (Figs. 2 and 3). While it is possible the activity of **8a** and **8b** toward LPA_1 could be overestimated, this does not alter our conclusion that **4a** and **4b** are better ligands for LPA_1.

OMPT, a stabilized phosphorothioate analog of LPA, was found to be a potent and long-lived LPA_3-specific agonist.[36] Synthesis of the 2*R* and 2*S* enantiomers of the 2-methoxy group of OMPT identified the unnatural 2*S* enantiomer as the more active isomer.[37] Since **4a** and **4b** are enantiomers, it is quite interesting that both are active agonists for $LPA_{1/2/3}$ receptors. It is likely that these alkyl LPAs will help elucidate the recognition mechanism of LPA and its analogs. Ligand recognition by GPCRs and most other binding or catalytic proteins generally shows a preference for the naturally-occurring enantiomer. However, recognition of LPA by its GPCRs has been cited as an exception, as both the natural L (*R*) and unnatural D (*S*) stereoisomers of LPA are equally active in some bioassays.[38] In contrast to the enantiomers of natural LPA, however, the activities of LPA analogs based on non-glycerol backbones show strong enantioselectivity. For example, *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.[39] Computational modeling and experimental results demonstrates that LPA pharmacophore has three substructural domains that are crucial for ligand–receptor interactions: (i) a negative charge (e.g. the phosphate head group), (ii) a hydrogen-bond acceptor in backbone or linker group (e.g. a glycerol or glycerol derivative), and (iii) a long hydrophobic tail (e.g. acyl or alkyl group).[40] More recently, it was demonstrated that LPA receptor subtype-selective recognition could be finely tuned by changing the relative three-dimensional arrangement of pharmacophores attached to a carbohydrate core.[41] Enantioselective responses will continue to be observed as non-LPA-like isoform-selective agonists and antagonists are developed.

In summary, we have developed an improved synthesis of alkyl LPA using an epoxide opening reaction. Moreover, we have applied this method to the synthesis of fluorinated alkyl LPA analogs. Efforts to prepare thiophosphate alkyl LPA analogs and alkenyl LPAs are in progress and will be reported in due course. This approach provides an efficient and convenient strategy for the production of alkyl LPA analogs. Alkyl LPA analogs induced cell migration with the same potency as natural oleoyl LPA, but this response was not enantioselective. An important finding is that the enantiomeric alkyl LPAs were potent agonists for all three LPA receptor isoforms.

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42. All new compounds were characterized by ^{1}H, ^{13}C, ^{31}P, (^{19}F) NMR, MS, and HRMS. Selected data: **4a**: ^{1}H NMR (CD_3OD): 5.33 (m, 2H), 4.00 (m, 1H), 3.92 (m, 2H), 3.47 (m, 4H), 2.02 (m, 4H), 1.56 (m, 2H), 1.29 (m, 22H), 0.89 (t, $J = 6.4$ Hz, 3H). ^{13}C NMR (CD_3OD): 129.47 (s), 128.81 (s), 70.67 (s), 70.50 (s), 68.48 (d, $J = 7.74$ Hz), 66.78 (d, $J = 5.43$ Hz), 31.06 (s), 28.87 (s), 28.84 (s), 28.80 (s), 28.77 (s), 28.71 (s), 28.64 (s), 28.55 (s), 28.46 (s), 28.35 (s), 26.16 (s), 26.13 (s), 25.20 (s), 21.74 (s), 12.51 (s). ^{31}P NMR (CD_3OD): 1.31 (s). MS (CI) *m/z* 407.3 (M^+ + 2-OH, 100.00). HRMS, M^+ + 2-OH, found: 407.2362, calcd for $C_{21}H_{44}O_5P$, 407.2373. $[\alpha]_D^{20} - 3.0$ (0.26, MeOH). **8a**: ^{1}H NMR (CD_3OD): 5.33 (m, 2H), 4.17 (m, 1H), 3.45–3.40 (m, 4H), 2.30–2.17 (m, 2H), 2.03 (m, 4H), 1.59 (m, 2H), 1.25 (m, 22H), 0.84 (t, $J = 6.4$ Hz, 3H).^{19}F NMR (CD_3OD): −108.33 (ddt, $J = 290.90$, 94.83, 22.20 Hz, 1F), −113.62 (ddt, $J = 290.90$, 94.83, 22.20 Hz, 1F). ^{13}C NMR (CD_3OD): 128.85 (s), 128.84 (s), 119.82 (td, $J = 260.45$, 215.20 Hz), 73.81 (s), 70.47 (s), 64.28 (m), 38.71 (m), 31.63 (s), 31.08 (s), 28.87 (s), 28.85 (s), 28.77 (s), 28.72 (s), 28.64 (s), 28.62 (s), 28.46 (s), 26.14 (s), 26.11 (s), 21.15 (s), 12.47 (s). MS (CI) *m/z* 439.2 ($M^+ - OH$, 3.34). HRMS, $M^+ - OH$, found: 439.2723, calcd for $C_{22}H_{42}PF_2O_4$, 439.2789.