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Activation of mTOR signaling by novel fluoromethylene phosphonate analogues of phosphatidic acid

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Abstract—Phosphonate analogues of phosphatidic acid (PA) were synthesized in which the bridging oxygen was replaced by an α-monofluoromethylene (–CHF–) or α-difluoromethylene (–CF_2–) moiety using hydrolytic kinetic resolution (HKR) of a racemic epoxide as the key step. Since PA activates signaling in the mTOR (mammalian target of rapamycin) pathway, these metabolically stabilized PA analogues were evaluated in quiescent HEK 293 cells. Most of these analogues surpassed PA in activating S6 kinase, a downstream target of mTOR signaling. The unnatural (2*R*) analogues were more slightly active than the natural (2*S*) enantiomers for both the mono- and difluoromethylene phosphonates.

1. Introduction

Phosphatidic acid (PA) constitutes a minor portion of the total phospholipid pool in resting cells. As an intermediate in phospholipid synthesis and as a target for lipid phosphate phosphatases, PA is a transient species. In addition, PA is an intracellular lipid second messenger that regulates a growing list of signaling proteins, including several protein kinases and phosphatases.[1] PA has also been implicated as a mediator of the mitogenic action of various growth factors and hormones in mammalian cells.[1] The concentration of PA in quiescent cellular membranes is less than 5% of that of phosphatidylcholine (PC);[2] stimulation of mitogenesis activates phospholipase D (PLD) giving rise to a corresponding increase in PA.[1]

Recently, PA and PLD have been revealed to be a critically involved in the mTOR pathway.[3,4] The mammalian target of rapamycin (mTOR; also named FRAP or RAFT1)[5,6] belongs to the family of phosphatidylinositol kinase-like kinases (PIKK).[7] The mTOR homologues in *Saccharomyces cerevisae*, Tor1p and Tor2p, control a wide range of growth-related cellular processes, including transcription, translation, and reorganization of the actin cytoskeleton.[8] The mTOR pathway similarly has pleiotropic and essential roles in the regulation of mammalian cellular functions.[9] For example, mTOR regulates translation initiation; its best known downstream effectors include the ribosomal subunit S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), two regulators of mitogen-stimulated translation initiation.[10] Both activation of S6K1 and phosphorylation of 4E-BP1 are stimulated by mitogens; mTOR is required for these responses.[11] Moroever, PA has been identified as a crucial mediator for mitogenic activation of mTOR. Extracellular concentrations of 100 μM PA stimulate S6K1 activation and 4E-BP1 phosphorylation in serum-starved human embryonic kidney (HEK) 293 cells.[3]

Interest in phosphatase-resistant phosphonolipids as phospholipid analogues has grown substantially with the recognition that lysophosphatidic acid (LPA) is an important mitogenic signal in ovarian cancer and in normal cell proliferation and migration.[12–16] In particular, analogues of natural phospholipids have been prepared in which the *sn*-3 oxygen of the diacylglycerol moiety was replaced with a –CH=CH–,[17] –CH_2–,[12] or a –CF_2– moiety.[18] The replacement of the phosphate

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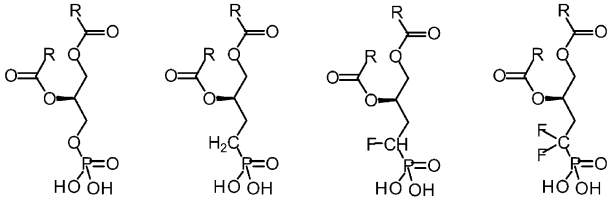


Figure 1. Phosphatidic acid (left, PA) and its phosphonate analogues.

group with a phosphonate reduces hydrolysis in biochemical, cell biological, and whole animal bioassays.[17,19] Herein we describe the synthesis and biological evaluation of enantiomerically homogeneous monofluoromethylene and difluoromethylene phosphonate analogues of di-oleoyl PA (Fig. 1)

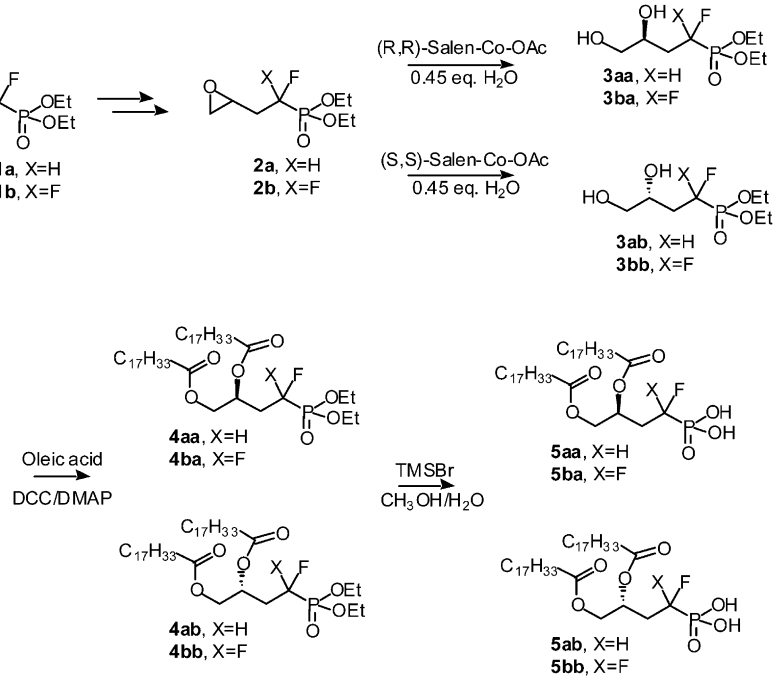
2. Synthetic chemistry

The isoelectronic and isosteric replacement of oxygen by difluoromethylene or monofluormethylene in phosphate analogues confer metabolic stability and impart important features for receptor binding.[20,21] Recently, we prepared α-fluorinated phosphonates analogues of LPA and found several to be isoform-selective agonists of the G-protein coupled LPA receptors.[18,22–24] Since the phosphate moiety of LPA is readily removed by lipid phosphate phosphatases, the phosphonates showed prolonged biological activity through alteration of pharmacokinetics, metabolism, and ligand binding. Consistent with our earlier strategy in preparing LPA analogues, we employed the ring-opening of one of two terminal epoxides, either diethyl 1-fluoro-3,4-epoxybutylphosphonate or diethyl 1,1-difluoro-3,4-epoxybutylphosphonate[18,23] as precursors to the desired α-fluorinated PA analogues.

The HKR (hydrolytic kinetic resolution) reaction[25,26] uses enantiomeric cobalt salen complexes as catalysts in order to convert terminal epoxides into enantiomerically-enriched diols. Scheme 1 shows the synthetic route for these fluorinated diol analogues required for PA analogue preparation. First, the desired epoxide was prepared from commercially-available diethyl dibromofluoromethyl phosphonate. Next, the reaction of racemic epoxides **2a** or **2b** with 0.45 equiv of H_2O in a minimum volume of THF, in the presence of 1.0 mol% of (*R*,*R*)-Salen-Co-OAc gave (*S*)-configuration diols **3aa** and **3ba** in 91–99% ee and 69–73% isolated yield. The ee values were determined by NMR of the MPTA esters. Similarly, using (*S*,*S*)-Salen-Co-OAc as the catalyst resulted in the opposite configuration® of diols **3ab** and **3bb** in 89–99% ee and 69–70% yield. Then, treatment of each of the diols with 2.2 equiv of oleic acid and 2.4 equiv of DCC and catalytic amount of DMAP (0.5 equiv) in CH_2Cl_2 at rt gave the corresponding diacylated protected PA analogue **4** in good yield (73–89%) after chromatography. Finally, each of the PA analogues **5** was obtained in essentially quantitative yield after dealkylation of the corresponding diethyl phosphonate **4** with excess bromotrimethylsilane (10.0 equiv) for 8 h at rt and subsequent hydrolysis by addition of 5% aq methanol.

3. Biological evaluation

HEK293 cells were stimulated with either 100 μM of PA or one of the dioleoyl PA phosphonates **5** (resuspended in buffer, without carrier) for 15 min or for 45 min. Cells



Scheme 1. Synthesis of PA phosphonates.

were then lysed, the mTOR downstream effector S6K1 was immunoprecipitated, and S6K1 kinase activity was measured.[3] Figure 2 shows the data from the 15-min stimulation and Figure 3 shows the 45-min stimulation. Each bar represents the average of at least three independent experiments. All data are normalized to the control 100 μM PA stimulation for a given experimental set, with 1-palmitoyl-2-oleoyl-*sn*-2-glycerol-3-phosphatidic acid used as the PA control. Each of the four fluorinated PA phosphonates **5** stimulated S6K1 to a similar or slightly greater extent relative to PA in quiescent HEK 293 cells. The concentrations of PA (100 μM) used in this study are similar to those used in other mammalian cell cultures reported in the literature.[27,28] We did not observe cell lysis or any other morphological changes under these conditions. Although we cannot definitively rule out the possibility of a stress response in the cells, the activation of the mTOR pathway appears to be specific for PA, as several other lipids including PE and PC similarly administered at the same concentrations had no effect on S6K1 activity.

The experimental results indicated that shorter stimulation times gave higher S6K1 activity for the PA analogues,

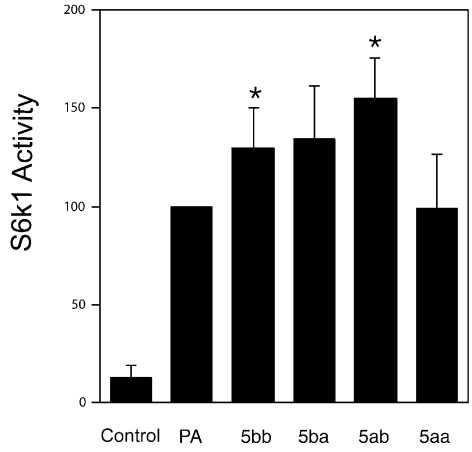


Figure 2. Fluorinated PA analogues stimulated mTOR signaling in quiescent HEK 293 cells at 15 min. *Indicates significant difference ($P < 0.05$) when compared to the data of PA.

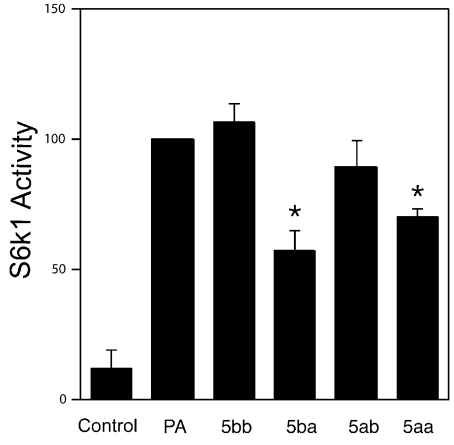


Figure 3. Fluorinated PA analogues stimulated mTOR signaling in quiescent HEK 293 cells at 45 min.

consistent with our speculation that PA is required during the early stage of mTOR activation. The phosphatase-resistant properties of the phosphonates are more crucial at this stage. Interestingly, a preliminary structure–activity relationship emerged that connected the phosphonate chemistry and the measured S6K1 activity. First, there was little difference between the difluorinated and monofluorinated PA analogues in the activation of the mTOR pathway, as both analogues were effective in increasing S6K1 activity. Second, the configuration at the *sn*-2 position had a clear effect on S6K1 activity. That is, the fluoromethylenephosphonate PA analogues with the unnatural (2*R*) configuration at *sn*-2 (**5ab** and **5bb**) were more active than those with the natural (2*S*) configuration (**5aa** and **5ba**). This result has two possible interpretations. The fluorinated (2*S*) PA analogues with could be more readily hydrolyzed by esterases or phophospholipases, Alternatively, the unnatural analogues may simply bind and activate mTOR more effectively. This difference becomes more obvious in the longer 45-min stimulation (Fig. 3), in the stimulation by **5aa** and **5ba** diminished relative to the activity at 15 min (Fig. 2), while **5ab** and **5bb** retained activities equivalent to the PA control.

While the mechanism of PA entry into the and the intracellular concentration of PA are not clear; neither can we rule out potential metabolites as mediators of mTOR signaling. However, four lines of evidence support the notion that PA and PLD1 are critical mediators of mTOR signaling: (i) mTOR signaling correlates with cellular PA levels; (ii) PA binds in vitro to a domain in mTOR; (iii) mitogens activate the mTOR target S6K1;[3] and (iv) PLD1 is required for mitogenic activation.[4] Furthermore, several PA metabolites, including LPA and DAG, have been examined and none activates mTOR signaling (Y. Fang and J. Chen, unpublished observations). The PA analogues described here all bind to the FRB domain in mTOR with similar affinity as PA (data not shown). Given that the binding assay is only semi-quantitative, the affinity of these analogues for FRB is consistent with their ability to activate mTOR signaling.

In conclusion, we present a concise route for the synthesis of enantiopure monofluorinated and difluorinated PA phosphonates from racemic epoxides. The synthetic strategy uses chiral salen–Co catalyzed HKR reactions of fluorinated epoxides. Additionally, these fluorinated PA analogues stimulated mTOR signaling to a similar or slightly higher extent as PA in quiescent HEK 293 cells. The SAR observed will facilitate further understanding of the mechanism by which PA activitates the mTOR signaling pathway.

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