

## Synthesis and Biological Activity of Phospholipase C-Resistant Analogues of Phosphatidylinositol 4,5-bisphosphate

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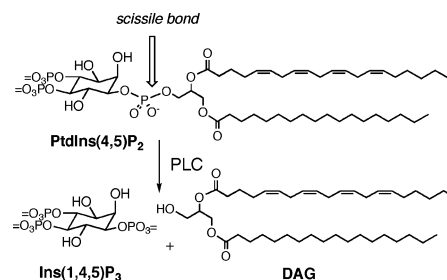
The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) is an important regulator of cytoskeletal organization during a plethora of cellular functions, such as vesicle trafficking, endocytosis, phagocytosis, focal adhesion formation, and cell migration.<sup>1</sup> PtdIns(4,5)P<sub>2</sub> binds to and affects the function of many actin-binding and actin-remodeling proteins<sup>2–4</sup> and is a cofactor in enzyme activation.<sup>5</sup> In addition, PtdIns(4,5)P<sub>2</sub> regulates the activity of many ion channels and transporters.<sup>6,7</sup> PtdIns(4,5)P<sub>2</sub> is also the source of three second messengers: Ins(1,4,5)P<sub>3</sub>, diacylglycerol (DAG),<sup>8,9</sup> and PtdIns(3,4,5)P<sub>3</sub>.<sup>10</sup> In many cases, it is the decrease in PtdIns(4,5)P<sub>2</sub>, resulting from hydrolysis by phospholipase C (PLC) (Scheme 1), and not the increase in Ins(1,4,5)P<sub>3</sub> and DAG that constitutes the physiologically relevant signal.<sup>11,12</sup> Hydrolysis of PtdIns(4,5)P<sub>2</sub> causes TRP channels to lose some activity.<sup>13–19</sup> Moreover, addition of PtdIns(4,5)P<sub>2</sub> restores sensitivity of TRPM4 and TRPM5 to activation by Ca<sup>2+</sup> and restores the sensitivity of TRPM8 and TRPV1 to thermal and chemical stimuli.<sup>15,16,18,19</sup>

The availability of a metabolically stabilized analogue of PtdIns(4,5)P<sub>2</sub>, that is, one that lacks the scissile P–O bond and thus could not be hydrolyzed by PLC activity, would have many applications in understanding the role of PtdIns(4,5)P<sub>2</sub> in cell physiology.  $\alpha$ -Fluoroalkylphosphonates have emerged as important nonhydrolyzable mimics for phosphoesters in the synthesis of biologically active “unnatural products”.<sup>20–23</sup> Herein we describe the first asymmetric total synthesis of isosteric and isoelectronic phosphonate analogues **1–5** of PtdIns(4,5)P<sub>2</sub> that cannot be hydrolyzed by PLC. The synthesis employs a Pd(0) coupling not previously exploited in phospholipid or phosphoinositide synthesis. Furthermore, we demonstrate that both saturated and unsaturated  $\alpha$ -fluorophosphonate analogues can substitute for exogenous PtdIns(4,5)P<sub>2</sub> in restoring the sensitivity of the TRPM4 channel to Ca<sup>2+</sup>.

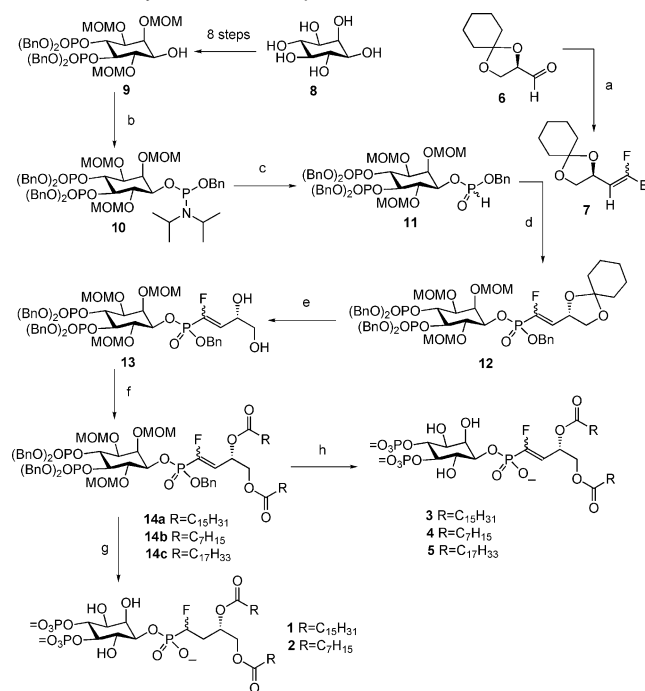
The synthetic sequence to the stabilized analogues **1–5** of PtdIns(4,5)P<sub>2</sub> is illustrated in Scheme 2. A variety of attempts to connect the intermediate **9**<sup>24</sup> with a fluoromethylenephosphonic acid synthon<sup>21</sup> failed. Eventually, we turned to the Pd(0)-catalyzed coupling of an *H*-phosphite with a vinyl bromide in order to form the desired C–P linkage. Thus, coupling the protected inositol **9** with dibenzyl *N,N*-diisopropylphosphoramidite gave the phosphoramidite intermediate **10**, which was converted to *H*-phosphonate **11** in 76% isolated yield in two steps.<sup>25</sup> The 1-bromo-1-fluoroolefin **7** (~1:1 *E/Z*) was separately prepared via a Et<sub>2</sub>Zn-promoted olefination reaction<sup>26</sup> of CBr<sub>3</sub>F/PPh<sub>3</sub> with glyceraldehyde **6** in excellent yield.

Few examples exist of Pd(0)-catalyzed formation of P–CF bonds, and in our hands, only traces of coupled compound **12** with a majority of the P–O cleaved compound **9** were obtained under standard conditions using Et<sub>3</sub>N or K<sub>2</sub>CO<sub>3</sub> as base. It appeared that

**Scheme 1.** Phospholipase C Catalyzes Hydrolysis of PtdIns(4,5)P<sub>2</sub> to Two Second Messengers, Ins(1,4,5)P<sub>3</sub> and Diacylglycerol



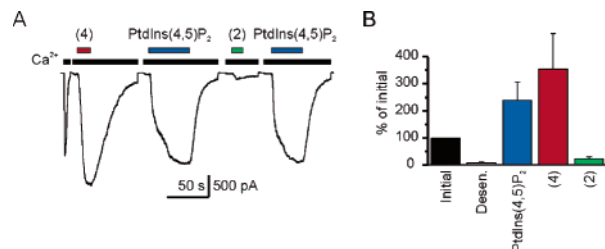
**Scheme 2.** Synthesis of Phosphonates **1–5**<sup>a</sup>



<sup>a</sup> Conditions: (a) CBr<sub>3</sub>F, PPh<sub>3</sub>, Et<sub>2</sub>Zn, THF, 76%; (b) (BnO)<sub>2</sub>P(NPr<sub>2</sub>-i)<sub>2</sub>, *N,N*-diisopropylethylammonium <sup>1</sup>H-tetrazole, CH<sub>2</sub>Cl<sub>2</sub>, rt; (c) H<sub>2</sub>O, 1*H*-tetrazole, rt, 1 h, CH<sub>2</sub>Cl<sub>2</sub>, 76% for two steps; (d) Pd(OAc)<sub>2</sub>, dppf, propylene oxide, THF, 70 °C, 62%; (e) 60% aqueous TFA, THF, 0 °C, 1 h, 86%; (f) EDCI, DMAP, fatty acid, CH<sub>2</sub>Cl<sub>2</sub>, rt; (g) H<sub>2</sub>, Pd/C, MeOH, 6 h, EtSH; (h) TMBR/TMSI (5:1), rt, 1.5 h; MeOH, 1 h.

the rate of decomposition was faster than the rate of coupling for the more hindered *H*-phosphonate **11**. To overcome this problem, we selected propylene oxide as a weak Lewis base and an effective scavenger of HBr.<sup>27</sup> Using this modification, treatment of the *H*-phosphonate **11** with Pd(OAc)<sub>2</sub>/dppf/propylene oxide in THF at 70 °C led to the formation of  $\alpha$ -fluorovinylphosphonate **12** in 62%

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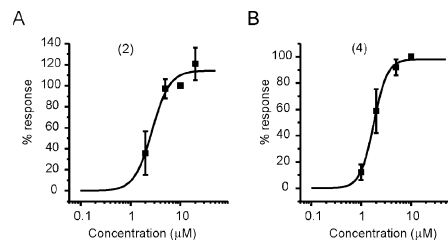
**Figure 1.** PtdIns(4,5)P<sub>2</sub> and analogues **2** and **4** restore TRPM4 currents following desensitization. (A) An excised inside-out patch from Chok1 cell expressing mouse TRPM4 (mTRPM4) shows activation and fast rundown of an inward current in the presence of 100  $\mu$ M Ca<sup>2+</sup> and recovery by dioctanoyl-PtdIns(4,5)P<sub>2</sub> and analogues **2** and **4** ( $V_m$  = 80 mV). (B) Initial magnitudes of the mTRPM4 currents, currents after rundown, and currents after recovery in response to 10  $\mu$ M each of PtdIns(4,5)P<sub>2</sub>, **2**, and **4** (averages,  $n$  = 8).

yield. Acetal **12** was selectively deprotected by treatment with 60% aqueous trifluoroacetic acid in tetrahydrofuran at 0 °C to give diol **13**. Next, acylation of **13** with either octanoic acid, palmitic acid, or oleic acid provided the fully protected phosphonates **14a**, **14b**, and **14c** in 80, 73, and 82% yields, respectively. Hydrogenolysis of **14a** and **14b** removed the benzyl groups, and then reaction with ethanethiol removed the MOM groups to give the  $\alpha$ -fluoromethylenephosphonate analogues **1** and **2**.<sup>28</sup> The  $\alpha$ -fluorovinylphosphonates **3**–**5**<sup>28</sup> were obtained by deprotection of benzyl and MOM groups simultaneously with TMSBr/TMSI (5:1).

Recently, the hydrolysis of the water-soluble dioctanoyl PtdIns(4,5)P<sub>2</sub> was found to be important in the desensitization of TRPM4 channel (activated by cytoplasmic Ca<sup>2+</sup>). Exogenous PtdIns(4,5)P<sub>2</sub> could restore the sensitivity of TRPM4 channels to Ca<sup>2+</sup>, demonstrating that PtdIns(4,5)P<sub>2</sub> was a general regulator for the gating of TRPM4 ion channels.<sup>15</sup> The ability of the two dioctanoyl-PtdIns(4,5)P<sub>2</sub> analogues **2** and **4** to restore TRPM4 currents following rundown is shown in Figure 1. Both analogues restored TRPM4 sensitivity following desensitization, but the  $\alpha$ -fluorovinylphosphonate **4** was more potent. Indeed, the unsaturated phosphonate **4** was even more effective than the hydrolyzable dioctanoyl-PtdIns(4,5)P<sub>2</sub> at restoring TRPM4 sensitivity. This provides further evidence that the regulation of TRPM4 by dioctanoyl-PtdIns(4,5)P<sub>2</sub> and the ability of dioctanoyl-PtdIns(4,5)P<sub>2</sub> to restore TRPM4 currents following rundown is not due to effects of products of PLC hydrolysis.<sup>15</sup>

To determine sensitivity of TRPM4 currents to **2** and **4**, we measured the effects of varying concentrations of both compounds on the recovery of TRPM4 currents in excised inside-out patches evoked in response to 100  $\mu$ M Ca<sup>2+</sup> (Figure 2). Maximal recovery of TRPM4 currents was observed upon reaching 10  $\mu$ M for both **2** and **4**, and half-activation was observed at  $\sim$ 2  $\mu$ M for both compounds, which is similar to the concentration of PtdIns(4,5)P<sub>2</sub> that promoted half-activation of TRPM4 (6  $\mu$ M).<sup>15</sup> The difference between the effectiveness of **2** and **4** in restoring TRPM4 currents (Figure 1) appears to result from differential abilities to promote activation of the TRPM4 channel. Taken together, these data suggest that the  $\alpha$ -fluorovinylphosphonate **4** is a biologically active, long-lived mimic of PtdIns(4,5)P<sub>2</sub>.

In conclusion, we developed an efficient synthesis of two nonhydrolyzable PtdIns(4,5)P<sub>2</sub> analogues, and we showed that  $\alpha$ -fluorovinylphosphonate **4** optimally restored the sensitivity of



**Figure 2.** Dose-response for recovery of TRPM4 currents by **2** and **4**. After TRPM4 desensitization, recovery was assessed. Data were normalized to the response to 10  $\mu$ M of each analogue in the same patch. (A) Averaged data ( $n$  = 5) for recovery of TRPM4 currents by **2** ( $EC_{50}$  =  $2.7 \pm 0.6$   $\mu$ M and  $n_H$  =  $2.5 \pm 1.2$ ). (B) Averaged data ( $n$  = 6) for **4** ( $EC_{50}$  =  $1.8 \pm 0.1$   $\mu$ M and  $n_H$  =  $3.2 \pm 0.5$ ).

TRPM4 currents. These results suggest that metabolically stabilized analogues of PtdIns(4,5)P<sub>2</sub> will have a wide variety of applications in separating the role of the phosphoinositide per se from activities that result when Ins(1,4,5)P<sub>3</sub>, DAG, Ca<sup>2+</sup>, or other downstream signals are generated from the hydrolysis of PtdIns(4,5)P<sub>2</sub> by PLC.

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**Supporting Information Available:** Experimental details for synthesis and characterization of new compounds, and protocols for TRPM4 channel activity measurement. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- Note on stereochemistry. Both compounds **1** and **2** are inseparable mixtures of diastereomers at the C–F stereocenter, and the chiral phosphorus atom is racemic. Similarly, compounds **3**, **4**, **5**, and **12**–**14** are inseparable *E/Z* mixtures.

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