Synthesis and Biological Evaluation of Imidazole-Bearing α-Phosphonocarboxylates as Inhibitors of Rab Geranylgeranyl Transferase (RGGT)

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In memory of Prof. Henryk Krawczyk

Rab geranylgeranyl transferase (RGGT) is an interesting therapeutic target, as it ensures proper functioning of Rab GTPases, a class of enzymes responsible for the regulation of vesicle trafficking. Relying on our previous studies, we synthesized a set of new α -phosphonocarboxylic acids as potential RGGT inhibitors, with emphasis on the elaboration of imidazole-containing analogues. We identified two compounds with activity similar to that of previously reported RGGT inhibitors, showing structural similarity to imidazo[1,2-*a*]pyridine-containing analogues in terms of their substitution pattern. Interestingly, analogues of the N-series, derived from another phosphonocarboxylate RGGT inhibitor, 2-fluoro-3-(1*H*-imidazol-1-yl)-2-phosphonopropanoic acid, turned out to be inactive in our model, indicating that an additional substituent localized at positions C2 or C4 of the imidazole ring, may adversely affect the potency against the targeted enzyme.

Introduction

Rab geranylgeranyl transferase (RGGT, Rab GGTase, GGT-II) is responsible for post-translational prenylation of Rab GTPases, enzymes from the Ras superfamily of small GTP-binding proteins, which are regulators of vesicle trafficking and their secretion into the extracellular matrix. Most Rab GTPases are modified by RGGT by formation of thioether bonds between two C-terminal cysteines and lipophilic geranylgeranyl chains (Figure 1). The abnormal activity of RGGT and some Rab proteins is associated with a number of diseases, including neurodegenerative and infectious disorders as well as several types of cancer.^[1-3]



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Figure 1. The isoprenoid biosynthesis pathway.

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The effect of disrupting geranylgeranylation is also associated with inhibition of upstream enzymes of the isoprenoid biosynthetic pathway, such as farnesyl pyrophosphate synthase (FPPS), whereas alternative prenylation of the oncogenic K-Ras GTPase by geranylgeranyl transferases might be responsible for the low cytotoxicity of farnesyl transferase (FT) inhibitors and their failure in clinical trials.^[4,5]

Numerous RGGT inhibitors have been developed in recent years, with possible application in the therapies of diverse dis-



eases.^[6-10] α -Phosphonocarboxylates (PCs) constitute one class of RGGT inhibitors, with the most active analogues containing imidazole in their structure, either in the N-substituted form (Figure 1: compound F-ZoIPC)^[11] or as built into the imidazo[1,2-*a*]pyridine ring (Figure 1: compound 3-IPEHPC and its analogues).^[12-14] The N-substituted imidazole ring seems to be advantageous for inhibitory activity against RGGT, as it is also present in the most potent RGGT inhibitors reported to date, bearing a tetrahydrobenzodiazepine scaffold.^[8]

In our previous studies we evaluated the influence of a substituent's type and localization in imidazo[1,2-*a*]pyridine on activity toward RGGT.^[14] We showed that the introduction of a substituent of different size, geometry, and electronegativity at position C6 of this heterocycle results in increased inhibitor activity, whereas substitution at any other location abolishes the potency of such analogues.

Our current efforts are focused on determining the structure-activity relationship for analogues of 2-fluoro-3-(1*H*-imidazol-1-yl)-2-phosphonopropanoic acid (F-ZoIPC). We studied both the influence of the point of attachment of α -phosphonopropionic acid to the imidazole ring (either via N- or C-substitution), as well as the effect of the substituent's localization in the imidazole ring on the compound's potency toward RGGT (Figure 2). For clarity, we discuss these two classes, Nand C-substituted analogues, separately.

Results and Discussion

Synthesis

We first synthesized six new analogues of the N-series, **1**a–**1**f, derived from F-ZoIPC, modified with a methyl or phenyl group, or bromine atom at either the 2- or 4(5)-position of the imidazole ring (Figure 2a).^[15] Target compounds **1**a–f were prepared according to a modified procedure previously developed by us.^[11] It involved aza-Michael addition of commercially available substituted imidazoles **4** to the ethyl 2-(diethoxyphosphoryl)-acrylate **3** (Scheme 1). Thus obtained triesters **5** were relatively unstable and spontaneously underwent subsequent reaction with an acceptor, forming so-called "double Michael addition" products (Scheme S1).^[16,17] To circumvent this side reaction, we



Figure 2. Structures of the studied compounds; *1d was obtained as a mixture of 4- and 5-methyl-substituted regioisomers (ratio 1.0:0.3).

subjected the Michael adduct **5** immediately to fluorination, using sodium hydride and the electrophilic fluorinating agent, *N*-fluorobenzenesulfonimide (NFSI). This new one-pot procedure led to triesters **6a**–**f** with significantly improved yields (76–87%) and purity. The use of Selectfluor as a fluorinating agent led to lower yields due to formation of side products, such as double Michael adducts mentioned above. Both desoxy-**5a**–**f** as well as fluoro analogues **6a**–**f** were subjected to hydrolysis in concentrated hydrochloric acid to afford target products with 11–91% yields.

Analogues of the C-series, **1** g–l, in which the phosphonocarboxylate group is connected to the imidazole ring via carbon, constituted the second class of studied compounds. For their synthesis we applied a method previously developed by us for imidazo[1,2-*a*]pyridine analogues,^[14] which is based on the condensation of appropriate aldehydes with triethyl phosphonoacetate (Scheme 2). We used N-methylated (**7** h and **7** j) as well as nitrogen-unsubstituted aldehydes (**7** g, **7** i, **7** k, **7** l) with



Scheme 1. Reagents and conditions: a) THF, RT, 30 min; b) 1. NaH, THF, -10 °C, 40 min, 2. NFSI, THF, -60 °C, 20 min, < -20 °C, 1 h; c) 12 M HCl, reflux, 4 h.

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Scheme 2. Reagents and conditions: a) Ph₃CCl, TEA, RT, (DMF, 48 h for 7g; CH₂Cl₂, 24 h for 7i); b) triethyl phosphonoacetate, TiCl₄, TEA, (CH₂Cl₂ for 8g, 8i and 7h,j,k; THF for 7l) $-20^{\circ}C \rightarrow RT$, 24 h; c) for 9g-i: NaBH₄, NiCl₂6 H₂O, MeOH, $-20^{\circ}C$, 15 min, for 9h,j,k,l: H₂, Pd/C, MeOH, RT, 5 h; d) Ph₃CCl, TEA, DMF, 60 °C, 48 h; e) 1. NaH, THF, $-10-5^{\circ}C$, 50 min, 2. NFSI, THF, $-60^{\circ}C$, 20 min, $< -20^{\circ}C$, 1 h; f) 12 M HCl, reflux, 4 h.

or without the additional C-attached methyl substituent. Some N-unsubstituted aldehydes have limited solubility in the reaction medium, CH_2Cl_2 , and therefore they were either tritylated prior to condensation (e.g., compounds **8g** and **8i** were obtained in 61 and 99% yields, respectively)^[18-20] or the condensation was carried out in THF (**7l**). Other aldehydes were used without prior tritylation.^[21]

The condensation of aldehydes with triethyl phosphonoacetate led to vinyl analogues 9, obtained as a mixture of E/Z isomers for compounds 9i-I (predominantly the E isomer) or as the single Z isomer (compounds 9g,h), with yields ranging from 30 to 78%. In the next step compounds 9 were subjected to hydrogenolysis on Pd-C or NaBH_4/NiCl_2 $\cdot H_2O,^{\scriptscriptstyle [22]}$ and thus obtained triesters **10** (49–91%) were fluorinated in the α -position, using NFSI along with NaH. Triesters 10 k,l, bearing two unsubstituted nitrogen atoms in the imidazole ring, required tritylation prior to fluorination. Otherwise only traces of products were detected, whereas tritylated analogues were fluorinated with 51-53% yields. The other fluorinated products 12 were obtained with good yields (65-87%). Thus obtained desoxy-10g-I and fluorinated esters 12g-I were subjected to acidic hydrolysis in concentrated hydrochloric acid, which simultaneously cleaved the trityl group, if present. Final acids were obtained with 41-94% yields.

NMR analysis

Synthesis of analogues by the aza-Michael reaction (so-called N-series), bearing substituents at the 4- and/or 5-positions of

the imidazole ring, could lead to a mixture of regioisomers, with preference for the formation of the less sterically hindered 4-substituted analogue. Such was the case in the reaction of ethyl 2-(diethoxyphosphoryl)acrylate **3** with 4(5)-methylimida-zole **4d** (ratio of regioisomers 1:0.3). We used this product mixture for confirmation of the structure of the major regioisomer by NMR analysis (COSY, HMQC, DEPT135, HMBC, NOESY). In the case of bromo- and phenyl-substituted imidazoles, only one regioisomer was formed. Our analysis was supported by previously reported structure determinations of τ - and π -regioisomers of histidinoalanines.^[23]

Because the formation of particular regioisomer(s) was determined in the aza-Michael addition, for NMR structural analysis we could choose the analogues among ester (6d) and/or acids (2 d/1 d), depending on the satisfactory separation of signals in ¹H and ¹³C NMR spectra. The most convenient model turned out to be the mixture of esters **6d** (Figure 3).^[24] We used the HMBC technique to assign the position of the methyl group in the imidazole ring, thanks to correlation of the corresponding quaternary aromatic carbon atom with protons in the β position. While in the minor regioisomer, C3 (128.3 ppm) shows strong correlation with protons in the β position (Figure 3 a, arrow a), the analogous correlation for the corresponding ¹³C signal in the major regioisomer, C6 (138.7 ppm), is not observed (Figure 3a, arrow h; see spectra in Figure S3). This observation indicates that the methyl group in the major isomer is connected to C6 (or position 4 according to the proper numbering of the imidazole ring). That assignment is supported by the fact that only in the case of the major



Figure 3. Key correlations observed in the a) HMBC and b) NOESY spectra acquired for the mixture of regioisomers **6 d**. For details see Figure S3–S4, Supporting Information.

isomer, two signals from aromatic tertiary carbon atoms (C5: 137.5 ppm and C8: 116.6 ppm) correlate with protons in the β position (Figure 3a, arrows **f** and **e**), whereas for the minor isomer only one tertiary carbon (C1, 137.8 ppm) shows such a correlation (arrow **b**). Additional confirmation comes from NOESY experiments (Figure 3b and Figure S2). In the minor regioisomer, the methyl group (2.19 ppm) and one aromatic proton (H-1, 7.44 ppm) interact with protons in the β position (Figure 3b, arrows **b** and **a**, respectively). In the case of the major regioisomer such correlation of the methyl group (2.16 ppm) was not observed, but instead both aromatic protons (H-5, 7.35 ppm and H-8, 6.64 ppm) interacted with protons in the β position (arrows **d** and **e**).

Based on comparative analysis of the correlations observed in the 2D spectra for bromo- and phenyl-substituted esters **6** and acids 1-2, which showed the same patterns as those observed for the major regioisomer **6**d', we determined that those analogues bear a substituent at the 4-position.

Biological studies

All synthesized fluorine-containing compounds **1** were screened for their biological activity, while desoxy analogues **2** were excluded from such tests as potentially less potent, as was indicated in our previous studies.^[13, 14]

Cytotoxicity assays with HeLa cells

Inhibition of prenyltransferases or enzymes in the mevalonate pathway may be associated with induction of cell death.^[25-27] Therefore, we investigated the antiproliferative activity of the newly synthesized phosphonocarboxylate analogues on human cervical carcinoma HeLa cells. Taking into account that activities of tested compounds could be attenuated or modulated by serum proteins,^[28] cell viability was assessed during serum deprivation as well as in the presence of FBS (Table 1).

All analogues 1a-f, in which the α -phosphonopropionic acid moiety is linked through N-substitution with the imidazole ring, did not influence the number of viable cells up to the maximum concentration tested (2 mm), except for compound 1 d, which showed negligible inhibition in fasting medium (IC_{50}) 1.88 mm). C-substituted analogues 1g,h and 1k did not show any effect on HeLa cell viability either, whereas 11 indicated insignificant cytotoxic effect only in serum-free medium (IC₅₀ 1.01 mm). However, analogues 1 i,j demonstrated cytotoxic effect under both medium conditions, with IC_{50} values of 566 and 546 μM in fasting medium and 1313 and 1277 μM in FBScontaining medium, respectively. Under serum-free conditions, they showed stronger effects than the reference compound F-ZoIPC (decrease in cell viability: $IC_{50} = 850 \ \mu M$)^[11] Relative to recently described analogues,^[14] these newly synthesized compounds showed slightly weaker antiproliferative activity, which was especially conspicuous in complete medium, but less pronounced in fasting medium.

Effect on prenylation of Rab11A and Rap1A/Rap1B

The novel PCs were screened for their ability to inhibit the activity of RGGT and GGT-1 in intact HeLa cells by detection of un- and misprenylated forms of Rab11A and Rap1A/Rap1B, which are modified with geranylgeranyl groups by RGGT and GGT-1, respectively. Inhibition of Rab11A prenylation alone is indicative of a specific RGGT inhibitor, whereas inhibition of prenylation of both types of GTPases at similar concentrations might be indicative of either unselective inhibition of both prenyl transferases (RGGT and GGT-1), or of inhibition of mevalonate pathway enzymes, such as GGPPS or FPPS, which play key roles in providing substrates for prenylation (GGPP and FPP, respectively).

The inhibitory activity of the set of newly synthesized compounds **1a**–**11** was initially tested at 50 μ M under serum-free conditions, and Rab11A enrichment in the cytosol fraction was examined by western blot analysis (Table 1).^[14] Interestingly, only two analogues of the C-series, compounds **1i** and **1j**, were found to be active at the tested concentration (Figure 4). They contain the α -phosphonopropionic residue connected to the imidazole ring through C5, whereas new analogues bearing such a linkage at the C2 or N1 positions were inactive. Notably, the active compounds **1i** and **1j** also had observable cytotoxic effects. None of the analogues of the N-series, **1a**–**f**, derived from the known RGGT inhibitor, F-ZoIPC, showed significant potency against RGGT, which demonstrates that addi-

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Table 1. Effects of synthesized PC analogues on HeLa cell viability in serum-supplemented (SS, 10% FBS) and serum-free (SF) medium, and inhibition	of
Rab11A prenylation.	

Compd Structure		R/R ¹ /R ² /R ³	HeLa IC	HeLa IC _{so} [µм] ^[a]		
			SS	SF		
1a	P(O)(OH) ₂	Me	NE	NE	_	
1b	СООН	Ph	NE	NE	-	
1c	N D	Br	NE	NE	-	
	K N R					
		$P^1 - M_0$, $P^2 - H$				
1 d	F	$R^{1} = H^{2} = Me$	NE	1880	-	
1e	COOH	$R^{1} = Ph, R^{2} = H$	NE	NE	_	
1 f	R ² N	$R^1 = Br, R^2 = H$	NE	NE	_	
	∭_Ń R ¹					
1g	P(O)(OH) ₂	н	NE	NE	_	
1h	F	Me	NE	NE	_	
1 i ^[c]	P(O)(OH) ₂	$R^1, R^2, R^3 = H$	1313	566	+	
1 j ^[c]	F		1277	546	+	
1 j-E1 ^[c,d]		$R^1 = Me; R^2, R^3 = H$	NE	1701	_	
1 j-E2 ^[c,d]	R ³ N ⁻ R'		728	297	+	
1k	N=<	$R^{1}, R^{2} = H; R^{3} = Me$	NE	NE	-	
11	R ²	$R^{1}, R^{3} = H; R^{2} = Me$	NE	1014	_	

[a] HeLa cells were treated with test compounds for 72 h and then viable cell number was determined. Data were calculated from the means of eight test concentrations from at least three independent experiments. NE stands for lack of response ("no effect") in cells treated with inhibitor up to 1 mm. [b] HeLa cells were treated for 48 h with compounds at 50 μм, then lysed and separated into cytosolic and membrane-rich fractions and western blotted for Rab11A and B-actin in cytosolic fractions. Data are from at least three independent experiments, and indicate for which compounds higher band intensity relative to control (untreated cells) was observed. [c] Compounds in bold were evaluated to inhibit Rab11A and Rap1A/Rap1B prenylation across a wide concentration range. [d] The enantiomers of 1 j are distinguished as E1 and E2, based on their retention times during chiral HPLC separation. The enantiomer with the shorter retention time is labeled as E1, and the one with the longer retention time, as E2.

Compound	control	1a	1d	1b	1e	1c	1f	control	1i	1g	1h	1j	1k	11
Rab11A	_		-	-		-		_	-			-	-	-
β-actin	-	-	-	-	-	-	-	-		-	-	-	-	

Figure 4. Effect of F-ZoIPC analogues (1 a-l) on Rab11A prenylation in HeLa cells. Cells were treated for 48 h with PCs (50 µm) in serum-free medium. Cells were then lysed and fractionated into cytosolic and membrane-rich fractions. Cytosolic fractions containing unprenylated proteins were separated by electrophoresis and western blotted for Rab11A and β -actin.

tional substitution at the C2 or C4 positions adversely affects the activity of this class of compounds.

Because 1d and 1l did not affect Rab11A prenylation, their effect on HeLa cell viability could be associated with a different mechanism of action. However, it cannot be excluded that in the case of the methyl-substituted analogue 1d, which had been obtained and tested as a mixture of 4- and 5-regioisomers, such activity stems from the presence of \approx 23% of the 5substituted analogue (\approx 12% of the more active stereoisomer), which seems to posses an advantageous substitution pattern that corresponds to 3-IPEHPC and 1 j.

Two selected analogues—1i and 1j—were subjected to a full-panel five-dose assay to determine the lowest effective dose (LED) inhibition of Rab11A and Rap1A/Rap1B prenylation (Table 2, Figure 5). Compound 1i, bearing no additional substituent in the imidazole ring, showed inhibitory activity against Rab11A prenylation at 25 µм, whereas analogue 1j, which has an additional methyl group at the N1 position of the imidazole ring, was found to be more active, as shown by a decrease in the LED value to 10 µm. Similar to previous findings,^[12, 14] only one enantiomer of **1 j**, compound **1 j-E2**, had the desired biological activity to inhibit Rab11A prenylation as

Table 2. Effects of selected PC analogues on the inhibition of Rab11A and Rap1A/Rap1B prenylation in HeLa cells.						
Compound	LED [μμ] ^[a]					
	Rab11A ^[b]	Rap1A/Rap1B				
1i	25	NE				
1j	10	NE				
1 j-E1	NE	NE				
1 ј-Е2	10	NE				
[a] LED = lowest effective dose; NE = LED is not included at a concentra-						

tion up to 50 μм. [b] The reference compound 3-(6-bromoimidazo[1,2a]pyridin-3-yl)-2-fluoro-2-phosphonopropanoic acid was evaluated in parallel, showing the same activity as reported previously.^[14]



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Figure 5. Effect of 1 i, 1 j, and their enantiomers (1 j-E1 and 1 j-E2) on Rab11A and Rap1A/Rap1B prenylation in HeLa cells. Cells were treated for 48 h with the indicated concentrations of PCs (μ M) and 10 μ M lovastatin (Lov) acting as a positive control in serum-free medium. Cells were then lysed and fractionated into cytosolic and membrane-rich fractions. Cytosolic fractions containing unprenylated proteins were separated by electrophoresis and western blotted for Rab11A, Rap1A/Rap1B, and β -actin.

measured by LED (Table 2, Figure 5). Stronger effect of **1j-E2** was also observed comparing the cytotoxic activity of enantiomers (Table 1, no effect and 728 μ M in full medium and 1701 μ M and 297 μ M in serum-free medium for **1j-E1** and **1j-E2**, respectively). Simultaneously, the selected compounds did not affect Rap1A/Rap1B prenylation at the concentrations tested (LED > 250 μ M, Figure S2).

Because the connection between imidazole and the α -phosphonopropionic acid's residue through the C5 atom is a preferable structural pattern for activity against RGGT, the influence of additional methyl substituents at other positions of the imidazole ring was analyzed. While addition of the methyl substituent at N1 slightly increased activity (as with compound 1 j), analogues with the methyl group at the C2 or C4 positions showed no potency toward RGGT at the concentrations tested. Interestingly, all compounds bearing a substituent at the C2 position (1a-c, 1g,h, 1l), regardless of the position connecting the α -phosphonopropionic acid with imidazole, did not show any potency, possibly due to steric bulk.^[29] The same applies to compounds bearing a substituent at position C4 of the imidazole ring (1 d-f, 1 k). These compounds bear structural similarity to 2-substituted imidazo[1,2-a]pyridine analogues, which were found to be inactive toward RGGT.^[14]

Our data indicate that **1***i,j* are selective micromolar RGGT inhibitors with similar or slightly weaker activity than the reference compound F-ZoIPC (LED for inhibition of Rab11A: 10 μ M)^[11] and α -fluorinated analogues of 3-IPEHPC (LED for inhibition of Rab11A: 10 or 25 μ M, depending on the substituent's character).^[14]

Conclusions

We elaborated convenient protocols for N- and C-functionalization of the imidazole ring with a 2-phosphonopropionic acid group. Among the new compounds, we identified two phosphonocarboxylates (**1i** and **1j**) that show micromolar potency as inhibitors of Rab11A prenylation, and which have the capacity to decrease HeLa cell viability. Both compounds structurally resemble the preferable substitution pattern found in other phosphonocarboxylate-derived RGGT inhibitors, confirming the structure–activity relationship disclosed recently for analogues bearing an imidazo[1,2-*a*]pyridine ring. Simultaneously, we found that the small imidazole ring constitutes the core scaffold necessary for the activity of phosphonocarboxylates against RGGT, and preferable points of modifications are localized solely at positions C5 and/or N1. The potentially different interaction mechanism between analogues bearing the 2phosphonopropionic acid moiety at either the N1 or C5 positions cannot be excluded. Studies on the synthesis (including asymmetric variant) and evaluation of analogues substituted at those two positions are in progress.

Experimental Section

All reagents were purchased from commercial sources and were used as obtained, unless specified otherwise. Thin-layer chromatography was performed with silica gel 60 with the F₂₅₄ indicator on alumina plates. Precursors of final compounds were purified using by liquid chromatography (Gilson PLC 2250) coupled with a mass spectrometer (Advion expression) and 40–63 μ m silica gel as stationary phase. Chromatography was performed with the reported solvent system with gradient elution. Preparative HPLC for purification of compounds 2c and 2f was performed using a Gilson Prep equipped with a UV/Vis-156 detector (237 nm) and semipreparative column Kromasil 100-5-C₁₈ (5 μm, 10×250 mm). Separation of enantiomers of compound 1j was performed on a Chiralpak QN-AX column (Chiral Technologies Europe; 0.46 cm×15 cm), according to a previously described method.^[12] The column was eluted isocratically (1 mLmin⁻¹) with 0.7 м TEAAc containing 75% MeOH at pH 5.8. Confirmation of the optical purity of enantiomers was evaluated based on HPLC analysis using a Chiralpak QN-AX column with a more sensitive detection method provided by a Gilson prepELS II detector (with temperatures set at 65 °C and 50 °C for drift tube and spray chamber, respectively). The enantiomer with the shorter retention time, 3.6 min, was termed 1j-E1, whereas that with the longer retention time, 4.6 min, was termed 1j-E2. Optical rotations of enantiomers 1j-E1 and 1j-E2 were measured on a PerkinElmer 241 polarimeter, and $[\alpha]_{D}^{20}$ values are given in deg·cm g⁻¹·dm⁻¹; concentration c = 1 for 1 g in 100 mL.

NMR spectra were measured at 250.13 or 700 MHz for ¹H NMR, 62.90 or 170 MHz for ¹³C NMR, and 283 or 101.30 MHz for ³¹P NMR on Bruker Avance DPX 250 and Bruker Avance II Plus 700 spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to: internal residual CHCl₃ in CDCl₃ (δ =7.26 ¹H NMR) or CDCl₃ signal in ¹³C NMR (δ =77.16); internal residual HDO in D₂O (δ =4.79 ¹H NMR) or external 85% H₃PO₄ (δ =0 ppm ³¹P NMR). ³¹P and ¹³C NMR spectra were proton-decoupled. Assignment of the signals in ¹H and ¹³C NMR was supported by two-dimensional experiments (COSY, HMQC, HMBC, DEPT135). For compounds obtained as mixtures of regioisomers (**5d**, **6d**, **1d**, **2d**) or isomers *E* and *Z* (**9g–I**), when two complementary signals overlap, the total integration of those two signals is given in the NMR description (e.g., in case of a mixture of isomers in a ratio of 1.0:0.6, total inte-



gration of overlapping signals from complementary CH_2 group equals $1.0 \times 2 + 0.6 \times 2 = 3.2$). All final compounds were obtained as white solids, decomposing before melting point was reached. Compounds used in biological testing possess a purity of no less than 95%.

PrestoBlue Cell Viability Reagent, Mem-PER Plus Membrane Protein Extraction Kit, and all reagents for cell culture were purchased from Life Technologies (Carlsbad, CA, USA). Mem-PERTM Plus Membrane Protein Extraction Kit was used for cell lysis and separation of cytosolic and membrane-rich fractions. Bradford Protein Assay and Clarity Western ECL Substrate were obtained from Bio-Rad (Hercules, CA, USA). Protease inhibitor cocktail and lovastatin were purchased from Sigma (St. Louis, MO, USA). Primary antibodies against Rab11A and Rap1A/Rap1B were obtained from Abcam (Cambridge, UK), and primary antibodies against β -actin along with secondary HRP-linked antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

General procedure for the synthesis of compounds 5a-f on the example of compound 5b

Reaction performed in dry flaks purged with Ar. To ethyl 2-(diethoxyphosphoryl)acrylate **3** (300 mg, 1.27 mmol) in THF (6 mL), imidazole **4b** (198 mg, 1.37 mmol, 1.1 equiv) at room temperature was added, and the resulting solution was stirred for 30 min. The obtained adduct was directly subjected to fluorination or, after evaporation of THF, to hydrolysis.

General procedure for the synthesis of compounds 6 a-f on the example of compound 6 b

Aza-Michael adduct **5 b** (obtained from 300 mg of **3** and 198 mg of **4 b**) in THF (6 mL) was added under argon to a cooled $(-10^{\circ}C)$ suspension of NaH (60 mg, 1.52 mmol, 1.2 equiv, 60% suspension in oil) in THF (9 mL) within 3 min. It was stirred for 40 min at $<5^{\circ}C$ and then cooled to $-60^{\circ}C$ followed by the addition of NFSI (480 mg, 1.52 mmol, 1.2 equiv) in THF (6 mL) within 3 min. It was stirred for 20 min at $-60^{\circ}C$ and then for 60 min at $<-20^{\circ}C$. The reaction was quenched by the addition of a saturated NH₄Cl solution (3 mL) and H₂O (3 mL). After addition of CHCl₃ (20 mL) the mixture was agitated, and the organic and aqueous phases were separated. The aqueous phase was additionally extracted with CHCl₃ (2×20 mL). The combined organic phases were dried over MgSO₄ and concentrated. The thus obtained oil was purified by column chromatography with gradient elution using CH₂Cl₂/MeOH system as eluent.

General procedure of the Knoevenagel reaction for the synthesis of compounds 9g–I on the example of compound 9i

In a dry and argon-purged double-neck flask equipped with thermometer and septum, triethyl phosphonoacetate was placed (1.09 g, 4.87 mmol) in 20 mL of CH₂Cl₂. The solution was cooled to -20 °C in a CO₂/acetone bath, and neat TiCl₄ (0.65 mL, 5.84 mmol, 1.2 equiv, 110 μ L/1 mmol TiCl₄) and TEA (1.9 mL, 13.6 mmol, 2.8 equiv) were then added. After 15 min, a solution of aldehyde **8i** (1.65 g, 4.87 mmol, 1 equiv) in CH₂Cl₂ (10 mL)¹ was added, and

the reaction mixture was stirred for 24 h at room temperature. Water (50 mL) was the added, and the solution was adjusted to pH 9 with a saturated Na₂CO₃ solution. The product was extracted with Et₂O (5×100 mL). Combined organic phases were dried over MgSO₄ and concentrated. Products were purified by column chromatography using a CH₂Cl₂/acetone eluent system, except for compounds **9***j*–**I**, for which a CHCl₃/MeOH system was used. For products containing triethyl phosphonoacetate after unsuccessful purification, the approximate yields were calculated for the quantity of the product determined based on ³¹P and ¹H NMR data.

General procedure for reduction of the double bond in compounds 9g and 9i under $NaBH_4/NiCl_2$ conditions on the example of the synthesis of compound 10i

To a solution of compound **9i** (1.07 g, 1.96 mmol) in MeOH (10 mL) NiCl₂·6H₂O (0.575 g, 2.42 mmol, 1.2 equiv) was added. The solution was cooled to -20 °C in a CO₂/acetone bath, and NaBH₄ (0.229 g, 6.06 mmol, 3 equiv) was carefully added so as to maintain temperature below -10 °C. The mixture was then stirred for 10 min. Then the cooling bath was removed, and when the temperature reached 10 °C, saturated NH₄Cl (20 mL) was added. The aqueous layer was extracted with CH₂Cl₂ (4×50 mL). The organic layer was dried over anhydrous Mg₂SO₄ and concentrated. The product was purified by flash liquid chromatography using CH₂Cl₂/MeOH system as eluent.

General procedure for reduction of the double bond in compounds 9h,j,k,l upon hydrogenolysis on the example of the synthesis of compound 10j

Reaction was carried out in a single-neck flask equipped with twoway stopcock, which enabled degassing the system (vacuumargon-vacuum, three times) prior to hydrogen supply. In a singleneck flask compound **9j** (0.9 g, 2.84 mmol) was placed in MeOH (20 mL). The flask was then equipped with a two-way stopcock. The system was degassed (see above). 10% Pd/C (110 mg, 40 mg/ 1 mmol substrate, 3.64 mol%) was carefully added, and the system was again degassed. Finally, the system was supplied with hydrogen atmosphere in the same manner as for argon (three times), and this suspension was stirred for 5 h at room temperature. The catalyst was then filtered off through a thin layer of Celite 500, and the filtrate was evaporated to dryness. The product was purified by flash liquid chromatography using CHCl₃/MeOH system as eluent.

General tritylation procedure for the synthesis of compounds 11 k–l on the example of compound 11 l

In a dry single-neck flask compound **101** was placed (280 mg, 0.88 mmol), and TEA (178 mg, 1.76 mmol, 2 equiv) dissolved in DMF (4 mL) was added via syringe. After 10 min of stirring at RT, trityl chloride was added (270 mg, 0.97 mmol, 1.1 equiv), and the mixture was stirred for a further 48 h at 60 °C. The mixture was then transferred to a separatory funnel in 40 mL EtOAc. It was washed with brine (15 mL), a saturated solution of Na₂CO₃ (15 mL) and H₂O (15 mL), dried over MgSO₄ and concentrated using a rotary evaporator. The crude product was purified by column chromatography using CHCl₃/MeOH system as eluent.

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¹ For the synthesis of compounds **9k** and **9l**, corresponding aldehydes were added without prior dissolution, and therefore the initial triethyl phosphonoacetate solution was prepared by using 35 mL CH₂Cl₂ and 40 mL THF, respectively.



General procedure for the synthesis of compounds 12g–1 on the example of compound 12i

Compound **10i** (400 mg, 0.732 mmol) was added under argon atmosphere to a cooled (-10°C) suspension of NaH (35 mg, 0.878 mmol, 1.2 equiv, 60% suspension in oil) in THF (6 mL, 1.5 mL/100 mg of substrate) within 4 min. It was stirred for 50 min at -10 to $+5^{\circ}\text{C}$ and then cooled to -60°C followed by the addition of NFSI (276 mg, 0.878 mmol, 1.2 equiv) in THF (4 mL) within 4 min. It was stirred for 20 min at -60°C and for 60 min at $< -20^{\circ}\text{C}$. The reaction was quenched by the addition of saturated NH₄Cl solution (4 mL) and H₂O (4 mL). After the addition of CHCl₃ (20 mL) the mixture was agitated and then the organic and aqueous phases were separated. The aqueous phase was additionally extracted with CHCl₃ (2×20 mL). Combined organic phases were dried over MgSO₄ and concentrated. Thus obtained oil was purified by column chromatography using CH₂Cl₂/MeOH system as eluent.

General procedure for the synthesis of compounds 1 a-f and 2 a-f on the example of 1 b

In a single-neck flask compound **6b** (190 mg, 0.477 mmol) was placed and 36% HCl (5 mL, ≈ 1 mL/0.1 mmol) was added. The mixture was held a reflux for 5 h. Excess HCl was evaporated, and the residue was co-evaporated with EtOH (3×1 mL). EtOH (1 mL) was then added, and the resulting precipitate was filtered off and rinsed with 0.5 mL ice-cold EtOH. It was then dried under vacuum, dissolved in water (3 mL) and lyophilized. Compounds **1c**, **1e** and **1 f**, which were insoluble in water, were instead lyophilized from suspension, followed by trituration of thus obtained powder. Compounds **2c** and **2f** required purification by preparative HPLC using H₂O as eluent followed by lyophilization. Remaining compounds **2** were only precipitated from EtOH.

2-Fluoro-3-(2-methyl-1H-imidazol-1-yl)-2-phosphonopropanoic

acid (1a): Yield: 64%. Obtained from 0.55 mmol (184 mg) of **6a**. ³¹P NMR (283 MHz, D₂O, pH 8): δ =8.67 ppm (d, ¹J_{PF}=65.9 Hz); ¹H NMR (700 MHz, D₂O): δ =2.54 (s, CH₃IM, 3H), 4.50 (ddd, ²J_{HH}= 15.1 Hz, ³J_{FH}=7.8 Hz, ³J_{PH}=4.7 Hz, CH_aH_bC(F)P, 1H), 4.87 (bdd, ³J_{FH}= 35.1 Hz, ²J_{HH}=15.1 Hz, CH_aH_bC(F)P, 1H), 7.06 (d, ³J_{HH}=1.8 Hz, CH_{ar(IM-4}), 1H), 7.21 ppm (bs, CH_{ar(IM-5)}, 1H); ¹³C NMR (176 MHz, D₂O): δ = 11.40 (s, CH₃IM, 1C), 50.87 (dd, ²J_{FH}=20.0 Hz, ²J_{PH}=8.0 Hz, CH₂C(F)P, 1C), 98.98 (dd, ¹J_{FC}=194.2 Hz, ¹J_{PC}=133.7 Hz, CH₂C(F)P, 1C), 121.74 (s, CH_{ar(IM-4)}, 1C), 121.97 (s, CH_{ar(IM-5)}, 1C), 146.62 (s, C_{ar(IM-2)}, 1C), 174.76 ppm (d, ²J_{FC}=21.3 Hz, CO₂H, 1C); Elemental analysis: C₇H₁₀FN₂O₅P(H₂O)_{1.1}, calcd: C 30.92, H 4.52, N 10.30, found: C 31.04, H 4.39, N 10.10.

2-Fluoro-3-(2-phenyl-1H-imidazol-1-yl)-2-phosphonopropanoic

acid (1b): Yield: 69%. Obtained from 0.48 mmol (190 mg) of 6c. ³¹P NMR (283 MHz, D₂O, pH 3): δ =8.68 ppm (d, ¹*J*_{PF}=67.8 Hz); ¹H NMR (700 MHz, D₂O): δ =4.65 (ddd, ²*J*_{HH}=15.0 Hz, ³*J*_{FH}=7.9 Hz, ³*J*_{PH}=4.9 Hz, CH_aH_bC(F)P, 1H), 4.97 (ddd, ³*J*_{FH}=34.3 Hz, ²*J*_{HH}= 15.0 Hz, ³*J*_{PH}=1.5 Hz, CH_aH_bC(F)P, 1H), 7.28 (d, ³*J*_{HH}=1.8 Hz, CH_{ar(IM-4}), 1H), 7.47 (bs, CH_{ar(IM-5)}, 1H), 7.63-7.66 (m, C₆H₅-, 3H), 7.70-7.73 ppm (m, C₆H₅-, 2H); ¹³C NMR (176 MHz, D₂O): δ =50.80 (dd, ²*J*_{FH}=19.7, ²*J*_{PH}=8.4 Hz, CH₂C(F)P, 1C), 98.24 (dd, ¹*J*_{FC}=194.6 Hz, ¹*J*_{PC}=134.9 Hz, CH₂C(F)P, 1C), 122.37 (d, ⁵*J*_{PC}=2.9 Hz, CH_{ar(IM-5)}, 1C), 123.74 (s, CH_{ar(IM-4)}, 1C), 127.00 (s, C₆H₅-, 1C), 129.01(s, C₆H₅-, 2C), 129.62 (s, C₆H₅-, 2C), 130.46 (s, C₆H₅-, 1C), 147.99 (s, C_{ar(IM-2)}, 1C), 174.00 ppm (bd, ²*J*_{FC}=21.1 Hz, CO₂H, 1C).

3-(2-Bromo-1*H***-imidazol-1-yl)-2-fluoro-2-phosphonopropanoic acid (1 c)**: Yield: 41%. Obtained from 0.38 mmol (153 mg) of **6 e**. ³¹P NMR (283 MHz, D₂O, pH 8): δ =8.85 ppm (d, ¹J_{PF}=66.1 Hz);

¹H NMR (283 MHz, D₂O): δ = 4.54 (ddd, ²J_{HH} = 15.0 Hz, ³J_{FH} = 7.6 Hz, ³J_{PH} = 4.9 Hz, CH_aH_bC(F)P, 1 H), 4.88 (bdd, ³J_{FH} = 35.8 Hz, ²J_{HH} = 15.0 Hz, CH_aH_bC(F)P, 1 H), 7.02 (d, ³J_{HH} = 1.6, Hz CH_{ar(IM-4)}, 1 H), 7.30 ppm (bs, CH_{ar(IM-5)}, 2 H); ¹³C NMR (283 MHz, D₂O): δ = 51.48 (dd, ²J_{FH} = 19.8, ²J_{PH} = 8.2 Hz, CH₂C(F)P, 1C), 98.58 (dd, ¹J_{FC} = 194.3 Hz, ¹J_{PC} = 134.4 Hz, CH₂C(F)P, 1C), 121.31 (s, C_{ar(IM-2)}, 1C), 123.66 (s, CH_{ar(IM-5)}, 1C), 128.49 (s, CH_{ar(IM-4)}, 1C), 174.60 ppm (d, ²J_{FC} = 22.0, CO₂H, 1C).

2-Fluoro-3-(4-methyl-1*H*-imidazol-1-yl)-2-phosphonopropanoic

acid (R¹) and 2-fluoro-3-(5-methyl-1H-imidazol-1-yl)-2-phosphonopropanoic acid (R²) (1 d): Yield: 63 %. Obtained from 0.54 mmol (182 mg) of **6b**. Mixture of regioisomers: $R^1/R^2 = 1.0:0.3$. ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 8.08 - 8.64$ ppm (2d overlapping, ¹J_{PF} pprox 65.0 Hz, R¹ and R², 1.3P); ¹H NMR (700 MHz, D₂O): δ = 2.27 (bs, CH₃IM, R¹, 3 H), 2.34 (bs, CH₃IM, R², 0.9 H), 4.50–4.57 (m, CH_aH_bC(F)P, R^1 and R^2 , 1.3 H), 4.87–5.98 (m, $CH_aH_bC(F)P$, R^1 and R^2 , 1.3 H), 7.03 (s, CH_{ar(IM-4)}, R², 0.3 H), 7.07 (s, CH_{ar(IM-5)}, R¹, 1 H), 8.18 (s, CH_{ar(IM-2)}, R¹, 1 H), 8.25 ppm (s, $CH_{ar(IM-2)}$, R^2 , 0.3 H); ¹³C NMR (283 MHz, D_2O): δ = 8.52 (bs, CH_3IM , R^2 , 0.3C) 10.27 (s, CH_3IM , R^1 , 1C), 49.60–49.81 (m, CH₂C(F)P, R², 0.3C), 52.65 (dd, ${}^{2}J_{FC} = 20.1$ Hz, ${}^{2}J_{PC} = 7.7$ Hz, CH₂C(F)P, R¹, 1C), 98.38 (dd, ${}^{1}J_{FC} = 193.6$ Hz, ${}^{1}J_{PC} = 133.6$ Hz, CH₂C(F)P, R¹, 1C), 97.71-99.55 (mutliplet overlapping with dd at 98.38, CH₂C(F)P, R², 0.3C), 118.50 (bs, $CH_{ar(IM-4)}$, R^2 , 0.3C), 118.72 (s, $CH_{ar(IM-5)}$, R^1 , 1C), 131.42 (s, $C_{ar(IM-5)}$, R^2 , 0.3C), 132.37 (bs, $C_{ar(IM-4)}$, R^1 , 1C), 135.66 (s, $CH_{ar(IM-2)}, \ R^1, \ 1C), \ 135.92 \ (bs, \ CH_{ar(IM-2)}, \ R^2, \ 0.3C), \ 174.19 \ ppm \ (m,$ CO_2H , R^1 and R^2 , 1.3C); Elemental analysis: $C_7H_{10}FN_2O_5P(H_2O)_{1.95}$, calcd: C 29.27, H 4.88, N 9.75, found: C 29.42, H 4.72, N 9.54.

2-Fluoro-3-(4-phenyl-1*H*-imidazol-1-yl)-2-phosphonopropanoic

acid (1e): Yield: 45%. Obtained from 0.41 mmol (165 mg) of 6d. ³¹P NMR (283 MHz, D₂O, pH 9): $\delta = 8.08$ ppm (d, ¹*J*_{PF} = 65.9 Hz); ¹H NMR (700 MHz, D₂O): $\delta = 4.52$ (ddd, ²*J*_{HH} = 15.0 Hz, ³*J*_{FH} = 8.7 Hz, ³*J*_{PH} = 4.6 Hz, CH_aH_bC(F)P, 1H), 4.93 (bdd, ³*J*_{FH} = 36.1 Hz, ²*J*_{HH} = 15.0 Hz, CH_aH_bC(F)P, 1H), 7.36–7.39 (m, C₆H₅-, 1H), 7.49–7.52 (m, C₆H₅-, 2H), 7.58 (bs, CH_{ar(IM-2 or 5)}, 1H), 7.76 (bs, CH_{ar(IM-2 or 5)}, 1H), 7.79 ppm (dd, ³*J*_{HH} = 8.4 Hz, ⁴*J*_{HH} = 1.2 Hz, C₆H₅-, 2H); ¹³C NMR (176 MHz, D₂O): $\delta = 51.86$ (dd, ²*J*_{FH} = 20.5 Hz, ²*J*_{PH} = 6.8 Hz, CH₂C(F)P, 1C), 99.16 (dd, ¹*J*_{FC} = 193.4 Hz, ¹*J*_{PC} = 133.2 Hz, CH₂C(F)P, 1C), 117.88 (s, CH_{ar(IM-2 or 5)}, 1C), 124.82 (s, C₆H₅-, 2C), 127.41 (s, C₆H₅-, 1C), 129.27 (s, C₆H₅-, 2C), 133.54 (s, C₆H₅-, 1C), 139.84 (s, CH_{ar(IM-2 or 5)}, 1C), 139.96 (s, C_{ar(IM-4)}, 1C), 175.06 ppm (bd, ²*J*_{FC} = 20.8 Hz, CO₂H, 1C).

3-(4-Bromo-1*H*-imidazol-1-yl)-2-fluoro-2-phosphonopropanoic

acid (1 f): Yield: 76%. Obtained from 0.38 mmol (152 mg) of 6 f. ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 8.43$ ppm (d, ¹*J*_{PF} = 66.5 Hz); ¹H NMR (700 MHz, D₂O): $\delta = 4.46$ (ddd, ²*J*_{HH} = 15.0 Hz, ³*J*_{FH} = 8.8 Hz, ³*J*_{PH} = 4.4 Hz, CH_aH_bC(F)P, 1H), 4.93 (bdd, ³*J*_{FH} = 35.7 Hz, ²*J*_{HH} = 15.0 Hz, CH_aH_bC(F)P, 1H), 7.20, 7.61 ppm (2bs, CH_{ar(M-2 and 5)}, 2H); ¹³C NMR (176 MHz, D₂O, residua signal of EtOH as a reference): $\delta =$ 52.65 (dd, ²*J*_{FH} = 20.2, ²*J*_{PH} = 7.8 Hz, CH₂C(F)P, 1C), 99.46 (dd, ¹*J*_{FC} = 193.2 Hz, ¹*J*_{PC} = 133.2 Hz, CH₂C(F)P, 1C), 113.06 (s, C_{ar(M-4)}, 1C), 121.15, 139.58 (2 s, CH_{ar(M-2 and 5)}, 2C), 175.30 ppm (bd, ²*J*_{FC} = 21.3, CO₂H, 1C); Elemental analysis: C₆H₇BrFN₂O₅P, calcd: C 22.73, H 2.23, N 8.84, found: C 22.66, H 2.33, N 8.75.

General procedure for the synthesis of compounds 1g-l and 2g-l on the example of compound 1i

In a single-neck flask compound **12i** (320 mg, 0.567 mmol) was placed and 36% HCl (5.5 mL, \approx 1ml/0.1 mmol) was added. The mixture was held at reflux for 3 h. Excess HCl was evaporated, and the residue was transferred to a separatory funnel in 5 mL of H₂O and 5 mL of CH₂Cl₂. The organic phase was discarded, and the aqueous

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phase was additionally washed with CH_2CI_2 (3×20 mL). Water was evaporated, and the residue was co-evaporated with EtOH (2× 1 mL). Then 1 mL of EtOH was added, and the resulting precipitate was filtered off and rinsed with 0.5 mL EtOH. The precipitate was dissolved in H₂O (2 mL) and lyophilized. When non-tritylated substrates were used (**10 h**, **j**, **k**, **l** and **12 h** or **12 j**), CH_2CI_2 washing was omitted.

2-Fluoro-3-(1*H***-imidazol-2-yl)-2-phosphonopropanoic acid (1 g):** Yield: 50%. Obtained from 0.41 mmol (229 mg) of **12**g. ³¹P NMR (283 MHz, D₂O, pH 8): δ =10.16 ppm (d, ¹*J*_{PF}=68.9 Hz); ¹H NMR (700 MHz, D₂O): δ =3.49 (ddd, ²*J*_{HH}=15.5 Hz, ³*J*_{FH}=10.5 Hz, ³*J*_{PH}=7.3 Hz, *CH*_aH_bC(F)P, 1 H), 3.77 (ddd, ³*J*_{FH}=36.6 Hz, ²*J*_{HH}=15.5 Hz, ³*J*_{PH}=4.1 Hz, CH_aH_bC(F)P, 1 H), 7.17 ppm (s, *CH*_{ar(IM-4 and 5)}, 2 H); ¹³C NMR (176 MHz, D₂O): δ =32.92 (d, ²*J*_{FH}=21.4 Hz, CH₂C(F)P, 1C), 97.77 (dd, ¹*J*_{FC}=192.2 Hz, ¹*J*_{PC}=138.1 Hz, CH₂C(F)P, 1C), 120.27 (s, CH_{ar(IM-4 and 5)}, 2 C), 144.16 (d, ³*J*_{PC}=14.2 Hz, *C*_{ar(IM-2)}, 1C), 175.91 ppm (dd, ²*J*_{FC}=20.6 Hz, ²*J*_{PC}=2.8 Hz CO₂H, 1C); Elemental analysis: C₆H₈FN₂O₅P(H₂O)_{1.15}, calcd: C 27.84, H 4.01, N 10.82, found: C 27.86, H 3.88, N 10.67.

$\label{eq:2-Fluoro-3-(1-methyl-1 \ensuremath{\textit{H}}\xspace{-1.5}\xspa$

acid (1 h): Yield: 80%. Obtained from 0.54 mmol (180 mg) of 12 h. ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 10.13$ ppm (d, ¹*J*_{PF}=70.6 Hz); ¹H NMR (700 MHz, D₂O): $\delta = 3.34$ (dt, ²*J*_{HH}=15.5 Hz, ³*J*_{FH}=7.8 Hz, ³*J*_{PH}=7.8 Hz, CH_aH_bC(F)P, 1 H), 3.64 (ddd, ³*J*_{FH}=37.7 Hz, ²*J*_{HH}= 15.5 Hz, ³*J*_{PH}=2.7 Hz, CH_aH_bC(F)P, 1 H), 3.64 (s, CH₃IM, 3 H), 6.92 (d, ³*J*_{HH}=1.6 Hz, CH_{ar(IM-4)}, 1 H), 7.01 ppm (d, ³*J*_{HH}=1.6 Hz, CH_{ar(IM-5)}, 1 H); ¹³C NMR (700 MHz, D₂O): $\delta = 30.66$ (d, ²*J*_{FC}=21.9 Hz, CH₂C(F)P, 1C), 34.37 (bs, CH₃IM, 1C), 97.19 (dd, ¹*J*_{FC}=193.7 Hz, ¹*J*_{PC}=137.7 Hz, CH₂C(F)P, 1C), 119.21 (s, CH_{ar(IM-4)}, 1C), 123.01 (s, CH_{ar(IM-5)}, 1C), 143.60 (d, ³*J*_{PC}=13.0 Hz, C_{ar(IM-2)}, 1C), 175.36 ppm (dd, ²*J*_{FC}=20.1 Hz, ²*J*_{PC}= 1.9 Hz, CO₂H, 1C).

2-Fluoro-3-(1*H***-imidazol-4(5)-yl)-2-phosphonopropanoic acid (1 i):** Yield: 70%. Obtained from 0.57 mmol (320 mg) of **12i**. ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 10.39$ ppm (d, ¹ $J_{PF} = 71.1$ Hz); ¹H NMR (700 MHz, D₂O): $\delta = 3.27$ (dt, ² $J_{HH} = 15.5$ Hz, ³ $J_{FH} = 7.6$ Hz, ³ $J_{PH} = 7.6$ Hz, C H_a H_bC(F)P, 1H), 3.59 (ddd, ³ $J_{FH} = 41.8$ Hz, ² $J_{HH} = 15.5$ Hz, ³ $J_{PH} = 2.2$ Hz, CH_aH_bC(F)P, 1H), 6.91 (s, CH_{ar(IM-4(5)]}, 1H), 7.70 ppm (s, CH_{ar(IM-2)}, 1H); ¹³C NMR (176 MHz, D₂O): $\delta = 30.41$ (dd, ² $J_{FC} = 21.5$, ² $J_{PC} = 3.3$ Hz, CH₂C(F)P, 1C), 98.47 (dd, ¹ $J_{FC} = 191.6$ Hz, ¹ $J_{PC} = 142.0$ Hz, CH₂C(F)P, 1C), 117.43 (s, CH_{ar(IM-4(5)]}, 1C), 129.20 (bd, ³ $J_{PC} = 14.2$ Hz, CO₂H, 1C); Elemental analysis: C₆H₈FN₂O₅P(H₂O)_{1.1}, calcd: C 27.94, H 3.99, N 10.86, found: C 28.02, H 4.00, N 10.69.

2-Fluoro-3-(1-methyl-1H-imidazol-5-yl)-2-phosphonopropanoic

acid (1j): Yield: 78%. Obtained from 0.46 mmol (156 mg) of 12j. 1j-E1: $[\alpha]_D^{20} = -18.1 (c = 0.5, H_2 O); 1j-E2: <math>[\alpha]_D^{20} = 23.5 (c = 0.5, H_2 O);$ ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 10.87$ ppm (d, ¹*J*_{PF} = 71.0 Hz); ¹H NMR (700 MHz, D₂O): $\delta = 3.33$ (dt, ²*J*_{HH} = 16.0 Hz, ³*J*_{FH} = 6.9 Hz, ³*J*_{PH} = 6.9 Hz, *CH*_aH_bC(F)P, 1H), 3.63 (ddd, ³*J*_{FH} = 41.0 Hz, ²*J*_{HH} = 16.0 Hz, ³*J*_{PH} = 1.9 Hz, *CH*_aH_bC(F)P, 1H), 3.74 (s, *CH*₃IM, 3H), 6.99 (s, *CH*_{ar(IM-4)}, 1H), 7.91 ppm (s, *CH*_{ar(IM-2)}, 1H); ¹³C NMR (176 MHz, D₂O): $\delta = 28.16$ (bd, ²*J*_{FC} = 20.9 Hz, *CH*₂C(F)P, 1C), 32.20 (bs, *CH*₃IM, 1C), 99.60 (dd, ¹*J*_{FC} = 190.9 Hz, ¹*J*_{PC} = 139.3 Hz, *CH*₂C(F)P, 1C), 123.10 (bs, *CH*_{ar(IM-4)}, 1C), 129.72 (bd, ³*J*_{PC} = 14.0 Hz, *C*_{ar(IM-5)}, 1C), 137.07 (s, *CH*_{ar(IM-2)}, 1C), 176.35 ppm (dd, ²*J*_{FC} = 22.3 Hz, ²*J*_{PC} = 3.9 Hz, *CO*₂H, 1C); Elemental analysis: *C*₇H₁₀FN₂O₅P(H₂O)_{0.35}, calcd: C 32.53, H 4.17, N 10.84, found: C 32.64, H 4.09, N 10.64.

2-Fluoro-3-(4(5)-methyl-1*H***-imidazol-5(4)-yl)-2-phosphonopropanoic acid (1 k)**: Yield: 41%. Obtained from 0.38 mmol (217 mg) of **12 k**. ³¹P NMR (283 MHz, D₂O, pH 8): δ =9.98 ppm (d, ¹*J*_{PF}=71.2 Hz); ¹H NMR (700 MHz, D₂O) δ =2.27 (s, C*H*₃IM, 3H), 3.27 (dt, ²*J*_{HH}=

15.6 Hz, ${}^{3}J_{FH} = 8.1$ Hz, ${}^{3}J_{PH} = 8.1$ Hz, $CH_{a}H_{b}C(F)P$, 1 H), 3.62 (ddd, ${}^{3}J_{FH} = 38.6$ Hz, ${}^{2}J_{HH} = 15.6$ Hz, ${}^{3}J_{PH} = 2.9$ Hz, $CH_{a}H_{b}C(F)P$, 1 H), 8.29 ppm (s, $CH_{ar(IM-2)r}$ 1 H); ${}^{13}C$ NMR (176 MHz, $D_{2}O) \delta = 8.71$ (s, $CH_{3}IM$, 1C), 29.46 (dd, ${}^{3}J_{FC} = 21.4$ Hz, ${}^{3}J_{PC} = 3.4$ Hz, $CH_{2}C(F)P$, 1C), 98.47 (dd, ${}^{1}J_{FC} = 191.2$ Hz, ${}^{1}J_{PC} = 140.3$ Hz, $CH_{2}C(F)P$, 1C), 124.02 (bdd, ${}^{3}J_{PC} = 13.3$ Hz, ${}^{3}J_{FC} = 2.4$ Hz, $C_{ar(IM-4(5))}$, 1C), 127.50 (s, Me- $C_{ar(IM-5(4))}$, 1C), 131.54 (s, $CH_{ar(IM-2)r}$ 1C), 176.03 ppm (dd, ${}^{2}J_{PC} = 19.9$ Hz, ${}^{2}J_{FC} = 2.4$ Hz, $CO_{2}H$, 1C).

2-Fluoro-3-(2-methyl-1*H***-imidazol-4(5)-yl)-2 phosphonopropanoic acid (11):** Yield, 74%. Obtained from 0.31 mmol (180 mg) of **12I**. ³¹P NMR (283 MHz, D₂O, pH 8): $\delta = 10.56$ ppm (d, ¹*J*_{PF} = 69.9 Hz); ¹H NMR (700 MHz, D₂O): $\delta = 2.53$ (s, C*H*₃IM, 3H), 3.26 (dt, ²*J*_{HH} = 15.7 Hz, ³*J*_{FH} = 7.7 Hz, C*H*_aH_bC(F)P, 1H), 3.58 (ddd, ³*J*_{FH} = 39.8 Hz, ²*J*_{HH} = 15.7 Hz, ³*J*_{PH} = 7.7 Hz, C*H*_aH_bC(F)P, 1H), 6.99 ppm (s, C*H*_{ar(IM-4(5)]}, 1H); ¹³C NMR (176 MHz, D₂O): $\delta = 10.92$ (s, C*H*₃-IM, 1C), 30.59 (dd, ²*J*_{FC} = 21.3, ²*J*_{PC} = 3.3 Hz, CH₂C(F)P, 1C), 99.10 (dd, ¹*J*_{FC} = 190.9 Hz, ¹*J*_{PC} = 139.2 Hz, CH₂C(F)P, 1C), 116.52 (s, CH_{ar(IM-4(5)}), 1C), 128.63 (d, ³*J*_{PC} = 20.7 Hz, ²*J*_{PC} = 2.6 Hz, CO₂H, 1C).

Biological studies

HeLa cell culture: The cervical epithelial carcinoma HeLa cell line was purchased from the American Type Cell Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 IU mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. For biological studies, PCs were dissolved in phosphate-buffered saline (PBS) at stock concentrations of 10 mM at approximately pH 7. Compounds were stored at 4 °C or -20 °C (for longer storage) prior to use.

Determination of cytotoxicity: HeLa cells were seeded into 96well cell culture plates at a density of 4×10^3 cells per well in 100 µL of complete culture medium. On the following day, cells were washed with PBS, and 100 µL fresh serum-containing or serum-free (fasting) medium was added. Subsequently HeLa cells were treated with PCs at eight concentrations; 72 h later PrestoBlue Cell Viability Reagent was applied. Following 50 min incubation time at 37 °C and 5% CO₂, cell viability was determined by measuring the fluorescence signal ($\lambda_{Ex}/\lambda_{Em} = 530/590$ nm) on a Synergy 2 microplate reader (BioTek, VT, USA). The obtained fluorescence magnitudes were used to calculate cell viability expressed as a percentage of untreated control cell viability. The data, expressed as the mean of at least four independent experiments, were used to calculate IC₅₀ values.

Assessment of inhibition of Rab11 and Rap1A/Rap1B prenylation: HeLa cells were seeded into six-well cell culture plates at a density of 4×10^5 cells per well in 3 mL of complete medium. On the following day, 1.5 mL of fresh serum-free medium was supplemented with PCs as well as lovastatin. After 48 h of incubation, cell monolayers were rinsed with PBS and detached using trypsin-EDTA solution. The cytosolic and membrane-rich fractions, containing protease inhibitor cocktail, were isolated from cell pellets using Mem-PER Plus Membrane Protein Extraction Kit according to the manufacturers' instructions. The protein concentration in both fractions was determined by Bradford Protein Assay. Equal amounts of protein (20 µg) from cytosolic fractions were resolved by 12% SDS-PAGE and transferred onto 0.2 µm nitrocellulose membranes. Membranes were probed with β -actin, Rab11A, or Rap1A/Rap1B antibodies and detected using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody, followed by electrochemiluminescence (ECL) assay. Visualization of the chemiluminescent pro-



tein bands was performed with a ChemiDoc MP Imaging System (Bio-Rad).

Abbreviations

RGGT (Rab GGTase, GGT-2): Rab geranylgeranyl transferase; FPPS: farnesyl pyrophosphate synthase; GGPPS: geranylgeranyl pyrophosphate synthase; FT: farnesyl transferase; GGT-1: geranylgeranyl transferase 1; FPP: farnesyl pyrophosphate; GPP: geranyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; 3-IPEHPC: 3-(3-pyrid-yl)-2-hydroxy-2-phosphonopropanoic acid; PC: phosphonocarboxy-late; F-ZoIPC: 2-fluoro-3-(1*H*-imidazol-1-yl)-2-phosphonopropanoic acid; NFSI: *N*-fluorobenzenesulfonimide; IM: imidazole ring.

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Conflict of interest

The authors declare no conflict of interest.

Keywords:inhibitors·Michaeladditionphosphonocarboxylates·prenylation·Rabgeranylgeranyltransferase

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FULL PAPERS

Vesicle traffic light: Novel phosphonocarboxylates bearing imidazole in their structure were synthesized and evaluated for inhibitory activity against Rab geranylgeranyl transferase (RGGT). The preferred positions for modifications in the heterocyclic ring, ensuring micromolar potency toward RGGT, harmonize with bicyclic analogues containing the imidazo[1,2-*a*]pyridine core.



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Synthesis and Biological Evaluation of Imidazole-Bearing α-Phosphonocarboxylates as Inhibitors of Rab Geranylgeranyl Transferase (RGGT)