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COMMUNICATION

Incorporation of a fluorous diazirine group into phosphatidylinositol 4,5-bisphosphate to illustrate its interaction with ADP-ribosylation factor 1†

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Phosphatidylinositides are one family of the most versatile signaling molecules in cells, yet how they interact with different proteins to regulate biological processes is not well understood. Towards a general strategy to identify phosphatidylinositide–protein interactions, a fluorous diazirine group has been incorporated into phosphatidylinositol 4,5-bisphosphate (PIP_2). The modified PIP_2 was effectively cleaved by phospholipase C, one signaling protein that utilizes PIP_2 as its endogenous substrate. Upon light illumination, the PIP_2 probe effectively crosslinks with small GTPase ADP-ribosylation 1 to form a complex, suggesting that the probe might be suitable to identify PIP_2-interacting proteins on the proteome level.

Dynamic regulation of the composition of cellular membranes, particularly the phosphatidylinositide (PI) family of lipids, is fundamental to many biological processes such as cell proliferation and migration.[1,2] The enzymes that regulate PI metabolism, such as phosphatidylinositol-3-kinase (PI3K) and phosphatase and tensin homolog (PTEN), have been directly linked to a number of diseases including cancer and diabetes.[3] Various PIs are not only integral components of cell membranes, but also one of the most versatile endogenous signaling molecules due to their dynamic interactions with a wide array of proteins. Understanding how different PIs interact with their binding proteins is thus highly significant because potential therapeutic intervention of PI-signaling could be designed.[4] Indeed, unbiased pulldown experiments have been carried out to identify interacting proteins of various PIs on the proteome level.[4–6] In these experiments, phosphatidylinositides are immobilized on solid supports or liposomes as baits to capture their interacting proteins through non-covalent interactions. However, the binding affinity of PIs with proteins is typically at the low μM range. In addition, some PI-interacting proteins such as protein kinase B (PKB, *aka* Akt) have low abundance in the cells. These features make the traditional affinity pulldown approaches likely fail to identify many PI-interacting proteins.

Fluorous molecules have seen increased applications in addressing various biological problems.[7,8] The fundamental principle behind these applications is that fluorous molecules tend to preferentially partition into the fluorocarbon enriched phase of a bi-phase or tri-phase system.[9–11] Some examples of the recent advancement include fluorous proteomics and metabolite enrichment,[12,13] fluorous small molecule microarrays,[14–16] and fluorous delivery agents.[17,18] These successes prompt us to couple fluorous chemistry with photocrosslinking to identify interacting proteins of endogenous PIs. As illustrated in Fig. 1A, the fluorous affinity group Rf is incorporated into a substrate to crosslink with its interacting proteins. The crosslinked proteins or protein complexes are digested with proteases along with other proteins in the cells. The resulting peptide mixture will then be separated through fluorous solid phase extraction (FSPE) and the fluorinated peptides will be enriched and analyzed. Because the enrichment process is on peptides instead of proteins, this approach will only identify fluorous group labeled interacting proteins, and with high sensitivity. This approach is simple and cost effective compared to other bioreagents, and orthogonal in biological systems. In addition, the fluorous tags are chemically inert and stable in tandem mass spectrometric analysis.[12]

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is one of the seven endogenous PIs and is a substrate or regulator for a number of important signaling proteins such as PI3K, phospholipase C (PLC), and ion channels. To illustrate the application of the fluorous photoaffinity labeling approach, we designed probe **1** (Fig. 1B) that incorporates a fluorous diazirine[19] unit into the side chain of PIP_2. In this design, the PIP_2 unit functions as the substrate for PIP_2-interacting proteins while the diazirine functions as the photoaffinity labeling group. As a control, the non-fluorous PIP_2 analog **2** (Fig. 1B) was also generated.

The synthesis of the fluorous probe **1** started with compound **3** (Scheme 1), which was synthesized from glycerol according to the literature.[20] Coupling of **3** with heptanoic acid in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) at 0 °C generated **4** in 98% yield. Removal of the *p*-methoxybenzyl (PMB) group in **4** with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in wet CH_2Cl_2 gave **5** without any detectable migration of the acyl group from the C2- to the

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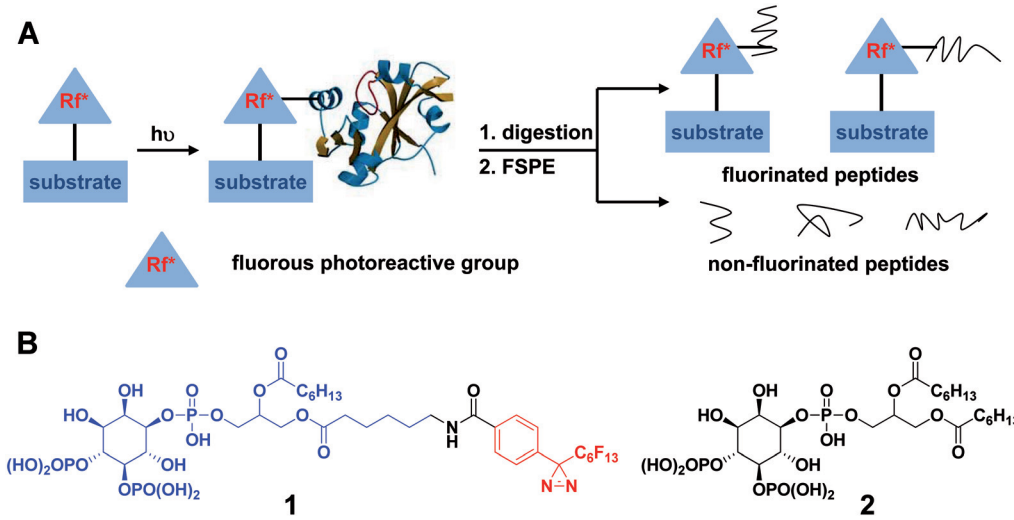
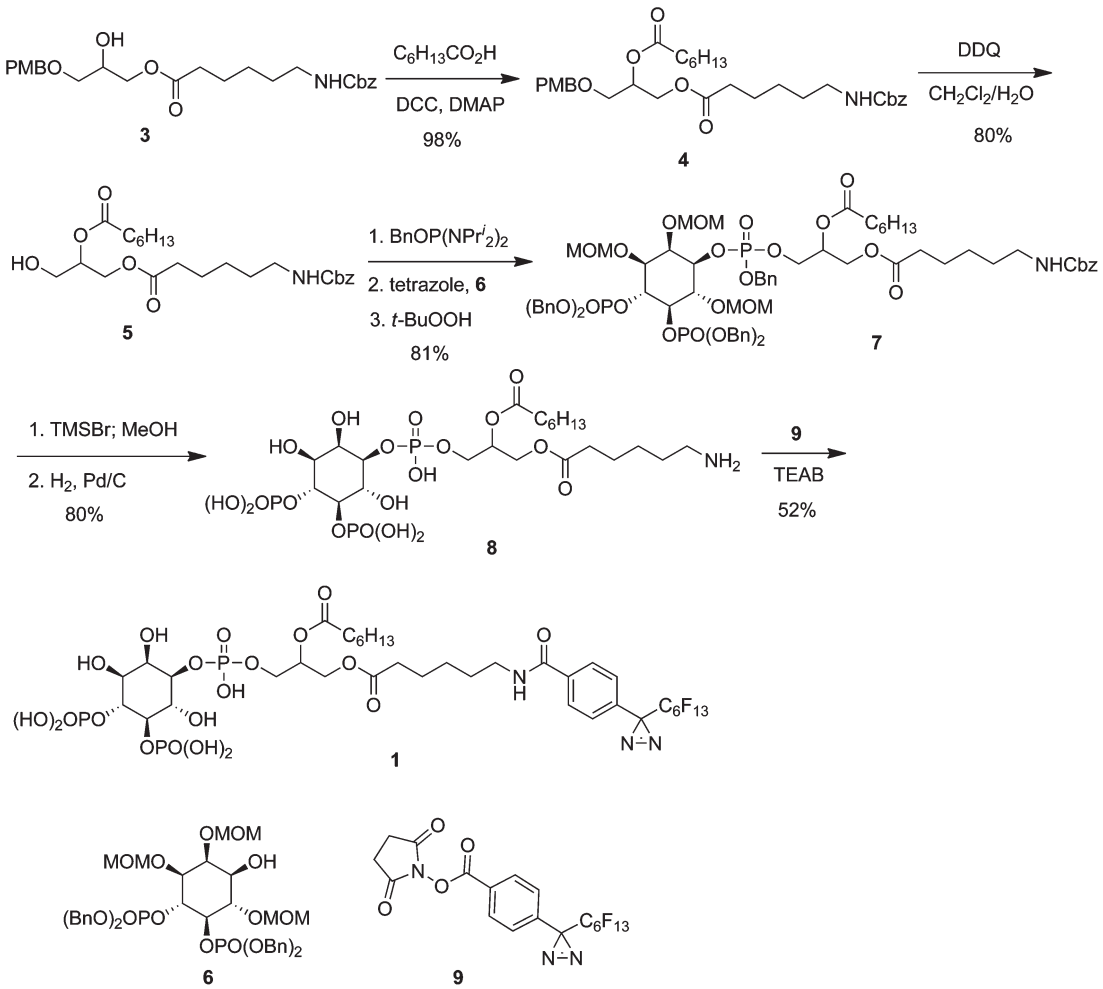


Fig. 1 (A) Fluorous photoaffinity labeling approach to identify small molecule interacting proteins. (B) Chemical structures of PIP_2–fluorous diazirine **1** and a non-fluorous PIP_2 analog **2**.



Scheme 1 Synthesis of the fluorous diazirine–PIP_2 analog **1**.

C3-position. Reaction of diacylglycerol **5** with dibenzyl diisopropylphosphoramidite, which was protonated by weakly acidic tetrazole to facilitate the subsequent nucleophilic addition, provided the phosphorylation reagent in 92% isolated yield. This reagent was not stable, and was used immediately after preparation for coupling to the protected inositol phosphate precursor

6.[21] Upon oxidation with *t*-butylperoxide, the phosphate **7** was synthesized in 81% yield. Removal of the methoxymethyl (MOM) protective group with trimethylsilyl bromide (TMSBr) in CH_2Cl_2 followed by hydrogenolysis to remove the benzyl and carboxybenzyl (Cbz) groups then provided **8** in 80% yield. The terminal amine at the *sn-1* position in **8** then coupled with the fluorous diazirine **9**, which was synthesized as described in the literature,[19] to generate the PIP_2 probe **1**. The PIP_2 derivative **2** was synthesized in a similar sequence of reactions.

Under physiological conditions, PIP_2 can be hydrolyzed by phospholipase C (PLC) isozymes to generate second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). To demonstrate that the fluorous PIP_2 derivative **1** retains functions of PIP_2, we tested its capacity as a PLC substrate and analyzed the reaction mixtures by liquid chromatography-mass spectrometry (LC-MS). When probe **1** was incubated with purified, wild type PLC-δ1, a new peak was observed (Fig. 2) and later confirmed as the diacylglycerol analog **10** (Fig. S1†) by mass spectrometry. In contrast, when the catalytically inactive mutant PLC-δ1 (E341A) was used, no formation of **10** was observed indicating that the cleavage of **1** is PLC-dependent. These results suggest that the fluorous PIP_2 derivative **1** still functions as an effective substrate for PLC.

Small GTPase ADP-ribosylation factor 1 (ARF1) has been implicated as a PIP_2-interacting protein.[5] Accordingly, we have expressed and purified ARF1 with the N-terminal 17 amino acid residues deleted as described in the literature.[22] To carry out the crosslinking experiment, the probe **1** (40 μM) was first incubated with purified ARF1 (20 μM) at 4 °C for 30 min. The complex of **1** and ARF1 was then irradiated at 365 nm for 10 min and the reaction mixture was subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 3A, new bands were formed on the gel suggesting that the crosslinking took place. In addition, these newly-formed

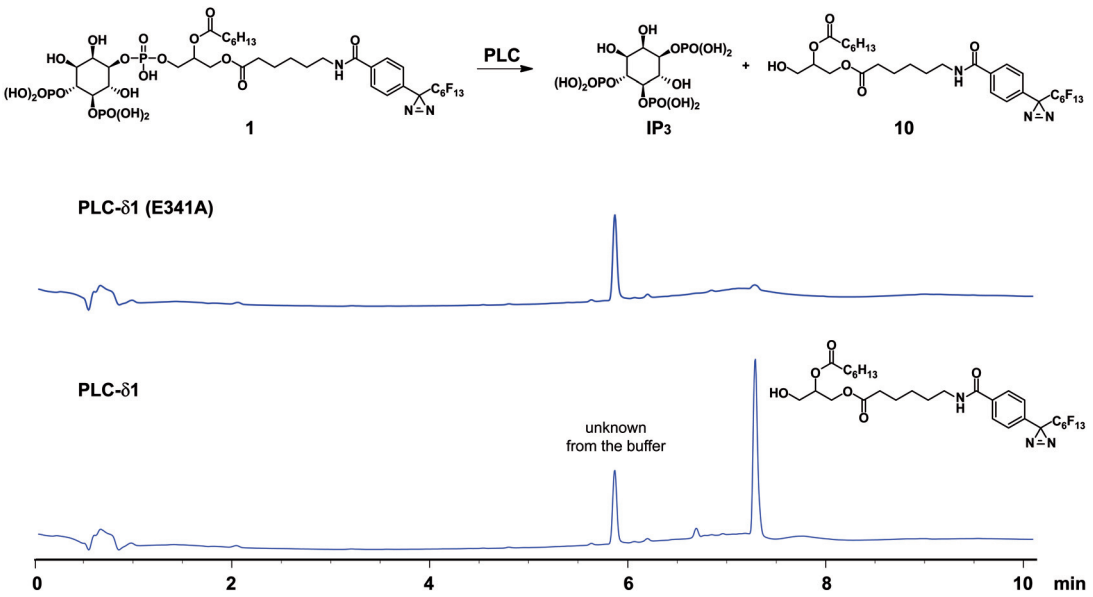


Fig. 2 Hydrolysis of fluorous PIP_2 derivative **1** by PLC-δ1.

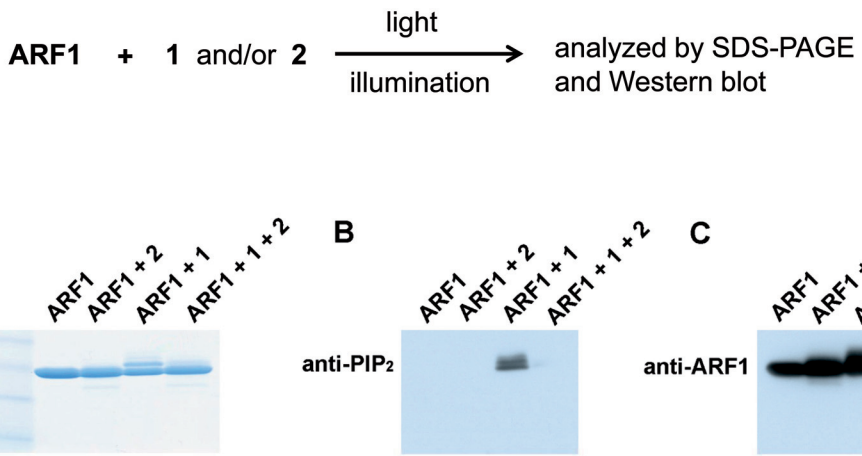


Fig. 3 Fluorous diazirine–PIP_2 analog **1** crosslinked with ARF1 in the presence or absence of **2** and analyzed by SDS-PAGE (A) and Western blot (B, C).

bands were competed off in the presence of soluble PIP_2 derivative **2** (Fig. 3A). The proteins on the SDS-PAGE gel were then transferred to a polyvinylidene fluoride (PVDF) membrane and probed with an antibody against PIP_2 (Fig. 3B). The newly-formed bands showed a strong signal on the Western blot. When the membrane was further probed with an antibody against ARF1, both pure ARF1 and the new bands showed a strong signal (Fig. 3C). Taken together, these results demonstrate that the new bands contain both PIP_2 and ARF1, and are the desired crosslinking products.

In conclusion, we have developed a fluorous diazirine-conjugated PIP_2 derivative as a novel probe to identify PIP_2-interacting proteins. The diazirine group will enable photoaffinity labeling of the interacting proteins while the fluorous tag will enrich peptides derived from the crosslinked proteins for analysis. These features will likely make the new probe suitable for identification of low abundant, low affinity interacting proteins. Although the fluorous photoaffinity group is attached to the *sn-1* position of PIP_2 in this work, it could also potentially be linked through the *sn-2* position or the inositol head group. In the long term, similar techniques should be able to apply to identification of interacting proteins of other endogenous PIs and possibly other families of small molecules.

Experimental

1 Synthesis of probe 1

16-(4-Methoxyphenyl)-3,10-dioxo-1-phenyl-2,11,15-trioxa-4-azahexadecan-13-yl heptanoate (4). The mixture of compound **3** (518 mg, 1.12 mmol), heptanoic acid (172 μL, 1.22 mmol), DCC (322 mg, 1.56 mmol) and DMAP (76 mg, 0.62 mmol) in anhydrous CH_2Cl_2 (20 mL) was stirred at room temperature overnight. After removal of the solvent, the residue was purified by column chromatography (hexane–ethyl acetate = 3 : 1) over silica to yield **4** (627 mg, 98%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.26–7.36 (m, 5H), 7.22 (d, J = 8.2 Hz, 2H), 6.86 (d, J = 8.6 Hz, 2H), 5.22 (m, 1H), 5.08 (s, 2H), 5.01 (brs, 1H), 4.45 (d, J = 11.4 Hz, 2H), 4.34 (dd, J = 11.8, 3.7 Hz, 1H), 4.15 (dd, J = 11.6, 6.8 Hz, 1H), 3.78 (s, 3H), 3.54 (d, J = 5.2 Hz, 2H), 3.16 (q, J = 6.7 Hz, 2H), 2.28 (m, 4H), 1.41–1.65 (m, 6H), 1.22–1.36 (m, 8H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.1, 173.0, 159.3, 156.4, 136.7, 129.7, 129.3, 128.4, 128.03, 128.01, 113.8, 72.9, 70.0, 67.8, 66.5, 62.8, 55.2, 40.8, 34.3, 33.8, 31.4, 29.6, 28.7, 26.1, 24.9, 24.4, 22.5, 14.0; ESI-HRMS for $[M + Na]^+$ $C_{32}H_{45}NO_8Na$: calcd 594.3043, found 594.3042.

1-(6-Benzyloxycarbonylaminhexanoyloxy)-3-hydroxypropan-2-yl heptanoate (5). To the solution of **4** (314 mg, 0.55 mmol) in wet CH_2Cl_2 (18 mL) was added DDQ (262 mg, 1.16 mmol). The reaction mixture was stirred at room temperature for 4 h, and then washed with 10% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$ and concentrated under vacuum. The resulting residue was purified by column chromatography (hexane–ethyl acetate = 2 : 1) over silica to generate **5** (199 mg, 80%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.31 (m, 5H), 5.05–5.17 (m, 3H), 4.33 (dd, J = 11.7, 4.1 Hz, 1H), 4.19 (dd, J = 12.1, 6.0 Hz, 1H), 3.71 (d, J = 5.1 Hz, 2H), 3.17 (q, J = 6.6 Hz, 2H), 2.99 (brs, 1H), 2.35 (m, 4H), 1.44–1.68 (m, 6H), 1.23–1.40 (m, 8H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.41, 173.40, 156.45, 136.60, 128.51, 128.09, 72.05, 66.63, 62.19, 61.44, 40.82, 34.28, 33.87, 31.42, 29.62, 28.73, 26.11, 24.88, 24.44, 22.47, 14.02; ESI-HRMS for $[M + Na]^+$ $C_{24}H_{37}NO_7Na$: calcd 474.2468, found 474.2472.

Compound 7. A solution of **5** (150 mg, 0.33 mmol) in anhydrous CH_2Cl_2 (1.5 mL) was added dropwise under argon to a flask that contained 1-(benzyloxy)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphinediamine (0.66 mmol) and tetrazole (24 mg, 0.34 mmol) in anhydrous CH_2Cl_2 (1.5 mL). The mixture was stirred at room temperature for 2 h before the solvent was removed by vacuum. The resulting residue was purified by flash column chromatography (hexane–ethyl acetate–triethyl amine = 100 : 20 : 3) to give the phosphoramidite intermediate as a colorless oil. The so-formed phosphoramidite (0.28 mmol) was dissolved in anhydrous CH_2Cl_2 and added to the solution of compound **6** (120 mg, 0.14 mmol) and 1*H*-tetrazole (39 mg, 0.56 mmol) in anhydrous CH_2Cl_2 (1.0 mL). The reaction mixture was stirred at room temperature overnight before *t*-BuOOH (5.5 M, 0.25 mL, 1.4 mmol) was added at −40 °C. The reaction mixture was then warmed to room temperature gradually and the solvents were removed. The residue was purified by column chromatography (hexane–acetone = 2 : 1) over silica to provide **7** (187 mg, 81% from compound **5**) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.20–7.40 (m, 25H), 4.82–4.55 (m, 15H), 4.65–4.80 (m, 3H), 4.62 (dd, J = 6.7, 6.9 Hz, 1H), 4.55 (d, J = 7.0 Hz, 1H), 4.02–4.48 (m, 8H), 3.54 (m, 1H), 3.39 (conformation 1) and 3.36 (conformation 2) (s, 3H), 3.32 (conformation 1) and 3.28 (conformation 2) (s, 3H), 3.24 (conformation 1) and 3.23 (conformation 2) (s, 3H), 3.18 (q, J = 6.6 Hz, 2H), 2.22–2.34 (m, 4H), 1.40–1.68 (m, 6H), 1.16–1.38 (m, 8H), 0.88 (t, J = 6.9 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.0, 172.9 and 172.8 (1C), 156.6, 136.8, 135.9, 135.8, 135.7, 135.5, 128.9, 128.8, 128.59, 128.56, 128.55, 128.52, 128.49, 128.44, 128.11, 128.07, 127.99, 98.9, 97.6, 97.0, 96.9, 78.9, 77.5, 77.2, 76.8, 76.5, 75.9, 74.7, 74.6, 74.5, 70.0, 69.8, 69.5, 69.3, 66.6, 65.80, 65.75, 65.57, 61.6, 56.74, 56.72, 56.69, 56.0, 55.8, 40.9, 34.1, 34.0, 33.9, 33.8, 31.5, 29.8, 29.7, 28.8, 26.2, 24.8, 24.4, 22.5, 14.1; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −0.18 (2P), −0.42 and −0.47 (1P); ESI-HRMS for $[M + H]^+$ $C_{71}H_{93}NO_{24}P_3$: calcd 1436.5300; found 1436.5282.

Compound 8. Freshly distilled TMSBr (0.6 mL) was added to a solution of compound **7** (20 mg, 13.9 μmol) in anhydrous CH_2Cl_2 (0.6 mL) at 0 °C under argon. The reaction mixture was stirred at room temperature for 1 h and the solvents were then removed under vacuum for 1 h. The residue was redissolved in MeOH (1.0 mL) and the resulting solution was stirred at room temperature for 1 h. After removal of the solvent, the residue was dried under vacuum for another 1 h. The residue was again redissolved in MeOH (3.0 mL) and 10% Pd/C (10 mg) was added. The mixture was stirred under H_2 at room temperature for 8 h before the Pd/C was filtered off. The filtrate was concentrated, washed with cold $CHCl_3$ three times (0.9 mL in total), and dried under vacuum to yield **8** (10 mg, 80%) as a white foamy solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 5.24 (m, 1H), 4.50 (dd, J = 9.2, 18.4 Hz, 1H), 4.39 (m, 1H), 3.95–4.26

(m, 7H), 3.66 (m, 1H), 2.96 (t, J = 7.5 Hz, 2H), 2.30–2.46 (m, 4H), 1.55–1.76 (m, 6H), 1.26–1.50 (m, 8H), 0.91 (t, J = 7.1 Hz, 3H); ^{13}C NMR (CD_3OD, 101 MHz) δ 174.64, 174.60, 81.0, 79.8, 78.3, 72.5, 72.0, 71.6, 65.6, 63.1, 63.0, 40.6, 35.0, 34.5, 32.6, 29.8, 28.2, 26.8, 25.9, 25.3, 23.6, 14.4; ^{31}P NMR (CD_3OD, 200 MHz) δ 2.19 (1P), 1.79 (1P), 0.33 (1P); MS (ESI) m/z 718.1 $[M - H]^-$. ESI-HRMS for $[M + H]^+$ $C_{22}H_{45}NO_{19}P_3$: calcd 720.1799; found 720.1798.

Compound 1. A solution of NHS ester **9** (5.1 mg, 10.2 μmol) in DMF (600 μL) was added to compound **8** (4.5 mg, 6.3 μmol) in TEAB buffer (0.25 M, 600 μL). The reaction mixture was stirred in the dark at room temperature overnight before the solvents were removed under vacuum. The residue was purified through fluorous solid phase extraction to yield **1** (3.9 mg, 52%) as a white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.80 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.3 Hz, 2H), 5.13 (m, 1H), 4.35 (dd, J = 11.0, 3.4 Hz, 1H), 4.28 (dd, J = 17.7, 7.8 Hz, 1H), 3.94–4.19 (m, 4H), 3.84–3.94 (m, 3H), 3.52 (dd, J = 9.7, 2.6 Hz, 1H), 3.28 (t, J = 6.7 Hz, 2H), 2.18–2.30 (m, 4H), 1.42–1.62 (m, 6H), 1.24–1.38 (m, 8H), 0.78 (t, J = 7.0 Hz, 3H); ^{13}C NMR (CD_3OD, 125 MHz) δ 174.9, 174.8, 168.8, 138.0, 132.9, 129.5, 129.4, 80.5, 78.4, 77.8, 72.8, 72.5, 72.2, 72.1, 72.0, 65.0, 63.8, 59.8, 41.0, 35.2, 34.8, 32.7, 30.1, 29.9, 27.6, 26.0, 25.7, 23.6; ^{31}P NMR (CD_3OD, 162 MHz) δ 3.7 (1P), 3.0 (1P), 1.0 (1P); ^{19}F NMR (CD_3OD, 376 MHz) δ −80.7 (t, J = 10.1 Hz), −108.8, −119.4, −121.2, −122.1, −125.6; MS (ESI) m/z 1080.3 $[M - H]^-$, 589.6 $[M - 2H]^{2-}$. ESI-HRMS for $[M + H]^+$ $C_{36}H_{48}F_{13}N_3O_{20}P_3$: calcd 1182.1836; found 1182.1833.

2 Crosslinking of ARF1 with probe 1

The protein ARF1 was expressed and purified as described in the literature.[22] A solution of ARF1 (20 μM) in the Tris buffer (50 mM, pH 8.0, 60 μL) was incubated with the fluorous diazirine **1** (40 μM) for 30 min at 4 °C. Subsequently, the reaction mixture was illuminated at 365 nm at 4 °C for 10 min. For competition experiments, PIP_2 derivative **2** (4 mM) was added to the mixture of ARF1 and probe **1**. The reaction mixture was analyzed by SDS-PAGE and Western blot. The Western blot was developed by using mouse anti-PIP_2 (1 : 2000) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1 : 5000) or anti-ARF1 (1 : 2000) and HRP-conjugated anti-rabbit IgG (1 : 5000).

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