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Development of activity-based probes with tunable specificity for protein tyrosine phosphatase subfamilies

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ABSTRACT

Herein we describe the development of activity-based probes toward protein tyrosine phosphatase (PTP) subfamilies. A novel phosphotyrosine analog serving as the latent trapping unit has been designed and explored. It allows addition of various amino acid residues to its C- and N-termini to extend the recognition element. As a proof-of-concept, we have synthesized three tripeptide probes, which carry the phosphotyrosine analog in the middle position and a leucinamide residue at the C-terminus. The three tripeptide probes differed only in their N-terminal amino acid (Glu, Phe, and Lys). The labeling properties of these probes were determined and the results showed the newly synthesized probes could selectively label PTPs in an activity-dependent manner. In addition, the probes' target specificity was also shown to be influenced by the amino acid residues flanking the phosphotyrosine analog.

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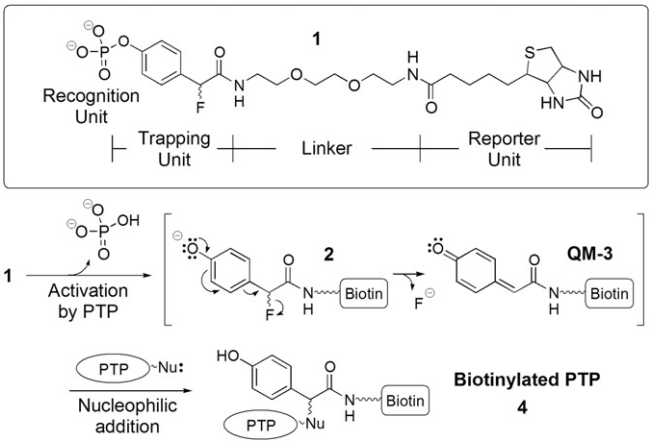
1. Introduction

Reversible protein tyrosine phosphorylation is one of the most important post-translational modifications that is utilized by living organisms to regulate protein functions.[1,2] The phosphorylation state of a protein not only determines its function, subcellular location, and distribution, but also relays the influence to other proteins through protein–protein interactions. The extent of tyrosine phosphorylation is tuned by the well-regulated actions of protein tyrosine kinases (PTks) and protein tyrosine phosphatases (PTPs). Dysfunctions of PTks and PTPs are usually associated with human diseases.[3] Although PTP-related research lags far behind that of PTKs, there is an increasing interest, in recent years, in PTPs as drug targets.[4,5] For example, PTEN is implicated as a product of a tumor suppressor gene[6–8] and PTP1B is associated with diabetes.[9]

Recently, development of activity-based probes for hydrolases has proven to be a powerful tool in modern chemical biology study.[10,11] We have previously developed probe **1** as a general probe for PTPs (Scheme 1).[12] Probe **1** consists of four structural units, including a biotin reporter unit. The labeling event of PTPs with probe **1** is a two-stage process, including activation and alkylation, as shown in Scheme 1. When the P–O bond between the recognition unit and the latent trapping unit is selectively cleaved with the



Scheme 1. The four structural units as well as the selective activation/labeling process of the activity-based probe **1** for PTPs.

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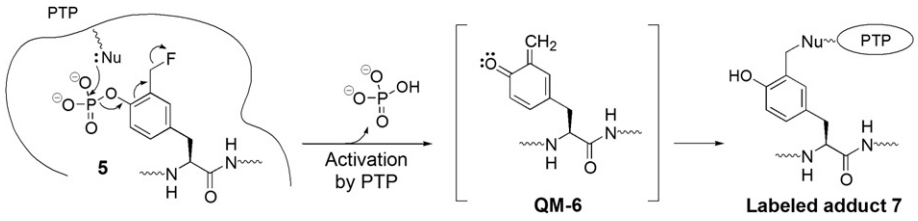
assistance of PTPs, it becomes activated with the release of *p*-hydroxybenzylic fluoride intermediate **2**. Intermediate **2** quickly undergoes 1,6-elimination to produce highly reactive quinone methide **3** (**QM-3**), which in turn alkylates nearby nucleophilic residues to form the biotinylated adduct **4**.[13]

In this report, we describe the development of activity probes that target subfamilies of PTPs. Since many PTPs showed different substrate specificity,[14] we envisioned that expansion of the recognition region by adding suitable interacting elements to the latent trapping unit would assist in tuning the target specificity.[15] However, the latent trapping unit of probe **1** does not have free ends to incorporate extra interacting elements. We thus designed phosphotyrosine analog **5** to serve as a novel latent trapping unit (Scheme 2), which would allow incorporation of various amino acid residues to its C- and N-termini to extend the recognition unit. As a proof-of-concept, we performed model synthesis on three tripeptide probes (**8a**–**c**) and evaluated their labeling performances. As shown in Figure 1, probes **8a**–**c** carry the phosphotyrosine analog in the middle position and a leucinamide residue at the C-terminus of the tripeptides. Probes **8a**–**c** differed only in the N-terminal residues (Glu for **8a**, Phe for **8b**, and Lys for **8c**). In this series of probes, a biotin reporter unit was attached through a hydrophilic linker to the N-terminus. The proposed activation/labeling mechanism is similar to that of probe **1**, except 1,4-elimination would occur for the formation of **QM-6** (Scheme 2).



Scheme 2. Novel phosphotryosine analog **5** serving as the latent trapping unit and the proposed activation/labeling process for PTPs.

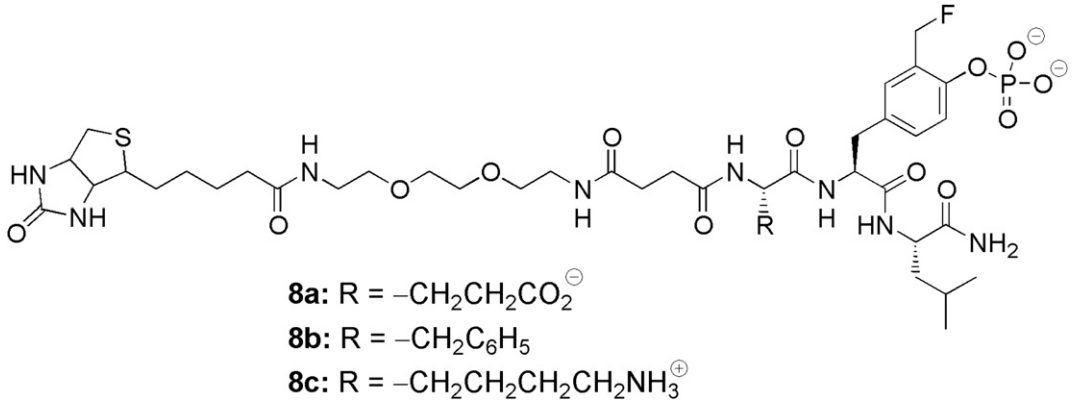


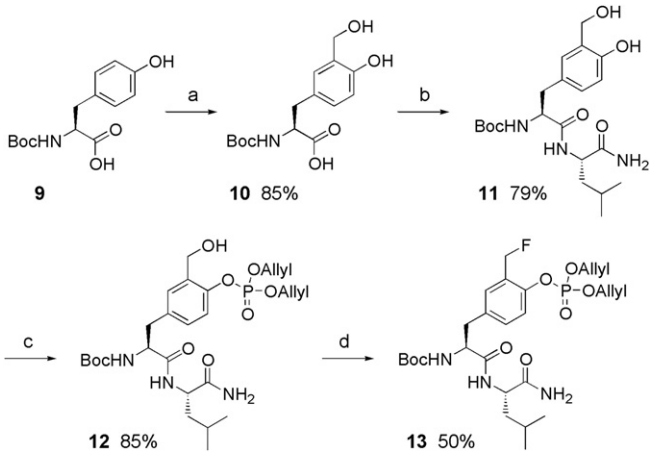
Figure 1. The structures for probes **8a**–**c**.

2. Results and discussion

The syntheses of tripeptide probes **8a**–**c** began with commercially available Boc-L-Tyr (**9**). As shown in Scheme 3, *ortho*-hydroxymethylation of **9** was accomplished by treating formaldehyde and borax under alkaline condition to give diol **10** (yield 85%). L-Leucinamide was then coupled to compound **10** under standard DCC/HOBt condition to give the dipeptide unit **11** (yield 79%). Selective phosphorylation of **11** at the phenolic OH was achieved with diallyl phosphite/CCl_4/DMAP to give the phosphorylated product **12** (yield 85%). The benzylic alcohol **12** was then converted to the corresponding fluoride by treatment with DAST to afford the orthogonally protected dipeptide intermediate **13** (yield 50%), which is a common key intermediate for the preparation of probes **8a**–**c**.

The Boc group of the dipeptide intermediate **13** was removed by treatment with TFA and the resultant ammonium salt was coupled to the three respective amino acid residues via the N-terminus (Scheme 4). The coupling reaction was accomplished with the standard DCC/HOBt procedure to give the fully protected tripeptides **14a**–**c** (yields 75–80%). For **14a** and **14c**, Fmoc protecting group was used for the α-amino group, whereas for **14b**, Boc was used. These protecting groups could be removed under basic and acidic conditions, respectively, allowing the attachment of a succinic acid spacer to offer compounds **15a**–**c** (yields 60–70%). The biotin reporter group with an ethylene glycol-type linker (**16**)[16] was then coupled to compounds **15a**–**c** to give the fully protected form of tripeptide probes **17a**–**c** (yields 60–65%). Final deprotection step was successfully carried out with TMSBr and BSTFA to provide the desired tripeptide probes **8a**–**c** (yields 80–90%).

To test if the newly designed and synthesized probes **8a**–**c** could label PTPs, we used the catalytic domain of PTP1B, a 39 kDa protein with 339 amino acid residues, as a model for the labeling experiments. In a typical labeling reaction, 3 μg each of PTP1B proteins were incubated with 0.25 mM of probe **8a** at 25 °C in the presence/absence of 1 mM Na_3VO_4. The incubation mixtures were divided into two parts and then monitored using two parallel SDS-polyacrylamide gels (Fig. 2). The gel on the left was stained with Coomassie blue, which showed the relative amount of loaded proteins. The gel on the right was visualized by immunoblotting analysis after transferring the reaction products onto a nitrocellulose membrane. As shown in Figure 2, intense biotinylated protein bands were observed in PTP1B that was treated with probe **8a**. In contrast, no biotinylated adduct was observed when Na_3VO_4, a phosphatase inhibitor, was present in the incubation mixture. Similar results were obtained when TCPTP was used as the labeling



Scheme 3. Synthesis of the orthogonally protected dipeptide intermediate **13**. Reagents and conditions: (a) CH_2O, $Na_2B_4O_7$, NaOH/H_2O; (b) L-Leucinamide, DCC, HOBt, DIEA, DMF; (c) $(AllylO)_2POH$, CCl_4, DMAP, DIEA, acetone; (d) DAST, CH_2Cl_2.

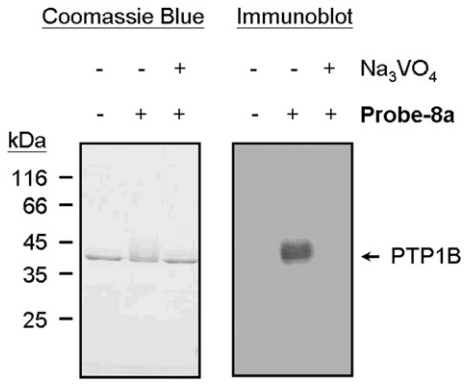
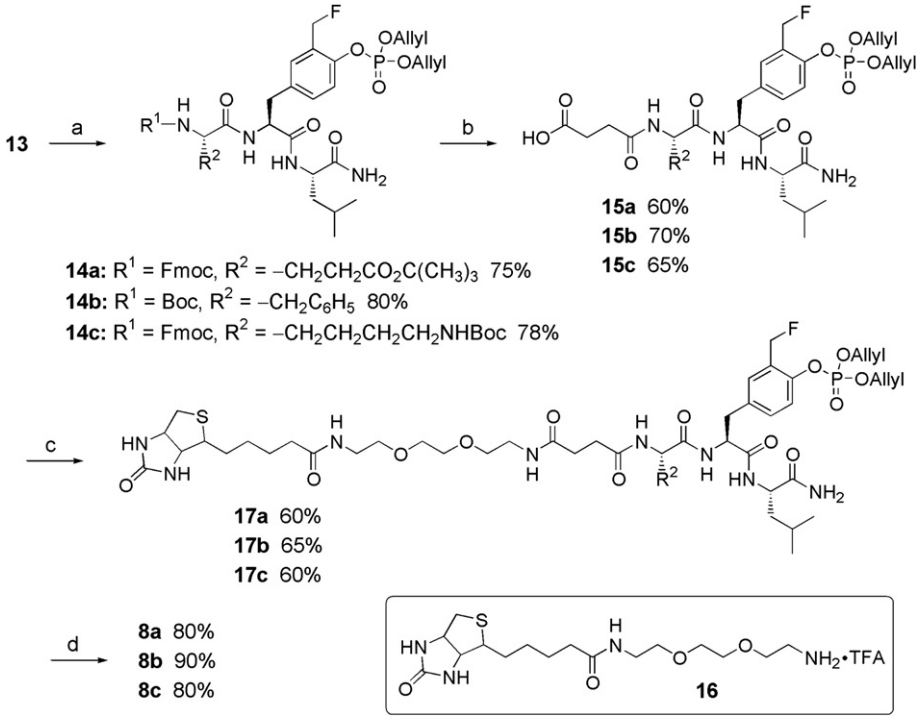


Figure 2. Activity-dependent labeling of PTP1B with probe **8a**. Each of PTP1B (3 μg) proteins were incubated with 0.25 mM of probe **8a** at 25 °C with or without 1 mM Na_3VO_4. The reaction mixtures were separated by 10% SDS-PAGE and then subjected to immunoblotting analysis using *anti*-biotin antibody. Labeled proteins were visualized by chemiluminescence using an ECL kit (Perkin–Elmer). The Coomassie blue-stained (left) and the ECL-developed gels (right) are presented.

target (data not shown). Since the probes themselves are also the substrates of the corresponding PTPs, the results clearly indicate that the newly developed latent trapping unit well mimics the phosphorotyrosine residue of the natural substrate. The results also indicate that the long tail containing the linker and the biotin reporter attached to the N-terminus of the tripeptide does not prevent the probe from entering the active site. Significantly, the labeling of PTPs with probe **8a** was activity dependent. It should be noted that the benzylic fluoride moiety in probes **8a**–**c** showed reasonable stability in the labeling buffer. In the absence of PTPs, they underwent hydrolysis slowly and the purity remained greater than 90% even after 1 h (as determined by HPLC).

To further confirm the group specificity of the activity probes, we compared the effect of probes **8a**–**c** on other proteins, including carbonic anhydrase, γ-globulin, phosphorylase b, RNase A, and lysozyme. The results obtained showed that probes **8a**–**c** did not label any of these proteins (data not shown). To test if probes **8a**–**c** could differentiate PTPs from other phosphatases, we conducted labeling experiments on nine phosphatases, including five PTPs, alkaline phosphatase (ALP), PTEN (dephosphorylating the phosphatidylinositol 3,4,5-trisphosphate), and two serine/threonine phosphtases (PPP1CA and PPM1A). The results showed that probes **8a**–**c** labeled all five PTPs and yet did not label any of the non-PTPs (Fig. 3), confirming that probes **8a**–**c** are indeed highly specific for PTPs.

In order to further study how well probes **8a**–**c** could differentiate between the various PTPs, we compared the labeling intensities on five PTPs (PTP1B, SHP2, TCPTP, VHR, and PTP-PEST) with those obtained from probe **1**. When the intensities of



Scheme 4. Synthesis of probes **8a**–**c** for PTPs. Reagents and conditions: (a) for **14a**: (i) TFA, CH_2Cl_2, (ii) Fmoc-Glu(O^tBu), DCC, HOBt, DIEA, DMF; for **14b**: (i) TFA, CH_2Cl_2, (ii) Boc-Phe, DCC, HOBt, DIEA, DMF; for **14c**: (i) TFA, CH_2Cl_2, (ii) Fmoc-Lys(Boc), DCC, HOBt, DIEA, DMF; (b) (i) 20% TFA (for **15b**)/50% Et_2NH (for **15a**, **15c**), (ii) succinic anhydride, DMAP, CH_2Cl_2; (c) **16**, DCC, HOBt, DIEA, DMF; (d) BSTFA, TMSBr, CH_3CN.

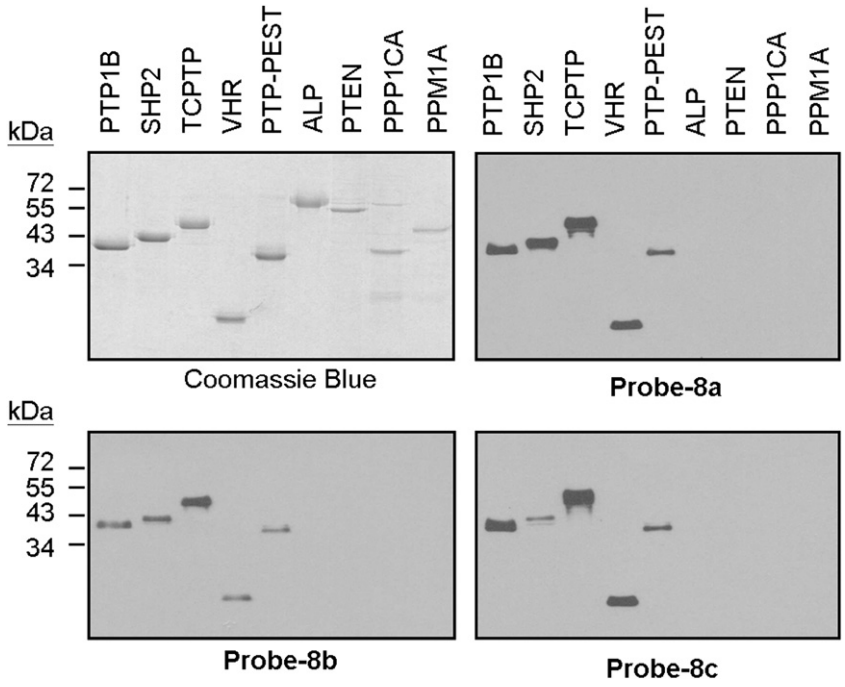


Figure 3. Probes **8a**–**c** specifically labeled PTPs. Each of (3 μg) different phsophatases were incubated with 0.25 mM of the probes **8a**–**c** at 25 °C for 20 min. For PPM1A, 60 mM of $MgCl_2$ was added to the reaction. The reaction mixtures were separated by 12% SDS-PAGE and then subjected to immunoblotting analysis using *anti*-biotin antibody.

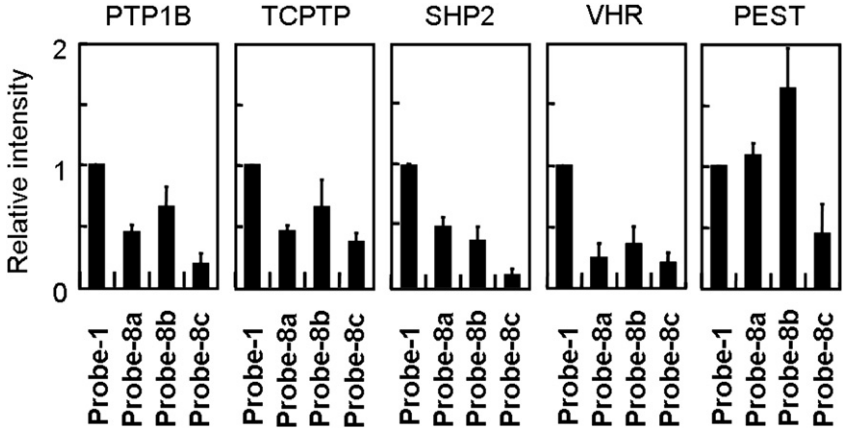


Figure 4. Determination of PTP substrate preferences by probes **8a**–**c**. Each of (3 μg) PTP1B, SHP2, TCPTP, VHR, or PTP-PEST were incubated with 0.1 mM probes at 25 °C for 20 min. The reaction mixtures were separated by 12% SDS-PAGE and subjected to immunoblotting analysis using *anti*-biotin antibody. Quantification of the western blots using Vilber Lourmat quantification tool. The results show the average value of three to four independent experiments.

the probes **8a**–**c**-labeled bands in each set of experiments were normalized relative to that of a probe **1**-labeled band, we were able to establish a quantitative comparison of their labeling preference (Fig. 4). The result strongly suggests that the labeling intensity also reflects the trend of substrate specificities for these PTPs. Of the five PTPs tested, the substrate specificities of PTP1B, TCPTP, and SHP2 were better studied than those of VHR and PTP-PEST.[17–21] The results for the former three PTPs showed that both PTP1B and TCPTP preferred probes with sequence Phe-pTyr-Leu over Glu-pTyr-Leu and Lys-pTyr-Leu, whereas SHP2 preferred Glu-pTyr-Leu and Phe-pTyr-Leu over Lys-pTyr-Leu, clearly supporting the observation that these probes indeed labeled different PTPs with varying efficiency. The trend of substrate preference obtained from this study was similar to those determined by other methods.[17–21]

We have also compared the labeling intensity data for the other two PTPs, VHR (a dual-specificity phosphatase) and PTP-PEST (a classical PTP). The results obtained suggest that PTP-PEST showed a substrate preference similar to that of PTP1B and TCPTP, whereas VHR did not show much substrate preference. This provides evidence to support the concept that probes with added amino acid residues flanking the latent trapping device were able to influence their target specificities. Although probes **8a**–**c** have not yet achieved exclusive labeling on a designated PTP, the underlying concept of expanding the recognition element presented in this paper is consistent with the recent trend in the development of inhibitors and chemical probes for PTPs.[22,23]

3. Conclusion

We have developed a novel phosphotyrosine analog as a latent trapping unit. It was incorporated into three tripeptide probes in the model synthesis. These probes could label PTPs in

an activity-dependent manner and the labeling was PTP-selective. The new trapping unit functioned as expected and that the amino acids flanking the trapping device could affect the target specificity. Therefore, our data support the concept that expansion of the recognition unit would help differentiate PTPs. Our ongoing studies aim to increase the variety of the recognition elements at the flanking positions in order to obtain probes capable of differentiating specific members in PTP subfamilies.

4. Experimental

4.1. Synthesis of the orthogonally protected dipeptide intermediate 13

All reagents and starting materials were obtained from commercial suppliers (Acros, Aldrich, and Merck) and were used without further purification. IR spectra were recorded on a Nicolet 550 series II spectrometer. ^{1}H, ^{19}F, and ^{13}C NMR were recorded using a Bruker Avance 400 spectrometer. The proton and carbon chemical shifts are given in parts per million using $CDCl_3$ (δ H 7.24 and 77.0) as internal standard. High resolution mass spectra were recorded with a JEOL-102A mass spectrometer. Analytical TLC (silica gel, 60F-54, Merck) were visualized under UV light and/or phosphomolybdic acid/ethanol stain. Column chromatography was performed with Kiesegel 60 (70–230 mesh) silica gel (Merck). Melting points are reported without correction.

*4.1.1. (S)-2-((tert-Butoxycarbonyl)amino)-3-(4-hydroxy-3-(hydroxymethyl)phenyl) propanoic acid (**10**).* To a solution of Boc-L-Tyr (**9**) (13.0 g, 46.2 mmol), 1 N NaOH (110 mL, 110 mmol), sodium borate decahydrate (44.1 g, 116 mmol) in 180 mL of water was added 35% formaldehyde (19.8 mL, 250 mmol). The reaction mixture was stirred at 40 °C for 3 days. When no more starting material was detected by TLC, the pH was adjusted to three with 1 N HC1. The solution was then extracted with EtOAc. The combined organic layer was washed with brine (×2), dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **10** was obtained (12.2 g, 85%) as an oil. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.13 (s, 1H, aromatic), 7.00 (dd, J=1.5, 8.9 Hz, 1H, aromatic), 6.72 (d, J=8.9 Hz, 1H, aromatic), 4.69 (s, 2H, benzylic), 4.30 (m, 1H), 3.08 (dd, J=5.0, 13.9 Hz, 1H), 2.91 (dd, J=8.4, 13.9 Hz, 1H), 1.35 (s, 9H, *t*-Bu). ^{13}C NMR (100 MHz, acetone-d_6): δ 173.9 (C), 156.3 (C), 154.8 (C), 129.6 (CH), 129.3 (CH), 128.8 (C), 127.8 (C), 115.8 (CH), 79.3 (C), 61.7 (CH_2), 55.9 (CH), 37.4 (CH_2), 28.5 (CH_3). IR (KBr): 3364, 2992, 2972, 1713, 1693, 1527, 1501, 1441, 1375, 1262, 1215, 1169, 1023 cm^{-1}. HRMS calcd for $C_{15}H_{21}NO_6Na$ $(M+Na)^+$ 334.1267, found 334.1275.

*4.1.2. tert-Butyl ((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl) amino)-3-(4-hydroxy-3-(hydroxy-methyl)phenyl)-1-oxopropan-2-yl) carbamate (**11**).* To an ice-cooled solution of compound **10** (704 mg, 2.26 mmol), HOBt (61 mg, 0.45 mmol), L-leucinamide hydrochloride (374 mg, 2.26 mmol), and DIEA (1495 μL, 9.05 mmol) in 20 mL of anhydrous DMF was added a solution of DCC (513 mg, 2.49 mmol) in 1 mL of DMF. The mixture was allowed to warm to rt and stirred for another 16 h. The white DCU precipitate was filtered off. The filtrate was concentrated to dryness and the residual oil was dissolved in EtOAc and then washed consecutively with 5% citric acid (×1), 5% $NaHCO_3$ (×3), H_2O (×3), and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **11** was obtained (760 mg, 79%) as a white solid after silica gel column chromatography eluted with $CHCl_3$/MeOH (94/6), mp 168–170 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 8.46 (br s, 1H, phenolic), 7.61 (d, J=8.3 Hz, 1H, NH), 7.11 (s, 1H, aromatic), 6.99 (dd, J=1.7, 8.2 Hz, 1H, aromatic), 6.96 (s, 1H, NH), 6.71 (d, J=8.2 Hz, 1H, aromatic), 6.68 (s, 1H, NH), 6.20 (d, J=7.6 Hz, 1H, NH), 4.67 (s, 2H, benzylic), 4.63–4.43 (m, 2H), 4.30 (m, 1H), 3.03 (dd, J=6.0, 13.8 Hz, 1H), 2.86 (dd, J=7.9, 13.8 Hz, 1H), 1.78–1.50 (m, 3H), 1.35 (s, 9H, *t*-Bu), 0.88 (d, J=6.0 Hz, 3H), 0.86 (d, J=5.7 Hz, 3H). ^{13}C NMR (100 MHz, acetone-d_6): δ 175.5 (C), 172.6 (C), 156.4 (C), 154.8 (C), 129.7 (CH), 129.5 (CH), 128.9 (C), 128.0 (C), 115.9 (CH), 79.5 (C), 61.8 (CH_2), 57.2 (CH_2), 52.0 (CH), 41.8 (CH_2), 37.8 (CH_2), 28.5 (CH_3), 25.1 (CH), 23.5 (CH_3), 21.9 (CH_3). IR (KBr): 3443, 3350, 3297, 3217, 2919, 2853, 1673, 1653, 1394, 1368, 1262, 990 cm^{-1}. HRMS calcd for $C_{21}H_{33}N_3O_6Na$ $(M+Na)^+$ 446.2267, found 446.2289.

*4.1.3. tert-Butyl ((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl) amino)-3-(4-((bis(allyloxy) phosphoryl)oxy)-3-(hydroxy-methyl) phenyl)-1-oxopropan-2-yl) carbamate (**12**).* To an ice-cooled solution of compound **11** (2.00 g, 4.72 mmol), DIEA (3.1 mL, 19 mmol), CCl_4 (4.5 mL, 47 mmol), and DMAP (115 mg, 0.943 mmol) in 50 mL of anhydrous acetone was added dropwise diallyl phosphite (1.4 mL, 9.4 mmol). The mixture was allowed to warm to rt. After stirring for 18 h the reaction mixture was concentrated under reduced pressure and the residual oil was dissolved in EtOAc and then washed consecutively with 5% citric acid (×3), H_2O (×3), and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **12** was obtained (2.34 g, 85%) as a colorless oil after silica gel column chromatography eluted with $CHCl_3$/MeOH (9/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43 (d, J=8.1 Hz, 1H, NH), 7.27 (s, 1H, aromatic), 7.09–7.00 (m, 2H, aromatic), 6.38 (s, 1H, NH), 6.26 (s, 1H, NH), 5.95–5.82 (m, 2H, =CH), 5.67 (d, J=8.1 Hz, 1H, NH), 5.34 (dt, J=17.1, 1.3 Hz, 2H, $=CH_2$), 5.23 (dd, J=10.4, 1.1 Hz, 2H, $=CH_2$), 4.68–4.52 (m, 6H), 4.52–4.30 (m, 3H), 3.00–2.78 (m, 2H), 1.62–1.45 (m, 2H), 1.45–1.26 (m, 10H), 0.80 (d, J=5.9 Hz, 3H), 0.79 (d, J=8.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.7 (C), 171.3 (C), 155.4 (C), 146.7 (d, J=7.1 Hz, C), 134.1 (C), 132.8 (d, J=5.7 Hz, C), 131.8 (dd, J=6.6, 3.1 Hz, C), 130.4 (CH), 129.7 (CH), 120.1 (CH), 118.8 (d, J=3.0 Hz, CH_2), 79.9 (C), 69.1 (d, J=3.0 Hz, CH_2), 59.4(CH_2), 55.5 (CH), 51.3 (CH), 40.3 (CH_2), 37.7 (CH_2), 28.2 (CH_3), 24.5 (CH), 22.8 (CH_3), 21.7 (CH_3). ^{31}P NMR (162 MHz, $CDCl_3$): δ −4.99. IR (KBr): 3310, 2966, 2933, 2878, 1673, 1534, 1501, 1361, 1428, 1255, 1182, 1109, 1130 cm^{-1}. HRMS calcd for $C_{27}H_{42}N_3O_9P$ Na $(M+Na)^+$ 606.2556, found 608.2368.

*4.1.4. tert-Butyl ((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl) amino)-3-(4-((bis(allyloxy) phosphoryl)oxy)-3-(fluoromethyl)phenyl)-1-oxopropan-2-yl) carbamate (**13**).* To an ice-cooled solution of compound **12** (1.470 g, 2.52 mmol) in 25 mL of anhydrous CH_2Cl_2 was slowly added DAST (463 μL, 3.78 mmol) through a syringe. The reaction mixture was allowed to warm to rt. When no more starting material was observed, it was cooled and quenched by adding 0.5 mL of MeOH and a small amount of silica gel. Silica gel was filtered off and the filtrate was concentrated under reduced pressure. The residual oil was dissolved in EtOAc and then washed consecutively with 5% $NaHCO_3$ (×3) and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. The desired product was purified by silica gel column chromatography eluted with $CHCl_3$/MeOH (95/5) to give compound **13** (738 mg, 50%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.27–7.18 (m, 2H, aromatic), 7.14 (d, J=8.4 Hz, 1H, aromatic), 8.30 (d, J=8.3 Hz, 1H, NH), 6.22 (br s, 1H, NH), 6.06 (br s, 1H, NH), 5.97–5.79 (m, 2H, =CH), 5.39 (d, J=7.6 Hz, 1H, NH), 5.38 (d, J=48.1 Hz, 2H, CH_2F), 5.34 (d, J=17.0 Hz, 2H, $=CH_2$), 5.23 (d, J=10.4 Hz, 2H, $=CH_2$), 4.60 (t, J=7.0 Hz, 4H, allylic), 4.50–4.25 (m, 2H), 3.03 (dd, J=13.8, 7.0 Hz, 1H), 2.94 (dd, J=13.0, 6.6 Hz, 1H), 1.66–1.49 (m, 2H), 1.45 (m, 1H), 1.35 (s, 9H, *t*-Bu), 0.84 (t, J=6.3 Hz, 3H), 0.83 (t, J=6.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3 (C), 171.2 (C), 155.5 (C), 147.2 (d, J=11.0 Hz, C), 133.9 (C), 131.8 (dd, J=6.8, 2.6 Hz, CH), 131.1 (CH), 130.5 (d, J=5.8 Hz, CH), 127.6 (dd, J=17.3, 6.6 Hz, C), 120.1 (CH), 118.9 (d, J=1.4 Hz, CH_2), 80.2 (C), 79.7 (d, J=167 Hz, CH_2F), 69.0 (dd, J=5.6, 1.3 Hz, CH_2), 55.7 (CH), 51.2 (CH), 40.5 (CH_2), 37.2 (CH_2), 28.2 (CH_3), 24.6 (CH), 22.9 (CH_3), 21.7

(CH_3). ^{31}F NMR (376 MHz, $CDCl_3$): δ −214.6 (d, J=47.0 Hz). ^{31}P NMR (162 MHz, $CDCl_3$): δ −5.84. IR (KBr): 3297, 2959, 2912, 1653, 1501, 1368, 1255, 1176, 1030, 963 cm^{-1}. HRMS calcd for $C_{27}H_{41}FN_3O_8P$ Na $(M+Na)^+$ 608.2513, found 608.2518.

4.2. Synthesis of probes 8a–c

*4.2.1. tert-Butyl ((S)-1-(((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl)amino)-3-(4-((bis (allyloxy)phosphoryl)oxy)-3-(fluoromethyl) phenyl)-1-oxopropan-2-yl)amino)-1-oxo-3-phenyl- propan-2-yl) carbamate (**14b**).* To a solution of the fluorinated compound **13** (1.00 g, 1.71 mmol) in 17 mL CH_2Cl_2 was added 3.4 mL of TFA. After stirring at rt for 30 min, the reaction mixture was concentrated under reduced pressure and then kept under high vacuum to remove the residual TFA. The resultant TFA salt was used for the coupling reaction without further purification. To an ice-cooled solution of the TFA salt, DIEA (1.2 mL, 6.8 mmol), HOBt (93 mg, 0.68 mmol), and Boc-L-Phe (454 mg, 1.71 mmol) in 15 mL of anhydrous DMF was added a solution of DCC (423 mg, 2.05 mmol) in 3 mL of DMF. The mixture was allowed to warm to rt and stirred for another 18 h. The white DCU precipitate was filtered off. The filtrate was concentrated to dryness. The residual oil was dissolved in $CHCl_3$ and then washed consecutively with 5% citric acid (×3), H_2O (×3), and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **14b** was obtained (1.102 g, 80%) as a white solid after silica gel column chromatography eluted with $CHCl_3$/MeOH (92/8), mp 143–145 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 7.42 (s, 1H, aromatic), 7.38–7.15 (m, 7H, aromatic), 6.08–5.92 (m, 2H, =CH), 5.48 (d, J=47.6 Hz, 2H, CH_2F), 5.42 (d, J=17.1 Hz, 2H, =CH_2), 5.30 (d, J=10.5 Hz, 2H, =CH_2), 4.75–4.61 (m, 5H), 4.44 (t, J=7.4 Hz, 1H), 4.36 (dd, J=9.6, 4.7 Hz, 1H), 3.19 (dd, J=13.5, 5.4 Hz, 1H), 3.09–2.73 (m, 2H), 2.77 (dd, J=13.7, 9.9 Hz, 1H), 1.77–1.57 (m, 3H), 1.38 (s, 9H, *t*-Bu), 0.97 (d, J=6.3 Hz, 3H), 0.94 (d, J=6.2 Hz, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 177.1 (C), 174.3 (C), 172.7 (C), 157.6 (C), 148.8 (C), 138.5 (C), 135.8 (C), 133.3 (CH), 132.6 (CH), 132.5 (CH), 130.3 (CH), 129.4 (CH), 128.9 (C), 127.7 (CH), 120.9 (CH), 119.2 (CH_2), 80.8 (d, J=164.4 Hz, CH_2F), 80.7 (C), 70.5 (CH_2), 57.4 (CH), 55.5 (CH), 52.8 (CH), 42.0 (CH_2), 39.2 (CH_2), 38.1 (CH_2), 28.7 (CH_3), 25.8 (CH), 23.5 (CH_3), 22.0 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.4 (t, J=48.0 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.11. IR (KBr): 3284, 2919, 2853, 1640, 1554, 1123, 1036 cm^{-1}. HRMS calcd for $C_{36}H_{50}FN_4O_9PNa$ $(M+Na)^+$ 755.3197, found 755.3203.

*4.2.2. (S)-tert-Butyl 4-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(((S)-1-(((S)-1-amino- 4-methyl-1-oxopentan-2-yl)amino)-3-(4-((bis (allyloxy)phosphoryl)oxy)-3-(fluoromethyl)phenyl)-1-oxopropan-2-yl) amino)-5-oxopentanoate (**14a**).* The same procedure as that for compound **14b** was used, except Fmoc-Glu(O^{t}Bu)-OH was used for the coupling. Yield 75%, mp 149–150 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 7.84 (d, J=7.5 Hz, 2H, aromatic), 7.70 (d, J=7.4 Hz, 2H, aromatic), 7.46–7.38 (m, 3H, aromatic), 7.38–7.30 (m, 3H, aromatic), 7.27 (d, J=8.4 Hz, 1H, aromatic), 6.04–5.90 (m, 2H, =CH), 5.45 (d, J=47.6 Hz, 2H, CH_2F), 5.40 (d, J=17.1 Hz, 2H, =CH_2), 5.29 (d, J=10.5 Hz, 2H, =CH_2), 4.74–4.58 (m, 5H), 4.45–4.32 (m, 3H), 4.25 (t, J=6.8 Hz, 1H), 4.10 (dd, J=8.6, 5.5 Hz, 1H), 3.22 (dd, J=13.9, 5.5 Hz, 1H), 3.03 (dd, J=13.9, 8.2 Hz, 1H), 2.28 (t, J=7.6 Hz, 2H), 2.03 (m, 1H), 1.85 (m, 1H), 1.73–1.55 (m, 3H), 1.48 (s, 9H, *t*-Bu), 0.93 (d, J=6.2 Hz, 3H), 0.92 (d, J=6.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3 (C), 172.5 (C), 171.8 (C), 170.2 (C), 156.5 (C), 147.2 (C), 143.8 (C), 143.5 (C), 141.1 (C), 133.5 (C), 131.7 (CH), 131.1 (CH), 130.5 (CH), 127.6 (CH), 127.0 (CH), 125.1 (CH), 120.1 (CH), 119.8 (CH), 118.8 (CH_2), 80.8 (C), 79.7 (d, J=165.5 Hz, CH_2F), 69.0 (CH_2), 67.1 (CH_2), 54.7 (CH), 54.2 (CH), 51.3 (CH), 46.8 (CH), 40.7 (CH_2), 37.7 (CH_2), 31.6 (CH_2), 27.9 (CH_3), 27.9 (CH_2), 24.7 (CH), 22.7 (CH_3), 21.9 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.7 (t, J=48.0 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −5.38. IR (KBr): 3277, 2926, 1640, 1540, 1454, 1262, 1156, 1043 cm^{-1}. HRMS calcd for $C_{46}H_{58}FN_4O_{11}PNa$ $(M+Na)^+$ 915.3721, found 915.3680.

*4.2.3. (9H-Fluoren-9-yl)methyl tert-butyl ((S)-6-(((S)-1-(((S)-1-amino-4-methyl-1-oxopentan- 2-yl)amino)-3-(4-((bis(allyloxy)phosphoryl) oxy)-3-(fluoromethyl)phenyl)-1-oxopropan-2-yl)amino)-6-oxohexane-1,5-diyl) dicarbamate (**14c**).* The same procedure as that for compound **14a** was used, except Fmoc-Lys(Boc)-OH was used for the coupling. Yield 78%, mp 148–150 °C. ^{1}H NMR (400 MHz, Acetone-d_6): δ 7.87 (d, J=7.6 Hz, 2H, aromatic), 7.72 (d, J=7.2 Hz, 2H, aromatic), 7.71 (s, 1H, NH), 7.56 (d, J=5.9 Hz, 1H, NH), 7.45–7.36 (m, 3H, aromatic), 7.36–7.23 (m, 4H, aromatic), 6.89 (d, J=5.9 Hz, 1H, NH), 6.68 (s, 1H, NH), 6.39 (s, 1H, NH), 6.04 (s, 1H, NH), 6.04–5.92 (m, 2H, =CH), 5.47 (d, J=49.1 Hz, 2H, CH_2F), 5.39 (d, J=19.0 Hz, 2H, =CH_2), 5.24 (d, J=10.4 Hz, 2H, =CH_2), 4.72–4.60 (m, 5H), 4.45–4.33 (m, 2H), 4.30–4.17 (m, 2H), 4.11 (m, 1H), 3.21 (dd, J=14.2, 5.1 Hz, 1H), 3.10–2.94 (m, 3H), 1.78–1.28 (m, 9H), 1.39 (s, 9H, *t*-Bu), 0.84 (d, J=5.0 Hz, 3H), 0.83 (d, J=5.0 Hz, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 177.1 (C), 175.0 (C), 172.9 (C), 158.5 (C), 145.2 (C), 142.6 (C), 136.0 (C), 133.3 (CH), 132.5 (CH), 132.4 (CH), 128.8 (CH), 128.2 (CH), 126.3 (CH), 121.0 (CH), 119.2 (CH_2), 80.8 (d, J=164.2 Hz, CH_2F), 79.9 (C), 70.4 (CH_2), 68.1 (CH_2), 56.7 (CH), 55.7 (CH) ,52.9 (CH), 48.4 (CH), 41.9 (CH_2), 41.0 (CH_2), 37.5 (CH_2), 32.7 (CH_2), 30.5 (CH_2), 28.8 (CH_3), 25.8 (CH), 24.1 (CH_2), 23.5 (CH_3), 21.9 (CH_3). ^{19}F NMR (376 MHz, $CDCl_3$): δ −216.8 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, Acetone-d_6): δ −5.77. IR (KBr): 3277, 3078, 2933, 2475, 2402, 1686, 1640, 1540, 1428, 1255, 1176, 1030 cm^{-1}. HRMS calcd for $C_{48}H_{63}FN_5O_{11}PNa$ $(M+Na)^+$ 958.4143, found 958.4147.

*4.2.4. 4-(((S)-1-(((S)-1-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl) amino)-3-(4-((bis(allyloxy) phosphoryl)oxy)-3-(fluoromethyl)phenyl)-1-oxopropan-2-yl)amino)-5-(tert-butoxy)-1,5-dioxopentan-2-yl) amino)-4-oxobutanoic acid (**15a**).* To a solution of compound **14a** (203 mg, 0.227 mmol) in 4 mL of CH_2Cl_2 was added 2 mL of Et_2NH. The mixture was stirred at rt for 30 min. When no more starting material was observed by TLC analysis, the reaction mixture was concentrated under reduced pressure and then kept under high vacuum to remove the residual Et_2NH. To a solution of the resultant Et_2NH salt in 2 mL of anhydrous CH_2Cl_2 was added succinic anhydride (46 mg, 0.46 mmol) at rt. After stirring for 18 h, $CHCl_3$ (20 ml) was added to dilute the reaction mixture. The diluted reaction mixture was then washed consecutively with 5% citric acid (×2), H_2O (×3), and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **15a** was obtained (105 mg, 60%) as an oil after silica gel column chromatography eluted with $CHCl_3$/MeOH (9/1). ^{1}H NMR (400 MHz, CD_3OD): δ 7.46 (s, 1H, aromatic), 7.38 (d, J=8.4 Hz, 1H, aromatic), 7.29 (d, J=8.4 Hz, 1H, aromatic), 6.06–5.86 (m, 2H, =CH), 5.46 (d, J=47.5 Hz, 2H, CH_2F), 5.40 (d, J=17.1 Hz, 2H, =CH_2), 5.28 (d, J=10.5 Hz, 2H, =CH_2), 4.75–4.60 (m, 4H), 4.53 (dd, J=10.2, 4.7 Hz, 1H), 4.37 (m, 1H), 4.18 (dd, J=8.3, 5.4 Hz, 1H), 3.25 (dd, J=14.1, 4.6 Hz, 1H), 3.09 (dd, J=13.9, 10.5 Hz, 1H), 2.76 (m, 1H), 2.69–2.50 (m, 2H), 2.43 (m, 1H), 2.30–2.10 (m, 2H), 1.93 (m, 1H), 1.79 (m, 1H), 1.72–1.55 (m, 3H), 1.43 (s, 9H, *t*-Bu), 0.97 (d, J=5.4 Hz, 3H), 0.90 (d, J=5.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.7 (C), 177.3 (C), 176.2 (C), 174.7 (C), 173.8 (C), 173.6 (C), 149.0 (C), 136.7 (C), 133.6 (CH), 132.6 (CH), 132.2 (CH), 129.3 (C), 121.3 (CH), 119.5 (CH_2), 82.0 (C), 80.8 (d, J=164.6 Hz, CH_2F), 70.7 (CH_2), 57.0 (CH), 55.4 (CH), 53.3 (CH), 41.8 (CH_2), 36.9 (CH_2), 32.7 (CH_2), 31.4 (CH_2), 30.3 (CH_2), 28.6 (CH_3), 27.8 (CH_2), 26.0 (CH), 24.0 (CH_3), 21.8 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.5 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.11. IR (KBr): 3271, 2926, 1726,

1639, 1540, 1421, 1249, 1156, 1017, 937 cm^{-1}. HRMS calcd for $C_{35}H_{52}FN_4O_{12}PNa$ $(M+Na)^+$ 793.3201, found 793.3228.

*4.2.5. 4-(((S)-1-(((S)-1-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl) amino)-3-(4-((bis(allyloxy) phosphoryl)oxy)-3-(fluoromethyl)phenyl)-1-oxopropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-4-oxobutanoic acid (**15b**).* To a solution of compound **14b** (607 mg, 0.828 mmol) in 5 mL of CH_2Cl_2 was added 1 mL of TFA. After stirring at rt for 30 min, the solvent and acid were removed under reduced pressure to give the TFA salt, which was used for the coupling reaction without further purification. To a solution of the TFA salt in 8 mL of anhydrous CH_2Cl_2 was added TEA (465 μL, 3.35 mmol), DMAP (20 mg, 0.16 mmol), and succinic anhydride (166 mg, 1.66 mmol). After stirring at rt for 18 h, $CHCl_3$ (50 ml) was added to dilute the reaction mixture. The diluted reaction mixture was then washed consecutively with 5% citric acid (×2), H_2O (×3), and brine (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **15b** was obtained (426 mg, 70%) as an oil after silica gel column chromatography eluted with $CHCl_3$/MeOH (9/1). ^1H NMR (400 MHz, CD_3OD): δ 7.50 (s, 1H, aromatic), 7.42 (d, J=8.5 Hz, 1H, aromatic), 7.33 (d, J=8.4 Hz, 1H, aromatic), 7.28–7.17 (m, 5H, aromatic), 6.08–5.95 (m, 2H, =CH), 5.50 (d, J=47.5 Hz, 2H, CH_2F), 5.42 (d, J=15.6 Hz, 2H, $=CH_2$), 5.31 (d, J=10.4 Hz, 2H, $=CH_2$), 4.75–4.65 (m, 4H), 4.56 (dd, J=10.0, 4.7 Hz, 1H), 4.47 (dd, J=9.5, 5.0 Hz, 1H), 4.40 (m, 1H), 3.27 (dd, J=14.1, 4.8 Hz, 1H), 3.13 (dd, J=13.9, 10.2 Hz, 1H), 3.06 (dd, J=14.1, 4.8 Hz, 1H), 2.83 (dd, J=14.0, 9.7 Hz, 1H), 2.71 (m, 1H), 2.62–2.48 (m, 2H), 2.36 (m, 1H), 1.80–1.60 (m, 3H), 1.0 (d, J=5.4 Hz, 3H), 0.95 (d, J=5.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.4 (C), 175.9 (C), 174.4 (C), 174.4 (C), 173.3 (C), 148.8 (C), 138.2 (C), 136.5 (C), 133.4 (CH), 132.4 (CH), 132.1 (CH), 130.0 (CH), 129.5 (CH), 129.1 (C), 127.8 (CH), 121.0 (CH), 119.3 (CH_2), 80.3 (d, J=164.4 Hz, CH_2F), 70.5 (CH_2), 57.2 (CH), 56.8 (CH), 53.1 (CH), 41.6 (CH_2), 38.0 (CH_2), 36.9 (CH_2), 31.3 (CH_2), 30.4 (CH_2), 25.8 (CH), 23.7 (CH_3), 21.6 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.4 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.14. IR (KBr): 3271, 2919, 2853, 1640, 1554, 1428, 1255, 1103, 1036 cm^{-1}. HRMS calcd for $C_{35}H_{46}FN_4O_{10}PNa$ $(M+Na)^+$ 755.2833, found 755.2809.

*4.2.6. 4-(((10S,13S,16S)-13-(4-((Bis(allyloxy)phosphoryl)oxy)-3-(fluoromethyl)benzyl)-16- carbamoyl-2,2,18-trimethyl-4,11,14-trioxo-3-oxa-5,12,15-triazanonadecan-10-yl)amino)-4-oxobutanoic acid (**15c**).* The same procedure as that for compound **15a** was used. Yield 65%. ^1H NMR (400 MHz, CD_3OD): δ 7.52 (s, 1H, aromatic), 7.45 (d, J=8.5 Hz, 1H, aromatic), 7.32 (d, J=8.4 Hz, 1H, aromatic), 6.10–5.95 (m, 2H, =CH), 5.50 (d, J=47.5 Hz, 2H, CH_2F), 5.44 (d, J=17.3 Hz, 2H, $=CH_2$), 5.32 (d, J=10.5 z, 2H, $=CH_2$), 4.78–4.66 (m, 4H), 4.52 (dd, J=10.7, 4.5 Hz, 1H), 4.40 (m, 1H), 4.11 (dd, J=8.2, 4.5 Hz, 1H), 3.29 (m, 1H), 3.15 (m, 1H), 2.99 (t, J=7.0 Hz, 2H), 2.81 (m, 1H), 2.70–2.55 (m, 2H), 2.44 (m, 1H), 1.78–1.53 (m, 5H), 1.53–1.44 (m, 2H), 1.47 (s, 9H, *t*-Bu), 1.44–1.33 (m, 2H), 1.02 (d, J=5.6 Hz, 3H), 0.94 (d, J=5.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 179.0 (C), 177.5 (C), 177.0 (C), 175.2 (C), 173.5 (C), 158.3(C), 148.6 (C), 136.7 (C), 133.3 (CH), 132.4 (CH), 132.1 (CH), 128.8 (C), 120.8 (CH), 119.2 (CH_2), 80.8 (d, J=164.4 Hz, CH_2F), 79.7 (C), 70.4 (CH_2), 57.0 (CH), 56.0 (CH) ,53.1 (CH), 41.6 (CH_2), 40.8 (CH_2), 36.5 (CH_2), 32.4 (CH_2), 32.4 (CH_2), 31.6 (CH_2), 30.5 (CH_2), 28.8 (CH_3), 25.7 (CH), 23.8 (CH_2), 23.7 (CH_3), 21.6 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −215.9 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.15. IR (KBr): 3277, 2919, 2860, 1640, 1540, 1255, 1136, 1036 cm^{-1}. HRMS calcd for $C_{37}H_{57}FN_5O_{12}PNa$ $(M+Na)^+$ 836.3623, found 836.3593.

*4.2.7. Diallyl (4-((2S,5S)-2-(((S)-1-amino-4-methyl-1-oxopentan-2-yl) carbamoyl)-5-benzyl-4,7,10,21-tetraoxo-25-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-14,17-dioxa-3,6,11,20-tetraazapentacosyl)-2-(fluoromethyl)phenyl) phosphate (**17b**).* To an ice-cooled solution of **16** (386 mg, 0.819 mmol), **15b** (600 mg, 0.819 mmol), DIEA (1.082 mL, 6.55 mmol) and HOBt (55 mg, 0.41 mmol) in 7 mL of DMF was added a solution of DCC (186 mg, 0.901 mmol) in 1 mL of DMF. The mixture was allowed to warm to rt and stirred for another 18 h. The white DCU precipitate was filtered off. The filtrate was concentrated to dryness. The residual oil was dissolved in $CHCl_3$ and then washed consecutively with 5% citric acid (×1) and brine (×1). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **17b** was obtained (580 mg, 65%) as a oil after silica gel column chromatography eluted with $CHCl_3$/MeOH (9/1). ^1H NMR (400 MHz, CD_3OD): δ 7.53 (s, 1H, aromatic), 7.46 (d, J=8.3 Hz, 1H, aromatic), 7.35 (d, J=8.4 Hz, 1H, aromatic), 7.28–7.19 (m, 3H, aromatic), 7.19–7.12 (m, 2H, aromatic), 6.10–5.95 (m, 2H, =CH), 5.52 (d, J=47.7 Hz, 2H, CH_2F), 5.43 (d, J=17.2 Hz, 2H, $=CH_2$), 5.32 (d, J=10.5 Hz, 2H, $=CH_2$), 4.74–4.67 (m, 4H), 4.58–4.48 (m, 2H), 4.45–4.36 (m, 2H), 4.32 (m, 1H), 3.64 (s, 4H), 3.61–3.55 (m, 4H), 3.46 (m, 1H), 3.42–3.35 (m, 2H), 3.35–3.26 (m, 2H), 3.25–3.17 (m, 2H), 3.06 (m, 1H), 2.95 (m, 1H), 2.85–2.63 (m, 3H), 2.59–2.45 (m, 2H), 2.34 (m, 1H), 2.28–2.20 (m, 2H), 1.83–1.56 (m, 7H), 1.52–1.42 (m, 2H), 1.01 (d, J=6.1 Hz, 3H), 0.94 (d, J=5.9 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.5 (C), 176.5 (C), 176.2 (C), 174.8 (C), 174.7 (C), 173.5 (C), 166.1 (C), 148.7 (C), 138.3 (C), 136.8 (C), 133.4 (CH), 132.4 (CH), 132.0 (CH), 130.0 (CH), 129.5 (CH), 127.8 (CH), 121.0 (CH), 119.3 (CH_2), 80.9 (d, J=164.3 Hz, CH_2F), 71.4 (CH_2), 71.2 (CH_2), 70.5 (CH_2), 70.5 (CH_2), 70.5 (CH_2), 63.4 (CH), 61.6 (CH), 57.6 (CH), 57.1 (CH), 57.0 (CH), 53.1 (CH), 41.6 (CH_2), 41.1 (CH_2), 40.5 (CH_2), 40.3 (CH_2), 37.9 (CH_2), 36.7 (CH_2), 31.9 (CH_2), 31.7 (CH_2), 30.8 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 26.8 (CH_2), 25.8 (CH), 23.7 (CH_3), 21.5 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.4 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.12. IR (KBr): 3430, 2913, 2853, 1646, 1554, 1467, 1255, 1096, 1036 cm^{-1}. HRMS calcd for $C_{51}H_{74}FN_8O_{13}PSNa$ $(M+Na)^+$ 1111.4715, found 1111.4746.

*4.2.8. (S)-tert-Butyl 4-(((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl)amino)-3-(4-((bis(allyl- oxy)phosphoryl)oxy)-3-(fluoromethyl) phenyl)-1-oxopropan-2-yl)carbamoyl)-6,9,20-trioxo-24-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-13,16-dioxa-5,10,19-triazatetracosan-1-oate (**17a**).* The same procedure as that for compound **17b** was used. Yield 60%. ^1H NMR (400 MHz, CD_3OD): δ 7.54 (s, 1H, aromatic), 7.46 (d, J=8.4 Hz, 1H, aromatic), 7.33 (d, J=8.4 Hz, 1H, aromatic), 6.10–5.96 (m, 2H, =CH), 5.51 (d, J=47.4 Hz, 2H, CH_2F), 5.43 (d, J=15.7 Hz, 2H, $=CH_2$), 5.33 (d, J=10.4 Hz, 2H, $=CH_2$), 4.77–4.68 (m, 4H), 4.58–4.50 (m, 2H), 4.39 (m, 1H), 4.34 (dd, J=7.9, 4.4 Hz, 1H), 4.16 (dd, J=8.4, 5.2 Hz, 1H), 3.70–3.63 (m, 4H), 3.63–3.54 (m, 4H), 3.54–3.35 (m, 4H), 3.28–3.20 (m, 2H), 2.96 (m, 1H), 2.83–2.70 (m, 2H), 2.65–2.52 (m, 2H), 2.43 (m, 1H), 2.34–2.13 (m, 4H), 1.98 (m, 1H), 1.90–1.55 (m, 9H), 1.55–1.40 (m, 11H), 1.01 (d, J=6.2 Hz, 3H), 0.94 (d, J=6.1 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 175.5 (C), 174.6 (C), 174.1 (C), 172.9 (C), 172.7 (C), 171.6 (C), 171.5 (C), 164.1 (C), 146.6 (C), 134.8 (C), 131.4 (CH), 130.2 (CH), 129.8 (CH), 127.1 (C), 119.0 (CH), 117.3 (CH_2), 79.8 (C), 78.8 (d, J=164.4 Hz, CH_2F), 69.4 (CH_2), 69.2 (CH_2), 68.6 (CH_2), 68.5 (CH_2), 68.5 (CH_2), 61.3 (CH), 59.6 (CH), 55.1 (CH), 55.0 (CH), 53.5 (CH), 51.2 (CH), 39.5 (CH_2), 39.1 (CH_2), 38.5 (CH_2), 38.2 (CH_2), 34.7 (CH_2), 34.5 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 29.7 (CH_2), 27.8 (CH_2), 27.5 (CH_2), 26.4 (CH_3), 25.4 (CH_2), 24.8 (CH_2), 23.8 (CH), 21.8 (CH_3), 19.5 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.6 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.09. IR (KBr): 3343, 2913, 2846, 1646, 1255, 1149, 1255, 1149, 1030 cm^{-1}. HRMS calcd for $C_{51}H_{80}FN_8O_{15}PSNa$ $(M+Na)^+$ 1149.5083, found 1149.5045.

4.2.9. tert-Butyl ((S)-21-(((S)-1-(((S)-1-amino-4-methyl-1-oxopentan-2-yl)amino)-3-(4-((bis (allyloxy)phosphoryl)oxy)-3-(fluoromethyl) phenyl)-1-oxopropan-2-yl)carbamoyl)-5,16,19-trioxo-1-((3aS,4S,6aR)-

*2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-9,12-dioxa-6,15,20-triazapentacosan-25-yl) carbamate (**17c**).* The same procedure as that for compound **17b** was used. Yield 60%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.55 (s, 1H, aromatic), 7.48 (d, J=8.6 Hz, 1H, aromatic), 7.33 (d, J=8.4 Hz, 1H, aromatic), 6.10–5.95 (m, 2H, =CH), 5.51 (d, J=47.5 Hz, 2H, CH_2F), 5.44 (d, J=17.1 Hz, 2H, =CH_2), 5.33 (d, J=10.5 Hz, 2H, =CH_2), 4.77–4.67 (m, 4H), 4.57–4.48 (m, 2H), 4.41 (m, 1H), 4.34 (dd, J=7.8, 4.4 Hz, 1H), 4.09 (m, 1H), 3.70–3.62 (m, 4H), 3.62–3.54 (m, 4H), 3.49 (m, 1H), 3.44–3.33 (m, 3H), 3.31 (m, 1H), 3.28–3.18 (m, 2H), 3.03–2.93 (m, 3H), 2.85–2.72 (m, 2H), 2.67–2.53 (m, 2H), 2.42 (m, 1H), 2.28–2.20 (m, 2H), 2.28–2.20 (m, 2H), 1.85–1.54 (m, 9H), 1.54–1.35 (m, 13H), 1.35–1.10 (m, 2H), 1.02 (d, J=6.3 Hz, 3H), 0.94 (d, J=6.2 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.3 (C), 176.6 (C), 175.9 (C), 175.8 (C), 175.3 (C), 174.4 (C), 173.4 (C), 165.8 (C), 158.2 (C), 148.4 (C), 136.7 (C), 133.2 (CH), 132.1 (CH), 131.7 (CH), 128.8 (CH), 120.8 (CH),119.2 (CH_2), 80.7 (d, J=164.4 Hz, CH_2F), 79.6 (C), 71.1 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 70.3 (CH_2), 69.5 (CH_2), 63.1 (CH), 61.4 (CH), 57.0 (CH), 56.9 (CH), 56.1 (CH), 53.0 (CH), 41.3 (CH_2), 41.0 (CH_2), 40.5 (CH_2), 40.1 (CH_2), 36.6 (CH_2), 36.2 (CH_2), 31.9 (CH_2), 31.6 (CH_2), 31.3 (CH_2), 30.5(CH_2), 29.6 (CH_2), 29.3 (CH_2), 28.8(CH_3), 26.7 (CH_2), 25.6 (CH), 23.8 (CH_3), 23.6 (CH_2), 21.4 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −214.6 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −6.15. IR (KBr): 3284, 2913, 2860, 1646, 1547, 1467, 1249, 1156, 1036 cm^{-1}. HRMS calcd for $C_{53}H_{85}FN_9O_{15}PSNa$ $(M+Na)^+$ 1192.5505, found 1192.5540.

*4.2.10. 4-((2S,5S)-2-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl)carbamoyl)-5-benzyl-4,7,10,21- tetraoxo-25-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-14,17-dioxa-3,6,11,20-tetraazapentacosyl)-2-(fluoromethyl)phenyl dihydrogen phosphate (**8b**).* To an ice-cooled solution of compound **17b** (20.0 mg, 0.0184 mmol) and BSTFA (99 μL, 0.37 mmol) in 1 mL of anhydrous CH_3CN was slowly added TMSBr (24 μL, 0.183 mmol). The reaction mixture was allowed to warm to rt and stirred further for 45 min. The reaction was quenched with 50% TEA in MeOH (1 mL). The organic solvents were removed under reduced pressure to give the crude product, which was purified by chromatography over Sephadex LH-20 eluted with MeOH. The fractions containing the product were pooled, concentrated and then lyophilized to afford compound **8b** (18 mg, 90%) as a colorless powder. ^{1}H NMR (400 MHz, CD_3OD): δ 7.44 (d, J=8.7 Hz, 1H, aromatic), 7.43 (s, 1H, aromatic), 7.32 (d, J=8.7 Hz, 1H, aromatic), 7.30–7.15 (m, 5H, aromatic), 5.58 (d, J=47.6 Hz, 2H, CH_2F), 4.57–4.48 (m, 2H), 4.45 (m, 1H), 4.42–4.18 (m, 2H), 3.64 (s, 4H), 3.62–3.52 (m, 4H), 3.47 (m, 1H), 3.42–3.36 (m, 3H), 3.28–3.13 (m, 9H), 3.10 (dd, J=14.2, 4.4 Hz, 1H), 2.96 (dd, J=12.8, 4.9 Hz, 1H), 2.83 (dd, J=14.1, 10.0 Hz, 1H), 2.77–2.26 (m, 2H), 2.59–2.45 (m, 2H), 2.34 (m, 1H), 2.25 (t, J=7.3 Hz, 2H), 1.83–1.53 (m, 8H), 1.53–1.37 (m, 3H), 1.33 (t, J=7.3 Hz, 9H, TEA), 1.00 (d, J=5.4 Hz, 3H), 0.93 (d, J=5.3 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.5 (C), 176.4 (C), 176.2 (C), 174.7 (C), 174.6 (C), 173.7 (C), 166.1 (C), 151.0 (C), 138.4 (C), 133.5 (C), 131.1 (CH), 130.1 (CH), 130.0 (CH), 129.7 (C), 129.5 (CH), 127.8 (CH), 121.8 (CH), 81.3 (d, J=162.3 Hz, CH_2F), 71.3 (CH_2), 71.2 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 63.4 (CH), 61.6 (CH), 57.5 (CH), 57.0 (CH), 53.1 (CH), 53.0 (CH), 47.7 (CH_2), 41.5 (CH_2), 41.1 (CH_2), 40.5 (CH_2), 40.2 (CH_2), 37.9 (CH_2), 36.9 (CH_2), 36.7 (CH_2), 31.9 (CH_2), 31.7 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 26.8 (CH_2), 25.7 (CH), 23.7 (CH_3), 21.5 (CH_3), 9.16 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −217.5 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −3.89. IR (KBr): 3377, 3291, 2853, 1633, 1547, 1467, 1255 cm^{-1}. HRMS calcd for $C_{45}H_{66}FN_8O_{13}PSNa$ $(M+Na)^+$ 1031.4089, found 1031.4070.

*4.2.11. (S)-4-(((S)-1-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl) amino)-3-(3-(fluoromethyl)-4- (phosphonooxy)phenyl)-1-oxopropan-2-yl)carbamoyl)-6,9,20-trioxo-24-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-13,16-dioxa-5,10,19-triazatetracosan-1-oic acid (**8a**).* The same procedure as that for compound **8b** was used. Yield 80%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.50–7.40 (m, 2H, aromatic), 7.31 (d, J=7.4 Hz, 1H, aromatic), 5.57 (d, J=47.6 Hz, 2H, CH_2F), 4.55–4.47 (m, 2H), 4.42–4.32 (m, 2H), 4.16 (m, 1H), 3.67 (s, 4H), 3.64–3.54 (m, 4H), 3.49 (m, 1H), 3.42–3.35 (m, 3H), 3.28–3.16 (m, 12H), 2.96 (dd, J=12.7, 5.1 Hz, 1H), 2.82–2.70 (m, 2H), 2.65–2.52 (m, 2H), 2.43 (m, 1H), 2.32–2.13 (m, 4H), 2.00 (m, 1H), 1.88 (m, 1H), 1.80–1.55 (m, 8H), 1.55–1.40 (m, 2H), 1.34 (t, J=7.3 Hz, 15H, TEA), 1.00 (d, J=6.0 Hz, 3H), 0.93 (d, J=6.0 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 178.2 (C), 177.6 (C), 176.6 (C), 176.2 (C), 175.1 (C), 174.6 (C), 173.9 (C), 166.1 (C), 151.0 (C), 134.0 (C), 130.8 (CH), 130.1 (CH), 129.7 (C), 121.7 (CH), 81.3 (d, J=162.6 Hz, CH_2F), 71.4 (CH_2), 71.2 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 63.3 (CH), 61.6 (CH), 57.6 (CH), 57.0 (CH), 56.0 (CH), 53.1 (CH), 47.5 (CH_2), 41.5 (CH_2), 41.1 (CH_2), 40.5 (CH_2), 40.2 (CH_2), 36.7 (CH_2), 36.7 (CH_2), 32.3 (CH_2), 31.8 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 27.5 (CH_2), 26.8 (CH_2), 25.7 (CH), 23.8 (CH_3), 21.4 (CH_3), 9.15 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −218.4 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −3.96. IR (KBr): 3278, 2919, 2846, 2661, 1733, 1633, 1547, 1262, 1030 cm^{-1}. HRMS calcd for $C_{41}H_{64}FN_8O_{15}PSNa$ $(M+Na)^+$ 1013.3831, found 1013.3817.

*4.2.12. 4-((2S,5S)-2-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl) carbamoyl)-5-(4-aminobutyl)- 4,7,10,21-tetraoxo-25-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-14,17-dioxa-3,6,11,20-tetraazapentacosyl)-2-(fluoromethyl)phenyl dihydrogen phosphate (**8c**).* The same procedure as that for compound **8b** was used. Yield 80%. ^{1}H NMR (400 MHz, D_2O): δ 7.38 (s, 1H, aromatic), 7.37–7.22 (m, 2H, aromatic), 5.50 (d, J=47.5 Hz, 2H, CH_2F), 4.62–4.51 (m, 2H), 4.36 (m, 1H), 4.27 (m, 1H), 4.09 (m, 1H), 3.64 (s, 4H), 3.64–3.52 (m, 4H), 3.43 (m, 1H), 3.38–3.30 (m, 3H), 3.30–3.24 (m, 2H), 3.18 (dd, J=14.7, 7.4 Hz, 1H, TEA), 3.19 (m, 1H), 2.93 (m, 1H), 2.79 (t, J=7.7 Hz, 2H), 2.72 (m, 1H), 2.65–2.43 (m, 4H), 2.22 (t, J=6.9 Hz, 2H), 1.75–1.30 (m, 15H), 1.25 (t J=7.3 Hz, 3H, TEA), 0.92 (d, J=5.7 Hz, 3H), 0.84 (d, J=5.6 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.7 (C), 177.2 (C), 176.1 (C), 175.2 (C), 174.6 (C), 174.0 (C), 166.1 (C), 151.3 (C), 133.8 (C), 131.3 (CH), 130.6 (CH), 129.0 (C), 121.3 (CH), 81.4 (d, J=163.4 Hz, CH_2F), 71.4 (CH_2), 71.2 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 47.6(CH_2), 41.5 (CH_2), 41.1 (CH_2), 40.6 (CH_2), 40.2 (CH_2), 36.7 (CH_2), 36.3 (CH_2), 32.1 (CH_2), 31.8 (CH_2), 30.7 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 28.0 (CH_2), 26.9 (CH_2), (CH_2) 25.8 (CH), 23.8 (CH_3), 22.5 (CH_2), 23.4 (CH_3), 9.16 (CH_3). ^{19}F NMR (376 MHz, CD_3OD): δ −218.0 (t, J=48.9 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ −3.28. IR (KBr): 3284, 2919, 2860, 1686, 1633, 1554, 1467, 1255, 1103 cm^{-1}. HRMS calcd for $C_{42}H_{69}FN_9O_{13}PS$ $(M+H)^+$ 990.4535, found 990.4563.

4.3. Typical procedures for the labeling of PTPs

4.3.1. Cloning of PTPs. To clone PTP1B and SHP-2, the cDNA encoding the catalytic domain of human PTP-1B (1–321) and SHP2 catalytic domains were obtained by RT-PCR using mRNA prepared from human lung cancer cell line H1299. The PCR products were then subcloned into pET6H to obtain plasmids pET6H-PTP1B and pET6H-SHP2. To clone TCPTP (Swiss prot: p17706) and VHR (BC002682), PPP1CA (BC00810), PPM1A (BC026691), PTP-PEST (BC050008), and PTEN (BC005821), primer pairs were used to PCR amplify cDNAs obtained from Meng TC (TCPTP and VHR; Institute of Biochemistry, Academia Sinica, Taipei, Taiwan) or Yang-Ming Genome Center (PPP1CA, PPM1A, PTP-PEST, and PTEN; NYMU, Taipei, Taiwan). The PCR products were then subcloned into pET6H. The DNA sequences of cloned genes were confirmed by sequencing analysis using dideoxy chain-termination methods.

4.3.2. Expression and purification of PTPs. E. coli BL21(DE3) or BL21 (DE3)-pLysS (Novagen) was used as a host to express 6-His tagged recombinant proteins. Similar procedures were used to purify these

phosphatases. Briefly, one-liter cultures of *E. coli* harboring expression plasmids were grown at 37 °C to an OD600 of 0.5 and then induced with the addition of 1 mM IPTG. The cells were grown at 25 °C for another 4 h before being harvested by centrifugation. Cells were resuspended in 10 mL of sonication buffer containing 50 mM NaH_2PO_4 pH 7.8, 300 mM NaCl, 1 mM DTT, 1× protease inhibitors (Calbiochem), and sonicated to release the cell contents. The sonicated cells were centrifuged at 13,000 g for 15 min at 4 °C to obtain total cell free extracts. The Ni Sepharose™ (Amersham Biosciences) was added to the total cell free extracts and incubated at 4 °C for 1 h. The resin was washed and eluted with buffer containing 50 mM NaH_2PO_4 pH 8.0, 20% glycerol and varying amounts of imidazole. Purified proteins were dialyzed against storage buffer (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol), aliquoted and frozen by dry ice/ethanol bath.

4.3.3. Sources of other recombinant proteins. Lysozyme (Sigma L6876), RNase from bovine pancreas (USB 21195), alkaline phsophatase (Sigma P7923), γ-globulin from bovine (Sigma G5009), and phsophorylase b (Sigma P6635) were purchased from Sigma.

4.3.4. Labeling reactions. Phosphatases were incubated with 0.1–0.25 mM of probes in reaction buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, and 150 mM NaCl at 25 °C for 20 min. The reaction products were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocelluose membrane. The membrane was blocked with 10% nonfat dry milk, washed with TTBS (0.05% Tween-20, 20 mM Tris pH 7.6, 137 mM NaCl), treated with *anti*-biotin antibody in TTBS with 1% nonfat dry milk for 2 h at 25 °C. Visualization of labeled proteins was achieved by treating the membrane with ECL chemiluminescence reagents (Perkin–Elmer) and exposed to film for 1–5 min before development. The images were quantified by Vilber Lourmat quantification tool.

*4.3.5. Stability test for probes **8a**–**c**.* Probes **8a**–**c** (0.05 mM) were incubated in reaction buffer (50 mM Tris pH 7.5, 1 mM EDTA, and 150 mM NaCl) at rt. At designated intervals, aliquots (100 μL) were taken for HPLC analysis. The reverse phase HPLC was performed on a Dionex UltiMate 3000 HPLC system with a Vydac C_{18} column (218TP54, 4.6×250 mm, 5 μm). Gradient elutions were run with a 0.1% aqueous solution of TFA as mobile phase A and 0.1% TFA in 90% acetonitrile as phase B, ranging from 0% to 100% B within 60 min. The flow rate was 1 mL/min and the eluents were monitored at 220 nm. The peak areas for the corresponding probes in each analysis were used to calculate the purity.

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Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2010.04.065.

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