# Journal of Medicinal Chemistry

# Discovery of SHP2-D26 as a First, Potent, and Effective PROTAC Degrader of SHP2 Protein

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Cite This: https://dx.doi.org/10.1021/acs.jmedchem.0c00471



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**ABSTRACT:** Src homology 2 domain-containing phosphatase 2 (SHP2) is an attractive therapeutic target for human cancers and other human diseases. Herein, we report our discovery of potent small-molecule SHP2 degraders whose design is based upon the proteolysis-targeting chimera (PROTAC) concept. This work has led to the discovery of potent and effective SHP2 degraders, exemplified by SHP2-D26. SHP2-D26 achieves DC<sub>50</sub> values of 6.0 and 2.6 nM in esophageal cancer KYSE520 and acute myeloid leukemia MV4;11 cells, respectively, and is capable of reducing SHP2 protein levels by >95% in cancer cells. SHP2-D26 is >30-times more potent in inhibition of phosphorylation of extracellular signal-regulated kinase (ERK) and of cell growth than SHP099, a potent SHP2 inhibitor, in KYSE520 and MV4;11 cancer cell lines. This study demonstrates that induced SHP2 degradation is a very effective approach to inhibit the function of SHP2. Further optimization of these SHP2 degraders may lead to the development of a new class of therapies for cancers and other human diseases.



# INTRODUCTION

Src homology 2 domain-containing phosphatase 2 (SHP2) is a protein tyrosine phosphatase. Mutations of SHP2 are prevalent in Noonan syndrome (50%) and LEOPARD syndrome (80%)<sup>1,2</sup> and activated mutations of SHP2 have also been identified in juvenile myelomonocytic leukemia (JMML, 35%), myelodysplastic syndrome (10%), B-cell acute lymphoblastic leukemia (7%), and acute myeloid leukemia (AML, 4%).<sup>3</sup> Somatic activating mutations in SHP2 have been associated with several types of solid tumors, including lung adenocarcinoma, colon cancer, neuroblastoma, glioblastoma, melanoma, hepatocellular carcinoma, prostate cancer, and breast cancer.<sup>4-8</sup> Accumulated evidence demonstrates that in cancer cells, SHP2 is involved in multiple signaling processes, such as RAS-ERK, JAK-STAT, PI3K-AKT, NF-KB, and mTOR pathways.<sup>9–13</sup> In the RAS-ERK pathway, SHP2 acts as a positive regulator at upstream to promote RAS-RAF-ERK kinase cascade signaling transduction. Therefore, SHP2 inhibition leads to dephosphorylation of ERK and suppression of the pro-oncogenic function of RAS-RAF-ERK pathway, resulting in cell growth inhibition and apoptosis induction in cancer cells.<sup>13</sup> Furthermore, SHP2 also participates in the programmed cell death pathway (PD-1/PD-L1) and inhibits T cell activation, thus contributing to immune evasion.<sup>14-16</sup> Hence, SHP2 is a very attractive cancer therapeutic target.

Due to the highly conserved and positively charged nature of its protein-tyrosine phosphatase (PTP) catalytic site, SHP2 has proved to be a difficult target in the discovery of small-molecule inhibitors.<sup>17,18</sup> Previously reported SHP2 inhibitors have not shown satisfactory selectivity and/or cellular activity, and this has prevented their development as useful therapeutic

agents.<sup>19–25</sup> A major breakthrough by Novartis scientists was the discovery of SHP099, a potent and allosteric SHP2 inhibitor, which was shown to selectively block SHP2 phosphatase activity and inhibit cancer cell growth *in vitro* and tumor growth in xenograft models in mice.<sup>26,27</sup> Subsequently, additional allosteric SHP2 inhibitors including SHP389 were reported and several of them have been advanced into clinical development for the treatment of human cancers.<sup>28–31</sup>

Although allosteric SHP2 inhibitors have been shown to be effective in preclinical models of Kirsten rat sarcoma (KRAS) mutant human cancer, we hypothesized that efficient depletion of SHP2 protein may provide an alternative and perhaps even more effective strategy for inhibition of the SHP2 activity. Mainardi et al.<sup>32</sup> demonstrated that SHP2 inactivation by CRISPR-Cas9 induces senescence and impairs tumor growth in xenograft models of KRAS mutant tumors. Furthermore, Ruess et al.<sup>33</sup> revealed that knockout of the PTPN11 gene, which encodes SHP2, in KRAS mutant human ductal adenocarcinoma (PDAC) cells results in reduction in cell proliferation and PTPN11-knockout cells are uniquely susceptible to mitogen-activated protein kinase (MEK) inhibitors. Such findings provide evidence that depletion of

Received: March 20, 2020



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Figure 1. Chemical structures of three previously reported SHP2 inhibitors SHP099 (1), SHP389 (2) and 3, and two designed SHP2 inhibitors (4, 5).



Figure 2. (A) Co-crystal structure of SHP2 in a complex with SHP099 (PDB ID: 5EHR), and (B and C) modeled structures of SHP2 in a complex with 4 or 5.

the SHP2 protein in tumor cells could be an effective therapeutic strategy for human cancers, particularly those carrying a KRAS-mutation.

In 2001, Deshaies and Crews formally demonstrated the proteolysis-targeting chimera (PROTAC) strategy to achieve targeted protein degradation.<sup>34</sup> A PROTAC molecule is a bifunctional small molecule consisting of a ligand that binds to the target protein of interest and another ligand that recruits an E3 ligase system. The ligands are tethered together through a chemical linker.<sup>35–37</sup> In recent years, the PROTAC approach has gained a momentum in the discovery and development of completely new classes of small-molecule therapeutic agents.<sup>38–45</sup> Two PROTAC molecules targeting the androgen receptor and estrogen receptor, discovered by Arvinas scientists, have been advanced into clinical development for the treatment of human cancers.<sup>46</sup>

In this paper, we report our design, synthesis, and evaluation of PROTAC SHP2 degraders. This study has resulted in the discovery of SHP2-D26, which induces rapid and efficient degradation of SHP2. This compound is also 10–100 times more potent than an allosteric SHP2 inhibitor in inhibition of ERK activity and cell growth in cancer cell lines. This study has laid a foundation for the development of a new class of therapeutics through targeted degradation of SHP2 protein.

# RESULTS AND DISCUSSION

In the design of PROTAC SHP2 degraders, it is important to identify both an SHP2 inhibitor and an E3 ligase ligand as well as suitable tethering sites to link them. A number of cocrystal structures of the SHP2 protein in complexes with potent SHP2 allosteric inhibitors have been reported (Figure 1). Examination of the cocrystal structures of SHP099 (PDB ID: SEHR) and compound 3 (PDB ID: 6MD7) showed that all the atoms in these SHP2 inhibitors are in close contacts with the SHP2 protein. However, in the cocrystal structure of SHP389 (2) (PDB ID: 6MDC) in complex with SHP2, the cyclopropyl group of SHP389 is exposed to the solvent, suggesting that this site can be used for a tether in potential SHP2 degraders.

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We designed compound 4 as a potential SHP2 inhibitor by combining a portion of SHP099 with a similar group found in compound 3 to facilitate our subsequent design and synthesis of PROTAC SHP2 degraders. The predicted binding model of 4 in a complex with SHP2 (Figure 2B) suggested that the hydrogen bond interactions between SHP099 and T108, F113, and E250 in SHP2 are preserved in the interactions of compound 4 and SHP2. The sulfur atom in 4 reorients the 2chloroaniline group in the binding site, while the chlorine atom in 4 interacts with the hydrophobic pocket formed by side chain atoms of L254, Q257, and Q495 in SHP2. The cationaromatic interaction between 4 and SHP2. Of significance, the amino group in 4 is exposed to the solvent, providing a potential tethering site for the design of PROTAC degraders.

We synthesized compound 4 and determined its inhibition of SHP2 enzymatic activity (see Figure S1). Compound 4 was found to be a potent SHP2 inhibitor with  $IC_{50} = 76.2$  nM. In the same assay, SHP099 is slightly less potent, with  $IC_{50} = 136.2$  nM.

To facilitate the convenient synthesis of PROTAC SHP2 degraders, we synthesized compound 5 by changing the amine group in 4 to an amide group and evaluated its inhibition of SHP2. Our data showed that 5 is a potent SHP2 inhibitor with  $IC_{50} = 98.7$  nM. The predicted binding model of compound 5

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# Table 1. PROTAC SHP2 Degraders Designed Using SHP2 Inhibitor 5 and a Previously Published Potent VHL-1 Ligand<sup>a</sup>





VHL-1 Ligand (6)

# PROTAC SHP2 Degraders

Compound	Linker	% SHP2 protein degradation in the KYSE520 cell line	
		0.1 (μM)	1 (µM)
7	× N N ***	4	0
8	* O O * N ***	9	7
9	0 0 *N H	13	6
10	× N N ***	0	3

<sup>*a*</sup>All the data are averages of three independent experiments with a treatment time of 6 h.

with SHP2 showed that the acetyl group extends further into the solvent exposed region (Figure 2C), making it a suitable site for tethering. We therefore employed compound  $\mathbf{5}$  as the SHP2 inhibitor and its amide group as the tethering site for the design of PROTAC SHP2 degraders.

The VHL-1/cullin 2 E3 ligase complex has been successfully used in the design of a large number of PROTAC degraders active against various proteins.<sup>47–57</sup> Based upon the cocrystal structures of VHL-1 ligands in a complex with VHL-1 and on

our previous studies of androgen receptor (AR) and estrogen receptor (ER) degraders, <sup>58,59</sup> we designed and synthesized a series of potential PROTAC SHP2 degraders using compound 5 and a potent and previously reported VHL ligand  $6^{60}$  (Table 1). Because the length of the linker plays a key role in the potency of PROTAC degrader molecules, we systematically varied the linker length in compounds 7–19 with the objective of determining the optimal linker length (Table 1). We first evaluated these compounds by Western blotting in the

KYSE520 esophageal cancer cell line, which was shown to be responsive to SHP2 inhibitors,<sup>26</sup> for their ability to induce degradation of SHP2 protein at 0.1  $\mu$ M and 1  $\mu$ M and obtained the data summarized in Table 1 and Figure S2.

Our Western blotting data showed that compounds 7–12 with different linker lengths, at 0.1 or 1  $\mu$ M, have little or no effect on the level of the SHP2 protein in the KYSE520 cell line. However, compound 13, which has one additional methylene group in the linker when compared to 12, reduces the SHP2 protein level by ~80% at 0.1 and 1  $\mu$ M. Increasing the linker length in 13 by one additional methylene yielded 14, which reduces the SHP2 protein level by >90% at 0.1 and 1  $\mu$ M. Compounds 15–19 with longer linkers than those in 14 are all potent and effective SHP2 degraders, capable of reducing the SHP2 protein level by >90% at 0.1 and 1  $\mu$ M.

Our previous studies showed that the linker composition in PROTAC molecules plays a key role in their degradative potencies and, accordingly, we performed further modifications of linker composition while keeping the linker length similar to that in **16**. The results are summarized in Table 2 and Figures S2 and S4.

Western blotting results showed that compounds 20-22 with a polyethylene glycol (PEG) unit embedded in the linker are less potent than compound 16. We replaced the amide bond in the middle of the linker in 16 with a positively charged piperazinyl group, which yielded compounds 23 and 24, both of which reduce the SHP2 protein level by ~80% at 0.1  $\mu$ M and >95% at 1  $\mu$ M in the KYSE520 cell line. Thus, while both 23 and 24 are very effective SHP2 degraders, they are less potent than 16. To examine the effect of the positively charged piperazinyl group in 23 and 24, we synthesized compound 25 containing a linker of 14 methylene groups. Compound 25 is less effective than either 23 or 24 in reducing the SHP2 protein level at both 0.1  $\mu$ M and 1  $\mu$ M in the KYSE520 cell line. Retention of the amide bond in the middle of the linker and introduction of a positively charged piperazinyl group resulted in compounds 26 and 27, both of which achieve near-complete SHP2 degradation at both 100 nM and 1  $\mu$ M. Introduction of a phenoxyl group in the middle of the linker led to compound 28, which reduces SHP2 protein by >95% at both 0.1  $\mu$ M and 1  $\mu$ M. Compound 29, containing a positively charged piperazinyl group in the central part of the linker, fails to induce any SHP2 degradation at 100 nM but reduces SHP2 protein by ~80% at 1  $\mu$ M.

The acute myeloid leukemia MV4;11 cell line was also shown to be very responsive to SHP2 inhibitors.<sup>26</sup> We next tested these compounds for their ability to reduce the SHP2 protein in the acute myeloid leukemia MV4;11 cell line at 100 nM with 24 h treatment time, obtaining the data summarized in Table 2. The data showed that 20, 22, 26, 27, and 28 all effectively reduce SHP2 protein by >95% at 100 nM, indicating that these compounds are potent and effective SHP2 degraders in the MV4;11 cell line. These linker modifications thus have led to a number of highly potent SHP2 degraders.

**Further Evaluation of Compound 26 (SHP2-D26).** In addition to its excellent degradation potency in both the KYSE520 and MV4;11 cell lines, compound **26** (SHP2-D26) has good aqueous solubility. We further investigated SHP2-D26 for its potency and mechanism of action in these two cell lines.

We examined the degradation of SHP2 by SHP2-D26 in a wide range of concentrations in KYSE520 and MV4;11 cell

Table 2. Optimization of the Linker Composition of SHP2 Degrader  $16^a$ 





"All of the data are the average of three independent experiments with a treatment time of 6 h in KYSE520 cell lines and 24 h in MV4;11 cell lines.

lines (Figure 3A–D). Western blotting results showed that SHP2-D26 effectively reduces SHP2 protein in a dosedependent manner. Quantification of the Western blotting data showed that the compound achieves  $DC_{50}$  values (concentration needed to induce targeted protein degradation by 50%) of 6.0 and 2.6 nM in the KYSE520 and MV4;11 cell lines, respectively (Figure 3B,D).

We next evaluated the kinetics of SHP2-D26 in induction of SHP2 degradation in the KYSE520 and MV4;11 cell lines (Figure 3E,F). In KYSE520 cells, SHP2-D26 at 100 nM effectively reduces the SHP2 protein level within 4 h and

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**Figure 3.** Further evaluation of SHP2-D26 for its degradation activity in the KYSE520 and MV4;11 cell lines. (A–D) Dose-dependent SHP2 degradation in the KYSE520 and MV4;11 cell lines, with SHP099 included as the inhibitor control. Cells were treated with SHP2-D26 for 12 h. SHP2 protein was examined by Western blotting, and the protein level was quantified by densitometry and normalized to the corresponding density of the GAPDH protein. (E,F). Degradation kinetics of SHP2-D26 in the KYSE520 and MV4;11 cell lines. Cells were treated with 100 nM of SHP2-D26 for different times. SHP2 protein was examined by Western blotting, and the GAPDH protein was used as the loading control. (G) KYSE520 cell line was treated with VHL-1 ligand 6 (10  $\mu$ M), compound 4 (at 10  $\mu$ M), MLN4924 (0.5  $\mu$ M), or MG132 (3  $\mu$ m) for 1 h and then treated with SHP-D26 (0.1  $\mu$ M) for 3 h. The protein level of SHP2 was examined by Western blotting, and the GAPDH protein was used as the loading control.



Figure 4. Compound 26 (SHP2-D26) suppresses the p-ERK pathway. KYSE520 or MV4;11 cells were treated as indicated with SHP2-D26 or SHP099 for 48 h. The protein levels of SHP2 (Bethyl Lab. A301–544), ERK (#9102, CST), and phospho-ERK (#4370, CST) determined by Western blotting. GAPDH was used as a loading control.

achieves essentially complete SHP2 depletion with 8 h treatment (Figure 3E). Similar kinetics was observed in the MV4;11 cell line (Figure 3F). These data show that SHP2 degradation induced by SHP2-D26 is fairly rapid in both KYSE520 and MV4;11 cells.

We examined if SHP2-D26 functions as a *bona fide* PROTAC degrader in the KYSE520 cell line. A VHL-1 ligand **6** (Table 1), compound 4 (an SHP2 inhibitor), MLN4924 (an E1 inhibitor), and MG132 (a proteasome inhibitor) all effectively block degradation of the SHP2 protein in KYSE520 cells (Figure 3G). Therefore, our data show that

SHP2 degradation induced by SHP2-D26 requires its binding to VHL-1 and SHP2 proteins and is also neddylation- and proteasome-dependent, demonstrating that SHP2-D26 is a *bona fide* PROTAC SHP2 degrader.

Since SHP2 protein is known to play an important role in the MAPK/ERK signaling pathway, we examined the impact of SHP2 degradation on the MAPK/ERK signaling pathway in the KYSE520 and MV4;11 cell lines, with SHP099 included as a control (Figure 4). Western blotting showed that both SHP2-D26 and SHP099 dose-dependently inhibit phosphorylation of ERK in the KYSE520 and MV4;11 cell lines.



Figure 5. Cell growth inhibition in KYSE520 and MV4;11 cells treated with SHP2 degrader 26 (SHP2-D26) and SHP2 inhibitors SHP099, 4 and 5. KYSE520 and MV4;11 cells were treated with indicated doses for 4 days, and cell viability was determined by a colorimetric WST-8 assay.

Scheme 1. Synthesis of the VHL Ligand  $(6)^{a}$ 



"Reagents and conditions: (a) Boc<sub>2</sub>O, NaHCO<sub>3</sub>, EtOAc/H<sub>2</sub>O, 2 h; (b) 4-methylthiazole, Pd(OAc)<sub>2</sub>, KOAc, DMA, 90 °C, 12 h; (c) 4 N HCl dioxane/MeOH, rt, 4h; (d) HATU, DIPEA, DMF, 0 °C to RT, 12 h; and (e) TFA, DCM, rt, 4h.

Scheme 2. Synthesis of the Key Intermediate  $(40)^{a}$ 



"Reagents and conditions: (a) *tert*-butylthiol, Cs<sub>2</sub>CO<sub>3</sub>, DMF, 120 °C, 36 h; (b) conc. HCl, 80 °C, 6 h; (c) K<sub>3</sub>PO<sub>4</sub>, CuI, 1,10-phenanthroline, dioxane, 90 °C, 16 h; and (d) DIPEA, DMSO, 100 °C, 3 h.

However, in both cell lines, SHP2-D26 is much more potent than SHP099 in inhibition of pERK. Specifically, in the KYSE520 cell line, while 100 nM of SHP2-D26 is effective in reducing the level of pERK, >3,000 nM of the SHP2 inhibitor SHP099 is needed to do so. In the MV4;11 cell line, while 100 nM of SHP2-D26 completely inhibits phorsphorylated ERK (pERK), >3,000 nM of the SHP2 inhibitor SHP099 is required to achieve complete inhibition of pERK. Therefore, our data indicate that SHP2-D26 is >30-times more potent than SHP099 in inhibition of pERK in both cell lines.

We evaluated the cell growth inhibition of SHP2-D26, and three SHP2 inhibitors (SHP099, compounds 4 and 5) in the

KYSE520 and MV4;11 cell lines (Figure 5). In the KYSE520 cell line, SHP2-D26 achieves  $IC_{50}$  values of 0.66  $\mu$ M (Figure 5A). In comparison, SHP099, compounds 4 and 5 have  $IC_{50}$  values of 18.2, 42.3, and 39.4  $\mu$ M, respectively. Hence, SHP2-D26 is 28-, 64-, and 60-times more potent than SHP099 and compounds 4 and 5 in inhibition of cell growth in the KYSE520 cell line. In the MV4;11 cell line, SHP2-D26, SHP099, 4 and 5 have  $IC_{50}$  values of 9.9 nM, 1.0  $\mu$ M, 3.9  $\mu$ M and 6.6  $\mu$ M, respectively, in inhibition of cell growth. Thus, SHP2-D26 is 100-, > 400 and >600-times more potent than SHP099, compounds 4 and 5, respectively in cell growth inhibition in the MV4;11 cell line.

# Scheme 3. Synthesis of Compounds $7-19^a$



<sup>&</sup>lt;sup>*a*</sup>Reagents and conditions: (a) 6, HATU, DIPEA, DMF, 0 °C to RT, 1 h; (b) 4 N HCl dioxane/MeOH, rt; (c) 40, DIPEA, DCM, 0 °C, 2 h; (d) LiOH·H<sub>2</sub>O, THF, MeOH, H<sub>2</sub>O, RT, 3 h; (e) HATU, DIPEA, DMF, 0 °C to RT, 1 h; and (f) TFA, DCM, rt, 2 h.





<sup>a</sup>Reagents and conditions: (a) **6**, HATU, DIPEA, DMF, 0 °C to rt, 1 h; (b) 4 N HCl dioxane, 2h; (c) **46b**, HATU, DIPEA, DMF, 0 °C to rt, 1 h; and (d) TFA, DCM, rt, 2h.

# CHEMISTRY

The compounds in Table 1 were synthesized from the VHL ligand (6) and the key intermediate (42). The synthesis of VHL ligand 6 follows a published procedure<sup>60</sup> and is outlined in Scheme 1. The commercially available (S)-1-(4-bromophenyl)ethan-1-amine (30) was protected by Boc<sub>2</sub>O, and a subsequent Heck coupling reaction with 4-methyl-thiazole afforded compound 31. Removal of the Boc group under acidic conditions gave compound 32, and amide

coupling with compound 33 led to 34, which after acidic deprotection afforded the VHL ligand (6).

The synthesis of the key intermediate (40) is shown in Scheme 2. The commercially available 2-chloro-3-fluoroaniline (35) was reacted with *tert*-butylthiol under basic conditions to produce compound 36, which was heated in concentrated hydrochloric acid to remove the *tert*-butyl group, yielding compound 37. A cross-coupling reaction of the intermediate 37 with 3-bromo-6-chloropyrazin-2-amine (38) was carried out in the presence of CuI and 1,10-phenanthroline as

Scheme 5. Synthesis of Compounds 23, 24, and 25<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) TCFH, NMI, MeCN/THF, rt, 6 h; (b) piperidine, MeCN, rt, 2 h; (c) K<sub>2</sub>CO<sub>3</sub>, KI, DMF, 60 °C, 8 h; (d) HATU, DIPEA, DMF, 0 °C to rt, 1 h; (e) TFA, DCM, rt, 2h; and (f) NaOH, THF-H<sub>2</sub>O.

catalysts, and potassium phosphate  $(K_3PO_4)$  as a base in 1,4dioxane at 90 °C for 16 h to obtain compound **39**, which was reacted with *tert*-butyl (4-methylpiperidin-4-yl)carbamate using DIPEA as a base in DMSO at 100 °C for 3 h to afford the key intermediate (**40**).

As shown in Scheme 3, compounds 7–19 were synthesized according to the following procedure. Using Boc-protected amine-terminated acids with different carbon chain lengths (41a-41j) as starting materials, the intermediates (43a-43j) were obtained by an amide condensation reaction followed by removal of the Boc protecting group under acidic conditions. The intermediates (46a-46c) were synthesized by the reaction of the key intermediate 40 with methyl-3-chloro-3-oxoproanoate esters with different carbon chain lengths (44a-44c) and subsequent hydrolysis of the methyl ester. Amide coupling between compound 43 and 46 followed by removal of the Boc protecting group afforded the final compounds (7–19) in high yields.

As shown in Scheme 4, compounds 20-22 were treated in a similar way to produce compounds 7-19. With a polyethylene glycol unit (PEG) embedded in their chains, Boc-protected amine-terminated acids (47, 49) were used as the starting material. The intermediates (48, 50) were obtained by amide condensation with the VHL ligand (6), and this was followed by removal of the Boc group. Employing compounds 48 and 50 as intermediates, compounds 20-22 were synthesized by condensation with the corresponding acid (48b) and subsequent removal of the Boc group.

The synthesis of compounds 23–25 is shown in Scheme 5. Because the starting material 40 is a non-nucleophilic amine, the formation of amide bonds in high yields with common coupling agents is difficult. After trying multiple reaction conditions, a combination of N,N,N,N-tetramethylchloroformamide hexafluorophosphate (TCFH) and N-methylimidazole (NMI) was found to be effective as a coupling reagent.<sup>61</sup> The intermediate (52) was obtained by amide condensation and subsequent removal of the Fmoc group. Following the substitution reaction of compound 52 with 11-bromoundecanoic acid or 12-bromododecanoic acid, (54a, 54b) were obtained. Following amide condensation with VHL ligand 6 and Boc removal, the final compounds (23, 24) were produced. Compound 25 was synthesized using the procedure described for the synthesis of compound 23 with 16-(benzyloxy)-16-oxohexadecanoic acid (55) as the starting material.

As shown in Scheme 6, compounds 26–29 were synthesized using the following procedure. Intermediates 59a/59b were synthesized by the substitution reaction of *tert*-butyl piperazine-1-carboxylate with methyl 9-bromononanoate or methyl 10-bromononanoate followed by hydrolysis with lithium hydroxide. The intermediates (60a, 60b) were obtained by an amide condensation reaction of 59a or 59b, respectively, with VHL ligand 6 followed by removal of the Boc group. Amide condensation of 60a or 60b with 46b followed by removal of the Boc group afforded compounds 26 and 27. Compounds 28 and 29 were synthesized using the procedure described for the synthesis of compound 26 with Scheme 6. Synthesis of Compounds 26, 27, 28, and 29<sup>a</sup>



"Reagents and conditions: (a)  $K_2CO_3$ , NaI, MeCN, 60 °C, 16 h; (b) LiOH.H<sub>2</sub>O, THF, MeOH, H<sub>2</sub>O, rt, 3 h (c) 6, HATU, DIPEA, DMF, 0 °C to rt, 1 h; (d) 4 N HCl dioxane, 2 h; (e) 46b, HATU, DIPEA, DMF, 0 °C to rt, 1 h; (f) TFA, DCM, rt, 2 h.

*tert*-butyl (4-hydroxyphenyl)carbamate (**61**) and *tert*-butyl (4-(piperazin-1-yl)butyl)carbamate (**65**), respectively, as the starting materials.

### CONCLUSION

We have designed, synthesized, and evaluated a series of PROTAC SHP2 degraders. Our study has resulted in the discovery of a number of highly potent SHP2 degraders, we obtained a number of highly potent SHP2 degraders, exemplified by compound **26** (SHP2-D26). SHP2-D26 achieves  $DC_{50}$  values of 6.0 nM and 2.6 nM in KYSE520 and MV4;11 cells, respectively, and >95% degradation at 30 nM in both cell lines. SHP2-D26 is much more potent and effective in inhibition of ERK phosphorylation and in inhibition of cell growth in KYSE520 and MV4;11 cells than three SHP2 inhibitors. This study provides the first proof-of-

concept that targeted degradation of SHP2 is a very effective strategy in inhibition of SHP2 activity. Further optimization of SHP2-D26 may lead to the development of a potent and effective SHP2 degrader for the treatment of human cancers. In addition, a potent and effective SHP2 degrader may also have a great therapeutic potential for the treatment of certain human genetic disorders caused by SHP2 mutation and activation, such as Noonan syndrome and LEOPARD syndrome.

# EXPERIMENTAL SECTION

**Chemistry.** *General Information.* All commercial reagents and solvents were used as supplied without further purification. Proton nuclear magnetic resonance (<sup>1</sup>H NMR) and carbon nuclear magnetic resonance (<sup>13</sup>C NMR) spectroscopy were performed on Bruker Advance 400 NMR spectrometers. <sup>1</sup>H NMR spectra are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). All

<sup>13</sup>C NMR spectra are reported in ppm and obtained with <sup>1</sup>H decoupling. In the spectral data reported, the format ( $\delta$ ) chemical shift (multiplicity, *J* values in Hz, integration) was used with the following abbreviations: s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. Electrospray ionization (ESI) mass spectral (MS) analysis was performed on a Thermo Scientific LCQ Fleet mass spectrometer. The final products were purified by reverse-phase HPLC (RP-HPLC) with solvent A (0.1% of TFSA in water) and solvent B (0.1% of TFA in CH<sub>3</sub>CN) as eluents with a flow rate of 45 mL/min. All final compounds have purity ≥95% as determined by Waters ACQUITY ultraperformance liquid chromatography (UPLC) using a reverse-phase column (SunFire, C18, 5  $\mu$ m, 4.6 × 150 mm2) and a solvent gradient of solvent A (H<sub>2</sub>O with 0.1% of TFA) and solvent B (CH<sub>3</sub>CN with 0.1% of TFA).

(2S,4R)-1-((S)-2-Amino-3,3-dimethylbutanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (6). HATU (4.56 g, 12.0 mmol, 1.2 equiv) was added to a solution of compound 32 (2.55 g, 10.0 mmol, 1.0 equiv), which was synthesized according to the reported procedure,<sup>60</sup> using (2S,4R)-1-((S)-2-((tertbutoxycarbonyl)amino)-3,3-dimethyl-butanoyl)-4-hydroxypyrrolidine-2-carboxylic acid (33) (3.45 g, 10.0 mmol, 1.0 equiv), and DIPEA (6.95 mL, 40.0 mmol, 4.0 equiv) in DMF (20 mL) at 0 °C under nitrogen. The mixture was stirred at ambient temperature for 12 h when LC-MS showed that the reaction was complete. The reaction mixture was quenched with H<sub>2</sub>O (100 mL) and extracted with EtOAc (100 mL  $\times$  2). The combined organic layers were washed with brine (200 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The organic solution was filtered and concentrated under reduced pressure to give a residue which was dissolved in DCM (30.0 mL), and TFA (15.0 mL) was added at 0 °C under nitrogen. The mixture was stirred at ambient temperature for 4 h, when LC-MS showed that the reaction was complete. The volatile components were removed on a rotary evaporator, and the residue was purified by reverse phase column chromatography to afford compound 6 as a TFA salt (3.12 g, 56%). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.01 (s, 1H), 8.61 (d, J = 8.0 Hz, 1H), 8.20 (brs, 3H), 7.45-7.37 (m, 4H), 4.95-4.90 (m, 1H), 4.54 (t, J = 8.0 Hz, 1H), 4.30 (brs, 1H), 3.90-3.87 (m, 1H), 3.76-3.73 (m, 1H), 3.52-3.47 (m, 1H), 2.45 (s, 3H), 2.14-2.09 (m, 1H), 1.78-1.72 (m, 1H), 1.38 (d, J = 7.2 Hz, 3H),  $\delta 1.02$  (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{23}H_{33}N_4O_3S$  [M + 1] <sup>+</sup>, 445.23; found, 445.44

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2vl)thio)-2-chlorophenvl)-N3-(3-(((S)-1-((2S,4R)-4-hvdroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-3-oxopropyl)malonamide (7). HATU (25 mg, 0.066 mmol, 1.1 equiv) was added to a mixture of (2S,4R)-1-((S)-2-(3-aminopropanamido)-3,3-dimethylbutanoyl)-4hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide hydrochloride (43a) (33 mg, 0.06 mmol, 1.0 equiv), 3-((3-((3-amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)-thio)-2-chlorophenyl)amino)-3-oxopropanoic acid (46a) (33 mg, 0.06 mmol, 1.0 equiv), and DIPEA (39 mg, 0.30 mmol, 5.0 equiv) in DMF (2 mL) at 0 °C under N2. The mixture was stirred at ambient temperature for 1 h. After being quenched with water (8 mL) and extracted with EtOAc (5 mL x 3), the organic layers were washed with brine (15 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The residue was dissolved in DCM (3 mL), and trifluoracetic acid (1 mL) was added at 0 °C. The reaction was stirred for 2 h, and the solvent was removed in vacuo. The residue was purified by reverse-phase chromatography over a C18 column to yield 7 (37 mg, 58%) as a white powder. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 10.31 (s, 1H), 9.00 (s, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.32-8.29 (m, 1H), 7.97 (brs, 3H), 7.80 (d, J = 7.2 Hz, 1H), 7.66 (s, 1H), 7.45–7.37 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 4.95–4.85 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.43 (t, J = 8.0 Hz, 1H), 4.29 (s, 1H), 4.06-4.02 (m, 2H), 3.64-3.57 (m, 2H), 3.38 (s, 2H), 3.33-3.26 (m, 2H), 2.45 (s, 3H), 2.43-2.33 (m, 2H), 2.04-1.99 (m, 1H), 1.81-1.77 (m, 1H), 1.73-1.70 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 0.92 (s, 9H); UPLC-

MS (ESI<sup>+</sup>): calculated for  $C_{45}H_{59}ClN_{11}O_6S_2 [M + 1]^+$ , 948.38; found, 948.31.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(4-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-4-oxobutyl)malonamide (8). This compound was prepared from 43b and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO $d_6$ ):  $\delta$  (ppm) 10.34 (s, 1H), 9.00 (s, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.29-8.27 (m, 1H), 7.96 (brs, 3H), 7.88-7.81 (m, 2H), 7.66 (s, 1H), 7.45–7.37 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.21 (brs, 1H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.42 (t, I = 8.0 Hz, 1H), 4.28 (s, 1H), 4.06–4.02 (m, 2H), 3.64-3.57 (m, 2H), 3.39 (s, 2H), 3.32-3.28 (m, 2H), 3.13-3.08 (m, 2H), 2.45 (s, 3H), 2.30-2.27 (m, 1H), 2.18-2.16 (m, 1H), 2.04-1.97 (m, 1H), 1.78–1.73 (m, 1H), 1.70–1.64 (m, 6H), 1.37 (s, 3H), 1.36 (s, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{46}H_{61}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 962.39; found, 962.33.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(5-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-5-oxopentyl)malonamide (9). This compound was prepared from 43c and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO $d_6$ ):  $\delta$  (ppm) 10.33 (s, 1H), 9.00 (s, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.27-8.25 (m, 1H), 7.99 (brs, 3H), 7.83-7.81 (m, 2H), 7.66 (s, 1H), 7.45–7.37 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.17 (brs, 1H), 4.95-4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05–4.02 (m, 2H), 3.64-3.56 (m, 2H), 3.39 (s, 2H), 3.33-3.28 (m, 2H), 3.13-3.08 (m, 2H), 2.45 (s, 3H), 2.29-2.23 (m, 1H), 2.16-2.13 (m, 1H), 2.01-1.97 (m, 1H), 1.78-1.70 (m, 4H), 1.54-1.41 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{47}H_{63}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 976.41; found, 976.45.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(6-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexyl)malonamide (10). This compound was prepared from 43d and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO $d_{\delta}$ :  $\delta$  (ppm) 10.33 (s, 1H), 9.00 (s, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.26-8.23 (m, 1H), 7.96 (brs, 3H), 7.81 (d, J = 8.8 Hz, 2H), 7.66 (s, 1H), 7.45–7.36 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.20 (brs, 1H), 4.95–4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.06-4.02 (m, 2H), 3.62-3.56 (m, 2H), 3.38 (s, 2H), 3.33-3.28 (m, 2H), 3.11-3.06 (m, 2H), 2.45 (s, 3H), 2.26-2.23 (m, 1H), 2.14-2.10 (m, 1H), 2.01-1.98 (m, 1H), 1.78-1.67 (m, 5H), 1.54-1.41 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.27-1.23 (m, 2H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{48}H_{65}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 990.42; found, 990.39.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(7-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-7-oxoheptyl)malonamide (11). This compound was prepared from 43e and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO $d_6$ ):  $\delta$  (ppm) 10.32 (s, 1H), 9.00 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 8.25-8.22 (m, 1H), 7.96 (brs, 3H), 7.82-7.79 (m, 2H), 7.66 (s, 1H), 7.47–7.36 (m, 4H), 7.15 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.20 (brs, 1H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.07–4.02 (m, 2H), 3.63-3.57 (m, 2H), 3.38 (s, 2H), 3.33-3.26 (m, 2H), 3.12-3.07 (m, 2H), 2.45 (s, 3H), 2.29-2.21 (m, 1H), 2.15-2.08 (m, 1H), 2.04-1.98 (m, 1H), 1.82–1.70 (m, 5H), 1.54–1.41 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.28-1.19 (m, 4H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{49}H_{67}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 1004.44; found, 1004.37.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(8-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-8-oxooctyl)malonamide (12). This compound was prepared from 43f and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSOd<sub>6</sub>):  $\delta$  (ppm) 10.33 (s, 1H), 9.00 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 8.25–8.23 (m, 1H), 7.96 (brs, 3H), 7.82–7.78 (m, 2H), 7.66 (s, 1H), 7.45–7.35 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J =1.2 Hz, 1H), 6.20 (brs, 1H), 4.95–4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.41 (t, J = 8.0 Hz, 1H), 4.27 (s, 1H), 4.06–4.02 (m, 2H), 3.63–3.57 (m, 2H), 3.38 (s, 2H), 3.33–3.26 (m, 2H), 3.12–3.07 (m, 2H), 2.45 (s, 3H), 2.28–2.21 (m, 1H), 2.14–2.07 (m, 1H), 2.04– 1.98 (m, 1H), 1.82–1.70 (m, 5H), 1.51–1.42 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.25–1.14 (m, 6H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>50</sub>H<sub>69</sub>ClN<sub>11</sub>O<sub>6</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1018.46; found, 1018.41.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(9-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-9-oxononyl)malonamide (13). This compound was prepared from 43g and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO $d_6$ ):  $\delta$  (ppm) 10.32 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 8.26-8.23 (m, 1H), 7.97 (brs, 3H), 7.82-7.78 (m, 2H), 7.66 (s, 1H), 7.45–7.36 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 0.8 Hz, 1H), 6.20 (brs, 1H), 4.95-4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.41 (t, J = 8.8 Hz, 1H), 4.27 (s, 1H), 4.05–4.02 (m, 2H), 3.63-3.57 (m, 2H), 3.38 (s, 2H), 3.33-3.26 (m, 2H), 3.12-3.07 (m, 2H), 2.45 (s, 3H), 2.28-2.21 (m, 1H), 2.13-1.98 (m, 2H), 1.82-1.70 (m, 5H), 1.47-1.40 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.28-1.17 (m, 8H), 0.93 (s, 9H); UPLC-MS (ESI+): calculated for  $C_{51}H_{71}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 1032.47; found, 1032.42.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(10-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecyl)malonamide (14). This compound was prepared from 43h and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  (ppm) 10.33 (s, 1H), 9.00 (s, 1H), 8.39 (d, J =8.0 Hz, 1H), 8.26–8.23 (m, 1H), 7.98 (brs, 3H), 7.83–7.78 (m, 2H), 7.67 (s, 1H), 7.47–7.33 (m, 4H), 7.15 (t, J = 8.0 Hz, 1H), 6.41 (dd, J =8.0 Hz, J = 1.2 Hz, 1H), 6.21 (brs, 1H), 4.96–4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.06–4.03 (m, 2H), 3.64–3.58 (m, 2H), 3.39 (s, 2H), 3.34–3.27 (m, 2H), 3.13–3.08 (m, 2H), 2.46 (s, 3H), 2.29–2.22 (m, 1H), 2.14–1.99 (m, 2H), 1.82–1.71 (m, 5H), 1.51–1.39 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.27–1.19 (m, 10H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>52</sub>H<sub>73</sub>ClN<sub>11</sub>O<sub>6</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1046.49; found, 1046.45.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(11-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-11-oxoundecyl)malonamide (15). This compound was prepared from 43i and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 10.31 (s, 1H), 8.99 (s, 1H), 8.36 (d, J =8.0 Hz, 1H), 8.24-8.22 (m, 1H), 7.95 (brs, 3H), 7.82-7.75 (m, 2H), 7.66 (s, 1H), 7.44–7.37 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.41 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.18 (brs, 2H), 4.95-4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05–4.02 (m, 2H), 3.64–3.57 (m, 2H), 3.38 (s, 2H), 3.33–3.26 (m, 2H), 3.12-2.97 (m, 2H), 2.45 (s, 3H), 2.28-2.21 (m, 1H), 2.13-1.98 (m, 2H), 1.82-1.70 (m, 5H), 1.50-1.40 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.29–1.15 (m, 12H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{53}H_{75}ClN_{11}O_6S_2 \ [M + 1]^+$ , 1060.50; found, 1060.51.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N3-(12-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecyl)malonamide (16). This compound was prepared from 43j and 46a by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) 10.30 (s, 1H), 8.98 (s, 1H), 8.36 (d, *J* = 8.0 Hz, 1H), 8.24–8.21 (m, 1H), 7.96 (brs, 3H), 7.83–7.75 (m, 2H), 7.66 (s, 1H), 7.44–7.37 (m, 4H), 7.16 (t, *J* = 8.0 Hz, 1H), 6.41 (dd, *J* = 8.0 Hz, *J* = 1.2 Hz, 1H), 6.15 (brs, 2H), 4.95–4.88 (m, 1H), 4.51 (d, *J* = 8.8 Hz, 1H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05–4.02 (m, 2H), 3.64–3.57 (m, 2H), 3.38 (s, 2H), 3.33–3.26 (m, 2H), 3.12–3.07 (m, 2H), 2.45 (s, 3H), 2.29–2.21 (m, 1H), 2.13–1.98 (m, 2H), 1.83–1.70 (m, 5H), 1.50–1.40 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.28–1.16 (m, 14H), 0.93 (s, 9H); <sup>13</sup>C NMR (100 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 172.03, 170.60, 169.59, 167.18, 165.73, 158.25, 157.90, 155.73, 153.52, 151.48, 147.72, 144.65, 137.25, 135.70, 131.11, 129.67, 128.81, 127.20, 126.37, 120.29, 119.71, 117.59, 114.67, 113.76, 68.74, 58.53, 56.31, 56.23, 52.13, 47.68, 43.05, 37.72, 35.19, 34.88, 34.10, 28.97, 28.88, 28.75, 28.66, 26.43, 26.37, 25.43, 22.43, 21.96, 15.97; UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>54</sub>H<sub>77</sub>ClN<sub>11</sub>O<sub>6</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1074.52; found, 1074.38.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2vl)thio)-2-chlorophenyl)-N4-(11-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-11-oxoundecyl)succinimide (17). This compound was prepared from 43i and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.54 (s, 1H), 8.98 (s, 1H), 8.36 (d, J = 8.0 Hz, 1H), 7.97 (brs, 3H), 7.84-7.75 (m, 2H), 7.66 (s, 1H), 7.49-7.37 (m, 4H), 7.14 (t, J = 8.0 Hz, 1H), 6.43 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.18 (brs, 2H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.27 (s, 1H), 4.05-4.02 (m, 2H), 3.64-3.57 (m, 2H), 3.33-3.26 (m, 2H), 3.05-3.00 (m, 2H), 2.63-2.59 (m, 2H), 2.45 (s, 3H), 2.41-2.37 (m, 2H), 2.28-2.21 (m, 1H), 2.13-1.98 (m, 2H), 1.82-1.70 (m, 5H), 1.52-1.40 (m, 4H), 1.38 (s, 3H), 1.36 (s, 3H), 1.28-1.17 (m, 12H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>54</sub>H<sub>77</sub>ClN<sub>11</sub>O<sub>6</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1074.52; found, 1074.47.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-(12-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecyl)succinimide (18). This compound was prepared from 43j and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.54 (s, 1H), 8.98 (s, 1H), 8.36 (d, J = 8.0Hz, 1H), 7.95 (brs, 3H), 7.84-7.75 (m, 2H), 7.66 (s, 1H), 7.48-7.37 (m, 5H), 7.14 (t, J = 8.0 Hz, 1H), 6.43 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.18 (brs, 2H), 4.95-4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05-4.02 (m, 2H), 3.63-3.57 (m, 2H), 3.33–3.26 (m, 2H), 3.05–3.00 (m, 2H), 2.63–2.59 (m, 2H), 2.45 (s, 3H), 2.41-2.37 (m, 2H), 2.28-2.21 (m, 1H), 2.13-1.98 (m, 2H), 1.82–1.70 (m, 5H), 1.52–1.41 (m, 4H), 1.37 (s, 3H), 1.36 (s, 3H), 1.27–1.16 (m, 14H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{55}H_{79}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 1088.53; found, 1088.49.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N5-(12-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecyl)glutaramide (19). This compound was prepared from 43j and 46c by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.52 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.96 (brs, 3H), 7.80-7.75 (m, 2H), 7.67 (s, 1H), 7.45-7.37 (m, 4H), 7.15 (t, J = 8.0 Hz, 1H), 6.43 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.19 (brs, 2H), 4.95-4.89 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.41 (t, J = 8.0 Hz, 1H), 4.27 (s, 1H), 4.06-4.02 (m, 2H), 3.64-3.57 (m, 2H), 3.33-3.26 (m, 2H), 3.04-2.99 (m, 2H), 2.45 (s, 3H), 2.39-2.35 (m, 2H), 2.28-2.21 (m, 1H), 2.14-1.98 (m, 4H), 1.82-1.70 (m, 6H), 1.47-1.41 (m, 4H), 1.37 (s, 3H), 1.36 (s, 3H), 1.27-1.18 (m, 16H), 0.93 (s, 9H); UPLC-MS (ESI+): calculated for  $C_{56}H_{81}ClN_{11}O_6S_2$  [M + 1]<sup>+</sup>, 1102.55; found, 1102.48.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-((R)-13-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecyl)succinamide (**20**). This compound was prepared from **48** and **46b** by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 10.29 (s, 1H), 9.00 (s, 1H), 8.45 (d, *J* = 8.0 Hz, 1H), 8.38 (t, *J* = 5.2 Hz, 1H), 8.07 (brs, 3H), 7.80 (dd, *J* = 8.0 Hz, 1H), 6.40 (dd, *J* = 8.0 Hz, *J* = 1.2 Hz, 1H), 6.21 (brs, 1H), 4.94– 4.87 (m, 1H), 4.54 (d, *J* = 8.8 Hz, 1H), 4.44 (t, *J* = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05–3.92 (m, 4H), 3.63–3.54 (m, 10H), 3.48–3.42 (m, 4H), 3.35–3.27 (m, 4H), 2.45 (s, 3H), 2.39–2.35 (m, 2H),2.08–1.99

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(m, 1H), 1.80–1.69 (m, 5H), 1.38 (s, 3H), 1.36 (s, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{51}H_{71}ClN_{11}O_9S_2$  [M + 1]<sup>+</sup>, 1080.46; found, 1080.21.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-((R)-14-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carbonyl)-15,15-dimethyl-12-oxo-3,6,9-trioxa-13-azahexadecyl)succinamide (21). This compound was prepared from 50a and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ (ppm) 10.28 (s, 1H), 9.00 (s, 1H), 8.40-8.36 (m, 2H), 8.06 (brs, 3H), 7.84 (d, J = 8.8 Hz, 1H), 7.80 (dd, J = 8.0Hz, J = 1.6 Hz, 1H), 7.66 (s, 1H), 7.45-7.33 (m, 4H), 7.16 (t, J = 8.0 Hz, 1H), 6.40 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.19 (brs, 1H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.27 (s, 1H), 4.05-4.00 (m, 2H), 3.63-3.55 (m, 4H), 3.51-3.42 (m, 12H), 3.34-3.26 (m, 4H), 2.57-2.52 (m, 1H), 2.45 (s, 3H), 2.40-2.32 (m, 3H), 2.07-1.99 (m, 1H), 1.80-1.69 (m, 5H), 1.38 (s, 3H), 1.36 (s, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{52}H_{73}ClN_{11}O_9S_2$  [M + 1]<sup>+</sup>, 1094.47; found, 1094.35.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-((R)-16-((2R,4S)-4-hydroxý-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carbonyl)-17,17-dimethyl-14-oxo-3,6,9,12-tetraoxa-15-azaoctadecyl)succinamide (22). This compound was prepared from 50b and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 10.29 (s, 1H), 9.00 (s, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.38-8.36 (m, 1H), 7.96 (brs, 3H), 7.80 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H), 7.66 (s, 1H), 7.46–7.33 (m, 5H), 7.16 (t, J = 8.0 Hz, 1H), 6.39 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.21 (brs, 1H), 4.94–4.87 (m, 1H), 4.54 (d, J = 8.8 Hz, 1H), 4.43 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05-4.02 (m, 2H), 3.96 (s, 2H), 3.62-3.53 (m, 14H), 3.47-3.42 (m, 4H), 3.33-3.26 (m, 4H), 2.45 (s, 3H), 2.38-2.33 (m, 2H), 2.07-2.02 (m, 1H), 1.87-1.70 (m, 5H), 1.38 (s, 3H), 1.36 (s, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{53}H_{75}ClN_{11}O_{10}S_2$  [M + 1]<sup>+</sup>, 1124.48; found, 1124.35.

(2R,4S)-1-((R)-2-(11-(4-(2-((3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-2-oxoethyl)piperazin-1-yl)undecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (23). This compound was prepared from 54a and 6 by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.79 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.97 (brs, 3H), 7.83-7.78(m, 2H), 7.66 (s, 1H), 7.44-7.36 (m, 4H), 7.19 (t, *J* = 8.0 Hz, 1H), 6.42 (dd, *J* = 8.0 Hz, *J* = 1.2 Hz, 1H), 6.18 (brs, 1H), 4.93-4.89 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.41 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05-4.02 (m, 2H), 3.61-3.51 (m, 4H), 3.36-3.26 (m, 4H), 3.15-2.99 (m, 6H), 2.73-2.61 (m, 2H), 2.45 (s, 3H), 2.33-2.22 (m, 2H), 2.13-1.98 (m, 3H), 1.82-1.63 (m, 7H), 1.49-1.45 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.32-1.21 (m, 10H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{56}H_{82}ClN_{12}O_5S_2$  [M + 1]<sup>+</sup>, 1101.57; found, 1101.62.

(2Ŕ,4S)-1-((Ŕ)-2-(12-(4-(2-((3-(((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-2-oxoethyl)piperazin-1-yl)dodecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (24). This compound was prepared from 54b and 6 by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ (ppm) 9.80 (s, 1H), 8.99 (s, 1H), 8.37 (d, J = 8.0 Hz, 1H), 7.97 (brs, 3H), 7.83–7.78 (m, 2H), 7.66 (s, 1H), 7.45–7.36 (m, 4H), 7.20 (t, J = 8.0 Hz, 1H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.18 (brs, 1H), 4.95–4.87 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.41 (t, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.05–4.02 (m, 2H), 3.63–3.52 (m, 4H), 3.38–3.26 (m, 4H), 3.15–2.99 (m, 6H), 2.73–2.63 (m, 2H), 2.45 (s, 3H), 2.29–2.22 (m, 2H), 2.13–1.98 (m, 3H), 1.82–1.63 (m, 7H), 1.52–1.43 (m, 2H),1.38 (s, 3H), 1.36 (s, 3H), 1.32–1.21 (m, 12H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>57</sub>H<sub>84</sub>ClN<sub>12</sub>O<sub>5</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1115.58; found, 1115.53.

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N16-((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3dimethyl-1-oxobutan-2-yl)hexadecanediamide (25). This compound was prepared from 56 and 6 by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.49 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.95 (brs, 3H), 7.81–7.78 (m, 2H), 7.66 (s, 1H), 7.45–7.34 (m, 4H), 7.15 (t, J = 8.0 Hz, 1H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.18 (brs, 2H), 4.95–4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.54 (s, 3H), 2.38–2.34 (m, 2H), 2.28–2.17 (m, 1H), 2.16–1.98 (m, 2H), 1.80–1.68 (m, 5H), 1.60–1.56 (m, 2H), 1.53–1.41 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.34–1.18 (m, 20H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>55</sub>H<sub>80</sub>ClN<sub>10</sub>O<sub>5</sub>S<sub>2</sub> [M + 1]<sup>+</sup>, 1059.54; found, 1059.51.

(2R,4S)-1-((R)-2-(9-(4-(4-((3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-4oxobutanoyl)piperazin-1-yl)nonanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phényl)ethyl)pyrrolidine-2-carboxamide (26). This compound was prepared from **60a** and **46b** by a procedure similar to that used for compound 7.  $^{1}$ H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.67 (s, 1H), 9.57 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.97 (brs, 3H), 7.79 (d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.47–7.35 (m, 4H), 7.15 (t, J = 8.0 Hz, 1H), 6.41 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.19 (brs, 1H), 4.95-4.89 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.45–4.39 (m, 2H), 4.28 (s, 1H), 4.14-4.02 (m, 3H), 3.63-3.56 (m, 2H), 3.50-3.45 (m, 2H), 3.40-3.26 (m, 3H), 3.10-3.05 (m, 3H), 2.96-2.83 (m, 2H), 2.72-2.63 (m, 4H), 2.45 (s, 3H), 2.29–2.20 (m, 1H), 2.13–1.99 (m, 2H), 1.82-1.70 (m, 5H), 1.69-1.58 (m, 2H), 1.53-1.42 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.30–1.19 (m, 8H), 0.93 (s, 9H); <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>): δ (ppm) 171.97, 170.87, 170.59, 170.18, 169.57, 158.28, 157.94, 155.78, 153.53, 151.51, 147.74, 144.63, 137.30, 135.95, 131.10, 129.69, 128.82, 126.95, 126.38, 122.27, 120.32, 117.80, 114.86, 113.77, 68.75, 58.54, 56.31, 55.55, 52.09, 50.92, 50.61, 47.69, 41.72, 38.15, 37.76, 35.23, 34.88, 34.12, 28.57, 28.51, 28.37, 27.30, 26.45, 25.90, 25.37, 23.13, 22.42, 21.98, 15.98; UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{56}H_{80}ClN_{12}O_6S_2$  [M + 1]<sup>+</sup>, 1115.54; found, 1115.29

(2R,4S)-1-((R)-2-(10-(4-((3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-4oxobutanoyl)piperazin-1-yl)decanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (27). This compound was prepared from **60b** and **46b** by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ (ppm) 9.73 (s, 1H), 9.57 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.99 (brs, 3H), 7.79 (d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.47–7.35 (m, 4H), 7.14 (t, J = 8.0 Hz, 1H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.19 (brs, 1H), 4.95-4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.46-4.39 (m, 2H), 4.28 (s, 1H), 4.14-4.02 (m, 3H), 3.63-3.56 (m, 2H), 3.50-3.45 (m, 2H), 3.37-3.26 (m, 3H), 3.10-3.03 (m, 3H), 2.97-2.84 (m, 2H), 2.72-2.61 (m, 4H), 2.45 (s, 3H), 2.29–2.21 (m, 1H), 2.13–1.99 (m, 2H), 1.82-1.70 (m, 5H), 1.69-1.57 (m, 2H), 1.52-1.41 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.31–1.20 (m, 10H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{57}H_{82}CIN_{12}O_6S_2$  [M + 1]<sup>+</sup>, 1129.56; found, 1129.38

N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-(4-((8-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-8-oxooctyl)oxy)phenyl)succinimide (28). This compound was prepared from 64 and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.84 (s, 1H), 9.60 (s, 1H), 8.99 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.95 (brs, 3H), 7.80 (d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.49-7.34 (m, 7H), 7.14 (t, J = 8.0 Hz, 1H), 6.87-6.82 (m, 2H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.19 (brs, 1H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.45-4.40 (m, 1H), 4.27 (s, 1H), 4.05-4.02 (m, 2H), 3.91-3.88 (m, 2H), 3.63-3.57 (m, 2H), 3.33-3.26 (m, 2H), 3.17-3.08 (m, 1H), 2.73-2.69 (m, 2H), 2.63-2.59 (m, 2H), 2.45 (s, 3H), 2.29-2.22 (m, 1H), 2.14-1.98 (m, 2H), 1.82-1.64 (m, 6H), 1.54-1.44 (m, 2H), 1.52-1.41 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.31–1.15 (m, 6H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{57}H_{75}ClN_{11}O_7S_2$  [M + 1]<sup>+</sup>, 1124.50; found, 1124.56.

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N1-(3-((3-Amino-5-(4-amino-4-methylpiperidin-1-yl)pyrazin-2yl)thio)-2-chlorophenyl)-N4-(4-(4-(5-(((R)-1-((2R,4S)-4-hydroxy-2-(((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-5-oxopentyl)-piperazin-1-yl)butyl)-succinamide (29). This compound was prepared from 68 and 46b by a procedure similar to that used for compound 7. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.56 (s, 1H), 8.99 (s, 1H), 8.35 (d, J = 8.0 Hz, 1H), 8.02 (brs, 3H), 7.94-7.91 (m, 1H), 7.86 (d, J = 8.4 Hz, 1H), 7.80 (d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.46-7.36 (m, 5H), 7.14 (t, I = 8.0 Hz, 1H), 6.87-6.82 (m, 2H), 6.43 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.17 (brs, 2H), 4.95-4.87 (m, 1H), 4.52 (d, I = 8.8 Hz, 1H), 4.43–4.37 (m, 1H), 4.23 (s, 1H), 4.05-4.01 (m, 2H), 3.67-3.56 (m, 3H), 3.47-3.27 (m, 5H), 3.10-3.05 (m, 8H), 2.65-2.61 (m, 2H), 2.45 (s, 3H), 2.42-2.39 (m, 2H), 2.30-2.27 (m, 1H), 2.22-2.15 (m, 1H), 2.07-1.99 (m, 1H), 1.83-1.71 (m, 5H), 1.63–1.49 (m, 6H), 1.48–1.40 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{56}H_{81}ClN_{13}O_6S_2 [M + 1]^+$ , 1124.50; found, 1124.47.

3-(tert-Butylthio)-2-chloroaniline (**36**). Cesium carbonate (84.0 g, 257.8 mmol, 2.5 equiv) was added at rt to a solution of 2-chloro-3-fluoroaniline (15.0 g, 103.1 mmol) and *tert*-butylthiol (41.0 mL, 32.8 g, 360.9 mmol, 3.5 equiv) in anhydrous DMF (160 mL). The reaction mixture was heated to 120 °C and stirred for 36 h under N<sub>2</sub>. After cooling, the reaction mixture was diluted with EtOAc (300 mL), washed with H<sub>2</sub>O, brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated *in vacuo* to afford crude 3-(*tert*-butylthio)-2-chloroaniline (19.8 g, 91.8 mmol) which was used directly in the next step. UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>10</sub>H<sub>15</sub>CINS [M + 1]<sup>+</sup>, 216.06; found, 216.10.

3-Amino-2-chlorobenzenethiol Hydrochloride (**37**). 3-(*tert*-Butylthio)-2-chloroaniline (19.5 g, 90.4 mmol) was suspended in conc. HCl (170 mL), and the reaction mixture was vigorously stirred for 6 h at 80 °C. After cooling down, the suspension was filtered, the white solids were washed with cold conc. HCl (15 mL) and hexane (30 mL), and dried under reduced pressure to give 3-amino-2chlorobenzenethiol hydrochloride (13.8 g, 78%). UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>6</sub>H<sub>7</sub>CINS [M + 1]<sup>+</sup>, 160.00; found, 160.10.

3-((3-Amino-2-chlorophenvl)thio)-6-chloropyrazin-2-amine (**39**). Potassium phosphate (K<sub>3</sub>PO<sub>4</sub>, 20.7 g, 97.6 mmol, 2.6 equiv) was added to a solution of 3-bromo-6-chloropyrazin-2-amine (7.8 g, 37.5 mmol, 1.0 equiv) and 3-amino-2-chlorobenzenethiol hydrochloride (9.6 g, 48.8 mmol, 1.3 equiv) in dioxane (120 mL). The reaction mixture was degassed and stirred at rt for 15 min, then CuI (1.4 g, 7.5 mmol, 0.2 equiv) and 1,10-phenanthroline (2.7 g, 15.0 mmol, 0.4 equiv) were added. After being degassed three times, the mixture was stirred at 90 °C under dry N2 for 16 h. The reaction mixture was cooled to rt, diluted with EtOAc (150 mL), and filtered through a pad of Celite followed by an EtOAc wash. The volatiles were removed under reduced pressure, and the residue was purified by silica gel chromatography eluting with 0-10% MeOH/DCM to afford 3-((3amino-2-chlorophenyl)thio)-6-chloropyrazin-2-amine (6.6 g, 61%) as a yellow solid. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 7.70 (s, 1H), 7.01–6.97 (m, 3H), 6.79 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.49 (dd, J = 7.6 Hz, J = 1.6 Hz, 1H), 5.51 (s, 2H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>10</sub>H<sub>9</sub>Cl<sub>2</sub>N<sub>4</sub>S [M + 1]<sup>+</sup>, 286.99; found, 287.05.

tert-Butyl (1-(6-amino-5-((3-amino-2-chlorophenyl)thio)pyrazin-2-yl)-4-methylpiperidin-4-yl (40). DIPEA (6.5 mL, 4.9 g, 37.5 mmol, 3.0 equiv) (1.0 mL) was added at rt to a solution of 3-((3-amino-2chlorophenyl)thio)-6-chloropyrazin-2-amine (3.6 g, 12.5 mmol, 1.0 equiv) and tert-butyl (4-methylpiperidin-4-yl)carbamate (5.4 g, 25.0 mmol, 2.0 equiv) in DMSO (50 mL). The reaction mixture was warmed to 100 °C and stirred for 3 h. After cooling, it was poured onto ice-cold water (200 mL). After extraction with EtOAc (100 mL  $\times$  2), the combined organic layers were washed with water, brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated under vacuum. The residue was purified by silica gelflash column chromatography with hexane:EtOAc (4:1-1:3) to afford tert-butyl (1-(6-amino-5-((3-amino-2chlorophenyl)thio)pyrazin-2-yl)-4-methylpiperidin-4-yl as a white solid (4.4 g, 76% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ (ppm) 7.59 (s, 1H), 6.83 (t, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.62 (s, 1H), 6.55 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 5.99 (s, 2H); 5.81 (dd, J = 8.0 Hz, J)

= 1.6 Hz, 1H), 5.41 (s, 2H), 3.85–3.81 (m, 2H), 3.23–3.18 (m, 2H), 2.09–2.05 (m, 2H), 1.46–1.41 (m, 2H), 1.39 (s, 9H), 1.25 (s, 3H);  $C_{21}H_{30}ClN_6O_2S [M + 1]^+$ , 465.18; found, 465.23.

(2S,4R)-1-((S)-2-(3-Aminopropanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phényl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (43a). HATU (295 mg, 0.66 mmol, 1.1 equiv) was added to a solution of 6 (335 mg, 0. 60 mmol, 1 equiv), 41a (125 mg, 0.66 mmol, 1.1 equiv), and DIEA (0.42 mL, 2.40 mmol, 4.0 equiv) in DMF (6 mL), and the resulting mixture was stirred at rt for 1 h. The solution was diluted with EtOAc and washed with H<sub>2</sub>O, saturated sodium bicarbonate aqueous solution, and brine and then dried over sodium sulfate. After removal of the solvent in vacuo, the residue was purified by HPLC and treated with 4 N HCl dioxane for 2 h to afford 43a as a hydrochloride salt (235 mg 71%). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.04 (s, 1H), 8.43 (d, J = 8.0 Hz, 1H), 8.23 (d, J = 8.8 Hz, 1H), 7.90 (s, 3H), 7.45–7.37 (m, 4H), 4.95-4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.44-4.40 (m, 1H)1H), 3.65-3.59 (m, 1H), 2.98-2.93 (m, 2H), 2.63-2.58 (m, 2H), 2.46 (s, 3H), 2.07–1.99 (m, 1H), 1.82–1.76 (m, 1H), 1.37 (d, J = 7.2 Hz, 3H), 1.30-1.26 (m, 1H), 0.95 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{26}H_{38}N_5O_4S [M + 1]^+$ , 516.26; found, 516.19

(25,4R)-1-((5)-2-(4-Aminobutanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((5)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)-pyrrolidine-2-carboxamide Hydrochloride (**43b**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.09 (s, 1H), 8.43 (d, J = 8.0 Hz, 1H), 8.01 (s, 3H),7.95 (d, J = 8.8 Hz, 1H), 7.46–7.37 (m, 4H), 4.95–4.88 (m, 1H), 4.50 (d, J = 8.8 Hz, 1H), 7.46–7.37 (m, 1H), 4.32–4.26 (m, 1H), 3.63–3.59 (m, 1H), 2.79–2.71 (m, 2H), 2.63–2.58 (m, 2H), 2.46 (s, 3H), 2.30–2.21 (m, 2H), 2.05–1.99 (m, 1H), 1.82–1.73 (m, 3H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.26 (m, 1H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>27</sub>H<sub>40</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 530.28; found, 530.19.

(25,4*R*)-1-((*S*)-2-(5-*Aminopentanamido*)-3,3-*dimethylbutanoyl*)-4-*hydroxy*-*N*-((*S*)-1-(4-(4-*methylthiazol*-5-*yl*)*phenyl*)*ethyl*)-*pyrrolidine-2-carboxamide Hydrochloride* (**43c**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.17 (s, 1H), 8.45 (d, *J* = 8.0 Hz, 1H), 8.02 (brs, 3H), 7.87 (d, *J* = 8.8 Hz, 1H), 7.49–7.35 (m, 4H), 4.93–4.89 (m, 1H), 4.50 (d, *J* = 8.8 Hz, 1H), 4.44–4.39 (m, 1H), 4.28–4.26 (m, 1H), 3.65–3.59 (m, 1H), 2.80–2.68 (m, 2H), 2.47 (s, 3H), 2.32–2.17 (m, 2H), 2.09–1.97 (m, 1H), 1.82–1.75 (m, 1H), 1.59–1.48 (m, 4H), 1.43–1.41 (m, 1H), 1.37 (d, *J* = 7.2 Hz, 3H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>28</sub>H<sub>42</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 544.30; found, 544.25.

(2R,4S)-1-((R)-2-(6-Aminohexanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (43d). This compound was prepared using a procedure similar to that used for compound 43a. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.07 (s, 1H), 8.42 (d, J = 8.0 Hz, 1H), 7.93 (brs, 3H), 7.83 (d, J = 8.8 Hz, 1H), 7.53–7.35 (m, 4H), 4.95–4.89 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.44–4.40 (m, 1H), 4.29–4.27 (m, 1H), 3.65–3.59 (m, 1H), 2.76–2.70 (m, 2H), 2.47 (s, 3H), 2.28–2.09 (m, 2H), 2.07–1.99 (m, 1H), 1.82–1.75 (m, 1H), 1.59–1.43 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.30–1.22 (m, 2H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>29</sub>H<sub>44</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 558.31; found, 558.27

(2R,4S)-1-((R)-2-(7-Aminoheptanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**43e**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.09 (s, 1H), 8.45 (d, J = 8.0 Hz, 1H), 7.96 (brs, 3H), 7.81 (d, J = 8.8 Hz, 1H), 7.49–7.34 (m, 4H), 4.95–4.88 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.44–4.40 (m, 1H), 4.30–4.26 (m, 1H), 3.61–3.56 (m, 1H), 2.75–2.70 (m, 2H), 2.46 (s, 3H), 2.28–2.09 (m, 2H), 2.05–2.00 (m, 1H), 1.81–1.74 (m, 1H), 1.54–1.42 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.21 (m, 4H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>30</sub>H<sub>46</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 572.33; found, 572.31.

(2R,4S)-1-((R)-2-(8-Aminooctanamido)-3,3-dimethylbutanoyl)-4hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine*2-carboxamide Hydrochloride* (*43f*). This compound was prepared using a procedure similar to that used for compound 43a. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.07 (s, 1H), 8.43 (d, J = 8.0 Hz, 1H), 7.94 (brs, 3H), 7.80 (d, J = 8.8 Hz, 1H), 7.46–7.37 (m, 4H), 4.95–4.88 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.44–4.40 (m, 1H), 4.29–4.25 (m, 1H), 3.61–3.56 (m, 1H), 2.76–2.71 (m, 2H), 2.46 (s, 3H), 2.28–2.08 (m, 2H), 2.04–1.99 (m, 1H), 1.81–1.75 (m, 1H), 1.53–1.42 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.26 (m, 6H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>31</sub>H<sub>48</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 586.34; found, 586.37.

 $(2R,4S)-1-((R)-2-(9-Aminononanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)-pyrrolidine-2-carboxamide Hydrochloride (43g). This compound was prepared using a procedure similar to that used for compound 43a. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): <math>\delta$  (ppm) 9.10 (s, 1H), 8.42 (d, J = 8.0 Hz, 1H), 7.95 (brs, 3H), 7.80 (d, J = 8.8 Hz, 1H), 7.49–7.36 (m, 4H), 4.95–4.87 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.46–4.39 (m, 1H), 4.30–4.24 (m, 1H), 3.61–3.56 (m, 1H), 3.12–3.05 (m, 1H), 2.75–2.70 (m, 2H), 2.46 (s, 3H), 2.28–2.07 (m, 2H), 2.04–1.99 (m, 1H), 1.82–1.76 (m, 1H), 1.55–1.41 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.25 (m, 8H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{32}H_{50}N_5O_4S$  [M + 1]<sup>+</sup>, 600.36; found, 600.33.

 $(2R,45)^{-1}$ - $((R)^{-2}$ -(10-Aminodecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N- $((R)^{-1}-(4-(4-methylthiazol-5-yl)phenyl)ethyl)$ -pyrrolidine-2-carboxamide Hydrochloride (**43h**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.07 (s, 1H), 8.42 (d, J = 8.0 Hz, 1H), 7.92 (brs, 3H), 7.79 (d, J = 8.8 Hz, 1H), 7.48–7.37 (m, 4H), 4.95–4.87 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.42–4.39 (m, 1H), 4.29–4.25 (m, 1H), 3.63–3.56 (m, 1H), 3.14–3.08 (m, 1H), 2.76–2.69 (m, 2H), 2.46 (s, 3H), 2.28–2.06 (m, 2H), 2.03–1.98 (m, 1H), 1.81–1.75 (m, 1H), 1.55–1.42 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.24 (m, 10H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>33</sub>H<sub>52</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 614.37; found, 614.35.

(25,4*R*)-1-((*S*)-2-(11-*A*minoundecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**43**i). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.02 (s, 1H), 8.39 (d, J = 8.0 Hz, 1H), 7.87 (brs, 3H), 7.79 (d, J = 8.8 Hz, 1H), 7.48–7.35 (m, 4H), 4.95–4.87 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.41–4.38 (m, 1H), 4.27–4.24 (m, 1H), 3.63–3.56 (m, 1H), 3.16–3.09 (m, 1H), 2.77–2.72 (m, 2H), 2.46 (s, 3H), 2.26–2.09 (m, 2H), 2.02–1.96 (m, 1H), 1.82–1.78 (m, 1H), 1.52–1.43 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.29–1.24 (m, 12H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>34</sub>H<sub>54</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 628.39; found, 628.31.

(2R,4S)-1-((R)-2-(12-Aminododecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**43***j*). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 8.99 (s, 1H), 8.37 (d, J = 8.0 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.64 (brs, 3H), 7.45–7.37 (m, 4H), 4.95–4.89 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.43–4.39 (m, 1H), 4.29–4.26 (m, 1H), 3.63–3.57 (m, 1H), 3.16–3.09 (m, 1H), 2.80–2.72 (m, 2H), 2.45 (s, 3H), 2.33–1.98 (m, 3H), 1.82–1.75 (m, 1H), 1.51–1.45 (m, 5H), 1.37 (d, J = 7.2 Hz, 3H), 1.30–1.24 (m, 14H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>35</sub>H<sub>56</sub>N<sub>5</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 642.40; found, 642.36.

Methyl 3-((3-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-3oxopropanoate (**45a**). Methyl 3-chloro-3-oxopropanoate (1.2 g, 8.9 mmol, 1.2 equiv) was added dropwise at 0 °C to a solution of *tert*butyl (1-(6-amino-5-((3-amino-2-chlorophenyl)thio)pyrazin-2-yl)-4methylpiperidin-4-yl (**40**) (3.4 g, 7.4 mmol, 1.0 equiv) and DIPEA (3.9 mL, 2.9 g, 22.2 mmol, 3.0 equiv) in DCM (50 mL). The reaction mixture was allowed to warm to rt and stirred for 1 h. It was poured into aq NaHCO<sub>3</sub> solution (50 mL). After extraction with DCM (30 mL × 2), the combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated under vacuum. The residue was used in the next step without further purification. UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>25</sub>H<sub>34</sub>ClN<sub>6</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 565.20; found, 565.33.

3-((3-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-3-oxopropanoic Acid (46a). Lithium hydroxide monohydrate (932 mg, 22.2 mmol) was added at 0 °C to a solution of residue obtained from up and was dissolved in THF/MeOH/H<sub>2</sub>O (15 mL/10 mL/5 mL). The reaction mixture was allowed to warm to rt and stirred for 2 h. After quenching with 1 N HCl to pH  $\sim$  3, the resulting mixture was extracted with EtOAc (40 mL  $\times$  2). The organic layer was washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and then concentrated under reduced pressure. The residue was then purified by silica gel column chromatography eluting with 0-5% MeOH/DCM to give 3-((3-((3-amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-3-oxopropanoic acid (2.5 g, 61%, two steps) as a white-yellow solid. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 12.72 (s, 1H), 9.85 (s, 1H), 7.62 (s, 1H), 7.58 (d, J = 7.6 Hz, 1H), 7.17 (t, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.63 (s, 1H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.11 (s, 2H); 5.81 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H), 3.86-3.84 (m, 2H), 3.49 (s, 2H), 3.24-3.19 (m, 2H), 2.10-2.06 (m, 2H), 1.46-1.41 (m, 2H), 1.39 (s, 9H), 1.25 (s, 3H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{24}H_{32}ClN_6O_5S [M + 1]^+$ , 551.18; found, 551.25.

4-((3-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-4-oxobutanoic Acid (**46b**). This compound was prepared using a procedure similar to that used for compound **46a** using methyl 4-chloro-4oxobutanoate as a starting material. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 12.14 (s, 1H), 9.55 (s, 1H), 7.62 (s, 1H), 7.44 (d, *J* = 7.6 Hz, 1H), 7.14 (t, *J* = 8.0 Hz,), 6.63 (s, 1H), 6.42 (dd, *J* = 8.0 Hz, *J* = 1.2 Hz, 1H), 6.10 (s, 2H); 3.87–3.83 (m, 2H), 3.36 (s, 2H), 3.24–3.19 (m, 2H), 2.64–2.62 (m, 2H), 2.10–2.05 (m, 2H), 1.46–1.41 (m, 2H), 1.39 (s, 9H), 1.25 (s, 3H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>25</sub>H<sub>34</sub>ClN<sub>6</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 565.20; found, 565.11.

5-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-5-oxopentanoic Acid (**46c**). This compound was prepared using a procedure similar to that used for compound **46a** using methyl 5-chloro-5-oxopentanoate as the starting material. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 12.15 (s, 1H), 9.53 (s, 1H), 7.62 (s, 1H), 7.40 (d, J = 7.6 Hz, 1H), 7.14 (t, J = 8.0 Hz,), 6.63 (s, 1H), 6.43 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.13 (s, 2H); 3.88–3.84 (m, 2H), 3.25–3.19 (m, 2H), 2.43–2.39 (m, 2H), 2.31–2.27 (m, 2H), 2.10–2.05 (m, 2H), 1.84–1.77 (m, 2H), 1.73–1.68 (m, 1H), 1.45–1.40 (m, 2H), 1.39 (s, 9H), 1.25 (s, 3H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>26</sub>H<sub>36</sub>ClN<sub>6</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 579.22; found, 579.16.

(2*R*,4*S*)-1-((*R*)-14-Amino-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3azatetradecanoyl)-4-hydroxy-*N*-((*R*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**48**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 9.05 (s, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 7.97 (brs, 3H), 7.47–7.34 (m, 4H), 6.58 (brs, 1H), 4.93–4.87 (m, 1H), 4.55 (d, *J* = 8.8 Hz, 1H), 4.47– 4.42 (m, 1H), 4.31–4.27 (m, 1H), 3.98 (s, 2H), 3.63–3.56 (m, 12H), 2.98–2.94 (m, 1H), 2.46 (s, 3H), 2.09–2.04 (m, 1H), 1.37 (d, *J* = 7.2 Hz, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>31</sub>H<sub>48</sub>N<sub>5</sub>O<sub>7</sub>S [M + 1]<sup>+</sup>, 634.33; found, 634.31.

(2R, 4S)-1-(R)-1-Amino-14-(tert-butyl)-12-oxo-3,6,9-trioxa-13azapentadecan-15-oyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**50a**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.11 (s, 1H), 8.44 (d, J = 8.0 Hz, 1H), 8.02 (brs, 3H), 7.89 (d, J = 8.0Hz, 1H), 7.46–7.36 (m, 4H), 4.95–4.87 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.44–4.40 (m, 1H), 4.29–4.26 (m, 1H), 3.62–3.46 (m, 15H), 2.97–2.93 (m, 1H), 2.46 (s, 3H), 2.40–2.33 (m, 1H), 2.05–2.00 (m, 1H), 1.81–1.74 (m, 1H), 1.37 (d, J = 7.2 Hz, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>32</sub>H<sub>50</sub>N<sub>5</sub>O<sub>7</sub>S [M + 1]<sup>+</sup>, 648.34; found, 648.25.

(2R,4S)-1-((R)-17-Amino-2-(tert-butyl)-4-oxo-6,9,12,15-tetraoxa-3-azaheptadecanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**50b**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 9.05 (s, 1H), 8.47 (d, *J* = 8.0 Hz, 1H), 7.96 (brs, 3H), 7.45–7.38 (m, 4H), 6.98 (brs, 1H), 4.94–4.87 (m, 1H), 4.54 (d, *J* = 8.8 Hz, 1H), 4.46–4.41 (m, 1H), 4.31–4.27 (m, 1H), 3.98 (s, 1H), 3.63–3.53 (m, 16H), 2.98–2.94 (m, 2H), 2.46 (s, 3H), 2.08–2.04 (m, 1H), 1.37 (d, *J* = 7.2 Hz, 3H), 0.94 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{33}H_{52}N_5O_8S$  [M + 1] <sup>+</sup>, 678.35; found, 678.31.

tert-Butyl (1-(6-Amino-5-((2-chloro-3-(2-(piperazin-1-yl)acetamido)phenyl)thio)pyrazin-2-yl)-4-methylpiperidin-4-yl)carbamate (52). tert-Butyl (1-(6-amino-5-((3-amino-2chlorophenyl)thio)-pyrazin-2-yl)-4-methylpiperidin-4-yl)carbamate (40) (930 mg, 2.0 mmol, 1.0 equiv), 2-(4-(((9H-fluoren-9yl)methoxy)carbonyl)piperazin-1-yl)acetic acid (51) (806 mg, 2.2 mmol, 1.1 equiv), and N-methylimidazole (574 mg, 7.0 mmol, 3.5 equiv) were dissolved in MeCN (8.0 mL) and THF (8.0 mL), and then TCFH (730 mg, 2.6 mmol, 1.3 equiv) was added in a single portion. The reaction was stirred until complete, judged by LC-MS. The reaction was then diluted with EtOAc (30 mL) and water (30 mL). The layers were separated and washed with water, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, the residue was dissolved in piperidine (2 mL) and MeCN (10 mL) after stirring at rt for 2 h, and the solvent was removed under vacuum and the residue was purified by pre-HPLC to afford 52 as a white solid (566 mg, 48% yield). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 10.00 (s, 1H), 8.01 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 7.62 (s, 1H), 7.19 (t, J = 8.0 Hz, 1H), 6.63 (brs, 1H), 6.39 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.10 (brs, 1H), 3.37-3.29 (m, 2H), 3.27-3.17 (m, 2H), 3.16 (s, 1H), 2.87 (t, J = 4.8 Hz, 2H), 2.58-2.51 (m, 2H), 2.13-2.09 (m, 2H), 1.47-1.42 (m, 2H), 1.39 (s, 9H), 1.25 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{27}H_{40}ClN_8O_3S$  [M + 1] <sup>+</sup>, 591.26; found, 591.21.

11-(4-(2-((3-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-2oxoethyl)piperazin-1-yl)undecanoic Acid (54a). K<sub>2</sub>CO<sub>3</sub> (3.0 equiv) and KI (1.2 equiv) were added to a solution of the intermediate 52 (118 mg, 0.2 mmol) and 11-bromoundecanoic acid (1.2 equiv) in DMF (5.0 mL). After the mixture was stirred for 12 h at 60 °C, the reaction was diluted with EtOAc (30 mL) and water (30 mL). The layers were separated and washed with water, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, and the residue was purified by pre-HPLC to afford 54a as a white solid (71 mg, 46% yield). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.80 (s, 1H), 9.44 (brs, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.20 (t, J = 8.0 Hz, 1H), 6.64 (brs, 1H), 6.44 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.09 (brs, 1H), 3.87-3.83 (m, 2H), 3.55-3.50 (m, 2H), 3.38 (s, 1H), 3.25-3.20 (m, 2H), 3.16-3.03 (m, 6H), 2.73-2.65 (m, 2H), 2.19 (t, J = 7.6 Hz, 2H), 2.13-2.06 (m, 2H), 1.66-1.58 (m, 2H), 1.53-1.45 (m, 4H), 1.39 (s, 3H), 1.32-1.24 (m, 15H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{38}H_{60}ClN_8O_5S$  [M + 1]<sup>+</sup>, 775.41; found, 775.32.

12-(4-(2-((3-((i3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-2oxoethyl)piperazin-1-yl)dodecanoic Acid (**54b**). This compound was prepared using a procedure similar to that used for compound **54a** using 12-bromododecanoic acid as the starting material. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.78 (s, 1H), 9.36 (brs, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.62 (s, 1H), 7.20 (t, *J* = 8.0 Hz, 1H), 6.64 (brs, 1H), 6.44 (dd, *J* = 8.0 Hz, *J* = 1.2 Hz, 1H), 6.09 (brs, 1H), 3.87–3.83 (m, 2H), 3.54–3.49 (m, 2H), 3.36 (s, 1H), 3.25–3.20 (m, 2H), 3.16–3.02 (m, 6H), 2.72–2.64 (m, 2H), 2.19 (t, *J* = 7.6 Hz, 2H), 2.12–2.05 (m, 2H), 1.68–1.59 (m, 2H), 1.52–1.42 (m, 4H), 1.39 (s, 9H), 1.33–1.21 (m, 17H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>39</sub>H<sub>62</sub>ClN<sub>8</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 789.42; found, 789.37.

16-((3-((3-((3-Amino-5-(4-((tert-butoxycarbonyl)amino)-4-methylpiperidin-1-yl)pyrazin-2-yl)thio)-2-chlorophenyl)amino)-16-oxohexadecanoic Acid (56). Compound 40 (558 mg, 1.20 mmol, 1.0 equiv), 16-(benzyloxy)-16-oxohexadecanoic acid (55) (542 mg, 1.44 mmol, 1.2 equiv), and N-methylimidazole (345 mg, 4.2 mmol, 3.5 equiv) were dissolved in MeCN (6.0 mL) and THF (6.0 mL), then TCFH (437 mg, 1.56 mmol, 1.3 equiv) was added in a single portion. The reaction was stirred until complete, judged by LC-MS. The reaction was then diluted with EtOAc (30 mL) and water (30 mL). The layers were separated and washed with water, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated; the residue was dissolved in THF (4 mL), MeOH (2 mL), and water (2 mL), and NaOH (192 mg, 4.8 mmol, 4.0 equiv) was added and stirred at rt for 8 h; the pH was adjusted to 6 and diluted with EtOAc (30 mL) and water (30 mL). The layers were separated and washed with water, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated, and the residue was purified by pre-HPLC to afford **56** as a white solid (228 mg, 26% yield). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 11.96 (s, 1H), 9.47 (s, 1H), 7.62 (s, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.14 (t, J = 8.0 Hz, 1H), 6.63 (brs, 1H), 6.42 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 6.09 (brs, 2H), 3.88–3.83 (m, 2H), 3.25–3.19 (m, 2H), 2.38–2.32 (m, 2H), 2.18 (t, J = 7.6 Hz, 2H), 2.11–2.03 (m, 2H), 1.63–1.54 (m, 2H), 1.52–1.42 (m, 4H), 1.39 (s, 9H), 1.35–1.19 (m, 23H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>37</sub>H<sub>58</sub>ClN<sub>6</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 733.39; found, 733.41.

9-(4-(tert-Butoxycarbonyl)piperazin-1-yl)nonanoic Acid (59a). A mixture of tert-butyl piperazine-1-carboxylate (57) (1.86 g, 10.0 mmol, 1 equiv), sodium iodide (1.49 g, 10.0 mmol, 1 equiv), potassium carbonate (4.14 g, 30.0 mmol, 3 equiv), and methyl 9bromononanoate (3.02 g, 12.0 mmol, 1.2 equiv) in MeCN (60 mL) was stirred at 60 °C for 16 h. After removal of solvent by rotary evaporation, the resulting residue was diluted with DCM and insoluble inorganics were filtered off, and the liquid filtrate was concentrated in vacuo. The residue was dissolved in THF/MeOH/ H<sub>2</sub>O (15 mL/9 mL/6 mL), and lithium hydroxide monohydrate (1.68 g, 40.0 mmol, 2 equiv) was added at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 2 h. After quenching with 1 N HCl to pH  $\sim$  3, the resulting mixture was extracted with EtOAc (100 mL  $\times$  2). The organic layer was washed with brine, dried over anhydrous Na2SO4, and then concentrated under reduced pressure, and the residue was purified by Biotage silica gel column chromatography to afford 59a as a white solid (1.43 g, 42%): <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 11.99 (s, 1H), 4.06-3.90 (m, 2H), 3.43-3.20 (m, 4H), 3.09-2.86 (m, 4H), 2.19 (t, J = 7.6 Hz, 2H), 1.73-1.65 (m, 2H), 1.52-1.47 (m, 2H), 1.41 (s, 9H), 1.27 (s, 8H); UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{18}H_{35}N_2O_4$  [M + 1] <sup>+</sup>, 343.26; found, 343.11.

10-(4-(tert-Butoxycarbonyl)piperazin-1-yl)decanoic Acid (**59b**). This compound was prepared using a procedure similar to that used for compound **59a** using methyl 10-bromodecanoate as the starting material. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ): δ (ppm) 11.98 (s, 1H), 4.07–3.92 (m, 2H), 3.47–3.34 (m, 2H), 3.26–3.13 (m, 2H), 3.09–2.98 (m, 2H), 2.96–2.85 (m, 2H), 2.18 (t, J = 7.6 Hz, 2H), 1.70–1.59 (m, 2H), 1.53–1.45 (m, 2H), 1.42 (s, 9H), 1.26 (s, 10H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>19</sub>H<sub>37</sub>N<sub>2</sub>O<sub>4</sub> [M + 1] <sup>+</sup>, 357.27; found, 357.16.

(2R,4S)-1-((R)-3,3-Dimethyl-2-(9-(piperazin-1-yl)nonanamido)butanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**60a**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.18 (brs, 2H), 8.99 (s, 1H), 8.35 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.48–7.37 (m, 4H), 4.93–4.90 (m, 1H), 4.52 (d, J = 8.8 Hz, 1H), 4.43–4.39 (m, 1H), 4.30–4.26 (m, 1H), 3.64–3.56 (m, 3H), 3.51– 3.21 (m, 6H), 3.16–3.09 (m, 3H), 2.45 (s, 3H), 2.33–1.99 (m, 3H), 1.84–1.77 (m, 1H), 1.66–1.57 (m, 2H), 1.51–1.45 (m, 2H), 1.37 (d, J = 7.2 Hz, 3H), 1.30–1.24 (m, 8H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>36</sub>H<sub>37</sub>N<sub>6</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 669.42; found, 669.37.

(2R,4S)-1-((R)-3,3-Dimethyl-2-(10-(piperazin-1-yl)decanamido)butanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**60b**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.24 (brs, 2H), 8.99 (s, 1H), 8.35 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.48–7.37 (m, 4H), 4.95–4.90 (m, 1H), 4.51 (d, J = 8.8 Hz, 1H), 4.43–4.39 (m, 1H), 4.31–4.27 (m, 1H), 3.63–3.55 (m, 3H), 3.52– 3.22 (m, 6H), 3.16–3.10 (m, 3H), 2.45 (s, 3H), 2.33–1.99 (m, 3H), 1.83–1.76 (m, 1H), 1.65–1.56 (m, 2H), 1.50–1.44 (m, 2H), 1.37 (d, J = 7.2 Hz, 3H), 1.31–1.25 (m, 10H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>37</sub>H<sub>59</sub>N<sub>6</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>, 683.43; found, 683.41. 8-(4-((tert-Butoxycarbonyl)amino)phenoxy)octanoic Acid (63). This compound was prepared using a procedure similar to that used for compound 59a using *tert*-butyl (4-hydroxyphenyl)carbamate and methyl 8-bromooctanoate as the starting material. UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{19}H_{30}NO_5$  [M + 1]<sup>+</sup>, 352.21; found, 352.10.

(2R,4S)-1-((R)-2-(8-(4-Aminophenoxy)octanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4-methylthiazol-5-yl)phenyl)-ethyl)pyrrolidine-2-carboxamide Hydrochloride (**64**). This compound was prepared using a procedure similar to that used for compound **43a**. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 9.92 (brs, 2H), 9.00 (s, 1H), 8.36 (d, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.8 Hz, 1H), 7.48–7.37 (m, 4H), 7.29–7.26 (m, 2H), 7.05–7.02 (m, 2H), 4.95–4.88 (m, 1H), 4.51 (d, *J* = 8.8 Hz, 1H), 4.44–4.40 (m, 1H), 4.31–4.28 (m, 1H), 3.98–3.94 (m, 2H), 3.64–3.57 (m, 2H), 2.45 (s, 3H), 2.33–1.98 (m, 3H), 1.83–1.76 (m, 1H), 1.73–1.66 (m, 2H), 1.54–1.42 (m, 2H), 1.37 (d, *J* = 7.2 Hz, 3H), 1.30–1.22 (m, 6H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>37</sub>H<sub>52</sub>N<sub>5</sub>O<sub>5</sub>S [M + 1]<sup>+</sup>, 678.37; found, 678.41.

5-(4-(3-((tert-Butoxycarbonyl)amino)propyl)piperazin-1-yl)pentanoic Acid (67). This compound was prepared using a procedure similar to that used for compound 59a using *tert*-butyl (3-(piperazin-1-yl)propyl)carbamate and ethyl 5-bromopentanoate as starting material. UPLC-MS (ESI<sup>+</sup>): calculated for  $C_{17}H_{34}N_3O_4$  [M + 1]<sup>+</sup>, 344.25; found, 344.17.

(2R, 45) - 1 - ((R) - 2 - (5 - (4 - (3 - Aminopropyl)piperazin - 1 - yl)pentanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((R)-1-(4-(4methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide Hydrochloride (**68**). This compound was prepared using a procedure similarto that used for compound**43a** $. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): <math>\delta$ (ppm) 9.08 (brs, 1H), 8.40 (d, J = 8.0 Hz, 1H), 7.96 (brs, 3H), 7.73 (d, J = 8.8 Hz, 1H), 7.43–7.36 (m, 4H), 4.93–4.87 (m, 1H), 4.50 (d, J = 8.8 Hz, 1H), 4.42–4.38 (m, 1H), 4.30–4.26 (m, 1H), 3.62–3.54 (m, 1H), 3.51–3.21 (m, 6H), 3.16–3.10 (m, 3H), 2.45 (s, 3H), 2.38–1.96 (m, 7H), 1.83–1.76 (m, 1H), 1.65–1.56 (m, 2H), 1.52– 1.41 (m, 6H), 1.37 (d, J = 7.2 Hz, 3H), 0.93 (s, 9H); UPLC-MS (ESI<sup>+</sup>): calculated for C<sub>35</sub>H<sub>56</sub>N<sub>7</sub>O<sub>4</sub>S [M + 1]<sup>+</sup>: 670.41; found, 670.27.

**Cell Lines and Cell Culture.** The MV4;11 cell line was purchased from the American Type Culture Collection (ATCC), cultured in Iscove's modified Dulbecco's media (IMDM). Esophageal cancer cell lines KYSE70 and KYSE520 were purchased from DSMZ (Braunschweig, Germany), grown in RPMI 1640 (Invitrogen). All of the cells were supplemented with 10% fetal bovine serum (Invitrogen) at 37  $^{\circ}$ C in a humidified 5% CO2 incubator.

**Cell Growth Assay.** Cell viability was evaluated with a WST-8 assay (Dojindo) following the manufacturer's instructions. Briefly, cells were seeded in 96-well cell culture plates at a density of 10,000–20,000 cells/well in 200  $\mu$ L for MV4;11 cell line or 2,000–3,000 for KYSE-520 cell line of culture medium containing serial dilution of testing compounds. After 4 days of treatment, cell growth was measured by a lactate dehydrogenase-based WST-8 assay (Dojindo Molecular Technologies) using a Tecan Infinite M-1000 multimode microplate reader (Tecan US, Morrisville, NC). The WST-8 reagent was added to each well, and cells were incubated for an additional 1–2 h and read at 450 nm. The readings were normalized to the vehicle-treated cells, and the IC<sub>50</sub> was calculated by nonlinear regression analysis using the GraphPad Prism 6 software.

**Western Blot Analysis.** Western blotting and quantification were performed with regular Western blot method or LI-COR Odyssey system. Treated cells were lysed by RIPA buffer supplemented with protease and phosphatase inhibitors. The cell lysates were separated by 4–12% SDS–PAGE gels and blotted into PVDF (polyvinylidene difluoride) membranes. Antibodies used in the study are indicated in the figure legends. The net protein bands and loading controls are calculated by deducting the background from the inverted band value. The final relative quantification values are the ratio of the net band to net loading control.

For the in vitro kinetics studies of SHP2 expression, cancer cells seeded in a 6-well plate overnight were treated with the compounds for another 2, 4, 8, 12, and 24 h. The treated cells were harvested, and

the level of SHP2 protein was examined by blot analysis. GAPDH was used as a loading control.

**Molecular Modeling.** The crystal structures of SHP2 with SHP099 (PDBID: 5EHR),<sup>26</sup> 9b (PDBID: 5XZR),<sup>28</sup> SHP099+SHP244 (PDBID: 6BMU),<sup>62</sup> SHP504 (PDBID: 6BMV),<sup>62</sup> and SHP099+SHP844 (PDBID: 6BMY)<sup>62</sup> were used to model the binding pose of compounds 4 and 5 with SHP2. SHP2 protein corrdinates from these crystal structures were extracted and protonated at pH 7.0 using the "protonate 3D" module in the MOE program (Molecular Operating Environment (MOE). Compounds 4 and 5 were created and optimized using MOE before performing an ensemble docking calculation using the GOLD program (version 5.2). Default parameters in the GOLD program were used in the docking calculation, and the PLP scoring function was used as the fitness function to rank the docked poses. The top ranked pose was used as the binding model.

# ASSOCIATED CONTENT

#### **1** Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00471.

- Western blotting analysis of SHP2 proteins in KYSE520 and MV4;11 cells treated with the SHP2 degraders and <sup>1</sup>H NMR, <sup>13</sup>C NMR, and UPLC-MS spectra of representative compounds (PDF)
- Molecular string files for all of the final target compounds (CSV)
- Modeled structures of compound 4 in complex with SHP2 (PDB)
- Modeled structures of compound 5 in complex with SHP2 (PDB)

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#### **Author Contributions**

<sup>II</sup>M.W. and J.L. contributed equally.

#### Notes

The authors declare the following competing financial interest(s): The University of Michigan has filed a patent application on these SHP2 degraders, which has been licensed by Oncopia Therapeutics Inc. S. Wang, M. Wang, J. Lu, M. Wang are co-inventors on the patent application. The University of Michigan has received a research contract from

Oncopia Therapeutics. S.W. is a co-founder of Oncopia, owns shares in Oncopia and is a paid consultant to Oncopia. The University of Michigan also owns shares in Oncopia.

# ACKNOWLEDGMENTS

This study was supported in part by a research contract from Oncopia Therapeutics Inc. and the Michigan Comprehensive Cancer Center (now Rogel Cancer Center) Core grant (P30 CA046592) from the National Cancer Institute (NCI), NIH.

# ABBREVIATIONS USED

SHP2, Src homology 2 domain-containing phosphatase 2; PTP, protein tyrosine phosphatase; NS, Noonan Syndrome; JMML, Juvenile myelomonocytic leukemia; ERK, extracellular signal-regulated kinase; PROTAC, proteolysis targeting chimera; HATU, 1-[bis(dimethylamino)-methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate; VHL, von Hippel–Lindau; TCFH, N,N,N,N-tetramethylchloroformamidinium hexafluorophosphate; NMI, *N*-methylimidazole; DIPEA, N,N-diisopropylethylamine; DMF, dimethylformamide; DCM, dichloromethane; HATU, 1-[bis-(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate; TFA, trifluoroacetic acid

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