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(*S*)-3-(Carboxyformamido)-2-(3-(carboxymethyl)ureido)propanoic Acid as A Novel PSMA Targeting Scaffold for Prostate Cancer Imaging

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Key words: prostate-specific membrane antigen (PSMA), novel inhibitors, prostate cancer, molecular imaging

ABSTRACT

In an effort to seek novel agents targeting prostate-specific membrane antigen (PSMA), sixteen ligands (**L1-L16**) with structural modifications in S1' binding pocket were synthesized and evaluated for PSMA inhibition. (*S*)-3-(Carboxyformamido)-2-(3-(carboxymethyl)ureido) propanoic acids proved potent PSMA ligands with K_i values ranging from 0.08 nM - 8.98 nM, which are in the range of or are higher in potency compared to previously published urea-based ligands. Computational docking was performed to study the binding mode of the two most potent ligands discovered. FITC-conjugated **L14** could selectively stain $PSMA^+$ LNCaP cells over $PSMA^-$ PC3 cells. IRDye800CW conjugated **L16** can effectively image tumors in a murine xenograft model of prostate cancer.

INTRODUCTION:

Prostate cancer (PCa) is the most common cancer and one of the leading causes of cancer-related death among men.[1] A variety of options exist but metastatic, castration-resistant disease (mCRPC) continues to portend a poor prognosis. Recently, radiopharmaceuticals targeting prostate-specific membrane antigen (PSMA) have showed significantly improved sensitivity and specificity over the conventional techniques of computed tomography and bone scan for imaging both primary and metastatic lesions.[2, 3] Such PSMA-targeted agents have been further adapted for radioligand therapy using therapeutic isotopes. Promising results have been reported and this new option may provide an effective way to control mCRPC.[4] The development of novel PSMA-targeted agents with superior pharmacokinetic properties, namely, those with fewer off-target tissue binding, is important to improve further the imaging and treatment of PCa, and has been an area of intensive investigation.

PSMA, also known as glutamate carboxypeptidase II (GCPII) and folate hydrolase 1 (FOLH1), is a type-II transmembrane protein and zinc-dependent metallopeptidase catalyzing the hydrolysis of *N*-acetylaspartylglutamate (NAAG) or other glutamate derivatives.[2, 5] Its expression is restricted to a few normal tissues such as kidneys, proximal small intestine and, to a lesser extent, to salivary glands, and has been reported to reach 1,000-fold enhancement in the epithelium of most PCa and the neovasculature of other solid tumors. Those properties make PSMA one of the most attractive targets for prostate cancer.[6] Low-molecular-weight targeting agents have been developed that bind tightly to the catalytic pocket of PSMA as inhibitors of its natural function. Many PSMA inhibitors have been reported over the past 20 years, and the most successful ones for imaging and therapy so far identified are those that provide modifications of the zinc-binding group in the catalytic pocket mimicking NAAG. Moderate to high potency can be achieved with the scaffolds of

phosphinates[7, 8]/phosphoramidates[9] (**1**, **2**), ureas[10, 11] (**3**) and carbamates[12] (**4**, **5**) as well as thiols[13, 14] and hydroxamates.[15] Phosphoramidates and ureas have been well studied for imaging applications. A variety of low-molecular-weight ligands have been labeled with radionuclides (^{11}C, ^{18}F, ^{64}Cu, ^{68}Ga, ^{86}Y, ^{99m}Tc, ^{111}In, $^{125/124}$I, ^{177}Lu, ^{161}Tb and ^{225}Ac) and studied for positron emission tomography (PET), single photon emission computed tomography (SPECT) or targeted radioligand therapy.[16-31] With high binding affinity and synthetic simplicity, urea-based inhibitors currently dominate in clinical trials for radiopharmaceutical imaging and radioligand therapy. Nevertheless, new targeting scaffolds with improved pharmacokinetic properties are still in demand to enhance non-targeting tissue clearance thereby decreasing potential toxicity in radioligand therapy.

Compared with the extensive studies of the PSMA ligand at the zinc-binding site and S1 site, only a few studies have reported modifications on the glutamate-like moiety, and those reported have met with little success.[11, 32] The S1' binding pocket of PSMA, which binds to the glutamate-like moiety, has strict structural requirements to enable productive interaction. To find a novel ligand with improved pharmacokinetic properties and to understand better the binding mode of PSMA ligands in S1' pocket, we designed a series of compounds under the general structure of **6**. There were several considerations in our initial design: 1) The modified structures were more hydrophilic than glutamate, which was expected to improve *in vivo* non-targeting clearance; 2) The ligands could be easily accessed and diversified from **7**. After we synthesized and evaluated 16 ligands, we identified (*S*)-3-(carboxyformamido)-2-(3-(carboxymethyl)ureido) propanoic acid as a novel PSMA targeting moiety with K_i values as low as 80 pM. Optical imaging was carried out as a proof-of-principle demonstration for its imaging potential.

RESULTS:

Following our initial design, five compounds (**L1** - **L5**) were synthesized with variations in the PSMA S1' pocket, and their syntheses are shown in Schemes 1 and 2. Commercially available **8** was first reacted with Fmoc-Cl to protect the free amino group and the α amino group was deprotected through hydrogenation to give compound **10**. After coupling of **10** with *tert*-butyl *L*-leucinate, compound **11** was obtained in suitable yield after three steps. After Fmoc deprotection, the intermediate **12** was applied to generate **L1** - **L4**. The yield ranged from 38 to 48% after HPLC purification. **L5** was synthesized in N^6-((benzyloxy)carbonyl)-L-lysine form to improve HPLC purification process with UV absorbance at 254 nm, as shown in Scheme 2. Intermediate **14** could be easily generated and used to access **L5** with a yield of 41%. The PSMA inhibitory activities of **L1** - **L5** were measured by florescence-based NAALDase assay[12] (Table 1). ZJ-43, a known PSMA inhibitor, was used as control inhibiting PSMA activity with a K_i of 3.53 nM. The (*S*)-2-amino-3-(carboxyformamido)propanoic acid variation **L1** showed comparable activity of 5.69 nM with ZJ-43, but all other compounds failed to show reasonable activity.[33]

We chose (*S*)-3-(carboxyformamido)-2-(3-(carboxymethyl)ureido) propanoic acid as the scaffold for further structural optimization. **L6** - **L15** were synthesized as shown in Scheme 3. Commercially available compound **8** first reacted with *tert*-butyl-2-chloro-2-oxoacetate and then the protecting group was removed under hydrogenation to afford **16** in good yield. **16** could be efficiently coupled with N^6-((benzyloxy)carbonyl)-L-lysine and after hydrogenation, compound **18** could be obtained in gram scale for further functionalization. **L6** - **L15** were synthesized and evaluated for PSMA binding affinity. The synthetic yield ranged from 21% to 66% after HPLC purification. As shown in Table 1, **L6** showed the most potent activity at a K_i of 0.08 nM and all other modifications **L7** - **L15** also showed good binding affinity to PSMA between 0.34 nM and

5.74 nM, which further proved that the (*S*)-3-(carboxyformamido)-2-(3-(carboxymethyl)ureido)propanoic acid ligand could be generally tagged with diversified functional groups for imaging and drug delivery purpose, without diminishing its PSMA binding potency.

As a proof-of-principle application, we modified **L12** with IRDye800CW dye and synthesized optical imaging ligand **L16**. The synthesis is shown in Scheme 4. The Cbz-group of **20** was removed under hydrogenation and then conjugated with a protected glutamic acid linker to afford **22**. After the deprotection steps, reactive intermediate **24** was synthesized and purified by HPLC in moderate yield. IRDye800CW NHS ester and **24** were allowed to react at room temperature for 2 h. **L16** was prepared in 40% yield after purification. It inhibited PSMA at a K_i of 0.65 nM, which is suitable for further biological evaluation.

We performed molecular docking studies of compounds **L1** and **L6** with the X-ray crystal structure of PSMA (PDB ID: 3D7H)[16] by using Surflex-Dock GeomX module of SYBYL-X 2.1.1 to elucidate their potential binding modes at the active site of PSMA (Figure 2). Both ligands made strong hydrogen-bonding interactions with Arg210 and Lys699, which are key amino acid residues of S1' pharmacophore subpocket. However, **L6** made more hydrogen bonds with the amino acid residue in the S1 subpocket such as Arg534, Arg 536, Asp 387 and Asp 453 as shown in Figure 2B. Furthermore, the hydrophobic interaction of 4-iodobenzoyl group on **L6** projected to S1 subpocket and enhanced its affinity towards PSMA, while L1 did not benefit from this because the short residue of Leu on **L1** could not fill the S1 subpocket effectively.

L14 with a green fluorescent dye (FITC) conjugated to the PSMA targeting moiety showed a K_i of 0.43 nM. It could selectively stain $PSMA^+$ LNCaP cells over $PSMA^-$ PC3 cells as shown in

Figure 3. The fluorescent signal mostly located on the cell surface, consistent with prior reports.[2] In addition, this fluorescent uptake could be successfully blocked with an established PSMA inhibitor DCIBzL at a concentration of 100 μM.[34]

The *in vivo* study was carried out with **L16** with a K_i of 0.65 nM (Figure 4). The tumor could be clearly visualized at 4 h post-injection and the image quality was improved at 10 h and 24 h with decreasing background signal. The *ex vivo* results further supported the observation, with tumor and kidney accumulating the most intensive signal. In addition, the tumor and kidney uptake could be significantly blocked with PSMA inhibitor DCIBzL.

DISCUSSION AND CONCLUSIONS:

PSMA inhibitors derived from glutamate-like scaffolds (**1** – **5**) have proved successful as targeting agents for PCa. With their high tumor-to-background contrast, PSMA targeting agents have been applied for radiopharmaceutical imaging and radioligand therapy in clinical studies. The potential of those agents demonstrated to date has attacted higher demand for novel targeting agents with improved pharmacokinetic properties, to improve further the imaging quality and reduce side effects previously demonstrated during the radioligand therapy, such as xerostomia, resulting from off-target effects. Compared with the active investigation of PSMA inhibitors deriving from the key modification at the PSMA Zn binding site, little progress has been made on modifying the glutamate moiety, due to strict structural requirements in the S1' binding pocket. Kozikowski et al. reported that incorporating even a methyl substitution into the glutamate weakened enzyme inhibitory activity dramatically.[35] Wang et al. systematically studied over 50 structural modifications in the S1' binding pocket and demonstrated that the allyl, alkynyl, furanyl, and

thiophenyl moieties were among the most effective. But those inhibitors still displayed over 500-fold lower potency than glutamate analogues ZJ-43 and DCIBzL, respectively.[32] To discover a novel replacement for the glutamate moiety, we designed a series of novel inhibitors with the general structure of **6** by introducing a nitrogen substitution on the backbone.

L1, with a (*S*)-3-(carboxyform amido)-2-(3-(carboxymethyl)ureido)propanoic acid structure, exhibited a similar potency with its close analogue ZJ-43 (K_i=5.69 nM vs 3.63 nM). That result suggested that the *N*-amide replacement could satisfy the stereoelectronic requirement in the binding pocket and a slightly extended anionic carboxylate at the far end could also be tolerated. In sharp contrast, **L2** to **L5**, with further increasing of the size or extension of the anion showed minimal inhibition of PSMA. This result again reflected the strict structural requirements of the S1' binding pocket. It is interesting to note that oxalyldiaminopropionic acid (ODAP) has been discovered as an effective natural product binding tightly to glutamate receptors,[36] and the novel ligand we discovered may reflect the biological similarity between Glu and ODAP. Computational docking supported our hypothesis as shown in Figure 2. Both **L1** and **L6** featured with the key interactions in the S1' binding pocket, including Tyr552, Tyr700, Arg210 to the carboxylic acid motif and Lys699 to oxalate motif, which were very similar. In the case of **L6**, the 4-iodobenzoic substitution not only helped with an extra "π-cation" interaction, but also improved the binding efficiency of oxalate to Lys699 (1.98 Å for **L1** vs 1.74 Å for **L6**). As a result, improved potency was observed.

Given that **L1** displayed high affinity to PSMA, we further synthesized and evaluated 10 derivatives to test whether (*S*)-3-(carboxyformamido)-2-(3-(carboxymethyl)ureido) propanoic acid could be applied as a general PSMA targeting moiety. Key intermediate **18** could be conveniently accessed from commercially available **8** in gram scale. From **18**, **L6-L15** were

synthesized and tested for their PSMA binding potency. As a close analog of DCIBzL, one of the most potent PSMA inhibitors reported to date,[16] **L6** inhibited PSMA with a K_i of 0.08 nM, compared with **DCIBzL** 0.20 nM with our assay. **L7-L9** substituted with the substitution of 4-fluorobenzamide,[16] 6-fluoronicotinamide,[37] 6-methoxy-2-naphthamide showed similar level or slightly improved potency compared with **L1**. One order of magnitude improvement could be achieved in **L10** and **L15**, with 4'-methoxy-[1,1'-biphenyl]-4-carboxamide and *N*-(4-bromobenzyl)acetamide substitution, which is also consistent with literature reports.[38] Overall, our results indicated that this novel scaffold could effectively target PSMA and be modified for imaging and therapeutic purposes. To balance the strong PSMA binding and synthetic cost, **L12** and **L13** with functional groups (NH_2 or COOH in protected form) for further bioconjugation applications were synthesized and tested with 1 nM potency.

To evaluate the cell targeting property, **L14** with a K_i of 0.43 nM was tested using $PSMA^+$ LNCaP and $PSMA^-$ PC3 cells. **L14** could selectively stain LNCaP cells with most of the signal located on the cell surface, consistent with the known PSMA expression pattern.[2] With this specificity proved, we performed *in vivo* imaging for a preliminary evaluation, using IRDye800CW conjugated ligand **L16** with a K_i of 0.65 nM. The result indicated the novel scaffold could effectively and specifically target tumor. At 4 h post injection, the tumor could be clearly visualized, with tumor and kidneys accumulating the most intensive signal, which is in agreement with reports on glutamate-based targeting agents (Figure 4).[39] After the ligand cleared from normal tissue and organs, target-specific imaging could be achieved at 10 h and 24 h. At 24 h post-injection, the mice were sacrificed and *ex vivo* imaging was performed on the organs for quantification. The results further confirmed the *in vivo* imaging with tumor and kidneys as the main organs for the signal uptake, which significantly higher than other organs (tumor-to-muscle 19.0 and tumor-to-kidney 0.19, see

details in supporting information Figure S4). Due to tumor model difference, direct comparison with literature could not be accurately achieved, but the biodistribution of **L16** at 24 h post-injection suggesting it's among the successful ones with high tumor-to-muscle and tumor-to-organ ratio.[39,40] Effort should be further spent on improving the tumor-to-kidney ratio and developing radiopharmaceuticals for more accurate quantification.

In summary, we discovered (*S*)-3-(carboxyformamido)-2-(3-(carboxymethyl)ureido) propanoic acids as a novel class of compounds targeting PSMA with similar or higher potency, compared with urea-based ligand 3. These ODAP-based ligands could selectively bind to PSMA over-expressing LNCaP cells. As a proof-of-principle study, IRDye800CW conjugated ODAP-based ligand succeeded in detecting tumor *in vivo* with high specificity. More detailed studies of this novel ligand are currently under investigation.

EXPERIMENTAL SECTION

General: Synthesis was carried out following Schemes 1-4. The final products and key intermediates were characterized with ^{1}H-NMR, ^{13}C-NMR and HRMS. All the final products were purified by HPLC to reach >95% purity before performing the binding assay. Generally, solvents and chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Analytical thin-layer chromatography (TLC) was performed using Merck Aluminum-backed silica gel 60 F254 (Billerica, MA). NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts (δ) were reported in ppm downfield by reference to proton resonances resulting from incomplete deuteration of the NMR solvent. High resolution ESI mass spectra were obtained on an Agilent 6545 Triple Quadrupole LC MS (Santa Clara, CA). HPLC analysis was performed using a Phenomenex C18 Luna 4.60 × 250 mm^2 column on a Shimadzu LC-20A chromatography system with solvent gradient A (water with 0.1% trifluoroacetic acid) and gradient B (acetonitrile with 0.1% trifluoroacetic acid) flowing at 1 mL/min and all analysis HPLC retention times are given for an eluent gradient of 5% B for the first 1 min, 5 – 95% B over 5 min, and 95% B for the final 10 min. HPLC purification was performed using a Phenomenex C18 Luna 10.0 × 250 mm^2 column on a Bonna-Agela Technologies Co., Ltd FL-H050G preparative chromatography system (Tianjin, China). The products were eluted using eluent A (water with 0.1% trifluoroacetic acid) and eluent B (acetonitrile with 0.1% trifluoroacetic acid). The general procedures, purification and characterization are provided in detail in the Supplemental Data.

Synthesis and Characterization:

Synthesis of (*S*)-*tert*-butyl-3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-(((benzyloxy) carbonyl)amino)propanoate (9). A mixture of (*S*)-tert-butyl-3-amino-2-(((benzyloxy)carbonyl) amino)propanoate hydrochloride **8** (1 g, 3.03 mmol) and Et_3N (1.23 g, 13.12 mmol) was mixed in anhydrous CH_2Cl_2 (20 mL) at 0°C. Fmoc-Cl (862 mg, 3.34 mmol) was added and the reaction mixture was stirred at room temperature for 6 h. Then, the solvent was evaporated under vacuum. 1.09 g compound **9** was isolated by silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 25%, vol/vol) as a colorless thick oil. The yield is 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.37 - 7.28 (m, 7H), 5.71 (s, 1H), 5.19 (s, 1H), 5.11 (s, 2H), 4.40 - 4.35 (m, 3H), 4.20 (t, J = 6.8 Hz, 1H), 3.63 (s, 2H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.31, 156.71, 156.25, 143.99, 143.85, 141.41, 136.24, 128.65, 128.34, 128.29, 127.83, 127.20, 125.19, 120.10, 83.21, 67.25, 67.17, 55.17, 47.26, 43.21, 28.03. HRMS calcd. for $C_{30}H_{32}N_2O_6$ $[M+H]^+$ 517.2333, found 517.2331.

Synthesis of (*S*)-*tert*-butyl 3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-amino propanoate (10). Compound **9** (900 mg, 1.74 mmol) and 10% dry Pd/C (35 mg) were mixed in MeOH (20 mL) under H_2 and stirred at room temperature for 12 h. After filtration, the solvent was removed under vacuum and 550 mg of product **10** was obtained after silica gel flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol) as a colorless oil. The yield was 83%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.33 (s, 1H), 4.40 (d, J = 6.9 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 3.57 - 3.47 (m, 2H), 3.31 - 3.24 (m, 1H), 1.47 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.14, 156.61, 144.07, 141.45, 127.82, 127.18, 125.19, 120.11, 82.09, 66.93, 54.86, 47.37, 44.65, 28.15. HRMS calcd. for $C_{22}H_{26}N_2O_4$ $[M+H]^+$ 383.1965, found 383.1959.

Synthesis of (6*S*,10*S*)-*tert*-butyl 6-(tert-butoxycarbonyl)-1-(9H-fluor-en-9-yl)-10-isobutyl-3,8-dioxo-2-oxa-4,7,9-triazaundecan-11-oate (**11**). Triphosgene (139 mg, 0.47 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) at -10°C. (*S*)-tert-butyl 2-amino-4-methylpentanoate hydrochloride (300 mg, 1.35 mmol) and Et_3N (545 mg, 5.38 mmol) in anhydrous CH_2Cl_2 (20 mL) was added within 5 min. The reaction was stirred at -10°C for 2 h. Then, a mixture of compound **10** (514 mg, 1.35 mmol) and Et_3N (408 mg, 4.03 mmol) in anhydrous CH_2Cl_2 (20 mL) was added in 30 min, and the reaction was further stirred for 5 h. After the solvent was removed, 620 mg of the product **11** was obtained as a colorless oil, after silica gel flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol). The yield is 77%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, J = 7.5 Hz, 2H), 7.63 (t, J = 7.9 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.4 Hz, 2H), 5.68 (s, 1H), 5.43 (s, 1H), 5.26 (s, 1H), 4.49 (s, 1H), 4.42 - 4.17 (m, 4H), 3.64 - 3.49 (m, 2H), 1.75 - 1.70 (m, 2H), 1.61 - 1.51 (m, 1H), 1.45 (s, 9H), 1.42 (s, 9H), 0.94 (d, J = 5.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.79, 170.35, 157.47, 156.84, 144.11, 141.53, 127.76, 127.22, 125.44, 120.03, 82.90, 82.04, 67.24, 54.51, 52.48, 47.29, 43.93, 42.45, 28.14, 28.01, 24.99, 22.97, 22.22. HRMS calcd. for $C_{33}H_{45}N_3O_7$ $[M+H]^+$ 596.3330, found 596.3321.

Synthesis of (*S*)-*tert*-butyl 2-(3-((*S*)-3-amino-1-(tert-butoxy)-1-oxopropan-2-yl)ureido)-4-methyl pentanoate (**12**). Compound **11** (400 mg, 0.67 mmol) and piperidine (2 mL) were dissolved in DMF (10 mL) and the reaction was stirred for 4 h at room temperature. The solvent was evaporated under vacuum. The crude product **12** was used for next step without purification. HRMS calcd. for $C_{18}H_{35}N_3O_5$ $[M+H]^+$374.2649. Found 374.2632

Synthesis of *tert*-butyl 2-chloro-2-oxoacetate. Oxoacetate (2 g, 15.76 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL) and a *tert*-Butanol (1.17 g, 15.76 mmol) solution in CH_2Cl_2 (20 mL) was added dropwise in 2 h. The reaction mixture was stirred at 0°C for another 24 h. After the

solvent was evaporated under vacuum, 2.08 g of 2-chloro-2-oxoacetate was obtained as a colorless oil and used for next step without further purification. The yield is 80%.

Synthesis of (*S*)-2-(3-((*S*)-1-carboxy-2-(carboxyformamido)ethyl)ureido)-4-methylpentanoic acid (L1). Compound **12** (50 mg, 0.13 mmol) and Et_3N (136 mg, 1.34 mmol) were mixed in anhydrous DCM (15 mL) at 0°C and tert-butyl 2-chloro-2-oxoacetate (66 mg, 0.40 mmol) was added dropwise in anhydrous DCM (5 mL). The reaction was stirred at room temperature for 5 h. After the solvent was removed under vacuum, CF_3COOH (5 mL) was added and the solution was stirred at room temperature for 3 h. After CF_3COOH was removed under vacuum, the product was purified by preparative HPLC. 20 mg of **L1** was obtained as a white solid with a yield of 44%. The analysis HPLC retention time t_R = 10.7 min. ^{1}H NMR (400 MHz, MeOD) δ 4.35 (t, J = 5.9 Hz, 1H), 4.16 (q, J = 5.0 Hz, 1H), 3.53 (d, J = 5.9 Hz, 2H), 1.67 - 1.59 (m, 1H), 1.53 - 1.37 (m, 2H), 0.83 (t, J = 7.3 Hz, 6H).^{13}C NMR (100 MHz, MeOD) δ 177.22, 173.84, 162.36, 160.52, 159.95, 53.90, 52.69, 42.58, 42.48, 25.98, 23.39, 22.01. HRMS calcd. for $C_{12}H_{19}N_3O_8$ $[M+H]^+$334.1245, found 334.1239.

Synthesis of 4-(((*S*)-2-carboxy-2-(3-((*S*)-1-carboxy-3-methylbutyl)ureido)ethyl)carbamoyl) benzoic acid (L2). 4-(*tert*-butoxycarbonyl)benzoic acid (29 mg, 0.13 mmol), DIPEA (55 mg, 0.43 mmol) and HATU (82 mg, 0.21 mmol) were mixed in DMF (20 mL) and stirred for 20 min at room temperature. Compound **12** (40 mg, 0.11 mmol) was then added and the reaction mixture was stirred for 5 h at room temperature. After the solvent was removed under vacuum, CF_3COOH (6 mL) was added and stirred at room temperature for another 5 h. The CF_3COOH was removed under vacuum and 21 mg **L2** was obtained by preparative HPLC as a white solid. The yield is 48%. The analysis HPLC retention time t_R = 10.8 min. ^{1}H NMR (400 MHz, DMSO) δ 8.74 (t, J = 5.6 Hz, 1H), 8.01 (d, J = 8.3 Hz, 2H), 7.89 (d, J = 8.3 Hz, 2H), 6.53 (d, J = 8.2 Hz, 1H), 6.32 (d, J

= 8.2 Hz, 1H), 4.35 (q, *J* = 7.0 Hz, 1H), 4.08 (q, *J* = 8.5 Hz, 1H), 3.55 - 3.49 (m, 2H), 1.67 - 1.59 (m, 1H), 1.52 - 1.33 (m, 2H), 0.86 (dd, *J* = 10.9, 6.6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO) δ 175.06, 172.90, 166.85, 166.06, 157.38, 138.18, 133.03, 129.28, 127.53, 52.59, 50.92, 41.43, 41.16, 24.28, 22.85, 21.69. HRMS calcd. for $C_{18}H_{23}N_3O_8$ $[M+H]^+$ 410.1558, found 410.1552.

Synthesis of 2-(((*S*)-2-carboxy-2-(3-((S)-1-carboxy-3-methylbutyl)ureido)ethyl)carbamoyl) benzoic acid (L3). Compound **12** (40 mg, 0.11 mmol), Et_3N (109 mg, 1.07 mmol) and isobenzofuran-1,3-dione (48 mg, 0.32 mmol) were mixed in CH_2Cl_2 (20 mL) and reacted at room temperature for 24 h. After the solvent was evaporated under vacuum, CF_3COOH (6 mL) was added, and stirred at room temperature for 5 h. 20 mg of **L3** was obtained as a white solid after preparative HPLC purification. The yield is 47%. The analysis HPLC retention time t_R = 10.8 min. ^{1}H NMR (400 MHz, DMSO) δ 8.39 (t, *J* = 5.8 Hz, 1H), 7.77 - 7.71 (m, 1H), 7.58 - 7.47 (m, 2H), 7.42 (d, *J* = 7.4 Hz, 1H), 6.49 (d, *J* = 8.1 Hz, 1H), 6.29 (d, *J* = 8.2 Hz, 1H), 4.32 (q, *J* = 7.0 Hz, 1H), 4.11 (q, *J* = 8.0 Hz, 1H), 3.44 - 3.38 (m, 2H), 1.71 - 1.59 (m, 1H), 1.50 - 1.37 (m, 2H), 0.87 (dd, *J* = 11.5, 6.6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO) δ 175.08, 172.92, 168.90, 167.93, 157.28, 138.26, 131.15, 130.68, 129.24, 129.11, 127.94, 52.61, 50.93, 41.18, 41.04, 24.28, 22.83, 21.77. HRMS calcd. for $C_{18}H_{23}N_3O_8$ $[M+H]^+$ 410.1558, found 410.1552.

Synthesis of (*S*)-2-(3-((S)-1-carboxy-2-(1H-1,2,3-triazole-4-carboxamido)ethyl)ureido)-4-methylpentanoic acid (L4). 1H-imidazole-2-carboxylic acid (20 mg, 0.17 mmol)，DIPEA (173 mg, 1.34 mmol) and HATU (102 mg, 0.27 mmol) were mixed and stirred in DMF (20 mL) for 10 min at room temperature. Compound **12** (50 mg, 0.13 mmol) was then added and the reaction mixture was stirred for 5 h. After the solvent was evaporated under vacuum, CF_3COOH (6 mL) was added and stirred at room temperature for another 5 h. The solvent was then removed under

vacuum, and 18 mg **L4** was obtained as a white solid after preparative HPLC purification. The yield is 38%. The analysis HPLC retention time t_R = 10.6 min. ^{1}H NMR (400 MHz, MeOD) δ 8.22 (s, 1H), 4.53 (t, J = 5.8 Hz, 1H), 4.29 (q, J = 5.1 Hz, 1H), 3.80 (d, J = 3.7 Hz, 2H), 1.81 - 1.72 (m, 1H), 1.65 - 1.52 (m, 2H), 0.96 (t, J = 6.9 Hz, 6H). ^{13}C NMR (100 MHz, MeOD) δ 175.22, 174.14, 174.06, 163.14, 160.01, 142.91, 54.36, 52.69, 42.47, 42.04, 29.11, 25.95, 23.38, 22.01. HRMS calcd. for $C_{13}H_{20}N_6O_6$ $[M+H]^+$357.1517, found 357.1505.

Synthesis of (*9S*,*13S*)-*tert*-butyl 13-(((((9H-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-9-(*tert*-butoxycarbonyl)-3,11-dioxo-1-phenyl-2-oxa-4,10,12-triazatetradecan-14-oate(13).

Triphosgene (84 mg, 0.28 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL) at -10°C. To the solution, a mixture of (*S*)-*tert*-butyl 2-amino-6-(((benzyloxy)carbonyl)amino)hexanoate hydrochloride (300 mg, 0.81 mmol) and Et_3N (326 mg, 3.23 mmol) in anhydrous CH_2Cl_2 (10 mL) was added in 5 min. The reaction mixture was stirred at -10°C for 2 h, and then a solution of compound **10** (308 mg, 0.81 mmol) and Et_3N (163 mg,1.61 mmol) in anhydrous CH_2Cl_2 (10 mL) was added over 30 min. The reaction was stirred for another 5 h. After the solvent was removed under vacuum, 430 mg of product **13** was obtained after silica gel flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol) as a colorless oil. The yield is 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, J = 7.5 Hz, 2H), 7.60 (dd, J = 7.3, 3.0 Hz, 2H), 7.38 - 7.27 (m, 9H), 5.66 – 5.57 (m, 2H),5.09 (s, 2H), 4.48 (q, J = 5.5 Hz, 1H), 4.37 - 4.26 (m, 3H), 4.19 (t, J = 7.1 Hz, 1H), 3.56 - 3.53 (m, 2H), 3.20 - 3.10 (m, 2H), 1.76 - 1.73 (m, 1H), 1.63 - 1.50 (m, 5H), 1.42 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.89, 170.56, 157.51, 156.77, 144.10, 144.07, 141.36, 136.78, 128.60, 128.19, 128.16, 128.12, 127.76, 127.19, 125.35, 120.03, 82.94, 82.08, 67.15, 66.71, 54.31, 53.45, 47.26, 43.90, 40.72, 32.61, 29.44, 28.11, 28.02, 22.36. HRMS calcd. for $C_{41}H_{52}N_4O_9$ $[M+H]^+$745.3807, found 745.3818

Synthesis of (*9S,13S*)-*tert*-butyl 13-(aminomethyl)-9-(*tert*-butoxyc-arbonyl)-3,11-dioxo-1-phenyl-2-oxa-4,10,12-triazatetradecan-14-oate (14). Compound **13** (400 mg, 0.54 mmol) was mixed with piperidine (2 mL) in DMF (10 mL) and the reaction was stirred for 4 h at room temperature. The solvent was removed under vacuum to get 241 mg crude product **14** as a yellow solid and used for next step without purification. The yield is 86%. HRMS calcd. for $C_{26}H_{42}N_4O_7$ $[M+H]^+$523.3126, found 523.3135.

Synthesis of (*9S,13S*)-3,11,16-trioxo-1-phenyl-2-oxa-4,10,12,15-tetraazaoctadecane-9,13,18-tricarboxylic acid (L5). Compound **14** (50 mg, 0.10 mmol), Et_3N (101 mg, 0.10 mmol) and dihydrofuran-2,5-dione (29 mg, 0.29 mmol) were mixed in CH_2Cl_2 (20 mL) and stirred at room temperature for 24 h. After the solvent was removed under vacuum, CF_3COOH (6 mL) was added. The solution was stirred at room temperature for 5 h and the solvent removed under vacuum. 20 mg of **L5** was obtained as a white solid after preparative HPLC purification. The yield is 41%. The analysis HPLC retention time t_R = 10.9 min. ^{1}H NMR (400 MHz, DMSO) δ 7.94 (t, J = 5.8 Hz, 1H), 7.39 - 7.28 (m, 5H), 7.23 (t, J = 5.5 Hz, 1H), 6.50 (d, J = 7.8 Hz, 1H), 6.24 (d, J = 8.0 Hz, 1H), 5.00 (s, 2H), 4.15 (q, J = 6.8 Hz, 1H), 4.03 (q, J = 7.1 Hz, 1H), 3.39 - 3.26 (m, 2H), 2.96 (q, J = 6.5 Hz, 2H), 2.40 (t, J = 6.7 Hz, 2H), 2.31 (t, J = 6.7 Hz, 2H), 1.68 - 1.60 (m, 1H), 1.56 - 1.47 (m, 1H), 1.43 - 1.36 (m, 2H), 1.31 - 1.25 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.53, 173.85, 172.91, 171.51, 157.25, 156.12, 137.32, 128.40, 127.76, 65.15, 52.79, 52.38, 40.56, 31.82, 30.01, 29.15, 22.51. HRMS calcd. for $C_{22}H_{30}N_4O_{10}$ $[M+H]^+$ 511.3025, found 511.3027.

Synthesis of (*S*)-*tert*-butyl 2-(((benzyloxy)carbonyl)amino)-3-(2-(*tert*-butoxy)-2-oxoacet amido)propanoate (15). (*S*)-*tert*-butyl 2-amino-4-methylpentanoate hydrochloride (2.0 g, 6.08 mmol) and Et_3N (3.08 g, 30.40 mmol) were mixed in anhydrous CH_2Cl_2 (30 mL) at 0°C. *Tert*-butyl 2-chloro-2-oxoacetate (2.58 g, 15.76 mmol) in anhydrous CH_2Cl_2 (10 mL) were added

dropwise over 30 min and the reaction mixture was stirred at room temperature for 10 h. After the solvent was removed under vacuum, 2.0g of **17** was obtained as a colorless oil after silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 30%, vol/vol). The yield is 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (s, 1H), 7.35 - 7.28 (m, 5H), 5.76 (d, J = 6.9 Hz, 1H), 5.10 (s, 2H), 4.38 (q, J = 6.1 Hz, 1H), 3.72 - 3.64 (m, 2H), 1.52 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.89, 159.16, 158.00, 156.31, 136.10, 128.54, 128.22, 128.15, 84.59, 83.35, 67.16, 54.39, 42.15, 27.87,27.71. HRMS calcd. for $C_{21}H_{30}N_2O_7$ $[M+H]^+$ 423.2126, found 423.2121.

Synthesis of (*S*)-*tert*-butyl 2-amino-3-(2-(tert-butoxy)-2-oxoacetam-ido)propanoate (16). A mixture of compound **15** (2 g, 4.74 mmol) and 10% dry Pd/C (15 mg) was stirred in MeOH (20 mL) under H_2 for 12 h at room temperature. The reaction mixture was filtered and washed with MeOH (10 mL) and CH_2Cl_2 (10 mL). The solvent was removed under vacuum to give compound **16** (1.27 mg, yield 93%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (s, 1H), 3.65 - 3.58 (m, 1H), 3.51 - 3.47 (m, 1H), 3.37 - 3.29 (m, 1H), 1.72 (s, 2H), 1.52 (s, 9H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.77, 159.51, 157.76, 84.57, 82.33, 54.27, 43.16, 28.06, 27.80. HRMS calcd. for $C_{13}H_{24}N_2O_5$ $[M+H]^+$ 289.1758, found 289.1741.

Synthesis of (*9S,13S*)-tri-*tert*-butyl 3,11,16-trioxo-1-phenyl-2-oxa-4,10,12,15-tetraazahexa decane-9,13,16-tricarboxylate (17). Triphosgene (360 mg, 1.22 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL) at -10°C. To the solution, a mixture of (*S*)-tert-butyl 2-amino-6-(((benzyloxy)carbonyl)amino)hexanoate hydrochloride (1.29 mg, 3.47 mmol) and Et_3N (1.41 g, 13.88 mmol) in anhydrous CH_2Cl_2 (15 mL) was added in 5 min. The reaction mixture was stirred at -10°C for 2 h, and then a solution of compound **16** (1 g, 3.47 mmol) and Et_3N (0.70 g, 6.94 mmol) in anhydrous CH_2Cl_2 (20 mL) was added over 30 min. The reaction was stirred for another 5 h. After the solvent was removed under vacuum, 1.62 mg of product **17** was obtained after silica

gel flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol) as a colorless oil. The yield is 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (t, J = 4.8 Hz, 1H), 7.35 - 7.25 (m, 5H), 5.87 (d, J = 6.7 Hz, 1H), 5.77 (d, J = 7.9 Hz, 1H), 5.10 - 5.50 (m, 2H), 4.49 (q, J = 6.9 Hz, 1H), 4.38 - 4.31 (m, 1H), 3.68 - 3.51 (m, 2H), 3.20 - 3.09 (m, 2H), 1.77 - 1.68 (m, 1H), 1.64 - 1.33 (m, 32H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.49, 170.09, 159.13, 158.09, 157.62, 156.70, 136.73, 128.51, 128.09, 128.06, 84.33, 83.12, 81.79, 66.58, 53.46, 53.35, 43.28, 40.70, 32.61, 29.32, 28.05, 27.92, 27.74, 22.31. HRMS calcd. for $C_{32}H_{50}N_4O_{10}$ $[M+H]^+$ 651.3600, found 651.3577.

Synthesis of (*S*)-*tert*-butyl 6-amino-2-(3-((*S*)-1-(tert-butoxy)-3-(2-(tert-butoxy)-2-oxoacet amido)-1-oxopropan-2-yl)ureido)hexanoate (18). A mixture of compound **17** (1 g, 1.54 mmol) and 10% dry Pd/C (50 mg) was stirred in MeOH (30 mL) under H_2 for 12 h at room temperature. The reaction mixture was filtered and washed with MeOH (10 mL) and CH_2Cl_2 (10 mL). The solvent was removed under vacuum to get 714 mg crude product **18** as a colorless oil and used for next step without purification. The yield is 90%. HRMS calcd. for $C_{24}H_{44}N_4O_8$ $[M+H]^+$ 517.3232, found 517.3241.

Synthesis of (*4S,8S*)-14-(4-iodophenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L6). A mixture of compound 2,5-dioxopyrrolidin-1-yl 4-iodobenzoate (50 mg, 0.15 mmol)，Et_3N (59 mg, 0.58 mmol) and compound **18** (50 mg, 0.10 mmol) was stirred in DMF (20 mL) for 12 h at room temperature. The solvent was removed under vacuum, and then CF_3COOH (6 mL) was added. The reaction mixture was stirred at room temperature for 5 h and the solvent was evaporated under vacuum. 27 mg of **L6** was obtained as a white solid after preparative HPLC purification. The yield is 48%. The analysis HPLC retention time t_R = 10.9 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 6.0 Hz, 1H), 8.50 (t, J = 5.5 Hz, 1H), 7.83 (d, J = 8.4

Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 6.50 (d, J = 8.1 Hz, 1H), 6.33 (d, J = 8.1 Hz, 1H), 4.26 (q, J = 6.7 Hz, 1H), 4.06 (q, J = 7.8 Hz, 1H), 3.41 (t, J = 6.3 Hz, 2H), 3.21 (q, J = 6.7 Hz, 3H), 1.70 - 1.60 (m, 1H), 1.60 - 1.46 (m, 3H), 1.38 - 1.26 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.47, 172.68, 165.47, 161.72, 158.34, 157.26, 137.15, 134.11, 129.21, 98.57, 52.34, 52.20, 40.92, 31.89, 28.76, 22.70. HRMS calcd. for $C_{19}H_{23}IN_4O_9$ $[M+H]^+$ 579.0582, found 579.0580.

Synthesis of (*4S,8S*)-14-(4-fluorophenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L7). A mixture of 4-fluorobenzoic acid (20 mg, 0.14 mmol), DIPEA (18 mg, 1.43 mmol) and HATU (109 mg, 0.29 mmol) was stirred in DMF (20 mL) for 20 min at room temperature. Then, compound **18** (50 mg, 0.10 mmol) was added and the reaction was stirred for 5 h. The solvent was evaporated under vacuum and CF_3COOH (6 mL) was added. The reaction was stirred at room temperature for 5 h. After the solvent was evaporated under vacuum, 18 mg of **L7** was obtained by preparative HPLC as a white solid and the yield is 40%. The analysis HPLC retention time t_R = 10.7 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 5.9 Hz, 1H), 8.46 (t, J = 5.5 Hz, 1H), 7.98 - 7.83 (m, 2H), 7.27 (t, J = 8.9 Hz, 2H), 6.51 (d, J = 7.3 Hz, 1H), 6.33 (d, J = 7.7 Hz, 1H), 4.27 (q, J = 6.1 Hz, 1H), 4.13 - 4.02 (m, 1H), 3.41 (t, J = 6.3 Hz, 2H), 3.22 (q, J = 6.8 Hz, 2H), 1.71 - 1.65 (m, 1H), 1.59 - 1.49 (m, 3H), 1.36 - 1.21 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.48, 172.69, 165.11, 165.03, 162.56, 161.72, 158.36, 157.27, 131.18, 131.16, 129.84, 129.75, 115.27, 115.06, 52.35, 52.21, 40.93, 31.91, 28.83, 22.71. HRMS calcd. for $C_{19}H_{23}FN_4O_9$ $[M+H]^+$ 471.1522, found 471.1514.

Synthesis of (*4S,8S*)-14-(6-fluoropyridin-3-yl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L8). A mixture of 6-fluoronicotinic acid (20 mg, 0.14 mmol)，DIPEA (18 mg, 1.43 mmol) and HATU (109 mg, 0.29 mmol) was stirred in DMF (20 mL) for 20 min at

room temperature. Then, compound **18** (50 mg, 0.10 mmol) was added and the reaction was stirred for 5 h. The solvent was evaporated under vacuum, and then CF_3COOH (6 mL) was added. The reaction was stirred at room temperature for 5 h. After the solvent was evaporated in vacuum, 15 mg of compound **L8** was obtained as a white solid after preparative HPLC purification and the yield is 33%. The analysis HPLC retention time t_R = 10.5 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 5.9 Hz, 1H), 8.68 - 8.65 (m, 2H), 8.36 (td, J = 8.3, 2.5 Hz, 1H), 7.28 (dd, J = 8.6, 2.6 Hz, 1H), 6.51 (d, J = 8.1 Hz, 1H), 6.33 (d, J = 8.1 Hz, 1H), 4.26 (q, J = 6.6 Hz, 1H), 4.07 (q, J = 7.8 Hz, 1H), 3.41 - 3.40 (m, 2H), 3.28 - 3.23 (m, 2H), 1.70 - 1.62 (m, 1H), 1.59 - 1.50 (m, 3H), 1.37 - 1.25 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.45, 172.66, 165.32, 163.53, 162.94, 161.70, 158.32, 157.24, 147.32, 147.16, 141.38, 141.29, 128.92, 128.87, 109.56, 109.19, 52.29, 52.18, 40.90, 31.86, 28.66, 22.66. HRMS calcd. for $C_{18}H_{22}FN_5O_9$ $[M+H]^+$ 472.1474, found 472.1477.

Synthesis of 2,5-dioxopyrrolidin-1-yl 6-methoxy-2-naphthoate. A mixture of 6-methoxy-2-naphthoic acid (100 mg, 0.50 mmol)，1-hydroxypyrrolidine-2,5-dione (85 mg, 0.74 mmol) and EDCI (285 mg, 1.49 mmol) was stirred in DMF (20 mL) for 20 h at room temperature. After the solvent was evaporated under vacuum, 119 mg of product was obtained as a white solid by silica gel flash colomn chromatography (ethyl acetate/petroleum ether, 0% to 50%, vol/vol) and the yield is 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (s, 1H), 8.04 (dd, J = 8.7, 1.7 Hz, 1H), 7.85 (d, J = 9.0 Hz, 1H), 7.80 (d, J = 8.7 Hz, 1H), 7.22 (dd, J = 9.0, 2.5 Hz, 1H), 7.16 (d, J = 2.3 Hz, 1H), 3.95 (s, 3H), 2.92 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.57, 162.25, 160.57, 138.30, 132.81, 131.36, 127.80, 127.53, 126.01, 120.36, 119.89, 105.95, 55.60, 25.84. HRMS calcd. for $C_{16}H_{13}NO_5$ $[M+Na]^+$ 322.0686, found 322.0683.

Synthesis of (*4S,8S*)-14-(6-methoxynaphthalen-2-yl)-1,6,14-trioxo-2,5,7,13-tetraazatetra decane-1,4,8-tricarboxylic acid (L9). A mixture of compound 2,5-dioxopyrrolidin-1-yl 6-methoxy-2-naphthoate (44 mg, 0.15 mmol)，Et_3N (98 mg, 0.97 mmol) and compound **18** (50 mg, 0.10 mmol) was stirred in DMF (10 mL) for 12 h at room temperature. The solvent was evaporated under vacuum, and then CF_3COOH (6 mL) was added. The reaction was stirred at room temperature for 5 h. After the solvent was evaporated in vacuum, 20 mg of **L9** was obtained as a white solid by preparative HPLC and the yield is 38%. The analysis HPLC retention time t_R = 10.9 min. ^{1}H NMR (400 MHz, DMSO) δ 8.73 (s, 1H), 8.53 (t, J = 5.4 Hz, 1H), 8.35 (s, 1H), 7.95 - 7.83 (m, 3H), 7.36 (s, 1H), 7.22 (dd, J = 8.9, 2.4 Hz, 1H), 6.52 (d, J = 8.0 Hz, 1H), 6.35 (d, J = 7.9 Hz, 1H), 4.27 (q, J = 6.9 Hz, 1H), 4.07 (q, J = 7.8 Hz, 1H), 3.89 (s, 3H), 3.38 - 3.21 (m, 4H), 1.78 - 1.67 (m, 1H), 1.62 - 1.53 (m, 3H), 1.42 - 1.33 (m, 2H).^{13}C NMR (100 MHz, DMSO) δ 174.47, 172.67, 166.24, 161.70, 158.46, 157.25, 135.67, 130.41, 129.77, 127.50, 127.20, 126.59, 124.74, 119.31, 105.86, 55.33, 52.37, 52.21, 40.91, 31.89, 28.94, 22.75. HRMS calcd. for $C_{24}H_{28}N_4O_{10}$ $[M+H]^+$ 533.1878, found 533.1852.

Synthesis of 2,5-dioxopyrrolidin-1-yl 4'-methoxy-[1,1'-biphenyl]-4-carboxylate. A mixture of 4'-methoxy-[1,1'-biphenyl]-4-carboxylic acid (66 mg, 0.29 mmol), 1-hydroxypyrrolidine-2,5-dione (50 mg, 0.43 mmol) and EDCI (111 mg, 0.58 mmol) was stirred in DMF (20 mL) for 20 h at room temperature. After the solvent was evaporated under vacuum, 66 mg of product was obtained as a white solid by silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 50%, vol/vol) and the yield is 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 8.2 Hz, 2H), 7.68 (d, J = 8.2 Hz, 2H), 7.59 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 8.5 Hz, 2H), 3.86 (s, 3H), 2.91 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.47, 161.95, 160.36, 147.35, 131.87, 131.25, 128.62,

126.93, 123.07, 114.62, 55.52, 25.82. HRMS calcd. for $C_{18}H_{15}NO_5$ $[M+H]^+$ 326.1023, found 326.1012.

Synthesis of (*4S,8S*)-14-(4'-methoxy-[1,1'-biphenyl]-4-yl)-1,6,14-trioxo-2,5,7,13-tetraazatetra decane-1,4,8-tricarboxylic acid (L10) A mixture of compound 2,5-dioxopyrrolidin-1-yl 4'-methoxy-[1,1'-biphenyl]-4-carboxylate (49 mg, 0.15 mmol)， Et_3N (98 mg, 0.97 mmol) and compound **18** (50 mg, 0.10 mmol) was stirred in DMF (10 mL) for 12 h at room temperature. The solvent was evaporated in vacuum, and then CF_3COOH (6 mL) was added. The reaction was stirred at room temperature for 5 h. After the solvent was evaporated under vacuum, 28 mg of **L10** was obtained as a white solid after preparative HPLC and the yield is 52%. The analysis HPLC retention time t_R = 11.1 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 5.9 Hz, 1H), 8.46 (t, J = 5.4 Hz, 1H), 7.89 (d, J = 8.3 Hz, 2H), 7.69 (dd, J = 10.9, 8.6 Hz, 4H), 7.04 (d, J = 8.7 Hz, 2H), 6.52 (d, J = 8.0 Hz, 1H), 6.35 (d, J = 8.1 Hz, 1H), 4.27 (q, J = 6.6 Hz, 1H), 4.07 (q, J = 7.8 Hz, 1H), 3.80 (s, 3H), 3.44 - 3.37 (m, 2H), 3.25 (q, J = 6.5 Hz, 2H), 1.75 - 1.63 (m, 1H), 1.62 - 1.50 (m, 3H), 1.38 - 1.33 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.47, 172.68, 165.83, 161.74, 159.37, 158.44, 157.27, 142.23, 132.75, 131.52, 128.02, 127.83, 125.84, 114.49, 55.26, 52.37, 52.21, 40.92, 31.92, 28.91, 22.73. HRMS calcd. for $C_{26}H_{30}N_4O_{10}$ $[M+H]^+$ 559.2035, found 559.2017.

Synthesis of (*9S,13S*)-3,11,16-trioxo-1-phenyl-2-oxa-4,10,12,15-tetraazahexadecane-9,13,16-tricarboxylic acid (L11). Compound **18** (40 mg, 0.06 mmol) was dissolved in CF_3COOH (6 mL) and stirred at room temperature for 5 h. After the solvent was removed under vacuum, 18 mg of **L11** was obtained as a white solid after preparative HPLC purification and the yield is 60%. The analysis HPLC retention time t_R = 11.0 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 6.0 Hz,

1H), 7.39 - 7.28 (m, 5H), 7.23 (t, J = 5.6 Hz, 1H), 6.49 (d, J = 8.1 Hz, 1H), 6.33 (d, J = 8.1 Hz, 1H), 5.00 (s, 2H), 4.26 (q, J = 6.6 Hz, 1H), 4.07 - 4.02 (m, 1H), 3.43 - 3.93 (m, 2H), 2.96 (q, J = 6.6 Hz, 2H), 1.66 - 1.61 (m, 1H), 1.54 - 1.49 (m,1H), 1.41 - 1.36 (m, 2H), 1.31 - 1.25 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.43, 172.65, 161.70, 158.32, 157.23, 156.10, 137.30, 128.38, 127.75, 65.13, 52.34, 52.18, 41.90, 31.83, 29.13, 22.46. HRMS calcd. for $C_{20}H_{26}N_4O_{10}$ $[M+H]^+$ 483.1722, found 483.1705.

Synthesis of tert-butyl 4-(2-(((benzyloxy)carbonyl)amino)ethoxy)benzoate. A mixture of *tert*-butyl 4-hydroxybenzoate (2 g, 10.31 mmol) and K_2CO_3 (2.14 g, 15.46 mmol) was stirred in DMF (20 mL) at 60°C for 1 h. Benzyl (2-bromoethyl)carbamate (2.66 g, 10.31 mmol) was then added and the reaction was stirred for 7 h at 60°C. After the solvent was evaporated under vacuum, 3.17g product was obtained as a white solid after silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 20%, vol/vol) and the yield is 83%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 8.8 Hz, 2H), 7.39 - 7.29 (m, 5H), 6.86 (d, J = 8.7 Hz, 2H), 5.11 (s, 2H), 4.08 (t, J = 5.0 Hz, 2H), 3.62 (q, J = 5.3 Hz, 2H), 1.58 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.61, 161.92, 156.52, 136.43, 131.55, 128.68, 128.33, 128.28, 125.05, 113.93, 80.75, 67.13, 67.05, 40.58, 28.36. HRMS calcd. for $C_{21}H_{25}NO_5$ $[M+Na]^+$ 394.1625, found 394.1609.

Synthesis of 4-(2-(((benzyloxy)carbonyl)amino*)ethoxy)benzoic acid.* *Tert*-butyl 4-(2-(((benzyloxy)carbonyl)amino)ethoxy)benzoate (2 g, 5.39 mmol) was dissolved in CF_3COOH (15 mL) and stirred for 5 h at room temperature. After the solvent was removed under vacuum, 1.44 g product was obtained as a white solid without further purification. The yield is 85%. ^{1}H NMR (400 MHz, DMSO) δ 12.60 (s, 1H), 7.88 (d, J = 8.6 Hz, 2H), 7.53 (t, J = 5.4 Hz, 1H), 7.39 - 7.29 (m, 5H), 7.01 (d, J = 8.7 Hz, 2H), 5.03 (s, 2H), 4.07 (t, J = 5.5 Hz, 2H), 3.40 (q, J = 5.4 Hz, 2H).^{13}C

NMR (100 MHz, DMSO) δ 167.01, 162.04, 156.32, 137.13, 131.38, 128.38, 127.79, 123.10, 114.31, 66.62, 65.38, 39.83. HRMS calcd. for $C_{17}H_{17}NO_5$ $[M+H]^+$316.1179, found 316.1108.

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(2-(((benzyloxy)carbonyl)amino)ethoxy)benzoate. A mixture of 4-(2-(((benzyloxy)carbonyl)amino)ethoxy)benzoic acid (1 g, 3.17 mmol), 1-hydroxypyrrolidine-2,5-dione (475 mg, 4.13 mmol) and EDCI (908 mg, 4.76 mmol) was stirred in DMF (20 mL) for 20 h at room temperature. After the solvent was evaporated under vacuum, 920 mg of product was obtained as a white solid after silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 50%, vol/vol) and the yield is 71%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 8.8 Hz, 2H), 7.37 - 7.30 (m, 5H), 6.93 (d, J = 8.7 Hz, 2H), 5.28 (s, 1H), 5.11 (s, 2H), 4.11 (t, J = 4.9 Hz, 2H), 3.63 (q, J = 5.2 Hz, 2H), 2.87 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.58, 163.87, 161.49, 156.50, 136.38, 133.03, 128.69, 128.37, 128.29, 117.64, 114.71, 67.38, 67.08, 40.45, 25.77. HRMS calcd. for $C_{21}H_{20}N_2O_7$ $[M+Na]^+$435.1163, found 435.1155.

Synthesis of (*4S,8S*)-tri-*tert*-butyl 14-(4-(2-(((benzyloxy)carbonyl)-amino)ethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylate (20). A mixture of compound 2,5-dioxopyrrolidin-1-yl 4-(2-(((benzyloxy)carbonyl)amino)ethoxy)benzoate (1.20 g, 2.91 mmol), Et_3N (784 mg, 7.75 mmol) and compound **18** (1 g, 1.94 mmol) was stirred in DMF (20 mL) for 12 h at room temperature. After the solvent was evaporated under vacuum, 945 mg of product **20** was obtained as a colorless oil after silica gel flash column chromatography (methanol/dichloromethane, 0% to 6%, vol/vol) and the yield is 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.77 (d, J = 8.7 Hz, 2H), 7.40 - 7.29 (m, 5H), 6.87 (d, J = 8.5 Hz, 2H), 6.65 (s, 1H), 5.76 (s, 1H), 5.66 (s, 1H), 5.33 (s, 1H), 5.11 (s, 2H), 4.43 - 4.37 (m, 1H), 4.33 - 4.28 (m, 1H), 4.07 (t, J = 4.9 Hz, 2H), 3.71 - 3.55 (m, 4H), 3.45 - 3.41 (m, 2H), 3.16 - 3.10 (m, 1H), 1.87 - 1.70 (m,

2H), 1.68 - 1.58 (m, 4H), 1.51 (s, 9H), 1.43 (s, 9H), 1.42 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.38, 169.97, 167.42, 161.14, 159.19, 158.21, 157.62, 156.56, 136.48, 129.09, 128.68, 128.33, 128.29, 127.52, 114.21, 84.60, 83.35, 82.02, 67.11, 67.03, 53.69, 53.45, 43.31, 40.59, 32.67, 29.82, 28.94, 28.14, 27.90, 27.84, 22.62. HRMS calcd. for $C_{41}H_{59}N_5O_{12}$ $[M+H]^+$ 814.4233, found 814.4254.

Synthesis of (*4S,8S*)-14-(4-(2-(((benzyloxy)carbonyl)amino)eth-oxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L12). Compound **20** (40 mg, 0.05 mmol) was dissolved in CF_3COOH (6 mL) and stirred at room temperature for 5 h. After the solvent was evaporated under vacuum, 19 mg of **L12** was obtained as white solid after preparative HPLC purification and the yield is 60%. The analysis HPLC retention time t_R = 10.4 min. ^{1}H NMR (400 MHz, DMSO) δ 8.74 (t, J = 5.8 Hz, 1H), 8.29 (t, J = 5.5 Hz, 1H), 7.79 (d, J = 8.7 Hz, 2H), 7.49 (t, J = 5.5 Hz, 1H), 7.38 - 7.29 (m, 5H), 6.97 (d, J = 8.7 Hz, 2H), 6.51 (d, J = 8.0 Hz, 1H), 6.35 (d, J = 8.1 Hz, 1H), 5.03 (s, 2H), 4.26 (q, J = 6.6 Hz, 1H), 4.09 - 4.04 (m, 3H), 3.43 - 3.37 (m, 4H), 3.21 (d, J = 6.1 Hz, 2H), 1.72 - 1.62 (m, 1H), 1.60 - 1.43 (m, 3H), 1.35 - 1.25 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.48, 172.68, 165.58, 161.77, 160.57, 158.70, 157.27, 156.33, 137.14, 128.99, 128.40, 127.83, 127.80, 127.05, 113.95, 66.51, 65.36, 52.37, 52.22, 40.90, 31.90, 28.97, 22.73. HRMS calcd. for $C_{41}H_{59}N_5O_{12}$ $[M+H]^+$646.2355, found 646.2338.

Synthesis of *tert*-butyl 4-(2-(benzyloxy)-2-oxoethoxy)benzoate. A mixture of *tert*-butyl 4-hydroxybenzoate (2 g, 10.31 mmol) and K_2CO_3 (2.14 g, 15.46 mmol) was stirred in DMF (20 mL) at 60°C for 1 h. Benzyl 2-bromoacetate (2.36 g, 10.31 mmol) was then added and the reaction was stirred for 7 h at 60°C. After the solvent was evaporated under vacuum, 3.00 g product was obtained as a white solid after silica gel flash column chromatography (ethyl acetate/petroleum ether, 0% to 20%, vol/vol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 9.0 Hz, 2H), 7.38 - 7.29 (m,

5H), 6.88 (d, J = 9.0 Hz, 2H), 5.23 (s, 2H), 4.70 (s, 2H), 1.58 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.36, 165.45, 161.08, 135.12, 131.56, 128.78, 128.75, 128.63, 125.71, 114.14, 80.82, 67.28, 65.30, 28.35. HRMS calcd. for $C_{20}H_{22}O_5$ $[M+H-56]^+$ 287.0914, found 287.0910.

Synthesis of 4-(2-(benzyloxy)-2-oxoethoxy)benzoic acid. *Tert*-butyl 4-(2-(benzyloxy)-2-oxoethoxy)benzoate (2 g, 5.85 mmol) was dissolved in CF_3COOH (15 mL) and stirred at room temperature for 5 h. After the solvent was evaporated under vacuum, 1.46 g product was obtained as a white solid and used for next step without further purification. The yield is 87%.

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(2-(benzyloxy)-2-oxoethoxy) benzoate. 4-(2-(benzyloxy)-2-oxoethoxy)benzoic acid (1 g, 3.50 mmol), 1-hydroxypyrrolidine-2,5-dione (804 mg, 6.99 mmol) and EDCI (2.01 g, 10.49 mmol) were stirred in DMF (20 mL) for 20 h at room temperature. After the solvent was evaporated under vacuum, 0.83 g product was obtained as a white solid after silica gel flash chromatography (ethyl acetate/petroleum ether, 0% to 50%, vol/vol) and the yield is 62%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, J = 8.9 Hz, 2H), 7.47 - 7.34 (m, 5H), 6.95 (d, J = 8.9 Hz, 2H), 5.24 (s, 2H), 4.74 (s, 2H), 2.89 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.49, 167.91, 162.99, 161.41, 134.98, 132.04, 128.87, 128.83, 128.71, 118.40, 114.93, 67.47, 65.32, 25.80. HRMS calcd. for $C_{20}H_{17}NO_7[M+Na]^+$ 406.0897, found 406.0893.

Synthesis of (*4S,8S*)-14-(4-(2-(benzyloxy)-2-oxoethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L13). A mixture of compound 2,5-dioxopyrrolidin-1-yl 4-(2-(benzyloxy)-2-oxoethoxy)benzoate (57 mg, 0.15 mmol)，Et_3N (98 mg, 0.97 mmol) and compound **18** (50 mg, 0.10 mmol) was stirred in DMF (10 mL) for 12 h at room temperature. After the solvent was evaporated under vacuum, CF_3COOH (6 mL) was added and stirred at room temperature for 5 h. The solvent was removed under vacuum. 26 mg of

L13 was obtained as a white solid after preparative HPLC purification. The yield is 43%. The analysis HPLC retention time t_R = 11.2 min. ^{1}H NMR (400 MHz, DMSO) δ 8.67 (d, J = 5.2 Hz, 1H), 8.32 (t, J = 5.6 Hz, 1H), 7.79 (d, J = 8.8 Hz, 2H), 7.41 - 7.30 (m, 5H), 6.98 (d, J = 8.8 Hz, 2H), 6.53 (d, J = 8.0 Hz, 1H), 6.39 (d, J = 7.9 Hz, 1H), 5.20 (s, 2H), 4.93 (s, 2H), 4.25 (q, J = 6.5 Hz, 1H), 4.06 (q, J = 7.8 Hz, 1H), 3.42 - 3.39 (m, 2H), 3.25 - 3.18 (m, 2H), 1.72 - 1.62 (m, 1H), 1.59 - 1.45 (m, 3H), 1.36 - 1.31 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.48, 172.70, 169.97, 168.50, 165.49, 161.91, 159.68, 157.26, 135.65, 128.92, 128.48, 128.23, 128.08, 127.68, 114.06, 66.12, 64.66, 52.38, 52.29, 40.87, 31.86, 28.94, 22.73. HRMS calcd. for $C_{28}H_{32}N_4O_{12}[M+H]^+$ 617.2089, found 617.2074.

Synthesis of (*S*)-6-amino-2-(3-((*S*)-1-carboxy-2-(carboxyformamido)ethyl)ureido)hexanoic acid. A mixture of compound **18** (80 mg, 0.16 mmol) was stirred in CF_3COOH (6 mL) at room temperature for 5 h, the solvent was evaporated in vacuum to get crude product 43 mg as a colorless thick oil, and used for next step without purification. The yield is 80%.

Synthesis of (*4S,8S*)-14-((3',6'-dihydroxy-3-oxo-3H-spiro[isob-enzofuran-1,9'-xanthen]-5-yl)amino)-1,6-dioxo-14-thioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (L14). A mixture of compound fluorescein isothiocyanate isomer 1 (22 mg, 0.06 mmol)， Et_3N (29 mg, 0.29 mmol) and compound **20** (20 mg, 0.06 mmol) was stirred in DMSO (1.5 mL) for 12 h at room temperature in dark. Then water (1.5 mL) was added and the mixture was purified with preparative HPLC to obtain **L14** (23 mg, yield 54%) as a solid. The analysis HPLC retention time t_R = 10.8 min. ^{1}H NMR (400 MHz, DMSO) δ 10.14 (s, 2H), 9.91 (s, 1H), 8.75 (s, 1H), 8.24 (s, 1H), 8.12 (s, 1H), 7.74 (d, J = 8.6 Hz, 1H), 7.16 (d, J = 8.3 Hz, 1H), 6.67 (d, J = 2.1 Hz, 2H), 6.62 - 6.56 (m, 6H), 6.35 (d, J = 8.1 Hz, 1H), 4.31 - 4.25 (m, 1H), 4.12 - 4.03 (m, 1H), 3.25 - 3.21 (m, 4H), 1.73 -

1.65 (m, 1H), 1.60 - 1.55 (m, 3H), 1.39 - 1.31 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 180.39, 174.43, 172.67, 170.56, 168.60, 161.72, 159.51, 158.41, 158.00, 157.26, 151.90, 147.07, 141.49, 129.69, 129.07, 126.48, 124.00, 118.18, 116.38, 115.22, 112.62, 109.76, 102.26, 53.60, 52.36, 52.21, 43.73, 40.91, 31.96, 28.13, 22.73. HRMS calcd. for $C_{33}H_{31}N_5O_{13}S$ $[M+H]^+$ 738.1712, found 738.1696.

Synthesis of (*4S,8S*)-tri-*tert*-butyl 14-(4-bromophenyl)-1,6-dioxo-2,5,7,13-tetraazatetra decane-1,4,8-tricarboxylate. A mixture of compound **18** (100 mg, 0.19 mmol), 4-bromobenzaldehyde (47 mg, 0.25 mmol), acetic acid (two drops) and sodium cyanoborohydride (18 mg, 0.29 mmol) was stirred in MeOH (10 mL) at room temperature for 15 h. After the solvent was evaporated under vacuum, 80 mg of product was obtained as a colorless oil after flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol). The yield is 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.52 (d, J = 8.3 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 6.11 (d, J = 7.0 Hz, 1H), 5.83 (d, J = 7.4 Hz, 1H), 4.37 - 4.31 (m, 1H), 4.19 - 4.12 (m, 1H), 4.10 (s, 2H), 3.70 - 3.63 (m, 2H), 2.91 (d, J = 6.2 Hz, 2H), 1.79 - 1.70 (m, 6H), 1.53 (s, 9H), 1.45 (s, 9H), 1.42 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.23, 170.17, 158.77, 158.68, 157.70, 132.43, 131.93, 130.20, 123.86, 85.50, 83.39, 82.15, 53.72, 53.57, 51.31, 47.34, 46.65, 42.42, 32.03, 31.70, 31.54, 30.30, 29.80, 29.47, 28.12, 28.05, 27.84, 25.87, 22.80, 22.62. HRMS calcd. for $C_{31}H_{49}BrN_4O_8[M+H]^+$ 685.2812, found 685.2813.

Synthesis of (*4S,8S*)-13-(4-bromobenzyl)-1,6,14-trioxo-2,5,7,13-tetraazapentadecane-1,4,8-tricarboxylic acid (L15). A mixture of (*4S,8S*)-tri-*tert*-butyl 14-(4-bromophenyl)-1,6-dioxo-2,5,7,13-tetraazatetra decane-1,4,8-tricarboxylate (60 mg, 0.09 mmol) and Et_3N (89 mg, 0.88 mmol) was stirred in anhydrous CH_2Cl_2 (10 mL) at 0°C. Acetyl chloride (14 mg, 0.18 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. After the solvent was

evaporated under vacuum, 38 mg of (*4S,8S*)-tri-*tert*-butyl 13-(4-bromobenzyl)-1,6,14-trioxo-2,5,7,13-tetraazapenta decane-1,4,8-tricarboxylate was obtained with a yield of 60%. (*4S,8S*)-tri-*tert*-butyl 13-(4-bromobenzyl)-1,6,14-trioxo-2,5,7,13-tetraazapenta decane-1,4,8-tricarboxylate (30 mg, 0.04 mmol) was stirred in CF_3COOH (6 mL) at room temperature for 5 h. After the solvent was removed under vacuum, 13 mg of **L15** was obtained as a white solid after preparative HPLC with a yield of 58%. The analysis HPLC retention time t_R = 10.9 min.^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, J = 6.0 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 8.3 Hz, 1H), 7.20 - 7.15 (m, 2H), 6.51 - 6.48 (m, 1H), 6.35 - 6.32 (m, 1H), 4.50 (s, 1H), 4.43 (s, 1H), 4.27 (q, J = 6.7 Hz, 1H), 4.12 - 4.02 (m, 1H), 3.40 (t, J = 6.0 Hz, 2H), 3.20 - 3.14 (m, 2H), 2.06 (s, 2H), 1.97 (s, 1H), 1.66 - 1.60 (m, 1H), 1.58 - 1.35 (m, 3H), 1.28 - 1.22 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.37, 172.66, 169.90,169.83, 161.72, 158.33, 157.22, 137.93, 137.45, 131.59, 131.23, 129.79, 128.80, 120.19, 119.94, 52.21, 52.07, 50.44, 47.61, 46.83, 44.92, 40.90, 31.83, 27.55, 26.70, 22.46, 22.26, 21.57, 21.18. HRMS calcd. for $C_{21}H_{27}BrN_4O_9[M+H]^+$ 559.1040, found 559.1032.

Synthesis of (*4S,8S*)-tri-*tert*-butyl 14-(4-(2-aminoethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylate (21). A mixture of **20** (500 mg, 0.62 mmol) and 10% dry Pd/C (25 mg) was stirred in MeOH (20 mL) under H_2 at room temperature for 12 h. The reaction was filtered and washed with MeOH (10 mL) and CH_2Cl_2 (10 mL). The solvent was evaporated under vacuum to give 372 mg crude compound **21** as a colorless oil and used for next step without purification. The yield is 89%.

Synthesis of (*4S,8S*)-tri-*tert*-butyl 14-(4-(2-((*S*)-4-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-5-(tert-butoxy)-5-oxopentanamido)ethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-

tetraazatetradecane-1,4,8-tricarboxylate (22). A mixture of (*S*)-4-amino-5-(*tert*-butoxy)-5-oxopentanoic acid (36 mg, 0.18 mmol), DIPEA (112 mg, 0.29 mmol) and HATU (380 mg, 0.96 mmol) was stirred in DMF(20 mL) for 20 min at room temperature. Compound **21** (100 mg, 0.15 mmol) was added and the reaction mixture was stirred for another 5 h. After the solvent was evaporated under vacuum, 96 mg of compound **22** was obtained as a colorless oil by silica gel flash column chromatography (methanol/dichloromethane, 0% to 10%, vol/vol). The yield is 60%. ^{1}H NMR (400 MHz, DMSO) δ 8.80 (t, J = 6.0 Hz, 1H), 8.27 (t, J = 5.4 Hz, 1H), 8.10 (t, J = 5.4 Hz, 1H), 7.88 (d, J = 7.5 Hz, 2H), 7.79 (d, J = 8.7 Hz, 2H), 7.71 (d, J = 7.5 Hz, 2H), 7.67 (d, J = 7.9 Hz, 1H), 7.41 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 6.51 (d, J = 8.1 Hz, 1H), 6.34 (d, J = 8.2 Hz, 1H), 4.34 - 4.25 (m, 2H), 4.05 - 4.01 (m, 2H), 3.94 - 3.86 (m, 1H), 3.62 - 3.60 (m, 1H), 3.44 - 3.42 (m, 2H), 3.25 - 3.20 (m, 2H), 3.16 (s, 2H), 3.16 - 3.11 (m, 2H), 2.03 - 1.85 (m, 2H), 1.83 - 1.73 (m, 2H), 1.66 - 1.52 (m, 4H), 1.46 (s, 9H), 1.38 (s, 9H), 1.35 - 1.33 (m, 20H). 13C NMR (101 MHz, DMSO) δ 172.23, 171.63, 171.42, 170.37, 165.54, 160.58, 159.51, 157.88, 157.07, 156.11, 143.81, 140.75, 128.96, 128.86, 127.68, 127.10, 125.28, 120.73, 113.90, 83.21, 80.88, 80.58, 80.32, 66.51, 65.64, 53.63, 53.03, 52.68, 46.66, 41.88, 40.96, 38.15, 31.88, 31.52, 29.04, 28.91, 27.65, 27.54, 27.38, 26.67, 22.62. HRMS calcd. for $C_{57}H_{78}N_6O_{15}$ $[M+H]^+$ 1087.5598, found 1087.5620.

Synthesis of (*4S,8S*)-tri-*tert*-butyl 14-(4-(2-((*S*)-4-amino-5-(tert-butoxy)-5-oxopentanamido) ethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylate (23). Compound **22** (96 mg, 0.09 mmol) was dissolved in DMF (5 mL), piperidine (1 mL) was added and the reaction mixture was stirred for 2 h at room temperature. After the solvent was evaporated under vacuum, 69 mg of crude product **23** was obtained and used for the next step without further purification. The yield is 90%. ^{1}H NMR (400 MHz, DMSO) δ 8.82 (t, J = 5.9 Hz, 1H), 8.29 (t, J

= 5.3 Hz, 1H), 8.21 (t, *J* = 5.3 Hz, 1H), 7.80 (d, *J* = 8.7 Hz, 2H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.53 (d, *J* = 8.0 Hz, 1H), 6.35 (d, *J* = 8.2 Hz, 1H), 4.22 (q, *J* = 7.0 Hz, 1H), 4.03 (t, *J* = 5.5 Hz, 2H), 4.01 - 3.94 (m, 1H) , 3.64 - 3.60 (m, 1H), 3.44 - 3.39 (m, 2H), 3.25 - 3.21 (m, 2H), 3.04 - 2.99 (m, 2H), 2.28 - 2.18 (m, 2H), 1.94 - 1.77 (m, 2H), 1.62 - 1.51 (m, 4H), 1.47 (s, 9H), 1.43 (s, 9H), 1.41 - 1.37 (d, *J* = 1.2 Hz, 20H). ^{13}C NMR (100 MHz, DMSO) δ 172.21, 171.51, 171.07, 170.35, 165.50, 160.55, 159.50, 157.86, 157.05, 128.95, 127.02, 113.87, 83.19, 81.83, 80.85, 80.30, 66.44, 53.01, 52.84, 52.66, 43.74, 40.95, 38.20, 31.85, 30.81, 29.03, 28.89, 27.64, 27.60, 27.53, 27.36, 22.21. HRMS calcd. for $C_{42}H_{68}N_6O_{13}$ $[M+H]^+$ 865.4917, found 865.4939.

Synthesis of (*4S,8S*)-14-(4-(2-((*S*)-4-amino-4-carboxybutanamido)ethoxy)phenyl)-1,6,14-trioxo-2,5,7,13-tetraazatetradecane-1,4,8-tricarboxylic acid (24). Compound **23** (40 mg, 0.05 mmol) was dissolved in CF_3COOH (6 mL) and stirred at room temperature for 5 h. After the solvent was evaporated in vacuum, 14 mg of compound **24** was obtained by preparative HPLC as a colorless oil and the yield is 47%. The analysis HPLC retention time t_R = 10.4 min. ^{1}H NMR (400 MHz, DMSO) δ 8.76 (t, *J* = 6.0 Hz, 1H), 8.30 - 8.24 (m, 4H), 7.80 (d, *J* = 8.8 Hz, 2H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.51 (d, *J* = 8.0 Hz, 1H), 6.34 (d, *J* = 8.1 Hz, 1H), 4.26 (q, *J* = 6.7 Hz, 1H) , 4.08 - 4.02 (m, 3H), 3.93 (s, 1H), 3.45 - 3.38 (m, 4H), 3.20 (q, *J* = 6.4 Hz, 2H) , 2.36 - 2.23 (m, 2H), 2.06 -1.93 (m, 2H), 1.70 - 1.63 (m, 1H), 1.60 - 1.47 (m, 3H), 1.36 - 1.30 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.47, 172.67, 171.28, 170.87, 165.57, 161.72, 160.55, 158.35, 157.26, 129.00, 127.08, 113.94, 66.42, 52.36, 52.20, 51.68, 40.91, 38.28, 31.92, 30.55, 28.95, 25.91, 22.73. HRMS calcd. for $C_{26}H_{36}N_6O_{13}$ $[M+H]^+$ 641.2413, found 641.2413.

Synthesis of 1-(6-(((S)-1-carboxy-4-((2-(4-(((S)-5-carboxy-5-(3-((S)-1-carboxy-2-(carboxy formido)ethyl)ureido)pentyl)carbamoyl)phenoxy)ethyl)amino)-4-oxobutyl)amino)-6-oxohexyl)-2-((E)-2-((E)-3-((E)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-

ylidene)ethylidene)-2-(4-sulfophenoxy)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (L16). A mixture of IRDye 800CW NHS Ester (0.45 mg, 0.39 μmol), Et_3N (0.39 mg, 3.85 μmol) and compound **24** (1 mg, 1.56 μmol) was stirred in DMSO (200 μL) for 2 h at room temperature in dark. Then, water (1.5 mL) was added to the reaction and the mixture was purified with preparative HPLC to get product **L16** (0.25 mg, yield 40%) as a blue solid. The analysis HPLC retention time t_R = 10.5 min. HRMS calcd. for $C_{72}H_{88}N_8O_{27}S_4$ $[M+H]^+$ 1625.4714, found.1625.4706

NAALADase assay: PSMA inhibitory activity was determined using a slightly modified method of the fluorescence-based Amplex Red Glutamic Acid Assay.[12] In the presence of 8 μM N-acetylaspartylglutamate (NAAG) (12.5 μL), lysates of LNCaP cell extracts (25 μL) were incubated with the inhibitor (12.5 μL, variable concentration covering 0.01 nM-1 μM) for 120 min. The glutamate concentration was measured by incubating with a working solution (50 μL) of the Amplex Red Glutamic Acid Kit for 30 min and the fluorescence was measured with a plate reader with excitation at 530 nm and emission at 590 nm. Inhibition curves were determined using semi-log plots and IC_{50} values were determined at the concentration at which enzyme activity was inhibited by 50%. Enzyme inhibitory constants (K_i values) were generated using the Cheng-Prusoff conversion. Assays were performed in triplicate. Data analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, California).

Molecular Modeling: The 2D and 3D chemical structures of **L1** and **L6** were generated by ChemBioDraw (ver. 11.0.1, PerkinElmer, Waltham, USA) and Chem3D Pro (ver. 11.0.1, PerkinElmer, Waltham, USA). Ligand preparation and optimization was performed by the 'Sanitize' protocol in SYBYL-X 2.1.1 (Tripos Inc., St. Louis, USA) to better present the initial

3-D structure. The PDB format protein structure was downloaded from the RCSB protein data bank (PDB ID: 3D7H).[10,16] The structure preparation tool in SYBYL-X 2.1.1 was utilized for the protein preparation. Under the application of 'TRIPOS' Force Field hydrogen atoms were added and protein minimization was performed through the 'POWELL' method. Initial optimization was set to SIMPLEX. The Surflex-Dock GeomX module in SYBYL-X 2.1.1 was used for performing docking studies. As an idealized representation of the potential binding site, 'Surflex-Dock protomol' guided docking to generate a maximum number of interactions with the binding site. Protomol was found and defined by the 'Ligand' method. Protomol generation factors Bloat and Threshould were set to 0.5 (Å) and 0, respectively. The maximum number of docked poses was set to 20. All other parameters followed the default setting.

Cell Lines and Mouse Models: Human prostate cancer cell lines, LNCaP and PC3, were obtained from the Chinese Academy of Sciences Typical Culture Collection (Shanghai, China) and were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% glutamax-I, and 1% sodium pyruvate. The cell's cultures were maintained in a 5% CO_2-humidified atmosphere at 37 °C. All animal studies were carried out in full compliance with the regulations on laboratory animals of the Beijing municipality. Five four-week-old male, BALB/c nu mice were implanted subcutaneously with LNCaP (10^7 cells/mouse in 50% BD Matrigel) cells at the forward left or right flanks, respectively. When the xenografts reached 6 to 8 mm in diameter mice were used for imaging.

Cell staining: LNCaP or PC3 cells (4×10^5) were plated onto 12-well culture plates and incubated at 37°C for 2 days. Cells were pretreated with KRB buffer with 1% FBS, with or without 100 μM DCIBzL[16] for 1 h at 37°C, and then incubated with the fluorescent ligand **L14** for 1 h at 37°C.

After incubation, the cells were washed twice with KRB buffer. Images were acquired with an Olympus IX71S1F-3 camera (Olympus Corporation, PA, USA).

***In vivo* imaging:** For *in vivo* studies, **L16** was imaged using the IVIS Spectrum Imaging System (IVIS®SPECTRUM, Caliper life Sciences, Hopkinton, MA) with excitation at 745 nm and emission at 800 nm. After image acquisition at baseline, each mouse was injected intravenously with 1.2 nmol of **L16** and images were acquired at 4 h, 10 h and 24 h. After the mice were sacrificed at 24 h by cervical dislocation, the tumor and other organs were collected and assembled on a Petri dish for image acquisition (animal number, n = 4). For blocking studies, **L16** was injected after 30 min of pre-injection of 1 μmol DCIBzL and images were acquired at 4 h, 10 h, and 24 h post-injection of the dye.

ANCILLARY INFORMATION

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org.

Supporting information includes PSMA inhibition results of the ligands tested, FACS study of **L14**, Optical Imaging and biodistribution of **L16**, ^{1}H-/^{13}C NMR Spectra of compounds **L1-L16** and other intermediates. Molecular formula strings for final compounds are also available.

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There is no conflict of interest.

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Abbreviations Used

PCa, prostate cancer; mCRPC, metastatic castration-resistant prostate cancer; PSMA, prostate-specific membrane antigen; GCPII, glutamate carboxypeptidase II; FOLH1, folate hydrolase 1; NAAG, N-acetylaspartylglutamate; NAALADase, N-acetyl-L-aspartyl-L-glutamate peptidase; FBS, fetal bovine serum; NHS, N-hydrosuccinimide; FITC, fluorescein isothiocyanate; ODAP, oxalyldiaminopropionic acid; HATU, 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; DIPEA, N,N-Diisopropylethylamine; EDCI, 1-ethyl-3(3-dimethylpropylamine) carbodiimide.

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Tables:

Table 1 PSMA Inhibitory Activity

Compounds	Ki (nM)	Compounds	Ki (nM)
ZJ-43	3.53	L8	5.74
DClBzL	0.20	L9	2.37
L1	5.69	L10	0.37
L2	>1000	L11	8.98
L3	>1000	L12	1.38
L4	>1000	L13	0.99
L5	>1000	L14	0.43
L6	0.08	L15	0.34
L7	3.81	L16	0.65

Figures:

Figure 1. PSMA binding scaffold design

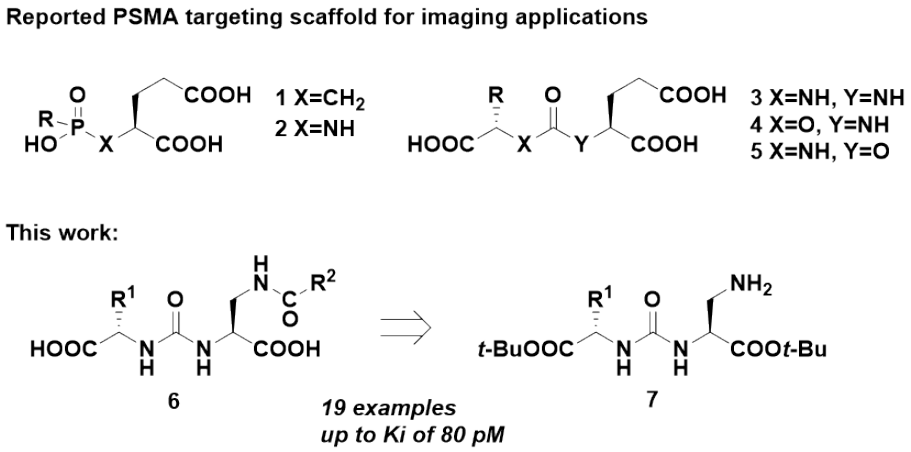
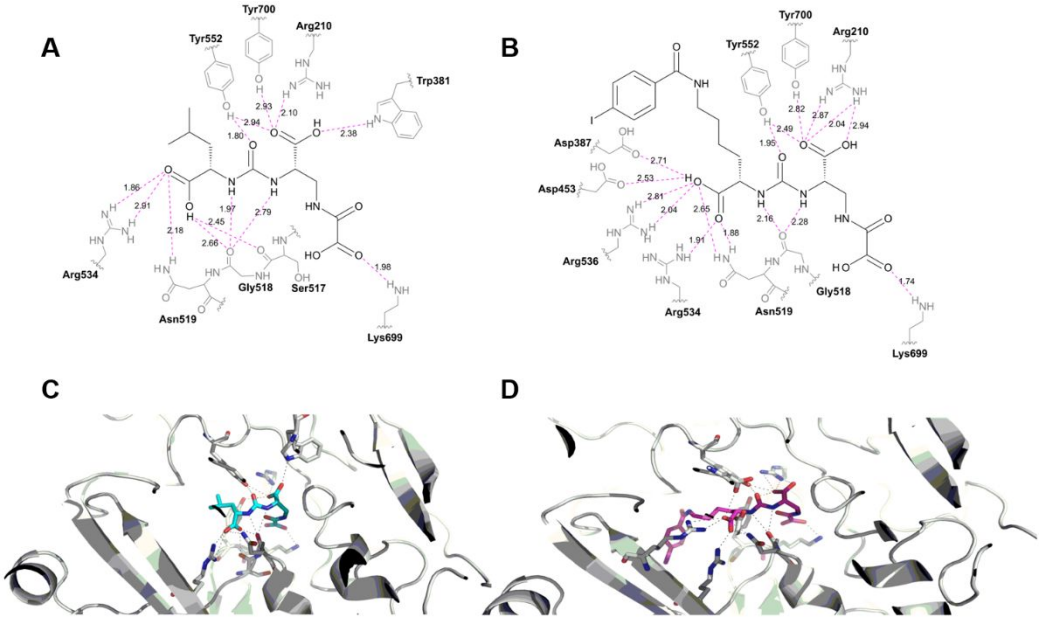


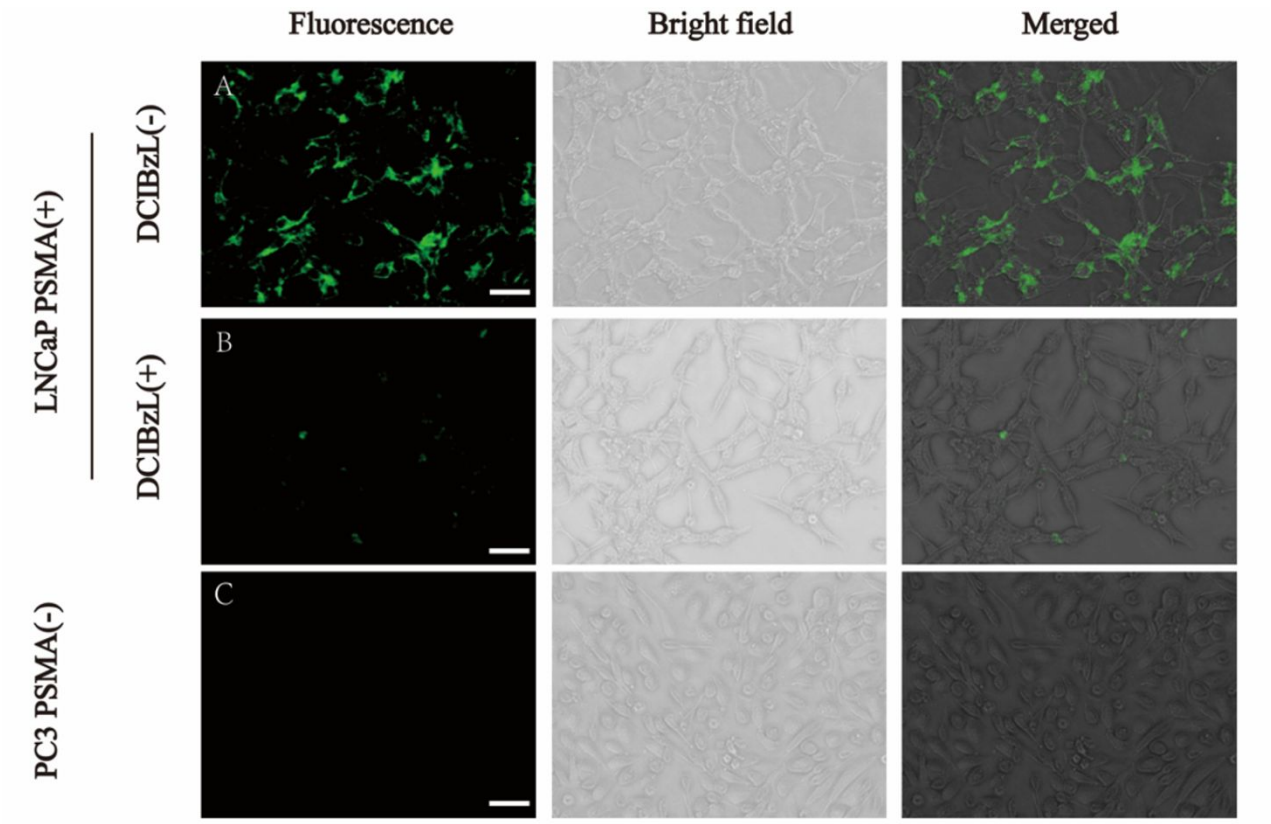
Figure 2. Docking results of L1 and L6 with PSMA protein



The best-docked poses of **L1** and **L6** in the active site of PSMA. (A) Interactions between **L1** and amino acid residues of the surrounding site, (B) Interactions between **L6** and amino acid residues

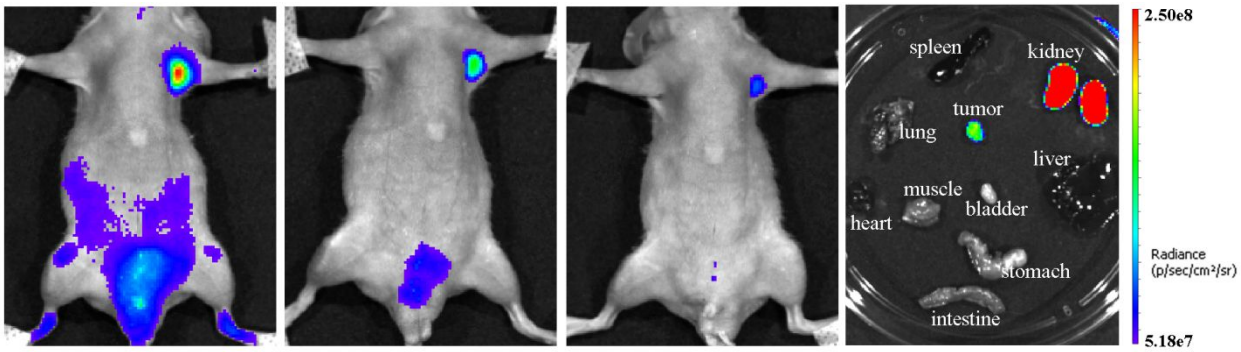
of the surrounding site, (C) 3-D view of **L1** in the active site of PSMA, (D) 3-D view of **L6** in the active site of PSMA.

Figure 3. L14 for cell imaging.



Fluorescence images of LNCaP (A and B) and PC3 (C) cells after incubation with 100 nM of **L14** for 1 hour, with or without pretreatment with 100 μM DCIBzL. Scale bars indicate 100 μm.

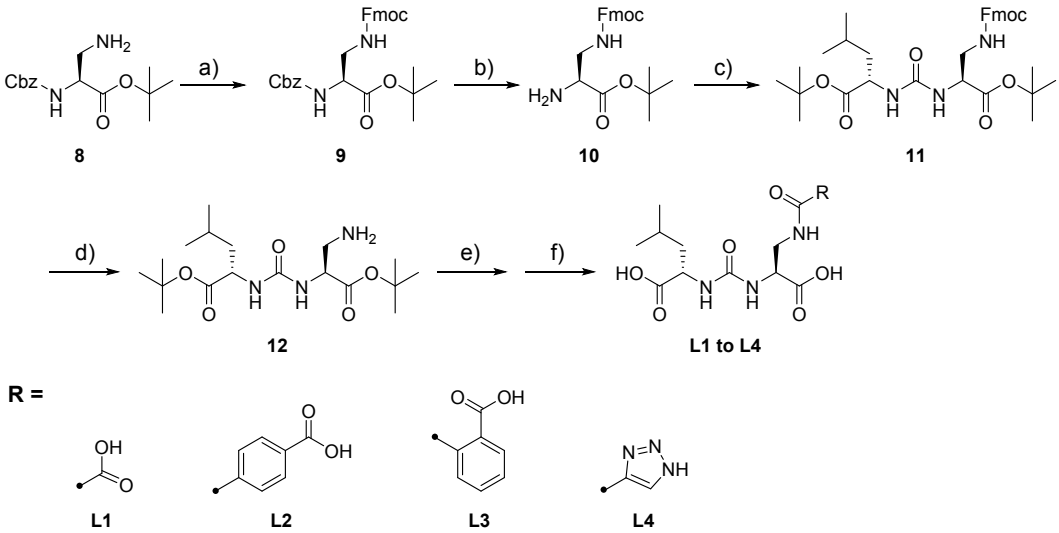
Figure 4. ***In vivo*** **optical imaging of L16.**



The images after administration of 1.2 nmol **L16** at 4, 10, and 24 h post-injection, and the image of the excised organs at 24 h post-injection.

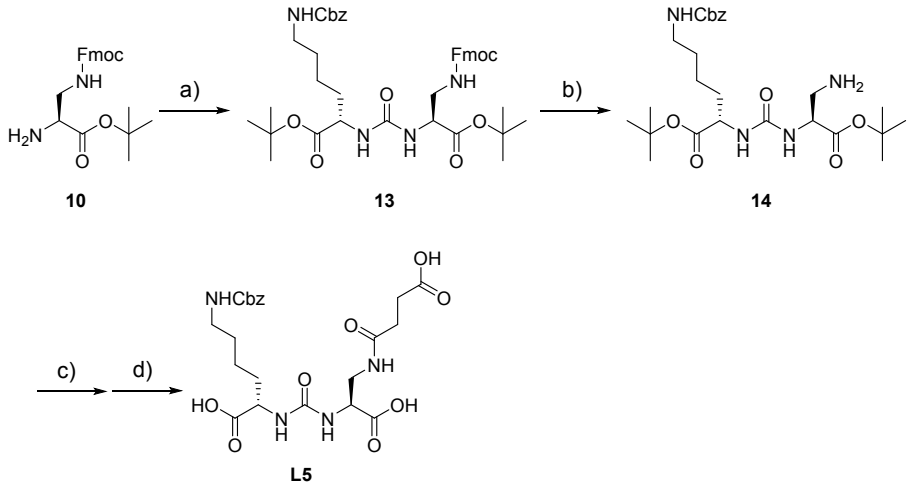
Schemes:

Scheme 1: The synthesis of **L1-L4**



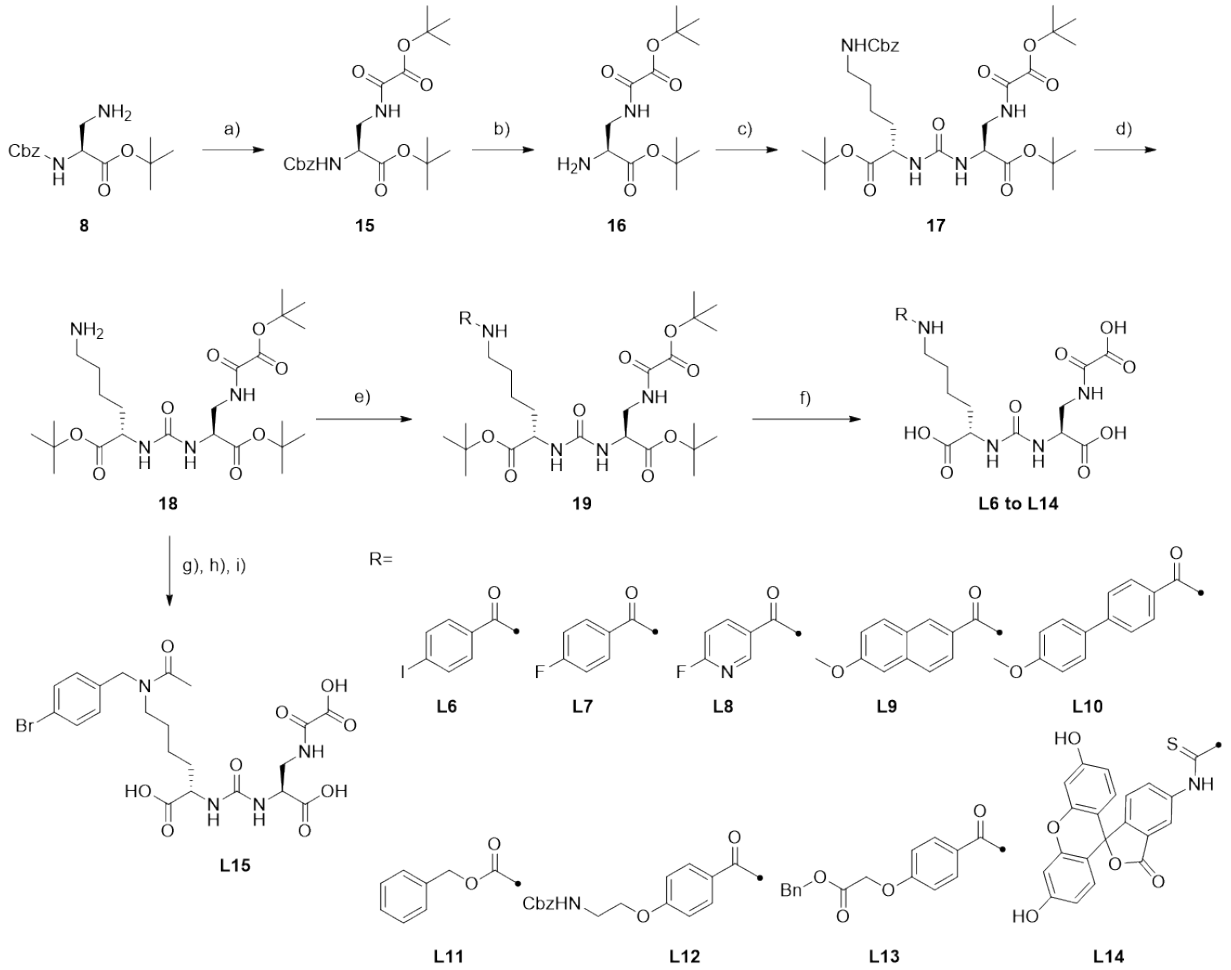
Reagents and condition: a) Fmoc-Cl, Et_3N, CH_2Cl_2, r.t.,6 h, 70% yield; b) Pd/C, H_2, MeOH, r.t., 12 h, 83% yield; c) H-Leu-*t*-Bu, triphosgene, Et_3N, CH_2Cl_2, -10°C, 2 h, 77%; d) Piperidine, DMF, r.t., 4 h; e) Acid chloride, anhydride or acid with coupling agents, as specified in experimental section; f) CF_3COOH, r.t., 5 h, 38-48% overall yield from **11**.

Scheme 2: The synthesis of **L5**



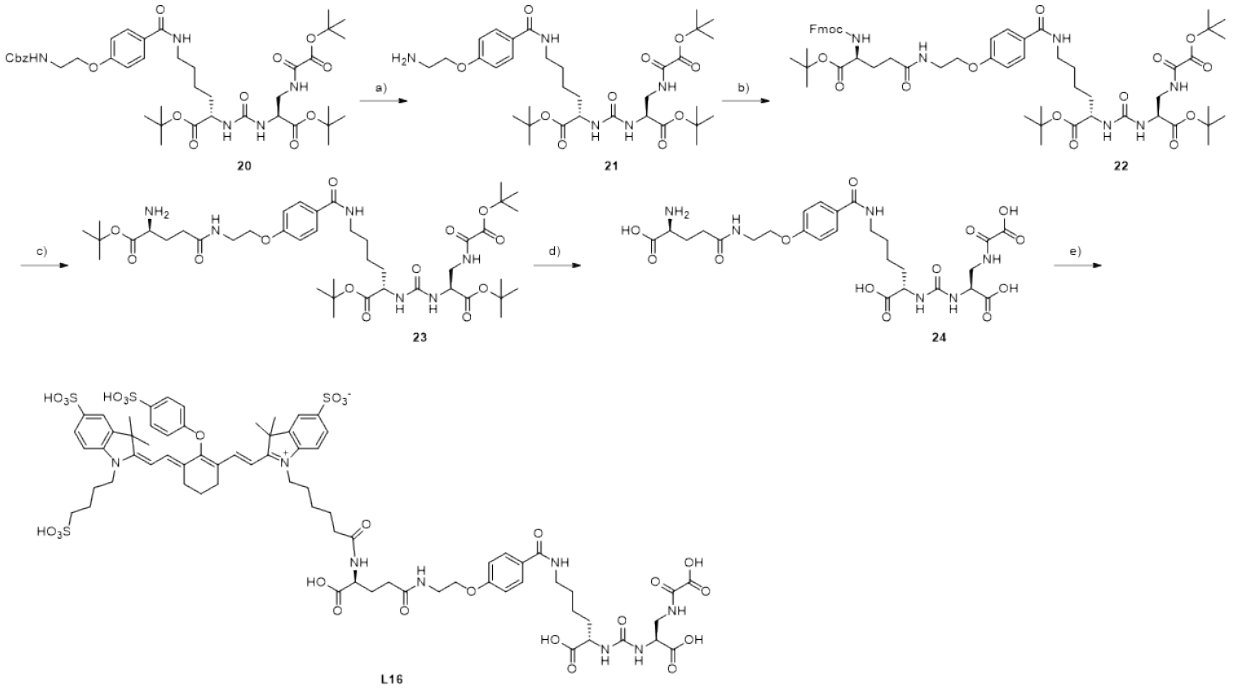
Reagents and condition: a) H-Lys(Cbz)-*t*-Bu, triphosgene, Et_3N, CH_2Cl_2, -10℃, 72% yield; b) Piperidine, DMF, r.t., 4 h; c) agents as specified in experimental section; d) CF_3COOH, r.t., 5 h, 35% overall yield from **13**.

Scheme 3: The synthesis of **L6-L15**



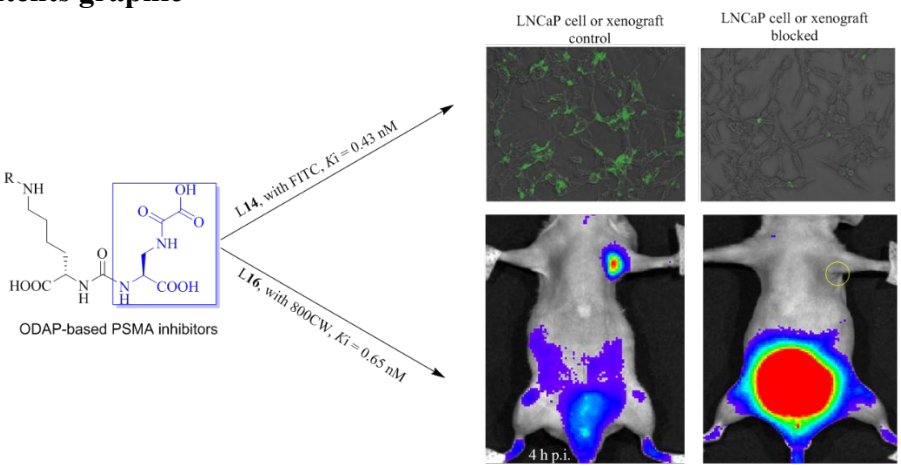
Reagents and condition: a) tert-butyl 2-chloro-2-oxoacetate, Et_3N, CH_2Cl_2, r.t., 10 h, 78% yield; b) Pd/C, H_2, MeOH, r.t. 12 h, 93%; c) H-Lys(Cbz)-*t*-Bu, triphosgene, Et_3N, CH_2Cl_2, -10°C, 5 h, 72% yield; d) Pd/C, H_2, MeOH, r.t. 12 h, without further purification; e) Conditions specified for each ligand in experimental section; f) CF_3COOH, r.t., 5 h, 33-60% overall yield from **17**; g) 4-bromobenzaldehyde, $NaBH_3CN$, AcOH, MeOH, r.t. 15 h, 61% yield; h) AcCl, Et_3N, CH_2Cl_2, r.t. 5 h, 60% yield; i) CF_3COOH, r.t., 5 h, 58% yield.

Scheme 4: The synthesis of optical imaging agent **L16**



Reagents and condition: a) Pd/C, H_2, MeOH, r.t. 12 h; b) Fmoc-Glu-*t*-Bu, HATU, DIPEA, DMF, r.t. 5h, 60% yield from **22**; c) piperidine, DMF, r.t., 2 h, 90% yield; d) CF_3COOH, r.t., 5 h, 47% yield; e) IRDye 800CW NHS Ester, Et_3N, DMF, r.t. 2 h in dark, 40% yield.

Table of Contents graphic



A novel ODAP-based PSMA ligand was discovered with high potency and applied successfully for cell and tumor imaging.