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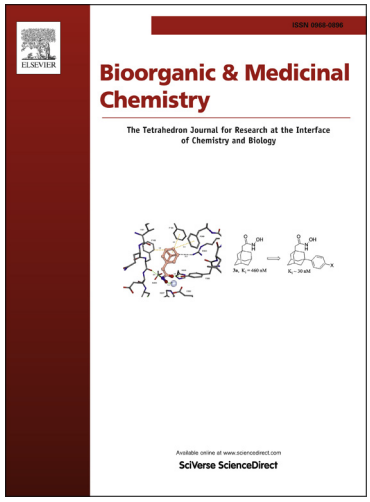

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Graphical Abstract

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Peptide conjugates of 4-aminocyclophosphamide as prodrugs of phosphoramide mustard for selective activation by prostate-specific antigen (PSA)

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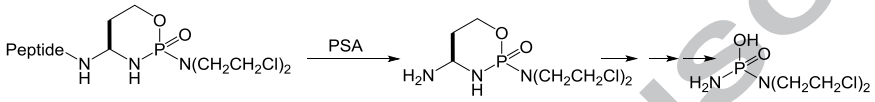

Peptide = *Succinyl*-Ser-Lys-Leu-Gln-
Succinyl-His-Ser-Ser-Lys-Leu-Gln-
Glutaryl-Hyp-Ala-Ser-Chg-Gln-

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ABSTRACT

In our continued effort to develop prodrugs of phosphoramide mustard, conjugates of 4-aminocyclophosphamide (4-NH_2-CPA) with three PSA-specific peptides were synthesized and evaluated as substrates of PSA. These include conjugates of *cis*-(2*R*, 4*R*)-4-NH_2-CPA with a tetrapeptide Succinyl-Ser-Lys-Leu-Gln-OH, a hexapeptide Succinyl-His-Ser-Ser-Lys-Leu-Gln-OH, and a pentapeptide Glutaryl-Hyp-Ala-Ser-Chg-Gln-OH. These conjugates were cleaved by PSA efficiently and exclusively after the expected glutamine residue to release 4-NH_2-CPA, the activated prodrug form of phosphoramide mustard. The cleavage was most efficient for the pentapeptide conjugate **3** (Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-CPA), which showed a half-life of 55 min with PSA, followed by the hexapeptide conjugate **2** (Succinyl-His-Ser-Ser-Lys-Leu-Gln-NH-CPA) and the tertrapeptide conjugate **1** (Succinyl-Ser-Lys-Leu-Gln-NH-CPA) with half-lives of 6.5 and 12 h, respectively. These results indicate a potential of the conjugate **3** as an anticancer prodrug of phosphoramide mustard for selective PSA activation.

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1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second-leading cause of cancer-related death in both American and European men.[1,2] Currently the castration-resistant prostate cancer remains a fatal disease, but new therapeutics have been developed or under development to improve survival.[3] Chemotherapy has primarily been used for the palliation of symptoms in patients who developed androgen-refractory prostate cancer. [4] Recently, several chemotherapeutic treatments also showed a survival benefit in the clinic and some have been approved by the U.S. FDA.[3] Because of the common dose-limiting systemic toxicity of most cancer drugs, the prodrug approach has been an attractive strategy in chemotherapy. Several biological features characteristic of prostate cancer cells have been extensively explored for this purpose.[5,6] In particular, prostate-specific antigen (PSA) has been considered an attractive target because of the following characteristics:[5] 1) it is a serine protease selectively expressed in prostate tissue and carcinoma in prostate cancer patients; 2) PSA is expressed at a high level (up to mg/g) in prostate carcinoma;[7] 3) the enzymatic activity of PSA is confined to the prostate and prostate-derived cancer cells; those leaked into systemic circulation are inactivated by plasma protease inhibitors. A number of prodrugs using PSA as the activating enzyme have been reported and some have entered clinical trials.[6]

Cyclophosphamide is an alkylating antitumor agent with activity against a broad spectrum of human cancers including slow-growing tumors.[8] However, the clinical application of cyclophosphamide is limited by its dose-related toxic side effects. This has resulted in considerable efforts in designing prodrugs that specifically release cytotoxic phosphoramide mustard at tumor sites.[9-11] We have recently proposed and demonstrated that 4-aminocyclophosphamide (4-NH_2-CPA) decomposes into phosphoramide mustard under physiological conditions and this feature enables its application as a prodrug moiety.[12,13] In an effort to develop phosphoramide mustard prodrugs which could selectively target PSA, recently we have reported the synthesis of tetrapeptide (Cbz-Ser-Ser-Phe-Tyr-) conjugates of 4-NH_2-CPA and the stereoselectivity of PSA-mediated cleavage of these conjugates.[14] In a continued effort, herein we wish to report the synthesis and the enzyme-catalyzed activation of three peptide conjugates of 4-NH_2-CPA which were designed for selective activation by PSA.

2. Design

PSA is a serine protease with chymotrypsin-like activities. The substrate specificity of PSA has been well characterized and several polypeptide sequences have been identified with specific activity toward PSA.[15-18] In particular, two short peptide sequences, the hexapeptide -His-Ser-Ser-Lys-Leu-Gln- (HSSKLQ) and a pentapeptide -Hyp-Ala-Ser-Chg-Gln-, showed high affinity and specificity toward PSA and both have been used to conjugate with various cytotoxins as prodrugs for treatment of prostatic cancer.[19-21] For a proof of concept study, we attached these two peptides with 4-NH_2-CPA in conjugates **2** and **3** as shown in Figure 1. The two capping groups, succinyl and glutaryl groups, have been used in the literature for the same purpose and were chosen because they could help with solubility of the resulting conjugates. In addition, the tetrapeptide sequence -Ser-Ser-Lys-Gln- (SKLQ) was reported to have less water solubility but comparable PSA substrate activity to the hexapeptide HSSKLQ.[16] This tetrapeptide was incorporated in the conjugate **1** in our design as the shortest peptide conjugate of 4-NH_2-CPA with specific and efficient PSA cleavage. Conjugates **1**-**3** were expected to be cleaved selectively by PSA after the peptide C-terminal glutamine residue to release 4-NH_2-CPA which would spontaneously decompose into cytotoxic phosphoramide mustard as shown in our previous publications.[12,13] The stereoselectivity of PSA on the 4-NH_2-CPA portion has previously been examined by the PSA assay of tetrapeptide-conjugated isomers of 4-NH_2-CPA with the *cis*-(2*R*,4*R*)-isomer showed the highest substrate activity for PSA.[14] Therefore, only this configuration was incorporated in our design. Compared to the natural products which have been attached to the same peptides for selective PSA activation,[19-21] 4-NH_2-CPA is a much smaller molecule so that it won't need extra linker such as Leu for efficient PSA cleavage.

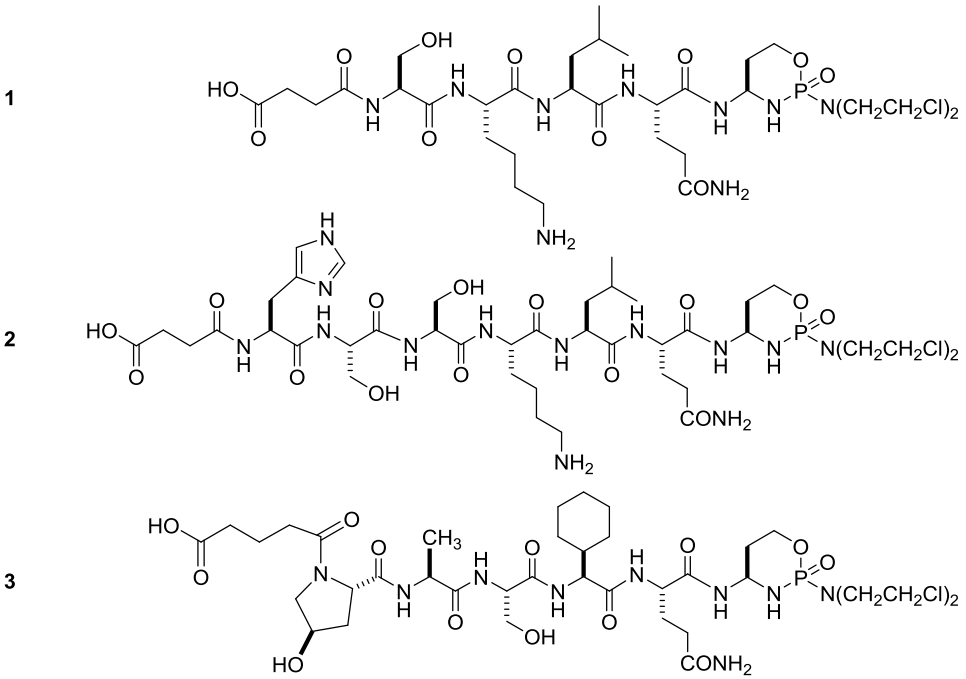


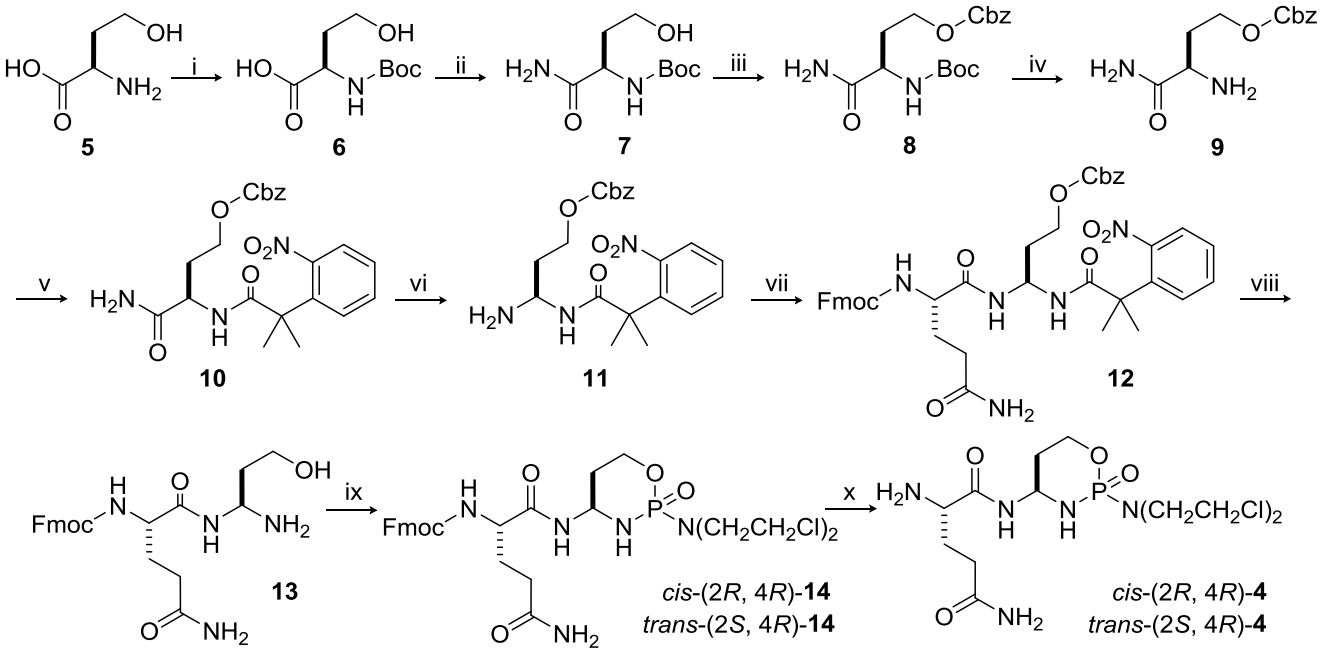
Figure 1. Peptide-conjugates of 4-NH_2-CPA designed for selective activation by PSA: Succinyl-Ser-Lys-Leu-Gln-NH-CPA (**1**), Succinyl-His-Ser-Ser-Lys-Leu-Gln-NH-CPA (**2**), and Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-CPA (**3**).

In addition, this relatively small molecule may also exhibit advantages in cell and tissue permeation which is an important factor in the chemotherapy of solid tumors.

3. Chemistry

Because the three peptide conjugates of 4-NH_2-CPA shared the same glutamine residue at the P1 subsite, the synthetic routes to these conjugates were designed using H-Gln-NH-CPA (**4**) as the common intermediate to couple with the corresponding peptide segments. Synthesis of **4** was outlined in Scheme 1. Previously, we synthesized phenylalanine and tyrosine conjugates of 4-NH_2-CPA by coupling the amino acid with homoserine first and then using the homoserine residue to construct the 4-NH_2-CPA moiety through a *gem*-diamine intermediate.[12,14] However, the same strategy could not be applied to the synthesis of the glutamine conjugate **4** because that would require protection of the side amide chain of the glutamine residue in generating the key intermediate of *gem*-diamine by Hofmann rearrangement reaction. Not only the protection strategies of a primary amide group are fairly limited,[22,23] but the subsequent deprotection after the yield-limiting cyclization step could also lead to significant decrease in overall yield. To solve this issue, we developed a new strategy to synthesize **4** using the 2,2-dimethyl-2-(o-nitrophenyl)acetyl (DMNA) group which is an assisted cleavage protecting group for amines previously developed in our group.[24] As shown in Scheme 1, instead of coupling glutamine to homoserine first, we protected the amino group of homoserine with the DMNA group and the coupling of glutamine was done after the formation of the *gem*-diamine intermediate. The DMNA protecting group showed two advantages in this new synthetic strategy: it could protect the *gem*-diamine as a stable acyl amide and be easily removed by acidic hydrogenolysis after the coupling of glutamine with the *gem*-diamine.

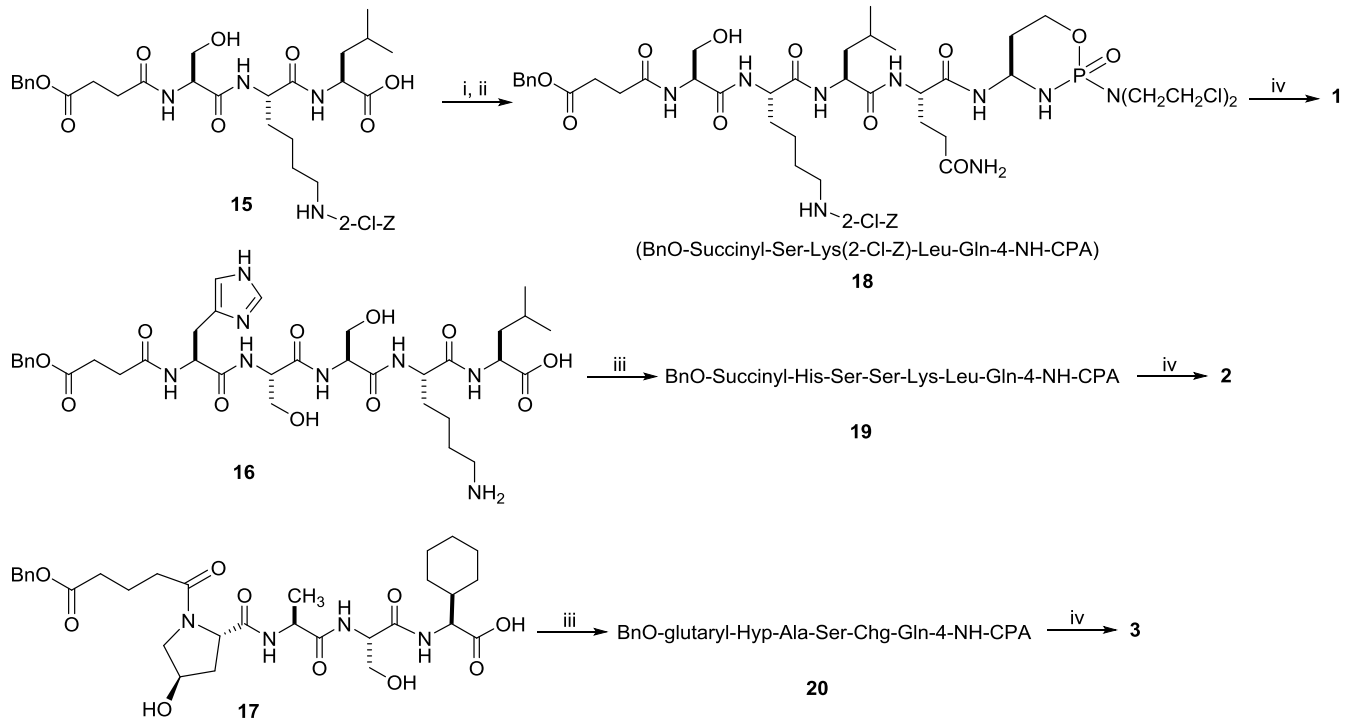
As shown in Scheme 1, synthesis of **4** started from D-homoserine (**5**) which was first protected with a Boc group by reacting with $(Boc)_2O$. The resulting Boc-D-Hse-OH (**6**) was converted to the corresponding amide 7 by treating the HOBt/EDC activated ester of **6** with saturated ammonium hydroxide.[25] The hydroxyl group in **7** was protected as a carbonate in **8** by reacting with Cbz-Cl/pyridine. The Cbz group was chosen because it could be removed together with the DMNA group under the same hydrogenation conditions. After removal of the Boc group by treatment with 30% TFA, the amino group of **9** was protected with the DMNA group using DMNA-Cl/DIEA. Synthesis of the reagent DMNA-Cl has been reported in our previous publications.[13,24] The BTI-mediated Hofmann rearrangement of **10** gave DMNA-protected *gem*-diamine **11**. Coupling of **11** with Fmoc-Gln-OH was done by using the HBTU-activated ester of Fmoc-glutamine to give the protected *gem*-diamine derivative **12**. The base-sensitive Fmoc instead of the acid-sensitive Boc group was chosen as the protecting group for the glutamine residue because the final 4-NH_2-CPA conjugates are labile to acid-catalyzed epimerization reaction.[26] In addition, the UV absorption of Fmoc would greatly facilitate the column separation of the intermediates in the following steps. Due to limited solubility of Fmoc-glutamine in CH_2Cl_2 or THF, the coupling reaction was done in N-methyl-2-pyrrolidone (NMP). The reaction was catalyzed by adding DIEA. Fmoc was found to be stable under these conditions with no deprotection products including fluorenyl methylene and its adducts observed on LC-MS. The Cbz and DMNA protecting groups in **12** were removed by hydrogenolysis over 10% Pd-C in 10% acetic acid/methanol. The Fmoc group was not affected under these conditions. The resulting Fmoc-Gln-gHse-NH_2 (**13**) was cyclized with bis(2-chloroethyl)phosphoramidic dichloride in the presence of TEA in THF with NMP as a cosolvent to help the solubility of **13**. Flash silica gel column chromatography afforded the faster-eluting diastereoisomer *cis*-(2*R*,4*R*)-**14** in 12% yield and the slower-eluting diastereoisomer *trans*-(2*S*,4*R*)-**14** in 6% yield. The degradation product Fmoc-Gln-NH_2 from **13** was also isolated in



Scheme 1. Synthesis of diastereomers of H-Gln-NH-CPA (**4**) from D-homoserine. Reagents and conditions: (i) Boc_2O, 1 M $KHCO_3$, THF, rt; (ii) HOBt, EDC, THF, rt, then sat'd NH_3 (aq), 46% (2-steps); (iii) Cbz-Cl, Pyridine, CH_2Cl_2, rt, 83%); (iv) 30% TFA, CH_2Cl_2, rt; (v) DMNA-Cl, DIEA, DMF, 75% (2-steps); (vi) BTI, CH_3CN/H_2O (1/1), ~100%; (vii) Fmoc-Gln-OH, HBTU, DIEA, DMF, rt, 76%; (viii) H_2, 10% Pd-C, 10% HOAc/MeOH, rt, 87%; (ix) n-BuLi, HMPA, THF, <-70 °C for 2 h, then $Cl_2PON(CH_2CH_2Cl)_2$, TEA, rt, 48 h; 12% for *cis*-**14** and 6% for *trans*-**14**; x) DEA, DMF, 91% for *cis*-**4** and 95% for *trans*-**4**.

18% yield. Other factors resulting in the low yields could include the low nucleophilicity of gemdiamines and the competing intermolecular cyclization.[12] The *cis*- and *trans*- configurations of ***14*** were conveniently assigned according to their chromatographic behavior, ^{1}H and ^{31}P chemical shifts.[12,13] The Fmoc group was found fairly stable in the presence of TEA and minimal side products due to the removal of the Fmoc group were observed on LC-MS. To remove the Fmoc protecting group, the two isomers of **14** were treated with diethyl amine (DEA) separately to obtain the corresponding two isomers of **4** in nearly quantitative yields. Compared to piperidine with a high boiling point (106 °C), DEA has a low boiling point (55.5 °C) and thus could be removed easily afterwards by rotary evaporation under reduced pressure.

The three peptide segments **15**-**17** were synthesized using solid phase synthesis on SASRIN resin.[27] The *N*-terminal capping group was introduced using the corresponding mono-benzyl ester of succinic acid or glutaric acid. The side chain amino group of Lys residue in **15** and **16** was protected by a 2-chlorobenzoxycarbonyl group (2-Cl-Z). Synthesis of the peptide conjugates **1**-**3** from *cis*-(2*R*,4*R*)-**4** were outlined in Scheme 2. All reactions were monitored by HPLC-MS. The peptide segment **15** was activated to a HOSu ester which was subsequently reacted with *cis*-(2*R*,4*R*)-**4** in the presence of DIEA in DMF. After the reaction was completed, the peptide conjugate **18** was isolated in 55% yield by flash silica gel column chromatography. The benzyl succinate in **18** was found to be unstable under the weak acidic silica gel conditions during column chromatographic separation, resulting in the formation of significant side products due to ester exchange, lactonization or hydrolysis. The benzyl group and 2-Cl-Z group in **18** were removed by hydrogenolysis over 10% Pd-C in methanol, affording the target compound **1** in 50% yield after preparative HPLC purification. Coupling of the peptide segment **16** with *cis*-(2*R*,4*R*)-**4** was first conducted by using the same HOSu/DCC activation method. However, to our surprise, we did not detect any desired product or any major side product when we monitored the reaction by LC-MS. The same result was also observed when HOSu/DCC was replaced by HOBt/EDC. We rationalize that this unexpected result could be due to the excess coupling agent DCC or EDC in the reaction mixture which might trigger the reaction of the imidazole group in histidine residue with the peptide carboxylic acid, and subsequently, the imidazole-activated carboxylic acid could react with the hydroxyl groups in the peptide sequence, resulting in complicated intermolecular coupling reactions. Accordingly, we switched to HBTU/DIEA activation method to avoid introducing excess coupling agent into the reaction. As we expected, the coupling reaction between **16** and *cis*-(2*R*,4*R*)-**4** was completed in 3 h as shown by LC-MS and the conjugated product **19** was identified as the major product on LC-MS. The conjugate **19** was isolated in 60% yield by flash silica gel column chromatography. The benzyl group and 2-Cl-Z group in **19** were removed by hydrogenolysis in DMF in the same step, affording the target compound **2** in 36% yield after preparative HPLC purification. Replacement of methanol with DMF as the solvent for the hydrogenolysis reaction was due to the limited solubility of **19** in methanol and to avoid the ester-exchange reaction of the terminal succinyl benzyl ester with methanol. Similarly, the peptide segment **17** was coupled with *cis*-(2*R*,4*R*)-**4** using HBTU/DIEA in DMF. The conjugated product **20** was isolated in 81% yield by crystallization from a mixture of ethyl acetate and 1 M $NaHCO_3$ (1:1). Because compound **20** showed poor solubility in methanol and isopropanol, the final hydrogenolysis step was conducted in DMF. The target compound **3** was isolated in 75% yield by crystallization from the mixture of ethyl ether and methanol after removal of DMF in vacuo.



Scheme 2. Synthesis of the peptide conjugates **1**-**3** from *cis*-(2*R*,4*R*)-**4**. Reagents and conditions: (i) HOSu, DCC, DME, rt, overnight, 65%; (ii) DIEA, DMF, rt, 55%; (iii) HBTU, DIEA, DMF, rt, 60% for **19**; 81% for **20**; (iv) H_2, 10% Pd/C, DMF, rt, 50% for **1**, 36% for **2**, 75% for **3**.

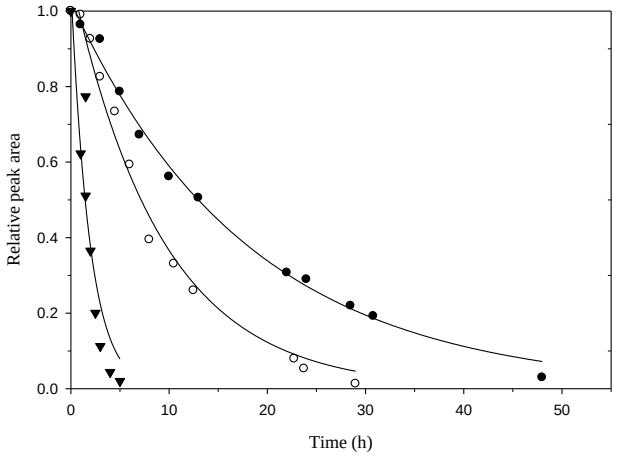


Figure 2. PSA digestion of the tetrapeptide conjugate **1** (•, $t_{1/2}$ = 12 h), the pentapeptide conjugate **2** (o, $t_{1/2}$ = 6.5 h), and the hexapeptide conjugate **3** (▼, $t_{1/2}$ = 55 min). Shown is the disappearance of the substrates.

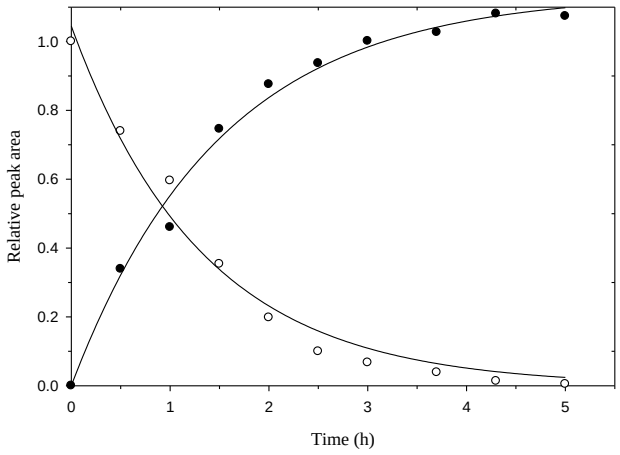


Figure 3. PSA digestion of the pentapeptide conjugate **3**. Shown are the disappearance of the substrate (o) and appearance of the peptide Glutaryl-Hyp-Ala-Ser-Chg-Gln-OH (•).

4. Substrate activity for PSA

The three peptide conjugates **1**-**3** of 4-NH_2-CPA were evaluated as substrates of PSA at an enzyme/substrate molar ratio of 1/100. All these conjugates were cleaved by PSA as the disappearance of the conjugates was observed by HPLC analysis (Figure 2). Half-lives of these conjugates under the assay conditions were calculated according to the disappearance of the substrate by nonlinear regression analysis. Among these three conjugates, the pentapeptide conjugate **3** had the shortest half-life of ~1 h, which is significantly shorter than the other two conjugates **1** and **2** with half-lives of 6.5 h and 12 h, respectively. Figure 2 indicated all three conjugates reached <10% in the presence of PSA, but the time interval ranged from 4 h for the pentapeptide conjugate **3** to 50 h for the tetrapeptide conjugate **1**. The 55 min half-life of the pentapeptide conjugate **3** was found comparable to the 30 min half-life of a peptide doxorubicin conjugate (Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox) which incorporated the same pentapeptide.[17] However, the PSA affinity of the peptide doxorubicin conjugate was enhanced by an additional two amino acids on the P' sites and the resulting dipeptide doxorubicin conjugate (H-Ser-Leu-Dox) upon proteolysis requires additional aminopeptidase cleavage to release the final cytotoxic species doxorubicin. In fact, several cytotoxic agents have been linked to peptides as prodrugs for selective PSA cleavage, but none of them could be linked directly to the C-terminus of the P sites with efficient PSA cleavage. Compared to these conjugates, the 4-aminocyclophosphamide conjugates have the advantage of releasing an intermediate that would undergo an efficient spontaneous chemical process to release the cytotoxic phosphoramide mustard directly after the proteolysis.

The enzyme incubated solutions of **1**-**3** were also examined using LC-MS. The expected peptide segments from **1** and **3** after PSA cleavage were observed unambiguously and they were identified as the only products shown on the UV chromatogram, indicating the exclusive cleavage after the glutamine residue by PSA. The half-life of **3** calculated from the appearance of the pentapeptide Glutaryl-Hyp-Ala-Ser-Chg-Gln-OH was found consistent with that from the disappearance of the substrate (Figure 3). The expected hexapeptide product from **2**, Succinyl-His-Ser-Ser-Lys-Leu-Gln-OH, was not observed using LC-MS, which might be due to the high polarity of this product eluted in the solvent front. Identification of the expected peptide products from these conjugates demonstrated that 4-NH_2-CPA was efficiently released from these conjugates upon PSA cleavage. The intact 4-NH_2-CPA molecule was not detected by LC-MS. Instead, a similar decomposition profile to products including 4-hydroxycyclophosphamide or aldophosphoramide was observed as described before.[13] These results indicate that these peptide conjugates of 4-NH_2-CPA could efficiently release cytotoxic phosphoramide mustard upon PSA cleavage.

5. Summary

Three PSA-specific peptide conjugates of 4-aminocyclophosphamide were successfully synthesized from D-homoserine and a new strategy has been developed to synthesize glutamine-conjugated 4-aminocyclophosphamides using a newly developed assisted cleavage protecting (DMNA) group for amines. All three conjugates demonstrated good substrate activity for PSA during the incubation with PSA and they were cleaved exclusively after glutamine residue by PSA. In particular, the pentapeptide conjugate, Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-CPA (**3**), showed the highest substrate activity for PSA with a half-life of 55 min at enzyme/substrate molar ratio 1/100, comparable to a doxorubicin conjugate which incorporated the same peptide together with a dipeptide linker. LC-MS analysis of the products from the incubation mixture of **3** with PSA suggested 4-aminocyclophosphamide was efficiently released from **3** upon proteolysis and quickly decomposed into phosphoramide mustard. These results suggested that the peptide conjugate **3** has a potential to be developed as an anticancer prodrug of phosphoramide mustard for selective PSA activation.

6. Experimental

General methods. Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon. Air- and moisture-sensitive materials were transferred by a syringe or cannula under an argon atmosphere. Solvents were either ACS reagent grade or HPLC grade. Tetrahydrofuran was dried over sodium/benzophenone. Triethylamine, dichloromethane, and ethyl acetate were dried over calcium hydride. Pyridine was dried over potassium hydroxide and distilled over calcium hydride. N,N-Dimethylformamide was dried over 4 Å molecular sieves at least for 1 week prior to use. Unless otherwise stated, all reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman precoated silica

gel plates. TLC plates were visualized using either 7% (w/w) ethanolic phosphomolybdic acid or 1% (w/w) aqueous potassium permanganate containing 1% (w/w) $NaHCO_3$. Flash silica gel column chromatography was performed using silica gel (Merck 230-400 mesh). Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous material, unless otherwise noted. All reagents were purchased at the commercial quality and used without further purification.

Melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer model 1600 series FTIR spectrometer using polystyrene as an external standard. Infrared absorbance is reported in reciprocal centimeters (cm^{-1}) with broad signals denoted by br. NMR spectra (^{1}H an d ^{13}C) were recorded at 200 MHz (^{1}H) or 50 MHz (^{13}C) on a 200 MHz Varian Gemini spectrometer using residual undeuterated solvents as the internal reference. ^{31}P NMR spectra were recorded at 121 MHz or 162 MHz using 5% H_3PO_4 in D_2O as an external standard. Chemical shifts are reported in parts per million (ppm) and coupling constants (J values) are given in hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High-resolution mass spectral (HRMS) data were obtained from the University of Kansas Mass Spectrometry Laboratory (Lawrence, KS). HPLC analysis were performed on an HP 1090 system equipped with a Phenomenex C18 column (5 µm, 4.6 mm × 250 mm) with gradient elution of 4-76% MeOH containing 0.1% formic acid in 15 min at a flow rate of 1 mL/min and a detection wavelength at 220 nm. HPLC purification was performed on a Beckmann system equipped with a Phenomenex C_{18} column (10 µm, 9.8 mm × 250 mm) with gradient elution of 4-76% MeOH containing 0.1% formic acid in 15 min at a flow rate of 5 mL/min and a detection wavelength at 220 nm.

Boc-D-Hse-OH (**6**). To a stirred solution of H-D-Hse-OH (2.39 g, 20 mmol) in 1 M $KHCO_3$ (26 mL) and ethanol (13 mL) at room temperature was added Boc_2O (4.81 mg, 22 mmol). The solution was stirred at room temperature overnight and ethanol was evaporated in vacuo. The aqueous residue was cooled in ice-water bath, acidified to pH 4 by adding 10% citric acid, extracted with ethyl acetate (50 mL x 4). The extractions were combined, washed with brine, dried over Na_2SO_4, and evaporated in vacuo to give a white solid (3.3 g, 75%); mp 123-124 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.70 (br s, 2H), 5.69 (d, J = 8.0 Hz, 1H), 4.44 (t, J = 8.8 Hz, 1H), 3.82-3.65 (m, 2H), 2.25-2.0 (m, 1H), 1.95-1.85 (m, 1H), 1.44 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.2, 156.6, 80.6, 58.6, 50.4, 35.4, 28.3; IR ($CDCl_3$) 3354.6 (br), 2978.9, 1774.8, 1683.5, 1532.0, 1368.9, 1160.5, 1064.2, 1012.7, 756.6 cm^{-1}; LC-MS (ESI-) 218.1 (100%).

Boc-D-Hse-NH_2 (**7**). To a solution of **6** (3.3 g, 15 mmol) in anhydrous THF (70 mL) was added HOBt (2.02 g, 15 mmol) and EDC (2.87 g, 15 mmol). The mixture was stirred at room temperature for 30 min. Saturated ammonia hydroxide solution (21 mL) was added dropwise as the reaction cleared. The reaction was stirred overnight after the addition was complete. Solvent was removed under vacuum, and the residue was taken up in ethyl acetate (200 mL), washed with saturated $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. Solvent was removed by evaporation to give a white solid. Flash silica gel column chromatography purification (CH_2Cl_2:CH_3OH, 20:1) gave a white solid (2.0 g, 61%); mp 119-110 °C; ^{1}H NMR (200 MHz, CD_3OD) δ 4.18 (dd, J = 8.8, 4.8 Hz, 1H), 3.66 (dd, J = 7.1, 5.4 Hz, 2H), 2.01-1.88 (m, 1H), 1.85-1.74 (m, 1H), 1.47 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.6, 156.6, 80.6, 58.8, 51.3, 36.3, 28.4; IR ($CDCl_3$) 3339.7 (br), 2977.4, 1674.2, 1513.7, 1367.5, 1252.2, 1167.8, 1053.9 cm^{-1}; LC-MS (ESI+) 256.9 $[M+K]^+$, 163.0, 119.0 (100%).

Boc-D-Hse(Cbz)-NH_2 (**8**). To a solution of **7** (975 mg, 4.47 mmol) in CH_2Cl_2 (20 mL) and pyridine (1 mL) at 0 °C was added dropwise Cbz-Cl (795 µL, 5.62 mmol). The solution was stirred at 0 °C for 30min and at room temperature for 6 h. After evaporation of solvent in vacuo, the residue was dissolved in EtOAc (100 mL), washed with 5% citric acid, saturated $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash silica gel column chromatography (CH_2Cl_2:CH_3OH, 50:1) to give a white solid (1.31 g, 83%); mp 130.5-131.5 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.42-7.34 (m, 5H), 6.39 (br s, 1H), 5.74 (br s, 1H), 5.32-5.29 (m, 1H), 5.18 (s, 2H), 4.29 (t, J = 6.2 Hz, 2H), 2.26-1.96 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.9, 155.7, 155.1, 135.2, 128.7, 128.4, 80.5, 69.8, 64.8, 51.4, 31.6, 28.4; IR ($CDCl_3$) 1742.5, 1674.7, 1267.3, 1164.4, 754.6, 700.0 cm^{-1}; LC-MS (ESI+) 375.0 $[M+Na]^+$, 338.0, 297.0 (100%), 253.0, 145.0.

N^{α}-DMNA-D-Hse(Cbz)-NH_2 (**10**). DMNA-OH (947 mg, 4.53 mmol) was dissolved in $SOCl_2$ (5 mL) and refluxed for 1 h. Evaporation of $SOCl_2$ in vacuo gave DMNA-Cl as a yellow oil which was used directly for the next step. Compound **8** (1.33 g, 3.78 mmol) was treated with 30% TFA in CH_2Cl_2 (30 mL). After evaporation of solvents in vacuo, the residue was pumped to dryness to give **9** which was dissolved in methylene dichloride (15 mL). To this solution at 0 °C was added DIEA (1.65 mL, 9.45 mmol) and a solution of DMNA-Cl in methylene dichloride (10 mL) in sequence. The reaction solution was stirred at room temperature for 3 h and then concentrated in vacuo. The residue was taken up in ethyl acetate (150 mL), washed with saturated $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. Flash silica gel column chromatography purification (CH_2Cl_2:CH_3OH, 20:1) gave a yellow solid (1.26 g, 75%); mp 55-56.5 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.74 (dd, J = 7.9, 0.6 Hz, 1H), 7.53-7.42 (m, 2H), 7.36-7.29 (m, 6H), 6.58 (br s, 1H), 6.38 (d, J = 7.2 Hz, 1H), 5.51 (br s, 1H), 5.07 (s, 2H), 4.49 (dd, J = 12.7, 7.0 Hz, 1H), 4.21 (t, J = 6.2 Hz, 2H), 2.13-1.97 (m, 2H), 1.58 (s, 3H), 1.55 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 173.1, 154.9, 149.4, 138.7, 135.2, 133.3, 128.7, 128.5, 128.2, 125.7, 69.9, 65.4, 51.0, 47.0, 30.7, 27.5, 27.4; IR ($CDCl_3$) 1745.8, 1673.6, 1528.6, 1359.5, 1265.6, 754.1 cm^{-1}; LC-MS (ESI+) 444.1 $[MH]^+$, 427.1 (100%).

N^{α}-DMNA-D-gHse(Cbz)-NH_2 (**11**). To a stirred suspension of 10 (1.24 g, 2.8 mmol) in 50 mL of acetonitrile and distilled water (1:1) was added bis(trifluoroacetate) iodobenzene (BTI) (1.25 g, 2.8 mmol). After 4 h of stirring under N_2 at room temperature, a clear colution resulted and no more starting material could be detected by TLC (CH_2Cl_2:CH_3OH, 20:1). The acetonitrile was removed under vacuum, and the aqueous layer was lyophilized. The resulting *gem*-diamine trifluoroacetate was triturated with Et_2O to give a yellow semi-solid (1.33 g, 100%); ^{1}H NMR (200 MHz, CD_3OD) δ 8.22 (d, J = 7.6 Hz, 1H), 7.87 (dd, J = 8, 0.8 Hz, 1H), 7.78-7.65 (m, 3H), 7.53-7.49 (m, 1H), 7.46-7.32 (m, 6H), 7.13 (t, J = 7.6 Hz, 1H), 5.24-5.13 (m, 3H), 4.26 (t, J = 5.6 Hz, 2H), 2.34-2.22 (m, 2H), 1.69 (s, 3H), 1.66 (s, 3H); ^{13}C NMR (50 MHz, CD_3OD) δ 177.4, 177.3, 154.3, 148.5, 137.5, 136.7, 135.0, 132.8, 129.5, 128.2, 127.7, 127.7, 127.6, 127.5, 124.8, 68.9, 62.4, 55.8, 55.7, 29.8, 25.9, 25.6; LC-MS (ESI+) 438.0 $[M+Na]^+$, 399.0, 247.0 (100%), 163.9.

Fmoc-Gln-gHse(Cbz)-NH-DMNA (**12**). To a suspension of Fmoc-Gln-OH (349 mg, 0.95 mmol) and HBTU (359 mg, 0.95 mmol) in DMF (4 mL) was added DIEA (166 µL, 0.95 mmol). The solution was stirred at room temperature for 30 min and added into a solution of **11** (266 mg, 0.95 mmol) in DMF (1 mL).

The reaction solution was stirred for 4.5 h at room temperature after the addition was complete and ethyl acetate (150 mL) was added. The solution was washed with 5% citric acid, saturated $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. Solvent was removed by evaporation in vacuo to give a yellow solid. Flash silica gel column chromatography purification (CH_2Cl_2:CH_3OH, 20:1) gave a yellow solid (456 mg, 76%); ^{1}H NMR (200 MHz, 1% CD_3OD/$CDCl_3$) δ 7.75-7.69 (m, 3H), 7.57-7.50 (m, 4H), 7.37-7.21 (m, 14H), 7.05 (d, J = 7.8 Hz, 1H), 5.40-5.36 (m, 1H), 5.05 (s, 2H), 4.30 (d, J = 6.6 Hz, 2H), 4.17-4.00 (m, 4H), 2.22-1.80 (m, 6H), 1.53 (d, J = 3.8 Hz, 6H); ^{13}C NMR (50 MHz, 1% CD_3OD/$CDCl_3$) δ 176.5, 176.1, 172.1, 156.7, 154.9, 149.0, 144.0, 143.8, 141.3, 138.5, 135.1, 133.4, 128.6, 128.4, 128.2, 127.8, 127.2, 125.7, 125.2, 120.0, 77.5, 69.8, 67.1, 64.5, 55.1, 54.4, 47.1, 47.0, 38.6, 32.2, 31.4, 28.2, 27.2; IR ($CDCl_3$) 1744.2, 1662.9, 1530.8, 1451.3, 1395.7, 1361.7, 1265.0, 755.0 cm^{-1}; LC-MS (ESI+) 788.2 $[M+Na]^+$, 766.2 $[MH]^+$, 558.1, 368.0 (100%).

Fmoc-Gln-gHse-NH_2 (**13**). To a solution of **12** (600 mg, 0.784 mmol) in 10 mL of acetic acid and methanol (1:9) was added 10% Pd-C. The solution was stirred under H_2 balloon at room temperature for 3 h. The catalyst was filtered through Celite and the filtrate was concentrated in vacuo. The residue was purified by flash silica gel column chromatography (CH_2Cl_2:CH_3OH, 20:1 to 5:1) to give a white solid (315 mg, 87%); mp 115 °C (dec.); ^{1}H NMR (200 MHz, CD_3OD) δ 7.84 (d, J = 1.2 Hz, 2H), 7.68 (d, J = 6.6 Hz, 2H), 7.45-7.30 (m, 4H), 5.22 (t, J = 6.6 Hz, 1H), 4.42 (d, J = 3.6 Hz, 1H), 3.39 (d, J = 3.6 Hz, 1H), 4.28-4.13 (m, 2H), 3.77-3.69 (m, 2H), 2.38 (t, J = 7.4 Hz, 2H), 2.10-1.92 (m, 4H); ^{13}C NMR (50 MHz, CD_3OD) δ 177.2, 172.8, 156.6, 143.4, 143.2, 140.7, 126.9, 126.3, 124.3, 119.1, 66.3, 56.2, 55.9, 53.8, 33.3, 30.1, 26.9; IR (KBr) 3298.6 (br), 1668.6, 1540.4, 1449.7, 1248.1, 1052.0, 739.4 cm^{-1}; LC-MS (ESI+) 463.1, $[M+Na]^+$, 424.1 (100%), 368.1.

Fmoc-Gln-4-NH-CPA (**14**). At 0 °C, to a stirred solution of bis(2-chloroethyl) phosphoramidic dichloride (185 mg, 0.693 mmol) in anhydrous THF (25 mL) under N_2 was added a solution of **12** (254 mg, 0.577 mmol) in NMP (25 mL) and TEA (195 μL, 1.39 mmol). The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 48 h. The solution was filtered to remove the white precipitate and evaporated at reduced pressure. The residue was purified by flash silica gel column chromatography (CH_2Cl_2:CH_3OH, 40:1) to give Fmoc-Gln-NH_2 and two diastereoisomers. The starting material was recovered in 15% yield. Fmoc-Gln-NH_2: white solid (38 mg, 18%); ^{1}H NMR (200 MHz, DMSO-d^6) δ 7.90 (d, J = 7.8 Hz, 2H), 7.74 (d, J = 7.5 Hz, 2H), 7.47-7.30 (m, 5H), 4.25 (s, 2H), 4.07 (br s, 2H), 3.94-3.80 (m, 2H), 3.36 (br s, 2H), 2.20-2.12 (t, J = 8.4 Hz, 2H), 1.95-1.74 (m, 2H); LC-MS (ESI+) 390.0, 368.0 $[MH]^+$ (100%); *cis-(2R,4R)*-**14** (the faster eluting isomer): white solid (44 mg, 12%); ^{1}H NMR (400 MHz, DMSO-d6) δ 8.44 (d, J = 3.4 Hz, 1H), 7.77 (d, J = 3.8 Hz, 2H), 7.63-7.60(m, 2H), 7.43 (d, J = 4.0 Hz, 1H), 7.30 (t, J = 3.6 Hz, 2H), 7.27 (s, 1H), 7.22 (t, J = 3.8 Hz, 2H), 6.64 (s, 1H), 5.13-5.11 (m, 1H), 4.99 (d, J = 8.6 Hz, 1H), 4.33 (dd, J = 9.2, 4.4 Hz, 1H), 4.15-4.07 (m, 3H), 4.05-3.98 (m, 1H), 3.87 (dd, J = 7.1, 3.6 Hz, 1H), 3.55 (t, J = 3.6 Hz, 4H), 3.23-3.14 (m, 4H), 2.05 (t, J = 4 Hz, 2H), 1.93-1.89 (m, 1H), 1.77-1.67 (m, 2H), 1.58 (d, J = 6.8 Hz, 1H); ^{13}C NMR (50 MHz, DMSO-d6) δ 174.5, 172.3, 156.6, 144.6, 144.6, 141.5, 128.4, 127.9, 126.1, 66.4, 63.8, 58.4, 54.6, 48.8 (d, J = 15.2 Hz), 47.4, 43.1, 32.1, 30.4, 28.7; ^{31}P NMR (162 MHz, DMSO-d^6) δ 9.15 (s); LC-MS (ESI+) 626.1 $[MH]^+$, 628.1, 406.0, 368.0 (100%); HRMS (FAB) m/z calc'd for $C_{27}H_{35}N_5O_6PCl_2$ $[MH]^+$ 626.1702, found 626.1721; *trans-(2S, 4R)*-**14** (The slower eluting isomer): white solid (20 mg, 6%); ^{1}H NMR (200 MHz, CD_3OD) δ 8.48 (d, J = 7.4 Hz, 1H), 7.90 (d, J = 7.2 Hz, 2H), 7.74 (d, J = 7.4 Hz, 2H), 7.54-7.30 (m, 6H), 7.03 (s, 1H), 6.78 (s, 1H), 5.24 (m, 1H), 4.94 (s, 1H), 4.25 (1H, covered by the solvent peak), 4.00-3.80 (m, 3H), 3.74-3.61 (m, 4H), 3.46-3.18 (4H, covered by the solvent peak), 2.50 (s, 2H), 2.12-2.09 (m, 2H), 1.88-1.60 (m, 4H); ^{13}C NMR (50 MHz, DMSO-d^6) δ 174.6, 174.4, 171.9, 156.7, 144.7, 144.6, 141.5, 128.4, 127.8, 126.1, 120.8, 66.5, 64.1, 58.3, 55.1, 49.1, 47.5, 32.3, 31.5, 28.5; LC-MS (ESI+) 626.1 $[MH]^+$, 628.1, 406.1, 368.0 (100%); HRMS (FAB+) m/z calc'd for $C_{27}H_{35}N_5O_6PCl_2$ $[MH]^+$ 626.1702, found 626.1680.

H-Gln-NH-CPA (**4**). *cis-(2R, 4R)*-**14** (24 mg, 0.038 mmol) or trans-(2R, 4R)-**14** (10 mg, 0.016 mmol) was treated with 10% diethyl amine (DEA) in DMF (3 mL) for 4.5 h. The solution was evaporated to dryness under reduced pressure at room temperature. The solid residue was washed with ethyl ether to give a white solid of *cis-(2R, 4R)*-**4** (13 mg, 91%) or *trans-(2S, 4R)*-**4** (14 mg, 95%); LC-MS (ESI+) 428.1, 426.1, 408.0, 406.0, 404.0 $[MH]^+$ (100%).

General methods for peptide synthesis. All of the peptides were synthesized by Merrifield solid-phase peptide synthesis protocols using a double-coupling protocol on a bubbler peptide synthesizer. The resins for the peptide assembly were 2-methoxy-4-alkoxybenzyl alcohol (SASRIN, 200-400 mesh) resin, purchased from Bachem (Torrance, CA), or 4-hydroxymethylphenoxy (WANG-type HMP) resin, purchased from Advanced Chemtech (Louisville, KY). Nα-Fmoc-protected amino acids of the L-configuration and reagents were purchased from Advanced Chemtech. Side-chain protection was Ser(But) and Lys(Cl-Z). The capping group was introduced via monobenzyl succinate or monobenzyl glutarate. C-terminal amino acid was loaded using DMAP/DIC protocol for SASRIN resin, and HOBt/DMAP/DIC protocol for WANG resin, and 3-fold excess of activated protected amino acid was used for each coupling and recoupling, using NMP as solvent. Following completion of the assembly on the resin support, the N-terminal Fmoc group was removed via the standard 25% piperidine/NMP protocol, followed by washing 5 times with NMP and introduction of N-terminal capping group. Deprotection and removal of the peptide from the resin support were effected using 90% TFA in CH_2Cl_2 for both SASRIN and WANG resin. The benzyl esters of the capping groups were not affected under these conditions. After removal of solvents under reduced pressure, the peptides were purified by preparative HPLC on reverse-phase radial compression C18 column (Waters, 15 μm, 100 Å, 5 x 30 cm). A step gradient was generated from 1 L each of successively increasing concentration of mobile phase (solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/CH_3CN). A flow rate of 9 mL/min was used to elute the peptide. Detection was performed by monitoring the UV absorbance at 220 nm. Homogeneous product fractions (>98% pure) were pooled and freeze-dried. The homogeneity of the peptides was demonstrated by analytical reverse phase HPLC using C_{18} column. Identities were confirmed by mass spectral analysis.

Monobenzyl Succinate. To a solution of succinic anhydride (1.0 g, 10 mmol) in DMF (4 mL) was added benzyl alcohol (0.94 mL, 9.09 mmol) and DIEA (1.93 mL, 11 mmol) at 0 °C. The reaction mixture was stirred at room temperature overnight and was evaporated in Speed-vac. The residue was dissolved in ethyl acetate (50 mL) and washed with saturated NaCl (10 mL x 2). The organic solution was extracted with aqueous $NaHCO_3$ (5 M, 5 mL x 3) and the aqueous extractions were combined, acidified to pH 4 by adding citric acid (5 M), extracted with ethyl acetate (30 mL x 3). The EtOAc extractions were combined, washed with saturated NaCl, and dried over Na_2SO_4. Solvent was

removed by evaporation in vacuo to give a white solid (1.66 g, 89%); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38 (s, 5H), 5.18 (s, 2H), 2.73-2.71 (m, 4H); LC-MS (ESI-) 207.0 (100%).

Monobenzyl glutarate. This compound was synthesized according to the same procedure described above from glutaryl anhydride (1.14 g, 10 mmol) and was purified by flash silica gel column chromatography (hexane:ethyl acetate, 6:1) as a white solid (1.49 g, 67%); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.37-7.30 (m, 5H), 5.12 (s, 2H), 2.48-2.38 (m, 4H), 2.04-1.93 (m, 2H); LC-MS (ESI-) 221.0 (100%).

Bn-succinyl-Ser-Lys(2-Cl-Z)-Leu-OH (**15**). The title compound was synthesized on SASRIN resin (1.0 g, 0.93 mmol) as described above and purified through preparative HPLC using a gradient of solvent B (30% for 1 min, 30-90% in 10 min, 90% for 3 min). The fractions containing the product were collected and lyophilized to dryness to give a white solid (538 mg, 82%); ^{1}H NMR (200 MHz, CD_3OD) δ 7.48-7.28 (m, 9H), 5.17 (s, 2H), 5.12 (d, J = 0.8 Hz, 2H), 4.43-4.37 (m, 3H), 3.85 (dd, J = 11, 5.4 Hz, 1H), 3.76 (dd, J = 11, 5.8 Hz, 1H), 3.14 (t, J = 6.2 Hz, 2H), 2.76-2.69 (m, 2H), 2.63-2.55 (m, 2H), 1.91-1.40 (m, 9H), 0.95 (d, J = 5.8 Hz, 3H), 0.91 (d, J = 5.8 Hz, 3H). ^{13}C NMR (50 MHz, CD_3OD) δ 173.8, 172.8, 172.6, 172.3, 170.7, 156.6, 135.5, 134.0, 132.2, 128.5, 127.6, 127.3, 127.2, 126.2, 65.7, 62.7, 61.0, 55.1, 52.7, 51.5, 50.2, 39.7, 39.6, 30.8, 30.4, 29.4, 28.5, 24.0, 22.1, 21.4, 19.9; LC-MS (ESI+) 705.20 (100%).

Bn-succinyl-His-Ser-Ser-Lys(2-Cl-Z)-Leu-OH (**16**). The title compound was synthesized on SASRIN (1.08 g, 1.1 mmol) as described above and purified through preparative HPLC using a gradient of solvent B (30% for 1 min, 30-90% in 10 min, 90% for 3 min). The fractions at 8.5-9.8 min were collected and lyophilized to dryness to give a white solid (459 mg, 45%); LC-MS (ESI+) 929.6 $[MH]^+$ (100%).

Bn-glutaryl-Hyp-Ala-Ser-Chg-OH (**17**). The title compound was synthesized on SASRIN resin (1.08 g, 1.1 mmol) as described above and purified by preparative HPLC using a gradient of solvent B (30% for 1 min, 30-70% in 12 min, 90% for 3 min). The fractions at 8.8-9.8 min were collected and lyophilized to dryness to give a white solid (403 mg, 58%); LC-MS (ESI+) 633.2 $[MH]^+$ (100%).

Bn-succinyl-Ser-Lys(2-Cl-Z)-Leu-Gln-NH-CPA (**18**). To a solution of 15 (18 mg, 0.0256 mmol) and HOSu (3 mg, 0.0256 mmol) in DME (0.5 mL) at 0 °C was added a solution of DCC (5.3 mg) in DME (0.5 mL). The solution was stirred at room temperature for 6 h and was evaporated in vacuo. The solid residue was recrystallized from 2-propanol to give the HOSu ester of 15 as a white solid (13.3 mg, 65%); LC-MS (ESI+) 802.2 $[MH]^+$ (100%). This activated ester intermediate (13 mg, 0.016 mmol) and *cis-(2R, 4R)*-**4** (6.6 mg, 0.016 mmol) were dissolved in DMF (1 mL) at room temperature. After addition of DIEA (3.5 μL, 0.02 mmol), the reaction mixture was stirred at room temperature for 6 h and ethyl acetate (30 mL) was added. The ethyl acetate solution was washed with 5% citric acid, saturated $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. Solvent was removed by evaporation to give a white solid (8.8 mg, 55%); LC-MS (ESI+) 1002, 1000 $[MH]^+$, 742.3 (100%), 744.3, 725.2, 727.2; HRMS (FAB+) m/z calc'd for $C_{39}H_{62}N_9O_{13}PCl_3$ $[MH]^+$ 1000.3270, found 1000.3263.

Succinyl-Ser-Lys-Leu-Gln-NH-CPA (**1**). To a solution of 18 (8.0 mg, 5.7 x 10-3 mmol) in methanol (5 mL) was added 10% Pd-C. The solution was stirred under H_2 balloon at room temperature for 30 min. After removal of catalyst by filtration through Celite® 545, the filtrate was concentrated in vacuo. The white solid residue was dissolved in 50% acetonitrile-water and purified by preparative HPLC using a gradient of solvent B (30-90%B in 15 min) to give a white solid (3.3 mg, 50%); LC-MS (ESI+) 832.2 $[MH]^+$ (100%), 834.2, 836.2, 574.1; HRMS (FAB+) m/z calc'd for $C_{31}H_{57}N_9O_{11}PCl_2$ $[MH]^+$ 832.3292, found 832.3287.

Bn-succinyl-His-Ser-Ser-Lys(2-Cl-Z)-Leu-Gln-NH-CPA (**19**). To a stirred solution of **16** (7.0 mg, 7.6 x 10-3 mmol) and HBTU (2.9 mg, 7.6 x 10-3 mmol) in DMF (0.5 mL) was added DIEA (2.6 μL, 1.5 x 10-2 mmol). After 15 min, the solution was added into a solution of *cis-(2R, 4R)*-**4** (3 mg, 7.6 x 10-3 mmol) in DMF (0.5 mL) and was stirred at room temperature for 2.5 h. The solution was poured into ethyl acetate (40 mL), washed with 5% citric acid, saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo. The white solid residue was washed with ethyl ether to give a white solid (6.0 mg, 60%); LC-MS (ESI+) 1340.2, 1338.1, 1336.0, 1318.3, 1316.2, 1314.2 $[MH]^+$, 475.3 (100%).

Succinyl-His-Ser-Ser-Lys-Leu-Gln-NH-CPA (**2**). To a solution of **19** (6.0 mg, 4.5 x 10-3 mmol) in DMF (4 mL) was added 10% Pd-C. The solution was stirred under a H_2 balloon at room temperature for 30 min. After removal of catalyst by filtration through Celite® 545, solvent was evaporated under reduced pressure at room temperature in Speed-Vac. The white solid residue was dissolved in 50% acetonitrile-water and was purified by preparative HPLC using a gradient of solvent B (10% for 2 min, 10-90% in 15 min, 90% for 3 min). The fractions at 10.5-11.5 min were collected and lyophilized to give a white solid (1.7 mg, 36%); LC-MS (ESI+) 1060.3, 1058.3, 1056.3 $[MH]^+$, 798.3 (100%); HRMS (FAB+) m/z calc'd for $C_{40}H_{69}N_{13}O_{14}PCl_2$ $[MH]^+$ 1056.4202, found 1056.4190.

Bn-glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-CPA (**20**). To a stirred solution of Bn-glutaryl-Hyp-Ala-Ser-Chg-OH (63 mg, 0.1 mmol) and HBTU (38.3 mg, 0.1 mmol) in DMF (1 mL) and methylene dichloride (3 mL) was added DIEA (17.4 μL, 0.1 mmol). The solution was stirred at room temperature for 20 min and added into a solution of *cis-(2R, 4R)*-**4** (0.1 mmol) and DIEA (17.4 μL, 0.1 mmol) in DMF (2 mL). The reaction mixture was stirred at room temperature for 5 h. Solvents were evaporated under reduced pressure, and the solid residue was recrystallized from the mixture of 0.5 M $NaHCO_3$ and ethyl acetate (1:1) to give a white solid (82.5 mg, 81%); ^{31}P NMR (121 MHz, DMSO-d^6) δ 9.1 (s); LC-MS (ESI+) 1020.0, 1018.0, 760.3 (100%), 743.3, 597.1.

Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-CPA (**3**). To a solution of 20 (55 mg, 0.054 mmol) in DMF (20 mL) was added 10% Pd-C. The solution was stirred under H_2 balloon at room temperature for 3 h. After removal of catalyst by filtration through Celite® 545, solvent was evaporated under reduced pressure at room temperature. The white solid residue was washed with ethyl ether and recrystallized from acetonitrile-water (1:1) to give a white solid (37.6 mg, 75%); LC-MS (ESI+) 930.2, 928.3 $[MH]^+$, 670.2 (100%), 653.3, 507.1; HRMS (FAB+) m/z calc'd for $C_{36}H_{61}N_9O_{13}PCl_2$ $[MH]^+$ 928.3504, found 928.3527.

Prostate-specific antigen (PSA) assay. PSA (100 μg, 2.45 mg/mL) was purchased from CALBIOCHEM (EMD Chemicals Inc). The substrate was mixed with PSA at a molar ratio of 100 to 1 in PSA buffer (50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1% Tween-20®). To a prewarmed PSA buffer solution (295 μL) at 37 °C was added 5 μL of a substrate stock solution in DMSO (10 mM). 245 μL of this solution was withdrawn and the reaction was initiated by adding PSA (5 μL). Aliquots (20 μL) were withdrawn at various time intervals and quenched with 5 μL of acetonitrile and store frozen prior to HPLC analysis. The half-life

was calculated based on the disappearance of the substrate and the appearance of the product if any.

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7. References and Notes

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