Bioorganic & Medicinal Chemistry Letters 19 (2009) 2587–2590

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and stereochemical preference of peptide 4-aminocyclophosphamide conjugates as potential prodrugs of phosphoramide mustard for activation by prostate-specific antigen (PSA)

Yongying Jiang [a,†], Robert S. DiPaola [b], Longqin Hu [a,b,*]

[a] Department of Pharmaceutical Chemistry, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA
[b] The Cancer Institute of New Jersey, New Brunswick, NJ 08901, USA

ARTICLE INFO

Article history:
Received 16 January 2009
Revised 3 March 2009
Accepted 4 March 2009
Available online 9 March 2009

Keywords:
Cyclophosphamide
Prostate-specific antigen
Proteolytic
Prodrug

ABSTRACT

In an effort to develop proteolytically activated prodrugs of phosphoramide mustard by prostate-specific antigen (PSA), a series of tetrapeptide (Cbz-Ser-Ser-Phe-Tyr)-conjugated 4-aminocyclophosphamide (4-NH_2-CPA) isomers were synthesized and evaluated as substrates of PSA. The cleavage of the conjugates by PSA were found to be stereoselective as only the two isomers with 4*R*-configuration were efficiently cleaved by PSA. The cis-(*2R,4R*)-isomer was the best substrate of PSA with a half-life of 12 min. LC/MS analysis of the incubation solution of this isomer with PSA suggests that 4-NH_2-CPA is released upon proteolysis and quickly degrades to cytotoxic phosphoramide mustard. These results clarified the stereochemical requirements of PSA on the peptide conjugates of 4-NH_2-CPA and demonstrated the potential of these conjugates as potential PSA-activated prodrugs targeting prostate cancer.

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, androgen deprivation therapy remains the mainstay of treatment for patients with metastatic prostate cancer. However, it eventually fails due to the development of hormone-refractory cancer.[3] Chemotherapy has primarily been used for the palliation of symptoms in patients with metastatic prostate cancer.[4] Recently, it has also been reported that chemotherapy improved overall survival in this group of patients.[5] Given the potency of many anticancer drugs against cancer cells, there has been a persisting interest to target these drugs selectively on cancer cells through a prodrug strategy to increase their therapeutic indices.[6] Several biological features characteristic to prostate cancer cells have been extensively explored for this purpose.[7–9] In particular, prostate-specific antigen (PSA) has been considered an attractive target because of the following characteristics:[9] (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;[10] (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.[9]

Cyclophosphamide is an alkylating antitumor agent with activity against a broad spectrum of human cancers including slow-growing tumors.[11] However, the clinical application of cyclophosphamide is limited due to its dose-related toxic side effects. This has resulted in considerable efforts in designing prodrugs that specifically release cytotoxic phosphoramide mustard at tumor sites.[12–14] 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.[15,16] In this Letter, we report the synthesis of two pairs of diastereomers of a tetrapeptide (Cbz-Ser-Ser-Phe-Tyr-) conjugated 4-aminocyclophosphamide (**1**) and the stereoselectivity of PSA-mediated cleavage of these conjugates. This is our initial report of our research toward developing phosphoramide mustard prodrugs utilizing PSA as the activating enzyme.

Previously, we have shown that four diastereomers exist for 4-NH_2-CPA and its conjugates (Fig. 1).[15–17] Results from the α-chymotrypsin digestion of *N*-phenylalanyl-4-aminocyclophosphamide isomers indicate that one of the cis isomers, the (*2R,4R*)-isomer, is the best substrate for α-chymotrypsin.[15] Although PSA is a member of the serine protease family and shares similar substrate specificity to chymotrypsin,[18] the stereochemical preference for PSA still needs to be confirmed. Among a number of amino acid sequences screened for PSA on its affinity and specificity, the se-

* Corresponding author. Tel.: +1 732 445 5291; fax: +1 732 445 6312.
E-mail address: LongHu@rutgers.edu (L. Hu).
† Present address: Hoffmann-La Roche Inc. Non-Clinical Safety, Nutley, NJ 07110, USA.

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.03.009

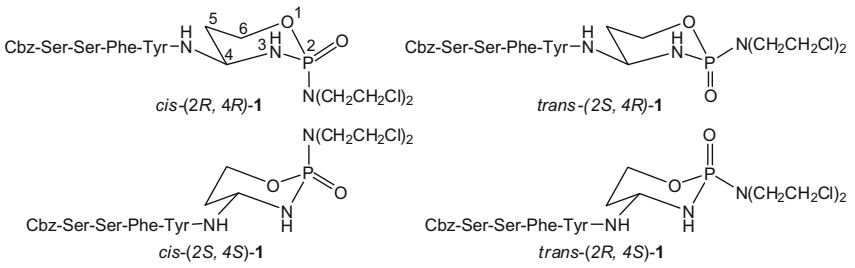


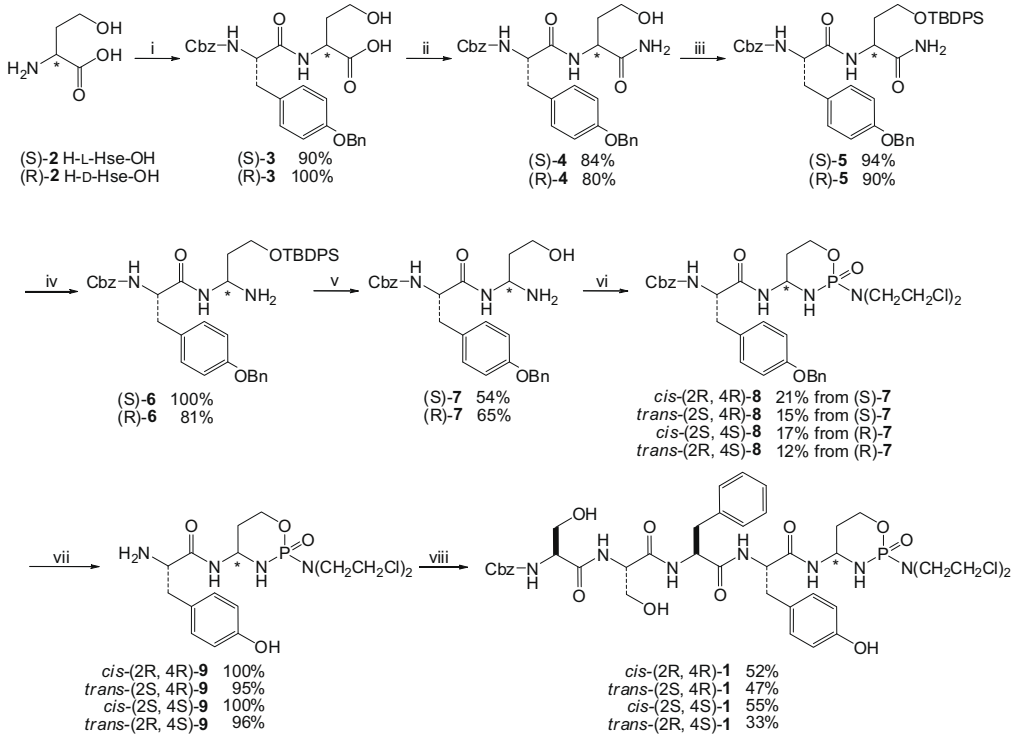
Figure 1. Four stereoisomers of Cbz-Ser-Ser-Phe-Tyr-NH-CPA (**1**).

quence SS(Y/F)Y↓S(G/S) was identified to be cleaved the fastest by PSA, with a catalytic efficiency as high as 3100 $M^{-1}s^{-1}$.[19] Therefore, we used this sequence and designed the tetrapeptide Cbz-Ser-Ser-Phe-Tyr-conjugate (**1**) of 4-NH_2-CPA in our initial efforts to screen for the stereochemical requirements of PSA-mediated cleavage of peptide conjugates of 4-NH_2-CPA (Fig. 1).

Synthesis of the four stereoisomers of **1** is outlined in Scheme 1. The two diastereomers of **1** with 4*R*-configuration were synthesized from L-homoserine (Hse) and the other two with *4S*-configuration were from D-Hse. Homoserine (**2**) was reacted with the preactivated HOSu ester of Cbz-Tyr(Bn)-OH to give the dipeptide **3** which was converted to its amide **4** through HOBt/EDC activation followed by aminolysis. The side chain hydroxyl group of **4** was protected with a TBDPS group using TBDPS-Cl/imidazole in DMF. BTI-mediated Hofmann rearrangement of the amide **5** was carried out in acetonitrile/water (1:1) with 20% DMF as a cosolvent, affording the *gem*-diamine derivative **6** in nearly quantitative yields. DMF was added to increase the solubility of **5** in the reaction solvents in order to expedite the reaction to reach completion within 4 h. Otherwise, the starting material would remain in the reaction mixture even after significantly extending the reaction time to overnight (~18 h). As a tertiary amide, DMF is stable under the Hofmann reaction conditions and has been used as a solvent for the BTI-mediated Hofmann rearrangement reaction.[20] The TBDPS group in **6** was removed by TBAF and the resulting γ-aminoalcohol **7** was used for the subsequent cyclization reaction.

Cyclization of (*S*)-**7** with bis(2-chloroethyl)phosphoramidic dichloride was initially carried out using TEA as a base in anhydrous EtOAc or THF at room temperature for 48 h. This condition has been generally used for the synthesis of cyclophosphamide analogs from γ-aminoalcohols.[17,21] Under this condition the faster-eluting diastereomer (*2R,4R*)-**8** (the cis isomer) was isolated in 16% yield and the slower-eluting diastereoisomer (*2S,4R*)-**8** (the trans isomer) in 5% yield after flash column chromatography. However, the degradation product from the starting *gem*-diamine, Cbz-Tyr(Bn)-NH_2, was isolated as the major side product in 25% yield. Assignment of configurations to these isomers has been extensively discussed in our previous publications.[15,17] The spectroscopic data used to assign the configuration of the four diastereomers of **8** are summarized in Table 1.

To improve the cyclization yield, we investigated several conditions which used NaH or *n*-BuLi in place of TEA. The stronger base



Scheme 1. Synthesis of diastereomers of Cbz-Ser-Ser-Tyr-Phe-NH-CPA (**1**) from D- or L-homoserine. Reagents and conditions: (i) Cbz-Tyr(Bn)-OSu, 1 M $KHCO_3$, THF, rt; (ii) HOBt, EDC, THF, rt, then satd NH_3 (aq); (iii) TBDPS-Cl, imidazole, DMF, rt; (iv) BTI, CH_3CN/H_2O (1/1), rt; (v) TBAF, THF, rt; (vi) *n*-BuLi, HMAP, THF, −70 °C for 2 h, then $Cl_2PON(CH_2CH_2Cl)_2$, TEA, rt, 48 h; (vii) H_2, Pd/C (10%), MeOH, rt; (viii) Cbz-Ser-Ser-Phe-OSu, DIEA, DME (dimethoxyethane).

Table 1
Analytical data of diastereomers of Cbz-Tyr-NH-CPA (**8**) and Cbz-Ser-Ser-Phe-Tyr-NH-CPA (**1**)

Compound	MS	NMR δ (ppm)		
		^{1}H (C-4, 1H)[c]	^{1}H (C-5, 2H)[c]	^{31}P[d]
Cis-(*2R,4R*)-**8**	663.1884[a]	5.38–5.08	2.30–1.80	9.8
Trans-(*2S,4R*)-**8**	663.1878[a]	5.41–5.30	2.10–1.70	13.4
Cis-(*2S,4S*)-**8**	663.1926[a]	5.30–5.10	2.02–1.50	9.2
Trans-(*2R,4S*)-**8**	663.1918[a]	5.45–5.25	1.90–1.44	11.0
Cis-(*2R,4R*)-**1**	894.2739[b]	5.34–5.20	2.21–1.80	8.8
Trans-(*2S,4R*)-**1**	894.2736[b]	5.45–5.30	2.18–1.90	12.1
Cis-(*2S,4S*)-**1**	894.2785[b]	5.40–5.25	2.20–1.76	10.7
Trans-(*2R,4S*)-**1**	894.2761[b]	5.40–5.30	2.00–1.70	13.3

[a] HRMS (FAB), *m/z* calcd for $C_{31}H_{38}C_{l2}N_4O_6P$ $[MH]^+$ 663.1906.
[b] HRMS (FAB), *m/z* calcd for $C_{39}H_{51}C_{l2}N7O_{11}P$ $[MH]^+$ 894.2761.
[c] The ^{1}H chemical shift was recorded at 200 MHz Varian Gemini spectrometer using residual undeuterated solvents as the internal reference.
[d] The ^{31}P chemical shift was recorded at 121 MHz using 5% H_3PO_4 in D_2O in a coaxial insert as an external standard.

was thought to facilitate the phosphorylation reaction of the hydroxyl group in **7** and thus the subsequent cyclization. One set of conditions using *n*-BuLi was found to increase the combined cyclization yield from 21% under the TEA conditions to 36%. Under the new conditions, (*S*)-**7** was treated with one equivalent of *n*-BuLi in a solvent mixture of HMPA and THF at −70 °C for 30 min before the addition of bis(2-chloroethyl)phosphoramidic dichloride. After 2 h at −70 °C, the reaction was allowed to warm up to room temperature and TEA (1.1 equiv) was added to complete the cyclization. The *n*-BuLi conditions produced (*2R,4R*) -**8** in 21% yield and (*2S,4R*)-**8** in 15% yield, which were 5–10% higher than that under the earlier TEA conditions. The side product, Cbz-Tyr(Bn)-NH_2, also increased slightly from 25% under the TEA conditions to 31%. The unreacted starting material was not recovered under both sets of conditions. The cis-(*2R,4R)*-**8** and trans-(*2S,4R*)-**8** isomers were readily separated by flash column chromatography on silica gel. However, we found that purification of the cis-(*2R,4R*)-**8** was often contaminated by Cbz-Tyr(Bn)-NH_2, the major side product of the cyclization reaction. This is because of their similar polarity on TLC. We finally resorted to BTI-mediated Hoffmann rearrangement to purify cis-(*2R,4R)*-**8**; this was accomplished by treating the mixture first collected with BTI to convert the amide side product Cbz-Tyr(Bn)-NH_2 to an unstable *gem*-diamine carbamate that would further decompose[22] before a second column purification. The Cbz and benzyl groups in **8** were removed by hydrogenolysis over 10% Pd–C, affording tyrosine-conjugated 4-aminocyclophosphamide **9** in nearly quantitative yields. The tripeptide Cbz-Ser-Ser-Phe-OH was synthesized through the standard solution peptide synthesis procedure using EDC/HOBt. For conjugation of the tripeptide with **9**, the HOSu ester of the tripeptide was synthesized using DCC/HOSu in DME and purified by recrystallization. The activated ester was then reacted with individual isomers of **9** in DMF and the reaction was monitored by LC/MS. The final product (**1**) was purified by flash column chromatography on silica gel and its identity was confirmed by its spectroscopic data. Other methods of conjugating the tripeptide with **8** using EDC/HOBt or HBTU/DIEA in DMF failed to give the desired peptide conjugate **1** as the major product. This unexpected result could be due to the involvement of the peptide side chain hydroxyl and phenol groups in the conjugation reaction. These side reactions were avoided by using the less active HOSu ester.

The four stereoisomers of **1** were evaluated as substrates of PSA by incubating each individual compound with purified PSA (Calbiochem, La Jolla, CA) in Tris/HCl buffer (50 mM, pH 8.0, 0.1% TWEEN-20®) at 37 °C. The enzyme/substrate molar ratio was 1/100. After the reaction was initiated by the addition of PSA, aliquots were withdrawn at various time intervals, quenched with acetonitrile, and analyzed by HPLC. The half-life of each compound under the reaction conditions was calculated based on the disappearance of the substrate. As shown in Figure 2, a significant difference of substrate activity exists between the stereomers of **1**. The diastereomers with 4*R*-configuration were efficiently hydrolyzed by PSA, with a half-life of 12 min for cis-(*2R,4R*)-**1** and 18 min for trans-(*2S,4R*)-**1**. On the other hand, the two diastereomers with the 4*S*-configuration were resistant to PSA hydrolysis. Under the same incubation conditions, no hydrolysis of cis-(*2S,4S*)-**1** was observed and only ~10% of trans-(*2R,4S*) -**1** was hydrolyzed after 60 min-incubation with PSA. These results demonstrate that the configuration of 4-NH_2-CPA is critical for the efficient hydrolysis of its peptide conjugate by PSA with strong preference for the (*2R,4R*)-configuration. Given that PSA is chymotrypsin-like serine protease, these results are consistent with our previous observation that α-chymotrypsin has the same stereochemical preference on the phenylalanine conjugate of 4-NH_2-CPA.[15] This is also consistent with the observation that substrate recognition by PSA is mediated by an extended binding pocket and subsites beyond subsite 1 (S1) play a critical role in defining its substrate specificity.[19] When the incubation mixture of cis-(*2R,4R*) -**1** with PSA was analyzed by LC/MS, the only product with UV absorption identified was the tetrapeptide Cbz-Ser-Ser-Phe-Tyr-OH, indicating that the PSA cleavage occurred only after tyrosine residue and 4-NH_2-CPA was released. However, we were not able to detect 4-NH_2-CPA using LC/MS. Instead, 4-hydroxylcyclophosphamide (or aldophosphoramide) was unambiguously identified. The degradation mechanism of 4-NH_2-CPA has been investigated and discussed in our previous publications.[15,16]

In summary, the four stereoisomers of Cbz-Ser-Ser-Phe-Tyr-conjugated 4-NH_2-CPA were synthesized stereospecifically from the D- and L-homoserine and were evaluated as substrates for PSA. Our results demonstrate that the configuration of 4-NH_2-CPA is critical for the proteolysis of the peptide conjugates by PSA. Only the stereoisomers with *4R*-configuration were efficiently hydrolyzed by PSA and cis-(*2R,4R*)-**1** was most efficiently cleaved by PSA with a half-life of 12 min followed by trans-(2*S,4R*)-**1**. LC–MS analysis of the proteolytic products of cis-(*2R,4R*)-**1** indicates that 4-NH_2-CPA was released upon proteolysis and disintegrates into cytotoxic phosphoramide mustard. These results suggest that peptide-conjugated 4-aminocyclophosphamides could potentially be used as prodrugs for proteolytic activation to improve the therapeutic effectiveness of cyclophosphamide in the treatment of cancer.

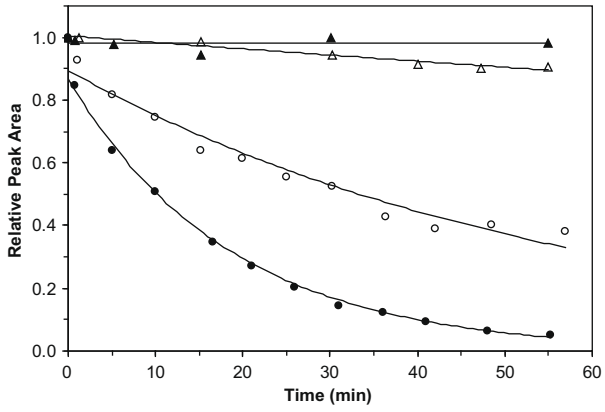


Figure 2. PSA digestion of compounds cis-(*2R,4R*)-**1** (●, $t_{1/2}$ = 12 min), trans-(*2S,4R*)-**1** (○, $t_{1/2}$ = 18 min), cis-(*2R,4S*)-**1** (▲, no cleavage within 60 min), and trans-(*2S,4S*)-**1** (△, <10% cleavage within 60 min). Shown is the disappearance of the substrates.

Acknowledgements

We gratefully acknowledge the financial support of grant SNJ-CCR 700-009 from the State of New Jersey Commission on Cancer Research and grant RSG-03-004-01-CDD from the American Cancer Society. This work was also partially supported by a pilot grant from the Gallo Prostate Cancer Center of the Cancer Institute of New Jersey that was supported by grant DAMD17-01-1-0755 from the Department of Defense.

References and notes

1. Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Murray, T.; Thun, M. J. *CA Cancer J. Clin.* **2008**, *58*, 71.
2. Ferlay, J.; Autier, P.; Boniol, M.; Heanue, M.; Colombet, M.; Boyle, P. *Ann. Oncol.* **2007**, *18*, 581.
3. Petrylak, D. P.; Tangen, C. M.; Hussain, M. H.; Lara, P. N., Jr.; Jones, J. A.; Taplin, M. E.; Burch, P. A.; Berry, D.; Moinpour, C.; Kohli, M.; Benson, M. C.; Small, E. J.; Raghavan, D.; Crawford, E. D. *N. Engl. J. Med.* **2004**, *351*, 1513.
4. Gilligan, T.; Kantoff, P. W. *Urol.* **2002**, *60*, 94.
5. de Wit, R. *BJU Int.* **2008**, *101*, 11.
6. Kratz, F.; Muller, I. A.; Ryppa, C.; Warnecke, A. *ChemMedChem* **2008**, *3*, 20.
7. Jayaprakash, S.; Wang, X.; Heston, W. D.; Kozikowski, A. P. *ChemMedChem* **2006**, *1*, 299.
8. Graeser, R.; Chung, D. E.; Esser, N.; Moor, S.; Schachtele, C.; Unger, C.; Kratz, F. *Int. J. Cancer* **2008**, *122*, 1145.
9. Atkinson, J. M.; Siller, C. S.; Gill, J. H. *Br. J. Pharmacol.* **2008**, *153*, 1344.
10. Lilja, H.; Abrahamsson, P. A.; Lundwall, A. *J. Biol. Chem.* **1989**, *264*, 1894.
11. Colvin, O. M. *Curr. Pharm. Des.* **1999**, *5*, 555.
12. Mulcahy, R. T.; Gipp, J. J.; Schmidt, J. P.; Joswig, C.; Borch, R. F. *J. Med. Chem.* **1994**, *37*, 1610.
13. Hernick, M.; Flader, C.; Borch, R. F. *J. Med. Chem.* **2002**, *45*, 3540.
14. Jain, M.; Kwon, C. H. *J. Med. Chem.* **2003**, *46*, 5428.
15. Jiang, Y.; Hu, L. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 517.
16. Jiang, Y.; Hu, L. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4059.
17. Jiang, Y.; Zhang, Z.; DePaola, R.; Hu, L. *Tetrahedron* **2007**, *63*, 10637.
18. Sokoll, L. J.; Chan, D. W. *Urol. Clin. North. Am.* **1997**, *24*, 253.
19. Coombs, G. S.; Bergstrom, R. C.; Pellequer, J. L.; Baker, S. I.; Navre, M.; Smith, M. M.; Tainer, J. A.; Madison, E. L.; Corey, D. R. *Chem. Biol.* **1998**, *5*, 475.
20. Yu, C.; Jiang, Y.; Liu, B.; Hu, L. *Tetrahedron Lett.* **2001**, *42*, 1449.
21. Jiang, Y.; Han, J.; Yu, C.; Vass, S. O.; Searle, P. F.; Browne, P.; Knox, R. J.; Hu, L. *J. Med. Chem.* **2006**, *49*, 4333.
22. Fletcher, M. D.; Campbell, M. M. *Chem. Rev.* **1998**, *98*, 763.