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Phenylalanyl-aminocyclophosphamides as model prodrugs for proteolytic activation: Synthesis, stability, and stereochemical requirements for enzymatic cleavage

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Abstract—4-Aminocyclophosphamide (4-NH_2-CPA, **7**) was proposed as a prodrug moiety of phosphoramide mustard. Four diastereomers of phenylalanine-conjugates of 4-NH_2-CPA were synthesized and their stereochemistry was assigned based on chromatographic and spectroscopic data. All diastereomers were stable in phosphate buffer but only the *cis*-(4*R*)-isomer of **15** was efficiently cleaved by α-chymotrypsin with a half-life of 20 min, which is much shorter than the 8.9 h to >12 h half-lives found for the other diastereomers. LC–MS analysis of the proteolytic products of *cis*-(4*R*)-**15** indicated that 4-NH_2-CPA was released upon proteolysis and further disintegrated to phosphoramide mustard. These results suggest the feasibility of using peptide-conjugated *cis*-(4*R*)-4-NH_2-CPA as potential prodrugs for proteolytic activation in tumor tissues.

Most anticancer agents suffer severe side effects from their marginal selectivity against cancer cells over healthy ones. The side effects limit the maximum dose of these agents to below the amount that can kill all viable tumor cells and the survived tumor cells could be more drug resistant and malignant.[1,2] Among strategies explored to increase the tumor selectivity of anticancer agents, tumor-targeted prodrug therapy has attracted much attention.[3–5] In this strategy, an inert prodrug form of an anticancer agent is selectively activated through a biochemical mechanism associated with tumor cells, such as hypoxic reduction, enzymatic action, or receptor recognition. Recently, several proteases have been identified to be unique for tumor growth and metastasis, including plasmin,[6,7] plasminogen activator,[8] matrix metalloproteinases,[9,10] and prostate specific antigen (PSA).[11,12] The specificity of these enzymes to tumor cells has provided novel opportunities that are being explored for tumor-targeted prodrug therapy.

Cyclophosphamide is one of the often used anticancer drugs in the clinic. Because of its activity against both cycling and non-cycling cells, it is one of the few anticancer agents effective in the treatment of slow-growing solid tumors.[13] However, the clinical use of cyclophosphamide is associated with a life-threatening side effect, hemorrhagic cystitis, in addition to bone marrow and GI tract toxicities which are commonly seen for most other anticancer agents. The existing knowledge of cyclophosphamide has facilitated the development of a variety of phosphoramide mustard prodrugs for tumor-targeted activation.[14–17] In the last few years, we have focused on improving the therapeutic efficiency of cyclophosphamide through the strategy of tumor-targeted enzyme prodrug therapy. In one approach, we developed a series of nitrobenzyl phosphoramide mustard prodrugs for *Escherichia coli* nitroreductase activation in enzyme prodrug therapy.[18,19] In this communication, we describe our efforts toward the conversion of cyclophosphamide into peptide-conjugated 4-aminocyclophosphamide prodrugs in the form of **6** for tumor-targeted proteolytic activation. To our knowledge, this is the first time that 4-aminocyclophosphamide (4-NH_2-CPA, **7**) is proposed as a prodrug moiety of phosphoramide mustard.

Keywords: Anticancer prodrugs; Cyclophosphamide; 4-Aminocyclophosphamide; Site-specific activation; Proteolytic activation; *gem*-Diamines; Phosphoramide mustard.
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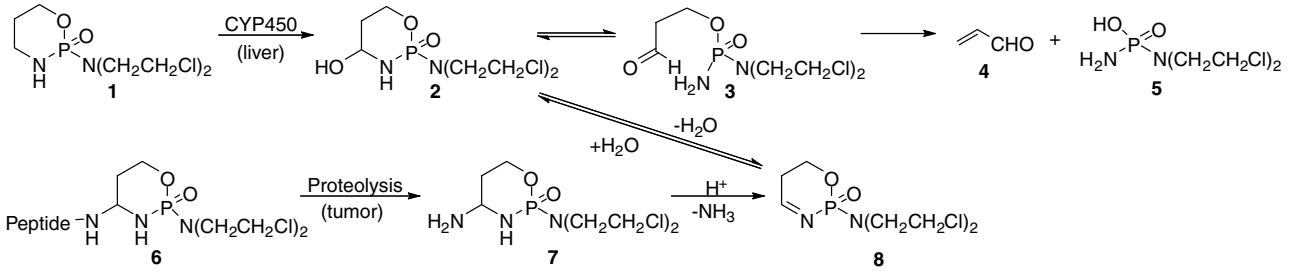
Cyclophosphamide (**1**) is a prodrug that requires activation by cytochrome P450 enzymes in the liver for its anticancer activity.[20,21] The enzymatic oxidation converts **1** to 4-hydroxycyclophosphamide (**2**) which generates acrolein (**4**) and phosphoramide mustard (**5**) after ring opening and β-elimination (Scheme 1).[20] Acrolein is responsible for the hemorrhagic cystitis associated with cyclophosphamide and phosphoramide mustard is the ultimate alkylating species that cross-links interstrand DNA.[22]

Our design of peptide-conjugates of 4-NH_2-CPA (**7**) as prodrugs for proteolytic activation takes advantage of the structural similarity of **7** to 4-hydroxycyclophosphamide (**2**) and the chemical instability of a *gem*-diamine. As illustrated in Scheme 1, proteolysis of conjugate **6** generates **7** and a peptide. Under physiological conditions, compound **7** is protonated and forms 3,4-dehydrocyclophosphamide (**8**) after elimination of ammonia. Compound **7** is known to be in equilibrium with **2** which is responsible for the generation of phosphoramide mustard in the activation mechanism of cyclophosphamide.[20] Chemically, **7** is a *gem*-diamine, which is known as a masked aldehyde.[23,24] *N*,*N*′-Diacylated or dicarbamylated *gem*-diamines are stable compounds and have been widely used in 'retro-inverso' peptide mimetics. Monoacyl or monocarbamyl *gem*-diamines are labile to acid- or base-catalyzed elimination reactions.

The phenylalanine-conjugated 4-NH_2-CPA (H-Phe-NH-CPA, **16**) and their benzyloxycarbonyl (Z)-protected analogs **15** were chosen as model prodrugs to examine the synthetic feasibility, chemical stability, and proteolytic activation of this class of compounds. Selection of the phenylalanine residue was based on the need of a chromophore to monitor reactions by UV and the known cleavage of amide bond after phenylalanine by α-chymotrypsin. Because of the presence of two chiral centers in the oxazaphosphorinane ring, compounds **15** and **16** exist as four configurational diastereomers. They are referred to as *cis*-(4*R*)-, *trans*-(4*R*)-, *cis*-(4*S*)-, and *trans*-(4*S*)- (Fig. 1) according to the oxazaphosphorinane C-4 configuration and the relative orientation of C-4 substituent to the oxygen atom of P═O bond in the oxazaphosphorinane ring (*cis* = *SR*/*RS*, *trans* = *RR*/*SS*).

The two diastereomers of (4*R*)-**16** were synthesized stereospecifically from L-homoserine ((*S*)-**9**) while the two diastereomers of (4*S*)-**16** were synthesized from D-homoserine ((*R*)-**9**) as shown in Scheme 2. Z-Phe-OH was preactivated using HOSu/DCC and the resulting activated ester reacted with **9** in a mixture of 1 M $KHCO_3$ and THF yielding the dipeptide **10**. Amidation of **10** was carried out using HOBt/EDC activation followed by treatment with saturated ammonium hydroxide,[25] and the hydroxyl group in **11** was protected with a TBDPS group. The bis(trifluoroacetoxy)iodobenzene (BTI)-mediated Hofmann rearrangement was employed to convert the amide **12** to the corresponding mono-acylated *gem*-diamine derivative **13**. This method was chosen over other methods such as the Curtius rearrangement because of its advantages of mild reaction conditions, high yield, and retention of stereochemistry.[26,27] The reaction was also conveniently monitored by the disappearance of the starting material on TLC. Protection of the hydroxyl group with TBDPS was necessary to avoid an intramolecular cyclization of the isocyanate intermediate during the Hofmann rearrangement to form 2-oxazolidinone.[28]

The TBDPS group in **13** was removed with TBAF at room temperature, which was accompanied by the formation of Z-Phe-NH_2 as a side product. Cyclization of 1,3-aminoalcohol **14** with bis(dichloroethyl)phospho-



Scheme 1. Proposed degradation mechanism of peptide-conjugated 4-aminocyclophosphamide as compared to the activation of cyclophosphamide.

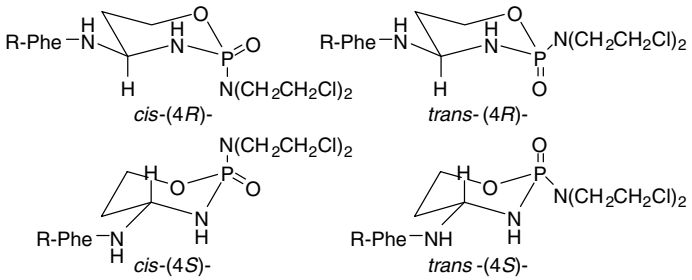
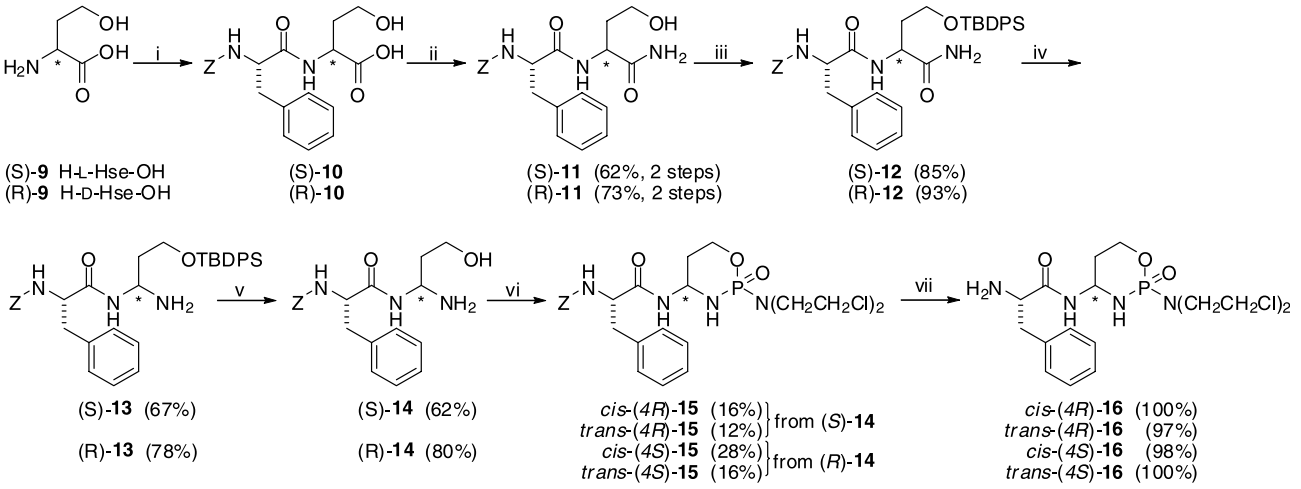


Figure 1. The four diastereomers of Z-protected and unprotected phenylalanine-4-aminocyclophosphamides **15** (R = Z) and **16** (R = H).



Scheme 2. Synthesis of diastereomers of H-Phe-NH-CPA (**16**) from D- or L-homoserine. Reagents and conditions: (i) Z-Phe-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) $Cl_2PON(CH_2CH_2Cl)_2$, TEA, THF, 0 °C, rt; (vii) H_2, 10% Pd/C, MeOH.

ramidic dichloride gave the *cis* and *trans* diastereomers of **15** that were easily separated by silica gel flash column chromatography, yielding a faster eluting *cis* diastereomer in 16–28% yield and a slower eluting *trans* diastereomer in 12–16% yield. The overall cyclization yield was apparently affected by significant degradation of **14** to Z-Phe-NH_2 during the lengthy reaction time (48 h) in addition to the low nucleophilicity of *gem*-diamines and the competing intermolecular cyclization, which have been observed in the synthesis of other similar cyclophosphamide analogs.[29,30] The *cis*- and *trans*-configurations of **15** were conveniently assigned according to their chromatographic behavior, ^1H and ^{31}P chemical shifts as shown in Table 1.[31,32] It should be noted that TLC on silica gel was used to help assign the *cis*/*trans*-diasteromers as the normal phase TLC is more reliable than reversed-phase HPLC in predicting relative polarity. Compared to the *trans*-diastereomer, the *cis*-isomer elutes more quickly on silica gel, has a more upfield C-4 proton signal, a more downfield C-5 proton signal, and a more upfield phosphorus signal. These differences between the *cis*- and *trans*-isomers of **15** are similar to those observed among the isomers of 4-nitrophenyl-cyclophosphoramide analogs.[18] The Z group in each isomer of **15** was removed by hydrogenolysis to give the corresponding unprotected isomer of **16**.

All four diastereomers of **16** were incubated in, pH 7.4, phosphate buffer (100 mM) at 37 °C. HPLC analysis of the incubation mixtures showed no significant changes over a period of 3 days (<10%), suggesting that the amino acid- or peptide-conjugated 4-NH_2-CPA is stable under these conditions.

The diastereomers of **15** and **16** were evaluated as substrates of α-chymotrypsin by incubating each compound (0.2 mM) in 100 mM phosphate buffer, pH 7.4, at 37 °C. N^{α}-Benzoyl tyrosine ethyl ester (BTEE) was used as a positive control of the activity of α-chymotrypsin. After the reaction was initiated by the addition of α-chymotrypsin, aliquots were withdrawn at various time intervals, quenched with acetonitrile, and analyzed by HPLC. The N-terminal capping group was found to be required for the proteolysis of these conjugates as none of compound **16** isomers were cleaved in the presence of α-chymotrypsin over an incubation time of 12 h.

Table 1. Analytical data of diastereomers of Z-Phe-NH-CPA (**15**) and H-Phe-NH-CPA (**16**)

Compound	R_f[a]/t_r[b]	MS	NMR δ (ppm)		
			^1H (C-4, 1H)	^1H (C-5, 2H)	^{31}P[e]
cis-(4*R*)-**15**	0.35[a]	557.3[c]	5.37–5.24	2.20–1.85	11.4
trans-(4*R*)-**15**	0.25[a]	557.3[c]	5.40–5.35	2.05–1.86	13.5
cis-(4*S*)-**15**	0.45[a]	557.3[c]	5.34–5.12	2.05–1.52	11.4
trans-(4*S*)-**15**	0.25[a]	557.3[c]	5.35–5.27	1.84–1.60	13.4
cis-(4*R*)-**16**	15.6[b]	423.1123[d]	5.37–5.22	2.27–1.85	11.0
trans-(4*R*)-**16**	16.2[b]	423.1135[d]	5.43–5.35	2.09–1.83	13.7
cis-(4*S*)-**16**	16.8[b]	423.1140[d]	5.33–5.25	2.31–1.44	11.6
trans-(4*S*)-**16**	16.1[b]	423.1135[d]	5.54–5.50	2.16–1.77	13.5

[a] TLC (on silica gel) developing solvents: CH_2Cl_2/MeOH = 20:1.
[b] HPLC retention time (min) on C18 column (5 μm, 4.6 × 250 mm) with a gradient elution of 4–76% methanol in 15 min.
[c] MH^+ recorded on LC–MS (ESI, isotopic peaks were not listed).
[d] HRMS (FAB), *m*/*z* calcd for $C_{16}H_{26}Cl_2N_4O_3P$ $[MH]^+$ 423.1120.
[e] The ^{31}P chemical shift was determined by using 5% H_3PO_4 in D_2O as an internal standard.

The absence of an N-terminal capping group not only decreases the affinity of the phenylalanine conjugate to α-chymotrypsin, but generates a positive charge close to the cleavage site, which may have resulted in the loss of substrate activity.[33] This was confirmed by the incubation of phenylalanine-conjugated homoserine amide with α-chymotrypsin, which was not cleaved without an N-terminal capping group.

All four isomers of **15** with a N^{α}-Z group were cleaved by α-chymotrypsin under near physiological conditions with the concurrent release of Z-Phe-OH. As shown in Figure 2, a significant difference of substrate activity exists between the diastereomers. The *cis*-(4*R*)-**15** was the best substrate of α-chymotrypsin with a half-life of 20 min. It was hydrolyzed by the enzyme about 27× more quickly than *trans*-(4*R*)-**15**, which has a half-life of 8.9 h. The two (4*S*)-**15** isomers were hydrolyzed much more slowly than their corresponding (4*R*)-isomers, both showing half-lives greater than 12 h. The proteolysis of *cis*-(4*R*)-**15** was also compared to that of the dipeptide Z-Phe-L-Hse-NH_2 ((*S*)-**11**) in Figure 3. *cis*-(4*R*)-**15** was hydrolyzed by the enzyme 1.6× more quickly than the dipeptide (*S*)-**11**, which has a half-life of 32 min, indicating that the C-terminal 4-NH_2-CPA rendered the conjugate an even better substrate to the enzyme. When the reaction mixtures of *cis*-(4*R*)-**15** with α-chymotrypsin were analyzed by LC–MS, Z-Phe-OH was clearly identified, but the released 4-NH_2-CPA (**7**) was not observed. Instead, we have observed compounds corresponding to 4-hydroxycyclophosphamide, phosphoramide mustard, and derivatives with their distinct isotopic patterns in MS (data not shown). This suggests that compound **7** was not stable under the assay conditions and may have disintegrated according to our proposed mechanism shown in Scheme 1.

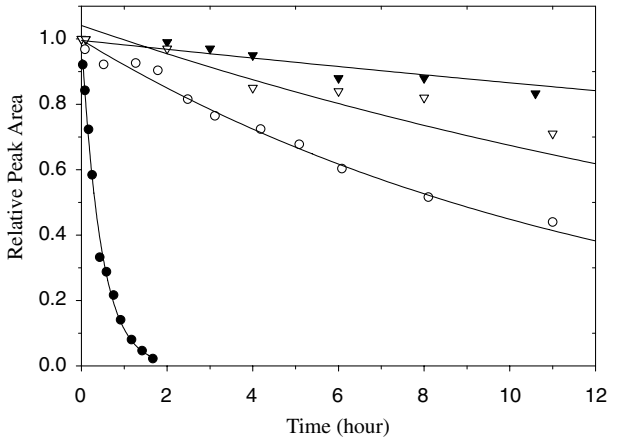


Figure 2. α-Chymotrypsin digestion of compound *cis*-(4*R*)-**15** (●, $t_{1/2}$ = 20 min), *trans*-(4*R*)-**15** (○, $t_{1/2}$ = 8.9 h), *cis*-(4*S*)-**15** (▼, $t_{1/2} \geqslant 12$ h), and *trans*-(4*S*)-**15** (▽, $t_{1/2} \geqslant 12$ h). Shown is the disappearance of the substrates.

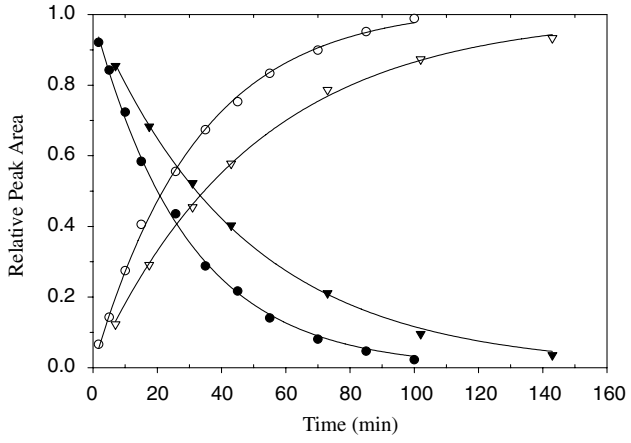


Figure 3. α-Chymotrypsin digestion of compounds *cis*-(4*R*)-**15** (● and ○, $t_{1/2}$ = 20 min) and Z-Phe-L-Hse-NH_2 ((*S*)-**11**, ▼ and ▽, $t_{1/2}$ = 32 min). Shown are the disappearance of the substrates (closed symbols) and appearance of Z-Phe-OH (open symbols).

In summary, 4-aminocyclophosphamide (4-NH_2-CPA, **7**) was shown for the first time as a prodrug moiety of phosphoramide mustard. The four diastereomers of phenylalanine-conjugated 4-NH_2-CPA were synthesized stereospecifically from the D- and L-homoserine and were found to be stable under physiological conditions. Proteolysis of the Z-Phe conjugates by α-chymotrypsin was dependent upon their stereochemical configurations. The *cis*-(4*R*) isomer was most efficiently cleaved by α-chymotrypsin with a half-life of 20 min, significantly shorter than the 8.9 to >12 h half-lives of the other three isomers. LC–MS analysis of the proteolytic products of *cis*-(4*R*)-**15** indicated that 4-NH_2-CPA was released, but disintegrated into phosphoramide mustard through elimination of ammonia and conversion to 4-hydroxycyclophosphamide. 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.

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