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Synthesis and epimerization of phenylalanyl 4-aminocyclophosphamides

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Abstract—Peptide and amino acid conjugates of (4*R*)- and (4*S*)-4-aminocyclophosphamides (4-NH_2-CPA, 3) were designed as prodrug forms of phosphoramide mustard. Four diastereomers of Boc-Phe-4-NH-CPA (**6**) were synthesized stereospecifically from homoserine (*R* or *S*) and the protection strategy was optimized for the homoserine hydroxyl group during the construction of the 1,3,2-oxazaphosphorinane ring. The Phe-4-NH-CPA isomers of the trans-configuration ((2*S*,4*R*)- and (2*R*,4*S*)-) were found to be less stable than the corresponding isomers of the cis-configuration ((2*R*,4*R*)- and (2*S*,4*S*)-) and to undergo epimerization of the C-4 chiral center in the presence of 25% TFA used during Boc deprotection. The synthetic route developed should be applicable to the synthesis of a variety of peptide and amino acid conjugates of 4-aminocyclophosphamide.
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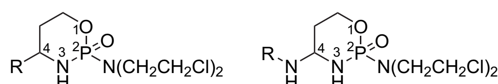
1. Introduction

The oxazaphosphorinane cyclophosphamide (**1**) is an alkylating antitumor agent with activity against a broad spectrum of human cancers including slow-growing solid tumors.[1] Over the past four decades, cyclophosphamide has become one of the most frequently used anticancer agents in clinic. The clinical significance as well as the unique conformational and stereochemical aspects of oxazaphosphorinane derivatives have attracted much interest in the chemistry community.[2–4] To elucidate the mechanism of action of cyclophosphamide and to enhance its efficacy as an antitumor agent, numerous oxazaphosphorinane derivatives were synthesized. The mechanism of action of cyclophosphamide has been well understood after decades of investigation.[5,6] Briefly, cyclophosphamide is oxidized by cytochrome P450 enzymes in the liver to 4-hydroxycyclophosphamide (**2**), which then decomposes into acrolein and the alkylating species phosphoramide mustard.[5,6] Acrolein is responsible for the hemorrhagic cystitis, a major dose-limiting side effect of cyclophosphamide. Tumor-targeted prodrug therapy is one of the strategies explored to improve the therapeutic index of cyclophosphamide.[7–12] In this strategy, phosphoramide mustard is incorporated with a biochemical activation mechanism specifically associated with tumor cells, such as hypoxic reduction, enzymatic action, and receptor recognition. Recently, we have reported for the first time that 4-aminocyclophosphamide (4-NH_2-CPA, **3**) can be used as a prodrug form of phosphoramide mustard because of its structural similarity to **2** and its spontaneous degradation as a mono-phosphorylated *gem*-diamine.[12] In that communication we had briefly described the synthesis of phenylalanyl 4-aminocyclophosphamide (H-Phe-4-NH-CPA, **4**) and its Cbz-protected derivative (**5**). Herein, we wish to report an alternative synthetic route to **4** through its Boc-protected derivative (**6**) and an unexpected but interesting epimerization reaction was observed for the trans-isomers of **4** under acidic de-Boc conditions. Protecting strategy for the hydroxyl group of homoserine was also optimized for the construction of the 1,3,2-oxazaphosphorinane ring. The synthetic routes developed can be easily adapted for the synthesis of various 4-aminocyclophosphamide conjugates, especially those of amino acids or peptides.



1 R = H
2 R = OH

3 R = H
4 R = H-Phe-
5 R = Cbz-Phe-
6 R = Boc-Phe-

Keywords: Cyclophosphamide; Prodrug; Proteolysis; Epimerization.
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2. Results and discussion

The four configurational diastereomers of compounds **4–6** are referred to as (2*R*,4*R*)-, (2*R*,4*S*)-, (2*S*,4*R*)-, and (2*S*,4*S*)- (Fig. 1) according to the C-2 and C-4 absolute configuration. Among them, (2*R*,4*R*)- and (2*S*,4*S*)- have been referred to as cis-isomers and (2*R*,4*S*)- and (2*S*,4*R*)- as trans-isomers according to the relative orientation of the C-4 substituent to the oxygen atom of P═O bond in the oxazaphosphorinane ring (cis=*RR*/*SS*, trans=*SR*/*RS*). Only the chair conformations are shown in Figure 1 to illustrate the relative stereochemistry; the actual conformations of the oxazaphosphorinane ring are more complicated and are discussed by others.[2,4,13] The initial synthesis of compound **6** started from *O*-benzyl protected Boc-homoserine (*S* or *R*) as shown in Scheme 1. The two diastereomers of **6** with *R* configuration at C-4 were synthesized stereospecifically from (*S*)-Boc-Hse(Bn)-OH ((*S*)-**7**) that has the same absolute stereochemistry found in natural amino acids while the two diastereomers of **6** with the *S* configuration at C-4 were synthesized stereospecifically from (*R*)-Boc-Hse(Bn)-OH ((*R*)-**7**). Amidation of **7** was carried out using HOBt/EDC activation followed by treatment with saturated ammonium hydroxide.[14,15] After removal of Boc group in **8**, the free amino group was coupled with Boc-Phe-OH to give the dipeptide amide **9**. The bis(trifluoroacetoxy)iodobenzene (BTI)-mediated Hofmann rearrangement was employed to convert the amide **9** to the corresponding mono-acylated *gem*-diamine derivative **10**. The BTI-mediated Hofmann rearrangement method was chosen over other methods such as the Curtius rearrangement for its mild reaction conditions, high product yields, and retention of the C_α-chiral center in homoserine.[16,17] The reaction was conveniently monitored by the disappearance of the starting material on TLC and the Boc group was stable under the mild acidic reaction conditions.

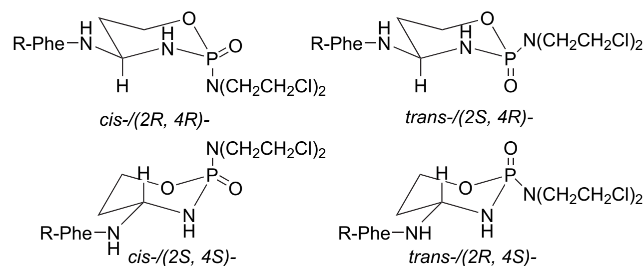
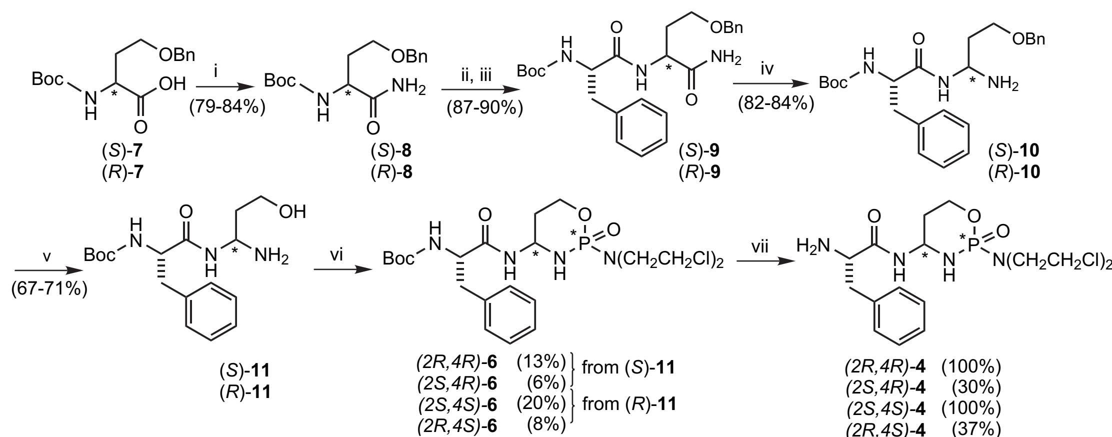


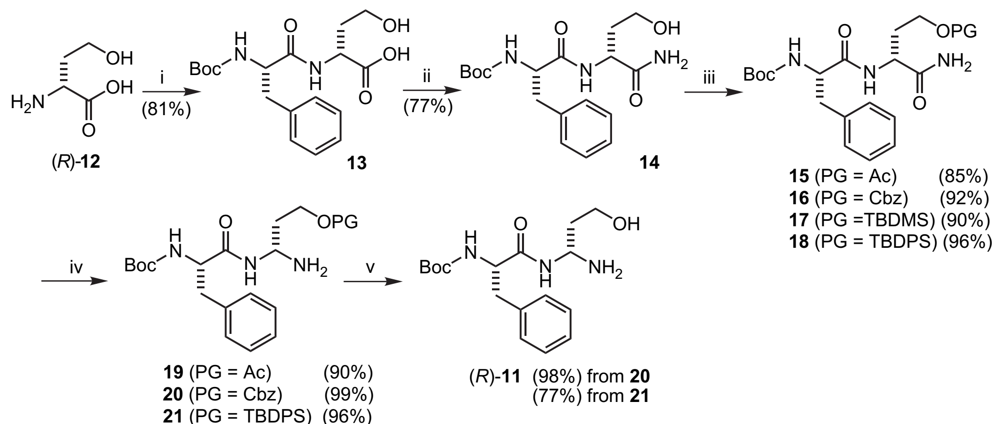
Figure 1. Four diastereomers of phenylalanyl 4-aminocyclophosphamides **4** (R=H), **5** (R=Cbz), and **6** (R=Boc).

The benzyl group in **10** was removed by catalytic hydrogenolysis at 50 psi at pH 3 for 1–2 days. The harsh conditions were necessary to overcome the problem of catalyst poisoning by the free amino group in the product[18] and to avoid an intramolecular O→N benzyl migration side reaction that would otherwise lead to the formation of a hard-to-remove benzyl amine side product.[19,20] LC–MS analysis indicated that the Boc group was not affected under these mild acidic conditions. However, Boc-Phe-NH_2 was isolated in 25–30% yield, indicating significant degradation of the *gem*-diamine **10** and **11** during the deprotection.[21] The amino alcohol **11** was isolated in 67–71% yield by a benzenesulfonic acid-based cation exchange column using 1% TEA/CH_3OH as the eluant. In an effort to avoid the difficulty of removing the benzyl group in the presence of an amino group, we removed the benzyl group in **10** prior to the Hofmann rearrangement. However, this process led to the formation of 1,3-oxazinan-2-one and was later developed into a useful method in the construction of 2-oxazolidinone libraries from β-hydroxylpropionamides.[22]

To facilitate the synthesis of 4-NH_2-CPA conjugates, we explored other reaction routes that avoided the difficulty of removing the benzyl group in **10** and could be more easily adapted for scaled-up synthesis. Using (*R*)-homoserine (**12**) as the starting material, we explored various protecting group strategies of the homoserine hydroxyl group and obtained the key intermediate (*R*)-**11** in much better yields. As shown in Scheme 2, the HOSu-activated ester of Boc-Phe-OH reacted with **12** in a mixture of 1 M $KHCO_3$ and THF (1:1) yielding the dipeptide **13** in 81% yield. The carboxylic acid **13** was subsequently converted to its corresponding amide **14** using the same amidation method as described above for compounds (*S*)- and (*R*)-**9**. Protection of the hydroxyl group in **14** was evaluated in parallel using acetyl, Cbz, TBDMS, and TBDPS groups in compounds



Scheme 1. Stereospecific synthesis of H-Phe-4-NH-CPA (**4**) from isomers of protected homoserine ((*S*)-**7** and (*R*)-**7**). Reagents and conditions: (i) HOBt, EDC, THF, rt, then added satd NH_3 (aq); (ii) 25% TFA, CH_2Cl_2, rt; (iii) Boc-Phe-OSu, 1 M $KHCO_3$/THF (1:1), rt; (iv) BTI, CH_3CN/H_2O (1:1), rt; (v) H_2 (50 psi), 10% Pd–C, add 0.2 N HCl to pH 3, MeOH, rt; (vi) $Cl_2PON(CH_2CH_2Cl)_2$, TEA, THF, 0 °C, rt; (vii) 25% TFA, CH_2Cl_2, rt.



Scheme 2. Use of different protecting groups during the synthesis of Boc-Phe-(*R*)-gHse-NH_2 ((*R*)-**11**) from (*R*)-homoserine (**12**). Reagents and conditions (PG=Protecting group): (i) Boc-Phe-OSu, 1 M $KHCO_3$/THF (1/1), rt; (ii) HOBt, EDC, THF, rt, then added satd NH_3 (aq); (iii) (a) for compound **15**: AcCl, pyridine, CH_2Cl_2, rt; (b) for compound **16**: Cbz-Cl, pyridine, CH_2Cl_2; (c) for compound **17**: TBDMS-Cl, imidazole, DMF, rt; (d) for compound **18**: TBDPS-Cl, imidazole, DMF, rt; (iv) BTI, CH_3CN/H_2O=1:1, rt; (v) (a) from **20**: H_2 (50 psi), 10% Pd–C, MeOH, rt; (b) from **21**: TBAF, THF, rt.

15, **16**, **17**, and **18**, respectively. The acetyl group was introduced using Ac_2O/pyridine; the Cbz group was introduced using Cbz-Cl/pyridine; and the two silyl groups were introduced using the corresponding silyl chloride/imidazole in DMF. The TBDMS protecting group in **17** was found to be unstable to the acidic Hofmann rearrangement conditions, resulting in the formation of the cyclized product, 1,3-oxazinan-2-one.[22] The other three protected dipeptide amides were successfully converted into their corresponding *gem*-diamine derivatives **19–21** in nearly quantitative yields. Subsequently, the Cbz group of **20** was completely removed to give (*R*)-**11** in quantitative yield by catalytic hydrogenolysis at 50 psi in 4 h without the need to add HCl. The TBDPS group of **21** was removed by TBAF at room temperature in 1 h to give (*R*)-**11** in 77% yield with the concurrent formation of Boc-Phe-NH_2 as a side product. Attempt to remove the acetyl group in **19** by LiOH-catalyzed hydrolysis gave a mixture of (*R*)-**11** and an acetamide resulting from O→N acetyl migration. The acetamide was resistant to hydrolysis under the same conditions. Thus, Cbz protection for the homoserine hydroxyl group gave the best yield in the synthesis of (*R*)-**11** and the carbonate was readily removed in high yield under mild conditions. TBDPS protection can be used as an alternative and was successfully used for the synthesis of Cbz-Phe-4-NH-CPA (**5**), when differentiation of the hydroxyl and the N-terminal amino protecting groups is necessary.[12]

Cyclization of the γ-amino alcohol in (*S*)- or (*R*)-**11** with bis(dichloroethyl)phosphoramidic dichloride gave a mixture of the corresponding cis- and trans-diastereomer of **6**, which were easily separated by silica gel flash column chromatography, yielding a faster eluting cis-diastereomer in 13–20% yield and a slower eluting trans-diastereomer in 6–8% yield. Boc-Phe-NH_2 was isolated in 22–32% yield. The low cyclization yield was attributed to the low nucleophilicity of *gem*-diamines and the significant degradation of the starting *gem*-diamines, a phenomenon that was observed during the synthesis of other cyclophosphamide analogs.[2,7,9,23] Efforts to improve the cyclization yield by using excess reagents or extending the reaction time to 72 h were unsuccessful and yielded more degradation products. The γ-amino alcohols (*S*)- and (*R*)-**11** were applied in either the HCl salt form or the free base form in the cyclization reaction. Organic carboxylic acid salts of **11** such as those of TFA were found to adversely affect the cyclization reaction by producing an *N*-acylated side product of **11**, presumably through activation and subsequent amidation of the carboxylic acid by the bis(dichloroethyl)phosphoramidic dichloride reagent used. The configurations of **6** as either cis or trans were unequivocally assigned according to their NMR data. The ^{31}P chemical shift for *cis*-2-oxo-1,3,2-oxazaphosphorinanes with an axial P-$N(CH_2CH_2Cl)_2$ is generally upfield as compared with that for the trans-isomers with an equatorial P-$N(CH_2CH_2Cl)_2$.[13,24] On ^1H NMR, the cis-isomers have a more upfield H-4 and a more down field H-5 as compared to the trans-diastereomers.[25] The spectroscopic data used to assign the configuration of the four diastereomers are summarized in Table 1.

To remove the N-terminal Boc group, each of the four diastereomers of **6** was treated with 25% TFA/CH_2Cl_2 for 30 min at room temperature and the deprotection reaction was monitored using HPLC and LC–MS. To our surprise, while the Boc group of each diastereomer was successfully removed under these conditions, the homogeneity of the deprotected products varied with the relative configuration of the starting material. Each of the cis-isomers gave a single product peak on HPLC, which was confirmed to be the corresponding amine **4** by LC–MS, but each of the trans-isomers gave two product peaks on HPLC and LC–MS, both having the same molecular ions and isotopic patterns corresponding to **4** (Fig. 2). Similarly, ^{31}P NMR demonstrated that deprotection of each cis-isomer gave a single peak, but that of each trans-isomer gave two peaks with chemical shifts of about 0.4–0.6 ppm apart (Table 1). The two products from each trans-isomer were separated by preparative HPLC and both fit the structure of **4** based on their ^1H NMR spectra. Interestingly, we noticed that each trans-isomer of **6** gave, upon deprotection, a product mixture that always contained one component having the same HPLC retention time and ^{31}P NMR chemical shift as the product from the cis-isomer with the opposite stereochemistry at C-4 under the same conditions. As shown in Figure 2, deprotection of trans-isomer (2*S*,4*R*)-**6** gave two peaks with retention time at 16.2 and 16.8 min; the latter overlapped with the peak corresponding to cis-isomer (2*S*,4*S*)-**4** (chromatogram a and b).

Table 1. Analytical data of diastereomers of Boc-Phe-NH-CPA (**6**) before and after TFA treatment

Before TFA treatment						After TFA treatment			
Compound	R_f[a]/t_R[b]	MS[c]	NMR δ (ppm)			Product[e]	t_R[b]	MS[g]	^{31}P NMR[d]
			^{1}H (C-4, 1H)	^{1}H (C-5, 2H)	^{31}P[d]				
(2*R*,4*R*)-**6**	0.6/22.5	523.1634	5.42–5.20	2.25–1.87	10.7	(2*R*,4*R*)-**4**	15.6 (100%[f])	423.1	11.0
(2*S*,4*R*)-**6**	0.2/22.8	523.1629	5.43–5.25	2.20–1.80	13.6	(2*S*,4*R*)-**4**	16.2 (52%[f])	423.1	13.7
						(2*S*,4*S*)-**4**	16.8 (48%[f])	423.1	11.6
(2*S*,4*S*)-**6**	0.45/22.6	523.1653	5.34–5.12	2.05–1.55	11.6	(2*S*,4*S*)-**4**	16.8 (100%[f])	423.2	11.6
(2*R*,4*S*)-**6**	0.2/22.7	523.1643	5.34–5.30	1.8–1.61	13.7	(2*R*,4*S*)-**4**	16.0 (41%[f])	423.3	13.5
						(2*R*,4*R*)-**4**	15.6 (59%[f])	423.1	11.0

[a] TLC (on silica gel) developing solvents: hexane/CH_2Cl_2/MeOH=1:1:0.1.
[b] HPLC retention time (min) on C_{18} column (5 μm, 4.6×250 mm) with a gradient elution of 4–76% methanol in 15 min.
[c] HRMS (FAB), *m/z* calcd for $C_{21}H_{34}Cl_2N_4O_5P$ $[MH]^+$ 523.1644.
[d] The ^{31}P chemical shift was determined using 5% H_3PO_4 in D_2O in a coaxial insert as an external standard.
[e] Product of **5** after TFA treatment.
[f] Relative composition as determined by peak areas on HPLC chromatogram monitored at 220 nm.
[g] MH^+ monoisotopic mass as recorded on ESI LC–MS (isotopic peaks were not listed).

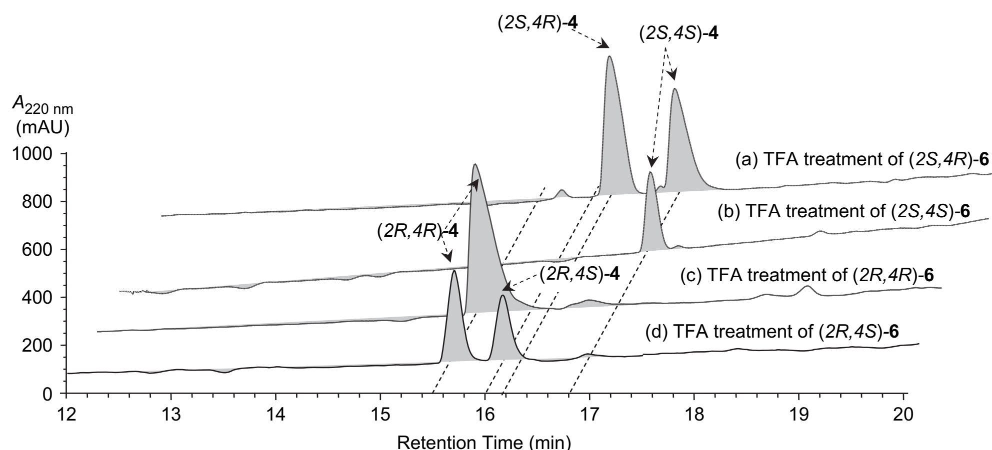
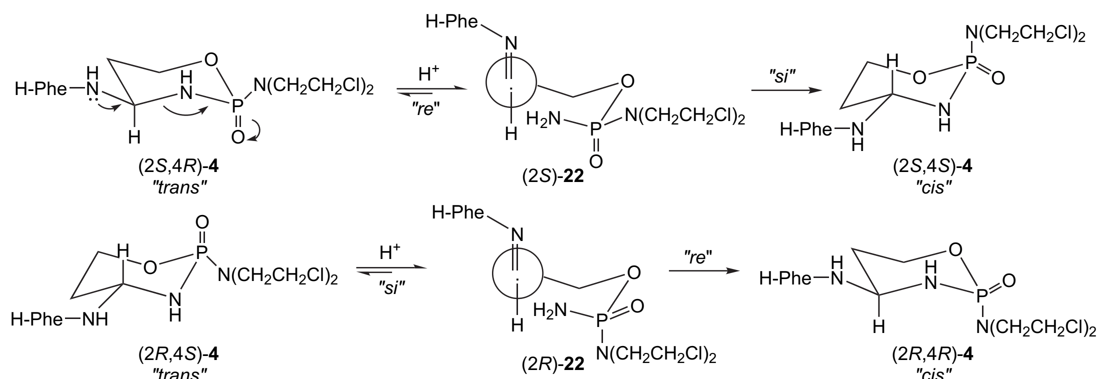


Figure 2. HPLC analyses of products after TFA treatment of each of the four diastereomers of Boc-Phe-NH-CPA (**6**).

Similarly, deprotection of trans-isomer (2*R*,4*S*)-**6** gave two peaks with retention time at 15.6 and 16.0 min; the former overlapped with the peak corresponding to (2*R*,4*R*)-**4**, the cis-isomer with opposite stereochemistry at C-4 (comparing chromatograms d and c, Fig. 2). Similarly, the ^{31}P NMR chemical shift of cis-isomer (2*R*,4*R*)-**4** at 11.0 ppm overlapped with one product from trans-isomer (2*R*,4*S*)-**6** and that of (2*S*,4*S*)-**4** at 11.6 ppm with one product from (2*S*,4*R*)-**6**. This was further confirmed by coinjections of these products into HPLC and ^{31}P NMR of the mixtures of these products.

Based on these evidences from the LC–MS, HPLC, and NMR, we concluded that deprotection of cis-isomers of **6** using 25% TFA gave only the corresponding cis-isomers of **4** but the two trans-isomers of **6** gave not only the expected trans-isomer of **4** but also another cis-isomer of **4** resulting from epimerization of the C-4 chiral center. Specifically, the deprotection product of trans-isomer (2*S*,4*R*)-**6** was a mixture of *trans*-(2*S*,4*R*)-**4** and *cis*-(2*S*,4*S*)-**4**, and that of *trans*-(2*R*,4*S*)-**6** was a mixture of *trans*-(2*R*,4*S*)-**4** and *cis*-(2*R*,4*R*)-**4**. This conclusion was further confirmed by the stereospecific synthesis of the four isomers of **4** from their Cbz-protected derivatives (**5**).[12] When the Cbz group was removed under neutral hydrogenolysis conditions, each trans-isomer of **4** was isolated as a single product and its identity in the above mixtures were confirmed by its HPLC retention time and ^{31}P NMR chemical shift. In addition, when each trans-isomer of **4** was treated with TFA (25–100%) in dichloromethane for 30 min, a mixture of trans- and cis-isomer were obtained as described above while the same treatment of the two cis-isomers of **4** did not cause any changes in the composition.

The epimerization of trans-isomers of H-Phe-NH-CPA (**4**) at the C-4 chiral center is rationalized by an acid-catalyzed ring opening of 4-aminocyclophosphamide to a ring-opened imide form, which would then cyclize to give the corresponding cis-diastereomer, similar to the epimerization of 4-hydroxycyclophosphamide through aldophosphoramide.[26] As shown in Scheme 3, under acidic conditions, *trans*-(2*S*,4*R*)-**4** undergoes a ring opening to form an imide (2*S*)-**22**, which then cyclizes to form either *cis*-(2*S*,4*S*)-**4** from *si* face or back to *trans*-(2*S*,4*R*)-**4** from *re* face. Similarly, *trans*-(2*R*,4*S*)-**4** forms *cis*-(2*R*,4*R*)-**4** through the cyclization of the imide intermediate (2*R*)-**22** from *re* face. The epimerization trend from trans to cis was further confirmed by the observation that an increasingly high conversion yield was obtained from trans-isomers of **4** to the corresponding cis-isomers of **4** when the TFA treatment was prolonged from 30 min to 9 h. The extended exposure to TFA also



Scheme 3. TFA-catalyzed epimerization of the trans-isomers of H-Phe-NH-CPA (**4**) at the C-4 chiral center.

promoted the degradation of **4** as H-Phe-NH_2 was observed as a side product and increased with the reaction time. The observation of epimerization only for the trans-isomers indicates that the cis-isomers of **4** are more stable than the trans-isomers and thus cyclization of **22** favors to the right side. Previous study has clearly shown that cis- and trans-isomer of 4-phenyl-cyclophosphamide exist in chair-like conformation with the phenyl substitute on an equatorial position, regardless of the phosphorus configuration.[24] As the phenylalaninamido group of **4** is more strictly demanding than the phenyl group, isomers of **4** should exist predominantly in the similar chair-like conformation with the C-4 substituent on the equatorial position as shown in Figure 1. Compared to the trans-isomers, the cis-isomers, therefore, are stabilized by a more efficient P(2)-N(3) anomeric interaction with $N(CH_2CH_2Cl)_2$ and a more efficient intramolecular H bonding between P=O and adjacent NH.[13,24] Attempts to avoid epimerization during Boc deprotection of the trans-isomers of **6** using milder acidic to neutral conditions included treatment with Me_3SiI in chloroform at room temperature,[27] bromocatecholborane in dichloromethane,[28] $AlCl_3/PhOCH_3$ in dichloromethane,[29] and $MeSO_3H$ in dioxane.[30] However, none of these conditions were satisfactory, resulting in either incomplete deprotection or decomposition of the product or the observed epimerization.

3. Conclusion

We successfully synthesized Boc-protected phenylalanine-conjugated 4-aminocyclophosphamide (**6**) isomers in a stereospecific manner from homoserine (*R* or *S*) and explored various protection strategies for the hydroxyl group of homoserine for the steps leading to the construction of the 1,3,2-oxazaphosphorinane ring. Among the protecting groups explored, Cbz was the best for the synthesis of the key intermediate **10** prior to the formation of the 1,3,2-oxazaphosphorinane ring and TBDPS can be used as an alternative when Cbz can not be used. The optimized synthetic route should be applicable to the synthesis of a variety of 4-aminocyclophosphamide conjugates. The trans-isomers of **6** were found to be unstable under the acidic conditions used to deprotect Boc and epimerized at the C-4 chiral center to the corresponding cis-isomers, which were stable under the same conditions. These findings suggested that acidic treatment of the trans-isomers of 4-aminocyclophosphamide conjugates should be avoided.

4. Experimental

4.1. General

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 one 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 column chromatography was performed using silica gel (Merck 230–400 mesh). Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogenous 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 and ^{13}C) were recorded 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 in a coaxial insert as an external standard. Chemical shifts are reported in parts per million (δ) 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 was performed on an HP 1090 system equipped with a Phenomenex C_{18} 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.

4.1.1. (*R*)-Boc-Hse(Bn)-NH_2 ((*R*)-8). To a solution of (*R*)-Boc-Hse(Bn)-OH (2.02 g, 6.54 mmol) in anhydrous THF (30 mL) were added HOBt (1.33 g, 9.8 mmol) and EDC (1.25 g, 6.54 mmol). The reaction solution was stirred at room temperature for 30 min. Saturated ammonium hydroxide solution (5 mL) was added dropwise as the reaction mixture cleared. The reaction was stirred for 3 h after the addition was complete. After the removal of solvent in vacuo, the residue was taken up in ethyl acetate (150 mL), washed with saturated $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. Solvent was removed by evaporation and the residue was purified by flash column chromatography (CH_2Cl_2/CH_3OH, 20:1) to give a white solid (1.69 g, 84%); mp 125–127 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.35–7.28 (m, 5H), 6.56 (br s, 1H), 6.22 (br s, 1H), 5.87 (d, *J*=7.0 Hz, 1H), 4.50 (s, 2H), 4.31–4.28 (m, 1H), 3.67–3.57 (m, 2H), 2.07–2.02 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.8, 155.8, 138.0, 128.5, 128.3, 127.9, 127.8, 127.5, 80.1, 73.4, 67.7, 53.1, 32.2, 28.4; IR ($CDCl_3$) 3334.1 (br), 2976.8, 1683.1, 1506.8, 1366.6, 1250.8, 1166.9 cm^{-1}; LC–MS (ESI) 347.0 $[M+K]^+$, 331.0 $[M+Na]^+$, 253.0, 209.0; HRMS (FAB) *m/z* calcd for $C_{16}H_{24}N_2O_4$ $[MH]^+$ 309.1809, found 309.1799.

4.1.2. (*S*)-Boc-Hse(Bn)-NH_2 ((*S*)-8). The compound was synthesized from (*S*)-Boc-Hse(Bn)-OH (3.0 g, 9.7 mmol) using the procedure described above in Section 4.1.1 as a white solid (2.37 g, 79%); mp 128–129 °C. ^1H NMR (200 MHz, CD_3OD) δ 5.86 (d, *J*=7 Hz, 1H), 4.50 (s, 2H), 4.29 (d, *J*=5.2 Hz, 1H), 3.62 (m, 2H), 2.05 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.1, 155.9, 138.0, 128.5, 127.8, 79.9, 77.9, 77.3, 76.7, 73.3, 67.5, 52.9, 32.3, 28.4; IR 3389.6, 3190.0, 2977.5, 2864.7, 1651.0, 1519.0 cm^{-1}; LC–MS (ESI) 331.0 $[M+Na]^+$, 253.0, 209.0.

4.1.3. Boc-Phe-(*R*)-Hse(Bn)-NH_2 ((*R*)-9). Compound (*R*)-**8** (1.40 g, 4.55 mmol) was treated with 50% TFA in CH_2Cl_2 (5 mL) for 30 min. After evaporation under vacuum the residue was pumped to dryness. The residue was dissolved in CH_2Cl_2 (8 mL) and DIEA (1.41 mL) was added. To this solution was added the preformed activated ester solution of Boc-Phe-OH, which was generated by Boc-Phe-OH (1.43 g, 4.55 mmol), HOBT (0.615 g, 4.55 mmol), and EDC (0.872 g, 4.55 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred at room temperature for 4 h and concentrated in vacuo. The residue was dissolved in EtOAc (150 mL), washed with 5% citric acid, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, dried over Na_2SO_4, and evaporated under vacuum. Flash column chromatography purification (CH_2Cl_2/CH_3OH, 20:1) gave a white solid (1.80 g, 87%); mp 60–61 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.33–7.23 (m, 10H), 7.08 (br d, *J*=4.8 Hz, 1H), 5.50 (br s, 1H), 5.06 (d, *J*=5.8 Hz, 1H), 4.54–4.36 (m, 4H), 4.10–3.90 (m, 1H), 3.70–3.45 (m, 2H), 3.10–2.85 (m, 2H), 2.15–1.75 (m, 2H), 1.40 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7, 171.5, 156.0, 137.9, 136.5, 129.2, 128.8, 128.6, 128.0, 127.0, 80.5, 73.5, 67.9, 57.1, 52.1, 37.7, 30.8, 28.3; IR (KBr) 3296.1, 1667.5, 1520.0, 1366.7, 1167.6 cm^{-1}; HRMS (FAB) *m/z* calcd for $C_{25}H_{34}N_3O_5$ $[MH]^+$ 456.2493, found 456.2467.

4.1.4. Boc-Phe-(*S*)-Hse(Bn)-NH_2 ((*S*)-9). The compound was synthesized using the procedure described above in Section 4.1.3 from (*S*)-**8** (2.06 g, 6.68 mmol) and Boc-Phe-OH (1.71 g, 6.68 mmol) as a white solid (2.74 g, 90%); mp 149–150 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.34–7.26 (m, 10H), 4.60–4.44 (m, 3H), 4.32–4.25 (m, 1H), 3.55 (t, *J*=5.0 Hz, 2H), 3.10 (dd, *J*=13.9, 5.6 Hz, 1H), 2.85 (dd, *J*=13.4, 8.4 Hz, 1H), 2.20–1.91 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.6, 171.4, 156.0, 137.8, 136.2, 129.6, 129.2, 128.6, 128.0, 127.7, 127.3, 80.8, 73.3, 68.3, 56.7, 52.3, 37.9, 30.7, 28.3; IR ($CDCl_3$) 3334.5, 1682.6, 1522.6, 1415.5, 1167.5 cm^{-1}; HRMS (FAB) *m/z* calcd for $C_{25}H_{34}N_3O_5$ $[MH]^+$ 456.2493, found 456.2467.

4.1.5. Boc-Phe-(*R*)-gHse(Bn)-NH_2 ((*R*)-10). To a stirred suspension of (*R*)-**9** (1.51 g, 3.32 mmol) in 20 mL of acetonitrile and distilled water (1:1) was added bis(trifluoroacetoxy)iodobenzene (BTI) (1.57 g, 3.65 mmol). After 4 h of stirring under N_2 at room temperature, a clear solution 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 phase was lyophilized. The resulting diamine trifluoroacetate was triturated with Et_2O to give a pale yellow solid (1.51 g, 84%); mp 52–54 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.36–7.23 (m, 5H), 5.30 (m, 1H), 4.53 (s, 2H), 4.35 (dd, *J*=9.2, 6 Hz, 1H), 3.6 (m, 2H), 3.1 (dd, *J*=13.6, 9.2 Hz, 1H), 2.8 (d, 2H), 2.2 (m, 2H), 1.35 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD) δ 173.4, 155.8, 137.4, 136.6, 128.7, 128.5, 127.6, 127.5, 127.0, 126.9, 125.9, 78.9, 72.3, 64.2, 55.7, 55.4, 31.2, 26.7; IR ($CDCl_3$) 2979.5, 1673.7, 1519.8, 1367.8, 1203.0, 1176.5, 1137.7 cm^{-1}; LC–MS (ESI) 450.1 [M+Na], 428.2 $[MH]^+$, 411.0 $[MH-17]^+$; HRMS (FAB) *m/z* calcd for $C_{24}H_{34}N_3O_4$ $[MH]^+$ 428.2544, found 428.2525.

4.1.6. Boc-Phe-(*S*)-gHse(Bn)-NH_2 ((*S*)-10). The compound was synthesized from (*S*)-**9** (3.36 g, 7.37 mmol) using the procedure described above in Section 4.1.5 as a pale yellow solid (2.95 g, 82%); mp 101.5–103 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.34–7.26 (m, 10H), 4.60–4.44 (m, 3H), 4.36–4.25 (m, 1H), 3.60–3.50 (m, 2H), 3.34–3.31 (m, 1H), 3.10 (dd, *J*=13.9, 5.6 Hz, 1H), 2.85 (dd, *J*=13.9, 8.4 Hz, 1H), 2.26–2.06 (m, 1H), 2.04–1.84 (m, 1H); ^{13}C NMR (50 MHz, CD_3OD) δ 173.3, 155.8, 137.4, 136.6, 128.5, 128.2, 127.6, 127.5, 127.4, 127.0, 126.9, 126.3, 126.1, 125.9, 125.8, 18.9, 72.4, 64.3, 63.3, 55.3, 36.8, 26.7; IR ($CDCl_3$) 1667.9, 1406.7, 1163.6, 699.2 cm^{-1}; LC–MS (ESI) 450.1 [M+Na], 428.1 $[MH]^+$, 411.2 $[MH-17]^+$; HRMS (FAB) *m/z* calcd for $C_{24}H_{34}N_3O_4$ $[MH]^+$ 428.2544, found 428.2520.

4.1.7. Boc-Phe-(*R*)-Hse-OH (13). To a solution of Boc-Phe-OH (5.0 g, 18.8 mol) in 1,2-dimethoxyethane (DME) (13 mL) at 0 °C were added HOSu (0.868 g, 18.8 mmol) and DCC (4.28 g, 21 mmol) sequentially. The reaction mixture was stirred at 0 °C overnight and was filtered to remove DCU. The filtrate was concentrated in vacuo and the resulting white solid was recrystallized from 2-propanol (20 mL) to give Boc-Phe-OSu as a white solid (5.72 g, 84%). To

a solution of H-(*R*)-Hse-OH (119 mg, 1 mmol) in 1 M $KHCO_3$ (2.2 mL) and THF (2.2 mL) was added Boc-Phe-OSu (362.4 mg, 1 mmol) at room temperature. The mixture was stirred at room temperature overnight. After THF was removed in vacuo, the resulting solution was acidified to pH 3 by adding 10% citric acid and then extracted with ethyl acetate (50 mL×4). The organic extracts were combined, washed with saturated NaCl, dried over Na_2SO_4, and evaporated to give a colorless oil (347.2 mg, 95%). The crude product was crystallized from a mixture of hexane and ethyl acetate to give a white solid (296 mg, 81%); mp 66–68 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.27–7.21 (m, 5H), 4.54–4.39 (m, 1H), 4.35–4.14 (m, 1H), 3.57–3.49 (m, 2H), 3.13 (dd, J=13.5, 6 Hz, 1H), 2.85 (dd, J=13.5, 9.2 Hz, 1H), 2.08–1.79 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.9, 172.1, 155.6, 136.6, 129.4, 128.8, 128.6, 127.1, 80.7, 65.9, 55.8, 49.0, 38.5, 30.0, 28.3; IR ($CDCl_3$) 3319.1 (br), 2978.2, 1660.5, 1519.7, 1167.9, 1052.0, 733.3 cm^{-1}; LC–MS (ESI) 733.4 $[2M+H]^+$, 367.2 $[M+H]^+$, 311.2. Boc-Phe-OSu: mp 149.5–151.5 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.32–7.24 (m, 5H), 4.75 (dd, J=9.9, 4.8 Hz, 1H), 3.78–3.28 (m, 1H), 3.04 (dd, J=13.9, 9.8 Hz, 1H), 2.86 (s, 4H), 1.38 (s, 9H); LC–MS (ESI) 363.1 [M+1] (100%).

4.1.8. Boc-Phe-(*R*)-Hse-NH_2 (14). The compound was synthesized from **13** (2.15 g, 5.87 mmol) using the procedure described above in Section 4.1.1. The crude product was purified by flash column chromatography (CH_2Cl_2/CH_3OH, 20:1 to 5:1) to give a white solid (1.65 g, 77%); mp 82–83.5 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.34–7.26 (m, 5H), 4.35–4.27 (m, 1H), 4.25–4.17 (m, 1H), 3.34 (m, 2H), 3.09–2.92 (m, 2H), 2.03–1.66 (m, 2H), 1.41 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.4, 172.4, 155.9, 136.7, 129.4, 128.7, 127.1, 80.5, 58.9, 56.6, 50.9, 38.3, 34.6, 28.3; IR ($CDCl_3$) 3303.1 (br), 2978.1, 1664.3, 1523.4, 1367.4, 1167.4 cm^{-1}; LC–MS (ESI) 731.5 $[2M+H]^+$, 366.3 $[MH]^+$, 310.2; HRMS (FAB) *m/z* calcd for $C_{18}H_{28}N_3O_5$ $[MH]^+$ 366.2023, found 366.2008.

4.1.9. Boc-Phe-(*R*)-Hse(Cbz)-NH_2 (16). To a solution of **14** (381.6 mg, 1.04 mmol) in CH_2Cl_2 (5 mL) and pyridine (2.5 mL) at 0 °C was added Cbz-Cl (450 μL, 3.13 mmol). The solution was stirred at 0 °C for 30 min 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 column chromatography (CH_2Cl_2/CH_3OH, 50:1) to give a white solid (481 mg, 92%); mp 156.5–158 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.39–7.09 (m, 12H), 5.36 (d, J=7.0 Hz, 1H), 5.15 (s, 2H), 4.53–4.43 (m, 1H), 4.22 (dd, J=14.6, 7.2 Hz, 1H), 3.98 (t, J=5.6 Hz, 2H), 3.03–2.96 (m, 2H), 2.13–2.03 (m, 1H), 1.95–1.82 (m, 1H), 1.38 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7, 172.3, 156.1, 155.0, 136.4, 135.2, 129.2, 128.7, 128.4, 127.0, 80.5, 69.8, 64.6, 56.6, 50.0, 38.1, 30.5, 28.2; IR ($CDCl_3$) 3296.6, 2977.6, 1747.0, 1666.9, 1498.1, 1455.3, 1266.3, 1167.0, 910.4 cm^{-1}; LC–MS (ESI) 500.3, 444.2, 400.2; HRMS (FAB) *m/z* calcd for $C_{26}H_{34}N_3O_7$ $[MH]^+$ 500.2391, found 500.2360.

4.1.10. Boc-Phe-(*R*)-Hse(TBDPS)-NH_2 (18). To a solution of **14** (318.5 mg, 0.87 mmol) in DMF (1 mL) at 0 °C were added imidazole (130 mg, 1.91 mmol) and TBDPS-Cl (249.3 μL, 0.96 mmol). The solution was stirred at room temperature for 5 h and was partitioned between ethyl acetate (50 mL) and water (10 mL). The organic phase was washed with 5% citric acid, saturated $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified through flash column chromatography (CH_2Cl_2/CH_3OH, 40:1) to give a white solid (506 mg, 96%); mp 71–72.5 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.68–7.03 (m, 15H), 4.64–4.60 (m, 1H), 4.02–3.99 (m, 1H), 3.85–3.66 (m, 2H), 3.08–2.90 (m, 2H), 2.06–1.79 (m, 2H), 1.42 (s, 9H), 1.09 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.8, 171.6, 162.6, 156.0, 136.4, 135.6, 133.4, 132.9, 129.2, 128.8, 127.9, 127.0, 80.5, 61.7, 57.2, 51.8, 37.8, 36.5, 33.1, 31.5, 28.3, 27.1, 19.3; IR ($CDCl_3$) 3293.5, 2931.1, 1668.2, 1497.7, 1169.6, 1111.5, 734.4, 701.9 cm^{-1}; LC–MS (ESI) 576.4 $[M+H]^+$ (100%), 559.4, 503.3, 459.3, 312.2; HRMS (FAB) *m/z* calcd for $C_{34}H_{46}N_3O_5Si$ $[MH]^+$ 604.3201, found 604.3163.

4.1.11. Boc-Phe-(*R*)-gHse(Cbz)-NH_2·TFA (20). The compound was synthesized from **16** (481 mg, 0.96 mmol) using the procedure described above in Section 4.1.5. The crude product was triturated with Et_2O to give a white solid (559.2 mg, 99%); mp 121–122.5 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.42–7.34 (m, 5H), 7.23–7.17 (m, 5H), 5.18 (s, 2H), 5.03 (dd, J=9.6, 5.2 Hz, 1H), 4.27 (t, J=7.8 Hz, 1H), 4.13–4.05 (m, 1H), 3.86–3.81 (m, 1H), 3.03 (dd, J=13.5, 7.2 Hz, 1H), 2.89 (dd, J=13.5, 8.0 Hz, 1H), 2.14–2.00 (m, 2H), 1.41 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$, 5% CD_3OD) δ 173.4, 155.9, 154.8, 136.3, 135.0, 129.3, 128.7, 128.5, 127.1, 80.7, 70.1, 63.0, 55.9, 50.2, 38.2, 31.4, 28.2; IR ($CDCl_3$) 2979.2, 1750.7, 1670.8, 1265.3, 1203.2 cm^{-1}; LC–MS (ESI) 494.3 $[M+Na]^+$, 472.3 $[M+H]^+$; HRMS (FAB) *m/z* calcd for $C_{25}H_{34}N_3O_6$ $[MH]^+$ 472.2442, found 472.2416.

4.1.12. Boc-Phe-(*R*)-gHse(TBDPS)-NH_2·TFA (21). The compound was synthesized from **18** (473.8 mg, 0.79 mmol) using the procedure described above in Section 4.1.5. The crude product was triturated with Et_2O to give a pale yellow solid (519.4 mg, 96%); mp 107–108.5 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.69–7.12 (m, 15H), 6.34 (br s, 1H), 5.08 (br s, 1H), 4.85 (dd, J=12.6, 5.8 Hz, 1H), 4.22–4.18 (m, 1H), 3.80–3.64 (m, 2H), 3.03–2.98 (m, 2H), 1.70–1.61 (m, 2H), 1.43 (s, 9H), 1.09 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.8, 171.6, 162.6, 156.0, 136.4, 135.6, 133.4, 132.9, 130.0, 129.2, 128.8, 127.9, 127.0, 80.5, 61.7, 57.2, 51.8, 37.8, 33.1, 28.3, 27.1, 19.3; IR ($CDCl_3$) 3316.3, 2932.0, 1674.4, 1531.8, 1429.1, 1203.0, 1114.0 cm^{-1}; LC–MS (ESI) 576.44 $[M+H]^+$, 559.4 $[MH]^+$ (100%), 503.3, 459.3, 312.2; HRMS (FAB) *m/z* calcd for $C_{33}H_{46}N_3O_4Si$ $[MH]^+$ 576.3252, found 576.3215.

4.1.13. Boc-Phe-(*R*)-gHse-NH_2 ((*R*)-11). *Method I.* To a solution of (*R*)-**10** (1.44 g, 2.66 mmol) in methanol (50 mL) was added 0.2 N HCl (~5 mL) until pH 3, followed by the addition of 10% Pd–C (0.15 g). The reaction mixture was shaken under 50 psi H_2 for 48 h and no more starting material was detected by TLC (CH_2Cl_2/CH_3OH, 5:1). The catalyst was removed by filtration through Celite 545 and the filtrate was concentrated in vacuo. The residue (a white foamy solid) was dissolved in 5 mL of methanol and passed through a Varian Bond Elut® cation exchange cartridge (1 g,

SCX, benzenesulfonic acid sorbent) according to the instructions in the manual. The cartridge was washed by methanol and the product was eluted using 1% TEA in methanol to give a white solid (601 mg, 67%) after solvent evaporation in vacuo. *Method II*. To a solution of **20** (435.2 mg, 0.743 mmol) in methanol (30 mL) was added 10% Pd–C. H_2 (60 psi) was established and maintained for 4.5 h. The catalyst was filtered through Celite 545 and the filtrate was concentrated in vacuo to give a white solid (307 mg, 98%). *Method III*. To a stirred solution of **21** (506 mg, 0.73 mmol) in THF (15 mL) under argon at 0 °C was added $Bu_4N^+F^-$ (2.57 mL, 1 M in THF). The solution was stirred at 0 °C for 5 min and at room temperature for 1 h. Solvent was evaporated in vacuo and the residue was purified by flash column chromatography (CH_2Cl_2/CH_3OH/TEA, 50:1:0.1) to give a white solid (95.7 mg, 77%); mp 70.5–72 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.38–7.24 (m, 5H), 5.18–5.06 (m, 1H), 4.33–4.26 (m, 1H), 3.60–3.40 (m, 3H), 3.03 (dd, J=13.9, 6.6 Hz, 1H), 2.90 (dd, J=13.9, 8.2 Hz, 1H), 2.01–1.80 (m, 2H), 1.40 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD) δ 172.1, 155.6, 136.6, 128.5, 127.6, 125.9, 78.8, 57.6, 57.0, 55.7, 37.6, 37.0, 26.8; IR (KBr) 3293.2 (br), 2976.9, 1653.4, 1532.8, 1251.0, 1168.3, 1057.0, 753.0, 699.9 cm^{-1}; LC–MS (ESI) 675.5 $[2M+1]^+$, 338.2 $[M+H]^+$, 265.1; HRMS (FAB) *m/z* calcd for $C_{17}H_{28}N_3O_4$ $[MH]^+$ 338.2074, found 338.2061.

4.1.14. Boc-Phe-(*S*)-gHse-NH_2 ((*S*)-11). The compound was synthesized from (*S*)-**10** (2.23 g, 4.12 mmol) according to the *method I* in Section 4.1.13 as a white solid (986 mg, 71%); mp 51–52.5 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.25 (m, 5H), 4.69 (t, J=6.5 Hz, 1H), 4.22 (dd, 1H, J=8.0, 6.0 Hz), 3.51 (t, J=6.6 Hz, 2H), 3.06 (dd, J=13.4, 6.3 Hz, 1H), 2.84 (d, J=13.4, 8.0 Hz, 2H), 1.69 (m, 2H), 1.30 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD) δ 172.2, 155.8, 136.9, 128.7, 127.8, 127.6, 125.9, 78.8, 57.7, 57.0, 55.7, 37.5, 37.2, 26.9; IR ($CDCl_3$) 3297.6 (br), 2977.8, 1657.8, 1531.4, 1367.0, 1250.7, 1168.9, 1055.7, 755.0 cm^{-1}; LC–MS (ESI) 675.5 $[2M+1]^+$, 338.5 $[M+H]^+$ (100%); HRMS (FAB) *m/z* calcd for $C_{17}H_{28}N_3O_4$ $[MH]^+$ 338.2074, found 338.2062.

4.1.15. Boc-Phe-(4*S*)-4-NH-CPA ((2*R*,4*S*)-6 and (2*S*,4*S*)-6). To a stirred solution of bis(2-chloroethyl)phosphoramidic dichloride (222 mg, 0.86 mmol) in anhydrous THF (20 mL) under argon at 0 °C was added a solution of (*R*)-**11** (263 mg, 0.78 mmol) and TEA (241 µL, 1.71 mmol) in anhydrous THF (20 mL). The reaction solution was stirred at 0 °C for 30 min and at room temperature for 48 h. The precipitate in the solution was filtered and the solution was evaporated in vacuo. The residue was purified by flash column chromatography (hexane/EtOAc/CH_3OH, 2:1:0.1) to give Boc-Phe-NH_2 (65 mg, 38%), (2*S*,4*S*)-**6** (the faster eluting isomer) (69 mg, 20%) and (2*R*,4*S*)-**6** (the slower eluting isomer) (27 mg, 8%). The yield was calculated after the recovery of 15% of the starting material.

Compound (2*S*,4*S*)-**6**: white solid; R_f (hexane/EtOAc/CH_3OH, 1:1:0.1) 0.45. ^1H NMR (200 MHz, CD_3OD) δ 7.34–7.19 (m, 5H), 5.24 (dt, J=20.6, 4.4 Hz, 1H), 4.35–4.25 (m, 1H), 4.13–4.04 (m, 2H), 3.68–3.62 (m, 4H), 3.51–3.37 (m, 4H), 3.19–2.78 (m, 2H), 2.05–1.93 (m, 1H), 1.64–1.55 (m, 1H), 1.42 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.2, 156.0, 136.7, 128.6, 127.6, 126.0, 78.9, 63.5 (d, J=5.2 Hz), 57.9, 55.2, 41.1, 37.5, 29.7, 26.8; ^{31}P NMR (121 MHz, CD_3OD) δ 11.6 (s); IR (KBr) 2974.4, 2379.5, 1666.7, 1410.3, 1364.1, 1230.8, 1164.1 cm^{-1}; LC–MS (ESI) 559.2, 557.2, 555.2 $[M+Na]^+$, 523.2 $[M+1]^+$; HRMS (FAB) *m/z* calcd for $C_{21}H_{34}N_4O_5PCl_2$ $[MH]^+$ 523.1644, found 523.1653.

Compound (2*R*,4*S*)-**6**: white solid; R_f (hexane/EtOAc/CH_3OH, 1:1:0.1) 0.20. ^1H NMR (200 MHz, CD_3OD) δ 7.30–7.22 (m, 5H), 5.34–5.30 (m, 1H), 4.25 (m, 3H), 3.68–3.62 (m, 4H), 3.50–3.40 (m, 4H), 3.04–2.90 (m, 2H), 1.80–1.61 (m, 2H), 1.42 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD) δ 172.0, 136.4, 128.6, 127.6, 126.0, 78.9, 63.5 (d, J=6.1 Hz), 57.7, 55.5, 41.1, 37.7, 29.9, 26.9; ^{31}P NMR (121 MHz, CD_3OD) δ 13.6 (s); IR (KBr) 3405.1 (br), 2974.4, 1671.8, 1364.1, 1251.3, 1215.4, 1164.1, 1046.2 cm^{-1}; LC–MS (ESI) 559.2, 557.2, 55.2 $[M+Na]^+$, 527.2, 525.3, 523.2 $[M+H]^+$ (100%); HRMS (FAB) *m/z* calcd for $C_{21}H_{34}N_4O_5PCl_2$ $[MH]^+$ 523.1644, found 523.1643.

4.1.16. Boc-Phe-(4*R*)-4-NH-CPA ((2*R*,4*R*)-6 and (2*S*,4*R*)-6). Compound (*S*)-**11** (178 mg, 0.53 mmol) was reacted with bis(2-chloroethyl)phosphoramidic dichloride (259 mg, 0.58 mmol) in the presence of TEA (163 µL, 1.2 mmol) according to the procedure described above in Section 4.1.15. The products were isolated by flash column chromatography (hexane/EtOAc/CH_3OH, 2:1:0.1) to give Boc-Phe-NH_2 (8.7 mg, 25%), (2*R*,4*R*)-**6** (34 mg, 13%) and (2*S*,4*R*)-**6** (14 mg, 6%). The yield was calculated after the recovery of 11% of the starting material.

Compound (2*R*,4*R*)-**6**: white solid; R_f (hexane/EtOAc/CH_3OH, 1:1:0.1) 0.60. ^1H NMR (200 MHz, CD_3OD) δ 7.34–7.20 (m, 5H), 5.32 (dt, J=20.6, 4.8 Hz, 1H), 4.40–4.18 (m, 3H), 3.76–3.64 (m, 4H), 3.57–3.36 (m, 4H), 3.20–3.10 (m, 1H), 2.92–2.80 (m, 1H), 2.25–2.13 (m, 1H), 1.95–1.87 (m, 1H), 1.40 (s, 9H); ^{31}P NMR (121 MHz, CD_3OD) δ 10.7 (s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.1, 171.5, 155.4, 136.9, 129.5, 129.4, 128.7, 128.6, 127.0, 126.9, 80.2, 63.7, 59.1, 55.6, 48.7 (d, J=5.0 Hz), 42.3, 38.5, 30.1, 28.4; IR (KBr) 3295.9, 2978.3, 2931.7, 1682.0, 1497.2, 1454.7, 1367.0, 1246.7 (P=O), 1168.4, 910.3, 734.2 cm^{-1}; LC–MS (ESI) 547.0, 545.25 $[M+Na]^+$, 523.1 $[M+H]^+$; HRMS (FAB) *m/z* calcd for $C_{21}H_{34}N_4O_5PCl_2$ $[MH]^+$ 523.1644, found 523.1634.

Compound (2*S*,4*R*)-**6**: white solid; R_f (hexane/EtOAc/CH_3OH, 1:1:0.1) 0.20. ^1H NMR (200 MHz, CD_3OD) δ 7.28 (s, 5H), 5.43–5.25 (m, 1H), 4.37–4.25 (m, 3H), 3.65–3.50 (m, 4H), 3.44–3.33 (m, 4H), 3.08 (dd, J=13.6, 5.4 Hz, 1H), 2.87 (dd, J=13.6, 8.6 Hz, 1H), 2.20–1.80 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD) δ 172.2, 155.8, 136.7, 128.6, 127.6, 126.0, 78.9, 63.6 (d, J=5.8 Hz), 57.9, 55.5, 41.1, 37.5, 29.9, 26.8; ^{31}P NMR (121 MHz, CD_3OD) δ 13.7 (s); IR ($CDCl_3$) 3277.5, 2976.5, 1670.4, 1498.2, 1455.0, 1366.8, 1218.3 (P=O), 1167.4, 988.3, 754.7 cm^{-1}; LC–MS (ESI) 549.1, 547.1, 545.1 $[M+Na]^+$, 527.2, 525.1, 523.2 $[MH]^+$ (100%); HRMS (FAB) *m/z* calcd for $C_{21}H_{34}N_4O_5PCl_2$ $[MH]^+$ 523.1644, found 523.1629.

4.1.17. H-Phe-(2*S*,4*S*)-4-NH-CPA·TFA ((2*S*,4*S*)-4). Compound (2*S*,4*S*)-**6** (8.1 mg, 0.016 mmol) was treated with 25% (v/v) TFA in CH_2Cl_2 for 30 min at room temperature.

After evaporation of solvents, the residue was dried under vacuum to give a white semi-solid (8.3 mg, 100%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.51–7.39 (m, 5H), 5.29 (dt, J=11.8, 2.0 Hz, 1H), 4.47–4.39 (m, 1H), 4.35–4.26 (m, 1H), 4.15 (t, J=3.8 Hz, 1H), 3.77 (t, J=3.6 Hz, 4H), 3.61–3.47 (m, 4H), 3.27 (dd, J=6.8, 4.0 Hz, 1H), 3.21 (dd, J=6.8, 3.8 Hz, 1H), 2.31–2.22 (m, 1H), 1.70–1.66 (m, 1H); ^{13}C NMR (50 MHz, CD_3OD) δ 167.7, 133.7, 128.6, 128.2, 127.0, 62.7 (d, J=6.4 Hz), 58.2 (d, J=3.0 Hz), 53.8, 40.9, 36.6, 28.2 (d, J=6.5 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ 11.8 (s); LC–MS (ESI) 847.2, 427.2, 425.2, 423.2 $[MH]^+$ (100%), 203.2; HRMS (FAB) m/z calcd for $C_{16}H_{26}N_4O_3PCl_2$ $[MH]^+$ 423.1120, found 423.1140.

4.1.18. H-Phe-(2*R*,4*R*)-4-NH-CPA·TFA ((2*R*,4*R*)-4). The compound was prepared from (2*R*,4*R*)-**6** (3.5 mg, 0.0067 mmol) according to the procedure described above in Section 4.1.17 as a semi-solid (3.6 mg, 100%). ^{1}H NMR (200 MHz, CD_3OD) δ 7.38–7.23 (m, 5H), 5.30 (dt, J=21.2, 4.8 Hz, 1H), 4.53–4.10 (m, 2H), 3.71–3.64 (m, 5H), 3.50–3.34 (m, 4H), 3.13 (dd, J=13.9, 5.0 Hz, 1H), 2.88 (dd, J=13.9, 8.2 Hz, 1H), 2.27–2.12 (m, 1H), 1.91–1.80 (m, 1H); ^{13}C NMR (50 MHz, CD_3OD) δ 173.2, 136.3, 128.7, 127.8, 126.1, 63.0 (d, J=6.5 Hz), 58.0 (d, J=2.3 Hz), 40.9, 39.4, 29.0 (d, J=6.8 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ 11.2 (s); LC–MS (ESI) 847.2 $[2M+1]^+$, 425.1, 423.1 $[M+1]^+$ (100%), 203.0; HRMS (FAB) m/z calcd for $C_{16}H_{26}N_4O_3PCl_2$ $[MH]^+$ 423.1120, found 423.1123.

4.1.19. H-Phe-(2*R*,4*S*)-4-NH-CPA·TFA ((2*R*,4*S*)-4). Compound (2*R*,4*S*)-**6** (19.5 mg, 0.0373 mmol) was treated with 25% (v/v) TFA/CH_2Cl_2 to give a mixture as a semi-solid (17 mg, 96%). The mixture was separated by preparative HPLC to give (2*R*,4*S*)-**4** (6 mg, 37%) and (2*R*,4*R*)-**4** (3.5 mg, 22%). The remaining amount was recovered as a mixture of the two compounds. (2*R*,4*S*)-**4**: ^{1}H NMR (200 MHz, CD_3OD) δ 7.52–7.39 (m, 5H), 5.54–5.50 (m, 1H), 4.48–4.41 (m, 1H), 4.36–4.27 (m, 1H), 3.78–3.74 (m, 4H), 3.58–3.51 (m, 4H), 2.27–3.2 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD) δ 167.3, 133.6, 128.6, 128.2, 127.0, 63.4 (d, J=5.6 Hz), 58.0, 53.7, 41.0, 36.8, 29.7 (d, J=4.6 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ 11.2 (s); LC–MS (ESI) 16.0 min, 847.1 $[2M+1]^+$, 455.3, 425.3, 423.3 $[MH]^+$, 203.3; HRMS (FAB) m/z calcd for $C_{16}H_{26}N_4O_3PCl_2$ $[MH]^+$ 423.1120, found 423.1135.

4.1.20. H-Phe-(2*S*,4*R*)-4-NH-CPA·TFA ((2*S*,4*R*)-4). Compound (2*S*,4*R*)-**6** (12 mg, 0.0229 mmol) was treated with 25% (v/v) TFA/CH_2Cl_2 to give a mixture as a white solid (11.3 mg, 89%). The mixture was separated by preparative HPLC to give (2*S*,4*R*)-**4** (3 mg, 30%) and (2*S*,4*S*)-**4** (2.5 mg, 25%). The remaining amount was recovered as a mixture of the two compounds. (2*S*,4*R*)-**4**: ^{1}H NMR (200 MHz, CD_3OD) δ 7.40–7.23 (m, 5H), 5.45–5.35 (m, 1H), 4.37–4.20 (m, 2H), 3.75–3.57 (m, 5H), 3.51–3.42 (m, 4H), 3.05 (dd, J=13.6, 6.2 Hz, 1H), 2.86 (dd, J=13.6, 7.8 Hz, 1H), 2.09–1.83 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD) δ 173.7, 136.5, 128.6, 127.8, 126.1, 63.5 (d, J=5.7 Hz), 57.8, 55.3, 41.0, 40.0, 29.8 (d, J=4.6 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ 15.5 (s); LC–MS (ESI) 847.2 $[2M+1]^+$, 425.1, 423.1 $[M+H]^+$, 203.1 (100%); HRMS (FAB) m/z calcd for $C_{16}H_{26}N_4O_3PCl_2$ $[MH]^+$ 423.1120, found 423.1135.

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