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Microwave-Assisted Solid-Phase Aza-peptide Synthesis: Aza Scan of a PKB/Akt Inhibitor Using Aza-arginine and Aza-proline Precursors

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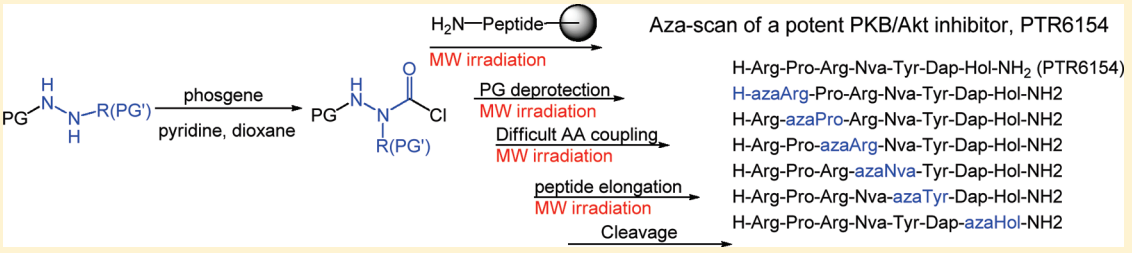
Supporting Information

ABSTRACT:



Aza-peptides are peptidomimetics in which one or more of the α-carbons, bearing the side-chain residues, has been replaced by a nitrogen. These peptidomimetics have been shown to be promising for the generation of drug leads and for structure−activity relationship studies. Aza-scan is the systematic replacement of amino acid residues in a given peptide with their aza counterparts. We report here an aza-scan of a potent, peptide-based PKB/Akt inhibitor, PTR6154. Procedures for microwave-assisted, Fmoc/t-Bu chemistry, solid-phase aza-peptide synthesis were developed which significantly reduce standard reaction time and are suitable for automation. Novel substituted hydrazines have been prepared for the straightforward incorporation of aza-arginine and aza-proline residues. This work will enable aza-scan to become a more common and standard method for structure−activity relationship studies of peptides.

INTRODUCTION

Proteins and peptides play diverse essential roles in biological systems and may be used as natural drug candidates. However, their therapeutic use is significantly limited by unfavorable pharmacokinetic and pharmacological properties. Peptidomimetics are designed to enhance biological activity while overcoming undesirable peptide properties such as rapid metabolism by proteolysis, poor bioavailability, and nonselective receptor binding.[1−7]

Many structural modifications of the amino acid side chains as well as the peptide backbone have been introduced and extensively studied.[1,8,9] Among these modifications, aza-peptides have been shown to be promising for the generation of peptidomimetic drug leads and for structure−activity relationship (SAR) studies.[10,11] Aza-peptides are peptides in which one or more of the α-carbons, bearing the side-chain residues, has been replaced by a nitrogen atom (Figure 1). Aza-amino acid residues impart spatial conformational changes to the parent peptide structure due to the loss of stereogenicity and reduction of flexibility.[12]

Aza-amino acid modifications affect a peptide both locally and globally. The incorporated aza-amino acid can adopt the proper local pharmacophore orientation for activity and selectivity yet confer resistance toward proteolytic degradation. The global reduction in flexibility that is conferred by the generation of hydrazide and urea structural elements has been shown to induce β-turn conformations.[13,14] The replacement of a particular Cα by N in a biologically active peptide can affect its overall conformation and hence its absorption, transport, distribution, enzyme or receptor binding, and metabolic stability. There have been several reports of an increase in the biological activity and/or improvement of the pharmacokinetic properties of aza-peptides, compared to the parent peptides.[2,10,13−15] Aza-scan is the systematic replacement of amino acid residues in a given peptide with their aza-counterparts.[14] This methodology can lead to the identification of significant backbone

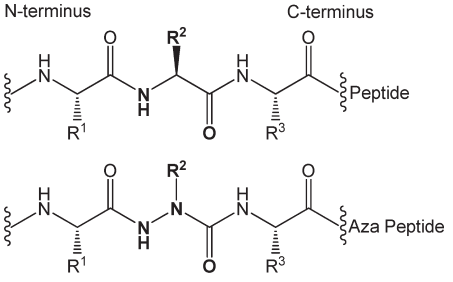


Figure 1. Peptide and aza-peptide.

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interactions and to bioactive β-turn conformations and is therefore a powerful tool for SAR studies and drug design. Although a method with very high potential, the synthetic challenges associated with solid-phase aza-peptide synthesis have limited the use of this method to experienced chemists. Overcoming these synthetic difficulties would allow more common use of aza-scan for SAR studies.

Compared to routine peptide synthesis, the synthesis of aza-peptides is rather challenging.[16] Introduction of an aza-amino acid residue into a peptide chain is usually achieved by the orthogonally protected hydrazine derivative which is activated with a carbonyl-donating reagent and subsequently applied to the free amino terminus of the peptidyl resin. The coupling of the following amino acid to the aza residue is more difficult than standard amino acid coupling, adding further synthetic challenges to aza-peptide synthesis.[17] In 2005, Boeglin et al. introduced an efficient method for solid-phase Fmoc chemistry aza-peptide synthesis utilizing *N*-Fmoc-aza-amino acid chlorides as building blocks.[13] Recently, we introduced *N*′-substituted Ddz-protected hydrazines and showed their application for Fmoc-chemistry solid-phase aza-peptide synthesis.[18] An alternative submonomer synthetic strategy for solid-phase aza-peptide synthesis has been recently reported.[19]

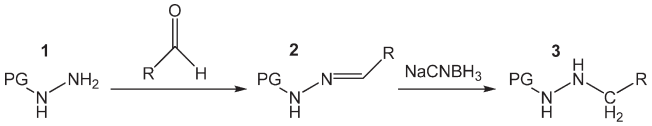
The use of microwave technology to enhance solid-phase chemical reactions frequently results in shorter reaction times and in increased product yield and/or purity.[20−22] Impressive improvements have been reported using microwave-assisted SPPS for the synthesis of difficult sequences, with regard to both coupling/deprotection speed and to product purity/yield.[23−30] Microwave-assisted SPPS has also achieved difficult couplings associated with peptidomimetic synthesis, facilitating the synthesis of attractive peptidomimetic compounds.[31−39] To the best of our knowledge, to date, no one has demonstrated microwave-assisted aza-peptide synthesis.

Persistently activated protein kinase B (PKB/Akt) is associated with many types of human cancer.[40] Consequently, PKB/Akt is an attractive target for potential cancer therapy. Small molecular weight ATP mimetic inhibitors, even those that have been claimed to be highly selective, frequently exhibit low selectivity toward the desired kinase.[41−44] Peptide inhibitors derived from the protein substrate have shown greater potential as selective inhibitors because of multiple and specific interactions with the protein kinase binding site.[45−47] Recently, the peptide Arg-Pro-Arg-Nva-Tyr-Dap-Hol (PTR6154), derived from the glycogen synthase kinase 3 (GSK-3) substrate peptide segment Arg-Pro-Arg-Thr-Ser-Ser-Phe, showed potential as a selective PKB/Akt inhibitor.[48] In previous peptidomimetic SAR studies of PTR6154, which included *N*-methyl peptides, peptoids, and backbone cyclic peptides, we pointed out the importance of the peptide backbone interactions.[49,50] In the current study, we performed an aza-scan of PTR6154 in order to gain more insight into the peptide−protein SAR and to hopefully obtain a more active drug lead. Novel procedures for Fmoc/t-Bu[51,52] microwave-assisted solid-phase aza-peptide synthesis were developed which significantly reduce the time of solid-phase aza-peptide synthesis. In addition, two novel protected substituted hydrazines were prepared to enable the incorporation of Aza-arginine and Aza-proline amino acids using Fmoc-chemistry solid-phase aza-peptide synthesis.

RESULTS AND DISCUSSION

Synthesis of Substituted Hydrazine. The synthesis of *N*′-substituted protected hydrazines suitable for solid-phase synthesis of aza-peptides was performed according to previous procedures for the synthesis of Fmoc- and Ddz-substituted hydrazines (Scheme 1).[13,18] Condensation of protected hydrazide **1** with the appropriate aldehyde readily gave the corresponding hydrazone **2**, which was reduced without further purification. Reduction was performed with sodium cyanoborohydride under mildly acidic conditions, achieved by acetic acid. The obtained $CNBH_2$ adduct[53] was subsequently hydrolyzed with aqueous NaOH in methanol for Ddz-protected hydrazines or by reflux in ethanol for Fmoc-protected hydrazines to give the desired *N*′-substituted protected hydrazines **3**.

Scheme 1. Synthesis of *N*′-Alkyl-*N*-protected Hydrazines



Synthesis of Aza-arginine Precursor. Aza-arginine (azaArg) peptides have been reported only twice.[13,54] The synthetic pathway reported previously for azaArg peptides involves the incorporation of an orthogonally protected aza-ornithine (Alloc) residue followed by selective Alloc deprotection and subsequent guanidinylation to obtain an azaArg peptide. In order to avoid these additional synthetic steps, we sought to prepare a substituted hydrazine which would enable us to incorporate an azaArg residue following the same procedures for the incorporation of other aza-amino acids. Several protecting groups for the guanidine moiety of arginine are used for routine Fmoc/t-Bu SPPS, the most common being Pbf.[55] The precursor for introduction of the Pbf group was unavailable to us. Instead, we used the more convenient and readily available Boc group for protection. Di-Boc-protected guanidine propane derivatives were readily obtained by guanidinylation of amines using *N*,*N*′-bis-Boc-methylisothiourea, with DMAP as a catalyst, as previously reported (Scheme 2).[56] However, our attempts to prepare 3-(diBoc)guanidino propylaldehyde **7**, for subsequent reaction with protected hydrazine as described above, encountered considerable synthetic difficulties. $LiAlH_4$ reduction of the corresponding 3-(diBoc)guanidino propanoic Weinreb amide **4** resulted in low yields of a mixture which proved difficult to purify. Our many attempts to prepare the aldehyde by hydrolysis of the corresponding 3-(diBoc)guanidino propylaldehyde diethyl acetal **5** were completely unsuccessful, giving no sign of the desired aldehyde. Our initial attempts to oxidize the corresponding 3-(diBoc)guanidino propanol **6** using PCC,[57,58] MnO_2,[59] and SO_3·Py complex[60,61] with a variety of catalysts were also completely unsuccessful, affording the pure starting material alcohol unaffected by the oxidation reagents. Finally, the desired aldehyde was obtained by oxidation with Dess−Martin periodinane.[62,63] The original procedure for the Dess−Martin oxidation offers two possible quenching and workup procedures: (1) the reaction mixture is washed with an aqueous mixture of sodium bicarbonate and sodium thiosulfate or (2) the reaction mixture is diluted with ether and washed with dilute aqueous sodium hydroxide. The authors also reported that the addition of pyridine to the reaction mixture did not affect the oxidizing performance of the reagent. In our hands, quenching and workup with the aqueous sodium bicarbonate and sodium thiosulfate mixture led to intramolecular cyclization and loss of one Boc protecting group. We assumed that the acetic acid byproduct released by the oxidation reaction and/or the quenching of the reaction with an aqueous sodium bicarbonate and sodium thiosulfate mixture was destructive to the aldehyde. To

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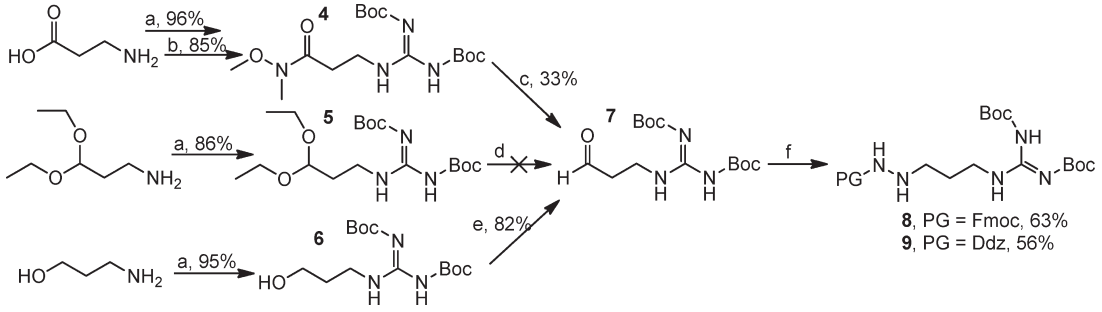
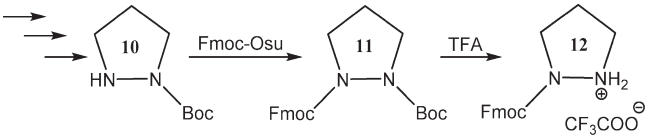
Scheme 2. Synthesis of azaArg Precursors[a]



[a] Reagents: (a) *N,N′*-bis-Boc-methylisothiourea, DMAP; (b) *N,O*-dimethylhydroxylamine hydrochloride, PyBop, DIPEA; (c) $LiAlH_4$; (d) $AcOH/H_2O$; (e) Dess−Martin periodinane; (f) (1) PG-NH-NH_2 then (2) $NaCNBH_3$, AcOH.

Scheme 3. Synthesis of azaPro Precursor



completely avoid acidity, we performed the Dess−Martin oxidation in the presence of pyridine and quenched the reaction with dilute aqueous sodium hydroxide to achieve full oxidation of the alcohol to the desired aldehyde with no side products. The aldehyde was employed without further purification to prepare Fmoc-azaArg-(diBoc) and Ddz-azaArg(diBoc) precursors, which were successfully used for introducing the azaArg residue using microwave-assisted SPPS.

Synthesis of Aza-proline Precursor. The synthesis of an azaPro precursor requires an alternative synthetic strategy due to the cyclic pyrazolidine structure. AzaPro-containing peptides have been previously synthesized;[14,64−72] however, to the best of our knowledge, the incorporation of azaPro residues using Fmoc SPPS strategy has been briefly reported only once.[73]

The azaPro precursor, *N*-Fmoc-pyrazolidine, was prepared according to Scheme 3. *N*-Boc-pyrazolidine **10** was prepared as previously reported[14,65] and treated with Fmoc-Osu to give diprotected pyrazolidine **11**. *N*-Boc-*N′*-Fmoc-pyrazolidine **11** was subsequently treated with neat TFA to effect Boc removal. Then Et_2O was added, and the resulting *N*-Fmoc-pyrazolidine **12** precipitated as a TFA salt.

Microwave-Assisted Solid-Phase Aza-Peptide Synthesis (Scheme 4). Our primary goal in this research was to perform an aza-scan of PTR6154 for SAR studies. Our first attempts to perform the aza-scan using standard protocols[13,18] were discouraging, with the single exception of the synthesis of the azaHol analogue. All of our attempts to synthesize the azaTyr and azaNva PTR6154 analogues by standard methods, using either Ddz- or Fmoc-substituted hydrazines, failed. We were hindered by incomplete coupling of the aza-residue to the growing peptide. Moreover, even when the degree of coupling of the aza-residue was sufficient, the next step in the synthesis, namely the coupling to the aza-residue, was in most cases completely unsuccessful. We suspected that these synthetic difficulties might be sequence related, and we reasoned that microwave-assisted aza-peptide synthesis might enhance the coupling of the difficult steps as well as reduce the overall synthesis time. To the best of our knowledge, this report is the first example of microwave-assisted aza-peptide synthesis. Our aza-synthesis efforts were directed at examining the feasibility of microwave-assisted aza-peptide synthesis, and we put extra thought into developing procedures that would facilitate automated microwave-assisted aza-peptide synthesis.

Activation and Coupling of *N′*-Substituted *N*-Protected Hydrazines. After thoroughly examining the aza-peptide synthesis literature, we decided to stick to the proven in situ phosgene activation[13,18,74,75] but replace the DCM with a microwave-compatible solvent. The two common solvents for microwave-assisted peptide synthesis are DMF and NMP. However, phosgene is highly reactive and can react with the amide group of DMF and NMP. Therefore, we activated the substituted hydrazine according to the published procedure in DCM and then evaporated the excess phosgene and the solvent. Subsequently, DMF and DIPEA were added to the activated aza-amino acid, and the mixture was introduced to the free *N*-terminal peptidyl-resin. Successful introduction of the aza-residue was confirmed by "small cleavage" and mass spectrometry analysis. Although the desired aza-peptide was readily obtained, this reaction lacks the potential for automation.

In order to make aza-peptide synthesis compatible with automation, we need a method for isolating the activated aza-amino acids. Unlike the automated synthesis of standard Fmoc-amino acids, in which the active ester is usually generated in situ, aza-amino acids would have to be available as preactivated species, similar to the commercially available Fmoc-amino acid pentafluorophenyl esters. Fortunately, in 1999, Gibson et al. achieved the isolation of aza-alanine amino acid chloride and reported it to be stable during long-term storage at 4 °C.[75] A significant finding in the study of Gibson et al. was the use of dioxane as a solvent for the activation of *N*-Fmoc-*N′*-methyl hydrazine with phosgene. Dioxane was found to be a good solvent for the activating procedure, showing no signs of formation of urea byproduct or of precipitation of *N*-Fmoc-*N′*-methyl hydrazine hydrochloride salt generated by the HCl released during activation. Following a slightly modified procedure, we were able to obtain both Fmoc-protected and Ddz-protected, activated aza-amino acid chlorides. To avoid unwanted deprotection of acid-sensitive Ddz and/or orthogonal Boc side-chain protection, pyridine was added to the reaction mixture. Upon complete activation, as determined by TLC, the pyridinium hydrochloride salt was filtered off and the solvent was evaporated to give sufficiently pure, activated aza-amino acid chloride (with minimal contamination of pyridinium hydrochloride salt) which was then dissolved in DMF. DIPEA was added, and the mixture

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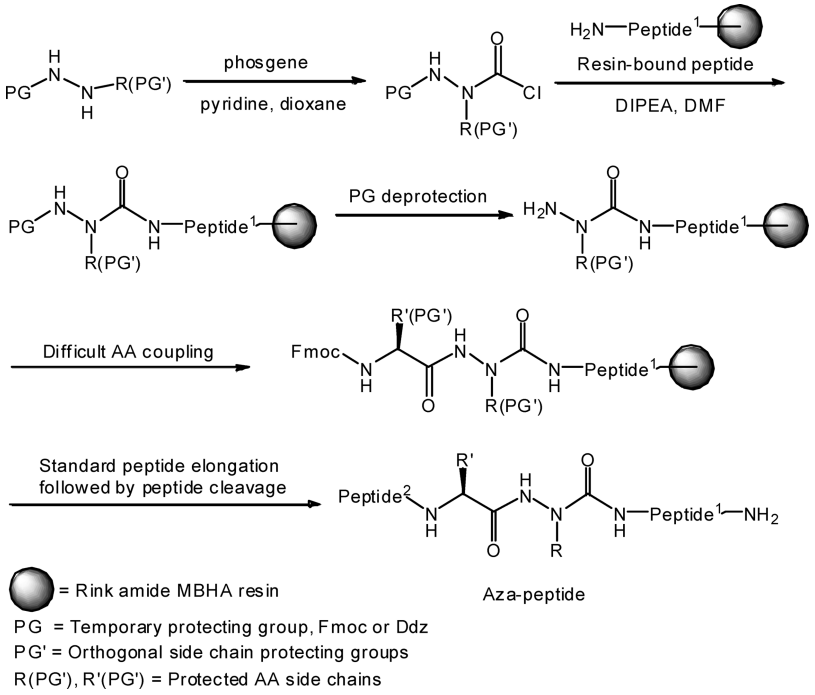
Scheme 4. Microwave-Assisted Solid-Phase Aza-peptide Synthesis[a]



[a] All reactions except for the phosgene activation and final cleavage were conducted under microwave irradiation. Reagents and conditions: Substituted hydrazine activation. PG-NH-NH-R(PG′) (3 equiv), pyridine (3 equiv), phosgene (6 equiv) in dry dioxane, 10 min. Aza amino acid chloride coupling. PG-aza-AA-Cl (3 equiv), DIPEA (3 equiv) in DMF 2 × 20 min, 25 W, 75 °C; PG deprotection. For Fmoc 20% piperidine in NMP 3 × 3 min, 50 W, 75 °C; for Ddz 10 equiv of $Mg(ClO_4)_2$ in ACN 2 × 15 min, 25 W, 70 °C. Difficult AA coupling. (1) Fmoc-AA-OH (2 equiv), DIPEA (6 equiv), HATU (2 equiv) in DMF 20 min, 25 W, 75 °C; if unsuccessful, proceed to (2) Fmoc-AA-OH (5 equiv), BTC (1.66 equiv), 2,4,6-collidine (23 equiv) in dibromoethane 20 min, 25 W, 75 °C; if unsuccessful, proceed to (3) activation in DCM and coupling in DMF: Fmoc-AA-OH (5 equiv), BTC (1.66 equiv), 2,4,6-collidine (23 equiv) in DCM. Evaporate, dissolve in DMF, and apply to resin coupling 20 min, 25 W, 75 °C.

was applied to the resin under microwave irradiation to conveniently obtain the desired aza-peptide. Importantly, the reaction time for aza-residue coupling was substantially reduced, from about 16 h at rt (2× overnight coupling) to two cycles of 20 min each under microwave irradiation at 75 °C. After aza-amino acid coupling, the temporary Fmoc or Ddz protecting group was conveniently removed under microwave irradiation conditions.

Difficult Coupling of the Following Amino Acid to the Aza-amino Acid. In recent studies, coupling to an aza-residue was achieved using superior activating reagents, such as HATU[75] or amino acid chlorides generated in situ by triphosgene activation,[13,18] or by the standard symmetric anhydride coupling procedure.[19] We have come up with three procedures for this coupling, all designed for microwave-assisted solid-phase aza-peptide synthesis. For each peptide, the procedures were attempted in the following sequence: first, standard HATU coupling in DMF; second, triphosgene activation and coupling in dibromoethane;[76] third, triphosgene activation in DCM, removal of the DCM by reduced pressure, dissolution in DMF, and application to the resin. After each coupling procedure "small cleavage" was performed, followed by MS analysis. If there was no sign of the starting material after HATU coupling in DMF, the elongation of the peptide was continued. If there was remaining starting material, we proceeded to triphosgene activation in dibromoethane. If MS analysis indicated that there was still remaining starting material, we performed triphosgene activation in DCM followed by solvent evaporation and coupling in DMF. After all three procedures were applied, the elongation of the peptide was continued, regardless of whether starting material remained.

This is the first use of triphosgene for coupling and of dibromoethane as a solvent using microwave-assisted SPPS. The reaction time for the coupling to the aza-residue was significantly reduced, from about 8 h at rt (overnight coupling) to a maximum of 60 min (3 × 20 min) under microwave irradiation at 75 °C.

The azaPro and azaArg precursors developed in this study, as well as the novel procedures introduced for microwave-assisted solid-phase aza-peptide synthesis, may be of great value to the establishment of aza-scan as a more popular and routine methodology for SAR studies. This study offers considerably shorter reaction times for obtaining aza-peptides and also exemplifies the potential for automated aza-peptide synthesis. Consistent with the report by Gibson et al.,[75] we found that Fmoc aza amino acid chlorides are not very labile and can be prepared and stored for future use. We envision that in the not too distant future these compounds will be commercially available and that aza-scan will become a more common practice and not restricted to skilled peptide chemists.

PTR6154 Aza-scan. Using the procedures described above, we performed a nearly complete aza-scan of the PKB inhibitor, PTR6154.[48] The aza-PTR6154 analogues were purified by RP-HPLC and tested for the ability to inhibit PKB/Akt. The characterization and inhibitory activities of the aza-PTR6154 analogues are summarized in Table 1.

Aza-peptide analogues are rather unique peptidomimetic structures. An apparently small change has both local effects,

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Table 1. Characterization and Inhibitory Activity of the Aza-PTR6154 Analogues

compd	structure	calcd mass[a]	obsd mass[b]	purity HPLC[c] (%)	inhibition[d] (50 μM) (%)	IC_{50} (μM)[e] (95% confidence)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2				98 ± 1	0.94 (0.78−1.14)
[azaArg[1]]PTR6154	H-**azaArg**-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	903.5635	452.2830	>85	95 ± 1	14.8 (11.9−18.5)
[azaPro[2]]PTR6154	H-Arg-**azaPro**-Arg-Nva-Tyr-Dap-Hol-NH_2	903.5635	903.5635	>95	97 ± 1	13.7 (13.1−14.5)
[azaArg[3]]PTR6154	H-Arg-Pro-**azaArg**-Nva-Tyr-Dap-Hol-NH_2	903.5635	903.5396	>95	N.I.	N.D.
[azaNva[4]]PTR6154	H-Arg-Pro-Arg-**azaNva**-Tyr-Dap-Hol-NH_2	903.5635	903.5436	>90	72 ± 5	N.D.
[azaTyr[5]]PTR6154	H-Arg-Pro-Arg-Nva-**azaTyr**-Dap-Hol-NH_2	903.5635	903.5630	>95	76 ± 2	N.D.
[azaHol[7]]PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-**azaHol**-NH_2	903.5635	903.5624	>95	50 ± 4	N.D.

[a] Calculated mass for $[M + H]^{1+}$. [b] For all peptides the main mass observed was $[M + 2H]^{2+}$. See the Supporting Information for complete mass observations. [c] Peptide purity determined at 220 nm after RP-HPLC purification. [d] PKB/Akt inhibition was determined according to radioactive kinase assay as described.[43] Inhibition at 50 μM is shown as the percent of reduction in PKB/Akt activity (0% inhibition = activity in the absence of inhibitor). [e] IC_{50} values and 95% confidence range (parentheses) were determined using Graphpad Prism 5 only for inhibitors that showed over 90% inhibition at 50 μM. N.I. = no inhibition. N.D. = not determined.

most significantly, rapid inversion of the stereogenic center, and global effects, most significantly increased backbone constraint and the possible generation of a β-turn conformation. Although aza-analogues are expected to be more metabolically stable than their parent peptides, one cannot predict their biological performance. All of our aza-PTR6154 analogues were found to be considerably less active than PTR6154. Of specific note, [azaArg[3]]PTR6154 did not inhibit PKB/Akt at all at 50 μM. The poor inhibition by the aza-PTR6154 analogues alongside the poor inhibition previously reported for *N*-methyl and peptoid analogues[50] suggests that the backbone interactions of PTR6154 are of paramount importance to the binding of PTR6154 to PKB/Akt and the consequent inhibition. This study also confirms our previous findings indicating that the two amino terminal residues (Arg^1 and Pro^2) are more tolerant toward backbone changes than the other residues, as can be deduced from the relative potencies of the various aza-analogues.[50]

CONCLUSIONS

We present an aza-scan of PTR6154 using microwave irradiation for solid-phase aza-peptide synthesis. The reduction in inhibitory potency observed for the aza-PTR6154 analogues agrees with our previous findings emphasizing the significance of the backbone interactions for PTR6154-PKB/Akt site recognition.

The procedures developed for the synthesis of the aza-PTR6154 analogues are important contributions to solid-phase aza-peptide synthesis. We developed precursors for azaPro and azaArg amino acid incorporation and used them successfully in aza-peptide synthesis. We introduced novel procedures for microwave-assisted solid-phase aza-peptide synthesis, which upon optimization will enable automated aza-peptide synthesis. We envision that in the future, aza-scan may become a routine tool for SAR study along with the already established Ala-scan, D-amino acid scan, and *N*-Me-amino acid scan.

EXPERIMENTAL SECTION

Hazards. Phosgene solution and triphosgene (BTC) are highly toxic and may cause death by inhalation. These substances should be handled in a well-ventilated hood with extreme caution.

Methods for Microwave-Assisted SPPS. Microwave-assisted SPPS was performed on a microwave peptide synthesizer generally following standard protocols.[28] Swelling: The resin was swelled for 2 h in DCM and washed successively with NMP to remove all the DCM before MW irradiation. Microwave-assisted Fmoc removal: The resin, swollen in NMP, was treated with a 20% solution of piperidine in NMP and heated with microwave irradiation (50 W) at 75 °C for 3 min. The procedure was repeated once, and the resin was washed with NMP (5 ×). Microwave-assisted HBTU coupling: Fmoc-protected amino acid (2 equiv) was dissolved in DMF and treated with DIPEA (6 equiv) and HBTU (2 equiv) for 1 min, and then the solution of activated amino acid was added to the resin, swollen in NMP, and heated with microwave irradiation (25 W) at 75 °C for 5 min. The resin was drained and washed with NMP (5 ×). Microwave-assisted HATU coupling: Fmoc-protected amino acid (2 equiv) was dissolved in DMF and treated with DIPEA (6 equiv) and HATU (2 equiv) for 1 min, and then the solution of activated amino acid was added to the resin, swollen in NMP, and heated with microwave irradiation (25 W) at 75 °C for 20 min. The resin was drained and washed with NMP (5 ×). Microwave-assisted BTC coupling in DBE: Fmoc-protected amino acid (5 equiv) was dissolved in DBE and treated with BTC (1.66 equiv), and the mixture was cooled to 0 °C. 2,4,6-Collidine (23 equiv) was added dropwise to the cooled solution for 1 min, and then the solution of activated amino acid was added to the resin, swollen in DBE, and heated with microwave irradiation (25 W) at 75 °C for 20 min. The resin was drained and washed with NMP (5 ×). Microwave-assisted BTC coupling in DMF: Fmoc-protected amino acid (5 equiv) was dissolved in DCM and treated with BTC (1.66 equiv), and the mixture was cooled to 0 °C. 2,4,6-Collidine (23 equiv) was added dropwise to the cooled solution for 1 min, and then the DCM was removed by reduced pressure, DMF was added, and the mixture was added to the resin, swollen in DMF, and heated with microwave irradiation (25 W) at 75 °C for 20 min. The resin was drained and washed with NMP (5 ×). Microwave-assisted aza-amino acid coupling: Fmoc-aza-amino acid chloride (3 equiv) was dissolved in DMF (2 mL) and treated with DIPEA (3 equiv) for 1 min, and then the solution of activated aza-amino acid was added to the resin, swollen in DMF, and heated with microwave irradiation (25 W) at 75 °C for 20 min. The coupling was repeated, and then the resin was drained and washed with NMP (5 ×). Microwave-assisted aza-amino acid Fmoc removal: The resin, swollen in NMP, was treated with a 20% solution of piperidine in NMP and heated with microwave irradiation (50 W) at 75 °C for 3 min. The procedure was repeated twice, and the resin was washed with NMP (5 ×). Microwave-assisted aza-amino acid Ddz removal: The resin was prewashed with ACN, treated with $Mg(ClO_4)_2$ (10 equiv) in ACN, and heated with microwave irradiation (25 W) at 70 °C for 15 min. The procedure was repeated once, and the resin was washed with ACN (5 ×) and NMP (5 ×). Cleavage (not under MW irradiation): The resin was washed with DCM (2 × 2 min) and thoroughly dried under vacuum. A freshly made solution of TFA/TDW/triisopropylsilane (TIPS) (95:2.5:2.5, v/v/v) was cooled to 0 °C (14 mL/g peptidyl-resin) and agitated for 3 h at room temperature. The resin was removed by filtration and washed with a small amount of neat TFA. The

TFA mixture was treated with a solution of ether:hexane 1:1, precooled to 0 °C, and the precipitated peptides were collected by centrifugation. The peptide precipitate was dissolved in ACN/TDW 1:1 and lyophilized. Small cleavage: A sample of 5−10 mg of peptidyl resin was treated with a TFA/TDW/TIPS (95:2.5:2.5, v/v/v) solution precooled to 0 °C. The solution was shaken for 0.5 h at rt. The resin was removed by filtration, and the solvents were evaporated by a stream of nitrogen. The residue was dissolved in ACN/TDW 1:1 solution and analyzed by mass spectrometry. The cleavage process as well as the small cleavage of aza-peptides containing an aza-amino acid with aromatic side chain were carried out at 0 °C as previously recommended.[13]

Chemistry. *3-(Di-tert-butyloxycarbonyl)guanidinopropanol* ***6***. To a solution of *N,N′*-bis(Boc)-*S*-methylisothiourea[56] (1.45 g, 5 mmol) in DMF (15 mL) were added 3-aminopropanol (1.52 mL, 20 mmol) and DMAP (60 mg, 0.5 mmol). The reaction mixture was stirred at rt until TLC (hexane/EtOAc, 7:3) showed complete consumption of the *N,N′*-bis(Boc)-*S*-methylisothiourea, at which point Et_2O (100 mL) was added. The solution was washed with dilute AcOH (0.1 M, 100 mL), and the aqueous layer was back-extracted with Et_2O (25 mL). The combined organic phase was washed with saturated $NaHCO_3$ (100 mL), brine (100 mL), and water (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to yield the pure product as a white solid (1.51 g, 4.76 mmol). Yield: 95%. Mp = 93−94 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.44 (s, 9H), 1.47 (s, 9H), 1.63−1.70 (m, 2H), 3.51−3.59 (m, 4H), 4.47 (brs, 1H), 8.42 (brs, 1H), 11.41 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 28.0, 28.1, 32.8, 36.8, 57.8, 79.4, 83.4, 153.1, 157.1, 162.8. HRMS: calcd for $C_{14}H_{28}N_3O_5$ 318.2023 (MH^+), found 318.2022.

3-(Di-tert-butyloxycarbonyl)guanidinopropylaldehyde ***7***. A solution of 3-(diBoc)guanidinopropanol **6** (1.51 g, 4.75 mmol) and pyridine (2.3 mL, 28.5 mmol) in DCM (6 mL) was added dropwise to a stirred solution of Dess−Martin periodinane (2.32 g, 5.46 mmol) in DCM (20 mL). After completion of the reaction as indicated by TLC (hexane/EtOAc 1:1), NaOH 1 M (50 mL) and Et_2O (20 mL) were added to the reaction mixture and stirring was continued for 10 min. Then Et_2O (80 mL) was added, and the layers were separated. The ether layer was washed with water (3 × 100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to provide a yellowish residue which was used without further purification. Yield: 82%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.46 (s, 9H), 1.47 (s, 9H), 2.76 (dt, J = 6.1 Hz, 0.9 Hz, 2H), 3.70 (q, J = 6.0 Hz, 2H), 8.54 (brt, J = 6.1 Hz, 1H), 9.80 (t, J = 0.9 Hz, 1H), 11.40 (brs, 1H).

N′-1-(3-(Di-tert-butyloxycarbonylguanidino)propyl)fluorenylmethyl Carbazate ***8***. To a suspension of 9-fluoren-9-ylmethyl carbazate[13,77] (0.54 g, 2.1 mmol) in dry THF (5 mL) was added 3-(di-*tert*-butyloxycarbonyl)guanidinopropylaldehyde **7** (0.66 g, 2.1 mmol). The reaction was stirred overnight and concentrated under reduced pressure. The resulting hydrazone was dissolved in dry THF (20 mL) and treated with $NaCNBH_3$ (0.53 g, 8.4 mmol) with stirring. To this mixture was added acetic acid (0.36 mL, 0.63 mmol), and the reaction was stirred overnight at rt. Additional $NaCNBH_3$ was added as necessary to ensure completion of the reaction, as verified by TLC (hexane/EtOAc 1:1 or DCM/MeOH/TEA 98.5:1:0.5). The solvent was removed by evaporation under reduced pressure, and the residue was partitioned between EtOAc (50 mL) and brine (50 mL). The organic layer was washed with 1 M $KHSO_4$ (50 mL), saturated aq $NaHCO_3$ (50 mL), and brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure to yield a solid that was dissolved in EtOH and heated at reflux for 1 h. The solvent was removed under reduced pressure to yield the title compound which was purified by flash chromatography on silica using 1% MeOH in DCM as eluent to give a white solid. Yield: 63% (0.73 g, 1.32 mmol). Mp = 50−55 °C. R_f = 0.51 (hexane/EtOAc 1:1). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.39 (s,9H), 1.46 (s, 9H), 1.52−1.67 (m, 2H), 2.60−2.81 (m, 2H), 3.26−3.42 (m, 2H), 4.22 (brt, J = 6.6 Hz, 1H), 4.32 (d, J = 6.7 Hz, 2H), 4.65 (brs, 1H), 7.32 (dt, J = 6.6 Hz, 1.0 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.68 (d, J = 7.5 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 8.31 (brt, J = 5.5 Hz, 1H), 8.61 (brs, 1H), 11.49 (s, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 26.7, 27.5, 27.9, 38.1, 46.6, 47.8, 65.3, 78.0, 82.7, 120.0, 125.1, 126.2, 127.5, 140.6, 143.7, 152.0, 155.1, 156.7, 163.0. HRMS: calcd for $C_{29}H_{40}N_5O_6$ 554.2973 (MH^+), found 554.2972.

N′-1-(3-(Di-tert-butyloxycarbonylguanidino)propyl)-2-(3,5-dimethoxyphenyl)propan-2-yl Carbazate ***9***. To a solution of Ddz hydrazide (0.2 g, 0.79 mmol) in dry Et_2O (4 mL) was added 3-(di-*tert*-butyloxycarbonyl)guanidinopropylaldehyde **7** (0.25 g, 0.79 mmol). The reaction was stirred until completion, as indicated by TLC (hexane/EtOAc 1:1), and concentrated under reduced pressure. The resulting hydrazone was dissolved in dry THF (20 mL) and treated with $NaCNBH_3$ (0.149 g, 2.38 mmol) with stirring. To this mixture was added acetic acid (0.136 mL, 2.38 mmol), and the reaction was stirred overnight at rt. Additional $NaCNBH_3$ was added as necessary to ensure completion of the reaction, as verified by TLC (hexane/EtOAc 1:1 or DCM/MeOH/TEA 98.5:1:0.5). The solvent was removed by evaporation under reduced pressure, and the residue was partitioned between EtOAc (50 mL) and brine (50 mL). The organic layer was washed with saturated aq $NaHCO_3$ (50 mL) and brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was dissolved in MeOH (10 mL), treated with 1 M NaOH (1.2 mol equiv), and stirred for 1 h at rt. The solvent was removed by reduced pressure, and the residue was dissolved in EtOAc (50 mL), washed with brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure to provide the desired compound which was purified by flash chromatography on silica using hexane/EtOAc 7:3 as eluent to give a white solid. Yield: 56% (0.24 g, 0.44 mmol), Mp = 53−57 °C. R_f = 0.27 (hexane/EtOAc 1:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.48 (s, 9H), 1.49 (s, 9H), 1.64−1.71 (m, 2H), 1.72 (s, 6H), 2.77−2.98 (m, 2H), 3.48 (t, J = 6.7 Hz, 2H), 3.75 (s, 6H), 4.00 (brs, 1H), 6.32 (t, J = 2.2 Hz, 1H), 6.48 (d, J = 2.2 Hz, 2H), 6.74, (brs, 1H), 8.48 (brs, 1H), 11.46 (brs, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 26.8, 28.0, 28.2, 28.9, 38.8, 48.9 55.1, 79.1, 81.5, 83.0, 98.4, 102.9, 148.7, 153.2, 156.0, 156.1, 160.6, 163.4. HRMS: calcd for $C_{26}H_{44}N_5O_8$ 554.3184 (MH^+), found 554.3179.

N-(tert-Butoxycarbonyl)-N′-(9-fluorenylmethoxycarbonyl)pyrazolidine ***11***. To a precooled to 0 °C solution of *N*-(*tert*-butoxycarbonyl)-pyrazolidine **10**[14,65] (1.71 g, 9.93 mmol) in ACN/H_2O 2:1 (210 mL) was added Fmoc-Osu (3.35 g, 9.93 mmol), and the reaction mixture was stirred overnight at rt. The reaction was concentrated under reduced pressure, taken up in EtOAc (150 mL), and washed with water (150 mL), 0.5 M HCl (150 mL), satd aq $NaHCO_3$ (150 mL), and brine (150 mL). The organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure to provide the desired compound, which was purified by flash chromatography on silica using hexane/EtOAc 7:3 as eluent to give a low melting point solid. Yield: 90% (3.53 g, 8.95 mmol). Mp = 28−32 °C. R_f = 0.63 (hexane/EtOAc 1:1). ^{1}H NMR ($CDCl_3$, 400 MHz): δ. 1.50 (s, 9H), 1.94−2.10 (m, 2H), 3.09−3.41 (2 brm, 2H), 3.78−4.06 (2 brm, 2H), 4.26 (t, J = 7.2 Hz, 1H), 4.32−4.60 (2 brm, 2H), 7.31 (t, J = 7.3 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.65 (d, J = 7.4 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 25.6, 28.2, 46.3, 46.8, 47.2, 68.0, 81.6, 119.9, 125.2, 127.0, 127.7, 141.2, 143.7, 156.3, 157.0. HRMS: calcd for $C_{23}H_{27}N_2O_4$ 395.1965 (MH^+), found 395.1963.

N-(9-Fluorenylmethoxycarbonyl)pyrazolidine ***12***. *N*-(*tert*-Butoxy carbonyl)-*N′*-(9-fluorenylmethoxycarbonyl)pyrazolidine **11** (2.14 g, 5.42 mmol) was dissolved in neat TFA (5 mL). After TLC (hexane/EtOAc, 7:3) showed complete consumption of the starting material precooled to 0 °C, Et_2O (50 mL) was added, and the TFA salt of the desired product precipitated as a white solid. The solid was collected and washed with cold Et_2O. Yield: 87% (1.93 g, 4.72 mmol). Mp = 142 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ. 2.06−2.22 (m, 2H), 3.35 (t, J = 6.9 Hz, 2H), 3.55 (t, J = 7.1 Hz, 2H) 4.30 (t, J = 6.9 Hz, 1H), 4.41 (d, J = 6.9 Hz, 2H), 7.34 (dt, J = 7.4 Hz, 1.0 Hz, 2H), 7.42 (t, J = 7.3 Hz, 2H), 7.70 (d, J = 7.4 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 10.83 (brs, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 25.00, 46.0, 46.2,

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46.4, 67.8, 116.4 (q, J = 294 Hz, TFA), 120.1, 125.2, 127.1, 127.8, 140.7, 143.4, 153.7, 159.9 (q, J = 34 Hz, TFA). HRMS: calcd for $C_{18}H_{19}N_2O_2$ 295.1441 (MH^+), found 295.1430.

Substituted Hydrazine Activation. Phosgene (2 mol equiv) was slowly added with stirring to a solution of substituted hydrazine (1 mol equiv) and pyridine (1 mol equiv) in dry dioxane (0.1 M). The reaction was stirred for 5−10 min until TLC (hexane/EtOAc 7:3) indicated complete consumption of the substituted hydrazine. The pyridine hydrochloride salt was filtered, and the solvent was removed under reduced pressure to yield the protected aza-amino acid chloride, which was used without further purification.

[**azaArg**^{1}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >85%, t_R 18.35. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2830; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5300.

[**azaPro**^{2}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95%, t_R 16.06. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{71}N_{16}O_8^{+}$ 903.5635 (MH^+), found 903.5635; calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2854; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5563.

[**azaArg**^{3}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95%, t_R 18.20. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{71}N_{16}O_8^{+}$ 903.5635 (MH^+), found 903.5396; calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2741; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5302.

[**azaNva**^{4}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >90%, t_R 16.26. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{71}N_{16}O_8^{+}$ 903.5635 (MH^+), found 903.5436; calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2824; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5344.

[**azaTyr**^{5}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95%, t_R 16.13. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{71}N_{16}O_8^{+}$ 903.5635 (MH^+), found 903.5630; calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2831; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5538.

[**azaHol**^{7}]**PTR6154** was prepared from 200 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95%, t_R 17.26. HRMS exact mass (ESI microTOF-LC): calcd for $C_{40}H_{71}N_{16}O_8^{+}$ 903.5635 (MH^+), found 903.5624; calcd for $C_{40}H_{72}N_{16}O_8^{2+}$ 904.5708 (MH_2^{2+}), found 452.2866; calcd for $C_{42}H_{72}F_3N_{16}O_{10}^{+}$ 1017.5564 ($MH(TFA)^+$), found 1017.5529.

PKB/Akt Assay. PKB/Akt kinase (HisΔPHPKBEEEFlag) was prepared as described by Klein et al.,[78] except that for routine screening the enzyme was only partially purified in one step on Ni-NTA agarose (Qiagen). The radioactive kinase assay was as described by Reuveni et al.,[43] except that the reaction mix comprised 50 mM Hepes pH 7.4, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate, 3-μM RPRTSSF peptide, 10 μM [γ^{32}P-ATP (1 μCi/assay well), the inhibitory compound, and 0.005 units HisΔPHPKBEEEFlag. A stock solution of each peptide was prepared and the concentration was determined by UV spectrophotometry as described.[79] For initial screening, compounds were tested at three to four concentrations. Compounds that showed significant inhibition at 50 μM were retested and IC_{50} values determined using Graphpad Prism 5. PTR6154 was included in every assay as a standard.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Synthetic procedures for preparation of compounds **4** and **5**, selected ^{1}H and ^{13}C NMR spectral data for compounds **4**−**12**, and complete mass spectra observations for the aza-peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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