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Exploring Side-Chain Diversity by Submonomer Solid-Phase Aza-Peptide Synthesis

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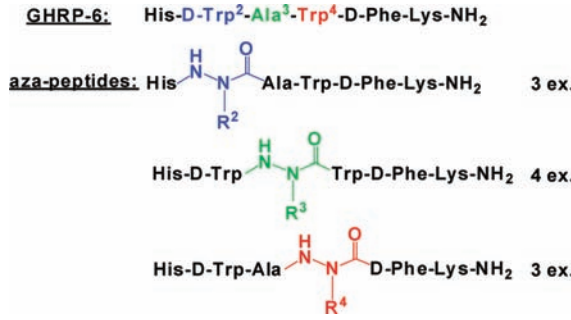
ABSTRACT



Submonomer synthesis of aza-peptides featuring regioselective alkylation of peptide-bound aza-Gly residues provided ten aza-analogues of the Growth Hormone Releasing Peptide-6 (GHRP-6) in 15−42% yield and purity generally ≥90%. Circular dichroism demonstrated that azaPhe-peptide 7a induced a β-turn conformation which may be responsible for its 1000-fold improvement in GHRP-6 selectivity for the CD36 receptor. This versatile method for making aza-peptides avoids solution-phase hydrazine synthesis and is well suited for studying side-chain-activity relationships of biologically active peptides.

The ability to introduce systematically various side-chain functionalities at different regions along a peptide or mimic offers power for investigating structure−activity relationships responsible for biological activity.[1] Ideally, different side chains could be attached directly to the growing peptide by a combinatorial method using solid-phase synthesis. For example, regioselective alkylation of supported glycine Schiff bases has allowed a variety of side-chain functional groups to be introduced onto amino esters as well as di- and tripeptide fragments.[2,3] Similarly, copper-catalyzed cross-coupling reactions in solution have been used to add vinyl, alkynyl, and aryl side chains onto *N*-*p*-methoxyphenyl glycine residues in simple di- and tripeptides.[4] Limited to the synthesis of amino acids and short peptides, these procedures are also generally not stereoselective, such that solution-phase chemistry using chiral phase-transfer catalysis has been employed to improve isomeric purity during glycine alkylation.[5,6] The issues of stereoselective C−H activation and modification of glycine residues have thus inhibited the

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general applicability of these routes for studying biologically relevant peptide sequences.[3,5,6]

Circumventing the issues related to Gly modification, we have explored adding side chains onto aza-Gly residues to provide aza-peptides. Aza-peptides possess one or more aza-amino acid residues, in which the α-carbon is substituted for nitrogen.[7] Insertion of aza-residues systematically along a sequence can identify the location and importance of β-turn conformations for peptide activity[8] and improve pharmacokinetic properties.[9]

The introduction of aza-amino acids into peptides involves a combination of hydrazine and peptide chemistry.[7] Typically, solid-phase aza-peptide synthesis strategies have necessitated the preformation of hydrazine precursors in solution prior to their incorporation on solid phase. For example, *N*-Fmoc protected *N*′-alkyl hydrazine[8e] and *N*-Boc aza-dipeptide fragments,[8c] as well as *N*-Fmoc-[8a] and *N*-2-(3,5-dimethoxyphenyl)propan-2-yloxy-carbonyl (Ddz)[8d]-protected aza-amino acid chlorides have been coupled to supported peptides to introduce the aza-residue. The scope of side-chain diversity is limited by these methods for aza-peptide synthesis because of the inherent difficulties of selectively differentiating the two nitrogens of the hydrazine moiety.[10] The regioselective alkylation of a supported aza-glycine moiety is specifically designed to surmount issues of solution-phase synthesis to introduce broader diversity of aza-residue side chains.

As a model peptide to demonstrate the utility of our method, we focused on modifying systematically each residue in the D-Trp-Ala-Trp tripeptide region of the growth hormone releasing peptide GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, **10**),[11] a challenging target because of potentially nucleophilic side chains at the His, Trp, and Lys residues. This hexapeptide stimulates growth hormone (GH) release by a pathway related to the G-protein coupled ghrelin receptor (GHS-R1a) and has been considered a target for developing treatments for GH secretory deficiency related to conditions such as cachexia and aging.[12] In addition to their endocrine activity, GHRP-6 analogues possess peripheral cardiovascular protective effects upon binding with the multifunctional CD36 scavenging receptor. CD36 plays a key role in the development of atherosclerosis by binding oxidized low-density lipoproteins (oxLDL)[13] and is involved in the down-regulation of angiogenesis in binding thrombospondin.[14a]

By modulating CD36 scanvenger receptor function, GHRP-6 analogues offer therapeutic potential in angiogenesis-related diseases such as age-related macular degeneration and diabetic retinopathy.[14] Moreover, the bioactive conformation of GHRP-6 has been suggested to adopt a turn-motif based on molecular modeling studies,[15] making aza-peptide analogues of GHRP-6 interesting targets for increasing potency and selectivity for binding to the CD36 target receptor.

A three-step process has been developed for aza-residue construction onto the peptide chain within a conventional Fmoc-based solid-phase peptide synthesis (SPPS):[16] (a) acylation of the supported peptide with a hydrazone-derived activated carbazate, (b) regioselective semicarbazone alkylation, and (c) chemoselective semicarbazone deprotection. Subsequent acylation of the aza-amino acid residue, completion of the SPPS sequence, deprotection, and cleavage provide the aza-peptide (Figure 1).

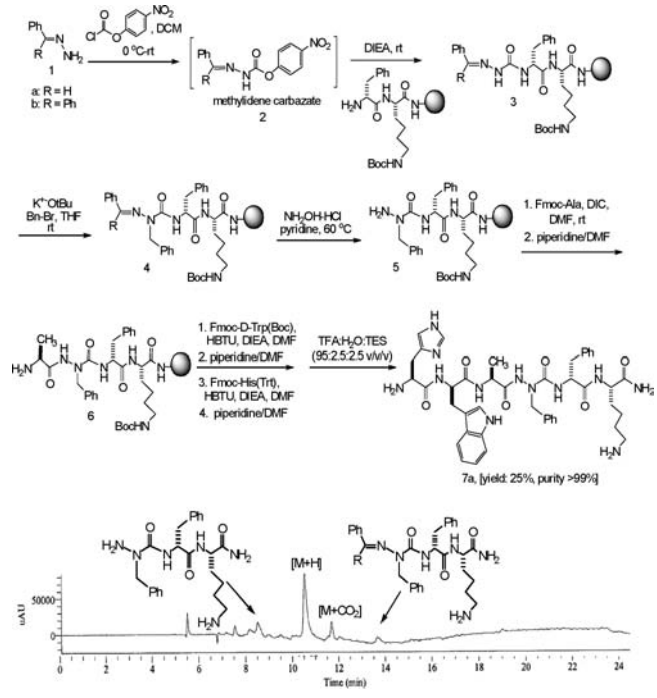

Figure 1. Submonomer synthesis and chromatogram at 254 nm of [aza-Phe[4]]-GHRP-6 (**7a**) after resin cleavage.

Rink amide polystyrene-divinyl benzene (PS-DVB) resin was used as a conventional support for SPPS, and the dipeptide D-Phe-Lys(Boc) constituted our starting sequence for aza-modifications at Trp[4]. [Aza-Phe[4]]-GHRP-6 (**7a**) was synthesized to demonstrate poof-of-concept. Aza-Gly was

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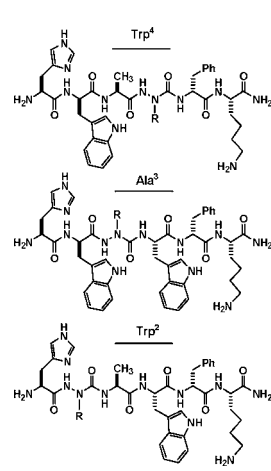
coupled to supported D-Phe-Lys(Boc) using methylidene carbazate **2** which was prepared in situ on mixing the respective hydrazone and *p*-nitrophenyl chloroformate. Better coupling was detected by LCMS analysis of product from benzylidene carbazate relative to its diphenylmethylidene counterpart (**3a** and **3b**, 95% and 89%, respectively, Figure 1). Similarly, in the regioselective alkylation[17] of the semicarbazone, benzylidene **3a** was more reactive than its diphenylmethylidene counterpart **3b**; i.e., treatment of **3** with KOtBu (300 mol %) and BnBr (300 mol %) gave resin samples, which after cleavage and LCMS analysis exhibited, respectively, 89% and 66% alkylated products **4a** and **4b** without any diastereomeric products from epimerization during alkylation.

Chemoselective semicarbazone deprotection necessitated mild conditions to avoid removal of side-chain protection and cleavage of peptide from resin. Commonly employed solution-phase acid-catalyzed hydrolysis conditions used for unmasking semicarbazones to aza-peptides were thus unsuccessful on the solid phase.[18] Considerable experimentation demonstrated that selective semicarbazone deprotection was effectively accomplished using trans-imination conditions,[19] employing NH_2OH·HCl in pyridine at 60 °C for 12 h. Again, benzylidene **4a** out-performed diphenylmethylidine **4b**; LCMS analyses showed, respectively, 81% and 64% conversions to semicarbazide **5**.

In our hands, efficient amino acid coupling to semicarbazide **5** was achieved using the symmetric anhydride method,[20] featuring activation of the Fmoc-amino acid with diisopropylcarbodiimide, DIC. Subsequent termination of the peptide sequence, deprotection, and cleavage gave [aza-Phe^4]-GHRP-6 (**7a**) in 73% crude purity. The major impurities in the chromatogram consisted of sequences generated from incomplete semicarbazone deprotection and failed acylation onto the aza-amino acid residue (Figure 1). With this method in hand, we investigated next further diversification at the peptide 4-position using *para*-substituted benzyl halides in the same sequence and produced respectively [aza-*p*-methoxy-Phe^4]- and [aza-*p*-trifluoromethyl-Phe^4]-GHRP-6 (**7b** and **7c**) in yields and purities similar to **7a** (Table 1).

Shifting to the 3-position, submonomer aza-residue synthesis was performed on the Trp(Boc)-D-Phe-Lys(Boc) sequence linked to Rink amide resin. Four aliphatic side chains were introduced by alkylation of the aza-Gly residue using iodomethane, allyl bromide, propargyl bromide, and *iso*-propyl iodide. Not surprisingly, lower conversion to alkylated product was detected using the latter secondary alkyl halide. Subsequent completion of the aza-peptide sequences gave the respective [aza-Ala^3]-, [aza-allylGly^3]-, [aza-propargylGly^3]-, and [aza-Val^3]-GHRP-6 analogues **8a**−**d**. Although the crude purity of aza-peptides **8** possessing various aliphatic side chains at the 3-position were lower than the benzyl analogues made at the 4-position, product was isolated in acceptable yield (14−28%) and high final purity (≥90%). The final [aza-Val^3]-GHRP-6 **8d** featured a 63:37 mixture of product contaminated with [aza-Gly^3]-GHRP-6.

Table 1. GHRP-6 aza-Peptides (**7**−**9**): Yields and Characterization Data



entry	R-X	crude purity[a]	isolated purity[b]	isolated yield[c]	mass[d]	RT (min)[e]	RT (min)[f]
7a		73%	>99%	25%	835.2(835.4)	16.3	13.3
7b		80%	>99%	27%	865.2(865.4)	16.9	13.2
7c		71%	>99%	15%	903.3(902.9)	20.6	15.1
8a	CH_3-I	49%	>99%	15%	874.2(873.9)	16.7	13.5
8b		51%	90%	17%	900.2(900.4)	17.2	13.8
8c		45%	96%	28%	898.5(898.4)	18.2	13.6
8d		32%	53%[g]	14%	902.5(902.5)	22.5	17.4
9a		45%	90%	20%	836.2(836.4)	10.5	9.91
9b		57%	>99%	25%	903.4(902.9)	21.1	14.9
9c		81%	>99%[h]	42%	861.5(861.5)	19.5	14.1

[a] Crude purity by LCMS at 254 nm using H_2O (0.1% FA) and MeOH (0.1% FA) or MeCN (0.1% FA) as eluent. [b] Isolated purity by LCMS at 254 nm using H_2O (0.1% FA) and MeOH (0.1% FA) as eluent. [c] Calculated from resin loading. [d] Observed mass (expected mass) as $[M + H]^+$ by LCMS. [e] Retention times using 2−40% MeOH/H_2O as eluent. [f] Retention times using 2−40% MeCN/H_2O as eluent. [g] 63:37 mixture of aza-Val^3 and aza-Gly^3 GHRP-6. [h] 77:23 mixture of His isomers.

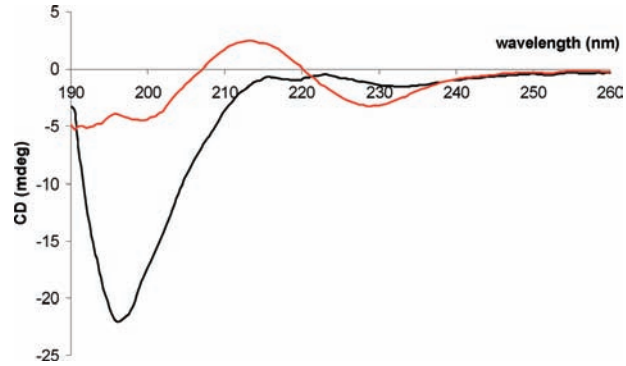


Figure 2. Circular dichroism spectra in water for GHRP-6 parent peptide **10** (black line) and aza-peptide **7a** (red line).

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Table 2. IC_{50} Binding Values for GHS-R1a and CD36 Receptors

entry	sequence	IC_{50} binding GHS-R1a	IC_{50} binding CD36
7a	His-D-Trp-Ala-azaPhe-D-Phe-Lys-NH_2	2.77×10^{-6} M	1.34×10^{-6} M
10	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	3.65×10^{-9} M	
11	His-D-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH_2		2.97×10^{-6} M

At the 2-position, D-Trp was replaced by a series of aryl aza-residues, using the general protocol. Aza-Gly was coupled to supported Ala-Trp(Boc)-D-Phe-Lys(Boc) and alkylated, respectively, with 2-(chloromethyl)pyridine, *p*-trifluoromethyl benzyl bromide, and cinnamoyl bromide. Completion of the SPPS protocol provided aza-peptides **9a**−**c** in isolated yields of 20−42% and purities of ≥90%. Coupling of Fmoc-His(Trt) onto the aza-residue was best completed with triphosgene and collidine,[21] albeit, **9c** was isolated as a mixture of diastereomers due to epimerization during coupling.[8a]

To examine peptide conformation in water, the CD spectrum of the parent peptide, **10**, was evaluated next to that of aza-peptide **7a** (Figure 2). The insertion of an aza-Phe motif into the GHRP-6 core had a pronounced effect on the CD curve indicative of a change in conformation relative to the native sequence. The CD curve for the parent peptide was characteristic of a random coil or disordered structure as suggested by the negative maximum band observed at 190 nm.[22] Alternatively, the CD signature for aza-peptide **7a** was indicative of an ordered β-turn conformer with characteristic negative maximum values located at around 190 and 230 nm and a positive maximum band near 215 nm.[23]

The binding affinity (IC_{50} values) was next evaluated for aza-peptide **7a** on the GHS-R1a and CD36 receptors. Aza-peptide **7a** was found to selectively bind to the CD36 receptor and lost affinity for the GHS-R1a receptor in competition binding studies (see Supporting Information for binding curves). More specifically, aza-peptide **7a** exhibited a 1000-fold drop in binding affinity to the GHS-R1a receptor relative to GHRP-6 (2.77 μM vs 3.65 nM) and retained comparable binding activity with the GHRP prototype ligand, hexarelin[24] (**11**), for the CD36 receptor (Table 2). Considering that aza-peptide **7a** induces a β-turn type conformation, then this geometry may be responsible for binding and differentiating the CD36 receptor from the GHS-1Ra receptor.[25]

An effective method for making aza-peptides without preformation of a hydrazine moiety in solution has been demonstrated by the solid-phase synthesis of 10 aza-analogues of GHRP-6. Preliminary conformational and biological analysis of [aza-Phe[4]]-GHRP-6 (**7a**) by CD spectroscopy and receptor binding studies demonstrated a preorganized β-turn geometry that bound discriminately for the CD36 receptor. In light of the antiangiogenic activity of GHRP-6 ligands that bind to the CD36 receptor, the selectivity of **7a** may be a promising lead for developing treatments for disorders such as age-related macular degeneration. Considering the scope of electrophiles that may be added to aza-Gly by this method, considerable opportunity now exists for studying side-chain-activity relationships at biologically active turn regions located along the peptide backbone.

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Supporting Information Available: Experimental procedures and characterization data of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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