Synthesis, Derivatization, and Conformational Scanning of Peptides Containing N-Aminoglycine

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Contents

Abstract

N-alkylated glycine residues are the main constituent of peptoids and peptoid-peptide hybrids that are employed across the biomedical and materials sciences. While the impact of backbone N-alkylation on peptide conformation has been extensively studied, less is known about the effect of N-amination on the secondary structure propensity of glycine. Here, we describe a convenient protocol for the incorporation of N-aminoglycine into host peptides on solid support. Amide-to-hydrazide substitution also affords a nucleophilic handle for further derivatization of the backbone. To demonstrate the utility of late-stage hydrazide modification, we synthesized and evaluated the stability of polyproline II helix and β-hairpin model systems harboring N-aminoglycine derivatives. The described procedures provide facile entry into peptidomimetic libraries for conformational scanning.

1. INTRODUCTION

Substitution of main chain amides in peptides can have a profound impact on their solubility, stability, bioavailability, preferred conformation, and interaction with receptors.[1] As a result, there is an increasing demand for synthetically convenient methods to diversify the peptide backbone.[2] Peptoids (N-alkylglycine polymers) and N-methylated peptides have thus been the subject of intense investigation in both biomedical and materials science.[3] Access to the N-alkylglycines within peptoids is typically accomplished on solid support through reactions between resin-bound haloacetamides and amine nucleophiles.[4] This operationally simple submonomer approach affords diverse libraries in short order from readily available building blocks. More targeted incorporation of N-alkyl groups into Cα-substituted peptides (peptide tertiary amides) is often carried out via primary amine sulfonylation, mono-alkylation, and de-sulfonylation to unmask the secondary amine.[5,6] Although less convenient than peptoid assembly, the combination of amide N-alkylation and Cα substitution enhances peptide backbone rigidity relative to N-alkylglycines.[7] Backbone-modification strategies that combine the synthetic accessibility of peptoids with the enhanced rigidity of peptide tertiary amides would likely find widespread applications in the development of chemical probes and therapeutics.

1.1. Conformational Effects of Gly N-Amination in Peptides

The incorporation of α-hydrazino acids into peptides has enabled several conformational studies on the impact of amide-to-hydrazide replacement. In the case of N-aminopeptides, Cα-substituted α-hydrazino acids stabilize extended conformations through a series of cooperative non-covalent interactions.[8-10] N-Aminoglycine (aGly), which lacks a Cα substituent, exhibits greater flexibility at the ψ and ϕ torsions (Figure 1). However, the hydrazide N'H_2 group may still engage in a rigidifying intra-residue H-bond analogous to that observed in conformationally extended residues within protein β-sheets.[11,8] This H-bonded conformer also experiences a lone pair repulsive effect that destabilizes the *cis* hydrazide rotamer.[12] Conversely, an H-bond between the hydrazide N'H_2 and the carbonyl O of the preceding residue can serve to stabilize the *cis* rotamer and relax aGly ϕ and ψ dihedral constraints.[13]

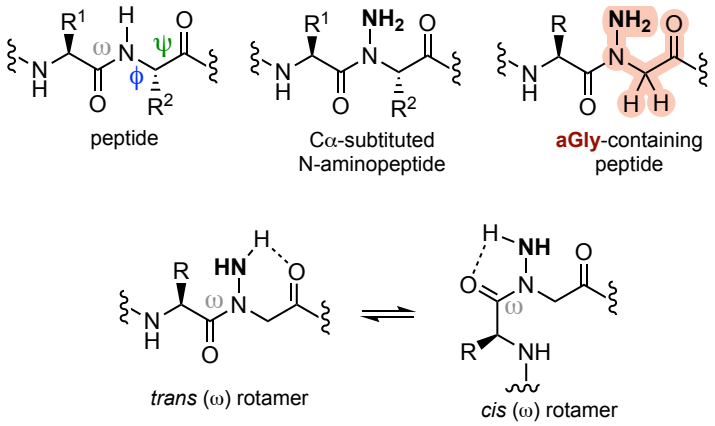


Figure 1. Structure and conformational characteristics of the aGly residue.

Amide *trans/cis* rotational equilibria are often significantly impacted by substitution of the peptide backbone. Similar to Pro, N-methyl amino acid residues in peptides typically give rise to an enhanced population of the *cis* amide rotamer.[5,6] This is in contrast to proteinogenic secondary amides that are found almost exclusively in the *trans* amide conformation. As shown in Figure 2, An N-acetylated Sar derivative exhibits diminished *trans* amide bias in water relative to Pro.[14] Although N-amination of Ala significantly enhances *trans* propensity, the same is not true for aGly. This may be due to less severe *cis* steric interactions, resulting in an increased propensity for aGly to engage in a *cis*-stabilizing inter-residue H-bond.[15]

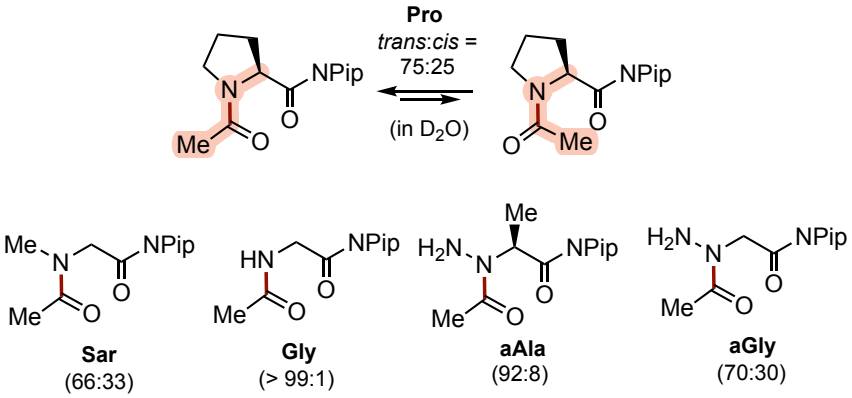


Figure 2. Equilibrium *trans*/*cis* rotamer ratios (given in parentheses) for N-acetyl piperidyl amides of selected residues, derived from ^{1}H NMR (in D_2O).

Amide isomerization rates are also sensitive to N-substitution. Amides of N-alkylated peptides, for example, were observed to isomerize more slowly than those derived from proteinogenic residues.[16] However, the barriers to rotation for peptide secondary and tertiary amides are often similar and highly dependent on flanking residues.[17] N-Amination of the peptide backbone introduces an electron-withdrawing substituent that significantly lowers the C-N rotational barrier relative to amides.[18] Replacement of Sar and other N-alkylated Gly residues with aGly is therefore expected to enhance the rate of isomerization in peptidomimetics that can adopt a significant population of the *cis* rotamer.

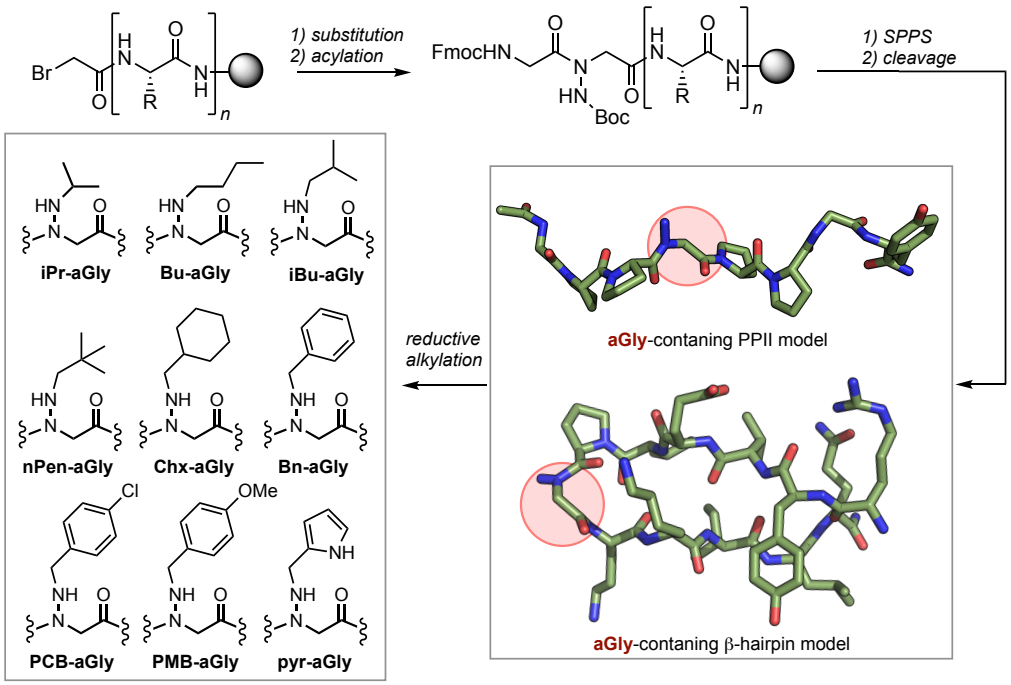
1.2. Late-Stage Modification of N-Aminopeptides

The hydrazide group in N-aminopeptides allows for chemoselective derivatization at a late stage. This contrasts with N-alkylated peptides where backbone substituent diversity is introduced during assembly on solid support. Burke and co-workers demonstrated diversification of an aGly-containing substrate through reductive alkylation reactions to generate a library of peptidomimetic HIV budding antagonists.[19] The hydrazone intermediates could also be isolated, characterized, and used in biological assays, though some peptide hydrazones are unstable in aqueous acid.[20]

Beyond applications in the diversity-oriented synthesis of bioactive peptidomimetics, N-aminopeptides can serve as convenient substrates for conformational scanning. Toward this end, secondary structure model peptides harboring one or more backbone hydrazides may undergo reductive amination to identify substituents that better stabilize a desired fold. Our group recently demonstrated this approach in the context of a polyproline II (PPII) helix.[14] An eight-residue Pro-rich model peptide was synthesized on solid support and its PPII helicity measured by circular dichroism. We then synthesized analogues in which the central Pro residue was substituted with aGly, N-aminoalanine (aAla), or N-aminophenylalanine (aPhe). Among these, Gly→aGly substitution enhanced PPII helicity without significant disruption of the native fold. The aGly analogue was then reacted with a small set of aldehydes in the presence of sodium cyanoborohydride to generate N'-alkylated derivatives. From this limited set, the N'-isopropylaminoglycine (iPr-aGly) derivative exhibited a considerable increase in PPII helicity relative both the aGly and parent Pro peptides. This initial study thus led to the rapid

identification of a new PPII-stabilizing residue and suggested that a similar strategy can be applied to other folded peptides and proteins.

In the current chapter, we describe an optimized protocol for the incorporation of aGly and its alkylated derivatives into host peptides. In addition to the synthesis of aGly and iPr-aGly-containing PPII helical peptides, we prepared a series of β-hairpin sequences in which a β-turn Gly residue is replaced with aGly or N'-alkylated aGly residues (Scheme 1). These peptides were then analyzed by CD and NMR to quantify the impact of specific hydrazide substitutions on secondary structure.



Scheme 1. General approach for incorporating aGly and N'-alkylated derivatives into secondary structure models.

2. PROTOCOLS FOR THE SYNTHESIS OF aGly-CONTAINING PEPTIDES

The incorporation of aGly into peptides is readily achieved through a submonomer approach. We use standard Fmoc solid-phase peptide synthesis (SPPS) to assemble the sequence preceding and following the aGly residue. To obtain a C-terminal amide we use Rink MBHA amide resin (high loading). Peptides can be synthesized by hand or using an automated synthesizer. Begin by synthesizing the peptide up to the residue

preceding aGly incorporation. Activate bromoacetic acid using diisopropylcarbodiimide (DIC) and add to the resin-bound peptide. Addition of *tert*-butyl carbazate to the resin-bound bromoacetamide affords the Boc-protected aGly peptide. Acylation of the deactivated N-terminal Boc-hydrazide is best carried out with a pre-formed Fmoc-protected amino acid chloride. Below, we include the procedure described by Carpino and co-workers to prepare the acid chlorides from Fmoc-protected amino acids using thionyl chloride.[21] Following aGly acylation, the remainder of the peptide is synthesized using standard SPPS protocols. The N-terminus can be capped or left as a free amine. The peptide is cleaved from resin and globally deprotected using a trifluoroacetic acid (TFA) cocktail. Addition of a desired aldehyde/ketone to the cleavage cocktail generates the aGly hydrazone *in situ*. This hydrazone can serve as a temporary protecting group (to inhibit undesired trifluoroacetylation during cleavage) or be reduced to the N'-alkylated product. At this stage, the crude peptide is lyophilized and purified by RP-HPLC to afford the desired N-aminopeptide.

2.1. Reagents and Equipment

- Rink MBHA amide resin (high-loading, 0.59–0.6 mmol/g)
- Standard Fmoc-protected amino acids suitable for solid-phase synthesis, with acid labile sidechain protecting groups.
- Solvents for synthesis: dichloromethane (DCM), N,N-dimethylformamide (DMF)
- N-terminal acetylation solutions: 10% acetic anhydride and 20% pyridine in DCM
- Coupling reagents: 2(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), N,N’-diisopropylcarbodiimide (DIC)
- Organic bases: N-methylmorpholine (NMM), 2,4,6-trimethylpyridine (collidine)
- Cleavage and deprotection solutions: 95 : 2.5 : 2.5 trifluoroacetic acid (TFA) : water : triisopropylsilane (TIPS), 20% piperidine (pip) in DMF
- Bromoacetic acid, thionyl chloride, *tert*-butyl carbazate, sodium cyanoborohydride ($NaBH_3CN$), aldehydes/ketones
- Automated peptide synthesizer
- Analytical (150 mm × 4.6 mm, 4 µm, 90 Å) and preparative (250 mm × 21.2 mm, 4 µm, 90 Å) C12 or C18 RP-HPLC columns

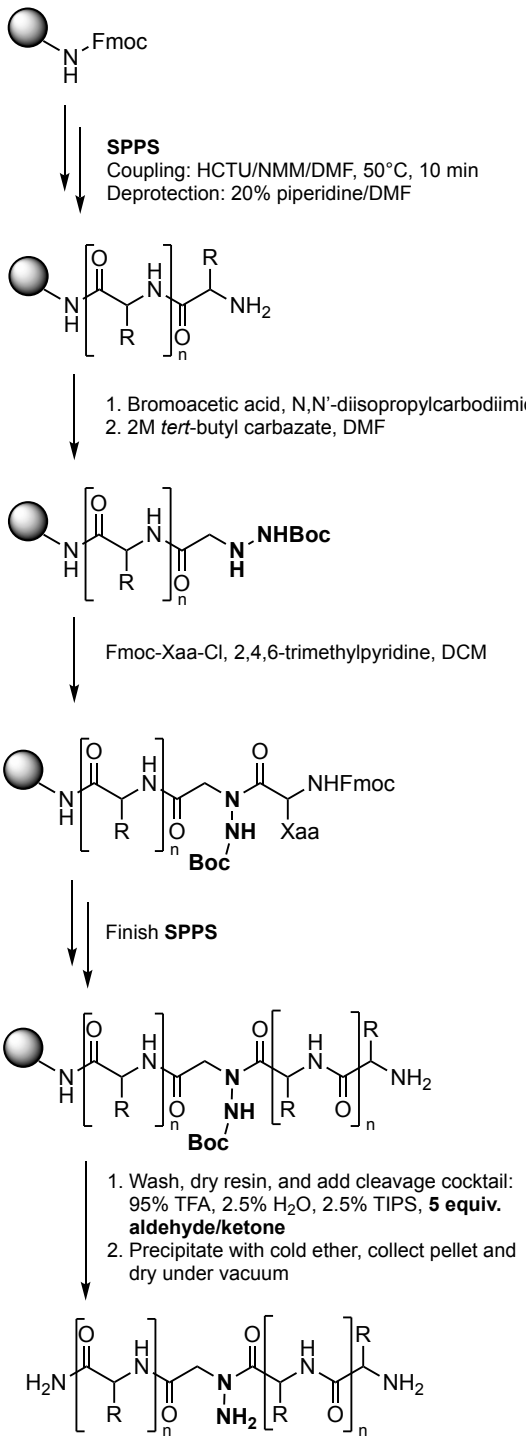
- Analytical (1 mL/min) and preparative (20 mL/min) RP-HPLC systems with UV detectors (220 and 280 nm)
- Lyophilizer

2.2. Synthesis of Fmoc-Protected Amino Acid Chlorides

1. Dissolve Fmoc-protected amino in DCM (0.2 M) and slowly add 10 equiv. of thionyl chloride via syringe. Heat the reaction to 60 °C and reflux for 2 h.
2. Concentrate the reaction mixture to remove excess solvent and thionyl chloride. Redissolve the residue in 1 mL of DCM.
3. Add 50 mL of cold hexanes and sonicate to precipitate the product. Centrifuge the mixture and decant the supernatant. Repeat precipitation with the resultant pellet.
4. Dry the product under vacuum.

Note: This method is only appropriate for amino acids without acid-labile side chain protecting groups. Pre-formed acid chlorides should be stored under vacuum to prevent hydrolysis to the carboxylic acid and used within two days.

2.3. General Procedure for Synthesis of aGly-Containing Peptides on Solid Support

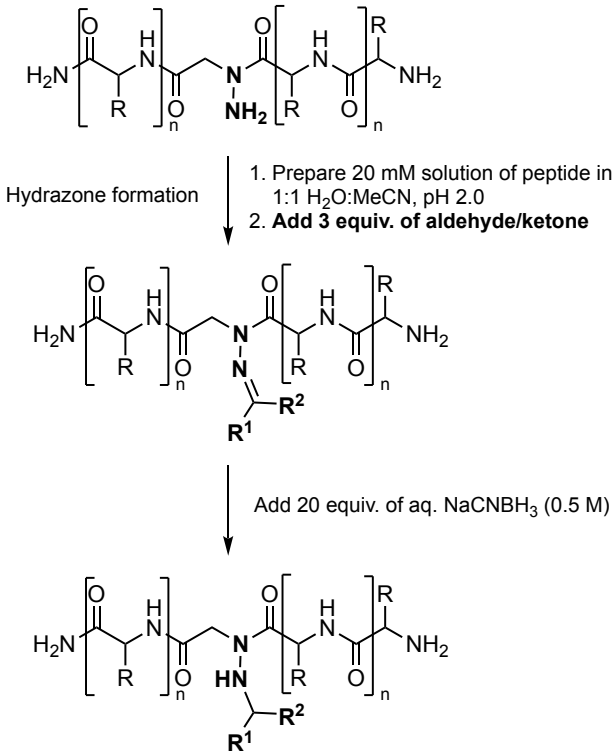


Scheme 2. Submonomer-based synthesis of aGly-containing peptides on solid support.

1. Add 0.1 mmol Rink MBHA amide resin to a solid-phase reaction vessel and swell in 10 mL of DMF for 5 min. Drain the solvent.

2. Remove the Fmoc protecting group by adding 8 mL of 20% pip/DMF to the resin and shaking for 5min. Drain the reaction vessel and wash with DMF (3 × 5 mL).
3. Add 5 equiv. (0.5 mmol) of the required Fmoc-protected amino acid, 5 equiv. (0.5 mmol) of HCTU, and 10 equiv. (1.0 mmol) of NMM as solutions in DMF to the resin and heat at 50°C via induction for 10 min. Drain the reaction vessel and wash the resin with DMF (3 × 5 mL).
4. Repeat steps 2 and 3 until the desired residues preceding the aGly residue are assembled. Carry out a final Fmoc deprotection (see step 2) and pause the synthesis.
5. Pre-activate 5 equiv. (0.5 mmol) of bromoacetic acid with 5 equiv. of DIC in 10 mL of DMF for 10 min. Add the solution to the reaction vessel and gently agitate for 30 min. Remove the solution with vacuum filtration and wash the resin with DMF (2 × 10 mL). Repeat this step.
6. Prepare a solution of 2 M tert-butyl carbazate in 10 mL of DMF and add to the reaction vessel. Gently agitate the reaction for 2 h. Drain the reaction vessel with vacuum filtration and wash the resin with DMF (2 × 10 mL).
7. Dissolve 5 equiv. (0.5 mmol) a preformed Fmoc-protected amino acid chloride 10 mL of DCM and add 10 equiv. (1 mmol) of collidine. Add the solution to the resin and gently agitate for 30 min. Drain the reaction vessel and wash the resin with DCM (2 × 10 mL).
8. Repeat step 7 then wash the resin with DMF (2 × 5 mL) before returning to the automated synthesizer.
9. Repeat steps 2 and 3 until the desired residue sequence is assembled. Carry out a final Fmoc deprotection (see step 2) to afford the side chain-protected resin-bound peptide.
10. For N-terminally acetylated peptides: wash the resin with DCM (2 × 5 mL) and treat with 10 mL of 10% acetic anhydride and 20% pyridine in DCM. Gently agitate for 10 min. Drain and repeat this step.
11. Wash the resin with DCM (3 × 5 mL) and shrink the resin by washing with MeOH (3 × 5 mL). Thoroughly dry the resin under vacuum.

2.4. Derivatization of aGly via Reductive Alkylation



Scheme 3. Reductive alkylation of crude aGly-containing peptides.

1. Add a 5 mL solution of 95% TFA, 2.5% TIPS and 2.5% H_2O treated with 5 equiv. (0.5 mmol) of the desired aldehyde/ketone to 0.1 mmol of dried resin-bound peptide in a fritted solid-phase reaction vessel. Gently agitate for 2-4 h depending on the amino acids in the sequence to effect cleavage.
2. Filter the resin and wash with 10 mL of DCM, collecting the filtrate in a 50 mL centrifuge tube. Concentrate the reaction under N_2 until the total volume is less than 5 mL. Precipitate the crude peptide with 45 mL of cold Et_2O. Centrifuge the mixture and decant the supernatant. Repeat precipitation with the leftover pellet. Dry the pellet under vacuum.

Note: For a non-alkylated N-aminopeptide, use acetone as the ketone additive and proceed to step 5.

3. Resuspend the crude peptide in 1:1 H_2O:MeCN (20 mM). Measure the pH of the resulting solution and, if needed, acidify to pH 2 by adding TFA dropwise. To ensure complete hydrazone formation, add an additional 3 equiv. of aldehyde/ketone and stir for 3 h.
4. Add 20 equiv. of $NaBH_3CN$ (2 mmol) as a 0.5 M aqueous solution and allow to stir for 1 h. Evaporate MeCN under N_2, freeze and lyophilize the reaction.

5. Add a minimal volume of the starting H_2O:MeCN gradient then vortex and sonicate until the peptide is fully dissolved. Filter the solution with a 22 μm syringe filter. Purify the peptide by RP-HPLC using a C18 column and a linear gradient of MeCN in water (solvents modified with 0.1% formic acid) over 30 min. Lyophilize the product-containing fractions to obtain the final peptide.

Tip: If the crude peptide solution remains turbid (other crude material from the reaction may be insoluble), centrifuge the solution and collect the supernatant. Filter as described.

3. TIPS and TROUBLESHOOTING

3.1. Condensation of Boc-protected α-Hydrazino Amides with Acid Chlorides

Submonomer synthesis represents a robust and efficient method to incorporate aGly into peptides. However, acylation of the resin-bound Boc-protected aGly residue is challenging due to the electronically deactivated Nα. We have found that reaction with Fmoc-protected acid chloride and $NaHCO_3$ in DCM results in the highest conversion to the Boc-hydrazide without detectable epimerization at Cα. Activation of the Fmoc-protected amino acid using standard uronium (HBTU; HCTU; HATU) and carbodiimide (DCC; DIC; EDC) reagents results in poor coupling efficiency except in the case of particularly unhindered residues (Fmoc-Gly-OH, Fmoc-Ala-OH).

In the protocol above the acid chloride is generated by heating with excess thionyl chloride.[21] This procedure is incompatible with residues harboring acid-labile sidechain protecting groups due to the production of HCl in DCM during the reaction. We have found Ghosez' reagent to be a suitable alternative to thionyl chloride for preparing Fmoc-protected amino acid chlorides from acid-sensitive substrates.[22] This method requires only a few minutes for conversion to the acid chloride and the resulting solution can be added directly to a mixture of the resin-bound Boc-hydrazine and excess $NaHCO_3$ in DCM.[23] Despite their reactivity, Fmoc-protected amino acid chlorides are known to be resistant to epimerization if organic bases and more polar solvents are avoided. We therefore employ excess $NaHCO_3$ in this step instead of the tertiary amine bases more commonly used for peptide synthesis. Since $NaHCO_3$ is only sparingly soluble in DCM, we perform this reaction manually (pausing the automated synthesizer) to ensure that residual salts are thoroughly removed in the washing steps. The resin is then returned to the automated synthesizer to complete the sequence.

Orthogonally-protected N-amino dipeptide subunits can also be prepared in solution prior to incorporation into host peptides.[24] This approach obviates the need for on-resin Boc-hydrazine acylation and allows for fully automated SPPS using standard Fmoc protocols.

3.2. Undesired Hydrazide Acylations During Peptide Cleavage and Deprotection

Cleavage and global deprotection of N-aminopeptides can be achieved using mixtures of TFA, water, and triisopropylsilane scavenger. However, a small degree of hydrazide trifluoroacetylation is sometimes observed in the crude reaction mixture (Figure 3). This undesired byproduct is typically higher in the case of aGly peptides, presumably due to reduced steric encumbrance. We found that the addition of excess aldehyde or ketone to the cleavage cocktail is generally effective to suppress aGly trifluoroacetylation. The hydrazone formed *in situ* serves as a temporary, albeit labile, protecting group. Reductive alkylation of the aGly residue can then be carried out by treating with additional aldehyde/ketone and $NaBH_3CN$. If the desired product is an unmodified aGly-containing peptide, acetone is the preferred additive due to its volatility. Incubation of the crude hydrazone in water/acetonitrile for ~ 1 h prior to HPLC purification is sufficient to effect hydrolysis, affording the aGly peptide in good yield.

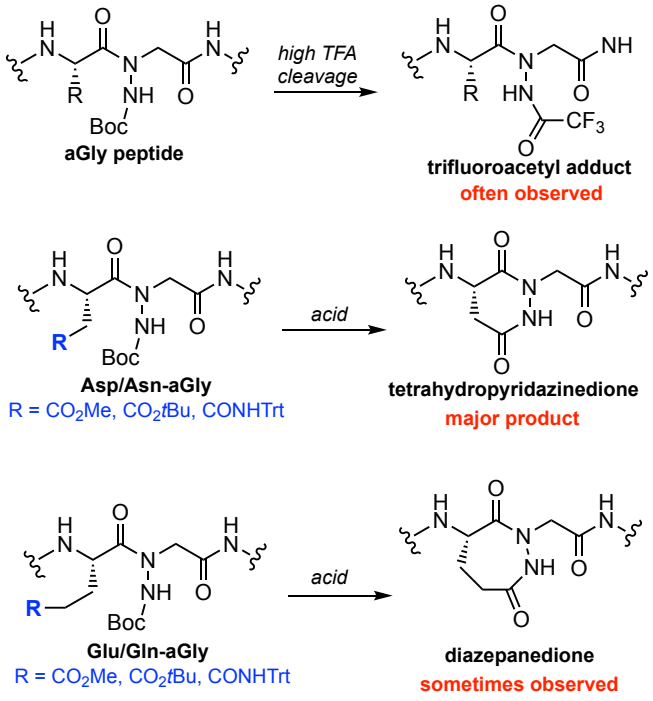


Figure 3. Hydrazide acylation reactions observed during synthesis of some aGly-containing peptides.

The ability to chemoselectively alkylate the aGly hydrazide in the presence of amines is due to its weak basicity. The hydrazide thus remains nucleophilic even at low pH. When aGly is positioned C-terminal to an Asp or Asn residue, formation the tetrahydropyridazinedione (Tpd) occurs rapidly during cleavage (Figure 3).[25,26] For this reason, peptides harboring an Asp/Asn-aGly motif are not accessible using the described protocol. We observed high conversion to the Tpd irrespective of the nature of the ester or amide protection on Asp or Asn. Monitoring of acid-mediated deprotection by HPLC-MS suggests that the hydrazide N'-Boc group is removed first, followed by cyclization onto the protected or unprotected sidechain carbonyl derivative. Formation of the corresponding 7-membered heterocycle from Glu/Gln-aGly motifs is less frequently observed, but byproducts resulting from this side reaction should be considered when analyzing crude cleavage mixtures.

3.3. Scope of Hydrazide Reductive Alkylation

Reductive alkylation of the aGly hydrazide proceeds with a variety of commercially available aldehydes. Below, we demonstrate the reaction of an aGly β-hairpin sequence with 8 different aldehydes as well as 1 ketone (acetone) to form the intermediate hydrazones in aqueous acid. While the hydrazone adduct of acetone forms readily, reaction of two different aGly peptides with benzophenone did not afford the alkylated product. Poor conversion in this case may be due to increased steric encumbrance or reduced hydrazone electrophilicity. Depending on the stability of the hydrazone formed *in situ*, analytical HPLC can be used to monitor the progress of both reaction steps. Hydrazones formed from benzaldehyde and its derivatives showed the greatest stability in aqueous acid. Aliphatic hydrazones, though not always observed by HPLC, were readily converted to N'-alkylated products following the addition of $NaBH_3CN$. The described procedure allows for the reductive alkylation of Cα substituted N-aminopeptides as well as peptides harboring multiple backbone hydrazides.

4. APPLICATIONS TO CONFORMATIONAL SCANNING

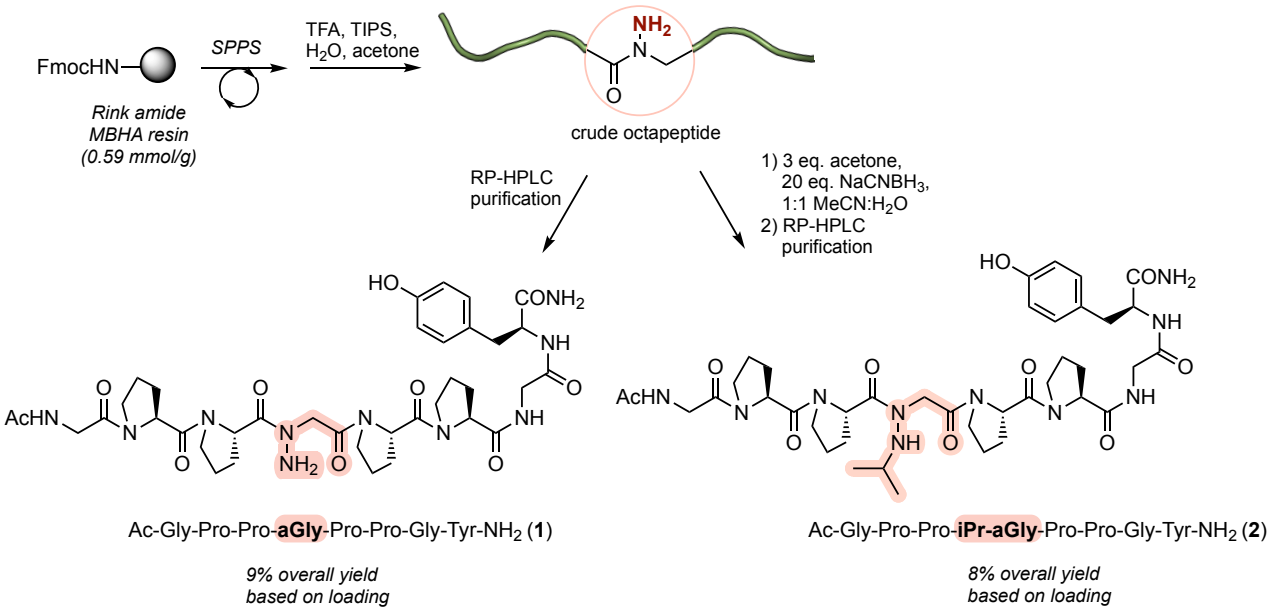
The conformational impact of backbone N-amination at Cα-substituted residues has previously been investigated in the context of β-sheet model peptides. The β-sheet stabilizing effect of α-hydrazino acids is supported by NMR, X-ray, and molecular dynamics simulations.[8-10] Substitution at Cα imparts additional torsional rigidity to α-hydrazino acids and contributes to the observed β-strand propensity of these residues. The conformational preferences of aGly are less well established. Moreover, the specific effect of aGly and its N'-alkyl derivatives on the stability of canonical protein folds has not been systematically investigated. To address this, we examined both the PPII helix and β-hairpin turn propensity of aGly and its derivatives using two well-established model systems. The studies below highlight the efficiency of late-stage reductive alkylation to generate libraries of aGly peptides for conformational scanning.

4.1 aGly Derivatives as Stabilizers of Polyproline II Conformation

The left-handed PPII helix is now considered to be one of the major classes of protein secondary structure and is an important motif for biomolecular recognition.[27,28] Unlike α-helices and β-sheets, PPII folds are stabilized primarily through inter-residue n→π* interactions rather than H-bonds involving the backbone amide NH.[29-31] The discovery of unnatural residues that stabilize PPII conformation could facilitate the development of new peptidomimetic strategies. Pro exhibits the highest PPII propensity among the 20 encoded amino acids and is a major component of PPII secondary structure. For this reason, several studies have examined PPII stability using substituted Pro and N-alkylglycine (peptoid) residues.[29,30,32,31] We hypothesized that N-amination of proteinogenic residues may increase their PPII propensity through enhancement of n→π* interactions. However, the preference for Cα-substituted α-hydrazino acids to adopt β-sheet conformation led us to focus on aGly as a potential Pro surrogate.

We employed the protocol described above to prepare an aGly analogue of the PPII octapeptide model reported by Brown and Zondlo.[33,34] The parent peptide is comprised of a pentaproline core flanked by Gly residues, and incorporates a C-terminal Tyr to facilitate quantitation by UV absorbance. As shown in Scheme 4, we prepared the aGly analogue of this octapeptide peptide in 9% overall yield following RP-HPLC purification. Alternatively, the crude peptide obtained following cleavage from the resin was subjected to reductive alkylation in the presence of acetone to provide iPr-aGly-containing peptide **2** in 8% overall yield following purification. Crude **1** was obtained without significant aGly trifluoroacetylation and in reasonably high

purity as determined by analytical RP-HPLC (Figure 4). Omitting acetone from the cleavage cocktail led to formation of the undesired trifluoracetyl derivative as the major product. The hydrazone intermediate following cleavage is unstable in acidic aqueous media, and we typically observe only the hydrazide product by RP-HPLC/MS. Additional acetone is added during the reductive alkylation step to maximize conversion to the N'-alkyl hydrazide.



Scheme 4. Synthesis of PPII sequences **1** and **2**.

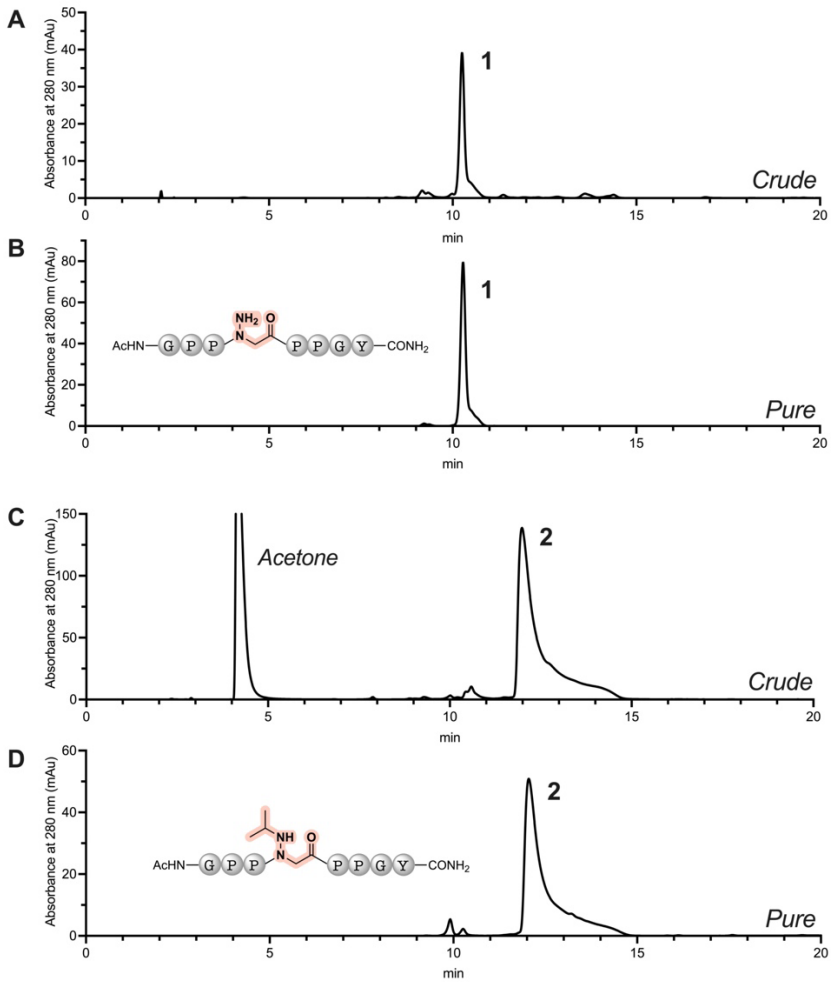


Figure 4. Analytical RP-HPLC chromatograms (monitored at 280 nm). Traces were generated on a C12 column and a linear gradient of 5-40% MeCN in water (solvents modified with 0.1% formic acid) over 20 minutes. (A) Crude aGly-containing **1** following precipitation. The hydrazone formed by cleavage in the presence of acetone is hydrolyzed by the HPLC mobile phase. (B) Peptide **1** following purification. (C) Crude **2** following addition of $NaCNBH_3$. The peak observed at retention time = 4.25 min is acetone. (D) Peptide **2** following purification.

Circular dichroism (CD) spectroscopy provides a convenient means by which to assess PPII helicity in this octapeptide model. The parent peptide (Ac-Gly-Pro_5-Gly-Tyr-NH_2; **3**) exhibits a small but clear positive Cotton effect near 228 nm that arises from the carbonyl n→π* electronic transition (Figure 5A). [35,36] A strong negative band at ~204 nm is also characteristic of PPII conformation. As expected, we found that a peptide harboring a Gly5 residue (**2**) exhibited near total loss of the positive band at 228 nm, indicating severe disruption of PPII

structure. In contrast, the aGly analogue **1** exhibited markedly enhanced PPII helicity as judged by a more intense maximum molar ellipticity at 228 nm. Remarkably, the PPII helical propensity of the aGly residue far exceeded that of Pro and Sar, which had been recently measured in the same model system.[32] We ruled out enhanced *trans* ω rotamer population as the reason for the increased PPII helicity of **1** based on the equilibrium ratios shown in Figure 2. Further enhancement of the PPII fold upon incorporation of aGly at the C-terminus of the pentaproline core suggests that the electron-withdrawing N-amino group strengthens n→π* interactions by enhancing carbonyl π* acceptor capacity.[14]

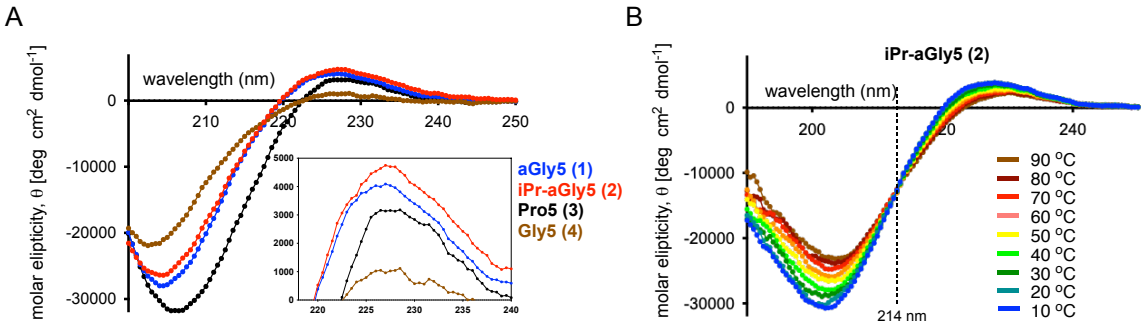


Figure 5. (A) CD spectra for PPII octapeptides containing aGly (**1**), iPr-aGly (**2**), Pro (**3**), and Gly (**4**), at position 5. (B) Variable temperature CD for iPr-aGly derivative **2**. CD spectra were measured at 150 μM in aqueous buffer (5 mM sodium phosphate, 25 mM KF).

In search of residues with even higher PPII helical propensity, we prepared both N'-acylated and alkylated derivatives of **1** following cleavage from the resin.[14] A small set of reductive alkylation products were synthesized as described in the protocol above. Out of these, isopropyl-substituted aGly analogue **2** emerged as the derivative with the highest maximum molar ellipticity. As with the Pro5 and aGly5 analogues, iPr-aGly5 peptide **2** demonstrated two-state denaturation behavior by variable temperature CD, with a clear isodichroic point at 214 nm (Figure 5B). Although the model peptide we employed is known to undergo a non-cooperative melting transition, we were able to estimate melting temperatures from first derivative analysis of maximum molar ellipticities across the temperature range.[29] Using this method, we calculated approximate T_m values of 25 °C, 48 °C, and 53 °C for the Pro5 (**3**), aGly5 (**1**), and iPr-aGly5 (**2**) analogues, respectively (Table 1).[14]

These data agree with the PPII helical propensities inferred from rt CD data and demonstrate that iPr-aGly is a remarkably effective stabilizer of the PPII fold.

Table 1. Maximum molar ellipticity at rt and estimated melting temperatures for PPII helix peptides **1**-**3**, based on the sequence Ac-Gly-Pro-Pro-Xaa-Pro-Pro-Gly-Tyr-NH_2. CD data obtained at 150 μM in aqueous buffer (5 mM sodium phosphate, 25 mM KF).

peptide	Xaa	$[\theta]_{228}$	T_m (from $\delta[\theta]/\delta t$)
1	aGly	4000	48 °C
2	iPr-aGly	4700	53 °C
3	Pro	3100	25 °C

4.2. Probing the Impact of N'-Alkyl aGly Residues on β-Hairpin Stability

Autonomously folded β-sheet model peptides have played an important role in determining the conformational propensities of natural and unnatural amino acid residues.[37] Understanding the factors that promote β-hairpin structure in these minimalist systems can inform strategies to stabilize more complex protein folds. A well-established β-hairpin model system designed by Gellman and coworkers (**5**, Figure 6) has been used to probe the impact of residue substitution on folding.[38,39] Linear dodecapeptide **5** folds into a stable β-sheet conformation in water as determined by NMR. Previous work with this and similar β-sheet models have related the chemical shifts of diagnostic α protons to folded population. Relative to random coil peptides, the extended ψ and ϕ dihedral angles in β-sheets result in downfield Hα shifts, or "secondary chemical shifts".[40] Comparing the Hα chemical shifts of H-bonded reporter residues (Figure 6) to the fully folded cyclic hairpin (**7**) and fully unfolded L-Pro mutant (**8**) allows for a straightforward calculation of β-sheet folded population. Our earlier work investigating the conformational impact of backbone N-amination found that introducing the N-amino group within the strand region of β-sheets enhances folded population.[8,9] However, the conformational effect of α-hydrazino acid substitution within the β-turn motif remains unexplored.

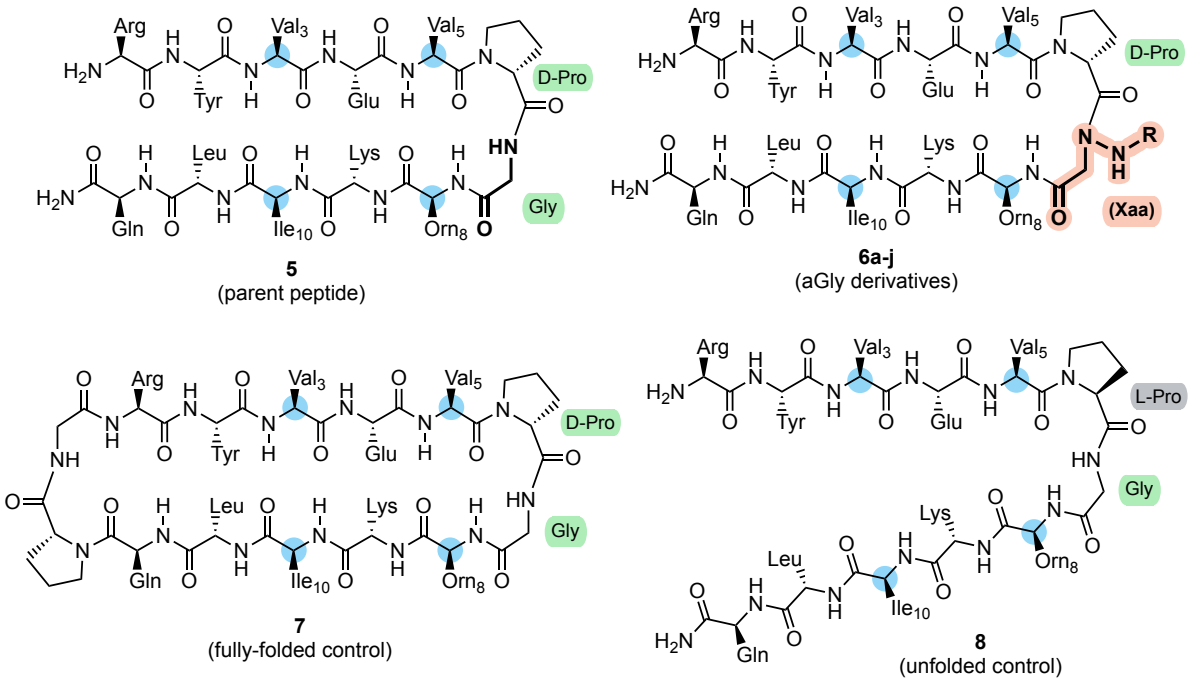


Figure 6. Structure of the parent model β-hairpin **5**, aGly peptides **6a-j**, cyclic control peptide **7**, and unfolded control peptide **8**. The blue circles indicate the reporter Hα protons.

Cognizant of the fact of Cα-substituted hydrazino acids promote an extended peptide conformation, we focused on N-amination of the type II' β-turn Gly7 residue in **5**. We employed the protocol described above to prepare **6a** and carried out late-stage reductive alkylation with various aldehydes/ketones to afford peptides **6b-j**. This conformational scanning approach thus generated 10 β-turn-modified analogues of peptide **5** from a single batch of crude substrate prepared on resin (1 mmol scale). Table 2 depicts each of the modified aGly peptides and their overall yields following purification. As with peptide **1,** crude **6a** was obtained in reasonably high purity after precipitation and in good overall yield. All peptides exhibited excellent purity following RP-HPLC (Figure 7).

Table 2. HRMS (ESI-TOF), and isolated yields for synthesized aGly β-hairpin peptides.

H-Arg-Tyr-Val-Glu-Val-**Xaa**-Orn-Lys-Ile-Leu-Gln-NH_2

peptide	Xaa	Calculated *m/z*	Observed *m/z*	Yield %
6a	aGly	1429.8565 $[M+H]^+$	1429.8624 $[M+H]^+$	60
6b	iPr-aGly	1471.9035 $[M+H]^+$	1471.9119 $[M+H]^+$	23

6c	Bu-aGly	1485.9191 $[M+H]^+$	1485.9269 $[M+H]^+$	12
6d	iBu-aGly	1485.9191 $[M+H]^+$	1485.9262 $[M+H]^+$	21
6e	Bn-aGly	1519.9035 $[M+H]^+$	1519.9107 $[M+H]^+$	6
6f	nPen-aGly	1499.9348 $[M+H]^+$	1499.9409 $[M+H]^+$	18
6g	Chx-aGly	1525.9504 $[M+H]^+$	1525.9585 $[M+H]^+$	15
6h	PCB-aGly	1553.8645 $[M+H]^+$	1553.8722 $[M+H]^+$	2
6i	PMB-aGly	1549.9140 $[M+H]^+$	1549.9239 $[M+H]^+$	2
6j	pyr-aGly	1508.8987 $[M+H]^+$	1508.9072 $[M+H]^+$	14

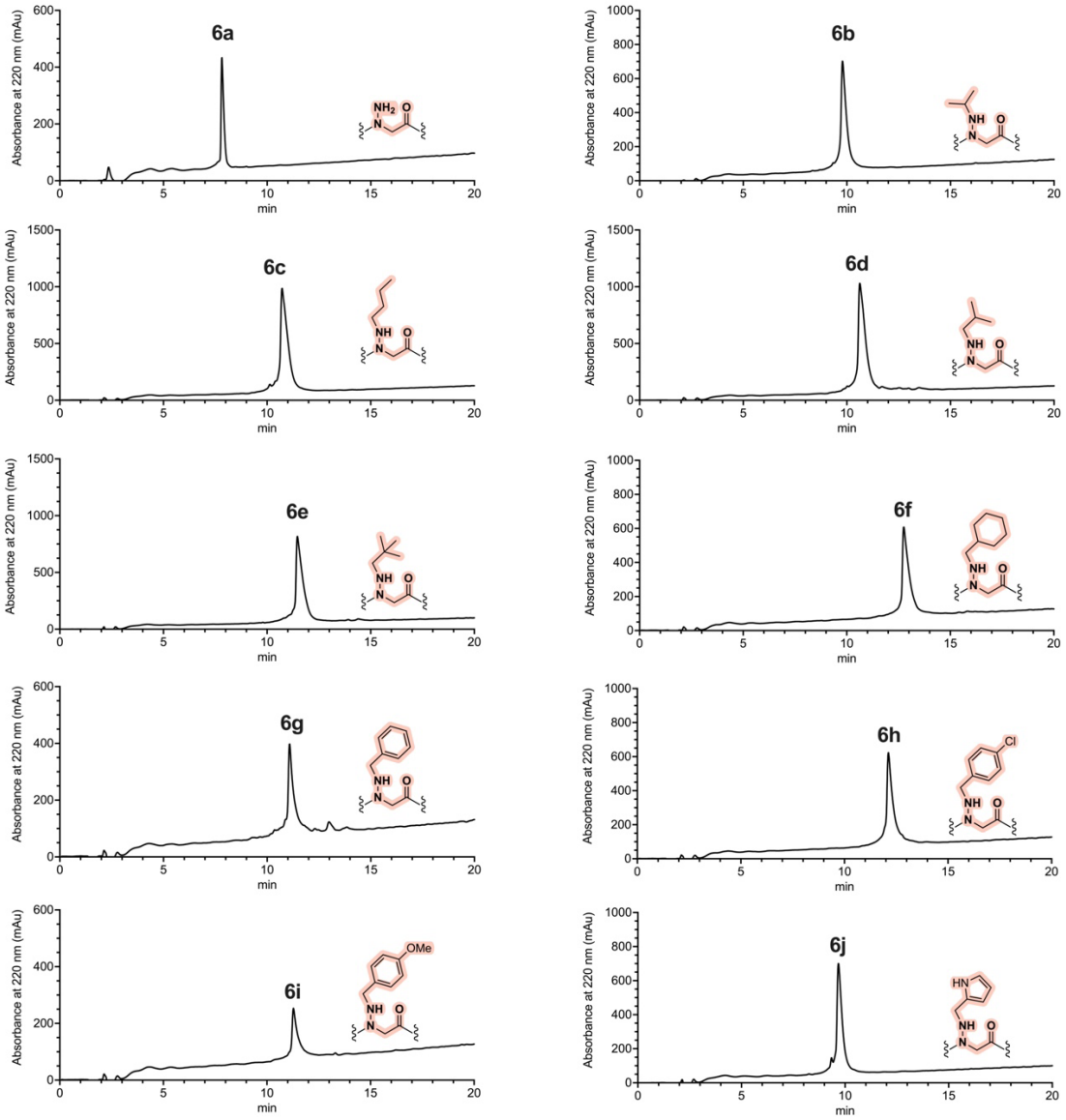


Figure 7. Analytical RP-HPLC chromatograms of peptides **6a – 6j** after purification. Traces were generated on a C12 column and a linear gradient of 5-40% MeCN in water (solvents modified with 0.1% formic acid) over 20 minutes.

The degree of folding of the aGly and N'-alkylated peptide hydrazides was determined by NMR, following previously established methods. For each of the peptides, the Hα chemical shifts of the Val3, Val5, Orn8, and Ile10 reporter residues were assigned using TOCSY experiments. Analogues with a high β-hairpin population exhibit a pronounced downfield shift of the Hα signal for these residues. The folded populations ($\%_f$) and equilibrium constants (K_f) for **6a-j** were calculated based on the extent downfield shift relative to fully folded (**7**) and unfolded (**8**) control analogues (Figure 8).[39] Values derived from each position were then averaged and converted to ΔG_f. Calculations and standard propagation of error was carried out as previously described.[38,39]

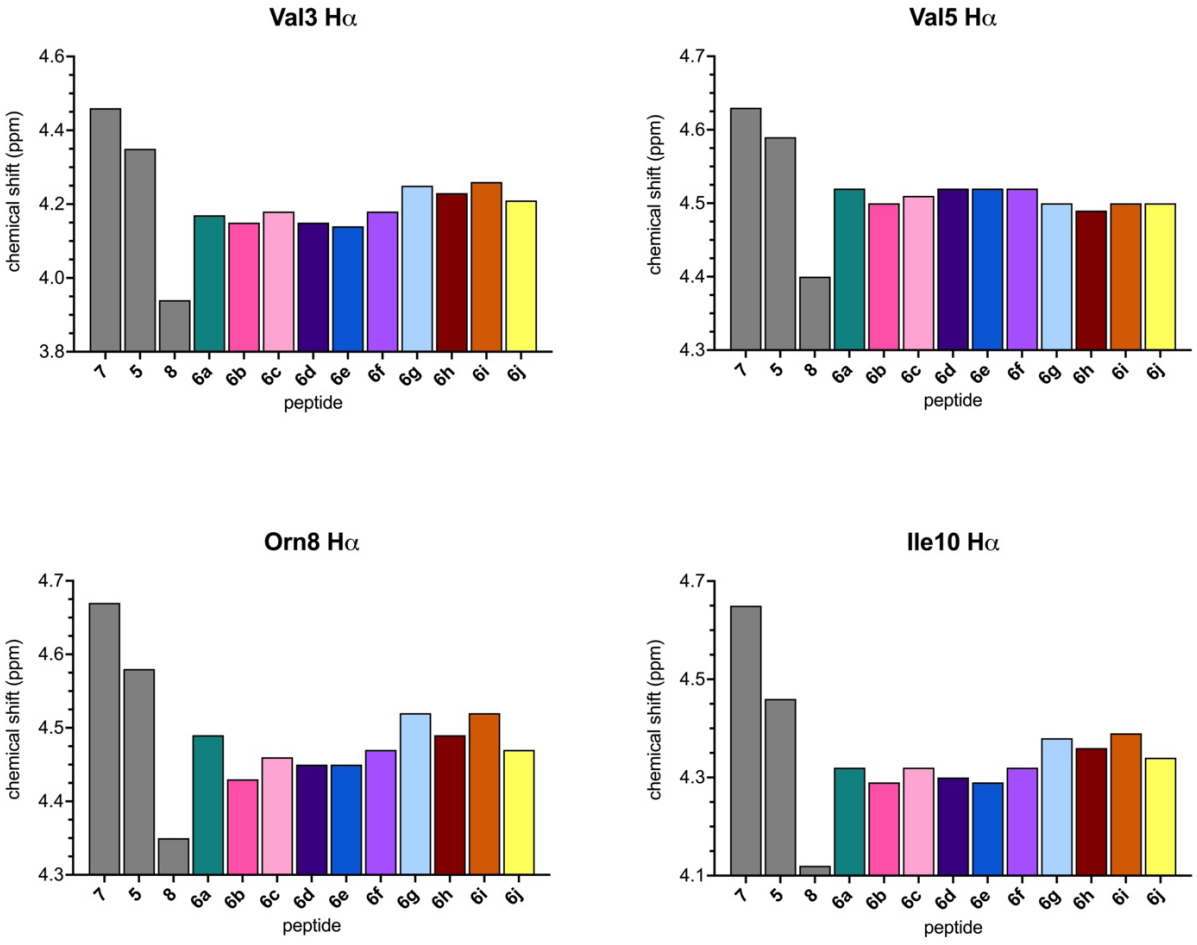


Figure 8. Hα NMR shifts of reporter residues in **6a-j** relative to fully folded control peptide **7,** unfolded control peptide **8**, and linear parent peptide **5**. Peptides were dissolved in D_2O (2 mM peptide concentration in 100 mM acetate buffer, pH 3.8, uncorrected) and spectra were collected at 4 °C on an 800 MHz NMR.

As shown in Table 3, N-amino and N-alkylamino substituents on the β-turn Gly residue compromised folding. Although differences between most of the substituted aGly derivatives were within the range of uncertainty, some trends are suggested by the data. The greatest energetic penalty was incurred upon incorporation of iPr-aGly at position 7 (**6b**). This destabilization is possibly due to steric interactions involving the branched alkyl hydrazide. The unbranched butyl group of **6c** also appeared better tolerated than the isobutyl or neopentyl substituents in **6d** and **6e**. Peptides **6f** and **6j**, featuring N'-cyclohexyl or N'-pyrrole substituents, exhibited folded populations similar to that of **6a**. Interestingly, incorporation of N'-benzyl substituents as in **6g-i** seems to restore stability relative to the unsubstituted hydrazide in **6a**. Although these analogues were still less folded than control peptide **5**, peptides **6g** and **6i** exhibited greater than 50% folded population at 4 °C. The Bn-aGly and PMB-aGly residues in **6g** and **6i** significantly enhanced β-hairpin folding relative to peptide **6b**, which harbors the PPII-promoting iPr-aGly residue. A representative TOCSY NMR expansion depicting each of the reporter residue correlations in **6i** is provided in Figure 9.

Table 3. Thermodynamic analysis of N'-alkyl aGly β-hairpins.[a]

peptide	$\%_f$	K_f	ΔG_f [kcal mol^{-1}]
5	75 ± 8	2.92 ± 0.46	–0.59 ± 0.09
6a	45 ± 6	0.80 ± 0.15	+0.12 ± 0.10
6b	35 ± 8	0.54 ± 0.18	+0.34 ± 0.18
6c	42 ± 7	0.71 ± 0.16	+0.19 ± 0.12
6d	39 ± 9	0.65 ± 0.22	+0.24 ± 0.18
6e	38 ± 10	0.62 ± 0.22	+0.26 ± 0.20
6f	44 ± 7	0.77 ± 0.17	+0.14 ± 0.12
6g	51 ± 7	1.05 ± 0.21	–0.03 ± 0.11
6h	46 ± 7	0.85 ± 0.19	+0.09 ± 0.12
6i	52 ± 8	1.09 ± 0.21	–0.05 ± 0.12
6j	44 ± 6	0.78 ± 0.15	+0.14 ± 0.11

[a] Folded fractions ($\%_f$) are reported as the mean of percentages calculated at each reporter residue from Hα data using the equation: $\%_f = (\delta_{obs} - \delta_u)/(\delta_f - \delta_u) \times 100$. Folded fraction error represents standard deviation from the mean. The fraction folded was converted to folding equilibrium constant (K_f) and folding free energy (ΔG_f) with standard propagation of error.

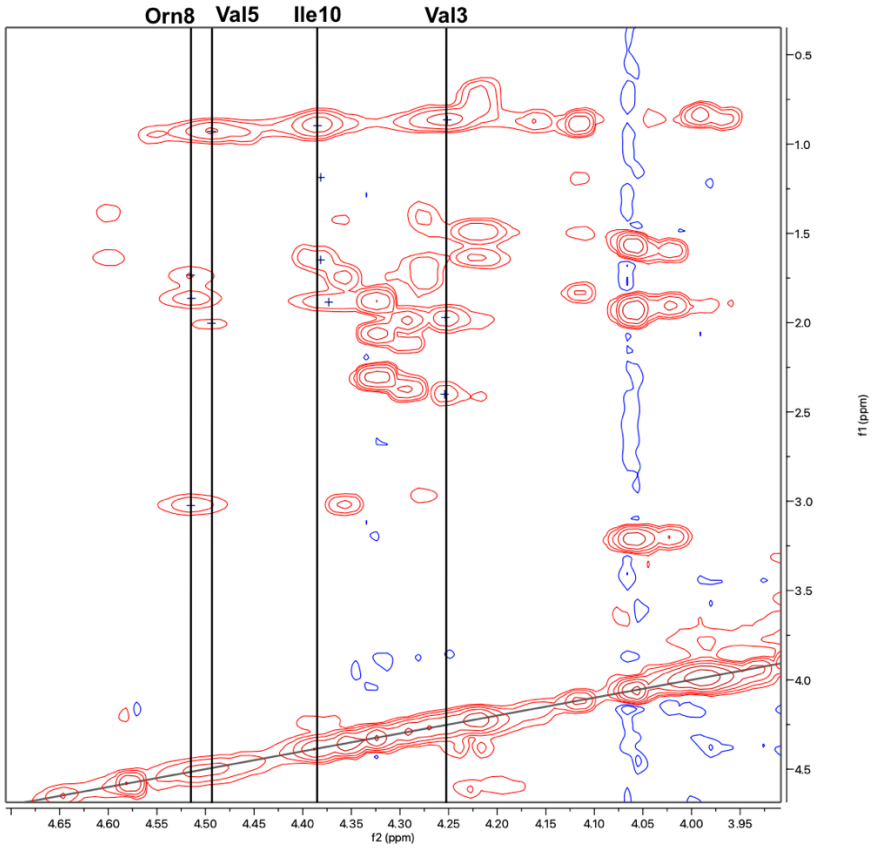


Figure 9. TOCSY of **6i** focusing on the Hα region. 2 mM **6i** was dissolved in D_2O (100 mM acetate buffer, pH 3.8, uncorrected) and spectra were collected at 4°C on an 800 MHz NMR. Reporter residue correlations are indicated with vertical lines.

5. SUMMARY

The above protocol details a convenient approach toward libraries of aGly peptide derivatives. Submonomer synthesis of aGly-containing peptides results in high crude purities and good overall yields. Subsequent reductive alkylation reactions can be carried out in water using crude unprotected substrates. Given access to a variety of commercially available aldehydes, the described protocol enables the synthesis of novel peptidomimetics with diverse backbone functionalities. These hybrid peptidomimetics can be used in a variety of applications including the rapid optimization of ligands for biological targets. Late-stage derivatization of peptides can also be leveraged for conformational scanning to identify motifs that stabilize a desired fold. Such studies can help to inform the design of new peptidomimetic strategies with broad applications. We demonstrated this approach in the context of PPII and β-hairpin models of folding. While an N'-alkylated aGly derivatives were found to strongly stabilize PPII conformation, replacement of a type II' β-turn residue with aGly

destabilized the β-hairpin fold. However, some N'-alkylated aGly derivatives were better tolerated in the hairpin, highlighting the utility of a diversity-oriented scanning approach.

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