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Post Translational Backbone Engineering through Selenomethionine Mediated Incorporation of Freidinger Lactams

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Abstract

Amino- γ -lactam (Agl) bridged dipeptides, commonly known as Freidinger Lactams, have been shown to constrain peptide backbone topology and stabilize Type II' β -turns. The utility of these links as peptide constraints has inspired new approaches to their incorporation into complex peptides and peptoids, all of which require harsh reaction conditions or protecting groups and limit their use on unprotected peptides and proteins. Here, we employ a mild and selective alkylation of selenomethionine in acidic aqueous solution, followed by immobilization of the alkylated peptide on to bulk reversed-phase C18 silica, and base-induced lactamization in DMSO. The utilization of selenomethionine, which is readily introduced by synthesis or expression, and mild conditions enable selective backbone engineering in complex peptide and protein systems.

Keywords

Freidinger Lactam; selective modification; Selenomethionine; Peptidomimetic; backbone constraint

In solution, peptides exist in an equilibrium of conformational states^[1] and backbone engineering has long been utilized to stabilize specific backbone topologies.^[1,2] Amino- γ -lactam (Agl) bridged dipeptides, commonly known as Freidinger lactams, have been shown to stabilize Type II' β -turns, a common motif in peptide structure.^[2] These peptidomimetic constraints restrict the peptide bond to a *trans*-confirmation limiting the conformational space accessible to the ψ_1 and ω_1 dihedral angles.^[1,2] Freidinger lactams were originally developed through a computational effort led by Rodger Freidinger at Merck in the 1980's.^[1] The original synthesis of amino- γ -lactams was accomplished through the use of protected methionine dipeptides and an intramolecular alkylation route was applied (Figure 1 A)^[1] where the thioether moiety is alkylated with methyl iodide converting it into the corresponding dimethyl sulfonium salt leaving group.^[1–3] Treatment with sodium hydride deprotonates the amide backbone, cyclizing to the Agl via a 5-exo-tet cyclization.^[1–3] These dipeptide lactams were then utilized in solid and solution phase peptide synthesis. Since its introduction the Freidinger lactam has been incorporated into numerous biologically relevant peptide systems.^[2] Although multiple methods have been developed to incorporate Amino- γ -lactams into chemically synthesized peptides and peptoids exist,^[4] including Kodadek's

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reductive amination based route,^[5] Lubell's cyclic sulfamidate facilitated approach^[6] and Amblard's solid phase adaptation (Figure 1B)^[3] of the methyl-sulfonium approach,^[1] most of these methods require protecting groups or harsh conditions, making them inapplicable for use on unprotected proteins or peptides.^[1–3,5,7]

Drawing inspiration from Freidinger's original work, we chose to pursue a selenomethionine (SeM, Z) mediated route as the selenoether moiety of selenomethionine is more nucleophilic than methionine, which allows for a milder alkylation.^[8,9] In addition, the resulting selenonium salt would be a better leaving group than the previously studied sulfonium salts, encouraging a facile cyclization. Since SeM can be incorporated into peptides by routine SPPS^[10] and auxotrophic expression,^[11–20] a chemoselective transformation of SeM into a lactam would facilitate generation of backbone modified peptidomimetics from unprotected protein precursors (Figure 1D). Here we present a method that allows for the straight forward incorporation of Freidinger lactams into unprotected peptides and proteins.

To determine the best path toward selenonium formation, alkylating agents were screened for their ability to alkylate SeM. Model peptide (**1**), Ac-GZGLYRAG-NH₂ (Z=selenomethionine), was treated with the following electrophilic alkylation agents under acidic aqueous conditions: benzyl bromide, iodoacetamide, methyl iodide, and benzyl chloride (Figure 2). Surprisingly, the original alkylating agent employed by Freidinger, methyl iodide, yielded no conversion to the methyl selenonium adduct under the tested aqueous conditions,^[1] while, benzyl bromide quickly alkylated selenomethionine resulting in complete conversion to the benzyl-selenonium adduct (**2**) in 1 hour.^[9,21,22] Upon determining that benzyl bromide was the preferred alkylating agent, conditions that would induce cyclization of the benzyl-selenonium adduct to the Freidinger lactam were explored. Initial attempts treating the benzyl selenonium adduct (**2**) in solution with 5% DBU in either water or DMF resulted in full reversion to the original peptide (**1**) and no lactam product. When the same peptide was treated with 5% DBU in DMSO, partial conversion to lactam (**3**), Ac-G-Agl-GLYRAG-NH₂ was observed. Although these conditions produce the desired species they presented a significant peptide handling challenge. High DBU concentrations experienced upon concentration in vacuo are likely responsible for peptide degradation. Also, the base/DMSO mixture is not ideal for preparative HPLC purification or concentration.

Recently, we introduced Reversible Adsorption to Solid Support (RASS) chemistry, a peptide handling method that facilitates the performance of multiple sequential reactions on unprotected peptides without the need for intermediate purification and handling steps (Figure 3).^[23] The unique polyvalent adsorption kinetics of peptides onto reversed-phase C₁₈ Silica allows the peptide to be adsorbed to the silica while small molecule reagents can be added or removed through washing.^[23–26] Taking advantage of the distinct differences between polyvalent vs monovalent binding kinetics, sequential reactions can be performed on an immobilized peptide while reagents can be washed away and solvents exchanged (Figure 3).^[23–26] Binding peptide substrates to reversed-phase C₁₈ Silica allows for facile solvent transfer, introduction of small molecule reagents, and removal of excess or consumed reagents, critically allows for multiple reactions to be performed without intermediate preparative HPLC purification.^[23] The utilization of reversed-phase C₁₈ silica,

which is ubiquitous for chromatographic separation in peptide labs, also allows for the determination of washing and elution conditions by RP-HPLC.^[23,24] As applied to this system a RASS procedure would consist of adsorbing peptide (**2**) to C18 silica at 5 wt. %, from the crude benzylation reaction mixture. Washing the resin with 10% ACN should remove excess BnBr and then, the adsorbed peptide could be transferred between aqueous and organic systems. After the cyclization reaction excess base could be washed with 10% ACN and finally the product peptide eluted and recovered from 90% ACN. The RASS system proved useful in washing the reaction mixture of excess small molecule reagents and amenable for adsorbed peptide transfer between aqueous and organic systems.

As expected, BnBr can be washed away from the crude reaction mixture with 10% ACN in water, and the peptide remains adsorbed to the solid support. After establishing that RAPS would facilitate peptide handling, we screened bases spanning a range of pK_as for their ability to induce lactam cyclization (Figure 4). Selenonium-peptide (**2**) immobilized onto reversed-phase C₁₈ Silica, was treated with 5% v/v base in DMSO. Although many believe C₁₈ silica is not compatible with organic solvents and aqueous base, we found the modern material robust to the conditions tested. At specified time points, aliquots of resin were taken from the reaction mixtures, quenched, washed, eluted and analyzed by HPLC. Unhindered bases such as, pyridine, N-methyl morphine, and triethylamine cleave the benzyl-selenonium adduct, resulting in reversion to the original peptide (**1**). Encouragingly, DIPEA was able to convert a small amount of peptide to lactam product (**3**), and the more hindered, highly basic, DBU efficiently converted the benzyl-selenonium adduct to the desired lactam product (**3**) at >60% conversion. These data suggest that a strong hindered base is necessary to deprotonate the amide backbone, while not attacking the selenonium adduct at the benzyl carbon, to induce lactam cyclization. Assuming that the methodology would be applicable to non-Gly amino acids at the i+1 position, as it is analogous to Freidinger's original methodology, the model peptide Ac-GZALYRAG-NH₂ (R=Me, as shown in Figure 4) was benzylation and cyclized to the corresponding lactam with similar ease to the SeM-Gly peptide (**1**) (Figure 4B).

The utility of this method for introducing β -turn stabilizing motifs into complex peptides was evaluated. The peptide MAX-1, developed in the Schneider Lab at the National Cancer Institute, is a amphiphilic β -turn containing peptide that can be induced to form viscoelastic hydrogels.^[27-31] Its β -turn motif is stabilized by a ^DPro-^LPro motif, a common Type II' β -turn stabilizer, at the i+1 and i+2 positions of the turn.^[27,29,30] Modification of this peptide to include SeM-Gly at the i+1 and i+2 positions of the turn, gave the parent sequence H-VKVKVKVKVZGTKVKVKVKV-NH₂. This peptide precursor was then efficiently transformed, on a 120 mg scale, as described above, into the lactam containing peptide, H-VKVKVKVKV-Agl-TKVKVKVKV-NH₂ (**4**) at 72% conversion by HPLC integration and 32% purified yield (by preparative HPLC). The secondary structure of both peptides were then probed by circular dichroism (CD) spectroscopy (Figure 5). Only in the lactam containing peptide was the distinct signature of extended β -fibril network formation observed, as indicated by the signal minimum at 215 nm (Figure 5)^[29,30] while Z containing peptide exhibits a spectrum consistent with a random coil. This indicates that the β -turn stabilizing lactam motif is required to induce gel formation. Also the peptide forms a

viscoelastic gel at 1 wt% in borate buffer at pH 9 (Figure 5). This technique easily allows us to chemically trigger β -turn stabilization, which can then be used for materials formation. The ease at which Z can be incorporated into peptides by SPPS and proteins by auxotrophic expression, suggests an interesting route for the large scale bioproduction of constrained peptide materials.

To test the applicability of the method toward larger protein domains, the WW-domain of the protein Pin1 was selected, since it has been widely studied to evaluate mimics of type-II' beta-turn systems.^[32–36] This protein domain was engineered and chemically synthesized with Z at the i+1 position of the turn (sequence -S-Z-S-S-) (**5**) to be modified to an amino- γ -lactam bridging the i+1 and the **serine** at the i+2 positions of the turn (sequence -S-**Agl**-S-S-) (**6**) (Figure 6). The endogenous methionine at position 10 was retained, allowing us to test the selectivity of the lactam cyclization at Z vs M (in an expressed system this methionine would likely be mutated to a leucine^[37–40]). The protein was benzylated (completely at the selenomethionine and to a lesser extent the methionine) but cyclized only at the Z position (SI). This protocol resulted in the generation of the desired product (**6**) with the Agl at position 12 and one major side product (~60%), which is the Agl containing WW-domain S-benzylated at methionine 10. The crude reaction mixture was purified by mass-directed preparative HPLC in which (**6**) and the S-benzyl met side product were collected separately and pure (**6**) was obtained in 12% recovered yield. Importantly, no product containing two lactams is observed, implying that the benzyl-sulfonium adduct is not a sufficient leaving group and Z is required for cyclization to the lactam product SI. Although not undertaken in this study the benzyl sulfonium adduct (side product) is known to be cleaved by treatment with a number of nucleophilic thiols,^[22] which could be used to generate the target peptide (**6**) which contains an unmodified methionine.^[22] Although it is not anticipated that both Met and SeM residues be incorporated into the same peptide, methods exist to generate an Agl containing peptide *via* SeM that also contains an unmodified methionine.^[22]

Biophysical properties of the resulting protein domain were then studied (Figure 6). CD spectra of the Z (not cyclized) (**5**) and Agl (**6**) containing peptide were taken (20 μ M peptide, 10 mM sodium phosphate buffer pH 7) (Figure 6). The spectra show similar behaviors in wavelength scans (between 190 and 250 nm) at 25° C and thermal denaturation curves (monitored at 227 nm) imply that the amino- γ -lactam unit does not perturb the protein structure (Figure 6). Finally, domains (**5**) and (**6**) were analyzed by trypsin digest MS/MS sequencing to further confirm the presence of the lactam. In both species the turn-inducing fragment were detectable by MS and were readily subjected to MS/MS analysis. In the Z containing species (**5**) the masses corresponding to the first three fragmentations (loss of Met, Ser, and SeM) were found and the expected masses corresponding to further single amino acid fragmentations are found at a lowered threshold. In the Agl-containing species (**6**) the first two fragmentations (loss of Met and Ser) are clearly seen and importantly, even at the lowered threshold, the masses corresponding to further single amino acid fragmentations are not seen. Instead, loss of the dipeptide Agl-Gly is observed, indicating that the amino- γ -lactam was incorporated into the protein domain (SI).

The chemical modification of proteins has become a routine approach for introducing functionality into a protein framework. However, such modifications are exclusively located at the termini and on amino acid side chains. In this work we demonstrate the backbone engineering of a protein through biocompatible transformation. This approach will facilitate the introduction of turn-inducing structures in peptide materials and folded proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Freidinger, 1982 **B.** Amblard, 2015 **C.** This Work **Figure 1:**

A^[1] and **B**^[3] Previously reported routes to Freidinger lactams. **C** this work

Figure 2:

A Selenomethionine alkylation with: benzyl bromide, methyl iodide, iodoacetamide (IAM), and benzyl chloride. **B** Conversion of **1** to corresponding selenonium **2**.

Figure 3:
Workflow for performing lactam cyclization on reversed-phase C₁₈ Silica

Figure 4:

A Lactam cyclization **B** Conversion of benzylated starting material **2** (Bn SM) to **3** (Lactam) with different bases, while adsorbed to bulk C₁₈ Silica.

Figure 5:

A Structure of expressible AgI containing hydrogel peptide. **B** CD of SeM containing precursor and **C** AgI-containing peptide **4** at pH 9 (150 μ M peptide, 125 mM Borate and 150 nM NaCl at 25°C) at specified time points after mixing.

Figure 6: **A** 5 SeM containing WW-domain and **6** Agl-containing WW-Domain. Structure rendered from PDB 2KCF.^[28] **B** CD wavelength scans and **C** CD thermal denaturation curves of **5** and **6** at 20 μ M, in 10 mM sodium phosphate pH 7