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Stabilization of Cyclic β-Hairpins by Ugi-Reaction-Derived *N*-Alkylated Peptides: The Quest for Functionalized β-Turns

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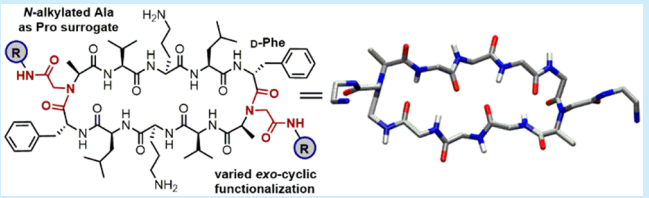
Supporting Information

ABSTRACT: A solid-phase approach including on-resin Ugi reactions was developed for the construction of β-hairpins. Various *N*-alkylated dipeptide fragments proved capable of aligning antiparallel β-sheets in a macrocyclic scaffold, thus serving as β-hairpin templates. Gramicidin S was used as the model β-hairpin to compare the Ugi-derived β-turns with the type-II′ β-turn. The results show that the multicomponent incorporation of such *N*-alkylated residues allows for the simultaneous stabilization and exo-cyclic functionalization of cyclic β-hairpins.



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Macrocyclization approaches based on multicomponent reactions (MCRs) have recently emerged as excellent tools for the incorporation of conformational constrains in peptide sequences.[1,2] In particular, Ugi-type macrocyclizations[3−6] have proven great success in accessing constrained cyclic peptides. At the same time, they allow for tunable exo- and endo-functionalization of the macrocyclic scaffold. Thus the Ugi macrocyclization has been employed for stapling peptides in α-helical[3] and turn[4] conformations, but so far, the Ugi moieties have not been responsible for imposing a specific conformation on the peptide backbone. A different scenario shows up in various MCR macrocyclizations developed by Yudin and coworkers,[5,6] in which either endo-cyclic heterocycles[7] or exo-cyclic amides,[8] formed during the MCR ring closure do have a determinant role in the peptide conformation by participating in intramolecular hydrogen bonds.

In an endeavor to demonstrate that the *N*-alkylated peptide fragment resulting from the Ugi macrocyclization is capable of enforcing a conformational bias, leading to a stable secondary structure, herein we describe a strategy for the construction of Ugi-derived cyclic β-hairpins. A β-hairpin is a class of secondary structure in which a reverse turn or loop is flanked by two antiparallel β-sheets.[9] This class of peptide motif, indeed comprising diverse conformations, is recognized as one of the most important protein regular structures and functional epitopes.[9] There are several hairpin-stabilizing templates known in the literature, such as the dipeptide D-Pro-L-Pro and combinations of Pro with other amino acids (AAs), for example, D-AA-L-Pro and Gly-L-Pro.[9] However, we are not aware of approaches based on Ugi or any other type of MCR macrocyclization targeting peptide mimics of β-hairpins.

We chose the antibacterial cyclic decapeptide Gramicidin S (GS, cyclo(Pro-Val-Orn-Leu-D-Phe)$_2$) as the model compound for β-sheet conformational mimicry.[10] Whereas our goal is not to develop novel antibacterials, GS is considered as a prototypical β-hairpin compound, and its structure has been previously used to design novel peptidomimetics[10−12] and sugar amino acid[13] turn inducers (Figure 1A). GS is a C2-symmetric

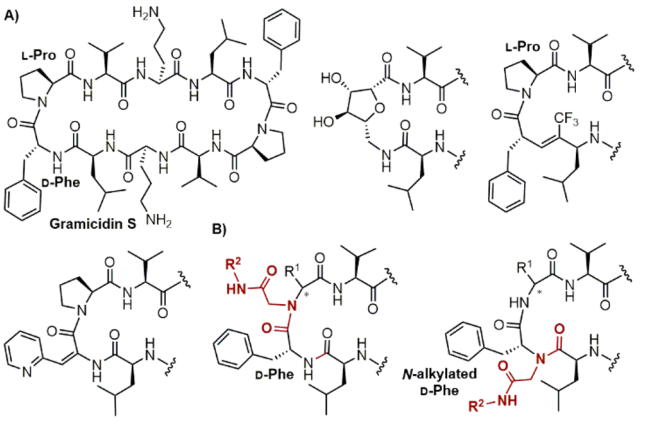

Figure 1. (A) Gramicidin S and some β-turn mimics used in its analogs. (B) Ugi-reaction-derived *N*-alkylated peptide fragments as novel β-hairpin-stabilizing templates.

β-hairpin in which two antiparallel β-strands are connected by two type-II′ β-turns induced by the dipeptide D-Phe-Pro. As depicted in Figure 1B, we envisioned that an *N*-alkylated peptide fragment created by the Ugi reaction could serve as a β-hairpin stabilizing template due to the conformational bias imposed by the turn-inducing effect of the tertiary amide, in a similar way as Pro and *N*-Me-AAs do in reverse turns.[9]

Our group has gathered previous evidence of the ability of Ugi-derived *N*-alkylated residues to fold oligopeptide chains[14]

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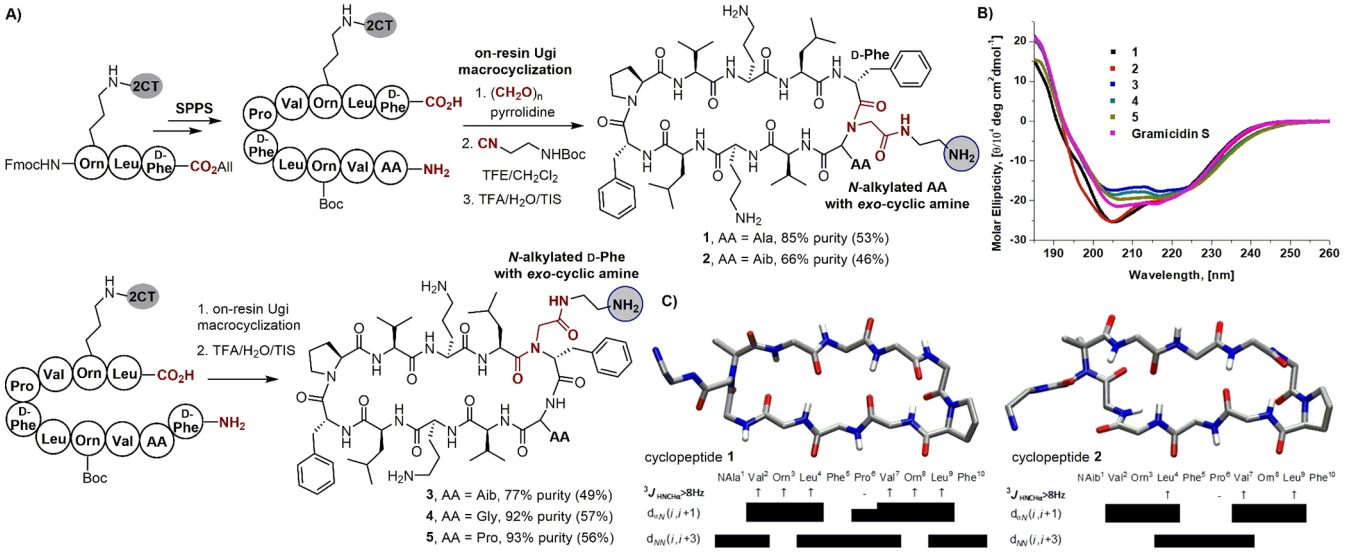
Scheme 1. (A) Synthesis of Cyclic Peptide β-Hairpins by On-Resin Ugi Macrocyclization,[a] (B) Circular Dichroism Spectra of Ugi-Derived β-Hairpins Compared with Gramicidin S, and (C) Average NMR-Derived Structures of Cyclic Peptides 1 and 2[b]



[a]In parentheses are the yields of crude products. An analytical sample was purified to >95% purity. [b]Most side chains are omitted for clarity.

and facilitate macrocyclization[15] by engaging the two termini. In addition, a recent study proved that the replacement of Pro by *N*-Me-Ala does not affect the activity of the GS analogs.[16] As a result, we initially focused on substituting one of the Pro residues in GS by an *N*-alkylated AA generated by the Ugi reaction, which simultaneously allows an exo-cyclic functional group to be installed, something that cannot be achieved with the use of Pro or *N*-Me-AAs.

Seeking to conduct most steps on resin, including the key Ugi multicomponent macrocyclization, an orthogonal solid-phase peptide synthesis (SPPS) strategy was envisioned. For this, it was important to achieve the previous attachment of the Orn side chain to a resin, thus leaving the two peptide termini free for macrocyclization. Albericio and coworkers were the first to develop an SPPS approach toward cyclic peptides, comprising the attachment of an amine-containing side chain to a resin.[17] In addition, the same group reported the synthesis of GS analogs by cyclization of the peptide at the Orn residue, whose side chain was anchored to a resin through a carbamate functionality.[18] However, nowadays it is known that the intrinsic folding of acyclic GS favors cyclization by residues opposed to one of the β-turns.[19] As a result, we designed the SPPS approach considering two dissimilar sites for incorporating the *N*-alkylation at one β-turn motif.

As depicted in Scheme 1, tripeptide Fmoc-Orn-Leu-D-Phe-OAll was produced in solution and next anchored to the2-clorotrityl (2CT) resin by the Orn side chain. Subsequent growth of the peptide sequence included the incorporation of either Ala or 2-aminoisobutyric acid (Aib) at the *N*-terminus, followed by the deprotection of the two termini. Thus the on-resin Ugi macrocyclization of the main-chain amino and carboxylic acid groups was conducted following a procedure recently developed for cyclizing peptide side chains.[3] The protocol comprises an initial transimination step using 4 equiv of paraformaldehyde and pyrrolidine, followed by washing of the resin-linked iminopeptide and a subsequent reaction with 4 equiv of the isocyanide in trifluoroethanol (TFE)/CH_2Cl_2 1:1 (v/v). Mini-cleavages and high-performance liquid chromatography (HPLC) analysis were carried out after 12 h of reaction, usually showing complete consumption of the linear peptide. In cases of incomplete macrocyclization after 12 h, a second cycle of imine formation and reaction with isocyanide is required (see the Supporting Information (SI)). It is worth mentioning that cleavage of the peptide from the 2CT resin was not detected during the course of the entire SPPS protocol, not even during the macrocyclization conducted in TFE/CH_2Cl_2.

Cyclic peptides **1** and **2** were produced by conducting the Ugi reaction ring closure with Ala and Aib, respectively, as the amino component and D-Phe as the carboxylic acid, thus forming an *N*-alkylated β-turn inducer opposed to the natural D-Phe-Pro sequence. The fact that even a peptide bearing a bulky Aib residue at the *N*-terminus undergoes, with two cycles, the Ugi macrocyclization in excellent yield corroborates the strength of the method. In parallel, cyclic peptides **3**, **4**, and **5** were prepared by choosing the other site recommended for cyclization, that is, between Leu and D-Phe. Once more, high crude purities were obtained for the three peptides, in which not only Pro (**5**) but also Gly (**4**) and Aib (**3**) were installed as preceding residues. GS was produced in a similar way in 67% crude purity using a final macrolactamization instead of the Ugi reaction ring closure.

An isocyanide bearing a protected amine was employed to introduce an additional exo-cyclic amino (cationic) group at the GS scaffold. In principle, any functionalization can be installed here in such a cyclo-ligation process. To address the effect of such structural variations on the stability of the β-hairpin, circular dichroism (CD) spectra were recorded and compared with those of GS. As shown in Scheme 1B, cyclic peptides **3**, **4**, and **5** exhibit CD spectra almost identical to those of GS, suggesting that the *N*-functionalization of D-Phe does not modify the type-II′ β-turn motif and keeps the stability of the β-hairpin. This result is reasonable for compounds **4** and **5**, in which the *N*-alkylated D-Phe-Gly and D-Phe-L-Pro fragments can be seen as mimetics of other type-II′ β-turns such as D-Pro-L-Pro and D-Pro-Gly. However, the fact that the *N*-alkylated D-Phe-Aib motif in **3** also induces this type of β-turn reproducing the β-hairpin conformation of GS is noteworthy.

However, in the case of cyclic peptides **1** and **2**, there is a small increase in the minimum at 205 nm as compared with the CD

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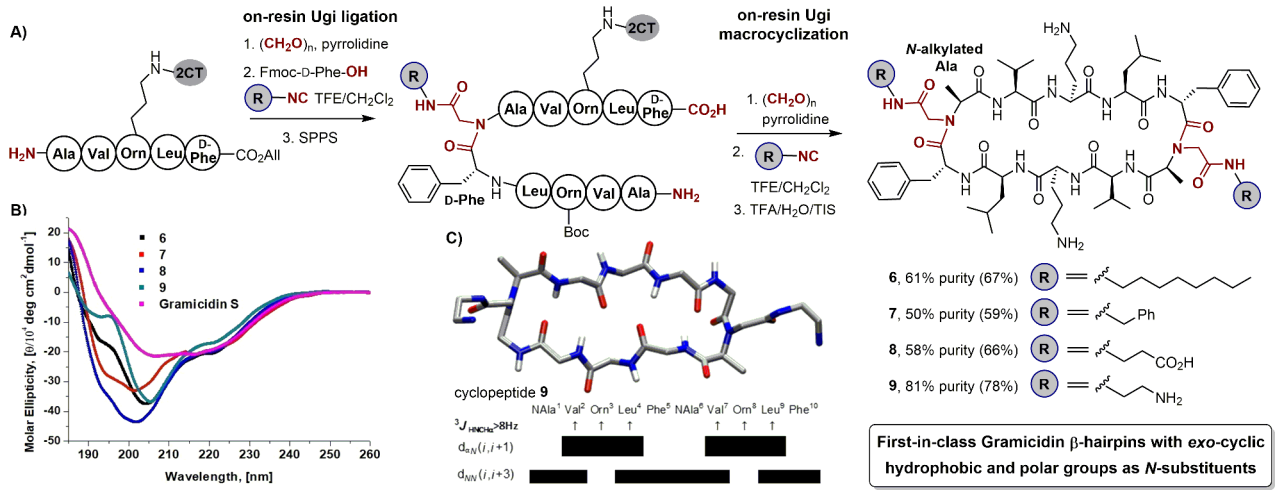
Scheme 2. (A) Synthesis of Cyclic β-Hairpins by an SPPS Approach Comprising Two Ugi Reactions,[a] (B) Circular Dichroism Spectra of Double Ugi-Derived β-Hairpins Compared with Gramicidin S, and (C) Average NMR-Derived Structure of Cyclic Peptide 9[b]



[a]In parentheses are the yields of crude products. An analytical sample was purified to >95% purity. [b]Most side chains are omitted for clarity.

spectrum of GS, proving a slight deviation from the model GS β-hairpin structure. To better understand this conformational change, the solution-phase structures of **1** and **2** were determined by means of nuclear magnetic resonance (NMR) and molecular dynamics (MD) simulations (see the SI). The NMR spectra of peptide **1** demonstrate an extended conformation for Val, Orn, and Leu residues (i.e., coupling constants $^3J_{NH\text{-}H\alpha}$ higher than 8 Hz and strong α_iN_{i+1} nuclear Overhauser effects (NOEs)) and two β-turns comprising the Leu-D-Phe-Pro-Val and Leu-D-Phe-*N*-(Alkyl)Ala-Val fragments, as confirmed by the N_iN_{i+3} NOEs between the amide protons of residues Val and Leu. In addition, cross-peaks indicative of the spatial proximity between the Hα of both Orn residues were observed.

As depicted in the NMR structure of **1** (Scheme 1C), this cyclic backbone occurs in a β-hairpin conformation, in which the exo-cyclic aminopeptidic moiety inserted as an amide *N*-substitution points to the opposite face of the Orn side chains. In this regard, Wishart and coworkers have shown that for type-II′ β-turns,[20] the side chains of residues *i*+1 and *i*+2 within the turn have equatorial and axial orientations, respectively, that is, D-Phe (*i*+1) and Pro (*i*+2) in GS. However, in cyclic peptide **1**, both the D-Phe (*i*+1) and *N*-(Alkyl)Ala (*i*+2) side chains have an equatorial disposition with respect to peptide backbone (see the side view in the SI), whereas it is actually the exo-cyclic *N*-substituent that is axially oriented. This analysis suggests that the Ugi-derived β-turn centered at D-Phe-*N*-(Alkyl)Ala is not of type II′. Nonetheless, this fact does not have a detrimental influence on the stability of the β-hairpin conformation; therefore, the combination of D-Phe with Ugi-derived *N*-alkylated L-AA could be considered as a new type of β-hairpin stabilizing template.

On the contrary, NMR evidence proves that the installation of an *N*-alkylated Aib does have a negative influence on the β-hairpin stability because the typical β-sheet characteristics are missing in cyclic peptide **2**. In this case, only the two Leu residues and one Val residue show coupling constants $^3J_{NH\text{-}H\alpha}$ higher than 8 Hz. Furthermore, the NOEs between Leu and Val amide protons are observed only for the residues enclosing the β-turn centered at D-Phe-Pro, whereas the dipeptide fragment D-Phe-*N*-(Alkyl)Aib does not induce a typical β-turn motif. As shown in Scheme 1C, the NMR structure of cyclic peptide **2** shows a distorted reverse turn at the D-Phe-*N*-(Alkyl)Aib corner, which certainly destabilizes the β-hairpin conformation.

We next wondered if the double substitution of the D-Phe-Pro fragments by the Ugi-derived D-Phe-*N*-(Alkyl)Ala would result in a stable β-hairpin conformation. As depicted in Scheme 2, an SPPS approach was employed in which an initial on-resin Ugi reaction enabled the introduction of the first *N*-alkylated Ala residue using Fmoc-D-Phe-OH as the carboxylic acid component and a variety of isocyanides. After the consecutive incorporation of the following AAs, a final Ugi macrocyclization was employed, as previously described, again coupling the terminal D-Phe and Ala with the introduction of a second amide *N*-substitution. The overall yields and purities of the crude peptides were acceptable considering the complexity of this SPPS sequence, which involved several coupling steps and two on-resin Ugi reactions, one of them for the ring closure. Thus cyclic peptides bearing long exo-cyclic aliphatic chains, phenyl rings, as well as anionic or cationic groups were readily produced.

The CD spectra of cyclic peptides **6**–**9** show a much more intense minimum around 205 nm for all compounds as compared with GS. This suggests a deviation from the native GS conformation, likely due to the presence of β-turn conformations different from that of GS. To get a deeper insight into this finding, we chose cyclic peptide **9** to determine the solution-phase structure based on NMR and MD simulations. Hence, all NMR evidence confirms that this type of cyclic peptide occurs in a β-hairpin conformation, albeit featuring β-turns different from the native type II′. The ^{1}H NMR spectrum of **9** proves the conformational symmetry expected for a cyclic C2-symmetric β-hairpin, in which there are only five amide protons (see the SI). Moreover, as expected for extended conformations, coupling constants $^3J_{NH\text{-}H\alpha}$ higher than 8 Hz and strong α_iN_{i+1} NOEs were detected for Val, Orn, and Leu residues (i.e., the β-sheet region), and N_iN_{i+3} NOEs between the amide protons of Val and Leu again confirmed the β-turn conformation at the two corners of the β-hairpin. The temperature coefficients of the amide protons were also determined (see the SI), showing low values for Leu and Val

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residues that further confirm the occurrence of closed β-turns.[20] Scheme 2C depicts the NMR structure of peptide **9**, featuring a β-hairpin conformation with the two amide *N*-substituents pointing toward the opposite faces of Orn side chains.

In conclusion, we have developed a new type of cyclic β-hairpin peptide by proving that the incorporation of *N*-alkylated AA residues by Ugi reactions may induce β-turn conformations and thereby stabilize the β-hairpin architecture. Our results suggest that the combination of *N*-alkylated D-Phe at *i*+1 with Pro, Gly, and even Aib at *i*+2 enables us to mimic type-II′ β-turn motifs, as found in GS. Thus the incorporation of these fragments opposite to the native D-Phe-Pro keeps the overall β-hairpin conformation of GS. The substitution of Pro (*i*+2) by *N*-alkylated Ala (but not Aib) also leads to a β-hairpin conformation, in which the D-Phe-*N*-(Alkyl)Ala sequence induces a β-turn, but not of type II′. Nonetheless, the Ugi-derived β-turn equally imposes the antiparallel alignment of the two β-strands in a β-sheet structure, thus forming a cyclic β-hairpin. In this sense, the Ugi reaction-based approach allows for the stabilization of the β-hairpin and the simultaneous functionalization of the β-turn motifs. This exo-cyclic modification can be used not only to modulate the bioactivity by adding additional cationic or hydrophobic tails but also to install bioconjugation handles, fluorescent and affinity tags, and so on.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02592.

Experimental procedures, RP-HPLC chromatograms, NMR, and HR-ESI-MS spectra of cyclic peptides (PDF)

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Notes

The authors declare no competing financial interest.

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