Organic LETTERS

Letter

pubs.acs.org/OrgLett

Hydrogen Bonded Squaramide-Based Foldable Module Induces Both β- and α-Turns in Hairpin Structures of α-Peptides in Water

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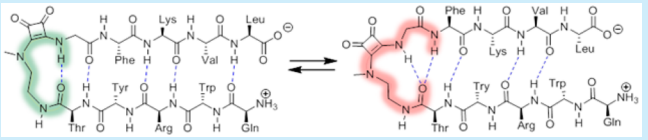
Supporting Information

ABSTRACT: A novel tertiary squaramido-based reverse-turn module SQ is reported, and its conformational properties are evaluated. This module is easily incorporated into a α-peptide sequence by conventional solid-phase peptide synthesis. The structure characterization of the hybrid squaramido-peptide **4** is described, showing that the turn segment induces the formation of hairpin structures in water through the formation of both αSQ- and βSQ-turns.



The hairpin structural motif, with two strands of α-peptides connected by a reverse turn forming an antiparallel β-sheet, is widespread among proteins and crucial to many biomolecular recognition events such as protein−protein and protein−nucleic acid interactions.[1] Reverse turns are classified by the number of amino acid residues involved in the turn segment. For instance, β-turns are formed by four amino acid residues and feature 10-membered hydrogen-bonded rings. Frequently observed in nature, peptides with this secondary structure display many biologically relevant properties such as antimicrobial and antiviral activities, among others.[2] α-Turns are larger structures formed by five amino acid residues and an ensemble of 13-membered hydrogen-bonded rings that usually are internally stabilized by tight smaller turns such as β-turns. Although the α-turn motif is rarely observed in peptides or proteins, it can be found in enzyme active sites, metal-binding domains,[3] and bioactive natural[3a,4] or non-natural cyclopeptides[5]and also plays a key role in relevant molecular recognition processes such as cell proliferation signaling[6] or reducing the HIV infection[7] capability. Therefore, numerous efforts have been made to design and synthesize non-natural reverse β-turn scaffolds that could induce autonomous folding of peptide or peptidomimetic strands with the aim of developing more selective and potent therapeutic agents,[8] new catalysts,[9] preorganized linear peptides,[10] etc. The continuous attempts made to develop modules that display α-turns in peptidomimetic compounds have afforded a limited number of successful examples.[11] However, it is still a challenge to overcome the drawbacks of the conformational flexibility of small peptides in water.

As part of our program aimed at studying the conformational properties of the squaramide compounds,[12] we have studied the folding capability of disecondary squaramide-based turn mimetic modules. Our folding segments rely on the relatively low rotational barrier of the squaramide unit and the hydrogen-bonding capabilities of the squaramide framework. These, combined with the presence of hydrogen-bonding donor atoms in one of the squaramide substituents, induce folding driven by the formation of an intramolecular hydrogen bond.[12]

Our attempts in the field have led us to preorganize linear oligosquaramide compounds in protic solvents to yield cyclic oligosquaramidic compounds with interesting antitumor properties.[13] Recently, we have described a squaramide-based module that can be easily combined with peptide strands and induce folding in organic solvents, although the conformational flexibility of the turn moiety is still considerable.[14]

Herein, we present the design, synthesis, and conformational studies of the squaramide-based scaffold SQ**1a**. The minimalist loop **1a**, formally an amino acid, is constructed from diethyl squarate and (2-aminoethyl)methylamine to give a tertiary squaramido-ethyl ester (Supporting Information). This module can be directly coupled with both the amino and the carboxylic acid groups of natural or non-natural amino acids to obtain peptidomimetic compounds, Figure 1.

Unlike secondary squaramides, tertiary squaramides such as *N*,*N*-dialkylsquaramides show little preference for any conformation in solution and exist as a mixture of *Z*,*Z* and *E*,*Z* conformers due to their lower rotational barrier energy (∼17 kcal mol^{-1}) for the squaramide C−N bond.[15] Therefore, the turn module **1a** was envisioned to adopt a high population of the *E*,*Z* conformation when conveniently functionalized driven by the formation of an intramolecular hydrogen bond and two additional C−H··O hydrogen bonds due the coplanar disposition of the squaramide substituents, Figure 2.[16]

Received: April 30, 2015
Published: June 2, 2015

DOI: 10.1021/acs.orglett.5b01268
Org. Lett. 2015, 17, 2980−2983

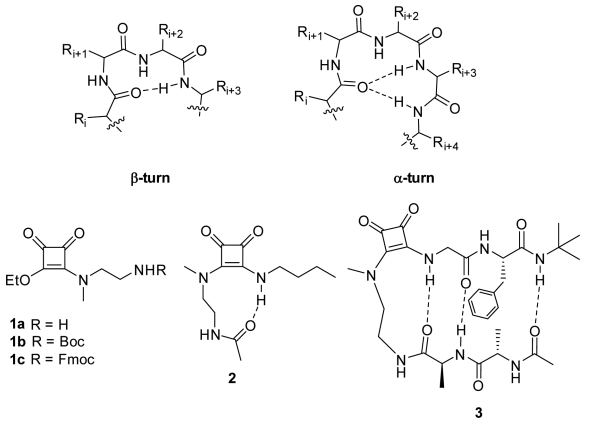


Figure 1. (Top) Schematic representation of typical β-turn and α-turn internally stabilized by a β-turn. (Bottom) Structure of the squaramide-based minimalist loop SQ (**1a**, **1b**, and **1c**) and the model compounds **2** and **3**.

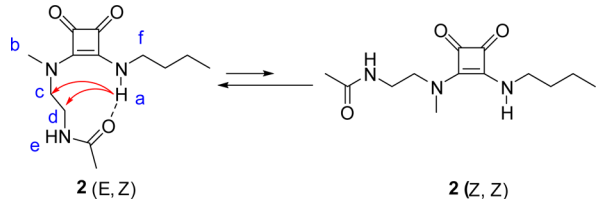


Figure 2. Representation of the conformational equilibrium of the squaramide-based turn module observed for **2**. Selected nonsequential NOEs observed for the *E,Z* conformation in chloroform are shown.

To evaluate the new loop design, we synthesized and studied the conformational properties of the model compounds **2** and **3** as a previous step to the incorporation of the novel turn sequence into a peptide strand, Figure 1.

Thus, in order to promote the formation of the intramolecular hydrogen-bonding interaction within the loop scaffold, compound **1b** was condensed with *n*-butylamine followed by the cleavage of the amino protecting group Boc and coupling with acetyl chloride to afford compound **2** (Supporting Information).

We ran NMR studies in chloroform to investigate the solution-state conformation of **2**. At 298 K, the squaramide proton NHa has little concentration dependence ($\Delta\delta$ = 0.34 ppm) in the 1−25 mM range. However, the amide proton NHe suffers an important downfield shift ($\Delta\delta$ = 0.80 ppm) at the same concentration interval, suggesting that NHe forms intermolecular hydrogen bonds upon concentration while NHa is shielded from others molecules. Below the coalescence temperature of 258 K, two different species are clearly observed in a 9:1 ratio and assigned to the folded and unfolded conformers, respectively. The addition of increasing amounts of DMSO up to 10% to a 1 mM solution of **2** in $CDCl_3$ induces the downfield chemical shift of the NHa ($\Delta\delta$ = 0.08) and NHe ($\Delta\delta$ = 1.5 ppm), respectively, confirming that unlike NHe, NHa is shielded from the solvent (Supporting Information).

NOESY studies of a 1 mM solution of **2** in $CHCl_3$ run at 244 K also evidence the folded state of **2** showing strong crosspeak signals between the nonadjacent protons NHa and the methylene groups (CH_2)c and (CH_2)d from the opposite substituent, Figure 2. Conformational studies performed in acetonitrile for **2**, in which self-aggregation is unlikely, show the same results as in chloroform (Supporting Information). Thus, the *E,Z* conformer induces the formation of a 10-membered ring driven by an intramolecular hydrogen bond mimicking natural β-turns (Supporting Information).

Single crystals obtained from **2** by slow evaporation of AcOEt yield the solid-state structure, revealing the folded arrangement of the turn framework in the crystals. X-ray data show the formation of the intramolecular hydrogen bonded 10-membered ring observed by NMR (N−H··O = 1.931 Å), and as in solution, the amide NHe is engaged in an intermolecular hydrogen bond (N−H··O = 2.015 Å) with one of the two squaramide carbonyls of a neighbor molecule, building hydrogen-bonded ribbons of folded molecules. Examination of the torsional angles reveals that the turn segment is almost planar as shown in Figure 3. Thus, the

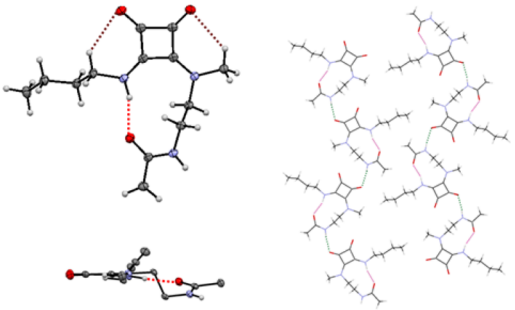


Figure 3. (Left) ORTEP front and side views of **2**. (Right) Crystal packing of **2** showing the intermolecular association of the folded squarmide molecules. All hydrogen bonds are represented by dotted lines. Some hydrogen atoms have been removed for clarity.

coplanar orientation of the *N*-methyl and N−CH_2 groups is favored by the formation of two additional C−H··O hydrogen bonds. Thus, distances C−Hf··O = 2.513 Å and C−Hb··O = 2.459 Å are 0.207 and 0.261 Å shorter than the sum of the van der Waals atoms radii, respectively.[16] As a result, the turn segment is arranged in a well-defined conformation suited to direct the formation of hairpin structures.

As a step forward in our study, we synthesized compound **3** to evaluate the suitability of the new loop segment to be incorporated in peptidomimetic structures. The conformational studies were performed in methanol, hydrogen-bonding competitive media, by NMR.

Compound **3** was synthesized from **1b**, first by a conventional squaramide condensation reaction with the amine group of the dipeptide H_2N-Gly-Phe-CONH^{t}Bu and followed by the cleavage of the Boc protecting group and the amide coupling reaction with the carboxylic group of the dipeptide AcNH-Ala-Ala-CO_2H (Supporting Information). Thus, upon folding, **3** may form two additional interstrand hydrogen bonds to stabilize the secondary structure.

At 233 K, solution-state NMR studies of **3** run in methanol show the existence of two equally populated conformers. These species were unambiguously assigned to the folded **3A** (*E,Z*) and unfolded **3B** (*Z,Z*) structures, respectively, Figure 4.

Selected nonsequential NOEs observed for **3** are shown in Figure 4. Thus, consistent with a *Z,Z* conformation, a strong NOE signal between the proton NHa′ and the N−(CH_3)b′ was observed, revealing the proximity of these groups in the unfolded structure **3B**. Alternatively, and as observed in **2**, the folded conformer **3A** shows a strong NOE signal between the NHa and the methylene group (CH_2)c pointing out the *E,Z* conformation of the squaramide module SQ. In addition, the folded structure shows several characteristic interstrand NOEs observed in hairpin-like structures.[18] These contacts are indicative of the

DOI: 10.1021/acs.orglett.5b01268
Org. Lett. 2015, 17, 2980−2983

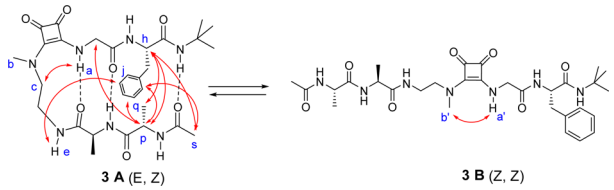


Figure 4. Nonsequential NOEs observed for **3** in methanol and representation of the assigned structures for conformers **3A** (*E*,*Z*) and **3B** (*Z*,*Z*).

antiparallel alignment of the two peptide strands. As a consequence, the juxtaposition of the residue side chains favors noncovalent interactions that contribute to the hairpin stabilization in the highly hydrogen bonding competitive media.

Motivated by this result, we investigated the conformational behavior of our turn module in water by its incorporation into a decapeptide strand using a conventional peptide solid-phase synthesis. For this purpose, we designed the hybrid peptido-squaramide compound **4**.

As shown in Figure 5, the structure follows the peptide sequence H_2N-Glu-Trp-Arg-Tyr-Thr-Xxx-Gly-Phe-Lys-Val-

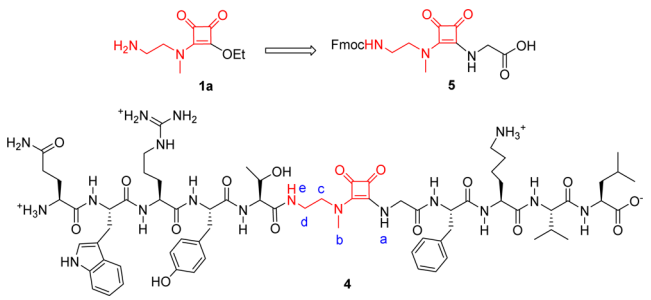


Figure 5. Turn module **1c** was conveniently modified to obtain the Fmoc-protected squaramido amino acid **5** as a suitable synthetic module for the solid-phase synthesis of the hybrid peptido-squaramide compound **4**. Turn module structure is highlighted in red.

Leu-CO_2H, where Xxx represents the squaramide-based turn module. Compound **4** was designed to fold, induced by the turn module, giving an antiparallel hairpin structure that can hold four additional interstrand hydrogen bonds. Inspired in natural hairpin structures, we conveniently distributed amino acids with hydrophobic side chains (Phe/Tyr and Trp/Val) to build a hydrophobic cluster that could stabilize the folded structure in water through interstrand side chain−side chain interactions. Furthermore, the peptide sequence was completed with amino acids with basic groups (Arg, Lys) that at physiological or acid pH are positively charged to enhance water solubility and avoid self-aggregation.[17]

In peptide solid-phase synthesis, amino acid coupling reactions usually are performed with the aid of coupling reagents using DMF as a solvent. The Fmoc-protected compound **1c** was condensed with the amino acid glycine in a water-buffered (BBS, borate buffered saline)−ethanol−mixture at pH 9.5 to give compound **5** in a 94% yield. Then, the vinylogous amino acid **5** was successfully incorporated into the peptide sequence by Fmoc solid-phase chemistry using the 2-chlorotrityl alcohol resin (1 mmol/g).[18] Squaramido-peptide **4** was obtained as the corresponding TFA salt in 95% of purity.

The secondary structure of the squaramido-peptide **4** was studied by NMR in H_2O at pH 2.5 and 5.2 and at 278 K.

At this temperature, compound **4** shows sharp and spread signals. Two main conformers **4A** and **4B** of equal proportion were clearly identified in both samples and conveniently assigned by NMR experiments (TOCSY, NOESY, and ROESY), Figure 5. Chemical shifts analysis reveals that pH does not significantly affect the turn module conformational behavior, since significant differences were observed only for the amino acids Val and Leu located at the carboxyl terminus (Supporting Information).

Interestingly, the main differences in the chemical shifts between the two conformers are observed for the NH groups located at the turn segment and nearby. Thus, NHa is shifted upfield ($\Delta\delta$ = −0.25 ppm) in **4B** when compared with **4A**. In contrast, the NH (Phe) and NH (Thr) signals are shifted downfield ($\Delta\delta$ = 0.55 ppm) in **4B**, which is indicative of their involvement in the formation in of stronger hydrogen bonding interactions than in **4A**. As anticipated, the 2D NMR (TOCSY and ROESY) studies of **4** run at pH 2.5 and 278 K show the characteristic contacts between nonadjacent residues, indicating the formation of a hairpin structure in both conformers. The detected critical cross-strand NOEs are represented in Figure 6.

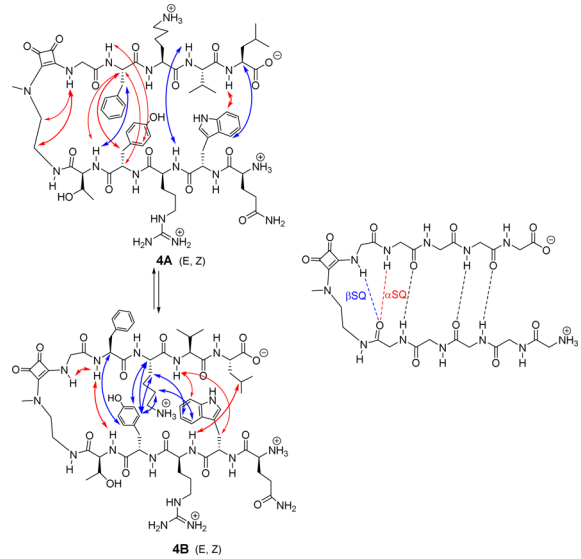


Figure 6. Relevant nonadjacent NOEs observed in the NOESY experiments of 4 (5 mM) in H_2O (5% D_2O) at pH 2.60 (red arrows) and pH 5.25 (blue arrows). Representation of the α- and β-turn moieties observed in the folded structure. Amino acid side chains have been removed for clarity.

In **4A**, NOE cross-peaks could be observed between NHa and (CH_2)c and (CH_2)d, confirming the formation of the squaramido-based reverse turn observed in compounds **2** and **3**. The antiparallel β-sheet structure of the peptide chains is reflected by backbone−backbone contacts between NH(Thr)--CHα(Phe) and CHα(Tyr)--CHα(Phe) and interstand contacts CHβ (Tyr)--CHα(Phe). Further evidence of the hairpin structure of conformer **4A** was found at pH 5.2 with the interstrand contacts NH(Thr)--CHβ(Phe), NH(Leu)-Trp side chain and CHα(Leu)--Trp side chain.

Conformer **4B** also shows interstrand NOE cross-peaks within the turn segment and along the peptide sequence. Interestingly, at pH 2.5 the corresponding NH(Phe) gives NOE contacts with the NH(Thr) and the squaramidic NHa signals showing their proximity. This, together with the downfield shift observed for NH(Phe) and NH(Thr) in **4B**, indicates that these two NH are

DOI: 10.1021/acs.orglett.5b01268
Org. Lett. 2015, 17, 2980−2983

involved in a different hydrogen bonding pattern within the turn. This situation is consistent with the formation of a bidentate hydrogen bonding interaction of the Thr carbonyl group with NHa and NH(Phe). This alternative folded structure forms an unusual 13-membered ring that mimics natural occurring α-turns with maintenance of the antiparallel strand registry. In this case as in nature, the αSQ-turn is internally stabilized by a βSQ-turn, Figure 6. The formation of the αSQ-turn is favored by the presence of glycine in the turn segment that allows fulfilling the geometry due its lack of bulky side chain.[19]

This supposition was confirmed by the addition of up to 70% of D_2O to a 5 mM sample of **4** at pH 2.6. As anticipated, we observed the fast disappearance of nonintramolecular hydrogen bonded amides oriented toward the solvent such as the free NH groups located at the N-terminal peptide strand of the two proposed folded structures **4A** and **4B** (Supporting Information). The rest of NH amide protons and the squaramide NHa remain unchangeable up to 4−10 h as expected for intramolecular hydrogen bonded signals. In compound **4**, the equilibrium between the two proposed conformers slows the exchange rate of all NH signals located at the C-terminal strand because those that do not participate in the intramolecular hydrogen bonds in conformer **4A** are bound in conformer **4B** and vice versa. Moreover, in spite of that secondary squaramides are more acidic than secondary amides, NHa shows an exchange rate comparable to amides which is indicative of the formation of the expected intramolecular hydrogen bond within the turn module. However, NHa shows a faster exchange rate in **4B** than **4A** as a consequence of the weaker hydrogen bond formed in this conformer as the chemical shift analysis reveals.

Further evidence of the highly populated hairpin structure of **4** in water was obtained from the circular dichroism spectrum of a 0.5 mM sample in this solvent (Supporting Information). The CD shows the characteristic intense exciton-coupled bands at 212 and 228 nm, indicating interaction between the aromatic chromophores as occurs in aromatic rich peptides folded in a hairpin structure.[20]

In conclusion, we have designed the efficient *N*-methylated squaramide based turn module **1a** that induces folding in small peptidomimetic structures. This module was easily incorporated into a α-peptide sequence by conventional solid-phase peptide synthesis obtaining the squaramide-decapeptide **4**. This hybrid compound folds in water, giving both αSQ- and βSQ-turns through a conformational equilibrium. Taking advantage of its dynamic conformational properties, this squaramide-based turn module could become a key scaffold to design bioactive peptidomimetic molecules.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and characterization data for the new compounds, NMR conformational studies, and CD spectrum of **4**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01268.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Ministry of Economy and Competitiveness (Grant Nos. CTQ2014-57393-C2-1-P and Consolider-Ingenio CSD2010-00065) and CAIB (Grant No. 23/2011, FEDER funds). We thank Dr M. Royo for synthesizing compound **4** at the Combinatorial Chemistry Unit of the Parc Científic of Barcelona.

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DOI: 10.1021/acs.orglett.5b01268
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