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PAPER

Synthesis and conformational studies of peptido-squaramide foldable modules: a new class of turn-mimetic compounds†

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The β-turn unit is one of the most important secondary structure elements in proteins. The access to new conformationally controlled foldable modules can afford compounds with interesting bioactivities. Here, we describe a new family of peptido-squaramide foldable modules based on the considerable potential of the squaramide unit as a hydrogen bond donor and acceptor as well as the low rotational barrier of the C–N bond. The conformational analysis by NMR of these modules in chloroform and acetonitrile solution shows that a disecondary squaramide with the 4-aminobutyric acid in one of its substituents can mimic the β-turn structure driven by the formation of an intramolecular hydrogen bonded ten-membered ring. This structure, although flexible, has been successfully combined with dipeptide chains to induce the formation of a hairpin-like structure driven by the formation of several cross-strand intramolecular hydrogen bonds.

Introduction

Proteins and peptides exert their biological activity through small regions of well-defined secondary structures such as α-helix, β-turn and β-sheet[1] which can be mimicked by means of several different foldable modules.[2] Unfortunately, small and medium size peptides and peptidomimetics are often unstructured in solution and rarely possess adequate function. Therefore, the access to new conformationally controlled foldable modules[3] is of great interest because they can be incorporated into peptide-like structures to afford compounds with compelling bioactivities and better pharmacokinetic properties than peptides.[4] Thus, hybrid sequences are acquiring increasing importance in the design of biologically active peptides,[5] because of the enhanced stability of these modified backbones to proteolytic cleavage. In this sense, unnatural scaffolds designed to reproduce structural protein features while retaining the property to bear the chemical diversity of the amino acid side chains have a potential application in drug discovery.[6]

The β-turn unit is one of the most important secondary structure elements in proteins. The initial interest on the studies of its conformational properties increased and focused on the synthesis of small molecules that mimic the β-turn structure when the biological activity of many peptides was related with a turn containing conformation.[7] This can be achieved through two different approaches: the use of covalent bonds[8] to introduce the conformational constrains that stabilise the turn geometry, and the induction of the folded state of linear molecules through the formation of intramolecular hydrogen bonds.[5,9] In the latter, when the conformational control is needed, one useful strategy is the incorporation of proline residues to stabilise the folded conformation due to its β-turn propensity.[10]

Alternatively, other foldable modules of a different nature can be used as proline substitutes. In this context, for example, β, γ, and δ-amino acids have proved to encourage turn formation[11] and a combination of several non-natural residues of an aliphatic or aromatic nature are well suited to serve as turn mimetics or β-hairpin initiators.

Many successful aliphatic folding modules contain a saturated carbon chain separating amide or urea groups. The squaramide scaffold, as well as the urea group, share with the amide linkage a number of interesting features, such as planarity, polarity, and hydrogen bonding capability[12] and represent an interesting surrogate.

Like amides, and due to the restricted rotation about the C–N bond, double secondary squaramides may exist, in principle, as a mixture of *anti*/*syn* conformers (Fig. 1). The extended *anti*/*anti* conformer allows the simultaneous participation of the two NH groups in the formation of hydrogen bonding interactions. However the *syn*/*anti* states lead to folded structures. On the other hand, the *syn*/*syn* conformer is not significantly populated due to the mutual steric hindrance of the substituents. It has been estimated that the rotation about the C–N bond for a secondary

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† Electronic supplementary information (ESI) available: Detailed experimental procedures and the characterisation data of new compounds. The ^{1}H and ^{13}C NMR spectra for compounds **1**–**4** and **9**–**15**. Stacked plots of ^{1}H RMN spectra obtained in concentration and temperature variation experiments for compounds **1**–**3**. Selected fragments of NOESY spectrum of **4**. See DOI: 10.1039/c2ob06715c

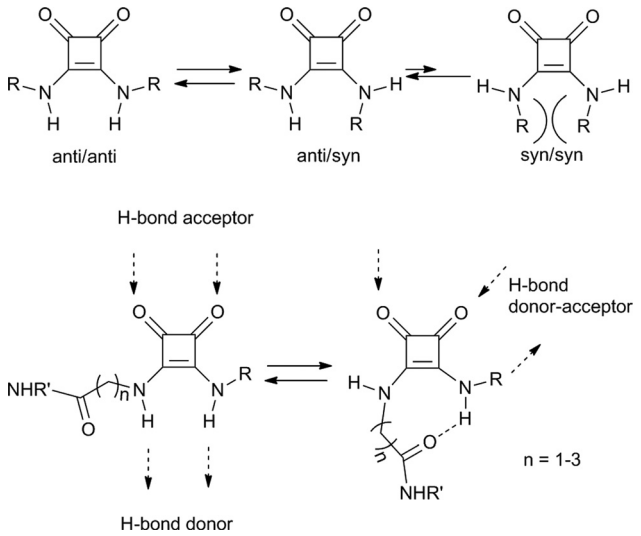


Fig. 1 Top: representation of the conformational equilibrium of disecondary squaramides. Bottom: hydrogen bonding options for the new squaramide-based turn fragment stabilised by intramolecular hydrogen bonding interaction.

squaramide has energetically accessible barriers to rotation (~ 63 kJ mol^{-1}), slightly lower than the barrier to rotation reported for tertiary squaramides (~ 71 kJ mol^{-1}) and are comparable to what is found for carbamates (~ 65 kJ mol^{-1}). Indeed, the C–N bond of a secondary squaramide also compares well with the C–N bond of proline derivatives. Secondary squaramides are easy to synthesise from squaric esters and amines. The combination of these two features convert to disecondary squaramides in a very interesting foldable module.

In this sense, we have described a squaramide-based foldable module that contains a donor atom located at the δ position of the alkyl chain of a disecondary squaramide.[13] The folding capability of this module is based on the low rotational barrier of the squaramide unit and driven by the intramolecular hydrogen bond formation of a nine-membered-ring. Oligosquaramide[14] compounds based on this module nicely fold in a hairpin-like structure and have been successfully used as preorganised linear precursors in macrocyclisation reactions in protic solvents.

In order to enrich the category of squaramide foldable modules and test their ability to promote hairpin structures, we describe here the synthesis and the conformational analysis of a new family of squaramide-based foldable modules that combine two motifs, a disecondary squaramide moiety and α-, β- or γ-amino acid residues (Fig. 1). The *syn*/*anti* conformers of these squaramide compounds driven by the formation of an intramolecular hydrogen bond between the carbonyl group of the amino acid residue and the squaramide NH proton of the neighbour substituent, result in the formation of eight-, nine-, and ten-membered-rings respectively. Moreover, the new squaramide-based turn module can be easily combined with peptide chains to build β-hairpin-like molecular structures mimicking the features of two antiparallel strands connected by a short loop segment. Consequently, these small turn-forming fragments could be a valuable tool pursuing bioactive ligand conformations in rational drug design.

Results and discussions

The conformational preferences of the new squaramide foldable modules were investigated on model compounds to identify those that may preferentially adopt adequate folding patterns.

Thus, compounds **1–3** (Chart 1) were designed for this purpose. In them, the squaramide moiety is linked to two different substituents, an *n*-butyl chain which is common to the three compounds, and a dipeptide formed by one of the three amino acids of different length, namely glycine, β-alanine and γ-aminobutyric acid (GABA) respectively, linked to the natural amino acid L-phenylalanine. To verify the propensity of compounds **1–3** to induce folded structures, we investigated their capability to form intramolecular hydrogen bonds between the oxygen of the carbonyl group of the amino acid and the squaramide NH proton of the *n*-butyl substituent.

Compounds **1–3** were synthesised according to conventional diethyl squarate and amine condensation reactions (Scheme 1). In short, diethyl squarate was condensed with one equivalent of *n*-buthylamine, as in a previously reported method,[13] to obtain the *n*-butylsquaramide ethyl ester **5**. Subsequently, **5** was separately condensed with the sodium salt of glycine, β-alanine and γ-aminobutyric acid (GABA) in ethanol to afford the three disquaramide compounds **6–8**. Finally, the terminal carboxylic groups of **6–8** were coupled with L-phenylalanine methyl ester using O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent, affording the three model compounds **1–3**.

We investigate the formation of well-defined folded structures in solution using a combination of NMR spectroscopic techniques. From our previous work on squaramide-based foldable modules[13] and due to the conformational properties and the hydrogen bonding propensity of disecondary squaramides, we expect to have in solution a mixture of *syn*/*anti* conformers that

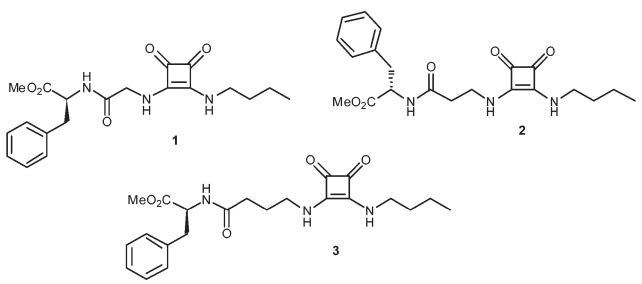
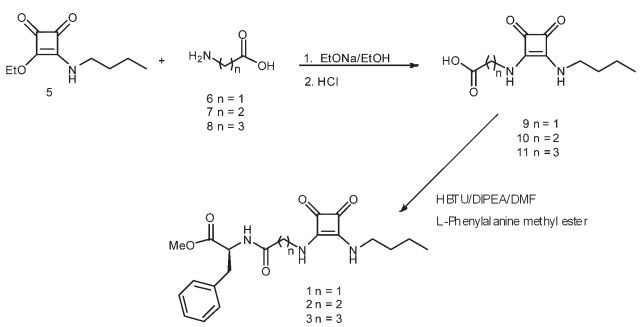


Chart 1 Structure of squaramides **1–3**.



Scheme 1 Synthesis of squaramide model compounds **1–3**.

can also aggregate to give higher order species depending on the concentration and solvent properties. All these different species in equilibrium can be characterised by analysing the effect on the NMR chemical shifts produced by variations in temperature, and concentration. Assignment of the stereochemistry of the species is based on the evidence that signals of *syn*-*N*-substituents are shifted downfield relative to those of *anti*, due to the paramagnetic influence of the squaramide carbonyl group. As in amides, the NMR chemical shift values for the squaramide NH protons are very sensitive to hydrogen bond formation. However, for small molecules like **1–3**, equilibration among non-hydrogen bonded and hydrogen-bonded states is usually rapid on the NMR time scale. Therefore, the observed NH values reflect a population-weighted average. Low temperatures allow slowing down of the interconversion processes among the different species in equilibrium. The interpretation of δ NH data in terms of intramolecular hydrogen bonding requires then, that the studied molecule be sufficiently dilute to prevent hydrogen bond-mediated aggregation. All the ^{1}H NMR spectra could be unequivocally assigned performing 2D NMR COSY and TOCSY experiments.

Conformational studies of 1

First, we studied the conformational properties of **1**. The ^{1}H NMR spectrum of a diluted solution of **1** (0.5 mM, 298 K) in a non-polar solvent like chloroform presents broad signals for the three NH protons of the molecule. Unexpectedly, the amide NH (c) proton is shifted downfield ($\delta \approx 7.2$ ppm) with respect to the two squaramide signals NH (a) and NH (b), which show similar chemical shifts at 6.4 ppm and 6.6 ppm respectively. These two NH protons are also shifted downfield relative to the usual region for non-hydrogen-bonded squaramide protons ($\delta \sim$ 6.0–6.2 ppm), (Fig. 2).

The ^{1}H NMR spectrum of **1** in $CDCl_3$ (1–100 mM) at 298 K is concentration dependent. The two squaramide signals NH (a) and NH (b) show relatively large and almost identical chemical shift changes upon dilution ($\Delta\delta$ = 0.8 ppm), which indicates the formation of intermolecular hydrogen bonds upon concentration. However, the amide signal NH (c) exhibits little change ($\Delta\delta$ = 0.3 ppm) under the same conditions. The expected chemical shift value for non-hydrogen-bonded NH amide protons at 298 K in $CDCl_3$ ranges from 5.8 to 6.4 ppm.[15] Therefore, the observed data suggest that the amide group is, to some extent, intramolecularly hydrogen bonded. To further investigate this assumption, we performed a variable temperature experiment.

The ^{1}H NMR spectrum of **1** (2 mM) in $CDCl_3$ is temperature dependent (313–237 K) with large temperature coefficients ($\Delta\delta/\Delta T = -11$ ppb K^{-1}) for the three NH protons of the molecule. This, together with the broadness observed for all the signals at 237 K, is indicative of the existence of a dynamic equilibrium among different species even at low temperature.[16] At 237 K, the signal NH (c) at 8.5 ppm is also clearly shifted downfield with respect to the two squaramide protons observed at 7.8 and 7.9 ppm.

Moreover, the NMR data obtained from these experiments evidence that the two squaramide signals as well as the two α-methylene protons CH_2 (e) and CH_2 (d) of the two squaramide N-substituents show quite similar chemical shifts and behaviour, independently of the concentration and temperature investigated.

Therefore, we assumed that the squaramide moiety prefers the extended *anti*/*anti* rather than the *syn*/*anti* conformation which is adequate to induce the sought folded structure. In the *anti*/*anti* conformation, the two squaramide NH groups are able to establish intermolecular hydrogen bonds. We expect that the aggregates formed in chloroform are mainly dimers due to the high solubility observed for compound **1** in organic non-polar solvents. The chemical shift displacements experienced by the two squaramide NH protons upon concentration are consistent with a homodimerisation process and fit[17] very well to a 1 : 1 dimerisation model, obtaining a dimerisation constant of $K_{dim} = 260 \pm 85$ M^{-1} at 298 K.

Additionally to this process, the behaviour observed by NMR for proton NH (c) upon concentration and temperature can be explained supposing that the N-Gly-Phe-OMe substituent of **1** folds, establishing an intramolecular hydrogen bond between the amide proton NH (c) and the nearest squaramide carbonyl group, inducing the formation of an eight-membered ring (Fig. 3). Similar conformations to this have been observed in the crystal structure of some proline squaramide derivates.[18] Moreover, the

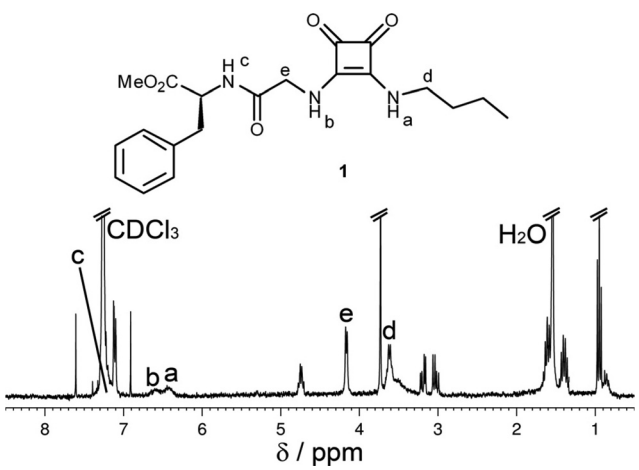


Fig. 2 Assigned ^{1}H NMR spectrum of **1** (0.5 mM) in $CDCl_3$ at 298 K.

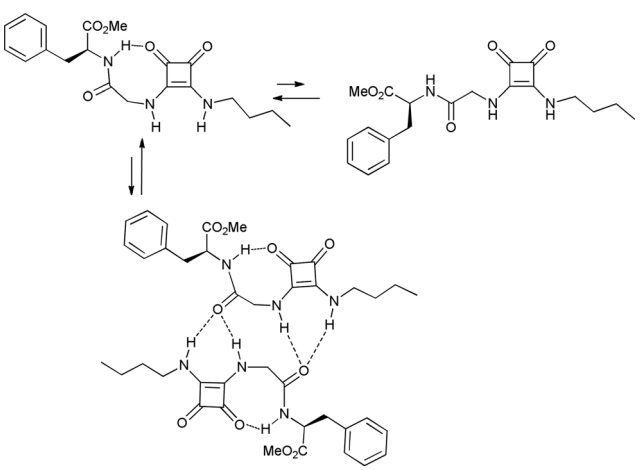


Fig. 3 Conformational equilibrium and homodimerisation of **1**.

proposed structure of the folded homodimer shown in Fig. 3 is consistent with those found for squaramide-related compounds described earlier in crystallographic structures of the *N*-carbamoyl squaramide dimer.[19]

To further investigate this assumption, we studied the conformational properties of compound **1** in acetonitrile. In this solvent, the hydrogen bonding interactions are less favoured than in chloroform, therefore hydrogen bond-mediated aggregation can be diminished and meanwhile the intramolecular hydrogen bonds are still favoured due its tightness.[20] In acetonitrile, at 298 K the chemical shift observed for NH (c) is 6.9 ppm. Even though the expected value for free amide protons in acetonitrile is approximately 6.1–6.3 ppm,[20] in this case, the downfield shift observed for this proton should be attributed to the paramagnetic effect of the carbonyl of the methylester group and not to the formation of hydrogen bond interactions.[21] On the other hand, the squaramide protons NH (a) and NH (b) appear at 6.2 ppm which is the usual spectral region for non-hydrogen-bonded squaramide protons. The monomeric state of **1** in acetonitrile was corroborated with a variable temperature experiment (313–237 K). The three NH signals showed small temperature coefficients ($\Delta\delta/\Delta T$ = −3 ppb K^{-1}) which can be interpreted in terms of these groups being either involved in intramolecular hydrogen bonds or fully solvated by the solvent.[14–22] Unlike in chloroform, at 237 K the ^{1}H NMR spectrum of **1** show sharp signals and some new low intensity signals can be observed. These new signals assigned to minor *syn*/*anti* conformations of the squaramide substituents.

These results indicate that, in acetonitrile, the prevalent species is the monomeric *anti*/*anti* conformer, in equilibrium with small amounts of unfolded *syn*/*anti* conformers.

We carried out a theoretical conformational analysis of compound **1**. Energies of the conformers were evaluated using the RB3LYP/6-311G* method. From all the structures generated, the two conformations of lowest energy correspond with the two conformers **A** and **B** that form the intramolecular hydrogen-bonded eight-membered rings. These structures are shown in Fig. 4 and indicate the tendency of compound **1** to form intramolecular hydrogen-bonded conformers. The two conformers **A** and **B** have almost identical relative energies. Conformer **A** can easily homodimerise retaining the folded conformation, establishing four additional hydrogen bonds. On the other hand, the alternative folded structure **B** prevents the formation of this kind of dimer, and consequently, additional intermolecular hydrogen bonds.

Taking all this together, the data obtained for compound **1** support the existence in solution of different species in equilibrium, with a significant percentage of the folded conformer that leads to the formation of an intramolecular hydrogen-bonded eight-membered ring between the amide proton NH (c) and the nearest squaramide carbonyl group.

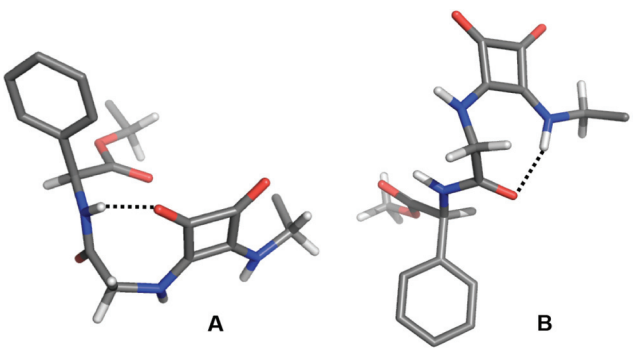


Fig. 4 Calculated conformations of **1**. The relative free energies (kcal mol^{-1}) were calculated at the RB3LYP/6-311G*level in the gas phase. The N–H···O hydrogen bonds are indicated.

Conformational studies of 2

In contrast to compound **1**, squaramide **2** showed low solubility in chloroform and prevented us running concentration-dependent studies in this solvent. At 298 K, a solution of **2** (1 mM) in $CDCl_3$ show two separated signals of different intensity for the three NH protons of the molecule, one at 6.1 ppm assigned to the squaramide NH (a) proton and the other at 6.4 ppm assigned to the squaramide NH (b) and the amide NH (c) protons (Fig. 5).

From these data we assumed that neither of these protons experience much hydrogen bonding interactions in these conditions because the values are consistent with the expected ones for free NH amide and squaramide protons in chloroform. We also observed that the signal of the α-methylene protons CH_2 (e) of the β-alanine substituent splits into two peaks at 3.8 and 3.9 ppm, respectively, due to the presence of *syn*/*anti* conformers or a restricted rotation along the N–C_{squ} bond of this substituent.

To further analyse the conformational properties of **2** we ran a variable temperature experiment. The chemical shifts of these signals are temperature dependent (237–313 K). The two squaramide protons NH (a) and NH (b) and the amide NH (c) proton considerably shift downfield upon decreasing temperature and show almost identical chemical shifts at any temperature investigated. The large temperature coefficients (15 ppb K^{-1}) obtained for all of them indicate that the three NH protons are involved in the formation of hydrogen bonding interactions.[16]

Taken together, these results suggest that the squaramide ring prevails in an *anti*/*anti* conformation, discarding the formation of any folded conformation. As a consequence, compound **2** exhibits a large tendency to form intermolecular hydrogen-

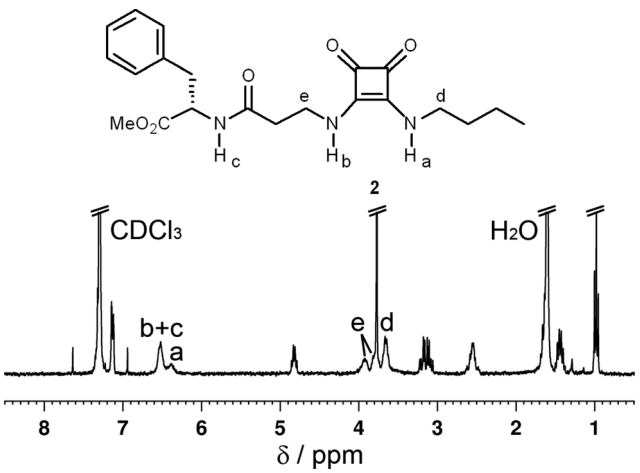


Fig. 5 Assigned ^{1}H NMR spectrum of squaramide **2** (1 mM) in $CDCl_3$ at 298 K.

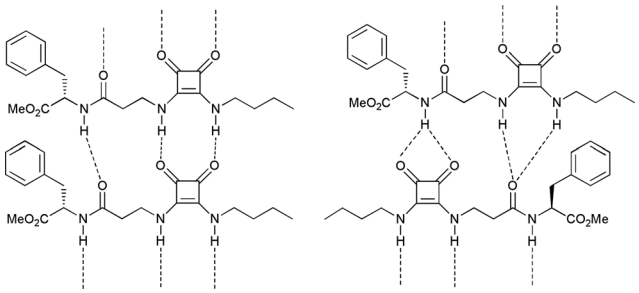


Fig. 6 Representation of two possible head-to-tail squaramide hydrogen-bonded aggregates of compound **2**.

bonded aggregates, in which the amide group is also involved. We can assume then, that compound **2** tends to form small–medium sized self-assembled structures.

In them, the molecules are organised in a head-to-tail fashion as disecondary squaramides usually assemble, either in solution or in solid phase, due to their double donor–acceptor hydrogen bonding capability[23] (Fig. 6).

This trend is the cause of the observed low solubility of many disecondary squaramides in non-competitive solvents, as is the case of squaramide **2**. Besides, in compound **2**, the tendency to form this kind of aggregate is strengthened by the presence of the amide linkage in the squaramide N-dipeptide substituent. Contrary to what happens in compound **1**, the NH (3) proton of the usually more stable *trans* isomer of the amide bond is oriented in the same direction as the two *anti*/*anti* squaramide NH protons. As a result, compound **2** can be tightly self-assembled through the formation of additional hydrogen bonds, instead of the formation of any folded conformer.

Conformational studies of 3

Finally, the conformational properties of the model compound **3** were also investigated. Unlike the previous studied compounds **1** and **2**, the ^{1}H NMR spectrum of **3** in $CDCl_3$ (2 mM, 298 K) shows significant evidence of the existence of a folded conformation of compatible features with the formation of a ten-membered ring induced by the formation of an intramolecular hydrogen bond (Fig. 7). The two NH squaramide protons NH (a) and NH (b) show quite different chemical shifts at 7.2 and 6.4 ppm respectively, as well as the α-methylene protons CH_2 (d) and CH_2 (e) at 3.7 and 3.4 ppm respectively. This can be attributed to a *syn*/*anti* conformation of the squaramide unit. The downfield shift observed for protons NH (b) and CH_2 (d) is due to the paramagnetic shielding of the squaramide carbonyl groups. Thus, the notable downfield shift observed for the squaramide NH (a) proton at 7.3 ppm can be attributed to the formation of the intramolecular hydrogen bond responsible for the formation of the turn. In this case, the chemical shift shown by the amide proton NH (c) at 6.3 ppm corresponds with the non-hydrogen-bonded state, and supports the formation of the expected ten-membered ring folded structure. To corroborate this evidence, the concentration dependence (1–100 mM) of the ^{1}H NMR spectrum of **3** in $CDCl_3$ was analysed.

The three NH signals show a downfield chemical shift upon concentration, suggesting the formation of aggregates. In this

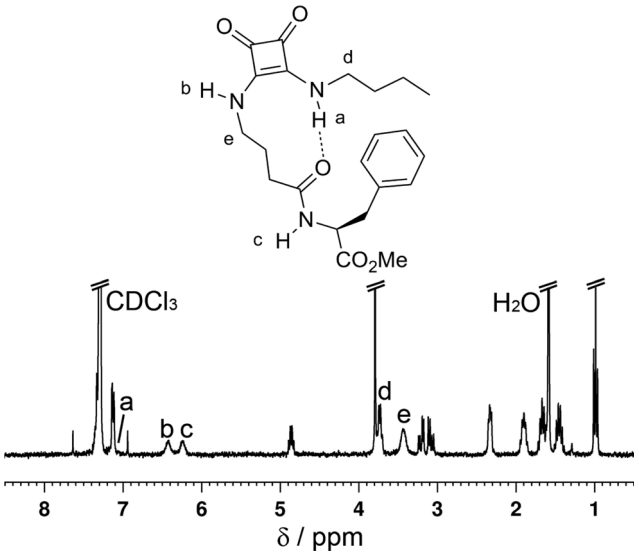


Fig. 7 Assigned ^{1}H NMR spectrum of squaramide **3** (2 mM) in $CDCl_3$ at 298 K.

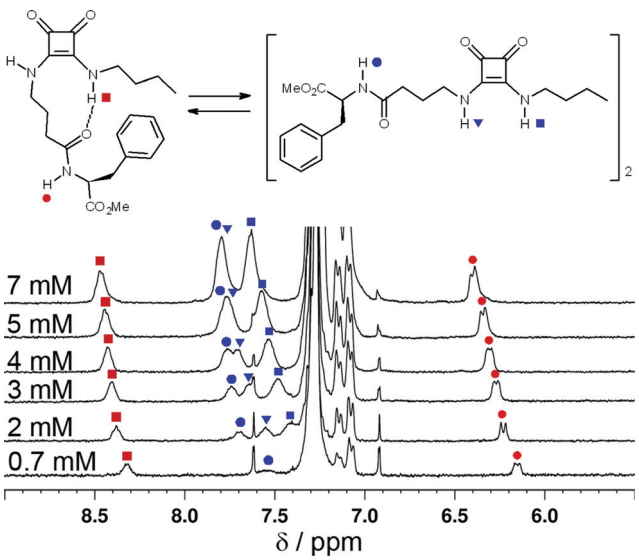


Fig. 8 Concentration dependence of **3** (2 mM) at 237 K in $CDCl_3$.

case, the chemical shift data observed also perfectly fit in a 1 : 1 dimerisation model, obtaining a dimerisation constant of $K_d = 50 \pm 7\ M^{-1}$, which is one order of magnitude lower than the dimerisation constant calculated for **1**. Therefore, the homodimers are in equilibrium with the monomeric folded structure that predominates at low concentrations. This could be corroborated when a 2 mM solution of **3** in chloroform was cooled to 237 K (Fig. 8). At this temperature, the signals corresponding to the NH protons split, indicating almost the existence of two different species in equilibrium in a 3 : 1 ratio. The majority species has signals at 8.4 and 6.3 ppm that were assigned to NH (a) and NH (c) respectively; meanwhile the corresponding NH (b) could be located at approximately 7.1 ppm shielded by the aromatic signals of the phenylalanine residue. From this data we can assume that the squaramide is in a *syn*/*anti* conformation and the

amide group NH (c) is not hydrogen bonded. The large downfield chemical shift exhibited by the NH (a) proton is indicative of the formation of an intramolecular hydrogen bond. All these results confirm the existence of the ten-membered ring folded structure. On the other hand, the second group of signals observed in the spectrum contains three peaks of very close chemical shift at 7.8, 7.6, and 7.5 ppm and were assigned to the protons labelled NH (c), NH (b), and NH (a) respectively. These chemical shift values are in agreement with intermolecular hydrogen-bonded aggregated structures like those observed at 298 K.

The aggregated nature of these structures was further confirmed with a concentration dependence experiment. Thus, the ^{1}H NMR spectrum of **3** changes upon concentration at 237 K. The relative intensity of the signals assigned to intermolecular hydrogen-bonded species increases upon concentration with respect to those assigned to the folded conformer (Fig. 8).

We can conclude that in diluted solutions of **3**, the monomeric intramolecular hydrogen-bonded structure that forms a ten-membered ring prevails over the aggregates but upon concentration, those are the prevalent species. This fact was confirmed in acetonitrile, where the presence of aggregates can be avoided. In these conditions, the intramolecular hydrogen-bonded structure could also be detected at 298 K[24]

At 244 K we observed the presence of unfolded conformers in equilibrium with the intramolecular hydrogen-bonded folded structure.

The conformational analysis performed for the three model compounds **1–3** evidenced that compound **3** presents the adequate features of a turn-forming module and is a good candidate to mimic natural β-turns that can be incorporated into hybrid structures.

Synthesis and conformational analysis of the squaramide-tetrapeptide 4

Once we had characterised the optimum new squaramide-based turn module consisting of a secondary squaramide scaffold combined with the γ-amino butyric acid in one of its substituents, we investigated the capability of this module to organise more complex structures. Therefore, the new loop structure was incorporated into the squaramide tetrapeptide **4** with the aim to obtain a minimal model of a β-hairpin structure (Chart 2). We assumed that the attachment of several natural amino acids, namely Gly, Phe and Leu and the linker ethylene diamine, to the basic foldable structure could lead to the formation of cooperative favourable interactions in the flanking regions of the loop segment, driven by the formation of two additional intramolecular hydrogen bonds in an antiparallel β-sheet fashion. This arrangement could overcome the observed flexibility of the turn segment in compound **3**. The carboxylic and amino terminal groups of this polypeptide-like compound were also conveniently functionalised by means of the formation of an amide and carbamate linkage, respectively, in order to increase the cross-strand hydrogen bonding capability.

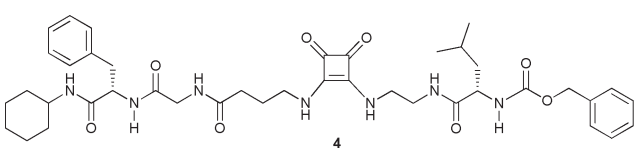
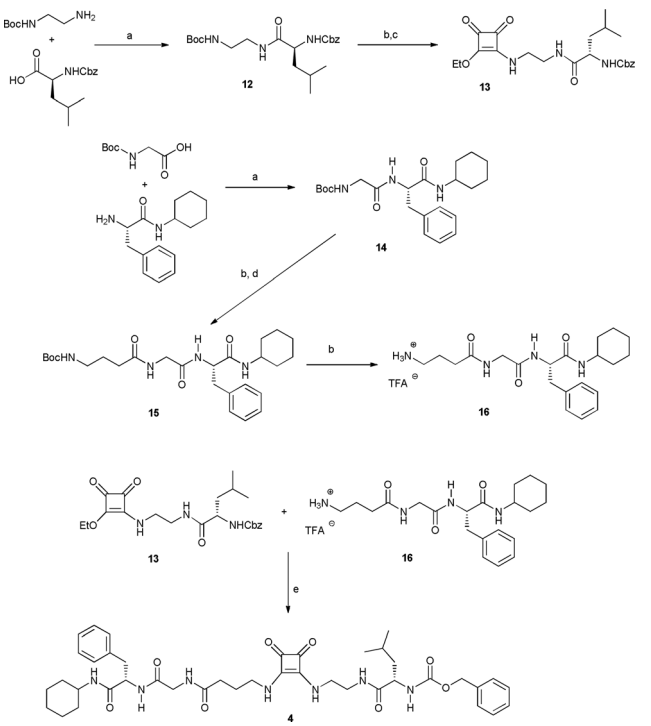


Chart 2 Structure of the squaramide-tetrapeptide **4**.

The squaramide tetrapeptide compound **4** was synthesised as shown in Scheme 2 and according to known procedures to obtain secondary squaramides and peptides. Briefly, the Boc-mono-protected ethylene diamine was coupled to L-Leu-Cbz assisted by the coupling reagent HBTU to give compound **12**. In parallel and under the same conditions, Gly-Boc and phenylalanineciclohexylamide were also coupled to give the dipeptide **14**. Subsequently, the amino protecting group of **14** was cleaved under acidic conditions and coupled to the amino Boc-protected γ-aminobutyric acid, affording the tripeptide **15**. Finally, the amino protecting groups of **12** and **15** were cleaved under acidic conditions to give the respective free amines. These were condensed in two steps with the squaramide moiety. First, the free amine of **12** was condensed in diethyl ether to obtain the squaramide ethyl ester **13**, which was further functionalised by condensation with the free amine of **15** in a 50 : 50 v : v mixture of ethanol–aqueous borax buffer (pH = 9) to give the new squaramide tetrapeptide model compound **4**.

Finally, the conformational properties of **4** were analysed. Due to the noticeable tendency of this compound to form aggregates in chloroform, **4** was studied in acetonitrile at 298 K. The 2D NMR (TOCSY, COSY) experiments performed (1mM, 298 K) allowed us to fully assign all the signals on the ^{1}H NMR spectrum.[25] In these conditions, the signals of the structural fragment ethylenediamine-Leu-Cbz split in two, suggesting the existence of different conformers in solution.[26]



Scheme 2 Synthesis of **4**. (a) DIPEA, HBTU, DMF; (b) TFA, CH_2Cl_2; (c) diethylsquarate, DIPEA, EtOH-Et_2O; (d) DIPEA, HBTU, DMF, 4-aminobutyric acid; (e) EtOH-Borax (pH = 9) 50 : 50 v : v.

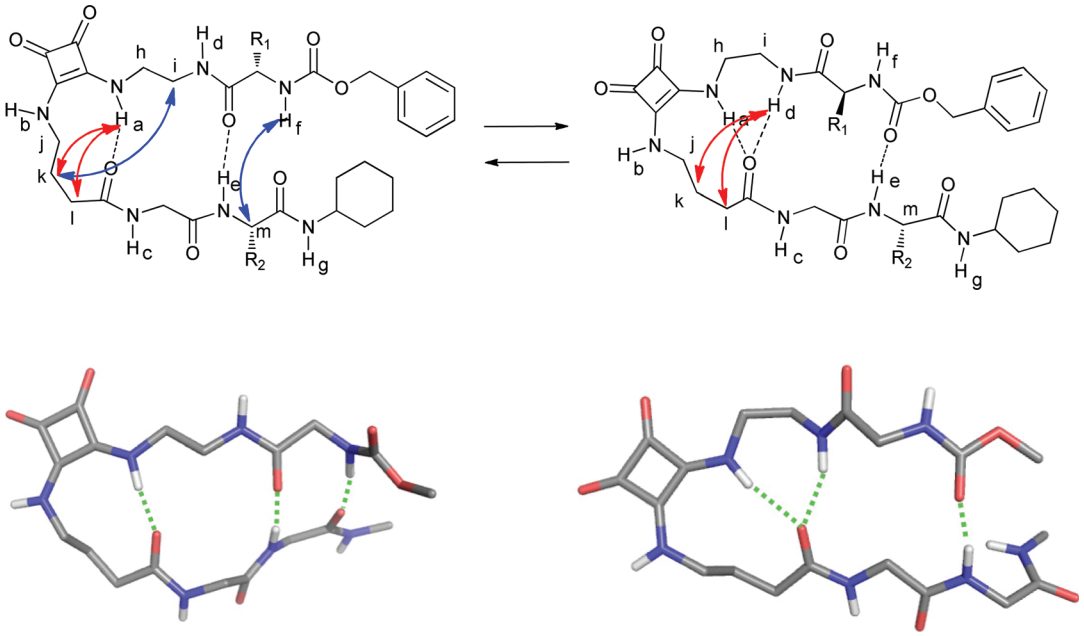


Fig. 9 Top: Folded conformations of **4** showing H atom labels and the corresponding NOE interactions observed in acetonitrile (1 mM, 298 K). Cross peaks represented by red arrows support the existence of the turn. The cross peaks represented by the blue arrows support the evidence of an equilibrium between conformers in solution. Bottom: Minimised structure of two conformers of **4** at RB3LYP/6-311G*level in the gas phase. The N–H···O hydrogen bonds are indicated. Non-acidic hydrogen atoms and bulky substituents have been omitted for clarity.

First of all, we observed that the squaramide NH protons named NH (a) and NH (b) show chemical shifts at 7.14 and 6.76 ppm, respectively, and are in agreement with a *syn*/*anti* conformation of the squaramide unit, where the NH (a) is intramolecularly hydrogen-bonded to the carbonyl group from the GABA fragment, and the NH (b) proton experiences the deshielding of the squaramide carbonyl group. This assumption is corroborated with the chemical shift of the signals corresponding to CH_2 (j) and CH_2 (h) at 3.58 and 3.77 ppm respectively. These data suggest the formation of an intramolecularly hydrogen-bonded ring system that features the turn architecture that we have characterised for the model compound **3**. The analysis of the chemical shift observed for the rest of the NH groups of the molecule indicates that they are also involved, to some extent, in hydrogen bonding interactions.[27] It is noteworthy that the NH (d) splits in two at 7.22 and 6.96 ppm, suggesting the existence of two conformations for this group participating in hydrogen bonding interactions in one of them.

To fully characterise the conformational state of **4**, we performed 2D-NOESY studies. We found several cross peaks between both chains supporting the existence of folding (see Supporting Information†). As shown in Fig. 9, we observed strong nOe signals between the amide protons NH (a) and the methylenic protons CH_2 (k) and CH_2 (l) of the GABA fragment, which are consistent with the turn conformation. However, the corresponding signal of NH (d) at 7.22 also gives strong nOe signals with the methylenic protons CH_2 (k) and CH_2 (l). These data, together with the downfield shift observed for NH (d), suggest that this group is also involved in the turn fragment by means of a hydrogen bonding interaction with the carbonyl group of the GABA residue. In this scenario, the ethylenediamine linker acts as a bi-dentate ligand and the turn is driven by the formation of two hydrogen bonding interactions. However, some nOe signals detected suggest the presence of a slightly different conformation of the molecule. For example, the cross-peaks observed, such as the one between the methylene protons CH_2 (i) and CH_2 (k) of both squaramide N-substituents and the carbamate NH (f) and the CHα (m) of the phenylalanine residue. These proximities are reasonably explained, considering that the ethylenediamine fragment adopts an extended conformation in which the corresponding NH (d) at 6.96 ppm faces outside of the folded molecule.

Minimised structures at RB3LYP/6-311G* level in the gas phase of **4** featuring both proposed loops show a good parallel alignment of the strands linked to them, favouring the cross-strand hydrogen bonding interactions that were detected by NOESY experiments.[28] The analysis of the 1- and 2D NMR spectra of **4** indicates that the two conformations of the loop are mostly equally populated.

Hence, the data obtained from this experiment support the existence of the hairpin-like folded conformation and show that the new turn-inducing module, due its conformational properties, is able to organise peptidomimetic structures in a hairpin fashion.

Conclusions

We have developed and characterised a new turn module based on the combination of a disecondary squaramide with the 4-aminobutyric acid moieties that form a 10-membered ring induced by the formation of intramolecular hydrogen bonds. Conformational studies based on ^{1}H NMR data for compound **3** showed that the new mimetic β-turn module is quite flexible.

Nevertheless, this system has been successfully combined with dipeptide chains to induce the formation of a hairpin-like structure driven by the formation of several cross-strand intramolecular hydrogen bonds. The conformational flexibility of this new loop is still considerable and leads to two slightly different conformations. These results indicate that this kind of hybrid structure that constitutes a new family of foldamers are good candidates to be considered, for example, in the development of conformational switches, or in drug design. Further work to fully control the conformational properties of this new module is under process in our laboratory.

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25 The low solubility of **4** either in chloroform or acetonitrile prevented us from running concentration- or temperature-dependent experiments by NMR techniques.
26 The split signals observed for this structural fragment are not only due to the quite probably also occurring interconversion equilibrium *E vs. Z* of the carbamate group. The carbamate is located at the end of the molecule and can freely rotate without provoking the splitting of the ethylendiamine NH (d) signal.
27 The chemical shifts observed for the NH groups of compound **4** are: NH (a) at 7.14 ppm; NH (b) at 6.76 ppm; NH (c) at 7.07 ppm; NH (d) at 7.22 and 6.96 ppm; NH (e) 6.83 and 6.15 ppm; NH (g) 6.68 ppm.
28 The minimised structures shown here were calculated to illustrate the experimental evidence of the existence of the two conformations observed by 2D RMN experiments. No further assumptions were made from them.