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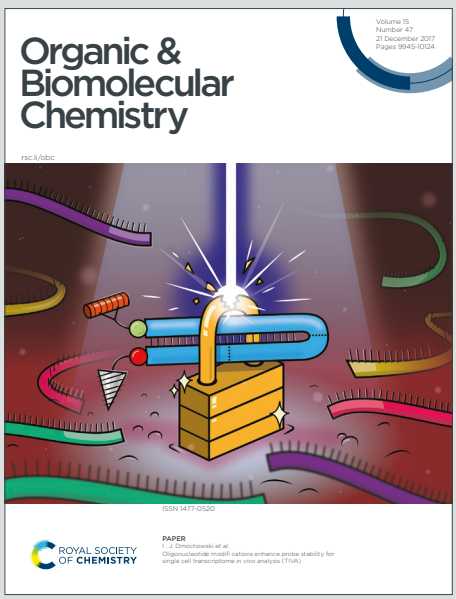

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COMMUNICATION

Synthesis of Amphiphilic Hydantoin-based Universal Peptidomimetics as Antibiotic Agents

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Three model hydantoin-based universal peptidomimetics are designed and synthetized. Their preferred amphiphilic β-turn conformation was assessed by molecular modeling and NMR experiments, and their antibacterial acivity tasted against Gram positive and Gram negative bacteria strains, demostrating that these compounds could be a captivating class of antibiotic to fight emergent drug resistance.

One of the most serious public health issues of the last four decades is antibiotics resistance.[1] Indeed, the capability of microbes to evolve mechanisms that protect them from the effects of antimicrobials along with the misuse and over-prescription of antibiotics are among the most important factors that caused an alarming increase of resistant strains.[2] Moreover, albeit the research activity driven to the discovery of new antibiotics is on the rise in both academia and industry, the discovery of new effective antibiotics is dramatically decreasing.[3] For these reasons, in the antibacterial research community there is a great interest in the search for new scaffolds that can overcome multidrug resistant infections. In medicinal chemistry, Nature is often the first source of inspiration to seek active scaffolds.[4] Among the different classes of natural antibiotics, such as aminoglycosides, β-lactams, macrolides, among others, antimicrobial peptides (AMPs) have witnessed a significant research interest to fight drug resistance.[5] There are different pros associated with AMPs, the most important being their range of activities,[6] the possibility to vary their chemical functionalities using the natural (or even not natural) amino acids, and lower capacity to develop resistance by the bacteria.[7] Nevertheless, AMPs are experiencing little clinical success mainly due to their scarce enzymatic proteolytic stability and bioavailability, high toxicity, and high cost of production.[8] In order to overcome these disadvantages, the design of small molecule peptidomimetics or short di- or tripeptides has recently emerged as a promising strategy to fight bacterial resistance.[9] Indeed, taking in consideration the general proposed mechanism of action of AMPs relying in a first electrostatic interaction between the negative charged bacterial membrane and the cationic moieties of the peptide followed by the intercalation of the hydrophobic residues into the lipophilic interior of the cell,[6] it is possible to rationally design small molecule peptidomimetics having a right balance and orientation of hydrophilic and hydrophobic residues. An outstanding example is brilacidin **1** (Figure 1), a defensin peptidomimetic that has completed phase II clinical trials for the treatment of bacterial skin infection, whose activity probably depends on its planar structure able to project the cationic moieties (guanidino groups) and the lipophilic substituents (trifluoromethyl groups) in position mimicking the secondary structure of defensins.[10] On the other end, short tripeptides able to assume turn conformations could cross Gram negative bacteria membrane thanks to oligopeptide transporters.[11] For instance, short arginine-based tripeptides having a central hydrophobic residue **2** (Figure 1) showed selective activity against *Pseudomonas aeruginosa* thanks to their ability to exist in β-turn conformation.[12]

Recently, we have introduced a hydantoin-based framework as a novel universal peptidomimetic scaffold able to mimic different protein secondary structures through rotation around a few of significant degrees of freedom.[13] The planar hydantoin framework is able to project the substituents in a position strictly related to α-helix and β-turn conformations, with the latter being favoured both in solution and in the solid state as evidenced by NMR and X-ray experiments. Taking inspiration from the structure and features of tripeptides **2**, we present herein the synthesis and antibacterial activity of three model hydantoin-based universal peptidomimetics **3** (Scheme 1) presenting two guanidino groups and a hydrophobic aromatic residue in positions related to the classical *i + n* side chain positions of typical protein secondary structures.

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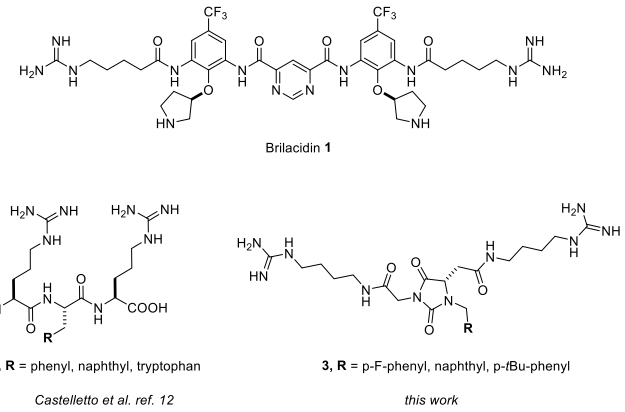

Figure 1. Structures of Brilacidin **1**, β-turn tripeptides **2** and hydantoin-based universal peptidomimetics **3**

It is well recognized that the activity of large AMPs depends on the fact that they often adopt protein secondary structures, such as α-helix and β-sheets, in solution or during the interaction with the cell membrane.[14] Also, small tripeptides **2** were designed because they are able to form turn structures.[12] This characteristic suggested us to exploit the conformational features of hydantoin-based universal peptidomimetics, thus to synthetize three model peptidomimetics **3** which could be able to project two hydrophilic guanidino substituents and a hydrophobic moiety in the opposite face of the β-turn mimicked secondary structure.

The synthetic pathway to synthetize **3** is very straightforward and is depicted in Scheme 1. Isocyanates **4a,b** reacted smoothly with aspartic acid **5** having the carboxylic functional group in α-position and in the lateral chain protected as methyl ester and tert-butyl ester, respectively, producing urea derivatives **6a,b**. We have previously shown that urea of type **6** with a further alkyl substituent tethered to the nitrogen of the aspartic acid cyclize, upon treatment with a base, regioselectively with the carboxylic ester in α-position forming the five-member hydantoin ring over the cyclisation on the carboxylic ester on the lateral chain which would form a six-member dihydrouracil ring.[13b] In the present case, being the two nitrogen atoms on the urea moiety mono-substituted, two cyclization reactions could occur, one on the methyl ester of the aspartic acid and the other on the benzyl or ethyl ester of the glycine, leading to the formation of two different regioisomeric hydantoins. To our delight, after treatment with a 1M solution of NaOH we obtained, after 5 minutes, the selective formation of hydantoins **7a,b** in very good yields.[15] Alkylation promoted by K_2CO_3 in DMF with benzyl halides such as p-tert-butyl benzyl bromide **8a**, p-F-benzyl bromide **8b** and 1-(bromomethyl)naphthalene **8c**, yielded fully substituted hydantoins **9a-c**, respectively very efficiently. The tert-butyl ester functional group of hydantoins **9a-c** was selectively hydrolysed under acid condition (TFA/DCM) and the resulting free carboxylic acids coupled with mono-Boc-1,4-butandiamine **10** producing hydantoin derivatives **11a-c** in very good yields. Diamine **10**, having the two amino moieties separated by four methylene groups, was chosen by similarity with the arginine side chain of tripeptides **2**. Next, ethyl ester of compounds **11a,b** was hydrolysed in basic condition (NaOH 1M solution), and the resulting carboxylic acids were coupled with a second molecule of **10** leading to the formation of "symmetric" hydantoins **12a,b** having the same arm attached to the imide-nitrogen and to the carbon of the ring. It is worth noting that hydantoins **9a-c** have two orthogonally protected carboxy functionalities, therefore the functionalization with different substituents would be feasible in future studies. Benzyl ester of intermediate **11c** was cleaved by catalytic hydrogenation and after coupling with **10** and deprotection of the Boc protecting group we recovered the free diamine **13c** which was used in the biological test for comparison between the amino groups with the guanidino groups (see below). Finally, after Boc-deprotection of hydantoins **12a,b**, the free amino groups of the resulting compounds, as well as the amino groups of **13c**, were transformed into guanidino groups by reaction with 1-[N,N'-(Di-Boc)amidino]pyrazole **14** providing the final peptidomimetics **3a-c** after Boc-deprotection.

In analogy with what we have done for the hydantoin-based peptidomimetics having hydrophobic substituents,[13] we have used a combined Monte-Carlo-Molecular Mechanics (MM) on amino and guanidino derivatives **13c**, **3c**, respectively, as model compounds to get insights on their propensity to adopt secondary structure, with particular interest in the β-turn conformation triggered by an intramolecular hydrogen bond involving the amidic NH_B hydrogen (Figure 1). The interatomic distance dα < 7 Å and the absolute value of the dihedral angle C1-C2-C3-N4 β < 30° were considered as a condition for the β-turn conformation, while interatomic distances between C*i*, C*i + 4* and C*i + 7*, related to the substituents *i*, *i + 4* and *i + 7* of an ideal α-helix, namely *i - i + 4* = 6.2 Å, *i – i + 7* = 10.3 Å and *i+ 4 – i + 7* = 5.8 Å were used as references for the α-helix conformation. In both cases, we considered the conformers within a 10 Kcal/mol range from the conformer at minimum energy.[16] In Table 1 we report the results as the percentage of conformers satisfying the requirements. For diamine derivative **13c** the α-helix conformation is slightly preferred, even if the minimum conformation turned out to be a β-turn, while for the bis-guanidino peptidomimetic **3c**, the β-turn geometry is the most favoured both at the ground-state and in the 10 kcal/mol range.

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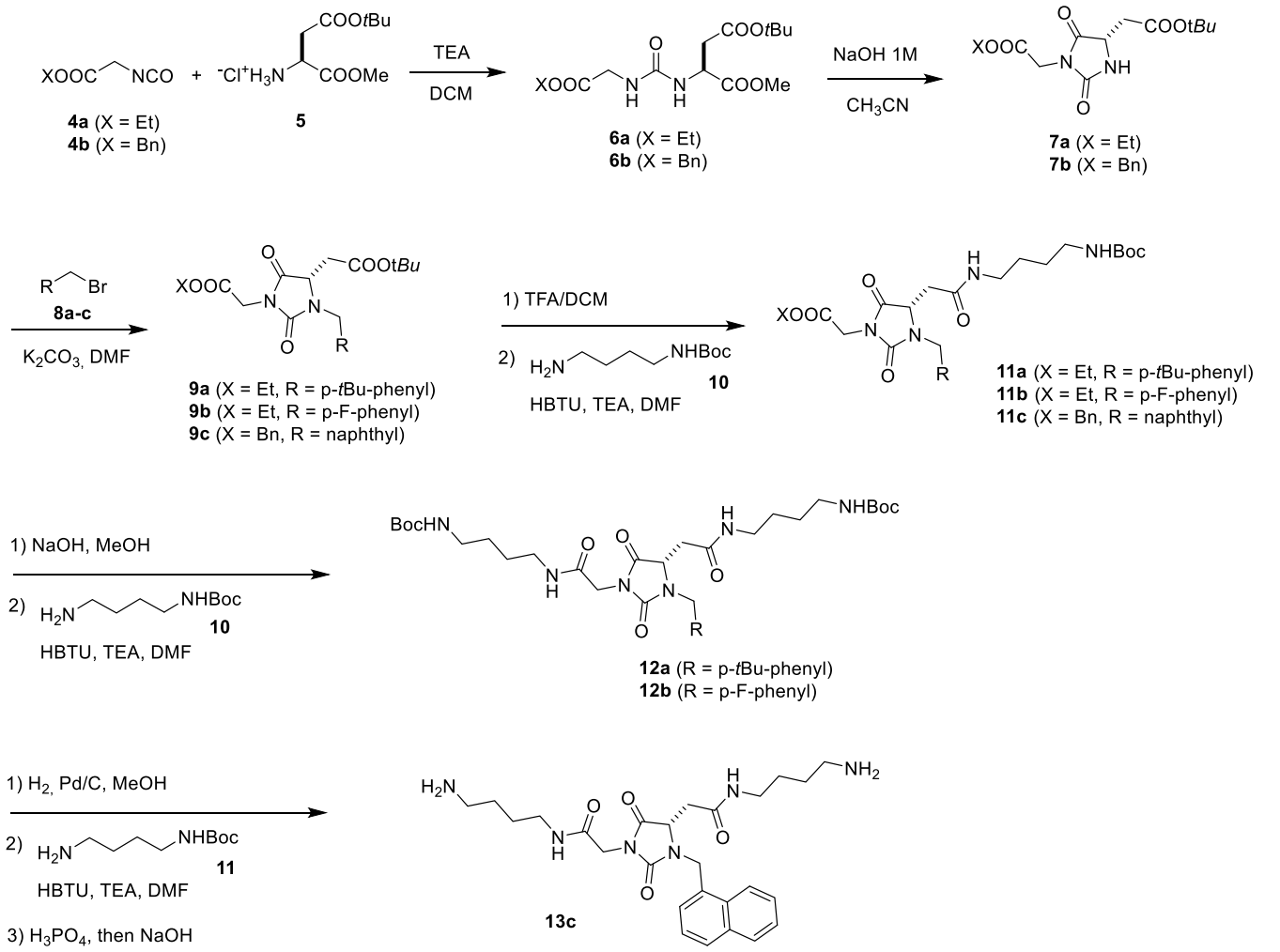

Scheme 1. Synthesis of hydantoin-based universal peptidomimetics **3**.

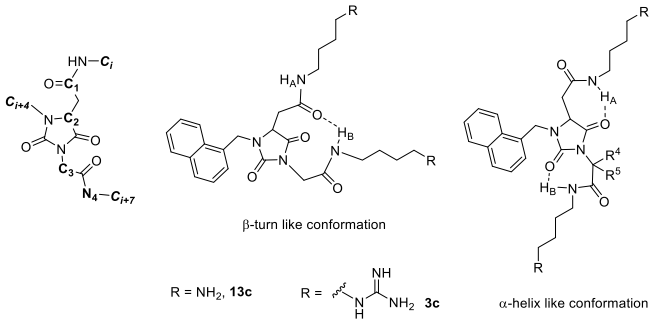

Figure 2. β-Turn and α-helix conformation of peptidomimetics **13c** and **3c**

This can be rationalized by considering that the hydrogen bond network is denser in **3c** due to the involvement of the guanidino groups.

Table 1. Results from Monte Carlo/MM conformational analysis[a].

Compound	β-turn (%)	α-helix %	Global minimum
13c	18	27	β-turn
3c	26	17	β-turn

[a] Results are reported as a percentage of conformers satisfying the geometrical requirements adopted

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In Figure 2 are reported the low-energy β-turn conformations and the fitting plots. Notably, the plot for **3c** shows a higher number of conformations due to the very dense hydrogen bond network that gives rise to many conformational possibilities. In the same pictures are highlighted also the hydrogen bonds (dotted blue lines) showing that together with the known C=O…H_B-N contacts, also new guanidino H-N…H-N connections further stabilize the conformations.

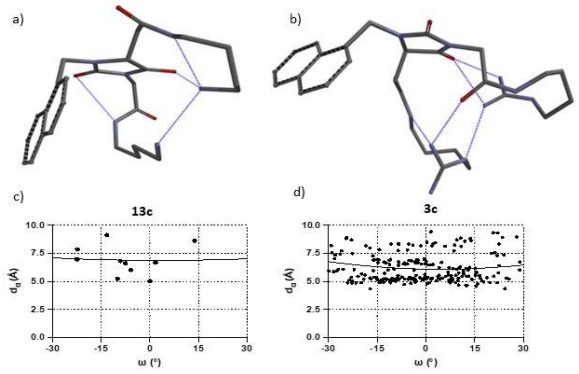

Figure 2. a) Low energy β-turn conformation of **13c**; b) low energy β-turn conformation of **3c**; c) fitting plots for **13c**; d) fitting plots for **3c**.

To check the presence of secondary structures in solution we investigated the strength of possible intramolecular hydrogen bonds involving the amidic protons NH_A and NH_B through ^{1}H NMR experiments. A first parameter to take in consideration is the chemical shift in a relatively nonpolar solvent according to which NH amidic protons resonating around 8.0 ppm are likely to be involved in intramolecular hydrogen bond. Unfortunately, guanidino derivatives **3a-c** were not soluble in deuterated nonpolar solvents, whereas diamine **13c** was soluble in $CDCl_3$. NH_B proton of **13c** (2.5 nM solution) resonates at 8.04 ppm while NH_A proton at 6.35 ppm (copy of the spectrum is reported in the Supporting Information), indicating the involvement of NH_B in the intramolecular hydrogen bond which trigger the β-turn conformation.[17] Moreover, for guanidino derivative **3c** the rate of exchange of NH_B with deuterium was very slow, as evidenced in the ^{1}H NMR spectrum recorded in deuterated methanol. Actually, proton NH_B resonating at 7.95 ppm integrates for one hydrogen (see spectrum in Supporting Information) while NH_A and the hydrogens belonging to the guanidino groups are not clearly detectable because, on the contrary, they exchange quickly with deuterium. This evidence further reflects the involvement of proton NH_B in intramolecular hydrogen bond. To investigate more in depth the possible presence of intramolecular hydrogen bonds we performed variable-temperature (VT) and DMSO titration ^{1}H NMR experiment on **13c** (Figure 3). As evidenced in Figure 3a and S1, with the increasing of the temperature the proton shifts for NH_A and NH_B are quite similar, with values of Δδ/ΔT equal to 9.0 and 5.9 ppb/K, respectively. These values are not very indicative since they are neither lower nor much higher than 2.4 ppb/K.[18] However, DMSO titration experiment, performed by gradually adding small aliquots (5 µL) of DMSO to a 2.0 mM $CDCl_3$ solution of **13c**, showed that the chemical shift of NH_B essentially did not change upon dilution because already involved in intramolecular hydrogen bond, while we registered a sizable increase of the chemical shift of amidic hydrogen NH_A with the increasing of the concentration of DMSO due to the forming of intermolecular hydrogen bond between the "free" NH_A with the titrating solvent (Figures 3b and S2).[19] All these experiments proved that also when the substituents on the hydantoin scaffold are polar, such as substituents bearing amino or guanidino moieties, the peptidomimetics show a great tendency to adopt secondary structures in solution, and in particular β-turn conformation triggered by the formation of a quite strong intramolecular hydrogen bond.

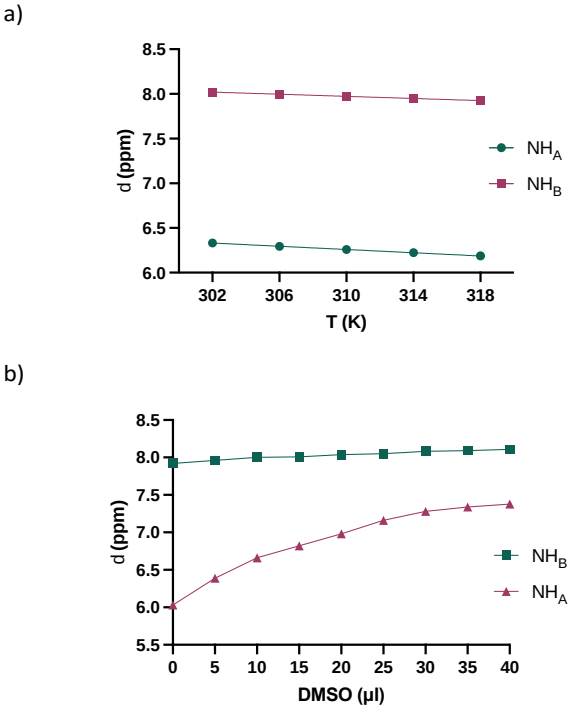

Figure 3. a) VT and b) DMSO titration ^{1}H NMR experiments on **13c**.

To assess the antimicrobial activity of the synthesized compounds, we used four bacterial species belonging to the ESKAPE group: the reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25293, *Acinetobacter baumannii* A17, and *Pseudomonas aeruginosa*.[20] Compounds **3a-c** bearing guanidino groups as terminal moieties of the extended arms and three different hydrophobic moieties, namely p-tert-butyl benzyl, p-F-benzyl, and 1-naphthyl-methylene group, respectively, and **13c** bearing two amino groups and 1-naphthyl-methylene group were tested at concentrations ranging from 4-0.06 µg/mL. The obtained results are summarized in Table 2.

Table 2. MIC (µg/mL) of compounds 3a-c and 13c[a]

Compound	*E. coli*	*S. aureus*	*P. aeruginosa*	*A. baumannii*

3a	4	0.25	2	1
3b	n.e[b]	n.e.[b]	n.e.[b]	n.e.[b]
3c	n.e[b]	2	n.e[b]	n.d.[c]
13c	n.e[b]	n.e[b]	n.e[b]	n.d.[c]

[a]Results determined in triplicate [b]Not effective in inhibiting bacterial growth. [c]Not determined.

Although designed as antibacterial agents against Gram negative bacteria, compound **3a** having a 4-tert-butylbenzyl group as hydrophobic moiety resulted to be the most potent peptidomimetic, showing a remarkable activity against the Gram-positive bacteria *Staphylococcus aureus* (MIC = 0.25 μg/mL), and a very good activity against Gram-negative bacteria *E. coli*, *P. aeruginosa*, and *A. baumannii*. The substitution on the benzene ring of the hydrophobic moiety exerts an important role on the antibacterial activity of the peptidomimetics. Indeed, derivative **3b** having a less hydrophobic fluoro substituent compared to the tert-butyl group of **3a**, resulted to be completely inactive against all the strains tested in the range of concentration measured. Interestingly, derivative **3c** having a bulky, planar and enhanced π–staking 1-naphthyl-methylene group, appeared effective only against *Staphylococcus aureus*, while the corresponding diammino derivative **13c** was inactive against the three strains tested.

Conclusions

In conclusion, we designed three model hydantoin-based universal peptidomimetics having two strands bearing terminal hydrophilic guanidino groups and differing for the hydrophobic aromatic moiety tethered to the N-1 of the heterocycle. Through molecular modelling and ^{1}H NMR experiments, we demonstrated that these peptidomimetics can project the hydrophilic guanidino groups and the hydrophobic moiety in the opposite face of the β-turn mimicked secondary structure. This feature is probably very important for the broad activity that compound **3a** showed against Gram-positive and Gram-negative bacterial strains and for the selective activity toward the Gram-positive *Staphylococcus aureus* of compound **3c**. Together with their facile synthesis, the results obtained demonstrate that these peptidomimetics are promising candidates for further structure-activity relationship studies to shed light on their mechanism of action and toxicity, and to optimize their activity/selectivity *in vitro* and *in vivo*. These studies are ongoing in our laboratories.

Author Contributions

A.C. and M.C.B. performed the chemical experiments. A.C. performed the computational modelling and the NMR studies. E.O. and S.A., performed the antibacterial tests. E.B. supervised the antibacterial tests. A.V. designed the scaffold, conceived the experiments and wrote the paper.

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Conflicts of interest

There are no conflicts to declare.

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