A Multicomponent Conjugation Strategy to Unique N-Steroidal Peptides: First Evidence of the Steroidal Nucleus as β-Turn Inducer in Acyclic Peptides

ARTICLE *in* CHEMISTRY - A EUROPEAN JOURNAL · OCTOBER 2014

Impact Factor: 5.73 · DOI: 10.1002/chem.201403773

7 AUTHORS, INCLUDING:

Daniel G Rivera
University of Havana
57 PUBLICATIONS **904** CITATIONS
SEE PROFILE

Aldrin Vasco Vidal
Leibniz Institute for Plant Biochemistry
2 PUBLICATIONS **6** CITATIONS
SEE PROFILE

Radell Echemendía Pérez
5 PUBLICATIONS **11** CITATIONS
SEE PROFILE

José A Gavin
Universidad de La Laguna
55 PUBLICATIONS **548** CITATIONS
SEE PROFILE

ChemPubSoc Europe

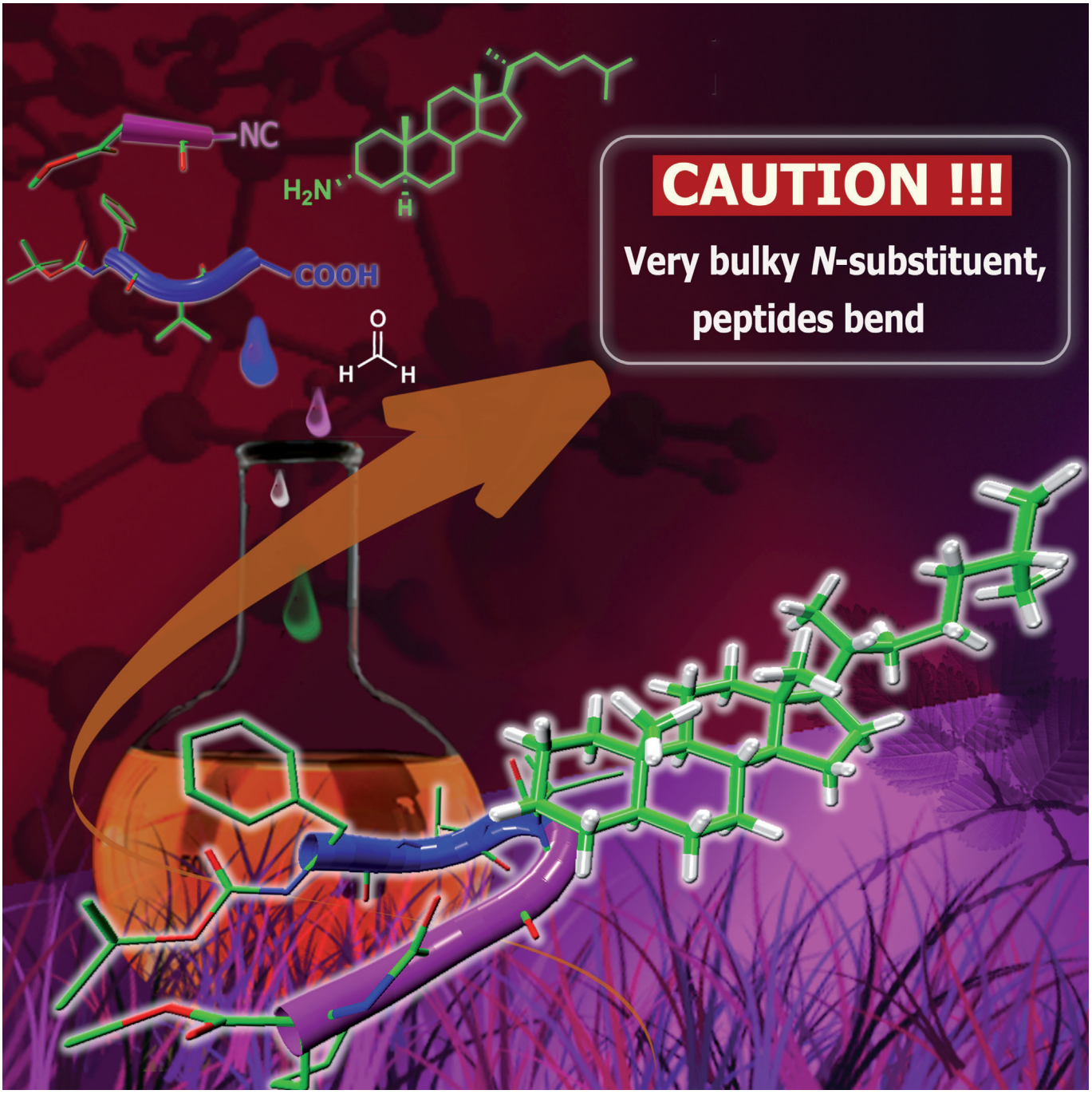
DOI: 10.1002/chem.201403773

CHEMISTRY
A European Journal
Full Paper

Peptides | *Very Important Paper*

VIP A Multicomponent Conjugation Strategy to Unique *N*-Steroidal Peptides: First Evidence of the Steroidal Nucleus as β-Turn Inducer in Acyclic Peptides

Daniel G. Rivera,*[a] Aldrin V. Vasco,[a] Radell Echemendía,[a] Odette Concepción,[a] Carlos. S. Pérez,[a] José A. Gavín,[c] and Ludger A. Wessjohann*[b]



Abstract: Constraining small peptides into specific secondary structures has been a major challenge in peptide ligand design. So far, the major solution for decreasing the conformational flexibility in small peptides has been cyclization. An alternative is the use of topological templates, which are able to induce and/or stabilize peptide secondary structures by means of covalent attachment to the peptide. Herein a multicomponent strategy and structural analysis of a new type of peptidosteroid architecture having the steroid as *N*-substituent of an internal amide bond is reported. The approach comprises the one-pot conjugation of two peptide chains (or amino acid derivatives) to aminosteroids by means of the Ugi reaction to give a unique family of *N*-steroidal peptides. The conjugation efficiency of a variety of peptide sequences and steroidal amines, as well as their consecutive head-to-tail cyclization to produce chimeric cyclopeptide–steroid conjugates, that is, macrocyclic lipopeptides, was assessed. Determination of the three-dimensional structure of an acyclic *N*-steroidal peptide in solution proved that the bulky, rigid steroidal template is capable of both increasing significantly the conformational rigidity, even in a peptide sequence as short as five amino acid residues, and inducing a β-turn secondary structure even in the all-*s-trans* isomer. This report provides the first evidence of the steroid skeleton as β-turn inducer in linear peptide sequences.

Introduction

Owing to its great rigidity, availability, chirality, and biological importance, the steroid skeleton has been traditionally considered as an amenable template for conjugating oligopeptide chains of biological interest. This unique polycyclic platform can be either highly lipophilic (e.g., cholesterol, steroidal hormones) or facially amphiphilic (e.g., bile acids), and these features can be used at will to modulate the physicochemical, structural, and biological properties of peptides on conjugation.[1] Notable applications are the conjugation of steroids: 1) to peptide drugs for improving their pharmacological properties,[2] 2) to protein active-site sequences for creating artificial protease-like enzymes[3] and receptors,[4] and 3) to epitope,[5] antibacterial,[6] and cell-surface active[7] peptide sequences for producing therapeutically useful conjugates, among other applications.

The mimicry of the intrinsic conformation of a bioactive peptide/protein sequence is a major goal in the design of synthetic peptide ligands and peptidomimetics. Thus far, the restriction of conformational space in small peptides has been achieved by cyclization or conjugation to topological templates.[8] A remarkable application of the conjugation of peptides to steroids has been the capability to reduce the conformational flexibility of very short peptide sequences. The pioneering works of Still et al.[4] and Wess, Kramer et al.[9] shed light on the possibility of accessing specific peptide conformations through conjugation of small peptides to steroids. Nevertheless, induction of peptide secondary structures such as loops and helices has only been achieved in cyclopeptidosteroids derived from the conjugation of both peptide termini to a steroidal template.[9,10] Recently, this idea was successfully implemented in the development of a cyclopeptidosteroid (structure **IV**, Figure 1 A) mimicking the conformation of a viral protein epitope.[5a]

On the other hand, the induction of defined secondary structures in acyclic peptides with only the aid of steroid conjugation has remained elusive so far. This may be due to the much lower control over the conformational space of the peptide if only one of its termini is linked to the steroid nucleus. We believe that greater control over such a rigidifying effect might be achieved by changing the traditional concept of how peptides are conjugated to steroids, and likely to any other type of topological template. A literature survey revealed that most common peptide–steroid conjugates have generic structures such as **I**,[11] **II**,[5b,c] **III**,[4,12] and **IV**[5a,13] (Figure 1 A), with the logical variation in the conjugation position, the steroid scaffold, and the peptide sequence. Indeed, the pursuit of novel peptidosteroid architectures requires alternative methods to those traditionally employed in the field, which are: 1) the sequential incorporation of *N*-protected amino acids by solution and solid-phase peptide coupling protocols[4–13] and 2) the direct conjugation of peptides by peptide coupling[2] or click chemistry.[14] In these conventional strategies, the peptide is always ligated at a terminus, a feature that is not sufficient to induce a stable secondary structure in acyclic short sequences.

We envisioned that a different scenario may occur if the steroidal template is an *N*-substituent of an internal amide bond. In this alternative case, the bulky and rigid steroid nucleus might be capable of inducing a turn motif in a short peptide chain. Whereas the synthesis of *N*-alkylated, especially *N*-methylated, peptides is currently established,[15] the incorporation of the steroid as internal peptide *N*-alkyl substituent poses a synthetic challenge that cannot yet be overcome by the

[a] *Prof. Dr. D. G. Rivera, A. V. Vasco, R. Echemendía, O. Concepción, Prof. Dr. C. S. Pérez*
Center for Natural Products Research
Faculty of Chemistry, University of Havana
Zapata y G, 10400, La Habana (Cuba)
E-mail: dgr@fq.uh.cu

[b] *Prof. Dr. L. A. Wessjohann*
Department of Bioorganic Chemistry
Leibniz Institute of Plant Biochemistry
Weinberg 3, 06120, Halle/Saale (Germany)
E-mail: wessjohann@ipb-halle.de

[c] *Prof. Dr. J. A. Gavín*
Instituto Universitario de Bioorgánica "Antonio González"
Departamento de Química Orgánica
Universidad de La Laguna
38206 La Laguna, Tenerife (Spain)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201403773.

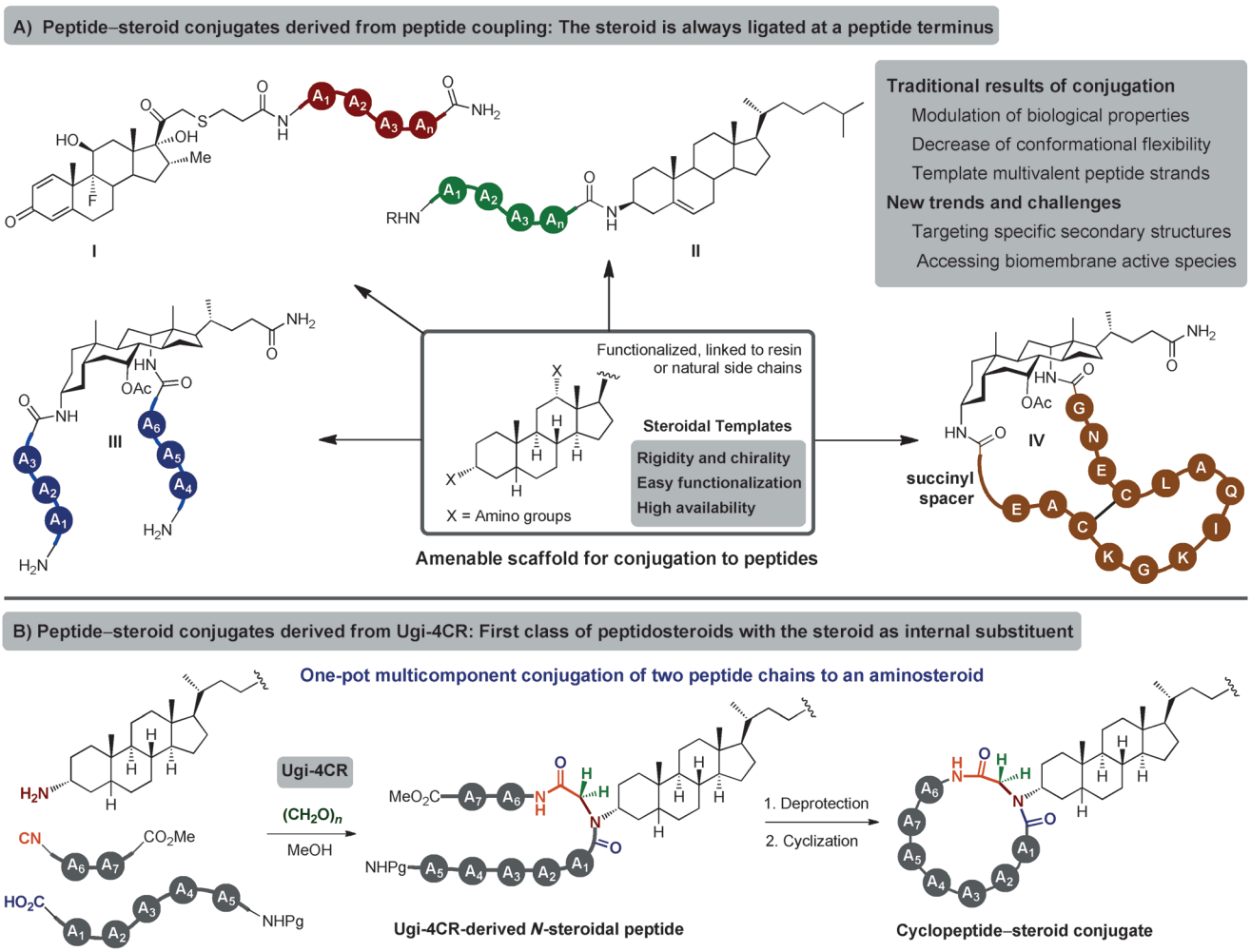


Figure 1. A) Generic structures of peptide–steroid conjugates produced by traditional peptide-coupling strategies. B) Multicomponent approach for the conjugation of two peptides to an aminosteroid, if desired followed by cyclization to a chimeric cyclopeptide–steroid conjugate.

most powerful peptide-coupling approaches. Thus, we focused on a multicomponent approach that can produce such a unique type of *N*-steroidal peptide in a one-pot conjugation procedure.

Herein we report on the use of the Ugi four-component reaction (Ugi-4CR) as a general multicomponent approach for the one-pot assembly of *N*-steroidal peptides covering a conformational space not previously explored by other peptidosteroid architectures. As depicted in Figure 1 B, the strategy relies on the ligation of two peptide derivatives (i.e., an isocyanopeptide or isocyanoacetate and a peptide acid) to an aminosteroid by using paraformaldehyde as oxo component. The result is a peptide with the steroid as *N*-substituent, and thus easier access to the *s-cis* isomer. In addition, it is likely that the bulky steroid substituent exerts a steric pressure on the peptide backbone, similar to the Thorpe–Ingold effect. Such *N*-steroidal peptides are further cyclized to chimeric cyclopeptide-steroid conjugates resembling the structures of natural lipocyclopeptides. To prove our hypothesis that the internal steroidal N substituent is capable of inducing a reverse turn motif in the peptide chain, we report a conformational study based on NMR and molecular dynamics simulation for a distinctive *N*-steroidal peptide. The aim is to disclose not only the three-dimensional structure of this novel type of conjugates, but also important details of the chemical efficiency, substrate scope, and potential of this powerful conjugation process.

Results and Discussion

Multicomponent peptide–steroid conjugation

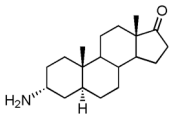
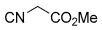
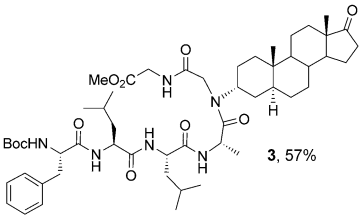
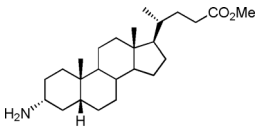
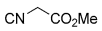
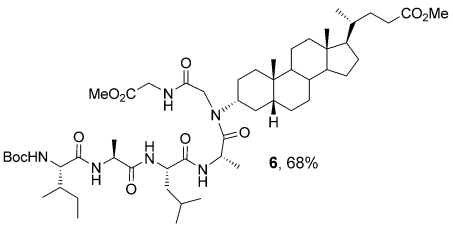
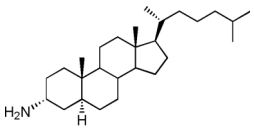
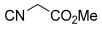
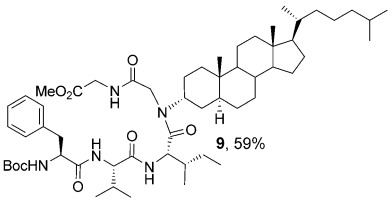
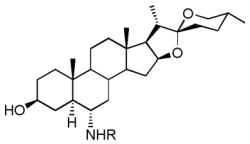
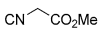
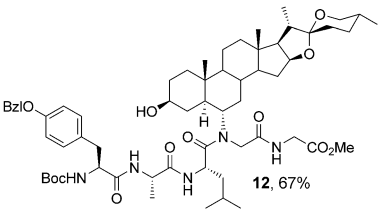
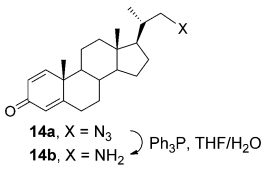
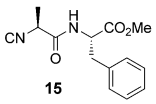
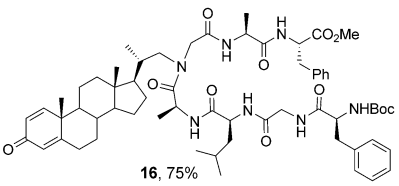
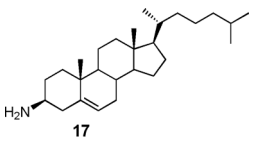
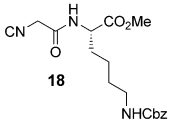
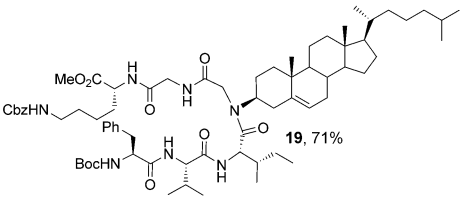
The Ugi-4CR[16] is the one-pot condensation of a primary amine, an oxo compound (i.e., ketone or aldehyde), a carboxylic acid, and an isocyanide to produce an *N*-substituted dipeptide backbone. Using steroids as substrates of this reaction, we have developed a variety of methods for the assembly of remarkably complex macro(multi)cyclic platforms.[17] Recently, we have addressed the possibility of ligating single amino acids (by their amino or carboxyl groups) to steroids functionalized as isocyanides[18] and carboxylic acids.[19] In addition, we have shown that, under certain constraints, multicomponent reactions can be used to build up sterically constrained peptoids

through ligation of two peptides.[20] These studies opened up the possibility to extend the methodology to the conjugation of peptides to aminosteroids, which is a promising scenario for the design of novel peptidomimetics.

The utilization of the Ugi-4CR for the conjugation of peptides to an aminosteroid provides a series of opportunities—not only structural, but also synthetic—which are not available with classic peptide-coupling strategies. Indeed, the most distinctive one is the possibility of ligating two (or even three) peptide chains to a steroidal template in a one-pot process.[18] In addition, the Ugi-4CR allows for the conjugation of peptides by their C terminus without erosion of the stereochemical integrity of the terminal amino acid, which is one of the major drawbacks of coupling peptide fragments.

One of our main concerns was the probable drop in the conjugation efficiency compared with peptide-coupling methods. Table 1 depicts the results of the multicomponent conjugation study aimed at assessing the chemical efficiency and

Table 1. Multicomponent conjugation of peptides to aminosteroids by the Ugi-4CR.[a]

Peptide	Aminosteroid	Isocyanide	*N*-Steroidal peptide[b]
Boc-Phe-Leu-Leu-Ala-OH (**1**)	 **2** **axial amine**		 **3**, 57%
Boc-Ile-Ala-Leu-Ala-OH (**4**)	 **5** **equatorial amine**		 **6**, 68%
Boc-Phe-Val-Ile-OH (**7**)	 **8** **axial amine**		 **9**, 59%
Boc-Tyr(Bzl)-Ala-Leu-OH (**10**)	 **11a**, R = Boc **11b**, R = H TFA/CH_2Cl_2 **equatorial amine**		 **12**, 67%
Boc-Phe-Gly-Leu-Ala-OH (**13**)	 **14a**, X = N_3 **14b**, X = NH_2 Ph_3P, THF/H_2O	 **15**	 **16**, 75%
Boc-Phe-Val-Ile-OH (**7**)	 **17** **equatorial amine**	 **18**	 **19**, 71%

[a] Conducted at room temperature in MeOH/CH_2Cl_2 (5:1) for 24 h. [b] Yield of isolated pure product.

substrate scope by variation of the peptide sequence and the nature of the aminosteroid. To this end, a variety of peptide acids were produced through typical solution-phase protocols,[21] focusing on the installation of more (e.g., Ile) and less (e.g., Ala and Leu) sterically hindered *C*-terminal residues. Methyl isocyanoacetate and isocyanopeptides[22] were considered as isocyanide components to preserve the native characteristic of the peptide backbone. A variety of known steroidal amines[14a,23] (i.e., androstanic, cholanic, cholestanic, spirostanic, and bis-norcholanic) was selected with the aim of assessing the influence of the stereochemistry (α or β) and position (steroidal nucleus or side chain) of the amino group on the chemical efficiency of the Ugi-4CR. For a suitable comparison, a typical Ugi-4CR protocol[16b] was implemented with 24 h of reaction time, paraformaldehyde as fixed component, and MeOH/CH_2Cl_2 (5:1) as solvent.

Table 1 shows that the multicomponent conjugation generally proceeds in good yields considering both the high complexity and steric demand of the resulting peptidosteroids as well as the formation of two new peptide bonds. Comparison of the conjugation of tetrapeptides **1** and **4** (both having an *C*-terminal Ala residue) to the axially oriented androstanyl amine **2** and the equatorially oriented cholanyl amine **5**, respectively, reveals that the equatorial amine is more reactive than the axial one. The same conclusion arises from comparison of the conjugation of tripeptide **7** to the axial 3α-cholestanyl amine **8** and the equatorial 3β-cholesteryl amine **17** leading to peptidosteroids **9** and **19** in 59 and 71% yield, respectively. Thus, the utilization of the same peptide acid (**7**) and similar isocyanides gave different results for two analogous cholestanyl amines **8** and **17**, again favoring the equatorial one (**17**). Conjugation of the equatorial 6α-spirostanyl amine **11b** to peptide **10** also proceeded in reasonable yield, which indicates that the β-oriented Me-19 does not greatly affect the reactivity of the α-oriented amine at position 6. A clear distinction shows up in the conjugation of peptide acid **13** and isocyanodipeptide **15** to the bis-norcholanyl amine **14b**, which furnished conjugate **16** in 75% yield, the highest among all conjugation processes. As a result of these experiments, we can conclude that neither the sequence of such short peptides nor the nature of the isocyanide derivative have a crucial influence on the efficiency of this conjugation process. In contrast, it is the reactivity of the steroidal amine, likely related to its steric hindrance, which plays a crucial role in this multicomponent approach.

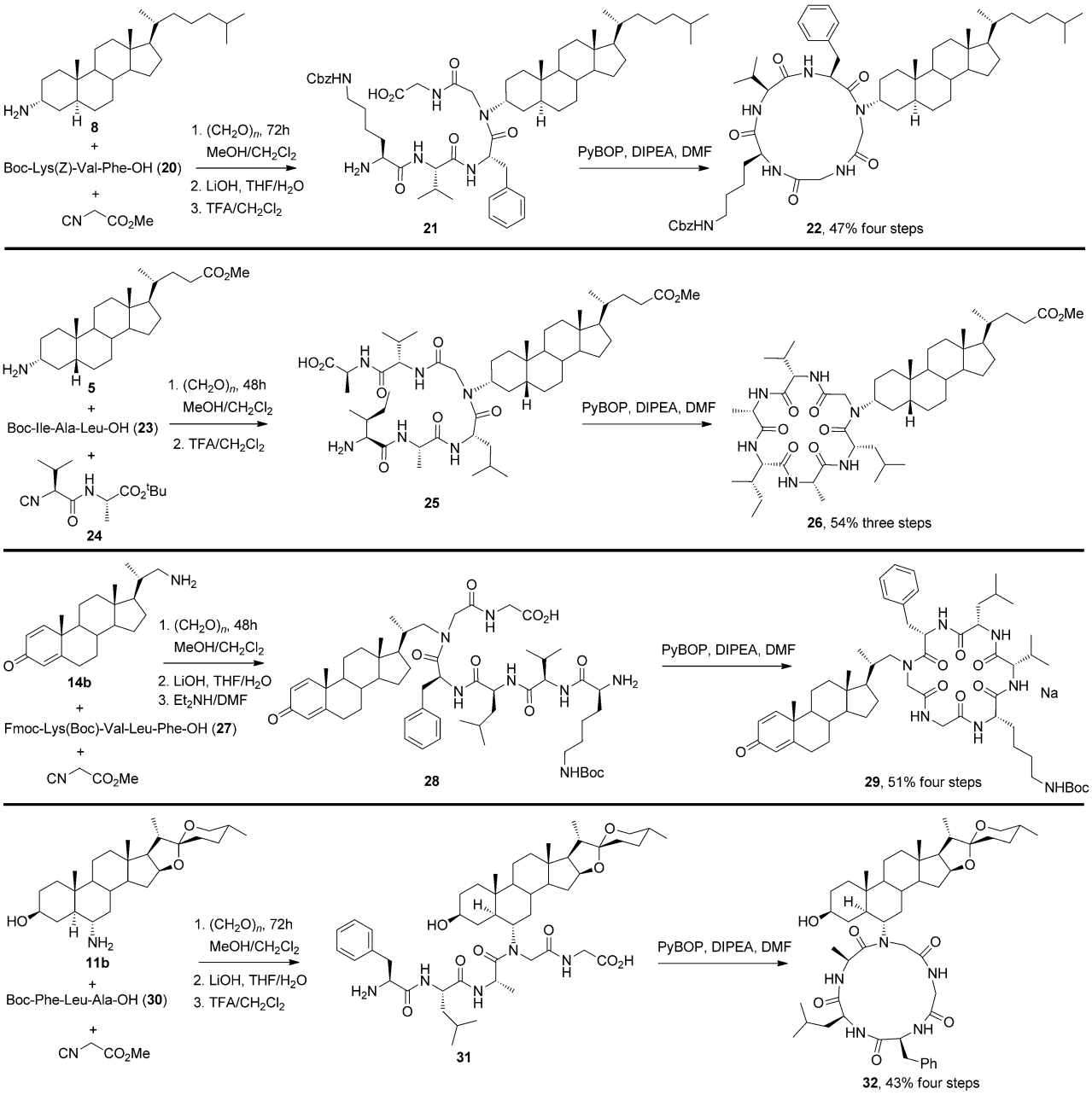
Since the Ugi-4CR proceeds via an intermediate α adduct that evolves through an intramolecular acylation step (Mumm rearrangement), migration of the amine should be easier with an equatorial disposition than with an axial one. To investigate whether the lower efficiency of the peptide conjugation to axial aminosteroids is due to the nature of Ugi-4CR per se, we performed a series of parallel experiments dealing with the conjugation of oligopeptides **1** and **4** to varied axial and equatorial aminosteroids by means of peptide coupling (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride/hydroxybenzotriazole, DMF, 24 h, RT). These experiments produced the equatorial peptidosteroids in about 70% yield of isolated pure products, generally 10–20% higher than that of the axial counterparts. This demonstrates that the multicomponent conjugation shows similar efficiency to classical methods of (terminal) peptide coupling while giving rise to peptidosteroid architectures not reported so far.

Inspired by a variety of antimicrobial cyclic lipopeptides such as surfactin and mycosubtilin bearing a fatty-acid chain directly linked to a cyclopeptide ring,[24] we envisioned utilization of the multicomponent conjugation in the development of a new class of lipocyclopeptides featuring chimeric cyclopeptide–steroid skeletons.[25] As shown in Scheme 1, the strategy comprises the deprotection of the N and C termini of the Ugi-derived *N*-steroidal peptide, followed by cyclization to afford a cyclopeptide scaffold having the steroid as exocyclic amide appendage. As detailed information was already gained regarding the efficiency of the conjugation, a sequential approach could be implemented that avoids isolation of pure intermediate peptidosteroids, which were only identified by ESI-MS, and then directly subjected to further deprotection and cyclization. In this sense, new peptide sequences were considered for conjugation to varied aminosteroids, once again combined either with isocyanoacetate or an isocyanopeptide. An important aim was to prove that the most common amino acid protecting groups (i.e., *tert*-butyloxycarbonyl (Boc), carboxybenzyl, 9-fluorenylmethyloxycarbonyl, CO_2Me, CO_2tBu) were suitable to implement this strategy by using simple orthogonal protection. Another crucial parameter was the reaction time required to run the initial conjugation step in nearly quantitative yield. For example, conjugation of axial aminosteroid **8** to peptide **20** needed 72 h for completion, whereas conjugations of the other equatorial amines were finished within 48 h. After deprotection, the *N*-steroidal peptides were subjected to cyclization with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) to give rise to diverse chimeric peptide–steroid conjugates in overall acceptable yields of isolated products.

We noted that, although no accurate comparison of the cyclization step was made, this class of *N*-steroidal peptide cyclizes more easily than nonsteroidal peptides of analogous sequence and length. This was considered to be a sign of the expected occurrence of a reverse turn conformation, or at least of easy access to the *cis* isomer of the *N*-steroidal amide, which facilitates the two peptide termini getting closer. As mentioned above, our initial hypothesis was that the location of a bulky steroid as internal amide substituent should force the peptide chain to fold back into a reverse turn.

Three-dimensional structure of a model *N*-steroidal peptide in solution

To seek further structural information, the prototypal *N*-steroidal peptide **9**, wherein a pentapeptide sequence is ligated with an axial disposition to steroid position 3, was chosen for solution-structure determination by means of NMR spectroscopy and molecular dynamics. For most peptidosteroids in which the peptide is directly attached to the steroid skeleton, ^{1}H NMR spectra are complex and show the presence of at least two conformers in appreciable abundance (see the Supporting



Scheme 1. Synthesis of chimeric cyclopeptide–steroid conjugates (lipocyclopeptides) by sequential multicomponent conjugation/deprotection/cyclization. Cbz = benzyloxycarbonyl, Boc = *t*-butoxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl.

Information). For compound **9**, three conformers in relative abundances of 10:8:1 were detected by exchange positive peaks in the ROESY spectrum (Figure 2). For the two major conformers (from here on referred to as conformers A and B), the $^{3}J_{NHCHa}$ values of Phe, Val, and Ile residues were larger than 8 Hz. Such values are indicative of high conformational rigidity of the peptide backbone and are characteristic for β-strand structures. Unambiguous assignment of the ^{1}H NMR resonances of the peptide and steroid portions was possible for these two major conformers, and thus enabled the accurate determination of the three-dimensional structure of peptidosteroid **9** in solution.

To this end, we started from a series of suppositions about the structural characteristics of compound **9**. Thus, we envisioned that the main conformational equilibrium should be due to the *s-cis*/*s-trans* isomerization around the *N*-alkylated Gly amide bond, in analogy to that found at proline or in *N*-alkylated polyglycines (i.e., peptoids).[26] Also, it was considered that the steroid skeleton should be responsible for the high rigidity of the peptide chain evidenced in the NMR analysis of both conformers. We had already supposed that—owing to the internal position of the bulky steroidal template—this *N*-substituent should be capable of folding back the peptide chain, and thus lead to a turn secondary structure. To prove

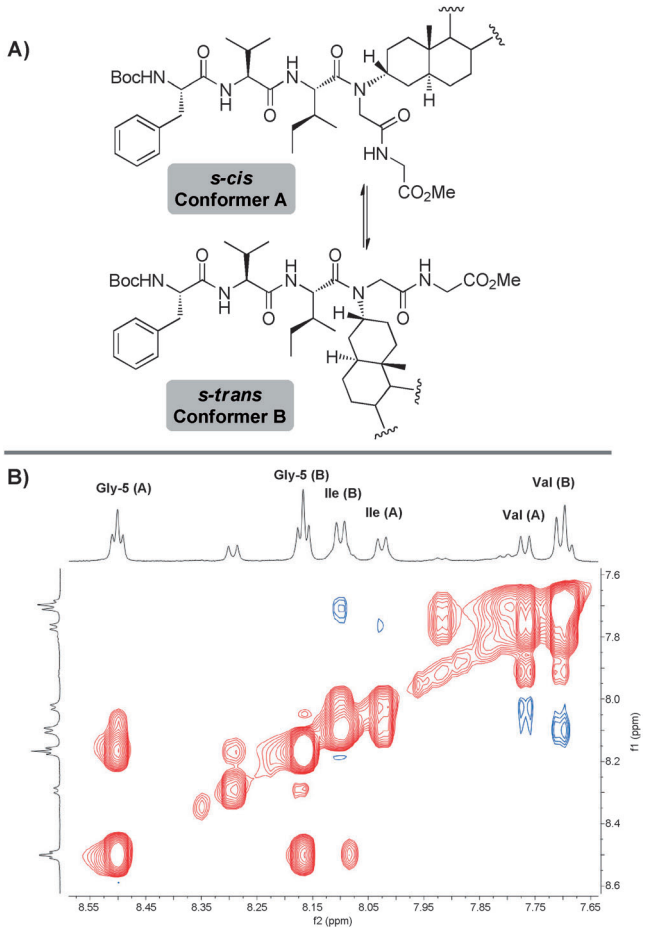


Figure 2. A) *cis/trans* isomerization of peptidosteroid **9** around the *N*-steroidal amide bond (for convenience, the *s-cis* and *s-trans* isomers refer to the peptide backbone). B) Amide region of the ROESY spectrum of peptidosteroid **9** showing the mixture of conformations. Positive (red) cross-peaks are indicative of conformational exchange, and the negative ones (blue) indicate ROE contact. The signals from the major conformers (A and B) are specified.

our hypothesis, the experimental data of each conformer were simulated twice, in one case by using a topology file with a fixed *cis* configuration and in the other one with the *trans* configuration at the *N*-substituted amide. For each case, the set of calculated structures best correlated with the experimental data was considered as the correct model.

Analysis of the ROESY spectrum of **9** resulted in a total of 47 distance constraints (5 strong, 13 medium, and 29 weak, 24 of which were inter-residual) for conformer A, which had an abundance of 42%. Four dihedral restraints were used in the MD simulation: three φ angles from the $^{3}J_{NHCH\alpha}$ data and one χ_1 angle from the Phe residue. In this case, it was found that structures derived from the *s-cis*-configured *N*-steroidal amide best fitted the experimental data. The final structures were obtained after two rounds of simulated annealing and refinement protocols in XPLOR-nih.[27] The first round of simulations allowed for the stereospecific assignment of methylene hydrogen atoms of Phe, Ile, and Gly^4 residues, although Val and Ile methyl groups could not be stereospecifically assigned. As shown in Figure 3, superimposition of the 20 lowest-energy structures of conformer A resulted in a very small value (0.037 Å) of average root-mean-square deviation (RMSD) to the mean structure of the peptide backbone, which corroborates the great conformational constraint imposed by the steroid onto the short peptide chain.

Similarly, the solution structure of conformer B (53% abundance) was determined from a total of 43 distance constraints (5 strong, 7 medium, and 33 weak, 23 of which were inter-residual) and four dihedral constraints. In this case, structures of the *s-trans*-configured *N*-steroidal amide best fitted the experimental data, confirming our idea that the two major conformers derive from the *cis/trans* isomerization of the *N*-substituted Gly^4 amide. Two rounds of simulated annealing and refinement protocols were required for the stereospecific assignment of Phe, Ile, and Gly^4 methylene hydrogen atoms. As before, methyl groups of Ile and Val remained ambiguous. Superimposition of the final 20 lowest-energy structures provided an average RMSD of 0.104 Å to the mean structure of the peptide backbone. As depicted in Figure 3, the structural definition at the steroid region of this conformer is lower than in conformer A. In this case, this likely originates from the lack of information on A-ring hydrogen atoms due to the ambiguous peak assignments in the high-field region. Otherwise, the mean deviations for the peptide region were found to be larger for residues away from the *N*-substituted amide, which supports the idea that the conformational constraint is provided by the steroid skeleton.

Among the most important contributions of this study is the confirmation that a steroid skeleton acting as an internal amide substituent is capable of inducing a β-turn structure in a short peptide chain. Tight turns are among the three most important secondary structures defining protein structure and

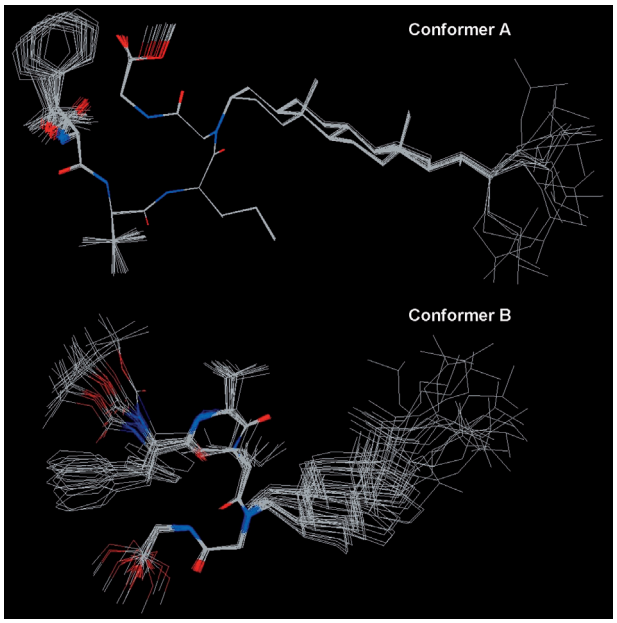


Figure 3. Superimposition of the 20 lowest-energy structures of conformers A and B of peptidosteroid **9** with no NOE violations greater than 0.3 Å and no dihedral violations greater than 2°.

function, as they allow the polypeptide chain to fold into compact globular structures.[28] Due to their topology and frequent location on exposed protein surfaces, these nonrepetitive motifs are crucial in biological events mediated by molecular-recognition processes, such as protein–protein and substrate–receptor interactions.[29] The most abundant type of tight turn is the β-turn, a secondary motif composed of four amino acid residues, usually stabilized by an intramolecular hydrogen bond between the carbonyl group of residue *i* and the NH group of residue $i+3$.[29,30] Whereas a wide variety of β-turns lack the ten-membered ring hydrogen bonding (i.e., open turns), the definition currently accepted is that a β-turn comprises four consecutive residues for which the distance between $C^{\alpha}(i)$ and $C^{\alpha}(i+3)$ is less than 7 Å and the tetrapeptide chain is not in an helical conformation. When flanked by two β-strands, β-turns allow for the formation of the biologically relevant β-hairpins, which, along with the α-helices, are the most important regular structures found in protein epitopes.[31]

The backbone representation for average peptide structures of conformers A and B of peptidosteroid **9** (Figure 4) shows

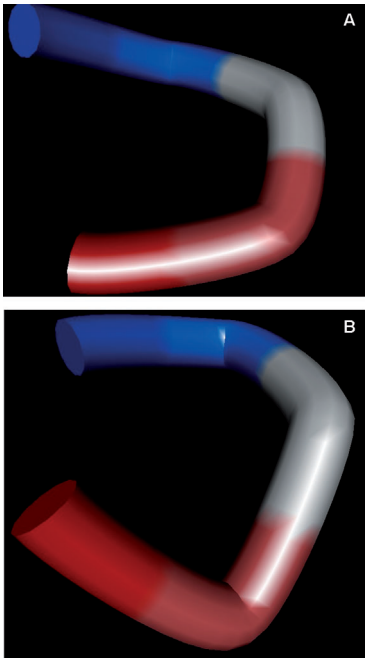


Figure 4. Representation of the peptide backbone for average structures of conformers A and B of peptidosteroid **9**, both of which feature a β-turn structure.

the occurrence of tight turns for both conformers, confirming our initial hypothesis regarding the effect of the steroid on the peptide topology. As shown in Table 2, distances between α carbon atoms of the *i* and $i+3$ residues (Val and Gly[5], respectively) in both conformers fit the definition of a β-turn, showing that the β-turn of conformer A is tighter than that of conformer B. Intriguingly, even the *s-trans*-configured conformer B occurs as a β-turn, which reveals that the folding-back effect imposed by the bulky steroid prevails over the configurational

Table 2. Dihedral backbone angles and distances between α carbon atoms of average structures for the two major conformers of peptido steroid **9**.

	Conformer A		Conformer B	
	φ [°]	ψ [°]	φ [°]	ψ [°]
residue $i+1$ (Ile)	−152	112	−154	68
residue $i+2$ (Gly[4])	−121	75	46	31
$d_{C^{\alpha}(i)\cdots C^{\alpha}(i+3)}$ [Å]	5.67		6.48	

effect of the *N*-substituted amide. Note that the backbone dihedral angles φ and ψ of the corner residues Ile and Gly[4] (Table 2) do not match those of any of the nine different types of β-turn found so far in native proteins.[30] Nevertheless, this is a reasonable result considering the completely novel architecture of this *N*-steroidal peptide, which distinct from those of any other types of known peptidomimetics. Certainly, this may be seen as a input for the design of new peptide ligands, lipopeptides, and peptidomimetics, since it provides access to a completely new conformational space, perhaps not available with the use of other topological templates.

Conclusion

We have re-engineered the role of the steroid skeleton as topological template in peptide secondary structures. For this purpose, a multicomponent approach based on the Ugi-4CR was applied to allow for one-pot conjugation of two peptides (or amino acid derivatives) to an aminosteroid, which led to a new class of peptidosteroids having the steroidal template linked to an internal amide bond. The conjugation efficiency of axial steroidal amines is lower than for equatorial ones, a behavior similar to that observed in conventional terminal conjugations by peptide coupling. A wide variety of *N*-steroidal peptides featuring diverse amino acid sequences and steroidal skeletons were produced, and eventually subjected to deprotection and head-to-tail cyclization to afford macrocyclic lipopeptides, that is, chimeric cyclopeptide–steroid conjugates, in good overall yields. An NMR/MD study on a model *N*-steroidal peptide proved that the internal *N*-steroidal substitution significantly increases the conformational rigidity of the peptide backbone while forcing it to adopt a β-turn secondary structure even with all-*s-trans* peptide bonds. To the best of our knowledge, this is the first evidence of the steroid nucleus as β-turn inducer in acyclic peptides. From both the synthetic and structural points of view, this simple strategy opens up a venue of opportunities for the utilization of the available steroid scaffold as template for the design of peptide ligands and protein epitope mimics based on tight turns (e.g., β-hairpins).

Experimental Section

Methods

^{1}H NMR and ^{13}C NMR spectra were recorded at 400/500 MHz for ^{1}H and 100/125 MHz for ^{13}C, in Bruker Avance spectrometers in $CDCl_3$

or CD_3OD solution at 300 K. Chemical shifts δ are reported in parts per million (ppm) relative to TMS by using the residual solvent signals as secondary standards. Coupling constants *J* are reported in Hertz. Splitting patterns that could not be easily interpreted are designated as multiplet (m) or broad singlet (brs). Carbon resonances were assigned by using information provided by DEPT spectra recorded with a phase angle of 135°. High-resolution ESI mass spectra were obtained from a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer with an external electrospray ion source. All reagents and solvents were used as received, with the exception of CH_2Cl_2, DMF, and *N*,*N*-diisopropylethylamine (DIPEA), which were dried by distillation from CaH_2 under argon prior to use as reaction solvents. Flash column chromatography was carried out with silica gel 60 (230–400 mesh) and analytical TLC was performed on silica-gel-coated aluminum sheets.

General procedure for the multicomponent conjugation of peptides to steroids

A suspension of the steroidal amine (0.5 mmol, 1 equiv) and paraformaldehyde (0.5 mmol, 1 equiv) in MeOH/CH_2Cl_2 (5 mL, 5:1 v/v) was stirred for 1 h at room temperature. The peptide acid (0.6 mmol, 1.2 equiv) and the isocyanide (0.6 mmol, 1.2 equiv) were then added and the reaction mixture was stirred at room temperature for a defined period of time. The volatile substances were concentrated under reduced pressure and the resulting crude product was dissolved in 50 mL of $CHCl_3$. The organic phase was washed sequentially with a saturated aqueous solution of citric acid (30 mL), aqueous 10% $NaHCO_3$ (30 mL), and brine (30 mL), and then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH) on silica to afford the corresponding *N*-steroidal peptide.

***N*-Steroidal peptide 3**: Androstanyl amine **2** (58 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **1** (168 mg, 0.3 mmol), and methyl isocyanoacetate (30 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography (CH_2Cl_2/MeOH 10:1) afforded conjugate **3** (109 mg, 57%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.91 (brs, 1H), 7.76 (brs, 1H), 7.45 (brs, 1H), 7.24–7.20 (m, 3H), 7.12–7.09 (m, 2H), 6.87 (brs, H), 5.32 (brs, 1H), 5.00 (m, 1H), 4.80 (m, 1H), 4.53 (m, 1H), 4.43 (m, 1H), 4.27 (m, 2H), 4.08 (d, 1H, *J* = 15.7 Hz), 4.04 (dd, 1H, *J* = 16.4/6.0 Hz), 3.77 (dd, 1H, *J* = 17.8/5.3 Hz), 3.70 (m, 1H), 3.67 (s, 3H), 3.07 (d, 1H, *J* = 10.5 Hz), 2.89 (dd, 1H, *J* = 13.3/7.9 Hz), 2.42 (dd, 1H, *J* = 19.2/8.7 Hz), 2.11–2.04 (m, 1H), 1.97–1.83 (m, 2H), 1.82–1.69 (m, 3H), 1.70–1.06 (m, 21H), 1.35 (s, 9H), 0.91–0.77 (m, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.4, 13.8, 18.9 (CH_3); 20.1, 21.7 (CH_2); 21.8, 22.0, 22.9, 23.0 (CH_3); 24.7, 24.8 (CH); 25.6 (CH_2); 28.2 (CH_3); 28.3, 30.4, 31.5, 32.7 (CH_2); 34.7 (C); 35.0 (CH_2); 35.1 (CH); 35.8, 38.5 (CH_2); 40.7 (CH); 40.9, 41.3, 41.6 (CH_2); 45.3 (CH); 47.7 (C); 47.8 (CH_2); 50.4, 51.3, 51.5, 52.0, 52.2 (CH); 54.8 (CH_3); 55.6 (CH); 80.4 (C); 127.0, 128.5, 129.2 (CH); 136.2 (C); 156.0, 169.5, 170.2, 171.7, 171.8, 173.3, 221.2 (CO). HRMS (ESI-FT-ICR): *m/z*: 985.5997 $[M+Na]^+$; calcd for $C_{53}H_{82}O_{10}NaN_6$: 985.5992.

***N*-Steroidal peptide 6**: Cholanyl amine **5** (78 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **4** (156 mg, 0.3 mmol), and methyl isocyanoacetate (30 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography (CH_2Cl_2/MeOH 10:1) afforded conjugate **6** (134 mg, 68%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.81 (brs, 1H), 7.35 (m, 2H), 6.91 (d, 1H, *J* = 6.8 Hz), 5.40 (brs, 1H), 4.95 (m, 1H), 4.67–4.63 (m, 1H), 4.52 (m, 1H), 4.17–4.02 (m, 2H), 3.87 (dd, 1H, *J* = 17.8/5.3 Hz), 3.70 (s, 3H), 3.67 (s, 3H), 2.38–2.33 (m, 2H), 2.25–2.20 (m, 2H), 2.08 (m, 2H), 1.95 (m, 2H), 1.87–1.77 (m, 3H), 1.74–1.45 (m, 13H), 1.43 (s, 9H), 1.41–1.29 (m, 13H), 1.25 (s, 3H), 1.12–1.02 (m, 6H), 0.97–0.85 (m, 23H), 0.65 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.4, 12.0, 15.6, 17.4, 18.2 (CH_3); 20.8 (CH_2); 23.4, 23.5 (CH_3); 24.1, 24.7, 26.9, 28.1 (CH_2); 28.3 (CH_3); 30.9, 31.0, 31.8, 31.9, 34.0 (CH_2); 34.7 (C); 35.0 (CH_2); 35.3 (CH); 35.6 (C); 35.8, 37.0 (CH); 39.9, 40.6 (CH_2); 40.9, 42.0, 42.6 (CH); 45.4, 46.2 (CH_2); 48.1, 48.8, 49.9 (CH); 51.2, 52.5 (CH_3); 55.8, 57.4, 59.4 (CH); 80.1 (C); 156.0, 169.2, 169.7, 170.8, 171.4, 171.5, 172.1, 174.7 (CO); HRMS (ESI-FT-ICR) *m/z*: 1009.6569 $[M+Na]^+$; calcd for $C_{53}H_{90}O_{11}NaN_6$: 1009.6560.

***N*-Steroidal peptide 9**: Cholestanyl amine **8** (78 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **7** (143 mg, 0.3 mmol), and methyl isocyanoacetate (30 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography (CH_2Cl_2/MeOH 10:1) afforded conjugate **9** (115 mg, 59%) as a white amorphous solid. A mixture of conformers was observed. The signals of the two major conformers are assigned. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28–7.18 (m, 5H), 7.04 (brs, 1H), 6.78, 6.62 (2×d, 1H, *J* = 8.0 Hz), 6.67, 6.54 (2×d, 1H, *J* = 7.8 Hz), 5.10, 5.02 (2 m, 1H), 4.91, 4.75 (m, 1H), 4.44–4.27 (m, 2H), 4.23–3.89 (m, 4H), 3.72, 3.70 (2 s, 3H), 3.15–3.01 (m, 2H), 2.15 (m 1H), 1.97–1.94 (m, 2H), 1.86–1.69 (m, 5H), 1.62–0.89 (m, 55H), 1.40, 1.39 (2 s, 9H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 13.9, 14.2, 14.5, 15.5, 17.5, 18.6, 19.3 (CH_3); 20.9 (CH_2); 22.5, 22.8 (CH_3); 23.5, 23.8, 24.1, 24.4, 25.0, 26.9 (CH_2); 28.0, 28.2 (CH_3); 28.5, 28.6 (CH_2); 29.7, 30.8 (CH); 31.5, 32.9 (CH_2); 34.4 (C); 35.0, 35.5, 35.8 (CH); 36.1, 37.7 (CH_2); 37.9, 38.0 (CH); 39.5, 39.9 (CH_2); 40.3, 40.5 (CH); 40.9, 41.0, 41.1 (CH_2); 42.2 (CH); 42.5 (C); 47.4, 47.6 (CH_2); 50.7, 50.8 (CH); 52.1, 52.3 (CH_3); 52.4, 53.5, 54.8, 55.0, 55.7, 55.8, 56.1, 56.2, 58.4 (CH); 80.3 (C); 126.9, 127.0, 128.6, 128.7, 129.2, 129.3 (CH); 136.5, 136.6 (C); 155.5 169.8, 170.2, 170.27, 170.9, 171.4, 171.6, 172.7, 173.1 (CO); HRMS (ESI-FT-ICR): *m/z*: 998.6902 $[M+Na]^+$; calcd for $C_{57}H_{93}O_8NaN_5$: 998.6916.

***N*-Steroidal peptide 12**: The Boc-protected spirostanyl amine **11a** (273 mg, 0.55 mmol) was dissolved in CH_2Cl_2 (7 mL) and treated with trifluoroacetic acid (TFA, 3 mL) at 0 °C. The reaction mixture was allowed to reach room temperature, stirred for 2 h, and then concentrated under reduced pressure. The resulting product was dissolved in CH_2Cl_2 (50 mL) and the solution was washed with saturated aqueous Na_2CO_3 (2×10 mL), dried over anhydrous Na_2SO_4 and concentrated to dryness. The resulting free spirostanyl amine **11b** (86 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **10** (167 mg, 0.3 mmol) and methyl isocyanoacetate (30 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography (CH_2Cl_2/MeOH 10:1) afforded conjugate **12** (220 mg, 67%) as a white amorphous solid. Mixture of conformers observed. The signals of the three major conformers are assigned. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 (m, 1H); 7.43–7.30 (m, 6H); 7.10 (d, 1H, *J* = 8.6 Hz); 7.09 (d, 1H, *J* = 8.6 Hz); 6.91 (d, 1H, *J* = 8.4 Hz); 6.89 (d, 1H, *J* = 8.6 Hz); 5.41 (d, 1H, *J* = 6.2 Hz); 5.04 (s, 2H); 4.96 (t, 1H, *J* = 6.9 Hz); 4.78 (m, 1H); 4.45 (m, 1H); 4.42 (m, 1H); 4.38–4.30 (m, 3H); 4.14 (m, 1H); 3.91 (m, 1H); 3.70 (s, 3H); 3.46 (m, 1H); 3.36 (t, 1H, *J* = 11.0 Hz); 3.03–2.91 (m, 2H); 2.05 (m, 1H); 1.40 (s, 9H); 1.30 (d, 3H, *J* = 7.2 Hz); 1.17 (s, 3H); 0.97 (d, 3H, *J* = 6.8 Hz); 0.92 (d, 3H, *J* = 6.4 Hz); 0.91 (d, 3H, *J* = 6.4 Hz); 0.87 (s, 3H); 0.78 (d, 3H, *J* = 6.0 Hz); 0.77 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1, 14.5, 16.3, 16.4, 17.1, 17.4, 18.0, 18.3, 18.6 (CH_3); 19.8, 20.9 (CH_2); 21.5, 21.6, 22.0 (CH_3); 22.6 (CH_2); 23.2, 23.3, 23.4, 24.4, 24.6 (CH_3); 24.8 (CH); 27.3 (CH_2); 28.2 (CH_3); 28.6, 28.9, 29.6

(CH_2); 30.2 (CH); 31.3, 31.5, 31.8, 33.0 (CH_2); 34.0, 34.5, 34.8 (C); 33.0, 35.2, 37.2, 37.9 (CH_2); 38.7 (C); 39.6, 40.8, 40.9, 41.0 (CH_2); 41.5, 43.0, 47.0, 47.7 (CH); 48.2 (CH_2); 48.5, 48.6, 48.8, (CH); 50.3, 50.9 (CH_2); 52.1, 52,15, 52.2 (CH_3); 52.5, 52.6, 55.1, 55.4, 55.7, 59.9, 62.0 (CH); 66.6, 69.8 (CH_2); 71.2 (CH); 80.2 (C); 80.4 (CH); 109.2 (C); 115.0, 127.4, 127.9 (CH); 128.3 128.4 (C); 128.5, 130.2, 130.3, 130.4 (CH); 136.8, 136.9, 137.0, 155.3, 155.4 (C); 157.7, 157.8, 157.9, 169.4, 169.7, 169.8, 170.0, 170.2, 170.6, 171.1, 171.3, 171.5, 172.0, 172.1, 172.4 (CO); HRMS (ESI-FT-ICR): *m/z*: 1120.6554 $[M+Na]^+$; calcd for $C_{62}H_{91}O_{12}N_5Na$: 1120.6556.

***N*-Steroidal peptide 16**: Azide **14a** (88 mg, 0.25 mmol) was dissolved in 3 mL of THF and PPh_3 (131 mg, 0.5 mmol) was added. The reaction mixture was stirred at room temperature for 8 h, treated with H_2O (180 μL, 10 mmol), and stirred for additional 48 h. The volatile substances were concentrated under reduced pressure and the crude product purified by flash column chromatography (CH_2Cl_2/Et_3N 10:0.1) to furnish bis-norcholanyl amine **14b** (74 mg, 91%), which was identified by ESI-MS and used without further characterization. Amine **14b** (65 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **13** (156 mg, 0.3 mmol), and isocyanodipeptide **15** (74 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography (CH_2Cl_2/MeOH 10:1) afforded conjugate **16** (166 mg, 75%) as a white amorphous solid. A mixture of conformers was observed. The signals of the major conformer are assigned. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (m, 1H), 7.30 (m, 5H), 7.18–7.04 (m, 6H), 7.06 (d, 1H, *J* = 10.2 Hz), 6.72 (br s, 1H), 6.24 (d, 1H, *J* = 10.0 Hz), 6.07 (s, 1H), 4.96 (br s, 1H), 4.75–4.43 (m, 2H), 4.40–4.10 (m, 2H), 4.04 (dd, 1H, *J* = 11.3/3.7 Hz), 3.96 (dd, 1H, *J* = 11.2/3.3 Hz), 3.68 (s, 3H), 3.57–3.55 (m, 1H), 3.14–3.10 (m, 2H), 2.98–2.93 (m, 2H), 2.46 (td, 1H, *J* = 13.1/3.9 Hz), 2.35 (d, 1H, *J* = 12.4 Hz), 2.04 (m, 1H), 194 (m, 2H), 1.80–1.57 (m, 9H), 1.54–1.13 (m, 17H), 1.39 (s, 9H), 1.10–0.92 (m, 6H), 0.84–0.75 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.1, 16.2, 16.5, 18.5, 18.6 (CH_3); 21.9 (CH); 22.8 (CH_2); 23.0 (CH_3); 24.4 (CH_2); 24.7, 28.2 (CH_3); 28.3, 29.7, 32.8, 33.4, 33.5 (CH_2); 35.2, 35.7 (CH); 39.1, 39.2, 40.8, 41.0 (CH_2); 43.0, 45.6 (C); 45.3, 52.0 (CH); 52.8 (CH_3); 53.4, 53.7, 54.4, 55.0 (CH); 62.1 (CH_2); 80.4 (C); 123.8, 126.6, 127.0, 127.4, 128.2, 128.6, 129.3 (CH); 136.2, 136.6 (C); 155.5 (CO); 155.9 (CH); 168.8, 169.3, 170.0, 170.5, 172.1, 172.3, 173.6, 186.4 (CO); HRMS (ESI-FT-ICR): *m/z*: 1128.6359 $[M+Na]^+$, calcd. for $C_{62}H_{87}O_{11}NaN_7$: 1128.6365.

***N*-Steroidal peptide 19**: Cholesteryl amine **17** (77 mg, 0.2 mmol), paraformaldehyde (6 mg, 0.2 mmol), peptide **7** (144 mg, 0.3 mmol), and isocyanopeptide **18** (108 mg, 0.3 mmol) were allowed to react in MeOH/CH_2Cl_2 (5 mL) for 24 h according to the general Ugi-4CR-based ligation procedure. Flash column chromatography purification (CH_2Cl_2/MeOH 8:1) afforded conjugate **19** (175 mg, 71%) as a white amorphous solid. A mixture of conformers was observed. The signals of the two major conformers are assigned. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.15 (br s, 1H), 7.88 (m, 1H), 7.69 (t, 1H, *J* = 5.5 Hz), 7.34–7.26 (m, 6H), 7.20–7.11 (m, 1H), 5.75 (m, 1H), 5.60 (br s, 1H), 5.47 (m, 1H), 5.09 (m, 1H), 5.04 (m, 2H), 4.75 (m, 1H), 4.58 (m, 2H), 4.38 (m, 1H), 4.09 (d, 1H, *J* = 11.3 Hz), 4.00 (m, 1H), 3.92 (m, 1H), 3.75, 3.74 (2 s, 3H), 3.67–3.60 (m, 5H), 3.10 (m, 2H), 3.00–2.85 (m, 2H), 2.00–1.95 (m, 2H), 1.79 (m, 2H), 1.64–1.53 (m, 3H), 1.48–1.25 (s, 16H), 1.14–0.98 (m, 3H), 0.91–0.64 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.5, 11.7, 15.1, 15.3, 18.2, 18.3, 18.5, 18.6, 19.9 (CH_3); 21.9 (CH_2); 22.5, 22.8 (CH_3); 23.4, 24.3, 25.1, 25.2, 25.9 (CH_2); 28.0 (CH); 28.3 (CH_3); 29.2, 29.3 (CH_2); 31.3 (CH); 31.6, 32.1, 33.2 (CH_2); 35.3, 35.7 (CH); 35.9 (C); 36.5, 37.8 (CH_2); 39.4 (CH); 40.3, 40.7, 40.9 (CH_2); 42.8 (C); 44.7, 45.6, 48.0 (CH_2); 48.2, 52.0, 52.2 (CH); 52.3 (CH_3); 55.2, 56.4, 57.3, 58.0, 58.9 (CH); 66.4 (CH_2); 79.7 (C); 121.1, 127.8, 128.0, 128.4, 129.3, 129.5 (CH); 136.7, 136.8, 143.9 (C); 156.7, 156.9, 169.4, 170.1, 170.9, 171.4, 171.7, 173.0 (CO); HRMS (ESI-FT-ICR) *m/z*: 1258.8087 $[M+Na]^+$, calcd. for $C_{71}H_{109}O_{11}NaN_7$: 1258.8082.

General cyclization procedure for cyclopeptide–steroid conjugates

The fully deprotected *N*-steroidal peptide (50 μmol, 1 equiv) was dissolved in DMF (50 mL) and treated with PyBOP (78 mg, 150 μmol, 3 equiv) and DIPEA (54 μL, 300 μmol, 6 equiv). The reaction mixture was stirred for 6 h at room temperature and then concentrated under vacuum. Water/acetonitrile (1:1, 20 mL) was added and the suspension sonicated and centrifuged. After removal of the supernatant, the resulting product was washed once by the same procedure, and then suspended in water/acetonitrile (1:1, 5 mL) and lyophilized. The resulting crude cyclopeptide–steroid conjugate was purified by flash column chromatography (CH_2Cl_2/EtOAc).

Cyclopeptide-steroid conjugate 22: *N*-Steroidal peptide **21** (as trifluoroacetate salt) was subjected to cyclization according to the general procedure. The crude product was purified by flash column chromatography (CH_2Cl_2/EtOAc 8:1) to furnish the cyclopeptide–steroid conjugate **22** (46 mg, 47%, based on amine **8**) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.44 (br s, 1H), 7.32 (m, 5H), 7.20–7.05 (m, 7H), 7.02 (br s, 1H), 5.36 (t, 1H, *J* = 5.6 Hz), 5.08 (m, 2H), 4.87 (m, 1H), 4.67 (d, 1H, *J* = 14.0 Hz), 4.52 (m, 2H), 4.43 (dd, 1H, *J* = 15.2/5.3 Hz), 4.22 (t, 1H, *J* = 6.1 Hz), 4.12 (d, 1H, *J* = 14.0 Hz), 4.04 (m, 1H), 3.80 (m, 1H), 3.24 (dd, 1H, *J* = 14.0/4.5 Hz), 3.17 (m, 2H), 2.95 (dd, 2H, *J* = 14.1/8.3 Hz), 2.27 (m, 1H), 0.89 (d, 3H, *J* = 6.5 Hz), 0.86 (d, 3H, *J* = 6.4 Hz), 0.85 (s, 3H), 0.85 (d, 3H, *J* = 6.4 Hz), 0.81 (d, 3H, *J* = 6.6 Hz), 0.75 (d, 3H, *J* = 6.4 Hz), 0.63 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.7, 12.0, 17.6, 18.6, 19.1 (CH_3); 20.7 (CH_2); 22.5, 22.8 (CH_3); 23.8, 24.0, 25.3 (CH_2); 27.9 (CH); 28.8, 29.4, 30.8, 31.5, 32.7, 33.1, 35.1 (CH_2); 35.2, 35.6, 35.7 (CH); 37.8 (C); 39.5 (CH_2); 39.6 (CH); 39.8, 39.9 (CH_2); 42.0 (C); 42.4, 43.2, 45.8 (CH_2); 47.1 (CH); 48.2 (CH_2); 53.7, 53.9, 54.1, 56.0, 56.1 (CH); 66.5 (CH_2); 126.6, 128.0, 128.3, 128.5, 129.0 (CH); 136.4, 138.8 (C); 156.9, 169.2, 170.3, 170.8, 170.9, 173.2 (CO); HRMS (ESI-FT-ICR): *m/z*: 1015.6620 $[M+Na]^+$; calcd for $C_{59}H_{88}O_7NaN_6$: 1015.6617.

Cyclopeptide–steroid conjugate 26: *N*-steroidal peptide **25** (as trifluoroacetate salt) was subjected to cyclization according to the general procedure. The crude product was purified by flash column chromatography (CH_2Cl_2/EtOAc 10:1) to furnish the cyclopeptide-steroid conjugate **26** (48 mg, 54% based on amine **5**) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.66 (d, 1H, *J* = 5.7 Hz), 7.15 (d, 1H, *J* = 7.2 Hz), 7.08 (d, 1H, *J* = 5.3 Hz), 6.97 (d, 1H, *J* = 5.9 Hz), 4.60 (t, 1H, *J* = 7.0 Hz), 4.53 (m, 1H), 4.46–4.41 (m, 2H), 4.37–4.23 (m, 1H), 3.94 (m, 1H), 4.14 (m, 1H), 4.11–4.06 (m, 1H), 4.00 (m, 1H), 3.81 (m, 2H), 3.63 (s, 3H), 2.35–2.28 (m, 2H), 2.22–2.14 (m, 2H), 1.96–1.90 (m, 2H), 1.82 (m, 4H), 1.52–0.80 (m, 66H), 0.61 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.4, 11.9, 15.5, 17.8, 18.2, 18.3, 19.4, 19.7, 19.9 (CH_3); 20.9 (CH_2); 21.8, 22.8, 23.4, 23.7 (CH_3); 24.1 (CH_2); 24.4 (CH); 24.5, 24.6, 26.0, 26.7 (CH_2); 28.1 (CH_2); 30.4, 30.7, 31.0 (CH_2); 34.9 (C); 35.3, 35.5, 37.2, 37.4, 37.6, 39.7 (CH); 40.0, 41.2 (CH_2); 41.9 (CH); 42.7 (C); 45.2, 45.4, 45.8, 48.8, 51.1 (CH); 51.4 (CH_3); 55.8, 56.4, 59.2 (CH); 167.8, 170.7, 171.3, 171.5, 171.9, 174.2, 174.7 (CO); HRMS (ESI-FT-ICR): *m/z*: 919.6251 $[M+Na]^+$; calcd for $C_{50}H_{84}O_8NaN_6$: 919.6249.

Cyclopeptide–steroid conjugate 29: *N*-steroidal peptide **28** was subjected to cyclization according to the general procedure. The crude product was purified by flash column chromatography (CH_2Cl_2/EtOAc 10:1) to furnish cyclopeptide–steroid conjugate **29**

(51 mg, 51%, based on amine **14b**) as a white amorphous solid. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.28–7.19 (m, 6H), 6.20 (dd, 1H, J = 10.1/1.8 Hz), 6.06 (s, 1H), 4.52 (d, 1H, J = 13.2 Hz), 4.51–4.40 (m, 4H), 4.40–4.36 (m, 1H), 4.37 (dd, 1H, J = 13.6/3.8 Hz), 4.34–4.30 (m, 1H), 4.26 (m, 1H), 4.14 (d, 1H, J = 7.3 Hz), 4.07 (dd, 1H, J = 13.6/8.9 Hz), 3.09 (dd, 1H, J = 13.9/4.7 Hz), 2.99 (m, 2H), 2.82 (dd, 1H, J = 13.9/9.5 Hz), 2.57 (td, 1H, J = 13.7/4.6 Hz), 2.40 (d, 1H, J = 12.5 Hz), 2.07–1.96 (m, 6H), 1.84–1.52 (m, 12H), 1.42 (s, 9H), 1.40–1.22 (m, 8H), 1.20–1.10 (m, 3H), 1.09–1.00 (m, 4H), 0.94–0.89 (m, 12H), 0.84 (s, 3H), 0.83 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ = 12.5, 17.4, 19.0, 19.8, 22.1, 23.5 (CH_3); 23.9, 24.2, 25.5 (CH_2); 25.5 (CH_2); 25.7 (CH); 28.7 (CH_3); 30.5 (CH_2); 31.8 (CH); 32.6, 33.9, 35.0, 35.8 (CH_2); 36.7 (CH); 38.9 (CH_2); 39.0 (CH); 40.6, 40.8, 41.1, 41.7, 43.2 (CH_2); 44.1, 45.4 (C); 49.0, 49.3, 53.6, 54.0, 54.8, 56.5 (CH); 56.9 (CH_2); 57.4, 60.4 (CH); 79.9 (C); 123.7, 125.0, 127.6, 129.4, 130.4 (CH); 138.5 (C); 157.8 (CH); 158.4 (CO); 169.7, 170.2, 172.5, 173.6, 174.1, 174.5, 174.8, 175.1, 188.7 (CO); HRMS (ESI-FT-ICR): m/z: 1034.6312 $[M+Na]^+$; calcd for $C_{57}H_{85}O_9NaN_7$: 1034.6306.

Cyclopeptide–steroid conjugate 32: *N*-Steroidal peptide **31** was subjected to cyclization according to the general procedure. The crude product was purified by flash column chromatography (CH_2Cl_2/EtOAc 10:1) to furnish cyclopeptide–steroid conjugate **32** (37 mg, 43%, based on amine **11b**) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.91 (d, 1H, J = 6.8 Hz), 7.71 (m, 1H), 7.51 (m, 1H), 7.42 (d, 1H, J = 7.6 Hz), 7.28–7.16 (m, 5H), 4.80 (m, 1H), 4.66 (m, 1H), 4.47–4.31 (m, 5H), 3.96 (dd, 1H, J = 16.6/5.8 Hz), 3.71 (m, 1H), 3.54 (m, 1H), 3.47 (m, 2H), 3.36 (t, 1H, J = 10.9 Hz), 3.14 (dd, 1H, J = 13.8/5.3 Hz), 2.90 (1H, J = 13.6/8.6 Hz), 2.78 (1H, J = 15.6/6.2 Hz), 2.56 (m, 1H), 2.42 (m, 1H), 2.22 (m, 1H), 1.30 (d, 3H, J = 7.1 Hz), 1.19 (s, 3H), 0.96 (d, 3H, J = 7.0 Hz), 0.90 (d, 3H, J = 6.2 Hz), 0.88 (d, 3H, J = 6.1 Hz), 0.78 (d, 3H, J = 6.8 Hz), 0.77 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): HRMS (ESI-FT-ICR): m/z: 882.5364 $[M+Na]^+$; calcd for $C_{49}H_{73}O_8N_5Na$: 882.5358.

Determination of the three-dimensional structure in solution

NMR spectra of *N*-steroidal peptide **9** were recorded on a Bruker 600 Avance III spectrometer in [D_6]DMSO at 300 K (15 mg/0.5 mL). NMR peaks were assigned by analysis of standard TOCSY (mixing time 60 ms), g-COSY, g-HSQC, HMBC, and Tr-ROESY (500 ms spin-lock) spectra. Cross-peaks in Tr-ROESY spectra were assigned and integrated in SparkyNMR.[32] For complete NMR assignment of both conformers A and B, see the Supporting Information. Distance constraints from ROE intensities were generated by using pseudo-atoms corrections where needed, and placed into three groups: strong (2.8 Å upper limit), medium (3.5 Å upper limit), and weak (5.0 Å upper limit). Backbone dihedral-angle restraints were inferred from $^3J_{NHCHa}$ coupling constants in the 1D spectrum at 300 K; ϕ was restrained to $-120\pm30^\circ$ for $^3J_{NHCHa} \geq 8$ Hz. Peptide bond angles ω were all set to *trans*, except for the *N*-steroidal Gly^4 amide bond. Structure calculations were carried out with the XPLOR-nih 2.33 package.[27] Starting structures were generated by using an ab initio simulated annealing protocol. Calculations were performed with the standard force field parameter set (PARALLHDG.PRO) and topology file (TOPALLHDG.PRO) in XPLOR-nih with in-house modifications to generate the cholestane substitution at the Gly^4 amide nitrogen atom and the *cis–trans* isomerization of the amide bond. Structures were refined by using the conjugate gradient Powell algorithm with 1000 cycles of energy minimization. Structures were visualized and analyzed with VMD-XPLOR 1.7.

Acknowledgements

We gratefully acknowledge financial support from the Land Sachsen-Anhalt, Germany (WZW project lipopeptides). We also thank the Nuclear Magnetic Resonance Service of Universidad de La Laguna, for allocating instrument time to this project.

Keywords: cyclization · macrocycles · multicomponent reactions · peptides · steroids

[1] a) D. B. Salunke, B. G. Hazra, V. S. Pore, *Curr. Med. Chem.* **2006**, *13*, 813–847; b) A. P. Davis, *Molecules* **2007**, *12*, 2106–2122; c) E. Virtanen, E. Kolehmainen, *Eur. J. Org. Chem.* **2004**, 3385–3399; d) A. Enhsen, W. Kramer, G. Wess, *Drug Discovery Today* **1998**, *3*, 409–418.

[2] a) W. Kramer, G. Wess, G. Schubert, M. Bickel, F. Girbig, U. Gutjahr, S. Kowalewski, K. H. Baringhaus, A. Enhsen, H. Glombik, *J. Biol. Chem.* **1992**, *267*, 18598–18604; b) W. Kramer, G. Wess, G. Neckermann, G. Schubert, J. Fink, F. Girbig, U. Gutjahr, S. Kowalewski, K. H. Baringhaus, G. Boger, A. Enhsen, E. Falk, M. Friedrich, H. Glombik, A. Hoffmann, C. Pittius, M. Urmann, *J. Biol. Chem.* **1994**, *269*, 10621–10627; c) P. W. Swaan, K. M. Hillgren, F. C. Szoka Jr., S. Øie, *Bioconjugate Chem.* **1997**, *8*, 520–525; d) S. Lee, K. Kim, T. S. Kumar, J. Lee, S. K. Kim, D. Y. Lee, Y. Lee, Y. Byun, *Bioconjugate Chem.* **2005**, *16*, 615–620.

[3] a) H. De Muynck, A. Madder, N. Farcy, P. J. De Clercq, M. N. Pérez-Payán, L. M. Öhberg, A. P. Davis, *Angew. Chem.* **2000**, *112*, 149–152; *Angew. Chem. Int. Ed.* **2000**, *39*, 145–148; b) A. Madder, L. Li, H. De Muynck, N. Farcy, D. Van Haver, F. Fant, G. Vanhoenacker, P. Sandra, A. P. Davis, P. J. De Clercq, *J. Comb. Chem.* **2002**, *4*, 552–562.

[4] a) Y. Cheng, T. Suenaga, W. C. Still, *J. Am. Chem. Soc.* **1996**, *118*, 1813–1814; b) R. Boyce, G. Li, H. P. Nestler, T. Suenaga, W. C. Still, *J. Am. Chem. Soc.* **1994**, *116*, 7955–7956.

[5] a) C. A. Bodé, T. Bechet, E. Prodhomme, K. Gheysen, P. Gregoir, J. C. Martins, C. P. Muller, A. Madder, *Org. Biomol. Chem.* **2009**, *7*, 3391–3399; b) S. Boonyarattanakalin, S. E. Martin, Q. Sun, B. R. Peterson, *J. Am. Chem. Soc.* **2006**, *128*, 11463–11470; c) S. E. Martin, B. R. Peterson, *Bioconjugate Chem.* **2003**, *14*, 67–74; d) H. Li, L.-X. Wang, *Org. Biomol. Chem.* **2003**, *1*, 3507–3513.

[6] B. Ding, U. Taotofa, T. Orsak, M. Chadwell, P. B. Savage, *Org. Lett.* **2004**, *6*, 3433–3436.

[7] a) A. Banerjee, E. Sergienko, S. Vasile, V. Gupta, K. Vouri, P. Wipf, *Org. Lett.* **2009**, *11*, 65–68.

[8] a) J. P. Schneider, J. W. Kelly, *Chem. Rev.* **1995**, *95*, 2169–2187; b) V. J. Hruby, *Nat. Rev. Drug Discovery* **2002**, *1*, 847–858; c) Y. Singh, G. T. Dolphin, J. Razkin, P. Dumy, *ChemBioChem* **2006**, *7*, 1298–1314.

[9] a) G. Wess, K. Bock, H. Kleine, M. Kurz, W. Guba, H. Hemmeric, E. Lopez-Calle, K.-H. Baringhaus, H. Glombic, A. Enhsen, W. Kramer, *Angew. Chem.* **1996**, *108*, 2363–2366; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2222–2224.

[10] a) D. Albert, M. Feigel, J. Benet-Buchholz, R. Boese, *Angew. Chem.* **1998**, *110*, 2855–2857; *Angew. Chem. Int. Ed.* **1998**, *37*, 2727–2729; b) D. Albert, M. Feigel, *Helv. Chim. Acta* **1997**, *80*, 2168–2181; c) M. Feigel, R. Ladberg, S. Engels, R. Herbst-Irmer, R. Fröhlich, *Angew. Chem.* **2006**, *118*, 5827–5831; *Angew. Chem. Int. Ed.* **2006**, *45*, 5698–5702.

[11] Y.-U. Kwon, T. Kodadek, *J. Am. Chem. Soc.* **2007**, *129*, 1508–1509.

[12] a) V. del Amo, L. Siracusa, T. Markidis, B. Baragaña, K. M. Bhattarai, M. Galobardes, G. Naredo, M. N. Pérez-Payán, A. P. Davis, *Org. Biomol. Chem.* **2004**, *2*, 3320–3328; b) D. G. Rivera, O. Concepción, K. Pérez-Labrada, F. Coll, *Tetrahedron* **2008**, *64*, 5298–5305.

[13] a) D. Verzele, S. Figaroli, A. Madder, *Molecules* **2011**, *16*, 10168–10186; b) S. Figaroli, A. Madder, *Tetrahedron* **2010**, *66*, 6912–6918; c) C. A. Bodé, C. P. Muller, A. Madder, *J. Pept. Sci.* **2007**, *13*, 702–708.

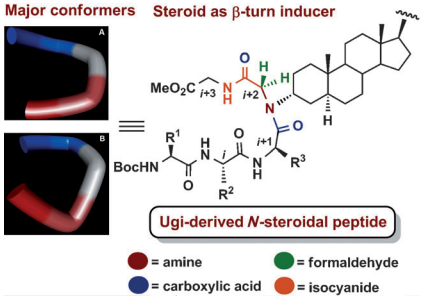
[14] a) R. Echemendía, O. Concepción, F. E. Morales, M. W. Paixão, D. G. Rivera, *Tetrahedron* **2014**, *70*, 3297–3305; b) P. M. Levine, K. Imberg, M. J. Garabedian, K. Kirshenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 6912–6915; c) N. V. Sokolova, G. V. Latyshev, N. V. Lukashev, V. G. Nenajdenko, *Org. Biomol. Chem.* **2011**, *9*, 4921–4926; d) J. M. Holub, H. Jang, K. Kirshenbaum, *Org. Biomol. Chem.* **2006**, *4*, 1497–1502.

[15] J. Chatterjee, C. Gilon, A. Hoffman, H. Kessler, *Acc. Chem. Res.* **2008**, *41*, 1331 – 1342, and references therein.
[16] a) A. Dömling, I. Ugi, *Angew. Chem.* **2000**, *112*, 3300 – 3344; *Angew. Chem. Int. Ed.* **2000**, *39*, 3168 – 3210; b) S. Marcaccini, T. Torroba, *Nat. Protoc.* **2007**, *2*, 632 – 639; c) I. Ugi, R. Meyr, U. Fetzer, C. Steinbrückner, *Angew. Chem.* **1959**, *71*, 386.
[17] a) D. G. Rivera, L. A. Wessjohann, *J. Am. Chem. Soc.* **2009**, *131*, 3721 – 3732; b) D. G. Rivera, O. Pando, R. Bosch, L. A. Wessjohann, *J. Org. Chem.* **2008**, *73*, 6229 – 6238; c) D. G. Rivera, L. A. Wessjohann, *Molecules* **2007**, *12*, 1890 – 1899; d) D. G. Rivera, L. A. Wessjohann, *J. Am. Chem. Soc.* **2006**, *128*, 7122 – 7123; e) L. A. Wessjohann, D. G. Rivera, F. Coll, *J. Org. Chem.* **2006**, *71*, 7521 – 7526; f) L. A. Wessjohann, B. Voigt, D. G. Rivera, *Angew. Chem.* **2005**, *117*, 4863 – 4868; *Angew. Chem. Int. Ed.* **2005**, *44*, 4785 – 4790.
[18] D. G. Rivera, F. León, O. Concepción, F. E. Morales, L. A. Wessjohann, *Chem. Eur. J.* **2013**, *19*, 6417 – 6428.
[19] D. G. Rivera, O. Pando, F. Coll, *Tetrahedron* **2006**, *62*, 8327 – 8334.
[20] O. Pando, S. Stark, A. Denkert, A. Porzel, R. Preusentanz, L. A. Wessjohann, *J. Am. Chem. Soc.* **2011**, *133*, 7692 – 7695.
[21] N. Sewald, H.-D. Jakubke, *Peptides: Chemistry and Biology*, Wiley-VCH, Weinheim, **2002**.
[22] a) A. V. Gulevich, A. G. Zhdanko, R. V. A. Orru, V. G. Nenajdenko, *Chem. Rev.* **2010**, *110*, 5235 – 5331; b) G. Zhao, C. Bughin, H. Bienaymé, J. Zhu, *Synlett* **2003**, *8*, 1153 – 1154.
[23] a) E. W. Sugandhi, C. Slebodnick, J. O. Falkinham III, R. D. Gandour, *Steroids* **2007**, *72*, 615 – 626; b) D. Albert, M. Feigel, *Tetrahedron Lett.* **1994**, *35*, 565 – 568; c) Q. Sun, S. Cai, B. R. Peterson, *Org. Lett.* **2009**, *11*, 567 – 570.
[24] R. M. Kohli, C. T. Walsh, *Chem. Commun.* **2003**, 297 – 307.
[25] For review on cyclic peptidomimetics derived from MCRs, see: a) G. Koopmanschap, E. Ruijter, R. V. A. Orru, *Beilstein J. Org. Chem.* **2014**, *10*, 544 – 598; b) L. A. Wessjohann, C. R. B. Rhoden, D. G. Rivera, O. E. Vercillo, *Top. Heterocycl. Chem.* **2010**, *23*, 199 – 226.
[26] a) Q. Sui, D. Borchardt, D. L. Rabenstein, *J. Am. Chem. Soc.* **2007**, *129*, 12042 – 12048; b) W. Brandt, T. Herberg, L. A. Wessjohann, *Biopolymers* **2011**, *96*, 651 – 668; c) J. A. Patch, K. Kirshenbaum, S. L. Seurynck, R. N. Zuckermann, A. E. Barron in *Pseudo-peptides in Drug Discovery* (Ed.: P. E. Nielsen), Wiley-VCH, Weinheim, **2004**, pp. 1 – 30.
[27] a) C. D. Schwieters, J. J. Kuszewski, N. Tjandra, G. M. Clore, *J. Magn. Reson.* **2003**, *160*, 65 – 73; b) C. D. Schwieters, J. J. Kuszewski, G. M. Clore, *Prog. Nucl. Magn. Reson. Spectrosc.* **2006**, *48*, 47 – 62.
[28] A. M. C. Marcelino, L. M. Gierasch, *Biopolymers* **2008**, *89*, 380 – 391.
[29] G. D. Rose, L. M. Gierasch, J. A. Smith, *Adv. Protein Chem.* **1985**, *37*, 1 – 109.
[30] K. C. Chou, *Anal. Biochem.* **2000**, *286*, 1 – 16.
[31] J. A. Robinson, *Acc. Chem. Res.* **2008**, *41*, 1278 – 1288.
[32] T. D. Goddard, D. G. Kneller, SPARKY 3, University of California, San Francisco.

Received: July 23, 2014
Published online on ■■ ■■, 0000

FULL PAPER

Size matters: When located as an internal *N*-substituent of a short peptide, the bulky steroid skeleton is capable of folding back a peptide chain to form a β-turn (see picture), even in the all-*s-trans* structure. Such unique *N*-steroidal peptides can be synthesized by a one-pot procedure based on the Ugi four-component reaction (Ugi-4CR) and subsequently cyclized to give cyclopeptide–steroid chimeras.

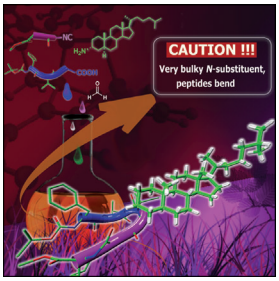


Peptides

D. G. Rivera, A. V. Vasco, R. Echemendía, O. Concepción, C. S. Pérez, J. A. Gavín, L. A. Wessjohann**

■■ – ■■

A Multicomponent Conjugation Strategy to Unique *N*-Steroidal Peptides: First Evidence of the Steroidal Nucleus as β-Turn Inducer in Acyclic Peptides



Size Matters

When located as internal *N*-substituent of a short peptide, the bulky steroid skeleton is capable of folding back a peptide chain leading to a β-turn, even in the all-*s-trans* structure. Such unique *N*-steroidal peptides can be synthesized in a one-pot procedure by means of a Ugi four-component reaction (Ugi-4CR) and subsequently cyclized to give cyclopeptide–steroid chimeras. For more details, see the Full Paper by D. G. Rivera, L. A. Wessjohann et al. on page ■■ ff.