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Synthesis of peptidomimetic-spirostane hybrids via Ugi reaction: a versatile approach for the formation of peptide–steroid conjugates

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Abstract—A general approach towards the preparation of peptide–steroid conjugates has been addressed. Utilizing the Ugi reaction, five peptidomimetic-steroid hybrids were achieved in good to excellent yields from carboxy- and amino-spirostanes and mono-protected α-amino acids. Diverse synthetic routes, specially focused on the formation of secosteroids, were implemented to introduce Ugi-type functionalities at different positions of the steroidal nucleus. This versatile approach is suitable for the formation of stable conjugates of steroids with other biologically relevant molecules.

1. Introduction

The conjugation of steroids to other chemically or biologically relevant molecules represents a valuable strategy to generate new properties in the resulting molecular hybrid. Similarly to the naturally occurring saponins,[1] many synthetic biomolecule-steroid conjugates have shown to possess physico–chemical and biological features arising from the junction of the two molecular entities. E.g., synthetic sugar–steroid conjugates have been synthesized to provide novel amphiphilic molecules capable to interact with phospholipid membranes.[2] Likewise, peptide–steroid conjugates have been employed as synthetic receptors of oligopeptide sequences,[3] as protease-like artificial enzymes,[4] and as mimics of the natural cationic peptide antibiotics.[5] Additionally, the attachment of detectable labels (e.g., fluorescent) to currently used steroidal hormones is an important approach in the development of clinical immunoassays. Indeed, this objective requires the production of novel reagents and methodologies toward improvements in the conjugation process.[6]

The formation of a chemically and metabolically stable linkage between a steroid and a biomolecule, a bioactive compound or a detectable tag is a crucial step for the potential applicability of the conjugate. By far the most commonly used chemical linkages in steroidal conjugates (i.e., glycosidic, amide, and ester) present an undeniable drawback: the sensitivity towards chemical or enzymatic hydrolysis. Another more stable and thereby widely exploited linkage, such as the oxime bond, is generally fixed to the presence of oxo-functions at the steroid.[6,7]

Herein we report on the use of the Ugi four-component reaction (Ugi-4CR) as a versatile approach towards the formation of peptide–steroid conjugates. The Ugi-4CR is the one-pot condensation of a primary amine, an oxo-component, a carboxylic acid, and an isocyanide to afford an *N*-substituted dipeptide backbone[8] (Scheme 1). This and other related scaffolds arising from the Ugi-4CR, or its variants, have found relevance in medicinal chemistry in the last decades.[9] Particularly interesting are oligomeric peptidomimetics containing *N*-substituted amide bonds (i.e., peptoids), which have shown a wide variety of biological applications[10] and a high resistance towards proteolytic degradation.[11]

The Ugi-4CR has been recently utilized to assemble very large cholane-based macrocycles tethered by highly diverse peptoid moieties.[12] However, a general conjugation process of biomolecules to steroids utilizing this approach has not been previously addressed. Apart from the accessible structural diversity arising from the possibility of utilizing carboxy, amino, isocyano, or oxo-steroids as building blocks, this strategy provides a very straightforward method to access stable peptide–steroid conjugates with potential biological activities.

Keywords: Steroids; Spirostanes; Steroid conjugates; Multicomponent reactions; Ugi reaction.
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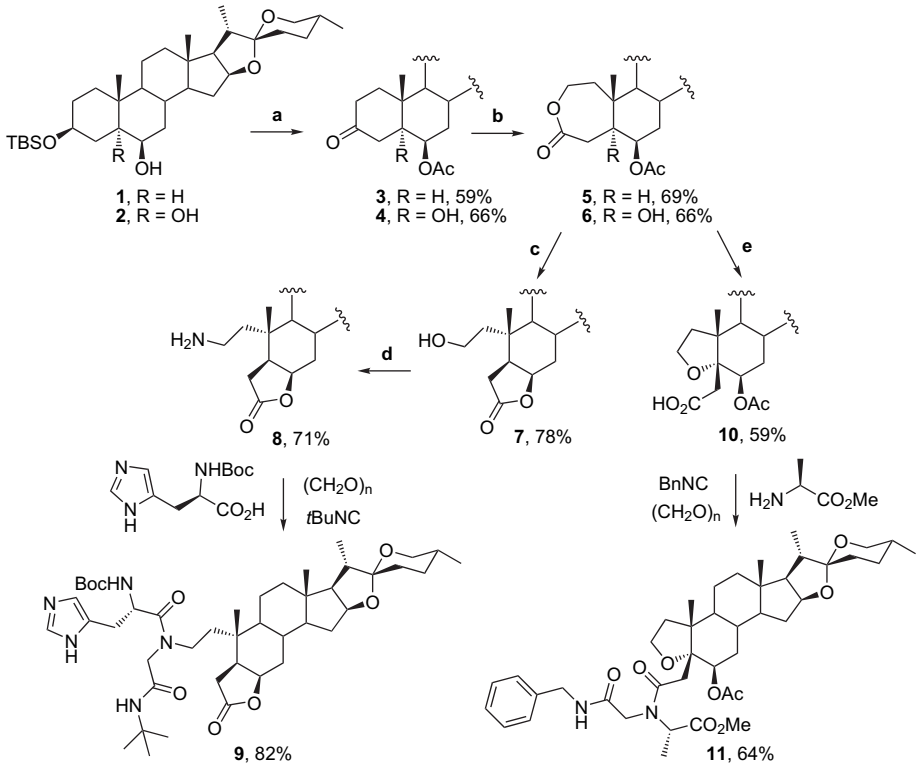


Scheme 1. Reagents and conditions: (a) i: Ac_2O, Py; ii: TBAF, THF; iii: PCC, CH_2Cl_2; (b) *m*CPBA/CH_2Cl_2; (c) from **5**, i: 5% KOH, MeOH, reflux; ii: Dowex 50W, set pH 3; (d) i: MsCl, CH_2Cl_2, Et_3N; ii: NaN_3, DMPU; iii: H_2, Lindlar catalyst; (e) from **6**; i: H_2SO_4, MeOH; ii: LiOH, THF/H_2O. TBAF=Tetrabutylammonium fluoride; DMPU=1,3-Dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidonone.

2. Results and discussion

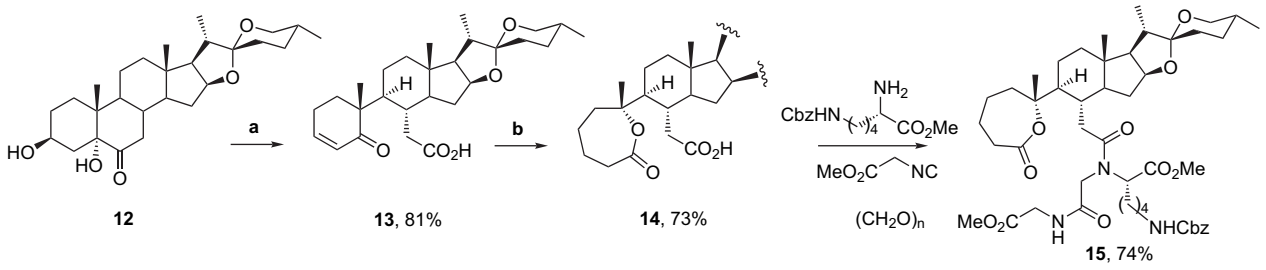
This article focuses on the conjugation process of interesting α-amino acids at different positions of the spirostane skeleton. However, we also aim to illustrate that this approach is suitable for introducing oligopeptides either by their C- or N-terminus at varied positions of the steroidal nucleus. Therefore, we concentrated on functionalizing steroids with carboxy and amino functions and taking advantage of the wide set of commercially available isocyanides (isonitriles) that can be employed to incorporate aromatic, aliphatic or other amino acid into the final hybrid compound. A key feature of these synthetic routes is the avoidance of the established succinate moiety as a source of carboxy groups. Instead, we concentrated on developing highly practical pathways that easily allow incorporating the above mentioned functional groups for the Ugi-4CR. These approaches are specially focused on secosteroid syntheses via lactone-ring opening and further activation of the Ugi-type functionality.

Spirostanes were chosen because of various features that make them amenable starting material to produce bioactive compounds. Derivatives of these sapogenins have exhibited a variety of biological activities depending on the functionalization pattern incorporated in their structures, e.g., ecdysteroid[13] and brassinosteroid[14] type activities have been reported upon introduction on rings A and B of functionalities typical of these natural products. Likewise, the general interest on these widely available steroids has been increased due to their unquestionable potential to access analogues[15] of cephalostatins[16] and ritterazines.[17]

Scheme 1 summarizes the synthesis of peptidomimetic-spirostane hybrids functionalized on ring A. Baeyer–Villiger oxidation of 3-oxosteroids **3** and **4** gave access to the corresponding lactones, which upon ring opening processes afforded varied secosteroids properly functionalized for the conjugation process. Thus, treatment of lactone **5** with basic conditions followed by acidification to pH 3 rendered the γ-lactone **7** in 78% yield after chromatography purification. The experimental condition were established to favor the kinetic product (5-exo-trig ring closing), thereby avoiding the regeneration of the ε-lactone. Further replacement of the primary hydroxyl group by an amino function allowed accomplishing the conjugation of the *N*-Boc-protected α-amino acid L-histidine in 82% yield by using *tert*-butylisocyanide. It must be mentioned that paraformaldehyde was always employed as the oxo-component to avoid the stereoisomers formation during the conjugation process.

The 5α-hydroxylated lactone **6** allowed an alternative pathway, in which an acid-catalyzed methanolysis led to dehydration at C-5 and subsequent nucleophilic attack of the primary hydroxyl at C-2 to yield a cyclic ether. Cleavage of the methyl ester function on the later intermediate furnished the final steroidal acid **10**. The *C*-protected α-amino acid L-alanine and benzylisocyanide were employed to achieve the formation of conjugate **11** in 64% yield. The lower isolated yield of this compound compared to **9** may be due to the less accessible character of the β-face directed carboxy function at C-3 because of typical steric effect.

As depicted in Scheme 2, the introduction on ring B of Ugi-type functionalities is accomplished by treatment of

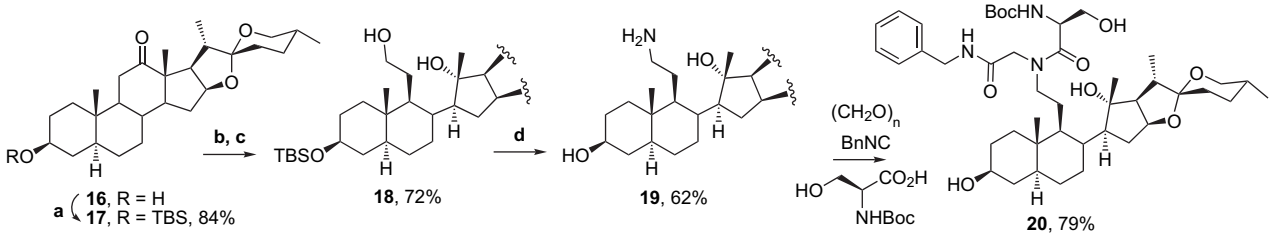


Scheme 2. Reagents and conditions: (a) i: Ac_2O, Py; ii: Jones, 60 °C; (b) i: H_2, Pd/C 10%; ii: *m*CPBA; CH_2Cl_2.

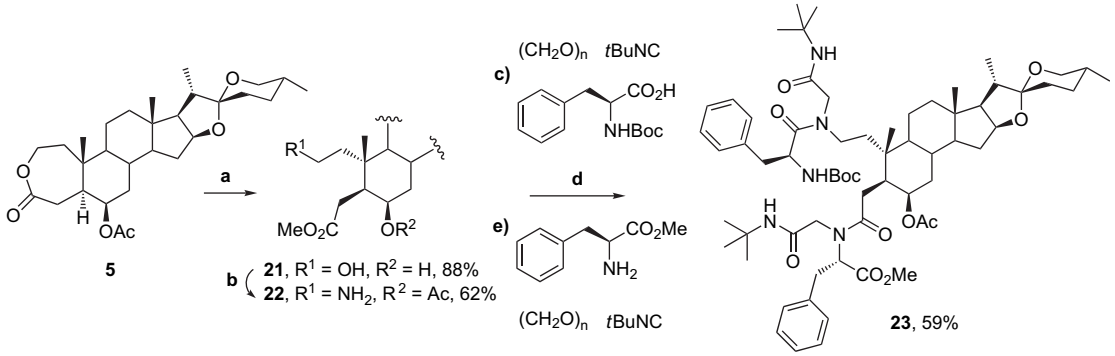
ketol **12** with strong oxidative conditions to allow C–C bond cleavage. Secospirostane **13** was then submitted to palladium-catalyzed hydrogenation and subsequent Baeyer–Villiger oxidation of the oxo-function at C-5 to afford the A-ring lactone **14** properly functionalized for the further Ugi-4CR. Thus, the carboxy functionality at C-6 was employed to achieve the conjugate **15** in 74% yield by using ε-*N*-Cbz-L-lysine methyl ester as the amino component and methyl isocyanoacetate. The use of the polyfunctional amino acid L-lysine and a functionalized isocyanide makes of the dipeptide–secospirostane hybrid **15** an amenable scaffold for further peptide coupling as well as for introducing other biologically relevant moieties. Indeed, this was possible due to the multicomponent nature of the Ugi-4CR, which easily allows the incorporation of multiple functionalized building blocks in a one-pot procedure.

To accomplish the formation of steroid conjugates functionalized on ring C, we turned to the use of hecogenin as starting material of the synthetic planning shown in Scheme 3. Protection of the hydroxyl at C-3 with *tert*-butyldimethylsilane (TBS) enabled further functionalizations on ring C without affecting the ring A. Once more, the Baeyer–Villiger oxidation of the oxo-function at C-12 allowed accessing Ugi-type functionalities via reductive ring opening with $LiAlH_4$ and subsequent replacement of the primary hydroxyl at C-12 by an amino group. Benzylisocyanide was then employed in the conjugation process of *N*-Boc-protected L-serine to the aminosteroid **19**, to afford the dipeptide–secospirostane hybrid **20** in 79% yield.

Having established the value of this strategy, we focused on extending it towards a double conjugation procedure of natural α-amino acids by either its amino or carboxy groups. In this sense, the *C*-protected secosteroidal amino acid **22** was produced by acid-catalyzed lactone-ring opening of intermediate **5** followed by incorporation of the required amino group at C-2 using a standard protocol. A sequential Ugi-4CR/deprotection/Ugi-4CR approach enabled the easy incorporation of two L-phenylalanine units to obtain the conjugate **23** in 61% yield (Scheme 4). Indeed, the use of both *C*- and *N*-protected α-amino acids as the carboxylic and amino components of the reaction sequence, respectively, confirms the versatile character of this methodology to produce highly functionalized conjugates with very low synthetic cost.



Scheme 3. Reagents and conditions: (a) TBSCl, imidazole, DMF; (b) *m*CPBA, CH_2Cl_2; (c) $LiAlH_4$, THF; (d) i: MsCl, CH_2Cl_2, Et_3N; ii: NaN_3, DMPU; iii: H_2, Lindlar catalyst; iv: TBAF, THF, TBS=*tert*-Butyldimethylsilane.



Scheme 4. Reagents and conditions: (a) i: H_2SO_4, MeOH, reflux; ii: NaOMe, MeOH; (b) i: MsCl, CH_2Cl_2, Et_3N; ii: NaN_3, DMPU; iii: Ac_2O, Py; iv: H_2, Lindlar catalyst; (d) LiOH, THF/H_2O.

3. Conclusions

As it has been demonstrated, various proteogenic amino acids could be conjugated to spirostanes via the Ugi-4CR to form peptidomimetic–steroid hybrids in yields ranging from 60% to 85%. Indeed, the high value of this synthetic approach lies on its potential applicability towards a general conjugation strategy of varied types of molecules with steroids. The multicomponent character of the reaction allowed utilizing the steroid and the α-amino acids either as the amino or the carboxylic component.

4. Experimental

4.1. General

Melting points were determined on a Stuart Scientific apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker ACF-250 spectrometer at 250.13 MHz and 62.9 MHz for ^{1}H and ^{13}C, respectively, using TMS as an internal standard. The high resolution ESI mass spectra were obtained from a Bruker Apex 70e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Infinity™ cell, a 7.0 tesla superconducting magnet. Reactions were monitored by thin-layer chromatography on precoated plates with silica gel (Merck) and spots were visualized with a 1% w/v spray of vanillin in perchloric acid and subsequent heating. 'Usual work-up' refers to dilution with an organic solvent, washing the extract consecutively with 5% HCl and/or 5% $NaHCO_3$, and brine, drying over anhydrous Na_2SO_4 and removal of the solvent under reduced pressure. The solid compounds were recrystallized from selected solvents for the melting point measurements. Flash column chromatography was performed on silica gel 60 (Merck, >230 mesh). Solvents were purified and dried according to standard procedures. Compounds **1**, **2**, and **12** were obtained from the widely available diosgenin as described in Ref. 13.

4.1.1. (25*R*)-6β-Acetoxy-5α-spirostan-3-one (3). Ac_2O (6 mL, 64 mmol) was added to a solution of compound **1** (5.8 g, 10.2 mmol) in anhydrous pyridine (60 mL) and the reaction mixture was stirred at room temperature for 18 h. The mixture was poured into 400 mL of cold water and the solid was filtered under reduced pressure and washed several times with water. The resulting crude product was dried and dissolved in anhydrous THF (60 mL). Tetrabutylammonium fluoride (TBAF) (4 mL, 1 M in THF) was added to the solution and the reaction mixture was stirred under nitrogen atmosphere for 6 h. The usual work-up (Et_2O) yielded a crude product, which was dissolved in CH_2Cl_2 (150 mL) and added to a suspension of pyridinium chlorochromate (PCC) (3.8 g, 17.6 mmol) in CH_2Cl_2 (200 mL) at 0 °C. The reaction mixture was stirred for 1 h at room temperature and then filtered through a pad of alumina. The solution was evaporated under reduced pressure and the crude product was purified by flash column chromatography (hexane/AcOEt, 4:1) to afford the ketone **3** (2.72 g, 59%). Mp (MeOH): 204–206 °C. ^{1}H NMR ($CDCl_3$): δ=0.79 (d, 3H, J=6.4 Hz, H-21); 0.81 (s, 3H, H-18); 0.96 (d, 3H, J=6.3 Hz, H-27); 1.35 (s, 3H, H-19); 2.05 (s, 3H, CH_3CO); 3.36 (t, 1H, J=10.8 Hz, H-26ax); 3.45 (dd, 1H, J=4.1/10.8 Hz, H-26eq); 4.40 (m, 1H, H-16α); 4.96 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=37.0 (C-1); 37.9 (C-2); 211.3 (C-3); 44.2 (C-4); 49.1 (C-5); 72.6 (C-6); 36.3 (C-7); 30.4 (C-8); 53.8 (C-9); 35.4 (C-10); 20.9 (C-11); 39.6 (C-12); 40.5 (C-13); 55.5 (C-14); 31.4 (C-15); 80.6 (C-16); 62.0 (C-17); 16.5 (C-18); 15.2 (C-19); 41.6 (C-20); 14.5 (C-21); 109.3 (C-22); 31.3 (C-23); 28.7 (C-24); 30.2 (C-25); 66.8 (C-26); 17.1 (C-27); 21.4 (*C*H$_3$CO); 170.2 (CH$_3$*C*O). HRMS (ESI-FT-ICR) *m*/*z*: 495.3084 [M+Na]$^+$ (Calculated for $C_{29}H_{44}NaO_5$: 495.3087).

4.1.2. (25*R*)-6β-Acetoxy-5-hydroxy-5α-spirostan-3-one (4). Diol **2** (3.0 g, 7.16 mmol) was treated in a similar way as described in Section 4.1.1 to give the ketol **4** (1.53 g, 66 %). Mp (MeOH): 212–213 °C. ^{1}H NMR ($CDCl_3$): δ=0.79 (d, 3H, J=6.2 Hz, H-21); 0.82 (s, 3H, H-18); 0.97 (d, 3H, J=6.2 Hz, H-27); 1.39 (s, 3H, H-19); 2.09 (s, 3H, CH_3CO); 3.37 (t, 1H, J=10.6 Hz, H-26ax); 3.45 (dd, 1H, J=4.1/10.8 Hz, H-26eq); 4.41 (m, 1H, H-16α); 4.73 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=31.6 (C-1); 37.7 (C-2); 211 (C-3); 48.4 (C-4); 76.9 (C-5); 75.6 (C-6); 33.6 (C-7); 30.1 (C-8); 45.2 (C-9); 38.9 (C-10); 20.9 (C-11); 39.8 (C-12); 40.5 (C-13); 55.4 (C-14); 31.4 (C-15); 80.6 (C-16); 62.0 (C-17); 16.5 (C-18); 15.9 (C-19); 41.6 (C-20); 14.5 (C-21); 109.2 (C-22); 31.3 (C-23); 28.7 (C-24); 30.2 (C-25); 66.8 (C-26); 17.1 (C-27); 21.4 (*C*H$_3$CO); 170.0 (CH$_3$*C*O). HRMS (ESI-FT-ICR) *m*/*z*: 511.3038 [M+Na]$^+$ (Calculated for $C_{29}H_{44}NaO_6$: 511.3036).

4.1.3. (25*R*)-A-Homo-2a-oxa-6β-acetoxy-5α-spirostan-3-one (5). A solution of *m*CPBA (2.0 g, 11.6 mmol) in CH_2Cl_2 (100 mL) was added to a stirred solution of ketone **3** (2.2 g, 4.6 mmol) in CH_2Cl_2 (100 mL) at 0 °C. The reaction was allowed to reach room temperature and stirred for 4 h in the dark. The mixture was diluted with $CHCl_3$ (150 mL) and washed sequentially with solutions of 10% Na_2SO_3 (2×200 mL) and 10% $NaHCO_3$ (2×200 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting crude product was purified by flash chromatography (hexane/EtOAc, 5:1) to yield the lactone **5** (1.57 g, 69%). Mp (EtOH): 202–205 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (d, 3H, J=6.3 Hz, H-27); 0.79 (s, 3H, H-18); 0.97 (d, 3H, J=6.6 Hz, H-21); 1.26 (s, 3H, H-19); 2.03 (s, 3H, CH_3CO); 3.36 (t, 1H, J=10.8 Hz, H-26ax); 3.47 (dd, 1H, J=4.2/10.9 Hz, H-26eq); 4.21 (m, 1H, H-2α); 4.39 (m, 1H, H-2β); 4.41 (m, 1H, H-16α); 4.94 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=41.0 (C-1); 63.9 (C-2); 175.0 (C-3); 29.0 (C-4); 52.1 (C-5); 72.1 (C-6); 36.5 (C-7); 30.0 (C-8); 53.3 (C-9); 35.7 (C-10); 21.0 (C-11); 39.6 (C-12); 40.3 (C-13); 55.4 (C-14); 31.5 (C-15); 80.7 (C-16); 62.1 (C-17); 16.5 (C-18); 15.1 (C-19); 41.6 (C-20); 14.5 (C-21); 109.3 (C-22); 31.3 (C-23); 28.7 (C-24); 30.2 (C-25); 66.8 (C-26); 17.2 (C-27); 170.1 (CH$_3$*C*O). HRMS (ESI-FT-ICR) *m*/*z*: 511.3039 [M+Na]$^+$ (Calculated for $C_{29}H_{44}NaO_6$: 511.3036).

4.1.4. (25*R*)-A-Homo-2a-oxa-6β-acetoxy-5-hydroxy-5α-spirostan-3-one (6). Ketol **4** (1.5 g, 3.1 mmol) was treated in a similar way as described in Section 4.1.3 to give the lactone **6** (1.02 g, 66%). Mp (MeOH): 218–220 °C. ^{1}H NMR ($CDCl_3$): δ=0.78 (d, 3H, J=6.3 Hz, H-27); 0.79 (s, 3H, H-18); 0.97 (d, 3H, J=6.4 Hz, H-21); 1.25 (s, 3H, H-19); 2.08 (s, 3H, CH_3CO); 3.37 (t, 1H, J=10.9 Hz, H-26ax);

3.47 (dd, 1H, *J*=4.2/10.9 Hz, H-26eq); 4.19 (m, 1H, H-2α); 4.38 (m, 1H, H-2β); 4.40 (m, 1H, H-16α); 4.93 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=36.2 (C-1); 65.1 (C-2); 169.8 (C-3); 42.9 (C-4); 76.6 (C-5); 72.1 (C-6); 31.7 (C-7); 29.7 (C-8); 44.7 (C-9); 41.5 (C-10); 20.9 (C-11); 39.7 (C-12); 40.3 (C-13); 55.4 (C-14); 31.5 (C-15); 80.7 (C-16); 61.9 (C-17); 16.5 (C-18); 15.8 (C-19); 41.6 (C-20); 14.5 (C-21); 109.3 (C-22); 31.3 (C-23); 28.7 (C-24); 30.2 (C-25); 66.8 (C-26); 17.1(C-27); 170.0 (CH_3CO). HRMS (ESI-FT-ICR) *m/z*: 527.2987 $[M+Na]^+$ (Calculated for $C_{29}H_{44}NaO_7$: 527.2985).

4.1.5. (25*R*)-2,3-Seco-2-hydroxy-3-carboxy-5α-spirostan-6β-yl (7). Lactone **5** (1.5 g, 3.1 mmol) was dissolved in a 2% solution of KOH in MeOH (70 mL). The reaction mixture was refluxed for 3 h, then acidified with resin Dowex 50W (H^+ form) until pH 3 and stirred at 0 °C for 2 h. The resin was then filtered off and the filtrate was evaporated under reduced pressure to give a white crude product. Recrystallization from AcOEt afforded the pure γ-lactone **7** (1.17 g, 78%). Mp (AcOEt): 202–203 °C. ^{1}H NMR ($CDCl_3$): δ=0.75 (s, 3H, H-18); 0.77 (d, 3H, *J*=6.3 Hz, H-27); 0.91 (s, 3H, H-19); 0.94 (d, 3H, *J*=6.8 Hz, H-21); 3.35 (t, 1H, *J*=11.0 Hz, H-26ax); 3.46 (dd, 1H, *J*=4.1/10.9 Hz, H-26eq); 3.65 (m, 2H, H-2); 4.39 (m, 1H, H-16); 4.53 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=35.0 (C-1); 58.4 (C-2); 178.1 (C-3); 42.2 (C-4); 46.2 (C-5); 79.9 (C-6); 33.1 (C-7); 30.3 (C-8); 41.3 (C-9); 36.8 (C-10); 20.9 (C-11); 39.6 (C-12); 40.2 (C-13); 56.5(C-14); 31.6 (C-15); 80.6 (C-16); 62.0 (C-17); 16.3 (C-18); 17.4 (C-19); 41.7 (C-20); 14.5 (C-21); 109.4 (C-22); 31.4 (C-23); 28.8 (C-24); 29.0 (C-25); 66.9 (C-26); 17.1 (C-27). HRMS (ESI-FT-ICR) *m/z*: 469.2932 $[M+Na]^+$ (Calculated for $C_{27}H_{42}NaO_5$: 469.2930).

4.1.6. (25*R*)-2,3-Seco-2-amino-3-carboxy-5α-spirostan-6β-yl (8). Mesyl chloride (0.44 mL, 3.7 mmol) was added dropwise at 0 °C to a solution of lactone **7** (1.1 g, 2.4 mmol) in anhydrous CH_2Cl_2 (80 mL) and Et_3N (3.8 mL, 30 mmol). The reaction mixture was stirred at 0 °C for 1 h and then washed with brine (2×100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduce pressure. The resulting crude product was dissolved in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidonone (DMPU, 30 mL) and the solution was treated with NaN_3 (403 mg, 7.2 mmol). The reaction mixture was stirred vigorously under an argon atmosphere at 50 °C for 48 h and then, the usual work-up (EtOAc) yielded a crude product, which was dissolved in 150 mL of absolute EtOH. Lindlar catalyst (350 mg) was added to the solution and the mixture was treated successively with hydrogen and vacuum, and finally stirred under hydrogen atmosphere for 36 h. The catalyst was removed by filtration and the resulting solution was evaporated under reduced pressure to give a crude product. Flash column chromatography purification ($CHCl_3$/MeOH/Et_3N, 10:1:0.5) furnished the amine **8** (758 mg, 71%). Mp (MeOH): 195–197 °C. ^{1}H NMR ($CDCl_3$): δ=0.76 (s, 3H, H-18); 0.77 (d, 3H, *J*=6.4 Hz, H-27); 0.90 (s, 3H, H-19); 0.94 (d, 3H, *J*=6.8 Hz, H-21); 3.36 (t, 1H, *J*=11.1 Hz, H-26ax); 3.46 (dd, 1H, *J*=4.2/11.0 Hz, H-26eq); 3.54 (m, 2H, H-2); 4.41 (m, 1H, H-16); 4.53 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=35.1 (C-1); 57.9 (C-2); 178.0 (C-3); 42.3 (C-4); 46.2 (C-5); 79.8 (C-6); 33.2 (C-7); 30.3 (C-8); 41.4 (C-9); 36.8 (C-10); 20.9 (C-11); 39.8 (C-12); 40.2 (C-13); 56.6 (C-14); 31.6 (C-15); 80.6 (C-16); 62.0 (C-17); 16.3 (C-18); 17.2 (C-19); 41.7 (C-20); 14.5 (C-21); 109.4 (C-22); 31.4 (C-23); 28.8 (C-24); 29.1 (C-25); 66.9 (C-26); 17.1 (C-27). HRMS (ESI-FT-ICR) *m/z*: 446.3272 $[M+H]^+$ (Calculated for $C_{27}H_{44}NO_4$: 446.3270).

4.1.7. Peptide-steroid conjugate 9. A solution of amine **8** (700 mg, 1.6 mmol) and paraformaldehyde (48 mg, 1.6 mmol) in MeOH (60 mL) were stirred at room temperature for 1 h to accomplish the formation of the corresponding imine. *N*-Boc-L-histidine (402 mg, 1.6 mmol) and *tert*-butylisocyanide (0.18 mL, 1.6 mmol) were then added and the reaction mixture was stirred for 6 h at room temperature. The solution was concentrated under reduced pressure and then, the usual work-up ($CHCl_3$) yielded a crude product. Flash column chromatography purification ($CHCl_3$/MeOH, 20:1) furnished the conjugate **9** (1.01 g, 82%). Mp (AcOEt): 226–227 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (s, 3H, H-18); 0.77 (d, 3H, *J*=6.5 Hz, H-27); 0.94 (s, 3H, H-19); 0.93 (d, 3H, *J*=6.8 Hz, H-21); 1.32 (s, 9H, $(CH_3)_3CNH$); 1.43 (s, 9H, $(CH_3)_3C$); 3.36 (t, 1H, *J*=11.0 Hz, H-26ax); 3.46 (dd, 1H, *J*=4.1/11.0 Hz, H-26eq); 3.57 (m, 2H, H-2); 4.40 (m, 1H, H-16); 3.72 (s, 3H, OCH_3); 4.02–3.94 (m, 6H, CH_2); 4.25–4.18 (m, 2H, CH_2); 4.45 (m, 1H, NC*H*); 4.54 (m, 1H, H-6α); 6.74 (m, 1H, C*H*-imidazole); 7.32 (m, 1H, C*H*-imidazole). ^{13}C NMR ($CDCl_3$): δ=14.5 (CH_3); 16.3 (CH_3); 17.1 (CH_3); 17.2 (CH_3); 20.9 (CH_2); 28.8 (CH_2); 28.5 (CH_3); 28.3 (CH_3); 29.1 (*C*H); 30.3 (*C*H); 31.4 (CH_2); 31.6 (CH_2); 33.2 (CH_2); 35.1 (CH_2); 36.8 (*C*); 39.8 (CH_2); 40.2 (*C*); 41.4 (*C*H); 41.7 (*C*H); 42.3 (CH_2); 45.6 (CH_2); 46.2 (*C*H); 49.6 (CH_2); 50.6 (CH_2); 51.8 (CH_3); 56.6 (*C*H); 57.9 (CH_2); 62.0 (*C*H); 66.9 (CH_2); 79.6 (*C*); 79.8 (*C*H); 80.6 (*C*H); 109.4 (*C*); 155.7 (*C*O); 168.5 (*C*O); 169.6 (*C*O); 178.0 (*C*O). HRMS (ESI-FT-ICR) *m/z*: 818.5044 $[M+Na]^+$ (Calculated for $C_{44}H_{69}NaN_5O_8$: 818.5045).

4.1.8. (25*R*)-2,3-Seco-6β-acetoxy-2(5)-oxa-5α-spirostan-3-oic acid (10). Fuming H_2SO_4 (2 mL) was added to a solution of lactone **6** (1.0 g, 1.9 mmol) in MeOH (50 mL) and the reaction mixture was stirred at reflux with the appearance of a precipitate after 10 min. The solid was then filtered under reduced pressure, washed with cold MeOH (2×20 mL) and dissolved in a mixture of THF/H_2O (2:1, 300 mL). LiOH (210 mg, 5.0 mmol) was added and the reaction mixture was stirred at 0 °C for 2 h and then acidified with aqueous 10% $NaHSO_4$ to pH 3. The usual work-up (AcOEt) gave a product, which was purified by flash column chromatography ($CHCl_3$/MeOH/AcOH, 15:1:0.1) to afford the acid **10** (583 mg, 59%). Mp (heptane/AcOEt): 255–256 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (d, 3H, *J*=6.8 Hz, H-21); 0.77 (s, 3H, H-18); 0.95 (d, 3H, *J*=6.9 Hz, H-27); 0.97 (s, 3H, H-19); 2.05 (s, 3H, CH_3CO); 2.41 (d, 1H, *J*=13.1 Hz, H-4); 2.63 (d, 1H, *J*=13.1 Hz, H-4); 3.35 (t, 1H, *J*=10.9 Hz, H-26ax); 3.44 (dd, 1H, *J*=4.1/10.8 Hz, H-26eq); 3.90 (m, 2H, H-2); 4.36 (m, 1H, H-16α); 5.21 (m, 1H, H-6α). ^{13}C NMR ($CDCl_3$): δ=37.9 (C-1); 63.9 (C-2); 169.3 (C-3); 36.4 (C-4); 83.8 (C-5); 70.5 (C-6); 30.8 (C-7); 29.3 (C-8); 45.7 (C-9); 46.6 (C-10); 22.7 (C-11); 40.6 (C-12); 39.8 (C-13); 56.7 (C-14); 31.6 (C-15); 80.7 (C-16); 62.2 (C-17); 16.4 (C-18); 15.1 (C-19); 41.6 (C-20); 14.5 (C-21); 109.4 (C-22); 31.5 (C-23); 28.6 (C-24); 30.4

(C-25); 66.7 (C-26); 17.2 (C-27); 169.7 (CH_3CO). HRMS (ESI-FT-ICR) *m/z*: 503.3009 $[M-H]^-$ (Calculated for $C_{29}H_{43}O_7$: 503.3008).

4.1.9. Peptide-steroid conjugate 11. A solution of L-alanine methyl ester hydrochloride (139 mg, 1.0 mmol), paraformaldehyde (30 mg, 1.0 mmol), and triethylamine (0.14 mL, 1.0 mmol) in MeOH (60 mL) were stirred at room temperature for 1 h to accomplish the formation of the corresponding imine. Acid **10** (500 mg, 1.0 mmol) and benzylisocyanide (0.12 mL, 1.0 mmol) were then added and the reaction mixture was stirred for 10 h at room temperature. The solution was concentrated under reduced pressure and then, the usual work-up ($CHCl_3$) yielded a crude product. Flash column chromatography purification ($CHCl_3$/MeOH, 20:1) furnished the pure conjugate **11** (471 mg, 64%). Mp (MeOH): 211–214 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (d, 3H, *J*=6.8 Hz, H-21); 0.77 (s, 3H, H-18); 0.94 (d, 3H, *J*=6.7 Hz, H-27); 1.14 (s, 3H, H-19); 1.53 (d, 3H, *J*=7.2 Hz, (CH_3)CHN); 2.03 (s, 3H, CH_3CO); 3.35 (t, 1H, *J*=10.8 Hz, H-26ax); 3.44 (dd, 1H, *J*=4.1/10.9 Hz, H-26eq); 3.83 (s, 3H, OCH_3); 3.92 (m, 2H, H-2); 4.15–4.08 (m, 2H, NC*H*); 4.29–4.26 (m, 2H, CH_2); 4.39 (m, 1H, H-16α); 4.50–4.56 (m, 2H, CH_2); 5.23 (m, 1H, H-6α); 7.13 (m, 5H, Ph). ^{13}C NMR ($CDCl_3$): δ=14.5 (*C*H_3); 15.1 (*C*H_3); 16.4 (*C*H_3); 17.2 (*C*H_3); 22.7 (*C*H_2); 28.6 (*C*H_2); 29.3 (*C*H); 30.4 (*C*H); 30.6 (*C*H_3); 30.8 (*C*H_2); 31.5 (*C*H_2); 31.6 (*C*H_2); 36.4 (*C*H_2); 37.9 (*C*H_2); 39.8 (*C*); 40.6 (*C*H_2); 41.6 (*C*H); 44.8 (*C*H_2); 45.7 (*C*H); 46.6 (*C*); 53.7 (*C*H_3); 55.7 (*C*H_2); 56.7 (*C*H); 62.2 (*C*H); 63.9 (*C*H_2); 66.7 (*C*H_2); 70.5 (*C*H); 80.7 (*C*H); 83.8 (*C*); 109.4 (*C*); 126.8 (*C*H); 127.3 (*C*H); 128.1 (*C*H); 131.2 (*C*H); 168.9 (*C*O); 169.6 (*C*O); 169.9 (*C*O); 174.2 (*C*O). HRMS (ESI-FT-ICR) *m/z*: 759.4199 $[M+Na]^+$ (Calculated for $C_{42}H_{60}NaN_2O_9$: 759.4197).

4.1.10. (25*R*)-5,6-Seco-5-oxo-3-spirostan-6-oic acid (13). Ketol **12** (2.5 g, 5.6 mmol) was dissolved in anhydrous pyridine (50 mL) and treated with Ac_2O (3 mL, 32 mmol) in a similar way as described in Section 4.1.1. The resulting crude product was dissolved in acetone (100 mL) and treated with 5 mL of Jones reagent. The reaction mixture was stirred at reflux for 2 h, concentrated until half volume and poured into 400 mL of cold water. The solid was filtered under reduced pressure, washed several times with water and then recrystallized from MeOH/H_2O (60 mL, 2:1) to afford the pure ketoacid **13** (2.03 g, 81%). Mp (MeOH/H_2O): 182–185 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (d, 3H, *J*=6.4 Hz, H-27); 0.80 (s, 3H, H-18); 0.95 (d, 3H, *J*=6.7 Hz, H-21); 1.11 (s, 3H, H-19); 3.33 (t, 1H, *J*=10.8 Hz, H-26ax); 3.46 (dd, 1H, *J*=10.8/4.0 Hz, H-26eq); 4.34 (m, 1H, H-16α); 5.84 (m, 1H, *J*=10.0/1.4 Hz, H-4); 6.75 (m, 1H, *J*=10.0 Hz, H-3). ^{13}C NMR ($CDCl_3$): δ=24.7 (C-1); 39.9 (C-2); 146.6 (C-3); 128.5 (C-4); 207.9 (C-5); 172.4 (C-6); 40.4 (C-7); 41.8 (C-8); 51.3 (C-9); 35.6 (C-10); 35.5 (C-11); 36.1 (C-12); 48.0 (C-13); 55.1 (C-14); 31.4 (C-15); 80.2 (C-16); 61.4 (C-17); 16.2 (C-18); 18.2 (C-19); 42.2 (C-20); 14.6 (C-21); 109.0 (C-22); 31.6 (C-23); 28.8 (C-24); 30.3 (C-25); 66.8 (C-26); 17.2 (C-27). HRMS (ESI-FT-ICR) *m/z*: 443.2797 $[M-H]^-$ (Calculated for $C_{27}H_{39}O_5$: 443.2796).

4.1.11. (25*R*)-5,6-Seco-A-homo-5(10)-oxa-5-oxo-spirostan-6-oic acid (14). Pd/C 10% (800 mg) was added to a solution of ketoacid **13** (2.0 g, 4.5 mmol) in absolute EtOH (100 mL). The reaction mixture was treated successively with hydrogen and vacuum and finally stirred under hydrogen atmosphere for 24 h. The catalyst was removed by filtration and the resulting solution was evaporated under reduced pressure to give a crude product. This product was dissolved in CH_2Cl_2 (80 mL) and treated with *m*CPBA (1.55 g, 9.0 mmol) in a similar way as described in Section 4.1.3 to give the lactone **14** (1.54 g, 73%). Mp (MeOH): 199–200 °C. ^{1}H NMR ($CDCl_3$): δ=0.78 (d, 3H, *J*=6.2 Hz, H-27); 0.81 (s, 3H, H-18); 0.96 (d, 3H, *J*=6.9 Hz, H-21); 1.28 (s, 3H, H-19); 3.34 (t, 1H, *J*=10.8 Hz, H-26ax); 3.46 (dd, 1H, *J*=10.8/4.0 Hz, H-26eq); 4.35 (m, 1H, H-16). ^{13}C NMR ($CDCl_3$): δ=24.7 (C-1); 39.9 (C-2); 29.2 (C-3); 41.2 (C-4); 176.1 (C-5); 172.5 (C-6); 40.8 (C-7); 41.6 (C-8); 50.9 (C-9); 79.1 (C-10); 35.6 (C-11); 36.7 (C-12); 48.2 (C-13); 55.1 (C-14); 31.4 (C-15); 80.4 (C-16); 61.8 (C-17); 16.2 (C-18); 22.3 (C-19); 42.2 (C-20); 14.6 (C-21); 109.0 (C-22); 31.6 (C-23); 28.8 (C-24); 30.3 (C-25); 66.8 (C-26); 17.2 (C-27). HRMS (ESI-FT-ICR) *m/z*: 461.2906 $[M-H]^-$ (Calculated for $C_{27}H_{41}O_6$: 461.2903).

4.1.12. Peptide–steroid conjugate 15. ε-*N*-Cbz-L-lysine methyl ester hydrochloride (596 mg, 1.7 mmol), paraformaldehyde (51 mg, 1.7 mmol), triethylamine (023 mL, 1.7 mmol), steroidal acid **14** (800 mg, 1.7 mmol), and methyl isocyanoacetate (0.2 mL, 1.7 mmol) were reacted in MeOH (80 mL) in a similar way as described in Section 4.1.9. Flash column chromatography purification ($CHCl_3$/MeOH, 15:1) furnished the pure conjugate **15** (1.1 g, 74%). Mp (heptane/AcOEt): 219–223 °C. ^{1}H NMR ($CDCl_3$): δ=0.77 (d, 3H, *J*=6.2 Hz, H-27); 0.84 (s, 3H, H-18); 0.95 (d, 3H, *J*=6.9 Hz, H-21); 1.21 (s, 3H, H-19); 3.34 (t, 1H, *J*=10.8 Hz, H-26ax); 3.46 (dd, 1H, *J*=10.8/4.0 Hz, H-26eq); 3.68 (s, 3H, OCH_3); 3.72 (s, 3H, OCH_3); 4.17 (m, 2H, CH_2); 4.26 (m, 1H, NC*H*); 4.39 (m, 1H, H-16); 5.10 (s, 2H, OCH_2); 7.32 (m, 5H, Ph–Cbz). ^{13}C NMR ($CDCl_3$): δ=14.7 (*C*H_3); 16.4 (*C*H_3); 17.2 (*C*H_3); 22.5 (*C*H_3); 24.9 (*C*H_2); 28.6 (*C*H_2); 28.8 (*C*H_2); 29.2 (*C*H_2); 29.6 (*C*H_2); 29.8 (*C*H_2); 30.4 (*C*H); 30.7 (*C*H_2); 31.4 (*C*H_2); 31.6 (*C*H_2); 35.6 (*C*H_2); 36.8 (*C*H_2); 39.5 (*C*H_2); 40.2 (*C*H_2); 41.3 (*C*H); 41.4 (*C*H_2); 42.2 (*C*H); 44.4 (*C*H_2); 44.8 (*C*H); 45.2 (*C*H_2); 48.5 (*C*); 50.5 (*C*H); 54.9 (*C*H); 61.8 (*C*H); 66.7 (*C*H_2); 66.9 (*C*H_2); 79.1 (*C*); 80.6 (*C*H); 109.0 (*C*); 127.7 (*C*H); 127.9 (*C*H); 128.4 (*C*H); 136.5 (*C*); 157.0 (*C*O); 169.4 (*C*O); 171.1 (*C*O); 174.9 (*C*O); 175.2 (*C*O); 176.1 (*C*O). HRMS (ESI-FT-ICR) *m/z*: 890.4780 $[M+H]^+$ (Calculated for $C_{47}H_{69}N_3NaO_{12}$: 890.4779).

4.1.13. (25*R*)-3β-(*tert*-Butyldimethylsilyloxy)-5α-spirostan-12-one (17). TBSCl (2.23 g, 17.5 mmol) and imidazole (1.23 g, 18.1 mmol) were added to a solution of hecogenin **16** (5.2 g, 12.1 mmol) in anhydrous DMF (80 mL). The reaction mixture was stirred under nitrogen atmosphere for 3 h and then, the usual work-up ($CHCl_3$) yielded a crude product, which was purified by flash column chromatography (hexane/AcOEt, 3:1) to give the ketone **17** (5.79 g, 84%). Mp (EtOH): 187–189 °C. ^{1}H NMR ($CDCl_3$): δ=0.07 (s, 3H, (CH_3)$_2$Si); 0.09 (s, 3H, (CH_3)$_2$Si); 0.76 (s, 3H, H-18); 1.17 (s, 3H, H-19); 0.78 (d, 3H, *J*=6.4 Hz, H-27); 0.92 (s, 9H, (CH_3)$_3$CSi); 0.94 (d, 3H, *J*=6.5 Hz, H-21); 3.36 (t, 1H, *J*=10.9 Hz, H-26ax); 3.49 (dd, 1H, *J*=10.8/3.9 Hz, H-26eq); 3.54 (br m, 1H, H-3α); 4.40 (m, 1H, H-16α). ^{13}C NMR ($CDCl_3$): δ=36.2 (C-1); 27.2 (C-2); 71.1 (C-3); 33.8

(C-4); 44.4 (C-5); 28.1 (C-6); 31.4 (C-7); 34.3 (C-8); 55.4 (C-9); 36.1 (C-10); 37.7 (C-11); 213.2 (C-12); 55.0 (C-13); 55.6 (C-14); 31.2 (C-15); 79.2 (C-16); 53.5 (C-17); 16.1 (C-18); 11.9 (C-19); 42.2 (C-20); 13.2 (C-21); 109.2 (C-22); 31.4 (C-23); 28.8 (C-24); 30.2 (C-25); 66.8 (C-26); 17.1 (C-27); 26.1 ((CH_3)$_3$CSi); 18.3 ((CH_3)$_3$*C*Si); −2.9 ((CH_3)$_2$Si). HRMS (ESI-FT-ICR) *m*/*z*: 567.3848 [M+Na]$^+$ (Calculated for $C_{33}H_{56}NaSiO_4$: 567.3846).

4.1.14. (25*R*)-12,13-Seco-3β-(*tert*-butyldimethylsilyloxy)-5α-spirostane-12,13-diol (18). Ketone **17** (4.0 g, 7.35 mmol) was treated with *m*CPBA (2.0 g, 11.6 mmol) for 36 h in a similar way as described for the synthesis of **5** to give the corresponding lactone. This crude product was dissolved in dry THF (100 mL) and added dropwise at 0 °C to a solution of $LiAlH_4$ (570 mg, 15 mmol) in dry THF (100 mL). The reaction mixture was stirred at room temperature under nitrogen atmosphere for 20 h. An aqueous 5% NaOH solution (100 mL) was added slowly to the reaction mixture and the stirring was continued for 30 min. The resulting white powder was then filtered off and washed with AcOEt (3×100 mL). The collected organic phase was washed with brine (100 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a crude product. Flash column chromatography purification ($CHCl_3$/MeOH, 10:1) afforded the compound **18** (2.97 g, 72%). Mp (AcOEt): 231–233 °C. ^{1}H NMR ($CDCl_3$): δ=0.08 (s, 3H, (CH_3)$_2$Si); 0.09 (s, 3H, (CH_3)$_2$Si); 0.80 (d, 3H, *J*=6.7 Hz, H-27); 0.82 (s, 3H, H-19); 0.92 (s, 9H, (CH_3)$_3$CSi); 1.04 (d, 3H, *J*=6.2 Hz, H-21); 1.10 (s, 3H, H-18); 3.36 (t, 1H, *J*=10.9 Hz, H-26ax); 3.41 (2H, m, H-12); 3.46 (dd, 1H, *J*=11.0/4.0 Hz, H-26eq); 3.53 (br m, 1H, H-3α); 4.41 (m, 1H, H-16α). ^{13}C NMR ($CDCl_3$): δ=37.9 (C-1); 29.0 (C-2); 70.8 (C-3); 35.8 (C-4); 44.8 (C-5); 30.6 (C-6); 32.8 (C-7); 34.3 (C-8); 52.1 (C-9); 37.8 (C-10); 38.0 (C-11); 64.5 (C-12); 78.5 (C-13); 48.6 (C-14); 31.2 (C-15); 79.8 (C-16); 51.7 (C-17); 20.3 (C-18); 12.4 (C-19); 43.7 (C-20); 14.1 (C-21); 109.2 (C-22); 31.6 (C-23); 28.8 (C-24); 31.2 (C-25); 67.5 (C-26); 17.4 (C-27); 26.1 ((CH_3)$_3$CSi); 18.3 ((CH_3)$_3$*C*Si); −2.9 ((CH_3)$_2$Si). HRMS (ESI-FT-ICR) *m*/*z*: 587.4107 [M+Na]$^+$ (Calculated for $C_{33}H_{60}NaSiO_5$: 587.4108).

4.1.15. (25*R*)-12,13-Seco-12-amino-5α-spirostane-3β,13-diol (19). Diol **18** (2.5 g, 4.4 mmol) was dissolved in CH_2Cl_2 (100 mL) and treated in a similar way as described in Section 4.1.6 to give a crude product that was identified by ^{1}H NMR analysis as the expected amine. This product was dissolved in THF (150 mL) and tetrabutylammonium fluoride (TBAF) (4 mL, 1 M in THF) was added. The reaction mixture was stirred under nitrogen atmosphere for 6 h and then the usual work-up (Et_2O) yielded a crude product. Flash column chromatography purification ($CHCl_3$/MeOH/Et_3N, 10:1:0.5) afforded the amine **19** (1.24 g, 62%). Mp (heptane/AcOEt): 218–220 °C. ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5): δ=0.79 (s, 3H, H-19); 0.81 (d, 3H, *J*=6.5 Hz, H-27); 1.01 (d, 3H, *J*=6.4 Hz, H-21); 1.08 (s, 3H, H-18); 3.36 (t, 1H, *J*=10.9 Hz, H-26ax); 3.37 (m, 2H, H-12); 3.44 (dd, 1H, *J*=10.8/4.0 Hz, H-26eq); 3.54 (br m, 1H, H-3α); 4.41 (m, 1H, H-16α). ^{13}C NMR ($CDCl_3$/CD_3OD, 95:5): δ=37.2 (C-1); 32.1 (C-2); 70.2 (C-3); 36.9 (C-4); 44.8 (C-5); 31.2 (C-6); 32.7 (C-7); 34.5 (C-8); 52.4 (C-9); 37.8 (C-10); 38.0 (C-11); 62.8 (C-12); 78.3 (C-13); 50.4 (C-14); 31.2 (C-15); 79.8 (C-16); 51.7 (C-17); 20.3 (C-18); 12.4 (C-19); 43.7 (C-20); 14.1 (C-21); 109.2 (C-22); 31.6 (C-23); 28.8 (C-24); 31.2 (C-25); 67.5 (C-26); 17.4 (C-27). HRMS (ESI-FT-ICR) *m*/*z*: 450.3586 [M+H]$^+$ (Calculated for $C_{27}H_{48}NO_4$: 450.3583).

4.1.16. Peptide–steroid conjugate 20. Steroidal amine **19** (800 mg, 1.8 mmol), paraformaldehyde (53 mg, 1.8 mmol), *N*-Boc-L-serine (365 mg, 1.8 mmol), and benzylisocyanide (0.22 mL, 1.8 mmol) were reacted in MeOH (100 mL) in a similar way as described in Section 4.1.7. Flash column chromatography purification ($CHCl_3$/MeOH, 15:1) furnished the conjugate **20** (1.10 g, 79%). Mp (AcOEt): 231–233 °C. ^{1}H NMR ($CDCl_3$): δ=0.78 (s, 3H, H-19); 0.81 (d, 3H, *J*=6.7 Hz, H-27); 1.04 (d, 3H, *J*=6.6 Hz, H-21); 1.12 (s, 3H, H-18); 1.43 (s, 9H, (CH_3)$_3$C); 3.36 (t, 1H, *J*=10.9 Hz, H-26ax); 3.39 (m, 2H, H-12); 3.44 (dd, 1H, *J*=10.8/4.1 Hz, H-26eq); 3.55 (br m, 1H, H-3α); 3.67–3.62 (m, 2H, CH_2OH); 4.25–4.23 (m, 1H, NC*H*); 4.28–4.32 (m, 4H, CH_2); 4.40 (m, 1H, H-16α); 7.13 (m, 5H, Ph). ^{13}C NMR ($CDCl_3$): δ=17.4 (*C*H$_3$); 12.7 (*C*H$_3$); 14.2 (*C*H$_3$); 20.2 (*C*H$_3$); 28.5 (*C*H$_3$); 28.8 (*C*H$_2$); 31.2 (*C*H$_2$); 31.3 (*C*H+*C*H$_2$); 31.6 (*C*H$_2$); 32.3 (*C*H$_2$); 32.6 (*C*H$_2$); 34.5 (*C*H); 36.7 (*C*H$_2$); 37.0 (*C*H$_2$); 37.9 (*C*); 38.2 (*C*H$_2$); 43.6 (*C*H); 44.9 (*C*H); 45.3 (*C*H$_2$); 46.8 (*C*H); 50.5 (*C*H); 51.6 (*C*H); 52.5 (*C*H); 62.9 (*C*H$_2$); 63.3, (*C*H$_2$); 66.8 (*C*H$_2$); 67.5 (*C*H$_2$); 70.3 (*C*H); 78.6 (*C*); 79.5 (*C*); 80.0 (*C*H); 109.3 (*C*); 126.8 (*C*H); 127.3 (*C*H); 128.2 (*C*H); 131.1 (*C*H); 155.8 (*C*O); 168.7 (*C*O); 170.1 (*C*O). HRMS (ESI-FT-ICR) *m*/*z*: 806.4933 [M+Na]$^+$ (Calculated for $C_{44}H_{69}NaN_3O_9$: 806.4932).

4.1.17. Methyl (25*R*)-2,3-seco-2,6β-dihydroxy-5α-spirostan-3-oate (21). H_2SO_4 (30%, 4 mL) was added dropwise to a solution of lactone **5** (2.0 g, 4.1 mmol) in MeOH (100 mL) and the reaction mixture was stirred at reflux for 8 h. The usual work-up (AcOEt) yielded a crude product, which dissolved in a 1 M solution of NaOMe in MeOH (200 mL). The reaction mixture was stirred for 2 h and then concentrated under reduced pressure. Flash column chromatography purification ($CHCl_3$/MeOH, 20:1) afforded the ester **21** (1.74 g, 88%). Mp (heptane/AcOEt): 242–243 °C. ^{1}H NMR ($CDCl_3$): δ=0.76 (s, 3H, H-18); 0.77 (d, 3H, *J*=6.5 Hz, H-27); 0.81 (s, 3H, H-19); 0.94 (d, 3H, *J*=6.6 Hz, H-21); 3.36 (t, 1H, *J*=11.0 Hz, H-26ax); 3.46 (dd, 1H, *J*=4.1/10.9 Hz, H-26eq); 3.72 (m, 2H, H-2); 3.85 (m, 1H, H-6α); 4.41 (m, 1H, H-16α). ^{13}C NMR ($CDCl_3$): δ=38.8 (C-1); 57.7 (C-2); 173.9 (C-3); 28.3 (C-4); 46.1 (C-5); 71.2 (C-6); 33.8 (C-7); 30.5 (C-8); 41.1 (C-9); 36.8 (C-10); 20.8 (C-11); 39.7 (C-12); 40.2 (C-13); 56.5 (C-14); 31.6 (C-15); 80.5 (C-16); 62.1 (C-17); 16.3 (C-18); 17.3 (C-19); 41.5 (C-20); 14.5 (C-21); 109.3 (C-22); 31.4 (C-23); 28.8 (C-24); 29.0 (C-25); 66.9 (C-26); 17.1 (C-27). HRMS (ESI-FT-ICR) *m*/*z*: 501.3192 [M+Na]$^+$ (Calculated for $C_{28}H_{46}NaO_6$: 501.3194).

4.1.18. Methyl (25*R*)-2,3-seco-6β-acetoxy-2-amino-5α-spirostan-3-oate (22). Compound **21** (1.6 g, 3.3 mmol) was dissolved in dry CH_2Cl_2 (100 mL) and submitted to mesylation (0.6 mL of MsCl and 6.2 mL of Et_3N) and subsequent nucleophilic replacement with NaN_3 (650 mg, 10 mmol) in a similar way as described in Section 4.1.6 to give the expected azide (identified by ESIMS analysis). This

intermediate was dissolved in pyridine (60 mL) and treated with Ac_2O (3 mL) exactly as described in Section 4.1.1. The resulting acetate was dissolved in absolute ethanol (100 mL) and submitted to catalytic hydrogenation (400 mg of Lindlar catalyst) as described in Section 4.1.6 to afford the crude amine **22**. Flash column chromatography purification (CH_2Cl_2/MeOH/Et_3N, 20:1:0.1) furnished the pure amine **22** (1.06 g, 62%). Mp (MeOH): 217–218 °C. ^1H NMR ($CDCl_3$): δ=0.77 (s, 3H, H-18); 0.77 (d, 3H, J=6.6 Hz, H-27); 0.82 (s, 3H, H-19); 0.96 (d, 3H, J=6.6 Hz, H-21); 2.02 (s, 3H, CH_3CO); 3.36 (t, 1H, J=11.0 Hz, H-26ax); 3.45 (dd, 1H, J=4.0/10.9 Hz, H-26eq); 3.60 (m, 2H, H-2); 4.38 (m, 1H, H-6α); 4.41 (m, 1H, H-16α). ^{13}C NMR ($CDCl_3$): δ=38.2 (C-1); 53.6 (C-2); 173.8 (C-3); 28.4 (C-4); 46.5 (C-5); 72.6 (C-6); 36.3 (C-7); 30.4 (C-8); 53.8 (C-9); 35.4 (C-10); 20.9 (C-11); 39.6 (C-12); 40.5 (C-13); 55.5 (C-14); 31.4 (C-15); 80.6 (C-16); 62.0 (C-17); 16.5 (C-18); 15.2 (C-19); 41.6 (C-20); 14.5 (C-21); 109.3 (C-22); 31.3 (C-23); 28.7 (C-24); 30.2 (C-25); 66.8 (C-26); 17.1 (C-27); 21.3 (*C*H_3CO); 170.0 (CH_3*C*O). HRMS (ESI-FT-ICR) *m/z*: 542.3458 $[M+Na]^+$ (Calculated for $C_{30}H_{49}NO_6Na$: 542.3456).

4.1.19. Peptide–steroid conjugate 23. Steroidal amine **22** (700 mg, 1.3 mmol), paraformaldehyde (41 mg, 1.3 mmol), *N*-Boc-L-phenylalanine (357 mg, 1.3 mmol), and *tert*-butylisocyanide (0.15 mL, 1.3 mmol) were reacted in MeOH (80 mL) in a similar way as described in Section 4.1.7. The resulting crude product was dissolved in a mixture of THF/H_2O (2:1, 200 mL), treated with LiOH (210 mg, 5.0 mmol) and stirred at 0 °C for 2 h. The reaction mixture was then acidified with aqueous 10% $NaHSO_4$ to pH 3 and extracted with AcOEt (2×100 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to dryness. The resulting acid reacted with L-phenylalanine methyl ester hydrochloride (280 mg, 1.3 mmol), paraformaldehyde (41 mg, 1.3 mmol), triethylamine (0.18 mL, 1.3 mmol), and *tert*-butylisocyanide (0.15 mL, 1.3 mmol) in MeOH (60 mL) in a similar way as described in Section 4.1.9. Flash column chromatography purification ($CHCl_3$/MeOH, 15:1) furnished the pure conjugate **23** (874 mg, 59%). Mp (AcOEt): 233–234 °C. ^1H NMR ($CDCl_3$): δ=0.78 (s, 3H, H-18); 0.77 (d, 3H, J=6.4 Hz, H-27); 0.85 (s, 3H, H-19); 0.95 (d, 3H, J=6.6 Hz, H-21); 1.32–1.35 (s, 18H, (CH_3)$_3$CNH); 1.44 (s, 9H, (CH_3)$_3$C); 2.03 (s, 3H, CH_3CO); 3.36 (t, 1H, J=10.9 Hz, H-26ax); 3.45 (dd, 1H, J=4.1/10.9 Hz, H-26eq); 3.62 (m, 2H, H-2); 3.71 (s, 3H, CH_3O); 4.12–4.27 (m, 4H, CH_2); 4.33–4.39 (m, 3H, NC*H*); 4.41 (m, 1H, H-16α); 7.21–7.25 (m, 10H, Ph). ^{13}C NMR ($CDCl_3$): δ=14.4 (*C*H_3); 15.4 (*C*H_3); 16.6 (*C*H_3); 17.1 (*C*H_3); 21.1 (*C*H_2); 21.2 (*C*H_3); 28.1 (*C*H_2); 28.3 (*C*H_3); 28.5 (*C*H_3); 28.8 (*C*H_2); 30.2 (*C*H); 30.7 (*C*H_2); 31.3 (*C*H_2); 31.4 (*C*H_2); 35.7 (*C*); 36.7 (*C*H_2); 38.0 (*C*H_2); 39.8 (*C*H_2); 40.7 (*C*); 41.5 (*C*H); 44.0 (*C*H_2); 44.2 (*C*H_2); 44.5 (*C*H_2); 45.8 (*C*H); 45.9 (*C*H); 46.1 (*C*H); 53.2 (*C*H_3); 53.3 (*C*H); 53.9 (*C*H_2); 55.2 (*C*H); 62.1 (*C*H); 66.8 (*C*H_2); 72.8 (*C*H); 79.5 (*C*); 80.4 (*C*H); 109.3 (*C*); 120.5 (*C*H); 121.1 (*C*H); 121.4 (*C*H); 127.1 (*C*H); 128.8 (*C*H); 129.4 (*C*); 155.8 (*C*O); 168.6 (*C*O); 168.8 (*C*O); 169.2 (*C*O); 169.5 (*C*O); 170.1 (*C*O); 170.3 (*C*O); 174.8 (*C*O). HRMS (ESI-FT-ICR) *m/z*: 1162.7036 $[M+Na]^+$ (Calculated for $C_{65}H_{97}N_5O_{12}Na$: 1162.7033).

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