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The Cu^{I}-catalyzed alkyne–azide cycloaddition as direct conjugation/cyclization method of peptides to steroids

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ABSTRACT

The Cu^{I}-catalyzed azide–alkyne 1,3-dipolar cycloaddition is implemented as a direct conjugation method of alkynyl peptides to azidosteroids, enabling the preparation of novel triazole-linked peptide–steroid conjugates in good to excellent yields. The process comprised the solution-phase synthesis of oligopeptides featuring varied chain length and amino acid sequence as well as the preparation of a small library of azidosteroids bearing the azido group either at the side chain or the steroidal nucleus. An alternative strategy relying on a sequential peptide coupling/CuAAC-based macrocyclization procedure was also developed to afford macrocyclic peptide–steroid conjugates featuring different sizes and topologies. Both methods showed great chemical efficiency and versatility, thus showing promise toward the future preparation of conjugates with potential pharmaceutical and biological applications.

1. Introduction

The conjugation of peptides to steroids has been a successful strategy in the modulation of the physicochemical and biological properties of both types of biomolecules.[1] One of the early and most remarkable examples of this strategy has been the conjugation of bile acids to peptidic drugs, which has allowed for the improvement of pharmacological properties of such drugs.[2] Alternatively, a variety of peptide–steroid conjugates have been designed as receptors for binding studies of specific oligopeptides sequences (e.g., opioid peptides),[3] as protease-like artificial enzymes,[4] and as facial amphiphiles mimicking the antibacterial activity of natural cationic peptide antibiotics.[5] Other important reports have proven that steroids can be utilized as templates for positioning peptide strands of great biological relevance. Examples are the conjugation of (i) multivalent epitope oligopeptide sequences to cholic acid derivatives,[6] (ii) the Arg-Gly-Asp sequence to spiroketal steroids in the design of new RGD mimics as integrin antagonists,[7] (iii) insulin to deoxycholic acid in the pursuit of orally active insulin derivatives,[8] and (iv) protein-binding peptide sequences to cholesterol derivatives in the design of artificial cell surface receptors for the cellular uptake of specific ligands.[9] Similarly, pharmaceutically useful steroidal drugs have been conjugated to *pseudo*-peptides, as proven by the conjugation of (i) dexamethasone to a variety of peptides and peptidomimetics as a mean of studying their cell permeability[10] and (ii) hydrocortisone and prednisolone to the urotoxin family of peptides with the aim to improve the immunosuppressive activity.[11]

An intrinsic feature of the steroidal skeleton that has prompted the conjugation to oligopeptides is its great conformational rigidity, a characteristic that has been utilized in the design and synthesis of macrocyclic peptide–steroid conjugates.[12] This idea has eventually led to cyclopeptide–steroid conjugates with biologically relevant types of peptide folding,[13] including a cyclopeptide–cholane conjugate designed to mimic the loop region conformation of a protein epitope (i.e., the measles virus hemagglutinin noose epitope).[14]

Upon designing peptide–steroid conjugates with biological or pharmaceutical applications, a crucial issue is the synthetic approach chosen for joining both molecules. Scheme 1 highlights two alternative strategies usually utilized for the assembly of peptide–steroid conjugates starting from aminosteroids: (A) the sequential incorporation of the required amino acids to assemble the

Abbreviations: Boc, *tert*-butoxycarbonyl; CuAAC, Cu^{I}-catalyzed alkyne–azide 1,3-dipolar cycloaddition; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; DIPEA, *N*,*N*-di-*iso*-propyl-*N*-ethylamine; DMF, dimethylformamide; DMPU, 1,3-dimethyl-3,4,5,6-tetrahydro-2(*1H*)-pyrimidinone; HOBt, 1-hydroxy-1*H*-benzotriazole; TFA, trifluoroacetic acid.

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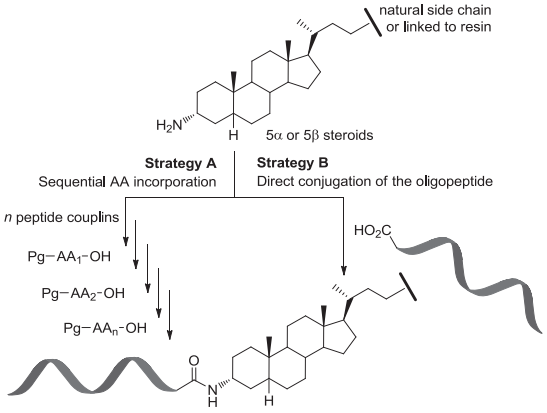
peptide at the steroidal skeleton and (B) the direct conjugation of a previously produced peptide to the steroid. Strategy A has been the method of choice in most cases, mainly due to the well-established solution- and solid-phase peptide coupling protocols[3,4 ,15] as well as the drawbacks concerning the possible racemization of the *C*-terminal amino acid upon carboxylate activation. Whereas new approaches relying on the Ugi reaction as conjugation method have been reported,[16] new strategies are in high demand in this active field of research.



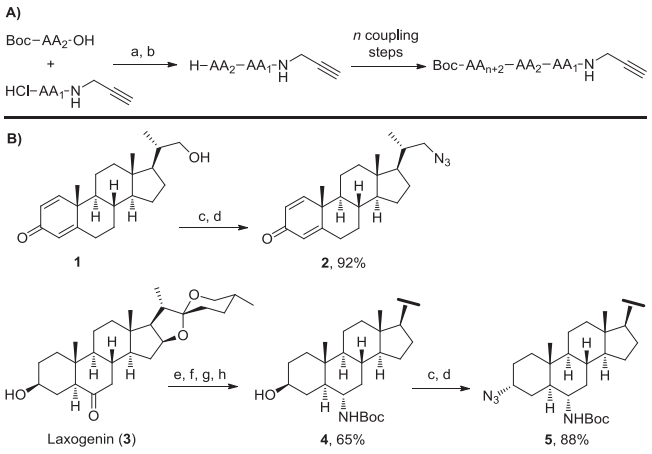
Scheme 1. Strategies for the assembly of peptide–steroid conjugates. Functionalization at other positions, e.g., 7 and 12, is equally possible. Pg-AA-OH: N^{α}-protected amino acid.

Herein we report on the utilization of the Cu^{I}-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC)[17] as direct conjugation method of oligopeptides to a variety of steroids. Similarly, we introduce this click approach as a suitable cyclization procedure for the preparation of macrocyclic peptide–steroid conjugates. As result of this conjugation/cyclization method, a triazole ring results as linkage of both molecular building blocks, which confer to these (cyclo)peptide–steroid conjugates desirable characteristics like: hydrolytic and metabolic stability, water solubility, and rigidity.

2. Results and discussion

The CuAAC approach has been extensively used in bioconjugation chemistry, with remarkable examples focusing on the ligation of sugars, peptides, and proteins.[18] Important features for the selection of this method are its great chemical efficiency and easy implementation under mild conditions, as well as the peptidomimetic character of the triazole linkage. The field of peptide–steroid conjugates has witnessed merely a few reports, albeit describing only the conjugation use of steroidal alkynes to *N*-alkylated peptides functionalized with azido groups.[19] However, a conjugation method based on the utilization of alkynyl peptides and azidosteroids has not been addressed so far, despite of the great advances achieved in the functionalization of peptides with alkynes[20] as well as steroids with azido groups.[21]

To implement the conjugation strategy based on the CuAAC between alkynyl peptides and azidosteroids, we first focused on the preparation of oligopeptides capped as propargylamide at the *C*-terminus. As shown in Scheme 2A, a typical solution-phase protocol—based on the utilization of the orthogonal Boc/Cbz protecting groups and the EDC/HOBt coupling system—was employed.[22] Thus, a series of alkynyl oligopeptides were prepared in good yields from propargylated amino acids by sequential coupling with Boc-N^{α}-amino acids followed by classic Boc removal upon treatment with hydrogen chloride in dioxane.



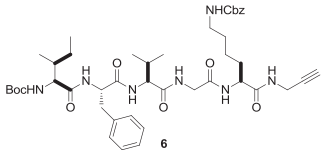
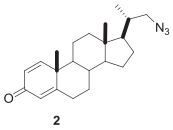
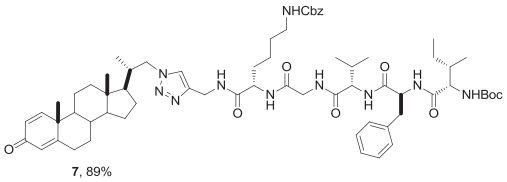
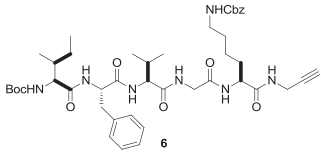
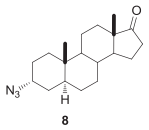
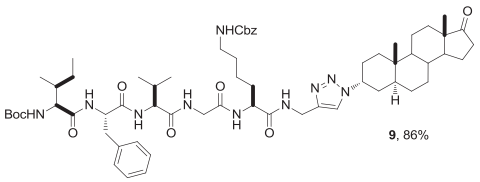
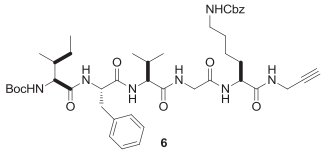
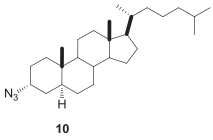
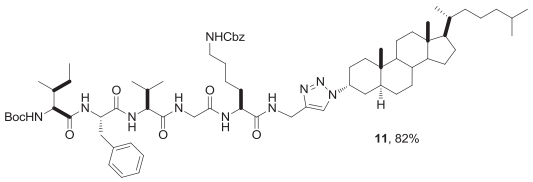
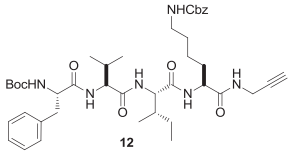
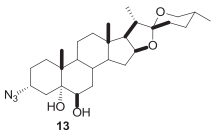
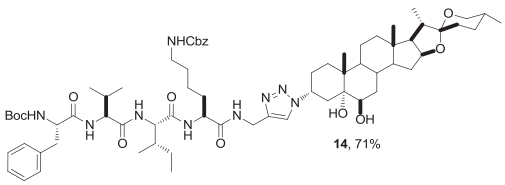
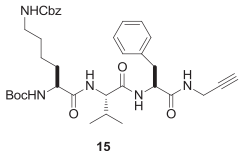
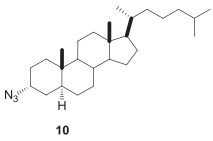
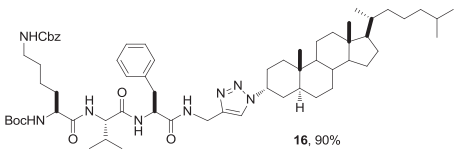
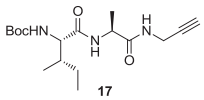
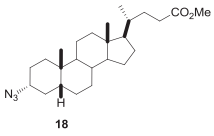
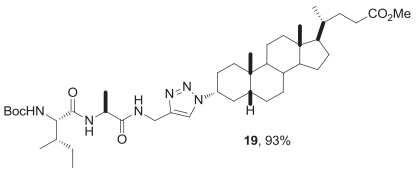
Scheme 2. (A) Solution-phase synthesis of alkynyl peptides: (a) EDC, HOBt, Et_3N, CH_2Cl_2; (b) HCl/Dioxane. (B) Synthesis of azidosteroids: (c) MsCl, Et_3N, CH_2Cl_2, 0 °C; (d) NaN_3, DMPU, 50 °C; (e) HCl·NH_2OH, NaOAc, MeOH, reflux; (f) H_2, PtO_2, HOAc; (g) Zn, AcOH, then aq $NaHCO_3$; (h) Boc_2O, $NaHCO_3$, THF/H_2O.

Alternatively, Scheme 2B depicts the synthesis of two new steroidal azides to be further conjugated to alkynyl peptides. Steroid **1**—having the classic A-ring functionalization of anti-inflammatory steroids—was subjected to mesylation and subsequent azide displacement to afford azidosteroid **2** in 92% over two steps. Alternatively, we turned to the synthesis of the bifunctional spirostane **5** bearing a 3α-azide and a Boc-protected 6α-amino function. For this, the natural sapogenin laxogenin (**3**) was converted into the corresponding oxime followed by reduction to amine and protection as carbamate, which enables further incorporation of the azido group at C-3. To ensure high stereoselectivity in the oxime reduction, PtO_2-catalyzed hydrogenation followed by further reduction of the resulting hydroxylamine with Zn/AcOH was employed to afford the 6α-aminospirostane, which upon standard amine protection as *tert*-butyl carbamate furnished compound **4** in 65% yield over four steps. Synthesis of the 3α-azidosteroid **5** was completed in 88% yield by mesylation of the equatorial 3β-OH followed by nucleophilic displacement with sodium azide. As will be shown later, the α disposition of both the amino and azido functionalities is required for the following conjugation/cyclization approach in which the two peptide termini will be ligated at positions 3 and 6 of the steroid.

To evaluate efficiency and versatility of the direct click peptide–steroid conjugation, it was important having not only a set of oligopeptides of different chain length and amino acid sequence but also a small library of azides derived from dissimilar steroidal skeletons. Thus, besides of azidosteroids **2** and **5**, a variety of previously described steroidal azides—including the androstanic one **8**,[21d] the cholestanic one **10**,[21e] the spirostanic one **13**,[21b] and the cholanic ones **18**[21f] and **23**[21g]—were prepared as reported in the literature. As shown in Table 1, azidosteroids were subjected to click conjugation with oligopeptides using the standard CuAAC conditions, i.e., 20 mol % of a Cu^{II} salt and 40 mol % of sodium ascorbate in a THF/H_2O mixture. Remarkably, the approach proved great efficiency on producing the triazole-linked conjugates, as the isolated pure products were obtained in good to excellent yields despite the highly bulky character of the two ligated building blocks.

To determine the effect of varying the structure of the steroid on the conjugation efficiency, alkynyl peptide **6** was first ligated to steroid **2**—having the azido group in the flexible side chain—and then to steroids **8** and **10** bearing the azide directed attached at C-3 of the steroidal nucleus. Nevertheless, in spite of the axial disposition of the 3α-azide in **8** and **10**, the conjugation yields were not significantly lower than for steroid **2** bearing the less hindered azide at C-22. On the other hand, the use of azidospirostane **13** dropped the

Table 1
Synthesis of triazole-linked peptide–steroid conjugates by CuAAC[a]

Alkynyl peptide	Azidosteroid	Peptide–steroid conjugates
 **6**	 **2**	 **7**, 89%
 **6**	 **8**	 **9**, 86%
 **6**	 **10**	 **11**, 82%
 **12**	 **13**	 **14**, 71%
 **15**	 **10**	 **16**, 90%
 **17**	 **18**	 **19**, 93%

[a] Conjugation conditions: 20 mol % $Cu(AcO)_2 \cdot H_2O$ and 40 mol % sodium ascorbate in THF/H_2O.

conjugation yield up to 71%, despite of being ligated to the shorter tetrapeptide **12**. An explanation for this may be the lower reactivity of the 3α-azido group of **13**, as consequence of the 1,3-diaxial interaction with the 5α-OH group that increases the steric congestion of the azido group. Alternatively, it was found that shortening the peptide chain leads to an increase in the conjugation yield, since reactions of tripeptide **15** and dipeptide **17** with the parent 3-azidosteroids gave conjugates **16** and **19** in 90% and 93% yield, respectively. It must be also noticed that the equatorial disposition of azide in steroid **18** may have influenced the high yield of conjugate **19**.

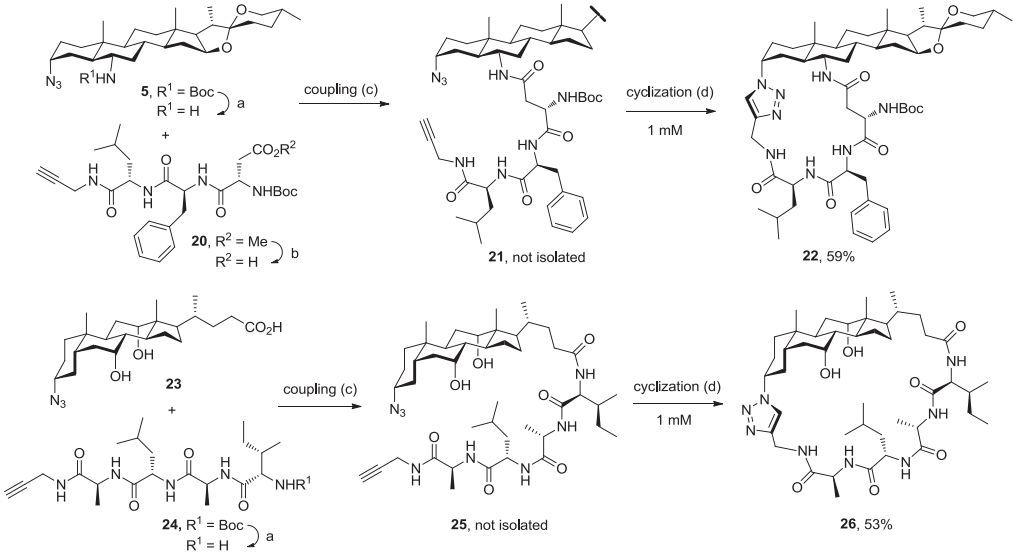
In general, the CuAAC proved to be a very efficient method for the conjugation of alkynyl peptides to azidosteroid, and thus becomes a reliable alternatively for the design of new conjugates with potential biological applications. In our opinion, this strategy complements well with solid-phase peptide synthesis (SPPS) protocols, wherein very large peptides may be conjugated on-resin to azidosteroids with similar or even higher efficiency, considering that large excess of reagents are usually employed to drive reactions to completion. To further assess stability of the triazole-linked conjugates under typical deprotection conditions, compounds **9**, **11**, and **16** were treated with 2% triisopropylsilane in TFA for 4 h. The integrity of the deprotected conjugates was analyzed by ESI-MS and RP-HPLC, showing that the triazole linkage is resistant to such drastic conditions usually employed in SPPS for cleaving protecting groups and releasing the peptide from the resin (see Supplementary data).

Whereas the availability and diversity of steroidal azides may be similar to that of steroidal alkynes, we believe there is an important

factor favoring the scope of this strategy over the previously reported method using azidopeptides and alkynyl steroids.[19] That is, the possibilities of peptides functionalization with alkyne groups are more diverse and synthetically feasible than with azido groups. It is known from literature[20,23] that oligopeptides—either from natural, synthetic or even recombinant origin—can be readily propargylated at the Glu, Asp, and Lys side chains as well as at the *C*- and *N*-termini in a highly selective manner upon standard coupling conditions. Similarly, alkyne-decorated non-proteinogenic aminoacids can be used in solution and solid-phase protocols, thus enabling site selective conjugation to azidosteroids.

After the grounds of the click conjugation approach were established, the focus was directed to implement a click cyclization procedure enabling the rapid access to triazole-linked macrocyclic peptide–steroid conjugates. Macrocyclic peptides comprise one of the most important classes of lead compounds in drug discovery and chemical biology,[24] as cyclization is one of the most effective ways to improve pharmacological properties (e.g., bioavailability and enzymatic stability)[25] and to decrease the entropy loss upon binding to receptors.[26]

A special class of macrocyclic peptides are those endowed with hybrid scaffolds including endocyclic motifs like aromatic (heterocyclic) rings[24] and steroidal skeletons,[12–14] rigid platforms used for the introduction of conformational constrains into short peptide sequences. Encouraged by the wide applications of cyclopeptide–steroid conjugates, we turned to addressing the scope of CuAAC as a useful method for the preparation of such hybrid architectures. Scheme 3 depicts the synthesis of two archetypal examples of macrocyclic peptide–steroid conjugates by a sequential peptide coupling/click macrocyclization procedure.



Scheme 3. Synthesis of (cyclo)peptide–steroid conjugates by sequential peptide coupling/CuAAC-based macrocyclization. (a) 30% TFA/CH_2Cl_2; (b) LiOH, THF/H_2O; (c) EDC, HOBt, DIPEA, DMF/CH_2Cl_2; (d) 20 mol % CuBr, DBU, toluene, reflux.

Bifunctional steroids **5** and **23**[21g] (shown above in chair conformation) were chosen as scaffolds for the assembly of the macrocyclic structures alternating a steroidal moiety and a short peptide chain. Initially, peptide **20** and **24**—prepared by solution-phase peptide synthesis as shown in Scheme 2—were deprotected according to standard protocols at the Asp side chain and the N-terminus, respectively, while spirostane **5** was also subjected to Boc cleavage. Accordingly, the two step approaches comprise the initial coupling of peptides **20** and **24** to aminospirostane **5** and azido-cholic acid **23** to furnish peptidosteroids **21** and **25**, respectively. Completion of the conjugation process by peptide coupling (EDC/HOBt) was confirmed by TLC analysis, while identity of peptidosteroids **21** and **25** was revealed by ESI-MS. These intermediates were not isolated by chromatography, but gently washed with acidic aqueous solution to remove the coupling by-products and next subjected to CuAAC macrocyclization. To avoid formation of cyclodimers and higher (cyclic) oligomers, click macrocyclization of **21** and **25** was accomplished at 1 mM concentration according to a procedure previously reported for the click cyclization of peptides,[27] i.e., 20 mol % of CuBr and DBU as additive in refluxing toluene for 24 h. Under these conditions, macrocyclic peptide–steroid conjugates **22** and **26** were obtained by chromatography in 59% and 53% yield, respectively, as the only cyclic products. However, the non-cyclized intermediates **21** and **25** were also recovered in 17% and 11% yield, respectively, showing that the cyclization yield can be improved with longer reaction times.

3. Conclusions

We have shown that the CuAAC is a powerful procedure for the conjugation of alkynyl peptides to azidosteroids, proving to be a reliable synthetic alternative for the preparation of pharmaceutically useful peptide–steroid conjugates. The approach demonstrated wide substrate scope, as varied steroidal azides were conjugated in good to excellent yields to oligopeptides of different chain length and amino acid sequence. The triazole-linked conjugates exhibited high stability under strong acidic conditions typically used in SPPS, which along with the great conjugation efficiency opens up the possibility of utilizing this strategy in solid-phase protocols. A sequential peptide coupling/CuAAC-based macrocyclization approach was also implemented with great success, enabling the synthesis of two archetypal macrocyclic peptide–steroid conjugates featuring different ring sizes and topologies. Further prospects may rely on the utilization of the click conjugation/cyclization method for the introduction of conformational constrains in biologically relevant peptide sequences as well as for their conjugation to steroidal carriers, hormones or cell membrane anchors.

4. Experimental

4.1. General

Melting points were determined on a Stuart Scientific apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 400 spectrometer at 399.94 MHz and 100.57 MHz,

respectively. Chemical shifts (δ) are reported in parts per million relative to the TMS (^{1}H NMR) and to the solvent signal (^{13}C NMR). 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 T superconducting magnet. Reactions were monitored by thin-layer chromatography on pre-coated plates with silica gel (Merck) and spots were visualized with a 1% w/v spray of vanillin in perchloric acid and subsequent heating. The solid compounds were recrystallized from selected solvents for the melting points measurements. Flash column chromatography was performed on silica gel 60 (Merck, >230 mesh). DMF, CH_2Cl_2, and DIPEA were dried by distillation from CaH_2.

*4.1.1. 22-Azido-23,24-bisnorcholane-1,4-dien-3-one (**2**).* Alcohol **1** (590 mg, 1.8 mmol) was dissolved in dry CH_2Cl_2 (60 mL) and treated with Et_3N (1.0 mL, 7.4 mmol) and mesyl chloride (0.29 mL, 2.45 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, then diluted with 100 mL of CH_2Cl_2, and washed with brine (2×50 mL). The organic phase was dried over anhyd Na_2SO_4 and concentrated under reduce pressure to dryness. The resulting crude methanesulfonate was dissolved in DMPU (50 mL) and the solution was treated with NaN_3 (185 mg, 3.3 mmol). The reaction mixture was stirred vigorously under nitrogen atmosphere at 50 °C for 24 h and then diluted with 200 mL of Et_2O. The organic phase was washed with aq 10% HCl (2×60 mL) and brine (100 mL), dried over anhyd Na_2SO_4, and evaporated under reduced pressure to dryness. The crude product was purified by flash column chromatography (*n*-hexane/EtOAc 5:1) to give the pure azide **2** (584 mg, 92%) as a white solid. Mp 165–166 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.75 (s, 3H); 1.04 (d, 3H, *J*=6.6 Hz); 1.23 (s, 3H); 2.10 (dt, 1H, *J*=12.9/3.5 Hz); 2.47 (dq, 1H, *J*=13.3/2.5 Hz); 2.47 (td, 1H, *J*=13.3/5.1 Hz); 3.04 (dd, 1H, *J*=11.9/7.2 Hz); 3.37 (dd, 1H, *J*=11.9/3.3 Hz); 6.07 (s, 1H); 6.23 (d, 1H, *J*=10.2 Hz); 7.05 (d, 1H, *J*=10.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.0, 17.6, 18.6 (*C*H$_3$); 22.7, 24.3, 27.7, 32.8, 33.5 (*C*H$_2$); 35.4, 36.8 (*C*H); 39.2 (*C*H$_2$); 42.7, 43.5 (*C*); 52.1, 53.0, 55.1 (*C*H); 57.8 (*C*H$_2$); 123.8, 127.4, 155.8 (*C*H); 169.2 (*C*); 186.3 (*C*O). HRMS (ESI-FT-ICR) *m*/*z*: 376.2370 $[M+Na]^+$; calculated for $C_{22}H_{31}ON_3Na$: 376.2365.

*4.1.2. (25R)-6α-[N-(tert-Butoxycarbonyl)amino]-6α-spirostan-3β-ol (**4**).* Sodium acetate (0.96 g, 11.6 mmol) and hydroxylamine hydrochloride (0.48, 7.0 mmol) were added to a solution of laxogenin (**3**, 1.01 g, 2.35 mmol) in 50 mL of MeOH. The reaction mixture was stirred at reflux for 8 h and then cooled to room temperature, poured into 200 mL of cold water, and the resulting precipitate was filtered under reduced pressure, washed with water, and dried at 60 °C to afford the corresponding oxime. This product was dissolved in glacial HOAc (30 mL) and platinum (IV) oxide hydrate (Adams's catalyst, 200 mg) was added to the solution. The reaction mixture was treated successively with vacuum and hydrogen and finally stirred under hydrogen atmosphere for 6 days. The suspension was then filtered under reduced pressure and the catalyst was washed with glacial HOAc (2×10 mL). Zinc powder was added to the combined filtrates and the suspension was stirred at room temperature for 24 h. Zinc was filtered off and the filtrate was evaporated under reduced pressure to dryness. The resulting crude product was dissolved in 150 mL CH_2Cl_2 and the solution was washed with satd aq $NaHCO_3$ (2×50 mL), dried over anhyd Na_2SO_4, and concentrated under reduced pressure to dryness. The resulting crude product was dissolved in a stirred mixture of THF (30 mL) and satd aq $NaHCO_3$ (15 mL) and treated with di-*tert*-butyl dicarbonate (Boc_2O, 0.93 g, 5.0 mmol). The reaction mixture was stirred for 30 h at room temperature and then, the layers were separated and the aqueous phase was extracted with EtOAc (2×50 mL). The organic extracts were combined, washed with brine (50 mL), dried over anhyd Na_2SO_4, and evaporated under reduced pressure to dryness. Flash column chromatography purification (*n*-hexane/EtOAc 3:1) afforded carbamate **4** (811 mg, 65%) as a white powder. Mp (EtOAc): 202–203 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.76 (s, 3H); 0.79 (d, 3H, *J*=6.4 Hz); 0.96 (d, 3H, *J*=6.8 Hz); 0.96 (s, 3H); 1.44 (s, 9H); 3.08 (br s, 1H); 3.37 (t, 1H, *J*=11.0 Hz); 3.47 (dd, 1H, *J*=10.4/2.8 Hz); 3.56 (m, 1H); 3.91 (br m, 1H); 4.43 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=14.4, 16.3, 17.0, 17.1 (*C*H$_3$); 20.1, 20.9, 26.1 (*C*H$_2$); 28.3 (*C*H$_3$); 28.7 (*C*H$_2$); 30.2 (*C*H); 31.3, 31.4, 31.7 (*C*H$_2$); 34.1 (*C*H); 35.0, 39.9 (*C*H$_2$); 40.7, 41.2 (*C*); 41.6, 42.7, 53.9, 56.0, 62.0 (*C*H); 66.8 (*C*H$_2$); 71.9 (*C*H); 80.6 (*C*H); 109.3, 157.4 (*C*). HRMS (ESI-FT-ICR) *m*/*z*: 554.3814 $[M+Na]^+$; calculated for $C_{32}H_{53}O_5N_1Na$: 554.3816.

*4.1.3. (25R)-3α-Azido-6α-[N-(tert-butoxycarbonyl)amino]-5α-spirostane (**5**).* Spirostanol **4** was submitted to mesylation and nucleophilic substitution with azide in a similar way as described in the synthesis of **2** to furnish azide **5** as a white solid. Mp (EtOAc): 177–178 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.76 (s, 3H); 0.78 (d, 3H, *J*=6.4 Hz); 0.96 (d, 3H, *J*=6.7 Hz); 0.92 (s, 3H); 1.44 (s, 9H); 3.09 (br s, 1H); 3.37 (t, 1H, *J*=11.1 Hz); 3.46 (dd, 1H, *J*=10.6/3.0 Hz); 3.61 (br m, 1H); 3.88 (m, 1H); 4.44 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.6, 14.3, 17.0, 17.1 (*C*H$_3$); 20.1, 20.9, 26.2 (*C*H$_2$); 28.3 (*C*H$_3$); 28.7 (*C*H$_2$); 30.2 (*C*H); 31.3, 31.4, 31.9 (*C*H$_2$); 34.2 (*C*H); 35.0, 39.9 (*C*H$_2$); 40.4, 41.2 (*C*); 41.6, 42.7, 53.9, 56.0, 57.8, 62.0 (*C*H); 66.8 (*C*H$_2$); 80.0 (*C*H); 109.3, 157.4 (*C*). HRMS (ESI-FT-ICR) *m*/*z*: 579.3883 $[M+Na]^+$; calculated for $C_{32}H_{52}O_4N_4Na$: 579.3886.

4.1.4. General peptide coupling procedure. The Boc-protected amino acid (1.0 mmol, 1.0 equiv), HOBt (168 mg, 1.1 mmol, 1.1 equiv), EDC (210 mg, 1.1 mmol, 1.1 equiv), and the *C*-propargylamide amino acid hydrochloride are suspended in dry CH_2Cl_2 (10 mL). Et_3N (0.15 mL, 1.1 mmol, 1.1 equiv) is syringed in one portion and the resulting solution is stirred at room temperature overnight (~12 h). The reaction mixture is then diluted with 100 mL EtOAc, transferred to a separatory funnel, and sequentially washed with 0.5 M aqueous solution of citric acid (2×50 mL) and saturated aqueous suspension $NaHCO_3$ (2×50 mL). The organic phase is dried over $MgSO_4$, filtered, and concentrated under reduced pressure.

4.1.5. General Boc removal procedure. The crude peptide is exposed to high vacuum for 1 h before dissolving it in a 4 M HCl solution in dioxane (2 mL) for Boc removal. As the material dissolved, gas evolution could be detected and the pressure that built up inside the reaction flask is regularly relieved by opening the reaction flask. After 30 min, usually no starting material is detected by thin layer chromatography and the reaction is concentrated under a stream of dry N_2 for about 30 min. The volatiles are then fully removed by concentrating the resulting thick oily residue under reduced pressure in the rotary evaporator and then placing the flask under high vacuum. If required, the hydrochloride salt can be crystallized from frozen diethyl ether.

*4.1.6. Peptide **6**.* *N*-Boc-Gly-OH (175 mg, 1.0 mmol) was coupled to HCl·Lys(Cbz)-NHC_3H_3 (353 mg, 1.0 mmol) according to the peptide coupling procedure. The resulting peptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. The same protocol was employed for the sequential coupling of *N*-Boc-Val-OH (217 mg, 1.0 mmol), *N*-Boc-Phe-OH (265 mg, 1.0 mmol), and *N*-Boc-Ile-OH (231 mg, 1.0 mmol) to afford the crude pentapeptide **6**. Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished the pure peptide **6** (583 mg, 58%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.86 (d, 6H, *J*=6.4 Hz); 0.90 (d, 6H, *J*=6.8 Hz); 1.24–1.32 (m, 2H); 1.43 (s, 9H); 1.52–1.61 (m, 4H); 1.67–1.72 (m, 2H); 2.06 (m, 1H); 2.38 (t, 1H, *J*=2.4 Hz); 3.06 (dd, 2H, *J*=14.0/6.4 Hz); 3.18 (m, 2H); 4.06 (m, 2H); 4.16 (m, 1H); 4.20 (m, 2H); 4.51 (m, 2H); 4.60 (m, 1H); 4.77 (t, 1H *J*=7.3 Hz); 4.86 (d, 1H *J*=7.6 Hz); 5.08 (s, 2H); 5.42 (m, 1H); 5.64 (t, 1H, *J*=7.2 Hz); 5.71 (d, 1H, *J*=7.6 Hz); 7.12–7.39 (m, 10H); 7.52 (br s, 1H);

7.80 (br s, 1H); 8.14 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.5, 16.2, 17.8, 18.9 (CH_3); 24.6 (CH); 24.7 (CH_2); 28.0 (CH_3); 28.3, 29.2, 30.5 (CH_2); 31.4 (CH); 38.6, 41.5 (CH_2); 47.8, 48.6, 51.6, 52.3 (CH); 53.6, 56.2, 59.4 (CH_2); 71.4 (CH); 78.2, 80.0 (C); 126.8, 127.4, 128.3, 128.4, 130.0, 135.7 (CH); 136.1, 138.3 (C); 156.6, 157.8; 158.3; 171.0, 171.4, 172.7, 173.2 (C=O). HRMS (ESI-FT-ICR) *m/z*: 856.4574 $[M+Na]^+$; calculated for $C_{44}H_{63}O_9NaN_7$: 856.4589.

*4.1.7. Peptide **12**.* *N*-Boc-Ile-OH (231 mg, 1.0 mmol) was coupled to HCl·Lys(Cbz)-NHC_3H_3 (353 mg, 1.0 mmol) according to the peptide coupling procedure. The resulting peptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. The same protocol was employed for the sequential coupling of *N*-Boc-Val-OH (217 mg, 1.0 mmol) and *N*-Boc-Phe-OH (265 mg, 1.0 mmol) to afford the crude tetrapeptide **12**. Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished the pure tetrapeptide **12** (567 mg, 73%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 95:5): δ=0.85 (d, 3H, *J*=6.8 Hz); 0.92 (d, 3H, *J*=7.0 Hz); 0.96–1.08 (m, 6H); 1.24–1.30 (m, 2H); 1.43 (s, 9H); 1.46 (m, 1H); 1.50–1.62 (m, 4H); 1.86 (m, 1H); 2.30 (t, 1H, *J*=2.5 Hz); 3.15 (m, 4H); 3.82 (m, 2H); 4.02 (m, 1H); 4.45 (m, 2H); 4.50 (t, 1H *J*=6.3 Hz); 4.72 (m, 1H); 5.08 (s, 2H); 5.68 (d, 1H, *J*=7.2 Hz); 7.14–7.48 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 95:5): δ=12.4, 16.2, 17.6, 18.9 (CH_3); 23.6 (CH); 24.7 (CH_2); 28.0 (CH_3); 29.2, 30.4 (CH_2); 31.7 (CH); 37.4, 40.6, 44.1 (CH_2); 47.8, 48.6, 52.1 (CH); 54.1 (CH); 58.4, 66.4 (CH_2); 74.2 (CH); 80.0, 81.2 (C); 126.9, 127.8, 128.3, 128.4, 129.0, 135.7 (CH); 136.4, 138.1 (C); 156.8, 157.7, 171.0, 171.7, 172.7, 173.1 (C=O). HRMS (ESI-FT-ICR) *m/z*: 799.4392 $[M+Na]^+$; calculated for $C_{42}H_{60}O_8NaN_6$: 799.4409.

*4.1.8. Peptide **15**.* *N*-Boc-Val-OH (217 mg, 1.0 mmol) was coupled to HCl·Phe-NHC_3H_3 (238 mg, 1.0 mmol) according to the peptide coupling procedure. The resulting peptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. The same protocol was employed for the coupling of *N*-Boc-Lys(Cbz)-OH (380 mg, 1.0 mmol) to afford the crude tripeptide **15**. Flash column chromatography purification (CH_2Cl_2/EtOAc 8:1) furnished the pure peptide **15** (510 mg, 77%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 95:5): δ=7.35–7.11 (m, 10H); 5.68 (d, 1H, *J*=7.6 Hz); 5.64 (t, 1H, *J*=7.2 Hz); 5.08 (s, 2H); 4.86 (d, 1H *J*=7.6 Hz); 4.78 (t, 1H *J*=6.3 Hz); 4.51 (m, 2H); 4.15 (m, 1H); 4.04 (m, 1H); 3.16 (m, 2H); 3.07 (dd, 2H, *J*=14/6.4 Hz); 2.23 (t, 1H, *J*=2.4 Hz); 2.06 (m, 1H); 154–1.49 (m, 4H); 1.43 (s, 9H); 0.90 (d, 3H, *J*=7.0 Hz); 0.88 (d, 3H, *J*=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 95:5): δ=17.6, 18.9 (CH_3); 28.0 (CH_3); 29.0, 30.5 (CH_2); 31.5 (CH); 37.5, 40.2 (CH_2); 52.1 (CH); 53.1 (CH_3) 54.1 (CH); 58.4, 66.4 (CH_2); 80.0 (C); 126.9, 127.8, 128.3, 128.4, 129.0, 135.7 (CH); 136.4 (C); 156.8, 157.7, 171.0, 171.7, 172.7 (CO). HRMS (ESI-FT-ICR) *m/z*: 686.3529 $[M+Na]^+$; calculated for $C_{36}H_{49}O_7NaN_5$: 686.3524.

*4.1.9. Peptide **17**.* *N*-Boc-Ile-OH (231 mg, 1.0 mmol) was coupled to HCl·Ala-NHC_3H_3 (162 mg, 1.0 mmol) according to the peptide coupling procedure to afford the crude dipeptide **17**. Flash column chromatography purification (CH_2Cl_2/EtOAc 8:1) furnished the pure peptide **17** (322 mg, 95%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 95:5): δ=0.92 (d, 3H, *J*=6.6 Hz); 0.96 (d, 3H, *J*=6.0 Hz); 1.34 (d, 3H, *J*=6.8 Hz); 1.43 (s, 9H); 1.46–1.58 (m, 2H); 2.08 (m, 1H); 2.23 (t, 1H, *J*=2.4 Hz); 4.16 (t, 1H, *J*=7.1 Hz); 4.40 (m, 2H); 4.62 (m, 1H); 7.44 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 95:5): δ=11.7, 16.4, 18.9, 28.2 (CH_3); 30.0 (CH_2); 32.6 (CH); 43.7 (CH_2); 50.6 (CH); 76.4 (CH); 79.2, 80.0 (C); 158.2, 169.7, 172.8 (C=O). HRMS (ESI-FT-ICR) *m/z*: 362.2042 $[M+Na]^+$; calculated for $C_{17}H_{29}O_4NaN_3$: 362.2065.

*4.1.10. Peptide **20**.* *N*-Boc-Phe-OH (265 mg, 1.0 mmol) was coupled to HCl·Leu-NHC_3H_3 (204 mg, 1.0 mmol) according to the peptide coupling procedure. The resulting peptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. The same protocol was employed for the coupling of *N*-Boc-Asp(Me)-OH (247 mg, 1.0 mmol) to afford the crude tripeptide **20**. Flash column chromatography purification (CH_2Cl_2/EtOAc 8:1) furnished the pure peptide **20** (397 mg, 73%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 95:5): δ=0.93 (d, 3H, *J*=6.6 Hz); 0.95 (d, 3H, *J*=5.8.0 Hz); 1.37 (s, 9H); 1.56 (m, 1H); 1.65–1.70 (m, 2H); 2.19 (t, 1H, *J*=2.6 Hz); 3.10 (m, 2H); 3.68 (s, 3H); 3.98 (m, 2H); 4.12 (m, 2H); 4.42 (m, 1H); 4.54 (d, 1H *J*=7.6 Hz); 4.64 (m, 1H); 7.12–7.28 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 95:5): δ=19.8, 21.4 (CH_3); 25.4 (CH); 28.3 (CH_3); 39.7, 41.2, 42.8 (CH_2); 48.6, 50.7, 51.8 (CH); 53.7 (CH_3); 60.5 (CH_2); 70.2 (CH); 79.6, 80.0 (C); 125.4, 126.8, 128.5 (CH); 136.4 (C); 156.8, 168.6, 170.8, 172.1, 173.8 (C=O). HRMS (ESI-FT-ICR) *m/z*: 567.2784 $[M+Na]^+$, calculated for $C_{28}H_{40}O_7NaN_4$: 567.2795.

*4.1.11. Methyl ester deprotection of peptide **20**.* Peptide **20** (190 mg, 0.35 mmol) was dissolved in THF/H_2O (2:1, 15 mL) and LiOH (38 mg, 0.9 mmol) was added at 0 °C. The mixture was stirred at 0 °C for 6 h and then acidified with aq 10% $NaHSO_4$ to pH 3. The resulting phases were separated and the aqueous phase was additionally extracted with EtOAc (2×20 mL). The combined organic phases were dried over anhyd Na_2SO_4 and concentrated under reduced pressure to afford the carboxylic acid free peptide **20**.

*4.1.12. Peptide **24**.* *N*-Boc-Leu-OH (231 mg, 1.0 mmol) was coupled to HCl·Ala-NHC_3H_3 (162 mg, 1.0 mmol) according to the peptide coupling procedure. The resulting peptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. The same protocol was employed for the sequential coupling of *N*-Boc-Ala-OH (189 mg, 1.0 mmol) and *N*-Boc-Ile-OH (231 mg, 1.0 mmol) to afford the crude tetrapeptide **24**. Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished the pure tetrapeptide **24** (360 mg, 69%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.87–0.94 (m, 12H); 1.35 (d, 3H, *J*=7.0 Hz); 1.38 (d, 3H, *J*=7.2 Hz); 1.43 (s, 9H); 1.46–1.58 (m, 2H); 1.62–1.76 (m, 2H); 1.81 (m, 1H); 2.25 (t, 1H, *J*=2.4 Hz); 4.12 (t, 1H, *J*=7.2 Hz); 4.61 (t, 1H, *J*=7.4 Hz); 4.68–4.76 (m, 2H); 5.62 (d, 1H, *J*=8.2 Hz); 7.48 (t, 1H, *J*=7.8 Hz); 7.64 (d, 1H, *J*=7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.5, 14.5, 17.2, 18.4, 22.4, 22.7 (CH_3); 24.6 (CH); 24.7 (CH_2); 28.2 (CH_3); 30.6 (CH_2); 41.4 (CH_2); 46.8, 48.6, 51.6, 52.9, 59.3, 73.6 (CH); 79.7 (C); 158.0, 171.7, 171.8, 172.6, 173.1 (C=O). HRMS (ESI-FT-ICR) *m/z*: 546.3262 $[M+Na]^+$, calculated for $C_{17}H_{29}O_4NaN_3$: 546.3285.

*4.1.13. Boc deprotection of peptide **24**.* Peptide **24** (262 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (7 mL) and treated with trifluoracetic 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. TFA was removed completely by repetitive addition and evaporation of CH_2Cl_2 to furnish quantitatively the TFA salt of peptide **24** as white foam, which was used without further purification.

4.1.14. Click approach for the conjugation of peptides to steroids. The peptidyl alkyne (0.2 mmol) and the azidosteroid (0.2 mmol) are dissolved in THF (5 mL) and treated with an 8 M aqueous a solution of $Cu(OAc)_2 \cdot H_2O$ (20 μL, 0.04 mmol) and a freshly prepared 8 M aqueous solution of sodium ascorbate (40 μL, 0.08 mmol). The reaction mixture is stirred at room temperature for 24 h and then diluted with 50 mL of EtOAc. The organic phase is washed with brine (20 mL), dried over anhyd Na_2SO_4, and concentrated under reduced pressure. The resulting crude product is purified by flash column chromatography.

*4.1.15. Peptide–steroid conjugate **7**.* Peptidyl alkyne **6** (165 mg, 0.2 mmol) and azidosteroid **2** (70 mg, 0.2 mmol) in THF (5 mL) were

reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **7** (211 mg, 89%) as a white solid. Mp (AcOEt): 203–204 °C. ^{1}H NMR (400 MHz, CD_3OD): δ=0.78 (d, 3H, J=6.8 Hz); 0.79–0.84 (m, 12H); 0.93 (d, 6H, J=6.6 Hz); 0.98–1.53 (m, 30H); 1.86–1.63 (m, 8H); 1.98–2.10 (m, 6H); 2.39 (d, 1H, 1H, J=12.3 Hz); 2.57 (td, 1H, J=13.3/4.9 Hz); 2.93 (dd, 1H, J=13.6/9.1 Hz); 3.07–3.12 (m, 4H); 3.73–3.95 (m, 4H); 3.99–4.10 (m, 2H); 4.26–4.31 (m, 2H); 4.34–4.40 (m, 1H); 4.45 (m, 2H); 4.61 (s, 1H); 4.72 (m, 1H); 5.05 (m, 2H); 6.05 (s, 1H); 6.20 (dd, 1H, J=10.1/1.9 Hz); 7.18 (m, 1H); 7.22–7.27 (m, 5H); 7.33 (m, 5H); 7.80 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.4, 16.6, 16.8, 18.0, 18.4, 19.1 (CH_3); 22.4, 24.6 (CH_2); 25.2 (*C*H); 26.4, 27.2 (CH_2); 28.2 (CH_3); 29.6, 30.2, 30.8 (CH_2); 31.6 (*C*H); 32.4, 34.1 (CH_2); 35.6, 36.4 (*C*H); 38.4, 39.2, 41.6 (CH_2); 42.6, 43.2 (*C*); 47.2, 48.4, 52.3, 52.8, 53.1 (*C*H); 53.8 (CH_2); 55.4 (*C*H); 56.6, 58.3, 59.4, 66.4 (CH_2); 81.2 (*C*); 123.4, 126.2, 127.1, 127.8, 128.3, 129.6, 130.4, 130.8 (*C*H); 136.2, 136.4, 138.8 (*C*); 155.8 (*C*H); 156.1, 156.3 (*C*=O); 169.4 (*C*); 171.0, 172.0, 172.7, 173.2, 186.3 (*C*=O). HRMS (ESI-FT-ICR) m/z: 1209.7042 $[M+Na]^+$; calculated for $C_{66}H_{94}N_{10}NaO_{10}$: 1209.7091.

*4.1.16. Peptide–steroid conjugate **9**.* Peptidyl alkyne **6** (165 mg, 0.2 mmol) and azidosteroid **8** (63 mg, 0.2 mmol) in THF (5 mL) were reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **9** (198 mg, 86%) as a white solid. Mp (AcOEt): 197–199 °C. ^{1}H NMR (400 MHz, CD_3OD): δ=0.78 (d, 3H, J=6.8 Hz); 0.82 (t, 3H, J=7.4 Hz); 0.85 (s, 3H); 0.93 (d, 6H, J=6.8 Hz); 0.94 (s, 3H); 1.04–2.15 (m, 38H); 2.40 (dd, 1H, J=11.0/8.5 Hz); 2.88–2.96 (m, 1H); 3.07–3.12 (m, 3H); 3.75 (m, 1H); 3.86–3.91 (m, 2H); 4.04 (d, 1H, J=7.1 Hz); 4.27 (m, 1H); 4.46 (m, 2H); 4.61 (m, 1H); 4.72 (d, 1H, J=7.1 Hz); 5.04 (m, 2H); 7.17–7.33 (m, 10H); 7.93 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.1, 12.2, 12.4, 16.2 18.2, 19.2 (CH_3); 20.3, 20.8 (CH_2); 21.4 (CH); 22.1, 23.3, 24.5 (CH_2); 24.6 (CH); 24.7, 26.8 (CH_2); 28.2 (CH_3); 28.6, 28.9, 29.0, 29.2 (CH_2); 31.3 (C); 32.1 (CH); 32.9 (CH_2); 35.4, 35.8 (CH); 36.3, 36.8, 38.6 (CH_2); 43.0 (C); 47.1, 47.8, 51.1, 52.3, 54.8 (CH); 56.2 59.4, 66.2 (CH_2); 71.4 (CH); 80.0 (C); 125.4, 126.8, 127.4, 128.3, 128.4, 130.0, 135.7 (CH); 131.4, 136.4, 138.2 (C); 156.6, 157.8; 158.3; 171.0, 171.4, 172.7, 173.2, 205.3 (C=O). HRMS (ESI-FT-ICR) m/z: 1171.6893 $[M+Na]^+$; calculated for $C_{62}H_{92}N_{10}NaO_{10}$: 1171.6924.

*4.1.17. Peptide–steroid conjugate **11**.* Peptidyl alkyne **6** (165 mg, 0.2 mmol) and azidosteroid **10** (83 mg, 0.2 mmol) in THF (5 mL) were reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **11** (204 mg, 82%) as a white solid. Mp (AcOEt): 212–214 °C. ^{1}H NMR (400 MHz, CD_3OD): δ=0.67 (s, 3H); 0.78 (d, 3H, J=6.4 Hz); 0.82 (d, 3H, J=7.2 Hz); 0.87 (d, 6H, J=6.8 Hz); 0.91 (s, 3H); 089–0.94 (m, 12H); 1.00–2.11 (m, 52H); 2.28–2.33 (m, 2H); 2.95 (m, 1H); 3.10 (m, 3H); 3.80 (m 1H); 3.88 (m, 1H); 3.92 (m, 1H); 4.03 (m, 1H); 4.27 (m, 1H); 4.45–4.50 (m, 2H); 4.61 (m, 2H); 5.05 (s, 2H); 7.23 (m, 5H); 7.32 (m, 5H); 7.93 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.4, 12.3, 14.6, 15.8, 16.5, 16.8, 17.8, 18.6, 19.1 (CH_3); 19.6 (*C*H); 20.4, 23.5, 23.8, 24.1 (CH_2); 24.8 (*C*H); 25.2, 28.1, 28.3 (CH_2); 28.6 (CH_3); 29.1, 29.4, 30.2, 30.6 (CH_2); 31.8, 32.6 (*C*H); 33.6 (*C*); 34.2 (*C*H); 35.4, 35.6, 35.8, 38.6, 39.2, 39.7, 41.5 (CH_2); 42.4 (*C*); 42.8, 47.4, 48.1, 48.6, 52.4 (*C*H); 53.6 (CH_2); 55.9, 56.2 (*C*H); 56.8, 59.4 (CH_2); 71.2 (*C*H); 80.1 (*C*); 124.2, 125.8, 126.4, 127.2, 128.6, 129.2, 130.0 (*C*H); 136.4 (C); 146.2, 148.6 (*C*); 156.4, 156.8, 158.3, 171.2, 171.4, 172.7, 173.2 (*C*=O). HRMS (ESI-FT-ICR) m/z: 1269.8352 $[M+Na]^+$; calculated for $C_{71}H_{110}N_{10}NaO_9$: 1269.8394.

*4.1.18. Peptide–steroid conjugate **14**.* Peptidyl alkyne **12** (155 mg, 0.2 mmol) and azidosteroid **13** (95 mg, 0.2 mmol) in THF (5 mL) were reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **14** (177 mg, 71%) as a white solid. Mp (AcOEt): 235–236 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.70–0.96 (m, 22H); 1.19–1.95 (m, 54H); 2.20 (m, 2H); 2.63 (m, 1H); 2.79 (m, 1H); 2.98 (m, 2H); 3.16 (m, 2H); 3.36 (t, 1H, J=10.8 Hz); 3.47 (m, 1H); 3.53 (m, 1H); 4.17–4.325 (m, 2H); 4.46–4.36 (m, 4H); 4.62 (m, 1H); 4.84 (m, 1H); 5.05 (s, 2H); 5.08 (m, 1H); 7.13 (m, 3H); 7.17 (m, 1H); 7.33–7.39 (m, 12H); 7.85 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.4, 14.6, 15.2, 16.7, 16.9, 17.4, 19.4, 19.8 (CH_3); 21.4 (CH_2); 23.6 (*C*H); 24.7, 25.0, 27.4 (CH_2); 28.2 (CH_3); 28.6, 29.1 (CH_2); 29.4, 30.6 (*C*H); 31.3, 31.6, 33.4, 35.2, 34.9, 36.4 (CH_2); 39.0 (*C*); 39.5, 40.3 (CH_2); 40.6 (*C*); 41.6 (CH_2); 42.6, 43.1(*C*H); 44.6 (CH_2); 46.8, 47.6 (*C*H); 51.3 (*C*); 52.8, 54.6, 59.4, 60.4, 62.6 (*C*H); 66.2, 68.4 (CH_2); 73.1 (*C*); 74.2, 77.6, 80.8 (*C*H); 81.2, 109.2 (*C*); 124.9, 125.8, 126.3, 127.2, 128.4, 129.0, 135.8 (*C*H); 136.4, 137.3 (*C*); 156.8, 157.7, 171.0, 171.7, 172.7, 173.1 (*C*=O). HRMS (ESI-FT-ICR) m/z: 1272.7623 $[M+Na]^+$; calculated for $C_{69}H_{103}N_9NaO_{12}$: 1272.7664.

*4.1.19. Peptide–steroid conjugate **16**.* Peptidyl alkyne **15** (132 mg, 0.2 mmol) and azidosteroid **10** (83 mg, 0.2 mmol) in THF (5 mL) were reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **16** (192 mg, 90%) as a white solid. Mp (AcOEt): 199–200 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.63 (s, 3H); 0.75 (d, 3H, J=6.2 Hz); 0.81 (d, 3H, J=6.6 Hz); 0.85 (d, 3H, J=6.8 Hz); 0.86 (d, 6H, J=6.4 Hz); 0.88 (s, 3H); 0.94–2.08 (m, 50H); 2.27 (d, 1H, J=13.0 Hz); 2.94 (dd, 1H, J=13.0/8.46 Hz); 3.17 (m, 2H); 3.23 (m, 1H); 4.04 (m, 1H); 4.22 (m, 1H); 4.43 (dd, 1H, J=15.1/5.2 Hz); 4.52 (m, 2H); 4.87 (m, 1H); 5.08 (m, 2H); 5.36 (t, 1H, J=5.6 Hz); 5.75 (d, 1H, J=5.0 Hz); 7.03 (m, 1H); 7.12–7.17 (m, 6H); 7.33 (m, 5H); 7.43 (m, 1H); 7.58 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.8, 12.2, 19.2 (CH_3); 20.7, 22.4 (CH_2); 22.5, 22.8 (CH_3); 23.9, 24.1, 25.3 (CH_2); 28.0 (CH); 28.2 (CH_2); 28.6 (CH_3); 29.4 (CH_2); 30.1 (CH); 31.6, 32.7, 33.1, 35.1 (CH_2); 35.3, 35.8 (CH); 36.1, 37.9, 39.5 (CH_2); 39.7 (*C*H); 39.8, 39.9 (CH_2); 42.5 (C); 53.9 (CH); 55.1 (C); 56.1, 56.2, 56.3 (CH); 66.6 (CH_2); 80.2 (*C*); 121.6, 126.6, 128.0, 128.3, 128.5, 130.0 (CH); 136.5, 136.8, 144.1 (*C*); 156.4, 156.9, 170.8, 171.0, 173.2 (*C*=O). HRMS (ESI-FT-ICR) m/z: 1099.7231 $[M+Na]^+$; calculated for $C_{63}H_{96}N_8NaO_7$: 1099.7298.

*4.1.20. Peptide–steroid conjugate **19**.* Peptidyl alkyne **17** (68 mg, 0.2 mmol) and azidosteroid **18** (83 mg, 0.2 mmol) in THF (5 mL) were reacted in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.04 mmol) and sodium ascorbate (0.08 mmol) according to the click conjugation procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 3:2) afforded conjugate **19** (140 mg, 93%) as a white solid. Mp (AcOEt): 188–189 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.65 (s, 3H); 0.85–0.89 (d, 6H); 0.92 (d, 3H, J=6.2 Hz); 1.01 (s, 3H); 1.17–1.47 (m, 14H); 1.14 (d, 6H, J=6.6 Hz); 1.37 (d, 3H, J=7.1 Hz); 1.43 (s, 9H); 1.54–1.60 (m, 2H); 1.74–1.98 (m, 10H); 2.17–2.25 (dd, 2H); 2.36 (m, 1H); 3.67 (s, 3H); 3.84 (m, 1H); 3.94 (t, 1H, J=7.0 Hz); 4.53 (m, 1H); 5.18 (d, 1H, J=7.6 Hz); 6.82 (d, 1H, J=7.5 Hz); 7.34 (br s, 1H); 7.58 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.1, 11.7, 12.2, 15.6, 18.2 (CH_3); 20.6 (CH_2); 23.4 (CH_3); 23.5 (*C*H); 24.1, 24.7, 26.3, 27.1, 27.9 (CH_2); 28.3 (CH_3); 30.9, 31.0, 33.9 (CH_2); 34.7 (*C*); 35.1 (CH_2); 35.3, 35.8, 37.1 (*C*H); 39.9 (CH_2); 40.5, 42.1, 42.7 (*C*H); 43.6 (*C*); 48.7 (CH_2); 51.5 (CH_3); 55.8, 56.2, 61.0 (*C*H); 80.1 (*C*); 120.2 (CH); 136.1 (*C*); 156.9, 171.4, 172.3, 174.9 (*C*=O). HRMS (ESI-FT-ICR) m/z: 777.5254 $[M+Na]^+$; calculated for $C_{42}H_{70}N_6NaO_6$: 777.5321.

*4.1.21. Cyclopeptide–steroid conjugate **22**.* Azidosteroid **5** (166 mg, 0.3 mmol) was dissolved in CH_2Cl_2 (7 mL) and treated with trifluoracetic acid (TFA, 3 mL) at 0 °C. The reaction mixture was

allowed to reach room temperature and stirred for 4 h. The solvent was evaporated under reduced pressure and the resulting product was dissolved in CH_2Cl_2 (50 mL), washed with satd aq Na_2CO_3 (2×10 mL), and dried over anhyd Na_2SO_4. Evaporation under reduced pressure gave the free amine as pale yellow foam. This compound was added to a solution of deprotected peptide **20** (159 mg, 0.3 mmol), EDC (57 mg, 0.3 mmol), and HOBt (40 mg, 0.3 mmol) in CH_2Cl_2/DMF (10 mL, 4:1, v/v). DIPEA (50 μL, 0.30 mmol) was added and the mixture was stirred at room temperature under nitrogen atmosphere for 12 h, then treated with further EDC (57 mg, 0.3 mmol) and HOBt (40 mg, 0.3 mmol), and stirred for additional 12 h. The mixture was diluted with CH_2Cl_2 (80 mL) and washed with aq 10% $NaHCO_3$ (2×50 mL), aq 10% HCl (50 mL), and brine (50 mL). The solution was dried over anhyd Na_2SO_4 and evaporated under reduced pressure. The resulting peptidosteroid **21** was dried under high vacuum and then suspended in 300 mL of dry toluene. The suspension was treated with DBU (0.12 mL, 1.0 mmol) and CuBr (8.5 mg, 0.06 mmol, 0.2 equiv), and then stirred vigorously at reflux under nitrogen atmosphere for 24 h. The volatiles were removed under reduced pressure and the resulting crude product was purified by flash column chromatography (CH_2Cl_2/EtOAc 3:2) to furnish the cyclopeptide–steroid conjugate **22** (171 mg, 59%) as pale yellow solid. Mp (AcOEt): 227–228 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.78 (s, 3H); 0.79 (d, 3H, J=6.4 Hz); 0.87 (d, 3H, J=6.1 Hz); 0.90 (d, 3H, J=6.2 Hz); 0.96 (d, 3H, J=6.8 Hz); 1.20 (s, 3H); 1.36 (s, 9H); 1.30–2.03 (m, 30H); 2.20 (m, 1H); 2.42 (m, 1H); 2.57 (m, 1H); 2.78 (dd, 1H, J=15.6/6.2 Hz); 2.90 (dd, 1H, J=13.6/8.7 Hz); 3.15 (dd, 1H, J=13.6/4.7 Hz); 3.36 (t, 1H, J=10.9 Hz); 3.47 (m, 1H); 3.48 (m, 1H); 3.96 (dd, 1H, J=16.6/6.0 Hz); 4.15 (m, 2H); 4.30–4.73 (br m, 4H); 4.80 (m, 1H); 5.35 (d, 1H, J=6.1 Hz); 7.17–7.30 (m, 5H); 7.42 (d, 1H, J=7.6 Hz); 7.51 (d, 1H, J=7.1 Hz); 7.70 (br s, 1H); 7.78 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.6, 12.4, 16.5, 16.9, 17.3, 18.4 (*C*H$_3$); 20.7 (*C*H$_2$); 21.6 (*C*H); 22.1 (*C*H$_2$); 22.7, 24.8 (*C*H); 25.4 (*C*H$_2$); 28.1 (*C*H$_3$); 28.7, 29.1 (*C*H$_2$); 29.6, 30.1 (*C*H); 30.8, 31.3, 34.5, 34.7, 35.0, 37.8 (*C*H$_2$); 39.5 (*C*); 40.7 (*C*H$_2$); 41.2 (*C*); 42.6 (*C*H); 42.9, 43.1 (*C*H$_2$); 45.0, 50.6, 52.3, 55.2, 57.9, 58.4, 61.8 (*C*H); 66.7 (*C*H$_2$); 870.4 (*C*H); 80.2, 109.0 (*C*); 122.8, 126.9, 128.5, 129.2 (*C*H); 136.4, 144.2 (*C*); 156.1, 169.7, 170.9, 172.5, 172.8 (*C*=O). HRMS (ESI-FT-ICR) m/z: 991.5992 $[M+Na]^+$; calculated for $C_{54}H_{80}N_8NaO_8$: 991.5997.

*4.1.22. Cyclopeptide–steroid conjugate **26**.* To a solution of azidocholic acid **23** (130 mg, 0.3 mmol), EDC (57 mg, 0.3 mmol), and HOBt (40 mg, 0.3 mmol) in CH_2Cl_2/DMF (10 mL, 4:1, v/v) was added the TFA salt of deprotected peptide **24** (156 mg, 0.3 mmol) and DIPEA (100 μL, 0.6 mmol). The reaction mixture was stirred at room temperature under nitrogen atmosphere for 12 h, then treated with further EDC (57 mg, 0.3 mmol) and HOBt (40 mg, 0.3 mmol), and stirred for additional 12 h. The mixture was diluted with CH_2Cl_2 (80 mL) and washed with aq 10% $NaHCO_3$ (2×50 mL), aq 10% HCl (50 mL), and brine (50 mL). The solution was dried over anhyd Na_2SO_4, evaporated under reduced pressure and dried carefully under high vacuum. The resulting peptidosteroid **25** was dried under high vacuum and suspended in 300 mL of dry toluene. The suspension was treated with DBU (0.12 mL, 1.0 mmol) and CuBr (8.5 mg, 0.06 mmol, 0.2 equiv), and then stirred vigorously at reflux under nitrogen for 24 h. The volatiles were removed under reduced pressure and the resulting crude product was purified by flash column chromatography (CH_2Cl_2/MeOH 8:1) to furnish cyclopeptide–steroid conjugate **26** (133 mg, 53%) as white solid. Mp (MOH): 196–198 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.67–0.73 (s, 6H); 0.85–1.11 (m, 24H); 1.19–1.60 (m, 28H); 1.70–1.93 (m, 12H); 2.34 (m, 2H); 2.37 (m, 1H); 2.81 (m, 1H); 4.01–4.22 (m, 3H); 4.35 (m, 1H); 4.53 (m, 1H); 4.78 (m, 1H); 4.86 (m, 1H); 5.03 (m, 1H); 5.15 (m, 1H); 7.58 (s, 1H); 8.43 (m, 1H); 8.74 (m, 1H); 9.17 (m, 1H); 9.52 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.2, 11.6, 12.4, 14.6, 16.2, 18.2 (*C*H$_3$); 20.2 (*C*H$_2$); 22.1, 22.6, 23.6 (*C*H$_3$); 24.6 (*C*H); 25.1, 25.7, 26.2, 26.8, 27.9, 28.1 (*C*H$_2$); 28.3 (*C*H$_3$); 30.2, 31.4, 32.9 (*C*H$_2$); 33.6 (*C*); 34.8 (*C*H$_2$); 35.8, 36.4 (*C*H); 40.9, 41.6 (*C*H$_2$); 42.5 (*C*H); 43.0 (*C*); 45.7, 46.8, 48.3 (*C*H); 51.8, 52.6, 53.4, 55.7, 56.8, 59.6, 62.4, 72.8, 73.2 (*C*H); 136.5 (*C*); 170.4, 171.2, 172.3, 172.5, 173.6 (*C*=O). HRMS (ESI-FT-ICR) m/z: 861.5592 $[M+Na]^+$; calculated for $C_{45}H_{74}N_8NaO_7$: 861.5578.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2013.10.032.

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