17α-Ethynylestradiol Peptide Labeling by 'Click' Chemistry

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Abstract: A synthesis of 17α-ethynylestradiol-labeled native peptides is reported. The peptide moiety is tethered to the steroid hormone by a 1,2,3-triazole bridge formed by a CuAAC reaction in which the azido group of the peptide combines with the terminal acetylenic moiety of ethynylestradiol to link the two bioactive molecules. Thus bioconjugates containing the hormone moiety at three positions within the peptide molecule could be useful targets for hormono–enzyme interaction studies.

Key words: cyclization, azides, alkynes, peptides, drugs, click chemistry

The steroidal estrogen (17*R*)-17-ethynylestradiol (17EE), a mechanism-based inactivator of cytochrome P450s (P450s) 2B1 and 2B6,[1] is useful for the identification of the active sites of proteins and enzymes.[2] The introduction of a labeled 17EE molecule into a cell could help determine if modification of the P450 apoprotein by a reactive 17EE intermediate is accompanied by loss of activity in the 2B enzyme.[2a,d] Although the relationship between membrane, cytosolic, and nuclear actions of 17EE has been investigated, the interaction between estrogen receptor mediated genomic and non-genomic pathways needs further study.[3] Literature synthetic and biochemical studies of 17EE labeled glycine peptoids,[2b,c,4] S-linked side-chain 17EE peptide conjugates,[2a] and 'miktoarm' core-crosslinked star copolymers[5a] have assisted the identification of the P450s enzymatic activity. Macromolecular derivatives of estradiol,[5b,c] including 17EE bovine serum albumin,[5d] cyclodextrin,[5e] and dendrimer conjugates,[5f] have previously assisted studies of the activation of the membrane-associated estrogen receptors,[3a] but the highly variable stability and activity of 17EE–macromolecule complexes requires further development of flexible and stable 17EE bioconjugates.

Click chemistry[6a] is very useful for the synthesis of peptidomimetics,[2b,c,4b] and bioconjugates[6b,c] and for the labeling of recombinant and membrane proteins in living bacteria.[6d–f] The high chemoselectivity,[7a,b] speed, green reaction conditions, and excellent yields[7c] of copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions have encouraged multiple applications in chemical biology.[7d,e] We now combine two biologically active molecules capable of cell recognition to prepare nontoxic bioconjugates through CuAAC-catalyzed reactions of native peptides with 17EE. We believe that successful and selective combination of tetrapeptide scaffolds with 17EE by a 1,2,3-triazole linkage should allow the generation of novel bioactive peptidomimetics ligands tailored for specific site-selective interactions with corresponding receptors.

We planned to label 17EE with azido-containing peptides **I**–**III** containing the azido group in diverse positions within the peptide moiety to access to a set of three 17EE steroid-enzyme conjugates **1**–**3** (Scheme 1).

Since organic azides are excellent reaction partners for a variety of transformations, numerous approaches have been developed to access peptides with an azido moiety ready for further functionalization.[8] The major routes as prior steps for introduction of azide into the molecule are: (i) nucleophilic substitution of a leaving group with an azide anion[9a,b] and (ii) diazo transfer from primary amines.[9c,d] The approach (i) was implemented to convert 2-chloroethylamine into the corresponding azide, which was then engaged in the synthesis of target **I**.[10] Target **II** was synthesized by route (ii) α-Boc-protected lysine **14** was converted into an ε-azide derivative by diazo transfer.[11] Enantiopure azidoalanine **23** for target **III** was synthesized from L-alanine using diazo transfer methodology developed earlier in our group.[11]

Conjugate **1** was synthesized by solution-phase coupling–deprotection methodology,[12a] starting from 2-azidoethylamine (**5**) (Scheme 2). Further reaction of **5** with Boc-protected tryptophan employing benzotriazole as a coupling additive[12b] generated **6**. A sequence of successive steps of Boc-amino acid couplings and the Boc group deprotections resulted in adding valine, phenylalanine, and alanine units to the peptide chain to afford azidopeptide **12**. Tetrapeptide **12** was then labeled with 17EE in the presence of copper(I) by application of the Sharpless and Fokin procedure,[7c] affording **13** which was deprotected under a positive pressure of hydrogen, using palladium on carbon as a catalyst, to give target conjugate **1**.

Synthesis of bioconjugate **2** required the introduction of ε-azido lysine **15** into a peptide chain. Further *N*,*N*′-dicyclohexylcarbodiimide-mediated coupling of azide **15** with L-phenylalanine methyl ester followed by the deprotection of **16** gave hydrochloride salt **17**. Stepwise solution-phase coupling–deprotection methodology[12b] was then successfully applied to convert **17** into **18**, **19**, and finally tetrapeptide **20**. Subsequent reaction of **20** with 17EE (Scheme 3) gave the Boc-protected conjugate **21**. Final deprotec-

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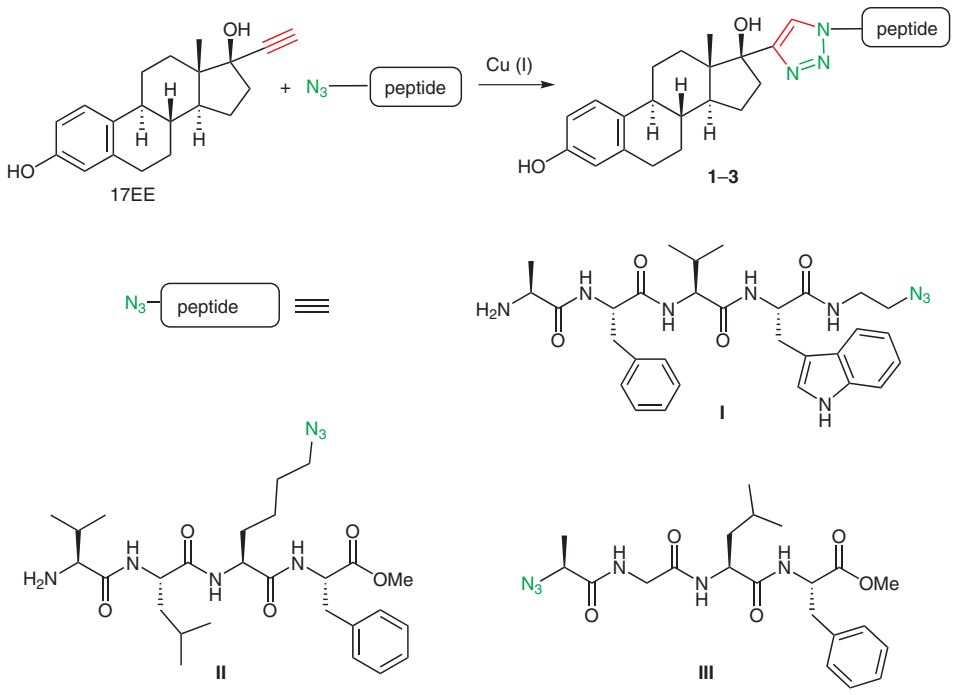
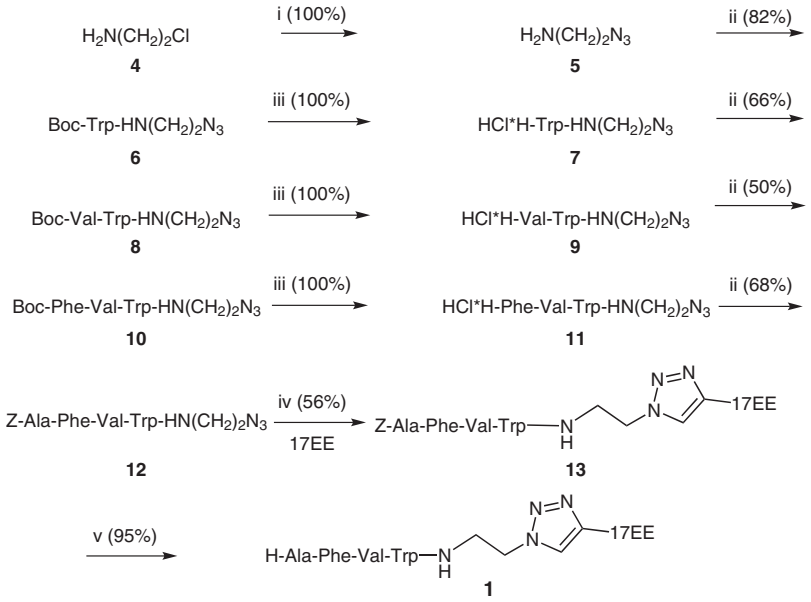


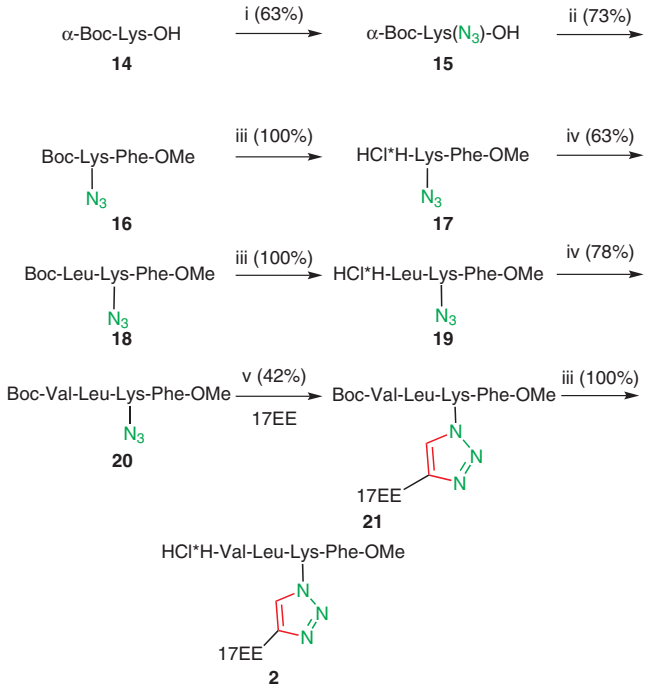
Scheme 1 General scheme for native peptide labeling with 17EE



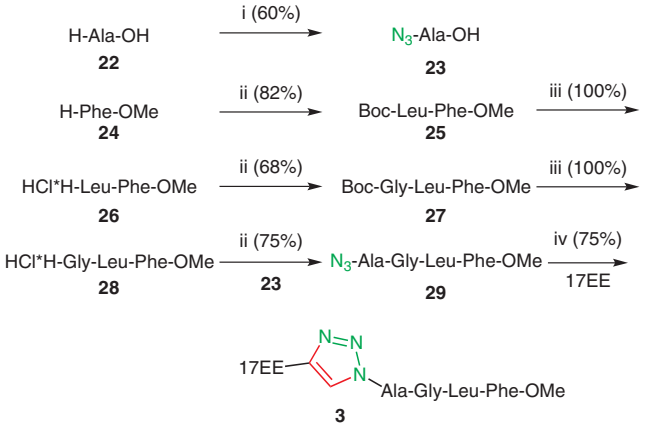
Scheme 2 Synthesis of conjugate **1**. *Reagents and conditions:* (i) NaN_3, DMF, r.t., 12 h; (ii) PG-AA-PH, DCC, BtH, DIPEA, MeCN, 0 °C to r.t.; (iii) HCl, MeOH, r.t., 2 h; (iv) Cu(I), *t*-BuOH, r.t., 72 h; (v) H_2, Pd, MeOH, r.t., 12 h.

tion resulted in target **2** with the peptide tethered to the steroid.

Azido-terminus peptide **29** was prepared by utilizing a systematic stepwise coupling–deprotection strategy.[12b] In this a way, leucine and glycine units were added to the starting L-phenylalanine methyl ester **24**. Introduction of the azido acid residue to the HCl·H-Gly-Leu-Phe-OMe (**28**) peptide chain was effected by coupling **28** with azidoalanine **23** (Scheme 4). Reaction of the **29** with 17EE was performed using a routine 'click' procedure[7c] which required four days to complete the reaction as monitored by TLC. The target peptide **3** was obtained in 75% yield and the enantiopurity of the product was confirmed by HPLC (99.3%).



Scheme 3 Synthesis of conjugate **2**. *Reagents and conditions:* (i) $BtSO_2N_3$, Et_3N, MeCN–H_2O, r.t., 6 h; (ii) HCl·H-Phe-OMe, DIPEA, DCC, BtH, MeCN, 0 °C to r.t.; (iii) HCl, MeOH, r.t., 2 h; (iv) Boc-AA-OH, DIPEA, DCC, BtH, MeCN, 0 °C to r.t.; (v) Cu(I), *t*-BuOH, r.t., 72 h.



Scheme 4 Synthesis of conjugate **3**. *Reagents and conditions:* (i) $BtSO_2N_3$, Et_3N, MeCN–H_2O, r.t., 6 h; (ii) PG-AA-OH, DIPEA, DCC, BtH, MeCN, 0 °C to r.t.; (iii) HCl, MeOH, r.t., 2 h; (iv) Cu(I), *t*-BuOH, r.t., 72 h.

All the target tethered peptides were characterized by HPLC and HRMS revealing purity not lower than 99.3% for **2** (see the Supporting information) (Table 1).

Conjugation of enantiopure versatile azido-enhanced oligopeptides with alkyne hormones is reported for the first time. We also provide different approaches for the introduction of azido moiety under solution-phase peptide synthesis conditions. The triazole bridge after 'click' conjugation is found to be stable under both Boc- and Cbz-deprotection conditions.

Table 1 HPLC and HRMS Data of Targets **1–3**

Target	Purity[a] (%)	HRMS $[M + Na]^+$	
		Found	Calcd
1	100.00	908.4778	908.4794
2	99.322	864.4986	864.4994
3	99.422	765.3927	765.3946

[a] Purity based on analytical HPLC (Phenomenex, mobile phase 100% MeOH, 0.5 mL/min^{-1}).

Melting points were determined on a capillary point apparatus equipped with a digital thermometer and are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra were recorded at 75 MHz on Gemini or Varian spectrometers at r.t. relative to TMS as internal standard (^{1}H NMR) or to the residual solvent peak (^{13}C NMR). Elemental analysis was performed on a Carlo Erba-1106 instrument. HRMS were recorded using Thermo Scientific LCQ Ion Trap. Column chromatography was performed on silica gel (230–400 mesh). HPLC analysis was performed on Shimadzu liquid chromatographic system equipped with UV detector. Detection was done at 254 nm using Phenomenex Luna C18 reversed-phase column (250 × 4.6 mm id) 5 micron.

N-Boc- and *N*-Cbz-protected L-amino acids, L-alanine (**22**), and L-phenylalanine methyl ester (**24**) were purchased from Peptides International Inc. All commercially available substrates were used as received without further purification.

2-Azidoethylamine (**5**) was synthesized according to Inverarity et al.[10] Azido-alanine **23** was prepared by our previously reported procedure.[11]

(*S*)-6-Azido-2-[(*tert*-butoxycarbonyl)amino]hexanoic Acid (15)

α-Boc-Lys-OH (2.46 g, 10.0 mmol) was dissolved in a mixture of H_2O–MeCN (1:1, 40 mL) and Et_3N (2.02 g, 20 mmol). Then $BtSO_2N_3$ (2.23 g, 10.0 mmol) was added as a solid at r.t. and the mixture was stirred overnight. The organic solvent was evaporated and residue was acidified with 0.33 M citric acid (60 mL) and extracted with EtOAc (2 × 30 mL). The organic layers were combined, dried (anhyd Na_2SO_4), and evaporated. The residue was then purified by column chromatography (EtOAc–hexanes, 1:2) to give **15** (1.71 g, 6.3 mmol, 63%) as a pale yellow oil. NMR spectra were in good compliance with previously reported spectra,[13] (mixture of two rotamers).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.65 (s, 1 H), 6.75 (br s, 0.3 H), 5.13 (d, *J* = 7.8 Hz, 0.7 H), 4.40–4.08 (m, 1 H), 3.29 (t, *J* = 6.6 Hz, 2 H), 1.98–1.81 (m, 1 H), 1.78–1.57 (m, 3 H), 1.54–1.34 (m, 11 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 177.3, 176.7, 157.1, 155.8, 82.1, 80.5, 54.5, 53.3, 51.3, 32.2, 31.1, 28.6, 28.5, 22.7.

Anal. Calcd for $C_{11}H_{20}N_4O_4$: C, 48.52; H, 7.40; N, 20.57. Found: C, 48.85; H, 7.97; N, 20.25.

Synthesis of 6, 8, 10, 12, 16, 18, 20, 25, 27, and 29; General Procedure

Equimolar amounts of amine or amine hydrochloride, N-protected L-amino acid, and benzotriazole together with DIPEA (1 equiv; 2 equiv in the case of the hydrochloride) were dissolved in MeCN and the mixture was cooled to 0 °C. DCC (1 equiv) was added and the mixture was stirred overnight at r.t. Then the solvent was evaporat-

ed, the residue was diluted with EtOAc and washed with 0.33 M citric acid soln (1 ×) and sat. Na_2CO_3 soln (2 ×). The organic layer was dried (anhyd Na_2SO_4), evaporated, and the residue was separated by column chromatography (EtOAc–hexanes, 1:4).

tert-Butyl {(*S*)-1-[(2-Azidoethyl)amino]-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}carbamate (6)
Light-yellow solid (3.05 g, 8.20 mmol, 82%); mp 140.0–141.8 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.64 (br s, 1 H), 7.62 (d, *J* = 7.5 Hz, 1 H), 7.36 (dd, *J* = 8.1, 0.9 Hz, 1 H), 7.22–7.16 (m, 1 H), 7.13–7.08 (m, 1 H), 7.00 (s, 1 H), 6.28 (s, 1 H), 5.30 (s, 1 H), 4.43 (s, 1 H), 3.25–3.00 (m, 6 H), 1.43 (s, 9 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 155.7, 136.4, 127.6, 123.4, 122.4, 119.8, 119.0, 111.5, 110.5, 80.5, 55.6, 50.6, 38.8, 28.7, 28.5.

Anal. Calcd for $C_{18}H_{24}N_6O_3$: C, 58.05; H, 6.50; N, 22.57. Found: C, 59.48; H, 7.05; N, 21.14.

Product **6** was isolated as a light-yellow solid and used crude as an intermediate for the preparation of **7** (see below).

tert-Butyl [(*S*)-1-({(*S*)-1-[(2-Azidoethyl)amino]-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}amino)-3-methyl-1-oxobutan-2-yl]carbamate (8)
White solid (2.33 g, 4.95 mmol, 66%); mp 175.1–176.8 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.54 (s, 1 H), 7.60 (d, *J* = 7.2 Hz, 1 H), 7.35 (d, *J* = 8.1 Hz, 1 H), 7.21–7.09 (m, 2 H), 7.01 (s, 1 H), 6.86 (d, *J* = 7.2 Hz, 1 H), 6.71 (br s, 1 H), 4.94 (d, *J* = 5.7 Hz, 1 H), 4.75 (q, *J* = 6.9 Hz, 1 H), 3.91 (t, *J* = 5.4 Hz, 1 H), 3.41–3.11 (m, 6 H), 2.17–2.11 (m, 1 H), 1.32 (s, 9 H), 0.91 (d, *J* = 6.6 Hz, 3 H), 0.81 (d, *J* = 6.9 Hz, 3 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 171.4, 156.2, 136.2, 127.4, 123.3, 122.3, 119.7, 118.4, 111.4, 109.9, 80.4, 60.5, 53.9, 50.1, 38.7, 30.1, 28.0, 27.5, 19.2, 17.3.

Anal. Calcd for $C_{23}H_{33}N_7O_4$: C, 58.58; H, 7.05; N, 20.79. Found: C, 58.30; H, 7.45; N, 20.63.

tert-Butyl ((*S*)-1-{[(*S*)-1-({(*S*)-1-[(2-Azidoethyl)amino]-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}amino)-3-methyl-1-oxobutan-2-yl]amino}-1-oxo-3-phenylpropan-2-yl)carbamate (10)
Light-yellow solid (1.33 g, 2.15 mmol, 50%); mp 128.1–130.6 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.21 (s, 1 H), 7.54 (d, *J* = 8.1 Hz, 1 H), 7.30–7.05 (m, 7 H), 6.98 (s, 1 H), 6.90–6.58 (m, 3 H), 4.94–4.86 (m, 1 H), 4.80 (q, *J* = 6.9 Hz, 1 H), 4.28–4.10 (m, 2 H), 3.42–3.23 (m, 5 H), 2.97–2.90 (m, 2 H), 2.16 (br s, 1 H), 1.90 (d, *J* = 10.2 Hz, 1 H), 1.75–1.62 (m, 1 H), 1.59 (d, *J* = 9.6 Hz, 1 H), 1.39–1.20 (m, 10 H), 0.94–0.80 (m, 3 H), 0.69 (d, *J* = 6.6 Hz, 1 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.3, 171.5, 155.9, 136.7, 136.2, 129.4, 128.6, 127.5, 126.9, 123.3, 122.1, 119.5, 118.7, 111.4, 110.3, 80.3, 58.8, 55.7, 53.9, 50.5, 49.5, 38.9, 33.9, 31.4, 28.4, 25.7, 25.1, 21.0, 19.3, 18.2.

Anal. Calcd for $C_{32}H_{42}N_8O_5$: C, 62.12; H, 6.84; N, 18.11. Found: C, 62.00; H, 7.54; N, 16.87.

Product **10** was isolated as light-yellow solid and used crude as intermediate for the preparation of **11** (see below).

Benzyl {(5*S*,8*S*,11*S*,14*S*)-1-Azido-11-benzyl-5-[(1*H*-indol-3-yl)methyl]-8-isopropyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazapentadecan-14-yl}carbamate (12)
Light-brown solid (1.06 g, 1.46 mmol, 68%); mp 187.1–188.8 °C.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.84 (s, 1 H), 8.16 (s, 1 H), 8.09 (d, *J* = 7.8 Hz, 1 H), 7.99 (d, *J* = 7.8 Hz, 1 H), 7.83 (d, *J* = 8.4 Hz, 1 H), 7.56 (d, *J* = 7.8 Hz, 1 H), 7.42 (d, *J* = 7.2 Hz, 1 H), 7.32–6.92 (m, 10 H), 4.98 (s, 2 H), 4.54 (br s, 2 H), 4.19 (t, *J* = 6.6 Hz, 1 H), 4.00 (t, *J* = 7.2 Hz, 1 H), 3.57–3.54 (m, 1 H), 3.37–3.31 (m, 2 H), 3.08–2.81 (m, 5 H), 2.10–1.86 (m, 2 H), 1.38–1.05 (m, 6 H), 0.95–0.70 (m, 6 H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.3, 171.5, 170.7, 170.4, 155.5, 137.7, 136.0, 136.0, 129.3, 128.3, 127.9, 127.7, 127.3, 126.1, 123.5, 120.8, 118.4, 118.2, 111.3, 109.8, 65.4, 57.6, 53.5, 53.3, 50.2, 49.8, 41.6, 38.7, 38.2, 37.1, 30.8, 27.9, 19.1, 18.2, 18.0.

Anal. Calcd for $C_{38}H_{45}N_9O_6$: C, 63.06; H, 6.27; N, 17.42. Found: C, 61.76; H, 6.94; N, 16.65.

Methyl (*S*)-2-{(*S*)-6-Azido-2-[(*tert*-butoxycarbonyl)amino]hexanamido}-3-phenylpropanoate (16)
Pale yellow solid (1.99 g, 4.60 mmol, 73%); mp 80.7–82.0 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32–7.20 (m, 3 H), 7.12–7.09 (m, 2 H), 6.56 (d, *J* = 7.5 Hz, 1 H), 5.04 (d, *J* = 8.1 Hz, 1 H), 4.85 (dd, *J* = 13.5, 6.3 Hz, 1 H), 4.09–4.04 (m, 1 H), 3.71 (s, 3 H), 3.24 (t, *J* = 6.9 Hz, 2 H), 3.19–3.03 (m, 2 H), 1.79–1.69 (m, 2 H), 1.60–1.50 (m, 4 H), 1.44 (br s, 9 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.8, 171.7, 155.9, 135.8, 129.4, 128.7, 127.3, 80.3, 54.4, 53.3, 52.5, 51.3, 38.0, 32.2, 28.6, 28.4, 22.8.

Anal. Calcd for $C_{21}H_{31}N_5O_5$: C, 58.18; H, 7.21; N, 16.16. Found: C, 58.24; H, 7.60; N, 16.25.

Methyl (2*S*,5*S*,8*S*)-5-(4-Azidobutyl)-2-benzyl-8-[(*tert*-butoxycarbonyl)amino]-10-methyl-4,7-dioxo-3,6-diazaundecanoate (18)
Pale yellow oil (1.38 g, 2.52 mmol, 63%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.22–7.12 (m, 3 H), 7.02 (d, *J* = 6.3 Hz, 1 H), 6.85–6.81 (m, 1 H), 5.08–5.03 (m, 1 H), 4.74–4.68 (m, 1 H), 4.40–4.34 (m, 1 H), 4.05–3.95 (m, 1 H), 3.60 (s, 3 H), 3.19–3.06 (m, 2 H), 3.02–2.96 (m, 2 H), 1.85–1.13 (m, 20 H), 0.83 (d, *J* = 6.3 Hz, 6 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.8, 171.7, 171.1, 155.8, 135.8, 129.2, 128.6, 127.1, 80.0, 53.4, 52.8, 52.3, 51.1, 41.2, 37.9, 32.0, 28.5, 28.4, 25.6, 24.8, 23.1, 22.5, 21.9.

Anal. Calcd for $C_{27}H_{42}N_6O_6$: C, 59.32; H, 7.74; N, 15.37. Found: C, 60.52; H, 8.69; N, 15.49.

Product **18** was isolated as a pale yellow solid and used crude as intermediate for the preparation of **19** (see below).

Methyl (2*S*,5*S*,8*S*,11*S*)-5-(4-Azidobutyl)-2-benzyl-11-[(*tert*-butoxycarbonyl)amino]-8-isobutyl-12-methyl-4,7,10-trioxo-3,6,9-triazatridecanoate (20)
White solid (1.27 g, 1.97 mmol, 78%); mp 174.1–174.8 °C.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.32 (d, *J* = 7.5 Hz, 1 H), 8.00–7.75 (m, 3 H), 7.26–7.17 (m, 5 H), 6.73 (d, *J* = 9.0 Hz, 1 H), 4.51–4.27 (m, 4 H), 3.79–3.73 (m, 1 H), 3.56 (s, 3 H), 3.28–3.23 (m, 2 H), 3.06–2.91 (m, 2 H), 2.00–1.82 (m, 1 H), 1.75–1.20 (m, 18 H), 0.92–0.80 (m, 12 H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 171.7, 171.6, 171.4, 171.1, 155.4, 137.0, 129.0, 128.2, 126.5, 78.0, 59.9, 54.8, 53.4, 51.8, 50.7, 50.6, 40.9, 36.6, 31.8, 30.3, 28.2, 27.9, 24.0, 23.1, 22.2, 21.5, 19.2, 18.2.

Anal. Calcd for $C_{32}H_{51}N_7O_7$: C, 59.51; H, 7.96; N, 15.18. Found: C, 59.70; H, 8.67; N, 15.02.

Methyl (*S*)-2-{(*S*)-2-[(*tert*-Butoxycarbonyl)amino]-4-methylpentanamido}-3-phenylpropanoate (25)
NMR spectra are in good compliance with those previously reported.[14] Pale yellow solid (2.86 g, 7.30 mmol, 82%); mp 82.4–83.2 °C (Lit.[14] 83.5–84.0 °C).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.31–7.20 (m, 3 H), 7.11 (d, *J* = 6.0 Hz, 2 H), 6.61 (d, *J* = 7.5 Hz, 1 H), 4.94–4.81 (m, 2 H), 4.13–4.08 (m, 2 H), 3.70 (s, 3 H), 3.20–3.00 (m, 2 H), 1.72–1.50 (m, 2 H), 1.44 (s, 9 H), 0.98–0.89 (m, 6 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.3, 171.8, 155.6, 135.9, 129.4, 128.7, 127.2, 80.2, 55.3, 52.5, 41.4, 38.1, 28.5, 24.8, 23.1, 22.1.

Anal. Calcd for $C_{21}H_{32}N_2O_5$: C, 64.26; H, 8.22; N, 7.14. Found: C, 64.50; H, 8.82; N, 7.52.

Methyl (2*S*,5*S*)-2-Benzyl-8-[(*tert*-butoxycarbonyl)amino]-5-isobutyl-4,7-dioxo-3,6-diazaoctanoate (27)
White solid (2.14g, 4.76 mmol, 68%); mp 143.4–144.5 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.29–7.21 (m, 3 H), 7.12–7.09 (m, 2 H), 6.89 (d, *J* = 7.8 Hz, 1 H), 6.78 (d, *J* = 7.2 Hz, 1 H), 5.38–5.33 (m, 1 H), 4.87–4.79 (m, 1 H), 4.50–4.47 (m, 1 H), 3.80–3.63 (m, 5 H), 3.17–3.00 (m, 2 H), 1.65–1.48 (m, 3 H), 1.45 (s, 9 H), 0.89 (t, *J* = 5.7 Hz, 6 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.9, 171.8, 169.7, 156.2, 136.0, 129.5, 128.7, 127.2, 80.4, 53.4, 52.5, 51.7, 44.4, 41.1, 38.0, 28.5, 24.8, 23.1, 22.2.

Anal. Calcd for $C_{23}H_{35}N_3O_6$: C, 61.45; H, 7.85; N, 9.35. Found: C, 61.22; H, 7.85; N, 11.44.

Methyl (*S*)-2-((*S*)-2-{2-[(*S*)-2-Azidopropanamido]acetamido}-4-methylpentanamido)-3-phenylpropanoate (29)
White solid (1.34 g, 3 mmol, 75%); mp 149.7–150.2 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52 (t, *J* = 4.8 Hz, 1 H), 7.45–7.38 (m, 2 H), 7.29–7.18 (m, 3 H), 7.11 (d, *J* = 7.5 Hz, 2 H), 4.86 (q, *J* = 6.8 Hz, 1 H), 4.68 (q, *J* = 7.4 Hz, 1 H), 4.08–4.00 (m, 2 H), 3.90–3.83 (m, 1 H), 3.71 (s, 3 H), 3.17–3.02 (m, 2 H), 1.69–1.55 (m, 3 H), 1.53 (d, *J* = 7.2 Hz, 3 H), 0.91 (d, *J* = 5.7 Hz, 3 H), 0.89 (d, *J* = 5.7 Hz, 3 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.1, 172.0, 170.8, 168.5, 136.0, 129.4, 128.6, 127.2, 58.5, 53.5, 52.5, 51.8, 42.9, 41.8, 38.0, 24.8, 22.9, 22.4, 17.2.

Anal. Calcd for $C_{21}H_{30}N_6O_5$: C, 56.49; H, 6.77; N, 18.82. Found: C, 56.74; H, 7.18; N, 18.72.

Synthesis of 2, 7, 9, 11, 17, 19, 26, and 28; General Procedure
Boc-protected peptide was dissolved in sat. HCl soln in MeOH and stirred at r.t. for 2 h. The mixture was then evaporated and dried under high vacuum.

Methyl (*S*)-2-((*S*)-2-{(*S*)-2-[(*S*)-2-Amino-3-methylbutanamido]-4-methylpentanamido}-6-{4-[(8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl]-1*H*-1,2,3-triazol-1-yl}hexanamido)-3-phenylpropanoate Hydrochloride (2)
Yellow solid (0.18 g, 0.20 mmol, 100%); mp 163.9–164.8 °C.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.29 (br s, 1 H), 8.05–7.96 (m, 1 H), 7.06–6.93 (m, 6 H), 6.73 (d, *J* = 8.4 Hz, 1 H), 6.29–6.24 (m, 2 H), 4.43–4.12 (m, 5 H), 3.54 (d, *J* = 5.1 Hz, 1 H), 3.07 (p, *J* = 1.5 Hz, 1 H), 2.89 (dd, *J* = 13.8, 5.4 Hz, 1 H), 2.75 (dd, *J* = 13.8, 8.4 Hz, 1 H), 2.50 (br s, 2 H), 2.18–2.06 (m, 1 H), 2.04–1.91 (m, 4 H), 1.83–1.73 (m, 3 H), 1.64 (br s, 3 H), 1.46–1.44 (m, 4 H), 1.39–1.29 (m, 3 H), 1.24–1.10 (m, 8 H), 0.81–0.76 (m, 10 H), 0.70 (q, *J* = 6.6 Hz, 8 H), 0.34 (m, 1 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 174.4, 173.9, 173.6, 169.9, 156.3, 153.3, 139.1, 138.3, 132.5, 130.7, 129.9, 128.3, 127.6, 127.1, 116.5, 114.2, 83.1, 59.8, 55.6, 54.4, 53.9, 53.8, 53.3, 45.0, 42.1, 41.3, 39.5, 38.7, 34.7, 33.0, 32.1, 31.1, 30.5, 29.0, 27.8, 26.2, 24.8, 23.9, 23.8, 22.6, 19.6, 18.4, 15.1.

HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{47}H_{67}N_7O_7Na$: 864.4994; found: 864.4986.

(*S*)-2-Amino-*N*-(2-azidoethyl)-3-(1*H*-indol-3-yl)propanamide Hydrochloride (7)
Yellow oil (2.47g, 8.00 mmol, 100%).

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.14 (s, 1 H), 9.00 (s, 1 H), 8.36 (br s, 3 H), 7.71 (d, *J* = 7.8 Hz, 1 H), 3.37 (d, *J* = 7.8 Hz, 1 H), 7.25 (s, 1 H), 7.10–6.96 (m, 2 H), 4.40 (br s, 2 H), 4.00–3.97 (m, 1 H), 3.33–3.16 (m, 4 H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.8, 136.3, 127.2, 124.9, 121.1, 118.6, 118.4, 111.5, 107.0, 52.9, 49.7, 38.2, 27.2.

Product **7** was used crude as an intermediate for the synthesis of **8** (see above); full spectral characterization is provided for **8**.

(*S*)-2-Amino-*N*-{(*S*)-1-[(2-azidoethyl)amino]-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}-3-methylbutanamide (9)
White solid (1.84 g, 4.50 mmol, 100%); mp 178.6–179.3 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.94 (s, 1 H), 8.72 (d, *J* = 7.8 Hz, 1 H), 8.41 (t, *J* = 5.4 Hz, 1 H), 8.35–8.15 (m, 2 H), 7.62 (d, *J* = 7.5 Hz, 1 H), 7.33 (d, *J* = 8.1 Hz, 1 H), 7.22 (d, *J* = 1.8 Hz, 1 H), 7.08–6.95 (m, 2 H), 4.60–4.52 (m, 1 H), 4.14 (br s, 2 H), 3.66–3.61 (m, 1 H), 3.39–2.97 (m, 4 H), 2.15–2.08 (m, 1 H), 1.23–0.89 (m, 6 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.3, 167.7, 136.1, 127.3, 123.9, 120.9, 118.4, 118.2, 111.4, 109.6, 57.1, 53.8, 49.8, 46.8, 38.2, 33.4, 29.9, 28.6, 27.8, 25.3, 24.5, 23.7, 18.4, 17.6.

Anal. Calcd for $C_{18}H_{26}ClN_7O_2$: C, 53.00; H, 6.42; N, 24.04. Found: C, 53.09; H, 6.16; N, 24.67.

(*S*)-2-[(*S*)-2-Amino-3-phenylpropanamido]-*N*-{(*S*)-1-[(2-azidoethyl)amino}-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}-3-methylbutanamide Hydrochloride (11)
Isolated as white solid (1.19 g, 2.15 mmol, 100%) and used crude as an intermediate for the preparation of **12** (see above); full spectral characterization is provided for **12**.

Methyl (*S*)-2-[(*S*)-2-Amino-6-azidohexanamido]-3-phenylpropanoate Hydrochloride (17)
Pale yellow oil (1.59 g, 4.31 mmol, 100%).

^{1}H NMR (300 MHz, CD_3OD): δ = 8.21 (br s, 1 H), 7.23–7.10 (m, 5 H), 4.64–4.58 (m, 1 H), 3.82–3.75 (m, 1 H), 3.60 (s, 3 H), 3.26–3.16 (m, 2 H), 3.14–3.07 (m, 1 H), 2.99– 2.92 (m, 1 H), 1.81–1.74 (m, 2 H), 1.55–1.47 (m, 2 H), 1.43–1.33 (m, 2 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 173.1, 170.3, 138.1, 130.3, 129.7, 128.1, 55.7, 54.2, 53.0, 52.2, 38.1, 32.3, 29.6, 22.9.

Anal. Calcd for $C_{16}H_{24}ClN_5O_3$: C, 51.96; H, 6.54; N, 18.94. Found: C, 53.03; H, 7.46; N, 18.41.

Methyl (*S*)-2-{(*S*)-2-[(*S*)-2-Amino-4-methylpentanamido]-6-azidohexanamido}-3-phenylpropanoate Hydrochloride (19)
Isolated as white solid and used crude as an intermediate for the preparation of **20**; full spectral characterization is provided for **20**.

Methyl (*S*)-2-[(*S*)-2-Amino-4-methylpentanamido]-3-phenylpropanoate Hydrochloride (26)
White solid (2.3 g, 7.0 mmol, 100%); mp 163.2–166.0 °C (Lit.[15] 162.3–163.0 °C).

Methyl (*S*)-2-[(*S*)-2-(2-Aminoacetamido)-4-methylpentanamido]-3-phenylpropanoate (28)
White solid (1.74 g, 4.49 mmol, 100%); mp 127.3–128.2 °C.

^{1}H NMR (300 MHz, CD_3OD): δ = 7.30–7.20 (m, 5 H), 4.98–4.92 (m, 1 H), 4.68–4.62 (m, 1 H), 4.44 (t, *J* = 7.5 Hz, 1 H), 3.71 (s, 3 H), 3.31 (t, *J* = 1.8 Hz, 1 H), 3.15 (dd, *J* = 14.0, 5.6 Hz, 1 H), 3.02 (dd, *J* = 13.4, 8.7 Hz, 1 H), 1.70–1.59 (m, 1 H), 1.51 (d, *J* = 7.2 Hz, 2 H), 0.99–0.88 (m, 6 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 174.5, 173.4, 167.2, 138.2, 130.4, 129.6, 128.0, 55.4, 53.3, 52.9, 42.2, 41.6, 38.3, 25.9, 23.5, 22.1.

Anal. Calcd for $C_{18}H_{28}ClN_3O_4$: C, 56.03; H, 7.31; N, 10.89. Found: C, 55.62; H, 8.00; N, 10.46.

Synthesis of 13, 21, and 3; General Procedure
17α-Ethynylestradiol (150 mg, 0.50 mmol) and azido-peptide (0.50 mmol) were suspended in *t*-BuOH–H_2O (3:1, 5 mL). A 1 M sodium ascorbate soln (0.1 mL) was added, followed by $CuSO_4 \cdot 5H_2O$ (3 mg) dissolved in H_2O (0.30 mL). The mixture was stirred for 3 d for **13** and **21**, and 4 d for **3** at r.t., solvent was evaporated and the residue was separated by column chromatography (EtOAc–hexanes).

Benzyl ((5*S*,8*S*,11*S*,14*S*)-11-Benzyl-1-{4-[(8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl]-1*H*-1,2,3-triazol-1-yl}-5-[(1*H*-indol-3-yl)methyl]-8-isopropyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazapentadecan-14-yl)carbamate (13)

White solid (285 mg, 0.28 mmol, 56%); mp 158.5–160.3 °C.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.00–7.72 (m, 3 H), 7.53 (d, *J* = 7.8 Hz, 1 H), 7.30–6.80 (m, 11 H), 4.46–6.40 (m, 2 H), 4.95 (s, 2 H), 4.62–4.56 (m, 2 H), 4.37–4.33 (m, 2 H), 4.12–4.00 (m, 2 H), 3.70–3.50 (m, 2 H), 3.47 (q, *J* = 7.2 Hz, 1 H), 3.10–2.90 (m, 2 H), 2.75–2.60 (m, 2 H), 2.50–2.37 (m, 1 H), 2.15–1.70 (m, 6 H), 1.65–1.10 (m, 9 H), 0.99 (s, 2 H), 0.95–0.87 (m, 2 H), 0.80–0.60 (m, 4 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 176.0, 174.5, 174.1, 173.6, 158.6, 155.9, 155.5, 138.9, 138.1, 138.0, 132.7, 130.5, 129.6, 129.2, 128.9, 128.7, 128.0, 127.3, 124.8, 124.7, 122.6, 120.0, 119.5, 116.1, 113.8, 112.5, 111.0, 99.5, 83.4, 68.0, 56.3, 56.1, 52.8, 50.2, 50.0, 44.8, 41.1, 40.8, 38.7, 38.2, 34.4, 31.6, 30.8, 29.0, 28.8, 27.6, 24.8, 19.7, 19.1, 18.1, 17.7, 15.6, 15.1.

Isolated as white solid and used crude as an intermediate for the preparation of **1** (see below); full spectral characterization is provided for **1**.

Methyl (2*S*,5*S*,8*S*,11*S*)-2-Benzyl-11-[(*tert*-butoxycarbonyl)amino]-5-(4-{4-[(8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl]-1*H*-1,2,3-triazol-1-yl}butyl)-8-isobutyl-12-methyl-4,7,10-trioxo-3,6,9-triazatridecanoate (21)

White solid (198 mg, 0.21 mmol, 42%); mp 158.2–159.0 °C.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.19 (d, *J* = 7.5 Hz, 1 H), 8.01 (t, *J* = 7.5 Hz, 2 H), 7.71 (s, 2 H), 7.19–7.07 (m, 5 H), 6.89 (d, *J* = 8.7 Hz, 1 H), 6.54–6.39 (m, 3 H), 4.59 (q, *J* = 5.7 Hz, 1 H), 4.39–4.25 (m, 4 H), 3.82 (t, *J* = 7.2 Hz, 1 H), 3.58 (s, 3 H), 3.04 (dd, *J* = 13.9, 5.7 Hz, 1 H), 2.91 (dd, *J* = 13.9, 8.3 Hz, 1 H), 2.66–2.64 (m, 2 H), 2.44–2.30 (m, 2 H), 2.02–1.20 (m, 31 H), 0.95 (s, 3 H), 0.90–0.79 (m, 10 H), 0.60 (t, *J* = 9.9 Hz, 2 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 174.6, 174.4, 173.7, 173.3, 158.1, 155.9, 155.4, 138.8, 137.9, 132.5, 130.3, 129.6, 127.9, 127.2, 124.0, 116.2, 113.8, 83.3, 80.6, 61.7, 55.2, 54.1, 53.2, 52.9, 51.0, 50.0, 44.9, 41.8, 41.1, 38.5, 34.4, 32.7, 32.0, 30.9, 28.9, 27.6, 25.8, 24.8, 23.7, 22.2, 20.1, 18.9, 15.1.

Anal. Calcd for $C_{52}H_{75}N_7O_9$: C, 66.29; H, 8.02; N, 10.41. Found: C, 65.94; H, 8.48; N, 10.32.

Methyl (*S*)-2-{(*S*)-2-[2-((*S*)-2-{4-[(8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-Dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl]-1*H*-1,2,3-triazol-1-yl}propanamido)acetamido]-4-methylpentanamido}-3-phenylpropanoate (3)

White solid (282 mg, 0.38 mmol, 75%); mp 140.0–141.8 °C.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.90 (br s, 1 H), 8.98 (br s, 1 H), 8.62 (t, *J* = 5.7 Hz, 1 H), 8.40 (d, *J* = 7.5 Hz, 1 H), 8.01 (d, *J* = 8.4 Hz, 1 H), 7.91 (s, 1 H), 7.28–7.17 (m, 4 H), 6.95 (d, *J* = 8.4 Hz, 1 H), 6.49–6.42 (m, 2 H), 5.55–5.45 (m, 1 H), 5.10 (br s, 1 H), 4.49–4.32 (m, 2H), 3.78 (d, *J* = 5.4 Hz, 2 H), 3.56 (s, 3 H), 3.44 (br s, 2 H), 3.04–2.95 (m, 2 H), 2.70 (br s, 2 H), 2.45–2.30 (m, 1 H), 2.12–1.21 (m, 14 H), 0.94–0.82 (m, 6 H), 0.70–0.52 (m, 1 H).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.0, 171.8, 169.1, 167.9, 154.9, 154.1, 137.2, 137.1, 130.4, 129.1, 128.3, 126.6, 126.1, 121.6, 114.9, 112.7, 81.3, 57.9, 53.6, 51.8, 50.7, 47.6, 46.8, 43.2, 41.9, 41.1, 39.4, 37.3, 36.5, 32.6, 29.4, 27.3, 26.2, 24.1, 23.6, 23.0, 21.8, 21.1, 18.3, 14.5.

Anal. Calcd for $C_{41}H_{54}N_6O_7$: C, 66.29; H, 7.33; N, 11.31. Found: C, 65.19; H, 7.90; N, 10.55..

HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{41}H_{54}N_6O_7Na$: 765.3946; found: 765.3927.

(*S*)-2-{(*S*)-2-[(*S*)-2-Aminopropanamido]-3-phenylpropanamido}-*N*-{(*S*)-1-[(2-{4-[(8*R*,9*S*,13*S*,14*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-17-yl]-1*H*-1,2,3-triazol-1-yl}ethyl)amino]-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl}-3-methylbutanamide (1)

Conjugate **13** (0.1 g, 0.09 mmol) was dissolved in MeOH (20 mL), and Pd/C (20 mg) was added. The mixture was kept under positive pressure of H_2 for 12 h. Then the suspension was filtered through Celite, and evaporated to give **1** as a white solid (86 mg, 0.09 mmol, 95%); mp 186.0–188.2 °C.

^{1}H NMR (300 MHz, CD_3OD): δ = 7.91–7.74 (m, 1 H), 7.62–7.60 (m, 1 H), 7.43 (d, *J* = 7.8 Hz, 1 H), 7.21–7.06 (m, 8 H), 6.99–6.80 (m, 3 H), 6.76 (d, *J* = 8.4 Hz, 1 H), 6.39–6.32 (m, 2 H), 4.44 (t, *J* = 6.3 Hz, 2 H), 4.27–4.22 (m, 2 H), 4.01–3.94 (m, 1 H), 3.58–3.38 (m, 3 H), 3.24 (s, 1 H), 3.16–2.80 (m, 3 H), 2.56 (br s, 2 H), 2.32 (br s, 1 H), 1.96–1.83 (m, 4 H), 1.80 (s, 2 H), 1.73–1.66 (m, 2 H), 1.55–1.30 (m, 3 H), 1.28–1.06 (m, 5 H), 0.90 (s, 3 H), 0.84–0.60 (m, 7 H).

^{13}C NMR (75 MHz, CD_3OD): δ = 174.7, 173.5, 172.4, 156.2, 155.7, 139.2, 133.0, 132.9, 130.8, 130.5, 130.3, 130.0, 129.6, 129.3, 129.1, 128.4, 127.7, 125.1, 123.0, 120.4, 119.9, 116.5, 114.1, 112.9, 111.1, 83.7, 61.2, 56.9, 56.3, 50.5, 45.2, 41.5, 41.1, 39.0, 34.7, 31.2, 29.2, 28.0, 25.1, 24.3, 20.2, 19.3, 16.8, 16.6, 15.4.

HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{50}H_{63}N_9O_6Na$: 908.4794: found: 908.4778.

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Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synthesis. HPLC, MS, ^{1}H and ^{13}C NMR data of compounds **1**–**29** are included.

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