Bioorganic & Medicinal Chemistry 18 (2010) 6127–6133

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Anhydrotetracycline–peptide conjugates as representatives for ligand-based transactivating systems

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ARTICLE INFO

Article history:
Received 10 March 2010
Accepted 16 June 2010
Available online 22 June 2010

Keywords:
Anhydrotetracycline
Peptide conjugates
TetR
Tetracycline
Transcriptional transactivation
Bioconjugation

ABSTRACT

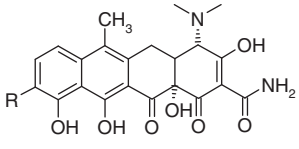
Bioconjugates of anhydrotetracycline and minimal activation sequences (VP1, VP2) derived from the Herpes simplex virus protein VP16 were synthesized. Different ligation strategies were applied and the resulting molecules tested in HeLa cells expressing the reverse transactivator rtTA-S3 for activity. The data clearly demonstrate that the atc-peptide conjugates are able to penetrate the cell membrane. Furthermore, binding to and induction of rtTA-S3 were detected. Structure–activity relationships indicated that the biological activity of the atc-peptide strongly depends on the specific linker used. The N-terminally linked oxime derivative 10 proved excellent activity when the increase of luciferase activity indicated a transcriptional activation substantially exceeding the inducing properties of anhydrotetracycline.

1. Introduction

Regulatory systems based on bacterial Tet repressor (TetR) are widely used to conditionally alter the expression of selected target genes in both model organisms and cultured cell lines.[1] Spatially and temporally controlled inducible expression allows in-depth analysis of an individual gene's contribution to normal cell physiology, development or disease.[2] TetR-based transregulators respond to tetracycline,[3] anhydrotetracyline (Fig. 1) and synthetic derivatives thereof,[4,5] which penetrate biological membranes non-specifically, including the placenta and the blood–brain barrier.[2a,6] Screening and selection of TetR mutants allowed to establish a regulatory system with a reversed phenotype (revTetR), that—in contrast to TetR—binds DNA only in the presence of tetracyclines.[7] For application in eukaryotic systems, TetR-based transactivators are typically constructed as artificial transcription factors by fusing an N-terminally located TetR or revTetR unit with an acidic transcriptional transactivation domain,[8] like the one derived from the Herpes simplex virus transactivator protein VP16.

As an alternative to these transcription factor-based systems, we aimed to create a ligand-based activating molecule by covalently attaching an activating peptide to the TetR-binding tetracycline derivative (Fig. 2). Such a synthetic platform could serve as first step for establishing highly flexible and versatile regulatory systems that can deliver many different kinds of biological readout, but only need to employ a single Tet-responsive protein. Short peptidic sequences that recruit proteins active in transcription regulation are widespread in proteins or are also easily selected by standard phage display techniques.[9] To realize this project, we chose to conjugate anhydrotetracycline (atc), which has high revTetR affinity, with the octapeptide DFDLDMLG (VP1), the minimal activating sequence derived from VP16 (Fig. 2b).[10]

As an attempt to improve the peptide's activating properties, VP2,[10] a 16-mer consisting of two VP1 units, should be attached as well. Since the crystal structure of TetR/glycylcycline[11] complexes showed a tunnel-like cavity around position 9 of the tetracycline core leading to the surface of the protein,[11a] we selected a 9-substituted atc-derivative for bioconjugation. 9-Aminoanhydrotetracycline[12] (H_2N-atc) was the precursor of choice, since we deemed the primary amine function most promising to allow direct introduction of protected peptides via their C-terminus or



R = H:
anhydrotetracycline (atc)

R = H_2N:
9-aminoanhydrotetracycline (H_2N-atc)

Figure 1. Anhydrotetracyclines.

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doi:10.1016/j.bmc.2010.06.061

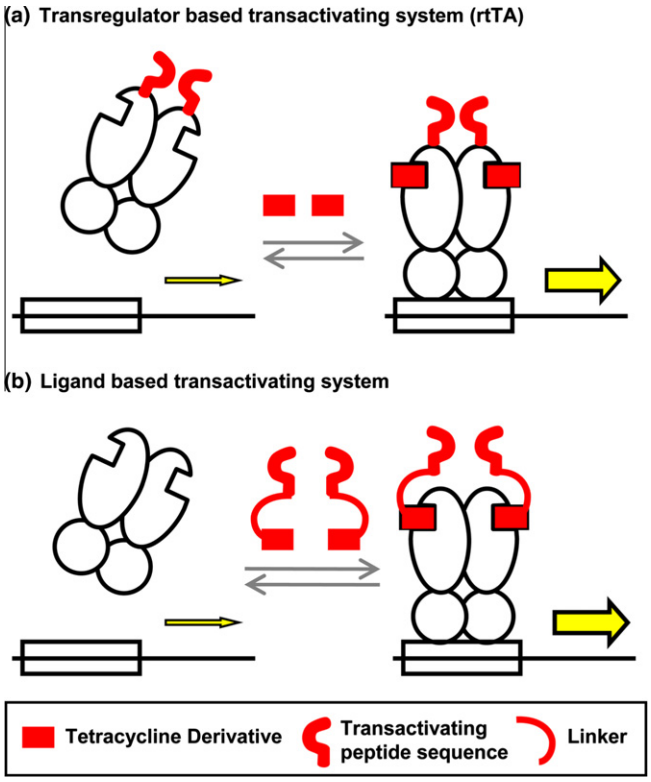


Figure 2. (a) Schematic representation of the revTetR-transactivator fusion protein rtTA binding to DNA in the presence of a tetracycline derivative; (b) revTetR binds to DNA in the presence of a tc-peptide conjugate and transcription is triggered by the transactivating peptide covalently bound to atc.

the attachment of suitable linkers for orthogonal ligation of unprotected peptides via their N-termini. To determine cell penetration, TetR interaction and transactivating potential of the conjugates, a test system consisting of a Tet-dependent luciferase reporter gene and the reverse transactivator rtTA-S3[7b] was used. Moreover, initial structure–activity relationships should help establish an effective ligand-based regulatory system.

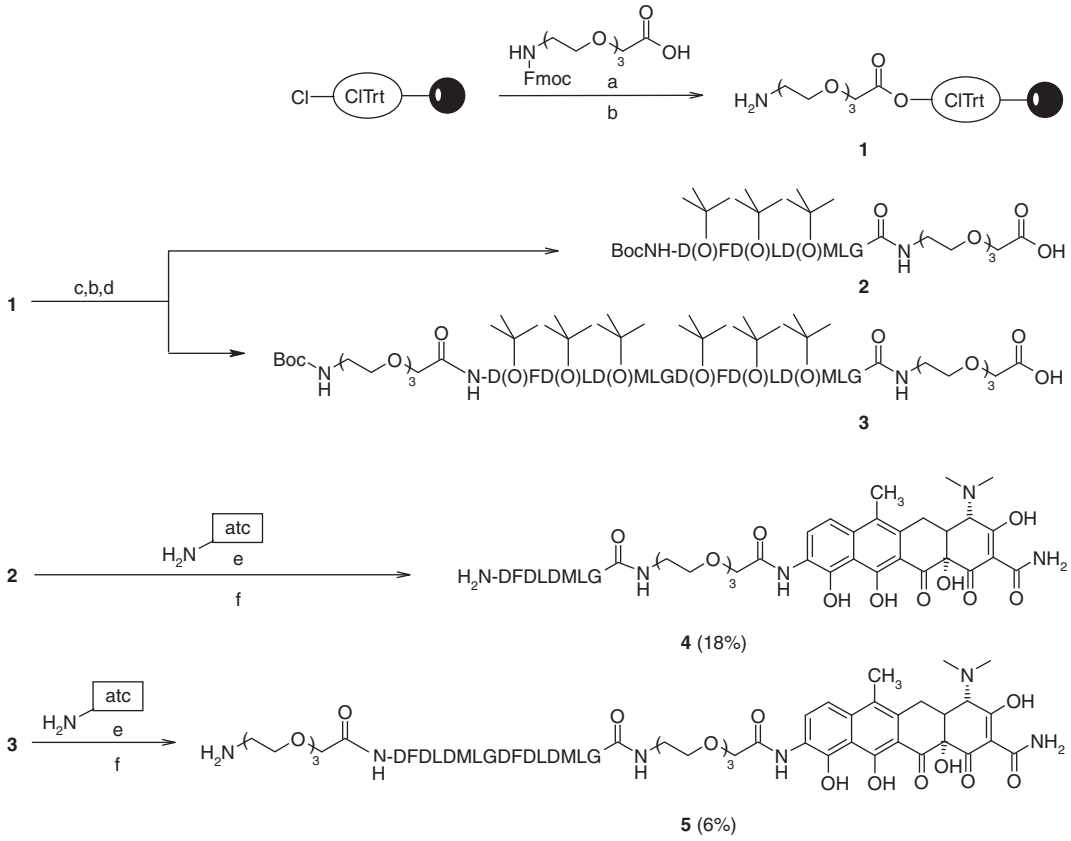
2. Results and discussion

To take advantage of the high binding and inducing activity of anhydrotetracycline,[13] our initial investigations were directed towards a solid phase supported peptide synthesis and a subsequent ligation in solution with a suitable atc-derivative to construct the anhydrotetracycline–peptide conjugates. We were aware of putative synthetic problems which conjugation of a peptide with the multifunctional tetracycline system might raise. To circumvent side reactions, we pursued the direct acylation strategy using protected peptides (Scheme 1). Starting from chlorotritylchloride resin, *N*-Fmoc-11-amino-3,6,9-trioxaundecanoic acid was attached by alkylation. Subsequent deprotection of the linker gave the loaded resin **1**. Repetitive cycles of peptide coupling reactions were performed using PyBOP/Fmoc-protected amino acids followed by Fmoc-deprotection, with microwave irradiation promoting both acylation and deprotection in a fast and effective way. Aspartic acid side chains were incorporated as *tert*-butyl esters and terminal amino acids were introduced as *N*-Boc protected derivatives. In particular, Boc-Asp(O*t*Bu)-OH was employed for the synthesis of the VP1 sequence and Boc-NH$(CH_2CH_2O)_3CH_2CO_2H$ for VP2, which should serve as solubility enhancer for the hexadecamer. The peptide resins were cleaved with hexafluoroisopropanol to obtain the fully protected peptide fragments **2** and **3** sufficiently pure for the following acylation reactions. The PyBOP-mediated coupling to 9-amino-atc was accelerated by microwave irradiation which overcame the low reactivity of the *ortho*-substituted amine moiety. Finally, cleavage of the *t*Bu-esters and the Boc-protecting group with TFA/DCM and RP18-HPLC purification allowed to obtain the atc-VP1 conjugate **4** and the VP2-conjugate **5**, each as two HPLC-separable isomers. In both cases, the major product displayed the higher retention time. Interestingly, solution of the purified compounds in methanol resulted in a conversion of the major to the minor isomer and vice versa. This was also observed upon re-investigating the VP2-conjugate solution in pyridine-d_6 after measuring the ^{1}H NMR spectrum. We excluded the formation of a 4-epi-derivative which might have been formed during the acidic peptide deprotection procedure, because the resonance of H-4a displayed the 10.6 Hz coupling constant typical for a non-epimerized spacial orientation of H-4 and H-4a (for comparison e.g., see atc-derivative **7**). A dynamic isomerization of an amino acid in the conjugated peptide chain can also be precluded. In our opinion, a plausible explanation is the co-existence of two slowly interconverting tautomers of the vinylogous carboxylic acid formed by the positions 1–3 of the tetracycline ring system.

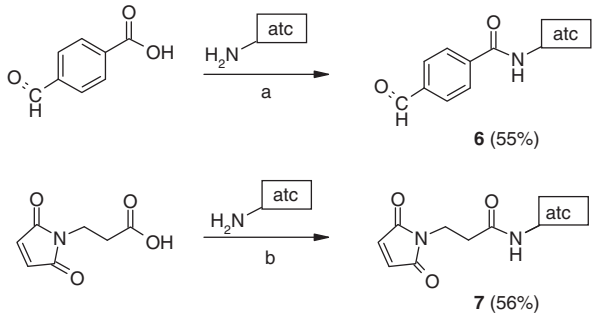
The synthesis of the N-terminally linked peptide conjugates followed the methodology of oxime[14] or maleimide/thiol[15] ligations. Acylation of 9-amino-atc with commercially available 4-formylbenzoic acid or 3-maleimidopropionic acid gave the functionalized atc-derivatives **6** and **7**, respectively (Scheme 2).

Peptide synthesis was performed starting from Fmoc-protected Rink-amide AM resin which was treated with piperidine solution and subsequently acylated with HATU-activated *N*-Fmoc-11-amino-3,6,9-trioxaundecanoic acid employing microwave irradiation (Scheme 3). After capping unreacted amine functions with acetic acid anhydride and subsequent deprotection of the Fmoc-function, the amino acid building blocks were incorporated as described above. At the *N*-terminus, either the hydroxylamine derivative bis-Boc-amino-oxyacetic acid or the protected thiol derivative tritylsulfamylpropionic acid was attached. Cleavage from the resin was achieved with a mixture of TFA and scavengers to yield the hydroxylamine functionalized peptide **8** and the thiol analogue **9**. Oxime formation with 9-(4-formylbenzoylamino)-atc **6** and thiol-Michael addition with the maleimido functionalized atc-derivative **7**, respectively, proceeded easily in buffered solution and we obtained the respective peptide-atc conjugates **10** and **11**, in good yields. In turn, both peptides were obtained as two isomers with the main product eluting at the higher retention time. Interestingly, the major isomer of the thiol-maleimide conjugate displayed two sets of ^{1}H NMR signals. We assume that the new stereogenic center of the succinimide ring did not form in a stereoselective manner and, thus, two diastereomers were generated.

To investigate the in vivo activity of the peptide–anhydrotetracycline conjugates, HeLa cells were cotransfected with the reporter plasmid pUHC13-3[8a] expressing firefly luciferase under Tet control and a plasmid expressing the transregulator rtTA-S3.[7b] This reverse transactivator shows relaxed effector specificity, that is, it binds tetracycline derivatives that are not or less efficiently recognized by other Tet transregulators (Pook, E.; Krueger, C.; Berens, C.; Hillen, W. unpublished observations). For induction of reporter gene expression, transfected cells were incubated 24 h with the respective atc-derivative (5 μM). The data in Table 1 clearly show that the atc-peptide conjugates investigated penetrate the cell membrane. Furthermore, they bind to the reverse transactivator and trigger its conformational change leading to DNA-binding and activation of transcription (Fig. 2b). Structure–activity relationships can also be deduced. Comparison of compounds **4**, **10** and **11** demonstrates, that the transactivating properties also strongly depended on the nature of the linking element and the point of peptide attachment, but not as significantly from the length of the peptide sequence.



Scheme 1. Reagents and conditions: (a) chlorotritylchloride resin, DIPEA, CH_2Cl_2, rt, 2 h, then successive washings with CH_2Cl_2/MeOH/DIPEA (17:2:1), CH_2Cl_2, DMF, CH_2Cl_2; (b) piperidine/DMF (1:4), 18 cycles microwave irradiation 100 W, each −10 °C to 35 °C for 5 s; (c) Fmoc-AA-OH/PyBOP/HOBt/DIPEA (5:5:7.5:5), DMF, 15 cycles microwave irradiation 50 W, each −10 °C to 35 °C for 10 s, N-terminal amino acid: Boc-Asp(OtBu)-OH (for peptide **2**) or Boc-NH$(CH_2CH_2O)_3CH_2CO_2H$ (for peptide **3**); (d) hexafluoroisopropanol/CH_2Cl_2 (1:4), rt, 30 min; (e) (1) PyBOP, HOBt, DIPEA, NMP, rt, then 9-H_2N-atc, 15 cycles microwave irradiation 100 W, each −10 °C to 35 °C for 10 s; (f) TFA/CH_2Cl_2 (1:1), 0 °C to rt, 2–4.5 h, then RP 18-HPLC.



Scheme 2. Reagents and conditions: (a) (1) $SOCl_2$, DMF_{cat}, toluene, $CHCl_3$, 60 °C, 30 min; (2) NEt_3, CH_2Cl_2, rt, 5 h; (b) HATU, DIPEA, NMP, rt, 3.5 h.

Thus, the N-terminally linked oxime derivative **10** proved excellent activity when a 400-fold increase of luciferase activity indicated a transcriptional activation substantially exceeding the inducing properties of anhydrotetracycline. On the other hand, the thiol-maleimide ligate **11** was almost devoid of function.

The 1.5 to 4-fold increased luciferase activity observed with compounds **4**, **5**, and **10** with respect to atc suggested that the coupled activating peptides contribute to overall transactivation. To address this question more stringently, we inactivated the VP16 transactivation domain present in rtTA-S3 by mutagenesis.[10a] HeLa cells were again cotransfected with the Tet-controlled reporter plasmid pUHC13-3[8a] and a plasmid expressing the variant rtTA-S3* with an inactivated activation domain. Induction was determined after a 24 h incubation with either atc or compound **5** bearing the VP2 domain. Addition of atc led only to a threefold increase in luciferase activity over the basal level indicating that the mutations introduced had indeed largely diminished VP16 domain functionality. Addition of compound **5** resulted in a larger, ninefold increase in luciferase activity demonstrating functionality of the peptide sequence in the atc-peptide conjugate in activating transcription.

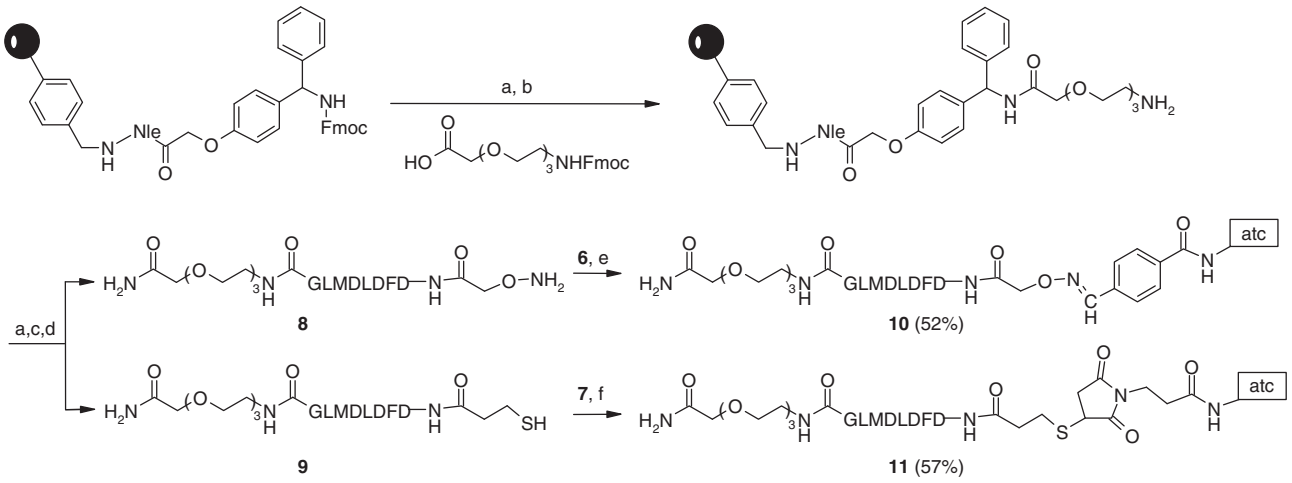
3. Conclusion

In conclusion, bioconjugates of anhydrotetracycline and the Herpes simplex derived minimal active sequences VP1 and VP2 were synthesized. Different ligation strategies were applied and a biological evaluation in HeLa cells expressing the reverse transactivator rtTA-S3 was performed. The data clearly displayed that (i) the structure of the linker unit contributes strongly to the biological activity of a conjugate, (ii) the atc-peptide conjugates are able to penetrate the cell membrane, (iii) they are biologically active as effectors for rtTA-S3 and that (iv) the VP2 sequence can activate transcription when coupled to atc. This demonstrates the general feasibility of ligand-based transactivation.

4. Experimental

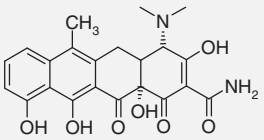
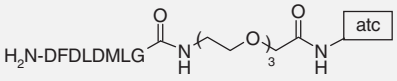
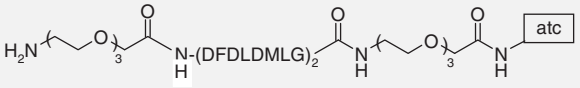
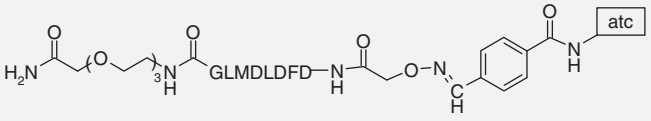
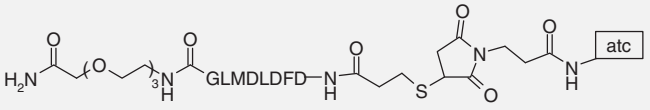
4.1. General

Reagents and solvents were obtained from Acros, Fluka, Aldrich and Novabiochem, and were used as received. 2-Chlorotrityl resin was purchased from IRIS Biotech or from Fluka. Fmoc-11-amino-3,6,9-trioxaundecanoic acid (Fmoc-mini-PEG-3®) and Boc-11-ami-



Scheme 3. Reagents and conditions: (a) piperidine/DMF (1:4), 18 cycles microwave irradiation 100 W, each −10 °C to 35 °C for 5 s; (b) (1) HATU, DIPEA, DMF, rt, 2 h, then 15 cycles microwave irradiation 50 W, each −10 °C to 35 °C for 10 s; (2) Ac_2O, 2,6-lutidine, DMAP, rt, 15 min; (3) piperidine/DMF (1:4), 18 cycles microwave irradiation 100 W, each −10 °C to 35 °C for 5 s; (c) Fmoc-AA-OH/PyBOP/HOBt/DIPEA (5:5:7.5:5), DMF, 15 cycles microwave irradiation 50 W, each −10 °C to 35 °C for 10 s, N-terminal amino acid: $Boc_2NOCH_2CO_2H$ (for peptide **8**) or Trt-$SCH_2CH_2CO_2H$ (for peptide **9**); (d) phenol/H_2O/thioanisole/triisopropysilane/TFA (0.25:0.25:0.25:0.125:0.15:5), rt, 2 h; (e) NaOAc/HOAc buffer 0.1 M (pH 4.5), THF, CH_3CN, rt, 20 h, then RP 18-HPLC; (f) KH_2PO_4/Na_2HPO_4 buffer (pH 5.5, Ph. Eur.), THF, CH_3CN, rt, 200 min, then RP 18-HPLC.

Table 1
In vivo activity of peptide–anhydrotetracycline conjugates

	Compound	Activity[a]
atc		100
4		150
5		190
10[a]		400
11		9

Luciferase activities determined from cell extracts represent the means of triplicate samples with standard deviations given in relative light units (RLU; defined as relative light units per μg of total cell protein corrected for transfection efficiency).

[a] % Activity with atc defined as 100%. Induction factors were calculated as $RLU_{inducer}/RLU_{basal}$ and varied in individual transfections between 107 and 525 for atc.

no-3,6,9-trioxaundecanoic acid × DCHA (Boc-mini-PEG-3®) were purchased from Peptides International (Louisville, Kentucky) and the DCHA salt was converted to the free acid according to the protocol of Peptides International. 3-Tritylsulfanyl-propionic acid was obtained from Bachem (Bubendorf, Switzerland). Unless otherwise noted, reactions were conducted without inert atmosphere. Microwave assisted (Discover® microwave oven, CEM Corp.) peptide synthesis was carried out in glass tubes loosely sealed with a silicon septum. Remark: the development of overpressure was avoided by using DMF as the solvent and intermittent cooling. Evaporations of final product solutions were done in vacuo with a rotary evaporator or by lyophilization. Reactions were monitored by HPLC-MS. ESI and APCI-MS were performed using a Bruker Esquire 2000 coupled with an Agilent 1100 analytic HPLC system. The NMR spectra were recorded on a Bruker Avance 600 (^1H at 600 MHz, ^{13}C at 150 MHz) or a Bruker Avance 360 (^1H at 360 MHz, ^{13}C at 90 MHz) spectrometer. Chemical shifts are reported relative to tetramethylsilane. Preparative RP-HPLC was performed using Agilent 1100 preparative series, column: Zorbax Eclipse XDB-C8, 21.2 × 150 mm, 5 μm particles [C8], flow rate [FR] as specified below, eluent: methods **P1–P3**: 0.1% TFA in acetonitrile (A) and 0.1% TFA in H_2O (B) applying a gradient system as described subsequently: **P1**: 25–42% A in 0–20 min, [FR] = 15 mL/min. **P2**: 25–45% A in 0–20 min and then 45–100% A in 20–23 min, [FR] = 16 mL/min. **P3**: 10–20% A in 0–5 min, 20–70% A in 5–20 min and then 70–95% A in 20–22 min, [FR] = 20 mL/min.

Purity and identity were assessed by analytical RP-HPLC (Agilent 1100 analytical series, column: Zorbax Eclipse XDB-C8 analytical column, 4.6 × 150 mm, 5 μm, flow rate: 0.5 ml/min, detection wavelength: 254 nm) coupled to a Bruker Esquire 2000 mass detector equipped with an ESI- or APCI-trap. Solvent system: x% CH_3OH in H_2O (A) + 0.1% HCO_2H (B): **A1**: 25–100% A in 0–12 min, 100–100% A in 12–18 min, 100–25% in 18–22 min. IR spectra were measured on a Jasco 410 apparatus, using KBr.

4.2. Synthesis of fully protected peptides

4.2.1. 11-Amino-3,6,9-trioxaundecanoyl-2-Cl-Trt-resin (1)

2-Chlorotritylchloride resin (chloro-2-Cl-Trt-resin, loading: 1.1–1.6 mmol/g) was stirred in a solution of Fmoc-11-amino-3,6,9-trioxaundecanoic acid (1.2 equiv)/diisopropylethylamine (DIPEA, 4 equiv) in CH_2Cl_2. After 2 h the resin was washed 3 × with CH_2Cl_2/CH_3OH/DIPEA (17:2:1), 3 × CH_2Cl_2, 2 × DMF, 2 × CH_2Cl_2 and dried in vacuo. The Fmoc group was removed by treating the resin 1 × with 20% piperidine in DMF (microwave irradiation: 5 × 5 s, 100 W). In between each irradiation step, cooling of the reaction mixture to a temperature of −10 °C was achieved by sufficient agitation in an ethanol-ice bath. Finally, the resin was washed with DMF (5×).

4.2.2. General procedure for the synthesis of protected peptides

α-Amino acids were incorporated as their commercially available derivatives: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Phe-OH and, depending on the sequence, either Boc-Asp(O*t*Bu)-OH or Boc-11-amino-3,6,9-trioxaundecanoic acid at the N-terminus. Peptide coupling was done employing 5 equiv of the respective amino acid (or linker)/PyBOP/DIPEA and 7.5 equiv HOBt, dissolved in a minimum amount of DMF (irradiation: 15 × 10 s, 50 W). In between each irradiation step, cooling of the reaction mixture to a temperature of −10 °C was achieved by sufficient agitation in an ethanol-ice bath. The Fmoc group was removed as described above. After each coupling and Fmoc-deprotection step, the resin was rinsed with DMF (4×). After the last acylation step the *N*-terminal Fmoc-residue was deprotected, the resin was rinsed with CH_2Cl_2 (10×) and dried in vacuo. Cleavage from the resin was performed by the treatment with hexafluoroisopropanol/dichloromethane 1:4 for 20 min followed by filtration and removal of the solvent in vacuo. The following crude peptides were obtained in sufficient purities and employed for the acylation reactions without further purification.

4.2.3. Boc-Asp(O*t*Bu)-Phe-Asp(O*t*Bu)-Leu-Asp(O*t*Bu)-Met-Leu-Gly-11-amino-3,6,9-trioxa-undecanoic acid (2)

The fully protected peptide was synthesized according to the general procedure starting from 11-amino-3,6,9-trioxaundecanoyl-2-Cl-Trt-resin (**1**). Boc-Asp(O*t*Bu)-OH was introduced as the last amino acid. For characterization, a small sample was fully deprotected using TFA/dichloromethane 1:1. ESI-MS: calcd for $C_{96}H_{149}N_{18}O_{37}S_2$ $[M+H]^+$: 1114.5, found: 1114.5.

4.2.4. Boc-11-Amino-3,6,9-trioxaundecanoyl-Asp(O*t*Bu)-Phe-Asp-(O*t*Bu)-Leu-Asp(O*t*Bu)-Met-Leu-Gly-Asp(O*t*Bu)-Phe-Asp(O*t*Bu)-Leu-Asp(O*t*Bu)-Met-Leu-Gly-11-amino-3,6,9-trioxa-undecanoic acid (3)

The fully protected peptide was synthesized according to the general procedure starting from 11-amino-3,6,9-trioxaundecanoyl-2-Cl-Trt-resin (**1**). Boc-11-amino-3,6,9-trioxaundecanoic acid was introduced as the last amino acid. For characterization, a small sample was fully deprotected using TFA/dichloromethane 1:1. ESI-MS: calcd for $C_{48}H_{76}N_9O_{19}S$ $[M+H]^+$: 2910.0, [(M+2H)/2]: 1105.5, [(M+3H)/3]: 737.3; found: 1105.6 $[(M+2H)/2]^+$, [(M+3H)/3]: 737.6.

4.3. General procedure for the acylation of 9-aminoanhydrotetracycline with protected peptides and subsequent deprotection

The protected peptide, PyBOP and HOBt·H_2O were dissolved in dry NMP and diisopropylethylamine was added. After stirring for 10–15 min, 9-aminoanhydrotetracycline in dry NMP was added and microwave irradiation (15 × 10 s, 100 W) with intermittent cooling in an ethanol-ice bath was performed. The solvent was removed and the protected peptide conjugate was either purified by preparative RP-HPLC or isolated by precipitation with H_2O. After lyophilization of the HPLC fractions or drying of the precipitate, the residue was dissolved in a chilled (0 °C) mixture of TFA/dichloromethane 1:1 and then stirred at rt for 3–5 h. The solvent was removed in vacuo and the residue was purified by RP-HPLC and lyophilized.

4.3.1. Conjugate 4·2CF_3COOH

The compound was synthesized using the fully protected peptide derivative Boc-NHD(O*t*Bu)FD(O*t*Bu)LD(O*t*Bu)MLGCONH$(CH_2CH_2O)_3CH_2CO_2H$ (**2**, 26.7 mg, 0.019 mmol), PyBOP (10.2 mg, 0.019 mmol), HOBt·H_2O (4.6 mg, 0.030 mmol), diisopropylethylamine (201 μl, 0.119 mmol) and NMP (0.5 mL). Preactivation time: 10 min. Addition of 9-aminoanhydrotetracycline[11] (12.9 mg, 0.029 mmol) in NMP (1.0 mL). Preparative RP-HPLC of the protected intermediate (**P1**, t_R: 14.3 min). Deprotection: TFA/CH_2Cl_2 1:1 (2 ml), 3 h. Preparative RP-HPLC (**P2**) furnished a main product (t_R: 12.9 min, 6.0 mg, 18%) and a second isomer (t_R: 12.2 min, 6.0 mg, 7%), both as an orange yellow powder. Analytical HPLC (**A1**): main product: t_r: 11.8 min, purity: 90% (contamination: 2nd isomer), second isomer: t_r: 11.0 min. ESI-MS: calcd for $C_{70}H_{96}N_{12}O_{25}S$ $[M+H]^+$: 1537.6, [(M+2H)/2]: 769.3; found: 1537.7 $[M+H]^+$, 769.6 $[(M+2H)/2]^+$.

4.3.2. Conjugate 5·2CF_3COOH

The compound was synthesized using the fully protected peptide derivative Boc-NH$(CH_2CH_2O)_3$CONH[D(O*t*Bu)FD(O*t*Bu)LD(O*t*Bu)MLG]$_2$CONH$(CH_2CH_2O)_3CH_2CO_2H$ (**3**, 70.2 mg, 0.027 mmol), PyBop (13.8 mg, 0.027 mmol), HOBt·H_2O (6.2 mg, 0.041 mmol), diisopropylethylamine (29 μl, 0.119 mmol) and NMP (1.0 mL). Preactivation time: 15 min (ultrasound bath). Addition of 9-aminoanhydrotetracycline[11] (14.7 mg, 0.033 mmol) in NMP (1.0 mL). Then the solvent was lyophilized, the residue redissolved in THF/methanol 1:1 (6 mL) and the protected intermediate precipitated by addition of H_2O (10 mL), decanted and centrifugated. The mother liquor was concentrated and the precipitation procedure was repeated with H_2O (5 mL). The combined precipitates were washed with H_2O (5 mL), then again decanted, centrifugated and dried in vacuo. Deprotection: TFA/dichloromethane 1:1 (4 ml), 5 h. Preparative RP-HPLC (**P2**) furnished a main product (t_R: 20.5 min, 4.2 mg, 6%) and a second isomer (t_R: 19.9 min, 1.3 mg, 2%), both as an orange yellow powder. Analytical HPLC (**A1**): main product: t_r: 13.1 min, purity: 88% (contamination: 2nd isomer), 2nd isomer: t_r: 12.5 min. ^{1}H NMR (600 MHz, C_5D_5N, two sets of signals were observed) δ 0.63–0.71 (m, 3H, CH_3 Leu), 0.72–1.16 (m, 21H, 7 × CH_3 Leu), 1.79–1.86 (m, 1H, C^βH Leu), 1.90–2.20 and 2.09, 2.11, 2.16, 2.17 (m and 4 × s, 13H, 5 × C^βH Leu, 2 × C^γH Leu and 2 × CH_3 Met), 2.25–2.35 (m, 2H, 2 × C^γH Leu), 2.36–2.46 and 2.40 (m and s, 5H, 2 × C^βH Leu und ar-CH_3), 2.47–2.61 and 2.58 (m and s, 7H, C^βH Met and $N(CH_3)_2$), 2.60–2.69 (m, 1H, C^βH Met), 2.69–2.97 (m, 7H, 2 × C^βH Met, 4 × C^γH Met and H-5), 3.06 (br d, 1H, *J* = 10.2 Hz, H-4a), 3.20–3.96 and 3.70 (m, 41H, H-4, H-5, CH_2 Gly, 5 × CH_2CH_2O, 13 × C^βH Asp/Phe, linker OCH_2CH_2NHCO and OCH_2-CO), 4.11–4.41 and 4.33 (m, 7H, CH_2 Gly, 3 × C^βH Asp/Phe and OCH_2CO), 4.52–4.65 (m, 3H, 3 × C^αH AS), 4.74–4.81 (m, 1H, C^αH AS), 4.86–4.95 (m, 2H, 2 × C^αH AS), 4.95–5.29 (m, 10H, 8 × C^αH AS and OCH_2CH_2NHCO), 7.25–7.28 (m, 1H, H-4′ Phe within the pyridine signal), 7.28–7.35 (m, 3H, H-4′ Phe and H-3′/5′ Phe), 7.38–

7.47 (m, 3H, H-7 and H-3′/5′ Phe), 7.48–7.54 (m, 2H, H-2′/6′ Phe), 7.60–7.69 (m, 3H, H-2′/6′ Phe and NH), 8.17–8.36 (m, 2H, 2 × NH), 8.54–8.71 (m, 3H, 3 × NH), 8.76–8.85 (m, 2H, NH and H-8), 8.88–9.19 (m, 9H, 9 × NH), 9.59–9.70 und 9.64 (m and s, 2H, NH und NH-9), 10.11–10.22 (m, 2H, $CONH_2$). ESI-MS: calcd for $C_{118}H_{170}N_{21}O_{43}S_2$ $[M+H]^+$: 2633.1, [(M+2H)/2]: 1317.1, [(M+3H)/3]: 878.4; found: 1317.2 $[(M+2H)/2]^+$, [(M+3H)/3]: 879.0.

4.4. Synthesis of the 9-acylaminoanhydrotetracycline precursors

4.4.1. 9-(4-Formylbenzoylamino)-anhydrotetracycline·CF_3COOH (6)

To a solution of 4-formylbenzoic acid (35.8 mg, 0.238 mmol) in a mixture of dry toluene (1.5 mL) and dry chloroform (1.5 mL) DMF (15 μL) and thionyl chloride (80 μL, 1.096 mmol) were added dropwise at rt and then the mixture was stirred for 30 min at 60 °C. The solvent was evaporated and the crude product was dried in vacuo. 9-Aminoanhydrotetracycline[11] (105.1 mg, 0.238 mmol) was dissolved in dry dichloromethane (2 mL) and added to the crude acid chloride, followed by the addition of triethylamine (33 μl, 0.237 mmol). After stirring for 5 h, the solvent was removed in vacuo and the crude product purified by preparative RP-HPLC. (**P3**, t_R: 16.7 min) **1** to afford 90.5 mg (55%) as a red powder, mp 173–176 °C (decomposition). Analytical HPLC (**A1**): t_r: 15.8 min, purity: 97%. IR (KBr) 3600–2700, 1698, 1671, 1650, 1599 cm^{-1}; ^1H NMR (600 MHz, C_5D_5N): δ 2.44 (s, 3H, ar-CH_3), 2.62 (s, 6H, $N(CH_3)_2$), 3.12 (ddd, 1H, J = 11.7, 4.5, 1.9 Hz, H-4a), 3.59 (dd, 1H, J = 16.6, 4.5 Hz, H-5α), 3.73–3.81 (m, 2H, H-4 and H-5β), 7.54 (d, 1H, J = 9.1 Hz, H-7), 8.02–8.09 (m, 2H, H-3′/5′), 8.38–8.44 (m, 2H, H-2′/6′), 8.91 (d, 1H, J = 9.1 Hz, H-8), 10.11–10.18 (br s and s, 2H, NH und CHO), 10.34 (br s, 1H, NH), 10.37 (br s, 1H, $CONH_2$).^{13}C NMR (150 MHz, C_5D_5N) δ 14.6 (ar-CH_3), 25.4 (C-5), 42.5 ($N(CH_3)_2$), 42.6 (C-4a), 65.7 (C-4), 78.3 (C-12a), 101.7 (C-2), 110.1 (C-11a), 113.5 (C-10a), 115.5 (C-7), 122.8 (C-6), 123.0 (C-9), 128.5 (C-8), 129.2 (C-2′/6′), 130.3 (C-3′/5′), 132.0 (C-5a), 136.2 (C-6a), 137.1 (C-1′), 139.2 (C-4′), 141.3 (C-10), 165.8 (C-11), 166.6 (ar-NHCO), 175.0 ($CONH_2$), 192.4 (ar-CHO), 194.1 (C-1), 198.2 (C-3), 200.0 (C-12). APCI-MS 574.7 $[M+H]^+$.

4.4.2. 9-(3-Maleimidopropionylamino)-anhydrotetracycline·CF_3-COOH (7)

To a solution of 3-maleimidopropionic acid (27.8 mg, 0.238 mmol) and HATU (62.7 mg, 0.165 mmol) in dry DMF (0.5 mL) diisopropylethylamine (57 μL, 0.332 mmol) was added dropwise at rt and then, after 15 min, a solution of 9-aminoanhydrotetracycline[11] (72.9 mg, 0.165 mmol) in dry DMF (1.5 mL). After stirring for 5 h, the solvent was removed in vacuo and the crude product purified by preparative RP-HPLC (**P3**, t_R: 15.0 min) **1** to afford 65.4 mg (56%) as a red powder, mp 166–170 °C (decomposition). Analytical HPLC (**A1**): t_r: 9.2 min, purity: 97%. IR (KBr) 3650–2900, 3103, 2954, 2922, 1772, 1710, 1674, 1640, 1599 cm^{-1}; ^1H NMR (600 MHz, C_5D_5N):δ 2.40 (s, 3H, ar-CH_3), 2.62 (s, 6H, $N(CH_3)_2$), 3.10 (ddd, 1H, J = 11.4, 4.5, 1.6 Hz, H-4a), 3.11 (t, 2H, J = 7.2 Hz, $C^{2'}H_2$), 3.55 (dd, 1H, J = 16.4, 4.5 Hz, H-5α), 3.73 (dd, 1H, J = 16.4, 1.6 Hz, H-5β), 3.75 (d, 1H, J = 11.4 Hz, H-4), 4.18 (t, 2H, J = 7.2 Hz, $C^{3'}H_2$), 6.81 (s, 2H, maleinimide), 7.45 (d, 1H, J = 9.2 Hz, H-7), 8.83 (d, 1H, J = 9.2 Hz, H-8), 10.11–10.16 (m, 2H, NH-9 and $CONH_2$), 10.37 ($CONH_2$).^{13}C NMR (150 MHz, C_5D_5N) δ 14.6 (ar-CH_3), 25.4 (C-5), 35.0 (C-2′/3′), 35.9 (C-2′/3′), 42.6 ($N(CH_3)_2$), 42.6 (C-4a), 65.8 (C-4), 78.3 (C-12a), 101.7 (C-2), 109.8 (C-11a), 113.2 (C-10a), 115.6 (C-7), 124.7 (C-9), 128.7 (C-8), 131.4 (C-5a), 136.5 (C=C, imide), 136.7 (C-6a), 151.1 (C-10), 166.1 (C-11), 166.2 (C=O, imide), 170.0 (ar-NHCO), 175.1 ($CONH_2$), 194.0 (C-1), 198.3 (C-3), 200.3 (C-12). APCI-MS 593.3 $[M+H]^+$.

4.5. General procedure for the synthesis of N-terminally linked peptides

The synthesis was accomplished starting from Rink-amide AM resin (200–400 mesh, Novabiochem, loading: 0.25 mmol/g). As the first amino acid, Fmoc-11-amino-3,6,9-trioxaundecanoic acid (1 equiv) was attached employing 1 equiv PyBOP/DIPEA and 1.5 equiv HOBt, dissolved in a minimum amount of DMF, followed by capping with a mixture of acetic acid anhydride/2,6-lutidine/4-dimethylaminopyridine 5:6:1 in DMF. α-Amino acids were incorporated as their commercially available derivatives: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Phe-OH and, depending on the sequence, either Bis-Boc-amino-oxyacetic acid or 3-tritylsulfanylpropionic acid were attached at the *N*-terminus. Fmoc-deprotection and elongation was performed as described above. Cleavage from the resin was performed by the treatment with a mixture of TFA/phenol/H_2O/thioanisole/EDT/triisopropylsilane 80:5:5:5:3:2 for 2 h followed by filtration and removal of the solvent in vacuo. The crude peptides were precipitated in *t*-butylmethylether, centrifugated and washed with *t*-butylmethylether (4×). They were obtained in sufficient purities and employed for the ligation reactions without further purification.

4.5.1. Aminooxyacetyl-Asp-Phe-Asp-Leu-Asp-Met-Leu-Gly-11-amino-3,6,9-trioxa-undecanoic amide (8)

The peptide was synthesized according to the general procedure. ESI-MS: calcd for $C_{50}H_{80}N_{11}O_{20}S$ $[M+H]^+$: 1186.5, found: 1186.5.

4.5.2. 3-Sulfanylpropionyl-Asp-Phe-Asp-Leu-Asp-Met-Leu-Gly-11-amino-3,6,9-trioxa-undecanoic amide (9)

The peptide was synthesized according to the general procedure. ESI-MS: calcd for $C_{51}H_{81}N_{10}O_{19}S_2$ $[M+H]^+$: 1201.5, found: 1201.5.

4.6. Ligation

4.6.1. Conjugate 10•CF_3COOH

The peptide H_2NOCH_2CO–DFDLDMLG–$NH(CH_2CH_2O)_3CH_2CO$-NH_2 (**8**, 11.4 mg, 8.7 μmol) was dissolved in a mixture of CH_3CN (0.5 mL) and 0.1 M acetate buffer pH 4.5 (1.0 mL). To a suspension of 9-(4-formylbenzoylamino)-anhydrotetracycline·CF_3COOH (**6**, 6.0 mg, 8.7 μmol) in CH_3CN (0.5 mL) and 0.1 M sodium acetate buffer pH 4.5 (1.0 mL) THF (q.s.) was added to result in the formation of a clear solution. This solution was slowly added to the peptide solution and stirred for 20 h under nitrogen. The solvent was removed by lyophilization and preparative RP-HPLC (**P3**) was performed to furnish a main product (t_R: 15.9 min, 5.3 mg, 33%) and a second isomer (t_R: 15.6 min, 3.1 mg, 19%), both as an orange yellow powder. Analytical HPLC (**A1**): main product: t_r: 13.1 min, purity: 95% (contamination: 2nd isomer), second isomer: t_r: 12.5 min, purity: 94%. ^1H NMR (600 MHz, C_5D_5N) δ 0.85 (d, 3H, J = 6.4 Hz, CH_3 Leu), 0.88 (d, 6H, J = 6.0 Hz, CH_3 Leu), 0.93 (d, 3H, J = 6.0 Hz, CH_3 Leu), 1.92–1.98 (m, 2H, $C^{\beta}H_2$ Leu), 2.00–2.11 and 2.02 (m and s, 7H, $C^{\beta}H_2$ Leu, 2 × H^{γ} Leu and SCH_3), 2.44 (s, 3H, ar-CH_3), 2.49 (m, 1H, H^{β} Met), 2.56–2.67 und 2.62 (m and s, 7H, H^{β} Met and $N(CH_3)_2$), 2.82 (ddd, 1H, J = 13.3, 7.1, 6.1 Hz, H^{γ} Met), 2.91 (ddd, 1H, J = 13.3, 8.9, 5.0 Hz, H^{γ} Met), 3.12 (ddd, 1H, J = 11.9, 4.7, 2.1 Hz, H-4a), 3.19 (dd, 1H, J = 16.6, 6.6 Hz, H^{β} Asp^1), 3.25 (dd, 1H, J = 16.5, 6.0 Hz, H^{β} Asp^2), 3.30 (dd, 1H, J = 13.9, 8.7 Hz, H^{β} Phe), 3.33 (dd, 1H, J = 16.3, 8.3 Hz, H^{β} Asp^3), 3.37 (dd, 1H, J = 16.5, 7.0 Hz, H^{β} Asp^2), 3.43 (dd, 1H, J = 16.3, 5.5 Hz, H^{β} Asp^3), 3.44 (dd, 1H, J = 16.6, 7.4 Hz, H^{β} Asp^1), 3.53 (dd, 1H, J = 13.9, 5.3 Hz, H^{β} Phe), 3.56–3.64 und 3.59 (m and dd, 7H, J = 15.6, 4.7 Hz, $(OCH_2CH_2)_3$ and H-5α), 3.65–3.70 (m, 6H, $(OCH_2CH_2)_3$), 3.76 (d, 1H, J = 11.9 Hz, H-4), 3.76 (dd, 1H, J = 15.6, 2.1 Hz, H-5β), 4.23 (s, 2H, H_2NCOCH_2O), 4.33 (dd, 1H, J = 16.5, 6.1 Hz, $C^{\alpha}H$ Gly), 4.36 (dd, 1H, J = 16.5, 5.9 Hz, $C^{\alpha}H$ Gly), 4.79 (m, 1H, $C^{\alpha}H$ Leu or Met),

4.89–5.00 and 4.91, 4.97 (m and 2 × d, 3H, 2 × J = 15.4 Hz, $C^{\alpha}H$ Leu or Met and C═N–OCH_2CO), 5.06 (ddd, 1H, J = 9.6, 7.0, 4.5 Hz, $C^{\alpha}H$ Leu or Met), 5.21 (ddd, 1H, J = 8.3, 7.4, 5.5 Hz, $C^{\alpha}H$ Asp^3), 5.38 (ddd, 1H, J = 8.0, 7.0, 6.0 Hz, $C^{\alpha}H$ Asp^2), 5.43 (m, 1H, $C^{\alpha}H$ Phe), 5.55 (m, 1H, $C^{\alpha}H$ Asp^1), 7.20 (m, 1H, H-4′ Phe), 7.30 (m, 2H, H-2′/6′ Phe), 7.38 (m, 2H, H-3′/5′ Phe), 7.54 (d, 1H, J = 9.1 Hz, H-7), 7.73 (m, 2H, BB′), 7.87 (br s, 1H, CH_2CONH_2), 8.19 (s, 1H, N═C*H*Ph), 8.24 (br s, 1H, CH_2CONH_2), 8.26 (m, 2H, AA′), 8.32–8.35 (m, 1H, OCH_2CH_2NH), 8.57 (d, 1H, J = 7.2 Hz, NH), 8.71 (d, 1H, J = 7.2 Hz, NH), 8.83 (dd, 1H, J = 6.1, 5.9 Hz, NH Gly), 8.93 (d, 1H, J = 9.1 Hz, H-8), 8.95 (d, 1H, J = 7.0 Hz, NH), 9.20 (d, 1H, J = 8.0 Hz, NH Asp^2), 9.26 (d, 1H, J = 6.2 Hz, NH), 9.48 (d, 1H, J = 7.4 Hz, NH Asp^3), 9.54 (d, 1H, J = 7.0 Hz, NH), 10.09 (s, 1H, C^9NH), 10.16 (br s, 1H, $CONH_2$ atc), 10.37 (br s, 1H, $CONH_2$ atc). ESI-MS: calcd for $C_{80}H_{105}N_{14}O_{28}S$ $[M+H]^+$: 1741.7, [(M+2H)/2]: 871.4; found: 1741.7 $[M+H]^+$, 871.7 $[(M+2H)/2]^+$.

4.6.2. Conjugate 11·CF_3COOH

9-(3-Maleimidopropionylamino)-anhydrotetra-cycline·CF_3COOH (**7**, 10.5 mg, 0.015 mmol) was dissolved in THF (volume least possible), then phosphate buffer pH 5.5, (Ph. Eur., 3.0 mL) was added. The peptide $HSCH_2CH_2O$–DFDLDMLG–$NH(CH_2CH_2O)_3CH_2CONH_2$ (**8**, 11.4 mg, 8.7 μmol) was dissolved in a mixture of CH_3CN (1.5 mL) and phosphate buffer pH 5.5 (Ph. Eur., 1.0 mL) and slowly added to the solution described above. After 3 h stirring under nitrogen, the solvent was removed by lyophilization and preparative RP-HPLC (**P3**) was performed to furnish a main product (t_R: 14.9 min, 13.3 mg, 47%) and a second isomer (t_R: 14.7 min, 2.7 mg, 10%), both as an orange yellow powder. Analytical HPLC (**A1**): main product: t_r: 13.7 min, purity: 91% (contamination: 6% 2nd isomer), second isomer: t_r: 12.7 min, purity: 81% (contamination: 17% 1st isomer). ^{1}H NMR (600 MHz, C_5D_5N, two sets of signals were observed, ratio 1:1) δ 0.86–0.92 (m, 9H, CH_3 Leu), 0.92–0.97 (m, 3H, CH_3 Leu), 1.91–2.15 and 2 × 2.03 (m and 2 × s, 9H, 2 × $C^{\beta}H_2$ Leu, 2 × $C^{\gamma}H$ Leu and CH_3 Met), 2.39 (s, 1.5H, ar-CH_3), 2.40 (s, 1.5H, ar-CH_3), 2.47–2.55 (m, 1H, $C^{\beta}H$ Met), 2.58–2.64 and 2 × 2.61 (m and 2 × s, 7H, $C^{\beta}H$ Met and $N(CH_3)_2$), 2.76–2.89 (m, 4H, H-4″, $C^{\gamma}H$ Met and SCH_2CH_2CO), 2.92 (ddd, 0.5H, J = 13.3, 8.7, 4.8 Hz, $C^{\gamma}H$ Met), 2.93 (ddd, 0.5H, J = 13.5, 8.5. 5.1 Hz, $C^{\gamma}H$ Met), 3.05–3.17 (m, 4H, H-4a, $C^{\alpha}H_2$ propionic acid and $C^{\beta}H$ Asp^2), 3.20–3.48 (m, 9H, H-5α, SCH_2CH_2CO, 2 × H-4 succinimide ring, 2 × $C^{\beta}H$ Asp und $C^{\beta}H$ Phe), 3.51–3.58 (m, 2H, H-5α and $C^{\beta}H$ Phe), 3.59–3.65 (m, 6H, $(OCH_2CH_2)_3$), 3.64–3.77 (m, 8H, $(OCH_2CH_2)_3$, H-4 and H-5β), 4.15–4.25 (m, 5H, CH_2CONH_2, $C^{\beta}H_2$ propionic acid and H-3 succinimide ring), 4.30–4.39 (m, 2H, CH_2 Gly), 4.74–4.81 (m, 1H, $C^{\alpha}H$ Leu^1), 4.93–5.00 (m, 1H, $C^{\alpha}H$ Leu^2), 5.02–5.09 (m, 1H, $C^{\alpha}H$ Met), 5.14–5.21 (m, 1H, $C^{\alpha}H$ Phe), 5.33–5.40 (m, 1H, $C^{\alpha}H$ Asp^3), 5.40–5.45 (m, 1H, $C^{\alpha}H$ Asp^2), 5.45–5.52 (m, 1H, $C^{\alpha}H$ Asp^1), 7.19–7.23 (m, 1H, H-4′ Phe), 7.27–7.32 (m, 2H, H-2′/6′ Phe), 7.37–7.43 (m, 2H, H-3′/5′ Phe), 7.45 (d, 1H, J = 9.1 Hz, H-7), 7.87 (br s, 1H, CH_2CONH_2), 8.24 (br s, 1H, CH_2CONH_2), 8.28–8.32 (m, 1H, OCH_2CH_2NH), 8.51–8.57 (m, 1H, NH Leu^2), 8.66–8.71 (m, 1H, NH Met), 8.78–8.85 and 8.82 (m and d, 2H, J = 9.1 Hz, NH Gly and H-8), 8.92 (d, 0.5H, J = 6.0 Hz, NH Asp^3), 8.92 (d, 0.5 Hz, J = 6.4 Hz, Asp^3), 9.03 (d, 0.5 Hz, J = 6.4 Hz, NH Leu^1), 9.07 (d, 0.5H, J = 6.0 Hz, NH Leu^1), 9.26 (d, 0.5H, J = 7.2 Hz, NH Asp^2), 9.30 (d, 0.5H, J = 7.2 Hz, NH Asp^2), 9.55 (d, 0.5H, J = 6.8 Hz, NH Phe), 9.57 (d, 0.5H, J = 6.4 Hz, NH Phe), 9.57 (d, 0.5H, J = 7.6 Hz, NH Asp^1), 9.60 (d, 0.5H, J = 7.6 Hz, NH Asp^1), 10.06 (s, 0.5H, NH-9), 10.08 (s, 0.5H, NH-9), 10.14 (d, 1H, J = 1.6 Hz, $CONH_2$ Atc), 10.36 (d, 1H, J = 1.6 Hz, $CONH_2$ Atc).

4.7. Bacterial strain, human cell line and cell culture methods

The bacterial strain DH5α[16a] was used for general cloning procedures[16b] and is derived from *Escherichia coli* K12. The cell line HeLa (ATCC #CCL-2) was cultured in high glucose Dulbecco's Modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco-BRL), 120 μg/mL penicillin, 120 μg/mL streptomycin and 2 mM L-glutamine in a humidified incubator at 37 °C under 7.5% CO_2. Transient transfections and luciferase activity determinations were performed as described.[4b]

4.8. Construction of rtTA-S3*

The amino acid exchanges SL439, FG442, SL444 and FA475 were introduced into the VP16 domain by site-directed mutagenesis using overlap extension PCR,[16c] four mutagenic oligonucleotides and two flanking oligonucleotides to introduce the restriction sites for cloning. The resulting DNA-fragment was incubated with the restriction enzymes XcmI/BamHI and cloned into likewise-restricted pWHE330.[4b] The correct insertion was verified by sequencing. All plasmid and primer sequences are available upon request.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 473). Andreas Schmidt, Iris Torres and Michaela Kettler are acknowledged for skillful technical assistance.

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