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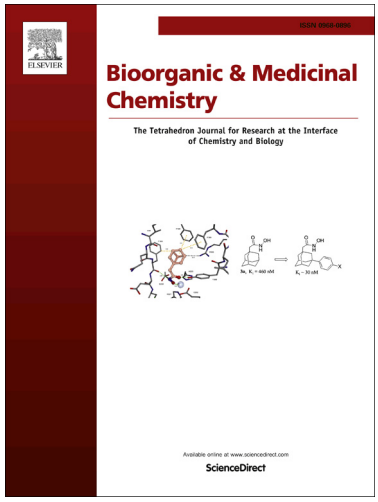

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Macrocyclic analogues of the diuretic insect neuropeptide helicokinin I show strong receptor-binding

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Abstract: Helicokinin I, a diuretic neuropeptide of the relevant cotton pest *Helicoverpa zea* represents a promising target for the design of insect neuropeptide mimetics. Using a ring-closing metathesis reaction, N-terminal bridged macrocyclic helicokinin I analogues with different rigidity were prepared and tested in a helicokinin receptor assay. A partially peptidomimetic helicokinin analogue, containing two structural modifications provides a deeper insight into the structural-requirements for receptor-binding.

INTRODUCTION

Neuropeptides control numerous vital functions in insects such as osmoregulation, oviposition muscle activity, development and pheromone production.[1] Insect neuropeptides are released in minute amounts from specialized neuroglandular cells and transported through the haemolymph to their target organs where they induce specific physiological reactions. Insect neuropeptides are small peptides, containing in general not more than 6 to 13 residues.[2] Within the family of insect neuropeptides the myokinins constitute a large class of multifunctional hormones expressing myotropic and potent diuretic activities. The myokinins are characterized by a highly conserved C-terminal pentapeptide sequence with the general formula Phe-X_1-X_2-Trp-GlyNH_2, where X_1 is Ser, His, Asn, Tyr and X_2 is Ser or Pro.[3] Helicokinins I-III have been isolated from *Heliothis zea* and were found to be highly diuretic.[4] In particular, helicokinin I (Tyr-Phe-Ser-Pro-Trp-GlyNH_2, **1**) stimulates Malpighian tubules with EC_{50} values in the range of 10^{-8} M and activates the helicokinin I receptor with an EC_{50} value as low as 2×10^{-9} M.[5] Furthermore it has been demonstrated that helicokinin I increases mortality after injection into larvae of *Heliothis virescens*, a serious pest of cotton.[6]

Since their discovery almost a century ago, insect neuropeptides have been discussed as lead structures for novel, environmentally beneficial and highly selective insecticides.[7] However, due to insufficient metabolic stability, missing cuticula penetration and poor solubility, the native peptides are particularly unsuited as crop protection agents. Despite of tremendous efforts during the recent past to improve the physicochemical properties by exchanging specific residues by unnatural amino acid analogues or by introduction of turn-mimetics, no insect neuropeptide analogue has entered the development phase or even the market, yet.[8,9,10]

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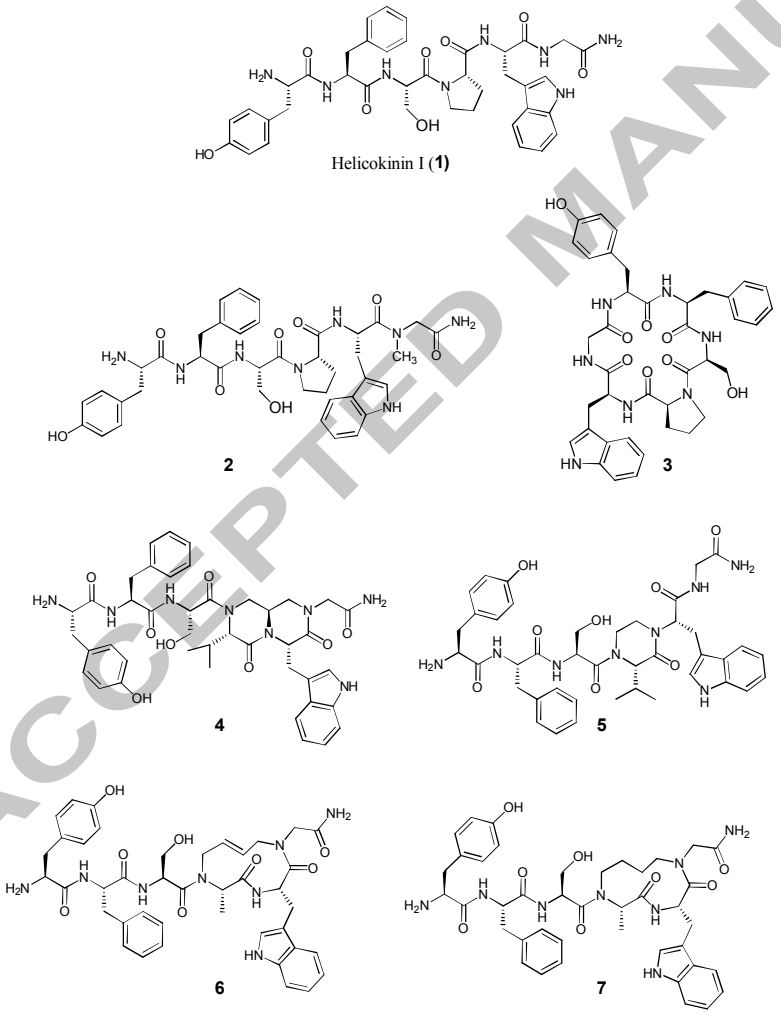
A complementary strategy for the replacement of single amino acids by nonpeptidic residues comprises a conformational design aiming at freezing the putative biologically active conformation or at least parts thereof into conformationally restricted macrocycles. Macrocyclization is one of the most widely used approaches to define the bioactive conformation of short peptides.[11] This strategy has been successfully applied to somatostatin, substance P, RGD peptides and other small peptides.[12] Basically, three different macrocyclization strategies can be distinguished: Firstly, cyclization by linking the C- and N-termini of the native peptide or a larger peptide containing additional residues. Secondly, cyclization by linking side-chains with suitable functional groups or thirdly, a backbone-cyclization, in which two amide nitrogens are connected by a spacer of appropriate length. The concept of backbone cyclization was first introduced to insect neuropeptides by Altstein and coworkers.[13] In all of those cyclization strategies, reduction of structural flexibility is the key to enhanced receptor-binding and improved metabolic stability.

Figure 1. Macrocyclic helicokinin I analogues



Helicokinin I (**1**) consists of two critical moieties, the C-terminal dipeptide Trp-GlyNH_2 and Phe2 (Figure 1). Already modest chemical modifications of these residues reduce receptor binding considerably.[5] For instance, a simple N-methylation of the α-amino group of Gly (**2**)

results in a complete loss of activity (Table 1). This can be attributed to the loss of a H-bond which is absolutely essential for the formation of the C-terminal β-turn required for receptor-binding. Also cyclohelicokinin I (**3**) as well as related, larger N- and C-terminally linked helicokinin I analogues were found inactive (Figure 1).[14] The same was demonstrated for rigid β-turn mimetics such as compounds **4** and **5**. Therefore, the more remarkable are our recent findings, that backbone-cyclized helicokinin I analogue **6** which contains a C4-linkage between Gly6 and Ala3, used as substitute for Pro3, retains considerable activity (Table 1). Obviously, it is possible to replace the hydrogen-bond required for the formation of a β-turn like structure in the critical C-terminal region by a macrocyclic backbone bridge. Also noteworthy, the saturated derivative **7** is less active by a factor of around 6 (Figure 1, Table 1). [15]

Table 1. EC_{50} values of helicokinin I analogues[5,14,15]

Compd.	Receptor assay EC_{50} [μM]
YFSPWG-NH_2 (**1**) (helicokinin I)	0.003
YFSPWNMeG-NH_2 (**2**) (NMe-Gly-helicokinin I)	>100
3	>100
4	>100
5	>100
6	1.9
7	12.5

These findings prompted us to conduct a similar study on the N-terminal region with the most critical Phe2 residue. Since we have already shown that the side-chains of Tyr1 and Ser3 are completely irrelevant for receptor binding, we constructed two macrocyclic analogues of helicokinin I by connecting the hydroxy groups of Tyr1 and Ser3 with a linker.[5] In addition, we were also interested in the question of the conformational flexibility of the Trp5 side-chain. Therefore, we envisaged a helicokinin I analogue consisting of a macrocyclic N-terminal bridge and an additional rigid Trp analogue**.**

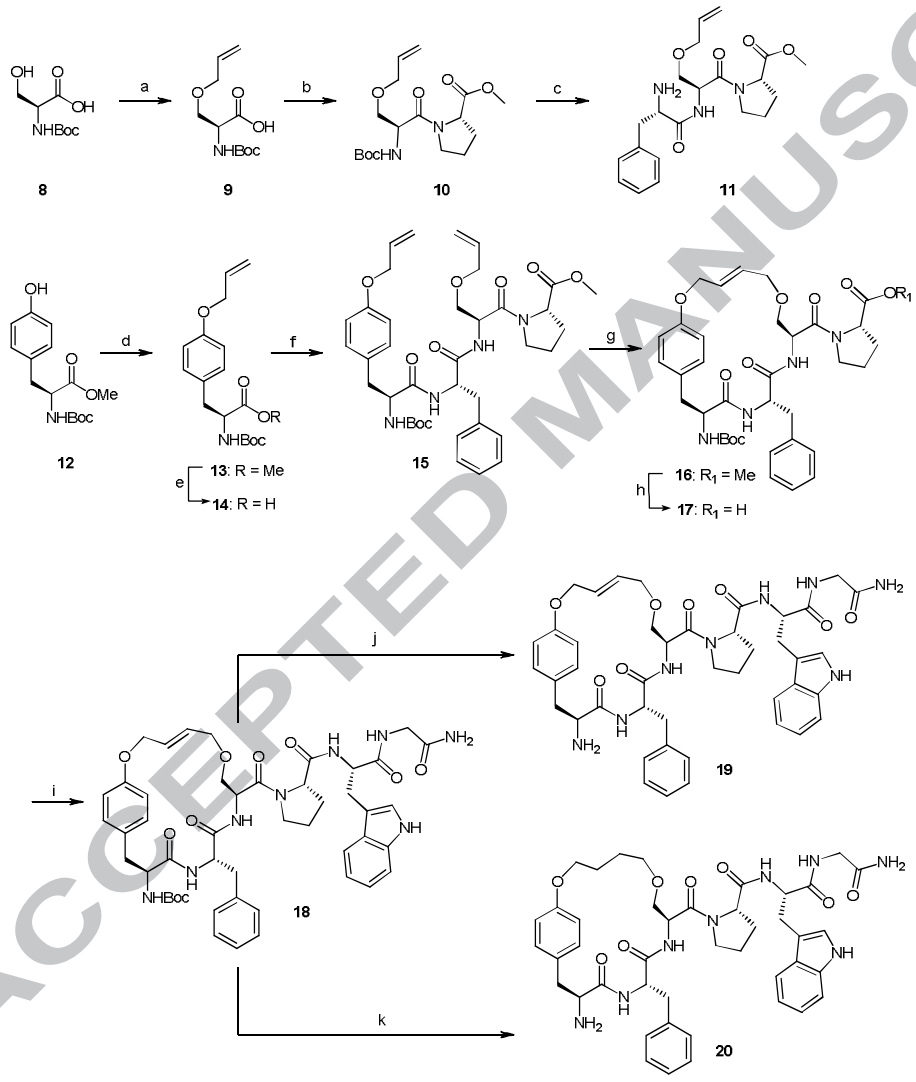
RESULTS AND DISCUSSION

Syntheses

Due to entropic reasons macrocyclizations are critical reactions which tend to intermolecular dimerizations instead of intramolecular ring closure. The dimerization reaction can be suppressed largely by either high-dilution cyclization conditions or by the template effect of a coordinating metal. To avoid impractical large amounts of solvent, we decided to accomplish the ring-closure not by a classical peptide bond formation but by a ruthenium catalyzed metathesis reaction.

Our synthesis commences with the selective *O*-allylation of Ser. Remarkably, the allylation of N-Boc-Ser-OMe using NaH as base, resulted in partial dehydration and decomposition of the starting material. In contrast, the sodium carboxylate formed when the free acid N-Boc-Ser-OH (**8**) was used, prevents the abstraction of the Ser α-proton and yielded 80% of the selectively side-chain allylated Ser derivative **9**. Dipeptide **10** was obtained in 93% yield from the reaction of N-Boc-*O*-allyl-Ser-OH (**9**) with L-Pro-OMe and subsequent protecting group removal under standard conditions. Intermediate **11** was prepared in 88% yield by TBTU (*O*-(benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate) mediated coupling of *O*-allyl-Ser-Pro-OMe with Boc-Phe-OH and acidic removal of the Boc-group (Scheme 1).

Scheme 1. Synthesis of macrocyclic helicokinin I analogues **19** and **20**



Reagents and conditions: a) allyl bromide, NaH, DMF, 80%. b) Pro-Ome x HCl, TBTU, DIPEA, DCM, 93%. c) (i) TFA/TIPS/H_2O (95:4:1), 3 h, 97%; (ii) Boc-Phe-OH, TBTU, DIPEA, DCM, 93%; (iii) TFA/TIPS/H_2O (95:4:1), 3 h, 98%. d) allyl bromide, K_2CO_3, DMF, 99%. e) 1N NaOH, MeOH, 18 h, rt, 98%. f) compound **11**, DIPEA, TBTU, DCM, 96%. g) Hoveyda-Grubbs 2nd catalyst (10 mol%), DCM, reflux, 50 h, 89%. h) LiOH, THF/H_2O (1/1), 18 h, 98%. i) H-Trp-Gly-NH_2, HATU, DIPEA, DMF, 98%. j) TFA/TIPS/H_2O (95:4:1), 0 ^{o}C, 4

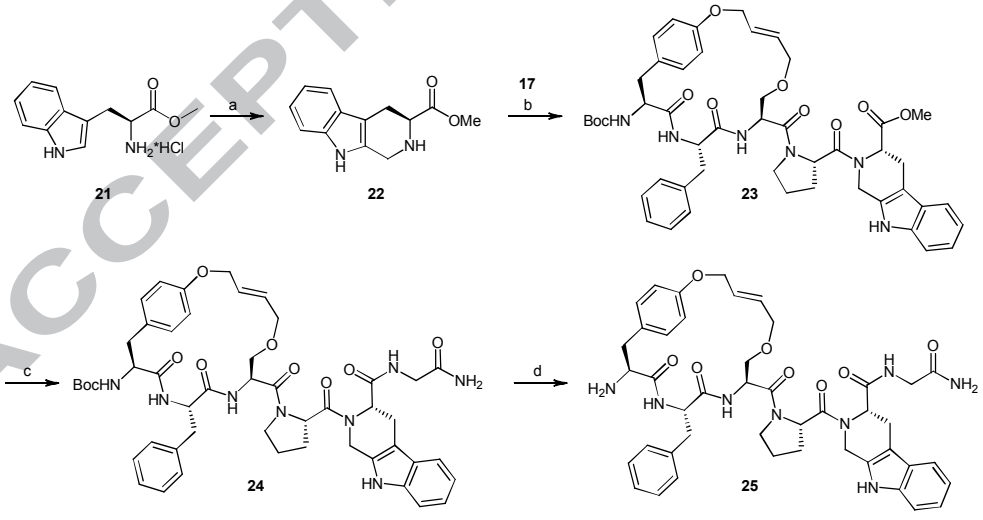
h, 94% (**19**). k) (i) Pd/C, H_2, MeOH/ EtOAc (2/1), rt, 24 h, 98%; (ii) TFA/TIPS/H_2O (95:4:1), 0 °C, 4 h, 94% (**20**).

Reaction of Boc-Tyr-OMe with allyl bromide in the presence of K_2CO_3 in DMF solution followed by basic ester cleavage afforded the *O*-allyltyrosine derivative **14** in 97% yield over two steps. Key metathesis precursor **15** was obtained in excellent yield (96%) by coupling of building blocks **11** and **14** with TBTU. The critical macrocyclization reaction was accomplished in an excellent yield (89%) with the Hoveyda-Grubbs second generation catalyst (10 mol%) after a reaction time of 50 h in refluxing DCM.[16] Upon treatment with 4% LiOH in THF/H_2O (1/1), methyl ester **16** was converted into the corresponding carboxylic acid **17**, which was then reacted with the dipeptide H-Trp-Gly-NH_2 using HATU (O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorphosphat) as coupling reagent. Removal of the Boc protecting group afforded the first macrocyclic helicokinin I analogue **19** in a yield of 92% over two steps.

The stereochemistry of the newly formed double bond was established based on NOESY and ^{1}H NMR decoupling experiments. A high coupling constant ($J_{Ha\text{-}Hb}$ = 16.1 Hz) between the two adjacent alkene protons was indicative for a *trans*-configuration. Remarkably, no *cis*-isomer was found.

In order to get additional information on the flexibility of the linker, the saturated derivative **20** was prepared by hydrogenation of the Boc-protected macrocyclic hexapeptide and subsequent deprotection. In a further approach, the conformationally rigid Trp-analogue **22** was introduced as a second structural modification to obtain a partially peptidomimetic helicokinin I analogue (Scheme 2).

Scheme 2: Synthesis of the twofold modified helicokinin I analogue **25**



Reagents and conditions: a) 36% HCHO, MeOH, rt, 4 h, 67%. b) **17**, HATU, DIPEA, DMF, rt, 20 h, 78%. c) (i) 4% LiOH, THF/H_2O (2/1), 5 h, 91%; (ii) H-Gly-NH_2 x HCl, TBTU, DMF, DIPEA, rt, 20 h, 77%. d) TFA/TIPS/H_2O (95:4:1), 0 °C, 3 h, 86%.

The tetrahydro-β-carboline **22** was prepared by the Pictet-Spengler reaction in 67% yield from Trp-OMe (**21**) following a literature procedure.[17] HATU mediated coupling of the macrocyclic intermediate **17** with the conformationally restricted Trp mimic **22** afforded the macrocyclic pentapeptide **23** in 78% yield. Subsequent saponification of the methyl ester with a 4% LiOH solution in THF/H_2O (2/1) followed by coupling with $GlyNH_2$ hydrochloride and removal of the Boc protecting group then gave the twofold modified helicokinin I analogue **25** with a yield of 60% over three steps.

Biological Data

The helicokinin analogues **19**, **20**, and **25** were tested in a *H. virescens* helicokinin receptor assay, functionally expressed in a CHO cell line.[18,19] As reference, the complete hexapeptide helicokinin I was used and not the pentapeptide core-sequence which lacks the N-terminal Tyr and already shows a significant reduced receptor-activation by a factor of around 100 (Table 2).

Table 2. EC_{50} values of helicokinin I analogues **19**, **20**, and **25**

Compd.	Receptor assay EC_{50} [μM]
YFSPWG-NH_2 (**1**) (helicokinin I)	0.003[15]
19	0.039
20	0.501
25	0.630

The unsaturated macrocyclic helicokinin derivative **19** was found somewhat less active (0.039 μM) as helicokinin I (0.003 μM) but 50 times more active than the C-terminal bridged helicokinin I analogue **6** (Table 1). Since it is well known that the side-chains of Ser and Tyr are not relevant for receptor-binding, it can be concluded that the reduced activity is caused by the macrocyclic bridge, which induces an orientation of the critical Phe residue, different from the native peptide. Not completely unexpected, the influence of conformational rigidification is less pronounced in the N-terminal region which is more flexible and not involved in a β-turn structure as the C-terminus. Similar to the backbone-cyclized macrocycles **6** and **7** (Table 1) hydrogenation of the alkenyl-bridge in compound **19** to the more flexible, saturated macrocycle **20** results in a reduction of receptor- activation by a factor of 13 compared to a factor of **6** for the backbone-cyclized compound **7**. However, the activity of the N-terminally bridged macrocycle **20** (0.501 μM) is higher by a factor of 25 as that of the C-terminally bridged macrocycle **7** (12.5 μM). These results demonstrate that even in the more flexible N-terminal region a highly specific orientation of the residues involved in receptor-binding is required for full activity.

While the effect of the N-terminal bridge is comparably small, the constrained Trp analogue **22** exerts a considerable influence on receptor-binding. The activity of helicokinin I analogue **25** declined by a factor of 16 (0.630 μM), but is still higher than that of the C-terminally bridged macrocycle **6** (1.9 μM). Taking into account that an exchange of Trp for Ala causes

only a reduction of receptor-activation to an EC_{50} of 0.050 µM it can be concluded that a strict conformational restriction, which directs the indole side-chain into a fixed orientation, is problematic for high-affinity receptor-binding.[5]

Summary and Conclusions

Three macrocyclic helicokinin I analogues were prepared by connecting the side-chain hydroxy groups of Tyr1 and Ser3 with a C4-spacer. The macrocyclization was accomplished in excellent yield by a cyclative metathesis reaction utilizing the template effect of the ruthenium catalyst. The biological activities of helicokinin I analogues **19**, **20**, and **25** were determined in a helicokinin receptor assay. The N-terminal region with the critical Phe2 residue tolerates conformational restriction significantly better than the C-terminus as exemplified with the C-terminally bridged helicokinin I analogues **6** and **7**. In contrast to the alkyl-bridged derivative **20**, the alkenyl-bridged macrocycle **19** showed almost the same activity as helicokinin I. In addition it was demonstrated, that conformational rigidification of the Trp5 side-chain (**25**) is disadvantageous for high-affinity receptor-binding. Taking these results together, it can be concluded that in both, the N-terminal and C-terminal region, a sensitive balance between rigidity and flexibility is required for optimal receptor activation.

Acknowledgement

We thank the "Vietnam international education development program" for providing Chien Tran Van with a generous grant.

Experimental Section

General

All chemical reagents were purchased from commercial suppliers and used without purification. Dichloromethane, acetonitrile, *N*,*N*-dimethylformamide (DMF) and *N*,*N*-diisopropylethylamine (DIPEA) were heated at reflux for 1 h with calcium hydride and distilled. Tetrahydrofuran (THF) was heated at reflux for several hours with $LiAlH_4$ and then distilled. The instrumentation used was as follows: ^{1}H NMR: Bruker Avance 400, Bruker Avance III 600; ^{13}C NMR: Bruker Avance 400, Bruker Avance III 600; Infrared (IR) spectra were obtained with a FTIR: Nicolet PROTÉGÉ 460 E.S.P. MS: Bruker microTOF; MS (ESI): Varian IT 500-MS Iontrap; LC: Preparative low-pressure chromatography was performed with silica gel 60 µm (230–400 mesh, Macherey–Nagel) The abbreviations are used for the proton spectra multiplicities are: s singlet, br. broad, br. s broad singlet, d doublet, dd double doublet, dt double triplet, t triplet, q quartet, m multiplet. TLC analyses were performed on silica gel 60 F254 (Merck). Detection was conducted with UV light (254 nm). Flash column chromatography was performed with silica gel (0.063–0.200 mm).

Receptor assay

The helicokinin receptor of *Heliothis virescens* was cloned from a Malpighian tubule cDNA library and showed about 50% homology (amino acids) to the leucokinin receptor from *Lymnea stagnalis*.[18,19] The helicokinin receptor (HKR) was functionally expressed in a CHO line. The activation of the HKR was analyzed in living cells by measuring the induced calcium ion flux in the cytosol after activation of the second messenger cascade via G-proteins [18,19].[20,21] Calcium ion dyes were obtained from Molecular Devices and were used according to the supplier's protocol. Cells were plated in 384-well plates ($4x10^3$ cells per well) and incubated overnight (37°C, 5% CO_2). Cells were removed from the incubator, allowed to reach room temperature over the course of 10 min, and then washed with 50 µl of HBSS. Subsequently, the medium was replaced with 50 µl of calcium ion dye solution in HBSS, and cells were loaded for 1 h in the dark. Then, the plates were read on a Flexstation fluorescence plate reader (Molecular Devices). Excitation and emission wavelengths were set to 485 nm and 525 nm, respectively. Measurements were made every 2.7s intervals for 100s. Basal fluorescence was determined for 15–30 s, followed by addition of 25µl test compounds (to assess agonist activity). Helicokinins I or II were used as standards and were tested in four independent experiments for evaluating EC_{50} values. Standard deviations

were 2 nM for helicokinin I, 3 nM for helicokinin II and 3 nM for helicokinin III. EC_{50} values were calculated after concentration-dependent induction of calcium flux analyzed in a Flexstation (Molecular Devices). Finally, EC_{50}-values were calculated as Fmax (maximal agonist signal) minus Fmin (baseline) according to Softmax-Pro 5.0 software (Molecular Devices). All tests compounds were measured in two independent experiments in ten dilution steps (in duplicates) from 0.5 nM to 10 µM.

Chemistry

(2*S*)-2-{[(*tert*-Butoxy)carbonyl]amino}-3-(prop-2-en-1-yloxy)propanoic acid (9)
To a stirred suspension of NaH (27 mg, 1.12 mmol) in DMF (0.5 mL) a solution of Boc-Ser-OH (105 mg, 0.51 mmol) in DMF (1 mL) was added dropwise at 0 °C. After 10 min, allyl bromide (51 µL, 0.59 mmol) was added and the reaction mixture was allowed to warm up to room temperature. After stirring for 20 h, the solvent was removed under reduced pressure. The residue was dissolved in H_2O (10 mL) and extracted with EtOAc (2x20 mL). The aqueous phase was acidified with 1M HCl at 0 °C to pH≈2 and extracted with EtOAc (3x40 mL). The combined organic phases were washed with brine solution and dried over $MgSO_4$. Removal of the solvent yielded the oily product **9** (100 mg, 80%). R_f = 0.45 (EtOAc). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 7.2 (br, 1H, OH), 5.91 (m, 1H), 5.45 (s, 1H, NH), 5.26 (m, 2H), 4.48 (br, 1H), 4.03 (dt, *J* = 1.5 Hz, *J* = 5.5 Hz, 2H), 3.92 (d, *J* = 8.3 Hz, 1H), 3.73 (dd, *J* = 3.5 Hz, *J* = 8.3 Hz, 1H), 1.47 (s, 9H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 174.8, 155.7, 134.1, 117.9, 80.3, 72.3, 69.6, 53.8, 28.7. **IR** (KBr) ν (cm^{-1}): 2986.9, 1705.9. **HPLC-ESI-MS**: m/z (%) = 268.1 (100) $[M+Na]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{11}H_{19}NO_5Na$ $[M+Na]^+$ 268.1156; found 268.1155.

Methyl-(2*S*)-1-[(2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}-3-(prop-2-en-1-yloxy)propanoyl]-pyrrolidine-2-carboxylate (10)
To a solution of compound **9** (162 mg, 0.66 mmol), Pro-OMe*HCl (131.3 mg, 0.79 mmol) and HATU (300 mg, 0.79 mmol) in DMF (4 mL) DIPEA (0.5 mL, 4.5 mmol) was added. After stirring at room temperature for 20 h, the solvent was removed under reduced pressure. Flash chromatography (eluent: CyH/EtOAc, 3/1) provided product **10** (220 mg, 93%) as an oil. R_f = 0.28 (CyH/EtOAc, 2/1). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 5.89 (m, 1H), 5.29 (d, *J* = 17.2 Hz, 1H), 4.67 (d, *J* = 7.1 Hz, 1H), 4.53 (dd, *J* = 4.0 Hz, *J* = 8.3 Hz, 1H), 4.05 (dd, *J* = 5.0 Hz, *J* = 17.6 Hz, 2H), 3.78 (m, 1H), 3.73 (m, 1H), 3.72 (s, 3H), 3.64 (d, *J* = 6.5 Hz, 2H), 2.21 (m, 1H), 2.05 (m, 1H), 1.99 (m, 2H, 1.43 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 172.2, 169.4, 134.6, 116.7, 79.7, 72.2, 70.3, 58.8, 52.2, 51.8, 46.7, 29.0, 28.1, 24.7. **IR** (KBr) ν (cm^{-1}): 2982.3, 1705.7, 1645.9. **HPLC-ESI-MS**: m/z (%) = 357.1 (50) $[M+H]^+$, 257.1 (100) $[M-Boc+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{17}H_{28}N_2O_6Na$ $[M+Na]^+$ 379.1840; found 379.1844.

(2*S*)-1-[(2*S*)-2-(Methoxycarbonyl)pyrrolidin-1-yl]-1-oxo-3-(prop-2-en-1-yloxy)propan-2-aminium-trifluoroacetate
Compound **10** (73 mg, 0.2 mmol) was treated with TFA/TIPS/H_2O (2 mL, 95/4/1) at 0 °C for 3 h. Afterwards, the reaction mixture was concentrated under reduced pressure to provide **(2*S*)-1-[(2*S*)-**2-(methoxycarbonyl)pyrrolidin-1-yl]-1-oxo-3-(prop-2-en-1-yloxy)propan-2-aminium-trifluoroacetate (51.1 mg, 97%) as a TFA salt. **^{1}H-NMR** (400 MHz, CD_3OD): δ = 5.90 (m, 1H), 5.30 (m, 2H), 4.55 (dd, *J* = 5.3 Hz, *J* = 8.7 Hz, 1H), 4.44 (dd, *J* = 4.0 Hz, *J* = 8.0 Hz, 1H), 4.13 (d, *J* = 5.4 Hz, 2H), 3.90 (dd, *J*= 4.0 Hz, *J* = 11.0 Hz, 2H), 3.75 (m, 1H), 3.73 (s, 3H), 3.70 (overlap, 1H), 3.66 (m, 1H), 2.32 (m, 1H), 2.06 (m, 2H), 2.02 (m, 1H). **^{13}C-NMR** (100 MHz, CD_3OD): δ = 173.4, 166.6, 133.2, 116.7, 72.6, 66.4, 59.2, 52.2, 51.3, 46.8, 28.3, 24.6. **IR** (KBr) ν (cm^{-1}): 2988.2, 1652.7. **HPLC-ESI-MS**: m/z (%) = 257.1 (100) $[M-CF_3COOH+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{12}H_{20}N_2O_4Na$ $[M+Na]^+$ 279.1340; found 279.1815.

Methyl-(2*S*)-1-[(2*S*)-2-[(2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}-3-phenyl-propanamido]-3-(prop-2-en-1-yloxy)propanoyl]pyrrolidine-2-carboxylate
DIPEA (0.38 mL, 2.26 mmol) was added dropwise to a stirred solution of Boc-Phe-OH (180 mg, 0.68 mmol), TBTU (218 mg, 0.68 mmol) and (2*S*)-1-[(2*S*)-2-(methoxycarbonyl) pyrrolidin-1-yl]-1-oxo-3-(prop-2-en-1-yloxy)propan-2-aminium-trifluoro-acetate (145 mg, 0.57 mmol) in DCM (8 mL). After stirring at room temperature for 18 h, the solvent was removed. The crude product was purified by silica gel column chromatography with CyH/EtOAc (5/2) as eluent to give methyl-(2*S*)-1-[(2*S*)-2-[(2*S*)-2-{[(*tert*-butoxy)carbonyl] amino}-3-phenyl-propanamido]-3-(prop-2- en-1-yloxy)propanoyl]- pyrrolidine-2-carboxylate (264 mg, 93%) as a solid. R_f = 0.18 (CyH/EtOAc, 5/2). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 7.28 (t, *J* = 7.0 Hz, 2H), 7.23 (t, *J* = 7.0 Hz, 1H), 7.19 (d, *J* = 7.7 Hz, 2H), 5.86 (m, 1H), 5.29 (d, *J* = 10.0 Hz, 1H), 5.20 (d, *J* = 11.0 Hz, 1H), 4.91 (d, *J* = 6.5 Hz), 4.49 (dd, *J* = 4.3 Hz, *J* = 8.0 Hz, 1H), 4.4 (bs, 1H), 3.8 (m, 1H), 3.72 (s, 3H), 3.68 (m, 1H), 3.64 (m, 1H), 3.58 (m, 1H), 3.09 (m, 2H), 2.2 (m, 1H), 2.0 (overlap, 1H), 2.06 (m, 1H), 1.42 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 172.1, 170.8, 168.5, 155.4, 134.2, 134.0, 129.4, 128.5, 126.9, 117.0, 80.1, 72.1, 69.5, 58.7, 55.5, 52.5, 50.7, 47.2, 38.1, 28.8, 28.0, 24.7. **IR** (KBr) ν (cm^{-1}): 2989.2, 1635.4. **HPLC-ESI-MS**: m/z (%) = 504.3 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{26}H_{37}N_3O_7Na$ $[M+Na]^+$ 526.2524; found 526.2528.

(1*S*)-1-{[(2*S*)-1-[(2*S*)-2-(Methoxycarbonyl)pyrrolidin-1-yl]-1-oxo-3-(prop-2-en-1-yloxy)-propan-2-yl]carbamoyl}-2-phenylethan-1-aminium trifluoroacetate (11)

A solution of methyl-(2*S*)-1-[(2*S*)-2-[(2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}-3-phenyl-propanamido]-3-(prop-2-en-1-yloxy)propanoyl]-pyrrolidine-2-carboxylate (70 mg, 0.14 mmol) in TFA/TIPS/H_2O (2 mL, 95/4/1) was stirred at 0 °C for 3 h. After that time, the mixture was concentrated under reduced pressure to afford product **11** (70.4 mg, 98%) as TFA salt. R_f = 0.23 (EtOAc/EtOH, 13/1). **^{1}H-NMR** (400 MHz, CD_3OD): δ = 7.37-7.31 (m, 5H), 5.96 (m, 1H), 5.34 (d, *J* = 17.0 Hz, 1H), 5.22 (d, *J* = 10.0 Hz, 1H), 4.91 (d, *J* = 6.5 Hz), 4.47 (dd, *J* = 4.3 Hz, *J* = 8.0 Hz, 1H), 4.22 (bs, 1H), 3.79 (m, 2H), 3.72 (s, 3H), 3.80 (m, 2H), 3.28 (m, 1H), 3.05 (m, 1H), 2.3 (m, 1H), 2.03 (overlap, 1H, 2.02 (m, 1H). **^{13}C-NMR** (100 MHz, CD_3OD): δ = 170.1, 169.8, 168.9, 136.8, 134.7, 131.6, 131.2, 129.9, 118.7, 74.9, 71.2, 61.5, 56.5, 53.8, 49.6, 39.4, 30.8, 27.3. **IR** (KBr) ν (cm^{-1}): 2992.5, 1669.5, 1627.1. **HPLC-ESI-MS**: m/z (%) = 404.3 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{21}H_{29}N_3O_5Na$ $[M+Na]^+$ 426.1999; found 426.1998.

Methyl-(2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}-3-[4-(prop-2-en-1-yloxy)phenyl]-propanoate (13)

Allyl bromide (0.28 mL, 3.18 mmol) was added dropwise to a suspension of Boc-Tyr-OMe (470 mg, 1.59 mmol) and K_2CO_3 (484 mg, 3.5 mmol) in DMF (8 mL) The reaction mixture was stirred for 20 h at room temperature. Then, the solid was removed by filtration through a Celite pad and washed with EtOAc. The filtrate was evaporated to provide allyl tyrosine **13** (526 mg, 99%) as an oily product. R_f = 0.36 (CyH/EtOAc, 5/1). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 7.0 (d, *J* = 8.5 Hz, 2H), 6.83 (d, *J* = 8.5 Hz, 2H), 6.05 (m, 1H), 5.4 (q, 1H), 5.26 (q, 1H), 4.54 (m, 2H), 4.50 (m, 1H), 3.70 (s, 3H), 3.0 (br, 2H), 1.41 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 172.0, 157.7, 133.3, 130.0, 128.1, 117.6, 114.8, 79.8, 69.6, 54.5, 52.3, 37.9, 28.5. **IR** (KBr) ν (cm^{-1}): 2986.9, 1705.9. **HPLC-ESI-MS**: m/z (%) = 358.1 (20) $[M+Na]^+$, 236.1 (100) $[M-Boc+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{18}H_{25}NO_5Na$ $[M+Na]^+$ 358.1630; found 358.1630.

(2*S*)-2-{[(*tert*-Butoxy)carbonyl]amino}-3-[4-(prop-2-en-1-yloxy)phenyl]propanoic acid (14)

Ester **13** (144 mg, 0.43 mmol) dissolved in MeOH (2 mL) was treated with 1N NaOH (0.64 mmol) at room temperature for 24 h. Afterwards, the solution was acidified with 1N HCl and extracted with EtOAc (3x40 mL). The combined organic phases were washed with brine and dried over $MgSO_4$. Removal of the solvent yielded acid **14** (135.6 mg, 98%) as an oily product. R_f = 0.29 (CyH/EtOAc, 6/1). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 7.1 (d, *J* = 8.0 Hz, 2H), 6.8 (d, *J* = 8.0 Hz, 2H), 6.1 (m, 1H), 5.4 (dd, *J* = 2.0 Hz, *J* = 17.6 Hz, 2H), 4.59 (br, 1H), 4.5 (d, *J* = 5 Hz, 2H), 3.1 (m, 1H), 3.0 (m, 1H), 1.4 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 176.4, 157.7, 155.5, 133.2, 130.0, 128.1, 117.4, 115.0, 80.2, 68.7, 54.4, 36.9, 28.2. **IR** (KBr) ν (cm^{-1}): 2930.8, 1713.4. **HPLC-ESI-MS**: m/z (%) = 344.2 (20) $[M+Na]^+$, 222.2 (100) $[M-Boc+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{17}H_{23}NO_5Na$ $[M+Na]^+$ 344.1474; found 344.1471.

Methyl-(2*S*)-1-[(2*S*)-2-[(2*S*)-2-[(2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}-3-[4-(prop-2-en-1-yloxy) phenyl]propanamido]-3-phenylpropanamido]-3-(prop-2-en-1-yloxy)propanoyl]-pyrrolidine-2-carboxylate (15)

To a solution of amino ester **11**(140 mg, 0.347 mmol), acid **14** (134 mg, 0.416 mmol) and TBTU (137 mg, 427 mmol) in DCM (4 mL) DIPEA (0.23 mL, 1.39 mmol) was added. After being stirred at room temperature for 20 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CyH/EtOAc, 3/1) to give the metathesis precursor **15** (236.4 mg, 96%) as a solid. R_f = 0.18 (CyH/EtOAc, 3/1). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 7.24-7.22 (m, 3H), 7.10 (overlap, 2H), 7.09 (d, *J* = 8.2 Hz, 2H), 6.84 (d, *J* = 8.2 Hz, 2H), 6.06 (m, 1H), 5.90 (m, 1H), 5.42 (d, *J* = 17.0 Hz, 1H), 5.29 (overlap, 1H), 5.18 (d, *J* = 11.0 Hz, 1H), 4.83 (q, *J* = 6.5 Hz, 1H), 4.65 (q, *J* = 6.5 Hz, 1H), 4.52 (m, 2H), 4.51 (overlap, 1H), 4.27 (bs, 1H), 4.0 (m, 2H), 3.78 (m, 1H), 3.72 (s, 3H), 3.67 (m, 1H), 3.59 (d, *J* = 6.5 Hz, 2H), 3.05 (m, 1H), 2.97 (overlap, 3H), 2.21 (m, 1H), 2.08 (m, 1H), 2.01 (overlap, 2H), 1.40 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 172.1, 171.1, 169.9, 168.3, 157.6, 156.1, 134.3, 134.1, 133.3, 130.4, 128.5, 126.8, 117.4, 116.8, 114.8, 129.2, 79.9, 72.2, 69.7, 68.7, 59.0, 55.8, 54.6, 52.1, 50.8, 47.0, 38.2, 37.2, 29.0, 28.3, 24.6. **IR** (KBr) ν (cm^{-1}): 3285.6, 1635.6. **HPLC-ESI-MS**: m/z (%) = 707.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{38}H_{50}N_4O_9Na$ $[M+Na]^+$ 729.3470; found 729.3466.

Methyl-(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-12-benzyl-15-{[(*tert*-butoxy)carbonyl]amino}-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraene-9-carbonyl]-pyrrolidine-2-carboxylate (16)

Hoveyda-Grubbs catalyst 2nd generation (14.5 mg, 10 mol%) was added to a solution of compound **15** (163 mg, 0.163 mmol) in degassed (for 40 min with argon) DCM (150 mL). The reaction mixture was refluxed for 50 h. After that time the solvent was removed. Flash chromatography with CyH/EtOAc (1/1) as eluent afforded **16** (140 mg, 89%) as a yellow solid product. R_f = 0.13 (CyH/EtOAc, 2/3). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ = 7.20-7.14 (m, 5H), 7.04 (m, 2H), 6.74 (d, *J* = 8.5 Hz, 2H), 5.70 (bs, 2H), 4.78 (t, *J* = 7.1 Hz, 1H), 4.62 (bs, 2H), 4.45 (t, *J* = 7.1 Hz, 1H), 4.41 (m, 1H), 4.24 (bs, 1H), 4.06 (d, *J* = 12.0 Hz, 1H), 3.84 (d, *J* = 11.0 Hz, 1H), 3.75 (m, 1H), 3.70 (s, 3H), 3.51 (m, 1H), 3.35 (m, 1H), 3.29 (t, *J* = 8.5 Hz, 1H), 2.97 (m, 1H), 2.92 (m, 2H), 2.89 (m, 1H), 2.17 (m, 1H), 2.03 (m, 1H), 1.97 (overlap, 2H), 1.44 (s, 9H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ = 172.1, 169.8,

168.5, 156.4, 156.1, 136.1, 130.8, 130.2, 129.6, 129.0, 128.2, 126.7, 115.1, 79.8, 70.1, 68.5, 66.7, 58.8, 55.2, 54.3, 52.3, 49.7, 46.8, 37.7, 28.8, 28.1, 24.4. **IR** (KBr): ν (cm^{-1}) 3286.1, 2971.8, 1635.3. **HPLC-ESI-MS**: m/z (%) = 679.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{36}H_{46}N_4O_9Na$ $[M+Na]^+$ 701.3157; found 701.3158.

(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-12-Benzyl-15-{[(*tert*-butoxy)carbonyl]amino}-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraene-9-carbonyl]pyrrolidine-2-carboxylic acid (17)

To methyl ester **16** (50 mg, 0.074 mmol) dissolved in THF/H_2O (3 mL, 1/1) an aqueous 4% LiOH solution (31 mg, 0.74 mmol) was added. The reaction mixture was stirred at room temperature for 18 h, then acidified with 1N HCl at 0 °C to pH≈4 and extracted with AcOEt (3x60 mL). The combined organic phases were washed with brine and dried over $MgSO_4$. Removal of the solvent gave **17** (46.5 mg, 98%) as a white solid. R_f = 0.41 (EtOAc). **^{1}H-NMR** (600 MHz, CD_3OD): δ = 7.24-7.19 (m, 5H), 7.07 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 8.2 Hz, 2H), 5.85 (m, 1H), 5.75 (dt, *J* = 5.0 Hz, *J* = 16.0 Hz, 1H), 4.78 (t, *J* = 7.1 Hz, 1H), 4.67 (d, *J* = 5.0 Hz, 2H), 4.54 (t, *J* = 7.1 Hz, 1H), 4.34 (t, *J* = 7.0 Hz, 1H), 4.31 (t, *J* = 7.0 Hz, 1H), 4.06 (d, *J* = 12.0 Hz, 1H), 3.85 (d, *J* = 11.0 Hz, 1H), 3.72 (br, 1H), 3.70 (s, 3H), 3.43 (m, 1H), 3.40 (m, 1H), 3.06 (t, *J* = 8.5 Hz, 1H), 3.04 (m, 1H), 2.96-2.90 (m, 2H), 2.89 (m, 1H), 2.21 (m, 1H), 2.02 (overlap, 2H), 1.93 (m, 1H), 1.44 (s, 9H). **^{13}C-NMR** (150 MHz, CD_3OD): δ = 174.5, 172.4, 169.5, 156.8, 154.1, 136.6, 130.5, 129.9, 129.8, 128.8, 127.9, 126.1, 114.7, 79.8, 69.9, 67.8, 66.8, 59.0, 55.2, 53.9, 50.0, 46.7, 37.5, 36.9, 28.7, 27.4, 24.1. **IR** (KBr) ν (cm^{-1}): 3309.7, 1637.9. **HPLC-ESI-MS**: m/z (%) = 665.3 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{35}H_{44}N_4O_9Na$ $[M+Na]^+$ 687.3001; found 687.3001.

***tert*-Butyl-*N*-[(4*E*,9*S*,12*S*,15*S*)-12-benzyl-9-[(2S)-2-{[(1S)-1-[(carbamoylmethyl)carbamo-yl]-2-(1H-indol-3-yl)ethyl]carbamoyl}pyrrolidine-1-carbonyl]-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraen-15-yl]carbamate (18)**

To a solution of **17** (32 mg, 48.14 µmol), H-Trp-Gly-$CONH_2$ (15 mg, 57.8 µmol) and HATU (22 mg, 57.8 µmol) in DMF (1 mL), DIPEA (40 µL, 241 µmol) was added. The reaction mixture was stirred at room temperature for 18 h and afterwards concentrated under reduced pressure. The residue was subjected to silica gel column chromatography with EtOAc/EtOH (10/1) as solvent to furnish hexapeptide **18** (43.6 mg, 100%) as a solid. R_f = 0.58 (EtOAc/EtOH, 8/1). **^{1}H-NMR** (400 MHz, DMSO-d_6): δ = 10.8 (s, 1H, NH), 8.25 (d, *J* = 8.5 Hz, 1H, NH), 8.0 (t, *J* = 6.0 Hz, 1H, NH), 7.95 (d, *J* = 7.0 Hz, 1H, NH), 7.70 (d, *J* = 7.7 Hz, 1H, NH), 7.53 (d, *J* = 8.0 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.18-7.12 (m, 5H), 7.14 (overlap, 1H), 7.05 (overlap, 1H), 7.00 (d, *J* = 8.5 Hz, 2H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.75 (d, *J* = 8.5 Hz, 2H), 5.83 (m, 1H), 5.69 (m, 1H), 4.68 (overlap, 3H), 4.50 (m, 1H), 4.46 (m, 1H), 4.23 (m, 1H), 4.17 (q, *J* = 7.0 Hz, 1H), 3.99 (dd, *J* = 5.0 Hz, *J* = 11 Hz, 1H), 3.75 (m, 1H), 3.65 (dd, *J* = 5.0 Hz, *J* = 17.0 Hz, 1H), 3.55 (dd, *J* = 5.0 Hz, *J* = 17.0 Hz, 1H), 3.50 (m, 1H), 3.29 (m, 2H), 3.27 (m, 1H), 3.19 (m, 1H), 2.98 (m, 1H), 2.88 (overlap, 3H), 2.79 (m, 1H), 1.90 (m, 1H), 1.68 (m, 1H), 1.66 (m, 2H), 1.41 (s, 9H). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ = 171.2, 170.7, 170.1, 169.7, 169.3, 168.4, 156.1, 154.4, 137.1, 136.0, 131.2, 130.9, 130.8, 129.7, 128.2, 127.3, 126.7, 124.0, 121.3, 118.7, 118.6, 111.7, 115.2, 78.2, 70.2, 68.4, 67.3, 60.2, 54.8, 53.7, 50.2, 47.4, 42.5, 38.0, 36.8, 29.2, 28.5, 24.4. **IR** (KBr) ν (cm^{-1}): 3433.9, 1653.1. **HPLC-ESI-MS**: m/z (%) = 907.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{48}H_{58}N_8O_{10}Na$ $[M+Na]^+$ 929.4168; found 929.4160.

***tert*-Butyl-*N*-[(9*S*,12*S*,15*S*)-12-benzyl-9-[(2*S*)-2-{[(1*S*)-1-[(carbamoylmethyl)carbamoyl]-2-(1H-indol-3-yl)ethyl]carbamoyl}pyrrolidine-1-carbonyl]-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),17,20-trie-15-yl]carbamate**

A suspension of hexapeptide **18** (18 mg, 19.85 µmol) and Pd/C (3.6 mg, 20 wt%) in MeOH/EtOAc (6 mL, 2/1) was stirred under a hydrogen atmosphere at room temperature for 20 h. The catalyst was removed by a filtration over a Celite pad and washed with EtOAc. Removal of solvent afforded *tert*-butyl-*N*-[(9*S*,12*S*,15*S*)-12-benzyl-9-[(2*S*)-2-{[(1*S*)-1-[(carbamoylmethyl)carbamoyl]-2-(1H-indol-3-yl)ethyl]carbamoyl}pyrrolidine-1-carbonyl]-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo [15.2.2]henicosa-1(19),17,20-trie-15-yl]carbamate (18 mg, 99%) as a brown solid. R_f = 0.37 (DCM/MeOH, 15/1). **^{1}H-NMR** (600 MHz, CD_3OD): δ = 7.58 (d, *J* = 8.6 Hz, 1H), 7.36 (d, *J* = 8.6 Hz, 1H), 7.18-7.13 (m, 5H), 7.13 (overlap, 2H), 7.07 (d, *J* = 7.5 Hz, 2H), 7.04 (t, *J* = 7.5 Hz, 1H), 6.78 (d, *J* = 7.5 Hz, 2H), 4.65 (t, *J* = 6.5 Hz, 1H), 4.56 (t, *J* = 7.5 Hz), 4.35 (m, 1H), 4.31 (m, 1H), 4.26 (t, *J* = 6.5 Hz, 1H), 4.1 (m, 2H), 3.87 (d, *J* = 17.0 Hz, 1H), 3.69 (d, *J* = 17.0 Hz, 1H), 3.58 (m, 1H), 3.50 (m, 1H), 3.43 (m, 2H), 3.39 (m, 2H), 3.37 (m, 1H), 3.25 (m, 1H), 3.04 (m, 1H), 2.93 (m, 1H), 2.82 (m, 1H), 2.80 (m, 1H), 2.07 (m, 1H), 1.78 (m, 3H), 1.74 (m, 2H), 1.62 (m, 2H), 1.43 (s, 9H). **^{13}C-NMR** (150 MHz, CD_3OD): δ = 172.3, 171.5, 170.2, 169.5, 168.4, 157.6, 156.8, 136.7, 129.7, 127.6, 127.5, 127.2, 126.2, 123.0, 121.2, 118.5, 117.8, 114.2, 110.0, 106.8, 79.8, 70.6, 68.9, 66.8, 60.5, 56.1, 54.7, 53.5, 51.3, 47.2, 42.0, 37.7, 37.1, 28.6, 28.3, 26.2, 25.3, 24.6, 24.). **IR** (KBr) ν (cm^{-1}): 3447.9, 1654.5. **HPLC-ESI-MS**: m/z (%) = 909.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{48}H_{60}N_8O_{10}Na$ $[M+Na]^+$ 931.4325; found 931.4328.

General procedure for *N*-Boc deprotection

Macrocyclic peptides were treated with TFA/TIPS/H_2O (2 mL, 95/4/1) at 0 °C for 4 h. The reaction mixtures were then concentrated under reduced pressure to dryness. The crude products were subjected to silica gel

column chromatography using DCM/MeOH (6/1, 0.1% TEA) to provide the pure products. White powders were obtained by dissolving the purified compounds in ACN/H_2O (3 mL, 1/1) and subsequent lyophilization.

(2*S*)-2-{[(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-15-Amino-12-benzyl-11,14-dioxo-2,7-dioxa-10,13-diaza-bicyclo [15.2.2]heicosa-1(19),4,17,20-tetraene-9-carbonyl]pyrrolidin-2-yl]formamido}-N-(carbamoyl methyl)-3-(1H-indol-3-yl)propanamide (19)

The alkenyl bridged compound **19** was obtained from the protected precursor **18** in 94% yield (20 mg). **^{1}H-NMR** (600 MHz, CD_3OD): δ = 7.65 (d, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.24-7.19 (m, 5H), 7.14 (s, 1H), 7.13 (overlap, 1H), 7.11 (d, *J* = 8.4 Hz, 2H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 2H), 5.84 (br, 1H), 5.71 (br, 1H), 4.71 (bs, 2H), 4.63 (t, *J* = 7.5 Hz, 1H), 4.24 (m, 1H), 4.60 (t, *J* = 8.0 Hz, 1H), 4.02 (d, *J* = 11.0 Hz, 1H), 3.84 (d, *J* = 17.0 Hz, 1H), 3.73 (d, *J* = 17.0 Hz, 1H), 3.66 (t, *J* = 11.0 Hz, 1H), 3.57 (m, 1H), 3.35 (overlap, 2H), 3.31 (m, 1H), 3.14 (m, 1H), 3.06 (d, *J* = 7.5 Hz, 2H), 3.04 (m, 1H), 2.03 (m, 1H), 1.78 (m, 1H), 1.72 (m, 1H), 1.64 (m, 1H). **^{13}C-NMR** (150 MHz, CD_3OD): δ = 174.2, 174.0, 173.9, 171.9, 171.1, 168.5, 159.3, 138.0, 137.7, 132.3, 130.4, 130.0, 129.1, 128.2, 126.5, 126.0, 123.2, 121.1, 118.5, 117.6, 111.0, 106.5, 70.2, 67.5, 66.9, 60.5, 54.7, 54.3, 53.2, 49.5, 47.6, 41.9, 37.3, 35.2, 28.6, 26.5, 24.6. **HPLC-ESI-MS**: m/z (%) = 708.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{43}H_{51}N_8O_8$ $[M+H]^+$ 807.3824; found 807.3853.

(2*S*)-2-{[(2*S*)-1-[(9*S*,12*S*,15*S*)-15-Amino-12-benzyl-11,14-dioxo-2,7-dioxa-10,13-diaza-bicyclo[15.2.2]henicosa-1(19),17,20-triene-9-carbonyl]-pyrrolidin-2-yl]formamido}-*N*-(carbamoylmethyl)-3-(1H-indol-3-yl)propanamide (20)

Starting material *tert*-butyl-*N*-[(9*S*,12*S*,15*S*)-12-benzyl-9-[(2*S*)-2-{[(1*S*)-1-[(carbamoyl-methyl)-carbamoyl]-2-(1H-indol-3-yl)ethyl]carbamoyl}pyrrolidine-1-carbonyl]-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]-henicosa-1(19),17,20-trie-15-yl]carbamate (18 mg, 26.46 µmol) afforded product **20** (15 mg, 94%). **^{1}H-NMR** (600 MHz, CD_3OD): δ = 7.58 (d, *J* = 8.6 Hz, 1H), 7.36 (d, *J* = 8.6 Hz, 1H), 7.18-7.10 (m, 5H), 7.13 (overlap, 2H), 7.08 (overlap, 2H), 7.04 (t, *J* = 7.5 Hz, 1H), 6.84 (d, *J* = 7.5 Hz, 2H), 4.64 (t, *J* = 6.5 Hz, 1H), 4.55 (t, *J* = 7.5 Hz), 4.44 (t, *J* = 6.5 Hz, 1H), 4.29 (m, 1H), 4.15 (m, 1H), 4.11 (m, 2H), 3.87 (d, *J* = 17.0 Hz, 1H), 3.68 (d, *J* = 17.0 Hz, 1H), 3.55 (m, 1H), 3.45 (m, 2H), 3.49 (m, 2H), 3.40 (m, 2H), 3.35 (m, 1H), 3.25 (m, 1H), 3.22 (m, 1H), 2.98 (m, 2H), 2.94 (m, 1H), 2.07 (m, 1H), 1.78 (m, 3H), 1.74 (m, 2H), 1.62 (m, 2H). **^{13}C-NMR** (150 MHz, CD_3OD): δ = 171.5, 170.3, 170.0, 169.2, 168.9, 155.8, 136.2, 130.1, 129.2, 127.7, 127.5, 126.5, 123.0, 121.3, 118.4, 117.9, 114.9, 110.0, 106.6, 70.5, 69.0, 66.7, 60.5, 54.7, 53.9, 53.5, 51.2, 47.4, 41.9, 38.0, 35.9, 28.7, 26.6, 25.2, 24.5, 24.5. **HPLC-ESI-MS**: m/z (%) = 809.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{43}H_{52}N_8O_8Na$ $[M+Na]^+$ 831.3800; found 831.3819.

(*S*)-Methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (22)

To a solution of L-Trp-OMe*HCl (800 mg, 3.14 mmol) in MeOH/H_2O (10 mL, 10/1) was added dropwise 36% formaldehyde (24 mg, 0.27 mmol) in MeOH (2 mL). After stirring at room temperature for 16 h, the reaction mixture was concentrated under reduced pressure to dryness. The residue was dissolved in H_2O (10 mL) and neutralized with $NaHCO_3$. The aqueous solution was extracted with EtOAc (3x50 mL), the combined organic phases were washed with brine solution (2x20 mL) and dried over Na_2SO_4. Removal of the solvent and subsequent flash chromatography (EtOAc/EtOH, 20/1) of the residue yielded tetrahydro-β-carboline **22** (400 mg, 55%) as a white solid. R_f = 0.12 (AcOEt/EtOH, 20/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 7.98 (s, 1H, NH), 7.5 (d, *J* = 7.5 Hz, 1H), 7.3 (d, *J* = 7.9 Hz, 1H), 7.18 (dt, *J* = 1.5 Hz, *J* = 7.1 Hz, 1H), 7.12 (dt, *J* = 0.9 Hz, *J* = 7.2 Hz, 1H), 4.1 (s, 2H), 3.83 (overlap, 1H), 3.82 (s, 3H), 3.14 (dd, *J* = 4.7 Hz, *J* = 15.2 Hz, 1H), 2.95 (dd, *J* = 9.6 Hz, *J* = 15.2 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 173.8, 135.9, 131.9, 127.2, 121.7, 114.5, 117.8, 110.7, 107.3, 55.9, 52.1, 42.1, 25.4. **IR** (KBr) ν (cm^{-1}): 3176.8 (NH), 1733.5 (CO). **HPLC-ESI-MS**: m/z (%) = 231 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{13}H_{15}N_2O_2$ $[M+H]^+$ 231.1134; found 231.1137.

Methyl-(3*S*)-2-[(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-12-benzyl-15-{[(*tert*-butoxy)carbonyl]amino}-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraene-9-carbonyl]pyrrolidine-2-carbonyl]-1,2,3,4,9-pentahydropyrido[3,4-b]indole-3-carboxylate (23)

To a solution of macrocycle **17** (40 mg, 60.17 µmol) and carboline **22** (18 mg, 78.23 µmol) in DMF (1.5 mL) HATU (29.7 mg, 78.23 µmol) and DIPEA (50 µL, 241 µmol) were added. After stirring at room temperature for 18 h, the solvent was removed and the crude residue was purified by silica gel column chromatography using EtOAc as eluent to afford product **23** (41 mg, 78%). R_f = 0.21 (EtOAc). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 7.37 (d, *J* = 8.2 Hz, 1H), 7.27 (d, *J* = 8.2 Hz, 1H), 7.22-7.13 (m, 5H), 7.11 (overlap, 1H), 7.05 (overlap, 1H), 6.98 (d, *J* = 8.0 Hz, 2H), 6.68 (d, *J* = 8.0 Hz, 2H), 5.66 (m, 1H), 5.56 (m, 1H), 5.05 (d, *J* = 17.1 Hz, 1H), 5.02 (m, 1H), 4.81 (t, *J* = 7.0 Hz, 1H), 4.76 (overlap, 1H), 4.72 (d, *J* = 14.2 Hz, 1H), 4.60 (m, 2H), 4.42 (t, *J* = 6.5 Hz, 1H), 4.21 (bs, 1H), 4.05 (m, 1H), 3.84 (m, 1H), 3.74 (br, 1H), 3.60 (m, 1H), 3.54 (s, 3H), 3.38 (m, 1H), 3.32 (m, 1H), 3.30 (overlap, 1H), 2.93 (m, 2H), 2.90 (overlap, 1H), 2.80 (m, 1H), 2.73 (m, 1H), 2.27 (m, 1H), 2.11 (m, 1H), 1.97 (m, 2H), 1.44 (s, 9H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 172.3, 171.5, 170.1, 169.6, 168.5, 156.3, 155.2, 136.5, 135.9, 130.7, 130.0, 129.5, 129.3, 128.5, 126.9, 121.8, 119.4, 118.0, 115.4, 111.1, 105.6, 70.5, 68.0, 66.8, 57.7, 55.5, 54.1, 52.3, 50.7, 50.2, 47.4, 42.2, 37.8, 37.6, 28.3, 27.9, 24.3, 22.1. **IR** (KBr) ν (cm^{-1}): 3421.3,

1653.1. **HPLC-ESI-MS**: m/z (%) = 877.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{48}H_{56}N_6O_{10}Na$ $[M+Na]^+$ 899.3950; found 899.3950.

(3*S*)-2-[(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-12-Benzyl-15-{[(*tert*-butoxy)carbonyl]amino}-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraene-9-carbonyl]-pyrrolidine-2-carbonyl]-1,2,3,4,9-pentahydropyrido[3,4-b]indole-3-carboxylic acid

Methyl ester **23** (34 mg, 38.7 µmol) in THF (1 mL) was treated with 4% aqueous LiOH (0.4 mL) at room temperature for 5 h. The reaction mixture was then acidified with 1N HCl at 0 °C to pH≈4 and extracted with EtOAc (3x40 mL). The combined organic phases were washed with H_2O, brine solution and dried over Na_2SO_4. Removal of the solvent yielded the free acid (30.5 mg, 91%) as a solid. R_f = 0.12 (EtOAc/EtOH, 7/1). **^{1}H-NMR** (400 MHz, CD_3OD): δ = 7.44 (d, *J* = 8.2 Hz, 1H), 7.27 (d, *J* = 8.2 Hz, 1H), 7.25-7.17 (m, 5H), 7.14 (overlap, 1H), 7.09 (overlap, 1H), 7.06 (d, *J* = 8.0 Hz, 2H), 6.78 (d, *J* = 8.0 Hz, 2H), 5.76 (m, 2H), 5.15 (m, 1H), 5.04 (m, 1H), 4.84 (overlap, 1H), 4.63 (overlap, 1H), 4.82 (overlap, 1H), 4.66 (br, 2H), 4.56 (t, *J* = 6.5 Hz, 1H), 4.31 (m, 1H), 4.10 (m, 1H), 3.81 (m, 1H), 3.76 (br, 1H), 3.63 (m, 1H), 3.41 (m, 1H), 3.35 (m, 1H), 3.36 (overlap, 1H), 2.98 (m, 2H), 2.90 (overlap, 1H), 2.84 (m, 1H), 2.78 (m, 1H), 2.31 (m, 1H), 2.21 (m, 1H), 1.97 (m, 2H), 1.44 (s, 9H). **^{13}C-NMR** (100 MHz, CD_3OD): δ = 171.3, 171.0, 170.6, 156.7, 154.9, 136.7, 135.2, 130.8, 130.3, 129.6, 128.5, 127.6, 122.5, 119.8, 118.4, 116.2, 111.7, 106.6, 70.0, 66.8, 66.7, 57.7, 55.3, 54.9, 50.3, 49.8, 47.6, 41.2, 37.4, 37.2, 28.6, 28.2, 24.4, 22.6. **IR** (KBr) ν (cm^{-1}): 3426.6, 1716.3, 1646.8. **HPLC-ESI-MS**: m/z (%) = 863.4 $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{47}H_{54}N_6O_{10}Na$ $[M+Na]^+$ 885.3794; found 885.3793.

***tert*-Butyl-*N*-[(4*E*,9*S*,12*S*,15*S*)-12-benzyl-9-[(2*S*)-2-[(3*S*)-3-[(carbamoylmethyl)carba-moyl]-1,2,3,4,9-pentahydropyrido[3,4-b]indole-2-carbonyl]pyrrolidine-1-carbonyl]-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo[15.2.2]henicosa-1(19),4,17,20-tetraen-15- yl]carbamate (24)**

To a stirred solution of the free acid (28 mg, 32.45 µmol), HCl*H-Gly-NH_2 (4.7 mg, 42.2 µmol) and TBTU (12.5 mg, 38.9 µmol) in DMF (2 mL) DIPEA (0.03 mL, 0.23 mmol) was added. The reaction was stirred at room temperature for 20 h. After that time the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography with EtOAc/EtOH (7/1) to obtain the hexapeptide **24** (23 mg, 77%) as a solid. R_f = 0.28 (EtOAc/EtOH, 7/1). **^{1}H-NMR** (400 MHz, CD_3OD): δ = 7.46 (d, *J* = 8.0 Hz, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.26-7.17 (m, 5H, 12-H, 13-H), 7.07 (overlap, 1H, 30-H, 7.01 (overlap, 1H), 6.78 (d, *J* = 8.0 Hz, 2H, 2-H), 5.76 (m, 2H, 36-H), 5.11 (m, 1H), 5.01 (m, 1H), 4.81 (overlap, 1H), 4.88 (overlap, 1H), 4.82 (overlap, 1H), 4.67 (br, 2H), 4.54 (m, 1H), 4.30 (m, 1H), 3.87 (m, 2H, 38-H), 3.71 (m, 2H, 38-H), 3.73 (br, 1H), 3.61 (m, 1H), 3.41 (m, 2H), 3.40 (overlap, 1H), 3.01 (m, 1H), 2.99 (m, 2H), 2.91 (overlap, 2H), 2.33 (m, 1H), 1.92 (m, 1H), 1.99-1.92 (m, 2H), 1.46 (s, 9H, 41-H). **^{13}C-NMR** (100 MHz, CD_3OD): δ = 174.1, 173.3, 172.1, 171.6, 170.9, 169.8, 158.1, 157.2, 138.9, 138.2, 130.0, 130.2 (36-C, 37-C), 129.0, 128.0, 126.4, 121.1, 118.5, 117.4, 114.9, 110.7, 106.4, 70.0, 66.9, 67.5, 57.8, 55.6, 54.2, 50.3, 49.2, 47.0, 42.2, 41.9, 37.3, 37.1, 28.6, 28.9, 24.9, 22.4 (25-C). **IR** (KBr) ν (cm^{-1}): 3421.0, 2932.8, 1662.5. **HPLC-ESI-MS**: m/z (%) = 919.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{49}H_{58}N_8O_{10}Na$ $[M+Na]^+$ 941.4168; found 941.4167.

2-{[(3*S*)-2-[(2*S*)-1-[(4*E*,9*S*,12*S*,15*S*)-15-Amino-12-benzyl-11,14-dioxo-2,7-dioxa-10,13-diazabicyclo-[15.2.2]henicosa-1(19),4,17,20-tetraene-9-carbonyl]pyrrolidine-2-carbonyl]-1,2,3,4,9-pentahydro-pyrido[3,4-b]indol-3-yl]formamido}acetamide (25)

The Boc-protecting group of macrocyle **24** (30 mg, 30 µmol) was removed according to the general procedure to yield after chromatographic purification (DCM/MeOH; 8/1 + 0.1% TEA) the final product **25** (23 mg, 86%). This was dissolved in ACN/H_2O (2 mL, 1/1) and lyophilized to afford a white powder. **^{1}H-NMR** (600 MHz, CD_3OD): δ = 7.47 (d, *J* = 8.0 Hz, 1/2H), 7.43 (d, *J* = 8.0 Hz, 1/2H), 7.29 (overlap, 1H), 7.26-7.18 (m, 5H), 7.08 (overlap, 1H), 7.00 (overlap, 1H), 6.82 (d, *J* = 8.0 Hz, 2H), 5.77 (m, 1H), 5.73 (m, 1H), 5.41 (m, 1H), 4.97 (m, 1H), 4.85 (overlap, 1H), 4.52 (m, 1H), 4.71 (overlap, 1H), 4.69 (br, 2H), 4.62 (m, 1H), 4.11 (m, 1H), 3.94 (m, 2H), 3.87 (m, 2H), 3.79 (m, 2H), 3.74 (m, 1H), 3.69 (overlap, 1H), 3.65 (m, 1H), 3.58 (m, 1H), 3.38 (m, 2H), 2.99 (m, 2H), 2.91 (overlap, 2H), 2.34 (m, 1H), 2.13 (m, 1H), 1.94 (m, 1H), 1.94 (m, 1H). **^{13}C-NMR** (150 MHz, CD_3OD): δ = 174.2, 173.7, 173.2, 172.9, 170.9, 169.8, 156.0, 138.4, 137.4, 132.0, 131.1, 129.8, 129.0, 128.0, 126.3, 121.1, 118.6, 117.2, 115.1, 110.6, 105.0, 69.9, 67.4, 67.1, 57.4, 54.6, 54.4, 53.9, 49.0, 47.0, 42.0, 39.0, 37.5, 36.8, 28.6, 24.7, 22.2. **HPLC-ESI-MS**: m/z (%) = 819.4 (100) $[M+H]^+$. **HR-ESI-MS** (m/z): calcd. for $C_{44}H_{51}N_8O_8$ $[M+H]^+$ 819.3824; found 819.3830.

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Graphical Abstract