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Synthesis and biological activity of N-terminal lipidated and/or fluorescently labeled conjugates of astressin as corticotropin releasing factor antagonists

Dirk T. S. Rijkers,[a,*] Jack A. J. den Hartog[b] and Rob M. J. Liskamp[a]

[a]*Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands*
[b]*Solvay Pharmaceuticals, Research Laboratories, C.J. van Houtenlaan 36, 1381 CP Weesp, The Netherlands*

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Abstract—This report describes the synthesis of eight N-terminally modified astressin analogs and their biochemical evaluation as corticotropin releasing factor (CRF) antagonists. The lipidated astressin derivatives were tested on rat CRF receptor type 1 and 2α and were found to be active as CRF antagonists (rCRFR1: pA_2 = 7.5–8.3; rCRFR2α: pA_2 = 7.5–9.0) with nearly equal activities as compared to unmodified astressin (rCRFR1: $pA_2 = 8.3 \pm 0.09$; rCRFR2α: $pA_2 = 8.7 \pm 0.08$).

1. Introduction

Transport of peptide hormones over phospholipid membranes is considered as rather difficult mainly as a result of their size and highly polar nature.[1,2] In the current literature several strategies have been described for membrane passage of large and polar biomolecules (e.g., peptide, proteins, and nucleic acids).[3] One strategy for peptide delivery through biological membranes involves the covalent coupling of a nontransportable peptide to a transportable vector peptide.[4–8] Among these are the nuclear transcription activator protein (Tat) encoded by HIV type 1,[9] the *Antennapedia* homeodomain-derived peptide penetratin,[10] polyarginine-based oligomers,[11] and guanidine-rich peptoids[12] or oligocarbamates.[13] A major limitation of such peptide chimeras in case of neuropeptides is the unpredictable influence of the vector peptide on receptor binding and (in)activation. However, lipidation of a peptide is a well-accepted approach to enhance peptide-membrane interaction and several examples exist in the literature, which describe the membrane passage of lipopeptides.[14,15]

Corticotropin releasing factor (CRF) is a peptide amide of 41 amino acids, which stimulates the release of adrenocorticotropic hormone (ACTH), β-endorphin and other pro-opiomelanocortin (POMC) derived products from the anterior pituitary gland and acts within the brain to modulate a wide range of stress responses.[16] Overexpression of CRF is related to psychiatric illnesses such as major depression and anxiety disorders.[17] Therefore the development of antagonists is of high interest to obtain insight into the physiological role of CRF in the body response to stress. With respect to this, approaches to increase the ability of these antagonists to penetrate into the desired brain regions, for example, by lipidation, are especially interesting.

The first peptide-based CRF antagonist, α-helical CRF(9–41),[18] formed the basis for an intensive search for better CRF antagonists, which ultimately led to the discovery of astressin {*cyclo*(30–33)[D-Phe12, Nle21,38, Glu30, Lys33]hCRF(12–41)}.[19,20] We decided to synthesize a series of lipidated astressin conjugates for future membrane transport studies. First, for these conjugates the influence of the N-terminal modification on CRF antagonistic potency was studied since derivatization of a neuropeptide, either at its N/C-terminus or at residues within the peptide sequence (e.g., serine/

Abbreviations: hCRF: human corticotropin releasing factor; rCRF: rat corticotropin releasing factor; dans: dansyl; Nle: norleucine; pal: palmitoyl; succ: succinimidyl

Keywords: Antagonists; Peptides and polypeptides; Peptide conjugates; Solid phase synthesis.

* Corresponding author. Tel.: +31-30-253-6916/7275; fax: +31-30-253-6655; e-mail: d.t.s.rijkers@pharm.uu.nl

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cysteine), can be to a large extent decisive for its biological activity. We found that lipidation of the α-amino functionality did not alter the potency of astressin as CRF antagonist.

2. Results and discussion

2.1. Synthesis

Our intention was to modify the N-terminal α-amino functionality of astressin with three small hydrophobic moieties leading to **5b–d**, three fatty acid derivatives of different length, and degree of saturation leading to **5e–g** and two lipidated amino acid residues affording **5h–i** as are shown in Figure 1. The latter lipidated amino acids were inspired by the work of Asakuma et al.[21] and Pakalns et al.[22] The dansyl moiety in derivatives **5d** and **5i** was used as a fluorescent probe to monitor the process of membrane translocation of the corresponding astressin conjugates.

For a rapid access to these astressin conjugates, our previously described[23] synthesis of astressin was adjusted as follows (Scheme 1). It was decided that the lactam bridge had to be introduced before modification of the N-terminus. Therefore, the α-amino functionality of the terminal D-Phe12 residue was temporarily protected with the orthogonal 9-fluorenylmethyloxycarbonyl (Fmoc) group. It was expected that this group was stable under the conditions used for allyl deprotection and ring closure. Allyl-based protecting groups of the glutamic acid30- and lysine33 side chains could be removed within 2 h in the presence of Pd(0) with phenylsilane as scavenger[24] and was a rapid and very clean reaction. To check the completion of the allyl deprotection and in order to verify that a premature loss of the Fmoc group had not occurred, a small amount of resin was treated with trifluoroacetic acid (TFA). Indeed, the N^{α}-Fmoc-protected linear astressin molecule (**6**) was found as the major product according to HPLC and electrospray ionization mass spectrometry (ESI MS, *m*/*z*, found: 3803.90; calcd: 3804.48). Ring closure was carried out by treatment with benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)/*N*-hydroxybenzotriazole (HOBt)[25] in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP). Formation of the lactam bridge was monitored by the Kaiser test[26] and was completed after 16 h.

After ring closure, a small portion of the resin was removed and treated with TFA to yield an Fmoc-protected astressin analog (**5c**). The remainder of the resin was treated with 20% piperidine in NMP to remove the Fmoc group. Subsequently, the resin was divided into eight portions (one control peptide, seven modified peptides) and in each portion the N-terminal α-amino group was modified according to procedures shown in Table 1.

Acetylation was complete within 15 min using the standard capping reaction conditions. Coupling of Fmoc-Ahx-OH (Ahx: 6-aminohexanoic acid) was easy and after deprotection subsequent coupling of the fluorescent dansyl group was performed in the presence of the mild base *N*-methylmorpholine (NMM) to avoid disubstitution as was found in the presence of the stronger base DIPEA.[27] The Kaiser test showed false positive colored resin beads during the coupling of the dansyl moiety due to the acidic character of the sulfonamide

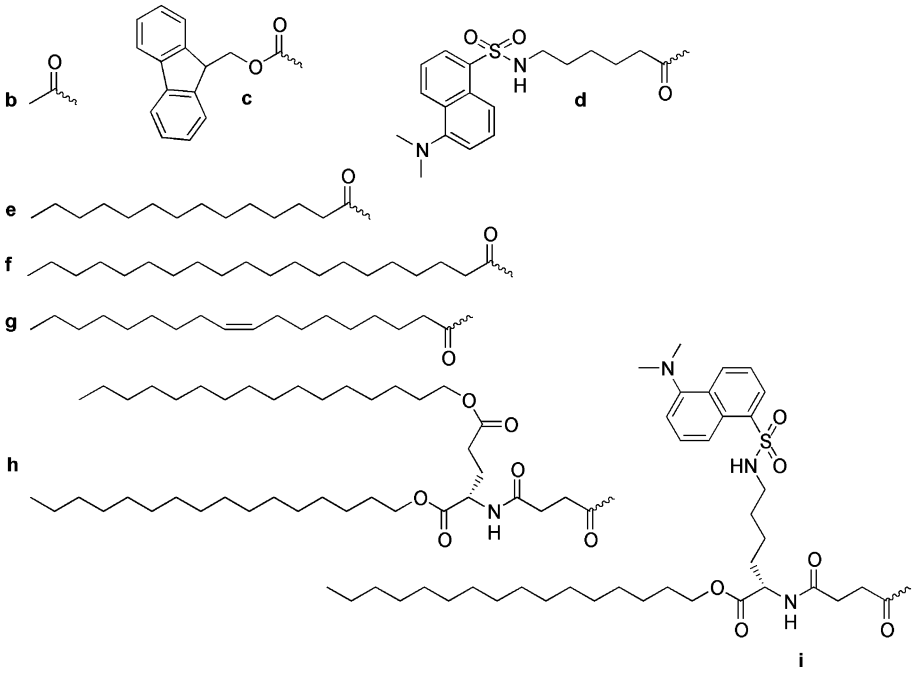
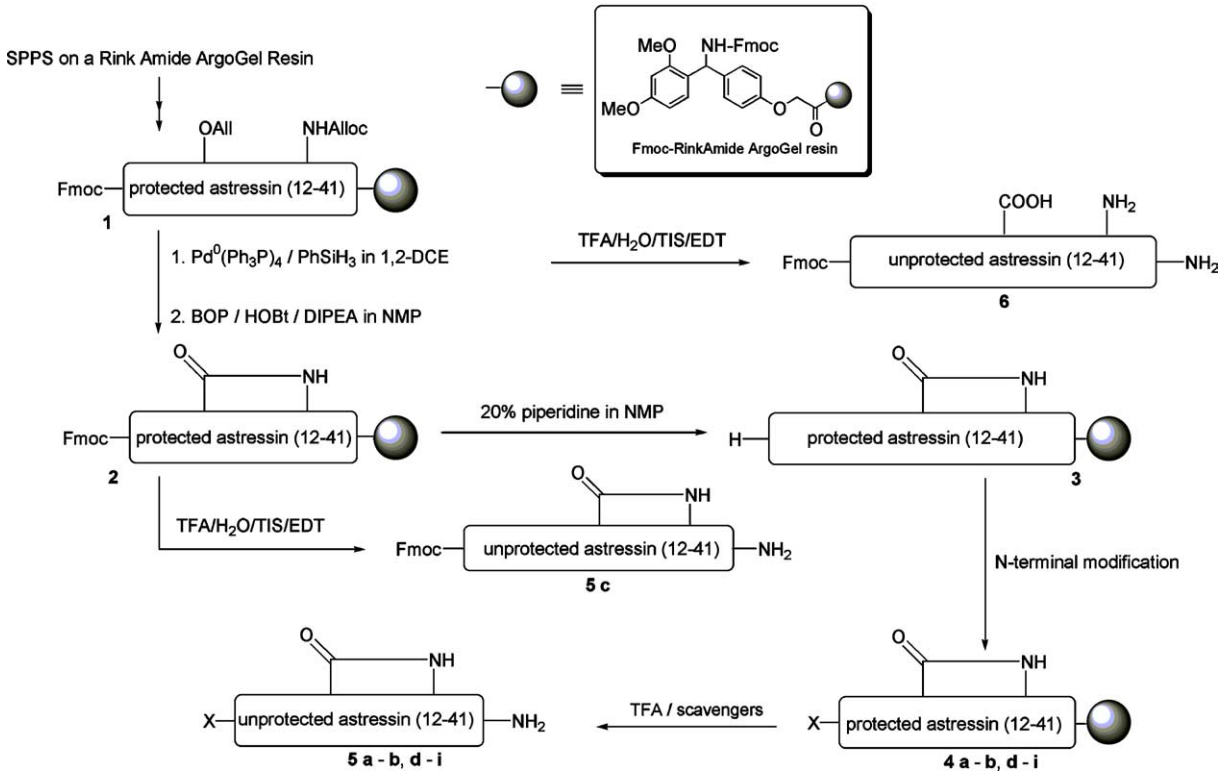


Figure 1. Chemical structures of the N-terminal astressin modifications: (a) none, free N-terminus (H); (b) acetyl (Ac); (c) 9-fluorenylmethyloxycarbonyl (Fmoc); (d) *N*-dansyl-6-aminohexanoyl (Dans-Ahx); (e) tetradecanoyl (Myr); (f) eicosanoyl (Ara); (g) *cis*-9-octadecenoyl (oleic); (h) $(PalO)_2Glu^{inv}$-Succ-OH; (i) $(PalO)Lys^{inv}$(Dans)-Succ-OH.



Scheme 1. Schematic overview of the synthesis of the lipo-astressin conjugates.

Table 1. Acylation conditions of the compounds synthesized in this study

	N-terminal modification	Compound	Acylation conditions	Cleavage
5a	None, free N-terminus (H)	Astressin	—	A
5b	Acetyl (Ac)	Ac-astressin	Ac_2O/DIPEA/HOBt in NMP, 15 min	A
5c	Fluorenylmethyloxycarbonyl (Fmoc)	Fmoc-astressin	Directly obtained from **2**	A
5d	*N*-dansyl-6-aminohexanoyl (Dans-Ahx)	Dans-Ahx-astressin	(i) Fmoc-Ahx-OH/HBTU/HOBt in NMP, 45 min (ii) 20% piperidine in NMP, 30 min (iii) Dansylchloride/NMM in DCE, 16 h	A
5e	Tetradecanoyl (Myr)	Myr-astressin	Myristic acid/BOP/HOBt/DIPEA in NMP/DCE 1:1, 16 h	A
5f	Eicosanoyl (Ara)	Ara-astressin	Arachidic acid/BOP/HOBt/DIPEA in NMP/DCE 1:2, 16 h	A
5g	*cis*-9-Octadecenoyl (Oleic)	Oleic-astressin	Oleic acid/BOP/HOBt/DIPEA in NMP/DCE 1:1, 16 h	B
5h	$(PalO)_2Glu^{inv}$-Succ-OH	$(PalO)_2Glu^{inv}$-Succ-astressin	**8**/BOP/HOBt/DIPEA in NMP/DCE 1:1, 16 h	A
5i	$(PalO)Lys^{inv}$(Fmoc)-Succ-OH	$(PalO)Lys^{inv}$(Dans)-Succ-astressin	(i) **11**/BOP/HOBt/DIPEA in NMP/DCE 1:1, 16 h (ii) 20% Piperidine in NMP, 30 min (iii) Dansylchloride/NMM in DCE, 16 h	A

A: TFA/H_2O/EDT/TIS 85:8.5:4.5:2 v/v/v/v; B: TFA/H_2O/EDT 90:5:5 v/v/v.

hydrogen compared to the NH of a normal amide bond. Therefore, the bromophenol blue test (BPB), which was more reliable in this case was used.[28] The use of Dans-Ahx-OH as a building block is not recommended, since protonation of the tertiary amine of the dansyl functionality hampered aqueous work-up. The fatty acid derivatives were coupled with BOP/HOBt in the presence of DIPEA. To prevent precipitation of the activated esters, a mixture of NMP/1,2-dichloroethane (DCE) 1:1 v/v was used. Coupling of the fatty acids was monitored by the Kaiser test and was found to be complete after 16 h. Synthesis of the lipophilic glutamic acid derivative **8** and lysine derivative **11** were carried out analogously the procedure of Asakuma et al.[21] and Pakalns et al.[22] (Scheme 2). As was mentioned above, functionalization of the ε-amino group of **11** with the dansyl moiety was carried out on the solid support.

The fatty acid astressin conjugates were deprotected and detached from the resin by treatment with

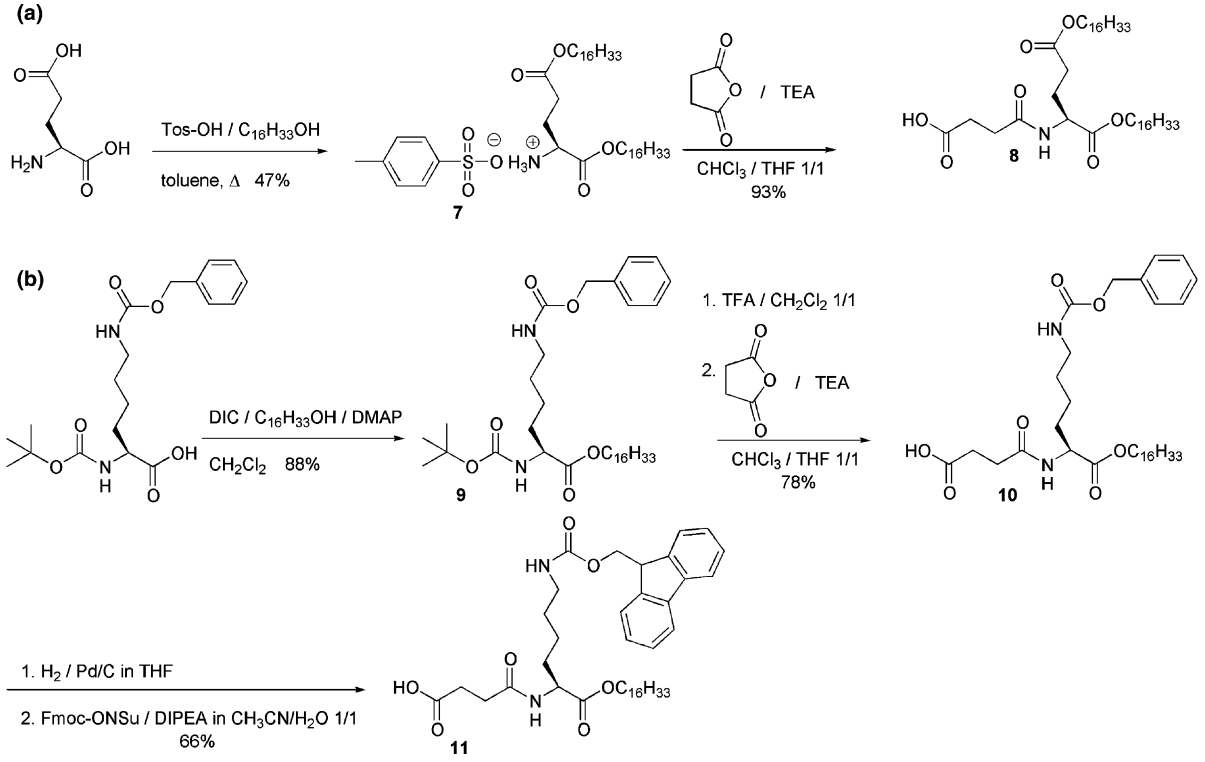
TFA/H_2O/1,2-ethanedithiol (EDT)/triisopropylsilane (TIS) 85:8.5:4.5:2 v/v/v/v. To avoid reduction of the double bond in the oleic acid residue of **4g**, TIS was not used as scavenger and TFA/H_2O/EDT 90:5:5 v/v/v was used instead.

It can be concluded that these synthetic procedures efficiently and successfully resulted in several N-terminally modified astressin derivatives (**5a–i**). The overall yields in all these cases were moderate (33–64%) and according to HPLC and MS analysis, the crude products consisted mainly of the desired astressin conjugates. We also tried to acylate the N-terminus with 1-adamantyl isocyanate, cholic acid and *cis*-4,7,10,13,16,19-docosahexaenoic acid but the synthesis of these conjugates failed mainly due to the intrinsic vulnerability of these N-terminal modifications with respect to the chosen synthesis/deprotection strategy, since the peptides could only be obtained after a final TFA-treatment.

Compounds **5a–i** were tested for their CRF antagonistic activity on two rat CRF receptor subtypes 1 and 2α.

2.2. Biological activity

Results of the biological activity studies are given in Table 2. The astressin conjugates **5a–i** were evaluated for their functional antagonistic activity (pA_2)[29,30] on the rat CRF receptor type 1 and 2α (rCRFR1, respectively, rCRFR2α). The functional assay in this study used a cloned rat CRFR1 and CRFR2α in a LVIP cell line furnished with a cAMP-dependent reporter gene for β-galactosidase. Compounds **5a–i** were tested for antagonistic properties assessing their ability to attenuate CRF-induced (against 1 nM CRF in rCRFR1; against 10 nM CRF in rCRFR2α) expression of β-galactosidase using Schild analysis. The given pA_2 values represent the mean value of six independent determinations.



Scheme 2. Schematic overview of the synthesis of the lipidated amino acid residues.

Table 2. Biophysical and biological data of the astressin derivatives

	Compound	$(M + H)^+$ calcd	$(M + H)^+$ found	HPLC C4 300 Å	HPLC CN 100 Å	pA_2[a] rCRFR1	pA_2[a] rCRFR2α
5a	Astressin	3564.23	3565.40	27.12	21.05	8.3 ± 0.09	8.7 ± 0.08
5b	Ac-astressin	3606.27	3606.21	31.18	22.03	8.3 ± 0.13	9.0 ± 0.1
5c	Fmoc-astressin	3786.47	3786.03	28.93	22.08	7.8 ± 0.03	8.2 ± 0.02
5d	Dans-Ahx-astressin	3910.68	3909.95	32.33	22.62	8.5 ± 0.05	8.9 ± 0.04
5e	Myr-astressin	3774.59	3775.70	39.34	23.93	8.0 ± 0.07	8.2 ± 0.07
5f	Ara-astressin	3858.75	3858.65	43.29	24.62	7.9 ± 0.04	8.1 ± 0.04
5g	Oleic-astressin	3828.68	3828.08	40.66	24.36	7.5 ± 0.07	7.8 ± 0.06
5h	$(PalO)_2Glu^{inv}$-Succ-astressin	4242.28	4242.10	Not eluted	26.15	7.3 ± 0.03	7.5 ± 0.03
5i	$(PalO)Lys^{inv}$(Dans)-Succ-astressin	4249.19	4249.98	Not eluted	25.51	7.7 ± 0.03	8.0 ± 0.03

[a] The pA_2 value is the mean value of six independent determinations.

Compounds **5b–i** were found to be active as CRF antagonists with nearly equal potency of (unmodified) astressin (**5a**). Generally, CRF antagonistic activity was found to be independent of the type of N-terminal acylation. These results agree well with our earlier observations that N-terminal truncation of astressin resulted in active CRF antagonists.[31] These lipidated CRF antagonists were designed with the purpose to obtain compounds capable of passage of membrane barriers, for example, present in the central nerve system, since CRF receptors are predominantly localized here. However, in view of earlier results obtained with myristoylated peptides[15] it is also very likely that in addition to binding extracellularly, the astressin conjugates have penetrated into cells or at least are present in cellular membranes. Apparently, this did not interfere with their CRF antagonistic action. Fluorescent microscopy studies using, for example, dansylated derivatives **5d** and **5i** might shed light on this issue and will be carried out in due course.

3. Experimental

3.1. Materials

N^{α}-9-Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids, and *N*-hydroxybenzotriazole (HOBt) were purchased from Advanced ChemTech Europe. The side chain protecting groups were chosen as: allyl (All) for glutamic acid, allyloxycarbonyl (Aloc) for lysine, *tert*-butyl (^{t}Bu) for glutamic acid, *tert*-butyloxycarbonyl (Boc) for lysine, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine, and trityl (Trt) for asparagine, glutamine, and histidine. Fmoc-Glu(OAll)-OH, Fmoc-Lys(Aloc)-OH, Fmoc-6-aminohexanoic acid (Fmoc-Ahx-OH), and Fmoc-D-Phe-OH were obtained from Neosystem Laboratoire. The coupling reagents, 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) were purchased from Richelieu Biotechnologies. Peptide grade *N*-methylpyrrolidone (NMP), dichloromethane (DCM), 1,2-dichloroethane (DCE) and trifluoroacetic acid (TFA) and HPLC-grade acetonitrile, hexane and methyl *tert*-butyl ether (MTBE) were from Biosolve. Piperidine, *N*,*N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM), triethylamine (TEA), 4-(*N*,*N*-dimethylamino)pyridine (DMAP), succinic anhydride, dansylchloride, and tetrakistriphenylphosphine palladium(0) were obtained from Acros Organics. Triisopropylsilane (TIS) and 1,2-ethanedithiol (EDT) were supplied by Merck. Phenylsilane, myristic acid, arachidic acid, oleic acid, and hexadecanol were obtained from Fluka. *N*,*N*′-diisopropylcarbodiimde (DIC) was from Aldrich. All other chemicals were at least analytical reagent grade. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[32] ^{1}H NMR spectra were recorded on a Varian G-300 (300.1 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS. ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. The optical rotations were measured at 20 °C using a Jasco P-1010 polarimeter. Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat (according to Dr. Tottoli) and are uncorrected. Combustion analyses were done by Kolbe Mikroanalytisches Labor (Mülheim an der Ruhr, Germany).

3.2. Peptide synthesis

3.2.1. Peptide assembly. The linear sequence of (fully protected) astressin (Fmoc-D-Phe-His(Trt)-Leu-Leu-Arg(Pbf)-Glu(O^{t}Bu)-Val-Leu-Glu(O^{t}Bu)-Nle-Ala-Arg-(Pbf)-Ala-Glu(O^{t}Bu)-Gln(Trt)-Leu-Ala-Gln(Trt)-Glu-(OAll)-Ala-His(Trt)-Lys(Aloc)-Asn(Trt)-Arg(Pbf)-Lys-(Boc)-Leu-Nle-Glu(O^{t}Bu)-Ile-Ile-NH-RinkAmide-ArgoGel) was synthesized as described earlier.[23,31] The allyl protective groups were removed by swirling the resin in DCE with phenylsilane (25 equiv) in the presence of 3 equiv tetrakistriphenylphosphine palladium(0) under an argon atmosphere for 2 h. The resin was extensively washed with DCM (2 × 2 min), NMP (2 × 2 min), 0.5% DIPEA/NMP (3 × 5 min), 0.02 M diethyldithiocarbamic acid sodium salt in NMP (3 × 15 min), NMP (5 × 5 min), and DCM (3 × 3 min), respectively, to remove the Pd catalyst.[33] Ring closure between the unprotected side chains of glutamic acid and lysine was achieved on the resin with 3 equiv BOP/HOBt in the presence of 9 equiv DIPEA in NMP for 16 h. Then, the resin was washed with NMP (3 × 2 min), DCM (3 × 2 min), and NMP (3 × 2 min). Deprotection and ring closure reaction were monitored by the Kaiser test.[26] After removal of the N-terminal Fmoc functionality by treatment with 20% piperidine in NMP (3 × 8 min), the resin was washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried in a vacuum desiccator.

3.2.2. N-terminal modification. Each portion of dry resin (160 mg, 0.02 mmol) was suspended in NMP (3 × 2 min) and DCE (3 × 2 min) to swell the resin before the reagents were added. The coupling and deprotection reactions were monitored by the Kaiser test.[26] The coupling reaction of dansylchloride was monitored by the bromophenol blue test.[28] If necessary, the coupling reaction was repeated once to achieve complete derivatization.

3.2.2.1. Ac-astressin (4b). The resin was treated with a solution of 0.5 M Ac_2O, 0.125 M DIPEA, and 0.015 M HOBt in NMP (2 mL) for 15 min. Then, the resin was washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.2. Dans-Ahx-astressin (4d). The resin was treated with Fmoc-Ahx-OH (85 mg, 0.24 mmol, 12 equiv), HBTU (91 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol, 12 equiv), and DIPEA (126 μL, 0.72 mmol, 36 equiv) in NMP (2 mL) for 45 min at room temperature. Then, the resin was washed with NMP (3 × 2 min), DCM (3 × 2 min), and NMP (3 × 2 min) and the Fmoc group was removed by treatment with

20% piperidine in NMP (2 mL, 3 × 10 min) subsequently followed by washing the resin with NMP (5 × 2 min) and DCM (5 × 2 min). Finally, dansylchloride (65 mg, 0.24 mmol, 12 equiv) dissolved in DCE (2 mL) was added to the resin followed by NMM (79 μL, 0.72 mmol, 36 equiv). After a reaction time of 16 h at room temperature, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.3. Myr-astressin (4e). Myristic acid (55 mg, 0.24 mmol, 12 equiv) was dissolved in NMP (1 mL) and BOP (106 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol) were added followed by the addition of DCE (1 mL). The clear solution was transferred to the resin and DIPEA (126 μL, 0.72 mmol, 36 equiv) was added. The reaction mixture was shaken for 16 h at room temperature. Then, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.4. Ara-astressin (4f). Arachidic acid (75 mg, 0.24 mmol, 12 equiv) was dissolved in NMP (1 mL) and BOP (106 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol) were added followed by the addition of DCE (1 mL). The clear solution was transferred to the resin and DIPEA (126 μL, 0.72 mmol, 36 equiv) was added. To prevent premature precipitation of the activated fatty acid an additional amount of DCE (1 mL) was added. The reaction mixture was shaken for 16 h at room temperature. Then, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.5. Oleic-astressin (4g). Oleic acid (76 μL, 0.24 mmol, 12 equiv) was dissolved in NMP (1 mL) and BOP (106 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol) were added followed by the addition of DCE (1 mL). The clear solution was transferred to the resin and DIPEA (126 μL, 0.72 mmol, 36 equiv) was added. The reaction mixture was shaken for 16 h at room temperature. Then, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.6. $(PalO)_2Glu^{inv}$-Succ-astressin (4h). Compound **8** (167 mg, 0.24 mmol, 12 equiv) was dissolved in NMP (1 mL) and BOP (106 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol) were added followed by the addition of DCE (1 mL). The clear solution was transferred to the resin and DIPEA (126 μL, 0.72 mmol, 36 equiv) was added. The reaction mixture was shaken for 16 h at room temperature. Then, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.2.7. (PalO)Lys^{inv} (Dans)-Succ-astressin (4i). Compound **11** (166 mg, 0.24 mmol, 12 equiv) was dissolved in NMP (1 mL) and BOP (106 mg, 0.24 mmol, 12 equiv), HOBt (37 mg, 0.24 mmol) were added followed by the addition of DCE (1 mL). The clear solution was transferred to the resin and DIPEA (126 μL, 0.72 mmol, 36 equiv) was added. The reaction mixture was shaken for 16 h at room temperature. Then, the resin was filtered and washed with NMP (3 × 2 min), DCM (3 × 2 min), and NMP (3 × 2 min) and subsequently treated with 20% piperidine in NMP (2 mL, 3 × 10 min) to remove the Fmoc group, followed by washing steps with NMP (5 × 2 min) and DCM (5 × 2 min). To this resin, dansylchloride (65 mg, 0.24 mmol, 12 equiv) dissolved in DCE (2 mL) was added to the resin followed by NMM (79 μL, 0.72 mmol, 36 equiv). After a reaction time of 16 h at room temperature, the resin was filtered and washed with NMP (5 × 2 min) and DCM (5 × 2 min) and subsequently dried on air.

3.2.3. Deprotection and cleavage from the resin. Resins **4a–f** and **4h–i** were treated with TFA/H_2O/EDT/TIS 85:8.5:2:4.5 v/v/v/v and resin **4g** was treated with TFA/H_2O/EDT 90:5:5 v/v/v for 3 h at room temperature. The peptides were precipitated with MTBE/hexane 1:1 v/v at −20 °C and finally lyophilized from acetonitrile/H_2O 1:1 v/v. The obtained yields were: **5a**: 64%, **5b**: 56%, **5c**: 35%, **5d**: 56%, **5e**: 50%, **5f**: 39%, **5g**: 49%, **5h**: 33%, and **5i**: 40%.

3.2.4. Tos-OH·H-Glu($OC_{16}H_{33}$)-$OC_{16}H_{33}$ (7). Glutamic acid (7.4 g, 50 mmol), hexadecanol (24.2 g, 100 mmol), and *p*-toluenesulfonic acid monohydrate (10.5 g, 55 mmol) were suspended in toluene (300 mL) and refluxed for 16 h in a Dean–Stark apparatus. Subsequently, the clear and colorless reaction mixture was evaporated to dryness. Pure **7** was obtained after crystallization from aceton in 47% yield (17.9 g). R_f (DCM/MeOH 98:2 v/v): 0.19; mp: 63 °C; $[\alpha]_D$ +2.5 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$): δ = 0.88 (t, 6H), 1.26 (br s, 52H), 2.18 (m, 2H), 2.34 (s, 3H), 2.46 (br m, 2H), 3.96–4.09 (m, 5H), 7.11/7.14–7.73/7.75 (dd, 4H), 8.29 (br s, 3H); ^{13}C NMR ($CDCl_3$): δ = 14.1, 21.3, 22.7, 25.2, 25.7, 25.9, 28.2, 28.5, 29.3, 29.4, 29.4, 29.6, 29.7, 29.7, 31.9, 52.4, 64.9, 66.6, 126.1, 128.8, 140.1, 141.4, 168.9, 172.2. Anal. calcd for $C_{44}H_{81}NO_7S$: C, 68.80; H, 10.63; N, 1.82; S, 4.17. Found: C, 68.72; H, 10.60; N, 1.86; S, 4.08; ESI MS *m*/*z* calcd for $C_{37}H_{74}NO_4$: $([M - Tos\text{-}OH] + H)^+$, 596.97; found: $([M - Tos\text{-}OH] + H)^+$, 596.65.

3.2.5. Succinimidyl-Glu^{inv}($OC_{16}H_{33}$)-$OC_{16}H_{33}$ (8). Compound **7** (15 g, 19.6 mmol) was dissolved in $CHCl_3$/THF 1:1 v/v (150 mL), and TEA (4.08 mL, 29.3 mmol, 1.5 equiv) followed by succinic anhydride (2.9 g, 29.3 mmol, 1.5 equiv) were added. After stirring for 16 h at room temperature, the reaction mixture was evaporated in vacuo. The residue was crystallized from aceton in 93% yield (12.6 g). R_f (DCM/MeOH 98:2 v/v): 0.08; mp: 59–61 °C; $[\alpha]_D$ +8.9 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$): δ = 0.88 (t, 6H), 1.26 (br s, 52H), 1.62 (m, 4H), 1.97–2.17 (dm, 2H), 2.55 (m, 2H), 2.66 (m, 2H), 3.06/3.09–3.11/3.14 (dd, 2H), 4.05/4.12 (dt, 4H), 4.59 (m, 1H), 6.82 (d, 1H); ^{13}C NMR ($CDCl_3$): δ = 8.4, 14.1, 22.7, 25.8, 25.9, 27.5, 28.5, 28.5, 29.2, 29.3, 29.3 29.49, 29.5, 29.6, 29.6, 29.7, 30.3, 30.6, 31.5, 31.9, 45.0, 51.7, 64.9, 65.7, 172.0, 172.5, 172.9, 176.9. Anal. calcd for $C_{41}H_{77}NO_7$: C, 70.75; H, 11.15; N, 2.01. Found: C, 70.68; H, 11.24; N, 2.08; ESI MS *m*/*z* calcd for $C_{41}H_{78}NO_7$: $(M + H)^+$, 696.58; found: $(M + H)^+$, 697.35.

3.2.6. Boc-Lys(Cbz)-$OC_{16}H_{33}$ (9). Boc-Lys(Cbz)-OH (3.8 g, 10 mmol), hexadecanol (2.4 g, 10 mmol), and DMAP (122 mg, 1 mmol, 0.1 equiv) were dissolved in DCM (100 mL). The solution was cooled on ice and DIC (2.2 mL, 14 mmol, 1.4 equiv) was added portionwise. The reaction mixture was stirred for 1 h at 0 °C followed by 16 h at room temperature. Subsequently, the turbid reaction mixture was filtered and the solvent was removed under reduced pressure. The residue was redissolved in EtOAc (150 mL) and subsequently washed with H_2O, 1 N $KHSO_4$, H_2O, 5% $NaHCO_3$, and brine (3 × 50 mL each). The EtOAc layer was dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was crystallized from aqueous aceton in 88% yield (5.3 g). R_f (DCM/MeOH 98:2 v/v): 0.50, R_f (DCM/MeOH 94:6 v/v): 0.88; mp: 55–59 °C; $[\alpha]_D$ +3.5 (*c* 0.99, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 0.88$ (t, 3H), 1.26 (br s, 26H), 1.43 (s, 9H), 1.12–1.80 (m, 8H), 3.18 (m, 2H), 4.11 (t, 2H), 4.26 (m, 1H), 4.83 (br s, 1H), 5.10 (br s, 3H), 7.35 (s, 5H); ^{13}C NMR ($CDCl_3$): $\delta = 14.1$, 22.4, 22.7, 25.8, 28.3, 28.5, 29.2, 29.3, 29.5, 29.56, 29.6, 29.7, 31.9, 32.5, 40.6, 53.2, 65.5, 66.6, 79.8, 128.1, 128.5, 136.5, 156.4, 172.8. Anal. calcd for $C_{35}H_{60}N_2O_6$: C, 69.50; H, 10.00; N, 4.63. Found: C, 69.44; H, 10.15; N, 4.56; ESI MS *m/z* calcd for $C_{35}H_{60}N_2O_6Na$: $(M + Na)^+$, 627.44; found: $(M + Na)^+$, 627.50, $([M - C_5H_8O_2] + H)^+$, 505.50.

3.2.7. Succinimidyl-Lys^{inv}(Cbz)-$OC_{16}H_{33}$ (10). Compound **9** (1.0 g, 1.7 mmol) was dissolved in TFA/DCM 1:1 v/v (20 mL) and stirred for 1 h at room temperature. The reaction mixture was evaporated under reduced pressure and coevaporated with DCM (3 × 20 mL) to remove any residual TFA. The resulting oil was dissolved in $CHCl_3$/THF 1:1 v/v (20 mL). This solution was neutralized with TEA and subsequently, TEA (350 μL, 2.5 mmol, 1.5 equiv) followed by succinic anhydride (250 mg, 2.5 mmol, 1.5 equiv) were added. After stirring for 16 h at room temperature, the reaction mixture was evaporated in vacuo. The residue was acidified with 1 N $KHSO_4$ and extracted into EtOAc (3 × 20 mL). The organic layer was washed with 1 N $KHSO_4$ and brine (twice 50 mL), dried (Na_2SO_4), filtered, and subsequently evaporated at reduced pressure. The residue was crystallized from aceton in 78% yield (782 mg). R_f (DCM/MeOH 98:2 v/v): 0.06, R_f (DCM/MeOH 94:6 v/v): 0.32; mp: 98–101 °C; $[\alpha]_D$ +36.1 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 0.88$ (t, 3H), 1.25 (br s, 26H), 1.39–1.91 (m, 8H), 2.54–2.92 (m, 4H), 3.16 (m, 2H), 4.12 (t, 2H), 4.63 (m, 1H), 5.09/5.14 (dd, 5H), 6.35 (d, 1H), 6.54 (m, 1H), 7.35 (s, 5H); ^{13}C NMR ($CDCl_3$): $\delta = 14.1$, 21.6, 22.1, 22.6, 25.8, 28.4, 29.1, 29.3, 29.5, 29.5, 29.6, 29.6, 30.6, 31.3, 31.9, 32.4, 40.6, 40.6, 51.7, 52.0, 65.7, 67.0, 67.3, 127.9, 128.0, 128.1, 128.5, 136.0, 136.4, 156.7, 158.5, 171.8, 172.5, 176.0, 176.8. Anal. calcd for $C_{34}H_{56}N_2O_7$: C, 67.52; H, 9.33; N, 4.63. Found: C, 67.34; H, 9.38; N, 4.52; ESI MS *m/z* calcd for $C_{34}H_{57}N_2O_7$: $(M + H)^+$, 605.42; found: $(M + H)^+$, 605.55, $(M + Na)^+$: 627.55.

3.2.8. Succinimidyl-Lys^{inv}(Fmoc)-$OC_{16}H_{33}$ (11). Compound **10** (410 mg, 0.7 mmol) was dissolved in THF (20 mL). The benzyloxycarbonyl functionality was removed by palladium on activated carbon (10% Pd) in a hydrogen atmosphere. After 2 h, the reaction mixture was filtered over Hyflo and the filtrate was evaporated to dryness. The residue was suspended in CH_3CN/H_2O 1:1 v/v (30 mL) and DIPEA was added to obtain a pH value of 8.5–9. Fmoc-ONSu (229 mg, 0.7 mmol) dissolved in CH_3CN was added in one portion and the pH was adjusted to pH 8 by adding DIPEA. After stirring for 2 h the reaction mixture became almost clear and 1 N $KHSO_4$ was added until a pH of 2 was reached. The turbid reaction mixture was extracted with EtOAc (3 × 20 mL) and the organic layer was washed with 1 N $KHSO_4$ and brine (twice 20 mL), dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was crystallized from aceton in 66% yield (312 mg). R_f (DCM/MeOH 98:2 v/v): 0.05, R_f (DCM/MeOH 94:6 v/v): 0.34; mp: 110–113 °C; $[\alpha]_D$ +32.1 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$): $\delta = 0.88$ (t, 3H), 1.25 (br s, 26H), 1.38–1.64 (br m, 7H), 1.90 (m, 1H), 2.37–2.97 (br m, 4H), 3.15 (m, 2H), 4.13 (dd, 2H), 4.22 (m, 1H), 4.41 (dd, 2H), 4.58–4.67 (dm, 1H), 5.17/6.48 (dm, 1H), 6.35/6.38–6.54/6.56 (dd, 1H), 7.31–7.40 (dt, 4H), 7.56–7.77 (dd, 4H); ^{13}C NMR ($CDCl_3$): $\delta = 14.1$, 21.5, 22.1, 22.7, 25.8, 28.2, 28.5, 29.2, 29.3, 29.5, 29.6, 29.6, 29.7, 30.8, 31.41, 31.9, 32.5, 40.6, 47.0, 51.6, 52.0, 65.7, 66.7, 67.5, 112.0, 125.0, 127.0, 127.7, 141.3, 143.9, 158.6, 171.8, 172.5, 176.8. Anal. calcd for $C_{41}H_{60}N_2O_7$: C, 71.07; H, 8.73; N, 4.04. Found: C, 70.85; H, 8.68; N, 3.95; ESI MS *m/z* calcd for $C_{41}H_{61}N_2O_7$: $(M + H)^+$, 693.45; found: $(M + H)^+$, 693.60, $(M + Na)^+$, 715.60.

3.3. Peptide purification and peptide characterization

HPLC runs were performed on a Gilson HPLC workstation. The crude peptides (30–100 mg) were dissolved in a minimum amount of 0.1% TFA in H_2O and loaded on a preparative HPLC column (Alltech Adsorbosphere™XL C4, 10 μm particle size, 300 Å pore size, *l*: 250 mm, id: 22 mm). The peptides were eluted with a flow rate of 10.0 mL/min using a linear gradient of buffer B (20–90% in 80 min) in buffer A (buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in CH_3CN/H_2O 8:2 v/v). Peptide purity was analyzed by analytical HPLC on an Alltech Adsorbosphere™XL C4 (5 μm particle size, 300 Å pore size, *l*: 250 mm, id: 4.6 mm) column and on a Merck LiChroSpher CN (5 μm particle size, 100 Å pore size, *l*: 250 mm, id: 4.6 mm) column at a flow rate of 1.0 mL/min using a linear gradient of buffer B (20–90% in 40 min) in buffer A (buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN/H_2O 8:2 v/v). Purity was determined to be 95% or higher. The peptides were characterized by mass spectrometry. Electrospray ionization mass spectrometry (ESI MS) and LC ESI MS was performed on a Shimadzu LC MS-QP8000 single quadrupole bench-top mass spectrometer operating in a position ion mode (deflector voltage was set on 50 V, which produces predominantly $(M + nH)^{n+}$ ions with little evidence of fragmentation for this type of peptides). Samples (typically 30 μL) were injected into a moving solvent (200 μL/min; 0.1% TFA in H_2O) using Shimadzu LC-10AD HPLC pumps and loaded onto an Alltech Adsorbosphere™XL C4 (5 μm particle size, 300 Å pore

size, *l*: 150 mm, id: 2.1 mm) column using a linear gradient of acetronitrile (0–90%) in 0.1% TFA in H_2O over 30 min. The mass of each analog was measured and the observed $(M + nH)^{n+}$ values were correlated with the calculated average $(M + nH)^{n+}$ values using MacBioSpec (Sciex Instruments, Thornhill, Ontario, Canada).

3.4. Biological testing

Rat CRF antagonistic activity (expressed as pA_2 values) was assayed by measuring the reduced induction of β-galactosidase in response to rat CRF in a stable CRF receptor 1, or receptor 2α expressing LVIP2.0Zc cell line that contain an exogenous cAMP-responsive β-galactosidase receptor gene product.[29] Formation of cAMP was stimulated with rat CRF (rCRFR1: 1 nM; rCRFR2α: 10 nM) for 3 h. The increase of cAMP resulted in an increase in the production of β-galactosidase capable of hydrolysis of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside resulting in a yellow color that was measured at 405 nm.[30] Antagonistic activity can be assessed after a 30 min preincubation with putative antagonists and subsequent incubation with CRF for 3 h (rCRFR1: 1 nM; rCRFR2α: 10 nM). Antagonistic potency is defined as the concentration of antagonist at which the agonist concentration needs to be doubled to arrive at the same effect as in the absence of antagonist. This pA_2 value was calculated from a Schild analysis. Schild analysis was done by measuring CRF-induced β-galactosidase expression in absence or presence of three different antagonist-peptide concentrations (10^{-5}–10^{-8} M). Antagonist-induced right-ward shifts of CRF-stimulation is a measure of antagonist potency and can be calculated as $pA_2 = \log(DR - 1) - \log[\text{antagonist}]$; where DR (dose ratio) is defined as the ratio between the EC_{50} of CRF (pEC_{50} [CRFR1]: 9.4; pEC_{50} [CRFR2α]: 8.9) in presence of a specified antagonist concentration, compared with control EC_{50} values. The given pA_2 values represent the mean value of six independent determinations.

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