Structure – Activity Studies on the Corticotropin Releasing Factor Antagonist Astressin, leading to a Minimal Sequence necessary for Antagonistic Activity

Dirk T. S. Rijkers,[a] John A. W. Kruijtzer,[a] Marja van Oostenbrugge,[b] Eric Ronken,[b] Jack A. J. den Hartog,[b] and Rob M. J. Liskamp*[a]

Corticotropin Releasing Factor (CRF) antagonists are considered promising for treatment of stress-related illnesses such as major depression and anxiety-related disorders. We report here the design, synthesis and biological evaluation of 91 truncated astressin analogues in order to deduce the pharmacophoric amino acid residues. Such truncated peptides may serve as valuable lead structures for the development of new small, non-peptide-based CRF antagonists. N-Terminal truncation of astressin led to active CRF antagonists that are substantially reduced in size and are selectively active at the human CRF receptor type 1 in vitro and in vivo. Subsequently, an alanine scan in combination with further truncated derivatives led to the proposal of a new pharmacophoric model of peptide-based CRF antagonists. It was found that the astressin(27 – 41)C sequence is the shortest active CRF antagonist. The first eight N-terminal amino acid residues were found to be an important structural determinant and were replaceable by alanine residues, thus enhancing the α-helical propensity. A covalent structural constraint is of utmost importance for the preorganization of the C-terminal amino acid residues. The C-terminal heptapeptide sequence, however, was found to be crucial for the antagonistic activity, since substitution or deletion of any residue led to inactive compounds.

Introduction

Corticotropin releasing factor (CRF) is a linear peptide amide consisting of 41 amino acid residues. CRF is synthesized in the hypothalamus and stimulates the release of adrenocorticotropic hormone (ACTH), β-endorphin and other proopiomelanocortin-derived (POMC-derived) peptides from the anterior pituitary gland.[1] Although the existence of CRF was postulated independently by Guillemin and Rosenberg[2a] and by Saffran and Schally,[2b] it took more than 25 years before it could be isolated from sheep hypothalami.[3] After its isolation, ovine CRF was sequenced and chemically synthesized,[3] subsequently followed by the identification of rat CRF[4] and human CRF[5] in 1983.

Recent clinical data suggest that CRF may be involved in endocrine illnesses such as feeding disorders[6] and in neurological and psychiatric illnesses[1] such as major depression[7] and anxiety-related disorders.[8] To obtain more insight into the physiological role of CRF, potent peptide antagonists were developed, mainly based on the amino acid sequence of CRF (e.g. α-helical CRF).[9] Further structure – activity relationship (SAR) studies[10] and a lactam bridge scan[10e] incorporating glutamic acid/lysine residues on position [*i*, *i*+3] ultimately led to the discovery of astressin (cyclo(30 – 33)[D-Phe12, Nle21,38, Glu30, Lys33]hCRF(12 – 41)),[10e, 11] the most potent peptide-based CRF antagonist so far described.

Corticotropin releasing factor binds to the CRF receptor family of seven transmembrane (G-protein coupled) receptors.[12] Molecular cloning studies indicate that at least two major classes of CRF receptor subtypes exist: CRF-R1[13] and CRF-R2.[14] These receptors differ in their sequences, tissue distribution and pharmacological profiles. The key CRF receptor involved in the release of ACTH and other POMC-derived peptides is thought to be of type 1. The receptor subtype mediating the role of CRF as the physiological regulator of stress is not yet known. Therefore, the development of antagonists for both CRF receptor subtypes is of great interest to the pharmaceutical industry as a potential treatment for anxiety-related disorders and depression. Several classes of small-molecule, non-peptide CRF antagonists showing high selectivity towards the CRF-R1 receptor have been developed.[15] Unfortunately, these classes of molecules are all closely related, and in addition, share a number of unfavorable properties, such as low solubility and low membrane permeability, and their narrow structure – activity relationship gives little room for optimization. Therefore, completely new lead structures would be very welcome.[16] Although peptides in

[a] *Dr. Ir. D. T. S. Rijkers, Dr. Ir. J. A. W. Kruijtzer, Prof. Dr. R. M. J. Liskamp*
Department of Medicinal Chemistry
Utrecht Institute for Pharmaceutical Sciences
Faculty of Pharmaceutical Sciences, Utrecht University
P.O. Box 80082, 3508 TB Utrecht (The Netherlands)
Fax: (+31) 30-253-6655
E-mail: r.m.j.liskamp@pharm.uu.nl

[b] *M. van Oostenbrugge, Dr. E. Ronken, Dr. J. A. J. den Hartog*
Solvay Pharmaceuticals, Research Laboratories
P.O. Box, 1380 DA Weesp (The Netherlands)

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general cannot be used as CNS drugs, due to their low bioavailability and high biodegradability, they may be valuable tools for deducing pharmacophoric information.

It was hypothesized that the high activity of astressin stems from the incorporation of an α-helical constraint by lactamization of the side chains of glutamic acid30 and lysine33.[11] Since the lactam bridge is an ideal starting point as a constraint in the design of peptidomimetics, we synthesized a series of truncated astressin analogues to obtain more insight into its pharmacophoric amino acid residues. This knowledge can in principle be used in the design of small-molecule peptidomimetics as CRF antagonists in which the pharmacophoric amino acid residues are scaffolded to mimic the bioactive conformation.[17]

In this report, astressin has been C-terminally, N-terminally, C/N-terminally and centrally truncated to arrive at smaller biologically active CRF antagonistic peptides. The deletion studies resulted in two astressin analogues that were significantly reduced in size but still retained CRF-R1 antagonistic activity both in vitro and in vivo. Furthermore, these two truncated peptides were subjected to an alanine scan and an additional series of centrally truncated peptides was synthesized in order to arrive at the smallest potentially active peptide-based CRF antagonist.

It has been found that the cyclic peptide astressin(27 – 41) is the smallest active (peptide-based) CRF antagonist, in which all amino acid residues are required for biological activity. Our studies described here have made it clear that this astressin derivative presumably adopts a stretched α-helical conformation when it interacts with the receptor. This implies that alternative scaffolding of the pharmacophoric amino acid residues in a β-turn-like conformation is not a feasible option to arrive at peptidomimetics that are active as CRF antagonists.

Results and Discussion

Chemistry

The synthetic methodology was designed to give access to a large diversity of astressin-derived peptides in order to elucidate the pharmacophoric amino acid residues.[18] Automated stepwise solid-phase chemistry incorporating the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group for N^{α}-amino protection, together with ArgoGel™ Rink-NH-Fmoc resin,[19] was used to assemble the linear sequences of astressin and congeners. Coupling reactions were mediated by HBTU/HOBt in the presence of DIPEA in NMP.[20] The linear synthesis of the peptides proceeded without any difficulties, and the crude peptides were obtained after cleavage and deprotection by TFA in excellent yields and high purities. Allyl and allyloxycarbonyl side-chain-protected glutamic acid and lysine derivatives[21] were incorporated at different positions of the astressin sequence, so that their orthogonal protection scheme would define the final position of the lactam bridge in the target cyclic peptide.

Deprotection of the allyl functionalities was performed as described by Kates et al.[22] with $Pd^0(Ph_3P)_4$ in $CHCl_3$/AcOH/NMM. Completion of the allyl removal was found to be strongly dependent on the length of the peptide, the nature of the amino acid residues and their TFA-labile protection groups, and the quality of the Pd^0 catalyst. Extending the deprotection time was found to be detrimental, since acetylated lysine residues were observed, due to the formation of allyl acetate from acetic acid and the deprotected amino group of lysine as a nucleophile in Pd-catalyzed deallylation. MS analysis indeed showed an additional compound with a mass 42 amu higher than the linear peptide ($\Delta = 60$ amu in relation to a cyclic peptide). FAB-MS-MS sequencing of the peptide additionally proved the formation of an acetylated lysine residue. This unwanted acetylation was circumvented by using phenylsilane[23] in the presence of $Pd^0(Ph_3P)_4$ in DCE, the allyl groups being smoothly removed, independent of peptide length and composition, without any other side reactions. Allyl deprotection was monitored by the Kaiser test.[24] In order to ensure that all allyl protecting groups had been removed, an aliquot of resin was treated with TFA and analyzed by HPLC and ESI-MS. Generally, removal of allyl protecting groups was complete after 2 h.

Ring closure leading to the lactam bridge was achieved with BOP/HOBt in the presence of excess DIPEA. The reaction as monitored by the Kaiser test, was complete within 16 hours. As anticipated from the low loading, favoring pseudodilution,[25] no dimers were detected either by LC-ESI-MS or FAB-MS.

The peptides were cleaved from the resin and deprotected by treatment with a TFA/H_2O/EDT/TIS mixture. Other cleavage mixtures led to unsatisfactory yields and purities. Typical yields of astressin and derivatives varied between 58 – 72%. After HPLC purification the peptides were obtained in 36 – 42% yields. Their identities and purities (> 95%) were verified by HPLC, LC-ESI-MS and FAB-MS. The peptides were stored as dry lyophilizates at 4 °C prior to biological testing.

Rationale for design and biological evaluation

C-Terminally (**3** – **13**), N-terminally (**14** – **32**) and C/N-terminally (**33** – **42**) truncated astressin derivatives were synthesized to unravel the importance of the C and N termini. To probe the possibility of a discontinuous interaction site in which amino acid sequences in both the C and the N termini of astressin interact with the receptor, a centrally truncated series (**43** – **49**) was synthesized. Both cyclic and linear derivatives were synthesized when residues glutamic acid30 and lysine33 were present. These derivatives were used to probe receptor affinity and selectivity (vide infra). The compounds (**1** – **49**) were screened for CRF-R1 and CRF-R2 antagonistic activity and affinity, and the biological data are summarized in Table 1.

As can be seen from Table 1, the cyclic constraint between the side chains of glutamic acid30 and lysine33 was found to be of utmost importance for CRF-R1 interaction, since the linear form of astressin (**1**) was almost 200 times less active than cyclic astressin (**2**). Furthermore, the C-terminal deletion study (**3** – **13**) revealed the importance of the isoleucine41 residue for maintenance of CRF antagonistic activity. C-Terminal deletion of only the Ile41 residue, as in **11**, resulted in a CRF-R1 antagonistic activity 200 times lower than in **2** or its equally active N-terminally acetylated full-length analogue **13**.

Table 1. Astressin derivatives, with their corresponding molecular masses, binding affinities and antagonistic potencies towards human CRF-R1.

Compound	Name[a]	Amino acid sequence[b]	Molecular mass found (calcd)	pA_2	pK_i
1	astressin L	fHLLREVLEBARAEQLAQEAHKNRKLBEII	3580.48 (3580.74)	6.85	7.77
2	astressin C	fHLLREVLEBARAEQLAQE*AHK*NRKLBEII	3562.73 (3562.05)	9.12	9.20
3	12 – 15	fHLL	570.30 (570.41)	< 5	< 5
4	12 – 20	fHLLREVLE	1196.60 (1196.68)	< 5	< 5
5	12 – 25	fHLLREVLEBARAE	1737.40 (1736.98)	6.62	6.90
6	12 – 26	fHLLREVLEBARAEQ	1865.16 (1865.04)	< 5	< 5
7	12 – 30	fHLLREVLEBARAEQLAQE	2306.90 (2306.26)	< 5	< 5
8	12 – 35L	fHLLREVLEBARAEQLAQEAHKNR	2912.97 (2912.60)	6.04	5.50
9	12 – 35C	fHLLREVLEBARAEQLAQE*AHK*NR	2894.87 (2894.59)	< 5	5.10
10	12 – 40L	fHLLREVLEBARAEQLAQEAHKNRKLBEI	3509.56 (3508.99)	< 5	5.50
11	12 – 40C	fHLLREVLEBARAEQLAQE*AHK*NRKLBEI	3491.64 (3490.98)	6.79	7.66
12	12 – 41L	fHLLREVLEBARAEQLAQEAHKNRKLBEII	3622.83 (3622.07)	6.87	6.40
13	12 – 41C	fHLLREVLEBARAEQLAQE*AHK*NRKLBEII	3604.82 (3604.06)	8.39	7.52
14	36 – 41	KLBEII	769.50 (769.52)	< 5	< 5
15	31 – 41	AHKNRKLBEII	1375.80 (1375.85)	< 5	5.10
16	30 – 41C	E*AHK*NRKLBEII	1486.33 (1486.89)	6.73	–
17	29 – 41C	QE*AHK*NRKLBEII	1614.27 (1614.94)	6.26	–
18	28 – 41C	AQE*AHK*NRKLBEII	1685.48 (1685.98)	6.47	–
19	27 – 41L	LAQEAHKNRKLBEII	1817.00 (1817.08)	< 5	5.40
20	27 – 41C	LAQE*AHK*NRKLBEII	1798.80 (1799.07)	7.40	8.56
21	26 – 41L	QLAQEAHKNRKLBEII	1945.20 (1945.13)	5.38	6.50
22	26 – 41C	QLAQE*AHK*NRKLBEII	1927.20 (1927.12)	7.00	8.38
23	25 – 41C	EQLAQE*AHK*NRKLBEII	2056.50 (2056.17)	6.00	–
24	24 – 41C	AEQLAQE*AHK*NRKLBEII	2127.65 (2127.20)	6.00	–
25	23 – 41C	RAEQLAQE*AHK*NRKLBEII	2282.72 (2283.30)	6.80	–
26	22 – 41C	ARAEQLAQE*AHK*NRKLBEII	2354.34 (2354.34)	6.53	–
27	21 – 41L	BARAEQLAQEAHKNRKLBEII	2485.40 (2485.44)	< 5	6.00
28	21 – 41C	BARAEQLAQE*AHK*NRKLBEII	2467.23 (2467.43)	7.75	8.38
29	16 – 41L	REVLEBARAEQLAQEAHKNRKLBEII	3112.28 (3111.78)	< 5	5.60
30	16 – 41C	REVLEBARAEQLAQE*AHK*NRKLBEII	3093.48 (3093.77)	5.97	7.85
31	13 – 41L	HLLREVLEBARAEQLAQEAHKNRKLBEII	3478.18 (3475.00)	< 5	6.00
32	13 – 41C	HLLREVLEBARAEQLAQE*AHK*NRKLBEII	3456.93 (3456.99)	7.76	9.10
33	24 – 29	AEQLAQ	700.40 (700.36)	< 5	< 5
34	22 – 31	ARAEQLAQEA	1127.70 (1127.58)	< 5	< 5
35	20 – 33L	EBARAEQLAQEAHK	1634.35 (1634.86)	< 5	< 5
36	20 – 33C	EBARAEQLAQE*AHK*	1617.08 (1616.85)	< 5	< 5
37	18 – 35L	VLEBARAEQLAQE*AHK*NR	2119.85 (2117.16)	< 5	< 5
38	18 – 35C	VLEBARAEQLAQE*AHK*NR	2099.25(2099.15)	< 5	6.00
39	16 – 37L	REVLEBARAEQLAQE*AHK*NRKL	2643.18 (2643.48)	< 5	< 5
40	16 – 37C	REVLEBARAEQLAQE*AHK*NRKL	2625.33 (2625.47)	< 5	6.50
41	14 – 39L	LLREVLEBARAEQLAQE*AHK*NRKLBE	3111.76 (3111.78)	< 5	< 5
42	14 – 39C	LLREVLEBARAEQLAQE*AHK*NRKLBE	3093.16 (3093.77)	< 5	5.00
43	des(15 – 38)	fHLEII	812.40 (812.47)	< 5	< 5
44	des(17 – 36)	fHLLRLBEII	1307.78 (1307.82)	< 5	< 5
45	des(19 – 34)	fHLLREVRKLBEII	1820.08 (1820.13)	< 5	5.50
46	des(21 – 32)	fHLLREVLEKNRKLBEII	2304.43 (2304.39)	< 5	5.50
47	des(23 – 30)	fHLLREVLEBAAHKNRKLBEII	2696.56 (2696.61)	< 5	5.20
48	des(25 – 28)L	fHLLREVLEBARAQEAHKNRKLBEII	3180.69 (3180.85)	< 5	5.40
49	des(25 – 28)C	fHLLREVLEBARAQE*AHK*NRKLBEII	3162.81 (3162.84)	6.87	7.77
50	30 – 41C I41A	E*AHK*NRKLBEIA	1444.70 (1444.84)	< 5	< 5
51	30 – 41C I40A	E*AHK*NRKLBEAI	1445.28 (1444.84)	< 5	< 5
52	30 – 41C E39A	E*AHK*NRKLBAII	1428.98 (1428.88)	< 5	< 5
53	30 – 41C B38A	E*AHK*NRKLAEII	1444.93 (1444.84)	< 5	< 5
54	30 – 41C L37A	E*AHK*NRKABEII	1444.95 (1444.84)	< 5	< 5
55	30 – 41C K36A	E*AHK*NRALBEII	1430.00 (1429.83)	6.18	
56	30 – 41C R35A	E*AHK*NAKLBEII	1401.80 (1401.82)	< 5	< 5
57	30 – 41C N34A	E*AHK*ARKLBEII	1443.78 (1443.88)		
58	30 – 41L K33A	EAHANRKLBEII	1447.85 (1447.84)	< 5	< 5
59	30 – 41C H32A	E*AAK*NRKLBEII	1420.98 (1420.86)	6.51	
60	30 – 41L E30A	AAHKNRKLBEII	1446.89 (1420.86)	< 5	< 5
61	27 – 41C I41A	LAQE*AHK*NRKLBEIA	1756.93 (1757.02)	< 5	< 5
62	27 – 41C I40A	LAQE*AHK*NRKLBEAI	1756.88 (1757.02)	6.66	
63	27 – 41C E39A	LAQE*AHK*NRKLBAII	1741.48 (1741.06)	7.23	
64	27 – 41C B38A	LAQE*AHK*NRKLAEII	1756.83 (1757.02)	< 5	< 5
65	27 – 41C L37A	LAQE*AHK*NRKABEII	1757.83 (1757.02)	< 5	< 5
66	27 – 41C K36A	LAQE*AHK*NRALBEII	1742.40 (1742.01)	< 5	< 5

Table 1 *(continued).*

Compound	Name[a]	Amino acid sequence[b]	Molecular mass found (calcd)	pA_2	pK_i
67	27 – 41C R35A	LAQE*AHK*NAKLBEII	1714.23 (1714.00)	< 5	< 5
68	27 – 41C N34A	LAQE*AHK*ARKLBEII	1758.15 (1756.06)	7.49	
69	27 – 41L K33A	LAQEAHANRKLBEII	1760.05 (1760.02)	< 5	< 5
70	27 – 41C H32A	LAQE*AAK*NRKLBEII	1733.10 (1733.04)	7.07	
71	27 – 41L E30A	LAQAAHKNRKLBEII	1758.80 (1759.07)	< 5	< 5
72	27 – 41C Q29A	LAAE*AHK*NRKLBEII	1741.88 (1742.04)	7.20	
73	27 – 41C L27A	AAQE*AHK*NRKLBEII	1757.00 (1757.02)	6.87	
74	30 – 35C	E*AHK*NR	777.40 (777.41)	< 5	< 5
75	30 – 41C des(36 – 39)	E*AHK*NRII	1003.58 (1003.58)	< 5	< 5
76	30 – 41C des(36 – 38)	E*AHK*NREII	1132.60 (1132.62)	< 5	< 5
77	30 – 41C des(37 – 39)	E*AHK*NRKII	1131.73 (1131.68)	< 5	< 5
78	30 – 41C des(37 – 38)	E*AHK*NRKEII	1260.93 (1260.72)	< 5	< 5
79	30 – 41C des38	E*AHK*NRKLEII	1374.15 (1373.80)	< 5	< 5
80	30 – 41C K36X des(37 – 39)	E*AHK*NRXII	1116.63 (1116.66)	< 5	< 5
81	30 – 41C K36G,L37G,B38G,E39G	E*AHK*NRGGGGII	1231.73 (1231.67)	< 5	< 5
82	34 – 41 cyclo(34 – 39)	*NRKLBE*II	979.70 (979.65)	< 5	< 5
83	34 – 41 cyclo(36 – 39)	NRK*LBE*II	1021.80 (1021.65)	< 5	< 5
84	30 – 41 cyclo(36 – 39)	EAHKNRK*LBE*II	1487.25 (1486.89)	< 5	< 5
85	30 – 41C K36L,B38L,I40E,I41A	E*AHK*NRLLLEEA	1446.05 (1445.79)	< 5	< 5
86	30 – 41 bicyclo(30 – 33,36 – 39)	E*AHK*NRK#LBE#II	1469.18 (1468.87)	< 5	< 5
87	30 – 41C N34n	E*AHK*nRKLBEII	1487.13 (1486.89)	< 5	< 5
88	30 – 41C R35r	E*AHK*NrKLBEII	1487.10 (1486.89)	< 5	< 5
89	30 – 41C R35G	E*AHK*NGKLBEII	1388.25 (1387.81)	< 5	< 5
90	30 – 41C N34n,R35G	E*AHK*nGKLBEII	1388.43 (1387.81)	< 5	< 5
91	30 – 41C N34p,R35G	E*AHK*pGKLBEII	1371.15 (1370.82)	< 5	< 5

[a] L and C denotes the linear and the cyclic forms of the molecule, respectively. [b] Each peptide is amidated at its C terminus and has an acetylated N terminus, except for compounds **1** and **2**, which each have a free N terminus; the amino acids marked with asterisks form the cyclic lactam moiety; amino acid residues (of the L configuration) are given as their one-letter codes; f: D-phenylalanine, n: D-asparagine, r: D-arginine, p: D-proline, B: L-norleucine (Nle), X: 6-aminohexanoic acid (Ahx). [c] The molecular masses are given as the monoisotopic values. [d] The pA_2 and pK_i values are mean values of six and three independent determinations, respectively.

In line with this observation, truncation on both termini of astressin resulted in inactive derivatives (**33** – **42**). Central truncation of astressin also proved to be deleterious for the biological activity (**43** – **49**). The importance of the C terminus of CRF had already been evidenced in studies by Vale et al.,[3] who found that modification of the amide functionality into a carboxylic acid or ester nullified the agonistic activity of CRF.[3] These studies and our data presented here make clear that an amidated isoleucine41 residue is obligatory. This was considerably different from observations on, for example, parathyroid hormone (PTH), in which the N-terminal 34-mer was found to constitute the functionally active domain of the 84-amino acid protein PTH.[26]

Noteworthy—and most surprising—was the fact that when astressin (**2**) was shortened from its N terminus (**14** – **32**) the CRF-R1 antagonistic activity sharply decreased, but was restored—although not regaining the activity of full-length astressin—if the peptide was further shortened by 16 to 19 amino acid residues. These deletion studies ultimately resulted in two peptides: astressin(30 – 41)C (**16**) and astressin(27 – 41)C (**20**), which were substantially smaller in size than astressin but retained CRF-R1 antagonistic activity. The cyclic constraint in these smaller peptides was found to be even more important for the biological activity than was the case for full-length astressin.

Generally, we found that the truncated peptides were inactive towards the CRF-R2α receptor. This was somewhat unexpected since astressin was found to bind CRF-R2 with almost equal potency (pA_2: 9.2; pK_i: 8.1 measured on human CRF-R2α). In a recent study in which a selective peptide CRF-R2 antagonist was described, it was shown that incorporation of a covalent α-helical constraint in a CRF-R2-selective antagonist had a deleterious effect on its activity.[27] From these literature data and from our own results it can be concluded that the peptide pharmacophores for the CRF receptor type 1 and type 2 are significantly different. On one hand, CRF-R1 is found to bind small peptide ligands under the strict condition that these peptide ligands are cyclized. On the other hand, CRF-R2 is found to bind longer linear peptide ligands, and affinity towards CRF-R2 is lost if a cyclic constraint is incorporated. Thus, N-terminal truncation was a feasible approach in the development of selective CRF-R1 peptide-based antagonists.

The truncated astressin analogues **16** and **20** were tested for their potency in inhibiting ACTH release in vivo (Table 2). It was found that, at a dose of 1 mg kg^{-1} intravenously, **20** is still active but **16** is almost inactive. To estimate the relative potency of **20**, the dose of astressin (**2**) was reduced to arrive at an equal inhibition of ACTH release and this was found to be 0.03 mg kg^{-1}. With regard to receptor selectivity, it must be stressed that current clinical data show that CRF-R1 mediates the ACTH release and the resulting regulation of stressful stimuli. Furthermore, **20** is not expected to displace CRF from its binding protein, since the Ala22 – Arg23 – Ala24 sequence—generally

*Table 2. In vivo ACTH-release inhibition by astressin (**2**) and by its truncated analogues 27–41C (**20**) and 30–41C (**16**).[a]*

Compound	Amino acid sequence	Concentration mg kg^{-1} i.v.	Inhibition of ACTH release [%]
2	fHLLREVLEBARAEQLAQE* AHK*NRKLBEII	1.0	100
2		0.1	100
2		0.03	40
2		0.01	<10
16	E*AHK*NRKLBEII	1.0	<10
20	LAQE*AHK*NRKLBEII	1.0	44

accepted as responsible for interaction with CRF binding protein—is absent.[28]

Although the truncated astressin analogues **16** and **20** were not as active as full length astressin, both astressin derivatives were subjected to an alanine scan (compounds **50**–**60** and **61**–**73**, Table 1) to obtain insight into the importance of each individual amino acid residue for CRF antagonistic activity. These results could be used for a further truncation of the peptide chain or for the incorporation of amino acid residues to ensure a secondary structural element such as an α-helix or a β-turn (vide infra). Furthermore, a series with stepwise internal deletion of the amino acid residues between the arginine35 and the isoleucine41 residues was also synthesized (compounds **74**–**81**). Conservation of Arg35 and Ile41 was required since the importance of the cyclic constraint and the C-terminal isoleucine41 for the CRF antagonistic activity has already been shown in this study and in studies in the literature. Incorporation of a cyclic constraint into linear astressin analogues strongly potentiates the CRF-R1 antagonistic activity (**2** versus **1** and **20** versus **19**). One option to increase the activity of the truncated astressin analogues **20** and **16** is to increase the stabilization of the putative α-helical structure of the C terminus. This concept was tested both by the use of a covalent constraint (compounds **82**–**84**, **86**) and by the incorporation of α-helix-inducing residues, resulting in an astressin/α-helical CRF chimera (**85**). NMR studies on CRF[29] and the Chou–Fasman[30] rules suggest the presence of a β-turn formed by the residues Asn34–Arg35. These two residues are highly conserved in peptides of the CRF family and could function as an important structural (β-turn-inducing) or functional (receptor interaction) feature. If these two residues function as a β-turn-inducing element, substitution by D-amino acid residues or strongly β-turn-inducing residues probably enhance the biological activity by preorganization of the bioactive conformation. Compounds **87**–**91** were therefore synthesized to validate this concept.

Generally, it can be said that all modifications of **16** and **20** (compounds **50**–**91**) resulted in inactive astressin derivatives (Table 1). Mainly N-terminal alanine substitutions were tolerated (Table 3). Although circular dichroism experiments on **16** and **20** showed that both peptides could adopt an α-helix in aqueous trifluoroethanol (Figure 1), the α-helix could not be stabilized by α-helix-inducing amino acid residues since all residues of **16** and **20** were found to be essential for biological activity. These results imply a novel pharmacophoric model for peptide-based CRF antagonists.

Implications for a novel pharmacophoric model

This study has shown that the first 15 N-terminal amino acid residues of astressin can be deleted (resulting in **20**) without significant loss of biological activity either in vitro or in vivo. Further terminal or internal truncation of **20** led to weak or inactive derivatives. Compound **20** thus represents the smallest sequence with CRF antagonistic activity (Scheme 1). Subsequently, the alanine scan showed that the remaining residues differ in their importance for CRF antagonistic activity. This difference led to the definition of either structurally or functionally important amino acid residues (Scheme 2). Structurally important amino acid residues could be replaced by alanine residues to maintain the biologically important α-helical propensity of the molecule. On the other hand, functionally important amino acid residues could not be replaced by alanine residues or by isosterically functionalized amino acid residues. Residues 27 to 34, including the covalent constraint between glutamic acid30 and lysine33 of **20**, comprise the structurally important residues. Its covalent α-helical constraint induces an α-helical conformation in the functionally important sequence from arginine35 to isoleucine41. In spite of the necessity for an α-helical conformation of the functionally important amino acid residues it could not be (covalently) stabilized. The conformation of the C-terminal amino acid residues of CRF is still unknown, since the NMR structure showed it to be random.[29] Presumably, the conformation of the functionally important amino acid

*Table 3. Alanine scan of 27–41C (**20**), 30–41C (**16**) and ovine CRF.[a]*

	C-terminal amino acid residues														
Peptide	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
27–41C (**20**)	Leu	Ala	Gln	cGlu	Ala	His	cLys	Asn	Arg	Lys	Leu	Nle	Glu	Ile	Ile
(antagonist)	+	o	+	−	o	+	−	+	−	−	−	−	+	+	−
30–41C (**16**)				cGlu	Ala	His	cLys	Asn	Arg	Lys	Leu	Nle	Glu	Ile	Ile
(antagonist)				−	o	+	−	+	−	+	−	−	+	+	−
ovine CRF	Leu	Ala	Gln	Gln	Ala	His	Ser	Asn	Arg	Lys	Leu	Leu	Asp	Ile	Ala
(agonist)	−	o	+	+	o	+	+	+	−	+	+	−	+	+	o

[a] Amino acid replacement by an alanine residue is: tolerated (+), native (o), not tolerated (−). The ovine CRF R35A and L38A derivatives are inactive both as agonists and as antagonists.[10a, 10c]

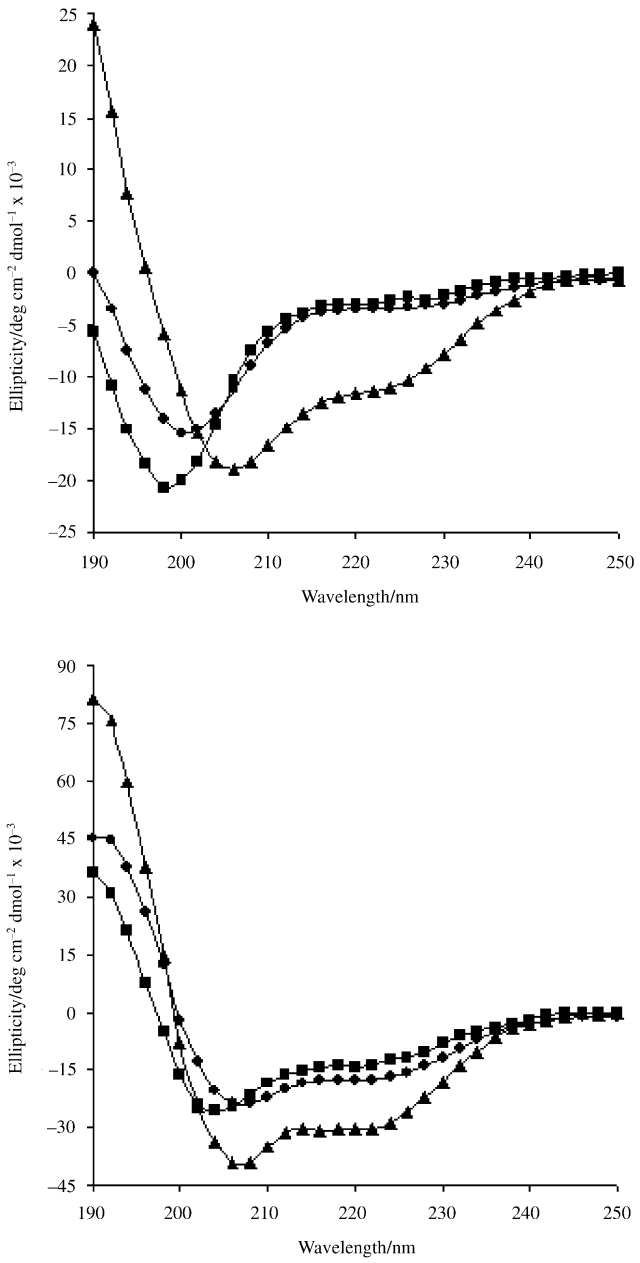


Figure 1. *Circular dichroism spectra of* ***2*** *(▲),* ***16*** *(■) and* ***20*** *(●) in water (A: upper frame) and in 50% aqueous trifluoroethanol (B: lower frame). The peptides were dissolved (1 mg mL^{-1}) in demineralized water and in TFE/H_2O 1:1 v/v at 25 °C. Spectra were measured at 1.0 nm intervals in the 190–250 nm range as the average of 20 runs.*

residues is α-helical rather than β-turn-like, since inserting β-turn-inducing amino acids resulted in inactive compounds.

Finally, scaffolding of the pharmacophoric amino acid residues to mimic the bioactive conformation, as had been successfully shown with somatostatin[17b,c] and melanocortin,[17d] for example, will be difficult in this case since all residual amino acids of the smallest peptide with CRF antagonistic activity are required for biological activity in which a stretched α-helix represents the bioactive conformation of peptide-based CRF antagonists.

Experimental Section

Instruments and methods: The peptides were synthesized automatically either on an Applied Biosystems 433 A Peptide Synthesizer (Foster City, CA, USA) or on a MultiSynTech Syro II Robot Synthesizer (MultiSynTech, Witten, Germany). Analytical and preparative HPLC runs were performed on a Gilson HPLC workstation (Middleton, Wisconsin, USA). Positive-ion fast atom bombardment mass spectrometry was performed with a Jeol JMS SX/SX 102 A four-sector mass spectrometer (Tokyo, Japan). Electrospray ionization mass spectrometry was performed on a Micromass Platform II (Altrincham, United Kingdom) single quadrupole bench-top mass spectrometer operating in a positive ionization mode. Liquid chromatography electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-QP8000 (Duisburg, Germany) single quadrupole bench-top mass spectrometer operating in a positive ionization mode.

Chemicals and reagents: ArgoGel™ Rink-NH-Fmoc resin (Argonaut Technologies, Muttenz, Switzerland) functionalized with a 4-((2′,4′-dimethoxyphenyl)aminomethyl)phenoxyacetamido moiety (Rink amide linker)[19] was used to obtain C-terminal peptide amides. The coupling reagents 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[31] and benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)[32] were obtained from Richelieu Biotechnologies Inc. (Montreal, Canada). *N*-Hydroxybenzotriazole (HOBt) and N$^{\alpha}$-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids were obtained from Advanced ChemTech (Machelen, Belgium). The side chain protecting groups were: All (allyl) for glutamic acid, Alloc (allyloxycarbonyl) for lysine, Boc (*tert*-butyloxycarbonyl) for lysine, *t*Bu for glutamic acid, Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl) for arginine and Trt (trityl) for asparagine, glutamine, and histidine. Fmoc-D-Asn(Trt)-OH, Fmoc-Glu(OAll)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-D-Phe-OH and Fmoc-D-Pro-OH were obtained from Neosystem Laboratoire (Strasbourg, France). Fmoc-D-Arg(Pbf)-OH and ovine corticotropin releasing factor (oCRF) were obtained from Bachem (Bubendorf, Switzerland). Peptide grade dichloromethane (DCM), 1,2-dichloroethane (DCE), *N*-methylpyrrolidone (NMP), trifluoroacetic acid (TFA) and HPLC grade acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Piperidine, *N*,*N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM) and tetrakistriphenylphosphine palladium(0) were obtained from Acros Organics ('s-Hertogenbosch, The Netherlands). Triisopropylsilane (TIS), 1,2-ethanedithiol (EDT) and HPLC grade TFA were obtained from Merck (Amsterdam, The Netherlands). Phenylsilane and 2,2,2-trifluoroethanol were purchased from Aldrich (Zwijndrecht, the Netherlands).

Peptide synthesis: Peptides **1**–**49** were synthesized on an Applied Biosystems 433 A Peptide Synthesizer by the FastMoc protocol on 0.25 mmol scales.[33] Each synthetic cycle consisted of N$^{\alpha}$-Fmoc removal by a 10 min treatment with 20% piperidine in NMP, a 6 min NMP wash, a 45 min coupling step with preactivated Fmoc amino acid (1.0 mmol) in the presence of 2 equiv DIPEA, and a 6 min NMP wash. N$^{\alpha}$-Fmoc amino acids were activated in situ with HBTU/HOBt (1.0 mmol, 0.36 M in NMP) in the presence of DIPEA (2.0 mmol). After the final Fmoc removal the free amine was acetylated with an excess of acetic acid anhydride/DIPEA/HOBt in NMP.

Peptides **50**–**91** were synthesized on a MultiSynTech Syro II robot synthesizer on 0.05 mmol scales in polypropylene reaction tubes. A synthesis cycle consisted of a double treatment with 20% piperidine in NMP (2 mL) for 8 min to remove the Fmoc group, followed by washing of the resin with NMP (5 × 2.5 mL, 2 min). Subsequently, an amino acid solution (0.2 M) in NMP (1 mL) was added, followed by a

Corticotropin Releasing Factor (CRF):[3]

H-Ser[1]-Glu-Glu-Pro-Pro[5]-Ile-Ser-Leu-Asp-Leu[10]-Thr-Phe-His-Leu-Leu[15]-Arg-Glu-Val-Leu-Glu[20]-Met-

Ala-Arg-Ala-Glu[25]-Gln-Leu-Ala-Gln-Gln[30]-Ala-His-Ser-Asn-Arg[35]-Lys-Leu-Met-Glu-Ile[40]-Ile-NH_2

Astressin:[11]

H-**D-Phe**[12]-His-Leu-Leu[15]-Arg-Glu-Val-Leu-Glu[20]-**Nle**-Ala-Arg-Ala-Glu[25]-Gln-

Leu-Ala-Gln-**Glu**[30]-Ala-His-**Lys**[33]-Asn-Arg[35]-Lys-Leu-**Nle**-Glu-Ile[40]-Ile-NH_2

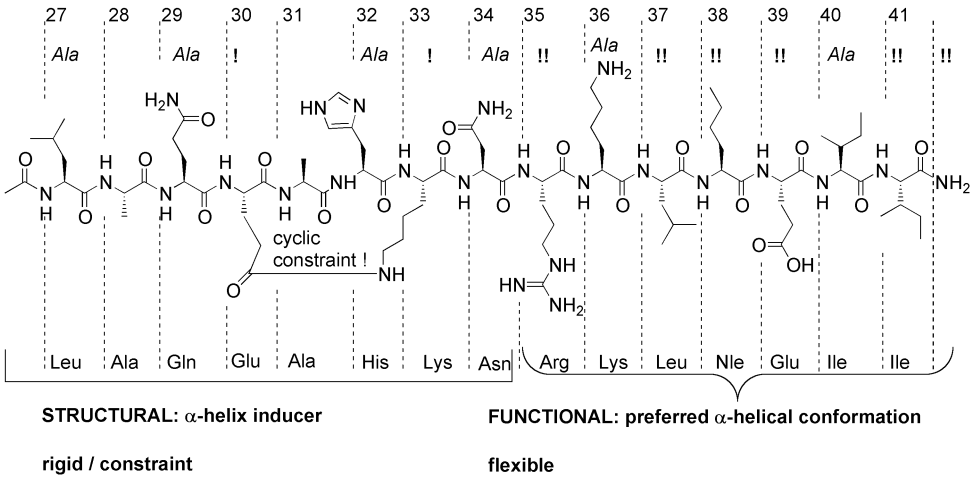
27-41C (**20**):

Ac-Leu[27]-Ala-Gln-Glu[30]-Ala-His-Lys[33]-Asn-Arg[35]-Lys-Leu-Nle-Glu-Ile[40]-Ile-NH_2

30-41C (**16**):

Ac-Glu[30]-Ala-His-Lys[33]-Asn-Arg[35]-Lys-Leu-Nle-Glu-Ile[40]-Ile-NH_2

Scheme 1. *CRF and astressin derivatives.*



Scheme 2. *Pharmacophoric model of peptide-based CRF antagonists. Ala: alanine substitution is allowed. !: important for CRF antagonistic activity. !!: necessary for CRF antagonistic activity.*

solution of HBTU/HOBt (0.267 M) in NMP (0.75 mL) and a solution of DIPEA (0.4 M) in NMP (0.5 mL). This suspension was allowed to react for 45 min. Residual coupling reagents were removed by washing the resin with NMP (5 × 2.5 mL, 2 min). During the resin swelling and washing steps, Fmoc deprotection and coupling reactions, the resin suspension was magnetically mixed by levitation stirring for 15 s per min. After the final Fmoc removal the free N terminus was acetylated by use of a mixture of acetic anhydride (0.50 M)/DIPEA (0.125 M)/HOBt (0.015 M) in NMP (2.2 mL) for 21 min. Finally, the resin was washed with NMP (5 × 2.5 mL, 2 min) and DCE (3 × 2.5 mL, 2 min).

After the linear synthesis was completed, allyl-based protective groups were removed by treatment with $CHCl_3$/acetic acid/NMM[22] or phenylsilane[23] in DCE in the presence of $Pd^0(Ph_3P)_4$ under argon, essentially as described by Kates et al.[22] Ring closure was performed on resin by using BOP/HOBt (3.0 mmol) in the presence of excess DIPEA (9.0 mmol) in NMP. The allyl removal and ring-closure reaction were monitored by the Kaiser test for the presence of free amine functionalities.[24]

The peptides were cleaved from the resin and deprotected by treatment with TFA/H_2O/TIS/EDT 85:8.5:2:4.5 *v/v/v/v* at room temperature for 3 h. The peptides were precipitated with diethyl ether/hexane 1:1 *v/v* at −20 °C. The precipitates were decanted and subsequently washed with cold diethyl ether/hexane 1:1 *v/v* (3 ×) and finally lyophilized from *tert*-BuOH/H_2O 1:1 *v/v*.

Peptide purification: The crude lyophilized peptides (30 – 60 mg) were dissolved in a minimum amount of 0.1% TFA in CH_3CN/H_2O 8:2 *v/v* and loaded onto the HPLC column (Adsorbosphere XL C-18, 300 Å pore size, 10 μm particle size, 2.2 × 25 cm). The peptides were eluted with a flow rate of 10.0 mL min^{-1} with a linear gradient of buffer B (70% in 80 min) from 80% 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 characterization: Peptide purity was analyzed by analytical HPLC on an Adsorbosphere XL C18 column (300 Å pore size, 5 μm particle size, 0.46 × 25 cm) at a flow rate of 1 mL min^{-1} with a linear gradient of buffer B (70% in 40 min) from 80% buffer A (buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN/H_2O 8:2 *v/v*). A second HPLC buffer consisting of buffer C (15 mM TEAP, pH 6.8) and buffer D (15 mM TEAP, pH 6.8/acetonitrile 40:60 *v/v*) was also used to assess peptide purity and was run with a gradient of 100% C to D in 40 min with a flow of 1 mL min^{-1}. The peptides were characterized by mass spectrometry. The mass of each analogue was measured and the observed monoisotopic $[M+H]^+$ values were correlated with the calculated $[M+H]^+$ values by use of MacBioSpec (Perkin Elmer Sciex Instruments, Thornhill, Ontario, Canada).

Functional assay: Human CRF-R1 antagonistic activity (expressed as pA_2 values) was assayed by measuring the reduced induction of β-galactosidase in response to hCRF in a stable hCRF-R1-expressing LVIP2·0Zc cell line containing an exogenous cAMP-responsive β-galactosidase receptor gene product.[34] Formation of cAMP was stimulated with hCRF (10 nM) for 3 h. The increase in cAMP resulted in an increase in the production of β-galactosidase capable of hydrolysis of the chromogenic *o*-nitrophenyl β-D-galactopyranoside substrate, resulting in a yellow color that was measured at 405 nm.[35] Antagonistic activity can be assessed after a 30 min preincubation with putative antagonists and subsequent incubation with hCRF for

3 h. 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 absence of the antagonist. This pA_2 value was calculated by Schild analysis. Schild analysis was performed by measuring CRF-induced β-galactosidase expression in the absence or the presence of three different antagonist/peptide concentrations. Antagonist-induced shifts to the right 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}: 9.4) in the presence of a specified antagonist concentration, compared with control EC_{50} values. The given pA_2 values each represent the mean value of six independent determinations.

Binding assay: The CRF receptor binding affinities (pK_i) of the antagonists were measured by their potency for displacement of ^{125}I-Tyr^0-oCRF from human CRF-R1 in membrane preparations of CC7-cells (CHO cells, stably transfected with the gene expressing the human CRF-R1). Separation of bound and free ligand was performed by filtration over glass-fiber filters essentially as described by Herdon et al.[36] Radioactivity on the filter was measured by liquid scintillation counting. The results are expressed as IC_{50} values and transformed into inhibitory constants (K_i) by Cheng – Prusoff analysis and expressed as pK_i values. The given pK_i values each represent the mean value of three independent determinations.

In vivo ACTH release inhibition assay

Animals: All in vivo experiments were carried out with adult male Wistar rats (200 – 300 g). The animals were housed under approved standard laboratory conditions and given access to unlimited food and water.

Reagents: Ovine CRF was dissolved in acetic acid (0.05 M) and diluted with NaCl (0.15 M) to a final concentration of 5.0 nmol/100 μL. Astressin derivatives (**16** and **20**) were dissolved in a 40% solution of hydroxypropyl-α-cyclodextrine (HPCD) at pH 2.5. Control vehicles consisted of 40% HPCD in NaCl (0.15 M) at pH 2.5.

ACTH assay: The animals were anaesthetized by an intraperitoneal injection of Nembutal® (Sanofi) at a dose of 70 mg kg^{-1}, and cannulation was directed to the left carotid artery. A control blood sample was taken just before the test compound was injected by cannula ($t = -10$ min). A second blood sample was withdrawn ($t = 0$ min), immediately followed by the administration of oCRF (1 nmol kg^{-1}). At $t = 15$ and 30 min the third and fourth blood samples, respectively, were taken. After this the animal was killed with an overdose of Nembutal®. A maximum of 250 μL of whole blood was withdrawn per sample and each blood sample was replaced with 250 μL sterile saline. Blood samples were stored on ice in tubes precoated with EDTA. All samples were centrifuged for 2 min (Beckmann Microfuge) and plasma samples were stored at −20 °C until the assay. ACTH concentrations were measured by a double antibody radio-immunoassay (ICN). If the ACTH concentration in the control sample exceeded 100 pg mL^{-1} or if the astressin derivative increased the ACTH concentration, these samples were then excluded from the calculation and statistical analysis. The inhibition of the ACTH release were measured at $t = 15$ min and statistically analyzed by a unilateral Wilcoxon test. The data each represent the mean value of two independent experiments with four to eight rats.

CD spectra: Circular dichroism spectra were recorded on an OLIS RSM 1000 CD spectrometer. Spectra were measured at 1.0 nm intervals in the 190 – 250 nm range as the average of 20 runs with a spectral bandwidth of 2.0 nm in 0.1 mm cuvettes thermostatted at 25 °C with the optical chamber continually flushed with dry N_2 gas. The spectra were measured in plain distilled water and in TFE/H_2O (1:1, *v/v*). The concentrations (1 mg mL^{-1}) were determined on the basis of the calculated molecular masses of the purified lyophilized peptides.

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