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Constrained Corticotropin-Releasing Factor Antagonists with *i*-(*i* + 3) Glu−Lys Bridges[†]

Antonio Miranda,[‡] Sabine L. Lahrichi, Jozsef Gulyas, Steven C. Koerber, Anthony G. Craig, Anne Corrigan, Catherine Rivier, Wylie Vale, and Jean Rivier*

Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, and Universidade Federal de Sao Paulo, Departamento de Biofisica, Rua 03 de Maio, 100, Sao Paulo, SP 04044-020, Brazil

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Hypothesis driven and systematic structure−activity relationship (SAR) investigations have resulted in the development of effective central nervous system (CNS) antagonists of corticotropin (ACTH)-releasing factor (CRF) such as α-helical $CRF_{(9-41)}$[3] and analogues of our assay standard [$DPhe^{12}$,$Nle^{21,38}$]$hCRF_{(12-41)}$.[4] On the other hand, equally potent CRF antagonists that block the hypothalamic/pituitary/adrenal (HPA) axis had not been described until recently.[5] Predictive methods, physicochemical measurements (nuclear magnetic resonance spectrometry and circular dichroism spectroscopy), and SAR studies suggest that CRF and its family members (urotensins and sauvagine) assume an α-helical conformation when interacting with CRF receptors.[6,7] To further test this hypothesis, we have systematically scanned the $hCRF_{(9-41)}$ or $hCRF_{(12-41)}$ sequences with an *i*−(*i* + 3) bridge consisting of the Glu-Xaa-Xbb-Lys scaffold which we and others had shown could maintain or enhance α-helical structure. From this series we have identified seven analogues that are either equipotent to, or 3 times more potent than, the assay standard; in addition, as presented earlier[5] cyclo(30−33)[$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}]$hCRF_{(12-41)}$ (astressin) is 32 times more potent than the assay standard in blocking ACTH secretion *in vitro* (rat pituitary cell culture assay). *In vivo*, astressin is also significantly more potent than earlier antagonists at reducing hypophysial ACTH secretion in intact stressed or adrenalectomized rats.[5] Since the corresponding linear analogues that were tested are significantly less potent, our interpretation of the increased potency of the cyclic analogues is that the introduction of the side chain to side chain bridging element (Glu^{30}---Lys^{33}, and to a lesser extent that of Glu^{14}---Lys^{17}, Glu^{20}---Lys^{23}, Glu^{23}---Lys^{26}, Glu^{26}---Lys^{29}, Glu^{28}---Lys^{31}, Glu^{29}---Lys^{32}, and Glu^{33}---Lys^{36}) induces and stabilizes in the receptor environment a putative α-helical bioactive conformation of the fragment that is not otherwise heavily represented. The effect of the introduction of two favored substitutions [(cyclo(20−23) and cyclo(30−33)] yielded **37** with a potency 8 times that of the assay standard but actually 12 times less than expected if the effect of the two cycles had been multiplicative. These results suggest that the pituitary CRF receptor can discriminate between slightly different identifiable conformations, dramatically illustrating the role that secondary and tertiary structures play in modulating biological signaling through specific protein−ligand interactions.

Introduction

Corticotropin-releasing factor (CRF) is a 41-residue peptide amide which stimulates the release of ACTH *in vitro* and *in vivo*[8,9] and acts within the brain to modulate a wide range of stress responses[9] as demonstrated by the extensive use of competitive antagonists such as the α-helical $CRF_{(9-41)}$[3] or analogues arising from the [$DPhe^{12}$,$Nle^{21,38}$]$hCRF_{(12-41)}$ lead.[4] Whereas antibodies to CRF were shown to be more effective after peripheral administration than competitive antagonists,[3] competitive antagonists will diffuse within the central nervous system (CNS) where antibodies are found to be less effective. This first generation of antagonists is very efficient at blocking effects of endogenous CRF when administered in the CNS but has significantly less affinity for the pituitary receptors and has some residual intrinsic activity.[3,4] Prior to the discovery of astressin {cyclo(30−33)[$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}]$hCRF_{(12-41)}$}, peripheral administration of CRF antagonists resulted in weak and short-lived effects *in vivo* (inhibition of release of ACTH). Our early observation that CRF antagonists had varying efficacy in different *in vivo* assays suggested the presence of several distinct CRF receptors.[10] CRF receptors that are pituitary/CNS[11−13] and heart/muscle[14,15] selective have now been characterized. Other members of this family

* Author for correspondence.

[†] Abbreviations: IUPAC rules are used for nomenclature of peptides including one letter codes for amino acids. Also, Ac = acetyl; ACTH = adrenocorticotropin hormone; assay standard, [$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12-41)}$; astressin = cyclo(30−33)[$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}]-$hCRF_{(12-41)}$; Boc = *tert*-butyloxycarbonyl; BOP = (benzotriazolyloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; CD = circular dichroism; CRF = corticotropin releasing factor (o = ovine, r = rat, h = human), rat and human sequences are identical; CZE = capillary zone electrophoresis; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DMF = dimethylformamide; Fmoc = 9-fluorenylmethoxycarbonyl; HF = hydrogen fluoride; GHRH = growth hormone releasing hormone; GnRH = gonadotropin releasing hormone; MBHA = methylbenzhydrylamine resin; NMR = nuclear magnetic resonance; OFm = *O*-fluorenylmethyl; PTH = parathyroid hormone; rms = root mean square; RP-HPLC = reverse phase high-performance liquid chromatography; SAR = structure-activity relationships; TBTU = *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TEAP 2.25 = triethylammonium phosphate, pH 2.25; TFA = trifluoroacetic acid; TFE = trifluoroethanol; UCN = urocortin; UT = urotensin. Results were presented in part at the 23rd European Peptide Symposium, 1994,[1] the Chinese Peptide Symposium, 1994,[2] and the 15th American Peptide Symposium, 1997 (in press).

[‡] Universidade Federal de Sao Paulo.

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Table 1. Members of the CRF Family

	5 10 15 20 25 30 35 40
CRF Human/Rat	S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I I ■
CRF Pig	S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I F ■
CRF Sucker	S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K M M E I F ■
CRF Xenopus	A E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M D I I ■
CRF Sheep/Goat	S Q E P P I S L D L T F H L L R E V L E M T K A D Q L A Q Q A H S N R K L L D I A ■
CRF Cow	S Q E P P I S L D L T F H L L R E V L E M T K A D Q L A Q Q A H N N R K L L D I A ■
UT Carp/GFHin2	N D D P P I S I D L T F H L L R N M E I M A R N E N Q R E Q A G L N R K Y L D E V ■
UT Sucker	N D D P P I S I D L T F H L L R N M E I M A R I E N E R E Q A G L N R K Y L D E V ■
UT Sole	S E E P P M S I D L T F H M L R N M I H R A K M E G E R E Q A L I N R N L L D E V ■
UT Flounder	S E D P P M S I D L T F H M L R N M I H M A K M E G E R E Q A Q I N R N L L D E V ■
UCN Rat	- D D P P L S I D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V ■
UCN Human	- D N P S L S I D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V ■
Sauvagine	-pE G P P I S I D L S L E L L R K M I E I E K Q E K E K Q Q A A N N R L L L D T I ■

■ indicates that the peptides are C-terminally amidated

of receptors which are coupled to guanine nucleotide stimulatory factor (Gs)-response pathways include receptors for growth hormone releasing factor,[16-18] calcitonin,[19] secretin,[20] and vasoactive intestinal peptide.[21] Additionally, the actions of CRF can be modulated by a 37 KDa CRF binding protein (CRF-BP).[22,23] Finally, efficacy of different analogues *in vivo* for one activity may be modulated by such factors as bioavailability, degradation, and elimination (pharmacokinetics), factors that may be very sensitive to structural modifications.

Several systematic approaches to understanding SAR of CRF have been followed (alanine scan,[24] single-point D-substitution of each residue in the sequence,[25] and single-point proteinogenic substitution[26]) that have led to a better definition of the contribution of each residue both in terms of its optimal composition and chirality. Additionally, all experimental and theoretical data point to the fact that CRF-like molecules assume an α-helical conformation upon binding to their receptors. For example, a recent NMR study of hCRF in TFE/water (66/34, v/v) identified a well-defined α-helix between residues 6 and 36 with an extended N-terminus and a disordered C-terminus.[7] The first half of the α-helix was clearly amphipathic as recognized earlier from model experiments.[6,27] Therefore, several options directed at stabilizing an α-helical conformation in solution have been attempted to increase the potency of CRF antagonists. The most successful approaches have been the introduction of α-helical-inducing residues within the sequence,[3] or the introduction of C-α-methylated amino acids[4] which are known to induce α-helicity. Significant improvement in potency was achieved, which translated, in several cases, into increased, although transient, duration of action *in vivo*.[4] Another means of achieving conformational stability is to create side chain to side chain covalent bonding. Salt bridges are known to stabilize tertiary structures in proteins, and examples can be found of $i-(i+3)$ and $i-(i+4)$ interactions (Glu or Asp to Lys, Arg or His). Some salt bridges can be replaced by a covalent amide bond forming a lactam bridge in molecules known to have a propensity for α-helix formation. Growth hormone releasing factor (GRF),[28,29] parathyroid hormone fragments (PTH),[30] and CRF[27] are three such molecules. On the other hand, the introduction of D-amino acids has been shown to stabilize turns. Because [DGlu^{20}]oCRF was significantly more potent than oCRF itself, the possibility of a turn in the middle of the CRF molecule was considered. On the other hand, the existence of a lysine residue at position 23 of oCRF was suggestive of a salt bridge stabilizing an α-helix (Glu is found in all characterized CRFs at position 20 while Lys is found at position 23 in ovine/caprine/bovine CRF and Arg in rat/human/porcine/sucker CRF) (Table 1). A systematic study that examined bridge length, chirality, and positioning of the lactam bond led to the identification of cyclo(20−23)[DPhe^{12},Glu^{20},Lys^{23},Nle^{21,38}]hCRF$_{(12-41)}$ as the most potent lead analogue (2.9 times the potency of **1**).[27] From these observations, we concluded that an $i-(i+3)$ Glu to Lys lactam bridge was compatible with α-helix stabilization and was an ideal means of investigating the effect of bridging on α-helix stabilization. To further test this hypothesis, we completed the synthesis of an $i-(i+3)$ bridge scan (Glu-Xaa-Xbb-Lys) of **1**.[4] Full chemical characterization and relative potency of the complete series are presented here (Table 2). Results of the biological properties of **27** and **28** were described in an earlier publication.[5]

Results and Discussion

All analogues shown in Table 2 were synthesized on a methylbenzhydrylamine resin and the Boc strategy with orthogonal protection of the side chains of lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[27,28] Main chain assembly was mediated in most

cases by diisopropylcarbodiimide. The best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and OFm protecting groups and when TBTU or BOP mediated the lactam formation.[27] The peptides were cleaved and deprotected in HF and purified using RP-HPLC and three aqueous buffers (TEAP 2.25, TEAP 6.5, and 0.1% TFA).[27,31,32] Peptides were characterized as shown in Table 2. Analogues were determined to be greater than 90% pure using RP-HPLC and CZE criteria except for **13**. The measured masses obtained using liquid secondary ion mass spectrometry were in agreement with those calculated for the protonated molecule ions.

CRF analogues were tested for antagonist activity in an *in vitro* assay measuring inhibition of CRF-induced release of ACTH by rat anterior pituitary cells in culture.[3,8,33] Relative potencies with 95% confidence limits in parentheses are shown using [DPhe^{12},Nle^{21,38}]-hCRF$_{(12-41)}$] (**1**) as the "assay standard" with a potency equal to 1.0 (Table 2). Three compounds (**2**, **21**, and **28**) were tested more than once to give consistent relative potencies thus giving an appreciation of the assay-to-assay variation that should be expected.

Considering that eight alanine-substituted analogues with substitutions between residues 9 and 41 are less than 10% as potent as oCRF (alanine at position 10, 12, 14, 15, 16, 19, 35, and 38)[24] and that substitutions were made at the same respective positions in 12 cyclic analogues reported here, we expected that many of the cyclic analogues would have drastically reduced potencies as compared to that of the assay standard. We did find that 13 cyclic analogues (**3**, **5**, **6**, **7**, **10**, **11**, **15**, **21**, **29**, **30**, **32**, **33**, and **36**) had potencies equal to or less than 10% that of **1**. Remarkably, **4**, **8**, and **9** with substitutions at residues 10, 16, and 14, respectively, had potencies significantly greater than that expected on the basis of the low potencies of [Ala^{10}]oCRF, [Ala^{16}]-oCRF, and [Ala^{14}]oCRF. In fact the potencies of **8** and **9** are statistically indistinguishable from that of the assay standard, while **4** has one-fourth the potency of **1**. Additionally, substitutions that had not been predicted to be deleterious on the basis of the alanine scan (**21**, **29**, and **32**) yielded analogues with low potency. This suggests that conformational stability conferred by the introduction of lactam bridges may result in potent analogues even when the substitution at any single position occupied by the bridging units will result in complete loss of potency. This has been well documented in the case of cyclic gonadotropin-releasing hormone (GnRH) analogues.[34,35]

More often than not, random amino acid substitution or the introduction of structural constraints in a native hormone will lead to analogues with reduced potency. This is also the case for a large number of analogues described here. Introduction of the Glu−Lys lactam bridge at positions 10/13 (**4**), 13/16 (**8**), 17/20 (**12**, **13**), 21/24 (**18**), 22/25 (**19**), 25/28 (**22**), 27/30 (**24**), 36/39 (**34**), and 37/40 (**35**) results in a 2−6-fold decrease in relative potency.

The question now remains as to why lactam bridge formation in other instances would result in maintenance [lactam bridge at positions 14/17 (**9**), 23/26 (**20**), and 33/36 (**31**)], tripling [lactam bridge at positions 20/23 (**16**), 26/29 (**23**), 28/31 (**25**), and 29/32 (**26**)], or considerable (>30-fold) increase in relative potency [lactam bridge at positions 30/33 (**27**)].

In a preliminary communication we suggested, on the basis of the large and unexpected increase of biological potency of **27** (compared to that of the assay standard or to its linear counterpart) and not of the corresponding linear or cyclic agonists {linear- or cyclo(30−33)[DPhe^{12},-Nle^{21,38},Glu^{30},Lys^{33}]hCRF$_{(4-41)}$}, that the lactam bridge at positions 30/33 stabilized an α-helical form of the peptide, a function fulfilled by the N-terminus in the agonists since linear and cyclic agonists have essentially the same potency.[5] The introduction of side chain bridging via disulfide, lactam, or other bond formation has been a powerful tool in peptide pharmacology, and we have previously exploited this technique in the development of CRF analogues. As reported previously[27] we proposed to use the activity profile of a D-amino acid scan to identify potential bridgeheads for the stabilization of α-helices. In this work, position 20 (Glu in hCRF) was chosen as a pivot point, and a series of 32 compounds was synthesized and evaluated for biological activity, the most potent of which featured a cyclo(20−23)[Glu^{20}−Lys^{23}] lactam bridge (**16**). Modeling studies of the influence of lactam size (i.e., Glu-Dpr, Glu-Dbu, ...) and span [i.e., $i-(i+3)$, $i-(i+4)$, $i-(i+5)$] were conducted on a tetradecaalanyl host in an α-helical conformation, and the most negligible perturbation employing L-amino acids was found to be the $i-(i+3)$ Glu−Lys bridge with an overall rms deviation after minimization of 0.17 Å over the backbone atoms of the host. The corresponding $i-(i+3)$ D-Glu−D-Lys bridge gave a slightly smaller rms statistic after minimization (0.14 Å). Two underlying structural assumptions were that (a) CRF and active analogues assume primarily α-helical conformations upon interaction with the CRF receptor;[6] and that (b) the $i-(i+3)$ Glu−Lys bridge would minimally perturb the molecule while facilitating helix maintenance via covalent constraints. The $i-(i+3)$ Glu−Lys lactam scan of [DPhe^{12},Nle^{21,38}]hCRF$_{(12-41)}$ was thus conducted and modeling predictions suggested that α-helicity should be enhanced in all cases irrespective of the bridgehead positioning. The question therefore remains as to what is so singular about the 30−33 lactam bridge.

While the choice of position 20 as a pivot point in our previous work[27] gained additional support from the observation that the naturally occurring residues (Glu^{20} and Arg^{23}) could be envisaged as participating in an electrostatically stabilizing interaction of the type observed for naturally occurring salt bridges, there was no expectation in the current work that Gln^{30} and Ser^{33}, the naturally occurring residues in CRF could be replaced so successfully in astressin by Glu^{30} and Lys^{33}. Whereas the strength of a Gln−Asp [$i-(i+4)$] hydrogen bond in peptide helices has recently been measured and defined,[36] [NMR experiments show that the aspartate carboxylate group interacts specifically with the *trans* amide proton (Hϵ) of glutamine] we have found no such study for a Gln−Ser [$i-(i+3)$] hydrogen bond of interest to us. In fact modeling experiments carried out *in vacuo* strongly suggest that hydrogen-bonding interactions of Gln and Ser side chains with an [$i-(i+3)$] spacing in a right-handed α-helical configuration are very unfavorable (>10 kcal/mol).

Table 2. Bridge Scan of r/hCRF Antagonists

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                         5         10        15        20        25        30        35        40
Human/Rat CRF   S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I I NH2
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no.	compound	HPLC[a]	CZE[b]	MS (mono) calcd/found[c]	relative potency *in vitro*[d]	% IA[e]
1	[DPhe^{12},Nle^{21,38}]hCRF$_{(12-41)}$	97	95	3538.01 3538.0	1.0	0−5
2	[DPhe^{12},Nle^{21,38}]hCRF$_{(9-41)}$	96	97	3867.17 3867.1	0.11 (0.060−0.200)[f] 0.28 (0.13−0.57)	31[f] 31
3	cyclo(9−12)[Glu^{9},DLys^{12},Nle^{21,38}]hCRF$_{(9-41)}$	99	98	3844.2 3844.3	0.0005 (0.000−0.002)	74
4	cyclo(10−13)[Glu^{10},DPhe^{12},Lys^{13},Nle^{21,38}]hCRF$_{(9-41)}$	82	94	3856.16 3856.4	0.27 (0.13−0.58)	27
5	cyclo(11−14)[Glu^{11},DPhe^{12},Lys^{14},Nle^{21,38}]hCRF$_{(9-41)}$	95	96	3892.17 3892.4	0.005 (0.001−0.016)	29
6	cyclo(12−15)[Glu^{12},Lys^{15},Nle^{21,38}]hCRF$_{(12-41)}$	93	95	3516.99 3517.0	0.005 (0.001−0.015)	14
7	cyclo(12−15)[DGlu^{12},Lys^{15},Nle^{21,38}]hCRF$_{(12-41)}$	92	95	3516.99 3516.9	0.010 (0.001−0.042)	9
8	cyclo(13−16)[DPhe^{12},Glu^{13},Lys^{16},Nle^{21,38}]hCRF$_{(12-41)}$	95	98	3483.98 3483.9	0.41 (0.17−0.92)	34
9	cyclo(14−17)[DPhe^{12},Glu^{14},Lys^{17},Nle^{21,38}]hCRF$_{(12-41)}$	91	97	3535.01 3534.9	1.25 (0.14−8.78)	21
10	cyclo(15−18)[DPhe^{12},Glu^{15},Lys^{18},Nle^{21,38}]hCRF$_{(12-41)}$	88	97	3564.99 3564.8	0.015 (0.001−0.13)	11
11	cyclo(16−19)[DPhe^{12},Glu^{16},Lys^{19},Nle^{21,38}]hCRF$_{(12-41)}$	89	97	3507.96 3508.2	0.034 (0.010−0.10)	25
12	cyclo(17−20)[DPhe^{12},Glu^{17},Lys^{20},Nle^{21,38}]hCRF$_{(12-41)}$	87	90	3519.06 3519.0	0.15 (0.070−0.31)	19
13	cyclo(17−20)[DPhe^{12},Glu^{17},DLys^{20},Nle^{21,38}]hCRF$_{(12-41)}$	75	92	3519.06 3519.2	0.16 (0.048−0.51)	5
14	cyclo(18−21)[DPhe^{12},Glu^{18},Lys^{21},Nle^{38}]hCRF$_{(12-41)}$	96	95	3564.99 3564.8	0.10 (0.029−0.34)	13
15	cyclo(19−22)[DPhe^{12},Glu^{19},Lys^{22},Nle^{21,38}]hCRF$_{(12-41)}$	94	90	3593.02 3592.9	0.036 (0.015−0.077)	17
16	cyclo(20−23)[DPhe^{12},Glu^{20},Lys^{23},Nle^{21,38}]hCRF$_{(12-41)}$	98	98	3491.99 3491.7	2.9 (1.3−6.7)	11
17	linear [DPhe^{12},Glu^{20},Lys^{23},Nle^{21,38}]hCRF$_{(12-41)}$	98	98	3510.01 3510.0	0.31 (0.14−0.65)	42
18	cyclo(21−24)[DPhe^{12},Glu^{21},Lys^{24},Nle^{38}]hCRF$_{(12-41)}$	98	98	3593.02 3593.7	0.25 (0.13−0.43)	1
19	cyclo(22−25)[DPhe^{12},Nle^{21},Glu^{22},Lys^{25},Nle^{38}]hCRF$_{(12-41)}$	96	99	3577.06 3577.2	0.46 (0.17−1.12)	2
20	cyclo(23−26)[DPhe^{12},Nle^{21},Glu^{23},Lys^{26},Nle^{38}]hCRF$_{(12-41)}$	99	98	3492.98 3492.9	1.75 (0.67−4.45)	19
21	cyclo(24−27)[DPhe^{12},Nle^{21},Glu^{24},Lys^{27},Nle^{38}]hCRF$_{(12-41)}$	90	97	3593.02 3592.8	0.07 (0.02−0.15)[f] 0.12 (0.04−0.28)	59[f] 69
22	cyclo(25−28)[DPhe^{12},Nle^{21},Glu^{25},Lys^{28},Nle^{38}]hCRF$_{(12-41)}$	98	98	3577.06 3577.0	0.52 (0.23−1.1)	15
23	cyclo(26−29)[DPhe^{12},Nle^{21},Glu^{26},Lys^{29},Nle^{38}]hCRF$_{(12-41)}$	99	$>$95	3521.02 3520.9	2.8 (0.35−3.4)	24
24	cyclo(27−30)[DPhe^{12},Nle^{21},Glu^{27},Lys^{30},Nle^{38}]hCRF$_{(12-41)}$	96	95	3536.00 3536.1	0.14 (0.073−0.26)	15
25	cyclo(28−31)[DPhe^{12},Nle^{21},Glu^{28},Lys^{31},Nle^{38}]hCRF$_{(12-41)}$	90	82	3635.07 3635.3	3.12 (1.1−10.1)	16
26	cyclo(29−32)[DPhe^{12},Nle^{21},Glu^{29},Lys^{32},Nle^{38}]hCRF$_{(12-41)}$	93	96	3512.02 3512.3	3.38 (1.2−12.1)	11
27	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12-41)}$ (astressin)	95	97	3562.13 3562.2	32[5]	0−5
28	linear [DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12-41)}$	96	94	3580.06 3580.1	0.10 (0.06−0.16)[f] 0.04 (0.01−0.12)	0[f] 5
29	cyclo(31−34)[DPhe^{12},Nle^{21},Glu^{31},Lys^{34},Nle^{38}]hCRF$_{(12-41)}$	96	97	3592.06 3592.2	0.020 (0.002−0.11)	5
30	cyclo(32−35)[DPhe^{12},Nle^{21},Glu^{32},Lys^{35},Nle^{38}]hCRF$_{(12-41)}$	97	97	3483.98 3483.94	0.063 (0.028−0.13)	25
31	cyclo(33−36)[DPhe^{12},Nle^{21},Glu^{33},Lys^{36},Nle^{38}]hCRF$_{(12-41)}$	85	96	3562.01 3562.3	0.81 (0.40−1.70)	21
32	cyclo(34−37)[DPhe^{12},Nle^{21},Glu^{34},Lys^{37},Nle^{38}]hCRF$_{(12-41)}$	88	94	3550.01 3550.3	0.003 (0.001−0.007)	18
33	cyclo(35−38)[DPhe^{12},Nle^{21},Glu^{35},Lys^{38}]hCRF$_{(12-41)}$	90	91	3507.96 3508.2	$<$0.001	15
34	cyclo(36−39)[DPhe^{12},Nle^{21},Glu^{36},Nle^{38},Lys^{39}]hCRF$_{(12-41)}$	95	95	3520.00 3520.2	0.21 (0.085−0.46)	26
35	cyclo(37−40)[DPhe^{12},Nle^{21},Glu^{37},Nle^{38},Lys^{40}]hCRF$_{(12-41)}$	96	96	3550.97 3551.1	0.12 (0.039−0.30)	8
36	cyclo(38−41)[DPhe^{12},Nle^{21},Glu^{38},Lys^{41}]hCRF$_{(12-41)}$	98	98	3550.97 3551.2	$<$0.001	3

Table 2 (Continued)

no.	compound	HPLC[a]	CZE[b]	MS (mono) calcd/found[c]	relative potency *in vitro*[d]	% IA[e]
37	dicyclo(20−23,30−33)[DPhe[12],Glu[20],Nle[21],Lys[23],Glu[30],Lys[33],Nle[38]]hCRF$_{(12-41)}$	96	98	3516.03 3516.0	8.3 (3.6−18.3)	49
38	linear[DPhe[12],Glu[20],Nle[21],Lys[23],Glu[30],Lys[33],Nle[38]]hCRF$_{(12-41)}$	98	96	3552.05 3552.1	0.09 (0.05−0.16)	37

[a] Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH_3CN/40% A with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm. 5 μm particle size, 300 Å pore size). Detection at 214 nm. [b] Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 Model 50Z and using a ChromJet integrator. Field strength of 15 kV at 30 °C; mobile phase 100 mM sodium phosphate (85:15 H_2O:CH_3CN) pH 2.50, on a Supelco P175 capillary (363 μm OD × 75 μm i.d. × 50 cm length). Detection at 214 nm. [c] The observed *m/z* of the monoisotope compared with the calculated $[M + H]^+$ monoisotopic mass. [d] Potencies are relative to that of [DPhe[12],Nle[21,38]]-hCRF$_{(12}$−41), in the *in vitro* rat pituitary cell culture assay, with 95% confidence limits in parentheses. [e] The percent intrinsic activity of each of the antagonists is calculated by determining level of secretion caused by the highest dose of antagonist (in the absence of oCRF) minus basal secretion and dividing that number by the level of secretion of 1nM oCRF minus basal secretion and multiplying the result by 100. [f] These particular compounds were tested twice.

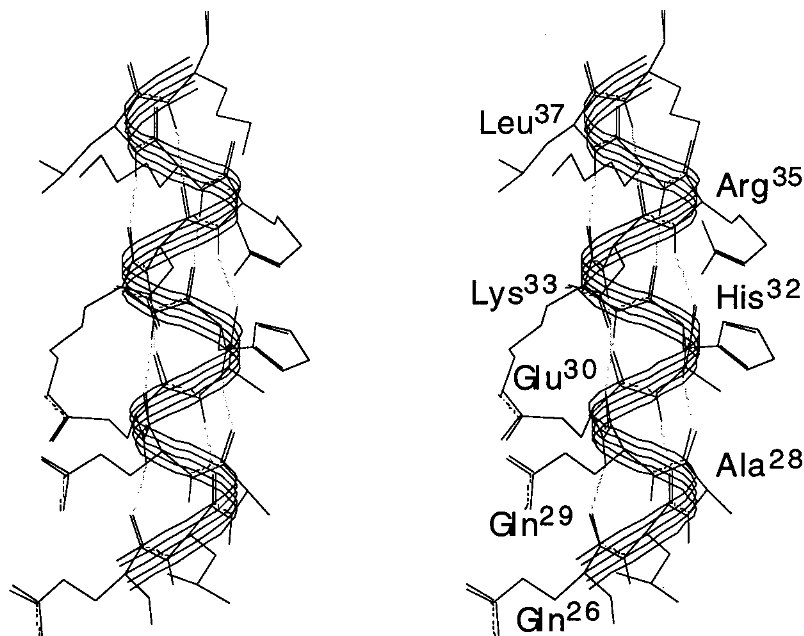

Figure 1. Stereoribbon diagram (heavy atoms and backbone protons only) of residues 26-38 of astressin, cyclo(30−33)[DPhe[12],Nle[21,38],Glu[30],Lys[33]]hCRF$_{(12-41)}$, in an α-helical conformation after minimization to allow the Glu[30]−Lys[33] bridge to relax after construction. Dotted lines show the requited hydrogen-bonding opportunities.

Multiple effects, both chemical and structural, attend the incorporation of side chain bridging. Our previous studies on the Ala and D-Xaa scans of oCRF, as well as the conceptually similar single-point proteinogenic substitution described by Beyermann *et al.*[26] can be analyzed by assigning to each residue a relative propensity for the manner (i.e., structural and through specific interactions with the receptor) in which the overall energetics of binding are affected. Although significant basic work remains to be done in the understanding of the influence of amino acid replacement in the peptide−receptor interaction, the role of a residue being structural or functional must be amended; in the case of side-chain bridging, the effect of the bridge must be included since it can virtually force a desired turn or helical secondary structure. Further, the side chains emanating from the bridgeheads of a Glu−Lys bridge have bulk and a certain amount of chemical binding reactivity, as e.g. in the polarized amide closing the bridge, which is nonetheless flexible. Consequently, a bridge may or may not modulate binding and/or transduction via a specific side chain interaction with the receptor but can have a marked effect on local structure. In this regard the SAR of **25**−**28** suggest an overwhelming structural role to residues 28−33, with increased α-helicity correlating with increased potency. The Ala-scan in this region, giving relative potencies of 1.0, 1.4, 0.8, 1.0, 4.5, and 2.0 to positions 28−33, respectively, strongly suggests that side chain-receptor interactions, with the partial exception of Gln[30], actually decrease potency, whereas replacement of the side chains of residue 28−31, 29−32, and 30−33 in **25**−**27** with the flexible yet helix-promoting Glu−Lys bridge increases potency from 3.1- to 32-fold. Whether this effect of helicity on potency is the result of a local structural modification in the region of residues 28−32, or a longer-range effect extending toward the C-terminus and/or N-terminus, remains to be determined. Elimination of the helix in the linear **28**, possibly in concert with the replacement of residue 30 and 33 with (unbridged) Glu[30] and Lys[33], results in negligible potency.

A stereo ribbon structure featuring residues 26−37 of astressin (i.e., including the Glu[30]−Lys[33] bridge) is shown in Figure 1. We observe that the side chain amide functionality of Gln[30] could be mimicked by the Glu[30]−Lys[33] bridge, but current modeling techniques do

not allow an unambiguous assignment of the role of this functionality in the peptide–receptor interaction, although we have seen that the closely related Asn^{30} substitution is poorly accepted.[26] Interestingly, whereas Gln^{30} is conserved in all CRFs and urotensins, it is neither in the two known urocortins nor is Ser^{33} even among mammalian CRFs. (See Table 1.) On the other hand it is noteworthy that the bracketing residues (Ala^{31}, Asn^{34}, and Arg^{35}) are conserved in CRFs, urotensins and urocortins throughout all species studied so far.

Finally, whereas four (positions 4, 7, 9, and 10) out of nine (additional positions are 15, 16, 31, 34, and 35) conserved residues of CRFs, urotensins, and urocortins throughout all species are to be found toward the N-terminus thought to be involved in receptor activation, two residues, Leu^{15} and Arg^{16}, must play an important role not yet uncovered. On the other hand the three conserved residues Ala^{31}, Asn^{34}, and Arg^{35} which are proximal to the propitious lactam bridge (Glu^{30}–Lys^{33}) must be responsible for both structure and function.

It is apparent that introduction of a lactam bridge beyond position 30 results in a very significant loss of potency. We and others had already shown that the integrity of the C-terminus was important for biological activity since the free acid as compared to the amide resulted in an inactive analogue.[8] Additionally, because a D-substitution scan clearly pointed to the importance of maintaining chiral integrity at the C-terminus in order to conserve potency,[25] we conclude that the C-terminus is more sensitive to changes in conformation than it is of composition (see also Beyermann *et al.*)[26] although there is consensus that an acidic residue at position 39 and two hydrophobic residues at positions 37 and 38 are important. On the other hand, in the 13 known CRF-like molecules (see Table 1), the only conserved residues are 4, 7, 9, 10, 15, 16, 31, 34, and 35, with significant diversity from residues 17 to 30 and toward the C-terminus.

Similarly, except for cyclo(14–17), cyclo(20–23), and cyclo(23–26), the central part of CRF (residues 12–25) is also quite sensitive to conformational manipulation. Interestingly cyclo(14–17) may mimic an i–(i + 4) salt bridge between residues 13 (His) and 17 (Glu), and cyclo(20–23) may mimic an i–(i + 3) salt bridge between residues 20 (Glu) and 23 (Arg). However, there is little literature precedent for the interaction of an arginine side chain with that of a glutamine [residue 26, i–(i + 3) interaction] or of a leucine [residue 27, i–(i + 4) interaction].

Other analogues reported here include **2**, the potency of which is to be compared to that of compounds **3**–**5**. Whereas cycles 9–12 and 11–14 result in significant loss of potency, the introduction of cycle 10–13 results in a doubling of potency. While the drop in potency resulting from increasing the size of the peptide antagonist from 30 (**1**) to 33 residues (**2**) was unexpected, it was not without precedent as some CRF analogues seem to gain intrinsic activity as fewer than the 12 N-terminal amino acids are deleted (unpublished results). Compound **7** emphasizes the importance of the introduction of a D-residue at position 12 which seems to be consistently favorable both as a single substitution (most potent CRF antagonists have a D-amino acid at position 12) and as a bridgehead (the potencies of **7** and **6** are not significantly different). Analogues **17** and **28** are the significantly less potent linear counterparts of our two most potent monocyclic analogues **16** and **27**, respectively. The rationale presented in an earlier publication to explain the loss of activity going from the cyclic **27** to the linear **28** most likely parallels what is seen with the pair **16** and **17**.[5] Whether the increase in potency for the cyclic analogues is based solely on stabilization of a favorable secondary structure or whether the loss of potency is due to the presence of additional charges in the linear analogues is still to be addressed. The problem that we encounter in trying to solve this dilemma is that while Glu and Gln are essentially isosteric, neutralization of the ϵ-amino function in Lys may result in significant electrostatic changes and steric hindrance even after acylation with the small formyl group. Finally, compounds **37** and **38** were synthesized in order to determine whether the beneficial effects of the introduction of a lactam bridge between residues 20 and 23 and 30 and 33 were additive. Results showing a 12-fold lower potency (8 times that of the assay standard) than that expected ($3 \times 32 = 96$) if the effect of each of the substitutions had been multiplicative suggest, in the absence of detailed structural information, that the pituitary CRF receptor can discriminate between slightly different identifiable conformations. Felix *et al.* also observed the deleterious effect on potency of the cumulation of two favorable cycles in a growth hormone releasing hormone analogue despite the fact that increased α-helicity had been observed.[28] These authors concluded that their bicyclic analogue was too rigid to best accommodate the receptor.

Finally, CRF antagonists that are effective in the CNS have been available for a number of years, but antagonists that would be potent and long acting on ACTH secretion have been lacking. Astressin is a significant improvement over previously available CRF antagonists due to its high potency and comparatively low intrinsic activity.[5] Whereas most antagonists reported here and in the past have intrinsic activities of less than 30%, we have identified **3**, **21**, and **37** with intrinsic activities equal to or superior than 50%. To the best of our abilities, we have excluded the possibility that these analogues were contaminated with a CRF agonist. Our present hypothesis is that the constraints imposed upon these analogues by the respective lactam bridges are conducive to receptor activation. Until now, full intrinsic activity has been observed with analogues 35 residues long or longer; **3** is 33 residues long and **21** and **37** are only 30 residues long.

Experimental Section

Synthesis of CRF Analogues. All analogues shown in Table 2 were synthesized either manually or on a Beckman 990 peptide synthesizer using the solid phase approach, a methylbenzhydryl amine resin,[27] and the Boc-strategy with orthogonal protection (Fmoc and OFm) of the side chains of residues to be cyclized.[28] Amino acid derivatives Boc-Ala, Boc-Arg(Tos), Boc-Asn(Xan), Boc-Asp(cHex), Boc-Gln(Xan), Boc-Glu(cHex), Boc-His(Tos), Boc-Ile, Boc-Met, Boc-Leu, Boc-Phe, Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Tyr(2,6-Br_2-Bzl), and Boc-Val were obtained from Bachem Inc. (Torrance, CA). Boc-Glu(Ofm) and Boc-Lys(Fmoc) were synthesized as described earlier.[37] All solvents were reagent grade or better. TFA, 60% in DCM, was used to remove the Boc group. Main chain

assembly was mediated by diisopropylcarbodiimide. Coupling time was 90−120 min following by acetylation (excess acetic anhydride in DCM for 15 min). A 3-fold excess of protected amino acid was used based on the original substitution of the methylbenzhydrylamine-resin. Deprotection of the Fmoc group was achieved using a solution of 20% piperidine/DMF (2 × 10 min) followed by sequential washes with DMF, MeOH, 10% TEA/DCM, and DCM. Lactam formation was mediated using TBTU or HBTU in DMF or NMP. Best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and Ofm protecting groups and cyclization as shown earlier.[27] The peptides were cleaved and deprotected in HF and purified using RP-HPLC and three solvent systems (TEAP at pH 2.25 and 6.5 and 0.1% TFA successively).[31,32]

Characterization of CRF Analogues. Peptides were characterized as shown in Table 2. Analogues were greater than 90% pure using independent HPLC and CZE criteria. Conditions are shown in the legend.

RP-HPLC. In addition to determining the purity of the peptides in an acidic system (see Table 2 legend), most of the analogues were also analyzed using 0.05% TEAP at pH 6.8 and a Vydac C_8 column (0.21 × 15 cm) at a flow rate of 0.2 mL/min with slightly varying gradient slopes. Retention times varied from 14 to 33 min with no correlation between apparent hydrophobicity and relative potency. Percent purity was in the range of that found with CZE or with HPLC under acidic conditions.

Capillary Zone Electrophoresis (CZE). Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE system 2050 controlled by an IBM Personal System/2 Model 50Z and using a ChromJet integrator. Electrophoresis was performed in 0.1 M sodium phosphate (pH 2.5) except for α-helical CRF_{9-41} which was measured in 0.1 M sodium borate (pH 8.5).

Mass spectroscopy. LSIMS mass spectra were measured with a JEOL JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and Cs^+ gun voltage between 25−30 kV were employed; for further details, see ref 27. Calculated values for protonated molecular ions were in agreement with those obtained using liquid secondary ion mass spectrometry.

***In Vitro* Pituitary Cell Culture Assay.** Rat anterior pituitary glands from male Sprague−Dawley rats were dissociated by collagenase and plated (0.16×10^6 cells/well in 48-well plates) in medium containing 2% fetal bovine serum (FBS).[33] Three days after plating, the cells were washed three times with fresh medium containing 0.1% bovine serum albumin (BSA) and incubated for 1 h. Following the 1 h preincubation, the cells were washed once more and the test peptides were applied in the absence (determination of intrinsic activity) or the presence (testing of antagonistic activity) of 1 nM oCRF. At the end of a 3 h incubation period, the media were collected and the level of ACTH was determined by radioimmunoassay (Diagnostic Products Corporation).

Molecular Modeling. Molecular modeling and visualization of the $i-(i+3)$ Glu−Lys lactam bridge scan of $[DPhe^{12},Nle^{21,38}]hCRF_{(12-41)}$ and other model compounds were conducted with DISCOVER and InsightII (MSI, San Diego, CA) on a Silicon Graphics workstation (Model 310 GTX) running the IRIX 5.0.3 operating system. Calculations were conducted *in vacuo* using the consistent valence force field.[38]

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