Single Point D-Substituted Corticotropin-Releasing Factor Analogues: Effects on Potency and Physicochemical Characteristics†,‡

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In an attempt to determine which conformational parameters are important for the biological activity of ovine corticotropin-releasing factor (oCRF), we have synthesized in significant amounts (50–200 mg) and characterized chemically, structurally (CD), and biologically, oCRF analogues with substitution of each amino acid by its corresponding D-isomer. Out of 37 of these analogues, three were found to be equipotent to, or twice as potent as, oCRF, 13 had potencies in the range from 10 to 60%, 17 had potencies ranging from 1 to 10%, and the four others had potencies less than 0.5%. None of the analogues antagonized oCRF-induced release of ACTH *in vitro* at concentrations ≥1000 oCRF. Since antagonists to CRF action can be generated by deletion of the first 8–14 residues, a series of CRF antagonists which exhibit significantly higher *in vitro* and *in vivo* biological potency than [Met^{18},Lys^{23},Glu^{27,29,40},Ala^{32,41},-Leu^{33,36,38}]h/rCRF, [α-helical-CRF$_{9-41}$], is also described. [D-Phe^{12},Nle^{21,38},Arg^{36}]h/rCRF, in particular, was found to be ca. 15 times more potent than α-helical-CRF$_{9-41}$ *in vitro*. In the rat, however, this analogue was about as effective as α-helical-CRF$_{9-41}$ in blocking CRF-induced decrease in mean arterial blood pressure and increase in heart rate. Its potency in blocking epinephrine release by CRF was not significantly different from that of α-helical-CRF$_{9-41}$. In the adrenalectomized rat, [Lys^{36}]α-helical-CRF$_{(9-41)}$ (1.7 mg/kg) blunted the effect of endogenous CRF over a 90-min period; by comparison, a similar dose of α-helical-CRF$_{9-41}$ was effective for less than 1 h.

Introduction

The release of ACTH induced by stressful stimuli involves the activation of several neural and humoral pathways including CRF, vasopressin, and catecholamines.[1-3] The essential role played by endogenous CRF in modulating ACTH secretion was demonstrated by the lowering of basal ACTH secretion in adrenalectomized rats or stress-induced ACTH release by intact animals injected with a CRF antiserum[4] or a specific CRF-antagonist.[5] CRF has also been shown to mediate stress-induced changes in the autonomic nervous system, in neuroendocrine functions, and in behavior.[6-11] Here, we describe the chemical, structural (CD), and biological (*in vitro* and *in vivo*) characterization of a complete series of ovine CRF (oCRF) analogues with substitution of each amino acid from residues 5 to 41 by its corresponding D-isomer in an attempt to determine which conformational parameters are important for the biological response (binding and activation). A similar study in which each residue was substituted by an alanine residue addressed the role played by individual amino acid side chains in the recognition and activation process.[12] Because two of the D substitutions (D-Phe^{12} and D-Glu^{20}) resulted in compounds with increased potency, we also report that some CRF antagonists, based on the sequence of human/rat-CRF (h/rCRF) and encompassing the D-Phe^{12} substitution, had significantly higher biological potency than [Met^{18},-Lys^{23},Glu^{27,29,40},Ala^{32,41},Leu^{33,36,38}]h/rCRF, [α-helical-CRF$_{9-41}$],[5] in both *in vitro* and *in vivo* studies.

Results and Discussion

We have previously shown that the elimination of residues 1–4 at the N-terminus of oCRF did not alter biological activities or potency in the systems tested.[1] In order to determine which conformational parameters are important for the retention of bioactivity of oCRF, we have first identified the smallest fragment [CRF$_{5-41}$] with CRF-like activity and comparable potency.[5,13] As a result, we have found the main CRF binding (residues 9–41) and activating (residues 4–8) regions.[5] In two other series of investigations, we have accumulated evidence suggesting that the CRF's binding region is likely to assume an α-helical structure.[14,15] Direct and indirect support for this hypothesis comes from recent structural (CD) and biological results derived from testing C$^{\alpha}$- and N$^{\alpha}$-substituted analogues of h/rCRF (Hernandez et al.;

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† **Abbreviations.** The abbreviations for the amino acids are in accord with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9–37). The symbols represent the one or three letter code of L-isomer amino acids except when indicated otherwise. In addition: 2ClZ, 2-chlorobenzyloxycarbonyl; α-helical-CRF$_{9-41}$, [Met^{18},Lys^{23},Glu^{27,29,40},Ala^{32,41},Leu^{33,36,38}]h/rCRF; ACTH, adrenocorticotropin hormone; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; CRF, corticotropin-releasing factor; CZE, capillary zone electrophoresis; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; OBzl, benzyl ester; DMF, dimethylformamide; EDT, ethanedithiol; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HR, heart rate; IA, intrinsic activity; icv, intracerebroventricularly; iv, intravenously; LSIMS, liquid secondary ion mass spectroscopy; MAP, mean arterial pressure; MeCN, acetonitrile; Nle, norleucine; oCRF, ovine CRF; h/rCRF, human/rat CRF; RV, retention volume; sc, subcutaneous; TEA, triethylamine; TEAP 2.25, 6.5, triethylammonium phosphate buffer 0.25 N in phosphate with pH adjusted respectively to pH = 2.25 or 6.5 using TEA; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

‡ A preliminary report of this work was presented at the Japan Symposium on Peptide Chemistry, Sept 28–Oct 2, 1987, in Kobe, Japan.[32]

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following article in this issue). These recent results confirm structural observations, which suggested that CRF may assume, at least in part, an α-helical secondary structure when interacting with its receptor[5,15] or in a TFE/water mixture when studied by NMR.[16] Introduction of structural modifications into native peptides allows the determination of the amino acid residues which play a functional role in the secondary structure of the peptide and in binding of the peptide to its receptor by demonstration of the retention of biological activity and potency. Biological and structural results (backbone conformation) derived from the systematic substitution of each residue in oCRF by its D-isomer are described here.

Because two of the D substitutions (D-Phe^{12} and D-Glu^{20}) resulted in compounds with increased potency, we also report that some CRF antagonists, based on the sequence of human/rat-CRF (h/rCRF) and encompassing the D-Phe^{12} substitution, exhibited higher biological potency than α-helical-CRF$_{9-41}$[5] in an *in vitro* assay while an analogue of α-helical-CRF$_{9-41}$ was shown to exhibit extended duration of action *in vivo*.

Peptide Synthesis and Characterization. Peptides were synthesized automatically on a Beckman 990 synthesizer using the amino acid derivatives and purification methods schedule described in the Experimental Section. No major synthetic problems were encountered under the reported protocol. However, when resin samples were collected after several residues had been added, dried, aliquoted, and submitted to further chain elongation (a procedure particularly helpful when N-termini modifications were being introduced), we found that the following coupling failed to occur quantitatively using normal protocols. We concluded that the drying of N$^{\alpha}$- and side chain protected amino acid peptido–resins should be avoided as a portion of the N-termini may become inaccessible even after TFA deblocking and TEA neutralization. Repeated coupling, however, could solve this problem, but particular care had to be exercised since N-terminally deleted major products were otherwise isolated as the acetylated truncated species since systematic acetylation was carried out automatically after each coupling. This seems to be a common problem which we encountered during the synthesis of several series of peptide analogues including analogues of neuropeptide Y[17] and of growth hormone releasing factors (manuscript in preparation). As shown in Table I, peptides purified by HPLC (greater than 95%) had the specific rotations shown. For each of the [D-amino acid] series of ovine CRF, an intense intact species present in the LSIMS spectra was observed at m/z consistent with (±0.4 Da) the calculated $[M + H]^+$ average mass of oCRF (4671.39 Da). As shown in Table II the observed m/z for each intense intact species present in the LSIMS spectra of the antagonists (α-helical-CRF$_{9-41}$, h/rCRF$_{9-41}$, and h/rCRF$_{12-41}$) were also consistent with the calculated $[M + H]^+$ average mass. Amino acid analyses gave the expected ratios of amino acids. It is noteworthy that $[\alpha]_D$ were not greatly influenced by introduction of a D-amino acid at any of the positions substituted in this study. Similarly (even though not obvious from the data reported here which were designed to secure good resolution for determination of the analogue's purity), all HPLC retention times under isocratic conditions fell within a narrow range surrounding that of oCRF. Under these conditions, it is recognized that contamination of the different peptides by oCRF itself, resulting from either racemization during coupling (an unlikely occurrence[18]) or contamination of the starting protected D-amino acid by the corresponding L-isomer, cannot be entirely excluded (see discussion below). Antagonists described in Table II were comparatively easy to synthesize because of their shorter size and the fact that it is considerably less likely that a contaminant would alter potency as dramatically as the presence of a L residue would in the D-series. Characterization also included HPLC, $[\alpha]_D$, mass spectral analysis (Table II), and amino acid analyses. Partial *in vivo* characterization was obtained to gain an appreciation of the role of some of the substitutions that were introduced on duration of action.

Bioassays. An *in vitro* assay similar to that used for the isolation and characterization of oCRF[1] was performed using synthetic oCRF as standard. Potencies were derived from analysis of dose–response curves (see Experimental Section). The *in vivo* assay used to test CRF antagonists measured plasma ACTH levels in adrenalectomized rats at different times after the administration of the peptides[4] (see also Experimental Section). This assay permits an evaluation of the duration of action of the analogues. Following the intravenous injection of CRF antagonists, their ability to block the lowering of arterial pressure and increase of heart rate was also measured. Finally, in order to test the efficacy of these antagonists, they were also tested for their ability to block the elevation of plasma catecholamines following the icv injection of CRF.

Structure–Activity Relationships. When synthesizing peptides containing the D-isomer of the corresponding L-amino acid, one should be particularly concerned by the possibility of contamination of the former by the latter, as it will be reflected by relatively high biological potency which may mask other biological properties of the desired peptides, such as partial agonism or antagonism. We have addressed this problem in several papers, at the occasion of the synthesis of D-amino acid containing analogues of neuropeptide Y,[17] Bombesin,[19] neurotensin,[20] and somatostatin.[21,22] Development of RP-HPLC of unprotected peptides[23-25] was instrumental in demonstrating desired optical purity of peptide analogues in most cases. Using such routine techniques, we can now exclude contamination in the order of 1 in 1000 to 1 in 100 000, depending upon how well resolved the two peptides are in a given system. As a peptide chain lengthens, resolution obtained on HPLC becomes less and less, and final proof of optical purity is more difficult. Use of capillary zone electrophoresis, often found to be superior to HPLC for the resolution of unprotected peptides, has also been used here to discriminate between stereoisomers. In a series like the one presented here, and where consistent synthetic protocols were applied, one can first rely on the accepted assumption that SPPS is racemization free,[18] which does not exclude the possibility of optically contaminated starting D-amino acids or resort to other criteria to exclude such contamination. Coelution experiments of oCRF and each [D-Xaa^n]oCRF using both HPLC at 37 °C and CZE was carried out (see Kirby et al.[17] for further chromatographic details on the separation of diastereomers). HPLC data were gathered at 37 °C based on our earlier observation that better separation could be obtained with the Vydac support at elevated versus ambient temperatures. Worthwhile mentioning was the observation that cooling μ Bondapak columns (ice bath) made in the late 1970s resulted in better recovery of CRF activity and better resolution.[26] We explained these differences particularly

Table I. Chemical and Biological Characterization of the D-Series of oCRF

oCRF sequence:

Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2

no.	D-Xaa-substituted analogue	purity[a] (%)	RT under gradient[b] isomer/oCRF	ΔRT[c] (min) isomer − oCRF	$[\alpha]^{20}_D$[d] (deg)	relative potency in vitro[e]
1	oCRF	98			−77	1.0 (standard)
2	[D-Pro^5]-oCRF	95	28.60/28.60	UR	−62	0.08 (0.0–0.2)
3	[DIle^6]]-oCRF	98	27.83/27.83	(+)*	−69	<0.005
4	[DSer^7]]-oCRF	99	27.04/27.04	UR	−72	0.02 (0.0–0.1)
5	[DLeu^8]-oCRF	99	26.12/27.70	−1.58	−70	<0.005
6	[DAsp^9]-oCRF	99	27.25/27.25	UR	−72	0.01 (0.0–0.0)
7	[DLeu^{10}]-oCRF	99	25.58/27.66	−2.08	−76	0.23 (0.1–0.5)
8	[DThr^{11}]-oCRF	99	26.83/28.08	−1.25	−78	0.08 (0.0–0.1)
9	[DPhe^{12}]-oCRF	99	27.37/27.37	UR	−76	2 (0.9–5.5)
10	[DHis^{13}]-oCRF	95	27.62/27.87	−0.25	−76	<0.005
11	[DLeu^{14}]-oCRF	98	25.00/27.75	−2.75	−76	0.03 (0.0–0.1)
12	[DLeu^{15}]-oCRF	99	26.16/27.54	−1.38	−77	0.26 (0.1–0.6)
13	[DArg^{16}]-oCRF	99	26.08/28.25	−2.17	−77	0.01 (0.0–0.0)
14	[DGlu^{17}]-oCRF	98	26.45/27.41	−0.96	−79	0.35 (0.1–1.0)
15	[DVal^{18}]-oCRF	99	25.33/28.00	−2.67	−77	0.57 (0.0–1.3)
16	[Dleu^{19}]-oCRF	99	27.37/28.00	−0.63	−83	0.11 (0.1–0.2)
17	[DGlu^{20}]-oCRF	98	27.00/27.41	−0.41	−78	1.5 (0.6–4.2)
18	[DMet^{21}]-oCRF	96	26.95/27.37	−0.42	−78	0.05 (0.0–0.1)
19	[DThr^{22}]-oCRF	99	25.83/28.25	−2.42	−78	0.06 (0.0–0.1)
20	[DLys^{23}]-oCRF	98	32.91/28.41	4.5	−76	0.09 (0.0–0.2)
21	[DAla^{24}]-oCRF	98	28.33/28.33	(+)*	−80	0.8 (0.4–1.6)
22	[DAsp^{25}]-oCRF	99	29.20/28.45	0.75	−73	0.45 (0.3–0.8)
23	[DGln^{26}]-oCRF	98	28.95/29.85	UR	−77	0.41 (0.1–0.9)
24	[DLeu^{27}]-oCRF	97	29.66/29.66	UR	−77	0.30 (0.1–0.8)
25	[Dala^{28}]-oCRF	98	28.00/28.00	(+)*	−77	0.21 (0.1–0.7)
26	[DGln^{29}]-oCRF	99	27.62/28.12	−0.50	−75	0.18 (0.1–0.5)
27	[DGln^{30}]-oCRF	99	35.12/35.66	−0.54**	−76	0.04 (0.0–0.1)
28	[DAla^{31}]-oCRF	99	27.79/27.79	UR	−64	0.01 (0.0–0.4)
29	[DHis^{32}]-oCRF	95	28.25/28.25	UR	−72	0.58 (0.4–0.9)
30	[DSer^{33}]-oCRF	98	28.16/28.16	UR	−70	0.35 (0.2–0.8)
31	[DAsn^{34}]-oCRF	93	28.41/28.41	UR	−71	0.06 (0.0–0.2)
32	[DArg^{35}]-oCRF	99	28.54/28.54	UR	−69	0.05 (0.0–0.1)
33	[DLys^{36}]-oCRF	99	27.75/27.75	UR	−73	0.09 (0.0–0.2)
34	[DLeu^{37}]-oCRF	98	29.04/28.08	0.96	−75	0.02 (0.0–0.04)
35	[DLeu^{38}]-oCRF	98	28.87/28.00	0.87	−73	<0.005
36	[DAsp^{39}]-oCRF	99	27.04/27.04	UR	−73	0.16 (0.1–0.4)
37	[DIle^{40}]-oCRF	97	35.95/35.50	0.45**	−74	0.01 (0.0–0.0)
38	[DAla^{41}]-oCRF	91	27.33/27.33	UR	−78	0.01 (0.0–0.0)

[a] Percent purity as determined by HPLC analysis performed on a Vydac C_{18} analytical column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) at 37 °C; UV detection at 210 nm; 0.8 mL/min flow rate; [A] = 0.1% TFA in 5% MeCN, and % [B] = 0.1% TFA in 80% MeCN; gradient = 25% B–50 min–70% B. [b] Retention times obtained with the conditions shown under *a* for both CRF and the respective analogues are given. [c] Differences in retention times (ΔRT) obtained under *b* between CRF and the analogues are given: UR = unresolved with conditions shown under *a*; * has a shoulder; ** analysis performed using a 75-min instead of a 50-min gradient. [d] Measured on a Perkin-Elmer Model 241 polarimeter. *C* = 0.5 in 1% HOAc at room temperature. [e] Agonist potencies in vitro, based on the ability of analogue to stimulate ACTH secretion by cultured pituitary cells, were determined from parallel dose–response curves versus oCRF of single bioassays and calculated using BIOPROG.

notable for CRF (and other amphipatic peptides) on the basis of the presence (μ Bondapak) or absence (Vydac) of free silanols on the columns and possibly on pore size (100 Å for the former and 300 Å for the latter). While the difference in separation of the different stereomers at room temperature and at 37 °C was notable in the present study, it was not critical that the separations be carried out at elevated temperatures as compared to room temperature to achieve separation. From these studies, it could be determined that compounds **5**, **7**, **8**, **11–15**, **19**, **20**, **22**, **27**, **34**, and **35** had less than 1% contamination. From the biological results showing that compounds **3**, **5**, **10**, and **35** had less than 0.005 the potency of oCRF, one can infer that any other peptide in this series which contains D-Ile as in **3**, D-Leu as in **5** and **35**, or D-His as in **10**, would be racemization free, thus suggesting that biopotencies of **3**, **5**, **7**, **10–12**, **16**, **24**, **29**, **34**, **35**, and **37** are correct within the limits of the assay. If one further excludes peptides **2**, **9**, **14–18**, **21–26**, **29–33**, and **36**, which have potencies greater than or equal to 5% of that of oCRF, as such an extent of racemization is unlikely, one is left with four compounds (**4**, **6**, **28**, and **38**) for which there may be some uncertainty as to the extent of possible contamination by a partially racemized amino acid, a fact that would result in a greater potency than that of the optically pure analogue or that would mask partial or complete antagonism. Of the four compounds of undetermined optical purity, two were resolved from oCRF by CZE (**6** and **28**). It should be noted that many separations achieved by HPLC were later confirmed using CZE and that in all cases when separation was obtained no contamination by CRF was observed. This leaves us with only two analogues (**4** and **38**) with potencies of ca. 1–2% which could not be resolved from oCRF by either HPLC (under the conditions tested) or CZE using three different buffers (see Experimental Section).

From the relative biopotencies presented in Table I and Figure 1, it can be suggested that recognition by and

Table II. Chemical and Biological Characterization of Selected CRF Antagonists

SQEPPISLDLTFHLLREMLEMAKAEQEAEQAALNRLLLEEA-NH_2 α-hel-CRF

SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH_2 Human/rat-CRF

no.	compd	HPLC[a] (%)	Iso RV (at % CH_3CN)[b]	$[\alpha]^{20}_D$[c]	calcd mass[d] (Da)	obsd mass (m/z)[d]	rel potency antagonist[e]	in vitro IA[e] (%)
39	α-Hel-CRF_{9-41}[1]	98	9.2 (44.0)	−2	3827.43	3827.2	1.0 (standard)	18
40	[$Nle^{21,38}$]h/$rCRF_{9-41}$	99	10.1 (39.6)	−18	3869.51	3869.3	6.7 (2.9–15)	5
41	[$Nle^{21,38}$]h/$rCRF_{12-41}$	97	9.5 (36.0)	−27		n/a	12 (6–26)	0
42	[Ile^{21},Leu^{38}]h/$rCRF_{12-41}$	99	9.5 (35.7)	−27	3540.16	3539.8	9 (3.4–23.4)	
43	[Lys^{36}]-α-Hel-CRF_{9-41}	99	10.3 (38.0)	−1	3842.44	3842.0	2 (0.4–14)	16
44	[$Nle^{21,38}$,Arg^{36}]h/$rCRF_{9-41}$	99	9.1 (39.0)	−16	3897.53	3897.3	2 (0.9–3.6)	0
45	[$Nle^{21,38}$,Arg^{36}]h/$rCRF_{12-41}$	98	10.1 (36.6)	−24	3568.17	3567.9	7 (3.7–13)	0
46	[D-Phe^{12},$Nle^{21,38}$]h/$rCRF_{12-41}$	98	9.9 (38.3)	−21	3538.01	3537.9	18 (7–60)	21
47	[D-$2Nal^{12}$,$Nle^{21,38}$]h/$rCRF_{12-41}$	98	8.9 (39.0)	−26	3590.22	3589.8	9 (3.9–23)	6
48	[D-Arg^{12},$Nle^{21,38}$]h/$rCRF_{12-41}$	98	10.5 (35.3)	−27	3549.17	3548.8	0.23 (0.1–0.8)	
49	[D-Glu^{12},$Nle^{21,38}$]h/$rCRF_{12-41}$	95	9.5 (36.8)	−25		n/a	0.08 (0.0–0.1)	
							0.26 (0.1–0.7)	5
50	[D-Phe^{12},$Nle^{21,38}$,Arg^{36}]h/$rCRF_{12-41}$	98	10.4 (42.6)	−28	3568.17	3567.8	15 (8.4–28)	9
51	[D-Phe^{12},$Nle^{21,38}$,Leu^{36}]h/$rCRF_{12-41}$	98	9.0 (45.0)	−23	3525.14	3524.8	3 (1.6–6.0)	6

[a] Percent purity as determined by HPLC analysis performed on a Vydac C_{18} analytical column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) at 37 °C; UV detection at 210 nm; 0.8 mL/min flow rate; [A] = 0.1% TFA in 5% MeCN, and % [B] = 0.1% TFA in 80% MeCN/water; gradient = 25%B–50 min–70%B. [b] RV = retention volume for isocratic elution at specific percentage of acetonitrile. HPLC analysis accomplished using a Vydac C_{18} analytical column (0.46 × 25 cm), 5-μm particle size, 300-Å pore size; UV detection at 210 nm; 2.0 mL/min flow rate; and [A] = 0.1% TFA. [c] Measured on a Perkin-Elmer Model 241 polarimeter. C = 0.5 in 50% HOAc at room temperature. [d] The observed m/z of the unresolved peak was compared with the calculated $[M + H]^+$ average mass in Da. [e] Antagonist potencies *in vitro* based upon abilities of analogues to inhibit oCRF-mediated ACTH secretion by cultured rat anterior pituitary cells were determined from parallel dose–response curves versus α-helical $CRF_{(9-41)}$ = 1 with 95% confidence limits in parentheses.

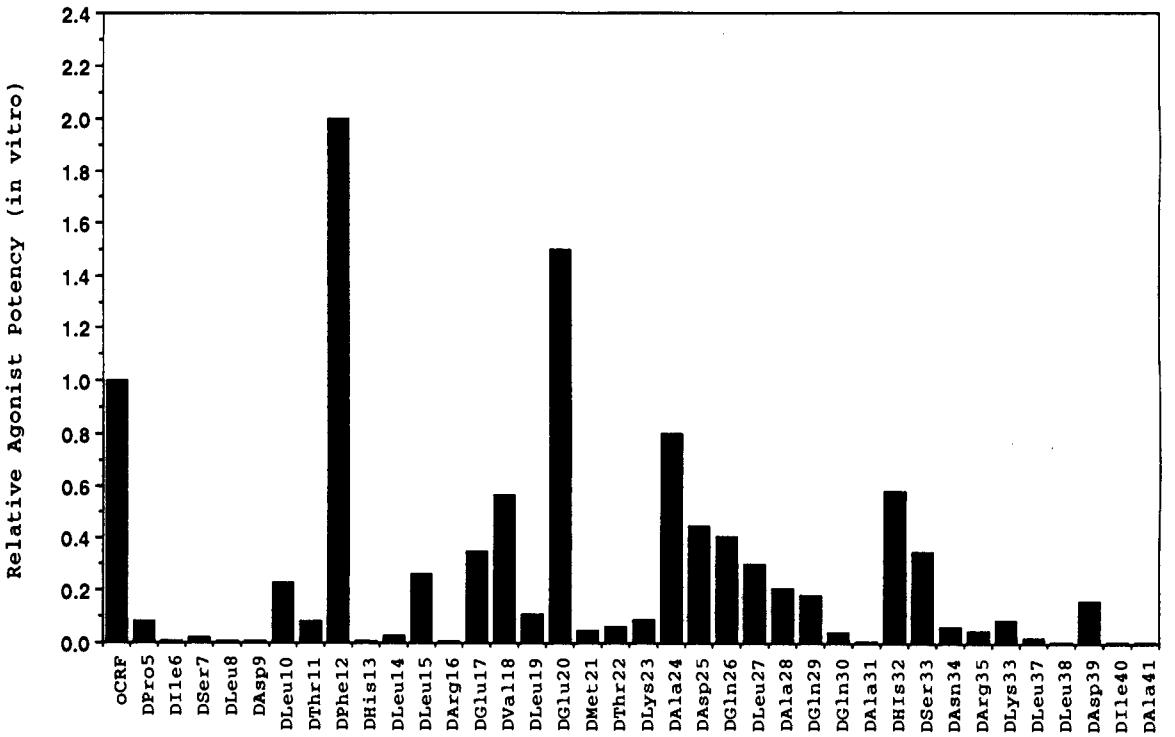

Figure 1. Graphic representation of the relative potency *in vitro* (oCRF = 1) of the agonists presented in Table I.

binding of the analogues to the CRF receptor (resulting in potencies greater than 20% but lower than 60% of that of oCRF) was conserved in [D-Leu^{10}]-, [D-Leu^{15}]-, [D-Glu^{17}]-, [D-Val^{18}]-, [D-Asp^{25}]-, [D-Gln^{26}]-, [D-Leu^{27}]-, [D-Ala^{28}]-, [D-Gln^{29}]-, [D-His^{32}]-, [D-Ser^{33}]-, and possibly [D-Asp^{39}]-oCRF. It has been suggested that increases in potency observed upon introduction of a D-amino acid within a sequence can be correlated with stabilization of a β-turn.[21] The corollary of this argument, one among several other possibilities, would be that CRF's conformation does not encompass a β-turn between residues 33 and 38, for example, since the D-substituted analogues are significantly less potent than oCRF. A β-turn in that stretch of the CRF structure had been postulated by Montecucchi et al.[27] and Pallai et al.[15] on the basis of Chou and Fassman's analysis. Recent NMR studies carried out in 66% trifluoroethanol/34% water suggested that the structure of hCRF comprised an extended N-terminal tetrapeptide connected to a well defined α-helix between residues 6 and 36. More specifically, residues 6–20 form an amphipatic α-helix. The five carboxy-terminal residues were found to be predominantly disordered.[16]

It is interesting to note that the aromatic residues Phe^{12} and His^{13} have previously been shown not to be involved in the receptor activation process of α-helical-CRF_{9-41}. However, introduction of the corresponding D-isomers into these positions has a marked effect on potency. The D-Phe^{12} analogue of oCRF is nearly twice as potent as the native peptide, whereas D-His^{13} shows no activity at all. All analogues reported in Table I exhibited full intrinsic

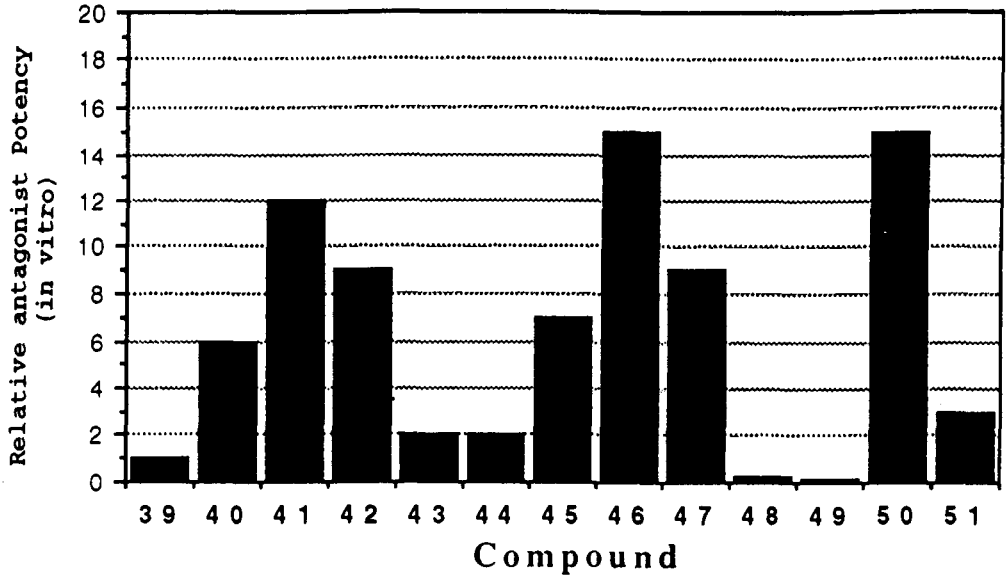

Figure 2. Graphic representation of the relative potency *in vitro* of the antagonists (α-helical-CRF$_{9-41}$ = 1) presented in Table II.

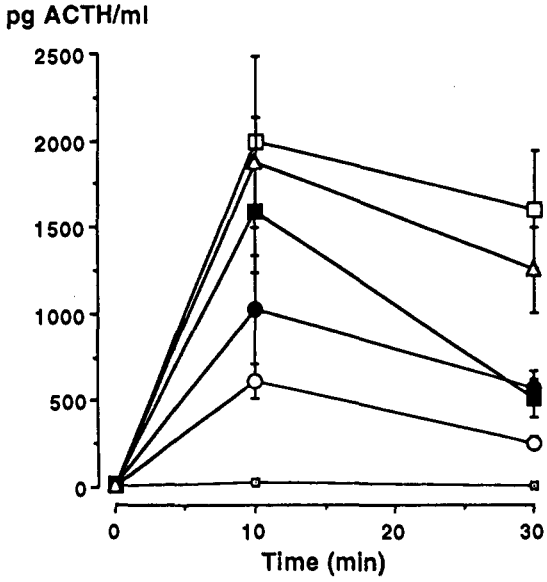

Figure 3. *In vivo* testing of agonists: control, small open square; 0.2 μg of oCRF (1), open circles; 1.0 μg of oCRF (1), closed circles; 5 μg of oCRF (1), open squares; 1.0 μg of **15**, closed squares; 1.0 μg of **9**, open triangles.

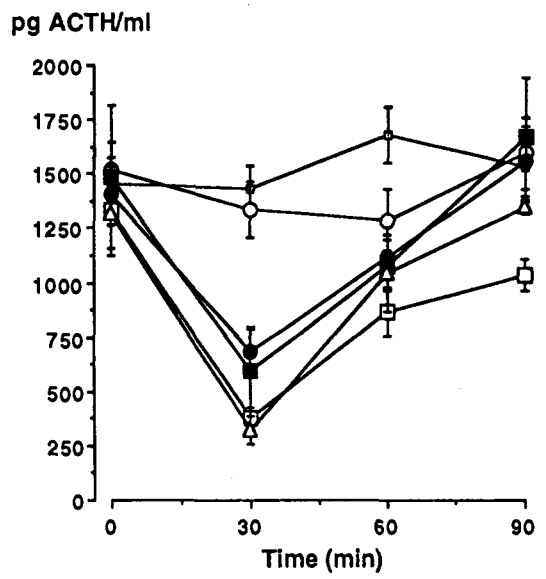

Figure 4. *In vivo* testing of antagonists: control, small open square; 0.1 mg of **39**, open circles; 0.5 mg of **39**, closed circles; 0.5 mg of **43**, open squares; 0.5 mg of **46**, closed squares; 0.5 mg of **50**, open triangles.

activity, thus excluding, *a priori*, the use of any of these modifications in the design of an antagonist. Two analogues in this series which showed retention of potency were tested *in vivo* for their ability to release ACTH (Figure 3), and **9** and **15** tested at 1 μg per rat intravenously (i.v.) were slightly more potent than oCRF with **9** being still active at 30 min, an activity that could only be matched by 5 μg of oCRF.

Antagonist structures presented in Table II and Figure 2 are derived from that of human/rat CRF (h/rCRF) with several substitutions introduced to answer particular questions. Replacement of Met at positions 21 and 38 by Nle and shortening of the chain from the N-terminus (**40**–**41**), resulted in an increase in potency. Substitution of Nle^{21,38} (**41**) by Ile^{21},Leu^{38} (**42**) has no significant effect on potency. An attempt to modulate a possible enzymatic cleavage site at position 36 by introduction of Lys in α-helical-CRF$_{9-41}$ resulted in a nonsignificant doubling of potency (**43**). Using the same rationale, Arg was introduced at position 36 to yield two analogues of different lengths (**44**–**45**). Here again, the shorter analogue is more potent.

We then investigated the effect of introducing the D-Phe^{12} substitution shown to increase potency of oCRF. We found **46** to be more potent than the parent **41** either as a result of a more favorable orientation of the Phe^{12} side chain or because of enzymatic stabilization against amino peptidases. We investigated whether the D-Phe^{12} side chain was the most favorable substitution by introducing new functionalities (increased hydrophobicity in **47**, basic functionality in **48**, and acidic functionality in **49** to arrive at the conclusion that all these modifications were highly detrimental). In compound **50** we introduced the Arg^{36} modification found in **45**; the biological data consistently showed that a basic residue in that position is favored which is further demonstrated by the loss of potency resulting from the introduction of Leu^{36} (**51**). In both series (CRF$_{9-41}$ and CRF$_{12-41}$), it is remarkable that introduction of Arg^{36} results in lowering of antagonist-potency, while introduction of D-Phe^{12} brings back high potency. Indeed, 75% inhibition of ACTH secretion by adrenalectomized rats upon iv administration of [D-Phe^{12}, Nle^{21,38},Arg^{36}]-h/rCRF$_{12-41}$ (**50**) (1.7 mg/kg mg) was ob-

Table III. Effects of α-Helical CRF_{9-41} (**39**) and [D-Phe^{12},Nle^{21,38},Arg^{36}]-CRF_{12-41} (**50**) on CRF-Induced Decrease in MAP and Increase of HR[a]

treatment	time (min)					
	−5	0	5	10	15	20
	MAP (mmHg)					
control	101 ± 3	103 ± 3	100 ± 2	100 ± 3	106 ± 3	104 ± 4
CRF, 2 nmol	99 ± 4	98 ± 3	81 ± 3**	80 ± 2**	84 ± 2**	90 ± 3
CRF + α-helical CRF, 2.4 nmol	97 ± 3	97 ± 4	98 ± 3	96 ± 2	98 ± 3	94 ± 2
CRF + **50**, 2.6 nmol	102 ± 4	103 ± 4	103 ± 2	100 ± 3	100 ± 4	96 ± 3
	HR (beats/min)					
control	382 ± 7	385 ± 12	373 ± 7	370 ± 14	380 ± 14	377 ± 18
CRF, 2 nmol	378 ± 6	386 ± 7	482 ± 11**	481 ± 10**	464 ± 11**	463 ± 7**
CRF + α-helical CRF, 2.4 nmol	397 ± 13	397 ± 12	388 ± 10	413 ± 8	419 ± 8	421 ± 9
CRF + **50**, 2.6 nmol	395 ± 11	387 ± 7	378 ± 8	425 ± 10	441 ± 17	446 ± 7

[a] Antagonist or vehicle was given iv 5 min prior to CRF injections (immediately after time = 0); *$P < 0.05$; **$P < 0.01$ compared to time = 0 values.

Table IV. Effects of α-helical CRF_{9-41} (**39**) and [D-Phe^{12},Nle^{21,38},Arg^{36}]-CRF_{12-41} (**50**) on CRF-Induced Increase of Plasma Concentrations of Epinephrine and Norepinephrine[a]

treatment	time (min)		
	0	10	20
	Epinephrine (pg/mL)		
control	132 ± 77	117 ± 48	107 ± 27
CRF, 1 nmol	145 ± 58	351 ± 74*	325 ± 51
CRF + α-helical CRF, 12 nmol	128 ± 51	207 ± 25	191 ± 38
CRF + **50**, 12 nmol	55 ± 22	128 ± 40	314 ± 100*
	Norepinephrine (pg/mL)		
control	290 ± 10	362 ± 44	381 ± 60
CRF, 1 nmol	355 ± 42	559 ± 77	705 ± 113*
CRF + α-helical CRF, 12 nmol	325 ± 47	486 ± 47*	412 ± 25
CRF + **50**, 12 nmol	344 ± 42	421 ± 54	520 ± 65*

[a] Antagonist or vehicle was given icv simultaneously with CRF after collection of the time zero sample; *$P < 0.05$; **$P < 0.01$ compared to time = 0 values.

tained within 30 min and lasted 90 min (Figure 4). The same dose of α-helical CRF_{9-41} (**39**) or [D-Phe^{12}, Nle^{21,38}]-h/$rCRF_{12-41}$ (**46**) caused a shorter lived (30 min) and less dramatic inhibition of ACTH secretion. In contrast, [Lys^{36}]α-helical-CRF_{9-41} (**43**) significantly ($P < 0.01$) decreased ACTH levels over a 90-min period.

Of some concern is the observation that most of these analogues still exhibit some residual intrinsic activity (IA) *in vitro*, the structural component(s) of which is (are) not yet well understood.

Because of the potential importance of CRF antagonists in the treatment of certain stress-related illnesses, it was important to characterize some of the most potent analogues in terms of their effects on blood pressure, heart rate, and CRF-induced increase in plasma concentrations of epinephrine and norepinephrine.

The effects of **39** and **50** on CRF-induced decrease of arterial pressure and heart rate are shown in Table III. In these experiments **39** and **50** given intravenously did not alter heart rate or arterial pressure but completely reversed the lowering of arterial pressure produced by CRF. It may be of some interest that **39** and to a lesser extend **50** prevented the increase of heart rate following intravenous administration of CRF.

The effects of **39** and **50** to affect CRF(icv)-induced increase of plasma concentrations of epinephrine and norepinephrine are shown in Table IV. At the doses tested **39** and **50** were effective to decrease epinephrine and norepinephrine following icv CRF.

Circular Dichroism Spectra. Each of the sequentially substituted D-isomer analogues of oCRF was studied using CD spectroscopy. Though not identical, the spectra produced from each analogue are quite similar. In an aqueous buffer, all exist predominantly in a random coil as seen by the spectrum of [D-Ala^{24}]oCRF (Figure 5) which is representative of the series. Successively increasing the amount of TFE in the solvent had the effect of increasing the helical content of each analogue (Figures 6 and 7). A protein secondary structure estimation program was used to analyze the spectra. Figure 8 shows the results of such an analysis plotted against the percentage (by volume) of TFE used in the solvent. The results shown for [D-His^{32}]-oCRF (**29**) are representative of the entire series. The fairly significant amount of β-sheet character in aqueous solution may be assigned to residues 6–16 which, by Chou-Fassman analysis, have a large potential for forming such structure. The final helical content obtained for the D-series analogues, i.e., that obtained in 100% TFE solvent, varied between 75 and 100% (±10%). It is not uncommon for a peptide to show a marked increase in helix content upon addition of an organic solvent (TFE) which facilitates intramolecular hydrogen bonding. For this reason, it is often argued that the helical form of a peptide is the biologically active form since a similar conformational change can be expected upon contact with a receptor site in a lipid-rich membrane environment. However, in the present study and within the certainty limits of our experimental data, no correlation has been found between the overall extent of helicity and biological activity.

Also, there does not seem to be any spectral indications of a β-turn within any of the analogues. One would expect that the inclusion of a type I or II β-turn region, several residues long, within a mostly helical peptide would produce a broadening and intensity reduction in the band centered at 192 nm along with a reduction in the intensity of the negative band located near 208 nm. This would be due to the weakly positive $\pi-\pi^*$ transition between 200 and 210 nm as well as the strong negative band around 190 nm which is usually associated with the CD spectra of β-turns.

In summary, oCRF analogues with monosubstitutions by D-amino acids have been synthesized and found to be between two times as potent to less than 0.005% as potent as CRF. On the other hand, shortened analogues of h/rCRF were found to be potent antagonists, and further studies are directed toward increasing the affinity of these molecules for the CRF receptor since it becomes evident that CRF antagonists could play a diagnostic, as well as a therapeutic, role in medicine.

Experimental Section

Peptide Synthesis. Peptides were made by the solid-phase approach. *tert*-Butyloxycarbonyl Nα protection (Boc) was used throughout. Side chains were protected as follows: benzyl esters (OBzl) for both aspartic and glutamic acids, benzyl ethers (OBzl)

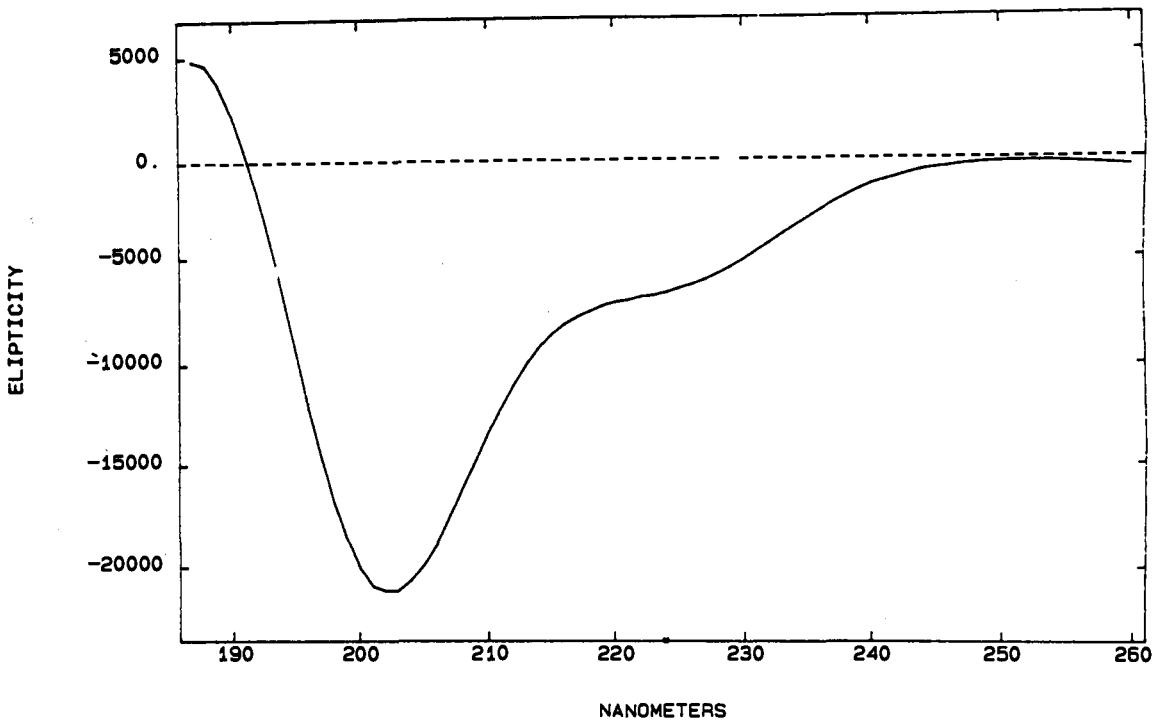

Figure 5. CD spectrum of [D-Ala[24]]oCRF in phosphate buffer. Units are mean residue elipticity.

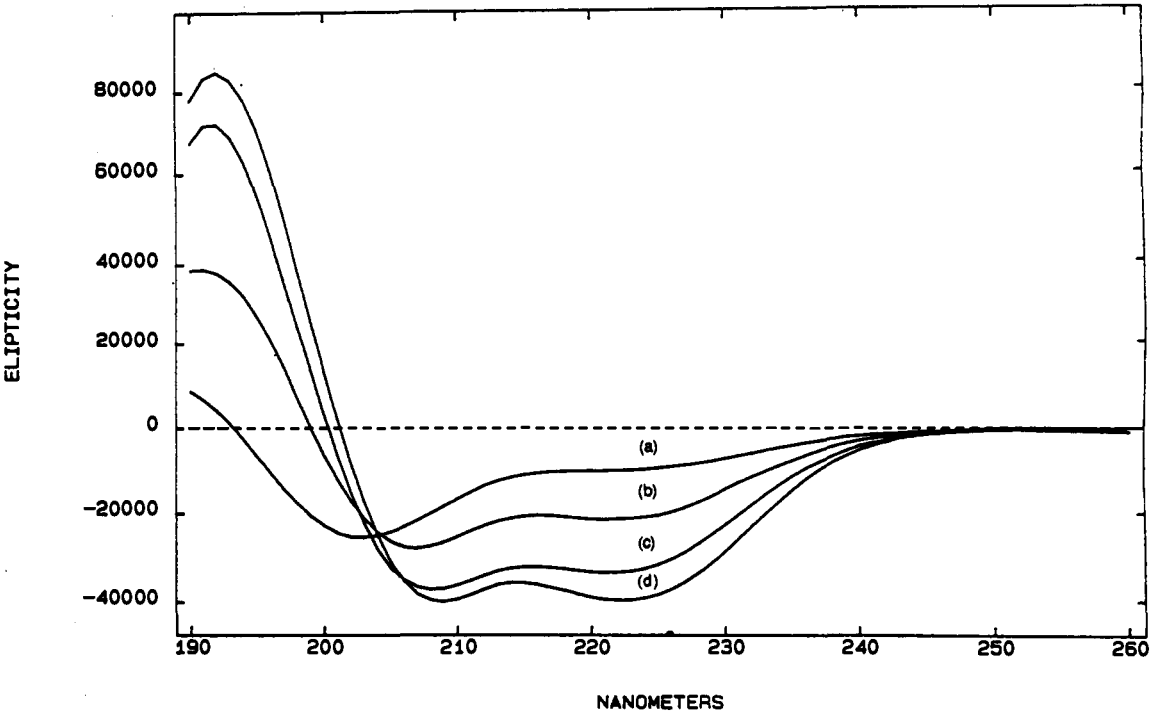

Figure 6. CD spectrum of [D-Phe[12]]oCRF in phosphate buffer/TFE: (a) 100% buffer; (b) 10% TFE; (c) 25% TFE; (d) 100% TFE.

for both serine and threonine hydroxyl groups, tosyl (Tos) for both histidine and arginine, and chlorobenzyloxycarbonyl (2ClZ) for lysine. Couplings (2 h) were mediated by dicyclohexylcarbodiimide in DCM or dimethylformamide in the presence of 2 equiv of HOBt for Asn, Gln, and Arg(Tos). A 3-fold excess of amino acid based on the original substitution of the resin was used. Double couplings were automatically performed at residues 14, 18, and 40. Two trifluoroacetic acid (60% in DCM, 5% ethanedithiol) deblockings were performed at each step (10 and 15 min, respectively). An isopropyl alcohol (1% ethanedithiol) wash followed TFA treatment prior to the two TEA (12% in DCM) neutralization steps (which included an intermediate MeOH wash). Each coupling step was followed by acetylation [10% $(CH_3CO)_2O$ in DCM for 15 min]. HF cleavage was performed in the presence of cresol (5–10%) and methyl ethyl sulfide (2–5%) for *ca.* 20 min at −20 °C and 40 min at 0 °C.

For purification of the synthetic peptides,[24,25] cartridges fitting Waters Associates preparative liquid chromatograph 500 (LC-500) were packed with 15–20-μm C_{18} silicas (300 Å, Vydac). A gradient of MeCN in TEAP buffer was generated. Crude peptides (directly after HF cleavage and lyophilization) were loaded (1–3 g per run) through the pump; cuts were made, analyzed, and often rerun under different conditions until pure product was obtained. Desalting of the purified fractions, independently checked for purity, was achieved by using a gradient of MeCN in 0.1% TFA. The center cut was lyophilized to give the desired product with a 10–15% yield.

Peptide Characterization. HPLC conditions are found in the legends of Tables I and II.

CZE analysis was accomplished using a fused silica capillary (50 cm × 75 μm) at 30 °C; UV detection at 214 nm; 20 kV constant, 102 μA; 0.1 M triethylammonium phosphate buffer, pH 2.50. In

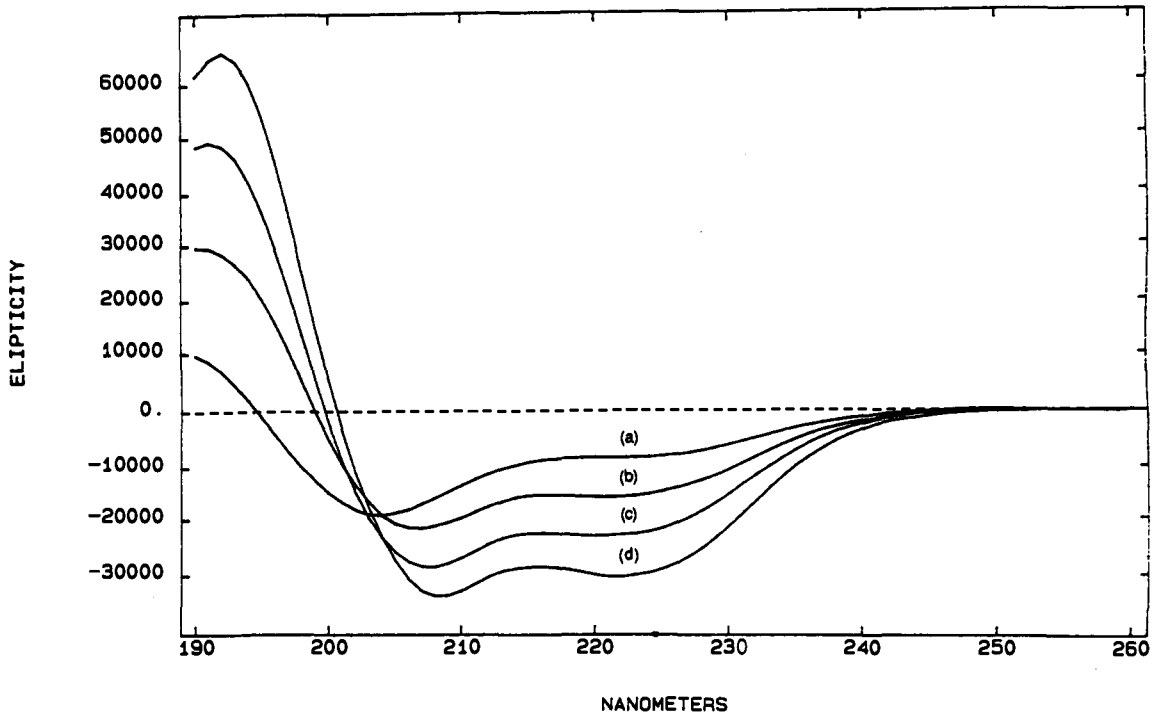

Figure 7. CD spectrum of [D-Lys[36]]oCRF in phosphate buffer/TFE: (a) 100% buffer; (b) 10% TFE; (c) 25% TFE; (d) 100% TFE.

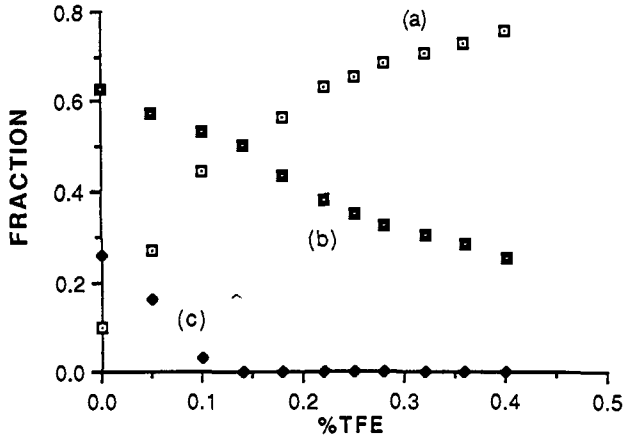

Figure 8. Conformational fraction versus percent TFE for [D-His[32]]oCRF: (a) α-helix; (b) unordered; (c) β-sheet.

addition to the above buffer the following were also used: 10 mM sodium phosphate/6 mM sodium borate, pH 7.0 (10 kV constant, 12 μA) and 0.1 M sodium phosphate, pH 3.50 (15 kV constant, 86 μA).

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 of 30 kV were employed. The sample was added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The centroid of $Cs(CsI)_n$ cluster peaks was used to calibrate the magnetic field scans. The spectra were measured at a nominal resolution of 1000. The observed m/z reported is based on a calculated peak centroid at a 5% threshold level determined using the Complement data analysis program (JEOL, Tokyo, Japan). The observed m/z for analogs 1–38 were within ±0.4 Da of the calculated average mass of 4671.39.

CRF *in Vitro* Assay. CRF analogues were tested for agonist and/or antagonist activity in an *in vitro* assay measuring basal or CRF-induced ACTH release.[28]

CRF *in Vivo* Assay. The effect of the intravenous injection of CRF agonists on ACTH secretion in the normal rat was carried out in intact male rats implanted with an indwelling venous catheter.[4,29] Data are shown in Figure 3. The effect of the intravenous injection of CRF antagonists on ACTH secretion by adrenalectomized rats is illustrated in Figure 4.

Cardiovascular Effects After Peripheral Administration. The effects of CRF antagonists on CRF-induced decrease of arterial pressure and increase of heart rate were tested in awake male Sprague–Dawley rats prepared with chronic iliac artery and jugular venous catheters. Pulsatile arterial pressure was recorded continuously with a Beckman Dynograph Recorder. Heart rate was obtained from direct counting of pulsatile wave forms. Mean arterial pressure was calculated using the formula MAP = (pulse pressure/3) + diastolic pressure. Peptides were dissolved in sterile saline and administered by an intravenous bolus.

Catecholamine Secretion After Central Administration. The effects of CRF antagonists on CRF-induced increase of catecholamines was tested in awake rats prepared with chronic lateral cerebroventricular (icv) cannulae and jugular venous catheters. Icv injections were made in 5-μL volumes. Mixed venous blood samples (400 μL) were collected 10 min after icv injection of test substances. Plasma levels of norepinephrine and epinephrine were measured with the single isotope radioenzymatic assay of Peuler and Johnson described by Fisher et al.[30]

Statistical Analyses. All experiments were performed using a randomized-block design with each groups consisting of six to eight rats. Data were evaluated using one-way analysis of variance, and significant treatment effects were assessed with the multiple range tests of Dunnett and Duncan.

Circular Dichroism Studies. Circular dichroism spectra were recorded at room temperature using an Aviv Associates (Lakewood, NJ) Model 62 DS spectrometer. Sample solutions were prepared by dissolving weighed amounts of peptide in either 0.01 M phosphate buffer (pH 7.2), 2,2,2-trifluoroethanol (TFE) (Aldrich 32,674-7), or a mixture of the two. Concentrations were determined using the same instrument by comparison of optical densities at 190 and 215 nm with those of selected analogues whose concentrations were known from amino acid analysis. Qualitative aspects of the spectra were found to be independent of peptide concentration within the range examined, 15–30 μM.

Peptide secondary structure was estimated using software available from Aviv and designed for use with their instruments. The program, which is derived from the algorithm of Yang et al.,[31] fits an idealized basis set of three spectra representing α-helix, β-sheet, and β-turn structure to recorded spectral data. The remaining structure is constrained to be random coil so that the molecular fractions of the four types of structure add to unity. Because of the inherent limitations of programs of this sort, e.g., assuming that each type of secondary structure has a single characteristic CD spectrum, its output is best used in looking at overall trends rather than in assigning absolute structure.

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