Synthesis and Relative Potencies of New Constrained CRF Antagonists†

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Two series of CRF antagonists with N^{α}- and C^{α}-methylated alanine and leucines were evaluated for their biological activities *in vitro* and *in vivo* in several systems. The poly-N-methylated analogue of α-helical-CRF_{9-41}, [N^{α}MeLeu^{10,15,27,37},N^{α}MeAla^{22,32,41}]-α-Hel-CRF_{9-41}, was found to be considerably less potent than the parent non-N-methylated analogue. This result was expected on the basis that α-helicity was thought to be required for biological activity and the prediction that backbone substitutions on the nitrogen have a tendency to break α-helices (a hypothesis that was confirmed by circular dichroism). Next, a series of constrained analogues of the potent CRF antagonist, [DPhe^{12},Nle^{21,38}]h/$rCRF_{12-41}$, was synthesized that contained C^{α}-methylleucine and/or C^{α}-methylalanine (Aib) residues at selected positions. Because C^{α}-methylation is recognized to increase α-helicity, and because there is now strong NMR data suggesting that residues 6–36 assume a well-defined α-helix, it was expected that these analogues would be more potent. Although usual solid-phase peptide synthesis procedures were followed, success in coupling the C^{α}-methyl amino acids was obtained only with a 1:1 mixture of BOP/HOBt. *In vitro* potencies of the synthesized compounds were measured in a collagenase-dispersed anterior pituitary cell culture bioassay. Monosubstituted analogues were shown to be twice to one fourth as potent as the parent compound; while the pluri-substituted peptides were slightly less potent. This decrease in potency might be correlated to an unexpected lower helical content of the pluri-substituted compounds (as determined by CD spectroscopy), as it was suggested that the bioactive conformation of the CRF was predominantly α-helical. Interestingly, one analogue, [DPhe^{12},Nle^{21,38},C^{α}-MeLeu^{37}]h/$rCRF_{12-41}$, was found to be more potent and longer acting than the parent compound in two *in vivo* assays measuring ACTH release after intravenous administration to adrenalectomized rats and reversal of stress-induced delay in gastric emptying in the rat after intracisternal administration. The molecular basis for this increased duration of action and potency is being investigated.

Introduction

CRF, whose presence in hypothalamus extracts was demonstrated in 1955 by Guillemin and Rosenberg[1] and Saffran and Schally,[2] was first isolated and characterized in 1981 from ovine hypothalamus by Vale et al.[3] This 41 amino acid, C-terminally amidated peptide was primarily recognized as the principal mediator for the stress-induced release of the adenohypophyseal peptides derived from the pro-opiomelanocortin, essentially ACTH and β-endorphin. However, the discovery of CRF in other regions of the central nervous system (CNS)[4] and in peripheral tissues[5] indicated other physiological functions. Therefore, a wide variety of effects on the autonomic nervous system and on various aspects of behavior interestingly related to the body's response to stress was established making CRF a general initiator and coordinator of this response.[6-11] Growing evidence indicates that CRF is also involved in mediating the effects of various stressors on gut motor function.[12-15] CRF injected into the cerebrospinal fluid mimics the effects of certain forms of stress on gastrointestional transit, most markedly by inhibiting gastric emptying of a non-nutrient meal and increasing colonic transit in rats and mice.[13,14,16,17] This CRF action is CNS-mediated through the autonomic nervous system independently from activation of the pituitary–adrenal axis.[14,16,18] Further, injection of the selective CRF antagonist, α-helical CRF_{9-41}, into the cerebrospinal fluid or specific hypothalamic nucleus prevented restraint and surgical stress-induced delays in gastric emptying in rodents.[13,19,20] In the present study, we compared the dose-related reversal of postoperative gastric ileus induced by intracisternal injection of α-helical-CRF_{9-41} and the newly developed [DPhe^{12},Nle^{21,38},C^{α}-MeLeu^{37}]h/$rCRF_{12-41}$.

While we initiated a program of analogue design and synthesis immediately after we discovered the structure of ovine CRF, there are very few reports on SAR of CRF. One major contribution was the discovery that deletion of the N-terminal 8–14 residues led to antagonists. The design of the first potent antagonist, α-helical-CRF_{9-41},[21] was based on the assumption that the biologically active form of the peptide was predominantly α-helical, as suggested by secondary structure predictions,[22,23] CD

† **Abbreviations.** The abbrevations 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 L-isomer except when indicated otherwise. In addition: ACTH, adrenocorticotropin hormone; Aib, aminoisobutyric acid; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; Bzl, benzyl ester; CD, circular dichroism; C_{18}, octadecyl; CNS, central nervous system; CRF, corticotropin releasing factor; CZE, capillary zone electrophoresis; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; EDT, ethanedithiol; α-Hel-CRF, α-helical CRF; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; h/rCRF, human/ratCRF; icv, intracerebroventricularly; ic, intracisternally; iv; intravenous; MBHA, 4-methylbenzhydrylamine; MeCN, acetonitrile; Nle, norleucine; OcHx, cyclohexyl ester; OPA, orthophthalaldehyde; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, retention time; SEM, standard error of the mean; TEA, triethylamine; TEAP 2.25, triethylammonium phosphate with pH adjusted to 2.25; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tos, *p*-toluenesulfonyl; 2ClZ, 2-chlorobenzyloxycarbonyl.

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studies,[23] lipid bilayer-binding experiments,[24] and recent NMR studies carried out in 66% TFE/34% water.[25]

In the preceding article, we described a series of antagonists derived from the human/rat CRF: one analogue, [DPhe^{12},Nle^{21,38}]h/rCRF$_{12-41}$, was shown to be ca. 15 times more potent *in vitro* than α-helical-CRF$_{9-41}$.[26] In order to maximize the α-helix forming potential of h/rCRF analogues, we substituted selected alanine and leucine residues by their C$^{\alpha}$-methyl derivatives. These α-helix inducing amino acids[27] could also provide the peptide with a higher resistance to biodegradation.[28-31] Results from the testing of 20 new antagonists *in vitro* and of a few selected ones in two *in vivo* systems after intravenous and intracisternal administration are described.

Results and Discussion

Synthesis and Characterization. Generally, the solid-phase synthesis of the C$^{\alpha}$-substituted CRF analogues followed usual procedures with the exception of the C$^{\alpha}$-methyl amino acids which needed special attention due to their particular properties. Firstly, coupling of these sterically hindered residues to the peptide-resin were much less efficient than for usual amino acids.[32] Furthermore, in the case of Boc-Aib, a secondary reaction due to the "*gem*-dimethyl" effect, which is known to accelerate intramolecular cyclization,[33,34] would explain the encountered difficulties.[35] There have been several reports on the synthesis of Aib or other C$^{\alpha}$-alkyl amino acid containing peptides. Methodologies for the introduction of these residues included DCC, DCC/HOBt,[36] the oxazolone method,[32] the use of the 2-(trifluoromethyl)-4,4-dialkyl-oxazolin-5(4*H*)-one,[37] preformed di- or tripeptides containing the hindered amino acid[36] in the case of solution synthesis and Woodward's reagent K,[38] preformed active ester with HOBt,[38] or two 4-h couplings with 6-fold excesses of Boc-amino acid and DCC[39] in the case of solid phase synthesis. In the present study, repeated couplings with Woodward's reagent K, DIC, BOP-Cl, or BOP (at room temperature or 40 °C) and a large excess of C$^{\alpha}$-methyl amino acid were not efficient enough for the synthesis of these 30 residue peptides, and in particular for the pluri-substituted ones, to obtain satisfactory yields. Finally, a 1:1 mixture of BOP/HOBt (pH 9–10, 2 h, 7 equiv of C$^{\alpha}$-methyl amino acid instead of 3.5 for usual residues) was shown to be the most suitable procedure, giving complete or almost complete peptide bond formation after the first coupling. Indeed, Hudson,[40] studying the relative efficiencies of a wide range of coupling reagents in solid-phase peptide synthesis, showed that the use of BOP/HOBt resulted in a dramatic improvement in rate and magnitude of activation compared to BOP alone or other reagents. The speed of coupling reactions is often critical as side reactions were reported when an attempt to couple Boc-Aib to Aib-OBzl with BOP-Cl gave a low yield of the dipeptide while the starting acid was quantitatively consumed.[35] Secondly, the α-amino function of the C$^{\alpha}$-methyl amino acids are significantly less reactive. This results in very low color yields with ninhydrin (under standard conditions, ninhydrin color yield of Aib and C$_{\alpha}$-MeLeu relative to Leu are 7.4 and 9.4%, respectively, as determined by Brückner et al.).[41] This property results in less accurate monitoring of the subsequent coupling step by the Kaiser test.[42] We systematically repeated this particular step three times with large excesses of Boc-amino acid and DIC and then deblocked an aliquot of the resin with TFA. Although the coupling extent was not quantified, observation of a strong positive Kaiser test and HPLC analysis of the final crude peptides indicated that this procedure was efficient enough and that, as observed by Hardy and Lingham[37] for dipropylglycine, less difficulties are encountered when coupling amino acids to the α-amino group of C$^{\alpha}$-alkyl amino acids than the other way around. The remaining free amino groups (when present) were finally blocked by acetylation. Thirdly, the low reactivity of the C$^{\alpha}$-methyl amino acids explains their particular behavior during amino acid analysis. Under the conditions used, amino acids are postcolumn derivatized with *o*-phthalaldehyde (OPA), and the reaction time is of the order of 1 min. However, Aib and C$^{\alpha}$-MeLeu react considerably more slowly than the α-amino acids and would need a 10- and 15-min reaction time, respectively, to reach their maximum fluorescence.[41] As a result, Aib gave a very low but quantifiable signal, while C$^{\alpha}$-MeLeu could not be detected in our system. A precolumn derivatization would appear to be necessary, but we envisage that other inefficiencies would result due to the relative instability of some OPA-adducts like OPA-glycine.[41] So, amino acid analysis of C$^{\alpha}$-methyl amino acid-containing peptides was coupled to mass spectrometry to confirm their structure (see Table I).

The peptides were obtained in highly purified form (greater than 97%). Generally, yields ranged from 3 to 17% (the lowest values were obtained for the pluri-substituted analogues). An exceptionally high 32% yield was obtained for one analogue, [DPhe^{12},-Nle^{21,38}C$^{\alpha}$-MeLeu^{37}]h/rCRF$_{12-41}$. This high yield and the high purity of the final product (99%) can be explained by a more efficient synthesis. For instance, building of the segments Gln^{29}-Gln^{30} and more particularly Asn^{34}-Arg^{35} of h/rCRF analogues was observed to be fastidious in our laboratory; with a C$^{\alpha}$-MeLeu in position 37, introduction of these residues (Gln^{29}-Gln^{30} and Asn^{34}-Arg^{35}) was completed without any recoupling, suggesting a favorable, sequence-dependent effect.

Biological Experiments. *In Vitro* Studies. The compounds were tested for their ability to antagonize CRF-induced release of ACTH by rat pituitary cells in culture. As summarized in Table I, the N$^{\alpha}$-methyl-containing peptide **2** is essentially inactive. It is clear that this inactivity could be explained by one single unfavorable substitution. However, from the alanine series we know that substitutions by an alanine at positions 22, 32, and 41 yield analogues that are three times, four times, or as potent as CRF, respectively, while the N$^{\alpha}$MeLeu at positions 10, 15, 27, and 37 replace leucines in the original structure. This suggests that the loss of activity results from the introduction of the N$^{\alpha}$Me substitution and not from the alteration of the side chain. This hypothesis was investigated using CD. Results presented in Figure 1 clearly demonstrate the lack of α-helicity even in the presence of 39% TFE which is known to induce such a structural motif. These results strongly suggest that while α-helicity may not be sufficient for biological activity, it may in fact be a necessary constraint. The positive control is to demonstrate that introduction of a C$^{\alpha}$-methylated residue results in either increased or unaltered biological potency. We therefore synthesized a series of mono C$^{\alpha}$-methyl-substituted analogues. With respect to their potencies, these analogues fell in three groups (see Table I). The first group consists of compounds **5** and **6** which are approximately twice as potent as the standard. The

Table I. Chemical and Biological Characterization of the CRF Antagonists

human/rat CRF

1 5 10 15 20 25 30 35 40

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

no.	compd	purity[a] (%)	RT[b] (min)[b]	$[\alpha]_D$[c]	calcd[d] (Da)	obsd[d] (m/z)	rel potency in vitro: antagonist[e]	rel potency in vitro: IA[f] (%)
1	α-HeI-CRF_{9-41}				3827.43	3827.2	0.12 (0.04–0.29)	14
2	[N^{α}MeLeu^{10,15,27,37},N^{α}MeAla^{22,32,41}]-α-Hel-CRF_{9-41}	99		-99	3909.66	3909.3	0.0 (0.0–0.0)	0
3	[DPhe^{12},Nle^{21,38}]h/$rCRF_{12-41}$		13.8	-70	3538.01	3537.9	1.0 (standard)	7
4	[DPhe^{12},C^{α}MeLeu^{14},Nle^{21,38}]h/$rCRF_{12-41}$	95	14.9	-54	3554.19	3553.8	1.0 (0.5–2.3)	7
5	[DPhe^{12},C^{α}MeLeu^{15},Nle^{21,38}]h/$rCRF_{12-41}$	95	16.4	-49	3554.19	3554.0	2.3 (1.0–6.0)	
6	[DPhe^{12},C^{α}MeLeu^{17},Nle^{21,38}]h/$rCRF_{12-41}$	99		-55	3538.23	3537.7	2.3 (1.2–4.7)	
7	[DPhe^{12},Nle^{21,38},C^{α}MeLeu^{37}]h/$rCRF_{12-41}$	99	14.5	-57	3554.19	3553.8	1.0 (0.5–2.0)	13
8	[DPhe^{12},C^{α}MeLeu^{17,37},Nle^{21,38}]h/$rCRF_{12-41}$	99	16.3	-43	3552.26	3552.2	1.0 (0.5–1.8)	9
9	[DPhe^{12},Nle^{21,38},Aib^{20}]h/$rCRF_{12-41}$	99		-37	3496.15	3495.1	0.23 (0.1–0.49)	9
10	[DPhe^{12},Nle^{21,38},Aib^{22}]h/$rCRF_{12-41}$	97	15.5	-61	3554.19	3553.9	0.74 (0.36–1.6)	16
11	[DPhe^{12},Nle^{21,38},Aib^{24}]h/$rCRF_{12-41}$	95	14.5	-55	3554.19	3554.0	0.65 (0.27–1.7)	14
12	[DPhe^{12},Nle^{21,38},Aib^{26}]h/$rCRF_{12-41}$	99		-37	3495.15	3495.1	0.04 (0.008–0.13)	29
13	[DPhe^{12},Nle^{21,38},Aib^{2}18]h/$rCRF_{12-41}$	98	14.3	-65	3554.19	3553.8	1.0 (0.44–2.4)	16
14	[DPhe^{12},Nle^{21,38},Aib^{29}]h/$rCRF_{12-41}$	95		-31	3495.01	3494.8	0.36 (0.2–0.7)	
15	[DPhe^{12},Nle^{21,38},Aib^{31}]h/$rCRF_{12-41}$	98	14.1	-54	3554.19	3553.9	0.35 (0.2–0.6)	21
16	[DPhe^{12},Nle^{21,38}Aib^{32}]h/$rCRF_{12-41}$	97		-27	3486.01	3485.7	0.6 (0.28–1.2)	
17	[DPhe^{12},Nle^{21,38},Aib^{33}]h/$rCRF_{12-41}$	98		-24	3536.03	3535.7	0.9 (0.48–1.9)	
18	[DPhe^{12},Nle^{21,38},Aib^{34}]h/$rCRF_{12-41}$	99		-24	3509.02	3509.0	0.69 (0.4–1.2)	18
19	[DPhe^{12},Nle^{21,38},Aib^{39}]h/$rCRF_{12-41}$	98		-27	3494.02	3493.8	0.28 (0.13–0.66)	
20	[DPhe^{12},Nle^{21,38}Aib^{40}]h/$rCRF_{12-41}$	98		-28	3509.98	3509.4	0.5 (0.2–1.3)	31
21	[DPhe^{12},C^{α}MeLeu^{14},Nle^{21,38},Aib^{24,28,31}]h/$rCRF_{12-41}$	99	17.0	-41	3596.27	3596.2	0.12 (0.1–0.2)	23
22	[DPhe^{12},C^{α}MeLeu^{15},Nle^{21,38},Aib^{24,28,31}]h/$rCRF_{12-41}$	99	15.1	-49	3596.27	3596.1	0.32 (0.2–0.7)	8

[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); UV detection at 210 nm; 1.5 mL/min flow rate; [A] = 0.1% TFA in H_2O, and % [B] = 0.1% TFA in MeCN/H_2O (60:40); gradient = 40–30 min–70% B. [b] Retention times obtained on 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; [A] = 0.1% TFA in H_2O, and % [B] = 0.1% TFA in MeCN/H_2O (60:40); gradient = 50–20 min–70% B.[c] c = 1.0 in 1% HOAc at room temperature. [d] The observed m/z of the unresolved peak was compared with the calculated $[M + H]^+$ average mass (Da). [e] Potencies expressed relative to [DPhe^{12},Nle^{21,38}]$rCRF_{12-41}$ with 95% confidence limits. [f] Intrinsic Activity.

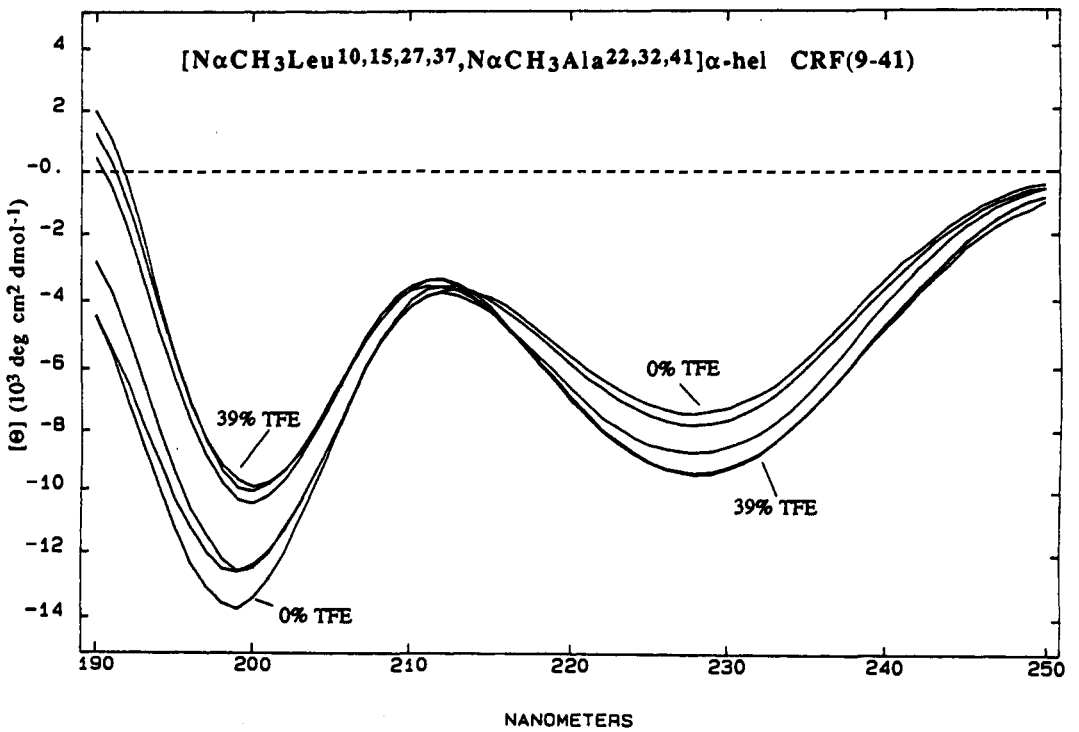

Figure 1. CD spectra of compound 2 in the absence and presence of increasing concentrations of trifluoroethanol (TFE).

second group consists of compounds **4**, **7**, **8**, **10**, **11**, **13**, **16–18**, and **20** which did not differ significantly in potency from their parent compound **3**, suggesting that the local conformational restrictions imposed by the α-methyl group were tolerated by the receptor. The third group consisting of compounds **9**, **12**, **14**, **15**, and **19** had potencies one third to one fourth that of **3**. This difference in potencies is relatively small and also suggests that such substitutions are tolerated relatively well. As one C^{α}-methyl amino acid may not influence the conformation of the entire peptide,[31,43] analogues **21** and **22** containing different combinations of supposedly tolerated substitutions were prepared. These compounds were 10 and three times less potent than **3**, indicating that the C^{α}-methyl moieties may hinder some contacts with the receptor or constrain the molecule in a less favorable conformation. In order to explain these results from a conformational point of view, CD measurements were undertaken. CD spectra of

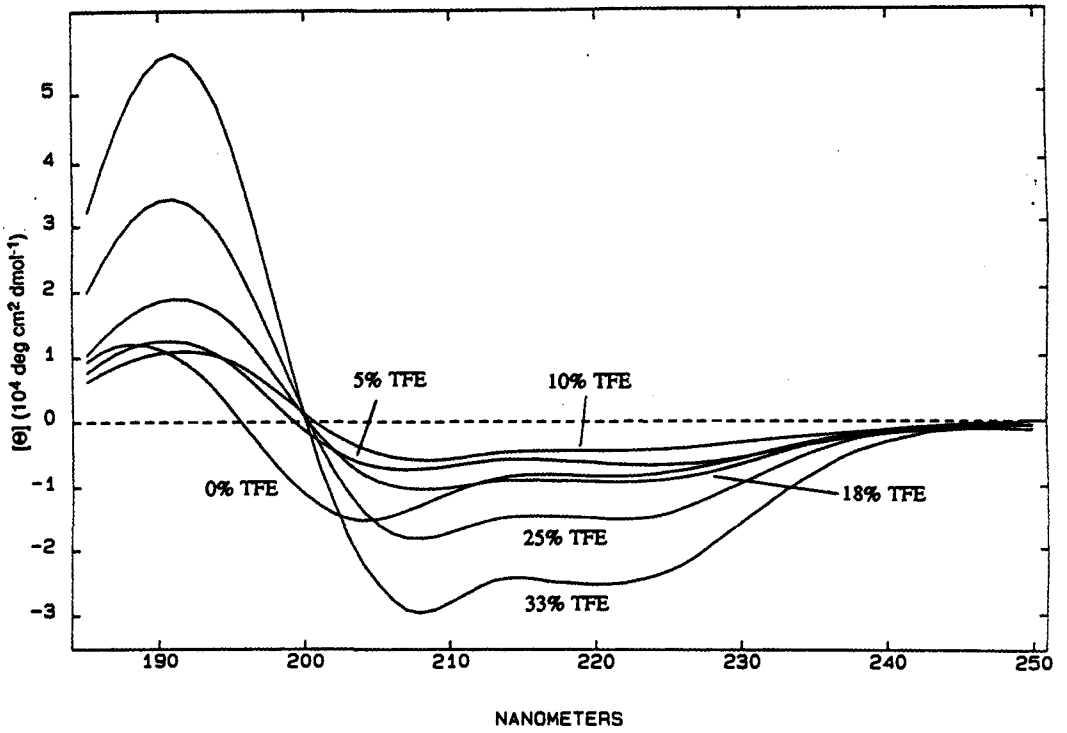

Figure 2. CD spectra of compound 3 in the absence and presence of increasing concentrations of trifluoroethanol (TFE).

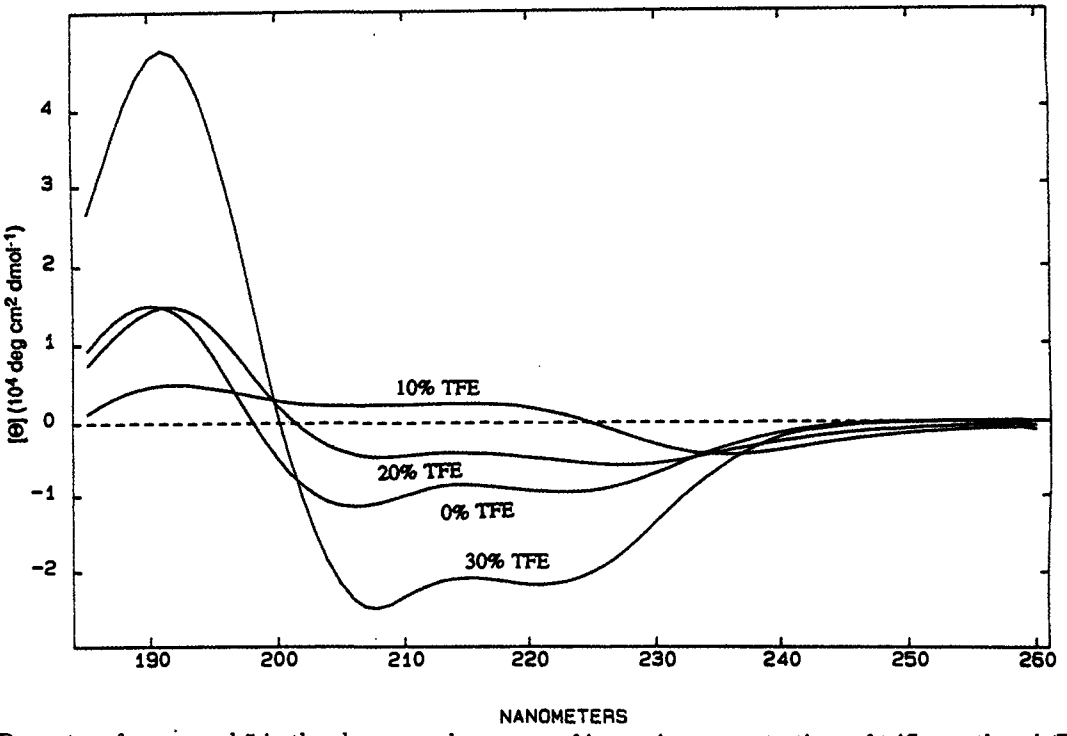

Figure 3. CD spectra of compound 5 in the absence and presence of increasing concentrations of trifluoroethanol (TFE).

compounds 3, 5, and 21 in the absence and in the presence of increasing concentrations of trifluoroethanol (TFE) are shown in Figures 2–4. The content of secondary structures was estimated from these spectra using Prosec, derived from a program described by Chang et al.,[44] and are presented in Table II. Although compounds 5 and 21 possess a slightly higher helical content than 3 in 0% TFE–buffer, they showed an unusual behavior when the TFE concentration was increased; at 10% TFE, a marked decrease in helical structure was observed for 5 with a concomitant increase in β-structure. This result might not be surprising as C^{α}-methyl amino acids were already implicated in the stabilization of β-conformation.[45,46] At higher TFE percentages, we observed an increase in the helical content, equivalent to that found in 3 in the case of the analogue 5 but at a lower degree for compound 21. This result could partly explain the lower potency of the pluri-substituted analogues. However, helical content is certainly not the only parameter responsible for these variations; for instance, pluri-substituted analogues of human GRF showed an important stabilization of their helical structure but possessed a low *in vitro* potency.[47] Another interesting point is the possible presence of a β-turn in 21 in 0% TFE–buffer, which disappears in 20% TFE. Stabilization of a β-turn by C^{α}-methyl amino acids,

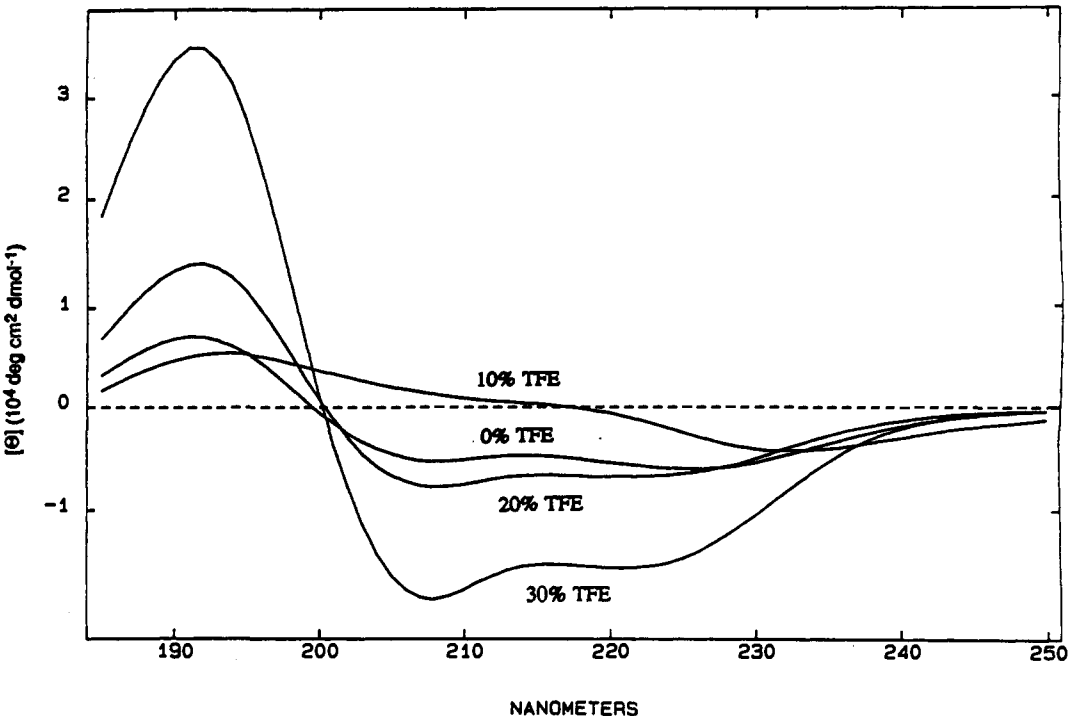

Figure 4. CD spectra of compound **21** in the absence and presence of increasing concentrations of trifluoroethanol (TFE).

Table II. Secondary Structure Estimation of Compounds **3**, **5**, and **21** by Using Prosec, Derived from a Program Described by Chang et al.[44]

compd	% TFE	helix (%)	β-sheet (%)	β-turn	random coil
3	0	13	38	0	50
	5	16	53	2	29
	10	12	69	0	20
	18	25	47	0	28
	25	45	18	0	37
	33	69	0	0	31
5	0	23	33	4	41
	10	3	81	4	12
	20	18	60	2	21
	30	63	0	0	37
21	0	18	40	12	30
	10	10	66	9	15
	20	17	59	0	24
	30	47	17	0	36

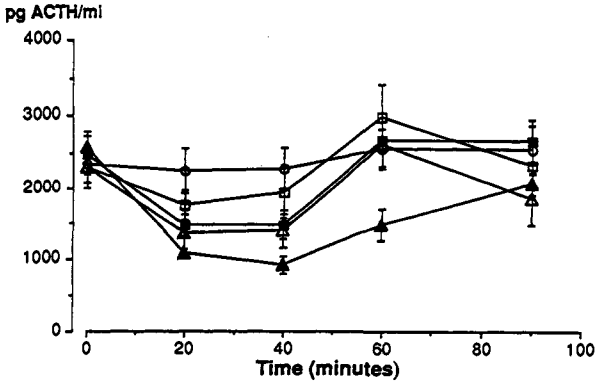

Figure 5. Effect of compounds **3** and **7** on ACTH secretion in ADX rats: control, open circles; 0.1 mg of **3**, open squares; 0.5 mg of **3**, closed squares; 0.1 mg of **7**, open triangles; 0.5 mg of **7**, closed triangles.

as in Aib-containing enkephalin analogues, has already been reported.[36,45,48-50]

In Vivo Studies. **ACTH Secretion in the Adrenalectomized Rat.** The compounds were tested *in vivo* in adrenalectomized rats for duration of action. Generally, the monosubstituted as well as the pluri-substituted analogues showed potency and duration of action similar to that observed for the parent compound (results not shown). As an exception to this series, compound **7**, possessing a C^{α}-methylleucine in position 37, was found to be longer acting than **3**. As shown in Figure 5, **7** (at a dose of 1.7 mg/kg) was still significantly active 60 min after injection while the ACTH level returned to control value with the same dose of **3**. The most likely explanation for this result would be a greater resistance of the peptide to biodegradation. Other C^{α}-methyl amino acid-containing peptides were shown to be stable to peptidases and proteases.[29,31] In addition, C^{α}-MeLeu[37] is adjacent to a basic doublet, Arg[35]-Lys[36] and this sequence motif is known to be an important target for enzymatic hydrolysis. Considering this basic doublet as a possible inactivation site of CRF, introduction of a C^{α}-methyl residue would impede efficient contact between this region and the enzyme. This assumption would need an *in vitro* and/or *in vivo* study of CRF inactivation to be confirmed as other unknown parameters might also account for the observed effect.

Reversal of Stress-Induced Delay in Gastric Emptying. In the control group receiving the oropharyngeal administration of methyl cellulose, the 20-min rate of gastric emptying was 60.0 ± 4%. Gastric emptying values were decreased to 16.5 ± 3.1% as measured 3 h after surgery in rats injected intracisternally with vehicle. The CRF antagonists, α-helical CRF_{9-41} (13 nmol) and **7** (2.6 nmol), injected intracisternally before abdominal surgery reversed by 55–59% the inhibitory effect of surgery (Figure 6). These results confirm the potent inhibitory effect of abdominal surgery on gastric emptying in rats[20,51] and the ability of central injection to normalize the postoperative gastric stasis.[20] In addition, the comparison of the antagonistic potency of **7** and **1** indicated that both have the same intrinsic activity as shown by the same levels of antagonistic effect to reverse stress-induced delay in gastric emptying; however, **7** is about 5-fold more potent than

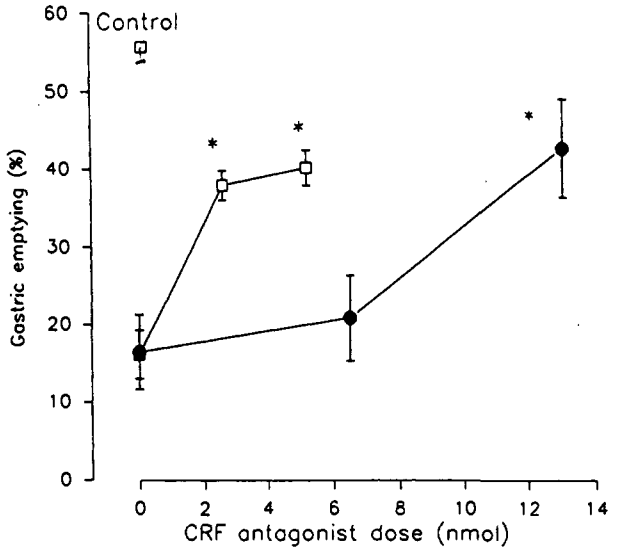

Figure 6. Partial reversal of abdominal surgery-induced gastric ileus in rats 3 h postsurgery. Dose response curve: α-hel-CRF_{9-41} (1) (closed circles) and 7 (open squares).

α-helical CRF_{9-41}. Intracisternal injection of 7 at 2.6 nmol induced a similar antagonistic effect as 13 nmol of 1 which was inactive at a dose of 6.5 nmol.

In conclusion, our attempts at developing more potent and long-acting CRF antagonists using C^{α}-methyl amino acids was successful in one instance. The design of even more potent analogues would benefit from the identification of the inactivation sites of CRF and the introduction at these sites of particular C^{α}-methyl amino acids. Of interest was the fact that these special residues did not bring detectable conformational changes in the expected way (i.e., stabilization of helical structure as monitored by CD).

Experimental Section

Peptide Synthesis. All reagents and solvents were analytical grade (Aldrich Chemical Co., Milwaukee, WI; Fluka Chemie AG, Switzerland; Fisher Scientific, Springfield, NJ; Mallinckrodt Inc., Paris, KY; Halocarbon, Hackensack, NJ) and were used without further purification. Boc-amino acids were purchased from Bachem, Torrance, CA, and included Aib, Asn, Gln, Arg(Nγ-tosyl), Asp(β-OcHex), Glu(γ-OcHex), Lys(ε2ClZ), Ser(Bzl), and His(N^{im}-tosyl). Boc-C^{α}-MeLeu was synthesized in three steps: DL-C^{α}-MeLeu was prepared by the method of Counsell et al.[52] and then resolved as the N^{α}-TFA derivative with a suspension of carboxypeptidase A (EC 3.4.17.1, type II from bovine pancreas, 70 units/mg protein purchased from Sigma Chemical Co., St Louis, MO),[53] and the resulting L-amino acid was N^{α}-protected with di-*tert*-butyl dicarbonate by the usual procedure. BOP reagent was synthesized as described by Castro et al.[54] MBHA resin (substitution of 0.43 mmol/g) was prepared by the method of Rivier et al.[55]

Peptides were manually synthesized on MBHA resin. Boc-amino acids were coupled via diisopropylcarbodiimide in DCM and/or DMF. Exception was the C^{α}-methyl amino acids which were introduced with an equimolar mixture of BOP/HOBt in DMF at pH 9–10 (3 equiv of DIPEA were used). Deblocking was accomplished with 60% TFA in DCM in the presence of 1% EDT for 25 min. Each step was monitored by the Kaiser test.[42] The protected peptide–resin was cleaved in liquid HF in the presence of anisole (1.5 mL/g of resin) 0 °C for 1 h. The crude peptides were precipitated with anhydrous diethyl ether and extracted from the resin with water and the aqueous solutions were then lyophilized.

Peptide Purification. Analytical-grade acetonitrile from Mallinckrodt, Paris, KY (for preparative RR-HPLC), acetonitrile UV from Burdick and Jackson, Muskegon, MI (for analytical RR-HPLC), distilled TFA (Halocarbon), and distilled TEA (Aldrich) were used in the preparation of buffers.

Crude peptides were purified by preparative reversed-phase HPLC in two or three steps as described earlier.[55,56] The gradient conditions used for purification were determined by analytical HPLC. The analytical system used comprised two Waters pumps M45, a Waters automated gradient controller, a SF 769Z Kratos Spectroflow 773 UV detector (detection was made at 210 nm), a Rheodyne 7125 injector, a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 30-nm pore size), and a Houston Instruments Omniscribe chart recorder. The preparative HPLC system comprised a Waters 500A Prep LC, a Waters preparative gradient mixer, a Waters 450 variable-wavelength detector (detection at 230 nm), a Waters 1000 PrepPak module, and an Omniscribe chart recorder. The cartridges were hand-packed in Waters polyethylene sleeves and frits and Vydac bulk C_{18} material, 15–20-μm particle size, 300-Å pore size. The crude lyophilized peptides (1–1.5 g) were dissolved in TEAP pH 2.25 buffer (ca. 200 mL), and the solutions were filtered prior to loading onto a C_{18} cartridge. Peptides were eluted with linear TEAP pH 2.25/MeCN gradients. Fractions (50–100 mL) were collected and monitored by isocratic analytical HPLC. Enriched fractions were pooled, diluted, and reloaded. In some cases, a second purification step (with different selectivity) in TEAP pH 5.2 was necessary. Peptide fractions greater than 95% pure were desalted with a linear gradient of 0.1% TFA/MeCN. Fractions containing the purified peptides were pooled and lyophilized.

Peptide Characterization. Purified peptides were subjected to HPLC analysis in two mobile-phase systems (0.1% TFA/MeCN and TEAP pH 2.25/MeCN) and were shown to be more than 95% pure.

Amino acid analysis of the peptides was performed following hydrolysis in 4 N methane sulfonic acid at 110 °C for 24 or 48 h as described[57] and gave the expected integer values for all amino acids except C^{α}-MeLeu.

Optical rotations of peptides were measured in 1% acetic acid (c = 1) on a Perkin-Elmer 241 polarimeter and were not corrected for peptide content.

Circular dichroism spectroscopy of the peptides was performed on an AVIV CD spectrometer, Model 62 DS (Lakewood, NJ).

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 were 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).

***In Vitro* Assay.** Peptides were tested for their ability to inhibit 1 nM CRF-induced ACTH secretion by rat anterior pituitary cells in monolayer culture.[3,21] Briefly, after 3–5 days, cultured rat pituitary cells were incubated with compounds at several doses for 3 h (n = 3). ACTH levels in the assay medium were determined by double antibody RIA. Relative potencies were derived from dose–response cruves using the BIOPROG program.

In Vivo Assays. Adrenalectomized (ADX) Rats. Adult male Sprague–Dawley rats (230–250 g) were adrenalectomized via a lombar approach under Metofane anesthesia. Their diet was supplemented with 0.9% NaCl in the drinking water and oranges. Two days prior to the experiments, the animals were equipped with jugular cannulae as previously described.[58] On the morning of the experiments, the iv cannulae were connected to a line filled with heparinized saline, the rats were placed in individual buckets, and left undisturbed for 2 h. For the experiment, a first blood sample was withdrawn (0.3 mL), the test solution was injected (in a 1-mL volume), and subsequent blood samples were obtained at times 20, 40, 60, and 90 min. The bloods were centrifuged and the separated plasma were kept frozen (−20 °C) until assayed for ACTH values. Plasma ACTH levels were measured by RIA.[59]

Peptides were first diluted in sterile distilled water, and the pH was adjusted to 7.0. Further dilutions were made in 0.04 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.01%

ascorbic acid. Results were analyzed by analysis of variance followed by Duncan's multiple range test.

Reversal of Stress-Induced Delay of Gastric Emptying. Adult male Sprague–Dawley rats (Bantin and Kingman, CA, and Harlan, 200–240 g) were housed in group cages, fed Purina rodent chow, and kept in bioprotected rooms with a 12-h light–dark cycle. All animals were quarantined for 1 week after shipping and then deprived of food but not water 15 h before each experiment.

Treatments. α-Helical CRF_{9-41} (1) and 7 were dissolved in doubly distilled HPLC water at pH 7.0 warmed at 37 °C. Peptides at various doses (10 μL volume) were injected immediately before abdominal surgery using an Hamilton syringe in a stereotaxic instrument into the cisterna magna by puncture of the occipital membrane. A control group received intracisternal injection of vehicle with surgery and another group no treatment or anesthesia.

Abdominal Surgery. The abdominal surgery consisted of a midline celiotomy and cecal exteriorization with cecal handling in saline soaked gauze for one minute. This standard procedure has been reported elsewhere.[21] The cecum was then returned to the abdominal cavity and the linea alba closed with 0000 sil suture. The skin was then closed in a second layer.

All procedures were performed under enflurane (Ethrane–Aanquest) vaporized anesthesia. Vapor concentration was 5.5%, and total anesthesia exposure was timed to 10 min.

Measurement of Gastric Emptying. Conscious rats received 160 min after the end of abdominal surgery a phenol red methyl cellulose solution, and gastric emptying was measured 20 min later as previously described.[20] In brief, a suspension of continuously stirred 1.5% methylcellulose (Sigma) and phenol red (0.5 mg/mL, Sigma) was given by oropharyngeal intubation (1.6 mL) to conscious rats. Following a 20-min emptying period the rats were sacrificed by CO_2 inhalation. After opening the abdominal cavity and clamping the gastroesophageal junction and pylorus, the stomach and the residual contents were removed. The stomach and contents were placed in 0.1 N NaOH (100 mL) and homogenized for 30 s (Polytron, Brinkmann Instruments). The suspension was allowed to settle for 45 min to allow air bubble removal. The supernatant (5 mL) was added to 20% trichloroacetic acid (wt/vol) (1 mL). Solid debris was removed by centrifugation at 3100 rpm at 4 °C for 20 min. The supernatant was mixed with 0.5 N NaOH (4 mL), and the absorbance of the sample was read at 560 nm (Shimazu 250 Spectrophotometer). Phenol red recovered from animals sacrificed immediately after delivery of the methylcellulose solution served as 0% emptying standards (reference). Percent emptying was calculated by: (A560 reference - A560 sample) 100/A560 reference.

Statistics. Comparisons of groups were performed using one-way ANOVA. All tests were performed using the NCSS statistics program. A P value of less than 0.05 was regarded as significant.

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