Human Growth Hormone-Releasing Hormone hGHRH(1−29)-NH_2: Systematic Structure−Activity Relationship Studies†

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Two complete and two partial structure−activity relationship scans of the active fragment of human growth hormone-releasing hormone, [Nle^{27}]-hGHRH(1−29)-NH_2, have identified potent agonists in vitro. Single-point replacement of each amino acid by alanine led to the identification of [Ala^8]-, [Ala^9]-, [Ala^{15}]- (Felix et al. *Peptides 1986* **1986**, 481), [Ala^{22}]-, and [Ala^{28},Nle^{27}]-hGHRH(1−29)-NH_2 as being 2−6 times more potent than hGHRH(1−40)-OH (standard) in vitro. Nearly complete loss of potency was seen for [Ala^1], [Ala^3], [Ala^5], [Ala^6], [Ala^{10}], [Ala^{11}], [Ala^{13}], [Ala^{14}], and [Ala^{23}], whereas [Ala^{16}], [Ala^{18}], [Ala^{24}], [Ala^{25}], [Ala^{26}], and [Ala^{29}] yielded equipotent analogues and [Ala^7], [Ala^{12}], [Ala^{17}], [Ala^{20}], [Ala^{21}], and [Ala^{27}] gave weak agonists with potencies 15−40% that of the standard. The multiple-alanine-substituted peptides [$MeTyr^1$,$Ala^{15,22}$,Nle^{27}]-hGHRH(1−29)-NH_2 (**29**) and [$MeTyr^1$,$Ala^{8,9,15,22,28}$,Nle^{27}]-hGHRH(1−29)-NH_2 (**30**) released growth hormone 26 and 11 times, respectively, more effectively than the standard in vitro. Individual substitution of the nine most potent peptides identified from the Ala series with the helix promoter α-aminoisobutyric acid (Aib) produced similar results, except for [Aib^8] (doubling vs [Ala^8]), [Aib^9] (halving vs [Ala^9]), and [Aib^{15}] (10-fold decrease vs [Ala^{15}]). A series of cyclic analogues was synthesized having the general formula cyclo(25−29)[$MeTyr^1$,-Ala^{15},Xaa^{25},Nle^{27},Yaa^{29}]-GHRH(1−29)-NH_2, where Xaa and Yaa represent the bridgehead residues of a side-chain cystine or [*i*-(*i*+4)] lactam ring. The ring size, bridgehead amino acid chirality, and side-chain amide bond location were varied in this partial series in an attempt to maximize potency. Application of lactam constraints in the C-terminus of GHRH(1−29)-NH_2 identified cyclo(25−29)[$MeTyr^1$,Ala^{15},$_D Asp^{25}$,Nle^{27},Orn^{29}]-hGHRH(1−29)-NH_2 (**46**) as containing the optimum bridging element (19-membered ring) in this region of the molecule. This analogue (**46**) was 17 times more potent than the standard. Equally effective was an [*i*-(*i*+3)] constraint yielding the 18-membered ring cyclo(25−28)[$MeTyr^1$,Ala^{15},Glu^{25},Nle,$^{27}Lys^{28}$]-hGHRH-(1−29)-NH_2 (**51**) which was 14 times more potent than the standard. A complete [*i*-(*i*+3)] scan of cyclo(*i*,*i*+3)[$MeTyr^1$,Ala^{15},Glu^i,$Lys^{(i+3)}$,Nle^{27}]-hGHRH(1−29)-NH_2 was then produced in order to test the effects of a Glu-to-Lys lactam bridge at all points in the peptide. Of the 26 analogues in the series, 11 had diminished potencies of less than 10% that of the agonist standard, 4 were weak agonists (15−40% relative potency), and 4 analogues were equipotent to the standard. The 7 most potent analogues ranged in potency from 3 to 14 times greater than that of the standard and contained the [*i*-(*i*+3)] cycles between residues 4−7, 5−8, 9−12, 16−19, 21−24, 22−25, and 25−28. The combined results from these systematic studies allowed for an analysis of structural features in the native peptide that are important for receptor activation. Reinforcement of the characteristics of amphiphilicity, helicity, and peptide dipolar effects, using recognized medicinal chemistry approaches including introduction of conformational constraints, has resulted in several potent GHRH analogues.

Introduction

Since its isolation from a human pancreatic islet tumor in 1982,[1,2] human growth hormone-releasing hormone (hGHRH, hGRH, hGRF) has been the target of several structure−activity relationship (SAR) studies. The quest for more potent and stable analogues of GHRH relies on information gained only through bioactivity and potency feedback since a representation of the drug−receptor interaction is not yet available. Sequence homologies vary from 93% to 40% for GHRH in human, bovine, porcine, ovine, caprine, murine, rat, carp, and salmon with greater homology among the higher mammals (see review).[3] GHRH interacts with a single high-affinity class of receptors,[4−6] and one study indicates the additional presence of a low-affinity, high-saturation site.[7] The receptor has recently been cloned and shown to be a G-protein-coupled 7-transmembrane helix receptor in the secretin family,[8−10] and the gene has been assigned to chromosome 7 (7p14).[11] Aside from abnormal, ectopic production of GHRH, normal distri-

† IUPAC rules are used for nomenclature of peptides. Additional abbreviations: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; Cpa, 4-chlorophenylalanine; CRF, corticotropin-releasing factor; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GH, growth hormone; GHRH, growth hormone-releasing hormone; HBTU, *O*-(benzotriazol-1-yl)-*N*, *N*, *N*′, *N*′-tetramethyluronium tetrafluoroborate; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; MBHA, methylbenzhydrylamine resin; NMP, *N*-methylpyrrolidinone; NMR, nuclear magnetic resonance; OFm, *O*-fluorenylmethyl; PTH, parathyroid hormone; RIA, radioimmunoassay; RPHPLC, reverse-phase high-performance liquid chromatography; SAR, structure−activity relationship; SDS, sodium dodecyl sulfate; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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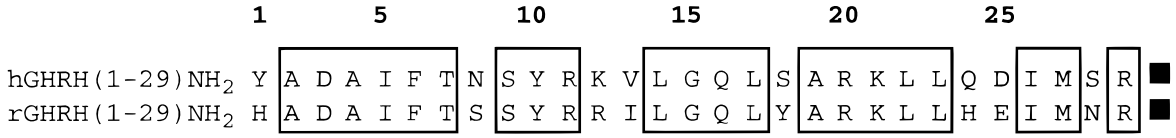

Figure 1. Sequences of human (h) and rat (r) GHRH(1−29)-NH_2 showing 70% homology (common residues are boxed).

bution is limited to a few sites in the hypothalamus.[12] It has been indicated that GHRH regulates its own receptor production[13] and induces growth hormone (GH) gene transcription independently of its stimulation of GH secretion.[14] Growth hormone secretion occurs in pulsatile fashion with GHRH directly controlling the GH pulse amount and somatostatin controlling the GH pulse frequency.[15] Other physiologic factors and chemical messengers mediate a complex feedback mechanism (i.e., insulin-like growth factor-I, IGF-I)[16,17] along the endocrine cascade that is still not fully understood (see reviews).[16,18,19] When the system goes awry, degenerative conditions such as acromegaly, somatotroph hyperplasia, dwarfism, and diabetes mellitus develop. Since GHRH is the primary physiological growth hormone secretagogue, it is a desirable target to study. Development of potent agonists/antagonists of GHRH would potentially facilitate reversal of GH-mediated disease states. Targeting conditions for controlled release of endogenous GH may also have the advantage of avoiding the negative side effects attributed to GH therapy.

The GHRH amino termini of 27 and 29 residues were found to be the shortest, fully bioactive fragments of the native peptide.[1,20] As a result, most subsequent research has been performed with GHRH(1−29)-NH_2 (Figure 1). Positions 1−4 are highly sensitive to substitution and most likely comprise the receptor activation site of the peptide.[21−26] The secondary structures of linear GHRH analogues have been evaluated by circular dichroism (CD) and 2D NMR, in combination with constrained molecular dynamics calculations, to be helical between residues 7−14 and 21−28 in water at pH 3 and between residues 4−26 in 75% aqueous methanol at pH 6.[27] Similarly, maximal helicity of the peptide occurs between residues 6−13 and 16−29 in 30% aqueous TFE at pH 4.[28,29] Several similar studies in aqueous organic solvents and aqueous SDS and phospholipid micelle environments are in agreement with these results.[30−34] These physicochemical data largely support the hypothesis that GHRH interacts with its receptor in a helical conformation; additionally, there is high probability that the amphiphilic nature of GHRH induces helical conformation in the receptor/membrane environment such as that mimicked by aqueous organic solvents in ^{1}H NMR and CD studies.

Results and Discussion

Synthesis. Peptides were assembled using the *tert*-butoxycarbonyl (Boc)-amino acid strategy on a *p*-methylbenzhydrylamine (MBHA) resin,[35,36] either manually or on a Beckman 990 synthesizer. Briefly, couplings were mediated by DIC, BOP, HBTU, or TBTU in dichloromethane (DCM), dimethylformamide (DMF), or *N*-methylpyrrolidinone (NMP) for 2 h. The N^{α}-Boc groups were removed with TFA, and the 9-fluorenylmethoxycarbonyl (Fmoc) side-chain protecting groups were removed with 20% piperidine in DMF or NMP. Lactam cyclization was performed after Fmoc deprotection of the side chains of the bridgehead residues by the method of Felix et al.[37] or by substituting HBTU or TBTU for BOP. All peptides were cleaved and deprotected with hydrogen fluoride. Air oxidation was used for disulfide bridge formation in dilute 25% acetonitrile−water at neutral pH. Purification was achieved to >95% purity, in most cases, via RP-HPLC on a C_{18} column with a gradient of TEAP/CH_3CN buffers at pH 2.25 and 6.5 followed by 0.1% TFA/CH_3CN buffers.[38] The purified peptides were characterized by quantitative HPLC, capillary zone electrophoresis (CZE), and mass spectral analysis (see Tables 1−4). Circular dichroism was performed on an AVIV model 62DS CD spectrophotometer as described in the Experimental Section. Biological testing was accomplished by treating collagenase-dispersed rat anterior pituitary cells with graded doses of a standard and the unknowns and then measuring the level of GH secretion by GH radioimmunoassay (RIA).[39] Potencies were determined against the reference standard hGHRH(1−40)-OH (agonist) or [MeTyr^1,D-Arg^2,Cpa^6,Nle^{27}]-rGHRH(1−29)-NH_2 (antagonist).

Alanine Scan. We first report the full characterization (Table 1) and the in vitro relative potencies (rat pituitary cell culture assay) of the alanine scan of [Nle^{27}]-hGHRH(1−29)NH_2, which were presented in preliminary form at the 12th American Peptide Symposium.[40] Of the 26 mono-Ala-substituted analogues synthesized, 11 showed potency greater than 100% when compared to the standard hGHRH(1−40)-OH. The Ala^8, Ala^9, Ala^{15}, Ala^{22}, and Ala^{28} substitutions resulted in the most potent analogues with 2−6 times the potency of the standard in vitro. Comparing the potencies of these five analogues to that of the parent peptide, [Nle^{27}]-hGHRH(1−29)NH_2 (**2**), we found **15** (first reported by Felix et al.)[41] and **21** to be the only two significantly more potent analogues. The Ala^{16}, Ala^{18}, Ala^{24}, Ala^{25}, Ala^{26}, and Ala^{29} substitutions resulted in analogues equipotent to the standard. Substitution of aromatic (Tyr^1, Phe^6, Tyr^{10}), polar (Asp^3, Arg^{11}), or certain hydrophobic (Ile^5, Val^{13}, Leu^{14}, Leu^{23}) residues by alanine resulted in analogues with drastically reduced potencies. None of these peptides (**3**−**6**, **10**, **11**, **13**, **14**, and **22**) were found to be antagonists when retested in an antagonist bioassay (see Experimental Section). The remaining six substituted residues (Ala^7, Ala^{12}, Ala^{17}, Ala^{20}, Ala^{21}, Ala^{27}) yielded weak agonists with potencies 15−40% that of the standard. The same qualitative trends were seen using a binding assay and different preparations of the Ala-substituted peptides by Lefrancois et al.,[42] while the IC_{50} values for Ala^7, Ala^{12}, Ala^{16}, Ala^{17}, Ala^{18}, Ala^{20}, Ala^{21}, Ala^{22}, Ala^{24}, Ala^{25}, Ala^{26}, and Ala^{28} were higher than expected when compared to our data. A lack of degradation by proteolysis in the cell culture assay was an explanation presented by the authors referenced above for the

Table 1. Alanine Scan of [Nle^{27}]-hGHRH(1−29)-NH$_2$

			mass[d]		HPLC purity, %[e]	
no.	substitution[a]	rel potency[b] in vitro	obsd	calcd	TEAP	TFA
1	hGHRH(1−40)-OH	1.00 (standard)	4542.4	4542.3	(94)[f]	>99
2	[Nle^{27}]-hGHRH(1−29)-NH$_2$	2.38 ± 0.41[c]	3338.9	3338.9	(97)[f]	99
3	Ala^{1}	0.01 (0.008−0.023)	3246.8	3246.8	97	98
4	Ala^{3}	0.05 (0.029−0.094)	3295.0	3294.9	98	95
5	Ala^{5}	0.10 (0.061−0.16)	3297.0	3296.8	99	98
6	Ala^{6}	0.00 (0.002−0.007)	3263.0	3262.8	>99	98
7	Ala^{7}	0.29 (0.16−0.50)	3308.9	3308.9	>99	98
8	Ala^{8}	2.1 (1.2−4.1)	3295.9	3295.9	>99	99
9	Ala^{9}	2.6 (1.5−4.6)	3323.0	3322.9	99	98
10	Ala^{10}	0.01 (0.008−0.025)	3246.8	3246.8	>99	99
11	Ala^{11}	0.01 (0.006−0.019)	3253.7	3253.8	>99	99
12	Ala^{12}	0.16 (0.10−0.24)	3281.8	3281.8	>99	99
13	Ala^{13}	0.06 (0.038−0.10)	3310.6	3310.8	>99	96
14	Ala^{14}	0.01 (0.01−0.023)	3296.7	3296.8	98	>99
15	Ala^{15}	5.6 (3.4−9.2)	3352.7	3352.9	>99	99
16	Ala^{16}	1.5 (0.73−2.9)	3281.7	3281.8	98	98
17	Ala^{17}	0.39 (0.12−1.1)	3296.9	3296.8	99	95
18	Ala^{18}	1.6 (0.50−5.3) 1.4 (1.1−1.8)	3322.9	3322.9	99	97
19	Ala^{20}	0.30 (0.12−0.72)	3253.9	3253.8	97	98
20	Ala^{21}	0.27 (0.16−0.44)	3281.7	3281.8	97	95
21	Ala^{22}	4.7 (2.9−8.6)	3297.0	3296.8	>99	98
22	Ala^{23}	0.07 (0.038−0.12)	3296.8	3296.8	>99	99
23	Ala^{24}	1.3 (0.60−2.1)	3281.9	3281.8	>99	99
24	Ala^{25}	1.3 (0.84−2.2)	3294.7	3294.9	99	99
25	Ala^{26}	1.1 (0.65−1.8)	3296.8	3296.8	99	>99
26	Ala^{27}	0.32 (0.20−0.53)	3296.8	3296.8	>99	99
27	Ala^{28}	1.9 (1.1−3.5)	3322.7	3322.9	>99	99
28	Ala^{29}	0.59 (0.38−0.93)	3253.9	3253.8	>99	>99
29	MeTyr^{1},Ala^{15,22}	25.6 (17.9−37.1)	3324.9	3324.8	98	(99)[f]
30	MeTyr^{1},Ala^{8,9,15,22,28}	11.2 (8.2−15.2)	3249.8	3249.9	99	(>99)[f]

[a] Ala^{2,4,9} are natural substitutions. [b] Potencies are relative to that of hGHRH(1−40)-OH in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. More than one potency value is listed for peptides that were retested. [c] Value reported is the mean ± standard error of the mean (SEM), where $n = 4$. [d] Mass data are reported as the $[M + H]^+$ monoisotopic species (m/z) for both observed and calculated values. [e] Percent purity was determined by HPLC using buffer system: A, TEAP (pH 2.5) or 0.1% TFA; B, 60% CH_3CN/40% A; gradient slope of 1% B/min; flow rate of 2.0 mL/min (or 1.5 mL/min with TFA) on a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size); detection at 214 nm. [f] Purity was assessed by CZE using a field strength of 10−20 kV at 30 °C, buffer of 100 mM sodium phosphate, pH 2.50, and detection at 214 nm.

differing results. Additionally, peptide content of the preparations from the two laboratories may have been different.

Evidence exists for GHRH binding to liposomes and interacting at the air−water interface.[43] Conformational models of secondary structure have emerged depicting GHRH in either a π- or α-helix for a large portion of the molecule which emphasizes the potential for amphiphilic character.[6,43−45] Figure 2 depicts the parent compound [Nle^{27}]-hGHRH(1−29)-NH$_2$ and Ala substitution sites in an Edmundson wheel projection[46] of an α-helix. The black areas represent hydrophobic residues[47] in the peptide which are segregated in the helix and suggest the possibility of amphiphilicity.[6] The shaded regions represent the individual Ala substitutions that were ≥100% potent and replace natural amino acids of neutral character with the exception of hydrophobic Leu^{22}, polar Asp^{25}, and basic Arg^{29}. These substitutions appear to extend the hydrophobic regions and would also be expected to enhance α-helicity in singly or multiply substituted analogues. If increased amphiphilicity were a condition for improved receptor−ligand interaction, then an extension of the hydrophobic arc would hypothetically enhance α-helicity in these analogues.[3] Analogues **29** and **30** were thus synthesized with multiple alanine substitutions in positions 15 and 22 or 8, 9, 15, 22, and 28. *N*-Methyltyrosine was also substituted for Tyr^{1} since it was shown to impart increased resistance of the peptide to enzymatic degradation in vivo.[23] Peptide **29** was found to be 26 times more potent than the assay standard hGHRH(1−40)-OH, while peptide **30** was 11 times more potent in vitro. It appears that multiple Ala replacement improves potency, yet the effect is not additive. A recent study by Coy et al.[48] has shown that the 11 alanine substitutions yielding analogues that were either equipotent or more potent than hGHRH(1−40)-OH (Table 1) could be

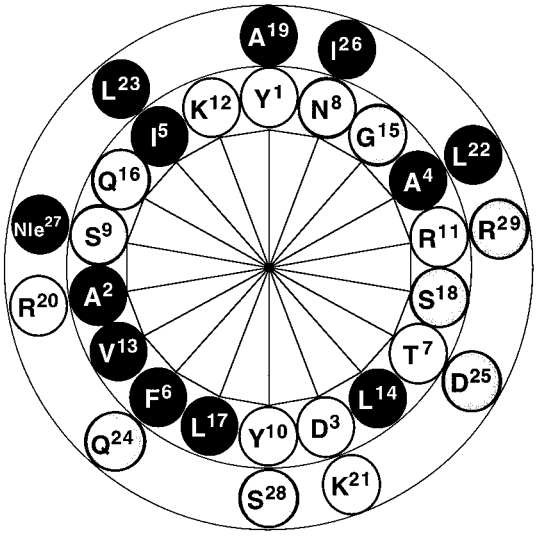

Figure 2. Helical wheel diagram of [Nle^{27}]-hGHRH(1−29)-NH$_2$. See text for description.

Table 2. Single-Point α-Aminoisobutyric Acid Replacement of the Parent Peptide [Nle[27]]-hGHRH(1−29)-NH_2

			mass[b]		purity, %	
no.	substitution	rel potency[a] in vitro	obsd	calcd	HPLC[c] (TEAP)	CZE[d]
1	hGHRH(1−40)-OH	1.00 (standard)	4542.4	4542.3	(>99)[e]	94
31	Aib[8]	4.5 (2.5−7.9)	3309.9	3309.9	99	99
32	Aib[9]	0.94 (0.56−1.6)	3337.0	3336.9	99	99
33	Aib[15]	0.47 (0.28−0.78)	3367.0	3366.9	>99	>99
34	Aib[16]	0.98 (0.54−1.8)	3295.8	3295.9	>99	>99
35	Aib[18]	1.4 (0.97−2.1)	3336.8	3336.9	99	97
36	Aib[22]	3.4 (2.0−5.6)	3310.8	3310.8	98	97
37	Aib[24]	1.2 (0.82−1.7)	3295.8	3295.9	>99	98
38	Aib[25]	1.1 (0.7−1.8)	3309.0	3308.9	>99	99
39	Aib[28]	2.2 (1.5−3.3)	3336.7	3336.9	99	96

[a] Potencies are relative to that of hGHRH(1−40)-OH in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. [b] Mass data are reported as the $[M + H]^+$ monoisotopic species (m/z) for both observed and calculated values. [c] Percent purity was determined by HPLC using buffer system: A, TEAP (pH 2.5) or 0.1% TFA (where indicated); B, 60% CH_3CN/40% A; gradient slope of 1% B/min; flow rate of 2.0 mL/min (or 1.5 mL/min with TFA) on a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size); detection at 214 nm. [d] Purity was assessed by CZE using a field strength of 10−20 kV at 30 °C, buffer of 100 mM sodium phosphate, pH 2.50, and detection at 214 nm. [e] Value indicated is percent purity determined by HPLC in 0.1% TFA.

introduced in a single analogue to yield a peptide equipotent to hGHRH(1−29)-NH_2.

α-Aminoisobutyric Acid (Aib) Scan. To test an hypothesis that inducing structural restraints may result in analogues with improved potency, Aib was employed to limit conformational freedom about both φ and ψ bonds so as to promote α-helix formation.[49] Nine of the positions of [Nle[27]]-hGHRH(1−29)-NH_2 that are most amenable to alanine replacement were then substituted with Aib; results are shown in Table 2. In most cases the potencies of the Aib analogues were similar to those of the corresponding Ala analogues, except at positions 8 (doubling vs **8**), 9 (halving vs **9**), and 15 (10-fold decrease vs **15**). Previous work indicates that chemical degradation of Asn[8] at physiological pH (albeit at slow rates) leads to rearrangement products possessing little potency.[50,51] A significant improvement of potency upon replacement of Asn[8] by Ala or Aib suggests, at least in part, an improved resistance to chemical degradation.

The increased helicity often associated with Aib incorporation[52] is facilitated in large measure by the conformational properties of the monomeric unit first elucidated by Marshall[53] investigating the $\varphi-\psi$ (Ramachandran) space of Aib. Aib can occupy two very restricted regions of this space corresponding to the right- and left-handed α-helixes, and the energetic cost of formation of nonhelical structure must be offset by the whole system, both peptide and medium, for nonhelical structure to be observed. However, a significant body of work shows that nonhelical structure, particularly the β-turn, can dominate the conformational manifold of short biologically active Aib-containing peptides.[54] Karle et al.[55] have shown the importance of solvent on secondary structure using model Aib-containing peptides with a central Gly-Gly feature, and corroboration has been found in model studies of α,α-dialkylated peptides[56] and the peptaibol antibiotics.[57] Thus, while the present hypothesis correlating enhanced helicity with biological potency may have served as a practical rationale for the synthesis of Aib analogues **31**−**39**, confirming the hypothesis awaits detailed structural (NMR) analysis.

In one method of analysis, circular dichroism spectra of the series were analyzed to indicate a possible effect of the substitutions on α-helicity. It appeared that the restraint imposed by a point substitution (Aib) did not grossly modulate the overall conformation of the region as evidenced by similar CD spectra in all cases under the conditions used (not shown). In an aqueous buffer solution at pH 7.0, the peptides existed in a largely random conformation and became increasingly helical as TFE was added until maximum helicity occurred near 20% TFE. The existence of a nearly isodichroic point where the spectra overlapped was indicative of a smooth transition between two major conformations, random and α-helix.

It is useful to compare the results obtained with analogues of the Ala series to those of the D-amino acid series previously reported[21,22,42,58] for [Nle[27]]-GHRH(1−29)-NH_2, since both systematic SAR components provide information to suggest residues or regions of the peptide where future structural manipulation may be focused. Difficulties exist in comparing the D-amino acid series data between the rat[40] and human[22,42,58] species due to differences in the in vitro bioassays used and the 30% incongruity in the two peptide sequences (Figure 1). However, there are some trends that may be identified from both the D-amino acid and Ala series. The first half of the molecule does not tolerate side-chain substitution as well as D-amino acid substitutions, except for Ala replacements for Asn[8] and Ser[9], as evidenced by the low potencies in the Ala series. A region where a large drop in potency occurs in both the D-amino acid and Ala series is seen for residues 5−7 and 13. A parallel increase in relative potency is also observed with DAsn[8] and Ala[8]. In general, the C-terminus of GHRH(1−29)-NH_2 may be substituted with D-amino acids or alanine without severe reductions in relative potency except for the exchange of Ala[23] for Leu[23].

C-Terminus [*i*-(*i*+4)] Lactam Scan. A recent development in SAR studies complementary to the classic alanine and D-amino acid scans is the systematic lactam scan of a peptide. Such a scan is based on studies of Glu-Lys ion pairs that effect helix stabilization when spaced four residues apart, *i* to (*i*+4), in short model peptides.[59] Synthetic applications of this method have used both cystine[21,25,60] and lactam[3,61,62] bridges along the sequence of GHRH(1−29)-NH_2. These secondary structural constraints were designed to retain the bioactive conformation of the peptide, to decrease flexibility, and to enhance helicity. Candidate peptides that

yield potent analogues with lactam constraints are those that are proposed to assume a helical conformation in their bioactive state (GHRH, CRF, PTH, and dynorphins, among others).[63−67]

Felix et al.[61] first synthesized GHRH analogues that contained [*i*-(*i*+4)] lactam bridges, formed through a BOP-mediated, side-chain-to-side-chain lactam cyclization reaction.[37] Of five peptides containing the cyclic motif between residues 4−8, 8−12, 12−16, 16−20, and 21−25, all but the cyclic (16−20) peptide were equipotent or had greater biological potency than hGHRH(1−44)-NH$_2$ in vitro.[62] These improvements were correlated with localized extensions of the peptide helical regions as evidenced by ^{1}H NMR and CD analyses (review).[3]

The studies with linear analogues had reinforced the hypothesis that enhancements in potency were due to increased amphiphilicity and conformational bias.[6,43] Referring to D-amino acid and alanine scans mentioned earlier, C-terminal sites 25, 28, and 29 tolerated both D-amino acid and Ala substitutions which targeted this region for further structural manipulation. Initial experiments[68] on the C-terminus of hGHRH(1−29)-NH$_2$ were designed to optimize the size and bridgehead backbone configuration of the constraint before it was uniformly used to scan the entire peptide. The first modification of the C-terminus was a disulfide linkage between residues 25−29 of rat GHRH(1−29)-NH$_2$.[60] The disulfide homologues of rat GHRH(1−29)-NH$_2$ were synthesized and found to be nearly equipotent to the assay standard. Since the configuration of DXaa^{25},LYaa^{29} was somewhat more potent than the other permutations, this scaffold was chosen as the template for a lactam bridge study of the C-terminal region of human GHRH(1−29)-NH$_2$ (Table 3). Molecular modeling studies also corroborated experimental data in the configuration choice of DCys-LCys in an [*i*-(*i*+4)] ring, since it yielded the least amount of strain in an idealized, acetylated tetradecaalaninamide α-helix model.[69]

The lactam ring sizes were then varied while keeping a constant [*i*-(*i*+4)] backbone bridging distance. Specifically, the side chain of DAsp in position 25 was linked to the *ω*-amino function of Dpr (**44**), Dbu (**45**), Orn (**46**), and Lys (**49**) (re. Yaa) in position 29 in cyclo(25−29)-[MeTyr^1, Ala^{15},DAsp^{25},Nle^{27},Yaa^{29}]-hGHRH(1−29)-NH$_2$. As the lactam ring size increased from 17 to 20 atoms, the relative potency ranged from 1 to 20 times that of the assay standard and maximized at the 19-atom ring size. Next, the lactam bridgehead configurations were permuted in this lactam study as was previously done with the disulfide analogues. Permuting the bridge amino acid configurations of potent analogue **46** did not improve upon the potency but yielded an equipotent analogue (**47**) with the cost-effective, all-L-amino acid configuration. The potency of **48** with DAsp^{25} and DOrn^{29} bridgehead residues was one-third that of **46** and **47** when tested in parallel in the same bioassay. Since the spatial location of the amide bond in the lactam may play a role in bioactivity,[70] we chose potent lactam analogue **46** and replaced the bridgehead residues while conserving the 19-membered ring size. Specifically, DGlu^{25} replaced DAsp^{25} and Dbu^{29} replaced Orn^{29} which translated the nonbackbone amide bond by one methylene group. The resulting analogue **50** was approximately equipotent to **46** and 11 times more potent than the assay standard.

Since the optimal distance of one turn in an ideal α-helix (3.6 amino acids) lies between [*i*-(*i*+4)] and [*i*-(*i*+3)] residue spans, both scans of the peptide are necessary to complete a thorough analysis with this approach. We therefore investigated bridging a shorter backbone segment by using an [*i*-(*i*+3)] lactam. Based upon a precedent with corticotropin-releasing factor (CRF) analogues,[69,71] the lactam bridge was formed using Glu^{25} and Lys^{28} to yield an 18-membered ring analogue (**51**) that was nearly equipotent to the potent [*i*-(*i*+4)] analogue **46** (Table 3). Substitution of DGlu^{25} yielded **52** that was only 2 times more potent than the standard. A decrease in the ring size to 17-membered **53** also decreased the potency by 50%.

The rationale for the incorporation of Aib in a scan of hGHRH(1−29)-NH$_2$ was based on the well-documented utility of Aib to enhance helicity, whereas the systematic bridge scan (**40**−**53**, **55**−**79**) (Tables 3 and 4) relied on both a structural basis and the observation that such constraints could yield potent peptides;[72] the identification of **51** (with 14 times the potency of the standard) would seem to validate this rationale. Although the [*i*-(*i*+3)] and [*i*-(*i*+4)] lactams can be well-accommodated in helixes by theoretical studies, lactam bridges have been found in a variety of nonhelical motifs. For example, Naider and co-workers[73] suggest that a distorted type I *β*-turn characterizes the lactam-bridged KPGQ sequence in *Saccharomyces cerevisiae* α-factor lyophylate. Similar findings of nonhelical structures in several media had been reported for this compound previously by Jelicks et al.[74] and Gounarides et al.[75] Hruby and co-workers[76] also found nonhelical structure about the DOrn^5−Glu^8 bridge in their studies of Ac-[Nle^4]α-MSH(4−11)-NH$_2$. The work of Taylor[77] on human calcitonin suggests that an Asp−Lys [*i*-(*i*+4)] bridge can accommodate a type I *β*-turn. Our modeling studies of cyclo(5−8)[Glu^5−Lys^8]- and cyclo(5−9)[Glu^5−Lys^9]-Ac-Ala^{12}-amide suggest that helixes and a variety of turn types can be accommodated with little energy penalty.

Figure 3 shows the CD spectra of five [*i*-(*i*+4)] lactam analogues (**44**−**46**, **49**, **50**) and the most potent [*i*-(*i*+3)] analogue (**51**) in phosphate buffer at pH 6. The observed spectral differences in aqueous solution suggest changes in the α and random components of the CD. The least helicity is exhibited by **44** (DAsp^{25}−Dpr^{29}) and **46** (DAsp^{25}−Orn^{29}). The observation that **45** (DAsp^{25}−Dbu^{29}), while lying between **44** and **46** in potency and ring size (Table 3), has an apparently elevated helical content reaffirms the lack of correlation of CD properties with potency for these molecules. Paradoxically, **46**, with an [*i*-(*i*+4)] bridge, is the most potent peptide presented in Table 3, yet the equally potent [*i*-(*i*+3)] **51** is the most helical lactam-containing peptide evaluated in this series in aqueous solution. Since [Nle^{27}]-hGHRH-(1−29)-NH$_2$ has been shown to become maximally α-helical at approximately 30% TFE,[28] we anticipated differences in helix-forming tendencies at the halfway point of 15% TFE. Spectral data analysis[78] does not find a quantitative linear correlation among the parameters of helicity, randomness, *β*-sheet character, and biological potency or ring size. However, all analogues lose random character and concomitantly gain α-helical

Table 3. Relative Potencies of [*i*-(*i*+3,4)] Lactam Analogues of Rat or Human Cyclo(25−28,−29)[MeTyr[1],Ala[15],Xaa[25],Nle[27],Yaa[28(29)]]-GHRH(1−29)-NH_2 and Effect of Ring Size and/or Bridgehead Backbone Configuration on Potencies

	substitution				mass[c]		purity, %	
no.	Xaa	Yaa	ring size	rel potency in vitro[a]	obsd	calcd	HPLC[d] (TEAP)	CZE[e]
1	hGHRH(1−40)-OH			1.00 (standard)	4542.4	4542.3	(>99)[f]	94
[*i*-(*i*+4)] rat GHRH analogues								
40	Cys^{25}	Cys^{29}	17	1.1 (0.72−1.7)	3426.9	3426.8	99	97
41	Cys^{25}	$DCys^{29}$	17	1.1 (0.69−1.8)	3426.9	3426.8	95	98
42	$DCys^{25}$	Cys^{29}	17	1.9 (1.3−2.9) 4.4 (2.4−7.9)	3426.8	3426.8	>99	98
43	$DCys^{25}$	$DCys^{29}$	17	1.5 (0.86−2.4)	3426.7	3426.8	92	93
[*i*-(*i*+4)] human GHRH analogues								
44	$DAsp^{25}$	Dpr^{29}	17	1.4 (0.61−3.6)	3279.0	3278.8	96	98
45	$DAsp^{25}$	Dbu^{29}	18	4.2 (2.4−7.5)	3292.7	3292.8	99	98
46	$DAsp^{25}$	Orn^{29}	19	16.7 (7.4−36.6) 6.3 (3.0−12.1)[b]	3306.9	3306.9	97	96
47	Asp^{25}	Orn^{29}	19	6.3 (2.9−12.3)[b]	3306.9	3306.9	98	99
48	$DAsp^{25}$	$DOrn^{29}$	19	2.2 (1.0−4.9)[b]	3307.0	3306.9	99	98
49	$DAsp^{25}$	Lys^{29}	20	8.4 (5.1−13.5)	3320.9	3320.9	98	99
50	$DGlu^{25}$	Dbu^{29}	19	10.7 (5.6−20.2)	3307.0	3306.9	95	90
[*i*-(*i*+3)] human GHRH analogues								
51	Glu^{25}	Lys^{28}	18	14.0 (6.8−28.5)	3403.9	3404.0	>99	>99
52	$DGlu^{25}$	Lys^{28}	18	1.9 (0.84−4.8)	3403.8	3404.0	92	91
53	Glu^{25}	Orn^{28}	17	6.6 (3.4−12.5)	3390.0	3390.0	99	95

[a] Potencies are relative to that of hGHRH(1−40)-OH in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. More than one potency value is listed for peptides that were retested. [b] Indicates peptides that were retested in parallel in the same bioassay. [c] Mass data are reported as the $[M + H]^+$ monoisotopic species (*m*/*z*) for both observed and calculated values. [d] Percent purity was determined by HPLC using buffer system: A, TEAP (pH 2.3); B, 60% CH_3CN/40% A; gradient slope of 1% B/min at 40 °C; 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. [e] Purity was assessed by CZE using a field strength of 10−20 kV at 30 °C, buffer of 100 mM sodium phosphate, pH 2.50, and detection at 214 nm. [f] Value indicated is percent purity determined by HPLC in 0.1% TFA (see Table 1).

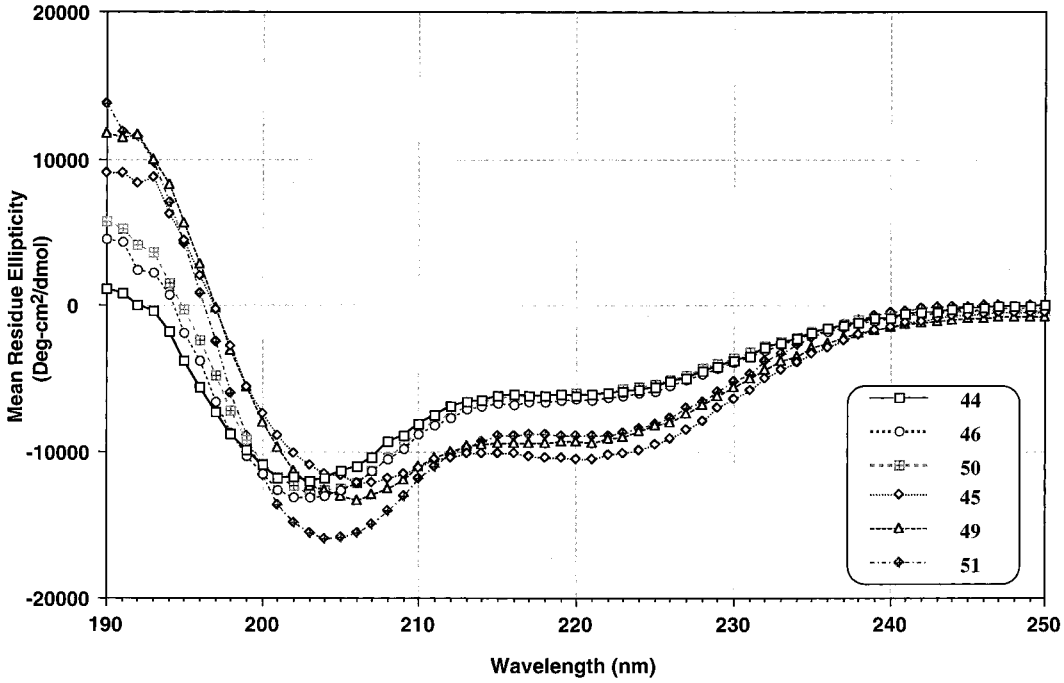

Figure 3. CD spectra of selected [*i*-(*i*+3)] and [*i*-(*i*+4)] analogues (from Table 3) in aqueous solution.

character in 15% TFE except for the [*i*-(*i*+3)] analogue **51** which retains constant helicity (not shown).

Complete [*i*-(*i*+3)] Lactam Scan. Summarizing the results shown in Table 3, Glu and Lys were found to be the optimal bridgehead residues in an [*i*-(*i*+3)] ring scaffold, since smaller lactam ring sizes (<18 atoms) led to molecules with lower relative potencies.[68] We then extended this model study to a complete [*i*-(*i*+3)] lactam scan of cyclo(*i*,*i*+3)[MeTyr[1],Ala[15],Glu^{i},$Lys^{(i+3)}$,Nle[27]]-hGHRH(1−29)-NH_2 based on our success with astressin, a CRF analogue.[79] Preliminary results were recently presented at the 15th American Peptide Symposium (Cervini et al., in press).

As depicted in Table 4, the [*i*-(*i*+3)] lactam scan of cyclo(*i*,*i*+3)[MeTyr[1],Ala[15],Glu^{i},$Lys^{(i+3)}$,Nle[27]]-hGHRH(1−29)-NH_2 yielded 26 analogues, the potencies of which were compared to that of the agonist standard hGHRH-(1−40)-OH in vitro. Eleven analogues had diminished potencies of less than 10% that of the agonist standard, and nine of these had substitutions that were located

Table 4. Relative Potencies and Mass Spectral Data for [*i*-(*i*+3)] Cyclic Analogues of [MeTyr^1,Ala^{15},Nle^{27}]-hGHRH(1−29)-NH$_2$

			mass[c]		purity, %	
no.	substitution	rel potency[a] in vitro	obsd	calcd	HPLC[d] (TEAP)	CZE[e]
1	hGHRH(1−40)-OH	1.00 (standard)	4542.4	4542.3	(>99)[f]	94
54	[MeTyr^1,Ala^{15},Nle^{27}]	5.8 (3.3−9.6)[b]	3366.9	3366.9	(>99)[f]	99
55	cyclo(1−4)[Glu^1,Lys^4]	0.004 (0.001−0.008)	3358.0	3357.9	91	97
56	cyclo(2−5)[Glu^2,Lys^5]	0.004 (0.001−0.008)	3422.0	3421.9	>99	99
57	cyclo(3−6)[Glu^3,Lys^6]	0.002 (0.023−0.004)	3343.9	3343.9	99	99
58	cyclo(4−7)[Glu^4,Lys^7]	4.8 (2.3−10.2)	3433.9	3433.9	97	99
59	cyclo(5−8)[Glu^5,Lys^8]	11.1 (5.5−22.3) 3.6 (2.1−5.8)[b]	3378.9	3378.9	99	99
60	cyclo(6−9)[Glu^6,Lys^9]	0.001 (0.000−0.002)	3372.0	3371.9	99	99
61	cyclo(7−10)[Glu^7,Lys^{10}]	0.008 (0.005−0.014)	3341.9	3341.9	>99	95
62	cyclo(8−11)[Glu^8,Lys^{11}]	0.014 (0.005−0.035)	3335.9	3335.9	97	98
63	cyclo(9−12)[Glu^9,Lys^{12}]	2.7 (1.2−7.1)	3390.9	3390.9	99	99
64	cyclo(10−13)[Glu^{10},Lys^{13}]	0.088 (0.056−0.14)	3343.7	3343.9	>99	99
65	cyclo(11−14)[Glu^{11},Lys^{14}]	<0.000	3336.8	3336.8	>99	97
66	cyclo(12−15)[Glu^{12},Lys^{15}]	1.2 (0.77−1.9)	3407.1	3406.9	>99	99
67	cyclo(13−16)[Glu^{13},Lys^{16}]	0.18 (0.12−0.27)	3378.9	3378.9	>99	>99
68	cyclo(14−17)[Glu^{14},Lys^{17}]	0.026 (0.011−0.056)	3380.1	3379.9	>99	>99
69	cyclo(15−18)[Glu^{15},Lys^{18}]	0.16 (0.084−0.31)	3448.1	3448.0	99	99
70	cyclo(16−19)[Glu^{16},Lys^{19}]	2.8 (1.4−5.5)	3407.0	3406.9	93	99
71	cyclo(17−20)[Glu^{17},Lys^{20}]	0.23 (0.12−0.40)	3336.8	3336.8	97	96
72	cyclo(18−21)[Glu^{18},Lys^{21}]	1.8 (1.2−2.7)	3390.9	3390.9	98	99
73	cyclo(19−22)[Glu^{19},Lys^{22}]	0.40 (0.22−0.72)	3421.9	3421.9	99	97
74	cyclo(20−23)[Glu^{20},Lys^{23}]	0.038 (0.021−0.065)	3337.7	3336.8	99	99
75	cyclo(21−24)[Glu^{21},Lys^{24}]	4.5 (2.9−7.1)	3349.8	3349.9	>99	>99
76	cyclo(22−25)[Glu^{22},Lys^{25}]	5.3 (3.4−8.1) 8.8 (4.0−17.9)[b]	3377.7	3377.9	91	93
77	cyclo(23−26)[Glu^{23},Lys^{26}]	<0.008	3380.0	3379.9	98	98
78	cyclo(24−27)[Glu^{24},Lys^{27}]	1.3 (0.60−2.6)	3364.9	3364.9	>99	99
51	cyclo(25−28)[Glu^{25},Lys^{28}]	14.0 (6.8−28.5) 5.2 (2.4−10.0)[b]	3403.9	3404.0	>99	>99
79	cyclo(26−29)[Glu^{26},Lys^{29}]	0.56 (0.31−0.97)	3336.9	3336.8	>99	99

[a] Potencies are relative to that of hGHRH(1−40)-OH in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. More than one potency value is listed for peptides that were retested. [b] Indicates peptides that were retested in parallel in the same bioassay. [c] Mass data are reported as the $[M + H]^+$ monoisotopic species (*m*/*z*) for both observed and calculated values. [d] Percent purity was determined by HPLC using buffer system: A, TEAP (pH 2.3); B, 60% CH_3CN/40% A; gradient slope of 1% B/min at 40 °C; 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. [e] Purity was assessed by CZE using a field strength of 10−20 kV at 30 °C, buffer of 100 mM sodium phosphate, pH 2.50 (100% H_2O or 85:15 H_2O/CH_3CN), and detection at 214 nm. [f] Values indicated are percent purity determined by HPLC in 0.1% TFA (see Table 1).

in the N-terminal half of the peptide. Four weak agonists (15−40% relative potency) had centrally located substitutions, and four analogues equipotent to the standard had substitutions located in the C-terminal half of the peptide. The seven most potent analogues ranged in potency from 3 to 14 times that of the standard with constraints uniformly distributed along the length of the molecule. These analogues contained [*i*-(*i*+3)] cycles between residues 4−7 (**58**), 5−8 (**59**), 9−12 (**63**), 16−19 (**70**), 21−24 (**75**), 22−25 (**76**), and 25−28 (**51**). The potencies of the three most potent analogues (**51**, **59**, and **76**) were then compared to each other and the parent peptide **54** in the same bioassay. A certain variability in the assay was observed; however, all values had overlapping 95% confidence limits. The potent analogues were thus determined to be equipotent to the parent, linear peptide **54**.

Evidence for a structural effect of the introduction of a cycle in GHRH analogues was sought through CD studies of the five C-terminal [*i*-(*i*+3)] analogues (**51**, **76**−**79**). These analogues ranged in potency from less than 0.1- to 14-fold that of the standard. We hypothesized that the cyclo(25−28) analogue (**51**) would show enhanced helicity relative to cyclo(24−27) (**78**), cyclo(26−29) (**79**), and linear parent peptide [MeTyr^1,Ala^{15},Nle^{27}]-hGHRH(1−29)-NH$_2$ (**54**). The introduction of the cycle (23−26) in **77** would be expected to be destabilizing and yield a CD spectrum with an increased random component; however, only subtle differences were seen among the five spectra in aqueous, acidic phosphate buffer (not shown). The trend of helicity loosely followed the order of increasing potency where the least helical peptide, cyclo(23−26) analogue **77**, was also the least potent. Although the peptides showed largely random and sheet character[78] in this aqueous environment, the possibility of facilitated helix formation in a membrane environment could not be ruled out.

Overall Data Summary. By comparing the effects of the introduction of a bridge (Tables 3 and 4), an alanine (Table 1), or a D-amino acid (see above and Cervini et al.[40]), we seek to understand the structural or functional role of each residue in the GHRH(1−29) sequence. As an exercise to assist in data analysis, we tabulated how modification(s) affect the potency of hGHRH(1−29)-NH$_2$ analogues, and these results are graded in Table 5. We define a sensitive residue as the amino acid which upon substitution contributes to a significant loss of potency. As an example, the effect of modifying position 6 is to lower the potency of the cyclic analogues whether the lactam bridge extends C-terminally (cycle 6−9) or N-terminally (cycle 3−6) (denoted X in Table 5). Therefore, both configurations are unfavorable. The same result also occurs when the native amino acid is substituted by its D-enantiomer or alanine (DPhe^6 or Ala^6).

Eleven residues are seen to be the most sensitive to substitution by a bridgehead: Tyr^1, Ala^2, Asp^3, Phe^6,

Table 5. Analysis of Data from Tables 1, 3, and 4 and Literature Reports[22,40,42,58,62] of D-Series and [*i*-(*i*+4)] Data[a]

residue	bridge scaffold[b]				Xaa	
i	*i*-(*i*-3)	*i*-(*i*+3)	*i*-(*i*-4)	*i*-(*i*+4)	Ala	D
Tyr[1]	n/a	X			X	=
Ala[2]	n/a	X			n/a	*P*
Asp[3]	n/a	X			X	=
Ala[4]	X	*P*		=	n/a	X
Ile[5]	X	*P*			X	X
Phe[6]	X	X			X	X
Thr[7]	*P*	X			w	X
Asn[8]	*P*	X	=	=	*P*	=
Ser[9]	X	*P*			*P*	w
Tyr[10]	X	X			X	=
Arg[11]	X	X			X	w
Lys[12]	*P*	=	=	=	w	=
Val[13]	X	w			X	X
Leu[14]	X	X			X	w
Gly[15]	=	w			*P*	n/a
Gln[16]	w	*P*	=	w	=	X
Leu[17]	X	w			w	X
Ser[18]	w	=			=	w
Ala[19]	*P*	w			n/a	X
Arg[20]	w	X	w		w	w
Lys[21]	=	*P*		=	w	=
Leu[22]	w	*P*			*P*	=
Leu[23]	X	X			X	w
Gln[24]	*P*	=			=	=
Asp[25]	*P*	*P*	=	*P*	=	=
Ile[26]	X	=			=	X
Met[27]	=	n/a			w	=
Ser[28]	*P*	n/a			=	=
Arg[29]	=	n/a	*P*		=	=

[a] Symbol definitions: X, modification caused diminished potency (potency ≤10% that of the standard); w, weak agonist (<50% relative potency to that of the standard); *P*, potent agonist (> twice the potency of the standard); =, equipotent; n/a, not applicable. [b] Only six [*i*-(*i*+4)] bridging regions have been scanned for hGRF(1−29)NH_2.

Tyr[10], Arg[11], Val[13], Leu[14], Leu[17], Arg[20], and Leu[23]. Whenever one of these residue positions is a lactam bridgehead substitution or alanine, the potency of the cyclic or linear analogue is diminished or weak. Studies on position 1 have shown that a hydrogen-bonding capacity of the amino acid is necessary for activity.[80] Although DAla[2] enhances agonist potency,[81] substitution of Ala[2] by proteinogenic or polar D-amino acids decreases the potency of the peptide.[58] Similarly, aromatic and basic D-amino acids are not well-tolerated in position 3.[58] The remaining eight sensitive residues may play a subtle role in the potency enhancement of cyclic analogues when the cycle places them in an (*i*+1) or (*i*+2) position (Table 4): (1) When the hydrophobic residues Phe[6], Tyr[10], Val[13], Leu[17], and Leu[23] are substituted in the (*i*+1) position of the lactam, the analogues are either equipotent or of greater potency than that of the standard (see cycles 5−8, 9−12, 12−15, 12−16, 16−19, and 22−25). (2) The (*i*+2) position is favored by the basic residues Arg[11,20] as well as the hydrophobic residues Phe[6], Leu[14], and Leu[23] (cycles 4−7, 4−8, 9−12, 12−15, 12−16, 18−21, 21−24, and 21−25). Hypothetically, the lactam bridge presents these key residues in a more favorable position for interaction with the receptor, or the cycles may structurally enhance the helical propensity of the linear peptide. In the above comparison Ile[5] is an anomalous residue. Upon DIle or Ala substitution, the potency of the resulting peptide is diminished relative to that of the standard. One would therefore expect that a lactam bridge containing residue 5 as a bridgehead would lead to a nonactive analogue. On the contrary, analogue **59** with the cyclo-(5−8) bridge configuration was one of the most potent analogues of the [*i*-(*i*+3)] series. However, when residue 5 is at the positive pole of the bridge configuration [cyclo-(2−5), **56**], the potency of the analogue is diminished. Therefore, the polar directionality of the bridging element aligned with the overall peptide dipole will also influence bioactivity as shown earlier.[70]

Conclusions

The present systematic studies highlight the current status of predictive methodology for determining which residues are more critical to receptor recognition and binding from the primary sequence of a peptide. Biological potencies obtained from fully characterized series of analogues suggest which residues are most sensitive to substitution. Identification of these residues then allows a more rational approach to the design of analogues with high potencies. The observation that 11 Ala-substituted analogues (mostly replacing hydrophilic residues) exhibited potency equal to or greater than that of the parent compound suggests that overall α-helicity and/or hydrophobicity are important factors in receptor binding. Other studies mentioned earlier[61−63] showed optimal lactam sizes of 20- and 21-membered rings for retention or gain in biological potencies. In contrast, we saw maximal potencies for a range of ring sizes from 18- to 20-membered [*i*-(*i*+4)] and 18-membered [*i*-(*i*+3)] lactam analogues in the C-terminus of GHRH(1−29)-NH_2. In summary, it becomes apparent that differences in the sizes of constraining rings have different effects on biological activity whether they are centrally located or introduced at the N- or C-termini.

Subtle differences in CD are observed among the analogues, yet the 18−20-membered lactam rings at the C-terminus do not impede the adoption of helical structure, nor do they drastically reduce potencies. These results also corroborate modeling studies which show that the bridges used in analogues **40**−**53** (Table 3) are compatible with an α-helix.[69] The conformational constraints applied in this study thus far yield subtle effects in the peptide−receptor interaction, and the bridging elements used may have other influences than structural.

Experimental Section

Instruments and Methods. The HF cleavage line was designed in-house and allowed for HF distillation under high vacuum. Preparative HPLC was run on a Waters Prep 500 instrument with model 500A preparative gradient generator, model 450 variable wavelength UV detector, PrepPAK 1000, and Fisher Recordall 5000 strip chart recorder. The 5- × 30-cm cartridge was packed in the laboratory with reversed-phase 300-Å Vydac C_{18} silica gel (15−20-μm particle size). Analytical HPLC screening was performed on a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) connected to a Rheodyne injector, two Waters M-45 pumps, a Waters automated gradient controller, a Kratos SF 7697 UV detector, a Shimadzu Chromatopac E1A integrator, and a Houston Instruments D-5000 strip chart recorder. Quality control HPLC was performed on one of two systems: (1) Peptides **1**−**50** were analyzed on a Waters Associates HPLC system comprised of two 6000A pumps, a WISP sample injector, a 300-Å Vydac C_{18} column as above, a Kratos Spectroflow model 773 UV detector (at 210 nm), and a Waters Associates data module integrator/recorder. (2) Peptides **51**−**79** were analyzed on a Hewlett-

Packard series II 1090 liquid chromatograph connected to a Vydac C_{18} column (0.21 × 15 cm, 5-μm particle size, 300-Å pore size), a controller model 362, and a Think Jet printer. Capillary zone electrophoresis (CZE) analysis for peptides **1**, **2**, and **29**−**79** was performed on a Beckman P/ACE system 2050 controlled by an IBM Personal System/2 model 50Z connected to a ChromJet integrator.

Starting Materials. The *p*-methylbenzhydrylamine resin (MBHA resin) with a capacity of 0.45−0.75 mequiv/g was obtained from a polystyrene cross-linked with 1% divinylbenzene (Biobeads SX-1, 200−400 mesh, Bio-Rad Laboratories, Richmond, CA) as previously published.[82] All *tert*-butyloxycarbonyl (Boc) N^{α}-protected amino acids with side-chain protection were purchased from Bachem Inc. (Torrance, CA) or Chem-Impex Intl (Wood Dale, IL). The side-chain protecting groups were as follows: Arg(Tos), Asp(β-OcHex or β-OFm), Cys(S-*p*-Mob), Dbu(γ-Fmoc), Dpr(β-Fmoc), Glu(γ-OcHex or γ-OFm), His(Tos), Lys(ϵ-2ClZ or ϵ-Fmoc), Orn(δ-Fmoc), Ser(OBzl), Thr(OBzl), Tyr(2BrZ), N^{α}-MeTyr(2,6-Cl_2Bzl). Reagents and solvents were analytical reagent grade.

Peptide Synthesis. Peptides were made by the solid-phase approach[83] either manually or on a Beckman 990 peptide synthesizer. Couplings on 1−2 g of resin/peptide were mediated for 2 h by DIC in CH_2Cl_2, DMF, or NMP and monitored by the qualitative ninhydrin test.[84] Difficult couplings were mediated with BOP, HBTU, or TBTU in DMF or NMP and adjusted to pH 9 with diisopropylethylamine (DIPEA). Boc-Asn and Boc-Gln were coupled in the presence of 1.5 equiv of HOBt. A 2.5-equiv excess of amino acid based on the original substitution of the resin was used in most cases. Coupling steps were followed by acetylation [10% $(CH_3CO)_2O$ in CH_2Cl_2 for 10−15 min] as necessary. Boc removal was achieved with trifluoroacetic acid (50% in CH_2Cl_2, 1−2% ethanedithiol, or *m*-cresol) for 20 min. An isopropyl alcohol (1% ethanedithiol or *m*-cresol) wash followed TFA treatment, and then successive washes with triethylamine solution (10% in CH_2Cl_2), methanol, triethylamine solution, methanol, and CH_2Cl_2 completed the neutralization sequence. The Fmoc groups were removed with 20% piperidine in DMF or NMP in two successive 10-min treatments. Lactam cyclization was performed after Fmoc deprotection of the side chains of the bridgehead residues by the method of Felix et al.[37] or by substituting HBTU or TBTU for BOP. HF cleavage occurred in the presence of 10% anisole and 2−5% dimethyl sulfide (for Cys-containing peptides) for 1.5 h at 0 °C. After HF distillation, the crude peptide was precipitated with diethyl ether, filtered, and dissolved in 10% aqueous acetic acid or 25% aqueous acetonitrile. The product was then shell-frozen and lyophilized. Disulfide bridge formation occurred after room-temperature air oxidation in dilute 25% acetonitrile−water adjusted to pH 7 with NH_4OH for 7 days.

Purification.[38] The crude, lyophilized peptides (1−3 g) were dissolved in a minimum amount (300 mL) of 0.25 N TEAP, pH 2.25, and acetonitrile and loaded onto the HPLC column. The peptides eluted with a flow rate of 100 mL/min using a linear gradient of 1% B/3 min increase from the baseline %B (eluent A, 0.25 N TEAP, pH 2.25; eluent B, 60% CH_3CN, 40% A). Generally, purifications in TEAP, pH 2.25, followed by TEAP, pH 6.5, were necessary to achieve the desired purity level. As a final step, the TEAP salt of the peptide was exchanged for the TFA salt using a gradient of 1% B/min, where A = 0.1% TFA.

Peptide Characterization. Peptide purity was determined in two systems by analytical HPLC in 0.1% TFA and TEAP buffer systems for peptides **3**−**28**. For peptides **1**, **2**, and **29**−**79** purity was assessed by analytical HPLC and CZE. CZE analysis employed a field strength of 10−20 kV at 30 °C with a buffer of 100 mM sodium phosphate, pH 2.5, on either a Beckman eCAP or a Supelco P175 fused silica gel capillary (363-μm o.d. × 75-μm i.d. × 50-cm length). Purity was determined to be >95% in most cases. Since sequence analysis of each analogue would not be practical, the laboratory relies on rigorous record keeping to achieve the proper sequence of addition of each amino acid during peptide assembly. Each amino acid was logged into a synthesis notebook, along with the manufacturer and lot number, date and time of usage, and duration of coupling. The method of reaction was also annotated, as well as Kaiser test results for coupling and deblock stages. Any other manipulations were also recorded. The analogue sequences were based on that of hGHRH(1−40)-OH which was originally verified by sequence analysis.[1] Liquid secondary ion mass spectra (LSIMS) were measured with a JEOL JMS-HX110 double-focusing mass spectrometer fitted with a Cs^+ gun. An accelerating voltage of 10 kV and Cs^+ gun voltage between 25 and 30 kV were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The mass of each analogue was measured, and the observed monoisotopic $(M + H)^+$ values were within 100 ppm of the calculated $(M + H)^+$ values. Circular dichroism was performed on an AVIV model 62DS CD spectrophotometer. The peptides were dissolved in an aqueous buffer and analyzed at ambient temperature in a 0.5- or 1-mm cell by one of three methods: (1) Peptides from the Ala and Aib series were dissolved in an aqueous buffer containing 5 mM potassium phosphate and 50 mM KCl at pH 7.0[31] and filtered through a 0.2-μm membrane before analysis. Peptide concentrations were determined by quantitative HPLC against a reference standard. Trifluoroethanol (TFE) was then added to give final TFE concentrations of 5.3%−21.7%. (2) Analogues **44**−**46** and **49**−**51** were analyzed in two aqueous buffer systems at a peptide concentration of 100 μM, 15 mM sodium phosphate at pH 6.0 and 10 mM sodium phosphate containing 15% trifluoroethanol at pH 6.0. (3) Analogues **51** and **76**−**79** were analyzed in 5 mM sodium phosphate at pH 3.0 with a peptide concentration of 1 mg/mL (~230 μM). Peptide concentrations in methods (2) and (3) were determined by using the calculated molecular weight of the TFA salt of the purified, lyophilized peptide assuming a 6% water content. Instrument parameters were set to average four or six repeated scans from 250 to 190 nm with a bandwidth of 2.0 nm and an averaging time of 2−4 s/data point. The spectra were then corrected for baseline noise by subtraction of the buffer spectrum.

Biological Testing. Primary cultures of rat anterior pituitary cells were incubated in triplicate with increasing concentrations of the standard or an analogue. For antagonist studies, the cells were incubated with increasing concentrations of the analogue in the presence of 1 nM GHRH(1−40)-OH. Media were collected after 3 h, and GH secretion was measured by RIA.[39] Potencies of agonists were determined against reference standard hGHRH(1−40)-OH or those of antagonists against standard [$MeTyr^1$,$DArg^2$,Cpa^6,Nle^{27}]-rGHRH(1−29)-NH_2 (manuscript in preparation) using the BIOPROG program,[85] which generates one curve for each analogue that was compared to that of the corresponding standard.

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References

(1) Rivier, J.; Spiess, J.; Thorner, M.; Vale, W. Characterization of a growth hormone-releasing factor from a human pancreatic islet tumor. *Nature* **1982**, *300*, 276−278.

(2) Guillemin, R.; Brazeau, P.; Bohlen, P.; Esch, F.; Ling, N.; Wehrenberg, W. B. Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science* **1982**, *218*, 585−587.

(3) Campbell, R. M.; Bongers, J.; Felix, A. M. Rational design, synthesis, and biological evaluation of novel growth hormone releasing factor analogues. *Biopolymers* **1995**, *37*, 67−88.

(4) Seifert, H.; Perrin, M.; Rivier, J.; Vale, W. Binding sites for growth hormone releasing factor on rat anterior pituitary cells. *Nature* **1985**, *313*, 487−489.
(5) Seifert, H.; Perrin, M.; Rivier, J.; Vale, W. Growth hormone-releasing factor binding sites in rat anterior pituitary: Modulation by glucocorticoids. *Endocrinology* **1985**, *117*, 424−426.
(6) Campbell, R. M.; Lee, Y.; Rivier, J.; Heimer, E. P.; Felix, A. M.; Mowles, T. F. GRF analogues and fragments: correlation between receptor binding, activity and structure. *Peptides* **1991**, *12*, 569−574.
(7) Abribat, T.; Boulanger, L.; Gaudreau, P. Characterization of [^{125}I-Tyr^{10}]human growth hormone-releasing factor (1−44) amide binding to rat pituitary: Evidence for high and low affinity classes of sites. *Brain Res.* **1990**, *528*, 291−299.
(8) Gaylinn, B. D.; Harrison, J. K.; Zysk, J. R.; Lyons, C. E.; Lynch, K. R.; Thorner, M. O. Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. *Mol. Endocrinol.* **1993**, *7*, 77−84.
(9) Mayo, K. E. Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone. *Mol. Endocrinol.* **1992**, *5*, 1734−1744.
(10) Lin, C.; Lin, S.-C.; Chang, C.-P.; Rosenfeld, M. G. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature* **1992**, *360*, 765−768.
(11) Gaylinn, B. D.; von Kap-Herr, C.; Golden, W. L.; Thorner, M. O. Assignment of the human growth hormone-releasing hormone receptor gene (GHRHR) to 7p14 by in situ hybridization. *Genomics* **1994**, *19*, 193−195.
(12) Gelato, M. C.; Merriam, G. R. Growth hormone releasing hormone. *Annu. Rev. Physiol.* **1986**, *48*, 569−591.
(13) Horikawa, R.; Hellmann, P.; Cella, S. G.; Torsello, A.; Day, R. N.; Muller, E. E.; Thorner, M. O. Growth hormone-releasing factor (GRF) regulates expression of its own receptor. *Endocrinology* **1996**, *137*, 2642−2645.
(14) Barinaga, M.; Bilezikjian, L. M.; Vale, W. W.; Rosenfeld, M. G.; Evans, R. M. Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. *Nature* **1985**, *314*, 279−281.
(15) Tannenbaum, G. S.; Ling, N. The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. *Endocrinology* **1984**, *115*, 1952−1957.
(16) Hartman, M. L.; Veldhuis, J. D.; Thorner, M. O. Normal control of growth hormone secretion. *Horm. Res.* **1993**, *40*, 37−47.
(17) Becker, K.; Stegenga, S.; Conway, S. Role of insulin-like growth factor I in regulating growth hormone release and feedback in the male rat. *Neuroendocrinology* **1995**, *61*, 573−583.
(18) Frohman, L. A.; Jansson, J. O. Growth hormone-releasing hormone. *Endocrinol. Rev.* **1986**, *7*, 223−253.
(19) Strobl, J. S.; Thomas, M. J. Human growth hormone. *Pharmacol. Rev.* **1994**, *46*, 2−34.
(20) Ling, N.; Baird, A.; Wehrenberg, W. B.; Ueno, N.; Munegumi, T.; Brazeau, P. Synthesis and in vitro bioactivity of C-terminal deleted analogues of human growth hormone-releasing factor. *Biochem. Biophys. Res. Commun.* **1984**, *123*, 854−861.
(21) Coy, D. H.; Murphy, W. A.; Lance, V. A.; Heiman, M. L. Strategies in the design of synthetic agonists and antagonists of growth hormone releasing factor. *Peptides* **1986**, *7*, 49−52.
(22) Coy, D. H.; Murphy, W. A.; Sueiras-Diaz, J.; Coy, E. J.; Lance, V. A. Structure−activity studies on the N-terminal region of growth hormone releasing factor. *J. Med. Chem.* **1985**, *28*, 181−185.
(23) Rivier, J.; Rivier, C.; Galyean, R.; Yamamoto, G.; Vale, W. Potent long-acting growth hormone releasing factor analogues. *Ann. N. Y. Acad. Sci.* **1988**, *527*, 44−50.
(24) Hernandez, J. F.; Theobald, P. G.; Yamamoto, G.; Andrews, J.; Rivier, C.; Vale, W.; Rivier, J. E. Synthesis and biological activity of growth hormone-releasing factor analogues. In *Peptides: Chemistry, Structure & Biology*; Rivier, J. E., Marshall, G. R., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1990; pp 236−238.
(25) Sato, K.; Hotta, M.; Kageyama, J.; Chiang, T. C.; Hu, H. Y.; Dong, M. H.; Ling, N. Structure−activity of growth hormone-releasing factor (GRF). *Pept. Chem.* **1989**, 85−90.
(26) Robberecht, P.; Coy, D. H.; Waelbroeck, M.; Heiman, M. L.; DeNeef, P.; Camus, J. C.; Christophe, J. Structural requirements for the activation of rat anterior pituitary adenylate cyclase by growth hormone-releasing factor (GRF): Discovery of (N-Ac-Tyr^1,D-Arg^2)-GRF(1−29)-NH$_2$ as a GRF antagonist on membranes. *Endocrinology* **1985**, *117*, 1759−1764.
(27) Fry, D. C.; Madison, V. S.; Bolin, D. R.; Greeley, D. N.; Toome, V.; Wegrzynski, B. B. Solution structure of an analogue of vasoactive intestinal peptide as determined by two-dimensional NMR and circular dichroism spectroscopies and constrianed molecular dynamics. *Biochemistry* **1989**, *28*, 2399−2409.
(28) Clore, G. M.; Martin, S. R.; Gronenborn, A. M. Solution structure of human growth hormone releasing factor. *J. Mol. Biol.* **1986**, *191*, 553−561.
(29) Brunger, A. T.; Clore, G. M.; Gronenborn, A. M.; Karplus, M. Solution conformations of human growth hormone releasing factor: comparison of the restrained molecular dynamics and distance geometry methods for a system without long-range distance data. *Protein Eng.* **1987**, *1*, 399−406.
(30) Stevenson, C. L.; Donlan, M. E.; Friedman, A. R.; Borchardt, R. T. Solution conformation of Leu^{27} hGRF(1−32)NH$_2$ and its deamidation products by 2D NMR. *Int. J. Pept. Protein Res.* **1993**, *42*, 24−32.
(31) Honda, S.; Morii, H.; Ohashi, S.; Uedaira, H. Structural study on human growth hormone-releasing factor fragment (1−29) in hydrophobic environment. In *Osaka, Japan, October 25−27, 1990*; Shimonishi, Y., Ed.; Protein Research Foundation: Minohshi, Osaka 562, Japan, 1990; pp 379−384.
(32) Kloosterman, D. A.; Scahill, T. A.; Hillman, R. M.; Cleary, D. L.; Kubiak, T. M. ^{1}H NMR analysis and in vitro bioactivity of Leu^{27}-bGRF(1−29)NH$_2$ and its D-Ala^2 and des-(Tyr^1-Ala^2)-analogues. *Pept. Res.* **1991**, *4*, 72−78.
(33) Kloosterman, D. A.; Scahill, T. A.; Friedman, A. R. Secondary structure of a human growth hormone-releasing factor fragment (Leu^{27}-hGRF(15−32)NH$_2$) in aqueous/SDS micelle environments. *Pept. Res.* **1993**, *6*, 211−218.
(34) Honda, S.; Ohashi, S.; Morii, H.; Uedaira, H. Solution structure of human growth hormone-releasing factor fragment (1−29) by CD: Characteristic conformational change on phospholipid membrane. *Biopolymers* **1991**, *31*, 869−876.
(35) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984; p 176.
(36) Marki, W.; Spiess, J.; Taché, Y.; Brown, M.; Rivier, J. E. Total solid-phase synthesis of porcine gut gastrin releasing peptide (GRP), a mammalian bombesin. *J. Am. Chem. Soc.* **1981**, *103*, 3178−3185.
(37) Felix, A. M.; Wang, C.-T.; Heimer, E. P.; Fournier, A. Applications of BOP reagent in solid-phase synthesis. II. Solid-phase side-chain to side-chain cyclizations using BOP reagent. *Int. J. Pept. Protein Res.* **1988**, *31*, 231−238.
(38) Hoeger, C.; Galyean, R.; Boublik, J.; McClintock, R.; Rivier, J. Preparative reversed phase high performance liquid chromatography. II. Effects of buffer pH on the purification of synthetic peptides. *Biochromatography* **1987**, *2*, 134−142.
(39) Vale, W.; Vaughan, J.; Jolley, D.; Yamamoto, G.; Bruhn, T.; Seifert, H.; Perrin, M.; Thorner, M.; Rivier, J. Assay of growth hormone releasing factor. In *Methods in Enzymology: Hormone Action: Neuroendocrine Peptides;* Conn, P. M., Ed.; Academic Press: Orlando, FL, 1986; Vol. 124, pp 389−401.
(40) Cervini, L.; Galyean, R.; Donaldson, C.; Yamamoto, G.; Koerber, S.; Vale, W.; Rivier, J. A SAR study of the complete Ala and partial Aib scans of the growth hormone releasing factor: [Nle^{27}]-hGRF(1−29)-NH$_2$. In *Peptides: Chemistry and Biology*; Smith, J., Rivier, J., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1992; pp 437−438.
(41) Felix, A. M.; Heimer, E. P.; Mowles, H. E.; Leung, P.; Lambros, T. J.; Wang, C.-T. Synthesis and biological activity of novel growth hormone releasing factor analogues. In *Peptides 1986*; Theodoropoulos, D., Ed.; Walter de Gruyter: Berlin, 1986; pp 481−484.
(42) Lefrancois, L.; Gaudreau, P. Identification of receptor-binding pharmacophores of growth-hormone-releasing factor in rat adenopituitary. *Neuroendocrinology* **1994**, *59*, 363−370.
(43) Kaiser, E. T.; Kezdy, F. J. Amphiphilic secondary structure: Design of peptide hormones. *Science* **1984**, *223*, 249−255.
(44) Tou, J. S.; Kaempfe, L. A.; Vineyard, B. D.; Buonomo, F. C.; Della-Fera, M. A.; Baile, C. A. Amphiphilic growth hormone releasing factor (GRF) analogues: Peptide design and biological activity in vivo. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 763−770.
(45) Velicelebi, G.; Patthi, S.; Kaiser, E. T. Design and biological activity of analogues of growth hormone releasing factor with potential amphiphilic helical carboxyl termini. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5397−5399.
(46) Schiffer, M.; Edmundson, A. B. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. *Biophys. J.* **1967**, *7*, 121−135.
(47) Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **1984**, *179*, 125−142.
(48) Coy, D. H.; Jiang, N.-Y.; Fuselier, J.; Murphy, W. A. Structural simplification of potent growth hormone-releasing hormone analogues: Implications for other members of the VIP/GHRH/PACAP family. In *VIP, PACAP, and Related Peptides;* Arimura, A., Said, S. I., Eds.; The New York Academy of Sciences: New York, 1996; Vol. 805, pp 149−158.
(49) Toniolo, C. Structure of conformationally constrained peptides: From model compounds to bioactive peptides. *Biopolymers* **1989**, *28*, 247−257.

(50) Felix, A. M.; Lambros, T.; Heimer, E. P.; Cohen, H.; Pan, Y.-C.; Campbell, R.; Bongers, J. Degradation of growth hormone releasing factor in aqueous solution: isolation and characterization of major metabolites. In *Peptides 1990*; Giralt, E., Andreu, D., Eds.; ESCOM: Leiden, The Netherlands, 1991; pp 732−733.
(51) Friedman, A. R.; Ichhpurani, A. K.; Brown, D. M.; Hillman, R. M.; Krabill, L. F.; Martin, R. A.; Zurcher-Neely, H. A.; Guido, D. M. Degradation of growth hormone releasing factor analogues in neutral aqueous solution is related to deamidation of asparagine residues. *Int. J. Pept. Protein Res.* **1991**, *37*, 14−20.
(52) Karle, I. L.; Balaram, P. Structural characteristics of α-helical peptide molecules containing Aib residues. *Biochemistry* **1990**, *29*, 6747−6756.
(53) Marshall, G. R.; Bosshard, H. E. Angiotensin II. Studies on the biologically active conformation. *Circ. Res., Suppl. II* **1972**, *30*−*31*, 143−150.
(54) Toniolo, C.; Bonora, G. M.; Bavoso, A.; Benedetti, E.; Di Blasio, B.; Pavone, V.; Pedone, C. Preferred conformations of peptides containing α,α-disubstituted α-amino acids. *Biopolymers* **1983**, *22*, 205−215.
(55) Karle, I. L.; Banerjee, A.; Bhattacharjya, S.; Balaram, P. Solid state and solution conformations of a helical peptide with a central Gly-Gly segment. *Biopolymers* **1996**, *38*, 515−526.
(56) Prasad, S.; Rao, R. B.; Balaram, P. Contrasting solution conformations of peptides containing α,α-dialkylated residues with linear and cyclic side chains. *Biopolymers* **1995**, *35*, 11−20.
(57) Monaco, V.; Formaggio, F.; Crisma, M.; Toniolo, C.; Shui, X.; Eggleston, D. S. Crystallographic structure of a multiple *β*-turn containing, glycine-rich heptapeptide: A synthetic precursor of the lipopeptaibol antibiotic trichodecenin I. *Biopolymers* **1996**, *39*, 31−42.
(58) Sato, K.; Hotta, M.; Kageyama, J.; Chiang, T. C.; Hu, H. Y.; Dong, M. H.; Ling, N. Synthesis and in vitro bioactivity of human growth hormone-releasing factor analogues substituted with a single D-amino acid. *Biochem. Biophys. Res. Commun.* **1987**, *149*, 531−537.
(59) Marqusee, S.; Baldwin, R. L. Helix stabilization by $Glu^{-}\cdots Lys^{+}$ salt bridges in short peptides of *de novo* design. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8898−8902.
(60) Rivier, J.; Rivier, C.; Koerber, S. C.; Kornreich, W. D.; Miranda, A.; Miller, C.; Galyean, R.; Porter, J.; Yamamoto, G.; Donaldson, C. J.; Vale, W. Structure activity relationships (SAR) of somatostatin, gonadotropin, corticotropin and growth hormone releasing factors. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J. E., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1992; pp 33−36.
(61) Felix, A. M.; Heimer, E. P.; Wang, C. T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. Synthesis, biological activity and conformational analysis of cyclic GRF analogues. *Int. J. Pept. Protein Res.* **1988**, *32*, 441−454.
(62) Felix, A. M.; Wang, C.-T.; Campbell, R. M.; Toome, V.; Fry, D. C.; Madison, V. S. Biologically active cyclic (lactam) analogues of growth hormone-releasing factor: Effect of ring size and location on conformation and biological activity. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J. E., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1992; pp 77−79.
(63) Fry, D. C.; Madison, V. S.; Greeley, D. N.; Felix, A. M.; Heimer, E. P.; Frohman, L.; Campbell, R. M.; Mowles, T. F.; Toome, B.; Wegrzynski, B. B. Solution structures of cyclic and dicyclic analogues of growth hormone releasing factor as determined by two-dimensional NMR and CD spectroscopies and constrained molecular dynamics. *Biopolymers* **1992**, *32*, 649−666.
(64) Romier, C.; Bernassau, J.-M.; Cambillau, C.; Darbon, H. Solution structure of human corticotropin releasing factor by ^{1}H NMR and distance geometry with restrained molecular dynamics. *Protein Eng.* **1993**, *6*, 149−156.
(65) Chorev, M.; Behar, V.; Yang, Q.; Rosenblatt, M.; Mammi, S.; Maretto, S.; Pellegrini, M.; Peggion, E. Conformation of parathyroid hormone antagonists by CD, NMR, and molecular dynamics simulations. *Biopolymers* **1995**, *36*, 485−495.
(66) Lung, F.-D. T.; Collins, N.; Stropova, D.; Davis, P.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Design, synthesis, and biological activities of cyclic lactam peptide analogues of Dynorphin A(1−11)-NH_2. *J. Med. Chem.* **1996**, *39*, 1136−1141.
(67) Tessmer, M. R.; Kallick, D. A. NMR and structural model of dynorphin A(1−17) bound to dodecylphosphocholine Micelles. *Biochemistry* **1997**, *36*, 1971−1981.
(68) Cervini, L. A.; Corrigan, A.; Donaldson, C. J.; Koerber, S. C.; Vale, W. W.; Rivier, J. E. Cyclic analogues of [$MeTyr^{1}$,Ala^{15},-Nle^{27}]GRF(1−29)-NH_2 with high potencies in vitro. In *Peptides: Chemistry, Structure and Biology*; Hodges, R. S., Smith, J. A., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1994; pp 541−543.
(69) Miranda, A.; Koerber, S. C.; Gulyas, J.; Lahrichi, S.; Craig, A. G.; Corrigan, A.; Hagler, A.; Rivier, C.; Vale, W.; Rivier, J. Conformationally restricted competitive antagonists of human/rat corticotropin-releasing factor. *J. Med. Chem.* **1994**, *37*, 1450−1459.
(70) Rivier, J.; Kupryszewski, G.; Varga, J.; Porter, J.; Rivier, C.; Perrin, M.; Hagler, A.; Struthers, S.; Corrigan, A.; Vale, W. Design of potent cyclic gonadotropin releasing hormone (GnRH) antagonists. *J. Med. Chem.* **1988**, *31*, 677−682.
(71) Miranda, A.; Lahrichi, S. L.; Gulyas, J.; Koerber, S. C.; Craig, A. G.; Corrigan, A.; Rivier, C.; Vale, W.; Rivier, J. Constrained corticotropin-releasing factor (CRF) antagonists with $i-(i+3)$ Glu−Lys bridges. *J. Med. Chem.* **1997**, *40*, 3651−3658.
(72) Gulyas, J.; Rivier, C.; Perrin, M.; Koerber, S. C.; Sutton, S.; Corrigan, A.; Lahrichi, S. L.; Craig, A. G.; Vale, W.; Rivier, J. Potent, structurally constrained agonists and competitive antagonists of corticotropin releasing factor (CRF). *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575−10579.
(73) Garbow, J. R.; Breslav, M.; Antohi, O.; Naider, F. Conformational analysis of the *Saccharomyces cerevisiae* tridecapeptide mating pheromone by ^{13}C,^{15}N rotational-echo double resonance nuclear magnetic resonance spectroscopy. *Biochemistry* **1994**, *33*, 10094−10099.
(74) Jelicks, L. A.; Broido, M. S.; Becker, J. M.; Naider, F. R. Interaction of the *Saccharomyces cerevisiae* α-factor with phospholipid vesicles as revealed by proton and phosphorus NMR. *Biochemistry* **1989**, *28*, 4233−4240.
(75) Gounarides, J. S.; Broido, M. S.; Becker, J. M.; Naider, F. R. Conformational analysis of [D-Ala^{9}]α-factor and [L-Ala^{9}]α-factor in solution and in the presence of lipid. *Biochemistry* **1993**, *32*, 908−917.
(76) Sugg, E. E.; de L. Castrucci, A. M.; Hadley, M. E.; van Binst, G.; Hruby, V. J. Cyclic lactam analogues of Ac-[Nle^{4}]α-MSH_{4-11}-NH_2. *Biochemistry* **1988**, *27*, 8181−8188.
(77) Kapurniotu, A.; Taylor, J. W. Structural and conformational requirements for human calcitonin activity: Design, synthesis, and study of lactam-bridged analogues. *J. Med. Chem.* **1995**, *38*, 836−847.
(78) Yang, J. T.; Wu, C.-S. C.; Martinez, H. M. Calculation of protein conformation from circular dichroism. In *Methods in Enzymology;* Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1986; Vol. 130, pp 228.
(79) Gulyas, J.; Rivier, C.; Perrin, M.; Koerber, S. C.; Sutton, S.; Corrigan, A.; Lahrichi, S. L.; Craig, A. G.; Vale, W.; Rivier, J. Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575−10579.
(80) Ling, N.; Baird, A.; Wehrenberg, W. B.; Ueno, N.; Munegumi, T.; Chiang, T. C.; Regno, M.; Brazeau, P. Synthesis and in vitro bioactivity of human growth hormone-releasing factor analogues substituted at position-1. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 304−310.
(81) Lance, V. A.; Murphy, W. A.; Sueiras-Diaz, J.; Coy, D. H. Superactive analogues of growth hormone-releasing factor (1−29)-amide. *Biochem. Biophys. Res. Commun.* **1984**, *119*, 265−272.
(82) Märki, W.; Spiess, J.; Taché, Y.; Brown, M.; Rivier, J. E. Total solid-phase synthesis of porcine gut gastrin releasing peptide (GRP), a mammalian bombesin. *J. Am. Chem. Soc.* **1981**, *103*, 3178−3185.
(83) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1969.
(84) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.
(85) Rodbard, D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **1974**, *20*, 1255−1270.

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