# Growth Hormone-Releasing Factor Analogs with Hydrophobic Residues at Position 19. Effects on Growth Hormone Releasing Activity in Vitro and in Vivo, Stability in Blood Plasma in Vitro, and Secondary Structure†

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To test the hypothesis that replacing Ala[19] in growth hormone-releasing factor (GRF) with more hydrophobic residues will increase growth hormone releasing activity, four GRF analogs were prepared and tested. The molecules were made by substituting Val, Ile, or Leu at position 19 of [Thr[2],Ala[15],Leu[27]]bGRF(1-29)$NH_2$. The compounds were evaluated for growth hormone (GH) releasing activity in vitro (rat anterior pituitary cells) and in vivo (steers). Additionally, their half-life in vitro was determined in bovine plasma, and their secondary structure was examined by circular dichroism. In pituitary cells, peptides with substitutions at position 19 had the following potencies: Ala (native), 0.37; Val, 1.16; Ile, 0.37; Leu, 0.043. When assayed in steers as a single iv bolus, over a 2-h period, the compounds gave the following integrated GH response: Ala, 2.75; Val, 2.67; Ile, 2.57; Leu, 1.55. Only the Leu analog was statistically different from the other three ($p = 0.05$). In bovine plasma, the half-lives (hours) were as follows: Ala, 4.9; Val, 6.6; Ile, 12.3; Leu, 14.7. In phosphate buffer the compounds were calculated to have the following percent helical content: Ala, 26; Val, 21; Ile, 27; Leu, 32. For these analogs, helicity in aqueous buffer is inversely related to their in vitro activity. Using a linear multiple regression model, the plasma half-life of the analogs positively correlated ($r^2 = 0.999$) with both the hydrophobicity of the residue at position 19 and the helicity of the analog. Although the Val analog had both increased inherent activity and increased plasma stability in vitro compared to the Ala analog, in this study we were unable to demonstrate an increase in activity in vivo. The in vivo GH releasing activity of the analogs was not simply related to a combination of their intrinsic GH releasing activity and their in vitro plasma half-life. This suggests that in vivo additional factors are moderating the expression of activity.

The pituitary hormone, somatotropin or growth hormone (GH) is an important regulator of growth in many species including humans,[1] pigs,[2] and cattle.[3] It is also a major regulator of lactation in ruminants.[4] GH secretion is largely controlled by the hypothalamic peptides somatostatin and growth hormone-releasing factor (GRF). GRF also enhances growth[5,6] and lactation.[7] Although the native molecules have a length of 42-44 residues, full activity is obtained from residues 1-29, both in vitro[9] and in vivo.[10] The sequence of bGRF(1-29)$NH_2$ is shown in Figure 1. Homology of the N-terminal region of GRF molecules from different species is high, with rat and mouse GRF being the most divergent from human, ovine, and bovine GRF molecules.[8] Native rat GRF and rat amino terminal fragments tend to be more active than native GRFs and amino terminal fragments from other species, including the human and the bovine when tested in rat pituitary cells.[11] Despite the differences between the rat and human and bovine sequences, there is a high correlation ($r = 0.9$)

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† This paper is dedicated to Professor Ralph Hirschmann on the occasion of his 70th birthday. You've led the way.

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1 5 10 15

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-

20 25 29

Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Asn-Arg-$NH_2$

**Figure 1.** The sequence of bGRF(1–29)$NH_2$.

between the potencies of 80 analogs, mostly derived from the human and bovine sequences, which were tested in dispersed rat and bovine pituitary cells.[12]

The intrinsic activity of GRF molecules is currently best measured in vitro, using a dispersed pituitary cell assay. The activity of an analog in vivo, however, is a composite of its intrinsic activity and pharmacokinetic behavior, which includes, among other factors, its proteolytic stability in blood and other organs. In our efforts to prepare GRF analogs with improved activity in vivo, we have focused our efforts at improving intrinsic activity and increasing in vitro proteolytic stability in plasma.

Native GRF molecules are enzymatically cleaved at the $Ala^2$–$Asp^3$ bond in humans,[13] in vivo, and in human,[13,14] bovine, and porcine[15] plasma in vitro by a dipeptidyl peptidase IV (DPP-IV) like enzyme. The N·terminal Asp peptide which is produced is essentially devoid of GH releasing activity.[13] Replacement of the $Ala^2$ residue by a number of residues including Thr yields compounds highly resistant to DPP·IV cleavage.[16] The doubly substituted $Thr^2$ $Ala^{15}$ analog, 1, has higher activity than the native compound in steers in vivo, despite its lower intrinsic activity in dispersed bovine pituitary cells.[17]

It is postulated that GRF binds to its receptor through prior or concomitant interaction of the GRF molecule with membrane phospholipids[18] and that this interaction may involve a helical conformation of the GRF molecule. The hGRF(1–29)$NH_2$ molecule, which has limited helical structure in water, becomes more helical upon binding to acidic phospholipids.[19] NMR studies in 30% aqueous trifluoroethanol[20] show $Nle^{27}$ hGRF(1–29)$NH_2$ to assume $\alpha$ helical structure between residues 6–13 and 16–29.

Modeling GRF in an $\alpha$·helical conformation reveals a twisted hydrophobic path comprising residues $Phe^6$, $Tyr^{10}$, $Val^{13}$, $Leu^{14}$, $Gly^{15}$, $Leu^{17}$, $Ala^{19}$, $Leu^{22}$, $Leu^{23}$, $Ile^{26}$, and $Met^{27}$. Replacement of the native $Gly^{15}$ residue by Ala can lead to analogs with increased helicity and activity.[21] Replacing the $Ala^{19}$ residue with a more hydrophobic one should increase the interaction with hydrophobic components such as phospholipids. Additionally, these replacements should alter the helical content of the analog, further testing the relationship between helicity and activity.

Only a few GRF analogs have been prepared with substitution at position 19. Sato[22] reported that D·$Ala^{19}$ hGRF(1–29)$NH_2$ is only 6% as active as the $Ala^{19}$ molecule. Velicelebi[23] prepared a hGRF(1–29)$NH_2$ analog with Ser replacing Ala.[19] This Ala to Ser substitution was only one of 13 modifications which were made to form an idealized $\alpha$-helix. In rat pituitary cells, the analog was equipotent to hGRF(1–40)OH. Although this study showed that GRF can be extensively altered and still maintain intrinsic activity, few conclusions can be drawn about how any single modification, including the substitution at position 19, affected biological activity.

We prepared four analogs of [$Thr^2$,$Ala^{15}$,$Leu^{27}$]bGRF·(1–29)$NH_2$ with the hydrophobic residues Leu, Ile, or Val replacing the native $Ala^{19}$ residue. We examined their GH releasing activity both in rat pituitary cell culture, and in steers, their stability in bovine plasma in vitro, and their circular dichroism (CD) spectra.

## Results and Discussion

Table I shows the GH releasing potency of the analogs relative to [$Leu^{27}$]bGRF(1–29)$NH_2$, 5, in cultured rat pituitary cells, GH release over a 2·h period in steers, their half·life in bovine plasma in vitro, and their helicity in aqueous buffer. In the rat pituitary cell assay, the Val analog, 2, was most active, with an estimated potency of 1.16. The Ile analog, 3, and the native Ala analog, 1, were equipotent with estimated potencies of 0.37. The Leu analog, 4, was least active with an estimated potency of 0.043. Table II presents in vitro GH release data analyzed by dose, and Figure 2 shows the dose–response curves.

When tested in steers, as an iv bolus at 10 pmol/kg, a dose at the low end of the dose response curve,[24] GH was

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**Table I.** Growth Hormone Releasing Activity, Plasma Half-Life, and Percent Helicity of GRF Analogs and Standards

| compd no. | compd name | in vitro potency[a] | GH release in vivo[b] | in vitro half-life (h)[c] | % helix[d] |
|---|---|---|---|---|---|
| 1 | [Thr$^2$,Ala$^{15}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 0.37 | 2.75[e] | 4.9[g] | 26 |
| 2 | [Thr$^2$,Ala$^{15}$,Val$^{19}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 1.16 | 2.67[e] | 6.6[h] | 21 |
| 3 | [Thr$^2$,Ala$^{15}$,Ile$^{19}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 0.37 | 2.57[e] | 12.3[i] | 27 |
| 4 | [Thr$^2$,Ala$^{15}$,Leu$^{19}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 0.043 | 1.55[f] | 14.7[i] | 32 |
| 5 | [Leu$^{27}$]bGRF(1–29)NH$_2$ | 1 | | 0.6[j] | |
| 6 | bGRF(1–44)NH2] | | 1.42[f] | | |
| 7 | [desNH$_2$Tyr$^1$,D-Ala$^2$,Ala$^{15}$]hGRF(1–29)NH$_2$ | | 2.67[e] | | |
| standard error of the mean | | | 0.21 | | |
| $p$ value | | | 0.001 | | |
| error mean squared | | | 526 | | |

[a] Potency in the rat pituitary cell assay versus the standard, compound 5. [b] Integrated area under the curve for GH release in steers ($n$ = 12) over the 2-h test period. [c] Half-life of compounds determined from first-order rate constants ($n$ = 3).[216] [d] Percent helix in 10 mM phosphate buffer, pH 7.4. [e–i] Compounds with different superscripts different at $p$ = 0.05. [j] Determined with the same plasma pool as compounds 1–4 but with time points 0, 15, 30, 45, 60, 90, 120 min.

**Table II.** GH Release from Rat Pituitary Cells

| compd no. | compd | GH released[a] at analog concn listed below | | |
|---|---|---|---|---|
| | | 0.1 nM | 1.0 nM | 10.0 nM |
| | basal (buffer only) | 1318[b] | 1318[b] | 1318[b] |
| 1 | [Thr$^2$,Ala$^{15}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 3900[d] | 11225[d] | 14292[e] |
| 2 | [Thr$^2$,Ala$^{15}$,Val$^{19}$Leu$^{27}$]bGRF(1–29)NH$_2$ | 6870[g] | 12115[e] | 13930[d,e] |
| 3 | [Thr$^2$,Ala$^{15}$,Ile$^{19}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 4223[e] | 10772[d] | 13730[d] |
| 4 | [Thr$^2$,Ala$^{15}$,Leu$^{19}$,Leu$^{27}$]bGRF(1–29)NH$_2$ | 1564[c] | 4696[c] | 11432[c] |
| 5 (std) | [Leu$^{27}$]bGRF(1–29)NH$_2$ | 6487[f] | 13608[f] | 14086[d,e] |
| SEM | | 134.7 | 246.6 | 353.0 |
| $p$ value | | 0.0001 | 0.0001 | 0.0001 |
| EMS | | 72600 | 243286 | 498533 |

[a] GH amount is given in ng/well per 4 h ($n$ = 4). [b–g] Within a column, compounds with different superscripts are different ($p < 0.05$).

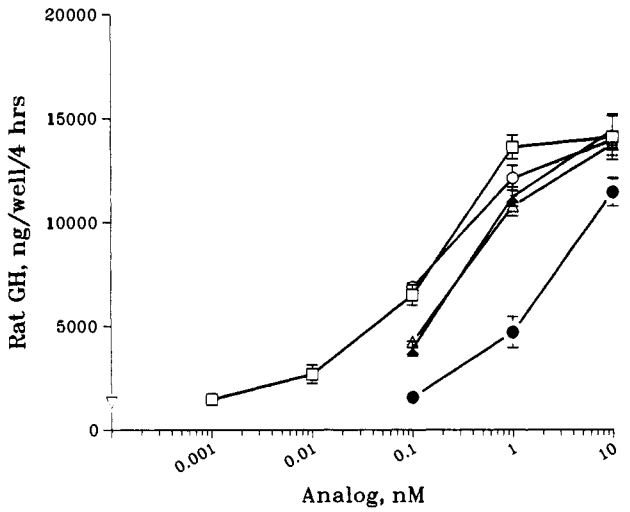


**Figure 2.** Growth hormone released in vitro in response to analogs and the standard in the rat pituitary cell assay ($n$ = 4). Compound 1 (△), compound 2 (○), compound 3 (△), compound 4 (○), compound 5 (□), basal release (▽). Error bar represents ∓1 standard deviation.

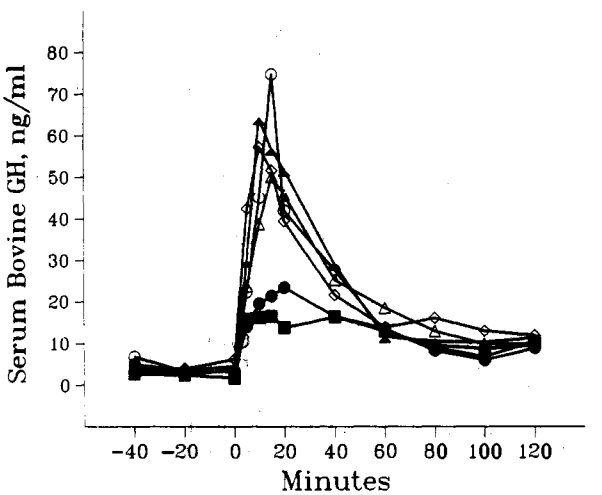


**Figure 3.** Growth hormone released in vivo over the test period to analogs and standards in the meal-fed steer assay ($n$ = 12). Compound 1 (△), compound 2 (○), compound 3 (△), compound 4 (○), compound 5 (□), compound 6 (■), compound 7 (◇).

released in the pattern shown in Figure 3. The Val analog, 2, the Ile analog, 3, and the Ala analog, 1, all released more GH than the Leu analog, 4, or the standard, bGRF(1–44)NH$_2$, 6. Compounds 1–3 had the same activity as compound 7, a compound reported to be a super potent GRF analog in vivo.[6,25] The Leu analog, 4, was as active in vivo as compound 6 despite its relatively low intrinsic activity in vitro. This observation is consistent with the report on related compounds with DPP-IV stabilizing modifications at position 2.[16,17] Modifications which improve both intrinsic potency and stability against enzymatic degradation by plasma enzymes should give improved performance in vivo. Although the Thr$^2$ modification protects against cleavage at the 2–3 bond, it was not known what additional effect the hydrophobic modifications at position 19 would have on the stability of the analogs in plasma in vitro, and how this would correlate with activity in vivo.

The half-lives of the test compounds in bovine plasma in vitro (determined from their first-order degradation

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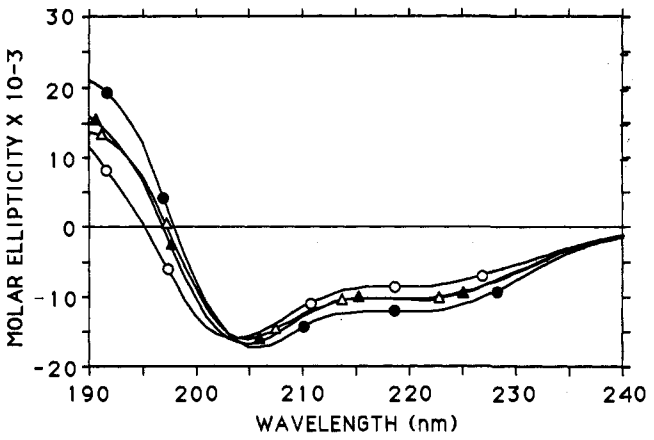


**Figure 4.** Circular dichroism spectra of analogs in 10 mM sodium phosphate buffer, pH 7.4. Compound 1 (△), compound 2 (○), compound 3 (▲), compound 4 (●).

rate constants)[26] showed a significant dependency on the residue at position 19 (Table I). As expected, compounds 1–4 were more stable in bovine plasma than compound 5, which has an Ala residue at position 2. The Leu analog, 4, and Ile analog, 3, had half·lives approximately 2 and 2.8 times longer than the Val analog, 2, and the Ala analog, 1, respectively. The enzymes in plasma responsible for the degradation of the Thr[2] analogs are not known. It was reported that porcine kidney derived DPP·IV cleaves compound 1 slowly.[17] However, in our HPLC analyses, no degradation products were observed. The GRF analogs contain five sites which would be susceptible to trypsin·like enzymes. Although Frohman reported trypsin·like activity in human plasma[14] and observed some trypsin·like degradation products from GRF analogs, Kubiak[15] did not observe trypsin·like degradation products of 5 in bovine plasma.

The CD spectra of compounds 1–4 were examined to look for relationships between secondary structure and activity or plasma stability. In aqueous buffer, all four compounds showed helical character by CD. This is observed by the CD spectra between 190 and 240 nm (Figure 4) and the calculated percent helicity (Table I). All four analogs have high helix forming potential, becoming more helical with increasing methanol concentration. All reached a helicity ranging from 86 to 91% in 80% methanol (data not shown). However, in the absence of methanol, differences in helicity were observed. The Leu analog, 4, was most helical, the Ala analog, 1, and the Ile analog, 3, intermediate, and the Val analog, 2, least helical. The increased helicity of the Leu analog, 4, over the Ala analog, 1, and the equal helicity of the Ala and Ile analogs is unexpected on the basis of either Chou and Fasman[27] or Garnier–Osguthorpe–Robson[28] algorithms which predict the Ala analog to be most helical. The observation that the Leu analog, 4, has higher helicity than the Ala analog, 1, and the equal helicity of 1 and the Ile analog, 3, is counter to the finding reported by Padmanabhan[29] for a series of 17 residue helical peptides. In that series, Ala and Leu had the highest helix forming tendencies, with Ile having a lower helix tendency. The lowest helicity of the Val substituted analog, 2, is consistent with prediction algorithms and with the effect of Val residues on helicity observed by Padmanabhan. The observations made here indicate the difficulty of predicting subtle differences in secondary structure for peptides.

In contrast to CD methods which present a global picture of secondary structure, NMR methods can reveal detailed structural information. In aqueous solution, no secondary structure was observed for the GRF analog, [Nle[27]]hGRF·(1–29)$NH_2$. However, upon addition of 30% trifluoroethanol, nuclear Overhauser effect signals diagnostic for α·helix were observed for the region 6–13 and 16–29.[20] The authors also proposed the presence of a half-turn between residues 13 and 16. Hydrogen bonding organic solvents such as methanol and trifluoroethanol have been suggested to be representative of a lipid environment. However, a CD study[19] of hGRF(1–29)$NH_2$ in trifluoroethanol, and in the presence of dimyristoyl phosphoglycerol liposomes, indicated differences in induced helicity, with lower helicity in the presence of liposomes. On the basis of the sequence of GRF and the lower helical content in the presence of phospholipids, the authors suggested that, on the surface of liposomes, GRF exists as two helical segments, but with a kink or twist between them near position 19.

Conflicting information exists on the correlation of helicity and activity in GRF analogs. An analog with the native Gly[15] replaced with an Ala residue had increased GH releasing activity and helicity, while a Gly to Sar modification had decreased activity and helicity, suggesting a positive correlation between activity and helicity.[21,30] Another report[31] suggested that there is a negative association between helicity (and rigidity) and GH releasing activity. Relative to hGRF(1–29)$NH_2$, [cyclo·(Glu[16]·Lys[20])·Ala[15]]hGRF(1–29)$NH_2$ has increased helicity and decreased GH releasing activity.

Although compounds 1–4 contain an Ala substituent at position 15 which would increase the helicity over the native sequence, 2–4 are modified at position 19 as well. For the compounds in this study, in vitro GH releasing potency was inversely related to helicity in aqueous solution. This finding suggests that for optimal receptor interaction, the residues in this region should not be in a helical conformation. However, the possibility of residue 19 being a receptor contact residue cannot be ruled out.

The Val analog, 2, Ala analog, 1, and the Ile analog, 3, all have much higher intrinsic activity than the Leu analog, 4. Other examples of diminished binding or activity of Leu containing peptides compared to their Ala, Val, or Ile

(26) Half-lives are log-normal distributed. Therefore, the analysis of variance (ANOVA) was carried out on the rate constants ($h^{-1}$) calculated from the disappearance of the compounds from bovine plasma. These were respectively: 1, 0.142[a]; 2, 0.105[b]; 3, 0.056[c]; 4, 0.047[c] (standard error of the mean = 0.002, $p$ = 0.0001, error mean square = 0.000 11). Values with different superscripts were different at $p$ = 0.05. The half-lives were determined from first-order rate constant ($h^{-1}$). The half-life of 5 was determined from its disappearance rate constant (1.12 $h^{-1}$) in a separate experiment using the same plasma pool.

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counterparts have been reported. Ala and Ile (as well as Ser and Thr) but not Leu could replace Val in binding the myohemerythrin peptide IleProGluProTyrVal to an antiserum generated using myohemerythrin as the immunogen.[32] This same pattern was observed for the activity of GRF analogs with these replacements at position 2.[16] Additionally, substitution of Ala for Leu in salmon calcitonin reduced helicity and enhanced activity.[33]

The plasma half-life of these position 19 analogs was highly correlated ($r^2$ = 0.999) with both the hydrophobicity[34] (Hy) of the residue[35] at position 19, and the helicity (He) of the analog in water. Half-life was described by the equation

$$\text{half-life (hours)} = -20.85 + 13.76(\text{Hy}) + 0.83(\text{He})$$

obtained by multiple linear regression analysis. One interpretation of this finding is that the peptides are protected from plasma enzyme proteolysis by hydrophobic binding to a plasma component such as albumin, and that the binding involves a helical conformation of the peptide.

## Conclusions

Clearly, the relation of biological activity both in vitro and in vivo with the biophysical and biochemical characteristics of a compound is complex. We were able to increase intrinsic potency with the Val analog 2 and to increase plasma stability in vitro with the Val, Ile, and Leu analogs 2, 3, and 4 over the Ala analog, 1. Although factors such as intrinsic activity and in vitro plasma stability are important, GH releasing activity in vivo is not simply related to a combination of these two factors. Our inability to demonstrate an increase in activity of compounds 2 and 3 over compound 1 in vivo may be related to the low resolution of the assay when only a single dose is administered. However, it may be related to other factors which operate in vivo. In vivo activity is a composite of a drug's intrinsic activity and its concentration at its receptor. In the case of GH release, counter regulation by somatostatin also is an important factor. Although we have measured intrinsic activity for these compounds, their concentration at the receptor was not measured and remains unknown. The receptor concentration is dictated by the dose of drug administered and its pharmacokinetic behavior, which includes metabolism by blood enzymes, but also includes metabolism by other organs, distribution, and excretion.

## Experimental Methods

bGRF(1–44)$NH_2$ was purchased from Bachem Inc. The other GRF analogs were synthesized by solid-phase Boc chemistry utilizing an Applied Biosystems 430A peptide synthesizer and reagents supplied by Applied Biosystems, Foster City, CA. Synthesis and cleavage protocols were identical to those described by Kubiak;[15] however, the ion-exchange step was omitted during purification. The following additional reagents were used: acetonitrile (Burdick and Jackson) and trifluoroacetic acid (TFA) (Pierce). Water was purified on a MilliQ system (Millipore). The peptides were purified by gradient reverse phase HPLC: Beckman 421 Controller; Model 110A pumps; Vydac C18 preparative column (The Separations Group, Hesperia, CA); water, acetonitrile, each containing 0.1% TFA; gradient 30–50% acetonitrile over 180 min; detection at 218 nm. Analytical HPLC: 15 cm Vydac C18 column; Perkin-Elmer 2600 system; gradient, 30–50% acetonitrile/water each containing 0.1% TFA over 30 min; detection at 215 nm. HPLC of samples for plasma stability: Hewlett-Packard Model 1090M; 5-cm Vydac C18 column; mobile phase, (A) 5% acetonitrile/95% water (v/v) with 0.045% TFA, (B) 80% acetonitrile/20% water (v/v) with 0.040% TFA; gradient, initial solvent composition (depending on the compound) varied from 30 to 35% with a gradient to 50% B in 5 min; flow rate, 2 mL/min; detection at 220 nm. For amino acid analysis, peptides were hydrolyzed with HCl in the vapor phase using a Pico-Tag Work Station (Waters Associates, Milford, MA). Hydrolysates were analyzed by ion-exchange chromatography on a Beckman 6300 amino acid analyzer. Mass spectral analysis: plasma desorption Cf-252 Time-of-Flight mass spectrometer (Bio Ion Model 20, Applied Biosystems). CD spectra of the compounds were measured at room temperature (20–22 °C) on a JASCO Model 600 or 720C CD spectropolarimeter using 1-mm quartz cells. The spectropolarimeter was calibrated at 290 nm with *d*-10-camphorsulfonic acid.

**[Thr$^2$,Ala$^{15}$,Leu$^{27}$]bGRF(1–29)NH2** (1). HPLC retention time = 20.2 min. Amino acid analysis: Asp 3.95 (4.0), Thr 1.82 (2.0), Ser 1.65 (2.0), Glu 2.04 (2.0), Ala 2.96 (3.0), Val 0.93 (1.0), Ile 1.88 (2.0), Leu 5.14 (5.0), Tyr 1.97 (2.0), Phe 0.98 (1.0), Lys 2.00 (2.0), Arg 3.08 (3.0). MS (M + H)$^+$ *m/z* 3414.1.

**[Thr$^2$,Ala$^{15}$,Val$^{19}$,Leu$^{27}$]bGRF(1-29)NH$_2$** (2). HPLC retention time = 23.1 min. Amino acid analysis: Asp 3.94 (4.0), Thr 1.56 (2.0), Ser 1.68 (2.0), Glu 2.03 (2.0), Ala 1.99 (2.0), Val 1.89 (2.0), Ile 1.82 (2.0), Leu 5.13 (5.0), Tyr 2.00 (2.0), Phe 1.00 (1.0), Lys 2.03 (2.0), Arg 3.07 (3.0). MS (M + H)$^+$ *m/z* 3440.9.

**[Thr$^2$,Ala$^{15}$,Ile$^{19}$,Leu$^{27}$]bGRF(1-29)NH$_2$** (3). HPLC retention time = 24.1 min. Amino acid analysis: Asp 3.97 (4.0), Thr 1.84 (2.0), Ser 1.67 (2.0), Glu 2.03 (2.0), Ala 1.98 (2.0), Val 0.94 (1.0), Ile 2.87 (3.0), Leu 5.13 (5.0), Tyr 1.97 (2.0), Phe 0.98 (1.0), Lys 2.01 (2.0), Arg 3.05 (3.0). MS (M + H)$^+$ *m/z* 3455.7.

**[Thr$^2$,Ala$^{15}$,Leu$^{19}$,Leu$^{27}$]bGRF(1-29)NH$_2$** (4). HPLC retention time = 23.4 min. Amino acid analysis: Asp 3.94 (4.0), Thr 1.84 (2.0), Ser 1.66 (2.0), Glu 2.01 (2.0), Ala 1.98 (2.0), Val 0.95 (1.0), Ile 1.89 (2.0), Leu 6.13 (6.0), Tyr 1.98 (2.0), Phe, 0.99 (1.0), Lys 2.00 (2.0), Arg 3.07 (3.0). MS (M + H)$^+$ *m/z* 3455.2.

**[Leu$^{27}$]bGRF(1–29)NH$_2$** (5). Amino acid analysis: Asp 4.09 (4.0), Thr 0.92 (1.0), Ser 1.81 (2.0), Glu 2.03 (2.0), Gly 1.01 (1.0), Ala 2.99 (3.0), Val 0.93 (1.0), Ile 1.81 (2.0), Leu 5.08 (5.0), Tyr 2.03 (2.0), Phe 0.93 (1.0), Lys 1.96 (2.0), Arg 3.07 (3.0). MS (M + H)$^+$ *m/z* 3370.9.

**[desNH$_2$Tyr$^1$,D-Ala$^2$,Ala$^{15}$]hGRF(1–29)NH$_2$** (7). Amino acid analysis: Asp 3.03 (3.0), Thr 0.97 (1.0), Ser 2.69 (3.0), Glu 2.06 (2.0), Ala 4.10 (4.0), Val 0.90 (1.0), Met 1.00 (1.0), Ile 1.86 (2.0), Leu 4.02 (4.0), Tyr 0.99 (1.0), Phe 0.93 (1.0), Lys 1.96 (2.0), Arg 3.05 (3.0). MS (M + H)$^+$ *m/z* 3358.6.

**In Vitro Rat Growth Hormone Release Assay.** All compounds were tested at 0.1, 1.0, and 10.0 nM in an in vitro dispersed rat pituitary cell assay as described.[36,37] Four replicates were run for each data point. Data were analyzed using the general linear models procedure (Proc-GLM, SAS)[38] by dose level with compound as the main effect. Compound means were compared within dose using least squares means from the GLM analysis. In order to estimate the potency of each analog, the maximum response level ($B_{max}$) was calculated for the standard, compound 5, using the procedure NLIN.[38] Responses at the 0.1 and 1.0 nM doses for compounds 1, 2, 3, and 5 and 1.0 and 10.0 nM doses for compound 4 were included in a regression analysis. Using the linear equation calculated for each compound, the concentration that would release one-half the $B_{max}$ of the standard

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was calculated as the $EC_{50}$. Compound potencies were obtained by taking the ratio of the $EC_{50}$ standard/$EC_{50}$ test compound.

**In Vivo Meal-Fed Steer GH Release Assay.** The compounds were tested as described previously[39] with minor modifications. Briefly, Holstein steers (225–300 kg) were meal-fed once daily at 1000 h and trained to consume their total daily feed intake in 2 h. In a 6 × 6 Latin Square design (replicated), 12 steers were injected (iv bolus) with stock solutions of the four test compounds (1–4) and two standards (bGRF(1–44)$NH_2$, 6, and [des$NH_2$Tyr$^1$,D-Ala$^2$,Ala$^{15}$] hGRF(1–29)$NH_2$, 7, at 10 pmol/kg bodyweight at 0800 h, 2 h before feeding. Blood samples (8 mL) were collected through a cannula in the jugular vein at 20-min intervals from 40 min before until 120 min after injection with additional samples collected at 5, 10, and 15 min after injection. Serum obtained from the samples was assayed for GH concentration by RIA.[40] Data was analyzed by analysis of variance with treatment, animal, and period as main effects.[41] Treatment means were compared using least squares means from GLM analysis.[41]

**Plasma Stability Assay.** Analogs and the standard were tested in triplicate in vitro in a bovine plasma stability assay[15] using freshly isolated, pooled plasma. The assay was performed with the following modifications: the plasma was buffered with 0.2 M sodium phosphate (pH 7.4) containing 0.01% sodium azide. The assay was linear for degradation of [Leu$^{27}$] bGRF(1–29)-$NH_2$, 5, for at least 48 h. The analogs were dissolved in plasma at a nominal concentration of 50 M. Plasma aliquots (0.05 mL) were taken and quenched at the following time points: 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 10.0, 14.0, 24.0, 30.0, and 48.0 h. The samples were extracted using Sep-pak C18 cartridges (Waters) as described, lyophilized, and reconstituted in 4 M guanidine hydrochloride containing 0.5% TFA and analyzed by HPLC. The concentration versus time data was fitted to a first-order kinetic model using the nonlinear least squares procedure NLIN.[38] The first-order rate constants, $k$, were estimated directly, and the half-lives[26] were computed.[42]

**Circular Dichroism Analysis.** Peptide stock solutions were prepared in water (~1.5 mg/mL). Stock solutions, 0.20 mL, were diluted to 4.0 mL with the appropriate methanol/sodium phosphate buffer (20 mM, pH 7.4) (0, 20, 40, 60, and 80 vol % methanol in buffer). This gives a nominal peptide concentration of 30 M. Actual concentrations were calculated from their UV spectra using 1 as the standard. The concentration of 1, determined by quantitative amino acid analysis, yielded a molar absorptivitiy of 82.0 per residue at 274.5 nm. Intensities were obtained from the distance of the line drawn between the wings of the absorption band to the maximum at 274.5 nm to correct for displaced base line and light scattering. To compensate for differences in peptide concentration, CD spectra were corrected by normalization of their UV curves to the curve of compound 1, as shown in Figure 5. The CD intensity, expressed as mean residue ellipticity, was calculated as described.[43] The percent helicity was calculated from the mean residue ellipticity at 222 nm, calculated by the method[44] of Chen et al.

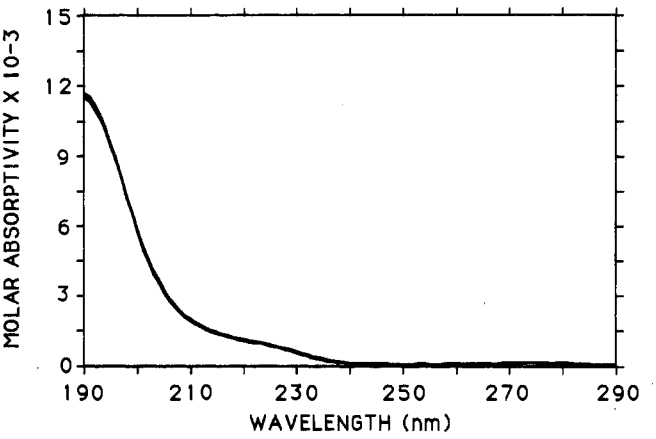


**Figure 5.** Normalized UV spectra of compounds 1–4 used for correcting CD curves.

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