benzyl chloride) was added to a stirred suspension of 2,4-lutidinium methiodide (25 g, 0.1 mol) in dry ether (100 mL) during 15 min at ambient temperature. The mixture was stirred for a further 2 h and then poured into a mixture of crushed ice (500 g) and 60% $HClO_4$ (60 mL). Crude 2-(4-methoxybenzyl)-1,4,6-trimethyl-1,2-dihydroxypyridine perchlorate (28.0 g) was collected from the center layer by filtration after 0.5 h, washed with EtOH, and dried. This intermediate was suspended in a mixture of MeOH (150 mL) and 2 N NaOH (200 mL) and treated with $NaBH_4$ (7.50 g) during 15 min. The mixture was kept at 55–60 °C overnight, diluted with water (200 mL), cooled, and extracted with ether (4 × 100 mL). The combined ether extracts were dried (Na_2SO_4), and the solvent was evaporated to give the corresponding tetrahydropyridine (19.20 g). This was dissolved in 47% aqueous HBr (150 mL), was heated under reflux for 22 h, and then poured into ice/water. The solution was basified with strong NH_4OH and extracted with $CHCl_3$ (4 × 100 mL). The combined extracts were dried (Na_2SO_4), and the solvent was removed to give a crude oil, which on washing with ether gave the product (6.9 g, 33%), mp 234–236 °C. Anal. ($C_{15}H_{21}NO$) C, H, N.

1,2,3,4,5,6-Hexahydro-4,6-dimethyl-8-hydroxy-2,6-methano-3-benzazocine (10g). A solution of **10b** (4.10 g, 0.018 mol) in acetic anhydride (10 mL) was heated at 100 °C for 3 h, and the excess acetic anhydride was removed in vacuo. The residue was dissolved in ethyl acetate (100 mL), the solution was washed with 5% aqueous Na_2CO_3 (3 × 50 mL) and dried (anhydrous K_2CO_3), and the solvent was removed to give the crude *O*-acetate (4.0 g). A solution of this intermediate in $CHCl_3$ (25 mL) was added gradually to a solution of CNBr (1.60 g) in $CHCl_3$ (25 mL) at room temperature with stirring. The mixture was heated under reflux, for 3 h, cooled, washed with 5% HCl (50 mL), and dried (Na_2SO_4). The solvent was removed to give crude *N*-cyano compound (4.07 g), which was dissolved in 7% HCl (80 mL), and the soluton was heated under reflux overnight. The cooled solution was made alkaline with aqueous ammonia and extracted with $CHCl_3$ (4 × 50 mL), and the combined, dried (anhydrous K_2CO_3) extracts were evaporated to give solid **10g** (2.27 g, 59%); mp 241–243 °C after recrystallization from 2-propanol. Anal. ($C_{14}H_{19}NO$) C, H, N.

1,2,3,4,5,6-Hexahydro-4,6-dimethyl-3-allyl-8-hydroxy-2,6-methano-3-benzazocine (10e). A mixture of **10g** (1.5 g, 6.9 mmol), allyl bromide (0.84 g, 6.9 mmol), and K_2CO_3 (0.5 g) in ethanol (50 mL) was heated under reflux for 24 h and then the solvent was removed. The residue was dissolved in water and extracted with $CHCl_3$ (3 × 100 mL), and the combined, dried (Na_2SO_4) extracts were concentrated to give the product (**10e**) (0.84 g, 48%). The hydrochloride crystallized from 2-butanone/MeOH and had mp 263–265 °C. Anal. ($C_{17}H_{24}NOCl$) C, H, N.

1,2,3,4,5,6-Hexahydro-4,6-dimethyl-3-(cyclopropylmethyl)-8-hydroxy-2,6-methano-3-benzazocine (10f). A solution of cyclopropylcarbonyl chloride (1.88 g, 18 mmol) in dry THF (30 mL) was added gradually to a solution of **10g**, (2.0 g, 9.2 mmol) and triethylamine (1.81 g, 19 mmol) in THF (50 mL) and the mixture stirred at room temperature for 3 h when the precipitated triethylamine hydrochloride was removed. Evaporation of the solvent gave crude cyclopropylamide (3.04 g), which was dissolved in THF (40 mL) and added dropwise to a suspension of LAH (0.97 g) in dry THF (50 mL). The mixture was heated under reflux for 8 h after which the excess LAH was destroyed cautiously. After removal of the precipitate, the filtrate was dried (Na_2SO_4) and the solvent evaporated to give the product (1.41 g, 61%), which solidified on standing. The hydrochloride crystallized from $EtOH/Et_2O$ and had mp 280–282 °C. Anal. ($C_{18}H_{26}NOCl$) C, H, N.

1,2,3,4,5,6-Hexahydro-3,5,6-trimethyl-8-hydroxy-2,6-methano-3-benzazocine (10d) was prepared by the procedure of Parfitt and Walters[2] for the nonphenolic derivative. The hydrochloride crystallized from ethanol–ether and had mp 283–285 °C dec. Anal. ($C_{15}H_{22}NOCl$) C, H, N.

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Structure–Activity Studies on the N-Terminal Region of Growth Hormone Releasing Factor

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In previous reports illustrating the effects of conformational restriction of the N-terminal region of human pancreatic growth hormone releasing factor, we demonstrated that D-amino acid substitutions in either of positions 1, 2, or 3 resulted in greatly increased growth hormone releasing activity both in vivo and in vitro. The most active compound, [D-Ala-2]GRF(1–29)NH_2, was 51 times more active than the parent 29 amino acid peptide in the sodium pentobarbital anesthetized rat. These observations have now been extended to analogues containing multiple D-amino acid replacements in these three positions. Once again, peptides with superagonist potencies ranging from 1200% to 3800% were obtained after solid-phase synthesis and purification by medium-pressure reverse-phase liquid chromatography. In addition, it was found that [D-Asn-8]- and [D-Ala-4]GRF(1–29)NH_2 were, respectively, 2.43 and 1.1 times more active than GRF(1–29)NH_2 itself. In contrast, [D-Phe-6] and [D-Thr-7] analogues were virtually inactive. Chou–Fasman structural predictions suggest that the first three residues of the peptide assume no fixed type of conformation but that a reverse turn could be present between residues 6 and 10. Attempts are made to rationalize the biological results with these calculations. The effects of other side chains on the D-amino acid in position 2 were also investigated. Both the Ac-[D-Phe-2]- and Ac-[D-Arg-2]peptides had very low activity. Several of the inactive peptides were tested as possible antagonists of GRF; however, none was able to block the stimulatory effects of GRF(1–29)NH_2 after combined administration.

Although the human pancreatic tumor,[2,3] rat,[4] and bovine[5] growth hormone releasing factors, possessing 40 or 44 amino acids, are well within range of rapid solid-phase techniques, it is much preferrable to work with as short

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VIP-SECRETIN FAMILY OF PEPTIDES

	1 2 3 4 5 6 7 8 9 10 11 12 13 14
VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-
SECRETIN	His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-
GLUCAGON	His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-
GIP	Tyr-Ala-Glu-Gly-Tyr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-
PHI	His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-
HPGRF	Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-

	15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
VIP	Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2
SECRETIN	Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
GLUCAGON	Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr
GIP	Asp-Lys-Ile-Arg-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln..
PHI	Gly-Gln-Leu-Ser-Ala-Lys-Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2
HPGRF	Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2

Figure 1. Amino acid sequences of peptides related to GRF (GIP has a total of 43 residues, the last 14 of which are not shown).

Table I. Percent Growth Hormone Releasing Activities of Analogues in the Anesthetized Rat Relative to hpGRF(1–29)NH_2

analogue	% GH-releasing activity (95% CL)
1. D-Tyr-1[a]	993 (556–1800)
2. D-Ala-2[a]	5146 (3365–7868)
3. D-Asp-3[a]	670 (392–1146)
4. D-Ala-4	110 (77–157)
5. D-Ile-5	<5
6. D-Phe-6	na[b]
7. D-Thr-7	na[b]
8. D-Asn-8	221 (169–290)
9. D-Ala-15	57 (37–88)
10. Ac-Tyr-1[a]	1224 (487–2050)
11. Ac-Tyr-1,D-Ala-2	2558 (1825–3586)
12. Ac-D-Tyr-1,D-Ala-2	3817 (2490–5850)
13. Ac-D-Tyr-1,D-Ala-2,D-Asp-3	1297 (889–1894)
14. Phe-1	14 (8–24)
15. Ac-His-1,D-Ala-2	817 (568–1175)
16. Ac-Tyr-1,D-Phe-2	na[b]
17. Ac-Tyr-1,D-Arg-2	na[b]
18. Phe-4	21 (15–30)
19. Ac-Tyr-1,*p*-Cl-Phe-6	52 (38–73)
20. Ac-Tyr-1,Trp-6	na[b]

[a] Taken from Lance et al.[6] [b] Not active at doses tested (see Table II).

a sequence as possible when structure–function studies involving numerous analogues are contemplated. For this purpose, the 1–29 amidated sequence appears to be an excellent choice since it retains much of the in vivo and in vitro GH releasing activity of the longer peptides. In addition, this portion of the sequences is preserved in species thus far examined (the rat peptide does have His in place of Tyr in position 1). Shorter analogues, such as the 1–24 amide[6,7] have greatly reduced biological activity.

The 1–29 sequence has recently[6] been utilized as a basis for synthetic and biological studies on analogues in which some degree of conformational restriction was built into the N-terminus by the replacement of L-amino acids in each of the first three positions by their D isomers. It was found that [D-Tyr-1]-, [D-Ala-2]-, and [D-Asp-3]GRF(1–29)NH_2 were all more active than the control peptide. The [D-Ala-2] analogue was at least 50 times more active in the rat and the pig[6] and also on preparations of cultured rat

Table II. Dose Responses of hpGRF(1–29)NH_2 (Control) and Analogues in the Anesthetized Rat

peptide	dose, μg/100 g of body wt	plasma GH, ng/mL ± SE
saline		362 ± 20
control	10	1184 ± 74
	25	2569 ± 121
D-Ala-4	5	805 ± 118
	25	2234 ± 457
D-Ile-5	50	435 ± 51
	125	670 ± 83
D-Phe-6	5	324 ± 79
	25	374 ± 66
D-Thr-7	5	213 ± 27
	25	328 ± 48
D-Ala-15	5	503 ± 52
	50	1751 ± 313
Ac-His-1,D-Ala-2	1	1447 ± 184
	5	2696 ± 456
Ac-Tyr-1,D-Phe-2	5	475 ± 65
	10	422 ± 72
Ac-Tyr-1,D-Arg-2	5	385 ± 121
	25	215 ± 42
Ac-Tyr-1,*p*-Cl-Phe-6	16	504 ± 96
	32	2378 ± 550
Ac-Tyr-1,Trp-6	5	469 ± 51
	25	516 ± 94
saline		366 ± 81
control	10	1272 ± 169
	25	2701 ± 177
D-Asn-8	4	1296 ± 118
	10	2397 ± 246
saline		189 ± 18
control	10	625 ± 112
	25	3076 ± 554
Ac-Tyr-1,D-Ala-2	0.4	1300 ± 329
	1	2496 ± 308
saline		420 ± 82
control	10	1533 ± 161
	25	2178 ± 173
Ac-D-Tyr-1,D-Ala-2	0.25	1235 ± 151
	0.62	2386 ± 349
saline		418 ± 83
control	10	1025 ± 191
	25	2191 ± 457
Ac-D-Tyr-1,D-Ala-2,D-Asp-3	1.2	1329 ± 185
	3	3504 ± 402
saline		225 ± 18
control	10	1187 ± 183
	25	2447 ± 298
Phe-1	25	519 ± 94
	62.5	1035 ± 130
saline		231 ± 33
control	10	948 ± 123
	25	1930 ± 305
Phe-4	37.5	796 ± 80
	93.8	1588 ± 176

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC Commission on biochemical nomenclature: *J. Biol. Chem.*, **247**, 977 (1971). Other abbreviation used are as follows: GRF(1–29), human pancreatic growth hormone releasing factor(1–29) amide; VIP, porcine vasactive intestinal peptide; GIP, gastric inhibitory peptide, PHI, intestinal peptide with histidine N-terminus and isoleucine amide C-terminus; LH-RH, luteinizing hormone-releasing hormone; MSH, melanocyte stimulating hormone; HPLC, high-performance liquid chromatography; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; Ac, acetyl.

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Table III. Lack of Effect of [D-Arg-2]GRF(1–29)NH_2 on the GH-Releasing Activity of GRF(1–29)NH_2 in the Anesthetized Rat

peptide	dose, μg/100 g of body wt	plasma GH, ng/mL
saline		372 ± 49
GRF(1–29)	20	1781 ± 219
GRF(1–29) + [D-Arg-2]GRF(1–29)	20 + 20[a]	2467 ± 253
GRF(1–29) + [D-Arg-2]GRF(1–29)	20 + 100[a]	2387 ± 201
GRF(1–29) + [D-Arg-2]GRF(1–29)	20 + 500[a]	2206 ± 286

[a] Peptides administered simultaneously.

pituitary cells.[8] It was also observed that simple acetylation of the α-amino terminus increased in vivo activity about 12-fold. In this paper we now report the results of combining these D-amino acid replacements in varying combinations in an effort to further increase activity. The D-amino acid substitution strategy was also extended to the amino acids in positions 4, 5, 6, 7, and 8. The effects of side-chain chemistry on the position 2 D-amino acid and substituents on the Phe ring in position 6 were also examined. The 1–29 sequence was subjected to the empirical Chou–Fasman method[9] of predicting secondary structure in an attempt to rationalize observed biological activities.

The GRF sequences are very closely related to those of the other five members of the VIP–secretin–glucagon family of peptides, the primary structures of which are shown in Figure 1. Thus analogue design approaches applied to one peptide can equally well be used on the others. This is particularly true of the N-terminal regions where maximum sequence homology exists. We recently described[10] a D-amino acid replacement strategy for glucagon which resulted in a highly active [D-Phe-4]peptide so that the value of the approach is evident. There is also evidence that secretin, GIP, and glucagon can effect pituitary responsiveness to GRF possibly due to receptor interactions.[11]

Experimental Section

Synthesis. Benzhydrylamine polystyrene resin cross-linked with 1% divinyl benzene was obtained from Bachem, Inc., Torrance, CA, and contained 0.5 mmol of free amino groups/g of resin. All amino acids were coupled as their N^{α}-Boc derivatives, which were obtained from the same source. Reactive side chains were protected as follows: Ser and Thr, benzyl ether; Lys, 2-chlorobenzyloxycarbonyl; His and Arg, tosyl; aspartic and glutamic, benzyl ester; Tyr, 2-bromobenzyloxycarbonyl. The amino acids were coupled to the growing peptide chain in 3 M excess in the presence of diisopropylcarbodiimide with a Beckman 990B automatic synthesizer. Boc-Asn and Boc-Gln were coupled in the presence of equivalent amounts of 1-hydroxybenzotriazole. Boc protection was removed at each step by two treatments with 33% TFA in methylene chloride for 1 and 25 min each. Couplings were monitored by the standard ninhydrin test[12] and repeated when necessary with preformed symmetrical anhydrides in DMF to which a catalytic amount of (dimethylamino)pyridine had been added.

Cleavage, Deprotection, Purification, and Characterization. The completed, protected peptide-resins (0.25 mmol) were treated with 20 mL of anhydrous hydrogen fluoride containing 10% anisole and 100 mg of dithiothreitol for 45 min at 0 °C. After removal of the HF under a stream of nitrogen (ca. 15 min), the free peptide amides were precipitated with either ether or ethyl acetate, filtered, and extracted with 2 M AcOH. At this stage, an HPLC profile of the crude material was obtained as a reference for future purification steps.

The extract was applied to a column (2.5 × 100 cm) of fine Sephadex G-50 and eluted with 2 M AcOH. Fractions were collected and examined individually by TLC on silica gel plates developed in 1-butanol/pyridine/acetic acid/water (15:10:3:12). The spots were visualized by ninhydrin followed by Cl_2/starch reagent and fractions pooled accordingly. Material thus obtained was then purified to >98% purity by preparative medium-pressure chromatography on a column (1.5 × 50 cm) of Vydac C_{18} silica (15–20-μm particle size; 300-Å pore size), which was eluted with a linear gradient (Eldex Chromatrol) of 25–55% acetonitrile/water (0.1% in TFA) at a flow rate of about 3 mL/min and a pressure of 80 psi. Eluent was monitored at 280 nm, and fractions were examined by TLC and analytical HPLC under conditions described in the next section. Fractions were pooled for maximum purity rather than yield and lyophilized repeatedly from dilute AcOH as white, fluffy powders.

Purified materials were examined for homogeneity by analytical HPLC with use of a Rainin machine with Apple II+ controller. Two types of column and conditions were normally used for each peptide as an added check for purity. The first analysis was carried out on a column (0.4 × 25 cm) of Vydac phenyl-silica (5-μm particle size; 300-Å pore size). Solvent 1 consisted of 20% acetonitrile/water (containing 0.05% TFA). Solvent 2 was 80% acetonitrile/water also containing 0.05% TFA. Peptide was eluted with use of a linear gradient of 25–55% solvent 1 in solvent 2 over a 30-min period. System B analysis was carried out on the same size column of Vydac C_{18}-silica (5-μm particle size; 300-Å pore size). Solvent 1 consisted of 20% acetonitrile/0.1 M triethylammonium phosphate (pH 2.0) and solvent 2 was 80% acetonitrile/0.1 M triethylammonium phosphate. Peptide was again eluted with use of a linear gradient of 25–55% of solvent 1 over 30 min. Peaks were detected at 215 nm with a Gilson Holochrome variable-wavelength monitor. Retention times are given in Table IV.

Peptides were also examined by silica gel TLC using the solvent system already described. R_f values are also given in Table IV.

Table IV. Chromatography Data for hpGRF(1–29) and Analogues

analogue no.[b]	TLC R_f[a]	HPLC retention time, min: system A[c]	system B[d]
GRF(1–29)	0.48	25.0	28.5
1	0.47	25.8	29.2
2	0.49	25.1	28.5
3	0.45	25.2	28.7
4	0.48	24.9	28.4
5	0.49	25.5	28.7
6	0.48	25.3	29.0
7	0.45	25.3	28.5
8	0.53	24.6	27.6
9	0.43	24.8	27.9
10	0.53	27.9	30.5
11	0.55	27.2	30.5
12	0.54	27.2	30.2
13	0.54	27.4	30.6
14	0.45	25.4	30.1
15	0.36	24.6	27.9
16	0.57	29.7	34.2
17	0.45	24.7	28.6
18	0.55	25.8	29.6
19	0.52	25.6	29.4
20	0.50	27.5	31.1

[a] Silica gel plates developed in 1-butanol/acetic acid/pyridine/water (15:10:3:12, v/v). [b] See Table I. [c] Phenyl-silica using the triethylammonium phosphate/acetonitrile system described in the text. [d] C_{18}-silica using the 0.1% trifluoroacetic acid/acetonitrile system described in the text.

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Table V. Amino Acid Ratios after Acid Hydrolysis of GRF Peptides

peptide	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	Arg
GRF(1–29)	3.00	0.95	2.97	2.19	1.04	2.96	1.02	0.75	1.92	4.14	1.95	0.93	1.93	2.84
1	3.00	1.00	2.84	2.34	1.05	3.23	0.99	1.02	2.01	4.00	1.99	0.96	2.02	3.17
2	3.15	1.00	2.92	2.20	1.07	3.10	0.95	1.02	1.96	4.22	1.82	0.95	1.97	2.93
3	2.83	0.98	2.77	2.31	1.01	3.02	0.96	1.03	1.91	3.57	1.93	0.95	1.93	3.13
4	2.81	0.91	2.73	2.15	1.04	2.87	0.89	1.00	1.81	3.86	1.90	0.93	1.90	2.93
5	3.01	0.98	2.92	2.24	1.10	3.00	0.96	0.97	1.74	4.23	2.06	0.90	1.86	3.13
6	3.00	0.93	2.71	2.09	1.06	1.99	1.02	0.93	1.89	4.02	1.83	0.93	1.90	2.93
7	2.82	0.92	2.73	2.15	1.00	2.85	0.91	1.02	1.73	3.77	1.96	0.90	1.92	3.13
8	2.85	0.94	2.81	2.11	1.01	2.95	0.97	0.98	1.92	4.01	1.90	1.91	1.98	3.00
9	2.99	1.00	2.92	2.27		4.00	0.99	1.09	1.92	4.03	1.94	0.94	2.06	3.20
10	2.92	1.00	2.80	2.20	1.05	3.06	0.95	1.02	1.90	3.80	1.93	0.91	1.96	3.07
11	3.00	0.95	2.78	2.09	1.03	2.95	0.90	0.97	1.87	4.03	1.82	0.96	1.95	2.79
12	2.95	1.00	2.91	2.20	1.08	3.02	0.93	1.04	1.90	3.97	1.88	0.98	2.00	3.10
13	2.89	1.00	2.76	2.19	1.05	3.04	0.96	1.02	2.19	3.73	1.88	0.99	1.94	3.04
14	2.92	0.92	2.74	2.02	1.00	2.92	0.75	0.95	1.89	4.00	0.93	1.95	1.84	2.83
15[a]	2.89	1.00	2.79	2.28	1.05	2.98	0.99	0.86	0.95	3.82	0.90	0.95	2.00	3.14
16	3.00	1.00	2.93	2.30	1.11	3.14	0.98	1.13	1.99	3.96	1.98	0.93	2.05	3.23
17	3.00	0.94	2.85	2.22	1.02	2.01	0.97	0.94	1.93	4.18	1.85	0.95	1.93	3.82
18	3.00	0.90	2.90	2.26	1.03	2.03	0.98	1.00	1.89	4.28	1.80	1.94	1.96	2.88
19[b]	3.00	0.94	2.84	2.21	1.00	2.97	0.98	0.98	1.89	4.18	1.80		1.93	2.93
20[c]	3.15	0.94	2.83	2.36	1.04	3.00	1.09	0.83	1.95	4.14	1.82		2.00	2.83

[a] His = 0.99. [b] p-Cl-Phe = 0.90. [c] 4 M methanesulfonic acid hydrolysate; Trp = 0.85.

Amino acid analyses of 6 M HCl hydrolysates (110 °C, 18 h) were carried out on a column-temperature programmable LKB 4150 automatic analyzer equipped with a 2220 recording integrator. Single column borate buffer methodology was employed and amino acid ratios obtained are given in Table V.

Biology. Peptides were tested as previously described[13] in male Charles River rats weighing 175–350 g, which were anesthetized with sodium pentobarbital (6 mg/100 g of body weight). Animals (6–9/group) were injected subcutaneously 30 min after the anesthetic with either saline, different doses of GRF(1–29)NH_2 (control), or two doses of an analogue in 0.5-mL volumes. Fifteen minutes after injection of test substance, a blood sample was taken from the jugular vein and plasma was analyzed for GH by radioimmunoassay with use of materials generously supplied by NIADDKD. Potencies and 95% confidence limits based on the four-point assay design were calculated by the method of Pugsley[14] using an Applesoft BASIC computer program developed in this laboratory.

Prediction of Secondary Structure. The 1–29 sequence of GRF was subjected to the empirical probability calculations of Chou and Fasman[9] in order to predict possible regions with either random coil, α-helical, or β-sheet characteristics. The potential location of reverse turns (β-bends) was also predicted and was considered particularly important for the design and rationalization of bioactivity of analogues. Calculations and graphics were carried out on an Apple IIe computer using the Applesoft BASIC program of Corrigan and Huang[15] employing the built-in data base compiled by Argos et al.[16]

Results and Discussion

Synthesis. The ability to synthesize the large number of analogues which are needed for worthwhile structure–activity studies on a molecule as complex as GRF is considerably aided when the 1–29 sequence rather than the longer 1–44 or 1–40 sequences is utilized. Standard HF cleavage of peptide-benzhydrylamine resin gave crude material of surprisingly good quality (Figure 2). Peptide of greater than 98% purity by integration of HPLC traces could generally be achieved in about 12% yield by gel filtration followed by one or two preparative reverse-phase liquid chromatography steps. For comparison, yields of the 1–40 sequence prepared in a similar fashion were less than half of this. It was found that phenyl-bonded silica with a 300-Å pore size provided an excellent separation medium for GRF as well as VIP, secretin, glucagon, and PHI. Alterations in relative elution rates of impurities compared to C_{18}-silica proved to be useful both for preparative work and also for demonstration of homogeneity. Analytical HPLC data were also obtained with use of two separate solvent systems as an added check for impurities. Retention times are given in Table IV. Amino acid analysis results are shown in Table V.

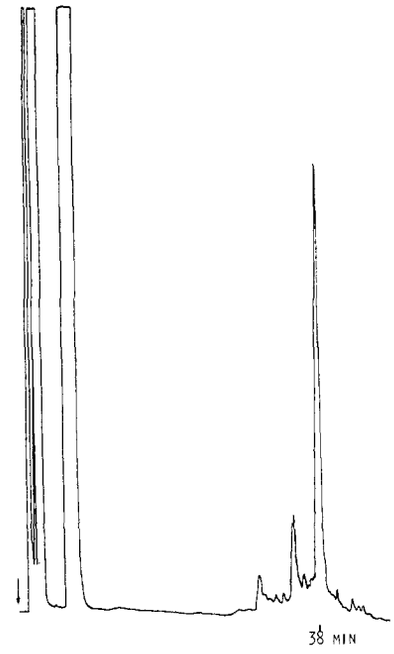

Figure 2. HPLC elution profile of crude hydrogen fluoride cleaved GRF(1–29)NH_2 on C_{18}-Vydac silica (5 μm, 300 Å; 0.4 × 25 cm), using solvent system B conditions (except gradient over 45 min).

Biological Activities. The N-terminal region of GRF and other related peptides is proving to be a fertile area for structure–function studies. Replacement of the first three residues of GRF(1–29) by the D isomers results in considerable increases in GH-releasing activity both in the rat[6] and in primary cultures of rat pituitary cells.[8] Obviously, conformational restriction of the N-terminus is

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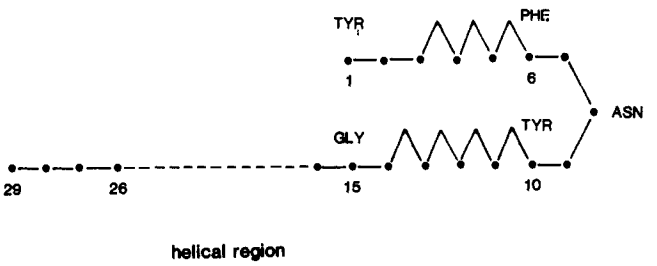

Figure 3. Secondary structural features predicted by Chou–Fasman calculations[15] on the GRF(1–29) amino acid sequence. Serrated lines indicate areas possessing a high probability of α-helical structure and diagonal lines β-sheet structure. A single reverse turn is indicated in the position 6–10 region.

favored but does not seem to be critical to one particular residue as is often the case with some other peptides such as LH-RH,[17] somatostatin,[18] or the enkephalins.[19] This is even more apparent in the present study where combinations of D-Tyr-1, D-Ala-2, and D-Asp-3 continue to produce increased activity, albeit not as high as acetyl[D-Ala-2]GRF. Also, [D-Ala-4]GRF exhibits full or even slightly higher activity compared to GRF itself. The in vitro activity of the D-Ala-2 analogue together with the lack of any observable prolongation of in vivo activity[6] seems to suggest that increases in activity are due to increases in receptor affinity rather than decreased enzymatic inactivation.

Moving further along the peptide chain from the N-terminus, D-amino acid replacements suddenly resulted in substantial loss of activity at residues 5 (Ile), 6 (Phe), and 7 (Thr) (see Table I). However, with the [D-Asn-8] analogue potency recovered since this analogue was more than twice as active as GRF. The Chou–Fasman method[9] for predicting possible secondary conformations of peptides and proteins can be extremely useful for providing some rationalization to this type of observation. The method predicts, for instance, that a reverse turn possibly exists in the active somatostatin conformation, in LH-RH, and in α-MSH,[20] all of which have been borne out by previous or subsequent analogue studies. The GRF(1–29) sequence was subjected to the Chou–Fasman procedure using the highly convenient computer program described and provided by Corrigan and Huang.[15] A qualitative representation of the result is illustrated in Figure 3. The first three residues appear to adopt no particular conformation. A β-sheet from residues 4 to 6 leads into a reverse turn from position 6 to 10. This picture seems to fit quite well with the analogue results. The presence of the β-bend could account for the low activities of the D-Phe-6 and D-Thr-7 analogues and also the high activity of the D-Asn-8 substitution assuming that this could stabilize the turn. In similar calculations (Coy et al., unpublished observations) on PHI, VIP, secretin, and glucagon, all have a similar β-bend in the 6–10 region. However, these peptides, unlike GRF, have a β-bend in the 1–4 region also. Thus, apparently small structural differences in the closely related N-terminal sequences could have profound effects on chain folding and biological activity. Residues 15–26 could possibly assume an α-helical configuration. Kaiser and Kezdy[21] have recently proposed an amphiphilic, helical secondary structure for GRF which would result in the formation of distinct hydrophilic and hydrophobic surfaces, a phenomenon not uncommon to many peptides. Their model, however, proposes that the whole 1–22 region of the peptide is helical. The lack of a β-bend associated with the Gly-15 residue would account for the deleterious effects of its replacement by D-Ala. A similar loss of activity has been reported for [D-Ala-15]GRF(1–32)NH_2.[22]

There was also the possibility that the size and structure of the side chain of the D-amino acid in position 2 could have important conformational effects. Indeed this is probably the case since both Ac[D-Phe-2]- and Ac[D-Arg-2]GRF were highly inactive.

[D-Thr-7]- and [D-Arg-2]GRF, in addition to having extremely low GH-releasing activity, actually resulted in significant lowering of rat GH levels relative to the saline groups at 5- and 25-μg dose levels, respectively (Table II). Since a possible explanation for this could be competitive inhibition of endogenous GRF, both peptides were tested for their abilities to block the action of GRF(1–29). The results after concomitant administration of GRF(1–29) and the D-Arg-2 analogue are shown in Table III and it is apparent that no blockade was detectable over a wide range of doses. The results (not shown) with Ac[D-Phe-2]- and [D-Thr-7]GRF were much the same.

Preliminary studies were also begun on the requirements of the aromatic ring side chains in positions 1 and 6. Replacement of Tyr-1 by His in the Ac-D-Ala-2 format also gave an highly active analogue which was, however, less active than the corresponding Tyr-1 analogue. It should be noted that rat GRF contains His in position 1.[5] The His analogue is proving to be an excellent peptide for ^{125}I-labeling and use in a GRF radioreceptor assay under development (Heiman et al., manuscript in preparation).

Replacment of Tyr-1 by Phe gave an analogue with 14% activity. [*p*-Cl-Phe-6]GRF retained about 50% activity; however, the Trp-6 analogue was not active at the doses tested, which indicates certain steric constraints are operative.

The results reveal many more possible approaches for the design of GRF analogues with even higher levels of activity and, hence, greater therapeutic potential. It is also possible that further conformational restraints, perhaps through the use of cyclic structures, might enable much shorter analogues to be developed which still retain useful levels of GH releasing activity.

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