Total Solid-Phase Synthesis of Porcine Gut Gastrin Releasing Peptide (GRP), a Mammalian Bombesin[1]

W. Märki, J. Spiess, Y. Taché, M. Brown, and J. E. Rivier*

Contribution from the Peptide Biology Laboratory, The Salk Institute for Biological Studies, San Diego, California 92138. *Received November 3, 1980*

Abstract: Recently, gastrin releasing peptide (GRP), Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, a mammalian bombesin, was isolated from porcine gastric mucosa and sequenced by McDonald et al.[9] This polypeptide was manually synthesized by solid-phase methodology, using a benzhydrylamine–styrene–1% divinylbenzene copolymer. Deprotection and cleavage from the resin were accomplished by HF. The crude peptide was purified by gel filtration and reverse-phase, high-performance liquid chromatography (RP-HPLC). Homogeneity of the synthetic peptide was demonstrated by RP-HPLC, sequence analysis, peptide mapping, and amino acid analysis. The peptide was further characterized by thin-layer chromatography, paper electrophoresis, optical rotation, ultraviolet spectroscopy, and 300-MHz Fourier transform proton nuclear magnetic resonance spectroscopy. The circular dichroism spectra of GRP indicated that the polypeptide chain was largely random with no evidence for α-helical structure. The primary structure was confirmed by amino acid analysis of the tryptic peptide fragments, sequence analysis of GRP and its Met(O) derivative using a modified 890 C spinning-cup sequencer, and C-terminal end group determination. GRP released gastrin when administered systemically and decreased gastric acid secretion when given intracisternally in rats. GRP also mimicked CNS-mediated actions of bombesin to influence thermoregulation or glucoregulation, most likely because of the common C-terminal homology of these peptides. This assumption was supported by the observation that the synthetic acetylated octapeptide [Ac-His^{20}]-GRP (20–27) showed pharmacological effects similar to those exhibited by GRP and amphibian bombesin.

Bombesin, a tetradecapeptide first isolated and characterized from the skin of the frog *Bombina bombina*[2] has been shown in mammals to influence various neural and visceral functions. Acting through the central nervous system, bombesin is a potent stimulus to disrupt thermoregulation,[3] to stimulate adrenal epinephrine secretion,[4] which results in lowering of plasma insulin and elevation of plasma glucagon and glucose,[5] and to suppress gastric acid secretion.[6] Bombesin when injected peripherally also stimulates gastrin release, gall bladder contraction, antidiuresis, and intestinal motor activity.[7] It is now recognized on the basis of immunologic data that bombesin has counterparts in mammalian brain and gastrointestinal tissues.[8]

McDonald et al.[9] have recently isolated and sequenced a porcine intestinal, 27 amino acid containing peptide, termed gastrin releasing peptide (GRP), which stimulates the release of gastrin from gastric mucosa. GRP has a C-terminal decapeptide which is identical with that of bombesin with the exception of one residue (Table I). It is noteworthy that structure–activity relationship studies of bombesin analogues had shown that the N-acetylated, C-terminal octapeptide of bombesin exhibited full biological action and potency.[10b,c] In view of the C-terminal homology between GRP and bombesin, it was suggested that GRP was a mammalian bombesin. Preliminary biological studies[11] confirmed this concept.

We report here in detail the first successful chemical synthesis of GRP, its physical characteristics, and its biological properties. These properties are, furthermore, qualitatively and quantitatively compared to those of synthetic bombesin and [Ac-His^{20}]-GRP (20–27).

Synthetic problems associated with conventional solid-phase peptide synthesis (SPPS) were related to the overall length of the peptide (27 amino acids) and to the presence of amino acids such as methionine, histidine, tryptophan, and tryrosine which may undergo side reactions during coupling, deblocking, final acidolytic cleavage, and deprotection or purification.[12a]

Results and Discussion

It was first attempted to synthesize GRP by solid-phase methodology (Merrifield)[13] using an automated program routinely applied in our laboratory[14] to the synthesis of peptides smaller than 15 amino acids. Purification included ion-exchange chromatography and partition chromatography on Sephadex G-50, followed by semipreparative, reverse-phase, high-performance liquid chromatography (RP-HPLC). The peptide thus obtained was found to be homogeneous by amino acid analysis, thin-layer chromatography (TLC), RP-HPLC in several systems, and peptide mapping of the tryptic digest. Sequence analysis of this material, however, showed the presence of 10% des-Pro^2-GRP which had remained undetected by the procedures mentioned above. A more controlled synthesis was therefore carried out, which, for practical purposes, was done manually.

(1) The conventions and nomenclature used are those of the IUPAC-IUB Commission on Biochemical Nomenclature ("Collected Tentative Rules and Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature", 2nd ed.; American Society of Biological Chemistry, Inc.: Bethesda, MD, 1974) and E. Wünsch ("Methoden der Organischen Chemie (Houben-Weyl)"; Müller, E., Ed.; Georg Thieme Verlag: Stuttgart, 1974; p 1-27). Additional abbreviations are as follows: GRP, porcine gut gastrin releasing peptide; DCC, *N,N*-dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; TFA, trifluoroacetic acid; TEAF, triethylammonium formate; TEAP, triethylammonium phosphate; DMOAP, dimethyloctylamine phosphate; HOBt, 1-hydroxybenzotriazole; TLC, thin-layer chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; RT, retention time; SPPS, solid-phase peptide synthesis; TPCK, L-[1-(tosylamido)-2-phenyl]ethyl chloromethyl ketone; CSF, cerebrospinal fluid; mol wt, molecular weight.

(2) Anastasi, A.; Erspamer, V.; Bucci, M. *Experientia* **1971**, *27*, 166-167.

(3) Brown, M.; Rivier, J.; Vale, W. *Science* **1977**, *196*, 998-1000.

(4) Brown, M.; Taché, Y.; Fisher, D. *Endocrinology* **1979**, *105*, 660-665.

(5) Brown, M.; Rivier, J.; Vale W. *Life Sci.* **1977**, *21*, 1729-1734.

(6) Taché, Y.; Vale, W.; Rivier, J.; Brown, M. *Proc. Natl. Acad. Sci. U.S.A.*, **1980**, *77*, 5515-5519.

(7) Erspamer, V.; Melchiorri, P.; Erspamer, G. F.; Negri, L. "Gastrointestinal Hormones and Pathology of the Digestive System": Grossman, M. E., Speranza, V., Basso, N., Lezoche, E., Eds.; Plenum Press: New York, 1978; p 51.

(8) Erspamer, V.; Melchiorri, P. "Gastrointestinal Hormones"; Thompson, J. C., Ed.; University of Texas Press: Austin, TX, 1975; pp 575-589. Polack, J. M.; Bloom, S. R.; Hobbs, S.; Solcia, E.; Pearse, A. G. E. *Lancet* **1976**, *1*, 1109-1110. Brown, M.; Allen R.; Villarreal, J.; Rivier, J.; Vale, W. *Life Sci.* **1978**, *23*, 2721-2728. Villarreal, J.; Brown, M. *Ibid.* **1978**, *23*, 2729-2734. Wharton, J.; Polack, J.; Bloom, S. R.; Ghatei, M.; Solcia, E.; Brown, M.; Pearse, A. G. E. *Nature (London)* **1978**, *273*, 769-770.

(9) McDonald, T. J.; Jörnvall, H.; Nilsson, G.; Vagne, M.; Ghatei, M.; Bloom, S. R.; Mutt, V. *Biochem. Biophys. Res. Commun.* **1979**, *90*(1), 227-233.

(10) (a) Rivier, J.; Brown, M. *Biochemistry* **1978**, *17*, 1766-1771. (b) Märki, W.; Brown, M.; Rivier, J., submitted for publication in *Biochemistry*. (c) Taché, Y.; Märki W.; Rivier, J.; Vale, W.; Brown, M., submitted for publication in *Gastroenterology*.

(11) Brown, M.; Märki, W.; Rivier, J. *Life Sci.* **1980**, *27*, 125-128.

(12) (a) Barany, G.; Merrifield, R. B. In "Peptides"; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. 2, pp 214-216. (b) *Ibid.*, pp 31-32.

(13) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149-2154.

(14) Rivier, J.; Kaiser, R.; Galyean, R. *Biopolymers* **1978**, *17*, 1927-1938.

0002-7863/81/1503-3178$01.25/0

Table I. Structures of GRP, Amphibian Bombesin and [Ac-His[20]]-GRP (20–27) and Their Relative Biological Potencies

compd	sequence[a]	rel potency, % effect on body temp[b]	rel potency, % effect on plasma glucose[b] levels
bombesin	pGlu-Gln-Arg-Leu-Gly-*Asn*-Gln-*Trp-Ala-Val-Gly-His-Leu-Met*-NH_2	100	100
GRP	Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-*Asn-His-Trp-Ala-Val-Gly-His-Leu-Met*-NH_2	28	35
[Ac-His[20]]-GRP (20–27)	Ac-*His-Trp-Ala-Val-Gly-His-Leu-Met*-NH_2	100	100

[a] Homologous residues are italic. [b] See Experimental Section for description of Biological tests.

The anchoring linkage was the methionine amide bond to a benzhydrylamine (BHA)–polystyrene–1% divinylbenzene copolymer prepared according to Rivier et al.[15] This linkage when used in conjunction with N^{α}-(*tert*-butyloxy)carbonyl protection has been shown to be suitable for the synthesis of various peptide amides; it may also be more stable to acidolysis for longer peptide amides terminating with methionine than the more acid-labile linkage derived from a *p*-methylbenzhydrylamine (MBHA resin) support.[16] A relatively low substitution level of the resin (0.2 mmol amine/g of resin) was used to comply with the suggestion of Barany and Merrifield[12b] that "the loading of a support for SPPS should be inversely proportional to the length of the desired target peptide in such a way that at the end of the synthesis, the weight increase due to the protected peptide is less than the original weight of the resin support".

All couplings except for Boc–asparagine were performed by the dicyclohexylcarbodiimide (DCC)[17] technique, which has been used successfully in numerous syntheses including those of somatostatin-28,[18] vasoactive intestinal polypeptide[19] (VIP), and glucagon[20] among others. The strategy followed for the GRP synthesis included the use of the N^{α}-Boc, a mild, acid-labile protection for the temporarily protected N^{α} amino group and permanent protection for the side-chain functionalities with a set of stable ethers and/or urethanes of substituted benzyl alcohols.[21] N^{α}-*tert*-butoxycarbonyl amino acids have been reported[22] to couple with minimal racemization to amino acids or peptide chains bound to the resin. Substantial racemization, however, may occur during coupling of certain N^{α},N^{im} derivatives of histidine[23] and should be suppressed by using the strong electron-withdrawing tosyl group for N^{im} protection.[24] The schedule used for the manual synthesis is reported in Table II.

After each cycle the completeness of the acylation reaction was tested with both the ninhydrin test[25] and the fluorescamine test[26] for free amine. The acylation was usually found to be complete after one or two couplings (see below). Coupling time was reduced to less than 1 h for certain amino acid residues such as glycine, alanine, valine, or leucine. Other amino acids such as proline, tyrosine, and lysine were coupled for a longer time. Toward the end of the synthesis, several cycles were routinely repeated, followed by acetylation in order to avoid the formation of deletion sequences. This, however, implied that complete acidolytic de-

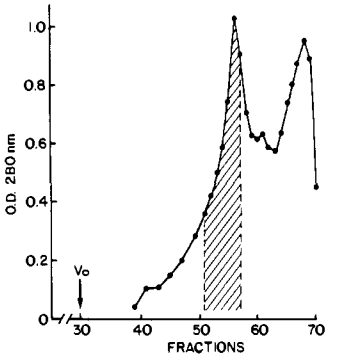

Figure 1. Gel filtration of crude GRP on Sephadex G-25 F. Crude GRP (420 mg) was suspended in 5% AcOH/0.02% ethanedithiol (12 mL) and centrifuged. The supernatant containing ca. 90% of the peptide was applied onto the column (2.5 × 100 cm). Elution was performed with the same solvent system at a flow rate of 13 mL/h. Fractions (6.5 mL) were collected. The yield (tubes 51–57) was 150 mg.

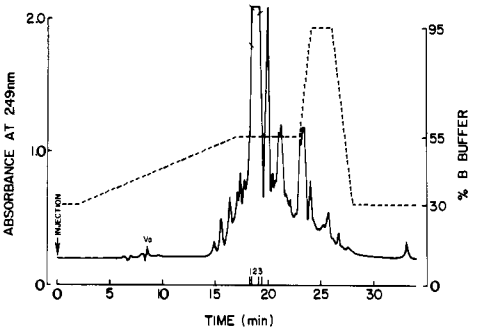

Figure 2. Semipreparative RP-HPLC purification of gel-purified GRP. Gel-purified GRP (25 mg) dissolved in buffer A (500 μL) was applied onto a C-18 column (1 × 25 cm) and eluted with a gradient of CH_3CN indicated by the dotted line: buffer A was TEAP pH 2.25; buffer B was 60% CH_3CN in A; flow rate was 2.0 mL/min; chart speed was 1 cm/min. Three fractions were collected. The yield of fraction 2 could only be determined after desalting and was estimated to be 7 mg.

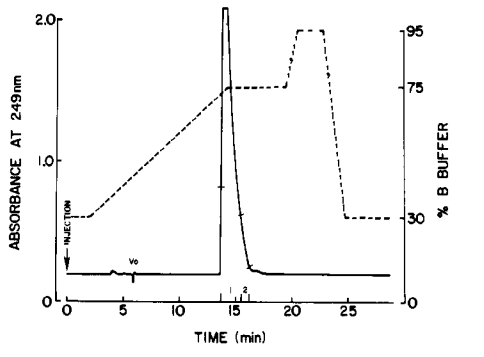

Figure 3. Desalting of RP-HPLC-purified GRP. RP-HPLC-purified GRP (~14 mg) dissolved in H_2O was applied onto a C-18 column (1 × 25 cm) and eluted with a gradient of CH_3CN indicated by the dotted line: buffer A was 0.1% TFA; buffer B was 60% CH_3CN in A; flow rate was 3.0 mL/min; chart speed was 1 cm/min. The yield of pure peptide (fraction 1) was 10 mg.

(15) Rivier, J.; Vale, W.; Burgus, R.; Ling, N.; Amoss, M.; Blackwell, R.; Guillemin, R. *J. Med. Chem.* **1973**, *16*, 545.
(16) Stewart, J. M.; Peña, C.; Matsueda, G. R.; Harris, K. "Peptides"; Loffet, A., Ed.; Editions de l'Universtiê de Bruxelles: Bruxelles, Belgium, 1976; pp 285–290.
(17) Sheehan, J. C.; Hess, G. P. *J. Am. Chem. Soc.* **1955**, *77*, 1067–1068.
(18) Ling, N.; Esch, F.; Davis, D.; Mercado, M.; Regio, M.; Böhlen, P.; Brazeau, P.; Guillemin, R. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 945–951.
(19) Coy, D.; Gardner, J. *Int. J. Pept. Protein Res.* **1980**, *15*, 73–78.
(20) Protein Synthesis Group, Shanghai Institute of Biochemistry *Sci. Sin. (Engl. Ed.)* **1975**, *18*, 745–768.
(21) Yamashiro, D.; Li, C. H. *J. Am. Chem. Soc.* **1978**, *100*, 5174–5179.
(22) Kent, S. B. H.; Mitchell, A. R.; Barany, G.; Merrifield, R. B. *Anal. Chem.* **1978**, *50*, 155–159.
(23) Windridge, G. C.; Jörgensen, E. C. *J. Am. Chem. Soc.* **1971**, *93*, 6318–6319.
(24) Lin, M. C.; Gutte, B.; Caldi, D. G.; Moore, S.; Merrifield, R. B. *J. Biol. Chem.* **1972**, *247*, 4768–4774.
(25) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.
(26) Felix, A. M.; Jimenez, M. H. *Anal. Biochem.* **1973**, *52*, 377–381.

Table II. Schedule for Manual SPPS of GRP on BHA Resin (6 g)

step	reagents and operations	mix time, min
1	CH_2Cl_2 wash (3×)	2
2	50% TFA/CH_2Cl_2–5% 1,2-ethanedithiol	1
3	50% TFA/CH_2Cl_2–5% 1,2-ethanedithiol	5[a]
4	50% TFA/CH_2Cl_2–5% 1,2-ethanedithiol	20
5	CH_2Cl_2 wash (3×)	2
6	10% Et_3N/CH_2Cl_2(1×)	2
7	MeOH wash (1×)	2
8	10% Et_3N/CH_2Cl_2 (1×)	2
9	MeOH wash (3×)	2
10	CH_2Cl_2 wash (3×)	2
11	6 equiv of Boc-amino acid in CH_2Cl_2[b] + 6 equiv of a 1 M solution, in 10% DMF/CH_2Cl_2 of DCC	30–180[c]
12	MeOH wash (3×)	2
13[d]	CH_2Cl_2 wash (3×)	2
14[e]	20% acetic anhydride/CH_2Cl_2 (1×)	15

[a] At step 3, amino acids at positions 7 to 3 were deblocked for 10 min and Pro (2) for 20 min. [b] Boc-Leu, Boc-Trp, and Boc-Arg(Tos) were dissolved in 5% DMF/CH_2Cl_2. [c] Boc-Asn-ONp (cycle 9) was dissolved in 50% DMF/CH_2Cl_2 and coupled with 4 equiv of 1-hydroxybenzotriazole (HOBt) for 10 h. [d] Aliquot taken for ninhydrin and fluorescamine test; if the test was negative, we started the next cycle at step 2, and if it was positive, another recoupling (steps 11–13) was introduced. Positive tests were ascertained at step 10. [e] After Asn (19), Arg (17), Tyr (15), Met (14), Val (10), Thr (9), Gly (6), Val (5), Val (3), and Pro (2) were coupled, an additional acetylation step (step 14) was performed.

Table III. Purification of Synthetic GRP

purification step	purity,[a] %	yield,[b] %	yield,[c] %	amt purified and isolated,[d] mg
crude material from HF cleavage	30	100	61	420
gel filtration, Sephadex G25F	45	36	22	150
RP-HPLC (TEAP/CH_3CN)	>98	~7	6	
RP-HPLC (0.1% TFA/CH_3CN)	>98	7	5	35

[a] Purity was assessed by analytical RP-HPLC of the different fractions. [b] Yield is based on the amount of crude material recovered after HF cleavage. [c] Yield is based on the amine content (substitution) per gram of starting resin [4.5 × 0.2 mM = 0.9 × 2.785 g = 2.50 g (100%): recovered after HF (1.52 g, 61%)]. Yields at the gel filtration and RP-HPLC steps are based on the purification of an aliquot (420 mg) of the crude material. [d] Absolute amount should be multiplied by 3.62 for normalization to 1.52 g of starting material.

blocking of the N^{α}-Boc group had taken place, an assumption that may need further investigation. The recouplings of the different amino acids provided significant improvement in completing the acylation reaction. In particular, proline (position 2) was coupled three times to valine (position 3) followed by an acetylation step since, as mentioned earlier, 10% of a des-Pro^2-GRP had been found and quantitated by sequencing (data not shown) in the product resulting from automated synthesis.

Cleavage from the resin and concomitant deprotection was achieved with one single treatment with hydrofluoric acid (HF) at the end of the synthesis.

A scheme for a fast isolation of the synthetic material is shown in Table III; recoveries for various steps are indicated. After HF cleavage, gel filtration of the crude material through Sephadex G 25 F (Figure 1) separated GRP (fractions 51–57) from shorter fragments (fractions 62–70) to yield a 45% pure peptide as shown by RP-HPLC (Figure 2). Fractions 62–70 (Figure 1) did not contain GRP on the basis of RP-HPLC and may represent terminated sequences due to acetylation and miscoupling. Final purification was achieved by a fast, semipreparative RP-HPLC

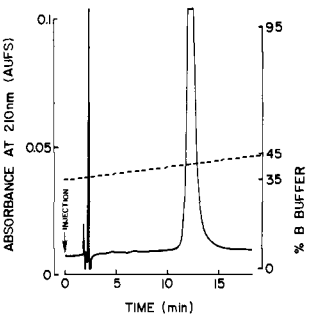

Figure 4. Analytical RP-HPLC of GRP. RP-HPLC-desalted GRP (10 μg) dissolved in buffer A (10 μL) was applied onto a SupelcoSil LC-18 column (4.6 × 150 mm) and eluted with a gradient of CH_3CN indicated by the dotted line: buffer A was TEAP pH 2.25; buffer B was 60% CH_3CN in A; flow rate was 1.0 mL/min; chart speed was 1 cm/min.

procedure using a C-18 column eluted with a triethylammonium phosphate (TEAP) buffer[27] (Table IV, Figure 2). Subsequent desalting was accomplished with the volatile 0.1% TFA/CH_3CN solvent system on the same column (Table IV, Figure 3). Fractions were pooled to favor purity rather than quantity. GRP was obtained after lyophilization as the TFA-salt in a 5% overall yield, based on the amine content (substitution) per gram of starting resin (Table III).

The homogeneity and the primary structure of synthetic GRP were investigated by RP-HPLC, amino acid analysis, sequence analysis, C-terminal end group determination, and peptide mapping. Lyophilized material of the final step of the isolation procedure (Table III) was analyzed by RP-HPLC on three different columns and eluted with four different solvent systems (Table IV). Under those conditions, one single peak without evidence for contaminating peptides was observed (Figure 4). The results of the amino acid analysis of GRP were in agreement with the expected values (Table V).

The digestion of GRP by TPCK–trypsin yielded three major components as expected from the location of lysine (position 13) and arginine (position 17) in the synthesized peptide chain. These three typtic fragments [GRP (1–13), GRP (14–17), and GRP (18–27)] were isolated by semipreparative RP-HPLC (Figure 5, upper panel). Their amino acid compositions were consistent with the expected compositions of the tryptic peptides from GRP. The minor side fractions (Figure 5, upper panel; RT = 14.5, 17.9, and 21.6 min) were also isolated and analyzed. Their amino acid compositions were consistent with chymotryptic fragments of GRP probably cleaved by residual chymotryptic activity of the TPCK–trypsin preparation used. As a control, synthetic GRP (14–27)[10b] was digested and gave a fingerprint on RP-HPLC similar to that for GRP, except for the absence of the peak assigned to the N-terminal 13-peptide (Figure 5 lower panel).

The sequence of GRP was determined by Edman degradation using a highly sensitive Beckman 890 C spinning-cup sequencer modified according to Wittmann-Liebold.[28] Whereas for natural peptides, of the size of GRP, only 0.6–1.5 nmol of peptide is usually subjected to sequence analysis with this instrument, 15 nmol of synthetic GRP was applied to the cup, thus allowing the detection of contaminating peptides representing as little as 2% of the main product. The sequence of the first 26 amino acid residues of the synthetic material was found to be identical with the corresponding primary structure of the natural hormone[9] (Figure 6). The C-terminal methioninamide could not be detected by spinning-cup sequencing since this amino acid was probably so hydrophobic after coupling to phenyl isothiocyanate that it was washed out of the cup before the final extraction. However, the presence of C-terminal methioninamide in GRP could be concluded from the chymotryptic end group determination and from the difference of the amino acid composition data obtained by amino acid

(27) Rivier, J. *J. Liq. Chromatogr.* **1978**, *1*(3), 343–366.
(28) Wittmann-Liebold, B. *Hoppe-Seyler's Z. Physiol. Chem.* **1973**, *354*, 1415–1431. Wittmann-Liebold, B.; Graffunder, H.; Kohls, H. *Anal. Biochem.* **1976**, *75*, 621–633.

Table IV. Reverse-Phase HPLC and Purity of GRP Peptides

compd	column[a]	load, mg	buffer A	% B buffer[b] (time, min)	flow, mL/min	AUFS,[c] nm	RT, min	purity, %
			Preparative					
GRP	A	25	TEAP	30–55 (15)	2.0	2.0^{249}	18.7	>98
GRP	A	1	TEAF	42–55 (20)	3.0	0.2^{280}	14.7	>97
GRP	A	14	0.1% TFA	30 (2)–75 (12)	3.0	2.0^{249}	14.5	>98
			Analytical					
GRP	B	0.010	TEAP	35–45 (20)	1.0	0.1^{210}	12.2	>98
GRP	B	0.005	DMOAP[d]	21 (iso)	1.5	0.1^{210}	10	>98
GRP	C	0.010	0.1% TFA	30–70 (25)	1.5	0.1^{210}	19.7	>98
GRP	D	0.003	35% TEAP + 30% CH_3CN + 35% H_2O	iso (50 °C)	1.0	0.1^{210}	19.1	>98
$[Met(O)^{14,27}]$-GRP	E	0.010	TEAP	30–55 (15)	1.5	0.1^{210}	10.6	
GRP	E	0.010	TEAP	30–55 (15)	1.5	0.1^{210}	14.6	

[a] Column specifications are as follows: A, C-18 column (1 × 25 cm) (semipreparative); B, LC-18 No. 956 SupelcoSil (0.46 × 15 cm); C, UE No. 1093 Altex Ultrasphere-ODS (0.45 × 15 cm); D, two PAC-I-125 (No. 093593 and No. 093594) gel-permeation columns (1 × 25 cm); E, LC-18 No. 177 SupelcoSil (0.46 × 15 cm). [b] Most peptides were eluted by increasing the concentration of B buffer throughout the run. Starting and final concentrations of B are given as well as the duration of the gradient in parentheses. Iso stands for isocratic (no change in the concentration of B buffer). [c] AUFS is absorbance units full scale. The superscript is the wavelength (in nanometers) at which the column eluate was monitored. [d] DMOAP: dimethyloctylamine phosphate buffer (pH 2.25).

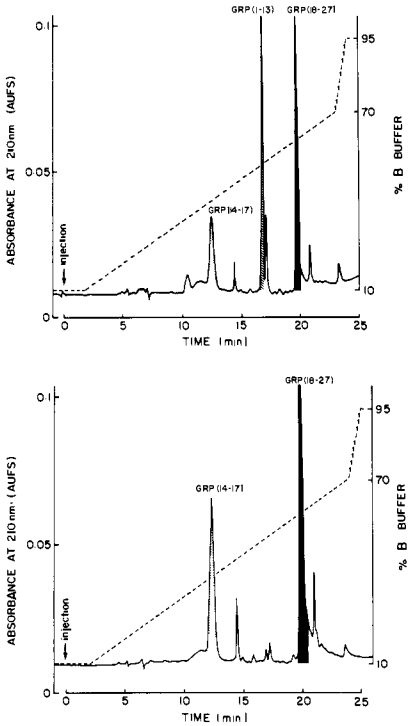

Figure 5. Analytical RP-HPLC of tryptic fragments of GRP and GRP (14-27).[10b] Upper panel: TPCK trypsin digested GRP (10 μg) dissolved in water (20 μL) was applied onto a C-18 column (1 × 25 cm) and eluted with a gradient of CH_3CN indicated by the dotted line; buffer A was TEAP pH 2.25; buffer B was 60% CH_3CN in A; flow rate was 2.5 mL/min; chart speed was 1 cm/min. An identical run at the 280-nm absorbance position gave a fingerprint similar to that in the lower panel. Lower panel: TPCK trypsin digested GRP (14-27) (~7 μg) dissolved in water (20 μL) was run under conditions identical with those described above.

analysis (Table V) and sequencing (Figure 6). No peptide contamination could be observed by sequence analysis.

It has been described that methionine sulfur atoms are susceptible to air oxidation, forming the corresponding sulfoxide.[29] We have therefore investigated the possibility that synthetic GRP was at least partially in the methionine sulfoxide form. This investigation was accomplished by characterization of $[Met(O)^{14,27}]$-GRP by RP-HPLC, amino acid, and sequence analyses. When synthetic GRP was treated with hydrogen peroxide[29] in dilute acetic acid, only methionine and methioninamide were

(29) Iselin, B. *Helv. Chim. Acta* **1961**, *44*, 61–77.

Table V. Amino Acid Analysis of GRP and $[Met(O)^{14,27}]$-GRP

amino acid	moles of amino acid/moles of Tyr		
	GRP[a]	$[Met(O)^{14,27}]$-GRP	intestinal GRP[b]
Met(O)	0.0	1.9	0
Asx	1.1	1.3	1
Thr	0.9	0.9	1
Ser	1.0	0.9	1
Pro	2.2	1.9	2
Gly	5.0	5.2	5
Ala	3.0	3.0	3
Val	3.7	3.9	4
Met	1.8	0.1	2
Leu	1.9	1.8	2
Tyr	1.0	1.0	1
Lys	1.0	0.9	1
His	1.9	2.1	2
Trp	1.0	1.0	1
Arg	1.0	1.0	1

[a] Peptide (2 nmol) was hydrolyzed for 24 h at 110 °C by 4 M methanesulfonic acid with 0.2% tryptamine. The values presented were obtained by means of three separate analyses. [b] See ref 9.

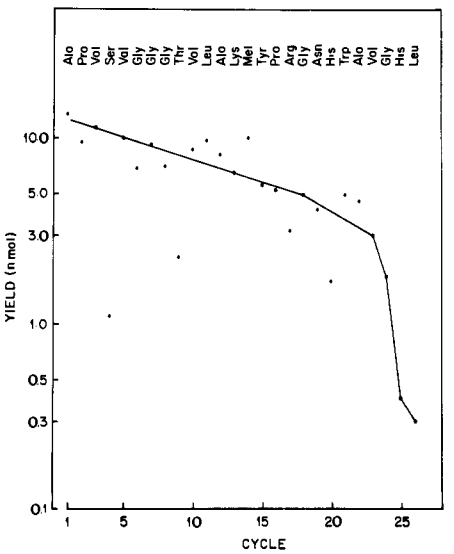

Figure 6. Sequence analysis of synthetic GRP. Yields of PTH-amino acids obtained by Edman degradation of 14.4 nmol of GRP in a modified spinning-cup sequencer were determined by RP-HPLC. Serine was determined as the predominant PTH-Ser derivative. Threonine was determined as PTH-dehydrothreonine. A second cleavage was performed in cycle 16.

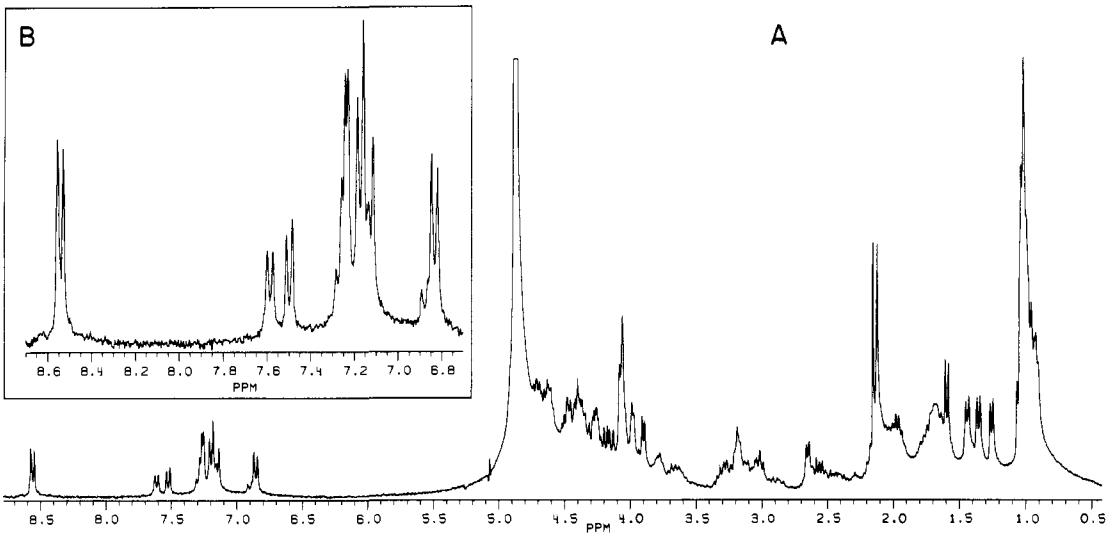

Figure 7. A: 300-MHz FT ^1H NMR spectrum of GRP in D_2O (concentration 10 mg/mL). B: detailed spectrum of the aromatic area (boxed); pH 4.1; temperature 23 °C. Chemical shifts are in parts per million relative to HOD (4.81 ppm) as an internal standard.

oxidized to the corresponding sulfoxide derivatives, whereas all the other amino acids of GRP including tryptophan were not affected as indicated by amino acid analysis. Sequence analysis of 2 nmol of [Met(O)^{14,27}]-GRP did not reveal any difference to the N-terminal sequence of GRP from residues 1–24. Residues 25–27 could not be detected in this particular run (data not shown). Under the sequencing conditions applied here, methionine sulfoxide (position 14) was reduced and determined as PTH–methionine, demonstrating that this sequencing procedure did not discriminate between methionine and methionine sulfoxide. [Met(O)^{14,27}]-GRP, however, eluted significantly earlier than GRP in RP-HPLC (Table IV). It was concluded on the basis of these results that synthetic GRP did not contain any methionine sulfoxide residue.

Reduction of [Met(O)^{14,27}]-GRP, as described by Savige and Fontana,[30] produced two main components as shown by RP-HPLC. One component was assigned to GRP on the basis of its retention time, and the other was probably due to a peptide having an altered tryptophan residue (oxindolylalanine derivative)[31] which may have been generated during the reduction.

In summary, on the basis of the three methods (RP-HPLC, sequence analysis, and peptide mapping of the tryptic digest) used to determine the homogeneity and primary structure of GRP, we conclude that the manually synthesized GRP preparation was more than 98% pure and contained the expected primary structure free of oxidized methionine residues.

The peptide was further characterized by thin-layer chromatography (TLC), paper electrophoresis, optical rotation and ultraviolet spectroscopy (UV). The tyrosine/tryptophan ratio was found to be 1 (expected 1) as determined by UV spectrophotometry[32] (see Experimental Section for other physical constants).

A routine 300-MHz Fourier transform (FT) ^1H NMR spectrum of GRP was taken (Figure 7). We attempted to assign the well-resolved aromatic region of the peptide in order to exclude, for example, the presence of aromatic protecting groups. The assignments were made by comparing the well-known random-coil ^1H NMR parameters (chemical shifts) of each amino acid residue (X) in the linear tetrapeptide H-Gly-Gly-X-Ala-OH[33] and the 300-MHz ^1H NMR spectrum of [Ac-His^{20}]-GRP (20–27) with the spectrum of GRP shown in Figure 7. The two C2 imide protons of histidine (positions 20 and 25) at 8.55 and 8.53 ppm,

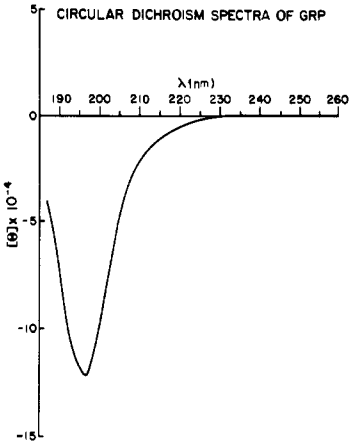

Figure 8. Circular dichroism spectra of synthetic porcine gut GRP in H_2O: concentration 0.1 M, 10 scans, 0.3-nm resolution, 0.1-mm cell, 23 °C.

the C4 (7.59 ppm) and C7 (7.50 ppm) protons of tryptophan (position 21) and the C3/5 protons of tyrosine (position 15) at 6.8/6.85 ppm were well resolved, thus allowing for tentative assignment of most of the signals of the aromatic amino acids in GRP. The two signals at 2.1 and 2.15 ppm were assigned to the two thiomethyl groups of the methionine and methioninamide. Resonances at 1.25, 1.35, and 1.44 ppm were assigned to the β-protons of the three alanines in the molecule. The strong signals at 4.0–4.1 ppm were assigned to the α-protons of several glycines. A more detailed investigation by NMR spectroscopy to determine tertiary structural features of GRP and some selected C-terminal analogues will be presented elsewhere (Von Binst et al., in preparation).

The circular dichroism (CD) spectrum of synthetic GRP was measured in dilute neutral aqueous solution and showed a negative Cotton effect with a minimum at 197 nm (Figure 8), indicating that the polypeptide chain was largely random, with no evidence for the presence of α-helical structure under those conditions.[34] It was shown by RP-HPLC that synthetic GRP partly decomposed during storage in slightly acidic aqueous solution or after repeated lyophilizations. A similar observation was also described for secretin.[35] The contaminating peptides which were observed may be due to air oxidation of the methionine residues[29] or partial deamidation.

(30) Savige, W. E.; Fontana, A. *Methods Enzymol. Part E* **1977**, *47*, 453–459.
(31) Savige, W. E.; Fontana, A. *Int. J. Pept. Protein Res.* **1980**, *15*, 285–297.
(32) Calculation according to: Beaven, G. H.; Holiday, E. R. *Adv. Protein Chem.* **1952**, *1*, 319.
(33) Bundi, A.; Wüthrich, K. *Biopolymers* **1979**, *18*, 285. Bundi, A., Ph.D. Thesis, Eidgenössische Technische Hochschule, Zürich, 1979, No. 6036.
(34) Holzwarth, G.; Doty, P. *J. Am. Chem. Soc.* **1965**, *87*, 218–228.
(35) Jäger, G.; König, W.; Wissmann, H.; Geiger, R. *Chem. Ber.* **1974**, *107*, 215–231.

Table VI. Pharmacologic Effects of Synthetic GRP, Bombesin, and [Ac-His[20]]-GRP (20–27) on Thermoregulation, Glucoregulation,[11] and Gastric Secretion[10c]

peptides	amt, μg	dose, pmol	temp,[a] °C	amt glucose,[a] mg/dL	amt plasma gastrin,[b] pg/mL	gastric secretion[c] mL/2 h/rat	gastric secretion[c] μequiv/2 h/rat
control			36.3 ± 0.4	150 ± 11	116 ± 24	2.2 ± 0.3	204 ± 3
GRP	0.01	3.6	36.2 ± 0.2	169 ± 6	108 ± 16	2.2 ± 0.3	170 ± 60
	0.1	36	34.8 ± 0.4	193 ± 10	229 ± 30	1.1 ± 0.2	40 ± 10
	1	360	32.8 ± 3	287 ± 17	402 ± 80	1.0 ± 0.2	34 ± 10
	10	3600			534 ± 78		
bombesin	0.01	6.2	34.4 ± 4	194 ± 2	131 ± 27	1.5 ± 0.2	86 ± 25
	0.03	18.5	33.8 ± 0.2	217 ± 15			
	0.1	61.7	32.8 ± 0.3	291 ± 30	289 ± 36	1.4 ± 0.2	30 ± 16
	1				323 ± 45	0.7 ± 0.2	0
[Ac-His[20]]-GRP (20–27)	0.01	10.1	34.3 ± 1	173 ± 10		2.0 ± 0.2	140 ± 10
	0.1	101	33.2 ± 0.4	257 ± 34		0.8 ± 0.2	40 ± 10
	1	1010	32.6 ± 6	259 ± 23		0.7 ± 0.2	19 ± 10

[a] The peptides were administered intracisternally to 12 cold exposed rats per point. [b] The peptides were administered intravenously to 12 rats held at room temperature. [c] The peptides were administered intrascisternally to 12 rats held at room temperature. Both volume (mL/2 h/rat) and output (μequiv of acid/2 h/rat) were measured.

The biological activities of synthetic GRP were compared in three bioassays to those of amphibian bombesin and its acetylated C-terminal peptide as summarized in Table VI. GRP, like bombesin, produced hypothermia and elevated plasma epinephrine and glucose.[11] GRP also decreased gastric acid secretion when given intracisternally and elevated plasma gastrin levels when administered intravenously.[10c] The affinity of synthetic GRP to GRP antibodies recently developed (M. Brown et al., in preparation) was found to be twice that of bombesin.

GRP exhibited characteristic bombesin-like activities. Since the C-terminal eight-peptide analogue [Ac-His[20]]-GRP (20–27) produced qualitatively the same biological effects, as did GRP, it is probable that this common partial sequence is essential for bombesin-like actions. More details on the structure–activity relationship of the C-terminal part of bombesin peptides were reported elsewhere.[10a,b] The biological functions of GRP (1–17) and bombesin (1–4) has not been established. In this context it was found that the N-terminal tryptic fragment GRP (1–13) was devoid of biological bombesin-like activity but may have other unrecognized functions.

In summary, a rapid purification scheme involving gel permeation followed by two resolutive HPLC steps has been applied to the isolation of GRP from a complex synthetic mixture. High resolution in HPLC could be obtained by employing state of the art C-18, reverse-phase columns[36] and the use of two buffers with different selectivities (i.e., the nonvolatile TEAP buffer and 0.1% TFA for desalting purposes). Similarly, a scheme for the characterization of the final product has been established. It involves (1) analysis by HPLC (reverse phase and gel permeation) using columns from several suppliers as well as solvent systems with different selectivities and (2) sequence analysis under conditions used in microsequencing which, at a relatively high load (~20 nmol), allow for the detection of minute amounts of contaminating peptides generated by synthetic errors which could not be detected by amino acid analysis, TLC, electrophoresis, or even HPLC. It is only then that, as suggested by Wünsch,[37] a variety of physicochemical, biological, and immunological data should be obtained to further ascertain the integrity of the peptide's primary structure.

Experimental Section

Materials. Derivatized amino acids used in the synthesis were of the L configuration and purchased from Bachem, Inc. TFA was distilled over P_2O_5 before use. HOBt was recrystallized from MeOH. All other solvents were reagent grade and were used without further purification. CM-52 cellulose was purchased from Whatman, Sephadex G 25 F from Pharmacia Fine Chemicals, and *N,N'*-dicycylohexylcarbodiimide from Pierce Chemicals. Bio Beads S-X1 (200–400 mesh) from Bio-Rad were subjected to a Friedel–Crafts acylation with benzoyl chloride, followed by a Leuckart reductive amination with ammonium formate and final hydrolysis of the formylated resin in 6 N HCl to yield the benzhydrylamine (BHA) resin previously described.[15] A substitution of 0.2 mequiv/g of resin[15] was obtained.

Reverse-Phase High-Performance Liquid Chromatography. The two systems used for RP-HPLC were as follows: (1) a Water's Associates Model U6K injector, a Model 660 programmer, two Model 6000A pumps, and a Model 450 variable wavelength detector; (2) a Spectra Physics "Minigrator", an Altex Model 322 MP LC system with a Model 420 microprocessor and a Model 210 injector, a Water's Associates 450 variable wavelength detector, and a Datamark SR 6253 Servocorder. The chart speed was 1 cm/min. Elution was accomplished by a binary buffer system. Buffer A consisted of 0.25 N H_3PO_4 or 0.25 N HCOOH adjusted, respectively, to pH 2.25 (TEAP) or pH 3.01 (TEAF) with triethylamine redistilled from thionylchloride. Buffer B contained 40% buffer A and acetonitrile. TEAP and TEAF buffers were filtered through a C-18 cartridge in Waters Associates Prep LC-500 as described earlier.[38] For the desalting of peptides, 0.1% TFA (pH 2.1) was used as eluent A and 40% A in acetonitrile as eluent B. Analytical RP-HPLC was performed on a SupelcoSil LC18 No. 956 column (0.46 × 15 cm) or on an Ultrasphere-ODS (0.46 × 15 cm) UE No. 1093 column from Altex. The columns were eluted under isocratic or gradient conditions by using buffers A and B (Table IV) with a flow rate of 1.0 or 1.5 mL/min. The load per injection was 5–10 μg of peptide. The semipreparative C-18 column (1 × 25 cm) was developed by Drs. R. Eksteen and B. Karger.[36]

Amino Acid Analysis. Peptides (0.2–0.5 nmol) were hydrolyzed and analyzed as described earlier.[39] Hydrolyzates with 100–500 pmol per amino acid were applied to a column (0.28 × 20 cm) packed with Beckman AA-10 resin in the sodium form or to a column (0.28 × 25 cm) containing the same resin in the lithium form.

UV spectra and absorption determinations were recorded on a Beckman Model 25 spectrophotometer.

Optical rotation was measured with a Perkin-Elmer Model 141 polarimeter.

Ascending thin-layer chromatography (TLC) on silica gel was performed on Schleicher and Schuell chromatogram sheets (no. F1500) by using the following solvent systems: (1) BAW; upper phase of 1-butanol–acetic acid–water (4:1:5) after 12 h of equilibration; (2) BPyA; 1-butanol–pyridine– 0.1 M acetic acid (5:3:11, upper phase); (3) BINET; 1-butanol–isopropyl alcohol–1 N NH_4OH–EtOAc (1:1:2.5:1). Iodine, ninhydrin spray, and Pauly reagent[40] were used to develop the TLC plates.

High-voltage paper electrophoresis was carried out on an electrophoresis apparatus of Savant Instruments, Inc., at 3000 V and 180 mA, in 0.03 M potassium phosphate (pH 7.1) or at 3000 V and 24 mA in 0.05 M formic acid (pH 2.7) for 20 min.

CD spectra were recorded on a Cary Model 61 spectropolarimeter on solutions of 1 mg/mL at 25 °C in a 0.1-mm path length cell; 10 scans were accumulated.

(36) Rivier, J. E.; Desmond, J.; Spiess, J.; Perrin, M.; Vale, W.; Eksteen, R.; Karger, B. In "Peptides: Structure and Biological Function"; Gross, E.; Meienhofer, J., Eds.; Pierce Chemical Co.: Rockford, Illinois, 1979; pp 125–128. Rivier, J.; Märki, W.; Eksteen, R.; Karger, B., in preparation.

(37) Wünsch, E. "Gut Hormones"; Bloom, S. R., Ed.; Churchill Livingstone: Edinburgh, London, and New York, 1978; pp 28–32.

(38) Rivier, J. *J. Chromatogr.* **1980**, *202*, 211–222.

(39) (a) Spiess, J.; Rivier, J.; Rodkey, J. A.; Bennett, C. D.; Vale, W. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2974–2978. (b) Spiess, J.; Villarreal; J.; Vale, W. *Biochemistry* **1981**, *20*, 1982–1988.

(40) Sanger, F.; Tuppy, S. A. *J. Biochem.* **1951**, *49*, 453.

The ^1H NMR spectrum was obtained by using a varian HR 300 magnet and probe equipped with home-built FT electronics. Free induction decays were accumulated on a Nicolet 1180 system. Samples (5 mg) were dissolved in 500 μL of 99.7% D_2O and lyophilized. This operation was repeated three times in order to exchange labile protons. Chemical shifts are given in parts per million relative to HOD (4.81 ppm) as an internal standard. The pH was measured with a microelectrode in the NMR-tube.

Biological Tests. Male Sprague–Dawley rats (180–200 g) obtained from Hilltop laboratories and fed tap water and Purina Rat Chow ad libitum were used in all experiments. GRP or bombesin was administered intracisternally in 10-μL volumes of artificial CSF or distilled water. The animals were then placed into an environmental chamber at 4 °C. Subsequently, rectal temperatures were measured by using a Yellowsprings Instruments thermal probe. Following rapid decapitation, blood was collected, and plasma levels of glucose were determined by using a Beckman glucose analyzer as previously described.[5]

Gastric secretion (volume and pH) of intracisternally treated animals and gastrin determinations after intravenous injection were measured as described in detail elsewhere.[6] A guinea pig antiserum to porcine gastrin at the final dilution of 1:100 000 (a generous gift of Dr. John Walsh, UCLA) was used for the radioimmunoassay with synthetic human [^{125}I]-gastrin-17 as a trace.

Following the analysis of variance, differences between groups were determined by the multiple-range tests of Dunnett and Duncan by using the computer program EXBIOL.

GRP (mol wt 2785). The synthesis was carried out manually on 6 g of BHA resin[41] (substitution, 0.2 mequiv/g of resin) in a filter-frit reaction vessel according to a previously described procedure.[15] The following protected amino acids were used: Boc-Met, Boc-Leu·H_2O, Boc-His(Tos), Boc-Gly, Boc-Val, Boc-Ala, Boc-Trp, Boc-Asn-ONp, Boc-Arg(Tos), Boc-Pro, Boc-Tyr (2,6-Cl_2-Bzl),[42] Boc-Lys(ε-2-Cl-Z),[43] Boc-Thr(Bzl), Boc-Ser(Bzl). Coupling of the Boc amino acids was accomplished by the DCC technique using 1 mmol of the Boc amino acid per gram of resin (fivefold excess) in CH_2Cl_2 except for Boc-Leu, Boc-Trp, and Boc-Arg(Tos), which were dissolved in 5–10% DMF/CH_2Cl_2. One mmole of DCC (1 M) in CH_2Cl_2/g of resin was added to the resin 2 min prior to the addition of the Boc amino acid. Coupling was performed for 1–10 h, depending upon the nature of the particular amino acid residue. (*tert*-Butoxycarbonyl)asparagine was coupled for 10 h as its *p*-nitrophenyl ester by using 1 equiv of HOBt[44] in 50% DMF/CH_2Cl_2. Acetylation after coupling was achieved with an excess of acetic anhydride (20% in CH_2Cl_2 for 20 min). TFA in CH_2Cl_2 (50%) was used in repetitive acid cleavage of the Boc protecting groups. 1,2-Ethanedithiol was added during all cleavages to protect the tryptophan residue from acid-catalyzed oxidation.[45] Since both tryptophan and methionine were unprotected, the last Boc group was removed with TFA prior to final HF cleavage to minimize *tert*-butylation.[46] The C-terminal methionine coupled quantitatively to the resin as shown by both ninhydrin[25] and fluorescamine[26] tests. Numerous aliquots of the growing peptide resin were taken to allow for those tests. The fourteen-peptide resin (0.2 mmol, 1.56 g) was taken out after cycle 14, in order to isolate and purify GRP (14–27).[10b] We have assumed that only 4.5 g of the original 6 g of BHA was recovered at the end of the synthesis. The protected GRP peptide resin (6.2 g) was treated with redistilled HF (70 mL) in the presence of anisole (9 mL, distilled over BaO) and methyl ethyl sulfide (3 mL) as scavangers at −20 °C for 30 min and at 0 °C for 60 min in the usual manner.[47] Subsequent removal of HF under high vacuum was accompanied by a discoloration of the resin from bright red to yellow. The resin was then washed several times with ether. The peptide was extracted with aqueous acetic acid (10%) and lyophilized. Approximately, 1.52 g of the crude, fluffy powder was obtained, which consisted of a major component representing 30% of the material detected by analytical RP-HPLC. The crude peptide (420 mg) was applied directly to a Sephadex G25F column (2.5 × 100 cm) which was equilibrated and run with 5% HOAc containing 0.2% ethanethiol (Eastman, v/v). The constant flow of 13 mL/h was obtained with a peristaltic pump from Pharmacia Fine Chemicals, Inc. The gel-filtered peptide (150 mg) was further purified by semipreparative RP-HPLC performed on a C-18 column (1 × 25 cm).[36]

The isolation of GRP was carried out by using TEAP buffer under the indicated conditions. The amount of peptide load in a typical experiment was 25 mg in 500 μL of buffer A per run. Peptides collected and pooled from several runs were concentrated by lyophilization and subsequently desalted with 0.1% TFA/CH_3CN. Lyophilization of the desalted material yielded 35 mg of peptide with a purity greater than 98% based on RP-HPLC. The purity estimate was based on the ratio of the integrated area under the main peak vs. the total integrated areas when recorded at 210 nm. The overall yield was 5% (see Table III for yields at each step of the purification). Detailed conditions of the semipreparative RP-HPLC purification are presented in Figures 2 and 3.

Physical constants obtained with this material were as follows: $[\alpha]^{23}_D$ −72.4°, (*c* 0.3, 1 N AcOH); $[\alpha]^{23}_{546}$ = −85.4° (c 0.3, 1 N AcOH). The Concentration (*c*) was calculated on the basis of dry weight and was not corrected for the presence of CF_3COOH or H_2O. R_f values on TLC were 0.31, 0.50, and 0.52 in solvent systems 1–3, respectively. Electrophoretic mobilities relative to aspartic acid were 0.25 at pH 7.1 and 4.1 at pH 2.7: UV (1% AcOH, (*c* 50 μg/mL) λ_{max} 280 nm (ε 3800), shoulder at 288 (3000); UV (0.1 N NaOH, *C* 50 μg/mL) shoulder at 242 nm (ε 9800), 282 (4800), shoulder at 289 (4600). The calculated Tyr to Trp molar ratio was 1.006 (theoretical value, 1).

Peptide Mapping. Synthetic GRP (3 mg) was digested with TPCK–trypsin (30 μg, Worthington) in buffer (0.1 M NH_4OAc, 0.001 M $CaCl_2$, pH 8.0, 300 μL) for 15 h at 37 °C. The reaction was terminated by glacial acetic acid (final concentration 10% v/v). GRP (14–27) (230 μg) was digested with TPCK–trypsin (3 μg) in 100 μL of buffer as described above. The tryptic fragments were isolated by RP-HPLC using the semipreparative C-18 column (1 × 25 cm; for detailed conditions, see the legend of Figure 5). The RP-HPLC-desalted tryptic fragments were subjected to amino acid analysis after 24 h of hydrolysis at 110 °C in 4 M methanesulfonic acid containing 0.2% tryptamine. NaOH (3.5 N)-neutralized hydrolysates were chromatographed through Beckman AA-10 resin in the sodium form. Results (mean of two independent analyses) were as follows: GRP (14–17) analyzed as $Pro_{0.9}$-$Met_{1.0}$-$Tyr_{1.0}$-$Arg_{1.0}$; GRP (1–13) analyzed as $Thr_{0.9}$-$Ser_{0.9}$-$Pro_{1.0}$-$Gly_{3.0}$-$Ala_{2.1}$-$Val_{2.9}$-$Leu_{0.9}$-$Lys_{1.0}$; Grp (18–27) analyzed as $Asx_{1.0}$-$Gly_{2.0}$-$Val_{1.0}$-$Met_{1.0}$-$Leu_{1.0}$-$His_{1.9}$-$Trp_{1.0}$.

C-Terminal End Group Determination by Chymotryptic Digestion. GRP (28 μg) was digested by 15 mL of chymotrypsin immobilized on Enzite Agarose beads (Enzite Chemicals, Inc.) in 50 μL of buffer (0.1 M NH_4OAc, 0.1 M $CaCl_2$, pH 9.5) for 18 h at 37 °C. The digestion was terminated by glacial acetic acid (final concentration, 20% v/v). The supernatant after removal of the bound enzyme was lyophilized. The resulting dry residue was extracted twice with anhydrous methanol. The methanol extract was freed of solvent and analyzed by amino acid analysis with AA-10 resin (Beckman) in the lithium form. Released methioninamide was eluted between histidine and 3-methylhistidine.

Oxidation of GRP to [Met(O)^{14,27}]-GRP. The methionine oxide derivative of GRP was prepared according to a modified procedure of Iselin.[29] The oxidation was performed with 5% H_2O_2 in 1 N acetic acid for 10 min at 0 °C followed by lyophilization. The lyophilization was repeated after addition of water.

Bombesin was synthesized by using solid-phase methodology.[10a] The synthetic material was found to have the expected amino acid composition. The specific optical rotation was $[\alpha]^{22}_D$ −57.9° (*c* 1, 1% HOAc). The degree of purity was 98% based on RP-HPLC.

[Ac-His^{20}]-GRP (20–27). The peptide was synthesized by using SPPS on a methylbenzhydrylamine resin (2 g)[16] and the methodology described for GRP. Cleavage and deprotection were achieved with HF in the presence of anisole and MeSEt as scavengers. Crude peptide (660 mg) was applied onto an ion-exchange column (1.7 × 20 cm, CM-52) and eluted with a linear gradient between 0.01 M NH_4OAc (300 mL) and 0.25 M NH_4OAc (300 mL) in 50% H_2O/MeOH. Fractions 23–30 (ca. 5 mL each) were collected, concentrated, and lyophilized twice from 0.1% AcOH (400 mg collected). Further purification was accomplished by partition chromatography on Sephadex G25F with an *n*-butanol–AcOH–H_2O (4:1:5) system. Fractions 33–46 (ca. 5 mL each) were pooled and lyophilized (233 mg, 84% pure peptide was collected). This last step was repeated, and the fractions (2.5 mL each) were analyzed by TLC and HPLC. Tubes 63–66 yielded 24 mg. This material was found to have the expected amino acid composition. The degree of purity was greater than 98% based on RP-HPLC with a SupelcoSil LC-18 column. The specific optical rotation was $[\alpha]^{22}_D$ −48.1° (*c* 0.5, 1 N HOAc) and $[\alpha]^{22}_{546}$ −57.3° (*C* 0.5, 1 N HOAc). Amino acid composition was as follows: $Gly_{1.0}$, $Ala_{1.2}$, $Val_{1.0}$, $Met_{1.1}$, $Leu_{1.1}$, $His_{1.9}$, $Trp_{1.0}$.

Carboxypeptidase A Digestion. GRP (65 μg) was digested with carboxypeptidase A (Worthington, 3 μL) in buffer (40 μL, 0.1 M

(41) Pietta, P. G.; Marshall, G. R. *Chem. Commun.* **1970**, 650–651.
(42) Erickson, B. W.; Merrifield, R. B. *J. Am. Chem. Soc.* **1973**, *95*, 3750–3756.
(43) Erickson, B. W.; Merrifield, R. B. *J. Am. Chem. Soc.* **1973**, *95*, 3757–3763.
(44) Upson, D. A.; Hruby, V. *J. Org. Chem.* **1976**, *41*, 1353–1358.
(45) Chino, N.; Masui, Y.; Sakakibara, S. "Peptide Chemistry 1977"; Shiba, T., Ed.; Protein Research Foundation: Osaka, Japan, 1978; pp 27–32. Sharp, J. J.; Robinson, A. B.; Kamen, M. D. *J. Am. Chem. Soc.* **1973**, *95*, 6097–6108.
(46) Noble, R.; Yamashiro, D.; Li, C. H. *J. Am. Chem. Soc.* **1976**, *98*, 2324–2328.
(47) Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2164–2167.

$NH_4OAc/(NH_4)HCO_3$, pH 8) for 62 h at 37 °C. C-terminally desamidated bombesin was also digested in a control experiment. The three C-terminal amino acids of desamidated bombesin were released under the above conditions as determined by amino acid analysis whereas no amino acid was released from GRP under those conditions.

Sequence Analysis. Sequence analysis based on the Edman degradation of peptides was performed with a Beckman 890C spinning-cup sequencer modified according to Wittmann-Liebold.[28] A Quadrol single-cleavage program was used. Prior to the application of peptide, purified Polybrene was applied to the cup as a peptide carrier[48] and subjected to 8-10 sequencer cycles. More details about the sequence method employed here was described elsewhere.[39b] The identification and quantitation of the 3-phenyl-2-thiohydantoin derivatives of the cleaved amino acids (PTH amino acids) were accomplished by RP-HPLC with a Hewlett-Packard Liquid chromatograph, Model 1084 B. Details of the PTH amino acid determination will be described elsewhere (Spiess and Heil, in preparation). The sequencing method employed here allows for direct sequence analysis of 25-30 residues of 0.6-1.5 nmol of peptide, if one-third of each sequencer cycle is injected per HPLC run. The homogeneity of synthesized GRP was tested by subjecting GRP (14.4 nmol) to sequence analysis so that contaminating peptides with free N termini representing as little as 2% of the applied GRP could be detected.

Acknowledgment. This work was supported in part by an NINCDS grant (NS-14263) and in part by The Clayton Foundation for Research, California Division. Drs. Spiess, Brown, and Rivier are Clayton Foundation Investigators. W.M. is a recipient of a Swiss National Foundation Fellowship. Y.T. is a Scholar of the Medical Research Council of Canada. We thank Dr. M. Goodman, University of California, San Diego (USCD), for the CD spectrum and are indebted to W. Leupin and J. Feigon in Dr. Kearn's laboratory, UCSD, for providing us with the 300-MHz FT ^1H NMR spectrum. We also thank B. Karger and R. Eksteen of Northeastern University for developing the semipreparative C-18 RP-HPLC column (1 × 25 cm). R. Kaiser, R. Lee, F. Lux, J. Heil, R. McClintock, R. Allen, W. Bartholomeus, M. Newberry, L. Pacheco, and S. McCall are acknowledged for technical assistance.

(48) Tarr, G. E.; Beecher, J. F.: Bell, M.; McKean, D. J. *Anal. Biochem.* **1978**, *84*, 622-627.

Synthesis of Deoxyoligonucleotides on a Polymer Support[1]

M. D. Matteucci and M. H. Caruthers*

Contribution from the Department of Chemistry, University of Colorado, Boulder, Colorado 80309. *Received September 18, 1980*

Abstract: The development of a new method for synthesizing deoxyoligonucleotides is described. The synthesis begins by derivatizing high-performance liquid chromatography grade silica gel to contain 5′-*O*-(dimethoxytrityl)deoxynucleosides linked through the 3′-hydroxyl to a carboxylic acid functional group on the support. This matrix is then packed into a column which is attached to a pump and a series of valves. The chemical steps for the addition of one nucleotide to the support are as follows: (1) detritylation using $ZnBr_2$ in nitromethane (30 min); (2) condensation of a 5′-*O*-(dimethoxytrityl)deoxynucleoside (3′-methoxytetrazoyl)phosphine with the support-bound nucleoside (60 min); (3) blocking unreacted, support-bound nucleoside hydroxyl groups with diethoxytriazolylphosphine (5 min); (4) oxidation of phosphites to phosphates with I_2 (5 min). Completed deoxyoligonucleotides are isolated by sequential treatment with thiophenol and ammonium hydroxide, purification by reverse-phase chromatography, and treatment with 80% acetic acid. The method is extremely fast (less than 2.5 h are needed for each nucleotide addition cycle), yields in excess of 95% per condensation are obtained, and isolation of the final product is a simple one-step column purification. The syntheses of d(C-G-T-C-A-C-A-A-T-T) and d(A-C-G-C-T-C-A-C-A-A-T-T) were carried out as a test of this method. Yields of support-bound deoxyoligonucleotides were 64% and 55%; the isolated yield of deoxydecanucleotide was 30%. Both synthetic products were homogeneous and biologically active by every criteria so far tested.

Synthetic deoxyoligonucleotides of defined sequence have been used to solve important biochemical[2,3] and biophysical[4,5] problems. Moreover, recent advances in chemical methods have led to the synthesis of genes[6-10] and of deoxyoligonucleotides useful for manipulating natural DNA and RNA.[11] Despite these achievements, the synthesis and isolation of deoxyoligonucleotides remains a difficult and time consuming task. Ideally, chemical methods should be simple, rapid, versatile, and completely automatic. In this way the rapid synthesis of genes and gene control regions can be realized and many important biochemical studies which are presently not possible can be initiated.

Our approach to solving this problem has involved developing methods for synthesizing deoxyoligonucleotides on polymer supports. This concept is not new and has been investigated extensively.[12,13] Recently several promising approaches have been

(1) This is paper IV in a series on nucleotide chemistry. Paper III: M. H. Caruthers, S. L. Beaucage, J. W. Efcavitch, E. F. Fisher, M. D. Matteucci, and Y. Stabinsky, *Nucleic Acids Symp. Ser.*, No. 7, 215 (1980). This research was supported by the National Institutes of Health (Grants GM 21120 and GM 25680).

(2) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 39 (1966).

(3) M. H. Caruthers, *Acc. Chem. Res.*, **13**, 155 (1980).

(4) A. H.-J. Wang, G. J. Quigley, F. J. Kolpak, J. L. Crawford, J. H. van Boom, G. vd Marel, and A. Rich, *Nature (London)*, **282**, 680 (1979).

(5) F. H. Martin and I. Tinoco, Jr., *Nucleic Acids Res.*, **8**, 2295 (1980).

(6) K. L. Agarwal, Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. van de Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature (London)*, **227**, 27 (1970).

(7) H. G. Khorana, *Science*, **203**, 614 (1979).

(8) K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science*, **198**, 1056 (1977).

(9) D. V. Goeddel, D. G. Kleid, F. Bolivar, H. L. Heyneker, D. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A. Riggs, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 106 (1979).

(10) D. V. Goeddel, H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. Seeburg, *Nature (London)*, **281**, 544 (1979).

(11) R. J. Rothstein, L. F. Lau, C. D. Bahl, S. A. Narang, and R. Wu, *Methods Enzymol.*, **68**, 98 (1979).

(12) C. B. Reese, *Tetrahedron*, **34**, 3143 (1978).

(13) V. Amarnath and A. D. Broom, *Chem. Rev.*, **77**, 183 (1977).

0002-7863/81/1503-3185$01.25/0