Bombesin, Bombesin Analogues, and Related Peptides: Effects on Thermoregulation†

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ABSTRACT: The synthesis and biological evaluation on thermoregulation of 39 peptides related to bombesin (structural analogues or other naturally occurring peptides) are described. The bioassay system reported measures the ability of peptides injected intracisternally to lower body temperature of cold (4 °C) exposed rats. The most potent analogues of bombesin were those in which positions one to five (not included) were altered, indicating that the decapeptide C terminal was sufficient for full potency. Gln at the seventh position and Gly at the 11th position could be replaced by D-Gln and D-Ala (but not D-Pro or D-Phe), respectively, without any change in potency. Methionine at the 14 position could be replaced with its D isomer with retention of 10% biological activity. Any other alteration of the C terminus (deletions or free acid with the exception of the *N*-methylamide) drastically reduced the biological potency of those peptides. Among other naturally occurring peptides, alytesin was found to have 100% of bombesin potency whereas litorin, neurotensin, xenopsin, substance P, physalaemin, and eledoisin were found to be in the order of 10^4 times less potent. The shortest peptide found to have full biological activity is the octapeptide des-Glp-Gln-Arg-Leu-Gly-Asn[D-Glp^7,D-Ala^{11}]-bombesin.

Bombesin, alytesin (Anastasi et al., 1971; Erspamer et al., 1970), ranatensin (Nakajima et al., 1970), and litorin (Anastasi et al., 1975) are peptides isolated from amphibean skin; their closely related structures are shown in Table I. All have a pyroglutamyl N-terminal and similar C-terminal octapeptide residues (Phe for Leu at the penultimate position of ranatensin and litorin). Two other families of closely related vasoactive peptides are: (1) neurotensin (Carraway & Leeman, 1975) and xenopsin (Araki et al., 1973) respectively isolated from bovine brain and gut and frog skin and (2) substance P (Chang et al., 1971), physalaemin (Anastasi et al., 1964), and eledoisin (Anastasi & Erspamer, 1963) respectively isolated from bovine, frog, and octopus tissues (see structures in Table I). Because of similarities of structure and biological actions, we have evaluated their activity in a variety of bioassay systems in an effort to determine their physiologic significance. One of these bioassay systems concerns the ability of these peptides to lower body temperature of cold exposed rats (Bissette et al., 1976; Brown et al., 1977a; Rivier et al., 1977). In these studies the peptides were given intracisternally and were found to produce a lowering of basal body temperature of rats or mice exposed to cold (4 °C). Bombesin and neurotensin were active at doses of 5 ng/kg body weight (BW)[1] and 5 μg/kg BW, respectively, to significantly lower the core temperature of cold exposed rats (4 °C). And, while certain synthetic analogues of neurotensin were also found to be 100% as active as neurotensin or even ten times more potent in that system (Brown et al., 1977a; Rivier et al., 1977; Lazarus et al., 1977a,b), peptides related to substance P, α-melanocyte stimulating hormone, luteinizing hormone releasing factor (LRF) and somatostatin, bradykinin and vasoactive intestinal polypeptide were found to be inactive. Although β-endorphin has also been reported to produce hypothermia in rats (Bloom et al., 1976; Tseng et al., 1977), it has been observed that this hypothermia is associated with significant alteration of the motor activity of rats receiving this peptide, resulting in muscular rigidity and ataxia (Tseng et al., 1977; Bloom et al., 1976; Jacquet & Marks, 1976). Since bombesin and neurotensin do not produce any obvious behavioral effect, the significance of β-endorphin on hypothermia becomes questionable (Brown et al., 1977b). Hypothermia induced by bombesin can be reversed by TRF, prostaglandin E_2 and by the specific opiate receptor antagonist naloxone. The hyperthermic effects of PGE_2 and TRF produced in rats kept at room temperature are also prevented by bombesin (Brown et al., 1977c). From these studies one could conclude that bombesin, in its effect of lowering core body temperature of cold exposed rats, is the most potent peptide to affect the central nervous system in vivo.

It was found that bombesin not only lowers body temperature of cold exposed rats, but also prevents cold induced TSH secretion while not preventing TRF induced TSH secretion: therefore bombesin may inhibit the secretion or delivery of TRF to the pituitary (Brown et al., 1977d).

Preliminary experiments indicate the presence of a high concentration of bombesin in the rat, sheep and pig brain (Brown et al., 1977b,d). It was thus of significant interest to us in our study of the possible mechanism involved in the control of body temperature to determine which part of the bombesin molecule was responsible for such striking property. It was also of interest to design analogues that would be possibly more potent, inactive or competitive antagonists, that could cross the blood brain barrier or be used for the raising of specific antibodies.

This paper reports on the synthesis of numerous analogues of bombesin and several known vasoactive natural peptides and on their relative biological potency in lowering the body tem-

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[1] Abbreviations used: Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [(1971) *J. Biol. Chem. 247*, 977]; TRF, thyrotropin releasing factor or thyroliberin; LRF, luteinizing hormone releasing factor or luliberin; BN, bombesin; PGE_2, prostaglandin E_2; CNS, central nervous system; CSF, cerebrospinal fluid; BW, body weight; HPLC, high pressure (or performance) liquid chromatography; TLC, thin-layer chromatography; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Bz, benzoyl; CMC, carboxymethylcellulose; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; (ε-2ClZ), 2-chlorobenzyloxycarbonyl; (2,6-Cl_2Bzl), 2,6-dichlorobenzyl.

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TABLE I: Sequences and Relative Potencies of Bombesin Analogues and Related Compounds.[a]

Compound	Potency relative to bombesin	Sequence of analogues
(1) Bombesin (BN)	100	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(2) [Lys^3]-BN	100	Glp-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(3) [Lys^4]-BN	95	Glp-Gln-Arg-Lys-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(4) [Tyr^4]-BN	100	Glp-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(5) [D-Ala^5]-BN	100	Glp-Gln-Arg-Leu-DAla-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(6) [Bz-Gly^5]-BN		Bz-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(7) [Ac-Gly^5]-BN	110	Ac-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(8) [Ac-Gly^5,D-Ala^{11}]-BN	100	Ac-Gly-Asn-Gln-Trp-Ala-Val-DAla-His-Leu-Met-NH_2
(9) [Ac-Gly^5,D-Gln^7,D-Ala^{11}]-BN	100	Ac-Gly-Asn-DGln-Trp-Ala-Val-DAla-His-Leu-Met-NH_2
(10) [D-Glp^7,-D-Ala^{11}]-BN	100	DGlp-Trp-Ala-Val-DAla-His-Leu-Met-NH_2
(11) [D-Asn^6]-BN	1	Glp-Gln-Arg-Leu-Gly-DAsn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(12) [D-Gln^7]-BN	100	Glp-Gln-Arg-Leu-Gly-Asn-DGln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(13) [D-Trp^8]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-DTrp-Ala-Val-Gly-His-Leu-Met-NH_2
(14) [D-Ala^9]-BN	5	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-DAla-Val-Gly-His-Leu-Met-NH_2
(15) [D-Val^{10}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-DVal-Gly-His-Leu-Met-NH_2
(16) [D-Ala^{11}]-BN	100	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-DAla-His-Leu-Met-NH_2
(17) [Ala^{11}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Ala-Ala-His-Leu-Met-NH_2
(18) [D-Phe^{11}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-DPhe-His-Leu-Met-NH_2
(19) [D-Pro^{11}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-DPro-His-Leu-Met-NH_2
(20) [Pro^{11}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Pro-His-Leu-Met-NH_2
(21) Des-His^{12}-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-...Leu-Met-NH_2
(22) [D-His^{12}]-BN	0.1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-DHis-Leu-Met-NH_2
(23) [Tyr^{12}]-BN	1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-Tyr-Leu-Met-NH_2
(24) [Phe^{13}]-BN	70	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(25) [D-Leu^{13}]-BN	<0.1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-DLeu-Met-NH_2
(26) [D-Met^{14}]-BN	10	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-DMet-NH_2
(27) Des-Leu^{13},-Met^{14}-BN	<1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-NH_2
(28) Alytesin	100	Glp-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(29) Ranatensin	20	Glp-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(30) Litorin	<1	Glp-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(31) Des-Gln^2-litorin	1	Glp-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(32) Des-Gln^2-[Leu^8]-litorin	1	Glp-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
(33) Neurotensin	$<10^{-2}$	Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH
(34) Xenopsin	$<10^{-2}$	Glp-Lys-Arg-Pro-Lys-Pro-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH
(35) Substance P	$<10^{-}$	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
(36) Physalaemin	$<10^{-4}$	Glp-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
(37) Eledoisin	$<10^{-4}$	Glp-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
(38) Bombesin-OH	<1	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-OH
(39) Bombesin-NHMe	70	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NHMe

[a] Sequences and relative potency values of bombesin (BN), analogues of BN, and other peptides to lower body temperature of cold-exposed rats, 60 min following intracisternal administration of peptide. Potency values were calculated using the four- or six-point parallel line bioassay method. Ninety-five percent confidence limits are of the order of ±10%.

perature of cold exposed rats (see Table I for structures and biological potencies).

Materials and Methods

Synthesis, Purification and Characterization of Peptides. All peptides reported in Table I were synthesized by the solid phase method in an automated synthesizer. The peptide-free acids were made starting with a chloromethylated resin to which the first protected amino acid was esterified by our modification of Monahan and Gilon's procedure whereby only 0.6 to 1.0 mequiv of the Boc-amino acid was used per mequiv of Cl on the resin. Furthermore, to avoid coloration of the solution, the potassium *tert*-butoxide was added to the solution of the protected amino acid in Me_2SO as a fluffy powder, and after addition of the resin to the clear solution, the suspension was warmed at 80 °C for 2 h (Monahan and Gilon, 1973; Rivier et al., 1976a). The peptide amides were synthesized using a benzhydrylamine resin (Pietta & Marshall, 1970; Rivier et al., 1973) or *p*-methylbenzhydrylamine resin (Stewart et al., 1976) to which the first amino acid was coupled using the standard dicyclohexylcarbodiimide reagent. Bombesin *N*-methylamide was synthesized starting with an N-

TABLE II: Physical Constants, Yield, and HPLC Results of Bombesin Analogues and Related Compounds.[a]

Compound	TLC systems BAW	BPyA	BINEt	2-BN	$[\alpha]^{22}$ [b] (deg)	Yield[c] (%)	HPLC[d] RT (min)	HPLC[e] % Impure (RT)	Remarks
(1) Bombesin (BN)	0.41	0.56	0.33	0.28	−57.9	33	16.9	<2	30% CH_3CN
(2) [Lys^3]-BN	0.44	0.56	0.36	0.28	−58.7	38	18.8	5 (23.1)	30% CH_3CN
(3) [Lys^4]-BN	0.31	0.41	0.26	0.12	−54.6	34	13.0	12(16.1)	30% CH_3CN
(4) [Tyr^4]-BN	0.43	0.59	0.35	0.29	−48.8	36	15.9	25 (19.9)	30% CH_3CN
(5) [D-Ala^5]-BN	0.41	0.56	0.36	0.28	−47.8	29	18.1	<3	30% CH_3CN
(6) [Bz-Gly^5]-BN	0.62	0.79	0.53	0.56	−28.4*	<9.5	22.0	<5	34% CH_3CN
(7) [Ac-Gly^5]-BN	0.57	0.70	0.47	0.43	−33.8*	20	16.1	11 (17.1)	33% CH_3CN
(8) [Ac-Gly^5,D-Ala^{11}]-BN	0.52	0.72	0.46	0.44	−29.6*	9	19.5	10 (21.8)	30% CH_3CN
(9) [Ac-Gly^5,D-Gln^7,D-Ala^{11}]-BN	0.50	0.76	0.58	0.43	−24.6*	17	21.6	<2	30% CH_3CN
(10) [D-Glp^7,D-Ala^{11}]-BN	0.60	0.80	0.60	0.58	−24.3	5	30.2	10 (34.1)	30% CH_3CN
(11) [D-Asn^6]-BN	0.39	0.54	0.32	0.29	−39.6	7	17.9	<4	30% CH_3CN
(12) [D-Gln^7]-BN	0.40	0.56	0.32	0.30	−48.7	29	18.9	9	30% CH_3CN
(13) [D-Trp^8]-BN	0.41	0.57	0.33	0.29	−42.4*	33	20.9	11 (24.9	30% CH_3CN
(14) [D-Ala^9]-BN	0.42	0.57	0.33	0.30	−44.3	30	23.3	<2	30% CH_3CN
(15) [D-Val^{20}]-BN	0.41	0.57	0.34	0.30	−47.1	6	10.3	15 (12.0)	33% CH_3CN
(16) [D-Ala^{11}]-BN	0.41	0.57	0.34	0.29	−44.9	48	18.2	<4	30% CH_3CN
(17) [Ala^{11}]-BN	0.40	0.56	0.34	0.28	−62.3	24	25.6	<4	30% CH_3CN
(18) [D-Phe^{11}]-BN	0.45	0.60	0.35	0.32	−50.9	16	35.7	<4	33% CH_3CN
(19) [D-Pro^{11}]-BN	0.41	0.56	0.34	0.31	−49.0	27	15.9	4 (20.2)	32% CH_3CN
(20) [Pro^{11}]-BN	0.41	0.56	0.33	0.29	−79.4	17	14.7	4 (18.5) 8 (29.4)	32% CH_3CN
(21) Des-His^{12}-BN	0.46	0.59	0.33	0.32	−44.9*	35	22.6	9 (33.2)	30% CH_3CN
(22) [D-His^{12}]-BN	0.41	0.57	0.32	0.29	−53.7	17	13.7	<4	30% CH_3CN
(23) [Tyr^{12}]-BN	0.47	0.57	0.33	0.31	−44.1	29	12.6	19 (18.8)	30% CH_3CN
(24) [Phe^{13}]-BN	0.40	0.55	0.31	0.29	−50.3	23	6.5	11 (8.6) 5 (14.2)	30% CH_3CN
(25) [D-Leu^{13}]-BN	0.40	0.56	0.33	0.30	−40.3	28	11.6	5 (16.0)	33% CH_3CN
(26) [D-Met^{14}]-BN	0.40	0.55	0.35	0.28	−45.3	9	12.7	6 (11.5)	33% CH_3CN
(27) Des-Leu^{13},Met^{14}-BN	0.38	0.56	0.30	0.10	−51.4	42	5.4	10 (7.0)	30% CH_3CN
(28) Alytesin	0.46	0.62	0.37	0.34	−37.5*	14	25.0	<4	30% CH_3CN
(29) Ranatensin	0.53	0.74	0.47	0.45	−59.6*	10	<8.3	5 (6.5)	33% CH_3CN
(30) Litorin	0.54	0.73	0.48	0.45	−32.3*	6	5.3	<5	33% CH_3CN
(31) Des-Gln^2-litorin	0.61	0.83	0.53	0.57	−28.6*	13	7.4	<5	33% CH_3CN
(32) Des-Gln^2[Leu^8]-litorin	0.61	0.85	0.51	0.57	−36.5*	2.5	6.6	<5	33% CH_3CN
(33) Neurotensin	0.43	0.44	0.25	0.14	−92.7	16	10.7	<2	30% CH_3CN
(34) Xenopsin	0.44	0.42	0.27	0.19	−82.3	22	7.6	<4	30% CH_3CN
(35) Substance P	0.31	0.24	0.18	0.07	−82.1	14	11.3	<2	30% CH_3CN
(36) Physalaemin	0.42	0.60	0.36	0.32	−76.4	30	<5.6	<2	34% CH_3CN
(37) Eledoisin	0.43	0.50	0.32	0.27	−66.5*	1.2	4.6	10 (9.1)	30% CH_3CN
(38) Bombesin-OH	0.31	0.68	0.42	0.19	−55.0	14	16.8	11 (20.7)	30% CH_3CN
(39) Bombesin-NHMe	0.37	0.72	0.37	0.22	−55.5	5	23.3	<2	30% CH_3CN

[a] BAW, 1-butanol–acetic acid–water (4:1:5, upper phase); BPyA, 1-butanol–pyridine–0.1 M acetic acid (5:3:11, upper phase); BINEt, 1-butanol–isopropyl alcohol–1 N NH_4OH–EtOAc (1:1:2.5:1 upper phase); 2-BA, 2-butanol–0.1 M acetic acid (1:1, upper phase); I_2, ninhydrin spray, and Pauly reagent were successively used. Loads varied from 20 to 40 μg per spot. Under those conditions very closely related impurities in the amount of up to 5% would not be detected. [b] Concentration in 1% AcOH = 1; * indicates concentration in 50% AcOH = 1. [c] Peptide yields are calculated on the basis of millimoles of peptides isolated after final purification relative to the total millimoles of starting *tert*-butyloxycarbonylamino acid, viz., as resin ester. Yields were not optimized. [d] HPLC were run using 30 to 34% acetonitrile in 0.01 N NH_4OAc buffer, pH 4.5, in an isocratic manner as indicated in column headed Remarks. Under the conditions reported here an unretarded material would elute with a retention time of 8 min. [e] In all cases where one amino acid of the original sequence of bombesin is replaced by its stereoisomer the impurities could be shown to be different from bombesin; their retention time is indicated in parentheses.

methylated benzhydrylamine resin as described by Rivier et al. (1977). The stepwise buildup of the peptide on the different resins was done on a Beckman Model 990 synthesizer using our standard program (Rivier et al., 1978). All couplings but those of Boc-Arg(tosyl) on one hand and Boc-D or L-Asn(xanthyl) and Boc-D- or L-Gln(xanthyl) on the other (Stewart et al., 1976) for which DMF and Me_2SO, respectively, were used, were carried out in CH_2Cl_2 + 5% DMF. The other protected amino acids [Z-Glp; Boc-Glu(γ-Bzl), Boc-D- or L-Leu·$1H_2O$; Boc-Gly; Boc-D- or L-Trp; Boc-D- or L-Ala; Boc-D- or L-Val; Boc-D- or L-His(tosyl); Boc-D- or L-Met; Boc-Lys (2-Cl-Z); Boc-Tyr (2,6-Cl_2-Bzl); Boc-D- or L-Phe; Boc-D- or L-Pro; Boc-Thr (OBzl); Boc-Ile·$\frac{1}{2}H_2O$; Boc-Asp (β-Bzl) and Boc-Ser (OBzl)] were also bought from BACHEM. Coupling times were variable depending on how easy the couplings were assessed to be (glycine being one of the easiest residues to couple) and also so that the synthesis of the peptides would be completed within 24 h. Coupling times, however, were never less than 30 min or more than 3 h. Benzoylation (benzoic acid and DCC in CH_2Cl_2) and acetylation (acetic anhydride: 50-fold excess in CH_2Cl_2) of the deblocked amino group of glycine at the fifth position were performed according to known procedures (Rivier et al., 1974).

After cleavage and deprotection by hydrofluoric acid (Sakakibara et al., 1976), the peptides were purified by ion-exchange chromatography on carboxymethylcellulose, partition chromatography on Sephadex G-25F (Yamashiro, 1964) or counter current distribution (Craig, 1943). R_f values on TLC in four different systems, optical rotation, yield, and percent contamination as measured by high pressure liquid chroma-

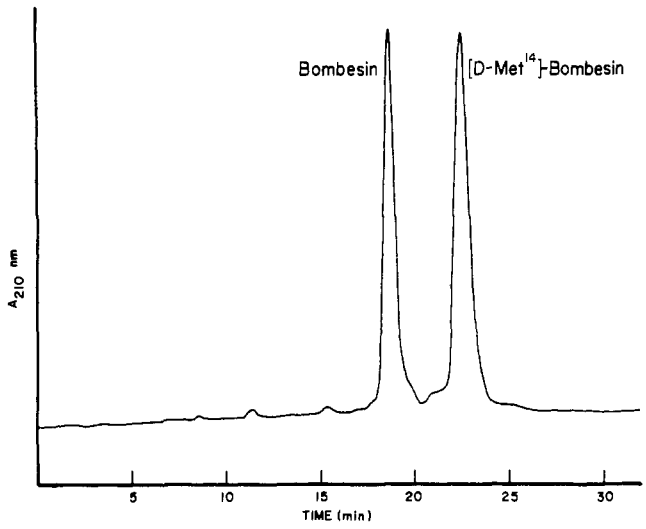

FIGURE 1: Analytical separation of bombesin from [D-Met^{14}]-bombesin by HPLC. Conditions: 0.7 × 120 cm C_{18} μ Bondapak column; 30 μg load of each peptide; isocratic runs, 30% CH_3CN/0.01 N NH_4OAc (pH 4.20); 6 mL/min; 4500 psi back pressure.

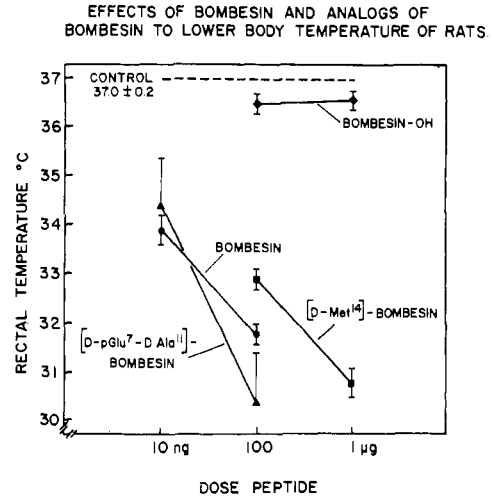

FIGURE 2: Dose response of bombesin and bombesin analogues to lower body temperature of cold exposed rats 60 min following intracisternal administration of peptide. Each point represents four animals.

tography (HPLC) are reported in Table II. A typical example of separations that can be achieved by HPLC is shown in Figure 1. Details on the equipment used as well as an evaluation of the method to determine the homogeneity of peptides have been reported previously (Burgus & Rivier, 1976; Rivier et al., 1977; see footnotes to Table II for pertinent experimental parameters).

Amino acid analyses were performed on peptide hydrolysates (6 N HCl containing 2.5% thioglycolic acid in evacuated sealed tubes at 110 °C for 20 h) using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. All analyses are consistent with the desired sequences and ratios for each amino acid are within 6% of unity.

Biological Test. The biological test used to evaluate the relative potencies of the different peptides reported in Table I gives an indication of the ability of the peptides to lower the body temperature of cold exposed rats (4 °C) (Brown et al., 1977a).

Male Sprague-Dawley rats weighing 200–300 g were used in the biological studies. Rats were fed Purina Rat Chow and tap water ad libitum and were housed in temperature and humidity controlled quarters with 14 h of light (0600–2000) and 10 h of dark. Substances were dissolved in artificial CSF (pH 7.2, 126 mM NaCl, 6 mM KCl, 1 mM NaH_2PO_4, 0.88 mM $MgSO_4$, 1.45 mM $CaCl_2$, 25 mM Hepes) and administered in a 10-μL volume via the cisterna magna into rats lightly anesthetized with ether. Animals were then placed in a cold room (4 °C) and rectal temperatures were recorded using a Yellowsprings Instrument Thermoprobe at 30-min time intervals following the injections. Experiments were carried out in randomized block design with four animals per treatment. Data were subjected to analysis of variance and differences between control and treatment groups were determined by the multiple range tests of Dunnett and Duncan. Potency values were calculated by four- and six-point bioassays with 95% confidence limits within ±10%. A typical graphical representation is shown in Figure 2.

Results and Discussion

Broccardo et al. (1975) have reported on the synthesis and biological properties (effects on smooth muscle preparations, blood pressure, gastric acid secretion, and intestinal myoelectric activity) of a large number of bombesin analogues. They found that the minimum length of the amino acid chain required for the first appearance of bombesin-like effects was represented by the C-terminal heptapeptide, and the minimum length for maximal effects by the C-terminal nonapeptide. The latter possessed approximately the same activity as bombesin and was considered a good substitute for biological studies. Furthermore, both the tryptophan-8 and histidine-12 residues seemed to be essential for bombesin-like activity. Subtle differences in relative length of action were observed when comparing bombesin or the C-terminal nonapeptide with the C-terminal octapeptide. This latter peptide is less potent and its action is more rapid in onset but less sustained. Whereas alytesin ([Gly^2,Thr^6]-bombesin) displayed the same biological actions of bombesin in the aforementioned systems (Anastasi et al., 1971), litorin closely mimics bombesin in its pharmacological effects, although there are again some notable differences (more potent on isolated smooth muscle preparations, for example, and less potent on gastrin release and acid secretion; Endean et al., 1975).

Our interest in bombesin and other vasoactive peptides which have all been suggested to be embryologically of neuroectodermal origin (Pearse, 1976), was triggered by the striking finding of the effect of bombesin on body temperature regulation (Brown et al., 1977a). Based on the earlier findings of Broccardo et al. (1975) and Endean et al. (1975) summarized earlier, we embarked on the design and synthesis of analogues that would satisfy us that the primary structural requirements for biological activity in our system were very close to those reported by Broccardo et al. (1975) in their biological systems, thus demonstrating a wide tissue distribution of closely related receptors, and that would give us an appreciation of the importance of the backbone conformation of the C-terminal nonapeptide for biological activity, as well as an appreciation of the importance of some selected side chains, while also giving us the opportunity to generate antibodies specific to certain partial sequence of bombesin. We also tested whether simple substitutions rendering the molecule more hydrophobic would make it cross the blood brain barrier. Certain analogues helped us develop specific antibodies used in radioimmunoassays and in passive immunization studies that will tell us about the possible physiological role of bombesin in mammals.

The low but significant biological activity of litorin (1%) in our system, inactivity of des-Gln^2-litorin, des-Gln^2[Leu^8]-litorin (corresponding to the C-terminal heptapeptide of bombesin), and des-Leu^{13},Met^{14}-bombesin (which has an altered C terminus), the higher potency of ranatensin (20%) (Pro^6 for Asn^6 and a common C-terminal octapeptide with bombesin), and the full potency of [acetyl-Gly^5]-bombesin (corresponding to the C-terminal decapeptide of bombesin) or [acetyl-D-Ala^5]-bombesin (having a common C-terminal nonapeptide with bombesin) are all consistent with the findings of Broccardo et al. (1975) who used different biological systems and analogues to delineate the primary structural requirements for biological activity as summarized earlier.

Introduction of a D-*Amino Acid in the Nonapeptide C-Terminal Sequence of Bombesin.* It is known that introduction of D-amino acids in a sequence can lead to longer acting ([D-Trp^6]-LRF (Vale et al., 1976)) and/or more potent analogues ([D-Arg^8]-vasopressin (Zaoral, 1966), [D-Trp^8]-somatostatin (Rivier et al., 1975)), perhaps either through resistance to enzymatic degradation and/or enhanced affinity. Also of interest was the observation that the introduction of D-cysteine at the 14th residue of somatostatin (Rivier et al., 1976b) would yield a compound with dissociated biological activities when comparing its potencies in vivo on the inhibition of the release of glucagon and insulin in the rat (Brown et al., 1977e). As shown in Table I, most bombesin analogues in which one amino acid was replaced by the corresponding D-amino acid were found to be inactive, <1% also indicating that contamination of those analogues by bombesin itself was nonsignificant. Two exceptions of great interest are the high potencies of [D-Gln^7]-bombesin (100%) (different from bombesin on HPLC) and [D-Ala^{11}]-bombesin (in lieu of Gly^{11}), while [L-Ala^{11}]-bombesin was inactive. This is reminiscent of the role of Gly^6 in LRF (Monahan et al., 1973). In contrast, whereas in LRF any D-amino acid at the 6th position whether natural or unnatural, protected or unprotected, gives a very potent and longer acting analogue of LRF with [D-Trp^6]-LRF being the most potent reported so far (Vale et al., 1976), results with other D- and corresponding L-amino acids at the 11th position in bombesin ([D- or L-Pro^{11}]-bombesin) and [D- or L-Phe^{11}]-bombesin) which were found inactive, would indicate that, if the D-alanine residue at the 11th position stabilizes a β turn (type II: Venkatachalam, 1968) as postulated and later substantiated by spectroscopic means (Donzel et al., 1977) for LRF (with the proviso that glycine occupy the third residue of the turn), the interaction in between bombesin and the receptor is so tight that it can, at the most, accommodate a substituent no larger than a methyl group. It is of interest that neither this later analogue nor [Ac-Gly^5,D-Ala^{11}]-bombesin nor [Ac-Gly^5,D-Gln^7,D-Ala^{11}]-bombesin were found to be long acting, although we have not thoroughly evaluated this possibility. The high potency of [Ac-Gly^5,D-Gln^7,D-Ala^{11}]-bombesin indicates that the two stereochemical changes introduced in the molecule are additive in their effects and compatible for high potency. The shorter analogue in this series with multiple changes to have full biological activity was found to be [D-Glp^7,D-Ala^{11}]-bombesin.

Whereas [D-His^{12}]-bombesin purified by conventional means was found to have 5% biological potency, after purification of an aliquot by HPLC, it was found to be completely inactive. We have shown in some earlier work (Rivier et al., 1976b) that contamination of [D-His^2]-TRF and [D-His^2]-LRF by TRF or LRF itself was very difficult to prevent. Only isolation of the final product using HPLC (Burgus & Rivier, 1976; Rivier et al., 1977) could definitely show that both of the above mentioned compounds were less than 0.1% as potent as TRF or LRF itself (Rivier, Vale, & Burgus, in preparation). On the other hand, the 5 and 10% biological potencies of [D-Ala^9]-BN and [D-Met^{14}]-BN cannot be attributed to the contamination of the materials by bombesin itself as shown by HPLC (see Figure 2). This would indicate a certain latitude in the receptor requirements at those two positions. It is noteworthy that the stereochemical changes at the 14th position seem less important to biological expression than a change in the functionality of the C terminus. Indeed, bombesin-free acid was found to be completely inactive while the noncharged bombesin *N*-methylamide analogue exhibited high activity (70%).

The synthesis of [Tyr^{12}]-bombesin for the raising of N-terminal directed antibodies (coupling to high molecular weight and antigenic proteins by the bisdiazotized benzidine method) and of des-His^{12}-bombesin which might have yielded an antagonist (refer to des-His^2-LRF: Vale et al., 1972), and the finding of the low biological activity of these peptides, confirms Broccardo's et al. (1975) statement that histidine was important for full biological activity. Tested at 1000 times the ED_{50} of bombesin in the presence of bombesin, des-His^{12}-bombesin was not found to inhibit the action of bombesin. As a matter of fact, at that dose, des-His^{12}-bombesin was found to be a weak agonist.

The synthesis of [Lys^3]- and [Lys^4]-bombesin for the raising of C-terminal directed antibodies (specific coupling to high molecular weight and antigenic proteins by the water soluble dicyclohexylcarbodiimide or glutaraldehyde procedure) and of [Tyr^4]-bombesin with the same aim in mind (bisdiazotized benzidine couplings) and the findings that they all had high potency in our biological tests, further indicate that the N-terminal pentapeptide of bombesin can be widely manipulated to yield molecules with full potency.

The details of the raising of antibodies to bombesin using the analogues described here and the development of a specific radioimmunoassay are not within the scope of this paper and will be described elsewhere (Brown & Rivier, in preparation).

[Phe^{13}]-Bombesin (corresponding to the substitution of phenylalanine for leucine at the 13th position) has a common C-terminal octapeptide with litorin and ranatensin. The fact that it exhibits 70% of bombesin activity emphasizes the earlier recognized more crucial role played by asparagine at the 6th position (replaced by pyroglutamic in litorin and proline in ranatensin) for full biological potency as compared with that of leucine, which can be replaced by phenylalanine at the 13th position with little effect.

Experiments whereby bombesin and a few selected analogues ([Ac-Gly^5,D-Ala^{11}]-bombesin, [D-Gln^7]-bombesin) were injected systemically to cold exposed rats showed that even the most hydrophobic ones (as measured by retention time on HPLC columns, Rivier et al., 1977) were inactive in both the temperature assay and in inhibiting cold induced TSH release. This would indicate that the peptides either could not reach (too labile in serum) or cross the blood brain barrier.

One can conclude that a good correlation in between different biological test systems has been demonstrated, that the new test introduced is highly quantitative, reproducible and specific, that molecules can be tailored for the raising of specific antibodies, and that the availability of a large number of analogues can allow for a better understanding of the receptor's requirements for biological expression.

Further studies evaluating the role of such peptides in areas as different as hibernation and the physiological control of cold induced TSH secretion are being investigated at the present time (Brown et al., 1977d).

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