Journal of Medicinal Chemistry, 1975, Vol. 18, No. 7 761

Bradykinin Analogs Containing β-Homoamino Acids

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Two new analogs of bradykinin, [8-β-homophenylalanine]- and [7-β-homoproline]bradykinin, have been synthesized by the solid-phase technique. In the anesthetized rat, [7-β-HPro]bradykinin was equipotent with bradykinin and 30–100 times more potent than [8-β-HPhe]bradykinin in vasodepressor activity. Both analogs were resistant to degradation in vitro by dipeptidylcarboxypeptidase from rabbit lung. Only the 7-β-HPro analog seemed to be resistant to this type of degradation in vivo, since its hypotensive effect in the anesthetized rat was not potentiated by SQ 20881 (BPP_{9a}), an inhibitor of dipeptidylcarboxypeptidase.

The biological activities of peptides are, in general, transient. Since the discovery of secretin and the elucidation of its mode of action, peptides have been considered paradigms of what physiological "messengers" should be; namely, carriers of the biological "mail" that disappear when the message has been delivered.

When peptide hormones became part of the medical armamentarium, a need arose to extend their biological lives. Several approaches have been taken to accomplish this, among others the use of peptide analogs that possess the desired biological activities and are not susceptible to enzymatic degradation, on the assumption that degradation by peptidase(s) is mainly responsible for the transient nature of the biological activities of peptides.[1]

In this paper, we describe a new example of this approach, based on the replacement with a β-aminoacyl bond of a peptide bond susceptible to enzymatic attack, maintaining the side chain of the amino acid replaced. To achieve this, we used the corresponding β-homoamino acid. We designate as β-homo-X the amino acid obtained by introduction of a CH_2 moiety between the α-carbon and the carboxyl of the amino acid X, and we use the abbreviation βHX for its residue.

β-Amino acid substitutions have been utilized in angiotensin,[2] gastrin,[3] and oxytocin[4] to explore structure–activity relationships. Analogs of ACTH[5] and angiotensin[6] with N-terminal β-amino acids have been synthesized to obtain derivatives that are resistant to degradation by aminopeptidases. It occurred to us that β-amino acids, with appropriate side chains, could also yield amide bonds resistant to enzymatic cleavage, if they replaced "normal" amino acids within biologically active peptides. In a previous paper[7] we described how the substitution of phenylalanine by β-homophenylalanine in a pentapeptide inhibitor of the angiotensin-converting enzyme yielded an active compound more resistant to enzymatic degradation. In the present studies, we selected the vasoactive nonapeptide bradykinin for β-homoamino acid replacements. Considerable work has been devoted to elucidating the mechanisms of inactivation of this kinin in vivo,[8] and the availability of analogs resistant to different types of enzymatic attack should certainly contribute to a better understanding of this process. The two new analogs described in this paper are [7-β-homo-L-proline]bradykinin (βHProB) and [8-β-homo-L-phenylalanine]bradykinin (βHPheB).

Arg-Pro-Pro-Gly-Phe-Ser-Pro-βHPhe-Arg
1 2 3 4 5 6 7 8 9

[8-β-HPhe]bradykinin (βHPheB)

Arg-Pro-Pro-Gly-Phe-Ser-βHPro-Phe-Arg
1 2 3 4 5 6 7 8 9

[7-β-HPro]bradykinin (βHProB)

The protected β-homoamino acid intermediates were obtained by homologation of the corresponding amino acid derivatives according to the Arndt–Eistert scheme. The preparation of the diazo ketones followed the technique described by Penke et al.[9] which we found to be very convenient when dealing with protected amino acids with such acid-sensitive protective groups as *tert*-butyloxycarbonyl. For the Wolff rearrangement, the procedure of Newman and Beal[10] was utilized. The reactions were followed by measuring the evolution of nitrogen and were usually finished in 2–3 hr.

The two nonapeptides were synthesized by the solid-phase technique, as described by Merrifield,[11] and the products, after removal of all protecting groups, were purified by ion-exchange chromatography on carboxymethylcellulose.

Results and Discussion

When βHPheB and βHProB were incubated with chymotrypsin, only the latter analog released arginine. However, cleavage at the Phe^5-Ser^6 bond was observed for both analogs. When each analog was incubated with angiotensin-converting enzyme (dipeptidylcarboxypeptidase) from rabbit lungs,[12] no liberation of either Phe-Arg or βHPhe-Arg was detected. Under the same conditions, bradykinin released the dipeptides Phe-Arg and Ser-Pro.

To determine the way in which the introduction of the β-homoamino acid residues into bradykinin had influenced activity in vivo, the effects of the two analogs on the blood pressure of the anesthetized rat were determined and compared with that of bradykinin.

In the rat βHProB was equipotent with bradykinin and approximately 30–100 times more potent than βHPheB in vasodepressor activity. βHProB produced a biphasic response, probably due to the release of catecholamines, since this biphasic pattern was eliminated after adrenergic blockade with phenoxybenzamine. The release of catecholamines was reported by Stewart et al.[13] with [des-9-Arg]bradykinin, an analog that has a penultimate proline residue and, hence, is not a substrate for dipeptidylcarboxypeptidase. The duration of the hypotensive action of βHProB was somewhat longer than that of bradykinin but was still quite transient, indicating that the analog had been destroyed by enzymes other than dipeptidylcarboxypeptidase.

The vasodepressor effect of βHPheB, 100 μg/kg, was potentiated by the dipeptidylcarboxypeptidase inhibitor SQ 20881[14] [<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; this nonapeptide is also designated as bradykinin potentiating peptide 9a (BPP_{9a}) by Greene et al.[15]] to about the same degree and with about the same duration as was that of bradykinin, 3 μg/kg, whereas the activity of βHProB at an equivasodepressor dose was neither prolonged nor enhanced by the test dose of SQ 20881 (Table I). These findings would indicate, on the one hand, that βHProB is not cleaved by rat dipeptidylcarboxypeptidase and, therefore, inhibition of this enzyme plays no role in prolonging the

Table I. Hypotensive Effects of Bradykinin and Analogs in the Anesthetized Rat, before and after the Administration of an Inhibitor of Dipeptidylcarboxypeptidase (SQ 20881)

Dose of test drug, μg/kg iv	Before SQ 20881			After SQ 20881, 0.125 mg/kg iv	
	N[d]	Max Δ BP, mmHg	Av duration, min	N[d]	Effect
Bradykinin					
1	3	15.7	0.5	0	Not determined
3	3	24.0	0.7	5	Potentiation[a]
10	2	36.0	1.5	0	Not determined
[8-β-HPhe]Bradykinin					
30	1	12.0	0.4	0	Not determined
100	3	20.7	0.8	4	Potentiation[b]
300	2	34.0	0.9	0	Not determined
[7-β-HPro]Bradykinin					
1	2	20.3[c]	2.1	0	Not determined
3	3	24.3[c]	1.8	1	No potentiation
10	2	32.5[c]	2.1	1	No potentiation

[a]Mean increase of 81.2% in maximal vasodepression with a mean duration of 68 min after the administration of SQ 20881, as compared with that preceding. [b]Mean increase of 98.6% in maximal vasodepression with a mean duration of 80 min after the administration of SQ 20881, as compared with that preceding. [c]Biphasic vasodepressor responses. [d]N is the number of animals; max Δ BP is the mean maximal decrease in mean arterial blood pressure.

hypotensive effect of this analog. On the other hand, βHPheB appears to be susceptible to degradation by this enzyme and its hypotensive activity can, therefore, be increased by such inhibition. However, data from the enzymatic digestions in vitro described above indicated that both analogs are equally resistant to the dipeptidylcarboxypeptidase from rabbit lungs. This discrepancy could, of course, be due to species difference (rabbit vs. rat), but it is tempting to speculate that it is due, rather, to the presence in vivo of at least two enzymatic systems, both capable of being inhibited by SQ20881, both unable to hydrolyze 7-βHProB, and only one able to hydrolyze 8-βHPheB. Stewart et al.[16] have reported that in rat lungs there is an enzymatic system that cleaves bradykinin at the bond Ser^6-Pro^7. It seems reasonable to assume that 7-βHProB would not be hydrolyzed by this enzyme, whereas 8-βHPheB, in which the hydrolyzable bond of bradykinin has not been changed, could be a substrate. The enzyme described by Stewart et al. and the dipeptidylcarboxypeptidase could constitute the two enzymatic systems hypothesized above. It should be pointed out that even though 7-βHProB is resistant to these enzymatic degradations, the duration of its hypotensive action is not as prolonged as that observed with bradykinin or 8-βHPheB after pretreatment with SQ 20881. This could be due to the prevalence of other degradative mechanisms either at the lungs or at the receptor compartment, but this point requires further investigation.

The results of the studies reported here demonstrate that replacement of an α-amino acid residue with the corresponding β-homoamino acid residue in biologically active peptides can yield analogs that retain significant activity and possess enhanced resistance to enzymatic degradation.

Experimental Section

Melting points were taken in capillary tubes and are uncorrected. The polystyrene resin used in the solid-phase synthesis was cross-linked with 2% divinylbenzene and had 2.0 mequiv/g of chlorine. *tert*-Butyloxycarbonyl-L-amino acids were obtained commercially, and their purity was checked by several analytical techniques. The homogeneity of the final products was confirmed by paper chromatography and electrophoresis. The peptide was spotted in 100–200-μg portions. Paper chromatography was performed in a descending fashion on Whatman 3MM paper: *n*-BuOH–pyridine–acetic acid–water (30:20:6:24). Low-voltage paper electrophoresis was carried out according to the technique described by Werum et al.;[17] mobilities are expressed as a function of the migration of the standard dyes for the five pH's: 3.3, 4.7, 7.2, 8.0, and 9.3. Acid hydrolyses were carried out at 110° for 16 hr in glass ampoules sealed under vacuum. Quantitative amino acid analyses were performed with a Jeolco amino acid analyzer. Optical rotations were determined with a Perkin-Elmer polarimeter, Model 141.

(2*S*)-*tert*-Butyloxycarbonyl-2-pyrrolidineacetic Acid (Boc-βHPro). *tert*-Butyloxycarbonyl-L-proline (10.8 g, 50 mmol) was dissolved in a mixture of ethyl ether (100 ml) and triethylamine (7.0 ml, 50 mmol). The solution was cooled and stirred in a Dry Ice–acetone bath at −5°, and ethyl chloroformate (4.9 ml, 50 mmol) was added. After 5 min, the triethylamine hydrochloride precipitate was filtered off, and, to the filtrate, an ethereal solution of diazomethane (prepared from 20 g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) was added. The reaction mixture was stirred for 16 hr at 5°, the solvent was removed in vacuo, and the residue was dissolved in ethyl acetate and then washed with saturated sodium hydrogen carbonate and water. The organic layer was dried over $MgSO_4$ and concentrated to dryness in vacuo. The crude diazomethyl ketone was purified by silica gel chromatography (R_f 0.21, silica gel–chloroform) yielding 8.3 g. The diazo ketone (8.3 g, 35 mmol) was dissolved in dry methanol (35 ml), and a few drops of a solution of silver benzoate (1 g) in triethylamine (9.1 g) were added with stirring and collection of the N_2 evolved. When the evolution of nitrogen had ceased (after 2–3 hr), the reaction mixture was treated with activated charcoal and concentrated to dryness in vacuo. The residue was dissolved in ethyl acetate and washed neutral. After drying, the solvent was removed in vacuo and the residue was chromatographed on silica gel: yield 5.0 g; R_f 0.45 (silica gel–chloroform).

The methyl ester (5.0 g, 20 mmol) was dissolved in a mixture of methanol (100 ml) and 1 *N* sodium hydroxide (30 ml) and stored at room temperature for 4 hr. The reaction mixture was neutralized and concentrated in vacuo to ca. one-third of the original volume, then diluted with ethyl acetate, and washed with aqueous sodium hydrogen carbonate. The aqueous phase was acidified and extracted with ethyl acetate. After the extract had been washed with water and dried over $MgSO_4$, the solvent was removed in vacuo and the residue was crystallized from hexane: yield 3.8 g (33% overall yield); mp 99–101°; $[\alpha]^{24}D$ −41.6° (*c* 1.9, DMF). Anal. C, H, N.

(3*S*)-3-*tert*-Butyloxycarbonylamino-4-phenylbutyric Acid (Boc-βHPhe). This compound was prepared by the procedure described above, starting from *tert*-butyloxycarbonyl-L-phenylalanine (13.2 g, 50 mmol): yield 5.5 g (40% overall yield); mp 98–100°; $[\alpha]^{24}D$ −13.3° (*c* 2, DMF). Anal. C, H, N. (This compound had been obtained as an oil by Chaturvedi et al.[2])

Arg-Pro-Pro-Gly-Phe-Ser-Pro-βHPhe-Arg. *tert*-Butyloxycarbonylnitro-L-arginylpolystyrene resin (5.0 g, 0.4 mequiv/g) was loaded into a mechanical shaker of the type described by Stewart

and Young[18] and submitted to the following cycle of operations: (1) methylene chloride wash (4 × 50 ml); (2) ethanol wash (4 × 5 ml); (3) acetic acid wash (4 × 50 ml); (4) 1 *N* HCl in AcOH wash (1 × 50 ml); (5) 1 *N* HCl in AcOH treatment (1 × 50 ml, 25 min); (6) acetic acid wash (4 × 50 ml); (7) ethanol wash (4 × 50 ml); (8) methylene chloride wash (4 × 50 ml); (9) triethylamine wash (6.5% in methylene chloride, 2 × 50 ml, 5 min); (10) methylene chloride wash (4 × 50 ml). (11) A solution of (3*S*)-3-*tert*-butyloxycarbonylamino-4-phenylbutyric acid (2.1 g, 7.5 mmol) in methylene chloride (35 ml) was added followed, after 25 min, by the addition of a solution of dicyclohexylcarbodiimide (1.55 g, 7.5 mmol) in methylene chloride (2.5 ml). After 3 hr of shaking, the reaction vessel was drained and the complete cycle was repeated for the introduction of each successive *tert*-butyloxycarbonylamino acid. For Boc-Arg(NO_2), a mixture of DMF–CH_2Cl_2 (1:1) was used in the coupling step. After the last amino acid had reacted, the resin was transferred to a sintered glass funnel, in which it was washed sequentially with methylene chloride, ethanol, acetic acid, and ethanol and then dried in vacuo. The peptide-resin (5.9 g) was suspended in trifluoroacetic acid, and hydrogen bromide was bubbled through for 1 hr. The resin was filtered and washed with a 1:1 mixture of trifluoroacetic acid and methylene chloride. The filtrate was evaporated to dryness in vacuo and the residue was triturated with ether, yielding 1.8 g. This crude bis(nitro-*O*-benzyl)nonapeptide was dissolved in a mixture of methanol–water–1 *N* HCl (940:70:36) and the solution was hydrogenated in the presence of 360 mg of 10% Pd/C until the disappearance of the nitroguanidine absorption at 270 nm. The catalyst was filtered off, the filtrate was concentrated to dryness in vacuo, and the residue (1.6 g) was chromatographed on a column of carboxymethylcellulose (CM52-100 g) in a gradient of ammonium acetate (0.005–0.5 *M*). The fraction containing the desired nonapeptide was lyophilized repeatedly to eliminate the ammonium acetate: yield 623 mg; $[\alpha]^{25}D$ −77.6° (*c* 1.03, 1 *N* AcOH); paper chromatography R_f 0.35 (ninhydrin, Sakaguchi, Rydon[19]); paper electrophoresis Am +45 (3.2), +35 (4.7), +31 (7.2), +31 (8.0), +20 (9.3) (ninhydrin, Sakaguchi). Quantitative amino acid analysis: Arg, 1.90; Ser, 0.80; Pro, 3.18; Gly, 0.95; Phe, 0.99. The presence of 3-*tert*-butyloxycarbonylamino-4-phenylbutyric acid in the acid hydrolysate was determined by paper chromatography in the solvent system described above.

Arg-Pro-Pro-Gly-Phe-Ser-βHPro-Phe-Arg. This nonapeptide was synthesized by the procedure described in the previous paragraph. The material obtained after CM-cellulose chromatography (903 mg) was chromatographed on G-25 Sephadex (with 0.2 *M* acetic acid as solvent): yield 685 mg; $[\alpha]^{25}D$ −71.7° (*c* 1.0, 1 *N* AcOH); paper chromatography R_f 0.35; paper electrophoresis Am +48 (3.2), +39 (4.1), +32 (7.2), +32 (8.0), +25 (9.3) (ninhydrin, Sakaguchi). Quantitative amino acid analysis: Arg, 2.0; Ser, 0.84; Pro, 1.90; Gly, 1.05; Phe, 2.10. The presence of 1-*tert*-butyloxycarbonyl-2-pyrrolidineacetic acid in the acid hydrolysate was determined by paper chromatography.

Enzymatic Degradations. Chymotrypsin. Bradykinin (1 mg) or 1 mg of the analog was dissolved in ammonium acetate buffer pH 7.5; 0.3 ml of chymotrypsin (Worthington, 2 mg/ml in ammonium acetate buffer) was added and the mixture was incubated at 37° for 16 hr. Acetic acid (0.2 ml) was added and the mixture was freeze-dried. The hydrolysate was examined by high-voltage paper electrophoresis (pH 1.9, 80 V/cm, 15 min). **Dipeptidylcarboxypeptidase.** The peptide (1 mg) was dissolved in 0.1 *M* ammonium acetate (3.8 ml), and 0.2 ml of dipeptidylcarboxypeptidase[12] was added. The mixture was incubated for 6 hr at 37°, then was diluted with acetic acid (1 ml), and freeze-dried. The residue was examined by high-voltage paper electrophoresis, as described above.

Biological Studies. The effects on blood pressure were studied in urethane-anesthetized, atropinized rats. All compounds were injected stat iv in saline solutions. In each experiment, injections of bradykinin were interspersed with injections of the peptide being investigated. Blood pressure from the right femoral artery was monitored continuously. In some animals adrenergic blockade was produced with phenoxybenzamine, 1 mg/kg iv. To determine potentiation by SQ 20881, a standard dose of this compound, 0.125 mg/kg, iv, was injected as a bolus, before and after which test doses of bradykinin or of one of the analogs were given at definite intervals.

Acknowledgment. The authors are very grateful to Mr. F. Russo-Alesi for the amino acid analyses, to Mr. O. Kocy for the electrophoretic data, and to Mr. T. R. Schaeffer for the vasodepressor assays. The enzyme dipeptidylcarboxypeptidase was kindly supplied by Dr. D. W. Cushman; it has been obtained by the procedure described in ref 12. The suggestions and comments of Dr. B. Rubin and the able technical assistance of Ms. Nina Williams are also gratefully acknowledged.

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