destroy the reagent. The aqueous reaction mixture was applied to a Amberlite IR-120 (pyridinium form) column (2.5 × 45 cm). The column was eluted with 0.1 *M* pyridine formate (pH 4.8) to give recovered material and subsequently with 0.4 *M* pyridine formate (pH 4.8) to afford the product. The fraction eluted by 0.4 *M* pyridine formate was evaporated to dryness after the pH of the solution was adjusted to 4.0 with formic acid. Repeated evaporation of the residue with ethanol gave a gum, which was dissolved in 5 ml of water. The solution was then passed through a column (2 × 4 cm) of Amberlite IR-400 (chloride form). The column was washed with 150 ml of water and the combined effluent and washing were evaporated to dryness under reduced pressure. Recrystallization of the residue from 90% ethanol afforded 50% of III hydrochloride: mp 241–243° dec: $[\alpha]^{25}D$ −167.7° (*c* 5.0, water); uv_{max} (water, pH 2.0) 268 nm (ε 10,800), 232 (9800): nmr (D_2O) δ 5.90 (1 H, br d, *J* = 8.5 Hz), 6.70 (1 H, d, *J* = 8.5 Hz), 6.85, 8.37 (AX type, *J* = 7.0 Hz). *Anal.* ($C_{19}ClH_{12}N_3O_3S$) C, H, N, S.

4′-Thio-1-β-D-arabinofuranosylcytosine (IV). III hydrochloride (100 mg) was dissolved in 3 ml of water and the solution was adjusted to pH 9 with aqueous ammonia. After 10 min at 25° the mixture was acidified with hydrochloric acid and applied to a column (1 × 2 cm) of Amberlite IR-120 (acid form). The column which was washed well with water was eluted with 100 ml of 1 *N* NH_4OH. The eluent was evaporated under reduced pressure and the residue crystallized from ethanol to produce 85 mg (90%) of pure IV: mp 221–222° dec; $[\alpha]^{25}D$ +72.8° (*c* 2.0, water); uv_{max} (water, pH 2.0) 280 nm (ε 12,100); nmr (D_2O) δ 2.48 (1 H, double t, *J* = 6.0, 8.0 Hz), 4.10 (2 H, d, *J* = 5.0 Hz), 4.15 (1 H, t, *J* = 8.0 Hz), 4.58 (1 H, double d, *J* = 6.0, 8.0 Hz), 6.41 (1 H, d, *J* = 6.0 Hz), 6.50, 8.78 (AX type, *J* = 8.0 Hz). *Anal.* ($C_9H_{13}N_3O_4S$) C, H, N, S.

Biological Assays. The *in vitro* antitumor assays were conducted according to the protocol cited in the Cancer Chemotherapy Report.[11] KB cells grown in monolayer cultivers were obtained from Flow Laboratories, Rockville, Md. The cells were grown in petri dishes (3 cm in diameter) with the minimum essential medium,[13,14] supplemented with 10% fetal calf serum and glutamine (2 m*M*). All tests were made during log phase. Aliquots (0.2 ml) of medium containing the various concentration of drugs were added to 2-ml portions of medium containing approximately 5 × 10^4 cells. 6-Mercaptopurine was used as a positive control. Incubation was conducted for 72 hr, after which time the cells were washed by Earl's balanced solution, trypsinized with EDTA, and counted in a Coulter counter. The base line of cell count was made after 24 hr from inoculation of cells. During a further 48 hr the cell number in the controls increased five- to sevenfold. The effective concentration of 6-mercaptopurine for 50% reduction of growth was shown to be 0.35 g/ml of medium.

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Hydroxyproline Analogs of Bradykinin†

John Morrow Stewart,*

Department of Biochemistry, University of Colorado School of Medicine, Denver, Colorado 80220

James W. Ryan, and A. H. Brady

Department of Medicine, University of Miami School of Medicine, Miami, Florida 33152. *Received November 19, 1973*

Alterations in the proline residues of the peptide hormone bradykinin (BK) (Figure 1) produce profound effects on the biological activities of the resulting analogs.

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

Figure 1. The structure of bradykinin.

This was first observed by Schröder[2] who found that replacement of proline 3 with alanine caused no change in the biological activity while [Ala²]-BK and [Ala⁷]-BK had about 1% of the potency of BK. Later, Stewart[3] found that the effect on biological activity of D-proline in positions 2 or 7 paralleled that of alanine in those positions, while [D-Pro³]-BK had only 1/10,000 the potency of bradykinin. Circular dichroism (CD) spectra showed[4] that the solution structures of BK and its highly potent analogs were quite similar, while several analogs with very low biological activities had solution conformations very different from that of BK. However, no unique correlation between conformation and biological activity could be made, since several inactive analogs had CD spectra very similar to that of BK. Among the proline analogs, [D-Pro⁷]-BK had a spectrum very similar to that of BK, differing only in the intensity of that 220-nm peak, while that of [D-Pro²]-BK was very different, being inverted in this region.

From these data it would appear that with respect to position 7 of BK the ring of proline plays a significant role in receptor interaction, since the loss in biological activity is similar upon replacement of proline with either alanine or D-proline, without a large alteration in conformation. In position 3, the ring clearly does not have a function in receptor interaction, since the alanine analog is fully potent. The case of the 2 position is less clear, since replacement by D-proline (which causes a large conformational change) or by alanine (which lacks the ring) causes a similar diminution of biological activity.

One might hope to shed additional light upon this question by examination of the biological activities and CD spectra of BK analogs containing substituted prolines. For this purpose 4-hydroxyproline is a logical candidate, since the size, hydrophilic character, and hydrogen bonding ability of the hydroxyl group might be expected to influence the biological activity and conformation of the peptides. These hydroxyproline analogs of BK are also of synthetic interest. Although hydroxyamino acids can often be used in classical peptide synthesis (where equimolar

† A preliminary report of this work has appeared; see ref 1.

Table I. Hydroxyproline Analogs of Bradykinin (BK)

Peptide	Partition coeff[a]	Amino acid analysis					
		Arg	Ser	Pro	Gly	Phe	Hyp
[Hyp²]-BK	0.70	2.08	1.03	1.88	1.00	1.96	0.96
[Hyp³]-BK	0.62	1.94	1.05	1.87	1.00	2.10	0.97
[Hyp⁷]-BK	0.52	1.91	0.98	1.90	1.00	2.06	0.92

[a] In the system 1-BuOH–1% TFA used for purification by CCD.

amounts of reactants are usually used) without protection of the hydroxyl group, work with the solid-phase method (where a large excess of the acylating moiety must be used) has shown that the hydroxyl group of threonine must be protected to avoid formation of ester branches on the hydroxyl group.[5] Serine and tyrosine have always been protected for solid-phase synthesis as ethers. Since a suitable ether of hydroxyproline was not readily available, it was of interest to see whether this amino acid could be satisfactorily incorporated into peptides by the solid-phase method without protection of the hydroxyl group.

Results and Discussion

Synthesis. Chemical data on BK and the three hydroxyproline analogs are given in Table I. The syntheses proceeded in a routine fashion and the expected products were obtained in good yield. The appearance of two spots on thin-layer chromatograms of the pure products does not indicate inhomogeneity of the material but is due to incomplete dissociation of the trifluoroacetate salts in the acetic acid chromatography system; if the salt is exchanged for acetate, only the lower R_f spot is seen. Similar behavior is seen with trifluoroacetate salts of other basic peptides.

Very careful examination of all the by-products from these three syntheses failed to show any evidence of the formation of branched chain peptides similar to those found earlier[6] when threonine was used in solid-phase synthesis without hydroxyl protection. This is surprising, since one would have thought *a priori* that the hydroxyl group of hydroxyproline, situated on a ring, would be less hindered sterically than that of threonine and consequently more susceptible to acylation. This result was confirmed recently by Felix, *et al.*,[7] who also synthesized [Hyp³]-BK by the solid-phase method.

CD Spectra. The CD spectra of the three hydroxyproline analogs of BK are shown in Figure 2. The spectrum of [Hyp³]-BK is identical with that of BK, while the other spectra differ only in the 222-nm band. Previously[8] the negative 234-nm band was assigned to a 3 → 1 hydrogen bond bridging the 7-proline residue of BK. Evidently substitution of hydroxyproline for proline does not significantly perturb this hydrogen bonded structure in any of the analogs. The positive 222-nm band is a composite of

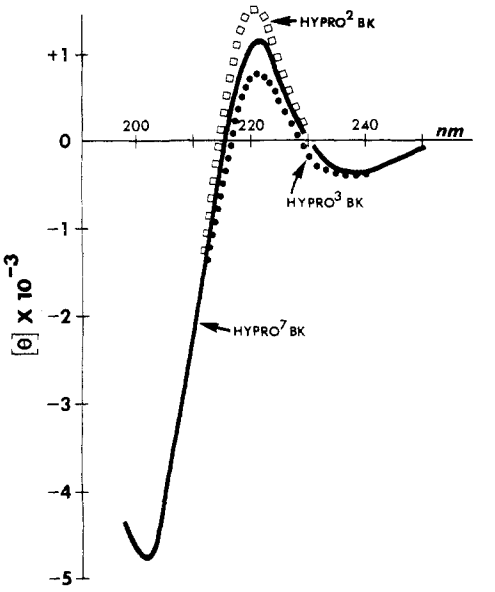

Figure 2. CD spectra of the hydroxyproline analogs of bradykinin. Ellipticities were calculated on the basis of a mean residue weight of 115. The spectrum of BK was identical with that of [Hyp³]-BK.

bands due to two chromophores, the characteristic aromatic band of the two phenylalanine residues overlying a band associated with the N-terminal Arg-Pro-Pro sequence. It is not surprising that the CD spectrum of [Hyp²]-BK is altered in this region, having greater intensity and a 2-nm blue shift. It is much more difficult to understand why the CD spectrum of [Hyp⁷]-BK is altered only in this same region and not in the 234-nm band; apparently the added hydroxyl group interacts configurationally or electronically to perturb only the N-terminal region. In longer wavelength regions, the spectra of BK and the three analogs are identical.

Biological Activity. The biological activities of the peptides are shown in Table II. The elevated potency of [Hyp²]-BK in the rat uterus and guinea pig vascular permeability assays is remarkable. Although this analog did appear to be slightly more resistant than BK to the action of pulmonary kininases, such resistance to enzymatic degradation is not likely to be responsible for the enhanced potency in rat uterus, where kininase activity cannot be readily demonstrated. More effective interaction of the peptide with BK receptors in uterus, due to H bonding, would seem to be a more plausible explanation. Previously, potency significantly higher than BK in this assay has been reported[9] only for analogs in which the phenylalanine residues were replaced by thienylalanine, although certain kallidin analogs, where the peptide chain is lengthened, were reported to be marginally more potent than BK.[10,11]

Table II. Biological Activities of the Peptides

Peptide	Rat uterus[a]	Guinea pig ileum[a]	Vascular permeability[b]	Rat blood pressure[c]	Pulmonary inactivation,[d] %
Bradykinin (BK)	3	50	5	80	97–99
[Hyp²]-BK	1.5	50	0.3	80–100	85–95
[Hyp³]-BK	3	150	7	90–100	92–96
[Hyp⁷]-BK	150	1500	25	1600–8000	96–98

[a] The concentration (ng/ml) required to produce a half maximal contraction. [b] The dose (ng) required by intradermal injection to produce a blue spot with a corrected diameter of 6 mm in guinea pigs previously injected intravenously with Evans Blue dye. [c] The amount (ng) required by intraaortic injection into a 200-g rat to produce a depressor response of 25 mm. [d] Calculated from the difference in the amounts needed by intravenous and intraaortic injection to produce the same depressor response in the rat.

The progressive loss of potency in all assay systems as the hydroxyproline residue is moved toward the carboxyl end of the chain is impressive. This effect is consistent with the suggestion[12] that the initial combination of BK with its receptors is at the carboxyl end of the peptide chain, followed by the "zippering up" of the remainder of the peptide with the receptor. Any molecular modification which interferes with this initial peptide-receptor interaction should be extraordinarily effective in lowering the biological activity of hormone analogs. The biological activities of the 7-alanine, 7-D-proline, and 7-hydroxyproline analogs are quite similar. The very low activity of [Hyp^7]-BK may mean either that there is no space on the receptor to accommodate the increased bulk of the hydroxyl group or that the hydroxyl group interacts strongly with areas of the tissue adjacent to the receptor to prevent normal peptide-receptor combination. In any case, the change in biological activity upon replacement or proline by hydroxyproline is apparently not due to gross alteration in the conformation of the peptide. Among these analogs, biological activity is not correlated with the height of the 220-nm CD maximum.

Experimental Section

Synthesis of peptides was by the solid-phase method[5] using an automatic instrument similar to the one previously described.[13] Polystyrene–2% divinylbenzene (200–400 mesh beads) was substituted with Boc-Arg(NO_2) to the extent of 0.31 mmol/g. *tert*-Butyloxycarbonylamino acids were used throughout, including Boc-Ser(Bzl), and were purchased from Schwarz BioResearch, except *tert*-butyloxycarbonyl-4-hydroxyproline, which was purchased from Fox Chemical Co. Boc groups were removed during synthesis by 30-min treatment with 4 *M* HCl in dioxane containing 1 mg/ml of 2-mercaptoethanol, following a prewash of the same reagent. Peptide hydrochlorides were neutralized by 10-min treatment with 10% Et_3N in $CHCl_3$ (v/v), following a prewash. DCC mediated coupling reactions were done in $CHCl_3$, using 2.5 equiv of *tert*-butyloxycarbonylamino acids and DCC per equivalent of peptide-resin. Boc-Arg(NO_2) was coupled in 50% DMF–$CHCl_3$ because of its solubility. Peptides were cleaved from the resin by 30-min treatment at 0° with anhydrous HF containing 10% $PhOCH_3$.

Peptides were purified by countercurrent distribution for 100 transfers in 1-BuOH–trifluoroacetic acid (1%); observed partition coefficients are given in Table I. Purified peptides were homogeneous by paper electrophoresis[5] at pH 5 (E_{Lys} = 0.64) and pH 2.8 (E_{Lys} = 0.70) and had the same mobility as BK. In tlc on cellulose in 1-BuOH–AcOH–H_2O (4:1:5) the pure peptide trifluoroacetates showed two spots, R_f 0.44 and 0.69; standard BK trifluoroacetate had R_f 0.49 and 0.72. The slower spot is peptide acetate, while the faster spot is undissociated peptide trifluoroacetate. Amino acid ratios were determined on hydrolysates (6 *N* HCl, 110°, 22 hr) with a Beckman 120 amino acid analyzer and are given in Table I.

CD spectra were determined on a Cary 60 spectropolarimeter with a CD attachment. Peptides were dissolved in 0.03 *M* K_2HPO_4 adjusted to pH 7.2 with HCl. The instrument was standardized with an aqueous solution of *d*-10-camphorsulfonic acid. Spectra were checked for conformity to Beer's law.

Bioassays on isolated rat uterus,[14] isolated guinea pig ileum,[14] and rat blood pressure[15] were done as previously described.

For determination of the effect of the peptides on vascular permeability, albino guinea pigs weighing 300–400 g were etherized lightly and injected intracardially with Evans Blue dye (10 mg/kg as 0.25% solution in 0.9% NaCl). The back of the animal was clipped closely, but not shaved or depilated. Graded doses of the peptides were injected intradermally into the back area in 0.05 ml of phosphate buffered saline, pH 7.25 (8.5 g of NaCl, 0.40 g of Na_2HPO_4, and 0.10 g of $NaH_2PO_4 \cdot H_2O$ per liter). New disposable No. 27 needles were used only once to minimize skin trauma. Saline blanks and BK controls were included in every animal. Twenty minutes after injection of the peptides, the major and minor diameter of each spot was measured with calipers, moistening the skin if necessary for better visualization. The major and minor diameters of each spot were multiplied together to give the "area product," and from this value was subtracted the area product of the blank, which was usually 2–3 mm^2. The square root of the remainder was the "corrected diameter," and a plot of this value against the logarithm of the dose gave the dose–response curve, which was linear over a log dose range of at least 3. Results from animals not showing linear dose–response curves were not used. The slopes of the curves from the Hyp-BK analogs were parallel to those for BK. To test analogs for inhibition, a mixture of the analog and BK was injected, due to the difficulty of making successive intradermal injections into exactly the same spot. Analogs were tested for inhibition at concentrations below the threshold of BK-like response, covering a log dose range of at least 4.

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Analogs of the Abortifacient Aminoglutethimide

Rolf Paul,* Richard P. Williams, and Elliott Cohen

Metabolic Disease Therapy Research Section, Lederle Laboratories, Division of American Cyanamid Company, Pearl River, New York 10965. *Received December 3, 1973*

Aminoglutethimide (1) has recently[1,2] been reported to induce abortions in pregnant rats by interfering with their cholesterol to Δ^5-pregnenolone conversion. The compound is nonsteroidal and nonestrogenic; thus it represents a new type of antifertility agent. Aminoglutethimide was found to clear from all tissues quickly with a half-life of 70 min. At suboptimal doses no gross abnormalities were noted in the surviving rat fetuses. **An** an abortifacient, aminoglutethimide might be used in humans only after a missed menses. This would mean it would be given less frequently than current steroidal combination drugs. The spread between efficacy (100 mg/kg) and toxicity (200 mg/kg), however, was not very great.[2] These reports prompted us to undertake an investigation to see if a more effective and less toxic analog could be made. A search of the literature revealed that most of the analogs reported were of