Alkylating Analogs of Peptide Hormones. 2. Synthesis and Properties of *p*-[*N*,*N*-Bis(2-chloroethyl)amino]phenylbutyryl Derivatives of Angiotensin II†

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The solid phase synthesis of *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylbutyryl-[Ile^5]angiotensin II (chlorambucil-angiotensin II) and of chlorambucil derivatives of 5 C-terminal fragments of angiotensin II are described. The compounds were assayed on rat blood pressure, isolated guinea pig ileum, and isolated rat uterus. Chlorambucil-angiotensin II and chlorambucil-[des-Asp^1,Ile^5]angiotensin II caused noncompetitive inhibition of the action of angiotensin on guinea pig ileum that was not reversed after 8 hr, while the response to histamine and bradykinin was slightly enhanced. It is concluded that the two chlorambucil peptides bind strongly to angiotensin receptors in the ileum, possibly by alkylating an anionic site. These compounds also showed pressor activity in the rat, but no irreversibility or inhibition was observed in this case. Effects of the peptides on rat uterus were variable.

The synthesis and study of numerous analogs of angiotensin II, recently reviewed by Schröder and Lübke,[1] have yielded much information about the dependence of biological activity on the covalent structure of this hormone. These studies, however, have afforded very little knowledge about the nature of the cellular receptor for angiotensin. One of the reasons for this is that only two synthetic analogs have been found to act as inhibitors of the biological activities of angiotensin. These antagonists are [Ile^5, Ala^8]angiotensin II[2] and [Phe^4, Ile^5, Tyr^8]angiotensin II,[3] recently reported to be competitive inhibitors of angiotensin II.

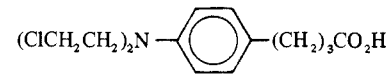
The characterization of angiotensin receptors would be greatly helped by the possibility of labeling them with strongly binding analogs. One step in this direction was reported by Lin and Goodfriend,[4] who found that radioactive monoiodoangiotensin binds to several tissues in what they interpreted as being a specific, reversible interaction with angiotensin receptors.

Perhaps greater promise of success lies in the incorporation into the angiotensin molecule of chemically reactive groups able to form covalent bonds with nucleophilic sites on the receptor. The first paper in this series[5] describes the application of this principle to the biologically active peptide bradykinin. Fragments of bradykinin and related peptides containing the nitrogen mustard chlorambucil were synthesized and found to produce irreversible effects on some enzymes which metabolize bradykinin, but irreversible blockage of bradykinin receptors was not obtained. In this paper we describe the application of this approach to angiotensin.

Results

Synthesis. Six peptides were synthesized containing C-terminal fragments of [Ile^5]angiotensin II with an alkylating moiety attached to their N-terminal ends. The alkylating moiety was provided by coupling the α-amino group of the peptide fragment with *p*-[*N*,*N*-bis(2-chloroethyl)amino]-phenylbutyric acid (chlorambucil, 1).

The chlorambucil-peptides that were prepared are listed in Table I. The chlorambucil derivatives of these angiotensin



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fragments were sol in $CHCl_3$, as had been observed with the bradykinin fragments.[5] They were homogeneous by tlc and showed the expected amino acid compositions, molar extinction coefficient, and rate of hydrolysis at physiological pH and temp.[5]

Isolated Guinea Pig Ileum. The administration of **6** and **7** to this preparation produced angiotensin-like responses that were about one-third as much as those produced by the corresponding free peptides (Table II). Qualitatively, there was no difference in the type of response: a contraction attaining a maximum after 2-3 min, followed by a return to normal tonus after 10-15 min. When high concentrations of angiotensin II or des-Asp^1-angiotensin II were left in contact with the ileum for 15-60 min and then washed off, there followed a period of low reactivity of the preparation toward angiotensin II. This tachyphylactic period, however, was transitory and the tissue returned to normal after a time that was proportional to the concentration of peptide that caused the refractory period.

The administration of high concentrations of **6** and **7**, washed off after 15-60 min of contact with the ileum, also produced an inhibition of the response to angiotensin. This inhibition, however, persisted after a time equivalent to complete disappearance of the tachyphylaxis produced by the corresponding free peptides in control experiments. This inhibition was observed up to 8 hr after administration of the chlorambucil-peptides and in none of about 20 experiments was there any significant recovery of the normal response to angiotensin by the tissue.

Figure 1 shows the inhibition of the ileum response to angiotensin II observed after contact with **6** for 15 min. Longer periods of contact, up to 60 min, did not cause a higher degree of inhibition. The inhibition was specific for angiotensin II; the response to histamine and bradykinin, after treatment with **6**, was usually slightly increased although not significantly.

The effect of **7** was qualitatively identical with that depicted in Figure 1 but it was less active an inhibitor than **6**. A 50% reduction of the maximum response to angiotensin

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Table I. Chlorambucil-Peptides Related to Angiotensin II

Compd	Structure[a]	R_f[b] tlc	Amino acid composition,[c] moles/mole of peptide[d]							
			Phe	Pro	His	Ile	Tyr	Val	Arg	Asp
2	Chl-His-Pro-Phe	0.68	*1.00*	1.08	1.02					
3	Chl-Ile-His-Pro-Phe	0.72	1.09	1.08	*1.00*	1.00				
4	Chl-Tyr-Ile-His-Pro-Phe	0.84	1.16	1.22	*1.00*	0.88	0.73			
5	Chl-Val-Tyr-Ile-His-Pro-Phe	0.96	1.06	1.13	0.97	0.86	0.83	*1.00*		
6	Chl-Arg-Val-Tyr-Ile-His-Pro-Phe	0.71	1.06	1.14	0.90	0.84	0.98	*1.00*	1.00	
7	Chl-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0.68	1.03	1.12	0.88	0.83	0.84	*1.00*	1.06	0.93

[a] Amino acids are abbreviated as recommended by IUPAC Commission on nomenclature; *J. Biol. Chem.*, 241, 2491 (1966). Chl = *p*-[*N*,*N*-bis-(2 chloroethyl)amino]phenylbutyryl. [b] Peptides were chromatogd on silica gel with fluorescent indicator (Brinkman) in $CHCl_3$-MeOH-AcOH-H_2O (65:30:4:1), and detected by uv absorption and reaction with Pauly and ninhydrin sprays.[9] [c] Peptide hydrolysates were made in 6 *N* HCl under N_2 for 72 hr at 110°. Each hydrolysate also contd 1 mg/ml each of 2-mercaptoethanol and phenol. Analysis was carried out on the Beckman 120C amino acid analyzer. [d] The residues used to calc the molar ratios are given in italics.

Table II. Biological Activity of Chlorambucil-Peptides

Compd	Angiotensin activity,[a] %		
	Rat pressor	Guinea pig ileum	Rat uterus
2	0	0	0
3	0	0	0
4	0	0	0
5	0.3	0.07	0.7
6	0.4	3	10
7	12	6	20

[a] Activity on the rat blood pressure, isolated guinea pig ileum, and isolated rat uterus compared on a molar basis, to that of [Ile^5]-angiotensin II, taken as 100%. Zero activity signifies that no response was detected in assays that would be sensitive to 0.01% pressor activity or 0.001% myotropic activity.

II was obtained by contact with 1.5×10^{-5} *M* **6** or 5×10^{-5} *M* **7**. The shorter peptides were inactive as antagonists.

Isolated Rat Uterus. Compds **5**, **6**, and **7** had agonistic activities upon this preparation that were about 20% as great as the corresponding free heptapeptide and angiotensin II, respectively. When left in contact with the uterus these compounds induced the rhythmic contractions that are characteristic of the action of angiotensin II upon that preparation.[6] In most uterus preparations, the tissue returned to its normal quiescent state after washing out of the peptide. In certain tissues, however, **6** and **7** produced permanent rhythmic contractions that could not be abolished by repeated washing, even over a period of several hours. Reasons for this variable response are not understood, and are under investigation.

Rat Blood Pressure. Compds **5**, **6**, and **7** had a low order of angiotensin-like pressor activity in the rat. The iv infusion of a total of 2 mg of any of these peptides, during 20 min, produced a moderate increase in the blood pressure that returned to normal a few minutes after the infusion ended. However, no permanent alteration of the response to angiotensin II or bradykinin was detected after the infusion.

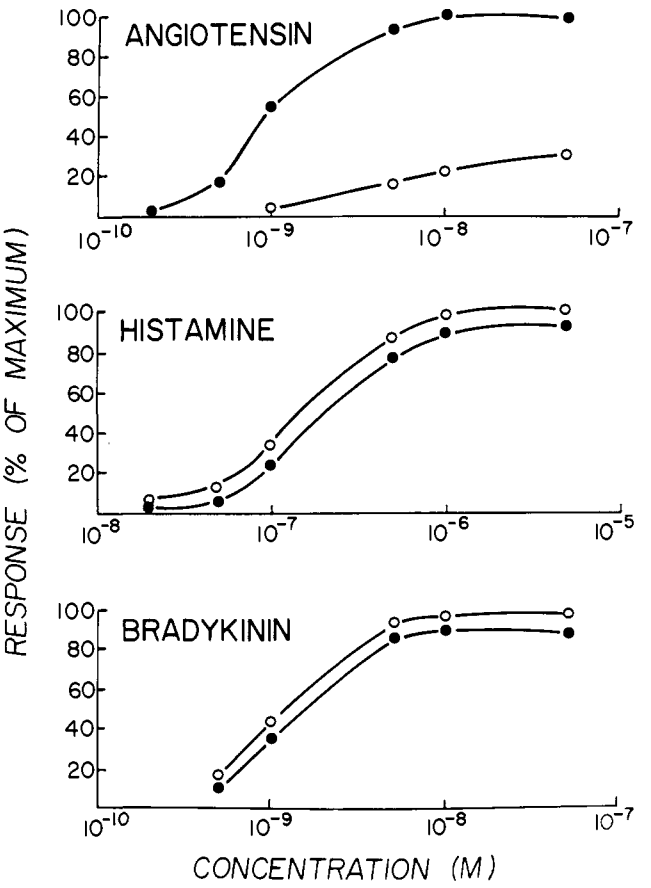

Figure 1. Dose-response curves for angiotensin II (upper), histamine (middle), and bradykinin (lower) on guinea pig ileum before (●–●) and after (○–○) treatment for 15 min with 10^{-5} *M* **6**.

Discussion

We interpret our results as showing that **6** and **7** are very strongly bound to the angiotensin receptors in the guinea pig ileum. This binding to the receptor was evidenced by a specific inhibition towards angiotensin II that was of the irreversible noncompetitive type (Figure 1). Since the response to a dose of angiotensin that normally produced a half-maximal response could be completely blocked, the inhibitor apparently alkylates those types of receptors responsible for the direct myotropic action of angiotensin II.

Although it was not possible to demonstrate an antiangiotensin action of the chlorambucil peptides on rat blood pressure, this failure may have been because the pressor activity and low water solubility of the peptides made it impractical to infuse large doses. It is possible that with the low concentrations and slow infusion rates employed, most of the alkylating moiety of the compounds reacted with water and the plasma proteins before it could reach the angiotensin receptors responsible for pressor activity.

It has been previously postulated that the angiotensin receptor contains an anionic site[7] that may interact with either the N-terminal ammonium group or the guanidinium group of the arginine in position 2 of the peptide chain.[8] It is conceivable that this anionic site may be alkylated by the chlorambucil-peptides in which the N mustard group is appropriately placed. Examination of molecular models showed that while the tertiary N of **5** and shorter peptides cannot replace either of the cationic groups of the N-terminus of angiotensin, this is possible with both **6** and **7**.

These chlorambucil derivatives show that it is possible to achieve tissue-specific, hormone-specific, irreversible inhibitors of peptide hormones. The selective inhibition of the response of guinea pig ileum to angiotensin II makes possible the unequivocal identification of this substance in unknown solutions. The synthesis of **6** containing a radioactive isotope should provide a useful tool for the isolation and characterization of the receptor substance of this tissue.

Experimental Section

The peptides were synthesized by the Merrifield solid phase method,[9] using *N-tert*-butyloxycarbonyl (Boc) derivatives of the L amino acids, in which the reactive side chains were protected with the usual groups, with the exception of histidine, where the imidazole was protected with the *p*-Ts group[10] recently introduced for this purpose into solid phase synthesis.[11] A substantial cleavage of the Ts group was observed during the usual deprotection with 4 *N* HCl in dioxane, though not with 25% (v/v) of trifluoroacetic acid (TFA) in $CHCl_3$. For this reason the latter reagent was used (for 30 min) in all the deprotection steps, with a prewash of the same reagent to avoid excessive dilution by solvent in the resin. Other details of the synthesis were as previously described.[5]

The Boc amino acids were purchased from Schwarz BioResearch, with the exception of Boc-N^{im}-Ts-L-His, obtd from Fox Chemical Co. The purity of all of intermediates was checked by tlc on silica gel in 3 solvent systems: A, $CHCl_3$-MeOH-AcOH (85:10:5); B, $CHCl_3$-AcOH (95:5); C, Me_2CO-AcOH (98:2).

Bioassays were carried out as previously described.[5]

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Synthesis of Di- and Tripeptides and Assay *in Vivo* for Activity in the Thyrotropin Releasing Hormone and the Luteinizing Releasing Hormone Systems†

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Remarkable structure-hormonal activity relationships for the tripeptidic thyrotropin releasing hormone (TRH) of the hypothalamus are emerging. TRH and certain analogs having only one structural change release thyrotropin in the pituitary, but at a dosage differential of 1 to 1000–10,000. Such effective biological activity over such an extreme range of dosage is apparently possible only because of the nanogram potency of TRH and the microgram potency of the analogs. Ten additional tripeptides and two dipeptides, each containing a pGlu moiety and representing two structural changes in the hormone have been synthesized. All these pGlu peptides are inactive even at levels as high as 5000–50,000 times greater than that of an effective dosage of TRH. Since the luteinizing releasing hormone (LRH) of the hypothalamus appears also to have a pGlu moiety, and since it was recently discovered that a synthetic tetrapeptide, pGlu-Tyr-Arg-Trp-NH_2, releases LH like the natural hormone, these same twelve pGlu peptides have been tested for release of LH. Although these pGlu peptide variants did not release LH even at very high dose levels, additional information on the structural specificity of LRH was gained.

Chang, *et al.*,[1] and Bowers, *et al.*,[2] have reported the synthesis and the hormonal activities of structural modifications of the His and Pro moieties of the thyrotropin releasing hormone (TRH), which is pyroglutamylhistidylprolinamide (pGlu-His-Pro-NH_2).[3] Although TRH is relatively specific in releasing TSH, it was found that several tripeptide analogs showed very low but otherwise similar release of TSH. However, some of the active analogs were not inactivated by serum, but the activity of all the active analogs was inhibited by triiodothyronine as is that of TRH.[1,2] These tripeptide analogs have about 0.1–0.3% of the activity of TRH which represents an unusual structure-activity relationship, where the same biological activity is found in an analog at dose levels up to 10,000 times that of the reference compound. Such very great differences in activity can be studied in compounds which are active in a dosage range of nanograms and micrograms, but not when the active dosage range is in milligrams or greater.

To learn more about the structure-activity relationships of TRH, which could indicate that the molecular environment of the functional site of TRH involves some very low incidence of molecular variation, 10 tripeptides and 2 dipeptides containing the pGlu moiety have been synthesized and assayed for TRH activity.

These additional tripeptides have the general structure, pGlu-X-Y-NH_2 where X and Y are 6 different amino acids (Ala, Gly, His, Phe, Trp, Tyr) and, in each case, at least one of the amino acids is His or Tyr. The two dipeptides pGlu-Tyr-NH_2 (**27**) and pGlu-His-NH_2 (**28**) were also synthesized and assayed. These 12 peptides are listed in Table I, for convenience of appraisal.

†Hypothalamic Hormones. 27.