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Alkylating Analogs of Peptide Hormones. 1. Synthesis and Properties of *p*-[*N*,*N*-Bis(2-chloroethyl)amino]phenylbutyryl Derivatives of Bradykinin and Bradykinin Potentiating Factor†

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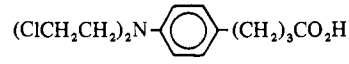
Peptides structurally related to bradykinin and to a bradykinin potentiating peptide from snake venom and bearing the N mustard chlorambucil were synthesized and tested for their ability to cause irreversible changes in receptors for bradykinin and angiotensin and in enzymes which metabolize these peptides. Certain of these permanently potentiated the action of bradykinin on isolated guinea pig ileum, while others permanently inhibited the action of angiotensin II on this tissue. Some peptides also permanently blocked certain of the pulmonary kininases in the rat *in vivo*.

For many years investigators have been searching for a potent and specific inhibitor of the peptide hormone bradykinin. Attempts in this laboratory,[1-4] as well as others,[5-9] to synthesize a structural analog with antibradykinin activity have been disappointing. Those few analogs which showed some inhibitory action at low doses uniformly showed bradykinin-like effects at higher doses, a property which, due to variations in tissue sensitivity and distribution of drug, would severely limit their usefulness *in vivo*.

Structure-activity studies[10,11] have shown that most of the functional groups in bradykinin (Figure 1) are necessary for significant biological activity and are, therefore, probably intimately involved in binding with functional groups on the receptor. The presence in one molecule of multiple sites for receptor-hormone interaction leads to very great sensitivity and structural selectivity. In this situation the incorporation into bradykinin and related peptides of chemically reactive groups capable of forming covalent bonds with receptors might be a fruitful approach to the synthesis of a bradykinin antimetabolite. This should lead not only to an irreversible inhibitor of the biological actions of the hormone but to a method for labeling, isolating, and identifying the receptor substance for this peptide. The presence of a significant portion of the peptide hormone in the alkylating derivative would hopefully confer a great deal of specificity and minimize the problem of nonspecific alkylation. Nonspecific alkylation has been a great problem in attempts to achieve labeling of adrenergic receptors.[12]

The studies reported here were carried out with a family of fragments and homologs of bradykinin containing *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylbutyric acid (chlorambucil, **1**), an N mustard type of alkylating agent. Since the C-terminal CO_2H of the bradykinin molecule is essential for biological activity[13,14] and may be important in initial receptor binding, the bradykinin fragments contained an intact carboxyl end and were acylated on their amino end with the carboxyl of the chlorambucil. A second group of peptides had structures related to a bradykinin potentiating peptide (BPP_{5a}) (Figure 1) found in *Bothrops jararaca* venom.[15] *In vitro*, this peptide potentiates the effect of bradykinin on guinea pig ileum, and *in vivo* it potentiates the hypotensive effect of bradykinin by inhibiting pulmonary kininases. It also inhibits the conversion of angiotensin I to angiotensin II.[16]



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Each of the alkylating peptides was tested for its ability to alter the response of the isolated guinea pig ileum and rat uterus to bradykinin and certain other spasmogens. In addition, each peptide was tested *in vivo* to determine if it might permanently alter the response of the cardiovascular system to bradykinin or angiotensin II or have an effect on the pulmonary inactivation of bradykinin and conversion of angiotensin I to angiotensin II. [Ile^5]angiotensin I and II were used in these studies.

Results

Synthesis. The chlorambucil peptides synthesized are listed in Table I. The presence of the chlorambucil residue conferred on the peptides a remarkable nonpolar character, making many of them freely soluble in $CHCl_3$. Since these were also partially soluble in EtOAc, Et_2O was used as a solvent to wash out anisole from the peptide-resin mixture following HF cleavage. All peptides reported herein were homogeneous on tlc and showed the proper amino acid composition (Table I). Also as would be expected the chlorambucil moiety of the peptide underwent hydrolysis in aq soln (pH 7.4; 38°) with the release of H^+ and Cl^-. Under these conditions chlorambucil and the chlorambucil-containing peptides had a half-life of approximately 90 min.

†This work was supported by U. S. Public Health Service Grant HE12325 and Population Council Grant M70.64C. All optically active amino acid residues are of the L configuration. Abbreviations used are: Boc = *tert*-butyloxycarbonyl; BK = bradykinin, Ang = angiotensin; PCA = pyrrolidonecarboxylic acid, Chl = chlorambucil.

Table I. Synthetic Alkylating Peptides

Compd	Structure[a]	R_f, tlc[b] Uv	Saka-guchi	Nin-hydrin	Amino acid composition,[c] moles/mole of peptide[d] Lys	Gly	Ser	Phe	Arg	Pro	Trp	Ala
1	Chlorambucil	0.96	Neg	Neg								
2	ε-Chl-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.10	0.10	0.12	*1.00*	1.15	1.12	2.04	0.90	3.34		
3	Chl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.34	0.33	Neg		*1.00*	0.97	2.22	1.95	2.99		
4	Chl-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.66	0.64	Neg		*1.00*	0.92	2.02	0.97	3.00		
5	Chl-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.65	0.65	Neg		*1.00*	0.96	2.14	0.90	1.98		
6	Chl-Gly-Phe-Ser-Pro-Phe-Arg	0.74	0.72	Neg		*1.00*	0.95	2.15	0.87	0.99		
7	Chl-Phe-Ser-Pro-Phe-Arg	0.77	0.75	Neg			*1.00*	2.10	0.94	1.07		
8	Chl-Ser-Pro-Phe-Arg	0.66	0.66	Neg			*1.00*	1.12	0.92	1.06		
9	Chl-Pro-Phe-Arg	0.68	0.65	Neg				*1.00*	0.89	1.01		
10	Chl-Phe-Arg	0.65	0.60	Neg				*1.00*	0.95			
11	Chl-Arg	0.61	0.61	Neg					*1.00*			
12	Chl-Lys-Trp-Ala-Pro	0.74	Neg	0.72	1.04					0.96	0.47	*1.00*
13	Chl-Trp-Ala-Pro	0.95	Neg	Neg						0.91	0.60	*1.00*
14	Chl-Ala-Pro	0.95	Neg	Neg						0.93		*1.00*
15	Chl-Lys-Trp-Ala-Pro-Phe	0.69	Neg	0.66	0.86			1.01		0.95	0.47	*1.00*
16	Chl-Trp-Ala-Pro-Phe	0.78	Neg	Neg				0.94		0.96	0.73	*1.00*
17	Chl-Ala-Pro-Phe	0.97	Neg	Neg				0.95		0.96		*1.00*
18	Chl-Lys-Trp-Ala-Pro-Phe-Arg	0.03	0.02	0.03	0.92			0.98	0.87	0.95	0.72	*1.00*
19	Chl-Trp-Ala-Pro-Phe-Arg	0.67	0.67	Neg				1.15	0.95	0.98	0.70	*1.00*
20	Chl-Ala-Pro-Phe-Arg	0.63	Neg	Neg				0.97	0.87	0.97		*1.00*
21	Chl-Pro-Lys-Trp-Ala-Pro	0.67	Neg	0.62	*1.00*					2.27	0.46	1.22

[a] Amino acids are abbreviated as recommended by IUPAC Commission on Nomenclature; *J. Biol. Chem.*, 241, 2491 (1966). Chl = *p*-[*N*,*N*-bis-(2-chlorethyl)amino]phenylbutyryl. [b] Peptides were chromatogd on silica gel with fluorescent indicator (Brinkmann) in $CHCl_3$–MeOH–AcOH–H_2O (65:30:4:1). Sakaguchi and ninhydrin sprays were prepd as described by Stewart and Young.[27] [c] Peptide hydrolysates were made in 6 *N* HCl under N_2 for 22 hr at 110°. For those peptides which contd Trp, 2-mercaptoethanol (1 mg/ml) and phenol (1 mg/ml) were included in the hydrolysis soln. Analysis was carried out on the Beckman 120C amino acid analyzer. [d] The residues used to calc the molar ratios are given in italics.

Bradykinin (BK)
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

Ile^5-Angiotensin I (Ang I)
H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH

Ile^5-Angiotensin II (Ang II)
H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH

Bradykinin Potentiating Peptide-$_{5a}$ (BPP_{5a})
PCA-Lys-Trp-Ala-Pro-OH

Figure 1. Structures of bradykinin, Ile^5-angiotensin I, Ile^5-angiotensin II, and bradykinin potentiating factor (BPP_{5a}).

Rat Blood Pressure. The results of the *in vivo* studies on the effect of the alkylating peptides on rat blood pressure are shown in Table II. Several of the chlorambucil-BK fragments (peptides **4**, **5**, **7–9**) were able to reduce significantly and permanently the inactivation of BK by the pulmonary kininases. This inactivation is normally 97–99% on a single passage through the pulmonary circulation.[17] The smaller C-terminal fragments (peptides **7–9**) were the most active in this regard. A typical experiment is shown in Figure 2. As can be seen in this experiment the iv dose of BK to produce a standard response decreased from 1.2 μg in the control situation to 0.2 μg following infusion of 100 μg/min of peptide **8** for a total of 40 min. The block persisted without change for at least 120 min (the maximum time tested) after stopping the infusion. There was also a slight but not significant increase in the sensitivity of the preparation to intraarterially administered BK in this experiment. There was no change in the conversion of Ang I to Ang II or in the basic pressor response to these peptides.

Other peptides (**19**, **20**) related to BPP_{5a} and containing the C-terminal dipeptide of BK (Phe-Arg) were also active in inhibiting the pulmonary inactivation of BK. This is not surprising since peptide **19** differs from the active peptide **8** only by replacement of the serine residue with an alanine. Similarly, **20** contains a Trp-Ala dipeptide which is closely related to the Phe-Ser dipeptide in **7**. The presence of an arginine at the C terminus seems to be necessary for pulmonary kininase inhibition since peptides **16** and **17**, which differ from the active peptides **19** and **20** only in that they lack the C-terminal arginine, were inactive. The necessity of a C-terminal arginine is not surprising since des-Arg^9-BK has been shown to be totally resistant to pulmonary degradation.[18] Chl-BPP_{5a} (**12**) and (Chl-Pro)-BPP_{5a} (**21**) showed

Table II. Effect of Alkylating Peptides on Pulmonary Metabolism of Bradykinin and Angiotensin I in the Rat

Compd	Bradykinin inactivation, %[a] Pre-infusion	During infusion[c,d]	Post infusion[d]	Angiotensin I conversion, %[b] Pre-infusion	During infusion[c,d]	Post infusion[d]
2*	97.0	93.4	93.4	100	100	100
3*	98.5	95.0	97.0	75.0	80.0	80.0
4	94.0	*87.0*	*92.0*	67.0	75.0	80.0
5	98.0	94.0	*90.0*	60.0	60.0	60.0
6	96.0	97.5	96.0	100	100	100
7	94.0	*80.0*	*75.0*	100	100	100
8	94.2	*83.0*	*80.0*	50.0	50.0	50.0
9	96.7	*80.0*	*75.0*	67.0	*18.0*	50.0
10	93.4	93.4	93.4	100	100	100
12	98.7	*83.0*	98.7	60.0	*20.0*	60.0
13	98.5	99.2	98.5	33.0	33.0	33.0
14	96.3	96.3	96.3	100	100	100
15	98.7	98.7	98.7	60.0	60.0	–
16	97.0	95.0	97.0	100	100	100
17	97.0	94.0	97.0	60.0	60.0	60.0
18	97.0	94.0	93.4	70.0	75.0	90.0
19	97.5	*90.0*	*92.0*	37.0	50.0	50.0
20	97.0	*90.0*	*87.0*	60.0	60.0	60.0
21	99.0	*38.0*	98.0	50.0		50.0

[a] Per cent bradykinin inactivation = 100 – [(intraarterial dose of BK)/(intravenous dose of BK) × 100]. [b] Per cent angiotensin I conversion = [(intraarterial dose of Ang. II)/(intravenous dose of Ang I) × 100]. [c] Alkylating peptides were infused at 100 μg/min per 0.1 ml except those marked with an asterisk which were infused at a rate of 10 μg/min per 0.1 ml. [d] Significant changes from control values are given in italics.

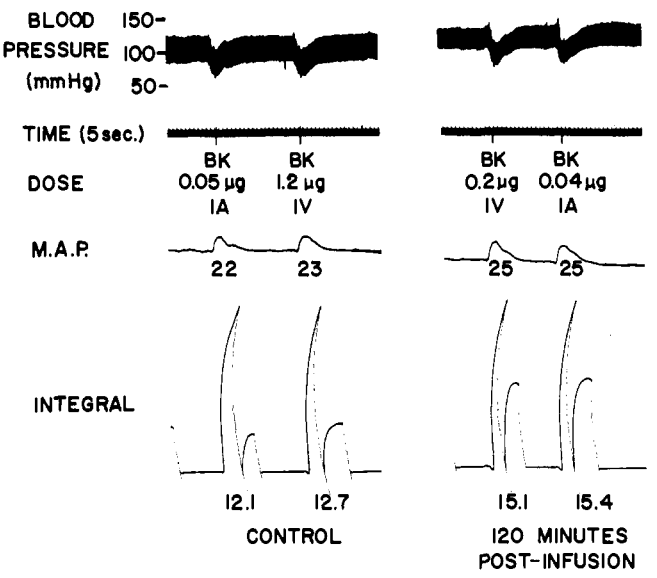

Figure 2. Effect of Chl-Ser-Pro-Phe-Arg (peptide 8) infusion on rat pulmonary kininases. Total dose of 8 was 4.0 mg infused at a rate of 100 µg/min per 0.1 ml of 0.9% NaCl. Post infusion record (right) was taken 120 min after stopping the infusion. MAP = mean arterial pressure. MAP trace is inverted to allow proper functioning of the Grass integrator.

competitive inhibition of pulmonary kininases during infusion, but produced no lasting effect. None of the peptides significantly altered the basic hypotensive response to BK. If the active peptide **8** was allowed to stand overnight at room temp in dil $NaHCO_3$ solution to inactivate the N mustard, it was no longer able to produce any lasting effect on the pulmonary kininases.

The relatively inactive decapeptide Ang I is rapidly converted to the active octapeptide Ang II by enzymes in the lung and in plasma.[19] Two of the peptides (**9**, **12**) showed temporary inhibition of this conversion. None of the peptides tested had any lasting effect on this conversion or on the direct pressor effect of Ang II.

Guinea Pig Ileum. None of the BK fragments showed any inhibition of the guinea pig ileum response to BK (Table III). On the contrary, many of the larger BK fragments (**4–8**) permanently potentiated the response of the tissue to BK. Compd **5** was the most active in this regard, causing a fourfold increase in sensitivity of the tissue (Figure 3b). As can be seen, there was no evidence of recovery for up to 180 min after removal of the alkylating derivative. Surprisingly, peptide **3** was not an active potentiator.

In contrast, the response of the tissue to Ang II was essentially unaffected by treatment with the larger BK fragments. Smaller fragments (**7–9**), however, showed a significant inhibition of the tissue response to Ang II, with **9** being most effective and most specific (Figure 3a). The nature of the inhibition is not clear, however, since the tissue could still be made to respond maximally by increasing the concentration of agonist. This would suggest a "competitive" inhibition although the persistance of the block for several hours, in spite of repeated washings of the tissue, suggests a "noncompetitive" block. None of the alkylating bradykinin fragments showed any significant effect on the response of the muscle to histamine. In addition, if the N mustard group was hydrolyzed by incubating the active chlorambucil-peptides in dil $NaHCO_3$ solution (1 mg of peptide/ml in 2.5% $NaHCO_3$) for 20 hr at room temp the peptides no longer had any significant effect on the guinea pig ileum response to either BK or Ang II.

Table III. Effect of Alkylating Peptides on Response of Guinea Pig Ileum and Rat Uterus to Bradykinin, Angiotensin II, and Oxytocin

	Guinea pig ileum, ED_{50}[a] before treatment/ED_{50} after treatment			Rat uterus, ED_{50} before treatment/ED_{50} after treatment			
Compd	Total dose, µg[b]	BK	Ang II	Total dose, µg[b]	BK	Ang II	Oxytocin
2	1.0	2.20	0.90	10	2.62	1.54	0.55
3	1.0	0.95	0.95	10	3.2	2.3	0.67
4	100	4.10	0.70	10	1.00	1.54	0.89
5	100	4.50	1.08	10	0.42	0.90	0.24
6	100	2.44	0.59	10	0.25	0.47	0.19
7	100	2.00	0.47	10	1.0	0.32	0.27
8	100	2.30	0.38	10	0.60	0.85	0.56
9	100	1.36	0.17	10	1.00	0.84	0.83
10	100	1.58	0.70	10	0.73	0.61	0.71
11	100	1.26	1.14	10	3.25	2.05	1.00
12	100	1.75	1.00	100	1.20	1.33	0.50
13	100	3.25	0.81	100	1.10	1.00	0.54
14	100	2.00	0.89	100	1.30	1.00	0.60
15	100	1.00	0.11	100	3.70	2.67	0.66
16	100	1.58	0.10	100	5.00	3.50	1.00
17	100	2.01	0.30	100	2.1	1.6	0.84
18	100	0.75	0.10	100	2.50	2.84	0.90
19	100	1.70	0.10	100	2.00	2.62	0.60
20	100	2.00	0.36	100	1.15	2.80	0.80
21	100	3.72	0.71	100	2.00	3.80	0.39

[a] ED_{50} = dose of agonist necessary to cause a 50% maximal contraction. [b] All doses of chlorambucil peptides were incubated with the tissue in a 10-ml tissue bath for 60 min.

In general, the alkylating peptides related to BPP_{5a} had little or no effect on the response of the guinea pig ileum to BK. The exceptions were **13** and **21**, which did significantly potentiate the responses to BK. Several of the BPP_{5a} analogs did, however, significantly inhibit the response of the tissue to Ang II. Only those compounds which contain the sequence Pro-Phe (the C-terminal dipeptide sequence of Ang II) were effective (**15–20**). Of these peptides those which also contained a Trp residue (**15**, **16**, **18**, **19**) were most effective and caused a tenfold decrease in the sensitivity of the tissue to Ang II. As with the BK fragments, the inhibition showed properties of both a competitive and noncompetitive inhibition.

Rat Uterus. As shown in Table III, none of the peptides tested showed any specific changes in the sensitivity of this tissue to either BK, Ang II, or oxytocin. When changes were seen they were always accompanied by a similar change in the sensitivity to one of the other agonists, which suggests a nonspecific change in tissue sensitivity.

Discussion

The results of this study are very encouraging, since they indicate that incorporation of alkylating groups into peptide hormones can result in irreversible changes in the sensitivity of the tissue to its agonist. Unfortunately, the initial goal of the study, which was to synthesize a specific and irreversible inhibitor of BK, was not realized. Several of the peptides did, however, cause permanent potentiation of the guinea pig ileum response to BK. The nature of this potentiation is unclear, although a logical explanation is that the peptides irreversibly inactivate kininases present in ileum and therefore effectively increase the concentration of BK in the area of the receptors. Such a mechanism has been suggested for the potentiation of the guinea pig ileum response to BK by bradykinin potentiation factors present in the venom of *Bothrops jararaca*.[20] The additional findings that certain of

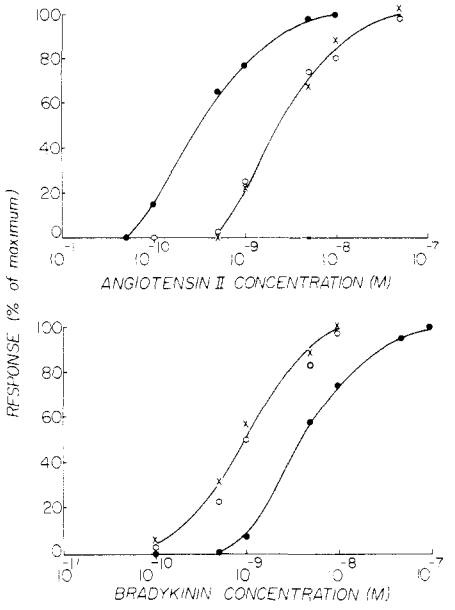

Figure 3. Effect of chlorambucil bradykinin fragments on guinea pig ileum dose-response curves for angiotensin II (upper) and bradykin (lower). Upper. Tissue was incubated for 60 min with 100 μg of peptide 9. (●–●) Preincubation dose-response curve for angiotensin II; (○–○) immediately following washout; (x–x) 180 min after washout. Lower. Tissue was incubated for 60 min with 100 μg of peptide **5**. (●–●) Preincubation dose-response curve for bradykinin: (○–○) 60 min after washout; (x–x) 180 min after washout.

the peptides could irreversibly block part of the pulmonary kininase activity and that no clear effect could be seen on the isolated rat uterus, which is relatively devoid of kininase activity, makes this mechanism a tempting one. Certain other observations, however, suggest that inhibition of kininases may not be the only mechanism capable of potentiating the guinea pig ileum response to BK. These include the findings that the isolated guinea pig ileum is not very efficient in destroying kinins,[21] and that other agents which potentiate the guinea pig ileum response to BK seem to do so by some mechanism distinct from their ability to inhibit kininases.[22] Recently a venom peptide has been isolated which potentiates the action of BK on rat uterus, but not on guinea pig ileum.[23] Also, BPP_{5a} potentiates the effect of BK on guinea pig ileum equally well whether added to the tissue bath before a dose of BK or after a BK-induced contraction has reached its peak. This effect is relatively rapid and suggests that BPP_{5a} may either displace BK from some nonreceptor binding sites on the tissue or it may in some way alter the receptor substance to give a more favorable interaction between BK and its receptor.‡ Similarly, the alkylating peptides found to potentiate the effect of BK on ileum could also work by irreversible blockade of nonreceptor binding sites or permanent modification of the receptor site, with a concomitant improvement in the ability of BK to interact with its receptor. It should also be noted that BPP_{5a}, which may act *via* a mechanism unrelated to its ability to inhibit ileum kininases, has no effect on the isolated rat uterus. In short, the irreversible potentiation of the guinea pig ileum to BK by these alkylating peptides may not be due to tissue kininase inhibition.

The inhibition of the guinea pig ileum response to Ang II by the chlorambucil C-terminal fragments of BK was at first surprising. On closer inspection, however, it is understandable since the active bradykinin fragments, as well as those active peptides related to BPP_{5a}, all contain the C-terminal dipeptide sequence of Ang II (Pro-Phe). This dipeptide sequence was also necessary for Ang II inhibition with the BPP_{5a} fragments. The inhibition caused by these peptides is paradoxical in that it could not be reversed by repeated washings of the tissue but could be reversed by increasing the concentration of angiotensin II in the bath. They, therefore, seemed to exhibit properties of both irreversible and reversible blockade. An explanation for this type of inhibition, which is also seen with some antiadrenergic and antihistamine agents, has recently been proposed.[24,25] It was suggested that an inhibitor may be bound very strongly by hydrophobic bonds (as with antihistaminics) or by covalent bonds (as may be the case with the alkylating peptides) to some biologically nonessential area of the receptor. Once bound, it then obstructs the access of the agonist to the biologically essential area of the receptor. Since the inhibitor does not alter the biologically essential area of the receptor, this type of inhibition could then be overcome by increasing the concentration of agonist and a maximal contraction could be elicited despite the permanent nature of the inhibition.

The results observed on the pulmonary kininases were considerably more straightforward. The smaller peptides related to the C-terminal portion of BK irreversibly blocked a significant portion of the pulmonary inactivation of BK. The essential requirements for this inactivation seemed to be the presence of the C-terminal Pro-Phe-Arg sequence. Compounds lacking the Pro residue (**10**, **11**) or the Arg residue (**12–17**) were not effective in inhibiting pulmonary kininases. Although some of the larger BK fragments were effective (**4**, **5**) they were not as good as the smaller peptides. The mechanism here must be inhibition of pulmonary kininases since this is the established mechanism for BK inactivation in the pulmonary circulation.[17] Indeed the evidence suggests that one of the pulmonary kininases cleaves the Pro^7-Phe^8 bond in BK. This might explain the observation that the smallest alkylating peptide to show any inhibition of pulmonary kininases was Chl-Pro-Phe-Arg (**9**).

The fact that many of the alkylating peptides caused permanent changes in the sensitivity of the guinea pig ileum to some peptide hormones and irreversible inhibition of a portion of the pulmonary kininase activity is encouraging and suggests that the basic idea of irreversible blockade of peptide hormone receptors by alkylating analogs is a sound one. Since it is not clear exactly how the derivatives are changing the guinea pig ileum sensitivity to the hormones, none of these peptides appears to be a likely reagent for introduction of a radioactive label into the receptor. Studies are now under way to incorporate aliphatic N mustards as well as other alkylating groups into bradykinin fragments, and to incorporate alkylating agents into other peptide hormones.

Experimental Section

Chemistry. Peptides used in this study were prepd by the automatic or manual Merrifield solid-phase method,[26] essentially as described by Stewart and Young.[27] Trp was protected by including 2-mercaptoethanol (3 mg/ml) in the HCl–dioxane reagent. In the automatic synthesis it was necessary to replace CH_2Cl_2 with $CHCl_3$ as the coupling solvent since difficulties in pumping the CH_2Cl_2 were experienced at the low atm pressure of Denver. The automatic synthesis was carried through the deprotection step in 4 *N* HCl–dioxane following coupling of the last Boc amino acid, at which time the peptide–resin was washed with EtOH, dried, and stored until

‡S. H. Ferreira, personal communication.

needed. For the synthesis of peptide **2** ε-Boc-α-Cbz lysine was used. Chlorambucil (Burroughs-Wellcome) was coupled to the peptide chain manually under conditions identical with those used to couple a Boc-amino acid residue. The deprotected peptide–resin was neutralized with Et_3N–$CHCl_3$ (1:9) and the chlorambucil coupling carried out with DCI in CH_2Cl_2. At the end of the coupling period the resin was washed with CH_2Cl_2, transferred to a fritted glass funnel with DMF, and dried.

Peptides were cleaved from the resin by stirring for 45 min at 0° in anhyd HF contg 5% (v/v) anisole. After distg off the HF *in vacuo*, the resin and peptides were transferred to a fritted funnel with either EtOAc (**2–12**, 19, **20**) or Et_2O (**13–18, 21**). The resin–peptide mixt was washed with the same solvent to remove both anisole and free chlorambucil and dried. Glacial AcOH (**2–7**, 9–**12**) or $CHCl_3$ (**8**, **13–21**) was used to ext the chlorambucil peptides from the resin. The $CHCl_3$ extn was inserted during the course of the study to eliminate any chance of contamination of the chlorambucil peptides with free peptide although this was not a problem with those peptides extd with AcOH. The $CHCl_3$ exts were taken to dryness *in vacuo*. The residue was redissolved in glacial AcOH and lyophilized. The AcOH-extd peptides were lyophilized directly. During all steps of the synthesis following coupling of the chlorambucil the peptides were protected from unnecessary exposure to light. Presence of chlorambucil in the peptides was demonstrated by following the rate of its hydrolysis at pH 7.4 and 38°. Release of H^+ was followed by continuous titration with a Radiometer TTT-1 titrator. Release of Cl^- was followed with a commercially available Cl^- kit. (Sigma Chemical).

Bioassays. In general, the chlorambucil peptides were not readily sol in H_2O (the exception was **5**). In order to assay the compds it was necessary to dissolve them in a minimal vol of propylene glycol and then dil the sample to the desired vol with 0.9% NaCl. This usually gave a fine milky suspension which was used immediately. No attempt was made to use these suspensions more than once. Propylene glycol had no effect on the assay prepns used in this study.

The isolated smooth muscle assays were carried out in 10-ml Sawyer–Bartlestone isolated tissue baths (Metro Scientific). Rat uterus assays were done with tissue from estrus virgin females in modified de Jalon's soln contg only 20 mg/l. of $CaCl_2$.[1] Isotonic contractions were measured under 1-g load. The bath temp was maintd at 29° and the soln was continually bubbled with 100% O_2. Guinea pig ileum assays were carried out with 2- to 3-cm strips of terminal ileum taken from starved (18 hr) animals. They were suspended in atropinized (100 μg/l.) Tyrode's soln and continually bubbled with 100% O_2. Bath temp was 29° and isotonic contractions were measured as above.

The protocol for testing the alkylating peptides was as follows. A standard dose–response curve was obtd for BK, Ang II, and histamine on the guinea pig ileum and for BK, Ang II, and oxytocin on the rat uterus. The tissue was then incubated with the desired dose (Table II) of alkylating derivative for 60 min. Following incubation the tissue was washed thoroughly and the dose–response curves were repeated. The results are expressed as the ratio of the dose of the agonist necessary to cause 50% maximal contraction of the tissue before treatment to the dose necessary to give a 50% maximal contraction following incubation with the alkylating peptide.

The rat blood pressure assay for the pulmonary metabolism of bradykinin and angiotensin I was carried out according to Stewart, *et al.*[18] The alkylating peptides being tested were infused in suspension into the femoral vein at the rate of 0.1 ml/min in 0.9% NaCl.

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