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62. Synthesis of Photoaffinity Labelling Analogues of the Peptide Hormone Bradykinin[1])

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Summary

Fourteen peptides, analogues of bradykinin and (des-Arg^9)bradykinin, have been synthesized by the solid-phase method or have been obtained through chemical modification. In all these peptides an aromatic residue has been substituted by (4′-NO_2)Phe, (4′-NH_2)Phe, (4′-N_3)Phe or (4′-NH_2-3′,5′-I_2)Phe. These peptides will be used as photoaffinity labelling or affinity labelling probes for peptide hormone receptors.

Introduction. - Photoaffinity labelling analogues of peptide hormones have become increasingly important in pharmacology and in biochemical receptor research. Several peptide hormone receptors have been studied recently, *e.g.* insulin [1] [2], gastro-intestinal hormones [2] [3], neurohypophyseal hormones [4] [5], angiotensin II [6] [7], enkephalins [8] [9], α-melanotropin [10] and others. Several analogues were synthesized by simple modifications of the parent peptide hormones with commercially available photolabelling reagents [5] [8]. Many of these peptides had drastically reduced biological activities. Moreover the labelling moiety was often far away from the part of the hormone which interacted with the receptor; therefore the results obtained with such peptides have to be taken with some reserve. However, if the photolabelling group was introduced on pharmacologically relevant side-chains of peptide hormones, highly active and specific compounds were obtained [11] [4].

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[1]) This work has been financed by grants from the *Medical Research Council of Canada*, by the *J.C. Edwards Foundation* and by the *Canadian Heart Foundation*. *E.E.* is a scholar of the *Canadian Heart Foundation*. Abbreviations follow the recommendations of the IUPAC-IUB Commission for biochemical nomenclature, see *E. Wünsch:* «Synthese von Peptiden», Vol. 15, part 1, of «Houben-Weyl, Methoden der Organischen Chemie», *E. Müller*, ed. G. Thieme, Stuttgart, GFR, 1974. Abbreviations: BK, bradykinin; OBK, (des-Arg^9)octa-bradykinin; BAW, butanol/acetic acid/water 10:2:3; BAWP, butanol/acetic acid/water/pyridine 30:6:20:12; BZL, benzyl; DCC, dicyclohexylcarbodiimide; DEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; ET, ethanethiol; HF, hydrogen fluoride; HPTLC, high performance thin layer chromatography; TFA, trifluoroacetic acid; TLC., thin-layer chromatography; Tos, *p*-toluenesulfonyl.

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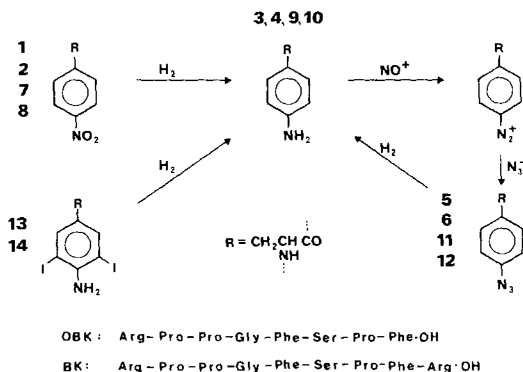
Scheme. *Modification scheme of aromatic residues in bradykinin analogues and sequence of bradykinins*



Owing to the encouraging results obtained with such an angiotensin II analogue [6] [12], we decided to look at a similar class of peptide hormones, the kinins, typical representatives of which being bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; BK) and (des-Arg^9)octa-bradykinin (OBK). These closely related peptides have distinctly different receptors on different smooth muscle tissues [13]. This class of peptide hormones has already been investigated with some attempted [14] and successful [15] affinity labelling analogues of BK, but the very high concentrations needed and the chemical properties of these compounds rendered this approach rather awkward. Potential photolabels of BK have already been published but no further details were disclosed [16]. For OBK we have reported [17] the first photoaffinity label and its weak biological properties. In order to collect more information and to possess several potential photolabelling peptides it was

Table

	Peptides	Formula	Molecular weight	TLC. BAW	TLC. BAWP	HPTLC. KC18
1	[(4'-NO_2)Phe^5]OBK	$C_{44}H_{60}N_{12}O_{12} \cdot C_2H_4O_2$	949.04 + 60.05	0.34	0.44	0.39
2	[(4'-NO_2)Phe^8]OBK	$C_{44}H_{60}N_{12}O_{12} \cdot C_2H_4O_2$	949.04 + 60.05	0.29	0.43	0.42
3	[(4'-NH_2)Phe^5]OBK	$C_{44}H_{62}N_{12}O_{10} \cdot C_2H_4O_2$	919.06 + 60.05	0.08	0.32	0.65
4	[(4'-NH_2)Phe^8]OBK	$C_{44}H_{62}N_{12}O_{10} \cdot C_2H_4O_2$	919.06 + 60.05	0.07	0.32	0.67
5	[(4'-N_3)Phe^5]OBK	$C_{44}H_{60}N_{14}O_{10} \cdot C_2H_4O_2$	945.06 + 60.05	0.39	0.45	0.29
6	[(4'-N_3)Phe^8]OBK	$C_{44}H_{60}N_{14}O_{10} \cdot C_2H_4O_2$	945.06 + 60.05	0.37	0.44	0.29
	OBK	$C_{44}H_{61}N_{11}O_{10} \cdot C_2H_4O_2$	904.05 + 60.05	0.33	0.35	0.43
7	[(4'-NO_2)Phe^5]BK	$C_{50}H_{72}N_{16}O_{13} \cdot C_4H_8O_4$	1105.23 + 120.11	0.16	0.38	0.27
8	[(4'-NO_2)Phe^8]BK	$C_{50}H_{72}N_{16}O_{13} \cdot C_4H_8O_4$	1105.23 + 120.11	0.12	0.36	0.32
9	[(4'-NH_2)Phe^5]BK	$C_{50}H_{74}N_{16}O_{11} \cdot C_4H_8O_4$	1075.25 + 120.11	0.02	0.25	0.48
10	[(4'-NH_2)Phe^8]BK	$C_{50}H_{74}N_{16}O_{11} \cdot C_4H_8O_4$	1075.25 + 120.11	0.02	0.24	0.54
11	[(4'-N_3)Phe^5]BK	$C_{50}H_{72}N_{18}O_{11} \cdot C_4H_8O_4$	1101.25 + 120.11	0.20	0.40	0.18
12	[(4'-N_3)Phe^8]BK	$C_{50}H_{72}N_{18}O_{11} \cdot C_4H_8O_4$	1101.25 + 120.11	0.17	0.38	0.20
13	[(4'-NH_2-3',5'-I_2)Phe^5]BK	$C_{50}H_{70}I_2N_{16}O_{11} \cdot C_4H_8O_4$	1327.04 + 120.11	0.27	0.38	0.21
14	[(4'-NH_2-3',5'-I_2)Phe^8]BK	$C_{50}H_{70}I_2N_{16}O_{11} \cdot C_4H_8O_4$	1327.04 + 120.11	0.27	0.38	0.22
	BK	$C_{50}H_{73}N_{15}O_{11} \cdot C_4H_8O_4$	1060.24 + 120.11	0.13	0.35	0.33

Figure. *Apparatus for hydrogenation of small quantities of peptide*

necessary to prepare a series of analogues similar to those of angiotensin [11]. We therefore introduced by the solid phase method L-(4'-NO_2)Phe in the aromatic positions 5 or 8 of BK and OBK. The nitro group was reduced to an amino group, diazotized and converted to an azido group. This modification pathway *(Scheme)* has been used to form the angiotensin II photolabels [11] and recently also for neurohypophyseal hormone analogues [4]. The intermediate products of the diazotation, the diazonium peptides (not characterized) have potential as affinity

Electro-	UV.		IR.	Yield	Amino-acid analysis						
phoresis	λ_{max} (nm)	log ε	[cm^{-1}]	%	Ser	Pro	Gly	Phe	Arg	(4'-NH_2)Phe	
0.36	278	4.11	-	29	0.90	3.14	1.03	1.02	1.00	0.94	**1**
0.33	278	4.09	-	67	0.91	3.10	1.03	1.03	1.00	1.08	**2**
0.65	285	3.00	-	77	0.89	3.14	1.04	1.03	0.99	0.91	**3**
0.67	285	3.02	-	75	0.91	3.25	1.11	1.08	1.02	0.89	**4**
0.36	280	3.25	2110	66	0.92	3.14	1.04	1.01	0.98	0.60	**5**
0.39	281	3.18	2110	37	0.86	3.17	1.06	1.04	0.99	0.94	**6**
0.33	-	-	-	-	-	-	-	-	-	-	
0.74	278	4.02	-	50	0.92	3.03	1.11	0.99	1.93	0.97	**7**
0.72	278	4.05	-	47	0.91	3.16	1.03	1.03	1.94	0.96	**8**
0.91	286	2.99	-	82	0.90	3.17	1.03	1.01	2.01	1.12	**9**
0.97	281	3.11	-	83	0.91	3.15	1.04	1.01	2.00	0.82	**10**
0.48	278/287	3.11/3.01	2110	58	0.91	3.14	1.03	1.02	1.99	0.80	**11**
0.57	278/287	3.32/3.23	2110	44	0.92	3.10	1.06	1.01	1.96	0.72	**12**
0.54	303	3.39	-	18	0.91	3.15	1.00	1.03	2.02	0.73	**13**
0.57	300	3.49	-	36	0.90	3.03	1.02	1.00	1.94	0.69	**14**
0.67	-	-	-	-	-	-	-	-	-	-	

labels and can be pharmacologically tested [18]. These and bromoacetyl derivatives [4] [19] of the peptides **3**, **4**, **9**, **10** will be used for this purpose. If a successful peptide emerged from this series it would be important to possess a radioactively labelled analogue for a possible receptor isolation. For this purpose two BK analogues were synthesized with (4'-NH_2-3',5'-I_2)Phe in the 5- or 8-position; subsequent tritiation and azide formation in the way described [20] is planned.

Results and Discussion. - The solid phase synthesis using N^{α}-BOC protected amino acids of the precursor peptides **1**, **2**, **7**, **8**, **13** and **14**, containing (4'-NO_2)Phe or (4'-NH_2-3',5'-I_2)Phe was performed without complications. As has been shown earlier, (4'-NH_2-3',5'-I_2)Phe did not need any side-chain protection owing to the steric hindrance from the iodine substituents [11]. The side-chain of Arg and Ser were protected by the Tos and the BZL group respectively. After anhydrous HF cleavage the peptides were purified by gel filtration and partition chromatography. Catalytic peptide hydrogenation was carried out under ~8 atm H_2-pressure with a simple system using disposable syringes, rubber balloons and reacti-vials with septum (see *Fig.*). Owing to the absorption of peptides on the catalyst, hydrogenation yields have been rather low [11]. In our syntheses washing the catalyst after the reaction with mercaptoethanol increased the yields. No problems were encountered during the formation of the azido peptides. These modified peptides were again purified by gel filtration and partition chromatography and their functional groups identified by UV. and IR. spectroscopy.

The purity of the peptides **1-14** was proved by TLC. in different solvent systems, HPTLC. and electrophoresis. In particular HPTLC. was essential to show clear differences between peptides that had identical Rf-values on TLC. (see *Table*).

The biological activities of these peptides in the dark and their photolabelling applications will be published elsewhere.

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Experimental Part

L-*t*-Butyloxycarbonylamido acids, peptide reagents, chloromethylated resin (copolystyrene - 1% divinylbenzene, 0.75 mmol of Cl/g of resin) were obtained from *Bachem* Fine Chemicals Inc., if not otherwise stated, and were used without further purification. DCC[1]) was purified by dissolving the commercial product (*Aldrich* Chemicals) in ether; the insoluble material was removed by filtration and the ether was evaporated *in vacuo*. All solvents and reagents used for solid-phase synthesis were of 'analytical reagent' quality and were redistilled before use. TLC. was performed on *Merck* precoated silica gel plates (type G60 - F254) in the solvent systems BAW, BAWP. HPTLC. was performed on *Whatman* reversed phase plates CK18 in the solvent system 0.5M NH_4OAc with 20% acetonitrile and 7% 2-propanol. Electrophoresis was performed with a *Beckman* model R electrophoresis apparatus using *Merck* precoated cellulose plates (F254, 0.10 mm). The buffer used for electrophoresis was prepared by diluting glacial acetic acid (59 ml) and formic acid (36 ml) to 2 l with distilled water (pH 2.1). Migrations are indicated by the ratio of the distance of migration of the peptides to that of L-arginine · HCl (m_{Arg}). The spots were visualized with UV. fluorescence, ninhydrin or a modified *Reindel-Hoppe* procedure [21]. All mixtures of liquids are given in *v/v*. Peptide samples for

amino-acid analyses were hydrolyzed during 24 h at 110° in 6N HCl + 1% ET[1]) in vacuum-sealed tubes. (4′-NO_2)Phe, (4′-N_3)Phe and (4′-NH_2-3′,5′-I_2)Phe were reduced during hydrolysis to (4′-NH_2)Phe and are recorded as such. The amino-acid analyses were performed on a *Technicon* TSM analyzer equipped with an *Autolab* integrator by Dr. *P. Schiller* of the Institute for Clinical Research in Montreal. UV. spectra were recorded on a *Beckman* 25 spectrophotometer with peptides dissolved in 0.9% NaCl. IR. spectra were recorded on a *Perkin-Elmer* 457 instrument in KBr pellets. Peptide synthesis was carried out with a *Burrel* shaker and glass reaction vessels using procedures previously described [22]. One mmol of dry BOC-amino-acid Cs-salt was coupled with one equivalent of chloromethylated resin according to *Gisin* [23], the substitution degree was measured with the picric acid test [24]. *N-α*-BOC protection was used for all the amino acids. This group was removed, prior to the next coupling, by reaction with TFA/CH_2Cl_2 1:2 for 20 min. A 5% solution of DEA[1]) in CH_2Cl_2 was used for neutralisation. The free base reacted with the symmetrical anhydride (following the method used by *Lemaire et al.* [25]) of the next amino acid; a 4–6 fold excess of the BOC-amino acid was used for each coupling. The completion of every coupling was checked after the reaction by the procedure of *Kaiser et al.* [26]. Simultaneous cleavage of the peptide from the resin and of the side-chain protecting groups was performed in liquid HF/anisole 5:1 for 60 min at 0°, using a Kel-F/Teflon home built apparatus. The crude peptides were extracted from the resin with 2N acetic acid and lyophilized.

Peptide purification. – Crude peptides were purified after HF-cleavage with gel filtration on *Sephadex* G 15. Columns of 3×73 or 3×116 cm were equilibrated with degassed 0.2M acetic acid. For partition chromatography G25 (same column size) was equilibrated with the aqueous layer of the degassed 2-phase system BAW 4:1:5 and eluted with the organic layer. For small peptide quantities (5–50 mg) columns of 0.8×75 cm Plexiglass tubing (G15) and 0.8×80 cm Teflon tubing (G25) were used with the same solvent systems. The columns were run by gravity, the fractions containing the peptides were detected by spotting 10-µl aliquots from each tube on TLC. plates and were revealed by UV. or *Reindel-Hoppe* procedure. The homogeneity of each purified peptide was verified by 2 TLC. systems (BAW, BAWP), HPTLC. and by electrophoresis. The analytical results of the peptides **1**–**14** are presented in the *Table*.

Peptide hydrogenation (see *Figure*). – Catalytic hydrogenation of nitro or iodopeptides was performed in the following way: in a 2 ml *Micro Product* vial with screw cap (*Wheaton Scientific*, New Jersey, USA) 1 to 50 mg peptide samples were dissolved in 1 ml of 50% AcOH; to this solution about 2 mg of Pd/C (10%, *Alfa Inorganics*) and a micro spin bar (5 mm) were added; the vial was closed with a silicone rubber septum; with an assembly of a hydrogen-filled rubber balloon, a 10 ml disposable syringe, a 22 gauge needle and 2 three way-luer-valves, the atmosphere inside the vial was exchanged, the syringe was then rinsed and filled with hydrogen; this gas was pressed into the vial and the syringe needle was drawn from the septum, leaving a H_2-pressure of about 8 atm inside the vial; the latter was immersed in a water bath at 20° (for temperature control and detection of leaks) and placed over a magnetic stirrer. Upon completion of hydrogenation (usually after 1 h, checked by TLC.) the catalyst was filtered off and washed with 10 ml 0.2N AcOH with 1% mercapto-ethanol. The united filtrates were lyophilized and used for further purification.

H-Arg-Pro-Pro-Gly-(4-NO_2)Phe-Ser-Pro-Phe-OH · AcO, [(4′-NO_2)Phe^5]OBK (**1**). Peptide **1** was synthesized from 1 g of BOC-Phe resin (0.53 mmol/g) and the following protected L-amino acids: BOC-Pro, BOC-Ser(BZL), BOC(4′-NO_2)Phe (from our laboratory), BOC-Gly, BOC-Arg(Tos). After HF-cleavage 270 mg of crude peptide were purified by gel filtration and by partition chromatography. The yield of pure peptide was 155 mg.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4′-NO_2)Phe-OH · AcO, [(4′-NO_2)Phe^8]OBK (**2**). Peptide **2** was synthesized from 1 g of BOC-(4′-NO_2)Phe resin (0.55 mmol/g) and the following protected L-amino acids: BOC-Pro, BOC-Ser(BZL), BOC-Phe, BOC-Gly, BOC-Arg(Tos). After HF-cleavage 570 mg of crude peptide were purified by gel filtration on G15 and partition chromatography. The yield of pure peptide was 370 mg.

H-Arg-Pro-Pro-Gly-(4′-NO_2)Phe-Ser-Pro-Phe-Arg-OH · 2AcO, [(4′-NO_2)Phe^5]BK (**7**). Peptide **7** was synthesized from 1.5 g of BOC-Arg(Tos) resin (0.37 mmol/g) and the following protected amino acids: BOC-Phe, BOC-Pro, BOC-Ser(BZL), BOC-(4′-NO_2)Phe (from our laboratory), BOC-Gly and BOC-Arg(Tos). After HF-cleavage 712 mg of crude peptide were purified by gel filtration and partition chromatography. The yield of pure peptide was 339 mg.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-NO$_2$)Phe-Arg-OH · 2AcO, [(4'-NO$_2$)Phe^8]BK (**8**). Peptide **8** was synthesized from 1.5 g BOC-Arg(Tos) resin (0.37 mmol/g) and the following protected amino acids: BOC-(4'-NO_2)Phe (our laboratory), BOC-Pro, BOC-Ser(BZL), BOC-Phe, BOC-Gly and BOC-Arg(Tos). After HF-cleavage 611 mg of crude peptide were purified by gel filtration and partition chromatography. The yield of pure peptide was 320 mg.

H-Arg-Pro-Pro-Gly-(4'-NH$_2$-3',5'-I$_2$)Phe-Ser-Pro-Phe-Arg-OH · 2AcO, [(4'-NH$_2$ 3',5'-I$_2$)Phe^5]BK (**13**). Peptide **13** was synthesized from 1.5 g BOC-Arg(Tos) resin (0.37 mmol/g) and the following protected amino acids: BOC-Phe, BOC-Pro, BOC-Ser(BZL), N^{α}-BOC-(4'-NH_2-3',5'-I_2)Phe (our laboratory), BOC-Gly, and BOC-Arg(Tos). After HF-cleavage 459 mg of crude peptide were purified by gel filtration and partition chromatography. The yield of pure peptide was 126.7 mg. Hydrogenation of 5 mg **13** gave a product identical to **9** in TLC. and HPTLC.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-NH$_2$-3',5'-I$_2$)Phe-Arg-OH · 2AcO, [(4'-NH$_2$-3',5'-I$_2$)Phe^8]BK (**14**). Peptide **14** was synthesized from 1.5 g of BOC-Arg(Tos) resin (0.37 mmol/g) and the following protected amino acids: N^{α}-BOC-(4'-NH_2-3',5'-I_2)Phe (our laboratory), BOC-Pro, BOC-Ser(BZL), BOC-Phe, BOC-Gly, and BOC-Arg(Tos). After HF-cleavage 623 mg of crude peptide were purified by gel filtration and partition chromatography. The yield of pure peptide was 290 mg. Hydrogenation of 5 mg **14** gave a product identical to **10** in TLC. and HPTLC.

H-Arg-Pro-Pro-Gly-(4'-NH$_2$)Phe-Ser-Pro-Phe-OH · AcO, [(4'-NH$_2$)Phe^5]OBK (**3**). Peptide **1** (30 mg) was hydrogenated for 1 h[3]). Pure peptide **3** (22.3 mg) was obtained.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-NH$_2$)Phe-OH · AcO, [(4'-NH$_2$)Phe^8]OBK (**4**). Peptide **2** (30 mg) was hydrogenated for 1 h[3]). Pure peptide **4** (21.7 mg) was obtained.

H-Arg-Pro-Pro-Gly-(4'-NH$_2$)Phe-Ser-Pro-Phe-Arg-OH · 2AcO, [(4'-NH$_2$)Phe^5]BK (**9**). Peptide **7** (47.9 mg) was hydrogenated for 2 h[3]). Pure peptide **9** (38.3 mg) was obtained.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-NH$_2$)Phe-Arg-OH · 2AcO, [(4'-NH$_2$)Phe^8]BK (**10**). Peptide **8** (46.9 mg) was hydrogenated for 1 h[3]). Pure peptide **10** (37.7 mg) was obtained.

H-Arg-Pro-Pro-Gly-(4'-N$_3$)Phe-Ser-Pro-Phe-OH · AcO, [(4'-N$_3$)Phe^5]OBK (**5**). To a solution of 29.2 mg of **3** in 2 ml 0.1N HCl at 0° was added 100 µl of 1N $NaNO_2$ under stirring. After 15 min the iodine-starch test was still positive and 110 µl of 1N sulfamic acid was added (in order to destroy excess nitrite) and the iodine-starch best became negative. After 5 min 68 µl of 1N NaN_3 was added and left for another 5 min. The pH of this solution was brought to 4 by addition of solid NH_4OAc and the mixture was directly applied to a G15 column for gel filtration, followed by partition chromatography. The yield of pure **5** was 19.9 mg. Hydrogenation of ~1 mg of **5** resulted in pure **3** (TLC.).

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-N$_3$)Phe-OH · AcO, [(4'-N$_3$)Phe^8]OBK (**6**). Compound **4** (30.0 mg) treated in the same way as described above for **3** yielded 11.3 mg pure **6**. Hydrogenation of ~1 mg **6** resulted in pure **4** (TLC.).

H-Arg-Pro-Pro-Gly-(4'-N$_3$)Phe-Ser-Pro-Phe-Arg-OH · 2AcO, [(4'-N$_3$)Phe^5]BK (**11**). Compound **9** (10.7 mg) was treated in the same way as above with the following aqueous solutions: 300 µl 0.1M $NaNO_2$, 335 µl 0.1M sulfamic acid and 200 µl 0.1M NaN_3. After neutralisation with 2 ml diluted $NaHCO_3$ solution, the product was lyophilized and directly subjected to partition chromatography. The yield of pure peptide **11** was 6.3 mg. Hydrogenation of ~0.5 mg of **11** resulted in pure **9** (TLC.).

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-(4'-N$_3$)Phe-Arg-OH · 2AcO, [(4'-N$_3$)Phe^8]BK (**12**). Compound **10** (10.1 mg) gave 4.5 mg of **12** with the procedures described above for **11**. Hydrogenation of ~0.5 mg of **12** resulted in pure **10** (TLC.).

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