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Synthesis of Some Analogs of Bradykinin Hormone Using Modified Solid Phase Peptide Synthesis and Microwave Technique (Part 1)

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THREE analogs of Bradykinin, (Lys^1) BK, (Lys^9) BK and ($Lys^{1,9}$) BK were synthesized by modified solid phase peptide synthesis with the application of microwave energy. The effect of the replacement of $Arg^{1,9}$ by Lys on the salt bridge between the guanidine group of Arg^1 and the carboxyl group of Arg^9, was investigated. The analogues will be tested *in vitro* for their effect on heart rate of rats and in isolated organ for the arterial pressure.

Keywords: Bradykinin, Modified solid phase peptide synthesis and Microwave energy.

Bradykinin (BK) is a nonapeptide, (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), involved in various physiological and pathophysiological processes, particularly as an initiator of inflamation . BK , a member of polypeptides called kinins, was discovered by Rocha *et al*.(1) and isolated later by Ellioe *et al*.(2) . It is produced in the body in response to many kinds of injuries and inflammatory insults. It is the most potent known elicitor of pain (3).

Activities of BK are mediated by kinin receptors expressed in almost all cells in the majority of species. These receptors belong to the G-protein coupled family and their activation stimulates smooth muscle cells, sensory nerve endings, causes vasodilatation and microvascular leakage and modulates the response of immunocompetent cells.

BK is also best known as a mediator of inflammatory responses and initiator of peripheral pain signal (4). Two types of receptors, designated B1 and B2, mediate the biological activities of BK. B2 receptors are very widely expressed in most tissues and require the entire BK sequence for recognition. B1 receptors recognize and bind des-Arg9-BK only and their expression is rapidly induced by inflammatory stimulation (4).

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The synthesis of BK analogues for structure-activity studies started shortly after the announcement of the structure of this hormone in 1960. However, the first report on bradykinin analogues able to antagonize the effects of BK in standard kinin assays, such as rat uterus, guinea pig ileum or rat blood pressure, only came 25 years later, with the description of [D-Phe7]BK and [Thi5,8,D-Phe7]BK [(5)].

Hundreds of analogues with single or multiple substitutions were later designed and synthesized in many laboratories. In the course of these studies the role of amino acid residues in all positions of BK, as well as the influence of various combinations of substitutions on the pharmacological activity of the resulting compounds were carefully investigated [(6)]. A major improvement in the potency of BK antagonists was achieved in 1991, when potent B2 blockers, carrying conformationally constrained amino acid residues of their C-terminal ends, were synthesized [(7,8)].

On the basis of comparison of the circular dichroism (CD) spectra of several analogs of BK, the presence of an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser6 and the amide proton of Phe8, an intramoleculr 4→1 hydrogen bond between the carbonyl oxygen of Pro2 and the amide proton of Phe5,and a salt bridge between the guanidino group of Arg1 and the carboxyl group of Arg9 was emphasized [(9)]. It was found that all of the peptides analogues of bradykinin of high biological activity, exhibited CD spectra like that of bradykinin, so it appears unlikely that highly ordered peptides of the same amino acids composition as bradykinin would possess bradykinin-like effects[(10)].

In the present study, analogues of bradykinin was synthesized using microwave assisted solid phase peptide synthesis[(11,12)]. The solid phase approach is a well established method for synthesizing peptides since the work of Bruce Merrifield in the 1960s. Since the first serious experiments for synthesizing peptides with the help of microwaves in 1992, the method and the instruments have been optimized a lot. Nevertheless many peptide chemists around the world still think of "cooking peptides" when they hear of microwave assisted peptide synthesis (MAPS) and fear the enhancement of side-reactions. Many examples of synthesized peptides from many laboratories show that MAPS is definitely a valuable tool for synthesizing peptides and that there is no need to fear the side-reactions[(13)].

The present work was carried out to further investigate the effect of the presence of the salt bridge in the structure of BK in solution. Moreover, the effect of Lys residue instead of Arg one on its stability and activity was studied. The synthesis using Microwave technique[(14)]and the biological activity of BK, Lys1 BK, Lys9 BK and Lys1, 9 BK are under investigation.

Materials and Methods

The synthesis was carried out on polystyrene – polyethylene glycol (PS-PEG3000) graft co-polymer as the polymeric support. The polymer was synthesized as described by Rapp *et al*.[15].

Fmoc amino acids were synthesized according to Carpino *et al*.[16]. The side chains of Arg, Ser and Lys were protected by methoxytrimethyl phenyl sulfonyl (MTR), t.Butyl (tBu), and tert-butyloxycarbonyl (Boc) groups, respectively.

The microwave oven was a 10% of its total power and with nitrogen bubbling (inert gas for stirring).

Peptide synthesis

Coupling steps were performed through the DIC/HOBT activation method using microwave irradiation [17] .

Attachment of the first amino acid to the resin

BKI [*H_2N-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH*]

The first amino acid Fmoc Arg (mtr)-OH was coupled to the polymer using a solution of (0.026 gm, 0.195 mmol) HOBt, (0.195 mmol) Fmoc-amino acid and (0.024 gm, 0.195 mmol, 0.03ml) DIC and a catalytic amount of DMAP in 2 ml DMF that was shacked at r.t. for 10min then it was added to a swelled suspension of (0.1 gm, 0.48 m eq) PS-PEG3000-NH2 in 2 ml DMF. The mixture was then subjected to MW irradiation until Kaiser Test showed the required result. Then, the resin was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether, respectively.

The Fmoc deprotection occurred by adding 2 ml of 25% piperidine/DMF solution to the Fmoc-Arg(mtr)- resin suspension in DMF and the mixture was then heated in MW oven till Kaiser test give positive result. The solution was then filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether. The capacity of coupling was checked by U.V detection of Fmoc group. It was about 98%.

BKII [H_2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH], and BKIII [H_2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH]

An anchor group (HMBA) was first attached to the resin by using a solution of (0.052 gm, 0.39 mmol) HOBt, (0.060 gm, 0.39 mmol) HMBA and (0.048 gm, 0.39 mmol, 0.06ml) DIC in 2 ml DMF that was shacked at r.t. for 10min then was added to a swelled suspension of (0.2 gm, 0.48 meq) PS-PEG3000-NH2 in 2 ml DMF. The mixture was then irradiated in MW oven until Kaiser Test showed the required result. Then, the resin was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether.

The first amino acid Fmoc Lys (Boc)-OH was coupled to the HMBA-polymer by using a solution of (0.026 gm, 0.195 mmol) HOBt, (0.195 mmol) Fmoc-amino acid and (0.024 gm, 0.195 mmol, 0.03ml) DIC and a catalytic amount of DMAP in 2 ml DMF was shacked at r.t. for 10min then it was added to a swelled suspension of (0.1 gm, 0.48 m eq) PS-PEG3000-NH2 in 2 ml DMF. The mixture was then subjected to MW irradiation until Kaiser Test showed the required result. Then, the resin was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether, respectively.

The Fmoc deprotection occurred by adding 2 ml of 25% piperidine/DMF solution to the Fmoc-Lys(Boc)-HMBA-resin suspension in DMF and the mixture was then heated in MW oven till Kaiser test gave positive result. The solution was then filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether. The capacity of coupling was checked by U.V detection of Fmoc group. It was about 96%.

Synthesis of the peptide sequences

The second amino acid Fmoc Phe-OH was coupled to the first amino acid attached to the polymer by adding a solution of (0.037g, 0.096mmol) Fmoc-Phe-OH, (0.013 gm, 0.096 mmol) HOBt, (0.024 gm, 0.096 mmol, 0.015ml) DIC and a catalytic amount of DMAP in 2ml DMF to a swelled suspention of amino freed first amino acid- polymer. The mixture was then heated in MW oven until Kaiser Test showed the required result. The resin was then filtered off and washed several times with DMF, DCM, MeOH and ether.

Coupling of Fmoc-Pro, Fmoc-Ser(tBut), Fmoc-Phe, Fmoc-Gly, Fmoc-Pro, Fmoc-Pro and Fmoc-Arg(pmc) in BKI (or Fmoc Lys (Boc)-OH in BKII and BKIII) were carried out as indicated above using 0.096mmol of each protected amino acid.

The coupling and deprotection steps were monitored by Kaiser Test[18] and UV test[19] was used for coupling capacity.

Cleavage of the synthesized peptides from the resin

The cleavage of the polymeric support and isolation of the free peptides were carried out using 1M aq. NaOH[20] after its treatment with 95% aq.TFA to remove the side-chain protecting groups by shaking the peptide resin at R.T. for one hour then isolation of the resin by filtration under reduced pressure and washing with TFA, the filtrate then discarded and the resin is washed with DCM, 10% DIPEA in DCM and DCM and left to dry under vacuum. The dry resin is then pre-swelled in dioxane and a cold 1M NaOH/ Dioxane (1:3, 20 ml/gm) solution is added and shacked for 15 min at R.T. the resin is then filtered into a flask contains 1M HCl (5 ml/gm)-this flask should be cooled in an ice bath to prevent warming as the base solution is neutralized- the resin is then washed with water and the pH of the filtrate is adjusted to 7.0. The filtrate is then washed with diethylether to get rid of any impurities and unwanted small peptides. The water residue is then lyophilized and the desired peptide was obtained.

The synthesized peptides were characterized using FAB mass and ESI mass spectroscopy, IR spectra and amino acid analysis as indicated in the next section.

Results and Discussion

The following peptide chains were synthesized using the modern solid phase peptide synthesis method with microwave technique application:

1. H_2N-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH
2. H_2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
3. H_2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH

The essential advantages of the microwave assisted solid phase peptide synthesis are that the reduction of coupling and deprotection required time, the decreasing of the racemization and the excellent purity of the crude peptide.

The time required for complete coupling and deprotection reactions for the first amino acids and percentage of the resin capacity in the synthesized sequences (I-III) by MSPPS using MW energy are indicated in the following table:

Fmoc-A.A	**% of maximum coupling capacity**	**Time required for maximum coupling in min**	**Deprotection**
Fmoc-Arg	94	10	4
Fmoc-Lys	96	7	4

9-Flurenyl methoxycarbonyl (Fmoc) group was used as N- terminal protecting group. It enables the UV spectroscopic monitoring of the coupling and deprotection reactions.

Time required for maximum coupling and deprotection reactions in MW oven for peptide sequences BKI, BKII and BKIII:

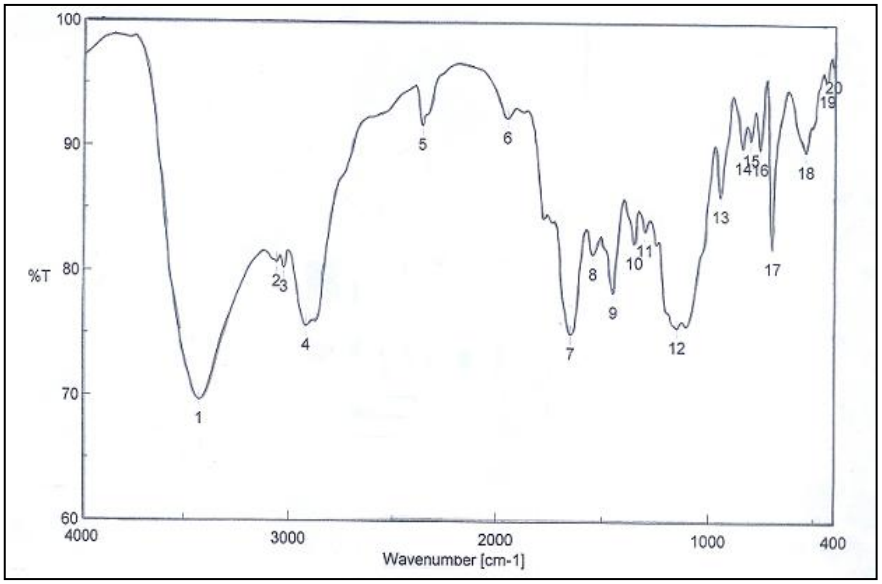
FMOC-A.A	**BK I**		**BK II**		**BK III**	
	C/min	**D/min**	**C/min**	**D/min**	**C/min**	**D/min**
Fmoc-Arg_1	---	---	7	6	---	---
Fmoc-Lys_1	8	6	---	---	5	4
Fmoc-Pro_2	4	3	9	5	4	3
Fmoc-Pro_3	7	3	10	5	3	2.5
Fmoc-Gly_4	4	4	5	5	2	2
Fmoc-Phe_5	8	4	8	5	3.5	3.5
Fmoc-Ser_6	8	4	6	10	6	3
Fmoc-Pro_7	6	4	5	4	3	2
Fmoc-Phe_8	8	3	6	4	4.5	3

The purity of the obtained peptide chains was proved by correct amino acid analysis and the mz values of the mass spectroscopy. The functionality of the characterized peptides was investigated using IR spectroscopy.

Amino acid analysis

	Arg	**Pro**	**Ser**	**Gly**	**Phe**	**Lys**
Calcd.	1	3	1	1	2	1
Found	0.93	1.85	0.7	0.8	1.9	1

IR spectroscopy

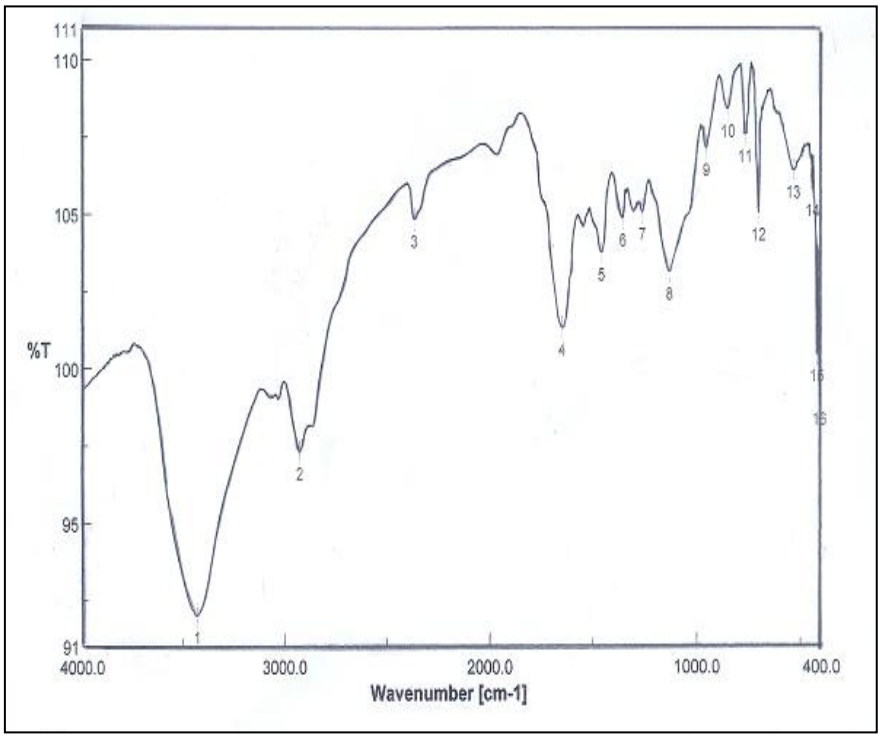


Peptide1. (BKI):H2N-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH (MS:m/z1032.19) .

Amino acid analysis

	Arg	**Pro**	**Ser**	**Gly**	**Phe**	**Lys**
Calcd.	1	3	1	1	2	1
Found	0.93	2.25	0.65	0.75	1.9	1

IR spectroscopy

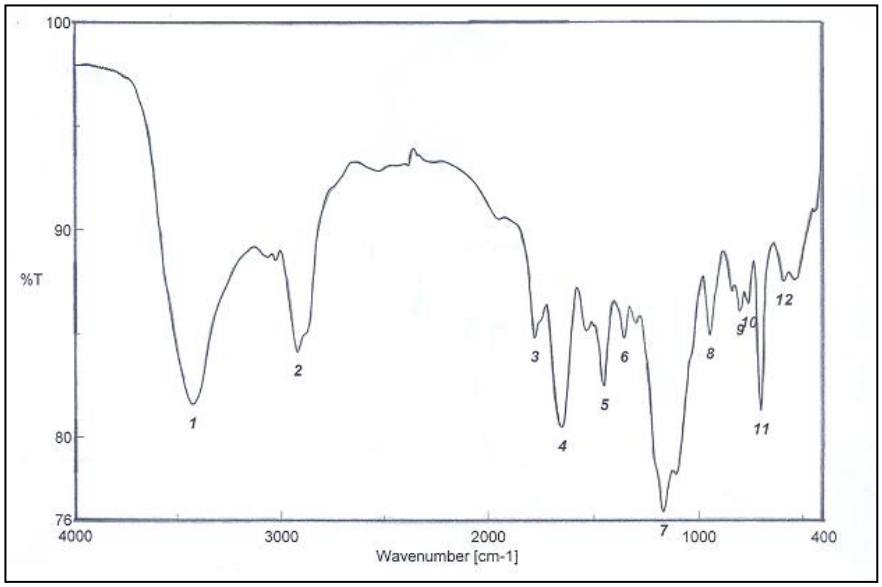


Peptide2. (BK2):H_2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (MS:m/z 1032.19) .

Amino acid analysis

	Pro	**Ser**	**Gly**	**Phe**	**Lys**
Calcd.	3	1	1	2	2
Found	2.34	0.72	0.93	1.7	1.9

IR spectroscopy



Peptide3 (BK3): H2N-Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys-OH (MS: m/z 1004.18).

The results indicated that the replacement of the first amino acid Arg with Lys amino acid affected the time of coupling and deprotection in microwave and the percentage of maximum coupling capacity of the amino acid.

The increased time taken by Fmoc-Arg in microwave for coupling, beside the effect of Fmoc group, due to the presence of guanidine group that leads to a steric hindrance that hinder the reaction between the resin beads and the amino acid.

Abbreviations

BK	Bradykinin
MSPPS	Modified Solid Phase Peptide Synthesis
Arg	Arginine
Pro	Proline
Gly	Glycine
Phe	Phenylalanine
Ser	Serine
Lys	Lysine
Thi	β-(2-thienyl)alanine
CD	Circular Dichroism
Mtr	4-Methylxy-2,3,6-trimethylphenyl-sulfonyl
tBu	tert. Butyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulfonyl
Boc	tert. Butyloxycarbonyl
Fmoc	9-Florenylmethyloxycarbonyl
DIC	Diisopropylcarbodiimide
HoBt	1-Hydroxybenzotriazol
DMAP	Dimethylaminopyridine
DMF	Dimethylformamide
DCM	Dichloromethane
MeOH	Methanol
TFA	Trifloroaceticacid
DIPEA	Diisopropylethylamine
UV	Ultraviolet
TLC	Thin Layer Chromatograghy
FAB-MS	Fast Electron Bombardment
ESI-MS	Electron Spray Ionization Mass Spectroscopy
HMBA	4-Hydroxymethyl benzewic acid

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استخدام طريقة السطح الصلب الحديثة فى تشييد بعض متشابهات هرمون البراديكينين مع تطبيق تقنية الميكروويف

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فى هذا البحث تم تحضير ثلاث متشابهات لهرمون البراديكينين بتطبيق أحدث الطرق فى تشييد الببتيدات وهى طريقة السطح الصلب الحديثه مع تطبيق تقنية الميكرويف.

وقد تم التحقق من التركيب الكيميائى للمركبات المحضره عن طريق اجراء التحاليل الدقيقة للأحماض الأمينيه وأطياف الكتله والأشعة تحت الحمراء. وكذلك تم التعرف على الفرق فى التشييد والسلوك بين الأحماض الأمينيه فى السلسله الأصليه للهرمون وفى المتشابهات المحضرة.

وقد تم تحضير المتشابهات بتغيير بعض الأحماض الأمينيه فى السلسلة الأساسيه للهرمون الأصلى بأخرى من نفس العائلة ولكن مختلفة فى الوزن الجزيئى لمعرفة مدى اهمية هذه الأحماض فى السلسله الأساسيه.

وجارى اجراء الجزء التطبيقى لهذا البحث والذى يتمثل فى اختبار تأثيرهذه المركبات على معدلات ضربات القلب وفى خفض ضغط الدم.