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Solid Phase Peptide Synthesis of Some Analogs of Bradykinin Hormone Using Microwave Energy Application (part II)

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THREE analogs of Bradykinin, ($Cys^{6,8}$) BK, ($Cys^{2,5}$) BK and ($Cys^{1,9}$) BK were synthesized by modified solid phase peptide synthesis with the application of microwave energy. The effect of the replacement of; Ser^6 and Phe^8 by $Cys^{6,8}$, Pro^2 and Phe^5 by $Cys^{2,5}$,and $Arg^{1,9}$ by $Cys^{1,9}$ on the biological activity of bradykinin was investigated. A continued work of a previously done study includes synthesis of three BK analogs, (Lys^1) BK, (Lys^9) BK and ($Lys^{1,9}$) BK, using the modified solid phase peptide synthesis with microwave energy. The six analogues have been tested *in vitro* for their effect on heart rate and in isolated organ for the arterial pressure of rats.

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[(1)], particularly as an initiator of inflamation . BK , a member of polypeptides called kinins, was discovered by Rocha *et al.* [(2)] and isolated later by Ellioe *et al.*[(3)] . 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 [(4)].

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 [(5)].

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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 [(6)].

Hundreds of analogues with single or multiple substitutions were later designed and synthesized in many laboratories[(7)]. 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. 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 [(8,9)].

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 [(10)]. 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[(11)].

In the present study analogues of bradykinin was synthesized using microwave assisted solid phase peptide synthesis [(12-14)]. The solid phase approach is a well established method for synthesizing peptides since the work of Bruce Merrifield in the 1962[(15,16)]. 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[(17)].

The present work was carried out to further investigate the effect of the presence of the intramolecular 3→1and 4→1 hydrogen bonds in the structure of

BK in solution. Moreover, the effect of Cys residues on bradkinin stability and biological activity was studied.

Chemistry

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.*[18].

Fmoc amino acids were synthesized according to Carpino *et al*.[19]. The side chain of Arg was protected by methoxytrimethyl phenyl sulfonyl (MTR), and of Ser and Cys, with t.Butyl (tBu).

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

Coupling steps were performed through the DIC/HOBT activation method using microwave irradiation [20].

Results and Discussion

The following peptide chains were synthesized using the modified solid phase peptide synthesis (MSPPS) method with microwave technique application :

1. H_2N-Arg-Pro-Pro-Gly-Phe-Cys-Pro-Cys-Arg-OH
2. H_2N-Arg-Cys-Pro-Gly-Cys-Ser-Pro-Phe-Arg-OH
3. H_2N-Cys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Cys-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 (IV-VI) by MSPPS using MW energy is 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-Cys	92	6	3

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

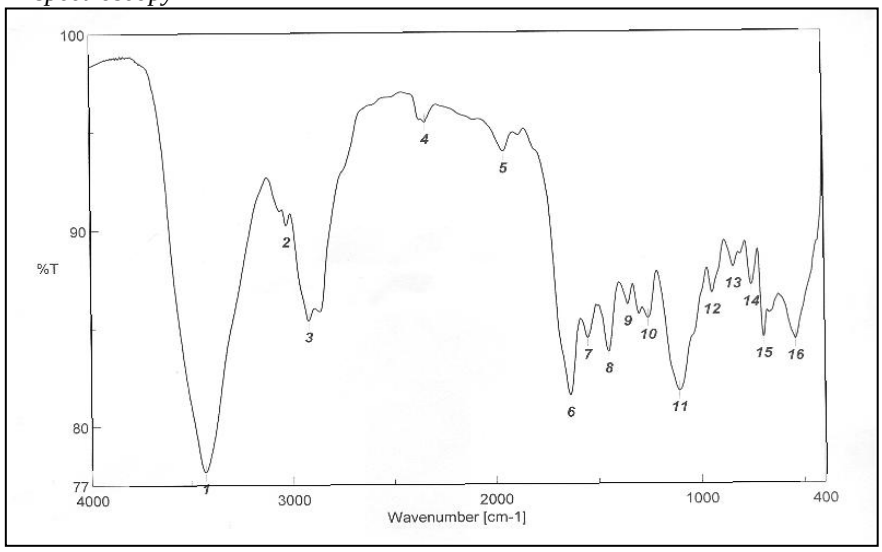
The purity of the obtained peptide chains was proved by correct amino acid analysis and the mz values of the mass spectroscopy. The functionality of the characterized peptides was investigated using IR spectroscopy.

Peptide4 (BKIV): H2N-Arg-Pro-Pro-Gly-Phe-Cys-Pro-Cys-Arg-OH
(MS: m/z 1032.24)

Amino acid analysis:

	Arg	**Pro**	**Gly**	**Phe**	**Cys**	**Arg**
Calcd.	2	3	1	1	2	2
Found	1.9	2.5	0.7	1	1.3	1.9

IR spectroscopy

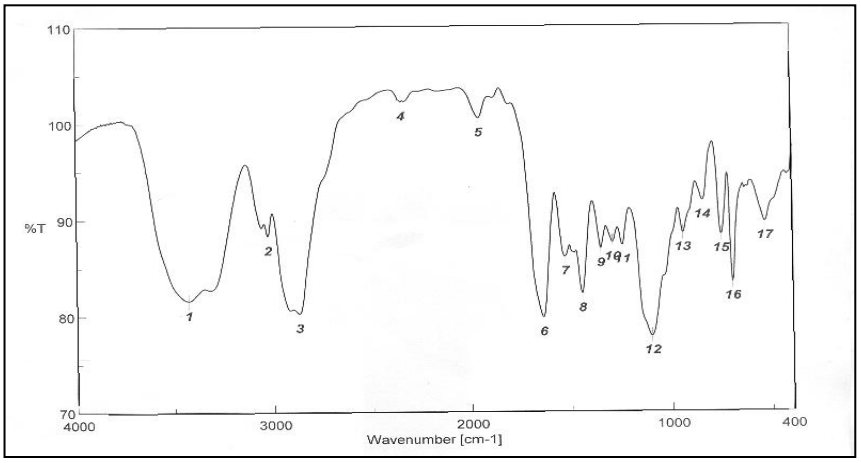


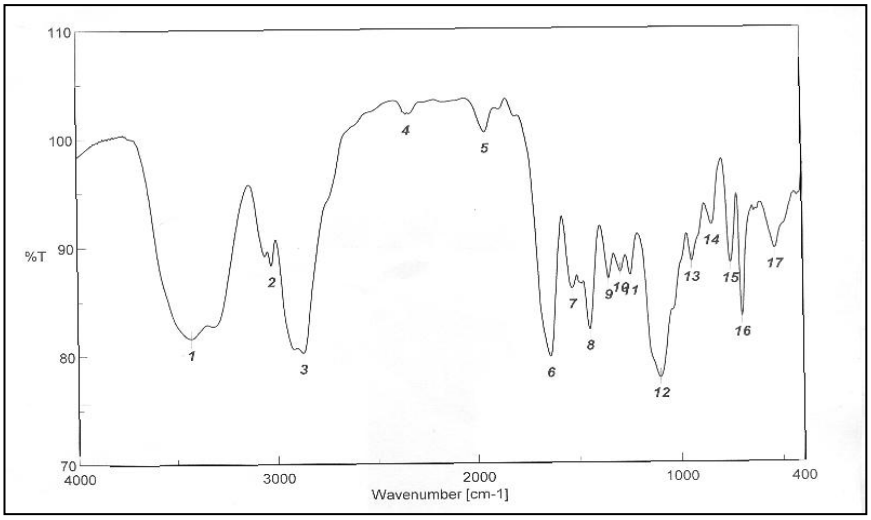
Peptide5 (BKV): H_2N-Arg-Cys-Pro-Gly-Cys-Ser-Pro-Phe-Arg-OH
(MS: m/z 1022.21)

Amino acid analysis

	Arg	**Cys**	**Pro**	**Gly**	**Ser**	**Phe**
Calcd.	2	2	2	1	1	1
Found	1.8	1.6	1.5	0.83	0.72	1

IR spectroscopy





Peptide6 (BKVI): H2N-Cys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Cys-OH
(MS: m/z 954.12)

Amino acid analysis

	Cys	Pro	Gly	Phe	Ser
Calcd.	2	3	1	2	1
Found	1.6	2.2	0.8	2	0.72

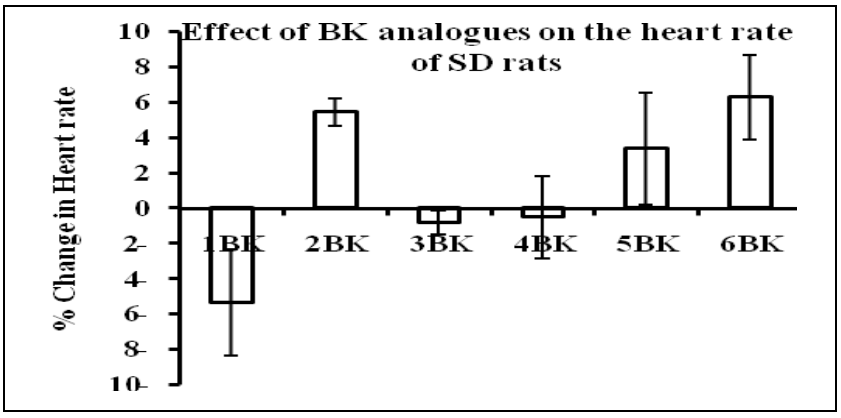
The results indicated that the replacement of the first amino acid Arg with Lys amino acid affected the time of coupling and deprotection in microwave and the percentage of maximum coupling capacity of the amino acid.

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 hinders the reaction between the resin beads and the amino acid.

Biological Evaluation

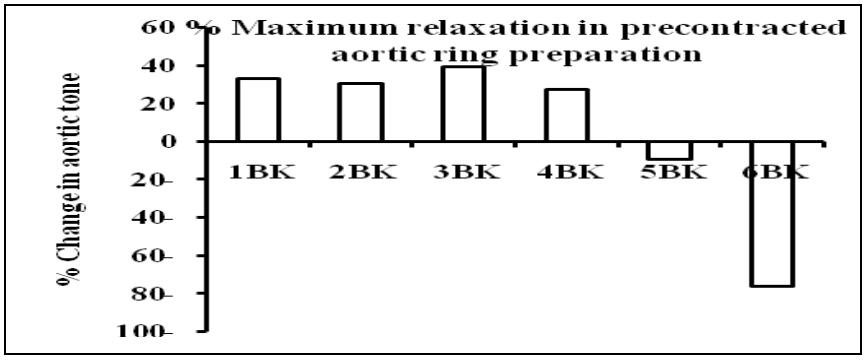
Investigation of the effect of the peptide candidates on the heart rate

The following chart represents the change in the heart rate for each peptide sequence:



Investigation of the effect of the peptide candidates on the smooth muscles

The following chart represents the maximum in the relaxation in aortic ring for each peptide sequence:



The results obtained from the two assays revealed the following significant criteria:

1. BKVI is considered the most potent vasodilator among all synthesized Bk analogs at dose range (10^{-4}-10^{-3}) using precontracted aoretic ring preparation.
2. BKII showed an accepted vasodilatation effect at dose range (10^{-4}-10^{-3}), using precontracted aoretic ring preparation.
3. BKV showed marginal vasodilatation effect at dose range (10^{-4}-10^{-3}), that might be improved at higher dose ranges.
4. BKII, BKV and BKVI induced vasodilatation resulted in natural physiological reflux tachycardia corresponding to its vasodilatation efficacy.
5. All other BK analogs failed to block nor-epinephrene enduced aortic ring contraction. However , they may block BK relaxation.
6. BKI induced bradycardia might be attributed to central effect (cardiovascular depression effect).

Approaching a qualitative structure/activity relationship for the peptide candidates

1. The peptide sequences BKI, BKII, BKIII and BKVI are designed to investigate the effect of the salt bridge between the guanidine group of Arg^1 and the carboxyl group Arg^9 in the main bradykinin sequence, thereby, the previous results revealed that:

i. Replacing of Arg^1 with Lys, as in BKI, shows bradykinin antagonistic effect, that may indicate that the salt bridge between Arg^1 and Arg^9 in the bradykinin sequence is important for its action and that bridge did not formed in the case of replacing of Arg^1 with Lys.

ii. Replacing of Arg^9 with Lys, as in BKII, shows bradykinin agonistic effect, that may be attributed to the salt bridge formed between the guanidino group of Arg^1 and the carboxyl group of Lys^9.

iii. Replacing of both Arg^1 and Arg^9 with Lys moieties, as in BKIII, did not show any bradykinin agonistic or antagonistic effects and that may be attributed to the disappearance of the salt bridge between Lys^1 and Lys^9.

iv. Replacing of both Arg^1 and Arg^9 with Cys moieties, as in BKVI, showed a significant agonistic effect that may be attributed to the disulfide bond formed between the two-thiol groups of Cys^1 and Cys^9.

2. The peptide sequence BKIV is designed to investigate the effect of the intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8 in the main bradykinin molecule, by that, the previous data indicate that:

- Replacing of both Ser^6 and Phe^8 with Cys moieties did not show any bradykinin agonistic or antagonistic effects that may attribute to the disappearance of the hydrogen bond and to the non-effective action of the

formed disulphide bond between the two-thiol groups of Cys^6 and Cys^8 in the synthesized peptide.

3. The peptide sequence BKV is designed to investigate the effect of intramolecular 4→1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5 in the main bradykinin molecule, thereby, the pre-mentioned data revealed that:

1. Replacing of both Pro^2 and Phe^5 with Cys moieties showed marginal potency and tends to have a bradykinin agonistic effect at higher doses. That may attribute to the formed disulphide bond between the two-thiol groups of Cys^2 and Cys^5 in the synthesized peptide.

Experimental

Attachment of the first amino acid to the resin

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 was added to a swelled suspension of (0.1 gm, 0.48 meq) PS-PEG3000-NH2 in 2 ml DMF. The mixture was then subjected to MW irradiation until Kaiser Test showed a negative result. Then, the resin was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and ether.

The Fmoc deprotection occurred by adding 2 ml of 25% piperidine/DMF solution to the Fmoc-A.A- 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 94% for Fmoc-Arg(mtr) and 92% for Fmoc-Cys(tBut).

Synthesis of the peptide sequences

The second amino acid Fmoc-A.A-OH was coupled to the first amino acid attached to the polymer by adding a solution of (0.096mmol) Fmoc-A.A-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-A.A's was carried out as indicated above using 0.096mmol of each protected amino acid.

The coupling and deprotection steps were monitored by Kaiser Test [21] and UV test[22] 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[23] 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 red 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.

Biological Evaluation

Determination of the effects of the synthesized peptide analogues [BKI, BKII, and BKIII], on the heart rate [HR] and on the smooth muscles (aortic ring) was realized in Pharmacology Department, National Research Centre.

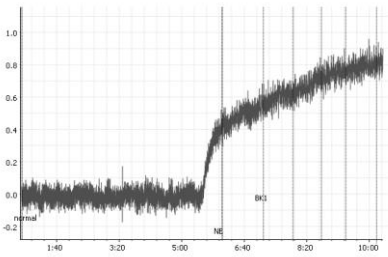
Evaluation of the action on the heart rate

About 300 gm SD rats were anesthetized with intrapretonial (i.p) injection of thiopental (100mg/kg). Tail veins were canulated and BK analogues were injected (600mg/kg). ECG [Electrocardiography] was recorded for about 5min. before and after drug administration and heart rates were compared. Confidence interval test was used to evaluate significance with α=0.05.

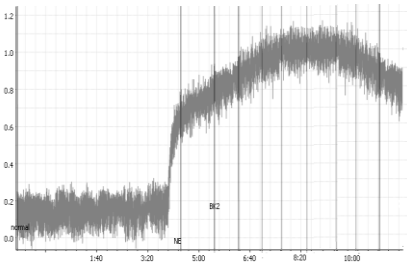
Evaluation of the action on the smooth muscles (aortic ring)

About 400 gm SD rats were euthanized by cervical dislocation and segments from the aorta were isolated and hanged in balanced Kreb's solution under static tension of 2 g/segment in thermostatic water jacketed isolated organ bath, Ugo-Basile, Italy. Aortic segments were precontracted with Nor-epinephrin (0.4 µg/ml) followed by application of the test compounds in the dose range of 10^{-4}-10^{-3} M [24]. Changes in N.E. induced contractile cascade in aortic ring were monitored and % change in muscle tones was calculated.

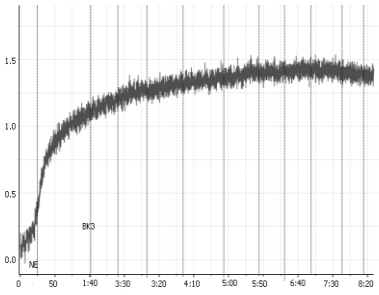
Thermostatic organ bath records before and after treatement with BK analogues are showed as followes:
BKIII: % Change in muscle tones = 39.3%



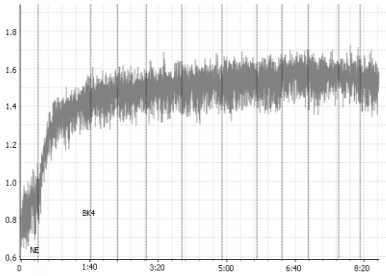
BKI: % Change in muscle tones = 33.1%



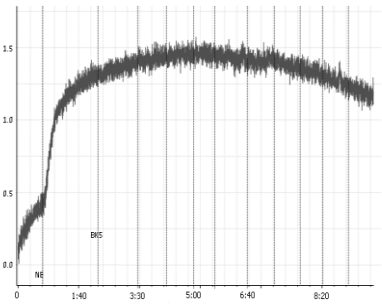
BKII: % Change in muscle tones = 30.6%



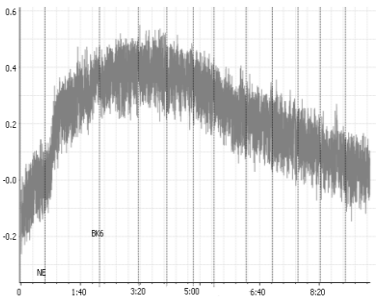
BKIII: % Change in muscle tones = 39.3%



BKIV: % Change in muscle tones =27.6%



BKV: % Change in muscle tones = -9.2%



BKVI: % Change in muscle tones = -76.2%

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إستخدام طريقة السطح الصلب الحديثة فى تشيد بعض متشابهات هرمون البراديكينين مع تطبيق تقنية الميكروويف

محمد على زويل ، أحمد نجلة و شيماء عثمان
قسم كيمياء الببتيدات – المركز القومى للبحوث – الجيزة – مصر .

يهدف هذا البحث الى تشييد بعض متشابهات هرمون البراديكينين نظرا لأهميته البيولوجية و تأثيره على انقباض العضلات اللإرادية وكذلك فى خفض ضغط الدم وغير ذلك من العمليات الحيوية داخل الجسم .

وقد تم تشييد السلاسل الببتيدية المعنية بإستخدام الطرق المستخدمة فى تحضير الببتيديات المعنية بإستخدام أحدث الاوساط الصلبة و بتطبيث تقنية الميكروويف الحديثة والتى ادت الى تحسن فى تحضير تلك المركبات من حيث زمن حدوث التفاعلات و درجة نقاوة المركبات المحضرة وكذلك كمية الناتج .

كما تم اختبار فاعلية المركبات المشيدة على معدل ضربات القلب وتأثيرها على ضغط الدم (إنبساط الاوعية)