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Synthesis of Peptides Histamine H2 Receptors in Solid-Phase Assisted by Microwave

Edgar A. Reynoso-Soto[1] and Ignacio A. Rivero[1,2]*

[1] Centro de Graduados e Investigación del Instituto Tecnológico de Tijuana. Apartado Postal 1166, Tijuana, B.C. 22000 México. irivero@tectijuana.mx.

[2] Instituto Nacional de Investigaciones Nucleares, Departamento de Química. Carretera México Toluca S/N, La Marquesa, Ocoyoacac 52750, México, D.F.

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Abstract: The synthesis of histamine H2 receptors peptides was conducted using the methodology of solid phase assisted by microwaves. Microwaves can reduce the reaction times during the coupling and deprotection steps to obtain the desired peptide sequence. The coupling reaction was carried out with a mixture of *N,N′*-diisopropylcarbodiimide (DIC) and *N,N,N′,N′*-tetramethyl-O-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU). The purity and yield are improved in peptide synthesis assisted by microwaves. Coupling reactions and deprotection on Rink resin were carried out in 5 min depending on amino acid and the length of the peptide chain.
Keywords: Solid Phase, Peptide, Microwave.

Resumen: La síntesis de péptidos receptores de histamina H2 fue realizada empleando la metodología de síntesis en fase sólida asistida por microondas. Las microondas permitieron disminuir los tiempos de reacción durante las etapas acoplamiento y desprotección para obtener la secuencia deseada de péptido. La reacción de acoplamiento se llevó a cabo con una mezcla de *N,N´*-diisopripilcarbodimida y *N,N,N′,N′*-tetrametil-O-(1*H*-benzotriazol-1-il)uronio hexafluorofosfato. La pureza y el rendimiento son mejorados en la síntesis de péptidos asistida por microondas. Las reacciones de acoplamiento y desprotección sobre la resina de Rink se llevaron a cabo en 5 min por protocolo y dependiendo del aminoácido son el número de protocolos empleados.
Palabras clave: Fase sólida, péptidos, microondas.

Introduction

While the deoxyribonucleic acid (DNA) is the vital part of living beings, so are the peptides and proteins which have the role of the construction, maintenance and support of them. Proteins and peptides are naturally occurring formed from a limited number of natural L-amino acids, however the diversity of structures, properties and the abundance of the protein compounds ensure its presence in all biological species on the planet. [1] Hence, proteins are present in nature to play vital roles in the functioning of almost all biological systems, acting as an extracellular messenger in animals and plants, or as hormones, neuromodulators and neurotransmitters, also have a major impact on vital functions such as metabolism, respiration and reproduction.

Several authors have devoted their research to the study, synthesis and sequence of peptides. These synthetic peptides in their three-dimensional conformers can be used as drugs or serve as antigens to stimulate the formation of specific antibodies [2].

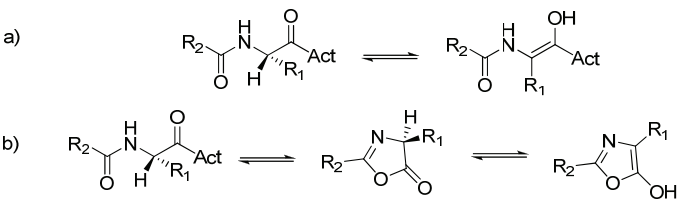
The solid phase synthesis (SPOS) has been widely used in the preparation of peptides, pseudopeptides, oligonucleotides and other kinds of organic compounds [3]. This methodology offers a number of advantages such as, efficiency, purification and handling. However, the main problem with this methodology is the difficulty for coupling agents to interact with active sites of the polymeric support at a slow reaction rate with very long reaction times, requires excess of reagents. A recent report showed significant improvement in reaction rate, purity and yield in SPOS reaction using irradiation with microwave [4].

The formation of a peptide bond occurs through a nucleophilic attack of an amine on the carbonyl of a carboxylic acid of the amino acid and the activation of this group is the first step [5-8]. When the acid has a stereogenic carbon in the α position with respect to the carbonyl, as in the α-amino acids, then there is a high probability this center can racemize. This process is a side reaction, which should be avoided in order to have an efficient synthesis. The racemization can occur through two mechanisms as shown in scheme **1** [9]:

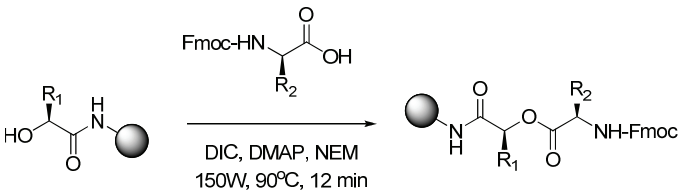
a) Enolization of active specie.
b) Formation and enolization of 5(4*H*)-oxazolone

The extent of racemization depends on the activation method employed in methods that use bases there will be trend for enolization by losing of α-hydrogen. Furthermore, if the activating group is a good leaving group, then there is a possibility of formation of oxazolone through an internal SN_2 displacement.

Previous investigations show the different coupling agents, *N,N′*-Diisopropylcarbodiimide (DIC), shown to be the best



Scheme 1. Mechanisms of racemization in the coupling reaction in the synthesis of peptides.



Scheme 2. Synthesis of pseudo-peptides in solid phase assisted by microwaves.

coupling agent for the esterification reaction in the synthesis of pseudopeptides in solid phase as shown in scheme **2** [10].

Here we report and efficient synthesis to peptide, a histamine receptor using microwave and HBTU.

The importance of histamine in regulation of gastric acid secretion was well established, the end-organ receptor mediating this action could not be defined until Black *et al.* [11] developed an antagonist for the H2 subtype of histamine receptor on the basis of simple analogy to *p* adrenergic receptor antagonists. The newly developed H2 histamine receptor antagonist has since become the cornerstone of peptidic ulcer therapy. Early pharmacological studies [12, 13] indicated that the bioactive form of histamine was a mono-cation and that imidazole tautomerism was important in binding of histamine to H2, but not H1 histamine receptors. From these studies, Weinstein *et al.* [13] proposed a three-site model predicting the requirement for negatively charged amino acid residues on the H2 histamine receptor to interact with the charged amine and N(3)H moieties of histamine and a third receptor amino acid residue which would serve as an H-bond donor to interact with the N(1) moiety of histamine. According with these results, studies in transmembrane region 3 by Gantz *et al.* [14], established what aspartic acid (position 107 in human H1 and position 98 in human H2 receptor) is essential for the binding of both histamine and basic antagonists for both receptors. Recently, success in cloning the gene encoding the H2 histamine receptor has permitted us to examine this model for histamine action at a molecular level.

Results and discussion

The design of the synthesize peptides sequences was made according to quantum mechanics study by Weinstein *et al.* [13]. They described that amino acid residues Tyr182, Asp186 and Thr190 are the responsible sites for interaction with histamine in histamine H2 receptor. We keep the amino acid position in peptide AA; the first position is for tyrosine what correspond to the amino acid to which was attached to the resin, position five will be aspartic acid and position nine is threonine varying the amino acids that are interspersed between these positions.

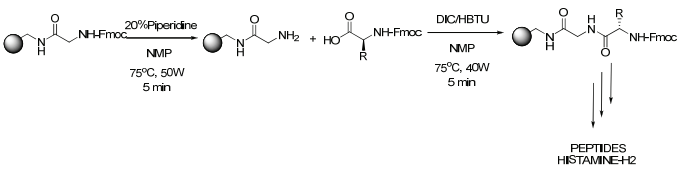
In this work we report the synthesis of peptides type histamine receptor on Rink resin assisted by microwaves; which were evaluated by molecular fluorescence spectroscopy [15] when they was immobilized on the polymer support (resin) and subsequently cleavage for their characterization by HPLC-ESI-MS. Cleavage from resin was making with a solution of TFA/TRIS/H_2O/dithiotreitol at room temperature stirring for 6 h; Then, the mixture was filtered and washed with CH_2Cl_2, MeOH. The filtrate was concentrated at a temperature not exceeding 35 °C under reduced pressure and finally precipitated peptides with cool ethyl ether.

Scheme 3 shows that the coupling reaction and deprotection were carried out in 5 min per protocol also coupling reaction occurred at 75 °C at 50 Watts and deprotection reaction occurred at 75 °C at 40 Watts. After complete reaction time the resins were assessed by the Kaiser test for the identification of free amino groups at the deprotection reaction or not react in the coupling reaction.

In Table 1 is described the abbreviation set by the IUPAC to identify the amino acids present in the sequences of peptides synthesized, such as 2-amino acetic acid whose generic name is glycine is abbreviated with the first three letters (Gly).

In Table 2 shows the percentage of purity and the molecular weight for each sequences of peptides synthesized. Peptide yields and sequences were analyzed by HPLC-UV-ESI-MS.

The peptide **Tyr**-Ala-Gly-Ile-**Asp** was characterized by HPLC-ESI-MS using as mobile phase a mixture of acetonitrile/water (20/80) with 0.1% formic acid, presenting a retention time of 1.0 minute.



Scheme 3. General methodology of peptide synthesis in solid phase coupling and desprotection reactions.

Table 1. Description of the abbreviations of the amino acids used.

Alanine	Ala	A	Leucine	Leu	L
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Threonine	Thr	T
Glycine	Gly	G	Tyrosine	Tyr	Y
Isoleucine	Ile	I			

Table 2. Molecular weight and % of purity of the peptide synthesized and cleavage.

Sequence	% yield	% purity	$[M + H]^+$
1. YAGID	92 %	93 %	537
2. YAGFDAGFT	89 %	90 %	946
3. YLGADLGAT	90 %	90 %	880
4. YLLLDLLLT	82 %	80 %	1076
5. YFFFDFFFT	70 %	85 %	1280
6. YGGGDGGGT	55 %	60 %	739
7. YAAADAAAT	85 %	95 %	823
8. YCCCDCCCT	75 %	60 %	1016

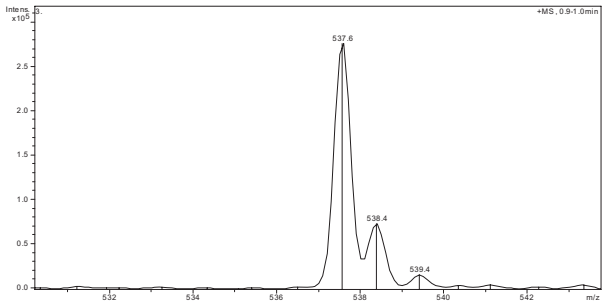


Fig. 1. Mass spectrum of peptide **Tyr**-Ala-Gly-Ile-**Asp**.

In the electrospray ionization mass spectra (Figure 1) was determined peak [M + H] at 537.6 m/z with a 100% isotopic abundance corresponding to the expected molecular weight of 537 also in the mass spectrum shows the peak [M + H] + 1 to 538.4 m/z with an isotopic abundance of 25.5% corresponding to the number of carbon atoms present in the peptide (Table 3).

The peptide **Tyr**-Ala-Gly-Phe-**Asp**-Ala-Gly-Phe-**Thr** which remains in the first position with a tyrosine residue, position five with aspartic acid residue and position nine with threonine residues obtained this sequence by the methodology of synthesis assisted by microwave was analyzed by HPLC using as mobile phase a mixture of acetonitrile/water (20/80) with 0.1% formic acid, presenting a retention time of 4.5 minutes. In the mass spectrum by electrospray ionization was determined peak [M + H] at 946.6 m/z with a 100% isotopic abundance corresponding to the expected molecular weight of 946 also in the mass spectrum (Figure 2) shows the peak [M + H] + 1 to 947.6 m/z with an isotopic abundance of 55.3% corresponding to the number of carbon atoms in the peptide and the peak [M + H] + 2 at 948.6 m/z with an isotopic

Table 3. Signals presents in the mass spectrum of peptide **Tyr**-Ala-Gly-Ile-**Asp**.

Signal	m/z	Intensity	% Relative isotopic abundance
[M+H]	537.6	183994	100
[M+H]+1	538.4	47096	25.5
[M+H]+2	539.4	11451	6.2

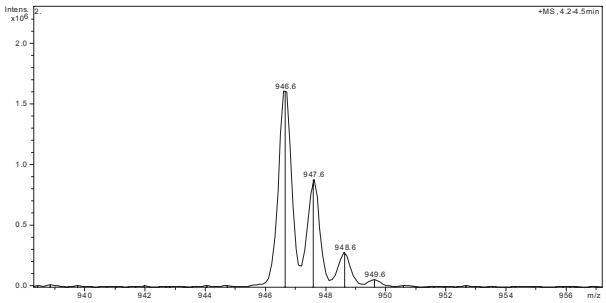


Fig. 2. Mass spectrum of peptide **Tyr**-Ala-Gly-Phe-**Asp**-Ala-Gly-Phe-**Thr**.

Table 4. Signals presents in the mass spectrum of peptide **Tyr**-Ala-Gly-Phe-**Asp**-Ala-Gly-Phe-**Thr**.

Signal	m/z	Intensity	% Relative isotopic abundance
[M+H]	946.6	1610231	100
[M+H]+1	947.6	881925	55.3
[M+H]+2	948.6	287796	17.8
[M+H]+3	949.6	60201	3.7

abundance of 17.8% corresponding to the number of nitrogen atoms (Table 4).

The peptide **Tyr**-Leu-Gly-Ala-**Asp**-Leu-Gly-Ala-**Thr** was analyzed by HPLC using as mobile phase a mixture of acetonitrile/water (20/80) with 0.1% formic acid, shows a retention time of 2.65 minutes. In the mass spectrum by electrospray insertion was determined peak [M + H] at 879.9 m/z with a 100% isotopic abundance corresponding to the expected molecular weight of 879 also in the mass spectrum shows the peak [M + H] + 1 to 880 m/z and [M + H] + 2 to 881 m/z. Peptide **Tyr**-Leu-Leu-Leu-**Asp**-Leu-Leu-Leu-**Thr** shows a retention time of 1.65 minutes with a yield of 80% for peptide **Tyr**-Phe-Phe-Phe-**Asp**-Phe-Phe-Phe-**Thr** the retention time is 2.746 minutes with a yield of 70% and purity of 85%. The sequence with high percent of residue of glycine **Tyr**-Gly-Gly-Gly-**Asp**-Gly-Gly-Gly-**Thr** has the lowest yield and purity because the flexibility in the liberty grades of the peptide can adopt different conformations.

The methodology was compared; microwave and RT. The result shows that purity was better in microwave conditions than RT. The low yield and purity of the peptide obtained at RT conditions was mainly due to small peptides, which are formed as byproducts.

Conclusions

The methodology for the synthesis of peptides on solid phase assisted by microwave has the advantage of reducing the reaction times for the peptide synthesis unlike the synthesis at RT, which was 24 h per protocol in the coupling reaction and deprotection reaction. Using the microwave assisted synthesis the time to obtain a peptide sequence of nine amino acid residue takes less than one day in high yield and purity.

Experimental Section

General. All reagents were purchased in the higher quality available and were used without further purification. The solvents used in column chromatography were obtained from commercial suppliers and used without further distillation. Infrared spectra (FTIR) were recorded on a Perkin Elmer FT-IR 1600 spectrophotometer. Liquid chromatograms (HPLC) were obtained on an Agilent 1100 Series LC with a reverse

phase ZORBAX sβ-C18 column (5 mm, 3 × 150 mm) and MSD Trap. Electrospray ionization mass spectra (ESI-MS) were obtained with an ion trap, and the intensities are reported as a percentage relative to the base peak after the corresponding *m*/*z* value. Microwave assisted synthesis was developed in a CEM Discover™ Focused Synthesizer.

Activation of Rink resin. To a mixture of Rink-Fmoc resin (1 g, 1.4 mmoles) in NMP (10 mL), was added 20 mL a solution of piperidine/Dicholomethane (20%) and stirred for 24 h at RT. The resin was filtered and washed (3 × 10mL) with DMF, MeOH and CH_2Cl_2 and dried for 2 h under reduced pressure. The resin was characterized by FT-IR (KBr): 3380, 3059, 2853, 1676, 1591, 1498, 1452, 1379, 1205, 1028, 751 cm^{-1} and the Kaiser's test procedure was carried out by dual protocol.

Incorporation of the first amino acid. To a mixture of Rink-NH_2 resin (1.0 g, 1.4 mmoles) in NMP (10 mL), was added; tyrosine (1.608 g, 7 mmol), HBTU (1.327 g, 7 mmoles), DIC (0.883 g, 7 mmol) and a solution of DIPEA (0.271 g, 4.2 mmol) in NMP (2 mL), the mixture was stirred for 24 h at RT. The resin was filtered and washed (3 × 10mL) with DMF, MeOH and CH_2Cl_2, and dried for 2 h under reduced pressure. The resin was characterized by FT-IR (3022, 2924, 1666, 1496, 1382, 1250, 1034, 763) cm^{-1}. Kaiser's test procedure was carried out by dual protocol.

Synthesis of peptide histamine H2 receptors on Rink-Tyrosine resins assisted by microwave. To a mixture of Rink-Tyrosine-Fmoc resin (0.2 g, 0.28 mmol) was removed the Fmoc group to be the amine free, with a solution of piperidine/Dicholomethane (20%). The mixture was assisted by microwave (75°C, 50 W, 5 minutes) with dual protocol, the resin was washed (3 × 10mL) with DMF and CH_2Cl_2. The resin was monitored with Kaiser's test procedure for primary amines. The next amino acid coupling was carried out. To the resin was added a solution of HBTU (0.2654 g, 1.4 mmol) in NMP (2 mL), the amino acid (1.4 mmol) in NMP (2 mL), DIC (0.035 g, 0.28 mmol) and DIPEA (0.055 g 0.84 mmol) in NMP (1 mL). The mixture was assisted by microwave (75 °C, 40 W, 5 min) with two protocols. The resin was washed (3 × 10 mL) with DMF and CH_2Cl_2. The resin was monitored with Kaiser's test procedure for primary amines. These procedures of coupling and deprotection were carried on until the desired peptide sequence was obtained. The yields were measured in the crude and the purification was carried out in cold ethyl ether (5 × 10 mL).

Acknowledgements

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