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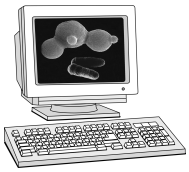


A convenient microwave-enhanced solid-phase synthesis of short chain *N*-methyl-rich peptides

Hortensia Rodríguez,[a,b]* Margarita Suarez[b] and Fernando Albericio[a,c,d]*

Structural modification of the peptide backbone via *N*-methylation is a powerful tool to modulate the pharmacokinetic profile and biological activity of peptides. Here we describe a rapid and highly efficient microwave(MW)-assisted Fmoc/tBu solid-phase method to prepare short chain *N*-methyl-rich peptides, using Rink amide p-methylbenzhydrylamine (MBHA) resin as solid-phase support. This method produces peptides in high yield and purity, and reduces the time required for Fmoc-*N*-methyl amino acid coupling. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: solid-phase peptide synthesis; microwave; Fmoc-*N*-methyl amino acids

Introduction

The replacement of natural amino acids for *N*-methyl amino acids in biologically active peptides has resulted in analogs with improved pharmacological properties, such as enzymatic stability [1], receptor selectivity [2], enhanced potency [3], and bioavailability [4,5]. The *N*-methylation of backbone confers high affinity toward the targets, proteolytic stability, membrane permeability, and conformational rigidity to the peptides. Thus, in peptide chemistry *N*-methylation is considered as one of the most attractive and subtle modifications of a peptide structure [6].

In SPPS, protocols to generate a very wide range of peptides are well established, although the coupling of *N*-methyl amino acids generally occurs in low yield and in many cases requires expensive coupling reagents and double coupling. The most promising reagents used to couple *N*-methylated amino acids are bis(2-oxo-3-ox-azolidinyl)phosphonic chloride (BOP-Cl) [7], HATU/HOAt [8] bis-(trichlororomethyl)carbonate (BTC) [9], and (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) or PyBOP/HOAt [10].

Furthermore, since 1986, microwave (MW) irradiation has been applied to promote difficult organic reactions [11] and a considerable number of articles have been published in the field of MW-assisted organic chemistry [12]. However, the use of MW irradiation in combination with SPPS has extended only in recent years [13] as a result of the commercial availability of specialized equipment for chemistry-dedicated MW applications with built-in direct temperature control, magnetic stirring, and software for temperature and pressure control.

On the basis of a previous report on the synthesis of difficult peptide sequences using the MW irradiation [14], and the improved results in terms of yields and purities with the use of standard HOBt/*N*,*N*-diisopropylcarbodiimide (DIPCDI) couplings [15], here we describe MW-assisted SPPS (**MW-SPPS**) with Fmoc/tert-butyl (tBu) strategy of short chain *N*-methyl-rich peptides.

Experimental Procedures

General

Resin preparation

Fmoc Rink amide MBHA resin (0.015 mmol) was placed in a peptide synthesis vessel, swollen in DMF, and deprotected with 5 ml of 20% piperidine/DMF for 4 min.

Washings between the first deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 0.5 min) and DCM (5 × 0.5 min) using 10 ml of solvent/g of resin each time.

MW-SPPS

Protected amino acid (3 eq.) and HOBt (3 eq.) in DCM (1–3 ml/g resin), followed by DIPCDI (3 eq.) were sequentially added to the resin and the mixture was irradiated in a *CEM Discover* MW for 20 min at 250 W with a maximum temperature of 35 °C.

* *Correspondence to: Hortensia Rodríguez, Laboratorio de Síntesis Orgánica, Facultad de Quimica, Universidad de La Habana, 10400-Ciudad Habana, Cuba. E-mail: horten@fq.uh.cu*

Fernando Albericio, Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain. E-mail: albericio@pcb.ub.es

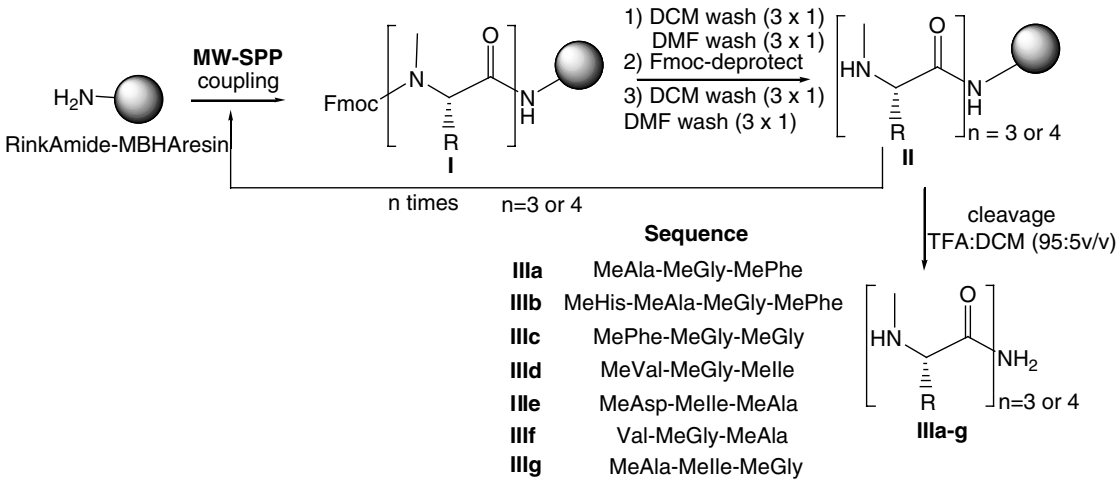
a *Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain*

b *Laboratorio de Síntesis Orgánica, Facultad de Quimica, Universidad de La Habana, 10400-Ciudad Habana, Cuba*

c *CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain*

d *Department of Organic Chemistry, University of Barcelona, MartíiFranqué 1-10, 08028-Barcelona, Spain*

Journal of PeptideScience



Sequence	
IIIa	MeAla-MeGly-MePhe
IIIb	MeHis-MeAla-MeGly-MePhe
IIIc	MePhe-MeGly-MeGly
IIId	MeVal-MeGly-MeIle
IIIe	MeAsp-MeIle-MeAla
IIIf	Val-MeGly-MeAla
IIIg	MeAla-MeIle-MeGly

Scheme 1. Fmoc/tBu SPPS of short chain *N*-methyl-rich peptides (**IIIa–g**).

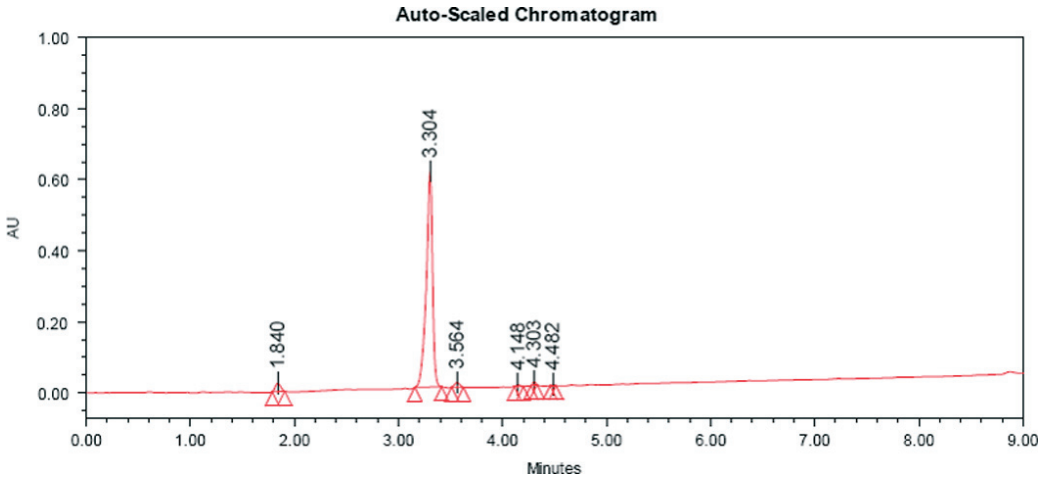


Figure 1. RP-HPLC of (NMe)Ala-(NMe)Gly-(NMe)Phe (**IIIa**).

Manual removal of the Fmoc-protecting group

Two treatments with piperidine : DMF (2 : 8, v/v) for 10 min, and two extra 5-min treatments with piperidine : DBU : toluene : DMF (5 : 5 : 20 : 70, v/v) were used.

Cleavage of the peptides

Final unprotected amide peptides were cleaved from the resin with the following cleavage cocktails : TFA : DCM (95 : 5 v/v) for 90 min (10 ml/g resin). Peptides were precipitated by addition of cold diethyl ether, the solution was decanted, and the solid was triturated with cold diethyl ether, which was decanted again. This process was repeated twice.

Results and Discussion

The short chain peptides were synthesized using a Fmoc/tBu-based strategy with stepwise coupling, and Rink amide MBHA was used as solid phase support (Scheme 1). MW irradiation was provided by a *CEM Discover* LabMate Focused Single Mode MW Synthesis System, which produced continuous stirring and irradiation with control of pressure and temperature [16].

According to standard SPPS protocols, the coupling time per residue at room temperature usually varies between 2 and 4 h. The coupling time was shortened to approximate 80 min by raising the temperature to 50 °C [17], and it has been reported that, under conventional coupling conditions, when only the acid component in the coupling is *N*-methylated, it proceeds well under standard coupling. However, when both components are *N*-methylated, as in our case, conventional couplings pose a challenge and can present some problems [9,10].

The **MW-SPPS** method provided a satisfactory coupling efficiency in 20 min and no secondary amines were detected by qualitative De Clercq [18] and chloroanyl tests [19]. Although, there have been a few reports stating that the peptide resin is well established on solid support at 50 °C [13e], we kept the reaction temperature below 40 °C under MW irradiation to prevent undesired side reactions.

The Fmoc removal of peptidyl resins can be accelerated by MW irradiation [20,14c]. However, to ensure complete Fmoc removal, we followed standard treatments with 20% piperidine in DMF solutions and two extra treatments of 5 min with piperidine : DBU : toluene : DMF (5 : 5 : 20 : 70, v/v) as a previous report showed that this treatment was optimal for avoiding

Table 1. Results from the MW-SPPS and theoretical and experimental masses of highly methylated short chain peptides

Product	Sequence	Theoretical MS	Experimental[a] MS (M+H)	Purity[b] %
IIIa	MeAla-MeGly-MePhe	334.20	335.20	93.7
IIIb	MeHis-MeAla-MeGly-MePhe	485.28	486.28	68.7
IIIc	MePhe-MeGly-MeGly	320.18	321.15	77.3
IIId	MeVal-MeGly-MeIle	328.25	329.25	97.3
IIIe	MeAsp-MeIle-MeAla	358.22	359.26	81.4
IIIf	MeVal-MeGly-MeAla	286.21	287.32	78.2
IIIg	MeAla-MeIle-MeGly	300.22	300.38	79.4

[a] Experimental masses obtained by HPLC-MS and exact masses.
[b] Purity of crude peptides (analytical RP-HPLC peak area, UV absorbance at 220 nm). The chromatograms are reproduced in Figure 1 and the supporting information.

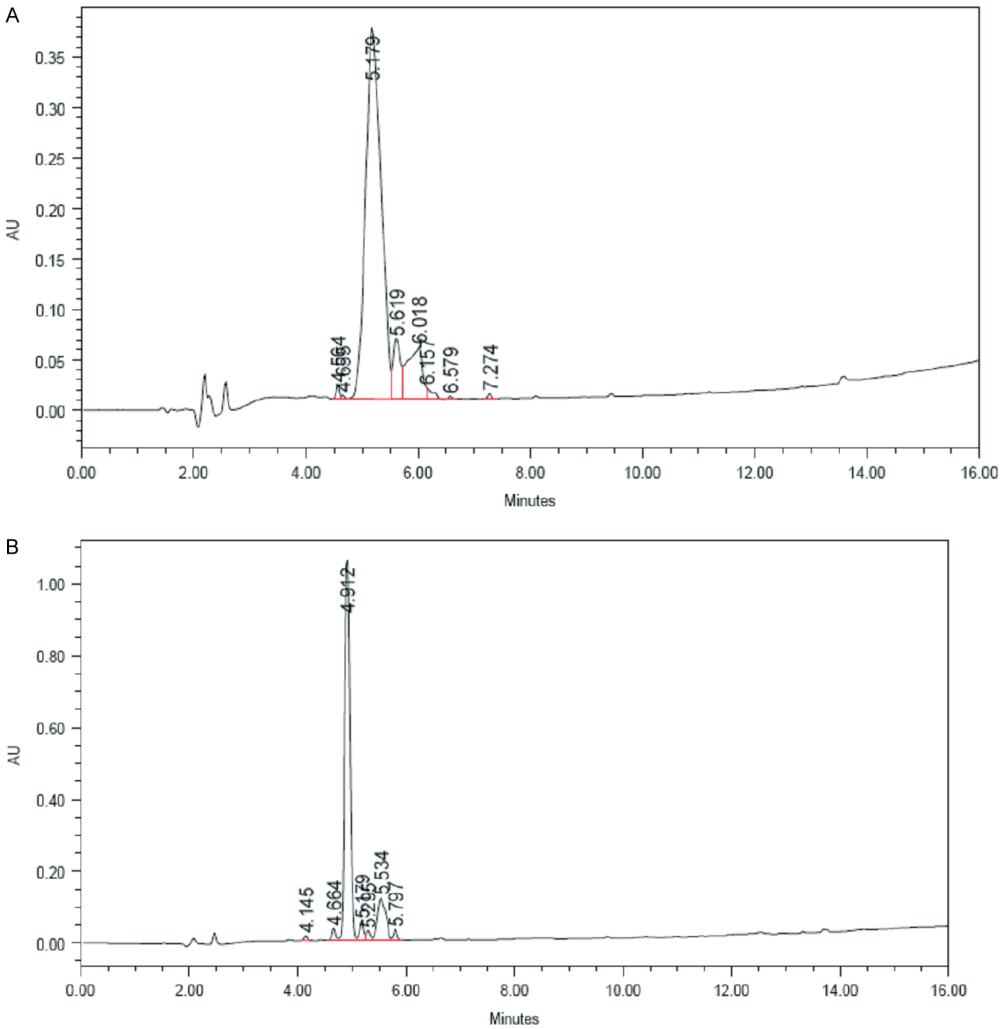


Figure 2. RP-HPLC of (NMe)Asp(NMe)Ile(NMe)Ala (**IIIe**) at (a) room temperature and (b) 60 °C.

incompleted deprotection for *N*-methylpeptidyl resins [10]. Final unprotected amide was cleaved from the resin by treatment with TFA : DCM (95 : 5 v/v) (Scheme 1, SI-1).

The crude peptides were analyzed by HPLC-MS. Table 1 shows the purity and the theoretical and experimental masses of the highly *N*-methylated peptides obtained. All *N*-methyl peptides were obtained in reasonable purity. See supporting information for full characterization. As an example see Figure 1.

In order to check the true effect of the MW heating mode on the solid-phase synthesis (SPS), we attempted to obtain at least two *N*-methyl-rich peptide sequences (**IIIa** and **IIIg**) by the conventional method, using the same conditions as under MW synthesis (coupling reagents, time of coupling, temperature, cleavage conditions, vessels). In both cases, peptides were obtained with very low purity, even after double and triple coupling, and using HOAt-based methods.

Journal of PeptideScience

Teixedó *et al*. [10] have described the SPS and characterization of *N*-methyl peptides by chromatography. They observed multiple peaks in chromatogram profiles for peptides containing clusters of three or more consecutive *N*-methylamino acids at the *C*-terminal as a result of the slow conversion between individual conformers.

The highly *N*-methylated tri- and tetra-peptides synthesized under MW-SPPS conditions generally showed profiles with narrow symmetrical peaks in RP-HPLC chromatograms (Figure 1 and supporting information). However, peptide **IIIe** (Sequence: MeAsp-MeIle-MeAla) presented a broad peak in the chromatogram profile, which may be due to the presence of high populations of conformers that differ in their hydrophobic character, and therefore in retention times (Figure 2(a)). This was confirmed by the dramatic effect on the peak shape for an increase in column temperature, since it changed from a broad to uniform sharp profile as a consequence of accelerated *cis*/*trans* isomerism (Figure 2(a) and (b)). The broad peak in the chromatogram profile of **IIIe** is a consequence of the presence of two very close bulky *N*-methyl-amino acid residues in this sequence and (NMe)Asp in the *N*-terminal position. The free carboxyl group present in this *N*-methyl amino acid could generate associations by hydrogen bonds and promote slower *cis*/*trans* interconversion of this peptide.

Our results indicate that the use of MW irradiation in the synthesis of small *N*-methyl-rich peptides dramatically reduces the reaction time and increases the purity of the product. One key feature in MW-assisted chemical reactions is the dipolar polarization mechanism. In this process, the alternating electric field from MW radiation provides the energy required for the rotation of the molecules with a dipole moment. Unlike conventional heating, MW energy activates any molecule with a dipole moment, thereby resulting in rapid heating at the molecular level. Since peptide backbones are polar, this mechanism is useful in preventing the aggregation of a growing peptide chain during the coupling reactions in SPPS, thereby improving the coupling efficiency. In addition, the technology used (*CEM Discover*) allows the control of temperature at 35 °C.

Conclusions

The benefits obtained by MW-SPPS using standard reagents compared to conventional SPPS for the synthesis of highly hindered *N*-methyl-rich peptides are as follows: reduction of reaction times (each coupling cycle can be efficiently performed in 20 min, instead of 1 or 2 h); higher yields in terms of purity, possibly because of the enhancement of the kinetics and thermodynamics of coupling steps in SPPS; and reproducible reaction conditions as a result of the control mechanism of reaction parameters, i.e. temperature and pressure. This procedure could be used for the future synthesis of long chain *N*-methyl-peptides and other difficult peptide sequences.

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Supporting Information

Principal results, characterization of short chain *N*-methyl rich peptides, details of HPLC-measurements. This material is available free of charge via the Internet.

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