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Solid-Phase Peptide Synthesis in Water Using Microwave-Assisted Heating

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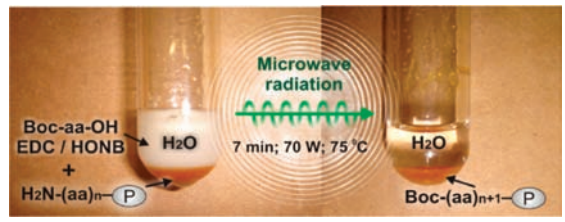
ABSTRACT



An approach using water as a solvent (coupling and deprotection) was developed for the solid-phase synthesis of peptides using the most common Boc-amino acid derivatives. Key aspects of this methodology are the use of a PEG-based resin, EDC-HONB as a coupling method, and microwave irradiation as an energy source.

Solid-phase synthetic (SPS) methods have made peptide synthesis simple, rapid, and easily subject to automation.[1] The solid-phase method is now the principal method for peptide synthesis, but it requires a large amount of organic solvent. As the safe disposal of organic solvent waste is an important environmental issue, a method for peptide synthesis in water would be desirable.[2] To carry out solid-phase peptide synthesis (SPPS) in water, researchers have so far focused on developing novel hydrophilic *N*-protecting groups for amino acids in order to improve the water solubility of the derivatives[3] because the conventional protecting groups (e.g., Z-, Boc-, and Fmoc- groups) are hydrophobic. Alternatively, more complicated synthesis methods have been reported such as enzymatic catalysis of coupling reactions[4] and the use of nanoparticulated derivatives.[5]

Typically, all steps in the SPPS cycle are carried out at room temperature. However, significant improvements for difficult peptide sequences have been obtained by performing peptide coupling steps at elevated temperatures (30−80 °C) using thermal heating[6] and more recently by MW irradiation.[7]

Herein we report the development of a water-based SPPS method using low cost commercially available Boc-derivatives and coupling reagents, without any preparation or modification, and microwave (MW) technology for rapid and efficient temperature elevation.

Initially, several coupling agents were tested and evaluated for their coupling efficiency in water using MW irradiation. Several protected/masked amino acids were tested at different

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pHs and various reaction times using Rink Amide Tentagel as solid support. In our hands, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydro-chloride (EDC hydrochloride)[8] in combination with *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide (HONB)[9] rendered the best yields when compared with other coupling agents such as *N*-hydroxybenzotriazole (HOBt),[10] *N*-hydroxysuccinimide (HOSu),[11] 2-mercaptobenzothiazole,[12] and ethyl cyanoglyoxalate-2-oxime (Oxyma)[13] (data not shown). However, the formation of the active esters in water was a rapid reaction, as expected decomposition occurred faster in water than in DMF, and it was further enhanced at high temperature. Therefore, a one minute short preactivation time[14] and relatively short reaction times (7 min) were used.

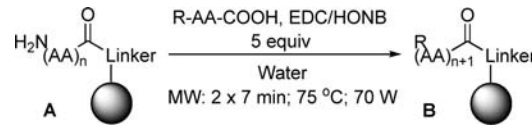
On the basis of the above results, Leu-Enkephalin (Leu-Enk; H-YGGFL-OH, an endogenous opioid peptide neurotransmitter found naturally in the brain of many animals) was used as a model to evaluate MW-assisted SPPS in water. Screening for the best protection/masking of the N^{α}-function of the amino acids was carried out. The most common protecting groups used in SPPS, Boc, and Fmoc, together with the N^{α}-azido modified residues, were investigated.[15] We reasoned that azido acids should be particularly useful due to their more hydrophilic character compared to Boc and Fmoc. For these preliminary experiments, each amino acid derivative was coupled (by classical SPPS using organic solvents at room temperature) to a presynthesized resin-bound oligopeptide (Table 1). Different commercially available supports (TentaGel, ChemMatrix, polystyrene, and NovaGel) and linkers (Rink Amide, hydrazinobenzoic, and HMBA) were compared.

Thus, each amino acid derivative was coupled twice to the resin-bound peptide sequence (9 μmol scale) using a 5-fold excess of the masked-AA/EDC/HONB coupling mixture in combination with a CEM Discover MW reactor with temperature control via a standard IR thermometer and applying 70 W power of MW radiation for 7 min at 75 °C (Table 1; see Supporting Information).

Extending the reaction time did not improve yields, and a temperature range of 70−80 °C was sufficient for complete coupling (data not shown). Coupling reactions at a lower temperature required an extended reaction time (>1 h), but the prolonged reaction times resulted in formation of several side-products. The reactions were performed in deionized water and the pH changed upon adding the reagents (approximately 3.5−4.5 initially, and 3.0−4.0 after the end of the reaction). Coupling reactions at higher pH values generated a variety of impurities.

Boc derivatives in combination with Rink Amide Tentagel rendered high coupling efficiency (Table 1, entries 2, 5, 8, and 17), providing products with a purity ranging from

Table 1. Screening of Amino Acid Protecting Groups for the MW-Assisted SPPS of Leu-Enk in Water



	coupling reaction				results[b]	
entry	AA	A[a]	B	X	% A[c]	% B[d]
1	X-F	H_2N-L-P	X-FL-P	Fmoc	nd	58.3
2				Boc	nd	78.4
3				N_3	nd	48.4
4	X-G	H_2N-FL-P	X-GFL-P	Fmoc	4.5	69.0
5				Boc	0.3	93.7
6				N_3	38.9	32.6
7	X-G	H_2N-GFL-P	X-GGFL-P	Fmoc	0	59.1
8				Boc	0	97.5
9				N_3	68.9	21.1
10	X-G	H_2N-GFL-P	X-GGFL-P	Fmoc	0	61.2
11				Boc	0	98.3
12				N_3	53.5	20.1
13	X-G	H_2N-GFL-P	X-GGFL-P	Fmoc	90.5	6.4
14				Boc	91.4	5.4
15				N_3	87.6	0
16	X-Y	H_2N-GGFL-P	X-YGGFL-P	Fmoc	2.2	74.6
17				Boc	0.9	83.5
18				N_3	31.6	27.0
19	X-Y	H_2N-GGFL-P	X-YGGFL-P	Fmoc	0.9	69.6
20				Boc	0	82.9
21				N_3	41.4	33.3
22	X-Y	H_2N-GGFL-P	X-YGGFL-P	Fmoc	81.4	2.8
23				Boc	83.2	3.7
24				N_3	88.8	0
25	X-G	H_2N-GFL-P	X-GGFL-P	Boc	0	97.9
26	X-Y	H_2N-GGFL-P	X-YGGFL-P	Boc	0	85.2
27	X-V	H_2N-GF-P	X-VGF-P	Boc	0	85.0
28	X-R[e]	H_2N-GF-P	X-RGF-P	Boc	0	100
29	X-C[f]	H_2N-GF-P	X-CGF-P	Boc	0	96.0

[a] Peptides were prepared using classical SPPS with organic solvents. P (in brown) = Rink Amide TentaGel; (in gray): P (in brown) = Rink Amide ChemMatrix; (in cyan): P (in brown) = Rink Amide Polystyrene; (in red): P (in brown) = Hydrazinobenzoyl NovaGel. [b] Yields were obtained by analytical RP-HPLC analysis and after proper workup and cleavage of the peptides. [c] Unreacted A oligopeptide. [d] Target synthesized oligopeptide. [e] Boc-Arg(Tos)-OH. [f] Boc-Cys(Acm)-OH.

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(14) Approximately one minute after the addition of the coupling reagents into the amino acid derivative solution, the solvent becomes nebulous due to the formation of the active ester (see Supporting Information).

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80−98% (yield 86−94%). Coupling reactions of Boc-derivatives provided almost complete coupling reactions as only traces of unreacted starting material (up to 0.9%) were detected. The Fmoc-protected amino acids (Table 1, entries 1, 4, 7, and 16) did not give such high coupling efficiency as observed with the Boc-protected amino acids, probably due to the bulky and more hydrophobic character of the Fmoc group compared to the Boc group. On the other hand, the azido acids (Table 1, entries 3, 6, 9, and 18) gave low coupling yields (purity: 21−48%), and significant amounts of unreacted starting material were detected (30−70%) in the individual coupling steps.

In comparison, using Rink Amide (RAM) polystyrene (instead of Tentagel) as a solid support resulted in very low coupling efficiency (Table 1, entries 13−15 and 22−24) regardless of the protection/masking of the *N*-function of the amino acids, while the RAM-ChemMatrix support (Table 1, entries 10−12 and 19−21) gave couplig efficiencies similar to those obtained with the use of RAM-Tentagel. Using Hydrazinobenzoyl NovaGel (HZB-NovaGel) in combination with Boc-protected amino acid derivatives (Table 1, entries 25 and 26) resulted in high coupling efficiency comparable with the results for RAM-Tentagel and RAM-ChemMatrix.

To study whether other more lipophilic Boc-protected amino acids could be solvated and give high coupling yields, Boc-protected valine, arginine (Tos-protected side chain), and cysteine (Acm-protected side chain) were coupled to a solid-phase bound dipeptide (Gly-Phe-RAM-TentaGel) using the same reaction conditions as described earlier (Table 1, entries 27−29). All the Boc-protected amino acids gave products with a purity of 85% or better. These results demonstrate that hydrophobic amino acids or derivatives with bulky side chain protecting groups are compatible with microwave-assisted SPPS synthesis in water when a hydrophilic solid support is used.

Once the best coupling conditions, protecting group and solid support were determined, the complete aqueous Boc SPPS of Leu-Enk was carried out with MW irradiation (scale: 0.042 mmol). The TFA-labile Rink Amide linker is not suitable for the preparation of peptides using the Boc strategy. Instead, hydrazinobenzoic acid (HZB) and 4-hydroxymethylbenzoic acid (HMBA) were used because both linkers are compatible with Boc chemistry.

The synthesis was carried out using the commercially available HZB-NovaGel resin or the preprepared HZB-ChemMatrix as the solid support due to the required mild cleavage conditions.[16] For comparison, an HMBA[17]- functionalized ChemMatrix solid support was also prepared and used. Each amino acid derivative was coupled (twice) on the solid phase using the MW reactor as described above (70 W MW power; 75 °C; 7 min).

The Boc group was removed using 1 N HCl in water (MW heating; 70 W; 3 × 7 min; 70 °C). Hydrochloric acid was

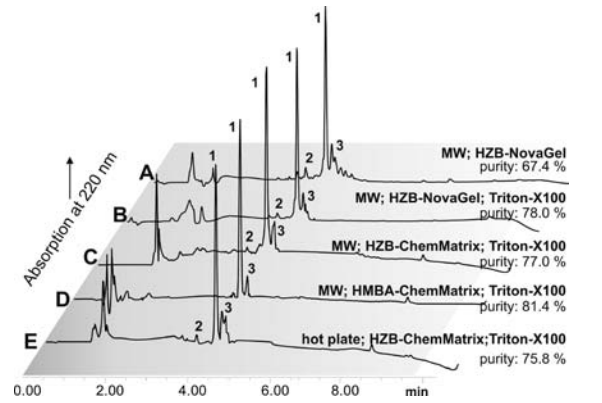

Figure 1. Analytical HPLC chromatograms of crude Leu-Enk synthesized by SPPS in water using: MW reactor, HZB-NovaGel resin without (A) and with (B) 0.5% Triton-X100, (C) MW reactor, HZB-ChemMatrix resin with 0.5% Triton-X100, (D) MW reactor, HMBA-ChemMatrix resin with 0.5% Triton-X100, and (E) hot plate at 83 °C, HZB-ChemMatrix resin with 0.5% Triton-X100. In all cases, the reaction time was set to 2 × 7 min. Numbered peaks correspond to: 1, Leu-Enk; 2, [desLeu]-Leu-Enk; 3, $[\text{Leu-Enk}]_{MW}$ + 135.0.[18] HPLC system: see Supporting Information.

first introduced by Merrifield for the removal of the Boc-group during SPPS using acetic acid instead of water as the solvent.[19] Diluted HCl in water can also be regarded as a more environmentally friendly reagent compared to the common use of TFA in the classical Boc -SPPS strategy. Careful stirring was used in both coupling and deprotection experiments, in order to avoid volumetric heating without gradients.[6,20]

The use of MW irradiation (2 × 7 min) at 75 °C (Figure 1A) using hydrazine-AM-NoveGel gave the target peptide at 67% purity. Small amounts of byproducts and failure sequences were observed. The main byproduct is the result of a Lossen rearrangement after the attack of a nucleophile at the Boc-AA-ONB active ester (3 in Figure 1), similar to the earlier proposed side reaction when HOSu is used in classical SPPS procedures.[18]

The same experiment was also repeated with the addition of 0.5% Triton-X100 detergent in the coupling reactions, as well as in the swelling and washing steps. This zwitterionic detergent is known to improve coupling efficiency in classical SPPS[21] as well as in the aqueous environment,[3a,e] due to its ability to improve both the aqueous solubility of the derivatives as well as the swelling properties of the resin. Indeed, the combination of MW-assisted heating with the

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zwitterionic detergent enhanced the synthesis efficiency of the pentapeptide to 78% purity (Figure 1B). Only traces of side products were detected. The peptide was obtained at 72% isolated yield after HPLC purification. A ^{1}H NMR spectra of this sample showed no traces of racemization (see Supporting Information).

MW-assisted SPPS of Leu-Enk using HZB- or HMBA-ChemMatrix as the solid support and the addition of 0.5% Triton-X100 detergent generated the target peptide at 77 and 81% purity, respectively (Figure 1C, D). This slightly enhanced synthetic yield following the use of the HMBA linker is the result of the attachment of the first Leu derivative onto the hydroxyl-functioned linker by traditional esterification using organic solvents (see Supporting Information). In this case, the [desLeu]LeuEnk byproduct is absent.

Although the MW technology covers the requisites for the rapid and efficient synthetic approach in water, the same sequence was prepared using thermal heating (hot plate) in order to investigate the generality of the strategy. Each amino acid coupling was carried out for 7 min. The Boc group was removed using 1 N HCl in water (hot plate; 3 × 7 min; 70 °C). The same type of vial and stirring was used in these experiments as for the experiments using MW-assisted heating.

The SPPS in water using thermal heating was first tested at 75 °C using HZB-NovaGel resin without Triton X-100, providing poor results compared to the relative synthesis by MW technology. When the temperature was increased to 83 °C (see Supporting Information, Figure S5) the target product was obtained with similar purity as for MW at 75 °C.

Thus, the synthesis of Leu-Enk was repeated using the HZB-ChemMatrix resin by thermal heating at 83 °C with the addition of 0.5% TritonX-100, yielding the peptide at 76% purity (Figure 1E), comparable with the MW irradiation at 75 °C (Figure 1C, 77%). The results demonstrate that the SPPS strategy is compatible with both the MW and thermal heating and are consistent with a previous study in which nearly identical results were obtained for SPPS in organic solvents when thermal heating and MW-assisted heating were compared.[7c] The observed differences between the MW and conventional heating are probably due to inaccurate temperature measurements and/or the use of different temperature monitoring systems. However, we find the MW reactor more easy to use and it is a more rapid technique.

The general protocol of the MW strategy is shown in Table 2. The protocol differs from standard SPPS in the almost complete absence of organic solvents; only small amounts of MeOH are required for optimal washing after Boc-removal.

In conclusion, MW-assisted SPPS with water as a solvent for coupling and deprotection, in combination with Boc-protected amino acids, EDC/HONB as coupling reagents and the use of zwitterionic detergent provided Leu-Enkephalin at high yield and purity, and free of racemization. This synthetic approach uses commercially available derivatives and agents without any specific pretreatment at any step of the procedure. This result is, to the best of our knowledge, the first reported SPPS in which the entire peptide has been synthesized using water as a solvent for coupling and deprotection.

Table 2. Optimized Synthetic Procedure for the SPPS in Water in the Presence of a Zwitterionic Reagent

step	reagents	time (min)
swelling	0.5% Triton X100 aq	5 × 1
coupling	Boc- aa/EDC/HONB (5 equiv)	2 × 7
	0.5% Triton X100 aq,	
	MW; 75 °C; 70 W	
washing	H_2O	5 × 1
	0.5% Triton X100 aq	10 × 1
	H_2O	5 × 1
Boc-removal	1N HCl aq, MW; 70 °C; 70 W	3 × 7
washing	H_2O	5 × 1
	MeOH: H_2O (1:1)	3 × 1
	H_2O	10 × 1
neutralization	10% DIEA in H_2O	4 × 5
washing	H_2O	10 × 1
	0.5% Triton X100 aq	3 × 1
	H_2O	10 × 1

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Supporting Information Available: Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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