Aqueous Microwave-Assisted Solid-Phase Peptide Synthesis Using Fmoc Strategy. III.
Racemization Studies and Water-Based Synthesis of Histidine-Containing Peptides

Keiko Hojo^{a,b,*}, Natsuki Shinozaki^a, Koushi Hidaka^a, Yuko Tsuda^a, Yoshinobu Fukumori^a, Hideki Ichikawa^a and John D. Wade^{b,c,*}

^a Faculty of Pharmaceutical Sciences & Cooperative Research Center of Life Sciences, Kobe Gakuin University, Chuo-ku, Kobe 650-8586, Japan; ^b The Florey Institute of Neuroscience and Mental Health, and ^c School of Chemistry, University of Melbourne, Victoria 3010, Australia

* Correspondence to: Keiko Hojo, E-mail: hojo@pharm.kobegakuin.ac.jp, Tel: 81-78-974-4005, Fax: 81-78-974-5689; John D. Wade, E-mail: john.wade@florey.edu.au, Tel: 61-3-8344-7285, Fax: 61-3-9348-1707.

Abstract

In this study, we describe the first aqueous microwave-assisted synthesis of histidine-containing peptides in high purity and with low racemization. We have previously shown the effectiveness of our synthesis methodology for peptides including difficult sequences using water-dispersible 9-fluorenylmethoxycarbonyl-amino acid nanoparticles. It is an organic solvent-free, environmentally friendly method for chemical peptide synthesis. Here, we studied the racemization of histidine during an aqueous-based coupling reaction with microwave irradiation. Under our microwave-assisted protocol using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, the coupling reaction can be efficiently performed with low levels of racemization of histidine. Application of this water-based microwave-assisted protocol with water-dispersible 9-fluorenylmethoxycarbonyl-amino acid nanoparticles led to the successful synthesis of the histidine-containing hexapeptide neuropeptide W-30 (10-15), Tyr-His-Thr-Val-Gly-Arg-NH₂, in high yield and with greatly reduced histidine racemization.

Keywords Histidine, microwave-assisted synthesis, nanoparticles, racemization solid-phase peptide synthesis, synthesis in water

Introduction

Solid-phase methods have made peptide synthesis simple, rapid, and easily adapted to automation. It is now the principal method for chemical peptide synthesis. However current solid-phase procedures generally require a large volume of organic solvents, the safe disposal of which is an important environmental issue. The development of synthesis methods that do not use organic solvents would be desirable (Anastas et al. 1998; Winterton 2001, Sheldon 2007). In recent years, we have been driven to focus on the development of a method for peptide synthesis in water which is an environmentally friendly solvent (Hojo et al. 2001, 2004a, 2004b, 2007, 2008, 2011, 2012, 2013). Solid-phase peptide synthesis (SPPS) is commonly performed *via* a base-labile 9-fluorenylmethoxycarbonyl (Fmoc) strategy. However, Fmoc-amino acids are sparingly soluble in water and have been considered inappropriate for in-water peptide synthesis. With the aim of achieving aqueous SPPS, we developed in-water synthesis protocols using water-dispersible nanoparticulate Fmoc-amino acids (Hojo et al. 2007, 2008, 2011, 2012, 2013). This technology uses suspended nanoparticle reactants for the coupling reaction to overcome the solubility problem. It offers many advantages in terms of reaction efficiency.

Microwave (MW)-assisted SPPS is already a widely accepted technology and automated peptide synthesizers equipped with MW capability are commercially available (Yu et al. 1992; Olivos et al. 2002; Erdélyi et al. 2002; Collins et al. 2003; Friligou et al. 2011; Wade et al. 2012; Echaliér et al. 2013). In previous work, we have been engaged in water-based MW-assisted SPPS using Fmoc amino acid nanoparticles

(Figure 1) and have established an efficient aqueous-based synthesis of the acyl carrier protein (65-74) peptide which is a well-known difficult sequence. Although MW irradiation represents an efficient means of accelerating the coupling reaction even in aqueous conditions, there are some reported cases of increased levels of amino acid racemization (Palasek et al. 2007; Loffrendo et al. 2009). As the configuration of proteins and peptides can critically affect their biological properties, the loss of chirality of even one amino acid residue can have a devastating effect. Therefore, it is imperative that racemization is minimized during MW-mediated chemical peptide synthesis.

Racemization has been noted as a serious issue during the synthesis of histidine (His)- (Jones et al. 1978; Sieber et al. 1978; Fletcher et al. 1979; Palasek et al. 2007; Isidro-Liobet et al. 2009) and cysteine (Cys)-containing peptides (Bodanszky et al. 1967; Kaiser et al. 1996; Han et al. 1997; Angell et al. 2002; Palasek et al. 2003). Recently, we reported the racemization of Cys residues under aqueous MW-assisted coupling conditions and of conditions that led to successful aqueous MW-assisted solid-phase synthesis of Cys-containing oxytocin analogue with a low Cys racemization level. The rate of racemization depends on the electron-withdrawing effect of the amino acid side chain, reaction temperature, solvent, coupling agents, and base. His is suspected to racemize through its own side chain, the weakly basic imidazole ring, and thus is highly prone to racemization in the course of the acylation reaction in conventional chemical peptide synthesis including SPPS (Angeletti et al. 1997). In MW-assisted SPPS, the level of racemization at His residue is significantly increased (Palasek et al. 2007). In this study, using a simple tripeptide model, we examined the

occurrence of the racemization during the aqueous MW-assisted coupling reaction of His residue. We also developed an efficient protocol for negligible His racemization as demonstrated by the successful synthesis of rat neuropeptide W-30 (NPW30) (10-15), Tyr-His-Thr-Val-Gly-Arg-NH₂ (Shimomura et al. 2002), by our aqueous-based MW-assisted method using water-dispersible Fmoc-amino acids nanoparticles.

Material and methods

Materials, instruments, and methods

Fmoc-amino acids were purchased from Watanabe Chemical Industries, Ltd. A planetary ball mill, model pulverisette 7 (Fritsch GmbH), was used for pulverization to prepare water-dispersible nanoparticles. The particle size of pulverized Fmoc-amino acids was determined by a dynamic light scattering analysis, model LB-500 (Horiba Instruments Inc.). The MW-assisted reaction system used in this study was the μ Reactor $\square$ Ex (2.45 GHz) (Shikoku Instrumentation Co. Ltd.) equipped with an internal fiber-optic temperature sensor for power delivery control. MW reactions were performed in open glass vessels (capacity 15 ml). Reaction mixture was stirred with a magnetic stirrer during the MW irradiation. Reversed phase high performance liquid chromatography (HPLC) was performed using a Waters model 600 instrument with a Cosmosil 5C₁₈-AR-II column and gradient system of acetonitrile / water containing 0.05% trifluoroacetic acid (TFA). Mass spectra were measured with a Bruker micrOTOF-Q II instrument using the time of flight (TOF) technique.

Preparation of water-dispersible Fmoc-amino acid nanoparticles

N^α -Fmoc- N^τ -trityl-histidine (Fmoc-His(τ -Trt)-OH) nanoparticles: An aqueous dispersion of nanoparticulate Fmoc-His(τ -Trt)-OH was prepared by grinding using a planetary ball mill. A 40 ml agate jar was charged with 0.5 mm diameter pre-cleaned zirconium oxide beads (80 g), Fmoc-His(τ -Trt)-OH (800 mg, 1.29 mmol), and 20 ml of aqueous 0.2% Triton X-100 solution. The batch was rolled at 246 rpm for 3 hr. After grinding, the beads were removed by filtration with 60 ml of aqueous 0.2% Triton X-100 solution. Particle size (mass median diameter): 556.6 ± 5.6 nm.

Nanoparticles of the following amino acids were also prepared as described above and had the observed particle size (mass median diameter):

N^α -Fmoc- N^π -methoxybenzyloxymethyl-histidine (Fmoc-His(π -MBom)-OH): 478.0 ± 10.4 nm. N^α -Fmoc-histidine Fmoc-His-OH nanoparticles: 348.7 ± 12.0 nm.

N^α -Fmoc-glycine (Fmoc-Gly-OH) nanoparticles: 419.5 ± 22.9 nm.

N^α -Fmoc-phenylalanine (Fmoc-Phe-OH) nanoparticles: 350.5 ± 2.6 nm.

N^α -Fmoc-*O*-*tert*-butyl-theronine (Fmoc-Thr(*t*Bu)-OH) nanoparticles: 255.2 ± 4.3 nm.

N^α -Fmoc-*O*-*tert*-butyl-tyrosine (Fmoc-Tyr(*t*Bu)-OH) nanoparticles: 462.7 ± 9.2 nm.

N^α -Fmoc-valine (Fmoc-Val-OH) nanoparticles: 629.0 ± 18.6 nm.

Synthesis of Phe-L-His-Gly-NH₂ by HBTU activation and MW irradiation in DMF

H-Rink amide resin (160 mg, amino group content, 100 μ mol) was swollen with DMF, Fmoc-amino acids (400 μ mol) were serially coupled onto the resin by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)

(151.6 mg, 400 μ mol) / *N,N*-diisopropylethylamine (DIEA) (139 μ l, 800 μ mol) in DMF at 70°C using MW (< 70 W) for 15 min. Fmoc deprotection was carried out by treatment with 20% piperidine/dimethylformamide (DMF), followed by washing with DMF. After completion of the synthesis, the resulting resin was treated with TFA-triisopropylsilane (TIPS)-water (92-4-4, 10 ml) for 1.5 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether. The crude peptides were directly analyzed by HPLC (Figure 3A) and ESI-MS.

Studies of His racemization during aqueous MW-assisted synthesis of Phe-L-His-Gly-NH₂

H-Gly-Rink amide-PEG grafted resin (150 mg, 35 μ mol) was swollen with 0.2% Triton X-100 solution, then an aqueous dispersion of nanoparticulate Fmoc-His(τ -Trt)-OH (16.3 ml, 175 μ mol), Fmoc-His(π -MBom)-OH (8.9 ml, 175 μ mol), or Fmoc-His-OH (5.9 ml, 175 μ mol) was coupled onto the resin by water-soluble carbodiimide (WSCl, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride) (34 mg, 175 μ mol) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (31 mg, 175 μ mol). WSCI was used in conjugation with 3-sulfo-*N*-hydroxysuccinimide (sulfo-HOSu) (38 mg, 175 μ mol). DIEA (31 μ l, 175 μ mol) or 4-methylmorpholine (NMM) (22 μ l, 204 μ mol) were used for base catalysts. Each reaction mixture was heated at 70°C by MW (<70 W) irradiation and kept for 10 min with stirring. After MW irradiation, the resins were washed with 0.2 % Triton X-100 solution and ethanol.

Fmoc deprotection was carried out by treatment with 20% piperidine/DMF for 10 min, followed by washing with DMF. Fmoc-Phe-OH (67 mg, 175 μ mol) was then coupled using *N,N'*-diisopropylcarbodiimide (DIC) (27 μ l, 175 μ mol) and 1-hydroxybenzotriazole (HOBt) (24 mg, 175 μ mol) in DMF. After removal of the Fmoc group, the resulting resin was washed with chloroform and dried *in vacuo*. Each resin was treated with TFA- TIPS-water (92-4-4, 15 ml) for 1.5 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether and lyophilized. The crude peptides were directly analyzed by HPLC (Figure 3B-D) and ESI-MS. The racemization ratios (D/L) were calculated from analytical HPLC profiles. Results are summarized in Table 1.

Synthesis of standard model peptides, Phe-L-His-Gly-NH₂ and Phe-D-His-Gly-NH₂

H-Rink amide resin (250 mg, amino group content, 155 μ mol) was swollen with DMF, Fmoc-amino acids (620 μ mol) were serially coupled onto the resin by DIC (96 μ l, 620 μ mol) and HOBt (83.7 mg, 620 μ mol) in DMF for 45 min. Fmoc deprotection was carried out by treatment with 20% piperidine/DMF for 10 min, followed by washing with DMF. After completion of the synthesis, the resulting resin was treated with TFA-TIPS-water (92-4-4, 10 ml) for 1.5 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether. The crude peptides were and directly analyzed by HPLC to confirm each retention time. These L- and D-peptide were

co-injected to the analytical HPLC (Figure 3E).

Phe-L-His-Gly-NH₂: ESI-MS (TOF) m/z: 359.3950 (C₁₇H₂₃N₆O₃ requires with calculated [M+1]⁺ 359.1915).

Synthesis of L-His^{II}-NPW30 (10-15) by aqueous MW-assisted solid-phase peptide synthesis using Fmoc-amino acid nanoparticles

The solid phase synthesis was carried out according to the protocol shown in Table 2. H-Arg(Pbf)-Rink amide-PEG grafted resin (200 mg, amino group content, 43 μmol) was swollen with 0.2% Triton X-100 solution, then water-dispersible Fmoc-amino acids nanoparticles (215 μmol) were serially coupled onto the resin. In-water coupling reaction was performed by DMTMM (38 mg, 215 μmol) and NMM (24 μl, 215 μmol) at 70°C using MW (< 70 W) for 10 min. Deprotection was carried out by treatment with 20% piperidine/DMF for 10 min. After completion of the synthesis, the resulting resin (H-Tyr(*t*Bu)-His(τ-Trt)-Thr(*t*Bu)-Val-Gly-Arg(Pbf)-Rink amide-PEG grafted resin) was washed with chloroform and dried *in vacuo*. The resin was treated with TFA-TIPS-water (92-4-4, 20 ml) for 2 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether and lyophilized. The crude peptides were analyzed by HPLC and ESI-MS. Yield (calculated from the amino group content of the used resin): 13 mg, 28%; ESI-MS (TOF) m/z: 731.3895 (C₃₂H₈₀N₁₂O₈ requires with calculated [M+1]⁺ 731.850).

Synthesis of standard peptides, L-His¹¹-NPW30 (10-15) and D-His¹¹-NPW30 (10-15)

H-Rink amide resin (250 mg, amino group content, 155 μ mol) was swollen with DMF, Fmoc-amino acids (620 μ mol) were serially coupled onto the resin by DIC (96 μ l, 620 μ mol) and HOBt (84 mg, 620 μ mol) in DMF for 45 min. Fmoc deprotection was carried out by treatment with 20% piperidine/DMF for 10 min, followed by washing with DMF. After completion of the synthesis, the resulting resin was treated with TFA-TIPS-water (92-4-4, 20 ml) for 2 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether. The crude product was purified by preparative HPLC to give an amorphous powder. These standards were co-injected to the analytical HPLC (Figure 4B).

Synthesis of L-His¹¹-NPW30 (10-15) by regular MW-assisted solid-phase peptide synthesis

H-Rink amide resin (250 mg, amino group content, 155 μ mol) was swollen with DMF, Fmoc-amino acids (620 μ mol) were serially coupled onto the resin by HBTU (227.4 mg, 620 μ mol) / DIEA (195 μ l, 1.24 mmol) in DMF at 70°C using MW (< 70 W) for 15 min. Fmoc deprotection was carried out by treatment with 20% piperidine/DMF, followed by washing with DMF. After completion of the synthesis, the resulting resin was treated with TFA-TIPS-water (92-4-4, 10 ml) for 2.0 hr at room temperature. The resin was removed by filtration and TFA was then evaporated to leave an oily material. The residue was dissolved in water and washed with diethylether. The crude peptides

were directly analyzed by HPLC.

Results and Discussion

His plays a key role in the active site of many proteins including enzymes. In chemical peptide synthesis, His is particularly prone to racemization (Veber 1975). This side reaction is one of the biggest concerns in the course of the total synthesis of biologically active peptides and their analogues. His has a basic imidazole ring that is well known to readily lead to serious racemization *via* the withdrawing α -proton of His (Mergler et al. 2001). Although many N^{im} -protecting groups for imidazole ring of His have been reported, none completely suppress racemization. Generally Fmoc-His(τ -Trt)-OH is the first choice building block for SPPS using Fmoc strategy (Sieber et al. 1978). There are many reports of studies using this derivative as a standard in racemization studies. Here we examined the racemization of His residue during the course of in-water coupling reaction using Fmoc-His(τ -Trt)-OH, the more recently developed Fmoc-His(π -MBom)-OH (Hibino et al. 2011, 2012) (Figure 2), and side chain protection-free Fmoc-His-OH. The literature lists several methods for the quantification of racemization of amino acids in general and of His in particular. Mergler and coworkers used the simple tripeptide, Phe-L-His-Gly-NH₂, as a suitable model to study the racemization of L-His during SPPS using the Fmoc strategy (Robertson et al. 1999; Mergler et al. 2001). Therefore, we used this tripeptide as a model in a racemization study of water-based SPPS using amino acid nanoparticles. Firstly, we examined the occurrence of this side reaction during the aqueous

MW-assisted coupling reaction of Fmoc-L-His(τ -Trt)-OH nanoparticles with H-Gly-Rink amide-PEG grafted resin. Water-dispersible Fmoc-L-His(τ -Trt)-OH nanoparticles were produced by a dispersion-based process with wet milling (Rainbow BE 2004). Fmoc-L-His(τ -Trt)-OH was ground with zirconia beads in aqueous 0.2% Triton X-100 solution using a planetary ball mill for 3 hr at room temperature. The beads were then removed by filtration to obtain water-dispersible Fmoc-L-His(τ -Trt)-OH nanoparticles (mass median diameter: 556.6 ± 5.6 nm). Several coupling methods and conditions were evaluated. WSCI (Sheehan et al. 1956) and DMTMM (Kaminski et al. 1998) were each examined. Couplings were conducted using DIEA or NMM with or without the additive sulfo-HOSu (Staros et al. 1986). The reaction temperature was set at 70°C using MW power of <70 W. The Fmoc group was removed by treatment with 20% piperidine/DMF without MW-heating. After introducing the Phe residue by DIC with HOBt in DMF, the target tripeptide was cleaved from the resin by treatment with TFA and the crude peptides were analyzed by HPLC. The HPLC profiles of crude Phe-L-His-Gly-NH₂ obtained by water-based synthesis methods are showed in Figure 3. The desired L-His-tripeptide and its D-His-epimer were identified by ESI-MS and HPLC analysis with standards Phe-L-His-Gly-NH₂ and Phe-D-His-Gly-NH₂ (Figure 3E). The D/L-His-epimer ratios are summarized in Table 1. In the conventional MW-assisted method using HBTU with DIEA (Dourtoglou et al. 1978, 1984), the D/L ratio is 13.8 % (Table 1, entry 1, Figure 3A). Interestingly, in the case of aqueous coupling reaction using Fmoc-His(τ -Trt)-OH nanoparticles by DMTMM, the D/L ratio was less than 3.3% (Table 1, entry 2, Figure 3B). Among the

factors that determine the level of racemization, polarity of the solvent is important. In general, racemization occurs more rapidly in highly polar solvents and more slowly in less polar solvents. Even in the highly polar solvent, water, the His racemization level was significantly lower than conventional MW-assisted method (Table 1, entry 1). It is well understood that the His racemization process is a base-catalyzed abstraction of α -carbon proton by imidazole ring. It is also well known that the level of racemization can be reduced by using a weaker base and eliminating the pre-activation process. In this case, the milder base NMM was used to maintain a neutral pH in reaction medium. During the coupling reaction using DMTMM, by keeping the pH between 6.7 and 7.2 with NMM it was possible to almost completely suppress the racemization. In the reaction using WSCI with DIEA (Table 1, entry 3, Figure 3C), the level of racemization is relatively higher than entry 2. We also applied water-dispersible Fmoc-His(π -MBom)-OH nanoparticles (mass median diameter: 478.0 ± 10.4 nm) to the water-based coupling using DMTMM method. Although MBom protection at π position of imidazole ring was reported to suppress the racemization better than Trt protection at τ position, it did not work well under aqueous conditions (Table 1, entry 4, Figure 3D). Surprisingly, in the case of Fmoc-His(π -MBom)-OH, the level of racemization is almost similar level to that of side chain-free Fmoc-His-OH nanoparticles (mass median diameter: 348.7 ± 12.0 nm) (Table 1, entry 5). The coupling reaction of Fmoc-His(π -MBom)-OH does not proceed smoothly and an extended reaction time is required to achieve complete acylation. This might be due to steric hinderance caused by the MBom group at the π -position very close to the carboxyl acid. The slow reaction

rate in aqueous media could easily lead to an increase in the level of racemization at the His residue.

Next, we demonstrated our optimized MW-assisted water-based SPPS of NPW30 (10-15), Tyr-His-Thr-Val-Gly-Arg-NH₂, using water-dispersible Fmoc-amino acid nanoparticles (Shimomura et al. 2002; Hibino et al. 2012). The reaction was carried out in an aqueous solution according to the protocol described in Table 2. The water-dispersible nanoparticles of Fmoc-amino acids were serially coupled onto a Rink amide-PEG grafted resin. Coupling reactions were carried out by DMTMM with MW irradiation set to a power of < 70 W for 10 min at 70°C. The completion of the coupling reaction was assessed by Kaiser test (Kaiser et al. 1970). After removal of the Fmoc groups, the partially protected peptide was cleaved from the resin by TFA treatment. The results of the HPLC analysis for crude L-His¹¹-NPW30 (10-15) obtained by solid-phase synthesis in water are shown in Figure 4A. We confirmed the retention times of L-His¹¹-NPW30 (10-15) and D-His¹¹-NPW30 (10-15) standards (Figure 4B). The level of racemization in L-His¹¹-NPW30 (10-15) prepared by the water-based method (Figure 4A) was < 6.2% at His position which was deemed to be acceptable. In contrast, in the case of regular MW-assisted SPPS using HBTU at 70°C, the level of racemization was over 20%. The overall yield of NPW30 (10-15) obtained by water-based method was 28% which was calculated from the amino group content of the used resin. This is the first achievement of MW-assisted water based solid-phase synthesis of His-containing peptide without serious racemization.

Conclusion

In the present study, we examined the racemization of His during MW-assisted aqueous-based coupling reactions. Under our MW-assisted protocol using DMTMM, coupling reactions can be performed with low level racemization of His. We also have demonstrated that MW radiation can be successfully applied to the synthesis of a 6 residue His-containing peptide in high purity. Moreover, this is the first documented example of a successful water-based solid-phase synthesis of a His-containing peptide. The present protocol should be generally applicable to the aqueous synthesis of a wide range of biological peptides, including His-containing peptides. Most solid-phase syntheses are currently carried out in organic solvents and few efforts have been made to carry out the reactions in water. The poor water solubility of reagents and building blocks has long been one of the obstacles in effort to carry out reactions in water. The present study provides a practical solution to address the poor solubility of building blocks and offers an efficient and practical new solid-phase synthesis method using the “environmentally friendly solvent water”.

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Ethical standards

The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Racemization of His during the synthesis of the model peptide, Phe-L-His-Gly-NH₂

Entry	Fmoc-His(X)-OH X =	Solvent	Reagents	Additives	Base	Temp. (°C)	Racemization (%) ^a
1	τ-Trt	DMF	HBTU	-	DIEA	70	13.8
2	τ-Trt	Water	DMTMM	-	NMM	70	3.3
3	τ-Trt	Water	WSCl	sulfo-HOSu	DIEA	70	16.6
4	π-MBom	Water	DMTMM	-	NMM	70	17.9
5	□	Water	DMTMM	-	NMM	70	16.8

^a Calculated from HPLC analysis profiles.

Table 2. Protocol for water based MW-assisted SPPS

Step	Reagents	Time
Washing	Water	3 min × 5
Coupling Reaction	Water-dispersible Fmoc-amino acids (5 eq), DMTMM (5 eq), NMM (5 eq)	10 min (MW: 70°C; 70W)
Washing	Water	3 min × 5
Washing	DMF	3 min × 3
Deprotection	20% piperidine / DMF	10 min
Washing	DMF	3 min × 3

Figure Legends

Figure 1. Aqueous MW-assisted solid-phase peptide synthesis using Fmoc-amino acid nanoparticles.

Figure 2. Structure of Fmoc-His(τ -Trt)-OH and Fmoc-His(π -MBom)-OH.

Figure 3. (A) Analytical HPLC profile of the crude Phe-L-His-Gly-NH₂ obtained by water based MW-assisted synthesis using Fmoc-His(τ -Trt)-OH, DMTMM with NMM.

(B) Analytical HPLC profile of the crude Phe-L-His-Gly-NH₂ obtained by water based MW-assisted synthesis using Fmoc-His(τ -Trt)-OH, WSCI and sulfo-HOSu with DIEA.

(C) Analytical HPLC profile of the crude Phe-L-His-Gly-NH₂ obtained by water based MW-assisted synthesis using Fmoc-His(π -MBom)-OH, DMTMM with NMM.

(D) Analytical HPLC profiles of the crude Phe-L-His-Gly-NH₂ obtained by MW-assisted synthesis using HBTU with DIEA in DMF. (E) Analytical HPLC profile of a mixture of standard Phe-L-His-Gly-NH₂ and standard Phe-D-His-Gly-NH₂. Column was cosmosil 5C₁₈-AR-II. Elution was carried out over 30 min at a flow rate of 1 ml/min with a linear gradient from 2:98 to 32:68 mixture of 0.05% aqueous TFA and 0.05% TFA in acetonitrile. a = desired Phe-L-His-Gly-NH₂, b = Phe-D-His-Gly-NH₂.

Figure 4. (A) Analytical HPLC profile of the crude L-His¹¹-NPW30 (10-15) obtained by water based MW-assisted synthesis using DMTMM with NMM. (B) Analytical HPLC profile of a mixture of L-His¹¹-NPW30 (10-15), and D-His¹¹-NPW30 (10-15). Syntheses were carried out separately by general Fmoc strategy in DMF. Column was Cosmosil 5C₁₈-AR-II. Elution was carried out over 60 min at a flow rate of 1 ml/min with a linear

gradient from 1:99 to 31:69 mixture of 0.05% aqueous TFA and 0.05% TFA in acetonitrile. I = L-His¹¹-NPW30 (10-15), II = D-His¹¹-NPW30 (10-15).

Figure 1

Figure 2