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ORIGINAL ARTICLE

Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy. III: Racemization studies and water-based synthesis of histidine-containing peptides

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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-NH_2, in high yield and with greatly reduced histidine racemization.

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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 and Warner 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, b, 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 and Gogoll 2002; Collins and Collin 2003; Friligou et al. 2011; Wade

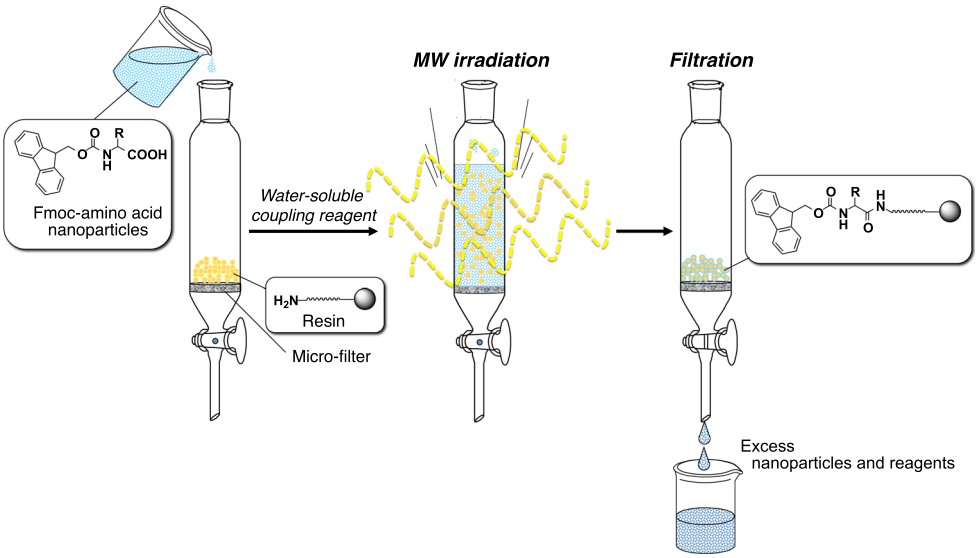

Fig. 1 Aqueous MW-assisted solid-phase peptide synthesis using Fmoc-amino acid nanoparticles

et al. 2012; Echalier et al. 2013). In previous work, we have been engaged in water-based MW-assisted SPPS using Fmoc-amino acid nanoparticles (Fig. 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 and Ramage 1978; Sieber and Riniker 1978; Fletcher et al. 1979; Palasek et al. 2007; Isidro-Liobet et al. 2009) and cysteine (Cys)-containing peptides (Bodanszky and Bodanszky 1967; Kaiser et al. 1996; Han et al. 1997; Angell et al. 2002; Palasek et al. 2007). 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 analog 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-NH_2 (Shimomura et al. 2002), by our aqueous-based MW-assisted method using water-dispersible Fmoc-amino acid nanoparticles.

Materials 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 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 $5C_{18}$-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 h. 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-threonine (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-NH_2 by HBTU activation and MW irradiation in DMF

H-Rink amide resin (160 mg, amino group content, 100 μmol) was swollen with DMF; Fmoc-amino acids (400 μmol) were serially coupled onto the resin by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (151.6 mg, 400 μmol)/*N*,*N*-diisopropylethylamine (DIEA) (139 μl, 800 μmol) 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 h 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 (Fig. 3a) and ESI–MS.

Studies of His racemization during aqueous MW-assisted synthesis of Phe-L-His-Gly-NH_2

H-Gly-Rink amide-PEG grafted resin (150 mg, 35 μmol) was swollen with 0.2 % Triton X-100 solution, then an aqueous dispersion of nanoparticulate Fmoc-His(τ-Trt)-OH (16.3 ml, 175 μmol), Fmoc-His(π-MBom)-OH (8.9 ml, 175 μmol), or Fmoc-His-OH (5.9 ml, 175 μmol) was coupled onto the resin by water-soluble carbodiimide (WSCI, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride) (34 mg, 175 μmol) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (31 mg, 175 μmol). WSCI was used in conjugation with 3-sulfo-*N*-hydroxysuccinimide (sulfo-HOSu) (38 mg, 175 μmol). DIEA (31 μl, 175 μmol) or 4-methylmorpholine (NMM) (22 μl, 204 μmol) was used as 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 μmol) was then coupled using *N,N'*-diisopropylcarbodiimide (DIC) (27 μl, 175 μmol) and 1-hydroxybenzotriazole (HOBt) (24 mg, 175 μmol) 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 h 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 (Fig. 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-NH_2 and Phe-D-His-Gly-NH_2

H-Rink amide resin (250 mg, amino group content, 155 μmol) was swollen with DMF; Fmoc-amino acids (620 μmol) were serially coupled onto the resin by DIC (96 μl, 620 μmol) and HOBt (83.7 mg, 620 μmol) 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 h 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.

Table 1 Racemization of His during the synthesis of the model peptide, Phe-L-His-Gly-NH_2

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	WSCI	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; 70 W)
Washing	Water	3 min × 5
Washing	DMF	3 min × 3
Deprotection	20 % piperidine/DMF	10 min
Washing	DMF	3 min × 3

These L- and D-peptide were co-injected to the analytical HPLC (Fig. 3e).

Phe-L-His-Gly-NH_2: ESI–MS (TOF) m/z: 359.3950 ($C_{17}H_{23}N_6O_3$ requires with calculated $[M+1]^+$ 359.1915).

Synthesis of L-His[11]-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, and then water-dispersible Fmoc-amino acid 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 h 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 ($C_{32}H_{80}N_{12}O_8$ requires with calculated $[M+1]^+$ 731.850).

Synthesis of standard peptides, L-His[11]-NPW30 (10–15) and D-His[11]-NPW30 (10–15)

H-Rink amide resin (250 mg, amino group content, 155 μmol) was swollen with DMF; Fmoc-amino acids (620 μmol) were serially coupled onto the resin by DIC (96 μl, 620 μmol) and HOBt (84 mg, 620 μmol) 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 h 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 (Fig. 4b).

Synthesis of L-His[11]-NPW30 (10–15) by regular MW-assisted solid-phase peptide synthesis

H-Rink amide resin (250 mg, amino group content, 155 μmol) was swollen with DMF; Fmoc-amino acids (620 μmol) were serially coupled onto the resin by HBTU (227.4 mg, 620 μmol)/DIEA (195 μl, 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 h 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 analogs. 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 and Riniker 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 and Nishiuchi 2011, Hibino et al. 2012) (Fig. 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-NH_2, 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 (Rabinow 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 h 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. Both WSCI (Sheehan and Hlavka 1956) and DMTMM (Kaminski et al. 1998; Kunishima et al. 1999) were 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-NH_2 obtained by water-based synthesis methods are showed in Fig. 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-NH_2 and Phe-D-His-Gly-NH_2 (Fig. 3e). The D/L-His-epimer ratios are summarized in Table 1. In the conventional MW-assisted method using HBTU with

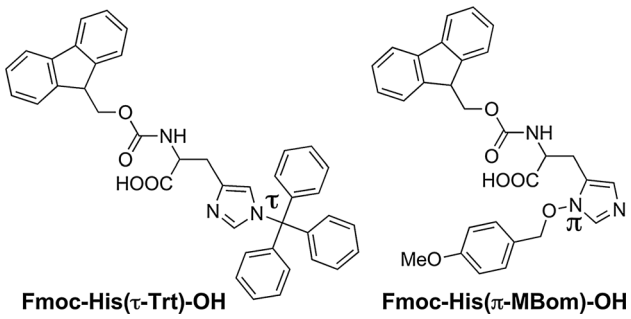

Fig. 2 Structure of Fmoc-His(τ-Trt)-OH and Fmoc-His(π-MBom)-OH

DIEA (Dourtoglou et al. 1978, 1984), the D/L ratio is 13.8 % (Table 1, entry 1, Fig. 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, Fig. 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 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, Fig. 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, Fig. 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

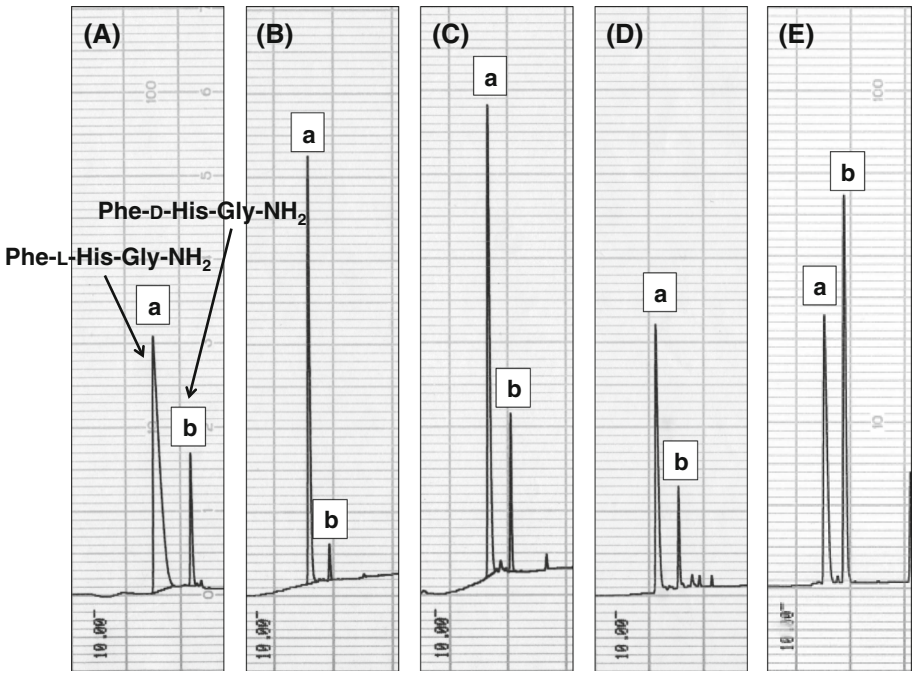

Fig. 3 **A** Analytical HPLC profile of the crude Phe-L-His-Gly-NH_2 obtained by water-based MW-assisted synthesis using Fmoc-His(τ-Trt)-OH, DMTMM with NMM. **B** Analytical HPLC profile of the crude Phe-L-His-Gly-NH_2 obtained by water-based MW-assisted synthesis using Fmoc-His(τ-Trt)-OH, WSCI and sulfo-HOSu with DIEA. **C** Analytical HPLC profile of the crude Phe-L-His-Gly-NH_2 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-NH_2 obtained by MW-assisted synthesis using HBTU with DIEA in DMF. **E** Analytical HPLC profile of a mixture of standard Phe-L-His-Gly-NH_2 and standard Phe-D-His-Gly-NH_2. Column was Cosmosil $5C_{18}$-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-NH_2, *b* Phe-D-His-Gly-NH_2

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-NH_2, 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-His^{11}-NPW30 (10–15) obtained by solid-phase synthesis in water are shown in Fig. 4a. We confirmed the retention times of L-His^{11}-NPW30 (10–15) and D-His^{11}-NPW30 (10–15) standards (Fig. 4b). The level of racemization in L-His^{11}-NPW30 (10–15) prepared by the water-based method (Fig. 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

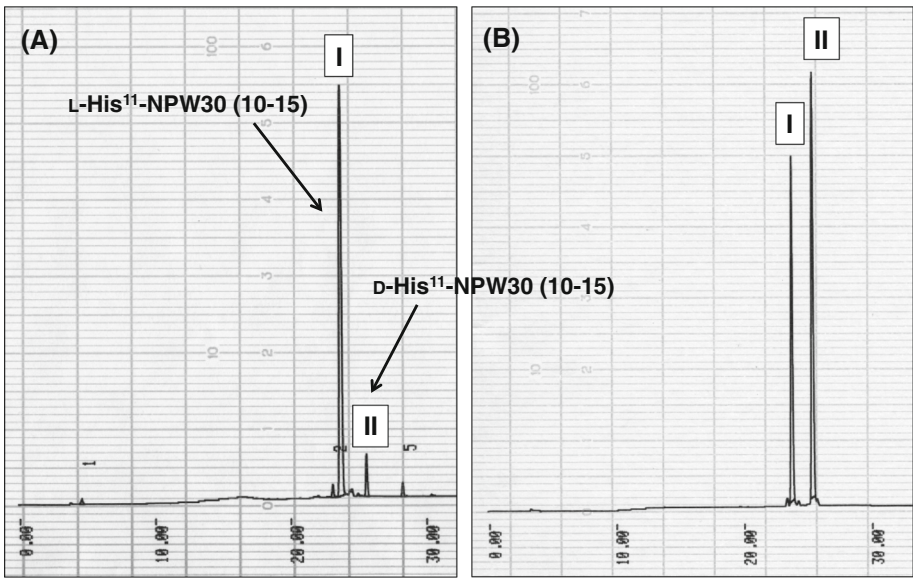

Fig. 4 **A** Analytical HPLC profile of the crude L-His[11]-NPW30 (10–15) obtained by water-based MW-assisted synthesis using DMTMM with NMM. **B** Analytical HPLC profile of a mixture of L-His[11]-NPW30 (10–15), and D-His[11]-NPW30 (10–15). Syntheses were carried out separately by general Fmoc strategy in DMF. Column was Cosmosil $5C_{18}$-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-His[11]-NPW30 (10–15), *II* D-His[11]-NPW30 (10–15)

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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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The manuscript does not contain clinical studies or patient data.

References

Anastas PT, Warner JC (1998) Green chemistry: theory and practice. Oxford University Press, New York

Angeletti RH, Bibbs L, Bonewald LF, Fields GB, Kelly JW, McMurray JS, Moore WT, Weintraub ST (1997) Analysis of racemization during "standard" solid phase peptide synthesis: a multicenter study. In: Marshak DR (ed) Techniques in protein chemistry III. Academic Press Inc, San Diego, pp 875–890

Angell YM, Alsina J, Albericio F, Barany G (2002) Practical protocols for stepwise solid-phase synthesis of cysteine-containing peptides. J Pept Res 60:292–299

Bodanszky M, Bodanszky A (1967) Racemization in peptide chemistry. Mechanism-specific models. Chem Commun 1967: 591–592

Collins JM, Collin MJ (2003) Novel method for enhanced solid-phase peptide synthesis using microwave energy. Biopolymers 71:361–366

Dourtoglou V, Ziegler JC, Gross B (1978) L'hexafluorophosphate de O-benzotriazoyl- N, N, N′, N′-tetramethyluronium: un reactif de couplage peptidique nouveau et efficace. Tetrahedron Lett 19:1269–1272

Dourtoglou V, Gross B, Lambropoulou C, Ziodrou C (1984) O-Benzotriazolyl-N, N, N′, N′-tetramethyluronium hexafluorophosphate as coupling reagent for the synthesis of peptides of biological interest. Synthesis 1984:572–574

Echalier C, Ai-Halifa S, Kreiter A, Enjalbal C, Sanchez P, Ronga L, Puget K, Verdié P, Amblard M, Martinez J, Subra G (2013) Heating and microwave assisted SPPS of C-terminal acid peptides on trityl resin: the truth behind the yield. Amino Acids 45:1395–1403

Erdélyi M, Gogoll A (2002) Rapid microwave-assisted solid-phase peptide synthesis. Synthesis 11:1592–1596

Fletcher AR, Jones JH, Ramage WI, Stachulski AV (1979) The use of the N^{π}-phenacyl group for the protection of the histidine side chain in peptide synthesis. J Chem Soc Perkin Trans 1(1979):2261–2267

Friligou I, Papadimitriou E, Gatos D, Matsoukas J, Tselios T (2011) Microwave-assisted solid-phase peptide synthesis of the 60–110 domain of human pleiotrophin on 2-chlorotrityl resin. Amino Acids 40:1431–1440

Han Y, Albericio F, Barany G (1997) Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis. J Org Chem 62:4307–4312

Hibino H, Nishiuchi Y (2011) 4-Methoxybenzyloxymethyl group as an N^{π}-protecting group for histidine to eliminate side-chain-induced racemization in the Fmoc strategy. Tetrahedron Lett 52:4947–4949

Hibino H, Miki Y, Nishiuchi Y (2012) Synthesis and application of N^{α}-Fmoc-N^{π}-4-methoxybenzyloxymethylhistidine in solid phase peptide synthesis. J Pept Sci 18:763–769

Hojo K, Maeda M, Kawasaki K (2001) A new water-soluble *N*-protecting group, 2-[phenyl (methyl) sulfonio] ethyloxycarbonyl tetrafluoroborate, and its application to solid phase peptide synthesis in water. J Pept Sci 7:615–618

Hojo K, Maeda M, Kawasaki K (2004a) A water-soluble N-protecting group, 2-[phenyl(methyl)sulfonio]ethoxycarbonyl tetrafluoroborate,

and its application to peptide synthesis. Tetrahedron 60: 1875–1866

Hojo K, Maeda M, Kawasaki K (2004b) 2-(4-Sulfophenyl) ethoxycarbonyl group: a new water-soluble *N*-protecting group and its application to solid-phase peptide synthesis in water. Tetrahedron Lett 45:9293–9295

Hojo K, Ichikawa H, Maeda M, Kida S, Fukumori Y, Kawasaki K (2007) Solid-phase peptide synthesis using nanoparticulate amino acids in water. J PeptSci 13:493–497

Hojo K, Ichikawa H, Fukumori Y, Kawasaki K (2008) Development of a method for solid-phase peptide synthesis in water. Int J Pept Res Ther 14:373–380

Hojo K, Hara A, Kitai H, Onishi M, Ichikawa H, Fukumori Y, Kawasaki K (2011) Development of a method for environmentally friendly chemical peptide synthesis in water using water-dispersible amino acid nanoparticles. Chem Cent J 5:49

Hojo K, Ichikawa H, Hara A, Onishi M, Kawasaki K, Fukumori Y (2012) Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy: in-water synthesis of "difficult sequences". Protein Pept Lett 19:1231–1236

Hojo K, Ichikawa H, Hara A, Onishi M, Kawasaki K, Fukumori Y (2013) Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy II: racemization studies and water-based synthesis of cysteine-containing peptides. Protein Pept Lett 20:1122–1128

Isidro-Liobet A, Álavarez M, Albericio A (2009) Amino acid-protecting groups. Chem Rev 109:2455–2504

Jones JH, Ramage WI (1978) An approach to the prevention of racemization in the synthesis of histidine-containing peptides. J Chem Soc Chem Commun 1978:472–473

Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid phase synthesis of peptide. Anal Biochem 1970:595–598

Kaiser T, Nicholson G, Kohlbau H, Volete R (1996) Racemization studies of Fmoc-Cys(Trt)-OH during stepwise Fmoc-solid phase peptide synthesis. Tetrahedron Lett 37:1187–1190

Kaminski ZJ, Paneth P, Rudzinski JA (1998) Study on the activation of carboxylic acids by means of 2-chloro-4,6-dimethoxy-1,3,5-triazine and 2-chloro-4,6-diphenoxy-1.3.5-triazine. J Org Chem 63:4248–4225

Kunishima M, Kawachi C, Morita J, Terao K, Iawasaki F, Tani S (1999) 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride: an efficient condensing agent leading to the formulation of amide and esters. Tetrahedron 55:13159–13179

Loffrendo C, Assunção NA, Gerhardt J, Miranda MTM (2009) Microwave-assisted solid-phase peptide synthesis at 60 °C: alternative conditions with low enantiomerization. J Pept Sci 15:808–817

Mergler M, Dick F, Sax B, Schwindling J, Vorherr TH (2001) Synthesis of Fmoc-His(3-Bum)-OH. J Pept Sci 7:502–510

Olivos HJ, Alluri PG, Reddy MM, Salony D, Kodadek T (2002) Microwave-assisted solid-phase synthesis of peptides. Org Lett 4:4057–4059

Palasek SA, Cox ZJ, Collins LM (2007) Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis. J Pept Sci 13:143–148

Rabinow BE (2004) Nanosuspensions in drug delivery. Nat Rev Discov 3:785–795

Robertson N, Jiang L, Ramage R (1999) Racemisation studies of a novel reagent for solid-phase peptide synthesis. Tetrahedron 54:14233–14254

Sheehan JC, Hlavka JJ (1956) The use of water-soluble and basic carbodiimides in peptide synthesis. J Org Chem 21:439–441

Sheldon RA (2007) The E factor: fifteen years on. Green Chem 9:1273–1283

Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, Watanabe T, Asami T, Kitada C, Mori M, Onda H, Fujino M (2002) Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 amd GPR8. J Biol Chem 277:35826–35832

Sieber P, Riniker B (1978) Protection of histidine in peptide synthesis. A reassessment of the trityl group. Tetrahedron Lett 28:6031–6034

Staros JV, Wright RW, Swingle DM (1986) Enhancement by *N*-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal Biochem 156:220–222

Veber DF (1975) Peptide synthesis from the practitioner's point of view. In: Walter R, Meienhofer J (eds) Peptides: chemistry, structure and biology. Ann Arbor Science Publishers, Michigan, pp 307–316

Wade JD, Lin F, Hossain MA, Dawson RM (2012) Chemical synthesis and biological evaluation of an antimicrobial peptide gonococcal inhibitor. Amino Acids 43:2279–2283

Winterton N (2001) Twelve more green chemistry principles. Green Chem 3:G73–G75

Yu HM, Chen ST, Wang JT (1992) Enhanced coupling efficiency in solid-phase peptide synthesis by microwave irradiation. J Org Chem 57:4781–4784