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ARTICLE TYPE

Microwave-assisted solution phase peptide synthesis in neat water

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An environmentally benign protocol for the solution phase peptide synthesis has been developed in neat water using TBTU/HOBt/DIEA as a coupling combination under microwave irradiation. Key features of this procedure are the replacement of commonly used toxic organic solvents like DMF and NMP, use of less amount of reactants, compatibility with both *N*-α-Boc- and *N*-α-Fmoc-protected amino acids and all commonly used side-chain protective groups, short reaction times, and racemization-free synthesis in high yield and purity.

Introduction

The condensation of two amino acids to form a peptide bond is considered as one of the most important reactions for sustaining life. Peptides play an important role in medicinal chemistry leading to discovery of a number of drugs.[1] Over the last few decades, significant research progress is achieved in identification of peptides having important therapeutic effects.[2-7] In general, most of the drugs show bioactivity by binding with the proteincious receptors or enzymes. Therefore, for significant interactions with receptors or enzymes, ligands, which are proteincious or peptidic in nature are ideal. For example, some of the peptides that are known to have specific receptor-based activity includes, bradykinin,[8] gonodotropin-releasing hormone (GnRH),[9] dermorphin,[10] enkephalins,[11] bombesin,[12] and thrombin.[13]

Peptides were synthesized using solution phase protocol till the discovery of solid phase peptide synthesis (SPPS) by Bruce R. Merrifield in 1963.[14] The SPPS offers several advantages over solution phase peptide synthesis that include, easy isolation and purification of large peptides. In SPPS, a library of peptides can be generated in short span of time, but synthesis requires 3-8 fold excess of amino acids and coupling reagents, in addition to expensive and non-reusable solid supports. The solution phase peptide synthesis requires 1.2-2.0 fold excess of the amino acids and coupling reagents, thereby offering significant advantage in the synthesis of short peptides, specifically those containing expensive and difficult to access modified/synthetic amino acids. The method could be equally useful for large peptides if synthesis is achieved using segment or chemical ligation approaches.[15] The Fig 1 displays structures of some short biologically active peptides.[16-20] Both solid phase and solution phase peptide syntheses generates a large volume of toxic waste, including a large amount of toxic organic solvents like *N,N*-dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP) and dichloromethane (CH_2Cl_2). Therefore, discovery of alternate environmentally friendly methods of peptide synthesis are desired.

The application of eco-friendly methodologies in peptide synthesis is not well explored. The first SPPS in water (H_2O) was reported by Kawasaki *et. al.*[21] The method used a PEG-based resin, a water-soluble amino protecting group, and EDC·HCl/HONB as the coupling combination. Grøtli *et al.* synthesized peptides in H_2O using Boc-protected amino acids, EDC·HCl/HONB and PEG-based resin under microwave (MW) irradiation.[22a] In both methods, aqueous 0.2% Triton X solution was added during the reaction to increase the swelling of resin and solubility of amino acids. More recently, Collins reported SPPS using amino acids bearing α,β-unsaturated sulfone-based α-amino protective groups in a solvent combination of H_2O-ethanol (EtOH) under microwave irradiation.[22b] None of these SPPS methods used neat water as the reaction medium and appeared limited in terms of exclusive amino protecting groups and expensive solid supports.

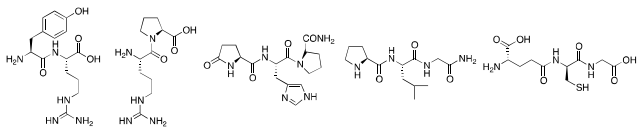

Fig. 1 Short biologically active peptides

Solution phase peptide synthesis has been performed in various solvents, but the application of H_2O as a reaction medium is not yet explored. Over the past few years water as a environmentally benign solvent has grabbed considerable interest.[23] Water as a solvent offers many advantages because it is abundant, non-toxic, non-flammable, and reduces the organic solvent waste in chemical industry.[24] Along with H_2O, the MW is another rapid and greener alternative compared to conventional heating.[25] The MW irradiation is much widely used in SPPS,[26] but has limited applicability in solution phase peptide synthesis.[27] More recently, we reported first generalized protocol for solution phase peptide synthesis under MW irradiation.[28] In some recent reports, formation of amide bond was also achieved using liquid-assisted and solvent-free ball-milling.[29] In our quest to discover eco-friendly peptide synthesis protocols, herein, we report solution

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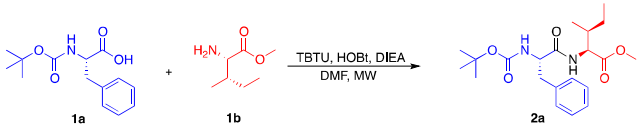
phase peptide synthesis in neat H_2O under MW irradiation. To the best of our knowledge, this is the first report on the synthesis of peptides in solution phase using neat H_2O as a reaction medium. The reaction employs commercially available Boc- and Fmoc-protected amino acids with commonly used coupling reagents for the formation of peptide bond. This protocol offers several benefits over the existing solution and solid phase peptide synthesis methodologies: (a) use of neat H_2O as the reaction medium, (b) full compatibility with both Boc- and Fmoc-protected amino acids, (c) no need for the special protection on the reactive side-chain of amino acids, (d) short reaction time, and (e) and racemization-free peptide synthesis in high yield and purity.

Results and discussion

To establish the protocol, we first carried out a model reaction with Boc-Phe-OH (1a) and Ile-OMe·2HCl (1b) using TBTU as a coupling reagent and HOBt as an auxiliary nucleophile in DMF as solvent (Scheme 1). The selection of the coupling reagent and auxiliary nucleophile was based upon their easy access, low cost and wide application in peptide synthesis.



Scheme 1 Generalized scheme for coupling reaction

Optimization of the protocol

Thereafter, we studied the influence of temperature and time on the formation of amide bond under MW irradiation. The graph in Fig. 2 shows monitoring of the coupling of (1a) with (1b) under varied sets of temperature and time. We observed that a temperature of 60 ˚C and reaction time of 30 min provides the best coupling conditions and gave 86% isolated yield of Boc-Phe-Ile-OMe (2a). The HPLC analysis indicated 97% purity for 2a, confirming the effectiveness of protocol.

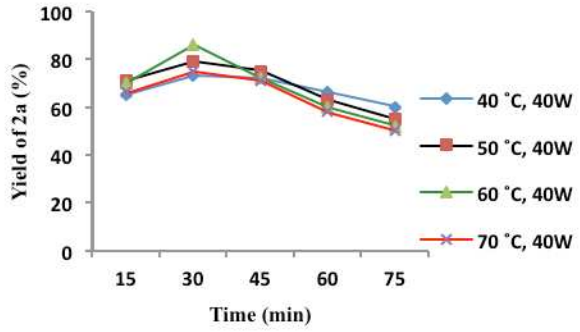


Fig. 2 Yield of peptide 2a as a function of time and temperature with TBTU/HOBt/DIEA under MW irradiation

The next step was to investigate the applicability of various solvents under the optimized conditions. The solvent screened during the optimization process included, DMF, acetonitrile (CH_3CN), NMP, H_2O, ethyl acetate (EtOAc), *tert*-butyl methyl ether (TBME), 2-methyltetrahydrofuran (2-Me-THF), 1-butanol (*n*-BuOH), EtOH and isopropyl alcohol (IPA), and the results are summarized in Table 1. The preliminary results indicate that the H_2O (entry no. 4) and TBME (entry no. 6) were comparable in terms of yield and purity. Also it was evident from the entry nos. 1 and 3 that the commonly used but toxic organic solvents (DMF and NMP) gave lesser yields compared to H_2O and TBME, which are considered greener alternative according to the GSK's solvent selection guide.[30] According to GSK's guide, the solvents are ranked on the basis of their waste, flammability, environmental, health and safety issues. Solvents such as DMF, NMP and CH_3CN come under the category of highly toxic and must be avoided. While H_2O, TBME, EtOAc, IPA, 2-Me-THF, and 1-butanol are considered to be the greener replacements of the toxic organic solvents.

Table 1 Effect of various solvents on the yield and purity of Boc-Phe-Ile-OMe (2a)[a]

Entry	Solvent	Yield	Purity
1	*N,N*-Dimethylformamide (DMF)	86	97
2	Acetonitrile (CH_3CN)	87	97
3	1-Methyl-2-pyrrolidone (NMP)	84	96
4	Water (H_2O)	90	98
5	Ethyl acetate (EtOAc)	85	96
6	*tert*-Butyl methyl ether (TBME)	88	97
7	2-Methyltetrahydrofuran (2-Me-THF)	79	95
8	1-Butanol (*n*-BuOH)	65	98
9	Ethanol (EtOH)	52	98
10	Isopropyl alcohol (IPA)	68	95

Reaction conditions: solvent (2.5 mL), 1a (1.2 equiv), 1b (1.0 equiv), TBTU (1.2 equiv), HOBt (1.2 equiv), DIEA (2 equiv) 60 ˚C, 30 min, MW.

[a]Isolated yield.

With these initial results in hand, H_2O and TBME appeared to be the promising greener replacement to the commonly used toxic organic solvents in solution phase peptide synthesis. To determine the most suitable coupling reagents, we explored the peptide bond formation under MW irradiation by screening various coupling reagent and auxiliary nucleophile combinations. The results of the study are shown in Fig. 3. As evident from the bar graphs, a coupling combination of TBTU/HOBt gave best results when H_2O was used as solvent. While, DIC/HONB emerged as the best coupling reagents combination when TBME was used as the solvent for the reaction.

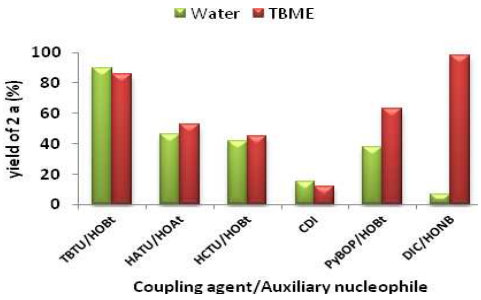


Fig. 3 A comparison of yield of 2a in H_2O and TBME in various coupling reagents

Finally, the scope and limitation of in-water peptide synthesis

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was further explored by synthesizing a number of peptide sequences in both TBME and H_2O (Fig. 4). We observed that the yield of Boc-protected peptides was higher in H_2O. However, slightly higher yields were observed when TBME was used in cases involving Fmoc-protected amino acids, which is in agreement with a substantial increase in their hydrophobicity.

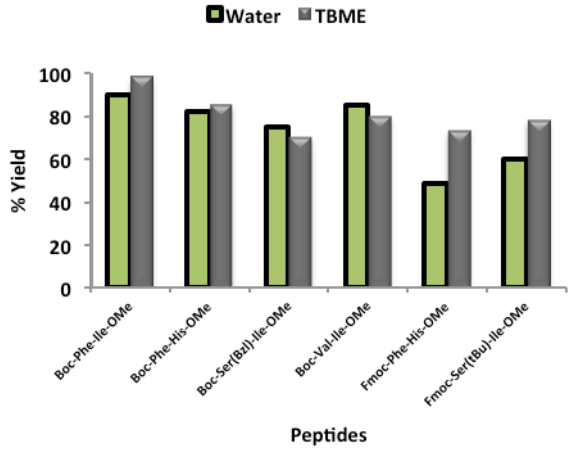


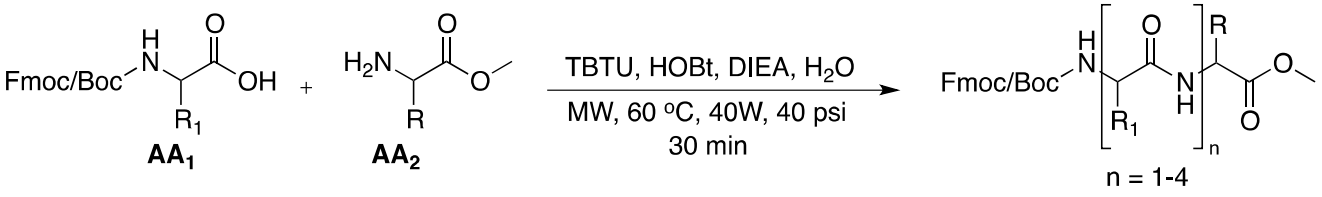
Fig. 4 Reaction conditions: (a) H_2O (2.5 mL), 1a (1.2 equiv), 1b (1.0 equiv), TBTU (1.2 equiv), HOBt (1.2 equiv), DIEA (2 equiv), 60 ˚C, 30 min, MW; (b) TBME (2.5 mL), 1a (1.2 equiv), 1b (1.0 equiv), DIC (1.2 equiv), HONB (1.2 equiv), DIEA (2 equiv), 60 ˚C, 30 min, MW.

The use of TBME is limited to the dipeptides synthesis, because of solubility problem of higher peptides in the reaction medium. In nutshell, experiments conducted indicate that neat H_2O can be preferred as a medium of choice for solution phase peptide synthesis under microwave irradiation.

Fig. 5 Progress of reaction: (a) beginning of reaction (b) end of reaction

With the optimized conditions in hand, the generality of the MW-assisted solution phase peptide in neat H_2O was explored. As shown in Fig. 5a, all reactants are partially soluble in the beginning of the reaction and the solution becomes clear on the exposure of MW irradiation at the end of reaction (Fig. 5b). We then synthesized a wide-variety of structurally diverse dipeptides, tripeptides and tetrapeptides consisting of Fmoc- and Boc-protected amino acids. The method was also extended to include amino acids without protection on the reactive side-chain and results were summarized in the Table 2. In all cases, excellent yields were obtained irrespective of the orthogonality of the α-amino and side-chain protecting groups. We have scaled-up the method by synthesizing peptides (≤ 1.0 g) without any difficulty.

Table 2 MW-assisted solution phase peptide synthesis in water



Entry	AA_1	AA_2	Product	Sequence	Yield	Purity
1	Boc-Phe-OH	H_2N-Ile-OMe	2a	Boc-Phe-Ile-OMe	90	98
2	Boc-Val-OH	H_2N-Ile-OMe	2b	Boc-Val-Ile-OMe	85	96
3	Boc-Phe-OH	H_2N-His-OMe	2c	Boc-Phe-His-OMe	82	97
4	Boc-Trp-OH	H_2N-Ile-OMe	2d	Boc-Trp-Ile-OMe	75	96
5	Boc-Met-OH	H_2N-Ile-OMe	2e	Boc-Met-Ile-OMe	75	95
6	Boc-Ser(Bzl)-OH	H_2N-Ile-OMe	2f	Boc-Ser(Bzl)-Ile-OMe	75	99
7	Boc-Trp-OH	H_2N-His-OMe	2g	Boc-Trp-His-OMe	82	100
8	Boc-His-OH	H_2N-His-OMe	2h	Boc-His-His-OMe	63	100
9	Boc-His(Bom)-OH	H_2N-Arg-OMe	2i	Boc-His(Bom)-Arg-OMe	69	99
10	Boc-Arg-OH	H_2N-Arg-OMe	2j	Boc-Arg-Arg-OMe	42	97
11	Boc-His(Bzl)-OH	H_2N-His-OMe	2k	Boc-His(Bzl)-His-OMe	75	98
12	Fmoc-Phe-OH	H_2N-Ile-OMe	2l	Fmoc-Phe-Ile-OMe	71	98
13	Fmoc-Ser(*t*-butyl)-OH	H_2N-Ile-OMe	2m	Fmoc-Ser(*t*-butyl)-Ile-OMe	60	99
14	Boc-Arg(Mtr)-OH	H_2N-Ile-OMe	2n	Boc-Arg(Mtr)-Ile-OMe	74	94
15	Boc-Val-OH	H_2N-Phe-Ile-OMe	3a	Boc-Val-Phe-Ile-OMe	86	94

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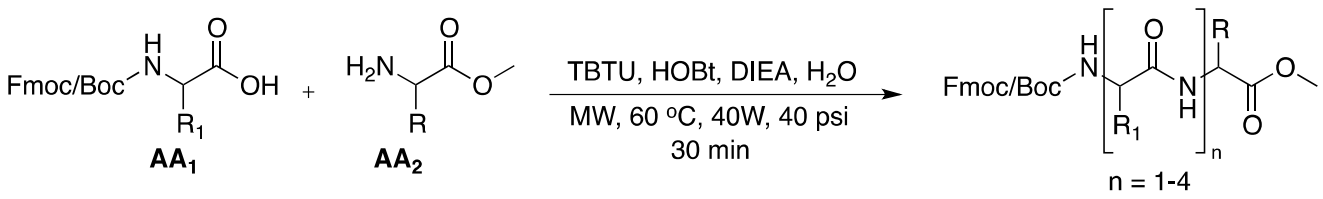
Table 2 (*Contd.*)



Entry	AA_1	AA_2	Product	Sequence	Yield	Purity[b]
16	Boc-Ile-OH	H_2N-Phe-Ile-OMe	3b	Boc-Ile-Phe-Ile-OMe	80	95
17	Boc-Abu-OH	H_2N-Phe-Ile-OMe	3c	Boc Abu-Phe-Ile-OMe	83	98
18	Boc-Phg-OH	H_2N-Trp-Ile-OMe	3d	Boc-Phg-Trp-Ile-OMe	65	95
19	Boc-Trp-OH	H_2N-Phe-Ile-OMe	3e	Boc-Trp-Phe-Ile-OMe	58	96
20	Boc-Ile-OH	H_2N-Ser(Bzl)-Ile-OMe	3f	Boc-Ile-Ser(Bzl)-Ile-OMe	75	94
21	Fmoc-Abu-OH	H_2N-Phe-Ile-OMe	3g	Fmoc-Abu-Phe-Ile-OMe	75	95
22	Fmoc-Leu-OH	H_2N-Phe-Ile-OMe	3h	Fmoc-Leu-Phe-Ile-OMe	83	98
23	Fmoc-Cys(*t*-butyl)-OH	H_2N-Ser(*t*-butyl)-Ile-OMe	3i	Fmoc-Cys(*t*-butyl)-Ser(*t*-butyl)-Ile-OMe	72	97
24	Boc-Phe-OH	H_2N-Ile-Phe-Ile-OMe	4a	Boc-Phe-Ile-Phe-Ile-OMe	71	99
25	Boc-Glu(OBzl)-OH	H_2N-Phg-Trp-Ile-OMe	4b	Boc-Glu(OBzl)-Phg-Trp-Ile-OMe	68	96
26	Boc-Ala(2-naphthyl)-OH	H_2N-Tyr(Bzl)-Phe-Ile-OMe	4c	Boc-Ala(2-naphthyl)-Tyr(Bzl)-Phe-Ile-OMe	74	97
27	Fmoc-Phe-OH	H_2N-Abu-Phe-Ile-OMe	4d	Fmoc-Phe-Abu-Phe-Ile-OMe	65	98
28	Boc-β-Ala-OH	H_2N-Trp-Met-Asp(Bzl)-Phe-NH_2	5a	Boc-β-Ala-Trp-Met-Asp-Phe-NH_2	57	99

[a] Reaction conditions: AA_1 (1.2 mmol), AA_2 (1.0 mmol), DIEA (2 mmol), TBTU (1.2 mmol), HOBt (1.2 mmol), H_2O (2.5 mL). [b]Purity was determined by HPLC analysis.

Lastly, the MW-assisted in-water peptide synthesis procedure was applied to synthesize pentagastrin (5a). Pentagastrin is a synthetic bioactive pentapeptide having the core active segment of gastrin.[31] The bioactive peptide stimulates the secretion of gastric acid, pepsin, and intrinsic factor, and is used as a diagnostic aid as the pentagastrin-stimulated calcitonin test. The seven steps total synthesis of pentagastrin (5a) in neat H_2O under MW irradiation is given in Scheme 2. The peptide was synthesized using *N*-α-Boc-protected amino acids in 42% overall yield. The HPLC chromatogram of the synthetic peptide is shown in Fig. 6. It can be safely concluded that peptide is synthesized in racemization-free high yield.

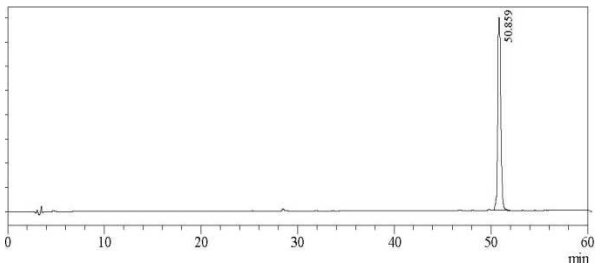
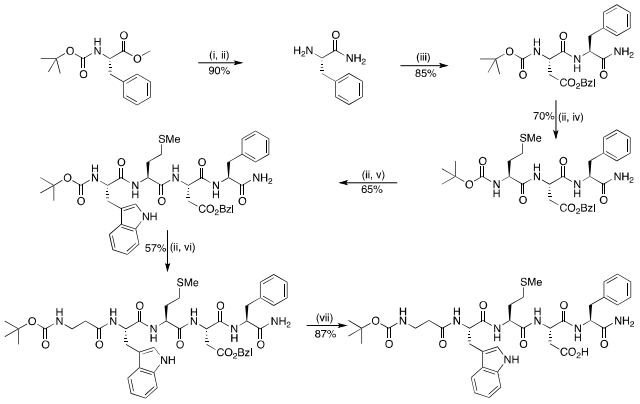


Fig. 6 HPLC chromatogram of pentagastrin (5a). Method: C-18, 300 Å, 5 µm, 250×4.6 mm column, run for 60 min with a flow of 1 mL/min, using a gradient of 95–5%, where buffer A was 0.1% TFA in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm



Reagents and conditions: (i) $HCONH_2$, NaOMe, 100 °C; (ii) 6N HCl, 15 min, 25 °C; (iii) Boc-Asp(Bzl)-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (iv) Boc-Met-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (v) Boc-Trp-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (vi) Boc-β-Ala-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (vii) 10% Pd/C, $HCOONH_4$, CH_3OH, reflux.

Scheme 2 Synthetic scheme for pentagastrin (5a)

Conclusion

In conclusion, we report the first MW-assisted solution phase peptide synthesis in neat H_2O. This rapid and environmental benign protocol for the preparation of racemization-free peptides is equally compatible with Fmoc- and Boc-protected amino acids, and completely eliminates the use of toxic organic solvents in peptide synthesis. The protocol proceeds smoothly under very mild reaction conditions in short reaction times, is cost effective (1.2-fold excess of reactants are used), and has compatibility with

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a wide-variety of side-chain protecting groups. Owing to the great diversity in amino acids, this protocol would be extremely useful in the synthesis of libraries of bioactive peptides.

Experimental Section

General experimental procedure for dipeptides (2a-n): In a 10 mL MW vial equipped with a magnetic stir bar, amino acid (AA_2) methyl ester·xHCl (1 mmol) and DIEA (2 mmol) was added H_2O (2.5 mL). Fmoc/Boc-AA_1-OH (1.2 mmol) was added, followed by TBTU (1.2 mmol) and HOBt (1.2 mmol). The reaction mixture was subjected to MW irradiation (CEM Discover® microwave reactor) with gas cooling (pressure of 40 psi was maintained during irradiation) for 30 min at 40W with magnetic stirring, and a temperature limit of 60 ˚C (reaction time refers to the hold time at the desired set temperature). H_2O was evaporated and the reaction mixture was purified on automated flash column chromatography system (Biotage®) to give Fmoc/Boc-AA_1-AA_2-OMe.

General experimental procedure for *N*-α-Boc-protected peptides (3a-f, 4a-c): In a 10 mL MW vial equipped with a magnetic stir bar, Boc-AA_1-AA_2-OMe (1 mmol) was reacted with 6N HCl (5 mL) at 25 ˚C for 15 min. Dihydrochloride salt of dipeptide was neutralized with DIEA (2 mmol). The resulting AA_1-AA_2-OMe (1 mmol) was dissolved in H_2O (2.5 mL). Boc-AA_3-OH (1.2 mmol) was added followed by TBTU (1.2 mmol) and HOBt (1.2 mmol). Mixture was subjected to MW irradiation (CEM Discover® microwave reactor) with gas cooling (pressure of 40 psi was maintained during irradiation) for 30 min at 40W with magnetic stirring, and a temperature limit of 60 ˚C (reaction time refers to the hold time at the desired set temperature). H_2O was evaporated and the reaction mixture was purified on automated flash column chromatography system (Biotage®) to afford tripeptides (**3a-f**). The deprotection and coupling cycle described above was repeated to obtain tetrapeptides (**4a-c**).

General experimental procedure for *N*-α-Fmoc-protected peptides (3g-i, 4d): In a 10 mL MW vial equipped with a magnetic stir bar, Fmoc-AA_1-AA_2-OMe (1 mmol) was reacted with 20% piperidine (2 mL) at 25 ˚C for 10 min followed by removal of excess piperidine. The resulting AA_1-AA_2-OMe (1 mmol) was dissolved in H_2O (2.5 mL). Fmoc-AA_3-OH (1.2 mmol) was added followed by TBTU (1.2 mmol), HOBt (1.2 mmol), and DIEA (2 mmol). Mixture was subjected to MW irradiation (CEM Discover® microwave reactor) with gas cooling (pressure of 40 psi was maintained during irradiation) for 30 min at 40W with magnetic stirring, and a temperature limit of 60 ˚C (reaction time refers to the hold time at the desired set temperature). H_2O was evaporated and the reaction mixture was purified on automated flash column chromatography system (Biotage®) to afford tripeptide (**3g-i**). The deprotection and coupling cycle described above was repeated to obtain tetrapeptide (**4d**).

Spectral data of Boc-Phe-Ile-OMe (2a): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.30-7.21 (m, 5H), 4.41-4.38 (m, 1H), 4.37-4.34 (m, 1H), 3.69 (s, 3H), 3.09 (dd, *J* = 5.8, 13.8 Hz, 1H), 2.82 (dd, *J* = 9.2, 13.7 Hz, 1H), 1.92-1.84 (m, 1H), 1.38 (s, 9H), 1.31-1.24 (m, 1H), 1.24-1.17 (m, 1H), 0.95-0.90 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.0, 171.9, 156.2, 137.1, 128.9, 127.9, 126.3, 79.3, 56.7, 55.8, 51.0, 37.6, 37.1, 27.2, 24.8, 14.5, 10.2; IR (neat) υ 3308.44, 2968.41, 2929.20, 2879.44, 1752.45, 1683, 1646.03, 1535.98, 1437.33, 1391.70, 1366.80, 1173.53, 1017.9, 989.31, 860.33, 716.14, 701; HRMS (ESI-TOF) calculated for [M+H$^+$] 393.2389, found 393.2384; HPLC: t_R= 47.80 min, 98%.

Spectral data of Boc-Val-Phe-Ile-OMe (3a): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.27-7.15 (m, 5H), 4.76 (t, *J* = 7.0 Hz, 1H), 4.35 (d, *J* = 6.3 Hz, 1H), 3.89 (d, *J* = 6.5 Hz, 1H), 3.65 (s, 3H), 3.06 (dd, *J* = 6.5, 13.6 Hz, 1H), 2.90 (dd, *J* = 8.2, 13.7 Hz, 1H), 1.97-1.89 (m, 1H), 1.86-1.78 (m, 1H), 1.41 (s, 9H), 1.30-1.26 (m, 1H), 1.23-1.14 (m, 1H), 0.89-0.83 (m, 12H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.7, 171.8, 170.3, 156.4, 136.7, 129.1, 128.0, 126.3, 79.1, 60.0, 56.8, 56.7, 54.1, 37.7, 29.3, 27.4, 24.8, 18.4, 17.2, 14.5, 10.3; IR (neat): υ 3277, 2966, 2414, 1648, 1527, 1458, 1366, 1246, 1175, 1017, 928, 878, 741, 697; HRMS (ESI-TOF): calculated for [M+H$^+$] 492.3073; found 492.3065; HPLC: t_R= 46.29 min, 95.78%.

Abbreviations: DIC, 1,3-diisopropylcarbodiimide; HONB, *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide; Bom, benzyloxymethyl; Bzl, benzyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt, 1-hydroxybenzotriazole; PyBOP, benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; EDC.HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; CDI, 1,1'carbonyldiimidazole; PEG, polyethylene glycol; DIEA, *N,N*-diisopropylethylamine.

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Notes and references

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Microwave-assisted solution phase peptide synthesis in neat water

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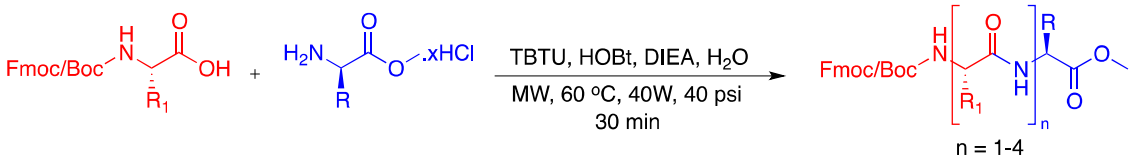

Text: An environmentally benign protocol for peptide synthesis is reported in neat water using TBTU/HOBt/DIEA under microwave irradiation.