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Solvent-free peptide synthesis assisted by microwave irradiation: environmentally benign synthesis of bioactive peptides†

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An efficient and facile, solvent-free peptide synthesis assisted by microwave irradiation, using DIC/HONB as the coupling reagent combination is reported. Key features of this original protocol are solvent-free synthesis, very short reaction time and scalability without affecting yield and purity. The versatility of the method was successfully demonstrated by synthesizing several biologically active peptides in high purity, yield and without racemization.

In recent years, the search for environmentally benign chemical processes and transformations has received much attention from chemists, as they are essential for the conservation of global ecosystems.[1] Solvent-free synthesis is probably the most efficient way to create eco-friendly complex organic syntheses, because organic solvents are generally the main source of toxic waste.[2] The omission of solvents from the synthetic cycle can address the increasing concern of the effect of harmful chemicals on the environment and human body.[3] In organic synthesis, several reactions have been performed under solvent-free conditions. Examples include, Mukaiyama-Aldol condensation,[4] Claisen and Cannizzaro reaction,[5] Wittig reaction,[6] Mannich reaction,[7] Suzuki-Miyara coupling reaction,[8] Passerini reaction,[9] and Prins cyclization.[10] The viability of solvent-free synthesis has become particularly very effective, when used in combination with microwave (MW) irradiation.[11] MW heating makes it convenient to perform reactions very effectively in neat conditions, and follow the twelve principles of green chemistry postulated by Anastas and co-workers.[12] In 1992, Sheldon *et al.*[13] coined the term "*E* factor" and defined it as the ratio of the weight of waste to the weight of product. In organic reactions, solvent contributes the most waste, and their elimination from the synthetic procedure reduces the *E* factor of the chemical reaction and enhances the sustainability of the chemistry. Thus, a reaction under solvent-free conditions in the presence of MW irradiation provides an ideal eco-friendly process in terms of reducing solvent cost and enhancement of the rate of reaction.

Amide bond formation is one of the most important reactions in organic synthesis due to its presence in a large number of natural and synthetic scaffolds having biological significance.[14] Among them, peptides represent an important class of compounds that are used as drugs in the treatment and prevention of several diseases.[15] Lately, apart from their biological importance, peptides have been used as catalyst in asymmetric synthesis,[16] biosensors,[17] molecular hydrogelators,[18] and biological markers in diagnostic tests.[19] Over the years, peptides have been synthesized by two strategies of solution phase and solid phase peptide synthesis (SPPS).[20] These procedures generate huge amount of toxic solvents like *N*,*N*-dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP), *N*,*N*-dimethylacetamide (DMA) and dichloromethane (CH_2Cl_2). Therefore, reducing and/or eliminating these solvents from the synthesis has been an ever-growing concern of the chemists.[21]

In quest for eco-friendly peptide synthesis, several research groups have reported SPPS in water (H_2O) as a solvent medium. The first SPPS in H_2O was reported by Kawasaki *et al.*[22] followed by Grøtli *et al.*[23] and Collins in 2012.[24] All these methods have limitations either in terms of coupling reagent, solid support or requirement of special protection on amino acids. Our efforts in the development of benign peptide synthesis have resulted in the disclosure of methods in neat water and under MW irradiation.[25,26] The important highlights of the "in-water" method were the use of commercially available amino acids, short reaction time, and elimination of hazardous solvents in the synthetic cycle. In continuation of our efforts to establish environmentally benign peptide synthesis, we now describe the first report on the solvent-free peptide synthesis assisted by MW irradiation. The scope of this eco-friendly synthetic transformation was established by illustrating its application in the synthesis of bioactive peptides.

Pursuing our interest in establishing solvent-free peptide synthesis, we found few interesting articles on the construction

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of amide bond in the presence of 1,1′-carbonyldiimidazole (CDI).[27] These reports use high temperature (250 °C) to form amide bonds; however could not be extended to peptide synthesis. More recently, Lamaty *et al.*[28,29] have reported a solvent-free construction of amide bond, and a liquid-assisted synthesis of peptide; both performed under ball milling conditions. The peptide synthesis method used pre-activated Boc-protected α-amino acid *N*-carboxy anhydrides or *N*-hydroxysuccinimide esters in the presence of ethyl acetate as the liquid grinding assistant.[29] To the best of our knowledge, till date no report exists on the synthesis of peptides under solvent-free conditions.

We initiated study by optimizing the microwave parameters under solvent-free conditions. For the model study, we took Boc–Phe–OH (**1a**) and Ile–OMe·HCl (**1b**) as the coupling amino acids, while DIC was used as the coupling reagent and HONB as an auxiliary nucleophile (Scheme 1).

The criterion for the selection of the coupling reagent and auxiliary nucleophile was based upon their low cost and wide application in peptide synthesis. A number of experiments were conducted under variable sets of temperature and time, keeping pressure and power constant, under MW. From the bar graph (Fig. 1), we envisaged that the temperature of 60 °C and reaction time of 15 min provide the best coupling conditions, and gave 97% isolated yield of Boc–Phe–Ile–OMe (**2a**).

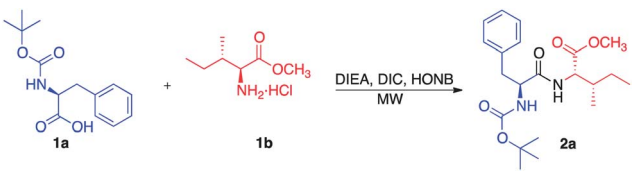
The HPLC analysis (Fig. 2) indicated a purity of 97% of **2a**, confirming the effectiveness of the protocol. It is important to note that, when reaction was conducted at a temperature higher than 60 °C, some racemization was observed with reduced yield of the peptide.

After establishing MW parameters, the role of DIEA, DIC and HONB was investigated by their sequentially addition in the reaction. From Table 1, it was evident that reaction does not proceed in the absence of coupling reagent and auxiliary nucleophile (entries 1 and 2). The reaction of the HCl salt of **1b** with **1a** using DIC gave 70% of **2a** (Table 1, entry 3), while *in situ* neutralization of the HCl salt with DIEA, and subsequent coupling with DIC enhanced the product yield to 90% (Table 1, entry 5). The coupling reaction with DIC and HONB gave only 47% yield (Table 1, entry 4). The best coupling combination was established by addition of DIEA to DIC and HONB, resulting in the synthesis of **2a** in highest yield and purity (entry 6).

To confirm the importance of MW irradiation, the peptide coupling using the same reactants and coupling combination was performed under conventional heating condition. The reaction was performed in a pre-heated oil bath at 60 °C for a duration between 15 and 45 min. We observed much lower yield of the peptide (14%), clearly demonstrating the importance of MW irradiation in the solvent-free synthesis of peptides.

We also explored the various sets of coupling combinations under the optimized conditions. It was interesting to note that all tested coupling combinations provide peptide under solvent-free conditions. The coupling combinations of PyBOP/HOBt, HATU/HOAt and TBTU/HOBt gave 63–81% yield of **2a** (Fig. 3). As observed from the chart graph, DIC/HONB gave the best result among all screened coupling combinations. As seen from the Fig. 2, DCC provides comparable yield of 91%, while the use of CDI as the coupling reagent furnished **2a** in 19% yield.

To explore the substrate scope of the reaction, a series of peptides were synthesized under solvent-free conditions. The results of this study are summarized in Table 2. The dipeptides (Table 2, entries 1–13) were synthesized in 75–97% yield. The



Scheme 1 Generalized scheme for coupling.

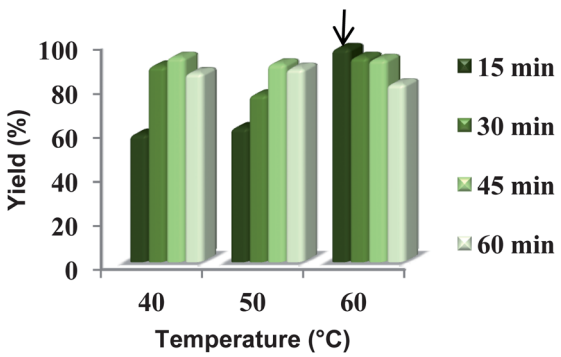


Fig. 1 Optimization of microwave parameters.

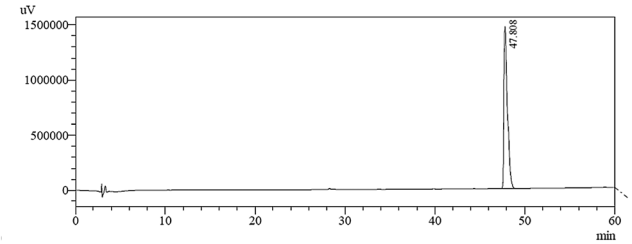


Fig. 2 HPLC chromatogram of **2a**. Method: C-18, 300 Å, 5 μm, 250 × 4.6 mm column, run for 60 min with a flow of 1 mL min^{-1}, using a gradient of 95–5%, where buffer A was 0.1% CF_3CO_2H (TFA) in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm.

Table 1 Various solvent-free reaction conditions[a]

Entry	Various solvent-free conditions	Yield (%)	Purity (%)
1	AA_1 + AA_2	No reaction	—
2	AA_1 + AA_2 + DIEA	No reaction	—
3	AA_1 + AA_2 + DIC	70	94%
4	AA_1 + AA_2 + DIC + HONB	47	96%
5	AA_1 + AA_2 + DIC + DIEA	90	95%
6.	AA_1 + AA_2 + DIC + HONB + DIEA	97	97%

[a] Reaction conditions: AA_1 (1.2 equiv.), AA_2 (1 equiv.), HONB (1.2 equiv.), DIEA (3 equiv.), DIC (1.2 equiv.), MW (60 °C, 15 min, 40 W); AA_1 represent **1a** and AA_2 represent **1b**.

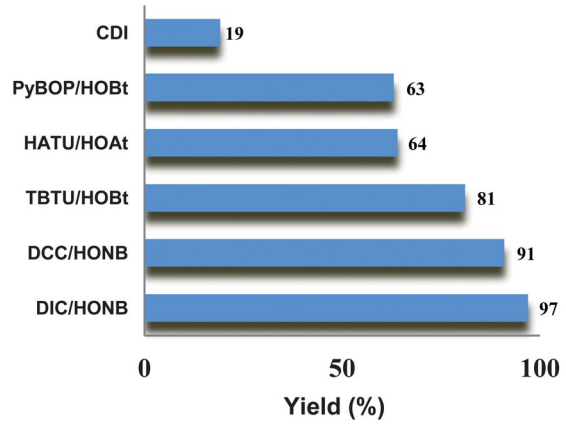
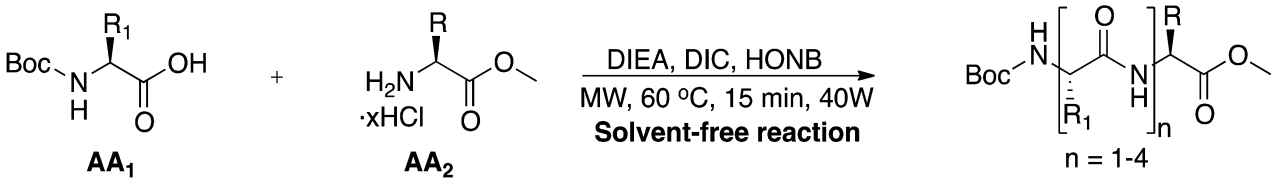


Fig. 3 Screening of coupling reagents under optimized parameters [AA_1 (1.2 equiv.), AA_2 (1 equiv.), DIEA (3 equiv.), MW (60 °C, 15 min, 40 W)].

electronic nature of the substituents present on the side-chain of the amino acid governed the yield of peptides. As observed from Table 2, the reaction proceeds smoothly even in cases where His, Pro, Trp and Arg were used without protection on the side-chain group (Table 2, entries 2, 4, 6, 7, 11, 19 and 23), confirming the reactive functional group tolerance of the process and racemization-free synthesis. The reaction is versatile to accommodate smooth coupling of bulky side-chain containing amino acids such as Leu, Ile, and Val.

Both hydrophobic (Table 2, entries 1, 4, 7, and 12) and hydrophlilic peptides (Table 2, entry 6), beside peptides containing both hydrophobic and hydrophilic residues were successfully synthesized. The removal of the α-Boc group was achieved by the reaction with aqueous 6N HCl for 15 min at ambient temperature. A set of tripeptides (Table 2, entries 14–19) was synthesized in 70–78%. The scope of the reaction was further extended with the successful synthesis of tetrapeptides

Table 2 Solvent-free peptide synthesis assisted by MW[a]



Entry	AA_1	AA_2	Product code	Sequence	Yield (%)	Purity[b] (%)
Dipeptides ($n = 1$)						
1	Phe	Ile	**2a**	Phe–Ile	97	97
2	Phe	His	**2b**	Phe–His	75	93
3	Ser(Bzl)	Ile	**2c**	Ser(Bzl)–Ile	99	96
4	Trp	His	**2d**	Trp–His	75	92
5	Val	Ile	**2e**	Val–Ile	75	92
6	His(1-Bzl)	His	**2f**	His(1-Bzl)–His	76	99
7	Trp	Ile	**2g**	Trp–Ile	98	95
8	Asp(Bzl)	Lys(Z)	**2h**	Asp(Bzl)–Lys(Z)	78	91
9	Met	Ile	**2i**	Met–Ile	96	98
10	Abu	Ile	**2j**	Abu–Ile	86	91
11	Lys(Z)	Pro	**2k**	Lys(Z)–Pro	76	93
12	Phg	Ile	**2l**	Phg–Ile	86	95
13	Val	Tyr(Bzl)	**2m**	Val–Tyr(Bzl)	87	99
Tripeptides ($n = 2$)						
14	Ile	Phe–Ile	**3a**	Ile–Phe–Ile	77	94
15	Thr(Bzl)	Phe–Ile	**3b**	Thr(Bzl)–Phe–Ile	78	92
16	Asp(Bzl)	Val–Tyr(Bzl)	**3c**	Asp(Bzl)–Val–Tyr(Bzl)	73	93
17	His(Bom)	Phe–Ile	**3d**	His(Bom)–Phe–Ile	71	95
18	Ala(2-naphthyl)	Phe–Ile	**3e**	Ala(2-naphthyl)–Phe–Ile	78	94
19	Pro	Met–Ile	**3f**	Pro–Met–Ile	70	98
Tetrapeptides ($n = 3$)						
20	Ala	Thr(Bzl)-Phe-Ile	**4a**	Ala–Thr(Bzl)–Phe–Ile	60	97
21	Lys(Z)	Asp(Bzl)-Val-Tyr(Bzl)	**4b**	Lys(Z)–Asp (Bzl)–Val–Tyr(Bzl)	65	95
22	Met	Ala(2-naphthyl)-Phe-Ile	**4c**	Met–Ala(2-naphthyl)–Phe–Ile	62	93
Pentapeptide ($n = 4$)						
23	Arg	Lys(Z)–Asp(Bzl)–Val–Tyr(Bzl)	**5a**	Arg–Lys(Z)–Asp(Bzl)–Val–Tyr(Bzl)	55	94

[a] Reaction Conditions: AA_1 (1.2 mmol), AA_2 (1.0 mmol), DIEA (3 mmol), DIC(1.2 mmol), HONB (1.2 mmol). [b] Purity was determined by HPLC analysis.

(Table 2, entries 20–22, yield: 60–65%) and a pentapeptide (Table 2, entry 23, 55% yield). In all cases, cleaner reaction and high yield were obtained irrespective of the orthogonality of the α-amino and side-chain protecting groups. It is important to mention that peptides in general were synthesized in higher yield compared to earlier reported MW-assisted approaches of peptide synthesis.[25,26]

To establish the application of the solvent-free synthesis in peptide-based drug discovery, we synthesized a number of peptides of pharmaceutical importance. The peptides selected for the solvent-free synthesis were carnosine (**6**, used in complications of diabetes),[30] aspartame (**7**, a commercially used artificial sweetener),[31] thyrotropin-releasing hormone (**8**, a regulatory neuropeptide),[32] thymopentin (**9**),[33] and Leu-enkephalin (**10**, an opioid neurotransmitter peptide)[34] (Fig. 4). The synthesis of thymopentin is discussed herein, while the synthetic schemes and discussion on the remaining four peptides is provided in the ESI.†

Thymopentin (TP-5), a pentapeptide derived from the thymopoietin hormone, possess a broad range of therapeutic activities and elicits all biological responses of hormone.[33] *In vitro* treatment of cells homologous to human T cells with thymopentin increases level of cGMP.[35] Whereas, *in vivo* activities of thymopentin finds it as immunomodulating agent in primary and secondary immune deficiencies like metastatic colorectal cancer (CC), generalized lymphadenopathy, atopic dermatitis, and rheumatoid arthritis adjuvant in vaccination against hepatitis B.[36]

The ten steps synthesis of thymopentin assisted by MW is illustrated in Scheme 2. The peptide was obtained in an overall yield of 53%, starting from Tyr(Bzl)–OMe·HCl. The key highlight of the synthesis was the successful coupling of Boc–Arg–OH (bearing a side-chain reactive group), in the penultimate step of the synthesis. Protected thymopentin **5a** upon deprotection (aqueous acidolysis to remove Boc group, base-catalyzed removal of ester group, and finally Pd-catalyzed removal of side-chain protective groups) afforded thymopentin **9** in the purity of 97% (Fig. 5).

Finally, we attempted to establish the versatility of the reaction by synthesizing peptides on a higher scale. The question of reaction scale was successfully answered by solvent-free synthesis of peptides in ≤1g, while maintaining the ratio of reactants and conditions intact, in an overall identical yield and purity.

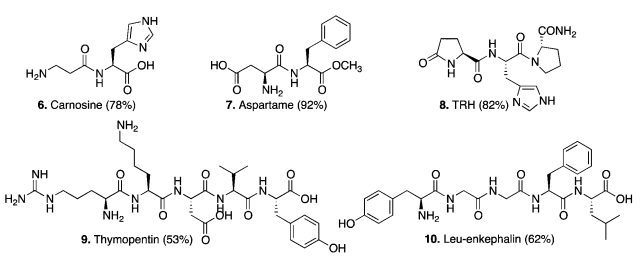
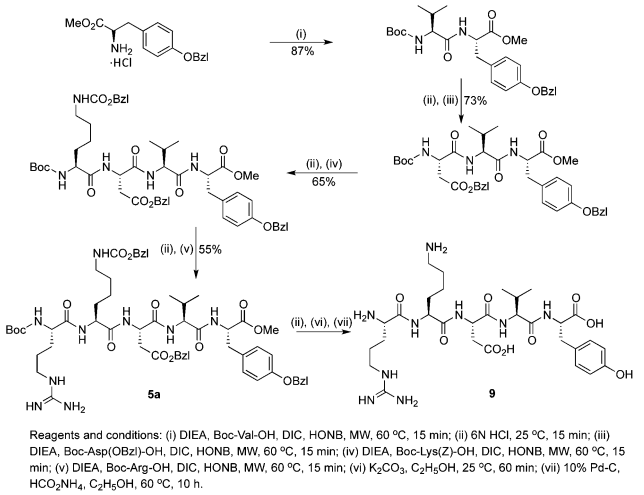


Fig. 4 Chemical structure of the bioactive peptides synthesized using solvent-free conditions (overall yield in parentheses).



Scheme 2 Synthesis of thymopentin (**9**).

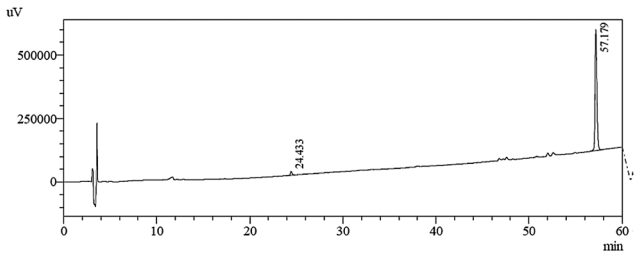


Fig. 5 HPLC chromatogram of **9**. Method: C-18, 300 Å, 5 μm, 250 × 4.6 mm column, run for 60 min with a flow of 1 mL min^{-1}, using a gradient of 95–5%, where buffer A was 0.1% TFA in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm.

Conclusion

We have developed an original and environmentally benign process for the synthesis of peptides under solvent-free conditions. This solvent-free synthesis was assisted by MW irradiation and requires about 1.2 fold excess of reactants to provide peptides in 15 min at 60 °C. The reaction is easily applicable to hydrophobic/hydrophilic amino acids containing a variety of orthogonal protective groups. The reaction also allows successful coupling of amino acids bearing side-chain reactive groups. The applicability and scope of this synthetic process was realized by synthesizing a varied number of bioactive peptides having significance in pharmaceutical industry. In summary, the synthesis of peptides under solvent-free conditions in reduced reaction time, high yield and purity, and without racemization, ably assisted by MW irradiation offers a new paradigm to sustainable chemistry.

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