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Optimization of solid-phase synthesis of difficult peptide sequences via comparison between different improved approaches

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Summary. Different approaches are applied to avoid the strong aggregation of the difficult peptide sequences, which is considered as the main reason for incomplete acylation and deprotection reactions hindering the synthesis of these sequences.

Temporary protection of amide nitrogen of peptide bond using 2-hydroxy-4-methoxybenzyl (Hmb) and 2,4,6-timethoxybenzyl (Tmob) amino acid derivatives, introduction of D-Ala or Pro residues in the peptide chain sequences and utilization of microwave energy are proved to be useful methods in the enhancement of solubility and in the hindrance of the aggregation during the solid-phase synthesis of oligoalanine. Oligoalanine is chosen to demonstrate the difficult sequences and to compare the efficiencies of these methods.

Keywords: Difficult peptide sequences – Solid-phase peptide synthesis – Oligo-alanine – N-amide protection – Microwave energy

Abbreviations: DCM, Dichloromethane; DIC, N,N-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; Hmb, 2-hydroxy-4-methoxybenzyl; HOBt, 1-hydroxybenzo-triazole; Ps–PEG, polystyrene–polyethyleneglycol; TFA, trifluoroacetic acid; Tmob, 2,4,6-timethoxybenzyl

Introduction

It has been frequently reported that ''difficult sequences'' are encountered during solid-phase peptide synthesis. Such difficulties occurring during the assembly of protected peptides are thought to be a result of internal aggregation of peptide chains within the peptide–resin matrix and are usually sequence dependent. Massive steric hindrance commonly results, leading to reduced reagent penetration and significantly reduced reaction rates in both acylation and deprotection steps. Purification of peptide synthesis was not carried out; even by using the most modern powerful chromatographic techniques, isolation of target peptide cannot be guaranteed (Kent, 1985, 1992).

Aggregation in batch synthesis is indicated by shrinkage of the resin matrix. In continuous flow synthesis it is detected by flattening and broadening of deprotection profile. The driving force for this intrachain and interchain association is regarded to be hydrogen bonding and hydrophobic forces (Mutter et al., 1981).

Aggregation can occur from as nearly as the fifth residue coupled. The tendency for aggregation depends on the nature of the peptide chain. Sequences containing a high proportion of Ala, Val, Ile, Asn, or Gln residues show particular propensity for this effect. Secondary amino acids such as Pro and Sar are thought to inhibit aggregations (Haack and Mutter, 1992; Wöhr et al., 1996).

Reported attempts to suppress the degenerative effect of such association during amino acylation reactions involve essentially ''external factors'' like solvent composition (Milton and Milton, 1990) as the employment of ''magic solvent'' (Zhang, 1994), elevated temperature (Lloyd et al., 1990), use of chaotropic and Cs salts (Abdel Rahman et al., 1992) or solubilizing protecting groups (Mutter et al., 1992) have been shown to have variable efficiencies. Hydrogen-bonded association has also been prevented by the introduction of nitrogen amide protecting group within the peptide chain (Johnson et al., 1993).

Over the last ten years, microwave synthesis has become widely accepted to increase reaction rates in organic synthesis up to 1000-fold. Unlike conventional heating, microwave energy directly activates any molecule with a dipole moment and allows for rapid heating at the molecular level (Hayes, 2002; Loupy, 2002). It has also been successfully used to increase the rate of peptide coupling reactions and to overcome intermolecular aggregation,

β-sheet formation, and steric hindrance (Erdélyi and Gogoll, 2002).

Materials and methods

Materials, instruments and methods

All protected amino acids were purchased from Calbiochem-Novabiochem AG (Laufelfingen, CH). The organic solvents and the chemicals used were obtained from Aldrich Chemical Company, E. Merck (Germany), Sigma (USA) and Fluka (Switzerland). PS–POE–NH_2 polymer was kindly supplied by Dr. Rapp W. (Tübingen, Germany). Solvents were used without further purification.

Infra-red spectra (KBr) were recorded on FT/IR 1650 Perkin-Elmer spectrometer. UV measurements were recorded on Perkin-Elmer (Lambda 1, UV/Vis) spectrophotometer.

Primary amines were detected by Kaiser test (Kaiser et al., 1970) and secondary amines by Chloranil test (Vojkovsky, 1995).

Synthesis of peptide chains

Coupling of PS–POE–NH_2 with the anchoring group (P-hydroxy methylbenzoic acid (HMBA)

It was carried out according to Sheppard and Williams (1982).

Coupling of the first amino acid to the PS–POE–NH–HMBA resin

To a suspension of 1 g polymer anchor (0.22 meq) in 2 ml (1:1) CH_2Cl_2/DMF, a solution of 0.684 g Fmoc-Ala-OH (2.2 meq), 0.227 g DIC (2.2 meq), 0.297 g HOBt (2.2 meq), and 11.4 mg DMAP (0.093 meq) in 3 ml (1:1) CH_2Cl_2/DMF was added. The mixture was shaken for 24 h at RT. It was filtered and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH, and ether.

*Coupling capacity measurement for the first amino acid was carried out according to Meinhofer et al. (1979).

Coupling of N,O-bis-Fmoc-N-(Hmb)Ala-OPfp to the growing peptide chain I

To a suspension of 1 g polymer-bound peptide (0.21 meq) in 3 ml DMF/CH_2Cl_2 (1:1), a solution of 0.526 g N,O-bis-Fmoc-N-(Hmb)Ala-Opfp (0.63 meq) and 0.085 g HOBt (0.63 meq) in 3 ml DMF was added. The mixture was then shaken at RT until Kaiser test became negative, and then filtered and washed as usual. The next Ala residue (after Hmb-Ala) was coupled using Fmoc-Ala-OH (20 meq)/DIC (10 meq) in DCM, until Chloranil test became negative.

Coupling of N-Fmoc-N-(Tmob)Ala-OH to the growing peptide chain II

To a suspension of 1 g polymer-bound peptide (0.21 meq) in 3 ml DMF/CH_2Cl_2 (1:1), a solution of 1.03 g N-Fmoc-N(Tmob)Ala-OH (2.1 meq), 0.283 g HOBt (2.1 meq), and 0.264 g DIC (2.1 meq) in 3 ml DMF/CH_2Cl_2 (1:1) was added. The mixture was then shaken at RT until Kaiser test became negative, and then filtered and washed as usual. The next Ala residue (after Tmob-Ala) was coupled by shaking Fmoc-Ala-OH (20 meq)/DIC (10 meq) in (1:1) CH_2Cl_2/DMF until Chloranil test became negative.

Coupling of Fmoc-Pro-OH to the growing peptide chain III

To a suspension of 1 g polymer-bound peptide (0.21 meq) in 3 ml DMF/CH_2Cl_2 (1:1), a solution of 0.70 g Fmoc-Pro-OH (2.1 meq), 0.283 g HOBt (2.1 meq) and 0.264 g DIC (2.1 meq) in 3 ml DMF/CH_2Cl_2 (1:1) was added. The mixture was then shaken at RT until Kaiser test became negative, and then filtered and washed as usual.

Coupling of Fmoc-D-Ala-OH to the growing peptide chain IV

To a suspension of 1 g polymer bound peptide (0.21 meq) in 3 ml DMF/CH_2Cl_2 (1:1), a solution of 0.653 g Fmoc-D-Ala-OH (2.1 meq), 0.283 g HOBt (2.1 meq) and 0.264 g DIC (2.1 meq) in 3 ml DMF/CH_2Cl_2 (1:1) was added. The mixture was then shaken at RT until Kaiser test became negative, then filtered and washed as usual.

Cleavage of the N-amide protecting groups

Cleavage of Hmb group

Polymer-bound peptide (50 mg) was placed in 2 ml syringe, and then 1 ml solution of 92% TFA/3% phenol/3% ethanedithiol/2% triethyl silane (v/w/v/v) was added. The reaction mixture was left to stand for 2 h, and then filtered and washed several times with DMF, CH_2Cl_2, MeOH, and ether.

Cleavage of Tmob group

Polymer-bound peptide (50 mg) was placed in 2 ml syringe, and then 1 ml solution of 95% TFA/5% H_2O/5% MeOH (v/v/v) was added. The reaction mixture was left to stand for 1 h, and then filtered slowly without suction, washed with 2 ml scavenger and filtered without suction. The precipitate was then washed several times with DMF, CH_2Cl_2, MeOH, and ether. One milliliter of piperidine/DMF (5:100) was added for neutralization. After 15 min the solution was filtered and washed several times with DMF, CH_2Cl_2, MeOH, and ether.

Microwave-assisted solid-phase peptide synthesis

Coupling of PS–PEG_{3000}–NH_2 with p-hydroxymethylbenzoic acid using MW energy

To a suspension of PS–PEG_{3000}–NH_2 (2 g, 0.44 meq) in 5 ml DMF, a solution of HOBt (0.297 g, 2.2 mmol), *p*-hydroxymethylbenzoic acid (0.334 g, 2.2 mmol), and DIC (0.277 g, 2.2 mmol) in 5 ml DMF was added. The mixture was then heated in MW oven until Kaiser test became negative. The reaction mixture was filtered and washed several times with DMF, CH_2Cl_2, MeOH, and ether.

Coupling of PS–PEG_{3000}–HMBA resin to the first Fmoc-Ala-OH

It was carried out manually out of the microwave oven as described before.

Coupling of the next Fmoc-Ala-OH to the peptide chain using MW energy

To a suspension of H-Ala-HMBA-resin (2 g, 0.42 meq) in 5 ml DMF, a solution of Fmoc-Ala-OH (0.653 g, 2.1 meq), HOBt (0.283 g, 2.1 meq) and DIC (0.264 g, 2.1 meq) in 5 ml DMF was added. The mixture was then heated in MW oven, until Kaiser test became negative. The solution was then filtered and washed several times with DMF, CH_2Cl_2, MeOH, and ether.

Deprotection of Fmoc N-protecting group using MW energy

Fmoc Ala-HMBA-resin (2 g, 0.42 mmol) was suspended in least amount of DMF. Then, 5 ml of 20% piperidine/DMF solution was added and the mixture was then heated in MW oven, until Kaiser test became positive. The solution was then filtered and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH, and ether.

Coupling of N,O-bis-Fmoc-N(Hmb)Ala-OPfp as fifth residue to the peptide chain using MW energy

To a suspension of polymer-bound peptide (1 g, 0.21 meq) in 3 ml DMF, a solution of N,O-bis-Fmoc-N-(Hmb)Ala-Opfp (0.526 g, 0.63 meq) and HOBt (0.085 g, 0.63 meq) in 3 ml DMF was added. The mixture was then heated in MW oven, until Kaiser test became negative, and then it was filtered and washed as usual.

Results and discussion

In the present work we studied the efficiency of different modified approaches for inhibiting the interchain association, preventing the β-structure formation and enhancing the rate of both coupling and deprotection reactions which hindered the synthesis of difficult peptide sequences. Oligoalanine was chosen to demonstrate the difficult sequences; this sequence is notorious for strong aggregation from the fifth residue onward, which makes it pointless to continue. Synthesis of oligo-$Ala_{(10)}$ gave an almost highly insoluble heterogeneous product with various deletion or termination sequences (Clausen et al., 1996). The selected approaches included the incorporation of N-Tmob-alanine, N-Hmb-alanine, D-Ala, and Proline every fourth residue during the batch solid-phase synthesis of resin-bounded oligoalanine. Microwave energy is also applied separately, aiming to decrease the intermolecular aggregation and β-sheet formation.

Synthesis of oligoalanine chains

The synthesis was carried out by using Fmoc SPPS. 4-Hydroxymethyl-benzoic acid (HMBA) was used as an anchoring group which binds the polymeric support (polystyrene–polyethylene glycol) to the first amino acid. This anchor group is base labile and TFA stable, so it enables the deprotection of Tmob and Hmb groups without cleavage of the polymeric support. The couplings were mediated by N,N-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT) in N,N-dimethylformamide (DMF). Deprotection of Fmoc group was carried out using 20% piperidine/DMF mixture.

All coupling and deprotection reactions were monitored by Kaiser test; Chloranil test was also used to detect the coupling of Ala residue after N-Tmob- and N-Hmb-Ala.

By means of applying the N-amide protecting groups and introducing D-Ala or Pro during the SPPS of oligoalanine, oligo-$Ala_{(21)}$ was synthesized without difficulties in coupling, deprotection, and purification steps. The coupling efficiency remained high as the length of resin-bound peptide increased and continued to show excellent incorporation for the addition of more Ala residues.

A major factor for such efficiencies is the powerful solubility of the growing peptide chains (Quibell and Johnson, 1995) which is observed especially when N-amide protecting groups such as N-Hmb and N-Tmob were used.

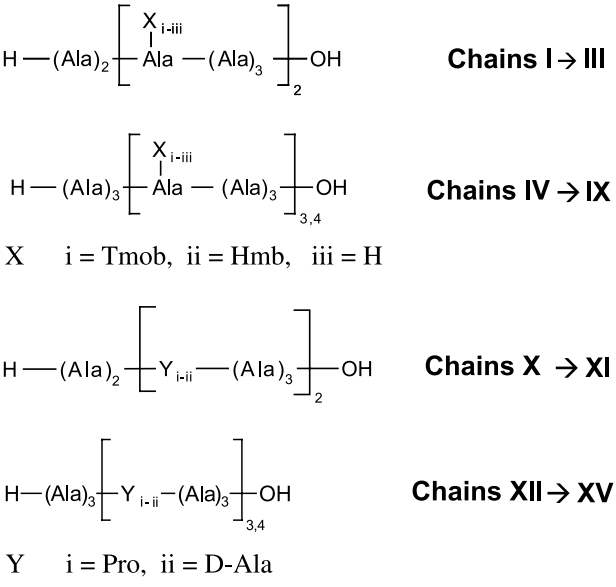
Hydrolysis of completed peptide resin followed by amino acid analysis revealed ratios in accord with expectation. Furthermore, comparison to a Nle ''internal reference'' showed that all amino acid incorporation steps were essentially quantitative.

The time of coupling reactions did not exceed one hour; except in case of coupling the Ala residues following Tmob-Ala directly the coupling time is increased to two hours as detected by Chloranil test; this may be due to the steric hindrance. In case of using Hmb-Ala no such increment in the time of coupling is observed. The deprotection of Fmoc group lasted for 20–30 min as usual.

Spectroscopic study on the synthesized peptide chains

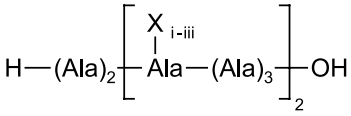
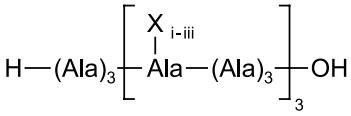
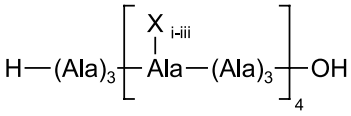
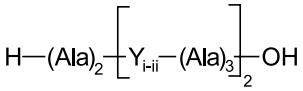
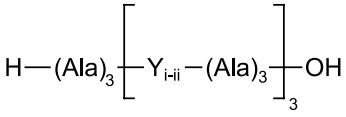
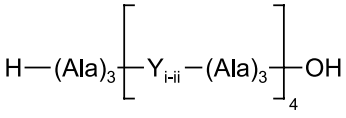
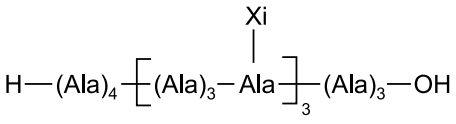
Spectroscopic study on the synthesized peptide chains was carried out by IR measurements. Chains containing N-Tmob and N-Hmb groups were subjected to the measurements before and after cleavage of these groups.

The following peptide chains were investigated:



The main goal of this study is to reveal the effect of replacing the alanine residue by N-Tmob-Ala, N-Hmb-Ala, Pro, and D-Ala, respectively, on the formation of the β-sheet which results in strong aggregation from the fifth residue in oligoalanine chain. The IR absorption spectra of the peptide under investigation are summarized in Table 1 at the regions of amide I and amide A which are the reported conformation diagnostic regions (Edelhoch et al., 1969).

Table 1. IR data of investigated peptides in the amide I and amide A regions

Chain structure	Chain number	X_{i-iii} & Y_{i-ii}	Amide I (cm^{-1})	Amide A (cm^{-1})
	I	i = Tmob	1659.8	3411.2
	II	ii = Hmb	1656.3	3422
	III	iii = H	1631	3444.6
	IV	I = Tmob	1655.2	3421.7
	V	ii = Hmb	1655.8	3422.5
	VI	iii = H	1630	3280
	VII	i = Tmob	1656	3411
	VIII	ii = Hmb	1655.4	3424
	IX	iii = H	1630	3283
	X	i = Pro	1655.6	3424.3
	XI	ii = D-Ala	1629.5, 1656.3	3422.9
	XII	i = Pro	1655.1	3422.8
	XIII	ii = D-Ala	1629.7, 1655.3	3426
	XIV	i = Pro	1656	3423.5
	XV	ii = D-Ala	1628.8, 1654.3	3425.7
	XVI	I = Tmob	1630, 1654.7	3424

According to Toniolo and Palumbo (1977) various types of β-structures are known to have strong bands near 1635–1624 cm^{-1} (amide I), 3280–3260 cm^{-1} (amide A) and a weak but distinct band at 705–720 cm^{-1} (amide V) whereas absorption near (1655 and 650 cm^{-1}) are associated with unordered conformation.

To avoid the ambiguity coming from the bands of carbamate transition of Fmoc group at 1715–1685 cm^{-1}, IR measurements were carried out on N-deprotected chains. Amide V band is excluded due to the interference of the aromatic ring stretching mode (of the resin).

The results show that peptide chains I, IV, and VII which contain Tmob-Ala residue every fourth residue exhibited strong bands at 1659.8, 1655.2, and 1656 cm^{-1} (amide I) and broad bands at 3411.2, 3421.7, and 3411 cm^{-1} (amide A), respectively, which revealed the presence of unordered structure. On the other hand, the spectra of peptide chains II, V, and VIII which contain Hmb-Ala residue every fourth residue showed the characteristic bands of unordered structure: strong bands at 1656.3, 1655.8, and 1655.4 cm^{-1} (amide I) and broad bands at 3422, 3422.5, and 3424 cm^{-1} (amide A region), respectively.

The spectra of $(oligo\text{-}Ala)_{10,15,19}$ chains III, VI, and IX which were produced by cleavage of Tmob and Hmb groups were found to exist in β-structure conformation. The IR spectra of these chains showed a strong band characteristic for β-structure at amide I region (1631, 1630, and 1630 cm^{-1}, respectively).

These results indicated clearly that the presence of Tmob-Ala and Hmb-Ala residues in oligoalanine chains disrupt the β-structure formation, while the cleavage of these N-protecting groups result in formation of β-structure.

This result is in good agreement with that obtained from the solid-phase NMR measurements of octamer III in the presence of Tmob group and after its cleavage (Fig. 1). The spectrum showed sharp peaks in the presence of Tmob group turned flat and broad after its cleavage which indicates the formation of β-structure in the latter case.

IR measurements also showed that the incorporation of Pro residue into polyalanine chain every fourth residue suppressed the formation of β-structure and inhibited chain aggregation (chains X, XII, and XIV).

Results also indicated that replacement of L-Ala by D-Ala residue every fourth residue (chains XI, XIII,

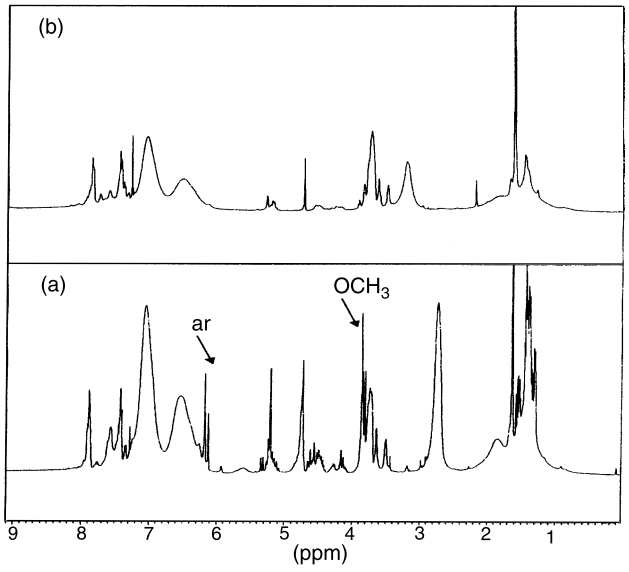


Fig. 1. The solid-phase NMR spectrum of octamer III: (**a**) in the presence of Tmob group and (**b**) after cleavage of Tmob group

and XV) in $(poly\text{-}Ala)_{10,15,19}$ results in the presence of both unordered and β-structure (amide I) at 1629.5, 1656.3, 1629.7, 1655.3, and 1628.8, 1654.3 cm^{-1}, respectively, while (amide A) at 3422.9, 3426, and 3425.7 cm^{-1}, respectively.

Results of IR spectroscopic measurements indicate generally that the incorporation of amide protecting groups as Tmob and Hmb groups prevented the formation of β-structure and markedly inhibited the aggregation which reduce the solvation and the rate of both coupling and deprotection reactions. The replacement of Ala by Pro every fourth residue showed the same effect as amide protecting groups while the introduction of D-Ala in the same manner is less efficient.

These results are in coincidence with the results obtained from the measurements of the time of coupling and deprotection reactions which showed that the temporary protection of amide nitrogen using groups such as Hmb and Tmob every fourth residue in difficult sequences proved to be a useful tool for disrupting the β-structure responsible for aggregation of these chains.

The incorporation of Pro residue in the same manner has the same useful effect but with the disadvantage of permanent presence of Pro residue in the synthesized sequence; D-Ala was found to be less efficient in preventing the β-structure formation.

The extent of the Tmob group protection is also tested by the IR measurements of the nonadeca chain XVI. It showed amide I band at 1630 and 1654.7 cm^{-1} and amide A band at 3424 cm^{-1}, which represents the formation of β-structure when seven alanine residues were added after the last Tmob-Ala residue. This indicates that the effect of Tmob group is weakened after six residues.

Solid-phase peptide synthesis of oligoalanine using microwave technique

We described here a novel application of microwave technology to enhance coupling efficiency in solid-phase peptide synthesis by decreasing β-sheet aggregation. According to the protocol described above, the synthesis was carried out inside a microwave oven in the nitrogen atmosphere.

The reaction vessel (syringe) was located in the middle of the microwave oven (operating at 200 W). A Teflon tube from the side arm of the reaction vessel was connected to a nitrogen source to introduce a steam of nitrogen during microwave irradiation. The nitrogen gas bubbles served also as a stirrer. The reaction solution was filtered off via the side arm by suction at the end of the reaction.

Octa-alanine chain was synthesized using fivefold excess of Fmoc-Ala instead of tenfold commonly used in normal SPPS.

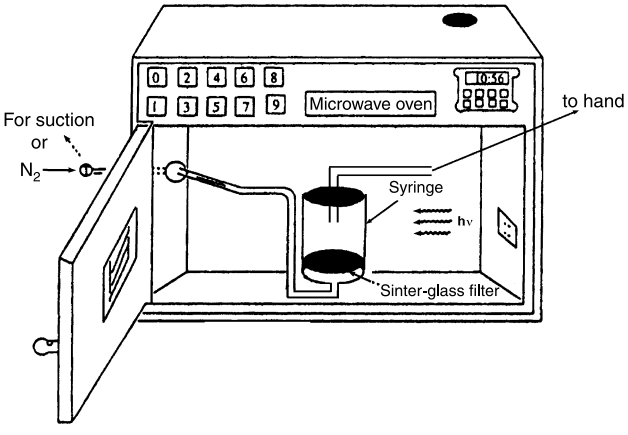


Fig. 2. The microwave reactor

Table 2. The time required for complete coupling and deprotection reaction in the synthesis of octa-alanine using MW energy

No. of residues	Time required for complete	
	Coupling/min	Fmoc deprotection/min
2	5	1
3	1.5	0.5
4	1.5	0.5
5	2	0.5
6	6	7
7	6	8
8	6	8

Complete coupling and deprotection reaction were followed up by Kaiser test. The time required for complete coupling and deprotection reactions in the synthesis of octa-alanine is represented in Table 2.

The above results indicated clearly that the rate of both coupling and deprotection reactions is markedly enhanced by using microwave energy. Although coupling reactions were carried out using only fivefold excess of amino acids, there is a drastic decrease in the time required for complete coupling (1.5–6 min) compared to 1–12 h in the conventional SPPS. Also, deprotection reactions as shown in Table 2 required only 30 sec to be completed till the fifth residue compared to 30 min required for Fmoc deprotection without microwave energy. Enhancement of solvation of peptide chain was also observed.

The nona-alanine chain H-$(Ala)_4$-Ala(Hmb)-$(Ala)_4$-OH containing one Hmb-Ala residue in position 5 was also synthesized using microwave energy as described before to demonstrate the effect of the temporary protection of N-amide bond on the time of coupling and deprotection reactions using the microwave energy.

Results indicated that the time required for complete coupling of Hmb-Ala is reduced from 2 h in conventional method to 1.5 min; also all alanine residues were coupled in time ranged between 1.5 and 7 min instead of 1–2 h.

These results provide a bird's eye view of the benefits of microwave SPPS in the enhancement of coupling and deprotection reactions especially in the synthesis of difficult sequences.

From the above results we can conclude that the application of microwave SPPS provides an efficient tool for synthesis of difficult sequences. Microwave energy can effectively disrupt intermolecular aggregation and prevent β-sheet formation which result in the enhancement of the rate of coupling and deprotection reactions. Microwave technique proved to be the best approach for synthesis of the difficult peptide sequences.

Table 3. The time required for complete coupling and deprotection reactions in the synthesis nano-peptide chain using MW energy

No. of residues	Time required for complete	
	Coupling/min	Fmoc deprotection/min
2	5	1
3	1.5	0.5
4	1.5	0.5
5	1.5	0.5
7	3	–
8	2	–
9	7	–

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