Protocol

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Conventional and microwave-assisted SPPS approach: a comparative synthesis of PTHrP(1–34)NH_2

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Attracted by the possibility to optimize time and yield of the synthesis of difficult peptide sequences by MW irradiation, we compared Fmoc/tBu MW-assisted SPPS of 1–34 *N*-terminal fragment of parathyroid hormone-related peptide (PTHrP) with its conventional SPPS carried out at RT. MWs were applied in both coupling and deprotection steps of SPPS protocol. During the stepwise elongation of the resin-bound peptide, monitoring was conducted by performing MW-assisted mini-cleavages and analyzing them by UPLC-ESI-MS. Identification of some deletion sequences was helpful to recognize critical couplings and as such helped to guide the introduction of MW irradiations to these stages. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

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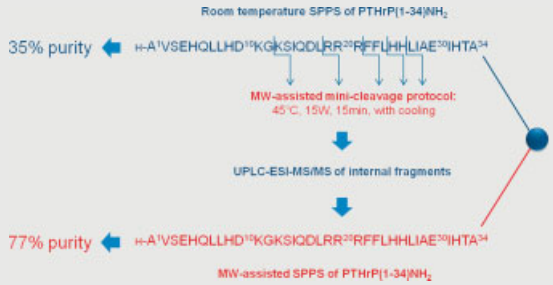
Keywords: microwave irradiation; solid-phase peptide synthesis; microwave-assisted mini-cleavages; difficult peptide sequences; parathyroid hormone-related peptide; UPLC-ESI-MS analysis

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

RT and MW-assisted SPPS of parathyroid hormone-related peptide (PTHrP)(1-34)NH_2 were performed using Liberty™ MW peptide synthesizer (CEM) on Rink-amide NovaSyn® TGR resin (0.2 mmol/g, 500 mg). After deprotection of the N-terminal Fmoc group with a 20% solution of piperidine in DMF, Fmoc-protected amino acids (5 equiv), TBTU (5 equiv) and DIEA (10 equiv) in DMF were used in the coupling steps. The RT-SPPS protocol consists of two consecutive deprotection steps of 5 and 10 min employing deprotecting reagent, and 20 min for the coupling steps. In MW-assisted SPPS protocol, the deprotection steps were performed at 75 °C using 35 W for 0.5 min for the first one and 60 W for 3 min for the second one, while the coupling steps were performed at 75 °C using 30 W for 5 min for all amino acids except Arg and His for which the conditions reported in Table 1 were used. The ongoing of synthesis was monitored by UPLC-ESI-MS of MW-assisted mini-cleavages of intermediate fragments. A small sample of Fmoc-protected peptide-resin beads (10 mg) was removed from the reactor vessel, weighted into a fritted polypropylene tube and treated with a 20% solution of piperidine in DMF (2 × 1 ml) for 5 min. The sample was then washed with DMF (2 × 1 ml) and DCM (3 × 1 ml), and dried under vacuum. Mini-cleavages were performed in a 10 ml glass tube containing 2 ml of cleavage mixture consisting of TFA/TIS/water solution (95 : 2.5 : 2.5 v/v/v). Tubes were inserted into the MW cavity of Discover™ S-Class (CEM) and the cleavage was carried out at 45 °C, using 15 W for 15 min assisted by external cooling of the reactor vessel. The crude fragments were analyzed by UPLC-ESI-MS with a Symmetry 300 C18 column (100 mm × 2.1 mm ID, 3.5 µm, Waters) using the following conditions: flow rate 0.15 ml/min with a linear gradient from 0 to 30% of B (solvent A: 2% ACN, 0.1% formic acid in H_2O; solvent B: 2% H_2O, 0.1% formic acid in ACN) in 7 min, 30-60% B in the next 3 min, then to 95% B for 2 min, and returned to initial conditions for 3 min for re-equilibration. The total run time per sample was 15 min

Scope and Comments

The introduction in 1992 of MW irradiation in peptide chemistry stimulated a host of interest followed by numerous efforts to use it to overcome synthetic difficulties resulting in sluggish reactions, low yields and complex reaction crude products that were hard to purify [1]. During the last decade the growing interest in MW field has been marked by the progress in developing scientific MW equipment to embrace specific research needs [2–5]. In general, introduction of MW technology led to the shortening of reaction time that resulted in higher purity of crudes and increased yields of the pure final product.

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Abbreviations used: *ACN, acetonitrile; MW, microwave; NMP, N-methyl-2-pyrrolidone; RT, room temperature; TIS, triisopropylsilane.*

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The application of MW irradiation in peptide chemistry has been reported in several publications, most of which describe case studies of successful syntheses of difficult peptide sequences [6–8]. However, a definitive comparison of conventional RT and MW-assisted-SPPS protocols must be performed on identical instruments. In fact, comparing conventional RT and MW-assisted SPPS carried out on different instruments that apply protocols that differ in the equivalent excess of reagents and their molar ratios, stirring techniques and the nature and number of washing steps, may be misleading. Moreover, the automated synthesizers are generally developed to produce peptides without monitoring of the progress of the synthesis. Therefore by-products caused by side reactions such as aspartimide and diketopiperazine formation, incomplete couplings and deprotections may be detected only after the final cleavage of the deprotected peptide from the resin.

In this study we compared the Fmoc/tBu RT-SPPS with the MW-assisted synthesis of the 1–34 *N*-terminal fragment of PTHrP using the same instrument (Liberty™, CEM, Matthews, NC, USA), and monitoring the progress of the synthesis by UPLC-ESI-MS of MW-assisted mini-cleaved fragments of the growing peptide chain.

PTHrP is an autocrine, paracrine and intracrine regulator of processes such as endochondrial bone formation and epithelial–mesenchymal interactions during the development of mammary glands. In analogy to PTH, most of the known biological functions are exerted by the *N*-terminal PTHrP(1–34) fragment that has 60% sequence similarity to PTH(1–34). The sequence of PTHrP(1–34)NH_2 is shown in Figure 1. In the past, this sequence was the subject of numerous structure–activity–conformation relationship studies [9,10]. Importantly, the presence of clusters of arginine, of sterically hindered and hydrophobic amino acid residues in the sequence represents a synthetic challenge that was the subject of our comparative study reported herein.

The synthesis was initially carried out following the conventional RT protocol using the Liberty™ automated peptide synthesizer excluding MW irradiations. As modern automated SPPS protocols allow the assembly of larger and increasingly complex peptides, a precise control of the coupling reactions is a crucial prerequisite in peptide synthesis. In fact monitoring the progress of synthesis allows the detection of undesirable products caused by side reactions, incomplete couplings or deprotections. Although different methods have been developed for monitoring of SPPS, we observed that the use of colorimetric monitoring or continuous-flow UV absorbance of the reaction column effluent was not informative enough to identify difficult steps in the synthesis. Therefore, we decided to monitor the progress of PTHrP(1–34)NH_2 synthesis by UPLC-ESI-MS analyses of small aliquots of cleaved peptide fragments obtained by MW-assisted mini-cleavages. The application of MW-assisted mini-cleavages of resin-bound peptides has been proposed as a fast, reliable method to monitor SPPS [11]. After specific coupling cycles, suspected to be difficult, we stopped the synthesizer and withdrew a small aliquot for analysis by UPLC-ESI-MS. In particular, we focused our attention on the PTHrP fragments related to the 19–28 sequence, characterized by clusters of Arg residues and highly hydrophobic residues (see Reaction Scheme and Figure 1).

By UPLC-ESI-MS analyses of intermediate fragments of the PTHrP(1–34)NH_2 included in the 19–34 sequence we noticed the presence of the desired peptide as well as of some by-products (Table 1 and Figure 2). The fragmentation patterns of these by-products in ESI-MS/MS allowed us to confirm the formation of deletion sequences as reported in Supporting Information (Figure S1).

As the length of the resin-bound peptide increases, the related UPLC-ESI-MS analyses become much more complex. We report as an example the characterization of the 12–34 fragment of PTHrP(1–34)NH_2. The deconvoluted spectrum obtained for the cleaved mixture of this sample resulted in several deletion sequences (Figure 3). The fragments desLys^{13}/Gln^{16}-f(12–34) and desLys^{13}/Gln^{16},Leu^{27}-f(12–34) were identified as two isobaric peptide sequences lacking either Lys^{13} or Gln^{16} residues.

The UPLC-ESI-MS/MS analyses of the intermediate resin-bound fragments obtained from the RT-SPPS of PTHrP(1–34)NH_2 confirm that it is a difficult sequence for SPPS. The desired peptide was usually present as the major component in the cleavage mixture, but it was accompanied by some deletion peptides mainly lacking Arg, Leu and His residues. It is well known that Arg-containing peptides are difficult to synthesize due to the sterically hindered Pbf group as side-chain protection and the tendency to form γ-lactam leading to low yield couplings [12].

With the above information in hand we sought to improve the synthesis of this difficult sequence of PTHrP(1–34)NH_2 by employing MW-assisted SPPS using Liberty™ automated peptide synthesizer with MW irradiations. To address the difficulties observed during the incorporation of Arg and His residues we applied the protocols reported in Table 2. Specifically, we have decreased the power of MW irradiation and lowered the temperature in order to avoid side reactions such as γ-lactam formation for Arg and racemization for His [13].

Indeed, after semi-preparative purification the MW-assisted SPPS of PTHrP(1–34)NH_2 yielded 27 mg of >95% pure peptide, whereas RT-SPPS gave only 18 mg of >95% of pure peptide. This improvement is attributed to the higher purity of the crude cleaved peptide mixture (Table 3 and Figure 4).

On the basis of the results of the analytical RP-HPLC of the crude PTHrP(1–34)NH_2, we conclude that the use of MW irradiations in SPPS has enhanced the efficiency of crucial coupling cycles improving the final yield and purity of crude peptide and speeding up the remaining coupling cycles. This improvement can be attributed to the prevention of peptide backbone aggregation and acceleration of deprotection and coupling steps.

In summary, although the application of MW-assisted SPPS to the synthesis of PTHrP(1–34)NH_2 led only to a moderate improvement in final yield (6.3% vs 4.4%), it allowed us to obtain a crude product of higher quality (77% vs 35%) and in a shorter time (20 h vs 34 h used for the RT and MW-assisted SPPS strategies). Moreover, we demonstrated the usefulness of the combination of an MW-assisted mini-cleavage protocol and the UPLC-ESI-MS analysis for monitoring the quality of the reaction step (see Supporting Information). Compared to the ninhydrin colorimetric monitoring, our strategy is faster and the UPLC-ESI-MS/MS analysis is more accurate and more informative. We think that application of the strategy presented in this report will help to improve many syntheses of difficult sequences.

Experimental Procedures

RT and MW-assisted SPPS of PTHrP(1–34)NH_2

All Fmoc-protected amino acids and TBTU were purchased from Iris Biotech. (Marktdrewitz, Germany). The following amino acid side-chain-protecting groups were used: OtBu (Asp, Glu), tBu (Ser, Thr), Pbf (Arg), Trt (Gln, His) and Boc (Lys). Rink-amide NovaSyn® TGR resin was purchased from Novabiochem (Laufelfingen, Switzerland). ACN, DCM and diethyl ether from Sigma-Aldrich

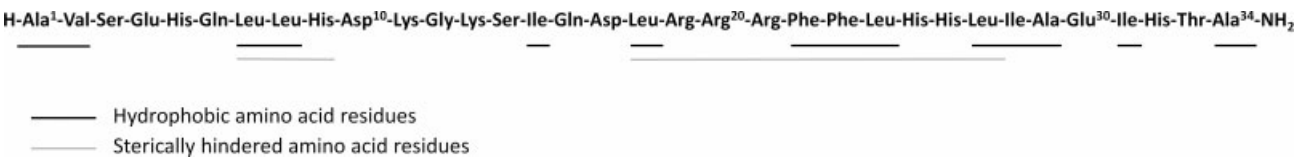

Figure 1. Characteristics of the PTHrP(1–34)NH_2 sequence are a cluster of arginine residues in the positions 19–21, sterically hindered and hydrophobic amino acid sequences.

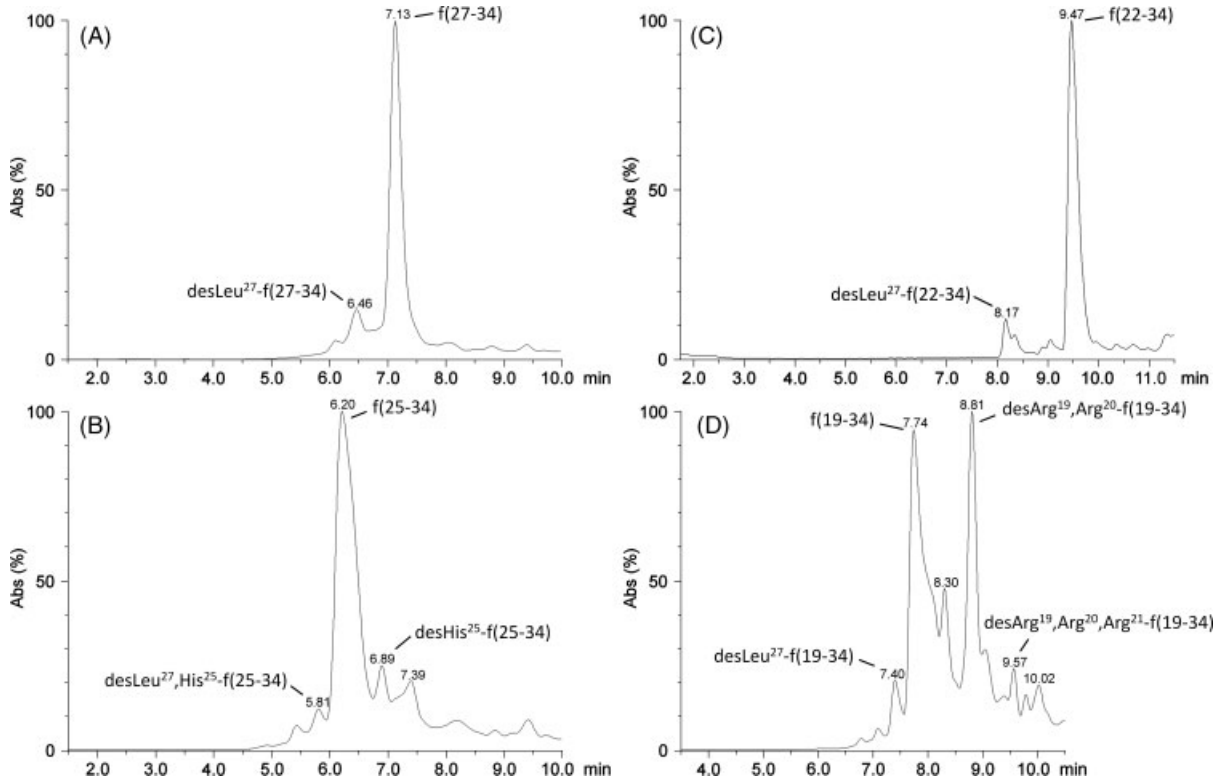

Figure 2. (A–D) TIC chromatogram of selected crude mixtures of intermediate resin-bound sequences obtained during the synthesis of PTHrP(1–34)NH_2 (Fmoc/tBu RT-SPPS) generated by the MW-assisted mini-cleavages. The related MS/MS spectra are shown in Supporting Information (Figure S1).

(St. Louis, MO, USA), DMF and NMP from Scharlau (Barcelona, Spain), DIEA and TFA from Acros Organics (Geel, Belgium) and TIS from Fluka/Aldrich (St. Louis, MO, USA). The synthesis of PTHrP(1–34)NH_2 was carried out using Fmoc/tBu SPPS strategy on a Liberty™ automated peptide synthesizer with a single-mode MW reactor (CEM), by RT and MW-assisted strategies. The reactions were performed in a Teflon vessel and mixed by nitrogen bubbling. Reaction temperatures were measured by an internal fiber-optic sensor. The RT-SPPS protocol consisted of two consecutive deprotection steps of 5 and 10 min, respectively, and a 20 min coupling step. In MW-SPPS protocol, the two deprotection steps were performed at 75 °C using 35 W for 0.5 min for the first one and 60 W for 3 min for the second one, whereas the coupling steps were performed at 75 °C, using 30 W for 5 min for all amino acids except for Arg and His residues that required specific coupling parameters performed in two steps (RT followed by MW irradiation, Table 1).

The syntheses were performed on Rink-amide NovaSyn® TGR resin (0.2 mmol/g, 500 mg), which was suspended in a solution of DMF/DCM (1 : 1 v/v) and swelled for 30 min. During the general coupling cycle, the *N*-terminal Fmoc-protecting group was removed with a solution of 20% piperidine in DMF. Fresh stock solutions of the Fmoc-protected amino acids (0.2 M) and TBTU (0.5 M) in DMF, and of DIEA (2 M) in NMP were prepared in separated bottles and used as reagents during the SPPS. In particular, the coupling cycles were performed using 2.5 ml of Fmoc-protected amino acids, 1 ml of TBTU and 0.5 ml of DIEA in NMP of stock solutions. The fully assembled peptide was cleaved from the resin by treatment with 7 ml of a TFA/TIS/water solution (95 : 2.5 : 2.5 v/v/v) for 3 h at RT. The resin was filtered and the combined filtrates were concentrated under a stream of nitrogen. The crude peptide was precipitated from the cleavage mixture by addition of ice-cold diethyl ether and stored for 30 min at −20 °C. The precipitated product was collected by centrifugation, washed with diethyl ether (3 × 7 ml) and centrifuged. The remaining solid was dried under a stream of nitrogen and lyophilized.

MW-assisted Mini-cleavage of PTHrP(1–34)NH_2 Fragments

Both RT and MW-assisted SPPSs were monitored by UPLC-ESI-MS analysis of MW-assisted mini-cleavages of intermediate resin-bound fragments using Discover™ S-Class single-mode MW reactor equipped with Explorer-48 autosampler (CEM). The mixing of the cleavage reaction was accomplished by magnetic stirring and the reaction temperature was monitored at the bottom of the reactor vessels by an IR sensor. A small sample of beads carrying

Table 1. Fmoc/tBu RT-SPPS of PTHrP(1–34)NH_2: list of fragments produced by MW-assisted mini-cleavages of intermediate resin-bound peptides

Analyzed resin-bound sequences	Sequence	Calculated monoisotopic mass	Intact sequences Qtof MS (ESI+) (*m/z*) found	Deletion sequences Qtof MS (ESI+) (*m/z*) found	Missing amino acid residues from deletion sequences	Amount of deletion sequences (%)
27–34	H-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-NH_2	865.51	866.39 $[M + H]^+$	753.41 $[M + H]^+$	Leu^{27}	7
25–34	H-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-NH_2	1139.62	1140.90 $[M + H]^+$	1003.95 $[M + H]^+$	His^{25}	2
				890.93 $[M + H]^+$	$Leu^{27}His^{25}$	5
22–34	H-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-NH_2	1546.84	1547.41 $[M + H]^+$	1434.44 $[M + H]^+$	Leu^{27}	8
19–34	H-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-NH_2	2015.15	1008.69 $[M + 2H]^{2+}$	952.19 $[M + 2H]^{2+}$ 852.72 $[M + 2H]^{2+}$	Leu^{27}	6
					$Arg^{19}Arg^{20}$	39
				774.73 $[M + 2H]^{2+}$	$Arg^{19}Arg^{20}Arg^{21}$	51
12–34	H-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-NH_2	2756.55	1379.24 $[M + 2H]^{2+}$	1322.29 $[M + 2H]^{2+}$	Leu^{27}	5
				1315.26 $[M + 2H]^{2+}$	Gln^{16}/Lys^{13}	55
				1258.81 $[M + 2H]^{2+}$	$Leu^{27}Gln^{16}/Lys^{13}$	3
				1065.43 $[M + 2H]^{2+}$	Gly^{12}-Lys-Ser-Ile-Gln-Asp^{17}	12

The relative amounts of the deletion sequences are calculated in percentage of area under the peak from the total area under the curve obtained from the TIC chromatogram.

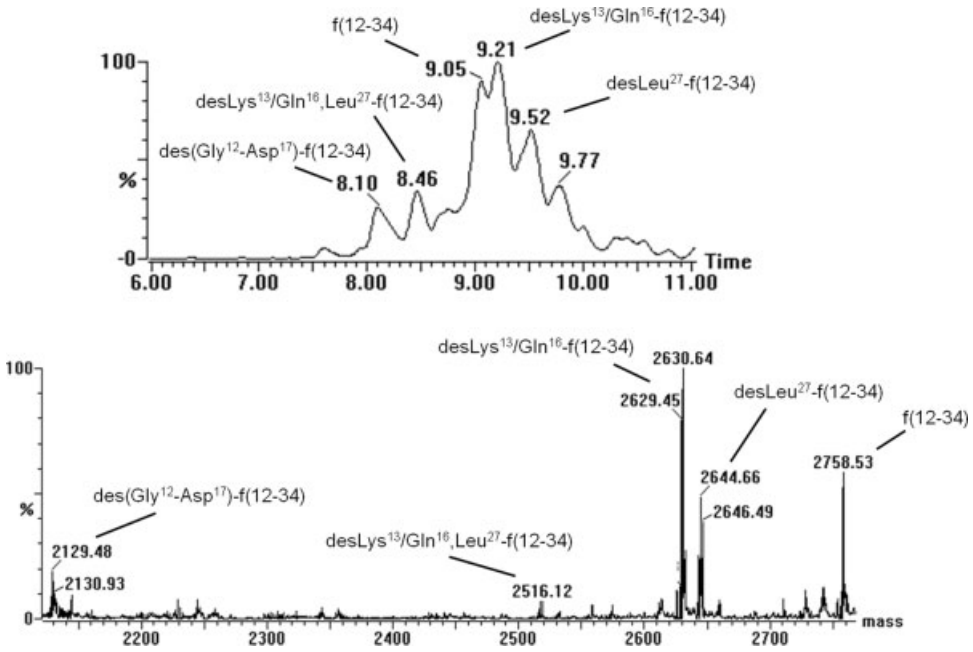

Figure 3. TIC chromatogram and deconvoluted spectrum of the cleaved mixture of the intermediate resin-bound sequence PTHrP(12–34)NH_2 (Fmoc/tBu RT-SPPS) generated by the MW-assisted minicleavages.

Table 2. Fmoc/tBu RT and MW-assisted SPPS deprotection and coupling protocols used for the synthesis of PTHrP(1–34)NH_2

	First deprotection			Second deprotection			Coupling		
Protocol	Time (min)	Power (W)	Temperature (°C)	Time (min)	Power (W)	Temperature (°C)	Time (min)	Power (W)	Temperature (°C)
RT-SPPS	5	–	20	10	–	20	20	–	20
MW-assisted SPPS	0.5	35	75	3	60	75	5	30	75
Arg[a]	Step 1				–		25	–	20
	Step 2				–		5	25	75
His	Step 1				–		2	–	20
	Step 2				–		4	30	50

[a] The Arg cycle (step 1 and 2) was performed twice refreshing the coupling solution.

Table 3. Yield of PTHrP(1–34)NH_2 obtained from the RT *versus* MW-assisted SPPS

SPPS strategy	Purity of crude peptide (%)	Yield of >95% pure peptide
RT	35	4.4% (18 mg)
MW-assisted	77	6.3% (27 mg)

Fmoc-protected resin-bound peptide (10 mg) was weighted into a fritted polypropylene tube and treated twice with a 20% solution of piperidine in DMF (1 ml) each time for 5 min. The beads were then washed with DMF (2 × 1 ml) and DCM (3 × 1 ml), dried under vacuum and transferred into a 10 ml glass tube containing the cleavage mixture that was placed into the MW cavity. The mini-cleavages were carried out with 2 ml of TFA/TIS/water solution (95 : 2.5 : 2.5 v/v/v) at 45 °C, using 15 W for 15 min with external cooling of the reactor vessel at the positions shown in the Reaction Scheme. All the parameters, i.e. pressure, temperature and power, involved in the MW-assisted mini-cleavage reactions were monitored as reported in Supporting Information (Figure S2). The reaction mixture was then filtered and the crude peptide was precipitated from the cleavage mixture by addition of ice-cold diethyl ether followed by cooling for 5 min at −20 °C. The product was collected by centrifugation and directly subjected to UPLC-ESI-MS analysis (see Supporting Information).

HPLC Analysis of PTHrP(1–34)NH_2

Crude PTHrP(1–34)NH_2 obtained from both the RT and MW-assisted SPPSs were analyzed by analytical RP-HPLC (Alliance 2695 HPLC system equipped with a 2996 photodiode array detector, Waters (Milford, MA, USA)) using a Jupiter C18 (5 µm, 250 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) at 1 ml/min. The solvents used were A (0.1% TFA in H_2O) and B (0.1% TFA in ACN).

RP-HPLC Semi-preparative of PTHrP(1–34)NH_2

Lyophilized crude peptide was prepurified by solid-phase extraction with an RP-18 LiChroprep silica column from Merck

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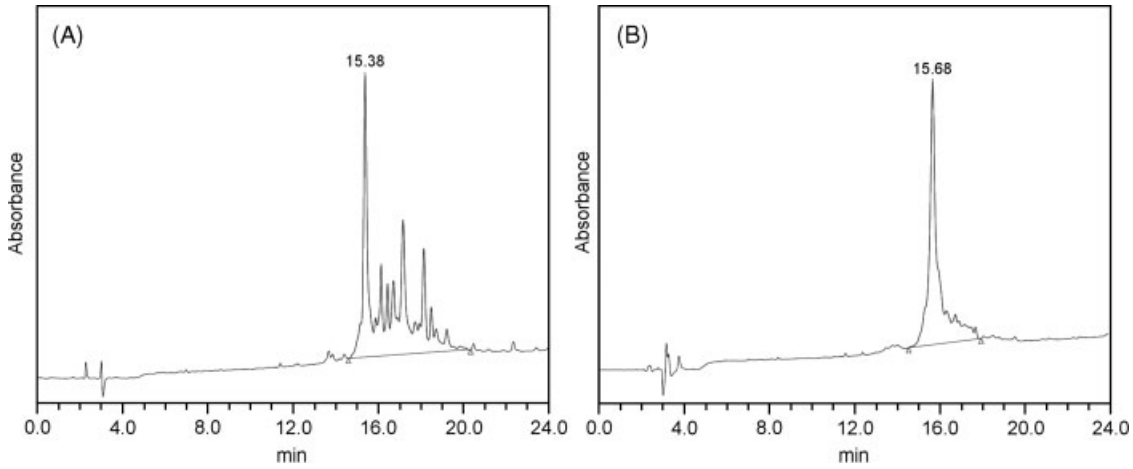

Figure 4. Analytical RP-HPLC of crude PTHrP(1–34)NH_2. Fmoc/tBu RT-SPPS (A) and Fmoc/tBu MW-assisted SPPS (B). HPLC: 10–60% B (0.1% TFA in ACN) in A (0.1% TFA in H_2O) over 20 min.

(Darmstadt, Germany) using H_2O/ACN as eluents. The purification of the peptide was performed by semi-preparative RP-HPLC on a Supelco C18 180 Å (250 × 10 mm, 5 µm) column (Sigma Aldrich, St. Louis, MO, USA); eluents: A 0.1% TFA in H_2O; B 0.1% TFA in ACN: flow 4 ml/min; gradient 30–60% of B in 30 min.

UPLC-ESI-MS Analysis of PTHrP(1–34)NH_2 Fragments

UPLC-ESI-MS system consisted of an ACQUITY™ UPLC system (Waters) coupled with a Micromass® Q-Tof MICRO™ mass spectrometer (Waters) equipped with an ESI source. The chromatographic separation was achieved on a Symmetry 300 C18 column (100 mm × 2.1 mm, ID 3.5 µm, Waters) with the column temperature set at 30 °C. The flow rate was 0.15 ml/min with a linear gradient running from 0 to 30% of B (solvent A: 2% ACN, 0.1% formic acid in H_2O; solvent B: 2% H_2O, 0.1% formic acid in ACN) in 7 min, followed by 30–60% B in the next 3 min, then by 95% B for 2 min, and returned to initial condition for 3 min for re-equilibration. The total run time per sample was 15 min. The ESI-MS analysis was carried out in the positive ESI mode, the optimal MS parameters were as follows: capillary voltage 3.2 kV, cone voltage 30 kV, source temperature 120 °C and desolvation temperature 320 °C. Nitrogen was used as desolvation and cone gas with a flow rate of 450 and 40 l/h, respectively. For MS/MS analyses, argon was used as collision gas, and the collision energy was set to 40 eV. Data were acquired and processed using MassLynx™ software (Waters).

Highlights and Limitations

This protocol describes two advantages of MW radiation in SPPS. The first one is monitoring of traditional RT-SPPS by MW-assisted mini-cleavages combined with fast, efficient and sensitive UPLC-ESI-MS analysis (15 min/analysis vs 30–45 min/analysis for traditional mini-cleavage). We use this procedure to confirm the presence of difficult coupling steps that result in truncated and deletion sequences. The second advantage is the specifically tuned MW-assisted SPPS that uses modified cycles targeting difficult couplings. In these couplings the first step is carried out at RT (without applying MW power, 20 °C) and for variable duration (25 or 2 min) and the second step is carried out in the presence of MW power (75 or 50 °C) for shorter time intervals (5 or 4 min). In the reported synthesis we applied such type of couplings for the three arginine residues (R^{19}-R-R^{21}) and for the two histidine residues (H^{25}-H^{26}). Evidently, carrying out the RT and MW-assisted SPPSs on the same instrument allowed unbiased side-by-side comparison and the conclusion that the latter procedure is superior to the RT-SPPS.

Although the modification of the coupling steps for Arg and His in the MW-assisted SPPS seems to be empirical in nature, it was guided by strong rational that took into account the distinct capacity of this methodology to overcome putative hydrophobic interactions and aggregation. The impact of these phenomena increases with the progression of the coupling reactions. We therefore decided to take advantage of the MW radiation only for a short duration after the bulk of the reaction has been already taken place. In this manner, the completion of the coupling reaction was facilitated without causing undesired side reactions. We propose this MW-based protocol as a general strategy for overcoming difficult coupling reactions. Admittedly, applying this strategy to different peptides will require fine tuning that will include the adjustment of parameters such as duration of coupling and recoupling steps as well as the level of MW energy employed in the recoupling step. We are confident that adapting the strategy outlined in this protocol will be advantageous over the 'one generic MW-assisted coupling cycle fits all'.

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Supporting information

Supporting information may be found in the online version of this article.

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