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Microwave-assisted Boc-solid phase peptide synthesis of cyclic cysteine-rich peptides

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Abstract: In this study we describe the first protocols for the synthesis of cystine-rich peptides in the presence of microwave radiation with Boc-solid phase peptide synthesis (SPPS). This method is exemplified for macrocyclic peptides known as cyclotides, which comprise ~30 amino acids and incorporate a cystine knot arrangement of their three disulfide bonds. However, the method is broadly applicable for a wide range of peptides using Boc-SPPS, especially for SPPS of large peptides via native chemical ligation. Microwave radiation produces peptides in high yield and with high purity, and we were able to reduce the time for the assembly of ~30 mer peptide chains to an overnight reaction in the automated microwave-assisted synthesis. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: microwave; Boc-SPPS; circular protein; cystine knot; native chemical ligation

INTRODUCTION

Microwave (MW) assisted methods have been the subject of much interest in the field of organic synthesis because they can decrease reaction times and increase yields [1]. There have been numerous studies on MW-assisted organic chemistry reactions since the first reports in 1986 [2,3], but until recently only a few reports about the use of MWs in solid phase peptide synthesis (SPPS) have appeared. However, in the last two years MW-assisted SPPS has been receiving increased attention [4–6] owing in part to the availability of new technology, including automated peptide synthesisers equipped with MW capability [7].

The first attempt at the acceleration of SPPS with MW radiation in a kitchen oven was published in 1991 [8]. For a while the field did not advance much because of a general belief that undesirable side reactions would be accelerated by MW heating and that some peptide-coupling reagents would be heat sensitive. However, it has been recently demonstrated that SPPS can be successfully accelerated with MW radiation and studies have been reported for small peptides [9,10], glycopeptides [11–13], phosphopeptides [14] and β-peptide libraries [15,16]. In addition, MW has been shown to be useful for making amino acid precursors for solid phase synthesis, including Fmoc-methylated amino acids [17]. Some work has been recently done also on resin supports compatible with MW-assisted chemistry [18,19], and the effects of coupling reagent, base and solvent choice have also been investigated [20].

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The main advantages of MW-assisted chemistry are shorter reaction times, higher yields and milder reaction conditions [21]. These advantages originate in part from the unique heat profiles of MW reactions, which cannot be reproduced by classical heating. However, the rate acceleration and increased yields cannot be explained by rapid heating alone and appear to be due to a combination of thermal and non-thermal factors [21–23]. The thermal effects arise from the different characteristics of MW dielectric heating and conventional heating, but the non-thermal effects are still not well understood. These effects are sometimes called ‘specific microwave effects’ and are defined as accelerations in chemical reactions that cannot be reproduced by conventional heating.

One important mechanism in MW-assisted chemical reactions is the dipolar polarisation mechanism, which is of particular importance for peptide synthesis. In this mechanism, the alternating electric field from MW radiation provides the energy for the rotation of the molecules having a dipole moment. Unlike conventional heating, MW energy activates any molecule with a dipole moment, resulting in rapid heating at the molecular level. Since peptide backbones are polar, this mechanism is useful in preventing the aggregation of a growing peptide chain during the coupling reactions in SPPS, thereby improving the coupling efficiency.

Some of the inherent difficulties of SPPS include intra- and inter-molecular aggregation, steric hindrance from protecting groups, premature termination of the sequence and premature secondary structure formation. Several accounts of MW-assisted Fmoc-synthesis have been reported [7] that overcome some of these difficulties, yielding significantly decreased coupling time and high yields. In particular, the

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common side-reactions observed in Fmoc-synthesis, including racemisation and aspartimide formation, have been reported to be minimised [6] via the use of MW reactions.

All the published applications of MW energy to solid phase synthesis have so far dealt with Fmoc-protection chemistry. We report here for the first time a MW-assisted method for Boc-SPPS. Boc-protected peptide synthesis is the method of choice for several applications, including native chemical ligation for the synthesis of large peptides and proteins, difficult peptide sequences and non-natural peptide analogues that are base sensitive. A significant proportion of manual and automated peptide synthesis is carried out by this method, and therefore the availability of MW-assisted protocols for Boc-SPPS should assist the application of this chemistry to high-throughput approaches. In this study, we devised protocols for the application of MW Boc methodology for the synthesis of the cyclotide family of macrocyclic peptides, but the approach is equally applicable to any Boc-SPPS application, including the synthesis of cysteine-rich peptides or thioester peptides for native chemical ligation [24–26].

The cyclotides are recently discovered plant proteins that are of great interest because of their high intrinsic stability towards thermal, enzymatic and chemical extremes [27,28]. Their stability makes them potentially useful molecules for drug design applications, either in their native form or modified via the grafting of novel bioactivities onto their stable scaffold [29]. Cyclotides have a macrocyclic peptide backbone and six conserved cysteine residues that form their signature cyclic cystine knot (CCK) motif [30]. There has been an attempt at Fmoc synthesis of these naturally occurring plant peptides, but the yields were low [31]. Although efforts are underway to improve Fmoc synthesis of cyclotides [32], Boc-SPPS in conjunction with native chemical ligation for the cyclisation of the peptide backbone has so far been the preferred method of production of this pharmaceutically interesting family of peptides [24,25].

The major aim of the current study was to maximise the yields and minimise reaction times for the synthesis of cyclotides to provide a high-throughput approach to the production of these peptides. In the process, we developed the first protocol for MW-assisted Boc-peptide synthesis that should be applicable to a wide range of cysteine-rich peptides. The broad applicability of this study is enhanced by the recent availability of a commercial MW-equipped automated peptide synthesiser [7], which, although so far used for mainly Fmoc chemistry, can now be used with Boc approaches.

MATERIALS AND METHODS

Peptide Synthesis

All peptides were assembled on phenylacetamidomethyl (PAM) resin (Applied Biosystems, Foster City, CA, U.S.A.) by SPPS on a Liberty (CEM, USA) automated peptide synthesiser with a single-mode MW reactor. Standard *in situ* neutralisation/2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) protocol for Boc chemistry was used [33]. The peptide chain was attached to the resin via a linker that generates a *C*-terminal thioester on HF cleavage [34]. The reagent volumes and concentrations delivered to the reaction vessel during the synthesis, as well as the MW radiation cycle conditions, are given in Tables 1 and 2. A power of 35 W was used for 5 min for the coupling of amino acids, whereas the MW radiation was switched off during the Boc-deptrotection step because of the high polarity of trifluoroacetic acid (TFA) and the potential for overheating. Amino acid side chain protection was as follows: Arg(Tos), Asn(Xan), Asp(OChx) Glu(OChx), Ser(Bzl), Thr(Bzl) and Trp-(CHO) (where Tos is tosyl, Xan is xanthyl, OChx is cyclohexyl ester, Bzl is benzoyl and CHO is formyl). The CHO protecting group on the tryptophan residue was not removed prior to cleavage, as the conditions required for deprotection are not compatible with the trityl-associated mercaptopropionic acid (TAMPAL) linker. Cleavage of the peptide from the resin was achieved using HF with *p*-cresol and *p*-thiocresol as scavengers (HF/*p*-cresol/thiocresol, 9 : 0.8 : 0.2 by vol). The reaction was allowed to proceed at −5 to 0 °C for 1 h; HF was removed under vacuum and the peptide precipitated with diethyl ether. Following cleavage, the peptide was dissolved in 50% acetonitrile containing 0.05% TFA and freeze-dried.

Table 1 Reagents used for microwave-assisted Boc-SPPS: the volumes and concentrations of reagents used for 0.1 mM scale Boc-SPPS. For syntheses on a larger scale, the reagents need to be linearly scaled up

	Reagent	Volume	Conc.	Solvent
Amino acid	Boc-	1.25 ml	0.4 M	DMF
Activator	HBTU	1 ml	0.5 M	DMF
Base	DIEA	0.5 ml	25% v/v	DMF
Deprotection	TFA	5 ml	100%	/

Table 2 Microwave cycles for Boc-SPPS protocols: Time, temperature and power of the microwave radiation of coupling and deprotection cycles of Boc-SPPS assisted by microwave radiation

	Coupling (time, MW power)	Max. temperature	
		MW	No MW
Coupling	5 min, 35 W	87 °C	27 °C
Deprotection	2 × 1 min, 0 W	27 °C	

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Peptide Purification and Analysis

Crude peptides were purified by RP-HPLC on a Phenomenex C18 column using a gradient of 0–80% B (Buffer A: water/0.05%TFA; Buffer B: 90% acetonitrile/10% water/0.045% TFA) over 80 min with the eluent monitored at 215 and 280 nm. Similar buffer conditions were used in subsequent purification steps. Analytical RP-HPLC and electrospray mass spectrometry (ES-MS) confirmed the purity and molecular mass of the synthesised peptides. The linear reduced peptides were cyclised and oxidised 'in one pot' by incubating in 0.1 M NH_4HCO_3 (pH 8.5)/propan-2-ol (50:50, v/v) with 1 mM reduced glutathione overnight at room temperature. The mixtures were then purified by RP-HPLC to yield the cyclic/oxidised peptides. Analytical RP-HPLC and ES-MS confirmed the purity of the final products and ^1H NMR was used to confirm correct folding.

RESULTS AND DISCUSSION

Synthesis of Kalata B1 with and without Microwaves

Our aim was to develop a rapid approach for cyclotide synthesis since these peptides have a range of potentially valuable applications as pharmaceutical templates [29]. We first optimised protocols for the synthesis of kalata B1, the prototypic cyclotide whose synthesis has been previously described [24,25]. Previous syntheses were carried out using manual Boc-SPPS approaches, which typically take around 10–14 days for chain assembly, cleavage and purification, depending on the resin substitution, quality and the efficiency of the coupling steps. The general approach for the Boc-SPPS of cyclotides is summarised in Figure 1.

To evaluate the advantages of coupling MW radiation to Boc-SPPS, we compared the yields of an automated Boc-SPPS protocol for kalata B1 with and without MW radiation. The 29-amino acid peptide was synthesised on a 0.1 mM scale on PAM-Gly-Boc resin to which a thioester linker (β-mercaptopropionic acid) was coupled with standard *in situ* neutralisation protocol with HBTU/DIPEA. After deprotection of the linker, the kalata B1 sequence was assembled, coupled to the free thiol group of the thioester, ending the synthesis with a cysteine residue at the *N*-terminus, as is required for thioester-based cyclisation [34]. In this case, the sequence was synthesised from Thr16 and finished with Cys17 (Figure 1), but in principle any of the six Cys residues could be chosen as the last residue of the synthesis since the final product is cyclic [35]. After cleavage of the peptide from the resin with hydrogen fluoride, cyclisation and oxidation of the cysteine residues occurred in a one-step reaction in solution, in which native chemical ligation was used to create the peptide bond between the *N*- and *C*-terminal residues of the assembled peptide chain.

The conditions used in the synthesis of kalata B1 are summarised in Table 1. Each coupling step lasted for 5 min and the deprotection steps were 1 min, repeated twice to deprotect the Boc-group from the reactive *N*-terminal of the growing peptide chain. The synthesis done in the presence of MW radiation employed the same *in situ* neutralisation chemistry and the same

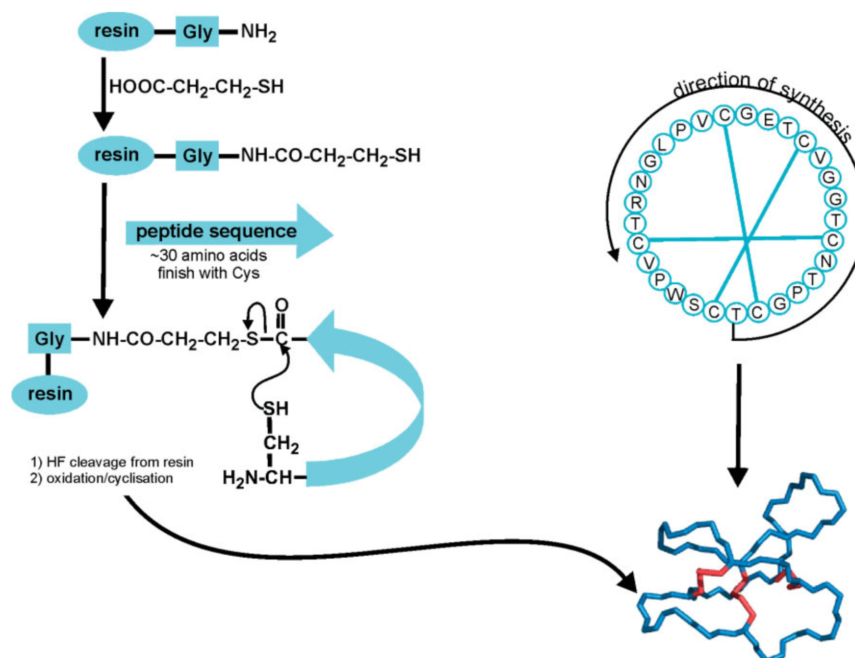

Figure 1 Boc-solid phase peptide synthesis of cyclotides: the schematic representation shows the strategy for synthesis of cyclotides, involving coupling of a thioester to the first amino acid, successively coupling the amino acid sequence and finishing with an *N*-terminal cysteine residue. After the sequence assembly, the peptide is cleaved from the resin and oxidised and cyclised in a single-step reaction in solution via native chemical ligation.

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coupling and deprotection times, but involved MW radiation at 35 W. The maximum temperature reached in the reaction vessel was 87 °C, representing a small overshoot with the MW radiation programmed to switch off at 75 °C. Nevertheless, we did not observe evidence of extensive side reactions or degradation of the peptide chain.

We compared the purity and yield of kalata B1 from syntheses with and without MW radiation by analysing the crude peptide mixtures on RP-HPLC and confirmed the identity of the final product with ESI-MS. As shown in Figure 2, the purity of product is clearly better for the peptide synthesised with MW radiation. Additionally, the yield is also higher for the MW-assisted synthesis. From 260 mg peptide resin cleaved from both the MW-assisted synthesis and the conventional synthesis, the total yield of crude peptide mixture was 45 mg for the former and 16 mg for the latter, which led to 9 mg and 1.2 mg of purified peptides, respectively. Thus, the overall yield of pure kalata B1 from the crude peptide mixture for MW-assisted synthesis was ~20%, compared to ~7% for the conventional synthesis. Figure 2(C) shows the RP-HPLC traces of the purified linear precursor compared to the purified cyclic peptide showing the characteristic change in retention time upon *N*- to *C*- terminal cyclisation and the formation of the cystine knot. The increase in retention time upon cyclisation and oxidation of kalata B1 is observed because there is a hydrophobic patch that is exposed on the surface of this CCK protein when it folds to the native structure. By contrast, most other peptides become more hydrophilic on folding and shift to a shorter retention time. We also confirmed by NMR that the peptide synthesised using MW radiation folded correctly to the CCK motif with the native structure [36]. Figure 2(D) shows the excellent dispersion of the peaks in the amide region of the ^{1}H NMR spectrum, and a comparison of the chemical shifts with those of the native plant-extracted peptide confirmed the native fold.

Boc-deprotection Step

In Boc-SPPS, the deprotection step is not well suited for MW radiation because TFA, used to cleave the Boc group protecting the reactive amine group, is highly polar and heats up rapidly. We found that the deprotection step was still highly efficient without applying MW radiation. Potentially, one could apply a low power, i.e. 5 W, of MW radiation to speed up the reaction and closely monitor the temperature inside the reaction vessel to avoid overheating.

One important improvement that we made to the software of the Liberty synthesiser in collaboration with the manufacturer was to deliver TFA in a timed rather than a looped approach. This meant that the TFA could be added rapidly in larger volumes. Prior to this modification, TFA was added by default in a

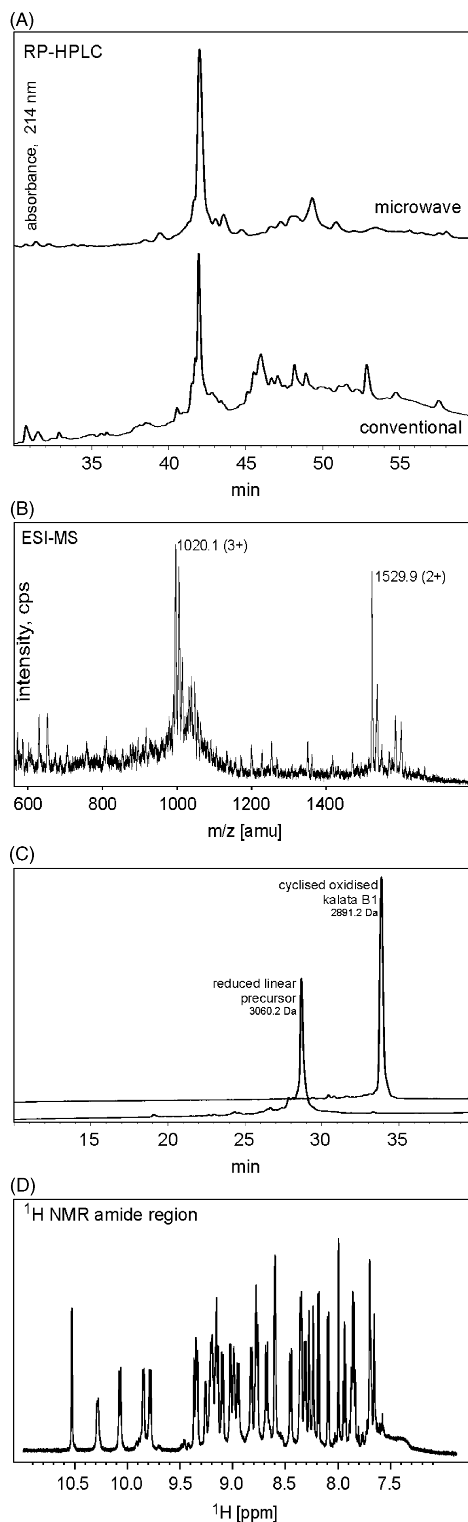

Figure 2 Boc-solid phase peptide synthesis of kalata B1 in the presence and absence of microwave radiation: (A) RP-HPLC profiles of the crude reaction mixture after cleavage from the resin of the prototypic cyclotide kalata B1 in the presence (top) and absence (bottom) of microwave radiation. (B) ESI-MS trace of the crude reaction mixture, demonstrating the correct molecular mass. (C) RP-HPLC profiles of the purified reduced linear precursor peptide (M_W 3060.2 Da, see Figure 1 for structure) and the cyclic, oxidised kalata B1 (2891.2 Da). (D) One-dimensional ^{1}H NMR spectrum of the amide region of kalata B1 obtained using microwave Boc-SPPS.

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1 ml loop, which took about 1 min to fill and deliver each aliquot. Thus, delivery of 5 ml of TFA took 5 min, which was unacceptable on the time scale allowed for Boc deprotection. This lengthy exposure of the peptide resin to TFA would have the potential to cause heating and side reactions. However, when TFA was added in a timed manner, 15 ml of TFA, which was necessary for deprotection in a 0.5 mM scale synthesis, was added in 20–30 s.

Another important feature that was optimised in this study relates to the deprotection of the trityl group of the thioester linker used in the synthesis of the cyclic. Normally this has to be done repeatedly with TFA and scavengers (H_2O, TIS) at least five times until the protecting group is completely removed from the thiol group. The effective removal of this protecting group is a prerequisite for a high yield. We noted that the use of pure TFA did not diminish the yield of the final product, although it is well known that the scavengers make the reaction more irreversible and remove the Trt group more effectively. The yield of cleavage of Trt group in this case is probably not diminished because of the large number (up to 10) of fast and repetitive exposures of the resin to TFA in the reaction vessel of the automated peptide synthesiser.

Scaling up Boc-Synthesis

Scale-up of the reaction was attempted for the synthesis of a new cyclotide, kalata B12, the only member of the cyclotide family discovered to date lacking a conserved Glu residue in loop 1 [37]. In other cyclotides this Glu residue has been proposed to play an important role in stabilising the CCK framework by hydrogen bonding its carbonyl side chain to the backbone amides of two residues in loop 3. It was of interest for future structural studies to obtain large quantities of kalata B12. The results for the kalata B12 synthesis on 0.5 mM scale are shown in Figure 3, including the RP-HPLC trace and ESI-MS spectrum for the crude reaction mixture. When the peptide was purified and cyclised in solution and its three-disulfide bonds were formed, a single product was obtained with a purity of >95%. Figure 3 shows the one-dimensional ^{1}H NMR spectrum of this peptide, which shows well-dispersed resonances in the amide region, signifying that the peptide has folded into a well-defined conformation.

Initially, when the synthesis of kalata B12 was scaled up from 0.1 mM to 0.5 mM scale, we used only three times the amount of reagents for five times the amount of resin, an approach often used in manual SPPS. A consequence of this was that the volume of the coupling solution was relatively small for the same amount of resin used previously. We observed a red-orange colouration of the resin at the end of the synthesis, which was not previously observed. To prevent the discolouration of the resin, which appeared

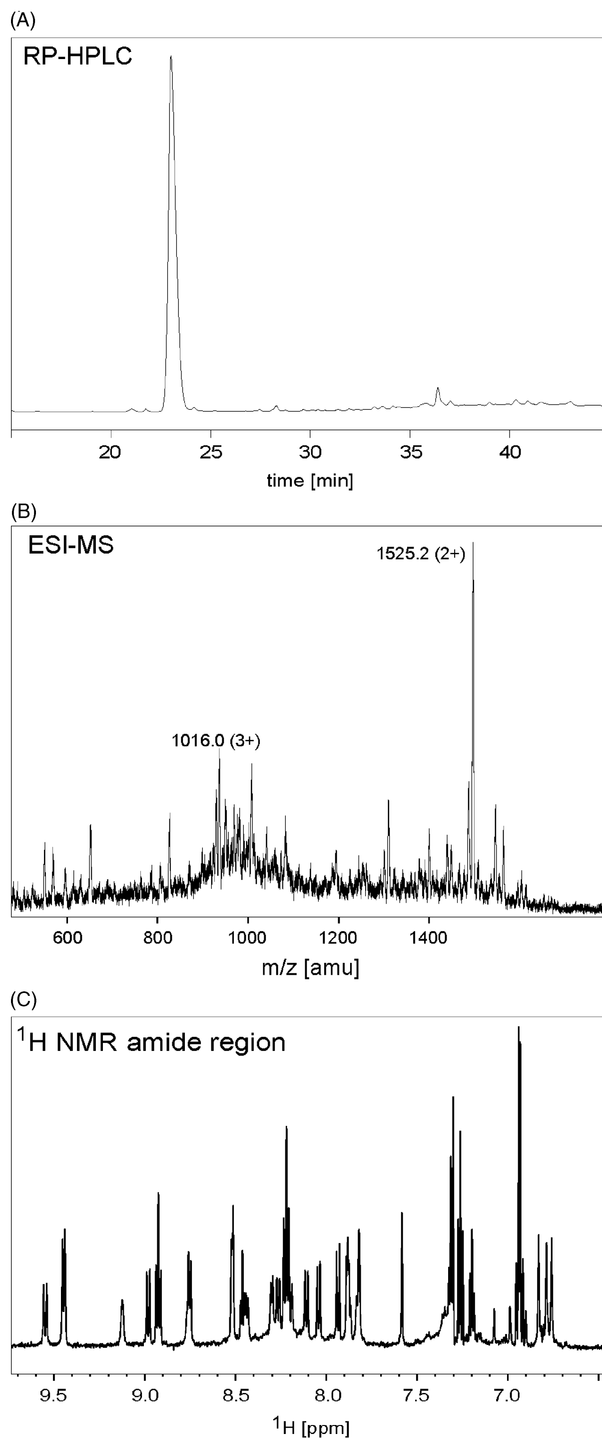

Figure 3 Boc-SPPS of kalata B12 on a 0.5 mM scale: (A) RP-HPLC profile of the crude reaction mixture after cleavage from the resin for the microwave Boc-SPPS of kalata B12 showing <95% yield. (B) ESI-MS trace of the crude reaction mixture for microwave Boc-SPPS of kalata B12, showing the correct mass of the linear, reduced peptide. (C) One-dimensional ^{1}H NMR spectrum of the amide region of kalata B12 obtained with microwave Boc-SPPS.

to be associated with an additional undesired mass in the ESI-MS spectrum (data not shown), we repeated the 0.5 mM scale synthesis by linearly scaling up the

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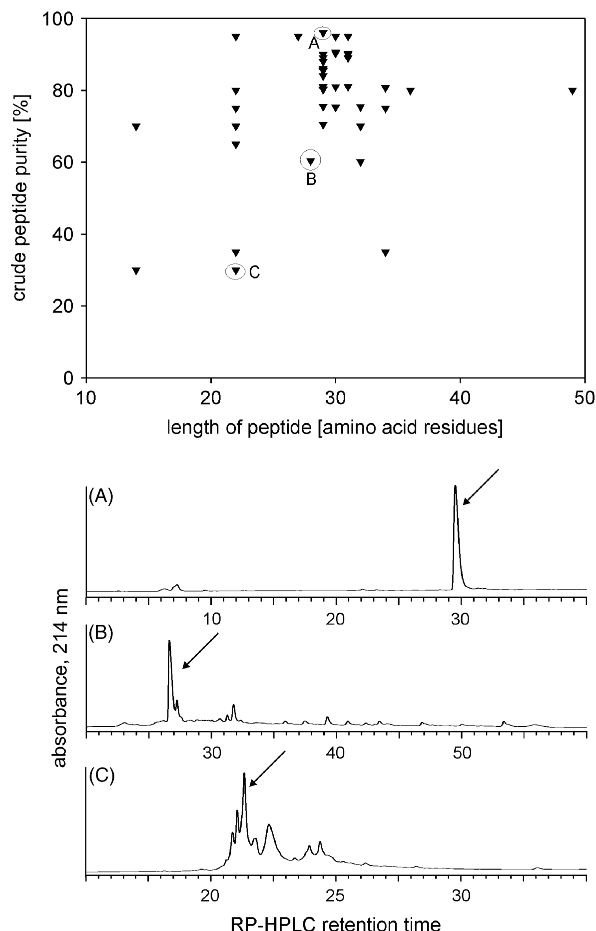

Figure 4 Representative results for the microwave-assisted synthesis of cyclic and disulfide-rich peptides: (Top panel) Purity of the crude peptide mixture from MW-assisted Boc-SPPS plotted against the length of the peptide. Purity is defined as the percentage area of the peak for the correct product relative to all other peptide peaks in the RP-HPLC trace. The majority of synthesis had crude peptide purities well above 60%, with only four syntheses (<10% of total experiments) having lower purities. Thus, independently of the length of the peptide, the protocol developed here for Boc-SPPS generally yielded crude peptide mixtures with high purity. (Bottom panel) RP-HPLC analysis of three examples MW-assisted synthesis: high (A), medium (B) and low (C) purity of the crude peptide mixture. The arrows indicate the correct product, which is the main peak in all cases.

amounts and volumes of the reagents from the 0.1 mM synthesis. In this case, there was no discolouration of the resin and no extra spurious masses were observed in the crude product, which was subsequently folded correctly. It therefore appears that caution must be used during the scale-up of MW-assisted reactions so as not to skimp on solution volumes.

Effectiveness of Boc-SPPS Assisted by MW Radiation

To assess the generality of the approach, the synthesis of a number of other cyclic peptides was attempted and a summary of the results is given in Figure 4(A). The test molecules are all cyclic cysteine-rich peptides and were synthesised by coupling a thioester linker to the PAM-Gly-Boc resin and terminating the assembled peptide sequence with a cysteine residue. The majority of the syntheses led to crude peptide mixtures of high purity, although in some cases the purity was better than that of others. The bottom panel in Figure 4 shows representative traces for low-, medium- and high-purity syntheses. The specific peaks corresponding to these peptides are marked on the upper panel of Figure 4 (labels A–C). It is clear that the MW-assisted method for automated Boc-synthesis is efficient and broadly applicable, with the average purity of the crude peptide mixture being >60% even without optimising the protocol for each specific sequence.

CONCLUSIONS

We have demonstrated that MW radiation can be successfully applied to the synthesis of complex cystine knot peptides using Boc chemistry and that it produces higher yields and purer products than without MW irradiation. The protocols developed here should be generally applicable to a wide range of disulfide-rich peptides, including members of the conotoxin [38] and defensin families, where milligram quantities of the peptides are often required for structural studies [39].

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