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Microwave-assisted solid-phase peptide synthesis at 60 °C: alternative conditions with low enantiomerization‡

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Several conditions have been used in the coupling reaction of stepwise SPPS at elevated temperature (SPPS-ET), but we have elected the following as our first choice: 2.5-fold molar excess of 0.04–0.08 M Boc or Fmoc-amino acid derivative, equimolar amount of DIC/HOBt (1 : 1) or TBTU/DIPEA (1 : 3), 25% DMSO/toluene, 60 °C, conventional heating. In this study, aimed to further examine enantiomerization under such condition and study the applicability of our protocols to microwave-SPPS, peptides containing L-Ser, L-His, L-Cys and/or L-Met were manually synthesized traditionally, at 60 °C using conventional heating and at 60 °C using microwave heating. Detailed assessment of all crude peptides (in their intact and/or fully hydrolyzed forms) revealed that, except for the microwave-assisted coupling of L-Cys, all other reactions occurred with low levels of amino acid enantiomerization (<2%). Therefore, herein we (i) provide new evidences that our protocols for SPPS at 60 °C using conventional heating are suitable for routine use, (ii) demonstrate their appropriateness for microwave-assisted SPPS by Boc and Fmoc chemistries, (iii) disclose advantages and limitations of the three synthetic approaches employed. Thus, this study complements our past research on SPPS-ET and suggests alternative conditions for microwave-assisted SPPS.

Keywords: high temperature; conventional heating; microwave technology; racemization; peptide isomers; peptide analysis

Introduction

It is well known that remarkable progress has been achieved in Biochemistry, Biology and Medicine with the use of synthetic peptides and oligonucleotides. In fact, these fine chemicals have been critical for generating knowledge of enzymology, protein and peptide chemistry, molecular biology, immunology, drug design and therapeutics.

Peptides were synthesized in solution [1] up to 1963, when Bruce R. Merrifield demonstrated that they could also be made using a new chemistry based on a polymeric support [2]. Subsequently, stepwise SPPS was impressively improved to the point of allowing the simultaneous production of hundreds or millions of relatively simple peptides in small quantities [3–6].

As the heterogeneity of the crude peptides resulting from solid-phase syntheses is strictly related to side reactions that may take place during the steps of peptide chain assembly on resin and/or during peptide cleavage from resin/full deprotection [7], both processes must be carried out under experimental condition that avoid or minimize side reactions. Special attention must be devoted to enantiomerization of the incoming amino acid [1,7,8] as (i) synthetic peptides can be used as drugs, additives in food industry, precursors for the preparation of bioactive compounds and tools in biological research, (ii) the presence of enantiomers in crude synthetic peptides are not easily revealed, (iii) the complete removal of such contaminants from the desired peptide by liquid chromatography may be impractical.

We have been studying the employment of elevated temperatures in manual SPPS as they abbreviate each of its steps (amino acid coupling, amino acid deprotection, amino acid acetylation and washings), reduce wastes and avoid the employment of DCM, an ozone-depleting organic solvent still used in traditional SPPS (all steps at room temperature). In previous works, although examining aspects such as optimum temperature, chemical strategies, swelling properties of resins, efficiency of coupling reagents and potential to minimize peptide aggregation, we found that the following combination was quite suitable for fast amino acid incorporation to a growing peptide-resin: 2.5-fold molar excess of Boc- or Fmoc-L-amino acid derivative, equimolar amount of N,N′-diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt) or 2-(1H- benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/diisopropylamine (DIPEA), 25% dimethylsulfoxide (DMSO)/toluene [9] and 60 °C with conventional heating [10,11]. Using very simple model peptides containing L-Ile, D-Tyr, L-Lys and L-Phe, we then addressed the key question of whether amino acid enantiomerization would be significantly enhanced under such reaction condition [12]. Having not observed that, we synthesized more complex peptides under similar experimental conditions

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[13]. Concomitantly, the use of microwave technology was successfully applied to SPPS [14,15], prompting us and other research groups to obtain a more accurate view of it [16–19].

In 2007, Collins and coworkers reported enantiomerization levels for microwave-assisted SPPS performed in the automated system CEM Liberty: when peptide chain assembly on resin was done by Fmoc strategy at a maximum temperature of 80 °C, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/DIPEA or HBTU/N-methyl morpholine (NMM) and *N*,*N*-dimethylformamide (DMF) and microwave energy of 40 W, the enantiomerization levels for L-His, L-Cys and L-Asn were 9.4, 9.6 and 4.5%, respectively; at 50 °C, the level for L-His was reduced to 1.6% [16]. Last year, Kappe and coworkers performed SPPS by Fmoc strategy on a 300 W single-mode manual microwave peptide synthesizer CEM Discover SPS set at 60 or 75 °C: using DIC/HOBt and N-methyl-2-pyrrolidinone (NMP) in the coupling reactions they found *ca.* 7 and 2% of enantiomerization for His and Cys, respectively, when microwave potency was 5–10 W [18].

Because in peptide synthesis changes in the reaction conditions may lead to minimization of enantiomerization, in this study we further examined the occurrence of this side reaction under our coupling condition at 60 °C using conventional heating [12] and investigated whether our protocols (that include our first-choice coupling condition) were applicable to microwave-assisted SPPS. The following peptides served as our new targets: Gly-Cys-Phe-NH_2 [20], Ac-Ala-Cys-Pro-Lys (acetylated fragment 12–15 of the human immunodeficiency virus-1 gp120 [21]; Ac-$Glic_{12-15}$), Ac-Pro-Ser-His-Arg (acetylated fragment 18–21 of unsulfated cholecystokinin [22,23]; Ac-CCK_{18-21}), Ac-His-Gly-Ser-Ala (acetylated fragment 50–53 of bovine α-hemoglobin [24]; Ac-Hb_{50-53}) and Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 (unsulfated fragment 24–33 of cholecystokinin [22,23]; CCK_{24-33}). For comparison, these target peptides were also synthesized by traditional SPPS. The following analytical techniques were used to inspect each crude product obtained: reversed phase-HPLC (RP-HPLC)/electron spray ionization mass spectrometry (LC/ESI-MS), capillary electrophoresis (CE) and/or gas chromatography-mass spectrometry (GC-MS) on a chiral column.

Materials and Methods

Trifluoroacetic acid (TFA), triethylamine (TEA), DIPEA and the solvents DCM, methanol (MeOH), DMSO and DMF were of synthesis grade and purchased from Merck (Darmstadt, Germany). Toluene was of synthesis grade and purchased from EM Science (Merck KGaA, Germany). Isopropanol (*i*PrOH) and diisopropyl ether were of analytical grade and purchased from Merck KgaA (Darmstadt, Germany) and Vetec (Rio de Janeiro, Brazil), respectively. Acetonitrile (CH_3CN) and NMP were of chromatography grade and purchased from EM Science (Darmstadt, Germany) and Applied Biosystems (Foster City, CA, USA), respectively. Anisole, 1,2- ethanedithiol (EDT), triisopropilsilane (TIS), phenol, 1,4 dithiothreitol (DTT) tri(hydroxymethyl) amino methane (Tris), dimethyl sulfide (DMS) and thioanisole were bought from Sigma Chemical Co. (St Louis, MO, USA). The following Boc- and Fmoc-L- or D-amino acid derivatives, aminoacyl-resins and resins were from: Bachem (Torrance, CA, USA) [Boc-L-Ser(Bzl)-OH, Boc-Gly-OH, Boc-L-His(tosyl; Tos)-OH, Boc-L-Arg(Tos)-PAM, Boc-L-Pro-OH, Boc-L-Cys(4-methylbenzyl; Mbzl)-OH, Boc-D-Ser(benzyl; Bzl)-OH, Boc-D-His(Tos)-OH, Boc-D-Cys(Mbzl)-OH, Boc-D-Ala-OH, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl) phenoxy (Rink amide) resin, Fmoc-L-Phe-OH, Fmoc-L-Cys(trityl; Trt)-OH, Boc-L-Phe-OH, Boc-D-Cys(Mbzl)-OH, Boc-D-Phe-OH, Fmoc-L-Tyr(terc-butyl; tBu)-OH, Fmoc-L-Arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl; Pmc)-OH)], Applied Biosystems [Boc-L-Ala-4-hydroxymethylphenylacetamidomethyl (PAM) resin, Boc-N^9-L-Arg(Tos)-PAM, Boc-L-Lys(2-chlorobenzyloxycarbonyl; 2-ClZ)-PAM)]; Protein Research Foundation (Osaka, Japan) [Boc-L-Ala-OH]; Peptides International (Louisville, KY, USA) [Fmoc-Gly-OH); Advanced ChemTech (Louisville, KY, USA) [4-methylbenzhydrylamine (MBHA) resin)]; Peptide Institute Inc. (Osaka, Japan) [Fmoc-L-Asp(OBut), Fmoc-L-Met-OH] and Novabiochem-Merck (Darmstadt, Germany) [Fmoc-L-Trp(tert-butyloxycarbonyl; Boc)-OH]. The coupling reagent HOBt was from Protein Research Foundation (Osaka, Japan), DIC was from Sigma Chemical Co. TBTU was purchased from Advanced ChemTech. The hydrogen fluoride (HF) used was from Quírios Produtos Químicos Ltda (São Paulo, Brazil). The (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid ($18C6H_4$) was from Aldrich Chem. Sigma Chemical Co. Other reagents used [37% HCl, acetic anhydride and propionic acid were purchased from Merck (Darmstadt, Germany), Applied Biosystems and Sigma Chemical Co.]. The water used was purified in a Milli-Q deionizer (Millipore, Bedford, MA, USA). All solvents and reagents were employed without further purification.

Peptide Synthesis

The peptides were manually synthesized by SPPS using Boc or Fmoc chemistry under atmospheric conditions at room temperature (traditional SPPS), at 60 °C using conventional heating and at 60 °C (set nominal temperature) using microwave heating. Traditional SPPS was carried out in a glass-fritted reaction vessel.

As in our previous works [10–13], all steps of SPPS at 60 °C using conventional heating were performed in a water-jacketed glass-fritted reaction vessel connected to a heated water-bath circulator (Polyscience 8001) under shaking.

As in previous reports [17,18,25], SPPS at 60 °C using microwave heating was performed in a 25 ml polypropylene vessel placed into the microwave cavity of the 300 W single-mode manual microwave peptide synthesizer (CEM, Discover SPS) set to such temperature; power pulsing sequences of 20 W were used for coupling and deprotection steps; the reaction temperatures were measured continuously with a fiberoptic probe inserted into the reaction vessel; the reaction volumes varied from 3.5 to 4.0 ml.

The protocols employed are those described in our previous works [10–12,22] with a few modifications (Table 1). Coupling and deprotection reactions were monitored by the Kaiser test of the growing peptide resins [26].

Peptide-resin Amino Acid Analysis

A small amount of the peptide-resin (1 mg) was suspended in 0.6 ml 6N HCl/propionic acid (1 : 1, v/v). Peptide bond hydrolysis was allowed to occur for 24 h at 130 °C in a Waters Pico-Tag workstation (Milford, CA, USA). The reaction was interrupted and the solvents were fully removed under vacuum. Amino acid analysis of the resulting hydrolysate was performed on a Beckman automated analyzer, model 7300 (Palo Alto, CA, USA) [27]. The results found for triplicates allowed calculating the amino acid content and, therefore, the substitution level (SL) of the peptide-resin.

Table 1. Summary of the experimental conditions employed for SPPS

Step	Traditional SPPS (room temperature)	SPPS at 60 °C – conventional heating[a]	Microwave-assisted SPPS[b]
Initial washing[c]	DCM, MeOH, 10% TEA/DCM (Boc)	25% DMSO/Toluene, dry MeOH, Toluene	25% DMSO/Toluene, dry MeOH, Toluene
	DCM, MeOH (Fmoc)		
Deprotection	50% TFA/DCM/anisole, 20 min (Boc)	50% TFA/Toluene, 10 min (Boc)	50% TFA/Toluene, 5 min, 20W (Boc)
	20% Piperidine/DMF, 10 min (Fmoc)	20% Piperidine/DMF, 5 min (Fmoc)	20% Piperidine/DMF, 5 min, 20W (Fmoc)
Washing post-deprotection [c]	DCM, 1% anisole/isopropanol, DCM, 10% TEA/DCM, MeOH (Boc)	25% DMSO/Toluene, dry MeOH, 10% TEA/Toluene, Toluene (Boc)	25% DMSO/Toluene, dry MeOH, 10% TEA/Toluene, Toluene (Boc)
	DCM and MeOH (Fmoc)	25% DMSO/Toluene, dry MeOH, Toluene (Fmoc)	25% DMSO/Toluene, dry MeOH, Toluene (Fmoc)
Coupling/recoupling	2.5-fold excess of Boc- or Fmoc-AA in DCM or DMF + DIC/HOBt or TBTU/DIPEA, 60 min	2.5-fold excess of Boc- or Fmoc-AA + DIC/HOBt in 25% DMSO/Toluene or TBTU/DIPEA in DMF, 30 min	2.5-fold excess of Boc- or Fmoc-AA + DIC/HOBt in 25% DMSO/Toluene or TBTU/DIPEA in DMF, 15 min, 20W
Washing post-coupling[c]	DCM, MeOH	25% DMSO/Toluene, dry MeOH, Toluene	25% DMSO/Toluene, dry MeOH, Toluene
N-terminal acetylation	50% acetic anhydride/DCM, 10 min	50% acetic anhydride/Toluene, 10 min	50% acetic anhydride/Toluene, 10 min
Washing post-acetylation [c]	DCM, MeOH	25% DMSO/Toluene, dry MeOH, Toluene	25% DMSO/Toluene, dry MeOH, Toluene

[a] Except for the acetylation step, all others were performed at 60 °C.
[b] Only the deprotection and coupling/recoupling steps were performed under microwave conditions; the other steps were carried out at room temperature.
[c] After the washing, the peptide-resin was submited to ninhydrin test. [26]

Peptide Cleavage From the Solid Support with Simultaneous Full Deprotection

Boc chemistry

Treatment with HF at 0 °C for 1.5 h in the presence of 1% anisole (model peptides lacking cysteine) or 1% anisole plus 1% DMS (cysteine-containing model peptides) was sufficient for generating the free unprotected peptide. After removing the acid under vacuum, the peptide was precipitated with cold diisopropyl ether and extracted with 0.1% TFA or CH_3CN/0.1% TFA mixture [3,28]. The resulting solution was lyophilized to produce the dried crude peptide.

Fmoc chemistry

The peptide-resin Gly-L-Cys-L-Phe-Rink amide (10 mg) was suspended in the mixture TFA/TIS/water/EDT (94/1/2.5/2.5; v/v/v/v), whereas the peptide-resin CCK_{24-33}-Rink amide (10 mg) was suspended in Reagent K (TFA/phenol/water/thioanisole/EDT; 82.5/5/5/5/2.5). Both suspensions were kept at 37 °C under shaking. Samples of the reaction media were analyzed by RP-HPLC after 2, 4 and 6 h of incubation. After 6 h, cold diisopropyl ether (800 µL) was added for peptide precipitation, then the suspensions were centrifuged and the supernatants discarded. This procedure was repeated three times. The resulting solids were dried under a steam of N_2. Peptide extraction from the dried solids was done by suspending them in 1 ml of 0.1% TFA/H_2O or in appropriate solution of CH_3CN/H_2O containing 0.09% TFA. The resulting solutions were lyophilized to give the crude peptides.

Peptide reduction

Solutions of the crude Ac-L-Ala-L-Cys-L-Pro-L-Lys (1 mg/ml) were incubated in 0.1% TFA/H_2O containing DTT (5-fold molar excess with respect to thiol groups) at 80 °C for 6 h. Reduction of cysteine was monitored by RP-HPLC.

Peptide Analysis by HPLC

Analytical RP-HPLC was performed in an LDC system composed of an automatic gradient controller, two pumps (LDC Analytical, a ConstaMetric 3500 and a ConstaMetric 3200 pump), a UV detector (Milton Roy SpectroMonitor 3100), a manual sample injector (Rheodyne 7125), an integrator (TermoSeparation Products, Data Jet integrator) and an analytical column (Vydac C_{18}, 5 µm, 300 Å, 0.46 × 25 cm). The condition employed was: 0.1% TFA/H_2O as solvent A, CH_3CN/H_2O containing 0.09% TFA as solvent B, linear gradient of solvent B, wavelength of 210 nm, flow rate of 1 ml/min. The percentage of CH_3CN in solvent B and the linear gradient employed were chosen according to the nature of the peptide analyzed.

Peptide Analysis by LC-ESI/MS

LC-ESI/MS was performed using an on-line coupling of a Shimadzu RP-HPLC system VP Series (Kyoto, Japan) [two LC-10AD pumps, an SDP-10-AV detector and a 7125 Rheodyne injector] to the Micromass Quatro II triple quadrupole mass spectrometer (Altrincham, UK). Analysis was achieved using the same column and the condition described above. The cone voltage used was 37 V. MassLynx for Windows NT software was used to analyze the spectra obtained.

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Peptide Analysis by CE

This type of analysis employed was a Beckman CE system, model 5510, using polyvinyl alcohol (PVA) coated capillaries of 55 cm × 50 μm (45 cm to detector). The buffer employed was composed of 18 mM Tris containing 6 mM $18C6H_4$ adjusted to pH 2.7 with citric acid. Samples were injected hydrodynamically [29] at 0.5 psi for 2 s and detected by UV absorbance at 214 nm. A potential of 20 kV was applied. The temperature was maintained at 25 °C.

Peptide Purification by RP-HPLC

Reduced Gly-D-Cys-L-Phe-NH_2 to be used as standard in RP-HPLC and CE spiking experiments was submitted to purification in analytical scale using the equipment described above and a Vydac C_{18} column (5 μm, 300 Å, 0.46 × 25.0 cm). The condition employed were: 0.1% TFA/H_2O as solvent A; 60% CH_3CN/H_2O containing 0.09% TFA as solvent B; linear gradients of solvent B from 5 to 95% in 30 min; wavelength of 210 nm and flow rate of 1 ml/min.

In-situ Deuteration and Chiral GC-MS of Amino Acids

About 100 nmol of the crude peptide were hydrolyzed in 0.3–0.5 ml 6 N DCl/D_2O for 8–24 h at 110 °C. After removing the excess of reagents by passing a steam of N_2 through the hydrolysis mixture, 300–500 μl 4 N deuterochloric acid-methyl alcohol were added to it. Esterification was allowed to occur at 110 °C for 15 min. Then, the reaction mixture was cooled to about 50 °C and evaporated by a stream of N_2. The residue obtained was dissolved in 250 μl TFA/DCM (1 : 9) to give a solution that was heated for 10 min to 130–140 °C. After cooling it to room temperature, the excess of reagents was removed by a stream of N_2 to furnish the final residue (for the analysis of histidine, 50 μl of isopropyl or butyl chloroformate was added to the sample and the vial was heated to 100 °C for 10 min and, again, the excess of reagent was removed by a stream of N_2).

The residue was dissolved in 150 μl of toluene and loaded on a GC Chirasil-Val column coupled to a mass spectrometer. Analytical condition employed were: detector, mass selective; split injector, 0.5 μl; carrier gas, H_2; flows, 1.5 ml/min (carrier gas), 35 ml/min (split) and 4 ml/min (purge); temperatures, 190 °C (injector), 65 °C isotherm for 3 min and 4 °C/min to 190 °C (oven). The amino acid derivatives were identified by their retention times and mass spectra [30,31].

Results

Except for CCK_{24-33}, the expected stereo isomers of all peptide models were manually synthesized by traditional SPPS (Table 1). The high quality of the crude peptides obtained was confirmed by analytical RP-HPLC and LC/ESI-MS (data not shown). Consequently, we used them as standards without prior purification. An exception was made for Gly-D-Cys-L-Phe-NH_2, which was purified by RP-HPLC in analytical scale.

Although the deprotection reactions (Boc or Fmoc removal) at 60 °C were accomplished in 5–10 min independently of the mode of heating used, several microwave-assisted amino acid couplings were not completed in 15 min (Table 1). In such cases, one or two recouplings were carried out (in some of these reactions DMF replaced 25% DMSO/toluene), thus making the time required for complete acylation of the growing peptide-resin equal to that required for the couplings performed at 60 °C using conventional heating.

Table 2. Peptide recoveries

Model peptide	SPPS approach	Peptide recoveries
Gly-Cys-Phe-NH_2	Traditional	0.24
	at 60 °C – conventional heating	0.21
	at 60 °C – microwave heating	0.16
Ac-$Glic_{12-15}$	Traditional	0.24
	at 60 °C – conventional heating	0.14
	at 60 °C – microwave heating	0.18
Ac-CCK_{18-21}	Traditional	0.29
	at 60 °C – conventional heating	0.31
	at 60 °C – microwave heating	0.27
Ac-Hb_{50-53}	Traditional	0.39
	at 60 °C – conventional heating	0.33
	at 60 °C – microwave heating	0.21
unsulfated CCK_{24-33}	Traditional	0.24
	at 60 °C – conventional heating	0.24
	at 60 °C – microwave heating	0.24

The reactions of full deprotection/peptide release from the resins, performed conventionally, were straightforward and provided the yields expected for the SPPS of short peptides (3–10 amino acid residues) starting from resins with substitution degrees varying from 0.4 to 0.8 mmol/g. Peptide recoveries (milligram of crude peptide/milligram of peptidyl-resin) for traditional SPPS, SPPS at 60 °C using conventional heating and microwave-assisted SPPS are described in Table 2.

Cysteine Containing- Tri- and Tetrapeptides

In 1997, Han and coworkers described the tripeptide amide Gly-L-Cys-L-Phe-NH_2 as a suitable model to study enantiomerization of L-Cys during peptide assembly on 5-(4-aminomethyl-3,5-dimethoxyphenoxy) valeric acid (PAL) resin by Fmoc chemistry at room temperature [20]. Therefore, in an initial step of this study, we used this tripeptide amide as target. Figure 1 shows the RP-HPLC profiles of the crude products obtained using the three synthetic approaches studied starting Rink amide resin (substitution level (SL) of 0.40 mmol/g). The desired L-peptide (**1**) and the D-Cys-epimer (**3**) were easily identified by LC/ESI-MS and RP-HPLC spiking experiments with the standards Gly-L-Cys-L-Phe-NH_2 and Gly-D-Cys-L-Phe-NH_2. No dimer was detected, indicating that this peptide was not prone to form intermolecular disulfide bridges under the working conditions. The D-Cys-epimer/L-peptide ratios found for the crude peptides resulting from traditional SPPS and SPPS at 60 °C using conventional heating were nearly equivalent and significantly lower than that obtained for the crude peptide resulting from microwave-assisted SPPS (Table 3). No attempt was made to separate and quantify the two other isomers probably formed during peptide chain assembly on resin (*LLD* and *LDD*) because we were explicitly interested in examining enantiomerization of L-Cys. Interestingly, the three synthetic approaches employed led to the formation of a significant amount of an analogue containing an additional phenylalanine residue (ESI-MS: calcd 471.2; found $[M+H]^+$ 472.1), which probably resulted from premature Fmoc removal of Fmoc-Phe-OH during its coupling to the Rink amide resin.

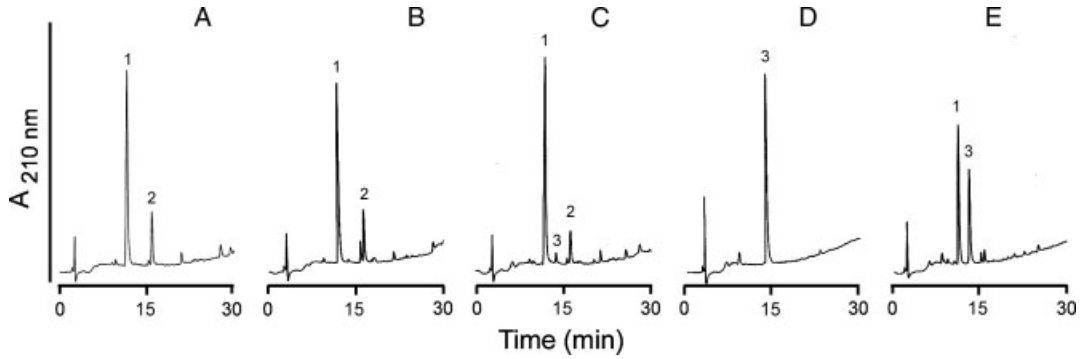

Figure 1. RP-HPLC profiles of the crude Gly-Cys-Phe-NH_2 (10 µg) resultant from traditional SPPS (A), SPPS at 60 °C using conventional heating (B) and microwave- assisted SPPS (C) as well as of the crude Gly-D-Cys-L-Phe-NH_2 (D). RP-HPLC identification of the isomer present in B spiked by D (E). Analytical condition: column: Vydac C_{18}, solvent A: 0.1% TFA/H_2O; solvent B: 90% CH_3CN/H_2O containing 0.09% TFA, Lλ: 210 nm, flow rate: 1.0 ml/min, linear gradient: 0–66% B in 33 min. Components identified by LC/ESI-MS: 1 = desired *LLL* peptide; 2 = analogue Gly-Cys-Phe-Phe-NH_2; 3 = peptide isomer *LDL*.

Table 3. Enantiomerization occurred during the SPPS of Gly-Cys-Phe-NH_2 and Ac-$Glic_{12-15}$

Cysteine-containing model peptide	SPPS approach	D-epimer/L-peptide[a] (%)
Gly-Cys-Phe-NH_2	Traditional	0.0[b]
	at 60 °C – conventional heating	0.8[b]
	at 60 °C – microwave heating	3.6[b]
Ac-$Glic_{12-15}$	Traditional	16.0[c]
	at 60 °C – conventional heating	12.0[c]
	at 60 °C – microwave heating	25.0[c]

[a] Calculated from the peaks of the RP-HPLC profiles obtained for the intact crude peptides.
[b] Only the D-Cys-epimer was considered.
[c] Only the sum of D-Ala-epimer and D-Cys-epimer was considered.

As to Ac-$Glic_{12-15}$, Ac-L-Ala-L-Cys-L-Pro-*L*-Lys, because the syntheses were performed by Boc chemistry starting from commercial Boc-L-Lys(2-chlorobenzyloxycarbonyl; 2ClZ) PAM resin (aminoacylation level of 0.52 mmol/g) and Boc-L-Pro, we did not expect isomers containing D-Lys and D-Pro to be formed during the coupling reactions [7], but only the following ones: *DDLL*, *LDLL* and *DLLL*. Figure 2 shows that all crude peptides obtained contained the desired peptide (ESI-MS: calcd 459.6; found $[M+H]^+$ 460.5), but also significant amounts of dimer resulting from partial Cys oxidation and, consequently, intermolecular formation of disulfide bridges. Therefore, they were incubated with DTT for reduction prior to analysis by RP-HPLC. As can be seen in the profiles shown in Figure 3, no evidence for the formation of the isomer *DDLL* was obtained. Because *LDLL* and *DLLL* were not baseline separated (although they were baseline separated from the L-peptide), a calculation of the ratio D-isomer/L-peptide was made considering the total area of *LDLL* and *DLLL* corresponding peaks. The results described in Table 3 are quite similar to those reported above for the previous cysteine-containing target peptide: the D-Cys-epimer/L-peptide ratios found for the crude peptides resulting from traditional SPPS and SPPS at 60 °C using conventional heating were nearly equivalent and significantly lower than that obtained for the crude peptide resulting from microwave-assisted SPPS.

Histidine and Serine-containing Tetrapeptides

In respect to Ac-CCK_{18-21}, Ac-L-Pro-L-Ser-L-His-L-Arg, it was impossible to achieve separation by RP-HPLC between the L-peptide and any isomer expected to be formed during its assembly by Boc chemistry starting from Boc-Arg(Tos)-PAM resin (Boc strategy; aminoacylation level of 0.42 mmol/g). Conveniently, modifications made to the CE condition previously determined by our group [12] allowed running spiking experiments with the standard isomers. The results, shown in Figure 4, indicated that syntheses furnished the desired peptide (ESI-MS: calcd 537.6; found $[M+H]^+$ 538.3) and suggested that the crude peptides obtained did not contain the isomers *LDLL* and *LDDL*. Suspecting that this was not correct, we submitted them to full acid hydrolysis, derivatization of the resulting amino acids, separation/identification/quantification of the derivatized amino acids by GC-MS using a chiral column (chiral amino acid analysis) [31]. The data collected (Table 4) revealed that the amino acids enantiomerization actually occurred, although in very low levels (less than 1%).

As to Ac-Hb_{50-53}, Ac-L-His-Gly-L-Ser-L-Ala synthesized by Boc strategy starting from Boc-L-Ala-PAM resin (aminoacylation degree of 0.80 mmol/g), all syntheses furnished the desired peptide (ESI-MS: calcd 412.4; found $[M+H]^+$ 413.3). Neither the analyses of the crude materials by RP-HPLC nor those by CE (Figure 5) were conclusive with respect to the presence of the isomers containing D-His. Therefore, again, the amino acid enantiomerization levels had to be calculated on the basis of the results provided by chiral amino acid analysis [31]. Table 4 shows that enantiomerization of Boc-L-His(Tos)-OH was significantly increased (6.5–8.5 times) when SPPS was carried out at 60 °C, independently of the mode of heating. However, in both cases, the levels did not exceed 2%.

Cholecystokinin (CCK)-derived Model Decapeptide

The syntheses by Fmoc chemistry starting from Rink amide resin (SL of 0.35–0.45 mmol/g) of CCK_{24-33} – a representative of peptides containing several trifunctional amino acids – was carried out to complement the study. In fact, in addition to enantiomerization of the incoming amino acids, this peptide model would allow for characterizing the crude peptides obtained at elevated temperature in terms of peptide recovery from peptide–resin and type of contaminants resulting from other side reactions typical of SPPS [8].

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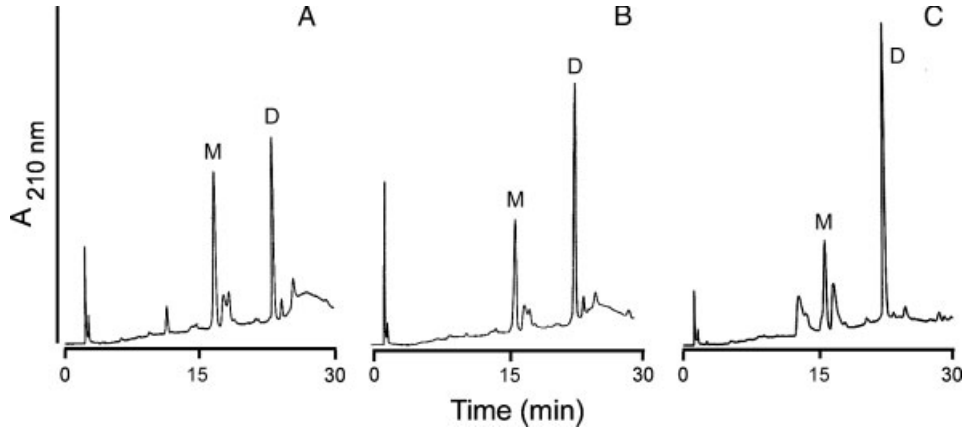

Figure 2. RP-HPLC profiles of the crude Ac-Ala-Cys-Pro-Lys (30 μg) resultant from traditional SPPS (A), SPPS at 60 °C using conventional heating (B) and microwave- assisted SPPS (C). Analytical condition: column: Vydac C_{18}, solvent A: 0.1% TFA/H_2O, solvent B: 10% CH_3CN/H_2O containing 0.09% TFA, λ: 210 nm, flow rate: 1 ml/min, linear gradient: 5–95% B in 30 min. Components identified by LC/ESI-MS: M: tetrapeptide monomer, D: tetrapeptide dimer.

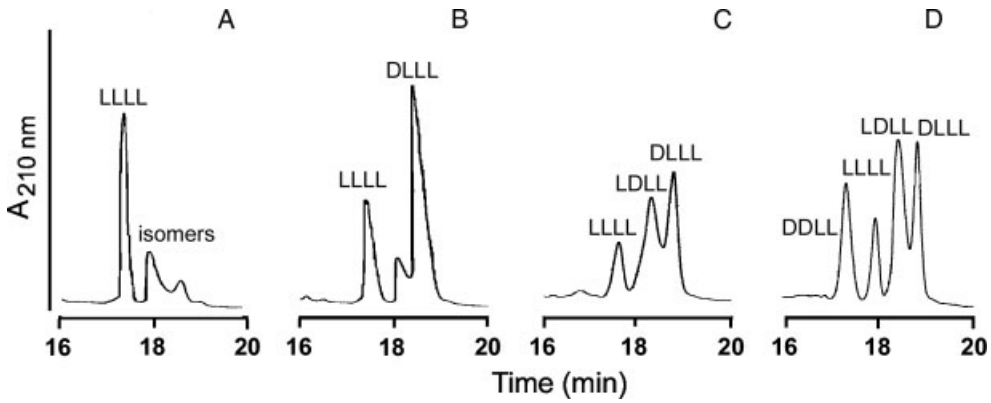

Figure 3. RP-HPLC identification of the isomers present in the crude Ac-Ala-Cys-Pro-Lys resultant from SPPS at 60 °C using conventional heating (A) through spiking experiments with the standard peptide isomers *DLLL* (B), *DLLL* and *LDLL* (C), *DLLL*, *LDLL* and *DDLL* (D). Analytical condition: column: Vydac C_{18}, solvent A: 0.1% TFA/H_2O, solvent B: 10% CH_3CN/H_2O containing 0.09% TFA, λ: 210 nm, flow rate: 1 ml/min, linear gradient: 5–95% B in 30 min. Identification was achieved by LC/ESI-MS and coelution with the standards employed.

Although peptide recoveries from peptide–resins were identical (0.24), we found that the microwave-assisted SPPS was the most advantageous synthetic approach used as it furnished the crude material with 33% of the desired L-peptide (**1** in Figure 6; ESI-MS: calcd 1334.5; found $[M+H]^+$ 1335.4, $[M+2H]^{+2}$ 668.0), a higher content than those found in the crude peptides resulting from SPPS at 60 °C using conventional heating (28%) and, surprisingly, for traditional SPPS (21%). The RP-HPLC profiles and the mass spectra obtained revealed that all three crude peptides contained des-Arg-CCK_{24-33} (**3**; ESI-MS: calcd 1178.3; found $[M+H]^+$ 1179.3, $[M+2H]^{+2}$ 590.2); the crude obtained from traditional and microwave-assisted SPPS also contained des-Tyr-CCK_{24-33} (**2**; ESI-MS: calcd 1171.3; found $[M+H]^+$ 1172.1, $[M+2H]^{+2}$ 586.2). Such byproducts probably resulted from incomplete coupling of Fmoc-L-Arg(Pmc)-OH and Fmoc-L-Tyr(tBu)-OH, respectively. Nevertheless, SPPS at 60 °C using conventional heating also furnished a Met(O) analogue of CCK_{24-33} (**4**; ESI-MS: calcd 1349.5; found $[M+H]^+$ 1350.4, $[M+2H]^{+2}$ 675.7).

Because the syntheses could produce crude peptides that were mixtures of the expected L-peptide with a large number of stereo isomers (maximum of 2^9), each crude was submitted to full hydrolysis/derivatization prior to analysis by the chiral amino acid analysis described above [31]. As shown in Table 4, the crude peptides synthesized by SPPS at 60 °C using conventional heating presented the highest contents of D-amino acids. However, again, none exceeded 2% (1.4% for Asp, 1.3% for Met, 1.5% for Trp).

Discussion

The results obtained in this study using 2.5-fold excess of 0.04–0.08 M Fmoc- or Boc-amino acid derivative, equimolar amount of DIC/HOBt (1 : 1) or TBTU/DIPEA (1 : 3, this last pair only when needed), 25% DMSO/toluene and conventional heating or microwave heating for the coupling step of SPPS confirmed that the enantiomerization of Boc- or Fmoc-L-Cys derivative is a quite relevant problem in microwave-assisted SPPS. Thus, our results support the previous recommendation that the incorporation of L-Cys to a growing peptide–resin should be carried out traditionally at room temperature or under microwave irradiation for 2 min at 0 W followed by 4 min at 40 W with a maximum temperature of 50 °C [16,32]. However, they show that this particular coupling reaction can be faster (in half of the time it normally takes at room temperature) and with low enantiomerization level if our condition at 60 °C using conventional heating is employed.

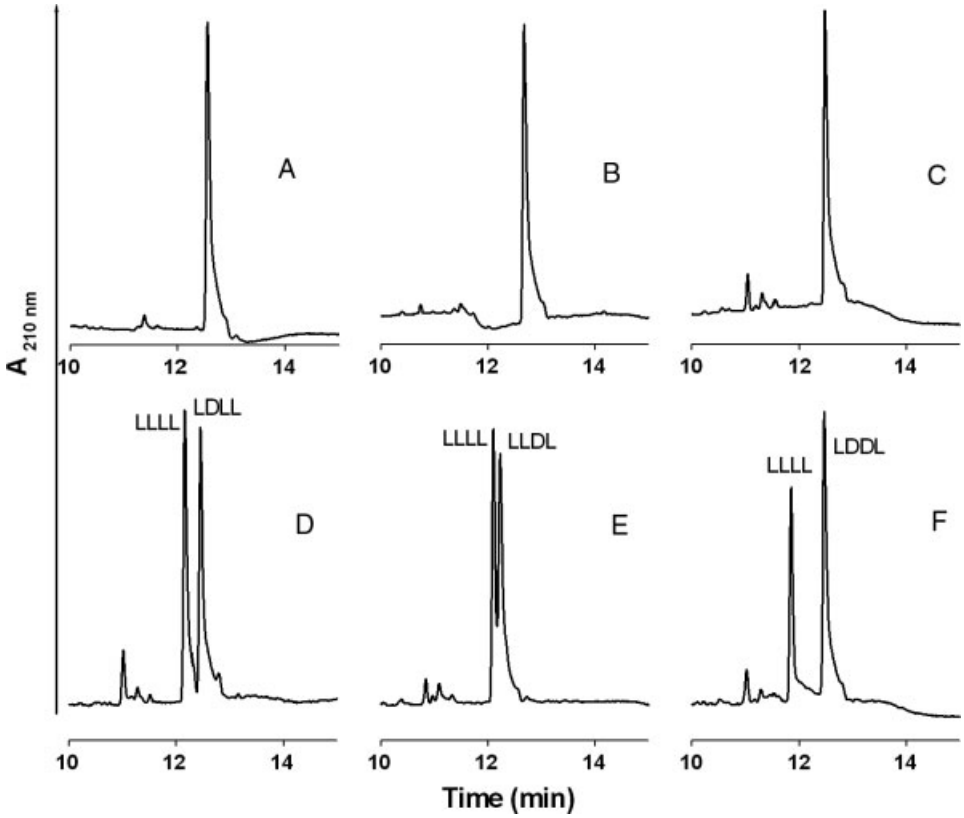

Figure 4. Eletropherograms of the crude Ac-Pro-Ser-His-Arg resultant from traditional SPPS (A), SPPS at 60 °C using conventional heating (B) and microwave-assisted SPPS (C). Electropherograms of B spiked by the peptide isomer *LDLL* (D), *LLDL* (E) or *LDDL* (F). Analytical condition: voltage: 20 kV, buffer: 18 mM Tris containing 6 mM $18C6H_4$ adjusted to pH 2.5 with citric acid, PVA-coated capillary length: 55 cm (45 cm to detector), capillary diameter: 75 mm, temperature: 20 °C, detection: 214 nm, injection: 2 s at 0.5 psi.

Table 4. Extension of enantiomerization occurred during the SPPS of Ac-CCK_{18-21}, Ac-Hb_{50-53} and unsulfated CCK_{24-33}

Peptide model	SPPS approach	D-amino acid (%)[a]									
		Pro	Ser	His	Arg	Ala	Asp	Met	Phe	Tyr	Trp
Ac-CCK_{18-21}	Traditional	<0.1	<0.1	0.5	<0.1	–	–	–	–	–	–
	at 60 °C – conventional heating	<0.1	0.2	0.8	<0.1	–	–	–	–	–	–
	at 60 °C – microwave heating	<0.1	0.4	0.9	0.2	–	–	–	–	–	–
Ac-Hb_{50-53}	Traditional	–	<0.1	0.2	–	<0.1	–	–	–	–	–
	at 60 °C – conventional heating	–	<0.1	1.7	–	0.2	–	–	–	–	–
	at 60 °C – microwave heating	–	<0.1	1.3	–	0.1	–	–	–	–	–
unsulfated CCK_{24-33}	Traditional	–	–	–	0.2	–	0.5	1.0	0.2	0.2	0.1
	at 60 °C – conventional heating	–	–	–	0.3	–	1.4	1.4	0.8	0.5	1.5
	at 60 °C – microwave heating	–	–	–	0.3	–	0.7	0.9	0.5	0.3	0.3

[a] Calculated from the GC-MS analyses of the hydrolyzates obtained in 6N DCl/D_2O.

Regarding the other Boc- and Fmoc-amino acids used in this study, our results evidenced that those derived from L-Pro, L-Ser, L-Arg, L-Ala and L-Tyr were practically not affected by temperature, solvent and mode of heating employed. Conversely, enantiomerization for those derived from L-Asp, L-Met, L-Phe, L-His and L-Trp was enhanced under our condition at 60 °C independently of the mode of heating used. Yet, the extents found were quite low as they varied from 0.8 to 1.7%. In fact, the highest one found was that for Boc-L-His(Tos)-OH (1.7%), which is similar to that measured by Palasek *et al*. at 50 °C for Fmoc-L-His(Trt)-OH and significantly lower than that recently reported by Bacsa *et al*. for the same Fmoc-amino acid derivative at 60–75 °C. Interestingly, these authors used considerably larger molar excess (5-fold) and higher concentration (0.18–0.20 M) of it [16,18].

The RP-HPLC and mass spectrometry analyses performed revealed that the crude peptides resulting from SPPS at 60 °C exhibited purities similar to those resulting from traditional SPPS, meaning that, under the experimental, conditions employed for peptide chain elongation on resin, many other side reactions typical of peptide chain assembly on resin and cleavage from

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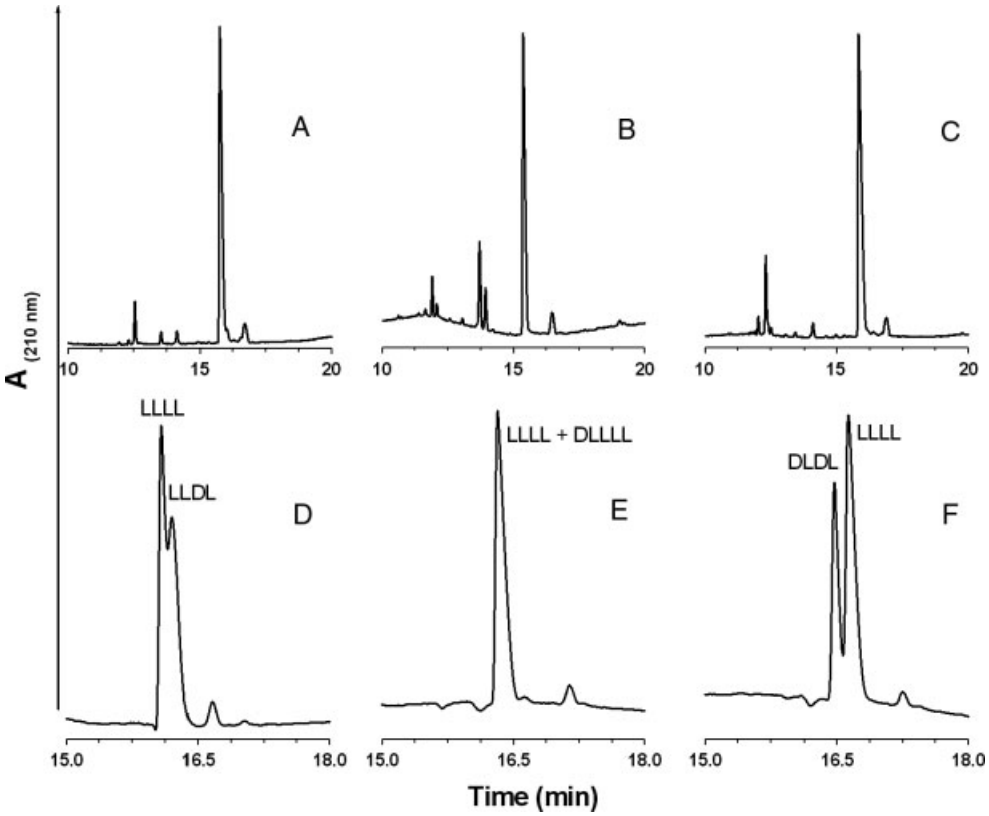

Figure 5. Eletropherograms of the crude Ac-His-Gly-Ser-Ala resultant from traditional SPPS (A), SPPS at 60 °C using conventional heating (B) and microwave-assisted SPPS (C). Electropherograms of B spiked by the peptide isomer *LLDL* (D), *DLLL* (E) or *DLDL* (F). Analytical condition: voltage: 20 kV, buffer: 18 mM Tris containing 6 mM $18C6H_4$ adjusted to pH 2.5 with citric acid, PVA-coated capillary length: 55 cm (45 cm to detector), capillary diameter: 75 mm, temperature: 20 °C, detection: 214 nm, injection: 2 s at 0.5 psi.

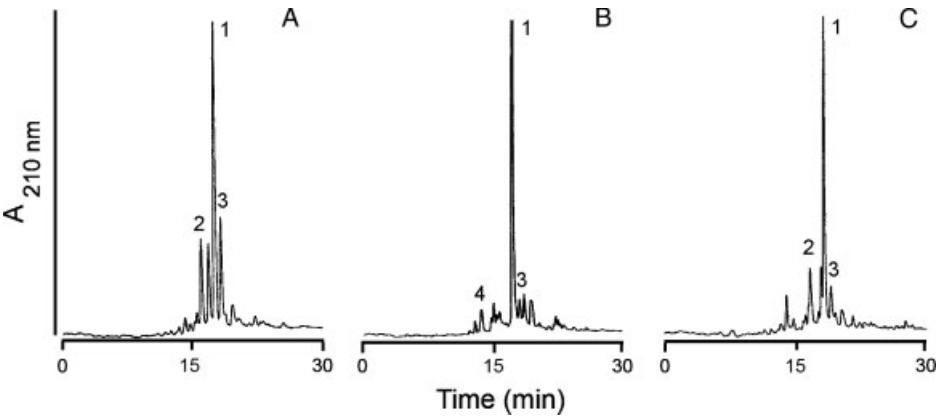

Figure 6. RP-HPLC profiles of the crude unsulfated CCK_{24-33} resulting from traditional SPPS (A), SPPS at 60 °C using conventional heating (B) and microwave-assisted SPPS (C). Analytical condition: column: Vydac C_{18}, solvent A: 0.1% TFA/H_2O, solvent B: 90% CH_3CN/0.09% TFA/H_2O, λ: 210 nm, flow rate: 1.0 ml/min, linear gradient: 5–95% B in 30 min. 1 = desired L-peptide, 2 = des-Tyr-CCK_{24-33}, 3 = des-Arg-CCK_{24-33}, 4 = Me(O)-analogue.

resin/full deprotection [7,8] were not significantly enhanced. An exception was made for Met oxidation as Met(O) analogues were detected in the crude unsulfated CCK_{24-33} resulting from SPPS at 60 °C using conventional or microwave heating. Although it may be argued that the formation of such oxidized byproducts during Fmoc-L-Met-OH coupling to the growing peptide–resin was mainly due to the use of the binary mixture 25% DMSO/toluene under atmospheric conditions, it must be noted that: (i) Met(O) analogues have also been detected in crude peptides resulting from microwave-assisted SPPS using DMF as solvent and CLEAR amide as resin (data to be published elsewhere); (ii) Met(O) can be reduced to Met after peptide cleavage from resin using *N*-methylmercaptoacetamide [33], ammonium iodide/DMS [34], ammonium fluoride/2-mercaptoethanol [35], $TiCl_4$ [36] or trimethylsilyl bromide/EDT [37] (obviously, we did not perform the cleavages from the resins under such conditions as we were interested in identifying all side reactions that could take place in the syntheses performed).

Therefore, it is reasonable to say that our new data support ours [10–13] and other author's [17] previous reports that peptides

can be synthesized with good yield and high purity at 60–65 °C employing Boc or Fmoc chemistry and conventional heating. In addition, these data reveal that our experimental protocols described in Table 1 (including our comparatively lower-cost coupling condition) are also compatible with Boc- and Fmoc-L-Cys, L-Ser and/or L-His derivatives. More importantly, they indicate that they are applicable for microwave-assisted synthesis of peptides, although the incorporation of L-Cys to the growing peptide chain on resin must be done traditionally or using conventional heating.

Because the above statements are strictly related to the fact that we employed the aprotic binary mixture 25% DMSO/toluene as basic solvent for the syntheses studied, it is essential to comment on it. Other solvent mixtures have been used for coupling and deprotection reactions in SPPS at high temperature [38], but 25% DMSO/toluene seems to be quite suitable for such purpose as it presents high thermal stability, does not evaporate significantly at 60–70 °C, dissolves well all Boc- and Fmoc-amino acid derivatives, swells well polystyrene/divinilbenzene based-resins, presents sufficiently low viscosity, appears to avoid peptide aggregation and, finally, it has been used successfully in SPPS at 60 °C using conventional heating [9,10] without significant enantiomerization of the incoming amino acids [12]. The following information strongly suggested that this aprotic binary mixture had the potential to absorb microwave energy: (i) dielectric constant (ε'), dielectric loss (ε''; amount of input microwave energy that is lost to the sample by being dissipated as heat) and tg δ ($\varepsilon''/\varepsilon'$) are all related to the ability of the common solvents to absorb microwave energy [39]; (ii) ε', ε'' and tan δ for NMP and DMF are: 32.2, 8.855 and 0.275 (NMP); 37.7, 6.070 and 0.161 (DMF); (iii) both aprotic solvents have been successfully employed for microwave-assisted SPPS [16–18,25]; (iv) ε' of 25% DMSO/toluene, estimated from a plot of ε *versus* the DMSO : toluene ratio [40], is 31.5; (v) ε'' and tan δ for toluene are 0.096 and 0.040 and for DMSO are 37.125 and 0.825, respectively, suggesting that ε'' and tan δ for the binary mixture 25% DMSO/toluene is relatively closer to those of DMSO, a high microwave absorbing solvent. In effect, all microwave-assisted syntheses described here using 25% DMSO/toluene as basic solvent were successful as they furnished the desired L-peptides as major products.

It is also important to mention that as we used only 2.5-fold excess of 0.04–0.08 M Boc- or Fmoc-amino acid derivative in the coupling step of SPPS, we chose to let the coupling/recoupling reactions at 60 °C using conventional heating to occur for 30 min (against 60 min for traditional SPPS at room temperature) and the microwave-assisted coupling/recoupling reactions to take place for 15 min (Table 1), reaction times higher than those generally reported for SPPS at temperatures higher than 50 °C [10,12,16,18,25]. Even though the time for peptide chain assembly on resin at elevated temperature was significantly reduced in comparison with traditional SPPS (almost 2-fold for SPPS using conventional heating and 4-fold for microwave-assisted SPPS). Attempts to optimize the coupling times, as well as deprotection times under the conditions described herein, are being undertaken in our laboratory.

Finally, it has to be noted that the amino acid couplings performed traditionally or at 60 °C using conventional heating occurred with very narrow temperature variation (maximum of 2 °C). Conversely, microwave-assisted reactions under our experimental conditions took place with higher temperature variation (maximum of 7 °C) as we employed a pulsed programming sequence typical of the CEM Discover SPS and in circumstances in which microwave irradiation was applied in short bursts throughout the reactions. Indeed, the following example illustrates such aspects of the microwave-assisted coupling reactions performed: when Fmoc-Phe-OH was coupled to Rink amide resin in 25% DMSO/toluene under our first-choice coupling condition, reaction temperature reached 60 °C after 16 s of microwave irradiation when the power went down to 0 W; as a result, reaction temperature quickly rose up to 67 °C and slowly decreased to 56 °C, when a short burst of microwave irradiation was provided again. As this cycle was repeated 15 min (Table 1), the sum of all bursts of microwave irradiation was 77 s, only 8.5% of the time given for the reaction to occur. Similar observations have been reported by Kappe and coworkers, who used the same microwave equipment/pulsed programming sequence [18]. Hence, our study also stimulates the discussion about the real effects of microwave on SPPS.

Conclusions

Our protocols and the combination of 2.5-fold molar excess of 0.04–0.08 M Fmoc- or Boc-amino acid derivative, equimolar amount of DIC/HOBt (1 : 1) or TBTU/DIPEA (1 : 3, this last pair only when needed), 25% DMSO/toluene and 60 °C with conventional heating are indeed suitable for SPPS with low enantiomerization of the incoming amino acids (including L-Ser, L-His and L-Cys). Under our conditions, the replacement of conventional heating by microwave heating speeds up the process radically with preservation of peptide yields and chirality of amino acids, except for L-Cys. In other words, our protocols are fully applicable for microwave-assisted SPPS and, therefore, they can be seen as a safe economical option for such purpose.

Acknowledgements

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