Research Article

Journal of PeptideScience

Received: 6 September 2011 Revised: 7 December 2011 Accepted: 4 January 2012 Published online in Wiley Online Library: 26 March 2012

(wileyonlinelibrary.com) DOI 10.1002/psc.2398

Racemisation of *N*-Fmoc phenylglycine under mild microwave-SPPS and conventional stepwise SPPS conditions: attempts to develop strategies for overcoming this

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We have been engaged in the microwave-solid phase peptide synthesis (SPPS) synthesis of the phenylglycine (Phg)-containing pentapeptide H-Ala-Val-Pro-Phg-Tyr-NH_2 (1) previously demonstrated to bind to the so-called BIR3 domain of the anti-apoptotic protein XIAP. Analysis of the target peptide by a combination of RP-HPLC, ESI-MS, and NMR revealed the presence of two diastereoisomers arising out of the racemisation of the Phg residue, with the percentage of the LLLDL component assessed as 49%. We performed the synthesis of peptide (1) using different microwave and conventional stepwise SPPS conditions in attempts to reduce the level of racemisation of the Phg residue and to determine at which part of the synthetic cycle the epimerization had occurred. We determined that racemisation occurred mainly during the Fmoc-group removal and, to a much lesser extent, during activation/coupling of the Fmoc-Phg-OH residue. We were able to obtain the desired peptide with a 71% diastereomeric purity (29% LLLDL as impurity) by utilizing microwave-assisted SPPS at 50 °C and power 22 Watts, when the triazine-derived coupling reagent DMTMM-BF_4 was used, together with NMM as an activator base, for the incorporation of this residue and 20% piperidine as an Fmoc-deprotection base. In contrast, the phenylalanine analogue of the above peptide, H-Ala-Val-Pro-Phe-Tyr-NH_2 (2), was always obtained as a single diastereoisomer by using a range of standard coupling and deprotection conditions. Our findings suggest that the racemisation of Fmoc-Phg-OH, under both microwave-SPPS and stepwise conventional SPPS syntheses conditions, is very facile but can be limited through the use of the above stated conditions. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: Fmoc-phenylglycine; racemisation; peptide diastereoisomers; DMTMM; super active esters; microwave-SPPS; conventional stepwise SPPS; RP-HPLC; ESI-MS; (1D) and (2D) NMR

Introduction

9-Fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) has become an important tool in peptide chemistry and has revolutionized the preparation of synthetic peptides for use in molecular biology, chemical biology, biochemistry, pharmacology, drug discovery, and medicinal chemistry [1–5]. With the increased demand for production of huge peptide libraries for high throughput screening, the use of microwave synthesizers has emerged as a complementary approach to conventional stepwise SPPS for accelerating the synthesis procedure and decreasing side products [6,7]. Although microwave-assisted synthesis can be highly efficient in enhancing the rates of deprotection and coupling steps, there are some reported cases of increased levels of racemisation of some amino acid residues [8,9].

In SPPS, the incidence of racemisation has been widely investigated for many urethane-type amino protected amino acid residues [8–14]. Racemisation mechanisms have been extensively studied and fall into two main types: direct enolization and oxazolone (azalactone) formation, which are both base-catalyzed mechanisms [15]. In the case of urethane-type amino protected amino acid residues, direct enolization is mostly the suggested mechanism, whereas the oxazolone mechanism occurs in coupling acyl-type amino protected amino acid residues and peptide fragments. The degree and rate of racemisation depend on many factors: inductive and mesomeric effects of amino acid side chain residues, the nature of activators/coupling reagents used, the basicity of tertiary amines used in activation/coupling steps and Fmoc protecting group removal, reaction duration, temperature, and synthesis mode whether conventional stepwise SPPS or microwave-SPPS.

Because racemisation of a single chiral amino acid residue may have a devastating effect on the biological action of a peptide, approaches to minimize racemisation of vulnerable residues are clearly essential.

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One such racemisation vulnerable residue is phenylglycine (Phg). Although not encoded genetically, Phg has been incorporated into a broad-spectrum of synthetic biologically active peptides such as anti-hepatitis c virus (HCV) genotype 1b replicon inhibitors [16,17] and HCV NS3 protease inhibitors [18], β-lactam antibiotics [19], gastrin-like peptides [20], platelet aggregation inhibitors [21], anticoagulant D-phenylglycinamide derived inhibitors of Factor X_a [22], vascularisation/angiogenesis inhibitors with possible use in cancer therapy [23], neurokinin inhibitors [24], D-phenylglycine-L-dopa conjugates that improved the bioavailability of the neurotransmitter L-dopa [25,26], and pro-apoptotic anticancer peptides [27].

In the course of our studies aimed at the synthesis of analogues of the pro-apoptotic peptide H-AVPPhgY-NH_2 (1), where the Phg is a crucial residue for the binding of the peptide to its target protein 'BIR3 domain of XIAP' [27], we have encountered extensive racemisation of this crucial residue when using 'standard' solid phase synthesis methods employing conventional and microwave-assisted approaches. This paper reports on the comparative degrees of racemisation engendered by using a range of synthesis conditions and the substantial suppression of racemisation of this residue by using the triazine-derived coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM-BF_4) with NMM as the activation base for the coupling steps and 20% piperidine/DMF for Fmoc-removal, under microwave-SPPS mild conditions.

Materials and Methods

Reagents

All amino acids were introduced as their Fmoc-protected derivatives. Fmoc-Ala-OH, Fmoc-Pro-OH, and Fmoc-Phe-OH were purchased from CEM (Buckingham, England, UK), whereas Fmoc-Val-OH, Fmoc-phenylglycine (Fmoc-Phg-OH), and Fmoc-Tyr-(tBu)-OH were obtained from Novabiochem (Darmstadt, Germany). *N*-Hydroxybenzotriazole (HOBT) was also obtained from CEM (Buckingham, England, UK). *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM-BF_4), and Rink amide MBHA resin were obtained from Iris Biotech GMBH (Marktredwitz,Germany). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM-Cl), diisopropylethylamine (DIEA), *N*-methylmorpholine (NMM), morpholine, piperidine, piperazine, triethanolamine, trifluoroacetic acid (TFA), triisopropylsilane, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), and diethylether were obtained from Sigma-Aldrich (Gillingham, Dorset, England, UK). HPLC-grade methanol and HPLC-grade trifluoroacetic acid were also supplied by Sigma-Aldrich. Double-distilled, deionised water was used throughout.

Liberty™ Microwave Synthesizer

The Liberty™ system (CEM Microwave Technology Ltd., Buckingham, England, UK) is an automated sequential peptide synthesizer that utilizes Pepdrive© software for controlling each step of the synthesis and can synthesize up to 12 different peptides in series. The Liberty™ contains a single-mode microwave (Discovery™) reactor in which a reaction vessel (30-ml glass fritted for 0.025–1.0 mmol syntheses scales, equipped with a spray head for reagent delivery and a fiber-optic temperature probe for power delivery control) is placed. The system is pressurized with nitrogen for conveying reagents, agitating the reaction mixtures, and providing an inert environment during synthesis. All reagents and amino acids are delivered through metered sample loops for accurate dispensing.

Peptide Synthesis Protocols

H-AVPPhgY-NH_2 (1), H-AVPFY-NH_2 (2), Fmoc-PhgY-NH_2 (3) and H-PhgY-NH_2 (4)

Peptides (1), (2), (3), and (4) were synthesized by the CEM Liberty automated microwave peptide synthesizer, utilizing either conventional or microwave-assisted modes. In essence, each peptide was synthesized on a 0.1-mmol scale, using Rink amide MBHA resin (0.154 g, substitution 0.65 meq/g). Fmoc removal was performed using two repeat cycles employing (i) 2% DBU, (ii) 20% piperidine, (iii) 5% piperazine (iv) 20% morpholine, or (v) 20% triethanolamine as solutions in DMF. For the microwave-assisted approach, a first deprotection cycle of 30 s at 50 Watts was employed, followed by a second deprotection cycle of 3 min at 50 Watts. Both cycles were carried out at 75 °C. The conventional approach employed a 10-min exposure to the base, followed by a 20-min exposure at room temperature, in the absence of any microwave-induced heating. Coupling steps were carried out by introducing each Fmoc-amino acid (0.2-M solution in DMF) at a fivefold excess over resin loading, together with various activation reagents and bases used in the molar ratios indicated, including (i) HBTU/DIEA/AA (1/2/1), (ii) HBTU/NMM/AA (1/2/1), (iii) DMTMM-Cl/NMM/AA (1/2/1), and (iv) DMTMM-BF_4/NMM/AA (1/2/1), using microwave-assisted synthesis or HBTU/HOBT/DIEA/AA (1/1/2/1) for conventional synthesis. Each coupling reaction was performed for 10 min at 22 Watts at a temperature of either 50 or 75 °C, for microwave-assisted synthesis, or for 45 min at room temperature, in the absence of microwave-induced heating for conventional synthesis. Finally, cleavage of the peptide from the solid support and concomitant removal of the side chain O-But protecting group of tyrosine was performed manually at room temperature, using TFA/water/triisopropylsilane (95/2.5/2.5, v/v/v) for two 1-h cycles, with washing with dichloromethane after every cycle. The collected cleavage reaction mixtures and washes were evaporated under vacuum, at 30 °C, cooled, and the products were precipitated by the addition of cold diethylether. The precipitates were collected by centrifugation (3–5 min at 2000–3000 g), and the pellet was washed thoroughly with diethylether. This process was repeated two to three times, each time the solid was collected by centrifugation. After a brief drying in a vacuum to remove all traces of diethyl ether, the peptide products were dissolved in a 10% TFA aqueous solution and freeze dried.

RP-HPLC Analysis

Peptide purity was checked through RP-HPLC, using a Waters HPLC system fitted with Waters 1525 binary HPLC pump and Waters 2489 UV/visible detector (λ_{216} nm) (Waters, Milford, Massachusetts, USA) and employing a Phenomenex Jupiter C12 column (250 × 4.66 mm; particle size, 10 μm). The runs were carried out on an analytical scale with a flow rate of 1 ml/min. An elution gradient was utilized to resolve the product components that went from 90% solvent A (0.05% TFA in H_2O)/10% solvent B (0.05% TFA in CH_3OH) to 10% solvent A/90% solvent B, in 60 min for peptides (1) and (2). Another elution gradient was used for peptide (3), which went from 100% solvent A to 10% solvent A/90% solvent B, in 35 min. Whereas, for peptide (4), an elution gradient that went from 80% solvent A/20% solvent B to 10% solvent A/90% solvent B in 45 min was applied.

ESI-MS Spectroscopy

Mass spectrometry was performed using a Thermo Finnigan LCQ Deca™ instrument (Thermo Electron Corporation, Hertfordshire, England, UK), which uses ElectroSpray Ionisation Ion-Trap Mass Spectrometry, and was operated in nanospray mode. The peptide samples were dissolved in 10% TFA in methanol to perform mass spec analysis.

NMR Spectroscopy

NMR experiments were performed using the 5-mm inverse probe head on Bruker DRX 500 (Bruker BioSpin, Germany) operating at a 1H resonance frequency of 500.13 MHz at 298 K. (1 mg in 600ul calculate, 0.35 mM). Peptide samples were prepared using a DMSO solvent. NMR spectra were internally referenced for *d*-DMSO resonating at 2.54 ppm, and the final volume of the peptide sample was 600 μl.

One-dimensional (1D) proton NMR spectra were acquired with a relaxation delay of 3.0 s and acquisition time of 2.34 s. Thirty-two scans were acquired over a 7-kHz spectral width, and 32 768 data points were collected. The data sets were zero-filled to 65 536 data points and was multiplied by 0.2-Hz line broadening. Two-dimensional (2D) total correlation spectroscopy (TOCSY) [28] and rotating frame Overhauser enhancement spectroscopy (ROESY) [29] data sets were acquired with a relaxation delay of 1.4 s, acquisition time of 0.14 s, and a spectral width of 7 kHz. The TOCSY data were acquired with an 80-ms mixing time, whereas the ROESY data were acquired with a 200-ms spin-lock. The 2D experiments were performed with 8 and 16 number of scans with 2048 (F2) and 1024 (F1) complex data points for the TOCSY and NOESY data, respectively. 2D data were zero-filled to 2048 data points in F1 prior to transformation. All data sets were apodised in both dimensions by using a shifted squared sine bell window function. All spectra were referenced internally to the residual 1-H signal of the *d*-DMSO, resonating at 2.54 ppm. Data were processed using the TOPSPIN program version 2.1 (Bruker BioSpin, Germany).

Results and Discussion

Microwave-SPPS synthesis of peptide H-AVPPhgY-NH_2 (1) was carried out under mild conditions, by employing an activation/coupling cycle of 10 min at 75 °C, at a power setting of 22 Watts. In the first instance, HBTU was employed as an activator in the presence of two equivalents of DIEA as an activator base. Fmoc group removal was performed using 2% 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) in DMF (Table 2, entry 1). The release of the target peptide from the resin and the removal of the O-But protecting group from tyrosine were carried out at room temperature, and the crude peptide was checked with RP-HPLC, as outlined above. The product was found to contain two major peaks, A (percentage area 51%) and B (percentage peak area 49%) with retention times differing by 4 min (Figure 1(a)). The ESI-MS analysis of Peak A (found m/z: 581.3 [M + 1]+, relative abundance 100%) and Peak B (581.2 [M + 1]+, relative abundance 100%) revealed identical m/z profiles corresponding to the calculated mass (580 Da) for the peptide.

This implies that Peaks A and B are isomers of a single compound, arising either out of distinct *cis–trans* configuration ratios of the proline residue or racemisation of, most likely, the Phg residue, resulting in the formation of two diastereoisomers, which we will designate as LLL**L**L and LLL**D**L (embolden residue corresponds to Phg). We thought the latter explanation to be the most likely, but we decided to perform 1D and 2D proton NMR on samples of purified material from Peaks A and B to confirm/establish the precise reason for the presence of the two forms of the peptide.

Figure 2 records the 1D proton, and the 2D TOCSY and ROESY spectra for each component. All spectra showed clear chemical shift dispersion and well-resolved peaks. Sequence-specific proton resonance assignment of the peptide sample was achieved through the analysis of TOCSY and ROESY spectra. Almost all the spin patterns were unambiguously assigned in the TOCSY spectrum. The presence of αiH/Ni + 1H and side chain connectivity cross peaks in the ROESY spectra led to the complete sequence-specific resonance assignment of the peptide. The presence of connectivities of neighboring amino acid residues supported the reliability of the resonance assignments (see Figure 2). Sequence-specific resonance assignments were carried out, which led to the full and unambiguous identification of all the individual spin systems.

Each of the proline residues in the purified samples of both peptides obtained from Peaks A and B exhibited an 8–9/1 *trans-cis* conformational ratio with no observable difference in chemical shift, whereas chemical shift differences of the α proton of the phenylglycine residue of each peptide showed a slight variation of 0.15 ppm. In contrast, the amide proton of the tyrosine residue of each peptide showed a remarkable chemical shift difference of 0.33 ppm and small chemical shift variations for α protons of 0.08 ppm. Aromatic protons of the phenylglycine and tyrosine also showed noticeable changes for both peptides (the chemical shifts are recorded in Table 1). This clearly indicates the twist of the amide bond between the phenylglycine and tyrosine residues at the C-terminus of the sequence. In common with many bioactive peptides, both peptide samples did not show any secondary structural features in the DMSO medium.

Consequently, the NMR analysis data are pinpointing that racemisation of the Phg residue is the main cause for the presence/generation of two diastereoisomers corresponding to our designation of LLL**L**L (Peak A) and LLL**D**L (Peak B) in the recorded RP-HPLC chromatogram (Figure 1(a)).

Our instinct was that the racemisation of the Phg residue occurred during the synthesis of the target peptide and not because of the optically impure starting material. However, we had to establish formally that the latter could be excluded as a possibility. Thus, the optical purity of Fmoc-Phg-OH was checked by measuring its optical polarization, which showed an [α]20/D of +85.4° (C = 1% in DMF), as reported in the supplier's certificate of analysis. This emphasizes the optical integrity of the starting Fmoc-protected amino acid and points to the racemisation of this residue occurring during the synthesis procedure.

Interestingly, when we synthesized the phenylalanine analogue, H-AVPFY-NH_2 (2), by using the same microwave-assisted conditions and reagents as utilized for the synthesis of H-AVPPhgY-NH_2, we obtained a single product according to RP-HPLC analysis (Figure 3). This product had a retention time of 29 min, and the ESI-MS m/z profile revealed a charged species of m/z: 617.7 [M + Na]+ of relative abundance 100% (calcd m/z: 594) (Figure 3). This suggests that the vulnerability of Phg towards racemisation is much more pronounced under the same conditions than it is for Phe.

Bodanszky and Birkhimer have demonstrated previously that the high racemisation tendency of aromatic amino acid derivatives in the presence of tertiary amines can be attributed to the abstraction of the α carbon proton, a mechanism know as direct

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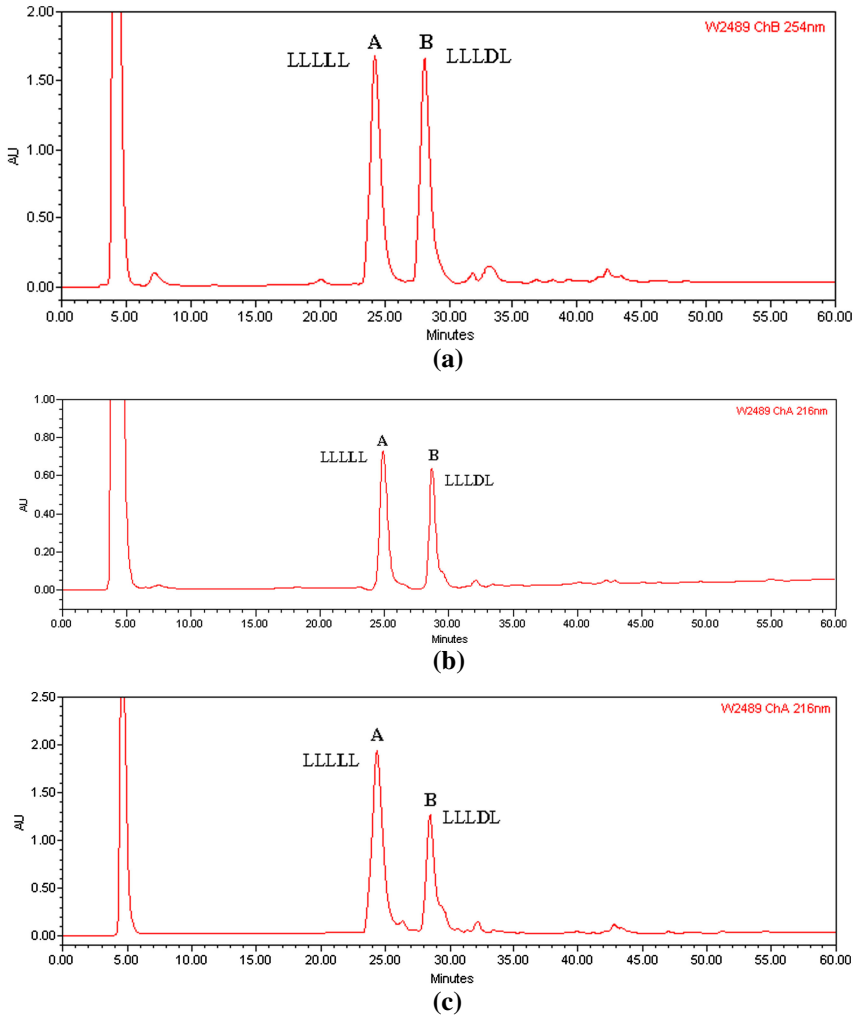

Figure 1. RP-HPLC profile of AVPPhgY-NH_2 (1) synthesized by (a) microwave-SPPS with conditions and reagents detailed in (Table 2, entry 1), the percentage of diastereoisomer LLL**D**L (Peak B) is 49% of the crude product; (b) microwave-SPPS with conditions and reagents detailed in (Table 2, entry 2), the percentage of diastereoisomer LLL**D**L (Peak B) is 43% of the crude product; (c) microwave-SPPS with NMM solution as activator base (Table 2, entry 3), the percentage of diastereoisomer LLL**D**L (Peak B) is 37% of the crude; (d) conventional stepwise SPPS with conditions and reagents detailed in (Table 2, entry 10), the percentage of diastereoisomer LLL**D**L (Peak B) is 36% of the crude product; (e) microwave-SPPS with conditions and reagents detailed in (Table 2, entry 8), the percentage of diastereoisomer LLL**D**L (Peak B) is 31% of the crude product; (f) microwave-SPPS with conditions and reagents detailed in (Table 2, entry 9), the percentage of diastereoisomer LLL**D**L (Peak B) is 29% of the crude product. The retention times for Peaks A and B are 25 and 29 min, respectively.

enolization [28]. In the case of Phg, the α carbon is a benzilic carbon, thus the carbanion generated from the release of the acidic α proton catalyzed by the tertiary amine is stabilized by the resonance effect of the phenyl ring. The stabilized carbanion can then be protonated from above or below the plane of the molecule. This is a possible explanation for the high racemisation tendency of the activated esters for such a residue (Figure 4). On the other hand, racemisation through the oxazolone (azalactone) mechanism cannot be a suggestion here, as the Phg residue is used as a urethane-type amino protected residue.

According to the previously suggested mechanisms, Phg would be susceptible to direct enolization at the benzilic α-proton either during the coupling step when exposed to the activator tertiary base N, *N*-diisopropylethylamine (DIEA) or during Fmoc removal for which the non-nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was used, initially, in our synthetic approach.

To investigate at which part of the synthetic cycle the epimerization had occurred, we synthesized the Fmoc-protected dipeptide Fmoc-PhgY-NH_2 (3) and its unprotected analogue H-PhgY-NH_2 (4) by using the same starting conditions used for the synthesis of the pentapeptide H-AVPPhgY-NH_2 (1) (see Table 2, entry 1). The RP-HPLC profile of the Fmoc-protected peptide (3) revealed only 3% **D**L diastereoisomer (Figure 5(a), Peak E), whereas that of the de-protected peptide (4) showed it contained 40% of the **D**L diastereoisomer (Figure 5(b), Peak G). These results demonstrate that Fmoc-removal is the largest contributing factor to Phg racemisation and that coupling/activation proceeds is of minor importance.

Thus, our first attempts to reduce the Phg racemisation focused on evaluating different bases for Fmoc-deprotection. In the original synthesis of (1), 2% DBU was used as a deprotect base (see Table 2, entry 1), which is one of the two most commonly used bases for the removal of the Fmoc group in Fmoc-based peptide synthesis [31–34], the other being the nucleophilic base, piperidine [35,36].

Because 2% DBU and 20% piperidine can be used interchangeably for the removal of Fmoc, we replaced the former with the latter under exactly the same conditions to examine the effect

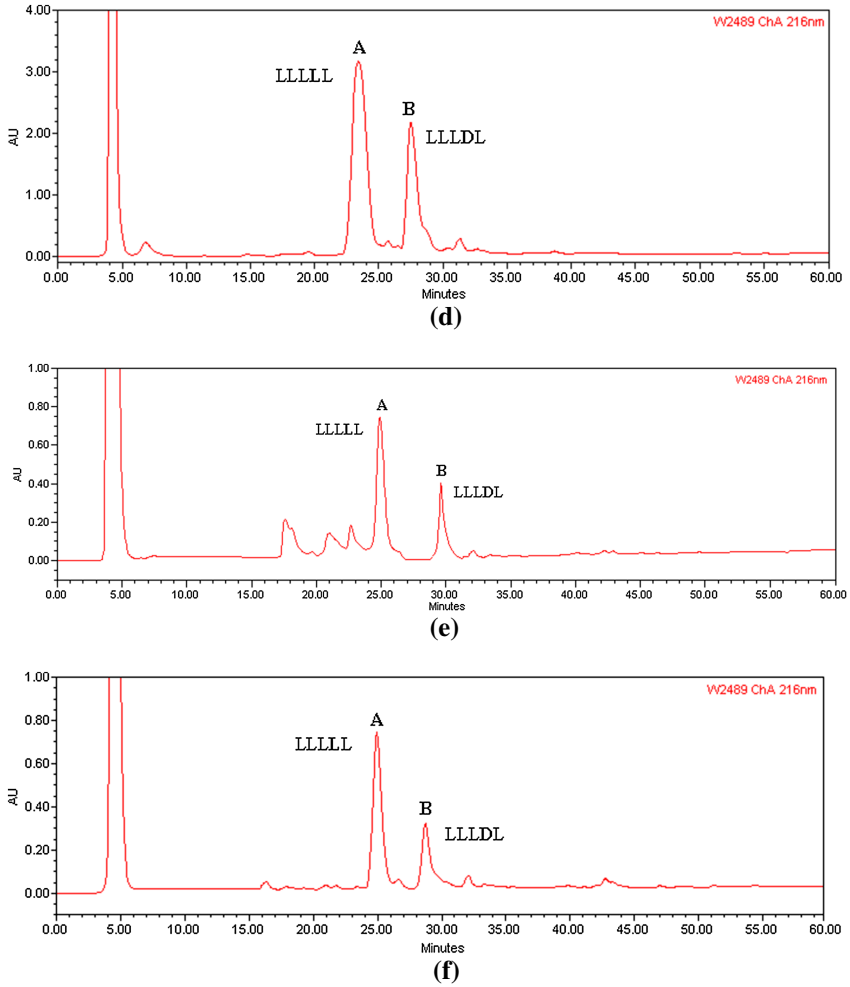

Figure 1. (continued)

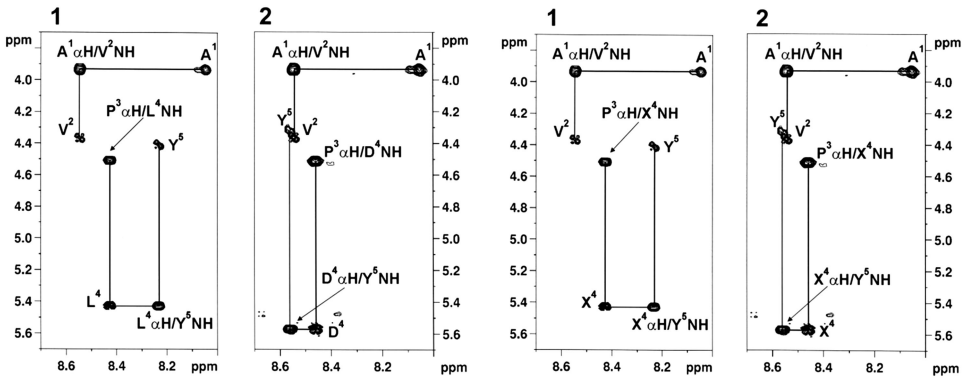

Figure 2. Fingerprint region with sequential connectivities of the ROESY spectra of *Y* and *Z* in DMSO at 500.13 MHz.

of the deprotection base on Phg racemisation. We observed a modest (6%) reduction in the percentage of LLL**D**L diastereoisomer formed (43% as compared to 49%; see Table 2, entry 2) when piperidine was used in place of DBU. The RP-HPLC traces of peptide (1) synthesized using these two bases are recorded in Figures 1(a) (DBU) and 1(b) (piperidine). Because DBU has a pKa of approximately 12 (in DMSO) and the weaker base piperidine has a pKa 11.2 [37], the use of the latter would be expected to result in a reduction of the abstraction of the Phg benzilic α-proton and a decrease in the formation of the LLL**D**L diastereoisomer.

We then started using combinations of milder deprotection and activation bases in an attempt at reducing racemisation in both Fmoc-removal and coupling/activation steps, respectively. When the switch in the use of piperidine for DBU for Fmoc group removal was combined with the use of an activator base with lower basicity, NMM (pKa 7.41) instead of DIEA (pKa 11), we observed a further (6%) drop in the proportion of the LLL**D**L diastereoisomer formed to 37% (Table 2, entry 3; RP-HPLC profile of peptide (1) synthesized using these conditions is shown in Figure 1(c)), regardless of the apparently lower contribution of

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Table 1. Chemical shift values of peptides obtained from Peaks A and B (in brackets) with reference to *d*-DMSO at 2.54 ppm. Data were collected on a Bruker 500.13 1H spectrometer at 25 C

Residue	NH	αH	βH	γCH2	δCH2	γCH3	Aromatic
1 Ala	8.05 (8.05)	3.94 (3.94)	1.32 (1.32)	—	—	—	—
2 Val	8.55 (8.55)	4.36 (4.36)	2.00 (2.01)	—	—	0.92 (0.92) 0.96 (0.97)	—
3 Pro	—	4.50 (4.51)	1.87, 2.05 (1.76, 2.09)	1.98, 1.98 (1.85, 1.91)	3.65, 3.74 (3.60, 3.77)	—	—
4 Phg	8.43 (8.46)	5.43 (5.58)	—	—	—	—	2,6 H; 7.70 (7.08) 3,5H; 7.37 (7.46) 4H; 7.31 (7.24)
5 Tyr	8.23 (8.56)	4.40 (4.32)	2.75, 2.92 (2.67, 2.88)	—	—	—	2,6 H; 7.00 (6.91) 3,5H; 6.64 (6.56)
Amide	9.19 (9.15)	—	—	—	—	—	—

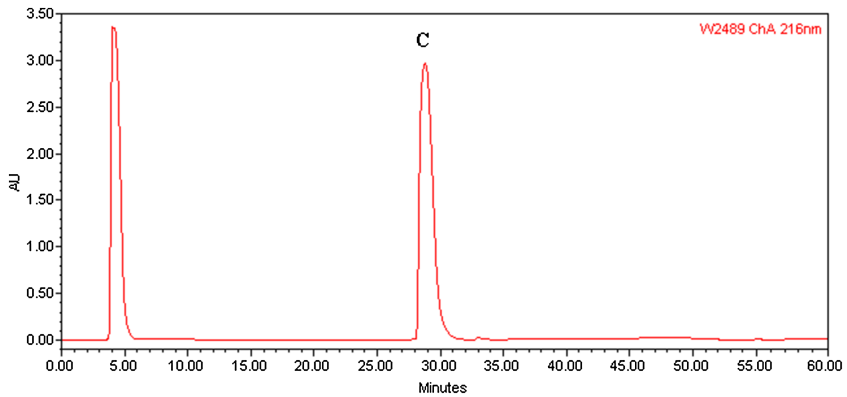

Figure 3. RP-HPLC profile of AVPFY-NH_2 synthesized by microwave-SPPS and by employing a 1-M solution of DIEA in DMF as an activator base. The retention time for Peak C is 29 min.

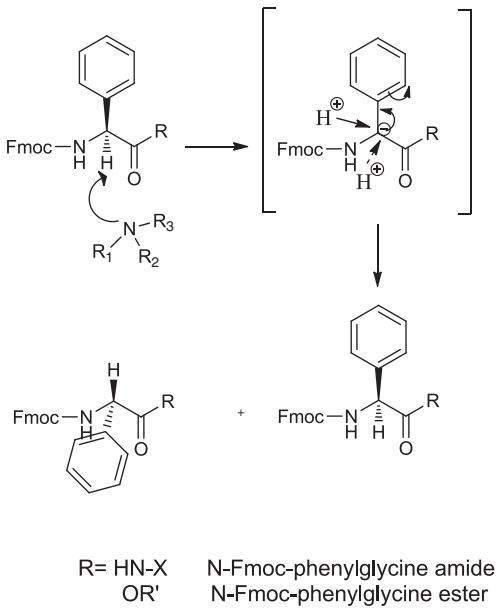

Figure 4. Base-catalyzed racemisation of *N*-Fmoc-phenylglycine derivatives through the direct enolization mechanism.

the coupling step in the racemisation process as observed from the Fmoc-PhgY-NH_2 RP-HPLC trace (only 3% **D**L diastereoisomer, Peak E in Figure 5(a)). This result is consistent with the work of Anderson *et al.*, [38] and Bodansky and Bodansky [39] which reported on the reduction in the rate of racemisation of Boc-Phg-OH-activated esters in DMF that used NMM in place of DIEA.

Given that the combination of the switch in 'activator' base and 'Fmoc-deprotection' base from DIEA + DBU to NMM + piperidine resulted in a 12% reduction in the formation of the LLL**D**L diastereoisomer (compare entries 1 and 3 in Table 2), we examined the effect of retaining NMM as the 'activator' base but replacing piperidine with two even milder bases, piperazine (pKa 9.8) and morpholine (pKa 8.36) [37], to see if we could achieve even further reductions of this isomer. However, the amount of this isomer formed with these combinations was not reduced any further (compare entries 3, 5, and 6 in Table 2). Finally, when an even weaker base triethanolamine (pKa 7.77) [33] was utilized, it proved ineffective at catalyzing the removal of the Fmoc group, and no peptide product whatsoever could be obtained (Table 2, entry 7).

To check the effect of microwave energy, reaction temperature, and coupling duration on the degree of Phg racemisation, peptide (1) was synthesized manually through conventional stepwise SPPS techniques at room temperature (Table 2, entry 10). The product purity was checked with RP-HPLC and, again, two diastereoisomers were obtained. However, the proportion of the LLL**D**L diastereoisomer formed was 36% (Figure 1(d)), which is some 7% lower than that formed in the microwave-assisted synthesis carried out at 75 °C, using identical activation reagents (HBTU + DIEA) and a deprotection base (piperidine) (Table 2, compare entry 2 with 10). Increased levels of racemisation at elevated reaction temperatures have been reported previously. For example, Bacsa *et al.* reported similar and

Table 2. Conditions used for the synthesis of peptide (1), and the %LLL**D**L in the crude product as determined by RP-HPLC. NP, no product was obtained using this base to remove the Fmoc group

Entry	SPPS technique	Temp. (°C)	Activator base	Base used in Fmoc-deprotection	Activator	Yield [%]	Purity [%]	%LLL**D**L
1	Microwave	75	DIEA	2% DBU	HBTU	53	96	49
2		75	DIEA	20% piperidine	HBTU	57	98	43
3		75	NMM	20% piperidine	HBTU	51	98	37
4		50	DIEA	20% piperidine	HBTU	51	97	36
5		50	NMM	5% piperidine	HBTU	47	95	36
6		50	NMM	20% morpholine	HBTU	48	95	35
7		50	NMM	20% triethanolamine	HBTU	—	—	NP
8		50	NMM	20% piperidine	DMTMM-Cl	58	86	31
9		50	NMM	20% piperidine	DMTMM-BF_4	65	97	29
10	Conventional	Room temp.	DIEA	20% piperidine	HBTU/HOBT	43	99	36

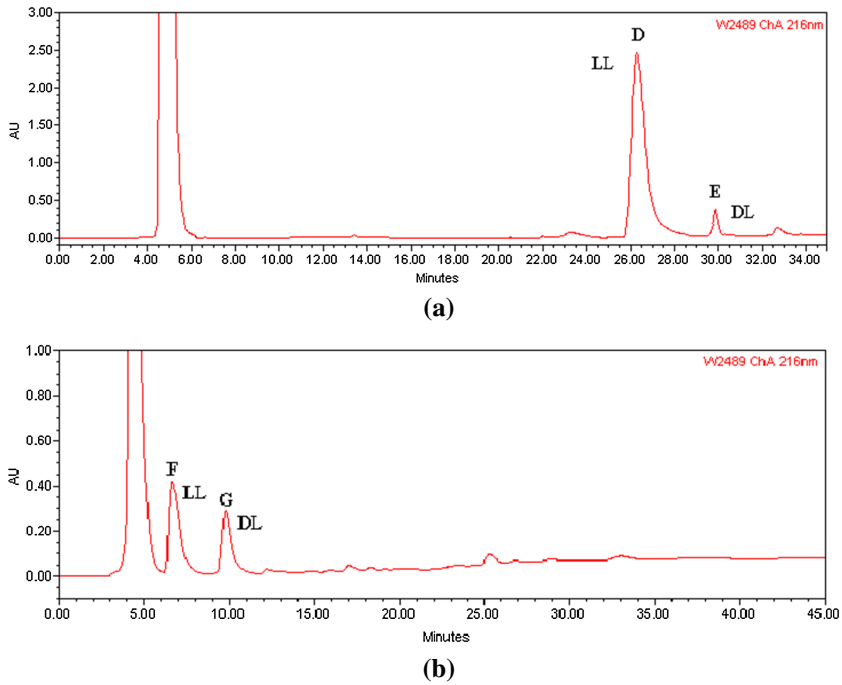

Figure 5. RP-HPLC profile of (a) Fmoc-PhgY-NH_2 (3) synthesized by microwave-SPPS with conditions and reagents detailed in (Table 2, entry 1), the percentage of diastereoisomer **D**L (Peak E) is 3% of the crude product. The retention times for Peaks D and E are 26.3 and 29.9 min, respectively. (b) PhgY-NH_2 (4) synthesized by microwave-SPPS with conditions and reagents detailed in (Table 2, entry 1), the percentage of diastereoisomer **D**L (Peak G) is 40% of the crude product. The retention times for Peaks F and G are 6.6 and 9.77 min, respectively.

significant levels of racemisation of histidine and cysteine when using both microwave and conventional SPPS at 86 °C, indicating that racemisation is a result of thermal effect, not the mode of heat production [40]. Also, Palasek *et al*. observed that the microwave-assisted coupling of the activated esters of the two residues resulted in significant racemisation at 80 °C, the extent of which could be reduced substantially if the coupling step temperature was reduced and conducted at 50 °C [8]. In light of those findings, we repeated the microwave synthesis of peptide (1) at 50 °C (Table 2, entry 4) but using exactly the same activation reagents, bases, and coupling times that had been employed at 75 °C. In doing so, we achieved a 7% decrease in the amount of LLL**D**L diastereoisomer formed (Table 2, compare entries 2 and 4), to a level (36%) identical to that formed during the conventional synthesis conducted at room temperature (Table 2, compare entries 4 and 10). So, it would appear that if the activation and coupling steps are carried out at temperatures up to 50 °C, then there is no difference in the extent of racemisation of the Phg residue through microwave-assisted synthesis and conventional SPPS approaches. Similar results have been reported by Loffrendo *et al*. whose work has demonstrated that virtually identical low levels of racemisation (<2%) were obtained during the coupling of many vulnerable residues at 60 °C (with the exception of L-cysteine) for both microwave and conventional stepwise SPPS [9]. Further confirmatory evidence that elevated reaction temperatures do not necessarily lead to enhancement of racemisation has been provided by Souza *et al*. who have investigated the utility of SPPS at elevated temperatures (SPPS-ET) and reported that the (nonmicrowave-assisted) syntheses of three simple model peptides at elevated temperature (55–75 °C) did not show significant increases in levels of racemisation compared to syntheses carried out at room temperature [10].

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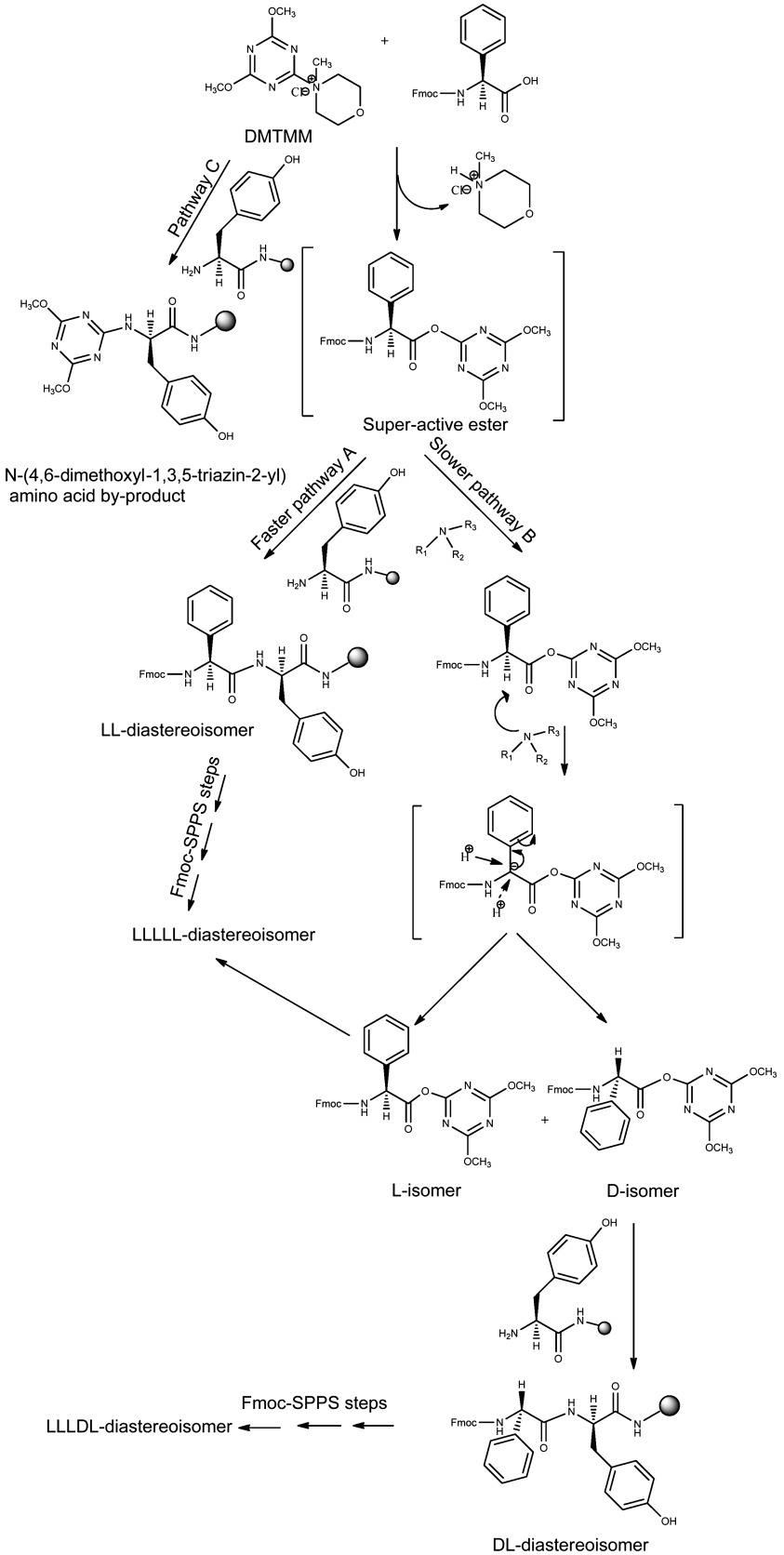

Figure 6. DMTMM-mediated super active ester formation (faster Pathway A), base-catalyzed racemisation of *N*-Fmoc-phenylglycine derivatives through the direct enolization mechanism (slower Pathway B) and N-(4,6-dimethoxy-1,3,5-triazin-2-yl) amino acid by product formation on the resin (Pathway C).

In the case of the Phg residue, the thermal effect played a role in increasing the degree of racemisation, leading to a 7% increase in the level of the LLL**D**L diastereoisomers (Table 2, compare entries 2, 4 and 10). So, we recommend using lower temperatures for the microwave-assisted SPPS of Phg-containing peptides (≤50 °C) or at room temperature for the conventional stepwise SPPS.

Despite the optimization in the selection of the bases used in the activation and Fmoc group removal steps, and reduction in reaction temperature, we were only able to achieve a modest overall reduction (~13%) in the formation of the 'undesired' LLL**D**L diastereoisomer, rather than the complete suppression we sought. Consequently, we decided to examine an alternative activator for HBTU in the hope of enhancing the rate of coupling of the Fmoc-Phg-OH residue in comparison to the rate of racemisation of this activated building block during the coupling step. Our attention was drawn to the triazine coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM-Cl) that was introduced by Kamiński *et al*. (1998) [41] and which has been applied to the synthesis of esters [42], amides [43], and simple peptides [44]. Of direct relevance to the present study are the reports that DMTMM-Cl yields peptide and peptidomimetic products with much lower levels of racemisation (in some instances, with complete suppression of the undesired formation of diastereoisomers) in comparison to those produced using other activating agents such as 2-chloro-4,6-dimethoxy-1, 3, 5-triazine (CDMT) and HBTU/HOBT [44–46].

When we utilized DMTMM-Cl as the reagent of choice for all of the coupling steps in the microwave-assisted synthesis of H-AVPPhgY-NH_2, at 50 °C, using NMM and piperidine as the bases for the activation and Fmoc group removal steps, respectively (Table 2, entry 8), we were able to achieve an additional 5% suppression for the formation of the LLL**D**L diastereoisomer (see Figure 1(e) for the RP-HPLC trace for this synthesis and see Table 2 to compare entries 4–6 and 10 with 8). We attribute this to the known ability of DMTMM-Cl to form super active ester derivatives of urethane-protected amino acids that are ideal acylating agents for amines. The formation of these super active esters was first postulated by Kamiński [47], and we suggest that this mechanism of amide bond formation (Figure 6, pathway A) proceeds much more rapidly than the direct epimerization of the Phg α proton (Figure 6, pathway B), thus, suppressing the incorporation of the D-enantiomer into the target peptide sequence. However, we noticed that the degree of purity for the crude peptide was lower than that of the HBTU-based synthesis (Figure 1(e), see also Table 2 to compare the crude peptide product purity for entries 1–6 and 10 with 8). This could possibly be due to the formation of resin-bound *N*-(4,6-dimethoxy-1,3,5-triazin-2-yl) derivatives at every coupling step (Figure 6, pathway C), a known side reaction to DMTMM-Cl. To overcome the formation of those side products, we replaced the DMTMM-Cl with its tetrafluoroborate salt analogue (DMTMM-BF_4) which was reported by Kamiński *et al*. to produce peptides with relatively high yields and higher degrees of purity [48]. The appearance of 'side product' peaks was substantially decreased (Figure 1(f)), and the percentage purity of the crude peptide was greatly improved (97%) compared to that of the crude peptide produced by DMTMM-Cl (86%), as anticipated (Table 2, compare entries 8 and 9).

In conclusion, we have identified a set of synthetic conditions that has enabled us to prepare our target peptide sequence H-AVPPhgY-NH_2, (1) with enhanced diastereomeric purity. We are of the opinion that microwave-SPPS in which mild bases for both Fmoc-removal (piperidine, piperazine or morpholine) and coupling/activation steps (NMM) are used, together with DMTMM-BF_4 as a coupling agent at 50 °C may, to provide significant advantages in the synthesis of peptides containing Phg and other amino acid residues with high propensity to undergo racemisation. Although we were able to achieve a 20% reduction in the racemisation level of Phg encountered, we believe that further investigations are needed to find a suitable base that can completely eliminate racemisation of this residue during Fmoc-group removal while retaining a high rate of efficiency in this deprotection step.

Acknowledgements

This work was supported by the generous donation of Dr John King (grant code: R9113PMY). Many thanks for Dr. Brendan Gilmore and Dr. Wei Chen in the School of Pharmacy, Queen's University of Belfast for their help in training us in the use of the Liberty™ microwave synthesis system, and to Adrienne Healy, School of Biological Sciences, Queen's University of Belfast, for performing the ESI-mass spectrometry analysis.

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