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# Solid-Phase Synthesis of a Dendritic Peptide Related to a Retinoblastoma Protein Fragment Utilizing a Combined boc- and fmoc-Chemistry Approach

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Abstract: Dendritic peptides, often presented as multiple antigen peptides (MAPs), are widely used in immunological-based fields of research, although their synthesis can be extremely challenging. In this paper, a tetrameric dendritic MAP-like presentation of the retinoblastoma protein [649-654] sequence ($4RB_{649\text{-}654}$) has been prepared using solid-phase peptide synthesis (SPPS) methods. During the synthesis of this dendritic molecule, numerous modifications to the synthetic protocols were examined. These modifications included the introduction of a combination Boc- and Fmoc-chemistry approach and also the use of 1,8-diazabicyclo[5.4.0]-undec-7-ene as a Fmoc-deprotection agent. The use in combination of Boc- and Fmoc-based synthetic strategies resulted in the production of the desired peptide molecule, $4RB_{649\text{-}654}$, in high purity and acceptable yields following purification by reversed phase HPLC. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dendritic peptides; diketopiperazine formation; deletion peptides; Fmoc-deprotection; retinoblastoma protein

## INTRODUCTION

Retinoblastoma protein (RB) is a tumour suppressor gene product that appears to play a central role in transcriptional control of cell differentiation [1]. Consequently, RB is a common cognate target for a variety of proteins with growth regulatory activity. All of these proteins share the amino acid sequence motif LXCXE, known to be critical for their binding to RB. A recent study has also suggested that insulin can also interact with RB. This conclusion was based on the identification [1] of the amino acid sequence LXCXE in the B-chain of insulin (i.e. the insulin $B_{[16\text{-}21]}$. . . -Tyr-Leu-Val-Cys-Glu-Glu-. . . ). The cognate recognition sequence within RB, namely $RB_{649\text{-}654}$, was deduced through application of hydropathic anti-complementarity theory [2] (Figure 1), and a multimeric peptide presentation of $RB_{649\text{-}654}$, termed $4RB_{649\text{-}654}$, was subsequently shown to bind insulin using various analytical methods, including an ELISA-type technique and affinity chromatography [1].

Following the characterization of these insulin-binding properties of $4RB_{649\text{-}654}$, we were interested in employing this multimeric peptide as a positive control in investigations examining the binding of insulin and/or insulinomimetics to their corresponding receptors, as well as in studies examining the molecular basis of the self-assembly of human insulin and its peptidic variants as dimers, hexamers and higher order multimers. The synthesis of $RB_{649\text{-}654}$ as the $4RB_{649\text{-}654}$ multimeric peptide was based on a dendritic Lys core, and was undertaken as part of these associated insulin-binding studies. As the preparation of $4RB_{649\text{-}654}$ in good yield and

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high purity was found to present a novel challenge in peptide synthesis methods, the results of these synthetic studies are reported here.

## MATERIALS AND METHODS

### Chemicals and Equipment

Peptide syntheses were performed in plastic columns with plastic sinters at their base for solvent removal under suction. Unless otherwise stated, solvents were of analytical grade. 4-Dimethylaminopyridine (DMAP) was obtained from Sigma Chem. Co., (St. Louis, USA). Acetic anhydride, dichloromethane (DCM), 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU), 1,3-diisopropylcarbodiimide (DIC), ethanedithiol, triethylamine (TEA), thioanisole and trifluoromethanesulfonic acid (TFMSA) were obtained from Aldrich Chemical Co. (Milwaukee, USA). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU), diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), 1-hydroxybenzotri-azole (HOBt), piperidine, trifluoroacetic acid (TFA), trinitrobenzene sulphonic acid (TNBSA), Wang-resin (0.9 mmol eq/g), Boc-Gly-PAM-resin (0.68 mmol eq/g), and the protected amino acids were all obtained from Auspep Pty. Ltd. (Melbourne, Australia).

### Peptide Synthesis

The peptides were synthesized using a combination of Fmoc- [3] and Boc-based [4] solid-phase techniques. Side-chain protecting groups for amino acids were as follows: Lys: Fmoc or Boc; Tyr: *t*Bu. Couplings were performed using HBTU/HOBt activation of the Fmoc- or Boc-amino acids in DMF. Completed peptide-resins were washed with dichloromethane (2 × 10 mL) and diethyl ether (2 × 10 mL) and dried under vacuum.

### Attachment of Fmoc-Gly-OH onto the Wang Resin

The Wang-resin (0.9 mmol eq/g) was reacted with Fmoc-Gly-OH (1 eq) and DMAP (0.5 eq) with either (a) HBTU/HOBt (1 eq) or (b) DIC (2 eq). The derived Fmoc-Gly-Wang-resin was used in subsequent syntheses (1 and 2) of $4RB_{649-654}$. Further details are given for each individual synthesis described below.

### Acetylation of Unreacted Amino Groups

At specified steps of the synthesis, remaining unreacted amino groups were blocked with acetic anhydride (500 μL)/triethylamine (100 μL) in DMF (5 mL) for 10 min before the capped peptide-resin was washed with DMF (3 × 5 mL) and then SPPS was resumed.

### TFA Cleavage Method

The dry peptide-resin (0.1–1.5 g) was placed in a round bottom flask and cooled on ice. A mixture of TFA (9.5 mL), 1,2-ethanedithiol (0.25 mL) and deionized $H_2O$ (0.25 mL) was prepared separately and cooled on ice for 10 min, before being added to the cooled peptide-resin. The mixture was stirred for 2 h at ambient temperature. The peptide/TFA cleavage mixture was filtered from the resin under vacuum. The resin was washed with TFA (2 × 3 mL), with the washings added to the filtered peptide/TFA solution. The filtrate was concentrated (reduced pressure) to about 2 mL and the crude peptide precipitated by the addition of chilled diethyl ether (30 mL). After storage overnight at 4°C, the diethyl ether was decanted, the crude peptide precipitate dissolved in 50% aqueous acetonitrile and lyophilized.

### TFMSA Cleavage Method

The dried peptide-resin was treated with thioanisole (1 mL) and ethanedithiol (0.5 mL) followed by TFA

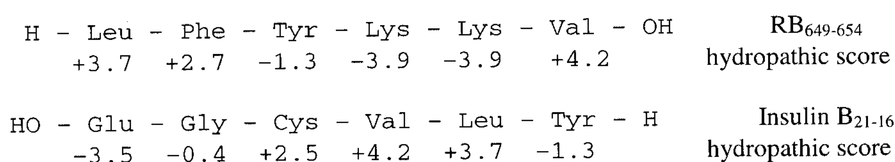


Figure 1 Antiparallel alignment of $RB_{649-654}$ and insulin B-chain$_{21-16}$ based on the hydropathic anti-complementarity theory.

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(10 mL) and the mixture stirred at ambient temperature for 5 min. The mixture was then cooled on ice and TFMSA (1 mL) added dropwise with stirring. The flask was sealed with a stopper and the mixture stirred at room temperature for 2 h. Ice-cold diethyl ether (50 mL) was added, the mixture stirred vigorously for 1 min and then filtered. The precipitated peptide was dissolved in TFA ($3 \times 3$ mL) and the solution concentrated under vacuum, followed by the addition of cold diethyl ether to precipitate the peptide. After storage at 4°C (overnight), the diethyl ether was removed by filtration, and the solid dissolved in 50% (v/v) water-acetonitrile before lyophilization.

### Peptide Purification and Analysis

The crude peptides products were analysed by RP-HPLC using gradient elution from 0 to 60% acetonitrile in water − 0.1% (v/v) TFA over 30 min with a TSK ODS-120T column ($150 \times 4.6$ mm ID). Chromatography was performed on a Waters Associates (Milford, MA) liquid chromatography system consisting of a model 600 solvent delivery pump, a Wisp model 712 sample processor and automated gradient controller with the eluant monitored at 214 nm. Electrospray mass spectra were acquired with a Perkin Elmer-SciEx mass spectrometer model PE Sciex API, with a scan range between 100–2400 amu. Amino acid composition was determined by hydrolysis of the peptide sample under vacuum in constant boiling 6N HCl for 22 h at 110°C. Samples were then analysed using the PICOTAG method [5].

### Synthesis 1 of $4RB_{649\text{-}654}$

The Wang resin was employed in this synthesis approach for the generation of $4RB_{649\text{-}654}$. Fmoc-Gly-OH (1 eq) was attached to swollen Wang-resin (0.27 mmol) by treatment with DMAP/HBTU/HOBt for 1 h in circa 60% yield. Unreacted sites on the resin were acetylated as described above. The Fmoc-Gly-Wang-resin was deprotected, followed by the addition of Fmoc-Lys(Fmoc)-OH. The deprotection of the resultant resin-bound dipeptide, Fmoc-Lys(Fmoc)-Gly-Wang-resin, was performed by $2 \times 10$ min treatment with 20% (v/v) piperidine in DMF. Further coupling of Fmoc-Lys(Fmoc)-OH to produce the Lys dendrite core with 4 free amino groups, was followed by the elongation of the peptide using an optimized HBTU/HOBt coupling method [6] with Fmoc-amino acids (Figure 2). The completed resin-bound peptide was cleaved by the TFA method. The crude products were analysed and purified by RP-HPLC techniques, with characterization of the major product recovered in purified yield of circa 40% subsequently achieved by ESI-MS. *Analytical RP-HPLC*: Retention Time (R.T.) of major peak: 16.8 min; *ESI-MS*: molecular mass: 4258 [calculated for $(H\text{-}LFYKKVGGG)_4\text{-}(K)_2\text{-}KG\text{-}OH$]; 967.6 (found).

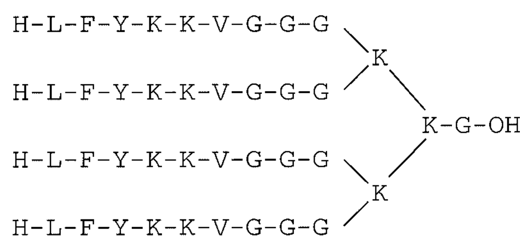


Figure 2 Schematic representation of the dendritic structure of the $4RB_{649\text{-}654}$ peptide prepared by the Fmoc- and Boc-based SPPS techniques.

### Synthesis 2 of $4RB_{649\text{-}654}$

The methodology for the second approach to the synthesis of $4RB_{649\text{-}654}$ followed that as described for *Synthesis 1*, except that Fmoc-Gly-OH was initially attached to free Wang-resin using DIC/DMAP (double coupling: 3 h/coupling) in a yield of circa 45% before continuation of SPPS. Deprotection of the dipeptide-resin, Fmoc-Lys(Fmoc)-Gly-Wang-resin, was also modified, with resin treated with 50% (v/v) piperidine/DMF for 5 min. Further synthesis proceeded using HOBt/HBTU (2eq.) with manual Fmoc methods. The TNBSA method [7] was used to monitor the completion of the coupling reactions, with the completed peptide-resin cleaved using TFA. The resultant products were analysed and purified by RP-HPLC. The two major products recovered in purified yields of ca. 10 % respectively were subsequently characterized by ESI-MS. *Analytical RP-HPLC*: R.T. of major peaks: 17.1, 18.9 min; *ESI-MS*: molecular mass: 4258 [calculated for $(H\text{-}LFYKKVGGG)_4\text{-}(K)_2\text{-}KG\text{-}OH$]; 4092, 4196 (found).

### Synthesis 3 of $4RB_{649\text{-}654}$

In all subsequent approaches to the synthesis of $4RB_{649\text{-}654}$ the Boc-Gly-PAM-resin (0.15 mmol) was utilized. The Boc protecting group was removed with 100% TFA treatment for 5 min, followed by neutralization with 10% (v/v) DIEA in DMF. Subsequent SPPS continued using Fmoc techniques with the attachment of Fmoc-Lys(Fmoc)-OH; double coupling with HOBt/HBTU (2 eq). Further addition of the other Fmoc-amino acids proceeded using the

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same methods with N-terminal Fmoc-group deprotections carried out with 20% (v/v) piperidine/DMF ($2 \times 5$ min). All reactions were performed under nitrogen with regular mixing of the peptide-resin. All reactions were monitored by the TNBSA test. The peptide products were cleaved from the resin with TFMSA, and the collected product analysed by RP-HPLC with the two major products recovered in purified yields of circa 5%, respectively, subsequently analysed by ESI-MS. *Analytical RP-HPLC*: R.T. of the major peaks: 18.7, 19.8 min; *ESI-MS*: 4258 [calculated for (H-LFYKKVGGG)$_4$-(K)$_2$-KG-OH]; 4146.8 and 4159 (found).

### Synthesis 4 of 4RB$_{649\text{-}654}$

The Boc-Gly-PAM-resin (0.15 mmol) was deprotected with 100% TFA as described for *Synthesis 3*. The Fmoc-Lys(Fmoc)-OH ($\times 2$) and Fmoc-Gly-OH ($\times 3$) were attached by double coupling of two equivalents with HBTU/HOBt. Each coupling was left to proceed for 1.5 h duration, after which acetylation was performed to block any free amino groups. The remaining six residues, comprising the actual RB$_{649\text{-}654}$ sequence, were attached, again using double coupling of each Fmoc-amino acid. Each coupling was allowed to continue for 3.5 h before capping of unreacted amino groups, as described above. The peptide products were cleaved from the resin with TFMSA. The crude product was purified and analysed with RP-HPLC. The three major purified products in purified yields of ca. 5 % respectively were subsequently characterized by ESI-MS. *Analytical RP-HPLC*: R.T. of the major peaks: 16.9, 18.9, 19.7 min; *ESI-MS*: 4258 [calculated for (H-LFYKKVGGG)$_4$-(K)$_2$-KG-OH]; 967.6; 4196 and 4030.2 (found).

### Synthesis 5 of 4RB$_{649\text{-}654}$

Boc-Gly-PAM-resin (0.28 mmol) was deprotected with TFA and neutralized before the addition of Boc-Lys(Boc)-OH (0.5 eq) with HBTU/HOBt (2 eq) for 1 h. Unreacted amino groups were blocked by acetylation, as described above. The synthesis continued based on Boc methodologies with the addition of Boc-Lys(Boc)-OH and Boc-Gly-OH (the latter $\times 3$). Each residue coupling involved 2 molar equivalents of the growing chain, with double couplings performed as standard before capping of any unreacted sites. A small sample of Boc-(GGG)$_4$-K$_2$-K-PAM-resin was taken for cleavage by TFMSA; with analysis of the product by RP-HPLC. The Boc-(GGG)$_4$-K$_2$-KG-PAM-resin was used to subsequently produce the complete 4RB$_{649\text{-}654}$ peptide product. Addition of the final 6 residues proceeded using Fmoc techniques with double couplings of each amino acid (3 eq) for 3.5 h each. The Fmoc-deprotection strategy involved treatment of the protected peptide-resin with 2% DBU/2% piperidine/DMF (5 min), followed by another 5 min treatment with 20% (v/v) piperidine in DMF. The peptide products were cleaved from the resin with TFMSA and crude products purified and analysed by RP-HPLC. The identity of the peptide products were confirmed by ESI-MS and amino acid analysis. *Analytical RP-HPLC*: R.T. of major peak: 19.6 min; *ESI-MS*: 4258 [calculated for (H-LFYKKVGGG)$_4$-(K)$_2$-KG-OH]; 4258.8 (found). *Amino acid analysis*: Tyr 3.55 (4); Val 3.8 (4); Leu 4.28 (4); Phe 4.2 (4); Lys 10.4 (11); Gly 13.1 (13). Yield after final RP-HPLC purification step: 11 mg, (10%).

## RESULTS AND DISCUSSION

In the initial description and characterization of the insulin binding properties of 4RB$_{649\text{-}654}$, the synthesis of (H-LFYKKVGGG)$_4$-(K)$_2$-KG-H, was achieved in very low yield by standard SPPS methodologies [1]. In an attempt to repeat this reported synthesis, a number of difficulties were encountered. Owing to their macromolecular structure and complexity, unambiguous synthesis of branched peptides, such as multiple antigen peptides (MAPs) often represent a challenging undertaking. A typical MAP comprises a central core component defining the branched architecture, which is employed to permit introduction of the multimerized dendritic peptide, giving them a cascade or pennant type of arrangement. In the cascade type [8] of MAP, the core matrix usually contains two or three levels of geometrically-branched Lys, whilst for the pendant type [9], a template-assembled peptide structure is typically employed.

The preparation of multiple antigen peptides can be accomplished by either (i) direct methods of stepwise solid-phase peptide synthesis, or (ii) indirectly, by protected/unprotected segment condensation methods [10]. In this study, the preparation of 4RB$_{649\text{-}654}$ was attempted using stepwise SPPS procedures, starting with the *C*-terminus core matrix using protected Lys residues to attain the desired branched level. The selected peptide multimer was then sequentially elongated onto the resin-bound Lys-core matrix to create the desired sequence. Ultimately, however, a number of key protocol

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modifications were required to achieve the desired molecule in acceptable yield. The steps progressively undertaken for this synthesis are listed in Table 1. Initially, the synthesis of $4RB_{649\text{-}654}$ was attempted using standard Fmoc-based SPPS protocols [11] with the Wang-resin (*Synthesis 1*). Upon TFA cleavage and analysis with RP-HPLC, the single product, however, was found to have a molecular weight of 967.6, corresponding to the linear sequence: H-LFYKKVGGG-OH, obtained in a purified yield of 20%, based on the initial resin-bound Fmoc-Gly-OH loading yield (Figure 3, Panel 1). The failed synthesis was attributed to base-catalysed 2,5-diketopiperazine formation, a common side reaction in Fmoc-Xaa-Gly-resin deprotection strategies [12]. The formation of the 2,5-diketopiperazines are particularly evident when hydroxymethylbenzyl resins are employed, as their use is prone to lead to substantial amounts of 2,5-diketopiperazine during the deprotection of the second amino acid in the sequence. Following deprotection, the free amino group can then undergo nucleophilic attack of the ester linkage of the dipeptide-resin itself, resulting in the release of the dipeptide from the resin as the cyclic 2,5-diketo-piperazine. This cyclization can be catalysed by either acids or bases used in SPPS deprotection. Moreover, the intramolecular aminolysis has been shown to be sequence dependent as the presence of Gly, Pro, D- or *N*-methyl amino acids in the dipeptide accelerates 2,5-diketopiperazine formation [12]. In the present case, the cyclization event appears to have occurred as a consequence of the extended exposure ($2 \times 10$ min) to the basic conditions of the deprotection step after the branched Lys core had been assembled.

For a susceptible sequence, such as that examined in the present study, the use of 50% (v/v) piperidine/DMF for 5 min as the deprotection step is recommended to minimize 2,5-diketopiperazine formation. In the next synthesis (*Synthesis 2*), the Fmoc-Lys(Fmoc)-Gly-Wang-resin was deprotected under these conditions. In this case, the Fmoc-Gly-Wang-resin was prepared using DMAP/DIC in place of DMAP/HBTU/HOBt in lower yield, indicating that these latter coupling conditions were more efficient and possible generates a Fmoc-Gly-Wang-resin with different distribution of Fmoc-Gly residues. However, following completion of the synthesis, a significant amount (approx. 15% yield based on the resin-bound Fmoc-Gly-OH content) of the linear peptide H-LFYKKVGGG-OH was still obtained when 50% (v/v) piperidine/DMF for 5 min was used as the deprotection step. Encouragingly though, two peptide products with molecular weights of 4092 and 4196 as determined by ESI-MS were present in circa 20% abundance in the crude mixture, and could be obtained as later eluting components by RP-HPLC procedures. Although these products represented deletion peptides incorporating the tetradentate core, the results nevertheless showed that retardation of 2,5-diketopiperazine formation could be achieved.

The main consequence of the formation of 2,5-diketopiperazines is a decrease in the overall yield of the dendritic peptide. Moreover, the results indicate that the hydroxyl sites formed on the polymer

Table 1 Outcomes Associated with the Synthesis of $4RB_{649\text{-}654}$ Achieved Using Several Different Modifications to the Synthetic Protocol

| Synthesis no. | Method details | Outcome |
|---|---|---|
| 1 | Fmoc-SPPS on Wang resin, with single couplings | Diketopiperazine formation, linear peptide recovered in 5% yield, no $4RB_{649\text{-}654}$ isolated |
| 2 | As per synthesis 1, with reduced exposure of Fmoc-Lys(Fmoc)-Gly-resin to piperidine | Reduced diketopiperazine formation, but still poor product mixture. No $4RB_{649\text{-}654}$ isolated |
| 3 | Fmoc-SPPS on PAM resin single couplings | No interfering diketopiperazine formation, but an extreme heterogeneous mixture of products, possible Val/Leu deletion; no $4RB_{649\text{-}654}$ isolated |
| 4 | Fmoc-SPPS on PAM resin; double couplings ; amino-group acetylation capping | Less heterogeneity of recovered products, no $4RB_{649\text{-}654}$ isolated; 4-Gly deletion peptide generated |
| 5 | Boc-SPPS of tetradentate core on PAM resin, followed by Fmoc-SPPS with DBU deprotection scheme | Major product of $4RB_{649\text{-}654}$; isolated yield of 10%. |

This Table summarizes the various methods and outcomes of each synthesis.

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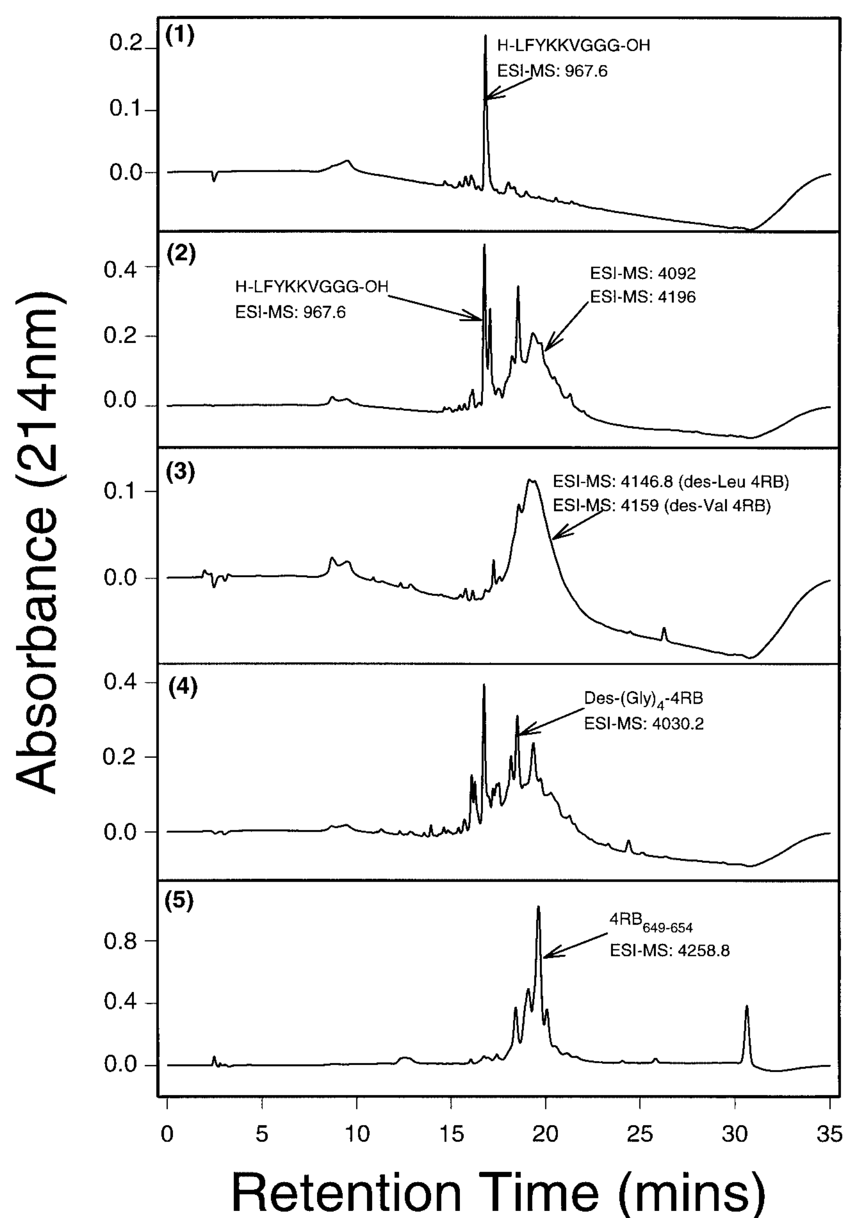


Figure 3 Analytical RP-HPLC chromatograms of the crude products obtained after cleavage of the synthetic peptides from the resin, as derived from the various procedures used for the synthesis of $4RB_{649\text{-}654}$. Panels (1)–(5) follow the different conditions outlined in Table 1. The RP-HPLC conditions are given in the 'Materials and Methods' section.

following release of the 2,5-diketopiperazine from the resin may be acylated by the incoming N-protected amino acid in the subsequent coupling reactions in the presence of DMAP, and this leads to the initiation of synthesis of a truncated peptide [13]. The isolation of the linear peptide H-LFYKKVGGG-OH was indicative of such re-attachment by one of the subsequent Fmoc-Xaa's onto the free resin. In fact, during these syntheses, it was the next but one, protected amino acid that re-attached to the resin starting a new peptide chain. Although the coupling of Fmoc-Lys(Fmoc)-OH onto the resin proceeded efficiently, re-attachment of Fmoc-Lys(Fmoc)-OH to the free sites on the Wang-resin appears to be too difficult, as no branching was detected in the peptide products. Although the modified deprotection strategy in *Synthesis 2* resulted in a reduction in 2,5-diketopiperazine formation, and consequently, a reduction in the amount of the linear peptide formed, there was still an

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unacceptable reduction in the yield of the dendritic peptide, i.e. $< 5\%$. In order to increase the yield, further attempts at the synthesis of $4RB_{649\text{-}654}$ utilized Boc-Gly-PAM-resin as the solid support, which was anticipated to be more resistant to diketopiperazine formation than Wang-resin. In this case, the cyclization to the diketopiperazine is expected to be less favoured owing to the differences in the nucleophilicity of the ammonium species arising from TFA deprotection compared to the amino-group that is generated under the 50% (v/v) piperidine/DMF deprotection conditions. After initial removal of the N-terminal Boc group with TFA, single couplings of Fmoc-amino acids/HOBt/HBTU to the Gly-PAM resin were then used throughout the synthesis (*Synthesis 3*). All reactions were performed under nitrogen with regular mixing of peptide-resin throughout. Following completion of the total synthesis, the peptide-resin was cleaved with TFMSA. Under these conditions, a broad range of peptide products was detected (Figure 3, Panel 3) using RP-HPLC analysis. Two products corresponding to a Leu deletion product (molecular weight of 4146.8) and a Val deletion product (molecular weight: 4159) of the parent molecule, $4RB_{649\text{-}654}$, were identified. This synthesis highlighted the problem of coupling inefficiencies when producing MAP structures by direct SPPS methods. Although, technically, there is not much difference in the synthetic steps required for the preparation of a linear peptide or a clustered peptide such as a MAP by SPPS, side products that accumulate during the assembly stages are amplified. A single inefficient coupling may give rise to deletion sequence lacking one, two, three or four copies of that residue. Because dendritic peptides are macromolecules, this increased microheterogeneity of the products leads to difficulties at the purification stages and lower yields [8].

To overcome these coupling inefficiencies and to reduce the formation of deletion peptides, the next synthesis (*Synthesis 4*) was performed using routine double couplings and acetylation or 'capping' after attachment of each amino acid residue. Despite a significant reduction in the number of byproducts, the desired product was still not identified amongst the main products analysed by RP-HPLC (Figure 3, Panel 4). The major characterized component corresponded to a $4RB_{649\text{-}654}$ deletion product with a molecular mass of 4030. The generation of this $4RB_{649\text{-}654}$ deletion product, lacking one Gly residue from each of the four peptide chains, during the synthesis occurred despite inclusion of capping steps and suggested a deprotection constraint for the growing chains using piperidine. Moreover, this problem was associated with the attachment of simple Gly residues to the tetradentate core.

Two strategies are accessible to overcome Fmoc-deprotection efficiencies. First, it was thought that using a Boc-based strategy for the construction of the tetradentate core would be feasible. Boc-deprotection using 100% TFA is generally very effective and with no side-chain protection in the final core product would present no complications later in the synthesis. However, the Fmoc strategy was retained for the assembly of the N-terminal hexapeptide sequence as side-chain deprotection would be simpler, and the preparation of the linear sequence had proven trivial in separate syntheses. To ensure complete deprotection of the Fmoc groups, the hindered base DBU has been successfully used in previous studies [14] on the synthesis of a number of 'difficult sequences'. Replacement of the standard piperidine-DMF (1:4 v/v) mixture with 2% DBU/2% piperidine/DMF was found to enhance the effectiveness of removal of the $N^{\alpha}$-Fmoc protecting groups. These results can be rationalized in terms of the greater basicity of DBU [14–16] and the increased DMF concentration, which would better solvate the support and allow for a more facile reaction.

At the fifth attempt, (*Synthesis 5*) our protocol modification combined both Boc- and Fmoc-based strategies. The tetradentate core and subsequent spacer Gly residues were attached onto Boc-Gly-PAM resin using Boc chemistry. A resin sample cleaved at this stage showed the desired intermediate in good yield. The final six residues, which encompassed the actual $RB_{649\text{-}654}$ sequence, were then attached using Fmoc chemistry with deprotection of the Fmoc groups performed by treatment of the peptide-resin with 2% DBU/2% piperidine/DMF for 5 min, followed by a 5 min treatment with 20% (v/v) piperidine/DMF. All residues were once again routinely double coupled and the peptide chains acetylated after each residue coupling; peptide-resin was regularly mixed under nitrogen. This strategy proved successful with the identification of desired product with the expected molecular weight of 4258 for $4RB_{649\text{-}654}$ in the crude cleaved mixture of products (Figure 3, Panel 5). After purification by RP-HPLC, $4RB_{649\text{-}654}$ was obtained as a homogeneous product in a final purified yield of approximately 10 % (based on the anticipated theoretical yield for the resin-bound peptide) with the composition confirmed by amino acid analysis.

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In summary, the synthesis of $4RB_{649\text{-}654}$ has been achieved through a combination of Fmoc- and Boc-based SPPS with the added inclusion of a stronger Fmoc deprotection agent whilst the distal amino acids were being attached. The use of this reagent was, in particular, more valuable during the latter stages of the synthesis, avoiding an increase in deletion products due to the comparative increase in aggregative effects as a consequence of the growing size of the multiple peptide chains.

## CONCLUSIONS

In this paper, the synthesis of a dendritic version of the retinoblastoma protein [649-654] sequence ($4RB_{649\text{-}654}$) is described. Similar dendritic peptides, as multiple antigen peptides, have been utilized extensively in immunological-based applications. Their synthesis however remains challenging with specialized chemistries being developed to facilitate their production [9]. In the case of the present investigation, $4RB_{649\text{-}654}$ could not be prepared by standard Fmoc-based SPPS techniques. By incorporating a number of modifications, including the construction of the tetradentate core using Boc-strategies, and also by optimization of the conditions of both the coupling and deprotection procedures, the target peptide was obtained as a homogeneous product in good yield.

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## REFERENCES

1. Radulescu RT, Bellitti RM, Ruvo M, Cassani G, Fassina G. Binding of the LXCXE insulin motif to a hexapeptide derived from retinoblastoma protein. *Biochem. Biophys. Res. Commun.* 1995; **206**: 97–102.
2. Blalock JE, Smith EM. Hydropathic anti-complementarity of amino acids based on the genetic code. *Biochem. Biophys. Res. Commun.* 1984; **121**: 203–207.
3. Colombo R, Atherton E, Sheppard RC, Woolley V. 4-Chloromethylphenoxyacetyl polystyrene and polyamide supports for solid-phase peptide synthesis. *Int. J. Peptide Protein Res.* 1983; **21**: 118–126.
4. Merrifield RB. Solid phase peptide synthesis: I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963; **85**: 2149–2154.
5. Cohen SA, Strydom DJ. Amino acid analysis utilizing phenylisothiocyanate derivatives. *Anal. Biochem.* 1988; **174**: 1–16.
6. Keah HH, O'Bryan M, de Kretser D, Hearn MTW. Synthesis of peptides related to the sperm tail protein tpx-1, a member of the CRISP superfamily of proteins. *J. Peptide Res.* 2001; **57**: 1–10.
7. Hancock WS, Battersby JE. A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitro-benzene-sulphonic acid. *Anal. Biochem.* 1976; **71**: 260–264.
8. Tam JP. Recent advances in multiple antigen peptides. *J. Immunol. Meths.* 1996; **196**: 17–32.
9. Mutter M, Vuilleumier S. A chemical approach to protein design. Template assembled synthetic proteins (TASP). *Angew. Chem. Int. Ed. Engl.* 1989; **28**: 535–554.
10. Tam JP, Spetler JC. Chemoselective approaches to the preparation of peptide dendrimers and branched artificial proteins using unprotected peptides as building blocks. *Biomed. Peptide Protein Nucleic Acids* 1995; **1**: 123–132.
11. Fields GB, Noble RL. Solid phase synthesis utilizing 9-fluorenylmethoxy-carbonyl amino acids. *Int. J. Peptide Protein Res.* 1990; **35**: 161–214.
12. Fields GB. Solid phase peptide synthesis. *Methods Enzymol.* 1997; **289**: 105–110.
13. Chiva C, Vilaseca M, Giralt E, Albericio F. An HPLC-ESMS study on the solid-phase assembly of C-terminal proline peptides. *J. Peptide Sci.* 1999; **5**: 131–140.
14. Kates SA, Sole NA, Beyermann M, Barany G, Albericio F. Optimized preparation of deca(L-alanyl)-L-valinamide by 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis on polyethylene glycol-polystyrene (PEG-PS) graft supports, with 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) deprotection. *Peptide Res.* 1996; **9**: 106–113.
15. Wade JD, Bedford J, Sheppard RC, Tregear GW. DBU as an *N*-$\alpha$-deprotection reagent for the fluorenylmethyloxycarbonyl group in continuous flow solid-phase peptide synthesis. *Peptide Res.* 1991; **4**: 194–199.
16. Dettin M, Pegoraro S, Rovero P, Bicciato S, Bagno A, DiBello C. SPPS of difficult sequences. *J. Peptide Res.* 1997; **49**: 103–111.

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