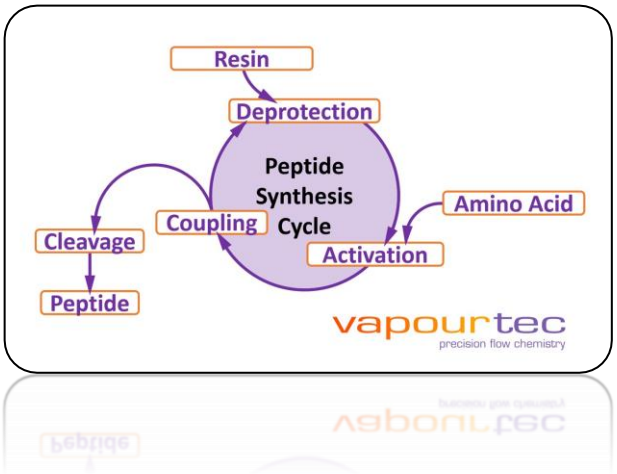

Continuous Flow-based Solid-phase Peptide Synthesiser

Introduction

As mainstream drug discovery is starting to shift from small molecules to peptide-based therapeutics, there is a growing demand for high efficiency peptide synthesis. Building on Merrifield's pioneering work, the Fmoc/tBu solid phase peptide synthesis (SPPS) has become the standard method of synthesising a range of peptides. Excitingly, this straightforward method opens the door for simple operation and fully automated synthesis methods.

Although a number of automated platforms exist for Fmoc/tBu SPPS, they often require complex set-up, microwave heating and/or significant excesses of reagents. Furthermore, existing microwave-assisted systems do not allow for in-line monitoring. Lastly, to the best of our knowledge, no system allows for direct observation of the physical properties of the resins during synthesis. The behaviour of resins during SPPS provides valuable information regarding the coupling efficiency and secondary structure of the growing peptide chain, which can be harnessed for subsequent optimisation work to give highly pure crude material.

As a result of a collaboration between Vapourtec and New Path Molecular Research, Vapourtec R-Series™ can now be used for highly efficient SPPS, where in-line UV detection, resin swelling and other reaction data can be directly monitored *via* the Vapourtec Flow Commander™ software. The Vapourtec SPPS system enables the automated synthesis of peptide sequences under highly controlled conditions, ensuring effective de-protection and coupling reactions that reduce cycle times and produce high purity peptides. Integrated autosampler technology and unique Vapourtec reactor capabilities combine to create a powerful system that can be applied to a wide range of diverse chemistries.

This white paper highlights the efficient synthesis of residues 65–74 of the acyl carrier protein (ACP, H_2N-VQAAIDYING-$CONH_2$), **1**. This peptide is well-known as a challenging sequence due to aggregation phenomena, and is commonly used to evaluate the performance of a synthesis platform. This example

illustrates the fast optimisation processes afforded by the Vapourtec system and straightforward scalability of the synthesis after small-scale optimisation.

Instrument & Reaction Setup

The continuous flow SPPS (CF-SPPS) is configured using a combination of two R2C+ pump modules and one R4 reactor module, as shown by **Fig. 1** & **2**. A Vapourtec Dual-Core™ PFA tube reactor and a column reactor are used for synthesis which can be heated using the R4 unit. This design allows for separation of activation and chain elongation steps. The inline UV detector is placed directly after the outflow of the column reactor and before a dynamic back pressure regulator (BPR), and the exit from the BPR is connected directly to the collection valve.

Figure 1 – The CF-SPPS setup, consisting of two R2 pump modules, one R4 reactor module, autosampler and in-line UV detector.

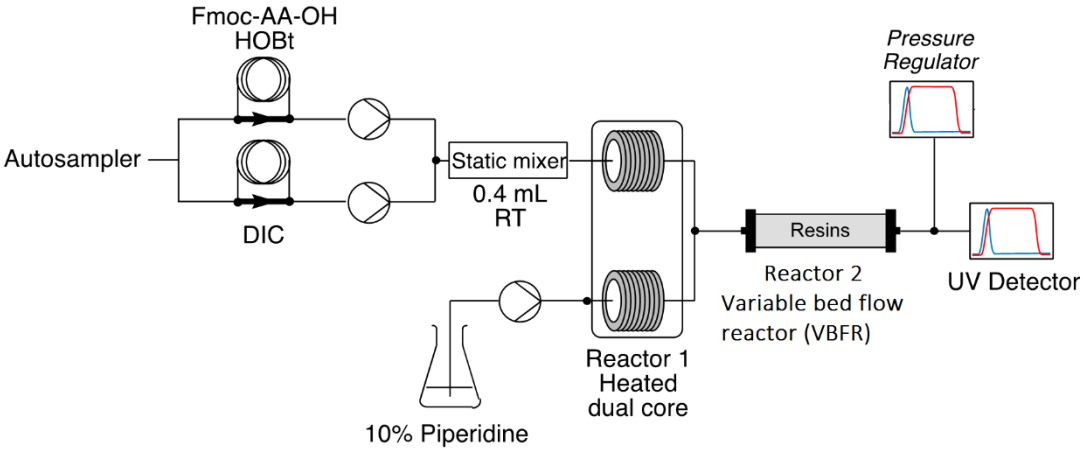

Figure 2: Schematic diagram of the CF-SPPS

The system can be used for both Boc or Fmoc chemistries although this application only describes a Fmoc/tBu approach. Peptide synthesis begins with the removal of Fmoc protecting group by 10% piperidine in DMF (Step 1, **Fig. 3**). This reagent is pumped directly to the Dual-Core™ tube reactor for pre-heating and passes through the resins in the heated column reactor. Subsequent coupling is accomplished by DIC/HOBt chemistry; however, the system flexibility allows for use of any common or novel combination of activating reagents. After removal of the Fmoc group and an exhaustive wash, the Fmoc-protected amino acid pre-mixed with HOBt is loaded automatically to an injection loop while DIC solution is loaded to a separate loop both *via* an autosampler. The loops then inject these reagents into the system which results in the activation of the amino acid in the Dual-Core™ tube reactor (Step 2) which is then passed through the resins in the column reactor for coupling and peptide chain elongation (Step 3). This sequence of deprotection and activation-coupling is then repeated until the peptide chain is completed.

Because both deprotection and activation/coupling steps are performed at elevated temperatures, both these steps are significantly accelerated compared to peptide synthesis at ambient temperatures. Also, since the resin is contained in a fully packed fixed-bed reactor, the washing can be performed very efficiently using the minimum of wash solvent. The combination of elevated temperatures and fixed-bed flow reactors significantly speed up the chain elongation process. For example, in a typical peptide synthesis experiment, a deprotection-activation/coupling cycle can be performed in under 15 minutes.

At each step, the reagents exiting the reactors are monitored by a UV-detector. This means that in the deprotection step the amount of cleaved Fmoc moiety (dibenzufulven or its piperidine adduct) can be monitored in up to four different wavelengths. The area under the curve can then be correlated to the loading of the resin and is a measure of the success of the previous coupling step. In the coupling step, however, the excess amino acid and coupling

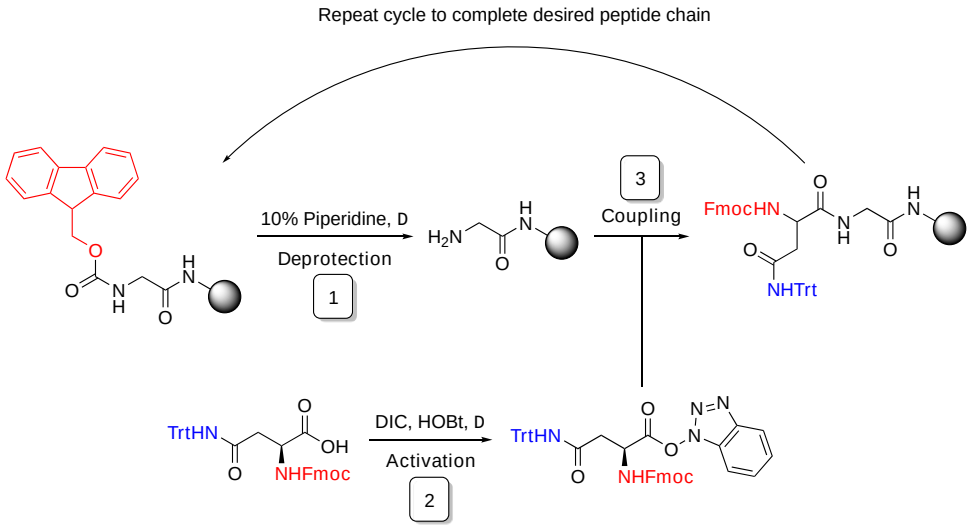

Figure 3 – Brief overview of the chemical steps in solid-phase peptide synthesis (SPPS).

reagents exit the reactor and are monitored by the detector, this in turn indirectly can be a measure for the success of the coupling reaction.

Separately to the inline UV detection, the Vapourtec variable bed flow reactor (VBFR) reactor detects and measures change in the volume occupied by the growing peptide chain. **Fig. 5** is an example of the SPPS measurement during an ACP synthesis. Each increase in volume detected corresponds to a coupling event, indicating growth of the peptide. The Vapourtec software constantly measures and records these changes in the reactor volume against time. Experimentally, these changes can be correlated to the chemistry that is happening on the surface of the resin and are a good indicator of the reaction success. For example, as the terminal residue on the polymer is deprotected, the volume of the reactor decreases. As the new amino acid is added to the resin, the volume starts to increase again till it gets to a plateau which indirectly indicates the completion of the coupling reaction. More importantly, if aggregation happens during the chain elongation, an instant decrease in the volume of the rector is detected. This is a very good indication that the subsequent couplings will be challenging and the problematic step can be remedied, for example by double-coupling of the amino acid or change of the solvent to a better solvating mixture.

Another advantage of the current configuration of the system is that the reactors for activation and coupling are fully independent of each other. As a result, each step can be performed in a different temperature. This can be of significant importance in suppression of side reactions, where for example the activation can be performed at a lower temperature to avoid some by-product formation but the coupling can be pushed to quick completion by increasing the temperature at this stage.

We therefore set out to demonstrate the usefulness of our current platform in the synthesis of ACP, a peptide sequence known for its difficult solid-phase synthesis due to collapsing and aggregation of the peptide chain during the process.

The synthesis starts with packing the flow reactor with Rink amide resin preloaded with the C-terminal Gly residue. The synthesis was then carried on with alternative deprotection and coupling steps as explained above. The outcome of each step was monitored by UV and the volume change of the variable bed reactor (VBFR) was plotted against time.

As observed below in **Fig. 4**, the VBFR expanded during the coupling reaction indicating peptide chain elongation; as each amino acid is added to the chain, the volume of the reactor increases. This expansion is most significant at the start of the synthesis as the effect of each added amino acid on the solvation of the polymer is more significant when the peptide chain is shorter. In the case of the ACP synthesis, the reactor volume eventually plateaus with increasing number of amino acid residues. However, a sudden reduction in reactor volume during Val coupling is observed, which potentially suggests peptide aggregation; this can be indicative of a lower coupling efficiency for the last amino acid. These data can be used in conjunction with the UV absorbance of the

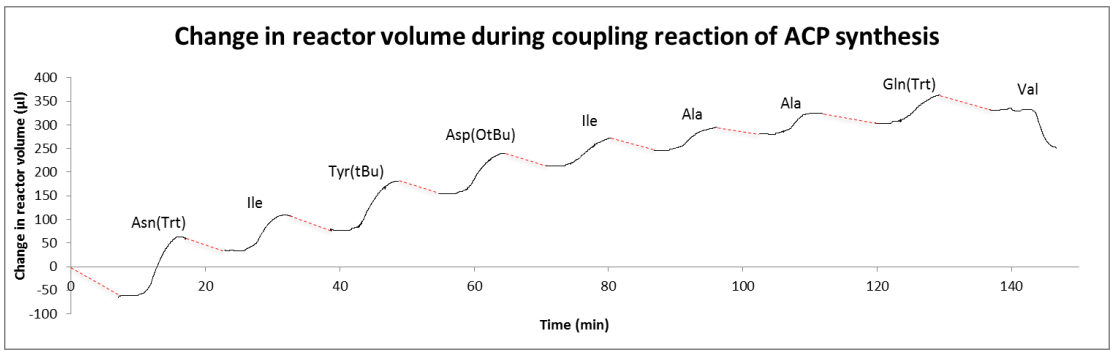

Figure 4 – Variable bed reactor (VBFR) data (black solid line) showing resin swelling during coupling reactions of ACP. The VBFR data during Fmoc-deprotection and washing steps are omitted (shown as red dotted lines). The reactor volume reduced significantly during Val coupling, suggesting potential peptide chain aggregation and lowering coupling efficiency.

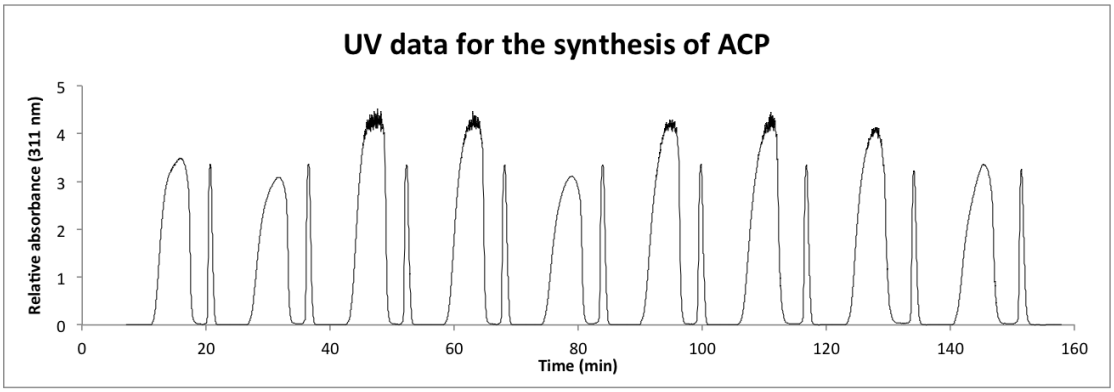

Figure 5 – UV data for synthesis of ACP.

cleaved Fmoc group during deprotection reactions **(Fig. 5)** to get a better understanding of reaction progress. As mentioned previously, the area under the UV trace during Fmoc group removal (deprotection) gives a semi-quantitative overview of the previous coupling's efficiency.

Results & Discussion

Modern peptide synthesizers are incredibly efficient at running repetitive coupling-deprotection steps. They have proved very useful for synthesis of "standard" peptides where no so-called difficult sequence is present and no problematic side reaction is happening. The challenge arises however, when the use of these machines results in low purity peptides due to various unforeseen reactions. In those situations, it becomes very difficult to pinpoint the problematic step and change the synthesis in a way that it can overcome the challenging step. The identification of what went wrong will then become the bottleneck in synthesis of such peptides.

Using Vapourtec platform will be of significant help in such situations as "live" analytical data will provide the chemist with valuable information, especially when a difficult step is encountered in peptide synthesis. The current project, synthesis of ACP (65-74) is a good showcase to demonstrate the usefulness of data created by the Vapourtec VBFR.

Initially, ACP (65-74) **(1)** was quickly synthesised on 0.2g of Rink amide resin using the standard conditions (see the experimental section for details). From this initial attempt, UV and VBFR data was used to determine amino acid residues with poor coupling efficiency and when aggregation occurred (see **Fig. 4 & 5**).

As can be inferred from the reactor volume (**Fig. 4**) the aggregation happens during coupling of Valine 65 (N-terminal residue), which negatively impacts its coupling efficiency. The area under the curve for the Fmoc released from this Val residue is visibly smaller than its predecessors (**Fig. 5**). The resulted deletion of this valine residue is further confirmed by HPLC analysis (**Fig. 6**) that shows the overall ~85% purity of ACP accompanied by ~5% des-V by-products (Entry 1, Table 1).

This information was clearly of significant help, as it enabled us to identify the problematic step and focus our optimisation efforts only on that step. The simplest way to overcome incomplete coupling is to double-couple the challenging residue. However, double-coupling valine resulted in similar overall purity and des-V was still present in > 2% (Entry 2, Table 1). As expected, despite using 6 equivalent excess of reagents and elevated temperature (80 °C), the coupling of valine was still hampered by peptide aggregation, as suggested by the VBFR.

One approach to disrupting aggregation is the use of TentaGel resins. These are polystyrene based matrices with grafted polyethylene glycol (PEG) groups. As PEG has both hydrophobic and hydrophilic properties, it can help minimise the degree of aggregation during peptide elongation in SPPS. Through the use of TentaGel and double-coupling V, ACP was synthesised in ~90% purity with < 2% des-V by-product (data not shown). Pleasingly, the reactor volume data showed no sudden collapse, confirming that use of Tentagel can suppress intera-chain H-bonds and peptide aggregation. However, one major drawback for TentaGel resin is the cost, where each mmol/g substitution on Fmoc-Gly-TentaGel is roughly 10 times more expensive than Fmoc-Gly-Rink amide. Since we were aiming to develop a synthesis suitable for larger scale preparation of peptides we investigated the possibility of alternative solutions to this problem.

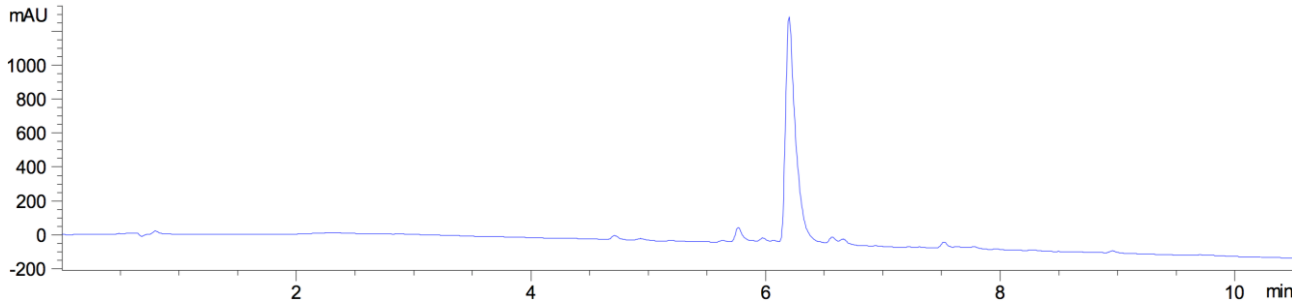

Figure 6 – HPLC trace of crude ACP (Entry 1, Table 1). Gradient = 5–40% B over 10 min; des-V by-product (rt ~5.8 min), ACP (rt ~6.2 min).

Table 1 – Optimisation process for ACP synthesis

Entry	Scale (mmol)	Double couple V	Solvent for V coupling	des-V by-product	Overall ACP purity
1 (TK147)	0.06–0.16	N	DMF	4.7	85.3
2 (TK144R)	0.06–0.16	Y	DMF	2.7	86.1
3 (TK148R)	0.06–0.16	N	50% DMSO in DMF	1.7	88.9
4 (TK149)	0.12–0.32	N	50% DMSO in DMF	2.3	88.1
5 (TK150)	0.30–0.80	N	50% DMSO in DMF	0.9	93.7

Another approach to prevent aggregation is to introduce elements that can disrupt hydrogen bonds in the environment in which the coupling reaction is performed. The addition of DMSO is known to disrupt such aggregation, and therefore the coupling solvent was switched for valine only from DMF to 50% DMSO in DMF (v/v). Remarkably, the VBFR measured an increase in column height during coupling, highlighting DMSO's effect on disrupting aggregation (**Fig. 7**). A comparison between **Fig. 4** and **Fig. 7**

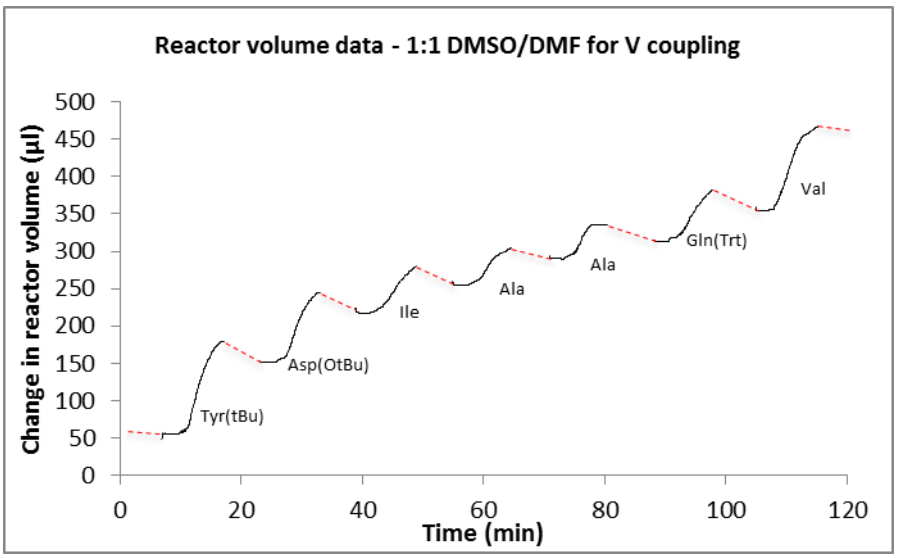

Figure 7 – Variable bed flow reactor (VBFR) data (black solid line) showing resin swelling for the last 7 coupling reactions of ACP. The VBFR data for the first two couplings, Fmoc-deprotection and washing steps are omitted (shown as red dotted lines). Using 50% DMSO/DMF (v/v) for Val coupling prevented the drop in column height and restored the characteristic swelling of the resins during coupling reaction

clearly shows the difference in resin's behaviour when DMSO was used in this step. The HPLC analysis also showed much lower level of des-V by-product (< 2%) and the overall purity of crude ACP was increased to nearly 90% without the need for double coupling (Entry 3, Table 1).

Scale up synthesis

Having found an efficient and economical way to supress des-valine formation, we were keen to evaluate the effectiveness of the platform in scaling up such challenging peptide sequences. To scale-up the synthesis of ACP peptide, conditions in Entry 3 of Table 1 were used. The flow parameters were simply extrapolated to accommodate scaled up synthesis on 0.4 g and 1.0 g Rink amide resin respectively.

The synthesis was followed using VBFR data which showed no collapse of resin during coupling of N-terminal valine and the reactor profile for both 0.4 and 1.0 g syntheses were similar to that of **Fig. 7**. Significantly, the purity of the crude peptide was not affected by the increase in scale, and ~94% purity was achieved for 1.0 g resin (0.3–0.8 mmol) scale as can be seen in the HPLC trace (**Fig. 8**). Only 0.9% des-valine impurity was detected in this scale (Entry 5, Table 1), presumably due to smaller error in a larger system.

Conclusion

We have developed a modular platform based on Vapourtec R-Series™ which can be used in continuous flow synthesis of peptides. The variable bed flow reactor (VBFR) that constantly monitors the physical characteristics of the resin and adjusts itself to maintain a true packed bed reactor proved to be a significant addition to simple continuous flow synthesis. The packed bed reactor, which can adapt to swelling and shrinking nature of the polymer, can improve reaction kinetics by suppressing channeling and a providing a uniform distribution of beads within the reactor. More importantly, the data acquired by VBFR can be used to detect problematic steps of the synthesis which in turn results in rapid optimisation of the process and access to purer peptides.

The incorporation of in-line UV detection can also be used to monitor the reaction in combination with VBFR data. The modular nature of the device makes it flexible so it can accommodate diverse reactors of various scales. In addition, by separating activation and coupling steps the versatility of the platform has significantly improved and allows for use of diverse chemistries which might not be available to current peptide synthesisers. Finally, using this reactor, rapid optimization and synthesis of difficult ACPv(65-74) peptide was achieved. The platform also

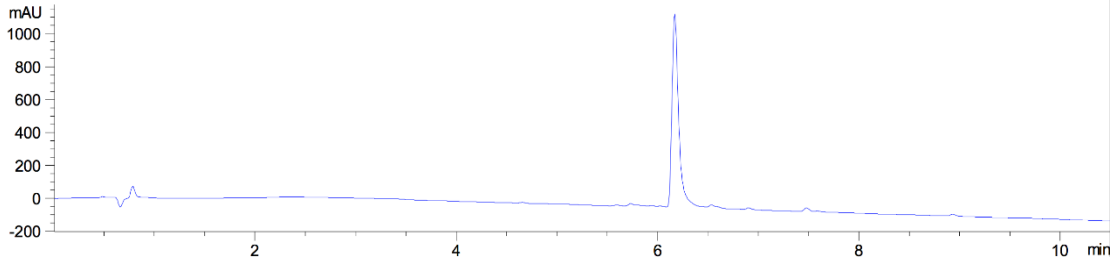

Figure 8 - HPLC trace of crude ACP (Entry 5, Table 1). Gradient = 5–40% B over 10 min. Purity = 93.7%.

demonstrated the potential for large-scale synthesis with relatively small footprint.

Experimental

Materials

All materials were obtained from commercial suppliers: Fluorochem (Fmoc-amino acids, 1-hydroxybenzotriazole hydrate (HOBt·H_2O), diisopropyl carbodiimide (DIC), triisopropylsilane (TIPS) and Fmoc-glycine Rink amide AM resin (0.3–0.8 mmol/g)), Sigma-Aldrich (1,2-ethanedithiol (EDT), piperidine, trifluoroacetic acid (TFA)), Fisher Scientific (DMF, DMSO, diethyl ether and acetonitrile).

N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) amino acids contained in the following side-chain protecting groups: Asn(Trt), Asp(OtBu), Gln(Trt), Tyr(tBu).

Method for CF-SPPS

<u>0.06–0.16 mmol scale (200 mg resin, 6.6 mm bore column reactor)</u>

Deprotection (80 °C)

- Pre-heating loop = 0.4 mL.
- 4 mL 10% piperidine in DMF @ 2.8 mL/min.
- 4 mL DMF @ 4 mL/min washing.

Coupling (80 °C)

- Fmoc amino acids and HOBt (0.24 M in DMF, 2 mL) in sample loop A.
- DIC (0.24 M in DMF, 2 mL) in sample loop B.
- Mixed in activation loop (0.8 mL). Final reagent conc. 0.12 M.
- 4 mL reagent vol. in DMF @ 0.7 mL/min.
- 8 mL DMF @ 4 mL/min washing.

<u>0.12–0.32 mmol scale (400 mg resin, 6.6 mm bore column reactor)</u>

Deprotection (80 °C)

- Pre-heating loop = 0.8 mL.
- 8 mL 10% piperidine in DMF @ 2.0 mL/min.
- 8 mL DMF @ 2 mL/min washing.

Coupling (80 °C)

- Fmoc amino acids and HOBt (0.24 M in DMF, 4 mL) in sample loop A.
- DIC (0.24 M in DMF, 4 mL) in sample loop B.
- Mixed in activation loop (1.6 mL). Final reagent conc. 0.12 M.
- 8 mL reagent vol. in DMF @ 1.4 mL/min.
- 16 mL DMF @ 2 mL/min washing.

<u>0.3–0.8 mmol scale (1 g resin, 15 mm bore column reactor)</u>

Deprotection (80 °C)

- Pre-heating loop: 2.0 mL.
- 20 mL 10% piperidine in DMF @ 10 mL/min.
- 20 mL DMF @ 10 mL/min washing.

Coupling (80 °C)

- Fmoc amino acids and HOBt (0.24 M in DMF, 10 mL) in sample loop A.
- DIC (0.24 M in DMF, 10 mL) in sample loop B.
- Mixed in activation loop (4 mL). Final reagent conc. 0.12 M.
- 20 mL reagent vol. in DMF @ 3.5 mL/min.
- 40 mL DMF @ 10 mL/min washing.

Final Cleavage and Analysis

After the synthesis of the peptide, the resins were washed with DMF (x3), DCM (x3) and dried down with MeOH (x3). The peptides were then cleaved by treatment with TFA:TIPS:EDT:H_2O (94:1:2.5:2.5, 10 mL/g resin) at room temperature for 1.5 hours. The resins were filtered off and the peptide isolated by precipitation with diethyl ether. The crude peptide was dried *in vacuo*, dissolved in 5:95 acetonitrile:H_2O and analysed by analytical HPLC (Agilent 1220).

XBridge® C18 2.5 μm column (4.6mm x 50mm, flow rate = 1.0 mL/min) was used for analysis. The following solvent system was used: solvent A, water containing 0.1% trifluoroacetic acid; solvent B, acetonitrile. The column was eluted using a linear gradient from 5–40% solvent B over 10 min. Mass analysis was carried out by ESI-MS (Advion expression CMS)

References

1. 1. R. B. Merrifield, *J. Am. Chem. Soc.,* 1963, 85 (14), 2149-2154