White Paper

A Holistic Approach to the Peptide Workflow in Drug Discovery

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Executive Summary

The study of peptides, especially for therapeutic use, has grown significantly in recent years. However, producing peptides for such research is challenging, not just in the synthesis. The molecules must be visualized to allow synthetic approaches to be designed, and after synthesis the purification and isolation of peptides is not straightforward. In order to address these challenges, Biotage has developed a holistic approach to the entire peptide workflow via an automated solution, designed for dedicated peptide researchers and those new to the field.

Introduction

Peptides are increasingly entering drug development pathways as potential active pharmaceutical ingredients due to their large chemical space, relative ease of synthesis, ready availability of low cost starting materials and general low toxicity. As a result, approximately 60 peptide-based drugs have been approved by the FDA, 140 peptidic drugs are currently under evaluation in clinical trials and a further 500 peptides are in development. Success stories include the prostate cancer drug Lupron™ from Abbott Laboratories, with global sales of more than US$2.3 billion in 2011. Understandably, research into the area of new peptide drugs is at an all-time high.[1]

Challenges in Synthesizing and Purifying Peptide Molecules

All peptides are manufactured by a step-wise addition of the correct amino acid to the peptide chain until the target molecule is achieved, through a series of deprotection and coupling steps. Amino acids may be naturally occurring or specific molecules created for such a purpose. Peptides may in fact be considered as polymers, but unlike most common plastics the monomers may each be different and must be placed in a particular part of the molecule to achieve the final peptide. The first degree of complexity in peptide synthesis is conceptualizing the peptide molecule and devising a suitable synthetic route by which it may be created.

This process may be very complex, as peptides may contain many tens of amino acids and some couplings may be difficult due to for example the growing hydrophobicity of the peptide chain. Despite the relative simplicity of most amino acids, peptide synthetic design is a complex and exacting process. An example of a complex peptide showing a cyclic domain is shown in Figure 1.

Once the molecule has been designed, the traditional method for synthesizing peptides involves complex solution-phase chemistry, and this methodology still finds favor in large scale synthesis. However, this approach is laborious because the peptide must be purified after each coupling step. A better approach to peptide synthesis on the laboratory scale is using a solid-phase approach where the peptide chains are grown from a solid resin, after first attaching the terminal amino acid. This approach has replaced the solution route in smaller scales, due to the ease in handling the growing peptide, for example washing a solid-supported peptide during synthesis is easily achieved through immersion and filtration. One of the biggest challenges in peptide synthesis is ensuring that the highest yield is achieved when adding an amino acid to the peptide chain, as low yields when propagated across the multiple coupling reactions results in a very poor final yield for the peptide, as illustrated in Figure 2.

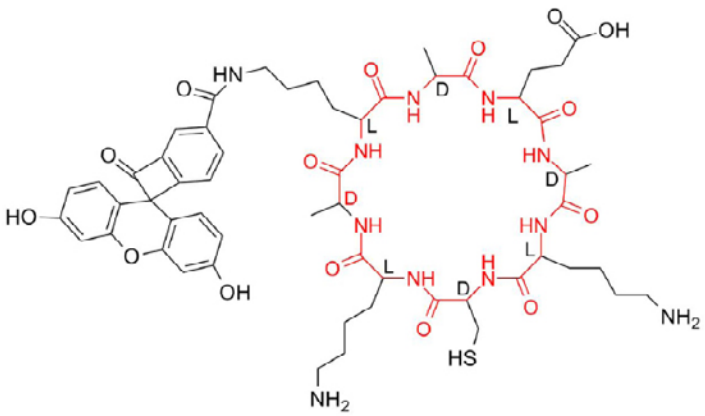

Figure 1. A peptide nanotube analogue as an example of a complex cyclic peptide.[2]

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Effect of Coupling Step Yields on Final Product Yield

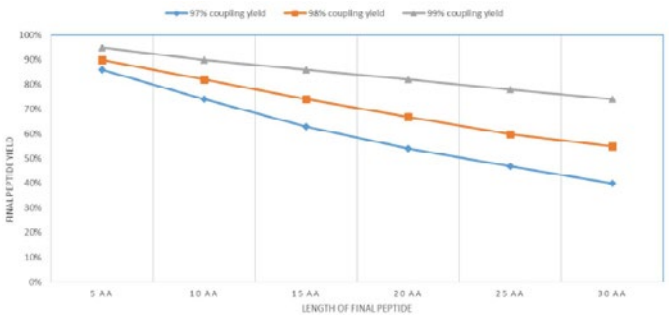

Figure 2. Effect of the yield of each coupling step on the final product yield as a function of final peptide sequence length.

Every effort must be made to ensure that each step of the synthesis is performed to the highest possible conversion in order to preserve a good yield of the final peptide at the end of the process.

The final challenge in the peptide workflow is to purify and isolate the peptide to the required specification prior to further testing. Peptide purity is typically assessed as the percentage of the target peptide to impurities that absorb at the peptide bond absorption wavelength (210–220 nm). The specification for target peptides depends very much on what they will be used for, as shown in Table 1.

Peptide Assessment	Typical Flash Purification
Quantitative studies, such as those involving NMR, monoclonal antibody production, in vivo studies, receptor-ligand binding studies, ELISA and RIA.	>95%
High-throughput screening work, non-quantitative enzyme-substrate studies, antibody affinity purification, non-quantitative blocking in immuno-histochemical (IHC) or Western blot analyses and plate coating for cell attachment.	>80%
ELISA standards, ELISPOT assays and polyclonal antibody production.	>70%

Table 1. Purity requirements for further assessment of peptides.

Producing peptides of the correct purity is a function of the synthetic route and also the purification method utilized to clean up the samples. Often in a peptide workflow the purification and isolation steps are the most time-consuming components of the entire process.

The Biotage Holistic Approach to the Peptide Workflow

Considering the workflow required to deliver a peptide suitable for further assay work, the steps involved concern conception of the target molecule, synthesis of that peptide, purification of the final product to the required specification and finally isolation. Biotage have developed a holistic approach to peptide drug discovery which targets each of these crucial steps in a single automated workflow approach:

1. Visualization software to help chemists understand approaches to synthesize conceptualized peptide molecules.
2. Synthesis of target peptide with an emphasis on the quality of the final product using ‘quality first’ and ‘right first time’ approaches.
3. Purification of the synthesized peptide to a level sufficient for further study using flash purification.
4. Isolation of the final peptide after purification.

Each of these considerations shall be discussed below, with examples illustrating the adoption of the Biotage workflow.

Conceptualizing Peptides and Designing Synthetic Routes

Conceptualizing peptides and designing suitable synthetic routes is a complex and demanding process, and many research projects are completely devoted to finding a successful pathway to synthesize a particular target peptide. Although mechanically simple, the order in which stepwise deprotection and coupling steps are combined is crucial to success, especially when one considers complex branched or cyclic peptides. Our approach to peptide design is illustrated by the Biotage® Initiator+ Alstra™ peptide synthesis software, which allows the chemist to fully visualize the peptide under construction. In the Biotage approach flexibility is key, peptide synthetic routes may be visualized and designed using standard amino acids, new amino acids and using industry standard deprotection and coupling chemistries or new chemistries. Importantly, the Biotage approach allows the visualization of complex peptides, allowing even very complex peptides to be broken down into key components. As example is shown in Figure 3 for the complex cyclic peptide shown in Figure 1.

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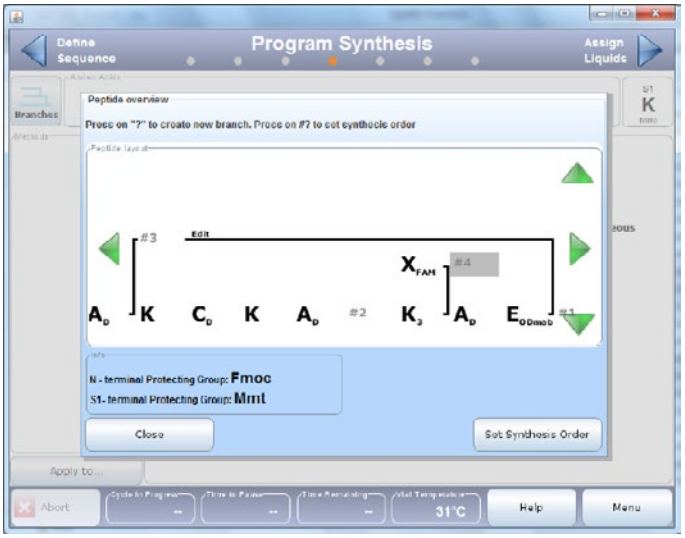

Figure 3. Biotage® Initiator+ Alstra™ software visualization of the synthetic route to a complex cyclic peptide (shown in Figure 1), showing the use of novel building blocks, cyclisation reactions and ordered synthetic approach.

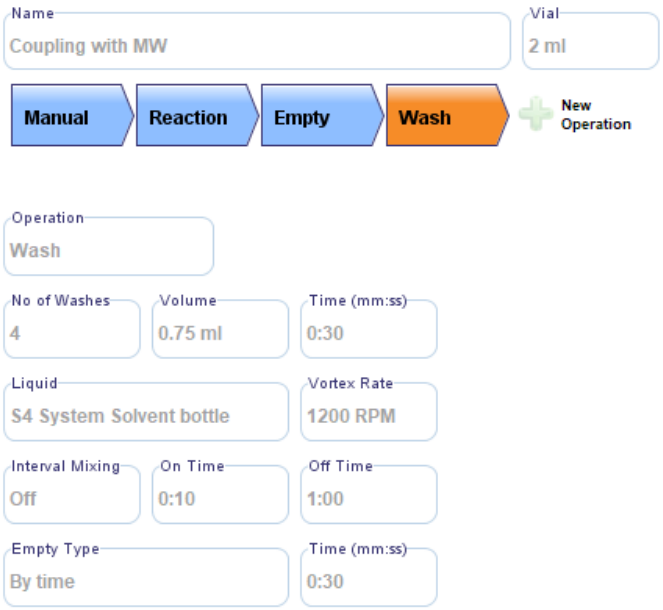

Figure 4. Examples of the steps that take place in a typical coupling operation, specifically the wash step, using the Biotage® Initiator+ Alstra™ software.

Target Peptide Synthesis with 'Quality First' and 'Right First Time' Approaches

Once the peptide has been conceptualized and the synthesis route designed, the chemical process can begin. The Biotage approach to peptide synthesis is based around 'quality first' – maximizing the quality of the final peptide. Care is taken over synthesis steps to ensure that the highest purity is obtained, with multiple washing steps to ensure quality is maintained. Often is a peptide the bottleneck to progress is final product purification, so taking time to ensure that the quality of the synthesis is maximized does not impact unduly on the overall workflow and is a worthwhile investment. The Biotage® Initiator+ Alstra™ automated peptide synthesis system exemplifies the quality-first approach. Figure 4 illustrates the steps that take place in a typical coupling operation, specifically the wash step. This may be altered as desired by the user, by as a default involves four washes of the solid-supported peptide to remove excess amino acids, reaction solvents and reagents. This attention to detail ensure that the best quality peptide is obtained from the synthesis.

As an example of the purities obtained for peptides using a quality-first approach, a known 'difficult' peptide, ACP 65-74 was synthesized overnight using the Initiator+ Alstra and the results are shown in Figure 5.

The quality-first approach to peptide synthesis ensures that the highest possible purity of product is obtained. It is possible to speed the synthesis, but this is typically either at the expense of washing steps resulting is a lower quality product, or by using very sequence-dependent chemistries. The Biotage 'right first time' approach is to ensure that sequence-dependent chemistries are minimized such that the final peptide is produced correctly the first time it is attempted, and no wasteful attempts are made using inappropriate techniques.

A further component of the quality first approach to peptide synthesis is the use of high concentration reagents such as the Fmoc-protected amino acids. This increases the yield and therefore coupling efficiency of peptide reactions, improving the quality of the peptide produced at the end of the synthesis. Too low a concentration, and final crude purities can be low, but too high a concentration and viscometric effects can hinder the chemistry. The Biotage® Initiator+ Alstra system employs digital syringe pumps to ensure that the accurate dispensing of even high concentration solutions is routine. The effect of reagent concentration on crude purity is shown in Table 2 for two peptides.

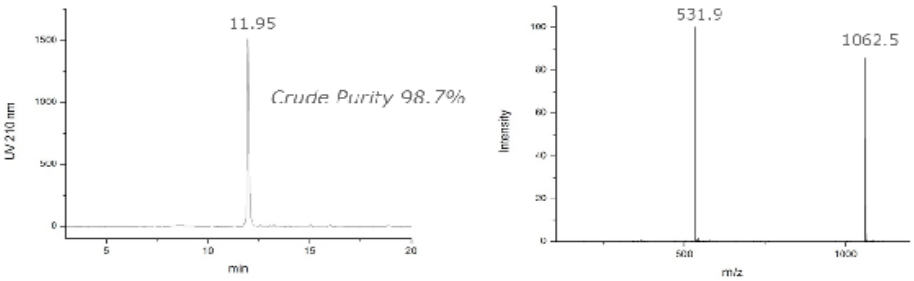

Figure 5. Reverse-phase HPLC (left) and mass spectrometry (right) after cleavage (crude product) of ACP 65-74, sequence: H-VQAAIDYING-NH2 (MW= 1062) synthesized on the Biotage® Initiator+ Alstra, scale: 0.1 mmol, resin: ChemMatrix Rink Amide, conditions: Single 5 min coupling (DIC/HOBt) at 75 °C.

Peptide: P E R V K V V F P L	
Concentration of Fmoc Amino Acid	**Crude Purity**
0.2M	88%
0.5M	92%

Peptide: I K P E A P G E D A S P E E L N R Y Y A S L R H Y L N L V T R Q R Y	
Concentration of Fmoc Amino Acid	**Crude Purity**
0.2M	61%
0.5M	74%

Table 2. The effect of reagent concentration on the crude purity to two peptide sequences.

Peptide Purification by Flash Chromatography

The traditional method of purifying peptides is by preparative reverse-phase HPLC. However, there are drawbacks to this technique – preparative HPLC is slow and has limited loading capacity, so for larger sample sizes multiple injection of material are required. Also, preparative HPLC systems and columns are expensive and often require specialist knowledge to be used correctly. In the Biotage approach, Flash purification is used. Flash systems are cheaper and easier to use than preparative HPLC systems, and specialist consumables for peptide separation are available with far higher loading capacities than preparative HPLC, allowing all of the peptide to be purified in one experiment. Biotage® SNAP Bio C18 and C4 flash purification columns have been designed specifically for the purification of peptides. Properties of the flash columns are shown in Table 3.

An example of the use of Flash in peptide purification is that of the antiparallel coiled coil peptide (44-mer) H-KRKKQKRKRKRAKQLRKRLQALEWQLAQIRKELQAAEKEEAQIE-NH2. After synthesis 150 mg sample of the crude peptide was dissolved in 2 mL 50% aq. Acetonitrile to give a 0.6% sample loading on a Biotage® SNAP Bio C18 300 Å 25 g cartridge. Figure 6 shows the Flash run of the peptide, with the product highlighted in green. Figure 7 shows the resulting analytical HPLC chromatogram of the product after flash purification, with the mass spectrometry data indicating purity.

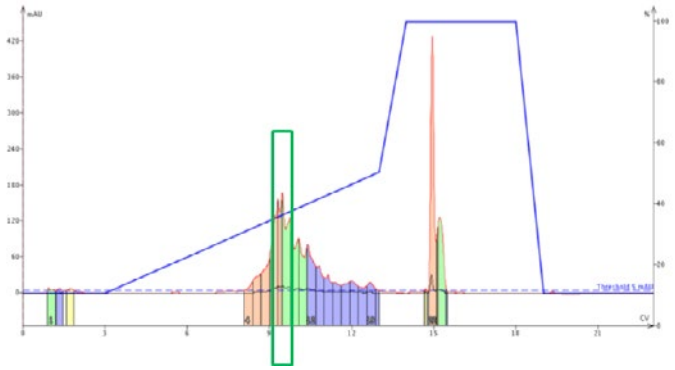

Figure 6. Flash run of the antiparallel coiled coil peptide (44-mer), acetonitrile/water gradient, with the peptide product highlighted in green.

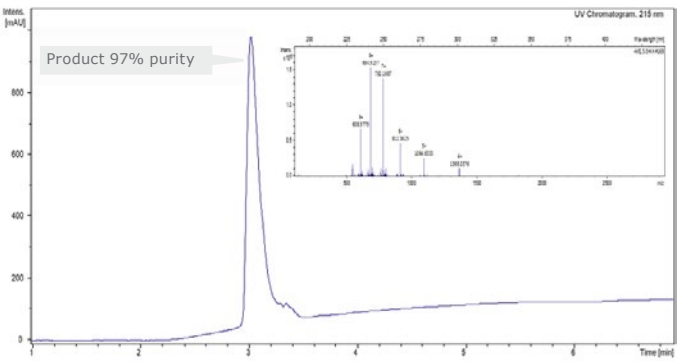

Figure 7. Resulting analytical HPLC chromatogram of the antiparallel coiled coil peptide (44-mer) after flash purification, with the mass spectrometry data indicating purity.

Cartridge	Silica	Particle Size (µm)	Pore Volume (mg/L)	Surface area m^2/g)	Pore Diameter (Å) Nominal
Biotage® SNAP Bio C18 300 Å	HP-Biosphere C18	20	0.95	150	300
Biotage® SNAP Bio C4 300 Å	HP-Biosphere C4	20	0.95	150	300

Table 3. Properties of Biotage® SNAP Bio C18 and C4 flash purification columns.

For the antiparallel coiled coil peptide (44-mer).
The performance benefit of Flash purification compared to a typical preparative HPLC purification are shown in Table 4, where 150 mg of the peptide was purified by both techniques.

Purification Technique	HPLC	HPFC
Column/Cartridge	Phenomenex Gemini® 5 µm NX-C18 110Å, 100 x 21.2 mm	Biotage® SNAP Bio C18, 25 g
Amount of Crude Peptide Purified	150 mg	150 mg
Number of Injections	4	1
Total Amount of Peptide Recovered	33 mg	30 mg
Total Solvent A (H_20)	1777 mL	1041 mL
Total Solvent B (CH_2CN)	1514 mL	477 mL
Total Time for Purification	160 min (40 min per run)	27 min

Table 4. Comparison of preparative HPLC and Flash purification techniques for the purifying 150 mg of the antiparallel coiled coil peptide (44-mer).

Clearly savings in time and in solvent usage can be achieved using Flash purification compared to a more traditional preparative HPLC approach. Most importantly multiple preparative HPLC purifications have been replaced by a single Flash purification using less solvent, resulting in a more concentrated peptide sample ready for isolation of the product.

Solvent	Volume (mL)	Method	Time (min)
NMP	12	Very High Boil	18
DMF	12	High Boil	7
DMSO	12	Very High Boil	15
Water	12	Aqueous	16
50% acetonitrile in Water	12	HPLC Fractions	15
20% Piperidine in DMF	8	Mixed Vol & HBP	7
TFA	5	Volatile	5

Table 5. Illustrative times to remove volumes of selected solvents, including typical Flash purification fraction solvents and peptide deprotection solutions.

Isolating Peptides

Once the peptide has been synthesized, the molecule must be cleaved from the resin. This achieved using a cleavage cocktail to break the bond between the peptide chain and the resin. After this step, the peptide is now in solution and must be isolated. Similarly, after purification of the final peptide, the peptide molecule must once again be isolated from the elution solvents, preferably as a solid.

Recovery of the final sample after purification is one of the most time consuming parts of any synthesis, especially if the sample is contained in a high boiling solvent which is difficult to remove, such as water. The traditional approach would be to use a rotary or centrifugal evaporator similar technology, which takes several hours to remove difficult samples and requires constant attention from the user. Alternatives such as freeze drying are also very time consuming and often require a critical number of samples before they can be employed.

Within the Biotage peptide workflow, evaporation is achieved using technologies specifically designed to allow the easy removal of high boiling solvents in very short timeframes, the Biotage® V-10 Touch evaporator. This approach makes use of three factors to drastically reduce the time taken to remove solvents:

1. Sample is spun in a vial to form a thin film of maximum surface area to aid evaporation and remove the possibility of sample 'bumping'
2. Heat is applied uniformly to the spinning sample
3. Vacuum is applied

All of these factors can be controlled independently to ensure the best evaporation conditions, resulting in extremely short evaporation times as shown below, especially in comparison to alternative techniques. Table 5 shows illustrative times to remove volumes of a selection of solvents, including typical flash purification fraction solvents and peptide deprotection solutions.

Automation is achieved through the use of a carousel to automatically load and unload samples, and a liquid handler to dispense solutions from one container to another, such as when concentrating down a large volume of sample into a small vial. An example of the removal of a cleavage cocktail (95% TFA; 2.5% TIS; 2.5% H_2O) from a peptide synthesis is shown in Figure 8.

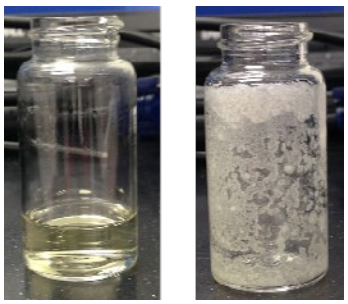

Figure 8. Removal of TFA cleavage cocktail from ACP synthesis, 5 mL (95% TFA; 2.5% TIS; 2.5% H_2O), approximate evaporation time 5 min at a temperature of 35 °C. First vial – peptide in cleavage cockatil, second vial – isolated peptide.

Summary

The preparation of peptide for research and development is increasing as new peptidic therapies become the focus of efforts in drug discovery. However, the challenges of manufacturing peptides begins with designing and visualizing the molecule, moves onto the synthesis itself and then in ways to purify and isolate the molecules. Biotage have adopted a 'quality first' and 'right first time' approach across all aspects of the peptide workflow, to ensure that the researcher produces the best quality peptides in the shortest time and with the highest confidence in success.

References

1. Peptide therapeutics: current status and future directions, Keld Fosgerau Torsten Hoffmann, Drug Discovery Today, volume 20, Issue 1, January 2015, Pages 122–128
2. Ghadiri el al. Nature, 1994, 364, 301–304

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