# A flow reactor process for the synthesis of peptides utilizing immobilized reagents, scavengers and catch and release protocols†

**Ian R. Baxendale, Steven V. Ley,* Christopher D. Smith and Geoffrey K. Tranmer**

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**A general flow process for the multi-step assembly of peptides has been developed and this procedure has been used to successfully construct a series of Boc, Cbz and Fmoc *N*-protected dipeptides in excellent yields and purities, including an extension of the method to enable the preparation of a tripeptide derivative.**

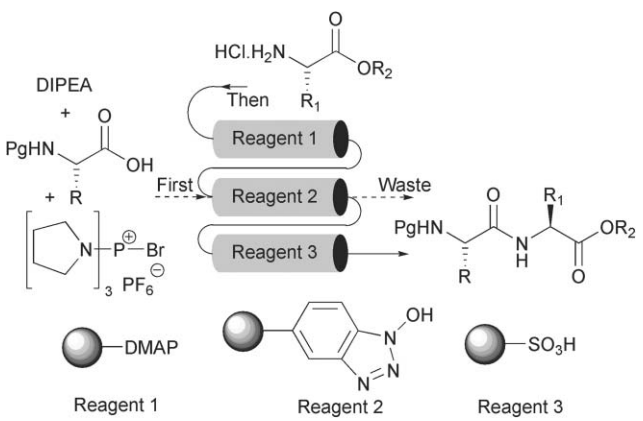
The preparation of peptides is largely considered to be a solved synthetic problem, yet one should remember that it took 25 years to develop reliable procedures for the formation of multiple amide bonds.[1] A significant proportion of the methodology developed has used solid-phase synthesis, as this is readily adapted to automated synthesiser systems.[2] Depending upon the length of the sequence, however, costs can escalate rapidly and scale up becomes an issue due to the linear nature of the processing. The inherent simplicity of the machinery also renders it hard to incorporate novel amino acids or adopt unusual chemical bond forming reactions, as required for peptidomimetics, in an automated fashion.[3]

Here we report an alternative method for the synthesis of di- and tripeptides using a simple flow process that should be extendable to give access to a variety of advanced amide products. The process is conducted by progressing *N*-protected amino acids through a microfluidic flow system that is comprised of various packed columns that contain supported reagents, scavengers and catalysts. The resulting peptide products can be collected upon elution from the flow system, and isolated in high purity by merely removing the solvent, thereby bypassing costly and time consuming purification procedures.

Previously we have developed continuous flow processes for the synthesis of the natural products grossamide[4] and oxomaritidine.[5] As part of the project on grossamide, an automated flow[6] reactor was developed and used to prepare a small focused library of amides through the coupling of various amines with a particular carboxylic acid. We have extrapolated upon this amide bond forming process and applied it to the synthesis of small Boc, Cbz and Fmoc *N*-protected dipeptides. In addition, we also report on an elongated flow process for the synthesis of a tripeptide derivative.‡

Our process makes use of a commercially available Syrris AFRICA® micro/mesofluidic pumping system,[7] however any HPLC-grade pump would be adequate to facilitate this flow process.[8] Initially, we placed the starting materials in reagent loops, which were then connected in-line with the flow system *via* an AFRICA® reagent store. The contents of the reagent loops are then introduced into the flow stream when needed using a manual switch and pumped through the columns containing the supported reagents, creating the peptides.

The synthesis of the Cbz and Boc protected dipeptides was accomplished by first packing a glass Omnifit® column[9] with polymer supported 1-hydroxybenzotriazole (HOBt, reagent 2), a useful reagent for amide bond synthesis.[10] This column was washed with DMF and a solution of the Cbz or Boc protected amino acid, DIPEA, and the phosphonium coupling reagent PyBroP® (bromo-tris-pyrrolidino-phosphonium hexafluorophosphate[11]) in DMF was pumped through the column. This allowed the PS-HOBt to sequester the activated amino acid, "catching" the substrate for later use, while also allowing any by-products of the reaction to be directed to waste. The column was then washed thoroughly with DMF leaving an activated amino ester which was covalently attached to the polymer support. The column was then switched in-line with prewashed/swelled columns (in DMF) of polymer supported dimethylaminopyridine (PS-DMAP, reagent 1) and polymer supported sulfonic acid resin (MP-$SO_3H$, reagent 3), linked in series (DMAP–HOBt–$SO_3H$, Scheme 1). A second amino acid, in the form of the HCl salt of the protected ester, was then passed through all three columns in DMF at a flow rate of 100 μL $min^{-1}$. The effect of this continuous flow process was to make use of PS-DMAP to liberate the amine from its salt, which then reacts with the HOBt-activated Cbz or Boc protected amino ester, forming a new peptide bond. The product of this reaction was then passed through the MP-$SO_3H$ column which scavenges any unreacted amine. The resulting solution was then evaporated




**Scheme 1** Synthesis of Boc and Cbz *N*-protected dipeptides.

*Innovative Technology Centre, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, UK CB2 1EW. E-mail: svl1000@cam.ac.uk; Fax: +441223 336442; Tel: +44 1223 336398*
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using a Vapourtec V-10® solvent evaporator,[12] an apparatus that can easily remove ~ 10 mL of DMF in 15 minutes, while only heating the mixture to 38 °C. It should also be noted that to ensure a uniform flow stream (~ 9 bar back pressure), a readily available and inexpensive back pressure regulator was also placed in-line at the exit of the flow stream.

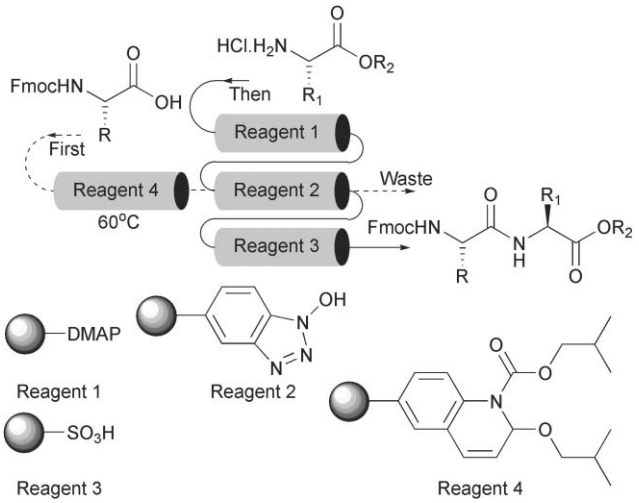
Table 1 displays the results of coupling the amino acids to create the corresponding dipeptides. Typically, the Boc protected amino acids were found to give the highest yields, > 80% isolated yield (entries 1–3), when compared to analogous Cbz protected dipeptides, 75–79% (entries 5–8). When the proline derived amino acids were used (entries 4 and 9), the isolated yields were found to be slightly lower at 66% and 61%, respectively, presumably due to the steric effects of the more hindered secondary amine. For the dipeptides which contained glycine derived amino acids, the optical purities were found to be in accordance with literature values (entries 2, 6 and 8), while all other products were found to be single diastereoisomers. In general, all products were found to be of > 95% purity by $^1$H NMR and LC-MS, and to display the same physical properties as those previously published in the literature. It is possible to achieve higher yields by loading the column with a greater equivalence of HOBt, although the current method uses the most practical flow rate and the most appropriate levels of reagents to give the most practical yields of product.

This same method was also used to couple Fmoc *N*-protected amino acids with the corresponding amino esters in an analogous fashion to that shown in Scheme 1. The products, however, were found to be of an unacceptable purity (~ 80%) by LC-MS and $^1$H NMR, most likely a result of the tertiary amine (DIPEA) degrading the Fmoc functionality by deprotection, a common process when using similar amine bases such as piperidine.[13] In order to improve the method with the aim of creating only high purity products, a separate route was developed. Scheme 2 details the flow process for the specific synthesis of Fmoc protected dipeptides. In this case, polymer supported IIDQ (2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline,[14] reagent 4) was first used to create an anhydride of the Fmoc protected amino acid which was then passed onto a column containing the PS-HOBt. This process acts as an activation step which allows for the resulting anhydride to react with the supported HOBt, generating an activated amino ester which is ready for the peptide coupling step. The by-products of this initial activation step are only 2-methylpropan-1-ol, carbon dioxide, and the residual Fmoc protected amino acid from the anhydride–HOBt reaction which can easily be collected and recycled. The PS-IIDQ column was

**Table 1** Yields of coupled dipeptides

| Entry | Pg | R | $R_1$ | $R_2$ | Yield (%)[a,b] |
|---|---|---|---|---|---|
| 1 | Boc | Me | $CH_2Ph$ | Et | 80 |
| 2 | Boc | Me | H | Et | 81 |
| 3 | Boc | Me | $CHMe_2$ | Me | 83 |
| 4 | Boc | Me | $[-CH_2-]_3$ | Me | 66 |
| 5 | Cbz | $CH_2Ph$ | $CHMe_2$ | Me | 79 |
| 6 | Cbz | $CH_2Ph$ | H | Et | 76 |
| 7 | Cbz | Me | $CH_2Ph$ | Et | 75 |
| 8 | Cbz | Me | H | Et | 78 |
| 9 | Cbz | Me | $[-CH_2-]_3$ | Me | 61 |

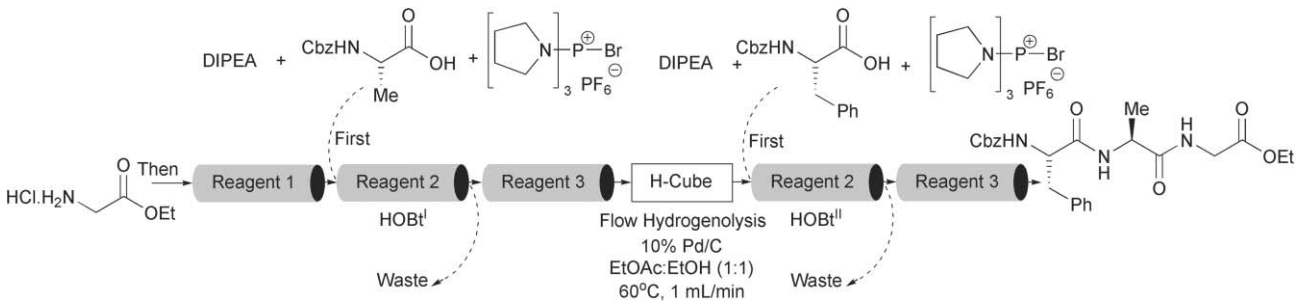
[a] Isolated yield. [b] Purities measured as > 95% by $^1$H NMR and LC-MS.




**Scheme 2** Synthesis of Fmoc *N*-protected dipeptides.

disconnected from the PS-HOBt column and prewashed/swelled PS-DMAP and MP-$SO_3$H columns were in turn connected in series to generate a flow stream that matches Scheme 1. Initially, the flow synthesis was carried out at room temperature and Fmoc-Ala-Phe-OEt was only isolated in 19% yield. The low yield was considered to be a result of incomplete generation of the anhydride from the IIDQ step. When the IIDQ column was heated to 60 °C using a Vapourtec R-4® column heater,[12] much more efficient activation of the HOBt column occurred and the product was isolated in an improved yield of 75%, while Fmoc-Ala-Gly-OEt was produced in a 71% isolated yield. As with previous examples, the spectral and physical data of these compounds were found to be consistent with those reported in the literature. Overall, this alternate method establishes a modified flow process for the synthesis of Fmoc protected dipeptides, which progresses with high purity (> 95%) and generates products as single diastereomers.

Our next step was to develop a process for the flow synthesis of tripeptides using the existing methodology, while also incorporating an additional deprotection step. We have previously reported on a method for the reduction of imines using a flow hydrogenator[15] (Thales H-Cube™)[16] and also for the deprotection of Cbz protected amines.[17] In an analogous fashion, it was our desire to use this piece of equipment to deprotect the Cbz group of the dipeptides. The resulting product could then be used in a subsequent coupling reaction generating a continuous flow process. Scheme 3 depicts how the flow process was implemented. Firstly, two separate columns of PS-HOBt were similarly activated with different Cbz protected amino acids, denoted $HOBt^I$ and $HOBt^{II}$. Prewashed/swelled columns of PS-DMAP and MP-$SO_3$H were then placed in series with the first activated HOBt column (DMAP–$HOBt^I$–$SO_3$H). The HCl salt of an ester-protected amino acid was then pumped through these columns (in DMF) and directed towards the flow hydrogenator. Due to solvent incompatibility, the DMF was removed using a Vapourtec V-10® solvent evaporator and the crude product from the $HOBt^I$ reaction was redissolved in a mixture of EtOAc : EtOH (1 : 1). The dipeptide was then subjected to flow hydrogenation for the deprotection of the Cbz group by the H-Cube[18] and the solvent removed using the Vapourtec V-10®, then redissolved in DMF for the last coupling step. This solvent switch procedure from DMF to EtOAc : EtOH,




**Scheme 3** Flow synthesis of tripeptide.

back to DMF is the only time product is handled by the user and is a result of solvent incompatibility and engineering, rather than a limitation of the flow process itself. Currently, we are working on methods to perform this solvent switch in a purely automated fashion.

In the final coupling process, the deprotected product was passed through the last two columns connected in series (PS-$HOBt^{II}$ and MP-$SO_3H$) and the product collected and solvent removed. The tripeptide Cbz-Phe-Ala-Gly-OEt was isolated in 59% yield as a single diastereoisomer, based on the longest linear flow process (HCl·Gly-OEt as limiting reagent) in greater than or equals to 95% purity.

One of the advantages of the flow process is that the starting materials and products spend very little time being exposed to the supported reagents while they are in solution. The products are part of a moving, dynamic flow stream and are quickly pumped beyond the reach of the reagents, making further chemical transformation, degradation or racemization negligible. An additional advantage of this flow process is the improvement in terms of time over conventional batch techniques, which usually take $\sim$ 24 hours.[19] A typical reaction for the flow synthesis of a dipeptide can easily be performed in 3 to 4 hours, while the synthesis of a tripeptide takes only 6 to 7 hours in total.

In this work we describe the development of several simplified flow methods that can be applied to the synthesis of dipeptides and tripeptides; we believe, however, that these procedures can be readily applied to the synthesis of a wide array of small polypeptides. This communication details only our preliminary efforts in the development of a flow process for the synthesis of peptides and we will eventually be extending our flow methodology to include longer peptide chains, a wider array of natural and unnatural amino acids, and peptidomimetics.

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## Notes and references

‡ General methods for the synthesis of Boc and Cbz protected dipeptides, for Fmoc protected dipeptides and a method for the synthesis of a tripeptide are located in the supplemental information.

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