Recent advances in the solid- and solution-phase synthesis of peptides and proteins using micro-flow technology

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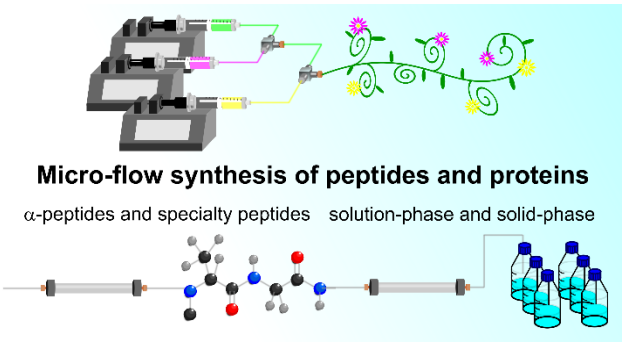

Micro-flow synthesis of peptides and proteins
α-peptides and specialty peptides
solution-phase and solid-phase

ABSTRACT

Peptides have become increasingly important as drugs and drug candidates. In particular, specialty peptides have garnered considerable attention in recent years owing to their improved metabolic stability, higher affinity, and biological target selectivity; some of them have the ability to enable cell membrane permeation and oral administration. Despite its long history, peptide synthesis has various limitations, such as high cost, generation of large amount of waste, and the requirement for hazardous reagents and solvents. Micro-flow synthesis, wherein the inner diameter of the reaction tube is ≤ 1 mm, presents several advantages compared with the conventional batch synthesis, such as precise control of the reaction time on a short scale, precise reaction temperature control, and facile scale-up with high reproducibility. Micro-flow technology has been utilized for peptide synthesis since the beginning of the 21st century. The advantages of micro-flow synthesis enable the use of highly active and unstable chemical species or high-temperature conditions that accelerate peptide synthesis. The combined use of micro-flow technology, automated synthesis, and online monitoring technologies has emerged in recent years. This approach not only improved the synthetic efficiency, but also afforded reliable and large amounts of data that can be used for training machine learning models. This review summarizes the solid- and solution-phase syntheses of α-peptides and specialty peptides, including *N*-methylated peptides, β-peptides, and cyclic peptides, reported mainly after 2017. The recently reported meso-flow peptide syntheses (inner diameter of the reaction channels: > 1 mm), the micro-flow automated syntheses, and in-line analysis are covered in this review.

KEYWORDS: micro-flow, α-peptides, *N*-methylated peptides, β-peptides, cyclic peptides, protein.

1. Introduction

A recent analysis of the functional groups employed in bioactive molecules in the medicinal chemistry literature between 1976 and 2018 revealed that amides are the most frequently used functionality.[1] Peptides significantly contribute to the highly frequent appearance of the amide functionality in bioactive molecules. Peptide drugs have garnered much attention in recent years for their ability to bring together the merits of both small molecule and antibody drugs.[2] There are about 80 approved peptide drugs in the global market, more than 150 peptides in clinical development, and 400–600 peptides in preclinical studies.[3] Several market studies forecasted a global growth of peptide sales from 29B$ in 2019 to 48B$ in 2025 (excluding insulin), with an annual 10% increase.[4] Specialty peptides such as *N*-methylated peptides[5], β-peptides[6], and cyclic peptides[7] have become increasingly important because they enhance metabolic stability, target selectivity, and affinity. Moreover, some of the specialty peptides enable cell membrane permeation and oral administration.

One of the challenges in the efficient peptide syntheses is the insufficient solubility of peptides. Solid-phase synthesis, developed by Merrifield in 1963, revolutionized peptide synthesis.[8] Solid-phase peptide synthesis (SPPS) enabled the treatment of long peptides with poor solubility and facilitated the work up process by removing the excess reagents and substrates by a simple process of washing and filtration. Merrifield reported the epoch-making synthesis of long peptides using an automated peptide synthesizer.[9] The 1980s witnessed the continuous-flow SPPS-enabled

peptide chain elongations by flowing solutions of reagents, amino acids, and washing solutions through resin filled columns.[10] Although SPPS is highly useful, it has some drawbacks, such as the need for large amounts of expensive condensation reagents, additives, and amino acids due to the slow coupling rates on the solid phase.[11] Moreover, the use of expensive resins and large amounts of hazardous solvents poses significant problems concerning sustainability and production costs.[11-14] For example, several metric tons of solvent and thousands of liters of water are typically necessary for producing 1 kg of a peptide.[15] This problem was highlighted in the ACS Green Chemistry Roundtable held in 2006, which announced that amide bond formation avoiding the poor-atom-economy reagents is the most important challenge for organic chemistry.[16] Ten years later, members of the roundtable again announced 10 key green chemistry research areas and "general methods for catalytic/sustainable (direct) amide or peptide formation" was selected as one of the key areas.[17] The development of low cost, high-yielding, and scalable synthetic approaches for peptide synthesis that are less wasteful and hazardous, remains an important objective.

Since the late 1990s, micro-flow synthesis has garnered considerable attention owing to its advantages over the conventional batch synthesis approaches.[18-22] In this review, “micro-flow” is defined as flow in reaction channels that have an internal diameter less than or equal to 1 mm. The advantages of micro-flow synthesis include the following.

(1) Precise control of the reaction time on a short scale (<1 s).

(2) Precise control of the reaction temperature.

(3) Higher light penetration efficiency compared with that of batch synthesis in photochemical reactions.

(4) Minimized risk while handling dangerous compounds.

(5) Facile scale-up.

(1) The very rapid mixing of solutions in a micromixer and reaction tube enables precise control of the reaction time on a short scale (<1 s). (2) The relatively large surface-to-volume ratios in the micro-flow reactor enable rapid heat transfer for precise reaction temperature control. These two attributes have enabled the practical use of highly reactive and unstable chemical species in organic synthesis.[23-29] (3) The intensity of light decreases exponentially with the length of the light path according to the Lambert–Beer law.[30-38] In this context, the thin reaction channels in micro-flow photoreactors enhance light penetration efficiency compared to that of batch photochemical reactions. (4) Micro-flow reactors also have small reaction spaces that can only contain small amounts of compounds. This attribute minimizes the risks when manipulating explosive and/or toxic compounds.[39-41] (5) Another important advantage is the facile scalability of processes either by continuous running or by numbering-up the reactors.[42-52] Micro-flow reactions are also highly amenable for use in automated synthesis,[53-55] as control of the pumping and temperature control devices is required to realize the micro-flow. Notably, a combination of the automated micro-flow synthesis[56, 57] with in-line monitoring technology allows high-throughput data acquisition that drives the autonomous optimization of reaction conditions using machine learning technology.[58, 59]

Micro-flow peptide synthesis emerged at the beginning of the 21st century[60] and a variety of efficient synthetic processes have been demonstrated. In 2014, Albericio et al. first reviewed a wide range of peptide syntheses that utilized micro-flow technology.[61] A focused review[62] and a comprehensive review[63] in 2018 nicely summarized solid-phase and solution-phase continuous-

flow peptide syntheses. In addition, the most recent review introduced selected continuous-flow syntheses and their combinations with in-line analysis and machine learning.[64] We also published a review of the solid-phase and solution-phase micro-flow synthesis of α-, β-, and cyclic peptides reported till 2017.[65] In this review, we focus on the micro-flow synthesis of α-peptides and specialty peptides, including *N*-methylated, β-, and cyclic peptides, reported mainly after 2017. The recently reported meso-flow peptide syntheses with reaction channel inner diameters > 1 mm, micro-flow automated syntheses, and in-line analysis are also introduced in this review.

2. Micro-flow α-peptide synthesis

In recent years, micro- and meso-flow syntheses technologies have enabled the acceleration of condensation and deprotection steps and reduced the waste in solid-phase peptide synthesis. In addition, automated synthesis and in-line monitoring technologies have been combined with flow synthesis technology. These powerful combinations allow high-throughput data acquisition that leads to the autonomous optimization of reaction conditions using machine learning technology.

2.1. Micro-flow α-peptide synthesis via generation of acyl azide in solution phase

Kappe et al. reported peptide bond formation via acyl azide generation in a micro-flow reactor.[66] The reactor used for the synthesis is shown in **Figure 1**. Syringe pumps (inlets A, B, D, and E), an HPLC pump (inlet C), and polyetheretherketone (PEEK) mixers (inner diameter: 0.5 mm or 1 mm) were connected to PTFE reaction tubes (inner diameter: 0.5 mm or 1 mm). In this method, hydrazide **1** and an aqueous solution of hydrogen chloride were injected into the cross-shaped

mixer from the inlet A, an aqueous solution of sodium nitrite was injected into the mixer from the inlet B, and toluene was injected into the mixer from inlet C. The acyl azide **2** was generated in the reaction tube 1 and it was injected into the T-shaped mixer 1. On the other hand, a solution of nucleophile **3** (hydrochloric salt of amine) and triethylamine in toluene were injected into T-shaped mixer 2 from inlets D and E, respectively. The resultant free amine **4** was injected into the T-shaped mixer 1. Peptide bond formation between **2** and **4** was performed in the reaction tube 2 at 0 °C for 16 min. Peptide formation proceeded efficiently without undesired epimerization (<1%) and/or Curtius rearrangement (**Figure 2**), and dipeptides **5-9** were obtained in good yields (70–80%). In addition, amidation via acyl azide formation allows the use of amino acids without protection of the side chains. The authors demonstrated the synthesis of dipeptide **6** containing a side-chain protection-free serine residue. The batch experiments were also carried out for comparison between the flow and batch results. In batch reaction, to the solution of hydrazide **1** in water and toluene were added aqueous solution of $NaNO_2$ and HCl at 0 °C. To the reaction mixture were added nucleophile **3** and triethylamine at the same temperature. The yields of batch conditions were shown in **Figure 2**.

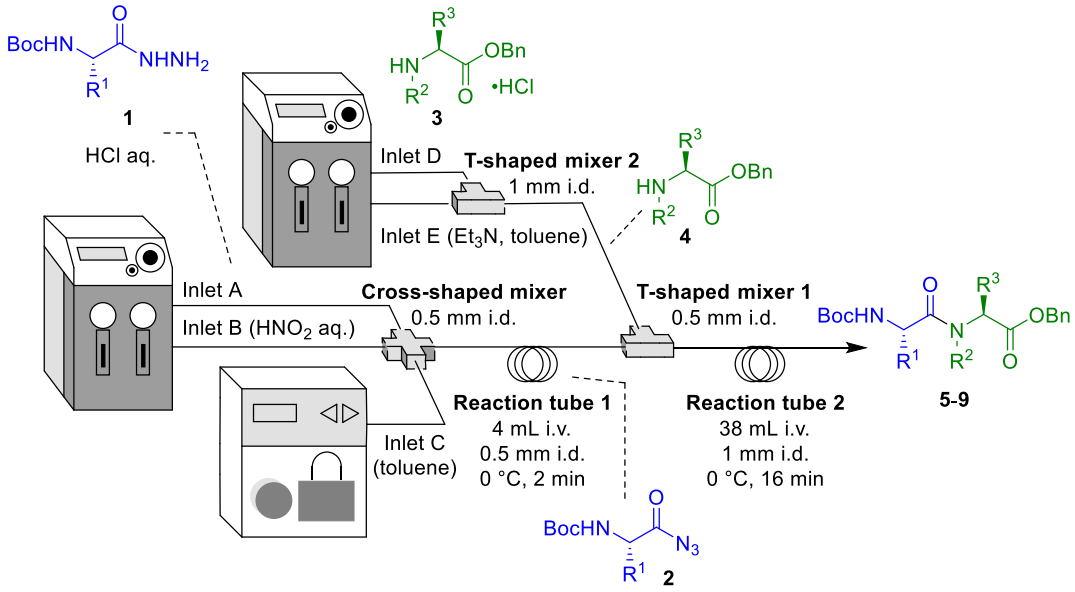


Figure 1. Micro-flow reactor developed by Kappe et al.

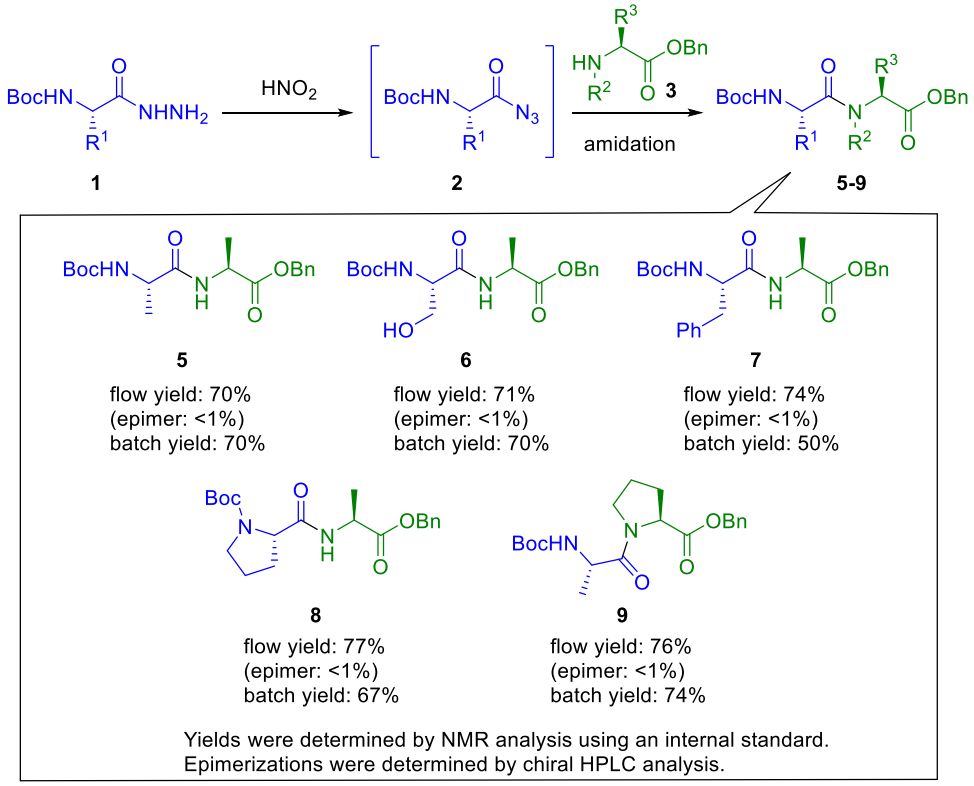


Figure 2. Scope of dipeptide synthesis via acy azide generation.

2.2. Automated micro-flow synthesis of proteins, covalent protein complexes, and peptide-PNA conjugates on solid phase

In 2014, Pentelute et al. reported fast solid-phase peptide synthesis using a micro-flow reactor.[67] Rapid peptide chain elongation (1.8 min/residue) was achieved using a micro-flow reactor equipped with a heat-exchange tube immersed in a water bath (60 °C) and a column reactor filled with the peptide-immobilized resin. The progress of the reaction was monitored by in-line UV detector that measured UV absorption of eliminated fluorenyl moiety during the removal of Fmoc group after each coupling cycle. Peptide chain elongation was performed by repeating rapid amidation (7 s) and rapid deprotection (10 s). In 2017, the group successfully accelerated intrinsically slow amidations on the solid phase by heating (90 °C) in a column reactor;[68] micro-flow technology enabled the rapid heating and cooling of the reaction. Thus, the heating time was precisely controlled to the shortest required period for reducing undesired epimerization. The epimerization of readily epimerizable residues, Cys and His, was suppressed by shortening the reaction time and optimizing the condensing agent. Their improved approach realized a higher speed (40 s/1 residue) of peptide chain elongation. Theoretically, it is possible to synthesize tens of thousands of individual 30-mer peptides per year using the developed approach.

Pentelute et al. developed an automated fast-flow peptide synthesis (AFPS) as shown in **Figure 3**. The authors achieved an impressive automated synthesis of the protein Sortase A consisting of 164 residues.[69] In this synthesis, a total of 327 amidation and deprotection steps were completed in 6.5 h only, and 6.4 mg of the target protein was synthesized with a total yield of 1%. The automated

micro-flow synthesis of eight other peptides/proteins consisting of 86–140 amino acid residues was also demonstrated in 3.5–5.7 h (**Figure 4**).

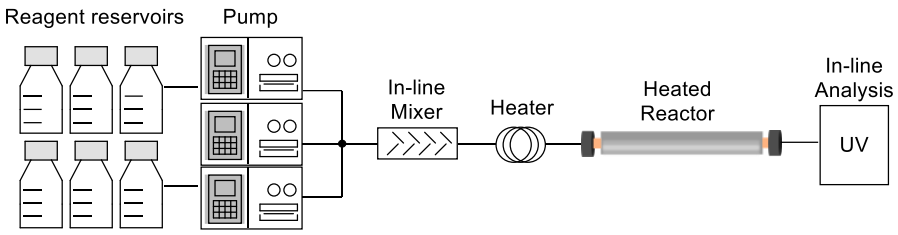


Figure 3. The synthesizer used for the automated fast-flow peptide synthesis (AFPS) developed by Pentelute et al.

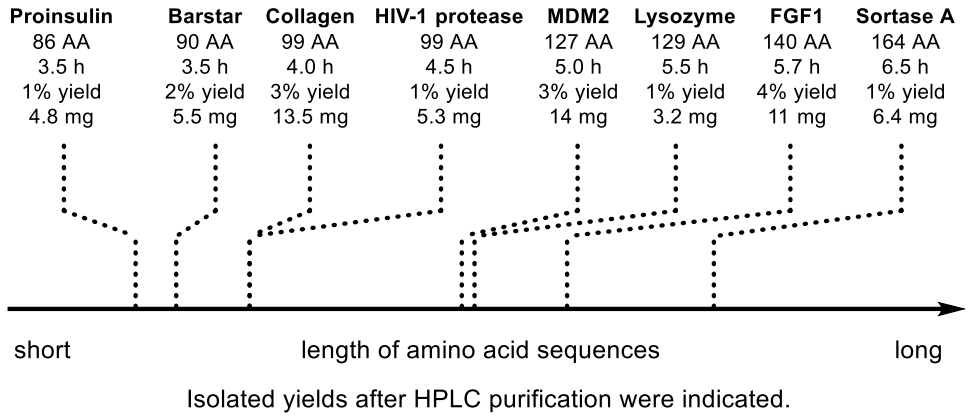


Figure 4. Peptides/proteins synthesized via AFPS approach.

Recently, Pentelute et al. reported the rapid (in a few hours) synthesis of covalent protein complexes by AFPS.[70] The transcription factor MAX forms both homodimer and heterodimer with the transcription factor MYC. The dimeric transcription factor complexes MAX/MAX and MYC/MAX activate or inhibit gene transcription, respectively, upon binding to the same enhancer box DNA. The syntheses of MAX/MAX and MYC/MAX complexes are shown in **Figure 5**. For

MAX/MAX synthesis, dual peptide chain elongation was performed using the two amino groups in **10**. On the other hand, in the case of the MYC/MAX synthesis, the sequence involved the first peptide chain elongation from the *N*-terminus of **12**, removal of Alloc group, and the subsequent second peptide chain elongation from the lysine side chain. The authors also synthesized peptide–PNA conjugates using this method.[71]

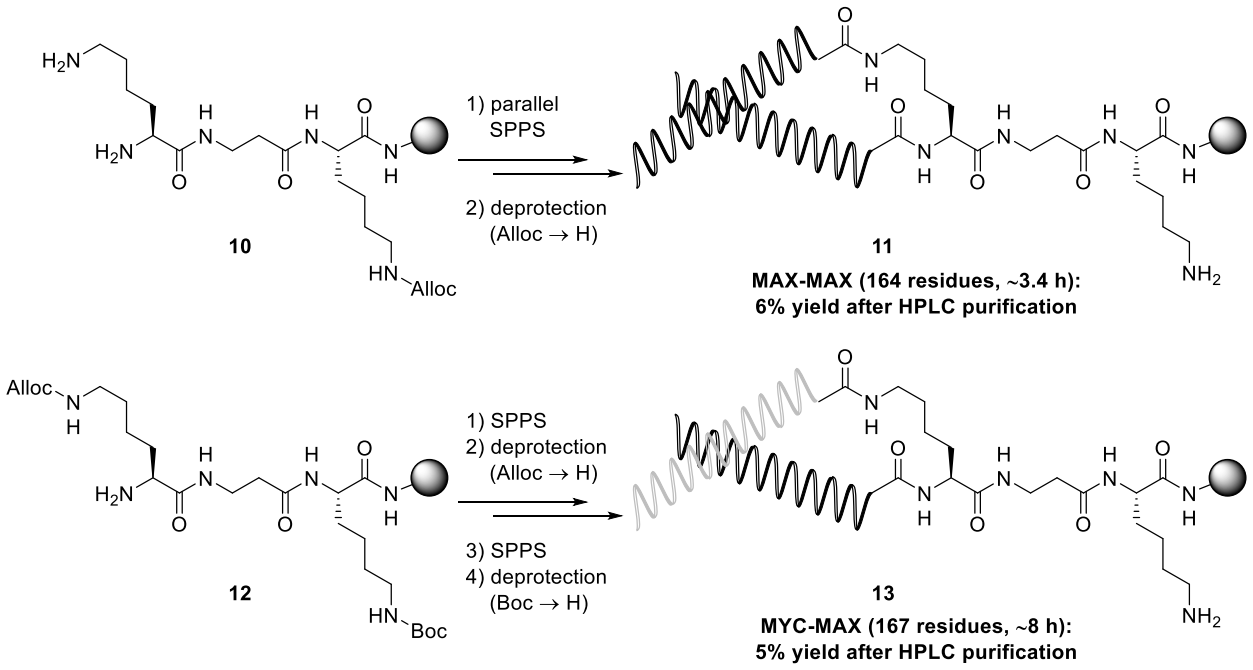
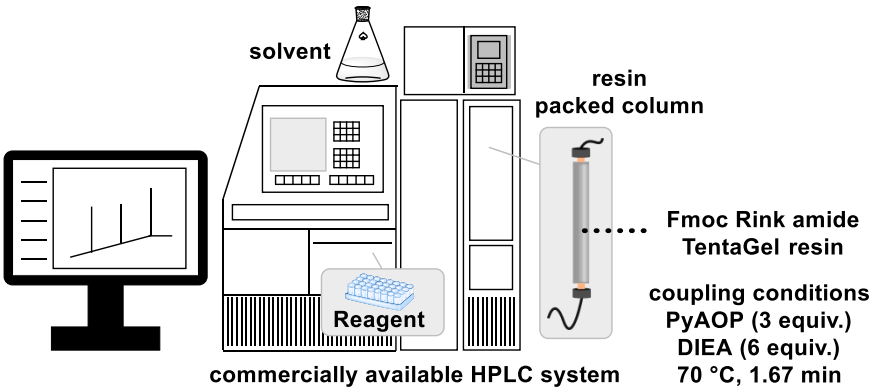


Figure 5. Synthesis of covalent protein complexes by AFPS.

2.3. Cost-effective meso-flow α-peptide synthesis on solid phase

Farkas et al. demonstrated α-peptide synthesis using a commercially available HPLC.[72] The meso-flow reactor used in this study is shown in **Figure 6**. The authors partially improved the commercially available HPLC system. Fmoc-protected amino acids were immobilized on

TentaGel resin via Rink amide, and the PEEK chromatography column was filled with resin. Equal or less than three equivalents of activated amino acid with respect to the immobilized amino acid was used for peptide chain elongation, and the amount of organic waste per cycle was 6 mL. This approach allowed economical and environmentally friendly peptide synthesis. It was possible to shorten the amino acid condensation time to 1.67 minutes by using a combination of PyAOP and DIEA under heating conditions (70 °C). The 10 mer- and 14 mer-peptides were synthesized in good yields and purities in 1.4 and 2.0 h, respectively. This method was also applicable to the synthesis of insulin chain B which is a difficult-to-link 30-mer peptide containing arginine in its sequence (FVNQHLCGSHLVEALYLVCGERGFFYTPKT). The approach could dramatically reduce the amount of organic solvents used compared to that used in microwave irradiation method although the coupling reagent, additive, and reaction time employed under the developed conditions were different from those employed under microwave irradiation conditions.



peptide sequence	total synthesis time	purity	yield
IFDPETGTWI (10 mer)	1.4 h	92%	62%
KRLFKKLLFSLRKY (14 mer)	2.0 h	82%	72%

insulin chain B synthesis

this study (flow)
PyAOP (3 equiv.)/DIEA
70 °C, 1.67 min
total time: 365 min
total solvent used: 220 mL
purity: 64%, yield: 78%

MW method
DIC (5 equiv.)/Oxyma
90 °C, 2 min
total time: 180 min
total solvent used: 1016 mL
purity: 62%, yield: 58%

Purities were determined by UV peak area of the crude peptides in HPLC analysis.
Yields were calculated from the theoretical values based on the volumes of the used resins.

Figure 6. Meso-flow peptide synthesizer developed by Farkas et al. based on the commercially available HPLC system and α-peptide synthesis

2.4. Computational analysis of the sequence-dependent property of peptides

The in-line UV-absorption detection of the previously described AFPS process afforded a large amount of reliable data that is useful for training machine learning models.[73] The integral and shape (width, height) of the UV signals afford sequence-dependent progress of Fmoc deprotection. The sequence-dependent properties, such as aggregation of peptides, significantly influenced the conditions required to complete the reactions. Pentelute et al. used deep learning technology to predict the sequence-dependent outcome of Fmoc-deprotection steps using extensive reliable data. Suitable reaction conditions were predicted with high accuracy using the machine learning approach (**Figure 7**).

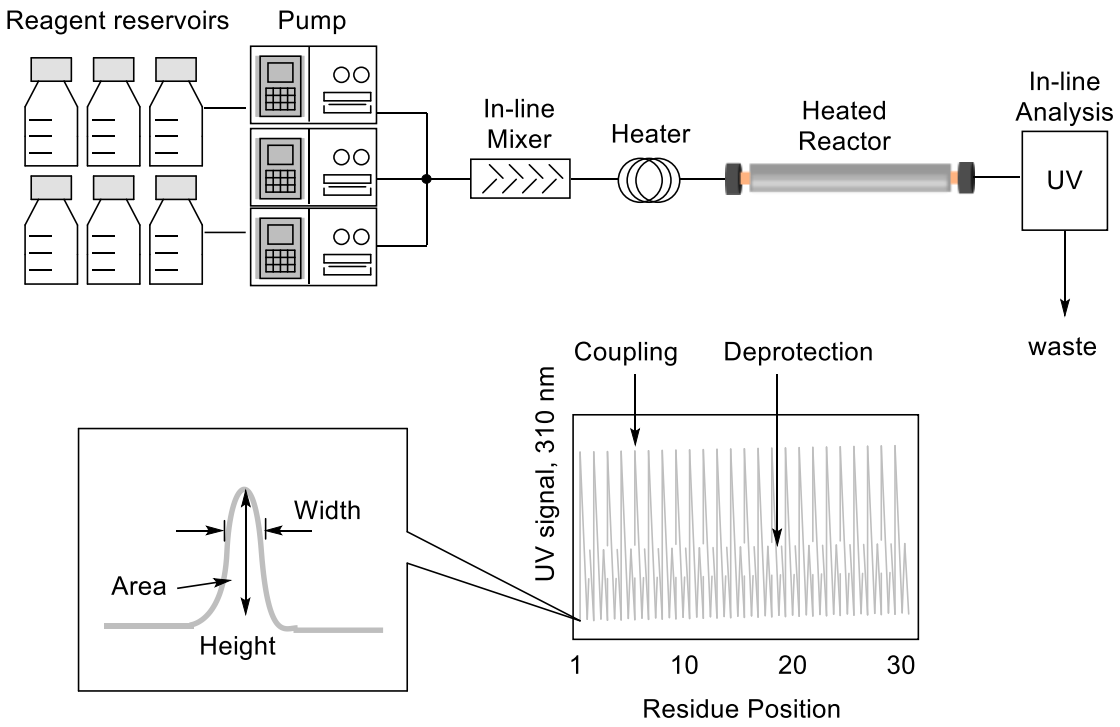


Figure 7. AFPS reactor and in-line data collection in the AFPS process developed by Pentelute et al.

2.5. Real-time monitoring of meso-flow, solid phase α-peptide synthesis

Seeberger et al. reported the use of a variable bed meso-flow reactor (VBFR) that enabled real-time monitoring of peptide elongation and resin aggregation in SPPS (inner diameter: > 15 mm).[74] In the conventional Fmoc SPPS, peptide chain elongation is monitored by UV–vis absorption of the fluorenyl group that is released upon removal of the Fmoc group. However, the obtained UV–vis absorption information cannot be fed back to improve the yield of prior amidation step. The VBFR solves this problem. The device developed by Seeberger et al. is shown in **Figure 8**. The volume change of the resin was measured by differential pressure before and after peptide bond

formation and deprotection. The authors examined the synthesis of peptides (AFLAFLA and WFTTLISTIM) using VBFR with an in-line monitoring system. Peptide synthesis with VBFR required three pumps, one for amino acids and activators, one for the coupling agent, and an isolated pump for piperidine delivery. The VBFR method enabled real-time monitoring of amidation progress that led to the saving of the amino acid used. Interestingly, when the sixth alanine was coupled in the oligo-alanine synthesis, the significant decrease in the volume of the resin bed was observed. This volume change came from aggregation of peptides caused by β-sheet formation. Therefore, VBFR allows the detection of peptide aggregations. This is another merit of using VBFR.

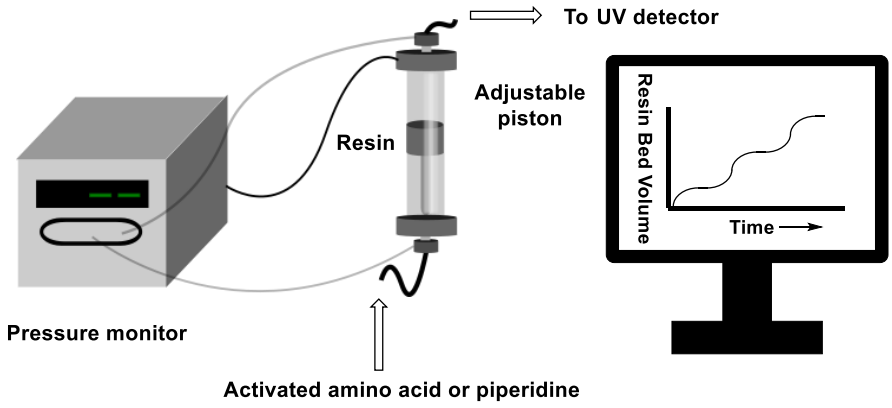


Figure 8. Variable bed flow reactor (VBFR) developed by Seeberger et al.

2.6. Micro-flow dipeptide synthesis via acid chloride generation

Hosoya et al. reported the flow synthesis of a dipeptide via acid chloride generation.[75] The progress of the acid chloride formation of Fmoc-Phe-OH using thionyl chloride was monitored by in-situ IR analysis equipment. The time-dependent conversion of Fmoc-Phe-OH observed by the in-situ

IR analysis equipment showed a good correlation with the simulated data based on the hypothesis that acid chloride formation is a second-order reaction. The appropriate residence time (56 s) for the flow acid chloride formation was determined from the estimated reaction rate at 90 °C based on Arrhenius plot. The flow reactor used in this study is shown in **Figure 9**. The pre-heated acetonitrile solution of Fmoc-Phe-OH and DMF was injected into the first T-shaped mixer from pump A, and an acetonitrile solution of thionyl chloride was injected into the first T-shaped mixer from pump B. Then, the acid chloride was generated in temperature controllable flow reaction system (i.d. = 1.0 mm) at 90 °C for 56 s, which was pre-cooled at 20 °C for 5 s for the following amidation at 20 °C. An acetonitrile solution of H-Phe-OMe hydrochloride and DIEA was injected into the second T-shaped mixer from pump C. Amidation occurred in the PFA tube reactor at 20 °C for 7 s, and the desired dipeptide was obtained in 94% isolated yield after crystallization.

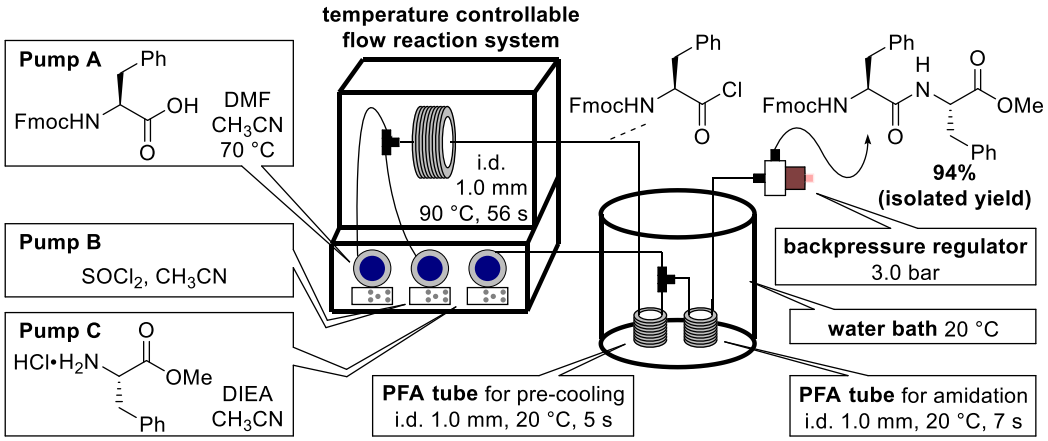


Figure 9. Flow system for the synthesis of dipeptide via acid chloride generation.

2.7. Micro-flow synthesis of α-dipeptides from urethane-protected α-amino acid *N*-carboxyanhydride (α-NCA) in solution phase

Fuse et al. reported micro-flow peptide syntheses via the generation of highly active electrophiles and synthesized natural products using the developed method.[76, 77] They recently reported the micro-flow synthesis of urethane-protected α-amino acid *N*-carboxyanhydride (UNCA) **17** by the rapid dual activation of both alkyl chloroformate **14** and α-NCA **15**.[78] They demonstrated a high-yielding one-flow synthesis of the two α-dipeptides **19** and **20** using the highly electrophilic UNCA (**Figure 10**).

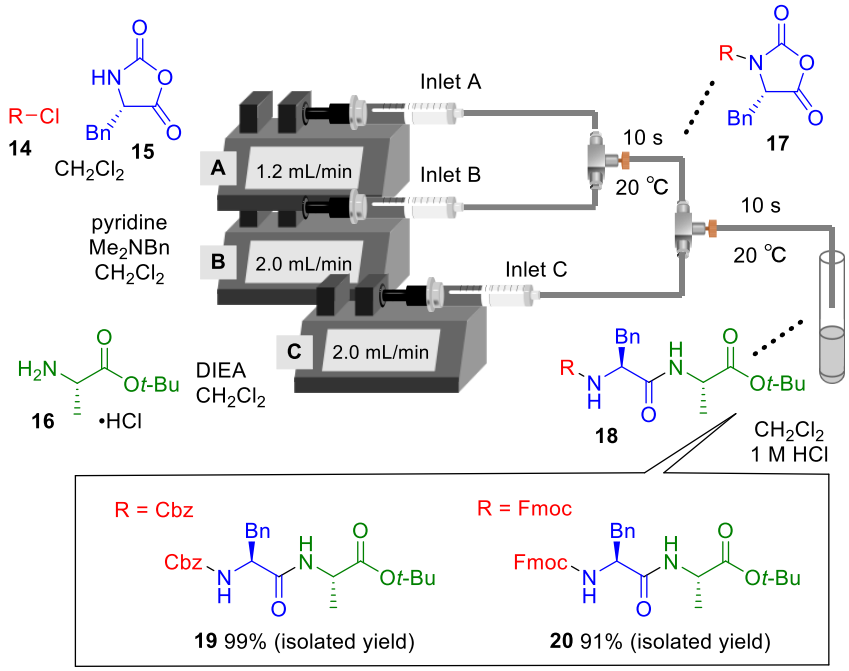


Figure 10. One-flow synthesis of dipeptides using UNCA **17**.

2.8. Micro-flow peptide chain elongation using unprotected α-amino acids in solution phase

Conventional peptide synthesis requires the use of protected amino acids for peptide chain elongation. Therefore, deprotection steps are indispensable for amide bond formation. This increases the number of synthetic steps and the production costs for peptide chain elongation. In

addition, although the average molecular weight of amino acids is *ca.* 110, the molecular weights of the frequently used protecting groups (Fmoc: 223, Boc: 101, Cbz: 135) are comparable or larger. The large amount of waste generated from protecting groups is another problem in peptide synthesis. The peptide chain elongation using unprotected amino acids is considered as an ideal approach in term of reducing the number of synthetic steps, waste, and cost. Unprotected amino acids are usually only soluble in water, on the other hand, *N*-protected amino acids or peptides (carboxylic acids) are usually only soluble in organic solvents. Therefore, the coupling reactions have been carried out in biphasic solvent system. The slow coupling rates of the biphasic reactions readily cause undesired epimerization/racemization.[79]

Fuse et al. reported peptide chain elongation from *N*-terminus to *C*-terminus using unprotected amino acids in a micro-flow reactor.[80] Micro-flow technology enabled the rapid mixing of an organic layer containing a protected amino acid and an aqueous layer containing an unprotected amino acid to accelerate the desired amidation without the undesired epimerization (0.4 %). The micro-flow reactor used in this study is shown in **Figure 11**. The acetonitrile solution of $ClCO_2i$-Bu was injected into the T-shaped mixer from syringe pump A, and an acetonitrile solution of carboxylic acid **21** and NMM/DIEA was injected into the T-shaped mixer from syringe pump B. Then, the mixed carbonate anhydride **22** was generated in 5 s. Subsequently, an aqueous solution of the unprotected amino acid **23** was injected into the second T-shaped mixer from syringe pump C. Amidation occurred by the rapid mixing of the organic phase (electrophile) and aqueous phase (nucleophile) in reaction tube 2 within 10 s. Various dipeptides **24**-**38** were obtained in high yields, as shown in **Figure 11**. The precise control of the reaction time and temperature, enabled by micro-flow technology, mitigated the undesired racemization/epimerization of the highly electrophilic mixed carbonic anhydride. In fact, use of the corresponding batch conditions resulted in a decrease

in both reproducibility and yield. Highly epimerizable tripeptide **36** and tetrapeptides **37** and **38** were successfully synthesized using this approach.

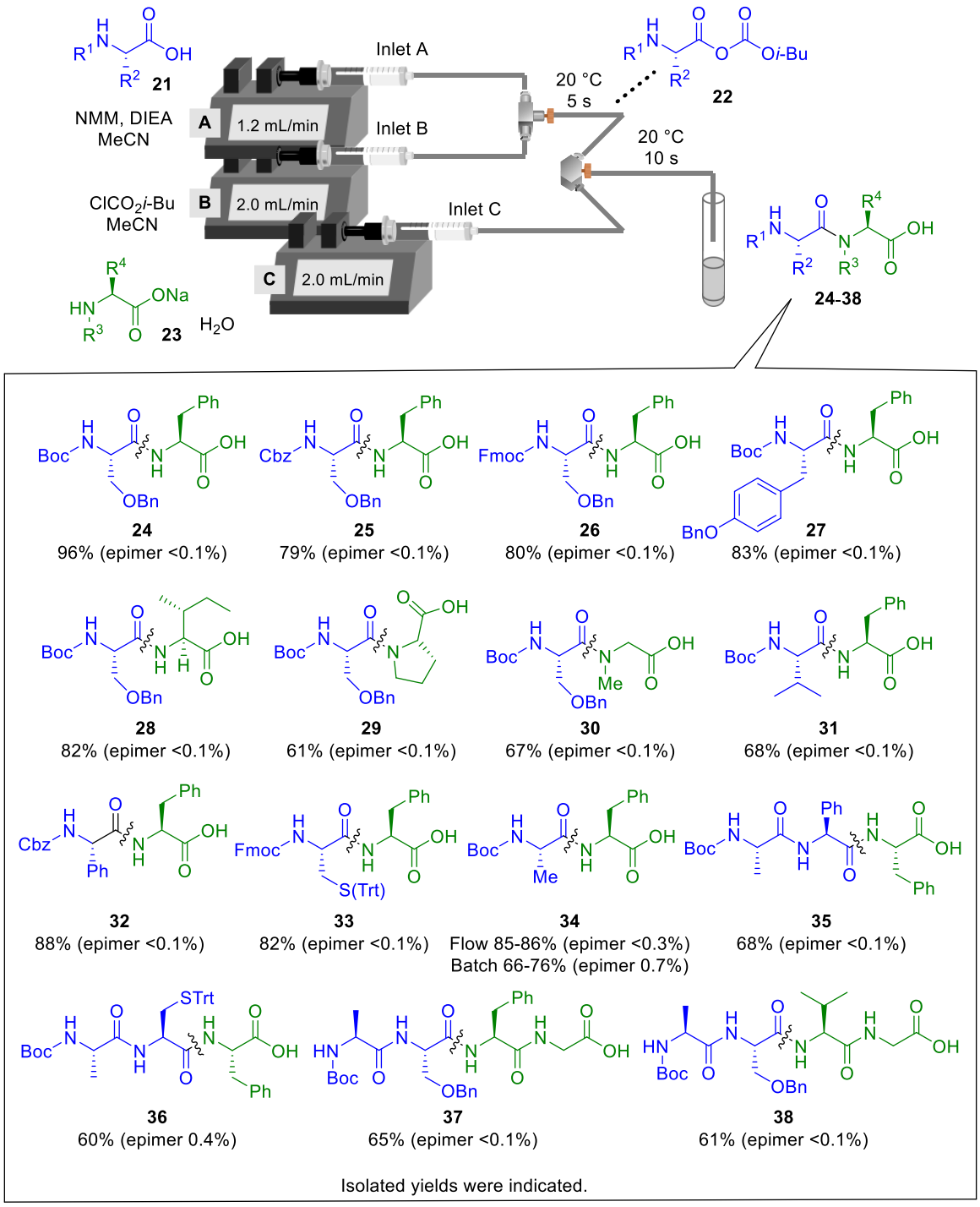


Figure 11. Scope of micro-flow α-peptide synthesis using unprotected amino acids.

3. Micro-flow syntheses of specialty peptides including *N*-methylated peptides, β-peptides, and cyclic peptides

α-Peptides comprising proteinogenic amino acids usually suffer from poor pharmacological properties, such as low metabolic stability and cell membrane permeability. Specialty peptides, including *N*-methylated peptides[5, 81-83], β-peptides[6, 84-88], and cyclic peptides[7, 89-91] generally have higher metabolic stability. In particular, it is believed that cyclic *N*-methylated peptides have better affinity and specificity against their biological targets because of their constrained chemical structure. In addition, they can exert cell membrane permeability and are available for oral administration. However, the amidation of sterically hindered *N*-methylated amino acids is not a simple task. Amide bond formation in a micro-flow reactor has recently been reported to overcome this difficulty.

3.1 Micro-flow synthesis of *N*-methylated peptides in solution phase

Fuse et al. succeeded in synthesizing *N*-methylated peptides via generation of highly electrophilic acyl *N*-methylimidazolium cations from the mixed carbonate anhydrides.[92] They used the micro-flow reactor shown in **Figure 12**. A 1,4-dioxane solution of carboxylic acid **39**, Me_2NBn, and DIEA was injected into the first T-shaped mixer from inlet A, and a 1,4-dioxane solution of $ClCO_2$*i*-Pr was injected into the first T-shaped mixer from inlet B. Mixed carbonate anhydride **40** was generated in the reaction tube 1 within 5 s, and the resultant solution was injected into the second T-shaped mixer. Subsequently, an acetonitrile/1,4-dioxane solution of **41**, HCl, and NMI

were injected into the second T-shaped mixer from inlet C. The highly electrophilic acyl *N*-methylimidazolium cation **42** was generated in the reaction tube 2, and was immediately coupled with an amino ester to afford the desired *N*-methylated peptide **43**-**58**. The developed approach enabled the synthesis of various dipeptides in high yields without severe racemization, as shown in **Figure 12**. The authors also achieved the first total synthesis of pterulamide I (**68**)–IV (**71**) containing multi-branched bulky *N*-methylamino acids (**Figure 13**).

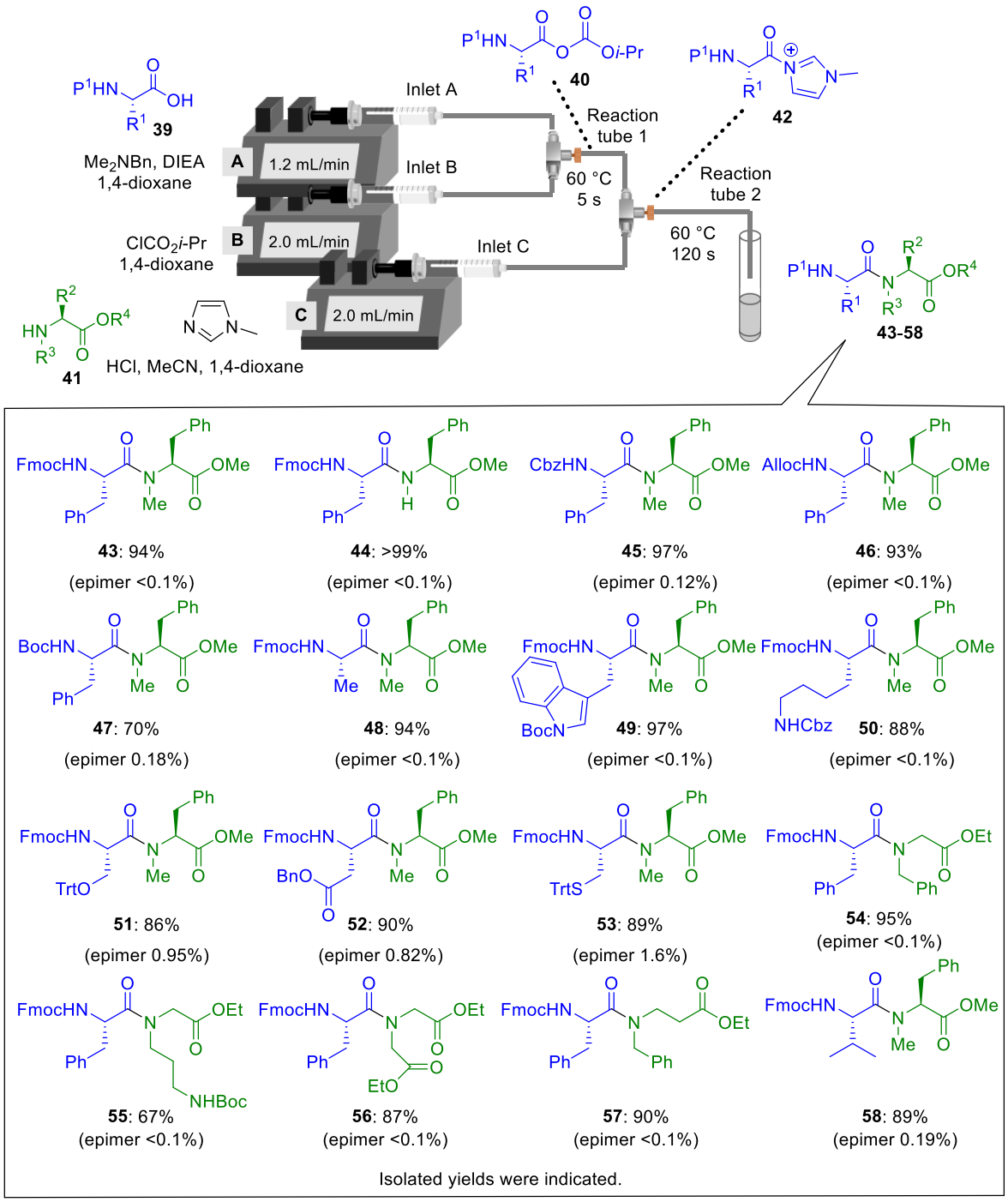


Figure 12. Micro-flow synthesis of *N*-methylated peptides via generation of highly electrophilic acyl *N*-methylimidazolium cations.

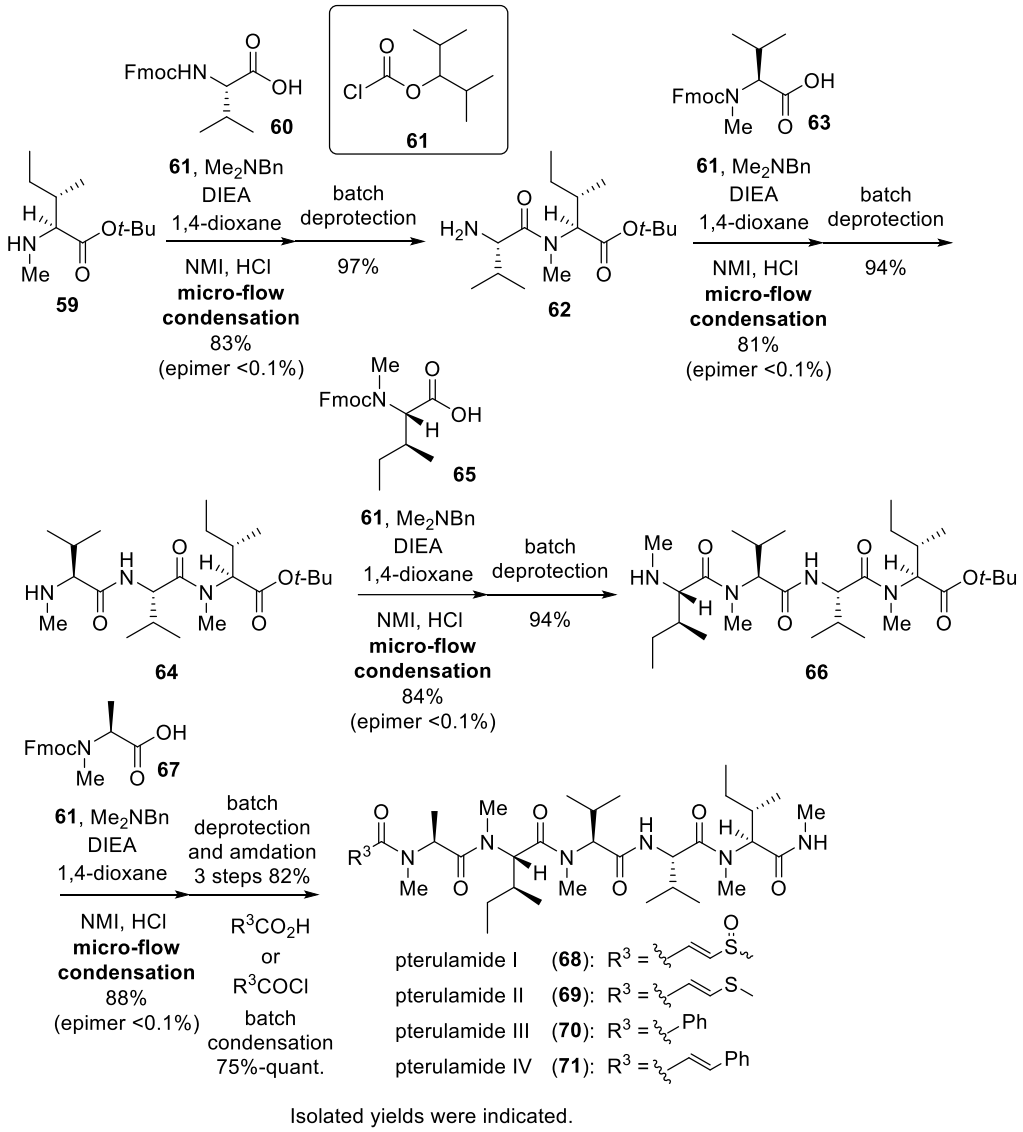


Figure 13. Total synthesis of pterulamide I–IV via micro-flow amide bond formation.

3.2 Meso-flow synthesis of β-peptides on solid phase

Mandity reported a meso-flow synthesis of six β-peptides that form an enantiodiscriminative helix.[93] The continuous-flow solid-phase peptide synthesizer was used for the synthesis of the 4 β-peptides containing bridged bicyclic residues (**72a**, **72b**, **73a**, and **73b**). The peptide chains were

elongated on Tentagel RAM resin using the Fmoc SPPS protocol on a 0.1 mmol scale. Couplings were performed using HATU and DIEA at 70 °C (yields are not mentioned). The obtained peptides **72** and **73** could be converted into the corresponding alkene **74** by retro Diels–Alder reaction. The reduction of dehydroalanine **74** was carried out in a continuous-flow hydrogenation reactor to afford **75** (**Figure 14**).

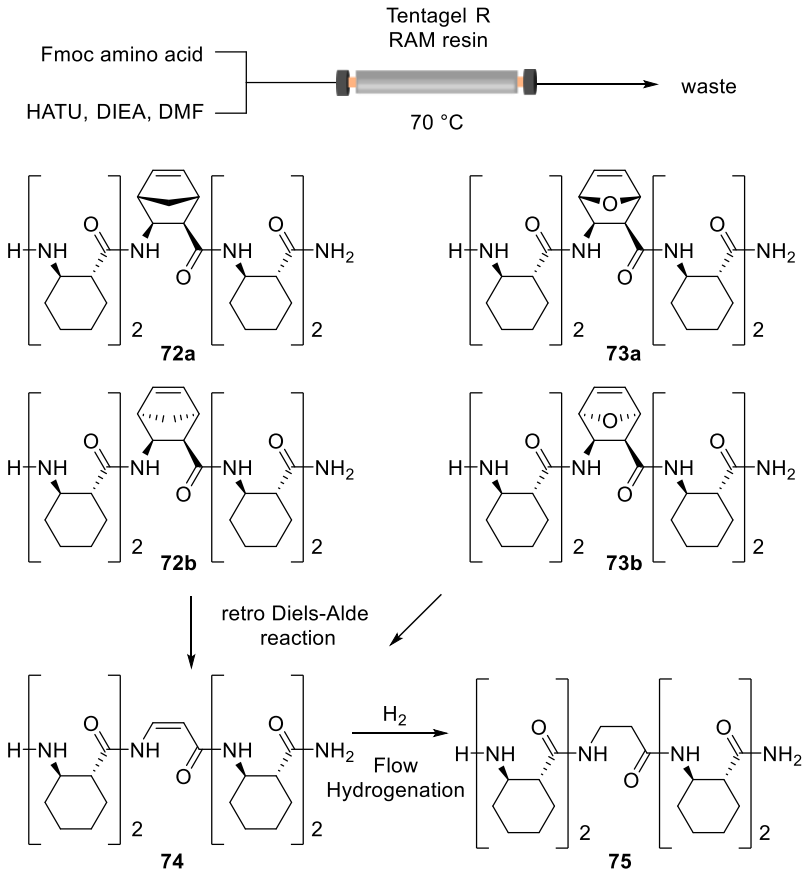


Figure 14. Flow synthesis of constrained β-peptides.

3.3 Micro-flow synthesis of β-peptide derivatives via dual activation of β-amino acid *N*-carboxyanhydrides (β-NCAs) and $ClCO_2i$-Bu in solution phase

Fuse et al. also reported the micro-flow synthesis of α- and β-NCAs via basic-to-acidic flash switching and flash dilution.[94, 95] They used β-NCAs for the micro-flow synthesis of β-peptide derivatives by rapid dual activation of both β-NCAs and $ClCO_2i$-Bu. The dichloromethane solution of β-NCA **76** and $ClCO_2i$-Bu was injected into the first T-shaped mixer from inlet A, and the dichloromethane solution of NMM was injected into the first T-shaped mixer from inlet B. The active intermediate **77** containing the two electrophilic functional groups, *i.e.,* the mixed carbonic anhydride and isocyanate moieties, was generated in situ in 3.3 s and it was injected into the second T-shaped mixer. Then, the dichloromethane solution of Nu^1 and DMAP was injected into the second T-shaped mixer from inlet C. The nucleophilic addition of **77** with Nu^1 occurred in 7.0 s and the resultant solution was poured into the mixture of Nu^2 and DIEA in a test tube. Then the second nucleophilic addition with Nu^2 occurred to afford β-peptide derivatives **78**-**98**. When the test tube was heated at 100 °C without second nucleophile Nu^2, dihydrouracil analogs **99** and **100** were obtained in good yields. Both $ClCO_2i$-Bu and β-NCA rapidly causes (< 1 min) undesired decomposition and oligomerization/polymerization, respectively, in the presence of an amine. Rapid (< 3.3 s) dual activation was key to suppressing the undesired reactions (**Figure 15**).

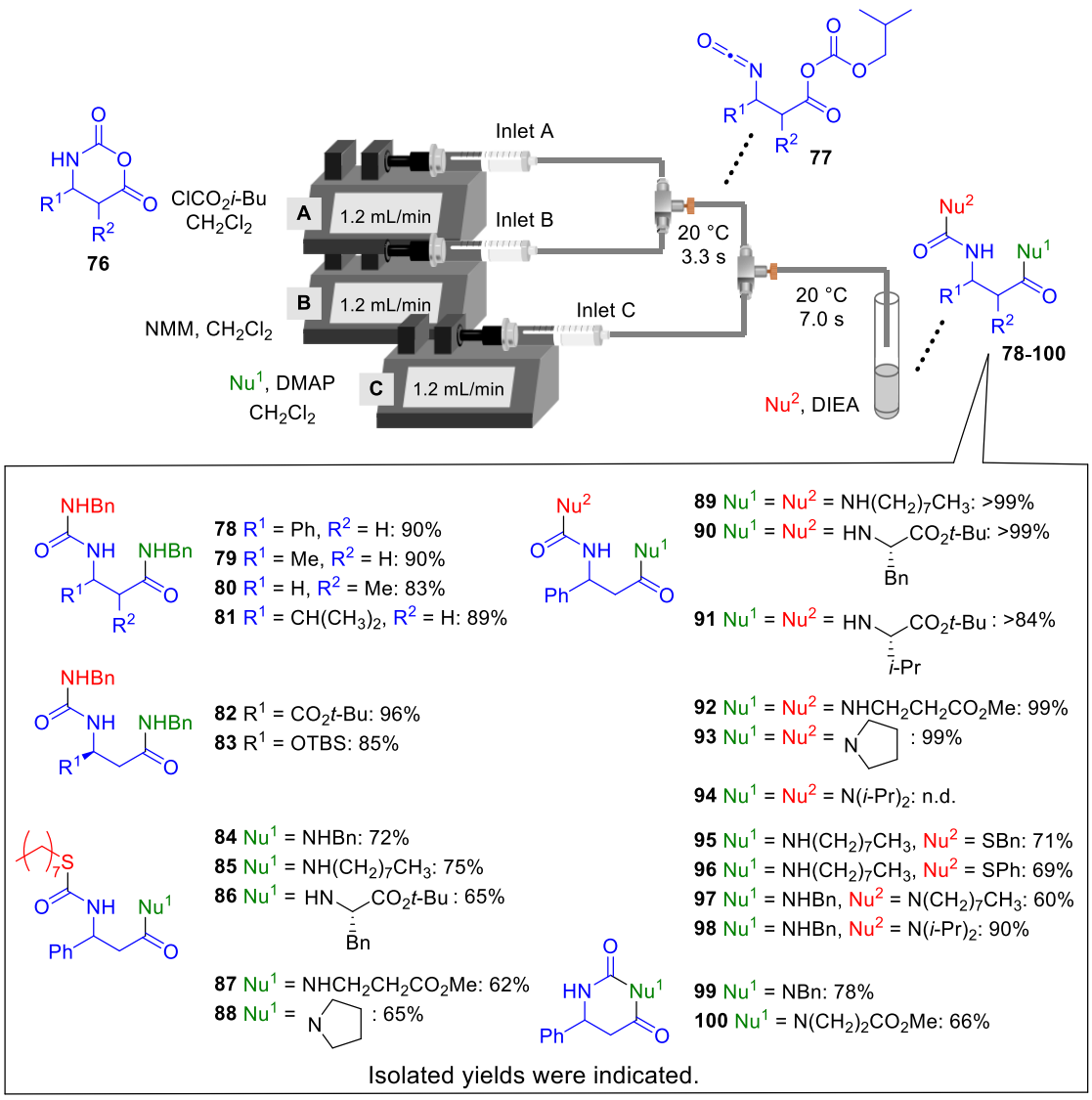


Figure 15. Micro-flow synthesis of β-peptide analogs via dual activation of β-NCA.

3.4. Rapid and mild synthesis of cyclic peptide using a micro-flow reactor in solution phase

Conventional lactamizations can be divided into two categories. The first category usually uses inexpensive Lewis acids; however, the requirement for heating and long reaction time conditions result in a narrow substrate scope. The other category uses condensing agents. This approach can

be performed under relatively mild conditions and is useful for the cyclization of acyclic substrates containing acid and/or heat-labile functional groups. In particular, phosphonium salts such as PyBOP, PyAOP, and BOP have been regarded as suitable choices for cyclization of peptides because they tend to mitigate the undesired nucleophilic attack at the *N*-terminus of the acyclic peptide.[96-98] However, these approaches require expensive and wasteful coupling agents that sometimes require tedious purification. Fuse et al. reported micro-flow lactam and cyclic peptide synthesis using the highly electrophilic triphosgene.[99] An acetonitrile solution of the cyclization precursor **101** and NMI was injected into the first V-shaped mixer from inlet A, and the acetonitrile solution of triphosgene was injected into the first V-shaped mixer from inlet B. The lactam formation occured in the reaction tube 1 only in 10 s. The authors demonstrated the rapid and mild synthesis of various lactams (not shown) and cyclic peptides **102** in a micro-flow reactor (**Figure 16**).

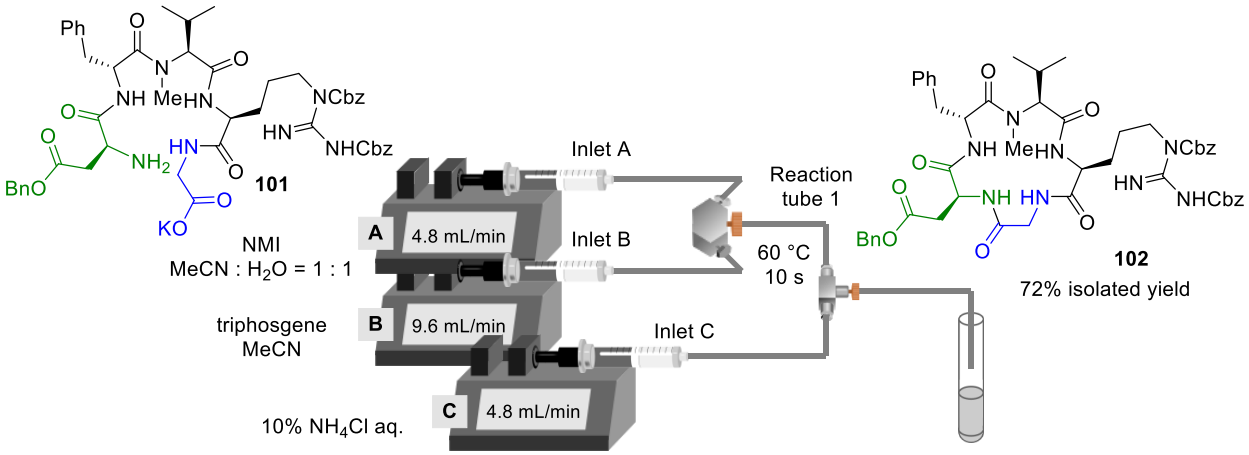


Figure 16. Micro-flow synthesis of a cyclic peptide.

4. Conclusion

The advantages of micro-flow technologies, including precise control of reaction time and temperature, enable the use of highly active chemical species or high-temperature conditions while avoiding the typical side reactions encountered during the traditional peptide synthesis. The developed approaches achieved high-speeds, generated less waste, and afforded low-cost synthesis of the peptides. However, despite the successful development of powerful micro-flow approaches, synthesis of specialty peptides such as *N*-methylated peptides and cyclic peptides pose some challenges. Continued efforts are necessary to develop efficient and green synthetic approaches to address such challenegs .

In recent years, automation of synthetic organic chemistry has attracted considerable attention. Essentially, organic synthesis experiments require a variety of manipulations; therefore, automation of the synthesis is not a simple task. However, peptide synthesis can be automated relatively easily because it requires repeated amidation and deprotection steps. In addition, the use of SPPS facilitates the work up process. This is a significant advantage in the automation of peptide synthesis. In fact, the history of automation of organic synthesis started with SPPS. The recently reported automated micro-flow synthesis of peptides/proteins has entered a new realm of research. The costs for operating synthetic organic chemistry experiments is relatively high compared to those of other fields. High-throughput acquisition of reliable and large amounts of data (>thousands) is not usually easy. In this context, the relavance and use of machine-learning techniques to gain maximum benefits in synthetic organic chemistry is still unclear. Conversely, the combination of micro-flow peptide synthesis, which has a high affinity for automated synthesis

and in-line analysis, allows high-throughput data acquisition. This approach is particularly useful for efficient reaction optimization.

Although significant progress has been made in the field of micro-flow peptide synthesis in recent years, the development of less wasteful and hazardous, low-cost, high-yielding, and scalable synthetic approaches for peptides remains an important pursuit. Approaching solutions to all such problems in the solution- and solid-phase peptide syntheses using a single approach is highly challenging. The authors hope that a variety of unique micro-flow synthesis approaches will be developed in the near future, which could dramatically improve the efficiency and sustainability of peptide synthesis.

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ABBREVIATIONS

HPLC, high performance liquid chromatography; PEEK, polyetheretherketone; PyAOP, (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; Oxyma, ethyl cyano(hydroxyimino)acetate; MW, micro wave; i.d., inner diameter; i.v. inner volume; DIEA, *N,N*-diisopropylethylamine; Fmoc, 9-fluorenylmethyloxycarbonyl; PTFE, polytetrafluoroethylene; PNA, peptide nucleic acid; UV, ultraviolet; Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; NMM, *N*-methylmorpholine; DMF, *N,N*-dimethylformamide; Bu, butyl; Me, methyl; Bn, benzyl; NMI, *N*-methylimidazole; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate; RAM, rink amide; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; BOP, (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate.

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