Introduction

Peptide-based drugs have become increasingly important over the past several decades.\(^1\) Reportedly, ca. 80% of peptide drugs on the market are short peptides consisting of less than or equal to 10 amino acids.\(^2\) Longer peptides can be readily prepared by linking these short peptides via ligation technologies.\(^3\) Therefore, efficient and scalable preparation of short peptides is extremely important both in academia and industry. Solid-phase peptide synthesis (SPPS) is a compelling option due to its facile purification.\(^4\) Pentelute and co-workers recently demonstrated a sophisticated solid-phase automated rapid-flow peptide/protein chain elongation.\(^5\) However, SPPS usually requires the use of a large amount of materials (i.e., expensive coupling agents, additives, a solid phase, amino acids, and solvents).\(^6\) Thus, production cost becomes high even for the synthesis of short peptides. Liquid-phase peptide synthesis (LPSS) has been frequently used for the production of short peptides because it does not require the use of an excess amount of materials. However, burdensome and costly purifications are usually required for the removal of remained and/or reacted materials from the target peptides after every step.\(^7\) Another problem both in SPPS and LPSS is the requirement of deprotection steps that account for almost half of the total number of synthetic steps. Researchers have long coveted an ideal process that enables fewer synthetic steps, low cost, mild, rapid, and scalable peptide chain elongation for short peptides without tedious purification.

One-pot/one-flow, multi-component coupling approaches are attractive because they skip intermediate purifications, and, thus, these can shorten the time for synthesis and reduce waste. However, as far as we could ascertain, there have been only three reports for peptide syntheses based on such an approach. Sampaio-Dias et al. used protection-free amino acid \(A\) as a scaffold in their one-pot, three-component coupling (3CC) approach (Scheme 1a).\(^8\) It is well known that the nucleophilicity of an amino group is higher than that of a carboxyl group in \(A\) under basic conditions. Therefore, amine \(A\) was coupled with activated ester \(B\), and the remaining carboxyl group was activated by TBTU for the following coupling with amine \(C\) to afford tripeptide \(D\). Fuller et al. used urethane-protected \(\alpha\)-amino acid \(N\)-carboxy anhydrides (UNCA) \(E\) as a scaffold in their one-pot, three-component coupling (3CC) approach (Scheme 1b).\(^9\) The UNCA \(E\) has an activated \(C\)-terminal moiety and a protected N-terminal moiety; thus, a coupling with amine \(C\) and removal of the Cbz group by Pd/C and \(H_2\) afforded amine. Coupling of the amine with \(F\) afforded the desired tripeptide \(D\). Yamamoto et al. used silyl-protected cyclic dipeptide \(G\) as a scaffold in their one-pot, 3CC approach (Scheme 1c).\(^10\) The N-terminus of \(G\) was selectively deprotected by \(n\)-Bu\(_4\)NF and coupled with an activated ester that was derived from carboxylic acid \(H\), EDCI, and HO\(_3\)t. Then, the \(C\)-terminus of the resultant dipeptide was again activated by EDCI and HO\(_3\)t, which was followed by coupling with amine \(C\) to afford the tetrapeptide \(I\). All the reported approaches demonstrated oligopeptide synthesis without
intermediate purifications. These approaches, however, require activation and/or deprotection steps using expensive reagents and additives as well as tedious column chromatographic purifications. In addition, although one-pot, multi-component Ugi reactions for peptide synthesis have been reported, these afford a diastereomeric mixture of peptides. Hirschmann et al. reported a one-pot, protecting group-free synthesis of short peptides (≤6 residues) using α-amino acid N-carboxy anhydride (α-NCA) J and J′ under carefully controlled conditions (pH = 10.2, 0–2 °C, high sheir mixing) and achieved an epoch for ribonuclease S synthesis by connecting the short peptides (Scheme 1d). Although α-NCA-based peptide chain elongation is ideal because it requires neither activation nor deprotection steps and emits only CO₂ during the coupling step, but the process has rarely been used due to difficulties in suppressing undesired reactions. In fact, Blacker et al. reported the synthesis of di- and tri-peptides using α-NCA J in 2017. Although they achieved peptide synthesis using α-NCA in a continuous stirred tank reactor, they pointed out the difficulties in purifying the obtained peptides due to the generation of undesired products. α-Dehydroamino acid NCAs are much more stable than α-NCA and have been used for peptide chain elongation via a one-pot, 3CC approach because the risk of undesired reactions is low.

Micro-flow technologies allow precise control of both the reaction time on a short scale (<1 s) by rapid mixing (<milliseconds) and reaction temperature. We reported a micro-flow peptide chain elongation using highly electrophilic species, as well as a one-step micro-flow synthesis of NCAs and the derivatizations. Herein, we report an efficient peptide chain elongation based on a one-flow, 3CC approach using α-NCA J (Scheme 1e). Although all the previously reported α-NCA-based approaches used α-NCA only as an electrophile (Scheme 1d), our approach is the first to use α-NCA J as both a nucleophile and an electrophile. The developed approach for the synthesis of various short peptides enables a reduction in the number of synthetic steps. The process also is mild (20 °C), rapid (<minutes), scalable, high-yielding, and column-chromatography-free.

Results and discussion

To the best of our knowledge, there has been no report of using α-NCA as a nucleophile in peptide bond formation. Therefore, we began by examining acylation of the H-Phe–NCA (1a). The initial examinations revealed that the use of acid chloride as an electrophile was suitable for the acylation, whereas the use of acid anhydride did not afford the desired amide product in a satisfactory yield (details are not shown). Thus, readily available pivaloyl chloride was used as a model for amino acid chloride. We connected two T-shaped mixers with Teflon tubing and immersed them in a water bath. A solution of pivaloyl chloride and 1a in CH₂Cl₂ was injected into the first mixer via syringe pump A. A solution of base in CH₂Cl₂ was injected into the first mixer via syringe pump B, which resulted in a nucleophilic acyl substitution. A solution of isopropylamine in CH₂Cl₂ was injected into the second mixer via syringe pump C to convert to the desired amino acid derivative 2. The resultant mixture including 2 was collected in test tubes and the results were evaluated via HPLC-UV analysis. The bases were examined (Table 1). The use of NMI, NMM, Me₂NBn, Et₃N, and i-Pr₂NET afforded the desired 2 in low yields (entries 1–5). The sole use of highly basic Et₃N or i-Pr₂NET generated white precipitates probably due to the undesired oligomerization/polymerization of 1a. The combined use of pyridine and i-Pr₂NET afforded 2 in a low yield (entry 6), but the yield was slightly higher than that from the sole use of i-Pr₂NET (entry 5). To our delight, the combined use of nucleophilic NMI or DMAP and i-Pr₂NET or Me₂NBn afforded the desired 2 in high yields (entries 7–9). In particular, the combined use of nucleophilic NMI and highly basic i-Pr₂NET afforded the desired 2 in an excellent yield (entry 7). This is probably because the nucleophilic NMI rapidly activated pivaloyl chloride via acyl N-methylimidazolium ion...
Table 1  Examination of bases for the synthesis of amino acid derivative 2

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base (pK_a)^a</th>
<th>Yield^b (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>NMI (7.0)^ab</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>NMM (7.4)^ab</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Me_2NBn (6.9)^ab</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>Et_3N (10.7)^ab</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>i-Pr_2NEt (11.4)^ab</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6^c</td>
<td>i-Pr_2NEt + pyridine (5.2)^ab</td>
<td>10</td>
</tr>
<tr>
<td>7^c</td>
<td>i-Pr_2NEt + NMI</td>
<td></td>
</tr>
<tr>
<td>8^c</td>
<td>i-Pr_2NEt + DMAP (9.7)^abc</td>
<td>99</td>
</tr>
<tr>
<td>9^c</td>
<td>Me_2NBn + NMI</td>
<td></td>
</tr>
</tbody>
</table>

^a pK_a of conjugated acid. ^b Yields were determined via HPLC-UV analysis. ^c The combined use of bases (1.0 equiv. + 1.0 equiv.).

formation and the highly basic i-Pr_2NEt rapidly deprotonate 1a to enhance its nucleophilicity. We previously reported urethane formation from z-NCAs and alkyl chloroformate, and the combined use of pyridine and tertiary amine afforded the best results. Interestingly, the optimal combination of bases (NMI and i-Pr_2NEt) for amidation in this study was different from that for urethane formation in the previous study.

Next, we examined a one-flow 3CC for tripeptide synthesis based on the developed conditions (Fig. 1). In order to avoid the isolation of a labile acid chloride, Fmoc-protected amino acid chloride 4a was in situ prepared from Fmoc-protected amino acid 3a and SOCl_2 in the presence of a base (for details of the optimization of bases, solvents and reaction times for acid chloride formation, see ESI Table S2†), which was used for the following coupling with z-NCa 1a. The coupling generated an intermediate 5a that was used for the subsequent coupling with amino acid ester 6a (for details of optimization of bases, amounts of substrate, and reaction times in the coupling between 5a and 6a, see ESI Table S3†) that afforded the desired tripeptide 7a. We successfully obtained the desired 7a in an 86% yield (30.5 s, 20 °C, 3 steps, Fig. 1). We were amazed that a rather pure peptide could be obtained after a simple aqueous work-up, and a simple recrystallization afforded a highly pure peptide without the need for column chromatographic purification. In addition, gram-scale (3.0 g) preparation of 7a was successfully achieved by simply extending the pumping time to 14 min 30 s (productivity: 12.3 g h^-1). To verify the importance of the micro-flow conditions, batch reactions were examined under the same reaction conditions with the exception of the residence time (0.5 s) of amino acid chloride 4a and z-NCa 1a (for details, see ESI Table S4†). Although the reaction mixture was vigorously stirred during the experiments, reproducible results were not observed due to batch-to-batch differences in mixing efficiency. Observed yields under batch conditions were ca. 15% lower than those under flow conditions. In particular, this reaction involves the generation of toxic gas such as sulfur dioxide and hydrogen chloride, and, therefore, scale-up of this reaction in a batch reactor should be avoided.

The substrate scope of our developed one-flow, 3CC approach was examined (Fig. 2). The use of Boc-protected amino acid was not suitable for this reaction due to z-NCa formation (for details, see ESI Table S6†), whereas the use of Cbz-protected amino acids afforded the desired tripeptides 7b–g in high yields (80–95%), although there also was a risk of z-NCa formation. The tripeptides 7h–p derived from Fmoc-protected amino acids afforded the desired products in high to excellent yields (83–94%). To our delight, the tripeptides 7h and 7i derived from sterically hindered Fmoc–Val–OH and Fmoc–Thr(Bn)–OH were obtained in high yields (86% and 87%). The tripeptides 7g and 7j derived from racemizable H–Cys(Bn)–NCA and Fmoc–Cys(t-Bu)–OH were also obtained in high yields (82% and 83%) without detectable racemization (<0.1%). Notoriously, none of the syntheses required column chromatographic purification, and the pure peptides were readily obtained by simple aqueous work-up with a subsequent recrystallization. A challenging synthesis of tripeptide 7q containing N-methyl amino acid residue was also examined (for
details of optimization of the amounts of N-methyl amino acid, reaction times, and temperatures for the coupling between 5a and H–MePhe–OMe, see ESI Table S5†), and 7q was obtained in an acceptable yield (51%).

We applied our developed approach for synthesis of the octapeptide 10b, which was shown in Scheme 2, is a beefy meaty peptide (H–Lys–Gly–Asp–Glu–Glu–Ser–Leu–Ala–OH). The preparation of amino acid chlorides from 3b–d and their couplings with α-NCA 1b–d was rapid (total 20.5 s). Thus, the process was carried out under micro-flow conditions. On the other hand, the following couplings with 6b, 7s, and 8b required a longer time (1–10 min), and, therefore, these were carried out under batch conditions. The first 3CC of 3b with 1b and 6b afforded crude tripeptide 7r, and the subsequent simple recrystallization afforded sufficiently pure 7r in 82% yield (HPLC purity 94%). We compared both the time and the cost of
Cbz group in 7r

available, inexpensive amino acids

a quantitative yield. The second 3CC of carbonic anhydride from 9b afforded a yield, HPLC purity 93%) and batch removal of the Cbz group via a one-flow 3CC approach from protected amino acids 3a, 3f, and 6b, which are inexpensive and both readily and commercially available.

Conclusions

We developed a one-flow 3CC approach to peptide chain elongation that is both rapid and mild. The proposed approach is the first to use α-NCAs as both electrophiles and nucleophiles. The key to success was the rapid generation of acid chloride and its rapid coupling with α-NCA in the presence of NMI and i-Pr₂NEt that suppressed undesired reactions of α-NCAs. The developed approach uses only readily removable reagents and substrates, which allows a simple aqueous work-up and recrystallization to afford the desired peptides in high yields and purities without tedious column chromatographic purifications. A scaled-up synthesis could be safely and readily achieved in a highly reproducible manner via simply extending the pumping time. In addition, we achieved a total synthesis of octapeptide, beefy meaty peptide, by repeating our developed 3CC approach with only the addition of one column chromatographic purification as the final step. The reductions in both time (ca. 1/31) and cost (ca. 1/7) were dramatic compared with that of typical solid-phase synthesis. Moreover, a one-flow tripeptide synthesis including in situ preparation of α-NCA was realized, which allowed a tripeptide to be rapidly prepared from readily and commercially available protected amino acid building blocks. The developed approach potentially produces longer peptides in one-flow fashion by injecting multiple NCAs. Although we challenged this powerful synthesis, we have not succeeded yet. The developed approach offers a novel and powerful option for the preparation of short peptides and should dramatically accelerate drug/material developments that are based on peptides/proteins.

Data availability

The synthetic procedures, characterization, and spectral data supporting this article have been uploaded as part of the ESI.

Author contributions

Naoto Sugisawa: conceptualization, data curation, formal analysis, methodology, investigation. Akira Ando: data curation, formal analysis, investigation. Shinichiro Fuse: conceptualization, data curation, project administration and supervision. All authors: writing – review & editing.
Conflicts of interest

There are no conflicts to declare.

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Notes and references