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## N-to-C peptide elongation by ammonia-Ugi reaction: synthesis of potent self-assembling elastin-like short peptides

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**The ammonia-Ugi reaction employing ammonium carboxylates of *N*-protected amino acids (or peptides), ketones, and  $\alpha$ -isocyano esters enabled N-to-C peptide elongation, together with *in situ* construction of  $\alpha,\alpha$ -disubstituted amino acid residues. This method offered an effective synthetic method of novel elastin-like short peptides, which exhibited highly potent self-assembling properties.**

Peptides possess considerable potential both as drug delivery vehicles and therapeutic agents against pharmacologically diverse and otherwise intractable biological targets.<sup>1</sup> Incorporation of unnatural  $\alpha,\alpha$ -disubstituted amino acid residues into peptides can significantly impact their metabolic resistance, hydrophobicity, membrane permeability, biological activity and selectivity, and immunogenicity, ultimately enhancing the druggability of peptide-based therapeutics.<sup>2</sup>

Peptide synthesis is typically performed *via* stepwise coupling of *N*-protected amino acids to a growing peptide chain anchored on a solid support using condensation agents.<sup>3</sup> Although such a solid-phase peptide synthesis (SPPS) works well with proteinogenic and other  $\alpha$ -monosubstituted amino acids, it often results in poor yields with sterically hindered  $\alpha,\alpha$ -disubstituted amino acids, requiring excessive amounts of coupling agents under harsh conditions such as heating or microwave irradiation.<sup>4</sup> Moreover, preparation of *N*-protected  $\alpha,\alpha$ -disubstituted amino acids generally requires multi-step and tedious processes. Alternatively, highly reactive condensation agents<sup>5</sup> and catalytic reaction systems<sup>6</sup> have offered efficient methods for unnatural peptide synthesis; however, these still use expensive unnatural  $\alpha,\alpha$ -disubstituted amino acids as substrates. These synthetic challenges have long

posed significant barriers to the discovery and development of bioactive peptides.

The ammonia-Ugi reaction, a variant of the Ugi reaction,<sup>7,8</sup> is a four-component coupling reaction that involves ammonia, an aldehyde or a ketone, a carboxylic acid, and an isocyanide, enabling a straightforward synthesis of peptides.<sup>9</sup> Although the ammonia-Ugi reaction had long been considered impractical and unsuccessful,<sup>10</sup> we recently reported an efficient synthetic protocol of unnatural dipeptides using the ammonia-Ugi reaction: by stirring ammonium carboxylates derived from *N*-protected amino acids **1**, ketones, and isocyanides in trifluoroethanol (TFE) at ambient temperature, a variety of dipeptides **2** were obtained in good yields (Scheme 1a).<sup>11</sup> Of note, these dipeptides **2** contained unnatural  $\alpha,\alpha$ -disubstituted amino acids, which were constructed *in situ* during the ammonia-Ugi reaction from readily available ketones as building blocks. Mechanistically, ammonium ( $\text{NH}_4^+$ ) dissociates into  $\text{NH}_3$  and  $\text{H}^+$ , which in turn facilitates the thermodynamically unfavourable *N*-unsubstituted imine formation (Scheme 1a).<sup>12</sup> With its success, however, the products **2** were obtained as amides at their C-termini, rendering them unsuitable for further N-to-C peptide elongation. To the best of our knowledge, neither the ammonia-Ugi reaction nor the Ugi reaction has ever been applied for N-to-C peptide chain elongation.<sup>13,14</sup> Here, this study proposes a novel strategy for N-to-C elongation of unnatural peptides by the ammonia-Ugi reaction employing  $\alpha$ -isocyano ester **3** as an elongation unit (Scheme 1b). The expected ammonia-Ugi adducts **4** possess ester moieties at their C-termini, providing a versatile platform for subsequent peptide chain elongation.

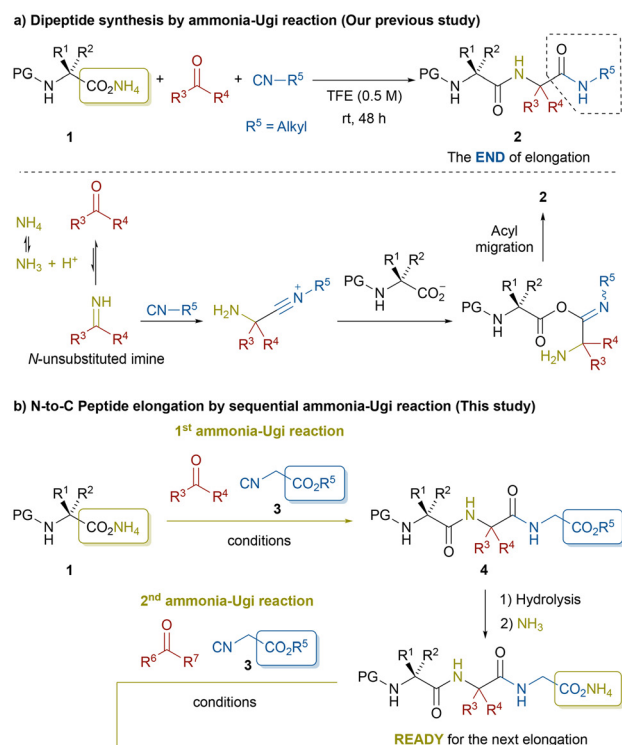
Using a commercially available ethyl isocyanoacetate (**3**, CN-Gly-OEt), a series of unnatural tripeptides **4** were synthesized (Scheme 2). The key ammonium carboxylates of *N*-protected amino acids **1** were prepared by stirring *N*-protected amino acids with aqueous ammonia in acetonitrile or THF at 0 °C (Table S1).<sup>11</sup> Then, the ammonia-Ugi reaction using Boc-Phe-ONH<sub>4</sub>, cyclopentanone, and CN-Gly-OEt (**3**) afforded the tripeptide Boc-Phe-Ac<sub>5</sub>C-Gly-OEt (**4a**) in

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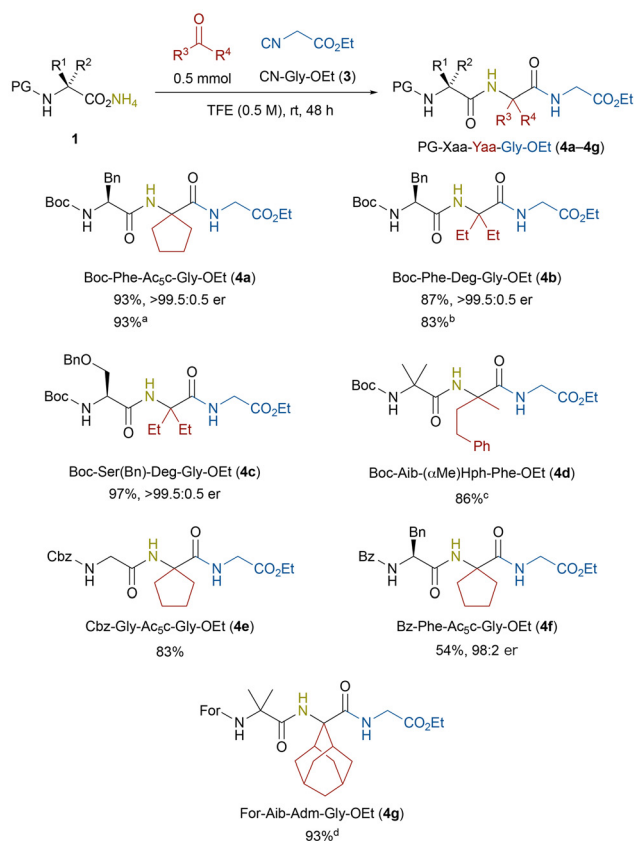
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**Scheme 1** (a) Dipeptide synthesis by ammonia-Ugi reaction, (b) N-to-C peptide elongation by sequential ammonia-Ugi reaction (this study).

93% yield, with no detectable racemization of the chiral  $\alpha$ -carbon of phenylalanine (>99.5:0.5 er). Herein, the unnatural 1-aminocyclopentane-1-carboxylic acid (Ac<sub>5</sub>c) residue in **4a** was constructed *in situ* from cyclopentanone as a substrate. In general, diethylglycine (Deg) is a challenging substrate to be incorporated into peptides under conventional SPPS conditions because of the steric hindrance,<sup>15</sup> whereas the present ammonia-Ugi reaction successfully delivered Boc-Phe-Deg-Gly-OEt (**4b**) in 87% yield. Again, no racemization was observed during this process (>99.5:0.5 er). The chiral  $\alpha$ -carbon of serine is known to be susceptible to racemization during peptide synthesis;<sup>16</sup> however, the present reaction conditions afforded **4c** in an excellent yield (97%) with a perfect stereochemical integrity (>99.5:0.5 er). Even the sterically demanding Boc-Aib was compatible with the present reaction conditions, affording the sterically congested tripeptide **4d**, composed of contiguous  $\alpha,\alpha$ -disubstituted amino acids [ $\alpha$ -aminoisobutyric acid (Aib) and  $\alpha$ -methylhomophenylalanine [( $\alpha$ Me)Hph]}, in 78% yield. In addition to Boc, various *N*-protecting groups, including Cbz, Bz, and formyl (For), were well tolerated, giving the tripeptides **4e**, **4f**, and **4g** in 83%–99% yields. Unfortunately, the stereochemical integrity of Bz-Phe was slightly lost (98:2 er), whereas that of Ac-Phe remained intact under the same conditions.<sup>11</sup> It is worth noting that the *N*-formyl group in **4g** can

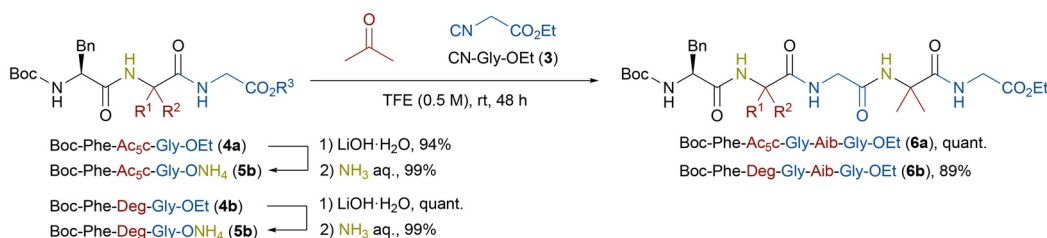


**Scheme 2** Synthesis of tripeptides **4a–4g** by ammonia-Ugi reaction using ammonium carboxylates of *N*-protected amino acids. <sup>a</sup> 5.4 mmol, <sup>b</sup> 3.5 mmol, <sup>c</sup> 1 mmol, <sup>d</sup> 4.5 mmol.

serve as a precursor of an isocyano group, potentially enabling inverse C-to-N peptide elongation.<sup>14</sup> Scale-up experiments (up to 5.4 mmol) proceeded smoothly without any detrimental effects on the reaction system, providing the corresponding products **4a**, **4b**, and **4g** in excellent yields (83%–93%). Overall, the ammonia-Ugi reaction using  $\alpha$ -isocyano ester and *N*-protected amino acids enabled the efficient synthesis of unnatural tripeptides containing  $\alpha,\alpha$ -disubstituted amino acids. These tripeptides possess ester groups at their C-termini, offering a platform for subsequent N-to-C peptide chain elongation.

Peptides containing glycine are frequently found in biomaterials<sup>17</sup> such as elastin,<sup>18</sup> collagen,<sup>19</sup> and silk fibroin.<sup>20</sup> Owing to glycine's small size and conformational flexibility, it plays a crucial role in modulating peptide structure and function. The development of efficient synthetic strategies for unnatural analogues of glycine-rich peptides is therefore of significant interest in both synthetic and biomedical research. In this study, we developed an efficient N-to-C peptide elongation method for synthesizing glycine-containing unnatural peptides by the ammonia-Ugi reaction employing CN-Gly-OEt (**3**) (Scheme 3). The C-terminal ester moieties of tripeptides **4a** and **4b** were hydrolyzed under basic conditions and subsequently treated with aqueous ammonia to give the corresponding ammonium carboxylates **5a** and **5b** in excellent yields. Under the present





**Scheme 3** Synthesis of pentapeptides **6a** and **6b** by ammonia-Ugi reaction starting from ammonium carboxylates of *N*-protected tripeptides **4a** and **4b**.

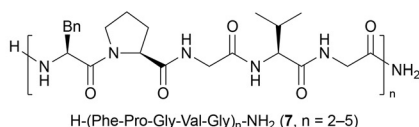
ammonia-Ugi reaction conditions, both tripeptides were successfully elongated into the pentapeptides Boc-Phe-Xaa-Gly-Aib-Gly-OEt **6a** and **6b** in good yields, together with *in situ* construction of Aib residue from acetone. The resulting peptides again possess ester moieties at C-termini, potentially providing a versatile platform for further N-to-C elongation or other chemical modifications. Totally, starting from *N*-protected amino acids **1**, the first ammonia-Ugi reaction furnished tripeptides **4** (Scheme 2), and the second ammonia-Ugi reaction extended them to pentapeptides **6** (Scheme 3). Each step elongated peptides by two amino acid residues at a time while constructing  $\alpha,\alpha$ -disubstituted amino acids *in situ*. This streamlined approach requires no condensation agents, thus providing an environmentally friendly synthetic method of glycine-containing unnatural peptides.

The obtained pentapeptides **6a** and **6b** share the repeating amino acid sequence of a short elastin-like peptide (sELP) H-(Phe-Pro-Gly-Val-Gly)<sub>n</sub>-NH<sub>2</sub> (**7**, Scheme 4a).<sup>21</sup> sELP exhibits reversible lower critical solution temperature (LCST)-type behaviour, being soluble at low temperatures and insoluble at high temperatures.<sup>22</sup> Such reversible temperature-responsive and self-assembling properties make sELP a promising candidate for drug delivery applications. The development of unnatural analogs with enhanced self-assembling properties is therefore of considerable interest. Herein, we designed a novel unnatural analogue, H-(Phe-Ac<sub>5</sub>c-Gly-Aib-Gly)<sub>2</sub>-NH<sub>2</sub> (**8a**), by modifying the original sELP sequence (Scheme 4b). Specifically, the Pro-2 in **7** was replaced with Ac<sub>5</sub>c, a noncanonical amino acid structurally related to Pro, and the Val-4, located at a guest position in the original sequence,<sup>23</sup> was substituted with Aib, a noncanonical amino acid analogous to Val.

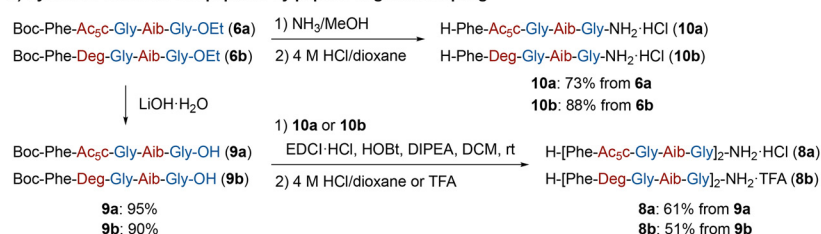
The decapeptide **8a** was synthesized in a good yield *via* segment coupling between Boc-Phe-Ac<sub>5</sub>c-Gly-Aib-Gly-OH (**9a**) and H-Phe-Ac<sub>5</sub>c-Gly-Aib-Gly-NH<sub>2</sub> (**10a**) under standard condensation conditions, followed by Boc deprotection under acidic conditions. We also designed and synthesized a structurally related analogue, H-(Phe-Deg-Gly-Val-Gly)<sub>2</sub>-NH<sub>2</sub> (**8b**), in which Ac<sub>5</sub>c residue in **8a** was replaced with Deg, a noncyclic analogue of Ac<sub>5</sub>c. Both **8a** and **8b** were obtained as TFA salts in pure form after purification by reversed-phase (RP)-HPLC (Fig. S1). Additionally, we designed and synthesized H-(Phe-Pro-Gly-Aib-Gly)<sub>2</sub>-NH<sub>2</sub> (**11**) as an Aib analogue of the original sELP **7** *via* a standard SPPS protocol (see, SI).

The self-assembling properties of peptides **8a**, **8b**, and **11** were then investigated under buffered aqueous conditions. Each peptide was dissolved in phosphate buffer containing NaCl (pH 7.4; 27.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 17.8 mM NaH<sub>2</sub>PO<sub>4</sub>, and 3M NaCl), and their turbidities at 400 nm were recorded upon increasing and decreasing temperatures (Fig. 1a, b and Table S2). The results revealed that these synthetic peptides **8a** and **8b** exhibited reversible LCST behaviour at concentrations of approximately 1–3.5 mM. The control peptide **11** showed the similar behaviour; however, it required significantly higher concentrations to initiate aggregation compared to the peptides **8a** and **8b** (Fig. 1c). Herein, the transition temperature (*T*<sub>t</sub>) was defined as the temperature at which turbidity reached half of its maximum value during heating. The relationship between *T*<sub>t</sub> and peptide concentration of **8a** and **8b** fitted well to a power function (*T*<sub>t</sub> = *aC*<sup>*b*</sup>), while that of **11** followed a conventional logarithmic function (*T*<sub>t</sub> = *a* log(*C*) + *b*) as previously demonstrated for ELPs, where *C* is peptide concentration and *a* and *b* are constants (Fig. 1d).<sup>24</sup> A clear correlation between

**a)** Short elastin-like peptide (sELP) with self-assembling ability

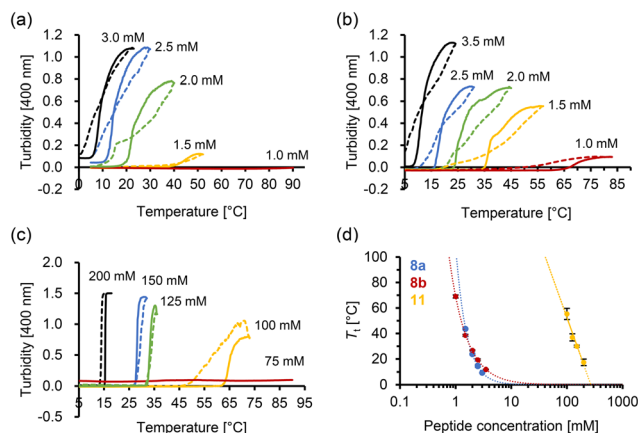


**b)** Synthesis of elastin-like peptides by peptide fragment coupling



**Scheme 4** (a) Chemical structure of short elastin-like peptide (sELP) **7**, (b) synthesis of unnatural sELP analogues **8a** and **8b** by conventional peptide segment coupling.





**Fig. 1** Turbidity measurements of peptides **8a** (a), **8b** (b), and **11** (c), and the correlation between  $T_t$  and concentration (d). In (a)–(c), the solid and dashed lines represent the turbidity profiles upon heating and cooling, respectively. Measured  $T_t$  values of peptides **8a** and **8b** were fitted with power functions, while that of **11** was fitted with conventional logarithmic functions.  $T_t$  values are shown with standard error.

peptide concentration and  $T_t$  demonstrated that the self-assembling capabilities of unnatural analogues **8a** and **8b** were approximately 100-fold stronger than that of reference peptide **11** (Fig. 1d). The reversibility of structural changes in **8a** and **8b** was roughly investigated by circular dichroism measurements (Fig. S2 and S3). Furthermore, the self-assembling behaviours were confirmed by bright-field microscopy (Fig. S4) and dynamic light scattering measurements (Fig. S5). Taken together, both peptides **8a** and **8b** were found to form aggregates above their respective  $T_t$  values. Ultra performance liquid chromatography (UPLC)-MS analysis showed peptides **8a** and **8b** were more hydrophobic than peptide **11** (Fig. S1), suggesting that the increased hydrophobicity of **8a** and **8b** may contribute to their enhanced self-assembling properties.

## Conclusions

This study, although preliminary, developed a streamlined N-to-C peptide elongation method for synthesizing unnatural peptides using the ammonia-Ugi reaction. Starting from readily available *N*-protected amino acids, ketones, and  $\alpha$ -isocyano ester as building blocks, a series of tripeptides **4a–4g** were synthesized in high yields and with excellent stereochemical integrity. Notably, the sterically demanding residues such as Aib, Ac<sub>5</sub>C, ( $\alpha$ Me)Hph, and Deg were constructed *in situ* from simple ketones, and successfully incorporated into peptides, overcoming limitations of conventional peptide synthesis. The tripeptides **4** were further elongated into pentapeptides **6** via the second ammonia-Ugi reaction, demonstrating the method's versatility. Interestingly, both **8a** and **8b** exhibited reversible LCST behaviour at approximately 100-fold lower concentrations than **11**, due probably to the increased hydrophobicity. Future work will focus on synthesis of longer peptides and more sterically congested peptides using  $\alpha$ -isocyano

$\alpha$ , $\alpha$ -disubstituted amino acids. Extensions in these directions will be reported from our laboratory in due course.

## Author contributions

KT, conceptualization, investigation, methodology, and writing – original draft; KT, NT, YK, MU, MA, HK, investigation; HN and TN, supervision and writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: experimental procedures, supplementary figures and tables, NMR spectra of all new compounds, and HPLC charts. See DOI: <https://doi.org/10.1039/d5ob01834j>.

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