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Sequence-Selective Decapeptide Synthesis by the Parallel Operation of Two Artificial Molecular Machines

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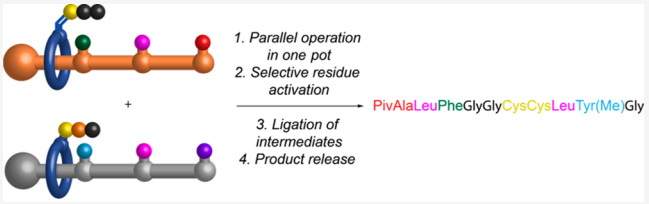
ABSTRACT: We report on the preparation of a decapeptide through the parallel operation of two rotaxane-based molecular machines. The synthesis proceeds in four stages: (1) simultaneous operation of two molecular peptide synthesizers in the same reaction vessel; (2) selective residue activation of short-oligomer intermediates; (3) ligation; (4) product release. Key features of the machine design include the following: (a) selective transformation of a thioproline building block to a cysteine (once it has been incorporated into a hexapeptide intermediate by one molecular machine); (b) a macrocycle-peptide hydrazine linkage (as part of the second machine) to differentiate the intermediates and enable their directional ligation; and (c) incorporation of a Glu residue in the assembly module of one machine to enable release of the final product while simultaneously removing part of the assembly machinery from the product. The two molecular machines participate in the synthesis of a product that is beyond the capability of individual small-molecule machines, in a manner reminiscent of the ligation and post-translational modification of proteins in biology.



INTRODUCTION

Chemists are adopting principles from the natural world to help develop artificial molecular machines capable of increasingly sophisticated operations.[1] Most examples of functional synthetic molecular machines[2−8] to date feature a single type working alone to achieve a task.[9−11] This contrasts with the complex and tightly regulated functional networks common in biology.[12] An example of the latter is ubiquitination, in which ubiquitin and a substrate peptide are made independently by ribosomes before being subsequently ligated by the cooperative action of enzymes.[13,14] This produces a protein product that is beyond the scope of synthesis by a single ribosome alone.

Rotaxane-based artificial molecular machines have previously been developed that produce short sequence-specific oligomers, most commonly peptides, in an approach inspired by aspects of the way the ribosome builds proteins.[15−32] However, a significant limitation of current track-based molecular synthesizers is that when each amino acid is extracted from the strand, the resulting cyclic transition state that transfers the amino acid to the terminus of the growing chain increases in size.[15] Every addition slows the transfer of further amino acids until eventually other processes (intermolecular reactions, hydrolysis, etc.) compete with the machine operation and the machine stops working. This effectively limits the size of product that can be made through such an approach. Inspired by the strategy evolution has selected for ubiquitination, we considered that a solution to the chain-length limitations of rotaxane-based molecular synthesizers might be to operate two different artificial molecular machines in one pot and then ligate their products *in situ* (Figure 1). In this way track-sequence information could be processed by multiple machines and then added together to generate a product not obtainable through the use of a single machine alone.

Molecular Machine Designs and Operation Pathway. The strategy behind such a molecular machine-driven synthesis is outlined in Figure 1. A decapeptide product of specific sequence is produced by a series of functional modules that make up two molecular machines and their tracks. The synthesis occurs in four consecutive stages: (1) Parallel operation of the two rotaxane-based molecular machines; (2) selective residue activation by release of the *C*-terminus hydrazide and *N*-terminus cysteine groups of the machine products; (3) ligation of the resulting short-sequence intermediate peptides; and (4) final product release through the activation of a glutamate residue installed in the machine **I** assembly module.

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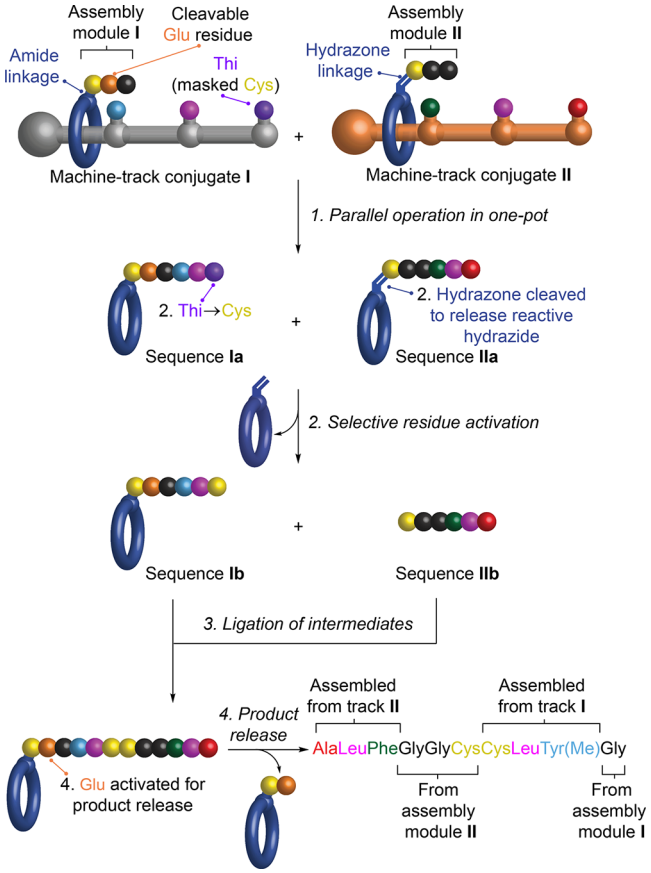

Figure 1. Decapeptide synthesis by a pair of modular artificial molecular machines: 1. Parallel operation of two molecular synthesizers with different assembly modules, assembly module-macrocycle linkages and track sequences; 2. Selective residue activation; 3. Ligation of short-sequence intermediates; 4. Release of the decapeptide product.

The molecular machine designs are related to previous rotaxane-based peptide synthesizers[15,19−21] and incorporate the following features:

(i) The rotaxane architecture confers processivity (the ring cannot dethread or exchange with others in the bulk until all the barriers have been removed).

(ii) The unreactive bulky "stopper" at the left-hand terminus of the axle provides the directionality necessary for sequence-specific synthesis (the component dynamics are not ratcheted, so the ring moves incessantly in both directions along the available section of track, but only movements away from the stoppered end of the track bring the ring-bound assembly module close enough to an axle-bound building block for a reaction to occur).

(iii) The reactive barriers are amino acyl derivatives, and the sequence they appear on the track is the sequence they are incorporated in the machine-made short-oligomer intermediate.

(iv) The macrocycle contains both a pyridine group, necessary for rotaxane formation of the machine-track conjugate by active template synthesis,[33−46] and an assembly module featuring a cysteine residue at one end of a tripeptide and an elongation site for the growing oligomeric chain at the other.

(v) The machines operate through multiple successive native chemical ligation (NCL) reactions,[47] the thiol of the cysteine abstracting a building block from the track to form a thioester, followed by transfer of the acyl group to the amino terminus of the elongation site, simultaneously forming a new peptidic bond and regenerating the catalytic thiol group for further barrier-removing reactions.

(vi) The lack of strong binding interactions between the ring and track, often a residual feature in rotaxanes not prepared by active template synthesis, ensures the ring movement between the track barriers is as unrestricted as possible.

(vii) Rigid spacers between the building blocks on the track inhibit track folding, preventing reactions out of sequence.

The following new features were introduced for the cooperative synthesis shown in Figure 1:

(viii) The machines were operated simultaneously in the same reaction vessel, but at high dilution to disfavor intermolecular reactions.

(ix) The assembly modules are connected to machine **I** and **II** by different functional groups (amide and hydrazone, respectively) to allow the chemoselective revealing of the *C*-terminus of one short-sequence intermediate.

(x) Track **I** contains a thioproline (Thi) residue that can be unmasked[48] following machine operation to provide a Cys residue that enables subsequent ligation[49] of the short-sequence intermediates.

(xi) Assembly module **I** contains a Glu as the second amino acid, enabling the first two amino acids arising from assembly module **I** to be removed from the final product during its liberation from the machine. Like many of our design solutions for artificial machines that work at the molecular level, this feature is inspired by considering how biology solves a related problem. In eukaryotic ribosomal protein synthesis, each sequence is initiated with a methionine residue codified by the start codon AUG. Following the ribosome operation the now-superfluous *N*-terminal methionine is cleaved by methionine aminopeptidase (MAP) to yield the final peptide product.[50]

In *C*-to-*N* sequence, the decapeptide product formed in Figure 1 consists of the following: one amino acid from assembly module **I** (AA1: Gly), AA2−4 incorporated in sequence from track **I** (AA2: Tyr(Me); AA3: Leu; AA4: transformed from Thi to Cys), AA5−7 from assembly module **II** (AA5: Cys; AA6: Gly; AA7: Gly), AA8−10 incorporated in sequence from track **II** (AA8: Phe; AA9: Leu; AA10: Ala).

RESULTS AND DISCUSSION

Synthesis of Molecular Machine-Track Conjugates 1 and 2. Molecular machine-track conjugates **1** and **2** were prepared in a rotaxane elongation strategy[19] via successive Cu(I)-catalyzed alkyne−azide cycloaddition (CuAAC) reactions of various synthons (Scheme 1). Active template CuAAC synthesis[33] generated one-barrier [2]rotaxanes **3** and **4** (see Supporting Information for synthesis and characterization, sections 3.1 and 3.2). The mechanically interlocked structure of each species was confirmed by mass spectrometry and ^{1}H NMR (see Supporting Information). Machine **1** was synthesized by CuAAC coupling of rotaxane **3** and the two-barrier fragment **5** (Scheme 1, route a). The assembly module for this molecular machine was attached to the macrocycle

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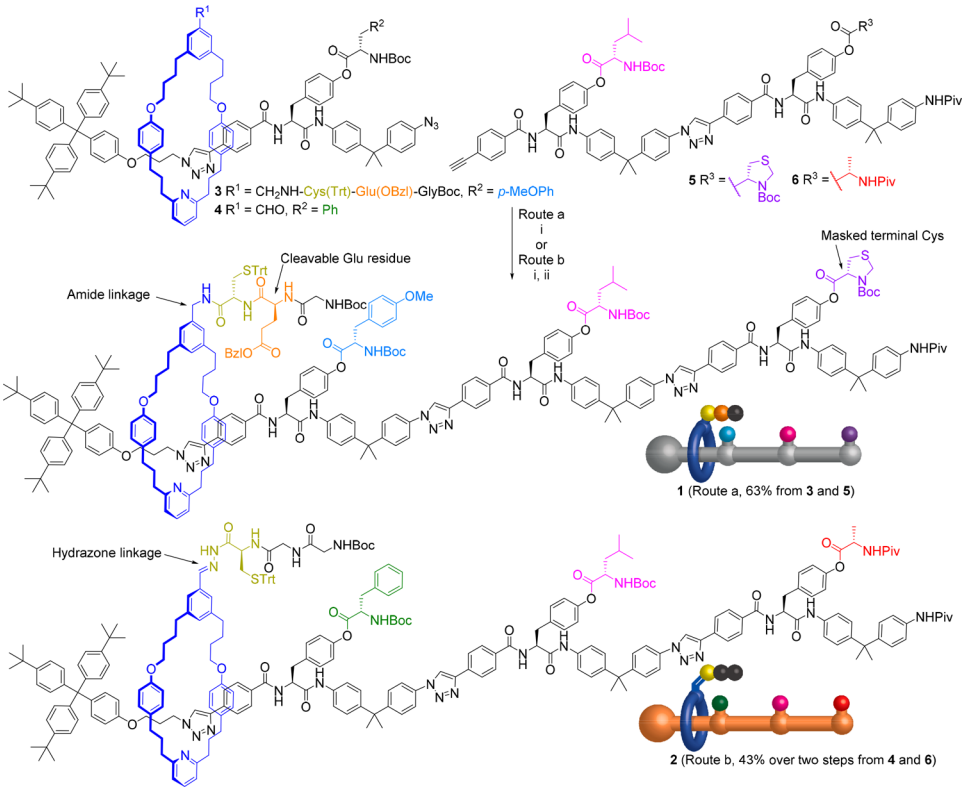
Scheme 1. Synthesis of 1 and 2 by Elongation of the Corresponding One-Barrier Rotaxane Synthons[a]



[a]Reagents and conditions: Route a: (i) **3** (1.0 equiv), **5** (1.5 equiv), $Cu(MeCN)_4PF_6$ (1.5 equiv), Tentagel TBTA resin (3.0 equiv), CH_2Cl_2, *t*-BuOH (5:1), rt, 48 h, 63%. Route b: (i) **4** (1.0 equiv), **6** (1.0 equiv), $Cu(MeCN)_4PF_6$ (0.45 equiv), Tentagel TBTA resin (0.5 equiv), CH_2Cl_2, *t*-BuOH (8:1), rt, 48 h, 86%. (ii) BocGlyGlyCys(Trt)$NHNH_2$, aniline, CH_2Cl_2, rt, 48 h, 50%.

prior to rotaxane formation (see Supporting Information section 3.1 and 3.2 for synthesis). Molecular machine-track conjugate **2** was assembled by the CuAAC reaction of **4** with the two-barrier extension unit **6**,[19] followed by condensation with the Boc-Gly-Gly-Cys(Trt)-$NHNH_2$ assembly module **II** (Scheme 1, route b).

Individual Operation of Machine-Track Conjugates 1 and 2. With both machines in hand, we investigated their operations individually (Figure 2A and 2B, and Supporting Information). Each machine-track conjugate was separately subjected to global deprotection with trifluoroacetic acid and triisopropylsilane (removing the Boc and trityl groups). The resulting protecting-group-free machines were then subjected to a standard set of operation conditions: Each machine was heated in a microwave in the presence of diisopropylethylamine (*i*-Pr_2NEt) and 4 Å molecular sieves in a mixture of acetonitrile/dimethylformamide at 75 °C for 15 h. The operation was monitored by mass spectrometry (Figure 2A and 2B; see Supporting Information, sections 3.3 and 3.4 for details) which in each case confirmed the formation of the desired peptide products (**7** and **8**, respectively). The sequence fidelity of these hexapeptide intermediates was confirmed by tandem mass spectrometry (Figure 2 A and 2B, bottom). In accordance with previous observations on small-molecule peptide synthesizers,[19,20] we also detected some truncated products arising from the hydrolysis of barriers, whose intensity in the mass spectrum does not necessarily correlate with their relative abundance in the product mixture.

Parallel Operation of Machines 1 and 2. Having confirmed the successful operation of each machine individually, we explored their contemporaneous operation within the same reaction vessel. The two machines were combined, deprotected, and operated under the standard conditions (Figure 2C, Supporting Information section 3.5). Pleasingly, the mass spectrum of the one-pot operation of **1** and **2** is virtually a superimposition of the spectra from the individual operation of each machine (Figure 2C, right), with both of the desired hexapeptide products (**7** and **8**) formed. Crucially, no byproducts were observed corresponding to intermolecular reactions (neither out of sequence nor higher order peptides), confirming that the two machines operate in parallel without effecting the outcome from the other's.

Selective Residue Activation of Sequences 7 and 8. Next we looked at coupling the two hexapeptide products of operation by a hydrazide ligation protocol. To do so the thioproline of **7** and hydrazone of **8** needed to be converted to the corresponding terminal cysteine, **9**, and hydrazide **10**, respectively (Scheme 2 and Supporting Information, section 3.7). Conveniently, the deprotection of thioproline is reported to proceed under mild conditions with methoxyamine hydrochloride,[51] while the same conditions should cleave the hydrazone in **8** via an exchange reaction. Accordingly, the mixture of **7** and **8** was treated with methoxyamine hydrochloride in MeOH at 50 °C. Pleasingly, hydrazide **10** was liberated from the macrocycle accompanied by deprotection of the thioproline to generate the *N*-terminal cysteine residue on the amide macrocycle (**9**).

Ligation of Intermediates 9 and 10. Following selective residue activation the resulting mixture was subjected to hydrazide ligation conditions.[52] Initially, the hydrazide is

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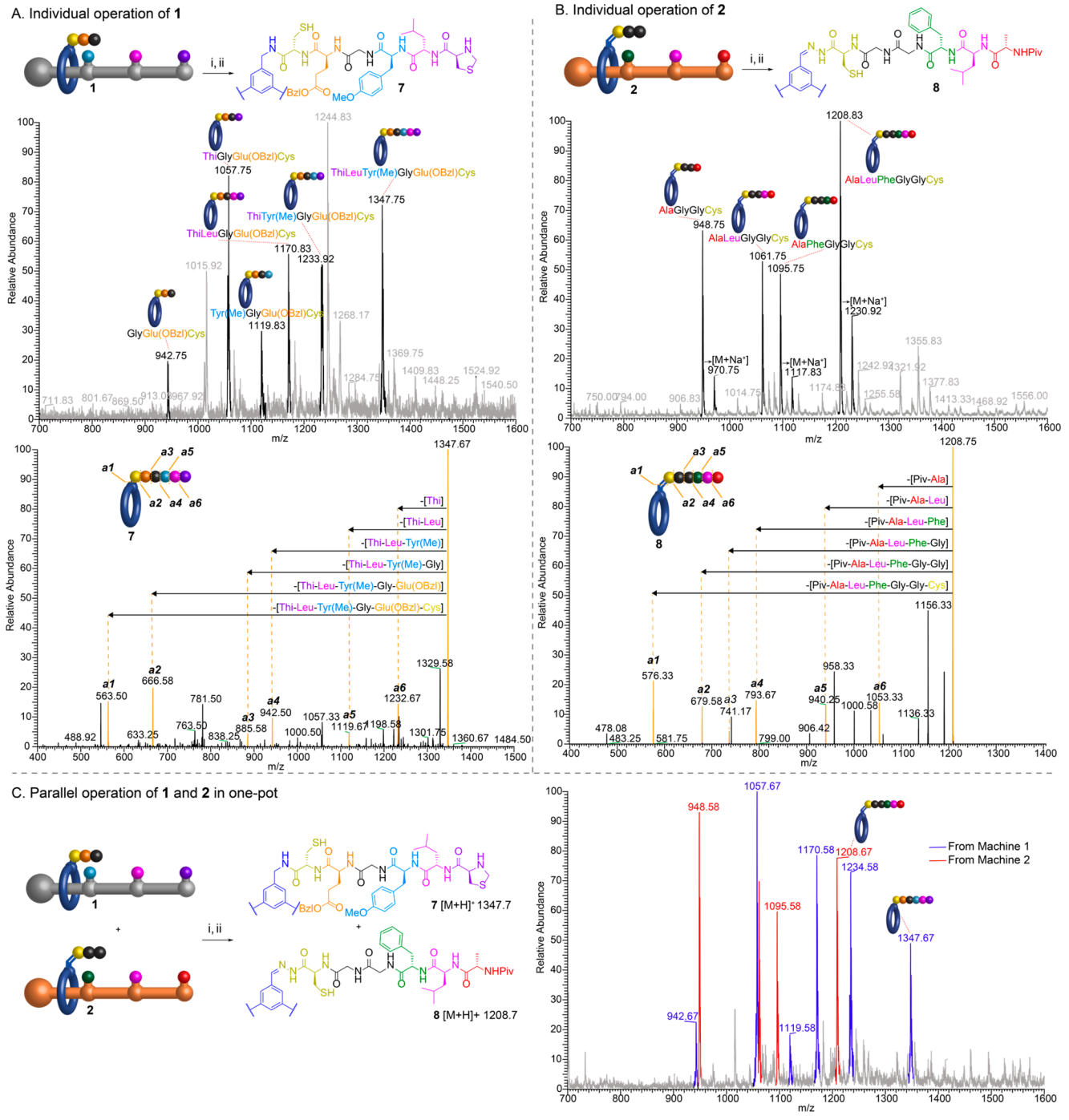

Figure 2. (A) Top: Mass spectrum of the reaction mixture following the operation of machine **1**. Signals in gray correspond to adducts with dimethylformamide (DMF; e.g. 1015 = 942 + DMF; 1244 = 1171 + DMF) and minor unidentified species. Bottom: Tandem mass-spectrometry sequencing of **1**. (B) Top: Mass spectrum of the reaction mixture following the operation of machine **2**. Bottom: Tandem mass-spectrometry sequencing of **2**. (C) Left: Parallel operation of both molecular machines, **1** and **2**, in the same reaction vessel ("one-pot" synthesis). Right: Mass spectrum of the reaction mixture following the one-pot operation of **1** and **2**. Peaks in blue correspond to products arising from the operation of machine **1**; peaks in red correspond to products arising from the operation of machine **2**. Reagents and conditions: (i) $(i\text{-Pr})_3SiH$, CF_3CO_2H, CH_2Cl_2, rt, 30 min. (ii) $i\text{-Pr}_2NEt$, MeCN/DMF (3:1), 4 Å molecular sieves, 75 °C, MW, 15 h.

oxidized at pH 3 by $NaNO_2$ to the corresponding acyl azide (Scheme 2B, hydrazide oxidation),[53] after which a large excess of 4-mercaptophenylacetic acid (MPAA) is added and the pH is adjusted to 7 by the use of buffer. The added MPAA has a multipurpose role: (i) It forms a thioester by reaction with the acyl azide (Scheme 2 B, thiolysis), (ii) it reduces any oxidized cysteine species back to the thiol, and (iii) it reacts with, thus removing, the residual $NaNO_2$.[49] Next, transthioesterification of the thioester and the terminal cysteine of **9** takes place to join the fragments (Scheme 2 B, transthioesterification). Finally, a 1,5-*S*,*N*-acyl shift completes the reaction (Scheme 2B, S to *N* acyl shift), rendering dodecapeptide **11**.

After 3 h, tris(2-carboxyethyl)phosphine (TCEP) was added to the reaction mixture to reduce any oxidized thiols formed

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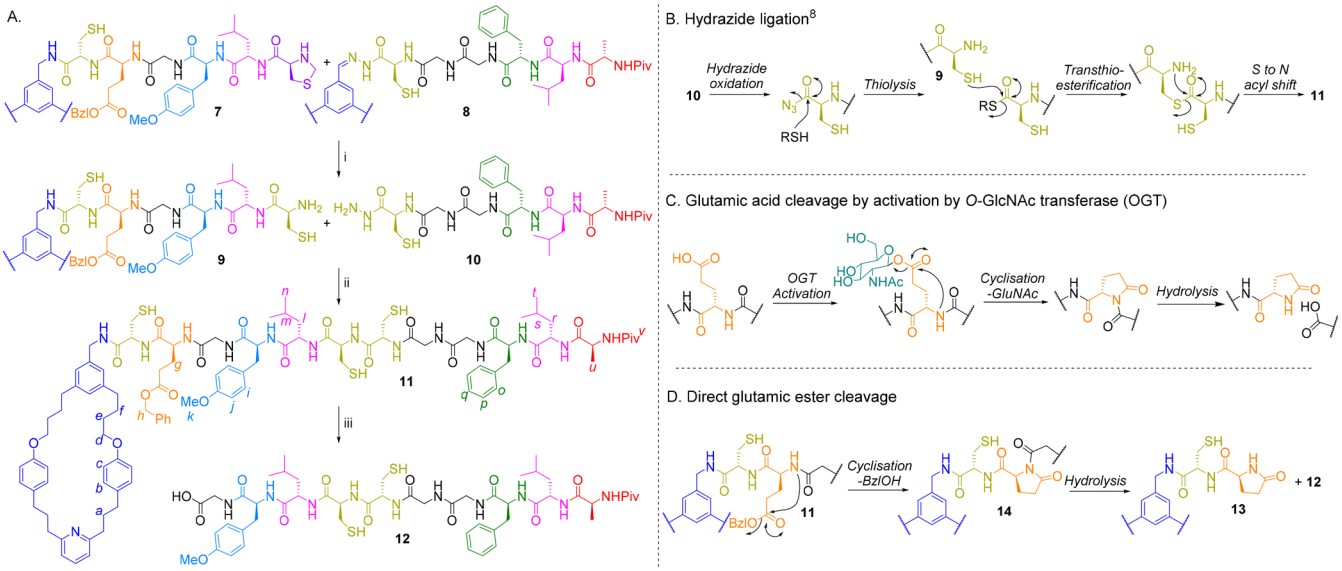
Scheme 2. (A) Ligation of Short Oligomer Intermediates to Form a Single Peptide; (B) Mechanism of Ligation; (C) Mechanism of Glutamic Acid Cleavage Mediated by OGT and (D) Direct Glutamic Ester Cleavage[a]



[a]Reagents and conditions: (i) $MeONH_2$·HCl, MeOH, 50 °C, 3 h. (ii) pH = 3, $NaNO_2$, 6 M guanidinium chloride, 0.2 M Na_2HPO_4, HFIP (hexafluoro-2-propanol), −10 °C, 20 min, then pH = 7, 4-mercaptophenylacetic acid, rt, 3 h, 25% over 2 steps. (iii) LiOH, THF/H_2O, rt, 30 min. The double-headed arrows are used as shorthand to indicate the formation and collapse of tetrahedral intermediates in the mechanism.

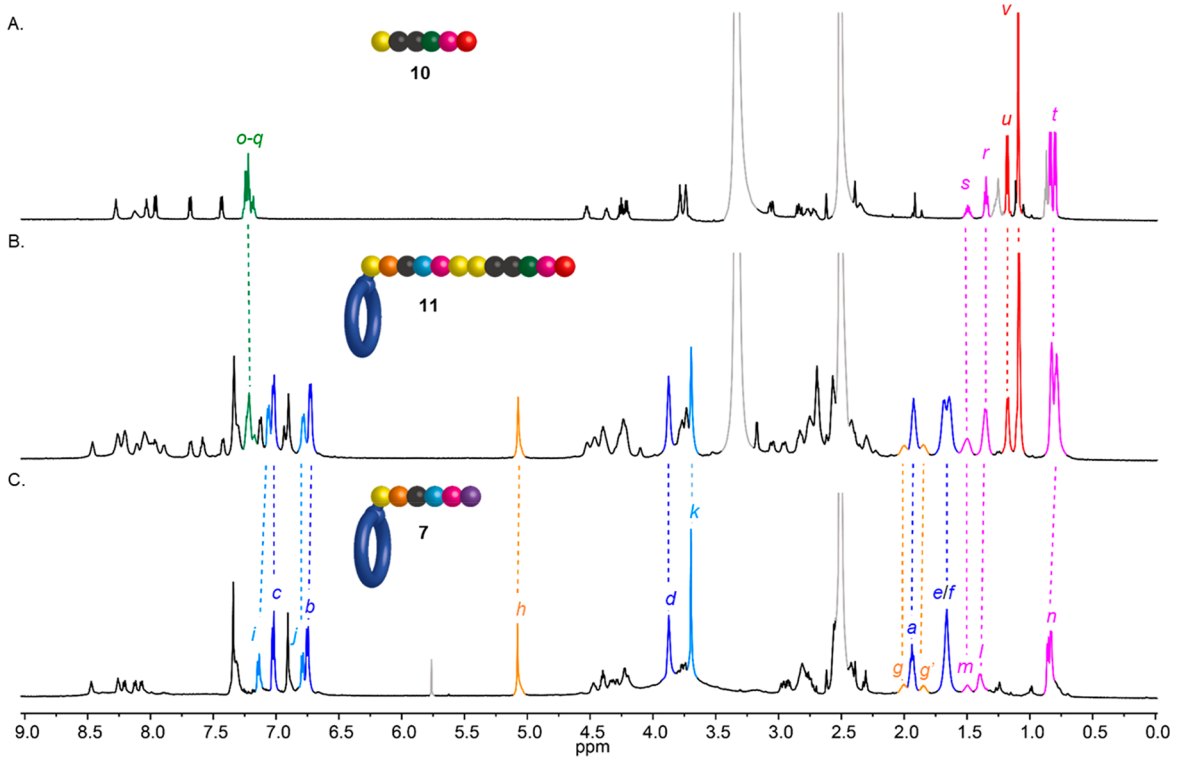


Figure 3. Partial ^{1}H NMR (600 MHz, DMSO-d_6, 298 K) of (A) hydrazide **10** (unpurified reaction product); (B) ligated peptide **11**; and (C) amide **7** (unpurified reaction product). Dashed lines indicate analogous proton resonances in the different molecules. Letters correspond to the labeling shown in Scheme 2. Peaks shaded gray correspond to residual solvent.

during the reaction. The ligation product was purified by precipitation in methanol and isolated in 25% yield. The identity of **11** was confirmed by HR-ESI(+) mass spectrometry and NMR (see Supporting Information, section 3.7). Qualitative comparison of the ^{1}H NMR spectrum of **11** with the intermediates **7** and **10** (Figure 3)[54] showed incorporation of diagnostic peaks from both fragments, such as signals from the amide-linked macrocycle (H_a–H_f), glutamic ester (H_g–H_h), and methyl tyrosine (H_i, H_j, and H_k), as well as from the phenyl alanine (H_o, H_p, and H_q) and alanine (H_u and H_v) originating from the hydrazide fragment, together with signals from both leucine residues (protons H_r–H_t and H_l–H_n).

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Release of Decapeptide 12 by Glutamic Ester Cyclization. Finally, we investigated removal of the ligated peptide from the remaining macrocycle and cysteine residue of assembly module **I** by selective glutamic acid cleavage.[55] We originally envisaged an approach based on the naturally occurring backbone protein cleavage of human cell factor 1 (HCF-1), where a glutamic acid is activated as the glycosyl glutamate ester by *O*-GlcNAc transferase (OGT) (Scheme 2C, OGT-activation). This ester is then converted to a transient pyroglutamate intermediate (Scheme 2C, cyclization and loss of GluNAc) that spontaneously undergoes hydrolysis to the carboxylic acid and lactam (Scheme 2C, hydrolysis).[55] Since the Glu residue in **11** is an *O*-Bzl ester, we reasoned it might undergo a similar transformation. Accordingly, **11** was reacted with LiOH for 30 min at room temperature (Scheme 2 D and Supporting Information, section 3.8) and we were pleased to observe direct formation of the desired decapeptide acid **12** together with the corresponding lactam **13**. Presumably the mechanism is analogous to HCF-1 cleavage, via an imide (**14**) formed by direct attack of the amide on the benzyl ester (benzyl alcohol as the leaving group, Scheme 2D, cyclization). Imide **14** is then cleaved under the basic aqueous conditions to give decapeptide **12** (Scheme 2D, hydrolysis), with *N*-to-*C* sequence PivAlaLeuPheGlyGlyCysCysLeuTyr(Me)Gly.[56−58]

CONCLUSIONS

Two artificial molecular machines, each composed of several functional modules, can work in parallel to carry out the sequence-selective synthesis of peptides in one pot without crossover or scrambling. The machine designs incorporate fragments that can be activated to allow intermediates to be ligated and released from the machine, the latter in a process reminiscent of the cleavage of a "starting codon". The overall synthesis generates a decapeptide of programmed sequence, a product length unreachable with the current generation of artificial molecular synthesizers acting alone. Nature's solutions to the challenges posed by operating machinery at the nanoscale continue to serve as useful examples to guide the design of increasingly complex artificial molecular machines capable of performing increasingly demanding tasks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c01234.

Experimental procedures, characterization of new compounds, and spectroscopic data (PDF)

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Notes

The authors declare no competing financial interest.

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