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Communication

Constructing Head-to-Tail Cyclic Peptide DNA-Encoded Libraries Using Two-Directional Synthesis Strategy

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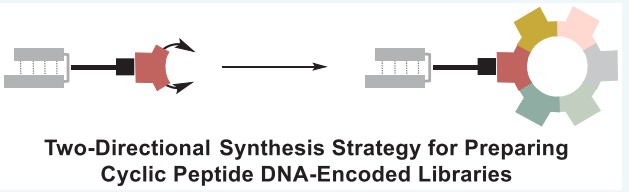
ABSTRACT: Macrocyclic peptides are an important class of therapeutic agents for the biological targets that are difficult to modulate by small-molecule compounds. Meanwhile, DNA-encoded library technology (DELT) provides a powerful platform for hits discovery. The unity of both fields has proven highly productive in finding cyclic peptide hits against diverse pharmaceutical proteins. Many researchers have extended the chemical toolbox for constructing head-to-tail macrocyclic DNA-encoded libraries with various ring sizes. However, the linear peptides of different lengths necessitate tuning the distance between closing sites and DNA-linked sites to perform the macrocyclization process, presumably due to the constrained conformation of linear precursors. To tackle this issue and streamline the synthetic workflow, we report a two-directional synthesis strategy. This method starts from a trifunctional reagent and prepares DNA-linked macrocyclic peptides of ring size between 15 (5-mer) and 24 (8-mer) via amide bond formation reaction, a common method to create macrocyclic peptides.



Cyclic peptides are frequently exhibited in many bioactive natural products as well as therapeutic candidates for modulating the challenging protein−protein interactions with high affinity and specificity.[1−4] Compared to their linear counterparts, the peptide macrocycles have increased resistance to proteolysis with decreased conformational freedom and therefore stabilize their three-dimensional assemblies. Modern drug discovery commonly adopts screening chemical compound collections by the high-throughput screening platform (HTS),[5,6] which is commonly followed by a general work flow of library production, separate biological assay, and hits optimization. However, HTS is flawed by high operational cost and time-intensive labor, making it only suitable to big pharmaceutical companies.

Delightedly, DNA-encoded library technology (DELT)[7] offers an alternative and efficient small molecule selection platform for hits identification. DELT is an organized campaign containing the preparation of DNA-encoded libraries (DEL), affinity selection against protein targets, data analysis, and off-DNA hit confirmation, enabling a streamlined workflow to easily assemble and interrogate billions of DNA-tagged compounds and successfully identifying numerous hits against various disease-relevant protein targets. Since the very early days of DELT, scientists have made enormous efforts to sample various DNA-encoded cyclic peptide libraries to accelerate the development of cyclic peptide hits[8] (Figure 1). Zhu et al. in GSK[9] reported a DNA-encoded macrocyclic library synthesized by six cycles of chemistry and cyclized via a CuAAc reaction, resulting in a library size of 2.4×10^{12} which is the largest DNA-encoded library ever reported. From this DEL, several hits were identified and confirmed against two protein−protein interaction targets, VHL and RSV N protein. Neri, Scheuermann, and co-workers[10] also published a DNA-encoded macrocyclic library cyclized by the CuAAc reaction. This encoded library utilized a single-stranded DNA coding strategy, allowing it to be screened by both affinity capture and photo-cross-linking methods. The selections yielded specific binders against serum albumins, carbonic anhydrases, and NKp46. Researchers from Ensemble Therapeutics and Bristol-Myers Squibb designed and synthesized a DNA-programmed library of 160,000 cyclic peptidomimetics and identified macrocyclic XIAP antagonists through affinity selection screening.[11] The Gillingham group[12] synthesized a macrocyclic library using the amide bond formation reaction to complete the macrocyclization. Its affinity selection against AGP generated a binder with a micromolar dissociation constant (7 μM). The Liu lab revealed their second-generation DNA-templated macrocycle libraries via Wittig cyclization, and the in vitro selections against various targets resulted in several potent inhibitors with nanomolar activities.[13−16] In 2021, Lu, Chen, and co-workers[17] developed an efficient palladium-catalyzed intramolecular S-arylation reaction for preparing an 8-million-member tetrameric cyclic peptide DNA-encoded library. An affinity selection of this library against p300 identified two

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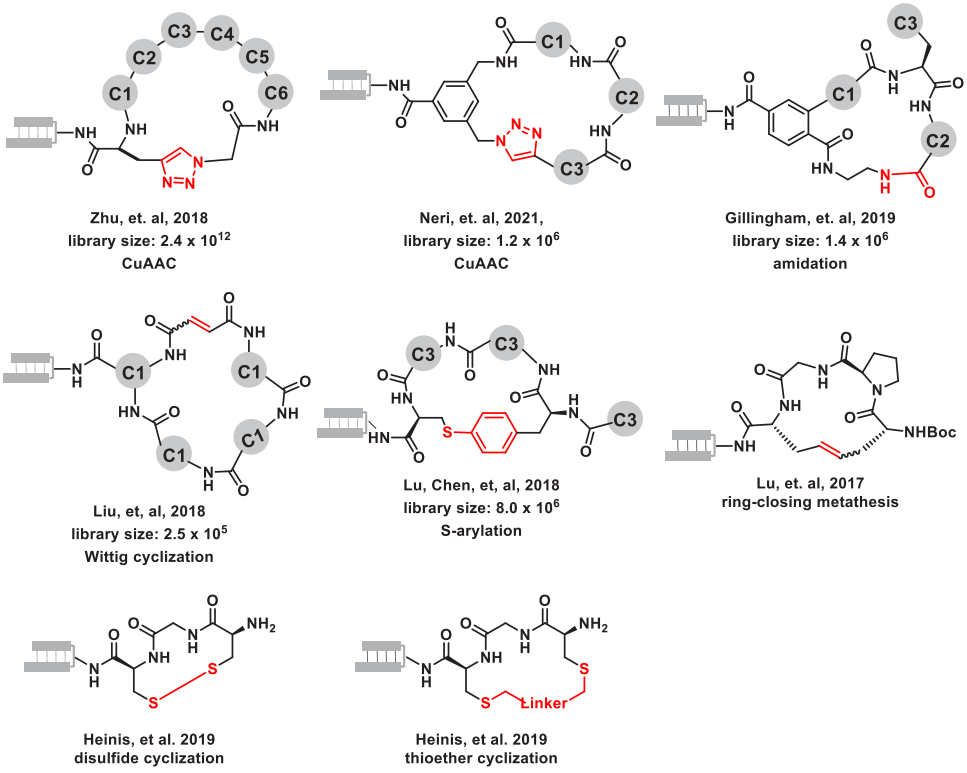
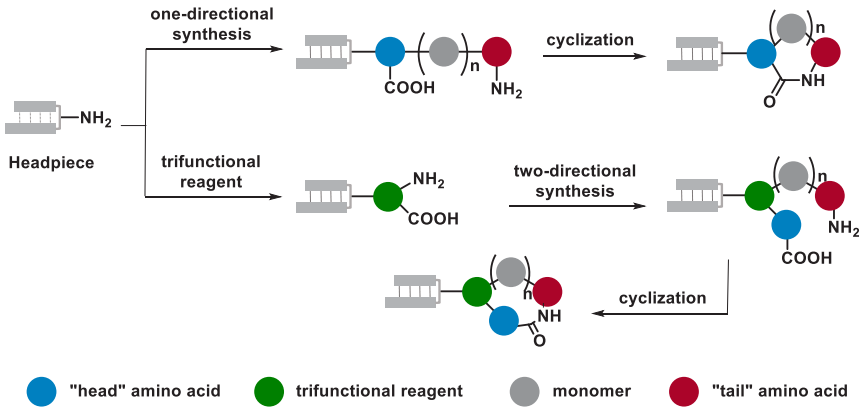


Figure 1. Reported DNA-encoded macrocyclic libraries and DNA-compatible ring-closure reactions.

Scheme 1. Schematic Workflows of One-Directional Synthesis and Two-Directional Synthesis



potential inhibitors with single-digit micromolar activity. In addition, the on-DNA Ru-promoted ring-closing metathesis[18,19] and thioether cyclization reaction[20] are also viable to be employed to prepare DNA-encode macrocyclic libraries.

The overall goal in DEL campaigns is to obtain as much selection information as possible to understand the structure-binding relationship (SBR) for hits identification and further optimization. It necessitates construction of the libraries with different ring sizes and various monomers. Our lab has been devoted to the macrocyclization of DNA-encoded libraries and has studied various cyclization methods, including the commonly used amide bond formation reaction. Conventionally, the DNA tag was linked to the "head" amino acid, and the linear peptide was constructed via one-directional workflow, followed by cyclization by cross-linking the functional side chains of both the "head" and "tail" amino acids. However, during the investigation, we found that some cyclic precursors with certain lengths failed to proceed in this way, presumably due to the improper conformations caused by the DNA tag. To address this issue, adjusting the conformation of the cyclic precursors by tuning the distance between closing sites and the DNA tag is necessary. It is going to be a tedious process for ensuring cyclic efficiency via a one-directional synthesis workflow. Herein, we revealed a two-directional synthesis strategy in this paper, in which the DNA tag was linked to a trifunctional reagent, followed by linking the monomers to either the N-terminal or C-terminal purposefully to build the linear precursors for macrocyclization (Scheme 1).

We commenced our study by treating the double-strand DNA "headpiece" with azidoacetic acid to produce DNA-linked azide **1** under the standard HATU/DIPEA acylation condition. Further transformation proceeded smoothly with the critical trifunctional reagent propargylglycine to generate the essential DNA-linked compound **2** with excellent conversion (Scheme 2A). **2** was then used as a common starting point to grow the peptide sequence from C/N

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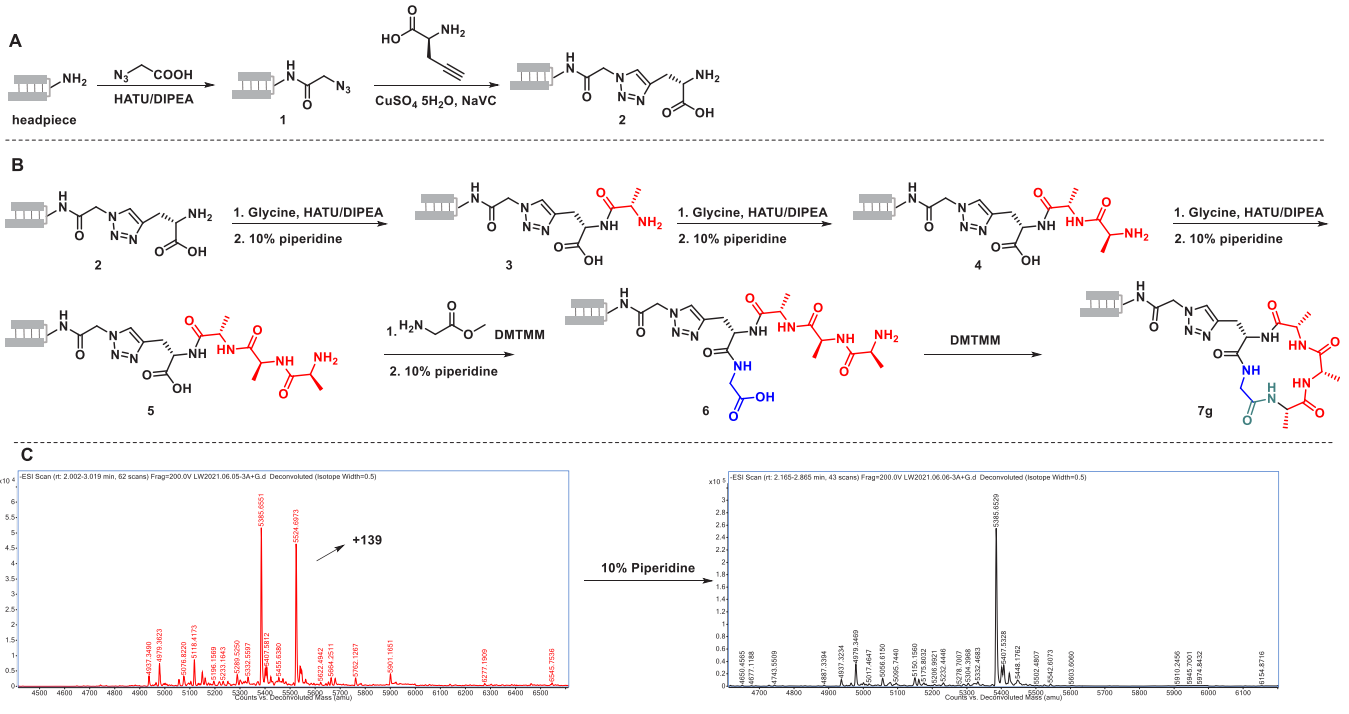
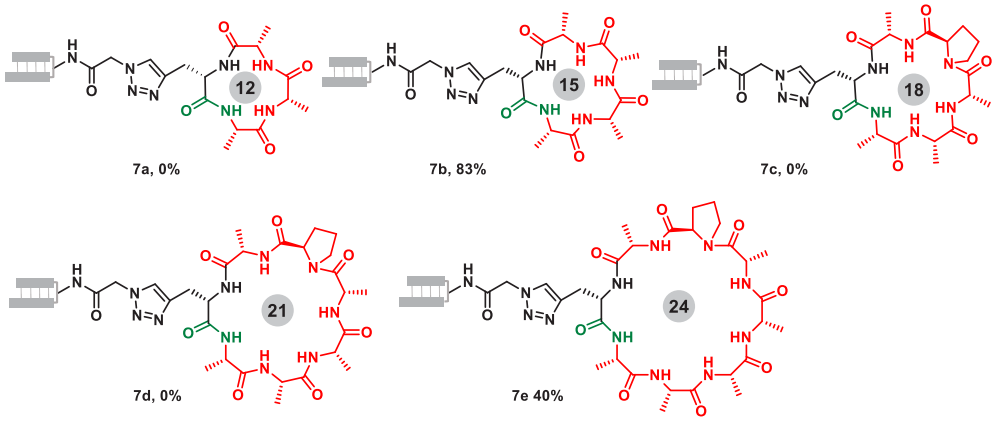
Scheme 2. (A) Synthesis of the Starting Point 2. (B) Streamlined Synthesis for an Exemplary DNA-Linked Cyclic Peptide 7g. (C) Final Treatment with 10% Piperidine to Yield the Final Product 7g



Scheme 3. Cyclization of the DNA-Linked Linear Peptides in Which All the Building Blocks Were Introduced from the N-Terminal



terminals which was followed by macrocyclization to furnish the DNA-conjugated cyclic peptides. The different distances between N/C terminals and DNA-linked site were achieved by BBs attached to the N/C terminals. The general on-DNA synthetic protocols were outlined in Scheme 2B through the production of an exemplary DNA-linked cyclic peptide **7g**. The linear peptide growing from the N-terminal was achieved by iterative acylation with Fmoc-protected amino acids under the standard HATU/DIPEA coupling−Fmoc deprotection protocol, which is marked in red. These synthetic reactions proceeded smoothly with excellent conversion. However, the acylation of the C-terminal presented inherent challenges for activating the DNA-linked acid functional group. Consequently, a large excess of reagents (1000 equiv of amino acid ester marked in blue, 1000 equiv of DMTMM, and 2100 equiv of NMM) with dilute reaction concentration (70 μM) were performed to completely modify the acid group. Hydrolysis of both the methyl ester and de-Fmoc could be achieved by piperidine, and the resulting primary amine and the carboxylic acid were used to close the peptide ring (marked in green) with large excess DMTMM (6000 equiv) at a highly diluted reaction concentration (52 μM). It is worth noting that the excess DMTMM reagent reacted with the 5′-OH of the headpiece to give a side product (Scheme 2C, +139), which was subsequently eliminated with 10% piperidine solution.

Having established the streamlined peptide construction procedure, we next explored the cyclization efficiency of DNA-linked cyclic peptides with ring sizes from 12 to 24 containing different distances between N/C terminals and DNA-linked site. As illustrated in Scheme 3, five exemplary DNA-linked cyclic peptides were initially prepared in which the trifunctional reagent provided both the DNA-linked site and the C terminal. Unexpectedly, the cyclic peptides **7a**, **7c**, and **7d**, with ring sizes 12, 18, and 21, respectively, failed to be produced.

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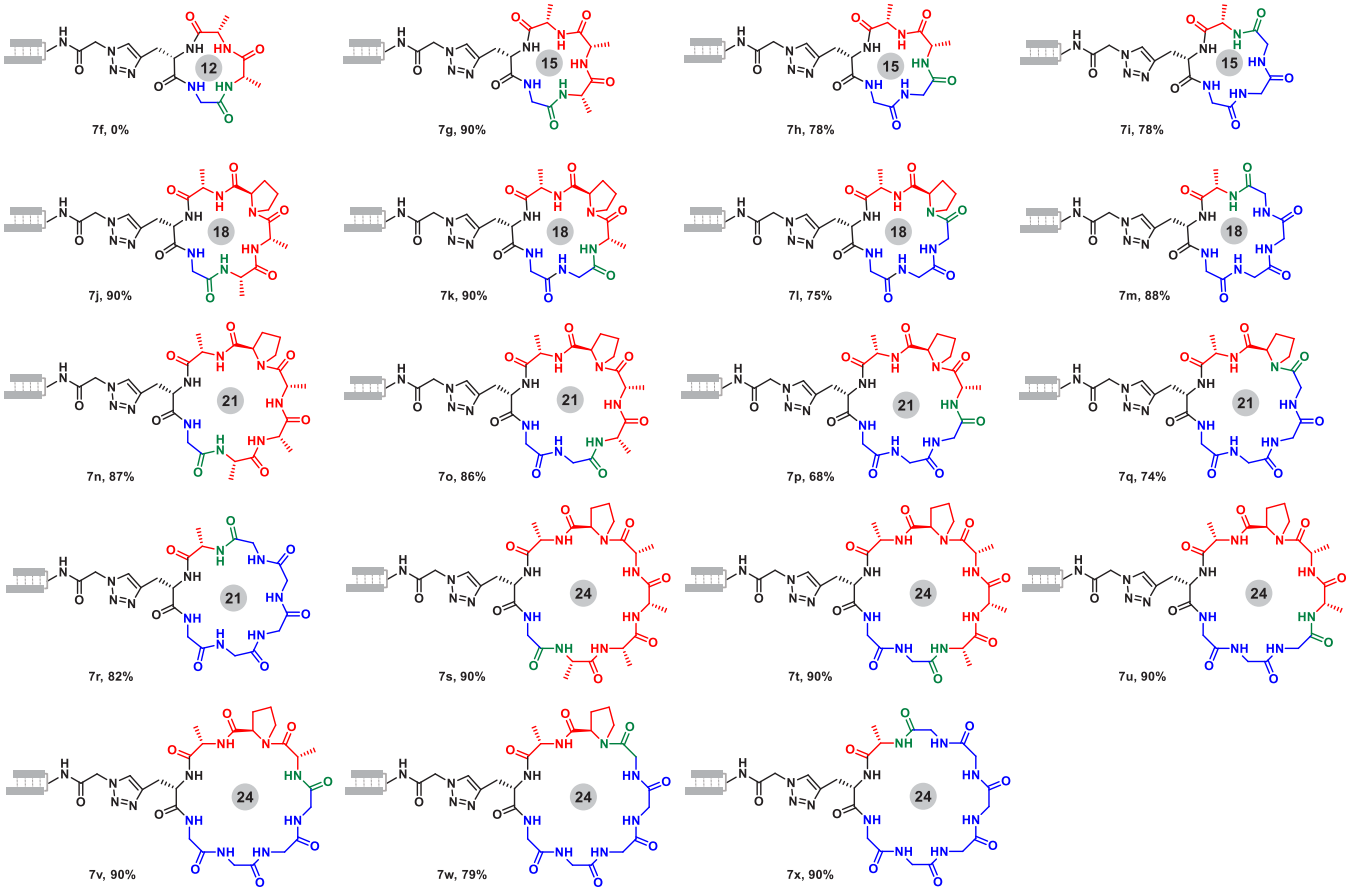
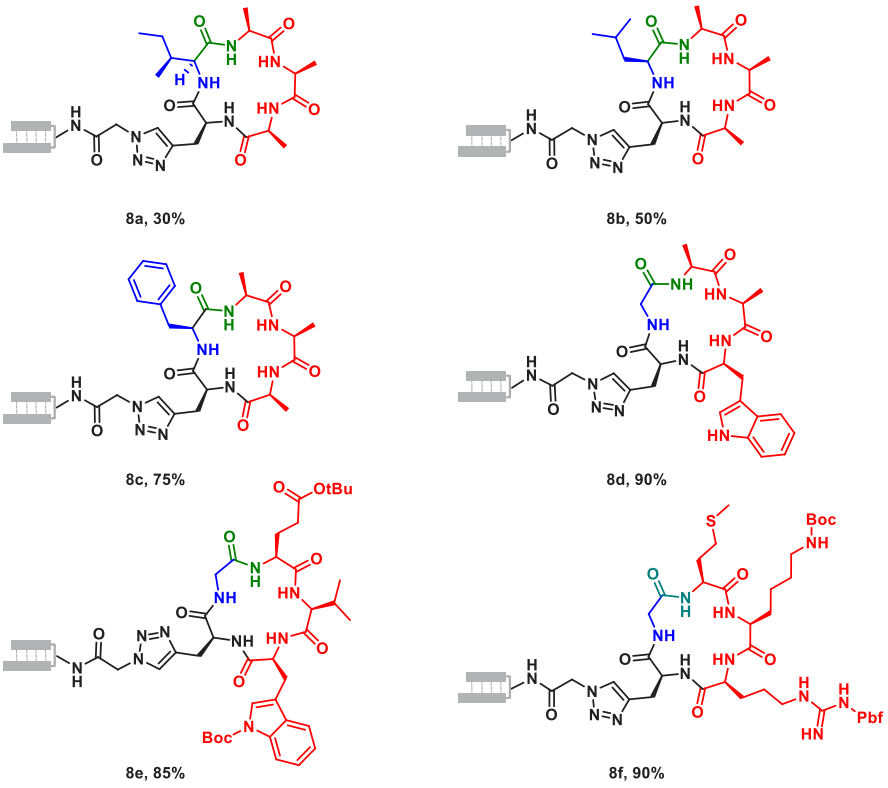
Scheme 4. Cyclization of a Wide Range of Model Macrocycles



Scheme 5. Proof-of-Concept Synthesis with Functionalized Amino Acids



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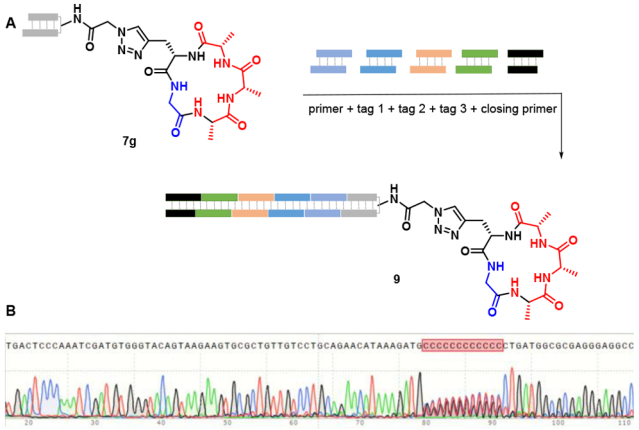
These cyclic precursors necessitated fine-tuning the distance by transferring at least one AA monomer from the N terminal to the C terminal.

As illustrated in Scheme 4, the exemplary linear peptides were prepared with at least one monomer linked to the C terminal. No desired tetrameric cyclic peptide **7f** was observed, presumably due to the constrained conformation of the small ring. To our delight, the macrocycles **7g**−**7x** of different ring sizes and distance between closing and DNA-linked sites were prepared with moderate to good yields. We believe both the ring size and the conformation of linear peptide precursors influenced the macrocyclization efficiency. These proof-of-concept results demonstrated the practical potential for preparing a wide range of DNA-encoded cyclic peptide libraries via a two-directional synthetic workflow.

Furthermore, we conducted a scope study for functionalized amino acids based on the skeleton of **7g** (Scheme 5). When changing the Gly to Ile, Leu, and Phe, **8a**−**8c** were prepared separately with moderate conversions. By contrast, **8d**−**8f** were produced with higher conversions when translating Ala to functionalized amino acids. This result inspired us to incorporate diverse amino acids into N-terminal for the design of macrocyclic DNA-encoded libraries. Meanwhile, the side chain indole in Trp (**8d**) was tolerated in the synthetic workflow, whereas nucleophilic groups such as carboxylic acid (Glu in **8e**), primary amine (Lys in **8f**), and guanidine (Arg in **8f**) need to be protected.

Finally, a proof-of-concept experiment was carried out to ensure its applicability in DEL synthesis (Scheme 6). An 8-bp

Scheme 6. (A) Ligation Efficiency Experiment. (B) DNA Amplification and Sequencing



double-stranded headpiece DNA was used as starting material to construct **7g** via optimized conditions. The product was then sequentially tagged with bar-coding DNA sequences (primer, tag 1, tag 2, tag 3, and closing primer) by enzymatic ligation to achieve a 52-bp-long DNA-linked product **10**. Each step afforded conversion of 90% by mass spectrometric analysis (see Figures S75−S83). The ligation product was then identified by Sanger sequencing, and no mutated base was found. Altogether, these results confirmed the feasibility of our on-DNA macrocyclization for DELs synthesis.

CONCLUSION

In summary, we have successfully demonstrated an operationally simple methodology to assemble macrocyclic peptide DNA-encoded libraries via two-directional synthesis. This strategy circumvents the issue caused by DNA tags and then enables the macrocyclization of linear precursors of different lengths. The scope exploration also showed that the reported protocol was reasonably broad, supporting its feasibility for library preparation. Our exploration exhibited here provides another tool to further expand the accessible chemical space by DNA-encoded libraries, with the potential to afford more comprehensive structure-binding information. Furthermore, we expect to expand this strategy to other fields, such as peptide optimization and PROTACs, and the corresponding work is currently ongoing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.2c00078.

Materials and methods; UV/Mass spectra for DNA-linked compounds (PDF)

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Notes

The authors declare no competing financial interest.

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