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# High Yield Branched Puromycin Linker Design Enables Efficient cDNA Display and Chemical Modification of Peptides

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## ABSTRACT

Peptide chemical modification is a valuable technique for improving peptide stability and bioactivity, particularly in drug discovery applications. Here, we report the development of a novel linker junction strategy using strain-promoted azide-alkyne cycloaddition (SPAAC) that enables the efficient formation of branched puromycin linkers with an average yield of 97%. This approach represents an improvement over traditional Michael-Addition methods, which typically yield ~15%–20%. The high yield of the SPAAC reaction and the near absence of by-products make the linkage by click reaction easy to purify. We demonstrate the utility of our linker design by successfully performing cDNA display and chemical modification strategies such as bicyclization of peptides. Our study demonstrates the functionality of the cDNA display system with the newly incorporated junction. In addition, the successful introduction of peptide bicyclization via tris-bromomethyl-benzene (TMBB) in cDNA display serves as a proof-of-concept for complex chemical modifications. Furthermore, the position of puromycin, which disrupts protein biosynthesis, has been determined. This approach offers novel insights into the discovery of chemically modified peptides and has the potential to accelerate the development of peptide-based therapeutics and diagnostics.

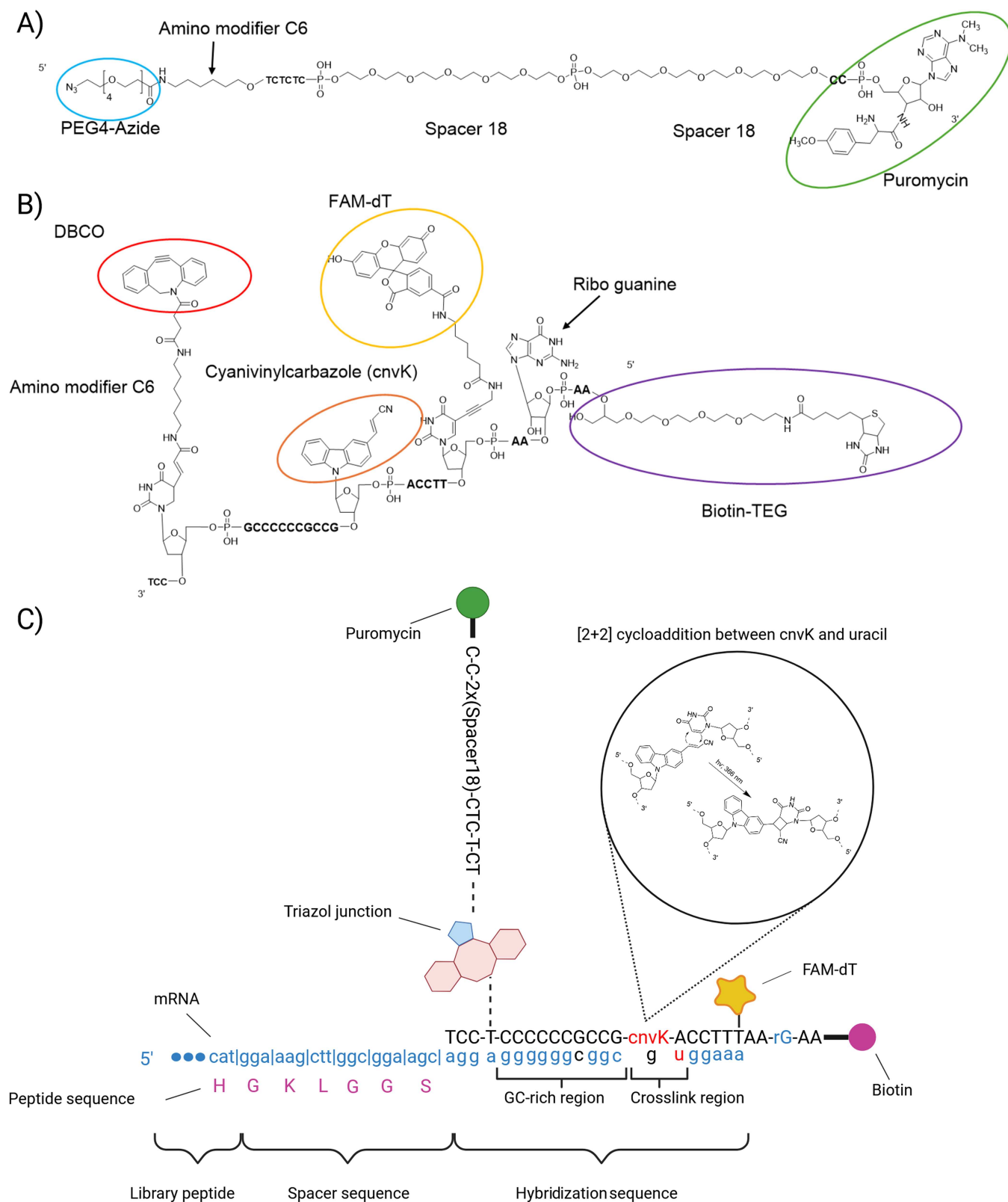
## 1 | Introduction

High target specificity and low attrition rates have made therapeutic peptides an attractive approach in medical development (Fosgerau and Hoffmann 2015). Consequently, they emerged as a significant contribution to modern medical development (Anand et al. 2022). Recent advances in drug discovery have highlighted the potential of cyclic peptides as powerful anticancer agents (Ramadhani et al. 2022). This exhibits enhanced structural rigidity increasing binding affinity and targeting properties (Zhang et al. 2021). Moreover, the cyclization process enhances stability, making them more resistant to degradation

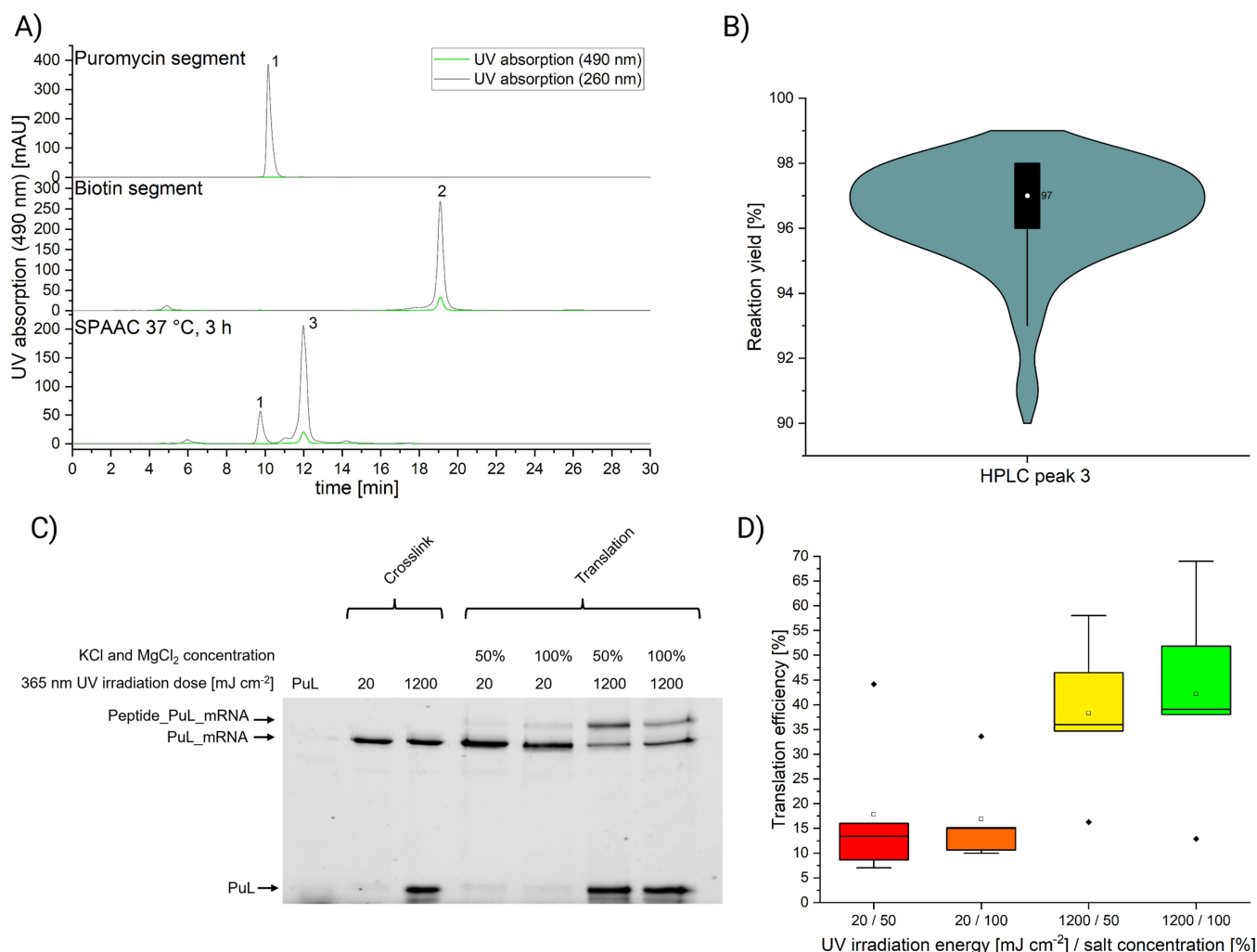
by peptidases (Mollica et al. 2015). Bicyclization, the formation of two or more rings within a peptide, has been shown to outperform the affinity and stability of single cyclized peptides (Angelini et al. 2012). For the de novo development of therapeutic peptides, display techniques rely on stable genotype-phenotype junctions, which link DNA libraries to their encoded peptides (Dotter et al. 2021). Of these methods, phage display has been widely used to search large peptides, but unfortunately, the transformation involved limits the library size in phage display to a diversity of  $\sim 10^{11}$  different peptides (Nixon et al. 2014). As a different approach, cDNA display uses

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**FIGURE 1** | Puromycin linker subsegments ready for SPAAC junction. (A) Puromycin segment. Blue circle: PEG4-azide modification; green circle: puromycin modification. (B) Biotin segment. Red circle: DBCO modification, orange circle: cyanovinylcarbazole (cnvK); yellow circle: FAM part of FAM-dT base; purple circle: biotin-TEG base. Bold capital letters mark standard DNA bases. All special bases and modifications are formulated in their Lewis structure formula. (C) Annealing and crosslink between the mRNA and the puromycin linker.



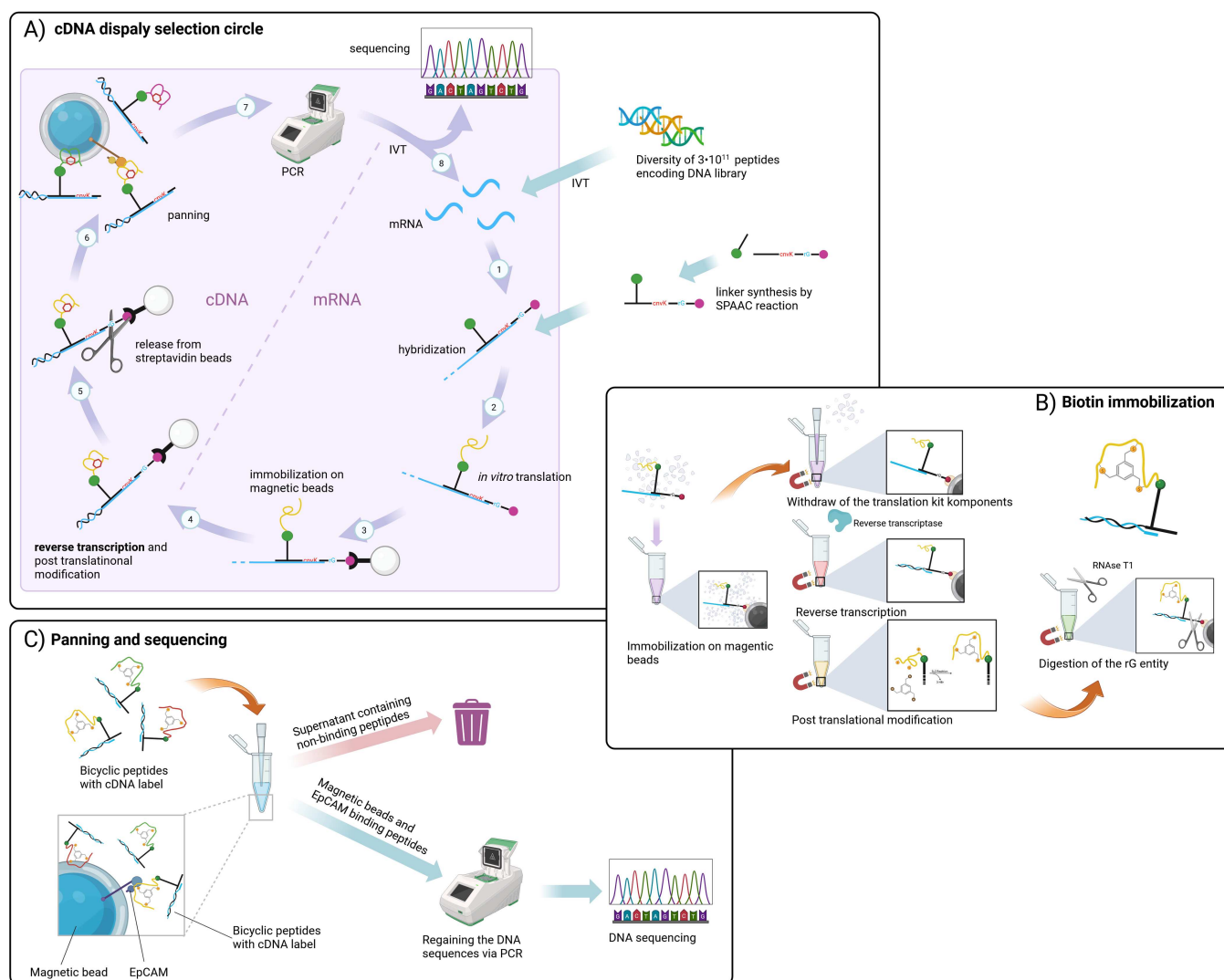
**FIGURE 2** | (A) Exemplary HPLC chromatograms of both ssDNA oligomer subsegments and after 3 h of SPAAC reaction at 37°C, indicating the near quantitative yield by full conversion of the biotin segment (Peak 2) to the reaction product (Peak 3). (B) Violin plot over the 36 analytic HPLC purifications of the isolated reaction product (A, Peak 3) from three SPAAC reactions. (C) Urea-PAGE for the determination of the conjugation efficiency comparing the different conditions. 100% salt concentration equals 750 mM KCl and 65 mM MgCl<sub>2</sub>. 50% salt concentration equals 375 mM KCl and 32.5 mM MgCl<sub>2</sub>. (D) Box blot over all five conjugation efficiency determinations. Salt concentrations as explained in (C).

the antibiotic puromycin to build the genotype–phenotype link, reaching library sizes  $> 10^{12}$  (Mochizuki et al. 2013, 2015; Yamaguchi et al. 2009). cDNA display's reverse transcription from mRNA to cDNA makes it chemically robust and suitable for posttranslational modifications or challenging environments (Lee et al. 2012). Usually, the conjugation of branched puromycin linkers' segments relies on Michael addition facilitated by EMCS (*N*- $\epsilon$ -maleimidocaproyl-oxy succinimide ester), resulting in yields of 15%–20% (Arai et al. 2020) and a plethora of by-products that complicate purification. Therefore, the synthesis of the puromycin linker is a costly and labor-intensive endeavor. To address this limitation, we have developed a novel strategy for connecting the subsegments via the strain-promoted azide-alkyne coupling (SPAAC), resulting in the highest yield of a branched puromycin linker that is amenable to purification by HPLC. Different from other novel puromycin linker designs using SPAAC (Zeng et al. 2023), a 5' biotinylation allows the solid phase immobilization of the entire cDNA-display hybrid molecule creating a robust working bench for posttranslational modifications like peptide cyclization.

## 2 | Material and Methods

### 2.1 | mRNA Library Preparation

A TRIM-codon ssDNA oligomer with precisely defined triplet sequences for encoding non-cysteine amino acids (Ella Biotech) was PCR-amplified using custom primers. The amplicons were purified and double digested for cloning into the pRDV\_hybr.-seq vector. The vector underwent identical digestion and gel purification. Inserts and vector were ligated. The final construct encoded TRIM-codons without stop or nonsense codons. The DNA library was transcribed to mRNA using the T7 RiboMAX kit, followed by DNase treatment and purification via Micro Spin G-50 columns, yielding a 222 bp mRNA at 576 ng/ $\mu$ L (Chapter S1.1). **Puromycin linker synthesis:** Puromycin and biotin ssDNA segments (Ella Biotech, Figure 1A) were synthesized with modifications for SPAAC reaction. In total, 100  $\mu$ L (500  $\mu$ M, 50 nmol) of the biotin segment and 200  $\mu$ L (500  $\mu$ M, 100 nmol) puromycin segment were incubated together at 37°C for 3 h. Product formation was verified and purified by HPLC. The purified puromycin linker was lyophilized and dissolved in

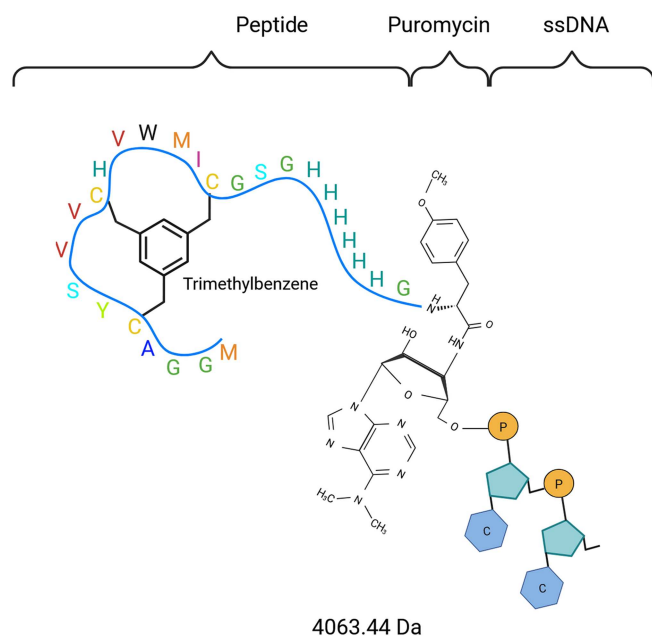


**FIGURE 3** | (A) DNA-encoded library is transcribed into mRNA and linked to a puromycin (Step 1). During translation, the nascent peptide covalently attaches to the linker, coupling genotype and phenotype (Step 2). The hybrid molecules are immobilized on streptavidin beads, enabling reverse transcription and peptide bicyclization (Steps 3–4). After release (Step 5), the library is subjected to EpCAM panning (Step 6). Bound species are amplified by PCR (Step 7) and either sequenced or recycled into the selection cycle (Step 8). (B) Utilization of magnetic streptavidin beads as solid phase. Each Eppendorf-tube color represents a different buffer solution. (C) Panning on EpCAM, regaining the cDNA sequences via PCR and sequencing.

nuclease-free water prior to use (Chapter S1.2.1). **Crosslink and translation:** The puromycin linker (4.8  $\mu$ L, 3.2 mM) was annealed to the mRNA (2  $\mu$ L, 7.62 mM) by thermal ramping and covalently crosslinked via UV-induced [2 + 2] cycloaddition (Figure 1B). The crosslinked constructs were translated using the PUREfrex 1.0 system (Gene Frontier). After translation, KCl to a final concentration of 750 mM and  $MgCl_2$  to 65 mM were added, and incubation continued. The efficiency of genotype (mRNA) to phenotype (peptide) conjugation, mediated by the mRNA-bound puromycin linker (PuL) capturing nascent peptides within the ribosome, was monitored by puromycin fluorescence on urea-PAGE (Chapter S1.2.2). **Reverse transcription:** Translated cDNA display complexes were immobilized on streptavidin magnetic beads (Figure 3B, NEW England Biolabs), washed, and reverse transcribed using the SuperScript IV kit (Invitrogen). Incubation and wash protocols are detailed in Chapter S1.2.3. **Peptide cyclization and purification:** Bead-bound cDNA display hybrids were washed and incubated with tris

(bromomethyl) benzene (TBMB, 10 mM) for peptide cyclization. Post-reaction, the hybrids were released from the beads and treated with RNase T1 to obtain the final construct (Chapter S1.2.4). Thereafter, the His-tagged cDNA display hybrids were captured using Dynabeads HIS Isolation, washed, and eluted with imidazole-containing buffer (50 mM  $Na_3PO_4$  pH 8, 300 mM NaCl, 0.01% Tween 20). The eluates were further cleaned using Bio Spin 6 columns (Bio Rad) (Chapter S1.2.5). **Panning and sequencing:** EpCAM-Fc (custom produced) was immobilized on Dynabeads Protein A (Invitrogen) and incubated with the cDNA display library. After binding for 1 h at 4°C, beads were washed with 1.5 mM  $MgCl_2$  buffer, and bound molecules were recovered for further analysis (Chapter S1.2.6). Bound cDNA display molecules were PCR-amplified, gel-purified, cloned into *Escherichia coli*, and sequenced via Sanger sequencing (Chapter S1.2.7). **MALDI-TOF analysis:** The identified clone HQ0318 was resynthesized, crosslinked, translated, and cyclized as before. The final peptide constructs were





**FIGURE 4** | Scheme of the bicyclic peptide measured with trimethylbenzene incorporated between the cysteine residues (yellow) and C-terminal puromycin including two remaining cytosine residues (ssDNA) after DNase RQ1 digestion. The theoretical mass of the molecule is estimated at 4063.44 Da.

purified, lyophilized, and prepared for MALDI-TOF MS using HCCA matrix (Chapter S1.4).

To assess the conversion of the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction between the biotin and puromycin segments, HPLC chromatograms of the reactants were compared to a 3 h reaction control (Figure 2A). To determine the optimal combination of ultraviolet (UV) irradiation energy and salt concentrations for crosslinking the puromycin linker with the mRNA strand during the translation UV doses (20 and 1200 mJ cm<sup>-2</sup> at 365 nm) and two salt conditions (KCl/MgCl<sub>2</sub>) combinations from Reyes et al. (2021) were tested. The highest mean conjugation efficiency of 42% (median of 38%) was achieved with 1200 mJ cm<sup>-2</sup> UV and 750 mM KCl/65 mM MgCl<sub>2</sub>, whereas the lowest mean conjugation efficiency of 17% (median of 15%) was observed at the combination of 20 mJ cm<sup>-2</sup> under the same salt conditions (Figure 2C,D). The irradiation dose has a pronounced effect on conjugation efficiency (20%–25%) when increasing from 20 to 1200 mJ cm<sup>-2</sup>, while doubling the salt concentration produced only minor changes (1%–4%). Thus, UV irradiation dose was identified as the dominant factor, with the optimal efficiency obtained at 1200 mJ cm<sup>-2</sup> UV irradiation and 750 mM KCl and 65 mM MgCl<sub>2</sub>. The method was validated by recovering sequence HQ0318 (MGGACYSVVVCHVWMICGSGHHHHHHH), which retained the 3Cys4N5N library architecture, after completing a full cDNA display selection cycle (Figure 3A). Therefore, a panning was conducted by incubating the hybrid molecules on magnetic bead immobilized EpCAM (Figure 3C). Thereafter, the named sequence HQ0318 was chosen for the cyclization validation experiments. Cyclization of the peptide trapped on the puromycin linker bound to magnetic beads was confirmed by digesting the linker with DNase RQ1. Owing to uncertainty ribosome stalling, puromycin could be incorporated at seven

positions, and the absence of a restriction site in DNase RQ1 allows up to two cytosine residues to remain 5' of the puromycin, yielding 21 possible variants for mass spectrometry analysis. MALDI-TOF mass spectrometry of TBMB-treated peptide produced a signal of 4064.42 Da (Figure S1), matching the theoretical [M + H]<sup>+</sup> mass of 4063.44 Da for “MGGACYSVVCHVWMICGSGHHHHHHG-puromycin-cytosine-cytosine” with TBMB incorporated between the cysteine residues (Figure 4). This confirmed puromycin interruption within the spacer sequence. Additionally, the 4064.42 Da signal indicated that only the first glycine of the 6-amino-acid spacer was translated, revealing that puromycin interrupts biosynthesis five residues before the hybridization region (Figure S3). Although binding affinity was not characterized in this method validation study, consistent HQ0318 recovery demonstrates successful cDNA display with chemical modifications, warranting future work on binding characterization and sequence diversity analysis. In summary, a novel linker junction strategy based on SPAAC was developed, enabling efficient formation of branched puromycin linkers with an average yield of 97%, compared to 15%–20% (Arai et al. 2020) from Michael Addition methods. The high yield, minimal and simplified purification make the approach cost-effective as a single library clone in our 3Cys4N5N-design requires only 0.5 pmol (0.02€/clone). During translation optimization the original MgCl<sub>2</sub> and KCl concentrations (Yamaguchi et al. 2009) proved more effective than the reduced concentrations that were superior in the experiments of Reyes et al. (2021), indicating that translation efficiency may depend on experimental context and warrants further study. Functional integrity of cDNA display with the new junction was confirmed by the successful recovery of DNA sequences after panning on EpCAM. Peptide bicyclization via TBMB, previously applied in phage display (Heinis et al. 2009), was successfully adapted to cDNA display and verified by MALDI-TOF analysis, representing the first demonstration of this chemical modification in this display format. The position of puromycin interrupting the protein biosynthesis was also determined. A recent study has described the use of the SPAAC reaction for the development of DARPins (Zeng et al. 2023). The improved branched linkers reduce costs, enhance purification, and preserve the ability of mRNA display to perform posttranslational modification through biotin-mediated immobilization on streptavidin beads. Owing to the exceptional stability of the streptavidin–biotin complex (dissociation constant up to 10<sup>-15</sup> M (Delgadillo et al. 2019; Deng et al. 2013)), cDNA display can withstand conditions incompatible with coordinative immobilization methods such as NiNTA-IMAC (Luong and Vashist 2020; Warnken et al. 2016). Consequently, the optimized cDNA display platform has been found to be a powerful and accessible tool for discovering chemically modified peptides, combining cost efficiency, stability and compatibility with in vitro chemical modifications during selection.

#### Author Contributions

Conceptualization: Simon Schneider, Melanie Boll, Matthias Eder, Ann-Christin Eder. methodology: Simon Schneider, Melanie Boll. formal analysis: Simon Schneider, Ann-Christin Eder. investigation: Simon Schneider, Melanie Boll. data curation: Simon Schneider. writing – original

draft preparation: Simon Schneider. writing – review and editing: Melanie Boll, Matthias Eder, Ann-Christin Eder. supervision: Matthias Eder and Ann-Christin Eder. project administration: Matthias Eder and Ann-Christin Eder. All authors have read and agreed to the published version of the manuscript.

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## Conflicts of Interest

German Cancer Research Center and University of Freiburg filed a patent application related to the process described in this article (EP25159422.2), naming S. Schneider, M. Eder, and A.-C. Eder as inventors.

## Data Availability Statement

The data underlying this article are available in Zenodo, at (<https://doi.org/10.5281/zenodo.15351542>).

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supplemental Figure 1:** MALDI-TOF mass spectrum of the TBMB treated peptide. The molecular mass peak of 4064 Da  $[m+H]^+$  is marked with a red label, the other one refers to an additional sodium ion  $[m+Na]^+$ . **Supplemental Figure 2:** Plasmid map of the pRDV\_hydr.seq vector including the 3Cys4N5N library. **Supplemental Figure 3:** Sequence of the NcoI – HindIII cloning site including the 3Cys4N5N library, spacer and hybridization sequences for the puromycin linker. ‘NNN’ represents a random codon and ‘X’ a random amino acid.