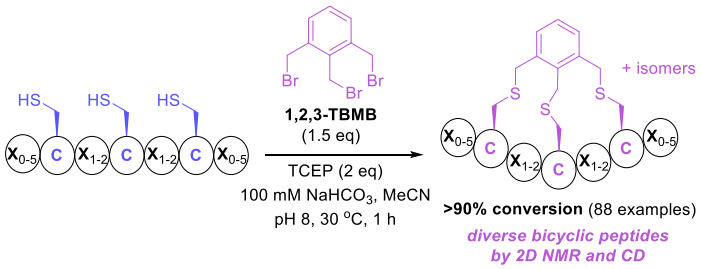
Accessing diverse bicyclic peptide conformations using 1,2,3-TBMB as a linker

Haritha Krishna Sudhakar,[1] Lisa J. Alcock,[1]* Yu Heng Lau[1,2]*

[1] *School of Chemistry, The University of Sydney, Camperdown, NSW 2006, Australia*
[2] *ARC Centre of Excellence for Innovations in Peptide and Protein Science, The University of Sydney, Camperdown, NSW 2006, Australia*

ABSTRACT

Bicyclic peptides are a powerful modality for the engagement of challenging drug targets such as protein-protein interactions. The most common crosslinkers used to generate bicyclic peptides are C3-symmetrical, with evenly positioned peptide loops facing radially outwards from a linker core to favour globular conformations. In contrast, linkers with alternative symmetries can potentially provide access to a more diverse conformational landscape of bicyclic peptides. Here, we use 1,2,3-tris(bromomethyl) benzene (**1,2,3-TBMB**) to access bicyclic peptides with multiple isomeric configurations, leading to conformations that differ substantially from both the parent linear peptides and the conventional bicyclization products formed with **1,3,5-TBMB**, as observed in 2D NMR and CD experiments. Bicyclization at cysteine residues proceeds efficiently under standard aqueous buffer conditions, with broad substrate scope, compatibility with high-throughput screening, and clean conversion (>90%) of linear precursors to bicyclic products for 88 of the 106 diverse peptide sequences tested. We envisage that the **1,2,3-TBMB** linker will be applicable to a variety of peptide screening techniques, thereby enabling the discovery of unconventional bicyclic peptides that can engage a broad range of novel drug targets.



INTRODUCTION

Over the past two decades, macrocyclic peptides have emerged as an important therapeutic modality. Macrocyclic peptides possess the appropriate size and conformational rigidity to engage challenging drug targets such as the flat extended binding interfaces of protein-protein interactions.[1-3] There is also great potential in using macrocyclic peptides as alternatives to antibodies for targeting receptors,[4] with their different molecular properties giving rise to distinct *in vivo* pharmacokinetics.[5] The discovery of macrocyclic peptide hits against such targets has been accelerated by the development of new macrocyclization chemistries and powerful library screening methods such as peptide display technologies and one-bead one-compound approaches.[6-12] As a testament to the therapeutic potential of macrocyclic peptides,

substantial translational progress has been reported for multiple lead compounds in clinical trials and pre-clinical studies,[12-18] such as Merck's oral PCSK9 inhibitor MK-0616 currently in Phase 3 trials.[15, 19-21]

Peptide bicyclization is a widely used macrocyclization strategy for increasing conformational rigidity relative to linear or monocyclic peptides.[22-24] Bicyclization can either be conducted stepwise using two independent macrocyclization reactions, or in a single reaction step where both cyclic constraints are generated using a trifunctional crosslinker.[25] Trifunctional crosslinkers can be advantageous in a screening context, as a single step reaction can increase the efficiency of the overall screening process due to the relative ease of upstream and downstream work-up processes, as well as the higher theoretical yields that can be obtained.

The most common single step approaches to peptide bicyclization involve reactions at three cysteine residues using C3-symmetric crosslinkers (Figure 1a), resulting in globular-like peptide conformations. The cysteine residues are typically spaced apart by multiple intervening residues (e.g. CX_nCX_nC, where n = 4-6), creating large peptide loops that extend from a core linker (Figure 1b). The linker itself most commonly consists of a cyclic scaffold that is 1,3,5-trisubstituted with electrophilic groups for reacting with cysteine thiols,[26] first reported by Timmerman and co-workers.[27] The biocompatibility of cysteine alkylation chemistry has been exploited for bicyclization of peptides in biological screening contexts, such as phage display using a 1,3,5-tris(bromomethyl)benzene linker (**1,3,5-TBMB**) established by Heinis and Winter.[28] Changes in the linker functionality (e.g. haloacetamide vs benzylic halide) and scaffold (e.g. benzene vs triazinane vs Bi^{3+}) can impart subtle conformational changes that may influence suitability towards different drug targets.[26, 29] However, greater diversity in bicyclic peptide conformation can potentially be achieved by using linkers with alternative substitution patterns and symmetries.

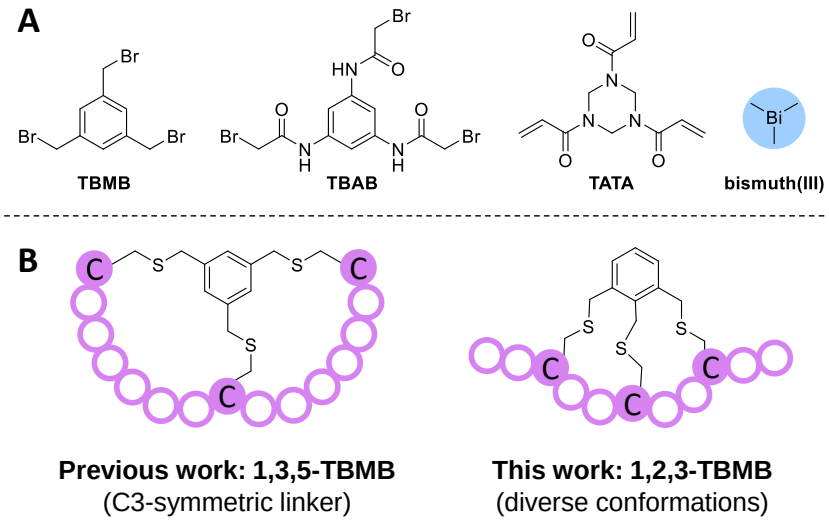


Figure 1. (A) Various C3-symmetric crosslinkers for bicyclization at cysteine residues. TBMB = 1,3,5-tris(bromomethyl) benzene, TBAB = N,N'-(5-(2-bromoacetimidamido)-1,3-phenylene)bis(2-bromoacetamide), TATA = 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)tris(prop-2-en-1-one). **(B)** Compared to the commonly used C3-symmetrical linker **1,3,5-TBMB** that favours globular conformations, we show that **1,2,3-TBMB** is a highly efficient linker that can generate diverse peptide conformations.

In this study, we show that 1,2,3-tris(bromomethyl)benzene (**1,2,3-TBMB**) can be used as a highly efficient and broadly compatible bicyclization linker for high-throughput access to a diverse range of non-canonical conformations arising from different isomeric configurations.

RESULTS AND DISCUSSION

During our investigation into stabilizing peptides with extended conformations,[30] we identified **1,2,3-TBMB** as a promising linker for the formation of smaller peptide loops with a bias towards one side of the linker (Figure 1b). While we expected the lack of C3 symmetry in the linker to result in different bicyclic peptide isomers, we reasoned that each of these isomers would generate a substantially different peptide conformation, therefore enabling access to greater overall conformational diversity. Synthesis of **1,2,3-TBMB** was afforded by allylic bromination of 1,2,3-trimethylbenzene using a modified literature protocol (Scheme S1).[31]

In an initial pilot study on ten model peptide sequences **P1**-**P10**, we found that **1,2,3-TBMB** was a highly effective bicyclization reagent, achieving clean and quantitative conversion of unprotected linear peptides to their corresponding bicyclic products (Figure 2). Each sequence was 8-11 residues in length, containing three cysteine residues placed at different positions within the peptides (i.e. *C*-terminal, *N*-terminal, central) and separated by either one or two randomly chosen intervening residues (i.e. CXCXC, CX_2CXC, CX_2CX_2C, $CXCX_2C$, see Table S1). Under standard bicyclization conditions consisting of 100 mM $NaHCO_3$ buffer at pH 8 with 50% MeCN and TCEP for disulfide reduction (henceforth referred to as Method A), addition of 1.5 equivalents of **1,2,3-TBMB** resulted in full conversion to the desired products in 1 h, as determined by LCMS analysis (Figure S3). Peptide **P2** is shown as an exemplar in Figure 2, where LCMS analysis shows complete disappearance of linear peptide **P2** and appearance of peaks corresponding to isomers with the expected mass for the bicyclic peptide **cP2**.

To confirm that all three cysteine thiol groups had reacted with **1,2,3-TBMB** as expected, we used Ellman's reagent on linear and bicyclic forms of three peptide sequences (**P2, P4, P6**) after HPLC purification. Free thiols were detected for all the linear peptides, while there was a lack of significant absorbance for all the bicyclic peptides tested as isomeric mixtures, indicating that all three cysteine residues had successfully reacted in each case (Figure S4).

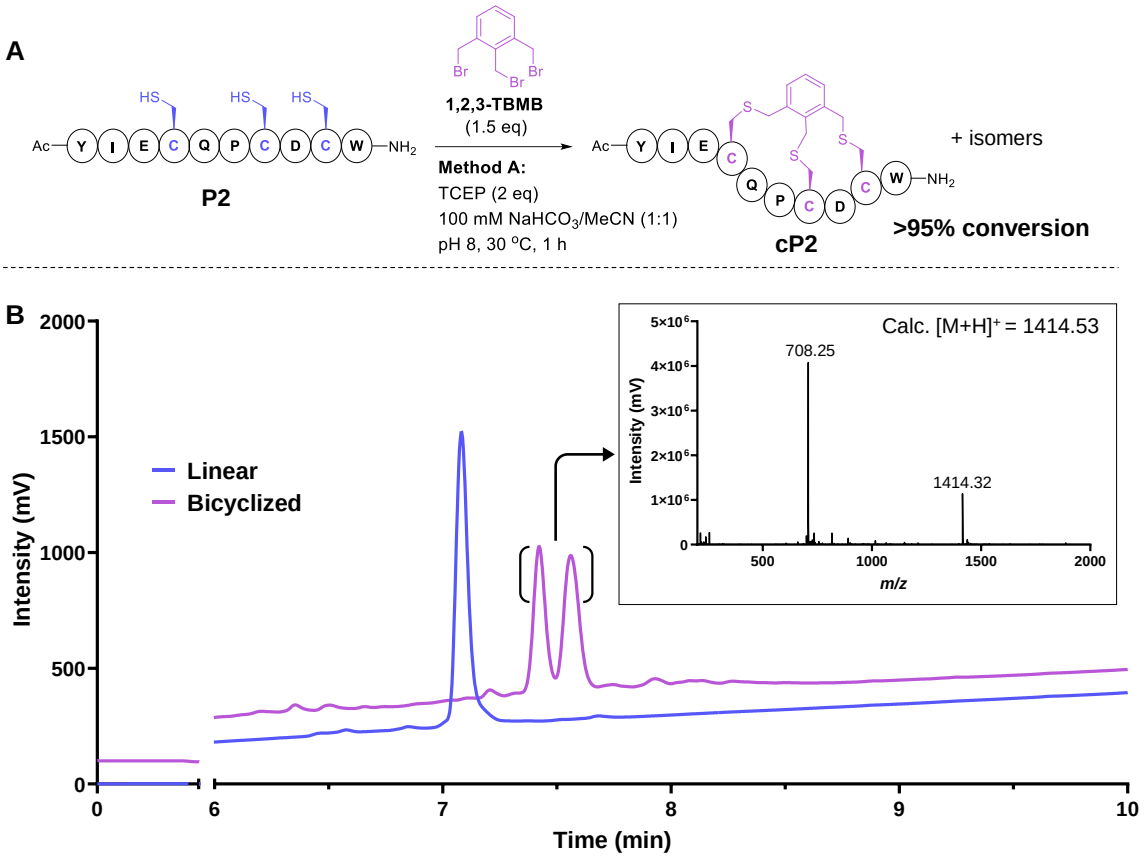


Figure 2. Exemplar peptide **P2** from a pilot study of ten bicyclization reactions with **1,2,3-TBMB**. Data for other peptides is available in SI Section 2. **(A)** Reaction with **1,2,3-TBMB** using Method A resulted in full conversion to the bicyclic product **cP2** as a mixture of isomers. **(B)** LCMS UV chromatograms monitored at 220 nm showing linear **P2** (blue) and crude bicyclization reaction mixture (purple). The crude bicyclized chromatogram shows two distinct UV peaks, each with identical *m/z* that matches the value expected for the bicyclized product **cP2**. No unreacted linear peptide was detected.

Different conformations were observed when comparing the bicyclization products with **1,2,3-TBMB** and the corresponding bicyclization product with the C3-symmetrical linker **1,3,5-TBMB**. Using **P7** as an exemplar in Figure 3, **1,3,5-TBMB** reacted with full conversion to the expected bicyclic product **cP7$_{1,3,5}$** as confirmed by LCMS as a single species. Meanwhile, **cP7** (bicyclized with **1,2,3-TBMB**) appeared as two distinct LCMS peaks consistent with the formation of isomers. CD spectroscopy was conducted after HPLC separation of the two peaks **cP7$_{pk1}$** and **cP7$_{pk2}$**, along with isolation of pure **cP7$_{1,3,5}$** (Figure 3c). Differences in the CD spectra of all three samples were observed, indicating that the different linker substitution patterns resulted in different conformations (Figure 3d).[32] Similar reactivity and CD trends were also observed for analogous studies on **cP3** and **cP3$_{1,3,5}$** (Figure S5).

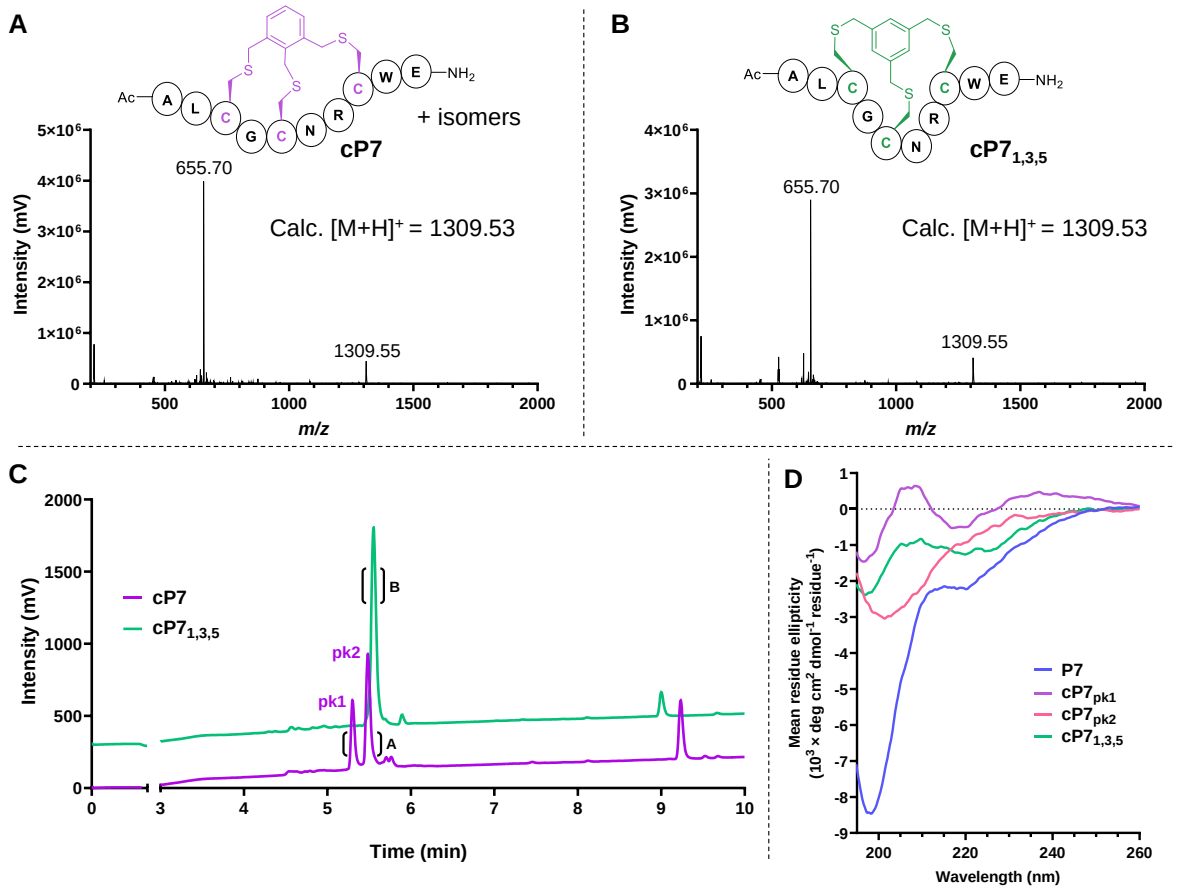


Figure 3. Comparison of **1,2,3-TBMB** and **1,3,5-TBMB** linker reactivity and resulting conformational changes on exemplar model peptide **P7**. **(A)** Structure and mass spectrum of **cP7**, with one possible isomer shown. **(B)** Structure and mass spectrum of **cP7**$_{1,3,5}$. **(C)** LCMS UV chromatograms monitored at 220 nm for crude bicyclization reaction mixtures of **P7** using Method A with either **1,2,3-TBMB** (purple) or **1,3,5-TBMB** (green). The **1,2,3-TBMB** reaction shows two distinct peaks with identical *m/z*, while the **1,3,5-TBMB** reaction has a single peak. No linear product is observed in either trace after bicyclization. Brackets indicate peaks that correspond to the mass spectra displayed in panels A and B. **(D)** CD spectra of linear **P7** (blue), bicyclized **cP7**$_{pk1}$ (purple), **cP7**$_{pk2}$ (pink), and **cP7**$_{1,3,5}$ (green).

We used 2D NMR spectroscopy to deconvolute the identity of the isomeric bicyclic forms that were observed by LCMS (Figure 4). Three isomers are theoretically possible, depending on which bromomethyl group reacts with each cysteine residue (Figure 4a). To simplify the interpretation of NMR data, we designed an 8-mer peptide **P11** to provide non-overlapping ^{1}H NMR signals for analysis. After bicyclization with **1,2,3-TBMB**, the resulting product **cP11** displayed two distinct peaks in a 2:1 ratio by LCMS (Figure 4a). After separation by HPLC to yield **cP11**$_{pk1}$ and **cP11**$_{pk2}$, the 2D ^{1}H-^{1}H TOCSY and COSY NMR spectra of **cP11**$_{pk1}$ had >9 distinct α-NH signals in the fingerprint region (only 8 amino acid and 1 *C*-terminal amide expected), suggesting that **cP11**$_{pk1}$ consists of more than one isomer. In comparison, the 2D ^{1}H-^{1}H TOCSY NMR spectrum of **cP11**$_{pk2}$ had the expected 9 α-NH peaks, indicating that the peak represents a single pure isomer (Figures S6-8). There was no significant overlap between the TOCSY spectra of **cP11**$_{pk1}$ and **cP11**$_{pk2}$, hence consistent with the formation of three different isomers of the bicyclized peptide.

^{1}H-^{1}H NOESY NMR cross peaks for isomerically pure **cP11$_{pk2}$** were consistent with the proposed chemical structure **cP11$_{b}$** (Figure 4b). The three benzylic protons at C1, C2 and C3 all displayed NOE cross peaks with the Cys7β and Cys7α protons, suggesting Cys7 most likely reacted with the central C2 position of **1,2,3-TBMB**. This inference was supported by NOE cross peaks of the C3 benzylic proton with the Cys4β and Ala6β protons, as well as additional cross peaks involving the aromatic CH protons on the linker (Figure S9). Taken together, this non-overlapping NOESY data further supports the conclusion that three distinct isomers with unique conformations are formed upon reaction with **1,2,3-TBMB**, with **cP11$_{b}$** isolated as an isomerically pure bicyclic peptide.

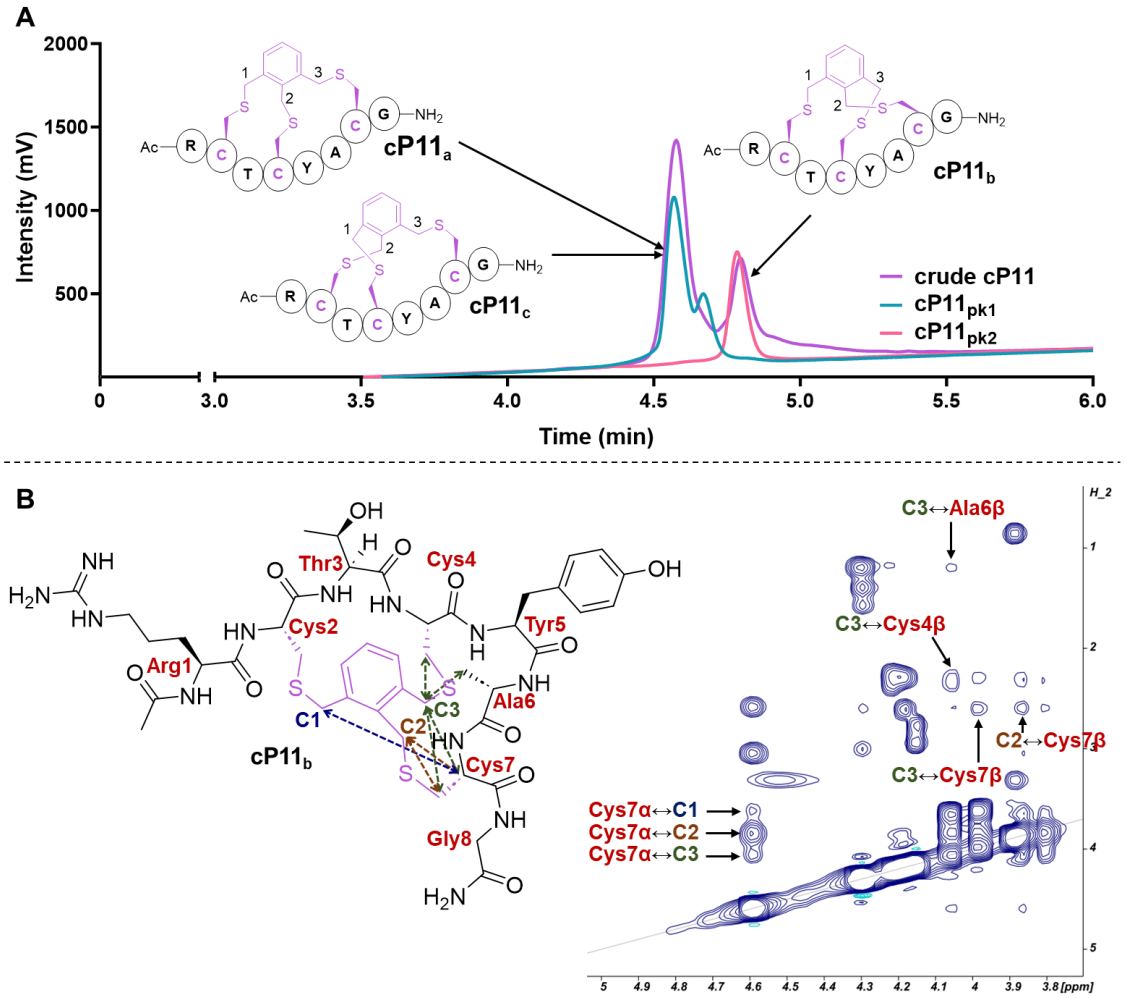


Figure 4. Separation and analysis of **cP11** isomers. **(A)** LCMS UV chromatograms monitored at 220 nm show two distinct peaks for crude **cP11** which were separated into **cP11$_{pk1}$** and **cP11$_{pk2}$** by HPLC. **cP11$_{pk2}$** contained only one isomer, while **cP11$_{pk1}$** was a mixture of two isomers. **(B)** 2D ^{1}H-^{1}H NOESY spectrum of **cP11$_{pk2}$** in the cysteine and linker cross peak region (3.5-5.0 ppm). Full structure of the corresponding isomer **cP11$_{b}$** is shown, with dashed arrows indicating NOE cross peaks between peptide protons and benzylic CH_2 protons at C1 (blue), C2 (brown), and C3 (green).

While bicyclization using Method A was effective for the pilot peptide sequences tested thus far, we noted certain hydrophobic sequences posed challenges under these buffered conditions likely attributable to poor solubility. In the case study of one peptide **P12** (Ac-DRRCVCFCLGF-NH_2), apparent poor solubility in the buffered aqueous conditions of Method A prompted switching the $NaHCO_3$ buffer component to water

with addition of DIPEA to deprotonate cysteine thiols. This enabled complete conversion to **cP12**, with no free thiols remaining according to Ellman's test (Figure S11).

Using Method B on variants of the **P12** sequence, we sought to probe the limits of these modified reaction conditions to variations in sidechain length, with the intention of fine-tuning the linker flexibility and hence peptide conformation. In the case of **P13** where all three cysteine residues were changed to homocysteine, only ~50% conversion was observed after overnight reaction at 30 °C (Table S4). Reverting the central amino acid back to cysteine in peptide **P14** resulted in complete conversion to the expected product (Table S4), suggesting a better match between linker geometry and peptide sequence. These results demonstrate that Method B tolerates longer sidechain lengths, however individual cases may require optimization.

Reactivity of **1,2,3-TBMB** with TCEP was noted to occur exclusively under the basic conditions of Method B. TCEP adducts of **1,2,3-TBMB** were observed when either excess TCEP was used (>10 equivalents) or if the bicyclization reaction was slow (e.g. **cP13**, Figure S12a). We confirmed adduct formation by reacting TCEP with **1,2,3-TBMB** and other commonly used crosslinkers in the absence of peptide, with the expected adduct masses observed by LCMS in all cases (Figure S13). Adduct formation has previously been reported and should be accounted for when using Method B.[33]

Given the desirable compatibility of Method A over Method B for downstream screening processes, we chose Method A to demonstrate that **1,2,3-TBMB** bicyclization can be conducted on diverse sequences in multi-well plate format (Figure 5). We obtained 96 crude 11-mer peptides from a commercial vendor, each with one of four possible defined cysteine spacings ($X_3CXCXCX_3$, $X_2CX_2CXCX_3$, $X_2CXCX_2CX_3$, $X_2CX_2CX_2CX_2$, where X is randomly chosen non-cysteine amino acid). For three of the spacing patterns ($X_2CX_2CXCX_3$, $X_2CXCX_2CX_3$, $X_2CX_2CX_2CX_2$), LCMS conversion rates of >90% were observed for 68 of the 72 sequences (94% success rate). In the case of the most constrained pattern however ($X_3CXCXCX_3$), only approximately half the sequences successfully reached >90% conversion (Figure 5a, Table S5), potentially due to conformational restriction and steric hindrance arising from the smaller ring sizes. As an exemplar, two LC UV chromatograms of the crude reaction mixtures are shown in Figure 5, with **$_{96w}$P15** achieving only 60% conversion (Figure 5b) while **$_{96w}$P51** achieved full conversion (Figure 5c). Overall, the data confirmed that **1,2,3-TBMB** linker is compatible with library screening methods, although care should be taken with narrow CXCXC spacing patterns.

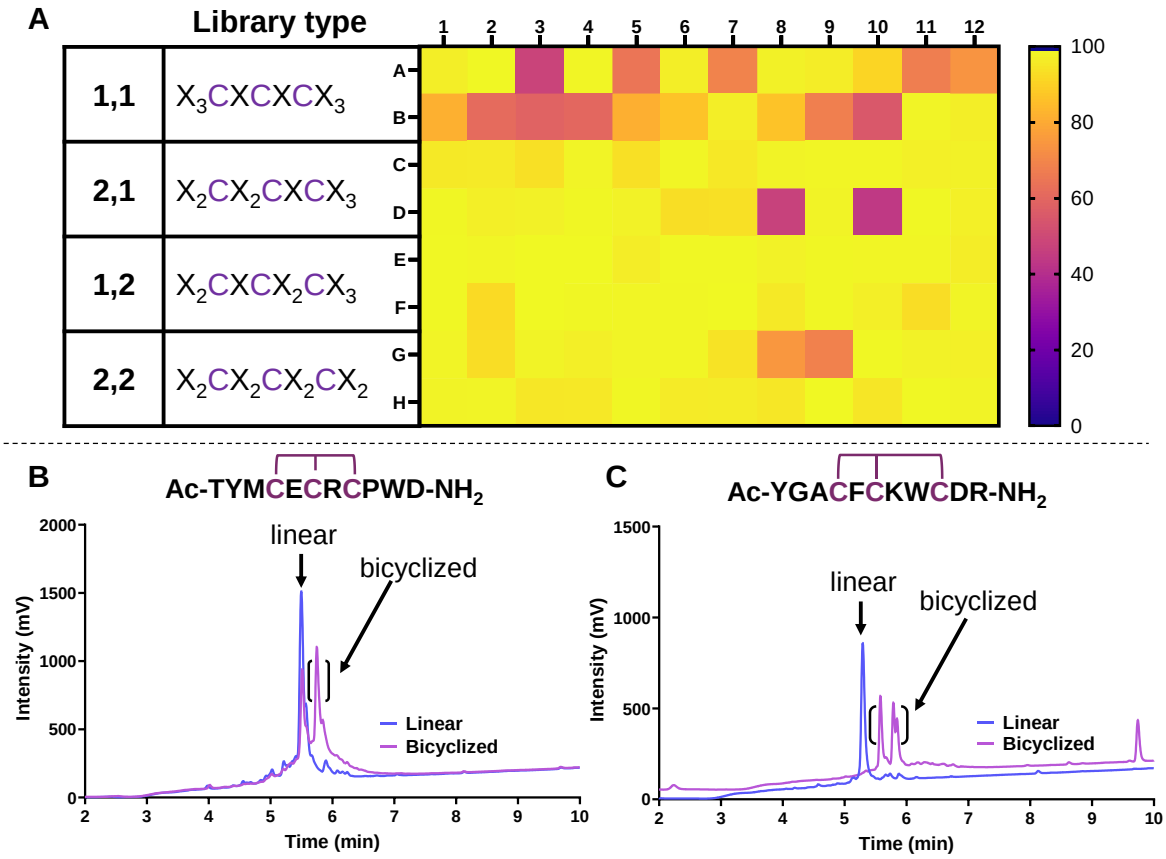


Figure 5. High throughput bicyclization with **1,2,3-TBMB** on 96 crude linear peptides using Method A. **(A)** Heatmap showing the percentage conversion by integration of extracted ion chromatogram peaks from LCMS of crude reaction mixtures, grouped according to the four different cysteine spacing patterns as indicated. **(B)** Exemplar crude LC UV chromatograms for peptide $_{96w}$**P15** with low conversion (60%) and **(C)** peptide $_{96w}$**P51** with high conversion (>95%).

We have reported that **1,2,3-TBMB** is a highly efficient linker for bicyclization of cysteine-containing peptides that is compatible with high-throughput screening processes. The optimized bicyclization conditions using aqueous buffer can accommodate a broad range of peptide sequences with high conversion rates (>90% conversion for 88 of the 106 sequences tested). The formation of three configurational isomers upon bicyclization was confirmed by 2D NMR experiments, with their different substitution patterns providing access to diverse peptide conformations that differ from the analogous linear or **1,3,5-TBMB** bicyclic peptides. In cases where isomers cannot be easily separated, we anticipate that close analogues will be synthetically accessible through a combination of orthogonal protecting group strategies and mixed chemistries to differentiate the reaction sites. Possibilities include orthogonal deprotection of one of the cysteines (e.g. MMT) for subsequent thiol-ene reaction with a bis(bromomethyl)styrene, or using diaminopimelic acid as the central amino acid for a subsequence reductive amination with the corresponding 2,5-dibromomethylbenzaldehyde to generate the amino analogue. In future work, we envision using **1,2,3-TBMB** to generate a broad diversity of bicyclic peptides in the

context of display screening technologies, leading to new hit compounds against a multitude of challenging drug targets.

AUTHOR INFORMATION

Corresponding Authors

*Email addresses: yuheng.lau@sydney.edu.au; lisa.alcock@sydney.edu.au

Notes

The authors declare no competing financial interest.

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