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Synthesis and Functionalization of Azetidine-Containing Small Macrocyclic Peptides

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Cyclic peptides are increasingly important structures in drugs but their development can be impeded by difficulties associated with their synthesis. Here, we introduce the 3-aminoazetidine (3-AAz) subunit as a new turn-inducing element for the efficient synthesis of small head-to-tail cyclic peptides. Greatly improved cyclizations of tetra-, penta- and hexapeptides (28 examples) under standard reaction conditions are achieved by introduction of this element within the linear peptide precursor. Post-cyclization deprotection of the amino acid side chains with strong acid is realized without degradation of the strained four-membered azetidine. A special feature of this chemistry is that further late-stage modification of the resultant macrocyclic peptides can be achieved via the 3-AAz unit. This is done by: (i) chemoselective deprotection and substitution at the azetidine nitrogen, or by (ii) a click-based approach employing a 2-propynyl carbamate on the azetidine nitrogen. In this way, a range of dye and biotin tagged macrocycles are readily produced. Structural insights gained by XRD analysis of a cyclic tetrapeptide indicate that the azetidine ring encourages access to the less stable, all-*trans* conformation. Moreover, introduction of a 3-AAz into a representative cyclohexapeptide improves stability towards proteases compared to the homodetic macrocycle.

Introduction

More than 60 peptide therapeutics have entered the market for clinical use with the majority of them being cyclic, highlighting the importance of macrocyclic peptides as scaffolds for drug development.[1–5] They are privileged structures with improved biological properties compared to their linear counterparts, including better cell permeability and reduced proteolytic digestion.[6–8] By constraining the peptide backbone into a macrocycle, conformational rigidity can be achieved which allows for high target affinity, whilst their large polar surface area enables cyclic peptides to bind difficult targets such as protein-protein interactions with antibody-like specificity.[9,10] Furthermore, the iterative synthesis of cyclic peptides by coupling of amino acid building blocks allows them to be easily modified, optimized and functionalized to tune their molecular properties.[11,12]

Despite the appeal of cyclic peptides as a class of therapeutics, there are major challenges in the development of these molecules as drugs.[13,14] Although they often exhibit enhanced cellular permeability and reduced proteolytic degradation compared to their linear counterparts, low oral bioavailability can still be challenging to overcome.[15,16] Moreover, small cyclic peptides of six or less amino acids are challenging to prepare from their linear peptide precursors. These tend to adopt an extended structure in solution where the *C*- and *N*-termini are not in close proximity hindering macrocyclization.[17–19] This often leads to unwanted side products resulting from cyclo-oligomerization and epimerization at the *C*-terminus. One way to alleviate these issues is to tune the conformational preferences of the linear peptide through replacement of one or more of the L-amino acids. Pseudoprolines, *N*-alkylated derivatives (*e.g.* peptoids), dehydroamino acids and D-amino acids have all been used to aid difficult macrocyclizations (Scheme 1a).[20–22] Other notable synthetic approaches to macrocyclization of peptides include the CyClick strategy, silver-mediated head-to-tail macrocyclization, and isocyanide mediated macrocyclizations.[23–25] However, a universal solution to reliably make small cyclic peptides remains elusive.

Recently, we reported the enhanced synthesis of small head-to-tail cyclic peptides by replacement of one of the backbone amide bonds with the turn-inducing 3-aminooxetane moiety (Scheme 1b).[26] Access to small cyclic peptides were improved compared to homodetic controls over a range of ring sizes measured by reaction rate, product distribution and isolated yields. However, their instability to acidic conditions has limited their utility.[27] To this end, we have developed an azetidine-based isostere of the amide C=O of an amino acid, which can be easily made and installed into cyclic peptide

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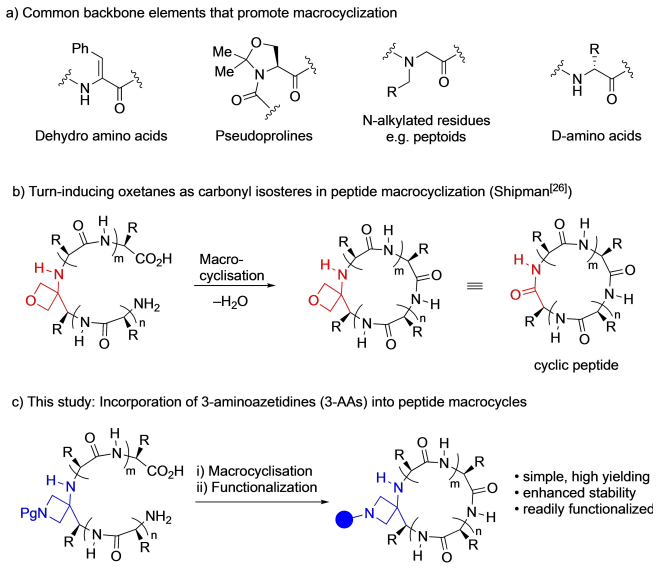


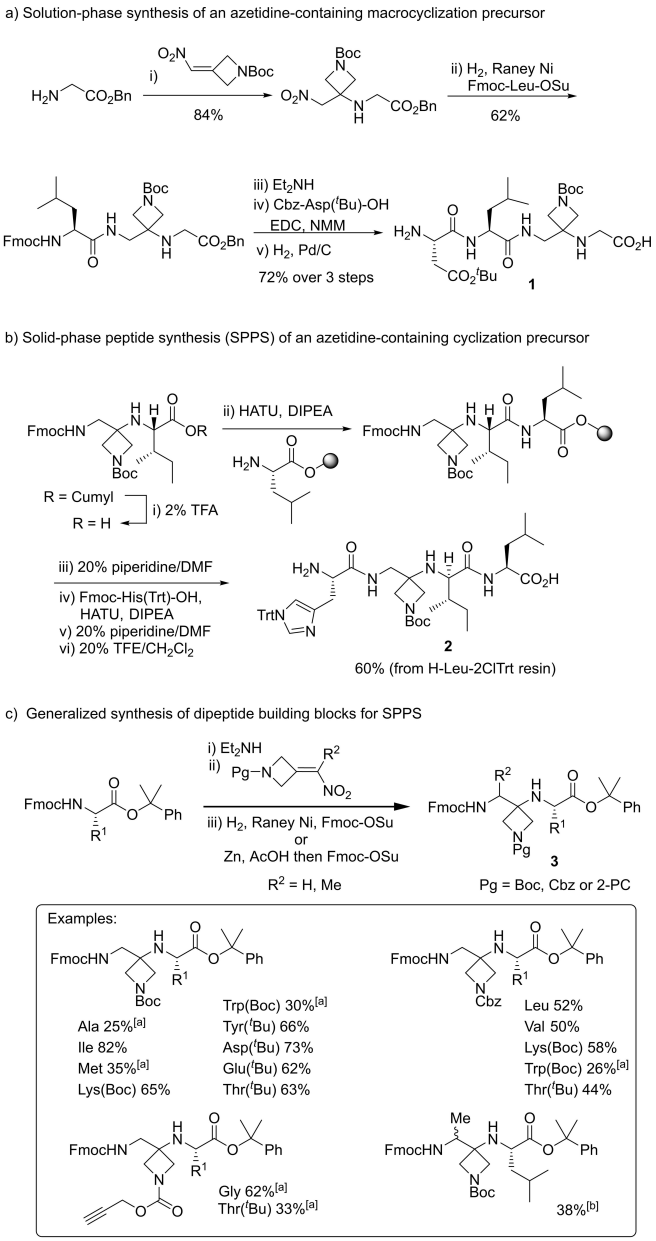
Scheme 1. a) Strategies for the synthesis of small macrocyclic peptides. b) Previous work involving the use of 3-aminooxetanes to improve the macrocyclization of small peptides. c) An overview of this work.

backbones (Scheme 1c). There are several reasons why this strategy may be an attractive approach. Azetidine-based systems were expected to be significantly more stable to acidic conditions whilst maintaining the turn-inducing abilities. In addition, we reasoned that late-stage, chemoselective modification of the azetidine ring within the macrocycle could be possible using a range of chemistries, given the plethora of methods available.[28,29] This would offer an approach to modify the backbone to modulate the physicochemical and biological properties, to enable structure-activity or property relationships, or for the preparation of derivatives with a variety of applications such as the attachment of dyes (for imaging), radionucleotides (for PET), drug conjugates (for receptor targeting or drug delivery), or additional peptide sequences (to aid cell penetration).[30–33] Common approaches for bioconjugation to cyclic peptides rely on sidechain groups, such as the amine of Lys, or require unnatural amino acids.[34] By using the azetidine nitrogen for derivatization, the side chains of the backbone amino acids are unoccupied and would be available to engage in target interactions. Finally, azetidines are important motifs in medicinal chemistry,[35] they can confer improved metabolic stability and pharmacokinetic properties,[36] are found in a number of drugs (e.g. baricitinib, cobimetinib)[37,38] and biologically active natural products (e.g. gelsemoxonine),[39] and small synthetic peptides.[40] Here, we report a new strategy employing a turn-inducing 3-aminoazetidine subunit designed to simultaneously address several of the difficulties associated with cyclic peptide development.

Results and Discussion

First, we required general methods for the synthesis of linear peptides containing a 3-aminoazetidine in place of one of the amide bonds in the peptide backbone. We began by exploring the development of a solution-phase approach as illustrated for the synthesis of tetrapeptide **1** (Scheme 2a). The approach relies on conjugate addition of an α-amino ester to an *N*-protected 3-(nitromethylene)azetidine, concomitant reduction of the nitro group with Raney nickel and coupling to the next amino acid as its preactivated succinimide ester, followed by further extension and *C*- and *N*-terminus deprotection using standard methods.

A second highly practical route was developed using Fmoc/*t*Bu solid-phase peptide synthesis (SPPS). This approach proved to be an efficient method to generate linear macrocyclization precursors, as exemplified by the synthesis of tetrapeptide **2** (Scheme 2b). Here, an azetidine-containing dipeptide building

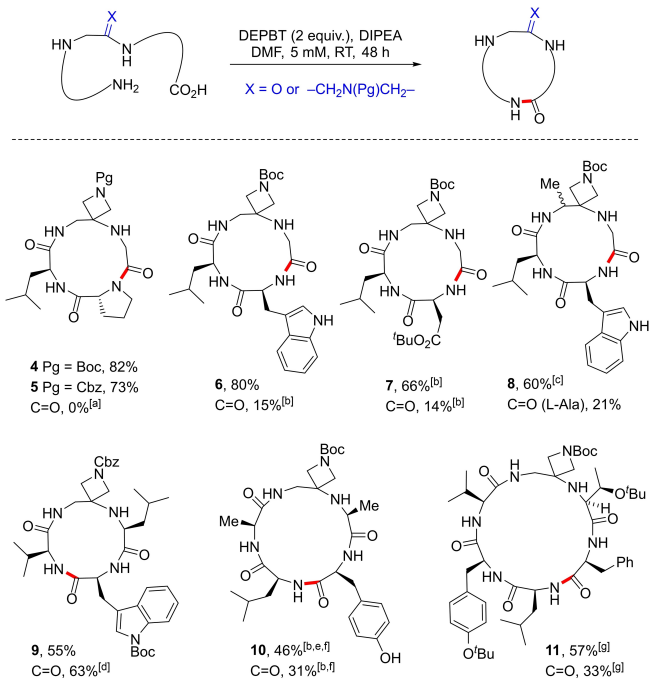


Scheme 2. Strategies for the synthesis of azetidine-modified linear peptides. For full details, see the ESI, pp5-58†. [a] Yield over 2 steps with isolation of the intermediate nitroalkane. [b] Isolated as a 2:1 mixture of diastereomers after column chromatography.

block with a *C*-terminal cumyl ester, removable under mildly acidic conditions, was coupled to a 2-chlorotrityl resin loaded amino acid.[27] Chain elongation at the *N*-terminus by conventional SPPS provided linear peptides such as **2** after final release from the support using 20% trifluoroethanol (TFE).

A wide variety of dipeptide building blocks **3** containing hydrophobic, acidic, basic and polar amino acid side chains were made and installed into linear peptides using this approach. These were made by conjugate addition of an amino 2-phenylisopropyl (cumyl) ester toward an *N*-protected 3-(nitromethylene)azetidine (Scheme 2c). This was followed by reduction of the pendant nitro group with concomitant Fmoc-protection of the *N*-terminus. For substrates which would not tolerate the Raney Ni reduction, a zinc-mediated reduction of the nitroalkane isolated after column chromatography proved to be a practical alternative. Besides conventional *N*-Boc and *N*-Cbz protected azetidines, the preparation of a 2-propynyl carbamate (2-PC) functionalized azetidine for late-stage click reactions was also realized (for full details, see the ESI, pp58†).

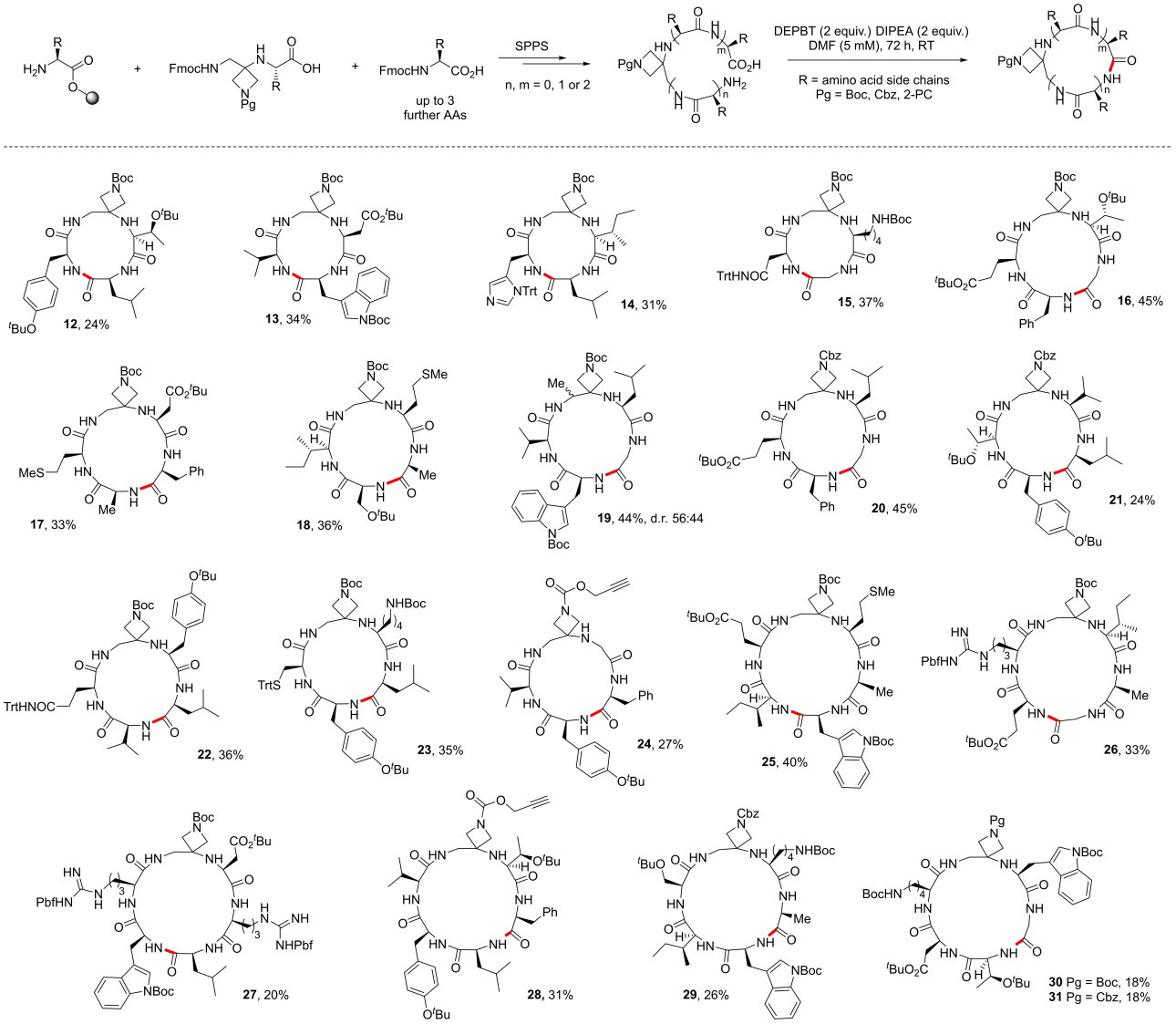
To assess the impact of azetidine modification on macrocyclization efficiency, eight different systems were examined across three different ring sizes (tetra-, penta- and hexapeptides) (Scheme 3). In each case, the macrocyclization efficiency was compared with that of the homodetic peptide sequence (X = O) under identical conditions using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) as activating agent. In all cases, the azetidine-modified cyclic peptides (AMCPs) **4**–**11** could be isolated in improved yields. Here, NMR analysis of a linear pentapeptide related to **10** showed that the azetidine is turn-inducing, providing an explanation for the observed improvement in macrocyclization compared to homodetic controls (see the ESI, pp10-26†). Little difference was seen in the isolated yields using either Boc or Cbz protection on the azetidine nitrogen (**4** *cf.* **5**). Ring closure for the tetrapeptide homodetic controls was very difficult, producing the macrocycles either in very low yield or not at all, with the dimeric octapeptide isolated instead. In contrast, the azetidine modification provided access to cyclic tetrapeptides **6** and **7** in four- to five-fold higher yields. A comparative study of azetidine incorporation against unmodified peptides highlights the strength of the modification to open up difficult to access chemical space. Whilst the azetidine-modified cyclic peptide **9** could be isolated as a single diastereomer in acceptable overall yield, cyclization of the identical sequence without modification provided the epimer as the major product alongside the desired macrocycle as an inseparable 55:45 mixture. Even placing the Gly residue in the sequence at the *C*-terminus to remove the chance for epimer formation was also not productive, this time leading to very low yields of the homodetic cyclic tetrapeptide (see the ESI, pp35-37†). We were also able to isolate **8** by macrocyclization of a linear precursor containing a 1:1 mixture of diastereomers at the 3-AAz center. The (*R*)–**8** and (*S*)–**8** diastereomers were readily separable by simple column chromatography.



Scheme 3. Comparison of the cyclization efficiency of azetidine-modified linear precursors with their homodetic sequences. C=O relates to macrocyclization of the parent peptide precursor from the literature under identical conditions unless otherwise stated.[26] Bond formed in the macrocyclization highlighted in red. [a] Yield of the dimeric cyclic octapeptide was 20%, cyclic tetrapeptide not observed. [b] Reaction concentration 1 mM. [c] Combined yield for 1:1 mixture of diastereomers, separated by column chromatography after cyclisation. [d] Isolated as an inseparable 55:45 mixture of diastereomers. [e] Isolated as an 8:1 mixture of diastereomers. [f] Reaction time 24 h. [g] Reaction time 72 h.[41]

It was clear that the azetidine modification enabled improved macrocyclization efficiency, so we sought to make a wider range of examples. A combined SPPS/solution phase macrocyclization protocol proved to be a practical way to produce a larger library of AMCPs. We generated twenty examples (**12**–**31**) across a range of different ring sizes incorporating Boc, Cbz and 2-PC protected azetidines in good overall yields (18–45%) based on the initial loading of the resin (Scheme 4). The azetidine-modified residue was positioned toward the middle of the linear starting sequence to gain the maximum benefit from any turn-inducing effect.[26] We focused on ring sizes containing four to six amino acids in length, as these are the most challenging examples to prepare. A variety of different *C*-terminal amino acids could be tolerated without extensive epimerization as judged by the isolation of single diastereomers. The cyclization worked well with various *N*-terminal amino acids including bulky residues, such as Asn(Trt), Ser(^{t}Bu) and Trp(Boc), which are often difficult to cyclize.[42] Synthesis of macrocycles containing azetidine-modified alanine residues was also possible using this methodology. Each of the AMCPs could be deprotected using TFA to reveal the azetidine amine as the TFA salt (see the ESI, pp45–53†). No evidence of azetidine ring opening was observed during the deprotections, even for macrocycles such as **27**, which required strongly acidic conditions (90:5:5 TFA/TIS/CH_2Cl_2) for deprotection of multiple Arg(Pbf) residues.

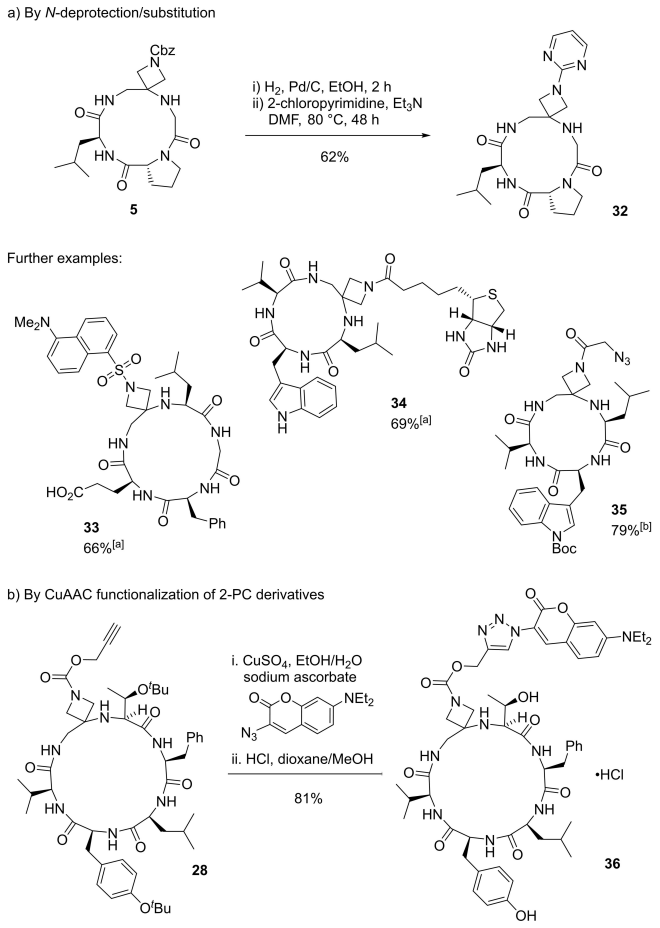
Next, our attention turned to using the azetidine nitrogen as a handle for late-stage functionalization of the macrocycles.



Scheme 4. Combined SPPS/solution phase macrocyclization approach to diverse AMCPs. Yield over the whole reaction sequence based on preloaded 2-ClTrt resin as the limiting reagent. Bond formed in the macrocyclization highlighted in red. For full experimental details, see the ESI, pp34-45†.

To allow orthogonal deprotection in the presence of the acid-labile side chain protecting groups used in the SPPS, we focused on macrocycles bearing Cbz-protected azetidines. This approach was tested with tetrapeptide **5**, which after hydrogenation was functionalized by nucleophilic aromatic substitution with 2-chloropyrimidine to **32** (Scheme 5a). Crucially, reaction at the more hindered secondary amine within the backbone of the macrocycle was not observed. Other simple chemistries such as acylation and sulfonylation also worked well. These reactions were used to produce dansyl, biotin and azide tagged derivatives **33**–**35** in good overall yields. A complementary approach that allows functionalization without the need to remove the azetidine protecting group was also explored. Macrocycles containing 2-propynyl carbamates (2-PC) proved to be suitable substrates for Cu-catalyzed azide–alkyne cycloaddition (CuAAC) chemistry. For example, **28** bearing the 2-PC moiety readily underwent click reaction with a coumarin-based azide[43] to generate a fluorescently conjugated macrocycle **36** after acidic deprotection of the side chains (Scheme 5b). UV/Vis spectroscopy verified the emission and extinction properties of the compound ($\lambda_{ex} = 407$ nm, $\lambda_{em} = 482$ nm, $\Delta\lambda = 75$ nm, MeOH). Access to macrocycle **35** containing an azide also enables the potential use of strain-promoted or Cu-based click reactions for late-stage functionalization of cyclic peptides.

Preliminary insights into the impact of azetidine substitution on the solid-state structure of the macrocyclic peptides were revealed through analysis of the crystal structure of cyclic tetrapeptide **35** (Figure 1).[44] Treating the azetidine ring as if it was an amide C=O, **35** is observed in a boat shape conformation, where all of the amides are in the *trans* conformation without additional stabilization by intramolecular hydrogen bonds. Due to the strained nature of twelve membered rings, their conformational landscape is often



Scheme 5. Strategies for the functionalization of azetidine-containing macrocycles. For conditions and full details, see the ESI, pp54-57†. [a] Yield over three steps for Cbz deprotection, functionalization and global deprotection. [b] Yield over two steps for Cbz deprotection followed by functionalization.

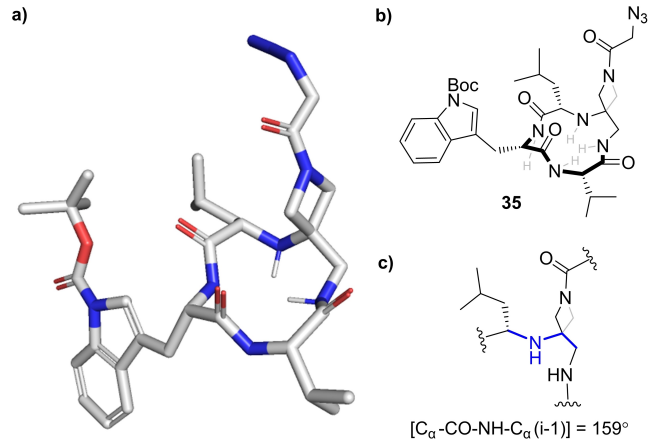


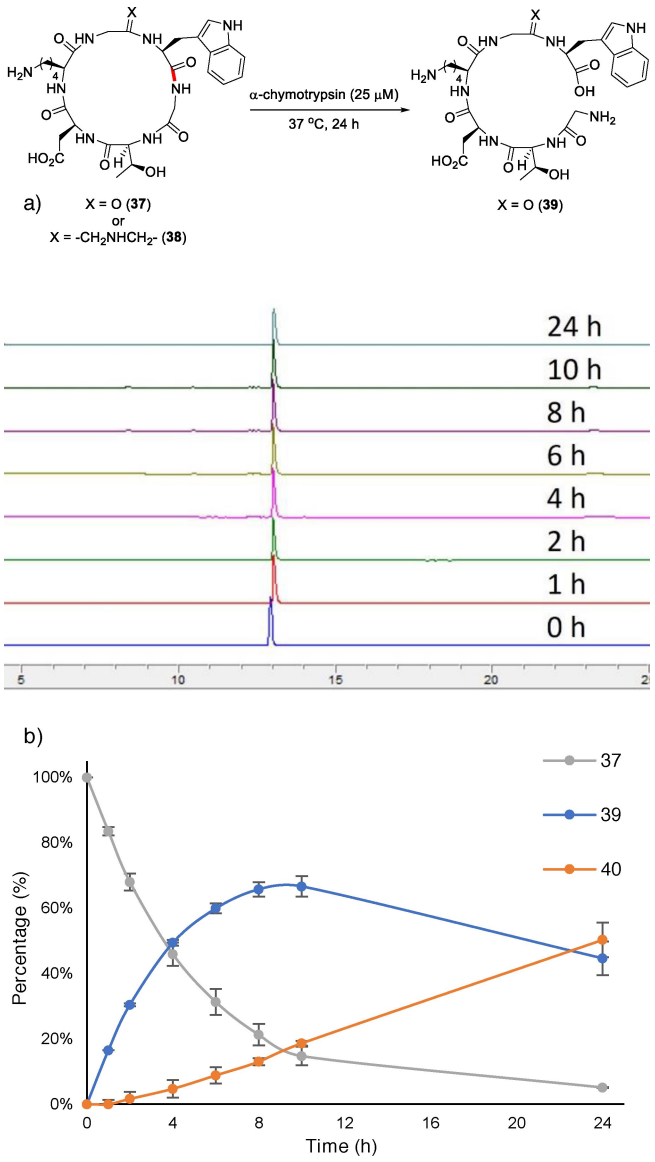
Figure 1. Structural insights from the crystal structure of cyclic tetrapeptide **35**. a) Structure determined by single crystal X-ray diffraction. b) Illustration of the observed all-*trans* conformation in the solid-state. c) Depiction of the Ω dihedral angle of the GAz residue in **35**.

complex. The all-*trans* configuration of the amide bonds within all-L cyclic tetrapeptides is uncommon because the lowest energy conformation often includes one or more *cis*-amide bonds to relieve strain.[45,46] Each of the amide bonds in **35** are slightly twisted from planarity with Ω [C_α-CO-NH-C_α(i-1)] dihedral angles 12–22° lower than the ideal value of 180°, which is a commonly observed feature for *trans* amide bonds within cyclic tetrapeptides.[47] The azetidine ring appears to mimic a *trans* amide bond well, as the dihedral angle measured for the azetidine-modified glycine in **35** is 159° (Figure 1c). Replacement of the sp^2 amide C=O with the sp^3 azetidine carbon appears to reduce angular strain in the macrocyclic product, which stabilizes the all-*trans* conformation.

To further probe the reactivity of the azetidine-modified macrocycles, we studied their proteolytic stability. A proteolytic assay was designed to compare the relative rate of α-chymotrypsin digestion of cyclo(Thr-Asp-Lys-Gly3-Trp-Gly1) (**37**) and **38**, in which the Gly3 residue is replaced by an azetidine-modified residue. These sequences contain a single amide target for α-chymotrypsin hydrolysis (the bond highlighted in red). At an α-chymotrypsin concentration of 25 μM, the azetidine-modified peptide **38** displayed impressive proteolytic stability, with no measured proteolysis over 24 h, as illustrated by the HPLC traces for the assay at regular time points (Scheme 6a). Even at elevated enzyme concentration of 125 μM, the cyclic peptide remained stable (Figure S4). In contrast, the unmodified cyclic peptide **37** showed near complete disappearance of the starting peptide peak within 24 h, with only 5% remaining and the formation of two new species (Scheme 6b). The two species were identified by LC–MS analysis as the macrocycle opened linear hexapeptide **39** and linear tetrapeptide **40**. Monitoring the three peaks over time, it can be inferred that **39** was formed first from an initial macrocycle opening, with **40** being generated following a second hydrolysis. Charged amino acid residues in the 2′ position are known to inhibit α-chymotrypsin activity, and we propose that the azetidine interferes with enzyme-substrate binding, preventing amide hydrolysis. This was further supported by analysis of the Cbz protected macrocycle **41**, where two different peptide digestion routes were observed: either macrocycle opening of the protected peptide at the targeted position, or removal of the Cbz group to give **38**, which did not undergo further degradation. In case cyclic **41** was opened, no further Cbz cleavage was observed (see the ESI, pp62-70†). It appears that the azetidine modification provides protection of the critical amide bond which would be otherwise proteolytically digested. This offers the opportunity to replace amide linkages that are found to be sensitive to degradation by a protease during hit-to-lead optimization of peptide therapeutics.

Conclusions

In summary, the 3-aminoazetidine unit has been explored as a tool for the efficient synthesis of small head-to-tail cyclic peptides containing four to six amino acids in length. Linear peptides can be synthesized using solution phase or SPPS techniques utilizing dipeptide building blocks, followed by subsequent macrocyclization in solution. In each of the examples studied, azetidine modification leads to up to five-



Scheme 6. Analysis of α-chymotrypsin degradation of cyclic peptides. a) HPLC data for cyclo(Thr-Asp-Lys-GAz-Trp-Gly) (**38**) over a period of 24 h with GAz being the azetidine-modified glycine. b) Peptide peak percentage for α-chymotrypsin assay over time for cyclo(Thr-Asp-Lys-Gly-Trp-Gly) (**37**) (grey) and linear degradation products H-Gly-Thr-Asp-Lys-Gly-Trp-OH (**39**) (blue) and H-Asp-Lys-Gly-Trp-OH (**40**) (orange). Peak intensity calculated using analytical HPLC traces. Assay conditions: hexapeptide (200 μL, 1.5 mM), α-chymotrypsin (200 μL, 50 μM), 37 °C, 24 h; final peptide concentration 750 μM, α-chymotrypsin concentration (25 μM). See ESI, pp62-70† for full assay development.

fold improvement in isolated yield of the cyclic peptides across a range of ring sizes, with particular enhancement for smaller ring sizes. A range of carbamate protecting groups can be employed on the azetidine nitrogen to enable the synthesis of different AMCPs: (i) Boc to unmask the azetidine as the free amine after acidic deprotection; (ii) Cbz which can be orthogonally removed in the presence of the side chain protecting groups to allow for chemoselective *N*-functionalization with a variety of different electrophiles; and (iii) a 2-propynyl carbamate, which serves as a functional handle for click reactions. Crystal structure analysis of a cyclic tetrapeptide indicates that the azetidine ring is a good isostere of a *trans* amide C=O in the solid state, which stabilizes conformations that would otherwise be difficult to access in the corresponding homodetic system due to ring strain. Protease stability studies suggest that the azetidine modification provides protection against enzymatic cleavage compared to the homodetic control, which is readily hydrolyzed. Further studies will aim to assess the structural effect of the azetidine modification in solution state and across larger ring sizes, as well as the utility of other functionalized azetidine-modified cyclic peptides towards drug development challenges, such as cell permeability and oral bioavailability.

Supporting Information

The authors have cited additional references within the Supporting Information.[26,27,43,48–52]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: macrocyclization · peptidomimetics · cyclic peptides · azetidine · functionalization

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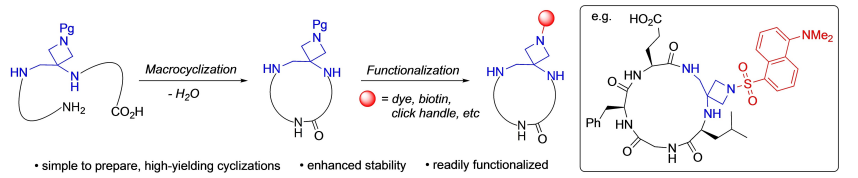

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1 – 8

Synthesis and Functionalization of Azetidine-Containing Small Macrocyclic Peptides

Incorporation of a 3-aminoazetidine (3-AAz) into peptide backbones improves head-to-tail cyclizations compared to unmodified peptides. The azetidine nitrogen can be readily functionalized using substitution or click chemistry. Crystal structure analysis reveals that a 3-AAz modified cyclic tetrapeptide adopts an uncommon all-*trans* conformation. The 3-AAz provides stability to protease degradation compared to the unmodified macrocycle.

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