Many natural cyclic peptides have potent and potentially useful biological activities. Their use as therapeutic starting points is often limited by the quantities available, the lack of known biological targets and the practical limits on diversification to fine-tune their properties. We report the use of enzymes from the cyanobactin family to heterocyclise and macrocyclise chemically synthesised substrates so as to allow larger-scale syntheses and better control over derivatisation. We have made cyclic peptides containing orthogonal reactive groups, azide or dehydroalanine, that allow chemical diversification, including the use of fluorescent labels that can help in target identification. We show that the enzymes are compatible and efficient with such unnatural substrates. The combination of chemical synthesis and enzymatic transformation could help renew interest in investigating natural cyclic peptides with biological activity, as well as their unnatural analogues, as therapeutics.
The measured maximum absorbance ($\lambda = 649$ nm) and emission ($\lambda = 671$ nm) properties of conjugate 8 were in good agreement with those of the parent Cy5 molecule (Figure S33). As these types of compounds could potentially be used for target identification by fluorescence microscopy, conjugate 8 (dark blue colour) was tested to ensure there was no unexpected behaviour (such as quenching or precipitation) in cells. When incubated with permeabilised HeLa cells, a diffuse staining pattern of 8 (red colour) throughout the cytoplasm and nucleus was visualised by fluorescent microscopy (Figure 2); this showed that the molecule behaves as expected in biological buffers.
Cyclic peptide 4 underwent a thio-Michael addition with the cysteine-containing glutathione peptide 9 (Scheme 4) with an excess of triethylamine in water and methanol.\(^\text{11b}\) The corresponding compound 10 was obtained in 43% yield. Following the successful addition of glutathione, we investigated whether the reaction could be carried out directly after the macrocyclisation reaction as a one-pot process. Once peptide 3 has been fully macrocyclised, 100 equivalents of mercaptoethanol were directly added into the reaction mixture, and this was left at 37°C overnight. The reaction was judged to be complete by MS, and the final compound 11 was obtained in 60% yield. The final product purifies as two separable peaks, which we attribute to different diastereoisomers (Figure S34).

As PatG\(_{\text{mac}}\) processes substrates with unnatural amino acids at similar rates to other sequences,\(^\text{9}\) we next tested the feasibility of introducing heterocycles into such unnatural substrates. The proline residue in peptide 1 was replaced with a cysteine (peptide 12) that could be enzymatically heterocyclised. Like PatG\(_{\text{mac}}\), the heterocyclase enzymes of the cyanobactin pathways (known as the D enzymes)\(^\text{3a, 19}\) have been shown to be tolerant of a wide range of sequences within the core peptide.

We incubated peptide 12 overnight with the engineered heterocyclase Lyndfusion (from the aestuaramide pathway (\textit{Lyngbya} sp.)) in the presence of ATP and MgCl\(_2\). The fully heterocyclised product 13 was detected by MS but not isolated (Scheme 5; Figures S35–S36). Subsequent addition of PatG\(_{\text{mac}}\).
to the reaction mixture afforded the patellamide-like analogue to 14 cyclo(-ITAA(NH)N(C-C-) in 58% yield.[20]

Milligram quantities of cyanobactin derivatives with fluorescent components will greatly facilitate the target identification of many of these natural biologically active products.[20] Target identification will not only provide a basis for redesign of the natural product but could also disclose new opportunities for therapy. The expense and complexity of these labels means in practical terms that they are better introduced late in the synthesis. In the case of macrocyclic peptides, this means ideally after the macrocycle is made. Introducing chemical diversity to probe or fine tune the pharmacokinetic and biological properties of natural products is likewise most desirable when performed as a final step on a common scaffold.

We have demonstrated that both the heterocyclases and macrocyclases from the patellamide (and a related) pathway can be used in vitro with entirely synthetic substrates that contain such chemically reactive unnatural amino acids. Moreover, we have shown that the resulting macrocycles can be derivatised with high efficiency. The ability to combine the diversity of chemical synthesis with the exquisite catalysis of enzymes is well known and recognised to be powerful in developing natural products into therapeutics.[21] This approach can be extended to peptidic macrocycles and might likewise enable their further development.

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[20] The stereochemistry of the alanine residue next to the heterocycle is unknown, as epimerisation is known to happen at some point during the biosynthesis. What we can confirm from the NMR data is the absence of any mixture of two diasteriomers.

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