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Biosynthesis **Hot Paper**

Cytochrome $P450_{Blt}$ Enables Versatile Peptide Cyclisation to Generate Histidine- and Tyrosine-Containing Crosslinked Tripeptide Building Blocks

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Abstract: We report our investigation of the utility of peptide crosslinking cytochrome P450 enzymes from biarylitide biosynthesis to generate a range of cyclic tripeptides from simple synthons. The crosslinked tripeptides produced by this P450 include both tyrosine-histidine (A–*N*–B) and tyrosine-tryptophan (A–*O*–B) crosslinked tripeptides, the latter a rare example of a phenolic crosslink to an indole moiety. Tripeptides are easily isolated following proteolytic removal of the leader peptide and can incorporate a wide range of amino acids in the residue inside the crosslinked tripeptide. Given the utility of peptide crosslinks in important natural products and the synthetic challenge that these can represent, P450 enzymes have the potential to play roles as important tools in the generation of high-value cyclic tripeptides for incorporation in synthesis, which can be yet further diversified using selective chemical techniques through specific handles contained within these tripeptides.

The crosslinking of peptides is common to many important, synthetically challenging natural products (Figure 1), including streptide,[1] the glycopeptide antibiotics (GPAs)[2] and arylomycin,[3] where it rigidifies and stabilises peptides as well as leading to potential restricted rotation and planar chirality.[4] Within the biosynthesis pathways of crosslinked peptides, one enzyme class commonly responsible for insertion of these side chain crosslinks is the cytochrome P450s (P450s).[5] P450s can perform an array of oxidative transformations in many biosynthetic pathways via activating molecular oxygen as a highly powerful intermediate (compound I),[6] making them capable of a wide range of transformations beyond C–H hydroxylation.[5] Given this synthetic utility and combination of oxidative power and regiochemical precision, P450s have been widely implicated as potential biocatalysts.[7] Beyond the use of natural P450s, many approaches have been explored to expand the scope of P450s yet further, including the use of alternate metalated enzymes as well as techniques to alter their substrate preference, such as library shuffling, ancestral variants, directed evolution and the use of decoy substrates.[8]

Whilst the power of P450s as biocatalysts is apparent, a challenge to their application in peptide crosslinking is that many pathways in which they occur are non-ribosomal and are difficult to engineer or exploit for synthesis due to their challenging (typically enzyme bound) substrates.[9] Ribosomal (RiPP) pathways offer greater potential to identify P450 (and other)[10] enzymes as biocatalysts due to their simpler substrates,[11] although these can still be long peptides due to the large leader sequences required for cyclisation (as seen in darobactin and cittilin).[12] Given this, we focussed on the biarylitides, a class of RiPPs that contain YxH tricyclic motifs installed by P450s.[13] Strikingly, this crosslink is found in a pentapeptide substrate, implying that the leader peptide is only two amino acids, and suggesting great biocatalytic potential for these P450s. We show that this pathway can

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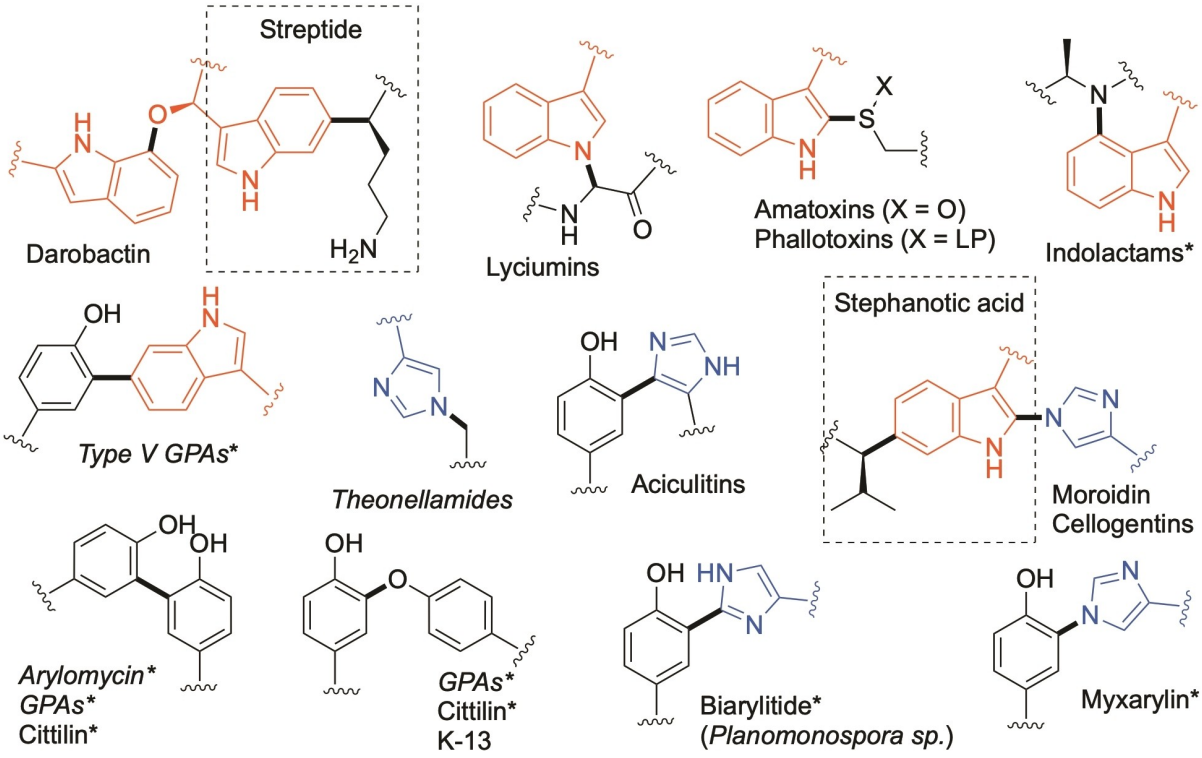

Figure 1. Peptide crosslinks found in RiPP and NRPS biosynthesis pathways. NRPS pathways shown in italics, crosslinks shown in bold, those installed by P450s indicated (*). LP = lone pair.

generate a range of cyclic tripeptides via P450-enabled biocatalysis and further that this allows incorporation of tryptophan in such crosslinked peptide building blocks.

To commence our study, we selected 9 P450s from a range of biarylitide pathways whose substrate sequences were MxYxH (Supporting Information Figure 1).[13] These P450s share a minimum sequence identity of 47 % and are from different families (Supporting Information Table 1, Supporting Information Figure 2). Expression of these constructs in *E. coli* demonstrated high level expression of 4, (Supporting Information Figure 3) with analysis of their catalytic competence and cyclisation activity towards MRLYH peptide **1** leading to the selection of CYP1251 C3 (*Micromonospora* sp. MW-13) for further investigation (referred to as $P450_{Blt}$). The CO complex of $P450_{Blt}$ showed a moderate proportion of catalytically competent enzyme (characterised by 450 nm absorption, Supporting Information Figure 4), and turnover of the **1** using the PuR/PuxB electron transport system from *Rhodopseudomonas palustris* CGA009[14] afforded >80 % conversion to the cyclic peptide under the conditions of our assay (Figure 2, Supporting Information Figure 5). We noted significant Met sulfoxidation in these assays, likely due to non-specific oxidation by side products of the $P450_{Blt}$ activation cycle, although this is unproblematic for generating cyclic tripeptides via tryptic digestion (see below). The binding of **1** to $P450_{Blt}$ displayed a relatively low spin state shift (5 %) and affinity in the low micromolar range (k_d = 2.1 μM, Supporting Information Figure 5), 10-fold tighter than P450 binding to related substrates (diketopiperazines, CYP121—21 μM;[15] CYP134A5 —24 μM)[16] and instead comparable to that seen with the peptidyl-PCP-X didomain substrates present in GPA crosslinking (k_d = 1.7 μM).[17] Liberation of the cyclic tripeptide $\mathbf{\Delta N\text{-}1_{linked}}$ from $\mathbf{1_{linked}}$ was performed via addition of trypsin following $P450_{Blt}$ assays, demonstrating a facile route to isolate cyclic tripeptide building blocks from such assays. Analysis of $\mathbf{\Delta N\text{-}1_{linked}}$ by NMR and the hydrogen/deuterium exchange (HDX) revealed that this crosslinked peptide contained an A–*N*–B crosslink (Supporting Information Figures 6–18), which is the same type of crosslink reported in myxarylin[18] but different to the A–B crosslink initially

Table 1: Exploring the substrate tolerance of $P450_{Blt}$ for alterations within the directly crosslinked residues (P3/P5) of pentapeptide substrates.

Entry	N-term	Peptide sequence	C-term	Conversion [%]
17	H–	MRYLh	–OH	6 (±1)
18	H–	MRyLH	–OH	2 (±1)
19	H–	mrylh	–OH	3 (±1)
20	H–	MRHLY	–OH	2 (±1)
21	H–	MRYL-Hpg	–OH	2 (±1)
22	H–	MR-Hpg-LH	–OH	8 (±1)
23	H–	MRYL-Thz	–OH	2 (±1)
24	H–	MRYLW	–OH	55 (±5)
26	H–	MRYGW	–OH	19 (±5)
27	H–	MRYAW	–OH	21 (±3)
28	H–	MRY-Nle-W	–OH	66 (±4)

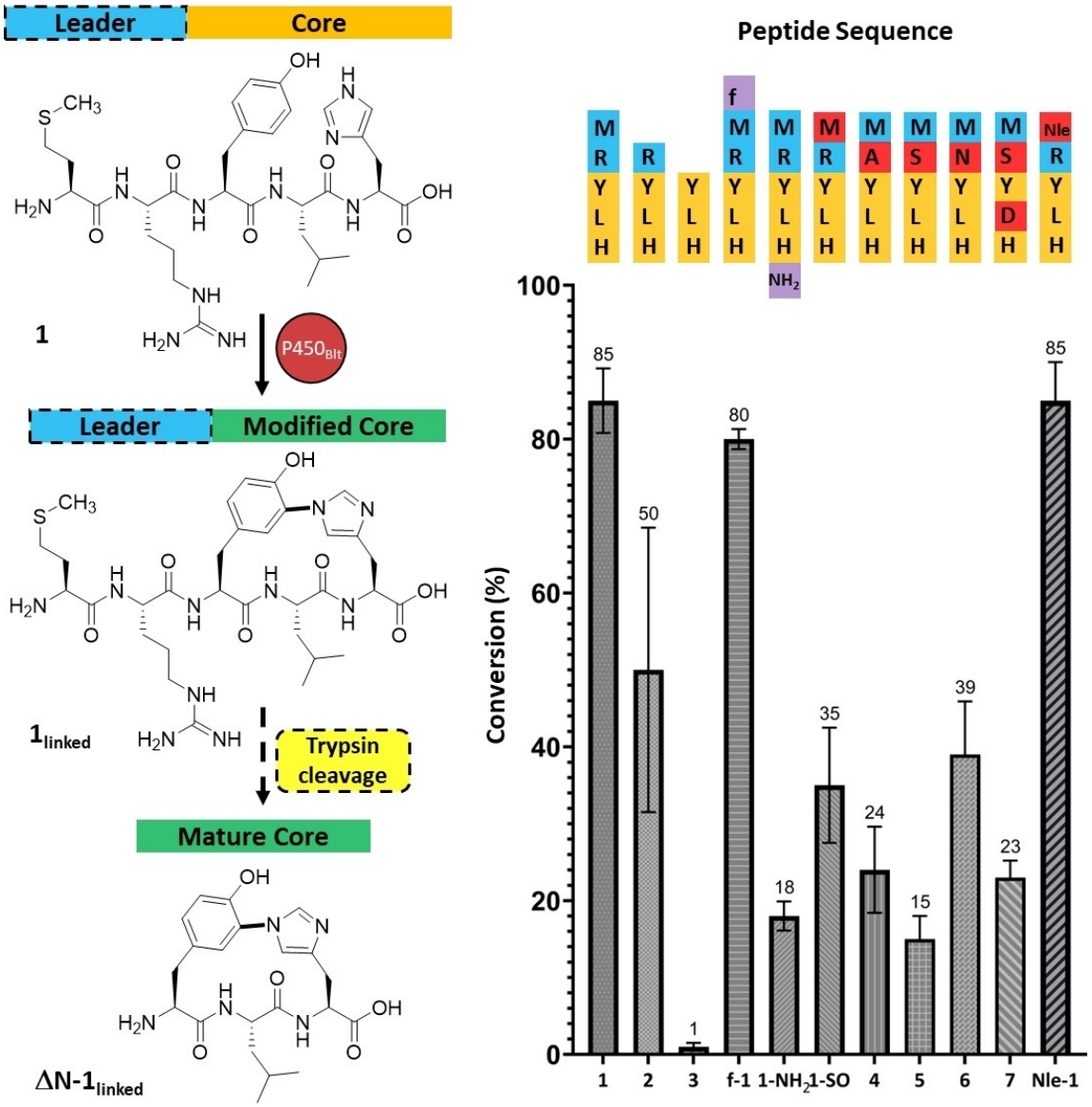

Figure 2. Peptide synthesis, P450-mediated cyclisation, and proteolytic cleavage to afford A–*N*–B linked cyclic tripeptides (left) with exploration of a range of modified biarylitide peptides (right). Reactions were conducted as described in small scale enzymatic cyclisation section (see Supporting Information). Conversions were calculated using AUC (area under curve) of the total ion current from LCMS analysis. Conversion (%) = (AUC_{cyclic} + $AUC_{cyclic(SO)}$)/(AUC_{linear} + $AUC_{linear(SO)}$ + AUC_{cyclic} + $AUC_{cyclic(SO)}$) X 100; for substrates without Met at position 1, $AUC_{linear(SO)}$ and $AUC_{cyclic(SO)}$ equal 0. Blue: leader, yellow: core, green: cyclised core, red: altered residue, purple: modification of N/C-termini.

reported for biarylitides.[13] Given this, we re-analysed the acetylated biarylitide YYH_{linked} produced by the P450 BytO (39 % sequence identity to $P450_{Blt}$) in *Planomonospora*[13] and compared the spectra obtained to **ΔN-1$_{linked}$** (Supporting Information Figure 16–17), which confirmed the difference in crosslinking pattern produced by these two related P450s and highlighting their impressive catalytic diversity.

With activity of $P450_{Blt}$ demonstrated towards **1**, we next analysed of the effect of alterations to the peptide substrate to examine the potential synthetic utility of this P450 (Figure 2, Supporting Information Figures 19–29). First, we investigated the effect of removing the (already minimal) leader sequence, which showed that removal of Met (**2**) significantly reduced activity and removal of Met-Arg (**3**) essentially abolished activity. This change appears due to the inability of these truncated substrates to generate the requisite spin state shift of the $P450_{Blt}$ heme iron (i.e., non-productive binding) rather than a loss in direct enzyme affinity. Modification of the peptide N-terminus through formylation (**f-1**) did not affect cyclisation, whilst truncation of the Met side chain (ARYLH, **4**) resulted in a major reduction of cyclisation (24 %), further supporting the importance of Met for effective $P450_{Blt}$-catalysed peptide cyclisation. Met sulfoxidation (**1-SO**) led to a reduction in total cyclisation by more than 50 %, indicating that these interactions are likely hydrophobic in nature. As significant

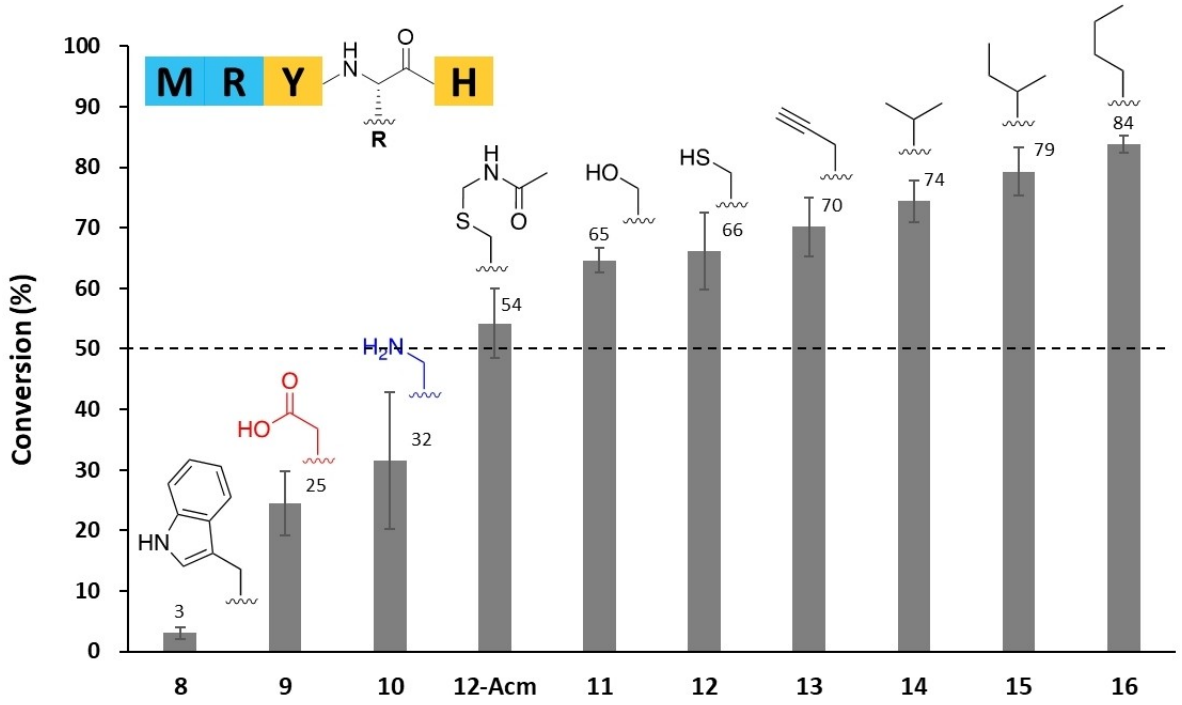

Figure 3. Exploration of substrate tolerance around P4 of the pentapeptide. Reactions conducted as described in the Supporting Information and conversions calculated as for Figure 2. Side chains indicated for each residue.

sulfoxidation is seen in most $P450_{Blt}$ assays, this further suggests that peptide cyclisation is rapid, whilst sulfoxidation is slower and likely caused through oxidation by reactive oxygen species generated during the P450 active cycle. To avoid sulfoxidation, we also tested the replacement of the Met residue with Nle within the wildtype peptide sequence (**Nle-1**), which showed that this replacement is well tolerated (85 % conversion) and also simplifies purification. Replacement of the C-terminal carboxylate of **1** with an amide (**1-NH_2**) revealed a 4-fold loss of $P450_{Blt}$ activity, attributable to a loss of two-orders of magnitude binding affinity (k_d = 179 μM, Supporting Information Figure 5) and suggestive of the loss of a salt bridging interaction.

Turning to position 2 (P2), we next replaced Arg with Ser (**5**) and Asn (**6**), which both showed reduced cyclisation activity (≈6-fold for **5** (15 %), ≈2-fold for **6** (39 %)). This further supports the importance of the Arg residue for peptide binding to $P450_{Blt}$ (Arg also being adventitious for isolation of cyclic tripeptides using trypsin), although curiously the cyclisation of the MSYDH peptide (**7**) showed no further reduction in cyclisation than for **5** alone (Figure 2, Supporting Information Figures 26–28). As a biarylitide crosslinking P450 (43 % sequence identity to $P450_{Blt}$) has recently been reported to introduce an A–*N*–B crosslink in a MNYLH pentapeptide,[18] we also carefully inspected the NMR data of myxarylin in comparison to that of **ΔN-1_{linked}**, with the differences observed suggesting that this altered A–*N*–B crosslinking pattern may be specific to each individual P450.

Given that the most interesting position outside of the crosslinking positions themselves is arguably P4 of the pentapeptide, we next studied the acceptance of a range of peptides with altered residues at P4 by $P450_{Blt}$ (Figure 3, Supporting Information Figures 30–39). We tested a range of peptides exploring the effect of side chain size (Val (**14**), Ile (**15**), Nle (**16**), Trp (**8**)) and charge (Asp (**9**)) on $P450_{Blt}$-catalysed cyclisation. These experiments showed that there is considerable tolerance for alterations in the size of P4, although charge appears problematic for $P450_{Blt}$. Curiously, whilst cyclisation of **14**–**16** showed no change in cyclisation compared to Leu (**1**), incorporation of Trp (**8**) almost totally abolished activity, showing that there is a limit to the size of residue that can be included inside the crosslink of the current (biosynthetic, not engineered) enzyme. The reduced level of cyclisation seen for **9** further revealed the "pocket" for P4 is likely hydrophobic in nature, although it is important note that P450s accepting peptides containing Trp_4 and Asp_4 residues have been reported.[13] Thus, future access to a peptide bound structure of $P450_{Blt}$ would be highly informative when combined with sequence data and could well enable the engineering of $P450_{Blt}$ to support activity towards such peptides.

Given the potential utility of an enzymatic route to a range of cyclic tripeptide building blocks, we further explored modifications of P4, concentrating on residues whose side chains contained moieties of use for chemical diversification (**10**–**13**, Figure 3, Supporting Information Figures 32–36). Peptides tested included those with an alkyne-containing Pra residue (**13**), alcohol containing Ser (**11**), thiol-containing Cys (both unprotected **12** and Acm-protected **12-Acm**) and amine-containing Dap (**10**) at P4. Peptides **11**–**13** were well accepted by $P450_{Blt}$, showing an impressive degree

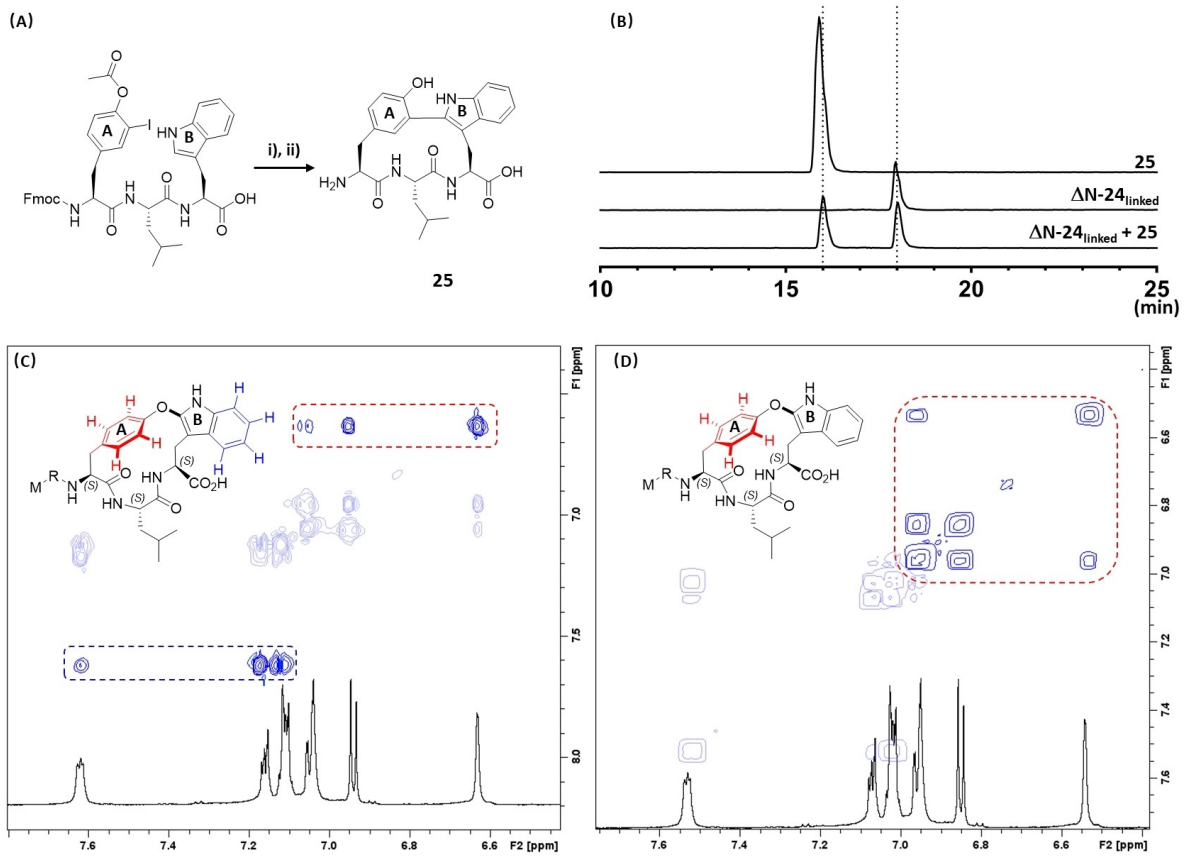

Figure 4. A) Synthesis of A–B crosslinked YLW standard **25**. i) 5 % mol % $Pd(OAc)_2$, 1.0 equiv of $AgBF_4$, 1.5 equiv of 2-NO_2BzOH in DMF: PBS (1 : 1), 80 °C (microwave), 12 h. ii) 0.5 M NaOH, 20 min. B) TIC analysis of enzymatically cyclised YLW peptide **ΔN-24$_{linked}$** and the synthetic A–B crosslinked YLW standard **25**. C) Superposition of ^{1}H-spectrum over a ^{1}H^{-1}H total correlation spectra (TOCSY) for **24$_{linked}$**, showing a zoomed view of the aromatic protons together with relevant interactions highlighted with red and blue; relevant cross peaks are boxed using dotted line with the corresponding colour. D) Superposition of ^{1}H-spectrum over a ^{1}H^{-1}H correlation spectrum (COSY) for **24$_{linked}$**, showing a zoomed view of the aromatic protons together with relevant interactions highlighted with red; relevant cross peaks are also boxed using dotted line.

of cyclisation despite the diversity of side chains present in these peptides. Even the Dap-containing peptide **10** was a moderate substrate; given that tryptic digestion allows for simple isolation of cyclic tripeptides, even a 30 % conversion of **10** allows this to remain a possible route to access such a range of cyclic synthons.

Next, we investigated the acceptance of peptides containing different residues within the site of the crosslink (Table 1, Supporting Information Figures 40–46). In these experiments, we explored the tolerance of $P450_{Blt}$ for altering stereochemistry within the peptide substrate (**17**–**19**), switching the positions of the Tyr/His residues (**20**) and replacing them with a more rigid 4-hydroxyphenylglycine (Hpg) residue (**21**–**22**), as well as ascertaining the importance of the NH moiety in these residues by replacement of the P5 imidazole side chain with a thiazoline moiety (**23**). As was perhaps expected for such a biosynthetic enzyme, peptides **17**–**22** were not well accepted—the highest conversions seen for **17** (D-His_5 residue) and **22** (Hpg_3 residue) were >10 % than that of **1**. This likely stems from the low affinity of $P450_{Blt}$ for these modified peptides, which is seen in the loss of affinity determined for **17** (k_d = 93 μM) and **18** (k_d = 530 μM) compared to **1** (k_d = 2.1 μM). Despite these low yields, the natural occurrence of a biarylitide A–(*X*)–B linked peptide with the sequence MRHEY shows that altering the ring substituents is possible,[13] further supporting the crucial importance of obtaining structural information of member/s of this versatile peptide crosslinking class of P450s. Turnover of **23** also supported the site of crosslinking present in the $P450_{Blt}$ reaction, as **23** is unable to be cyclised by $P450_{Blt}$ despite it being able to bind to the enzyme (k_d = 59 μM).

Finally, we tested the exchange of the His_5 residue for Trp, given that such crosslinks are widely reported in cyclic peptides (see Figure 1). $P450_{Blt}$-catalysed cyclisation of MRYLW (**24**) was very effective, showing 2/3^{rds} of the level of cyclisation of **1** despite the alteration of a crosslinking residue and the large increase in size of this residue (Supporting Information Figure 47). Such differences are seen in the spectral response of $P450_{Blt}$ upon binding of **24**, where the spectrum no longer resembles the activation spectra seen for **1** and displays a slight reduction in affinity (k_d = 4.2 μM, Supporting Information Figure 5). We further explored the tolerance of $P450_{Blt}$ in accepting alterations in the MRYxW peptide (**26**–**28**) and observed a similar trend as had been seen for MRYxH peptides with higher activity

seen with larger, hydrophobic substrates (19 % MRYGW ≈21 % MRYAW < 66 % MRY-Nle-W; Supporting Information Figures 48–50). Cyclisation of **24** by $P450_{Blt}$ raised the important question of the nature of the crosslink in this peptide given that the type of linkage could affect the ring size of the crosslinked peptide. To address this, we synthesised a cyclic YLW tripeptide standard (**25**) containing an A–B linkage (Figure 4)[19] and compared the retention time of this standard to the product formed by $P450_{Blt}$-catalysed cyclisation of **24** with subsequent tryptic digestion (Δ**N-24$_{linked}$**). Having seen a different retention time in this analysis, we next performed extensive NMR characterisation of both **24$_{linked}$** and **25** to understand the nature of the crosslink in **24$_{linked}$** (Supporting Information Figure 51–60). These analyses revealed that—in addition to not containing an aryl A–B crosslink—**24$_{linked}$** retained all four Tyr aromatic protons, implying the crosslink is not through the Trp indole nitrogen to the Tyr ring and it does not resemble the crosslinking seen in pseudosporamide.[20] This led to the conclusion that the crosslink installed in this case is an A–*O*–B crosslink to the Trp indole ring through the Tyr phenol oxygen. HDX analysis of Δ**N-24$_{linked}$** and **25** further supported a heteroatom containing crosslink for Δ**N-24$_{linked}$** (Supporting Information Figure 18). The ability of $P450_{Blt}$ to generate **24$_{linked}$** shows that there is significant plasticity in $P450_{Blt}$ for the generation of alternate rings linkages in tripeptides and supports the potential of this P450 class as a general tool for the generation of a range of cyclic tripeptide building blocks containing A–B,[13] A-*N*-B[18] and A–*O*–B crosslinks.

In summary, we have performed a detailed analysis of the substrate tolerance of the biarylitide peptide crosslinking $P450_{Blt}$. We have shown that the minimal leader sequence of these pentapeptides remains important for catalysis, although this can be removed through proteolysis to allow potential access to A–*N*–B crosslinked cyclic tripeptides. Furthermore, we have demonstrated that generating various crosslinked tripeptides is highly feasible. Perhaps most impressive is the ability of this enzyme to install a crosslink in which the natural His residue is replaced by Trp. This, combined with altered crosslinking and substrates reported for other members of this biosynthetic pathway demonstrates that biarylitide biosynthetic P450s could well play important roles as future biocatalysts for the generation of a diverse range of cyclic tripeptide building blocks. In this endeavour, a structure of the substrate-bound P450 remains a priority for the field to allow rational engineering of these versatile enzymes and to address unanswered mechanistic questions concerning P450-mediated peptide crosslinking, an important biosynthetic process central to the generation of many crosslinked peptide natural products.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Amino Acids · Biocatalysis · Cytochrome P450 · Metalloenzymes · Peptide Crosslinking · Peptide Cyclisation · Peptides

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