Design and use of de novo cascades for new benzylisoquinoline alkaloid biosynthesis

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Abstract: The benzylisoquinoline alkaloids (BIAs) are an important group of secondary metabolites from higher plants and have been reported to show significant biological activities. The production of BIAs through synthetic biology approaches provides a higher-yielding strategy than traditional synthetic methods or isolation from plant material. However, the reconstruction of BIA pathways in microorganisms by combining heterologous enzymes can also give access to BIAs through cascade reactions. Most importantly, non-natural BIAs can be generated through such artificial pathways. In the current study, we describe the use of tyrosinases and decarboxylases and combine these with a transaminase enzyme and norcoclaurine synthase for the efficient synthesis of several BIAs, including six non-natural alkaloids, in cascades from L-tyrosine and analogues.

Benzylisoquinoline alkaloids (BIAs) are a structurally diverse group of natural products showing potent pharmaceutical significance. Their well-studied therapeutic fields cover antibacterials, anti-inflammatories and anti-virals, and they are also used as a coronary vasodilator and microtubule disrupter.[1,3] Further applications are being discovered, including the known antimicrobial berberine, which has been reported to downregulate β-catenin signaling in colon tumor cells, and have cholesterol lowering effects.[3,4]

In higher plants, natural BIAs share a common central precursor (S)-norcoclaurine (S)-1, which is synthesized from the condensation of dopamine 2 and 4-hydroxyphenylacetaldehyde (4-HPAA 3) via a Pictet-Spengler reaction catalyzed by norcoclaurine synthase (NCS).[5] Dopamine 2 is generated by the ortho-hydroxylation and decarboxylation of L-tyrosine 4 by a tyrosine hydroxylase and DOPA decarboxylase (DODC), respectively. In a parallel pathway, 4-HPAA 3 is formed from the deamination of L-tyrosine 4 to 4-hydroxyphenylpyruvate by a tyrosine aminotransferase and is then decarboxylated to 4-HPAA 3 by a 4-hydroxyphenylpyruvate decarboxylase.[5,6] Through the Pictet-Spenglerase reaction, dopamine 2 becomes the origin of the isoquinoline moiety, and 4-HPAA 3 is incorporated into the benzyl moiety, resulting in the benzylisoquinoline nucleus of (S)-1. Recent reports using recombinant NCSs from Thalictrum flavum (TNCs), Coptis japonica (CjNCS) and Papaver bracteatum (PbNCS) have highlighted their versatility in BIA and tetrahydroisoquinoline (THIA) synthesis using a range of aldehydes and several ketones.[7-11] X-ray crystallographic and modelling studies have also enhanced a mechanistic understanding.[12-14] In addition, their incorporation into enzymatic and chemoenzymatic cascades to give THIAs in high yields and stereoselectivities has demonstrated their potential in sustainable synthesis.[15-18]

To date, BIAs are mainly obtained by plant extraction. However, the yield is normally not high due to the low accumulation levels in native plant cells along with other complex metabolites and challenging isolation procedures.[19] The structural complexity of many BIAs also means that chemical synthesis via multistep procedures are not viable on an industrial scale.[20-23] Synthetic biology has provided a new approach to BIA biosynthesis in recent years. Indeed, the reconstruction of BIA pathways in microorganisms, such as E. coli or S. cerevisiae, with bacterial or plant enzymes has enabled the production of BIAs via fermentation in vivo (Scheme 1A), although racemic NCS products were noted in a number of cases.[23-29] Additionally, such heterologous pathways can also be achieved by combining enzymes into cascade reactions in vitro (Scheme 1B). This highly valuable strategy enables the introduction of greater structural diversity to produce novel alkaloids, as alternative substrates or enzyme steps can be incorporated that are not compatible with natural in vivo pathways.

Scheme 1. (A): Representation of previous in vivo approaches to alkaloid natural products. (B): Representation of in vitro approaches to novel alkaloids in this work.

In this study, we describe de novo BIA cascades to synthesize non-natural BIAs using the amino acid L-tyrosine 4 and analogues. To achieve this, tyrosinases, and decarboxylases were identified and incorporated with transaminase (TAm) and NCS enzymes in several high yielding one-pot multi-step cascade reactions.
The first step in the conversion of tyrosine or analogues to meta-hydroxylated phenethylamines, required for the Pictet-Spenglerase reaction, is an ortho-hydroxylation. Several enzymes have been reported to do this, mammalian tyrosine hydroxylases (TyrHs), tyrosinases (TyrRs) and recently a cytochrome P450 oxidase.\textsuperscript{[23,26,30]} Since mammalian TyrHs require a tetrahydrobiopterin cofactor that \textit{E. coli} cannot produce, it was decided to investigate the use of TyrRs. \textit{Ralstonia solanacearum} tyrosinase (Gene: \textit{RsSc0337}) has previously been reported to have high monophenolase activity,\textsuperscript{[11]} and has been incorporated into the fermentative production of racemic NCS products.\textsuperscript{[24,28]} Based on sequence homology to the reported \textit{Rs} TyrR, Cu-dependent TyrRs from \textit{Ralstonia solanacearum} (Rs, Gene: \textit{RsSc1501}, 25\% identity to \textit{RsSc0337}), \textit{Bacillus megaterium} (Bm), \textit{Rhizobium mellioti} (Rm), and \textit{Candidatus nitrosopumilus} (Cn) were selected and expressed in \textit{E. coli} (18-41\% sequence identity to \textit{Rs} TyrR (\textit{RsSc0337}; SI Fig. S1 for sequence alignments). In addition, two Cu/co-factor protein (CoF) TyrRs from \textit{Streptomyces avermitilis} (Sav) and \textit{Streptomyces antibioticus} (San) and one para-hydroxybenzoate hydroxylase (PHBH) from \textit{Pseudomonas aeruginos}a (Pa) were also chosen. Enzymes were readily expressed and used as crude cell lysates with L-Tyr 4 (2.5 mM) to give L-DOPA 5, and tyramine 6 (2.5 mM) to give dopamine 2 using optimized conditions of pH 5.5 and 25 °C with a 500 μL reaction volume (Figure 1). The addition of sodium ascorbate 7 was found to reduce problems with quinone formation so was used in TYR assays and cascades.

Both the new \textit{Rs} TyrR and \textit{Cn} TyrR gave 5 and 2 in high yield (\textgreater95\%), highlighting them as useful tyrosinases in biocatalytic reactions and one \textit{Cn} TyrRwas selected for further study. Three enzymes, \textit{Pa} PHBH, SavTyr and SanTyr gave 5 and 2 only in low yields (< 20\%). Enzymes \textit{Bm} TyrR and \textit{Rm} TyrR showed a preference towards 4, giving 5 in 30\%-75\% yield and 2 in lower yields (15\%-45\%). Using purified \textit{Cn} TyrR the apparent \textit{Km} app and \textit{kcat} app towards L-tyrosine 4 were 1.78 mM and 31.6 s\(^{-1}\) (\textit{kcat} app/\textit{Km} app 1.78 x 10\(^4\) s\(^{-1}\) M\(^{-1}\)) and tyramine 6 3.43 mM and 55.2 s\(^{-1}\) (\textit{kcat} app/\textit{Km} app 1.61 x 10\(^4\) s\(^{-1}\) M\(^{-1}\)), respectively; although \textit{Cn} TyrR showed greater affinity towards 4, the \textit{kcat} app was higher with 6. Sequence identity to a related TYR from \textit{Cn} koreensis\textsuperscript{[32]} is 54\%, where the \textit{Km} and \textit{kcat} towards 4 were 9.2 mM and 4.3 s\(^{-1}\) (\textit{kcat}/\textit{Km} 4.7 x 10\(^4\) s\(^{-1}\) M\(^{-1}\)); a higher apparent monophenolase activity with the \textit{Cn} TyrR identified here may be due to the addition of 7.

The decarboxylation of \textit{l}-DOPA 5 to give dopamine 2 has been reported in BIA pathways by \textit{Psuedomonas putida} DOPA decarboxylase (\textit{Pp}DODC) where it was required to have higher activity towards 5 rather than 4 to avoid side product formation.\textsuperscript{[24,26]} \textit{Pp}DODC was selected for use here together with new tyrosine decarboxylases (TyrDC) from \textit{Lactobacillus brevis} (Lb) and \textit{Enterococcus faecalis} (Ef).\textsuperscript{[33,34]} Good substrate tolerance was also sought with non-natural substrates. Sequence identities for both \textit{Ef}TyrDC and \textit{Lb}TyrDC to \textit{Pp}DODC are 21\% (SI Fig. S2 for sequence alignments). Two of the enzymes, \textit{Pp}DODC, \textit{Lb}TyrDC showed low levels of decarboxylation (<10\%) (Figure 2). \textit{Ef}TyrDC gave the best performance towards both 4 and 5 (2.5 mM) (> 90\% yield by HPLC) at optimised conditions compatible with the TyR of pH 5.5 and 25 °C. The apparent \textit{Km} and \textit{kcat} of \textit{Ef}TyrDC with 4 were 1.58 mM and 39.0 s\(^{-1}\) (\textit{kcat}/\textit{Km} app 2.47 x 10\(^4\) s\(^{-1}\) M\(^{-1}\)) and with 5 were 2.31 mM and 60.2 s\(^{-1}\) (\textit{kcat}/\textit{Km} app 2.61 x 10\(^4\) s\(^{-1}\) M\(^{-1}\)), respectively. Due to the promising activities displayed by the new enzymes \textit{Cn}TyrR and \textit{Ef}TyrDC, they were selected for use in \textit{in vitro} BIA pathways with natural and non-natural substrates.

![Figure 1](image1.png)  
Figure 1. Reactions A and B with the TYRs and \textit{Pa}PHPB, 0.4 mg/mL cell lysate (containing 15-40\% of the recombinant protein), HEPES (50 mM), 7 (4 equiv.), CuSO\(_4\) (0.4 equiv) (or FAD and NADPH with PHBH), 4 or 6 (2.5 mM), at pH 5.5, 25 °C, 250 rpm, 8 h, and a total reaction volume of 500 μL. Yields were determined by HPLC analysis at 280 nm of the products formed (5 and 2); ( ) L-DOPA 5 yield reaction A; ( ) Dopamine 2 yield reaction B.

![Figure 2](image2.png)  
Figure 2. Reactions C and D with the DCs, 0.4 mg/mL cell lysate (20% of the recombinant protein), 4 or 5 (2.5 mM), PLP, at pH 5.5, 25 °C, 250 rpm, 8 h. Reaction volume: 500 μL. Conversion yields were determined by HPLC analysis at 280 nm of the products 6 and 2. ( ) Tyramine 6 yield reaction C; ( ) Dopamine 2 yield reaction D.

The substrate tolerance of \textit{Cn} TyrR towards analogues of 4, \textit{meta}-1-tyrosine 8, 3-F-L-tyrosine 9, 3-CI-L-tyrosine 10 and 3-L-tyrosine 11 was investigated. No conversions were observed for the halogenated tyrosines 10 and 11, indeed 11 has been reported to be a mixed type inhibitor, which is competitive and non-competitive for some TYRs.\textsuperscript{[35]} 2-Chlorophenol has also been described to act as a competitive inhibitor towards TYRs.\textsuperscript{[36]}
Interestingly, 3-F-L-tyrosine 9 (2.5 mM) was readily accepted, with an 80% conversion yield (48 h) to give the corresponding F-DOPA analogue 12. Indeed, some previous work has indicated that 9 can be accepted by a tyrosinase. The substrate tolerance of E TyrDC was also investigated using meta-L-tyrosine 8, and the halogenated tyrosine analogues 9-11, 13 (at 2.5 mM, Table 1). Decarboxylated products were readily formed in all cases giving 14-18 in 90-100% conversion yields, and 7 was added to avoid substrate or product oxidation. Overall the reactions highlighted E TyrDC as an extremely efficient and versatile decarboxylase, providing a novel route to 14, 15, 17 and 18.

Table 1. Decarboxylation of tyrosine analogues by E TyrDC

<table>
<thead>
<tr>
<th>Substrate/ R group</th>
<th>Product</th>
<th>Conversion yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tyr 4</td>
<td>tyramine 6</td>
<td>quantitative</td>
</tr>
<tr>
<td>L-DOPA 5</td>
<td>dopamine 2</td>
<td>quantitative</td>
</tr>
<tr>
<td>8</td>
<td>HO</td>
<td>95%</td>
</tr>
<tr>
<td>9</td>
<td>HO</td>
<td>90%</td>
</tr>
<tr>
<td>10</td>
<td>HO</td>
<td>quantitative</td>
</tr>
<tr>
<td>11</td>
<td>HO</td>
<td>95%</td>
</tr>
<tr>
<td>13*</td>
<td>HO</td>
<td>90%</td>
</tr>
</tbody>
</table>

Reaction conditions: E TyrDC 0.4 mg/mL cell lysate (22% of the recombinant protein), substrate (2.5 mM), PLP, 7 (10 mM), pH 5.5, 25 °C, 250 rpm, 8 h, reaction volume: 500 μL. Conversion yields were determined by HPLC analysis.

The construction of multi-step one-pot in vitro enzyme cascades were then established using CnTyr, E TyrDC, together with a versatile transaminase from Chromobacterium violaceum (CvTam) and wild-type Δ29 T NCS that have been used in some enzyme cascades and demonstrated good substrate tolerances. Initially, three cascades were developed using L-tyrosine 4 (at 2.5 mM, Table 2, entries 1-3). First the one-pot reaction was developed using CnTyr, E TyrDC to produce dopamine 2, followed by the addition of phenylacetaldehyde 19 and T NCS. It was necessary to ensure that the TYR and DC reactions were not performed in phosphate buffer, due to the reported competing chemical Pictet-Spengler reaction. In addition, it was noted that while T NCS did not accept L-DOPA 5, due to its high reactivity some background chemical Pictet-Spengler reaction was also noted in HEPES buffer requiring the full conversion of 5 into 2 prior to the addition of 19. The TYR and DC reactions were optimized in a one-pot cascade at 30 °C and pH 5.5 for 12 h, with subsequent adjustment of the reaction pH to 7.5, and addition of the aldehyde and T NCS for a 6 h reaction at 37 °C. This gave the non-natural BIA (S)-20 in 66% isolated yield (99% yield by analytical HPLC) and >97% e.e. via a three-enzyme cascade. The lower isolated yields reflected the challenges of purifying such alkaloids that has been noted in the literature. The four-enzyme cascade to the natural BIA (S)-norlauandonololene (S)-21 was then established combining CnTyr, E TyrDC, CvTAm and T NCS, utilizing only 4 as the starting material to extend the previously reported triangular cascade. The dopamine 2 formed was converted to 2-(3,4-dihydroxyphenyl)- acrylicdehyde 22 by CvTAm and addition of T NCS gave (S)-21 in 53% isolated yield (98% yield by analytical HPLC) and >97% e.e. Furthermore, to demonstrate the general applicability of this approach the reaction was scaled to 1 g giving (S)-21 in 43% isolated yield (85% HPLC yield). A different one-pot four-enzyme cascade was also established towards (S)-norcoalaurine (S)-1 combining CnTyr, E TyrDC, CvTAm and T NCS, but using a different order of addition, exploiting the ability of the TYR to hydroxylate tyramine 6 (entry 3). Firstly, 4 was decarboxylated to 6 by E TyrDC, then hydroxylated to 2 by CvTAm, while 6 was also converted to 4-HPAA 3 by adding CvTAm. Finally, 2 was reacted with 3 by the addition of T NCS, giving (S)-1 in 62% isolated yield (85% yield by HPLC) and >97% e.e.

One-pot cascades using meta-L-tyrosine 8 (10 mM) were then established which is an efficient method of generating 14 (entries 4-6). The first, was a two-step reaction using E TyrDC to convert 8 into 14, which was then reacted with 19, yielding (S)-23 in 25% isolated yield (82% yield by HPLC) and >97% e.e. Using an analogous approach and 2-bromophenylacetaldehyde 24, (S)-25 was formed in 28% isolated yield (45% yield by HPLC) and 75% e.e., presumably reflecting the effect of introducing a bulkier aryl group at C-1. Using 8 (entry 6) a three-enzyme cascade was constructed with E TyrDC to generate 14, which was converted into 3-hydroxynaphthaldehyde 26. A subsequent reaction between 14 and 26 by the addition of T NCS gave (S)-27 in 32% isolated yield (78% yield by HPLC) and 95% e.e. Again, this reaction was demonstrated on a larger scale using approximately 0.5 g of 8 to give (S)-27 in 39% isolated yield (72% by HPLC) which was comparable to the smaller scale reaction. Using an alternative starting material to demonstrate the versatility of the strategy 9 (15 mM), which has higher water solubility than 4, was hydroxylated using CnTyr to give 12, then decarboxylated with E TyrDC to give amine 28 and subsequently reacted with 19 via the addition of T NCS to give the novel BIA (S)-29 in 23% overall isolated yield (35% yield by HPLC) and 90% e.e. (entry 7). Finally, to show that the cascades can be applied to non-amino acids, (rac)octopamine 30 (40 mM) was hydroxylated using CnTyr to give noradrenaline 31 and then reacted with T NCS and 19 to give (1S,4R)-32 in 47% isolated yield (65% yield by HPLC) and a ratio of 5:3 (1S,4R):(1S,4S) (entry 8). The use of other NCS variants did not significantly affect the isomeric ratio of 32 (see SI) and it was noted that 32 readily dehydrated under acidic conditions.
Yields were determined by HPLC analysis at 280 nm against products standards. For preparative-scale reactions products were purified by preparative HPLC or by using an extraction method (see the Supporting Information). ee values were determined by chiral HPLC (>97% indicates that no minor isomer was detected and reflects the limits of detection). Reaction conditions: Details are provided in the Supporting Information and are specific to each cascade. As an example, for entry 1, a 50 mL reaction consisting of HEPES (50 mM), CnTYR and EfTyrDC (10% lysate (v/v)) and 4 (2.5 mM) in 10% DMSO (v/v), at pH 5.5, 25°C, 7 (4 equiv), PLP, CuSO₄ was run for 8 h at 250 rpm. The pH was then adjusted to pH 7.5, aldehyde and T/NCS (50 mg/mL) were added and the reaction was performed at 37°C, 250 rpm for 6 h.

Table 2. Enzyme cascade reactions using CnTYR, EfTyrDC, CvTAm and T/NCS.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Aldehyde added</th>
<th>Cascade route</th>
<th>Product</th>
<th>Yield by HPLC[a] (isolated product)[b]</th>
<th>ee or (1S,4R)/(1S,4S)[c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>19</td>
<td></td>
<td></td>
<td>99% (66%)</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>No (22 made in situ)</td>
<td></td>
<td></td>
<td>98% (53%; 1 g scale 43%)</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>No (3 made in situ)</td>
<td></td>
<td></td>
<td>85% (62%)</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>19</td>
<td></td>
<td></td>
<td>82% (25%)</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>24</td>
<td></td>
<td></td>
<td>45% (28%)</td>
<td>75%</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>No (14 made in situ)</td>
<td></td>
<td></td>
<td>78% (32%; 0.544 g scale 39%)</td>
<td>95%</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>19</td>
<td></td>
<td></td>
<td>35% (23%)</td>
<td>90%</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>19</td>
<td></td>
<td></td>
<td>65% (47%)</td>
<td>5:3</td>
</tr>
</tbody>
</table>

[a] Yields were determined by HPLC analysis at 280 nm against products standards. [b] For preparative-scale reactions products were purified by preparative HPLC or by using an extraction method (see the Supporting Information). [c] ee values were determined by chiral HPLC (>97% indicates that no minor isomer was detected and reflects the limits of detection). Reaction conditions: Details are provided in the Supporting Information and are specific to each cascade. As an example, for entry 1, a 50 mL reaction consisting of HEPES (50 mM), CnTYR and EfTyrDC (10% lysate (v/v)) and 4 (2.5 mM) in 10% DMSO (v/v), at pH 5.5, 25°C, 7 (4 equiv), PLP, CuSO₄ was run for 8 h at 250 rpm. The pH was then adjusted to pH 7.5, aldehyde and T/NCS (50 mg/mL) were added and the reaction was performed at 37°C, 250 rpm for 6 h.
In conclusion, two enzymes, tyrosinase CN Tyr and tyrosine decarboxylase ETyrDC, were cloned and used with a range of substrates to highlight their use with nonnatural analogues. When combined together in different combinations with 4, 8, 9, and 30 and the additional enzymes CV TAm and TT NCs, artificial cascades were successfully constructed, giving BIA in 23–66% yields of isolated product (35–95% yields by HPLC) and high stereoselectivity over up to 4 enzyme steps. Moreover, selected reactions were scaled up successfully to 1 g. Overall, this work highlights the versatility of the “mix and match” strategy with enzymes in vitro to generate two natural and six non-natural BIA. Interestingly, in parallel with this work, the synthesis of noscapine and halogenated BIA in yeast has been reported using a reported TyrH and DODC, together with other downstream biosynthetic enzymes in vivo.[43] While some halogenated BIA were detected, it was unclear how much was produced or what the stereoselectivity was. Here, the one-pot in vitro cascades demonstrate an extremely powerful strategy for introducing molecular diversity using sustainable catalysts.

Acknowledgements

We gratefully acknowledge UCL (Dean’s Prize) for funding to Y.W. We also thank the BBSRC (BB/N01877X/1) for funding D.M.S., and EPSRC (EP/K014897/1) for funding M.B. We thank K. Karu (UCL Mass Spectrometry Facility) and A. E. Aliev (UCL NMR Facility) in the Department of Chemistry.

Keywords: alkaloids • biocatalysis • enzyme cascades • tyrosinase • tyrosine decarboxylase

References


