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

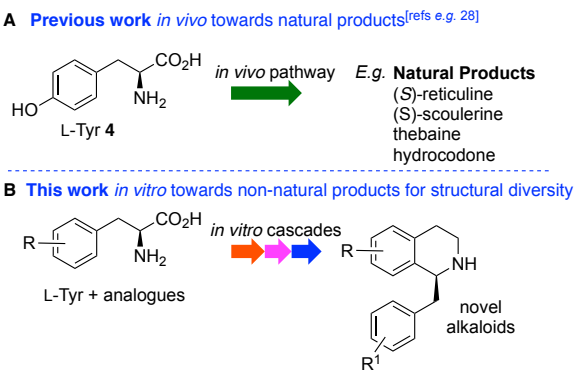
Yu Wang[a], Nadine Tappertzhofen[a], Daniel Méndez-Sánchez[a], Maria Bawn[b], Boyu Lyu[b], John M. Ward*[b] and Helen C. Hailes*[a]

Abstract: The benzylisoquinoline alkaloids (BIAs) are an important group of higher plant secondary metabolites, which have been reported to show significant pharmaceutical activities. The production of BIAs via synthetic biology approaches provides a higher yielding strategy compared to traditional synthetic methods and plant isolation methods. However, the reconstruction of BIAs pathways in microorganisms combining heterologous enzymes can also give access to BIAs via cascade reactions. Most importantly, non-natural BIAs can be generated through such artificial pathways. In the current study, we describe new 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. The well-studied therapeutic fields cover anti-bacterials, anti-inflammatories and anti-virals, and they are also used as a coronary vasodilator and microtubule disrupter.[1,2] 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* (*Tf*NCS), *Coptis japonica* (*Cj*NCS) and *Papaver bracteatum* (*Pb*NCS) 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.[20-22] 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.

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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 (TYRs) and recently a cytochrome P450 oxidase.[23,26,30] Since mammalian TyrHs require a tetrahydrobiopterin cofactor that *E. coli* cannot produce, it was decided to investigate the use of TYRs. *Ralstonia solanacearum* tyrosinase (Gene: *RSc0337*) has previously been reported to have high monophenolase activity,[31] and has been incorporated into the fermentative production of racemic NCS products.[24,26] Based on sequence homology to the reported *Rs*TYR, Cu-dependent TYRs from *Ralstonia solanacearum* (*Rs,* Gene: *RSc1501,* 25% identity to *RSc0337*), *Bacillus megaterium* (*Bm*), *Rhizobium meliloti* (*Rm*), and *Candidatus nitrosopumilus* (*Cn*) were selected and expressed in *E. coli* (18-41% sequence identity to *Rs*TYR (*RSc0337*); SI Fig. S1 for sequence alignments). In addition, two Cu/co-factor protein (CoF) TYRs from *Streptomyces avermitilis* (*Sav*) and *Streptomyces antibioticus* (*San*) and one *para*-hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas aeruginosa* (*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 ºC (Figure 1). The addition of sodium ascorbate **7** was found to reduce problems with quinone formation so was used in TYR assays and cascades.

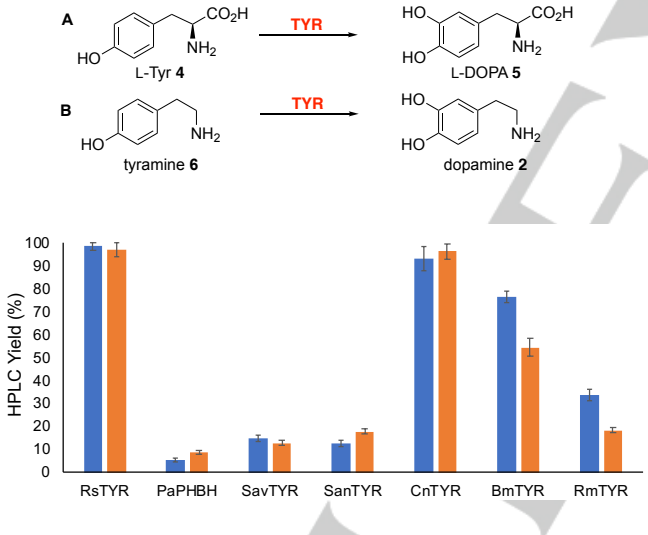

Figure 1. Reactions **A** and **B** with the TYRs and *Pa*PHPB, 0.4 mg/mL cell lysate (containing 15-40% of the recombinant protein), HEPES (50 mM), **7** (4 equiv.), $CuSO_4$ (0.4 equiv) (or FAD and NADPH with PHBH), **4** or **6** (2.5 mM), at pH 5.5, 25 ºC, 250 rpm, 8 h, and a total reaction volume 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**.

Both the new *Rs*TYR and *Cn*TYR gave **5** and **2** in high yields by HPLC (>95%), highlighting them as useful tyrosinases in biocatalytic reactions and one, *Cn*TYR, was selected for further study. Three enzymes, *Pa*PHBH, *Sav*TYR and *San*TYR gave **5** and **2** only in low yields (< 20%). Enzymes *Bm*TYR and *Rm*TYR showed a preference towards **4**, giving **5** in 30%-75% yield and **2** in lower yields (15%-45%). Using purified *Cn*TYR the apparent kinetic parameters K_m and k_{cat} towards L-tyrosine **4** were 1.78 mM and 31.6 s^{-1} ($k_{cat,app}/K_{m,app}$ 1.78 x 10^4 s^{-1} M^{-1}) and tyramine **6** 3.43 mM and 55.2 s^{-1} ($k_{cat,app}/K_{m,app}$ 1.61 x 10^4 s^{-1} M^{-1}), respectively: although *Cn*TYR showed greater affinity towards **4**, the $k_{cat,app}$ was higher with **6**. Sequence identity to a related TYR from *Cn koreensis*[32] is 54%, where the K_m and k_{cat} towards **4** were 9.2 mM and 4.3 s^{-1} (k_{cat}/K_m 4.7 x 10^4 s^{-1} M^{-1}): a higher apparent monophenolase activity with the *Cn*TYR identified here may be due to the addition of **7**.

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

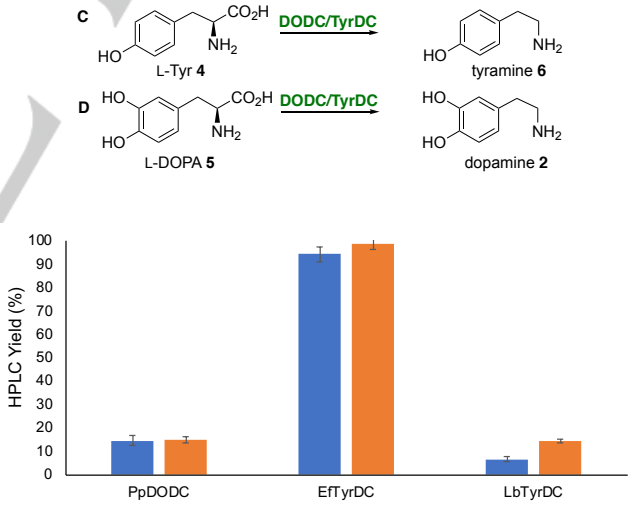

Figure 2. Reactions **C** and **D** with the DCs, 0.4 mg/mL cell lysate (containing 20-23% of the recombinant protein), **4** or **5** (2.5 mM), PLP, at pH 5.5, 25 ºC, 250 rpm, 8 h and a total reaction volume of 500 µL. Yields were determined by HPLC analysis at 280 nm of the products formed (**6** and **2**). (■) Tyramine **6** yield reaction **C**; (■) Dopamine **2** yield reaction **D**.

The substrate tolerance of *Cn*TYR towards analogues of **4**, *meta*-L-tyrosine **8**, 3-F-L-tyrosine **9**, 3-Cl-L-tyrosine **10** and 3-I-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.[35] 2-Chlorophenol has also been described to act as a competitive inhibitor towards TYRs.[36]

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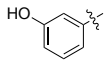
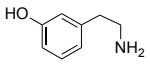
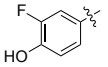
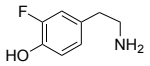
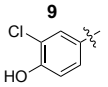
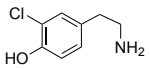
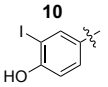
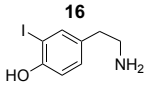
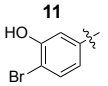
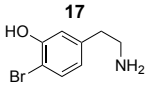
Interestingly, 3-F-L-tyrosine **9** (2.5 mM) was readily accepted, with an 80% conversion yield (48 h) to give the corresponding F-DOPA analogue **12**. Indeed, some previous work has indicated that **9** can be accepted by a tyrosinase.[37] The substrate tolerance of *Ef*TyrDC was also investigated 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 *Ef*TyrDC as an extremely efficient and versatile decarboxylase, providing a novel route to **14**, **15**, **17** and **18**.[38]

Table 1. Decarboxylation of tyrosine analogues by *Ef*TyrDC

4, **5**, **8**, **9**-**13**

Substrate/ R group	Product	Conversion yields
L-Tyr **4**	tyramine **6**	quantitative
L-DOPA **5**	dopamine **2**	quantitative
 **8**	 **14**	95%
 **9**	 **15**	90%
 **10**	 **16**	quantitative
 **11**	 **17**	95%
 **13***	 **18**	90%

*Reaction conditions: Ef*TyrDC 0.4 mg/mL cell lysate (containing 22% of the recombinant protein), substrate (2.5 mM), PLP, **7** (10 mM), pH 5.5, 25 °C, 250 rpm, 8 h, and a total reaction volume of 500 µL. Conversion yields were determined by HPLC analysis at 280 nm, based on substrate consumption. *Reaction required 7 d.

The construction of multi-step one-pot *in vitro* enzyme cascades were then established using *Cn*TYR, *Ef*TyrDC, together with a versatile transaminase from *Chromobacterium violaceum* (*Cv*TAm) and wild-type Δ29*Tf*NCS that have been used in some enzyme cascades and demonstrated good substrate tolerances.[16,17,39,40] 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 *Cn*TYR, *Ef*TyrDC to produce dopamine **2**, followed by the addition of phenylacetaldehyde **19** and *Tf*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.[41] In addition, it was noted that while *Tf*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 *Tf*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.[42] The four-enzyme cascade to the natural BIA (*S*)-norlaudanosoline (*S*)-**21** was then established combining *Cn*TYR, *Ef*TyrDC, *Cv*TAm and *Tf*NCS, utilizing only **4** as the starting material to extend the previously reported triangular cascade.[16] The dopamine **2** formed was converted to 2-(3,4-dihydroxyphenyl)-acetaldehyde **22** by *Cv*TAm and addition of *Tf*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*)-norcoclaurine (*S*)-**1** combining *Cn*TYR, *Ef*TyrDC, *Cv*TAm and *Tf*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 *Ef*TyrDC, then hydroxylated to **2** by *Cn*TYR, while **6** was also converted to 4-HPAA **3** by adding *Cv*TAm. Finally, **2** was reacted with **3** by the addition of *Tf*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 *Ef*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 *Ef*TyrDC to generate **14**, which was converted into 3-hydroxyphenylacetaldehyde **26**. A subsequent reaction between **14** and **26** by the addition of *Tf*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 *Cn*TYR to give **12**, then decarboxylated with *Ef*TyrDC to give amine **28** and subsequently reacted with **19** via the addition of *Tf*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 *Cn*TYR to give noradrenaline **31** and then reacted with *Tf*NCS and **19** to give (1*S*,4*RS*)-**32** in 47% isolated yield (65% yield by HPLC) and a ratio of 5:3 (1*S*,4*R*):(1*S*,4*S*) (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.

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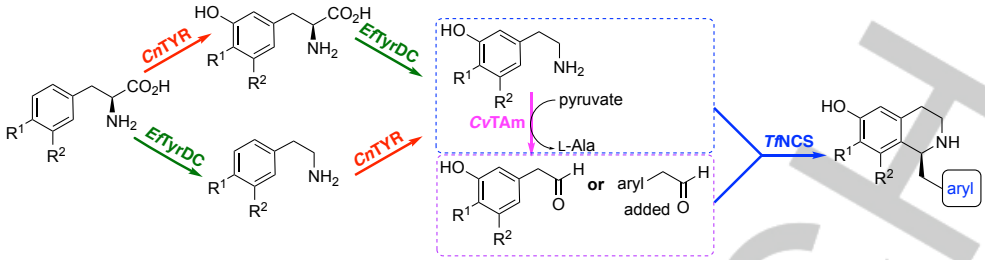
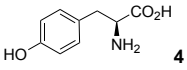
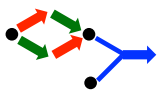
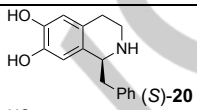
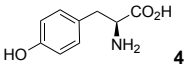
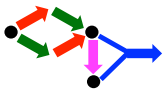
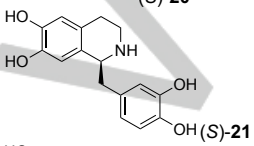
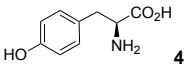
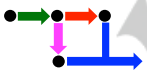
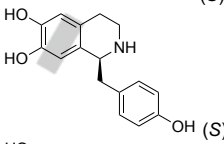
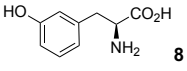
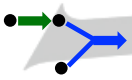
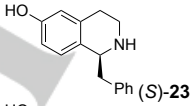
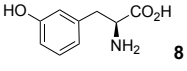
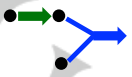
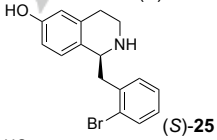
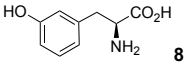
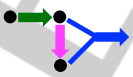
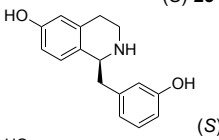
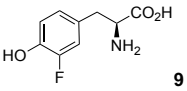
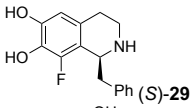
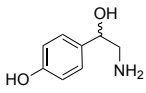
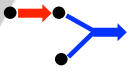
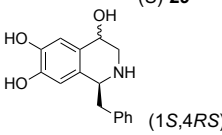
Table 2. Enzyme cascade reactions using *Cn*TYR, *Ef*TyrDC, *Cv*TAm and *Tf*NCS.



Entry	Substrate	+ aldehyde	Cascade route	Product	[a]Yield by HPLC ([b]isolated yield)	[c]*e.e* or ratio.
1	 **4**	**19**		 Ph (*S*)-**20**	99% (66%)	>97%
2	 **4**	No (**22** made *in situ*)		 (*S*)-**21**	98% (53%; 1 g scale 43%)	>97%
3	 **4**	No (**3** made *in situ*)		 (*S*)-**1**	85% (62%)	>97%
4	 **8**	**19**		 (*S*)-**23**	82% (25%)	>97%
5	 **8**	**24**		 (*S*)-**25**	45% (28%)	75%
6	 **8**	No (**14** made *in situ*)		 (*S*)-**27**	78% (32%; 0.544 g scale 39%)	95%
7	 **9**	**19**		 (*S*)-**29**	35% (23%)	90%
8	 **30**	**19**		 (1*S*,4*RS*)-**32**	65% (47%)	(1*S*,4*R*):(1*S*,4*S*) 5:3

[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 via an extraction protocol (see SI). [c]*E.e.*s 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 SI and are specific to each cascade. As an example for entry 1, the reaction mixture (50 mL) consisted of HEPES (50 mM), *Cn*TYR and *Ef*TyrDC (10% lysate (*v*/*v*)) and were used with **4** (2.5 mM) in 10% DMSO (*v*/*v*), at pH 5.5, 25 °C, **7** (4 equiv.), PLP, $CuSO_4$, for 8 h at 250 rpm. The pH was then adjusted to pH 7.5, aldehyde and *Tf*NCS (50 μg/mL) were added and the reaction performed at 37 °C, 250 rpm for 6 h.

In conclusion, two new enzymes, a tyrosinase *Cn*TYR and tyrosine decarboxylase *Ef*TyrDC were cloned and used with a range of substrates to highlight their use with non-natural analogues. When combined together in different combinations with **4**, **8**, **9**, **30**, *Cv*TAm and *Tf*NCS, artificial cascades were successfully constructed, giving BIAs in 23-66% isolated yields (35-99% yields by HPLC) and high stereoselectivities in 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 BIAs. Interestingly, in parallel with this work, the synthesis of noscapine and halogenated BIAs in yeast has been reported using a reported TyrH and DODC together with other downstream biosynthetic enzymes *in vivo*.[43] While some halogenated BIAs 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.

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Keywords: benzylisoquinoline alkaloids • Cascade reactions • biocatalysis • tyrosinase • tyrosine decarboxylase • transaminase • norcoclaurine synthase

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