Enzymatic synthesis of benzylisoquinoline alkaloids using a parallel cascade strategy and tyrosinase variants

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Benzylisoquinoline alkaloid derived pharmaceuticals are widely applied in modern medicines. Recent studies on the microbial production of benzylisoquinolines have highlighted key biological syntheses towards these natural products. Routes to non-natural benzylisoquinolines have been less explored, particularly halogenated compounds which are more challenging. Here, we show the use of a tyrosinase, tyrosine decarboxylase, transaminase, and norcoclaurine synthase which are combined in a parallel cascade design, in order to generate halogenated benzylisoquinoline alkaloids in high enantiomeric excess. Notably, mutagenesis studies are applied to generate tyrosinase mutants, which enhance the acceptance of halogenated tyrosines for use in the biocatalytic cascades developed.

Benzylisoquinoline alkaloids (BIAs) play an important role in the field of natural products due to their pharmaceutical properties. The use of plants containing BIAs dates back to 1500 BC, when the ancient Egyptians used opium as a painkiller1. Modern medicine revealed the biologically active components as codeine and morphine, both of which exhibit analgesic properties2,3. Recent studies on alkaloids have largely accelerated the use of BIA-derived pharmaceuticals, such as the anti-cancer agents noscapine and berberine4, anti-HIV agents cochlaurine and norcoclaurine6, and anti-inflammatory drugs berbamaine and coptisine7,8. Indeed, amongst all approved small-molecule based new drugs from 1981 to 2019, over 30% were based on natural products and 82% of these were semi-synthesised from natural product skeletons9.

Naturally-derived BIAs include around 2500 known compounds derived from the key intermediate (S)-norcoclaurine, (S)-1. In plants, (S)-1 is produced by the condensation between dopamine 2 and 4-hydroxyphenylacetaldehyde (4-HPAA) 3 via a norcoclaurine synthase (NCS) mediated Pictet-Spengler reaction. Both 2 and 3 are derived from L-tyrosine 4, via L-DOPA 5 and 4-hydroxyphenylpyruvic acid 6 by tyrosine hydroxylase (TH)/DOPA decarboxylase (DDC) and tyrosine aminotransferase (TyrAT)/4-hydroxyphenylpyruvate decarboxylase (4-HPPDC) respectively (Fig. 1a together with some examples of alkaloids formed)10,11,12.

The reconstruction of heterologous BIA pathways into microbes is an exciting area of research, with significant progress being made in recent years: the in vivo BIA pathway in Escherichia coli and Saccharomyces cerevisiae have been reported with the production of natural BIAs13,14,15. Pathways to BIAs have also been achieved using enzyme cascade reactions in vitro in high yields19. The NCS Pictet-Spengler reaction is the key step to construct the BIA skeleton in such approaches19,20,21. Previous work has established the NCS mechanistic requirement for the arylethylamine meta-hydroxyl group, which binds into the NCS active site, and is deprotonated to enable ring formation20,21. We have reported a recombinant tyrosinase from Candidatus Nitrosopumilus salaria BD31 (Cn TYR) able to effectively convert 4 to L-DOPA 5 without the requirement for expensive co-factors, generating the required meta-hydroxyl moiety21. Additionally a tyrosine decarboxylase from Enterococcus faecalis DC32 (Ef TyrDC) was developed for the decarboxylation of 5 and these were used in cascades to generate BIAs (Fig. 1b). However, such cascades do not have to follow the natural pathway starting from L-tyrosine 4. They can be designed combining other enzymes in different orders, or other substrates, to enable the production of non-natural BIAs.

Here, we show the design of a route to differentially halogenated non-natural BIAs through a parallel cascade and combination strategy, in which arylethylamines and aroylacetaldehydes are derived from two
different amino acids. To achieve this, a TYR is used to provide the necessary meta-hydroxyl group and a TyrDC to produce the amine from the amino acid. Also, a transaminase (TAm) for aldehyde formation, and finally NCS are used for BIA formation (Fig. 1b). Importantly, as well as defining the parallel cascade sequence, to extend the enzyme cascades into other substrate capabilities, Cn TYR variants are generated with good monophenolase activities towards halogenated tyrosines, enabling the production of a range of halogenated BIA.

Results and discussion

Designing and implementing parallel cascades

To generate the required arylethylamine moiety in the upper branch of the parallel cascade (Fig. 2a), the first step when using L-tyrosine or analogues is the addition of a meta-hydroxyl group. In this one-pot process in initial studies, dopamine was formed in a quantitative yield from 4. Tyramine could also be formed as an intermediate with both enzymes, if the decarboxylation occurs prior to the hydroxylation. Sodium ascorbate was added to prevent the oxidation of substrates and products, and enzyme lysates were used for ease of production and as these are normally used in industry. Studies confirmed that Ef TyrDC could also be used with meta-tyrosine to produce meta-tyramine in >95% yield.

For aldehyde production in the lower half of the cascade (Fig. 2b and Table 1), readily available starting materials were required, and amino acids were an ideal starting point. Arylacetaldehydes are particularly challenging to prepare as they are oxidatively sensitive and can self-condense via aldol reactions. Here, to produce aldehydes, the transaminase from Chromobacterium violaceum DSM30191 (Cv TAm) was selected, in combination with Ef TyrDC, as they have both been reported to have broad substrate tolerances.

Amino acids 4, 5, 9, 10, and 11 are condensed by the Pictet-Spenglerase norcoclaurine synthase (NCS), generating (S)-norcoclaurine, (S)-1, which is converted into BIA, such as morphinans, aporphines and protoberberines.
3-F-L-tyrosine 11, 3-Cl-L-tyrosine 12 and 3-L-tyrosine 13 were investigated for aldehyde production and the amine 3-phenyl-L-propylamine 14 was also used. Decarboxylation (other than for 14) was followed by deamination with sodium pyruvate 15 as the amine acceptor and pyridoxal 5'-phosphate (PLP) as cofactor. All of the selected substrates were readily accepted by EfTyrDC and CvTAm, giving compounds 16–22 in overall conversions of 70–95% (Table 1). Enzyme lysates were used in all cases.

Table 1 | Lower branch conversion of amino acids and 14 into aldehydes 16–22

| Substrate (R) | Lower branch product | Conversions |
|---------------|----------------------|-------------|
| 4             | 16                   | >95%        |
| 5             | 17                   | >95%        |
| 6             | 18                   | 85%         |
| 9             | 19                   | 77%         |
| 11            | 20                   | 80%         |
| 12            | 21                   | 72%         |
| 13            | 22                   | 70%         |

Reaction conditions: a 1 mL reaction mixture (50 mM HEPES, pH 7.5) containing amino acid substrate 4–9, NCS, 0.4 mg mL⁻¹ EfTyrDC cell lysate (containing 5% of recombinant protein), 0.2 mg mL⁻¹ CvTAm cell lysate (containing 80% of recombinant protein), 8 (1 equiv.), PLP (5 mM) and 15 (1 equiv.) at 37°C for 6 h and quenched by adding 1 μL of TFA. Conversions were determined by HPLC analysis at 280 nm based on starting material consumption.

The final step in this five enzyme-step parallel cascade was combining the amine and aldehyde moieties with addition of a wild-type (WT) TfNCS enzyme that has displayed good substrate tolerances. Overall, two amino acids were used as starting materials (other than when utilising 14), one for the amine generation (upper branch) and the other for aldehyde formation (lower branch). Nine cascades were constructed, the first five using L-tyrosine 4 (Fig. 3, Table 2, entries 1–5) with CvTyr and EfTyrDC for amine production (25°C, 6 h). In parallel, meta-L-tyrosine 7 (Fig. 3, Table 2, entry 1) was used with EfTyrDC and CvTAm (37°C, 6 h), followed by the addition of TfNCS (37°C, 8 h). This produced the non-natural BIA (S)-23 in 47% isolated yield (82% yield by analytical HPLC) and 90% enantiomeric excess (ee). The isolation of BIAs, as reported previously can result in the loss of product, so the conversion yields are also given (determined by HPLC against product standards). The cascade to (S)-24 was achieved with the aldehyde formed from 14 (Table 2, entry 2) using CvTAm, giving (S)-24 in 14% isolated yield (21% yield by HPLC) and 90% ee. The lower yield of (S)-24 suggested that the wild-type TfNCS may not accept phenyl propionaldehyde as readily as arylacetaldelydes. Halogenated tyrosines were also adopted into the cascades where 11, 12 and 13 (Table 2, entries 3–5) were converted into the corresponding halogenated arylacetaldelydes 19–21, which were then condensed with the amine using TfNCS, forming halogenated BIAs (S)-25–27 in 40–42% isolated yield (84–86% yields by analytical HPLC) and up to 96% ee.

In a similar fashion, four parallel cascades starting from meta-L-tyrosine 9 (Table 2, entries 6–9) to produce the amine moiety were established. In the first of these approaches the starting materials were swapped, compared to entry 1, with L-tyrosine 4 producing the aldehyde 4-HFPA 3, giving (S)-28 in 42% isolated yield (76% yield by HPLC) and 92% ee. Similarly, halogenated L-tyrosines 11–13 (Table 2, entries 7–9) were used to produce the aldehyde and matched with the amine component derived from 9, giving halogenated BIAs (S)-29–31 in 27–35% isolated yield (58–66% yield by analytical HPLC). The ee of (S)-29 was 85%, slightly lower than for (S)-30 (95% ee) and (S)-31 (91% ee). In general, lower yields for the halogenated BIAs (S)-29–31 derived from 9 were noted, compared to BIAs (S)-25–27 generated from 4, presumably reflecting the more activated dopamine catechol ring that interacts with Lys122 in the NCS active site.

Some halogenated BIAs have been reported previously and were produced via a chemoenzymatic route. Maresh et al. used a novel enzyme, EfTyrDC utilised the oxidant NaOCl for aldehyde generation to produce 26 and 27. However, enzymes can react under milder and more environmentally friendly conditions and only relative rates with TfNCS were described. In addition, in recent work on the synthesis of noscapine in yeast, some C-8 halogenated BIAs were detected by liquid-chromatography mass spectrometry analysis (LC-MS). Here, the parallel in vitro cascades highlight a versatile amino-acid derived route to halogenated BIA synthesis in high ees.
| Entry | Substrate 1 | Substrate 2 | Cascade route | Product | Yield by HPLC* (Isolated yield) | ee* |
|-------|-------------|-------------|---------------|---------|-------------------------------|-----|
| 1     | 4           | 9           |               | (S)-23  | 82% (47%)                     | 90% |
| 2     | 4           | 14          |               | (S)-24  | 21% (14%)                     | 90% |
| 3     | 4           | 11          |               | (S)-25  | 84% (42%)                     | 96% |
| 4     | 4           | 12          |               | (S)-26  | 86% (40%)                     | 94% |
| 5     | 4           | 13          |               | (S)-27  | 85% (40%)                     | 92% |
| 6     | 9           | 4           |               | (S)-28  | 76% (42%)                     | 92% |
| 7     | 9           | 11          |               | (S)-29  | 58% (27%)                     | 85% |
| 8     | 9           | 12          |               | (S)-30  | 67% (35%)                     | 95% |
| 9     | 9           | 13          |               | (S)-31  | 68% (35%)                     | 91% |

*Yields were determined by HPLC analysis against products standards. **For preparative-scale reactions, products were purified by preparative HPLC or extraction method (Supplementary Information). ee's were determined by chiral HPLC. Reaction conditions: Details are specific to each cascade. E.g. (entry 1) - 50 mL reaction mixture A (RMA) - HEPES (50 mM, pH 5.5), 4 (2.5 mM, 1 equiv.), CnTYR and ETFpdC (10% (v/v) lysates), 8 (4 equiv.), PLP (0.5 equiv), CuSO4 (40 μM) at 25 °C for 6 h. A 50 mL reaction mixture B (RMB) - HEPES (50 mM, pH 7.5), 9 (7.5 mM, 3 equiv), ETFpdC and CvTam (10% and 5% (v/v) lysates, respectively), 8 (3 equiv), PLP (1.25 mM), 15 (3 equiv) at 37 °C for 6 h. RMA + RMB were combined, TINCS (10% (v/v) lysates) added, and the reaction run at 37°C for 8 h.
The tyrosinase (CoTYR) used in the enzyme cascades was found to be limiting as L-tyrosine 4 and tyramine 7 were well accepted but 3-F-L-tyrosine 11 was poorly accepted\cite{11}. For wider applications it was desirable to expand the substrate range, however, the iodo-analogue 3-I-L-tyrosine 13 has been reported to be a ‘mixed type’ inhibitor (a competitive and non-competitive inhibitor) for some tyrosinases\cite{13}. 2-Chlorophenol has also been described to act as a competitive inhibitor towards tyrosinases\cite{12}. Therefore, to expand the capability of these cascades using tyrosinases, mutagenesis of CoTYR was investigated.

**CoTYR mutagenesis**

The narrow substrate range of CoTYR is likely due to steric interactions in the active site, precluding access by the halogenated tyrosines. To probe this, several CoTYR variants were proposed based on the DNA alignment of CoTYR with reported engineered tyrosinase variants described by Kanteev et al.\cite{10}. Using the crystal structure of BmTYR (PDB code: 3NPY)\cite{3NPY} as a template, homology modelling (SWISS-MODEL)\cite{SM} was used to develop a model of wild-type CoTYR. CoTYR variants were generated in silico using Chimera\cite{Chimera}. Residues at 63, 185 and 201 positions of CoTYR were all located at the entrance of the catalytic site. G63 is already a smaller residue so was not modified (Supplementary Fig. 85a), while E185 was not modified due to concern of its remote distance to the catalytic site (Supplementary Fig. 87b). The N201 is a conserved residue in tyrrosinases, and is believed to be important for stabiilisation of the orientation of the nearby H200 imidazole for Cu coordination; mutation in this residue 5,6,11 faced away from the di-copper centre in 9

![Fig. 4](https://doi.org/10.1038/s41467-022-33122-1)

Preliminary molecular docking studies with L-tyrosine 4 and 3-F-L-tyrosine 11 suggested they readily fitted into the active sites of both the wild-type CoTYR and N201S, with the para-hydroxyl group bound to one of the Cu$^{2+}$ ions in the di-copper centre of tyrosinases, allowing the meta-carbon to be hydroxylated by the other Cu$^{2+}$ (Fig. 4a, d). In addition, meta-L-tyrosine 9 and 3-Cl-L-tyrosine 12 could access the enzyme active sites but not in a productive orientation, which agreed with the reported competitive inhibitor behaviour of some substrates with tyrosinases. With the wild-type CoTYR, 12 was orientated with the para-hydroxyl group and meta-chloro group facing towards the di-copper centre, while the other meta-carbon was rotated with the other copper and the meta-carbon near to the copper centre can be hydroxylated (Fig. 4f). Similarly, substrate 9 was orientated with the meta-hydroxyl group towards the Cu$^{2+}$ ion, so the nearby ortho-carbon can be hydroxylated (Fig. 4e). Similarly, the meta-hydroxyl group of 9 faced away from the di-copper centre in the wild-type CoTYR (Fig. 4b). However, with variant N201S, 3-Cl-L-tyrosine 12 was rotated with the meta-chloro group facing away from the di-copper centre, while the para-hydroxyl group and the other meta-carbon faced towards the catalytic copper. Therefore, here the para-hydroxyl group can bind to one of the coppers and the meta-carbon near to the copper centre can be hydroxylated (Fig. 4i).

Kinetic studies with CoTYR-N201S revealed the apparent kinetic parameters $K_{m, app.}$ and $k_{cat, app.}$ towards L-tyrosine 4 were 1.89 mM and 182.9 s$^{-1}$ ($k_{cat, app.}/K_{m, app.} = 9.68 \times 10^4$ M$^{-1}$ s$^{-1}$) and the corresponding values for tyramine 7 were 1.78 mM and 197.5 s$^{-1}$ ($k_{cat, app.}/K_{m, app.} = 1.11 \times 10^4$ M$^{-1}$ s$^{-1}$), respectively. Compared to the wild-type CoTYR $k_{cat, app.}/K_{m, app.} = 1.78 \times 10^4$ M$^{-1}$ s$^{-1}$ for 4 and 1.61 \times 10^4$ M$^{-1}$ s$^{-1}$ for 7), the catalytic efficiencies of CoTYR-N201S towards both 4 and 7 were 6-fold higher. This could be due to the larger size of the entrance into the active site, enhancing access for substrates 4 and 7, but would also make it easier for the catechol substrates to access the active site, boosting the diphenolase reaction and over-oxidation of catechols to quinones. While the highest yield of L-DOPA 5 produced by the variant with 4 was 76% (using optimised conditions), 10 equivalents of sodium ascorbate were required. The large amounts of ascorbate added could cause problems during product purifications so the generation of new variants maintaining a higher monophenolase activity was explored.

Directed evolution based on CoTYR-N201S were then carried out for this purpose. Random mutagenesis was performed with E. coli XL1 Red cells and twenty-six stable and positive colonies resulted after three rounds of re-transformation and colorimetric selection (see the methods section). Compounds 3-F-L-tyrosine 11 and 3-Cl-L-tyrosine 12 were screened with sodium ascorbate under different concentrations (0, 4, and 10 equiv.) and used to select appropriate variants. The enzyme monophenolase activities were initially estimated based on the colorimetric assay; if the reaction turned black without 8 and slightly brown with 4 equiv. of 8, this suggested potentially a better monophenolase activity towards the substrate. All negative controls gave rise to no colour change. Variants M1 and M23–M25 were estimated to display a better monophenolase activity towards 11, while M8 and M26 gave higher monophenolase activities towards 3-Cl-L-tyrosine 12 (Fig. 5a).
The quantification of products generated by the selected variants with 4 equiv. of ascorbate 8 was then carried out. Variant M1 gave the highest yield of the catechol product 3-F-L-DOPA 43, with a 90% yield by HPLC analysis (Fig. 5b), followed by variant M25 and wild-type CnTYR (70% yield). Variants M8 and M26 gave the highest amount of 3-ChL-DOPA 44 in 55–60% yield (Fig. 5c). Variants M1, M8, M25, and M26 were sequenced as N201S/G205R/V206I, N201S/H202N, N201S/G205R and N201S/G205K, respectively. This indicated that on changing Gly205 to Ser, Arg or Lys, the diphenolase activities were decreased, possibly due to steric reasons, with these groups obstructing the catechol products from re-entering the active sites. A similar effect was also observed in a previous study on the mutagenesis of BmTYR: when they mutated the residues Met61, Met184, and Phe197, which are located at the entrance to the active site, to the smaller residues Leu and Ala the diphenolase activity was enhanced 33.

Electrostatic interactions may also be important. It has been reported that the Asn residue may stabilise the nearby His residue and coordinate with Cu2+ for its uptake, and the substitution of this to either Ala or Asp decreased CnTYR activities. However, the substitution of Asn201 to Ser in CnTYR increased the enzyme activities in this study. This could possibly be due to the Ser201 forming a hydrogen bond with the imidazole ring of the nearby His200 residue, orientating the His residue into a position for better Cu2+ uptake. Meanwhile, the smaller size of the Ser201 may allow bulkier substrates to access the active site. Although N201S is a successful candidate for the acceptance of 3-F-L-tyrosine 11 and 3-Ch-L-tyrosine 12, preliminary docking analysis was insufficient to reveal the function of Ser201. In the future, further experiments using MD simulation studies may provide further insights. The variants generated were then used in the BIA cascades.

**Use of CnTYR variants in BIA synthesis**

Initially the TYR were tested in cascades based upon those previously established, providing (S)-45 (in 35% HPLC yield)19 and (S)-46 (in 27% HPLC yield) 46 from 11 using wild-type CnTYR. E/TyrDC, phenylacetaldehyde 47 and T/NCS, and for (S)-46 a methyltransferase (MT). The tyrosinase reaction previously limited the yields in these enzyme cascades. Notably, when replacing wild-type CnTYR with the variants N201S, M1(N201S/G205R/V206I), and M25 (N201S/G205R) the yield of (S)-45 increased to 66, 89, and 79% by HPLC analysis, respectively (Fig. 6a). The highest yield reached was 89% with the variant N201S/G205R/V206I. Meanwhile, the yield of (S)-46 reached 77–86% (by HPLC analysis) using CnTYR-M1 (N201S/G205R/V206I) (Fig. 6b). For the final methylation step, three O-MTs were trialled, the catechol-O-MTs from *Rattus norvegicus* (*RnCOMT*)19 and *Coptis japonica* (*Cj-6-OMT*), and *SaC* from *Myxococcus xanthus* (*MxSaC*)48 with all giving the 6-OMe product 46 in good yields. 46 Due to the high cost of the cofactor (S)-adenosylmethionine (SAM), a methionine adenosyltransferase (MAT) E.C. 2.5.1.6 was used to generate SAM from ATP and L-methionine, and a methylthioadenosine nucleosidase (MTAN, E.C. 3.2.2.9) to remove the inhibitory by-product (S)-adenosylhomocysteine (SAH), both from *E. coli* (Ec)48. Reaction times for the tyrosinase steps were also

**Table 3 | Comparison of the conversion yields of the wild-type CnTYR and CnTYR_N201S towards different substrates**

| Substrate (R) | WT-CnTYR Conversion yield | CnTYR_N201S Conversion yield | Substrate (R) | WT-CnTYR Conversion yield | CnTYR_N201S Conversion yield |
|---------------|----------------------------|------------------------------|---------------|----------------------------|------------------------------|
| L-tyrosine 4  | quantitative               | quantitative 10-times faster | tyramine 7    | quantitative               | quantitative 10-times faster |
| meta L-tyrosine 9 | no conversion             | 55%                          | meta-tyramine 10 | no conversion             | 64%                          |
| 11            | 27%                        | quantitative                 | 34            | 51%                        | quantitative                 |
| 12            | no conversion              | 60%                          | 35            | 34%                        | quantitative                 |
| 13            | no conversion              | 10%                          | 36            | 32%                        | 90%                          |
| 32            | 24%                        | quantitative                 |               |                            |                              |

Reaction conditions: HEPES buffer (50 mM, pH 5.5), substrates (2.5 mM), CuSO4 (5 μM) and enzyme cell lysates (10% v/v) in a total volume of 500 μL were run at 25 °C for 3 h. Reactions were quenched by adding 1 μL trifluoroacetic acid. Conversions were analysed using analytical HPLC at 280 nm based on substrate consumption.
were reacted with 2-bromo-phenylacetaldehyde \(50\), giving double halogenated BIAs \(S\)-\(51\) and \(S\)-\(52\) in 83 and 16\% yield by HPLC and >92\% ee, respectively (Table 4, entries 1 and 2). The low yield of \(S\)-\(52\) was most likely due to the lower monophenolase activity of the \(CN\) variant with \(2\) and the effect of the more sterically hindered 3-Cl-dopamine derived intermediates in the \(CN\) active site. More halogenated BIAs were then produced using the ‘parallel cascade’ strategy. The amine moiety was generated from \(11\) as before, and aldehydes produced from \(11\) to \(13\) and \(4\) (Table 4, entries 3–7). This gave BIAs \((S)\)-\(54\–\(57\) in 26–78\% yields and >90\% ee. Although some of the products were formed in lower yields due to the difficulties of using more sterically challenging halogenated analogues and unknown side-products, it nevertheless highlights a very valuable and flexible strategy to non-natural BIAs in high enantioreactivities.

In summary, \(EF\) and \(CT\) are capable of accepting a broad range of aromatic amino acids, and so were used here to generate aldehydes for coupling reactions using Pictet-Spenglerases. Such arylacetaldheydes are difficult to synthesise using traditional chemical routes as the aldehydes are prone to oxidations and aldol self-condensations. Enzyme cascades were then developed using parallel reaction strategies and nine non-natural BIAs were initially produced in good yields and ee. Mutagenesis studies were then applied with \(CN\) to expand its substrate scope towards halogenated amino-acids. Several \(CN\) variants were generated that displayed better monophenolase activities towards 3-F-tyrosine \(11\) and accepted \(meta\)-L-tyrosine \(9\) and 3-Cl-L-tyrosine \(12\), which are known inhibitors for wild-type tyrosinases. Then extended enzyme cascades using the \(CN\) and \(T\) variants were carried out to give 14 halogenated (and 13 non-natural) BIAs in good stereoselectivities. Importantly, this is the first time that double halogenated BIAs have been reported, highlighting the abilities of enzyme cascades to ‘mix and match’ arylethylamines and aldehydes to give different BIAs. This parallel enzyme
cascade strategy together with enzyme mutagenesis is a powerful synthetic approach for alkaloid synthesis.

**Methods**

**Chemicals**

Dopamine, L-tyrosine, L-DOPA, tyramine, sodium ascorbate, 3-Cl-L-tyrosine, 3-I-L-tyrosine, 3-phenyl-1-propylamine, sodium pyruvate, 3-NH₂-L-tyrosine, 3-NO₂-L-tyrosine, octopamine, synephrine, 4-(2-aminoethyl)aniline, 4-F-L-phenylalanine, 4-Cl-L-phenylalanine, 4-Br-L-phenylalanine, 4-Ome-L-phenylalanine, 4-NH₂-L-phenylalanine, 4-NO₂-L-phenylalanine, PLP, CuSO₄·5H₂O and kanamycin were purchased from Sigma-Aldrich (Germany). Meta-L-tyrosine, meta-tyramine, 3-F-L-tyrosine, phenylacetaldehyde, 2-bromophenylacetaldheyde and IPTG was purchased from Alfa Aesar (Thermo Fisher Scientific, USA). All chemicals were purchased in the highest purity available. Thin layer nano-columns were purchased from Merck (Germany).
chromatography was performed using plates with a silica gel matrix on an aluminium support. Ultraviolet light (254 nm) and ninhydrin stain was used to visualise the plates.

HPLC methods
Analytical methods were performed with a Dionex™ UltiMate™ 3000 HPLC System, with a Dionex™ UltiMate™ 3000 RS Pump, a Dionex™ UltiMate™ 3000 Autosampler, a Dionex™ UltiMate™ 3000 Column Compartment and a UltiMate™ 3000 RS Diode Array Detector (ThermoFisher Scientific, US). Preparative methods were developed with an Agilent 1260 Infinity™ HPLC System, with a 1260 Infinity™ Preparative Pump, a 1260 Infinity™ Preparative-scale Fraction Collector, a 1260 Infinity™ Multiple Wavelength Detector, and a 1260 Infinity™ Preparative Autosampler.

Analytical HPLC method 1 (achiral). Achiral quantitative analyses adopted a reverse phase analysis method. Separation was achieved with an ACE 5 C18 column (150 × 4.6 mm) with a flow speed of 1 mL/min at 30 °C. The injection volume was 10 μL. Substrates and products were measured via UV absorbance at 280 nm. Eluent A (H2O with (v/v) 0.1% TFA) and eluent B (acetonitrile) were used as a mobile phase over 10 min. The gradient was as: 0.0 min (10% B)-1.0 min (10% B)-6.0 min (70 % B)-6.1 min (100% B)-6.5 min (100% B)-6.6 min (100% B)-10.0 min (10% B).

Analytical HPLC method 2 (chiral). The chiral separation was achieved with an Supelco Astec Chirobiotic™ T column (25 cm × 4.6 mm) or a Supelco Astec Chirobiotic™ T2 column (25 cm × 4.6 mm), and a flow speed of 1 mL/min at 30 °C. The injection volume
was 5 μL. Products were measured via UV absorbance at 230 nm. Methanol (0.2% AcOH, 0.1% TEA) was used as a mobile phase over 40 min or 80 min.

Analytical HPLC method 3 (chiral). The chiral separation was achieved with a Supelco Astec Chirobiotic™ T column (25 cm × 4.6 mm) and a flow speed of 0.2 mL/min at 30 °C. The injection volumes were 5 μL. Compounds were detected by UV absorbance at 230 nm. An isocratic mobile phase 20 mM NH₄OAc (pH 4.0):MeOH (70:30) was used over 120 min.

Preparative HPLC method 4. The separation was achieved with a Vydac™ 218TP1022 (C18, 10 μm, 2.2 cm ID × 25 cm L) preparative column or a Supelco™ Discovery BIO wide pore (C18, 10 μm, 2.12 cm × 25 cm) preparative column and a flow speed of 8 mL/min at 25 °C. The injection volume was 900 μL. Products were identified via UV absorbances at 214 nm and 280 nm. Eluent A (H₂O with 0.1% (v/v) TFA) and eluent B (acetonitrile with 0.1% (v/v) TFA) were used as a mobile phase over 28 min. The gradient was as: 0.0 min (5% B)-3.0 min (5% B)-20.0 min (70% B)-22.0 min (70% B)-23.0 min (5% B)-28.0 min (5% B).

Table 4 | Parallel cascades with CnTYR variants, EfTyrDC, CvTam, and TfNCS-A79I to halogenated BIAs

| Entry | Substrate 1 | Substrate 2 | Cascade route | Product | Yield by HPLC (Isolated yield) | ee |
|-------|-------------|-------------|---------------|---------|--------------------------------|----|
| 1     | 11          | 50          |               | (S)-51  | 83% (57%)                      | 95 |
| 2     | 12          | 50          |               | (S)-52  | 16% (7%)                       | 92 |
| 3     | 11          | made in situ from 11 | | (S)-53  | 26% (16%)                      | 95 |
| 4     | 11          | 11          |               | (S)-54  | 43% (31%)                      | 90 |
| 5     | 11          | 12          |               | (S)-55  | 47% (33%)                      | 95 |
| 6     | 11          | 13          |               | (S)-56  | 42% (28%)                      | 95 |
| 7     | 11          | 4           |               | (S)-57  | 78% (52%)                      | 95 |

*Yields were determined by HPLC analysis at 280 nm against products standards. For preparative-scale reactions products were purified by preparative HPLC or an extraction method. *ees were determined by chiral HPLC. Reaction conditions: Details are specific to each cascade. E.g., entry 4, 50 mL reaction mixture A (RMA) - HEPES (20 mM, pH 5.0), 11 (10 mM, 1 equiv.), CnTYR-M1 (N201S/G205R/V206I) and EfTyrDC (10% (v/v) cell lysates), 8 (4 equiv.), PLP (0.5 equiv.), CuSO₄ (5 μM) was stirred at 25 °C for 8 h. A 20 mL reaction mixture B (RMB) - HEPES (50 mM, pH 7.5), 11 (20 mM, 1 equiv.), EfTyrDC and CvTam (10% and 5% (v/v) cell lysates, respectively), 8 (4 equiv.), PLP (1 equiv.), 15 (1 equiv.) was stirred at 37 °C for 8 h. Afterwards, RMA and RMB were combined, TfNCS-A79I (10% (v/v) cell lysates) added, and the reaction stirred at 37 °C for 16 h.
Article

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Preparative HPLC method 5. The separation was achieved with a VydaCTM 218TPI022 (C18, 10 µm, 2.2 cm ID × 25 cm L) preparative column or a SupelcoTM Discovery BIO wide pore (C18, 10 µm, 2.12 cm × 25 cm) preparative column and a flow speed of 8 mL/min at 25 °C. The injection volume was 900 µL. Products were identified via UV absorbances at 214 and 280 nm. Eluent A (H2O with 0.1% (v/v) TFA) and eluent B (acetonitrile with 0.1% (v/v) TFA) were used as a mobile phase over 25 min. The gradient was as: 0.0 min (25% B)-3.0 min (25% B)-20.0 min (90% B)-22.0 min (90% B)-23.0 min (25% B)-28.0 min (25% B).

Chemical analytics

1H and 13C NMR spectra were obtained using a Bruker Advance III 700 MHz spectrometer. Chemical shifts specified are relative to trimethylsilylane (set at 0 ppm) and referenced to the residual, protonated NMR solvent. Coupling constants in 1H-NMR spectra (J) are given in Hertz (Hz) and described as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m). Mass spectrometry data of compounds were measured on an Agilent 1100 Series System with a Finnigan LTQ mass spectrometer. An ACE 5 C18 reverse phase column of compounds were measured on an Agilent 1100 Series System with a Finnigan LTQ mass spectrometer. An ACE 5 C18 reverse phase column (50 mm × 2.1 mm, 5 µm) was adopted with a mobile phase of eluent A (H2O with 0.1% (v/v) formic acid) and eluent B (acetonitrile) over 5 min with a flow rate of 0.6 mL/min. The sample injection volume was 10 µL. Chemical compounds were measured in a positive ion mode, and the operating conditions of the ESI interface were set to a capillary temperature 300 °C, capillary voltage 9 V, spray voltage 4 kV, sheath gas 40, auxiliary gas 10, sweep gas 0 arbitrary units. The gradient of eluents was as: 0.0 min (5% B)-4.0 min (90% B)-4.5 min (5% B)-5.0 min (5% B).

Recombinant protein expression in E. coli BL21 (DE3)

Selected enzyme glycerol stocks (E. coli BL21 (DE3)) were plated out on agar plates supplemented with 50 µg/mL kanamycin. A single colony was then picked to inoculate into 10 mL of LB media supplemented with 50 µg/mL kanamycin and grown at 37 °C and 250 rpm overnight (8–16 h). 1 mL of the overnight cultures were inoculated into a 500 mL baffled shaking flask containing 100 mL of TB media supplemented with 50 µg/mL kanamycin at 37 °C, 250 rpm until an OD600 = 0.6. The selected mutants were sequenced by Qiagen Mini Prep Kit (Qiagen, Germany) and further transformed with E. coli BL21 (DE3) competent cells. After cultivation at 37 °C for 1 h, cells were plated on the LB agar plate with 50 µg/mL kanamycin and 500 µM IPTG. The plates were cultivated at 37 °C overnight. Colonies shown black colour were selected and inoculated in 10 mL of TB broth with 50 µg/mL kanamycin at 37 °C overnight. As a single colony may contain various plasmids, to obtain the stable mutants, again, plasmids were extracted and transformed with E. coli BL21 (DE3) competent cells, which was then plated on the LB agar plates with 50 µg/mL kanamycin and 50 µM IPTG and grown overnight. The above steps were repeated twice. Afterwards, colonies shown black were chosen and further cultivated in TB broth. Mutated proteins were expressed, and cell lysates were used for enzyme screening with 3-F-L-tyrosine 11 and 3-ChL-tyrosine 12 based on the colorimetric reaction. The DNA of the selected mutants were sequenced by ‘DNA Sequencing Service’ from Eurofins Scientific (Belgium).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data to support this work is available from the corresponding authors upon request.

Calibration curves - all calibration curves used for determination of conversions to give products are given in the supplementary information.

Complete chemical syntheses and analyses - complete synthetic methods, characterisation of THIQ products and corresponding chiral HPLC data are given in the supplementary information.

AutoDock Vina - experiment details of the docking study using AutoDock Vina are given in the supplementary information.

1. The protein sequence for enzymes used in this study are available in the Genbank database under accession code list below:
   1) Tyrosinase from Candidatus Nitrosopumilus salaria BD3k(CnTYR), accession code: EJ654321.1
   2) Tyrosine decarboxylase from Enterooccus faecalis (EfTyrDC), accession code: AFO43338.1
   3) Transaminase from Chromobacterium violaceum (CvTAm, accession code: AAASQ9973.1)
   4) (S)-Norcoclaurine synthase from Thalictrum flavum (T297TNCs, accession code: AAR22502.1)
   5) Catechol-O-methyltransferase from rat liver (RnCOMT, accession code: AAAS0881.1)
   6) Catechol-O-methyltransferase from Myxococcus xanthus (MxSafC, accession code: AAC44130.1)
   7) Norcoclaurine 6-O-methyltransferase from Coptis japonica (G6OMT, accession code: BAB08004.1)
   8) S-Adenosylmethionine synthetase from E. coli (EcMAT, accession code: AAB24164.1)
   9) Methylthioadenosine/SAH nucleosidase from E. coli (EcMTAN, accession code: AAB08389.1)

2. The protein sequence for CnTYR variants and TNCs variants are available in the supplementary information.
3. The crystal structure data for enzymes in this study are provided in the RCSB Protein Data Bank (PDB) under accession code list below:

1. Crystal structure of tyrosinate from Bacillus megaterium (BzTYR, accession code: 3NPY)
2. Crystal structure of (S)-norcoclraine synthase from Thalictrum flavum (Δ297NCS, accession code:5N8Q)

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Author contributions
Y.W. performed enzymatic reactions, chemical characterisation, and created figures. F.S. provided enzymes and advice for enzyme assays. E.C. performed chemical syntheses. The project was supervised by T.D.S., J.M.W., and H.C.H. and the manuscript was written by Y.W. and H.C.H. The manuscript has been approved by all contributing authors.

Competing interests
The authors declare no competing interests.

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