The Catalytic Potential of \textit{Coptis japonica} NCS2 Revealed – Development and Utilisation of a Fluorescamine-Based Assay

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\begin{abstract}
The versatility and potential of a norcochlorine synthase (NCS) from \textit{Coptis japonica} NCS2 has been investigated, together with the development and application of a novel fluorescence-based high-throughput assay using nearly forty amines/aldehydes. The stereocontrol exerted by CjNCS2 on selected non-natural substrates has been determined, where the tetrahydroisoquinolines (THIAs) were formed as the (1S)-isomer in >95\% ee, as observed with the natural product norcochlorine. Docking calculations involving THIA mechanism intermediates, utilising the reported \textit{Thalictrum flavum} NCS X-ray crystallographic structure, were carried out and combined with the CjNCS2 screening results to further understand the mode of action of NCS. These findings suggested that in addition to the key active-site residues K122 and E110, D141 is also mechanistically essential for the enzymatic transformation. The exceptional tolerance of NCS towards aldehyde substrates is furthermore supported by our proposed mechanism in which the aldehydes protrude out of the enzymatic pocket.

\textbf{Keywords}: alkaloids; biotransformations; norcochlorine synthase; Pictet–Spengler reaction; tetrahydroisoquinolines
\end{abstract}

\section*{Introduction}

The benzylisoquinoline alkaloids (BIAs) are a large and structurally diverse group of secondary metabolites that have a key role in chemical plant defense. Many BIA natural products have been studied for their pharmacological properties including magnoflorine (anti-HIV), the antibiotic berberine, papaverine (coronary vasodilatation), and the well-known analgesic morphine (Figure 1). A variety of synthetic BIAs including dihydro- and tetrahydroisoquinoline alkaloids (DHIA and THIA, respectively) have also been identified as bioactive molecules against a wide array of diseases. This myriad of biological activities has highlighted the THIA scaffold as a versatile pharmacophore, and the BIA natural products and structural analogues as sought after pharmaceutical candidates.

The chemical synthesis of chiral BIAs is often challenging as it requires the regioselective functionalisation of phenols and amines, and the formation of cyclic structures in a stereocontrolled manner. Despite the development of elegant chemical strategies for their production, syntheses of THIAs remain either step-intensive or lack stereocontrol to generate single isomers.

Interesting recent reports have described the use of a recombinant monoamine oxidase and nanoscale...
The enzyme catalyses the stereochemical synthesis of BIA alkaloids is mediated by norcoclaurine synthase (NCS). The enzyme catalyses the stereoselective coupling of dopamine 1 and 4-hydroxyphenylacetalddehyde (4-HPAA) 2 to generate (S)-norcoclaurine (S)-3, the key biosynthetic precursor to BIA natural products (Scheme 1). The enzyme NCS therefore has significant potential as a tool for the stereoselective production of libraries of THIA and BIA compounds.

Early NCS studies focused on identifying the natural precursors involved in BIA alkaloid biosynthesis. The subsequent use of cultured plant cells enabled NCSs to be purified and characterised in more detail, particularly from Thalictrum flavum. More recently, the cloning and over-expression of T. flavum NCS (TfNCS) led to high levels of expression and then elegant mechanistic, X-ray crystallographic and NMR studies, as well as investigations into substrate tolerance with a small number of analogues. This was followed by a scale-up of the reaction using TfNCS for the generation of (S)-norcoclaurine. However, in some of these studies the enzymatic reactions were performed under phosphate buffer reaction conditions, favouring the undesirable chemical background reaction which can complicate interpretation of the results. In parallel with our current studies, TfNCS has been recently used to generate a range of THIAs to expand its application in biocatalytic transformations. Notably, in TfNCS studies to date, limited attention has been focused on the stereoselectivity of the reaction. The CD spectra in some reports has indicated that one isomer was formed preferentially but here ee values were not determined. However, in the scale-up study using TfNCS with 1 and 2 an ee for norcoclaurine 3 of 93% was reported. In the recent publication to generate non-natural THIAs using TfNCS no stereoselectivities were reported.

For biocatalytic applications enzymes need to be readily expressed, stable under the conditions used, accept a broad range of substrates, and give high stereoselectivities. To develop these properties, high-throughput assays are invaluable to allow the rapid characterisation of native and mutant enzyme activities against a range of substrates. In addition, chiral assays as well as methods to confirm absolute configurations are vital. When developing enzyme variants with improved properties such as solvent tolerance again these assay tools are essential. Here we describe the versatility and potential of an NCS from Coptis japonica, the development and application of a novel fluorescence-based high-throughput assay, the absolute stereochemistry of the norcoclaurine formed, and for the first time detail the stereocontrol that NCSs can exert on non-natural substrates. In addition, docking calculations have been performed and combined with screening results to further understand the mode of action of NCS.

Results and Discussion

A previous study investigating norcoclaurine synthase in Coptis japonica revealed NCS activity from cultured C. japonica cells when using substrates 1 and 2. Recombinant NCSs were generated in E. coli and designated as C. japonica NCS1 (CjNCS1) and C. japonica PR10A (CjPR10A) since the protein was initially classified as pathogenesis-related 10 (PR10). Both enzymes were reported to produce (S)-3, although the LC-MS elution time of the products differed, which was surprisingly suggested to be due to a different mechanism of norcoclaurine production. In subsequent work, recombinant CjPR10A was selected for incorporation into alkaloid pathways, rather than CjNCS1, to give (S)-reticuline. More recently, CjNCS1 was further studied and found not to form norcoclaurine 3. As part of a study to use little explored NCSs in biocatalytic studies with non-natural substrates CjPR10A, denoted here as CjNCS2 (accession number A2A1A1), was obtained as a synthetic gene, subcloned into pET29a and expressed in E. coli. After purification of the his-tagged protein 0.3 mg/mL of protein were isolated (4.4 U/mg of protein). When used in a bioconversion (5 mM scale) with 1 and 2, the formation of norcoclaurine 3 was confirmed by HPLC and comparison to racemic 3 generated by a biomimetic reaction.

In order to evaluate the catalytic versatility of CjNCS2 and validate it as tool for the synthesis of novel THIAs several assays were considered, including HPLC and GC. However, to enhance throughput, particularly for future library screening, fluorescent or colorimetric screens were considered. Fluorescamine 4, is a water soluble spirolactone that exclusively generates fluorescent pyrrolidinones upon reaction with primary amines. This chemoselectivity for primary amines (e.g., dopamine 1) to generate fluores-
cent species over secondary amines (e.g., norcoclaurine 3) that give non-fluorescent furanones was utilised to monitor NCS activity by quantifying the depletion of dopamine 1 (Scheme 2). Fluorescence intensities resulting from the reaction of 4 with dopamine 1 and norcoclaurine 3 were measured and the signal was 1000-fold greater for the pyrrolidinone, than for the furanone (Scheme 2; see the Supporting Information). Fluorescamine auto-fluorescence was also observed but at a negligible level. Purified CjNCS2 in HEPES buffer was reacted with dopamine 1 and increasing concentrations of 4-HPAA 2 at 37°C over 1 h. The addition of fluorescamine 4 after 1 h generated fluorescence by reaction with the residual dopamine 1. A drop in fluorescence intensity was observed which was dependent on the concentration of 4-HPAA used, showing a typical dose-response correlation (Scheme 2, Figure 2). It should be noted that in the absence of NCS, but in a suitable medium (phosphate buffer), dopamine can readily react with 4-HPAA to yield norcoclaurine.[12] However, the use of HEPES buffer minimised the non-enzymatic reaction (<1%).

Several control reactions were performed to confirm that the consumption of dopamine in the assay was due to a CjNCS2-mediated reaction. Tyramine 5 (a dopamine analogue inert towards NCSs) instead of dopamine was used with CjNCS2 and a high fluorescence signal observed irrespective of the concentration of 4-HPAA, indicating no reaction had occurred (Scheme 2, Figure 2). When no enzyme was used in the assay an elevated and constant fluorescence signal was observed, whereas in the absence of 4, no fluorescence was noted. A significant fluorescence signal was observed due to CjNCS2 C-terminal amine and side chain reactions with 4. Indeed, incubation with 0.4 mg/mL of CjNCS2 yielded more than 50% of background fluorescence, compared to a 25% level with 0.1 mg/mL of the protein (Figure 2). This led to the selection of CjNCS2 at concentrations of 0.1 mg/mL for use in the assay as it gave a rapid rate of consumption of dopamine, but also limited the background fluorescence signal. Overall, these results demonstrated that a fluorescamine-based assay could be used to quantitate the consumption of dopamine in an NCS-mediated reaction.

After validating and establishing procedures for the fluorescamine-based assay it was used for screening libraries of amine and aldehyde substrate analogues. While several substrates were obtained from commercial sources, many compounds such as arylacetaldehydes and variously decorated phenethylamines were commercially unavailable so were synthesised. The aryl and heteroaromatic acetaldehydes were readily prepared from either the corresponding terminal alkenes via ozonolysis or the corresponding alcohols via a Parikh–Doering oxidation.[34] This enabled access to a variety of electron-rich, electron-deficient, carbocyclic and polyfunctionalised aldehydes (6–32) (Table 1). Commercially available amines (1, 33–37) (Table 2) were used, together with 3-substituted phenethylamines (38–42) prepared from the corresponding phenylacetonitriles via standard reduction reactions. Using the assay procedure established above, the CjNCS2-mediated conversion of dopamine was ini-
Table 1. Aldehydes used in the fluorescence assay and the conversions observed.

<table>
<thead>
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<th>Compound number</th>
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<th>Conversion&lt;sup&gt;a,b&lt;/sup&gt; [%]</th>
<th>Compound number</th>
<th>Aldehyde</th>
<th>Conversion&lt;sup&gt;a,b&lt;/sup&gt; [%]</th>
<th>Compound number</th>
<th>Aldehyde/ketone</th>
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<sup>a</sup> In each well was 20 μL 1 (0.1 mM in H₂O), 20 μL aldehyde/ketone (1 mM in CH₃CN) and 160 μL of purified CjNCS2 (140 mU in pH 7.4 HEPES). The fluorescence microplates were incubated for 1 h at 37°C and reactions quenched with 50 μL of 4 (2 mM in CH₃CN). Reactions were performed in triplicate.

<sup>b</sup> Conversions represented by + + + (>80%), + + (40–80%), + (25–40%), trace (10–25%), 0 (<10%) and % in parentheses (see the Supporting Information), standard deviation ±5%.
initially explored with aldehydes 2 and 6–32 (Table 1). In order to detect any non-enzymatic reactions, controls with buffer replacing the CjNCS2 enzyme solution were performed alongside each enzymatic reaction. Experiments in the absence of dopamine 1 gave the background fluorescence level, and in the absence of 2 corresponded to a 0% conversion (no consumption of dopamine). These controls enabled comparability between plates (see the Supporting Information). Data from the assays indicated the tolerance of CjNCS2 to aldehydes and % conversions were calculated (Table 1). The assay demonstrated that all aryl and heteroaromatic acetaldehydes studied (2, 6–19) were substrates for CjNCS2. Substitution at the para-position on the aromatic ring was particularly well tolerated, with almost complete enzymatic conversion (Table 1; ++ +) after 1 h with electron-donating groups (2, 12, 17) and electron-withdrawing moieties (15, 16). The conversions were higher than for phenylacetaldehyde 19, perhaps reflecting aldehyde solubilities.

**meta**-Substituted arylacetaldehydes were also accepted, though less readily, with conversions towards 50% (Table 1; + +) (9, 10). The combination of **meta**- and para-substitution lead to reduced levels of conversion in the range 10–40% (Table 1; trace, +) (6, 7, 14, 18). Previous studies using NCS from cultured C. japonica cells reported that 18 and 19 produced the corresponding THIAs (no data shown), and more recently CjNCS was successfully used with substrate 18 in a pathway to (S)-reticuline, consistent with our results using CjNCS2. The pentafluorophenyl analogue 13 gave low conversions with CjNCS2 (Table 1; trace). Our data using arylacetaldehydes suggested that unfavourable steric interactions may occur in the active site when going from 4-, to 3- to further substituted substrates. Interestingly, in recent work with TjNCS, several 2-, 3-, 4-, and 3,4-arylacetaldehydes were used with 1 and conversions determined using an HPLC dopamine consumption-based assay.[24] All conversions were in the range 51–71%, although a 3-h reaction time was used, perhaps enabling less differentiation of substrate tolerances.[24] With CjNCS2 the heteroaromatic acetaldehydes 8 and 11 gave moderate conversion levels (Table 1; +), highlighting the potential of using this enzyme with a range of functionalised aldehydes. Crucially, under the reaction conditions used non-enzymatic conversions were low with the aryl and heteroaromatic acetaldehydes (2, 6–19).

Benzaldehydes (21, 22, 26, 27) and 2-naphthaldehyde (30) were poor substrates for CjNCS2, with low or no enzymatic activities compared to the non-enzymatic reaction, which was still evident even in HEPES buffer (see the Supporting Information). Short aliphatic aldehydes showed more varied activities. Formaldehyde 24 is a highly reactive substrate that is fully converted in the absence of enzyme and was not explored further. CjNCS2 was inactive towards ethanol 20 and cyclopentane-carboxaldehyde 23, however isobutyraldehyde 28 was accepted at detectable levels. For TjNCS, no products were reported when using formaldehyde, ethanol 20 or benzaldehyde 21, consistent with our data, however competing non-enzymatic conversions were not noted.[24] The longer chain aliphaticdehyde heptanal 25 and hydrocinnamaldehyde 32 proved to be good substrates for CjNCS2 with enzymatic conversions of approximately 60% (Table 1; + +). The more sterically constrained cinnamaldehyde 29 and substitution of the aldehyde moiety in 2 for a ketone (31) precluded both the enzymatic and non-enzymatic reactions. These results overall demonstrated that the fluorescent assay was able to provide data on the differential acceptance of

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**Table 2.** Amines used in the fluorescence assay and the conversions observed.

<table>
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<tr>
<th>Compound number</th>
<th>Amine</th>
<th>Conversion[a,b,c] [%]</th>
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</table>

[a] In each well was 20 μL amine (0.5 mM in H2O), 20 μL 2 (1 mM in CH3CN) and 160 μL of purified CjNCS2 (140 mM in pH 7.4 HEPES). The fluorescence microplates were incubated for 1 h at 37°C and reactions quenched with 50 μL of 4 (2 mM in CH3CN). Reactions were performed in triplicate.

[b] Conversions represented by + + + (>80%), + + (40–80%), + (25–40%), trace (10–25%), 0 (<10%) and % in parentheses; see the Supporting Information, sd ±5%.

[c] Amine 42 was consumed in the assay; a secondary LC-MS assay confirmed no THIA product was formed.
a range of aldehyde analogues by CjNCS2. It simultaneously provided information on the non-enzymatic reaction, which must be assessed for each new substrate. In general arylacetaldehydes were good to excellent substrates with dopamine for CjNCS2 and some aliphatic aldehydes could be used as substrates by the enzyme.

The substrate tolerance of CjNCS2 towards a selection of amines was also investigated with 4-HPAA 2 (Table 2; see the Supporting Information). Out of the amines (1, 33–42) tested, CjNCS2 displayed significant activity towards compounds 1, 37 and 38. The common feature uniting these three analogues is a meta-phenol group. The lack of a phenol at this position (33, 34) or substitution with an electron-donating amine, or electron-withdrawing halide group (39–41) resulted in no product formation. In previous work aniline 39 gave THIA products in a biomimetic phosphate-mediated reaction. Here the lack of acceptance of 39 compared to 1 by CjNCS2 may be due to the higher reactivity of the phenolate intermediate generated via deprotonation in the active site. The presence of substituents on the ethylamine moiety reduced amine consumption with CjNCS2 from approximately 80% with 38 down to 35% with metaraminol (37), probably due to unfavourable steric or hydrogen-bonding interactions of 37 in the active site. A complete loss of fluorescence was observed with 2-nitrophenethylamine 42, in both the enzymatic and non-enzymatic reaction. This reaction is mechanistically unlikely due to the strong electron-withdrawing effect exerted by the nitro group. In light of this result, enzymatic and non-enzymatic reactions for the amine and aldehyde substrates were performed and reactions analysed by LC-MS (see the Supporting Information). For 42, neither reaction led to THIA product formation, highlighting an example of a false positive result in the fluorescamine-based assay.

To study the application of CjNCS2 as a biocatalyst the biotransformations were then performed on a larger scale (5 mM amine, 10 U CjNCS2). Five substrates were selected from the hits identified in the fluorescamine-based assay. Dopamine 1 was reacted with 4-HPAA 2, 2-bromophenylacetaldehyde 15 and heptanal 25, and 2 was also reacted with 3-hydroxyphenethylamine 38. All four enzymatic reactions yielded THIA products (3, 43–45) in good to excellent isolated yields, comparable to conversion yields calculated from the fluorescamine-based assay (Table 1 and Table 2). Negligible conversions were observed in enzyme-free control reactions, demonstrating that these were exclusively produced via CjNCS2-mediated reactions. The reaction between 1 and heptanal 25 also generated the ortho-addition product 44a in 11% isolated yield (Table 3). One of the essential advantages biocatalysts have is their ability to catalyse stereoselective transformations. It was therefore crucial to determine the optical purity of not just norcoclaurine 3, but also compounds 43–45, and additionally to establish the absolute configurations which are essential for the bioactivity of THIAs.

Previous studies using TjNCS have either indicated that one isomer of 3 was formed preferentially using CD (no quantitative stereochemical data), phosphate buffers were used enabling the non-enzymatic reaction to occur, or stereoselectivities of products other than 3 were not reported. When cultured C japonica cells were used, the results indicated that (S)-3 was the major isomer resulting from the bioconversion, but no ee was reported. The synthesis of 45 has also been reported using TjNCS, although no yield or selectivity were given and reaction times were over 24 h in phosphate buffer, which is known to catalyse the non-enzyme-mediated coupling of these amines and aldehydes.

To facilitate the routine determination of product stereoselectivities from NCS-mediated reactions, and absolute configurations, Mosher’s derivatisation of the product followed by analysis of the 1H NMR spectra was performed. Initially the stereoselectivity of norcoclaurine 3 produced using CjNCS2 was investi-
tigated. Racemic 3 and (S)-3 (> 80% ee) by chiral HPLC of N-Boc-3 and Mosher's derivatisation standards were synthesised using a phosphate-mediated reaction, and by modification of a published procedure using a chiral auxiliary Bischler–Napieralski cyclisation and reduction strategy (see the Supporting Information).[11,12] These were reacted with (R)-MTPA chloride. Analysis of the 1H NMR spectrum of the rac-3 derivative highlighted differences between the (1S,2'S) and (1R,2'S) diastereoisomers, particularly at 1-H, which was observed at 5.70 ppm and 5.89 ppm [Figure 3, rac-3 (middle)]. Analysis of (S)-3 (> 80% ee) indicated that the major 1-H signal at 5.70 ppm [Figure 3, (1S)-3 (bottom)] corresponded to the (1S,2'S)-isomer, and that at 5.89 ppm to the (1R,2'S)-isomer. The derivatisation was repeated with CjNCS2-3, and the 1-H signal was observed exclusively at 5.70 ppm [Figure 3, CjNCS2-3 (top)], indicating that CjNCS2 catalysed the stereoselective synthesis of (S)-norcoclaurine in > 95% ee, as observed with TjNCS.[23]

With the conserved environment at 1-H in 3 and 43–45, the same approach was used for the other THIAs. Compounds 43, 44 and 45 were derivatised with (R)-MTPA chloride and analysis of the 1H NMR spectra revealed that for all three analogues, a unique 1-H signal was observed between 5.5 ppm and 5.7 ppm with no other diastereoisomer detected at a higher chemical shift. This indicated that again the (1S)-enantiomer of 43–45 was formed in high stereoselectivities of > 95% ee.

While the fluorescamine-based assay demonstrated that recombinant NCSs such as CjNCS2 are attractive catalysts for the stereoselective production of THIAs,
it also highlighted some catalytic limits of the enzyme regarding substrate acceptance. In order to develop more versatile NCS catalysts, for instance via site-directed mutagenesis, a thorough understanding of the topology and mechanism of action of NCS are essential. To date, there have been few reports of the catalytic mode of action of NCS. The tertiary structure of TNCNCs has recently been investigated by Rösch et al. via CD and NMR titration experiments and Boffi et al. using X-ray crystallography.\textsuperscript{[19,21,22,39]} In the crystallographic studies, a stacked configuration was observed between the non-productive aldehyde used and 1, with the aldehyde carbonyl hydrogen-bonded to the mechanistically important residue Lys122.\textsuperscript{[22]} However, the stacked configuration reported would suggest the formation of (R)-norcoclaurine, unless there is substantial reorientation within the active site. This may be because the 4-HPAA non-productive analogue 4-hydroxybenzaldehyde used, has a more electron-withdrawing aryl ring than 2 and may therefore bind to NCS in an unproductive orientation. Glu110 and Tyr108 were also highlighted as potentially important residues mechanistically. The NMR experiments used methyl(4-hydroxyphenyl) acetate and (4-hydroxyphenyl)ethanol as HPAA 2 substitutes, and suggested that Glu75 may be a catalytically important residue.\textsuperscript{[39]} With further mechanistic insights required, computational docking experiments were performed (with autodock4.2 and autodock vina\textsuperscript{[40]} using the reported crystal structure of T/NCS (PDB 2VQ5).\textsuperscript{[22]} T/NCS has a high 77% sequence homology to CjNCS2 (see the Supporting Information), and therefore provides a suitable model for the investigation of CjNCS2. The aminol productive intermediates 46a–d (Figure 4, a) which lead to the THIAs 3 and 43–45 were docked: analysis of these docking calculations highlighted two putative binding modes. A first binding mode (binding mode A, Figure 4, b and c) was the lowest in energy, and characterised by the aminol adopting an L-shape conformation to fit the enzymatic cavity with the catechol group buried in the cavity and the R1 aldehyde moiety occupying the entrance to the active site. In the second putative binding mode (binding mode B, not shown), aminols 46 also adopted an L-shape topology but with a flipped conformation in which the aldehyde group R1 is buried in the enzyme cavity and the catechol occupies the entrance to the active site. These two new binding modes are in contrast to the stacked X-ray crystallographic configuration.\textsuperscript{[22]}

The fluorescence assay results showed that CjNCS2 tolerates aldehydes that are significantly more sterically challenging than 4-HPAA 2, the natural substrate (e.g., 2-indolecarboxaldehyde 8 and 3-methoxyphenylacetalddehyde 10), or possess longer alkyl chains (e.g., heptanal 25 and hydrocinnamaldehyde 32). This suggests that the aldehyde substrate, and by extension the R1 end of the corresponding aminol intermediate, occupies a large space in the active site with limited steric constraints. In this respect, the stacked model proposed by Boffi and co-workers is disfavoured.\textsuperscript{[22]} Similarly, the putative binding mode B with the R1 of the aminol deeply buried in the enzymatic cavity is less likely. In the proposed binding mode A, the end of the R1 substituent occupies the entrance to the active site. As a result, bulkier substrates and those possessing longer alkyl chains could more readily be tolerated by the enzyme, as they

Figure 4. a) Structure of aminol intermediates 46a–d. b) Lowest energy docking conformations of binding mode A and aminols 46a–d in red (46a and d), blue, yellow, respectively (2VQ5 in grey ribbon and water channel is highlighted with red spheres). c) T/NCS1 active site with compound 46a represented as an electronic density surface around a green core (binding mode A). Amino acid residues located within 5 Å to the putative aminol intermediates are highlighted in grey with catalytically active residues highlighted in cyan.
would protrude out of the enzymatic cavity towards the solvent.

The fluorescence screen also highlighted a differential in CjNCS2 tolerance towards arylacetaldehyde substituents with 4-substituted arylacetaldehydes (2, 12, 15–17) being excellent substrates, while 3-arylacetaldehydes and heteroaromatic acetaldehydes (8–11) were moderate to good substrates, and 3,4-disubstituted arylacetaldehydes (6, 7, 14, 18) and pentafluorophenylacetaldehyde 13 were generally poor substrates. This reactivity is in accordance with binding mode A which results in the aldehyde 4-aryl substituents directed towards the solvent, 3-aryl substituents slightly oriented towards active site residues and poly-substituted arylacetaldehydes facing major steric challenges with hydrophobic residues (Phe80, Phe99 and Tyr108).

The poor substrate tolerances observed with the benzaldehydes 21, 22, 26, 27 could, according to binding mode A, be due to the aromatic ring being too deeply buried in the enzymatic cavity resulting in unfavourable steric interactions with residues from within the enzymatic pocket. In addition 23 was not accepted and 28 was a very poor substrate, both possessing substitution at the α-position, giving rise to unfavourable steric interactions. Poor substrate acceptance of α-substituted aldehydes has been noted for TfNCS.[24] Binding mode A furthermore supports the observed poor substrate tolerance towards the amines, where metamaraminol 37 was a poorer substrate for CjNCS2 than dopamine 1 or 3-hydroxyphenethylamine 38. In binding mode A, the phenethylamine group in 46 docks into a sterically hindered binding pocket. Analysis of CjNCS2 substrate tolerances from our fluorescent screen therefore strongly support binding mode A. Similar substrate tolerance studies recently reported for TfNCS and other mechanistic studies are also are in accordance with binding mode A.[20,24,25]

The combined analysis of docking (theoretical) and assay (empirical) results has lead to the formulation of a putative mechanism for the catalytic activity of NCS, building upon earlier studies (Figure 5). Initially, binding of the phenethylamine substrate to the catalytic pocket via hydrogen bonding could occur between the 3-hydroxy moiety and Lys122 (~2.5 Å). Anchoring of the amine substrate could be further enhanced by hydrogen bonding of the amine functionality with Asp141 (~2.4 Å). Subsequent entry by the aldehyde substrate into the enzymatic cavity, could be held in place via hydrogen bonding with Glu110 (~2.8 Å). Then Glu110 and Asp141 residues assist the nucleophilic attack of the primary amine onto the aldehyde. Due to their proximity to one another, Glu110 and Asp141 could complex to both amine and aldehyde functions and orientate them into a productive conformation. Asp141 would also enhance the nucleophilicity of the amine functionality by deprotonation of the ammonium cation (predominant at neutral pH). The aminol intermediate resulting from condensation of the amine with the aldehyde subsequently undergoes dehydration to form an iminium ion, stabilised by an electrostatic interaction with Asp141 or potentially Tyr108, highlighted as an important residue in previous work.[22] The putative dehydration would then be promoted by the evacuation of a water molecule via a water channel (Figure 4, b, Figure 5). The formation of the isoquinoline bond could then be assisted by residues Lys122 and Asp141, in a concerted manner: abstraction of the 3-hydroxy proton on 1 by Lys122 would generate a reactive phenolate anion that can cyclise onto the iminium cation (stabilised by Asp141) and assist the electrophilic addition reaction to form the 1,2-hydroquinoline ring (Figure 5, a). The stereoselectivity associated with the cyclisation step
would be attributed to the topology of the active pocket orientating the iminium intermediate for the formation of the (S)-enantiomer. Re- aromatisation of the 1,2-hydroquinoline into the tetrahydroisoquinoline product is mediated by the abstraction of 8α-H (Figure 5, b). A possible candidate for this step is Glu110 in agreement with previous work,[22] which according to docking calculations is 2.2 Å away from 8α-H (see the Supporting Information), and could also be assisted by Tyr108. Following rearomatisation, the tetrahydroisoquinoline product leaves the active site. This proposed mode of action is consistent with the putative role of amino acid residues bearing carboxylates such as Asp141 and Glu110 for the enzymatic catalysis of Pictet–Spengler reactions is further supported by studies on strictosidine synthase, which catalyses the condensation of the aldehyde secologanin with tryptamine.[41] Here residue Glu309 was identified as essential for the Pictet–Spengler condensation. Not only is it involved in holding together the amine and aldehyde residues, but it is also believed to enhance the nucleophilicity of tryptamine and facilitate the final rearomatisation step by the abstraction of proton 2-H.[41,42] Our putative mechanism which complies with previous empirical results, suggests that the catalytic activity observed with NCS is based on the key amino acid residues Glu110, Lys122 and Asp141. However, our docking calculations highlighted many other residues that are likely to be catalytically inactive, but involved in sculpting the topology of the active site (Figure 5).

**Conclusions**

A novel fluorescence assay based on the chemoselective reactivity of fluorescamine for primary amines has been developed. The assay allowed us to map CjNCS2 tolerance for both amine and aldehyde substrates. A limited set of dopamine analogues were tolerated by the enzyme, and highlighted the requirement of a meta-hydroxy moiety in dopamine, consistent with previous reports. In contrast, CjNCS2 showed surprising versatility towards aldehyde substrates. Variously decorated arylacetaldehydes were excellent substrates for CjNCS2, which can also accommodate some aliphatic aldehydes. These results were validated by four successful biotransformations with selected substrates, and stereoselectivities established (>95% ee). Overall these results demonstrated that CjNCS2 has significant potential as a biocatalyst, and can be used to generate novel THIAs in high stereoselectivities.

Examination of the fluorescence assay results combined with docking calculations also leads to the formulation of an alternative catalytic mechanism for NCS, with the aldehyde protruding out of the enzymatic pocket, explaining the versatility towards aldehyde substrates. Our putative mechanism revealed three key enzymatic residues (E110, K122 and D141) that are essential for the catalytic activity of NCS. This, in turn, may help engineer NCS mutants with enhanced activities and substrate tolerances, and provide an essential tool for the regio- and stereoc controlled synthesis of THIAs.

**Experimental Section**

**Heterologous Expression and Purification of CjNCS2**

CjNCS2 (also known as CjPR10 A, accession A2A1A1) was obtained as a synthetic gene with the addition of six C terminal histidine residues from DNA 2.0. The synthetic construct was subcloned into pET29a (Novagen) using NdeI and HindIII, and the resulting construct was used to transform *Escherichia coli* BL21 (DE3) pLysS (Promega). Terrific broth with 50 μg/mL kanamycin was inoculated with 4% overnight culture (grown in terrific broth, 37 °C, 250 rpm) and was incubated for 2 h at 37 °C, shaking at 250 rpm, before lowering the temperature to 30 °C for 1 h. The cultures were then induced with 0.5 mM IPTG and grown for a further 3 h. Cells were harvested by centrifugation (10000× g, 10 min) and lysed using sonication (20×15 s at 10 μ amplitude) before being purified by nickel affinity chromatography. Purified protein was then desalted using a PD10 desalting column (GE Healthcare).

**General Procedure for the CjNCS2-Mediated Synthesis of THIAs**

To a mixture of the amine in water (1 equiv.) and aldehyde in acetonitrile (1.5 equiv.) was added purified CjNCS2 (7.5 mL, 10 U) in HEPES buffer (0.1 M, pH 7.4). The reaction mixture was stirred at 37 °C for 3 h, and subsequently quenched by the addition of HCl (1 M; 1 mL). The crude mixtures were filtered to remove enzyme precipitate and purified by semi-preparative HPLC according to either gradient 1 or gradient 2. Semi-preparative HPLC were performed on a Varian Prostar instrument equipped with an autosampler, a UV-visible detector and a DiscoveryBIO wide Pore C18–10 Supelco column (25×2.12 cm). Elutions were monitored at 280 nm and carried out according to either of the following gradients. **Gradient 1**: 5% to 40% of acetonitrile/water (0.1% TFA) and **gradient 2**: 5% to 90% of acetonitrile/water (0.1% TFA). Fractions containing the desired product were combined and concentrated under reduced pressure. Iterative co-evaporation of the residue with methanol (3×5 mL) allowed the complete removal of any residual TFA and yielded the product as pale yellow to colourless glassy solids.
Synthesis of 3, 43–45 using C/NCS2

Determination of the absolute configuration is described in the Supporting Information via Mosher’s derivatisation.

(15)-1-(4-Hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol ([S]-norcoaurine: (S)-3): Compound (S)-3 was prepared according to the general procedure from dopamine 1 (40 µmol in 400 µL water) and 4-hydroxyphenylacetaldehyde 2 (60 µmol in 120 µL acetonitrile). The product was purified by semi-preparative HPLC using gradient 1 (retention time: 11.8 min) to give (S)-3 as a colourless glassy solid; yield: 10.7 mg (99%). Spectroscopic characterisation data was identical to that previously described and for (S)-3 (see the Supporting Information). [23]

Compound (S)-4 as a colourless glassy solid; yield: 10.3 mg (77%). IR (neat): νmax = 2950 br, 1670, 1598, 1532 cm⁻¹; ¹H NMR (600 MHz; CD3OD): δ = 2.90–3.03 (m, 2H, 4-H), 3.08 (dd, J = 14.4 and 8.5 Hz, 1H, CH2CH), 3.28 (app. quint, J = 6.4 Hz, 1H, 3-HH), 4.65 (dd, J = 8.2 and 6.3 Hz, 1H, 1-H), 6.54 (s, 1H, 8-H), 6.63 (s, 1H, 5-H), 7.25 (d, J = 8.4 Hz, 2H, 2’-H and 3’-H); ¹³C NMR (150 MHz; CD3OD): δ = 25.6, 40.6, 40.8, 57.4, 114.2, 116.2, 122.6, 123.6, 132.6, 133.3, 136.0, 145.8, 147.0; MS (ES+): m/z = 336 [M(HBr)⁺], 334 [MHBr⁺], 30 (50), 317 (52), 273 (100); HR-MS (ESI+): m/z = 334.0443, calcd. for C13H23BrNO₂ [MH⁺]: 334.0433.

Compound (S)-44 (yield: 5.6 mg, 56%) (retention time: 11.1 min) to give (S)-4 as a colourless glassy solid; yield: 4.8 mg (73%). IR (neat): νmax = 3475, 3008, 1671, 1530 cm⁻¹; ¹H NMR (600 MHz; CD3OD): δ = 2.95–3.11 (m, 3H, CH2CH2H), 3.29–3.32 (m, 1H, 4-H), 4.34 (dd, J = 8.9 and 5.8 Hz, 1H, 1-H), 6.65 (d, J = 2.5 Hz, 1H, 5-H), 6.69 (dd, J = 8.5 and 2.5 Hz, 1H, 7-H), 6.81 (d, J = 8.5 Hz, 2H, 2’-H and 3’-H), 7.03 (d, J = 8.5 Hz, 1H, 8-H), 7.13 (d, J = 8.5 Hz, 2H, 2’-H and 6’-H); ¹³C NMR (150 MHz; CD3OD): δ = 26.4, 40.4, 40.6, 58.0, 115.6, 116.0, 126.9, 129.2, 131.7, 133.9, 158.3, 158.5; HR-MS (ESI+): m/z = 256.1342, calcd. for C16H21NO2 [MH⁺]: 256.1338.

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References
