Norcoclaurine Synthase Mediated Stereoselective Synthesis of 1,1'-Disubstituted, Spiro- and Bis-Tetrahydroisoquinoline Alkaloids

Jianxiong Zhao,[‡,1] Daniel Méndez-Sánchez,[‡,1] Rebecca Roddan,[1,2] John M. Ward,[3] and Helen C. Hailes[,1]*

[1]Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK.

[2]Institute for Structural and Molecular Biology, Department of Biological Sciences, Birkbeck, University of London, Malet Street, London, WC1E 8HX, UK.

[3]Department of Biochemical Engineering, University College London, London, Gower Street, WC1E 6BT, UK.

***Corresponding author:** Helen C. Hailes (h.c.hailes@ucl.ac.uk)

[‡]These 2 authors contributed equally

ABSTRACT: The Pictet-Spenglerase norcoclaurine synthase (NCS) catalyzes the formation of (*S*)-norcoclaurine, an important intermediate in the biosynthetic pathway of benzylisoquinoline alkaloids. NCS has been used as a biocatalyst with *meta*-hydroxy phenethylamines and aldehydes for the preparation of single-isomer tetrahydroisoquinoline alkaloids (THIAs). Recently, it was also reported to accept some ketones as substrates including 4-substituted cyclohexanones and phenyl acetones. Here we report the use of wild-type NCS and selected variants with aliphatic, cyclic, α-substituted cyclic, heterocyclic and bicyclic ketones to access challenging non-natural THIAs. Remarkably, fused bicyclic ketones as well as diketones could also be accepted by some of the NCS variants and *in silico* modelling was used to provide insights into a rationale for this.

KEYWORDS: biocatalysis, norcoclaurine synthase, Pictet-Spengler, tetrahydroisoquinoline, *in silico* modelling

INTRODUCTION:

Alkaloids are found primarily in plants and are particularly prevelant in certain families of flowering plants. Tetrahydroisoquinoline alkaloids (THIAs) are a structurally diverse class of compounds, with a history of human use dating back thousands of years.[1] In addition to their prominent role in traditional medicine THIAs have a wide variety of pharmacological applications for example as anti-tussives, anti-microbials and anti-spasmodics.[1-3] Recently preliminary studies have also uncovered new potential applications in treating cancer, malaria, and many other diseases.[2-9] Amongst all THIAs a particularly interesting group are the 1,1'-spiro-tetrahydroisoquinolines, such as the natural products ochotensine **1**[10] and related *N*-oxide **2**,[6] as well as the anti-plasmodial compound **3** (Figure 1).[8] Indeed, when **3** was used in *in vitro* assays against *P. falciparum*, compared to a range of other THIAs the anti-malarial activity significantly increased and it was suggested that this was due to the rigidity in this spiro analogue.[8] The structurally related *Erythrina* class of alkaloids, also spiro-THIAs, has furthermore been used for a plethora of for example ethnomedicine applications.

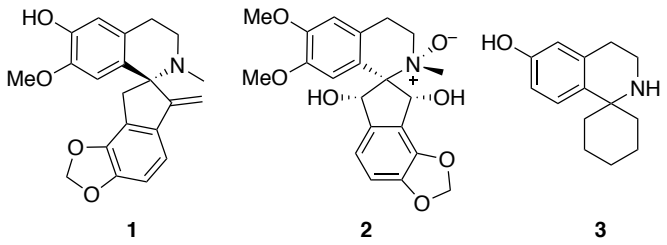
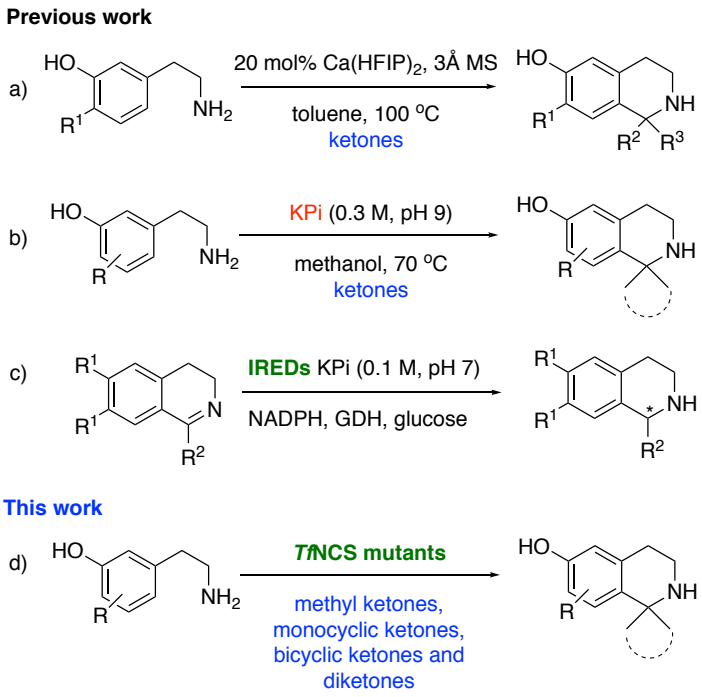


Figure 1. Examples 1,1'-spiro tetrahydroisoquinoline alkaloids **1**-**3**.

Traditionally, many alkaloids such as morphine have been extracted from plant material. However, typically they are present as part of a mixture of components and low extraction yields are obtained by the end of the process.[11] To overcome these limitations, different synthetic strategies have been developed. To date, there are many reported approaches to access THIAs, although the Bischler-Napieralski cyclization/reduction sequence[12] or Pictet

Spengler reactions (PSRs)[13,14] have been most frequently utilized. For asymmetric synthetic strategies, transfer hydrogenation with transition metals and chiral ligands have been successfully employed with a range of substrates for the synthesis of a variety of THIAs.[15,16] Although many of these methods are very useful, the protection of phenolic groups is typically required, reaction efficacy varies as does the stereoselectivities achieved, and the approach can not be used to generate 1,1'-disubstituted THIAs. In order to access these, Brønsted acid or Lewis acid catalysts have been utilised (Scheme 1a) however harsh reaction conditions are normally required.[17-20] More recently, the use of aqueous phosphate media[21] (Scheme 1b) has enabled access to a range of 1,1'-disubstituted THIAs under mild conditions.[22] Here, the main limitation is that racemic products are formed, unless single-isomer chiral substrates are used, which could also lead to the formation of diastereoselective products.



Scheme 1. Non-enzymatic and enzymatic routes to 1-monosubstituted and 1,1'-disubstituted PSRs. Parts (a) and (b) previous routes to 1,1'-disubstituted THIAs, (c) the use of imine reductases to enantiopure THIAs and (d) this work using NCS to 1,1'-disubstituted THIAs.

In order to overcome these issues, the use of biocatalysis has emerged as a powerful tool to generate high value single isomer products. For instance, imine reductases (IREDs) have been used to produce enantiopure THIAs from the corresponding dihydroisoquinoline (Scheme 1c).[23] Pictet-Spenglerases are lyases (EC 4) that have been use as biocatalysts for the stereoselective synthesis of a range of alkaloids.[24-26] The main advantage of this group of enzymes is that no co-factor such as NADPH is required, which reduces the overall cost of processes compared to other biocatalysts that require, for example, glucose dehydrogenase (GDH) redox co-factor recycling systems. The synthesis of functionalised starting materials for enzymes such as IRED is also required.

The Pictet Spenglerase norcoclaurine synthase (NCS) catalyses the PSR between dopamine and 4-hydroxylphenylacetaldehyde (4-HPAA) to form the key compound (*S*)-norcoclaurine, which is then biosynthetically converted into more than 2500 other alkaloids such as morphine. Despite the acceptance of a range of aldehydes by NCS,[25,26,27-32] very few ketones have been tolerated. Previously some α-ketoacid acceptance was described [33] and recently it was reported that several unactivated ketones have been accepted in high yields.[34] For this wild-type (WT) *Thalictrum flavum* NCS (*Tf*NCS) was used together with selected single-point active site mutants, with some variants leading to higher product yields with cyclic ketones (e.g. A79F) while others were more productive towards the methyl ketones (e.g. A79I), and computational substrate docking experiments highlighted putative orientations of the imine intermediates in the active site.[34] *In silico* docking experiments and protein x-ray crystallographic studies have also been performed to understand better the NCS mechanism and role of key active site residues.[32,35,36]

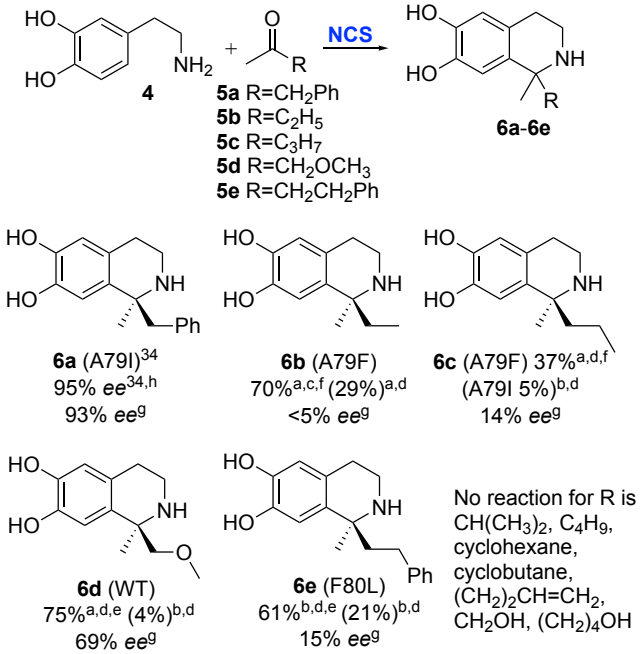
Here, our aim was to fully explore the ketone substrate promiscuity of NCSs to enhance the applications of using this PSR more widely synthetically. As part of this a more effective

method for determining reaction stereoselectivities was required. Remarkably, using a wide range of cyclic, acyclic, fused bicyclic ketones and diketones, THIAs were synthesised and the reaction conditions were optimized and successfully applied to several phenethylamines (Scheme 1d). Importantly, some of the THIAs synthesized also have important bioactivities.

RESULTS AND DISCUSSION:

In previous work it was reported that NCS enzymes could accept phenyl acetones.[34] Initial studies here focussed on NCS reactivities towards dopamine **4** with phenylketone **5a** and aliphatic methyl ketones **5b**-**e** to give THIAs **6a**-**6e**, to understand the structure-activity trends, and also methods were developed to help determine the stereoselectivities of the reactions. While **5a** readily formed **6a** as previously described (87% isolated yield),[34] for methyl ketones **5b**-**e** they were screened against WT-*Tf*NCS and variants A79I, A79F, L76A, L76V, F80L, M79F and Y108F as cell lysates. From this the highest performing variant was identified (Supporting Information Figure S1.1), reaction conditions optimised, including the addition of the antioxidant sodium ascorbate to avoid side-reactions due to the oxidation of catechols,[37] and THIAs generated (Scheme 2). Reactions were monitored to determine conversion yields by HPLC analysis (dopamine depletion or product formation) and/or ^{1}H NMR spectroscopy using an internal standard as indicated, because of the reported challenges of product isolation which frequently lowers the isolated yield: larger scale reactions were also performed for product characterisation purposes.[18,22] It was clear that regardless of the NCS variant used, increasing the length of the alkyl chain decreased the THIA yield. For example with **5b**, **6b** was formed in 70% conversions, while with **5c** the conversion decreased to 37% for **6c**, with the best variants A79F and A79I respectively (Scheme 2). Indeed, no reaction was observed with 3-methylbutan-2-one, longer aliphatic chain methyl ketones and 1-cyclic-methylketones, presumably due to unfavourable steric interactions (Scheme 2, Supporting Information Figure S1.2). Interestingly, a 75% conversion was observed with methoxyacetone **5d** to give **6d**, although problems were encountered during its purification, while hydroxylated methyl ketones were not accepted. Benzylacetone **5e** gave **6e** in conversions of 61%, perhaps reflecting its structural similarity to the natural substrate 4-HPAA. Overall, isolated yields were in the range of 4-29%.

Previously, the stereoselectivity of the 1,1'-disubstituted THIAs produced by NCS were determined by chiral HPLC, compared to racemic standards. The absolute stereoselectivity of the major isomer was also assigned based upon the known selectivity with aldehydes.[34] However, for **6b**-**6e** chiral separations could not be achieved using a range of chiral HPLC columns and methods. Alternative methodologies were therefore investigated using **6a** as the *ee*s had previously been established,[34] including the preparation of Moshers's amide which has been reported with THIAs formed using NCS and aldehydes.[25] However, with the more sterically congested C-1 centre the ^{1}H NMR spectrum of the Mosher's products could not readily be assigned due to the amide rotamers formed. An alternative method to determine *ee*s is the use of Marfey's reagent,[38,39] which avoids the amide rotamer problems and is attached via an S_NAr reaction. Using (*rac*)-**6a**, formed via the recently reported basic potassium phosphate (KPi) reaction conditions,[22] and (1*S*)-**6a** generated using *Tf*NCS (95% *ee* via chiral HPLC analysis[34]) protocols were developed using Marfey's reagent which confirmed an *ee* of 93% (Supporting Information Figure S1.3).



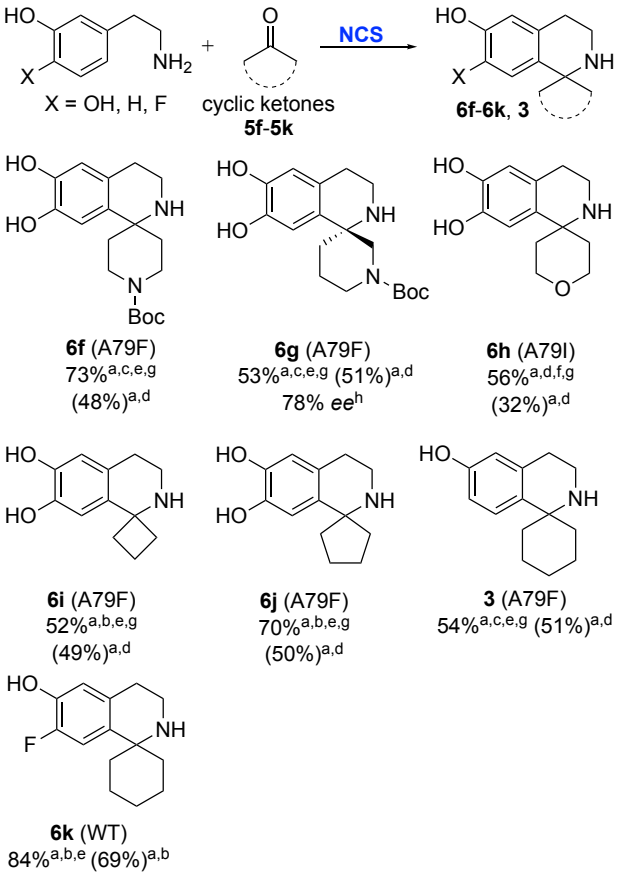
Scheme 2. Use of *Tf*NCSs with methyl ketones **5a**-**5e** to synthesise **6a**-**6e**.

Reaction conditions: Small-scale reactions were performed using **4** (10-15 mM), ascorbate (10-15 mM), methyl ketone (10-150 mM), DMSO (10%) at 37 °C in HEPES buffer (100 mM pH 7.5) using the NCS and time indicated. *NCS*: [a]purified NCS (1.6 mg/mL), [b]NCS lysate (1.7 mg/mL NCS). *Reaction time*: [c]1 day; [d]7 days or more. *Reaction conversions* by: [e]dopamine depletion by HPLC analysis, [f]HPLC analysis against product standards. *Determination of ees*: [g]Marfey's reagent and ^{1}H NMR spectroscopy, [h]chiral HPLC. Isolated yields are given in parenthesis. For further details and larger scale reactions for characterisation purposes see the Supporting Information.

Reaction stereoselectivities were then established using this method for the NCS reactions with the most productive variants towards **5b**-**5e** (Scheme 2) and for this racemic compounds were also synthesised.[22] With 2-butanone **5b**, **6b** was formed in a negligible *ee*, while **5c** with a slightly longer aliphatic chain gave **6c** in a 14% *ee*. Clearly, where similar sized groups are present either side of the carbonyl moiety there is little stereodifferentiation in the active site. The incorporation of the methyl ether in **5d** improved the *ee* in **6d** to 69% reflecting that the impact of possible hydrogen bonding in the side chain on the stereochemistry. Surprisingly benzyl acetone **5e** only gave **6e** in 15% *ee*. Following the stereopreference for the WT and NCS variants with aldehydes the major isomers were assigned as having the (1*S*)-stereochemistry, other than **6d** which due to the Cahn-Ingold-Prelog priority rules was assigned as (1*R*)-**6d**.

Previously it was reported that NCS enzymes could readily accept several 4-substituted cyclohexanones, but we were curious as to whether the presence of different heteroatoms on the cyclohexane ring or different ring sizes could affect the reaction. For the cyclic ketones leading to **11-15**, as before they were screened against WT *Tf*NCS and available variants as lysates to identify the highest performing variant for use in reactions (Supporting Information Figure S1.4). In previous work using the 4-substituted cyclohexanones A79F was noted as a productive variant as was the case here using **4** and 1-*N*-Boc-4-piperidone **5f**: with A79F **6f** was formed, with a large Boc group at the 4-position relative to the carbonyl group, in a conversion yield of 73% after 72 h (48% isolated yield) (Scheme 3). When 1-*N*-Boc-3-piperidone **5g** was reacted with **4**, again with A79F, the conversion to give **6g** after 72 h was

53% (51% isolated yield) and an *ee* of 78% was observed. Following the general stereochemical preference for aldehydes, the major isomer in **6g** due to the Cahn-Ingold-Prelog priority rules was assigned as (1*R*)-**6g**. Despite the good reactivities observed with **5f** and **5g**, the corresponding analogues with *N*-benzyl groups did not seem to be accepted which may have been due to substrate solubility issues (Supporting Information Figure S1.2). Next, tetrahydro-4*H*-pyran-4-one **5h** was investigated with **4** and several variants readily gave **6h** including A79I in 56% conversion yield (32% isolated yield). Alternative ring sizes using **4** with cyclobutanone **5i** and cyclopentanone **5j** gave **6i** and **6j** after 24 h in 52% and 70% conversions respectively using A79F, and both were isolated in ~50% yield. Interestingly, the five membered ring analogue of **5g**, 1-*N*-Boc-3-pyrrolidinone was not accepted or the analogue 1-*N*-benzyl-3-pyrrolidinone (Supporting Information Figure S1.2). In addition to using alternative cyclic ketones, selected substituted phenethylamines were investigated. 2-(3-Hydroxyphenyl)ethylamine and a fluorinated analogue were prepared following reported procedures,[40] and reacted with cyclohexanone **5k** and A79F to give **3** and WT to give **6k**, in 54% and 84% conversion yields (51% and 69% isolated yields) respectively. Compound **3** is particularly interesting as it has been reported to possess good anti-malarial properties compared to other THIAs screened.[8] Overall it was clear that a range of cyclic ketones were accepted by the NCSs and cell free lysates could readily be used for these reactions, however rings possessing bulkier side groups were not tolerated, most likely due to steric factors, so this was explored in more detail with α-substituted cyclohexanones.

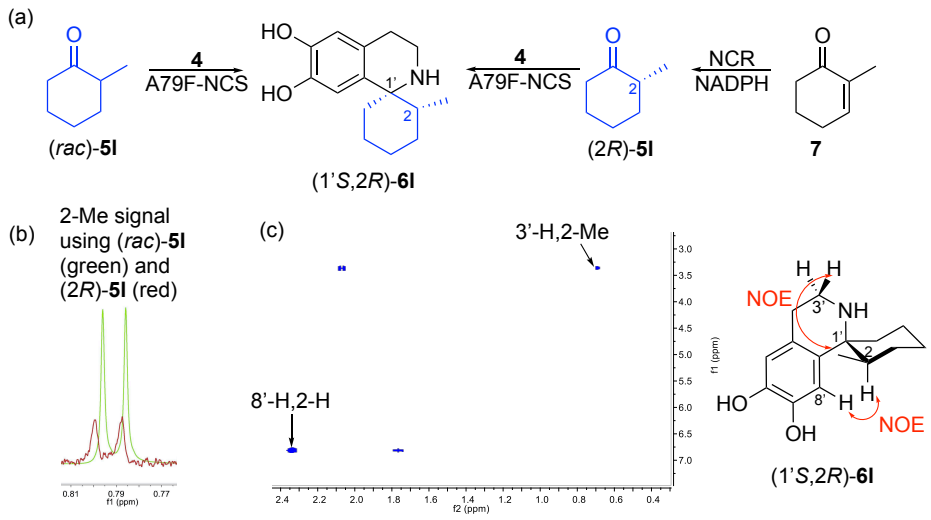


Scheme 3. Use of *Tf*NCSs with cyclic ketones **5f**-**5k** to synthesise **6f**-**6k** and **3**.

Reaction conditions: Small-scale reactions were performed using **4**, 2-(3-hydroxyphenyl)ethylamine or 5-(2-aminoethyl)-2-fluorophenol (10-15 mM), ascorbate (10-15 mM), ketone (10-150 mM), DMSO (10%) at 37 °C in HEPES buffer (100 mM pH 7.5) using the NCS and time indicated. *NCS*: [a]NCS lysate (1.7 mg/mL NCS; for **3**, **6k** 8.5 mg/mL). *Reaction time*: [b]1 day, [c]3 days, [d]7 days. *Reaction conversions* by: [e]amine depletion by HPLC analysis, [f]HPLC analysis against product standards, [g]^{1}H NMR spectroscopy against an internal standard. *Determination of ees*: [h]Marfey's reagent and ^{1}H NMR spectroscopy. Isolated yields are given in parenthesis. For larger scale reactions for characterisation purposes see the Supporting Information.

In previous work it was reported that α-substituted cyclic ketones yielded no products most likely due to steric reasons.[34] However, as α-methyl substituted aldehydes can be accepted by NCS,[32] and the acceptance of α-substituted cyclohexanones by NCS could be useful in developing routes to the erythrina alkaloids, this was explored further. Initial attempts using 2-chlorocyclohexanone lead to substrate degradation and no THIA product was generated. However with 2-methylcyclohexanone **5l** and WT-NCS, THIA formation was noted by LC-

MS. Reactions were optimized, particularly using higher equivalents of ketone, higher enzyme concentrations, longer reactions times, different co-solvents (DMSO gave the highest yields) and enzyme variants. Variants A79I, A79F, Y108F and WT that gave rise to some of the higher conversions after 24 h were used in reactions for longer periods of time leading to improved conversions: a preparative scale reaction for example with *rac*-**5l** (A79F, 7 d) gave **6l** in 15% isolated yield: longer reaction times reported higher conversions of >70% but they were evaluated as unsatisfactory for synthetic applications (Supporting information Figure S1.5). In theory, up to 4 stereoisomers could be generated, but chiral HPLC analysis suggested there was predominantly one diastereoisomer present. To confirm whether one isomer of **5l** was accepted, a 2 step one-pot reaction cascade was developed. Compound (2*R*)-**5l** was prepared from 2-methyl-2-cyclohexen-1-one **7** using the ene-reductase NCR from *Zymomonas mobilis* expressed in *E. coli*[41] (and NADPH and G6PDH co-factor recycling system) and then A79F-*Tf*NCS was added to give **6l** (Scheme 4a). Both products formed using (*rac*)-**5l** and (2*R*)-**5l** gave the same single product by ^{1}H NMR spectroscopy (see the methyl signal region in Scheme 4b). ^{1}H NMR spectroscopic analysis revealed key NOEs between 2-H and 8'-H and 3'-H and 2-CH_3 and since (2*R*)-**5l** was readily accepted by A79F-*Tf*NCS, and the equatorial C-2-methyl substituent is more likely, the stereochemistry of **6l** was assigned as (1'*S*,2*R*), consistent with the NOE data (Scheme 4c) and the *S*-selectivity normally observed with aldehydes and NCS. Interestingly in previous work, (3*R*)-methylcyclohexanone was accepted by A79F-*Tf*NCS to give the (1*R*,3'*R*)-product, but with the methyl group at the 3-position different steric restaints will be acting within the active site.[34]

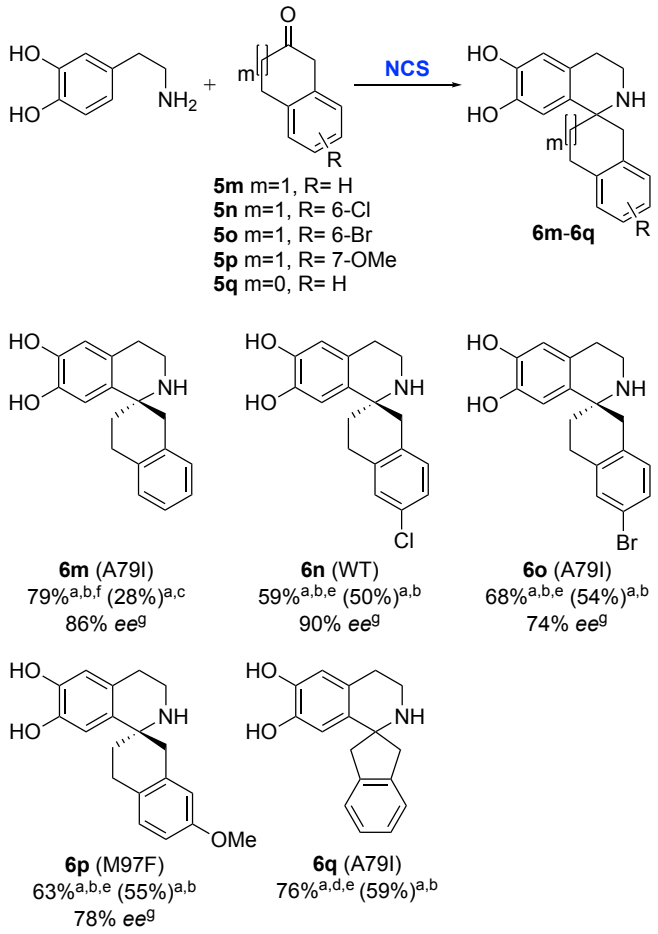


Scheme 4. Use of **5l** and key NMR analyses to establish the stereochemistry of **6l**. (a) A79F-*Tf*NCS catalysed PSR between **4** and (*rac*)- **5l** or (2*R*)-**5l** generated from 2-methyl-2-cyclohexen-1-one and the ene-reductase NCR. (b) C-2 Me signals in the ^{1}H NMR spectra of **6l** starting from (*rac*)-**5l** (green) and (2*R*)-**5l** (red). (c) Key ^{1}H-^{1}H NOESY correlations in **6l** produced from (*rac*)-**5l**.[42]

A computational docking study also suggested that the imine intermediate formed between dopamine and (2*R*)-**5l** was preferred in the active site, compared to (2*S*)-**5l**, with the methyl group adopting an equatorial conformation (Supporting Information Figure S1.6). Since A79 is located at the entrance of the active site, the improved reactivity of A79F towards **5l** could be explained by the bulkier amino acid residue helping to orientate the imine intermediate into a reactive conformation, promoting the cyclization to give **6l**. The acceptance of **5l** by NCS highlights its ability to achieve both enantioselective and stereoselective PSRs, which is chemically challenging to achieve by other methods. In addition to **5l**, 2-ethylcyclohexanone was also used and a small amount of the corresponding THIA was detected by LC-MS but could not be isolated.

Next, more challenging bicyclic ketone substrates were explored affording a family of otchosentine **1**[10] natural product analogues. Again the use of a range of NCS variants was explored and the highest yielding variants used are given in Scheme 5 (also see Supporting Information Figure S1.7). Remarkably, when using β-tetralone **5m**, **6m** was formed in 79%

conversion yield and 86% *ee* using A79I. Using other β-tetralones **5n**-**5p**, good conversions and isolated yields were achieved, together with similar *ee*s. Indeed, 6-substituted-β-tetralones with electron withdrawing chloro- or bromo-groups gave **6n** with WT-NCS and **6o** with A79I in 50% and and 54% isolated yields, respectively. The use of **5p** with an electron donating group at the 7-methoxy-position was also readily accepted, giving **6p** in 55% isolated yield with M97F. The purification of THIAs **6m**-**6p** was readily achieved by acid-base extraction procedures without the requirement for chromatographic purifications, enabling the facile scalability of such approaches. All NCS variants used however failed to accept β-tetralones bearing methoxy groups at C-5 or C-8 (Supporting Information Figure S1.2), presumably due to steric reasons when entering the active site. Assignment of the major enantiomer shown (Scheme 5) was tentatively made, based upon preference for formation of the (*S*)-isomer with aldehydes.[25,26] 2-Indanone **5q** was readily accepted to give **6q** in 76% conversion yield and 59% isolated yield.



Scheme 5. Use of *Tf*NCSs with dopamine and tetralones **5m**-**5p** and indanone **5q** to synthesise spiro-THIA alkaloids.

Reaction conditions: Small-scale reactions were performed using **4**, ascorbate (10-15 mM), ketone **5m**-**5q** (10-150 mM), DMSO (10%) at 37 °C in HEPES buffer (100 mM pH 7.5) using the NCS and time indicated. *NCS*: [a]NCS lysate (1.7 mg/mL: for **6n-q** 8.5 mg/mL). *Reaction time*: [b]1 day, [c]3 days, [d]7 days. *Reaction conversions* by: [e]dopamine depletion by HPLC analysis, [f]HPLC analysis against product standards. *Determination of ees*: [g]Marfey's reagent and ^{1}H NMR spectroscopy (compared to racemic standards). Isolated yields are given in parenthesis. For further details and larger scale reactions for characterisation purposes see the Supporting Information.

To understand better the results with these challenging bicyclic ketones some docking experiments were performed. Here, both the affinity energies and key distances of the five NCS mutants used (A79I, A79F, M97V, F80L, L76V) and the WT-NCS (Supporting information Figures S1.8 and S1.9) with imine intermediates towards **6m** and **6o** were determined. Previous work has described the 'dopamine-first mechanism' where the Pictet-Spengler cyclisation is

triggered by the deprotonation of the (dopamine derived) *meta*-OH by the Lys122 and the crystal structure containing a mimic highlighted this key interaction with bond distances of ~3 Å.[35,36] Notably, with both intermediates modelled (Figure 2), they fitted into the active site of all the structures but A79I gave either the shortest distance (~3 Å) from the *meta*-OH to the Lys122 residue combined with a folded conformation (**6o**) or one of the best binding affinities and a folded conformation (**6m**) with the *meta*-OH to Lys122 approaching ~ 4.0 Å when a weak interaction can occur. Indeed, the modelling with L76V to give **6m** gave a less favourable binding affinity which was consistent with the lower conversions observed. The modelling overall revealed that productive conformations of the imine intermediates can remarkably fit into the WT-NCS active site and that of variants, with the *meta*-OH proximal to Lys122, and the bicyclic system occupying the entrance into the active site, thus highlighting many interesting potential applications of these enzymes.

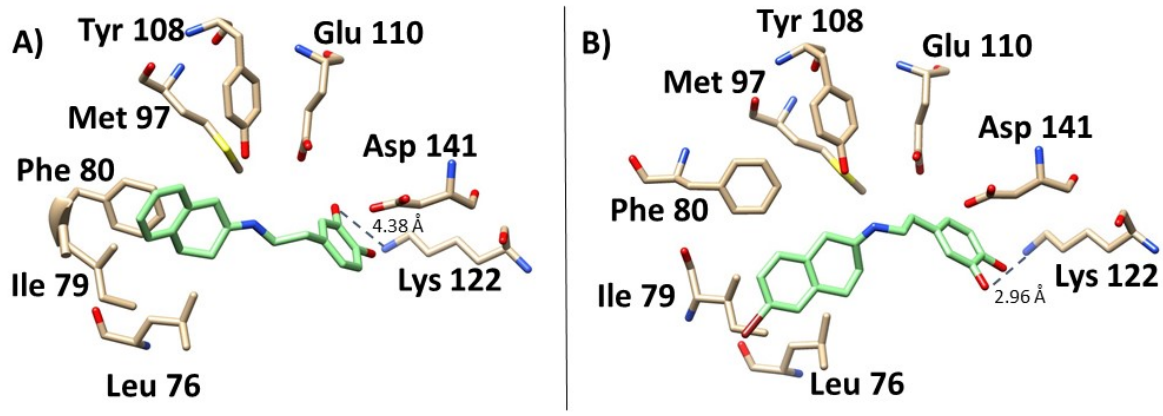
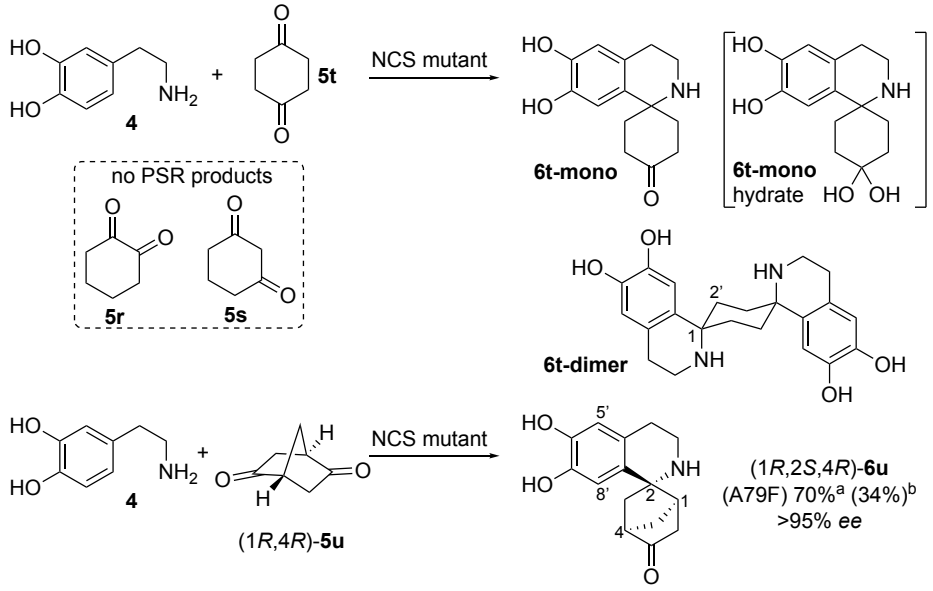


Figure 2. A) Predicted conformation of the imine-β–tetralone intermediate to give **6m** in the A79I-*Tf*NCS (modelled) active site and key residues. B) Predicted conformation of the imine-β–bromotetralone intermediate in the A79I-*Tf*NCS active site to give **6o** (subunit A, 5N8Q). Reaction intermediates were docked in the *Tf*NCS active site (subunit A, 5N8Q) using AUTODOCK VINA[43] and UCSF Chimera.[44] For further details see the Supporting Information.

Finally, it was decided to explore the use of diketones **5r**-**5t**, 1,2-, 1,3- and 1,4-cyclohexanedione respectively to establish whether mono-PSR products could be formed with ketone functionalities for further derivatisation. With **5r** and **5s**, no PSR products were

observed (Supporting Information Figure S1.2). With cyclohexan-1,4-dione, surprisingly, we observed formation of both mono- and a symmetrical di-PSR-product, with the selectivity depending on the NCS variant used (Scheme 6). Moderate conversions were recorded in all cases (20-35%). Notably, WT-*Tf*NCS gave mono-product **6t-mono**: **6t-dimer** in a ratio of 8:1 and **6t-mono** was isolated in 28% yield. Enzyme variants with larger residues at positions towards the entrance to the active site (A79I, A79F) gave similar product ratios of 9:1. However, replacement of the bulky phenylalanine residue with leucine in F80L reversed the selectivity for **6t-mono**: **6t-dimer** to 1:3, enabling **6t-dimer** to be isolated more easily and the structure shown, which was the same with all mutants, was consistent with the spectroscopic data. Both the formation of di-PSR products and changes in such reaction selectivities are unprecedented, and control reactions gave no PSR products. Compound **6t-mono** was also observed to form some of the hydrate by NMR spectroscopy.



Scheme 6. Application of the *Tf*NCS catalysed PSR between dopamine and different di-ketones to synthesise 1,1'-disubstituted and spiro-THIA alkaloids.

Reaction conditions: Reactions were performed using **4** (10-15 mM), ketone **5r**-**5u** (10-150 mM), purified NCS (1.6 mg/mL), sodium ascorbate (1 eq. with respect to **4**), 10% (v/v) DMSO in HEPES buffer (pH 7.5, 100 mM) at 37 °C for 1 day. [a]Conversion yield, [b]isolated yield.

As an alternative diketone (1*R*,4*R*)-**5u** was also used. Up to 70% yields of **6u** were observed after 20 h with the most productive variant A79F (Supporting Information Figure S1.10) and the formation of one isomer was observed. The product **6u** was purified by preparative HPLC and isolated in 34% yield. Based upon the selectivity observed with aldehydes and the α-methyl cyclohexanone, the isomer formed was assigned as (1*R*,2*S*,4*R*)-**6u** with the new (2*S*)-spiro-sterocentre at C-2/C-1'. No disubstituted PSR products were formed with any of the mutants screened, presumably due to steric effects with **5u**.

In order to probe further the results with these diketones, some molecular docking experiments with the **6t-dimer** were carried out. Using WT-*Tf*NCS (subunit A, 5N8Q) **6t-dimer** did not fit into the active site presumably reflecting the preference for the **6t-mono** that was formed. Furthermore, modelling with F80L (homology model) allowed entrance of the imine-diproduct intermediate into the active site which must occur after the first reaction to afford **6t-dimer** (Figure 3A): similar modelling with WT-*Tf*NCS (Figure 3B) revealed no suitable potential binding modes due to long distances between the mechanistically important *meta*-OH and key residue Lys122. This could explain the preference of the WT-*Tf*NCS for **6t-mono** formation. Also, modelled A79I and A79F with the dimer intermediate showed either poorer binding affinities or longer *meta*-OH to Lys122 distances, or both.

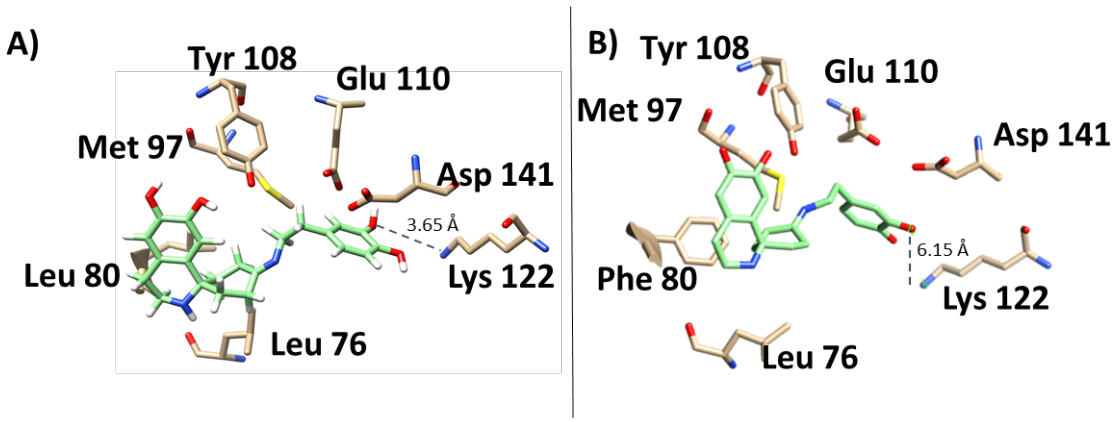


Figure 3. A) Predicted conformation of the imine-diproduct intermediate to give **6t-dimer** in the F80L-*Tf*NCS (modelled) active site and key residues. B) Predicted conformation of the imine-diproduct intermediate to give **6t-dimer** in the WT-*Tf*NCS active site (subunit A, 5N8Q). eaction intermediates were docked in the *Tf*NCS active site (subunit A, 5N8Q) using AUTODOCK VINA[43] and UCSF Chimera.[44] For further details see the Supporting Information.

In summary, the Pictet-Spenglerase norcoclaurine synthase was found to accept a large range of unnatural substrate ketones, to synthesize 1,1'-substituted and spiro-THIAs. Several selected NCS variants, in particular A79I/F, F80L, M97F and Y108F were identified to effectively catalyse the synthesis of more than 20 diverse THIAs from linear aliphatic, α-substituted, cyclic and bicyclic ketones and diketones. The key parameter for reactivity with these substrates is, as well as a folded productive conformation in the active site and good binding affinity, a short distance between the mechanistically important Lys122 and (dopamine or more generally the phenethylamine) *meta*-OH to initiate the reaction. If steric or other factors prevent this key interaction then it is not possible for the reaction to proceed. This study also highlights the high degree of enzyme promiscuity displayed by NCSs and good stereoselectivities that can be achieved. NCS is proving to be a very useful, sustainable catalyst to access 1,1'-substituted and spiro-THIAs which are chemically challenging to synthesize.

AUTHOR INFORMATION

Corresponding Author

*Helen. C. Hailes. Email: h.c.hailes@ucl.ac.uk

Author Contributions

J. Z. and D. M.-S. performed chemical syntheses, chemical characterization, and enzymatic assays and R.R. expressed *Tf*NCS mutants and performed enzymatic assays. The project was supervised by J.M.W and H.C.H. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Funding Sources

This work was funded by a UCL Dean's Prize and UCL-China Scholarship Council Joint Research Scholarship to J. Z. Funding from the Biotechnology and Biological Sciences Research Council (BBSRC) to D. M.-S. (BB/N01877X/1) is gratefully acknowledged. This work was also funded by a Birkbeck Anniversary PhD scholarship to R.R as part of the London Interdisciplinary Doctoral Programme. We also acknowledge 700 MHz NMR equipment support by EPSRC (EP/P020410/1).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

NCS, norcoclaurine synthase; PSR, Pictet-Spengler reaction; THIA, tetrahydroisoquinoline alkaloids; *Tf*, *Thalictrum flavum*; 4-HPAA, 4-hydroxyphenylacetaldehyde; IRED, imine reductase; NMR, nuclear magnetic resonance; *de*, diastereomeric excess; *ee*, enantiomeric excess; GDH, glucose dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; DMSO, dimethylsulfoxide; LC-MS, liquid chromatography-mass spectrometry; WT, wild-type.

SUPPORTING INFORMATION

The supporting information is available free of charge on the ACS Publications website at DOI: xxxx. Experimental methods, supporting Figures and Tables and chemical characterization are given (PDF).

ACKNOWLEDGMENTS

We thank K. Karu (UCL Mass Spectrometry Facility) and A. E. Aliev (UCL NMR Facility) in the Department of Chemistry at UCL.

REFERENCES

1. Dastmalchi, M.; Park, M. R.; Morris, J. S.; Facchini, P. Family portraits: the enzymes behind benzylisoquinoline alkaloid diversity. *Phytochem. Rev.* **2018,** *17*, 249-277, DOI: 10.1007/s11101-017-9519-z.

2. Singh, I. P.; Shah, P. Tetrahydroisoquinolines in therapeutics: a patent review (2010-2015). *Expert Opin. Ther. Pat.* **2017,** *27*, 17-36, DOI: 10.1080/13543776.2017.1236084

3. Hagel, J. M.; Facchini, P. J. Benzylisoquinoline alkaloid metabolism: A century of discovery and a brave new world. *Plant Cell Physiol.* **2013,** *54*, 647-672, DOI: 10.1093/pcp/pct020.

4. Zou, Z. H.; Lan, X. B.; Qian, H.; Huang, W. L.; Li, Y. M. Synthesis and evaluation of furoxan-based nitric oxide-releasing derivatives of tetrahydroisoquinoline as anticancer and multidrug resistance reversal agents. *Bioorg. Med. Chem. Lett.* **2011,** *21*, 5934-5938, DOI: 10.1016/j.bmcl.2011.07.077.

5. Stermitz, F. R.; Lorenz, P.; Tawara, J. N.; Zenewicz, L. A.; Lewis, K. Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Proc. Natl. Acad. Sci. U S A* **2000,** *97*, 1433-1437, DOI: 10.1073/pnas.030540597.

6. Yin, X.; Zhao, F.; Feng, X.; Li, J.; Yang, X.; Zhang, H.; Tu, P.; Chai, X. Four new spirobenzylisoquinoline N-oxide alkaloids from the whole plant of Corydalis hendersonii. *Fitoterapia* **2018,** *128*, 31-35, DOI: 10.1016/j.fitote.2018.04.016.

7. Kashiwada, Y.; Aoshima, A.; Ikeshiro, Y.; Chen, Y. P.; Furukawa, H.; Itoigawa, M.; Fujioka, T.; Mihashi, K.; Cosentino, L. M.; Morris-Natschke, S. L.; Lee, K. H. Anti-HIV benzylisoquinoline alkaloids and flavonoids from the leaves of Nelumbo nucifera, and structure-activity correlations with related alkaloids. *Bioorg. Med. Chem.* **2005,** *13*, 443-448, DOI: 10.1016/j.bmc.2004.10.020.

8. Hanna, J. N.; Ntie-Kang, F.; Kaiser, M.; Brun, R.; Efange, S. M. N. 1-Aryl-1,2,3,4-tetrahydroisoquinolines as potential antimalarials: Synthesis, *in vitro* antiplasmodial activity and in silico pharmacokinetics evaluation. *RSC Adv.* **2014,** *4*, 22856-22865, DOI: 10.1039/c3ra46791k.

9. Scott, J. S.; Bailey, A.; Davies, R. D. M.; Degorce, S. L.; MacFaul, P. A.; Gingell, H.; Moss, T.; Norman, R. A.; Pink, J. H.; Rabow, A. A.; Roberts, B.; Smith, P. D. Tetrahydroisoquinoline Phenols: Selective Estrogen Receptor Downregulator Antagonists with Oral Bioavailability in Rat. *ACS Med. Chem. Lett.* **2016,** *7*, 94-99, DOI: 10.1021/acsmedchemlett.5b00413.

10. Irie, H.; Kishimoto, T.; Uyeo, S. The total synthesis of (±)-ochotensine and related compounds. *J. Chem. Soc. C* **1968,** 3051-3057, DOI: 10.1007/978-1-4757-0937-7_6.

11. Bulduk, I. .; Gezer, B.; Cengiz, M. Optimization of ultrasound-assisted extraction of morphine from capsules of papaver somniferum by response surface methodology. *Int. J. Anal. Chem.* **2015**, 796349, DOI: 10.1155/2015/796349.

12. Bischler, A., Napieralski, B. Zur Kenntniss einer neuen Isochinolinsynthese. *Ber. Dtsch. Chem. Ges.* **1893,** *26*, 1903-1908, DOI: 10.1002/cber.189302602143.

13. Pictet A.; Spengler T. First Discovery of the Pictet-Spengler Reaction. *Berichte der Dtsch. Chem. Gesellschaft* **1911**, *44*, 2030–2036, DOI: 10.1002/cber.19110440309.

14. Horiguchi, Y.; Kodama, H.; Nakamura, M.; Yoshimura, T.; Hanezi, K.; Hamada, H.; Saitoh, T.; Sano, T. A Convenient Synthesis of 1,1-Disubstituted 1,2,3,4-Tetrahydroisoquinolines via Pictet–Spengler Reaction Using Titanium(IV) Isopropoxide and Acetic-Formic Anhydride. *Chem. Pharm. Bull.* **2002,** *50*, 253-257, DOI: 10.1248/cpb.50.253.

15. Luu, H. T.; Wiesler, S.; Frey, G.; Streuff, J. A Titanium(III)-Catalyzed Reductive Umpolung Reaction for the Synthesis of 1,1-Disubstituted Tetrahydroisoquinolines. *Org. Lett.* **2015,** *17*, 2478-2481, DOI: 10.1021/acs.orglett.5b00987.

16. Chrzanowska, M.; Grajewska, A.; Rozwadowska, M. D. Asymmetric Synthesis of Isoquinoline Alkaloids: 2004–2015. *Chem. Rev.* **2016**, *116*, 12369-12465, DOI: 10.1021/acs.chemrev.6b00315.

17 Yokoyama, A.; Ohwada, T.; Shudo, K. Prototype Pictet-Spengler reactions catalyzed by superacids. Involvement of dicationic superelectrophiles. *J. Org. Chem.* **1999,** *64*, 611-617, DOI: 10.1021/jo982019e.

18. Vanden Eynden, M. J.; Kunchithapatham, K.; Stambuli, J. P. Calcium-promoted pictet-spengler reactions of ketones and aldehydes. *J. Org. Chem.* **2010,** *75*, 8542-8549, DOI: 10.1021/jo1019283.

19. Youn, S. W., The Pictet-Spengler reaction: Efficient carbon-carbon bond forming reaction in heterocyclic synthesis. *Org. Prep. and Proced. Int.* **2006,** *38*, 505-591, DOI: 10.1080/00304940609356447.

20. Morita, S.; Ito, T.; Tono, T. Syntheses of isoquinolines by condensation of dopamine with carbonyl compounds. *Agr. Biol. Chem. Tokyo* **1975,** *39*, 547-549, DOI: 10.1080/00021369.1975.10861631.

21. Pesnot, T.; Gershater, M. C.; Ward, J. M.; Hailes, H. C. Phosphate Mediated Biomimetic Synthesis of Tetrahydroisoquinoline Alkaloids. *Chem. Commun.* **2011**, *47*, 3242-3244, DOI: 10.1039/C0CC05282E.

22. Zhao, J.; Mendez-Sanchez, D.; Ward, J. M.; Hailes, H. C. Biomimetic Phosphate-Catalyzed Pictet–Spengler Reaction for the Synthesis of 1,1′-Disubstituted and Spiro-Tetrahydroisoquinoline Alkaloids. *J. Org. Chem.* **2019**, *84*, 7702-7710, DOI: 10.1021/acs.joc.9b00527.

23. Zhu, J.; Tan, H.; Yang, L.; Dai, Z.; Zhu, L.; Ma, H.; Deng, Z.; Tian, Z.; Qu, X. Enantioselective synthesis of 1-Aryl-substituted tetrahydroisoquinolines employing imine reductase. *ACS Catal.* **2017,** *7*, 7003-7007, DOI: 10.1021/acscatal.7b02628.

24. Pressnitz, D.; Fischereder, E. M.; Pletz, J.; Kofler, C.; Hammerer, L.; Hiebler, K.; Lechner, H.; Richter, N.; Eger, E.; Kroutil, W. Asymmetric Synthesis of (R)-1-Alkyl-Substituted Tetrahydro-ß-carbolines Catalyzed by Strictosidine Synthases. *Angew. Chem. Int. Ed. Engl.* **2018,** *57*, 10683-10687, DOI: 10.1002/anie.201803372.

25. Pesnot, T.; Gershater, M. C.; Ward, J. M.; Hailes, H. C. The catalytic potential of *Coptis japonica* NCS2 revealed - Development and utilisation of a fluorescamine-based assay. *Adv. Synth. Catal.* **2012,** *354*, 2997-3008, DOI: 10.1002/adsc.201200641.

26. Roddan, R.; Ward, J. M.; Keep, N. H.; Hailes, H. C. Pictet–Spenglerases in alkaloid biosynthesis: Future applications in biocatalysis. *Curr. Opin. Chem. Biol.* **2020,** *55*, 69-76, DOI: 10.1016/j.cbpa.2019.12.003.

27. Ilari, A.; Franceschini, S.; Bonamore, A.; Arenghi, F.; Botta, B.; Macone, A.; Pasquo, A.; Bellucci, L.; Boffi, A. Structural basis of enzymatic (S)-norcoclaurine biosynthesis. *J. Biol. Chem.* **2009,** *284*, 897-904, DOI: 10.1074/jbc.M803738200.

28. Bonamore, A.; Rovardi, I.; Gasparrini, F.; Baiocco, P.; Barba, M.; Molinaro, C.; Botta, B.; Boffi, A.; Macone, A. An enzymatic, stereoselective synthesis of (S)-norcoclaurine. *Green Chem*. **2010,** *12*, 1623-1627, DOI: 10.1039/c0gc00036a.

29 Panjikar, S.;Stoeckigt, J.; O'Connor, S.; Warzecha, H. The impact of structural biology on alkaloid biosynthesis research. *Nat. Prod. Rep*. 2012, *29*, 1176-1200, DOI: 10.1039/c2np20057k.

30. Bonamore, A.; Calisti, L.; Calcaterra, A.; Ismail, O. H.; Gargano, M.; D'Acquarica, I.; Botta, B.; Boffi, A.; Macone, A. A Novel Enzymatic Strategy for the Synthesis of Substituted Tetrahydroisoquinolines. *ChemistrySelect* **2016,** *1*, 1525-1528, DOI: 10.1002/slct.201600134.

31. Lichman, B. R.; Lamming, E. D.; Pesnot, T.; Smith, J. M.; Hailes, H. C.; Ward, J. M. One-pot triangular chemoenzymatic cascades for the syntheses of chiral alkaloids from dopamine. *Green Chem*. **2015,** *17*, 852-855, DOI: 10.1039/c4gc02325k.

32 Roddan, R.; Gygli, G.; Sula, A.; Méndez-Sánchez, D.; Pleiss, J.; Ward, J. M.; Keep, N. H.; Hailes H. C. Acceptance and Kinetic Resolution of α-Methyl-Substituted Aldehydes by Norcoclaurine Synthases. *ACS catal*. **2019**, *9*, 9640-9649, DOI: 10.1021/acscatal.9b02699.

33 Minami, H.; Dubouzet, E.; Iwasa, K; Sato, F. Functional Analysis of Norcoclaurine Synthase in Coptis japonica. *J. Biol. Chem*. **2007**, *282*, 6274-6282, DOI: 10.1074/jbc.M608933200.

34. Lichman, B. R.; Zhao, J.; Hailes, H. C.; Ward, J. M. Enzyme catalysed Pictet-Spengler formation of chiral 1,1'-disubstituted- and spirotetrahydroisoquinolines. *Nature Commun*. **2017,** *8,* 14883, DOI: 10.1038/ncomms14883.

35. Lichman, B. R.; Gershater, M. C.; Lamming, E. D.; Pesnot, T.; Sula, A.; Keep, N. H.; Hailes, H. C.; Ward, J. M. Dopamine-first mechanism enables the rational engineering of the

norcoclaurine synthase aldehyde activity profile. *FEBS J*. **2015,** *282*, 1137-1151, DOI: 10.1111/febs.13208.

36. Lichman, B. R.; Sula, A.; Pesnot, T.; Hailes, H. C.; Ward, J. M.; Keep, N. H. Structural Evidence for the Dopamine-First Mechanism of Norcoclaurine Synthase. *Biochemistry* **2017,** *56*, 5274-5277, DOI: 10.1021/acs.biochem.7b00769.

37 Zhao, J.; Lichman, B. R.; Ward, J. M.; Hailes, H. C. One-pot chemoenzymatic synthesis of trolline and tetrahydroisoquinoline analogues. *Chem. Commun*. **2018**, *54*, 1323-1326, DOI: 10.1039/c7cc08024g.

38. Marfey, P. Determination of o-amino acids. ii. use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun*. **1984,** *49*, 591-596, DOI: 10.1007/BF02908688.

39. Bhushan, R.; Bruckner, H. Marfey's reagent for chiral amino acid analysis: A review. *Amino Acids* **2004,** *27*, 231-247, DOI: 10.1007/s00726-004-0118-0.

40. Claudi, F.; Cardellini, M.; Cingolani, G. M.; Piergentili, A.; Peruzzi, G.; Balduini, W. Synthesis and dopamine receptor affinities of 2-(4-fluoro-3-hydroxyphenyl)ethylamine and N-substituted derivatives. *J. Med. Chem*. **1990,** *33*, 2408-2412, DOI: 10.1021/jm00171a014.

41. Dobrijevic, D.; Benhamou, L.; Aliev, A. E.; Méndez-Sánchez, D.; Dawson, N.; Baud, D.; Tappertzhofen, N.; Moody, T. S.; Orengo, C. A.; Hailes H. C.; Ward, J. M. Metagenomic ene-reductases for the bioreduction of sterically challenging enones. *RSC Adv*. **2019**, *9*, 36608-36614, DOI: 10.1039/C9RA06088J.

42. Zhao, J, Synthesis of Tetrahydroisoquinoline Alkaloids using Norcoclaurine Synthase and Phosphate Buffer mediated Pictet-Spengler Reactions, PhD thesis, **2020**, University College London.

43. Trott, O.; Olson, A. AutoDock Vina: improving the speed and accuracy of docking with

a new scoring function, efficient optimization andmultithreading. J. *J. Comput. Chem.* **2010**, *31*, 455–461, DOI: 10.1002/jcc.21334.

44. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera-A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612, DOI: 10.1002/jcc.20084.

TABLE OF CONTENTS GRAPHIC