Pictet-Spenglerases in Alkaloid Biosynthesis: Future Applications in Biocatalysis

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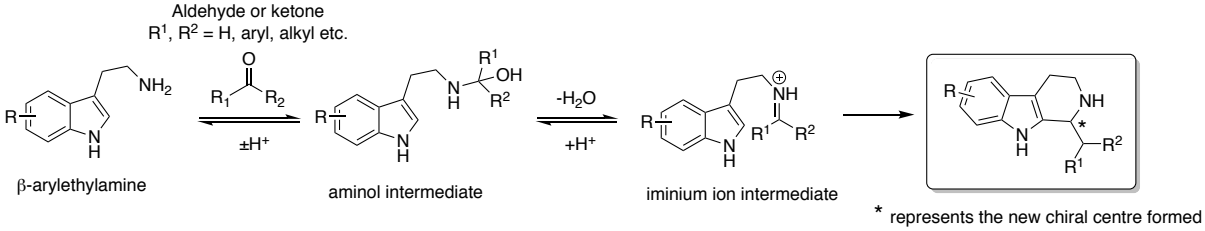
Abbreviations:
THIQ, tetrahydroisoquinoline; NCS, norcoclaurine synthase; BIA, benzylisoquinoline alkaloid; *Tf*, *Thalictrum flavum*; *Cj, Coptis japonica*; NMR, nuclear magnetic resonance; DIS, deacetylipecoside synthase; DIIS, deacetylisoipecoside; STR, strictosidine synthase; MIA, monoterpenoid indole alkaloid, *Rs*, *Rauvolfia serpentia*; *Cr*, *Catharanthus roseus.*

Abstract
Pictet-Spenglerases provide a key role in the biosynthesis of many biologically-active alkaloids. There is increasing use of these biocatalysts as an alternative to traditional organic synthetic methods as they provide stereoselective and regioselective control under mild conditions. Products from these enzymes also contain privileged drug scaffolds (such as tetrahydroisoquinoline or β-carboline moieties), so there is interest in the characterisation and use of these enzymes as versatile biocatalysts to synthesize analogues of the corresponding natural products for drug discovery. This review discusses all known Pictet-Spenglerase enzymes and their applications as biocatalysts.

1.1 Introduction

Alkaloids are a group of naturally-occurring, nitrogen-containing molecules, many of which are pharmacologically relevant. Some widely known examples are quinine (anti-malarial), berberine (anti-bacterial), ergotamine (childbirth) and morphine (analgesic) [1]. Alkaloids have been used for medicinal and recreational purposes for millennia and there continues to be significant interest in isolating novel bioactive compounds from plants. There is also research activity focussed on the enzymes involved in alkaloid biosynthesis to understand their mechanism of action and replicate syntheses *in vitro* or *in vivo*.

The Pictet-Spengler reaction involves an intermolecular cyclisation reaction between a β-arylethylamine and an aldehyde or ketone. Synthetic approaches include the use of an acid or inorganic phosphate catalyst and the reaction proceeds via the condensation of the two substrates to form an iminium ion via an aminol intermediate [2,3]. Intramolecular nucleophilic attack of the aromatic ring onto the iminium ion provides ring closure and generates a stereogenic centre in the product, thus providing a significant increase in product molecular complexity in a single step (Scheme 1).[4]



Scheme 1: The general scheme for the Pictet-Spengler reaction. A β-arylethylamine (e.g. an indole or aryl ring) is condensed with an aldehyde or ketone to give an iminion ion intermediate. An intramolecular cyclisation then occurs to give the product. A new chiral centre is formed in the product if $R^1 \neq R^2$.

The Pictet-Spengler reaction is a key step in the biosynthesis of many alkaloids and a variety of Pictet-Spenglerases (also known as PSases) involved in alkaloid production have been characterised and used in the syntheses of pharmacologically relevant molecules.[5] Pictet-Spenglerases are classified as lyases (EC 4) but there is no subclass dedicated to them. This review article discusses current progress in the identification and characterisation of Pictet-Spenglerases and applications in the synthesis of alkaloids, with a view to highlighting future biocatalytic opportunities.

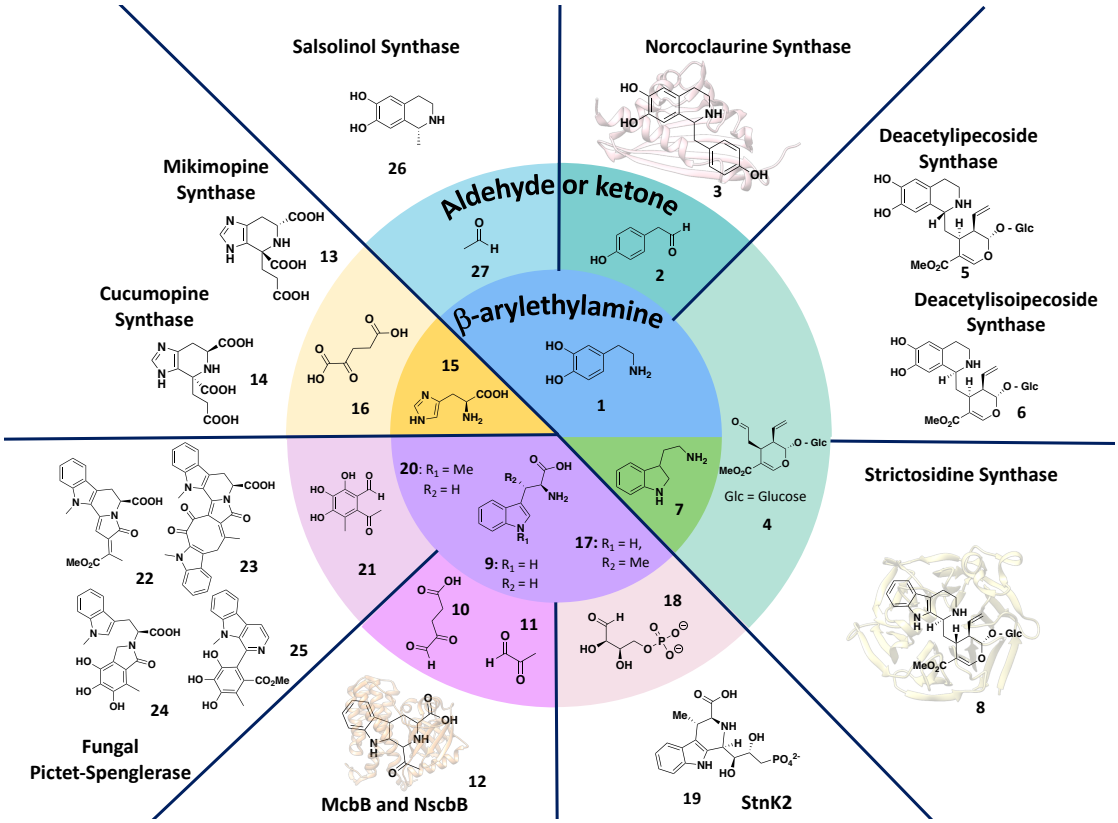


Figure 1: The natural substrate scope of known Pictet-Spenglerases. Each segment corresponds to an enzyme, which condenses a β-arylethylamine and an aldehyde or ketone to give the product. Protein structures, where known, are represented. The structures of NCS (PDB: 5N8Q), strictosidine synthase (PDB: 2FPC) and McbB (PDB: 3X27) are behind **3**, **8** and **12**.

1.2 Plant Pictet-Spenglerases

1.2.1 Norcoclaurine synthase

Norcoclaurine synthase (NCS) performs a stereoselective Pictet-Spengler reaction between two tyrosine-derived molecules, dopamine (**1**) and 4-hydroxyphenylacetaldehyde (**2**) to give (*S*)-norcoclaurine (**3**), the first committed intermediate to benzylisoquinoline alkaloids (BIAs) [6]. Many BIAs are pharmaceutically relevant and widely used, such as the analgesic, morphine and noscapine, used as an anti-tussive [7].

Two NCSs, isolated from *Thalictrum flavum* (*Tf*NCS) and *Coptis japonica* (*Cj*NCS) have been most widely characterised and early work on the enzymes isolated from plants established the native substrates and enzyme kinetics [8,9]. Since then recombinant enzymes have been generated in *E. coli* and a variety of structural studies have been performed with *Tf*NCS. Initially a mechanism was proposed whereby the aldehyde binds first to the active site based upon NMR studies and a co-crystallised structure with a non-substrate benzaldehyde [10,11]. However, this did not account for the diverse aldehyde substrate scope observed and mechanistically an active site residue is required to deprotonate the catechol, for the intramolecular cyclisation with the iminium ion intermediate. More recent computational docking experiments and crystallographic studies, using a non-productive mimic of an advanced reaction intermediate co-crystallised with *Tf*NCS (Figure 2a), provided new

mechanistic insights. These suggested that dopamine binds first to the active site, followed by the aldehyde [12,13]. A subsequent crystal structure and molecular dynamics studies using novel aldehyde substrates, as well as a new computational study have further supported the 'dopamine first' mechanism [13,14]. This mechanism also accounts for the impressive substrate scope observed by *Tf*NCS.

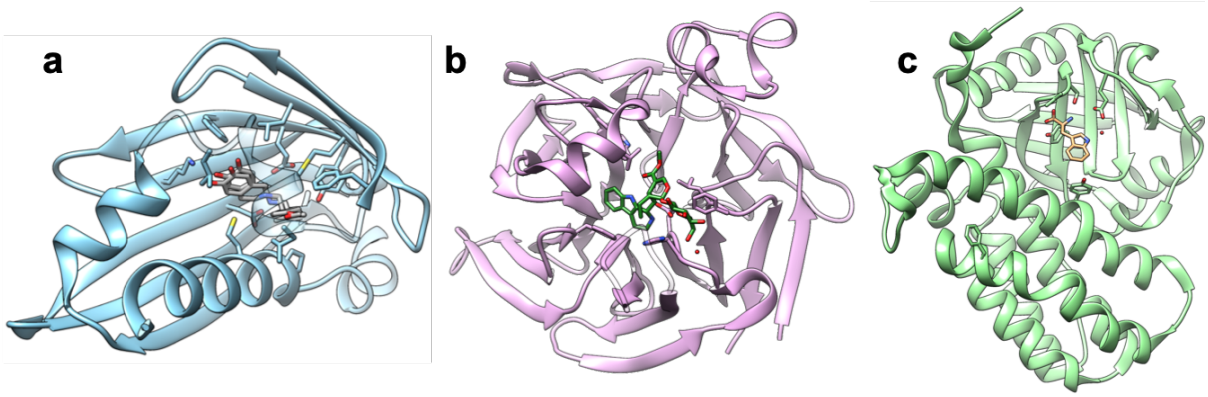
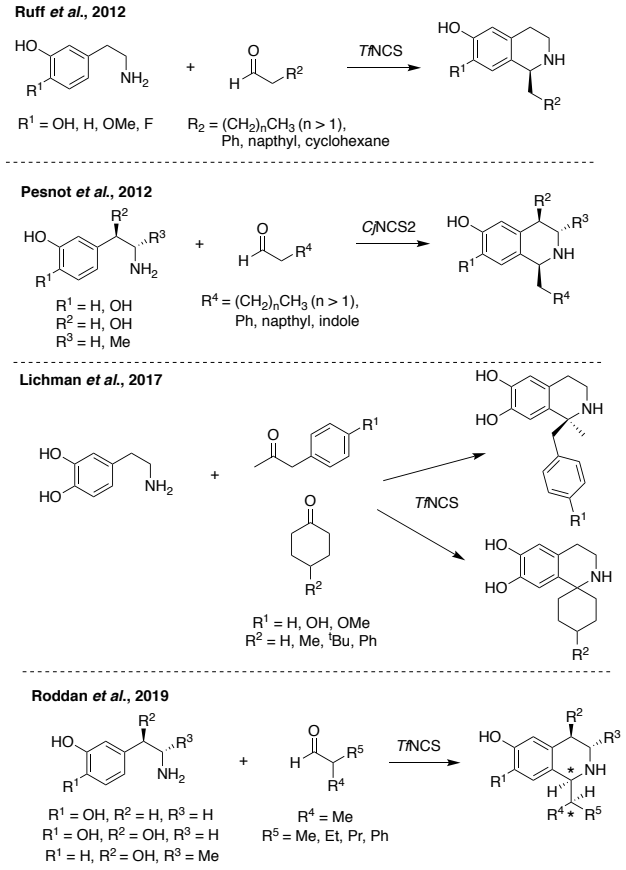


Figure 2: a) Co-crystallised structure of *Tf*NCS with an intermediate analogue in a productive and non-productive binding-mode (PDB: 5NON, chain A). **b)** Co-crystallised structure of *Rs*STR with strictosidine (PDB: 2V91, chain A). **c**) Co-crystallised structure of McBb with L-tryptophan (PDB: 3X27, chain A). For structures key active site residues are represented.

Initial substrate screens showed that a variety of different aldehydes were accepted as substrates but that the amine substrate scope was narrower, with the meta-hydroxyl moiety of dopamine (**1**, figure 1) being essential for a productive reaction [15,16]. Moreover, when chiral α-methyl substituted aldehydes were used, a kinetic resolution of the aldehyde occurred, resulting in the preferential acceptance of the (*R*)-aldehyde by *Tf*NCS, giving (1*S*,1'*R*)-tetrahydroisoquinoline (THIQ) products in high diastereomeric ratios (98:2) using an active site mutant M97V [17]. Remarkably, ketones have also been accepted as substrates, leading to the biocatalytic production of chiral 1,1'-disubstituted and spiro-THIQs [18]. Indeed, a variety of linear aliphatic aldehydes have been accepted and this has been exploited in chemoenzymatic cascades to produce trolline derivatives, (*S*)-benzylisoquinoline and (*S*)-tetrahydroprotoberberine alkaloids [19–21]. A summary of the reported NCS substrate scope is given in scheme 2. *In vivo* pathways incorporating NCS, to the alkaloids (S)-reticuline and thebaine have also been reported in *E. coli* and *S. cerevisiae* [22–25]. This in particular highlights the exciting opportunities of using NCS and variants in the preparation of a significant range of alkaloids.

The substrate scope of *Cj*NCS2 (59% identity, 78% homology to *Tf*NCS) has been explored less extensively. To date, it accepts a range of aldehydes as substrates but not ketones [18]. The key active site residues in *Tf*NCS and *Cj*NCS2 are mostly conserved, other than A(*Cj*)79I(*Tf*) and there is an extra alanine in this loop in *Cj*NCS2. However, the variant A79I in *Tf*NCS does not alter the ketone acceptance observed, suggesting that perhaps the extra alanine in the loop is responsible for the differing activities [18]. NCSs from other plants (such as *Argemone mexicana, Papaver bracteatum* and *Corydalis saxicola*) have also been expressed recombinantly, characterised and found to give THIQ products in high yield with high enantiomeric excesses in the products [26].

NCSs used in biocatalytic applications have been shown to produce tetrahydroisoquinolines with (*S*)-stereochemistry at the C-1 carbon. However, there have been reports of the isolation of (*R*)-norcoclaurine from the seed embryo of the sacred lotus plant, *Nelumbo nucifera* [27–29]. This suggests that the NCS that can either perform an (*R*)-selective reaction or a norcoclaurine epimerase may be present [30–32]. Recently, seven different genes encoding for NCS have also been identified in the sacred lotus genome [33]. Further details are discussed in a comprehensive review on sacred lotus alkaloid biosynthesis [34].



Scheme 2: Selected biocatalytic reactions with NCSs derived from *Thalictrum flavum* (*Tf*NCS) and *Coptis Japonica* (*Cj*NCS2).

1.2.2 Deacetylipecoside synthase

Two different Pictet-Spenglerases have been identified in *Alangium lamarckii* with the ability to condense dopamine (**1**) and secologanin (**4**), a glucosylated monoterpene. Deacetylipecoside synthase (DIS) forms the (*R*)-enantiomer at C-1 to give **5** while deacetylisoipecoside (DIIS) forms the (*S*)-enantiomer in **6**. Both undergo spontaneous lactamization followed by subsequent enzymatic modifications to give alangiside and isoalangiside-type glucosides respectively. DIS has been successfully isolated and purified from *A. lamarckii* and found to be 30 kDa in molecular weight however, DIIS was found to be too labile for purification [35].

1.2.3 Strictosidine synthase

Strictosidine synthase (STR) catalyses the Pictet-Spengler reaction between tryptamine (**7**) and secologanin (**4**) to form 3-α(*S*)-strictosidine (**8**), which is the biosynthetic precursor to monoterpenoid indole alkaloids [36]. There are over 2,000 different monoterpenoid indole

alkaloids (MIAs), many of which have important medicinal activities, such as quinine (anti-malarial), camptothecin (anti-tumour agent) and ajmaline (anti-arrhythmic) [37]. Two STRs have been most extensively characterised, isolated from *Rauvolfia serpentia* (*Rs*STR) and *Catharanthus roseus* (*Cr*STR) [38,39].

The mechanism of action was elucidated by Maresh *et al.* in 2008 [40] A crystal structure of recombinant *Rs*STR co-crystallised in the presence of secologanin and tryptamine was solved (PDB: 2V91). The structure of STR is unusual, being the first example of a β-propeller protein found in the plant kingdom (Figure 2b). Sequence homology to similar structures is low [39]. Mutagenesis identified the key active site residues as E309, Y151 and H307. Kinetic isotope effects and pH dependence of the reaction suggested that formation of the iminium ion intermediate is acid-catalysed and that the final deprotonation step is base-catalysed. *Ab initio* calculations have indicated that the reaction mechanism does not go *via* a spiroindolenine intermediate [40].

The amine donor substrate scope is not limited to tryptamine and screens have been performed with wild-type and mutated STR [41–43]. Analogues with hydroxyl and methoxy groups at C-5 and C-6 respectively of tryptamine were accepted [44]. Activity was also retained when the tryptamine benzene ring was substituted for a pyridine moiety or if the tryptamine pyrrole ring was exchanged for a furan ring [45]. The aldehyde substrate scope has also been altered using active site mutants [43,44] and a recent review discusses the substrate scope in detail [46].

Despite these successes, recombinant expression and isolation of STRs has proven challenging. A recent paper by Pressnitz *et al*. improved the expression and activity of clarified cell lysates 100-fold via optimisation of the expression protocol by using synthetic, codon-optimised genes in *E. coli* and the removal of signal peptides [47]. Interestingly, a variety of non-natural, aliphatic aldehydes have been accepted by four different STRs to give single enantiomer products with (*R*)-stereochemistry at the C-1 position. This led to the enantioselective chemoenzymatic synthesis of (*R*)-harmicine, in an analogous method to the synthesis of trolline-derivatives using *Tf*NCS [47]. STR has also been employed in the stereoselective synthesis of *N*-substituted strictosidine derivatives as novel topoisomerase inhibitors and other alkaloids via chemoenzymatic cascades [42,48]. These examples highlight its potential application in future biocatalytic reactions and cascades.

1.2.4 Putative Pictet-Spenglerase from *Lophocereus schottii*

THIQ alkaloids with an isobutyl group at C-1 (lophocereine) have been isolated from *L. schotti,* a desert cactus [49,50]. No other naturally-occurring THIQs with an aliphatic group at the C-1 position have been identified. Feeding studies have suggested that both leucine and mevalonic acid are precursors to lophocereine. It is however known that leucine is not incorporated *via* mevalonic acid [51,52]. Although the enzymes present in *L. schottii* have not been identified, 3-methylbutanal was also incorporated during feeding studies suggesting that lophocerine may be formed by a Pictet-Spenglerase [53,54].

1.3 Bacterial Pictet-Spenglerases

1.3.1 McbB

The enzyme McbB (isolated from *Marinactinospora thermotolerans*) has been found to perform the Pictet-Spengler reaction between L-tryptophan (**9**) and oxaloacetaldehyde (**10**). Subsequent oxidation and decarboxylation steps give a β-carboline scaffold, found in many pharmacologically active molecules including benzodiazepine inverse agonists [55,56]. The enzyme has been shown to accept the non-natural substrates, 5-methyl-DL-tryptophan, 7-methyl-DL-tryptophan and smaller aldehydes such as methylglyoxal (**11)**, formaldehyde, acetaldehyde, propanal and isobutyraldehyde instead of **10**. The co-crystallised structure of McbB with L-tryptophan was obtained by Mori *et al*. in 2015 (PDB: 3X27). The active site is formed by a homodimerization where each monomer adopts a slightly different conformation. The catalytically important active site residues have been determined and site-directed mutagenesis resulted in the formation of various active mutants. Mutations of two bulky residues, H87A and R72A, located at the entrance of the active site, led to the acceptance of the unnatural aldehyde, phenylglyoxal and condensation with L-tryptophan [56].

1.3.2 NscbB

Using genome mining, a new Pictet-Spenglerase enzyme, NscB, was discovered (identified in *Nocardiopsis synnemataformans*, derived from a kidney transplant patient). This comprehensive bioinformatics analysis using McbB as a search gene identified homologues based on existing microbial genomic data. NscbB catalyses the Pictet-Spengler reaction between L-tryptophan (**9**) and methylglyoxal (**11**) to give 1-acetyl-3-carboxy-β-carboline (**12**) i.e. the same reaction as with McbB. Both enzymes have high sequence identity (66%) and homology (80%) with a conserved active site residue E97 suggesting that that both operate *via* similar reaction mechanisms. NscbB has *ca.* a 30 fold higher k_{cat}/K_M than McbB, but NscbB is less thermally stable [57].

1.3.3 Mikimopine synthase and cucumopine synthase

Mikimopine (**13**) and cucumopine (**14**) are opines, formed via the Pictet-Spengler condensation of histidine (**15**) and α-ketoglutaric acid (**16**). Opines are found in plant tumours induced by the parasite, *Agrobacterium*. The T-DNA encoding for the enzymes responsible for opine biosynthesis are passed to the plant via horizontal gene transfer. Opines are then synthesised by the plant cells and provide carbon and nitrogen sources for the invading bacteria. There has been little characterisation of the two enzymes; however the genes encoding mikimopine synthase (mis) have been isolated from *A. rhizogenes* and both enzymes have been expressed recombinantly in *E. coli* and enzymatic activity confirmed [58–60]. Interestingly, the carbonyl substrate is an α-ketoacid, whereas other Pictet-Spenglerases typically have an aldehyde as the natural substrate.

1.3.4 StnK2

Stnk2 is a Pictet-Spenglerase involved in the biosynthesis of streptonigrin, an alkaloid antibiotic with antitumor activity and it has high sequence identity with McbB (41%). The enzyme performs a stereospecific reaction between (2*S*,3*S*)-β-methyl-tryptophan (**17**) and D-erythrose-4-phosphate (**18**), with the newly formed chiral centre **19** in the (*R*)-configuration. (*S*)-Stereochemistry at C-3 in tryptophan is essential for its reactivity. The aldehyde substrate scope is limited: methylglyoxal and ethyl glyoxalate were not accepted. Interestingly, several fluoro-substituted L-tryptophan analogues were accepted and improved affinity was observed with 5- and 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan compared to the natural

substrate. This is therefore a promising strategy for generating fluorinated analogues of streptonigrin [61].

1.3.5 Non-ribosomal peptide synthetase SfmC (NRPS)

The NRPS SfmC module has been found to perform seven sequential reactions, including two Pictet-Spengler condensation in the biosynthesis of Saframycin C, an anti-tumour antibiotic with a THIQ scaffold [62]. A full discussion of the NRPS SfmC Pictet-Spenglerase reaction is given in a recent review article [63].

1.4 Fungal Pictet- Spenglerases

Comparative genetic analysis has been used to identify a silent Pictet-Spenglerase in the fish-derived fungi, *Chaetomonium globosum*. It was found that 1-methyl-L-tryptophan (**20**) can upregulate the expression of the Pictet-Spenglerase which results in the condensation of 1-methyl-L-tryptophan (**20**) with the aldehyde, flavipin (**21**) . The product is then altered by other fungal enzymes to give a novel class of alkaloids the 'chaetoglines' (**22-25**) and pharmacological activities have been assessed [64]. Two have anti-bacterial activities and another acts as an inhibitor of acetylcholinesterase [65].

1.5 Mammalian Pictet-Spenglerases

1.5.1 Salsolinol synthase

Salsolinol (**26**) has gained significant interest due to links with Parkinson's disease and alcoholism [66]. It is a THIQ alkaloid, formed by the condensation of dopamine (**1**) and acetaldehyde (**27**). Higher levels of (*R*)-salsolinol (**26**) have been found in the human brain, than the (*S*)-enantiomer from chiral HPLC studies [67,68], suggesting that salsolinol is formed enzymatically [69]. An enzyme, isolated from *Rattus norveticus,* has been overexpressed recombinantly in rat PC21 cells and expression correlated with an increased production of salsolinol. Chiral HPLC analysis of isolated salsolinol gave an enantiomeric excess of 20% (*R*-isomer) [70].

1.6 Conclusions

Pictet-Spenglerases are valuable biocatalysts for synthetic applications that can perform the Pictet-Spengler reaction in a stereoselective and regioselective manner under mild reaction conditions. Several Pictet-Spenglerases have been documented in the literature, however only two (NCS and STR) have been widely characterized and used to synthesize a variety of novel alkaloids. The biocatalytic use of other Pictet-Spenglerases has been limited by a narrow substrate scope, challenging isolation methods or poor enzyme stability, however there is significant scope for further investigations. In time, genome mining and bioinformatics techniques are likely to reveal further novel enzymes. Enzyme engineering based upon ligand-bound crystal structures and directed evolution methods are also likely to widen the substrate scope of the known Pictet-Spenglerases. Thus, the applications of recombinant Pictet-Spenglerases *in vitro* or *in vivo* will continue to expand to generate diverse portfolios of alkaloids.

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Keywords
Biocatalysis, Pictet-Spengler, alkaloid, biosynthesis.

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