Article:
Grogan, Gideon James orcid.org/0000-0003-1383-7056, Patil, Mahesh and Yun, Hyungdon (2018) Biocatalyzed C-C bond formation for the production of alkaloids. ChemCatChem. ISSN 1867-3899

https://doi.org/10.1002/cctc.201801130

Heterogeneous & Homogeneous & Bio- & Nano-

CHEMCATCHEM

CATALYSIS

Accepted Article

Title: Biocatalyzed C-C bond formation for the production of alkaloids

Authors: Mahesh D. Patil, Gideon Grogan, and Hyungdon Yun

To be cited as: *ChemCatChem* 10.1002/cctc.201801130

Link to VoR: http://dx.doi.org/10.1002/cctc.201801130

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemcatchem.org

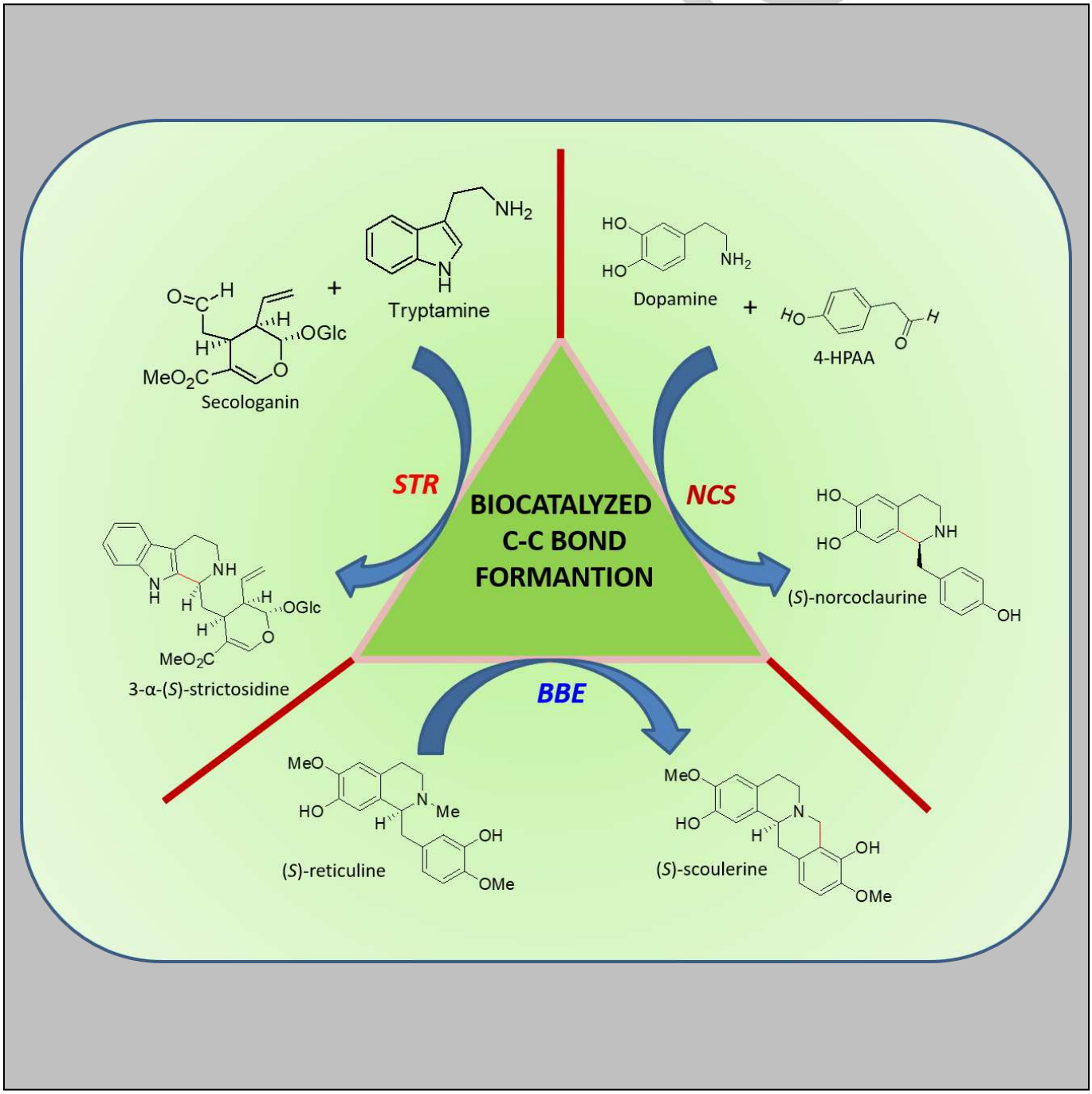
10.1002/cctc.201801130

WILEY-VCH

REVIEW

Biocatalyzed C-C bond formation for the production of alkaloids

Mahesh D. Patil,[a] Gideon Grogan,[b] and Hyungdon Yun*[a]



Abstract: Traditional methods of chemical synthesis of alkaloids exhibit various problems such as lack of enantioselectivity, the use of toxic chemical and intermediates, and multiple numbers of synthetic steps. Consequently, various enzymatic methods for the formation of C-C bonds in the alkaloid skeleton have been developed. Herein, we report advances achieved in the enzymatic or chemo-enzymatic synthesis of pharmaceutically important alkaloids that employ three C-C bond forming enzymes: two Pictet-Spenglerases and the oxidative C-C bond forming flavoenzyme Berberine Bridge Enzyme. Protein engineering studies, improving the substrate scope of these enzymes, and thereby leading to the synthesis of non-natural alkaloids possessing higher or newer pharmacological activities, are also discussed. Furthermore, the integration of these biocatalysts with other enzymes, in multi-enzymatic cascades for the enantioselective synthesis of alkaloids, is also reviewed. Current results suggest that these enzymes hold great promise for the generation of C-C bonds in the selective synthesis of alkaloid compounds possessing diverse pharmacological properties.

1. Introduction

Plant secondary metabolites of the alkaloid class are indispensable constituents of natural products possessing a diverse range of pharmacological activities.[1,2] Alkaloids comprise about 15.6% of known natural products, and constitute almost 50% of pharmaceutically important natural products of plant origin.[3] Of their many important structural types, monoterpene indole [MIAs] and benzylisoquinoline alkaloids are of special interest owing to the diverse array of pharmacological activities exhibited by their members. For instance, the FDA-approved antihypertensive agent reserpine, anti-tumor drugs such vinorelbine, vinblastine and vincristine all belong to the MIA class of alkaloids.[4,5] Similarly, members of the benzylisoquinoline class, atracurium, mivacurium and cisatracurium have been approved as muscle relaxants by the FDA.[4,6]

Owing to their plant origin, alkaloids are not always accumulated at high concentrations. Therefore, isolation and purification of trace amounts of pharmaceutically important alkaloids from their natural hosts is limited by cost factors.[7] Thus, the chemical synthesis of these natural products and their analogues is of special interest. Though significant developments have been achieved in recent years for chemical transformations for the synthesis of alkaloids,[8,9] these methods display numerous disadvantages from the perspective of sustainable chemistry. Furthermore, the highly complex structures of alkaloids, and the presence of multiple chiral centers within the structures, also present challenges to the chemical synthesis of these compounds. It has also been reported that many pharmaceutical companies have given up on the screening of chemically synthesized libraries of natural products as potential drug hits consequent of two reasons, - 'Poor synthetic yields' and 'impracticality of scale-up'.[10-12]

Despite the potential challenges, there is a resurgence of interest in the drug discovery process regarding natural products owing to the unmatched potential of these compounds.[11-13] Moreover, with recent developments in molecular biology, many of the biosynthetic pathways for alkaloid production in plants have been studied at the molecular level.[6] This has enabled the design of various biocatalytic synthetic strategies by employing important enzymes involved in the alkaloid production.[14] Enzymatic strategies reduce the use of hazardous toxic chemical and intermediates, thereby providing a 'greener' alternative to the traditional chemical syntheses.[15-19] Furthermore, a surge in demand for enantiopure pharmaceuticals, owing to the regulations imposed by various regulatory agencies, can be addressed using the exquisite mixture of enantio-, regio- and, chemoselectivity displayed by enzymes.[20,21] Enzymes can also be employed in multi-enzymatic one-pot cascades which can execute the synthesis of complex compounds from cheaper starting materials, most importantly, avoiding the purification of the intermediates.[22-25]

This review focuses on recent studies on the role of C-C bond forming enzymes such as Pictet-Spenglerases *i.e.* Strictosidine synthase (STR) and Norcoclaurine synthase (NCS) and an oxidative C-C bond catalyzing enzyme Berberine bridge enzyme (BBE), in the chemo-enzymatic synthesis of pharmaceutically important alkaloids. The review also takes a closer look at recent advances in the understanding of protein structure and protein engineering, which have been shown to improve the substrate scope of these enzymes, leading to the synthesis of non-natural alkaloids possessing superior or alternative pharmacological activity. The synthetic applications of these biocatalysts along with other enzymes, in multi-enzymatic cascades, are also discussed.

[a] Dr. Mahesh D. Patil, Prof. Dr. Hyungdon Yun*
Department of Systems Biotechnology,
Konkuk University,
Seoul 143-701, Korea
E-mail: hyungdon@konkuk.ac.kr
[b] Prof. Dr. Gideon Grogan
Department of Chemistry,
University of York,
Heslington, York, YO10 5DD, UK

Mahesh D. Patil completed his M. Tech (Pharmaceutical Biotechnology) in 2012 at National Institute of Pharmaceutical Education and Research, Mohali, India under the supervision of Prof. U.C. Banerjee. He continued his research under Prof. Banerjee and completed Ph.D in 2017. Since August 2017, he joined Prof. Hyungdon Yun and currently working as 'Research Professor' at Konkuk University, Seoul. The research work of Dr. Mahesh is mainly focused on biochemical engineering, protein biochemistry and enzymatic synthesis of pharmaceutically important compounds.

Prof. Grogan works on the identification, characterisation and application of new enzymes that have potential applications in biotechnology, including those for the production of chiral pharmaceutical precursors and the bioprocessing of renewable materials. The research of Prof. Grogan is mainly focused on a multidisciplinary approach involving synthetic chemistry, microbiology, molecular biology, and X-ray crystallography, with a view to engineering important enzymes in chiral amine synthesis such as imine reductases, amine oxidases and Cytochromes P450s for altered or improved characteristics.

Prof. Yun's major research is focused on method development of protein engineering based on unnatural amino acid mutagenesis and biotechnological production of chiral compounds, bioactive molecules and bioplastics by using enzyme technology, enzyme engineering, whole-cell biocatalyst and synthetic biology. The specific research interest of Prof. Yun's lab is the process development for enantioselective synthesis of unnatural amino acids, nylon monomers, chiral amines, and other pharmaceutically important compounds .

2. Pictet-Spenglerases

The Pictet-Spengler reaction is the acid-catalyzed electrophilic addition of an iminium ion to a substituted aryl species (Figure 1A) and was first reported in 1911 by Amé Pictet and Theodor Spengler.[26] The Pictet–Spengler reaction consists of two steps: In the first step, an iminium intermediate is generated by the acid catalyzed condensation between the aldehyde and an electronic-rich aromatic amine; In the second step a Mannich-type electrophilic aromatic substitution reaction occurs, in which intra-molecular attack of the activated aromatic ring to the new double bond yields a positively charged intermediate, which is subsequently deprotonated to yield the *β*-carboline products[27-32] (Figure 1B).

The Pictet-Spengler reaction has been utilized for the synthesis of a diverse range of alkaloids and key intermediates thereof, such as β-carboline and isoquinoline derivatives. These alkaloids are the constituents of many important pharmaceuticals. For instance, the β-carboline scaffold is a part of numerous agonists and inverse agonists of benzodiazepine receptor.[28,29,33,34] Benzodiazepine receptors play a pivotal role in the control of important physiological phenomena such as, anxiety,[35] memory learning,[36] and sleep.[37,38] While agonists of Benzodiazepine receptor are clinically used as anticonvulsants and myorelaxants, inverse agonists are used as anxiogenics and samnolytics. Similarly, the isoquinoline scaffold is a constituent of many pharmaceutically important alkaloids, such as the analgesic codeine, the muscle relaxant papaverine, the antibiotic sanguinarine, and the antineoplastic noscapine.[39]

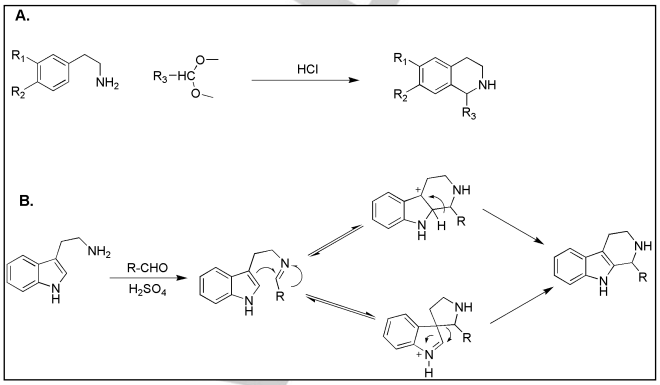


Figure 1. A) Acid catalyzed Pictet-Spengler condensation reaction; and B) Mechanism of the Pictet-Spengler reaction

While significant achievements in the chemical synthesis of alkaloid synthesis using the Pictet–Spengler condensation were achieved in subsequent years, the first enzyme catalyzing the Pictet–Spengler reaction was discovered in 1977.[40,41] Pictet-Spenglerases are unique enzymes *per se* because the chirality in alkaloid products is introduced with the formation of an asymmetric C-C bond.[42-44] The Pictet-Spenglerase group of enzymes consists of four important enzymes: Deacetylipecoside synthase (DAPS), Deacetylisoipecoside synthase (DAIPS), Norcoclaurine synthase (NCS) and Strictosidine synthase (STR).[44,45] While both Deacetylipecoside synthase and Deacetylisoipecoside synthase have not been well characterized, both Norcoclaurine synthase and Strictosidine synthase have been the subject of intensive studies with respect to their roles, structure, mechanism and application, in the synthesis of chemically and pharmaceutically important alkaloids.

2.1. Strictosidine synthase

Strictosidine synthase (STR; E.C. 4.3.3.2) was the first Pictet-Spenglerase to be identified, and was partially purified from *Catharanthus roseus*.[46,47] Although subsequent studies purified the STR in homogenous form from *Catharanthus* cell suspensions, the preparation consisted of isozymes having similar physical and kinetic properties.[48] The STR purified from cell cultures of *Rauvolfia serpentina*, by contrast, was shown to be a single enzyme.[49] STR, the first enzyme of the terpene-indole alkaloid pathway, is known to catalyze a Pictet-Spengler reaction between secologanin and tryptamine to form strictosidine with the 3-α-(*S*)-configuration.[50,51] Isotopic labeling of strictosidine has demonstrated that strictosidine serves as the sole precursor in the biosynthesis of more than 2,000 monoterpene indole alkaloids

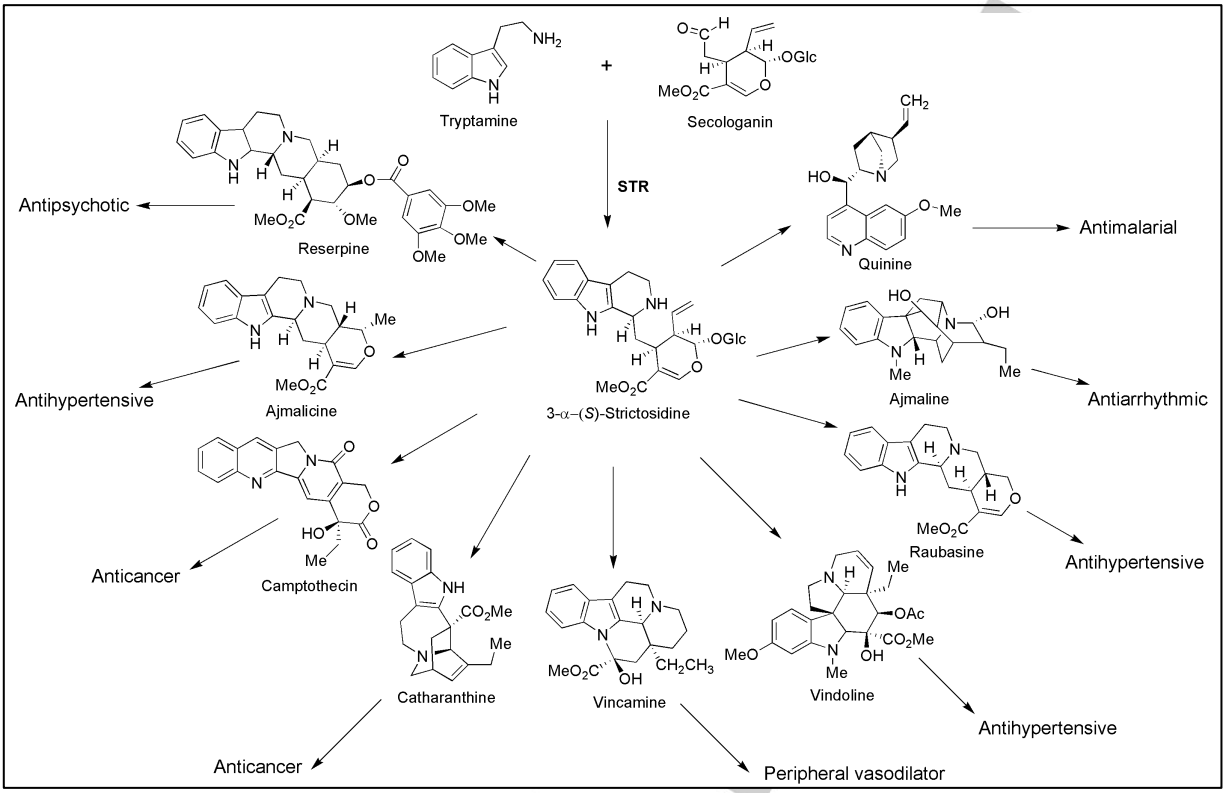


Figure 2. Role of strictosidine as a central precursor in the synthesis of diverse range of pharmacologically important MIAs

[MIAs], a structurally diverse family of natural products that includes pharmaceutically valuable compounds, such as ajmaline, ajmalicine, vinblastine, vincristine, and camptothecin[42,48,52,53] (Figure 2). A taxonomic survey of STR distribution later confirmed the exclusive occurrence of STR in plants producing indole alkaloids.[54]

2.1.1 Structure of STR

The first crystal structure of an STR was obtained for the enzyme from the Indian medicinal plant *R. serpentina* by Ma *et al*.[55] In these studies, the crystal structures of *R. serpentina* STR (STR-*Rs*) and its complex with the natural substrates tryptamine [PDI ID: 2FPB] and secologanin [PDI ID: 2FPC] were reported. The structure of STR-*Rs* was reported to contain a six-bladed four-stranded β-propeller fold and all the six blades are radially arranged around a pseudo six-fold symmetry axis (Figure 3A). Each blade was also reported to contain a twisted four-stranded antiparallel β-sheet. STR-*Rs* also contains three helical segments. The first two helices are located in the loop that connects the two blades (blade-1 and blade-2). The third helix is a part of blade-3, and together with the loop region of blade-5, was presumed to form a cap over the catalytic center of STR.[56]

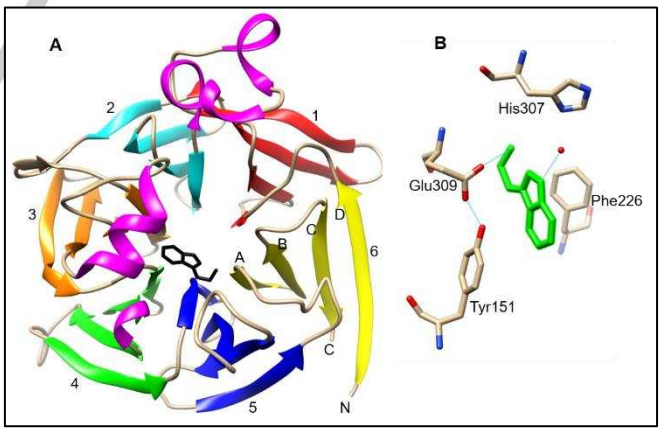


Figure 3. A) Structure of six-bladed β-propeller fold of STR-*Rs* in complex with tryptamine [PDI ID: 2FPB]; and B) The interaction of catalytic residues of STR with tryptamine substrate [55]

These crystal structures provided deeper insights into the architecture of the active site, and the nature of the substrate binding pocket. The active site of STR-*Rs* contains three ionizable residues, Tyr151, His307, and Glu309[55] *(R. serpentina*

numbering) (Figure 3B). To establish the specific role of these residues, a site-directed mutagenesis approach was employed wherein it was found that a change of Tyr151 to phenylalanine did not significantly alter the catalytic activity, implying that the ionizable hydroxyl group does not play a key role in catalysis.[55] Furthermore, the decrease of catalytic activity by 900-fold, following site-directed mutagenesis of Glu309, supported the involvement of this residue in enzyme catalysis.[55] Also, the crystal structure revealed that His307 is involved in the binding to the glucose moiety of secologanin and site-directed mutagenesis of His307 to alanine resulted in a significant increase in the *K*m values for secologanin.[55]

Further *ab initio* and crystallographic studies by Maresh *et al*.[57] provided insight into the nature of the binding site, and the productive transition states involved in the reaction of STR from *C. roseus* (STR-*Cr*; 89% homology to STR-*Rs*). The pH dependence of the solution and enzymatic reactions provided evidence for a sequence of acid-base catalysis steps in the STR-catalyzed reaction. These studies suggested that Glu309 plays the important role of general acid catalyst in STR-mediated catalysis and is responsible for protonation of the carbinolamine to result in iminium formation. Protonated tryptamine subsequently enters the active site, and transfers its proton to Glu309, thereby supplying the proton for acid-catalysis.[57] Also, Glu309 was presumed to be responsible for the final deprotonation step, since no other appropriate residues appeared to perform this role.

The Kinetic Isotope Effect (KIE) of the enzymatic reaction in these studies reported that the primary isotope effect was not observed with [2-^{2}H]-tryptamine and established the fact that the aromaticity is broken during the cyclization step, thus establishing cyclization as the rate-controlling step. Since the Pictet-Spengler reaction is principally an electrophilic aromatic substitution reaction, some non-enzymatic electrophilic aromatic substitution reactions have been reported to exhibit small KIE values,[58] indicating that the final deprotonation step can often be a rate-limiting in non-enzymatic electrophilic aromatic substitution reactions.[57]

2.1.2 Protein engineering of STR for improved substrate scope

Protein engineering approaches are highly promising with respect to expanding the substrate scope, and to produce 'unnatural' natural products that exhibit new or improved biological activities.[59-64] Genes encoding STRs have been successfully isolated from *Rauvolfia serpentina*,[65] *C. roseus*,[66] *O. pumila*[67] and *O. japonica*.[68] With increasing knowledge about the catalytic mechanism and the residues that play important roles in STR-mediated biosynthesis of alkaloids, further efforts were devoted to broadening the substrate scope of the enzymes. STRs have been known to accept the analogs of their natural substrates tryptamine and secologanin.[69] Chen *et al*.[70] engineered an STR-*Cr* overexpressed in *E. coli* BL 21(DE3) and the resultant mutants were screened for their activity towards structural analogs of the natural substrates. These substrate analogs were prepared by modifications at the methyl ester (R_1) and vinyl (R_2) positions of secologanin (Figure 4). The mutant Asp177Ala showed a significant increase in specificity towards the pentynyl secologanin derivative. Another mutant, Asp177Gly, also moderately improved the specificity towards pentynyl secologanin derivative. Also, the mutant Asp177Ala in these studies accommodated the bulkier and more hydrophobic substrate analogs and the ratio of product formation for wild-type strictosidine to strictosidine pentinyl ester was 5.4:1.[70]

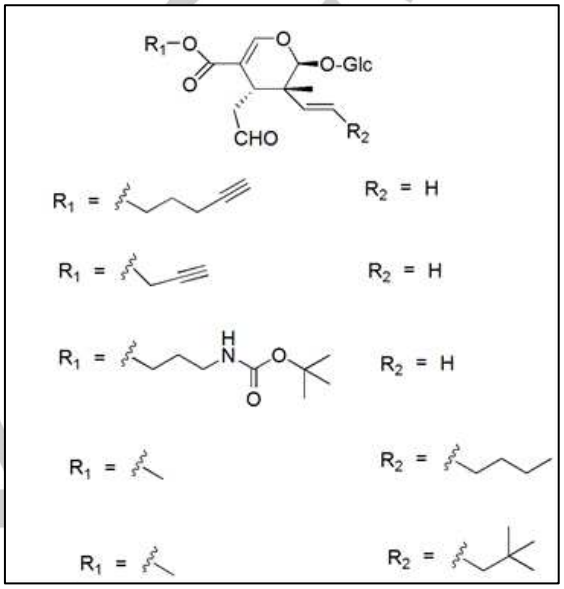


Figure 4. Substrate analogs of secologanin tested for the activity towards STR-*Cr* variants [70]

It has been reported that positions 5 and 6 of tryptamine moiety play a crucial role in the pharmacological activities. However, only the tryptamine derivatives with relatively small substituents such as -fluoro and, -hydroxyl were principally accepted as substrates by STRs. Conversely, substrates with 5-methoxy, 5-methyl or 6-methoxy exhibit much lower, or no transformation by native STR.[71] To modulate the substrate scope of STR-*Rs*, Loris *et al*.[72] carried out rational site-directed mutagenesis studies. In these studies, the bulky amino acid residue Val208, known to shield the steric positions of strictosidine, was replaced with alanine. The resultant mutant Val208Ala could successfully convert 5-methoxy- and 5-methyltryptamine to the corresponding substituted strictosidine in the presence of secologanin. Also, the affinity of 5-hydroxytryptamine was improved towards the mutant, as exhibited by the lower *K*m values for the Val208Ala mutant compared to the wild-type. Kinetic analysis also showed the efficient conversion of 6-methyl and 6-methoxytryptamine by Val208Ala mutant. It is worth emphasizing that the new STR mutant Val208Ala retained its enantioselectivity and thereby the 3α-(*S*) configuration was retained in the new strictosidine analogues obtained by this protein engineering method. Nevertheless, the recent findings by Pressnitz *et al*.[73] reported an unexpected access to (*R*)-tetrahydro-β-carbolines. STR-

catalyzed Pictet-Spengler reaction of tryptamine with small-molecular-weight aliphatic aldehydes enabled their successful transformation to the corresponding 1,2,3,4-tetrahydro-β-carbolines products, importantly with (*R*)-configuration. Furthermore, these studies presented the shortest and highest yielding biocatalytic route till date for the synthesis of natural alkaloid (*R*)-harmicine. STR-*Rs*-catalyzed transformation of commercially available methyl 4-oxobutanoate and tryptamine led to the formation of Pictet-Spengler product, which spontaneously converted to the lactam product (*R*)-1,2,5,6,11,11b-Hexahydro-3*H*-indolizino[8,7-*b*]indol-3-one. Chemical reduction of this lactam produced (*R*)-harmicine with good yield of isolated product (62%) and excellent enantioselectivity (>98%).[73]

Site-saturation mutagenesis strategy has also been applied to expand the substrate scope of STR-*Cr*. The sequence alignment studies between STRs from *C. roseus* and *R. serpentina* suggested that residue Val214 in the STR-*Cr*, important for substrate recognition, was equivalent to the Val208 of STR-*Rs*. Accordingly, saturation mutagenesis was carried out at this position, and a newly developed high-throughput screening method was utilized to detect the activity of newly generated mutants. One of the most important mutations identified by the screening was Val214Met, which allowed the formation of halogenated indole alkaloids, previously unacceptable as substrate by wild-type STR-*Cr*.[74] Owing to the importance of halogens in improving the pharmacological properties of pharmaceutical compounds, the formation of halogenated indole alkaloids provides the important consideration of incorporating a halogen into all MIAs *via* the intermediate strictosidine.[75,76] These studies suggested that engineering of the indole part of the strictosidine molecule can be very useful strategy to generate large libraries of novel indole alkaloid analogs possessing novel biological properties.[72] Recently, a novel Pictet-Spenglerase function of the STR has been reported, which permitted enzymatic synthesis of the less frequently described piperazino [1,2-a]indole scaffold.[77] Owing to the wide range of biological activities of the compounds containing pyrazino- and piperazino[1,2-a]indole scaffolds, the generation of an STR-mediated synthetic method has great potential.

2.1.3 Chemoenzymatic synthesis of alkaloids using STR

The Pictet-Spengler condensation catalyzed by STRs is a very attractive strategy for carbon–carbon bond formation due to its advantages of high stereoselectivity, efficiency and mild reaction conditions.[78,79] Owing to the important pharmaceutical properties of the alkaloids synthesized by STR, research efforts have been devoted towards the chemo-enzymatic synthesis of various derivatives of the alkaloids. In the studies carried out by Zou *et al*.[80] STR-*Rs* was over-expressed in *E. coli* M15 and was immobilized on nickel nitrilotriacetate (Ni-NTA). This immobilized STR was utilized to synthesize the important alkaloid intermediates strictosidine and 12-aza-strictosidine, by enzymatic coupling of secologanin with tryptamine and 7-aza-tryptamine, respectively. These intermediates were further used to explore a possible simple chemoenzymatic synthesis of novel MIAs with molecular diversity and multiple chiral centers, such as nacycline, strictosidine lactam, tetrahydroalstonine, and their aza-analogues (Figure 5). While the lactams were obtained by the conversion of strictosidine and 12-aza-strictosidine under mild basic conditions, Nacycline and its 12-aza-derivative were synthesized using simple acidic catalyzation *i.e.* 2M H_2SO_4. To generate tetrahydroalstonine and its aza analogue, a one-pot chemoenzymatic reaction was initiated using β-glucosidase-catalyzed deglucosylation of strictosidine and 12-aza-strictosidine to form the active intermediate with ring closure. Further reduction of these intermediates and regiospecific electrophilic addition led to the generation of tetrahydroalstonine and its aza analogue.[80] Furthermore, these newly synthesized alkaloids were evaluated *in vitro* for their cytotoxic effects towards the human alveolar adenocarcinoma cell line A549, and it was concluded that newly incorporated nitrogen of the indole moiety renders higher cytotoxicity than the carbon atom at the corresponding positions in newly synthesized alkaloids.[80]

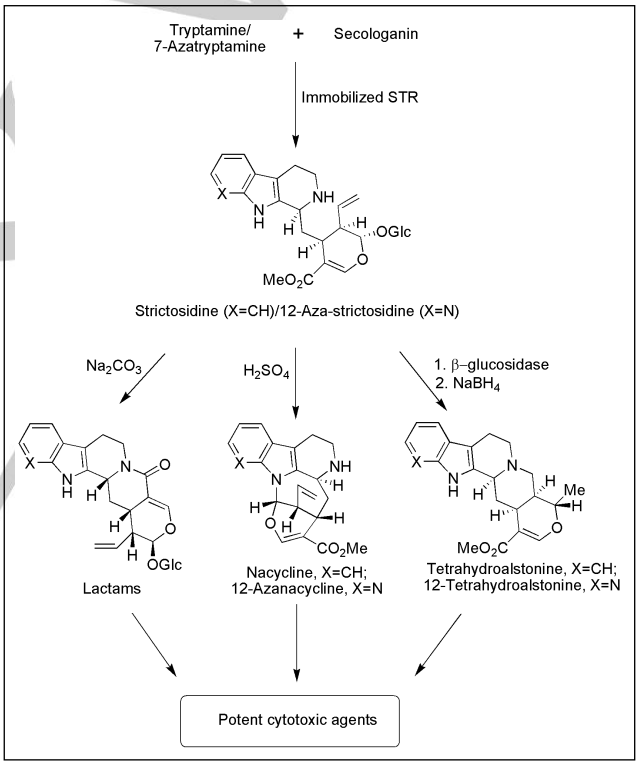


Figure 5. STR-catalyzed chemo-enzymatic synthesis of novel MIAs [80]

Further studies by Bernhardt *et al* demonstrated the first example of aldehyde substrate promiscuity of STR from *Ophiorrhiza pumila* (STR-*Op*) for the Pictet–Spengler condensation of a range of simple achiral aldehydes and substituted tryptamines to form highly enantioselective (*ee* >98%) tetrahydro-β-carbolines.[81] These studies demonstrated that STR-*Op* preferred the aldehyde substrates, however, unsubstituted at the alpha position. Furthermore, the absolute configuration of the enzymatic products was 3-(*S*), which was the same as to that of the natural substrate secologanin. It is

noteworthy that the enantioselectivity of the product formed by the reaction between tryptamine and secologanin was preserved with achiral aldehydes substrates, implying that the enantioselectivity of the product formed does not rely on the chirality of the secologanin substrate.[81]

Most recently, Cai *et al.*[78] reported the enantioselective synthesis of three *N*-substituted (*S*)-3,14,18,19-tetrahydroangustine (THA) derivatives.[78] Previous studies have established the camptothecin and evodiamines as potential Topoisomerase I inhibitors.[82,83] As the structural arrangement of THA derivatives was similar to camptothecin and the evodiamines, it was postulated that the *N*-acylated THA derivatives designed in this study would also exhibit potential antitumor activity. Also, to evaluate the differential effect of different configurations, 3-(*S*)-THAs were prepared by an STR-mediated chemo-enzymatic approach (Figure 6), and their corresponding (*R*)-enantiomers were chemically synthesized using vincosamide (Figure 6; upper panel). The preliminary inhibitory screening studies suggested that the 3-(*S*)-THA derivatives were about 3-fold more potent for both *in vitro* HepG2 cytotoxicity and Top1 inhibition, compared to their corresponding (*R*)-enantiomers.

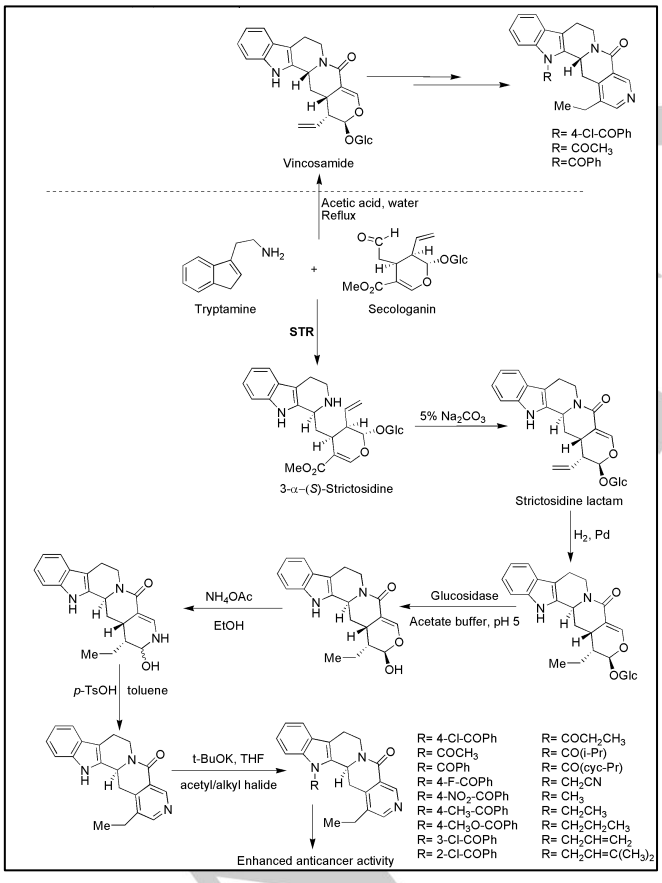


Figure 6. STR-catalyzed chemo-enzymatic synthesis of *N*-acylated THA derivatives [78]

MD simulations of the enantiomers with topoisomerase 1 (PDB ID: 1k4t) suggested that (*R*)-enantiomers were pushed out of the binding pocket of Top1, while in the case of the corresponding *S*-enantiomer, an induced fit was observed with π-stacking interactions that remained strong throughout the simulation. Furthermore, the lack of potency of the (*R*)-enantiomers was correlated to the out-of plane angle between the indole ring and the rest of the ring system. In contrast, the (*S*)-enantiomer was found to be much more planar, facing more empty room in the pocket, thereby allowing for free substitution of the indole nitrogen in (*S*)-enantiomers. This extended flexibility was also proposed as a plausible reason for the higher cytotoxic effects of the (*S*)-enantiomers and their substituents.[78]

Fischereder *et al.* recently demonstrated the formation of (*S*)-strictosidine derivatives possessing an additional stereogenic center at C3 of the tetrahydro-β-carboline core using a biocatalytic cascade involving ω-TA and STR.[84] In these studies, a stereoselective amination of prochiral ketones was performed using ω-TA to generate α-methyltryptamine derivatives. In the second step, a Pictet – Spengler condensation of α-methyltryptamine derivatives with secologanin was catalyzed by STR to form optically pure C3-methyl-substituted strictosidine derivatives. Furthermore, both the enantiomers of α-methyltryptamine derivatives could be synthesized using stereocomplementary ω-TAs, which ultimately allowed the preparation of both the epimers of C3-methyl-substituted strictosidine derivatives.[84] (Figure 7).

2.2 Norcoclaurine synthase

Norcoclaurine synthase (NCS; E.C. 4.2.1.78) catalyzes the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) through the formation of a C–C bond to form (*S*)-norcoclaurine. NCS plays a rate-limiting role in the biosynthesis of benzylisoquinoline alkaloids in plants, as norcoclaurine serves as a precursor for over 5,000 benzylisoquinoline alkaloids (BIQs).[85,86] (Figure 8). Thus, the role of Norcoclaurine synthase in the biosynthesis of benzylisoquinoline alkaloids is comparable to that played by strictosidine synthase in the biosynthesis of MIAs.[86] Owing to the diverse array of BIQs, these alkaloids exhibit a broad range of pharmacological activities, for example, codeine and morphine are used as analgesic, while papaverine is used as a muscle relaxant.[87] (Figure 8).

NCS was originally known as norlaudanosoline synthase because of its ability to convert dopamine and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) to (*S*)-norlaudanosoline.[88] As NCS accepted both the substrates i.e. 4-HPAA or 3,4-DHPAA, it was wrongly concluded that (*S*)-norlaudanosoline serves as a common pathway intermediate in the biosynthesis of benzylisoquinoline alkaloids. However, further detailed studies established that only (*S*)-norcoclaurine was found in plants.[88-90]

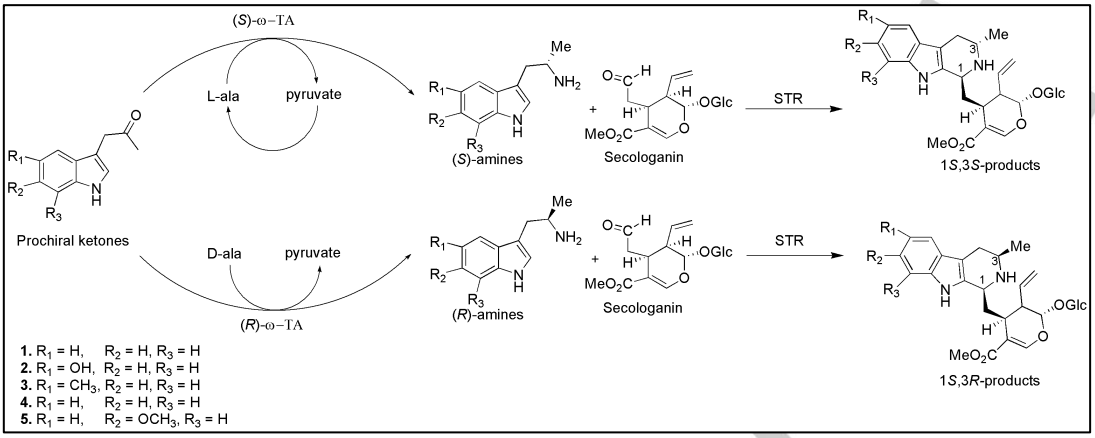


Figure 7. Biocatalytic cascade employing ω-TA and STR for the chemo-enzymatic synthesis of C3-methyl-substituted enantiopure strictosidine derivatives [Reprinted with the permission from Fischereder *et al.*,[84] Copyright 2016, *American Chemical Society*]

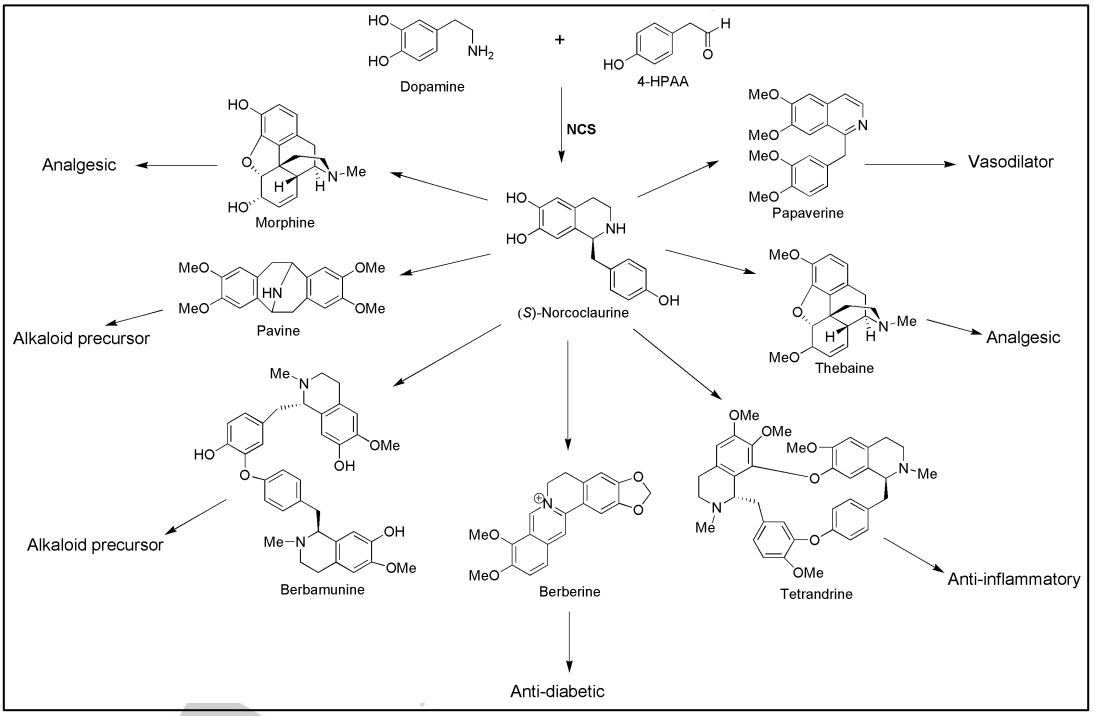


Figure 8. Role of (*S*)-norcoclaurine as a central precursor in the synthesis of diverse range of pharmacologically important benzylisoquinoline alkaloids

2.2.1 Structure, and mechanistic of NCS-catalyzed reaction

The presence of NCS was first detected in plants belonging to three families: the Berberidaceae, Papaveraceae, and Ranunculaceae. In early 1980s, Zenk and co-workers reported the presence of NCS in the cell material of ten plant genera that were unique in producing BIQ-type alkaloids. Furthermore, it was detected that phenylpyruvate and its derivatives, such as 4-hydroxy and 2,4-dihydroxy, do not serve as substrates for NCS.[42,88-92] Further studies by Facchini *et al.* in the next decade gained deeper insights into NCS.[93-95] In their studies, Samanani *et al.* purified NCS 1590-fold from the cell suspension cultures of the medicinal plant *Thalictrum flavum* (Meadowrue), belonging to the family Ranunculaceae and known to accumulate the BIQ alkaloids, such as berberine and magnoflorine.[94] The purified NCS was reported to exist in four isoforms, two major and two minor charge isoforms that showed pI values of 5.5 and 6.2,

respectively.[94] It is notable that kinetic analysis of the product inhibition exhibited uncompetitive binding pattern for 4-HPAA and noncompetitive binding for dopamine, implying that aldehyde substrate in NCS-mediated catalysis binds to the enzyme before its amine counterpart.[94]

Further research by Samanani *et al.*[95] allowed them to isolate a full-length cDNA, containing a 630-bp open-reading frame, from the cell culture library of *T. flavum*. However, the expression of this full-length ORF resulted in very low heterologous expression of the recombinant NCS. Nevertheless, the construct encoding truncation of the first 19 N-terminal amino acids was established later, permitting the production of C-terminal His-tagged polypeptides in *E. coli.*[95] Further optimization of the expression system in *E. coli* allowed the production of 15 mg of homogenous and active protein per liter of bacterial culture.[93]

Subsequently, Minami *et al.* carried out the functional analysis of an NCS as well as a pathogenesis-related homologous protein (PR-10) from *Coptis japonica*.[96] These two enzymes are completely dissimilar to one another; however, both exhibited NCS activity. Substrate specificity studies of NCS from *C. japonica* [NCS-*Cj*] suggested that among the various amine substrates, such as dopamine, tyramine, tryptamine, noradrenaline, 2-phenylethylamine, and 3-hydroxy-4-methoxyphenethylamine, with 4-HPAA as an aldehyde donor, only dopamine was accepted as a substrate and produced the condensation product norcoclaurine.[96] Nevertheless, various aldehydes and carboxylic acids were accepted as substrates, and produced condensation products when reacted with dopamine as an amine substrate. For example, the reactions of dopamine with phenylacetaldehyde, 3,4-dihydroxyphenylacetaldehyde, and 4-HPAA produced 1-benzyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, norlaudanosoline, and norcoclaurine, respectively.[96]

The aim of determining the NMR solution structure of NCS from *T. flavum* [NCS-*Tf*] served as a vehicle to optimize the overexpression, and establish the purification protocol, yielding pure and isotopically labelled protein in sufficient amounts. In studies performed by Berkner *et al.*, two heterologous *E. coli* expression systems had been developed, resulting in truncated His-tagged NCS.[97] The two- and three-dimensional NMR studies and CD spectroscopic studies performed by Berkner *et al.* indicated the high similarity of NCS to a birch pollen allergenic protein, Bet v 1.[98] In these studies, 86% of the amino acid sequence of the truncated NCS was assigned and the homology model was built using the crystal structure of Bet v1 as a template. Chemical shift index (CSI) analysis suggested that not only the secondary structure content of NCS but also the distribution of secondary structure elements was similar to that of Betv1. The NCS has been reported to consist of seven-stranded antiparallel β-sheets wrapped around a long C-terminal helix (α3), and two smaller α-helical segments (α1 and α2). Also, a series of hydrophobic residues and a polar patch located at the entrance of the cavity forms an accessible cleft in each monomer of NCS.[93,99] The homology modelling studies, along with CSI and NMR titration experiments, suggested that while Phe71 can form hydrophobic interactions with the aromatic ring of the dopamine substrate, Met155 was associated with the aliphatic part.[98]

Structural studies of NCS were extended by crystallization and X-ray analysis[100,101] (Figure 9A). These studies suggested that the geometry of the NCS active site is dominated by four important amino acid residues; three strong proton exchangers, Lys122, Asp141, and Glu110, and one hydrogen bonding donor, Tyr108 (Figure 9B).

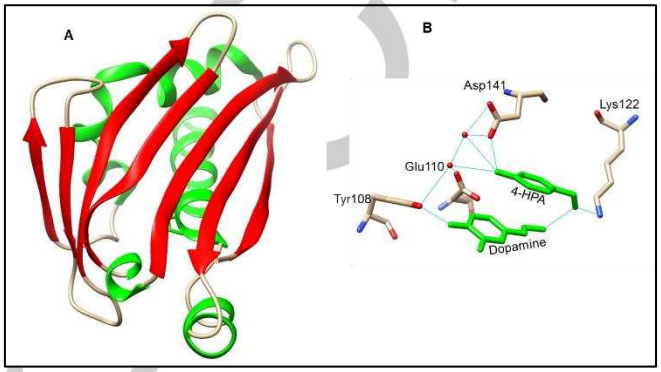


Figure 9. A) Structure of NCS from *T. flavum* [PDI ID: 2VNE]; and B) Interaction of catalytic residues of NCS with dopamine and 4-HPA substrates [100].

To further establish the role of active side residues, this group also generated the site-specific mutants of the catalytically competent amino acid residues.[100] A Lys122Ala mutant was found to be completely inactive, implying that Lys122 is the key catalytic residue. In contrast, replacement of Tyr108 and Glu110 with Phe or Ala, respectively, resulted in diminished NCS activity. It is notable that the catalytic mechanism was proposed based on the arrangement of the catalytic residues and NCS was proposed to adopt the different mechanism to catalyze the Pictet-Spengler cyclization compared to that of STR.[99,100] The mechanism proposed by these studies concluded that the aldehyde substrate (4-HPAA) binds to the NCS prior to dopamine (the 'HPAA-first' mechanism). Subsequently, an alternative mechanism was proposed using computational techniques, wherein dopamine binds to the NCS prior to the aldehyde substrate (the 'dopamine-first' mechanism).[102] Recently, Lichman *et al.*[103] compared the two proposed mechanisms: 'the HPAA-first mechanism' was studied using *holo*-X-ray crystal structure, and 'the dopamine-first mechanism' using computational docking studies. In these studies, 'the HPAA-first mechanism' was proposed based on the arrangement of the substrates in subunit B.[99,100,103] The *holo*-crystal structure in these studies failed to explain the reactivity of NCS towards various aldehyde substrates. Also, the simultaneous incorporation of large aldehydes such as citronellal and amine substrate dopamine in the proposed stacked formation with NCS active site could not be explained.

On the other hand, 'the 'dopamine-first' mechanism' was validated using the docking results of the substitutions at selected amino acid, Tyr108, Glu110, Lys122, and Asp141. The replacement of Lys122 with Leucine resulted in the loss of NCS activity towards 4-HPAA, corroborating the previous results which

suggested that Lys122 plays a crucial role in the rate-limiting cyclization step. Also, the role of Asp141 was established in the general electrostatic stabilization. Furthermore, the dual role of Tyr108, contributing to the electrostatic properties of the active site, but also in defining the shape of the cavity entrance was established.[103] Most recently, the studies by Lichman *et al.*[104] presented new high-resolution X-ray crystallography data describing NCS-*Tf* bound to a mechanism-inspired ligand, which supported the key features of 'the dopamine-first mechanism'.

Recent years have achieved seen significant advances in understanding of enzyme structure and the mechanism of NCS-mediated cyclization of amine and aldehyde substrates. This improved understanding of NCS mechanism will prove useful in the rational engineering approaches to extend the substrate scope for the synthesis of novel alkaloids possessing the '*privileged scaffold*', THIQ, and thereby benzylisoquinolines.[105]

2.2.2 Extended substrate scope and chemo-enzymatic synthesis of benzylisoquinoline alkaloids using NCS

As numerous biologically active substances that act as important pharmaceutical drugs include the THIQ scaffold, they are considered as important secondary metabolites.[106] Also, the product of the reaction catalyzed by NCS, *i.e* (*S*)-norcoclaurine, is a potent alternative to dobutamine, the best current β1-adrenergic drug. However, the clinical development of (*S*)-norcoclaurine is limited by its low availability in Nature and further by the expensive stereoselective methods required for synthesis. Although the catalytic mechanism of NCS-mediated condensation has been established, the strategy for the multigram synthesis of (*S*)-norcoclaurine was unavailable until recently. Also, the previous reactions for the synthesis of (*S*)-norcoclaurine have been reported to suffer from insufficient yields and higher production of byproducts. In 2010, Bonamore *et al.*[107] reported the first successful example of a green, enzymatic synthesis of the key precursor of benzylisoquinoline alkaloids (*S*)-norcoclaurine. In those studies, (*S*)-norcoclaurine was synthesized from the cheap tyrosine and dopamine substrates in a one-pot, two-step process employing NCS-*Tf.* As aldehyde substrate 4-HPAA is highly unstable in aqueous solution and commercially unavailable, it was synthesized by the oxidative decarboxylation of tyrosine in the presence of an equimolar amount of hypochlorite, and was immediately used in the enzymatic reaction.[107] The subsequent addition of dopamine substrate and the use of multipurpose activated charcoal afforded the extraction of (*S*)-norcoclaurine with high yield (80%) with excellent enantioselectivity (93% *ee*) (Figure 10).

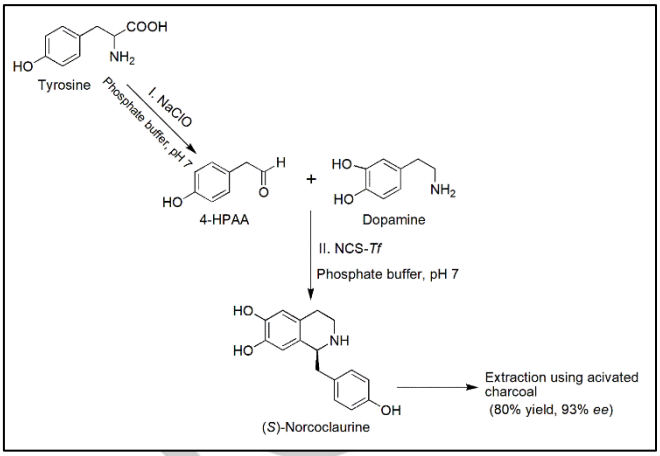


Figure 10. One-pot chemo-enzymatic synthesis of (*S*)-norcoclaurine from tyrosine and dopamine [Adapted with permission from Bonamore *et al.*[107] Copyright 2010, *The Royal Society of Chemistry*]

To develop a biocatalytic strategy for the synthesis of various THIQ derivatives, Ruff *at al.*[108] described the substrate scope of NCS-*Tf*. NCS-*Tf* was found to successfully catalyze the reaction between normal amine substrate dopamine, and various aldehyde substrates, such as acetaldehydes containing more than 3 carbon atoms and those unsubstituted at the α-position (Figure 11). Also, aromatic acetaldehydes were efficiently converted to their respective products with higher rates compared to the aliphatic compounds. This substrate flexibility exhibited by NCS-*Tf* suggested an efficient enzymatic method for the synthesis of a diverse array of THIQ derivatives, such as 1-(*ortho*-, *meta*- and *para*-substituted benzyl)- THIQs.[108]

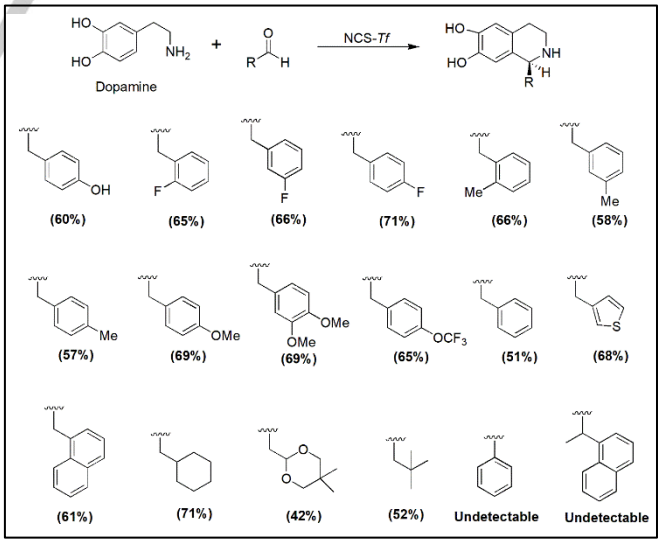
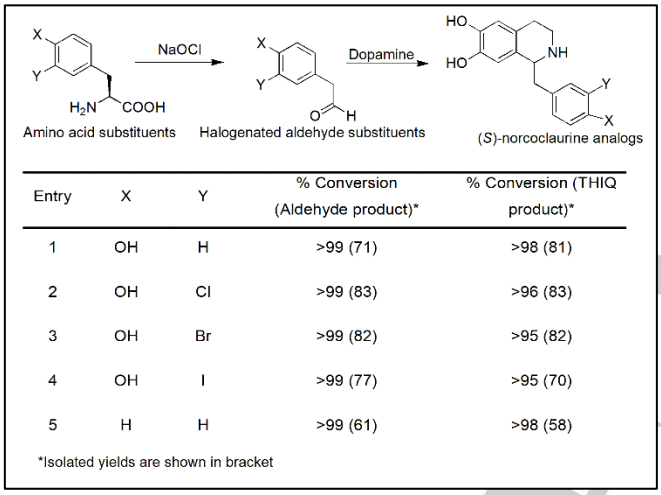


Figure 11. Aldehyde substrate specificity of NCS-*Tf* [108] (% conversion to corresponding products is given in parenthesis)

Several other 1-substituted-1,2,3,4-THIQs were also stereoselectively synthesized using NCS-*Cj*.[109] NCS-*Cj* proved to be a promising biocatalyst that could synthesize 6,7-dihydroxy-1-phenethyl-1,2,3,4-THIQ and 6,7-dihydroxy- 1-propyl-1,2,3,4-THIQ with higher molar yields (86.0 and 99.6%, respectively) with excellent enantioselectivities of 95.3 and 98.0% ee, respectively.[109]

Recently, Maresh *et al.* reported the generation of halogenated 4-HPAA and norcoclaurine from amino acids in aqueous media, avoiding the use of toxic reagents. Furthermore, these halogenated aldehyde substrates could be utilized for the enzymatic synthesis of enantioselective (*S*)-norcoclaurine analogs.[110] It is worth mentioning that these studies extensively expanded the substrate scope of NCS and proposed for the first time that halogenated substituents of the natural phenol substrate can be accepted by NCS (Figure 12).



Entry	X	Y	% Conversion (Aldehyde product)*	% Conversion (THIQ product)*
1	OH	H	>99 (71)	>98 (81)
2	OH	Cl	>99 (83)	>96 (83)
3	OH	Br	>99 (82)	>95 (82)
4	OH	I	>99 (77)	>95 (70)
5	H	H	>99 (61)	>98 (58)

*Isolated yields are shown in bracket

Figure 12. The stereospecific chemo-enzymatic synthesis of halogenated derivatives of (*S*)-norcoclaurine [110]

Lichman *et al.* in 2015 reported a one-pot two-enzyme '*triangular'* cascade for the synthesis of (*S*)-norlaudanosoline, an endogenous BIA precursor for morphine, using the enzymes transaminase and NCS. Furthermore, the subsequent addition of formaldehyde triggered the second Pictet-Spengler reaction to form (*S*)-10,11-dihyroxy tetrahydroprotoberberine[111] (Figure 13). In a preparative scale synthesis, dopamine substrate was converted to (*S*)-norlaudanosoline with 86% conversion (62% isolated yield; >95% *ee*). Subsequently, (*S*)-10,11-dihyroxy tetrahydroprotoberberine was formed with 47% conversion (42% isolated yield; >95% *ee*).[111]

Recently, Bonamore *et al.*[112] developed a fully enzymatic strategy for the synthesis of substituted THIQs. The first step consisted of the transformation of a variety of aliphatic and aromatic amines to the corresponding aldehydes by diamine oxidase enzyme from *Lathyrus cicera*. In the subsequent step, aldehyde obtained in the first step was mixed with the dopamine to yield (*S*)-configured THIQs using NCS-*Tf*. Notably, excellent conversion (95-98%), yields (72-83%) and enantiopurity (> 99% *ee*) could be achieved for all the products formed by this strategy (Figure 14).

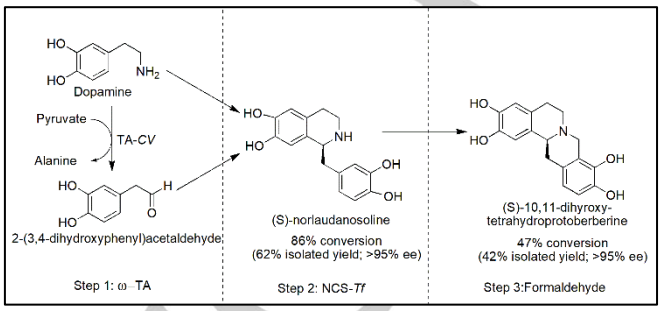


Figure 13. The biocatalytic 'triangular' cascade employing ω-TA-*Cv* and NCS-*Tf* for the synthesis of (*S*)-norlaudanosoline [Adapted with permission from Lichman *et al.*,[111] Copyright 2015, *The Royal Society of Chemistry*]

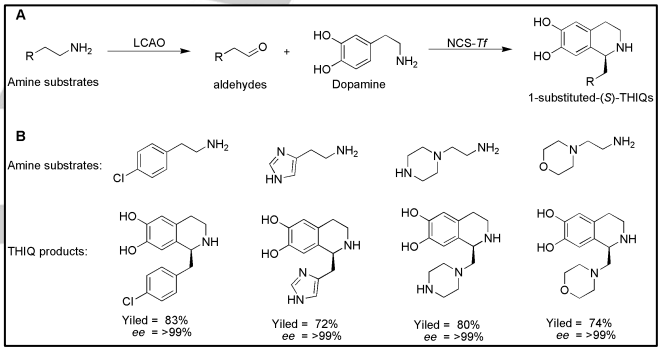


Figure 14. A) Biocatalytic cascade for the stereoselective synthesis of 1-substituted-(*S*)-THIQs using LCAO and NCS-*Tf* and B) Amine substrates tested and the synthesis of corresponding THIQ products [112]

The applicability of NCS was further demonstrated by Erdmann *et al.*[113] in the enzymatic and chemoenzymatic synthesis of 1,3,4-trisubstituted THIQs with three chiral centers using the cascade of three reactions. In the first reaction, carboligation of 3-hydroxybenzaldehyde and pyruvate was carried out using carboligase to generate (*R*)-1-hydroxy-1-(3-hydroxyphenyl)propan-2-one. In the subsequent reaction, 2-amino-1-(3-hydroxyphenyl) propan-1-ol was generated using transaminase-mediated biotransformation using IPA as an amine donor. Finally, the product of the previous reaction underwent NCS-mediated Pictet-Spengler condensation with the carbonyl co-substrate phenylacetaldehyde to generate THIQ product (*1S,3S,4R*)-1-benzyl-3-methyl-1,2,3,4-tetrahydroisoquinoline-4,6-diol with an excellent yield of 92% (Figure 15A). It is notable that the chemical cyclization catalyzed by phosphate gave opposite stereoselectivity in the synthesis of C1-substituted THIQs (Figure 15B). Thus, enzymatic and chemo-enzymatic methods provided access to both orientations of the THIQ substituents.[113]

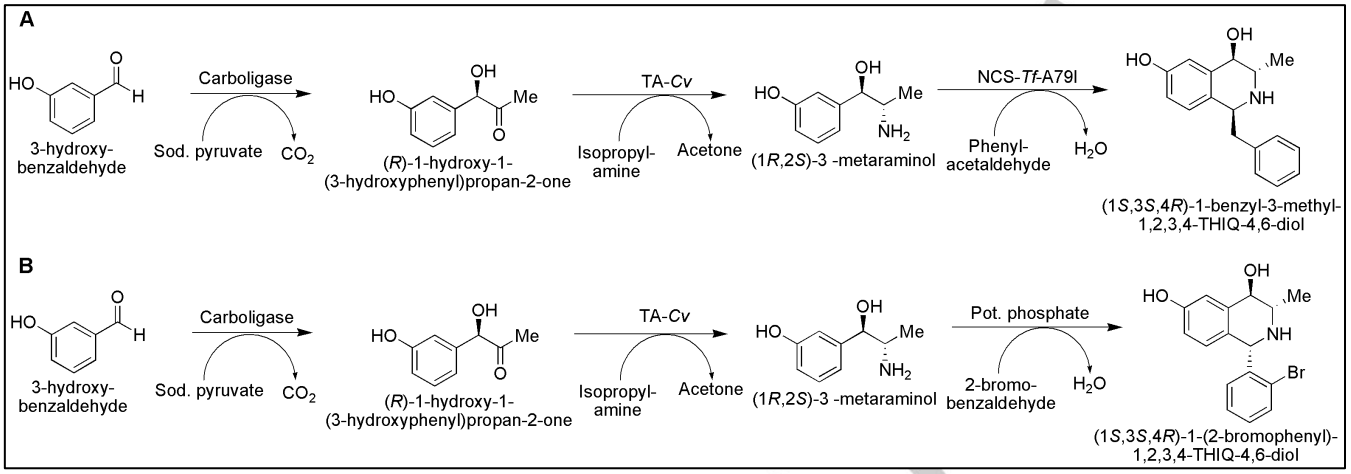


Figure 15. A) Enzymatic synthesis of 1-benzyl-THIQs employing the cascade of carboligase, ω-Transaminase and NCS; and B) Chemo-enzymatic synthesis of 1-benzyl-THIQs [Reprinted with permission from Erdmann *et al.*,[113] Copyright 2017, *John Wiley and Sons*]

Although NCSs exhibit wide tolerance for various aldehyde substrates, a strategy for the stereoselective Pictet-Spengler condensation of unactivated ketones was unavailable until very recently. Lichman *et al.*[114] have presented the first example of NCS-catalyzed Pictet-Spengler condensation with unactivated ketones (Figure 16). In their studies, wild type NCS-*Tf* was utilized for the condensation of dopamine and 4-hydroxyphenylacetone into a 1,1'-disubstituted THIQ. Acceptance of a ketone substrate 4-hydroxyphenylacetone served as a starting point to further investigate the extended scope of ketone substrates using variants of WT-NCS-*Tf.* Along with 4-hydroxyphenylacetone, several other ketones such as phenylacetone, cyclohexanone, and 4-methyl-, 4-*tert*-butyl- and 4-phenyl- substituted cyclohexanones were accepted as substrates and converted to their corresponding products. Notably, the mutation of the amino acid residue Ala79 most significantly affected the acceptance of ketone substrates. For instances, the product formation in Ala79Ile was twice as that of WT for the methyl ketone substrates. Another substitution, Ala79Phe, improved the conversion for all the cyclohexanone substituent substrates. Furthermore, various chiral and spiro-1,1'-disubstituted THIQs were biocatalytically synthesized using NCS-*Tf* variants (Figure 16). The extension of the substrate scope of NCS was followed by the most recent studies by Lechner *et al.*,[115] who reported the extension of the panel of Pictet-Spenglerase NCS and their immobilization for the biocatalytic generation of isoquinoline products.[115] The library of nine various NCSs was initially tested for their ability to condense the dopamine and 4-HPAA substrate using lyophilized whole cells as biocatalysts. Furthermore, the most active enzyme, NCS from *Papaver bracteatum* (NCS-*Pb*) was purified and immobilized on 10 different carriers. The immobilized catalyst was used in the flow-based system, wherein it resulted in high optical purity and significant product concentrations.

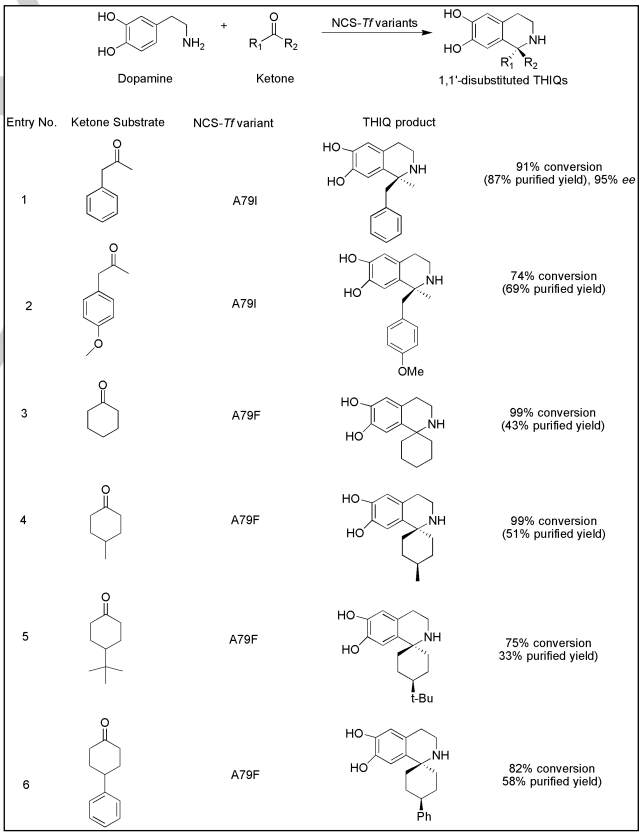


Figure 16. Biocatalytic synthesis of 1,1'-disubstituted THIQs using NCS-*Tf* variants [114]

The last few years have witnessed the extensive utilization of Pictet-Spenglerase enzymes for the stereoselective synthesis of a diverse array of alkaloids. Significant achievements have been achieved in the understanding of the catalytic mechanisms and substrate scope of the enzymes carrying Pictet-Spengler condensation. To this end, the most recent advance in NCS-mediated synthesis of THIQs is the detection of variants accepting substituted ketones as substrates. Furthermore, recent years have seen a trend of rational engineering of metabolic pathways in microbial species for the synthesis of alkaloids of pharmaceutical importance.[4,7,9,114,116-118] Recent advances in the improvement of substrate scope of the Pictet-Spenglerase enzymes can form the foundation of biosynthetic pathways in the microbial systems for the generation of novel alkaloids with improved and novel pharmaceutical activities. Additionally, a handful of new enzymes carrying the Pictet-Spengler reaction such as salsolinol synthase,[119] McbB from *Marinactinospora thermotolerans*,[120] deacetylisoipecoside synthase, and deacetylipecoside synthase,[121] two novel NCSs from *Argemone mexicana* (AmNCS1, AmNCS2) and one new NCS from *Corydalis saxicola*[115] have recently been identified. The development in the understanding of these enzymes will further enrich the biocatalytic synthesis of a myriad of alkaloids with diverse pharmaceutical functions.[42,122-126]

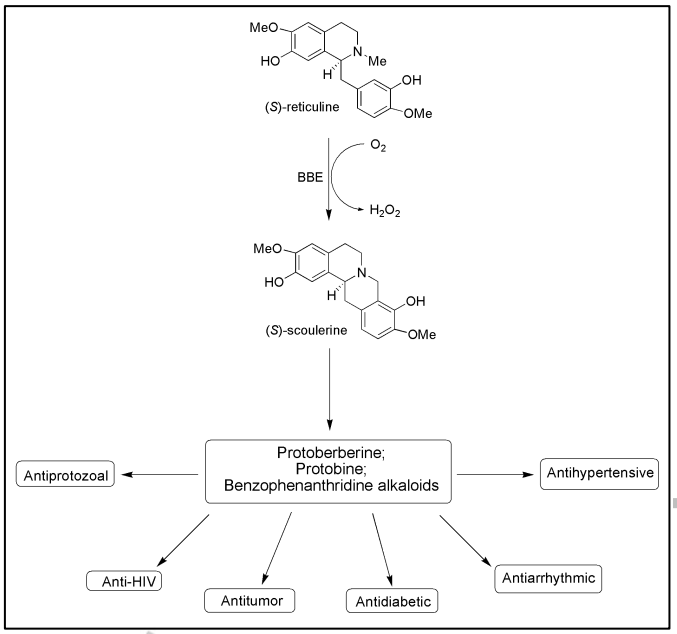


Figure 17. A natural reaction catalyzed by BBE and the role of (*S*)-scoulerine in the synthesis of pharmaceutically important alkaloids

3. Berberine bridge enzyme (BBE)

The berberine bridge enzyme (BBE; E.C. 1.21.3.3), a flavin-dependent oxidase, catalyzes the oxidative cyclization of THIQ (*S*)-reticuline to (*S*)-scoulerine by the formation of intramolecular C-C-bond, the so-called berberine-bridge.[127] (*S*)-scoulerine is the branch point intermediate in the biosynthetic pathway that leads to the formation of protoberberine, protobine and benzophenanthridine alkaloids.[128,129] These alkaloids and their substituents have been demonstrated to possess antimicrobial,[130] antiprotozoal,[131] antibacterial,[132] antitumor,[133-137] and anti-HIV properties,[138] along with several other pharmacological properties, such as antihypertensive, antiarrhythmic, and anti-diabetic.[139] Berberine has also been shown to possess a cholesterol-lowering property, thereby exerting a protective role in atherosclerosis.[140] Furthermore, berberines are known to possess central nervous system activities and might act as an herbal antidepressant.[127] Most recently, berberine derivatives have been reported to exhibit anti-influenza activity through the blocking of neuraminidase [141,142] (Figure 17).

Though BBE was first isolated from *Berberis beaniana* in 1985,[143] its activity was first detected a decade earlier in 1975 from *Macleaya microcarpa*.[144] Steffen *et al*.[143] attempted to develop a convenient assay method, and reported the presence of active BBE in 66 differentiated plants and cell suspension cultures, mainly from the Papaveraceae and Fumariaceae families.

In light of the mechanism of BBE, it has been established that the ring closure in the conversion of (*S*)-reticuline to (*S*)-scoulerine is carried out by the intramolecular C-C bond coupling between the *N*-methyl group and the 2'-carbon of the benzyl moiety of (*S*)-reticuline.[145] However, the characterization of BBE was previously limited owing to the quantities of purified enzyme available.

Kutchan and Dittrich[146] isolated the cDNA encoding the BBE followed by its overexpression in insect cell culture. In their studies, it was demonstrated that in the heterologously expressed enzyme, the FAD cofactor is covalently linked to a His104 residue via its 8α-position.[146] Also, it was proposed that the reaction mechanism of BBE-mediated cyclization of (*S*)-reticuline to (*S*)-scoulerine proceeds in two steps. The first step consists of the formation of the methylene iminium ion. The second step, ionic ring closure forming the berberine bridge of (*S*)-scoulerine, is stereospecific in nature.[146] The following section highlights the advancements in the heterologous expression, characterization and catalytic mechanism of BBE-mediated conversion of (*S*)-reticuline to (*S*)-scoulerine.

3.1 Heterologous expression of BBE

With the aim of gaining deeper insights into the structure and catalytic mechanism, various expression systems have been

developed to heterologously express the BBE.[147] Kutchan *et al*.[148] expressed a gene encoding BBE from California poppy (*E. californica*) in *Spodoptera frugiperda* Sf9 cells using the baculovirus-based expression system. A maximum production of 4 mg of purified protein could be achieved using this expression system that allowed basic characterization of substrate specificity. Similar to BBE from California poppy, three unique genomic clones (*bbel*, *bbe2*, and *bbe3*) were isolated from opium poppy.[149] These studies reported that not all BBE homologs in Opium poppy are functional, as only *bbel* was expressed and encoded a functional BBE protein.[149] However, the heterologous expression of BBE in bacterial cells or in *S. cerevisiae* led to an insoluble protein or to very small amounts of soluble and active enzyme.[150] The high expression of BBE was limited by the presence of N-terminal signal peptide and vacuolar sorting determinan.[150,151] Also, an active form of BBE was *N*-glycosylated and requires covalently bound FAD.

Subsequently, a high-level expression system for BBE from California poppy was developed in the methylotrophic yeast *Pichia pastoris* employing the secretory pathway of the host.[147] 120 mg of BBE per liter of fermentation broth were obtained in these studies using a two-step chromatographic purification protocol. This quantity of BBE achieved was 30 times more than that previously reported for the enzyme isolated from insect cell culture.[148]

3.2 Crystal structure, and mechanism of BBE-catalyzed reaction

The N-terminal amino acid sequencing and mass spectrometric analysis of the isolated flavin-containing peptide provided evidence for the bi-covalently attached FAD cofactor and also covalent linkage between the 6-position and the thiol group of a cysteine residue (Cys-166) in BBE.[147] The sequence similarity of BBE with glucooligosaccharide oxidase (GOOX) and 1-tetrahydrocannabinolic acid synthase implied that BBE belongs to a novel group of flavoproteins containing a bi-covalently attached flavin cofactor.

The importance and role of covalent linkages in BBE was further addressed by Winkler *et al*.[152] Amino acid residues involved in the bi-covalent attachment of the cofactor to BBE, i.e. His104 and Cys166 were mutated. The replacement of Cys166 with alanine reduced the turnover rate of BBE to 6% compared to that of wild-type. The measurement of redox potentials in these studies suggested that 6-*S*-cysteinyl linkage is a critical determinant of the rate of flavin reduction, and ultimately of the oxidation of (*S*)-reticuline to (*S*)-scoulerine. Furthermore, the removal of 6-*S*-cysteinyl linkage obtained a similar redox potential to that of other flavoproteins, implying that the deviations of kinetic properties in BBE are due to the FAD-6-*S*-cysteinyl bond. It is worth emphasizing that the results of rapid reaction stopped-flow experiments supported a hydride transfer mechanism from the *N*-methyl group of the substrate to the flavin cofactor.[152]

Since 1995, when Kutchan and Dittrich proposed a two-step process for the formation of berberine bridge that initiates with the oxidation of the *N*-methyl group to the corresponding iminium ion, followed by an ionic ring closure initiated by the deprotonated C3'OH group of the benzyl moiety,[146] various studies have suggested that the mechanism of BBE-mediated reactions is similar to those of other flavin-dependent enzymes.[153,154]

Subsequent research efforts by Winkler *et al*.[155] elucidated the three-dimensional X-ray crystal structure of the BBE from *E. californica* in complex with the natural substrate (*S*)-reticuline, which provided the ultimate proof for the bi-covalent binding of the flavin cofactor[155] (Figure 18). The BBE enzyme structure was reported to consist of two domains; a FAD binding domain, and an α/β domain with a seven-stranded, antiparallel β-sheet that forms a substrate binding domain.[155] The substrate, (*S*)-reticuline, is sandwiched between the flavin cofactor and amino acid residues extending from the β-sheet of the central domain, consisting of β-sheet.[129,155]

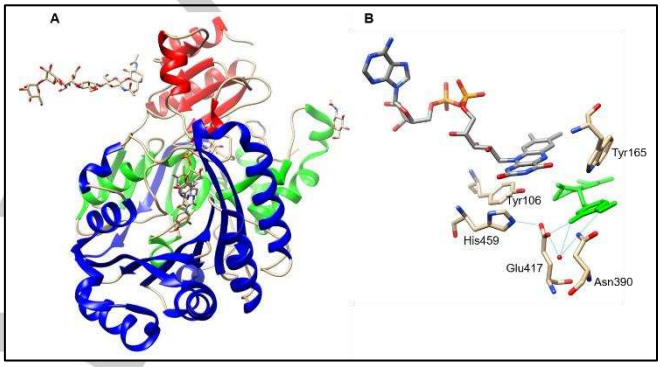


Figure 18. A) Crystal structure of BBE in complex with (*S*)-reticuline [PDB ID: 3D2D]; B) Interactions between (*S*)-reticuline substrate and active site amino acids [155]

For better understanding of the formation of the Berberine Bridge, these studies involved the site-directional mutational analysis of the active site amino acid residues Glu417, Tyr106, and His459 with Gln, Phe, and Ala, respectively. Eventually, the concerted mechanism for the formation of the berberine bridge has been proposed, wherein Glu417 has important mechanistic implications by acting as a catalytic base. This catalytic base deprotonates the C3' hydroxy group of the substrate and thereby increases the nucleophilicity of the C2' atom, which further facilitates the SN_2-nucleophilic attack on the *N*-methyl group of the substrate. Consequently, the C-C methylene bridge is formed, and hydride is concomitantly transferred to the flavin cofactor[129,155] (Figure 19). Recently, a series of substrate deuterium and solvent kinetic isotope effect studies provided additional evidence in support of the concerted mechanism of BBE-catalyzed formation of the berberine bridge.[156]

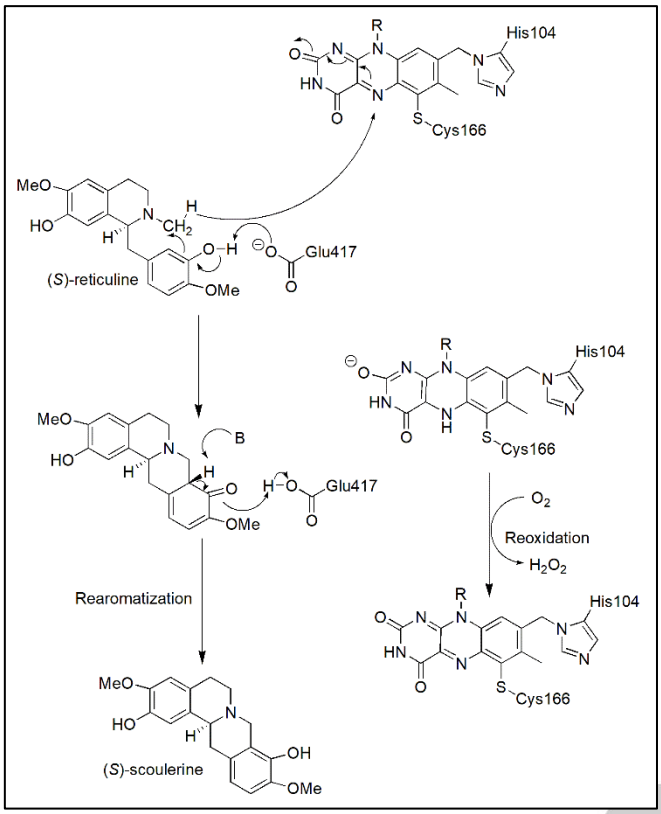
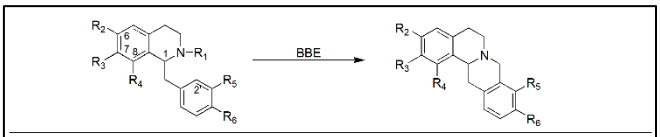


Figure 19. A concerted mechanism of BBE-catalyzed oxidative cyclization of (*S*)-reticuline [Reprinted with permission from Winkler *et al.*,[155] Copyright 2008, *Springer Nature*]

3.3 Substrate scope of BBE

The studies by Kutchan and Dittrich,[146] which expressed the BBE in insect cell culture, also demonstrated the substrate scope of BBE. Among the 19 tetrahydrobenzylisoquinolines, BBE catalyzed the formation of berberine bridge in five compounds, i.e. (*S*)-reticuline, (*S*)-protosinomenine, (*R,S*)-crassifoline, (*R,S*)-6-*O*-methyllaudanosoline, and (*R,S*)-laudanosoline, leading to conversion to corresponding protoberberines. Though the conversion rates for racemic forms of these substrates were low, it should be noted that enzyme is specific only for the *S*-epimer. Among the tested tetrahydroprotoberberine alkaloids, a double bond between the nitrogen and C8 was introduced in (*S*)-coreximine and (*S*)-norsteponine. It was concluded that the presence of the (*S*)-configuration at C1 and the substitution of the aromatic carbon *ortho*- to C2' of the ring closure with a hydroxyl group were structural requirements for the formation of berberine bridge in *N*-methyltetrahydrobenzylisoquinoline. Moreover, the substitution pattern of the aromatic ring moiety of the isoquinoline nucleus did not affect the formation of the berberine bridge. Also, the hydroxyl group of benzyl moiety was presumed to play a crucial role in the bridge formation, as the enzyme was inactive towards the substrates lacking the hydroxyl group (Figure 20).



Entry	Substrate	R_1	R_2	R_3	R_4	R_5	R_6	Conversion (%)
1	(*S*)-reticuline	CH_3	OCH_3	OH	H	OH	OCH_3	13
2	(*S*)- protosinomenine	CH_3	OH	OCH_3	H	OH	OCH_3	83
3	(*R,S*)-crassifoline	CH_3	H	OCH_3	OH	OH	OCH_3	44
4	(*R,S*)-6-*O*-methyllaudanosoline	CH_3	OCH_3	OH	H	OH	OH	10
5	(*R,S*)-laudanosoline	CH_3	OH	OH	H	OH	OH	8
6	(*R,S*)-*N*-methylcoclaurine	CH_3	OCH_3	OH	H	H	OH	2
7	(*R*)-reticuline	CH_3	OCH_3	OH	H	OH	OCH_3	0
8	(*S*)-Nnorreticuline	H	OCH_3	OH	H	OH	OCH_3	0
9	(*S*)- coclaurine	H	OCH_3	OH	H	H	OH	0
10	(*R,S*)-orientaline	CH_3	OCH_3	OH	H	OCH_3	OH	0

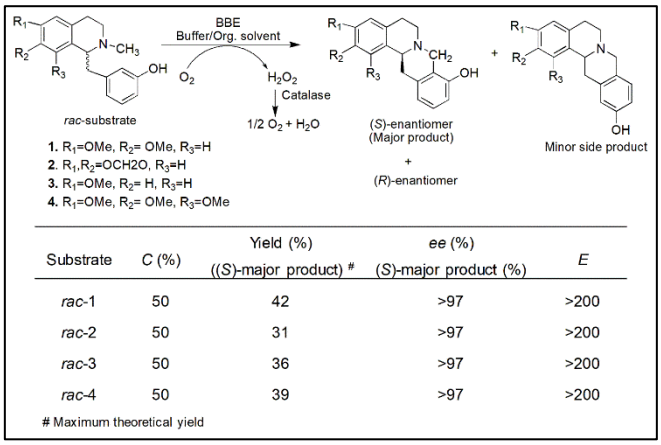
Figure 20. Substrate scope of BBE [146]

3.4 Extension of substrate scope and BBE-catalyzed chemo-enzymatic synthesis of alkaloids

As the berberine family of alkaloids consists of many important pharmaceutically important modalities, various chemical and enzymatic methods have been developed for the synthesis of novel unnatural products. Despite the advances in various chemical methods, such as metal-catalyzed asymmetric hydrogenation,[157] intramolecular allylic amination[158] and metal- or organo-catalyzed asymmetric alkylation reactions,[159] enantiopure products (>99% *ee*) were scarcely produced. Furthermore, chemical methods also have been reported to be time consuming and with low productivity and thereby yielding low product level.[145] Also, the isolation of these alkaloids from their natural source is very time consuming.[28,129]

Schrittwieser *et al.*[160] initiated studies to check the promiscuity of the BBE and synthesized unnatural substrates with a yield of 40% in a 5-step synthesis process. These studies suggested that four non-natural racemic THIQ substrates, lacking the methoxy group at 4'-position of reticuline and possessing a methoxy group in position 7 of the benzylisoquinoline backbone, could be accepted by BBE[160] (Figure 21). Furthermore, it was also demonstrated that these substrates could be synthesized on a higher scale, and 1 g/L of BBE successfully turned over all the substrates at concentrations of 20 g/L. This large quantity of substrates could be accommodated by employing 0.05 g/L catalase, to prevent the inhibitory effect of hydrogen peroxide, and by performing the reaction in the presence of organic co-solvents to improve the solubility of the substrates. Among the various organic solvents tested, the BBE was stable in the biphasic reaction employing toluene, benzene, and diphenyl ether. Furthermore, it was found that while the (*R*)-enantiomers were inactive, only (*S*)-enantiomers of these non-natural THIQs could be accepted as substrates by BBE, implying the high stereoselectivity of the enzyme. BBE catalyzed the kinetic

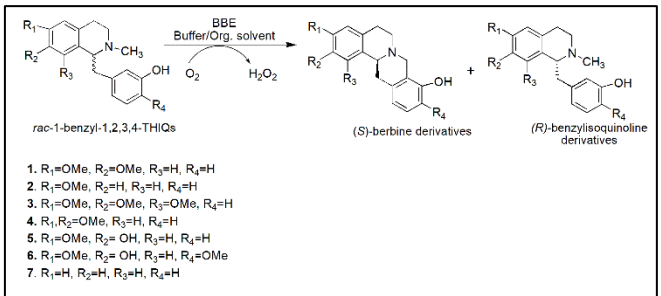
resolution of all the racemic substrates to yield optically pure (*S*)-products [>97% *ee*], with a minor side product wherein the C-C bond was formed using the 6'-carbon atom instead of the 2'-carbon atom[160] (Figure 21).



Substrate	*C* (%)	Yield (%) ((*S*)-major product) #	*ee* (%) (*S*)-major product (%)	*E*
rac-1	50	42	>97	>200
rac-2	50	31	>97	>200
rac-3	50	36	>97	>200
rac-4	50	39	>97	>200

Maximum theoretical yield

Figure 21. Biocatalytic enantioselective oxidation of non-natural THIQs catalyzed by BBE and the preparative scale (500 mg) kinetic resolution of racemic substrates [160]



Substrate	*C* (%)	Yield of (*S*)-berbine derivatives (%)#	*ee* of the (*S*)-product (%)	Yield of (*R*)-benzylisoquinoline derivative (%)#	*ee* of the (*R*)-product (%)	*E*
rac-1	50	42	>97	50	>97	>200
rac-2	50	36	>97	36	>97	>200
rac-3	50	39	>97	47	>97	>200
rac-4	50	31	>97	46	>97	>200
rac-5	50	22	>97	49	>97	>200
rac-6	50	47	>97	37	>97	>200
rac-7	50	46	>97	49	>97	>200

Maximum theoretical yield = 50%

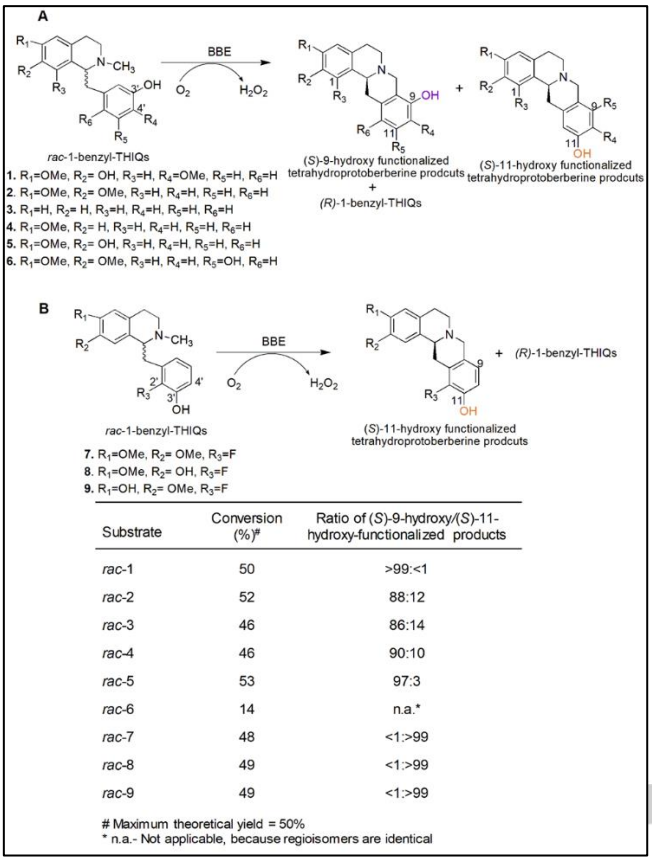
Figure 22. BBE-catalyzed oxidative kinetic resolution of racemic 1-benzyl-THIQs by C-C bond formation for the synthesis of enantiopure (*S*)-berbine derivatives [162]

Nevertheless, considering the diverse pharmacological profile of the (*S*)-scoulerine derivatives, newly synthesized non-natural THIQ products served as a vehicle for the discovery of new pharmaceutically important alkaloids. The chemoenzymatic synthesis of non-natural THIQ products was further optimized for the reaction conditions such as pH, temperature and solvents.[161]

Schrittwieser *et al.*[162] further extended the chemoenzymatic synthesis of berberine and benzylisoquinoline derivatives. In their studies, racemic 1-benzyl-1,2,3,4-THIQs were synthesized using two distinct pathways, i.e. Bischler Napieralski cyclization, or by alkylation of Boc-protected tetrahydroisoquinoline. An enantioselective oxidative C-C coupling, mediated by BBE, generated enantiopure (*R*)-benzylisoquinoline and (*S*)-berbine alkaloids with excellent enantioselectivity [>97% *ee*; E > 200] and yields ranging from 22-50% (Maximum theoretical yield-50%)[162] (Figure 22). It was notable that the synthetic route demonstrated by Schrittwieser achieved the asymmetric synthesis of (*S*)-scoulerine for the first time on a preparative scale of 0.5 g, wherein 230 mg of enantiomerically pure (*S*)-scoulerine was yielded by 9 linear steps.[162]

A follow-up study by Resch *et al.*[163] demonstrated that the regioselectivity of the products formed as a consequence of BBE-catalyzed cyclization is influenced by the substitution pattern of the substrate, the pH of the reaction medium and the type and amount of co-solvents used. It is worth noticing that the 9-hydroxy, and not 11-hydroxy regioisomers of tetrahydroprotoberberine derivatives are produced from racemic 1-benzyl-THIQ substrates following their cyclization by BBE (Figure 23). Resch *et al.*[163] attempted to completely switch the regioselectivity of BBE by medium engineering and by blocking the usual site of C–C coupling with a fluoro moiety. The blocking of the "normal" reaction centre with a fluoro moiety achieved a complete switch in regioselectivity, leading to the formation of excellent yields (32–50%) of the (*S*)-11-hydroxy functionalized protoberberines and the remaining (*R*)-benzylisoquinolines. It was notable that notwithstanding the change in regioselectivity, the enantioselectivity of the products was fully conserved (>97% *ee*) and such strategies hold considerable promise for the generation of enantiomerically pure novel berberine and benzylisoquinoline derivatives[163,164] (Figure 23).

10.1002/cctc.201801130

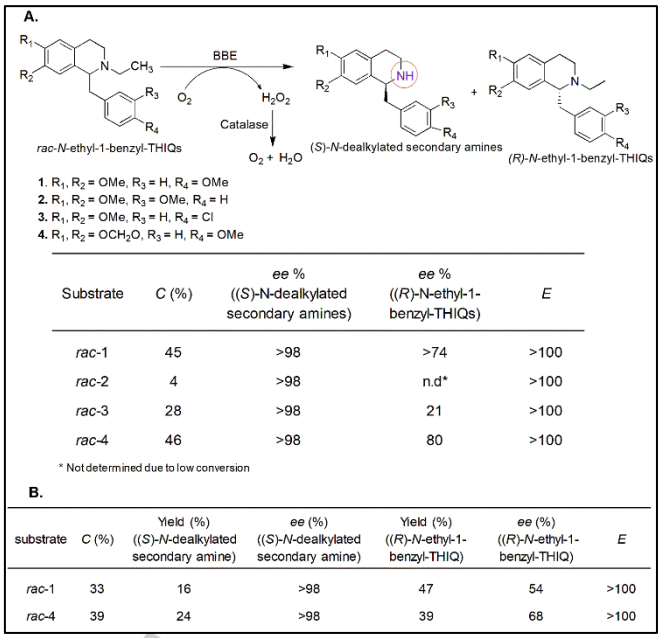


Substrate	Conversion (%)#	Ratio of (*S*)-9-hydroxy/(*S*)-11-hydroxy-functionalized products
rac-1	50	>99:<1
rac-2	52	88:12
rac-3	46	86:14
rac-4	46	90:10
rac-5	53	97:3
rac-6	14	n.a.*
rac-7	48	<1:>99
rac-8	49	<1:>99
rac-9	49	<1:>99

Maximum theoretical yield = 50%
* n.a.- Not applicable, because regioisomers are identical

Figure 23. BBE-catalyzed oxidative kinetic resolution of racemic 1-benzyl-THIQs by C-C bond formation to generate enantiopure (*S*)-9-hydroxyprotoberberines as major products and B) complete switch in the regioselectivity of BBE by blocking the "normal" reaction center with a fluoro moiety [163]

Recently, Gandomkar *et al.*[165] have demonstrated the novel *N*-dealkylation function of BBE. Since the previous studies have shown that BBE could enatioselectively transform the various *N*-methyl reticulines to the corresponding berberines,[160] the authors expected that *N*-ethyl derivatives could also be accepted as substrates. Surprisingly, the reaction of BBE with *N*-ethyl-1-benzyl-1,2,3,4-THIQ derivatives did not form a new C-C-bond, however, BBE catalyzed the *N*-dealkylation of these substrates to form the corresponding secondary amines, leaving behind the unreacted (*R*)-substrate in enantiopure form (Figure 24). Furthermore, the enantioselective *N*-dealkylation of two substrates was performed on a preparative scale (171 and 81 mg), which yielded corresponding secondary amine products with good yields and excellent stereoselectivity [*ee* >98%][165] (Figure 24).



Substrate	*C* (%)	*ee* % ((*S*)-N-dealkylated secondary amines)	*ee* % ((*R*)-N-ethyl-1-benzyl-THIQs)	*E*
rac-1	45	>98	>74	>100
rac-2	4	>98	n.d*	>100
rac-3	28	>98	21	>100
rac-4	46	>98	80	>100

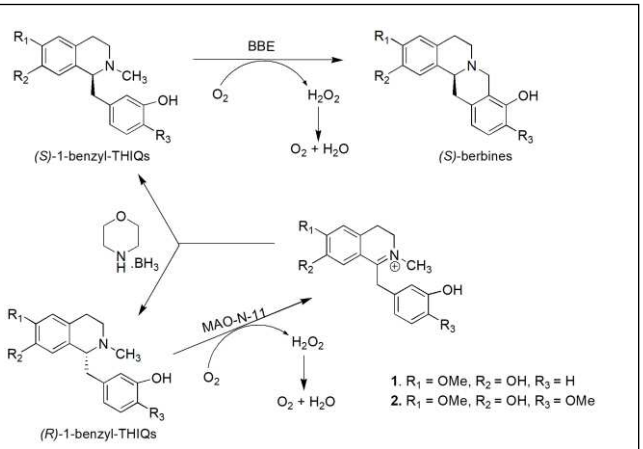
* Not determined due to low conversion

B.

substrate	*C* (%)	Yield (%) ((*S*)-*N*-dealkylated secondary amine)	*ee* (%) ((*S*)-*N*-dealkylated secondary amine)	Yield (%) ((*R*)-*N*-ethyl-1-benzyl-THIQ)	*ee* (%) ((*R*)-*N*-ethyl-1-benzyl-THIQ)	*E*
rac-1	33	16	>98	47	54	>100
rac-4	39	24	>98	39	68	>100

Figure 24. A) BBE-catalyzed enantioselective dealkylation of *rac*-*N*-ethyl-1-benzyl-THIQs to corresponding (*S*)-*N*-dealkylated secondary amines and (*R*)-*N*-ethyl-1-benzyl-THIQs; B) Preparative scale enantioselective N-dealkylation by BBE [165]

Though various non-natural substrates have been reported to be successfully transformed to their corresponding (*S*)-berberine derivatives by BBE, it must be noted that BBE is highly stereoselective in nature. As BBE can accept only (*S*)-epimers of the substrates, the maximum theoretical yield of product formation from the racemic substrates is 50%.[127] To overcome the limitation of kinetic resolution, Schrittwieser *et al.* recently performed a chemo-enzymatic deracemization strategy by employing simultaneous kinetic resolution and stereoinversion using a cascade of MAO and BBE.[166] Transformation of *rac*-benzylisoquinolines to optically pure (*S*)-berbines was reported using this strategy. Initially, *(R)*-benzylisoquinoline substrates were oxidized by MAO-N-11 to form the corresponding achiral iminium intermediates. These iminium intermediates were non-stereoselectively reduced by chemical reductant morpholine borane to form the (*S*)-epimers of the substrates, which could subsequently be transformed to the corresponding (*S*)-berberine products by BBE-catalyzed cyclization. Furthermore, this process could be successfully carried out on a preparative scale (150-165 mg substrate), wherein enantiomerically pure (*S*)-berberine derivatives were produced with excellent yields (up to 98% conversion and up to 88% isolated yields) and enantioselectivity (*ee* > 97%)[127,166](Figure 25)



1-benzyl-THIQ Substrate	% Conversion ((*S*) product)	% yield ((*S*) product)	*ee* % ((*S*) product)
rac-1	92	88	>97
rac-2	98	80	>97

Figure 25. Deracemization of *rac*-1-benzyl-THIQs to enantiopure (*S*)-berbines using a cascade of MAO/BBE/ boranes [166]

The formation of C-C bonds plays a critical role in the connection of smaller substructures in order to synthesize complex organic structures and the carbon backbone of organic molecules.[20,167,168] Although challenging, the generation of multifunctional products is of enormous value.[127] Subsequently, various sophisticated enzymatic and chemo-enzymatic for the synthesis of complex compounds, such as pharmaceutically important alkaloids have been developed.[127]

The nomenclature committee of the International Union of Biochemistry and Molecular Biology maintains a database of enzymes (http://www.enzyme-database.org) that categorizes the enzymes possessing unique catalytic activities. BBE, a member of this enzyme database, is an important enzyme that catalyzed an important reaction in the biosynthesis of the alkaloids belonging to the '*prestigious scaffold*' THIQ.[145,169]

Advances achieved regarding the BBE crystallization, and structural and mechanistic insights have significantly broadened the catalytic scope of the BBE, which in turn can be used for the synthesis of large libraries of unnatural alkaloids with improved or novel biological activities.[34] The enzymatic reactions catalyzed by BBE have been successfully integrated for the development of the total chemical syntheses of alkaloids and their precursors.[86]

Further improvement such as enantioselective dealkylation[165] and deracemization using enzyme cascades,[166] in order to expand the scope of C-C bond formation, has recently been achieved. These approaches have also been verified at the preparative scale implying their applicability at industrial scale.

The structural details of BBE have revealed the presence of typical FAD-binding domain and substrate binding domain ['BBE-domain'], a feature of this family that distinguishes it from the other sub-families of the superfamily of FAD-linked oxidases. The research efforts in the recent years have discovered many homologous proteins, the 'BBE-like enzymes'. The ever-increasing number of uncharacterized BBE-like enzymes will witness an expansion of reactions involved in the biosynthesis of important alkaloids in the near future.[170,171]

4. Summary and outlooks

The formation of C-C bond plays a critical role in the connection of smaller substructures in order to synthesize complex organic structures such as alkaloids.[167] The last few years have witnessed tremendous achievements in the understanding of the catalytic mechanisms and substrate scope of C-C bond forming enzymes such as the Pictet-Spenglerases and BBEs. Protein engineering techniques have been progressively used for the synthesis of large libraries of unnatural alkaloids with improved or novel biological activities.[34] Furthermore, structure-guided engineering also has been successfully utilized to extend the synthesis of alkaloids in microbial systems.[4,7,9,117,118] For instance, some of the steps in industrial production of terpenoid artemisinin are conducted in engineered yeast.[172] The near future of alkaloid syntheses should see the judiciously designed metabolic pathways as an opportunity to advance in this 'golden age' of natural products drug discovery.[173] The well-known plasticity of few of the C-C bond forming enzymes is now being exploited. For instance, despite of the previously reported exclusive formation of *S*-configured products by STRs, the recently reported access to *R*-configured products will further strengthen the enzymatic toolbox for the synthesis of pharmaceutically important alkaloids.[73] With advances in the protein engineering techniques, and discovery of novel enzymes, it can be anticipated that major bottlenecks, for instance, restricted scope of amine substrates for NCSs towards amines, issues related with scalability, can be tackled efficiently.[174] Although many of the biocatalytic reactions employing these C-C bond forming enzymes have been demonstrated at smaller scales, few of them have also been successfully carried out at liter-scales,[107] implying their potential at industrial scales in near future.

Recent years have seen the applications of elegant multi-enzymatic strategies, wherein the C-C bond formation reactions catalyzed by these enzymes have been successfully integrated with other enzymatic reactions or chemical synthetic methods for the generation of a diverse array of alkaloids or their precursors. Moreover, the successful demonstration of these enzymatic approaches at the preparative scale has already proven their industrial potential. The enzymatic pool for the synthesis of pharmaceutically important enantiopure alkaloids has been significantly widened and C-C bond forming enzymes such as Pictet-Spenglerases and BBEs are currently serving as effective alternatives to the traditional approaches of alkaloid synthesis.

Acknowledgements

This work was supported Konkuk University (Grant no: 2016-A019-0270).

Keywords: alkaloids • berberine bridge enzyme • biocatalysis • C-C bond formation • Pictet-Spenglerases

[1] J.W. Li, J. C. Vederas, *Science* **2009**, *325*, 161-165.
[2] H. Yun, B-G. Kim, *Biotechnol. Bioprocess Eng.* **2008**, *13*, 372.
[3] G. A. Cordell, M. L. Quinn-Beattie, N. R. Farnsworth, *Phytother. Res.* **2001**, *15*, 183-205.
[4] A. M. Ehrenworth, P. Peralta-Yahya, *Nature Chem. Biol.* **2017**, *13*, 249-258.
[5] S. Mijatović, A. Bramanti, F. Nicoletti, P. Fagone, G. Kaluđerović, D. Maksimović-Ivanić, *Biotechnol. Adv.* **2018**, *In Press.* DOI: 10.1016/j.biotechadv.2018.04.001
[6] S. E. O'Connor, J. J. Maresh, *Nat. Prod. Rep.* **2006**, *23*, 532-547.
[7] I J. Trenchard, M. S. Siddiqui, K. Thodey, C. D. Smolke, *Metab. Eng.* **2015**, *31*, 74-83.
[8] D. Ravelli, S. Protti, M. Fagnoni, *Chem. Rev.* **2016**, *116*, 9850-9913.
[9] A. Ruiz-Olalla, M. A. Würdemann, M. J. Wanner, S. Ingemann, J. H. Van Maarseveen, H. Hiemstra, *J. Org. Chem.* **2015**, *80*, 5125-5132.
[10] B. David, J. L. Wolfender, D. A. Dias, *Phytochem. Rev.* **2015**, *14*, 299-315.
[11] S. M. Pearsall, C. N. Rowley, A. Berry, *ChemCatChem*, **2015**, *7*, 3078-3093.
[12] L. Narcross, E. Fossati, L. Bourgeois, J. E. Dueber, V. J. Martin, *Trends Biotechnol.* **2016**, *34*, 228-241.
[13] M. Lahlou, *Pharmacol. Pharm.* **2013**, *4*, 17-31.
[14] A. Khalil, in *Catharanthus roseus* (Eds.: M. Naeem, T. Aftab, M. Khan), Springer, Cham, **2017**, pp. 59-70. DOI: https://doi.org/10.1007/978-3-319-51620-2_4.
[15] G. Grogan, *Curr. Opin. Chem. Biol.* **2018**, *43*, 15-22.
[16] S. Mathew, H. Yun, *ACS Catal.* **2012**, *2*, 993-1001.
[17] S. Mathew, S. P. Nadarajan, U. Sundaramoorthy, H. Jeon, T. Chung, H. Yun, *Biotechnol. Lett.* **2017**, *39*, 535-543.
[18] D. Ghislieri, A. P. Green, M. Pontini, S. C. Willies, I. Rowles, A. Frank, G. Grogan, N. J. Turner, *J. Am. Chem. Soc.* **2013**, *135*, 10863-10869.
[19] M. D. Patil M. J. Dev, A. S. Shinde K. D. Bhilare, G. Patel, Y. Chisti, U. C. Banerjee, *Process Biochem.* **2017**, *63*, 113-121.
[20] P. Durairaj, J. S. Hur, H. Yun, *Microbi. Cell Fact.* **2016**, *15*, 125.
[21] Y. Ravikumar, S. P. Nadarajan, T. Hyeon Yoo, C. S. Lee, H. Yun, *Biotechnol. J.* **2015**, *10*, 1862-1876.
[22] S. Wu Z. Li, Whole-Cell Cascade Biotransformations for One-Pot Multistep Organic Synthesis. *ChemCatChem* **2018**, *10*, 2164-2178.
[23] M. M. Ahsan, H. Jeon, S. P. Nadarajan, T. Chung, H-W. Yoo, B-G. Kim, M. D. Patil, H. Yun, *Biotechnol. J.* **2018**, *13*, 1700562. DOI: 10.1002/biot.201700562.
[24] M. M. Ahsan, S. Sung, H. Jeon, M. D. Patil, T. Chung, H. Yun, *Catalysts*, **2017**, *8*, 4. DOI: 10.3390/catal8010004.
[25] M. D. Patil, G. Grogan, A. Bommarius, H. Yun, *Catalysts*, **2018**, *8*, 254. DOI: 10.3390/catal8070254.
[26] A. Pictet, T. Spengler, *Ber. Dtsch. Chem. Ges.* **1911**, *44*, 13.
[27] V. Cechinel-Filho, Plant *bioactives and drug discovery: principles, practice, and perspectives*, John Wiley & Sons, 2012.
[28]. M. Chrzanowska, M. D. Rozwadowska, *Chem. Rev.* **2004**, *104*, 3341-3370.
[29] E. D. Cox, J. M. Cook, *Chem. Rev.* **1995**, *6*, 1797-1842.
[30] W. S. Glenn, W. Runguphan, S. E. O'Connor, *Curr. Opin. Biotechnol.* **2013**, *24*, 354-365.
[31] E. L. Larghi, M. Amongero, A. B. Bracca, T. S. Kaufman, *Arkivoc* **2005**, *12*, 98-153.
[32] B. E. Maryanoff, H, C, Zhang, J. H. Cohen, I. J. Turchi, C. A. Maryanoff, *Chem. Rev.* **2004**, *104*, 1431-628.
[33] B. Kovács, R. Savela, K. Honkala, D. Y. Murzin, E. Forró, F. Fülöp, R. Leino, *ChemCatChem. In press*, 10.1002/cctc.201800293
[34] H. Kries, S. E. O'Connor, *Curr. Opin. Chem. Biol.* **2016**, *31*, 22-30
[35] K. A. Yonkers, K. Gilstad-Hayden, A. Forray, H. S. Lipkind, *JAMA Psychiatry* **2017**, *74*, 1145-1152.
[36] M. Ebrahimi-Ghiri, M. Rostampour, M. Jamshidi-Mehr, M. Nasehi, M. R. Zarrindast, *Brain Res.* **2018**, *1678*, 164-173.
[37] P. H. Luppi, C. Peyron, P. Fort, *Sleep Med. Rev.* **2017**, *32*, 85-94.
[38] W. Wisden, X. Yu, N. P. Franks in *Handbook of Experimental Pharmacology* Springer, Berlin, Heidelberg, **2017**. DOI: 10.1007/164_2017_56.
[39] W. C. Hillmann, Ph.D. thesis, Massachusetts Institute of Technology (U.S.A.), **2008**.
[40] J. Stöckigt, M. H. Zenk, *FEBS Lett.* **1977**, *79*, 233-237.
[41] J. Stöckigt, M. H. Zenk, *J. Chem. Soc. Chem. Commun.* **1977**, *18*, 646–648.
[42] J. Stöckigt, A. P. Antonchick, F. Wu, H. Waldmann, *Angew. Chem. Int. Ed.* **2011**, *50*, 8538-8564.
[43] H. Kohls, Ph.D. thesis, University of Greifswald (Germany). **2015**
[44] G. Lesley-Ann, Ph.D. thesis, Massachusetts Institute of Technology (U.S.A.), 2011.
[45] V. Resch, J. H. Schrittwieser, E. Siirola, W. Kroutil, *Curr. Opin. Biotechnol.* **2011**, *22*, 793-799.
[46] H. Mizukami, H. Nordlov, S. L. Lee, A. I. Scott, *Biochemistry*, **1979**, *18*, 3760-3763.
[47] J. F. Treimer, M. H. Zenk, *FEBS J.* **1979**, *101*, 225-233.
[48] U. Pfitzner, M. H. Zenk, *Planta Medica* **1989**, *55*, 525-530.
[49] N. Hampp, M. H. Zenk, *Phytochemistry* **1988**, *27*, 3811-3815.
[50] J. Stöckigt, J. Treimer, M. H. Zenk, *FEBS Lett.* **1976**, *70*, 267-270.
[51] A. I. Scott, S. L. Lee, *J. Am. Chem. Soc.* **1975**, *23*, 6906-6908.
[52] A. De Waal, A. H. Meijer, R. Verpoorte, *Biochem. J.* **1995**, *306*, 571.
[53] K. Fesko, M. Gruber-Khadjawi, Biocatalytic Methods for C-C Bond Formation. *ChemCatChem* **2013**, *5*, 1248-1272.
[54] J. F. Treimer, M. H. Zenk, *FEBS Lett.* **1979**, *97*, 159-162.
[55] X. Ma, S. Panjikar, J. Koepke, E Loris, J. Stöckigt, 2006. *Plant Cell*, **2006**, *18*, 907-920.
[56] E. Fischereder, D. Pressnitz, W. Kroutil, S. Lutz, *Bioorg. Med. Chem.* **2014**, *22*, 5633-5637.
[57] J. J. Maresh, L. A. Giddings, A. Friedrich, E. A. Loris, S. Panjikar, B. L. Trout, J. Stöckigt, B. Peters, S. E. O'Connor, *J. Am. Chem. Soc.* **2008**, *130*, 710-723.
[58] H. Zollinger in *Advances in Physical Organic Chemistry* (Eds.: V. Gold), Academic Press: London, **1964**.
[59] Y. Ravikumar, S. P. Nadarajan, T. H. Yoo, C. S. Lee, H. Yun, *Trends Biotechnol.* **2015**, *33*, 462-470.
[60] K-Y. Choi, E. O. Jung, H. Yun, Y.-H. Yang, B.-G. Kim, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 8191-8200.
[61] I. V. Pavlidis, M. S. Weiß, M. Genz, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *Nat. Chem.* **2016**, *8*, 1076-1082.
[62] F. F. Chen, G. W. Zheng, L. Liu, H. Li, Q. Chen, F. L. Li, C. X. Li, J. H. Xu, *ACS Catal.* **2018**, *8*, 2622−2628.
[63] G. A. Aleku, S. P. France, H. Man, J. Mangas-Sanchez, S. L. Montgomery, M. Sharma, F. Leipold, S. Hussain, G. Grogan, N. J. Turner, *Nature Chem.* **2017**, *9*, 961–969
[64] J. M. Woodley, *Phil. Trans. R. Soc. A.* **2018**, 376, 20170062. DOI: 10.1098/rsta.2017.0062.
[65] T. M. Kutchan, *FEBS Lett.* **1989**, *257*, 127-130.
[66] T. D. McKnight, C. A. Roessner, R. Devagupta, A. I. Scott, C. L. Nessler, *Nucleic Acids Res.* **1990**, *18*, 4939.
[67] C. Canel, M. I. Lopes-Cardoso, S. Whitmer, L. van der Fits, G. Pasquali, R. van der Heijden, J. H. Hoge, R. Verpoorte, *Planta* **1998**, *205*, 414-419.
[68] Y. Lu, H. Wang, W. Wang, Z. Qian, L. Li, J. Wang, G. Zhou, G. Kai, *Mol. Biol. Rep.* **2009**, *36*, 1845-1852.

[69] E. McCoy, M. C. Galan, S. E. O'Connor, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2475-2478.
[70] S. Chen, M. C. Galan, C. Coltharp, S. E. O'Connor, *Chem. Biol.* **2006**, *13*, 1137-1141.
[71] H. Y. Lee, N. Yerkes, S. E. O'Connor, *Chem. Biol.* **2009**, *16*, 1225-1229.
[72] E. A. Loris, S. Panjikar, M. Ruppert, L. Barleben, M. Unger, H. Schübel, J. Stöckigt, *Chem. Biol.* **2007**, *14*, 979-985.
[73] D. Pressnitz, E. M. Fischereder, J. Pletz, C. Kofler, L. Hammerer, K. Hiebler, H. Lechner, N. Richter, E. Eger, W. Kroutil, *Angew. Chem.* **2018**, *130*, 10843 –10847. DOI: 10.1002/anie.201803372.
[74] P. Bernhardt, E. McCoy, S. E. O'Connor, *Chem. Biol.* **2007**, 14, 888-897.
[75] Z. Xu, Z. Yang, Y. Liu, Y. Lu, K. Chen, W. Zhu, *J. Chem. Inf. Model.* **2014**, *54*, 69-78.
[76] H. Zhu, P. Kercmar, F. Wu, C. Rajendran, L. Sun, M. Wang, J. Stockigt, *Curr. Med. Chem.* **2015**, *22*, 1880-1888.
[77] F. Wu, H. Zhu, L. Sun, C. Rajendran, M. Wang, X. Ren, S. Panjikar, A. Cherkasov, H. Zou, J. Stöckigt, *J. Am. Chem. Soc.* **2012**, *134*, 1498-1500.
[78] Y. Cai, H. Zhu, Z. Alperstein, W. Yu, A. Cherkasov, H. Zou, *ACS Chem. Biol.* **2017**, *12*, 3086-3092.
[79] D. L. Wang, X. C. Shi, Y. Y. Wang, J. Ma, *Chin. Chem. Lett.* **2016**, *27*, 261-264.
[80] H. B. Zou, H. J. Zhu, L. Zhang, L. Q. Yang, Y. P. Yu, J. Stöckigt, *Chem. Asian J.* **2010**, *5*, 2400-2404.
[81] P. Bernhardt, A. R. Usera, S. E. O'Connor, *Tetrahedron Lett.* **2010**, *51*, 4400-4402.
[82] G. Dong, C. Sheng, S. Wang, Z. Miao, J. Yao, W. Zhang, *J. Med. Chem.* **2010**, *53*, 7521-7531.
[83] D. Wu, D. W. Zhao, Y. Q. Li, W. G. Shi, Q. L. Yin, Z. K. Tu, Y. Y. Yu, B. H. Zhong, H. Yu, W. G. Bao, *Oncol. Rep.* **2018**, *39*, 871-879.
[84] E. M. Fischereder, D. Pressnitz, W. Kroutil, *ACS Catal.* **2016**, *6*, 23-30.
[85] M. Chrzanowska, A. Grajewska, M. D. Rozwadowska, *Chem. Rev.* **2016**, *116*, 12369-12465.
[86] J. Stöckigt, Z. Chen, M. Ruppert, in *Natural Products via Enzymatic Reactions* Springer (Eds.: J. Piel), Berlin, Heidelberg, **2010**, pp. 67-103.
[87] M. Weid, J. Ziegler, T. M. Kutchan, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13957-13962.
[88] M. Rueffer, H. El-Shagi, N. Nagakura, M. H. Zenk, *FEBS Lett.* **1981**, *129*, 5-9.
[89] H. M. Schumacher, M. Rüffer, N. Nagakura, M. H. Zenk, *Planta Medica.* **1983**, *48*, 212-220.
[90] R. Stadler, T. M. Kutchan, S. Loeffler, N. Nagakura, B. Cassels, M. H. Zenk, *Tetrahedron Lett.* **1987**, *28*, 1251-1254.
[91] M. Rueffer, M. H. Zenk, *Zeitschrift für Naturforschung* C. **1987**, *42*, 319-332. DOI: https://doi.org/10.1515/znc-1987-0402
[92] R. Stadler, M. H. Zenk, *Eur. J. Org. Chem.* **1990**, *1990*, 555-562.
[93] L. Y. Luk, S. Bunn, D. K. Liscombe, P. J. Facchini, M. E. Tanner, *Biochemistry*, **2007**, *46*, 10153-10161.
[94] N. Samanani, P. J. Facchini, *J. Biol. Chem.* **2002**, *277*, 33878-33883.
[95] N. Samanani, D. K. Liscombe, P. J. Facchini, *Plant J.* **2004**, *40*, 302-313.
[96] H. Minami, E. Dubouzet, K. Iwasa, F. Sato, *J. Biol. Chem.* **2007**, *282*, 6274-6282.
[97] H. Berkner, J. Engelhorn, D. K. Liscombe, K. Schweimer, B. M. Wöhrl, P. J. Facchini, P. Rösch, I. Matečko, *Protein Exp. Purif.* **2007**, *56*, 197-204.
[98] H. Berkner, K. Schweimer, I. Matecko, P. Rösch, *Biochem. J.* **2008**, *413*, 281-290.
[99] A. Bonamore, M. Barba, B. Botta, A. Boffi, A. Macone, *Molecules* **2010**, *15*, 2070-2078.
[100] A. Ilari, S. Franceschini, A. Bonamore, F. Arenghi, B. Botta, A. Macone, A. Pasquo, L. Bellucci, A. Boffi, *J. Biol. Chem.* **2009**, *284*, 897-904.
[101] A. Pasquo, S. Bonamore, A. Franceschini, A. Macone, A. Boffi, A. Ilari, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2008**, *64*, 281-283.
[102] T. Pesnot, M. C. Gershater, J. M. Ward, H. C. Hailes, *Adv. Syn. Catal.* **2012**, *354*, 2997-3008.
[103] B. R. Lichman, M. C. Gershater, E. D. Lamming, T. Pesnot, A. Sula, N. H. Keep, H. C. Hailes, J. M. Ward, *FEBS J.* **2015**, *282*, 1137-1151.
[104] B. R. Lichman, A. Sula, T. Pesnot, H. C. Hailes, J. M. Ward, N. H. Keep, *Biochemistry*, **2017**, *56*, 5274-5277.
[105] M. E. Welsch, S. A. Snyder, B. R. Stockwell, *Curr. Opin. Chem. Biol.* **2010**, *14*, 347-361.
[106] M. K. Pyo, D. H. Lee, D. H. Kim, J. H. Lee, J. C. Moon, K. C. Chang, H. S. Yun-Choi, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4110-4114.
[107] A. Bonamore, I. Rovardi, F. Gasparrini, P. Baiocco, M. Barba, C. Molinaro, B. Botta, A. Boffi, A. Macone, *Green Chem.* **2010**, *12*, 1623-1627.
[108] B. M. Ruff, S. Bräse, S. E. O'Connor, *Tetrahedron Lett.* **2012**, *53*, 1071-1074.
[109] M. Nishihachijo, Y. Hirai, S. Kawano, A. Nishiyama, H. Minami, T. Katayama, Y. Yasohara, F. Sato, H. Kumagai, *Biosci. Biotechnol. Biochem.* **2014**, *78*, 701-707.
[110] J. J. Maresh, S. O. Crowe, A. A. Ralko, M. D. Aparece, C. M. Murphy, M. Krzeszowiec, M. W. Mullowney, *Tetrahedron Lett.* **2014**, *55*, 5047-5051.
[111] B. R. Lichman, E. D. Lamming, T. Pesnot, J. M. Smith, H. C. Hailes, J. M. Ward, *Green Chem.* **2015**, *17*, 852-855.
[112] A. Bonamore, L. Calisti, A. Calcaterra, O. H. Ismail, M. Gargano, I. D'Acquarica, B. Botta, A. Boffi, A. Macone, *ChemistrySelect*, **2016**, *1*, 1525-1528.
[113] V. Erdmann, B. R. Lichman, J. Zhao, R. C. Simon, W. Kroutil, J. M. Ward, H. C. Hailes, D. Rother, *Angew. Chem. Int. Ed.* **2017**, *56*, 12503-12507.
[114] B. R. Lichman, J. Zhao, H. C. Hailes, J. M. Ward, *Nature Commun.* **2017**, *8*, 14883.
[115] H. Lechner, P. Soriano, R. Poschner, H. C. Hailes, J. M. Ward, W. Kroutil, *Biotechnol. J.* **2018**, *13*, 1700542. DOI: 10.1002/biot.201700542.
[116] S. Brown, M. Clastre, V. Courdavault, S. E. O'Connor, *Proc. Natl. Acad. Sci.* **2015**, *112*, 3205-3210.
[117] A. Diamond, I. Desgagné-Penix, *Plant Biotechnol. J.* **2016**, *14*, 1319-1328.
[118] S. Galanie, K. Thodey, I. J. Trenchard, M. F. Interrante, C. D. Smolke, *Science* **2015**, *349*, 1095-1100.
[119] M. Naoi, W. Maruyama, P. Dostert, K. Kohda, T. Kaiya, *Neurosci. Lett.* **1996**, *212*, 183-186.
[120] T. Mori, S. Hoshino, S. Sahashi, T. Wakimoto, T. Matsui, H. Morita, I. Abe, *Chem. Biol.* **2015**, *22*, 898-906.
[121] W. De-Eknamkul, N. Suttipanta, T. M. Kutchan, *Phytochemistry* **2000**, *55*, 177-181.
[122] F. Ghirga, A. Bonamore, L. Calisti, I. D'Acquarica, M. Mori, B. Botta, A. Boffi, A. Macone, *Int. J. Mol. Sci.* **2017**, *18*, 2464.
[123] F. Ghirga, D. Quaglio, P. Ghirga, S. Berardozzi, G. Zappia, B. Botta, M. Mori, I. D'Acquarica, *Chirality* **2016**, *28*, 169-180.
[124] J. M. Hagel, P. J. Facchini, *Plant Cell Physiol.* **2013**, *54*, 647-672
[125] C. Ingallina, I. D'Acquarica, G. Delle Monache, F. Ghirga, D. Quaglio, P. Ghirga, S. Berardozzi, V. Markovic, B. Botta, *Curr. Pharm. Des.* **2016**, *22*, 1808-1850.
[126] S. Vimolmangkang, X. Deng, A. Owiti, T. Meelaph, C. Ogutu, Y. Han, *Sci. Rep.* **2016**, *6*, 26323.
[127] N. G. Schmidt, E. Eger, W. Kroutil, *ACS Catal.* **2016**, *6*, 4286-4311.
[128] D. K. Liscombe, P. J. Facchini, *Curr. Opin. Biotechnol.* **2008**, *19*, 173-180.
[129] S. Wallner, C. Dully, B. Daniel, P. Macheroux, in *Flavoproteins.* De Gruyter, Berlin, **2012**, pp. 1-30
[130] D. Yan, C. Jin, X. H. Xiao, X. P. Dong, *J. Biochem. Biophys. Methods* **2008**, *70*, 845-849.
[131] M. Bahar, Y. Deng, X. Zhu, S. He, T. Pandharkar, M. E. Drew, A. Navarro-Vázquez, C. Anklin, R. R. Gil, R. W. Doskotch, K. A. Werbovetz, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2606-2610.
[132] S. Q. Wen, P. Jeyakkumar, S. R. Avula, L. Zhang, C. H. Zhou, *Bioorg. Med. Chem. Lett.* **2016**, *26*, 2768-2773.
[133] C. Y. Lo, L. C. Hsu, M. S. Chen, Y. J. Lin, L. G. Chen, C. D. Kuo, J. Y. Wu, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 305-309.
[134] W. Ma, M. Zhu, D. Zhang, L. Yang, T. Yang, X. Li, Y. Zhang, *Phytomedicine* **2017**, *25*, 45-51.

[135] F. Morceau, S. Chateauvieux, M. Orsini, A. Trécul, M. Dicato, M. Diederich, *Biotechnol. Adv*. **2015**, *33*, 785-797.
[136] C. R. Naveen, S. Gaikwad, R. Agrawal-Rajput, *Phytomedicine* **2016**, *23*, 736-744.
[137] N. Wang, H. Y. Tan, L. Li, M. F. Yuen, Y. Feng, *J. Ethnopharmacol*. **2015**, *176*, 35-48.
[138] H. S. Bodiwala, S. Sabde, D. Mitra, K. K. Bhutani, I. P. Singh, *Eur. J. Med. Chem*. **2011**, *46*, 1045-1049.
[139] R. Li, J. Wu, Y. He, L. Hai, Y. Wu, *Bioorg. Med. Chem. Lett*. **2014**, *24*, 1762-1765.
[140] W. Kong, J. Wei, P. Abidi, M. Lin, S. Inaba, C. Li, Y. Wang, Z. Wang, S. Si, H. Pan, S. Wang, *Nature Med*. **2004**, *10*, 1344-1351.
[141] G. Enkhtaivan, P. Muthuraman, D. H. Kim, B. Mistry, *Bioorg. Med. Chem*. **2017**, *25*, 5185-5193.
[142] A. Kumar, K. Chopra, M. Mukherjee, R. Pottabathini, D. K. Dhull, *Eur. J. Pharmacol*. **2015**, *761*, 288-297.
[143] P. Steffens, N. Nagakura, M. H. Zenk, M.H., *Phytochemistry* **1985**, *24*, 2577-2583.
[144] E. Rink, H. Böhm, *FEBS Lett*. **1975**, *49*, 396-369.
[145] J. E. Vick, C. Schmidt-Dannert, *Angew. Chem. Int. Ed*. **2011**, *50*, 7476-7478.
[146] T. M. Kutchan, H. Dittrich, *J. Biol. Chem*. **1995**, *270*, 24475-24481.
[147] A. Winkler, F. Hartner, T. M. Kutchan, A. Glieder, P. Macheroux, *J. Biol. Chem*. **2006**, *281*, 21276-21285.
[148] T. M. Kutchan, A. Bock, H. Ditrrich, *Phytochemistry* **1994**, *35*, 353-360
[149] P. J. Facchini, C. Penzes, A. G. Johnson, D. Bull, *Plant Physiol*. **1996**, *112*, 1669-1677.
[150] H. Dittrich, T. M. Kutchan, *Proc. Natl. Acad. Sci. U.S.A*. **1991**, *88*, 9969-9973.
[151] D. A. Bird, P. J. Facchini, *Planta* **2001**, *213*, 888-897.
[152] A. Winkler, T. M. Kutchan, P. Macheroux, *J. Biol. Chem*. **2007**, *282*, 24437-24443.
[153] D. E. Edmondson, C. Binda, A. Mattevi, *Arc. Biochem. Biophys*. **2007**, *464*, 269-276.
[154] N. S. Scrutton, *Nat. Prod. Rep*. **2004**, *21*, 722-730.
[155] A. Winkler, A. Łyskowski, S. Riedl, M. Puhl, T. M. Kutchan, P. Macheroux, K. Gruber, *Nature Chem. Biol*. **2008**, *4*, 739-741.
[156] H. M. Gaweska, K. M. Roberts, P. F. Fitzpatrick, *Biochemistry* **2012**, *51*, 7342-7347.
[157] P. C. Yan, J. H. Xie, G. H. Hou, L. X. Wang, Q. L. Zhou, *Adv. Synth. Catal*. **2009**, *351*, 3243-3250.
[158] C. Shi, I. Ojima, *Tetrahedron* **2007**, *63*, 8563-8570.
[159] C. Dubs, Y. Hamashima, N. Sasamoto, T. M. Seidel, S. Suzuki, D. Hashizume, M. Sodeoka, *J. Org. Chem*. **2008**, *73*, 5859-5871.
[160] J. H. Schrittwieser, V. Resch, J. H. Sattler, W. D. Lienhart, K. Durchschein, A. Winkler, K. Gruber, P. Macheroux, W. Kroutil, *Angew. Chem. Int. Ed*. **2011**, *50*, 1068-1071.
[161] V. Resch, J. H. Schrittwieser, S. Wallner, P. Macheroux, W. Kroutil, *Adv. Synth. Catal*. **2011**, *353*, 2377-2383.
[162] J. H. Schrittwieser, V. Resch, S. Wallner, W. D. Lienhart, J. H. Sattler, J. Resch, P. Macheroux, W. Kroutil, *J. Org. Chem*. **2011**, *76*, 6703-6714.
[163] V. Resch, H. Lechner, J. H. Schrittwieser, S. Wallner, K. Gruber, P. Macheroux, W. Kroutil, *Chem. Eur. J*. **2012**, *18*, 13173-13179.
[164] J. H. Schrittwieser, V. Resch, *RSC Adv*. **2013**, *3*, 17602-17632.
[165] S. Gandomkar, E. M. Fischereder, J. H. Schrittwieser, S. Wallner, Z. Habibi, P. Macheroux, W. Kroutil, *Angew. Chem. Int. Ed*. **2015**, *54*, 15051-15054.
[166] J. H. Schrittwieser, B. Groenendaal, V. Resch, D. Ghislieri, S. Wallner, E. M. Fischereder, E. Fuchs, B. Grischek, J. H. Sattler, P. Macheroux, N. J. Turner, *Angew. Chem. Int. Ed*. **2014**, *53*, 3731-3734.
[167] S. Van de Vyver, Y. Román-Leshkov, *Angew. Chem. Int. Ed*. **2015**, *54*, 12554-12561.
[168] J. Schückel, E. L. Rylott, G. Grogan, N. C. Bruce, *ChemBioChem* **2012**, *13*, 2758-2763.
[169] Enzyme nomenclature 1992: Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes (Eds: E. Webb), Academic Press, New York.
[170] B. Daniel, B. Konrad, M. Toplak, M. Lahham, J. Messenlehner, A. Winkler, P. Macheroux, *Arch. Biochem. Biophys*. **2017**, *632*, 88-103.
[171] B. Daniel, S. Wallner, B. Steiner, G. Oberdorfer, P. Kumar, E. van der Graaff, T. Roitsch, C. W. Sensen, K. Gruber, P. Macheroux, *PloS One* **2016**, *11*, e0156892.
[172] E. Kim, B. S. Moore, Y. J. Yoon, *Nature Chem. Biol*. **2015**, *11*, 649-659.
[173] B. Shen, *Cell* **2015**, *163*, 1297-1300.
[174] C. K. Winkler, K. Faber, M. Hall, *Curr. Opin. Chem. Biol*. **2018**, *43*, 97-105

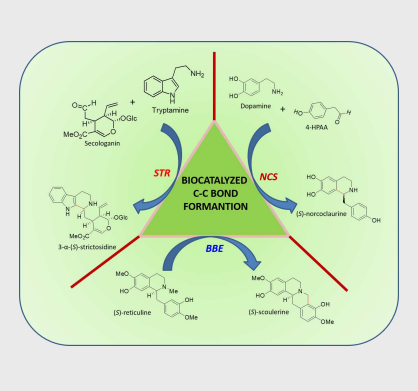
10.1002/cctc.201801130

WILEY-VCH

Table of Contents

REVIEW

This review provides a comprehensive account of three important C-C bond forming enzymes, the Pictet-Spenglerases Strictosidine synthase (STR) and Norcoclaurine synthase (NCS), and the oxidative C-C bond forming flavoenzyme Berberine Bridge Enzyme (BBE). Advances achieved in the enzymatic and chemo-enzymatic synthesis of pharmaceutically important alkaloids are discussed.



*Mahesh D. Patil, Gideon Grogan, and Hyungdon Yun**

Page No. – Page No.

Biocatalyzed C-C bond formation for the production of alkaloids