Terpene Indole Alkaloid Biosynthesis

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TABLE OF CONTENTS

INTRODUCTION

Microbes and plants have evolved and produce a large array of complex molecules secondary metabolites.[1-3] Secondary metabolites– natural products– are some of the most structurally interesting molecules found in nature, and serve as effective pharmaceutical agents.

Alkaloids, nitrogen containing compounds produced by higher plants, are arguably some of the most chemically complex molecules found in nature, and exhibit a diverse array of potent biological activities.[4] However, the biosynthetic pathways of these molecules are more challenging to elucidate then the polyketide and peptide

pathways found in microbial organisms.[5] First, eukaryotic plant hosts are slower growing and developmentally more complex than prokaryotes, and genomic sequence data of medicinal plants are unavailable. Furthermore, the genes of a given metabolic pathway are physically clustered on the genome of a microbial organism; once a fragment of the metabolic pathway has been identified, it is a relatively straightforward process to sequence the adjoining DNA that contains the remainder of the pathway. Plant pathways are generally not clustered, meaning that each plant enzyme of a pathway must be individually isolated and cloned independently of one another. For these reasons, despite the medicinal importance of plant natural products, plant pathways are not as well understood as bacterial natural product pathways.

To date, comparatively few enzymes involved in plant alkaloid biosynthesis have actually been cloned, though more enzymes have been purified.[6] Many plant enzymes are characterized by "reverse genetics" in which the enzymes are isolated from plants or plant cell culture by traditional biochemical chromatography techniques.[6] More recently, plant cDNA libraries have been screened for the presence of P450 enzyme or acetyl transferase homologues.[7,8] If the appropriate DNA libraries or arrays are available, cDNA subtraction techniques can be used to compare the differences in gene expression between alkaloid producing and non-producing plants.[9] These sequencing and proteomics technologies have been sufficiently refined such that they can be used to address the challenges associated with plant pathways. Therefore, although study of plant alkaloid pathways remains a challenging prospect, modern technologies are beginning to transform the study of plant secondary metabolism.

TERPENE INDOLE ALKALOIDS

The terpene indole alkaloids, produced primarily by the *Apocynaceae* and *Rubiaceae*, effectively illustrate the complexity of alkaloid biosynthesis. The terpene indole alkaloids are a particularly diverse class of natural products, comprising approximately 3000 members possessing a range of chemical structures and a wealth of biological activities (Fig. # 1).[10,11] A number of terpene indole alkaloids are used as anti-cancer, anti-malarial and anti-arrhythmic agents (Fig. # 1).[4] In the US, vinblastine (Velban) and vincristine (Oncovin)[12] are used clinically to treat cancers including Hodgkin's disease,[13] non-Hodgkin's lymphoma [14] and Kaposi's sarcoma.[15] Notably, 500 kilograms of the plant *Catharanthus roseus* are required to produce 1 gram of vincristine (a yield of 0.0002%),[16] and total synthesis of this compound is not practical on an industrial scale.[17]

EARLY ENZYMES OF TERPENE INDOLE ALKALOID BIOSYNTHESIS

The first few steps of TIA biosynthesis are well known and are outlined in Fig. # 2. Terpene indole alkaloids are derived from tryptophan,[18] which is decarboxylated to yield tryptamine.[19] The involvement of the monoterpene iridoid secologanin [20-24] has been established. Strictosidine (*S* stereochemistry at C5) is a common intermediate for all terpene indole alkaloids. [25-30]

The enzymes that catalyze these first steps of terpene indole alkaloid biosynthesis are known and have been cloned. Tryptophan decarboxylase, a pyridoxal dependent enzyme,[31,32] converts tryptophan to tryptamine. Strictosidine synthase catalyzes the stereoselective Pictet-Spengler condensation [33,34] of tryptamine (**1**, Fig. # 2) and the aldehyde secologanin (**2**) to yield strictosidine (**3**).[35-47] Secologanin is a natural product in its own right, and a few of the enzymes responsible for secologanin biosynthesis have

also been isolated.[48-50] Previous studies with strictosidine synthase from the plants *Catharanthus roseus* and *Rauwolfia serpentina* have reported K_m values of 20-200 μM for tryptamine (no reported K_m for secologanin) and a range of V_{max} values. A limited number of alternate substrates have been tested with strictosidine synthase, including *N*-substituted tryptamine, tryptophan, phenylethylamine, tyramine, and a variety of iridoid aldehydes.[36]

Strictosidine Synthase Tryptamine Substrate Specificity[51]

The Pictet-Spengler cyclization is critical for the biosynthesis of thousands of alkaloids. We sought to expand our understanding of strictosidine synthase by systematically probing the electronic and steric requirements of the indole substrate and quantifying the steady state kinetics for each of these substrates. Both the 3-(2-aminoethyl)-benzofuran (**4**) [52] and benzothiophene (**5**) [53] analogs have been assayed. Precedence exists for benzofuran and benzothiophene heterocycles with interesting biological properties.[54,55] Both compounds are turned over by strictosidine synthase in the presence of the aldehyde substrate secologanin **2**, and a single diastereomer is observed, indicating that enantioselective enzymatic catalysis is not compromised (Table # 1). The alternate heterocycles are turned over by strictosidine synthase at a diminished rate relative to the tryptamine **1** substrate. Although the low activity of the thiophene substrate precluded a quantitative comparison of **4** and **5**, the rate of reaction of benzothiophene **5** is significantly slower than benzofuran **4**. Notably, no chemical reaction of 3-(2-aminoethyl)-benzofuran (**4**) and 3-(2-aminoethyl)-benzothiophene (**5**) occurred at 40 mM concentration under mild acidic conditions, demonstrating that the

enzyme can catalyze product formation with relatively chemically inactive substrates with complete enantioselective control.

Since the Pictet-Spengler cyclization is inherently dependent on an electron rich aminoethylarene substrate,[34] the decreased electron density of the benzofuran and benzothiophene rings may cause the slower rate compared to indole. Alternatively, strictosidine synthase may utilize a specific hydrogen bonding interaction to the indole nitrogen. The benzofuran **4** exhibits a K_m value close to that of tryptamine, but displays a significantly reduced k_{cat}, suggesting that the electron deficient nature of the heterocylic ring is slowing catalysis (Table # 2). *N*-methyl tryptamine **6** (Table # 1) was not a competent substrate, suggesting that the enzyme tolerates only small steric perturbations at the indole nitrogen. To further explore the effect of electron density on catalysis, the indole ring was substituted with electron-withdrawing substituents (fluoro=F) at each of the indole ring positions (Table # 1). Substitution with a fluoro moiety results in a decrease in k_{cat} in each case, suggesting that the enzymatic reaction is inherently dependent on the electron density of the substrate.

Early qualitative studies, performed after strictosidine synthase was first isolated, indicated that some substitution on the indole ring was tolerated.[36] To rigorously quantify the effect of indole ring substitution on catalysis, each position of the indole ring was systematically substituted with a methyl group (compounds **11**-**14**, Table # 1), and the kinetic parameters of active substrates were measured. In general, reactivity of substrates with methyl substitutions in the 4 (compound **11**) and 7 (compound **14**) indole positions was significantly higher than substrates with substitutions at the 5 (compound **12**) and 6 (compound **13**) positions (Tables # 1 and # 2). The K_m for the 4-substituted tryptamine

analog **11** was approximately 2-fold lower than the K_m for 7-substituted compound **14**, while substrates with methyl moieties in the 5 and 6 positions– compounds **12** and **13**– were completely inactive. Substitution with a hydroxyl group in the 5 position (compound **15**, Table # 1 and Table # 2) did yield an active substrate, although the K_m was the highest measured in this series– a 60-fold increase compared to the native substrate tryptamine. Therefore, the binding pocket of the enzyme can better tolerate a hydrophilic hydroxyl substituent than a hydrophobic methyl group at the 5 position.

The 2-pyrrole-3-ethylamine analog (**19**) has been shown to undergo a non-enzymatically catalyzed Pictet-Spengler reaction.[56] Surprisingly, this smaller substrate along with the isosteric histamine (**20**) were not turned over by strictosidine synthase, indicating that the benzyl moiety is absolutely required for recognition by the enzyme (Table # 1). It was previously established that tryptophan, phenylethylamine and tyramine are not accepted by strictosidine synthase,[36] and since pyrrole substrates are also not tolerated, we conclude that the basic indole framework is required for recognition by this enzyme. Interestingly, the only other sequenced "Pictet-Spenglerase" (norcoclaurine synthase), which utilizes tyrosine derived amine and aldehyde substrates, exhibits no sequence homology to strictosidine synthase.[57]

Strictosidine Synthase Secologanin Substrate Specificity

Several naturally occurring iridoid terpenes had been previously shown to fail to serve as competent aldehyde substrates in place of secologanin.[36] Therefore, we modified two of the key functional groups of the secologanin substrate to assess the aldehyde substrate requirements. A streamlined gram-scale isolation protocol of secologanin from a local source of *Lonicera tatarica* enabled a semisynthetic approach to yield secologanin

derivatives. Olefin cross metathesis was used to introduce a variety of alkyl groups at the vinyl position of secologanin (i.e. compounds **21** and **22**, Table # 1).[58] Since a reduced version of secologanin, in which the vinyl group is hydrogenated to yield a saturated single C-C bond, had been previously shown to be a competent substrate,[36] we were optimistic that this position could be derivatized. However, our assays indicated that bulkier groups at the vinyl position completely prevented turnover (Table # 1). In contrast, trans-esterification at the methyl ester with larger alkyl groups[58] gave substrates **23** and **24** that were turned over by the enzyme to yield the corresponding strictosidine analogs, suggesting that this is a more promising position for derivatization (Table # 1).

ENZYMES AFTER STRICTOSIDINE SYNTHASE

In the first enzymatic step after strictosidine formation, the glucose of strictosidine is enzymatically hydrolyzed to reveal a reactive hemi-acetal (Fig. # 2). In essence, the glucose moiety is serving as a protecting group to mask a reactive species, a strategy that is utilized in other plant biosynthetic pathways such as the cyanogenic glucosides and the glucosinolates.[2] The dedicated glycosidase, strictosidine-β-glucosidase has been isolated and cloned from *Catharanthus roseus* and *Rauwolfia serpentina*.[59-63] Based on its amino acid sequence, strictosidine glucosidase is predicted to be a type 1 beta glycosyl hydrolase with a retaining mechanism.[64] Some biochemistry of the glucosidase from the plants *C. roseus* and *R. serpentina* has been investigated.[59-63,65] K_m values of ~100-200 μM for strictosidine, a pH optimum of 5-8.5, and a range of V_{max} values have been reported for this enzyme.

Fig. # 3 summarizes much of what is known about the enzymes of alkaloid biosynthesis that act after strictosidine deglycosylation.[6] In ajmaline biosynthesis, at least

eight enzymes are predicted to catalyze the subsequent steps after strictosidine deglycosylation. Two of these enzymes have been cloned,[66,67] and the remainder have been either purified or detected in crude cell extracts.[68-75] The pathway for ajmaline biosynthesis is arguably the best-characterized terpene indole alkaloid pathway. Five enzymes are predicted to catalyze the transformations leading from tabersonine **(23**) to vindoline **(24**) [76]. Three of these enzymes have been cloned[77-80] and the remainder have been partially purified.[81-84]

STRUCTURAL DIVERSITY OF TERPENE INDOLE ALKALOIDS

A number of examples of the terpene indole alkaloid classes, each arising from rearrangement of strictosidine, are shown in Fig. # 4. How the wealth of TIA structures each derive from the deglycosylated strictosidine intermediate remains one of the most fascinating problems in secondary metabolism. Extensive feeding studies and biomimetic syntheses executed in the 1960's and 1970's yielded chemical information about how this branching process might occur. A figure summarizing some of the conclusions is presented below (Fig. # 5). After deglycosylation of strictosidine (**3**) the resulting aglycone (**25**) opens to form an intermediate often referred to as a dialdehyde (**26**). The resulting aldehyde then reacts with the secondary amine to form a six membered ring to yield dihydrocorynanthe aldehyde (**27**). Dihydrocorynanthe aldehyde can undergo allylic isomerization and enolization to produce either the enol (**28**) or keto (**29**) forms of dehydrogeissoschizine. Dehydrogeissoschizine is believed to be a central intermediate in TIA biosynthesis. Dehydrogeissoschizine can be reduced by a dehydrogenase enzyme to yield geissoschizine (**30**), an intermediate that may also play a role in TIA biosynthesis.[85-87]

It is likely that the enol form of dehydrogeissoschizine **28** will undergo 1,4 conjugate addition to produce the heteroyohimbine cathenamine (**33**). Early biomimetic syntheses support the hypothesis that cathenamine can be produced from dehydrogeissoschizine.[88] An equilibrium between cathenamine and dehydrogeissoschizine has also been observed.[89,90] Stereoselective reduction of cathenamine will yield ajmalicine **34**, and further oxidation will yield serpentine **35**.[91-94] Cathenamine **9** is the major product isolated after incubation of strictosidine with strictosidine-β-glucosidase *in vitro*.[60] Therefore, an enzymatic pathway to the corynanthe skeleton from strictosidine appears to be relatively straightforward.

The enzymatic conversion of deglycosylated strictosidine to the other classes of alkaloids remains much less clear. For yohimbine (**38**) formation, a direct biosynthetic route could involve homoallylic isomerization of the keto dehydrogeissoschizine **29** followed by 1,4 conjugate addition.[95] The structurally more complex aspidosperma, strychnos and iboga alkaloids may each be derived from the corynanthe alkaloids.[96] This hypothesis is indirectly supported by observation that the corynanthe alkaloids are produced early in the lifetime of the *Catharanthus roseus* plant, with the aspidosperma and iboga alkaloids appearing after the plant ages.[96] Deglycosylated strictosidine can rearrange to form the strychnos, aspidosperma and iboga alkaloids. Although the details of the pathway are not absolutely certain, it is generally agreed that dehydrogeissoschizine (**28**) can rearrange to form a strychnos-like intermediate termed preakkumacine (**39**).[97] Stemmadenine (**40**) could then in turn rearrange to form an acrylic ester (**41**) that could serve as a common intermediate for the aspidosperma (*i.e.* tabersonine **43**) and the iboga skeletons (*i.e.* catharanthine **42**). A few of the key

experiments and hypotheses are described in the following selected references.[20,97-117] (Some of these findings are reviewed.[115,118-121])

The branching among these enzymes is in part controlled by the species of plant. While the corynanthe, iboga, and aspidosperma alkaloids are observed in *Catharanthus roseus* plants, strychnine (*Strychnos nux vomica*) and ajmaline (*Rauwolfia*) are not. Moreover, the aspidosperma and iboga alkaloids appear to be concentrated in the ariel portions of *C. roseus*, while the corynanthe appear primarily in the roots [122]. The coexistence of multiple pathways–the corynanthe, aspidosperma and iboga– makes *Catharanthus* an intriguing system to monitor alkaloid biosynthesis.

TURNOVER OF STRICTOSIDINE ANALOGS BY STRICTOSIDINE GLUCOSIDASE

Chemical synthesis of complex natural products is often impractical on a commercial scale, and isolation of these compounds from the environment can also be an expensive and low yielding process. Furthermore, isolation procedures provide limited opportunities to modify the chemical and biological properties of the natural product. Understanding the enzymes that catalyze natural product synthesis may enable production in more tractable host organisms and may also facilitate reprogramming of biosynthetic pathways to produce "unnatural" natural products with potentially improved pharmacological activities. Natural products from polyketide,[123,124] non-ribosomal peptide,[125] terpene,[126] and saccharide [127] biosynthetic pathways have been heterologously expressed in organisms that are faster growing or easier to culture. Although metabolic engineering has proven remarkably successful in polyketide biosynthetic and nonribosomal peptide pathways,[123,128,129] in terpene indole alkaloid biosynthesis, the

backbone of strictosidine is significantly rearranged over the course of several steps, whereas polyketides and nonribosomal peptides are synthesized by an iterative, "assembly-line" process, in which a linear chain is successively elongated.[130] Can a "non-modular" pathway process unnatural substrates to yield novel alkaloids?

While initial results suggest that strictosidine synthase can produce a range of strictosidine analogs, it remains to be established whether these alternate intermediates can be processed by the downstream terpene indole alkaloid machinery to produce novel, biologically active alkaloids. In the next step of the pathway, a dedicated glucosidase hydrolyzes the glycosidic linkage of strictosidine to yield cathenamine **33** (Fig. # 5).[59,60]

To evaluate whether cathenamine derivatives could be enzymatically produced from the corresponding strictosidine analogs, we incubated all enzymatically generated strictosidine derivatives (Table # 1, column 4) with the second enzyme of the pathway, strictosidine-β-glucosidase. All strictosidine derivatives were processed by strictosidine glucosidase as monitored by the disappearance of the strictosidine derivative peak by HPLC. These results suggest that the substrate specificities of strictosidine synthase and glucosidase are sufficiently complementary to produce a variety of terpene indole alkaloid intermediate analogs.

SUMMARY AND FUTURE DIRECTIONS

The terpene indole alkaloids are a diverse family of plant-derived compounds that exhibit numerous potent pharmaceutical properties. Strictosidine synthase catalyzes a Pictet-Spengler reaction in the first step in the biosynthesis of terpene indole alkaloids to generate strictosidine. This β-carboline intermediate is next turned over by strictosidine

glucosidase to yield a reactive intermediate that rearranges to form the fused 5-ring cathenamine structure. Subsequent enzymes are responsible for converting this early intermediate into a structurally diverse set of alkaloids. We have systematically probed the substrate requirements for strictosidine synthase and shown that the enzymatically generated unnatural strictosidine intermediates are processed by the glucosidase to yield analogs of the corynanthe type. As more enzymes in the terpene indole alkaloid pathways are cloned, the substrate specificity of these enzymes can also be evaluated to determine whether this biosynthetic pathway can potentially be used to produce novel alkaloid derivatives.

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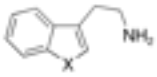
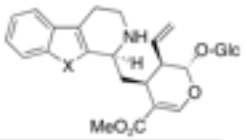
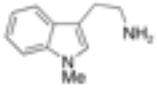
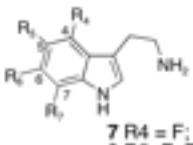
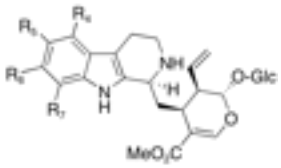
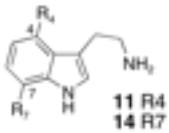
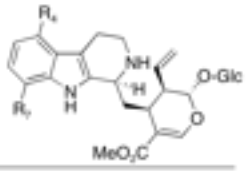
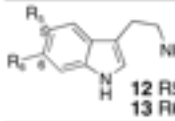
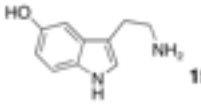
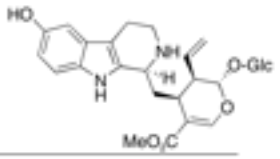
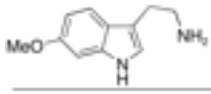
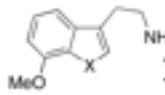
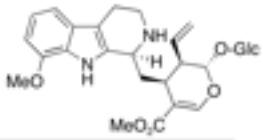
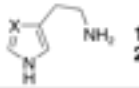
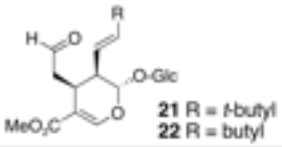
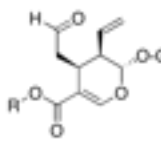
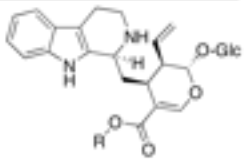
Table 1. Substrates tested with strictosidine synthase and the resulting products.

Amine Substrate	Aldehyde Substrate	Strictosidine Analog
 **4** X = O **5** X = S	**2**	
 **6**	**2**	**no reaction**
 **7** R4 = F; R5, R6, R7 = H **8** R5=F; R4, R6, R7 = H **9** R6=F; R4, R5, R7 = H **10** R7=F; R4, R5, R6 = H	**2**	
 **11** R4 = Me; R7 = H **14** R7 =Me; R4 = H	**2**	
 **12** R5 = Me; R6 = H **13** R6 = Me; R5 = H	**2**	**no reaction**
 **15**	**2**	
 **16**	**2**	**no reaction**
 **17** X = NH **18** X = O	**2**	
 **19** X = CH **20** X = N	**2**	**no reaction**
1	 **21** R = *t*-butyl **22** R = butyl	**no reaction**
1	 **23** R = ethyl **24** R = allyl	

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1}/s^{-1})
1	7.4	0.9	2,030
4	7.7	0.023	50
7	42	0.35	139
8	7.1	0.043	101
9	8.9	0.056	105
10	13	0.11	141
11	80	0.19	40
14	198	0.29	24
15	1,200	0.096	1.3

Table 2. Kinetic parameters for the most highly active amine strictosidine synthase substrates. k_{cat} and K_m were measured using a purified *E. coli* preparation of strictosidine synthase.

FIGURE LEGENDS

Figure 1. Representative terpene indole alkaloids.

Figure 2. The early steps of terpene indole alkaloid biosynthesis. Strictosidine synthase sets the stereochemistry at C5.

Figure 3. This scheme summarizes much of the current knowledge of TIA enzymes that act after the deglycosylation of strictosidine. **A**. Ajmaline biosynthesis. SB, sarpagan bridge enzyme; PAE, polyneuridine aldehyde reductase; VS, vinorine synthase; VH, vinorine hydroxylase; VR, vomilenine reductase(s); AE, 17-*O*-acetyl-ajmalan acetylesterase; NMT, norajmaline-*N*-methyltransferase. Only polyneuridine aldehyde reductase and vinorine synthase have been cloned. **B**. Vindoline biosynthesis from tabersonine. T16H, tabersonine-16-hydroxylase; HTOM, 16-hydroxytabersonine-16-O-methyltransferase; NMT, N-methyltransferase; D4H, desacetoxyvindoline-4-hydroxylase; DAT, desacetylvindoline O-acetyltransferase. Tabersonine-16-hydroxylase,

desacetoxyvindoline-4-hydroxylase and desacetylvindoline O-acetyltransferase have been cloned.

Figure 4. Representative classes of the terpene indole alkaloids. The name of the alkaloid class is given in parentheses below the name of the molecule. Each of these alkaloids is derived from the common intermediate strictosidine.

Figure 5. Summary of some of the key rearrangements of the intermediates of the terpene indole alkaloid biosynthetic pathway to yield the corynanthe, aspidosperma, and iboga skeletons. The pathways are hypothesized from evidence derived from feeding experiments, biomimetic syntheses and transformations with crude cell extracts.

FIGURES

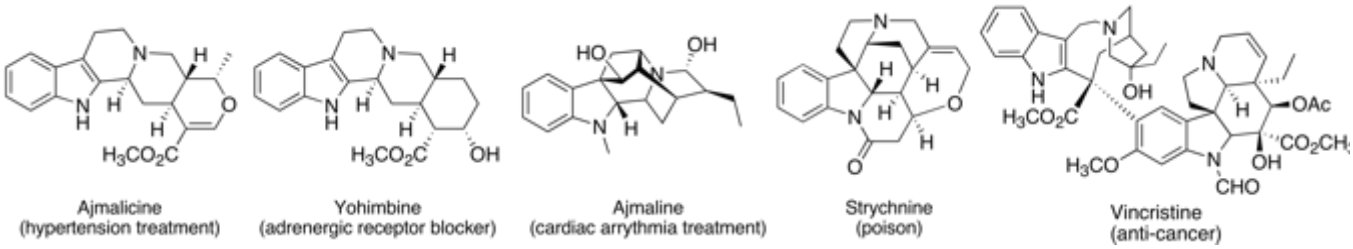


Figure 1.

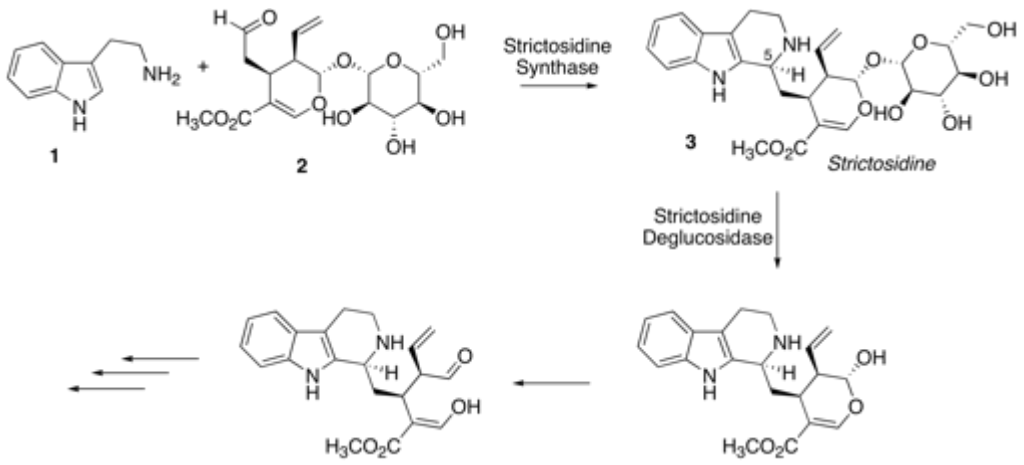

Strictosidine
Synthase
Strictosidine
Deglucosidase
1
2
3
Strictosidine

Figure 2.

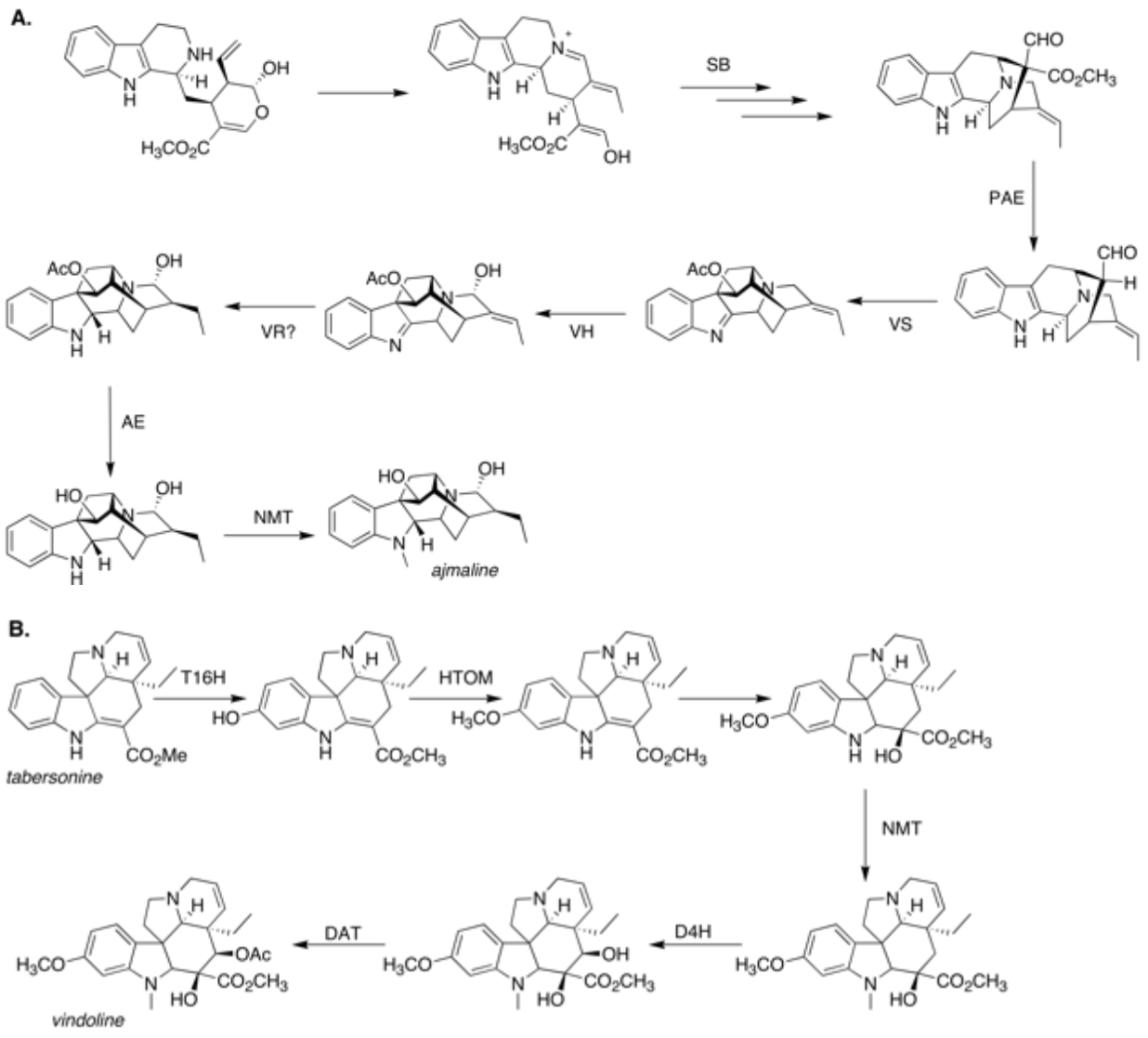

A.
SB
PAE
VS
VH
VR?
AE
NMT
ajmaline
B.
T16H
HTOM
NMT
D4H
DAT
tabersonine
vindoline

Figure 3.

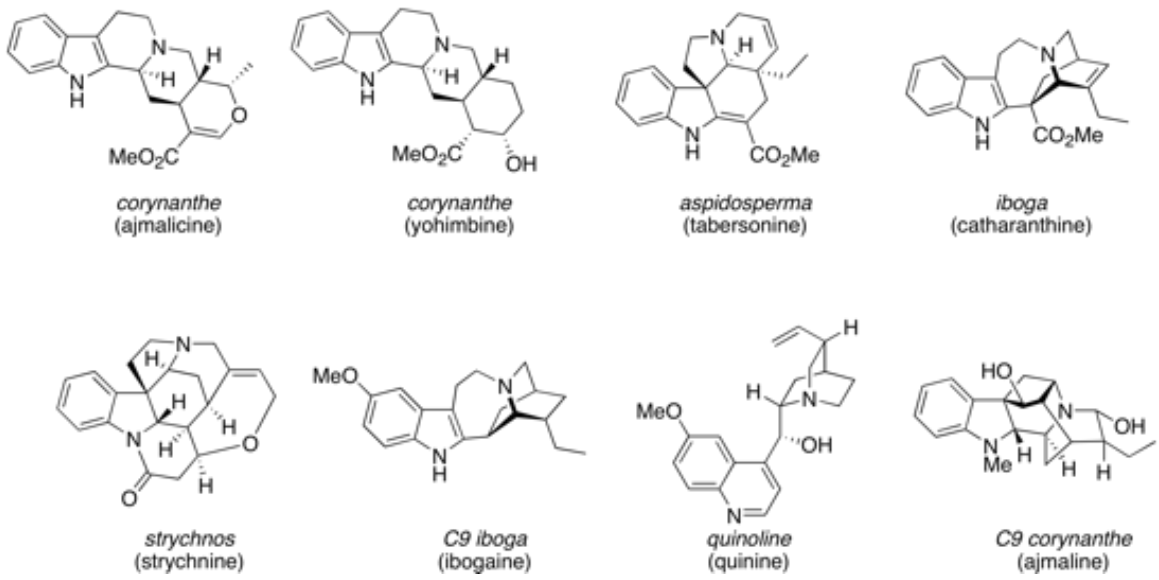

corynanthe
(ajmalicine)
corynanthe
(yohimbine)
aspidosperma
(tabersonine)
iboga
(catharanthine)
strychnos
(strychnine)
C9 iboga
(ibogaine)
quinoline
(quinine)
C9 corynanthe
(ajmaline)

Figure 4.

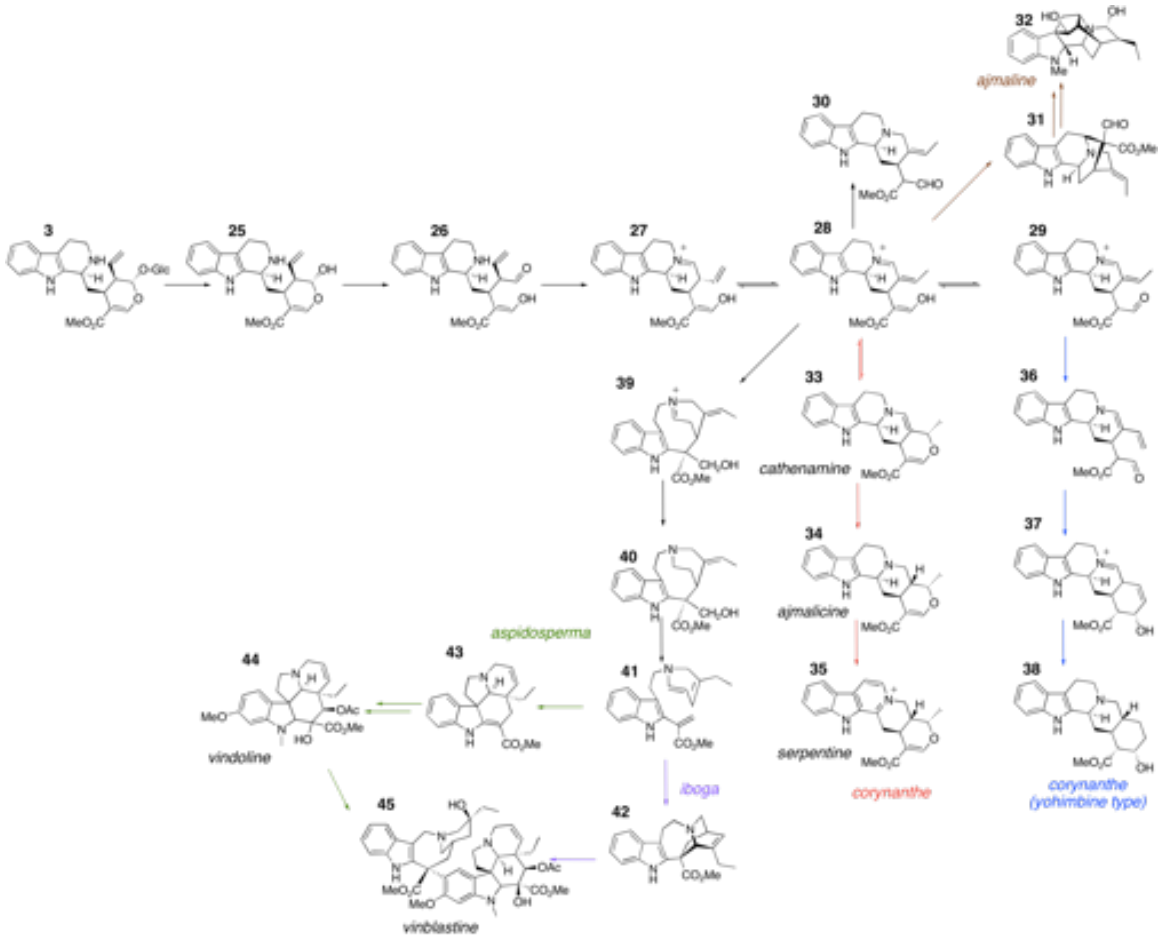

3
25
26
27
28
29
30
31
32
ajmaline
cathenamine
ajmalicine
serpentine
corynanthe
corynanthe (yohimbine type)
39
40
41
42
33
34
35
36
37
38
aspidosperma
iboga
43
44
45
vindoline
vinblastine

Figure 5.

KEYWORDS

terpene indole alkaloid, biosynthesis, Pictet-Spengler