Biosynthesis of the α-D-Mannosidase Inhibitor (–)-Swainsonine

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Abstract (150 words)

(–)-Swainsonine is a polyhydroxylated indolizidine alkaloid with potent inhibitory activity against α-D-mannosidases. In this work, we successfully reconstituted swainsonine biosynthetic pathway both in vivo and in vitro. Our study unveiled a surprising epimerization mechanism involving an imine reductase (SwnN) and two non-heme iron and α-ketoglutarate-dependent oxygenases (SwnH2 and SwnH1). SwnH2 is a multifunctional enzyme responsible for both C2-hydroxylation and amine desaturation, while SwnN completes the oxidation-reduction sequence to achieve the net redox neutral epimerization. Notably, SwnN exhibits substrate-dependent stereospecificity and the stereochemical outcome of SwnN-catalyzed iminium reduction is determined by SwnH1-catalyzed C8-hydroxylation. We also discovered that an *O*-acetyl ester can serve as a detachable protecting/directing group, altering the site-selectivity of SwnH2-catalyzed hydroxylation while maintaining the stereoselectivity. Insights gained from the biochemical characterization of these tailoring enzymes enabled biocatalytic synthesis of a new polyhydroxylated indolizidine alkaloid, opening doors to the biosynthesis of diverse natural product-based glycosidase inhibitors.

Introduction

Polyhydroxylated alkaloids, also known as iminosugar alkaloids, are a class of natural products recognized for their potent and highly selective inhibitory activity against glycosidases (**Figure 1A**).[1] These compounds structurally mimic the transition state of glycoside-cleavage reactions, allowing them to bind tightly to the active sites of glycosidases, effectively inhibiting their function.[2] This distinctive mode of action makes polyhydroxylated alkaloids valuable chemical tools for investigating glycosidase-related cellular processes, and offers opportunities to develop therapeutic agents for treating diseases where glycosidase is a key drug target, such as cancer, diabetes, viral infections, and metabolic disorders.[3]

(–)-Swainsonine (SW) is a polyhydroxylated indolizidine alkaloid that was initially isolated from Darling pea (*Swainsona canescens*)[4] and the fungus *Rhizoctonia leguminicola*.[5] It is also found in spotted locoweed (*Astragalus* and *Oxytropis*) and has been identified as the causative agent of "locoism" in grazing animals.[6] The toxicosis of SW is attributed to its inhibition of lysosomal α-mannosidase, which results in the accumulation of mannose-rich oligosaccharides, a symptom similar to that of the genetic disorder α-mannosidosis.[7] SW is also a potent inhibitor of Golgi α-mannosidase II, an enzyme essential for the maturation of *N*-linked oligosaccharides in newly synthesized glycoproteins.[8,9] As a result, SW was the first glycoprotein processing inhibitor to be considered for clinical trials for cancer treatment.[10] Furthermore, SW has also been utilized in developing therapeutic agents for treating viral infections and immunological disorders.[11,12]

Due to its remarkable biological activities and unique structure, SW has garnered significant interest as a synthetic target, leading to the development of numerous synthetic approaches for the total synthesis of SW and its analogues.[13-19] In contrast, despite decades of biosynthetic investigations, the underlying mechanism of SW biosynthesis remains obscure. The Harris group first demonstrated that SW is derived from L-pipecolic acid (PA)

and malonate, and observed an enigmatic epimerization process at the ring fusion (i.e. C8a position) during SW biosynthesis.[20-22] The inverted stereo-configuration of C8a contributes to SW's potent inhibition of α-D-mannosidase, as the inhibitory activity of chemically synthesized (–)-8a-*epi*-swainsonine decreases by 7% compared to SW.[23,24]

In 2017, Schardl and colleagues identified a conserved biosynthetic gene cluster (BGC) for SW in fungi, comprising a nonribosomal peptide-polyketide synthase (NRPS-PKS) hybrid, two non-heme iron and α-ketoglutarate (Fe/αKG)-dependent oxygenases, two short-chain dehydrogenases/reductases (SDRs), a transporter, and a pyridoxal 5'-phosphate-dependent aminotransferase (**Figure 1B**).[25] Based on the predicted gene functions, Schardl et al. proposed a plausible biosynthetic pathway for SW, but the exact function of each enzyme remains to be characterized (**Figure 1C**). Recently, Wang and coworkers conducted gene-deletion experiments and proposed a multibranched biosynthetic pathway for SW (**Figure 1D**).[26] Although this study provided new biosynthetic insights, many mechanistic questions remain unanswered. For instance, several indolizidine compounds have been observed (e.g. **3a/3b, 4a/4b**, and **5**), but their biosynthetic relevance is poorly understood. Furthermore, very little is known about the timing and mechanism of epimerization at the ring fusion. Additionally, the multibranched biosynthetic proposal does not fully align with some literature results. For example, the early feeding experiments indicated that the C1-proton from intermediate **3a** is retained during SW biosynthesis,[21] whereas the multibranched pathway suggests the involvement of various C1-epimer intermediates (e.g. **3a**/**3b** and **4a**/**4b**).

To address these long-standing biosynthetic questions, we reconstituted the SW biosynthetic pathway both in vivo and in vitro. We provided the biochemical basis for nearly every enzymatic transformation and resolved the decades-old puzzle regarding the epimerization mechanism at the ring fusion during SW biosynthesis. Our study unveiled a

unique process involving two Fe/αKG-dependent oxygenases (SwnH2 and SwnH1) and an NADPH-dependent reductase SwnN. We demonstrated that the net redox-neutral epimerization is achieved through an oxidation-reduction cascade, interrupted by a critical hydroxylation event that ultimately determines the stereochemical outcome of the final reduction step. Furthermore, we discovered that an *O*-acetyl ester can serve as a detachable protecting/directing group, altering the site-selectivity of SwnH2-catalyzed hydroxylation while preserving its stereoselectivity. Insights gained from the biochemical characterization of these tailoring enzymes enabled the biocatalytic synthesis of a new polyhydroxylated indolizidine alkaloid, paving the way for future biosynthesis of diverse natural product-based glycosidase inhibitors.

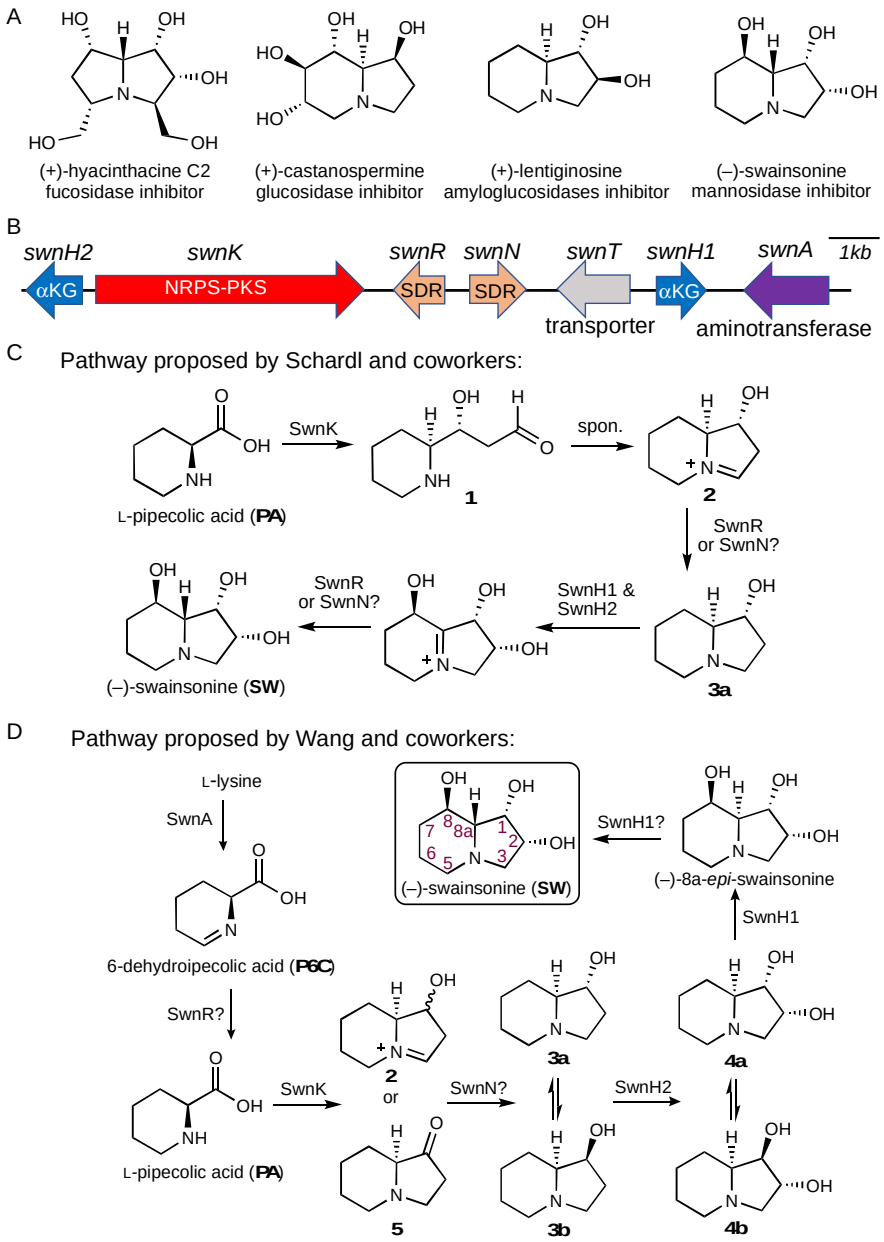

Figure 1. (–)-Swainsonine and other polyhydroxylated alkaloids. (A) Examples of polyhydroxylated alkaloids. (B) Swainsonine biosynthetic gene cluster from *Metarhizium robertsii* ARSEF 23 characterized in this study. (C) Swainsonine biosynthetic pathway proposed by Schardl et al.[25] (C) A multibranched swainsonine biosynthetic pathway proposed by Wang et al. [26]

Results

Biosynthesis of the indolizidine scaffold in SW

We began by reconstructing SW biosynthetic pathway in vivo. *Aspergillus nidulans* LO7890 was selected as the heterologous host for stepwise reconstitution.[27] Overexpression of the NRPS-PKS hybrid SwnK alone did not yield any detectable product **(Figure 2A**). However, co-expressing SwnK with the aminotransferase SwnA led to the production of **3a (**see **Table S2** for structural characterization). Introducing the two SDRs, SwnN and SwnR, into the co-expression system significantly increased the yield of **3a**, with the highest titer (~110 mg/L) achieved by co-expressing SwnR. It is important to note that we did not observe any spontaneous conversion of **3a** to **3b** in our experiments, as opposed to the interconversion reported by Wang et al.[26] It is likely that in the previous study, the authors may have misidentified these compounds, as **3a** can indeed exist in two forms (a salt form and a free-base form) with distinct NMR chemical shifts (**Figure S1**).

Analysis of the metabolite profile by GC-MS revealed a previously unidentified metabolite, 5,6,7,8-tetrahydroindolizine (**6**), in the absence of SwnN/SwnR (**Figure 2B**). Conversely, no significant amount of **6** was observed when either of the reductases was provided. We reasoned that **6** is likely a shunt product derived from **2**, the expected intermediate released from the NRPS-PKS assembly line. If the cyclic iminium moiety in **2** is not promptly reduced by an imine reductase, **2** may undergo dehydration and isomerization to give **6** (**Figure 2D**).

To confirm the enzymatic functions of SwnK, SwnN and SwnR, we set out to characterize their enzymatic activities in vitro. We purified recombinant SwnK from *Saccharomyces cerevisiae* BJ5464-npgA strain,[28] and purified SwnN and SwnR from *E. coli* (**Figure S2**). When all necessary substrates and cofactors (i.e. malonyl-CoA, NADPH, and pipecolic acid) were provided, SwnK readily synthesized **3a** in vitro, showing a preference for L-PA over D-PA as the amino acid precursor (**Figure S3**). Neither compound **2** nor the shunt

product **6** was observed in this assay, and the addition of SwnN or SwnR had no effect on the reaction outcome. These findings suggest that SwnK alone can catalyze the reduction of **2** to **3a**, either through its ketoreductase (KR) domain or the thioester reductase (R) domain. This result explains why trace amounts of **3a** was observed in vivo even in the absence of SwnN/SwnR (**Figure 2A, 2B**). However, for efficient product of **3a**, we propose that an imine reductase, such as SwnR, is still needed in order to minimize the formation of the shunt product **6**.

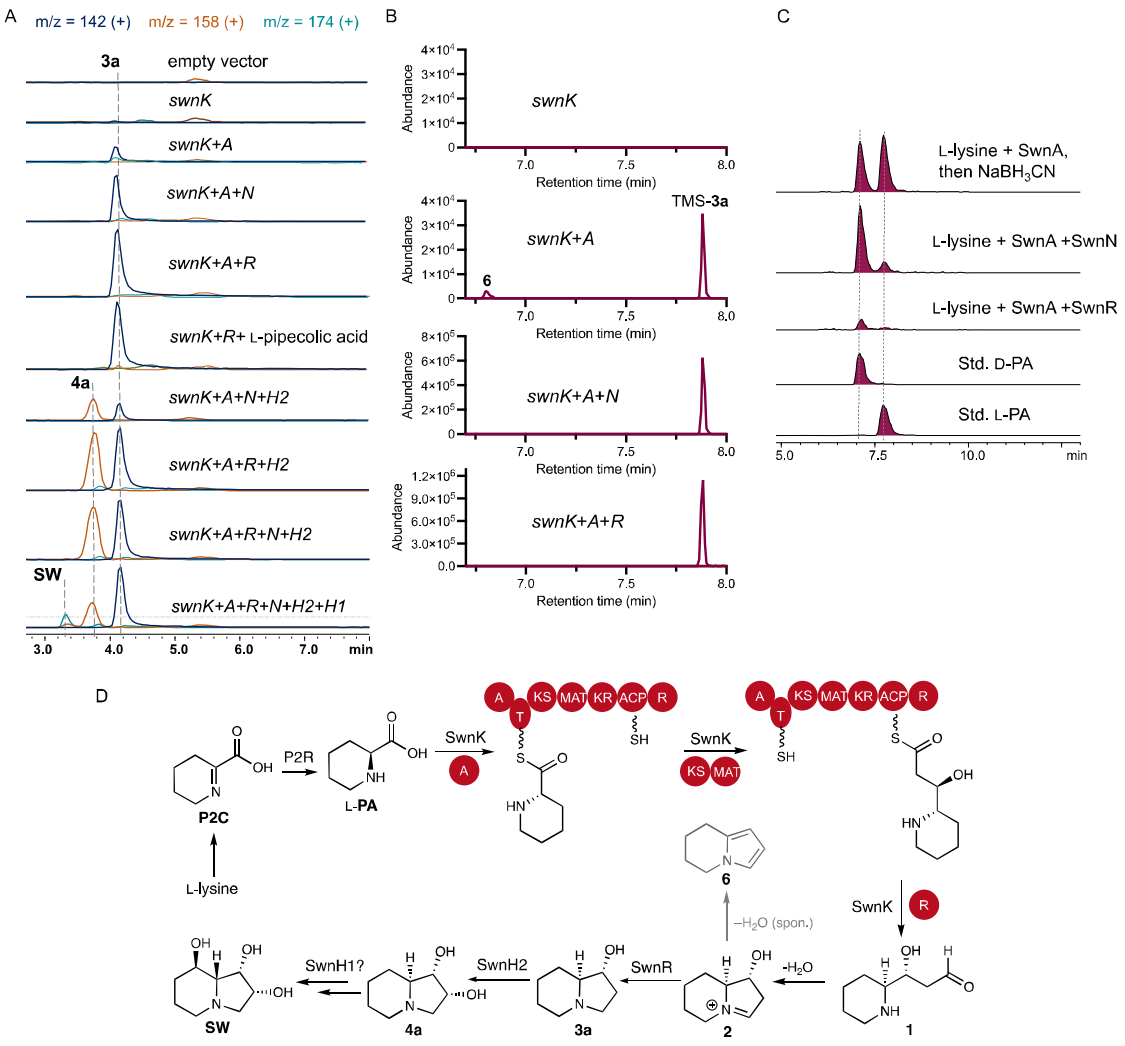

Figure 2. Reconstitution of swainsonine biosynthesis in *Aspergillus nidulans*. (A) LC-MS analysis of product profile of heterologous expression of different combinations of *swn* genes; (B) GC-MS analysis of product profile of heterologous expression of different combinations of *swn* genes. To ease the detection of **3a**, its hydroxyl group was derivatized by trimethylsilyl (TMS) group. (C) In vitro biochemical characterization of SwnA, SwnN, and SwnR. (D) Proposed early biosynthetic pathway for SW.

Our in vivo heterologous reconstitution study also showed that the loss of the *swnA* gene can be chemically complemented by supplementing the cell culture with L-PA, confirming that SwnA is solely involved in the biosynthesis of this noncanonical amino acid precursor (**Figure 2A**). To precisely determine its enzymatic function, we purified recombinant SwnA protein from *E. coli* and characterized its activity in vitro. Incubating SwnA with L-lysine and αKG resulted in the formation of dehydropepcolic acid, and subsequent chemical reduction by $NaBH_3CN$ yielded a mixture of racemate pipecolic acids (**Figure 2B**). The loss of L-configuration indicated that SwnA functions as a lysine 6-aminotransferase, producing 2-dehydropipecolic acid (P2C), rather than the previously proposed P6C.

To investigate whether SwnN or SwnR could serve as the requisite P2C reductase, we performed SwnA-coupled enzymatic assays (**Figure 2C**). Both SwnN and SwnR were capable of reducing P2C to PA. However, the catalytic efficiency of SwnR is too low to be physiologically relevant; while SwnN can efficiently reduce P2C, it predominantly produced D-PA, rather than the desired L-PA. These findings indicate that neither of these two enzymes is the P2C reductase dedicated for L-PA biosynthesis, and suggesting that some endogenous reductases from *A. nidulans* must be co-opted for P2C reduction. This hypothesis in accord with our fermentation results showing that overexpressing SwnA alone is sufficient to supply L-PA in vivo.

Oxidative tailoring steps and epimerization in SW biosynthesis

Following the elucidation of the biosynthesis of the indolizidine scaffold in SW (**Figure 2D**), we shifted our focus to the tailoring phase of SW biosynthesis. The addition of the Fe/αKG-dependent oxygenase SwnH2 into the heterologous expression system led to the formation of **4a** (**Table S3**), a known intermediate in SW biosynthesis.[17] Further inclusion of the Fe/αKG-dependent oxygenase SwnH1 enabled the de novo production of SW, though

intermediates **3a** and **4a** accumulated (**Figure 2A**). This in vivo reconstitution study indicates that SwnH2 is responsible for C2 hydroxylation while SwnH1 is essential for the conversion of **4a** to SW. However, the molecular mechanism underlying this transformation remains ambiguous.

To unravel the enzymatic functions of these two oxygenases and their roles in SW biosynthesis, we purified recombinant SwnH2 and SwnH1 from *E. coli* and assayed their activities in vitro using isolated biosynthetic intermediates **3a**/**4a**, along with all the necessary cofactors and co-substrates (e.g. αKG, iron sulfate, sodium ascorbate). Compound **3a** was oxidized by SwnH2, but not by SwnH1, yielding **4a** and two new compounds, **7** and **8** (**Figure 3A**). Following the reaction progress revealed that **4a** and **7** were formed concurrently, with an approximate ratio of 4.5:1 (**Figure S4**), while **8** emerged after a lag phase. Additionally, **4a** could be further converted to **8** by SwnH2, but not SwnH1. Isolation and structural characterization of **7** and **8** from the reaction mixtures identified **7** as the iminium derivative of **3a** (**Table S4**), and **8** as the iminium derivative of **4a**. These results suggest that SwnH2 catalyzes consecutive oxidation reactions transforming **3a** to **8** via **4a**.

We reasoned that **8** is two steps away from SW: first, an iminium reduction is required to set the *R*-configured stereocenter at C8a, fulfilling the net redox-neutral epimerization process; second, a stereospecific hydroxylation reaction at C8 is needed to complete the polyhydroxylated substitution pattern. To test whether **8** serves as an intermediate en route to SW, we used isolated **8** as a substrate to assess its reactivity with the two BGC-encoded reductases, SwnR and SwnN. No reaction occurred between **8** and SwnR, but SwnN was able to catalyze the reduction of **8** in an NADPH-dependent fashion, producing a species with a similar molecular weight and retention time of **4a** (**Figure 3B**). Isolation and structural characterization of this potentially new diol species revealed that it is actually a mixture of two stereoisomers in nearly a 1:1 ratio, consisting of **4a** and a diol isomer that we

tentatively assigned to *ent*-lentiginosine **9** (**Figure S5**). Although the absolute stereochemistry of **9** remains to be confirmed, its formation is likely an artifact caused by spontaneous epimerization during our purification of the unstable iminium compound **8**. Nevertheless, SwnN was shown to reduce **8** to form **4a**, rather than the initially hypothesized C8a-epimer intermediate **4c** (**Figure 3D**), suggesting that even though **8** is a substrate for SwnN, this reaction does not proceed in the direction of SW formation.

We then considered an alternative reaction sequence, proposing that C8-hydroxylation proceeds iminium reduction. As shown in **Figure 3B**, clear consumption of **8** was observed in the presence of SwnH1 and the necessary co-substrates. When SwnN and NADPH were included in this assay, the desired final product SW was obtained from this biocatalytic cascade. These results strongly indicate that: (1) SwnH1 catalyzes C8-hydroxylation to produce an iminium triol intermediate **10**; (2) SwnN catalyzes the reduction of the in-situ generated **10** and completes the enigmatic epimerization at the ring fusion. More importantly, our findings suggest that SwnN is a rare imine reductase displaying substrate-dependent stereospecificity: SwnN can catalyze iminium reduction on two similar substrates (e.g. **8** and **10**) to yield completely opposite stereochemistry. To our knowledge, reductases demonstrating such substrate-dependent stereospecificity are scarce. Only a few ketoreductases are known to exhibit this behavior, and they are embedded within PKS assembly lines.[29,30] Therefore, SwnN represents the first example of a standalone imine reductase with substrate-dependent stereospecificity.

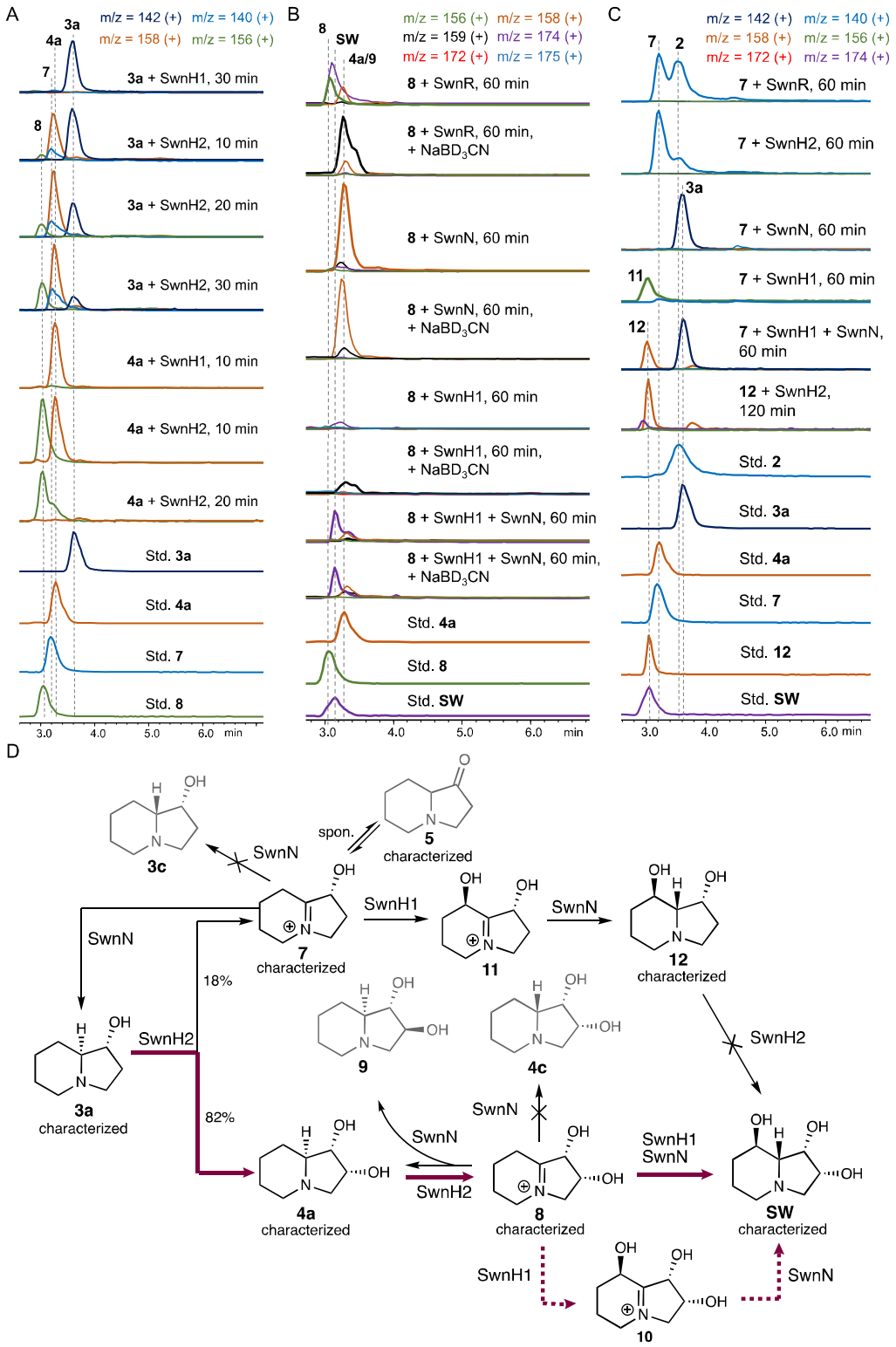

Figure 3. Elucidating the tailoring phase of swainsonine biosynthesis involving enzymes SwnH2, SwnH1, and SwnN. (A) Enzymatic reaction with intermediates **3a** and **4a**. (B)

Enzymatic reaction with intermediate **8**. To verify if the observed species are iminium compounds, we performed $NaBD_3CN$ reduction to derivatize any iminium species after each enzymatic reaction. (C) Enzymatic reaction with intermediate **7**. (D) Proposed late-stage biosynthetic way for swainsonine starting from intermediate **3a**. The colored arrows indicate the verified linear pathway for swainsonine, while the dashed arrows indicate proposed transformation as compound **10** was not directly observed in our study.

Timing of epimerization is determined by C8-hydroxylation

Having established the intermediacy of **8** in SW biosynthesis and elucidated the epimerization mechanism, we next turned our attention to the other iminium derivative, **7**, a minor product isolated from the SwnH2-catalyzed oxidation of **3a**. We wondered if **7** might also be an on-pathway intermediate towards SW and thus assayed its reactivity with all tailoring enzymes (**Figure 3C**). Compound **7** did not undergo C2-hydroxylation with SwnH2 to give intermediate **8**, nor could it be reduced by SwnR. Instead, we observed tautomerization of **7** to the ketone isomer **5** under these conditions. In comparison, SwnN could reduce **7** to reform **3a**, without yielding any C8a-epimer product (e.g. **3c** in **Figure 3D**). While this reaction does not advance towards SW formation, it demonstrates that SwnN has the ability to recycle the by-product **7** in order to regenerate **3a**.

Interestingly, SwnH1 oxidized compound **7** to produce a new iminium product, **11**, which was subsequently reduced by SwnN to yield a stable diol, **12**. Isolation and structural characterization of **12** indicates that SwnH1 catalyzes hydroxylation at C8, with the same stereochemistry observed in SW (**Figure 3D**). The fact that SwnH1 only catalyzes hydroxylation reaction with substrate **7** and **8** strongly suggest that SwnH1 has a very stringent substrate specificity and only recognize the iminium substrates. The epimerized C8 stereocenter in **12** also indicates that the net redox-neutral epimerization process at the ring-fusion is once again achieved through an oxidation-reduction cascade, interrupted by C8-hydroxylation. Taken together, these findings suggest that although SwnN controls the stereochemical configuration at the ring fusion, the stereochemical outcome is ultimately determined by the gatekeeper, SwnH1. With the C8-hydroxyl group installed by SwnH1, SwnN preferentially accesses the substrate from the β-face, leading to epimerized C8a in both **12** and SW. On the other hand, without the C8-hydroxyl group, SwnN catalyzes iminium reduction from the opposite α-face, preserving the original stereo-configuration.

The stereochemistry of **12** fully matches that of SW, raising the question of whether this route (i.e. from **3a** to **12**) could also lead to SW. We incubated isolated **12** with SwnH2. However, only trace amounts of a triol product were observed after an extended period (i.e. 2 hours) of incubation (**Figure 3C**). This experiment indicates that **12** is not a kinetically competent intermediate in SW biosynthesis. The irrelevance of **12** to SW biosynthesis is also supported by the observation that the SwnN-catalyzed reduction of **7** occurs more rapidly than the SwnH1-catalyzed hydroxylation of **7**, as re-formed **3a** was actually the major product instead of **12** unless the 10-fold more SwnH1 was used in the reaction. Given that the starting material **7** was also a minor by-product, these results collectively explain why diol **12** has never been isolated from any SW-producing organism before.

Directing the site-selectivity of SwnH2 by a protecting/directing group

Since the pathway branching from **7** has now been demonstrated to be an off-pathway, the formation of **7** appears to result from an error by SwnH2 during its hydroxylation reaction with **3a**. This prompted us to speculate whether the lack of catalytic specificity in SwnH2 might be due to **3a** being an inappropriate substrate. This speculation was inspired by our recent biosynthetic study of loline alkaloids.[31] In that pathway, the intermediate 1-aminopyrrolizidine must be acetylated by a yet-unidentified *N*-acetyltransferase in order to be recognized by the Fe/αKG-dependent oxygenase LolO for the installation of the characteristic ether bridge.[32] Subsequently, the acetyl group is proposed to be removed by the BGC-encoded deacetylase, LolN.[33] Although the exact reason why acetylation is required in loline biosynthesis is not fully understood, we hypothesized that a similar cryptic acetylation event might also take place in swainsonine biosynthesis, making *O*-acetylated **3a** (**Ac-3a**) a more suitable substrate for SwnH2.

To examine this hypothesis, we synthesized **Ac-3a** and tested its reactivity with SwnH2 (**Figure 5**). An acetylated diol product (**Ac-13**) was indeed produced, but the formation of **7** appeared to be inevitable. In fact, the percentage (~30%) of this by-product was substantially higher compared to the reaction with **3a**. Surprisingly, isolation and structural characterization of the diol product revealed that SwnH2 installed the hydroxyl group stereospecifically at C7 instead of C2 on **Ac-3a**. These results clearly ruled out the possibility of **Ac-3a** being an intermediate in SW biosynthesis, supporting the linear pathway highlighted in **Figure 3D** as the only productive route for SW biosynthesis. Nonetheless, this result serendipitously demonstrated that the artificially attached *O*-acetyl group could act not only as a protecting group to prevent hydroxylation at C2, but also as a directing group, altering the site-selectivity of SwnH2 and resulting in unexpected hydroxylation at C7,

Because the *O*-acetyl group can be easily detached, we then subjected the deprotected diol, **13**, to SwnH2 for further oxidative modifications. We also included SwnN and NAPDH to quench any iminium cations resulted from SwnH2-catalyzed amine desaturation. To our delight, a triol compound, **14**, was successfully obtained from this one-pot reaction (**Figure 5**). Isolation and structural characterization of **14** revealed a new polyhydroxylated indolizidine alkaloid isomeric to SW, bearing the same C2-hydroxyl group but retaining the stereochemistry at C8a. The absence of epimerization corroborates our proposed gatekeeping mechanism, which suggests that SwnH1-catalyzed C8-hydroxylation dictates the timing of epimerization.

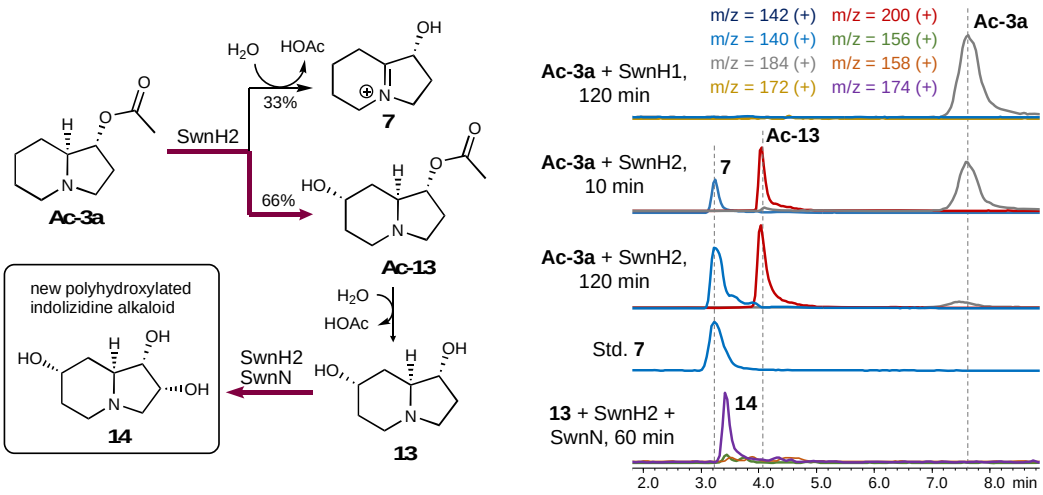

Figure 4. Biocatalytic synthesis of a novel polyhydroxylated indolizidine alkaloid enabled by a protective acetyl group-directed biosynthesis.

Discussion

Polyhydroxylated alkaloids are renowned for their potent glycosidase inhibitory activities and potential therapeutic applications, yet their biosynthesis remains largely unexplored. Our study of the SW biosynthesis marks the first complete elucidation of the biosynthetic pathway of any polyhydroxylated alkaloid to date. Contrary to the multibranched pathway proposed by Wang et al.,[26] our findings support a linear biosynthetic mechanism in agreement with the feeding results reported forty years ago.[21] Briefly, the NRPS-PKS hybrid SwnK constructs the indolizidine backbone from L-pipecolic acid, while the tailoring enzymes (SwnH2, SwnH1, and SwnK) catalyze a sequence of late-stage modifications, following the order of C2-hydroxylation, amine desaturation, C8-hydroxylation, and finally imine reduction to complete the biosynthesis.

The epimerization process in SW biosynthesis is reminiscent of the stereoinversion of (*S*)-reticuline to (*R*)-reticuline via 1,2-dehydroreticuline, the first committed step in morphine biosynthesis.[34] Unlike reticuline epimerase, a fusion protein composed of a cytochrome P450 domain and an aldo-keto reductase domain, SW biosynthesis recruits a discrete Fe/αKG-dependent oxygenase (SwnH2) to facilitate the desaturation of amine, followed by a separate reductase (SwnN) that catalyzes the stereospecific imine reduction. The inversion of unactivated stereocenters through such a direct oxidation-reduction process is a common chemical logic in both chemical synthesis and biosynthesis.[35-37] However, SW biosynthesis employs a striking hydroxylation-directed epimerization process, where the typical oxidation-reduction sequence is interrupted by SwnH1-catalyzed C8-hydroxylation. More importantly, this C8-hydroxylation determines the stereochemical outcome of the subsequent imine reduction catalyzed by SwnN. We propose that this additional level of control is necessary for utilizing the same enzyme, SwnN, to recycle the seemingly derailed iminium by-product, **7**. It should also be noted that this substrate-dependent stereospecificity of SwnN differs from the nonstereospecific hydride transfer catalyzed by many sugar epimerases, such

as the well-characterized UDP-hexose-4-epimerase, which reduces a transient keto intermediate in a nonstereospecific manner to yield either UDP-galactose or UDP-glucose.[38]

Aside from the unusual epimerization process, another surprising discovery is the multifunctional Fe/αKG-dependent oxygenase SwnH2. We demonstrated that SwnH2 catalyzes both stereospecific hydroxylation and regiospecific tertiary amine desaturation. The exact mechanism of the amine desaturation step remains unclear, but it is conceivable that it proceeds through stereospecific hydroxylation at C8a, followed by spontaneous dehydration of the hemiaminal intermediate, yielding the observed iminium compounds. This mechanistic proposal is supported by the fact that SwnH2-catalyzed amine desaturation is also stereospecific, as all four substrates (e.g. **3a**/**4a**/**Ac-3a**/**13)** possess the same *S*-configuration at C8a, while the epimerized compounds (i.e. *R*-configured C8a), such as **12** and SW, are no longer readily desaturated by SwnH2. This substrate stereospecificity conforms to a hydroxylation mechanism involving a stereospecific hydrogen atom abstraction reaction at C8a during SwnH2-catalyzed amine desaturation, and is consistent with the stereochemistry of SwnH2-catalyzed C2-hydroxylation. We propose that all substrates approach the non-heme iron center in SwnH2 via the α-face, and only the pseudo-axial C-H bonds at C2 and C8a are accessible by the oxoiron (IV) ferryl intermediate.[39]

This proposal also explains the stereochemical outcome of SwnH2-catalzyed C7-hydroxylation with the unnatural substrate **Ac-3a**. It is likely that the bulky acetyl group induced a slight change in substrate binding, rendering the C2-H bond inaccessible while positioning the C7 site closer to the ferryl intermediate. Although a comprehensive mechanistic understanding of SwnH2 awaits further study, the successful use of an acetyl group to alter its site-selectivity is inspiring. We suggests that deploying a detachable protecting group to direct the biocatalytic activity of enzymes, instead of engineering the respective enzymes, could become a general strategy in biocatalysis.[40]

Finally, our study of SW biosynthesis also sheds light on the biosynthetic pathway of slaframine, a structurally related indolizidine alkaloid mycotoxin (**Figure 5**). Since slaframine is also derived from L-pipecolic acid and the fungus *R. leguminiocola* produces both SW and slaframine, Harris et al. proposed that slaframine and SW share the same early biosynthetic pathway with ketone indolizidine **5** serving as the common precursor.[21] However, our study of SW biosynthesis clearly demonstrates that **5** is not a biosynthetic intermediate for SW, and the stereo-configuration of C1-OH in **3a** is determined by SwnK. These results indicate that there must be a separate NRPS-PKS hybrid dedicated to slaframine biosynthesis in *R. leguminiocola*, with its product being either **3b** or its iminium precursor like **2**. Furthermore, this NRPS-PKS hybrid must be highly similar to SwnK, except for its KR domain, which sets the C1-OH in the opposite stereochemistry. Based on these insights, we now propose a revised biosynthetic pathway for slaframine in light of the discoveries made in SW biosynthesis (**Figure 5**).

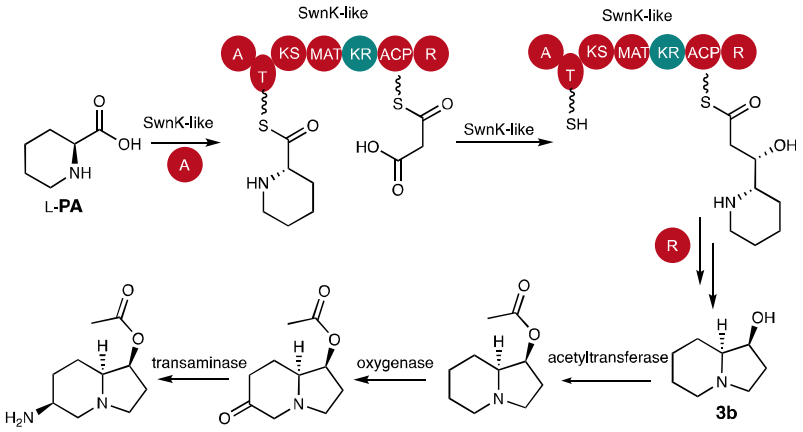

Figure 5. Proposed biosynthetic pathway of slaframine.

Conclusion

In conclusion, we have elucidated the complete biosynthesis of swainsonine, solving a biosynthetic puzzle that has persisted for decades. Our comprehensive characterization of SwnH2, SwnH1, and SwnN unveiled an unusual epimerization mechanism: it follows a classic oxidation-reduction cascade but is interrupted by a hydroxylation event that ultimately determines the stereochemical outcome of the reduction step. We demonstrated that SwnH2 is a multifunctional enzyme catalyzing both C2-hydroxylation and amine desaturation, while SwnH1 is a C8-hydroxylase specific to iminium substrates and controls the stereospecificity of the imine reductase, SwnN. Additionally, we discovered that the site-selectivity of SwnH2-catalyzed hydroxylation can be directed by deploying a protective acetyl group on the substrate. This strategy enabled the biosynthesis of a new polyhydroxylated alkaloid. Our study underscores the potential of harnessing these biosynthetic enzymes for the discovery and development of natural product-based glycosidase inhibitors.

Data availability

All data supporting the findings of this study are presented in the main text and the Supplementary Information file. NCBI (https://www.ncbi.nlm.nih.gov/) accessions codes are referenced in the Supplementary Information file. All data is available from the corresponding author upon request.

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Author contributions:

Y.H. and S.L. designed the overall research. S.L. and Z.B. performed all biochemical experiments. Y.H. and S.L. wrote the manuscript.

Competing interests

The authors have no competing interests to declare.

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