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Review

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The Biosynthesis Pathway of Swainsonine, a New Anticancer Drug from Three Endophytic Fungi

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Swainsonine (SW) is the principal toxic ingredient of locoweed plants that causes locoism characterized by a disorder of the nervous system. It has also received widespread attention in the medical field for its beneficial anticancer and antitumor activities. Endophytic fungi, *Alternaria* sect. *Undifilum oxytropis* isolated from locoweeds, the plant pathogen *Slafractonia leguminicola*, and the insect pathogen *Metarhizium anisopliae*, produce swainsonine. Acquired SW by biofermentation has a certain foreground and research value. This paper mainly summarizes the local and foreign literature published thus far on the swainsonine biosynthesis pathway, and speculates on the possible regulatory enzymes involved in the synthesis pathway within these three fungi in order to provide a new reference for research on swainsonine biosynthesis by endophytic fungi.

Keywords: Swainsonine, biosynthesis, *Alternaria oxytropis*, *Slafractonia leguminicola*, *Metarhizium anisopliae*

Introduction

Swainsonine (SW) is an indolizidine alkaloid that was first identified from *Swainsona canescens*, a toxic legume that is found in Australia [1]. In China, the morbidity and death rate of livestock caused by SW have increased annually and led to large economic losses in the western rangelands of China in the last 50 years [2]. Researchers found that the half-chair conformation of the SW cation is similar to that of the mannosyl cation and it is highly compatible with α-mannosidase [1]. SW, a strong inhibitor of α-mannosidase, mainly inhibits golgi α-mannosidase II (MAN2A1), lysosomal α-mannosidase (MAN2B1), and endoplasmic reticuluma/cytoplastn α-mannosidase (MAN2C1) activities, resulting in the loss of enzymatic hydrolysis activity as well as lysosomal storage disease and altering glycoprotein processing [3–5].

However, more remarkably, SW has significant anti-cancer and antitumor activities. In 1985, Hino *et al*. [6] were the first to report that SW inhibited tumor cell growth and metastasis. Subsequently, researchers have explored the effect of SW on lymph cancer [7], colorectal cancer [8], Ehrlich ascites carcinoma [9], human hepatoma [10], and leukemia [11] and showed that all patients received a good therapeutic effect. SW is a specific inhibitor of α-mannosidase II in the golgi complex, which can affect the synthesis of various carbolydrates, glycoproteins, and glycolipids, thereby directly killing tumor cells [12–14]. Furthermore, SW can promote tumor cell apoptosis in order to achieve the purpose of cancer treatment. In SGC-7901 cells, Sun *et al*. [15] observed the expression of p53 protein and found that SW inhibited the expression of mtp53 protein and increased the expression of wtp53 protein to induce apoptosis. Intracellular calcium (Ca^{2+}) concentration change is an important information system of cells. Intracellular Ca^{2+} overload is the ultimate pathway of cell death. Ca^{2+} directly activates the endonuclease to induce apoptosis of SGC-7901 cells. The inhibitory effect of SW on tumor cells is partly attributable to its enhancement of immune function in tumor patients, which indirectly affects the growth and metastasis of tumor cells [16]. The putative anticancer mechanisms of SW are shown in Fig. 1. Compared with the traditional chemical anticancer drugs, SW can enhance the immune system and the immunological activity of the immune cell, and promote the proliferation of bone marrow cells, and plays an important role in the

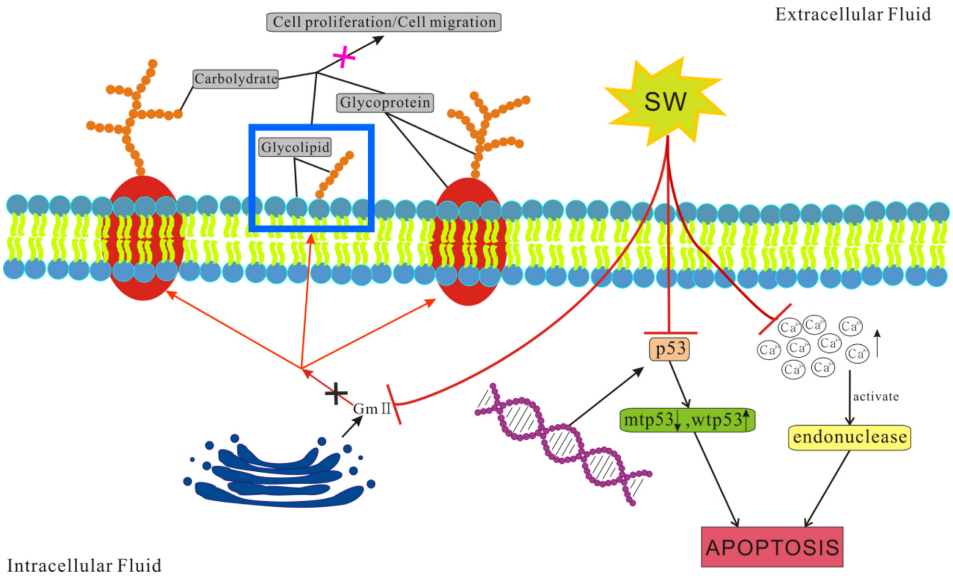

Fig. 1. The putative anticancer mechanisms of swainsonine (SW).

Table 1. The research history of swainsonine (SW).

Year	Event	Reference
1979	SW first isolated and identified from *Swainsona canescens*	Colegate *et al.* [1]
1980	SW found as an inhibitor of lysosomal α-mannosidase	Dorling *et al.* [3]
1982	SW confirmed as the poisonous ingredient of locoweed	Molyneux *et al.* [20]
1983	*S. leguminicola* found to produce SW	Schneider *et al.* [21]
1984	Chemical synthesis of SW for the first time	Ali *et al.* [22]
1985	*M. anisopliae* found to produce SW	Hino *et al.* [6]
1985	SW found to inhibit tumor cell growth and metastasis	Hino *et al.* [6]
1989	SW first extracted and identified from *O. ochrocephala* of China	Cao *et al.* [23]
1989	SW found as the only toxic substance that causes locoism	James *et al.* [24]
1995	Identification of SW from *Ipomoea* sp. plants	Molyneux *et al.* [25]
2002	Identification of SW from *Sida carpinifolia*	Colodel *et al.* [26]
2003	SW found to be highly correlated with endophytes infection in locoweed	Braun *et al.* [27]
2007	Identification of SW from *Turbina cordata*	Dantas *et al.* [28]
2009	*Undifilum oxytropis* found to be responsible for the synthesis of SW in locoweed	Cook *et al.* [29]
2016	The whole-genome sequencing of *Undifilum oxytropis* producing SW	Lu *et al.* [30]
2017	SW biosynthesis genes found in fungi	Cook *et al.* [31]

antitumor effect [8, 16–19]. SW thus has great potential as an anticancer drug. The acquisition of SW can provide sufficient material resources for medical research. The research history of SW is shown in Table 1 [1, 3, 6, 20–31].

Currently, there are three sources for SW production: extraction from infected plants, chemical synthesis, and fermentation from fungi. The first method is time consuming and yields are low. Tong *et al.* [32] extracted SW from *Oxytropis kansuensis* using a sublimation method, but the yield of the plant was only 14 μg/g. Moreover, reagents used for extraction may cause environmental pollution, and excessive harvesting of plants containing SW could damage the grasslands and lead to ecological imbalance. The second method is chemical synthesis. Ever since Ali *et al.* [22] first achieved chemical synthesis of SW in 1984, about more than 40 different synthetic routes have been

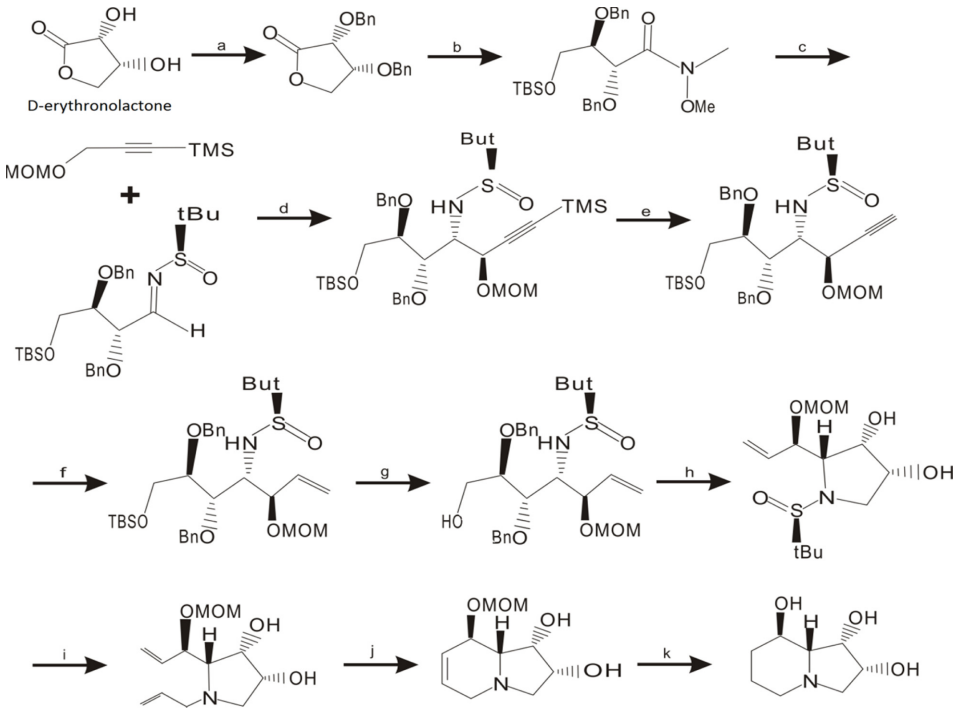

Fig. 2. The chemical synthesis process of swainsonine.

Reagents and conditions: (a) BnBr, Ag_2O, $CaSO_4$, MeCN, rt., 36 h; (b) 1) AlMe, HN(Me)OMe·HCl, THF, 0°C-rt., 15 h; 2) TBSC 1, imidazole, DMF, 0°C, 1 h; (c) 1) Dibal-H, THF, -78°C, 30 min; 2) (Rs)-TBS-sulfinamide, $Ti(OEt)_4$, THF, rt., 15 h; (d) 1) SBuLi, Et_2O, TMEDA, –80°C, 1 h; 2) $ZnBr_2$, –80°C, 20 min; (e) K_2CO_3, MeOH, 0°C, -rt., 2 h; (f) CpZr(H)Cl, THF, 0°C-rt., 30 min; (g) TBAF, THF, rt., 1 h; (h) 1) MsCl, Et3N, CH_2Cl_2, 0°C, 30 min; 2) NaH, 15-C-5, THF, 0°C, 30 min; (i) HCl, MeOH, 0°C-rt., 1 h, then Et_3N, allybromide, 0°C-rt., 3 h; (j) Grubbs 11, toluene, 100°C, 3 h; (k) 1) HCl, MeOH; 2) Pd/C, H_2, MeOH, 36 h. (Abbreviations: THF: Tetrahydrofuran; TBSC1: T-Butyldimenhylsilyl Chloride 1; DMF: N,N-Dimethylformamide; TBS: T-Butyldimethylsilys; TMEDA: Tetramethylethylenediamine; TBAF: Tetrabutylammonium fluoride; MOM: Methoxymethyl; TMS: Trimethylsiyl).

reported. In 2001, Wardrop *et al*. [33] synthesized SW by using D-erythronolactone as the starting material, followed by 15 steps in the total yield of 11% (see Fig. 2). Owing to the presence of four chiral carbon atoms of SW, all methods require the synthesis of precursors and intermediate derivatives and conversion by several steps to synthesize SW. Moreover, chemical synthesis of SW is cumbersome and the reaction conditions are difficult to achieve. The third method is fermentation and extraction from fungi. SW could be produced by the clover pathogen *Slafractonia leguminicola* (= *Rhizoctonia leguminicola*) [21, 34], the insect pathogen and plant symbiont *Metarhizium robertsii* [6], locoweed symbionts belonging to *Alternaria* sect. *Undifilum oxytropis* [35], and a recently discovered morning glory symbiont belonging to order Chaetothyriales [36]. SW production through biological fermentation has advantages of low cost, easy control of fermentation conditions, no destruction of grassland ecology, and no pollution of the environment, so this method has a great potentiality in the biological pharmaceutical field.

The initial steps of the SW biosynthetic pathway have been extensively studied in *S. leguminicola* and *M. anisopliae* [37–39]. SW is a metabolite of lysine via saccharopine, pipecolic acid, and 1-piperideine-6-carboxylic acid. In *S. leguminicola*, starting from lysine, there is a branch in the pathway. One way is to synthetize SW and the other way to synthetize slaframine (SF). In *M. anisopliae*, there are two pathways in the first half of SW synthesis, and both pathways synthesize the SW precursor 1,6-piperidine carboxylic acid, and then generate SW. The similar steps in the SW biosynthesis pathway of *A. oxytropis* are not clear yet. In this review, we reference the SW biosynthesis pathway of *S. leguminicola* and *M. anisopliae* as well as some key enzymes that regulate SW synthesis. Combining the genome sequencing and functional analysis of *A. oxytropis* [30], we hypothesize the early steps of the SW biosynthesis pathway in *A. oxytropis* and some key enzymes that may be involved in the regulation of its synthesis. Production of a

mutant *A. oxytropis* strain that lacks SW could help solve locoism of animals. In contrast, production of an *A. oxytropis* mutant with high production of SW could be used for the mass production of SW by biological fermentation for its medical research.

Slafractonia leguminicola

Slafractonia leguminicola is a plant pathogenic fungus that falls within Fungi, Ascomycota, Dothideomycetes, Pleosporomycetidae, Pleosporales, *Incertae sedis*, *Slafractonia* [34]. After ingesting red clover (*Trifolium pretense* L.) and other legume hays and silages, the grazing animals (often horses) suffer from extreme salivation or "slobbers." Early scientific literature showed that slobbers was caused by SF, which is produced by the plant pathogenic fungus *S. leguminicola* as a metabolic product of lysine and pipecolic acid [40–43]. Subsequently, Schneider *et al.* [21] studied metabolic products of *S. leguminicola*, and isolated another compound, SW. In 1995, Croom *et al.* [44] extracted pure SW from the mycelia of *S. leguminicola*. Yang and Cao [45] isolated 10 strains of *S. leguminicola* from leguminous plants from China, and found that the SW content in dry mycelia was 12.3–29.9 μg/g.

Since the discovery of SF predated that of SW in *S. leguminicola*, more is known about SF biosynthesis. Previous studies have shown that pipecolic acid is a precursor substance of SF biosynthesis. Wickwire *et al.* [37, 38] discovered a reaction chain beginning with the conversion of lysine to saccharopine by saccharopine dehydrogenase. Saccharopine is converted to δ1-piperideine-6-carboxylic acid (P6C) by an oxidative reaction, and then the P6C produces pipecolic acid. With the clarification of the stereochemical structures of SW and SF, epimerization was demonstrated in the 8α position of SF and SW; the carbon atom conformation of SF is S type, whereas SW is R type. Thus, later steps of the same synthesis pathway diverge generating different metabolites [46]. In 1986, Broquist [47] proposed that 1-oxygen-8-hydrogen indolizidine may be the branching point of SF and SW in the biosynthetic pathway of *S. leguminicola*. Harris *et al.* [48] studied the synthesis of SF and SW in the late synthesis process of *S. leguminicola* by using a chiral precursor labeled with tritium and confirmed that the branching point of the biosynthetic pathway to SF and SW is certainly 1-oxoindolizidine. 1-Oxoindolizidine was reduced to (1S, 8aS)-*cis*-1-hydroxy indolizidine and (1R, 8aS)-*trans*-1-hydroxy indolizidine. (1S, 8aS)-*cis*-1-hydroxy indolizidine produced SF through 1,6-dihydroxy indolizidine and 1-hydroxy-6-oxoindolizidine; however, (1R, 8aS)-*trans*-1-hydroxy indolizidine ultimately formed SW through 1,2-dihydroxy indolizidine, imineion-1,2-dihydroxy indolizidine, and imineion-1,2,8-trihydroxy

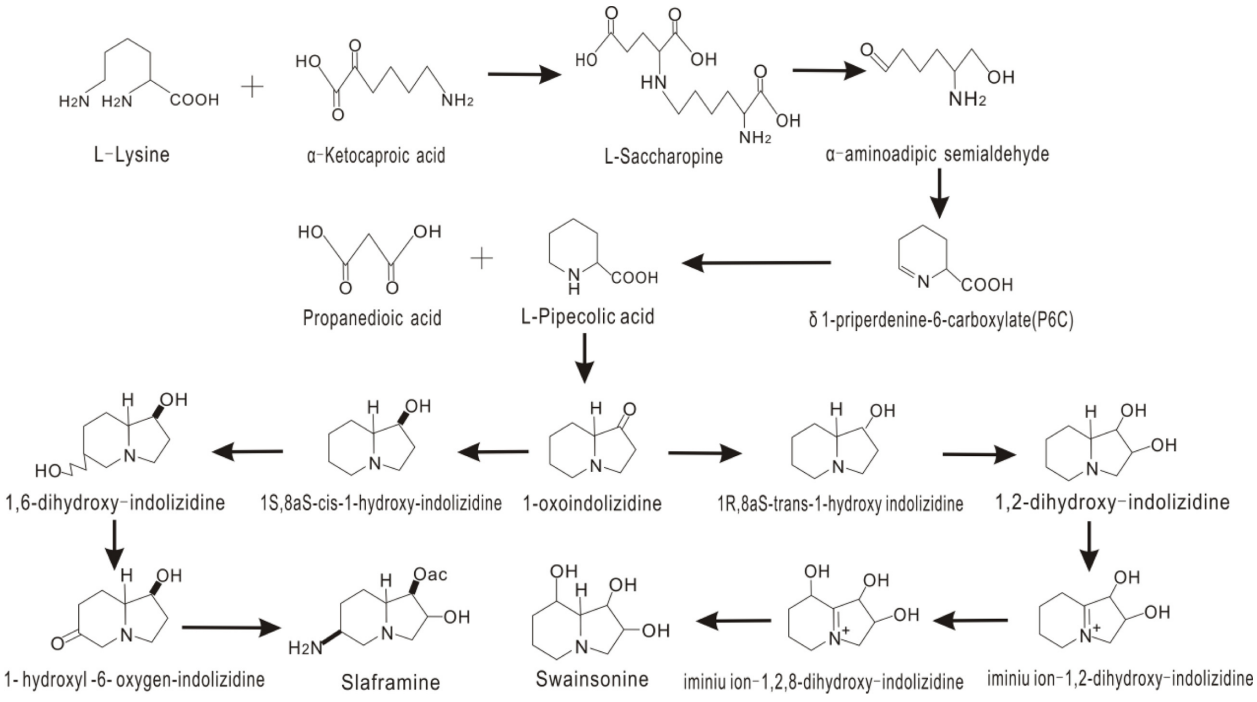

Fig. 3. The biosynthesis pathway of swainsonine and slaframine in *Slafractonia leguminicola*.

indolizidine. The theorized biosynthetic pathway for SW in *S. leguminicola* is shown in Fig. 3.

Metarhizium anisopliae

Metarhizium anisopliae is a common insect pathogen, which belongs to Fungi, Ascomycota, Sordariomycetes, Hypocreales, Clavicipitaceae, *Metarhizium* [49, 50]. Since 1985, when SW was first isolated by Hino *et al*. [6] from a fermentation broth of *M. anisopliae*, many scholars have studied the physiological properties of *M. anisopliae* to improve SW production. Tamerler *et al*. [51] improved SW production by changing the pH of the culture medium from 6.5 to 3.8, increasing the yield of SW up to 45.5 mg/l. Sim and Perry [52] assessed SW using an α-mannosidase enzyme method. Under the anoxic conditions, SW production began while the pH was low. Addition of L-lysine to *M. anisopliae* cultures grown in Czapek Dox medium enhanced SW production by 4-fold, suggesting that genetic modifications to the lysine biosynthetic pathway may prove useful in increasing yields, leading to a hypothesized biosynthetic pathway of SW in *M. anisopliae*. This result also suggested that lysine could be a metabolite precursor of SW.

M. anisopliae has not been reported to produce SF, but is presumed to have a similar biosynthetic pathway to SW as *S. leguminicola*. Saccharopine is produced from a L-lysine substrate in *M. anisopliae*, which is oxidized by saccharopine oxidase to 1,6-piperidine formic acid. This could also be generated through another pathway, whereby α-ketoglutarate is converted to α-aminoadipic acid semialdehyde through a series of reactions [53], and then 1,6-piperidine formic acid is produced via a non-enzymatic cyclization reaction. Then, 1,6-piperidine carboxylic acid is converted to L-pipecolic acid, and finally L-pipecolic acid produces SW through several undetermined steps [39]. Comparing the SW biosynthetic pathway in *S. leguminicola* and *M. anisopliae*, it was determined that L-lysine could be a precursor in both fungi. In *S. leguminicola*, 1,6-piperidine leads to 1-oxoindolizidine and then two pathways diverge. One pathway acted by reductase to produce SW, whereas the other pathway generated SF. In *M. anisopliae*, there are two sources of 1,6-piperidine: L-lysine and α-ketoglutarate.

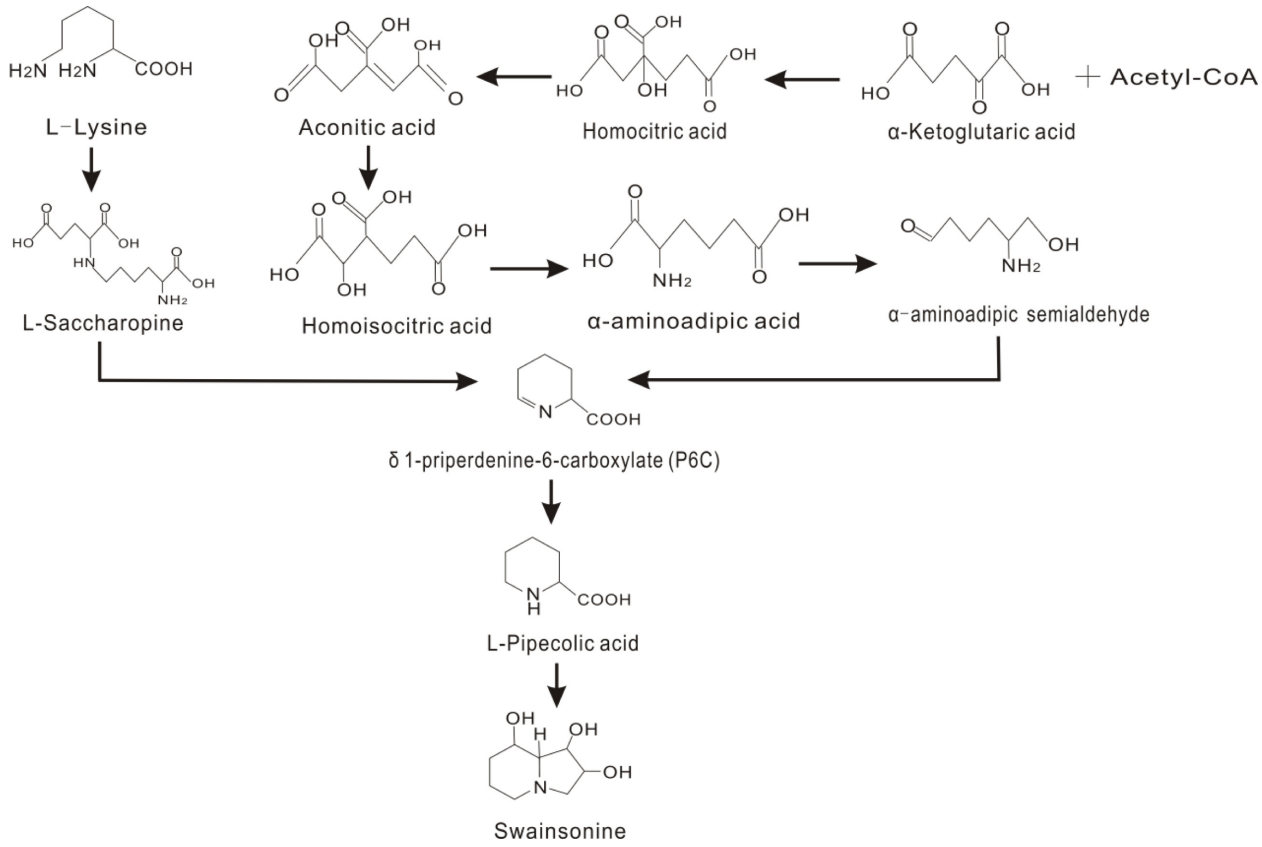

Fig. 4. The biosynthetic pathway of swainsonine in *Metarhizium anisopliae*.

Both generate the intermediate metabolite 1,6-piperidine and eventually generate SW. The theorized biosynthetic pathway for SW in *M. anisopliae* is shown in Fig. 4.

Alternaria sect. *Undifilum oxytropis*

Alternaria sect. *Undifilum oxytropis*, a locoweed symbiont, belongs to Ascomycota, Pleosporales, Pleosporaceae, *Alternaria,* section *Undifilum*. It has been isolated from leaves, stems, flowers, and seeds (maternally transmitted through the coat but not the embryo) of toxic locoweed species [35, 54–58]. Harris *et al*. [59] found SW and the precursors of SW synthesis in locoweed (*Astragalus oxyphysus*) through the isotopic tracer method and preliminarily discussed the synthesis mechanism of SW. They hypothesized that the SW synthesis pathway from locoweed plants was similar to that from *S. leguminicola* and *M. anisopliae*. *A. oxytropis* strains cultured in vitro produce SW, but SW cannot be detected in locoweeds without such endophytic fungus, indicating that SW in plants is completely derived from *A. oxytropis.* Plants with high levels of SW yield fungi that produce high levels of SW [60, 61]. Extrinsic factors such as growth environment, pH value of culture medium, precursor substances, other nutrient elements, and genetic characteristics can affect SW synthesis. Oldrup *et al*. [55] found that *A. oxytropis* grew best at pH 5.5 and temperature of 28°C, but SW levels were increased by 5.5-fold and 3.9-fold, respectively, in ULTO-PEG media and low pH (4.5) media, whereas high temperature, and high nitrogen and phosphorus concentration or potassium deficiency did not change SW production. These results show similar trends to *M. anisopliae*, which produces greater concentrations of SW in a medium containing pH 7.0 rather than pH 9.0 [52]. Zhang [62] found that L-piperidine acid, L-lysine, and α-ketoglutarate in a certain concentration range could increase the synthesis of SW, suggesting that these three compounds may be involved in SW biosynthesis in *A. oxytropis*. In 2016, Xue *et al*. [63] obtained SW from the fermentation broth of *A. oxytropis* and its content was 5.17×10^{-3} g/l.

At present, there are few studies on the SW biosynthesis pathway in *A. oxytropis.* Previous studies of non-endophytic ascus fungi have shown that saccharopine and L-pipecolic acid are the key intermediate metabolites in the synthesis of SW. Saccharopine is the main metabolites of lysine, which is the substrate for the synthesis of SW. The biosynthesis of pipecolic acid in plants and animals has been widely studied because it is closely related to lysine metabolism and some lysine-related diseases [64, 65]. Lysine is converted to pipercolic acid through two routes: l-piperideine-2-carboxylic acid (P2C) pathway and P6C [66]. Synthesis of P2C from D-lysine via piperacine has been partly studied in *Pseudomonas* [67, 68]. Howerer, there is no report on the P2C pathway being involved in the biosynthesis of SW. This could be due to the lack of key enzymes required for this pathway in the fungus producing SW or other reasons. Moreover, in a tracer test, the nitrogen atom in the alpha position of L-lysine (labeled with N15) could be converted to L-pipecolic acid, suggesting that the L-lysine to L-pipecolic acid conversion goes through the P6C pathway in *A. oxytropis* [69]. The P6C pathway was studied in depth in *S. leguminosae*, because pipecolic acid is synthesized by this route in *S. leguminosae.* In *Shodotorula glutinis*, pipecolic acid reverse produces lysine through P6C, α-AASA, and saccharopine [70, 71]. L-Pipecolic acid could be catalyzed to form 1-carbonyl indolizidine under a certain enzyme, followed by the formation of 1,2,8-trihydroxyl indolizidine, SW.

In 2016, the genome of *A. oxytropis* was sequenced and assembled into a 70.05 Mb draft genome, which had 11,057 protein-coding genes and 54% of them were similar to current public sequences. The *A. oxytropis* genes were annotated and we identified enzymes, such as saccharopine dehydrogenase (SDH), saccharopine oxidase (FAP2), pyrroline-5-carboxylate reductase (P5CR), polyketide synthase (PKS), cytochrome P450, saccharopine reductase (SR), and pipecolate oxidase (PIPOX), that might have a role in the SW biosynthesis pathway [30]. Recently, Cook *et al*. [31] analyzed the genome sequence of *S. leguminicola*, *M. robertsii*, *A. oxytropis*, and *I. carnea* endophyte (which it has phylogenetic affinity to the order Chaetothyriales, but it is an undescribed species) and revealed that these fungi share orthologous gene clusters, designated "SWN." This further confirmed that there are similar SW biosynthesis pathways in these fungi, but little research has been done into regulatory enzymes of the SW synthetic pathway in *A. oxytropis*. Mukhejee *et al*. [72] demonstrated that saccharopine reductase plays a role in SW metabolism in *A. oxytropis*. They knocked out the saccharopine reductase gene through homologous recombination, and the mutant strains produced increased levels of P6C and SW in fermentation liquid of the mutant strains, while the levels of saccharopine and lysine were decreased. These results suggested that P6C and pipecolic acid are precursors in SW synthesis in *A. oxytropis*, and the pathway is similar to that of *S. leguminicola*. SDH catalyzes lysine to saccharopine, but saccharopine can also be formed by saccharopine reductase catalyzing P6C. So far, the regulatory enzyme has not been reported for the

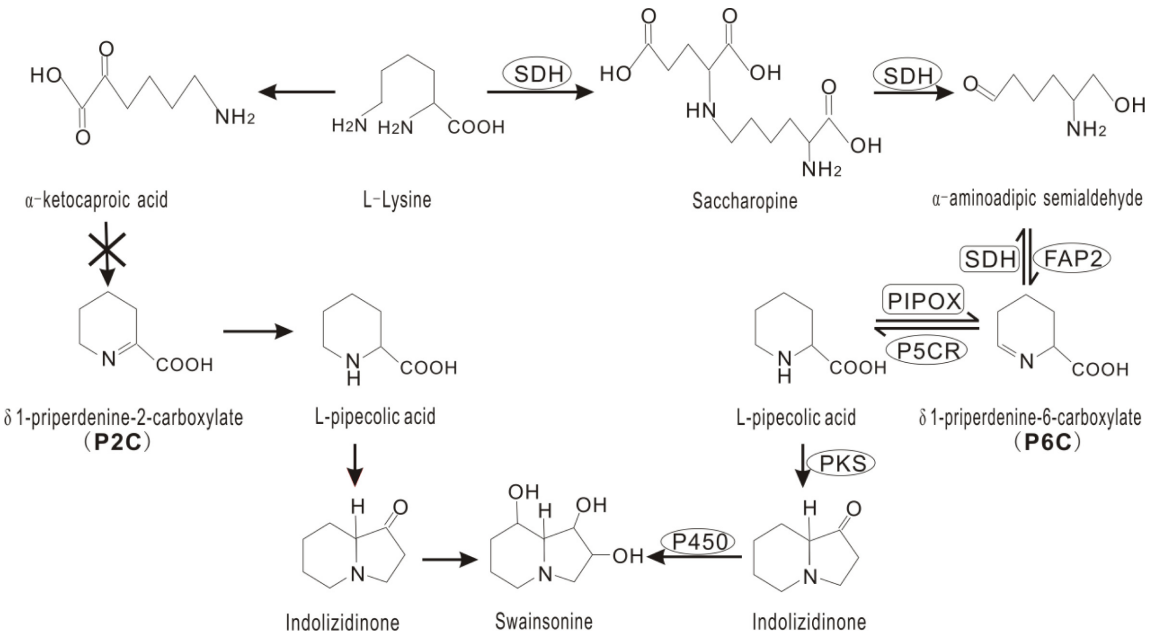

Fig. 5. The putative swainsonine biosynthetic pathway in *Alternaria* sect. *Undifilum oxytropis*.
Enzymes involved in this pathway: SDH: saccharopine dehydrogenase (EC:1.5.1.7) K00290, (EC:1.5.1.9) K00292, (EC:1.5.1.10) K00293; FAP2: saccharopine oxidase (EC: 1.5.3.1) K00301; PIPOX: L-pipecolate oxidase (EC:1.5.3.1 1.5.3.7) K00306; P5CR: pyrroline-5-carboxylate reductase (EC:1.5.1.2) K00286; PKS: polyketide synthase (EC: 2.3.-.-); P450: cytochrome P450 (EC: 1.14.-.-).

reaction of P6C reduced to pipecolic acid. However, Fujii *et al*. [73] found that *E. coli* pyrroline-5-carboxylate reductase (P5CR) acted efficiently with *Flavobacterium lutesens* LAT to convert L-lysine into L-pipecolic acid. It is possible that in the microbes that produce L-pipecolic acid via the P6C pathway, the universally conserved P5C reductase is actually responsible, at least in part, for the reduction of P6C into L-pipecolic acid. In *Penicillium chrysogenum*, pipecolate oxidase converts pipecolic acid into piperideine-6-carboxylic acid, and the saccharopine reductase transforms piperideine-6-carboxylic acid into saccharopine [74]. PKS is a large class of compounds produced by bacteria, actinomycetes, fungi, and plants [75]. This natural product plays an important role in anti-infectivity, mold resistant, antitumor, and immunosuppression activities. 1-Carbonyl indolizidine is a ketone compound. Whether PKS catalyzes L-pipecolic acid to 1-carbonyl indolizidine still requires further research to confirm it. In addition, cytochrome P450 is everywhere and participates in some drug biotransformations, chemical carcinogen metabolism, and some important compound biosynthesis such as steroids and fatty acid. Cytochrome P450 has carbon hydroxylation and aromatic hydroxylation [76]. In *S. leguminicola*, the conversion of 1-carbonyl indolizidine to SW needs to go through hydroxylation twice, and in the biosynthesis of alkaloids with ornithine, lysine, and nicotinic acid as precursors, 1-indolizine is hydroxylated to form SW, so we speculate that P450 might play an important role in the biosynthesis of SW in the fungus, followed by the formation of 1,2,8-trihydroxyl indolizidine, SW via hydroxylation, but the enzymes involved in these steps are not known. The hypothetical SW synthesis pathway and some key enzymes regulating its synthesis in *A. oxytropis* are shown in Fig. 5.

Summary

Currently, studies on SW biosynthesis have mostly been performed on *S. leguminicola* and *M. anisopliae*. However, the detailed biosynthesis pathway is still unclear. In *A. oxytropis*, the gene sequences of some putative key enzymes involved in the SW biosynthesis pathway have been obtained and some gene knockout experiments are being carried out. The purpose of these studies is mainly related to two aspects; on the one hand, SW production would be increased by gene knockout of putative reverse regulatory enzymes, so that sufficient SW will be provided as a source for medical research for its significant roles in immune regulation and anticancer activity. On the other hand, genes of some putative forward regulatory enzymes will be knocked out to obtain mutants of *A. oxytropis*,

which do not produce SW. On this basis, a new locoweed species will be gained that does not contain SW. This will fundamentally resolve the locoism of animals caused by SW and implement the comprehensive utilization and management of locoweeds of China's western grasslands.

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