# Total Biosynthesis of Cotylenin Diterpene Glycosides

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**Abstract:**

Cotylenins (CNs) are bioactive fungal diterpene glycosides that exhibits stabilizing activity on 14-3-3 protein-protein interactions (PPIs), which has significant therapeutic potential. Despite its importance, the biosynthetic pathway of CNs have remained unclear. Here, we report the identification of the biosynthetic gene cluster and elucidation of the biosynthetic pathway of CNs. Our investigation reveals the roles of glycosyltransferase, methyltransferase, and prenyltransferase enzymes in the assemble and modification of the glycoside moiety, as well as the multifunctional oxidation activity of the P450 enzyme CtyA. We also demonstrate the synthesis of an active unnatural CN derivative through combinatorial biosynthesis, showcasing the potential of pathway enzymes as catalytic tools to expand the structural diversity of the diterpene glycosides. Additionally, we highlight the stabilization effects of pathway intermediates on 14-3-3 PPIs, providing insights into the evolutionary optimization of bioactivity

within the biosynthetic pathway. These findings pave the way for future efforts to achieve the production Cotylenin A and related compounds with enhanced bioactivity through synthetic biology or semisynthesis.

## Introduction:

Cotylenins (CN) represent a class of fungal-derived diterpene glycosides renowned for their diverse biological activities[1]. Among them, Cotylenin A (CN-A, **1**) stands as the flagship molecule, derived from the Fusicoccane-type diterpene Cotylenol (**7**), featuring a distinct 5-8-5 tricyclic ring system with intricate sugar moiety modifications (Figure 1). Originally isolated from the plant pathogenic fungus *Cladosporium* sp. 501-7W, CN-A exhibits plant hormone-like activity and shows promise in inducing leukemia cell differentiation and enhancing the efficacy of various anticancer drugs[2, 3]. Besides **1**, additional CN derivatives, such as CN-C (**2**) and F (**3**) were isolated from the same strain[4]. Other structurally and biosynthetically related molecules include Fusicoccin A (FC-A)[1], Brassicicene I (BC-I, **8**)[5] and their derivatives.

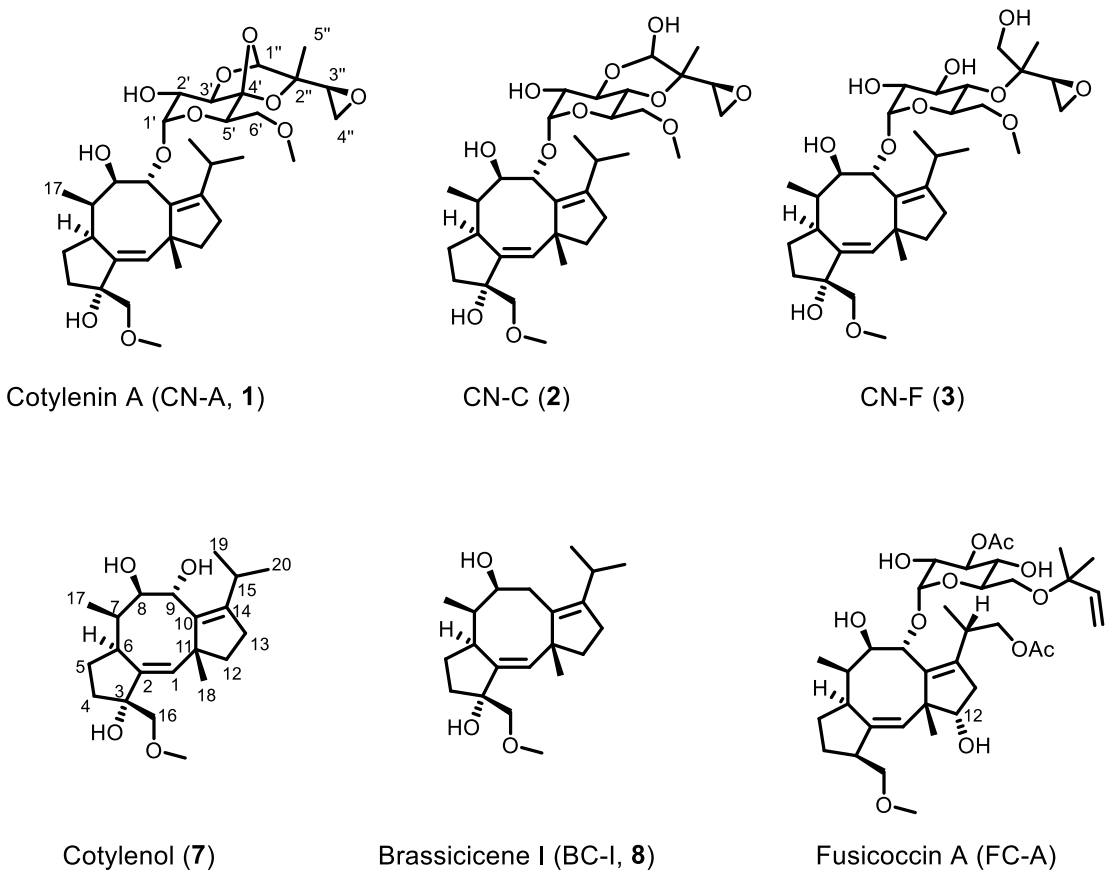



**Figure 1.** Chemical structures of representative CN natural products and biosynthetically related FC-A and BC-I

**1** exerts its biological effects by stabilizing protein-protein interactions (PPIs) involving the 14-3-3 protein, impacting cancer-relevant pathways such as C-RAF signaling[6]. This unique mode of action positions **1** as a promising therapeutic agent for cancer and other diseases. Given the integral role of PPIs in cellular processes and disease pathogenesis, **1**'s ability to target 14-3-3 PPIs offers a novel approach for therapeutic intervention. Furthermore, structurally related FC glycosides also demonstrate varying activity and selectivity towards 14-3-3 PPIs[7, 8]. However, studies on the activity of intermediates in the biosynthetic pathway of **1** against 14-3-3 protein PPIs have not been reported, possibly due to the inability to isolate these molecules.

Despite its biological significance, the preparation of **1** remains challenging due to several factors. The original producing organism, *Cladosporium* sp. 501-7W, has lost its ability to proliferate in culture[9], rendering microbial fermentation unfeasible. Additionally, the complexity of **1**'s molecular structure poses a significant hurdle, with

the first total synthesis reported by Masahisa Nakada's research group in 2020 requiring a 25-step process starting from Geraniol[10]. Many studies have attempted to shorten the total synthesis steps by employing concise synthesis or enzymatic synthesis of its aglycone **7**[11, 12]. However, even with these efforts, the intricate sugar modifications continue to present difficulties in chemical synthesis.

These challenges underscore the need for alternative approaches, such as identifying the biosynthetic gene cluster (BGC) from other strains or employing synthetic biology strategies to develop more efficient and scalable methods for **1** production and derivative synthesis. One such attempt involved knocking out genes in the production organism of FC-A, thereby generating structurally similar glycosylated precursors for semisynthetic production[9].

In this study, we successfully identified a BGC and heterologously expressed it, leading to the production of crucial intermediates such as CN-C (**2**) and F (**3**). Additionally, we identified glycosyltransferases and modifying enzymes for sugar moieties, enabling enzymatic synthesis of active intermediates and unnatural CN derivatives. Our research will make significant contributions to the ultimate biosynthesis of **1** and the preparation of its derivatives.

**Identification of the Cotylenin BGC**

The original producing strain of CNs has lost its ability to proliferate, and there are no reports of its genome, thus making the search for its BGC challenging. Considering the structural similarity between CNs and FCs, the BGC of CNs should have a high degree of similarity with the *Pa* cluster of FCs. The fusicoccadiene synthase (FS) and glycosyltransferase, as well as prenyltransferase, are its indispensable components. At the same time, the BGC of CNs should also have its uniqueness, possessing a methyltransferase on the glycoside and lacking the acetyltransferase found in the *pa* cluster.

Based on these characteristics, we identified an *Pa*-like cluster from the genomic library of our own fungus *Talaromyces adpressus* (TA), which contains the fusicoccadiene synthase (FS) and glycosyltransferase. However, it lacks the prenyltransferase. Since this cluster is located at the end of the scaffold, it is suspected

to be incomplete. Using cblaster[13], we found a highly similar but longer biosynthetic gene cluster (BGC) in the *Talaromyces verruculosus* TS63-9 strain (Figrue S1), which contains both a prenyltransferase and a methyltransferase. Subsequently, we performed a local BLAST search for these two enzymes in our strain and found two homologous enzymes located next to each other at the end of another scaffold. By connecting the ends of these two scaffolds in our strain, we obtained a more complete BGC, which we named the *cty* cluster (Figure 2).We then conducted a comparative analysis of this cluster with the *Pa* cluster and the Brassicicenes BGC *abn* cluster[14](Figure 2).

BC-I (**8**) is a common intermediate for both CN and BC compounds, formed by the five genes *abnABCDE*[14] (Figure 2), which have highly similar counterparts *ctyDFGHI* in the *cty* cluster (Table S3). After the formation of BC-I, the subsequent steps of 9-hydroxylation, glycosylation, and prenylation are similar for FCs and CNs. Not surprisingly, we found that the three modifying enzymes involved in the FC pathway, Pa-orf6 (glycosyltransferase), Pa-orf7 (C-9 hydroxylase), and Pa-orf11 (prenyltransferase), have highly similar homologs in the *cty* cluster: ctyD, ctyB, and ctyJ, respectively. Additionally, we identified two genes unique to the *cty* cluster, including *ctyE,* which encodes a methyltransferase similar to those found in bacteria, and *ctyA*, a P450 enzyme with low homology to the P450 enzymes in the other two clusters, possibly catalyzing a novel oxidation reaction. Based on this information, we propose that the *cty* cluster is likely responsible for the biosynthesis of CNs.

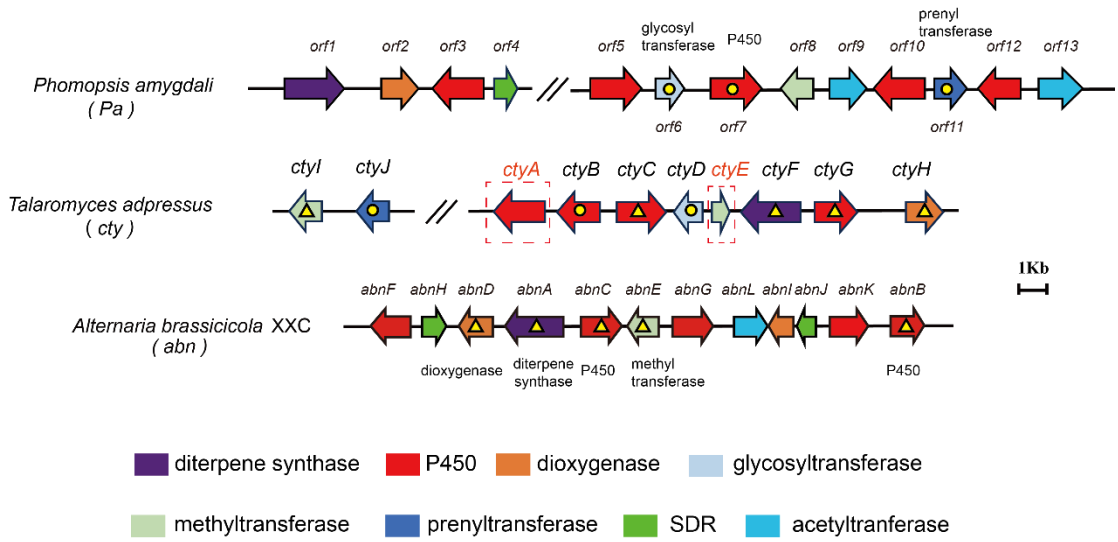



**Figure 2:** Comparison of the *Pa* (FC BGC), *cty* (CN BGC), and *abn* (BC BGC). Yellow triangles mark the homologous genes in the *abn* and *cty* clusters that synthesize BC-I. Yellow circles indicate the homologous genes in the *Pa* and *cty* clusters involved in the later stage of FC and CN biosynthetic pathways after BC-I formation. Unique genens (*ctyA* and *ctyE*) are marked in red boxes.

### Heterologous Expression of *cty* Cluster

We cultured *Talaromyces adpressus* on various media but did not observe the production of CNs (Figure S2), suggesting that this cluster is likely silent. Therefore, we employed heterologous expression techniques to study it.

We first heterologously expressed the terpene synthase CtyF and confirmed it produced fusicoccadiene (Figure S14). Given the high homology between CtyDFGHI and the BC-I (**8**)-producing enzymes AbnABCDE, we hypothesized that *AO-ctyDFGHI* also produces **8**. Therefore, instead of constructing *AO-ctyDFGHI*, we assumed **8** as its product and used it to feed the transformants of other *cty* genes to quickly verify their functions. We constructed the following transformants: *AO-ctyB, AO-ctyBD, AO-ctyBDE, AO-ctyBDEJ,* and *AO-ctyABDEJ*, and analyzed the feeding products using LC-MS. In AO-ctyB fed with **8**, we detected a new peak with a molecular weight (mw) increase of 16 (Figure 3, lane ii), which was isolated and identified as compound **7** through NMR spectroscopy (Figure S15-16 and Table S4). In AO-ctyBD adding **8**, we detected a new peak with a mw increase of 162 (Figure 3, lane iii), which was isolated and identified as the glycosylated product **6** (Figure S17-19 and

Table S5). We then introduced the methyltransferase *ctyE*, and after feeding **8**, LC-MS detected a new peak with a mw increase of 14 (Figure 3, lane iv). Scale up fermentation and isolation identified this compound as the methylated product **5** (Figure S20-23 and Table S6). To verify the function of the prenyltransferase, we further introduced *ctyJ* into the previous transformant, and after feeding **8**, LC-MS detected a new peak with a mw increase of 68 (Figure 3, lane v). Scale up fermentation and isolation identified this compound as the prenylated product **4** (Figure S24-26 and Table S7). Next, we introduced the last uncharacterized P450 oxidase, CtyA and expected to obtain products with oxidative modifications on the glycosyl moiety. After feeding **8**, LC-MS detected two new peaks with mw increases of 32 and 30 (Figure 3, lane vii). Scale up fermentation and isolation identified these compounds as **3** (Figure S27-30 and Table S8) and **2** (Figure S31-34 and Table S9), respectively. Additionally, in the *AO-ctyBDEJ* transformant lacking the methyltransferase *ctyE*, feeding **8** resulted in small amounts of compounds **12** (mw 619) and **13** (mw 635) (Figure 3, lane vi). Compound **12** was isolated, but due to its small quantity, it was difficult to purify completely. However, 1D and 2D NMR allowed structural assignment (Figure S35-40 and Table S10), revealing a C3"-4" epoxy structure, with no oxidation at the C1". This indicates that CtyJ and CtyA can accept substrates without methylation at the C6' position, forming a monooxygenated epoxy product by CtyA. Due to the low yield, **13** could not be isolated for NMR analysis., but based on its molecular formula and elution time, we infer that its structure is a further hydroxylation product of compound **12** at the 1" position (Scheme 1).

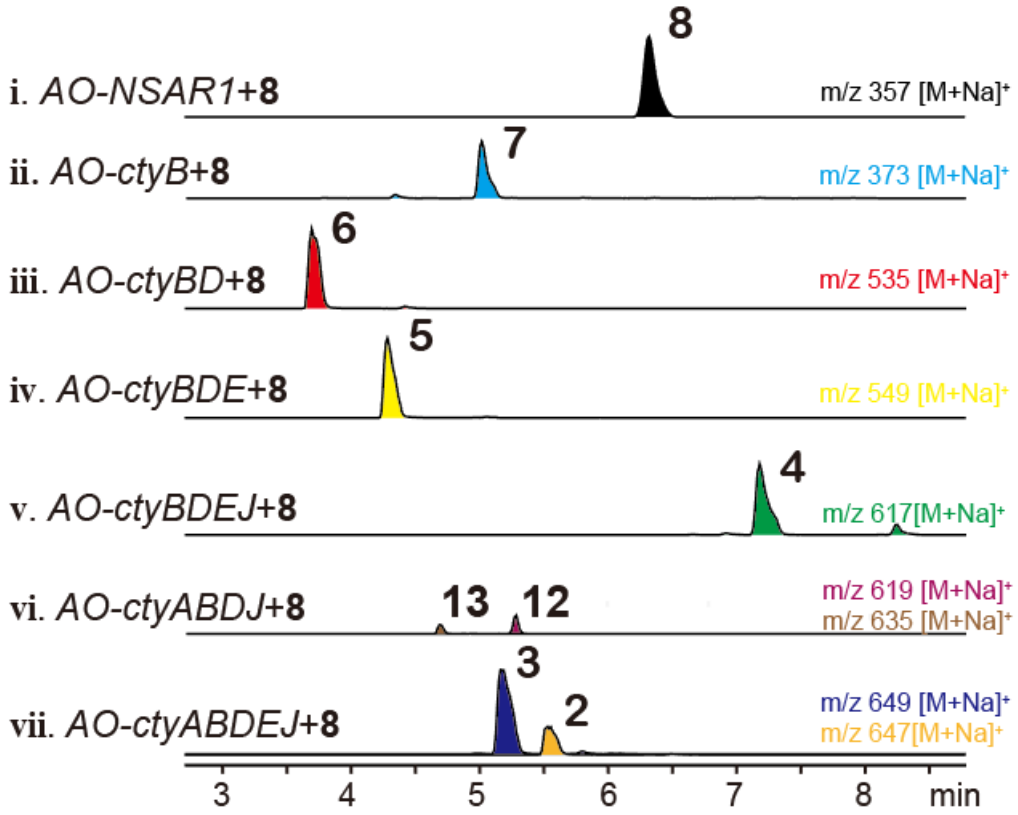



**Figure 3**. EIC chromatography of extracts from *AO* transformants of different *cty* gene combinations fed with **8**.

The feeding results indicate that CtyA is multifunctional, capable of introducing an epoxide on the prenyl C3"-4" double bond and oxidizing the prenyl C1" to a hydroxyl and aldehyde group (Figure 4A). To further investigate the order of its modifications, we constructed *AO-ctyA* and examined the biotransformation products over a time gradient. The results showed that feeding **4** to *AO-NSAR1* did not result in any detectable oxygenated products, and the substrate remained unconsumed (Figure 4B, lane iv). In *AO-ctyA*, small amounts of a monooxygenated compound **11** were observed at 1.5 and 2.5 days (Figure 4B, lanes i and ii), but **2** and **3** were more predominant. After 3.5 days, **11** almost disappeared, with only **2** and **3** remaining (Figure 4B, lanes iii). To confirm that the conversion from **3** to **2** was not caused by the heterologous host, we fed **3** to *AO-NSAR1* and did not observe the formation **2** (Figure 4B, lane vi). The mw of **11** corresponds to the molecular formula $C_{32}H_{52}O_{10}$, but it could not be isolated due to its low yield. Based on the structure of **12**, we speculate that **11** is the epoxidized

product of **4** at the C3"-4" double bond (Figure 4B, lanes i and ii). These in vivo experiments demonstrate that CtyA is a multifunctional oxidase, capable of both epoxidizing the terminal double bond on the glycosylated prenyl group and performing 4-electron oxidation on the methyl group.

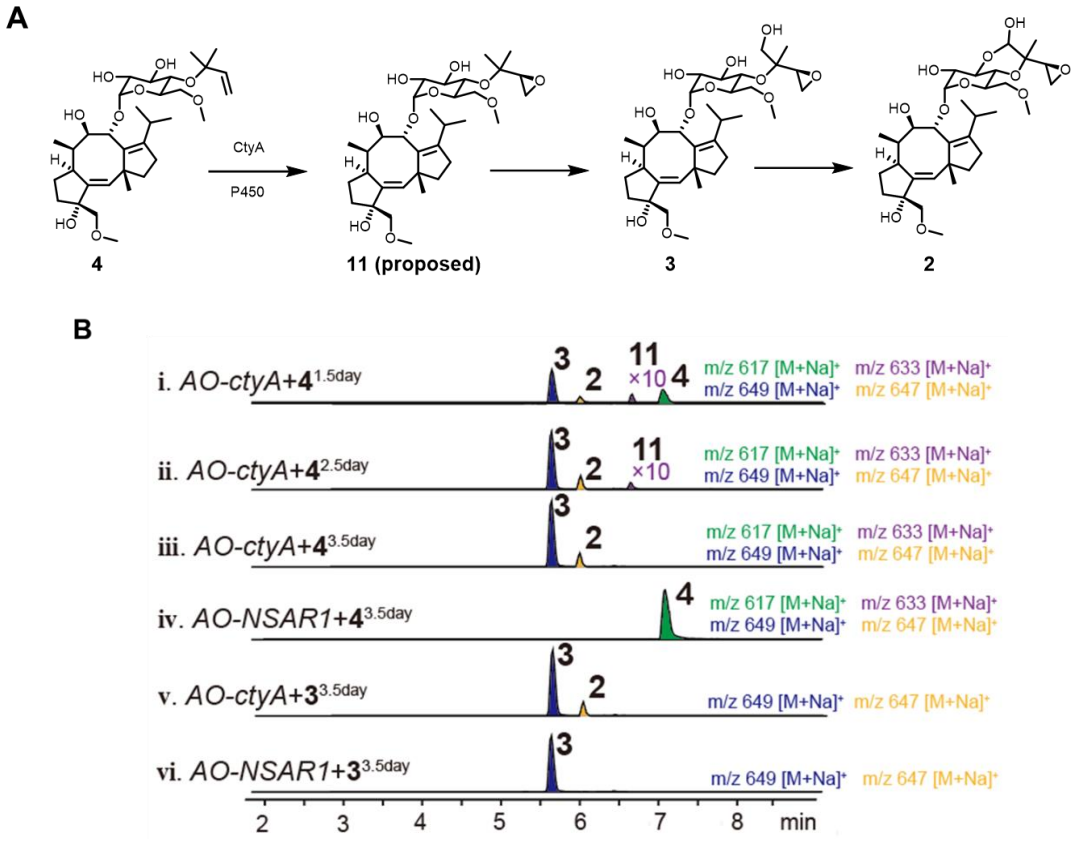



**Figure 4.** EIC chromatograms of extracts from *AO-ctyA* and *AO-NSAR1* at different days of biotransformation: i-iii, products detected from *AO-ctyA* fed with **4** at 1.5, 2.5, and 3.5 days; iv, products detected from *AO-NSAR1* fed with **4** at 3.5 days; v-vi, products detected from *AO-ctyA* and *AO-NSAR1* fed with **3** at 3.5 days.

Converting **2** to **1** requires only one more oxidation step to form an oxygen bridge between C4' and C1". We proceeded by introducing genes from the extended region of the *cty* cluster (Figure S3, lane I, ii, iii and iv, Table S3), particularly focusing on the FAD enzyme *hp5* with potential oxidase functions. Unfortunately, none of the transformants produced CN-A (Figure S3, lane v). This suggests two possibilities: either the final catalytic enzyme is outside the *cty* cluster, or the enzyme required for the formation of **1** is absent in the *TA* strain. To verify these possibilities, we fed the previously obtained pathway intermediates **8, 6, 4**, and **2** to *TA* in multiple medium, but

**1** was still not detected (Figure S2). This result supports the notion that although the *TA* strain possesses the BGC for CNs production, it lacks the capability to form **1**. Further efforts will be needed to identify new strains or novel oxidases to achieve the complete biosynthesis of CN-A.

**Functional Characterization of Pathway Enzymes In Vitro**

CNs and FCs undergo extensive modifications beyond glycosylation, with CNs involving prenylation, methylation, and oxidation, and FCs involving prenylation and acetylation. We proceeded to examine their in vitro activities.

Biotransformation results indicates the glycosyltransferase CtyD converts **7** into **6**. Alphafold2 modeling indicates that it belongs to the GT-A family of glycosyltransferases[15] (Figure S4). We purified CtyD and performed in vitro reactions with **7**. In the presence of the divalent metal ion $Mg^{2+}$, CtyD introduces glucose at the C9 hydroxyl position (Figure 5, lane i), while no activity was observed in the presence of $Zn^{2+}$(Figure 5, lane ii). The poor in vitro activity is likely due to the low solubility of CtyD expressed in *E. coli* (Figure S13).

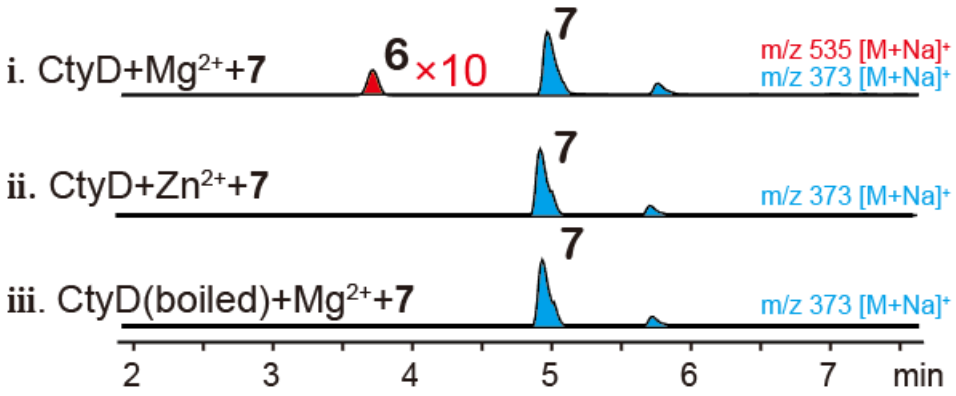



**Figure 5.** EIC chromatogram of in vitro enzymatic activity of CtyD

In vivo experiments showed that the prenyltransferase CtyJ catalyzes the prenylation of the C4'-hydroxyl group on the glycoside of **5**, whereas the Pa-orf11 (H7CE84.1) from the *Pa* cluster prenylates the C6'-position on the glycoside. To investigate whether these PTs have substrate specificity and to prepare artificial combinatorial molecules, we reacted CtyJ and Pa-orf11 with **6** separately. Both enzymes recognized the substrate, but the prenylation sites remained regioselective, producing **9** (Figure S41-46 and Table S11) and an unnatural molecule **10**(Figure S47-

52 and Table S12), respectively (Figure 6A). Prenylation on glycosides is rare; a recent example is the work by Zou's group, which reported that the glycosyltransferase CosD can introduce a prenyl group onto linear saccharide[16]. Phylogenetic analysis revealed that CtyJ, Pa-orf11, and CosD belong to typical fungal prenyltransferases and share high homology (Figure S5).

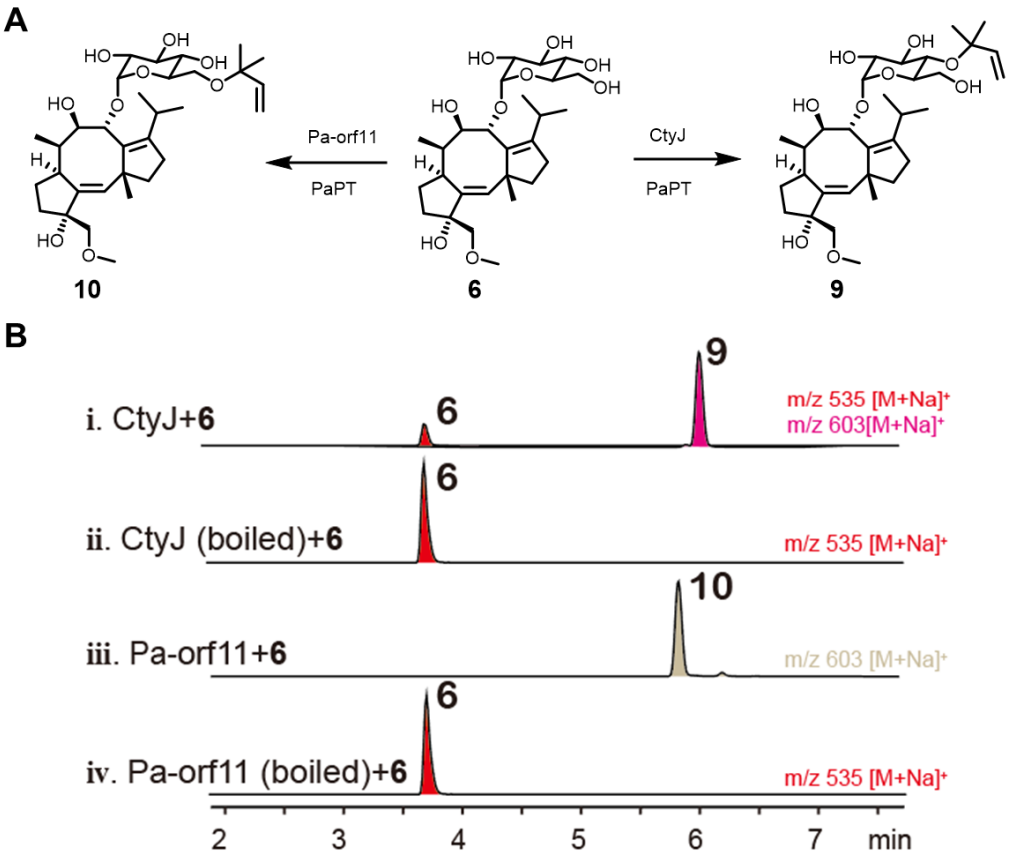



**Figure 6.** In vitro enzymatic activity of CtyJ and Pa-orf11: A, Reactions catalyzed by CtyJ and Pa-orf11; B. EIC chromatograph of the in vitro reaction of CtyJ and Pa-orf11 with **6.**

In vivo experiments showed that the methyltransferase CtyE converts **6** into **5**. Purified CtyE exhibited the same activity, but it cannot recognize the prenylated product **9** (Figure 7). This result clarifies that methylation occurs before prenylation in the pathway (Scheme 1). According to the three-dimensional structure predicted by Alphafold2, CtyE belongs to the Type I MTase family (Figure S6). Methyltransferases acting on glycosides are rare in fungi but are more common in bacteria, appearing in pathways such as the polyketide glycoside elloramycin[17] and the macrolide

mycinamicin[18]. Phylogenetic analysis of representative MTases from fungal and bacterial secondary metabolites showed that CtyE has higher homology with bacterial MTases (Figure S7). This interesting phenomenon suggests that bacteria might have first evolved methyltransferases for metabolizing **6**, and this capability was later acquired by fungi through gene transfer.

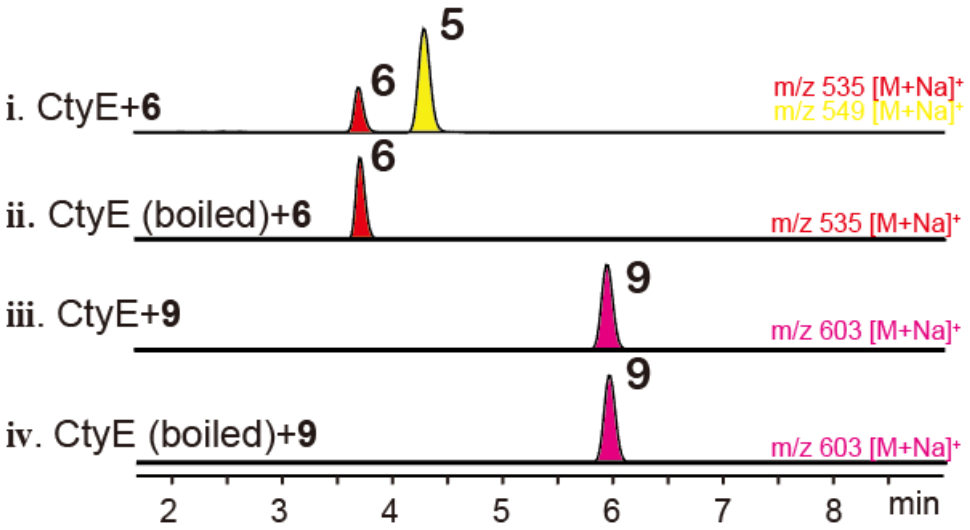



**Figure 7.** EIC chromatograph of in vitro reaction of CtyE with **6** and **9**

The P450 enzyme CtyA exhibited consecutive oxidation activity on the prenyl group in vivo. We introduced its cDNA into yeast, extracted the microsomes, and tested the activity (Figure 8). The results showed that CtyA can oxidize **4** to **11** and **3** in vitro, but the efficiency is much lower than in *AO*, and **2** was not detected. The in vitro results confirmed CtyA's ability to oxidize different positions on the prenyl group, suggesting that after the formation of the epoxide, a change in the relative position brings the C1" position closer to the Heme iron center (Figure S8). We also examined CtyA's activity on **10**, which has a shifted prenyl position, but no conversion was observed (Figure S9).

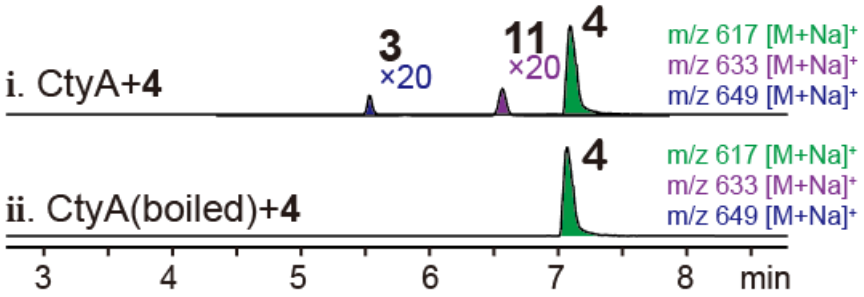
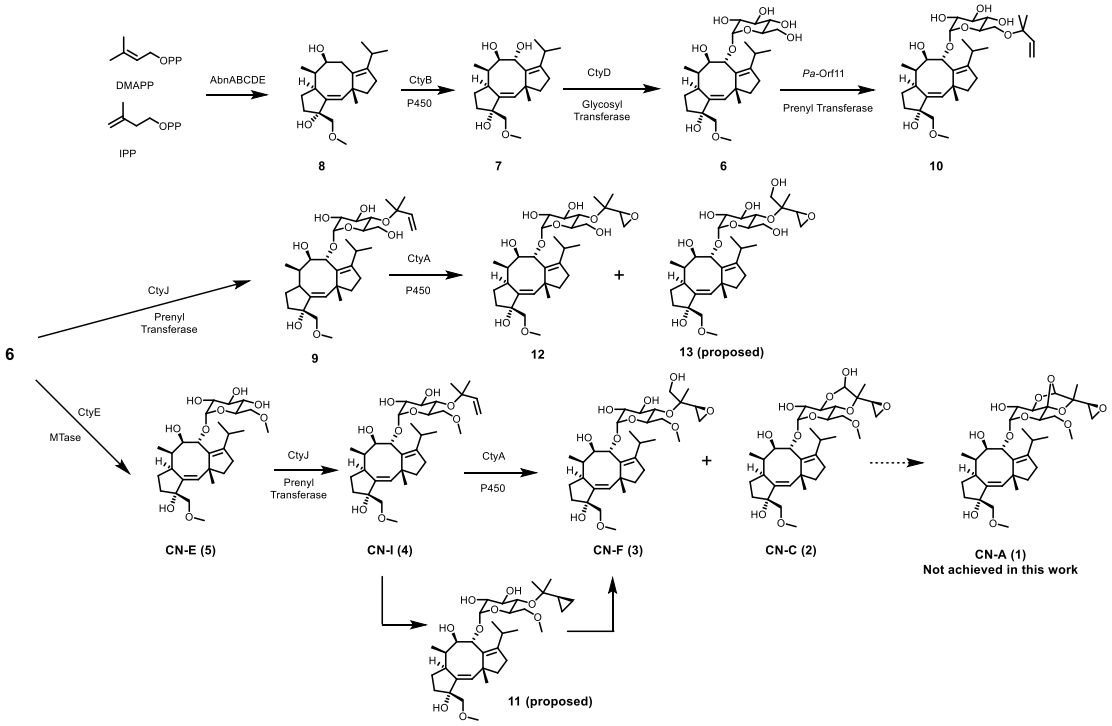



**Figure 8.** EIC chromatography of microsome fraction from yeast expressing CtyA reacting with **4**

Based on the results from the in vivo and in vitro experiments, we have elucidated for the first time the biosynthetic pathways of several CN type diterpene glycosides, including Cotylenin E (**5**), I (**4**), F (**3**), and C (**2**) (Scheme 1), remaining only one oxidation step away from forming the oxygen bridge in CN-A (**1**). Additionally, by recombining the relevant genes in *AO*, we have isolated and identified previously unreported natural intermediates **6, 9**, and **12** in the CNs pathway, as well as a new molecule **10** by borrowing the prenyltransferase from *Pa* cluster.

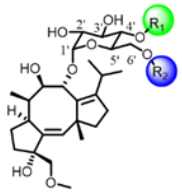



**Scheme 1.** Reconstitution of the biosynthetic pathway of CNs in this work

**Stabilizing Activity of CNs on 14-3-3 PPIs**

CN-A is known as a 14-3-3 protein-protein interaction (PPI) stabilizer and has been reported to stabilize the interaction between the C-RAFpS233pS259 peptide segment and 14-3-3ζ protein in vitro[6]. We attempted to obtain CN-A from various sources, but were unsuccessful. Therefore, we tested the stabilizing effects of other purified CN compounds from this work on the C-RAFpS233pS259/14-3-3ζ PPI using a fluorescence polarization assay (Figure 9). The results showed that the aglycone **7** had the weakest stabilizing activity, while the glycosylated products exhibited significantly improved activity (Figure 9A). Compounds **2-6** and **9** are intermediates in the CN pathway, and the trend observed is that the closer the structure is to **1**, the better the stabilizing effect. Notably, Molzan *et al.* obtained an $EC_{50}$ value of 65.4 ± 7.7 μM for CN-A using the same method and conditions that we adopted in our study[6](Figure 9B). Although a direct comparison is not possible, our results indicate that **2** exhibits a stabilizing capability on par with CN-A. Interestingly, the unnatural molecule **10** had activity better than **2**. Specific structural-activity relationship is proposed as follows: compound **10**, with a prenyl group at the C6' position, had an $EC_{50}$ value of 46.2 ± 5.5 μM. When the prenyl group was shifted to the C4' position, the $EC_{50}$ value of **9** sharply increased to 938.1 ± 258.9 μM. This significant difference suggests that introducing a hydrophobic group at the glycosyl C6' position is crucial for stabilizing activity. **6** had an $EC_{50}$ value of 323.1 ± 98.7 μM, and after methylation at the C6'-position, the $EC_{50}$ of **5** decreased to 168.1 ± 24.6 μM, supporting the previous hypothesis. On the other hand, the oxidation of the C4' prenyl group plays a beneficial role in PPI stabilization, as reflected in the increasing $EC_{50}$ values from compounds **2, 3**, to **4**. We further tested the impact of these compounds on the apparent affinity ($K_d$) of the C-RAFpS233pS259/14-3-3ζ interaction, which showed a trend consistent with the $EC_{50}$ values (Figure 9B). These findings suggest that the enzymatic steps in the biosynthetic pathway have evolved to enhance the bioactivity of the metabolic products.

A

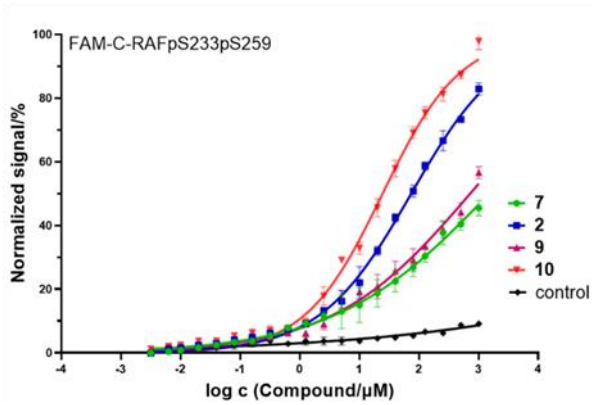


R1=Oxidized prenyl group
R2=Hydrophobic group
Good stabilizing effect

B

| | 9 | 7 | 6 | 5 | 4 |
|---|---|---|---|---|---|
| $EC_{50}$ (μM) | 938.1±258.9 | 3313± 1239 | 323.1±98.7 | 168.1± 24.6 | 165.2±21.7 |
| Apparent $K_d$ (nM) | 36.0 | 64.7 | 19.1 | 11.8 | 19.0 |
| | **3** | **2** | **10** | reported **1** | control |
| $EC_{50}$ (μM) | 133.4±10.6 | 91.7±15.1 | 46.2± 5.5 | 65.4± 7.7 | |
| Apparent $K_d$ (nM) | 19.2 | 9.7 | 9.6 | 20.0 | 71.1 |

C




D

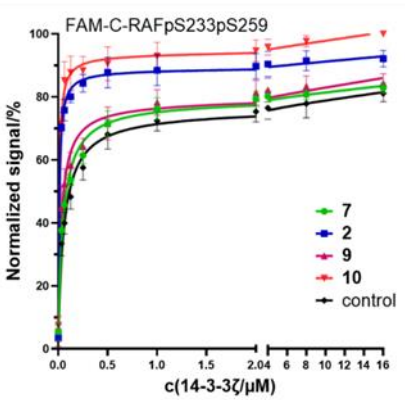



**Figure 9.** Stablizing effect of CN derivatives on 14-3-3 PPIs. A, Summary of SARs of CNs as PPI stabilizer; B, $EC_{50}$ and apperant $K_d$ results of the CN derivatives; C, FP measurements of FAM-labeled C-RAFpS233pS259 peptide (20 nM) and 14-3-3ζ (0.25 μM) titrated with different CNs to obtain $EC_{50}$ values (control: sample without CNs); D, FP measurements of FAM-labeled C-RAFpS233pS259 peptide (20 nM) in the absence or presence of 150 μM of the different CNs titrated with 14-3-3ζ to obtain the apparent $K_d$ (control: sample without CNs).

## Conclusion:

In this study, through heterologous expression and functional characterization of enzymes within the *cty* cluster, we elucidated the biosynthetic pathways of several natural diterpene glycosides, including cotylenin E (**5**), I (**4**), F (**3**), and C (**2**). We identified and confirmed the roles of key enzymes responsible for assembling and modifying the sugar moiety. Additionally, we demonstrated the multifunctional oxidation activity of the P450 enzyme CtyA on the prenyl group, highlighting its ability to perform sequential oxidation steps.

Our investigation extended to exploring enzyme substrate promiscuity and

regioselectivity, leading to the creation of the unnatural Cotylenin derivative **10**. This demonstrates the value of these enzymes as tools for combinatorial biosynthesis and enzymatic synthesis to prepare more diverse diterpene glycoside derivatives. Furthermore, we revealed for the first time the stabilizing activity of CN biosynthetic intermediates on 14-3-3 PPIs. Our studies suggest that the biosynthetic pathway has evolved to enhance the bioactivity of the metabolic products, as evidenced by the systematic increase in PPI stabilization activity observed from intermediate compounds to CN-A.

In summary, our comprehensive functional characterization and synthetic biology approach have provided insights into the intricate biosynthesis of diterpene glycosides and identified potential pathways for further engineering to enhance bioactive properties. Future efforts will focus on identifying new strains or novel oxidases to achieve the complete biosynthesis of CN-A, thereby unlocking its full therapeutic potential.

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