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Total biosynthesis of cotylenin diterpene glycosides as 14-3-3 protein–protein interaction stabilizers†

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Cotylenins (CNs) are bioactive fungal diterpene glycosides that exhibit stabilizing activity on 14-3-3 protein–protein interactions (PPIs), which has significant therapeutic potential. Although CNs were isolated as early as 1970, their biosynthetic pathway has remained unclear, and their limited supply has hindered further research. Here, we report the identification of the biosynthetic gene cluster *cty* and elucidation of the biosynthetic pathway of CNs. Our investigation reveals the roles of glycosyltransferase, methyltransferase, and prenyltransferase enzymes in the assembly and modification of the saccharide moiety, as well as the multifunctional oxidation activity of the P450 enzyme CtyA. We leveraged this knowledge to achieve the total biosynthesis of not only key intermediates such as CN-C, E, F, and I, but also a novel, unnatural CN derivative using heterologous expression. This showcases the potential of pathway enzymes as catalytic tools to expand the structural diversity of diterpene glycosides. Furthermore, the stabilization effects of pathway intermediates on 14-3-3 PPIs underscore the importance of saccharide modifications in bioactivity. These findings provide a foundation for future rational synthesis of cotylenin A and other structurally diverse derivatives, broadening the scope of diterpene glycoside production.

Introduction

Cotylenins (CNs) represent a class of fungal-derived diterpene glycosides renowned for their diverse biological activities.[1–3] 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 saccharide moiety modifications (Fig. 1). Originally isolated from the plant pathogenic fungus *Cladosporium* sp. 501-7W in 1970, CN-A exhibits plant hormone-like activity and shows promise in inducing leukemia cell differentiation and enhancing the efficacy of various anticancer drugs.[4,5] Besides **1**, additional CN derivatives, such as CN-C (**2**) and F (**3**) were

isolated from the same strain.[6] Other structurally and biosynthetically related molecules include fusicoccin A (FC-A),[1] brassicicene I (BC-I, **8**)[7] and their derivatives.

Compound **1** exerts its biological effects by stabilizing protein–protein interactions (PPIs) involving the 14-3-3 protein,
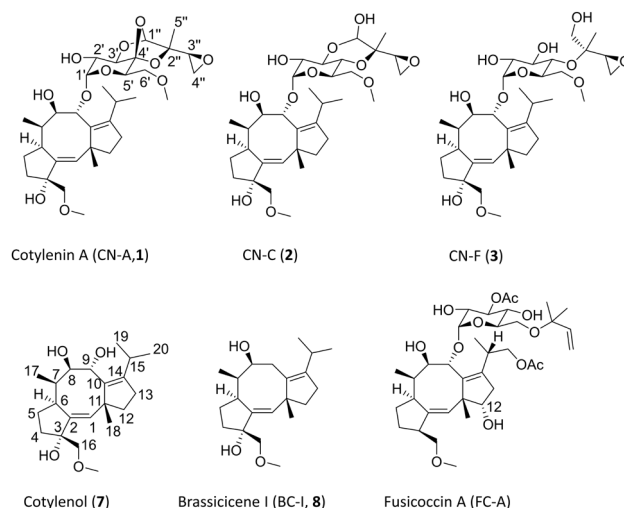

Fig. 1 Chemical structures of representative CN natural products and biosynthetically related FC-A and BC-I.

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a family of regulatory proteins that bind phosphorylated targets to modulate their function. This action impacts cancer-relevant pathways such as C-RAF signaling.[8] Given the integral role of PPIs in cellular processes and disease pathogenesis,[9] compound **1**'s ability to target 14-3-3 PPIs offers a novel approach for therapeutic intervention of cancer and other diseases. Furthermore, structurally related FC glycosides also demonstrate varying activity and selectivity towards 14-3-3 PPIs.[10,11] However, studies on the activity of other natural CN products on 14-3-3 PPIs have not been reported, likely due to the difficulty in isolating 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,[12] 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.[13] Many studies have attempted to shorten the total synthesis steps by employing concise synthesis or enzymatic synthesis of its aglycone **7**.[14,15] However, even with these efforts, the intricate saccharide 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 to produce **1** and other CN derivatives. One such attempt involved knocking out genes in the FC-A BGC, thereby generating analogous glucosylated precursors for semisynthetic production.[12] FC-A, which is structurally similar to CN-A, benefits from an available producing strain, and its BGC and biosynthetic pathway have been extensively studied by the Dairi group.[7,12,16,17]

In this study, we identified a BGC and achieved the production of crucial intermediates such as CN-C (**2**) and F (**3**) through heterologous expression in *Aspergills oryzae* NSAR1 (*AO*).[18,19] Additionally, we characterized the glycosyltransferase and the modifying enzymes for the saccharide moiety, enabling enzymatic synthesis of previously unidentified intermediates and unnatural CN derivatives. Our research will make significant contributions to the ultimate biosynthesis of **1** and the preparation of its derivatives.

Results

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 known BGC of FCs (*Pa* in Fig. 2).[12] 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 saccharide methyltransferase (MTase) gene and lacking the acetyltransferase gene 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 encodes the 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,[20] we found a highly similar but longer BGC in the *Talaromyces verruculosus* TS63-9 strain (Fig. S1†), which encodes both a prenyltransferase and a MTase. Subsequently, we performed a local BLAST search for these two enzymes in our *TA* 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, we obtained a more complete BGC, which we named the *cty* cluster (Fig. 2). We then conducted a comparative analysis of this cluster with the *Pa* cluster and the brassicicenes BGC *abn* (Fig. 2).[21]
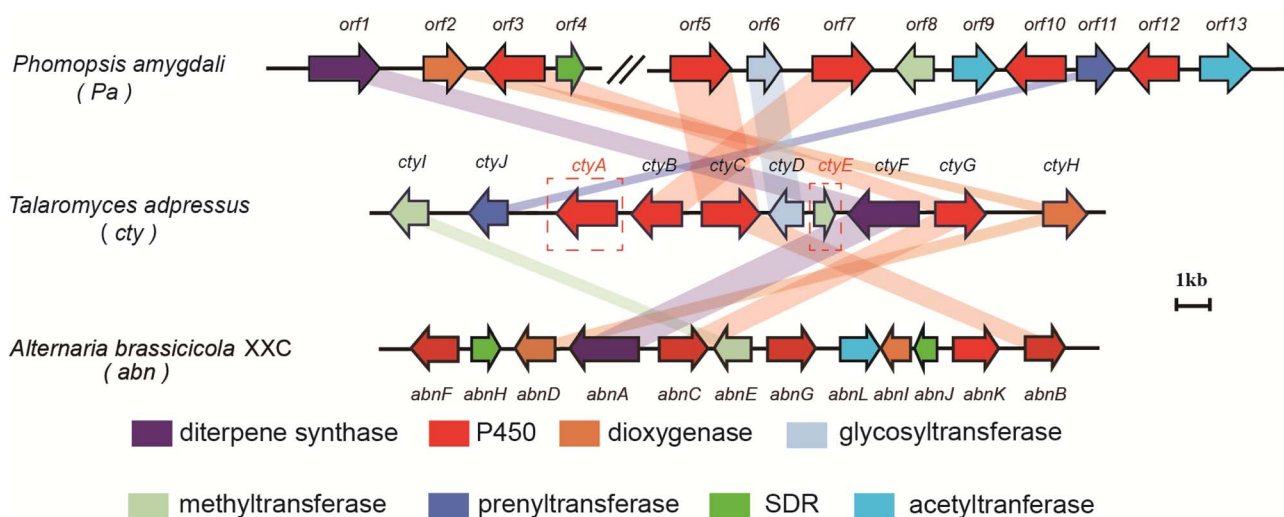

Fig. 2 Comparison of the *Pa* (FC BGC), *cty* (CN BGC), and *abn* (BC BGC). Homologous gene pairs with >40% identity to genes in the *cty* cluster are shaded. Unique genes (*ctyA* and *ctyE*) are marked in red boxes.

BC-I (**8**) is a common intermediate for both CN and BC compounds, formed by the five genes *abnABCDE*[21,22] in *abn* cluster (Fig. S2 and S16†), which have highly similar counterparts *ctyCFGHI* in the *cty* cluster (Fig. 2 and Table S3†). After the formation of BC-I, the subsequent steps of 9-hydroxylation, glucosylation, and prenylation are similar for FCs and CNs. Not surprisingly, we found that the three modifying enzymes in the FC pathway, Pa-orf6 (glycosyltransferase), Pa-orf7 (C-9 hydroxylase), and Pa-orf11 (prenyltransferase), have highly similar homologous genes in the *cty* cluster: *ctyD*, *ctyB*, and *ctyJ*, respectively (Fig. 2 and Table S3†). Additionally, we identified two genes unique to the *cty* cluster, including *ctyE*, which encodes a MTase annotated as a ribosomal RNA small subunit methyltransferase, and *ctyA*, a P450 enzyme with low homology to the P450 enzymes in the other two clusters, possibly catalyzing a novel oxidation reaction (Table S3†). Based on this information, we propose that the *cty* cluster is likely responsible for the biosynthesis of CNs.

Heterologous expression of *cty* cluster

We cultured *TA* on various media but did not observe the production of CNs (Fig. S3†), suggesting that this cluster is likely silent. Therefore, we employed heterologous expression techniques in *AO* to study it.

We first heterologously expressed the gene encoding terpene synthase CtyF and confirmed it produced fusicoccadiene (Fig. S15†). Given the high homology between CtyCFGHI and the BC-I (**8**)-producing enzymes AbnABCDE, we hypothesized that *AO-ctyCFGHI* also produces **8**. Therefore, instead of constructing *AO-ctyCFGHI*, we assumed **8** as its product and used it to feed the transformants of other *cty* genes to quickly verify their functions. Taking the modification sequence for biosynthesizing FCs as a reference, 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 Da (Fig. 3A(ii)), which was isolated and identified as compound **7** through NMR spectroscopy (Fig. S17, S18 and Table S4†). Recent years have seen progress in achieving C9 beta-hydroxylation of CN glycosides through organic catalysis due to the research by Nakada, Shenvi, and Renata, who have continuously improved the stereoselectivity of the reaction.[13–15] We recently reported that Orf7 in the *Pa* cluster can efficiently catalyze this reaction enzymatically.[23] Here, we discovered that CtyB has the same function. In *AO-ctyBD* adding **8**, we detected a new peak with a mw equals to **7** + 162 Da (Fig. 3A(iii)), which was isolated and identified as the glucosylated product **6** (Fig. S19–S21 and Table S5†). We then introduced the MTase *ctyE*, and after feeding **8**, LC-MS detected a new peak with a mw equals to **6** + 14 Da (Fig. 3A(iv)). Scale up fermentation and isolation identified this compound as the methylated product **5** (Fig. S22–S25 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 equals to **5** + 68 Da (Fig. 3A(v)). Scale up fermentation and isolation identified this compound as the prenylated product **4** (Fig. S26–S28 and Table S7†). Next, we introduced the last uncharacterized P450 oxidase CtyA, and expected to obtain products with oxidative modifications on the glucosyl moiety. After feeding **8**, LC-MS detected two new peaks with mw equals to **4** + 32 Da and + 30 Da (Fig. 3A(vii)). Scale up fermentation and isolation identified these compounds as **3** (Fig. S29–S32 and Table S8†) and **2** (Fig. S33–S36 and Table S9†), respectively. Additionally, in the *AO-ctyABDJ* transformant lacking the MTase *ctyE*, feeding **8** resulted in small amounts of compounds **12** (mw 619 Da) and
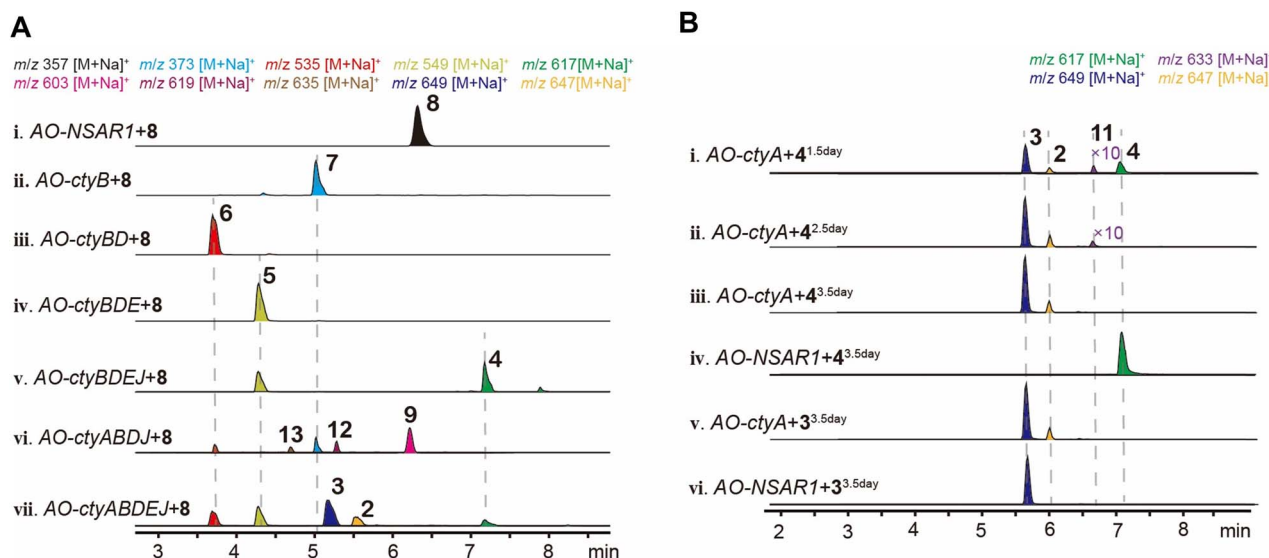

Fig. 3 Heterologous expression of *cty* cluster and the function of *ctyA*. (A) EIC chromatography of extracts from *AO* transformants of different *cty* gene combinations fed with **8**. (B) EIC chromatograms of extracts from *AO-ctyA* and *AO-NSAR1* at different days of biotransformation: (i–iii), 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 and vi), products detected from *AO-ctyA* and *AO-NSAR1* fed with **3** at 3.5 days.

13 (mw 635 Da) (Fig. 3A(vi)). Compound **12** was isolated, but due to its small amount, it was difficult to purify completely. However, 1D and 2D NMR allowed structural assignment (Fig. S37–S42 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 C1″ position (Fig. 4).

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. 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 (Fig. 3B(iv)). In *AO-ctyA*, small amounts of a monooxygenated compound **11** were observed at 1.5 and 2.5 days (Fig. 3B(i and ii)), but **2** and **3** were more predominant. After 3.5 days, **11** almost disappeared, with only **2** and **3** remaining (Fig. 3B(iii)). To investigate if CtyA can also transform the C1″ hydroxyl group to an aldehyde

group, **3** was fed into *AO-ctyA* for 3.5 days, resulting in the production of **2** (Fig. 3B(v)), while feeding **3** to *AO-NSAR1* did not observe the formation of **2** (Fig. 3B(vi)). The mw of **11** corresponds to the molecular formula $C_{33}H_{54}O_{10}$, 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 (Fig. 3B(i and ii)). These *in vivo* experiments demonstrate that CtyA is a multifunctional oxidase, capable of both epoxidizing the terminal double bond on the glucosylated prenyl group and performing 4-electron oxidation on the methyl group.

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 (Fig. S4(i, ii, iii, iv) and Table S3†), particularly focusing on the FAD enzyme *hp5* with potential oxidase functions. Unfortunately, none of the transformants produced CN-A (Fig. S4(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 (Fig. S3†). This result suggests that while the *TA* strain harbors the BGC for CNs, the exact capability of the strain to
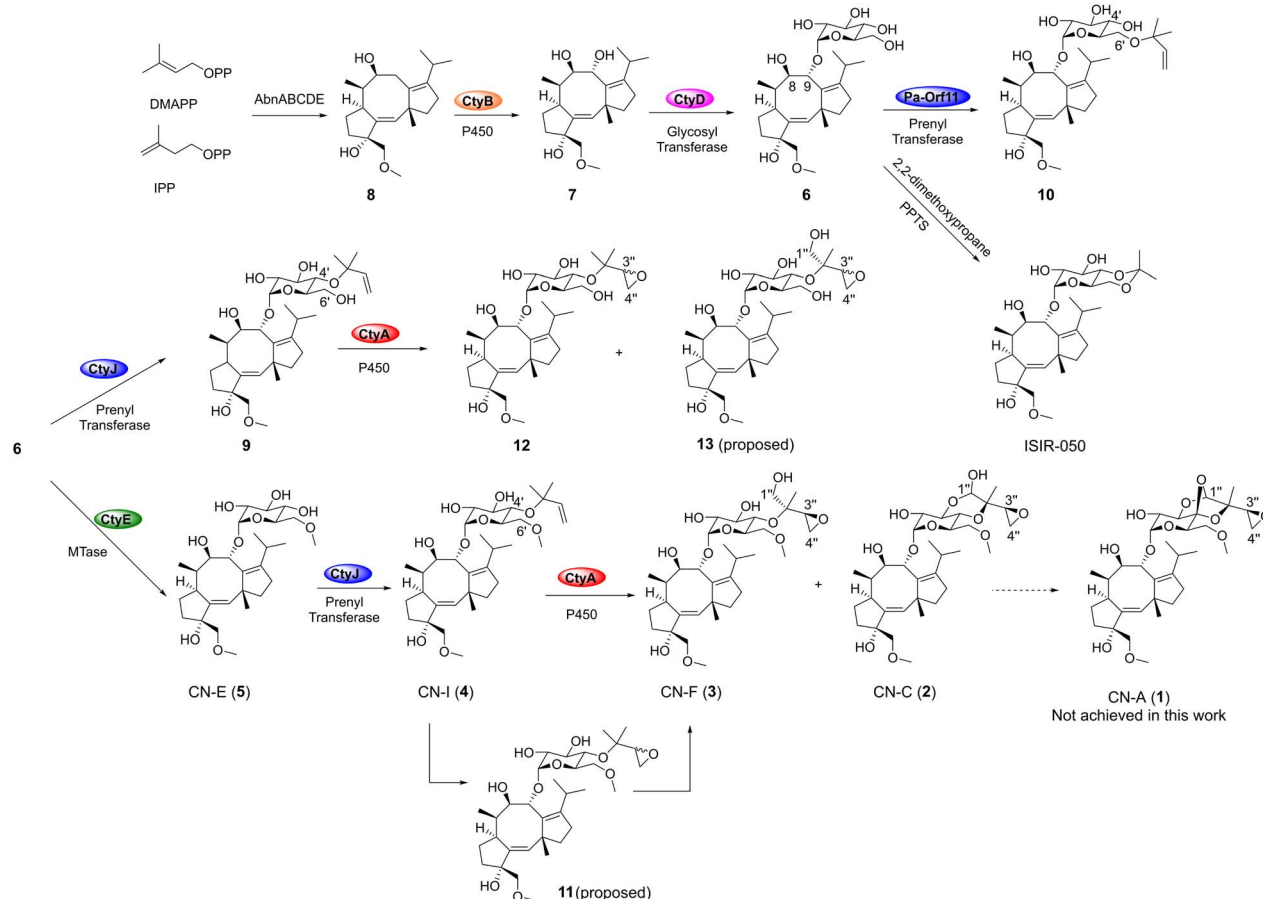

Fig. 4 Reconstitution of the biosynthetic pathway of CNs in this work and the semi-synthesis of ISIR-050.

produce CN-A remains unclear. 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 glucosylation, with CNs involving prenylation, methylation, and oxidation, and FCs involving prenylation and acetylation. We proceeded to examine their *in vitro* activities. Biotransformation results indicate the glycosyltransferase CtyD converts **7** into **6**. Alphafold2 modeling indicates that it belongs to the GT-A family of glycosyltransferases (Fig. S5†).[24] 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 (Fig. 5A(i)), while no activity was observed in the presence of Zn^{2+} (Fig. 5A(ii)). The poor *in vitro* activity is likely due to the low solubility of CtyD produced in *E. coli* (Fig. S14†).

In vivo experiments showed that the prenyltransferase CtyJ catalyzes the prenylation of the C4′-hydroxyl group on the saccharide of **5**, whereas the Pa-orf11 (H7CE84.1) from the *Pa* cluster is reported to prenylate the C6′-position on the FC's saccharide moiety. To investigate the substrate specificity of these PTs and try to prepare artificial combinatorial molecules, we reacted CtyJ and Pa-orf11 with **6** separately. Both enzymes exhibited regioselectivity by recognizing the substrate and catalyzing prenylation reactions at specific sites, producing **9** (Fig. S43–S48 and Table S11†) and an unnatural molecule **10**

(Fig. S49–S54 and Table S12†), respectively (Fig. 5B). Prenylation on saccharides is rare; a recent example is the work by Zou's group, which reported that the prenyltransferase CosD can introduce a prenyl group onto linear saccharide.[25] Phylogenetic analysis revealed that CtyJ, Pa-orf11, and CosD belong to typical fungal prenyltransferases and share high homology (Fig. S6†).

In vivo experiments showed that the MTase CtyE converts **6** into **5**. Purified CtyE exhibited the same activity, but it cannot recognize the prenylated product **9** (Fig. 5C). This result clarifies that methylation occurs before prenylation in the pathway (Fig. 4). According to the protein structure predicted by Alphafold2, CtyE belongs to the Type I MTase family (Fig. S7†).[26] MTases acting on saccharides are rare in fungi but are more common in bacteria, appearing in pathways such as the polyketide glycoside elloramycin[27] and the macrolide mycinamicin.[28] Phylogenetic analysis of representative MTases from fungal and bacterial secondary metabolites showed that CtyE has higher homology with bacterial MTases (Fig. S8†). This interesting phenomenon suggests that bacteria might have first evolved MTases for metabolizing **6**, and this capability was later acquired by fungi through gene transfer.

The P450 enzyme CtyA exhibited consecutive oxidation activity on the prenyl group *in vivo*. We introduced its cDNA into *S. cerevisiae* RC01 which contains an integrated copy of cytochrome P450 reductase (AtCPR) from *Aspergillus terreus*,[29] extracted the microsomes, and tested the *in vitro* activity (Fig. 5D). The results showed that CtyA can oxidize **4** to **11** and **3**, but the efficiency is much lower than that in *AO*, and **2** was not
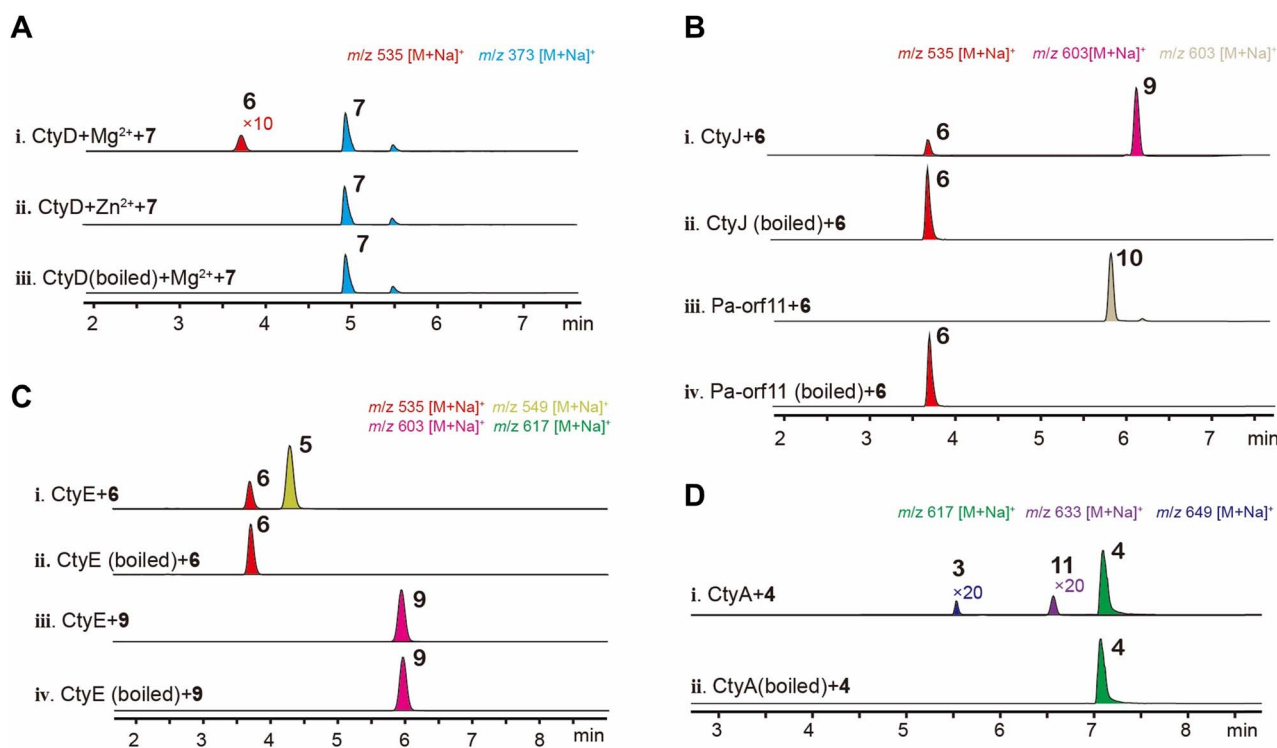

Fig. 5 The function study of CtyD, CtyJ, Pa-orf11, CtyE and CtyA. (A) EIC chromatogram of *in vitro* enzymatic activity of CtyD. (B) EIC chromatograph of the *in vitro* reaction of CtyJ and Pa-orf11 with **6**. (C) EIC chromatograph of *in vitro* reaction of CtyE with **6** and **9**. (D) EIC chromatography of microsome fraction from yeast expressing *ctyA* reacting with **4**.

detected. The *in vitro* results confirmed CtyA's ability to oxidize two distinct positions on the prenyl group, suggesting that after the formation of the epoxide, a change in the relative position of the intermediate brings the C1″ position closer to the Heme iron center (Fig. S9†). We also examined CtyA's activity on **10**, which has a shifted prenyl position, but no conversion was observed (Fig. S10†).

Based on the results from the *in vivo* and *in vitro* experiments, we have elucidated for the first time the biosynthetic pathways of several reported CN diterpene glycosides, including cotylenin E (**5**), I (**4**), F (**3**), and C (**2**) (Fig. 4), 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 intermediates **6**, **9**, and **12** in the CNs pathway, as well as an artificial molecule **10** by borrowing the prenyltransferase from *Pa* cluster.

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* by Molzan *et al.*[8] We attempted to request CN-A from various sources, but were unsuccessful. Instead, starting from compound **6**, we rapidly synthesized ISIR-050 (Fig. 4, S55 and S56†), a semi-synthetic CN-A mimic. This mimic has been reported to exhibit interferon-α (IFNα)-dependent growth

inhibitory activity and PPI stabilization similar to CN-A.[30] Through our experiments, we confirmed its ability to stabilize the interaction between the C-RAFpS233pS259 peptide segment and the 14-3-3ζ protein (Fig. 6). Using ISIR-050 as a positive control, we tested the stabilizing effects of the CN derivatives obtained in this study on the C-RAFpS233pS259/14-3-3ζ PPI using a fluorescence polarization (FP) assay method (Fig. 6). The results showed that the aglycone **7** had the weakest stabilizing effect, while the glucosylated intermediates **2–6** and **9** exhibited significantly improved activity with **2** outperforming ISIR-050 (Fig. 6B). 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 (Fig. 6B).[8] Although a direct comparison is not possible, our results indicate that **2** exhibits a stabilizing capability close to CN-A. Interestingly, the unnatural molecule **10** had activity better than **2**. We further analyzed the impact of different modifications on the glucosyl group on compound activity: compound **10**, with a prenyl group at the C6′ position, had an EC_{50} value of 43.5 ± 4.8 μM. When the prenyl group was shifted to the C4′ position, the EC_{50} value of **9** sharply increased to 823.9 ± 120.9 μM. This significant difference suggests that introducing a hydrophobic group at the glycosyl C6′ position is crucial for stabilizing activity (Fig. 6A). Compound **6** had an EC_{50} value of 313.4 ± 71.2 μM, and after methylation at the C6′-position, the EC_{50} of **5** decreased to 111.9 ± 12.5 μM, supporting the previous hypothesis. On the other hand, the oxidation of the
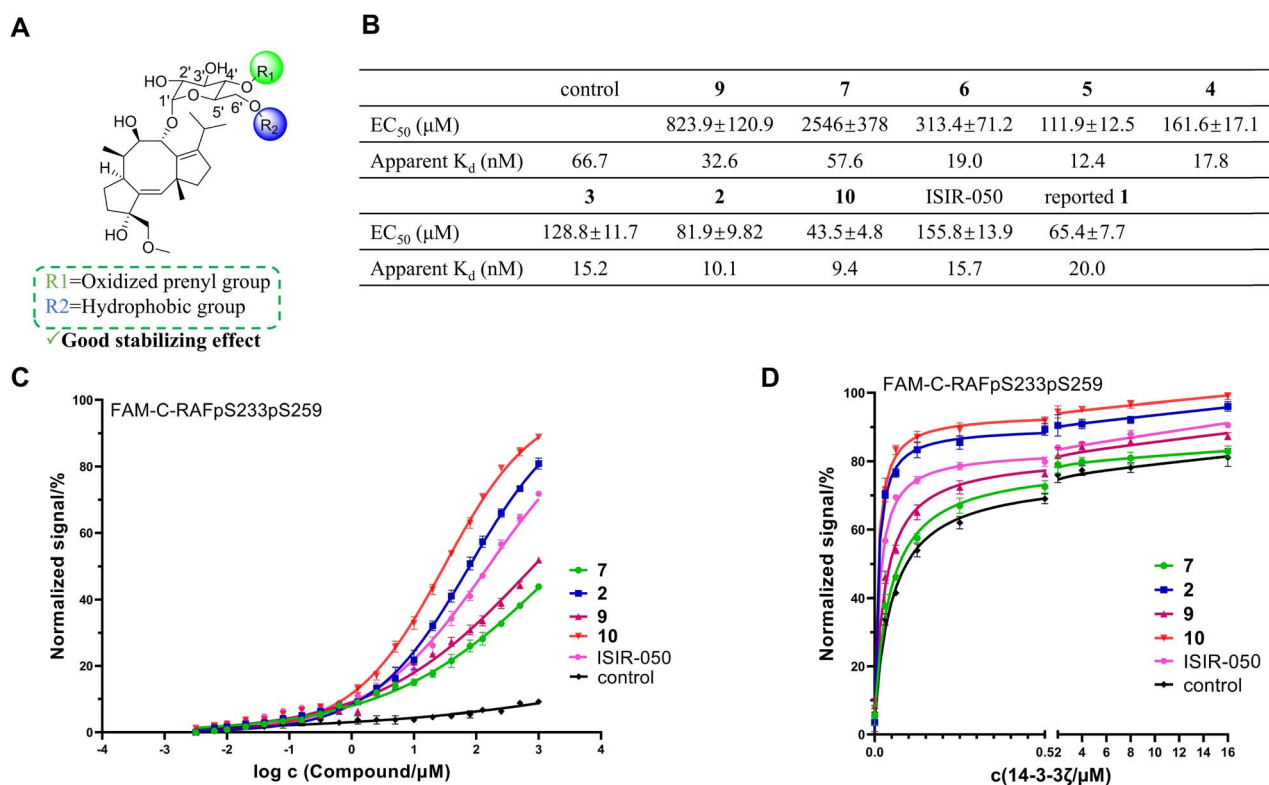

Fig. 6 Stablizing effect of CN derivatives on 14-3-3 PPIs. (A) Summary of structure–activity relationships of CNs as PPI stabilizer; (B) EC_{50} and apperant K_d results of the CN derivatives; (C) FP measurements of FAM-labeled C-RAFpS233pS259 peptide (20 nM) and 14-3-3ζ (0.25 μM) titrated with different 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).

	control	9	7	6	5	4
EC_{50} (μM)		823.9±120.9	2546±378	313.4±71.2	111.9±12.5	161.6±17.1
Apparent K_d (nM)	66.7	32.6	57.6	19.0	12.4	17.8
	3	2	10	ISIR-050	reported 1	
EC_{50} (μM)	128.8±11.7	81.9±9.82	43.5±4.8	155.8±13.9	65.4±7.7	
Apparent K_d (nM)	15.2	10.1	9.4	15.7	20.0	

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

C4′ prenyl group plays a beneficial role in PPI stabilization (Fig. 6A), 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 (Fig. 6B). Taken together, these findings indicate that glycosylation and the specific modifications of the saccharide moiety are essential for stabilizing the C-RAFpS233pS259/14-3-3ζ interaction, with the unnatural derivate **10** exhibiting the most potent activity among the tested CNs.

Discussion

Modulating protein–protein interactions (PPIs) is challenging due to their flat and large interfaces, making them difficult drug targets.[31,32] Natural products like CNs and FCs, which stabilize PPIs, are crucial for mechanistic studies and drug development. Our discovery of the BGC for CNs and the elucidation of the biosynthetic pathways for various intermediates with PPI-stabilizing activity represent a significant advancement, particularly given the structural complexity and chemical synthesis challenges of these molecules. This is even more critical as the producing strain is no longer available.

We have identified the CN BGC for the first time, enabling comparison with the known brassicicene and fusicoccin BGCs to understand the genetic basis of their structural differences. In comparing the *abn* and *cty* clusters, both share homologous genes involved in the production of BC-I (**8**). The *abn* cluster lacks glycosylation genes but contains a unique P450 enzyme for oxidative rearrangement,[22] leading to the formation of the 5-9-5 scaffold as revised by our group;[33] On the other hand, in the comparison between the *Pa* and *cty* clusters, both contain C9-hydroxylase and glycosyltransferase genes, facilitating similar glucosylation, but distinct modifying enzymes specific to the aglycone and sugar units drive structural variations between them. It is noteworthy that FCs primarily affect stomatal opening by stabilizing the interaction between plant 14-3-3 protein and H^+-ATPase, whereas CNs promote cell elongation and division.[34] The difference between FC and CN derivatives is reported to be due to the 12-hydroxyl group in FCs, which prevents complex formation with mode-1 ligands.[35] Additionally, the unique enzymes CtyA (P450) and CtyE (methyltransferase) in the *cty* cluster for sugar moiety modifications have shown important activity-enhancing functions in our preliminary structure–activity relationship studies. These comparisons reveal critical insights into how genetic variations within BGCs influence the structure and bioactivity of these diterpenoids. Furthermore, our cblaster analysis indicates that the *cty* cluster is not widely distributed (Fig. S1†), which may help explain why CN-producing strains are relatively rare.

The discovery of the *cty* cluster provides a rich set of catalytic elements, facilitating the biosynthesis and chemoenzymatic synthesis of CNs. Additionally, combinatorial biosynthesis with the *Pa* cluster can generate more derivative molecules (such as compound **10** in this study). These derivatives can be employed to investigate whether their structural differences enhance the selectivity of 14-3-3 proteins for various target proteins, which is crucial for developing highly selective, druggable PPI stabilizers.

Unfortunately, the final step leading to CN-A remains unresolved in this study. Despite our attempts using various fermentation conditions and intermediate feeding in our *TA* strain, CN-A was not observed. This raises the possibility that the entire BGC may be silent. The cluster lacks obvious regulatory elements such as BGC-specific transcription factors, suggesting the need to explore various stimuli to activate it. Understanding the regulatory mechanisms of CN biosynthesis will provide further insight into their physiological and ecological functions.

Conclusions

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 C (**2**), F (**3**), I (**4**) and E (**5**). We identified and confirmed the roles of key enzymes responsible for assembling and modifying the saccharide 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.

Beyond simply elucidating the biosynthesis, our work revealed the potential of pathway enzymes as tools for rational design and the total biosynthesis of new compounds. This is exemplified by our successful creation of the unnatural cotylenin derivative **10**, achieved through leveraging enzyme substrate promiscuity and regioselectivity across homologous BGCs. Furthermore, we revealed for the first time the stabilizing activity of CN biosynthetic intermediates on 14-3-3 PPIs. Future efforts will focus on identifying new strains or novel oxidases to achieve the complete biosynthesis of CN-A.

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

Y. Z., Y. Y., and Z. X. designed the research. Z. G., N. Y., Y. X., Y. X., Mewlude Rehmutulla, W. Y., and F. L. performed the experiments. C. C., H. Z., Y. Z., and Q. T. analyzed the data. Z. G., and Y. Y. wrote the manuscript. All the authors have approved the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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