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Digest

Biosynthetic approaches to creating bioactive fungal metabolites: Pathway engineering and activation of secondary metabolism

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ABSTRACT

The diversity of natural products is greater than that of combinatorial chemistry compounds and is similar to that of drugs. Compounds rich in sp^3 carbons, such as natural products, typically exhibit high structural complexity and high specificity to molecular targets. Microorganisms can synthesize such sp^3 carbon-rich compounds and can be used as excellent factories for making bioactive compounds. Here, we mainly focus on pathway engineering of two sp^3 carbon-rich bioactive indole alkaloids, fumitremorgin C and terpendole E. We also demonstrate the importance of activation of secondary metabolism by focusing on tenuazonic acid, a bioactive tetramic acid compound, as an example.

Importance of the biosynthetic approach: In 1997, Lipinski described a general rule (Lipinski's rule of five) for the solubility and permeability of drugs.[1] This rule may be applicable to synthetic compounds but not natural products. Chemical space analysis has revealed that the diversity of natural products is greater than that of combinatorial chemistry (combichem) compounds. Moreover, the diversity pattern of natural products is similar to that of drugs.[2] Compounds rich in sp^3 carbons, such as natural products, have high complexity and high specificity to target proteins.[3,4] Thus, natural products can become potent drugs with minor side effects. Chemical synthesis is a powerful approach used to generate natural products, and sp^3-rich scaffolds have been synthesized through this approach.[5] Microorganisms can synthesize many sp^3 carbon-rich compounds (natural products) and can be used as excellent sources of these bioactive compounds. Actinomycetes (prokaryotes) and filamentous fungi (eukaryotes) are well known as producers of various bioactive metabolites. However, production of secondary metabolites is unstable in many cases. Controlling the biosynthetic gene clusters responsible for generation of secondary metabolites is important for producing bioactive metabolites. Pathway engineering of secondary metabolites enables us to achieve improved, stable production of natural products.[6] Pathway engineering is also used to create new derivatives with better biological activities.[7] Furthermore, activation of secondary metabolism is also required in many cases because of poor expression of biosynthetic gene clusters.[8]

Here, we introduce two examples of sp^3 carbon-rich fungal secondary metabolite production. We describe pathway engineering of the biosynthetic gene clusters for two fungal metabolites, fumitremordin C (FTM-C) and terpendole E.[9–11] We also focus on tenuazonic acid, a bioactive tetramic acid compound, as an example of activation of secondary metabolism.[12]

FTM-C biosynthesis: FTM-C (**1**, Fig. 1), a prenylated indole alkaloid produced by the human pathogen *Aspergillus fumigatus*, is a potent and specific inhibitor of breast cancer resistance protein (BCRP; also known as ABCG2), a member of the ABC transporters involved in multidrug resistance in cancer cells.[13,14]

The involvement of the megasynthases polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) is a characteristic of the biosynthesis of microbial secondary metabolites. The biosynthetic pathway of FTM also involves the megasynthase FtmA, an NRPS.[16] In general, the backbone compounds created by megasynthases do not show potent biological activity. Tailoring enzymes, which catalyze modifications of the backbone structure after release from megasynthases, are required for the generation of bioactive compounds. Thus, in the development of novel drug leads, analysis of such tailoring steps is important. The analysis of gene knockout mutants is the key for identifying gene functions

Abbreviations: FTM-C, fumitremordin C; BCRP, breast cancer resistance protein; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; α-KG, α-ketoglutarate; SAR, structure-activity relationship; KSP, kinesin spindle protein; TAS1, tenuazonic acid synthetase 1; PCP, peptidyl carrier protein; AT, acyltransferase; KS, ketosynthase; ACP, acyl carrier protein.

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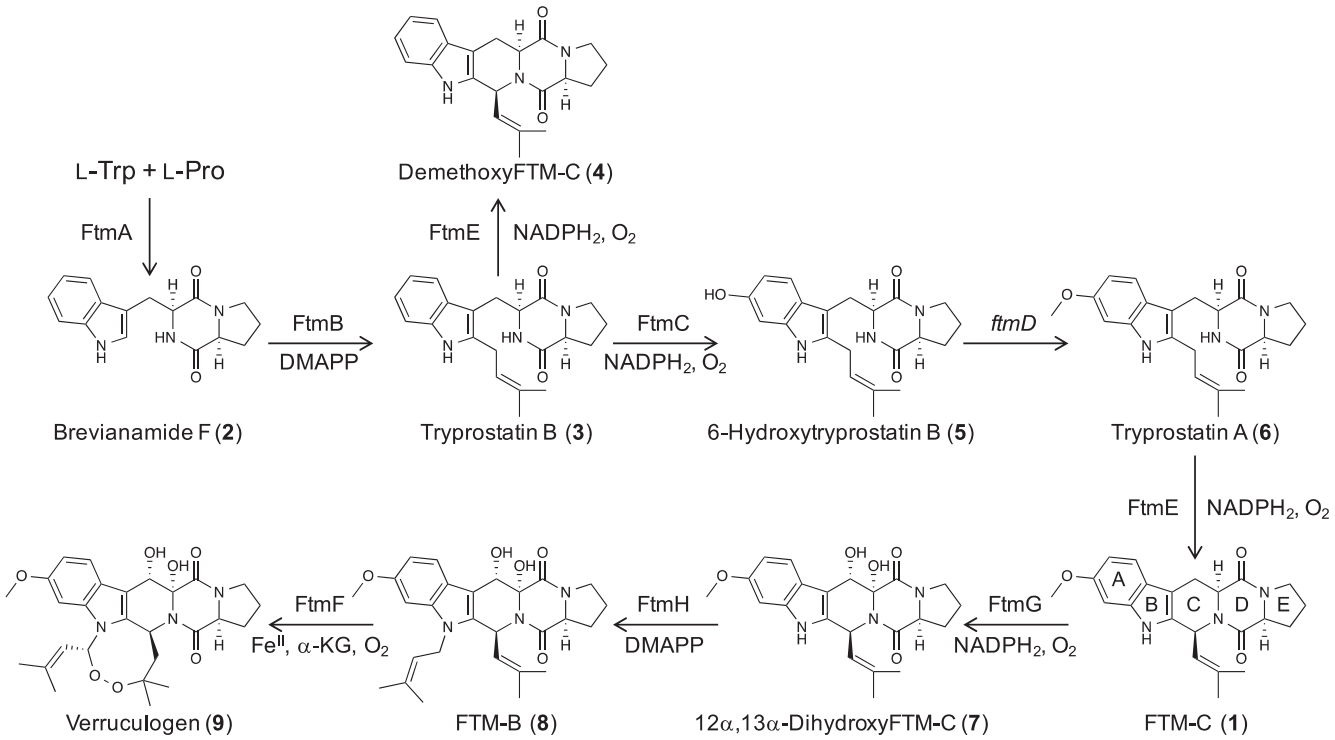

Fig. 1. Fumitremorgin (FTM) biosynthesis in *Aspergillus fumigatus* BM939. All the biosynthetic steps in the proposed pathway, except for the methylation of **5**, which most likely involves *ftmD*, were confirmed experimentally.[15] DMAPP, dimethylallyl diphosphate; α-KG, α-ketoglutarate.

in secondary metabolite gene clusters. Gene knockout mutants accumulate biosynthetic intermediates that normally exist in small amounts in wild-type cultures. Structural determination of such intermediates enables us to predict the functions of disrupted genes. Furthermore, we can evaluate the biological activity of the isolated biosynthetic intermediates.

In the biosynthesis of secondary metabolites, oxygenation can be one of the most important tailoring steps. Oxygenation can be carried out more easily by biosynthetic enzymes than by chemical methods. Biosynthetic enzymes can introduce oxygen into nonactivated carbon atoms in a sterically and optically selective manner, which is difficult or impossible to achieve by chemical methods.[17] There are three cytochrome P450 genes and one α-ketoglutarate (α-KG)-dependent dioxygenase gene in the FTM biosynthetic gene (*ftm*) cluster. Their functions in FTM biosynthesis have been elucidated by gene knockout and subsequent biochemical analysis (Fig. 1).[9,18,19] FtmF, an α-KG-dependent dioxygenase, catalyzes the unique endoperoxide bond formation of **9**,[18,19] whereas cytochrome P450s are involved in the key biosynthetic steps required for the biological activity of FTMs.[9]

Genetic engineering of the FTM-C biosynthetic gene cluster: By constructing *ftm* knockout mutants, we obtained nine compounds (**1**–**9**) that are biosynthetic intermediates or shunt metabolites in the FTM biosynthetic pathway (Fig. 1), and their biological activities, e.g., cytotoxicity and BCRP inhibitory activity, were evaluated (Table 1).[9] All compounds had a diketopiperazine scaffold, but were structurally diverse. Thus, structure-activity relationship (SAR) studies using these compounds may be useful. Our SAR study showed that **1** was the strongest BCRP inhibitor and that FtmE-mediated cyclization for formation of the C ring was the most important step for conferring inhibitory activity against BCRP (Table 1). The biological activities changed along with the biosynthetic pathway (Fig. 1). Cytotoxicity was detected in **8**, which indicated that further modification of **1** weakened the BCRP inhibitory activity but conferred a novel activity by targeting other proteins.

Table 1
Evaluation of the biological activities of FTM biosynthetic intermediates.

Compound[a]	Cytotoxicity	BCRP
Brevianamide F (**2**)	–	–
Tryprostatin B (**3**)	–	–
DemethoxyFTM-C (**4**)	–	++
6-Hydroxytryprostatin B (**5**)	–	–
Tryprostatin A (**6**)	–	–
FTM-C (**1**)	–	+++
12α,13α-DihydroxyFTM-C (**7**)	–	+
FTM-B (**8**)	+	+
Verruculogen (**9**)	+/–	+

[a] The cytotoxicity of FTMs was measured using five mammalian cell lines: Jurkat, HeLa, HL60, MCF-7, and HT29 (+, IC_{50} of 5–15 μM; +/–, IC_{50} of 20–30 μM). The BCRP inhibitory activity of FTMs was assessed by inhibition of drug efflux in K562/BCRP cells and inhibition of BCRP-dependent ATPase activity.[9]

Fumitremorgins (**8**, **9**, and fumitremorgin A) are known as harmful tremorgenic mycotoxins produced by *A. fumigatus* and related fungi.[20–22] This activity seems to be physiologically important. Some biosynthetic intermediates show interesting biological activities. As demonstrated here, compounds **1** and **4** showed stronger BCRP inhibitory activities than compounds **8** and **9**. The isolation of biosynthetic intermediates is not always useful for obtaining more bioactive metabolites, but is applicable to future screening programs and may lead to the discovery of different biological activities.

Terpendole E: We rediscovered terpendole E (**10**, Fig. 2A) as the first natural product inhibitor of mitotic kinesin Eg5 (also called kinesin spindle protein [KSP]).[23] Kinesin Eg5 inhibitors can arrest the cell cycle of cancer cells at the M phase by interfering with the separation of centrosomes during mitosis. Eg5 inhibitors are predicted to be good anticancer drugs with fewer side effects than conventional mitotic inhibitors that directly inhibit the polymerization of microtubules.[24,25]

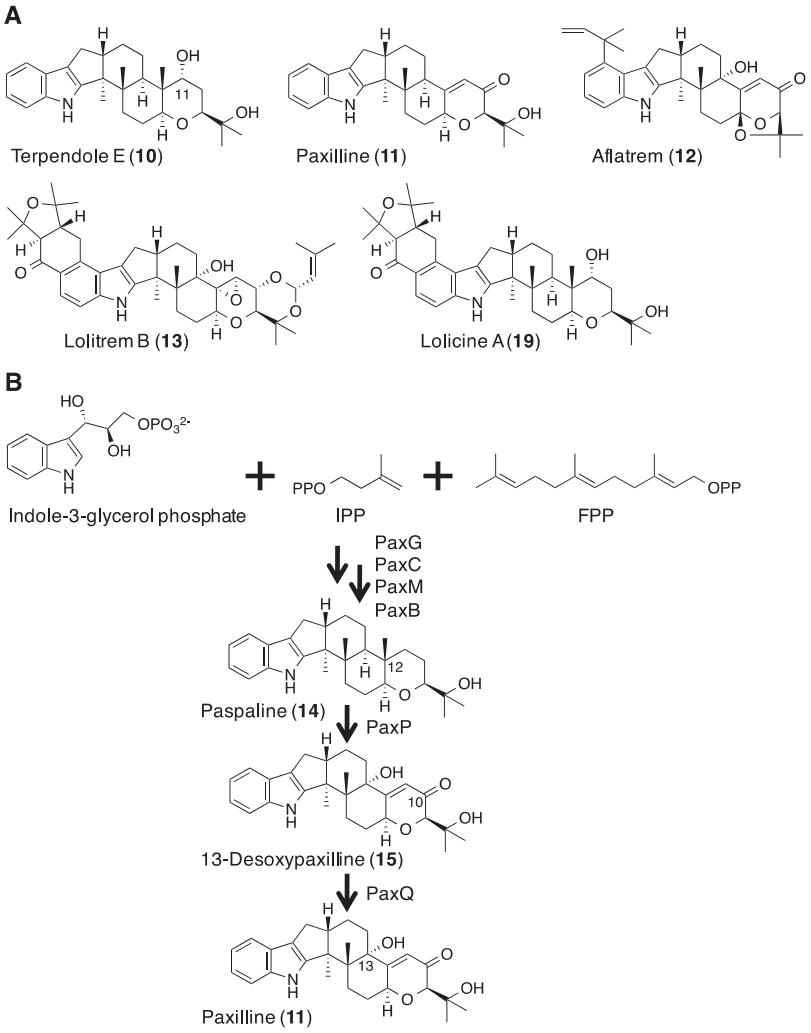

Fig. 2. Terpendole E is an indole-diterpene compound similar to paxilline and related compounds. (A) Terpendole E and related indole-diterpene compounds. (B) The paxilline biosynthetic pathway.

However, production of terpendole E is unstable, and isolation of this compound is difficult. Because of its complex structure, total synthesis of terpendole E is also difficult,[26–28] although one study reported total synthesis of this compound very recently (Fig. 3).[29] Thus, further analysis of the activity of terpendole E has not been possible. We isolated the terpendole E biosynthetic gene cluster and performed pathway engineering, resulting in stable production of terpendole E and its new analog.[10,11]

Isolation and analysis of the terpendole E biosynthetic gene cluster: Terpendole E is an indole-diterpene secondary metabolite produced by the Ascomycota fungus *Tolypocladium album* (syn. *Chaunopycnis alba*, *Albophoma yamanashiensis*). Some indole-diterpenes are tremorgenic mycotoxins produced to control mammalian and insect predators.[30] These tremorgenic mycotoxins are produced by several Ascomycota filamentous fungi belonging to the evolutionarily separated Eurotiomycetes and Sordariomycetes groups.[31] *T. album* belongs to the Sordariomycetes class of fungi. Analysis of indole-diterpene biosynthetic gene clusters in four filamentous fungi (*Penicillium paxilli* [paxilline (**11**) producer, Eurotiomycetes], *Aspergillus flavus* [aflatrem (**12**) producer, Eurotiomycetes], *Neotyphodium lolii*, and *Epichloë festucae* [lolitrem B (**13**) producer, Sordariomycetes]) revealed the presence of similar biosynthetic pathways (Fig. 2A, B).[31–34] In the paxilline biosynthetic gene cluster (Fig. 2B), four proteins (PaxG, PaxC, PaxM, and PaxB) are involved in the biosynthesis of paspaline (**14**), the first stable intermediate. Paspaline is converted to 13-desoxypaxilline (**15**) by the P450 enzyme PaxP, and 13-desoxypaxilline is then converted to paxilline by another P450 enzyme, PaxQ. *A. flavus*, *N. lolii*, and *E. festucae* also have homologs of the six Pax enzymes, suggesting that the four filamentous fungi have similar indole-diterpene

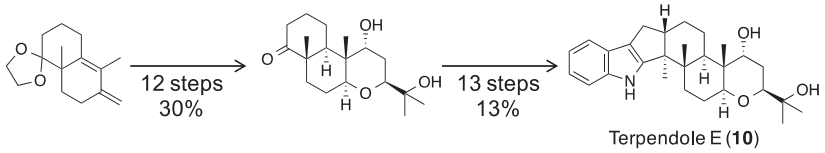

Fig. 3. Chemical synthesis of terpendole E.[29]

Table 2
Comparison of indole-diterpene biosynthetic gene clusters.

P. paxilli (Paxilline)	*A. flavus* (Aflatrem)	*N. lolii* (Lolitrem B)	*T. album* (Terpendole E)	Proposed function
paxG	*atmG*	*ltmG*		GGPP synthase
paxC	*atmC*	*ltmC*	*terC*	Prenyltransferase
paxM	*atmM*	*ltmM*	*terM*	FAD-dependent monooxygenase
paxB	*atmB*	*ltmB*	*terB*	Terpene cyclase
paxP	*atmP*	*ltmP*	*terP*	P450 monooxygenase
paxQ	*atmQ*	*ltmQ*	*terQ*	P450 monooxygenase
paxA	*atmA*			Unknown (membrane protein)
paxD	*atmD*			Prenyltransferase
		ltmF	*terF*	Prenyltransferase
		ltmK	*terK*	P450 monooxygenase
		ltmE		Prenyltransferase
		ltmJ		P450 monooxygenase

biosynthetic pathways. From structural comparisons, we propose that terpendole E may be biosynthesized by C11 hydroxylation of the common intermediate, paspaline. Although terpendole E may be a biosynthetic intermediate in indole-diterpene biosynthesis, it has not been isolated from other indole-diterpene producers.

We isolated the terpendole E biosynthetic gene cluster, which consisted of seven genes encoding three P450 monooxygenases (TerP, TerQ, and TerK), an FAD-dependent monooxygenase (TerM), a terpene cyclase (TerB), and two prenyltransferases (TerC and TerF; Table 2). The deduced amino acid sequences of the seven genes showed highest similarity to homologs from a lolitrem B producer *N. lolii*, suggesting high similarity in the biosynthetic pathway. Five of the seven genes, i.e., the genes encoding TerC, TerM, TerB TerP, and TerQ, were common to all the clusters. TerC, TerM, and TerB are homologs of enzymes required for the biosynthesis of paspaline.[35–37] TerP and TerQ are homologs of P450s involved in the downstream metabolism of paspaline.[38] Two of the seven genes are specific to the terpendole producer (encoding TerF and TerK) and the lolitrem B producers (encoding LtmF and LtmK). LtmF and LtmK are candidate enzymes for conversion of terpendole I (**16**) to terpendole C (**17**).[39] The terpendole E cluster was found to lack a gene for geranylgeranyl diphosphate synthase (GGPPS), an enzyme that is essential for indole-diterpene biosynthesis, suggesting that the GGPPS gene may be located outside of the cluster.

We revealed the terpendole E biosynthetic pathway through gene knockout and feeding experiments (Fig. 4).[10] Terpendole E was produced by the C11-hydroxylation reaction of TerQ from paspaline. TerP, a PaxP homolog, converted terpendole E to 13-desoxyterpendole I (**18**, a new compound), which is an important intermediate in the biosynthesis of terpendoles. TerP also converted paspaline to 13-desoxypaxilline, a shunt metabolite. 13-Desoxyterpendole I was converted by TerQ to terpendole I (**16**), a biosynthetic intermediate of terpendole C (**17**). Our data indicated that terpendole C, a tremorgenic mycotoxin, was biosynthesized via terpendole E.

Comparison of indole-diterpene biosynthetic pathways: Homologs of TerQ have important roles in creating chemical diversity from paspaline, a common biosynthetic intermediate of paxilline-like indole-diterpenes (Fig. 5). All the analyzed TerQ homologs have the common C13 hydroxylating activity. Some homologs also have specific activities (e.g., C11 hydroxylation) for creating structural diversity. The C13 hydroxyl group is important for tremorgenic activity.[40] In the biosynthesis of paxilline and aflatrem, TerP homologs (PaxP and AtmP) function first, and 13-desoxypaxilline is created. Then, this compound is converted to different compounds by TerQ homologs (PaxQ and AtmQ), creating structural diversity.[41] In contrast, in terpendole biosynthesis, TerQ functions first, and terpendole E is created. This reaction is a unique C11 hydroxylation reaction. This C11 hydroxyl group is required for creation of the

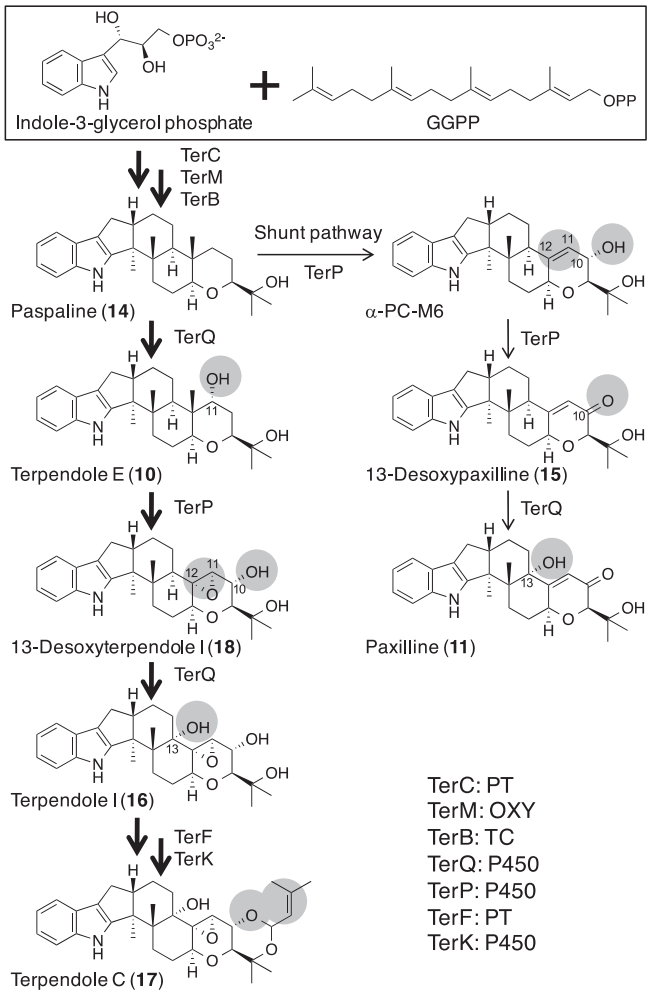

Fig. 4. Terpendole biosynthetic pathway. Seven gene products are proposed to be involved in the biosynthesis. Paspaline can be converted to pathway intermediates or shunt metabolites.

C11–C12 epoxy structure formation. Then, TerP converts terpendole E to 13-desoxyterpendole I. Subsequently, TerQ functions again (C13 hydroxylation), and 13-desoxyterpendole I is converted to terpendole I. Thus, TerQ functions at two steps in terpendole biosynthesis, representing a key enzyme in this pathway. The C11-C12 epoxy structure is also observed in lolitrem B, suggesting that LtmQ, a TerQ homolog, also has C11-hydroxylation activity.

There are at least two biosynthetic pathways for paxilline-like indole-diterpenes (Fig. 5). The first, used in *P. paxilli* and *A. flavus*, converts paspaline to the C11-C12 double bond-containing 13-des-

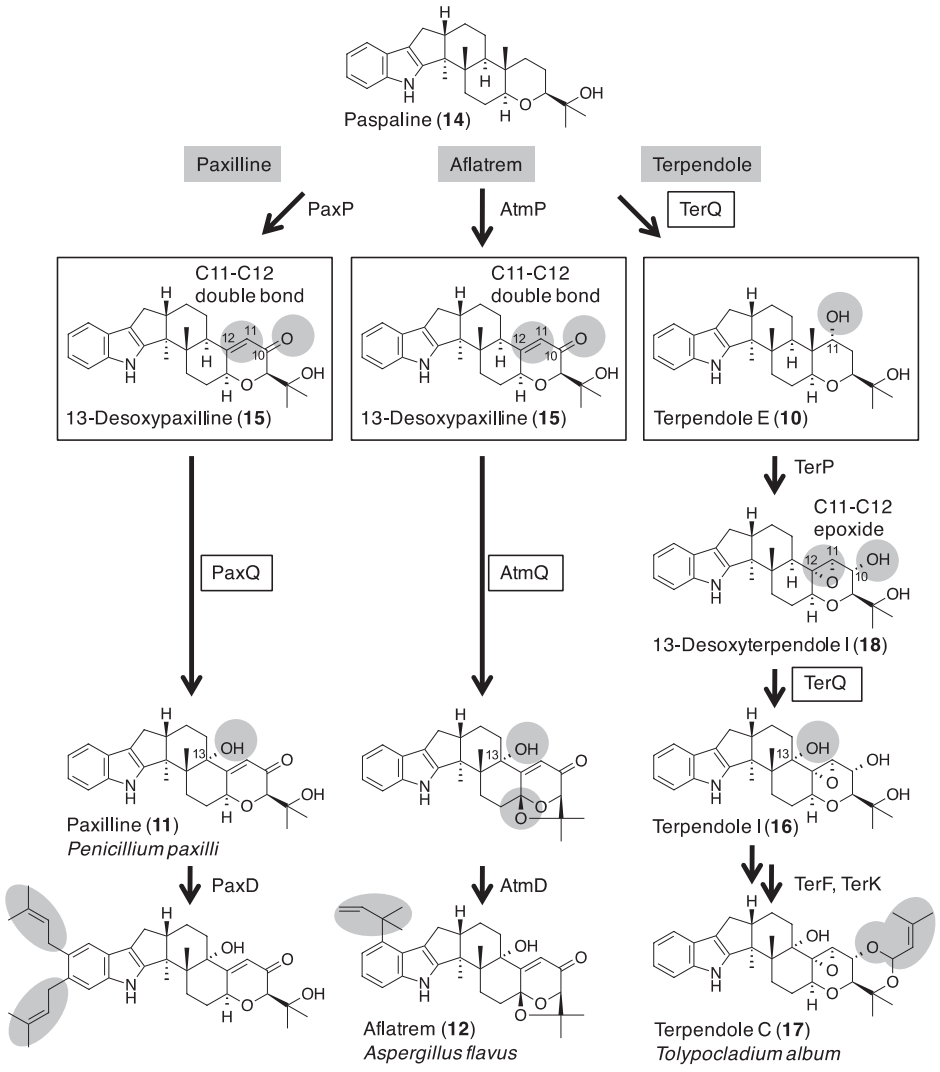

Fig. 5. Comparison of paxilline-like indole-diterpene biosynthetic pathways. Paspaline is a common intermediate in all of the indicated pathways. Terpendole E, a key biosynthetic intermediate of terpendole biosynthesis, is produced from paspaline by the key enzyme TerQ. 13-Desoxypaxilline is a key biosynthetic intermediate of paxilline and aflatrem.

oxypaxilline, which serves as a key biosynthetic intermediate. The alternative route, identified in *T. album*, instead converts paspaline to 11-OH-containing compounds, such as terpendole E, which is used as a key biosynthetic intermediate. 13-Desoxypaxilline is a shunt metabolite in *T. album*, but acts as a key biosynthetic intermediate in paxilline and aflatrem producers. Terpendole E is converted to 13-desoxyterpendole I, which has a C11–C12 epoxy structure. Demethylation of C12 in the presence or absence of 11-OH produces a C11–C12 epoxy structure and a C11–C12 double bond, respectively. It appears that *N. lolii* also uses terpendole E or a similar compound as a key intermediate because this fungus produces a terpendole E-like compound (lolicine A [**19**, Fig. 1A]), and lolitrem B also has a C11–C12 epoxy structure. Thus, we propose that the biosynthetic pathways for paxilline-like indole-diterpenes may differ between Sordariomycetes (*T. album*, *N. lolii*, and *E. festucae*) and Eurotiomycetes (*P. paxilli* and *A. flavus*). Recently, the biosynthetic pathway for penitrem A was elucidated.[42] Penitrem A is produced by an Eurotiomycetes fungus (*Penicillium simplicissimum*) via paxilline, supporting our hypothesis.

Pathway engineering of the terpendole E biosynthetic gene cluster: In the wild-type *T. album* strain, terpendole E accumulates only transiently during early culture. We successfully overproduced terpendole E by disrupting the *terP* gene, which encodes the bispecific enzyme TerP. *terP* disruption blocks two points: conversion of terpendole E to 13-desoxyterpendole I and conversion of paspaline to a shunt metabolite 13-desoxypaxilline (Fig. 6). Inhibition of the latter step caused accumulation of paspaline and its conversion to terpendole E by the action of TerQ. Inhibition of the former step caused stable accumulation of biosynthesized terpendole E. Thus, *terP* disruption was an efficient technique for overproduction of terpendole E. Our data indicated that terpendole E production was unstable because terpendole E was a biosynthetic intermediate in terpendole biosynthesis.

Isolation of a terpendole E analog with stronger kinesin Eg5 inhibitory activity: Overproduction of bioactive compounds may cause production of shunt metabolites with improved bioactivity. Indeed, a new terpendole E analog was isolated from a *terP*-knockout strain that stably overproduced terpendole E.[11] After prolonged culture, a *terP* knockout strain accumulated some other metabolites in addition to terpendole E. One major metabolite was isolated, and its structure was determined. The isolated compound, 11-ketopaspaline (**20**), was a new oxidized analog of terpendole E (Fig. 6). This oxidization reaction occurs in late cultures. Because terpendole E is an early biosynthetic intermediate that accumulates transiently in early cultures,[10] 11-ketopaspaline production should be limited in the wild-type strain. 11-Ketopaspaline

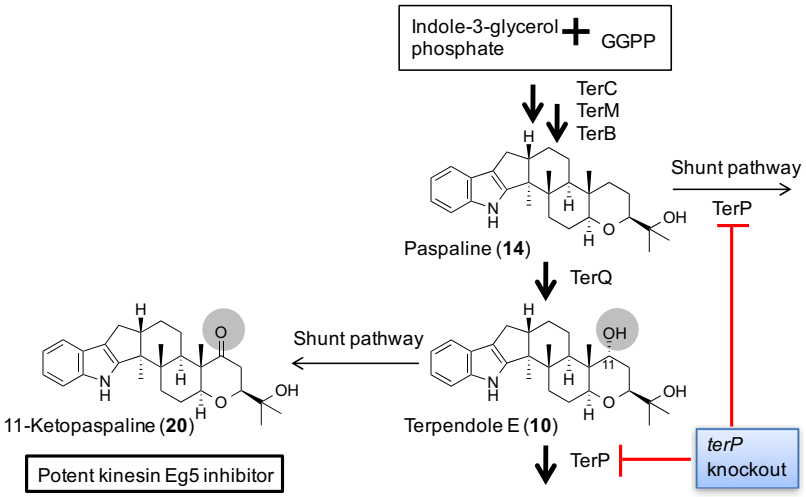

Fig. 6. Pathway engineering of the terpendole biosynthetic pathway. Terpendole E can be overproduced by disruption of the *terP* gene, encoding a bispecific P450 monooxygenase. The *terP* knockout strain produces a potent kinesin Eg5 inhibitor 11-ketopaspaline as a shunt metabolite in late cultures.

Table 3
Evaluation of the biological activities of terpendole E and 11-ketopaspaline.

Compound	Cytotoxicity[a]	Microtubule-stimulated ATPase activity[b]	
		Kinesin Eg5	Kinesin-1
Terpendole E (**10**)	12.1	34.7 ± 2.6	>100
11-Ketopaspaline (**20**)	2.9	16.5 ± 4.3	>100

[a] Cytotoxicity was measured using HeLa cells, and IC_{50} values (μM) were determined.
[b] The ATPase activity of kinesins was measured using the Eg5 motor domain (amino acids 1–368) or kinesin-1, and IC_{50} values (μM) were determined.

showed stronger cytotoxicity and kinesin Eg5 inhibitory activity than terpendole E (Table 3), suggesting that the C11 ketone, the only difference between the two compounds, increases the specificity to the target protein kinesin Eg5.[11] In contrast, terpendole I and terpendole C do not show kinesin Eg5 inhibitory activity.[23] TerP and TerQ are involved in conversion from terpendole E to terpendole I (Figs. 4 and 5). Terpendole I is then converted to terpendole C. Some of the modifications, i.e., C12 demethylation, C11-C12 epoxide formation, C10 hydroxylation, and C13 hydroxylation, catalyzed by TerP or TerQ should cause the loss of kinesin Eg5 inhibitory activity. Terpendole E and 11-ketopaspaline only partially inhibited kinesin Eg5-microtubule interactions, unlike other Eg5 inhibitors, such as GSK-1 and S-trityl-l-cysteine (STLC). Furthermore, terpendole E and 11-ketopaspaline inhibited several kinesin Eg5 mutants that are resistant to GSK-1 or STLC, but with the same extent of inhibition against wild-type kinesin Eg5. Our results suggest that terpendole E and 11-ketopaspaline have inhibitory mechanisms different from those of L5 loop-binding-type kinesin Eg5 inhibitors, such as GSK-1 and STLC.

T. album also produces terpendoles that have tremorgenic activity[43] or acyl-CoA: cholesterol acyltransferase (ACAT) inhibitory activity.[44–46] Therefore, pathway engineering of the terpendole biosynthetic gene cluster will enable us to isolate new terpendoles with diverse biological activities.

From an evolutionary point of view, we expect that biosynthetic intermediates may have unidentified biological activities. By using strains that produce secondary metabolites and their gene knockout mutants, we can easily obtain natural product scaffolds that are otherwise difficult to prepare by chemical synthesis; these scaffolds can be deposited into chemical libraries used for the screening of bioactive compounds.

Activation of secondary metabolism: Filamentous fungi have been a rich source of secondary metabolites for drug development. Whole genome sequencing analyses have revealed that filamentous fungi have much more secondary metabolism genes than expected. Thus, most secondary metabolism genes are poorly expressed under laboratory conditions. Activation of secondary metabolism genes is important for making full use of fungal secondary metabolites. Therefore, activation of secondary metabolism has been carried out through many approaches, including overexpression of pathway-specific transcription factors, manipulation of global regulators, co-culture, epigenetic control, ribosome engineering, and heterologous expression of secondary metabolite gene clusters.[8,47–49] Some indole-diterpene compounds, including paxilline, aflatrem, penitrem A, and shearinine D, have been successfully produced using *Aspergillus oryzae* as a heterologous host.[37,42,50,51] Interestingly, *A. oryzae* has aflatrem biosynthetic gene clusters with a critical mutation in the *atmQ* gene.[41] *A. oryzae* is a suitable host for heterologous production of fungal secondary metabolites, particularly indole-diterpenes.

Recently, we succeeded in activating secondary metabolism and stably producing tenuazonic acid (TeA; Fig. 7A).[12] TeA production was induced by disturbance of the two-component signal transduction system involved in environmental responses. TeA is a mycotoxin produced by some phytopathogenic fungi.[52–54] TeA also has antitumor, antiviral, antibacterial, and plant disease-controlling activities.[55–57] We identified the TeA biosynthetic enzyme TAS1, which was a megasynthase consisting of a condensation (C) domain, an adenylation (A) domain, a peptidyl carrier protein (PCP) domain, and a ketosynthase (KS) domain (C-A-PCP-KS; Fig. 7B). TAS1 was a unique NRPS-PKS hybrid enzyme that begins with an NRPS module (C-A-PCP). This domain structure was quite different from that of conventional fungal PKS-NRPS enzymes, which begin with a PKS module (Fig. 7B). The PKS part of TAS1 had only a KS domain with a unique sequence. We found that TeA was synthesized from isoleucine and acetoacetyl-CoA (diketide) by TAS1 (Fig. 7C).[12] The unique KS domain was involved in the final cyclization step for TeA release, although involvement in diketide biosynthesis has been predicted.[58] These results indicated that TAS1 was a unique type of biosynthetic enzyme and could be

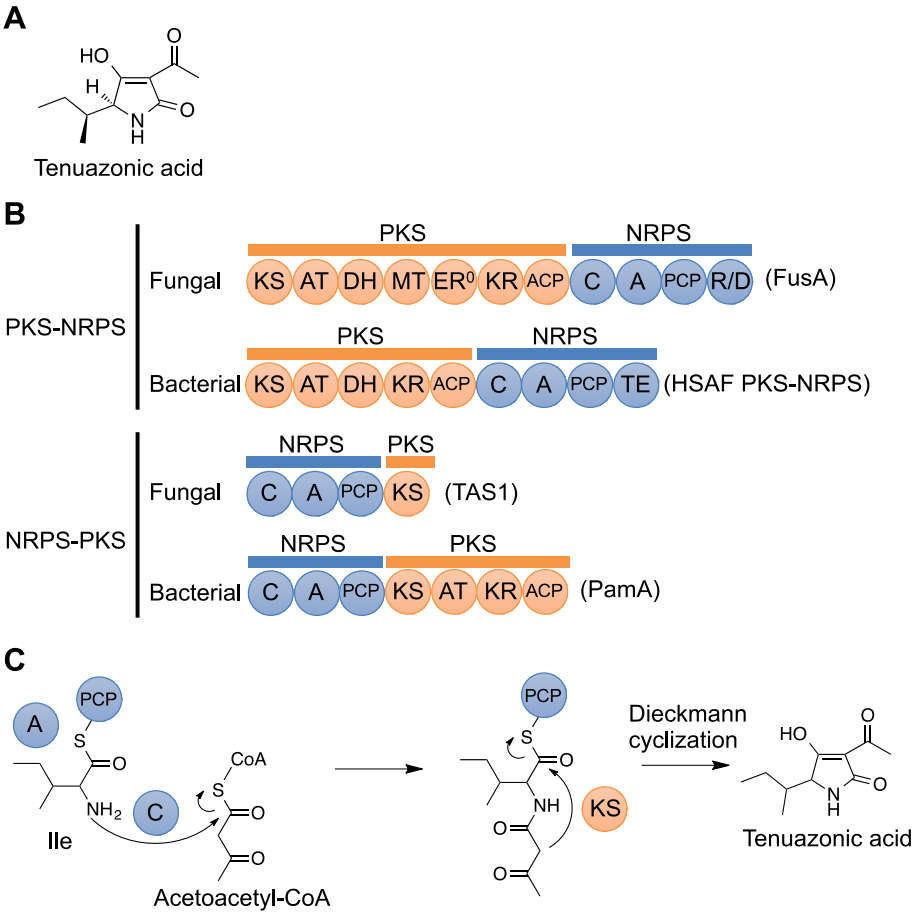

Fig. 7. Tenuazonic acid (TeA). (A) Chemical structure of TeA. (B) Tenuazonic acid synthetase 1 (TAS1), the first reported fungal NRPS-PKS hybrid enzyme, and related enzymes. Domain structures of a typical fungal PKS-NRPS, a bacterial PKS-NRPS, a fungal NRPS-PKS (TAS1), and a bacterial NRPS-PKS hybrid enzyme are shown. FusA: fusarin synthetase A from *Fusarium moniliforme*, HSAF PKS-NRPS: heat-stable antifungal factor (dihydromaltophilin) synthesis core enzyme from *Lysobacter enzymogenes*, PamA: paenilamicin synthetase A from *Paenibacillus larvae*. (C) Biosynthesis of TeA. TeA is biosynthesized from isoleucine and acetoacetyl-CoA by TAS1. The unique KS domain catalyzes the final cyclization step.

used for production of various compounds. The development of methods for the activation of secondary metabolism is important for the isolation of bioactive natural products.

Conclusions: Biosynthetic approaches are powerful ways to create and produce bioactive compounds. Biosynthetic approaches are particularly useful when producing sp^3 carbon-rich and complex natural products, such as FTM-C and terpendole E, for which chemical synthesis is difficult. Filamentous fungi are useful hosts in biosynthetic approaches and can produce such compounds. Elucidation of the biosynthetic pathways of these products will enable us to produce and isolate biosynthetic intermediates and shunt metabolites with high structural diversity. Some of these metabolites may be chemically stable and could have improved biological activity. We isolated one such metabolite, 11-ketopaspaline. However, in many cases, activation of secondary metabolism is required because most secondary metabolism genes are poorly expressed under laboratory conditions. We succeeded in activating secondary metabolism and discovering a unique biosynthetic enzyme, TAS1. Pathway engineering, activation of secondary metabolism, use of unique enzymes, and their combinations will facilitate the production of diverse natural products.

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