

Cite this: *Chem. Sci.*, 2018, 9, 2589

Asperphenamate biosynthesis reveals a novel two-module NRPS system to synthesize amino acid esters in fungi†

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Amino acid esters are a group of structurally diverse natural products with distinct activities. Some are synthesized through an inter-molecular esterification step catalysed by nonribosomal peptide synthetase (NRPS). In bacteria, the formation of the intra-molecular ester bond is usually catalysed by a thioesterase domain of NRPS. However, the mechanism by which fungal NRPSs perform this process remains unclear. Herein, by targeted gene disruption in *Penicillium brevicompactum* and heterologous expression in *Aspergillus nidulans*, we show that two NRPSs, ApmA and ApmB, are sufficient for the synthesis of an amino acid ester, asperphenamate. Using the heterologous expression system, we identified that ApmA, with a reductase domain, rarely generates dipeptidyl alcohol. In contrast, ApmB was determined to not only catalyse inter-molecular ester bond formation but also accept the linear dipeptidyl precursor into the NRPS chain. The mechanism described here provides an approach for the synthesis of new small molecules with NRPS as the catalyst. Our study reveals for the first time a two-module NRPS system for the formation of amino acid esters in nature.

Received 27th May 2017

Accepted 23rd January 2018

DOI: 10.1039/c7sc02396k

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Introduction

Amino acid esters and their derivatives represent a huge group of pharmaceutically important natural products. These products include the cytotoxic asperphenamate (**1**),¹ anticancer taxol,² antibiotic daptomycin,³ and the recently identified antibiotic teixobactin, which kills bacteria with no detectable resistance (Fig. 1).⁴ **1** is a rare linear amino acid ester derived from fungal nonribosomal peptide that exhibits antitumor activity towards a number of cell lines.^{5,6} It was first isolated from *Aspergillus flavipes* in 1977⁷ and then identified from a wide range of *Aspergillus*⁸ and *Penicillium* species.^{9,10} The structure of **1** contains two subunits, *N*-benzoylphenylalanine (**2**) and *N*-benzoylphenylalaninol (**3**), which are connected by an inter-molecular ester bond. An experiment performed by feeding the labeled precursor [U-¹⁴C]-**4** into *P. brevicompactum*

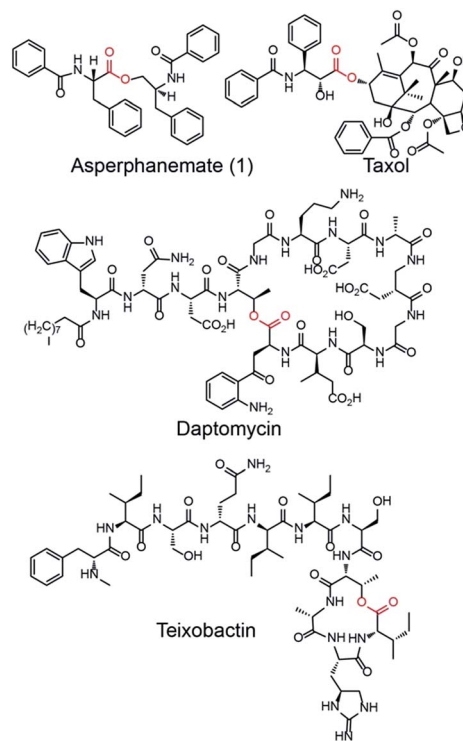


Fig. 1 Representations of bioactive amino acid esters from nature: asperphenamate (**1**), taxol, daptomycin, and teixobactin. The ester bond connecting the subunit of each compound is highlighted in red.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7sc02396k

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proved that the benzoyl groups of **1** are derived from the precursor phenylalanine (**4**).¹ However, no genetic study has been reported for the biosynthesis of **1**. The motivation to study the biosynthesis of **1** is not only because of its biological activity but also because of the structural formation of the rare linear amino acid ester bond. The elucidation of the biosynthesis of **1** at the molecular level will provide an opportunity to produce **1** using an engineered microorganism. Moreover, it will provide new insights into the biosynthesis of related natural products.

NRPSs usually have modular structures that are responsible for peptide elongation. Typically, an NRPS consists of an adenylation (A) domain, a thiolation (T) domain or PCP (peptidyl carrier protein), and a condensation (C) domain. A previous study demonstrated that bacterial NRPSs utilize thioesterase (TE) or C domains to perform esterification reactions.¹¹ For example, the NRPS subunit DptD with a termination TE domain is involved in the biosynthesis of daptomycin in *Streptomyces roseosporus*. In this process, the TE domain is required for the formation of the daptomycin lariat structure and releasing of the peptide from the synthetase.³ SgcC5 is a free-standing C-domain. Interestingly, in *Streptomyces globisporus* this domain catalyses both C–O ester bond and C–N amide bond formations in the biosynthesis of antitumor antibiotic C-1027.¹² In contrast, little is known about the mechanism by which NRPS catalyses ester bond formation in fungi. One example is FUM14p, an NRPS containing only PCP and C domains from *Fusarium verticillioides*. This NRPS catalyses the esterification of fumonisins instead of the typical amide bond formation. Therefore, characterisation of the esterification mechanism will provide useful knowledge for mycotoxin reduction and new insight into understanding the reactions catalysed by NRPS.¹³

Results and discussion

To investigate the biosynthetic pathway of **1**, we worked on the **1** producing strain *P. brevicompactum*.¹ The genome of this strain was sequenced in 2013. First, compound **1** was isolated from a 3 day culture of *P. brevicompactum* on YES medium by repeated column purification and semi-preparative HPLC (see ESI† for further details). **1** was elucidated by NMR identification and the data corresponded well to the previously published data.¹⁴ Analysing the structure of **1** showed that it consisted of two subunits, **2** and **3**. Therefore, we postulated that an NRPS containing at least two A-domains was required for catalysing its formation. By searching the genome of *P. brevicompactum*, we found 73 biosynthetic gene clusters (BGCs), including 14 NRPSs. The NRPS domain prediction was performed using the service NRPSpredictor2.¹⁵ The prediction indicated that 6 NRPSs containing two A-domains matched our previous predictions. Interestingly, cluster 1 contained two NRPSs harbouring four A-domains. It was also possible that **1** needed four A-domains for the synthesis because its structure was not symmetric. To narrow down the NRPS target, we assessed their expressions in transcriptional levels by reverse transcriptase PCR (RT-PCR). Five NRPSs, NRPS1.1, NRPS1.2, NRPS9, NRPS10, and NRPS49 were normally expressed under the tested

conditions. These NRPSs are indicated as possibly involved in the biosynthesis of **1** (Fig. S1†).

To verify which NRPSs were involved in the biosynthesis of **1**, we implemented a genetic deletion approach in *P. brevicompactum*. First, we examined the strain's antibiotic resistance to hygromycin (hph) and determined the growth inhibition concentration of 100 $\mu\text{g ml}^{-1}$. Next, we made the protoplasts with modifications according to the previously reported method to improve the quality of the protoplasts.^{16,17} After that, the constructed NRPS deletion cassettes were transformed into *P. brevicompactum* protoplasts. Following the hygromycin selection and PCR verification, corrected transformants were created (Fig. S2†). HPLC analysis showed that **1** was completely abolished in both NRPS1.1 (renamed ApmA) and NRPS1.2 (renamed ApmB) deletion mutants. HPLC analysis also showed the essential roles of ApmA (KX443597) and ApmB (KX443596) in **1** biosynthesis (Fig. 2A). Therefore, these two NRPSs in cluster 1 (named the *apm* cluster) were shown to be involved in the biosynthesis of **1**.

Next, we predicted the *apm* biosynthetic genes by bioinformatics analysis across the sequenced fungal genomes (JGI, <http://genome.jgi.doe.gov>). The *apm* cluster was conserved in the genomes of three fungi: *P. brevicompactum*, *A. terreus*, and *A. aculeatus*. The cluster consisted of two NRPS genes, *apmA* and *apmB*, one putative aldolase gene (*apmC*) and one putative epimerase/dehydratase gene (*apmD*) (Fig. 2B). The genes *apmA*–*D* from *P. brevicompactum* shared 68–80% sequence identities at the amino acid level with their orthologues from *A. terreus*; they

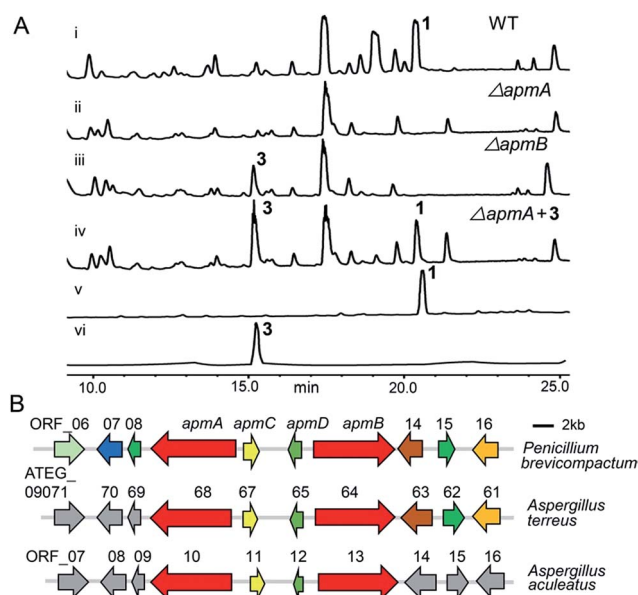


Fig. 2 Identification of asperphenamate (*apm*) biosynthetic gene cluster. (A) HPLC analysis of crude extracts from wild type (WT) strain (i) and *apm* deletion mutants (ii, iii): *apmA* and *apmB* deletion strains eliminated **1** production; $\Delta apmB$ accumulated **3** production (iii); feeding compound **3** into the *apmA* deletion strain restored **1** production (iv); traces v, vi show the pure compounds **1**, **3**. (B) Organization of *apm* biosynthetic genes in *P. brevicompactum* and their homologues in *A. terreus* and *A. aculeatus*.



Table 1 Asperphenamate (1) biosynthetic genes in *P. brevicompactum* and their homologues in *A. terreus* and *A. aculeatus*

<i>P. brevicompactum</i>	<i>A. terreus</i>	Coverage/identity	<i>A. aculeatus</i>	Coverage/identity	Putative function
ORF_06	ATEG_09 071	—	ORF_07	—	C6 zinc finger protein
ORF_07	ATEG_09 070	—	ORF_08	—	RTA-like protein
ORF_08	ATEG_09 069	—	ORF_09	—	HHE domain protein
<i>apmA</i>	ATEG_09 068	99/71	ORF_10	99/71	NRPS
<i>apmC</i>	ATEG_09 067	100/80	ORF_11	100/75	Phospho-2-dehydro-3-deoxyheptonate aldolase
<i>apmD</i>	ATEG_09 065	39/80	ORF_13	96/76	NAD dependent epimerase
<i>apmB</i>	ATEG_09 064	96/68	ORF_14	94/70	NRPS
ORF_14	ATEG_09 063	—	ORF_15	86/65	Kinesin-like protein klpA
ORF_15	ATEG_09 062	—	ORF_16	99/72	Coronin -binding protein
ORF_16	ATEG_09 061	—	ORF_17	90/45	Zinc finger protein

shared 70–76% sequence identities with their orthologues from *A. aculeatus* (Table 1). Scanning the flanking regions of *apmA-D*, no biosynthetic genes were found on either side. Furthermore, no homologues were found in the flanking regions of *A. aculeatus* or upstream of *A. terreus*. Though there were three homologues in the down stream of *A. terreus*, they were putative kinesin-like or zinc finger proteins (Table 1). In addition, neither **1** nor its derivative was reported from *A. terreus* or *A. aculeatus*. These results indicated that four genes in the cluster are possibly sufficient for the synthesis of **1**. To address the question of whether *apmC* and *apmD* are involved in the biosynthesis of **1**, the same strategy was used for gene deletion, and the extracts from the deletion mutants were analysed (Fig. S2†). HPLC analysis showed that **1** still existed in both *apmC* and *apmD* mutants, indicating that they are not essential for the production of **1** (Fig. S3†). Taken together, these results demonstrate that *apmA* and *apmB* would be sufficient for the biosynthesis of **1**.

To probe the roles of ApmA and ApmB in the biosynthesis of **1**, we identified the intermediates from the *apm* gene deletion mutants. By analysing Δ *apmB* strains, we found that a peak (named compound **3**) was accumulated in 15.2 min (Fig. 2A, trace (iii)). We hypothesized that **3** is the product of ApmA. To identify the structure of **3**, it was isolated by a scale-up fermentation of Δ *apmB* mutant. Its structure was elucidated as *N*-benzoylphenylalaninol by NMR and MS analysis (see ESI† data), which corresponded well with the previously published data.¹ Feeding **3** into the Δ *apmA* mutant restored **1** production, which confirmed that **3** was a product of ApmA (Fig. 2A, trace iv). It is rare for an NRPS to generate an alcohol. Using antiSMASH analysis, we identified ApmA as an unusual NRPS module consisting of A-T-C-A-T-R with a C-terminal reductase (R)-domain.¹⁸ Several studies have demonstrated the function of the R-domain in natural product biosynthesis.^{19–23} For example, Cox and co-workers revealed that the R domain of PKS-NRPS TENS functions as a Dieckmann cyclase in the formation of tenellin.²⁴ Walsh and Liu demonstrated the role of the R-like domain of PKS-NRPS CpaS for releasing the product in the biosynthesis of cyclopiazonic acid.²⁵ Recently, the R domain in PKS-NRPS was identified as an aryl-aldehyde generator from an aryl-acid in the formation of meroterpenoid LL-Z1272 β and 2,4-dihydroxy 5,6-dimethyl benzaldehyde.^{26,27} Differences of R-

domains in NRPSs from bacteria and fungi were demonstrated by a phylogenetic tree analysis (Fig. S4†). Thus, the hypothesis is that the R domain acts in the reductive release of the shunt product **3** in the two-modular NRPS ApmA. **4** and benzoic acid (**5**) are the possible substrates of ApmA.

To confirm this, *apmA* was expressed in both *Saccharomyces cerevisiae* and *A. nidulans* systems.^{17,28} HPLC analysis of ApmA-expressing *A. nidulans* showed a new peak at 12.2 min. This peak was isolated and the structure was elucidated as **3**. The final titer was 20 mg L⁻¹ by the scale-up fermentation using **4** and **5** as substrates (Fig. 3A and ESI†). LC-MS analysis of crude

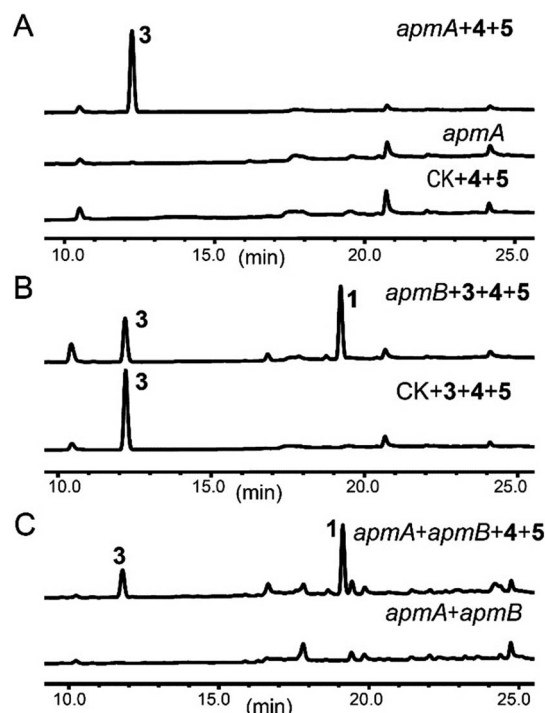


Fig. 3 Roles of ApmA and ApmB in the biosynthesis of asperphenamate. ApmA and/or ApmB were expressed in *A. nidulans*. (A) HPLC analysis of metabolites produced from ApmA-expressing strains with feeding of precursors **4** and **5**. (B) HPLC analysis of metabolites produced from ApmB-expressing strains with feeding of precursors **3**, **4** and **5**. (C) **1** was obtained by co-expressions of ApmA and ApmB.



extracts from *apmA* expressed strains in yeast and in *A. nidulans* revealed its molecular weight as 256.1395 $[M + H]^+$, confirming **3** production (Fig. S5†). Two additional peaks were also observed with masses of 270.2131 $[M + H]^+$ and 254.0875 $[M + H]^+$ by LC-MS analysis. These molecular weights suggested the formations of the acid form of **2** (molecular formula $C_{16}H_{15}NO_3$, calculated molecular weight 269.1052) and the aldehyde form of *N*-benzophenylalaninol (**6**, molecular formula $C_{16}H_{15}NO_2$, calculated molecular weight 253.1103) (Fig. S5†). These data indicate that ApmA catalyses the carrier protein-bound thioester-intermediate, releasing it from the acid form of **2** to the primary alcohol through an aldehyde intermediate **6**. Since ApmA harbored an R domain in the C-terminal, we assumed that it had a reduction function. To test this hypothesis, we fed the chemically synthesized *2*-*S*-*N*-acetylcysteamine (*2*-SNAC) into ApmA-expressing *A. nidulans*. **3** was detected by LC-MS analysis (Fig. S6†). Our data confirmed that ApmA is a *N*-benzophenylalaninol (**3**) synthetase using **4** and **5** as substrates and revealed for the first time a two-module NRPS system that acts in the reductive release in the biosynthesis of natural product.

Next, we assessed the role of ApmB by LC-MS analysis of its expression mutants in both *S. cerevisiae* and *A. nidulans*, but no product was detected (data not shown). Bioinformatic analysis showed that ApmB is a regular two modular NRPS with six domains, A-T-C-A-T-C. Analysing the structure of **1**, we proposed that ApmB may activate the same substrates as does ApmA, **4** and **5**, then we performed a two-step cascade reaction: tethering the linear *N*-benzophenylalaninepeptidyl to the ApmB chain and waiting for the attack of **3** before moving to the next step.²⁹ Accordingly, we fed the proposed substrates **3**, **4**, and **5** into ApmB-expressing strains along with the control strains under the same conditions. Notably, HPLC analysis demonstrated that the peak of **3** decreased, while a new peak with the same retention time and UV spectra as **1** appeared in the ApmB transformant (Fig. 3B). LC-MS analysis of the extracts further confirmed that the compound was **1** (molecular formula $C_{32}H_{30}N_2O_4$, measured molecular weight 507.2293 $[M + H]^+$, calculated 506.2206) (Fig. S7†). The feeding of **4** in the culture media greatly increased the yield of **1** (Fig. S8†). Considering all results, we concluded that ApmB acts in the ester bond formation and release of the final product **1**. Since substrate **5** is a product of primary metabolism, the biosynthesis of **5** in fungi is rarely reported. One case is the squalestatin biosynthesis in *Phoma* sp. C2932 using benzoic acid as the substrate. In this example, biosynthesis may have been catalysed by a gene encoding phenylalanine ammonia lyase (PAL) mfm7 as the first step of **4** degradation.³⁰ Searching the *P. brevicompactum* genome with M7 as a probe, we found the homologue of PAL gene without annotation (identity of 39.0% in amino acid). Interestingly, the PAL encoding gene was not located in the *apm* cluster. It may be involved in benzoate production.

Because of the unusual function of ApmB, it is logical to ask whether ApmB can use linear dipeptides such as **2** and **3** as substrates. This question was addressed by feeding **2** and **3** into the *apmB*-expressing *A. nidulans* strain. **1** was detected by LC-MS analysis (Fig. 4). To exclude the residual **4** in the medium as

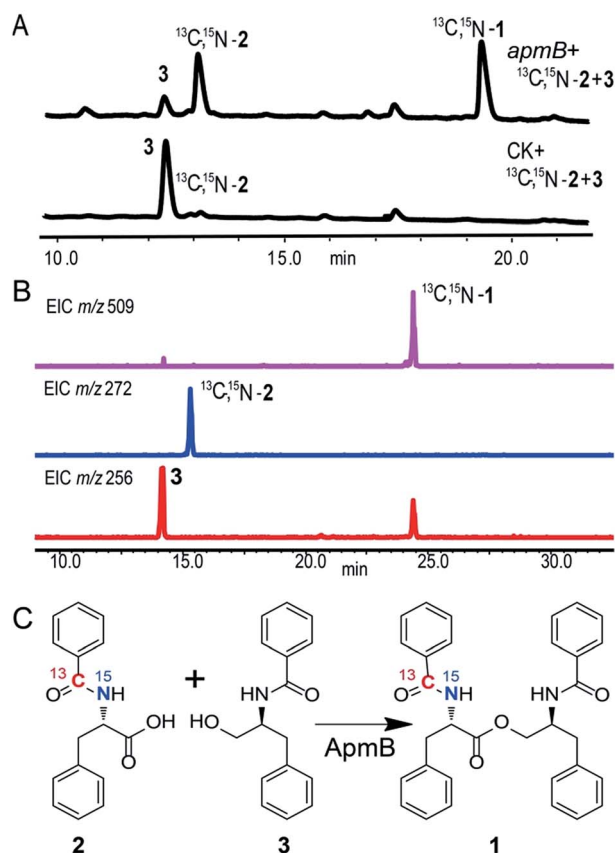


Fig. 4 ApmB utilizes linear dipeptidyl precursor to synthesize asperphenamate. (A) HPLC analysis of metabolites produced from ApmB expressing-strain with feeding $^{13}C,^{15}N$ -**2** and **3**. $^{13}C,^{15}N$ -**1** was obtained. (B) LC-MS confirmation of labeled $^{13}C,^{15}N$ -**2** integration into final product of **1**. (C) ApmB-catalysed reactions by using $^{13}C,^{15}N$ -**2** and **3** as substrates. Labeled $^{13}C,^{15}N$ -**1** was characterised by NMR and MS analysis.

substrate, $^{13}C,^{15}N$ -**2** (Fig. S10–S11†) was synthesized chemically and used as a substrate for the feeding experiments. LC-MS analysis of the crude extract clearly demonstrated that $^{13}C,^{15}N$ -**1** was the major product with a determined molecular weight of 509.2282 $[M + H]^+$ (Fig. S9†). To characterise the structure of $^{13}C,^{15}N$ -**1**, large scale fermentations were conducted by feeding labelled $^{13}C,^{15}N$ -**2** and **3** as substrates, and the structure was elucidated by NMR analysis (Fig. S12–S13†). This result suggested that ApmB activates and accepts the linear dipeptide **2** as a substrate. Finally, we coexpressed *apmA* and *apmB* genes in *A. nidulans*. A clear final product peak **1** was observed by feeding the substrates of **4** and **5** in the transformant (Fig. 3C).

Based on the gene disruption results and feeding studies in heterologous hosts, we proposed a biosynthetic pathway for **1** (Fig. 5). Using **4** and **5** as substrates, ApmA catalysed amide bond formation and tethered the intermediate into the NRPS chain. Then, the terminal R domain of ApmA catalysed the reduction reaction to get the shunt product **3** through the acid form **2** and the aldehyde form **6** (Fig. 5). Subsequently, ApmB activated the same substrates as did ApmA and waited for the



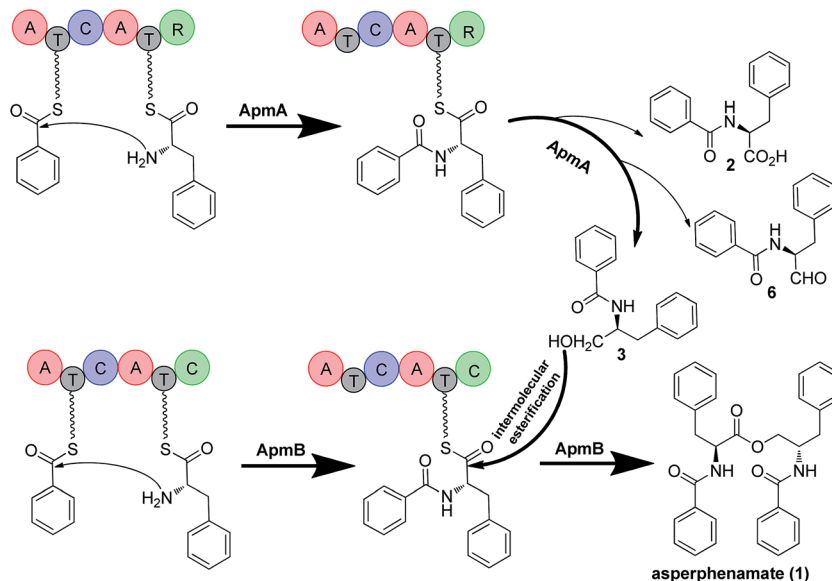


Fig. 5 Proposed biosynthetic pathway of **1** and mechanism for reduction and esterification steps in *P. brevicompactum*. Bold arrow indicates the main steps of ApmA and ApmB coordination for the formation of **1**. **2**, **6** are marked by light arrows indicating that ApmA catalyses the formation of **3** through reduction steps.

attack of **3**. After the attack of **3** on the peptidyl-*S*-*N*-benzoyl-phenylalanine, the inter-molecular ester bond was formed and the final product **1** was released by ApmB (Fig. 5). The whole process was completed by the coordination of two NRPSs, ApmA and ApmB. This is the first case of a simple compound formation by the machinery of two NRPSs shown in nature.

Conclusions

In summary, we identified the biosynthetic gene cluster of asperphenamate (**1**) and characterised the two NRPSs function using a heterologous expression approach in *A. nidulans*. We demonstrated that the NRPS enzyme ApmA had an R-domain, which was responsible for the formation of alcohol reduced from the acid through the aldehyde intermediate. Meanwhile, we also elucidated another NRPS enzyme, ApmB, with functions for ester bond formation and the release from the synthetase to form the final product. More interestingly, ApmB was able to accept the linear dipeptide to form the final product **1**. Therefore, our findings not only provide new insights into fungal NRPS machinery for the formation of amino acid esters, but also demonstrate a synthetic approach to develop new small molecules catalysed by NRPS.

Materials and methods

The strains, plasmids and primers used in this work are described in the ESI Tables S1, S2 and S3.† The HPLC analyses of the feeding experiment in *apmB*-expressed *A. nidulans* are listed in the ESI Fig. S7 and S8.† The LC-MS spectra of feeding experiment in *apmB*-expressed *A. nidulans* with ¹³C,¹⁵N-**2** and **3** as substrates are listed in the ESI Fig. S9.† The NMR spectra for compound ¹³C,¹⁵N-**1**, ¹³C,¹⁵N-**2**, **3** and **2**-SNAC are listed in the

ESI Fig. S10–S15†. Detailed descriptions of experimental procedures and reagents are provided in detail in the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank Drs Jinwei Ren, Wenzhao Wang and Haining Lyu (Institute of Microbiology, CAS) for NMR and MS data collection. We thank Dr Xuebing Li for generously providing *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and 1-hydroxysuccinimide. We thank Dr Georgina Salazar at the University of Texas Health Science Center at Houston for her careful and critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China (31470178 to W. B. Y. and 81502964 to Z. L.) and the State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Grant GTZK201506), and the Welch Foundation (Grant AU00024 to Z. A.). W. B. Y. is a scholar of “the 100 Talents Project” of CAS.

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