Biosynthesis of a New Benzazepine Alkaloid Nanangelenin A from *Aspergillus nanangensis* Involves an Unusual L-Kynurenine-Incorporating NRPS Catalyzing Regioselective Lactamization

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ABSTRACT: 1-Benzazepine is a pharmaceutically important scaffold but is rare among natural products. Nanangelenin A (**1**), containing an unprecedented 3,4-dihydro-1-benzazepine-2,5-dione-*N*-prenyl-*N*-acetoxy-anthranilamide scaffold, was isolated from a novel species of Australian fungus, *Aspergillus nanangensis*. Genomic and retrobiosynthetic analyses identified a putative nonribosomal peptide synthetase (NRPS) gene cluster (*nan*). The detailed biosynthetic pathway to **1** was established by heterologous pathway reconstitution in *A. nidulans*, which led to biosynthesis of intermediates nanangelenin B-F (**2**–**5** and **7**). We demonstrated that the NRPS NanA incorporates anthranilic acid (Ant) and L-kynurenine (L-Kyn), which is supplied by a dedicated indoleamine-2,3-dioxygenase NanC encoded in the gene cluster. Using heterologous *in vivo* assays and mutagenesis, we demonstrated that the C-terminal condensation (C_T) and thiolation (T_3) domains of NanA are responsible for the regioselective cyclization of the tethered Ant-L-Kyn dipeptide to form the unusual benzazepine scaffold in **1**. We also showed that NanA-C_T catalyzes the regioselective cyclization of a surrogate synthetic substrate, Ant-L-Kyn-*N*-acetylcysteamine, to give the benzazepine scaffold, while spontaneous cyclization of the dipeptide yielded the alternative kinetically favored benzodiazepine scaffold. The discovery of **1** and the characterization of NanA have expanded the chemical and functional diversities of fungal NRPSs.

INTRODUCTION

Benzazepines are an important class of heterocyclic compounds with interesting biological and pharmacological activities, and are found in a number of clinical drugs, including the vasodilator fenoldopam and the heart rate-lowering agent ivabradine.[1] Specifically, 1-benzazepine is featured in the angiotensin-converting enzyme (ACE) inhibitor benazepril[2] and the cholesteryl ester transfer protein (CETP) inhibitor drug candidate evacetrapib (Figure 1).[3] While 2- and 3-benzazepines are quite common in Nature (e.g. aurantioclavine and communesin from fungi[4] and rhoeadine and chilenine from plants[5-6]), 1-benzazepine-containing natural products are relatively rare, with the only reported examples being the two related plant alkaloids, goniomine[7] and kopsiyunnanine L,[8] and penioxalamine A[9] from *Penicillium oxalicum* (Figure 1).

Continuing our natural product discovery strategy targeting rare Aspergilli,[10-11] we recently reported the isolation of a family of drimane sesquiterpenes, nanangenines, from the Australian fungus *Aspergillus nanangensis*.[12] During the course of the study, we isolated an additional unrelated compound with a UV-vis spectrum distinct from the nanangenines. Herein, we describe the structural characterization and heterologous biosynthesis of this novel compound, named nanangelenin A (**1**), which contains an unprecedented 3,4-dihydro-1-benzazepine-2,5-dione-*N*-prenyl-*N*-acetoxy-anthranilamide scaffold (Figure 1). A nonribosomal peptide synthetase (NRPS) gene cluster (*nan*) was identified to be responsible for the biosynthesis of **1**. The biosynthetic pathway to **1** was then characterized by heterologous pathway reconstitution in *A. nidulans*, from which five biosynthetic intermediates nanangelenins B–F (**2**–**5** and **7**) were characterized.

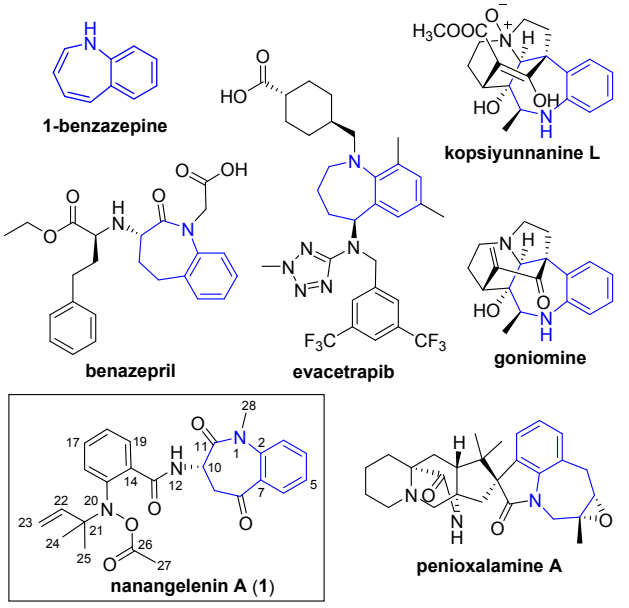

Figure 1. Representative pharmaceutical molecules and natural products known to contain 1-benzazepine, and the structure of nanangelenin A (**1**) (box) reported in this study.

Furthermore, we demonstrated that the NRPS NanA incorporates anthranilic acid (Ant) and L-kynurenine (L-Kyn), and its C-terminal condensation domain (C_T) is capable of regioselectively cyclizing the tethered Ant-L-Kyn dipeptide into the 1-benzazepine-dione in **1**.

RESULTS AND DISCUSSION

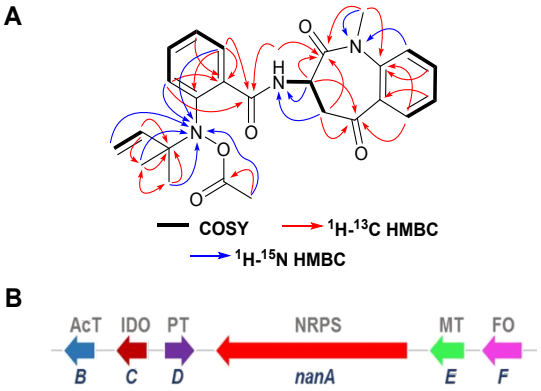

Figure 2. (A) Selected 2D NMR correlations for **1**. (B) The identified *nan* gene cluster responsible for the production of **1**. AcT, acetyltransferase; PT, prenyltransferase; MT, methyltransferase; FO, FAD oxidoreductase. A, adenylation; T, thiolation; C, condensation.

Discovery and Structural elucidation of nanangelenin A (1). Compound **1** was first detected in the culture of *A. nanangensis* grown on jasmine rice (Supporting Information). A search of the UV–vis profile of **1** against our in-house metabolite library (>7,500 standards) failed to provide a tentative assignment. Similarly, no fit was identified from our fungal spectral libraries (>25,000 spectra from 2,000 type species and >60,000 spectra from 3,000 talented fungal strains) suggesting **1** was a hitherto unknown secondary metabolite. Compound **1** was subsequently purified from the EtOAc fraction of a scaled-up culture of *A. nanangensis* on jasmine rice (Supporting Information). HRESI(+)MS analysis of HPLC-purified **1** revealed an adduct ion $[M + Na]^+$ at *m/z* 472.1848, indicative of a molecular formula $C_{25}H_{27}N_3O_5$ (Δmmu 0.5) requiring 14 double bond equivalents. The ^{1}H and ^{13}C NMR spectra of **1** in DMSO-d_6 (Table S6) revealed four discrete spin systems attributable to two 1,2-disubstituted aromatic rings, one reverse *N*-prenyl group and one CH_2–CH–NH fragment. The resonances associated with the *N*-prenyl group were significantly broadened at 25 °C, but sharpened on heating to 60 °C, suggesting the presence of slowly interconverting conformers arising from restricted rotation of the bulky prenyl group. Additional isolated resonances were observed for one *N*-methyl (δ_H 3.33, s; δ_C 35.7), one acetate (δ_H 2.12, s; δ_C 170.2 and 18.2), one ketone (δ_C 199.8), one tertiary amide (δ_C 169.4) and one secondary amide (δ_H 10.25, br s; δ_C 164.5), accounting for all atoms in the molecular formula and 13 of 14 DBE, thus requiring **1** to be tricyclic. Detailed analysis of the 2D NMR data for **1** (Figure 2A) established the 3,4-dihydro-1-benzazepine-2,5-dione-anthranilamide core of **1**, while key ^{1}H-^{15}N HMBC correlations positioned the *N*-prenyl and *N*-acetoxy groups on the aromatic amine nitrogen of the anthranilamide and the *N*-methyl group on the benzazepine nitrogen. The single chiral center in **1** was postulated to arise from L-tryptophan (L-Trp) and was tentatively assigned a 10*S* configuration, which was later confirmed by single crystal X-ray diffraction analysis of its immediate biosynthetic precursor, nanangelenin F (**7**) (see below).

Bioinformatic analysis targeted the putative biosynthetic gene cluster. Compound **1** is only the third reported natural product featuring a 1-benzazepine scaffold. Additionally, the unusual *N*-prenyl-*N*-acetoxy-anthranilamide moiety has not been reported previously, while only one fungal metabolite, antrocinnamomin A,[13] has been reported to contain an *N*-acetoxy group. The unique structure of **1** prompted us to investigate the molecular genetic basis for its biosynthesis. Retrobiosynthetic investigation of **1** suggested that the benzazepine scaffold is derived from the non-proteinogenic amino acid L-Kyn, which forms an amide bond with Ant. L-Kyn is the first stable intermediate metabolite in L-Trp metabolism and is the precursor to important biochemicals such as nicotinamide adenine dinucleotide (NAD^+) and the neuroinhibitor kynurenic acid.[14] Natural conversion of L-Trp to L-Kyn is catalyzed by the Fe^{2+}/heme-dependent enzyme tryptophan-2,3-dioxygenase (TDO) or indoleamine-2,3-dioxygenase (IDO), leading to *N*-formyl-L-Kyn, which is then hydrolyzed by kynurenine formamidase to produce L-Kyn.[15]

We initially proposed that a putative Ant-L-Kyn dipeptide precursor of **1** could be derived from a bimodular NRPS, like the acetylaszonalenin NRPS, AnaPS.[16] We further postulated that the NRPS gene likely clustered with gene(s) required for L-Kyn biosynthesis. Given that TDO is absent from fungal genomes,[15] we reasoned that the gene cluster may encode an IDO. The structural features of **1** suggested the gene cluster should also encode an acetyltransferase and a methyltransferase. Using these criteria and the strategy described previously,[17] we found a candidate gene cluster (*nan*) in the *A. nanangensis* genome[12] containing a NRPS gene *nanA*, a gene *nanC* encoding a putative IDO (Figures S4-6), an acetyltransferase gene *nanB*, a prenyltransferase gene *nanD*, a methyltransferase gene *nanE* and a gene *nanF* encoding a FAD-dependent oxidoreductase (Figure 2B). Thus, the putative *nan* gene cluster encodes all the biosynthetic enzymes required for biosynthesis of **1**, except for a kynurenine formamidase. Considering a previous study showed that *N*-formyl-L-Kyn is unstable and can undergo spontaneous hydrolysis to form the more stable L-Kyn,[18] we reasoned that IDO alone may be sufficient to supply L-Kyn from L-Trp.

Deciphering biosynthetic pathway to nanangelenin A. To unequivocally link the *nan* cluster to the biosynthesis of **1**, we applied a whole pathway reconstruction approach in a characterized host *A. nidulans* LO7890,[19] using a hybrid yeast-fungal artificial chromosome (pYFAC) expression system.[20-21] We first expressed only the NRPS gene *nanA* in *A. nidulans*, however, the resulting transformant did not produce any new compounds (Figure 3A, trace i). We then co-expressed *nanA* together with the IDO gene *nanC*, which led to the production of the dipeptide product nanangelenin B (**2**) (Figure 2A, trace ii). The structure of **2** was unambiguously confirmed by NMR (Table S9) and single crystal X-ray diffraction analysis (Figure 4). These results suggested that the NRPS NanA can activate Ant and L-Kyn, and that the IDO NanC is required to supply L-Kyn. To confirm that L-Kyn was formed before loading into the A domain of NanA, we fed the *A. nidulans* expressing *nanA* with L-Kyn, which successfully led to the production of **2** (Figure 5A, trace vi). Thus, we propose that NanC catalyzes the conversion of L-Trp to *N*-formyl-L-Kyn, followed by spontaneous hydrolysis to produce L-Kyn, which is then activated and captured by the NRPS NanA.

Coexpression of *nanAC* with the methyltransferase gene *nanE* led to partial conversion of **2** to nanangelenin D (**4**) (Figure 3A, trace iii). Structural elucidation by NMR analysis revealed **4** as the *N*-methylated analogue of **2**. Coexpression of *nanAC* with the prenyltransferase gene *nanD* resulted in the exclusive production of nanangelenin C (**3**) (Figure 3A, trace iv), which is the *N*-prenylated derivative of **2**. However, when coexpressing *nanAC* with *nanF*, encoding a FAD-oxidoreductase, the metabolite profile still showed the production of the immediate NRPS

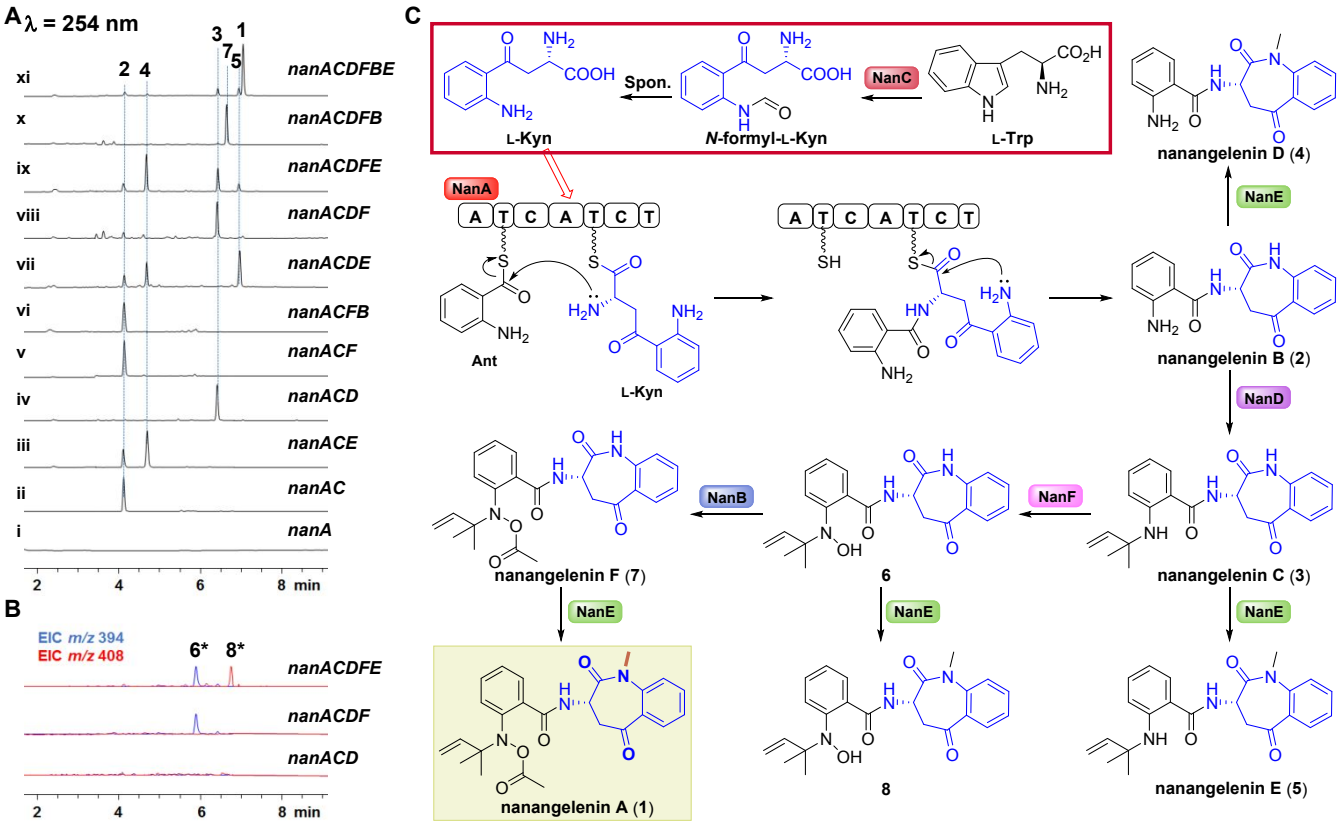

Figure 3. Characterization of nanangelenin A (**1**) biosynthesis. (A) HPLC traces of *A. nidulans* transformants showing the production of intermediates. (B) Extracted ion chromatograms (EIC) showing the putative MS peaks corresponding to **6** and **8**. * means not isolated. (C) Proposed biosynthetic pathway to **1**.

product **2** (Figure 3A, trace v). Further coexpression of the acetyltransferase gene *nanB* together with *nanACF* still led to the production of **2** (Figure 3A, trace vi). Altogether, the results suggest that *N*-hydroxylation cannot happen before prenylation, which must occur early in the biosynthesis of **1**. Coexpression of *nanACDE* led to the production of a new major peak, nanangelenin E (**5**), together with **2** and **4** (Figure 3A, trace vii). Compound **5** was shown by NMR to be the *N*-methylated analogue of **3** (Figure 3C). These results indicate that NanE is an *N*-methyltransferase that methylates the amide nitrogen of 1-benzazepine, with substrate tolerance for both **2** and **3**.

An NCBI BLAST search revealed that the FAD oxidoreductase NanF shares homology with the indolic nitrone synthase OxaD (50.2%)[22] and the putative *N*-hydroxylase TqaE (32.7%).[23] Thus, we proposed that NanF serves as an *N*-hydroxylase to facilitate *N*-acetoxylation. We reasoned that hydroxylation of **2** did not occur when coexpressing *nanACF* due to the inability of *nanF* to process the non-prenylated substrate. To confirm this hypothesis, we coexpressed *nanF* together with *nanACD*. However, the resulting *A. nidulans* still produced **3** as the major metabolite (Figure 3A, trace viii). Interestingly, searching the extracted ion chromatogram (EIC) revealed a putative MS peak corresponding to **6** (Figure 3B). Likewise, when coexpressing *nanACDFE*, we could detect a putative MS peak for **8** in the EIC along with **6** (Figure 3A, trace ix and Figure 3B), but were unable to purify either compound due to the very low yields. We reasoned that this is likely due to the unstable nature of the *N*-prenyl-hydroxylamine moiety on **6** and **8**, which could easily degrade back to the *N*-prenyl-amine precursors.[24] Thus, we coexpressed the acetyltransferase gene *nanB* together with *nanACDF*, which led to production of a new peak nanangelenin F (**7**) as the major product (Figure 3A, trace x). The structure of **7** was elucidated by MS and NMR analyses to contain the *N*-prenyl-*N*-acetoxy functional group (Figure 3C), and the absolute configuration was confirmed by single crystal X-ray diffraction analysis (Figure 4). Finally, introduction of the methyltransferase gene *nanE* into *nanACDFB* yielded nanangelenin A (**1**) as the major product, thus achieving the total biosynthesis of **1** in *A. nidulans* (Figure 3A, trace xi). The complete depletion of **7** suggests that although NanE can methylate **2**, **3** and **6**, it prefers **7** as a substrate. This also suggests that, *in vivo*, the biosynthesis of **1** most likely does not proceed via a strictly linear pathway.

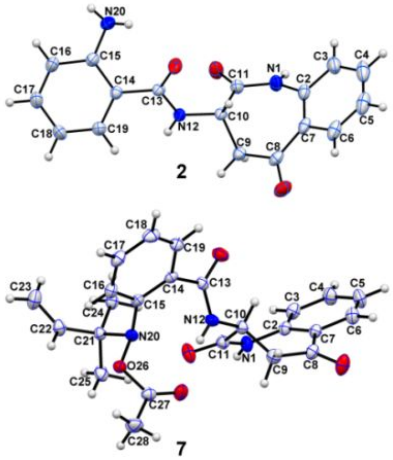

Figure 4. Single crystal X-ray structures of nanangelenin B (**2**) and nanangelenin F (**7**).

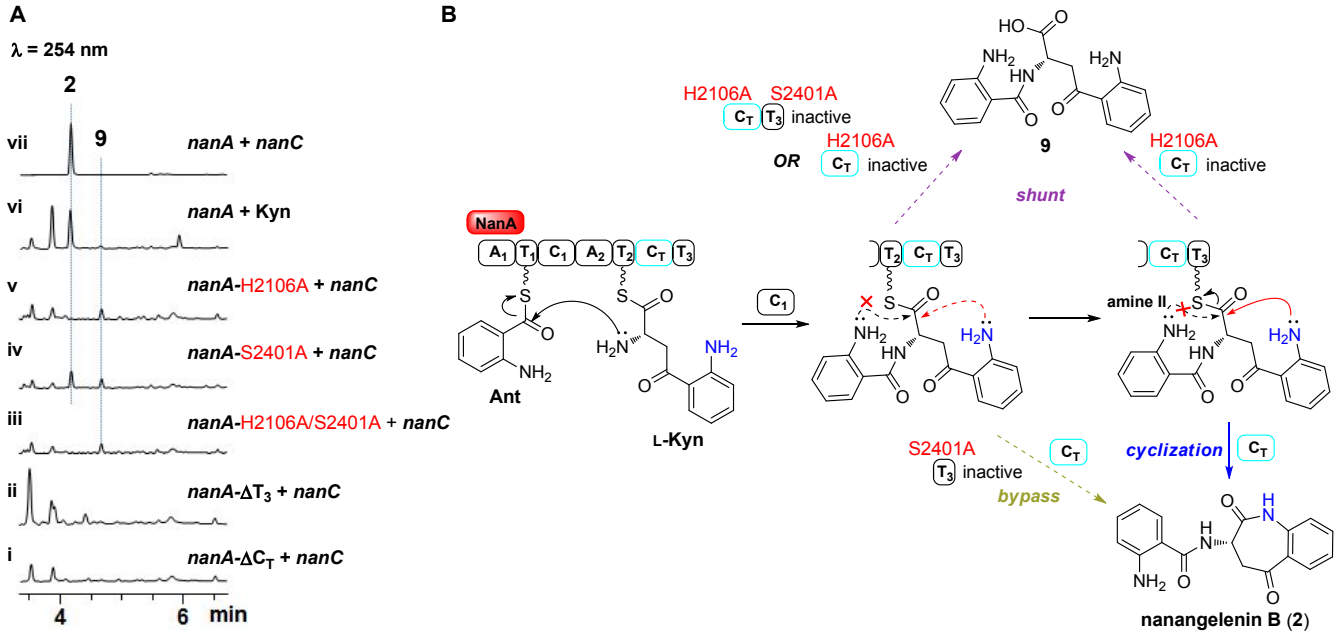

Figure 5. In vivo verification of NanA C-terminal C-T domain involvement in the formation of **2**. (A) HPLC traces from the extracts of *A. nidulans* expressing NanA wild-type or mutants together with the IDO NanC. (B) Proposed cyclization mechanism catalyzed by NanA.

C-terminal condensation and thiolation domains involved in the formation of 2. Despite the biosynthesis of **1** being successfully reconstituted heterologously, some ambiguity remained regarding the mechanism of NanA-mediated 1-benzazepinedione formation. Since two primary aromatic amines exist on the tethered Ant-L-Kyn, cyclization could proceed via the amine on Ant to yield cyclo-(Ant-L-Kyn)-benzodiazepinone (**11**), or via the amine on L-Kyn, resulting in **2**. However, *nanAC* yielded only a single product **2**, suggesting NanA has the ability to control the regioselectivity of the cyclization. NanA is a bimodular NRPS harboring a A-T-C-A-T-C_T-T domain organization. A previous study by Tang and coworkers showed that the bimodular NRPS AnaPS (C*-A-T-C-A-T-E) activates Ant and L-Trp and the corresponding tethered dipeptide forms a benzodiazepinedione viaspontaneous cyclization (Figure S3).[25] Recently, Schroeder and coworkers demonstrated that the gliotoxin synthetase bimodular NRPS GliP (A-T-C-A-T-C_T-T) utilizes the C-terminal C_T-T domains to catalyze diketopiperazine formation (Figure S3).[26] They showed that the activity of C_T was dependent on a histidine in the SHXXXD catalytic moiety, which was originally reported on the C-terminal of some fungal multimodular NRPSs and is responsible for macrocyclization.[25] This conserved sequence was also observed in the NanA-C_T domain (Figure S7), suggesting that the C_T domain in NanA may be involved in the regioselective cyclization of the tethered Ant-L-Kyn dipeptide.

To investigate the putative function of NanA-C_T and NanA-T_3 domains, we first constructed two NanA mutants: NanA-ΔC_T with the C_T domain deleted but the T_3 domain intact and NanA-ΔT_3 with the T_3 domain truncated but the C_T domain intact (Supporting Information). We then expressed each of the NanA mutants together with NanC in *A. nidulans* to assess the impact of these deletions in the biosynthesis of **2**. Examination of the metabolite profiles of *A. nidulans* expressing either *nanA*-ΔT_3/*nanC* or *nanA*-ΔC_T/*nanC* failed to identify any detectable **2** (Figure 5A, trace i and ii). These data suggest that normal production of **2** requires both the C_T and T_3 domains. To exclude the possibility that a truncated NanA failed to function *in vivo* due to misfolding, we further constructed NanA carrying point mutations in C_T and T_3 active sites. We first constructed a point mutation of NanA lacking the putative catalytic histidine (H2106A) in the C_T domain (Figure S7). When we coexpressed the NanA-H2106A together with NanC, the *A. nidulans* transformant lost production of **2** and accumulated a small amount of the linear Ant-L-Kyn (**9**) (Figure 5A, trace v). While we were unable to isolate **9** due to the low yield, the structure of **9** was supported by the identical UV spectrum, retention time and MS to synthetic Ant-L-Kyn. To confirm whether the T_3 domain of NanA is functional, we then mutated the predicted phosphopantetheinyl active site serine 2401 into alanine (S2401A) (Figure S8). Coexpression of NanA-S2401A and NanC in *A. nidulans* resulted in the production of a small amount (< 3% of WT) of **2**, as well as even lower amounts of **9**. Furthermore, expression of NanA carrying both point mutation sites in C_T and T_3 (NanA-H2106/S2401) with NanC also yielded a small amount of **9** (Figure 5A, trace iii).

These *in vivo* NanA point mutation results demonstrated the following: (1) The C_T domain is indeed required for the formation of **2** from a tethered Ant-L-Kyn precursor, and the histidine residue (H2106) is essential for the cyclization. (2) The terminal T_3 domain can enhance the catalytic efficiency of C_T, suggesting the T_2-tethered Ant-L-Kyn is transferred to T_3 prior to cyclization by C_T domain. In the absence of T_3, C_T could catalyze the cyclization of T_2-tethered Ant-L-Kyn, but with low efficiency. When the C_T domain is inactive, the tethered Ant-L-Kyn precursor can be spontaneously hydrolyzed from the T_2 or T_3 domain as a linear dipeptide **9**. However, we cannot completely rule out the involvement of endogenous host enzymes in the accumulation of **9**. (3) When the T_3 domain is inactive, the T_2 domain can partially complement the function of T_3 and the T_2-tethered-Ant-L-Kyn can still be cyclized to yield **2**.

Formation of 2 involves a regioselective lactamization catalyzed by the C_T domain of NanA. To further explore the mechanism of the benzazepine formation in **2**, we synthesized Ant-L-Kyn dipeptide *N*-acetylcysteamine thioester (Ant-L-Kyn-SNAC; **10**) as a surrogate substrate to mimic the T domain *S*-phosphopantetheinyl(Ppant-)-tethered Ant-L-Kyn. We then assayed the activity of the standalone NanA-C_T using a recombinant *N*-His-tagged NanA-C_T overexpressed in *Escherichia coli* BL21 (DE3) (Figure S9). In the presence of NanA-C_T in Tris-

HCl buffer (pH 7.0), the conversion of **10** to **2** could be clearly detected after 5 h (Figure 6A). However, when NanA-C_T was inactivated by boiling, the production of **2** was not observed under the same conditions. Instead, a new compound **11** was formed, which had the same MW as **2** (*m/z* 310 $[M + H]^+$) but a different UV spectrum and HPLC retention time (Figure 6B). Extensive NMR analysis combined with DFT calculations (Tables S14-S15) confirmed **11** to be cyclo-(Ant-L-Kyn)-benzodiazepine, which we named isonanangelenin B. These results confirmed that **10** can be partially converted to **11** non-enzymatically in the absence of NanA-C_T (Figure S10). Tang and coworkers demonstrated that formation of benzodiazepinedione from the AnaPS-T_2-tethered Ant-L-Trp is a non-enzymatic reaction (Figure S3).[25] A previous synthetic study also revealed that, at the melting point, the linear Ant-Kyn undergoes rapid thermal cyclization to form the 1,4-benzodiazepinedione product (70%) in preference to the 1-benzazepinedione (9%),[27] which indicates cyclization via the aromatic amine of Ant is more facile than via the Kyn amine. Taken together, these results suggest NanA-C_T catalyzes the regioselective cyclization of **10**, specifically directing the conversion of **10** to **2** over the competing facile cyclization to **11**.

In nonribosomal peptide biosynthesis, C-terminal C_T domains, or thioesterase (TE) domains, have been widely demonstrated to catalyze head-to-tail macrocyclization of the tethered linear peptide to form the mature peptides.[25, 28] This includes examples where the cyclic peptide contains a lysine residue, which include an additional free primary amine.[29] In the biosynthesis of the surugamides, a standalone *trans*-TE SurE has also been shown to catalyze the macrocyclization step.[30] Here, NanA provides the first example of a C_T domain that selectively cyclizes the L-Kyn residue of tethered Ant-L-Kyn dipeptide to form Ant-cyclo-(L-Kyn) **2**, instead of the typical head-to-tail macrocyclization to form the kinetically favored cyclo-(Ant-L-Kyn) **11**.

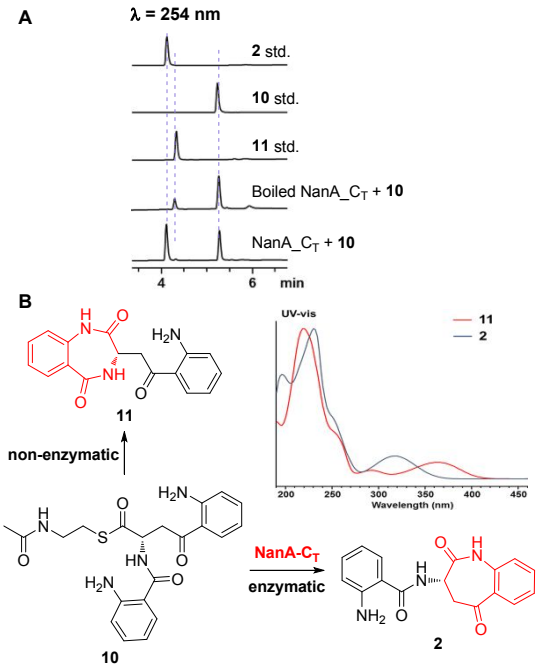

Figure 6. In vitro verification that NanA-C_T catalyzes a regioselective cyclization. (A) LC-MS analysis of enzymatic reaction of NanA-C_T with **10**. (B) Structures of Ant-L-Kyn-SNAC (**10**), NanA-C_T-catalyzed cyclization product nanangelenin B (**2**), the non-enzymatic cyclization product isonanangelenin B (**11**), and the UV-vis spectra of **2** and **11**.

Bioactivity screening and mining of putative 1-benzazepinone gene clusters in other Aspergilli. Bioactivity screening of **1-5** and **7** against a panel of microorganisms and cells revealed that nanangelenins A (**1**) and C (**3**) exhibit moderate in vitro cytotoxicity against mouse myeloma (NS1) cells (IC_{50} 12.5 μg/mL), but no significant antibacterial, antifungal or antiparasitic activities were detected (Table S17). Nonetheless, given the pharmaceutical interest in 1-benzazepines (e.g. as potential ACE inhibitors[2]) and the rarity of the scaffold in natural products, we were interested in whether other fungi also encode the production of similar 1-benzazepinediones. A cblaster (https://zenodo.org/record/3660769) search using bimodular NRPS NanA and IDO NanC as query sequencesagainst the public genome databases, we found homologs of *nanA* and *nanC* in several other *Aspergillus* species (Figure 7 and Table S16). Some of these gene clusters also encode other tailoring enzymes, such as methyltransferases and P450s. Thus, these homologous gene clusters provide a source of discovery for novel 1-benzazepine-containing metabolites.

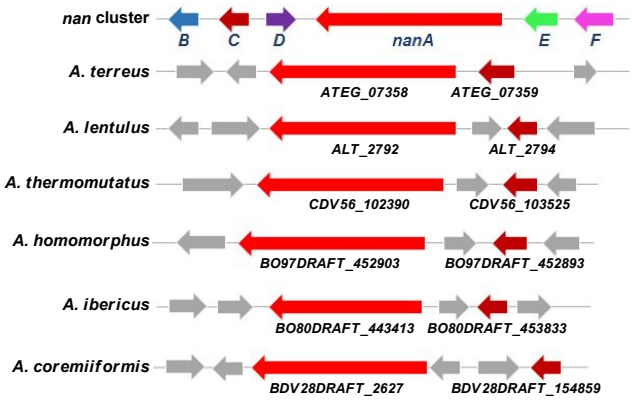

Figure 7. Biosynthetic gene clusters containing co-clustered bimodular NRPS and IDO genes conserved in other fungi.

CONCLUSION

In conclusion, we have discovered a new peptidyl alkaloid, nanangelenin A (**1**), featuring an unprecedented 3,4-dihydro-1-benzazepine-2,5-dione-*N*-prenyl-*N*-acetoxy-anthranilamide skeleton, along with five heterologously produced biosynthetic intermediates. We identified the *nan* gene cluster for **1**, which encodes a dedicated IDO to supply the unusual building block L-Kyn. Moreover, we demonstrated that the formation of the L-Kyn-derived 1-benzazepine ring involves a unique C_T domain that can control the regioselectivity of the cyclization release. To date, only the antibiotic daptomycin has been reported to incorporate L-Kyn as a building block.[31] The related amino acid 4-Cl-L-Kyn, an oral prodrug for the treatment of major depressive disorder, has been observed only in the cyclic lipopeptide taromycins (Figure S1).[32-34] Here, the use of a primary metabolite, L-Kyn, by the NRPS NanA to generate a new cyclic scaffold, cyclo-(L-Kyn), highlights another of Nature's innovative approaches for increasing chemical diversity, and is analogous to the repurposing of homoserine to homoserine lactones in actinomycetes.[35] Given the structural complexity and novelty, the biosynthesis of **1** is highly concise. This study opens up new avenues to uncover additional 1-benzazepine metabolites via fungal genome mining.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, NMR and MS spectra, tabulated 2D NMR data (PDF)
X-ray crystallographic data for nanangelenins B (**2**) and F (**7**) (CIF). CCDC 1981524 and 1981525 contain the supplementary crystallographic data for this paper.

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Notes

The authors declare no competing financial interests.

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