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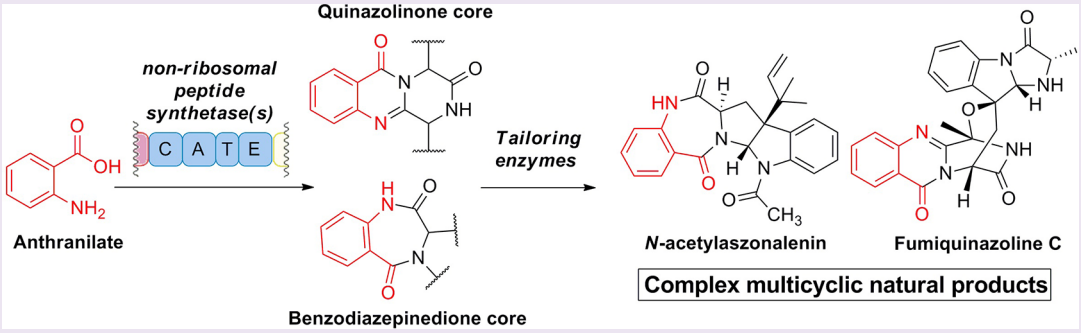
Short Pathways to Complexity Generation: Fungal Peptidyl Alkaloid Multicyclic Scaffolds from Anthranilate Building Blocks

Christopher T. Walsh,*[,†] Stuart W. Haynes,[†] Brian D. Ames,[†,∥] Xue Gao,[‡] and Yi Tang[‡,§]

[†]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts

[‡]Department of Chemical and Biochemical Engineering, [§]Department of Chemistry and Biochemistry University of California, Los Angeles (UCLA), 405 Hilgard Avenue, Los Angeles, California



ABSTRACT: Complexity generation in naturally occurring peptide scaffolds can occur either by posttranslational modifications of nascent ribosomal proteins or through post assembly line tailoring of nonribosomal peptides. Short enzymatic pathways utilizing bimodular and trimodular nonribosomal peptide synthetase (NRPS) assembly lines, followed by tailoring oxygenases and/or prenyltransferases, efficiently construct complex fungal peptidyl alkaloid scaffolds in *Aspergilli*, *Neosartorya*, and *Penicillium* species. Use of the nonproteinogenic amino acid anthranilate as chain-initiating building block and chain-terminating intramolecular nucleophile leads efficiently to peptidyl alkaloid scaffolds with two to seven fused rings.

1. INTRODUCTION TO NONRIBOSOMAL PEPTIDE MYCOTOXIN BIOSYNTHETIC LOGIC

Fungi, especially Ascomycota, are prolific producers of peptidyl alkaloids, often isolated because of their toxic activities toward mammalian cells and tissues.[1] A characteristic feature of many of these conditional peptidic metabolites is the multicyclic, constrained architectures that can lead to high affinity for biological targets.

Genome sequencing of several *Aspergillus* and *Penicillium* strains in the past few years have indicated two dozen to three dozen nonribosomal peptide synthetase (NRPS)-associated gene clusters per fungal genome.[2,3] Only a fraction of these gene clusters have been matched to known metabolites, reflecting cryptic biosynthetic capability under laboratory growth conditions.[2,4] An equivalent number of polyketide synthase gene clusters are also found in these fungal genomes as the second major class of natural product encoding genes.[2]

The fungal NRPS genes typically encode from one to six NRPS modules (although the cyclosporin synthase involves eleven modules[5,6]), correlating to assembly lines that can activate and modify single amino acids to those that can assemble hexapeptide scaffolds. Some of these fungal NRPS assembly lines release the free acid form of peptide products such as ACV synthetase,[7] which yields the aminoadipyl-Cys-Val tripeptide that is the immediate substrate for isopenicillin N synthetase and is the gateway to penicillins and subsequently cephalosporins. Other fungal NRPSs release head to tail cyclic lactams as exemplified by the synthetase for the immunosuppressant cyclosporin[8] and also for echinocandin B,[9] the scaffold for clinical antifungal drugs.[10]

Many of the well-studied fungal toxins arise from bimodular NRPS assembly lines; the nascent products released in those cases are the cyclic diketopiperazines (DKPs). The Phe-Ser-DKP is the building block for gliotoxin maturation (Figure 1).[11] The DKP formed between L-Trp and L-Pro is brevianamide F[12] that gets further elaborated to the trypostatins and fumitremorgins by a series of prenylations and oxygenations effected by dedicated tailoring enzymes that modify the indole side chain of the Trp residue.[13] A variety of other Trp-X-DKPs, including the symmetric Trp-Trp-DKP[14] are similarly precursors for further elaboration of the nascent DKPs.

The fungal NRPS subset of interest for this minireview encode two module to four module assembly lines that release the nascent products as bicyclic to tetracyclic nitrogen-containing frameworks (Figure 2). The key building blocks are anthranilate,[15] a nonproteinogenic amino acid, and its granddaughter primary metabolite L-tryptophan;[16,17] the products are fused nitrogen heterocycles, not diketopiperazines. These bi- to tetracyclic nascent

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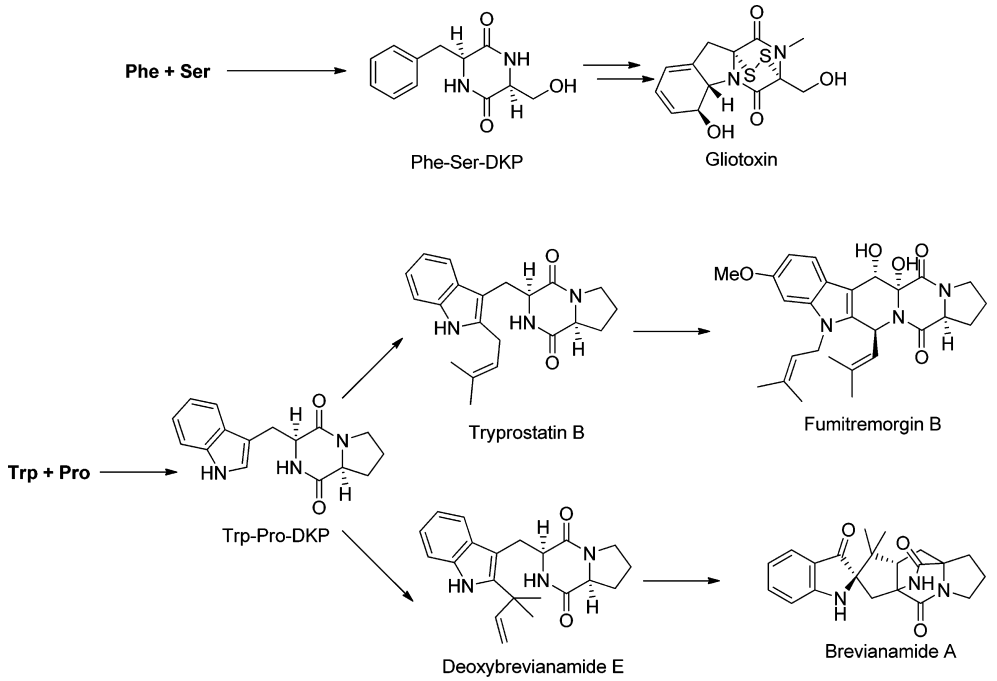

Figure 1. NRPS-mediated diketopiperazine (DKP) formations in the biosynthesis of gliotoxin and brevianamide and fumitremorgin C.

products then undergo tailoring reactions of oxygenation and/or prenylation that create additional fused ring systems, ranging from bicyclic to heptacylic frameworks (also shown in Figure 2)

2. FUNGAL METABOLITES BUILT BY NRPS ASSEMBLY LINES THAT USE ANTHRANILATE AS A KEY BUILDING BLOCK

NRPS assembly lines are freed from the restrictions to use proteinogenic amino acid building blocks, mandated by the inventory of aminoacyl-tRNAs and their cognate aminoacyl-tRNA synthetases that enable ribosomal protein synthesis.[18] One of the hallmarks of NRPSs is the ability to select, activate, and incorporate nonproteinogenic amino acids as building blocks.[19] The use of aminoadipate for penicillin biosynthesis by ACV synthetase[20] is among the most celebrated examples of this catalytic versatility.

A subset of fungal metabolites containing from two to seven fused nitrogen heterocycles (Figure 2) have incorporated the noncanonical amino acid anthranilate (*ortho*-aminobenzoate) as a key building block. In fact, in the 12 sets of fungal peptidyl alkaloids discussed below, anthranilate is always the chain-initiating building block in two module or three module NRPS assembly lines. We argue that the use of anthranilate sets up the dipeptidyl and tripeptidyl intermediates, tethered in typical thioester linkage to thioester domains on the bimodular and trimodular assembly lines (Figure 3), for intramolecular cyclization as the release mechanism. The result is the remarkable generation of bicyclic, tricyclic, and even tetracyclic nascent products from such anthranilate-utilizing NRPS assembly lines. This is in contradistinction to the diketopiperazine frameworks noted above that result when anthranilate is not a building block.

2.1. Economic Catalytic Inventory. These anthranilate-derived core frameworks generated by the bi- and trimodular NRPS get released as bicyclic 6,7-benzodiazepinediones, 6,6,6-tricyclic pyrazinoquinazolinediones, or as 6,6,7,6-diazepinone-containing cyclic scaffolds. They are then processed in short, complexity-generating pathways that involve only four kinds of tailoring enzymes (Figure 4). Two of these four are FAD-dependent oxygen-utilizing enzymes: one set is made up of FAD-dependent indole epoxygenases; the second set oxidize the pyrazinone ring to a cyclic imine that undergoes intramolecular addition reactions by N, and O nucleophiles. A third set is acylation enzyme catalysts that transfer acyl groups from acyl thioester substrates. These can be as simple as acetyl CoA, or they can be aminoacyl thioesters tethered to thiolation domains of monomodular NRPS enzymes.[21,22] This monomodular NRPS subset carry out annulations of tryptophan-derived indole rings during maturation of nascent products containing both Ant and Trp building blocks. The fourth set of tailoring enzymes is prenyl transferases adding dimethylallyl groups to the beta position of the indole ring of such scaffolds and acting thereby as heterocyclization catalysts.

2.2. Metabolic Generation and Utilization of Anthranilate. Anthranilate is a primary metabolite in organisms that can synthesize the proteinogenic amino acid tryptophan. Clearly this is the case for fungi, since tryptophan also serves as a building block for many of the signature indole alkaloids that are fungal metabolites. Anthranilate is generated by diversion of some of the cellular flux of chorismate from its routing to phenylalanine and tyrosine biosynthesis.[23] Anthranilate synthase carries out a two step reaction.[24] In step one, ammonia released from glutaminase action by one of the enzyme subunits attacks bound chorismate in the other enzyme subunit to yield a 2-amino-deoxychorismate. That is then subjected to aromatizing elimination of the 3-enolpyruvyl substituent to yield anthranilate (Figure 5a).

To generate the essential amino acid tryptophan, anthranilate is then coupled enzymatically with 5-phosphoribosyl-1-pyrophosphate to yield, after a series of rearrangements, indole glycerol-3-P.[16] In turn, this metabolite, along with cosubstrate serine, is converted to L-tryptophan by the well studied pyridoxal-P dependent enzyme tryptophan synthase (Figure 5b).[16] Anthranilate in parallel serves as precursor to a wide variety of heterocyclic natural product scaffolds, reflecting the chemical versatility of this 2-aminobenzoate building block. This includes

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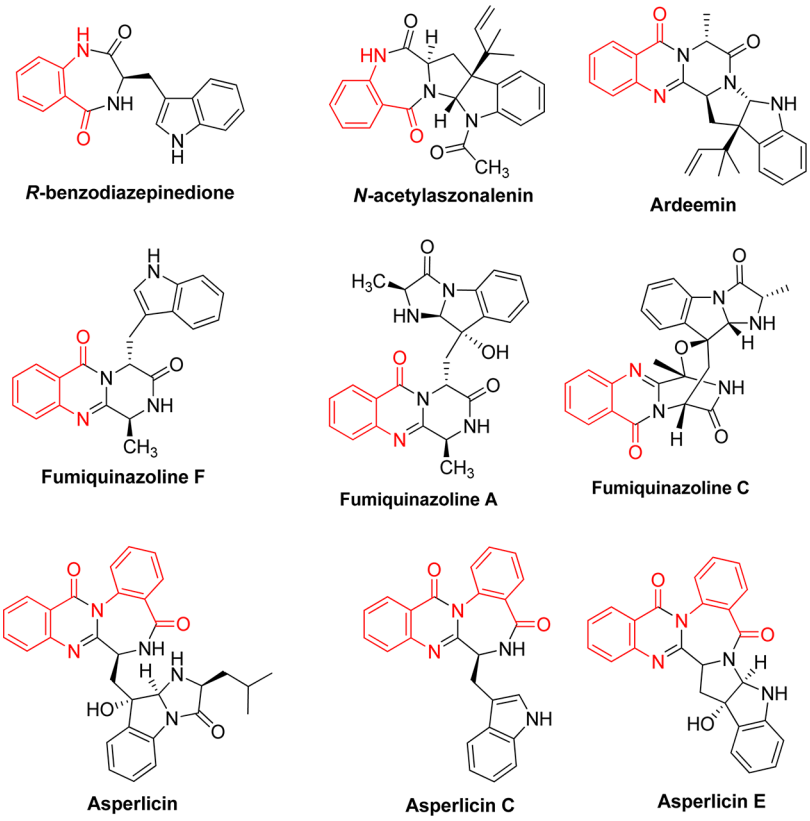

Figure 2. Bicyclic to heptacyclic peptidyl alkaloid scaffolds from fungi utilizing anthranilate as building block(s) (in red).

the 6,7-benzodiazepinediones and 6,6,6-tricyclic quinazolinones that are the subjects of this review but also benzodiazepinones in the tomaymycin and sibiromycin class of antibiotics,[25−27] the tricyclic phenoxazine in actinomycins[28] and the corresponding phenazines,[29] the quinolone units in aurachins,[30,31] and the bicyclic benzoxazolinate in the enediyne natural product C1027.[32−34] One can imagine there are many metabolic demands on the anthranilate pool. In *Aspergillus fumigatus*, which make fumiquinazolines constitutively, there is a second, dedicated anthranilate synthase in the fumiquinazoline biosynthetic gene cluster[35] to ensure an adequate supply of anthranilate for these conditional pathways.

3. PATHWAYS UTILIZING TETHERED ANT-X DIPEPTIDYL INTERMEDIATES

Two examples of fungal alkaloids that have incorporated anthranilate as the amino terminal unit of a dipeptide intermediate are Ant-Trp for aszonalenin scaffolds and Ant-Phe for the cyclopenin class of metabolites. In each case, the dipeptidyl intermediates, tethered in thioester linkage to the pantetheinyl prosthetic group of a posttranslationally modified NRPS module, are proposed to undergo release via intramolecular cyclization initiated by the N-terminal anthranilyl free-NH_2. The nascent bicyclic scaffolds are then processed by tailoring enzymes to very distinct mature molecular frameworks.

3.1. Ant-Trp for Aszonalenin Biosynthesis. The aszonalenin pathway (Figure 6a) was the key to deciphering the chemical logic and enzymatic machinery for the whole class of anthranilate-derived multicyclic alkaloid scaffolds in fungal metabolism. The first enablement was the availability of the genome sequence of *Neosartorya fischeri*.[36] The second was the investigation by S. M. Li and colleagues who have characterized the regiospecificity and mechanism of the class of Mg^{2+}-independent prenyltransferases that act on distinct sites on the indole ring of tryptophan-based fungal metabolites.[37−39] They heterologously overproduced both the prenyltransferase and the neighboring acetyltransferase from *N. fischeri* in *E. coli*. Using synthetic *R*- and *S*-benzodiazepinedione, which they intuited should be the prenyltransferase substrate if this were the aszonalenin pathway, they indeed observed efficient conversion of *R*-benzodiazepinedione to aszonalenin.[40] In turn, they demonstrated that the acetyltransferase would then acetylate the indole nitrogen and yield the known acetylaszonalenin.[40] The aszonalenin prenyltransferase shows specificity for transfer to C_3 of the indole moiety of the *R*-benzodiazepinedione substrate. The resultant prenylated indole is now electron deficient at C_2 and is captured intramolecularly by one of the two amide nitrogens of the diazepinedione ring. This leads to construction of the fused 6−7−5−5−6 five ring system characteristic of the aszonalenin framework. Such prenyl transfers are paradigmatic for complexity generation not only in the molecules described below but also in Trp-X-DKP derivatives noted above.

The studies by Li and colleagues[40] did not address the biosynthesis of the benozdiazepinedione but did note that an adjacent ORF was a predicted bimodular NRPS. In principle the bicyclic benzodiazepinedione could arise from either a tethered Trp_1-Ant_2-S-enzyme, released by attack of the NH_2 of the Trp moiety on the Ant thioester carbonyl, or from an Ant_1-Trp_2-S-enzyme, released by attack of the Ant-NH_2 on the activated Trp-thioester carbonyl. Our bioinformatic analysis of the bimodular ORF AnaPS (C*-A_1-T_1-C_2-A_2-T_2-E_2) suggested that module 2 contained a canonical amino acid activating Adenylation domain A_2 for activation of Trp, but the corresponding A_1 domain had an anomalous signature.[35] We predicted that would be an

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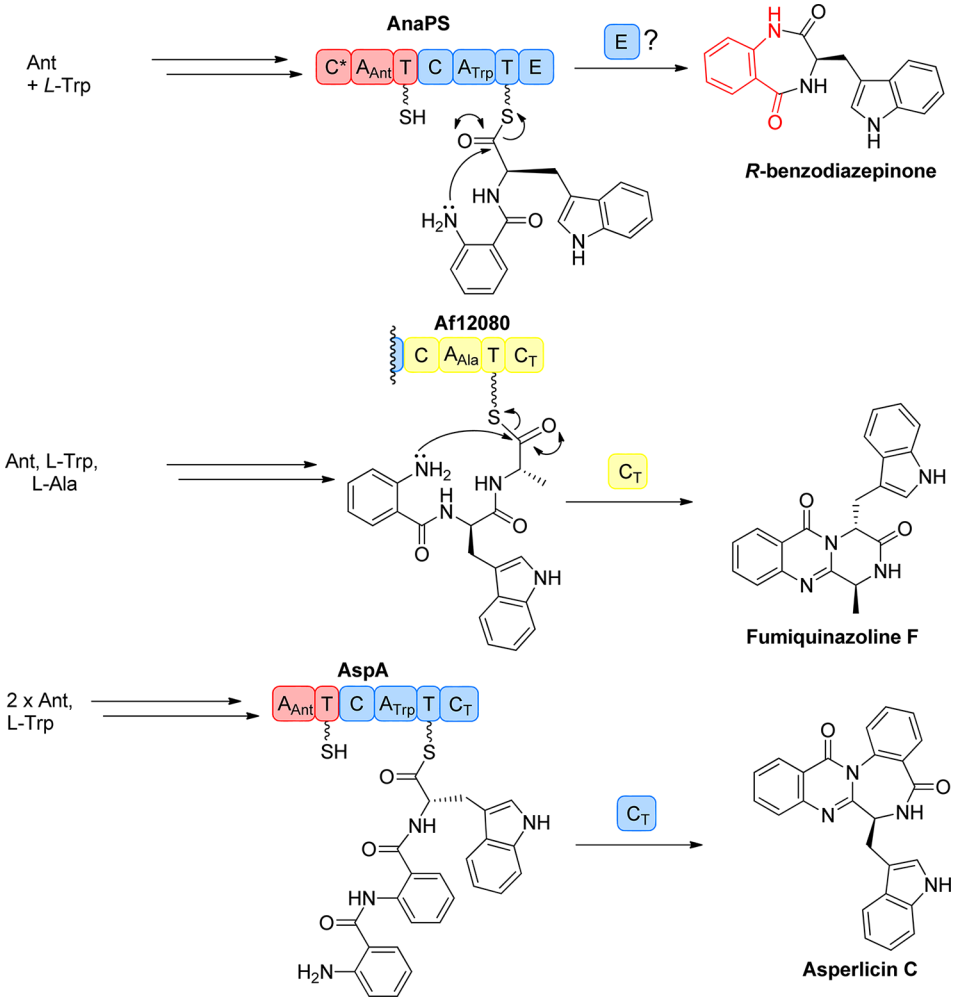

Figure 3. Bicyclic, tricyclic, and tetracyclic fused heterocyclic ring scaffolds from anthranilate-utilizing bimodular and trimodular NRPS assembly lines: AnaPS makes R-6,7-benzodiazepinedione; Af12080 makes fumiquinazoline F; AspA makes asperlicin C. *A* = adenylation, *T* = thiolation, *C* = condensation, *E* = epimerization.

anthranilate-activating domain and heterologously expressed C*-A_1-T_1 in *E. coli* and indeed demonstrated reversible activation of anthranilate as anthranilyl-AMP and its subsequent covalent tethering in thioester linkage to the pantetheinyl arm posttranslationally introduced on T_1 (Figure 6b). This demonstration allowed us to predict a 10 residue code for anthranilate activation in fungal adenylation domains of NRPS enzymes[35] and opened the door for all the subsequent biosynthetic genetic and enzymatic studies noted in the following sections.

Expression of active full length bimodular AnaPS was then achieved in a yeast expression system,[41] bearing a phosphopantetheinyl transferase gene in the chromosome to generate the posttranslationally modified *holo* forms of the two T domains of AnaPS required for covalent loading of Ant_1 and Trp_2. Encouragingly, the 280 kDa bimodular holoprotein was soluble and active, producing *R*-benzodiazepinedione as product.[41] Mutagenesis to inactivate the epimerase (E) domain should block the "on enzyme" epimerization of L-Trp to D-Trp. Indeed, from that mutant a small amount of *S*-benzodiazepinedione was released but none of the *R*-product. Thus, one can write the mechanism for AnaPS, as shown in Figure 6c. Anthranilate and L-Trp are separately selected, activated as mixed AMP anhydrides and then attached as thioesters to the thiol of the phosphopantetheinyl arms of T_1 and T_2, respectively. The C_1 condensation domain makes the Ant-Trp peptide bond, creating the Ant-Trp dipeptidyl thioester on T_2. From work on epimerase domains in other NRPS assembly lines,[42] we predict epimerization at C_α of the N-acylated Trp thioester occurs at this stage, generating Ant-D-Trp-*S*-T_2. Now the release step occurs, all the flux proceeding by intramolecular amide formation from the Ant-NH_2 group. There is no detectable competing hydrolysis to liberate the linear Ant-D-Trp-COOH. This generates the 6,7-bicyclic scaffold, constructing in that step the unusual 7-member diazepinedione ring. The size of the 7-member ring is dictated by the fact that the anthranilyl unit is a β- not an α-amino acid, and it is a planar aromatic β-amino acid at that, one which is able to snap shut in both this and in tricyclic and tetracyclic-generating reactions described in the next sections. Anthranilate is the key noncanonical building block that drives the framework diversity of this class of peptidyl alkaloids. Crucially, also, it appears as though the epimerization of L-Trp to D-Trp on the AnaPS assembly line controls the exclusive generation of the *R*-benzodiazepinedione.

To make the pentacyclic scaffold of aszonalenin from the two primary metabolites anthranilate and L-tryptophan only two enzymes are required, AnaPS and the prenyltransferase AnaPT. On the one hand, it is a dramatic lesson in chemical efficiency and

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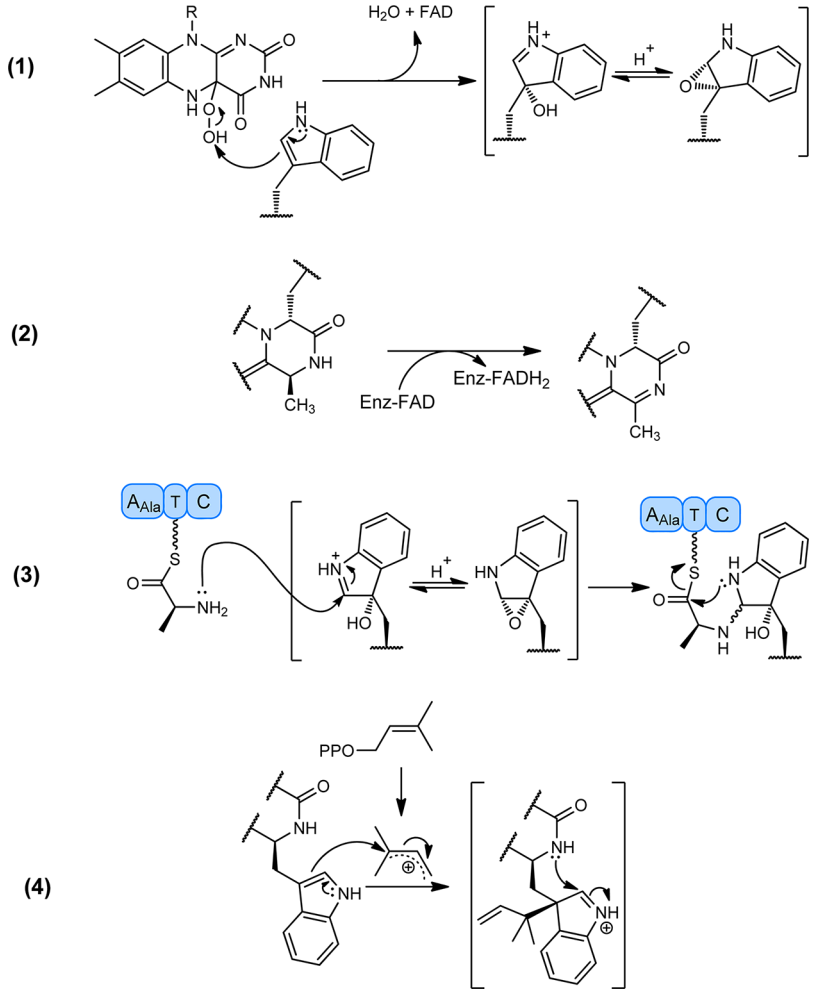

Figure 4. Four kinds of tailoring enzymes that act on anthranilate-derived nascent peptidyl alkaloids: (1) FAD-dependent indole epoxygenases; (2) FAD-dependent pyrazinone oxidases; (3) annulating NRPS; (4) indole C_3-dimethylallyl transferases.

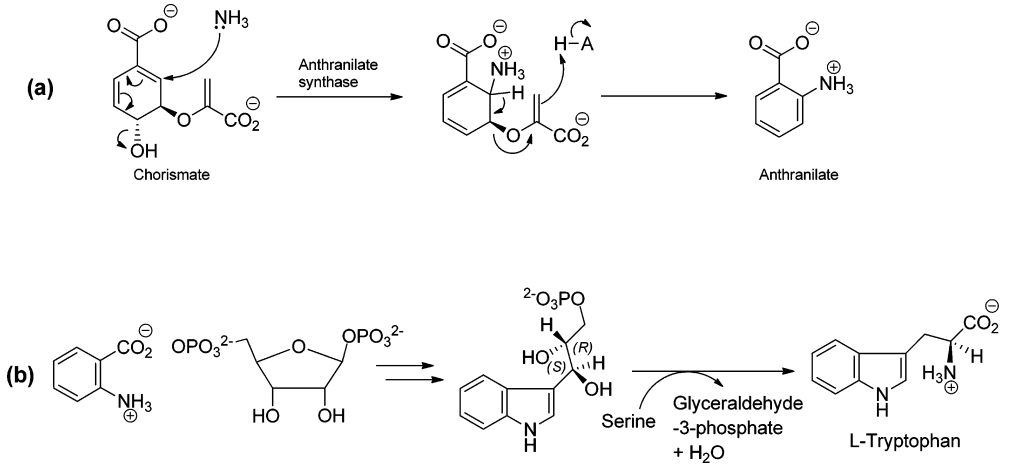

Figure 5. Anthranilate metabolism: (a) chorismate to anthranilate via anthranilate synthase; (b) anthranilate to indoleglycerol-3-P to tryptophan.

on the other hand presages the utility of anthranilate as a key unit in more complex multicyclic scaffolds.

3.2. Ant-Phe for Cyclopenin Biosynthesis. The findings on aszonalenin scaffold biosynthesis are predictive for how cyclopeptine, dehydrocyclopeptine, and cyclopenin are assembled (Figure 7a) in *Aspergillus nidulans* and *Penicillium sp*. Although the relevant orfs have not yet been identified genetically, it is known that Ant and Phe are activated in extracts as the AMP derivatives and the methyl group on the benzodiazepinedione nitrogen derives from S-adenosylmethionine.[43] Thus, a bimodular NRPS with thioesterified Ant-L-Phe-S-T_2 is expected and comparable intramolecular capture will generate the 6,7-bicyclic N-methyl benzodiazepinedione scaffold of the known metabolite cyclopeptine (Figure 7b).

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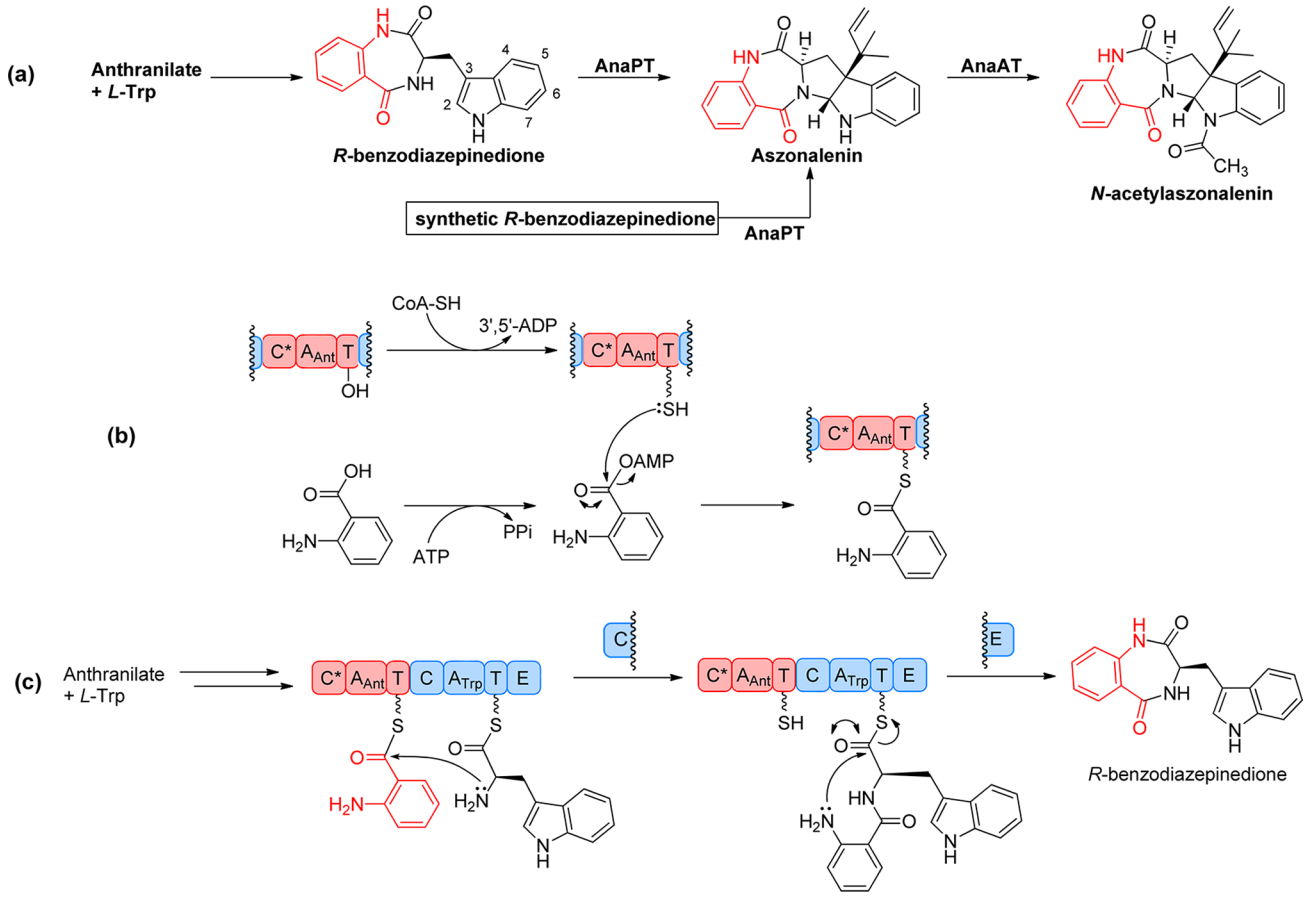

Figure 6. (a) Aszonalenin three step pathway. (b) Selection, activation, and covalent tethering of anthranilate by the Adenylation (A) domains of AnaPS. (c) Mechanism for *R*-6,7-benzodiazepinedione formation by AnaPS.

Subsequent modifications by tailoring enzymes prime the bicyclic scaffold for subsequent rearrangement. Enzymatic studies[43−49] from *Penicillium cyclopium* indicate that cyclopeptin-(e) dehydrogenase desaturates the Phe-derived side chain to the olefin, which is then epoxidized by an FAD-dependent dehydrocyclopeptin(e) epoxidase.[46,49] There is also an attendant hydroxylation of the phenyl ring to a *para*-substituted phenol, yielding cyclopenol in three steps.[47,48] At this stage, an enzyme known as cyclopenase[50,51] converts the epoxy-6,7-bicyclic framework to the 6,6-bicyclic quinolone viridicatol (Figure 7c). Viridicatol formation is attended by production of CO_2 and methyl amine (thought to arise from methyl isocyanate, which forms as coproduct when this conversion is carried out non-enzymatically[52,53]). Viridicatol is processed further in *Penicillium* species by tandem *O*-methylation of the two −OH groups, introduction of two additional −OH groups (perhaps by hydratase and hydroxylase action, respectively), followed by prenyl transferase action to yield peniprequinolones.

The cyclopenase-mediated rearrangement is a remarkable scaffold morphing and can also be effected nonenzymatically in acid or base conditions.[50,52,53] The mechanism proposed below in Figure 7c shows the C_3 hydroxyl group of viridicatin is derived from the epoxide oxygen of cyclopenin. It features neighboring group participation from the anthranilyl-derived amide nitrogen to assist the π electrons of the adjacent benzene ring to open the epoxide. The 6,7-epoxy tricyclic framework is thus rearranged to the phenyl-substituted 6,6-quinolone.

4. PATHWAYS USING TETHERED ANT-TRIPEPTIDYL INTERMEDIATES

From the identification that the A_1 domain in module one of AnaPS specifies selection and activation of anthranilate for the aszonalenin assembly line, we were able to predict a 10 residue code that should specify anthranilate-specific A domains in other fungal NRPS enzymes. To that end, we focused on a trimodular NRPS Af12080 in the *A. fumigatus* Af293 genome that should use module one for anthranilate, module two for D-Trp (with "on assembly" line epimerization to D-Trp), and module three should select L-Ala (figure 8a).[23,35] Expression of the C_1-A_1T_1 module in *E. coli* validated Ant activation and so confirmed the prediction experimentally.[21] This algorithm has been used by us subsequently in tryptoquialanine (also in Figure 8a),[54] ardeemin,[55] and asperlicin[56] biosynthetic studies to identify NRPS assembly lines that incorporate anthranilate. This approach also allows attention on neighboring ORFs that form a biosynthetic cluster. Generally, these include tailoring enzymes to convert the nascent scaffolds released from the NRPS bi- and trimodular assemblies into more complex, mature multicyclic scaffolds.

4.1. Ant-Trp-Ala to Fumiquinazolines and Tryptoquialanine: The Trimodular NRPS Assembly Lines Generate a Tricyclic Pyrazinoquinazolinedione Scaffold. The two pathways that start with trimodular NRPSs, utilizing anthranilate as the chain-initiating amino acid, that have been most extensively deconvoluted to date are those for fumiquinazolines from *A. fumigatus* and tryptoquialanine from *Penicillium aethiopicum*. Each of these pathways utilizes cognate NRPSs that activate

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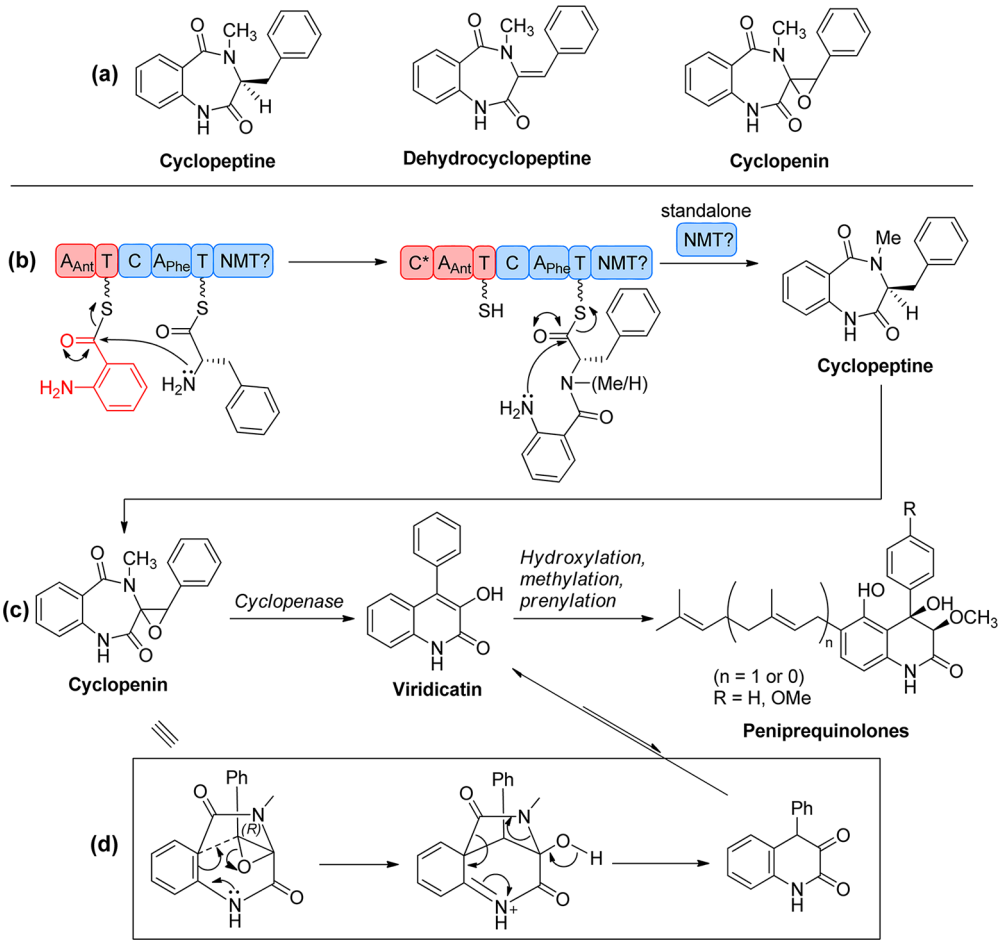

Figure 7. (a) Structures of cyclopeptine, dehydrocyclopeptine, cyclopenin. (b) Conversion of Ant-Trp-S-enzyme to cyclopeptine. (c) Conversion of epoxy-cyclopeptine to the quinolone viridicatol and conversion of viridicatol to peniprequinolones. (d) Mechanistic proposal for rearrangement catalyzed by cyclopenase.

anthranilate in module one, tryptophan in module two, and alanine in module three. The identical tricyclic fumiquinazoline F (FQF) is released as product by both Af12080 and TqaA (Figure 8b).[35,41] It is the subsequent processing that creates distinct mature frameworks.

TqaA has been expressed in yeast and the three module enzyme obtained in purified, active soluble form,[41] a remarkable feat considering the 450 kDa size (C^*AT_1-CAT_2E-CAT_3C_T). Purified TqaA indeed releases the tricyclic 6,6,6 FQF (a pyrazinoquinazolinedione), representing a dramatic morphing of the presumed linear Ant-D-Trp-L-Ala-S-T_3 tripeptidyl-*S*-enzyme intermediate. A double cyclization of that tethered linear tripeptidyl-*S*-enzyme must ensue. There are two possible routes. One involves initial formation of the Ant-diketopiperazine (path b in Figure 8b) and then its subsequent capture by the free NH_2 of the anthranilyl moiety followed by dehydration. A second route involves intramolecular attack of the Ant-NH_2 on the Ala_3 thioester carbonyl as the initial release step (path a in Figure 8b). This would generate a 6,10-bicyclic system. Transannular attack and dehydration by the amide nitrogen (from the Trp residue) on the newly formed amide bond would yield the 6,6,6-framework of FQF.[57,58] While one might be hesitant to propose the formation of a 10 member ring as kinetic product, data from variant tripeptide analogs are consistent with this route[41] as is a proposed, comparable 6,11 bicycle pathway in asperlicin biogenesis[56] noted below in section 4.3. Mutagenesis studies on the C_T (terminal condensation) domain of TqaA indicated that this domain was the cyclization/release catalyst.[41] The chain-initiating anthranilate is crucial for generation of the tricyclic FQF: substitution by salicylate or benzoate is not accepted by the NRPS.

4.1.1. From FQF to FQA to FQC to FQD. There are three tailoring enzymes encoded in the fumiquinazoline biosynthetic cluster that catalyze three consecutive transformations of FQF: the flavoenzyme oxygenase Af12060, the monomodular NRPS Af12050, and a flavoprotein oxidase Af12070 (Figure 9a). Af12060 acts formally as an epoxygenase, in functional analogy to the dehydrocyclopeptin epoxidase noted in section 2.1.2. Af12060 uses O_2 as cosubstrate and likely generates a typical FAD-4a-OOH as the proximal donor of an electrophilic oxygen atom.[21] The nucleophilic partner in the substrate is FQF at the $C_{2,3}$ olefin of the pyrrole ring of the indole side chain. The product can be written as the epoxyindole or the hydroxy iminium species and is metastable. In the absence of the next enzyme, Af12050, the epoxide/hydroxyiminium adduct will decay to the diol, which is a dead end metabolite.[22] The partner Af12050 is an alanine-activating monomodular NRPS that tethers the alanine in thioester linkage to its T domain.[21,22] It also then delivers that alanyl unit for net annulation of the epoxyindole to create the product fumiquinazoline A (FQA), a known *A. fumigatus* metabolite. The exact timing of the two bonds in forming the new five member ring in FQA is not known:

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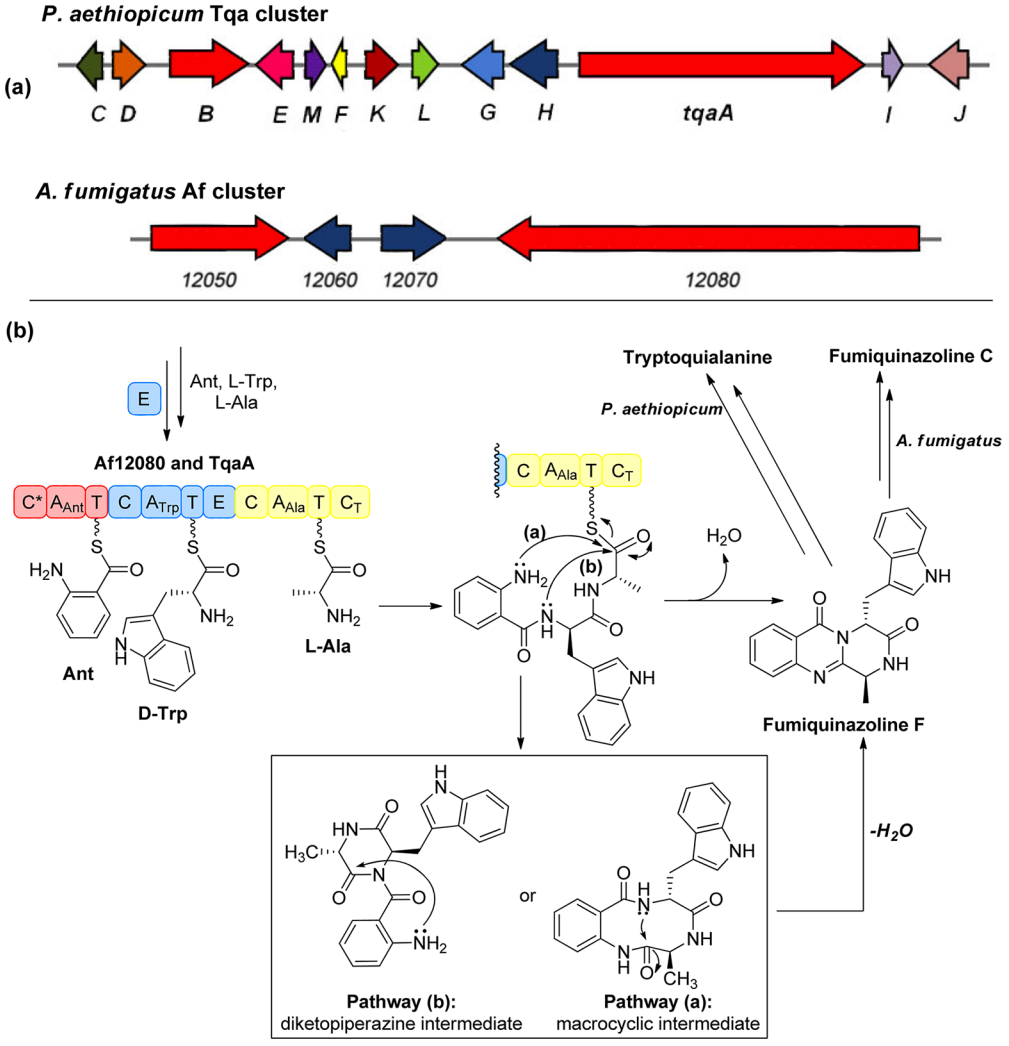

Figure 8. (a) Domain organization and predicted selectivity of Af12080. (b) Common formation of FQF by Af12080 and TqaA. Different end products (FQC vs tryptoquialanine).

one could formulate alanylation of the indole NH to bring the alanyl-NH_2 into proximity to attack C_2 of the epoxyindole intermediate. The oxygen introduced during epoxidation/hydroxyimine formation ends up as a C_3 alcohol with α-stereochemistry while the newly formed C_2−N bond form the alanyl capture is β in FQA. The net chemical result is annulation of the indole to a tricyclic imidazoindolone ring system.

At this point, three enzymes into the FQ pathway, there are a pair of tricyclic moieties, the pyrazinoquinazolinedione and the imidazoindolone connected by a bridging CH_2 (originally the C_β of the L-Trp_2 building block). The fourth and last enzyme in the pathway enables the joining of that pair of tricyclic ring systems into a fused heptacyclic framework in the FQ metabolites FQC and FQD. Af12070 purifies from *E. coli* heterologous expression as an FAD-containing oxidase.[59] It works on FQA to produce FQC as the kinetic product. Over hours to days, FQC (with its cyclic hemiaminal ether linkage) equilibrates to FQD non-enzymatically.[59] FQD with an alternate cyclic aminal linkage is apparently more stable thermodynamically than the hemiaminal ether in FQC. One can explain these observations by Af12070 acting as an amine oxidase at C_α of the L-Ala_3 moiety incorporated into the 6,6,6 tricycle of FQF by the first enzyme in the pathway (the trimodular NRPS). The resultant imine is apparently captured kinetically by the −OH group that was introduced in the tandem epoxygenase/annulation reactions by enzymes two and three in the pathway. The shape change in going from FQA substrate to the heptacyclic FQC product is dramatic (Figure 9b). There must be a finite equilibrium between this heptacyclic hemiaminal ether FQC and that nascent imine product because attack by the amido nitrogen of the imidazoindolone ring then generates an alternate heptacyclic scaffold FQD, with an aminal linkage. In contrast to the FQC framework, the ring connecting the tricyclic pyrazinoquinazolinedione and imidazoindolone ring systems is now an eight member ring rather than the seven member ring in FQC. The four enzyme FQ pathway is breathtakingly efficient at building constrained three-dimensional molecular frameworks full of nitrogen heterocycles, all from the simple readily available starting materials Trp, Ala, and anthranilate.

An equivalent Ant-Trp-Ala tripeptidyl NRPS enzyme intermediate is the likely precursor of alantrypinone (Figure 10a), which accompanies FQF as a metabolite of *Penicillium verrucosum*.[60] The FQF scaffold could undergo oxidation of the indole substituent to the 2-indolone. Then, action by an Af10270-type of FAD amine oxidase would yield the equivalent imine in the pyrazinone ring. Intramolecular capture by the indolone C_3 carbanion (rather than −OH or −NH in FQC and FQD formation) would yield the new C−C bond in the hexacyclic

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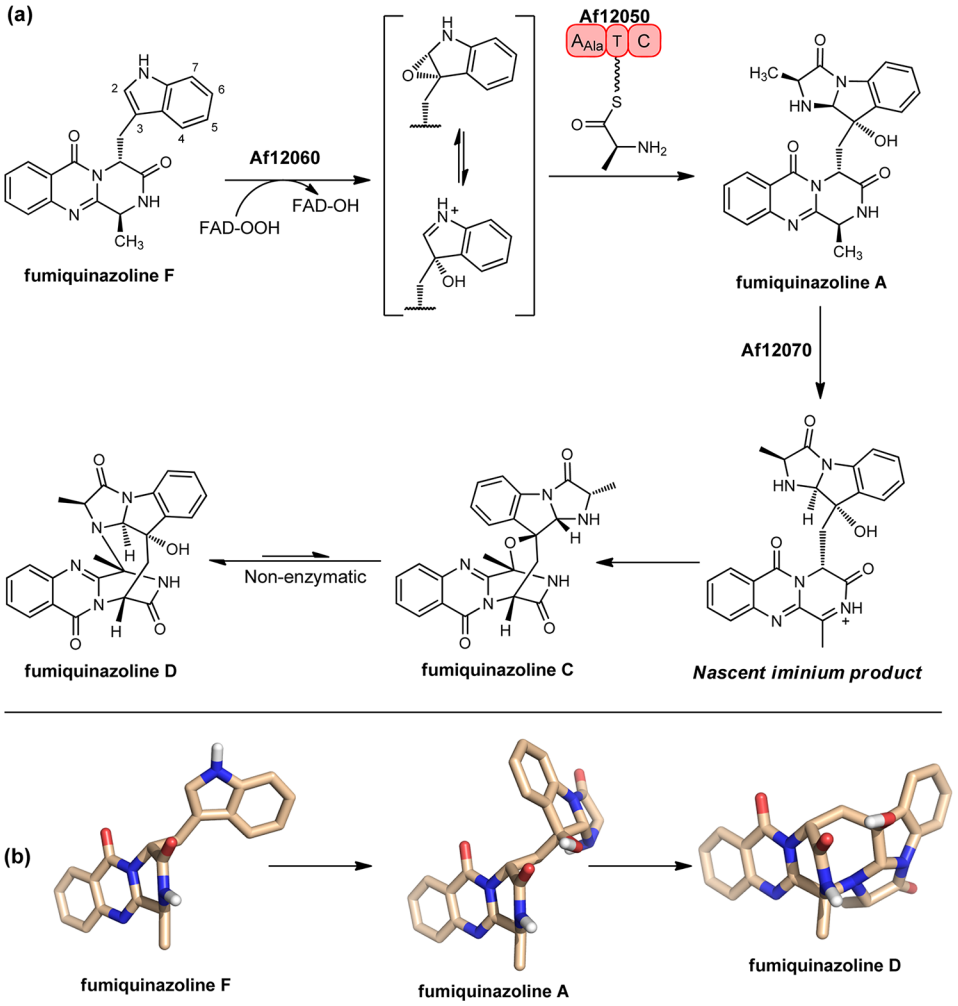

Figure 9. (a) Three-step pathway from FQF to FQC and subsequent equilibration to FQD. (b) Shape changes in FQ scaffold with sequential processing.

alanyltrypinone final product. A related serantrypinone (figure 10b) would arise from a comparable Ant-Trp-Ser-NRPS thioacyl enzyme intermediate in *Penicillium thymicola*.[61] Another related example is found in cottoquinazoline A from *Aspergillus versicolor*[62] that has a heptacyclic scaffold analogous to that in FQA.

4.2. Rerouting FQF to Tryptoquialanine Tremorgenic Toxins. Given that FQF is the first released product in the tryptoquialanine (TQA) pathway, the question arose as to when the scaffolds subsequently diverge. From identification of the TQA biosynthetic gene cluster,[54] there are two homologues to Af12060 and Af12050 in the TQA gene cluster, namely TqaH and TqaB. It was possible to show that the tailoring enzyme pair acted analogously but with one difference. They generated the 2-*epi* FQA, differing from FQA only in the stereochemistry of annulation at C_2 in the resultant imidazoindolone ring: the C_2 and C_3 now have *cis* rather than *trans* substituents (Figure 11a). That altered stereochemistry is controlled by the C domain in the single A-T-C module of TqaB.[22] Asperlicins and chaetominines, discussed in subsequent sections, have the *trans* 2,3-stereochemistry seen in the fumiquinazolines, while lumpidin[63] and fiscalins[64] instead have the *cis* stereochemistry, as seen in the tryptoquialanine scaffold, suggesting successful evolutionary dispersion of the two types of connectivities.

While Ala will work in the TqaB-mediated annulation the preferred substrate in the producing *P. aethiopicum* is aminoisobutyrate, yielding 15-dimethyl-2-*epi*-FQA as the intermediate (figure 11b).[54] The FAD-dependent oxidase TqaG makes a comparable imine in the pyrazinoquinazoline tricycle, in analogy to Af12070 action. However, this imine then undergoes ring-opening deamination to the keto acid that is proposed to undergo spirolactonization to yield the framework of dehydrotryptoquialanone.[54] The pathway concludes with enzymatic reduction of the ketone to the alcohol, acetylation of that alcohol, and N-hydroxylation of the original indole nitrogen.[54] Note that the oxidative cleavage of the pyrazinoquinazolinone by TqaG and subsequent spirolactonization to the dimethyl-hydroxy-imidazoindolone to create the core of the tryptoquialanine scaffold dramatically alters the framework from the advanced FQA and FQC metabolites.

4.3. Ant-Ala-Trp to Ardeemins. The ardeemins were originally isolated in activity screens in cancer cell lines overexpressing multidrug resistant (MDR) export pumps.[65,66] These tumor cells become resistant to chemotherapeutic drugs such as the antitumor vinca alkaloids by such pumping action. Ardeemins can bind to the MDR membrane proteins and resensitize some tumor cells to vinca alkaloids by up to 700 fold.[67] The hexacyclic natural products were named ardeemins based on this function: the **a**bility to **r**everse **d**rug insensitivity (Figure 12a). Inspection of ardeemin and its 15-N-acetyl derivative suggest features of both aszonalenin and fumiquinazoline assembly logic. Indole N-acetylation is presumably a last

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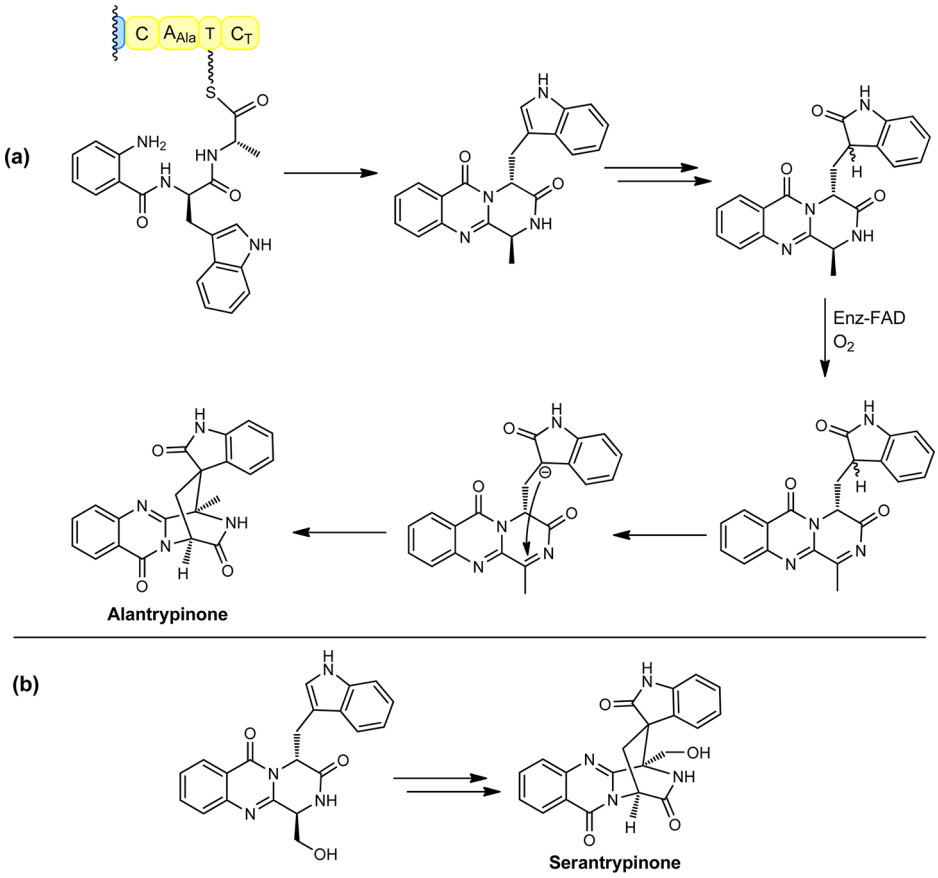

Figure 10. (a) Alanytrypinone formation from Ant-Trp-Ala-S-Enzyme intermediate. (b) Seranyltrypinone formation.

step, preceded by a ring-closing reverse prenylation at C_3 of an indole moiety, concomitant capture at C_2 by an amide nitrogen, generates the N−C bond as a five member ring forms, while the indole is converted to a tricyclic pyrroloindole functionality. The substrate for such a cyclizing prenylation would be a tricyclic 6,6,6-pyrazinoquinazoline (Figure 12b), reminiscent of the scaffold found in FQF, with one distinct difference. The substitution pattern on that 6,6,6-tricycle must have arisen from an Ant-D-Ala-L-Trp tripeptidyl-*S*-NRPS intermediate rather than the Ant-D-Trp-L-Ala-S-NRPS just discussed for Af12080 and TqaA.

With this retrobiosynthetic logic in mind, a producer *Aspergillus fischeri* strain was subjected to genomic sequencing.[55] The resultant bioinformatic analysis turned up one set of three adjacent genes that encoded such a trimodular NRPS, a prenyl transferase, and an acetyltransferase. Genetic disruption of the NRPS and the prenyl transferase validated their identity as the ardeemin gene cluster and that the product of the NRPS (ArdA) was indeed the 6,6,6-tricyclic pyrazinoquinazolinedione, termed ardeemin FQ (Figure 12b).[55] The mechanism of the double cyclization of the Ant-D-Ala-L-Trp-S-T_3 is likely the same as shown for TqaA or Af12080 (Figure 12c). The altered product regiochemistry arises from an evolutionary swap in the order of modules two and three from that seen in TqaA and FQF. Combinatorial biosynthesis could arise from such mixing and rematching of NRPS modules in an assembly line. Studies on the purified ardeemin prenyltransferase ArdB validated its cyclization activity as it converted ardeemin FQ to ardeemin and completed the hexacyclic framework of this class of natural products.[55] It is one ring more extended than the corresponding aszonalenins (6−7−5−5−6 array). The altered size of the second ring (6− vs 7−) in the ardeemin 6−6−6−5−5−6 array of rings is a consequence of a three module vs a two module NRPS assembly line that initiates with anthranilate.

4.4. Other Fungal Alkaloids from Related NRPS Assembly Lines. *Aspergillus clavatus* is known to make glyantrypine (Figure 13a) from anthranilate, tryptophan, and, as the third amino acid, the simplest of the proteinogenic amino acids glycine. There are two trimodular NRPS clusters (ACLA_076770 and ACLA_095980) in the *A. clavatus* genome that are predicted to activate anthranilate as chain-initiating building block, but it is not yet known if either is the glyantrypine cluster. The precedents noted in the previous sections suggest a simple trimodular NRPS and an Ant-Trp-Gly-S-enzyme intermediate, released by a double cyclization to glyantrypine without further tailoring.

Neosartorya fischeri strains produce fiscalins A, B, and C, isolated initially as ligands to human neurokinin receptor by displacement of radiolabeled substance P.[64] Fiscalin B is the simplest framework (Figure 13b) and clearly arises from an Ant-D-Trp-Val-tripeptidyl-NRS assembly line by the molecular logic presented (NFIA_057960). Fiscalin C would then result from tandem action of a flavoenzyme epoxygenase and a monomodular NRPS (NFIA_057990) activating aminoisobutyrate, such as the tryptoquialanine pathway) to yield the annulated dimethylimidazolone. The 2 and 3 substituents are *cis*, in analogy to the 2′-epi FQA produced in the tryptoquialanine pathway. The third metabolite fiscalin A differs from fiscalin C only in the use of D-Ala instead of AIB in the annulation step. Thus, there are intersections with both the FQ and TQA pathways. Other

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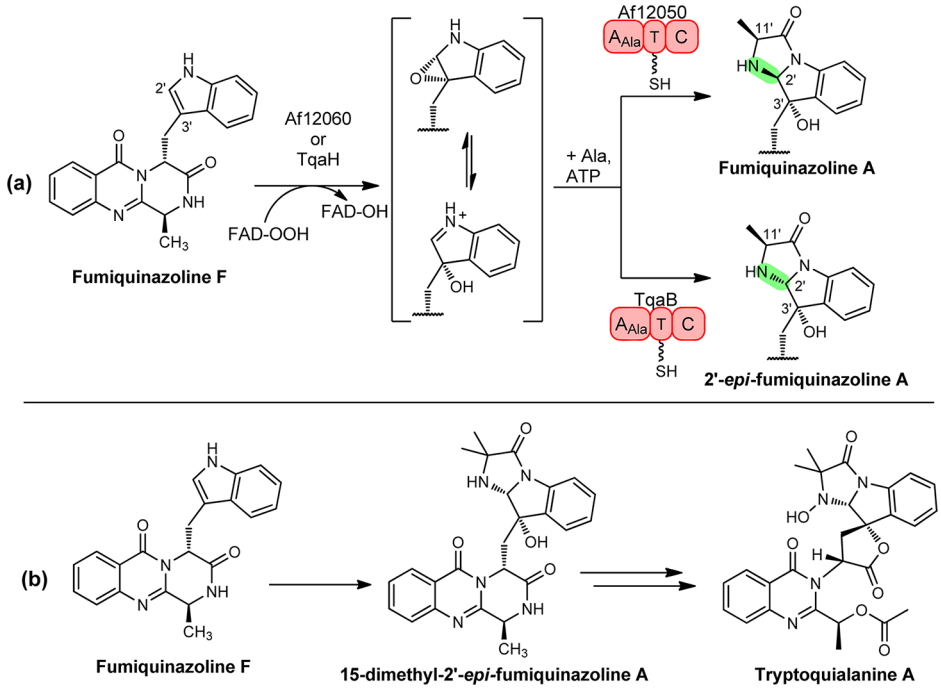

Figure 11. (a) FQF to 2′-*epi*-FQA; (b) 15-dimethyl-2′-*epi*-FQA formation and processing to tryptoquialanine.

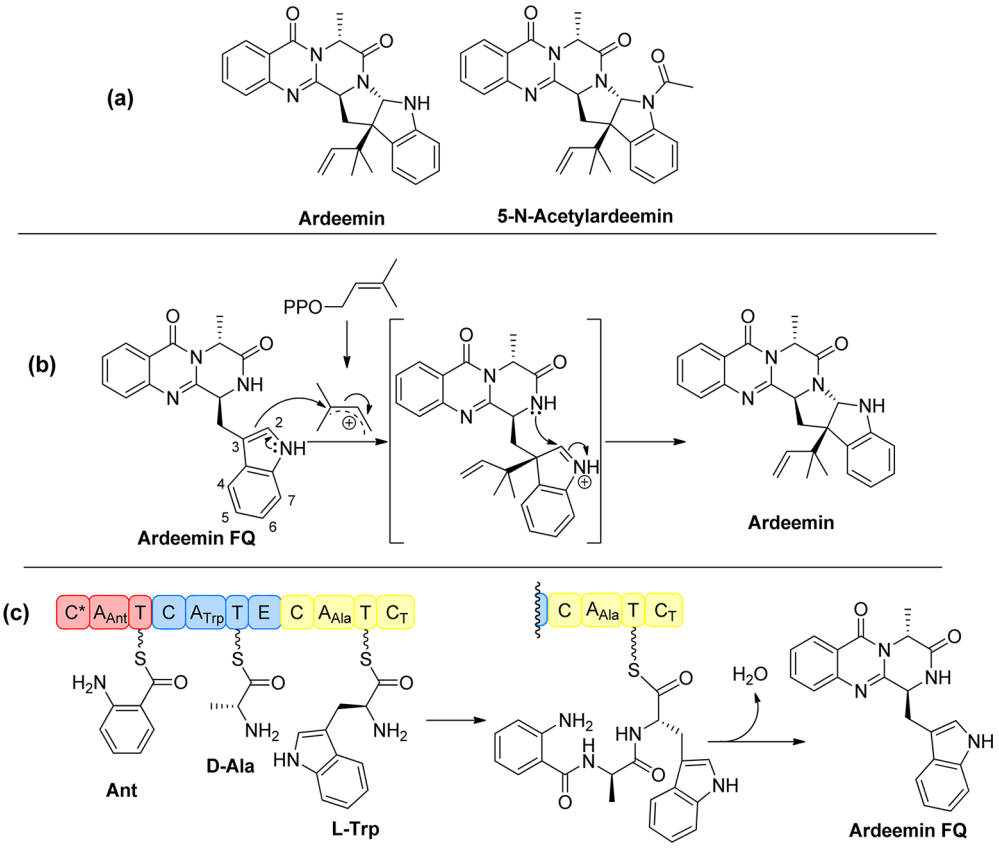

Figure 12. (a) Ardeemin family of metabolites. (b) Formation of ardeemin FQ. (c) Ardeemin FQ to ardeemin via prenyl transfer.

fiscalins have now been discovered: *epi*, *neo*, etc.[68] Verrucines A and B and anacine have been isolated from *Penicillium verrucosum* (Figure 13c).[69] They likely form via Ant-L-Gln-L-Phe-, Ant-L-Gln-D-Phe-, and Ant-L-Gln-D-Leu-thioester intermediates on related trimodular NRPSs with the same double cyclization/release process to yield the characteristic pyrazinoqinazolinone scaffold.

Chaetominine from an endophytic *Chaetomium sp.* (IFB-E015)[70] seems to offer two variations on the biosynthetic themes to date. It has a bicyclic quinazolinone ring, analogous to that in

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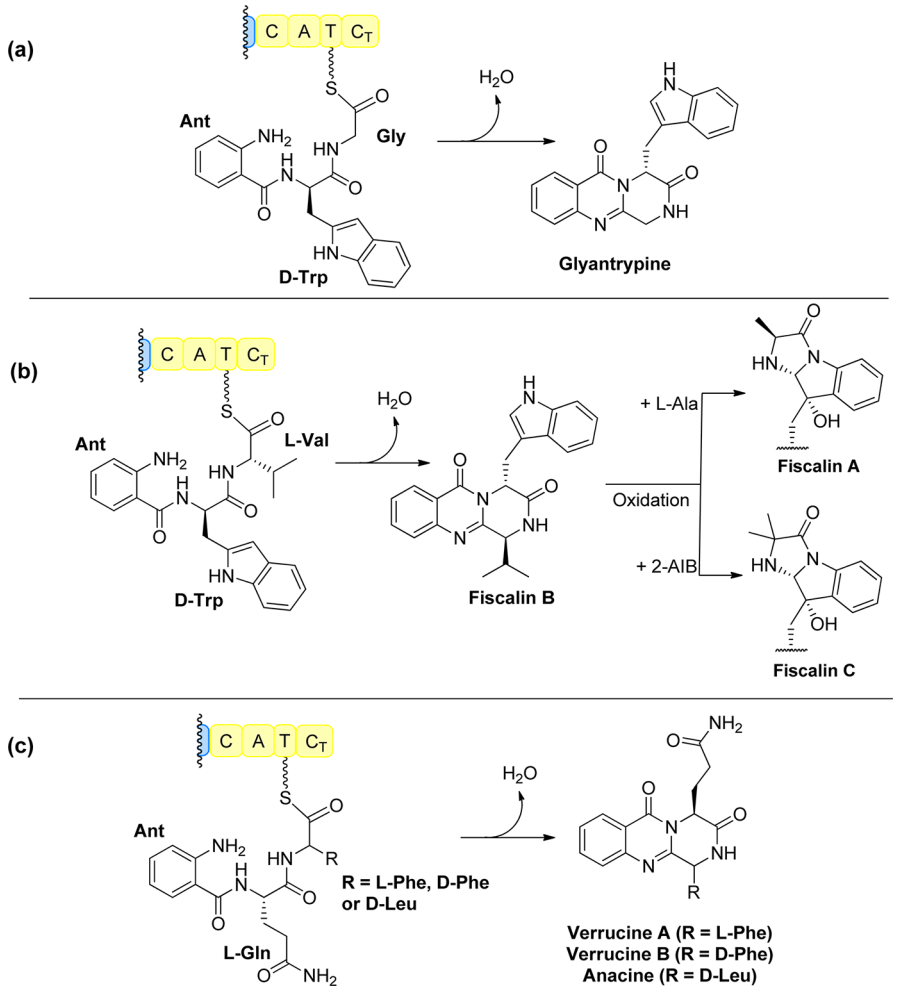

Figure 13. (a) Glyantrypine formation; (b) fiscalin B, A, and C; (c) verrucines A and B and anacine.

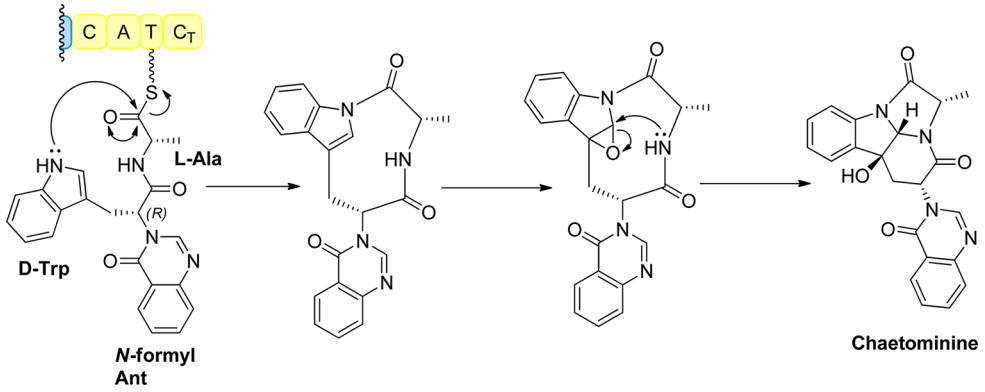

Figure 14. Chaetominine and proposed biosynthetic route.

the tryptoquialanine and tryptoquivaline frameworks and an angular 6−5−5−6 tetracyclic core. However, in the chaetominine case, it may be that N-formyl anthanilate rather than anthranilate is the starter unit in an N-formyl-Ant-Trp-Ala assembly line (Figure 14). Chain release is proposed by intramolecular attack of the indole nitrogen on the thioester bond. The indole side chain of the Trp residue could then be epoxidized by the kind of flavoprotein epoxygenase noted in previous examples. Now, the epoxide ring-opening/annulation could be intramolecular, with the amide nitrogen as nucleophile, rather than an incoming alanine, aminoisobutyrate, or valine group as in previous examples. The capture by the indole nitrogen would create a new fused 5,6-bicyclic ring as the tetracyclic core of chaetominine is assembled. Another route to tetracyclic core scaffolds is taken up in the next section.

5. TWO ANT RESIDUES IN A TRIPEPTIDYL-NRPS

A distinct subset of molecular frameworks in the anthranilate-derived fungal alkaloids includes asperlicins,[71−73] circumdatins,[74−79] benzomalvins,[80] and sclerotigenin[81] (Figure 15). Examination of their structures suggests they arise from tethered tripeptidyl intermediates on NRPS assembly lines that have two

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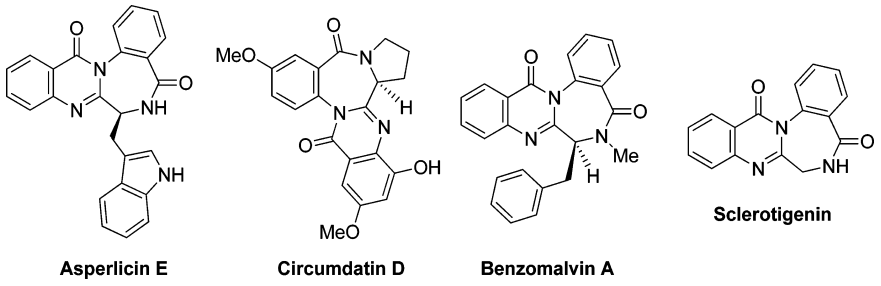

Figure 15. Asperlicins, benzomalvins, circumdatins, and sclerotigenin.

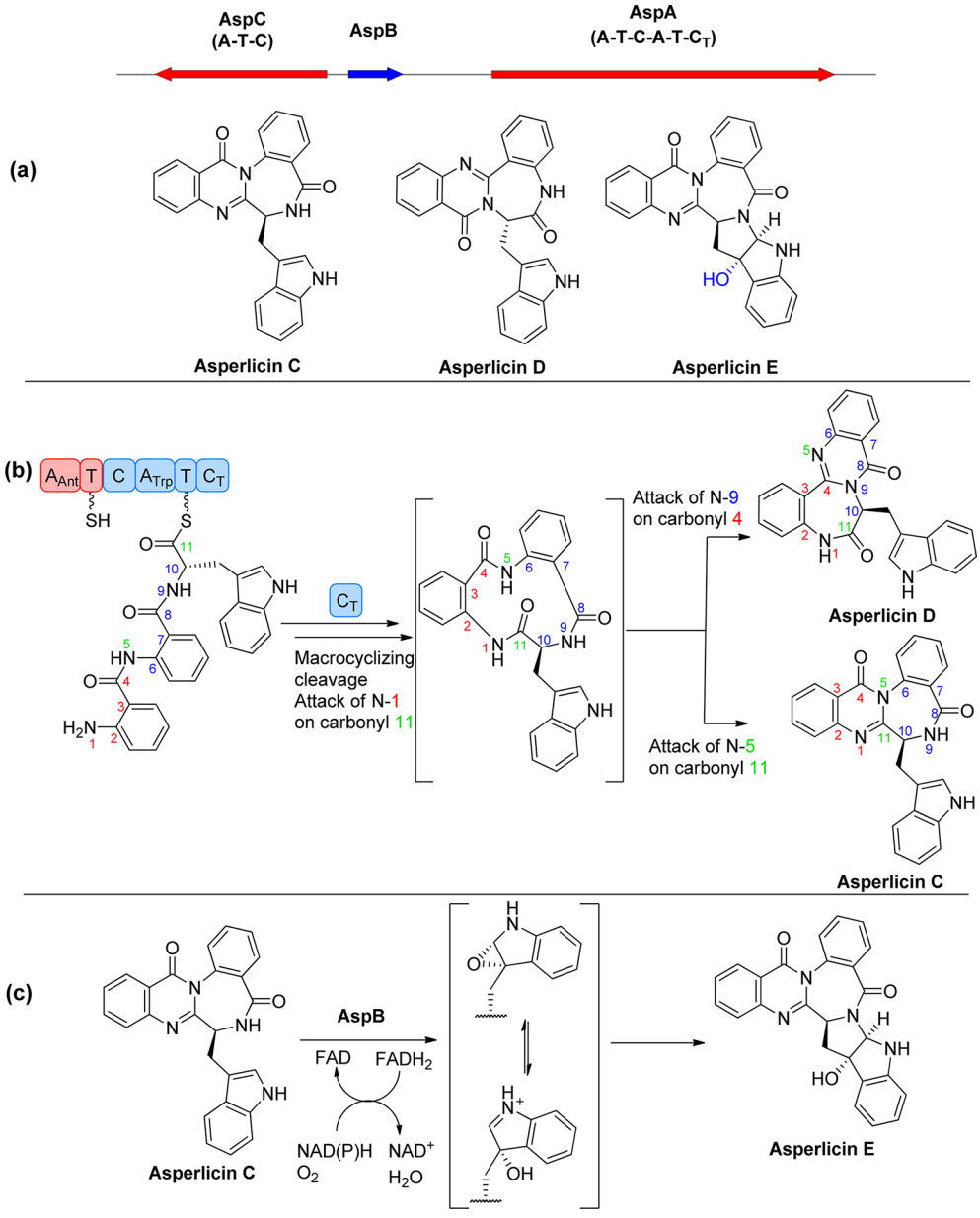

Figure 16. (a) Asperlicins C, D, E; (b) asperlicin C formation from Ant-Ant-Trp-S-Enzyme; (c) asperlicin E formation.

anthranilate building blocks as residues one and two. We noted that chain initiation with one anthranilate in a dipeptidyl NRPS assembly line releases 6,7-bicyclic benzodiazepinediones. Chain initiation with anthranilate in trimodular NRPS assembly lines yields the 6,6,6-tricyclic pyrazinoquinazolonediones. The tandem selection and incorporation of two anthranilates by the NRPS assembly lines below instead leads to release of an angular 6,6,7,6 tetracyclic scaffold.

5.1. Ant-Ant-Trp ⇒ Asperlicins C, D, E, and Asperlicin. To validate the predictions, we sequenced the genome of an *Aspergillus alliaceus* producer of the asperlicin metabolites asperlicin C, D, E and mature asperlicin (Figure 16a), looking

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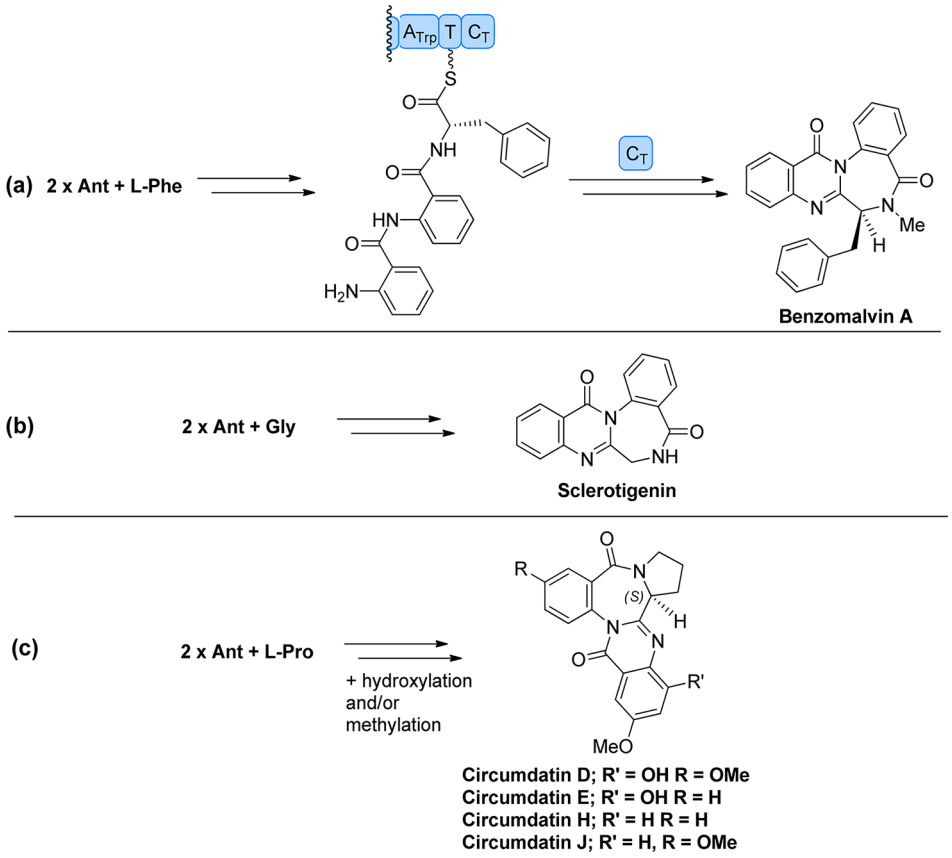

Figure 17. (a) Benzomalvin assembly; (b) sclerotigenein assembly; (c) circumdatins D, E, H, and J.

for candidate biosynthetic gene clusters.[56] Although we anticipated a trimodular NRPS, the most likely candidate turned out to be a bimodular NRPS, predicted to activate Ant in module one and Trp in module two. A predicted flavoprotein epoxygenase and monomodular NRPS were adjacent ORFs, the tailoring enzymes one would need to get to asperlicin. Inactivating insertion into the gene encoding the bimodular NRPS termed AspA confirmed that it was required for generation of asperlicins.[56] We suggest that module one activates anthranilate iteratively, generating an Ant-Trp-*S*-T_2 dipeptidyl enzyme while it reloads HS-T_1 with another anthranilate (Figure 16b). Chain elongation would then generate a tripeptidyl intermediate (Ant-Ant-Trp-) tethered as a thioester to T_2. Chain release by cyclization could then occur. If the free NH_2 on Ant_1 acts as nucleophile on the Trp_3 thioester carbonyl, a 6,11-bicyclic nascent product would be released. Transannular capture and dehydration could in principle proceed by two routes as shown. One gives aspericin C, the other asperlicin D. Both metabolites are seen in producer cultures. We believe asperlicin D is a dead end metabolite, reflecting an unwanted post-NRPS release cyclization pathway.

When the flavoenzyme AspB is purified from heterologous expression in *E. coli*, it works on asperlicin C but not asperlicin D.[56] The by now anticipated indole 2,3-epoxide formation could then be followed by intramolecular capture by the amide nitrogen in the 7-member diazepinedione ring of Asperlicin C. The result is the extended 6,6,7,6,5,5,6 heptacylcic framework of asperlicin E (Figure 16c). The underlined 5 member ring is the newly formed one that knits tetracyclic 6,6,7,6 core to the 5,6-bicyclic indole moiety. AspA and AspB comprise a spectacularly efficient two enzyme pathway for complexity generation.

5.2. Related Ant-Ant-X Derived Alkaloids: Benzomalvins, Circumdatins, Sclerotigenin. Comparable assembly line logic is presumed operant for benzomalvins.[80] (Figure 17a) In that series, an Ant-Ant-Phe- tethered tripeptidyl intermediate is anticipated (whether on a trimodular or again a bimodular NRPS assembly line is yet to be determined). Benzomalvin A has the same tetracyclic core as in asperlicins C and D, but the diazepinone ring has a side chain derived from L-Phe_3, and the amide nitrogen has been N-methylated, presumably in an S-adenosylmethionine-derived reaction by a tailoring methyltransferase. Analogously, an Ant-Ant-Gly-NRPS would be precursor to sclerotigenin (Figure 17b) and Ant-Ant-Pro- for circumdatins D, E, and H (Figure 17c). Given that Pro is a cyclic amino acid, these circumdatins have a pentacyclic framework. For circumdatin F, [75] the assembly line could begin with hydroxyanthranilate instead of anthranilate or the aromatic hydroxylations could occur after the polycyclic scaffold has been released from its generating NRPS assembly line.

Auranthine, a nephrotoxin isolated from *Penicillium aurantogriseus* (Figure 18)[82] appears to arise from two anthranilates and one glutamine building block but offers two new features. One is that the connectivity would suggest an Ant-Gln-Ant-NRPS intermediate, with Ant in the third rather than second position in contrast to all the examples noted. This could reflect evolutionary mixing of NRPS modules to create new scaffolds. The second feature of the benzodiazepindione-containing (6−7−6−6−6) scaffold of auranthine is that the regiospecificity of linkage of the building blocks is also unusual. It will require study with purified NRPS but a possible path would be for an Ant-Gln-*S*-T_2 to be captured by Ant-*S*-T_3 on the side chain carboxamide while the Gln thioester carbonyl is captured by the NH_2 of Ant.

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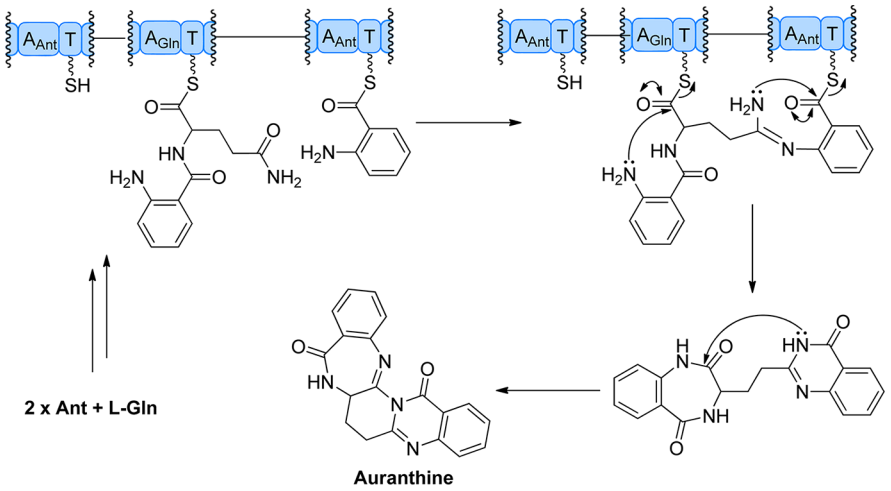

Figure 18. Auranthine, proposed biosynthetic route.

6. SUMMARY REMARKS

The fungal peptide alkaloids discussed here are a subset of a larger universe of fungal metabolomes but they are now well understood in terms of molecular logic and chemical strategies for assembly. A combination of fungal genomics, insertional mutagenesis, and *in vitro* assays of some of the heterologously expressed enzymes have led to clarity about the molecular assembly line machinery and the small number of chemical transformations by dedicated tailoring enzymes.

The core strategy for biosynthesisis starts with bi- or tri-modular NRPS enzymes acting in assembly line format. Anthranilate, a primary metabolic precursor of the proteinogenic amino acid L-tryptophan, is the signature building block for this natural product class. Each of the NRPS examined to date initiate dipeptidyl or tripeptidyl chain growth with anthranilate as non-canonical building block. The cyclization chemistry is enviably efficient, and they release 6,7-bicyclic scaffolds, 6,6,6-tricyclic scaffolds, and angular 6,6,7,6 tetracyclic scaffolds.

Then, a small number of tailoring enzymes are brought into play and carry out three kinds of maturation chemistry: oxygenations, alkylations (prenyl transfers), and (amino) acylations. This limited set of chemical transformations means that short (two to four enzyme) pathways account for production of fused polycyclic scaffolds ranging from bicyclic to heptacyclic rings.

In addition to the cyclization behavior of activated anthranilates on the bi- and tripeptidyl-NRPS enzyme intermediates, the directed reaction of the pyrrole ring of the indole side chain of a Trp unit with two kinds of electrophiles, FAD-OOH or dimethylallyl cation, serve as the second key reaction type in this peptidyl alkaloid class. Intramolecular capture of the 3-substituted indolyl cations at C_2 by amide nitrogens are complexity generating reactions used to build the final fused ring frameworks.

Combinatorial biosynthetic strategies from NRPS module exchanges, evolution of adenylation domains to distinct monomer selection, and mix and match tailoring with oxygenases, annulating enzymes, and prenyltransferases yield a diversity of mature frameworks and architectures with consequent differences in biological activities. The ability to search for and recognize anthranilate-activating adenylation domains of fungal NRPS modules should allow scanning of fungal genomes to see how far the ability to create these kinds of peptidyl alkaloid scaffolds has spread beyond *Neosartorya*, *Aspergillus*, and *Penicillium* fungal species and give some insight into potential utility of these molecules to producers in different niches The historical isolation of these fungal peptidyl alkaloids on the basis of their activities against vertebrates as tremorgens, CCK4 antagonists, MDR pump inhibitors, and cytotoxic agents may not be directly reflective of the roles they play in fungal life cycles.

AUTHOR INFORMATION

Corresponding Author

*E-mail: christopher_walsh@hms.harvard.edu.

Present Address

[‖]Novartis Institutes for Biomedical Research, Inc., 100 Technology Square, Cambridge, Massachusetts

Notes

The authors declare no competing financial interest.

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