Divergent pathways in the biosynthesis of bisindole natural products

Katherine S. Ryan¹ and Catherine L. Drennan¹*

¹Departments of Chemistry and Biology and the Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA
*Correspondence: Email: cdrennan@mit.edu; Phone: 617-253-5622; Fax: 617-258-7847

RUNNING TITLE Bisindole natural product biosynthesis

SUMMARY

Two molecules of the amino acid L-tryptophan are the biosynthetic precursors to a class of natural products named the ‘bisindoles.’ Hundreds of these bisindole molecules have been isolated from natural sources, and many of these molecules have potent medicinal properties. Recent studies have clarified the biosynthetic construction of six bisindole molecules, revealing novel enzymatic mechanisms and leading to combinatorial synthesis of new bisindole compounds. Collectively, these results provide a vantage point for understanding how much of the diversity of the bisindole class is generated from a small number diverging pathways from L-tryptophan, as well as enabling identification of bisindoles that are likely derived via completely distinct biosynthetic pathways.

Introduction

The rich chemical ecology of organisms such as microbes, fungi, and plants is due in large part to secondary metabolism (Austin et al., 2008). This secondary branch of biochemistry stands in contrast to the primary biochemical processes that enable organisms to convert organic molecules into chemical energy and to build up carbohydrates, lipids, nucleotides, and amino acids from simpler precursors (Dewick, 2001a). Secondary metabolism instead entails making
molecules, for instance, for defense and communication, often in response to changing environmental conditions. Secondary metabolites, or natural products, are characteristically diverse, with a cornucopia of new molecules isolated each year from natural sources. Further, while primary metabolites are often found throughout the kingdoms of life, constituting largely conserved biochemical processes, secondary metabolites can be highly restricted in their distribution, and are thought to play roles in enabling organisms to thrive in niche environments (Austin et al., 2008). Humans have used these secondary metabolites since pre-historical times for their own purposes, from mining organisms for medicines to gathering dye molecules and poisons.

One major class of secondary metabolites are alkaloids, which are nitrogen-containing molecules derived from amino acids or purines (Dewick, 2001b). Particularly infamous among alkaloids are those derived from L-tryptophan molecules, with notable examples including ergot alkaloids and the synthetic LSD, whose structure was inspired by the indole alkaloid D-lysergic acid. A more recently characterized class of L-tryptophan derived alkaloids consists of the ‘bisindoles’ (Table 1). These molecules, generated by the fusion of two molecules of L-tryptophan, contain two indole rings in their structures (structure of indole 1 in Figure 1A). The first reported ‘observation’ of a bisindole molecule was of violacein 21 in the 1880’s (Boisbaudran, 1882); the violacein molecule was purified (Strong, 1944) and synthesized by 1958 (Ballantine et al., 1958). Subsequent mining of microbial organisms for anti-cancer natural products led to the discovery of staurosporine 18 (Omura et al., 1977), a kinase inhibitor (Rüegg and Burgess, 1989), and, later, rebeccamycin 17 (Bush et al., 1987; Nettleton et al., 1985), analogues of which are DNA-topoisomerase I inhibitors (Bailly et al., 1997). More recently, terrequinone A 24, structurally related to antitumor, antiviral, and antidiabetic compounds
(Schneider et al., 2007), was isolated from a fungus dwelling in the rhizosphere of an Arizona desert plant (He et al., 2004), and molecules AT2433-A1 38 (Matson et al., 1989) and K252a 39 (Kase et al., 1986) and have been reported (Table 1). These six molecules represent the only bisindoles for which complete biosynthetic information is now available. They share the presence of two indole rings, but differ in how these indole rings are connected and in the types of chemical ornamentation, for instance prenylation, chlorination, hydroxylation, and glycosylation, that decorates their skeletons (Table 1).

Beyond these six molecules, hundreds of bisindole molecules have been isolated and characterized (Higuchi and Kawasaki, 2007), but the mechanisms of their biosyntheses are largely unexplored. Bisindoles have been isolated from tunicates, sponges, plant leaves, roots, bark, and flowers, algae, fungi, and myxomycetes (Higuchi and Kawasaki, 2007), and are most commonly isolated from actinomycetes (Higuchi and Kawasaki, 2007), a class of bacteria known for production of secondary metabolites (Piepersberg, 1994). Although there are many reports on bisindole molecules, the focus of research is clear: most scientists are interested in how bisindoles can be used as drug molecules. They work to synthesize derivatives, test medicinal properties, and isolate new molecules from natural sources (Higuchi and Kawasaki, 2007; Prudhomme, 2004). Hence, despite the large interest in bisindoles, there is a paucity of studies on why organisms make bisindoles at all, which may represent a rich area for study in the next years.

In this Review, we discuss the known, diverging pathways that lead from L-tryptophan molecules to bisindoles. We first discuss L-tryptophan as a biosynthetic precursor. We then turn our attention to the biosynthetic pathways which are now well-understood, starting with a discussion of a major class of bisindoles, the indolo[2,3-a]pyrrolo[3,4-c]carbazoles 4 (Figure
1D), which includes rebeccamycin 17, AT2433-A1 38, staurosporine 18, and K252a 39, and then turn to a discussion of a second class consisting only of violacein 21 and a third class, the bisindolylquinones, which includes terrequinone A 24 (Table 1). Our focus in these sections is on the enzymes used to make bisindoles. One recurring theme that we find is that these biosynthetic enzymes have evolved to utilize, stabilize, and control the inherent reactivity of bisindole intermediates. Simply put, a key to bisindole biosynthesis is the prevention of spontaneous decomposition of reactive intermediates to undesired side products and the coordination of enzymatic activities to shuttle highly reactive species to more stable biosynthetic intermediates on route to the desired natural products. Using principles discovered from investigation of these three classes of bisindoles, we then discuss some of the hundreds of bisindoles whose biosynthesis is still unexplored, and postulate routes for construction of some of these molecules. Finally, we reflect on major questions that still remain about indolo[2,3-a]pyrrolo[3,4-c]carbazole, violacein, and bisindolylquinone biosynthetic pathways, and on future directions of research for biochemists and microbiologists.

**L-tryptophan as a biosynthetic building block**

Like other indole alkaloids (O'Connor and Maresh, 2006), naturally occurring bisindoles are generally thought to be derived from the amino acid L-tryptophan (Sánchez et al., 2006b), and this assumption has indeed proven true for all characterized bisindole biosynthetic pathways. L-tryptophan provides an excellent scaffold for dimerization and derivatization, and consequent production of bisindole molecules which, via distinct chemical properties, give rise to distinct biological properties.
The pathways described in this Review show how enzymes take advantage of the inherent reactivity of L-tryptophan to generate divergent natural products. One feature of the indole ring of L-tryptophan (Figure 1A and F) is the potential reactivity of the C2, as well as the C4, C5, C6, and C7, carbons in electrophilic aromatic substitution reactions (Figure 1F). Enzymatic derivatization, including chlorination, hydroxylation, and prenylation, occurs at a subset of these carbons in each of the biosynthetic pathways described in this Review.

Oxidation of L-tryptophan to either indole-3-pyruvate imine or to indole-3-pyruvic acid increases its reactivity, and this strategy is used in making each of the three classes of compounds described in this Review. In both the indolo[2,3-α]pyrrolo[3,4-c]carbazole and the violacein pathways, conversion to indole-3-pyruvate imine generates a substrate suitable for an enzymatically catalyzed oxidative dimerization of two indole-3-pyruvate imine molecules at the Cβ carbons (Figure 1G). This intermediate 10 (Figure 1H) can spontaneously collapse, giving rise to chromopyrrolic acid 11 (Figure 1I), a precursor suitable for a series of subsequent enzymatic transformations (Figure 1I and J). Alternately, as in the violacein pathway, the same intermediate 10 can break down through an alternate route. Instead of formation of a pyrrolo ring, a cyclopropyl ring can form and collapse (Figure 1K), with the process driven by a decarboxylation. This alternate pathway leads to the [1,2]-migration of an indole ring, generating a new, rearranged carbon skeleton and a violacein precursor.

Additional opportunities for dimerization and cyclization emerge via formation of indole 3-pyruvate from L-tryptophan. As exemplified by the terrequinone A biosynthetic cluster, tethering of the carboxylate of indole-3-pyruvate as a thioester on a carrier protein generates an electrophile at the carbonyl carbon, which can be subjected to nucleophilic attack by another
indole-3-pyruvate molecule (Figure 1L). Hence, the rich chemistry of L-tryptophan provides a template for production of a diversity of bisindole natural products.

**Indolo[2,3-a]pyrrolo[3,4-c]carbazoles**

The most frequently isolated bisindole molecules are indolocarbazoles (structure of carbazole 2 in Figure 1B), and, among indolocarbazoles, the most common are indolo[2,3-a]carbazoles 3 (Figure 1C). Among this subgroup, the most widespread are indolo[2,3-a]pyrrolo[3,4-c]carbazoles 4 (Figure 1D). Dimerization of L-tryptophan molecules via their Cβ carbons gives rise to a connectivity that favors formation of this core indolo[2,3-a]pyrrolo[3,4-c]carbazole structure, which may explain its prevalence (Figure 1G-I). Recently, gene clusters encoding proteins needed to biosynthesize four indolo[2,3-a]pyrrolo[3,4-c]carbazoles – rebeccamycin 17, AT2433-A1 38, staurosporine 18, and K252a 39 – have been isolated and characterized (Table 1). Rebeccamycin and AT2433-A1, on one hand, and staurosporine and K252a, on the other, represent two types of indolo[2,3-a]pyrrolo[3,4-c]carbazoles. Rebeccamycin and AT2433-A1 are chlorinated, contain a fully oxidized C-7 carbon (numbering, Figure 1D), and bear a β-glycosidic bond to only one indole nitrogen. Staurosporine and K252a, by contrast, are not halogenated, contain a fully reduced C-7 carbon, and contain a sugar attached to both indole nitrogens (Table 1). Three parts of the biosynthetic pathways are thus distinct between the two groups: (1) halogenation, (2) oxidation of the C-7 carbon, and (3) glycosylation. Because the rebeccamycin and staurosporine biosynthetic pathways are the best studied, only details for these pathways will be reviewed here (see pathways in purple, red, and blue in Figure 2), although initial information about the molecular details of AT2433-A1 and K252a construction is now available (Gao et al., 2006; Kim et al., 2007).
Chlorination of L-tryptophan is the first step in rebeccamycin biosynthesis, however, the presence of a chlorine atom is apparently not essential to substrate recognition in later steps in rebeccamycin biosynthesis, as des-chloro substrates are utilized by all of the downstream rebeccamycin biosynthetic enzymes (Sánchez et al., 2005). Additionally, many of the subsequent enzymes in the pathway are also able to accept alternately halogenated substrates, however, yields are lower with such modifications (Sánchez et al., 2005). In rebeccamycin biosynthesis, chlorination occurs via the FADH$_2$-dependent halogenase RebH, with FADH$_2$ generated by the partner flavin reductase, RebF (Figure 2) (Yeh et al., 2005). The crystal structure of RebH reveals that there are two active sites in RebH: one, where FADH$_2$, Cl$^-$, and O$_2$ bind, and ~11 Å away, a second site where L-tryptophan binds. Occluding the tunnel between the active sites is the conserved amino acid lysine-79 (Figure 3A) (Bitto et al., 2008; Yeh et al., 2007). Biochemical analysis of RebH suggests that chlorination occurs in two kinetic phases (Yeh et al., 2006), with generation of a stable intermediate on RebH. It has been proposed that reduced flavin reacts with oxygen and then a chloride ion, to generate hypochlorite (HOCl). Based on the stability of the intermediate in the protein, with a half-life of 28 h at room temperature (Yeh et al., 2007), but the rapid decomposition of HOCl in solution, it has been suggested that RebH may use lysine-79 to generate a chloramine intermediate on its terminal $\varepsilon$-NH$_2$. This chloramine intermediate would then act as a transferring agent to bring HOCl generated from FADH$_2$ in the first reaction to L-tryptophan bound at a distant site in the protein (Yeh et al., 2007). Structural and mechanistic studies of the highly related tryptophan chlorinase PrnA have also provided considerable insight into RebH, and led to suggestion of an alternate reaction mechanism (Dong et al., 2005; Flecks et al., 2008). RebH provides an example of the interesting chemistry one finds in the bisindole biosynthetic pathways.
RebO, the next enzyme in the pathway, uses FAD to convert 7-chloro-L-tryptophan to its corresponding imine, with a 57-fold greater $k_{cat}/K_m$ for 7-chloro-L-tryptophan than for L-tryptophan itself (Nishizawa et al., 2005). Identification of 7-chloro-L-tryptophan as the preferred substrate suggests that RebO provides a control point between primary and secondary metabolism by utilizing a non-proteogenic amino acid as a substrate (Nishizawa et al., 2005). StaO, the corresponding enzyme from the staurosporine pathway (Onaka et al., 2002), is likely to carry out an identical reaction, but its physiological substrate is L-tryptophan, and StaO is not likely to provide such a control point between primary and secondary metabolism.

The indole-3-pyruvate imine 8 generated by RebO, which is not stable in solution, is thought to be channeled to the next enzyme RebD, which is a ~113 kDa protein and putative dimer that contains three equivalents of iron, with two as heme iron (Howard-Jones and Walsh, 2005). Isotopic labeling experiments demonstrated that the product of RebO, indole-3-pyruvate imine, rather than its decomposition product, indole-3-pyruvic acid, is indeed the preferred substrates for RebD (Howard-Jones and Walsh, 2005). RebD’s use of the reactive substrate indole-3-pyruvate imine is an example of a bisindole biosynthetic enzyme that stabilizes and channels a reactive intermediate to a desired product. It is thought that RebD, as well as its functional homologues, StaD, from the staurosporine pathway, and VioB, from the violacein pathway, acts to install a carbon-carbon bond between the two Cβ carbons of two molecules of indole-pyruvate imine, generating the Cβ benzylically coupled iminophenylpyruvate dimer 10, with spontaneous chemistry resulting in production of chromopyrrolic acid 11, the final product and substrate for subsequent enzymes (Balibar and Walsh, 2006; Howard-Jones and Walsh, 2005; Nishizawa et al., 2006).
Both StaP and RebP, enzymes with 54% identity from the staurosporine and rebeccamycin biosynthetic pathways, respectively, are functionally equivalent cytochrome P450 enzymes that react with chromopyrrolic acid \( \text{11} \) (Sánchez et al., 2005). Only one of the two enzymes, StaP, has been studied extensively, but all results are thought to extend to its homologue, RebP. The crystal structure of StaP (Figure 3B) shows the bound substrate chromopyrrolic acid in the active site of StaP with its indole rings turned away from one another (Makino et al., 2007). The orientation of chromopyrrolic acid \( \text{11} \), which appears to be ‘locked’ into place by extensive interactions with the StaP active site, strongly suggests that the reaction occurs at the C10 carbon of chromopyrrolic acid. The heme iron and the C10 atom are 4.7 Å apart (Makino et al., 2007), a distance that is within range of substrate-iron distances in other cytochrome P450 enzymes (Cupp-Vickery and Poulos, 1995; Poulos et al., 1985). By contrast, the carboxylates are > 8Å from the heme iron, which is too far for interaction (Makino et al., 2007). A proposed reaction scheme involves generation of two indole cation radicals, with subsequent steps enabling aryl-aryl coupling to occur between the two C2 carbons of the indole rings of chromopyrrolic acid (Makino et al., 2007).

StaP must also be considered collectively with downstream enzymes RebC, from the rebeccamycin pathway, and StaC, from the staurosporine pathway. Co-incubation of StaP with StaC results in formation of the 4-electron oxidation product, the staurosporine aglycone \( \text{12} \), and co-incubation of StaP with RebC results in formation of the 8-electron oxidation product, the rebeccamycin aglycone \( \text{13} \) (Howard-Jones and Walsh, 2006). While both enzymes RebC and StaC contain sequence homology to flavin-dependent oxygenases, only RebC is co-purified with FAD (Howard-Jones and Walsh, 2006). The crystal structure of RebC, solved with a decomposition product of aryl-aryl coupled chromopyrrolic acid trapped in the active site, shows
flavin in an appropriate position to carry out flavin-based chemistry on the C7 carbon (Figure 3C). The intermediate trapped in the RebC active site decomposes within 30 min in solution (Howard-Jones and Walsh, 2007), yet a RebC crystal is able to stabilize the same molecule over a week-long time span, suggesting the role of RebC is to stabilize a reactive intermediate as well as to carry out oxidative chemistry at the C7 carbon (Ryan et al., 2007). StaC, which is purified without FAD (Howard-Jones and Walsh, 2006), may be an enzyme that acts without flavin-based hydroxylation chemistry and primarily acts in stabilizing reactive intermediates. In any case, stabilization of reactive intermediates is likely a key function of both enzymes.

After formation of the rebeccamycin or staurosporine aglycones, further modification occurs via glycosylation and methylation. In the rebeccamycin pathway, a β-glycosidic bond is installed between one indole nitrogen and a molecule of glucose by the enzyme RebG (Figure 2) (Zhang et al., 2006). Subsequently, the glucose is methylated by RebM (Zhang et al., 2006). RebG exhibits flexibility in the indoles it can glycosylate, and RebM exhibits flexibility in the sugars it can methylate (Zhang et al., 2006). In the staurosporine pathway, glucose is converted via the action of enzymes StaA, StaB, StaE, StaJ, Stal, and StaK to NTP-L-ristasamine (Onaka et al., 2002; Salas et al., 2005). L-ristasamine is then installed on the staurosporine aglycone at one indole nitrogen by the action of StaG; re-orientation of the sugar into the unfavored 1C4 conformation is followed by attachment to the second indole nitrogen by the action of StaN (Figure 2) (Salas et al., 2005). Both StaG and StaN are able to use alternate sugars as substrates, suggesting flexibility in substrate specificity (Salas et al., 2005). Following attachment, O-methylation at the 4'-amine of L-ristasamine and N-methylation at the 3'-hydroxyl of L-ristasamine occurs by the action of StaMA and StaMB, respectively (Salas et al., 2005). More details on sugar biosynthesis and attachment are available in an earlier review of bisindole
biosynthesis (Sánchez et al., 2006b). Production of rebeccamycin in an *E. coli* host has also been described (Hyun et al., 2003).

**Violacein**

Although the violacein (Table 1) biosynthetic operon was originally annotated as a four-gene cluster (VioABCD) in a number of separate studies (August et al., 2000; Brady et al., 2001; Brazilian National Genome Project Consortium, 2003), it was later discovered that an additional gene, VioE, is necessary for pigment production (Balibar and Walsh, 2006; Sánchez et al., 2006a). Recent studies have shown that VioA is the first enzyme in the five enzyme violacein biosynthetic pathway (see purple and green pathways in Figure 2) (Balibar and Walsh, 2006; Sánchez et al., 2006a). VioA, which has been shown to be functionally equivalent to RebO and StaO from the rebeccamycin and staurosporine biosynthetic pathways (Sánchez et al., 2006a), is a flavin-dependent L-amino acid oxidase, converting L-tryptophan to indole-3-pyruvate imine (Balibar and Walsh, 2006). VioB, the second enzyme in the violacein biosynthetic pathway, is a close homologue of RebD and StaD (Balibar and Walsh, 2006), suggesting a relationship, as for VioA, with indolo[2,3-α]pyrrolo[3,4-c]carbazole biosynthetic pathways. Multiple studies have established that this sequence homology translates to functional equivalence, and the combination of VioA and VioB results in the same product (chromopyrrolic acid 11) as the combination of RebO and RebD (Balibar and Walsh, 2006; Sánchez et al., 2006a). Additionally, incubation of RebO, RebD, and later enzymes of the violacein pathway – VioCDE – results in violacein production (Sánchez et al., 2006a), further demonstrating the equivalence of VioAB with RebOD, and suggesting a close evolutionary relationship between the two pathways. However, production of chromopyrrolic acid 11 by VioAB is not on pathway to violacein.
Chromopyrrolic acid 11 is a shunt product and not a precursor to violacein (Balibar and Walsh, 2006; Sánchez et al., 2006a). A filtration-HPLC assay established that VioE, the next enzyme in the pathway acts on a yet-uncharacterized intermediate produced by VioB to generate prodeoxyviolacein 19 (Balibar and Walsh, 2006). Based on the ability of VioE to produce prodeoxyviolacein 19 from the VioB product, a likely reaction for both VioB and RebD to catalyze is the dimerization of two molecules of indole-3-pyruvate imine to give the Cβ-Cβ benzylically coupled iminophenylpyruvate dimer 10, a molecule which can spontaneously decompose to chromopyrrolic acid 11 in the absence of VioE. If the coupled iminophenylpyruvate dimer is indeed the substrate of VioE, then VioE is required to mediate a 1,2 shift of an indole ring, closure of the pyrrolo ring, two decarboxylations, and additional oxidative chemistry (Figure 2). Despite carrying out these many reactions, VioE, a protein with few sequence homologues, lacks any detectable cofactors or metals, as judged by biochemical (Balibar and Walsh, 2006) as well as subsequent crystallographic analysis (Hirano et al., 2008; Ryan et al., 2008). The crystal structures of VioE reveal a dimeric, 11-stranded antiparallel β sheet. Two sets of site-directed mutagenesis experiments demonstrate six residues in the active site that are important, but not essential, to catalysis (Figure 3D), based on the decrease in product formation upon mutation of any of these residues to alanine. From crystallographic analysis, it appears that VioE may function by orienting a substrate in the cleft of its surface, such that steric effects promote production of prodeoxyviolacein and suppress formation of the shunt product chromopyrrolic acid 11 (Hirano et al., 2008; Ryan et al., 2008).

The product of VioE-mediated catalysis, prodeoxyviolacein 19, is in turn the substrate for VioD, a flavin-dependent enzyme. VioD hydroxylates prodeoxyviolacein in the presence of NAD(P)H, generating proviolacein 20 (Balibar and Walsh, 2006). VioC, another flavin-
dependent enzyme that requires NAD(P)H for activity, is then responsible for oxygenation in the oxindole ring. Although VioC is able to react with either prodeoxyviolacein 19 (the VioE product) or proviolacein 20 (the VioD) product, VioD is only able to react with prodeoxyviolacein 19 (the VioE product), suggesting that VioD acts before VioC in the biosynthetic pathway (Balibar and Walsh, 2006). This pathway order is also consistent with \textit{in vivo} results (Sánchez et al., 2006a).

**Bisindolylquinones**

Terrequinone A (Table 1), the only bisindoylquinone for which biosynthetic information is available, was first isolated from the fungus \textit{Aspergillus terreus} residing in the rhizosphere of canyon ragweed (\textit{Ambrosia ambrosioides}) from the Tucson Mountains of Arizona (He et al., 2004). One cluster of five genes was identified from the related species \textit{Aspergillus nidulans}, and found to encode five enzymes, TdiABCDE, which were shown to biosynthesize terrequinone A (Bok et al., 2006).

The first step in terrequinone A construction (see pathway in black in Figure 2) is the pyridoxal-5’-phosphate (PLP)-dependent reaction of TdiD with L-tryptophan, generating indole pyruvic acid 9 (Balibar et al., 2007; Schneider et al., 2007). The co-substrate is phenylpyruvic acid (Schneider et al., 2007). This first biosynthetic step immediately separates terrequinone A biosynthesis from indolocarbazole and violacein biosynthesis, as these pathways use indole-3-pyruvate imine 8 as a precursor, rather than indole-3-pyruvic acid 9. One key chemical issue in production of indole pyruvic acid (as for the imine) is its instability. However, TdiA, the next enzyme in the terrequinone A pathway reacts with indole-3-pyruvic acid with a $K_M$ of 1.2 ± 0.3
µM, suggesting that the instability of the indole-3-pyruvic acid produced by TdiD is countered by the high affinity for this molecule by TdiA (Balibar et al., 2007).

TdiA represents a novel enzyme. Bioinformatic analysis suggests that TdiA is a non-ribosomal peptide synthetase (NRPS) (Figure 4A) with the following three domains: adenylation (‘A’), thiolation (‘T’), and thioesterase (‘TE’). However, TdiA has many unusual features, and since it does not catalyze the formation of peptide bonds, it may be appropriate to term this type of enzyme a ‘quinone synthetase,’ rather than an ‘NRPS.’ Also, in contrast to most NRPS enzymes, it is a single module, lacks the canonical condensation (or replacement cyclization) domain, and may use a new mechanism of release from the thioesterase domain. A new mechanism of release from the thioesterase domain is needed because terrequinone A lacks an acid, amide, or ester group that would be generated by a hydrolysis or cyclization reaction on the thioesterase (Figure 4A) (Balibar et al., 2007). Additionally, its putative substrate is not, as is typical, an amino acid, but instead is an α-keto acid (Figure 4B).

The holo-enzyme TdiA, which has a phosphopantetheinyl arm loaded on the T domain (indicated by squiggly line attached to -SH in Figure 4), produces the bisindole product, didemethylasterriquinone D 22 from two molecules of indole-3-pyruvic acid (Balibar et al., 2007; Schneider et al., 2007). Both the T and the TE domains of TdiA are necessary for this activity, as neither an unloaded T domain with an active TE domain, nor a TE domain with a mutation at a conserved serine with an active T domain, produce didemethylasterriquinone D 22 (Balibar et al., 2007). With two molecules of indole-3-pyruvate loaded onto TdiA (one at the phosphopantetheinyl arm of the T domain, and the other at the serine of the TE domain), a plausible reaction scheme involves two mixed Claisen condensations to give rise to the final product (Figure 4B). This type of condensation reaction is a yet-unknown chemistry for NRPS
domains and represents the first reported NRPS-like protein that can catalyze carbon-carbon bond formation (Balibar et al., 2007; Schneider et al., 2007). The ability of TdiA to dimerize two indole pyruvate molecules by tethering them on adjacent T and TE domains of a single module enzyme explains both the need for only one NRPS-like module, as well as the lack of amide, ester, or acid linkages connecting two indole pyruvate molecules in the final product didemethylasterriquinone D 22.

Subsequent enzymes in the biosynthetic pathway have been shown to be even more unusual than TdiA. While these final three enzymes, TdiC, TdiB, and TdiE, are able to generate terrequinone A 24 from didemethylasterriquinone D 22 in a reaction mixture containing NAD(P)H, dimethylallyl pyrophosphate (DMAPP), and divalent metal ions, none of the three enzymes are able to react with didemethylasterriquinone D 22 alone (Balibar et al., 2007). TdiC, a putative NADH-dependent oxidoreductase, is proposed to transfer a hydride to didemethylasterriquinone D 22, converting this compound into an electron-rich nucleophile. TdiC, however, is likely to work in concert with TdiB and TdiE, and together these enzymes generate the mono-prenylated compound, ochrindole D 23. TdiB is thought to be the prenyltransferase, and TdiE is thought to impart specificity. Without TdiE present, an off-pathway prenylation occurs at one of the hydroxyls; with TdiE present, however, only ochrindole D 23 is observed. TdiB is then able to react again, on its own, with ochrindole D 23 to install a second prenyl group, generating terrequinone A 24 (Balibar et al., 2007).

One of the most fascinating features of TdiB is its ability to install prenyl groups at two unique sites, at one site through a ‘regular’ prenylation reaction and at the other site through a ‘reverse’ prenylation reaction, while avoiding prenylation at near-identical sites elsewhere on the didemethylasterriquinone 22 scaffold. That is, prenylation occurs at C5 of the quinone core and
C2 of an indole ring (2'), but not at C2 of the quinone core or C2 of the other indole ring (2'"")
(numbering, Figure 1E). Since 22 is symmetric prior to prenylation, this differentiation must
occur after prenylation. How a single prenyltransferase can specifically catalyze a prenylation on
two unique sites of the same molecule, and avoid prenylation on near-equivalent sites, is still
unknown.

It should be noted that two groups came to distinct conclusions regarding the role of TdiB
in the biosynthesis of terrequinone A. While both groups agree that TdiB catalyzes a prenylation
at the C2 of one indole carbon, they disagree on whether the substrate is
didemethylasterriquinone 22 or ochrindole 23. One group showed data to suggest a single-
enzyme mediated prenylation of didemethylasterriquinone D 22 at the C2 of one indole carbon
(Schneider et al., 2008), but the other group was unable to observe a reaction between TdiB and
didemethylasterriquinone 22. Instead, they observed that TdiB could carry out the same
prenylation reaction on the monoprenylated derivative, ochrindole D 23, generating the bisindole
terrequinone A 24 (Balibar et al., 2007). Despite the differences in observed activity, it is clear
that only one prenylation, on the C2 of one indole ring, occurs by the action of TdiB alone
(Balibar et al., 2007; Schneider et al., 2008), and the presence of TdiB, TdiC, and TdiE does
allow prenylation at the C5 of the quinone core to occur (Balibar et al., 2007). These results
suggest a complicated and yet-unelucidated relationship between TdiB, TdiC, and TdiE.

Other bisindole molecules

The studies described in this Review have revealed the biosynthetic steps that lead to
construction of the indolo[2,3-a]pyrrolo[3,4-c]carbazole core, the violacein skeleton, and the
bisindolylquinone skeleton. A number of related molecules may be biosynthesized through
similar pathways, and it is worthwhile to consider the similarities and differences that are likely
to exist between the biosynthesis of rebeccamycin 17, AT2433-A1 38, staurosporine 18, K252a
39, violacein 21, and terrequinone A 24 and other, related molecules. Based on the variety of
bisindole molecules isolated from nature, however, it is also clear that some bisindole molecules
cannot be understood in terms of indolo[2,3-a]pyrrolo[3,4-c]carbazole, violacein, or
bisindolylquinone biosynthesis, and these molecules are likely to be formed by unique pathways.
The study of bisindole biosynthesis, therefore, will be a rich area of investigation for many more
years.

One example of a variation on rebeccamycin is the molecule indocarbazostatin B 25
(Figure 5A) (Matsuura et al., 2002), which contains a similar aglycone core to rebeccamycin, but
differs in the glycosylation, with glycosylation partially resembling that of K252a 39. Indocarbazostatin B is also hydroxylated at the C5 position of one indole ring and aminated at
the C4 position of the same ring. Therefore, it is reasonable to anticipate that bacteria encoding
the biosynthetic cluster for indocarbazostatin B will contain homologues to RebO, RebD, RebP,
and RebC but will differ from the rebeccamycin gene cluster in the presence of a distinct set of
enzymes devoted to hydroxylation and amination reactions. Additionally, glycosylation enzymes
in this pathway may largely resemble those from the K252a pathway, with some modifications.
More dramatically distinct from rebeccamycin and staurosporine is a molecule such as
arcyriacyanin A 26 (Figure 5B) (Steglich, 1989), but it is possible that the unusual linkage in its
core structure results from an alternate aryl-aryl coupling by a StaP substitute (Figure 5B). This
step would likely use a similar chemical strategy to StaP, but with the chromopyrrole acid
substrate held in an alternative orientation to that seen with StaP to facilitate radical coupling
between distinct carbons on the two indole rings. The biosynthesis of granulatimide 27 (Figure
5C) (Britton et al., 2001), may also be similar to the indolo[2,3-a]pyrrolo[3,4-c]carbazole biosynthetic pathways but would use one molecule of L-tryptophan and one molecule of L-histidine as biosynthetic precursors. Molecules related to terrequinone A include semicochliodinol B 28 (Figure 5D) (Fredenhagen et al., 1997), whose biosynthesis should be identical to terrequinone A biosynthesis through the intermediate didemethylasterriquinone D 22. However, its biosynthetic pathway will differ in the type of prenyltransferase, as it is prenylated at a distinct site from either prenylation site in terrequinone A. Further, the molecule fellutanine A 29 (Figure 5E) (Kozlovskiĭ et al., 2000) may also, like terrequinone A, be constructed using NRPS chemistry, but would use two modules specific for L-tryptophan, with formation of an amide bond to release the product. A two module NRPS system is used to assemble the diketopiperazine cyclomarazine dipeptides (Schultz et al., 2008). However, NRPS-independent pathways have been described for the production of a diketopiperazine in *Streptomyces noursei*, raising the possibility that fellutanine may also be generated through an NRPS-independent pathway (Lautru et al., 2002).

While the studies presented in this Review do clarify the biosynthetic construction of some bisindole natural products, a number of unusual bisindole molecules are unlikely to be constructed via the same types biosynthetic pathways. For instance, although the molecule nortopsentin A 30 (Sakemi and Sun, 1991) (Figure 5F) appears to have a similar core skeleton to that of violacein, nortopsentin A must be generated through an alternate process: unlike violacein, whose biosynthesis involves loss of a nitrogen, nortopsentin A contains two nitrogens in its central ring. Other molecules (Figure 6), including ancorinazole 31 (Meragelman et al., 2002), the cytotoxin cladoniamide G 32 (Williams et al., 2008), the DNA intercalating agent fascaplysin 33 (Hormann et al., 2001; Kirsch et al., 2000), tjipanazole I 34 (Bonjouklian et al.,
1991), rhopaladin A 35 (Sato et al., 1998), and the cytotoxin iheyamine B 36 (Sasaki et al., 1999), whose indole rings may be derived from L-tryptophan, are likely to be generated by yet-unexplored pathways (Figure 6A). A recent, exciting result is the finding that spontaneous oxidative reaction between L-tryptophan and indole-3-pyruvate is responsible for the production of a variety of pigments including pityriacitrin 37 (Zuther et al., 2008). Inactivation of the Tam1 gene, which encodes an L-tryptophan aminotransferase in the plant pathogen Ustilago maydis, abolishes production of each of these pigments (Zuther et al., 2008). Many of the pigments, including pityriacitrin 37, can be produced, however, by simply incubating the product of Tam1 catalysis, indole-3-pyruvate, with L-tryptophan (Zuther et al., 2008) (Figure 6B). This unexpected discovery suggests that spontaneous chemistry, already thought to play a role in key steps of indolo[2,3-a]pyrrolo[3,4-c]carbazole, violacein, and bisindolylquinone biosynthesis, can be primarily responsible for production of a series of bisindole pigments. The specific routes to spontaneous production of these pigments should be a rich area for future study, and it is tempting to speculate that spontaneous chemistry may play a role in production of a variety of yet-unexplored bisindole biosynthetic pathways.

Future directions in bisindole biosynthesis

Although studies have clarified the role of many of the enzymes involved in the construction of the three types of bisindole molecules presented in this Review, a number of questions remain about the details of these enzymatic reactions. In rebeccamycin biosynthesis, spectroscopic or structural elucidation of the proposed Lys-79-chloramine intermediate in RebH is still lacking, as is an understanding of how FADH$_2$ produced by the partner reductase RebF is transferred to RebH. The structure of RebD, which is not yet available, would represent a largely
uncharacterized enzyme. It also might shed light on how RebD, which has 3 equivalents of iron (Howard-Jones and Walsh, 2005), can be functionally identical to VioB, which has less than 1 equivalent of iron (Balibar and Walsh, 2006), as well as provide details about potential routes for the proposed channeling of indole-3-pyruvate imine from RebO to RebD. For the two enzyme RebP-RebC system, although a series of studies suggest that an unstable intermediate is produced by RebP and is the substrate of RebC (Howard-Jones and Walsh, 2006, 2007; Ryan et al., 2007), questions of intermediate channeling, the precise identity of the substrate of RebC, and the mechanism of flavin based hydroxylation chemistry are areas of active study. Further, in the staurosporine pathway, questions remains about the role of StaC, which is a close homologue of RebC but is purified without bound FAD and carries out a distinct reaction (Howard-Jones and Walsh, 2006). In the violacein pathway, a number of questions are also outstanding. Although an intermediate has been shown to be transferred from VioB to VioE, the identity of this intermediate has only been suggested (Balibar and Walsh, 2006). Further, while structural characterization now provides a model of VioE (Hirano et al., 2008; Ryan et al., 2008), the lack of an essential residue in VioE is puzzling, and the mechanistic chemistry involved in converting the Cβ-Cβ benzylically coupled iminophenylpyruvate dimer 10 to prodeoxyviolacein 19 is largely unexplored. Next, VioD, while apparently a ‘standard’ flavin hydroxylase, will hydroxylate prodeoxyviolacein but not the related molecule oxyviolacein (Balibar and Walsh, 2006), suggesting either steric or electronic effects modulate its substrate specificity. In the bisindolylquinone pathways, exemplified by the terrequinone A biosynthetic pathway, only TdiD is reasonably well-understood. For TdiA, the mechanistic details of how didemethylasterriquinone 22 is generated still await exploration, as the mixed Claisen condensations could occur with either the T-domain tethered or the TE-domain tethered indole-
3-pyruvate acting as a nucleophile. For TdiB, TdiC, and TdiE, available data suggest a complicated relationship between the three enzymes (Balibar et al., 2007), and further studies will be needed to delineate how TdiE accelerates the TdiB-TdiC-mediated production of terrequinone A 24 from didemethylasterriquinone D 22. For many of the bisindole biosynthetic enzymes, whose roles include enzymatic reactions and stabilization of reactive pathway intermediates, standard biochemical approaches are often difficult, particularly when putative substrates and products decompose quickly in solution.

Major questions also remain about the roles of these molecules for their producing organisms. For instance, staurosporine is often isolated as a natural product (Sánchez et al., 2006b); why do many actinomycete bacteria invest biochemical energy to produce a protein kinase inhibitor such as staurosporine? Additionally, what is the physiological role of violacein; is it related to its various biochemical properties, including anti-bacterial, anti-viral, and anti-protozoan properties (Durán and Menck, 2001; Matz et al., 2008), to its apparent regulation with quorum sensing pathways (McClean et al., 1997), or to its distinctive purple color? Finally, why do plant associated fungi such as Aspergillus nidulans produce molecules such as terrequionone A, which has unknown properties? Is there a yet unexplored role for terrequinone A that is relevant to its role in its ecological niche? One frontier in the basic science research of bisindole natural products is a detailed investigation of the ecological interactions mediated by natural products such as rebeccamycin, staurosporine, violacein, and terrequinone A.

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Table 1. Bisindole natural products with characterized biosynthetic clusters.

<table>
<thead>
<tr>
<th>Compound</th>
<th>#</th>
<th>Structure</th>
<th>Class</th>
<th>Source organism used to isolate biosynthetic genes</th>
<th>Known medicinal properties</th>
<th>Biosynthetic enzymes</th>
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<td>Violacein</td>
<td>21</td>
<td><img src="image5.png" alt="Image" /></td>
<td>Violacein</td>
<td><em>Chromobacterium violaceum</em></td>
<td>Anti-bacterial, antiviral, anti-cancer, antileishmanial, antiulcerogenic</td>
<td>VioA, VioB, VioE, VioD, VioC</td>
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<tr>
<td>Terrequinone A</td>
<td>24</td>
<td><img src="image6.png" alt="Image" /></td>
<td>Bisindolylquinone</td>
<td><em>Aspergillus nidulans</em></td>
<td>Unknown</td>
<td>TdiD, TdiA, TdiC, TdiB, TdiE</td>
</tr>
</tbody>
</table>
**Figure Legends**


**Figure 2.** Divergent pathways in bisindole biosynthesis. These pathways are responsible for generating three classes of bisindole molecules. Indolo[2,3-α]pyrrolo[3,4-c]carbazole is represented here by rebeccamycin 17 and staurosporine 18 in red and blue, respectively, violacein 21 in green, and bisindolylquinone by terrequinone A 24 in black. Intermediates shared by more than one pathway are in purple, and the L-tryptophan precursor is in tan. R=Cl for rebeccamycin precursors; R=H for staurosporine and violacein precursors. Compounds in boxes are characterized intermediates, whereas compounds that are not boxed are proposed but yet-uncharacterized intermediates. Double boxes indicate final products or the L-tryptophan starting material. The proposed mechanism for TdiA-catalyzed production of didemethylasterriquinone D is shown in Figure 4B. 6, L-tryptophan; 7, 7-chloro-L-tryptophan; 8, indole-3-pyruvate imine;
9, indole-3-pyruvate; 10, Cβ-Cβ benzylically coupled iminophenylpyruvate dimer; 11, chromopyrrolic acid; 12, K252c (staurosporine aglycone); 13, dichloro-arcyriaflavin A (rebeccamycin aglycone); 14, holyrine A; 15, 4'-demethyl-rebeccamycin; 16, O-demethyl-N-demethyl-staurosporine; 17, rebeccamycin; 18, staurosporine; 19, prodeoxyviolacein; 20, proviolacein; 21, violacein; 22, didemethylasterriquinone D; 23, ochrinolide D; 24, terrequinone A.

**Figure 3.** Active sites of bisindole biosynthetic enzymes. A. RebH, an FADH₂-dependent chlorinase, contains two active sites, separated by ~11 Å. FAD (in green; PDB ID 2OAL), L-tryptophan (in orange, PDB ID 2E4G), and Lys-79 (in magenta) are shown (Yeh et al., 2007). B. StaP (PDB ID 2Z3U) (Makino et al., 2007), a cytochrome P450 enzyme, binds chromopyrrolic acid (shown in yellow) with a distance of 4.7 Å from one C10 carbon to its heme iron (brown sphere; porphyrin ring in red). C. RebC (PDB ID 2R0G) (Ryan et al., 2007), a putative flavin hydroxylase (FAD shown in blue), binds a tautomer of 7-carboxy-K252a (shown in pink). Two arginines (in gray) hydrogen bond to the out-of-plane carboxylate of the 7-carboxy-K252c tautomer. D. VioE, an enzyme with no cofactors, metals, or single essential side-chain, has been co-crystallized with PEG (in green, from PDB ID 3BMZ (Ryan et al., 2008)) and two molecules of phenylpyruvic acid (from PDB ID 2ZF4 (Hirano et al., 2008), not shown) in its putative active site. Nearby are 6 residues (in gray) which play a role in the enzymatic acceleration of prodeoxyviolacein production (Hirano et al., 2008; Ryan et al., 2008).

**Figure 4.** NRPS chemistry and TdiA. A. ‘Standard’ non-ribosomal peptide synthetases (NRPS’s) construct peptides using an assembly-like construction. The enzymes are modular, with each
module containing domains, which can include ‘A’ (adenylation domain, responsible for activating an amino acid), ‘T’ (thiolation domain, which tethers the amino acid or peptide via a phosphopantetheinyl arm, shown as a squiggly line), ‘C’ (condensation, responsible for amide bond formation), and ‘TE’ (thioesterase, responsible for release of the final peptide product). Long peptides can be generated by iterative rounds of amide bond formation, with each step catalyzed by a distinct module. In a final stage, the elongated peptide is transferred to an active site serine on the TE domain. Release of the peptide from the enzyme can occur by hydrolysis, giving rise to an acid, or cyclization, giving rise to either an ester or an amide linkage. B. TdiA is thought to use a non-standard NRPS-like chemistry to generate the terrequinone A precursor. Instead of utilizing amino acids, TdiA reacts with an α-keto acid, indole-3-pyruvic acid, although, as for ‘standard’ NRPS’s, activates it via an adenylation reaction. Rather than using one module per substrate, TdiA tethers two indole-3-pyruvate substrates on adjacent domains of one module. Finally, TdiA lacks a condensation domain, and instead is thought to use two mixed Claisen condensation reactions to generate the didemethylasterriquinone 22 product.

**Figure 5.** Underexplored bisindole products. Possible biosynthetic routes are shown. A. Indocarbazostatin B 25. B. Arcyriacyanin A 26. C. Granulatimide 27. D. Semicochliodinol B 28. E. Fellutanine A 29. NRPS domains are shown in boxes (A=adenylation, T=thiolation, C=condensation, TE=thioesterase). F. Nortopsentin A 30.

**Figure 6.** Unusual bisindole molecules. A. A number of unusual molecules are unlikely to be constructed by processes that generate indolo[2,3-a]pyrrolo[3,4-c]carbazole cores, the violacein skeleton, or the bisindolylquinone skeleton. Each bisindole molecule shown below, however,
could be derived from two dimerized L-tryptophan molecules. To emphasize this point, the molecules are drawn with atoms in blue, representing atoms from one L-tryptophan molecule; red, representing atoms from a second L-tryptophan molecule. Note, however, that labeling studies have not established the biosynthetic origins of any of these molecules. B. A proposed mechanism for pityriacitrin A biosynthesis includes the spontaneous reaction of L-tryptophan 6 with indole-3-pyruvate 9 (Zuther et al., 2008).