Reviews

Cloning and Characterization of the Bleomycin Biosynthetic Gene Cluster from *Streptomyces verticillus* ATCC15003[1]

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Bleomycin (BLM) biosynthesis has been studied as a model for hybrid peptide−polyketide natural product biosynthesis. Cloning, sequencing, and biochemical characterization of the *blm* biosynthetic gene cluster from *Streptomyces verticillus* ATCC15003 revealed that (1) the BLM hybrid peptide−polyketide aglycon is assembled by the BLM megasynthetase that consists of both nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules; (2) BlmIX/BlmVIII/BlmVII constitute a natural hybrid NRPS/PKS/NRPS system, serving as a model for both hybrid NRPS/PKS and PKS/NRPS systems; (3) the catalytic sites appear to be conserved in both hybrid NRPS/PKS and nonhybrid NRPS or PKS systems, with the exception of the KS domains in the hybrid NRPS/PKS systems that are unique; (4) specific interpolypeptide linkers may play a critical role in intermodular communication to facilitate the transfer of the growing intermediates between the interacting NRPS and/or PKS modules; (5) post-translational modification of the BLM megasynthetase has been accomplished by a single PPTase with broad carrier protein specificity; and (6) BlmIV/BlmIII-templated assembly of the BLM bithiazole moiety requires intriguing protein juxtaposition and modular recognition. These results lay the foundation to investigate the molecular basis for intermodular communication between NRPS and PKS in hybrid peptide−polyketide natural product biosynthesis and set the stage for engineering novel BLM analogues by genetic manipulation of genes governing BLM biosynthesis.

Introduction

Nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) use a very similar strategy for the assembly of peptides and polyketides, two distinct classes of natural products. The bleomycins (BLMs) are a family of glycopeptide-derived antibiotics, isolated from several *Streptomyces* species. The naturally occurring BLMs differ structurally primarily at the C-terminus of the glycopeptide (Figure 1A). Structurally and biosynthetically related to the BLMs are the phleomycins and tallysomycins. BLMs exhibit strong antitumor activity and are currently used clinically in combination with a number of other agents for the treatment of several types of tumors. BLMs are thought to exert their biological effects through a sequence selective, metal-dependent oxidative cleavage of DNA and RNA in the presence of oxygen. Almost uniquely among anticancer drugs, BLMs do not cause myelosuppression, promoting their wide application in combination chemotherapy. Early development of drug resistance and cumulative pulmonary toxicity are the major limitations of BLMs in chemotherapy. Consequently, there have been continuing attempts to develop new BLM congeners to define the fundamental functional roles of BLM's individual domains and to search for anticancer drugs with better clinical efficacy and lower toxicity. Readers are referred to several excellent reviews appearing in the recent literature on BLM synthesis[2] and mechanism of action.[2a,3]

The biosynthesis of BLMs has been extensively studied by feeding isotope-labeled precursors. These results unambiguously established the hybrid peptide−polyketide origin of the BLMs, the aglycon of which is derived from nine amino acids, an acetate, and two molecules of *S*-adenosyl methionine (AdoMet). Subsequent isolation and structural determination of a series of biosynthetic intermediates and shunt metabolites, such as P-3, P-3A, P-3K, P-4, P-5, P-5m, P-6m, P-6mo, and the BLM aglycon, from fermentation cultures led to the hypothesis of a processive pathway for the assembly of the BLM hybrid peptide−polyketide backbone (Figure 1B). These early studies also have been extensively reviewed.[3a,4]

We have been studying the biochemistry and genetics of BLM biosynthesis as a model for the biosynthesis of hybrid peptide−polyketide natural products.[5] In this review, we will emphasize primarily our findings on cloning and characterization of the BLM biosynthetic gene cluster from *Streptomyces verticillus* ATCC15003. These results shed light on the molecular basis of a functional hybrid nonribosomal peptide synthetase (NRPS)−polyketide synthase (PKS) system and set the stage for rational engineering of BLM biosynthesis to make new anticancer drugs.

BLM as a Model for Hybrid Peptide−Polyketide Natural Product Biosynthesis. Nonribosomal peptides and polyketides are synthesized from amino acids and short carboxylic acids by NRPSs and PKSs, respectively. NRPSs and PKSs use a very similar strategy for the assembly of these two distinct classes of natural products (Figure 2A,B).[6] Both NRPSs and type I PKSs are multifunctional proteins that are organized into modules. The number and order of modules on each NRPS or PKS protein determine the structural variations of the resulting peptide and

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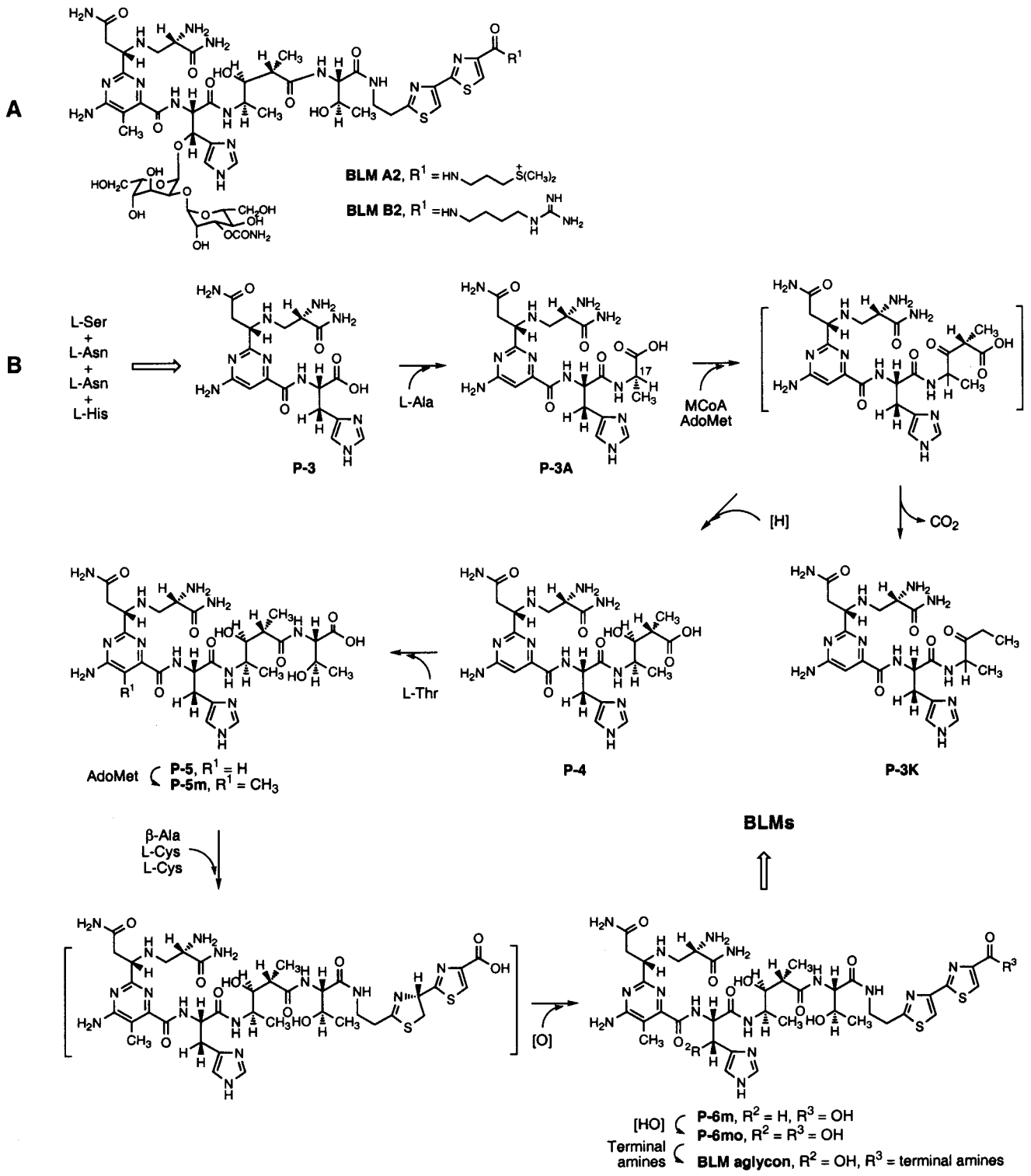

Figure 1. (A) Structures of BLMs and (B) proposed biosynthetic pathway for BLMs. Intermediates except those in brackets were identified from *S. verticillus*. [H], reduction; [OH], hydroxylation; [O], oxidation, AdoMet, *S*-adenosylmethionine; MCoA, malonyl coenzyme A.

polyketide products. Both systems use carrier proteins, peptidyl carrier protein (PCP) for NRPS and acyl carrier protein (ACP) for PKS, to activate substrates and to channel the growing peptide or polyketide intermediates during the elongation processes. ACP and PCP are post-translationally modified by the same 4′-phosphopantetheine prosthetic group, catalyzed by the 4′-phosphopantetheineyl transferases (PPTases) (Figure 2C).[7] The modular structure of NRPS and PKS has greatly facilitated rational engineering of metabolic pathways for peptide and polyketide biosynthesis. Both NRPS and PKS modules are considerably tolerant toward genetic engineering, resulting in the combinatorial biosynthesis of complex peptide and polyketide libraries.[8]

Hybrid peptide−polyketide metabolites refer to natural products that are biosynthetically derived from amino acids and short carboxylic acids, catalyzed by hybrid NRPS−PKS systems (Figure 2D,E). Thus, while the condensation domain (C) catalyzes the nucleophilic substitution between the peptidyl-S-PCP from the upstream NRPS module and the amino acyl-S-PCP of its cognate NRPS module to form the C−N bond in peptide biosynthesis (Figure 2A), the C

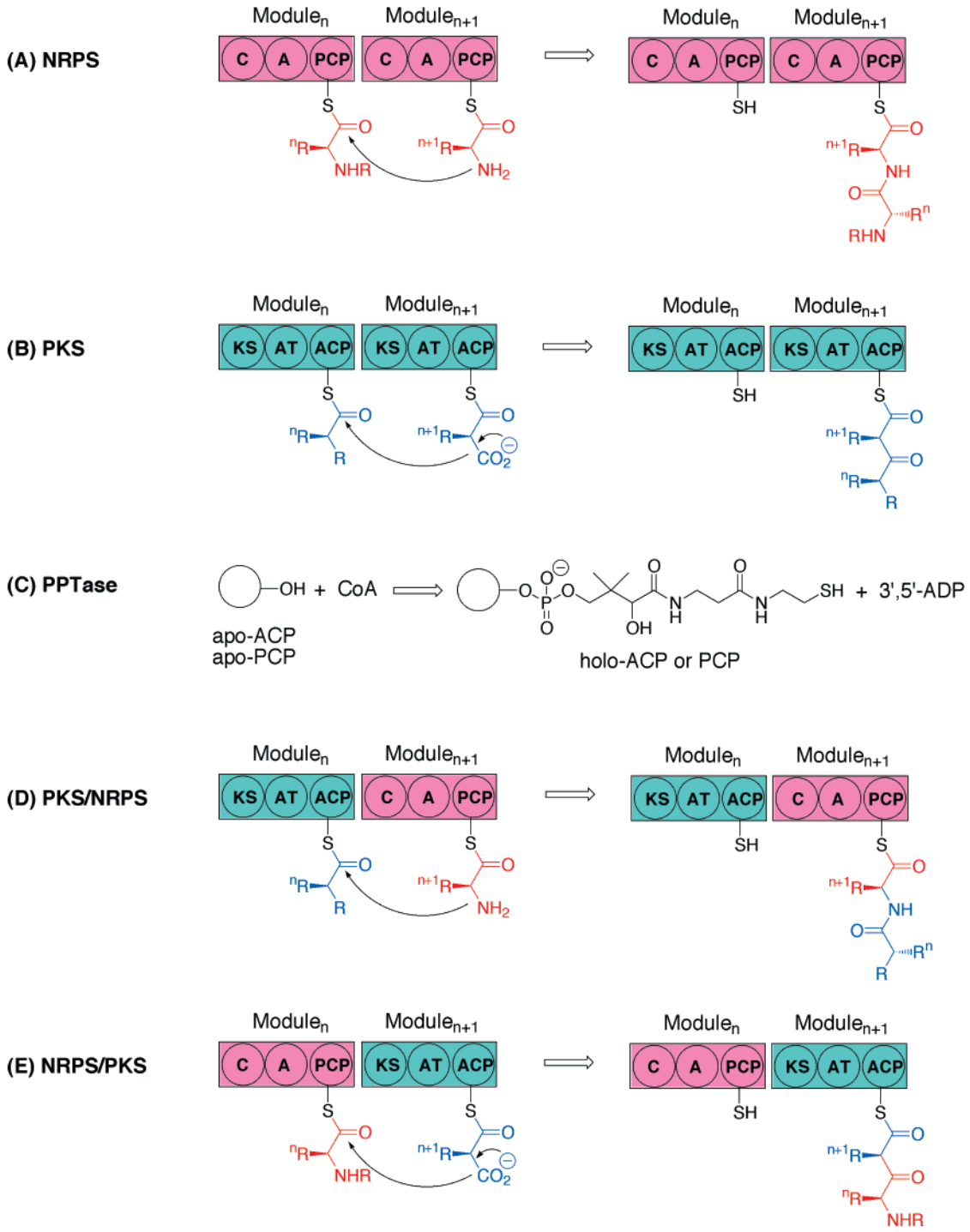

Figure 2. Modular organization of NRPS (A), PKS (B), hybrids PKS/NRPS (D) and NRPS/PKS (E), and post-translational modification of apo-ACP or apo-PCP into holo-ACP and holo-PCP by a PPTase (C). Hypothetical NRPS (pink) and PKS (blue) modules are shown with core domains. A, adenylation; ACP, acyl carrier protein; AT, acyltransferase, C, condensation; KS, ketoacyl synthase; PCP, peptidyl carrier protein.

domain in a hybrid PKS/NRPS system has to catalyze the nucleophilic substitution between the acyl-S-ACP of the PKS module and the amino acyl-S-PCP of the NRPS module to form the C−N bond in hybrid polyketide/peptide biosynthesis (Figure 2D). Similarly, the KS domain catalyzes (1) transfer of the acyl-S-ACP from the upstream PKS module to the active site cysteine of KS and (2) decarboxylic condensation between the resulting acyl-S-KS and the malonyl-S-ACP of its cognate PKS module to form the C−C bond in polyketide biosynthesis (Figure 2B). In contrast, the KS domain in a hybrid NRPS/PKS has to catalyze the transfer of the peptidyl-S-PCP from the upstream NRPS module to the active site cystine of KS and the subsequent decarboxylative condensation between the resulting peptidyl-S-PKS and its cognate malonyl-S-ACP to form the C−C bond in hybrid peptide/polyketide biosynthesis (Figure 2E). Therefore, hybrid peptide−polyketide natural product biosynthesis provides an excellent opportunity to investigate intermodular communication between NRPS and PKS modules.[5,6] The BLMs serve as an excellent model for such study because their aglycon has been established unambiguously to be of hybrid peptide−polyketide origin.[3a,4] We could easily envisage the biosynthesis of the BLM aglycon in three stages: (1) NRPS-

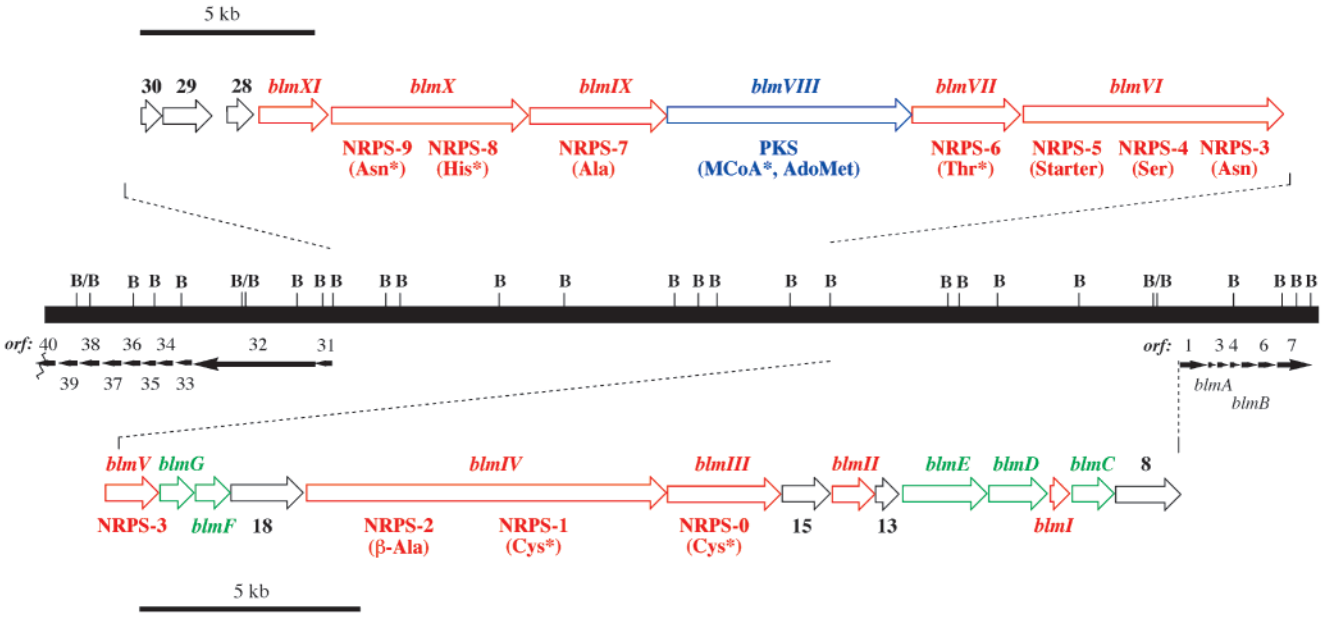

Figure 3. Genetic organization of the BLM biosynthetic gene cluster (red for NRPS genes, blue for PKS genes, green for sugar biosynthesis genes, and black for all other genes). Modules for individual NRPS and PKS are given along with their predicted substrates in parentheses, and * indicates substrates that have been confirmed biochemically. AdoMet, *S*-adenosylmethionine; MCoA, malonyl coenzyme A; B, *Bam*HI.

mediated formation of P-3A from Ser, Asn, Asn, His, and Ala; (2) PKS-mediated elongation of P-3A by malonyl CoA and AdoMet to yield P-4; and (3) NRPS-mediated elongation of P-4 by Thr to P-5 that is further elongated by β-Ala, Cys, and Cys to afford P-6m (Figure 1B). Therefore, the transition between stage 1/2 or 2/3 potentially could represent a natural hybrid PKS/NRPS (Figure 2D) or NRPS/PKS (Figure 2E) system, respectively.

Cloning and Identification of the *blm* Gene Cluster from *S. verticillus*. Given the precedent that antibiotic production genes commonly occur as a cluster in actinomycetes, we adopted an approach combining chromosomal walking from the *blmAB* resistance locus and DNA hybridization with NRPS or PKS probes to clone and identify the *blm* biosynthetic gene cluster.[9] Sugiyama and coworkers previously cloned two BLM resistance genes, *blmAB*, from *S. verticillus* ATCC15003.[10] BlmA is a BLM-binding protein, conferring BLM resistance by drug sequesting,[10b,c] and BlmB is an *N*-acetyltransferase, inactivating BLM by *N*-acetylation of BLM at the primary amine of the β-aminoalaninamide moiety in the presence of acetyl CoA.[10d,e] Calcutt and Schmidt[11] and our group[5a] subsequently sequenced a 15-kb DNA fragment flanking the *blmAB* genes, revealing 14 open reading frames (orfs). However, none of them were found to encode putative NRPS or PKS enzymes. Since our hypothesis for BLM biosynthesis calls for at least nine NRPS modules and one PKS module, we were concerned by the fact that neither NRPS nor PKS genes were found in the 15-kb *blmAB* locus. We decided to clone NRPS and PKS genes directly from *S. verticillus* by PCR and use them as probes to screen the vicinity of the *blmAB* locus before additional sequencing. Using primers designed according to the conserved sequences found in the KS domains of PKS,[12] we cloned at least four KS domains. However, none of these KSs are clustered with the *blmAB* locus, suggesting that they belong to polyketide gene cluster(s) not related to BLM biosynthesis. In contrast, using primers designed according to the conserved core II and VI motifs found in the A domains of NRPS,[13] we cloned at least three A domains.[5a] We mapped the cloned A domains to three independent NRPS loci, but none of them are clustered with the *blmAB* genes, arguing against their involvement in BLM biosynthesis. However, we noticed weak signals upon southern hybridization of one of the A domains to cosmids within the 100-kb DNA region upstream of the *blmAB* genes. Cloning and sequencing of one of the A domain-hybridizing fragments revealed two incomplete NRPS modules. Encouraged by the fact that these NRPS modules are clustered with the *blmAB* genes, albeit not immediately, we proceeded to determine a total of 77.5-kb DNA sequences directly upstream of *blmAB*. Among the *orfs* identified from the cloned cluster, we indeed found 10 NRPS genes encoding nine NRPS modules, a PKS gene encoding one PKS module, five sugar biosynthesis genes, as well as genes encoding other biosynthesis, resistance, and regulatory proteins (Figure 3).[9]

Substrate Specificity of Individual NRPS and PKS Modules. Inspection of the *blm* gene cluster (Figure 3) showed that the Blm NRPS and PKS modules are not organized along the chromosome in a linear order that parallels the order of the amino acid and carboxylic acid precursors incorporated into the BLM aglycon; that is, they do not follow the so-called "collinearity rule" for peptide and polyketide biosynthesis gene clusters.[6,14] Realizing that BLM biosynthesis cannot be rationalized simply according to the "collinearity rule", we determined the substrate specificity of individual NRPS and PKS modules to shed light on the modular organization of the Blm megasynthetase complex.

For NRPS modules, we adopted the methods of Marahiel and co-workers[15] and Townsend and co-workers[16] that predict amino acid specificity of an NRPS module according to the specificity-conferring codes of the A domain. Although the two groups used a slightly different set of amino acid residues to define the specificity-conferring codes, both methods were based on the same X-ray structure of PheA[17] and yielded consistent predictions. Using these codes and the updated codes developed by carrying out additional sequence analysis,[9c] we were able to predict the amino acid specificity for all NRPS modules. They, in fact, accounted

for all the amino acids required for BLM biosynthesis (Figure 3).[9c]

The method of specificity-conferring codes to predict the amino acid specificity of an NRPS is based on the assumptions that (1) the main chain conformation of the A domains in all NRPSs is likely to be very similar and (2) substrate specificity of individual NRPS modules will be mainly determined by the nature of the amino acids lining the substrate binding pocket.[15−17] While this method has been very useful, and many of the predictions have been confirmed biochemically, we were concerned by the fact that it depends primarily on a few amino acid residues that putatively line the substrate binding pocket rather than the entire region between core sequences A3 and A6 of an A domain that represents the substrate specificity determinant of an NRPS module.[17] Given the latter structural information and the vast amount of NRPS sequences available in the GenBank, we developed a complementary approach to predict substrate specificity for an NRPS module by comparing the overall sequence between the A3 and A6 region. The nine Blm NRPS modules were compared with 84 modules from various bacterial and fungal NRPSs available at the GenBank, including those with known or putative specificity for amino acids present in BLM. A table of overall similarities/identities was generated by PILEUP analysis of the A3 to A6 regions. The percentage similarities for each Blm NRPS module were plotted against the rest of the NRPS modules to display the overall sequence homology between the A3 and A6 regions. While a constant level of similarities (30−40%) was evident among all the NRPS modules analyzed, most of the Blm NRPS modules showed striking similarities (50−60%) to a particular cluster of NRPS modules as exemplified in Figure 4 for NRPS-1 and NRPS-6. Close examination of these modules clustered with higher similarities revealed that they activate the same or very similar amino acid, based on which putative substrate for the NRPS in question could be predicted; that is, NRPS-1 and NRPS-6 activate L-Cys and L-Thr, respectively. These results agree well with the predictions made according to the specificity-conferring codes of A domains.

Finally, we verified the predicted amino acid specificities of individual NRPS modules by overproducing the A domains, followed by examining their substrate specificity according to the amino acid-dependent ATP-PPi assay.[9,18] While we have encountered many difficulties in overproducing soluble, functional proteins, we have successfully confirmed the amino acid specificity for five of the nine NRPS modules identified within the *blm* gene cluster (Figure 3).

The *blmVIII* gene encodes a PKS module consisting of a KS, an acetyl transferase (AT), a methyltransferase (MT), a ketoreductase (KR), and an ACP domain, and the AT domain specifies the substrate for the PKS module. The AT domain of BlmVIII is predicted to be specific for malonyl CoA, on the basis of sequence comparison with ATs of known substrate specificity,[19] indicative that the BlmVIII PKS module uses malonyl CoA as the preferred extender unit. To biochemically confirm the predication, we overproduced the BlmVIII PKS protein and converted it in vitro into the holo-form.[20] Upon incubation of the holo-BlmVIII PKS in the presence of [2-^{14}C]malonyl CoA, BlmVIII is specifically labeled as evident by autoradiographic analysis. We also overproduced the BlmVIII-ACP domain alone and converted it in vitro into the holo-form. Interestingly, upon incubation of the holo-BlmVIII PKS and holo-BlmVIII-ACP in the presence of [2-^{14}C]malonyl CoA, autoradiographic analysis showed both the BlmVIII PKS and BlmVIII-ACP proteins were labeled. These results not only confirmed that BlmVIII specifies malonyl CoA as a substrate but also revealed that the AT domain is capable of both *cis* and *trans* loading of malonyl CoA to the ACP domain (Figure 5).[21]

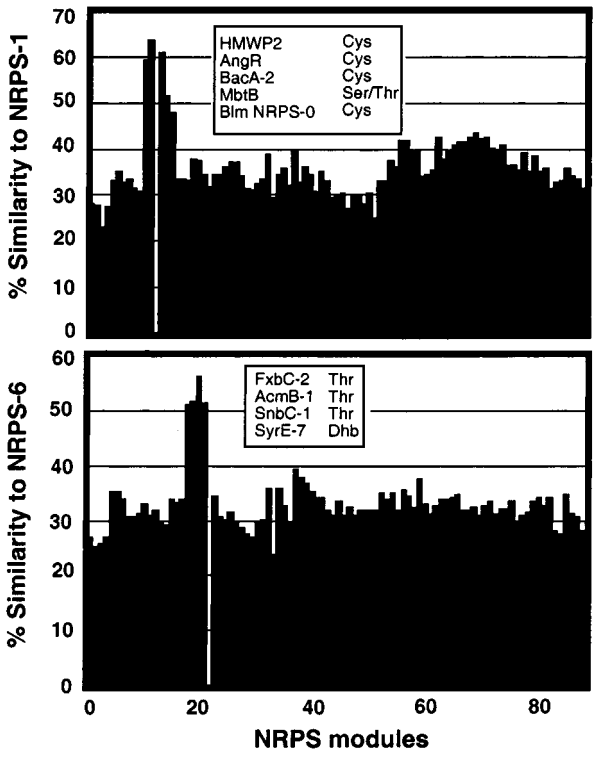

Figure 4. Determination of substrate specificity for NRPS-1 and NRPS-6 by comparison of the A3 to A6 region of the A domain to 84 NRPS modules available at GenBank that activate various amino acids. Shown in the boxes are those modules that exhibit more similarity to the NRPS in question, and the number following the protein name indicates the order of a particular A domain in the multimodular NRPS protein. The protein accession numbers are P48633 (HMWP2), P19828 (AngR), AAC06346 (BacA-2), CAB03756 (MbtB), AAC80285 (SyrE−7), AAC38442 (AcmB-1), CAA67248 (SnbC-1), AAC82550 (FxbC-2), AAG02358 (Blm NRPS-6), AAG02364 (Blm NRPS-1), and AAG02365 (Blm NRPS-0). Dhb stands for 2,3-dehydroaminobutyric acid.

BLM Megasynthetase as an Example of Natural Hybrid NRPS/PKS Systems. Using the substrate specificity of individual NRPS and PKS modules as a guide, we have been able to propose a linear model for the BLM megasynthetase-templated assembly of the BLM peptide/polyketide/peptide aglycon from nine amino acids and one acetate (Figure 6).[9c] The individual modules are first primed with the amino acid or short carboxylic acid precursors, the choice of which is determined by the A domains of the NRPS modules or the AT domain of the PKS module, respectively. The Blm megasynthetase-mediated N-to-C directional assembly of the BLM hybrid peptide/polyketide/peptide aglycon proceeds by sequential condensation of the amino acid and carboxylic acid precursors. Although we are yet to provide direct evidence supporting the specific molecular recognition and interaction between the neighboring proteins, it is striking to note that all the biosynthetic intermediates isolated to date are derailed from NRPS or PKS modules at the junctions between the interacting proteins.[3a,4] Since it is not difficult to imagine that an intermediate is more likely to fall off the enzyme complex when it is subjected to interpeptide transfer rather than intrapeptide transfer, we view the

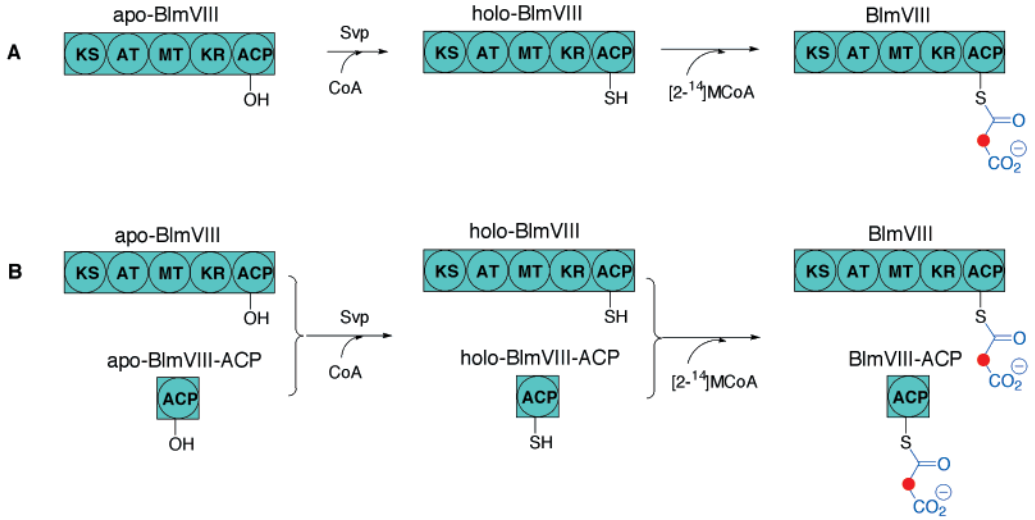

Figure 5. In vitro 4′-phosphopantetheinylation of apo-ACP by the Svp PPTase and AT-catalyzed in *cis* (A) and in *trans* (B) loading of the resultant holo-ACP with malonyl CoA. ACP, acyl carrier protein; AT, acyltransferase; KR, ketoreductase; KS, ketoacyl synthase; MCoA, malonyl coenzyme A (red dot indicates the ^{14}C-label); MT, methyltransferase.

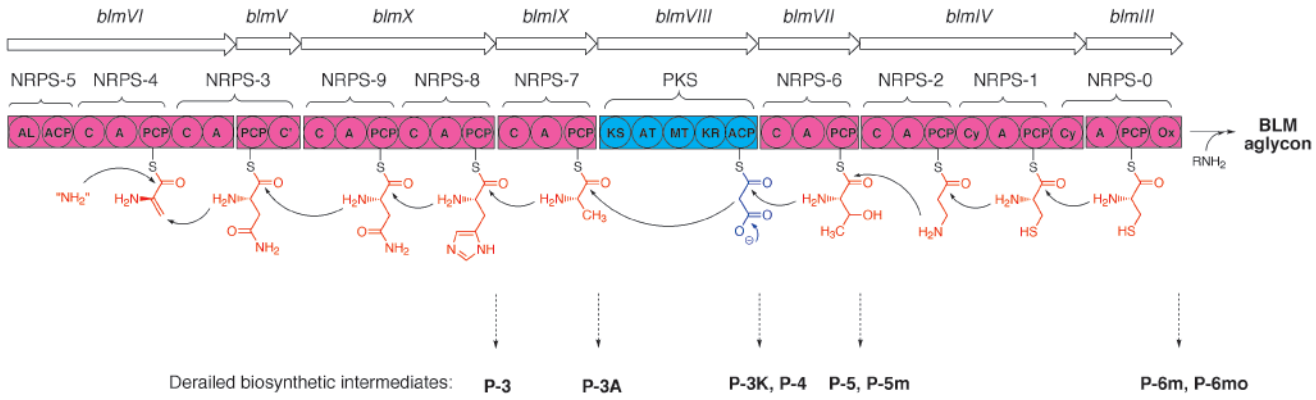

Figure 6. Linear model for the BLM megasynthetase-templated assembly of the BLM peptide/polyketide/peptide aglycon from nine amino acids and one acetate. A, adenylation; ACP, acyl carrier protein; AL, acyl CoA ligase, AT, acyltransferase, C and C′, condensation; Cy, condensation/cyclization; KR, ketoreductase, KS, ketoacyl synthase; MT, methyltransferase; Ox, oxidation; PCP, peptidyl carrier protein; "NH_2", an unspecified amino group donor.

latter observation as strong evidence supporting the current model of the Blm megasynthetase.

Our model of the Blm megasynthetase indicates that BlmIX/BlmVIII/BlmVII constitutes a natural hybrid NRPS/PKS/NRPS system, combining features of both hybrid NRPS/PKS (Figure 2E) and PKS/NRPS (Figure 2D) systems. For BlmIX/BlmVIII, we found that the PCP domain of BlmIX seems to have no relevant difference as compared to those in nonhybrid NRPS systems. In contrast, the KS domain of BlmVIII is unique in comparison with those in nonhybrid PKS systems. Upon phylogenetic analysis, the KS domain of BlmVIII can be grouped into a subfamily of KSs from all other known hybrid NRPS/PKS systems regardless of their origins.[5b] Therefore, the BlmVIII PKS may have evolved a KS domain with an altered substrate specificity to adapt the BlmIX-bound peptidyl-S-PCP intermediate (Figure 7, step a). Once the peptidyl intermediate is transferred to the active site cysteine of KS, BlmVIII appears to keep the same catalytic sites conserved in all other PKS domains to execute the decarboxylative condensation with malonyl-S-ACP (Figure 7, step b) and the associated modifications (Figure 7, steps c and d) to yield the hybrid peptide/polyketide product. For BlmVIII/BlmVII, we found that the ACP domain of BlmVIII is more similar to PCPs than to ACPs, but the C domain of BlmVII shows no unique feature as compared to those in nonhybrid NRPSs.[5b] These findings are in accord with (1) the proposed function for the carrier proteins of both PKS and NRPS as workstations without any selectivity[22] and (2) the C domain of NRPS has a low selectivity toward the upstream substrate, also known as donor site substrate.[23] Physical proximity of the active sites may be sufficient for the C domain to accept an acyl-S-ACP, instead of peptidyl-S-PCP, and to catalyze its elongation with the amino acyl-S-PCP to afford the hybrid polyketide/peptide product (Figure 7, step e).

Intermodular communications in PKS have been attributed to intermodular or interpolypeptide linkers, and polyketide chain transfer between modules appears to be permissive as long as the linkers can provide the connectivity between the adjacent modules.[24] Inspired by the linker hypothesis for PKS, we have also carried out similar sequence analyses for hybrid NRPS−PKS systems and identified putative interpolypeptide linkers for both hybrid NRPS/PKS and hybrid PKS/NRPS systems.[5b] They are located at the C-termini of the NRPS proteins and N-termini of the PKS proteins for hybrid NRPS/PKS systems and at the C-termini of the PKS proteins and N-termini of the NRPS proteins for PKS/NRPS systems. The linkers vary from 5 to 76 amino acids and are rich in basic and acidic residues.[5b] It is tempting to propose that these linkers may play a critical role in protein−protein recogni-

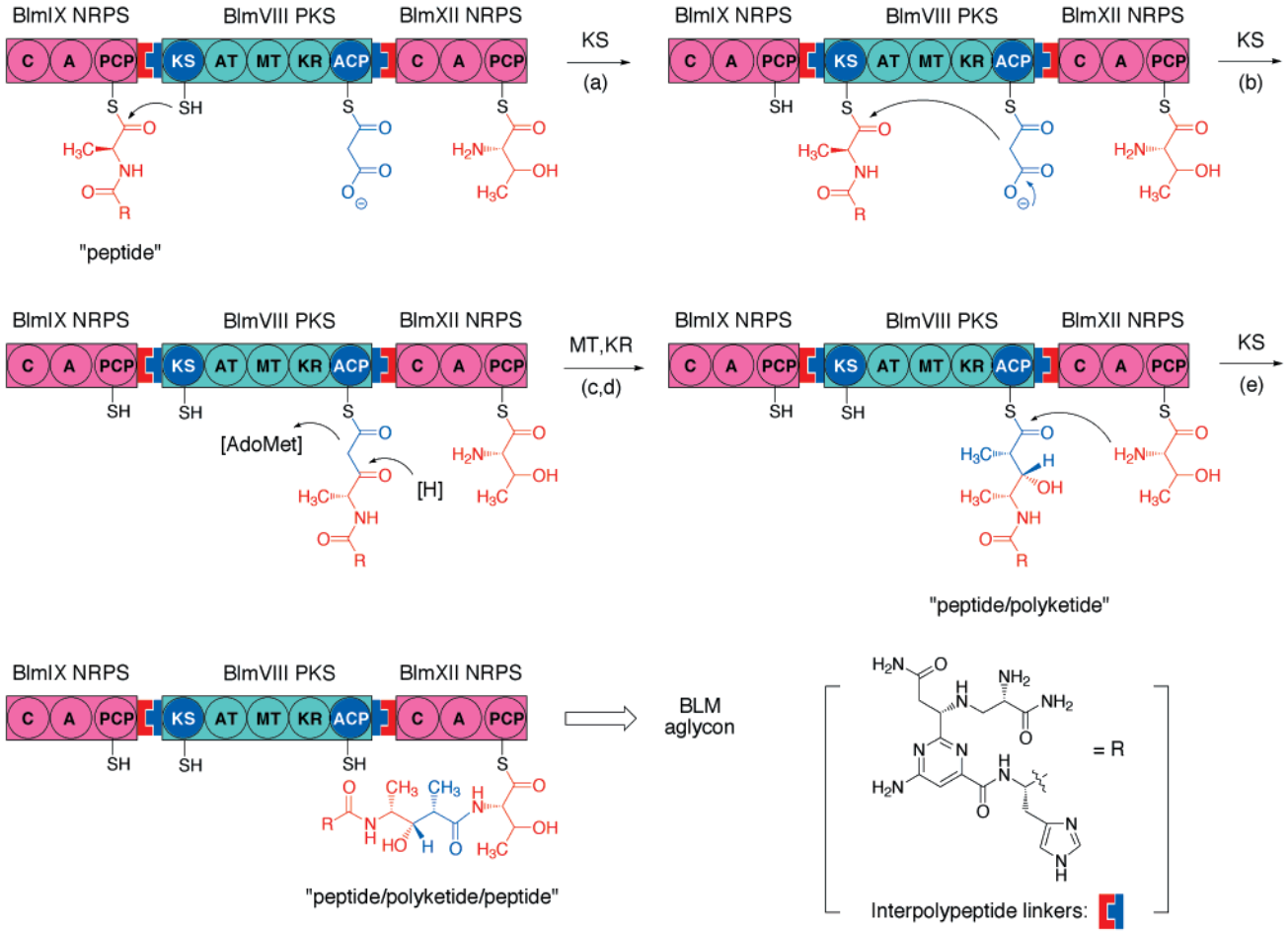

Figure 7. Schematic representation of the BlmIX/BlmVIII/BlmVII system as a model for channeling the growing intermediate between NRPS (pink) and PKS (blue) modules or vice versa for the biosynthesis of hybrid peptide−polyketide natural products. The KS and ACP domains of BlmVIII and the putative interpolypeptide linkers between BlmIX/BlmVIII and BlmVIII/BlmVII are shaded in darker color to emphasize their roles in facilitating interactions between NRPS and PKS to constitute a functional hybrid NRPS/PKS system. A, adenylation; ACP, acyl carrier protein; AT, acyltransferase, C, condensation; KR, ketoreductase, KS, ketoacyl synthase; MT, methyltransferase; PCP, peptidyl carrier protein.

tion to constitute a functional hybrid NRPS−PKS system, as depicted for BlmIX/BlmVIII/BlmVII (Figure 7). However the latter conclusion is based purely on sequence analysis and will need to be assessed experimentally in the future.

Bithiazole Biosynthesis Requires Both *cis* and *trans* Adenylation, Cyclization, and Oxidation. Five-membered heterocycles, such as the bithiazole moiety of BLM, are common structural units of many natural products.[25] Two mechanisms are known for the biosynthesis of these heterocycles from peptide precursors. One is exemplified by microcin B17 biosynthesis, where the heterocycle-forming steps occur post-translationally, catalyzed by the microcin B17 synthase complex that consists of three discrete proteins, McbBCD.[26] The other emerges from nonribosomal peptide biosynthesis where the peptide elongation and heterocycle-forming steps proceed processively, catalyzed by NRPSs that are characterized by the cyclization (Cy) and oxidation (Ox) domains.[9b,27]

We have proposed that the BlmIV and BlmIII NRPS proteins are responsible for the biosynthesis of the bithiazole moiety of BLM on the basis of their predicted amino acid specificity (BlmIV for β-Ala and Cys and BlmIII for Cys) and the characteristic Cy and Ox domains (Figure 8).[9b,c] However, close examination of the A_0 domain of BlmIII revealed that it lacks several of the highly conserved motifs, indicative that it is not functional.[9c] The latter was confirmed by overproducing and biochemically characterizing the BlmIII-A_0 protein. This raises the question as to how BlmIII-PCP_0 is charged with Cys. We overproduced the BlmIV-(A_1-PCP_1-Cy_0) and BlmIII proteins and converted them into the corresponding holo-NRPS proteins in vitro in the presence of the Svp PPTase and CoA.[20] Upon incubation of BlmIV-(A_1-PCP_1-Cy_0) and BlmIII in the presence of ATP and [^{35}S]Cys, we observed covalent loading of Cys not only to PCP_1 but also to PCP_0, as evidenced by autoradiographic analysis, confirming that the A_1 acts both in *cis* (for PCP_1) and in *trans* (for PCP_0) to activate and load Cys to both PCPs (Figure 8, step a). After the two Cys are loaded to PCP_1 and PCP_0, the two Cy domains, Cy_1 and Cy_0, catalyze the transpeptidation and cyclization to form the two thiazoline rings (Figure 8, steps b and c). All Cy domains known to date are located upstream of its cognate A domain, as exemplified by the NRPS-1 module of BlmIV, but the NRPS-0 module is unique, whose Cy_0 (on BlmIV) and A_0-PCP_0-O_x domains (on BlmIII) are located on two separate proteins (Figure 8). This suggests that the formation of the first thiazoline ring involves a *cis* transpeptidation and cyclization step, while a *trans* transpeptidation and cyclization step is necessary for the second thiazoline ring formation (Figure 8, step b vs c) (Du, L.; Shen, B. Unpublished results). This mechanism, in fact, raises a very interesting stereochemistry question for the chiral carbon atom of the thiazolinylthiazole moiety (shown in Figure 8 with the *S* configuration). While this carbon atom is stereochemically cryptical in BLM biosynthesis, it has been established to have the *R* configuration for phleomy-

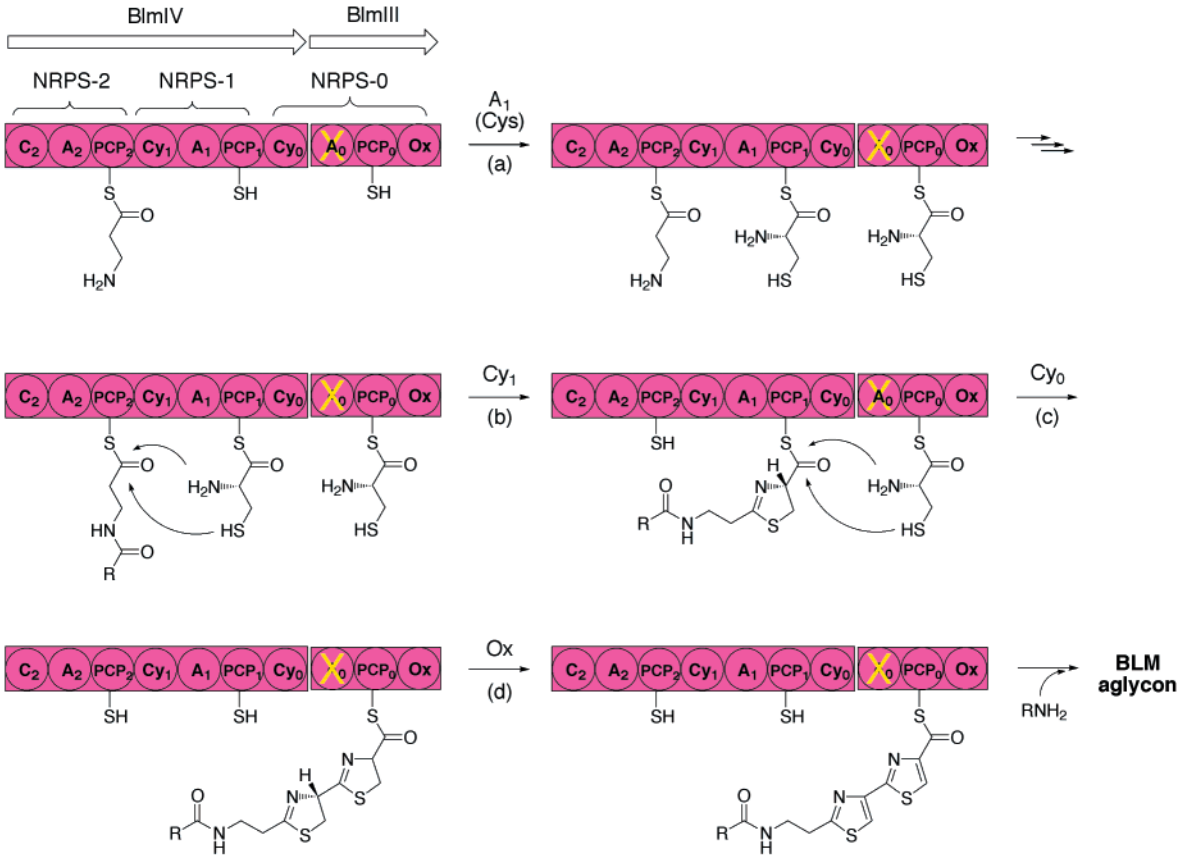

Figure 8. Schematic representation of the BlmIV/BlmIII-templated biosynthesis of the bithiazole moiety of BLM. A, adenylation; AL, acyl CoA ligase, C, condensation; Cy, condensation/cyclization; Ox, oxidation; PCP, peptidyl carrier protein.

cin,[28] which has been thought to be biosynthesized via a common pathway as BLM.[3a,4] In a mechanistic analogy, phleomycin biosynthesis could involve either a D-Cys or an additional epimerization step for L-Cys to furnish the *R* configuration. Experiments are in progress to characterize the phleomycin biosynthesis gene cluster to shed light on these issues.

Finally, the oxidative conversion of a thiazoline to a thiazole in thiazole-containing natural product biosynthesis requires an Ox domain.[9b,27f] The Ox domain has been identified from all thiazole-forming NRPS modules known to date,[9b,c,25b,c,29] and NRPS-templated thiazole biosynthesis proceeds according to the processive mechanism of Cys incorporation, transpeptidation, cyclization, and oxidation. The latter hypothesis is supported by the occurrence of two Ox domains for the bithiazole biosynthesis observed from the myxothiazol gene cluster.[29] We have overproduced both the BlmIII and BlmIII-Ox proteins and characterized the Ox domain as an oxidoreductase containing 1 molar equiv of noncovalently bound FMN as a prosthetic group.[9b] However, the NRPS-1 module of BlmIV lacks an Ox domain, raising the question as to how the first thiazoline ring is oxidized into a thiazole ring in BLM biosynthesis. We have previously proposed ORF8 as a possible candidate for this activity on the basis of an early cell-free study,[4c] suggesting the presence of a discrete oxidase.[5a,9c] Overproduction of ORF8 protein and biochemical investigation of ORF8 in vitro under all conditions tested so far, however, failed to confirm this activity (Du, L.; Shen, B. Unpublished results). In the absence of experimental data supporting the involvement of ORF8 in thiazole biosynthesis, we now have to consider an alternative hypothesis that the single Ox domain might be responsible for the BLM bithiazole biosynthesis by oxidizing both thiazoline rings (Figure 8, step d). Again, the Ox domain will have to act in *trans* and in *cis* to oxidize the first and the second thiazoline ring, respectively. Biosynthesis of the BLM bithiazole moiety by BlmIV and BlmIII, therefore, requires the intriguing juxtaposition and molecular recognition between the BlmIV and BlmIII proteins. We indeed found that BlmIV-A_1 alone, while functional in activating Cys, can no longer load Cys to the PCP_0 domain of BlmIII, presumably due to the lack of the precise interaction between the BlmIV and BlmIII proteins (Du, L.; Shen, B. Unpublished results).

Post-translational Modification of the Hybrid Blm NRPS−PKS by the Svp PPTase with Broad Carrier Protein Specificity. For NRPS and PKS to be functional, their carrier proteins must be converted from the apo-forms to the holo-forms by covalent attachment of the 4′-phosphopantetheine group to a highly conserved serine residue. This post-translational modification is catalyzed by PPTases that derive the 4′-phosphopantetheine group from CoA (Figure 1C).[7] Most of the PPTases characterized to date exhibit high carrier protein specificity, leading to the proposal that each 4′-phosphopantetheine-requiring pathway has its own post-translational modifying PPTase activity.[7b] In order for a hybrid NRPS−PKS to be functional, both PCP and ACP have to be modified. This raises an interesting question if a PPTase from a hybrid peptide−polyketide natural product producing organism exhibits relaxed substrate specificity toward both ACPs and PCPs.

Sequence analysis of the *blm* biosynthetic gene cluster failed to reveal an associated PPTase gene, suggesting that the *blm* NRPS and PKS genes are not clustered with their PPTase gene. We subsequently developed a PCR method for cloning PPTases of actinomycete origin and cloned the

Svp PPTase gene from *S. verticillus*.[20] The *svp* gene is mapped to an independent locus not clustered with any of the NRPS or PKS loci known to *S. verticillus*. We overproduced and purified the Svp protein and showed that Svp can efficiently 4′-phosphopantetheinate both type I and type II ACPs and PCPs from either *S. verticillus* or other *Streptomyces* species. As compared to Sfp,[7c,d] the only other PPTase known to accept ACPs, aryl carrier proteins (ArCPs), and PCPs from various organisms, Svp displays a similar catalytic efficiency for PCPs but a 346-fold increase in catalytic efficiency for the tested ACP.[20] We argue that the broad substrate specificity of Svp toward various carrier proteins is an intrinsic property for PPTases that are generally responsible for secondary metabolite biosynthesis. Pending experimental confirmation, we further speculate that a PPTase with broad carrier protein specificity might be a general solution to provide functional hybrid NRPS−PKS systems for hybrid peptide−polyketide natural product biosynthesis.[20]

Future Prospects. The availability of the *blm* gene cluster now has set the stage for investigating the molecular basis for intermodular communication between NRPS and PKS in hybrid peptide−polyketide natural product biosynthesis and for engineering novel BLM analogues by genetic manipulation of genes governing BLM biosynthesis. BlmIX/BlmVIII/BlmVII, a natural hybrid NRPS/PKS/NRPS system, serves as an excellent model for both hybrid NRPS/PKS and PKS/NRPS systems,[9c] and Svp, a PPTase with broad carrier protein specificity, should greatly facilitate the mechanistic characterization of hybrid NRPS−PKS.[20] Although sequence analysis and genetic studies are beginning to shed light on hybrid peptide−polyketide biosynthesis, revelation of the basic catalytic and molecular recognition features and structure−function relationship of the Blm megasynthetase most likely will rely on its in vitro biochemical and mechanistic characterization. The prerequisites for such studies are the expression of the *blm* NRPS and PKS genes, purification of the Blm NRPS and PKS proteins, reconstitution of the Blm megasynthetase from individual components, access to biosynthetic intermediates as enzyme substrates or mimics, and development of sensitive methods to assay enzyme activities. While progress has been made in improving heterologous expression in *E. coli* and *Streptomyces* hosts, overproduction of NRPS and PKS in functional form remains to be one of the greatest challenges in NRPS and PKS enzymology.[27,30]

Efficient genetic systems have to be developed for rational engineering of BLM biosynthesis in vivo. Genetic manipulation of BLM biosynthesis in *S. verticillus* in vivo has heretofore met with little success despite the exhaustive effort by other laboratories and our own. Although significant progress has been made in the past decade in introducing foreign DNA into *Streptomyces* species, it is far from certain if efficient genetic systems could be developed for every desired natural product-producing organism. The lack of a practical genetic system remains one of the major obstacles preventing us from applying the genetic principles to meet the biotechnological challenge for drug discovery and development in many organisms that are known to produce biologically or clinically important natural products but are poorly characterized physiologically and genetically. Methods to circumvent the latter difficulties are emerging, taking advantage of the genetically amenable host strains such as *S. coelicolor* or *S. lividans*.[31] For example, gene clusters smaller than 40-kb DNA can be directly introduced into these heterologous hosts for expression, on either replicating or integrating plasmids.[32] The latter plasmids can specifically integrate into the host chromosome, providing stable recombinants that often can be propagated without detectable loss of the plasmid even in the absence of antibiotic selection. Gene clusters larger than 40-kb DNA can be cloned into two or more coexisting plasmids—replicating, integrating, or the combination of both.[25b,33] Co-introduction of these plasmids to the heterologous hosts yields a recombinant strain expressing the entire gene cluster. Bacterial artificial chromosomes (BAC) that can be shuttled between *E. coli* (replicating) and *Streptomyces* (integrating), called ESAC, also have been developed.[34] The ESAC vectors can be used not only to generate genomic libraries with large inserts (up to 140-kb DNA insert) but also to reconstruct an entire gene cluster from existing overlapping clones through the iterative use of homologous recombination in *E. coli*. The ESAC constructs can be introduced into a *Streptomyces* host and integrated into the host chromosome. The resulting recombinant strains have been found to be rather stable, even without antibiotic selection, making it possible to carry out genetic analysis of large gene clusters from genetically intractable organisms in a suitable *Streptomyces* host. Complementary to the ESAC strategy, a stepwise homologous recombination method for direct integration of the entire gene cluster from overlapping clones into a neutral site of the heterologous host chromosome also has been reported.[35] This method in theory allows integration of the gene cluster into any desired positions of the host chromosome without leaving traces of the vector sequence, which could interfere with future genetic analysis in the resultant recombinant strain. Given the difficulty we have encountered so far with *S. verticillus*, we have now shifted our effort to express the entire *blm* gene cluster in a heterologous host. Once established, we hoped that genetic engineering of BLM biosynthesis will result in the production of novel BLM analogues, difficult to prepare by other means, some of which could lead to the development of anticancer drugs with improved therapeutic efficacy.

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