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The Chemical Versatility of Natural-Product Assembly Lines

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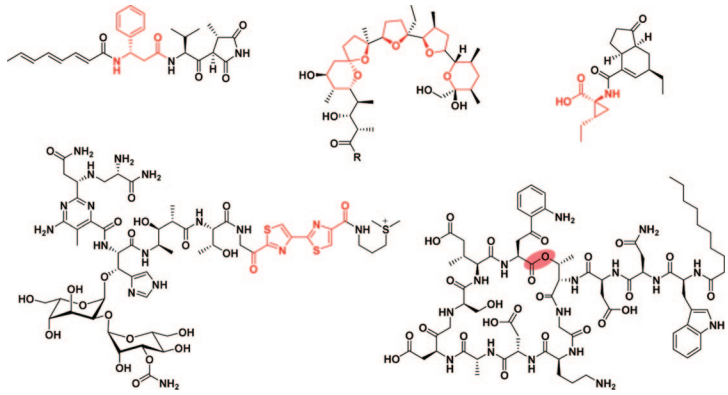

Microbial natural products of both polyketide and nonribosomal peptide origin have been and continue to be important therapeutic agents as antibiotics, immunosupressants, and antitumor drugs. Because the biosynthetic genes for these metabolites are clustered for coordinate regulation, the sequencing of bacterial genomes continues to reveal unanticipated biosynthetic capacity for novel natural products. The re-engineering of pathways for such secondary metabolites to make novel molecular variants will be enabled by understanding of the chemical logic and protein machinery in the producer microbes.

This Account analyzes the chemical principles and molecular logic that allows simple primary metabolite building blocks to be converted to complex architectural scaffolds of polyketides (PK), nonribosomal peptides (NRP), and NRP-PK hybrids. The first guiding principle is that PK and NRP chains are assembled as thioesters tethered to phosphopantetheinyl arms of carrier proteins that serve as thiotemplates for chain elongation. The second principle is that gate keeper protein domains select distinct monomers to be activated and incorporated with positional specificity into the growing natural product chains. Chain growth is via thioclaisen condensations for PK and via amide bond formation for elongating NRP chains. Release of the full length acyl/peptidyl chains is mediated by thioesterases, some of which catalyze hydrolysis while others catalyze regiospecific macrocyclization to build in conformational constraints. Tailoring of PK and NRP chains, by acylation, alkylation, glycosylation, and oxidoreduction, occurs both during tethered chain growth and after thioesterase-mediated release.

Analysis of the types of protein domains that carry out chain initiation, elongation, tailoring, and termination steps gives insight into how NRP and PK biosynthetic assembly lines can be redirected to make novel molecules.

1. Introduction

Many natural products of polyketide and nonribosomal peptide origin and hybrids thereof have useful therapeutic activities ranging from immunosuppression (cyclosporine, rapamycin, and FK506) to cancer (adriamycin and bleomycin) to infectious disease (erythromycin, β-lactams, and tetracycline).[1−8] Remarkably, complex chemical scaffolds can be fashioned from simple building blocks, such as acyl-CoAs (polyketides) and amino acids, both proteinogenic and nonproteinogenic (nonribosomal peptides), achieving high functional group density and restricted conformations for

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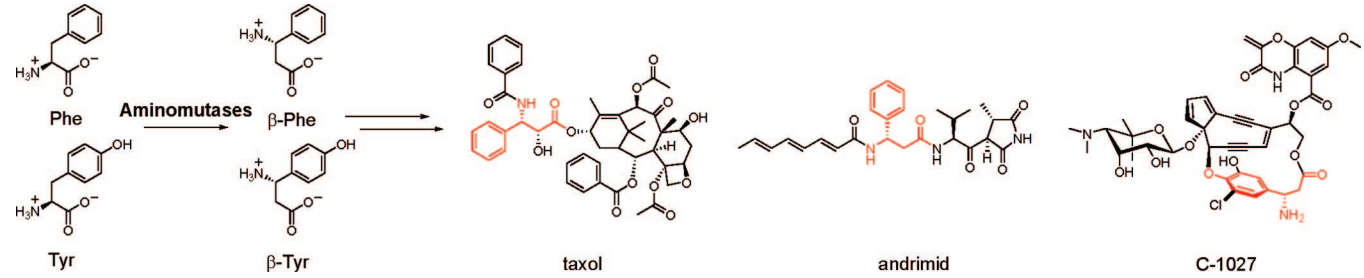
SCHEME 1



selective recognition by biological targets. Nonribosomal peptide synthetases (NRPS) and type-I polyketide synthases (PKS), as well as hybrid NRPS–PKS enzymes, build the growing chains as a series of elongating acyl-*S*-proteins, covalently tethered to the terminal thiol of phosphopantetheinyl arms, with themselves tethered to side chains of serine residues of carrier protein domains in the assembly line.[9]

The logic of NRPS and PKS assembly lines is modular, and the encoding genes in microbial genomes are almost always clustered. Natural-product acyl chains grow from an N-terminal initiation enzyme module through a series of elongation modules, one for each monomer added; then, a C-terminal termination module releases the full-length acyl chain from its covalent thioester linkage. Each module has at least three protein domains: (1) a catalytic domain for selection and loading of the monomer, e.g., malonyl- or methylmalonyl-CoA for PKS and amino acids for NRPS, (2) the pantetheinylated carrier protein domain to hold the thioesterified monomer, and (3) a catalytic domain that does chain extension, consisting of C–C bond formation to a β-keto thioester (a thioclaisen condensation) for PKS and C–N amide bond formation for NRPS. With these rules that govern acyl chain initiation, elongation, and termination, one can translate the DNA sequence into predicted assembly lines and sometimes predict the backbone structures of the encoded natural products.

In the past decade, the combination of microbial genome sequencing and *in vivo* and *in vitro* characterization of the chemical steps performed by NRPS and PKS assembly lines have led to insights into mechanisms for the formation of dedicated and unusual monomers, for tailoring chemistries. These create good prospects for the controlled generation of novel variants of natural products with optimized properties.

2. Generation and Utilization of Unusual Building Blocks

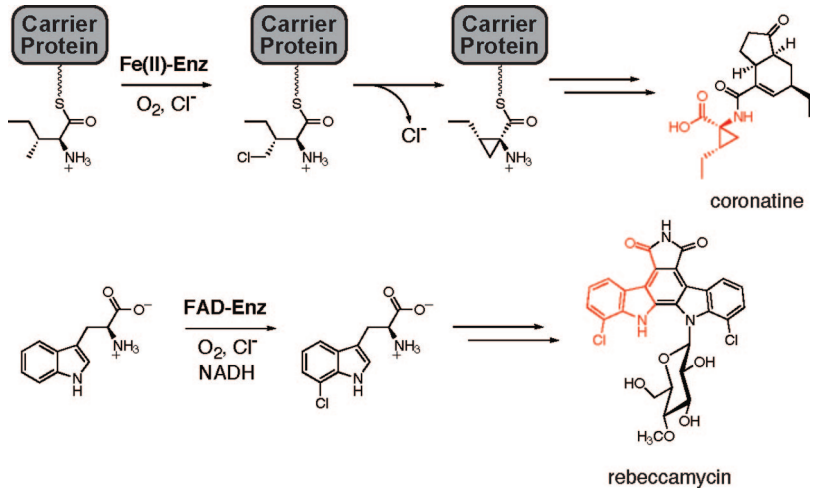
A diagnostic feature of nonribosomal peptides is the presence of amino acid monomers that are not found in proteins, including the aminoadipoyl moiety in the biosynthetic precursor to all penicillins and cephalosporins and the pipecolate moiety in the backbones of rapamycin and FK506. Most of the genes encoding enzymes for the synthesis of nonproteinogenic amino acid monomers are clustered with the NRPS assembly line genes for coordinate regulation for "just in time" inventory control. This has allowed for the investigation of biosynthetic diversion of primary metabolites to these dedicated monomers and augurs for effective portability of genes to make such unusual monomers in engineered microbes. Four recent cases are noted for diversification of NRP and NRP–PK scaffold backbones.

The first is represented by β-amino acid incorporation to modulate backbone connectivity in the natural products. The enediyne antitumor antibiotic C-1027 has a 3-Cl-4,5-$(OH)_2$-β-Phe residue,[10] while the widely used taxol also has a β-Phe moiety.[11] The pseudopeptide antibiotic andrimid[12] also has a β-Phe moiety.[13] In all three cases, it is now established that amino acid mutases containing a covalent methylene imidazolone cofactor convert Tyr or Phe, respectively, into the β-amino acids and then the assembly line domains specifically recognize and load only the β-amino acids (Scheme 1).

The lipopeptidolactone daptomycin was recently approved for the treatment of bacterial infections, including those caused by vancomycin-resistant enterococci.[14] Daptomycin and related natural products have a β-methyl Glu residue. An enzyme encoded in the daptomycin biosynthetic cluster has been shown to methylate the corresponding 2-keto acid with an electrophilic methyl group from *S*-adenosylmethionine followed by transamination to yield the 3-methyl-Glu that is specifically incorporated.

The plant phytotoxin coronatine, a bacterial mimic of the plant hormone jasmonic acid, is an NRP–PK hybrid, with 1-amino-1-carboxy-2-ethylcyclopropane (coronamic acid) as the amino acid moiety. The three-membered ring is formed from a γ-Cl-L-*allo*-Ile moiety,[15] which is in turn produced by the chlorination of a L-*allo*-Ile-*S*-carrier protein by a mononuclear nonheme iron halogenase.[16] Other cyclopropyl amino acid moieties may be generated in natural products by such

SCHEME 2



cryptic chlorination paths, and the nonheme iron halogenases are a novel class of O_2 and α-ketoglutarate-requiring catalysts, which can halogenate unactivated sp^3 carbon centers.[17] Electron-rich side chains of amino acids, such as tryptophan and tyrosine, can similarly be chlorinated but require less potent halogenation reagents; therefore, flavin adenine dinucleotide (FAD)-dependent halogenases are used for ortho-hydroxylation of Tyr side chains in vancomycin biogenesis and for forming 7-Cl-Trp in rebeccamycin[18] (Scheme 2).

There are many natural-product peptidolactones, including daptomycin noted above. We shall note below that chain termination steps can involve macocyclization and generate lactones by participation of Ser, Thr, or Tyr side chains. However, in such molecules as the K^+-ionophore valinomycin or the emetic toxin cereulide with multiple oxo ester linkages, α-hydroxy acid rather than α-amino acid monomers are required.[19] Recent studies have shown that these assembly lines actually select, activate, and covalently load α-keto acid monomers and then carry out chiral reduction on the assembly lines to yield the α-OH-acyl-*S*-pantetheinyl intermediates as nucleophiles required for chain elongation.[19] Portability of such enzymes to other systems is under investigation for backbone diversifications.

Monomer units for PKS assembly lines have been more restricted. In addition to the most common malonyl and methylmalonyl-CoAs, a variety of other starter units, including cyclohexenoyl and benzoyl CoAs, have been characterized. As extender units, ethylmalonyl- and methoxymalonyl-CoAs are used much less frequently than malonyl and methylmalonyl donors. Recent studies on the glycoyl and ethanolamine moieties of zwittermicin have revealed aminomalonyl- and hydroxymalonyl-*S*-carrier proteins as donors.[20]

3. Tailoring on Assembly Lines

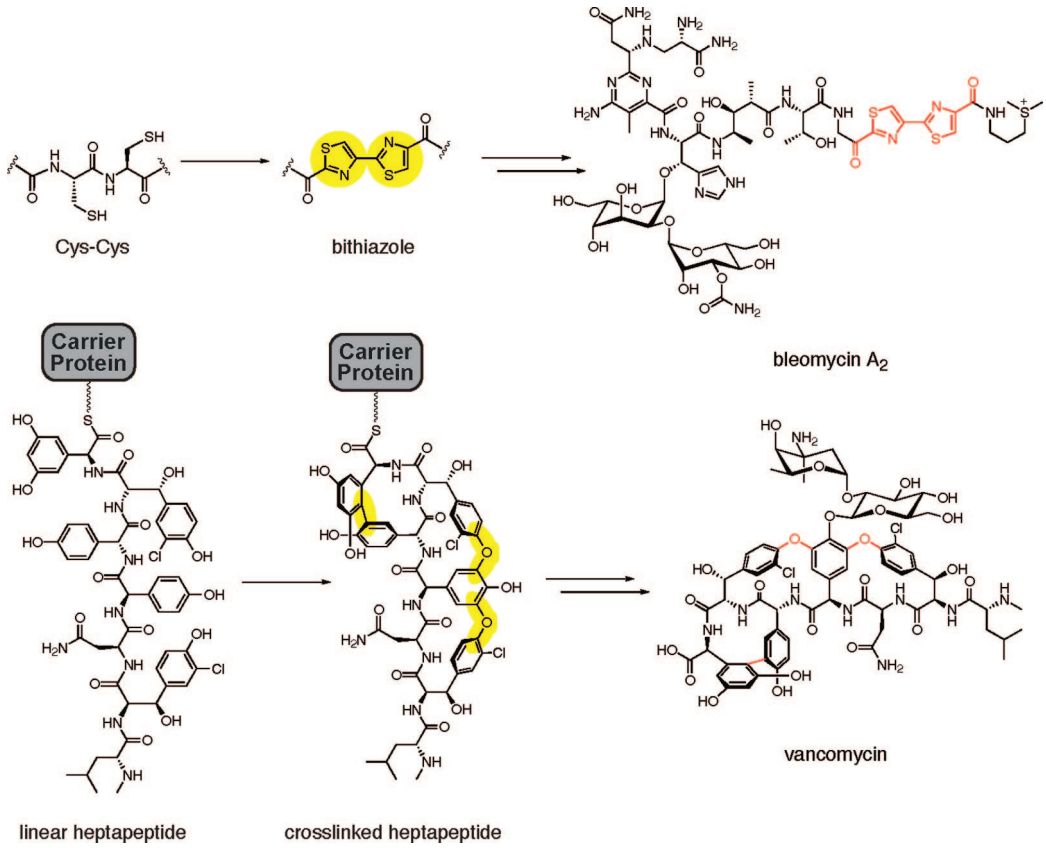
A variety of tailoring reactions during chain growth can occur on assembly lines. Most common is the insertion of additional protein catalytic domains *in cis* in one or more modules, exemplified by the presence of ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains in PKS type-I modules. The failure of one or more such auxiliary domains to act controls whether a β-CH_2, an α,β-enoyl, a β-OH, or a β-keto functionality gets carried forward to the next chain-elongation step in the assembly line.

Analogously, many NRPS modules contain epimerization domains to generate D-aminoacyl moieties or *N*-methyl transferase domains (7 of the 11 modules in cyclosporine synthetase) to control chirality and the N-methylation state, respectively, and thus modulate the protease susceptibility and water solubility of the products.[9]

A remarkable alteration of backbone connectivity in NRP scaffolds occurs when cysteine, serine, or threonine side chains are intramolecularly cylized and dehydrated to the thiazoline and oxazoline rings, respectively, by variant NRPS condensation domains. Redox adjustments can be catalyzed to reduce the thiazoline to thiazolidine or oxidize it to the stable thiazole. For example, tandem conversion of a pair of Cys residues during action of the bleomycin assembly line yields a bithiazole that is a DNA-intercalating moiety in the final bleomycin product (Scheme 3) and may account for some of its targeting to DNA.[21]

While many polyketides, such as erythromycin, have methyl branches introduced at C_α positions during chain elongation by use of methylmalonyl CoA as a nucleophilic mmonomer unit, other polyketides, such as jamaicamide,

SCHEME 3



bacillaene, and the antibiotic myxoviriscein have β branches. It has recently been determined that such β branches represent the intersection of isoprene and polyketide biosynthetic pathways with a generation of a branched β-hydroxymethylglutaryl-*S*-pantetheinyl-carrier protein as an intermediate that undergoes dehydration and then decarboxylation to the Δ^2-prenyl-*S*-carrier protein, ready for the next round of PKS-mediated chain elongation.[22] If the branching enzyme machinery, acting here *in trans*, is portable, this might allow for the tailoring of polyketide scaffolds at both α and β positions during chain elongations.

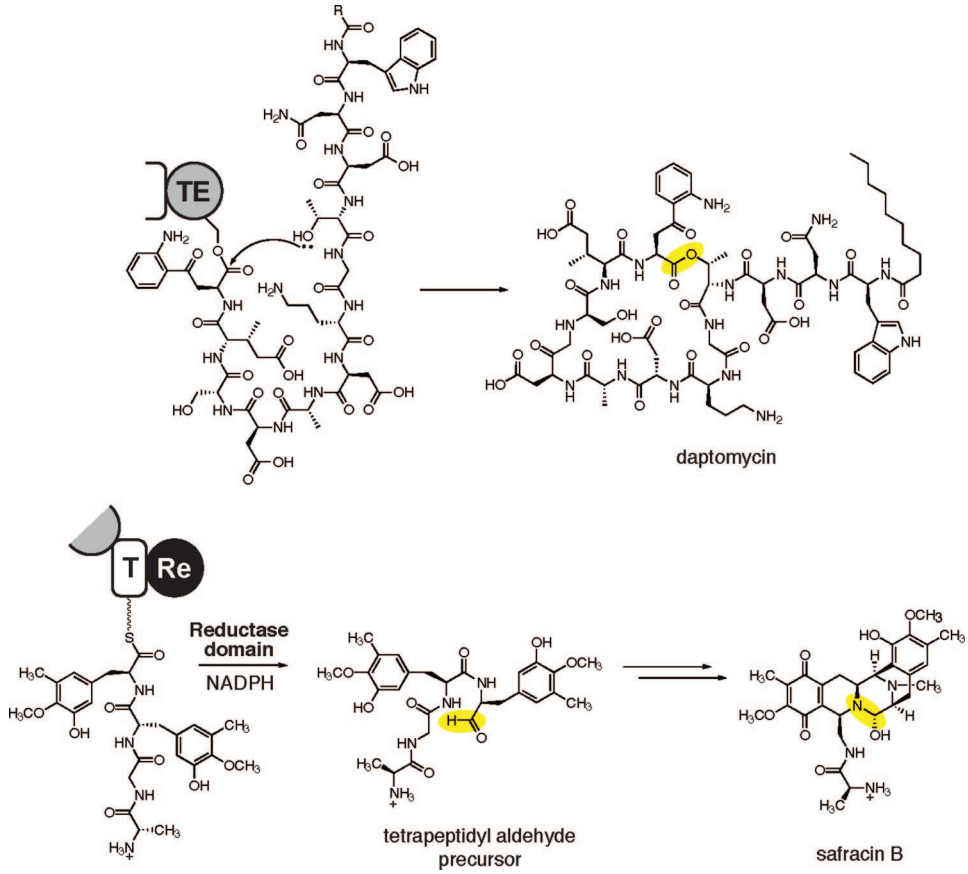
Additional tailoring enzymes that work *in trans* on assembly lines are now known and have been characterized to different extents. One set is the FAD-dependent aromatic halogenases noted above in the context of chlorination of Tyr_2 and Tyr_6 during elongation of the heptapeptide scaffold of vancomycin. In the comparable gene cluster for a related glycopeptide bahlimycin, there are three heme iron monoxygenases, OxyABC, that act to cross-link the heptapeptidyl-*S*-carrier protein, generating two aryl ether linkages between Tyr_2 and $PheGly_4$ and $PheGly_4$ and Tyr_6 and a direct C–C cross-link between $PheGly_5$ and $PheGly_7$.[23,24] These rigidifying cross-links create the conformation required for vancomycin and related glycopeptide antibiotics to bind bacterial peptidoglycan D-Ala-D-Ala termini with high affinity (Scheme 3). A comparable conformation-restricting aryl ether cross-link is analogously installed during arylomycin maturation.[25]

4. Chain-Release Mechanisms

Three major chemical routes for disconnection of the covalent thioester linkages of PKS and NRPS acyl chains when they reach the most downstream carrier protein domains on assembly lines have been observed.[26,27] The first is hydrolytic, typically involving prior transfer of the full-length acyl, peptidyl, or hybrid chain from carrier protein pantetheinyl thiol to the active site CH_2OH of a Ser residue in a thioesterase (TE) domain. The acyl-*O*-TE intermediate can then undergo catalyzed hydrolysis to release the free acid, e.g., the triply cross-linked heptapeptide acid scaffold in vancomycin.

The second route and the one of most medicinal interest is the intramolecular capture of the acyl-*O*-TE by a nucleophile within the growing acyl chain. In a NRP, this can be the N-terminal NH_2 (tyrocidine) or a side chain $-NH_2$ (bacitracin) to give cyclic lactams. When a side chain –OH is the selected nucleophile, a macrolactone results, as in the antibiotic dap-

SCHEME 4



tomycin, where a Thr_4-*O*-kyneurinine$_{13}$ ester link occurs[27] (Scheme 4). TE domains can also be macrolactonizing in PKS assembly lines, as is the case for erythromycin and hundreds to thousands of other macrolides. Excised TE domains retain macrocyclization activity and have been utilized to generate variant macrocyclic natural-product structures.[28]

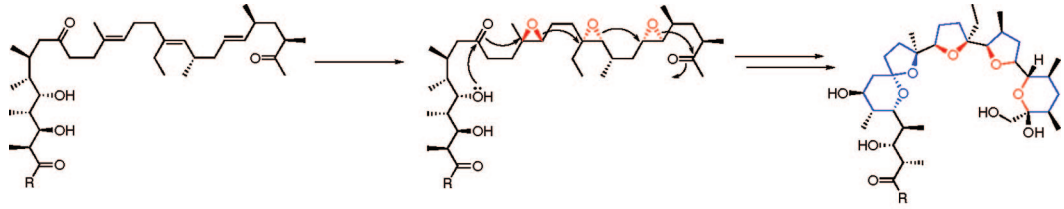
A third route of chain release is reductive, utilizing NAD(P)H as a hydride-transfer cosubstrate. Usually, the most downstream catalytic domain in such assembly lines is a reductase domain rather than a TE domain (Scheme 4). The two-electron reduction of an acyl thioester generates the thioacetal, which spotaneously decomposes to release the product aldehyde. In some cases, the aldehyde appears to be held in the reductase active site and undergo a second round of reduction to the alcohol. Nascent peptide aldehydes can undergo intramolecular cyclization as seen in the formation of safracin B and nostocyclopeptide.[29] The potential to control the route of peptide or polyketide chain disconnection by engineering/swapping of C-terminal catalytic domains in assembly lines offers prospects for useful manipulation of nascent natural-product structures.

5. Post-Assembly-Line Tailorings

Many of the molecules released by hydrolytic, reductive, or macrocyclizing TE domains on NRPS and PKS assembly lines undergo further tailoring by dedicated enzymes to create the final structures with potent and specific biological activities. Three main types of enzyme-mediated modifications occur: glycosylations, acylations, and oxidations by enzymes in biosynthetic gene clusters.[3,9]

Glycosylations, using nucleotide diphosphosugar cosubstrates, occur widely on nonribosomal peptide and polyketide frameworks.[30,31] This holds both in macrolides, such as erythromycin, which becomes doubly glycosylated in steps essential for the gain of antibiotic activity, and in aromatic polyketide antitumor drugs, such as daunomycin. Although O-glycosylations predominate, N-glycosylations and C-glycosylation tailoring events are known. Recent studies by Thorson and colleagues have show that antibiotic glycosyltransferases have equilibrium constants usefully close to unity, allowing for the transfer of specific sugars regioselectively between enediyne and glycopeptide scaffolds.[32]

SCHEME 5



N- and O-acylations catalyzed by acyltransferases titrate the hydrophobic/hydrophilic balance in natural products, as exemplified by the decanoyl chain on the glucosyl moiety in the glycopeptide antibiotic teicoplanin and in the the acyl chains of the macrolide carbomycins. Variation of the acyl chains can control physical properties. In the commercial fermentation of daptomycin, decanoic acid is added to overwhelm endogenous fatty acid pools and produce a homogenously acylated product.[14]

Oxygenative tailoring of PK and NRP metabolites is a common maturation step. We noted the cross-linking of the heptapeptide scaffolds of the vanomycin family NRPs while on the assembly lines, but there are many oxidative transformations that occur post-assembly-line release. Among the most spectacular are the tandem enzyme-mediated epoxidations of an acyclic triene in polyether biosynthesis that set up a cascade of epoxide openings (Scheme 5) that lead to the five cyclic ether rings in monensin.[33]

There are many other late-stage enzymatic tailoring steps whose chemistry is incompletely worked out but where the functional-group changes would be useful to have in a natural-product enzymatic toolbox. Among these are the methylation and rearrangement of a transannular disulfide to a methylthioacetal during echinomycin maturation.[34] Also, there are dozens of NRP and PK metabolites with tetramic-acid-derived rings that may be useful pharmacophores.[35] The antibiotic andrimid has such a methylsuccinimide moiety at its C terminus, and this is thought to be a recognition element for the interaction with its target, bacterial acetyl-CoA carboxylase.

6. Concluding Remarks

Natural products continue to be of great interest for drug design and new therapeutics, either in their natural form, as scaffolds for semisynthetic variation, or as templates for simplification while retaining pharmacophores that confer potency and specificity. Nonribosmal peptides, polyketides, and natural-product hybrids represent a large class of such bioactive molecules. NRPS and PKS assembly lines create estimable chemical diversity from a few builiding blocks, modular acyl-transfer chemistry, and a set of *in cis* and *in trans* reactions for pre- and post-assembly-line tailoring. Great progress has recently been achieved in deciphering the capabilities of the enzymatic machinery and the molecular logic of the chemical steps involved. This has opened the door for both *in vivo* and *in vitro* manipulations of programmed and combinatorial biosynthesis to redirect the natural-product assembly lines of nature.

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BIOGRAPHICAL INFORMATION

Christopher T. Walsh is the Hamilton Kuhn Professor of Biological Chemistry and Molecular Pharmacology (BCMP) at Harvard Medical School (HMS). He has had extensive experience in academic administration, including Chairmanship of the Massachusetts Institute of Technology (MIT) Chemistry Department (1982–1987) and the HMS Biological Chemistry and Molecular Pharmacology Department (1987–1995), as well as serving as President and CEO of the Dana Farber Cancer Institute (1992–1995). His research has focused on enzymes and enzyme inhibitors, with recent specialization on antibiotics. He and his group have authored over 600 research papers; a text, *Enzymatic Reaction Mechanisms*; and two books, *Antibiotics: Origins, Actions, Resistance*, and *Posttranslational Modification of Proteins: Expanding Nature's Inventory*. He is a member of the National Academy of Sciences, the Institute of Medicine, and the American Philosophical Society.

FOOTNOTES

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