Published in final edited form as:

Curr Opin Pharmacol. 2013 October ; 13(5): 678–687. doi:10.1016/j.coph.2013.07.002.

Emerging Trends in the Discovery of Natural Product Antibacterials

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Abstract

This article highlights current trends and advances in exploiting natural sources for the deployment of novel and potent anti-infective countermeasures. The key challenge is to therapeutically target microbial pathogens exhibiting a variety of puzzling and evolutionary complex resistance mechanisms. Special emphasis is given to the strengths, weaknesses, and opportunities in the natural product antimicrobial drug discovery arena, and to emerging applications driven by advances in bioinformatics, chemical biology, and synthetic biology in concert with exploiting the microbial phenotype. These orchestrated efforts have identified a critical mass of lead natural antimicrobials chemical scaffolds and discovery technologies with high probability of successful implementation against emerging microbial pathogens.

Keywords

multidrug resistance; natural antimicrobials; high throughput technologies; phenotypic based discovery; natural product biosynthesis; bioinformatics; genomics; synthetic biology

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Introduction

Multidrug resistant microbes account for a substantial percentage of nosocomial infections and represent a serious threat to human health. In addition, their refractoriness to commonly used drugs poses a significant therapeutic challenge. These include established threat organisms such as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) as well as emerging pathogens such as carbapenem-resistant *Klebsiella pneumoniae* CRKP, the New Delhi metallo-beta-lactamase containing Enterobacteriaceae, [1–4].

Inappropriate or unnecessary antibiotic prescriptions, their excessive use in the agricultural and livestock industries, and the lack of patient adherence to full antibiotic regimens, all of which select for resistant bacteria, appear to be the key contributors to the emergence of antibiotic resistance. Because of the increasing threat of multidrug resistant pathogens and the continuing evolution of resistance, there is a pressing need to develop new antibacterial therapies.

Beginning with the discovery of penicillin in 1929, natural products have been the main source of new antibacterial drugs and have revolutionized the treatment of infectious diseases. In the past 80 years numerous classes of natural product antimicrobials (Fig 1) have been discovered. Natural products and their analogs continue to play a prominent role in medicine, accounting for two-thirds of new antibacterial therapies approved from 1980–2010 [5] as well as several antibacterials currently in clinical trials (Table 1, Fig 2). The efficacy of natural products as antibacterial agents likely stems from the fact that they have been honed by evolutionary processes to be bioactive, thereby giving the organisms capable of making the most efficacious compounds a selective advantage in the environment. In spite of the promise of natural products, the discovery and development of new antibacterial drugs by large pharmaceutical companies has waned over the last two decades. Companies with active antibacterial discovery programs have gradually diminished these efforts [6]. This de-emphasis on industrial development of new antimicrobials may be due to the perceptions that the market is saturated, that new drug targets are scarce, and that obtaining regulatory approval is increasingly difficult [7]. This gloomy reality is often described as the “*end of the antibiotic era*”. Interestingly, this landscape has generated a vacuum in which smaller biotechnology firms can innovate and compete. For example, in 1995, the 10 largest pharmaceutical companies had only 3 antibacterials in clinical development compared to 17 compounds being developed by the biotechnology industry [8,9]. However, these efforts have only occasionally yielded novel classes of antimicrobials. Rather, the majority of new drugs have arisen by alteration of existing drugs to improve efficacy, reduce resistance, and minimize toxicity [7].

Discovery and development of new antibacterial agents is experiencing a renaissance brought on by the confluence of diverse fields and expertise, and once again natural products are coming to the forefront. The speed by which new antibacterial drug targets and mechanisms of action are being discovered is likely to increase due to improvements in existing methodologies and the introduction of new technologies. An improved understanding of the phenotypic determinants of microbial virulence and pathogenicity, including drug efflux, biofilm formation, and quorum sensing, is setting the stage for development of new phenotype-based antibacterial screens that have the potential to identify new drugs previously overlooked by conventional antibiotic discovery platforms. Rapid advances in our understanding of and ability to manipulate natural product biosynthesis pathways, increasingly driven by informatics, chemical biology, and synthetic biology, is

beginning to allow us to more efficiently translate the genetic information encoding natural products into clinically useful chemical output – new drugs [10].

This article highlights some of the recent advances as well as the emerging challenges of natural product antibacterial drug discovery and development. Natural product drug discovery, slowly marginalized by the biopharmaceutical industry over the last 20 years, is poised to make a come-back, supported by new legislative and funding initiatives to spur development of antibacterial discovery programs and thereby ensure a continued supply of the new drugs needed to combat today's emerging infectious disease threats.

1. Advances in Bioactivity-Guided Discovery

1.1. New Targets

Both academic and industrial settings have identified a list of targets that have been rediscovered, revitalized, or exploited from scratch. The synthesis of the bacterial peptidoglycan has been recognized for over 50 years as fertile ground for antibacterial discovery. Initially, empirical screening of natural products for inhibition of bacterial growth detected many chemical classes of antibiotics whose specific mechanisms of action were eventually determined [11,12]. The filamentous temperature-sensitive mutant Z (FtsZ), a protein essential for cell division, is highly conserved among both bacteria and archaea. It is present in almost all species of bacteria and is the ancestor of the eukaryotic tubulin. The three-dimensional structures of both proteins are remarkably similar despite low-level (10–18%) amino acid similarity [13]. Recent evidence that the plant antimicrobial berberine can also target FtsZ in the nematode *Onchocerca volvulus* is also intriguing [14].

Inhibiting translation via the therapeutically unexploited with respect to natural products A-site of the bacterial ribosome is a potentially promising new avenue for antibacterial therapy. Bottromycins, modified peptides that bind to the A-site and are active against Vancomycin-Resistant Enterococci (VRE), have recently been the target of bioengineering studies aimed at lead optimization [15].

Recent work on pyridomycin, a compound with antimycobacterial activity produced by *Dactylosporangium fulvum* illustrates the power of genomics in determining mechanism of action. Examination of pyridomycin-resistant mutants of *Mycobacterium tuberculosis* by whole-genome sequencing and subsequent genetic confirmation identified the NADH-dependent enoyl-acyl carrier protein reductase InhA as the principal target of pyridomycin. Structural studies of InhA identified it as a new drugable target. The same study also provided evidence for the increased susceptibility of isoniazid-resistant mycobacterial clinical isolates to pyridomycin [16].

Antimetabolites, natural mimics of essential metabolites such as coenzymes, are also considered lead structures for the development of new antibiotics. Roseoflavin, an antimetabolite of riboflavin (vitamin B2), identified in *Streptomyces davawensis*, inhibits *Listeria monocytogenes* at very low concentrations [17]. Similarly, the natural product CJ-15,801, which shares structural similarity with pantothenic acid, the vitamin precursor of coenzyme A (CoA), and therefore likely blocks a step in CoA biosynthesis or utilization, was found to inhibit *S. aureus* [18].

1.2. New Assays

Advances in microbial cell physiology shed light on a series of pathways and phenotypes that may serve as attractive targets for antimicrobial drug discovery. A common theme is an attempt to dissect at the molecular level the social (intercellular) interaction in both synthetic and natural microbial populations. It is thought that these interactions are important

determinants of antibiotic resistance and tolerance [19]. A few examples illustrate the importance of social interaction in natural antibiotic discovery and the variation of antagonistic behaviors between bacteria “in the wild”. A screen of approximately 35,000 possible mutual interactions among *Vibrionaceae* ocean isolates showed a direct correlation between genotypic clusters that participate in natural antibiotic production and resistance and cohesive habitat association. Genetic analysis of these populations indicates that a small proportion of the population are antibiotic producers while the majority were resistant. These results support the idea that conspecifics interact cooperatively and that natural antibiotics are used as signal-mediators for competition with other species rather than the means for individual genotypes to achieve dominance [20].

Studies of the diversity of natural products made by bacterial communities in soil-and plant-associated environments and their activity patterns against coexisting microorganisms has revealed multiple activities beyond the historical perception of these compounds as weapons used in microbial conflicts [21]. Many biological processes are regulated by antibiotics, including changes in development that promote survival in adverse conditions, virulence toward host plants, and modulation of multi-trophic interactions. These recent studies challenge us to develop innovative phenotype-based antibacterial assays that are responsive to the dynamic nature of microbial communities. We highlight four such assay platforms that utilize natural products as lead compounds.

1. *Inhibition of drug efflux using natural product efflux pump inhibitors (EPIs)* in combination with other drugs has emerged as a potentially fruitful avenue for antibacterial therapy. Both novel and previously known natural products that are active against several microbial efflux systems have recently been reported [22,23]. The concept of restoring and enhancing the utility of antimicrobials by employing EPIs is appealing but is not yet at a therapeutic stage. Discovery efforts are multidimensional and ongoing but the gaps beyond the methodology as well as the main concepts of efflux are barring identification of lead EPIs for clinical implementation [24].
2. *Quorum sensing inhibition (QSI)* has been proposed as an attractive way to combat pathogens since QS systems are known to be key regulators of virulence factor production. QSI may target the synthesis of QS signal molecules, interfere with signal secretion or degradation, inhibit binding of QS signal molecules to their receptors, or inhibition downstream signal transduction cascades. Research in identifying natural products QSIs has recently intensified both conceptually as well as the wealth of available lead chemotypes. Two distinct examples are provided here. Natural product honaucins A-C isolated from the cyanobacterium *Leptolyngbya crossbyana* exhibited potent inhibition of both bioluminescence, a QS-dependent phenotype, in *Vibrio harveyi* BB120 and lipopolysaccharide-stimulated nitric oxide production in murine macrophages [25]. Bioassay-guided fractionation of garlic extracts, led to the identification of ajoene, a sulfur-containing compound as a QSI that directly interferes with QS-related genes in *P. aeruginosa* [26].
3. *Eradication of persister cells*, specialized non-dividing survivor cells protecting bacterial populations from antibiotic killing, is also a potential route to antibacterial therapies. Persisters may survive challenge with antibacterials by virtue of their metabolic inactivity. Eradication of these cells is currently challenging because the exact mechanisms of persister formation are still unclear. However transcriptomic analysis of isolated persisters suggests that toxin/antitoxin modules play an important role in the phenomenon [27]. Interestingly, both natural product

antibiotics mitomycin and rifampin were found to enhance killing of microbial populations through mechanisms consistent with persister eradication [27].

4. *Protection of the host from infection* is primarily based on recognition of pathogenic cells by the immune system. Toll-like receptors (TLRs) are an integral pattern-recognition component of the host immune response. It was shown that the orphan receptor TLR13 in mice recognizes the region of bacterial ribosomal 23S rRNA containing the binding site for macrolide, lincosamide, and streptogramin group (MLS) antibiotics (including erythromycin). Importantly, 23S rRNA from clinical isolates of erythromycin-resistant *S. aureus* and oligoribonucleotide-based ligands mimicking MLS resistant 23S rRNA failed to stimulate TLR13, suggesting that mutations in the 23S rRNA may allow pathogens to evade the immune response [28]. Immuno-therapeutics are also a potential means of antibacterial therapy as illustrated by the recent report of raising human monoclonal antibodies and polyclonal sera against the common bacterial surface polysaccharide, poly-1,6-*N*-acetylglucosamine (PNAG) [29].

2. Advances in Biosynthesis-Guided Discovery

Synthetic and biosynthetic chemists endeavor to produce natural products and their analogs cheaply and efficiently. Understanding the details of natural product biosynthetic pathways is key to accomplishing this goal. To date ~700 bacterial and fungal natural product biosynthetic gene clusters, many of which encode antibacterial compounds, have been cloned and sequenced, leading to functional elucidation of many of the biosynthetic steps in common natural product biosynthetic modes: type I [30] and type II polyketide synthases (PKS-I and PKS-II, respectively), non-ribosomal peptide synthases (NRPS) [31], and ribosomal peptide (RiPP) biosynthetic systems [32]. These studies have revealed that natural product pathways are modularly organized and have been combinatorially rearranged through evolution to produce the staggering array of observed structures. The diversity-oriented nature of these pathways implies that many new enzymatic activities, biosynthetic pathways, and biosynthetic paradigms remain to be discovered.

Our understanding of natural product biosynthesis allows combined bioengineering and chemical approaches for constructing natural product analogs. Both *in vivo* unnatural amino acid mutagenesis and chemoenzymatic synthesis strategies were recently used to create unnatural amino acid-bearing analogs of ribosomal peptides cyanobactin [33], an inhibitor of drug efflux, and lactacin 481 [34], an antibacterial compound targeting peptidoglycan biosynthesis. In the latter case, analogs with improved potency were identified. Precursor-directed biosynthesis, in which synthetic compounds mimicking a biosynthetic intermediate are fed to a producing organism, continues to be an effective strategy for generating natural product analogs. This strategy was recently used for production of an equipotent erythromycin analog bearing an easily derivatizable alkyne functional group [35]. The ability to biosynthetically install such “chemical handles” into natural products will be invaluable in medicinal chemistry and probe development for mechanism of action studies and high throughput screening.

Our extensive knowledge of natural product structure, bioactivity, and biosynthesis is now being applied in a holistic manner to discover new bioactive natural products. StreptomeDB [36], a database that integrates from literature mining these various types of information on >2400 bioactive natural products from *Streptomyces* species, facilitates *in silico* screening for new bioactivities. BioMAP [37], an experimentally obtained database of activity profiles of a panel of natural product structural classes against clinically relevant bacterial strains, establishes bioactivity fingerprints useful for identifying new classes of antibacterial compounds in crude extracts.

It is apparent from analysis of microbial genome sequence data that thousands of unstudied natural product gene clusters are encoded in microbial genomes. The ability to reliably identify these gene clusters and to accurately predict the structures of encoded compounds from sequence would enable rapid *in silico* screening for the presence of novel natural product biosynthetic capability antiSMASH [38], a software package designed for this purpose, is a move in this direction. However, because many natural product gene clusters are “cryptic”, or transcriptionally silent in particular culture conditions, experimental techniques such as transcriptomics combined with methods for activating cryptic clusters such as variation of culture conditions, co-culture with other microbes, disruption or overexpression of cluster-specific regulatory proteins (CSRs), and insertion of strong constitutive promoters [39] will be required to reap the full benefits of *in silico* predictions. Nevertheless, we expect rapid advances in this area in the near future.

The vast majority of microbes are not amenable to laboratory cell culture, leaving a huge portion of natural product diversity unexplorable by traditional culture-dependent methods. Recently metagenomics has been applied to discovery of new antibacterial compounds tetarimycin [40] and fasamycin, an inhibitor of the fatty acid biosynthesis protein (FabF) [41]. In these studies, cosmid libraries were constructed from soil environmental DNA, heterologously expressed in *Streptomyces albus*, and the transformants screened for antibacterial activity. Cosmids from active clones were sequenced and the structures of the encoded compounds were determined by NMR and X-ray crystallography. This approach holds promise for accessing compounds from uncultivable organisms. However, limitations of this approach lie in the fact that most gene clusters are not active when heterologously expressed and many natural product gene clusters are too large to be captured intact on a single cosmid.

The large volume of data linking organisms with the natural products they produce is beginning to reveal that natural product biosynthesis is unevenly distributed in nature. A recent study on Cyanobacteria correlated natural product biosynthesis with only a few clades within the phylum [42]. Such analyses can facilitate phylogenetics-based bioprospecting. Phylogenetic analysis of individual natural product gene amplicons can also be used to predict the presence of specific biosynthetic subclasses and to differentiate novel versus known compounds in new organisms or environmental samples. **Natural Product Domain Seeker**, NaPDoS [43], a sequence-based phylogenetic classifier of PKS-I ketosynthase domains and NRPS condensation domains, is a move in this direction.

Experimental “-omics” tools such as transcriptomics, proteomics, and metabolomics are beginning to be applied to discovery of natural products and the gene clusters that encode them. These approaches have the advantage that they detect only transcriptionally active gene clusters and their downstream protein and metabolite products. A transcriptome mining approach [44] was used to discover previously unknown biosynthesis of four ansamycin class compounds in *Streptomyces flaveolus*, a strain whose genome had not been sequenced. A cDNA library from *S. flaveolus* was probed by PCR for PKS-I and NRPS-encoding cDNAs. Amplicon sequencing, gene disruption, and metabolite HPLC profiling of mutants led to identification of the compounds and their encoding gene cluster.

Another example [45] involves the discovery of the novel non-ribosomal peptide koranimine from an environmental *Bacillus* species using direct peptide sequencing tandem mass spectrometry of high molecular weight protein bands of PKS-I and NRPS. The resulting peptide sequences were used to identify the gene cluster which was then sequenced and analyzed in order to predict the structure of the encoded compound whose structure was confirmed to be koranimine by small molecule mass spectrometry.

Finally, the therapeutically promising antibacterial agent arylomycin A2 was identified in *Streptomyces roseosporus* using an innovative combined imaging mass spectrometry (IMS)/short sequence tagging (SST) strategy [46]. *S. roseosporus*, whose genome had been sequenced, was spotted onto lawns of *S. aureus* and *Staphylococcus epidermidis*. IMS, a mass spectrometric technique that spatiotemporally detects intact masses of small molecules produced by a bacterial colony, was used to identify natural product ions associated with zones of inhibition. Mass fragmentation patterns of the molecules provided short sequence tags that, together with bioinformatics analysis, unambiguously identified the NRPS gene cluster encoding the compound and led to structural assignment of the compound as that of arylomycin A2.

Most antibacterial natural products and their gene clusters studied thus far are from Actinobacteria, although Cyanobacteria, Proteobacteria, Firmicutes, and Fungi have emerged as additional sources. However, because many of these organisms grow slowly and are difficult to manipulate genetically, heterologous production of natural products in laboratory strains such as *E. coli* is advantageous. The marked differences in codon usage, gene regulation, levels of precursor metabolites, and compound self-resistance between most producing organisms and *E. coli* are so great that heterologous expression is extremely challenging [47]. Successful examples, such as those described above, are typically limited to small gene clusters that use ubiquitous precursors, and/or involve both optimizing gene expression and increasing precursor supplies by engineering. Successful heterologous expression of two out of ten natural product gene clusters from the closely related α -proteobacterium *Photorhabdus luminescens* in non-metabolically engineered *E. coli* was recently achieved. The 80% attrition rate was attributed to toxicity and/or lack of expression [48]. Steps toward optimizing *E. coli* for production of polyketide compounds by heterologous expression of pathways for key precursors propionyl-CoA and methylmalonyl-CoA, which are absent in wild-type *E. coli*, were successful, resulting in intracellular levels comparable to endogenous acyl-CoAs [49]. Advances in metabolic engineering and the decreasing cost of gene synthesis suggest that production of a variety of natural products in laboratory strains such as *E. coli* will soon become a reality.

Conclusions and future perspectives

Some of the early chemotherapeutic antimicrobials originated from synthetic chemistry (e.g., dye research), as embodied by sulfa drugs, in the early 1930s. Indeed, some of these chemotherapeutics are among the most widely used bactericidal (e.g., quinolones) and bacteriostatic (e.g., sulfonamides) compounds. However, innovation in natural products research remains the main driver in antibiotic discovery. Since the introduction of antibiotics, we have witnessed advances in a variety of scientific fields and concurrent appearance of changes in the global socioeconomic environment that have led to complex clinical problems such as the emergence of microbial multidrug resistance. Effective countermeasures to this adaptive response are not trivial and there is certainly no single solution to the problem. This article outlines evidence for lead antimicrobial structures and technologies that are stemming from exploiting nature. Some of these are closer to the bench and others closer to the bedside. Yet significant changes to the current regulatory environment are essential for robust and timely clinical implementation of new therapies.

Acknowledgments

Funding

Work in the authors laboratories was supported by the NIH (grant 5U54MH084690-02 to GPT), DTRA (contract number HDTRA1-13-C-0005 to GPT), NCRR (5P20RR016480-12 to CGB) and NIGMS (8P20GM103451-12 to CGB)

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Highlights

- Bioactivity based discovery has yielded an array of new antibacterial targets
- Emphasis in exploiting phenotypic determinants and intra- and inter-species interactions
- Efflux, quorum sensing, and host-based therapeutics are appealing discovery platforms
- Biosynthesis-guided discovery integration with informatics chemical and synthetic biology

Figure 1. Natural versus synthetic classes of natural product antimicrobials. Natural products are shown in pink and red and synthetics are shown in white and grey. The year of discovery of the first representative of each class is indicated in parentheses.

Figure 2.
Chemical structures of natural antibacterials detailed in this article, natural and natural-derived antibiotics recently approved or under clinical investigation

Figure 3.
The key elements, tools, and outcomes of biosynthesis-guided natural products discovery using both natural and engineered biological systems.

Table 1

Recently approved/under clinical investigation natural and natural derivative antibiotics (American Medical Association <http://www.ama-assn.org/ama> and Food and drug administration <http://www.fda.gov>)

Structure	Activity Range	Regulatory status	Class	Target/MOA
Fidaxomicin	broad spectrum	FDA (5/2011)	tiacumicin/macrolactone	RNA synthesis
Daptomycin	Gram-positives	FDA (11/2003)	lipopeptide	peptidoglycan cell wall
Retapamulin	Gram-positives	FDA (4/2007)	pleuromutilin	protein synthesis
Telavancin	Gram-positives	FDA (9/2009)	lipoglycopeptide/vancomycin deriv.	peptidoglycan cell wall
Telithromycin	broad spectrum*	FDA (4/2004)	ketolide	protein synthesis
Omadacycline	broad spectrum*	Phase III trials	aminomethylcicline/tetracycline deriv.	protein synthesis
Oritavancin	Gram-positives	Phase III	lipoglycopeptide	peptidoglycan cell wall
Dalbavancin	broad spectrum*	Phase III complete	lipoglycopeptide	peptidoglycan cell wall
Cethromycin	broad spectrum	filled for FDA NDA	ketolide	protein synthesis