New Strategies and Methods in the Discovery of Natural Product Anti-Infective Agents: The Mannopeptimycins

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Received April 12, 2007

Introduction

Greater than 25% of deaths worldwide are directly related to infectious disease, and much of this mortality is due to infections arising from resistant organisms.[1] Clearly, there is an urgent need to discover and develop new anti-infective agents possessing novel mechanism of action and enhanced activity profile. However, the past 3 decades have seen only two new classes of antibiotics introduced into clinical use, represented by the natural product daptomycin and the oxazolidinone linezolid.[2,3] During the past 10 years, only nine antibiotic NMEs[a] or new indications for antibiotics have been approved by the FDA, and only a half-dozen key antibacterials are currently in late stage clinical development.[4,5] Natural products have been the mainstay of antibiotics discovery since the beginning of the field in the 1940s. There are compelling reasons why natural products derived from microorganisms should continue to be a rich source of novel antimicrobial agents,[6] for it can be argued that the methods employed and environments explored so far in the search for anti-infectives and other agents have uncovered only a minor fraction of the potential biological and chemical diversity.[7] Furthermore, advances in biosynthetic engineering of natural products, particularly polyketides and nonribosomal peptides, are at the stage where significant, unique structural transformations can be achieved on these very complex structures.[8,9] Why then are there so few new anti-infectives? The answer lies partly in the aggressive timelines now imposed upon natural products discovery programs and in an unfavorable commercial and regulatory atmosphere.[4,10] This Miniperspective provides a viewpoint that is focused on the chemical challenges surrounding today's natural products-based antibiotic discovery programs and presents chemical and biological strategies to meet those challenges. The discovery of the mannopeptimycins, a promising new class of potent anti-infective agents, is provided as an example of one of these strategies.

Natural Products and High-Throughput Screening

Drug discovery programs rely in large part on a process-driven strategy that incorporates target-directed, high-throughput screening (HTS) of chemical libraries followed by rapid hit-to-lead and lead optimization phases.[11] For natural products to be successful in this paradigm the active compound(s) must be detected, extracted from the source, purified, and identified, often along the same timeline as synthetic compounds. The first hurdle is to detect robust hits and identify known or otherwise uninteresting compounds (dereplication). The second hurdle in the process, de novo structure determination of novel molecules, is now readily surmounted with the aid of modern spectroscopic techniques, particularly high resolution NMR. While many approaches have been developed to lower the third hurdle, that being the isolation and purification of the active principle(s) (often a minor component) from a complex matrix, it remains as a major bottleneck that continues to lengthen natural product discovery timelines. Thus, traditional natural products programs that rely on screening of extract libraries, bioassay-guided isolation, structure elucidation, and subsequent production scale-up remain challenged by the rapid cycle times that are a characteristic of the modern HTS approach. This has led to the demise of natural products programs in many pharmaceutical companies.[10] It is essential that new strategies and technologies be employed in the HTS approach to facilitate rapid navigation of natural product hits through the detection, isolation, hit-to-lead, and lead optimization phases.

Complications in high-throughput screening of natural products libraries against molecular targets arise from multiple sources. The first is detection interference. For example, in assays based on readout of fluorescence, difficulties are encountered when natural product samples contain compounds that either emit or absorb radiation at excitation or emission wavelengths of the fluorophore (typically flourescein) or when insoluble components cause light scattering.[12] This liability is shared with synthetic screening libraries as well, but the issue is exacerbated with natural products because the presence and nature of the interfering compounds may not be fully characterized. Recent developments in kinase, protease, and phosphatase assays have shown that fluorescence and light scattering interferences can be overcome by increasing the fluorophore concentration in the assay, by using red-shifted wavelength dyes[13] or by the technique of lifetime discriminated polarization.[12] Second, in order to detect competitive inhibitors, it is best to screen at a compound concentration close to the K_m of the enzyme for the substrate. This poses a challenge with natural products libraries where the relative concentrations of individual components in a sample are often not precisely known and may vary by 3 orders of magnitude or more. At any given assay dose, the levels of trace components may not be high enough for detection. At the same time, highly abundant, but inactive components may exhibit inhibition via nonspecific binding, perturbation of the assay pH, or other physical properties. Alternatively, the amount of active compounds present may be in great excess, leading to dose independent nonspecific inhibition. This problem can be overcome with new approaches such as quantitative high-throughput screening (QHTS). QHTS is a titration based approach that can be effective in screening libraries of compounds that vary greatly in potency or abundance and can furnish concentration–response curves in the primary screen. The QHTS approach was demonstrated on a 60 000-member library screened against pyruvate kinase.[14]

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[a] Abbreviations: NME, new molecular entity; FDA, Food and Drug Administration; HTS, high-throughput screening; QHTS, quantitative high-throughput screening; FabF/B, β-ketoacyl-(acyl-carrier-protein) synthase I/II; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; MRSA, methicillin resistant *Staphlococcus aureus*; SAR, structure–activity relationship.

10.1021/jm070432l CCC: $40.75
Published on Web 04/05/2008

Prefractionated natural product libraries are an effective strategy to reduce interferences encountered with crude libraries and to shorten the time needed to identify the active principle.[15] There are many variations of the prefractionation strategy, and each offers advantages of expediency or purity gained at the cost of up-front partial purification.[16] Interferences are reduced because of the fact that compounds at the extremes of the polarity scale are separated from the bulk of the library samples. Samples produced by the prefractionation approach are simpler mixtures, and the final resolution of active components requires fewer purification steps. Moreover, the relative concentration of minor components is increased over that in the crude, thereby enhancing the opportunity to uncover novel biologically active metabolites. Advances in automated purification methods have made modest (a few thousand compounds) purified natural product libraries feasible as well,[17] alleviating many HTS drawbacks of traditional natural products libraries.

In the case of antibiotics, a lead generation strategy evolved in the 1990s based on high-throughput screening of synthetic compound libraries against microbial targets identified via bacterial genomics. Unfortunately, success with this approach has been somewhat limited. The GSK group leveraged bacterial genomics information to design and execute 70 HTS antibacterial assays of which 67 were against molecular targets, with disappointing results.[18] Payne et al. attribute their limited success in finding suitable anti-infective leads in significant part to the limited chemical diversity of their synthetic screening library. They proposed that screening natural products libraries with cell based phenotypic assays would enjoy greater success. Indeed, high throughput screening of traditional natural products libraries by cell-based assay using engineered strains that underexpress key targets has been demonstrated to detect novel, therapeutically promising antibacterial leads. To discover platensimycin, investigators at Merck screened a library of 250 000 microbial extracts using a whole-cell antisense differential sensitivity assay. These efforts led to the isolation of a new class of potent and selective inhibitors of β-ketoacyl-(acyl-carrier-protein) synthase I/II (FabF/B), a key enzyme in the biosynthetic pathway of fatty acids in bacteria.[19] Additional screening using the same whole-cell approach led to the isolation of phomalenic acids A−C, which do not possess suitable druglike properties but which further validate the approach.[20] The recent approval of the first-in-class lipopeptide antibiotic daptomycin demonstrates that once identified, natural product leads can in fact be chemically optimized and scaled within today's timelines to yield clinically effective therapeutics.[2] Furthermore, recent work on second-generation daptomycin analogues demonstrates one of the unique attributes of microbial natural products, the ability through biosynthetic engineering to produce analogues not accessible via chemical synthesis and in quantities sufficient for advancement.[21,22]

Looking Back to Earlier Antibiotics Discoveries

The evolving clinical picture of bacterial resistance has altered the profile of what are desirable antibiotics and creates opportunities for new strategies to discover them. Advances in HPLC, NMR, and mass spectrometry make it possible to isolate and structurally characterize what were previously intractable molecules. Therefore, on the basis of the emerging need for agents effective against resistant strains of Gram-negative organisms such as vancomycin resistent *enterrocci* and methicillin resistant *S. aureus*, Wyeth implemented in the late 1990s a strategy of re-examining legacy antibiotics discovered over the recent 50-year period and applying contemporary analytical, chemical, and biological methods to more thoroughly explore their potential. This so-called "look-back" program was successful in the identification of several novel antibiotics with highly diverse structures and mechanism.[23–25]

An example is the mannopeptimycin family of antibiotics (Figure 1).[26] The mannopeptimycin complex was originally isolated in the late 1950s from *Streptomyces hygroscopicus*, strain LL-AC98 where it showed potent activity against Gram-positive organisms.[27] At that time, the chemical complexity of the complex coupled with the lack of broad-spectrum activity reduced the prospects to further develop the antibiotic. However, under the look-back program, interest in the complex was revived because of its activity against clinically important Gram-positive pathogens, including antibiotic-resistant strains such as methicillin resistant *S. aureus* (MRSA), penicillin resistant *Streptococcus pneumoniae*, and vancomycin resistant *enterococci*.[28] Antibacterial and mechanistic studies with Gram-positive and Gram-negative bacteria demonstrated that the primary cellular target was bacterial cell wall biosynthesis, with a specific targeted step different from those targeted by known agents.[28] It was later shown that the mannopeptimycins act by inhibiting cell wall biosynthesis via a unique mode of action, by binding the membrane bound cell wall precursor lipid II (C_{35}-MurNAc-peptide-GlcNAc) in a fashion different from other lipid II binders such as ramoplanin, mersacidin, and vancomycin.[29] In this binding mode, the mannopeptimycins inhibit the essential transglycosylation steps necessary for incorporation of lipid II into nascent peptidoglycan but in a manner not circumvented by mechanisms of vancomycin resistance (Figure 2).[29] The mannopeptimycins also bind lipoteichoic acid in a manner that serves to concentrate the antibiotic on the cell surface. These findings further validate lipid II as a viable target for continued antibiotic discovery in that no fewer than four different classes of antibiotics are now known to target lipid II, each with a different detailed mechanism.[30] It is also important to note that this novel mechanism would not have been uncovered using a molecular target-based screen.

The mannopeptimycin complex is composed of five major members, designated mannopeptimycin $\alpha-\epsilon$ (**1**−**5**, Figure 1). The series is based on a cyclic hexapeptide core containing D,L stereoisomers of an unprecedented amino acid, α-amino-β-[4′-(2′-iminoimidazolidinyl)]-β-hydroxypropionic acid (Aiha, also termed β-hydroxyenduracididine). Mannopeptimycin α, χ, δ, and ϵ are O-glycosylated with mannose monosaccharide and disaccharide moieties on the tyrosine phenolic OH and N-glycosylated on the D-Aiha residue. In manopeptimycin $\chi-\epsilon$ (**3**−**5**) the terminal mannose bears an isovalerate ester.[26] The unprecedented structures of these molecules illustrate the challenges facing structure elucidation and lead optimization. The major components of the fermentation product complex mannopeptimycin α (**1**), which lacks esterification, and mannopeptimycin β (**2**), which lacks glycosylation, have poor activity.[28] However, esterification of an isovaleryl group of the terminal mannose at C2, C3, and C4 enhanced activity, with C4 being the preferred position. This natural SAR was used to guide a semisynthetic analogue program that sought to improve potency and in vivo activity. In the first stages of investigation, it was clear that the structural complexity of the mannopeptimycins would require multiple approaches to achieve the desired structural variation for lead optimization. The approaches employed included selective chemical synthesis, directed biosynthesis, and biosynthetic engineering. The first approach at semisynthesis sought to provide additional SAR information by derivatization. Since the parent (**1**) bears 14 hydroxyl groups and several basic nitrogen atoms, it might be postulated that

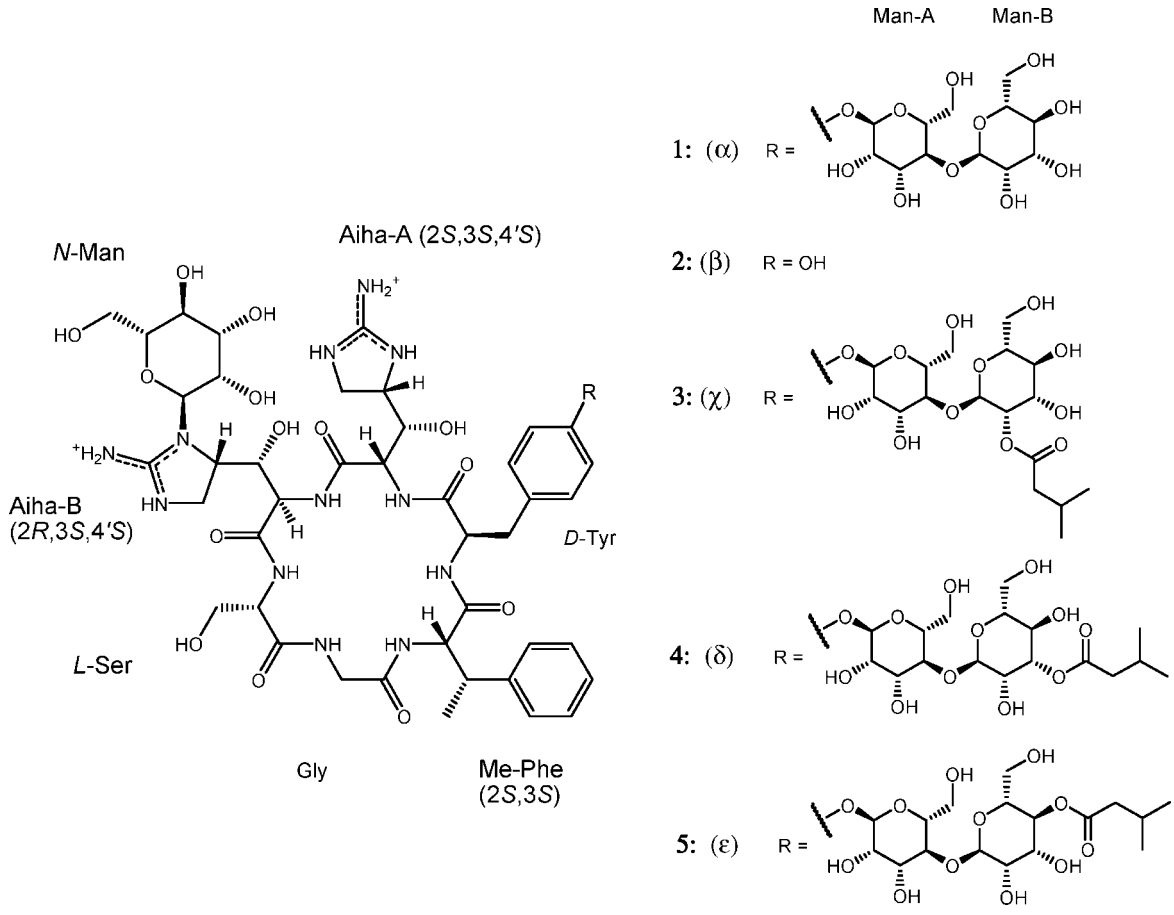

Figure 1. Structures of mannopeptimycins α−ϵ (**1–5**).

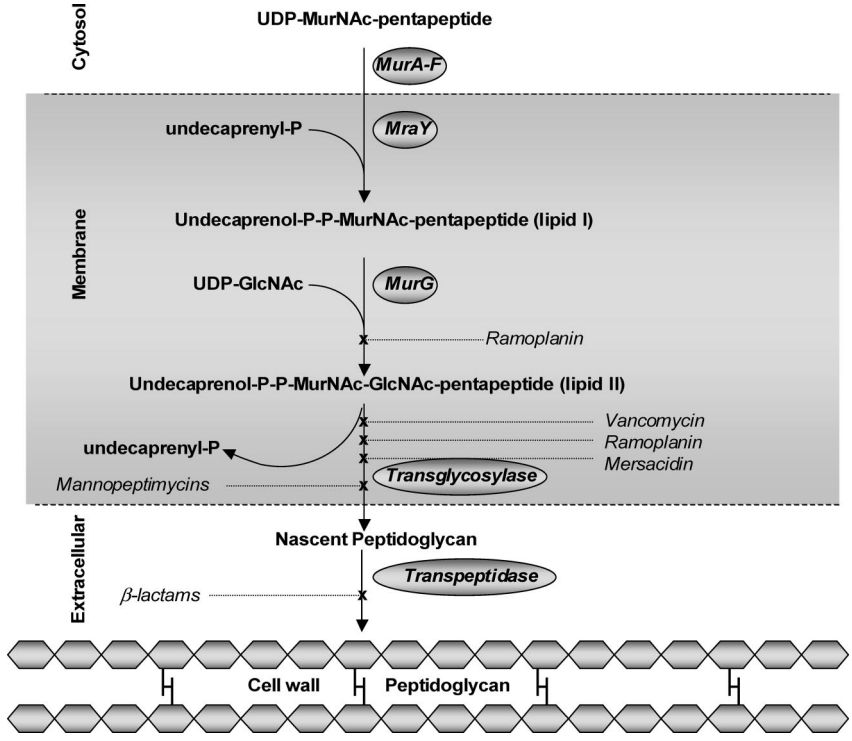

Figure 2. Lipid II pathway and site of action of mannopeptimycins. Biosynthesis of the bacterial cell wall building blocks begins in the cytosol with assembly of the UDP-MurNAc-pentapeptide, which under catalysis by MraY is linked to undecaprenyl phosphate in a membrane associated process to form lipid I. MurG then catalyzes the addition of UDP-GlcNAc to form lipid II which, as it crosses the membrane, undergoes removal of the undecaprenyl unit and transglycosylation onto the nascent peptidoglycan chain into the extracellular space. Final incorporation into the bacterial cell wall is accomplished via catalysis by transpeptidases. Various lipid II-based antibiotics (in italics) are shown where they inhibit this process. The mannopeptimycins block the transglycolylation steps by binding the lipid II substrate.

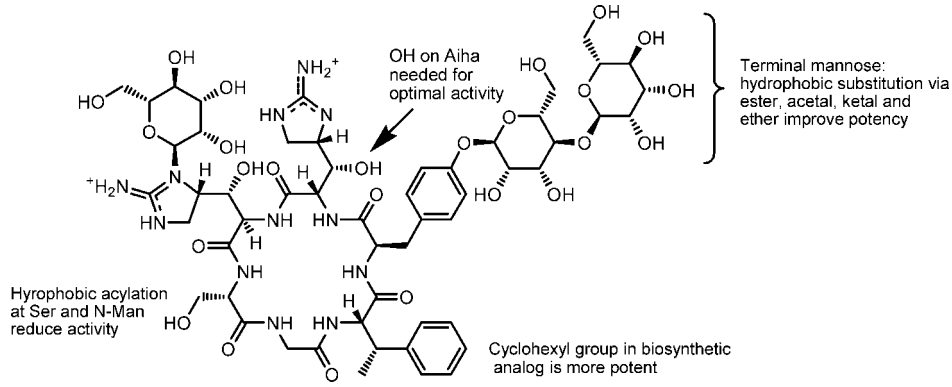

Figure 3. Structure–activity relationships of mannopeptimycin.

so-called "shotgun" derivatization methods might yield overly complex mixtures of products. However, approaches using separation and structure elucidation methods developed in the isolation of the natural products were successfully applied to these "shotgun" reaction products. Thus, random acylation followed by HPLC isolation and NMR and MS characterization furnished a series of 30 fully characterized monoester products.[31] When assayed against a bacterial panel, it was apparent that substitution of a hydrophobic ester group on the N-Man or serine moieties suppressed antibacterial activity, whereas hydrophobic acylation on either of the two O-mannoses, particularly the terminal mannose, significantly enhanced activity. Carbonate analogues showed similar SAR. The results of these studies confirmed the SAR observed in the natural analogues, namely, that that hydrophobic substitution at the terminal mannose residue of **1** enhances potency. Subsequent studies explored hydrophobic acetal and ketal derivatives of **1**,[32] ether derivatives,[33] halogenated derivatives,[34] and benzoxazole derivatives.[35] Structural assignment of these derivatives relied heavily on NMR and FTMS methods.[36] Overall, these studies led to a solid SAR picture (Figure 3) and furnished numerous mannopeptimycin analogs with increased potency and serum stability. One of the most potent compounds obtained was the terminal mannose 4,6 adamantyl ketal **6** (Figure 4).[32]

In parallel to the chemical semisynthesis, directed biosynthetic and biosynthetic engineering techniques were used to elaborate novel analogues. These approaches were based on the successful cloning, sequencing, and manipulation of the mannopeptimycin biosynthetic gene cluster.[37] In studies investigating the biosynthetic origins of the D and L forms of the β-hydroxyenduracididine, it was found that the product of the mmpO gene in the mannopeptimycin gene cluster resembled several known nonheme iron α-ketoglutarate-dependent oxygenases, and it was surmised that it played a role in the introduction of the OH group. Inactivation of the mppO gene yielded a strain that produced dideoxymannopeptimycins, confirming the biosynthetic function.[38] Importantly, the dideoxy compounds showed significantly reduced potency. Directed biosynthetic studies with the producing organism led to a number of derivatives. One of these was analogue **7**, prepared by feeding the culture cyclohexyl alanine (Figure 4). When the terminal mannose 4,6 adamantyl ketal of **7** was prepared, the resulting compound AC98-6446 (**8**) showed superior antimicrobial potency and properties, both in vitro[39] and in vivo.[40] The example of **8** demonstrates how the convergence of semisynthetic and biosynthetic chemistry efforts on a complex structure can result in an optimized lead.

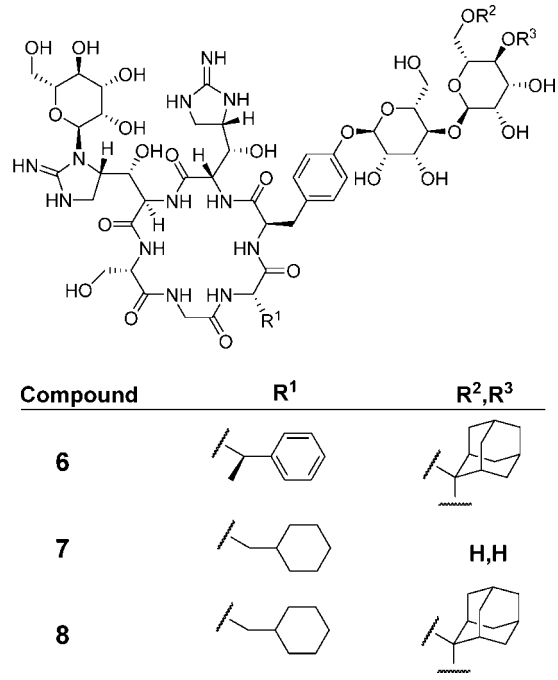

Figure 4. Semisynthetic analogues of mannopeptimycin.

Conclusions

It is clear that new antibiotics are needed to combat the growing threat of bacterial resistance. But despite significant advances in bacterial genomics, high throughput screening, and synthesis, antibiotics discovery over the most recent 30 years has not kept pace with the demand for new agents. Reduced emphasis in industrial antibiotic discovery programs has no doubt played a major role in the present situation. In the opinion of the author it is also likely that reduced emphasis on general natural products discovery has contributed as well. Timelines and therapeutic objectives have changed considerably from the days when natural products provided the basis for the entire Golden Age of antibiotic discovery, and newer approaches are needed to overcome some of the special hurdles faced by natural products programs. Fortunately, there is ample evidence to support the premise that if pursued vigorously with newer strategies of screening, lead optimization, and biosynthetic chemistry, natural products derived from microbial fermentation can again assume a prominent place in the search for new antibiotics.

Biography

Frank E. Koehn is Director of Natural Products Discovery and Discovery Analytical Chemistry at Wyeth Research, Pearl River, New York. He received his Ph.D. in Biochemistry in 1977 from the University of Wisconsin—Madison and went on to 2 years of postdoctoral training in Natural Products Chemistry at the University of Pennsylvania. From there he served the next 10 years as a Senior

Investigator at Harbor Branch Oceanographic Institution, Division of Biomedical Marine Research, in Fort Pierce, FL, working in the field of marine natural products drug discovery. In 1994 he joined Wyeth Research where he leads a research program aimed at the discovery and development of microbial natural products as therapeutic agents.

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JM070432L