Discovery of novel antibiotics from actinomycetes by integrated metabolomics & genomics approaches

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Chapter 12

Summary and Discussion

1. Summary and discussion of the experimental results

Since Fleming's ground-breaking discovery of penicillin,[1] antibiotic usage has increased enormously. This has led to a concomitant sharp rise in infectious diseases associated with drug-resistant pathogens since the 1980s, which rapidly spread across the globe.[2] Many multi-drug resistant (MDR) bacteria have developed resistance to most if not all antibiotics discovered to date, which imposes a massive challenge on society.[3] Soon after new anti-infectives enter the market, including those representing our last line of defense (e.g. vancomycin), resistance rapidly emerges, which has led to the return of diseases once considered nearly eradicated, with tuberculosis as clear example. Nosocomial pathogens collectively known as the ESKAPE pathogens,[4] namely *Pseudomonas aeruginos*a, *Enterobacteriaceae*, *Klebsiella penumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Enterococcus spp*. are also a constant threat to human health care.[5] The increase in resistance comes at a time where drug-discovery pipelines are drying out,[6] primarily caused by the fact that finding new drugs is time consuming and hence expensive.[7,8] New ideas and insights should be brought in to revitalize natural products research, and improve our chances of finding new antimicrobial drugs, particularly those with novel modes of action and/or unique chemical skeleton are of paramount importance to human health.

Should we lose faith that we can overcome these challenges? An old Chinese philosophy is "things in the world are allelopathy". If so, what can curb the raging tiny bugs? As always, the answer should come from Nature. Billions of years of evolution has resulted in an extraordinary and diverse library of chemical structures with huge functional variety. In other words, it is like a textbook encoded in the language of chemistry. Modern analytical tools now enable us to understand how structure relates to bio- or physiological function. With the advances made in synthetic biology and chemistry,[9] we can not only make copies of naturally occurring molecules, but also design new bio-inspired compounds, some with the functions unprecedented in the natural world - compounds that will influence, if not shape, every facet of our life.[10]

To come back to antibiotics, where can we find new molecules to replenish the pipelines of the pharma industry? Although scientists have been trying to tap the huge repository of microorganism in nature, the biosynthetic potential of microbes is still largely underestimated, and probably just a tip of iceberg is discovered.[11,12] Several causes can be discriminated. Firstly, while the number of filamentous microbes in soil and marine environments is enormous, the bulk of them resists cultivation in the laboratory and thus escape screening and exploitation.[13] Recent work has shown that enabling growth of these 'uncultivable' microorganisms might open up a new area of the chemical space of natural products.[14,15] Secondly, many natural product gene clusters of cultivable microbes remain silent under standard laboratory cultivation. As an example, genome sequencing of actinomycetes revealed that the producing capacity of even the best-studied model organisms has been grossly underestimated.[16,17] And thirdly, those gene clusters that are expressed, in particular if expression is relatively low compared to other bioactive compounds produced by the same organism, often escape discovery due to the very demanding phytochemical analysis or chromatographic purification that is required.[18]

Actinomycetes are industrially and medicinally important microorganisms because of their ability to produce a plethora of secondary metabolites. Indeed, more than two-thirds of

known antibiotics are produced by these filamentous bacteria, the majority of which by *Streptomyces* species. The advent of next-generation sequencing (NGS) technologies has uncovered that even the best-studied model actinomycete *Streptomyces coelicolor* A3(2) still possesses many yet underexplored resources for NPs discovery.[16] New approaches are therefore required to exploit the unexplored reservoir of bioactive natural products, and to rejuvenate the drug-discovery pipelines.[19]

In this thesis, approaches to aid the discovery of novel antibiotics from actinomycetes are presented, focusing on new approaches and ideas from the perspectives of both chemistry and biology. Many efforts aim to elevate the chances of finding new lead compounds with novel modes of action, whereby the emphasis lies on unlocking cryptic biosynthetic pathways in microbes, followed by efficiently prioritizing the elicited molecules. Various proofs of concept are provided that NMR-based metabolomics integrated with different antibiotics-eliciting strategies is a very efficient method to facilitate the discovery of new antibiotics from actinomycetes.

In recent years, the implementation of the microbial co-cultivation strategy attracted considerable interest and application, because it proved to be an effective way to harvest unique structures with pronounced biological activities.[20] In Chapter 4, it is shown that co-cultivation of *Streptomyces coelicolor* and the filamentous fungus *Aspergillus niger* has a major impact on their respective secondary metabolism. NMR-based metabolomics revealed several compounds that correlated specifically to co-cultures, including the cyclic dipeptide cyclo(Phe-Phe), and 2-hydroxyphenylacetic acid. These compounds were produced by *A. niger* in response to the chemicals produced by *S. coelicolor*. Inspired by this co-culture metabolomics study, biotransformation of hydroxycinnamic acids resulted in the specific production of the novel molecule (2*E*,4*E*)-3-(2- carboxy-1-hydroxyethyl)-2,4-hexadienedioxic acid by the co-cultures microorganisms.

Selection for streptomycin-resistant mutants of actinomycetes is another pleiotropic approach to enhance the production of antibiotics.[21] In Chapter 5, a strategy based on NMR-based metabolomics combined with the introduction of streptomycin resistance in *Streptomyces* sp. MBT28, led to the identification of the new compound 7-prenylisatin that showed antimicrobial activity against *Bacillus subtilis*. Furthermore, the metabolite-guided genome mining combined with Natural Product Proteomining platform[22] identified an orphan gene cluster (*isa*) with an indole prenyltransferase that transforms tryptophan into 7-prenylindole. However, the biosynthesis genes for the final product 7-prenylisatin are not confined to a single cluster in the genome of *Streptomyces* sp. MBT28, as indispensable oxygenases responsible for the oxidation of 7-prenylindole into the7-prenylisatin were not found within or in close proximity of the *isa* gene cluster. This study underlines that combination of various ‘omics’ approaches can facilitate the discovery of novel molecules, whilst offering a straightforward way to characterize the gene cluster responsible for the identified compounds.

Secondary metabolites are often produced in a growth phase-dependent manner by actinomycetes, and finding the optimal harvesting time is crucial for successful isolation of the desired bioactive metabolites. In Chapter 6, the growth phase-dependence of secondary metabolite production by *Streptomyces* sp. MBT76 was analyzed using NMR-based metabolomics. A group of methoxylated isocoumarins were characterized, including a

tetra-methoxylated compound 5,6,7,8-tetramethoxyl-3-methyl-isocoumarin. Multivariate data analysis indicated that methoxylation led to an increase in the antimicrobial activity of isocoumarin-type antibiotics, which was confirmed by antimicrobial assays using the methoxylated and non-methoxylated isocoumarins. Furthermore, metabolomics-guided biotransformation of isoflavone genistein resulted in a suite of methoxylated isoflavones, undergoing exactly the same modification pattern of isocoumarin. Interestingly, while isocoumarin-type metabolites are frequently isolated from actinomycetes,[23,24] the underlying genetic basis for this type of polyketide is still poorly understood. In consideration of the structural similarity between 6,8-dihydroxy-3-methyl-isocoumarin and genistein, it is conceivable that the same *O*-methyltransferase is involved in the methylation of both substrates, and this putative *O*-methyltransferase seems to have strong methylation capacity. Therefore, it will make sense to characterize the genetic basis for isocoumarin biosynthesis in *Streptomyces* sp. MBT76. This should shed light on the nature of the methyltransferase responsible for the post-PKS modification, since the genes for PKS assembly and post-PKS decoration are always clustered in the genome of actinomycetes. Furthermore, this effort will also benefit further genetic engineering of this type of important antibiotics, such as combinatorial biosynthesis.

In Chapter 7, an approach was followed whereby the pathway-specific activator of a biosynthetic gene cluster (BGC) was over-expressed, leading to the activation of a poorly expressed type II PKS gene cluster (*qin*) in *Streptomyces* sp. MBT76. NMR-based metabolic profiling then efficiently linked the chemotype to the genotype of interest in the recombinant strain, whereby we shed first light on the intriguing architecture of qinimycins A-C, a group of novel pyranonaphthoquinones with structural features of 8-glycosylation, 5,14-epoxidation, and 13-hydroxylation. The methodology exemplified in Chapter 7 is particularly useful when information pertaining to the structural novelty of the products of particular BGCs can be gleaned from predictive bioinformatics analysis. NMR profiling of the molecules produced in highly complex matrices thereby allows rapid linkage of chemotype to genotype, greatly streamlining the downstream isolation and structure elucidation. However, there are also intrinsic limitations in this pathway-specific strategy, such as lack of high throughput, in some cases difficulty in recognition of pathway-specific activators, and the fact that many wild isolates are not genetically tractable. Selected novel compounds discovered in the course of the NMR-based metabolomics studies are displayed in Figure 1.

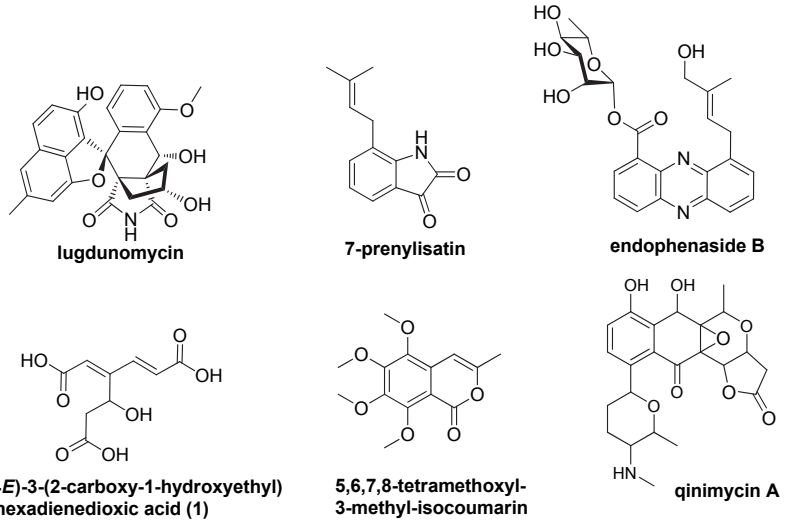

Figure 1. Representative new compounds described in this thesis. Especially, lugdunomycin is a angucycline-derived with unprecedented chemical scaffold.

Metabolomics-driven drug discovery offers advantages over the routine bioassay-guided screening. The major shortcoming in the traditional method is lack of prior structural information on the bioactivity of interest prior to the labor-intensive and time-consuming compound isolation. It should be noted that deficiencies exist in NMR-based metabolomics for handling microbial extracts. Routinely in this thesis, actinomycetes were fermented in the liquid or solid minimal media. Many components from growth media are inevitably extracted by ethyl acetate, and in most cases are more abundant relative to the true secondary metabolites produced by actinomycetes. As NMR spectroscopy is a quantitative but relatively insensitive technique as compared to Mass Spectrometry (MS), the proton signals belonging to the compound of interest are often in low abundance and therefore obscured by the signals assignable to culture media. Therefore, NMR-based metabolomics works better when combined with an efficient antibiotics-eliciting approach. Alternatively, one can remove the unwanted interfering compounds in the initial step and accordingly highlight the signals of interest. For this purpose, exploitation of a comprehensive extraction method other than single organic solvent (ethyl acetate) liquid-liquid partition awaits further investigation, whereby solid-phase extraction seems promising (see below), because a wide range of solid sorbents with various physiochemical properties are available. Moreover, the disadvantage of the insensitivity and congestion problems in NMR-based metabolic profiling could potentially be overcome by HPLC-NMR hyphenation techniques.[25] The major advantages of on-flow HPLC-NMR lie in the real-time access to structure information of chromatographic peaks by using NMR spectrometers as detector.[26] To leverage the inherent lower sensitivity of NMR spectra and the limited injection volume onto the HPLC unit, solid-phase-extraction (SPE) is coupled to trap the analytes onto an identical SPE cartridge when repeated HPLC analysis, followed by final elution with a deuterated solvent into miniaturization tube for NMR analysis.[27] By using this LC-SPE-NMR approach,[28] comprehensive structural information about chemical composition of crude extracts can be obtained online, thus enabling early and prospective assessment of the valuable components of microbial extract or fraction.[29] Furthermore, when integrating MS into the system, like LC-DAD-SPE-MS-NMR instrumentation,[30] the architecture of novel compounds can be elucidated *de novo* and online without laborious purification of the analyte(s) from the highly complex matrix.

There are perhaps no strictly silent biosynthetic gene clusters. The compounds will undoubtedly be produced under some conditions in the original habitat where the bacteria live, as else the BGCs would have been lost during the course of evolution, and most likely would never have evolved in the first place. However, many microbial secondary metabolites are produced in trace amounts, which are out of the detection limit of NMR technique, and thus missed in the NMR-based metabolomics strategy. Logically, minor compounds are more likely to have been missed by routine screening efforts in the past, and hence have a better chance of being novel over those expressed abundantly under normal laboratory culturing conditions. A logical approach to unveil these minor compounds is up-scale fermentation to accumulate enough material for follow-up repeated chromatography isolation and structure elucidation. Though time- and labor- extensive, it has been fruitful to discover novel chemical skeletons by isolation of all the detectable compounds in any biological sample, in other words a systematic isolation method, which is prevalent in the field of traditional natural products chemistry. Actually, it is common that a single microbial source produces multiple

secondary metabolites,[31] and systematic isolation is unambiguously the most efficient way to bring light the chemical diversity of a given actinomycete. Besides the discovery of new compounds, systematic isolation often aids our understanding of the biosynthetic pathway of a given natural product with complex architecture, because the structures of the co-isolated intermediates or side products could provide clues to elucidate the bioassembly of final target product. Furthermore, the fact that changes in the cultivation conditions can completely shift the metabolic profile of many microorganisms, resulted in the OSMAC (one strain many compounds) strategy for drug discovery from microbial sources.[32] A microorganism never produces its entire arsenal of compounds at the same time under one specific environmental condition, which would be energetically and metabolically too costly. Rather, each compound is produced when needed, ensuring a competitive advantage when environmental conditions change. Consequently, varying culturing conditions is a logical approach to attempt to induce or optimize the production of secondary metabolites. In this context, systematic alteration of cultivation parameters (media composition, aeration, pH, temperature, addition of chemicals, etc.) of a given microorganism greatly increases the likelihood of finding new metabolites from a single strain. Of particular note is the static cultivation for diversification of secondary metabolites in the laboratory, because this is more consistent with their natural survival environment. It was recognized previously that the coproduction by actinomycetes in nature of two or more secondary metabolites is necessary to synergistically or contingently beat biological competitors, and that synergy and contingency may be common driving forces for the evolution of multiple secondary metabolite production by these sessile saprophytes.[31] The OSMAC strategy was applied to discover the natural products produced by *Streptomyces* sp. QL37 (Chapter 8) and *Kitasatospora* sp. MBT66 (Chapters 9-10). Perturbed liquid NMMP media and solid MM, supplemented with different carbon sources (glycerol, mannitol, glucose, rhamnose, *N*-acetylglucosamine), additional additives (yeast extract, potato extract, peptone, starch, soy flower), or high alkalinity (pH 10), were compared, and solid fermentation was found better for secondary metabolites production in these two actinomycetes.

Systematic isolation of *Streptomyces* sp. QL37 cultured in MM supplemented with mannitol and glycerol as the carbon sources, led to the production of the lugdunomycins, which are natural products with a completely new chemical scaffold (Figure 1). Their chemical structure indicates that the lugdunomycins are derived from angucyclines, but the basic structure is then modified to generate a highly complex molecule with an unprecedented three dimensional structure. Lugdunomycins possess a benzaza[4,3,3]propellane skeleton adorned with a spirocyclic 2*H*-naphtho[1,8-bc]furan moiety and two all-carbon quaternary centers embedded within five contiguous stereogenic carbons. The striking backbone of benzaza[4,3,3]propellane-6-spiro-2'-2*H*-naphtho[1,8-bc]furan has a so far unprecedented chemistry. Further fermentation of *Streptomyces* sp. QL37 in R5 agar plates with peptone followed by systematic isolation, resulted in the characterization of many additional rearranged angucyclines (Chapter 8). As a result, in total nearly 30 angucycline derivatives were characterized, and roughly classified into four different chemical backbones, including the seven-ring lugdunomycins, three-ring anthraquinones, four-ring benz[a]nthraquinones, and the five-ring limamycins. The metabolite-guided genome mining identified a type II PKS (*lug*) gene cluster for angucycline production in *Streptomyces* sp. QL37, which was confirmed

by genetic disruption of minimal PKS genes. However, it was possible that a single *lug* is insufficient for the synthesis of all the discovered angucyclines, whereby outside genes (clusters) were needed to construct the complex architecture of lugdunomycin. More intriguingly, all the characterized rearranged and/or unrearranged angucycline derivatives in QL37 involve an oxidative C-C cleavage in the post-PKS stage to substantially change the basic backbone of benz[a]nthraquinone. It seems likely that more than one oxygenase is involved in the post-PKS modification in the lugdunomycin pathway, because multiple oxidations were observed in the isolated angucyclines. In particular, the oxygenase responsible for the quinone ring opening via Bayer-Villiger oxidation was of utmost importance for lugdunomycin biosynthesis, which has the potential to modify other polyketide antibiotics, like tetracycline.

In Chapter 9, systematic investigation of *Kitasatospora* sp. MBT66 enabled the discovery of five novel rhamnosylated phenazines (termed as endophenasides A—E),[33] as well as the rhamnosylated plecomacrolide leucanicidin. Metabolite-guided genome mining of MBT66 identified the endophenaside gene cluster that is nearly identical to those of the endophenazine biosynthetic gene cluster (*epa*) of *Kitasatospora* sp. HKI 714.[34] However, genes responsible for the glycosylation of the endophenazine aglycones was unresolved in Chapter 9, as no such genes were found within or in close proximity of the *epa* gene cluster. The genetic basis for this rhamnosylation was subsequently resolved in Chapter 10.[35] Interestingly, the *de novo* rhamnosylation of both types of structure cores in *Kitasatospora* sp. MBT66, via phenazine and plecomacrolide, originated from decoration by the same glycosyl transferase and methyltransferase, LeuAB. This inherent substrate flexibility of LeuAB evoked us to reinvestigate the structural diversity of endophenasides in MBT66, whereby MS/MS based molecular networking facilitated the characterization of nine new endophenasides by the OSMAC strategy, including an unprecedented tautomeric glyceride phenazine (Chapter 10). Molecular networking tool is an effective tool for chemical dereplication strategy and a way of prioritizing novel compounds,[36] and its fundamental principle is based on the fact that structurally related natural products are characterized by similar MS/MS fragmentation patterns. In view of the glycosylation has profound impact on pharmaceutical properties, the promiscuity of LeuAB (or other glycosyltransferases) by accepting totally different chemical skeletons as substrates can be exploited to glycorandomize a broad range of natural products. This offers an alternative way to develop new antibiotics based on post-biosynthetic modification strategies.

Genes for secondary metabolite production in actinomycetes are typically clustered in the genomes, which is the basis of bioinformatics-guided discovery of natural products. However, the crosstalk of the *epa*, *baf,* and *rml* gene clusters for endophenasides production in *Kitasatospora* sp. MBT66 (Chapter 10), the joint contribution of *isa* and putative oxygenase for 7-prenylisatin production in *Streptomyces* sp. MBT28 (Chapter 5), as well as the genetics for lugdunomycin production in *Streptomyces* sp. QL37 (Chapter 8), is intriguing and led us conclude that a non-clustered or subclustered genome arrangement for secondary metabolites biosynthesis in actinomycetes is more common than previously suggested. The combinational biosynthesis of a single compound employing several distant regions of the genome is also seen for the biosynthesis of sioxanthin[37] in the actinomycete genus *Salinispora,* of prodiginines in *Streptomyces coelicolor*[38]*,* and pyrrolamides in *Streptomyces netropsis*.[39] In

other cases, single one gene cluster tends to deliver a multitude of structurally diverse products. For instance, the type II PKS *act* gene cluster in *S. coelicolor* synthesize different aryl scaffolds, including pyranonaphthoquinone,[40,41] anthraquinones[42,43], naphthoquinones[22,44], and mutactin.[45] The metabolic profile of a recombinant *Streptomyces* strain expressing the *S. coelicolor whiE* minimal PKS led to the production of ~30 polyketides with varying chain lengths and cyclization patterns.[46] In this thesis, the *epa* gene cluster of *Kitasatospora* sp. MBT66 encodes the synthesis of more than 20 phenazines (Chapters 9 and 10), while the *lug* cluster of *Streptomyces* sp. QL37 results in the production of at least four kinds of chemical skeletons, with several tens of different metabolites (Chapter 8). Taken together, though synthetic biology approaches will be a major trend for future antibiotics discovery (Chapter 1),[47] it may be less effective for the handling of this type of intertwined metabolic pathways, and thus traditional systematic isolation should not be abandoned.

Whatever pipeline is developed for the discovery of new antibiotics, a common feature is the eventual phytochemical analysis of microbial cultures to characterize the molecules of interest. A rational extraction procedure is thus very important to remove the interfering components (such as components of the culture media), and to concentrate the widest possible range of specialized secondary metabolites with diverse polarities. In Chapters 4—10, the organic solvent-ethyl acetate was uniformly used to extract metabolites produced by all the investigated actinomycetes. As mentioned above, many media components are inevitably extracted by ethyl acetate, and in many cases these are more abundant relative to the true secondary metabolites produced by actinomycetes. In particular, a group of apolar chemicals from our routinely used culture media, including 2,5-di-tert-butylphenol, 3,4-di-tert-butylphenol, and (3,5-di-tert-butyl-4-hydroxyphenyl)-propionic acid octadecyl ester, were extracted by ethyl acetate in large amounts, which tended to be "common impurities or contaminants" in almost all our isolated compounds, and severely affected the final structure elucidation and bioactivity tests when the compounds of interest were in low yield. Another drawback of ethyl acetate is that it is less appropriate for the extraction of polar compounds, such as water-soluble aminoglycosides. In Chapter 11, we therefore explored the application of the natural polymer cellulose as a solid adsorbent of antibiotics in an aqueous matrix. This work showed that indeed cellulose-based solid-phase extraction can be considered as a complementary approach to conventional liquid-liquid extraction to uncover a wide range of secondary metabolites produced by actinomycetes, especially for the easily-overlooked water-soluble antibiotics.

2. Perspectives

With the rapid increase in bacterial resistance, it is of ultimate importance to develop new antibiotics. Historically, antibiotic discovery or other natural product research, has originally been dominated by chemistry, focusing on the isolation of pure compounds, structural characterization, and total or semi- synthesis. However, as connecting natural products to their biosynthetic genes becomes more important to exploit the full potential of genome and metagenome sequencing, the biology plays an increasingly important role in natural product research in the current (post-)genomics era. The recent explosion of publications dealing with genomics approaches to discover novel natural products has demonstrated that the

"genotype-to-chemotype" roadmap is a rewarding approach that has already revolutionized traditional natural product research.[6,48,49] This field of research now merges multiple disciplines such as whole genome sequencing, bioinformatics, transcriptomics, proteomics, synthetic biology, systems biology, chemical ecology, and others.[50] Synthetic biology has a great potential to reinvigorate drug discovery through engineering biosynthetic pathways.[9] As summarized by Jon Clardy and co-workers,[51] with known compounds like artemisinin, synthetic biology supports natural products by improving supply; with 'known unknowns' like the products of cryptic biosynthetic gene clusters identified in the bacterial genomes, synthetic biology will likely support natural products by enabling their biosynthesis and elucidation; and with 'unknown unknowns', natural products can provide new insights into the biosynthetic knowledge and new tools for synthetic biology. The advances in the modern genetics tools,[52] such as transformation-associated recombination (TAR),[53] Red/ET recombination,[54] and CRISPR-Cas9 genome editing[55,56], will accelerate synthetic biology and hence natural product research. In this context, it is imperative that a pharmacognosist committed to find antibiotics, has the appropriate biological knowledge and practical genetic tools. Extensive collaboration between scientific experts from various disciplines is the best way forward to accelerate the discovery of the new antibiotics.

Microorganisms occupy almost all the observed ecological niches, playing a pivotal role in maintaining the sustainable run of macroscopic or microscopic ecosystems. Many ecological phenomena are directly derived from natural roles of microbial natural products, such as rhizoxin causing rice seedling blight.[57] It might be fruitful to follow "ecology-inspired" approaches for antibiotic discovery, in view of the tremendous biodiversity on the earth. In this sense, nature is always generous to offer inspiration. What is important is to direct our attention to the interesting natural phenomena in life, consider the underlying principles, link this to the hidden microbes, and use various "OMICS" tools for bioprospecting. Auguste Rodin already said: "It's not the lack of beauty in life, but the lack of the eyes to find the beauty".

The central dogma of molecular biology—the transmittance of information from DNA to RNA to protein— has now been extended to encompass the metabolome.[58,59] There is corresponding "OMICS" developed in every step of the extended central dogma to facilitate natural products discovery (Figure 2). The fact that all natural products have a genetic basis suggests a new concept that I would like to refer to as "Genometabolomics", which unifies the universally available knowledge of biosynthetic genes and biosynthetic protein reactivity, and can be exploited to predict and also characterize new natural products — and *vice versa* — by harnessing biosynthetic logic.

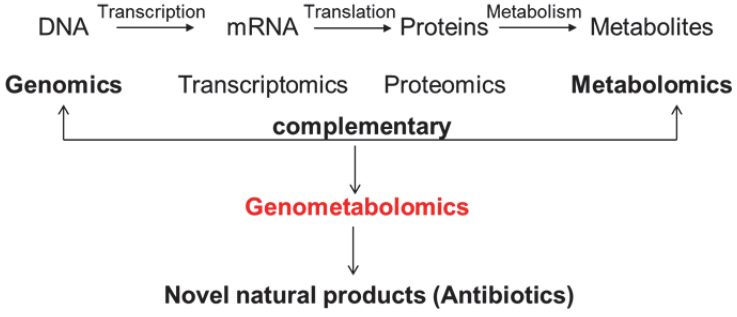

Figure 2. Genometabolomics for natural product research in actinomycetes.

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