Bioprospecting for antituberculosis leads from microbial metabolites

Elizabeth Jane Ashforth,†[a] Chengzhang Fu,†[ac] Xiangyang Liu,†[a] Huanqin Dai,[a] Fuhang Song,[a] Hui Guo[ac] and Lixin Zhang*[ab]

Received 24th June 2010
DOI: 10.1039/c0np00008f

Covering: 2005 to 2010

Microbial metabolites have been an important source of tuberculosis (TB) therapeutics, but the last truly novel drug that was approved for the treatment of TB was discovered 40 years ago. In light of the growing threat of multi-drug resistance, recent advances have been made to accelerate the discovery rate of novel TB drugs including diversifying strategies for environmental strains, and high-throughput screening assays. This review will discuss the approaches used in biodiversity- and taxonomy-guided microbial natural product library construction, specific cell-based and target-based high-throughput screening assays and early-stage dereplication processes by liquid chromatography–mass spectrometry (LC–MS). New antituberculosis natural products that have been recently discovered are highlighted.

1 Introduction

Bioprospecting is the search and discovery of natural products that have a useful pharmacological or biological application. Natural products from microbial secondary metabolites have a long history in the treatment of TB (*Mycobacteria tuberculosis* H37Rv and related strains). Streptomycin, from the actinobacterium *Streptomyces griseus*, was first introduced in 1946, and became the standard treatment by 1955 in combination with synthetic drugs *p*-aminosalicylic acid and isoniazid. However, in the early 1990s, after decades of decline, the incidence of TB began to increase due to inadequate treatment regimens and a diminished public health system allowing the emergence of resistant strains. In addition, the onset of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) epidemic complicated the potentially problematic drug–drug interactions of TB treatment regimes. A flurry of screening programmes and structure–activity relationship studies were implemented in the mid-1990s, but due to the lack of rationality behind the approaches used, there are concerns that the resulting compounds may be unsuitable for TB treatment.[1]

First-line treatment of TB now consists of a combination of 4 drugs – isoniazid, rifampin, pyrazinamide, streptomycin and/or ethambutol. While successful when implemented under the 'Directly Observed Therapy Short-course', a World Heath Organisation strategy to help stop the spread of TB, only one-third of TB patients worldwide have access and receive this treatment.[2] Consequently, poor compliance and premature discontinuation of treatment programs is the main cause of the rapid emergence of multi-drug-resistant TB (MDR-TB), resistant to isoniazid and rifampin, and extensively drug-resistant TB (XDR-TB). The latter is resistant to isoniazid and rifampin as well as to any member of the quinolone family and at least one of the following second-line anti-TB injectable drugs: kanamycin, capreomycin, or amikacin.[2] TB has a profound innate resistance to host defence mechanisms as well as intrinsic tolerance to chemotherapeutic reagents, accumulating resistance plasmids or transposons, for multi-drug efflux pumps, each of which can pump out more than one drug type (see ref. 3 for a full review). Estimates are that one person dies of TB every 20 s, and given that the cases of MDR-TB in 2008 were as high as 300 000,[4] new affordable therapeutics are urgently required. The challenge for bioprospecters is to find novel natural products that can provide a shorter, simpler and safer treatment for patients, avoiding multiple injections and unwanted drug–drug interactions. In addition, drugs need to be found that can successfully eradicate

[a]*Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, People's Republic of China. E-mail: zhanglixin@im.ac.cn; Fax: +86-10-62566511; Tel: +86-10-62566511*
[b]*South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, 510301, P. R. China*
[c]*Graduate School of Chinese Academy of Sciences, Beijing, 100049, P. R. China*
† These authors contributed equally to the work.

Table 1 Microbial natural products with antituberculosis activities (adapted from ref. 107 with permission.)

Antibiotic class	Year	Example	Strain	Mode of action	Ref.
Aminoglycoside	1940	Actinomycin	*Streptomyces antibioticus*	Protein synthesis	108
Aminoglycoside	1943	Streptomycin	*Streptomyces griseus*	Protein synthesis	109
Macrolide	1949	Erythromycin	*Saccharopolyspora erythraea*	Protein synthesis	110
Pleuromutilins	1951	Pleuromutilin	*Pleurotus mutilus*	Protein synthesis	111
Cycloserine	1955	Cycloserine	*Streptomyces orchidaceus*	Bacterial cell wall	112
Aminoglycoside	1957	Kanamycin	*Streptomyces kanamyceticus*	Protein synthesis	113
Ansamycin	1959	Rifamycin	*Amycolatopsis mediterranei*	RNA synthesis	114
Aminoglycoside	1960	Capreomycin	*Streptomyces capreolus*	Protein synthesis	115
Bacterial peptide	1969	Bacitracin	*Bacillus subtilis* var. Tracy	Bacterial cell wall	116
Thiolactone	1982	Thiolactomycin	*Nocardia* sp.	Fatty acid synthesis	117
Lipo-nucleoside	1986	Capuramycin	*Streptomyces griseus* 446-S3	Bacterial cell wall	118
Lipo-nucleoside	2005	Caprazamycin	*Streptomyces* sp. MK730-62F2	Bacterial cell wall	119
Cyclic heptapeptide complex	2005	GE23077	*Actinomadura* sp.	RNA synthesis	120

the dormant (but highly resilient) slow-growing TB cells that have made carriers of a third of the world's population.[2]

Despite future predictions that by 2020, nearly 1 billion people will become infected with TB,[2] there is still hope. Natural products possess an enormous structural and chemical diversity, and provide important structural motifs and pharmacophores that can be manipulated by chemical or genetic means to yield semi-synthetic drugs with improved properties *e.g.* cephalosporins and rifampicin.[1] The rich functionality and stereochemistry of natural products is without doubt one of their great strengths, providing both potency and selectivity,[5] and with current advances in strain improvement, high titres of product can be produced.[6,7]

Of the 22 500 biologically active compounds that have been obtained so far from microbes, 38% are from fungi, 17% are from unicellular bacteria and 45% are produced by actinomycetes,[8] warranting actinomycetes reputation as one of the most prolific producers of bioactive natural products known to date. Natural products are already among the most important anti-TB agents (see Table 1), with over 60% of approved and yet-to-be-approved drug candidates either natural products or related to them.[9] However, it is estimated that a mere 1–3% of all antibiotics have been discovered.[10]

Lixin Zhang

Lixin Zhang has more than 16 years of experience in microbiology and biotechnology, with a current focus on natural product based drug discovery. Having been awarded funding from the Hundred Talents Program, he moved from USA back to China, and is currently a professor at the Institute of Microbiology, Chinese Academy of Sciences. He has co-edited a book with Arnold Demain on natural products, published in 2005 by Humana Press, holds eleven patents and has written seven books and more than eighty publications. He has been appointed on the editorial board of 5 peer-reviewed journals.

This review will discuss the challenges of finding novel microbial metabolites with anti-TB activity. The emphasis of this review is on natural product bioprospecting, and focuses on two aspects: 1) biodiversity- and taxonomy-guided library construction: the sourcing and de-replication of microbial metabolites from novel species isolated from a variety of environments, including extreme and marine habitats, and those species in symbiosis with other organisms, and 2) synergistic high-throughput screening (HTS): robust HTS assays for the de-replication and identification of active anti-TB microbial metabolite extracts from marine natural product libraries that work in synergy with known antimicrobial compounds. New anti-TB natural products that have been recently discovered will also be highlighted.

2 High-quality microbial natural product libraries

2.1 Biodiversity- and taxonomy-guided library construction

Diversifying the microbial sources of natural compounds is of primary concern in library development. Microorganisms of both terrestrial and marine origin have proven to be excellent sources of novel natural products, and recent advances in microbial genomics (genome sequencing, microbial ecology and metagenomics) have unequivocally demonstrated that the biosynthetic potential of natural products in bacteria is much higher than previously appreciated. Amongst microorganisms, the bacterial members of the suborders Micromonosporineae, Pseudonocardineae, Streptomycineae and Streptosporangineae of the order Actinomycetales of the class Actinobacteria are currently the most prolific sources of novel secondary metabolites, notably antibiotics.[8] The actinomycete genome, for example, contains approximately 8000 genes coding for 20–50 proteins from secondary metabolite synthetic gene clusters.[11,12] These organisms are already responsible for three-quarters of the antibiotics used in clinical and agricultural applications,[8] and are still a primary target of potentially useful compounds (Table 1).

Undiscovered species inhabiting unique environments with differing environmental constraints have been thought to be resources of novel compounds.[13,14] For example, pristine environments hold a larger diversity of species, including actinomycetes, than areas suffering from the impact of man,[15] while conditions of extreme temperature,[16] pressure[17] and pH[18] offer

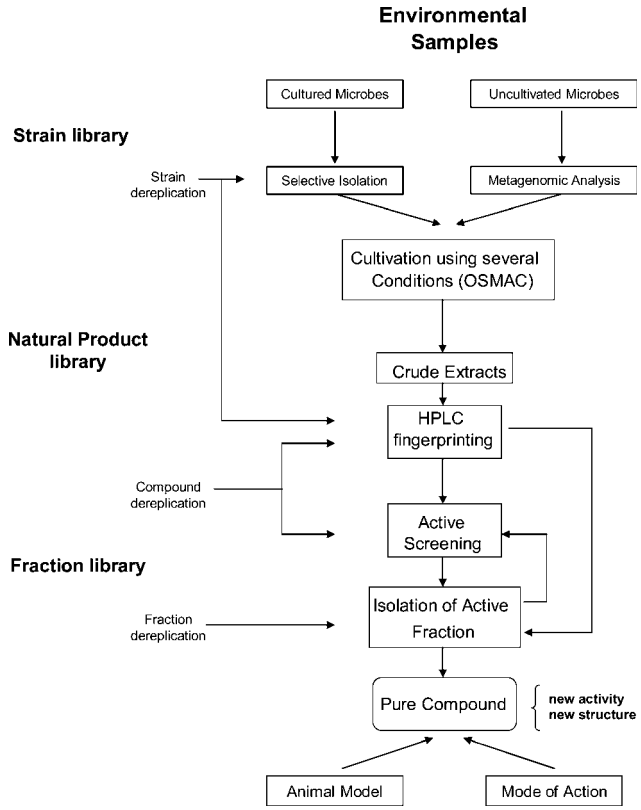


Fig. 1 The chemotaxonomy based screening approach. Steps used by the Institute of Microbiology, Chinese Academy of Sciences (IMCAS), in creating a high-quality natural product library with diversifying and de-replicating steps to ensure that microbial metabolites with novel actions and/or structures may be discovered. The initial aim is to diversify the compound base with samples rich in rare and unique organisms, with the challenge being to selectively isolate the common species or specifically target productive genera. Once a range of strains have been cultured, then high-throughput colony picking is needed to establish a large library of isolates and de-replication of those strains by morphology, characteristics, metagenomic data, molecular data, and chemical data. Following this, taxonomic characterisation and preservation of the strains that produce new bioactive compounds and biocatalysts must be developed, and testing of new culture media for maximum expression of secondary metabolites determined (OSMAC philosophy).[121] Solvent extraction of crude extracts from the chosen strains in the most productive media can then be de-replicated by HPLC–diode array detection and rapidly compared with a database of known compounds. De-replicated microbial metabolite crude extracts can then be used in HTS assays to test for anti-TB hits, as well as synergistic co-drug hits. Generation of semi-purified fractions from the positive hits, containing 1–3 compounds each, are also verified in biological assays. Confirmed hits are isolated and the total structure elucidated.

unique, competitive environments conducive to the production of biologically interesting small molecules.[19,20]

Marine environments and materials, such as the sea surface microlayer (home to neustonic organisms), the water column, 'marine snow', and sediments (including the 'deep biosphere', and sediments bearing methane hydrates), have already attracted a lot of attention as the source of new actinomycetes.[21,22] We have focused on constructing a marine natural product library[23,24] (Fig. 1), and have found novel and rare actinobacterial isolates in areas such as the accessible Qiongdongnan basin, South China Sea,[21] many of which show antimicrobial properties (Table 2).

Table 2 Recent hits from various screening assays of a marine natural product library Institute of Microbiology, Chinese Academy of Sciences, Beijing

Assay	Samples	Hits
BCG	~12 900	233
MRSA	~10 000	200
Antifungal	~12 900	165
14-3-3 inhibitors	~12 000	80
BPL	~8000	46

While many novel marine isolates have been collected from sediments and water samples, there is a growing interest in novel bacteria that are in symbiotic/mutualistic relationships with higher organisms. New marine actinomycete isolates have been found in sponges, tunicates and brittlestars.[25–27] Terrestrial actinomycete symbionts are also a source of novel species and compounds, as in the case of gut-specific organisms of the order Actinomycetales in the termite *Nasutitermes corniger*,[28] and those associated with fungus-growing ants.[29] Plant endophytes are also of interest, with researchers in China looking into the possibility that novel natural products of endophytic microbes may be behind many of the medicinal qualities of plants used in traditional Chinese medicines.[30]

As the previous examples demonstrate, there is value in focusing on ecology- and taxonomy-based approaches to strain selection. To increase the chance of finding novel compounds, a prospecting strategy that includes a good range of geographic areas and/or multiple environmental conditions increases the diversity of microbes, which can be confirmed using molecular methods (below). While many new isolates have been discovered, many containing antimicrobial natural products, few have been tested specifically on TB strains.[31] Therefore there is a huge resource ready and waiting to be screened for anti-TB activity.

2.2 Isolation and de-replication of rare and indigenous microbes

A major issue concerning strain libraries is redundancy of commonly isolated organisms. Special media are important for the isolation of rare or indigenous microbes, for example, the use of seawater-based isolation media to specifically allow the growth of marine organisms.[32] Additionally, some microorganisms may not grow in pure culture despite being provided with the proper nutrients (carbon, nitrogen, trace metals) or the correct temperature, aeration and time of cultivation. However, stimulation by signalling molecules, such as pyruvate, cyclic adenosine monophosphate and homoserine lactones, has been demonstrated to generate greater numbers of microorganisms.[33]

'Harsh' conditions or pre-treatments involving extremes of temperature or pH and specifically optimised media may be required to increase the number and diversity of extreme actinomycetes whilst decreasing the occurrence of other species.[34] Cycloheximide, nystatin, nalidixic acid mixture, and potassium dichromate have all been used as a means of reducing unwanted fungi and Gram-negative bacteria,[35,36] and dilutions to extinction or agar overlain with a membrane and a solid substrate have

been used to specifically isolate low-abundance actinobacteria.[37] Despite the wide variety of different media and pre-treatments, many of the isolates picked using high-throughput colony-picking technology will be clones with identical metabolic potential; therefore, further de-replication of genetically identical strains and/or compounds at an early stage in the bioprospecting process is necessary.

Strain de-replication can be successfully achieved with molecular profiling such as Restriction Fragment Length Polymorphism (RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S/18S genes. Detecting rare and novel actinomycetes has become easier with increasing availability of sound classifications based on the integrated use of genotypic and phenotypic data.[38] Morphological discrimination can be achieved with an expert eye; alternatively, highly automated colour and texture recognition platforms have been developed, accurate to 98% when compared with the identification results based on DNA fingerprinting.[39] Screening of the 'metabolome' as a means of fingerprinting strains to de-replicate clones is achieved by characterizing the nature of mixtures of compounds, in crude extracts using analytical platforms incorporating mass spectrometry (MS), ultraviolet light (UV) and nuclear magnetic resonance (NMR) data, and will be discussed briefly in Section 4.

2.3 Prediction of novel genes: culture independent methods

Maximizing diversity within the microbial groups is one of the cornerstones of any rational strategy designed to find new microbial metabolites with biological activity, but it remains an imperfect science and a critical challenge.[40] It is well understood that 99% of bacteria and 95% of fungi are currently uncultivated in the laboratory; therefore, molecular methods can verify the taxonomic diversity of housekeeping genes, such as 16S rRNA, independently or in tandem with culture-dependent techniques.[41] In addition, the diversity of large multi-enzymatic, multi-domain mega-synthases, nonribosomal peptide synthetases (NRPSs) or polyketide synthases (PKSs), involved in the biosynthesis of natural products such as cyclosporin, rifamycin and erythromycin can be determined. New PCR primers have been designed to specifically target and amplify the NRPS and PKS-I gene sequences from actinomycetes biosynthetic systems.[42] As a result, discrimination between cDNA libraries or strains based on the novelty of genes, potential productivity (compared to well-known productive species such as *Streptomyces*), and even the likely chemical structures produced can be achieved using comprehensive databases.

Genome mining has been recently used to induce a silent metabolic pathway in the important model organism *Aspergillus nidulans*, leading to the discovery of novel PKS-NRPS hybrid metabolites.[43] Expressing these novel biosynthetic genes in a host bacterium allows access to a vast untapped reservoir of genetic and metabolic diversity whilst avoiding the culture element,[44,45] and may be particularly important for environments where bioactive compounds can only be isolated in extremely low yields or where organisms are difficult to culture. While a discussion of genetic engineering is beyond the scope of this paper, reviews can be found discussing advances in actinomycete genome mining[46] and the success of molecular biology in identifying novel bioactive compounds.[47] These references highlight the fact that the metagenomic approach is coming into its own and is likely to be a major constituent of bioprospecting in the future.

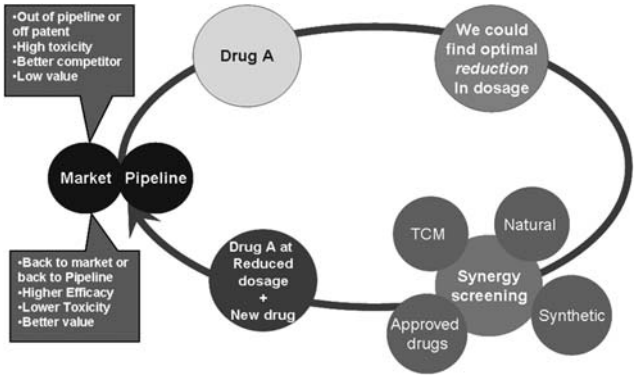


Fig. 2 The mechanism for discovery of synergistic drugs (TCM – Traditional Chinese Medicine). Adapted from ref. 122

3 High-throughput screening to evaluate the potential of new compounds

3.1 Synergistic high-throughput screening

Using automation, miniaturized assays and large-scale data analysis, high-throughput screening (HTS) aims to accelerate the pace of discovery of new drugs by screening hundreds of thousands of compounds. Targets include known classes of compounds (macrolides, pleuromutilins, quinolones and 2-pyridones and oxazolidinones[48]) and novel compounds with unknown action. Many TB thereputics suffer from having serious nephro- and oxotoxicity side effects and lengthy treatment times. Synergistic HTS uses quantitative data to discover those compounds that are effective under non-toxic concentrations when combined with other classes of TB drugs (Fig. 2). For example, a combination of 2 μg/mL of the natural plant product beauvericin (BEA) and 0.01 μg/mL ketoconazole (KTC) showed a greater inhibition of the fungal pathogen *Candida parapsilosis* ATCC 22019 than 1 μg/mL KTC alone.[24,49] Another example is the synergistic inhibition of multi-drug resistance pumps in flavonolignan 5′-methoxyhydnocarpin and the berberine alkaloids.[50]

HTS has now evolved into a mature discipline that is a crucial source of chemical starting points for drug discovery. The assays described in this review are used to screen the marine natural product library, and include those that focus on: the targeted screening of enzymes and pathways; the effect of a compound on the whole cell of the microbe; and the validation of compounds in intracellular tests.

3.2 Target-based assays

Natural product libraries are screened for prime anti-TB targets including those essential to the viability of TB, absent in the host cells, and believed to be responsible for resistance mechanisms. Current targets of interest include component systems driven by histidine phosphorylation,[51,52] eukaryotic serine/threonine kinases,[53] such as PknG (required for growth in animals),[54] and PknB,[55] protein tyrosine phosphatases PtpA and PtpB (essential

for persistence in the host cells), and polyphosphate kinases PPK1 and PPK2.[56]

Using a colorimetric assay, utilizing *p*-nitrophenyl phosphate as a substrate,[57,58] the inhibition of PtpA by natural products can be determined in a HTS format. Protein kinase assays, on the other hand, currently rely on incorporation of radiolabeled [g^{32}P]-ATP or [g^{32}P]-GTP as the phosphate donor, but we are adapting it to a non-radioactive fluorescence resonance energy transfer technology assay, performed on the Fusion® AlphaScreen apparatus (Perkin Elmer). Protein expression and purification are both essential for advancing this type of research. High amounts of protein can be expressed in *E. coli* using a T7 transcription vector,[59] and different forms of recombinant enzyme with or without fusion to GST, a C-terminus His tag, and maltose binding protein, can be constructed to facilitate rapid purification.

An approach to discover likely targets uses proteomic analysis to measure up-regulation and over-expression of proteins in response to sub-lethal quantities of a specific inhibitor. Cellular extracts of the proteome of test and control TB strains can be compared using two-dimensional gel electrophoresis.[60] Up-regulated proteins excised from gels have their identity determined by MS.[60] Each corresponding candidate gene can be cloned and then its proposed activity analysed biochemically in the presence of the inhibitor. This strategy, however, is limited to soluble proteins, and therefore membrane protein solubilisation may need to be employed.[61,62]

3.3 Whole-cell assays

A particular problem in enzyme-based assays is that while natural products, such as fosmidomycin, may show good inhibition of pure enzymes, they may also show a limited inhibitory effect on the growth of TB bacilli due to their inability to translocate through the membrane.[63,64] To screen the large numbers of compounds for growth/inhibition kinetics, simple HTS techniques are needed. Alamar Blue, validated against slow-growing TB in 1997, is a rapid and inexpensive way to measure cell growth by the extent of pink fluorescence in an oxidation/reduction reaction.[65] The quantitative nature of the results makes it ideal for synergistic studies against target and non-target cells.

The green fluorescence protein (GFP) assay[66,67] measures bacterial growth by direct readout of fluorescence from the expression vector (pUV3583c-GFP). We have used this assay to screen the effect of marine microbial natural products on the growth of *Mycobacterium bovis* ATCC35743 (BCG) (a slow-growing non-virulent strain closely related to *M. tuberculosis* H37Rv in terms of drug susceptibility profile and genetic composition) and TB, with throughput reaching 60 000 wells/day in an adapted 384-well format. Results from screening an initial 5000 extracts, showed 80 as having >90% inhibition against exponentially growing BCG, and 46 anti-TB activity, 8 at dilutions of $^1/_{16}\times$, and 1 at $^1/_{128}\times$ dilution. A quantitative comparison of the activity of the natural product extracts on *in vitro* growth in TB, and erythrocyte (RBC) lysis revealed 26 compounds where RBC lysis was less than 10% and growth inhibition was greater than 80%, at one or more concentrations (not shown). Recent improvements in GFP using an acetamidase promoter,[67] have increased the signal-to-background ratios, making it preferable over the alternative, Luciferase.[68] Several other advantages of GFP include its intrinsic fluorescent nature precluding the need for a substrate and better biosafety (if minimal bactericidal concentration is not being determined), as the microplate can remain closed after inoculation. Also, GFP measurements in intracellular environments preclude the need for host cell lysis (since a substrate is not required), allowing direct and repeated measurements of cell viability, thus offering easy kinetic monitoring and low cost.[67,68]

3.4 Intracellular assays

Infection studies are critical to enable testing of compounds within the human macrophage to determine their activity in the target environment. Infection of the human macrophage THP1 cell line can be achieved using well established methods of infection[69,70] and inhibitor tests.[71] Using an assay based on fluorescence-activated cell sorting (FACS),[72] the initial toxicity of lead compounds can be determined by monitoring survival of exposed macrophages, and the effect on phagosome maturation has been assayed using latex beads coated with the lead compounds.[73]

It is worth noting that specific and challenging experimental methodologies must be employed in the bioprospecting of novel compounds as connecting antimicrobial activity with mode of action is one of the most significant hurdles for cell-based small-molecule screening. Recently, a novel chemical genomic approach has been used to identify a new molecule, MAC13243, as a novel target against *Pseudomonas aeruginosa*.[74] Unfortunately, these methods are still limited in that antibiotic resistance frequently occurs for particular targets; therefore, multi-target screening models are required to obtain the most efficient results from compound screening.

It is also the case that many natural products have their own innate fluorescence, which can interfere with particular assays, resulting in false results. Of the positive hits found in the GFP assay example described above, only half of the hits remained positive when a dilution series of the extracts was tested in an erythrocyte haemolysis assay. Some innovative researchers are using the innate fluorescence of compounds to develop assays to screen strains directly for particular products.[75] For a more detailed review of the pitfalls of many high-throughput bioassay and natural product chemistry techniques, see ref. 68.

4 De-replication of anti-TB compounds

In order to avoid the re-discovery of known natural products, we have generated several specific antibiotic-resistant *Mycobacterium smegmatis* strains. Those extracts which demonstrate activity against a susceptible strain, but not the resistant strain, are presumed to have produced a natural product that corresponds to the respective antibiotic resistance marker. This allows us to screen out streptomycins or rifampin, two of the most frequent natural-product-derived antibiotics produced by actinomycetes. Cubist Pharmaceuticals has initiated a program to miniaturize fermentations by using calcium-alginate macro-droplet beads as fermentation vessels.[76] These are screened against a multi-drug-resistant *Escherichia coli* strain that carries

Table 3 Minimum inhibitory concentrations of microbial metabolites platensimycin, polymyxin B and polymyxin E[88,91,92,93]

Strain	Platensimycin **8**	Polymyxin B **9**	Polymyxin E **10**
Mycobacterium tuberculosis	12 μg/mL	5 mg/mL	—
Mycobacterium smegmatis	14 μg/mL	30 mg/mL	32 mg/mL
Mycobacterium avium	—	5 mg/mL	>500 mg/mL

resistance markers to 17 of the most frequently produced antibiotics by microorganisms, again allowing rapid de-prioritising of extracts.

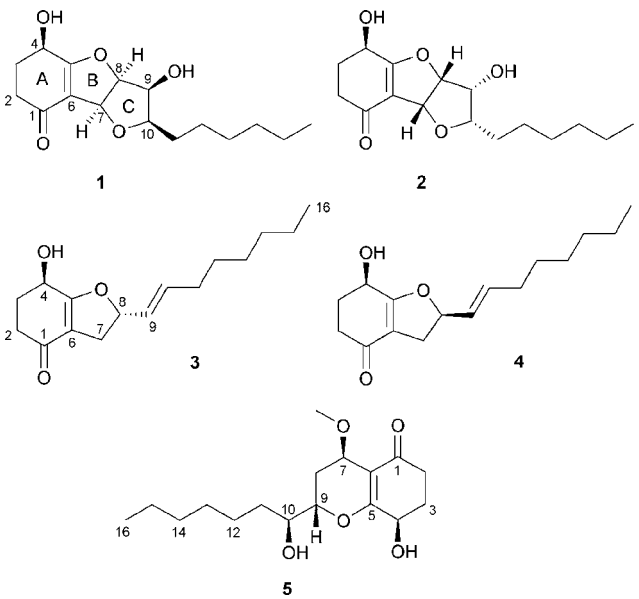
The second way to de-replicate known compounds is with rapid profiling/fingerprinting techniques and target analysis. Many hyphenated systems can: elucidate structures of new molecules; identify impurities and contaminants; analyze metabolites and biomarkers to evaluate biological pathways; and predict accurate structural details. In the past few years, advances in technology have allowed the development of tandem analytical techniques, such as LC–MS–MS[77] and LC–NMR.[78] HPLC–DAD–NMR–MS‡ is the most versatile analytical platform for complex mixture analysis.[79] Several recent improvements have been introduced to increase the sensitivity of NMR detection, bring it more into line with MS sensitivity and permitting the analysis of compounds at low concentration, a most important aspect in de-replication of natural products. The future of this hybrid technology will be the fully comprehensive HPLC–DAD–NMR–IT-TOF-MS‡, which will determine accurate molecular weight and NMR spectrum in one injection, and by evaluating the retention time, UV spectrum, NMR spectrum and molecular weight, represents a powerful all-inclusive tool for de-replication of crude extracts. However, most of these approaches can be performed only by the pharmaceutical industry or biotech companies with strong financial backing, leaving academic researchers behind.

For a more modest sum, our de-replication process for bioactive extracts is initiated by HPLC analysis using an analytical reverse-phase column, elution with a standardized methanol–water gradient, and detection at 254 nm. In the first pass the UV spectra and retention times recorded for each bioactive peak are compared to an in-house library of previously isolated and known microbial metabolites identified under the same parameters (Agilent 1200). Unidentified compounds are then further evaluated against natural product databases including the Dictionary of Natural Products, SciFinder, Antibase, and more sophisticated software packages such as Structure Elucidator developed by ACD Labs.[80] With HTS formats available, these approaches are – and will continue to be – important for de-replication of culture collections in order to avoid redundancy in the selection of species and maximize the chemical diversity of the microbial natural product library.

‡ DAD – diode array detection; IT-TOF-MS – ion-trap time-of-flight mass spectrometry.

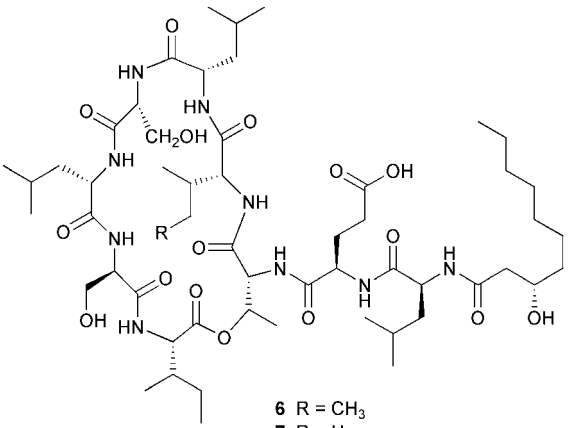
5 New microbial secondary metabolites discovered

The indication of quality control in any natural product library is in the novelty of the active natural products identified.[35] We find that around 30% of our purified compounds have novel structures, and have recently identified 20 natural product scaffolds of chemotherapeutic interest (unpublished). During a pilot screen of 5000 crude extracts from the library against the exponentially growing TB vaccine strain BCG, the known compounds isonitrile, nucleosidyl peptide and ansamycin were identified as active chemicals present in the library.[81] In addition, one crude extract from fungal plant pathogen *Trichoderma koningii* MF349 showed anti-BCG activity, and from the HPLC profile and UV spectrum, it was deduced that there were novel compounds in the extract. Ten polyketides were purified from this broth, including 5 novel structures, **1–5**.[82] However on re-fermentation, the compounds did not reproduce the same effects. Marine bacterial strain LS247, most closely related to type strain *Streptomyces puniceus*, also showed anti-BCG and anti-TB activity. Two active compounds were identified, and found to be cyclo-D-Pro-D-Leu and cyclo-D-Pro-D-Val with MIC80s against TB of 7.1 and 18.5 mg/mL, respectively.[83] Results such as this confirm the success of the screening program, the diversity of the samples, and the efficiency of the assays.



Novel anti-TB microbial metabolites have recently been found from a variety of aquatic and terrestrial sources, including marine *Pseudomonas* species isolated from a marine alga and tube worm.[84] The *Pseudomonas* strains produced the cyclic depsipeptides massetolide A **6** and viscosin **7**. When tested against TB and *Mycobacterium avium-intracellulare*, **6** displayed MIC values of 5–10 and 10–20 mg/mL, respectively, while **7** had activities of 2.5–5 and 5–10 mg/mL.[85] A novel endophytic fungus of the wild pineapple, *Ananas ananassoides* found growing in the Bolivian Amazon basin, has recently been reported to produce anti-TB metabolites. The volatiles of *Muscodor crispans* inhibited several susceptible and resistant TB isolates; however, the specific compounds responsible, the mode of action, and MIC or IC_{50} values are as yet unknown.[86] Several anti-TB ambiguine

isonitriles (structures unpublished) from the cyanobacterial extracts of freshwater *Nostoc muscorum* and terrestrial/freshwater *Fischerella ambigua* (MIC <100 μg/mL and 2.7 μg/mL, respectively) have been found using the Alamar Blue assay.[87] These examples show the range of habitats where anti-TB microbial metabolites are being found. The mode of action for many is as yet unknown, but they represent potential unaccompanied or synergistic drug leads.

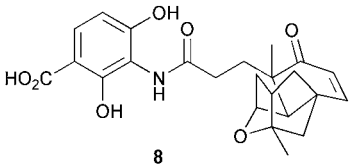


6 R = CH_3
7 R = H

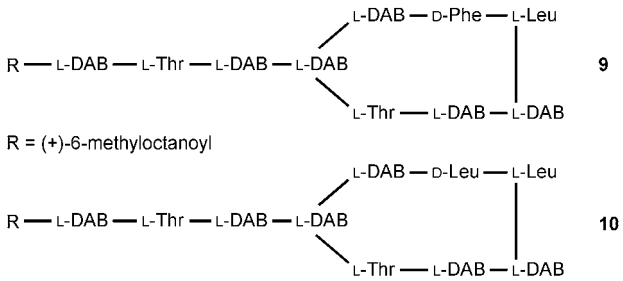
Two natural products, platensimycin and polymyxin, are prime candidates for synergistic testing. Platensimycin **8**, from a strain of *Streptomyces platensis*, was found through systematic screening of 250 000 natural product extracts using a combination of target-based, whole-cell and biochemical assays.[88] Compound **8** demonstrated strong, broad-spectrum Gram-positive antibacterial activity by selectively inhibiting mycolic acid biosynthesis, specifically the β-ketoacyl-ACP syntheses KasA and KasB.[88] However, poor permeability meant that it was inactive against TB.[88] Polymyxins, which were considered too toxic for use, have been re-discovered as inhibitors of TB by using an enzyme-based screening of a natural antibiotics library. The polymyxins (non-ribosomally synthesized in *Bacillus polymyxa*) bind between the cationic polypeptides and anionic lipopolysaccharide molecules in the outer membranes of MDR Gram-negative bacteria, increasing the permeability of the membrane.[89] Polymyxins B **9** and E **10** differ only in the amino acid at position 6, but showed differing potency against *Mycobacterium* strains.[89,90] The bacteristatic activity of platensimycin,[88] polymyxin E[91,92] and polymyxin B[93] (Table 3) could well be increased (and the toxicity decreased) at lower doses with a suitable synergist, potentially each other. The three compounds are already suitable in some respects, as their targets are not present in mammalian cells.

Table 4 Minimum inhibitory concentrations of microbial metabolites platensimycin, polymyxin B and polymyxin E[88,91,92,93]

Strain	Platensimycin **8**	Polymyxin B **9**	Polymyxin E **10**
Mycobacterium tuberculosis	12 μg/mL	5 mg/mL	—
Mycobacterium smegmatis	14 μg/mL	30 mg/mL	32 mg/mL
Mycobacterium avium	—	5 mg/mL	>500 mg/mL



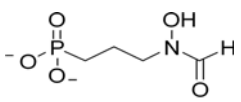
8



9

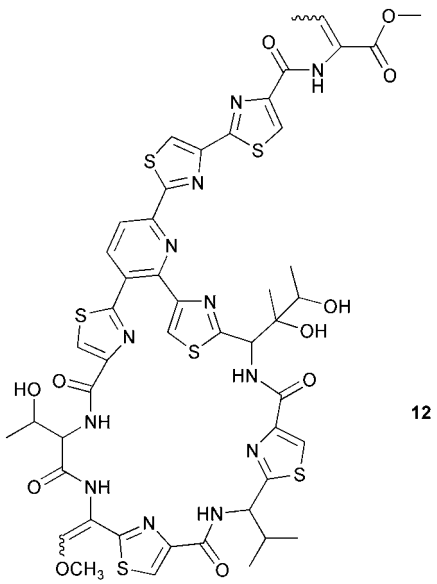
10

The phosphonate drug fosmidomycin, compound **11**, isolated from *Streptomyces lavendulae*, has been found to inhibit the 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway.[94] The MEP pathway of TB is an attractive target, as the final products of the MEP pathway are obligatory precursors of all isoprenoids, which are involved in many of the essential biochemical transformations required for TB survival, including biosynthesis of the cell wall and production of ATP. As yet, the TB MEP pathway has not been targeted by any promising new anti-TB drug; thus, the opportunities are ripe for the identification of inhibitors. A review of the genes in the MEP pathway and proposed HTS-amenable assays can be found.[95]



11

The use of rifampicin-resistant strains has recently led to the discovery of nocardithiocin **12**, isolated from the pathogenic *Nocardia pseudobrasiliensis* strain IFM 0757. This compound was found to be highly active against rifampicin-resistant as well as rifampicin-sensitive TB strains, with the majority of the resistant strains inhibited at concentrations ranging from 0.025 to 1.56 μg/mL.[96] Under investigation, its activity was found to be higher than that of fluoroquinolone and levofloxacin, though the compound was unstable in light. Nocardithiocin **12** was found to be a new cyclic thiopeptide (a group known to be activators of thiostrepton-induced protein A) containing 1- and 8-methyl groups, a 10-carbonyl group and novel carbon-methylated amino sugar constituent.[96] Mukai *et al.* have also reported a new siderophone and a benzonoid compound which also have anti-mycobacterial activity. They suggest that the high similarity of *Nocardia* to *Mycobacterium* strains, in terms of physiological characteristics (cell membrane/cell wall permeability), may make *Nocardia* a productive source of anti-mycobacterial compounds.[96]



12

6 Future prospects

Recently, a more systemic biological approach has been taken to drug discovery, taking into account the complex interactions found in biological systems and environments at multiple levels. Researchers are investigating the interactions between drugs and cascades of metabolic pathways,[97] growing promising strains under multiple culture conditions to fully explore secondary metabolite potential,[98] and developing co-cultivation of relevant natural co-existing organisms to simulate the natural environment and trigger secondary metabolite production.[99] An excellent example of the importance of interactions between organisms and this role in drug discovery is quorum sensing (QS). Chemical hormone-like autoinducers, used in cell-to-cell signaling mechanisms, were discovered to be responsible for controlling a plethora of virulence genes in several bacterial pathogens, specifically peptides in TB.[100] Antagonists to these autoinducers intercept bacterial intercellular communication, hindering their ability to act in a coordinated manner to express virulence traits. Moreover, because QS is not directly involved in essential processes, such as growth of the bacteria, one can reason that inhibition of QS will not yield a selective pressure for development of resistance. Therefore, screening for the inhibitors of signals of quorum sensing in TB could also yield novel anti-TB drugs.[100] We believe that a balanced approach incorporating ecological studies with the bioprospecting process could give valuable insights into the productivity of habitats and strains used in our screening programs, and that the significance of novelty can not be underestimated in the light of multi-resistant TB.

This drive is supported by the ever-increasing sophistication of new technologies and molecular methods (as discussed above). A relatively new addition to targeted screening of natural products is fragment-based lead discovery, which identifies small molecular fragments in the 120–300 Da range. In screening these small compounds, central scaffolds that can fit into the target binding site are identified.[101] These fragments are then evolved into larger compounds, either by linking or merging, or by incorporating additional interactions. This fragment evolution relies on access to experimentally determined structures of fragments bound to the target either by X-ray crystallography or by high-field NMR techniques, and despite the infancy of this computational approach, compounds identified have already progressed to clinical trials.[102]

According to the Global Alliance for TB Drug Development, the ideal new TB treatment will shorten the duration of therapy for all TB patients, including those with drug sensitive and drug resistant disease as well as those co-infected with HIV and receiving anti-retroviral therapy. New estimates suggest we have discovered only 10–20% of the small molecules produced by screened strains and only 1–2% of the global molecules available;[10,46] therefore, the possibility of finding new structures, which are less likely to cause unwanted drug–drug interactions, is still high. The need for drugs has meant that previously unsuitable β-lactam and aminoglycosides have been re-evaluated,[103] and drug analogues with improved efficacy and/or toxicity characteristics have been developed.[104,105] The significant improvement in activity and reduced toxicity that can be found in combinations involving natural products makes this area of study of major importance in finding new TB therapeutics. These studies will allow us to utilize our knowledge of drug discovery and screening to find novel and more effective ways to treat TB. Compounds found in natural products will be used in combination with current chemotherapies and in synergy with drugs currently deemed ineffective against TB, providing the opportunity to re-utilise known and clinically tested drugs.

What is most promising is the growing number of academic institutes that are joining with pharmaceutical and biotechnology companies to benefit from each other's merits – in other words, the productivity potential and focus of large companies, and the diversity of knowledge and innovation of academic institutes.[106] Large international organisations, such as TB Alliance, represent international partnerships and collaborations of experts all working towards a common goal and increasing the success rate in a long, difficult and expensive battle. The cost of treatment is one of major hurdles in TB drug development programs that aim to provide drugs to third world countries. Thus, the ability to prescribe cost-effective natural drugs to TB patients provides hope for the many who carry MDR and XDR strains, and may provide us with therapies against the as-yet untreated latent stages of the disease.

7 Acknowledgements

Part of this work was performed under research collaboration between the Global Alliance for TB Drug Development (TB Alliance) and the Institute of Microbiology of the Chinese Academy of Sciences (IMCAS). We acknowledge Drs. Zhenkun Ma, Anna Upton and Christopher B. Cooper from the TB Alliance for their scientific input during the performance of this work. We thank Debra Hung and Sarah Stanley for helping confirming BCG hits on MTB. This work was supported in part by grants from the National 863 Project (2007AA09Z443), Key Project for International Cooperation (2007DFB31620), National Natural Science Foundation of China (30973665, 30873129, 30901849, 30911120484, 81011120046 and 30911120483), National Key Technology R&D Program

(2007BAI26B02), the Chinese Academy of Sciences Innovation Project (KSCXZ-YW-G-013) and Pillar Program (KSCX2-YW-R-164), Important National Science & Technology Specific Projects (2008ZX09401–05, 2009ZX09302–004). L. Z. received funding from the Hundred Talents Program.

8 References

1 Y. L. Janin, Antituberculosis drugs: Ten years of research, *Bioorg. Med. Chem.*, 2007, **15**(7), 2479–2513.
2 *Tuberculosis: Fact sheet no. 104*, World Health Organization, 2003; http://www.who.int/medicentre/factsheets/who104/en/print.html (accessed 10th January 2010).
3 H. Nikaido, Multidrug Resistance in Bacteria, *Annu. Rev. Biochem.*, 2009, **78**, 119–146.
4 *Global tuberculosis control, short update to the 2009 report*, World Health Organization, 2009; www.who.int (accessed 10th January 2010).
5 G. L. Verdine, The combinatorial chemistry of Nature, *Nature*, 1996, **384**, 11–13.
6 A. D. Demin, From natural products discovery to commercialization: A success story, *J. Ind. Microbiol. Biotechnol.*, 2006, **33**, 486–495.
7 H. Gao, X. Zhou, Z. Gou, Y. Zhuo, C. Fu, M. Liu, F. Song, E. Ashforth and L. Zhang, Rational design for over-production of desirable microbial metabolites by precision engineering, *Antonie van Leeuwenhoek*, 2010, **98**(2), 151–163.
8 J. Berdy, Bioactive microbial metabolites, *J. Antibiot.*, 2005, **58**(1), 1–26.
9 A. L. Demain and S. Sanchez, Microbial drug discovery: 80 years of progress, *J. Antibiot.*, 2009, **62**, 5–16.
10 M. G. Watve, R. Tickoo, M. M. Jog and D. Bhole, How many antibiotics are produced by the genus *Streptomyces*?, *Arch. Microbiol.*, 2001, **176**, 386–390.
11 U. Galm and B. Shen, Expression of biosynthetic gene clusters in heterologous hosts for natural product production and combinatorial biosynthesis, *Expert Opin. Drug Discovery*, 2006, **1**, 409–437.
12 S. G. Van Lanen and B. Shen, Progress of combinatorial biosynthesis for drug discovery, *Drug Discovery Today: Technol.*, 2006, **3**, 285–292.
13 P. R. Jensen and W. Fenical, Marine bacterial diversity as a resource for novel microbial products, *J. Ind. Microbiol. Biotechnol.*, 1996, **17**(5), 346–351.
14 A. T. Bull, M. Goodfellow and J. H. Slater, Biodiversity as a source of innovation in biotechnology, *Annu. Rev. Microbiol.*, 1992, **46**, 219–252.
15 Y. Cao, Y. Jiang and X. Li-Hua, Great Shangri-La composition analysis of soil actinomycetes and biological activity determination, *Acta Microbiol. Sinica*, 2009, **49**, 105–109.
16 T. Zhu, J. Li, L. Li, H. Ma, Q. Che and Q. Gu, Isolation and bioactive metabolites research of Antarctic actinomycetes, *15th International Symposium on the Biology of Actinomycetes*, Shanghai, China, 20th–25th August 2009, p. 56.
17 M. Wagner, A. Gierth, W. Abdel-Mageed, M. Jaspers, M. Goodfellow, A. T. Bull, K. Horikoshi and H.-P. Fiedler, Dermacozines: Drugs from the abyss, *15th International Symposium on the Biology of Actinomycetes*, Shanghai, China, 20th–25th August 2009, p. 43.
18 A. T. Bull and J. E. M. Stach, Marine actinobacteria: New opportunities for natural product search and discovery, *Trends Microbiol.*, 2007, **15**(11), 491–499.
19 A. A. Stierle, D. B. Stierle and K. Kelly, Berkelic acid, a novel spiroketal with selective anticancer activity from an acid mine waste fungal extremophile, *J. Org. Chem.*, 2006, **71**, 5357–5360.
20 A. A. Stierle, D. B. Stierle and K. Kemp, Novel sesquiterpenoids with matrix metalloproteinase-3 inhibitory activity from an acid mine waste extremophile, *J. Nat. Prod.*, 2004, **67**, 1392–1395.
21 J. Bian, Y. Li, J. Wang, F.-H. Song, M. Liu, H.-Q. Dai, B. Ren, H. Gao, X. Hu, Z.-H. Liu, W.-J. Li and L. Zhang, *Amycolatopsis marina* sp. nov., an actinomycete isolated from an ocean sediment, *Int. J. Syst. Evol. Microbiol.*, 2009, **59**, 477–481.
22 A. C. Ward and N. Bora, Diversity and biogeography of marine actinobacteria, *Curr. Opin. Microbiol.*, 2006, **9**(3), 279–286.
23 L. Zhang, R. An, J. P. Wang, N. Sun, S. Zhang, J. Hu and J. Kuai, Isolation of novel bioactive compounds from marine microbes, *Curr. Opin. Microbiol.*, 2005, **8**, 276–281.
24 L. Zhang, K. Yan, Y. Zhang, R. Huang, J. Bian, C. Zheng, H. Sun, Z. Chen, N. Sun, R. An, F. Min, W. Zhao, Y. Zhuo, J. You, Y. Song, Z. Yu, Z. Liu, K. Yang, H. Gao, H. Dai, X. Zhang, J. Wang, C. Fu, G. Pei, J. Liu, S. Zhang, M. Goodfellow, Y. Jiang, J. Kuai, G. Zhou and X. Chen, High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 4606–4611.
25 A. Schirmer, R. Gadkari, C. D. Reeves, F. Ibrahim, E. F. DeLong and C. R. Hutchinson, Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge, *Appl. Environ. Microbiol.*, 2005, **71**, 4840–4849.
26 L. Gram, J. Melchiorsen and J. Bruhn, Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms, *Mar. Biotechnol.*, 2010, **12**, 439.
27 T. P. Anand, A. W. Bhat, Y. S. Shouche, U. Roy, J. Siddharth and S. P. Sarma, Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of south east India, *Microbiol. Res.*, 2006, **161**(3), 252–262.
28 T. Lefebvre, E. Miambi, A. Pando, M. Diouf and C. Rouland-Lefèvre, Gut-specific actinobacterial community structure and diversity associated with the wood-feeding termite species, *Nasutitermes corniger* (Motschulsky) described by nested PCR-DGGE Analysis, *Insectes Soc.*, 2009, **56**(3), 269–276.
29 U. G. Mueller and R. Sen, A Re-Evaluation of the symbiosis between fungus-growing ants and actinomycetes bacteria, *15th International Symposium on the Biology of Actinomycetes*, Shanghai, China, 20th–25th August 2009, p. 6.
30 G. Strobel, B. Daisy, U. Castillo and J. Harper, Natural products from endophytic microorganisms, *J. Nat. Prod.*, 2004, **67**, 257–268.
31 S. Ramesh and N. Mathivanan, Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes, *World J. Microbiol. Biotechnol.*, 2009, **25**(12), 2103–2111.
32 W. Pathom-aree, J. E. M. Stach, A. C. Ward, K. Horikoshi, A. T. Bull and M. Goodfellow, Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench, *Extremophiles*, 2006, **10**, 181–189.
33 V. Knight, J. J. Sanglier, D. DiTullio, S. Braccili, P. Bonner, J. Waters, D. Hughes and L. Zhang, Diversifying microbial natural products for drug discovery, *Appl. Microbiol. Biotechnol.*, 2003, **62**, 446–458.
34 W. Hozzein, M. Ali and W. Rabie, A new preferential medium for enumeration and isolation of desert actinomycetes, *World J. Microbiol. Biotechnol.*, 2008, **24**(8), 1547–1552.
35 J. S. Fry, in: *Microbial Diversity and Bioprospecting*, ed. A. Bull, ASM Press: Washington, D.C.; 2004, pp. 80–97.
36 M. Hayakawa, Studies on the isolation and distribution of rare actinomycetes in soil, *Actinomycetologica*, 2008, **22**, 12–19.
37 M. Polsinelli and P. G. Mazza, Use of membrane filters for selective isolation of actinomycetes from soil, *FEMS Microbiol. Lett.*, 1984, **22**(2), 79–83.
38 A. T. Bull, A. C. Ward and M. Goodfellow, Search and discovery strategies for biotechnology: The paradigm shift, *Microbiol. Mol. Biol. Rev.*, 2000, **64**, 573–606.
39 M. E. Hansen and J. M. Carstensen, Density-based retrieval from high-similarity image databases, *Pattern Recognit.*, 2004, **37**(11), 2155–2164.
40 S. B. Singh and F. Pelaez, Biodiversity, Chemical diversity and drug discovery, *Prog. Drug Res.*, 2008, **65**, 142–174.
41 Y. Hu, C. Fu, Y. Yin, G. Cheng, F. Lei, X. Yang, J. Li, E. J. Ashforth, L. Zhang and B. Zhu, Construction and preliminary analysis of a metagenomic fosmid library from a deep-sea sediment from the Qiongdongnan basin, South China Sea, *Mar. Biotechnol.*, 2010, DOI: 10.1007/s10126–010–9259–1.
42 A. Ayuso-Sacido and O. Genilloud, New PCR Primers for the screening of NRPS and PKS-I systems in actinomycetes: Detection and distribution of these biosynthetic gene sequences in major taxonomic groups, *Microb. Ecol.*, 2005, **49**(1), 10–24.

43 S. Bergmann, J. Schumann, K. Scherlach, C. Lange, A. A. Brakhage and C. Hertweck, Genomics-driven discovery of PKS–NRPS hybrid metabolites from *Aspergillus nidulans*, *Nat. Chem. Biol.*, 2007, **3**(4), 213–217.

44 R. R. Colwell, Fulfilling the promise of biotechnology, *Biotechnol. Adv.*, 2002, **20**, 215–228.

45 B. Gaudilliere, P. Bernardelli and P. Berna, in: *Annual Reports in Medicinal Chemistry*, ed. A. M. Doherty, Academic Press: San Diego, 2001, vol. 36, pp. 293–318.

46 R. H. Baltz, Renaissance in antibacterial discovery from actinomycetes, *Curr. Opin. Pharmacol.*, 2008, **8**(5), 557–563.

47 X. Lia and L. Qin, Metagenomics-based drug discovery and marine microbial diversity, *Trends Biotechnol.*, 2005, **23**(11), 539–543.

48 M. K. Spigelman, New tuberculosis therapeutics: A growing pipeline, *J. Infect. Dis.*, 2007, **196**, S28–S34.

49 M. E. Falagas, A. P. Grammatikos and A. Michalopoulos, Potential of old-generation antibiotics to address current need for new antibiotics, *Expert Rev. Anti-Infect. Ther.*, 2008, **6**, 593–600.

50 F. R. Stermitz, P. Lorenz, J. N. Tawara, L. A. Zenewicz and K. Lewis, Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 1433–1437.

51 S. T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead and B. G. Barrell, Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence, *Nature*, 1998, **393**, 537–544.

52 Y. Av-Gay and V. Deretic, in: *Tuberculosis*, ed. J. W. S. Cole, ASM Press: Washington, D.C., 2004, pp. 359–366.

53 Y. Av-Gay and M. Everett, The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*, *Trends Microbiol.*, 2000, **8**, 238–244.

54 S. Cowley, M. Ko, N. Pick, R. Chow, K. J. Downing, B. G. Gordhan, J. C. Betts, V. Mizrahi, D. A. Smith, R. W. Stokes and Y. Av-Gay, The *Mycobacterium tuberculosis* protein serine/threonine kinase Pkng is linked to cellular glutamate/glutamine levels and is important for growth *in vivo*, *Mol. Microbiol.*, 2004, **52**, 1691–1702.

55 P. Fernandez, B. Saint-Joanis, N. Barilone, M. Jackson, B. Gicquel, S. T. Cole and P. M. Alzari, The Ser/Thr protein kinase Pknb is essential for sustaining mycobacterial growth, *J. Bacteriol.*, 2006, **188**, 7778–7784.

56 K. Surekha, S. Dey, P. Datta, A. K. Singh, A. Dasgupta, S. Rodrigue, J. Basu and M. Kundu, Polyphosphate kinase is involved in stress-induced Mprab-Sige-Rel signaling in mycobacteria, *Mol. Microbiol.*, 2007, **65**, 261–276.

57 S. C. Cowley, R. Babakaiff and Y. Av-Gay, Expression and localization of the *Mycobacterium tuberculosis* protein tyrosine phosphatase Ptpa, *Res. Microbiol.*, 2002, **153**, 233–241.

58 H. Bach, K. G. Papavinasasundaram, D. Wong, Z. Hmama and Y. Av-Gay, *Mycobacterium tuberculosis* virulence is mediated by Ptpa dephosphorylation of human vacuolar protein sorting 33b, *Cell Host Microbe*, 2008, **3**, 316–322.

59 F. W. Studier, A. H. Rosenberg, J. Dunn and J. W. Dubendorff, Use of T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol.*, 1990, **185**, 60–89.

60 N. S. Dosanjh, M. Rawat, J. H. Chung and Y. Av-Gay, Thiol specific oxidative stress response in mycobacteria, *FEMS Microbiol. Lett.*, 2005, **249**, 87–94.

61 J. Blonder, K. C. Chan, H. J. Issaq and T. D. Veenstra, Identification of membrane proteins from mammalian cell/tissue using methanol-facilitated solubilization and tryptic digestion coupled with 2D-LC-MS/MS, *Nat. Protoc.*, 2006, **1**, 2784–2790.

62 S. Mikami, T. Kishimoto, H. H. and T. Mitsui, Technical improvement to 2D-PAGE of rice organelle membrane proteins, *Biosci., Biotechnol., Biochem.*, 2002, **66**, 1170–1173.

63 R. K. Dhiman, M. L. Schaeffer, A. M. Bailey, C. A. Testa, H. Scherman and D. C. Crick, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (Ispc) from *Mycobacterium tuberculosis*: Towards understanding mycobacterial resistance to fosmidomycin, *J. Bacteriol.*, 2005, **187**, 8395–8402.

64 L. M. Henriksson, T. Unge, J. Carlsson, J. Aqvist, S. L. Mowbray and T. A. Jones, Structures of *Mycobacterium tuberculosis* 1-deoxy-D-xylulose-5-phosphate reductoisomerase provide new insights into catalysis, *J. Biol. Chem.*, 2007, **282**, 19905–19916.

65 L. A. Collins and S. G. Franzblau, Microplate Alamar Blue assay versus Bactec 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*, *Antimicrob. Agents Chemother.*, 1997, **41**(5), 1004–1009.

66 L. A. Collins, M. N. Torrero and S. G. Franzblau, Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*, *Antimicrob. Agents Chemother.*, 1998, **42**(2), 344–347.

67 C. Changsen, S. G. Franzblau and P. Palittapongarnpim, Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter, *Antimicrob. Agents Chemother.*, 2003, **47**, 3682–3687.

68 G. F. Pauli, R. J. Case, T. Inui, Y. Wang, S. Cho, N. H. Fischer and S. G. Franzblau, New perspectives on natural products in TB drug research, *Life Sci.*, 2005, **78**, 485–494.

69 K. G. Papavinasasundaram, B. Chan, J. H. Chung, M. J. Colston, E. O. Davis and Y. Av-Gay, Deletion of the *Mycobacterium tuberculosis* Pknh gene confers a higher bacillary load during the chronic phase of infection in Balb/C mice, *J. Bacteriol.*, 2005, **187**, 5751–5760.

70 A. L. Hestvik, Z. Hmama and Y. Av-Gay, Kinome analysis of host response to mycobacterial infection: A novel technique in proteomics, *Infect. Immun.*, 2003, **71**, 5514–5522.

71 B. Hegymegi-Barakonyi, R. Szekely, Z. Varga, R. Kiss, G. Borbely, G. Nemeth, P. Banhegyi, J. Pato, Z. Greff, Z. Horvath, G. Meszaros, J. Marosfalvi, D. Eros, C. Szantai-Kis, N. Breza, S. Garavaglia, S. Perozzi, M. Rizzi, D. Hafenbradl, M. Ko, Y. Av-Gay, B. M. Klebl, L. Orfi and G. Keri, Signalling inhibitors against *Mycobacterium tuberculosis* – Early days of a new therapeutic concept in tuberculosis, *Curr. Med. Chem.*, 2008, **15**, 2760–2770.

72 N. Pick, S. Cameron, D. Arad and Y. Av-Gay, Screening of compounds toxicity against human monocytic cell line Thp-1 by flow cytometry, *Biol. Proced. Online*, 2004, **6**, 220–225.

73 R. Singh, V. Rao, H. Shakila, R. Gupta, A. Khera, N. Dhar, A. Singh, A. Koul, Y. Singh, M. Naseema, P. R. Narayanan, C. N. Paramasivan, V. D. Ramanathan and A. K. Tyagi, Disruption of Mptpb impairs the ability of *Mycobacterium tuberculosis* to survive in guinea pigs, *Mol. Microbiol.*, 2003, **50**, 751–762.

74 R. Pathania, S. Zlitni, C. Barker, R. Das, D. A. Gerritsma, J. Lebert, E. Awuah, G. Melacini, F. A. Capretta and E. D. Brown, Chemical genomics in *Escherichia coli* identifies an inhibitor of bacterial lipoprotein targeting, *Nat. Chem. Biol.*, 2009, **5**(11), 849–856.

75 J. S. Sandler, W. Fenical, B. M. Gulledge, A. R. Chamberlin and J. J. La Clair, Fluorescent profiling of natural product producers, *J. Am. Chem. Soc.*, 2005, **127**, 9320–9321.

76 R. H. Baltz, Antimicrobials from actinomycetes: Back to the future, *Microbe*, 2007, **2**, 125–131.

77 J. L. Wolfender, P. Waridel, K. Ndjoko, K. R. Hobby, H. J. Major and K. Hostettmann, Evaluation of Q-TOF-MS/MS and multiple stage IT-MSN for the dereplication of flavonoids and related compounds in crude plant extracts, *Analusis*, 2000, **28**, 895–906.

78 S. C. Bobzin, S. Yang and T. P. Kasten, LC–NMR: A new tool to expedite the dereplication and identification of natural products, *J. Ind. Microbiol. Biotechnol.*, 2000, **25**, 342–345.

79 O. Corcoran and M. Spraul, LC–NMR–MS in drug discovery, *Drug Discovery Today*, 2003, **8**(14), 624–631.

80 C. Steinbeck, Recent developments in automated structure elucidation of natural products, *Nat. Prod. Rep.*, 2004, **21**, 512–518.

81 C.-Z. Fu, F.-H. Song, H. Guo, S. Stanley, H. Dai, H.-L. Chen, D. Hung and L.-X. Zhang, in: Prospecting for anti-tuberculosis drugs from the natural products library, *15th International Symposium on the Biology of Actinomycetes*, Shanghai, China, 20th–25th August, 2009, p. 89.

82 F. Song, H. Dai, Y. Tong, B. Ren, C. Chen, N. Sun, X. Liu, J. Bian, M. Liu, H. Gao, H. Liu, X. Chen and L. Zhang, Trichodermaketones A–D and 7-*O*-methylkoninginin D from the marine fungus *Trichoderma koningii*, *J. Nat. Prod.*, 2010, **73**, 806.

83 F.-H. Song, G. Pei, C.-Z. Fu, N. Sun, H. Guo, S. Stanley, H. Dai, H.-L. Chen, D. Hung and L.-X. Zhang, in: Diketopiperazines with antituberculosis activity from *Streptomycices puniceius* strain Ls247, *15th International Symposium on the Biology of Actinomycetes*, Shanghai, China, 20–25th August, 2009, p. 48.

84 J. Gerard, R. Lloyd, T. Barsby, P. Haden, M. T. Kelly, R. J. Andersen and A.-H. Massetolides, Antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats, *J. Nat. Prod.*, 1997, **60**, 223.

85 K. A. El Sayed, P. Bartyzel, X. Shen, T. L. Perry, J. K. Zjawiony and M. T. Hamann, Marine natural products as antituberculosis agents, *Tetrahedron*, 2000, **56**, 949–953.

86 A. M. Mitchell, G. A. Strobel, E. Moore, R. Robison and J. Sears, Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus, *Microbiology*, 2010, **156**, 270–277.

87 G. Chlipala, S. Mo, E. J. Carcache de Blanco, A. Ito, S. Bazarek and J. Orjala, Investigation of antimicrobial and protease-inhibitory activity from cultured cyanobacteria, *Pharm. Biol.*, 2009, **47**, 53–60.

88 A. K. Brown, R. C. Taylor, A. Bhatt, K. Fütterer and G. S. Besra, Platensimycin activity against mycobacterial β-ketoacyl-ACP synthases, *PLoS One*, 2009, **4**, e6306.

89 T. Mogi, Y. Murase, M. Mori, K. Shiomi, S. Omura, M. P. Paranagama and K. Kita, Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: Quinone oxidoreductase from the Gram-positive bacterium *Mycobacterium smegmatis*, *J. Biochem.*, 2009, **146**, 491–499.

90 P. R. Schindler and M. Teuber, Action of polymyxin B on bacterial membranes: Morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B, *Antimicrob. Agents Chemothe.r*, 1975, **8**(1), 95–104.

91 N. Rastogi, M. C. Potar and H. L. David, Antimycobacterial spectrum of colistin (polymyxin E), *Ann. Inst. Pasteur Microbiol.*, 1986, **137A**, 45–53.

92 H. L. David and N. Rastogi, Antibacterial action of colistin (polymyxin E) against *Mycobacterium aurum*, *Antimicrob. Agents Chemother.*, 1985, **27**, 701–707.

93 J. A. McGarvey and L. Bermudez, Phenotypic and genomic analyses of the *Mycobacterium avium* complex reveal differences in gastrointestinal invasion and genomic composition, *Infect. Immun.*, 2001, **69**, 7242–7249.

94 A. T. Koppisch, D. T. Fox, B. S. Blagg and C. D. Poulter, *E. coli* MEP synthase: Steady-state kinetic analysis and substrate binding, *Biochemistry*, 2002, **41**, 236–243.

95 H. Eoh, P. J. Brennan and D. C. rick, The *Mycobacterium tuberculosis* MEP (2C-methyl-D-erythritol 4-phosphate) pathway as a new drug target, *Tuberculosis*, 2009, **89**, 1–11.

96 A. Mukai, T. Fukai, Y. Hoshino, K. Yazawa, K. Harada and Y. Mikami, Nocardithiocin, a novel thiopeptide antibiotic, produced by pathogenic *Nocardia pseudobrasiliensis* IFM 0757, *J. Antibiot.*, 2009, **62**, 613–619.

97 P. K. Naoghare, H. A. Ki, S.-M. Paek, Y. K. Tak, Y.-G. Suh, S. Kim, K.-H. Lee and J. M. Song, Simultaneous quantitative monitoring of drug-induced caspase cascade pathways in carcinoma cells, *Integr. Biol.*, 2010, **2**, 46–57.

98 T. O. Larsen, J. Smedsgaard, K. F. Nielsen, M. E. Hansen and J. C. Frisvad, Phenotypic taxonomy and metabolite profiling in microbial drug discovery, *Nat. Prod. Rep.*, 2005, **22**, 672–695.

99 V. Schroeckh, K. Scherlach, H.-W. Nützmann, E. Shelest, W. Schmidt-Heck, J. Schuemann, K. Martin, C. Hertweck and A. A. Brakhage, Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**(34), 14558–14563.

100 V. Sperandio, Novel approaches to bacterial infection therapy by interfering with bacteria-to-bacteria signalling, *Expert Rev. Anti-Infect. Ther.*, 2007, **5**, 271–276.

101 M. N. Schulz and R. E. Hubbard, Recent progress in fragment-based lead discovery, *Curr. Opin. Pharmacol.*, 2009, **9**, 615–621.

102 S. Howard, V. Berdini, J. A. Boulstridge, M. G. Carr, D. M. Cross, J. Curry, L. A. Devine, T. R. Early, L. Fazal, A. L. Gill, M. Heathcote, S. Maman, J. E. Matthews, R. L. McMenamin, E. F. Navarro, M. A. O'Brien, M. O'Reilly, D. C. Rees, M. Reule, D. Tisi, G. Williams, M. Vinković and P. G. l. Wyatt, Fragment-based discovery of the pyrazol-4-yl urea (At9283), a multitargeted kinase inhibitor with potent aurora kinase activity, *J. Med. Chem.*, 2009, **52**, 379–388.

103 H. F. Chambers, D. Moreau, D. Yajko, C. Miick, C. Wagner, C. Hackbarth, S. Kocagoz, E. Rosenberg, W. K. Hadley and H. Nikaido, Can penicillins and other β-lactam antibiotics be used to treat tuberculosis?, *Antimicrob. Agents Chemother.*, 1995, **39**(12), 2620–2624.

104 T.-Y. Tsai, S.-C. Chang, P.-R. Hsueh, N.-H. Feng and J. T. Wang, *In vitro* activity of isepamicin and other aminoglycosides against clinical isolates of Gram-negative bacteria causing nosocomial bloodstream infections, *J. Microbiol. Immunol. Infect.*, 2007, **40**, 481–486.

105 M. E. Falagas and P. Kopterides, Old antibiotics for infections in critically ill patients, *Curr. Opin. Crit. Care*, 2007, **13**(5), 592–597.

106 C. J. Tralau-Stewart, C. A. Wyatta, D. E. Kleyna and A. Ayada, Drug discovery: New models for industry–academic partnerships, *Drug Discovery Today*, 2009, **14**(1–2), 95–101.

107 M. S. Butler and A. D. Buss, Natural products – the future scaffolds for novel antibiotics?, *Biochem. Pharmacol.*, 2006, **71**, 919–929.

108 S. A. Waksman and H. B. Woodruff, Bacteriostatic and bacteriocidal substances produced by soil actinomycetes, *Proc. Soc. Exp. Biol.*, 1940, **45**, 609–614.

109 S. A. Waksman and E. Bugie, Strain specificity and production of antibiotic substances. II. *Aspergillus flavus-oryzae* group, *Proc. Natl. Acad. Sci. U. S. A.*, 1943, **29**, 282–288.

110 J. M. McGuire, R. L. Bunch, R. C. Anderson, H. E. Boaz, E. H. Flynn, H. M. Powel and J. W. Smith, Ilotycin a new antibiotic, *Antibiot. Chemother.*, 1952, **2**, 281–283.

111 F. Kavanagh, A. Hervey and W. J. Robbins, Antibiotic substances from basidiomycetes. VIII. *Pleurotus multilus* (Fr.) Sacc. and *Pleurotus passeckerianus* Pilat., *Proc. Natl. Acad. Sci. U. S. A.*, 1951, **37**, 570–574.

112 I. G. Epstein, K. G. Nair and L. J. Boyd, Cycloserine, a new antibiotic, in the treatment of human pulmonary tuberculosis: A preliminary report, *Antibiot. Med. Clin. Ther.*, 1955, **2**, 80–93.

113 H. Umezawah, M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and T. Takeuchi, Production and isolation of a new antibiotic: kanamycin, *J. Antibiot.*, 1957, **10**(5), 181–188.

114 P. Sensi, P. Margalith and M. T. Timbal, Rifomycin, a new antibiotic—preliminary report, *Farmaco Sci. Ed.*, 1959, **14**(2), 146–147.

115 E. B. Herr, M. E. Haney, G. B. Pittenger and C. E. Higgens, Isolation and characterization of a new peptide antibiotic, *Proc. Indiana Acad. Sci.*, 1960, **69**(134).

116 M. Rieber, T. Imaeda and I. M. Cesari, Bacitracin action on membranes of mycobacteria, *J. Gen. Microbiol.*, 1969, **55**(1), 155–159.

117 H. Oishi, T. Noto, H. Sasaki, K. Suzuki, T. Hayashi, H. Okazaki, K. Ando and M. Sawada, Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties, *J. Antibiot.*, 1982, **35**(4), 391–395.

118 H. Yamaguchi, S. Sato, S. Yoshida, K. Takada, M. Itoh, H. Ssto and N. Otake, Capuramycin, a new nucleoside antibiotic. Taxonomy, fermentation, isolation and characterization, *J. Antibiot.*, 1986, **39**(8), 1047–1053.

119 M. Igarashi, Y. Takahashi, T. Shitara, H. Nakamura, H. Naganawa, T. Miyake and Y. Akamatsu, Caprazamycins, novel lipo-nucleoside antibiotics, from *Streptomyces* sp. II. Structure elucidation of caprazamycins, *J. Antibiot.*, 2005, **58**, 327–337.

120 R. Mariani, G. Granata, S. I. Maffioli, S. Serina, C. Brunati, M. Sosio, A. Marazzi, A. Vannini, D. Patel, R. White and R. Ciabatti, Antibiotics Ge23077, Novel inhibitors of bacterial RNA polymerase. Part 3: Chemical derivatization, *Bioorg. Med. Chem. Lett.*, 2005, **15**(16), 3748–3752.

121 H. B. Bode, B. Bethe, R. Höfs and A. Zeeck, Big effects from small changes: Possible ways to explore Nature's chemical diversity, *ChemBioChem*, 2002, **3**(7), 619–627.

122 X. Liu, E. Ashforth, B. Ren, F. Song, H. Dai, M. Liu, J. Wang, Q. Xie and L. Zhang, Bioprospecting microbial natural product libraries from the marine environment for drug discovery, *J. Antibiot.*, 2010, **63**, 415–422.