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Bioautography with TLC-MS/NMR for Rapid Discovery of Anti-tuberculosis Lead Compounds from Natural Sources

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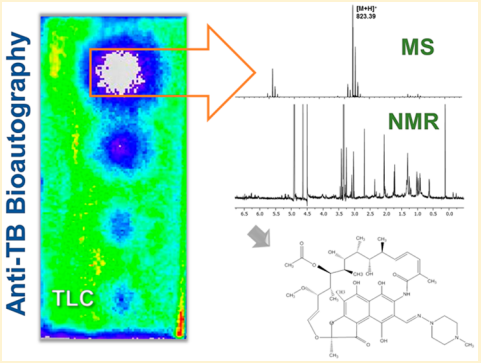
ABSTRACT: While natural products constitute an established source of lead compounds, the classical iterative bioassay-guided isolation process is both time- and labor-intensive and prone to failing to identify active minor constituents. (HP)TLC-bioautography-MS/NMR, which combines cutting-edge microbiological, chromatographic, and spectrometric technologies, was developed to accelerate anti-tuberculosis (TB) drug discovery from natural sources by acquiring structural information at a very early stage of the isolation process. Using the avirulent, bioluminescent *Mtb* strain mc^{2}7000 luxABCDE, three variations of bioautography were evaluated and optimized for sensitivity in detecting anti-TB agents, including established clinical agents and new leads with novel mechanisms of action. Several exemplary applications of this approach to microbial extracts demonstrate its potential as a routine method in anti-TB drug discovery from natural sources.



KEYWORDS: *bioautography, drug discovery, Mycobacterium tuberculosis, natural products, TLC-MS/NMR*

Tuberculosis (TB) continues to be one of the world's major health problems, ranking as the second leading cause of death from an infectious disease, with 9.6 million new cases and 1.5 million deaths in the year 2014.[1] One-third of the world's population is infected with latent TB, and approximately 5−10% might develop active TB during their lifetime.[2]

Natural products have been a fruitful source of bioactive leads in drug discovery,[3] including the search for new anti-TB compounds.[4−7] Despite the historical success of natural products, major pharmaceutical industries have ceased their natural products screening programs.[8] This is in part due to the inefficiency of the classical bioassay-guided isolation process which precedes the determination of structure and requires monitoring of biological activity throughout the process. In order to enhance the efficiency of discovery of active principles, the development of alternative, interdisciplinary approaches that integrate cutting-edge microbiological and analytical methods may provide strategies for substantially shortening the bioassay-guided isolation process and facilitating the identification of otherwise overseen active minor constituents.

Bioautographic detection, first described in 1946,[9] is a simple, rapid, and inexpensive method that utilizes thin-layer chromatography (TLC) as a basic methodology to detect various activities such as antibacterial, antifungal, antioxidant, antiestrogenic, and enzymatic.[10] One foremost advantage of bioautography is its capability to be directly connected with other chromatographic and/or spectrometric technologies. Applications of TLC combined with bioautography detection can be found in the rapid screening of a large number of samples for bioactivity and in the target-directed isolation of active compounds.[11]

Responding to the demand for new anti-TB lead compounds, and as an alternative to the bioassay-guided isolation procedure, a new technology—(HP)TLC-bioautography-MS/NMR—was developed as a means of discovering new anti-TB agents. This method circumvents the lengthy bioassay-guided isolation process, enables the detection of active, even minor constituents, and minimizes the risk of being side-tracked by synergy-dependent activity. By using an avirulent, bioluminescent *Mtb* strain, mc^{2}7000 luxABCDE, this method also overcomes the *Mtb*-specific issues of slow growth and the requirement of a biosafety level 3 laboratory.

To ensure the detection of compounds covering a wide range of polarities, three slightly different approaches were evaluated, using different anti-TB agents: (i) contact bioautography, (ii) agar-overlay bioautography, and (iii) direct bioautography. The applicability of this new approach was demonstrated by using

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(HP)TLC-bioautography-MS/NMR to evaluate actinomycete and *Aspergillus* extracts for the identification and/or isolation as well as for the dereplication of anti-TB lead compounds.

MATERIALS AND METHODS

Bacterial Strain, Media, and Growth Conditions. The *Mycobacterium tuberculosis* (*Mtb*) mc^{2}7000 strain was received from William Jacobs' laboratory (Albert Einstein College of Medicine, USA), and the luxABCDE cassette was received from Siouxsie Wiles' laboratory (University of Auckland, New Zealand) and introduced in our own laboratory (University of Illinois at Chicago, USA). *Mtb* mc^{2}7000 luxABCDE was grown in 7H9-GlyT80-OADC-PAA medium (Middlebrook 7H9 broth, Difco, Detroit, MI, USA) supplemented with 10% oleic acid−albumin−dextrose−catalase (OADC; Difco), 0.5% glycerol, 0.05% Tween 80, and pantothenic acid (PAA) at a final concentration of 100 μg/mL (Sigma-Aldrich Corp., St. Louis, MO, USA). The strain was grown at 37 °C in an incubator shaker at 220 rpm until the optical density (OD) of the bacterial suspension had reached 0.2−0.3 at 570 nm. The bacterial stocks were stored in 15% glycerol at −80 °C.

For individual experiments, 200 mL of 7H9-GlyT80-OADC-PAA medium was inoculated with 1.5 mL of frozen glycerol bacterial stock and incubated at 37 °C in an incubator shaker at 220 rpm until achieving an OD of 0.2−0.3 at 570 nm.

Chemicals. Isoniazid (INH), rifampin (RMP), ethambutol dihydrochloride (EMB), capreomycin sulfate (CAP), moxifloxacin hydrochloride (MOX), clofazimine (CLF), palmitic acid (PAL), and gliotoxin were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Pretomanid (PMD) and bedaquiline (BDQ) were received from The Global Alliance for TB Drug Development. The actinomycete and *Aspergillus* extracts used in this study were obtained from the Extract Collection of Useful Microorganisms (ECUM) at Myongji University, Republic of Korea. Ecumicin was isolated from a *Nonomuraea* sp. MJM5123,[12] hytramycins I and V from the *Streptomyces hygroscopicus* strain ECUM14046,[13] xylamycin from the *Streptomyces* sp. strain ECUM8412,[14] and gliotoxin from the *Aspergillus fumigatus* strain ECUM40602.

Stock Solution and Sample Preparation. Individual stock solutions of INH, RMP, EMB, CAP, MOX, CLF, PMD, PAL, and BDQ were prepared at 5 mg/mL by dissolving CAP and PAA in milli-Q water, CLF in chloroform, and the other antibiotics in methanol. The solutions were stored at −20 °C. Working solutions were freshly prepared at concentrations from 0.001 to 100 MIC. The standard solutions of ecumicin, hytramycins V and I, xylamycin, and gliotoxin, as well the actinomycete and *Aspergillus* fermentation extracts were prepared by dissolving the materials in methanol.

Thin-Layer Chromatography. TLC glass-backed plates precoated with silica gel (20 × 20 cm; no. 809013), silica gel 60 with a fluorescence indicator (20 × 20 cm; no. 809023), nanosilica gel 60 with a fluorescence indicator (10 × 20 cm; no. 811023), nanosilica gel RP-2 with a fluorescence indicator (20 × 20 cm; no. 811082), nanosilica gel C18-50 with a fluorescence indicator (10 × 10 cm; no. 811064), and nanosilica gel C18-100 with a fluorescence indicator (10 × 10 cm; no. 811062) were purchased from Macherey-Nagel (Bethlehem, PA, USA). The TLC plates were predeveloped with methanol and dried at 120 °C for 20 min on a TLC plate heater, cut to an appropriate size, wrapped in aluminum foil, and stored in a desiccator to prevent contamination.

The standard solutions/extract samples were applied to the (HP)TLC plate by using an automatic TLC sampler ATS4 (CAMAG, Muttenz, Switzerland) with the following application conditions: filling speed: 15 μL/s, predosage volume: 200 nL, dosage application speed: 150 nL/s, rinsing cycle 1 with methanol/water (9:1, v/v), rinsing vacuum time 4 s: filling vacuum time: 1 s.

The TLC plates were developed in an appropriate solvent system in a classical TLC chamber (CAMAG) or in a CAMAG automated multiple development chamber AMD 2 (CAMAG). The TLC plates were prepared in duplicate; one plate was subjected to bioautography, while the second was used for TLC-MS experiments.

Bioautography. **Direct:** 7H9-GlyT80-OADC-PAA medium was mixed with *Mtb* mc^{2}7000 luxABCDE working bacterial suspension (1:4, v/v) and poured into a dipping chamber. The TLC plate was dipped into the working bacterial suspension with a chromatogram immersion device (CAMAG) for 8 s with an immersion speed of 30 mm/s. The plate was then placed in a sterile Petri dish, which was sealed with tape, and incubated for 24 h at 37 °C. **Contact:** 7H11-Gly-OADC-PAA agar (i.e., Middlebrook 7H11 agar; Difco, Detroit, MI, USA) supplemented with 1% glycerol, 10% OADC, and PAA at a final concentration of 100 μg/mL (Sigma-Aldrich Corp., St. Louis, MO, USA) was mixed with *Mtb* mc^{2}7000 luxABCDE working bacterial suspension (1:5, v/v) and then poured into a Petri dish. After the agar solidified, a TLC plate was placed face-down on the surface of the agar and the plate was cooled to 4 °C for 15 min. The TLC plate was then removed, and the Petri dish was sealed with tape and incubated for 24 h at 37 °C. **Agar-overlay:** The TLC plate was placed in a Petri dish and covered with a thin layer of 7H11-Gly-OADC-PAA agar inoculated with *Mtb* mc^{2}7000 luxABCDE working bacterial suspension (1:5, v/v). After solidification of the agar layer, the Petri dish was sealed with tape and incubated for 24 h at 37 °C.

Bioluminescence Imaging. Bioluminescence was assessed with an IVIS Spectrum system (Caliper Life Sciences, Alameda, USA). After incubation, the inhibition zones were visualized on agar/TLC plates as a reduction in bioluminescence intensity. Bioluminescence was measured for 10 s and expressed as radiance (photons/s/cm^2/sr).

Spectrometry. *TLC-MS:* Areas corresponding to the inhibition zones on a bioautogram were marked with a pencil on the duplicate chromatogram. The TLC-MS interface (CAMAG) equipped with an oval elution head (4 × 2 mm) was used to extract the pencil-encircled zones. The eluent acetonitrile was pumped by an HPLC pump at a rate of 0.4 mL/min. The eluates were collected in vials and analyzed by LCMS-IT-TOF (Shimadzu, Tokyo, Japan). The ^{1}H NMR spectra used for identification of compounds from TLC plates and/or by bioassay-guided isolation were obtained on a Bruker Avance 600 MHz NMR spectrometer (Bruker Inc., Bremen, Germany).

Biosafety. All procedures were performed inside a biosafety cabinet in a biosafety level 2 laboratory. All biohazardous waste was decontaminated as follows: liquid wastes were treated with 10% final concentration bleach (Clorox) for at least 15 min followed by drain disposal. All solid biohazard wastes were autoclaved.

RESULTS AND DISCUSSION

Selection of the *Mycobacterium tuberculosis* Strain. Because of the challenge of maintaining absolute containment

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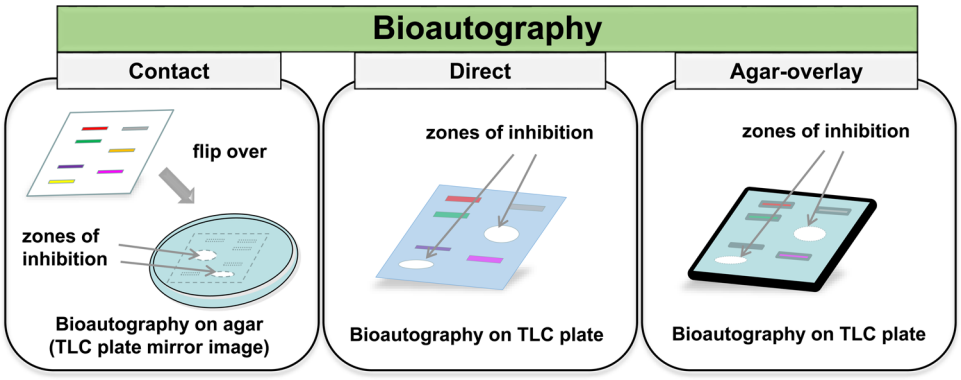

Figure 1. Variations of the bioautography methods. **Contact bioautography:** a developed (HP)TLC plate is placed on the surface of inoculated agar, removed after 15 min, and the agar plate is incubated at 37 °C for 24 h. **Direct bioautography:** a developed (HP)TLC plate is dipped in inoculated broth and incubated at 37 °C for 24 h. **Agar-overlay bioautography:** a developed (HP)TLC plate is covered with a layer of inoculated agar and, after solidification, incubated at 37 °C for 24 h.

during all procedures involved in bioautography, we chose to work with the avirulent strain *Mtb* mc^{2}7000.[15] To overcome the long generation time of *Mtb* (ca. 16−24 h), which precludes the use of most diffusion-based methods that rely upon visual observation, we introduced a luxABCDE reporter into this strain. This allows bacterial growth determination and distinguishing of inhibition zones within 24 h by luminescence imaging. This strain demonstrates identical sensitivity to that of *Mtb* mc^{2}7000 against all anti-TB drugs tested to date and can, therefore, serve as a surrogate.[16]

The mc^{2}7000 strain (*Mtb* H37Rv Δ*RD1* Δ*panCD*) is an unmarked version of mc^{2}6030;[15] the *hyg sacB* cassette was removed by transduction with phAE87 containing the *γδ* resolvase, and a screening for sucrose-resistant and hygromycin-sensitive isolates was applied.[17] The strain has an independent deletion in the *panC* and *panD* genes that are required for the de novo biosynthesis of pantothenate and an additional deletion in the entire *RD1* region. All of these regions contribute to the virulence of *Mtb*.[15,18] The strain mc^{2}7000 has been approved for use in biosafety level 2 containment by the Institutional Biosafety Committees of the Albert Einstein College of Medicine (where it was constructed) and the University of Illinois.

The luciferin−luciferase system utilized for bioluminescence is based on the bacterial luciferase found in *Photorhabdus luminescens*.[19] These are heterodimeric enzymes that catalyze the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde to yield flavin mononucleotide (FMN) and a long chain fatty acid. As a consequence, light is emitted at a wavelength of 490 nm. The luciferase enzyme is encoded by the genes luxAB that form an operon together with the genes luxCDE whose products synthesize the long-chain aldehyde.

The highly bioluminescent *Mtb* mc^{2}7000 luxABCDE strain was constructed by cloning luxABCDE into the expression vector pMV306 that contains the P_{hsp60} promoter and by electroporating the construct into mc^{2}7000. The assessment of bioluminescence was performed using an IVIS Spectrum system.

Development and Optimization of Bioautography Methods. Bioautography can be regarded as an effect-directed analysis method, providing simple but critical "yes/no" information about the presence of desired active principles in the sample.[20] TLC-bioautography allows the localization of the active principles during the separation process of even complex extracts directly on the TLC plate. Although bioautography appears to be increasingly utilized to detect antimicrobial activities,[21,22] there are no reports of its use with *Mtb*.

There are three basic variations of the bioautography process: contact, direct, and agar-overlay/immersion (Figure 1). The present study adapted all three methods for the use with the mc^{2}7000 luxABCDE strain, under various chromatographic conditions (sorbents, solvents, additives, sample volume, resolution, and developing mode) and microbiological parameters such as the composition of the culture medium and incubation conditions. Bioluminescence detection with the IVIS Spectrum imaging system shortened the read-out and total assay time to 24 h.

Contact Bioautography. The developed TLC plate is placed face-down on the surface of inoculated agar to allow diffusion of the compounds into the agar layer. TLC plates are carefully removed, and the agar plate is incubated for 24 h at 37 °C. The zones of inhibition (decrease in bioluminescence signal) appear where the anti-TB active compounds had diffused into the agar layer. Bacterial concentration and contact time are the two main factors that influence the results and required optimization.

Two different bacterial concentrations of inoculated agar were tested (A) 3.4×10^5 CFU/mL and (B) 1.76×10^5 CFU/mL. The mean values of bioluminescence measured as radiance (photons/s/cm^2/sr) obtained for the region of interest (the area of contact between agar and TLC plate) equaled (A) 2.11×10^3 and (B) 1.31×10^3. The inoculated agar (A) was regarded as optimal when the bioluminescence signal was sufficient to differentiate zones of inhibition from the background, homogeneous for the entire area of interest, and when the growth inhibition zones were clearly defined. The TLC plates spotted with polar, midpolar, and nonpolar standards (CAP, MOX, and BDQ, respectively) were placed on the inoculated agar surface for 5, 15, 45, 60, 120, 180, and 240 min. A contact time of 15 min was determined as optimal.

The major shortcomings of this method are the difficulty of gaining sufficient contact between the TLC plate and the agar layer and the requirement of careful handling to avoid adherence of the TLC sorbent layer to the agar surface. Other issues may result from differences in the diffusion of compounds of varying polarity from the TLC plate into the agar layer. These limitations lead to potentially reduced sample

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concentration in the agar and blurred zones of inhibition growth.

Direct Bioautography. The TLC plate is dipped into a suspension of mc^{2}7000 luxABCDE in a broth medium and incubated for 24 h at 37 °C. The bacteria grow and cause bioluminescence signals directly on the surface of the TLC plate, except in areas where anti-TB active compounds are present.

The quality of TLC-direct bioautography largely depends on the viscosity of the bacterial suspension. If the suspension density is too high or low, the resulting bacterial film/luminescence is too uneven to yield clear inhibition zones. Bacterial suspensions in 7H9 broth were tested (i) without addition of agarose, (ii) with 0.05% agarose, and (iii) with 0.1% agarose. The last resulted in a decrease in sensitivity, whereas suspensions with 0.05% agarose or without any agarose resulted in TLC plates containing a uniform bacterial layer. All TLC-direct bioautography experiments on normal phase (NP) TLC plates described below were, therefore, performed without agarose. The effects of dipping time and concentration of the inoculated bacterial suspension on the TLC plate in direct bioautography were examinedtoo. The plates were dipped into bacterial suspensions using a CAMAG immersion device for 1, 3, 8, 15, 30, and 60 s. Effects of single and double dipping were compared. Best results were achieved with a single dip in a time frame of 3−8 s for the NP-TLC plates to maintain a thin bacterial film on the TLC plate and to minimize compound diffusion. For reverse-phase (RP) TLC plates, a double dip, each dip for up to 60 s, and addition of 0.05% agarose to the broth seem to improve homogeneity of the bacterial film.

Additional factors to be optimized are incubation time and humidity. Insufficient incubation time of the inoculated TLC plates and a resulting low bacterial concentration can cause irregular inhibition zones, while excessive incubation times can effect a decreased sensitivity to active compounds. Comparing incubation times of 24 and 48 h for all bioautography methods resulted in a sufficient bioluminescence signal and well-defined inhibition zones for the shorter period of 24 h. For TLC-direct bioautography, incubation in a humidified atmosphere achieved a more homogeneous bacterial layer compared to plates that were incubated in a nonhumidified incubator.

Agar-Overlay Bioautography. This method can be considered a hybrid of contact and direct bioautography, where the TLC plate is covered with *Mtb*-seeded molten agar. After solidification of the agar, the plate is incubated for 24 h at 37 °C. The compounds diffuse from the chromatogram into the agar medium. This is comparable to the contact method, but the agar layer remains on the surface of the TLC plate during incubation, and therefore, compound transfer is not limited by contact time. Zones of growth inhibition, read as a decrease in the bioluminescence signal, are visible on the agar layer in areas where anti-TB active compounds are present on the TLC plate. The downside of this method is the influence of the compound polarity on the adhesion of the agar to the TLC plate surface. Very apolar compounds are known to cause the agar layer and the TLC surface to be disconnected.

Two bacterial concentrations of inoculated agar were tested (A) 3.4×10^5 CFU/mL and (B) 1.76×10^5 CFU/mL, and the mean values of bioluminescence measured as radiance (photons/s/cm^2/sr) obtained for the TLC plates covered with suspension equaled (A) 3.46×10^3 and (B) 1.63×10^3. Once again, the higher density suspension (A) yielded superior results. Another crucial factor in the agar-overlay bioautography is the thickness of the agar layer. The NP-TLC plates spotted with CAP, MOX, and BDQ were placed in "cuvettes" made of aluminum foil and covered with (A) 10 mL and (B) 5 mL of inoculated agar. Plate (C) was placed inside the Petri dish and overlaid with just enough molten inoculated agar (less than 5 mL) to cover the entire plate, with just a small amount of overflow. After solidification, the plates were incubated for 24 h at 37 °C. The highest sensitivity was obtained for plate (C), confirming that the agar layer covering the TLC plate should be as thin as possible to reduce the diffusion/dilution factor, while being sufficiently thick to obtain a satisfactory bioluminescence signal.

Factors Influencing the Bioautographic Detection. *Selection of TLC Sorbent.* While there is a broad variety of TLC layer materials commercially available, the selection of a proper TLC sorbent can be a challenge because both chromatographic and microbiological requirements need to be fulfilled. A limit of detection analysis was performed for CAP, MOX, and CLF in order to compare the biocompatibility of various (HP)TLC sorbents: silica gel 60, nanosilica gel NH_2, nanosilica gel RP-2, nanosilica gel C18-50, and nanosilica gel C18-100, all of which with and without a fluorescence indicator. Bioluminescence on reverse-phase layers (C18-100, C18-50, and RP-2) was subdued without the presence of an inhibitor, whereas a distinct bioluminescence background could be observed on silica gel NH_2, and an excellent background was observed on the most commonly used standard NP silica gel plates. Moreover, for economic reasons, most experiments were performed using NP-TLC plates.

Selection of TLC Solvent System. Solvents and/or their residues in the adsorbent layer carry the risk of bacterial growth inhibition. Thus, it is highly beneficial to use low boiling, highly volatile solvents that can be removed readily and completely from the TLC layer prior to the bioautography process. Unfortunately, this is not the case for most acids and bases, which are common additives to TLC solvent systems. Evaluation of 10 solvents commonly used in TLC (MeOH, $CHCl_3$, CH_2Cl_2, Tol, MeCN, EtOAc, Hex, Ace, NH_4OH, and FA) confirmed that the addition of FA and NH_4OH to the mobile phase may inhibit bacterial growth/luminescence in a direct bioautography setup.

Bioautography Visualization Methods. The most commonly used visualization method in the general bioautography process employs a tetrazolium salt spray, such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Dehydrogenases of living microorganisms convert these salts into colored formazans, and as a result, for MTT, yellow zones of inhibition are observed on a purple background. The preliminary experiments with MTT and resazurin were unsatisfactory for *Mtb*, which resulted in the development of the bioluminescent strain mc^{2}7000 luxABCDE, allowing the detection of growth and inhibition after only 24 h of incubation.

Evaluation of Bioautography Methods Using Established Anti-TB Agents. The optimized methods were tested with eight anti-TB agents: INH, RMP, EMB, CAP, MOX, CLF, PMD, and BDQ. The limit of detection (LOD) for each compound was determined as the lowest amount of the antibiotic that resulted in a zone of any size of reduced luminescence (Table 1). Among the three bioautography methods, direct bioautography demonstrated the highest overall sensitivity. This may be due, at least in part, to the decreased likelihood for compound diffusion/dilution com-

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Table 1. Limit of Detection Values of Anti-TB Compounds Assessed by Contact, Agar-Overlay, and Direct Bioautography

analyte	clog *P*	MIC (μg/mL)	bioautography LOD (μg per spot)		
			direct	contact	agar-overlay
capreomycin	−9.609	0.27	13.5	27	13.5
isoniazid	−0.64	0.03	0.03	0.3	0.03
ethambutol	−0.14	1.02	1.02	10.2	1.02
moxifloxacin hydrochloride	2.033	0.11	0.11	5.5	0.55
pretomanid	3.393	0.13	0.0065	0.065	0.0065
rifampin	3.719	0.03	0.003	0.3	0.03
clofazimine	7.132	0.08	0.04	0.08	0.08
bedaquiline	7.52	0.09	0.0009	0.0045	0.0045

pared to the contact and agar-overlay bioautography methods. Another important aspect is the selectivity of the method, that is, the ability to differentiate and quantify the analyte in the presence of other constituents in the matrix. In TLC-bioautography, the selectivity can be evaluated by characterizing the positive and negative control together with the blank matrix or background/blind control. The zones of inhibition on the bioautogram should be significantly different from other interference spots, including inactive compounds. In order to create a positive control, samples of an inactive actinomycete extract (previously tested with the Microplate Alamar Blue Assay MABA against *Mtb* H37Rv)[23] were individually spiked with eight anti-TB antibiotics. MeOH was used as negative control, and the inactive extract served as a blank matrix or background/blind control. These samples were applied to TLC plates and subjected to bioautography for comparison. The zones of inhibition were visible only in places where the positive control samples were applied. The negative control sample and matrix blank did not result in luminescence reduction.

Application of (HP)TLC-Bioautography-MS/NMR to Natural Product Samples. The optimized (HP)TLC-bioautography methods for *Mtb* mc^{2}7000 luxABCDE were aligned with MS and NMR for the analysis of bacterial and fungal fermentation extracts using a three-step approach (Figure 2) as follows. **Step 1. (HP)TLC**: as this method enables parallel separation of multiple samples, it can be used for cost-efficient detection of active compounds. To increase reproducibility and produce the highest resolution, the sample application and the development of the TLC plates were carried out with the automatic CAMAG ATS 4 and AMD 2 devices, respectively. **Step 2. Bioautographic Detection**: The use of the *Mtb* luminescent strain allows the specific and rapid readout of the TLC plates. After localization of the inhibition zones on the bioautogram, the zones of interest were marked on the duplicate TLC plate, and these zones were extracted for MS and NMR analysis. **Step 3. LC−MS**: (HP)TLC-bioautography coupled with LC−MS and NMR analysis allows the acquisition of structural information on active compounds at the time of initial screening.

Dereplication of Ecumicin. A high-throughput screening campaign of >65 000 actinomycete extracts against *Mtb* H37Rv identified the *Nonomuraea* species strain MJM5123 as a promising source for anti-TB active lead compounds and led to the isolation of the new anti-TB macrocyclic tridecapeptide ecumicin.[12] Ecumicin exerts potent, selective bactericidal activity against *Mtb* in vitro (MIC 0.26 μg/mL), including nonreplicating cells. It also retains activity against isolated multiple-drug-resistant and extensively drug-resistant strains, by virtue of a new mode of action.[24] Ecumicin targets the ClpC1 ATPase complex in *Mtb* by enhancing the ATPase activity of WT ClpC1 and preventing activation of proteolysis by ClpC1.

The (HP)TLC-bioautography-MS/NMR (Figure 3) approach was applied on the *Nonomuraea* extract in order to confirm the capability to identify the previously isolated ecumicin and gain sufficient structural information about the active principle(s) at the beginning of the isolation procedure. The ecumicin reference material and the enriched Sephadex

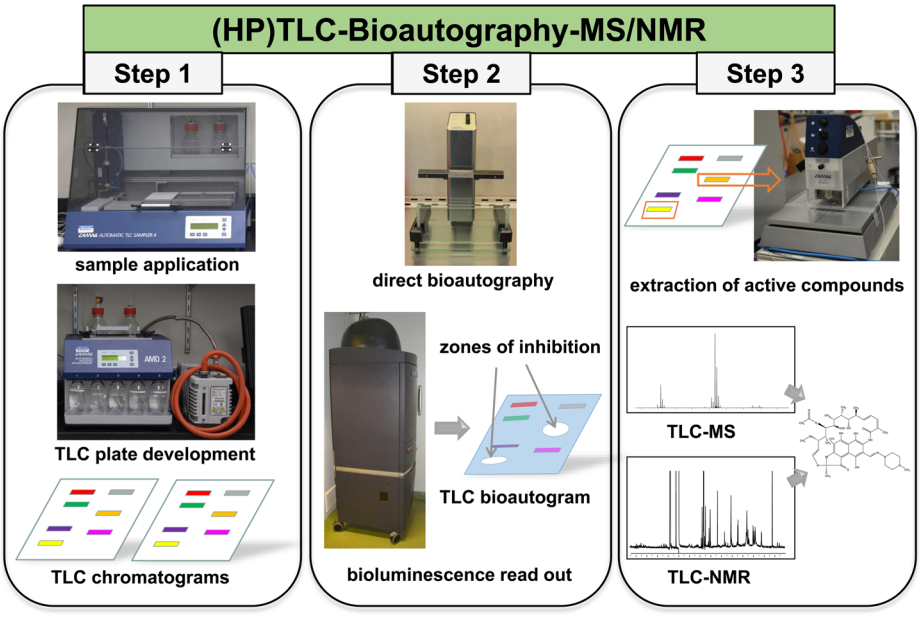

Figure 2. Workflow of the (HP)TLC-bioautography-MS/NMR approach.

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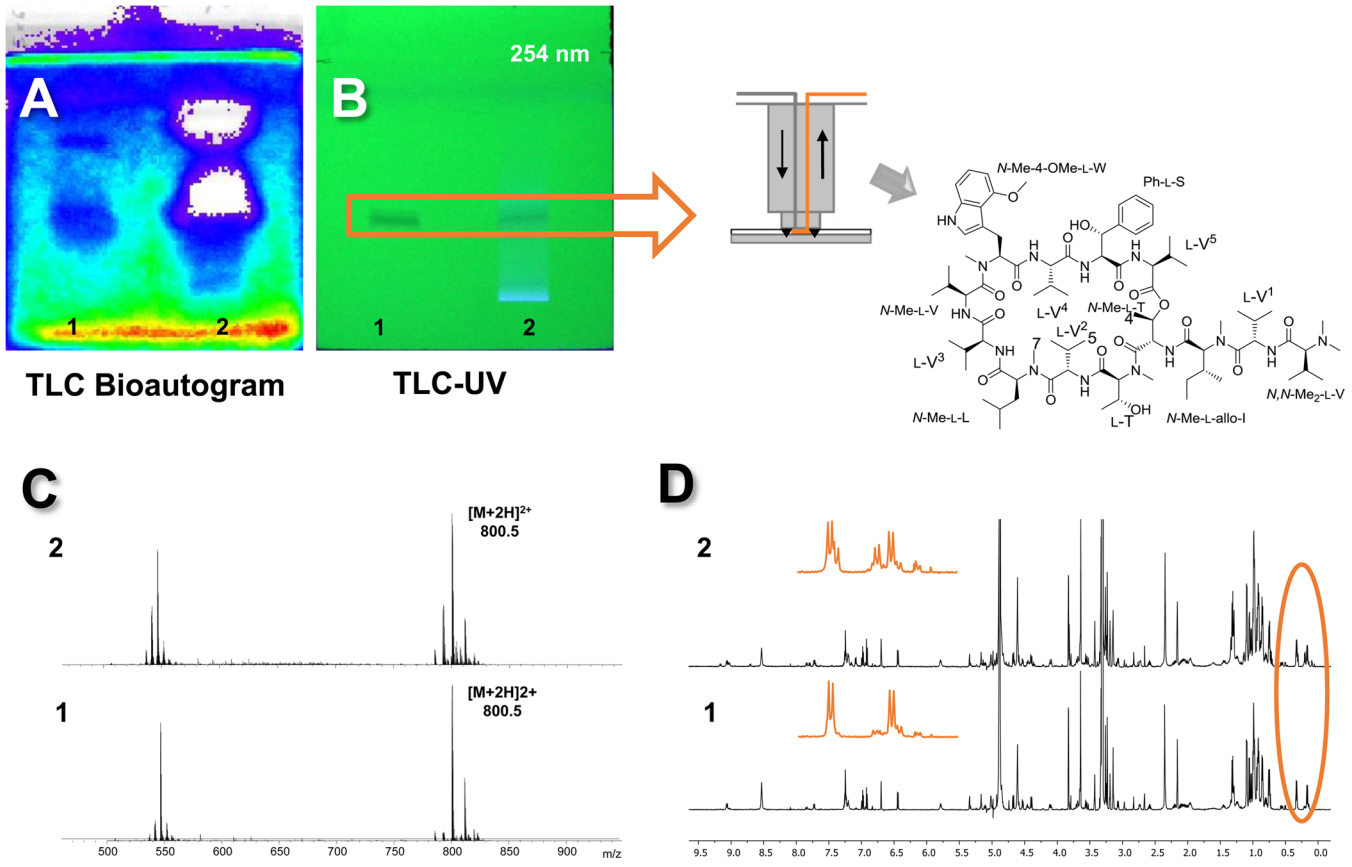

Figure 3. Dereplication of ecumicin with (HP)TLC-Bioautography-MS/NMR. (1) ecumicin, (2) MJM5123 Sephadex LH-20 fraction; (A) bioautographic confirmation of anti-TB activity; (B) localization of the anti-TB active lead on the TLC plate and extraction; confirmation of the structural information by (C) TLC-MS and (D) TLC-NMR analysis.

LH-20 fraction were both spotted on a NP-TLC plate, developed with $CHCl_3/CH_2Cl_2$/MeOH (8:1:1, v/v/v), and subjected to direct bioautography. After the growth inhibition zone was detected on the bioautogram, the zone of interest was marked on the duplicate chromatogram plate (R_f 0.35) and extracted with the TLC−MS interface. The structural dereplication was performed by LCMS-IT-TOF and ^{1}H (HR)NMR analysis.

Detection and Dereplication of Gliotoxin. The same high-throughput screening campaign that led to the isolation of ecumicin showed high activity against *Mtb* H37Rv (MIC < 0.02 μg/mL) in the chloroform extract of the *Aspergillus fumigatus* strain ECUM40602. Initial fractionation with C18 vacuum liquid chromatography, followed by separation with RP open-column chromatography resulted in the active fraction F3.

At this point, TLC-direct bioautography was used to identify and isolate the primary active principle. With bioautography, the activity within F3 could be assigned to one primary active compound (NP-TLC plate developed with $CHCl_3/CH_2Cl_2$/MeOH (8:1:1, v/v/v); R_f 0.66) (Figure 4A). NMR and LCMS-IT-TOF analysis confirmed the identity of the active principle as gliotoxin, a previously isolated active and toxic (VERO cell IC_{50} = 4.3 μg/mL) compound commonly produced by fungi and especially by *Aspergillus fumigatus*.

Dereplication of Hytramycins V and I. Another high-throughput screening campaign, with 35 000 actinomycete extracts against *Mtb* H37Rv, led to the isolation of two new anti-TB active hexapeptides, hytramycin V (MIC 11.3 μg/mL) and hytramycin I (MIC 6.0 μg/mL) from *Streptomyces hygroscopicus* ECUM14046.[13] The ethyl acetate extract of ECUM14046 and the daughter fraction F3 were spotted on the NP-TLC plate, developed with EtOAc/Tol/MeOH/FA (95%) (60:40:5:1, v/v/v/v), and subjected to contact bioautography (Figure 4B). The zones of interest corresponding to the zones of inhibition on the agar plate were localized on the duplicate TLC plate (R_f 0.44 and 0.53). Based on the bioautography and MS analysis, these bands could be assigned to the previously isolated hytramycins V and I.

Isolation of Minor Active Components. Two isoflavone isolates with NMR purities of >90% obtained from the actinomycete extract *Streptomyces* sp. ECUM8412 demonstrated MICs of 0.75 and 0.11 μg/mL against *Mtb* H37Rv. However, when the compounds were synthesized and their structures were confirmed by NMR, neither of the isoflavones demonstrated any detectable anti-TB activity. This indicated that minor components/impurities could be responsible for the observed bioactivity.[25]

Contact bioautography of the active mother fraction revealed an inhibition zone and confirmed its association with minor impurities in both samples (Figure 4C). The active spots were extracted from the plate (NP-TLC plate developed with $CHCl_3$/MeOH (9:1, v/v); R_f 0.28), and the MS spectra indicated the presence of the cyclic peptides, which likely exhibited MIC values against *Mtb* H37Rv of <0.1 μg/mL. One such peptide was subsequently isolated using a counter-current chromatography-based isolation scheme, applying the "generally useful estimate for solvent systems in CCC" (GUESS) principle of solvent system selection,[26] and the structure was

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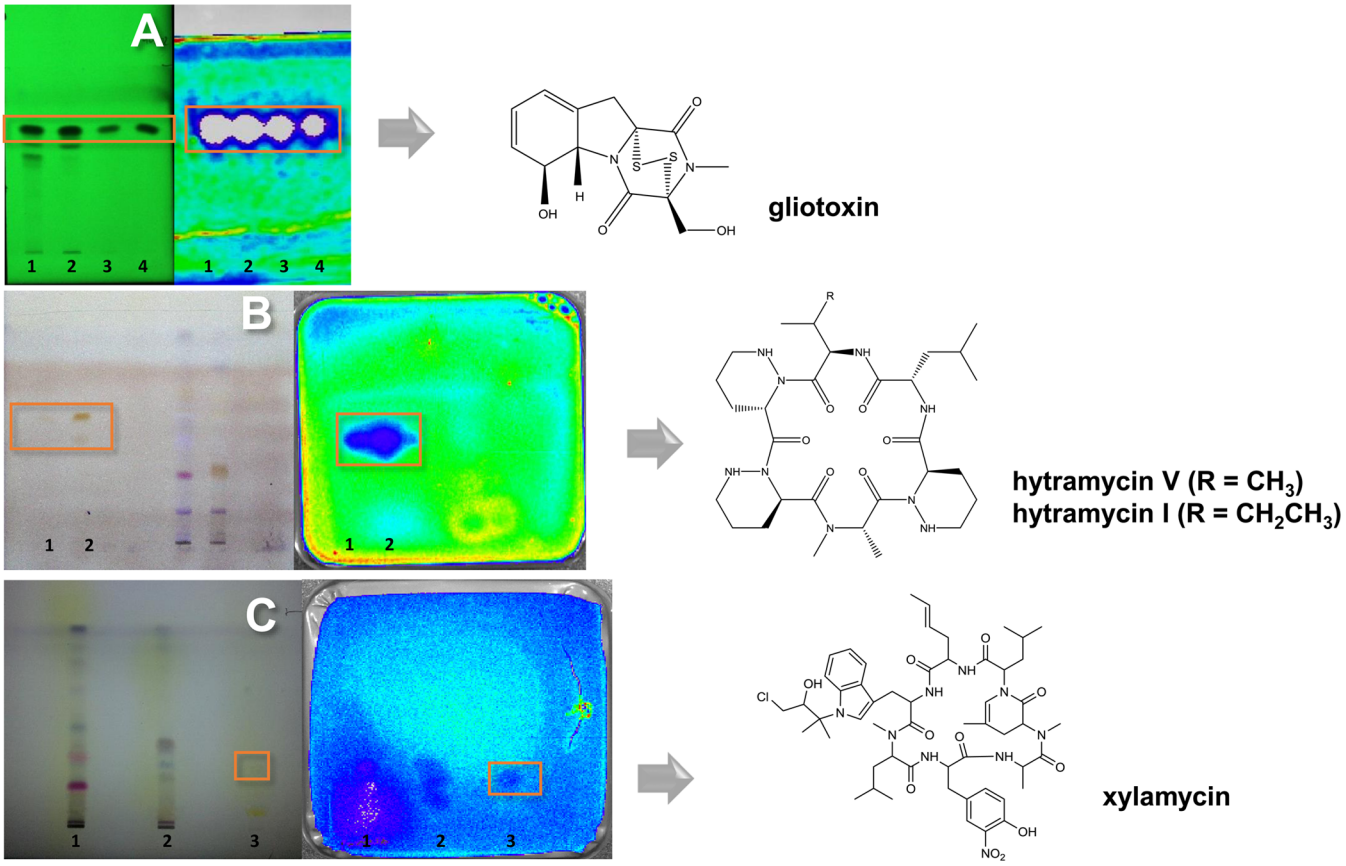

Figure 4. Bioautography of extracts of the actinomycete strains: (A) ECUM40602, **1** chloroform extract, **2** fraction 3 of second fractionation, **3** and **4** gliotoxin standard; (B) ECUM14046 **1** ethyl acetate extract, **2** fraction 3 of first fractionation; (C) ECUM8412 **1** ethyl acetate extract, **2** fraction 5 of first fractionation, **3** fraction 1 of fourth fractionation.

elucidated with high-resolution NMR as the cyclic heptapeptide, xylamycin, a compound previously described in a patent by Eli Lilly and company in 1999.[27]

CONCLUSION

This study demonstrates the potential of a method that aligns (HP)TLC-bioautography using the strain *Mtb* mc^{2}7000 luxABCDE with MS/NMR analysis as a powerful tool for screening diverse natural extracts and fractions for anti-TB lead compounds. TLC is a well-developed, simple, and rapid chromatographic technique that allows simultaneous separation of numerous samples. The agar-diffusion-based bioautography methods (contact and agar-overlay) are particularly applicable to polar and moderately polar compounds and are more suitable for the screening of pure substances. The direct method can be used for screening of polar and nonpolar samples, complex extracts, as well as pure substances. Among the three optimized *Mtb* bioautography methods, the direct method proved to be the most sensitive. With capreomycin as the only exception, the LOD values for anti-TB standard drugs were in the same range as, or even lower than the MIC values obtained with the commonly used MABA against *Mtb* H37 for the same agents.

The combination of TLC with MS and/or NMR analysis allows the chemical characterization of the separated compounds, and in combination with bioautography, the anti-TB activity can be directly assigned to individual compounds in the sample. Utilization of an avirulent, luminescent *Mtb* strain overcomes the challenges of working with a slow-growing, biohazardous organism.

(HP)TLC-bioautography-MS/NMR aids the prioritization of extracts in screening campaigns for further processing and facilitates dereplication at a very early stage in the bioassay-guided isolation workup. In order to yield a compound with >90% purity, a classical bioactivity-guided isolation procedure might require up to 20 isolation and purification steps, while the chemical nature of active principle remains unknown. With TLC-bioautography-MS/NMR, however, structural information of the active principle can be gained at an early stage, and the isolation scheme can be adapted to target the active compound.[28] Moreover, monitoring the anti-TB active compounds with bioautography throughout their isolation process permits the detection of minor but highly active compounds that would otherwise be overseen.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) WHO. *Global Tuberculosis Report*, 20th ed.; Geneva, Switzerland, 2015.
(2) Dye, C., and Williams, B. G. (2010) The population dynamics and control of tuberculosis. *Science 328* (5980), 856−861.
(3) Newman, D. J., Cragg, G. M., and Snader, K. M. (2003) Natural products as sources of new drugs over the period 1982−2002. *J. Nat. Prod. 66* (7), 1022−1037.
(4) Newton, S. M., Lau, C., and Wright, C. W. (2000) A review of antimicrobial natural products. *Phytother. Res. 14* (5), 303−322.
(5) Copp, B. R. (2003) Antimycobacterial natural products. *Nat. Prod. Rep. 20* (6), 535−557.
(6) Okunade, A. L., Elvin-Lewis, M. P., and Lewis, W. H. (2004) Natural antimycobacterial metabolites: current status. *Phytochemistry 65* (8), 1017−1032.
(7) Pauli, G. F., Case, R. J., Inui, T., Wang, Y., Cho, S., Fischer, N. H., and Franzblau, S. G. (2005) New perspectives on natural products in TB drug research. *Life Sci. 78* (5), 485−494.
(8) Potterat, O., and Hamburger, M. (2013) Concepts and technologies for tracking bioactive compounds in natural product extracts: generation of libraries, and hyphenation of analytical processes with bioassays. *Nat. Prod. Rep. 30* (4), 546−564.
(9) Goodall, R. R., and Levi, A. A. (1946) A microchromatographic method for the detection and approximate determination of the different penicillins in a mixture. *Nature 158* (4019), 675−676.
(10) Marston, A. (2011) Thin-layer chromatography with biological detection. *J. Chromtogr. A 1218* (19), 2676−2683.
(11) Marston, A., and Hostettmann, K. (1999). Biological and Chemical Evaluation of Plant Extracts and Subsequent Isolation Strategy. In *Bioassay Methods in Natural Products Research and Drug Development* (Bohlin, L., and Bruhn, J. G., Eds), pp 67−80, Kluwer Academic Publishers, Dordrecht, The Netherlands.
(12) Gao, W., Kim, J. Y., Chen, S. N., Cho, S. H., Choi, J., Jaki, B. U., Jin, Y. Y., Lankin, D. C., Lee, J. E., Lee, S. Y., McAlpine, J. B., Napolitano, J. G., Franzblau, S. G., Suh, J. W., and Pauli, G. F. (2014) Discovery and characterization of the tuberculosis drug lead ecumicin. *Org. Lett. 16* (23), 6044−6047.
(13) Cai, G., Napolitano, J. G., McAlpine, J. B., Wang, Y., Jaki, B. U., Suh, J. W., Yang, S. H., Lee, I. A., Franzblau, S. G., Pauli, G. F., and Cho, S. (2013) Hytramycins V and I, anti-mycobacterium tuberculosis hexapeptides from *Streptomyces hygroscopicus*. *J. Nat. Prod. 76* (11), 2009−2018.
(14) Cai, G. (2014) Closing the In Vitro/In Vivo Gap in Tuberculosis Drug Discovery. Ph.D. Thesis, University of Illinois, Chicago, IL.
(15) Sambandamurthy, V. K., Derrick, S. C., Hsu, T., Chen, B., Larsen, M. H., Jalapathy, K. V., Chen, M., Kim, J., Porcelli, S. A., Chan, J., Morris, S. L., and Jacobs, W. R., Jr. (2006) Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. *Vaccine 24* (37−39), 6309−6320.
(16) Ioerger, T. R., O'Malley, T., Liao, R., Guinn, K. M., Hickey, M. J., Mohaideen, N., Murphy, K. C., Boshoff, I. M., Mizrahi, V., Rubin, E. J., Sassetti, C. M., Barry, C. E., III, Sherman, D. R., Parish, T., and Sacchettini, J. C. (2013) Identification of new drug targets and resistance mechanisms in *Mycobacterium tuberculosis*. *PLoS One 8* (9), e75245.
(17) Ojha, A. K., Baughn, A. D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jacobs, W. R., Jr., and Hatfull, G. F. (2008) Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol. 69* (1), 164−174.
(18) Sambandamurthy, V. K., Wang, X., Chen, B., Russell, R. G., Derrick, S., Collins, F. M., Morris, S. L., and Jacobs, W. R., Jr. (2002) A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat. Med. 8* (10), 1171−1174.
(19) Andreu, N., Zelmer, A., Fletcher, T., Elkington, P. T., Ward, T. H., Ripoll, T., Parish, T., Bancroft, G. J., Schaible, U., Robertson, B. D., and Wiles, S. (2010) Optimisation of bioluminescent reporters for use with mycobacteria. *PLoS One 5* (5), e10777.
(20) Choma, I. M., and Grzelak, E. M. (2011) Bioautography detection in thin-layer chromatography. *J. Chromatogr. A 1218* (19), 2684−2691.
(21) Jamshidi-Aidji, M., and Morlock, G. E. (2015) Bioprofiling of unknown antibiotics in herbal extracts: Development of a streamlined direct bioautography using *Bacillus subtilis* linked to mass spectrometry. *J. Chromatogr. A 1420*, 110−8.
(22) Taha, M. N., Krawinkel, M. B., and Morlock, G. E. (2015) High-performance thin-layer chromatography linked with (bio)assay and mass spectrometry - a suited method for discovery and quantification of bioactive components? Exemplarily shown for turmeric and milk thistle extracts. *J. Chromatogr. A 1394*, 137−147.
(23) Collins, L., and Franzblau, S. G. (1997) Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother. 41* (5), 1004−1009.
(24) Gao, W., Kim, J. Y., Anderson, J. R., Akopian, T., Hong, S., Jin, Y. Y., Kandror, O., Kim, J. W., Lee, I. A., Lee, S. Y., McAlpine, J. B., Mulugeta, S., Sunoqrot, S., Wang, Y., Yang, S. H., Yoon, T. M., Goldberg, A. L., Pauli, G. F., Suh, J. W., Franzblau, S. G., and Cho, S. (2015) The cyclic peptide ecumicin targeting ClpC1 is active against *Mycobacterium tuberculosis* in vivo. *Antimicrob. Agents Chemother. 59* (2), 880−889.
(25) Rodriguez Brasco, M. F., Jaki, B., Becker, B., Kim, Y., Cho, S., Lankin, D. C., Friesen, J. B., Klein, L., Suh, J., Yoon, T., Lee, I., McAlpine, J., Franzblau, S. G., and Pauli, G. F. (2010) P-24: Partition-based isolation and characterization of anti-TB drug leads from cultured actinomycetes. The Joint Annual Meeting of the American Society of and the Phytochemical Society of North America; Tradewinds Island Grand St. Petersburg Beach, Florida, July 10−14, 2010.
(26) Friesen, J. B., and Pauli, G. F. (2005) G.U.E.S.S.- a generally useful estimate of solvent systems for CCC. *J. Liq. Chromatogr. Relat. Technol. 28* (17), 2777−2806.
(27) Eli Lilly and Company (2000). Rufomycin derivatives useful as antibiotics. WO2000078797 A1, pp 1−92.
(28) Grzelak, E. M., Liu, Y., Nam, J. W., Friesen, J. B., Chen, S. N., Lankin, D. C., McAlpine, J. B., Suh, J. W., Yang, S. H., Cheng, J., Lee, H., Kim, J. Y., Cho, S. H., Pauli, G. F., Franzblau, S. G., and Jaki, B. U. (2015) TLC-bioautography linked with guess: A truely targeted active compound isolation process. *Planta Med. 81* (11), PJ5.

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ACS Infect. Dis. 2016, 2, 294−301