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Direct mass spectrometric screening of antibiotics from bacterial surfaces using liquid extraction surface analysis

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RATIONALE: There is a need to find new antibiotic agents to fight resistant pathogenic bacteria. To search successfully for novel antibiotics from bacteria cultivated under diverse conditions, we need a fast and cost-effective screening method.
METHODS: A combination of Liquid Extraction Surface Analysis (LESA), automated chip-based nanoelectrospray ionization, and high-resolution mass or tandem mass spectrometry using an Orbitrap XL was tested as the screening platform. Actinobacteria, known to produce well-recognized thiazolyl peptide antibiotics, were cultivated on a plate of solid medium and the antibiotics were extracted by organic solvent mixtures from the surface of colonies grown on the plate and analyzed using mass spectrometry (MS).
RESULTS: LESA combined with high-resolution MS is a powerful tool with which to extract and detect thiazolyl peptide antibiotics from different Actinobacteria. Known antibiotics were correctly detected with high mass accuracy (<4 ppm) and structurally characterized using tandem mass spectra. Our method is the first step toward the development of a novel high-throughput extraction and identification tool for antibiotics in particular and natural products in general.
CONCLUSIONS: The method described in this paper is suitable for (1) screening the natural products produced by bacterial colonies on cultivation plates within the first 2 min following extraction and (2) detecting antibiotics at high mass accuracy; the cost is around 2 Euro per sample. Copyright © 2012 John Wiley & Sons, Ltd.

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Antibiotics have become indispensable agents for human health. The discovery and development of antibiotics has substantially increased life expectancy by suppressing infectious diseases caused by bacterial pathogens.[1] Nevertheless, most bacteria acquire resistance to various antibiotics.[2] The resistance of pathogenic bacteria must be countered with new antibiotic agents.[3,4]

The naturally occurring thiazolyl peptides (e.g. thiostrepton, sulfomycins, thioplabins, thiazomycins, philipimycin) are some of the most potent *in vitro* growth inhibitors of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE), and are widespread among Actinobacteria.[5–9] However, the poor water solubility of thiazolyl peptides complicates their application as antibiotic drugs. Chemical modifications of thiazolyl peptides can enhance water solubility and in the process convert them into medical agents. For that reason new thiazolyl peptide structures represent a promising new area of exploration.

Although in the past bioassay-based inhibitory tests have yielded a large amount of antibiotics, recently this screening method delivered few novel antibiotics or antibiotic leads.[10] To identify new antibiotic structures, the conditions under which microorganisms produce antibiotics in natural habitats have to be simulated. In addition, the role of signaling molecules in the function of antibiotics needs to be clarified[11–13]; such molecules help regulate the complex interactions among microorganisms in their ecological habitat. In the environment, microorganisms live in communities and produce both antibiotic compounds as well as signaling molecules probably due to complex interactions with other organisms in their ecological habitat.[14–18] We can stimulate the production of novel antibiotics by exposing organisms to different nutrient sources and modulating cultivation conditions (pH value, temperature, etc.), and we can alter the producer strain to enhance yields of antibiotics or co-cultivate antagonistic bacterial strains.[19–22] However, merely studying the process by which well-known antibiotic-producing microorganisms combine different nutrient sources is sufficiently promising if the investigation is based on a fast, sensitive and cost-effective screening technique.

The classic way to search for antibiotics involved the extraction, chromatographic separation and subsequent analysis of bacterial natural products using mass spectrometric and other structural analysis techniques (e.g.[23]). The results were, however, disappointing.[20] In contrast, liquid extraction surface analysis (LESA) using Triversa Nanomate technology is a promising and highly reproducible extraction infusion technique[24] based on an automated chip-based nanoelectrospray ionization technique applied to food surfaces,[25] blood spots,[26] plant roots,[27] and body tissue sections.[28]

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In this report, we demonstrate that LESA combined with high-resolution mass spectrometry is a powerful tool to extract and detect thiazolyl peptide antibiotics from different Actinobacteria. The extraction, performed directly from bacterial surfaces, presents a fast and simple method for detecting and characterizing antibiotic compounds. It is the first step towards the development of a novel high-throughput extraction and identification tool for dereplication of known antibiotics and expediting the rapid discovery of novel antibiotics.

EXPERIMENTAL

Bacteria

The bacteria we used were selected according to their ability to produce thiazolyl peptides. They were obtained from the Fundación MEDINA culture collection. The non-*Streptomyces* isolate F-169.583 was isolated from the rhizosphere of an *Acacia karroo* at the Karoo National Park, in the Western Cape, South Africa, and produces multhiomycin. The *Streptomyces*

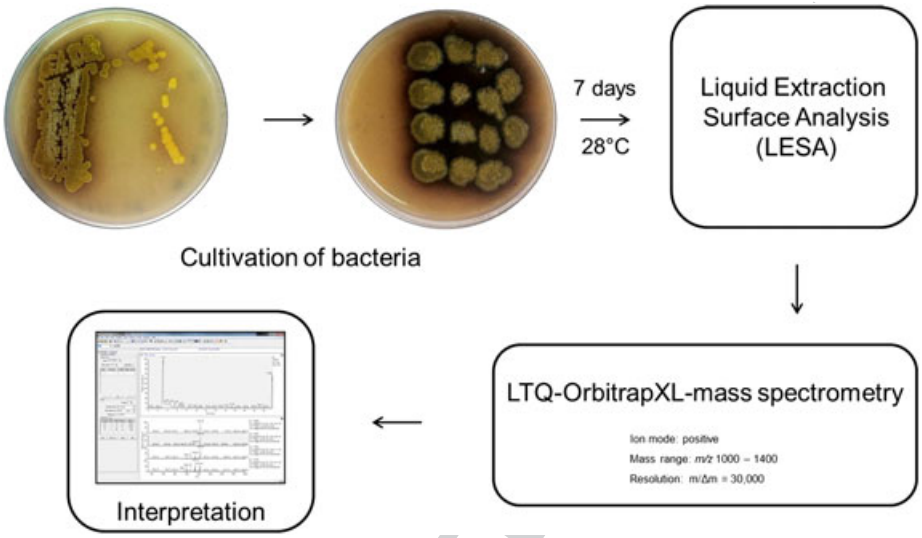

Figure 1. Workflow for directly screening bacterial colonies with LESA.

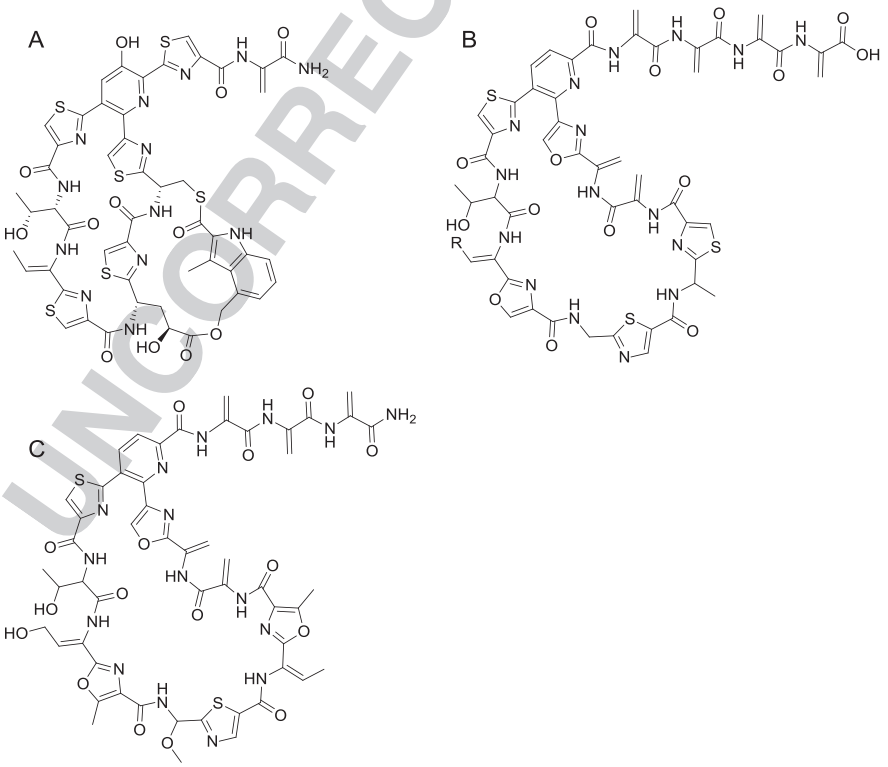

Figure 2. Molecular structures of multhiomyin (A); thioplabins (B) – thioplabin A (R = CH_3), thioplabin B (R = CH_2CH_3); sulfomycin III (C).

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sp. F-179.539 produces sulfomycin III and was isolated from a mangrove area in Sri Lanka. *Amycolatopsis* sp. F-136.425 produces a thioplabin derivative (A10255) and was isolated from the rhizosphere of a specimen of *Junipenus oxycedrus* growing on siliceous soil in Córdoba, Spain.

Strains were cultivated using the same media formulation, namely, the thiazolyl peptides were originally detected after liquid cultivation and standard solvent extraction. Strains F-179.539 and F-136.425 were grown in a medium of glycerol (Carl Roth, Karlsruhe, Germany) 60 g L^{-1}, oat flour (neuform international, Zarrentin, Germany) 15 g L^{-1}, and tomato paste (Beste Ernte, Maxhütte-Haidhof, Germany) 5 g L^{-1}, pH 7.0) (GOT), and strain F-169.583 was grown in a medium of cornmeal (Sigma Aldrich, St. Louis, MO, USA) 40 g L^{-1}, lactose (Merck, Darmstadt, Germany) 40 g L^{-1}, and yeast autolysate (Sigma Aldrich, St. Louis, MO, USA) 5 g L^{-1}) (CLA). Both media were supplemented with 15 g L^{-1} agar. After an incubation of 7 days at 28 °C, bacteria were kept at 4 °C in the dark.

F1

Liquid extraction surface analysis (LESA) of bacteria (Fig. 1)

Bacteria were spotted from a pure culture on plastic Petri dishes containing GOT- or CLA-based agar media using an inoculation loop. Plates were incubated for 7 days at 28 °C in the dark. Subsequently, LESA using Triversa Nanomate technology (Advion, Ithaka, NY, USA) was performed. For the analysis, the bacterial mycelium was stuck from the surface of the Petri dish in the Nanomate. The size of the Petri dish was reduced with a razor blade to accommodate the size of the LESA platform. Thiazolyl peptides were extracted using the solvent mixture ethyl acetate/acetone (10 µL aspirated to the tip, 65:35, v/v) containing 0.1% formic acid. A selected spot on the bacterial colony was extracted three times using the same tip. A volume of 2 µL of solvent mixture were dispensed, and, after 2 s, 2.2 µL were aspired into the tip every extraction cycle. Ca. 1 mm^2 was sampled during the extraction cycle. After the colony had been extracted, the tip was moved to the ESI chip equipped with 400 nozzles (20 × 20) and sprayed into the orifice of the instrument. One nozzle was used for one analysis to avoid cross-contamination. The applied spray voltage was 2 kV with a helium gas pressure of 1 psi providing ca. 1 nL/min solvent flow and typically 50–70 pA ion current. MS analysis of the formed ions was performed on a LTQ-OrbitrapXL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in positive ion mode. Capillary temperature and capillary voltage were set to 275 °C resp. 35 V. Full-scan mass spectra were acquired in a mass range of *m/z* 1000–1400 at a mass resolution of $m/\Delta m$ 30 000. The measurements were performed on at least three randomly selected bacterial spots of similar appearance and color in two independent experiments. Blanks were obtained using GOT- or CLA-based medium without bacteria. Tandem mass spectra were acquired from an orbitrap analyzer using a mass resolution of $m/\Delta m$ 30 000. Precursors were selected using a 7 Da isolation window in the LTQ and fragmented there using 5–15 V instrument settings.

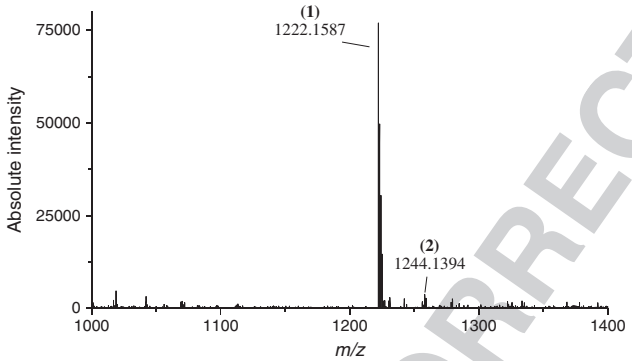

Figure 3. MS spectrum of non-*Streptomyces* isolate F-169.583 with the protonated multhiomycin (1) and the sodium adduct of multhiomycin (2).

RESULTS AND DISCUSSION

Fast, sensitive and cost-effective screening methods are required to identify new antibiotics from bacteria cultivated under diverse conditions. The present study is the first part of an evaluation of LESA using automated chip-based nanoelectrospray ionization as a direct screening technique to detect antibiotics. Known producer strains of different members of the thiazolyl peptide family (multhiomycin, sulfomycin III, and thioplabin A and B) were chosen (Fig. 2). To establish a fast screening procedure, the preparation of the bacterial samples was simplified. Several spots were plated from a pure culture on a new Petri dish. After 7 days of incubation, the spots were analyzed by liquid extraction

F2

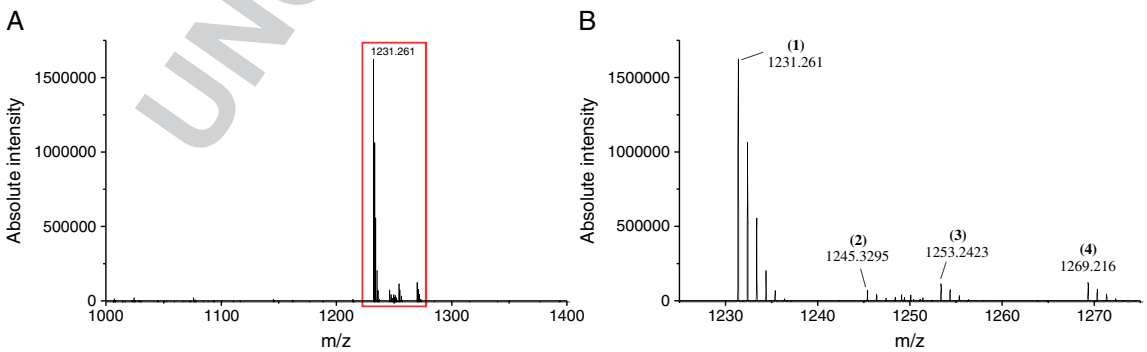

Figure 4. (A) MS spectrum of *Amycolatopsis* sp. F-136.425 (mass range *m/z* 1000–1400). (B) Peaks in the enlarged red-framed box in (A) represent the protonated (1), the sodium adduct (3) and the potassium adduct (4) of thioplabin A and the protonated thioplabin B (2).

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directly from the bacterial surface using Nanomate technology. During an early development stage, solvents, volume of extraction solvent, number of solvent depositions and extraction time were optimized. It was found that solvent composition is critical for effective extraction of antibiotics and for avoiding extraction of nutrients, vitamins, and additional media compounds from agar plate. Water, aqueous formic acid, methanol, acetonitrile, ethyl acetate and acetone were tested either individually or in mixtures. For our system ethyl acetate/acetone (65:35, v/v) containing 0.1% formic acid was optimal. Multiple spotting of ca. 2 μL of the mixture, short extraction time, and multiple extraction

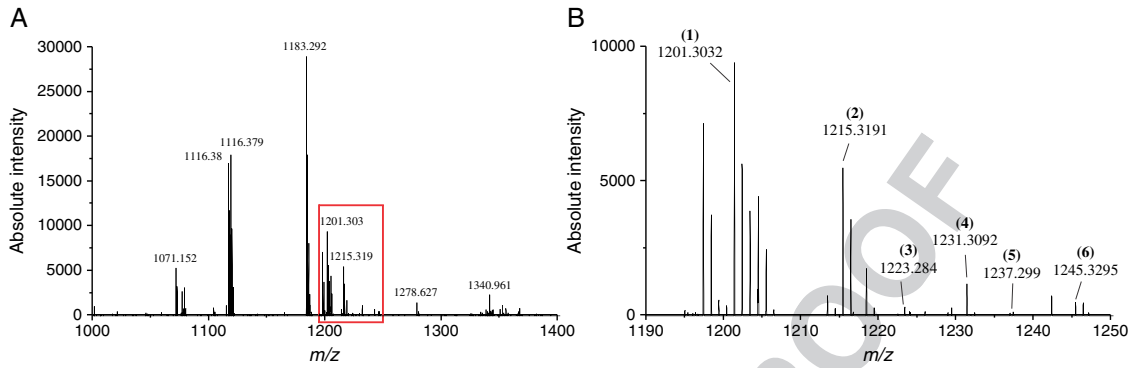

Figure 5. (A) MS spectrum of *Streptomyces* sp. F-179.539 (mass range *m/z* 1000–1400). (B) Peaks in the enlarged red-framed box in (A) represent the protonated antibiotic 10381Z2 (1), the protonated demethoxysulfomycin I (2) the protonated sulfomycin III (4), and the protonated sulfomycin I (6). Sodium adducts of demethoxysulfomycin I (5) and antibiotic 10381Z2 (3) were also observed.

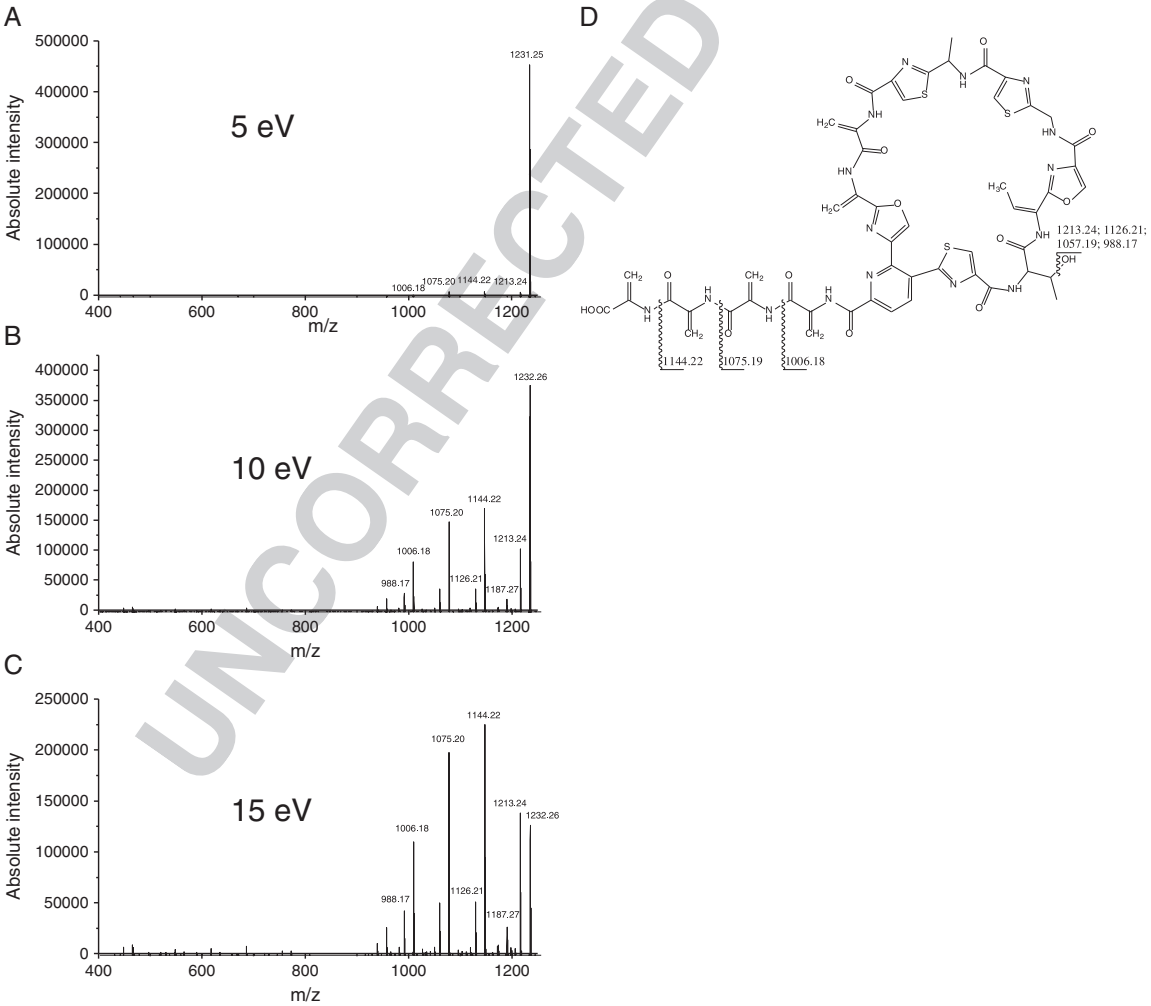

Figure 6. MS^2 spectra from thioplabin A using collision-induced dissociation. Spectra were acquired using different collision energies: 5 eV (A), 10 eV (B), and 15 eV (C). Assumed fragmentation of thioplabin A (D).

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from the same spot using the same tip were additionally beneficial for obtaining rich mass spectra. The most intense ion detected from the non-*Streptomyces* isolate F-169.583 has been assigned to the monoisotopic mass of protonated multhiomycin at *m/z* 1222.1587 with a mass difference of 2.95 ppm from the exact mass of multhiomycin (Fig. 3). Additionally, the monoisotopic mass of the sodium adduct of multhiomycin was detected (*m/z* 1244.1394, 1.92 ppm). Additional minor peaks were also present in the spectrum; however, they were not the focus of this study.

The analysis of the *Amycolatopsis* strain F-136.425 revealed monoisotopic masses at *m/z* 1231.261 and 1245.3295, which matched to protonated thioplabin A (3.79 ppm), resp., thioplabin B (3.2 ppm) (Fig. 4). Additionally sodium and potassium adducts of thioplabin A were detected at *m/z* 1253.2423 resp. *m/z* 1269.216). The accurate mass data were used to calculate the molecular formulas. Theoretical isotopic patterns for possible compositions were compared to the measured data. Supplementary Fig. S1 shows coinciding patterns for thioplabin A and multhiomycin. The standard deviation falls in a range from 0.07 to 0.62% (for thioplabin A, n = 3). Clearly, Nanomate technology can be used to acquire tandem mass spectra (Fig. 6). The mass spectrum obtained from the *Streptomyces* isolate F-179.539 LESA extract was characterized by several masses; however, we were interested in sulfomycin III and other tentatively identified sulfomycin derivatives (Fig. 5). Four monoisotopic masses were observed which coincided with the exact masses of protonated sulfomycin I (*m/z* 1245.3295, 3.4 ppm), sulfomycin III (*m/z* 1231.3092, –1.28 ppm), demethoxysulfomycin I (*m/z* 1215.3191, 3.57 ppm) and antibiotic 10381Z2 (*m/z* 1201.3032, 3.24 ppm). Furthermore, sodium adducts of demethoxysulfomycin I and antibiotic 10381Z2 were observed at *m/z* 1237.299 (0.91 ppm) resp. *m/z* 1223.284 (1.12 ppm), and the potassium adduct of antibiotic 10381Z2 at *m/z* 1239.252 (1.54 ppm). Sulfomycins were first described in 1969 as an antibiotic complex subgroup in the family of thiazolyl peptides and produced by a single bacterial isolate.[29] Whereas *Streptomyces* sp. F-179.539 has been described only as a sulfomycin III producer, we have detected possible additional sulfomycin derivatives. However, until now, the tandem mass (MS^2) spectra from multhiomycin and sulfomycin derivatives have been insufficient to dereplicate fully the antibiotic structure[30] as too few mass fragments have been created using collision-induced fragmentation (typically only from side chain, see Fig. 6). Additionally, using high-collision dissociation (HCD) MS^2 using nitrogen gas for collision did not provide better data. Currently, we are optimizing solvents, time of extraction, and cultivation parameter to increase the concentration of antibiotics. As CID and HCD fragmentation did not provide sufficiently rich MS^2 spectra due to cyclic antibiotic nature we hope to obtain richer fragmentation spectra by electron transfer dissociation (ETD) to break the cyclic-peptide-like ring.[31]

We have successfully demonstrated that the liquid extraction technique detects antibiotics directly from bacterial surfaces, providing answers to questions concerning not only antibiotic production, but also bacterial signaling molecule production, occurrence and function. The developed protocol can be used for fast screening of other bacterial strains, fungus and other microorganisms growing under laboratory conditions on an artificial medium or on natural substrates. In cases where rich MS^2 spectra are obtained, fragmentation tree clustering using BLAST algorithms can be employed to classify extracted compounds according to biosynthetic classes and ultimately to identify them.[32] To increase method throughput MS^E acquisitions might be employed as well.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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