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Chapter 11

Utilization of cellulose as a solid sorbent for antibiotic extraction

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ABSTRACT

Rational phytochemical analysis plays an important role in drug discovery approaches. Here, we exploited the natural polymer cellulose as a solid adsorbent of antibiotics in an aqueous matrix. Cellulose was shown to selectively bind several antibiotics with different chemical scaffolds, with preference towards those rich in hydrogen bonding such as aminoglycosides and glycopeptides. The binding selectivity of cellulose was also seen in the practical extraction of antibiotics specialized by actinomycetes. Cellulose preferentially absorbed polar compounds that were missed in conventional organic solvent fractionation such as using ethyl acetate extraction. This allowed the characterization of a group of structurally novel catechol-type siderophores (**12—14**) in *Streptomyces* sp. MBT76, which were exclusively extracted by cellulose. In summary, cellulose extraction can be considered as a complementary approach to conventional liquid-liquid extraction (LLE) to cover the widest range of secondary metabolites produced by actinomycetes, especially for the easily-overlooked water-soluble antibiotics.

1. INTRODUCTION

During the isolation of natural products from cultures, a rational extraction procedure is very important in terms of the removal of interfering components, such as from the culture medium, and to concentrate the widest range of specialized secondary metabolites with diverse polarities. Conventionally, the extraction of secondary metabolites from microbial cultures (predominantly in aqueous broth) is conducted with the organic solvents immiscible with water, such as ethyl acetate, chloroform. However, this conventional extraction method does not make use of all the metabolites contained in actinomycetes, since microbial NPs cover a tremendous chemical diversity with diverse chemical polarity. Particularly, many water-soluble polar antibiotics, such as aminoglycosides, are not amenable to extraction with ethyl acetate, which instead results in the extraction of a considerable amount of apolar lipids. On the other hand, extraction specificity in some cases is needed to simplify the separation procedure of compounds with specific structural scaffolds or chemical functionalities,¹ which is challenging to achieve in liquid-liquid extraction (LLE) that adversely consume large amounts of environment-unfriendly organics.

Various types of solid sorbents (e.g. C18 silica, polymeric resins, etc) have been employed to extract microbial NPs, among them, Diaion HP-20 resin is the most used, but it shows relatively low recovery for polar compounds. It is cost-ineffective to manufacture these artificial materials, and what is worse that they impose potential adverse effect on the environment. Alternatively, there has been an explosion of interest in the use of green biomass as a renewable resource for extraction purpose. Because of the omnipresence of interacting surface hydroxyl groups, the hydrophilic polymer cellulose is saturated with water when immersed in water or conditioned in moist atmosphere, which is the basis of the paper thin-layer chromatography. The retention of small molecules by cellulose is also observed in daily life, such as the irreversible adsorption of pigments by white filter paper after use and vegetables obtaining the flavors of meat when cooked together. Furthermore, Wagman and co-workers experimentally confirmed filtration materials and feces adsorb aminoglycoside-type antibiotics.^{2,3} All of these facts reveal that cellulose might have great potential as a solid adsorbent for extraction of some suitable antibiotics. However, to the best of our knowledge, there have been no reports on this topic.

Herein, the applicability of cellulose as trapping adsorbent for antibiotics was investigated. Eight commercial antibiotics with diverse chemical skeletons were first compared for their interaction with cellulose. Then, cellulose was employed for the extraction of polar metabolites produced by three actinomycetes, in comparison with the more traditional ethyl acetate extraction.

2. RESULTS AND DISCUSSION

2.1. Cellulose selectively binds antibiotics with different chemical scaffolds

An examination of the degree of physical association of eight commercial antibiotics with cellulose was investigated, namely the aminoglycosides hygromycin B, spectinomycin and streptomycin, the (glyco-)peptides vancomycin, capreomycin, and actinomycin D and the polyketides oxytetracyclin and chloramphenicol. All of these antibiotics except actinomycin D bound well to cellulose to give nonspecific cellulose-antibiotic complexes. The binding

efficiency of each of these antibiotics was manifested by the quantity of the cellulose-trapped molecules, in a setup where 10 mg of compound was added to 1 g of absorbent. Cellulose efficiently (>10%) absorbed aminoglycosides (Figure 1), with hygromycin B (22.6%) binding particularly well, in agreement with previous reports.^{2,3} In addition to the aminoglycosides, the peptide antibiotic vancomycin (14.8%) bound well too, with an efficiency comparable to those of the aminoglycosides streptomycin (17.5%) and spectinomycin (13.7%). Of the capreomycin, 8.1% was retained by the cellulose. Oxytetracyclin (2.5%) and chloramphenicol (0.5%) are deficient in H-bonding and were not captured well by cellulose. In this sense, cellulose could be reconsidered as a natural material for solid phase extraction and/or isolation of antibiotics.

The observed selectivity in Figure 1 prompted us to hypothesize a plausible binding mechanism of cellulose. Hydrogen bonding should be the principal factor, which explains aminoglycosides rich in –OH, and –NH₂ much more captured by residual –OH on cellulose surface than polyketide oxytetracyclin. Besides, size effects might also be involved. Cellulose is derived from D-glucose units condensed through $\beta(1\rightarrow4)$ -glycosidic bonds, to form a straight-chain polymer. The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen atoms on the same or on a neighbor chain, holding the chains firmly together side-by-side. The complicated three-dimensional hydrogen-bonding network presumably brings about an additional “microcage” among the glucose units. The size of this type of pores fits a six-membered glucose ring. Therefore, if a suitable ring or residue is rich in hydrogen-bonding groups, it would fall into the “microcage” and thus be trapped by –OH on the cellulose. This theory would explain why vancomycin with two glycosyl moieties was extracted much more efficiently by cellulose than actinomycin D, though they are both cyclic peptides. In light of these findings, it is recommended that caution prevail in filtration of aminoglycosides that are tenaciously bound to cellulose, especially when low concentrations of these antibiotics are used, large losses may occur.³

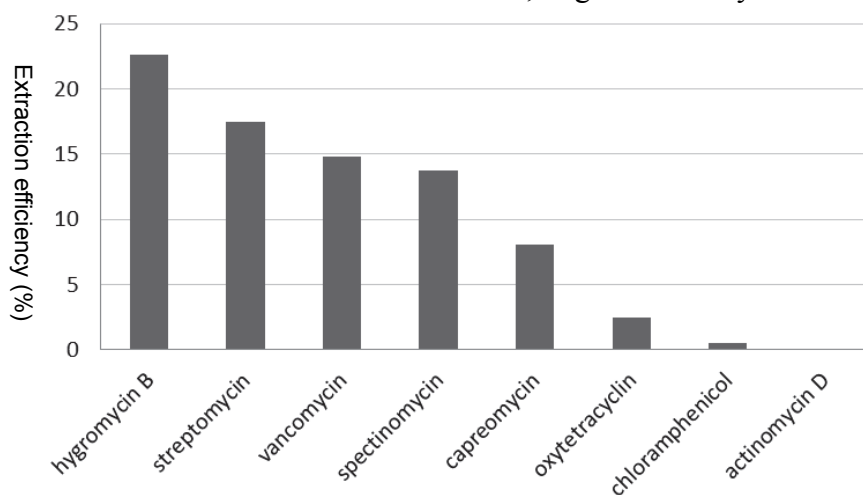


Figure 1. Cellulose selectively binds antibiotics with different chemical scaffolds. The extraction efficiency (in %) of the eight tested antibiotics is displayed in descending order.

2.2. Cellulose extracts antibiotics from the culture broth of actinomycetes

We then wondered if cellulose could be used to expedite antibiotic discovery from actinomycetes, especially those easily-neglected polar compounds? *Streptomyces* sp. MBT76 was previously identified as a prolific producer of antibiotics,⁴ and recent NMR-based

metabolomics revealed it could produce a diversity of secondary metabolites, such as isocoumarins, prodiginines, acetyltryptamine, and ferverulin, *etc.*⁵ In view of the intensive chemical investigation, this strain thus formed an ideal model for cellulose extraction of metabolites produced by a natural isolate. After 5 days of fermentation in perturbed NMMP medium, the culture broth of MBT76 was subjected to cellulose extraction and ethyl acetate (EtOAc) liquid-liquid partition, respectively. The extracts were compared by NMR profiling⁶ to compare these two extraction methods, whereby marked differences were observed (Figure 2). The compounds **1**–**14** (Figure 3) were identified with ¹H NMR technique and UHPLC-ToF-MS analysis (positive mode). EtOAc resulted in the extraction of a wide range of compounds (**1**–**9**) with diverse scaffolds and polarities, the identification of which were effortless due to the prior phytochemical knowledge of MBT76⁵ and the common components, via *di*-*tert*-butylphenols **8** and **9** in routine NMMP culture medium.^{7,8} In contrast, cellulose preferentially extracted polar chemicals from the culture broth of MBT76, *e.g.* a considerable amount of water-soluble mannitol (**10**), and glycerol (**11**), which avoided the apolar compounds **8** and **9** that oftentimes interfere with the purification of the desired natural products. Furthermore, the ¹H NMR spectrum of the cellulose extracts presented characteristic aromatic signals for catechol-type siderophores, including a suite of resonances at δ 7.34 (brd, $J = 7.8$ Hz), 7.03 (brd, $J = 7.8$ Hz), and 6.77 (t, $J = 7.8$ Hz) for 2,3-dihydroxybenzoic acid residue, a multitude of protons in the region δ 3.10–4.70 for *O*- or *NH*-substituted methylene or methine, and highly overlapped signals in the saturated region δ 1.70–2.10. UHPLC-ToF-MS analysis of cellulose crude extract presented a molecular feature at m/z 777.3157 for a formula of C₃₂H₄₅N₁₀O₁₃ (calculated for 777.3162), which confirmed the existence of divanchrobactin (**12**). Another mass peak at m/z 770.3408 established the molecular formula of C₃₁H₄₇N₉O₁₄ (calculated for 770.3315) for **14**, with one more unsaturation compared with that of compound **13**. Further MS/MS fragmentation indicated the unsaturation located in the hOrn-5 moiety, and was tentatively assigned as a double bond as depicted (Figure 4).

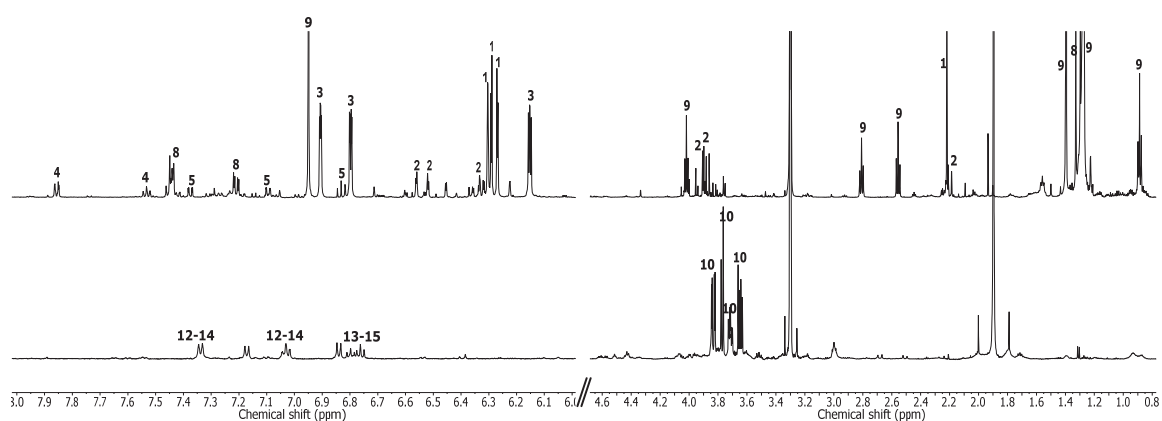


Figure 2. NMR comparison of extracts obtained from the culture broth *Streptomyces* sp. MBT76 using EtOAc (top) or cellulose (bottom). Proton signals were assigned to compounds numbered in Figure 3. EtOAc extracted a wide range of chemicals (**1**–**9**) with different scaffolds and diverse polarities, while cellulose showed specificity towards siderophores (**12**–**14**). Though the extraction of lipids **8** and **9** in EtOAc liquid partition could be avoided, a considerable amount of supplemented mannitol (**10**) in the NMMP culture medium was bound by cellulose.

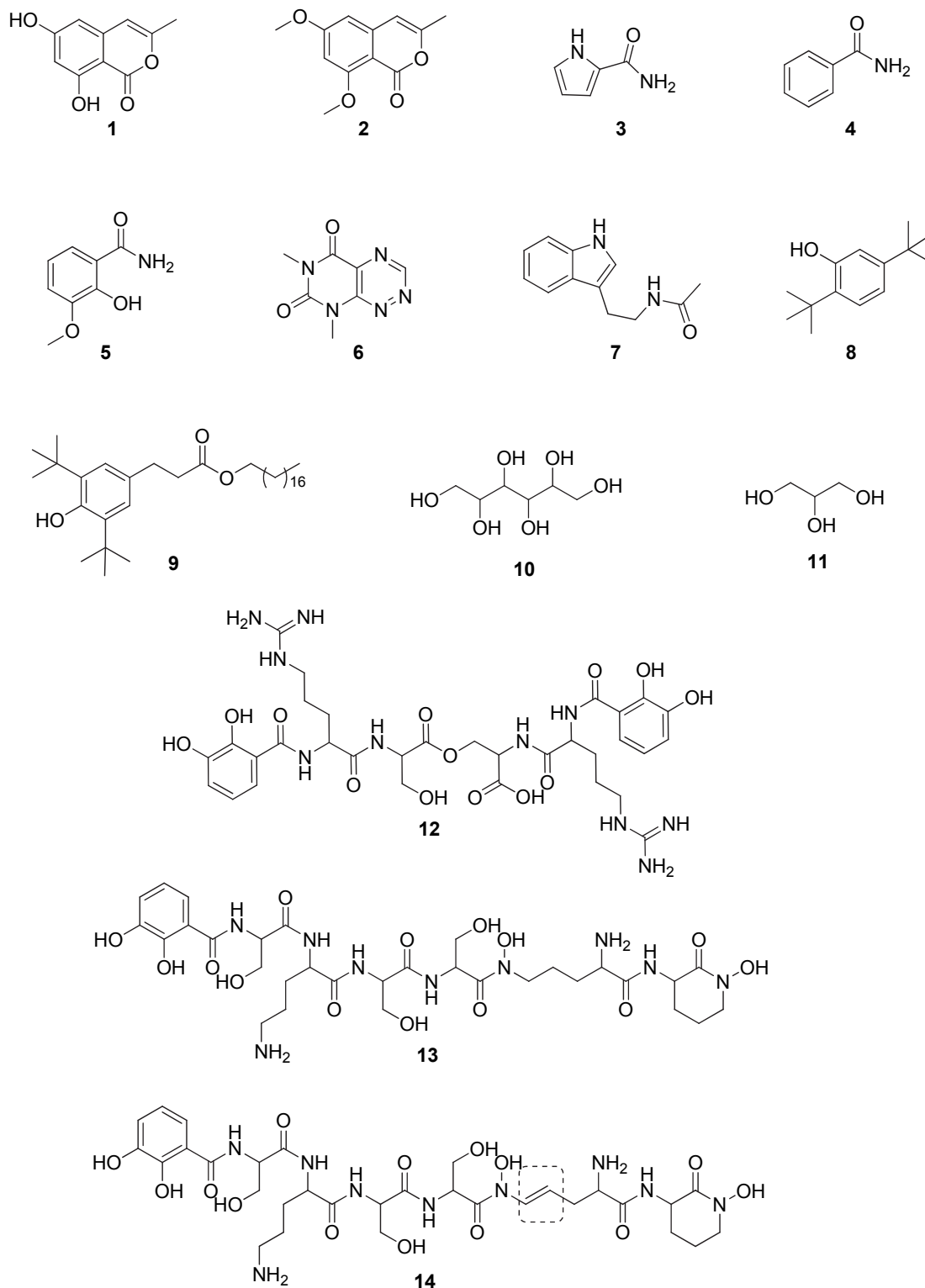


Figure 3. Compounds identified in the NMR profiling of *Streptomyces* sp. MBT76. Compounds 1–9 were extracted by ethyl acetate as previously described,⁵ while cellulose extraction gave rise to characterization of compounds 12–14.

As a further proof of concept, similar experiments were then conducted for *Streptomyces* sp. MBT18, a microbial isolate without any prior phytochemical investigation. ¹H NMR profiling not unexpectedly confirmed that cellulose exclusively coupled a considerable

amount of mannitol and glycerol, while EtOAc extracted a broad spectrum of compounds, one of which was identified as 9-methylstreptimidone (Figure S1). The extraction selectivity of cellulose was also seen for *Streptomyces* sp. MBT73,⁴ whereby more yellow pigment resistomycin than the apolar red pigment undecylprodiginine was bound, as visually detected by thin-layer chromatography (TLC) analysis (Figure S2).

The large genomes of actinomycetes (>8 Mb) contain a plethora of gene clusters for natural products with diverse polarities.⁹ The classical liquid-liquid extraction (LLE) employing ethyl acetate tends to miss water-soluble antibiotics, and lack the extraction selectivity towards specialized metabolites, especially for valuable chemicals produced in trace titers. Besides the comparative advantage in green chemistry, cellulose effectively avoids the metabolic complexity and unwanted lipids in LLE (Figure 2), but instead shows preference towards specific polar metabolites. In view of the notorious difficulty to purify water-soluble compounds,¹⁰ ¹H NMR profiling tool affording structural information in an early stage, can rationalize the downstream isolation workflow of compounds extracted by cellulose. For instance, NMR-revealed catechol-type siderophores (**12–14**) in the cellulose extraction of *Streptomyces* sp. MBT76 could be potentially separated from the abundant sugars (mannitol and glycerol) by using Fe₃O₄ magnetic nanoparticles that selectively bond salicylic acid moiety¹.

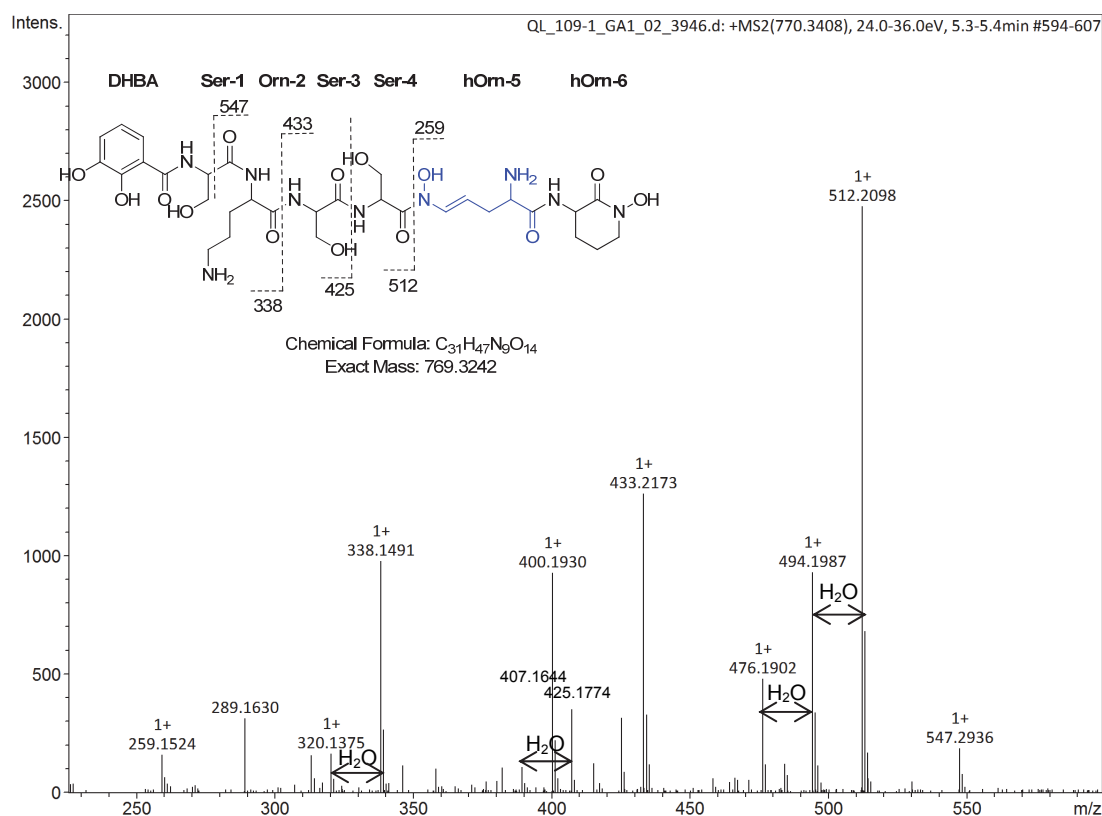


Figure 4. ESI-tandem mass spectrum (MS²) of the siderophore **14** in the cellulose extraction of *Streptomyces* sp. MBT76.

3. CONCLUSION

Our work shows that cellulose solid-phase extraction (CL-SPE) is complementary to conventional LLE, thus giving a broader coverage of metabolites to be retrieved from cultures

of actinomycetes, and in particular aiding in isolating water-soluble metabolites that readily escape EtOAc extraction. Therefore, the binding selectivity of cellulose in combination with NMR-based profiling will expedite the discovery of some novel chemical scaffolds and low-abundant metabolites from actinomycetes because it does not require prior knowledge of biosynthetic potential. It is important to note that cellulose is the most abundant natural polymer on the planet, which has far reaching implications for the ecological role of this polysaccharide. For example, we envision that natural products will accumulate around cellulose as they do in our extraction procedure and this may for example explain why water-soluble antibiotics are retained in the soil and not easily washed away. Thus, the antibiotic-binding nature of cellulose could provide novel ecological insights with implications for among others soil microbiology. Studies in this direction are currently underway in our laboratory.

4. EXPERIMENTAL SECTION

4.1. General Experimental Procedures

Cellulose was used as microcrystalline powder (Sigma, St. Louis, MO, USA). Silica gel 60 F254 (Merck, Darmstadt, Germany) was used for analytical TLC. Antibiotics were: spectinomycin dihydrochloride pentahydrate (Duchefa, Haarlem, Netherland), streptomycin sulfate (Boehringer Mannheim GmbH, Germany), vancomycin hydrochloride (Duchefa, Haarlem, Netherland), oxytetracyclin hydrochloride (Duchefa, Haarlem, Netherland), capreomycin sulfate (Sigma), hygromycin B (Duchefa), chloramphenicol (Sigma), actinomycin D (Sigma). All organic solvents were of analytical or HPLC grade, depending on the experiment.

4.2. Cellulose extraction of commercially obtained antibiotics

10 mg of hygromycin B, spectinomycin, streptomycin, vancomycin, capreomycin, oxytetracyclin, and chloramphenicol were dissolved in 25 ml of distilled water, except actinomycin D which was dissolved in ethyl acetate. After adding 1 g of cellulose powder, the mixture was stirred under room temperature for 2 h. The suspension was centrifuged and the supernatant was discarded. The cellulose in each sample was then rinsed three times with 20 ml of fresh solvents. Desorption of antibiotics from cellulose was conducted with three cycles of 15 ml acidified water (1% acetic acid), by stirring for 1 h. The combined acid extract was lyophilized using a freeze-dryer (Edwards Ltd., Crawley, UK). The obtained residue was dissolved in a mixture of $\text{CH}_3\text{OH-}d_4$ (0.4 ml) and KH_2PO_4 buffer in D_2O (pH 6.0, 0.4 ml) containing 0.01% (w/v) 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium (TMSP) as internal standard for ^1H NMR analysis. The extraction efficiency of cellulose was a ratio that the amount of acid-recovered antibiotics was divided by initially added amount (10 mg). Quantitative NMR was used to calculate the quantity of antibiotics recovered from cellulose, based on the relative ratio of intensity of the well-separated NMR peak to the known amount of TMSP as reference.¹¹

4.3. Cellulose extraction of metabolites from culture broth

Streptomyces sp. MBT76, MBT73, and MBT18 from the actinomycete collection⁴ of

Molecular Biotechnology, IBL, Leiden University was maintained in 20% glycerol at -20 °C. The liquid culture medium employed for fermentation was Minimal Liquid Medium (NMMP),¹² containing (NH₄)₂SO₄ (2 g), Casaminoacid (5.0 g), MgSO₄·7H₂O (0.6 g), minor element solution (1 mL), Na⁺-K⁺ phosphate buffer pH 6.8 (150 mL), 10% mannitol solution (50 mL), 20% glycerol solution (50 mL), peptone (8 g),^{4,5} and distilled water up to one liter. Additional 0.8% peptone was supplemented for MBT76, and 0.5% yeast extract for MBT73 according to previous study.⁴ Spores suspension was inoculated into 50 mL culture medium, and the culture broth was continuously shaken at 220 g and 30 °C for 5 days.

Cultures were harvested by centrifugation at 4000xg for 10 min. The culture broth (50 ml) was divided into two equal portions, one of which was extracted three times with 10 ml of ethyl acetate (EtOAc). The organic phases were pooled and washed with 30 ml of water and subsequently dried with 5 g of anhydrous Na₂SO₄. Finally, the EtOAc was removed under vacuum at 40 °C and the residue dissolved in 2.0 ml of EtOAc in a microtube (Eppendorf type-5415C, Hamburg, Germany). The solvent was then evaporated at room temperature under nitrogen gas, and subsequently dipped into liquid nitrogen and lyophilized using a freeze dryer (Edwards Ltd., Crawley, England). Cellulose (1 g) extraction of another one portion of microbial culture broth (25 ml) followed the same procedure for commercial antibiotics as described above. The obtained residue was dissolved in 0.3 ml of CH₃OH-*d*₄ for ¹H NMR analysis. All experiments were conducted in triplicate.

4.4. NMR measurement

NMR sample preparation and measurements were done according to our previously published protocol.⁶ Briefly, 500 µl of methanol-*d*₄ was added to the freeze-dried sample. Subsequently, the mixture was vortexed for 10 s and sonicated for 20 min at 42 kHz using an Ultrasonicator 5510E-MT (Branson, Danbury, CT, USA), followed by centrifugation at 13,000 g at room temperature for 5 min. The supernatant (300 µl) was transferred to a 5 mm micro NMR tube and analyzed. The ¹H NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. Deuterated methanol was used as the internal lock. Each ¹H NMR spectrum consisted of 128 scans using the following parameters: 0.16 Hz/point, pulse width (PW) = 30 (11.3 µs) and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to residual methanol-*d*₄ at 3.30 ppm, using MestReNova 8.1.

4.5. UHPLC-ToF-MS analysis

UHPLC-ToF-MS analyses were performed on an UHPLC system (Ultimate 3000, ThermoScientific, Germany) coupled to an ESI-IIQ-ToF spectrometer (microTOF-QII, Bruker Daltonics, Germany) in the positive mode. The chromatographic separation was done using a Kinetex C₁₈ UPLC 2.6 µm particle size column 150 × 2.0 mm (Phenomenex, Torrance, CA, USA) at a flow rate of 0.3 ml/min and a column temperature of 30 °C. Samples (3 µl) were eluted using a gradient of solvent A (water) and B (acetonitrile), both with 0.1% formic acid (v/v). The initial percentage of B was 5%, which was linearly increased to 90% in 19.5 min,

followed by a 2 min isocratic period and, then re-equilibrated with original conditions in 2 min. Nitrogen was used as drying and nebulizing gas. The gas flow was set at 10.0 l/min at 250 °C and the nebulizer pressure was 2.0 bar. The MS data were acquired over m/z range of 100–1000. The capillary voltage was 3.5 kV. For internal calibration, a 10 mM solution of sodium formate (Fluka, Steinheim, Germany) was infused. Formic acid, water and acetonitrile were LCMS grade, Optima (Fisher Scientific, Waltham, MA, USA).

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