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Sulfated phenolic acids from Dasycladales siphonous green algae

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ABSTRACT

Sulfated aromatic acids play a central role as mediators of chemical interactions and physiological processes in marine algae and seagrass. Among others, *Dasycladus vermicularis* (Scopoli) Krasser 1898 uses a sulfated hydroxylated coumarin derivative as storage metabolite for a protein cross linker that can be activated upon mechanical disruption of the alga. We introduce a comprehensive monitoring technique for sulfated metabolites based on fragmentation patterns in liquid chromatography/mass spectrometry and applied it to Dasycladales. This allowed the identification of two new aromatic sulfate esters 4-(sulfooxy)phenylacetic acid and 4-(sulfooxy)benzoic acid. The two metabolites were synthesized to prove the mass spectrometry-based structure elucidation in co-injections. We show that both metabolites are transformed to the corresponding desulfated phenols by sulfatases of bacteria. In biofouling experiments with *Escherichia coli* and *Vibrio natriegens* the desulfated forms were more active than the sulfated ones. Sulfatation might thus represent a measure of detoxification that enables the algae to store inactive forms of metabolites that are activated by settling organisms and then act as defense.

1. Introduction

Sulfated metabolites of diverse biosynthetic origin are widely distributed in marine organisms. Recent reports indicate that, in particular, sulfated phenolic metabolites play a role as storage form for more active metabolites with multiple ecological functions (Welling et al., 2009). For example psammaplin A sulfate from the sponge *Aplysinella rhax* is converted upon tissue disruption to its desulfated form thereby increasing its activity as a defensive metabolite (Thoms and Schupp, 2008). Recently, we also revised the role of the sulfated metabolite zosteric acid in the seagrass *Zostera marina* (Kurth et al., 2015). Zosteric acid has been considered as an anti-fouling metabolite, but in-depth investigation of its activity revealed that again, this sulfated form is only a precursor to the more active coumaric acid that is released by sulfatases.

Motivated by this series of reports on the activation of sulfated aromatic metabolites and on a preliminary survey, we undertook a structure mining approach based on LC/MSMS analysis of extracts from green algae of the order Dasycladales to identify novel sulfated metabolites. These ancient algae have a unique morphology with an unusual unicellular thallus architecture that might seem otherwise prone to fatal injury in comparison to multicellular organisms (Welling et al., 2009). Having a fast and reliable mechanism to mitigate mechanical damage both from herbivore attack and mechanical forces occurring in the environment is one way to succeed. This is the case with *Dasycladus vermicularis* (Scopoli) Krasser 1898, which copes with injury by sealing its wounds using a sophisticated series of biopolymerisation reactions and signaling events (Ross et al., 2006; Welling et al., 2011). In this green alga dihydroxycoumarin sulfate is a storage form for the more reactive trihydroxycoumarin (Welling et al., 2011). Upon wounding sulfatases are activated, liberating this metabolite that is immediately further oxidized and serves as protein cross-linker enabling the formation of a wound sealing co-polymer with the proteins from the alga (Fig. 1). We chose representative species from three of the six genera in the Dasycladaceae for analysis of sulfated secondary metabolites and wound responses. The species were *D. vermicularis*, *Cymopolia barbata* (Linnaeus) J.V. Lamouroux 1816 and *Neomeris annulata* Dickie 1874 that are phylogenetically closely related (Verbruggen et al., 2009; Zechman, 2003). All algae were collected in the Florida Keys from two distinct habitats (mudflats and mangroves) and produce wound plugs following cell wall disruption. Here we report novel sulfated phenolic acids and address their potential ecological function as precursors for biofilm inhibitors.

2. Results and discussion

2.1. Sulfated metabolites of D. vermicularis

Three candidate molecules for which sulfatation was indicated by the presence of a fragment of $[M-H-80]^-$ in the mass spectrum

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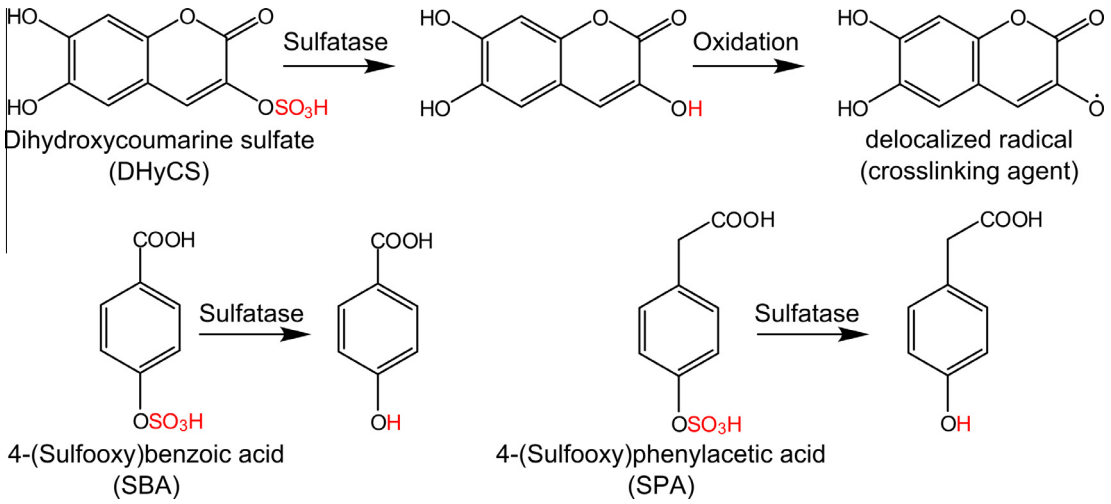

Fig. 1. Structures and transformation of the sulfated aromatic metabolites from Dascycladales.

were detected by UPLC–MS/MS measurements in extracts of *D. vermicularis*. The metabolite with a mass of 273 $[M-H]^-$ and a fragment with $m/z = 193$ could readily be assigned to dihydroxycoumarin sulfate (Fig. 1) based on previous results and co-injection with a synthetic standard (Welling et al., 2009). For identification of the two unknown potentially sulfated metabolites ($m/z = 217$ $[M-H]^-$ and 231 $[M-H]^-$, respectively) synthetic standards were prepared. Based on mass spectra and polarity in UPLC–MS (Fig. 2A, D and F) we selected 4-(sulfooxy)benzoic acid (SBA) and 4-(sulfooxy)phenylacetic acid (SPA) as likely candidates. After estimation of the content of the metabolites in the algal extract, co-injection experiments with algal extract and the synthetic standards were performed (Fig. 2C and E). Peak symmetry was important since the short retention times and strong solvent effects of the samples required a rigorous quality control of co-eluting peaks. SBA showed the same retention time and mass spectrum to the first sulfated metabolite in the *D. vermicularis* extract. When added in co-injection experiments, an increase of intensity of the first signal was observed (Fig. 2C). The mass spectrum remained unaffected by the co-injection. The *ortho*- and *meta*-isomers of (sulfooxy)benzoic acid eluted at different retention times (data not shown). The same procedure was applied for co-injection of SPA (Fig. 2E). No significant change in peak symmetry was observed upon addition of SBA or SPA, which unambiguously confirms the identity of the natural and synthetic products. Besides the occurrence as catabolic products in mouse urine, these metabolites have to our knowledge not been reported as natural products before (Manna et al., 2011; van der Hooft et al., 2012).

2.2. Sulfated metabolites of C. barbata

In *C. barbata* chromatograms two dominant signals exhibiting an $[M-H-80]^-$ fragment were observed. Retention times and mass spectra were in accordance with dihydroxycoumarin sulfate and SBA. Co-injection experiments as described above proved these structural suggestions (Fig. 3). In the extracts of slightly stressed algae we could still detect the ions corresponding to SBA and dihydroxycoumarin sulfate. We also found trihydroxycoumarin indicative for a wound activated desulfatation, a mechanism previously shown to control wound plug formation of *D. vermicularis* (Fig. 1) (Welling et al., 2011). In addition in non-stressed algal material extracted directly after harvesting we observed a signal with a molecular ion of 289 $[M-H]^-$ (data not shown) that also exhibited the loss of 80, which would be in accordance with a methoxylated dihydroxycoumarin sulfate.

2.3. Sulfated metabolites of N. annulata

In *N. annulata* we detected two closely eluting metabolites, with identical pseudomolecular ions of $[M-H]^- = 259$ and each with a fragment of $[M-H-80]^-$. These metabolites were not detectable anymore shortly after wounding. Molecular weight and MS/MS spectra would be in accordance with a mono sulfated dihydroxycinnamic acid, such as caffeic acid or a positional isomer. We therefore synthesized the sulfated 3,4-, 2,3-, 2,4- and 2,5 dihydroxycinnamic acid derivatives, but despite very similar retention times and MS/MS fragmentation patterns identity to the natural products could not be confirmed.

2.4. Biofilm inhibition by 4-(sulfooxy)benzoic acid, 4-(sulfooxy)-phenylacetic acid and their desulfated derivatives

The novel metabolites show structural similarity to zosteric acid (4-(sulfooxy)coumaric acid), a natural product produced by the seagrass *Z. marina* (Todd et al., 1993). Zosteric acid has previously been intensively investigated due to its alleged anti-fouling activity (see for example Barrios et al., 2005; Boopalan and Sasikumar, 2011; Newby et al., 2006; Xu et al., 2005a,b; Villa et al., 2010). However, a critical re-examination revealed that anti-fouling activity in bacterial biofilm assays with *Escherichia coli* and *Vibrio natriegens* is not caused by zosteric acid but rather by the corresponding desulfated *p*-coumaric acid (Kurth et al., 2015). Due to their structural similarity to zosteric acid, we investigated the biofilm inhibition activity of SBA and SPA as well as of the corresponding desulfated 4-(hydroxy)benzoic acid (HBA) and 4-(hydroxy)phenylacetic acid (HPA). We employed a rapid microtiter dish and a longer term PTFE plate assay. For the survey we selected a high, but non-toxic initial concentration of 2.05 mM of all metabolites in our tests. This concentration was chosen to facilitate comparison with the activity of the zosteric acid/*p*-coumaric acid system, which was active but not toxic at this concentration in previous assays (Kurth et al., 2015). All four tested metabolites did not negatively affect growth of *E. coli* and *V. natriegens* in the microtiter dish assay before the onset of biofilm formation, indicating that no direct bactericidal activity is caused by the compounds (Figs. 4A and 5A). In fact a slight growth promoting effect of SPA HPA and HBA was observed for *V. natriegens*. Biofilms of *V. natriegens* formed at the air–liquid interface during this assay could not be evaluated due to floc formation. UPLC–MS monitoring revealed that transformation of SPA and SBA to corresponding alcohols by bacterial sulfatase occurs during incubation

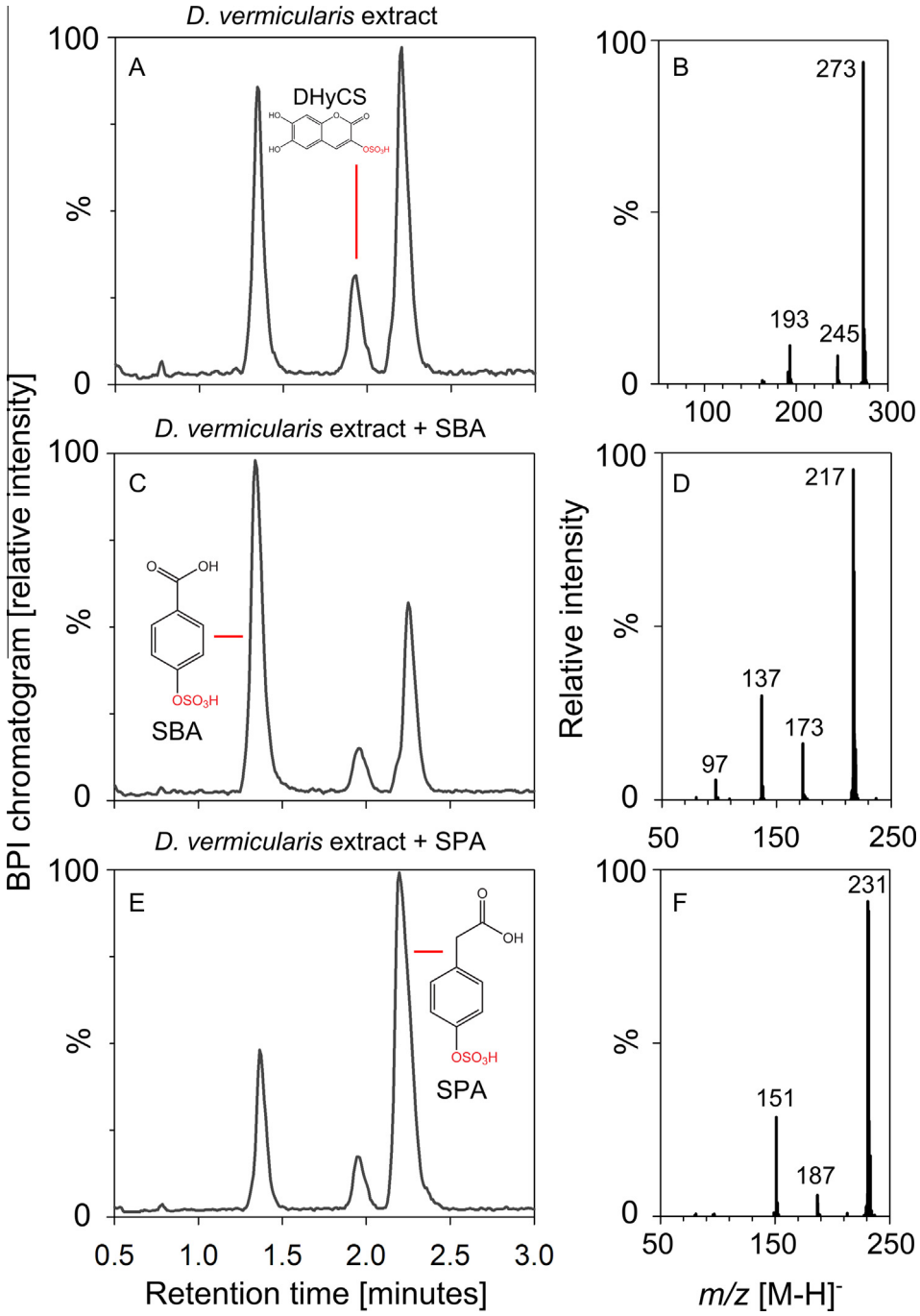

Fig. 2. LC/MS chromatograms (base peak intensity, BPI) of (A) a *D. vermicularis* extract. The labeled peak indicates dihydroxycoumarin sulfate (DHyCS). (B) MS/MS of DHyCS in ESI-negative ionization mode. (C) Co-injection with 4-(sulfooxy)benzoic acid (SBA). (D) MS/MS of SBA in ESI-negative ionization mode. (E) Co-injection with 4-(sulfooxy)phenylacetic acid (SPA). (F) MS/MS of SPA in ESI-negative ionization mode.

(Fig. 5b). In the *E. coli* microtiter dish biofilm assay the non-sulfated metabolites caused a more pronounced biofilm inhibition within the first hours compared to the sulfated forms (Fig. 4B). In more matured biofilms formed later during the assay this activity was not observed any more despite the fact that the test compounds were stable over the entire time (Fig. 4C). Biofilms can serve among others as a protection against harmful substances (Stoodley et al., 2002). The protective function has been shown to be dependent on biofilm architecture (Mah and O'Toole, 2001). Thus it can be assumed that the phenolic acids can defer *E. coli* biofilm development but not prevent its formation. The mature biofilm matrix might function then as a protective barrier against these compounds. In the *V. natriegens* bioassay on submerged PTFE plates a transient inhibition of biofilm formation was observed. Here again the non-sulfated HBA was significantly more active than the sulfated parent compound, (Fig. 6A). Biofilm inhibition of HBA and its sodium salt has been observed for fresh water bacteria before (Al-Juhni and Newby, 2006). In general, the active concentrations were relatively high and in-depth investigations would be required to prove the function for the producer. High concentrations might also limit their potential in further developments as commercial anti-fouling metabolites, but the general stabilization/activation

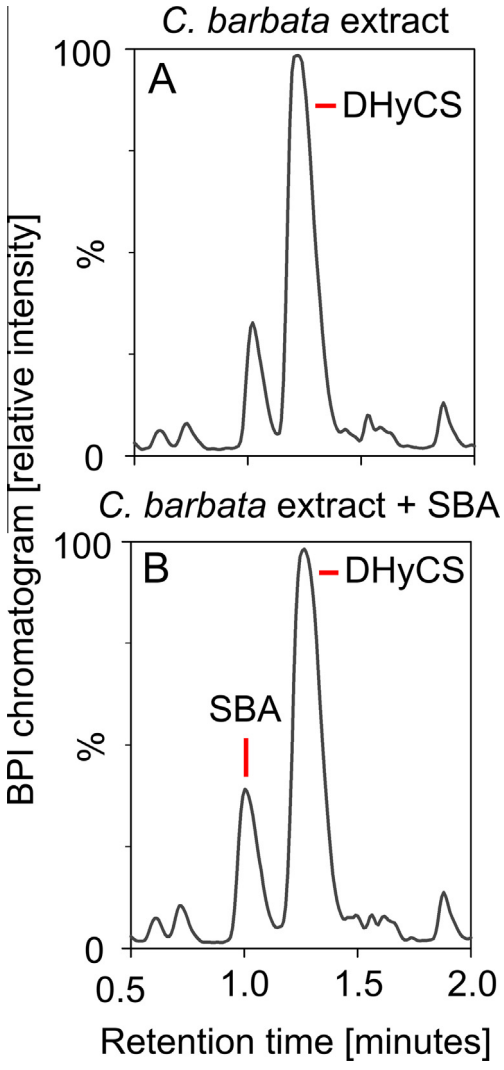

Fig. 3. LC/MS chromatograms of (A) a *C. barbata* extract, the labeled peak indicates dihydroxycoumarin sulfate (DHyCS). (B) Co-injection with 4-(sulfooxy)benzoic acid (SBA) in ESI-negative ionization mode.

concept involving sulfatation might prove worthy to pursue in this context. In contrast to *E. coli*, *V. natriegens* transformed the added metabolites during both assays (Figs. 5B and 6B). Both SBA and SPA were desulfated, by a sulfatase activity of the bacteria. No transformation of the metabolites was observed in sterile medium (data not shown). Released HBA was degraded during prolonged incubation, which could explain the loss of biofilm inhibiting activity towards the end of the experiments. The metabolites produced by the algae could thus serve as storage form for more active products released by sulfatase activity. Sulfatation/desulfatation can thus be seen as means to facilitate storage of less active forms that can be activated during or after release, as has been observed for other sulfated metabolites of marine origin (Welling et al., 2009; Thoms and Schupp, 2008; Welling et al., 2011). If however, in addition to certain bacteria, the algae themselves have means for desulfatation of the sulfated metabolites remains open. Such a mechanism could represent an activated defense if the required active concentrations would be delivered locally (Pohnert et al., 2007). To clarify the ecological role of the newly identified metabolites, future experiments will include the quantification of sulfated metabolites, the localization of potential storage vesicles and the survey of exsudation and activation mechanisms.

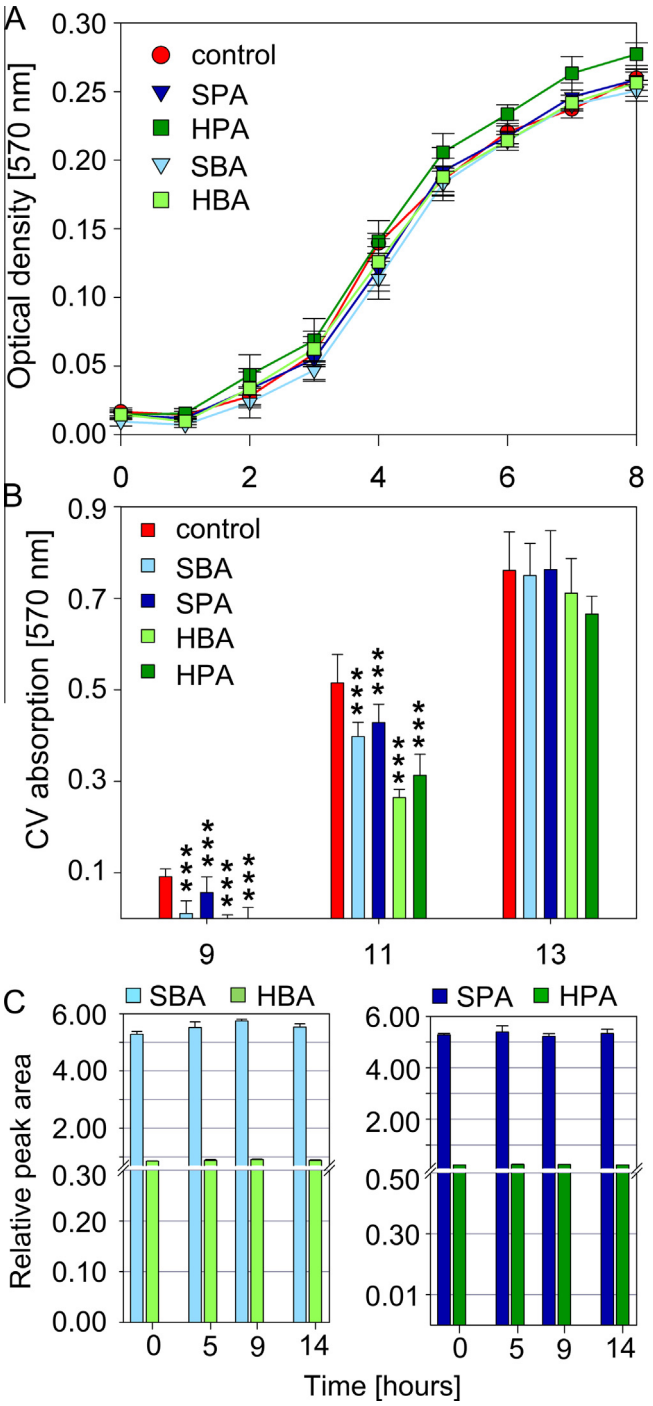

Fig. 4. Mictrotiter dish biofilm assay with *E. coli*. (A) Growth of *E. coli* until the onset of biofilm formation in the presence of different test-compounds. (B) Biofilm formation and (C) chemical stability of the additives upon incubation in *E. coli* cultures for up to 14 h. 4-(Sulfooxy)benzoic acid (SBA), 4-(sulfooxy)phenylacetic acid (SPA), 4-(hydroxy)benzoic acid (HBA), 4-(hydroxy)phenylacetic acid (SHA). Mean ± SD, $n = 8$ for A, B and $n = 3$ for C; asterisks indicate One Way ANOVA (Holm-Šídák test): ***significantly different with $p < 0.001$ to the control.

3. Concluding remarks

We demonstrate that sulfated phenolic acids are widely distributed in Dasycladales. Two new metabolites are described and their function as potential inhibitors of biofilm formation is evaluated. We conclude that the novel sulfated metabolites serve as storage metabolites for more active desulfated forms that can

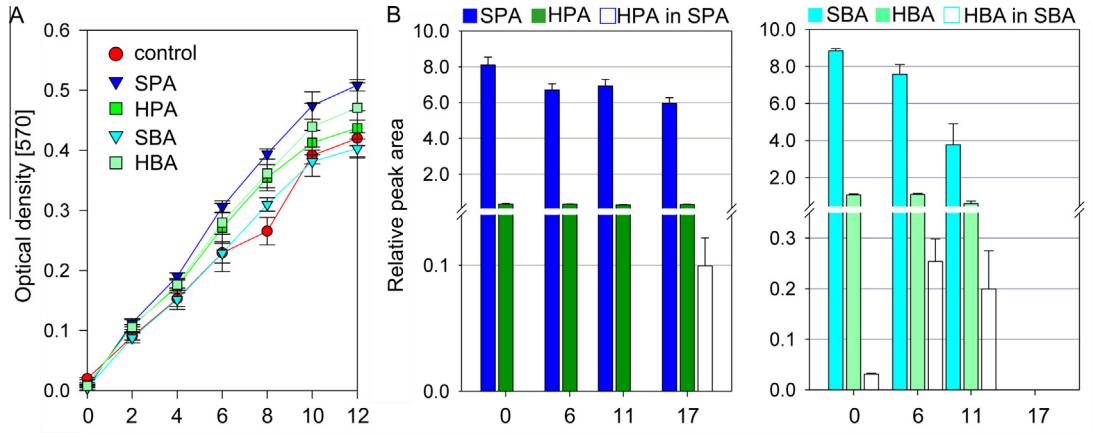

Fig. 5. Microtiter dish biofilm assays with *V. natriegens*. (A) Growth of *V. natriegens* until the onset of biofilm formation in presence of different test-compounds. (B) Chemical stability of the additives during the assay. 4-(Sulfooxy)benzoic acid (SBA), 4-(sulfooxy)phenylacetic acid (SPA), 4-(hydroxy)benzoic acid (HBA), 4-(hydroxy)phenylacetic acid (SHA). Mean ± SD, $n = 8$ for A and $n = 3$ for B.

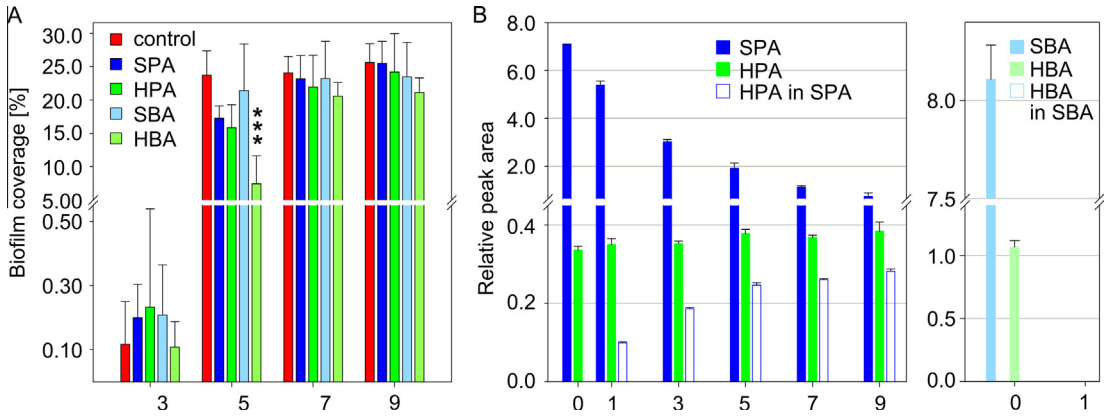

Fig. 6. Biofilm assays on submerged PTFE plates with *V. natriegens*. (A) Biofilm formation in presence of different test compounds. (B) Chemical stability of the additives during the assay. 4-(Sulfooxy)benzoic acid (SBA), 4-(sulfooxy)phenylacetic acid (SPA), 4-(hydroxy)benzoic acid (HBA), 4-(hydroxy)phenylacetic acid (SHA). Mean ± SD, $n = 3$; asterisks indicate One way ANOVA (Holm-Šídák test): ***significantly different with $p < 0.001$ to the control.

be released by bacterial activity. Sulfatation/desulfatation of aromatic metabolites to modulate activity might be a general strategy among Dasycladales and might be also found in other aquatic organisms such as the seagrass *Z. marina*.

4. Experimental

4.1. Algal material

D. vermicularis, *C. barbata*, and *N. annulata* were collected at the Florida Keys (USA) in the Bahia Honda area by snorkeling. Algae were carefully detached from their substratum and directly transported to the lab. Extraction of the fresh material was performed within 24 h after collection. Additional specimens of *C. barbata* were ordered from www.live-plants.com (Bradenton, FL, USA).

4.2. Extractions

Algal biomass (200 mg) was blot dried using paper towels, ground in a pre-cooled (−20 °C) mortar and pestle containing cold (−20 °C) MeOH (1 mL) and the slurry was transferred to an Eppendorf tube (1.5 mL). Samples were centrifuged (3 min, 16,700*g*, 20 °C) and the supernatant was collected. The solvent was removed under reduced pressure and the frozen samples were stored. Immediately before measurement, samples were taken up in 200 µL MeOH.

4.3. Ultra performance liquid chromatography–mass spectrometry (UPLC–MS) analysis

For identification of the unknown sulfated metabolites Ultra High-Performance Liquid Chromatography tandem mass spectrometry (UPLC–MS/MS) runs were directly performed with the MeOH extracts. UPLC–MS/MS was performed on an Acquity® Ultra-Performance LC system (Waters, Milford, MA, USA), coupled to a quadrupole time-of-flight (QTof) MicroMass spectrometer (Waters Micromass, Manchester, England). Separation was performed on a UPLC® C18-BEH column (Waters, 2.1 mm × 50 mm, particle size 1.7 µm) or on a Kinetex C18 column (Phenomenex, 2.1 mm × 50 mm, particle size 1.7 µm). All samples were eluted using a gradient solvent composition. Solvent A was composed of 98% water and 2% acetonitrile plus 0.1% formic acid, solvent B solely contained acetonitrile plus 0.1% formic acid. Only UPLC grade solvents from Fisher Scientific (UK) were used. Electrospray-ionization (ESI) tandem mass spectra were recorded with a cone gas flow of 10 L h^{-1} and a desolvation gas flow of 700 L h^{-1}. The desolvation temperature was set to 300 °C, the source temperature to 120 °C. For all experiments a collision energy of 10 eV was applied. The capillary voltage was set to 2700 V in

negative ionization mode. The gradient started with 100% A (held for 0.2 min) and was ramped to 82% A (1.8 min) and to 0% A (4.5 min) before re-equilibration with 100% A started at 6 min. Co-injection experiments were performed to confirm the identity of synthetic and natural products. Therefore, the concentrations of metabolites in the algal samples were roughly estimated using an external calibration with the synthetic standards (10–80 μg mL^{-1} in steps of 10 for SBA and 50–400 μg mL^{-1} in steps of 50 for SPA). Then, algal extracts were mixed with the synthetic standards in similar concentrations. Samples were immediately measured by UPLC–MS using a modified gradient. This started with 100% A (held for 0.2 min) and was ramped to 91% A (2.5 min) and to 0% A (5 min) before re-equilibration with 100% A started at 6.5 min. If peak areas were doubled without major changes in peak symmetry, peaks were considered as co-eluting, confirming the identity of the tested metabolites.

4.4. Screening for sulfated metabolites

Sulfated metabolites show, other than the pseudo molecular ion $[M-H]^-$ a characteristic mass of $[M-H-80]^-$ due to loss of the SO_3. This characteristic property of the mass spectra was used to identify candidate peaks for further structure elucidation. Since in no cases could enough material be obtained for a direct NMR structure elucidation we reverted to the synthesis of standards and co-injection experiments to prove the identity of the natural products.

4.5. Synthesis of 4-(sulfooxy)benzoic acid

15 g 4-hydroxybenzoic acid (108 mmol, Sigma Aldrich, Germany) and 17.2 g sulfur trioxide pyridine complex (Pyr^*SO_3) (108 mmol, Alfa Aesar, Germany) were dissolved in 200 mL water free pyridine and the reaction mixture was stirred in a round neck flask at RT for 48 h. Subsequently, pyridine was removed by rotor evaporation to yield a yellow to brown oil. The oil was dissolved in as little water as possible and the pH adjusted to ~6–7 with a 25% potassium hydroxide (KOH) solution. The aqueous solution was then washed three times with the same volume of ethyl acetate. During the first two washing steps a precipitate formed, not containing product, which was filtered off after phase separation. The ethyl acetate phase was discarded, the aqueous phase concentrated by rotor evaporation to yield a brown to yellow, slightly damp raw product. The raw product was dissolved in as little water as possible and pH was adjusted to 10 with 25% KOH. The solution was stirred for 1 h at 60 °C to cleave the anhydride side products, providing sulfated product and educt. The solution was neutralized with diluted H_2SO_4 and water was removed by rotary evaporation. The yellowish powder was dissolved in as little water as possible at 40 °C and twice the volume of methanol was added to precipitate salts. The precipitate was filtered off and washed with a further single amount of methanol, followed by unification of the methanolic solutions. The batch was then left standing at RT over night without stirring and the newly formed crystalline precipitate was filtered off and discarded. After evaporation of the solvent to dryness the powder was taken up in 1/10 the amount of methanol used to precipitate salts and placed into an ultrasonic bath until a fine suspension of a white powder and yellowish solution was obtained. The solution was filtered off, evaporated again and the suspension step repeated with half the amount of methanol. The white precipitate was collected, washed with acetone and dried at 70 °C to give 13.5 g of 4-(sulfooxy)benzoic acid (SBA) as a fine white powder (57%). ^1H NMR (400 MHz, DMSO [D_6] + 0.8% formic acid) δ(ppm): 7.22 (d, 2H); 7.84 (d, 2H), ^{13}C NMR δ(ppm): 119.33, 126.59, 130.45, 156.93, 164.64, Elemental analysis found: C 38.44, H 1.62, S 14.32. Calc. for $C_8H_7O_6SKa$ C 38.50%, H 2.70%, S 14.70%. UPLC–MS m/z $[M-H]^-$: 216.99.

4.6. Synthesis of 4-(sulfooxy)phenylacetic acid

7.5 g of 4-hydroxyphenylacetic acid (52 mmol, Sigma Aldrich, Germany) and 8.3 g Pyr^*SO_3 (52 mmol) were dissolved in 100 mL water free pyridine. The synthesis and work-up followed that described for SBA synthesis. ^1H NMR (400 MHz, DMSO [D_6] + 0.8% formic acid) δ(ppm): 3.11 (s, 2H), 6.98 (d, 2H), 7.08 (d, 2H), ^{13}C NMR δ(ppm): 45.93, 119.89, 129.30, 135.26, 150.82, 174.06. Elemental analysis as potassium salt found: C 32.24, H 1.87, S 10.83. Calc. for $C_7H_5O_6SKa$ C 35.55, H 2.61, S 11.86. UPLC–MS m/z $[M-H]^-$: 231.00.

4.7. Synthesis of other standards

Additionally, the synthetic standards 2-(sulfooxy)benzoic acid, 3-(sulfooxy)benzoic acid, 2,3- and 4-sulfooxy methoxyphenol, as well as the isomers of mono sulfated 2,3- and 3,4-dihydrocycinnamic acids were prepared as described for SBA. Dihydroxycoumarin sulfate was prepared as described elsewhere (Welling et al., 2011).

4.8. Bioassays on bacterial growth and biofilm formation

Bioassays were conducted with SBA and SPA as previously described (for full experimental details see Kurth et al. (2015)). Microtiter dish assay with *E. coli* and *V. natriegens*: Briefly, solutions of the test compounds (2.05 mM) were added to bacterial cultures (ATCC 25404 and ATCC 14048, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) in 96-well plates and incubated at 30 °C. When biofilm formation started a first plate was removed from the incubator and from then on hourly samples were taken by collecting additional plates for biofilm evaluation and UPLC–MS analysis. Therefore 190 μL sample were collected in triplicates per assayed compound and centrifuged (15 min, 13,000 rpm). 100 μL of supernatant were then transferred into glass vials containing 900 μL of a methanolic solution with 0.1 mg mL^{-1} *trans*-cinnamic acid as internal standard and stored at −26 °C until UPLC–MS analysis. For biofilm evaluation, the remaining liquid contents were poured quickly into a waste container, and then plates were immediately washed in phosphate saline buffer and water. After drying, 200 μl of a 0.1% aqueous crystal violet (CV) solution were added into each biofilm well and stained for 15 min. After washing with deionized water and drying 200 μl of a 80:20 mixture of ethanol/acetone were filled in each stained well and left for 15 min until CV was dissolved. CV absorption was then measured in a Mithras plate reader at 570 nm (30 °C), with CV staining of untreated wells as reference. Biofilm of *V. natriegens* was not evaluable due to floc formation under the given experimental conditions. Growth was monitored until the onset of biofilm formation by OD measurements.

Biofilm assay on submerged polytetrafluoroethylene (PTFE) plates with *V. natriegens*: Briefly, 1.5 cm long PTFE tubes were each cut twice to 1/3 of the diameter and two PTFE plates with a diameter of 12 mm were fixed in the cuts. 5–10 of the set-ups were autoclaved added to Erlenmeyer flasks and incubated with 50 mL *V. natriegens* (ATCC 14048) cultures with or without test compounds (2.05 mM). Flasks were sealed and put on a shaker (150 rpm) at 30 °C. For biofilm sampling, one PTFE set-up from each flask was removed and washed with water, air dried and dyed in 0.1% CV solution for 15 min. After washing and air-drying, tubes of the PTFE set-ups were cut, such that PTFE plates could be removed and biofilm formation was monitored by microscopy (for full experimental details see Kurth et al. (2015)).

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