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# Identification of fungal fossils and novel azaphilone pigments in ancient carbonised specimens of *Hypoxylon fragiforme* from forest soils of Châtillon-sur-Seine (Burgundy)

Frank Surup<sup>1,2</sup>  · Abolfazl Narmani<sup>1,3</sup> · Lucile Wendt<sup>1,2</sup> · Sebastian Pfütze<sup>1,2</sup> · Robin Kretz<sup>1</sup> · Kevin Becker<sup>1,2</sup> · Clément Menbrivès<sup>4</sup> · Alain Giosa<sup>4</sup> · Michelle Elliott<sup>4</sup> · Christophe Petit<sup>4</sup> · Manfred Rohde<sup>5</sup> · Marc Stadler<sup>1,2</sup> 

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## Abstract

Fungal stromata were recently discovered in association with charcoal and burnt soil aggregates during an archaeological survey in the Châtillon-sur-Seine forest massif. The wood and soil in the samples were dated to the medieval period (between 738 and 1411 AD). Light microscopy and scanning electron microscopy revealed that a few of the stromatal fragments still contained ascospores. Their macromorphological characters were described and secondary metabolite profiles were generated using high performance liquid chromatography with diode array and mass spectrometric detection (HPLC–DAD/MS). The combination of these two data lines then allowed species identification. Most of the fragments were assigned to *Hypoxylon fragiforme*, the type species of the Hypoxylaceae (Xylariales). Two further species whose stromata grew on the fossil charcoal could be tentatively identified as *Jackrogersella cohaerens* and (more tentatively) as *Hypoxylon vogesiacum*. These three species are still commonly encountered in the forests of Central Europe today. Furthermore, the HPLC–HRMS data of *H. fragiforme* suggested the presence of unknown azaphilone dimers and of further new pigments. These archaeological compounds were compared to fresh stromata of *H. fragiforme* collected in Germany and subjected to the same analytical protocol. While the major components in both samples were identified as the known mitorubrin type azaphilones and orsellinic acid, the chemical structures of seven novel complex azaphilone pigments, for which we propose the trivial names rutilins C–D and fragirubins A–E, were elucidated using spectral methods (NMR and CD spectroscopy, high resolution mass spectrometry). It appears that these pigments had indeed persisted for millennia in the fossil stromata.

**Keywords** Biodiversity · Chemotaxonomy · Phylogeny · Sordariomycetes · Xylariales · Structure elucidation

## Introduction

The family Hypoxylaceae (Xylariales, Sordariomycetes) is characterised by the presence of manifold stromatal pigments (see recent overview by Helaly et al. 2018), most of

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✉ Marc Stadler  
marc.stadler@helmholtz-hzi.de

<sup>1</sup> Dept. Microbial Drugs, Helmholtz-Zentrum für Infektionsforschung GmbH, Inhoffenstrasse 7, 38124 Braunschweig, Germany

<sup>2</sup> German Centre for Infection Research (DZIF), Partner Site Hannover-Braunschweig, 38124 Braunschweig, Germany

<sup>3</sup> Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

<sup>4</sup> University of Paris 1 Panthéon-Sorbonne, UMR 7041, Archéologies Environnementales, Paris, France

<sup>5</sup> Helmholtz-Zentrum für Infektionsforschung GmbH, Central Facility for Microscopy, Inhoffenstrasse 7, 38124 Braunschweig, Germany

which belong to the chemical type of azaphilones. In particular the genera *Annulohypoxylon*, *Hypoxylon* and *Jackrogersella* have been thoroughly studied for secondary metabolites, and some of their chemotaxonomic markers have been detected in type specimens and historical herbarium materials originally collected up to over 250 years ago (Stadler et al. 2008a, b). Together with meticulous studies of the morphology of both their asexual and sexual states, these molecular data provided the basis for a taxonomic rearrangement of the major lineages of stromatic Xylariales, resulting in the segregation of the Xylariaceae *s. lat.* into separate families (Daranagama et al. 2018; Wendt et al. 2018). However, the secondary metabolism of these fungi is so diverse that most compounds produced during their life cycle have not yet been identified. Many metabolites that exhibit prominent biological activities are indeed only produced by the cultures (Surup et al. 2014) or in the growing stromata bearing the anamorph (Stadler et al. 2006), and several were only identified after large-scale biotechnological production and extensive chromatographic work (Kuhnert et al. 2015; Surup et al. 2018).

Other metabolites seem to accumulate in either the stromata or the cultures, and the former compounds in particular can be detected even in ancient specimens.

Recently, some remnants of charred fungal elements were encountered in association with numerous charcoals and burnt soil aggregates during an archaeopedological survey in the Châtillon-sur-Seine forest massif. Macroscopically, these fungi were reminiscent of certain species of the Hypoxylaceae. We chose to study these ancient specimens for the presence of phenotypically informative features, such as the presence of ascospores, by the use of light microscopy, scanning electron microscopy (SEM) and high performance liquid chromatography coupled with diode array detection and mass spectrometry (HPLC–DAD/MS) profiling. However, the HPLC profiles displayed peaks that did neither correspond to known secondary metabolites of *H. fragiforme* to any other mitorubrin derivative derived from previous work (e.g. Hellwig et al. 2005; Sir et al. 2016).

In order to find out whether these metabolites constitute artefacts or genuine secondary metabolites that had potentially persisted in the stromata over many centuries, fresh material of the species in question was collected in the field from Germany and their metabolites were isolated to purity and identified by spectral methods. This paper presents the identification the fossil species and the structure elucidation of their secondary metabolites.

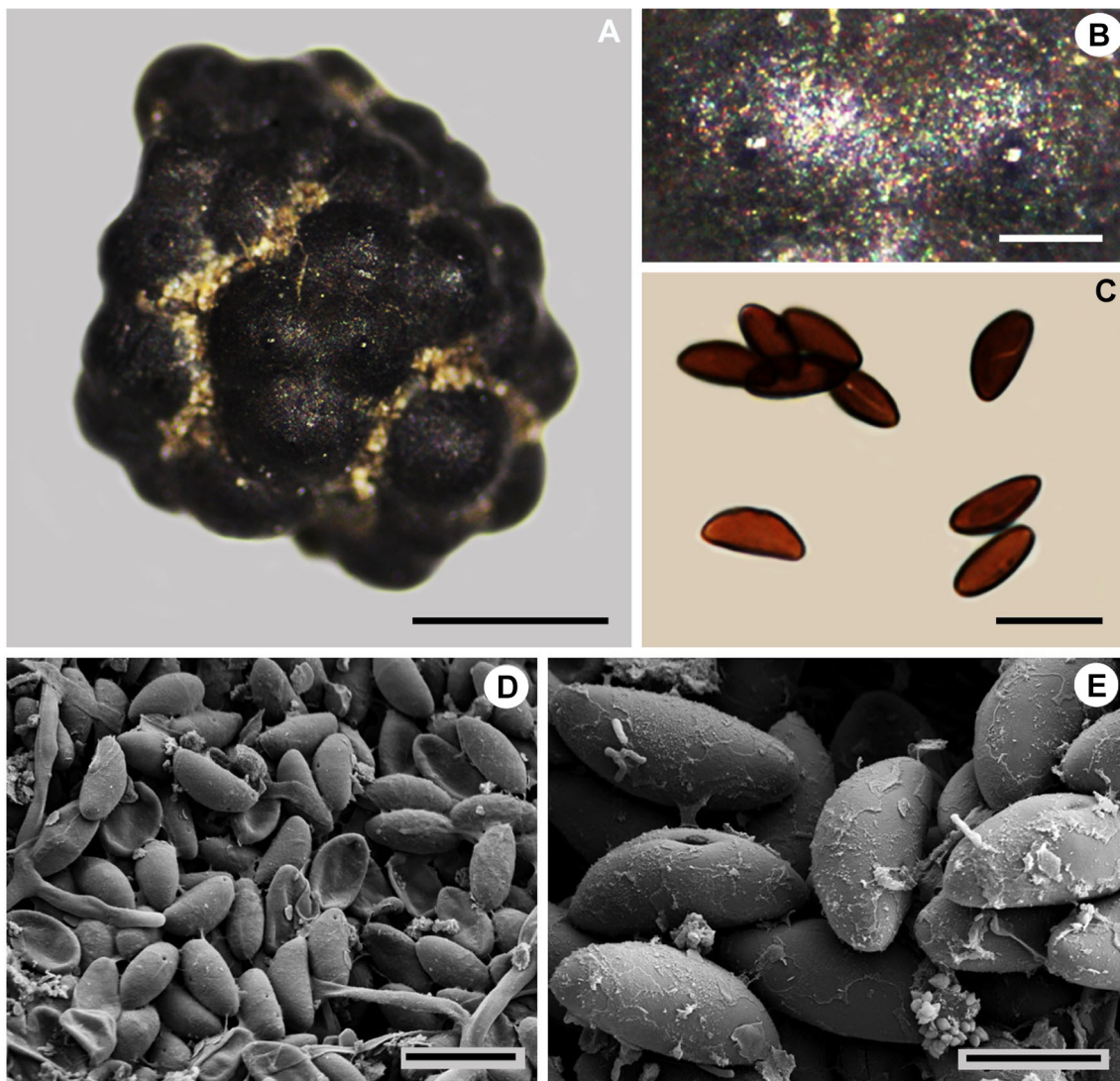
## Materials and methods

### General

NMR spectra were recorded with a Bruker Avance III 700 spectrometer with a 5 mm TCI cryoprobe ( $^1\text{H}$  700 MHz,  $^{13}\text{C}$  175 MHz) and a Bruker Avance III 500 ( $^1\text{H}$  500 MHz,  $^{13}\text{C}$  125 MHz) spectrometer (Bruker, Bremen, Germany). Chemical shifts  $\delta$  were referenced to the solvents chloroform-*d* ( $^1\text{H}$ ,  $\delta$  = 7.27 ppm;  $^{13}\text{C}$ ,  $\delta$  = 77.0 ppm), acetone-*d*<sub>6</sub> ( $^1\text{H}$ ,  $\delta$  = 2.05 ppm;  $^{13}\text{C}$ ,  $\delta$  = 29.9 ppm), pyridine-*d*<sub>5</sub> ( $^1\text{H}$ ,  $\delta$  = 7.22 ppm;  $^{13}\text{C}$ ,  $\delta$  = 123.9 ppm) and acetonitrile-*d*<sub>3</sub> ( $^1\text{H}$ ,  $\delta$  = 1.94 ppm;  $^{13}\text{C}$ ,  $\delta$  = 1.39 ppm). Optical rotations were determined using a 241 MC polarimeter (Perkin Elmer, Waltham, USA). UV/Vis spectra were measured with a Shimadzu UV–Vis spectrophotometer UV-2450. CD spectra were recorded with a Jasco J-815 CD spectrometer.

### Archaeological material

The archaeological fungi (Figs. 1, 2 and 3) were collected during an archaeopedological survey of the surroundings of a rural roman settlement known as the “villa de la Pépinière” in the Châtillon-sur-Seine forest massif (Provost 2009). An Airborne Laser Scanner (ALS) using Light Detection and Ranging technique (LiDAR) had indeed revealed a dense network of linear structures all around the archaeological site, which prompted fieldwork to study ancient the agrarian activities of the area, mainly through the analysis of soil characteristics and their biological remains (ecofacts). To do so, the soils of five archaeological contexts were sampled using regular grids of 25 m; 103 pedological pits were described and sampled (10 l per individualised horizon in each pit). Soil samples were sieved through a 4 mm and 2 mm column, and artefacts and ecofacts were recovered, among which a few carbonized fungal elements. The carbonized fungal elements have not been directly dated; however, charcoals and reddish burnt soil recovered in the same sample are dated by radiocarbon and thermoluminescence to the medieval period (between 738 and 1411 AD 2 sigma, 11 dates). As the material is not dated directly and comes from an unstratified context, the fungal fragments could potentially be misdated. We assume nevertheless that the fungi were burnt during the same fire event(s) that produced the charcoal and burnt soil aggregates, and are therefore medieval in date. The rationale for this hypothesis is that the hypoxylaceous fungi, and in particular, *Hypoxylon fragiforme*, are known to form stromata only on fresh woody substrates in which they even live as endophytes (Chapela et al. 1993; Helaly et al. 2018; Stadler 2011) and



**Fig. 1** *Hypoxylon fragiforme* (Specimen Fo4-2): **a** Stromatal habit. **b** Ostioles. **c** Ascospores by light microscopy. **d–e**. SEM images of Ascospores. Scale bars: **a** 1000  $\mu\text{m}$ , **b** 200  $\mu\text{m}$ , **c**, **d** 10  $\mu\text{m}$ , **e** 5  $\mu\text{m}$

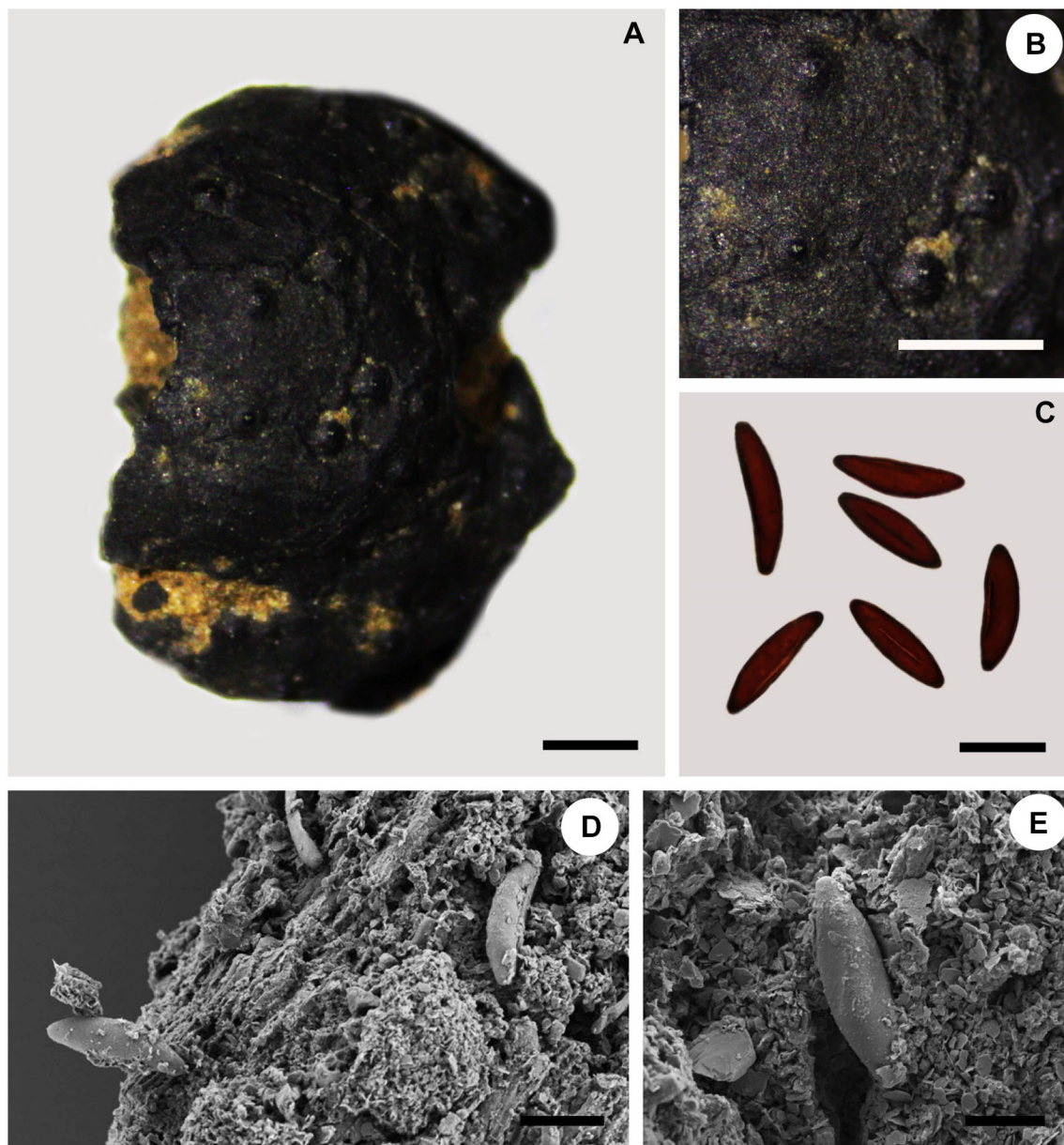
could not possibly have grown from the charcoal or the soil.

### Microscopy and scanning electron microscopy

The morphology of macroscopic and microscopic features of the stromata were determined as described by Stadler et al. (2014) and Fournier et al. (2010), also using the key to European species of *Hypoxylon* s. lat. provided in the latter paper. Photos were taken with a Canon EOS 650D camera through a brightfield microscope at 400–1000  $\times$  magnification or a stereo microscope, respectively. SEM of ascospores were recorded using a field-emission scanning electron microscope (FE-SEM Merlin, Zeiss, Germany), in a similar fashion as reported previously (Sir et al. 2016; Kuhnert et al. 2017).

### HPLC profiling

The stromatal fragments were examined under a dissecting microscope and pieces that obviously contained intact perithecia were singled out and examined using light microscopy. Selected samples that contained ascospores or fragments thereof were chosen for subsequent SEM investigations and also analysed for the presence of secondary metabolites. For this purpose, stromatal extracts were obtained by removing small amounts of the surface and perithecial layer, which were transferred to 1.5 ml reaction tubes. Subsequently, 50–100  $\mu\text{l}$  of acetonitrile were added and the material was extracted for 15 min at 40  $^{\circ}\text{C}$  in an ultrasonic bath. The tubes were then centrifuged and the supernatant was used for chemical analyses using high performance liquid chromatography coupled

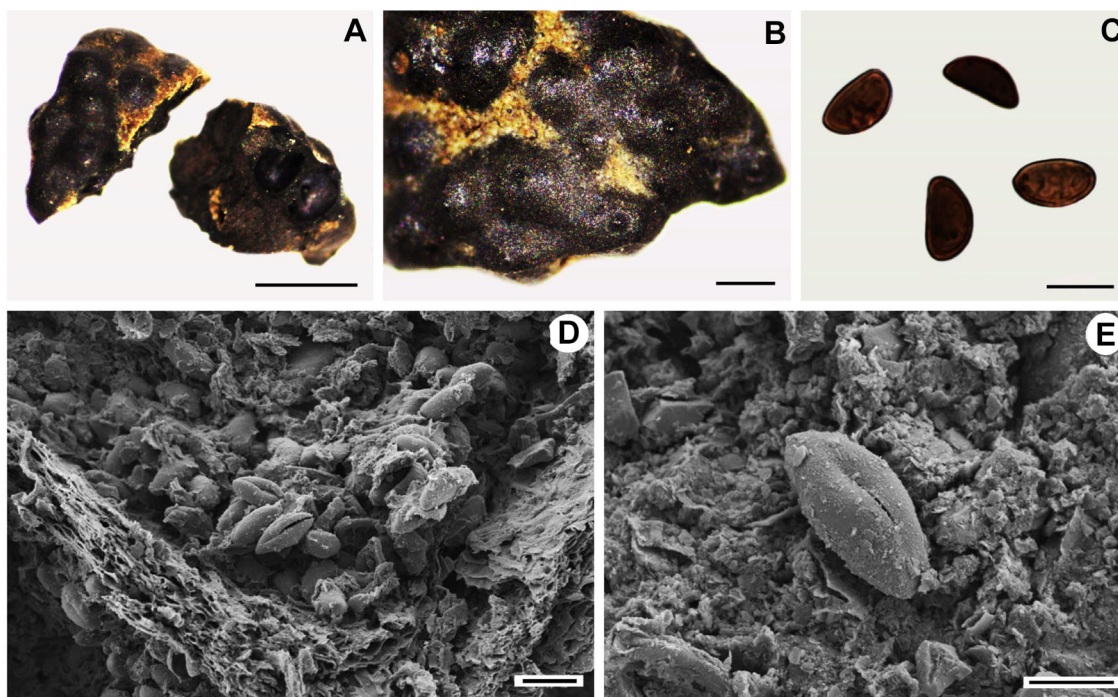


**Fig. 2** *Hypoxylon* cf. *vogesiacum* (Specimen Fo4-5): **a** Stromatal fragment. **b** Ostioles. **c** Ascospores by light microscopy. **d, e** SEM images of ascospores. Scale bars: **a** 1000  $\mu\text{m}$ , **b** 200  $\mu\text{m}$ , **c, d** 10  $\mu\text{m}$ , **e** 5  $\mu\text{m}$

with diode array and electrospray mass spectrometric detection (HPLC/DAD-ESIMS). The instrumental settings and conditions were as described in Kuhnert et al. (2014b). The mass spectra, UV-visible spectra and retention times were compared with an internal database comprising standards of known compounds from Xylariales (cf. Bitzer et al. 2007; Kuhnert et al. 2014a).

#### **Preparation of stromatal extracts for identification of the new pigments and preparative HPLC**

Circa 65 g of air-dried stromata of *Hypoxylon fragiforme* were collected by L. Wendt from *Fagus sylvatica* in the vicinity of Braunschweig, Germany. A voucher specimen, which showed the characteristics of the species, is deposited at the herbarium of M. Stadler (HZI, Braunschweig, Germany; Acc. No. STMA18022). The stromata were extracted with acetone for  $3 \times 30$  min in an ultrasonic bath and the extracts were combined and evaporated to dryness



**Fig. 3** *Jackrogersella cohaerens* (Specimen Fo2-1): **a** Stromata. **b** Ostioles. **c** Ascospores by light microscopy. **d, e** SEM images of ascospores. Scale bars: **a** 1000  $\mu\text{m}$ , **b** 200  $\mu\text{m}$ , **c, d** 10  $\mu\text{m}$ , **e** 5  $\mu\text{m}$

as described previously (Kuhnert et al. 2015). The crude extract (6 g) was subjected to normal phase Flash chromatography (GRACE Reveleris<sup>®</sup>X2 flash system), using a Reveleris<sup>®</sup> 40 g silica cartridge (particle size: 40  $\mu\text{m}$ ). The column was eluted with a ternary gradient mobile phase generated from three solvent mixtures [solvent A: dichloromethane (DCM), solvent B: DCM/acetone (9/1, v/v), solvent C: acetone] at a flow rate of 40 mL/min. After isocratic conditions (100% A) for 4 min, the first gradient (AB system) was operating from 0% B to 100% B in 25 min, followed by a second gradient (BC system) from 0% C to 100% C in 20 min. UV detection was conducted at 220, 254, and 280 nm. Fractions were combined according to the major UV-absorbing peaks to yield 13 main fractions (I–XIII), which were combined and concentrated in vacuo (40 °C) to dryness.

Main fractions obtained this way were further separated via RP-LC with deionized water (solvent A) and acetonitrile (B). A Gilson RP-HPLC system (Middleton, Wisconsin, USA) equipped with a GX-271 Liquid handler, a diode array detector (DAD) 172 and a 305 and 306 Pump was utilised to process fractions I, II, and V with a VP Nucleodur C18ec (150  $\times$  40 mm, 7  $\mu\text{m}$ ; Macherey-Nagel, Düren, Germany) column. The process was performed with a flow rate of 40 ml/min. Prior to the preparative LC experiments, the crude extracts were dissolved in acetonitrile and filtered through a Strata X-33  $\mu\text{m}$  Polymere Reversed Phase Tube (Phenomenex, Aschaffenburg,

Germany) to remove fatty acids and debris. The LC fractions were collected according to the UV absorption at 210 nm. Acetonitrile was evaporated in vacuo, and the aqueous residues were frozen. An Alpha 1–4 LSC freeze dryer (Christ, Osterode, Germany) was used to remove the remaining water.

Fraction V was separated on a gradient from 45 to 80% solvent B in 40 min with an increase to 100% B in 5 min, followed by isocratic conditions at 100% B for 15 min to yield 17.2 mg of **1** and 3.89 mg of **8**. These were eluted as pure compounds after 27.5 min and 51.5 min, respectively.

Fraction I yielded **3–5** as pure compounds on a gradient starting with isocratic conditions for 5 min at 60% B, followed by a gradient from 60 to 100% B in 45 min, isocratic conditions for 5 min, and a decrease of B to 60% in 2 min. **3** was gained as 37.47 mg with a RT of 48 min, **4** yielded 1.58 mg and eluted after 56 min, while **5** yielded 9.11 mg with a RT of 43 min.

Compounds **6** (1.90 mg) and **7** (3.42 mg) were isolated from fraction II on a gradient from 60 to 100% B in 45 min at RTs of 18.5 min and 19 min, respectively.

Fraction VI, however, was separated via RP-MPLC (column 480  $\times$  30 mm, ODS/AQ C18 [Kronlab, Sinsheim, Germany]) with a flow rate of 30 mL/min to yield 15.4 mg of **2**. It eluted after 56 min with conditions starting isocratically at 10% B for 5 min, followed by a gradient to 100% B in 60 min, and isocratic conditions at 100% B for 30 min.

Rutilin C (**1**): Red oil;  $[\alpha]_D^{25} = +1062^\circ$  ( $c = 0.1$  CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN)  $\lambda_{\max}$  (log  $\epsilon$ ): 215 nm (4.61), 266 (4.50), 308 (4.34), 371 (4.60), 442 (4.33) nm; CD (CH<sub>3</sub>CN)  $\lambda$  ( $\Delta\epsilon$ ): 214 (+ 7.9), 260 (− 3.5), 312 (+7.9), 365 (− 3.9), 450 (+ 1.3) nm; <sup>1</sup>H NMR (700 MHz, acetone-*d*<sub>6</sub>):  $\delta_H$  10.78 (s, 2'-b-OH), 10.72 (s, 2'-a-OH), 9.27 (s, 4'-b-OH), 9.24 (s, 4'-a-OH), 8.21 (s, 1a-H), 8.13 (dd,  $J = 15.1$  Hz, 11.6 Hz, 13a-H), 7.64 (d,  $J = 11.6$  Hz, 14a-H), 7.04 (d,  $J = 15.1$  Hz, 12a-H), 6.90 (s, 4a-H), 6.57 (dq,  $J = 15.5$  Hz, 7.0 Hz, 13b-H), 6.38 (br s, 5'-b-H), 6.35 (br s, 5'-a-H), 6.24 (m, 3'-b-H), 6.24 (s, 4b-H), 6.23 (m, 3'-a-H), 6.17 (dq,  $J = 15.5$  Hz, 1.0 Hz, 12b-H), 5.74 (s, 6a-H), 5.11 (d,  $J = 13.5$  Hz, 1b-H<sub>a</sub>), 4.97 (d,  $J = 13.5$  Hz, 1b-H<sub>b</sub>), 2.64 (s, 7'-b-H<sub>3</sub>), 2.60 (s, 7'-a-H<sub>3</sub>), 1.90 (dd,  $J = 7.0$  Hz, 1.0 Hz, 14b-H), 1.73 (s, 11b-H<sub>3</sub>), 1.66 (s, 11a-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, acetone-*d*<sub>6</sub>):  $\delta_C$  194.1 (C, C-7b), 192.8 (C, C-9a), 192.3 (C, C-7a), 188.9 (C, C-9b), 170.7 (C, C-8'a), 170.5 (C, C-8'b), 166.5 (C, C-2'b), 166.2 (C, C-2'a), 164.1 (C, C-4'b), 164.0 (C, C-4'a), 162.5 (C, C-3b), 155.4 (C, C-3a), 155.3 (CH, C-1a), 144.94 (C, C-6'b), 144.89 (C, C-6'a), 144.5 (C, C-5b), 143.0 (C, C-5a), 140.8 (CH, C-14a), 135.9 (CH, C-13b), 135.6 (CH, C-12a), 131.5 (CH, C-13a), 129.2 (C, C-6b), 125.9 (CH, C-12b), 116.1 (C, C-10b), 115.9 (CH, C-4a), 115.6 (C, C-10a), 112.8 (CH, C-5'b), 112.7 (CH, C-5'a), 110.0 (CH, C-6a), 105.0 (C, C-1'a), 104.8 (C, C-1'b), 101.81 (CH, C-3'b), 101.76 (CH, C-3'a), 98.5 (CH, C-4b), 86.82 (C, C-8b), 86.75 (C, C-8a), 64.8 (CH<sub>2</sub>, C-1b), 24.3 (CH<sub>3</sub>, C-7'b), 24.1 (CH<sub>3</sub>, C-7'a), 22.73 (CH<sub>3</sub>, C-11b), 22.69 (CH<sub>3</sub>, C-11a), 18.9 (CH<sub>3</sub>, C-14b); ESIMS  $m/z$  763.22 [M + H]<sup>+</sup>, 761.22 [M - H]<sup>-</sup>; HRESIMS  $m/z$  763.2014 [M + H]<sup>+</sup> (calcd. for C<sub>42</sub>H<sub>35</sub>O<sub>14</sub>, 763.2012); R<sub>t</sub> = 13.5 min.

Rutilin D (**2**): Yellow oil;  $[\alpha]_D^{25} = +6^\circ$  ( $c = 0.1$  CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN)  $\lambda_{\max}$  (log  $\epsilon$ ): 215 (3.66), 266 (3.45), 310 (3.29), 366 (3.40), 441 (3.13) nm; CD (CH<sub>3</sub>CN)  $\lambda$  ( $\Delta\epsilon$ ): 262 (− 3.7), 319 (+ 11.2), 366 (− 0.4), 416 (+ 2.9) nm; <sup>1</sup>H NMR (700 MHz, CH<sub>3</sub>CN-*d*<sub>3</sub>):  $\delta_H$  10.59 (s, 2'-b-OH), 10.54 (s, 2'-a-OH), 8.04 (s, 1a-H), 8.04 (m, 13a-H), 7.42 (d,  $J = 11.8$  Hz, 14a-H), 6.88 (d,  $J = 15.1$  Hz, 12a-H), 6.74 (s, 4a-H), 6.51 (dd,  $J = 15.6$  Hz, 5.5 Hz, 13b-H), 6.35 (m, 12b-H), 6.32 (m, 5'-a-H, 5'-b-H), 6.23 (d,  $J = 2.6$  Hz, 3'-b-H), 6.21 (d,  $J = 2.6$  Hz, 3'-a-H), 6.19 (s, 4b-H), 5.70 (d,  $J = 0.9$  Hz, 6a-H), 5.11 (br d,  $J = 14.0$  Hz, 1b-H<sub>a</sub>), 4.99 (br d,  $J = 14.0$  Hz, 1b-H<sub>b</sub>), 4.74 (br d,  $J = 5.5$  Hz, 14b-H<sub>2</sub>), 2.60 (s, 7'-b-H<sub>3</sub>), 2.57 (s, 7'-a-H<sub>3</sub>), 2.09 (s, 16b-H<sub>3</sub>), 1.68 (s, 11b-H<sub>3</sub>), 1.64 (s, 11a-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CH<sub>3</sub>CN-*d*<sub>3</sub>):  $\delta_C$  194.4 (C, C-7b), 193.3 (C, C-9a), 192.9 (C, C-7a), 189.4 (C, C-9b), 171.4 (C, C-15b), 174.6 (C, C-8'a, C-8'b), 166.2 (C, C-2'b), 165.9 (C, C-2'a), 163.8 (C, C-4'a), 163.7 (C, C-4'b), 161.4 (C, C-3b), 155.6 (CH, C-1a), 155.5 (C, C-3a), 145.31 (C, C-6'b), 145.25 (C, C-6'a), 144.3 (C, C-5b), 143.7 (C, C-5a), 141.2 (CH, C-14a),

135.6 (CH, C-12a), 133.2 (CH, C-13b), 131.4 (CH, C-13a), 129.1 (C, C-6b), 125.6 (CH, C-12b), 116.9 (C, C-10b), 116.1 (CH, C-4a), 115.6 (C, C-10a), 112.84 (CH, C-5'b), 112.75 (CH, C-5'a), 109.7 (CH, C-6a), 105.2 (C, C-1'a), 105.0 (C, C-1'b), 101.83 (CH, C-3'a), 101.80 (CH, C-3'b), 100.7 (CH, C-4b), 87.2 (C, C-8a, C-8b), 65.0 (CH<sub>2</sub>, C-1b), 64.2 (CH<sub>2</sub>, C-14b), 24.2 (CH<sub>3</sub>, C-7'b), 24.0 (CH<sub>3</sub>, C-7'a), 22.9 (CH<sub>3</sub>, C-11a), 22.8 (CH<sub>3</sub>, C-11b), 21.1 (CH<sub>3</sub>, C-16b); ESIMS  $m/z$  763.22 [M + H]<sup>+</sup>, 761.22 [M - H]<sup>-</sup>; HRESIMS  $m/z$  821.2063 [M + H]<sup>+</sup> (calcd. for C<sub>44</sub>H<sub>37</sub>O<sub>16</sub>, 821.2076); R<sub>t</sub> = 12.7 min.

Fragirubrin A (**3**): Yellow oil;  $[\alpha]_D^{25} = +447^\circ$  ( $c = 0.1$  CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN)  $\lambda_{\max}$  (log  $\epsilon$ ): 218 (4.27), 326 (4.26) nm; CD (CH<sub>3</sub>CN)  $\lambda$  ( $\Delta\epsilon$ ): 199 (− 7.1), 231 (+ 1.8), 248 (− 1.3), 273 (+ 4.8), 322 (− 4.9) nm; <sup>1</sup>H NMR (700 MHz, CHCl<sub>3</sub>-*d*):  $\delta_H$  7.85 (d,  $J = 0.9$  Hz, 1-H), 6.14 (s, 4-H), 5.54 (d,  $J = 0.9$  Hz, 5-H), 5.17 (dq,  $J = 7.4$ , 6.5, 5.3 Hz, 11-H), 2.69 (dd,  $J = 15.0$ , 7.4 Hz, 10-H<sub>a</sub>), 2.61 (dd,  $J = 15.0$ , 5.3 Hz, 10-H<sub>b</sub>), 2.45 (t,  $J = 7.5$  Hz, 16-H<sub>2</sub>), 1.63 (m, 17-H<sub>2</sub>), 2.45 (t,  $J = 7.5$  Hz, 16-H<sub>2</sub>), 2.06 (s, 14-H<sub>3</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.33 (d,  $J = 6.5$  Hz, 12-H<sub>3</sub>), 1.33 (m, 18-H<sub>2</sub>), 1.24–1.31 (m, 19-H<sub>2</sub>–29-H<sub>2</sub>), 0.89 (t,  $J = 7.1$  Hz, 30-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CHCl<sub>3</sub>-*d*):  $\delta_C$  193.2 (C, C-8), 192.8 (C, C-6), 173.1 (C, C-15), 170.3 (C, C-13), 157.8 (C, C-3), 153.7 (CH, C-1), 141.9 (C, C-4a), 115.2 (C, C-8a), 110.8 (CH, C-4), 107.5 (CH, C-5), 84.1 (C, C-7), 67.5 (CH, C-11), 39.4 (CH<sub>2</sub>, C-10), 33.2 (CH<sub>2</sub>, C-16), 31.9 (CH<sub>2</sub>, C-28), 29.68, 29.67, 29.66, 29.64, 29.63, 29.61, 29.41, 29.35, 29.26 (9 × CH<sub>2</sub>, C-19–C-27), 28.9 (CH<sub>2</sub>, C-18), 24.6 (CH<sub>2</sub>, C-17), 22.7 (CH<sub>2</sub>, C-29), 22.1 (CH<sub>3</sub>, C-9), 21.2 (CH<sub>2</sub>, C-14), 19.8 (CH<sub>3</sub>, C-12), 14.1 (CH<sub>3</sub>, C-30); ESIMS  $m/z$  531.32 [M + H]<sup>+</sup>, 529.27 [M - H]<sup>-</sup>; HRESIMS  $m/z$  531.3316 [M + H]<sup>+</sup> (calcd. for C<sub>31</sub>H<sub>47</sub>O<sub>7</sub>, 531.3316); R<sub>t</sub> = 18.8 min.

Fragirubrin B (**4**): Yellow oil;  $[\alpha]_D^{25} = -86^\circ$  ( $c = 0.1$  CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN)  $\lambda_{\max}$  (log  $\epsilon$ ): 219 (4.16), 327 nm (4.16); CD (CH<sub>3</sub>CN)  $\lambda$  ( $\Delta\epsilon$ ): 199 (− 1.8), 232 (+ 0.7), 249 (− 0.4), 274 (+ 1.6), 323 (− 1.4) nm; <sup>1</sup>H NMR (700 MHz, CHCl<sub>3</sub>-*d*):  $\delta_H$  7.85 (br s, 1-H), 6.14 (s, 4-H), 5.54 (br s, 5-H), 5.17 (dq,  $J = 7.4$ , 6.5, 5.3 Hz, 11-H), 2.69 (dd,  $J = 15.0$ , 7.4 Hz, 10-H<sub>a</sub>), 2.61 (dd,  $J = 15.0$ , 5.3 Hz, 10-H<sub>b</sub>), 2.45 (t,  $J = 7.5$  Hz, 16-H<sub>2</sub>), 2.06 (s, 14-H<sub>3</sub>), 1.63 (m, 17-H<sub>2</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.33 (d,  $J = 6.5$  Hz, 12-H<sub>3</sub>), 1.33 (m, 18-H<sub>2</sub>), 1.24–1.31 (m, 19-H<sub>2</sub>–31-H<sub>2</sub>), 0.89 (t,  $J = 7.0$  Hz, 32-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CHCl<sub>3</sub>-*d*):  $\delta_C$  193.2 (C, C-8), 192.8 (C, C-6), 173.1 (C, C-15), 170.2 (C, C-13), 157.8 (C, C-3), 153.7 (CH, C-1), 141.9 (C, C-4a), 115.2 (C, C-8a), 110.8 (CH, C-4), 107.5 (CH, C-5), 84.1 (C, C-7), 67.5 (CH, C-11), 39.4 (CH<sub>2</sub>, C-10), 33.2 (CH<sub>2</sub>, C-16), 31.9 (CH<sub>2</sub>, C-30), 29.68, 29.67, 29.66, 29.64, 29.63, 29.61, 29.42, 29.35, 29.26 (11 × CH<sub>2</sub>, C-19–C-29), 28.9 (CH<sub>2</sub>, C-18), 24.6 (CH<sub>2</sub>, C-17), 22.7 (CH<sub>2</sub>, C-

31), 22.1 (CH<sub>3</sub>, C-9), 21.2 (CH<sub>2</sub>, C-14), 19.8 (CH<sub>3</sub>, C-12), 14.1 (CH<sub>3</sub>, C-32); ESIMS *m/z* 559.42 [M + H]<sup>+</sup>, 557.32 [M - H]<sup>-</sup>; HRESIMS *m/z* 559.3635 [M + H]<sup>+</sup> (calcd. for C<sub>33</sub>H<sub>51</sub>O<sub>7</sub>, 559.3629); R<sub>t</sub> = 19.8 min.

**Fragirubrin C (5):** Yellow oil; [α]<sub>D</sub><sup>25</sup> = + 1206° (*c* = 1.0 CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN) λ<sub>max</sub> (log ε): 219 (4.34), 327 nm (4.34); CD (CH<sub>3</sub>CN) λ (Δε): 201 (- 6.2), 232 (+ 1.6), 249 (- 1.1), 272 (+ 4.6), 322 (- 4.6) nm; <sup>1</sup>H NMR (700 MHz, CHCl<sub>3</sub>-*d*<sub>6</sub>): δ<sub>H</sub> 7.85 (br s, 1-H), 6.14 (s, 4-H), 5.54 (br s, 5-H), 5.30-5.41 (m, 23-H, 24-H, 26-H, 27-H), 5.17 (dq, *J* = 7.4, 6.5, 5.3 Hz, 11-H), 2.77 (t, *J* = 7.0 Hz, 25-H<sub>2</sub>), 2.69 (dd, *J* = 15.0, 7.4 Hz, 10-H<sub>a</sub>), 2.61 (dd, *J* = 15.0, 5.3 Hz, 10-H<sub>b</sub>), 2.44 (t, *J* = 7.5 Hz, 16-H<sub>2</sub>), 2.05 (s, 14-H<sub>3</sub>), 2.07-2.02 (s, 22-H<sub>2</sub>, 28-H<sub>2</sub>), 1.63 (m, 17-H<sub>2</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.32 (d, *J* = 6.2 Hz, 12-H<sub>3</sub>), 1.24-1.38 (m, 18-H<sub>2</sub>-21-H<sub>2</sub>, 28-H<sub>2</sub>-31-H<sub>2</sub>), 0.89 (t, *J* = 6.8 Hz, 32-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CHCl<sub>3</sub>-*d*): δ<sub>C</sub> 193.2 (C, C-8), 192.8 (C, C-6), 173.1 (C, C-15), 170.2 (C, C-13), 157.8 (C, C-3), 153.7 (CH, C-1), 141.9 (C, C-4a), 130.2 (CH, CH-23)\*<sup>1</sup>, 130.1 (CH, C-27)\*<sup>1</sup>, 127.94 (C, CH-24)\*<sup>2</sup>, 127.92 (CH, C-26)\*<sup>2</sup>, 115.2 (C, C-8a), 110.8 (CH, C-4), 107.5 (CH, C-5), 84.1 (C, C-7), 67.5 (CH, C-11), 39.4 (CH<sub>2</sub>, C-10), 33.2 (CH<sub>2</sub>, C-16), 31.49 (CH<sub>2</sub>, C-28)\*<sup>3</sup>, 31.48 (CH<sub>2</sub>, C-30)\*<sup>3</sup>, 29.6, 29.3, 29.14, 29.06, 28.9 (5 × CH<sub>2</sub>, C-18-C-21, C-29), 27.18 (CH<sub>2</sub>, C-22)\*<sup>4</sup>, 27.16 (CH<sub>2</sub>, C-28)\*<sup>4</sup>, 25.6 (CH<sub>2</sub>, C-25), 24.6 (CH<sub>2</sub>, C-17), 22.5 (CH<sub>3</sub>, C-31), 22.1 (CH<sub>3</sub>, C-9), 21.2 (CH<sub>3</sub>, C-14), 19.8 (CH<sub>3</sub>, C-12), 14.1 (CH<sub>3</sub>, C-31), \*<sup>1</sup>, \*<sup>2</sup>, \*<sup>3</sup>, \*<sup>4</sup> assignments might be interchanged; ESIMS *m/z* 555.39 [M + H]<sup>+</sup>; HRESIMS *m/z* 555.3322 [M + H]<sup>+</sup> (calcd. for C<sub>33</sub>H<sub>47</sub>O<sub>7</sub>, 555.3316); R<sub>t</sub> = 18.1 min.

**Fragirubrin D (6):** Yellow oil; [α]<sub>D</sub><sup>25</sup> = + 56° (*c* = 1.0 CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN) λ<sub>max</sub> (log ε): 221 (4.11), 327 (4.08) nm; CD (CH<sub>3</sub>CN) λ (Δε): 202 (- 2.3), 230 (+ 1.1), 248 (- 0.3), 273 (+ 2.0), 323 (- 1.9) nm; <sup>1</sup>H NMR (700 MHz, CHCl<sub>3</sub>-*d*): δ<sub>H</sub> 7.86 (d, *J* = 0.9 Hz, 1-H), 6.14 (s, 4-H), 5.54 (d, *J* = 0.9 Hz, 5-H), 5.30-5.41 (m, 23-H, 24-H, 26-H, 27-H), 5.17 (dq, *J* = 7.4, 6.5, 5.3 Hz, 11-H), 3.81 (m, 29-H), 2.78 (t, *J* = 6.2 Hz, 25-H<sub>2</sub>), 2.69 (dd, *J* = 14.8, 7.5 Hz, 10-H<sub>a</sub>), 2.61 (dd, *J* = 14.8, 5.4 Hz, 10-H<sub>b</sub>), 2.45 (t, *J* = 7.4 Hz, 16-H<sub>2</sub>), 2.09 (m, 28-H<sub>2</sub>), 2.06 (s, 14-H<sub>3</sub>), 2.03 (s, 22-H<sub>2</sub>), 1.63 (m, 17-H<sub>2</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.48 (m, 29-H<sub>a</sub>, 30-H<sub>2</sub>), 1.41 (m, 29-H<sub>b</sub>), 1.33 (d, *J* = 6.2 Hz, 12-H<sub>3</sub>), 1.24 - 1.38 (m, 18-H<sub>2</sub> - 21-H<sub>2</sub>), 1.20 (d, *J* = 6.5 Hz, 32-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CHCl<sub>3</sub>-*d*): δ<sub>C</sub> 193.2 (C, C-8), 192.9 (C, C-6), 173.1 (C, C-15), 170.3 (C, C-13), 157.8 (C, C-3), 153.7 (CH, C-1), 142.0 (C, C-4a), 130.2 (CH, CH-23)\*<sup>1</sup>, 129.6 (CH, C-27)\*<sup>1</sup>, 128.4 (C, CH-24)\*<sup>2</sup>, 127.8 (CH, C-26)\*<sup>2</sup>, 115.2 (C, C-8a), 110.8 (CH, C-4), 107.5 (CH, C-5), 84.1 (C, C-7), 68.5 (CH, C-31), 67.5 (CH, C-11), 39.4 (CH<sub>2</sub>, C-10), 38.9 (CH<sub>2</sub>, C-30), 33.2 (CH<sub>2</sub>, C-16), 29.6, 29.3, 29.14, 29.06, 28.9 (5 × CH<sub>2</sub>, C-

18-C-21, C-29), 27.18 (CH<sub>2</sub>, C-22)\*<sup>3</sup>, 27.12 (CH<sub>2</sub>, C-28)\*<sup>3</sup>, 25.8 (CH<sub>2</sub>, C-29), 25.6 (CH<sub>2</sub>, C-25), 24.6 (CH<sub>2</sub>, C-17), 23.5 (CH<sub>3</sub>, C-32), 22.1 (CH<sub>3</sub>, C-9), 21.2 (CH<sub>3</sub>, C-14), 19.8 (CH<sub>3</sub>, C-12), 14.1 (CH<sub>3</sub>, C-32), \*<sup>1</sup>, \*<sup>2</sup>, \*<sup>3</sup> assignments might be interchanged; ESIMS *m/z* 571.32 [M + H]<sup>+</sup>, 569.30 [M - H]<sup>-</sup>; HRESIMS *m/z* 571.3264 [M + H]<sup>+</sup> (calcd. for C<sub>33</sub>H<sub>47</sub>O<sub>8</sub>, 571.3265); R<sub>t</sub> = 14.4 min.

**Fragirubrin E (7):** Yellow oil; [α]<sub>D</sub><sup>25</sup> = + 124° (*c* = 1.0 CH<sub>3</sub>CN); UV (CH<sub>3</sub>CN) λ<sub>max</sub> (log ε): 218 (4.08), 326 nm (4.00); CD (CH<sub>3</sub>CN) λ (Δε): 198 (- 4.9), 231 (+ 1.6), 249 (- 1.1), 273 (+ 3.9), 323 (- 4.1) nm; <sup>1</sup>H NMR (700 MHz, CHCl<sub>3</sub>-*d*): δ<sub>H</sub> 7.85 (br s, 1-H), 6.14 (s, 4-H), 5.54 (br s, 5-H), 5.36-5.43 (m, 23-H, 26-H, 27-H), 5.33 (m, 24-H), 5.17 (dq, *J* = 7.4, 6.5, 5.3 Hz, 11-H), 3.56 (m, 30-H), 2.80 (t, *J* = 6.7 Hz, 25-H<sub>2</sub>), 2.69 (dd, *J* = 14.8, 7.5 Hz, 10-H<sub>a</sub>), 2.61 (dd, *J* = 14.8, 5.4 Hz, 10-H<sub>b</sub>), 2.45 (t, *J* = 7.5 Hz, 16-H<sub>2</sub>), 2.21 (m, 28-H<sub>a</sub>), 2.17 (m, 28-H<sub>b</sub>), 2.06 (s, 14-H<sub>3</sub>), 2.04 (s, 22-H<sub>2</sub>), 1.63 (m, 17-H<sub>2</sub>), 1.55 (m, 29-H<sub>a</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.52 (m, 31-H<sub>a</sub>), 1.49 (m, 29-H<sub>b</sub>), 1.46 (m, 31-H<sub>b</sub>), 1.41 (m, 29-H<sub>b</sub>), 1.33 (d, *J* = 6.2 Hz, 12-H<sub>3</sub>), 1.25-1.38 (m, 18-H<sub>2</sub>-21-H<sub>2</sub>), 0.95 (t, *J* = 7.4 Hz, 32-H<sub>3</sub>); <sup>13</sup>C NMR (175 MHz, CHCl<sub>3</sub>-*d*): δ<sub>C</sub> 193.2 (C, C-8), 192.8 (C, C-6), 173.1 (C, C-15), 170.3 (C, C-13), 157.8 (C, C-3), 153.7 (CH, C-1), 142.0 (C, C-4a), 130.3 (CH, CH-23), 129.5 (CH, C-27), 128.6 (C, CH-26), 127.7 (CH, C-24), 115.2 (C, C-8a), 110.8 (CH, C-4), 107.5 (CH, C-5), 84.1 (C, C-7), 72.9 (CH, C-30), 67.5 (CH, C-11), 39.4 (CH<sub>2</sub>, C-10), 36.7 (CH<sub>2</sub>, C-29), 33.2 (CH<sub>2</sub>, C-16), 30.2 (CH<sub>2</sub>, C-31), 29.5 (CH<sub>2</sub>, C-21), 29.1, 29.0 (2 × CH<sub>2</sub>, C-19, C-20), 28.9 (CH<sub>2</sub>, C-18), 27.2 (CH<sub>2</sub>, C-22), 25.6 (CH<sub>2</sub>, C-25), 24.6 (CH<sub>2</sub>, C-17), 23.6 (CH<sub>3</sub>, C-28), 22.1 (CH<sub>3</sub>, C-9), 21.2 (CH<sub>3</sub>, C-14), 19.8 (CH<sub>3</sub>, C-12), 9.9 (CH<sub>3</sub>, C-32); ESIMS *m/z* 571.32 [M + H]<sup>+</sup>, 569.30 [M - H]<sup>-</sup>; HRESIMS *m/z* 571.3260 [M + H]<sup>+</sup> (calcd. for C<sub>33</sub>H<sub>47</sub>O<sub>8</sub>, 571.3265); R<sub>t</sub> = 14.5 min.

**Lenormandin F (8):** Yellow oil; UV (CH<sub>3</sub>CN) λ<sub>max</sub> (log ε): 222, 328 nm; CD (CH<sub>3</sub>CN) λ (Δε): 199 (- 6.5), 234 (+ 2.8), 250 (- 1.0), 273 (+ 4.8), 323 (- 4.1) nm; <sup>1</sup>H NMR (500 MHz, CHCl<sub>3</sub>-*d*): δ<sub>H</sub> 7.88 (br s, 1-H), 6.20 (s, 4-H), 5.55 (br s, 5-H), 4.17 (dq, *J* = 7.4, 6.5, 5.3 Hz, 11-H), 2.54 (m, 10-H<sub>2</sub>), 2.44 (t, *J* = 7.5 Hz, 14-H<sub>2</sub>), 1.63 (m, 15-H<sub>2</sub>), 1.53 (s, 9-H<sub>3</sub>), 1.32 (d, *J* = 6.5 Hz, 12-H<sub>3</sub>), 1.23-1.35 (m, 16-H<sub>2</sub>-27-H<sub>2</sub>), 0.89 (t, *J* = 6.9 Hz, 28-H<sub>3</sub>); <sup>13</sup>C NMR (125 MHz, CHCl<sub>3</sub>-*d*): δ<sub>C</sub> 193.4 (C, C-8), 192.9 (C, C-6), 173.1 (C, C-13), 159.0 (C, C-3), 153.8 (CH, C-1), 142.2 (C, C-4a), 115.2 (C, C-8a), 110.6 (CH, C-4), 107.3 (CH, C-5), 84.0 (C, C-7), 65.4 (CH, C-11), 42.5 (CH<sub>2</sub>, C-10), 33.2 (CH<sub>2</sub>, C-14), 31.9 (CH<sub>2</sub>, C-26), 29.68, 29.67, 29.66, 29.64, 29.63, 29.61, 29.41, 29.35, 29.26 (9 × CH<sub>2</sub>, C-17-C-25), 28.9 (CH<sub>2</sub>, C-16), 24.6 (CH<sub>2</sub>, C-15), 23.6 (CH<sub>3</sub>, C-12), 22.7 (CH<sub>2</sub>, C-27), 22.1 (CH<sub>3</sub>, C-9), 14.1 (CH<sub>3</sub>, C-28); ESIMS *m/z* 489.32 [M + H]<sup>+</sup>, 487.32 [M - H]<sup>-</sup>;



HRESIMS  $m/z$  489.3217  $[M + H]^+$  (calcd. for  $C_{29}H_{45}O_6$ , 489.3211);  $R_t = 17.8$  min; data are in good agreement with the literature (Kuhnert et al. 2014c).

## Mosher's analyses

For the preparation of the (*S*)-MTPA ester 0.2 mg of **8** was dissolved in 600  $\mu$ l of pyridine- $d_5$ , and 10  $\mu$ l of (*R*)-MTPA chloride was added. The mixture was incubated at 25 °C for 15 min before measurement of  $^1H$ , COSY, TOCSY and HSQC NMR spectra:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ):  $\delta_H$  8.22 (1-H), 6.32 (4-H), 5.76 (5-H), 5.55 (11-H), 2.79 (10- $H_2$ ), 1.73 (9- $H_3$ ), 1.33 (7- $H_3$ ). The (*R*)-MTPA ester was prepared in the same manner by the addition of 10  $\mu$ L of (*S*)-MTPA chloride:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ):  $\delta_H$  8.13 (1-H), 6.04 (4-H), 5.63 (5-H), 5.56 (11-H), 2.72 (10- $H_2$ ), 1.72 (9- $H_3$ ), 1.38 (12- $H_3$ ).

Fragirubrin D ( $2 \times 0.2$  mg, **6**) was converted analogously. (*S*)-MTPA ester of **6**:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ): similar to **6**, but  $\delta_H$  5.29 (31-H), 1.63 (30- $H_a$ ), 1.53 (30- $H_b$ ), 1.31 (32- $H_3$ ); (*R*)-MTPA ester of **6**:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ): similar to **6**, but  $\delta_H$  5.28 (31-H), 1.70 (30- $H_a$ ), 1.58 (30- $H_b$ ), 1.24 (32- $H_3$ ).

Fragirubrin E ( $2 \times 0.2$  mg, **7**) was converted analogously. (*S*)-MTPA ester of **7**:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ): similar to **7**, but  $\delta_H$  5.22 (30-H), 2.13 (28- $H_a$ ), 2.05 (28- $H_b$ ), 1.71 (29- $H_a$ ), 1.65 (29- $H_b$ ), 1.68 (31- $H_2$ ), 0.90 (32- $H_3$ ); (*R*)-MTPA ester of **7**:  $^1H$  NMR (700 MHz, pyridine- $d_5$ ): similar to **7**, but  $\delta_H$  5.23 (30-H), 2.26 (28- $H_a$ ), 2.22 (28- $H_b$ ), 1.81 (29- $H_a$ ), 1.70 (29- $H_b$ ), 1.61 (31- $H_2$ ), 0.79 (32- $H_3$ ).

## Results

Over 100 fragments that were reminiscent of xylarialean stromata were studied microscopically, but most of those did not contain any material that would allow for their concise identification. Seven samples that contained more or less intact ascospores that allowed for determination of their dimensions and shape were submitted concurrently to high performance chromatography coupled with diode array and mass spectrometric detection (HPLC–DAD/MS) and also studied using scanning electron microscopy.

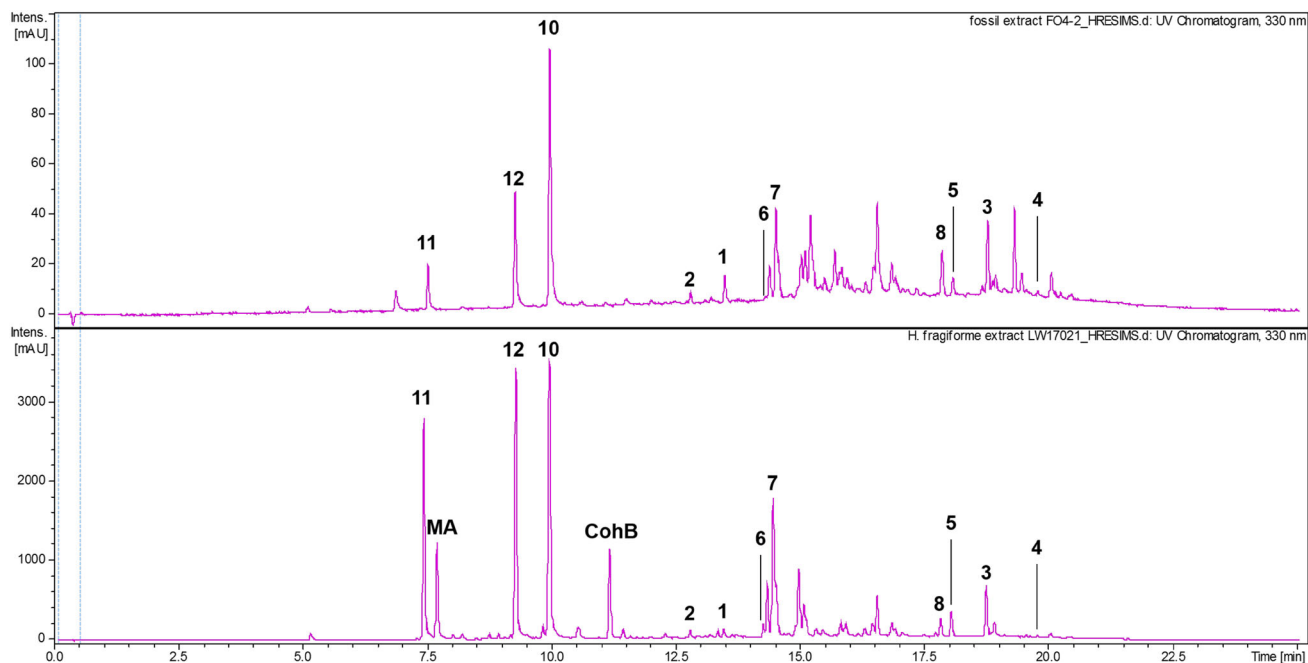
Four of these samples were identified as *Hypoxylon fragiforme*, two were assigned to *Jackrogersella cohaerens* and one was identified tentatively as *Hypoxylon cf. vogesiacum*. The *Jackrogersella cohaerens* specimens were identified by their characteristic stromatal and ascospore morphology and also contained traces of the characteristic pigments, cohaerins A and B (Quang et al. 2005b). Other cohaerins, such as the compounds described by Quang et al. (2006) and Surup et al. (2013) were not detected.

The specimen of *H. cf. vogesiacum* was determined by morphology only, since the HPLC profiles were inconclusive. However, the rather large ascospores strongly pointed toward the identity of this species, and at least from Europe, no other species except for the arctic-alpine *H. macrosporum* has ascospores of similar size. The latter species also features olivaceous stromatal pigments, due to the presence of daldinin type azaphilones (Stadler et al. 2008b), whereas the stroma we studied did not give any pigment reaction, as is often the case even in freshly collected *H. vogesiacum*.

The *H. fragiforme* specimens all contained the typical metabolite patterns, including orsellinic acid (**9**) and the azaphilones, mitorubrin (**10**), mitorubrinol (**11**), and mitorubrinol acetate (**12**) as major metabolites. Furthermore, the extracts contained many peaks corresponding to hitherto unknown metabolites (*cf* Fig. 4), which could not be assigned to any known metabolites of the Xylariales according to our database search. Since these metabolites were also encountered in fresh material, large quantities of stromata were collected in the field. Subsequently, the isolation of sufficient amounts allowed for the structure elucidation of the novel metabolites **1–7**. Compound **8** was found to be lenormandin F (Kuhnert et al. 2014c).

Rutilin C (**1**) was isolated as a red oil; its molecular ion cluster in the HRESIMS spectrum revealed the molecular formula  $C_{42}H_{34}O_{14}$ . The proton and  $^1H,^{13}C$  HSQC NMR spectra of **1** showed the presence of five methyls, one methylene and 13 olefinic/aromatic methines. The  $^{13}C$  and  $^1H,^{13}C$  HMBC spectra furthermore indicated four conjugated ketones, two carboxyls, and 15  $sp^2$  hybridized plus two  $sp^3$  hybridized further carbons without bound protons. By  $^1H,^{13}C$  HMBC correlations, two orsellinic acid and two azaphilone subunits were identified. COSY and TOCSY correlations connected methines 12a–H/13a–H/14a–H and a propenyl side chain comprising 12b–H/13b–H/14b– $H_3$ , which were linked to C–3a and C–3b by HMBC correlations, respectively. HMBC correlations from 14a–H to C–7b/C–6b/C–5b and 4b–H to C–5b/C6b revealed the planar core structure. The CD spectrum of **1** with its negative cotton effect at 365 nm and positive effects at 260 and 450 nm confirmed the 8a*R*/8b*R* stereochemistry alike the biosynthetically related mitorubrins (Clark et al. 2008). Taken together, the compound constitutes the 7a-dehydro-14b-desacetoxy derivative of rutilin B, which is known from *Hypoxylon rutilum*. Due to the structural similarity to rutilin B, we chose to name **1** as rutilin C.

The red oil rutilin D (**2**) was analyzed for a molecular formula  $C_{44}H_{36}O_{16}$ , indicating the formal addition of a  $C_2H_2O_2$  fragment in comparison to **1**. The NMR spectra of **2** were highly similar to those of **1**. The key difference was the replacement of methyl 14b by an oxymethylene, and signals for an additional acetyl moiety, which was



**Fig. 4** HPLC-UV/VIS chromatogram (at 330 nm) of the fossil sample Fo4-2, depicted in Fig. 1 (upper part) and the large scale extraction from fresh stromatal material STMA18022 (lower part).

connected to 14b due to the HMBC correlation from 14b-H to C-15. Therefore, **2** was identified as the 14b-acetoxy derivative of **1** and named rutilin D (Fig. 5).

Fragirubrin A (**3**) was isolated as yellow oil. Its HRESIMS data indicated the molecular formula  $C_{31}H_{46}O_7$ , and thereby nine degrees of unsaturation. The  $^1H$  and  $^1H,^{13}C$  HSQC NMR spectra of **3** showed four methyls, three olefinic and one aliphatic methines and 15 methylenes. In addition, the  $^{13}C$  spectrum provided evidence for two conjugated ketones, two ester carbonyls, one oxygenated  $sp^3$  hybridized and three  $sp^2$  hybridized carbons devoid of bound protons.  $^1H,^{13}C$  HMBC correlations from 1-H to C-3, C-4a, C-8, C-8a; from 4-H to C-3, C-4a, C-5, C-8a; from 5-H to C-4, C-4a, C-7, C-8a; and from 9-H<sub>3</sub> to C-6, C-7, C-8 identified a highly conjugated azaphilone core structure. COSY correlations between 10-H<sub>2</sub>, 11-H and 12-H<sub>3</sub> assigned a 2-hydroxypropyl moiety, attached to C-3 due to HMBC correlations from 10-H<sub>a/b</sub> to C-3, C-4 and H-4 to C-10. Another entity was assigned as palmitinyl based on COSY and TOCSY correlations from both 17-H<sub>2</sub>/18-H<sub>2</sub> and 19-H<sub>2</sub>/30-H<sub>2</sub> to the unresolved CH<sub>2</sub> region. The fatty acid side chain was connected to C-7 based on the missing valences, finalizing the planar structure of **3**. The stereochemistry of C-7 was addressed by CD spectroscopy. The CD spectrum of **3** showed a negative cotton effect at 347 and positive effects at 273, indicating a 7*R* stereochemistry alike to the structurally closely related mitorubrins (Clark et al. 2008). The absolute

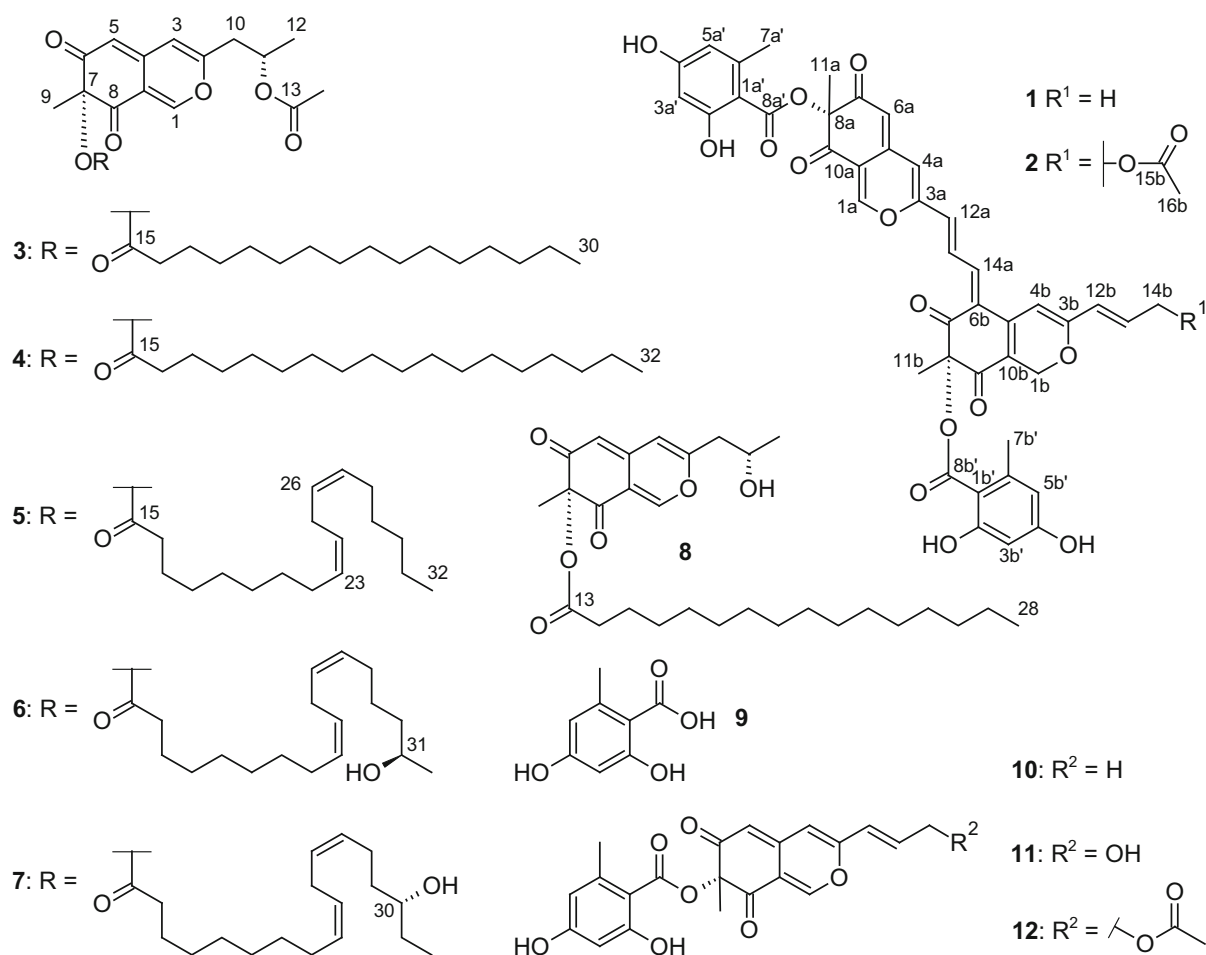
Orsellinic acid (**9**) cannot be observed at 330 nm but elutes after RT = 3.63 min. Numbers correspond to compound identifiers used in this article. *MA* mitorubrinic acid, *CohB* cohaerin B

stereochemistry of C-11 was assigned by Mosher's method (Hoye et al. 2007), utilizing **8**, the deacetyl-derivative of **3**. Negative  $\Delta\delta^{SR}$  values of 11-H and 12-H<sub>3</sub> and positive ones for all other protons indicated a 11*S* configuration of the metabolite family; the same stereochemistry had been determined for lenormandin F isolated from *H. lenormandii* (Kuhnert et al. 2014c).

Fragirubrin B (**4**) had the molecular formula  $C_{33}H_{50}O_7$ , indicating the formal addition of a  $C_2H_4$  fragment compared to **3**. Since the NMR spectra of **4** were virtually identical to those of **3**, the presence of a stearyl fatty acid side chain was deduced.

For fragirubrin C (**5**) a molecular formula  $C_{33}H_{46}O_7$  was identified by HRESIMS data, indicating two additional degrees of unsaturation compared to **3**. The proton spectrum of **5** was similar to that of **3**, with the exception of four additional olefinic methines between  $\delta_H$  5.30–5.40. The chemical shift of 25-H<sub>2</sub> ( $\delta_H$  2.77) indicated that this methylene is surrounded by two double bonds. The double bond geometry was deduced as *Z* for both ones, since ROESY correlations were observed between 25-H<sub>2</sub> and 22-H<sub>2</sub>/28-H<sub>2</sub>, but not between 22-H<sub>2</sub>/28-H<sub>2</sub> and 24-H/26-H. Due to HMBC correlations from 28-H<sub>2</sub> to 29-H<sub>2</sub>/30-H<sub>2</sub> and 32-H<sub>3</sub> to 30-H<sub>2</sub>/31-H<sub>2</sub>, the fatty acid side chain was identified as linoleic acid.

The molecular formula of fragirubrin D (**6**) was determined as  $C_{33}H_{46}O_8$ , indicating an additional oxygen atom compared to **5**. The key difference between the highly



**Fig. 5** Stromatal metabolites detected in the current study. **1**: rutilin C; **2**: rutilin D; **3**: fragirubrin A; **4**: fragirubrin B; **5**: fragirubrin C; **6**: fragirubrin D; **7**: fragirubrin E; **8**: lenormandin F; **9**: orsellinic acid; **10**: mitorubrin; **11**: mitorubrinol; **12**: mitorubrinol acetate

similar  $^1H$  and  $^{13}C$  NMR spectra of **6** and **5** was the replacement of a methylene by an oxymethine. Since the additional oxymethine 31-H showed a COSY correlation to 32- $H_3$ , whose triplet of **3** was replaced by a doublet in **6**, **6** was identified as the 31-hydroxy derivative of **5**. The absolute stereochemistry of C-31 was assigned by Mosher's method. Negative  $\Delta\delta^{SR}$  values of 30- $H_a$  and 30- $H_b$  and a positive one for 32- $H_3$  indicated a 31*R* configuration.

As shown by its HRESIMS data, fragirubrin E (**7**) has the same molecular formula  $C_{33}H_{46}O_8$  as **6**. The NMR spectra of **7** were highly similar to those of **6**, with the major difference of methyl 32- $H_3$  showing a triplet instead of a doublet. Due to the HMBC correlation of 32- $H_3$  to oxymethine 30-H, **7** was identified as the 30-hydroxy derivative of **5**. The absolute stereochemistry of C-30 was again assigned by Mosher's method. Negative  $\Delta\delta^{SR}$  values of 28- $H_a$ , 28- $H_b$ , 29- $H_a$  and 30- $H_b$  and positive ones for 31- $H_2$  and 32- $H_3$  indicated a 30*R* configuration.

## Discussion

The current study is to the best of our knowledge the first one where secondary metabolites from fungi have been detected in ancient materials that are estimated to be many centuries old. It was already surprising to see that the specimens still contained ascospores, and this may be due to the fact that the Xylariales often have the ability to form rather persistent spores. However, the spores were not apparently intact (e.g., the perispore could not be seen) and hence, their examination and the macroscopic characters of the stromata still left some doubt as to the identity of the material and only the fact that the secondary metabolites were rather well preserved allowed for a safe identification of the species.

Some metabolites involved were already known to be relatively stable and had repeatedly been detected in herbarium specimens that had been kept in museums. For instance, mitorubrins and the related rubiginosins have been detected in various specimens of *Hypoxylon* by

Stadler et al. (2008a) and *Entonaema* (Stadler et al. 2008b) that were collected during the nineteenth century.

The present study reports on the occurrence of conjugated bisazaphilones in *H. fragiforme* for the first time. However, structurally related compounds have already been obtained previously from different species of the Hypoxylaceae, including the rutilins from *H. rutilum* (Quang et al. 2005a) and entonaemin C from a fungus that was referred to as *Entonaema splendens* collected in Japan (Hashimoto and Asakawa 1998). The identification of the latter species was probably not correct, since *E. splendens* is a name referring to a Caribbean species whose type features immature stromata, but similar compounds were detected by HPLC–DAD/MS in both, *E. cinnabarinum* and *E. liquescens* (Stadler et al. 2008b). It is difficult to tell why these compounds have persisted in the stromata for such a long time because in particular the azaphilones are regarded to be rather reactive, and the trivial name of this metabolite class even relates to their ability to spontaneously incorporate nitrogen (Gao et al. 2013). On the other hand, as shown by the illustrations provided by Stadler and Fournier (2006) and Stadler et al. (2006), they are contained in the mature stromata in rather large amounts and are embedded in a waxy layer beneath the stromata surface, surrounding the perithecia, where they are normally not exposed to the environment. This feature seems to have determined the longevity of the secondary metabolites of many type and historical specimens and now it has even been proven that the metabolites remain stable for up to a millenium. We had been hoping to find evidence, e.g. for the presence of new taxa, or of tropical fungi that may have dominated the forests of Central France during medieval times, but according to the evidence provided in this study, we have mostly found the same fungi that still dominate the beech forests of the temperate Northern hemisphere. However, our studies are going on, and we have not yet studied many of the fragments that resulted from the excavation.

We are not aware of any previous study on the presence of secondary metabolites in ancient fungal specimens, and do not think this could be a general option to establish in palaeontology. After all, most of the petrified fossil fungal specimens that have been dealt with previously are not very likely to yield conclusive data on secondary metabolites, even if the most sensitive methods of analytical chemistry will be employed. Due to the fact that we only observed highly carbonised stromata, deflated hyphae and no intact ascospores, even by SEM, we can exclude that it would still be possible to obtain DNA from the ancient material. It should be very interesting to see whether a similar approach will succeed with other samples that have been collected during excavations that are intended to study the recent human history.

Our methodology is well-suited to study further fungal specimens including those that have been dated and proven to be much older than the current material as outlined by Knoll (2014), palaeobiology does already rely on microscopic studies including SEM, but the reconstruction of the early evolution of Eukaryotes is hampered by the lack of data on fossils. In particular, the newly arising Molecular Clock studies (Divakar et al. 2017; Hongsanan et al. 2017) that rely on temporal phylogenies would certainly work better if more fossils with reliably determined age were available. The future will show whether chemotaxonomic traits can be employed in such endeavours.

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