




Stachybotrychromenes A–C: novel cytotoxic meroterpenoids from *Stachybotrys* sp.

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Abstract

In the course of gaining new insights into the secondary metabolite profile of various *Stachybotrys* strains, in particular concerning triprenyl phenol-like compounds, so far, unknown metabolites with analogous structural features were discovered. Three novel meroterpenoids containing a chromene ring moiety, namely stachybotrychromenes A–C, were isolated from solid culture of the filamentous fungus *Stachybotrys chartarum* DSMZ 12880 (chemotype S). Their structures were elucidated by means of comprehensive spectroscopic analysis (1D and 2D NMR, ESI-HRMS, and CD) as well as by comparison with spectroscopic data of structural analogues described in literature. Stachybotrychromenes A and B exhibited moderate cytotoxic effects on HepG2 cells after 24 h with corresponding IC₅₀ values of 73.7 and 28.2 μM, respectively. Stachybotrychromene C showed no significant cytotoxic activity up to 100 μM. Moreover, it is noteworthy that stachybotrychromenes A–C are produced not only by *S. chartarum* chemotype S but also *S. chartarum* chemotype A and *Stachybotrys chlorohalonata*.

Keywords Mycotoxins · *Stachybotrys* · Meroterpenoids · Isolation · Structure elucidation · Cytotoxicity

Introduction

The fungal genus *Stachybotrys* is ubiquitously present in the environment, especially on commodities rich in cellulose, such as straw, wood, and paper as well as gypsum board, and was first isolated from a moldy wallpaper in Prague (Bisby 1943). The occurrence of *Stachybotrys* species has been reported frequently, particularly in water-damaged buildings (Johanning et al. 1996; Jarvis et al. 2000; Bloom et al. 2009). In this context, the species *Stachybotrys chartarum* (*atra*) received attention in the 1990s, within cases of building-related illnesses and idiopathic pulmonary

hemosiderosis in infants (Etzel et al. 1998; Dearborn et al. 1999; Page and Trout 2001; Pestka et al. 2008). Approximately 150 secondary metabolites from *Stachybotrys* species are known and might be related to the observed adverse human health effects in the cases mentioned above (Jarvis et al. 1998; Hossain et al. 2004). Important representatives are macrocyclic trichothecenes, including the highly toxic satratoxins, atranones, and phenylspirodrimanens (Fig. 1) (Jarvis 2003). The phenylspirodrimanens occur in considerably higher levels and are therefore designated to be the most characteristic and dominant group of mycotoxins among this genus (Jarvis et al. 1995). Additionally, the latter compounds are produced by the majority of *Stachybotrys* species, whereas the macrocyclic trichothecenes are only produced by *S. chartarum* chemotype S (Andersen et al. 2003).

Phenylspirodrimanens are part of the group of triprenyl phenols, which in turn belong to the broad group of meroterpenoids. The latter represent natural products whose molecular structures contain moieties originating from the polyketide pathway as well the terpenoid pathway (Geris and Simpson 2009; Matsuda and Abe 2016). This structurally unique class of substances shows a broad diversity of biological activities. For instance, disruption of the complement system, immunotoxicity, neurotoxicity, cytotoxicity, fibrinolysis,

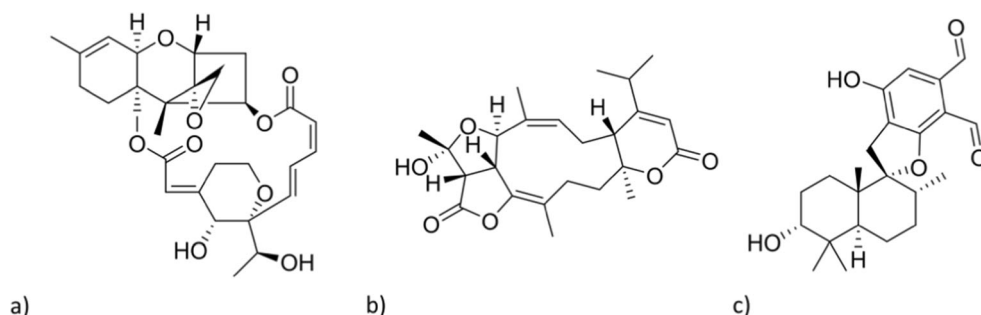
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Fig. 1 Main classes of *Stachybotrys* metabolites. **a** Macrocytic trichothecenes (satratoxin H). **b** Atranones (atranone A). **c** Phenylspirodrimanes (stachybotrydial)



and plasminogen activation, as well as antiviral and antiplasmodial activity, have been shown (Sawadjoon et al. 2004; Hasumi et al. 2007; Sasaoka et al. 2007; Wang et al. 2015).

Particularly in the last years, the genus *Stachybotrys* revealed tremendous potential for the isolation of novel meroterpenoid derivatives with remarkable properties concerning biological activity, e.g., inhibitory effects against the dengue virus (Li et al. 2014a, b; Bao et al. 2015; Ma et al. 2015; Chunyu et al. 2016; Ding et al. 2017a, b; Li et al. 2017; Liu et al. 2017; Yin et al. 2017; Zhang et al. 2017; Zhao et al. 2017a, b, c).

In the course of HPLC-MS/MS profiling of secondary metabolites from *Stachybotrys* species, the triprenyl phenol-like compounds stachybotrychromenes A–C (1–3) (Fig. 2) were identified as three novel meroterpenoid metabolites. Herein, we describe the isolation and structure elucidation and give first insights into cytotoxicity of these compounds.

Materials and methods

General experimental procedures

Analytical thin-layer chromatography (TLC) was performed with pre-coated silica gel plates (Silica gel 60 F254, Merck, Darmstadt, Germany), and UV irradiation was used for visualization (254 and 360 nm, Herolab, Wiesloch, Germany).

Semi-preparative reversed-phase high-performance liquid chromatography with ultraviolet detection (RP-HPLC-UV) was carried out by using a Jasco system (Jasco, Groß-Umstadt, Germany) equipped with a 1-mL sample loop (Rheodyne, CA, USA) at a wavelength of 360 nm with an Agilent Eclipse XDB-C 18 column (250 × 9.4 mm, 5 μm, Agilent, Waldbronn, Germany).

1D and 2D nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz for ¹H NMR and 150 MHz for ¹³C NMR, respectively, using an Agilent DD2 600 MHz spectrometer (Agilent, Waldbronn, Germany). Chemical shifts are reported in parts per million (ppm), coupling constants (*J*) are given in Hertz (Hz), and CD₃CN was used as the solvent.

High-resolution mass spectrometry with electrospray ionization (ESI-HRMS) data were obtained using an LTQ Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany). UV spectra were obtained with a J-750 Spectrophotometer (Jasco, Groß-Umstadt, Germany) in acetonitrile (MeCN).

For circular dichroism (CD) measurements, a J-600 Spectropolarimeter (Jasco, Groß-Umstadt, Germany) with a 0.1-cm cell in MeCN at room temperature was used.

Chemicals and culture media

Ethyl acetate (EtOAc, analytical grade) was purchased from Grüssing (Filsum, Germany). Cyclohexane (synthesis grade) and MeCN (HPLC grade) were purchased from Roth (Karlsruhe, Germany) and Fisher Scientific (Schwerte,

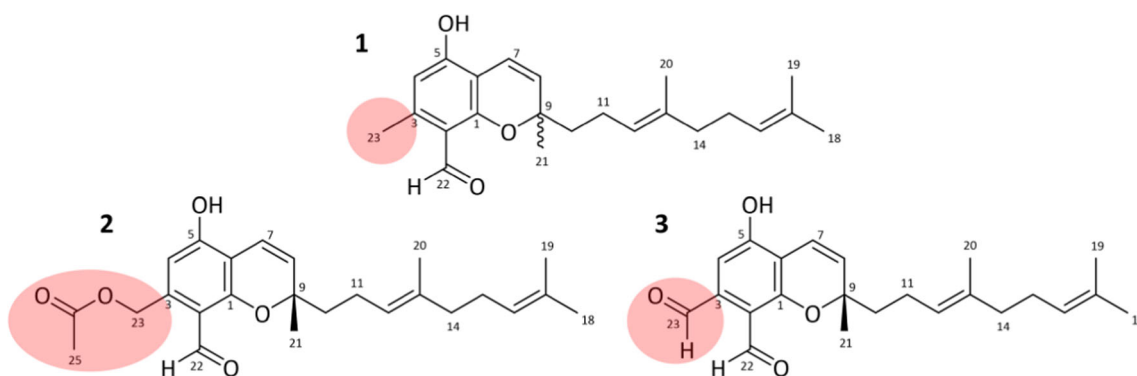


Fig. 2 Chemical structures of the isolated stachybotrychromenes A–C (1–3) from *S. chartarum*

Germany), respectively. Dichloromethane was of analytical grade (Roth, Karlsruhe, Germany). Potato dextrose agar (PDA) was purchased from Sigma-Aldrich, Steinheim, Germany. Dimethyl sulfoxide (DMSO) of bioscience grade was purchased from Roth, Karlsruhe, Germany. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (v/v) fetal bovine serum (FBS) was purchased from Gibco, Thermo Fisher, Darmstadt, Germany.

Fungal material, cultivation, extraction and isolation

The fungal strain *S. chartarum* DSM 12880 (chemotype S) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). After reviving the freeze-dried culture according to the instruction of the DSMZ, the strain was maintained on slants of PDA medium at 4 °C. Seed cultures were prepared in Erlenmeyer flasks (500 mL) containing 200 mL of liquid PD medium on a laboratory shaker at 150 rpm at 25 °C for 3 days in darkness. The media were autoclaved at 121 °C for 15 min. Petri dishes (92 × 16 mm, Sarstedt, Nümbrecht, Germany) were prepared with PDA for main cultivation, three-point inoculated with 1 mL seed culture, and incubated for 21 days at 25 °C protected from light in an incubator.

After 21 days, the agar plates were extracted exhaustively three times using EtOAc, yielding a crude extract. Afterwards, the crude extract was filtered through Miracloth (Merck, Darmstadt, Germany) to remove spores and agar and evaporated to dryness at 40 °C using a rotary evaporator. The residue was redissolved in dichloromethane, subjected to silica gel column chromatography (CC) (Silica gel 60, 230–400 mesh, Merck, Darmstadt, Germany) and eluted applying a cyclohexane-ethyl acetate gradient of increasing polarity (from 1:0 to 0:1) with 3 mL/min. Fractions were checked by TLC, and similar fractions containing **1–3** were combined and further separated and purified by semi-preparative RP-HPLC eluting with 100% MeCN at 4 mL/min to yield **1** (3 mg), **2** (5 mg), and **3** (8 mg).

Cell culture experiments

For the cytotoxicity assay, 20 mM stock solutions and 200-fold concentrations of the used concentration range (0.1–100 µM) of **1–3** were prepared in DMSO. These stock solutions were diluted to the respective concentrations with serum containing culture medium. Human hepatic cancer cells (HepG2, ACC 180, DSMZ, Braunschweig, Germany) were cultivated in DMEM using standardized culture conditions (37 °C, 5% CO₂, saturated humidified atmosphere). For evaluation of cytotoxic effects, the resazurin reduction assay (Alamar Blue assay) based on O'Brien was performed in 96-

well plates (O'Brien et al. 2000). Cells were treated with **1–3** (0.1–100 µM) and incubated for 24 h, whereas 0.01% (w/v) saponin from *Quillaja* bark (Sigma-Aldrich, Steinheim, Germany) served as positive control. Viability was calculated after blank subtraction as test over control (T/C). Six replicates of all experiments were performed for each of three independent passages ($n = 3$). The data are presented as the mean ± standard deviation (SD) in Fig. S22 (Supplementary Material). The IC₅₀ and significance values were calculated with OriginPro 2016G (OriginLab Corporation, Northampton, USA): significant at * $p \leq 0.05$ and highly significant at ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results and discussion

Isolation and structure elucidation

The filamentous fungus *S. chartarum* DSM 12880 (chemotype S) was cultured on PDA for 3 weeks and extracted with EtOAc. The crude extract was fractionated by silica gel column chromatography and semi-preparative RP-HPLC-UV to yield compounds **1–3**.

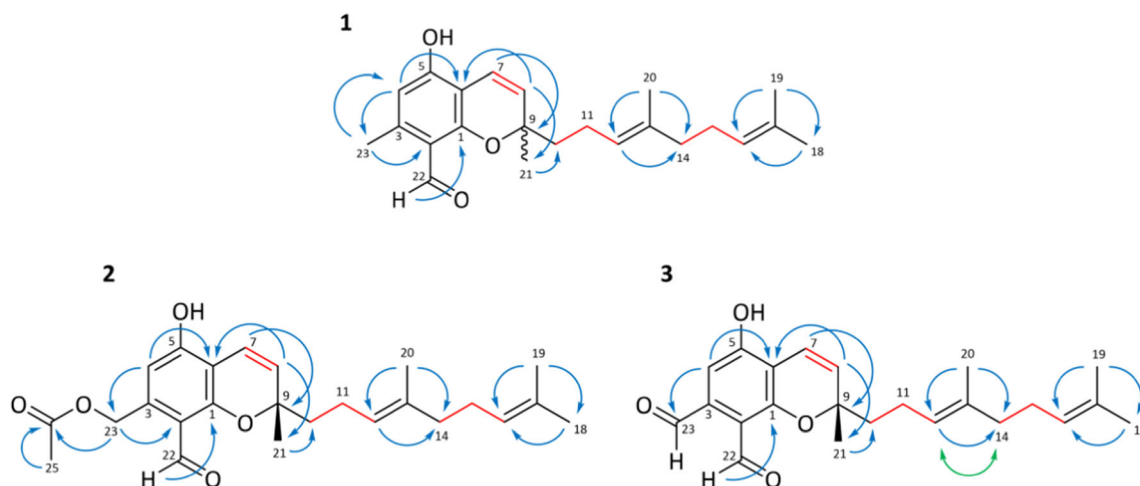
Stachybotrychromene A (**1**) was isolated as a brownish oil. Its molecular formula was determined to be C₂₃H₃₀O₃ by ESI-HRMS ([M-H]⁻ m/z 353.2115, calc. 353.2122), corresponding to the ¹H and ¹³C NMR data (Table 1) and HSQC spectrum, which indicated nine degrees of unsaturation. The ¹³C and HSQC spectra revealed 23 carbon signals. Briefly, one aldehyde carbon (δ_C 195.17), six aromatic carbons (δ_H 161.6, 161.1, 145.7, 114.1, 111.6, 107.6), one oxygen-linked quaternary carbon (δ_C 81.6), four olefinic carbons (δ_C 128.0, 125.2, 124.8, 116.1), two quaternary carbons (δ_C 136.3, 132.2), four methylene groups (δ_C 42.1, 40.3, 27.3, 23.3), and five methyl groups (δ_C 27.4, 25.5, 18.4, 17.7, 16.0) (Table 1). The ¹H NMR spectrum displayed the presence of a phenol proton at δ_H 12.76 (1H, s, OH-5), an aldehyde proton at δ_H 10.05 (1H, s, H-22), an aromatic proton at δ_H 6.20 (1H, s, H-4), and an aromatic methyl group at δ_H 2.49 (3H, s, H-23). Further examination of the spectrum disclosed a couple of *cis*-configured olefinic protons, δ_H 6.64 (1H, d, $J = 10.2$ Hz, H-7) and 5.61 (1H, d, $J = 10.2$ Hz, H-8), whereas the characteristic COSY correlation established the partial structure (Fig. 3 and Supplementary Material Fig. S3). The HMBC interactions from the olefinic proton at δ_H 5.61 (H-8) with carbons at δ_C 107.6 (C-6), 27.4 (C-21), and 42.1 (C-10) and the olefinic proton at δ_H 6.64 (H-7) with carbons at δ_C 161.1 (C-1) and 81.6 (C-9) confirmed a chromene moiety attached to a side chain. According to the ¹H NMR data, this side chain contains four methyl groups; one of them directly linked between the chromene ring and the side chain (δ_H 1.39 (3H, s, H-21)) as confirmed by HMBC experiments as well. The three remaining methyl groups at δ_H 1.54 (3H, s, H-20), δ_H 1.57 (3H, s,

Table 1 ^1H and ^{13}C NMR data of stachybotrychromenes A–C (1–3) in CD_3CN (δ in ppm, J in Hz)

No.	1		2		3	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
1	161.1, C		161.2, C		161.5, C	
2	114.1, C		115.9, C		113.1, C	
3	145.7, C		142.0, C		138.8, C	
4	111.6, CH	6.20, s	111.3, CH	6.40, s	117.5, CH	6.90, s
5	161.6, C		161.2, C		160.4, C	
6	107.6, C		109.6, C		114.0, C	
7	116.1, CH	6.64, d (10.2)	113.1, CH	6.66, d (10.2)	115.8, CH	6.71, d (10.2)
8	128.0, CH	5.61, d (10.2)	129.1, CH	5.67, d (10.2)	131.8, CH	5.82, d (10.2)
9	81.6, C		82.0, C		82.4, C	
10	42.1, CH_2	1.69, m	42.1, CH_2	1.72, m	42.1, CH_2	1.77, m
11	23.3, CH_2	2.07, m	23.3, CH_2	2.09, m	23.2, CH_2	2.12, m
12	124.8, CH	5.11, t (7.2)	124.7, CH	5.12, t (7.2)	124.6, CH	5.12, t (7.2)
13	136.3, C		136.3, C		136.5, C	
14	40.3, CH_2	1.94, m	40.3, CH_2	1.93, m	40.3, CH_2	1.94, m
15	27.3, CH_2	2.03, m	27.3, CH_2	2.02, m	27.3, CH_2	2.03, m
16	125.2, CH	5.07, t (7.0)	125.2, CH	5.07, t (7.0)	125.2, CH	5.07, t (7.0)
17	132.2, C		132.2, C		132.2, C	
18	17.7, CH_3	1.57, s	17.8, CH_3	1.57, s	17.7, CH_3	1.57, s
19	25.8, CH_3	1.64, s	25.8, CH_3	1.64, s	25.8, CH_3	1.64, s
20	16.0, CH_3	1.54, s	16.0, CH_3	1.54, s	16.0, CH_3	1.54, s
21	27.4, CH_3	1.39, s	27.5, CH_3	1.41, s	27.5, CH_3	1.45, s
22	195.2, CHO	10.05, s	194.9, CHO	10.01, s	196.6, CHO	10.63, s
23	18.4, CH_3	2.49, s	63.3, CH_2	5.26, s	193.5, CHO	10.02, s
24			171.2, C			
25			21.1, CH_3	2.04, s		
OH-5		12.76, s		12.72, s		12.95, s

H-18), and δ_{H} 1.64 (3H, s, H-19), in addition to the two deshielded CH triplets at δ_{H} 5.11 (1H, t, $J=7.2$ Hz, H-12) and δ_{H} 5.07 (1H, t, $J=7.0$ Hz, H-16), imply the presence of an isoprene unit. As assigned from COSY and HMBC

resonances, the remaining four methylene signals at δ_{H} 1.69 (2H, m, H-10), δ_{H} 2.07 (2H, m, H-11), δ_{H} 1.94 (2H, m, H-14, overlapped by solvent), and δ_{H} 2.03 (2H, m, H-15) completed the structural features of the two prenyl groups derived from

**Fig. 3** Key ^1H - ^1H COSY (red lines), HMBC (blue \rightarrow), and NOE (green \leftrightarrow) correlations of 1–3

the isoprene side chain and concluded the presence of 32 protons in total. The determination of the absolute configuration was not possible, most likely due to tiny impurities adversely affecting the CD properties of the molecule. The compound was designated to be (*E*)-2-(4,8-dimethylnona-3,7-dien-1-yl)-5-hydroxy-2,7-dimethyl-2*H*-chromene-8-carbaldehyde.

The molecular formula of stachybotrychromene B (**2**) was in accordance with C₂₅H₃₂O₅ (ESI-HRMS, [M-H]⁻ *m/z* 411.2177 (calc. *m/z* 411.2175)) and the compound was obtained as brownish oil. The degree of unsaturation was designated to be 10. The analysis of 1D and 2D NMR (HSQC, COSY, and HMBC) data proved **2** to have a similar core structure as described for **1**, with the exception of an acetyl group (δ_C 171.2 (C-24), δ_C 21.1 (C-25)/ δ_H 2.04, 3H, s, H-25) replacing the methyl group at C-23 of **1**. The downfield shift of the methylene protons (δ_H 5.26, 2H, s, H-23) and the respective carbon shift (δ_C 63.3, C-23) suggest the attachment of the acetyl group to C-23 via an ester bond (Table 1). HMBC resonances between H-25 and C-24, H-23 and C-24, H-4 and C-23, and H-23 and C-2 confirmed the assignment (Fig. 3). Lastly, an in-source acetate loss (*m/z* 351.1960 [M-CH₃COOH]⁻) observed in the ESI-HRMS spectrum established the structure unequivocally (Supplementary Material Fig. S12).

The absolute configuration of the stereocenter at C-9 was determined as (*S*) based on the weak but characteristic positive Cotton effect at 260–290 nm observed in the CD spectrum (Fig. 4), which is in agreement with reported analogues (Li et al. 2014b; Bao et al. 2015; Zhang et al. 2017). Therefore, **2** was identified to be (*S*, *E*)-2-(4,8-dimethylnona-3,7-dien-1-yl)-8-formyl-5-hydroxy-2-methyl-2*H*-chromen-7-yl)methyl acetate.

Stachybotrychromene C (**3**) was obtained as a yellow oil, and its molecular formula was determined to be C₂₃H₂₈O₄ based on ESI-HRMS ([M-H]⁻ *m/z* 367.1915, calc. 367.1907) and NMR data (Table 1), corresponding to 10 degrees of unsaturation. The ¹H, ¹³C, HSQC, COSY, and HMBC spectroscopic data are

highly comparable to those of **1** and **2**, except for the additional aldehyde group with corresponding signals at δ_H 10.02 (1-H, s, H-23) and δ_C 193.5 (C-23) instead of a methyl or acetoxymethylene group, respectively.

Although the chemical shifts of the increment system of the prenyl scaffold of the H-12 proton at δ_H 5.11 and the C-20 signal δ_C 16.00 (analogously to **1** and **2**, Table 1) already indicate *E* configuration (Nishino and Bowers 1976; Ma et al. 2015), the configuration of the isoprene unit of **3**, as representative example, was in addition investigated by extensive and selective 1D and 2D NOE experiments. Moreover, the herein presented compounds **1–3** are closely related to the recently published stachybonoids A–C (Zhang et al. 2017), which were interestingly described to possess a *cis*-configured isoprene unit. However, selective NOE experiments with saturation on the proton of the C-12 (δ_H 5.12) enhanced the methylene unit at δ_H 1.94 (H-14) (Fig. 3 and Supplementary Material Fig. S18 and Fig. S19), thus confirming the *E* configuration of the double bond between C-12 and C-13.

The absolute configuration of the C-9 was also assigned as (*S*) based on CD measurement as described above (Fig. 4). Hence, the structure was determined to be (*S*, *E*)-2-(4,8-dimethylnona-3,7-dien-1-yl)-5-hydroxy-2-methyl-2*H*-chromene-7,8-dicarbaldehyde.

Proposed biosynthesis of **1–3** and occurrence in other *Stachybotrys* strains

Meroterpenoids are hybrid biosynthesized natural products, partially derived from terpenoids (Geris and Simpson 2009). Regarding compounds **1–3**, this hybrid structure is comprised of an additional tetraketide part. Key intermediate is *o*-orsellinic acid (OA), which is formed from acetyl-CoA and 3 units of malonyl-CoA after cyclization. Afterwards, a prenylation step leads to the attachment of the prenyl side chain and the carboxy function of OA undergoes reduction to an aldehyde group to form LL-Z1272 β , also known as ilicicolin B (Li et al. 2016). This compound is well known as a key intermediate within the biosynthetic pathway of many meroterpenoids and is therefore assumed to be the precursor for compounds **1–3**.

Ultimately, **1** bears the aromatic methyl group at C-23, derived from *o*-orsellinaldehyde, which is further transformed into an oxidized acetoxymethylene group yielding **2**. Subsequently, the final oxidation at C-23 leads to the formation of **3**, possessing the respective aldehyde group. HPLC-MS/MS analysis of the metabolic profile of *S. chartarum* (DSM 12880) showed that **1** and **2** are the main compounds formed during the first days of incubation. The signals of **1** and **2** decrease over time and **3** occurs as the major compound at later time points (data not shown). Thus, **1** and **2** are most likely intermediates, whereas **3** is the final product within this biosynthetic pathway. These observations are in agreement

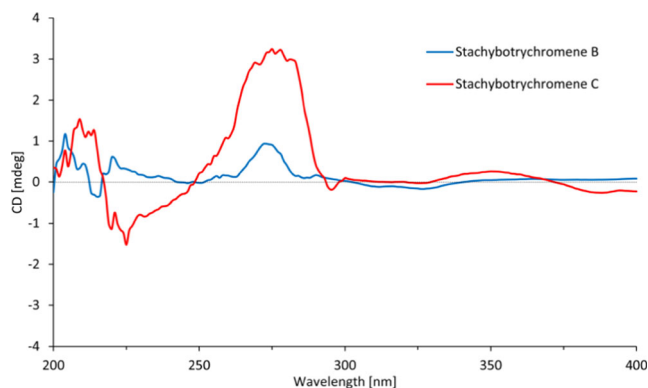


Fig. 4 CD spectra of **2** and **3**

with reported (proposed) biosynthetic pathways of other meroterpenoids among the genus *Stachybotrys* (Zhang et al. 2017; Zhao et al. 2017c).

1–3 are produced by a diversity of *Stachybotrys* strains. Within metabolite profiling, it was observed that also *S. chartarum* chemotype A (IBT 40288, CBS 109291), S (IBT 40293, CBS 109290), and *Stachybotrys chlorohalonata* (IBT 40285, CBS 109283) are capable producers, as well on different media, such as malt extract agar. Only *S. chartarum* DSMZ 63425 appeared with a different profile, and no stachybotrychromenes were detected (data not shown).

Cytotoxicity

For assessment of cytotoxic effects, compounds 1–3 were tested on HepG2 cells, using the resazurin reduction assay. For compound 1, the results indicated that cell viability is significantly reduced with treatment of 50 μM ($p < 0.05$) after 24 h of incubation. Compound 2 was more potent and showed significantly reduced cell survival rates at 25 μM ($p < 0.05$), whereas for compound 3, no significantly reduced cell viability up to 100 μM was observed (Supplementary Material, Fig. S22). For 1 and 2, IC_{50} values were determined to be 73.7 and 28.2 μM , respectively.

Due to the fact that chromenes and chromanes have been reported to possess antiprotozoal and antiviral properties (Harel et al. 2013; Li et al. 2014b; Presley et al. 2017), further investigations regarding those biological activities of the newly isolated compounds might be of interest.

The present work discovered three novel meroterpenoid derivatives (1–3), named stachybotrychromenes A–C, isolated from *S. chartarum* DSM 12880 (chemotype S) containing a chromene ring backbone structure with an attached isoprenoid side chain. In view of the biosynthetic pathway, it can be assumed that 3 is the final product and is biosynthesized via the intermediates 1 and 2. In terms of cytotoxicity, 1 and 2 showed moderate cytotoxic effects on HepG2 cells after 24 h of exposure, whereas 3 exhibited no cytotoxicity within the investigated concentration range (0.1–100 μM). It should be noticed that these compounds are produced by both chemotypes of *S. chartarum* as well as *S. chlorohalonata* and contribute to the toxic profile of this genus.

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Compliance with ethical standards

Conflict of interest None

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