Functionalized para-substituted benzenes as 1,8-cineole production modulators in an endophytic Nodulisporium species

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A Nodulisporium species (designated Ti-13) was isolated as an endophyte from Cassia fistula. The fungus produces a spectrum of volatile organic compounds (VOCs) that includes ethanol, acetaldehyde and 1,8-cineole as major components. Initial observations of the fungal isolate suggested that reversible attenuation of the organism via removal from the host and successive transfers in pure culture resulted in a 50 % decrease in cineole production unrelated to an overall alteration in fungal growth. A compound (CPM1) was obtained from Betula pendula (silver birch) that increases the production of 1,8-cineole by an attenuated Ti-13 strain to its original level, as measured by a novel bioassay method employing a 1,8-cineole-sensitive fungus (Sclerotinia sclerotiorum). The host plant produces similar compounds possessing this activity. Bioactivity assays with structurally similar compounds such as ferulic acid and gallic acid suggested that the CPM1 does not act as a simple precursor to the biosynthesis of 1,8-cineole. NMR spectroscopy and HPLC-ES-MS indicated that the CPM1 is a para-substituted benzene with alkyl and carboxyl substituents. The VOCs of Ti-13, especially 1,8-cineole, have potential applications in the industrial, fuel and medical fields.

INTRODUCTION

Endophytes are micro-organisms that live within the tissues of virtually all plants on earth, generally without causing disease symptoms or displaying outward signs of habitation (Wilson, 1995). In the case of fungal endophytes, the host may benefit by virtue of the secondary metabolites produced by the fungus (Strobel & Daisy, 2003). Frequently these secondary metabolites are antimicrobial substances, but compounds able to deter herbivory and increase rates of nutrient uptake have also been reported (Clay, 1996; Malinowski et al., 2000). The variety of secondary metabolites produced by endophytic fungi reflects the diversity of the organisms themselves, and includes compounds such as taxol, novel antibiotics, quorum-sensing inhibitors and fuel-associated hydrocarbons (Castillo et al., 2003; Ezra et al., 2004c; Mends et al., 2012; Li et al., 2001; Stierle et al., 1993; Strobel et al., 2008).

In 2010, an endophytic Hypoxylon species was identified as the first non-plant source of 1,8-cineole, a monoterpen with fuel potential (Tomsheck et al., 2010). Since then many other isolates of Nodulisporium sp., the imperfect stage of this organism, have been isolated and shown to produce cineole (Strobel, 2014). As a stand-alone automotive fuel, cineole possesses performance characteristics similar to biodiesel, but with reduced exhaust emissions (Tamilvendhan et al., 2011; Tarabet et al., 2012; Takeda & Hoki, 1982). Moreover, a 90 % (v/v) mixture of cineole in petrol has an octane rating of 95 and adjusting the cineole concentration to 70 % (v/v) provides a fuel with nearly

Abbreviations: AS, attenuated strain; BGF, bioassay-guided fractionation; COSY, correlated spectroscopy; CPM, cineole production modulator; GBA, gas bioactivity assay; HMBC, heteronuclear multiple bond correlation; HSOC, heteronuclear single quantum coherence; ITS, internal transcribed spacer; OS, original strain; PTR, proton transfer reaction; RS, recovered strain; SEM, scanning electron microscopy; VOC, volatile organic compound.

The GenBank/EMBL/DDBJ accession number for the ITS1–5.8S–ITS2 ribosomal gene sequence of isolate Ti-13 is KJ 558391.
identical performance to pure petrol, but with much lower total fuel consumption and carbon monoxide output (Sugito & Takeda, 1981). Finally, addition of cineole to petrol prevents the problem of phase separation when water is encountered in the fuel system (Barton & Tjandra, 1989; Takeda & Hoki, 1982). A recent United States Department of Energy report confirmed the approach of finding, studying and using endophytic fungi as potential producers of fuels (Gladden, 2013).

For cineole to be implemented as a biofuel, more efficient and higher yielding methods for its production must be developed (Gladden, 2013; Tomsheck et al., 2010). Presently, the only industrial-scale cineole collection method involves a relatively inefficient distillation of crude eucalyptus oil obtained from plant materials (Babu & Singh, 2009). Cineole-producing fungi represent a new source of the compound and can be cultivated on minimally processed and easily available feedstocks in a solid-state fermentation system to cheaply and quickly produce economically feasible amounts of cineole (G. Strobel and B. Blatt, unpublished, 2013).

This report describes the modulation of cineole production in an isolate of *Nodulisporium* sp. obtained as an endophyte from *Cassia fistula* sampled from the highlands of Thailand. GC/MS and proton transfer reaction mass spectrometry (PTR-MS) confirm the presence of cineole in the spectrum of volatile organic compounds (VOCs) produced by this organism when grown on potato dextrose agar (PDA). Initial observations of the fungal isolate suggested that reversible attenuation of the organism via removal from the host and successive transfers in pure culture resulted in a decrease in cineole production unrelated to an overall alteration in fungal growth.

Differential cineole production, which is seemingly unrelated to the overall viability of the fungus, and which depends on the presence of the host, suggests the existence of a cineole production modulator (CPM) in the host. Identification of the CPM and characterization of the mechanism by which it alters fungal cineole production will facilitate the development of fermentation methods using this organism. This report discusses the purification of the CPM via bioassay-guided fractionation (BGF) and proposes a nearly complete structure for the molecule. As the sole purpose of this study was to identify the CPM, no additional attempts were made to elucidate a mechanism for its biological effects.

**METHODS**

**Fungal isolation, cultivation and storage.** The fungal species used in this study (designated Ti-13) was obtained as an endophyte from a *C. fistula* sample from the Wang Pha resort area in Tong Pha Poon, Kanchanaburi province, Thailand. Other plants sampled from the area included *Tectona grandis*, *Uncaria tomentosa*, *Citrus grandis*, *Acacia sp.*, *Mangifera indica* and *Artocarpus heterophyllus*, and none of these harboured Ti-13.

Initial isolation of the fungus followed the procedures of Tomsheck et al. (2010). In summary, plant samples were surface sterilized by exposure to 70% ethanol, and placed on water agar and glycerol arginine medium. Fungi growing out of these plant samples were picked by removing an agar plug supporting mycelial growth and reculturing on PDA. All transfers and fungal growth occurred at 22 ± 2 °C. Long-term storage of Ti-13 strains was accomplished by growing the organisms on barley seeds and storing them at −70 °C. Three strains of the fungus were produced and maintained, and the procedures to obtain them are as follows. The original strain (OS), as initially isolated from the plant host, was immediately stored at −70 °C on barley seeds. During transfers, sterile *C. fistula* stems (~20 mg) were placed on the surface of the PDA to mimic natural conditions and to encourage fungal growth on host tissues. The attenuated strain (AS) was generated by serial weekly transfers on to PDA without adding plant material for a period of 5 weeks. The AS was then stored as described above. The recovered strain (RS) was obtained by placing the AS on PDA in the presence of plant materials (original host or others). The RS also was stored as described above. In each case, the level of cineole production of each strain was quantified by the standard bioassay method or PTR-MS as described below.

**Taxonomic identification.** Sequencing of the internal transcribed spacer (ITS) region of the fungus and morphological observations were used for taxonomic identification (Ezra et al., 2004a). Genetic information was acquired by using the Prepmann Ultra sample preparation reagent (Applied Biosystems) to extract genomic DNA from mycelium collected from a 7-day-old OS culture. The ITS region was amplified by PCR using the universal ITS primers ITS1 (5'-TCGTAAGTGAACCTGCGG-3') and ITS4 (5’-TCCCTCCGTTA-TTGATATGC-3’). The primer pair, the PCR and DNA sequencing were all carried out as described by Tomsheck et al. (2010). The amplified products were sequenced and aligned with sequences in GenBank by the BLASTN program (Altschul et al., 1997). Relevant sequences were downloaded and the evolutionary position of the organism was ascertained by reconstructing a phylogenetic tree according to Tamura et al. (2004, 2007).

In addition, scanning electron microscopy (SEM) was performed on Ti-13 to acquire morphological data. The fungus was grown on gamma-irradiated carnation leaves, to encourage spore formation, for 3 weeks, and the samples were then slowly dehydrated in ethanol, critically point dried, coated with gold and examined with an FEI XL30 scanning electron microscope/field emission gun with high vacuum mode using an Everhart–Thornley detector (Tomsheck et al., 2010). The fungus was also observed under a standard Zeiss light microscope and drawings were made by camera lucida techniques.

**PTR-MS.** To confirm the attenuation phenomenon of cineole production by Ti-13, the production of 1,8-cineole by the OS, AS and RS isolates was quantitatively measured by PTR-MS following previously described procedures using authentic cineole as a standard (Ezra et al., 2004b; Tomsheck et al., 2010). One culture of each strain (OS, AS, RS) was grown on an individual 500 ml PDA slant in a 1 litre bottle at 22±2 °C for 10 days. The bottles were topped with a specially designed O-ring-sealed cap possessing sealable inlet and outlet tubes with 10 cm2 min−1 of compressed air (Booth et al., 2011). Air space analysis of AS, RS and OS was done by passing a small flow of medical-grade compressed air through the culture bottles and then diluting with air of the same quality. A 1 : 20 to 1 : 10 dilution kept the measurements within the linear dynamic range of the instrument and prevented water from condensing in the sample lines. The sample lines were constructed entirely from perfluoroalkoxy alkane Teflon tubing and fittings. During analysis, all ions in the spectrum were followed for a given strain from baseline level to baseline level before a different strain was analysed. Analysis time varied from 15 to 20 cycles, with each cycle corresponding to
1.67 min (2 Da s⁻¹). Mass spectral scans were acquired from 20 to 220 Da. As cineole fragmentations to m/z 81, m/z 137 and m/z 155 under PTR-MS conditions, the areas of these peaks were used to calculate relative concentrations in each sample using equations derived from reaction kinetics and assuming a reaction rate coefficient of 2 × 10⁻⁹ ml s⁻¹.

Note that PTR-MS is used to detect ions of organic molecules in the gas phase by the reaction of the parent molecule with H₂O⁺. Typically, a proton is transferred from H₂O⁺ to any molecule with a proton affinity higher than that of water, producing an ionized organic molecule that can be detected by a quadrupole mass spectrometer. PTR-MS can be applied to standard air samples with or without dilution because the primary components of ambient air have proton affinities less than that of water and are thus not ionized. Nearly all organic molecules besides alkanes have a proton affinity greater than that of water and can be effectively detected by this technique (Ezra et al., 2004b; Tomsheck et al., 2010).

**Qualitative determination of Ti-13 cineole production via a bioassay method.** Cineole is inhibitory to Sclerotinia sclerotiorum as well as Fusarium solani in sealed Petri plate assays (Tomsheck et al., 2010). Therefore, a biological assay was devised that allowed for the qualitative determination of cineole production by any strain of Nodulisporium using S. sclerotiorum as a test organism according to a previously described gas bioactivity assay (GBA) (Tomsheck et al., 2010). A half-inch strip of agar was removed from the centre of a PDA plate, creating two distinct halves. The fungus (AS) was cultivated on half of the plate and their growth measured after 7 days. After 7 days, the other half was inoculated with test the organism, S. sclerotiorum, via agar plugs (Fig. 1). After two additional days the radial growth of the test organism was determined by averaging three measurements taken from the edge of the agar plug to the edge of mycelial growth in three different directions. Inhibition of the test organism was determined by comparing average growth values with controls grown in the absence of AS and is directly proportional to the amount of cineole present in the gas phase. This assay was established using varying amounts of authentic cineole in the assay instead of the fungus itself. Unless otherwise indicated, all GBAs were conducted in triplicate.

**Establishing a standardized bioassay for the CPM.** To determine whether the CPM was present in individual plant specimens and extracts during the purification, it was necessary to modify the bioassay test system described above. Sterile plant material (20 mg) was placed on top of the PDA and an agar plug supporting mycelial growth (AS) was placed directly on top of the plant material. For plant extracts, a known mass of the extract was dried, resuspended in methanol and pipetted onto the surface of half of the PDA plate. The methanol was then allowed to evaporate from the plate before an agar plug of AS mycelia was placed directly on top of the dried extract material. Then the amount of cineole produced by the AS would directly affect the test organism (S. sclerotiorum) as described above.

With the exception of C. fistula all plants were sampled from various locations across the Montana State University Bozeman campus. Sampling was done in a non-invasive manner by clipping the end of a single, small, leaf-bearing branch from each plant. Note that the plants sampled were selected at random and there was no reason to predict the effects of any one plant prior to testing. The plant giving the greatest biological effect was chosen for further study.

**Purification of the CPM.** Purification of the CPM began with water extraction and proceeded via organic solvent extraction, silica column chromatography, TLC and a final step in silica gel column chromatography. A GBA was employed following each purification step as a means of BGF to determine which fraction from a purification procedure possessed the most bioactivity. In summary, 30 g silver birch (Betula pendula) stems was boiled in 2 litres distilled H₂O for 2 h. The extract was vacuum filtered and evaporated to one-fifth its original volume via rotary evaporation [30 °C/20 ± 3 p.s.i. (138 kPa)]. An extract of the host plant was prepared in the same manner. The water extract was thoroughly mixed with 1 : 1 (v/v) dichloromethane and the solvent was removed via rotary evaporation [30 °C/20 ± 3 p.s.i. (138 kPa)]. The dry extract was resuspended in 2 ml methanol and applied to a 10 x 2.5 cm silica gel column. The column was rinsed with 100 ml dichloromethane, then eluted successively with 50 ml portions of methanol/dichloromethane (v/v) proceeding through 1 : 99, 5 : 95, 10 : 90, 20 : 80, 50 : 50 and 100 : 0. The active fractions were resuspended in 1 ml methanol and separated on EMD silica gel 60 F₂₅₄ glass plates (20 x 20 cm) with dichloromethane/methanol (8.5 : 1.5, v/v) (solvent A). Other TLC solvent systems included hexane/ethyl acetate (1 : 1, v/v) (solvent B) and chloroform/acetonitrile (8.5 : 1.5, v/v) (solvent C). Bands visible in solvent system A at 254 nm were collected and the products were removed from the silica by washing with methanol. The most active silver birch TLC product (Rᵣ=0.76) was ultimately placed on a silica column with dichloromethane/methanol (98 : 2, v/v), yielding approximately 1 mg of the final product, termed CPM₁. A portion of the final product was used for solution NMR and the remainder was tested for bioactivity in the GBA.

**NMR.** Solution NMR analysis was performed on the CPM₁ using a 5 mm NMR tube that was susceptibility matched to methanol to allow a restricted sample volume of roughly 0.25 ml to be used. All NMR analyses were performed on a Varian Inova 500 MHz spectrometer. Data obtained include a 1D ¹H spectrum, and heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond
correlation (HMBC) and correlated spectroscopy (COSY) spectra. A recycle time of 1 s was used for all analyses. The HSQC analysis was performed using \( \omega_1 \) and \( \omega_2 \) spectral widths of 25.13 and 8.01 kHz, respectively, together with 512 evolution increments of 16 scans each. Total analysis time was 7.1 h. The HMBC spectrum was obtained using \( \omega_1 \) and \( \omega_2 \) spectral widths of 30.17 and 8.01 kHz, respectively, together with 400 evolution increments of 16 scans each. Total analysis time was 5.7 h. The COSY spectrum was acquired using 400 evolution increments of eight scans each together with a spectral width of 8.01 kHz in both dimensions. The analysis time for COSY was 1.4 h.

HPLC-ES-MS. HPLC-ES-MS with a reversed-phase column was used to obtain the molecular masses and purity of compounds obtained from various steps in CPM purification. An Agilent Zorbax C18 column with 1.8 \( \mu \)m particles and dimensions of 50 mm length and 2.1 mm diameter was used for separation, with an Agilent 1290 HPLC system. The column was maintained at 50 °C with a flow rate of 800 \( \mu \)l min \(^{-1}\). Chromatography was as follows: the solvent consisted of water with 0.1% (v/v) formic acid for channel 'A' and acetonitrile with 0.1% formic acid for channel 'B'. Following column equilibration at 5% B, the sample was injected via autosampler, and the column was flushed for 0.5 min to waste. From 0.5 min to the end of the run, the column eluant was directed to the MS source. From 0.5 to 7.5 min, the gradient was linearly ramped from 5 to 95% B. From 7.5 to 9.5 min, the gradient was held at 95%, and from 9.5 to 10.0 min the gradient was held at 5% for re-equilibration. A UV detector was used in-line following the column and prior to the mass spectrometer.

The mass spectrometer used was an Agilent 6538 Q-TOF with dual-ES source; resolution is approximately 20 000 and accuracy is 1 p.p.m. Source parameters were as follows: drying gas 12 l min \(^{-1}\), nebulizer 55 psi (379.5 kPa), capillary voltage 3500 V, capillary exit 120 V. Spectra were collected in positive mode from 50 to 1000 m/z at a rate of 2 Hz. The compounds of interest were detected using a combination of UV detection at 254 nm, the MS intensity of ions above background, and comparison between various analytical samples and blanks.

Fragmentation studies of the compounds were carried out on a second mass spectrometer, a Bruker 'maXis Impact' Q-TOF. Elution fractions from the 1290 HPLC system were collected manually and introduced via direct infusion through the maXis ES source at a rate of 180 \( \mu \)l h \(^{-1}\). The ion of interest was selected for isolation, and source parameters and fragmentation parameters were screened for optimal ion production; energies ranged between 5 and 60 eV.

RESULTS

Hypoxylon sp., the subject of this study

The fungal isolate (Ti-13) herein designated Nodulisporium sp. (perfect stage=Hypoxylon sp.) was obtained from the highlands of western Thailand as an endophyte of C. fistula. No signs or symptoms existed on the plant from which the sample was acquired. Identity of the organism was based on physical characteristics using SEM, light microscopy, molecular techniques via partial sequencing of its ITS 5.8S rRNA gene and comparison of these data with observations of comparable organisms described in the literature (Hanlin, 1998; Tomshcek et al., 2010). A 10-day-old OS culture on PDA was circular and a uniform creamy white throughout. The outermost 0.5–0.75 cm of mycelia formed a dense band approaching pure white. As viewed by SEM, the mycelium produced erect, branching conidiophores possessing elongated conidiogenous cells attached in irregular patterns (Fig. 2a). Conidiogenous cells terminated in clusters of one-celled ellipsoid to clavate–ellipsoid conidia hyaline (5–6 \( \times \) 2.5–4 \( \mu \)m) at the apical end (Fig. 2a, b). Based on morphology, the fungus appeared as a Nodulisporium species, the anamorphic stage of Hypoxylon sp.

While SEM depicted the anamorphic stage of the isolate, Nodulisporium sp., comparisons based on the ITS1–5.8S–ITS2 ribosomal gene sequence gave the closest match of the organism as the teleomorph, Hypoxylon sp. Highest sequence similarity (99%) was with Nodulisporium sp. CMUUP-E34 (accession no. JN558831.1) and Hypoxylon sp. WHCS-8 (accession no. JQ362418.1) (Fig. 3). However, the highest sequence similarity at the species level was only 90% with Hypoxylon anthochroum (sequence coverage of 95%). Thus, species-level identification could not be ascertained for this organism. The ITS1–5.8S–ITS2 ribosomal gene sequence of Ti-13 has been deposited in GenBank with accession number KJ 558391. Attempts to produce the perfect stage of this fungus were not successful, and thus a species description of the teleomorphic stage of this organism cannot be made with certainty at this point.

Effects of attenuation on cineole production by Ti-13

Initial bioactivity and olfactory-based observations suggested a decrease in cineole production by Ti-13 (OS) following attenuation, as well as a subsequent increase in production following recovery of the AS. This observation was confirmed quantitatively by examining the VOC profile of each strain with PTR-MS (Fig. 4). VOC concentration estimates based on the cineole peaks (m/z 155, 137 and 81) indicated that attenuation of the OS to the AS results in an approximately 50% drop in cineole concentration in the headspace of 10-day-old Ti-13 cultures. Recovery of the AS to the RS was associated with complete reversal of this effect, shifting cineole concentration above that observed even for the OS (Fig. 4a). Ethanol and acetaldehyde were major species, together accounting for 65–85% of the total VOC profiles of each strain (Fig. 4b). Unexpectedly, the total VOC concentration for the RS was substantially lower than for both the OS and the AS. The explanation for this observation is unknown. The PTR result substantially confirmed the observation that it was possible, via serial transfer of the OS, to produce an AS that was much reduced in cineole production. Then ultimately, the AS could be reversed by growing the fungus on plant material (possessing a CPM) that would restore cineole production to the RS.

Bioactivity of cineole

The inhibitory effects of cineole on the growth of S. sclerotiorum were assessed to provide a basis for a qualitative measurement of cineole production by Ti-13 (Fig. 5). This
method was deemed more accurate and less time consuming than the solid phase microextraction technique that is commonly used to acquire composition analysis of VOCs (Strobel, 2014). Cineole was added to sterile microcups in a GBA instead of inoculating the plate with the AS and the assay was effective in a linear range from 0 to 5 μl of cineole per Petri plate (with 50 ml dead air volume above the agar surface). S. sclerotiorum exhibited a gradual decline in growth and was not killed over a range of cineole concentrations resembling those produced by the AS, indicating that it was a suitable test organism for the qualitative determination of cineole production by Ti-13 via GBAs.

Plants as a source of the CPM molecule

Initially, the host plant (C. fistula) of the fungus was examined for the CPM using the methods described above. When subjected to TLC the active column fraction yielded bands with $R_f$ values of 0.76 and 0.84 at 254 nm. These were collected and tested for potential modulatory effects on cineole production by the fungus (AS). The assays (GBA) indicated that 60% inhibition of S. sclerotiorum could be achieved when the AS was grown on 0.16 mg of the $R_f = 0.84$ product and less than 0.10 mg of the $R_f = 0.76$ product (Table 1). HPLC-ES-MS of the host extract TLC products revealed a complex mixture intractable to further purification efforts in that more than 20 compounds were present in this fraction.

As the host plant extract possessed CPM activity, but was a chemically complex mixture, a readily available and simpler source of the activator was sought. Of nine plants tested, silver birch (B. pendula) stems exhibited the greatest repeatable modulatory effect on the bioactivity of the AS. TLC of the active column fraction (silver birch) produced the same banding pattern as the host fraction, although the bands were fainter. These TLC products also had modulatory
effects on bioactivity of the AS, but more product was needed to achieve the same level of inhibition of the test organism (Table 1). Nevertheless, the CPM of silver birch was sought given that it seemed to be less chemically complex.

Successive fractionation of silver birch consistently produced a product with more specific bioactivity than the starting material, as determined by the GBAs (Table 1). The different fractions were compared based on 60% inhibition of \textit{S. sclerotiorum} because this number was within experimental observations for each bioassay and was substantial enough to eliminate experimental artefacts. A GBA with the column-cleaned TLC product remaining after NMR (CPM1) indicated a high level of purity and bioactivity (Table 1). Only 0.31 mg of this product was needed to achieve 60% inhibition of \textit{S. sclerotiorum}, and cultivation of the AS on 0.40 mg of the product resulted in 81% inhibition of \textit{S. sclerotiorum}.

The greatest increase in purity was achieved following dichloromethane extraction, and cultivation of the AS on only 8 mg of the dichloromethane extract of silver birch was sufficient to induce complete inhibition of \textit{S. sclerotiorum}. As the bioactivity of the AS increased, its radial growth decreased, suggesting that increased bioactivity due to modulator effects is not simply a secondary effect resulting from increased viability.

### Characteristics of the CPM

Two CPMs were isolated from the stems of silver birch. The products had $R_f$ values of 0.76 and 0.84 under the TLC conditions outlined, and this was the same TLC banding pattern observed for extracts of the host plant (Table 2). Although the banding pattern was the same, product yields

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**Fig. 4.** PTR-MS quantification of VOC production by Ti-13. Concentrations are given in parts per billion expressed per volume and have not been adjusted for dilution (dilution factor = 11). (a) Cineole production by Ti-13 based on $m/z$ 155, 137 and $m/z$ 81. Areas beneath the peaks represent cineole concentration as a function of sampling time (one cycle = 1.67 min). (b) Total VOC concentration in the head space of 10-day-old OS, AS and RS cultures. The relative contributions of acetaldehyde and ethanol to the total are shown.

**Fig. 5.** Inhibitory effects of cineole on growth of \textit{S. sclerotiorum}. Each data point corresponds to a mean of three measurements of radial growth extending from the edge of the agar plug to the edge of mycelial growth in three directions in a GBA in which cineole was added to sterile microcups in 1, 2, 3, 4 and 5 μl volumes in lieu of inoculation with the AS. Linear regression was performed to derive a standard curve for the qualitative assessment of cineole production by the AS in other GBAs ($R^2$ = 0.91).
and UV absorbancies were different (Table 2). The two silver birch TLC products were analysed by two additional TLC systems, namely B and C. In each case again, only two bands were visible at 254 nm (Table 2b). Of the two products, the one with \( R_f \) of 0.76 in the TLC solvent A was cleaned on a silica column and termed CPM1 and was selected for structural studies as it possessed the greatest specific bioactivity relative to the modulation of cineole production (Table 1).

A \(^1\)H-NMR analysis of CPM1 indicated that peaks at 7.11 and 6.78 were suggestive of a \( \text{para} \)-substituted benzene component while signals up-field of this (0.5–2.2 p.p.m.) were assignable to methyl and methylenes (Silverstein et al., 1974) (Fig. 6c). Commonly, protons on carbons bearing oxygen resonate at 3.3–4.0 p.p.m. (Silverstein et al., 1974). Further 2D NMR analyses by HMBC and HMQC revealed that the structure is an asymmetrical ether containing a \( \text{p} \)-substituted benzene with a \( \text{CH}_2–\text{R} \) at position 4 on the benzene ring. However, on position 1 there is an ether oxygen linked to a \( \text{CH}(	ext{CH}_3)(\text{CH}_2)–\text{R} \) as the second functional group on the benzene ring (Fig. 6a). Also, the HMBC evidence suggests the presence of one or two carboxyl groups and they could be associated with either one or the other or both of the \( \text{R} \) functionalities (Fig. 6b). Finally, HPLC-ES-MS on CPM1 revealed that, despite all of the purification steps taken to yield a pure product, a peak with UV absorbancies at 210, 280 and 350 nm appeared with a retention time of 3.43 min and a mass of 194.94 Da. This is consistent with an earlier LC/MS analysis of a CPM1 preparation that produced a major peak at or near this retention time. However, as suggested by the \(^1\)H-NMR analysis, a number of other compounds (in small amounts) were present in CPM1 and appeared in the LC/MS analysis with retention times, among others, at 5.5, 7.0 and 7.4 min. Note that the relatively small mass defect in all of these analyses suggests that the compounds belong to the same class of chemical substances, which is in agreement with the NMR analysis. Overall, it appears that the product is recalcitrant to standard purification procedures and that it

### Table 1. Comparison of the host and silver birch fractions at various steps throughout the purification process

The small amount of crude host plant material available precluded detailed analysis of all but the TLC fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mass needed for 60% ( S. ) ( \text{sclerotiorum} ) inhibition (mg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( C. ) ( \text{fistula} )</td>
</tr>
<tr>
<td>Crude plant material</td>
<td>–</td>
</tr>
<tr>
<td>Dichloromethane extract</td>
<td>–</td>
</tr>
<tr>
<td>Active column fraction</td>
<td>–</td>
</tr>
<tr>
<td>TLC, solvent A (( R_f )=0.84)</td>
<td>0.16</td>
</tr>
<tr>
<td>TLC, solvent A (( R_f )=0.76)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CPM1</td>
<td>–</td>
</tr>
</tbody>
</table>

### Table 2. Characteristics of the CPMs

(a) Absorbancies and product yields for the host and silver birch TLC products. (b) \( R_f \) values for the silver birch TLC products under three solvent systems. UV absorption data were obtained using a Beckman DU 50 spectrophotometer with the compounds suspended in 1 ml methanol. Path length was 1 cm. Product recovery values were obtained by performing the outlined purification procedure using 15 g \( B. \) \( \text{pendula} \) and \( C. \) \( \text{fistula} \) plant material. All TLC was done on EMD silica gel 60 \( F_{254} \) glass plates.

<table>
<thead>
<tr>
<th>(a) Fraction (( R_f ))</th>
<th>Wavelength (nm)</th>
<th>Absorbance</th>
<th>Recovery ([\text{mg product (g plant material)}^{-1}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C. ) ( \text{fistula} ) (0.76)</td>
<td>316.75</td>
<td>1.335</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td>52.5</td>
<td>1.935</td>
<td>–</td>
</tr>
<tr>
<td>( C. ) ( \text{fistula} ) (0.84)</td>
<td>120.75</td>
<td>1.94</td>
<td>0.257</td>
</tr>
<tr>
<td>( B. ) ( \text{pendula} ) (0.76)</td>
<td>336</td>
<td>0.9114</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>269.5</td>
<td>1.576</td>
<td>–</td>
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<tr>
<td>( B. ) ( \text{pendula} ) (0.84)</td>
<td>325.5</td>
<td>0.9408</td>
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<tr>
<td></td>
<td>269.5</td>
<td>1.876</td>
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</table>

<table>
<thead>
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<th>(b) ( R_f ) values</th>
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<tbody>
<tr>
<td>Solvent A</td>
</tr>
<tr>
<td>0.84</td>
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<tr>
<td>0.76</td>
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</table>

J. Nigg and others
contains a base structure of a \( p \)-substituted benzene ether having alkyl and carboxyl substituents, but it is too early to make structural assignments that are more extensive than that presented (Fig. 6). Finally, further work is needed to refine the purification and characterization of one or more of the constituents in CPM1. Other efforts will be needed to confirm the nature of the components of CPM2.

**Other compounds as bioactivity modulators**

As the spectral studies revealed that the CPM1 is a \( p \)-substituted benzene, it was deemed fitting to test already-known compounds as potential modulators of cineole production. Three readily available substituted benzene compounds and one non-benzene compound with plant origins (giberellic acid) were used in a GBA as described for plant extracts to determine whether the effects of the CPM were limited to the plant extract or whether they could be reproduced, to some degree, by commercially available compounds. This was done to rule out the possibility that the putative CPM was merely a metabolic precursor to cineole. Ferulic acid, gallic acid, vanillin and giberellic acid were used, and each had an increasing effect on the bioactivity of the AS (Table 3). Giberellic acid had the strongest effect, with cultivation of the AS on 25 \( \mu \)mol giberellic acid resulting in an estimated cineole production of 7.10 \( \mu \)l, compared with only 2.74 \( \mu \)l when the AS was grown on PDA alone. Ferulic acid also had a strong effect, treatment with 25 \( \mu \)mol resulting in an estimated cineole production of 5.92 \( \mu \)l. The results suggest that the cineole modulation effect is not entirely restricted to substances that are \( p \)-substituted benzenes, as giberellic acid was an effective promoter of cineole production.

**DISCUSSION**

The observation of reversible attenuation of cineole production by Ti-13 upon removal from the host suggested the presence of one or more CPMs in the host. Two CPMs were purified from silver birch by BGF and their biological activities were confirmed by bioassays. When cultivated on less than 0.5 mg of either product, cineole production of the AS could be restored to levels comparable with those of the OS and changes in cineole production did not correlate with changes in the growth stage of the organism. NMR and HPLC-ES-MS of CPM1 revealed that the compound is a mixture of \( p \)-substituted benzenes with a major

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**Fig. 6.** NMR data for CPM1. (a) Proposed structure for CPM1 based on NMR data. (b) Assignments and HMBC correlations for CPM1. (c) \(^1\)H-NMR spectra of CPM1. Negative peaks at 3.2, 4.6 and 4.8 p.p.m. represent partially suppressed solvent resonances.
component having a mass of 194.94 Da with other related compounds present presumably having greater or lesser numbers of carbons in the aliphatic side chain and one or two carboxyl moieties.

To rule out the possibility that CPM₁ is simply a metabolic precursor to cineole, rather than a modulator, four other compounds were examined for potential modulatory effects on cineole production. A GBA using pure cineole instead of inoculation with the AS indicated that the growth of *S. sclerotiorum* is inhibited by 60% when cultivated alongside 5.5 ml cineole (Fig. 5). This level of cineole production could be achieved when the AS was cultivated on just 1.59 mmol CPM₁, but GBAs with giberellic acid, ferulic acid, vanillin and gallic acid indicated that 15.0, 19.8, 28.9 and 34.7 mmol of each compound, respectively, was required to increase the production of cineole by the AS to this level (Table 3). The heightened activity of CPM₁ relative to these other compounds suggests that it truly has modulatory effects and is not merely a precursor to cineole.

Regulation of endophytic secondary metabolism by the host is not a novel phenomenon. For example, Fleetwood et al. (2007) identified a gene cluster in *Neotyphodium lolii*, an endophyte of grasses of the subfamily Pooidae, that is putatively involved in ergovaline biosynthesis and highly expressed in planta but, for reasons as yet undetermined, is either unexpressed or expressed only at low levels in axenic cultures. Another study showed that production of herbivory-deterring loline alkaloids by endophytic *Neotyphodium uncinatum* and *Neotyphodium siegelii* isolates is regulated in planta by precursor availability (Zhang et al., 2009). Indeed, regulation of endophytic secondary metabolism by the host is a commonly observed phenomenon and this regulation can be based on both gene expression and substrate availability (Kusari et al., 2012). In the case of substrate availability, some studies have successfully elucidated the precursors provided by the plant that are required for fungal synthesis of the compound of interest (Zhang et al., 2009). Host-derived compounds involved with the host-mediated modulation of the expression of genes involved with fungal secondary metabolism, however, remain elusive.

The results of GBAs using giberellic acid, vanillin, ferulic acid and gallic acid suggest that in the present study, regulation of fungal secondary metabolism by CPM₁ is based on gene expression rather than substrate availability. The information about CPM₁ presented here thus provides a first glimpse at a non-substrate, host-derived genetic regulator of fungal endophyte secondary metabolism.

Recent genome sequencing efforts have revealed that previous estimates of the diversity of fungal secondary metabolism do not approach the enormous diversity that is probably present (Brakhage, 2013; Wiemann & Keller, 2014). The majority of secondary metabolite biosynthesis gene clusters are silent under standard cultivation conditions and a lack of knowledge regarding how these clusters might be activated is currently a major factor limiting the discovery of novel and useful fungal secondary metabolites (Brakhage, 2013). Investigation into how CPM₁ modulates cineole production by Ti-13 could reveal genetic mechanisms of the

### Table 3. Inhibitory effects of the AS against *S. sclerotiorum* when supplemented with the indicated compounds in GBAs

The AS was cultivated on the indicated amount of each compound as described in the GBA procedure. Inhibition values represent the median inhibition of *S. sclerotiorum* in three trials as compared with controls grown alone. Estimated cineole production is based on the equation of the trend line shown in Fig. 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (μmol)</th>
<th>Median inhibition (%)</th>
<th>Estimated cineole production (μl)</th>
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<td>Ferulic acid</td>
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<td>5</td>
<td>41.2</td>
<td>3.80</td>
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<tr>
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<td>52.9</td>
<td>4.86</td>
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<tr>
<td></td>
<td>15</td>
<td>52.9</td>
<td>4.86</td>
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<tr>
<td></td>
<td>25</td>
<td>64.7</td>
<td>5.92</td>
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<td>Gallic acid</td>
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<td>2.74</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>41.2</td>
<td>3.80</td>
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<td>25</td>
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<tr>
<td>Giberellic acid</td>
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regulation of secondary metabolite biosynthesis in this organism and such findings could potentially be extrapolated to other systems. Furthermore, the present data suggest that modification of the genetics of the organism via the stated or other techniques may offer a mechanism to enhance cineole production.

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REFERENCES


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