









Cite this: *Org. Biomol. Chem.*, 2017, **15**, 5357

The formation of sesquiterpenoid presilphiperfolane and cameroonane metabolites in the *Bcbot4* null mutant of *Botrytis cinerea*†

Gabriel Franco dos Santos,  ‡^{a,b} Javier Moraga,  ‡^a Jacqueline A. Takahashi,  ^b Muriel Viaud,  ^c James R. Hanson, ^d Rosario Hernández Galán  ^a and Isidro G. Collado  *^a

Botrytis cinerea is a polyphagous fungal parasite which causes serious damage to more than 200 plant species and consequent economic losses for commercial crops. This pathogen produces two families of phytotoxins, the botryanes and botcinins, which are involved in the infection mechanism. The *B. cinerea* genome has provided a complete picture of the genes involved in the biosynthesis of its secondary metabolites. The botrydial biosynthetic gene cluster has been identified. This cluster consists of seven genes, where the genes *BcBOT1*, *BcBOT3* and *BcBOT4* encode three mono-oxygenases. A study of the *Bcbot4Δ* null mutant revealed that this mono-oxygenase was involved in the hydroxylation at C-4 of the probotryane skeleton (C-11 of the presilphiperfolane skeleton). A detailed study of the *Bcbot4Δ* null mutant has been undertaken in order to study the metabolic fate of the presilphiperfolan-8-ol intermediate biosynthesized by this organism and in particular by this strain. As a result three new presilphiperfolanes and three new cameroonanes have been identified. The results suggest that the absence of the oxygen function at C-11 of the presilphiperfolane skeleton permits rearrangement to a cameroonane whilst hydroxylation at C-11 precludes this rearrangement. It is possible that the interactions of the C-11 hydroxylated derivatives perturb the stereo-electronic requirements for the migration of the C-11:C-7 sigma bond to C-8.

Received 4th May 2017,
Accepted 31st May 2017

DOI: 10.1039/c7ob01088e

rsc.li/obc

Introduction

Fungi from the genus *Botrytis* are widespread, having been identified in cold regions such as Alaska and Canada as well as in sub-tropical regions such as Egypt. They are necrotrophic parasites which cause serious economic losses to commercial crops. The polyphagous species *Botrytis cinerea* has been identified as a pathogen of more than 200 plant species, often attacking damaged parts of the plant before spreading to healthy tissues. The most common symptoms involve the

development of necrotic lesions on the leaves.^{1–4} The initiation and progress of the infection by *B. cinerea* involves a complex sequence of biological events including both enzymatic attack and the production of phytotoxic secondary metabolites.^{5,6} However in accordance with the broad range of host of this pathogen, there is no evidence for the production of host-specific toxins. The toxins produced by *B. cinerea* not only reproduce the symptoms of a fungal infection but have also been detected in plant tissues infected by *B. cinerea*.^{5,7–9} Two major families of phytotoxic metabolites are sesquiterpenes related to botrydial^{5,9} (**1**) and the polyketides, 3-acetylbotcinic and 3-acetylbotcinic acids (**2**, **3**) and their botcinin (**4**, **5**) relatives, Scheme 1.^{10–12} Botrydial (**1**) is produced during the infection of a plant and induces chlorosis and cell collapse.¹³ 3-Acetylbotcinic acid (**2**) and its derivatives also induce chlorosis and necrosis and have some anti-fungal activity, (Scheme 1).¹²

The sequencing of the *B. cinerea* genome has led to the identification of the botrydial biosynthetic gene cluster, Fig. 1.^{14,15} Such gene clusters are typical of the secondary metabolism in filamentous fungi.^{16,17} This cluster consists of seven genes (*BcBOT1* to *BcBOT7*) coding for a sesquiterpene cyclase (*BcBOT2*), an acetyltransferase (*BcBOT5*), a transcription

^aDepartamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain. E-mail: isidro.gonzalez@uca.es

^bDepartamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, MG, Brazil

^cUMR BIOGER, INRA, AgroParisTech, Université Paris-Saclay, 78850 Thiverval-Grignon, France

^dDepartment of Organic Chemistry, University of Sussex, Brighton, Sussex, BN1 9QJ, UK

† Electronic supplementary information (ESI) available: Details of spectroscopic and characterization data, and reproduction of ¹H- and ¹³C-NMR spectra. CCDC 1547406. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c7ob01088e

‡ These authors contributed equally to this work.



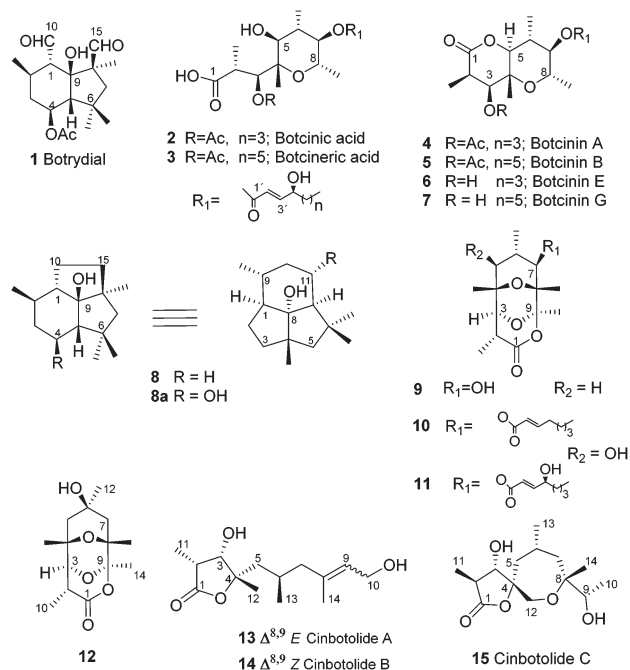
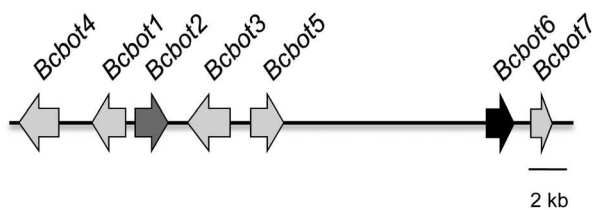
Scheme 1 Some metabolites isolated from *B. cinerea*.

Fig. 1 The botrydial biosynthetic gene cluster.

factor (*BcBOT6*), a putative dehydrogenase (*BcBOT7*) and three mono-oxygenases (*BcBOT1*, *BcBOT3* and *BcBOT4*).^{17–19}

The functional role of these genes in botrydial biosynthesis has been identified using mutants.^{15,18,19} The metabolites which were produced by the null mutants *Bcbot3Δ* and *Bcbot4Δ* were analysed after five days fermentation¹⁹ at the time of maximum botrydial production (Scheme 1).²⁰ The fermentation of the *BcBOT4* mutant revealed that the related mono-oxygenase was involved in the hydroxylation at C-11 (C-4 in botrydial numbering, see 8) of the presilpiperfolan-8-ol intermediate (8) in the biosynthesis. No other intermediates in the botrydial pathway were detected and, at this stage in the fermentation, compound 8 accumulated. When botrydial production was disrupted, larger quantities than normal of the polyketide metabolites were formed including compounds (2–7), the botrydialones (9–12) and the cinbotolides A–C (13–15).¹⁹

Since a large amount of compound 8 was isolated after the five-day fermentation of the *Bcbot4Δ* null mutant,¹⁹ it was of interest to study its metabolic fate after longer periods of fermentation. The structures of these metabolites form the subject of this paper.

Results and discussion

The mutant *Bcbot4Δ* was grown on a malt agar medium (1 L) for 10, 17 and 21 days. A detailed TLC analysis of the extracts revealed significant differences between the 10 and 17 day fermentations when compared to a 5 day fermentation. The mutant was then grown on a malt agar medium (6 L) for 17 days at 23 °C under white light. The metabolites were recovered from the medium by extraction with ethyl acetate and the constituents of the extract (6.7 g) were separated by chromatography. A small amount of compound 8 was obtained from the less polar fraction. The polyketides 2–7 and 9–15 were also isolated and identified by comparison of their spectroscopic data with those of authentic samples.¹⁹ Six new compounds (16–21) were isolated from the fractions of medium polarity and purified by further chromatography including HPLC. The ¹H and ¹³C NMR spectra suggested that they possessed sesquiterpenoid structures.

The HRMS and ¹³C NMR data for compound 16 which were obtained as a colourless crystalline solid, corresponded to a molecular formula of C₁₅H₂₆O₃. It possessed IR absorption at 3430 cm⁻¹ (OH). The ¹³C NMR spectrum, aided by DEPT and HSQC experiments, (see ESI, Table S1†) exhibited three methyl group signals, five methylenes including one hydroxymethyl group, four methines including one secondary alcohol and three quaternary carbons including a tertiary alcohol. The three methyl group signals in the ¹H NMR spectrum were a doublet and two singlets, whilst the hydroxymethyl group signal only contained a geminal coupling. The secondary alcohol resonance was a doublet of doublets. These data suggested that compound 16 was a dihydroxypresilpiperfolan-8-ol. The HMBC spectrum revealed correlations between the signal for the secondary alcohol at δ_H 3.25 and C-7, C-9, C-11 and C-15 and between the hydroxymethyl group at δ 3.35 and C-6 thus establishing the location of the extra hydroxyl groups. The proposed structure for compound 16 was confirmed by extensive HMBC, HSQC and NOESY experiments (see Table S1 in the ESI†). In particular there was a NOESY correlation between the signal at δ_H 3.25 (H-10) and H-7α and H-15 indicating a β configuration for this hydroxyl group. Finally the structure was confirmed by X-ray crystallography (Fig. 2). On acetylation with acetic anhydride in pyridine, compound 16 formed a diacetate 16a (OAc δ_H 2.05 and 2.08) in which the signals for H-14 and H-10 were shifted to δ_H 4.0 and 4.6 respectively.

The minor metabolites 17 and 18 showed ¹H NMR signals which were similar to those of compound 16 retaining the signals for the three methyl groups, the tertiary alcohol and the hydroxymethyl group. The principle difference in the spectra of 17 was the lack of the CH(OH) signal at δ_H 3.25 and the presence in the ¹³C NMR spectrum of a carbonyl signal at δ_C 213.7. The H-11 signals were deshielded to δ_H 2.34 and 2.52. Consequently compound 17 was the oxidation product of 16 at C-10, a conclusion supported by 2D COSY, HMBC and HSQC experiments (see Table S2, ESI†). The major difference in the NMR spectra of compound 18 compared to that of com-



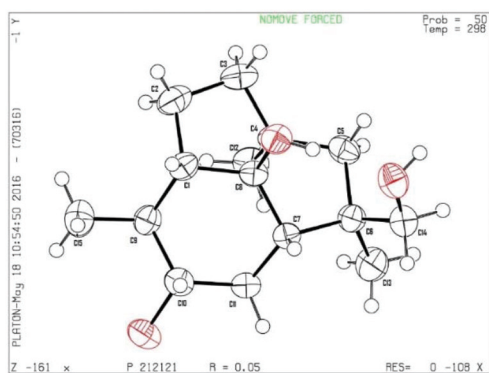


Fig. 2 X-Ray structure of compound **16** with the presilphiperfolane carbon numbering.

Compound **16** was the presence of alkene signals at δ_{H} 4.94 (dd, J 2.1 and 2.5 Hz) and δ_{C} of 148.7 and 116.2 corresponding to a trisubstituted alkene. The location of the alkene between C-1 and C-2 was established by the deshielding of the methyl group doublet and the H-3 signals at δ_{H} 2.40 (1H, dd, J 17 and 2.1 Hz) and 2.25 (1H, dt, J 17 and 2.5 Hz). In the HMBC spectrum (see Table S3, ESI[†]) there were correlations between H-2 and C-3, C-4 and C-8 and between H-15 and C-1. Thus the structures of **16–18** were presilphiperfolan-8 α ,10 β ,14-triol (**16**), 8 α ,14-dihydroxypresilphiperfolan-10-one (**17**) and 8 α ,10 β ,14-trihydroxypresilphiperfol-1-ene (**18**).

The HRMS and ^{13}C NMR data for compound **19** corresponded to a molecular formula, $\text{C}_{15}\text{H}_{26}\text{O}_3$. The ^1H and ^{13}C NMR spectra, see Table S4,† revealed the presence of one secondary methyl group and two tertiary methyl groups, four methylene and one hydroxymethyl group, two secondary alcohols and two further methine carbons and three quaternary carbons. Whilst there were similarities to the spectra of the presilphiperfolanetriol **16**, there was a major difference in the absence of the tertiary alcohol. However the spectra were in accordance with a cameroonane carbon skeleton. Cameroonane-7 α -ol has previously been obtained by the rearrangement of presilphiperfolan-8 α -ol.^{21,22} The ^1H NMR signal of one of the secondary alcohols was a singlet (δ_{H} 3.89) indicating that this alcohol was situated between two quaternary carbons typical of the cameroonane-7 α -ol. The other secondary alcohol was a doublet of doublets (δ_{H} 3.72, ddd, J 11, 9.7 and 6.0 Hz). Two of these couplings were assigned to a methylene (δ_{H} 1.73 and 1.65). Apart from their geminal coupling (J 12.2 Hz), these resonances possessed no additional couplings. Together with the HMBC spectra, this was consistent with the location of this hydroxyl group at C-10 (see Table S4, ESI[†]). The stereochemistry of the alcohols was established by a NOESY correlation between the H-10 resonance and H-15, between H-7 and H-12 and H-9, and between H-14 and H-1. Furthermore when compared to the ^{13}C NMR spectrum of cameroonane-7 α -ol there were γ -*gauche* shieldings by the C-10 β hydroxyl group of the resonances of C-1, C-8 and C-15 and by the C-14 hydroxyl group of C-5. This compound formed a triacetate (**19a**), the spectra of which were consistent

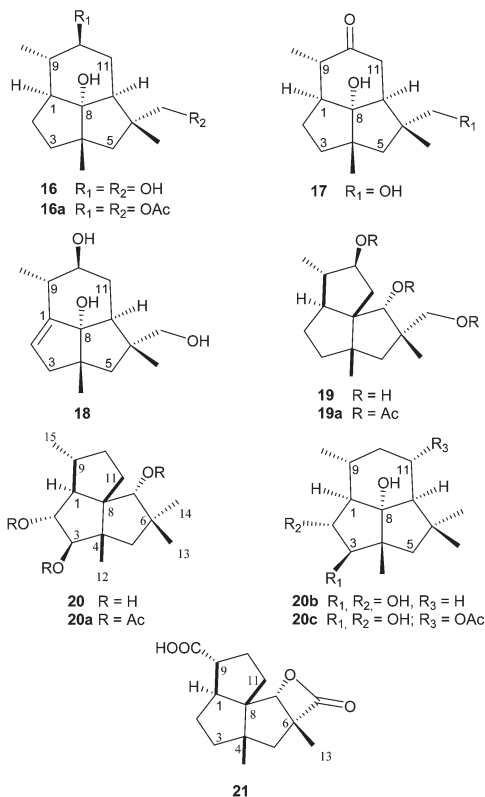
with the formulation of the parent alcohol as cameroonane-7 α ,10 β ,14-triol (**19**).

The ^{13}C NMR spectrum (Table S5[†]) of compound **20** contained signals assigned to four methyl groups, three methylenes, five methines, three of which were secondary alcohols and three quaternary carbon atoms. The pattern of the ^1H NMR signals was similar to that of compound **19** particularly in the presence of the singlet assigned to H-7. However the signal assigned to H-10 was replaced by a double-doublet (δ 3.49, J 8.6 and 9.1 Hz) and a doublet (δ 3.81, J 9.1 Hz) which were assigned to two secondary alcohols. Their vicinal relationship was revealed by the COSY spectrum whilst their location at C-2 and C-3 followed from HMBC correlations between H-2 and C-9 and C-3 and between H-3 and C-12 and C-5. Their stereochemistry followed from the magnitude of the vicinal coupling constants H-1:H-2 (J 8.6 Hz) and H-2:H-3 (J 9.1 Hz) which corresponded to *trans* relationships between these protons. There were nOe correlations between H-2 and H-9 and between H-3 and H-1, H-5 α and H-14. The stereochemistry of the alcohol at C-7 followed from the nOe enhancements of H-11 β and H-13 on irradiation of H-7. Acetylation of the triol gave a triacetate (**20a**) with NMR data (see Experimental) that was consistent with the structure of cameroonane-2 α ,3 β ,7 α -triol for the parent alcohol. It is interesting to note that the stereochemistry of this vicinal diol (**20**) corresponds to that formed from presilphiperfolan-8 α -ol (**20c**) prior to the cleavage of the five-membered ring to form botrydial (**1**). The oxygenases that mediate this step are coded by the genes *BcBOT1* and *BcBOT3*. Molecular models reveal a structural similarity between the presilphiperfolan-8 α -ol substrate and a cameroonane-7 α -ol in which a presilphiperfolanol 11 α -hydroxyl group and a cameroonane-7 α -hydroxyl group would also be juxtaposed.

Compound **21** was the most polar metabolite. The ^1H and ^{13}C NMR spectra, (Table S6[†]) contained signals assigned to two tertiary methyl groups, five methylenes, three methines and three quaternary carbon atoms and two carbonyl groups at δ_{C} 180.6 and 175.7. These were assigned to a carboxylic acid and an ester function. The characteristic cameroonane-7 α -ol, H-7, singlet was shifted to δ_{H} 4.27 and the presence of IR absorption at 1810 cm^{-1} was indicative of the presence of a β -lactone. There were HMBC correlations between H-12 and C-3, C-4, C-5 and C-8 and between H-13 and C-5, C-6, C-7 and C-14 thus locating the carboxyl group at C-15. Compound **21** was therefore assigned the structure of 7- α -hydroxycameroonane-14,15-dioic acid 14,7-lactone.

Biosynthetically these cameroonane metabolites may be formed by the rearrangement of presilphiperfolan-8 α -ol which has laboratory precedent.²² The loss of the 8 α -hydroxyl group may generate a C-8 carbocation (**A**) which may be followed by the rearrangement of the C-11:C-7 bond to C-8 to yield a C-7 carbocation (**B**), Scheme 3. This is then discharged by a hydroxyl group to form the cameroonane-7 α -ol (**22**). The geometrical requirements for this rearrangement have been examined. An X-ray crystal structure of the *p*-nitrobenzoate of presilphiperfolan-8 α -ol revealed that there is a *trans*-relationship





Scheme 2 Presilphiperfolanes, cameroonanes and their derivatives.

between the departing 8α -hydroxyl group and the migrating C-11:C-7 bond in a chair six-membered ring. It is likely that this rearrangement leads to the formation of the cameroonanes in *B. cinerea*.²²

In the same line compound 19, Scheme 3, should be formed from the isolated compound 16, while compound 20 must arise from the rearrangement of the hypothetical precursor 20b.

Compounds with the cameroonane carbon skeleton have not been detected previously as the metabolites of *B. cinerea*. Fermentation of the intact *B. cinerea* for 15 and 17 days led to the isolation of botryanes and the presilphiperfolane 20c, Scheme 2, the precursor of botrydial (1) by oxidative cleavage of the C10–C15 bond.¹⁹ The presence of the C-11 hydroxyl

group (C-4 in botryane numbering, see 8) which is introduced by the enzyme system mediated by *BcBOT4*, appears to inhibit the rearrangement. It could be that transannular hydrogen bonding to the C-8 hydroxyl group and interactions between the C-11 hydroxyl group and the methyl groups at C-4, lead to a distortion of the chair form of the six-membered ring and so reduces the facility with which the ring-contraction occurs.

Experimental

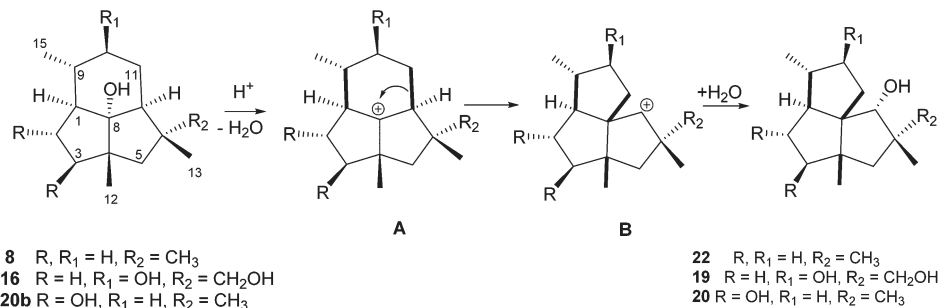
General experimental procedures

Purification by semipreparative and analytical HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a differential refractometer detector (RI-7490). A LiChrospher® Si 60 (5 μm) LiChroCart® (250 mm \times 4 mm) column and a LiChrospher® Si 60 (10 μm) LiChroCart® (250 mm \times 10 mm) were used in isolation experiments. Silica gel (Merck) was used for column chromatography. TLC was performed on Merck Kieselgel 60 F254, 0.25 mm thick. Melting points were measured with a Reichert-Jung Kofler block and are uncorrected. Optical rotations were determined with a digital polarimeter. Infrared spectra were recorded on a FT-IR spectrophotometer and reported as wave number (cm^{-1}). ^1H and ^{13}C NMR measurements were recorded on Agilent 500 and 600 MHz spectrometers with SiMe_4 as the internal reference. Chemical shifts were referenced to CDCl_3 (δ_{H} 7.25, δ_{C} 77.0). NMR assignments were made using a combination of 1D and 2D techniques. Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quarter; quint = quintuplet; sext = sextuplet; m = multiplet, br = broad. High-Resolution Mass Spectroscopy (HRMS) was performed either with a double-focusing magnetic sector mass spectrometer in a QTOF mass spectrometer in positive or negative ion ESI or APCI modes (APGC+ for samples analysed by GC chromatography).

Fungal material

Fungal strains. Strain B05.10 of *Botrytis cinerea* Pers. Fr. was isolated from a *Vitis* field sample.²³

Media and culture conditions. Standard procedures¹⁹ for culturing and maintaining of *B. cinerea* strains and the mutant were performed at 23 $^\circ\text{C}$ with 12 h daylight per day. Cultures



Scheme 3 Rearrangement of presilphiperfolane to the cameroonane skeleton.



were grown on malt agar medium (20 g per L malt extract, 5 g per L yeast extract, 15 g per L agar), and minimal medium (20 g per L glucose, 2 g per L NaNO₃, 1 g per L KH₂PO₄, 500 mg per L MgSO₄·7H₂O, 500 mg per L KCl, 10 mg per L FeSO₄·7H₂O, 15 g per L agar). The transformation medium was a minimal medium supplemented with saccharose (20 g per L glucose, 200 g per L saccharose, 2 g per L NaNO₃, 1 g per L KH₂PO₄, 500 mg per L MgSO₄·7H₂O, 500 mg per L KCl, 10 mg per L FeSO₄·7H₂O, 15 g per L agar). The *bcbot4* mutant was selected with nourseothricin fungicide (Werner Bioagent, Germany- 70 µg ml⁻¹).

For the isolation of metabolites, the null mutant $\Delta Bcbot4$ of *Botrytis cinerea* was cultured in 200 mm Petri dishes containing 100 mL of malt agar medium (6 L) for 17 days at 23 °C under white light. After the growth time, the media were extracted with ethyl acetate. The extracts were dried over sodium sulfate, and the solvent was evaporated to give 6.7 g of extract.

Gene deletion of the cytochrome P450-encoding gene *bcbot4*

The *B. cinerea* gene *bcbot4* (GenBank: AY277723.2) was deleted by homologous recombination. The 5' genomic non-coding regions of *bcbot4* (1442 pb) was amplified by PCR. In the same way, we obtained the 3' regions of *bcbot4* (866 pb) and the nourseothricin resistance gene (*nat*). For each gene, a Knock-Out (KO) cassette consisting of the 5' region of *bcbot*, the *nat* resistance gene and the 3' region *bcbot* was generated by double-joint PCR fusion²⁴ as previously described for *B. cinerea*.²⁵ All the PCR primers have been reported.¹⁹

Protoplasts from the B05.10 strain were prepared and transformed as previously described.^{19,26} Additionally, the genetic purity of the KO transformants was checked by the absence of the *bcbot4* WT allele. Information about the *B. cinerea* KO mutant used in this study and other published mutants is available at <http://botbioger.versailles.inra.fr/botmut/>.

Isolation of the metabolites

Fractionation of the extract was carried out on a silica column to give six fractions using 100% hexane (5.3 mg), hexane : ethyl acetate (70 : 30) (1.7 g), hexane : ethyl acetate (40 : 60) (2.1 g), hexane : ethyl acetate (10 : 90) (1.25 g), ethyl acetate : methanol (70 : 30) (1.3 g) and 100% methanol (303.8 mg).

In addition to the known compounds 2–15,¹⁹ which were obtained from the fractions of medium polarity, the sesquiterpenes presilphiperfolan-8 α ,10 β ,14-triol (**16**) (93 mg), 8 α ,14-dihydroxypresilphiperfolan-10-one (**17**) (1.5 mg), 8 α ,10 β ,14-trihydroxypresilphiperfol-1-ene (**18**) (1 mg), cameroonan-7 α ,10 β ,14-triol (**19**) (102 mg) and 7 α -hydroxycameroonane-14,15-dioic acid 14-7-lactone (**21**) (6.71 mg) were isolated and characterized. Compounds **16** (2.03 mg), **19** (1.27 mg) and **20** (1.54 mg) were acetylated. For acetylation, compounds were dissolved in the minimum quantity of dry pyridine (0.5 mL), and acetic anhydride (30 mg) was added to the solution. The mixture was kept at room temperature with stirring for 24 h. The solvent was evaporated to give derivatives **16a** (2.20 mg), **19a** (1.32 mg) and **20a** (1.49 mg).

Presilphiperfolan-8 α ,10 β ,14-triol (16). Colourless solid; mp 162 °C; $[\alpha]_D^{20}$ -1.02 (*c* 10.5 in CH₃OH); IR (film) ν_{\max} 3293, 2948, 2874, 1455, 1047 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 3.35 (2H, d superimposed, *J* = 10 Hz, H-14), 3.23 (1H, ddd, *J* = 11, 9.4, 3.4 Hz, H-10), 2.34 (1H, m, H-2), 2.02 (1H, d, *J* = 12.1 Hz, H-5), 1.92 (2H, m, H-2', H-3), 1.86 (1H, m, H-7), 1.82 (1H, m, H-11), 1.59 (1H, ddd, *J* = 12, 11 Hz, H-11'), 1.43 (1H, m, H-1), 1.41 (1H, m, H-9), 1.34 (3H, s, H-12), 1.16 (3H, s, H-13), 1.13 (1H, m, H-3'), 1.01 (1H, d, *J* = 12.1 Hz, H-5'), 0.97 (3H, d, *J* = 5.9 Hz, H-15); ¹³C NMR (125 MHz, CDCl₃) δ 95.3 (C, C-8), 76.3 (CH, H-10), 72.5 (CH₂, H-14), 57.9 (C, C-4), 54.4 (C, C-6), 47.6 (CH, C-9), 45.9 (CH, C-7), 44.9 (CH, C-1), 43.5 (CH₂, C-5), 36.9 (CH₂, C-11), 34.3 (CH₂, C-11), 34.1 (CH₂, C-3), 29.0 (CH₃, C-12), 22.8 (CH₃, C-13), 18.0 (CH₃, C-15); HRMS (ESI): calcd for C₁₅H₂₃O [M - 2H₂O + H]⁺ 219.1749, found 219.1744.

10 β ,14-Diacetoxypresilphiperfolan-8 α -ol (16a). Colourless oil, ¹H NMR (500 MHz, CDCl₃) δ 4.61 (1H, td, *J* = 11.1, 3.5 Hz, H-10), 4.0 (s, 2H, H-14), 2.41 (1H, m, H-2 β), 2.08 (s, 3H, CH₃CO), 2.05 (s, 3H, CH₃CO), 1.98 (1H, m, H-2 α), 1.94 (1H, d, *J* = 12.1 Hz, H-5 α), 1.90 (1H, m, H-3), 1.88 (1H, m, H-9), 1.88 (1H, m, superimposed, H-11), 1.68 (1H, td, *J* = 11.0, 6.2 Hz, H-1), 1.58 (1H, m, H-11), 1.46 (1H, ddd, *J* = 11.2, 9.1, 3.9 Hz, H-7), 1.34 (s, 3H, H-12), 1.24 (1H, m, H-3'), 1.21 (s, 3H, H-13), 1.14, (1H, d, *J* = 12.1 Hz, H-5 β), 0.89 (d, *J* = 6.3, 3H, H-15). ¹³C NMR (125 MHz, CDCl₃) δ , 170.9 (C, CH₃CO), 170.7 (C, CH₃CO), 94.44, (C, C-8), 77.11 (CH, C-10), 74.7 (CH₂, C-14), 56.13 (C, C-4), 51.64 (C, C-6), 46.9 (CH, C-7), 44.6 (CH, C-9), 43.61 (CH₂, C-5), 41.0 (CH, C-1), 33.23 (CH₂, C-2), 33.07 (CH₂, C-3), 32.4 (CH₂, C-11), 28.32 (CH₃, C-12), 22.53 (CH₃, C-13), 21.2 (CH₃CO), 21.0 (CH₃CO), 17.20 (CH₃, C-15). HRMS (APGC⁺): calcd for C₁₉H₃₁O₅ [M + H]⁺ 339.2171, found 339.2162.

X-ray crystallographic analysis of 16. Crystals of **16** were obtained from *n*-hexane/EtOAc by slow evaporation. X-ray diffraction data were recorded on a Bruker Smart CCD diffractometer with graphite monochromated Mo K α radiation (λ = 0.71073 Å) at 298(1) K. Data collection was based on three ω -scan runs (starting = -34°) at values of φ = 0°, 120°, and 240° with the detector at 2θ = -32°. For each of the runs, 606 frames were collected at 0.3° intervals and 10 s per frame. An additional run at φ = 0° of 100 frames was collected to improve redundancy.

The diffraction frames were integrated using the program SAINT, and the integrated intensities were corrected for Lorentz_polarization effects and absorption with SADABS. The structure was solved by direct methods and refined to all 3237 unique F_o^2 by full matrix least-squares calculations using SHELXL-97. All the hydrogen atoms, except the hydrogens of the -OH groups, were placed at idealized positions and refined as rigid atoms. The positions of the OH hydrogens are found in the diffraction map and refined as rigid atoms. Final R_1 indices [2699 with $I > 2\sigma(I)$] 0.0465, wR_2 = 0.1074. R indices (all data) R_1 = 0.0578, wR_2 = 0.1150. $S[F^2]$ 1.050 for 173 refined parameters. Crystal data: C₁₅H₂₆O₃, M = 254.36, orthorhombic, a = 9.9836(8) Å, b = 11.1199(9) Å, c = 12.6582(11) Å, α = β = γ = 90°, V = 1405.3(2) Å³, dimensions 0.50 × 0.28 × 0.10 mm, space group: $P212121$, Z = 4, $F(000)$ = 560, μ = 0.081 mm⁻¹.



Crystallographic data of presilphiperfolan-8 α ,10 β ,14-triol have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 1547406.†

8 α ,14-Dihydroxypresilphiperfolan-10-one (17). Colourless oil; $[\alpha]_D^{22} = -33.75$ (c 0.20 in CHCl_3); IR (film) ν_{max} 3303, 2918, 2874, 1707, 1455, 1037 cm^{-1} ; $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 3.44 (2H, s(br), H-14, H-14'), 2.52 (1H, dd (br), $J = 13.1$ Hz, H-11 β), 2.50 (1H, m, H-2'), 2.43 (1H, dq, $J = 12.4, 6.3$ Hz, H-9), 2.34 (1H, dd, $J = 13.1, 8.7$ Hz, H-11 α), 2.20 (1H, m, H-7), 2.17 (1H, d, $J = 12.1$ Hz, H-5 α), 2.05 (1H, m, H-2), 1.71 (1H, m, H-1), 1.98 (1H, m, H-3), 1.54 (3H, s, H-12), 1.26 (1H, m, H-3'), 1.14 (1H, d, $J = 12.1$ Hz, H-5 β), 1.14 (3H, s, H-13), 0.98 (3H, d, $J = 6.4$ Hz, H-15). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ , 213.7 (C, C-10), 92.4 (C, C-8), 71.5 (CH₂, C-14), 57.8 (C, C-4), 54.0 (C, C-6), 48.13 (CH, C-9), 48.09 (CH, C-1), 46.2 (CH, C-7), 42.2 (CH₂, C-11), 41.7 (CH₂, C-5), 34.9 (CH₂, C-2), 32.7 (CH₂, C-3), 28.14 (CH₃, C-12), 22.5 (CH₃, C-13), 13.5 (CH₃, C-15). HRMS (ESI⁻): C₁₅H₂₄O₃ $[\text{M} - \text{H}]^-$ 251.1647, found 251.1655.

8 α ,10 β ,14-Trihydroxypresilphiperfol-1-ene (18). Colourless oil; $[\alpha]_D^{22} = -20.39$ (c 0.32 in CHCl_3); IR (film) ν_{max} 3380, 2961, 2928, 2879, 1454, 1035 cm^{-1} ; $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 4.94 (1H, dd, $J = 2.1, \text{H-2}$), 3.33 (2H, d(br), $J = 4$ Hz, H-14, H-14'), 3.18 (1H, ddd, $J = 10, 8.7, 5.3$ Hz, H-10), 2.46 (1H, m, H-9), 2.40 (1H, dd, $J = 17, 2.1$ Hz, H-3), 2.25 (1H, dt, $J = 17, 2.5$ Hz, H-3') 1.94 (1H, m, H-11) 1.87 (1H, d, $J = 13.3$ Hz, H-5) 1.86 (1H, m, H-11') 1.56 (1H, d, $J = 13.3$ Hz, H-5') 1.36 (1H, m, H-7) 1.20 (3H, d, $J = 6.5$ Hz, H-15), 1.11 (3H, s, H-12), 1.07 (3H, s, H-13). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ , 148.7 (C, C-1), 116.2 (CH, C-2), 93.5 (C, C-8), 79.8 (CH, C-10), 71.0 (CH₂, C-14), 53.43 (CH₂, C-5), 53.4 (CH, C-7), 50.25 (CH₂, C-3), 47.1 (C-C-4), 46.4 (C, C-6), 39.7 (CH, C-9), 30.5 (CH₂, C-11), 29.7 (CH₃, C-12), 18.9 (CH₃, C-13), 14.15 (CH₃, C-15). HRMS (ESI⁺): C₁₅H₂₄O₃ $[\text{M} + \text{H}]^+$ 253.1804, found 253.1788.

Cameroonan-7 α ,10 β ,14-triol (19). Colourless oil; $[\alpha]_D^{20} = -6.47$ (c 9.15 in CHCl_3); IR (film) ν_{max} 3364, 2957, 2870, 1455, 1043, 756 cm^{-1} ; $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 3.89 (1H, s, H-7), 3.82 (1H, d, $J = 10.9, \text{H-14}$), 3.72 (1H, ddd, $J = 11, 9.7, 6.0$ Hz, H-10), 3.21 (1H, d, $J = 10.9, \text{H-14}'$), 2.17 (1H, t, $J = 9.0$ Hz, H-1), 1.80 (1H, m, H-2), 1.73 (1H, dd, $J = 12.2, 6$ Hz, H-11), 1.65 (1H, dd, 12.2, 11 Hz, H-11'), 1.49 (2H, m, H-3, H-3'), 1.39 (1H, d, $J = 14.5$ Hz, H-5), 1.34 (1H, m, H-2'), 1.27 (1H, d superimposed, H-5'), 1.22 (1H, m, H-9), 1.20 (3H, s, H-13), 1.05 (3H, d, $J = 6.5$ Hz, H-15), 1.00 (3H, s); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 91.52 (CH, C-7), 77.45 (CH, C-10), 69.61 (CH₂, C-14), 62.85 (C, C-8), 49.96 (CH, C-9), 48.47 (C, C-4), 47.85 (CH, C-1), 47.42 (CH₂, C-5), 44.83 (CH₂, C-11), 42.35 (C, C-6), 38.93 (CH₂, C-3), 28.62 (CH₂, C-2), 27.39 (CH₃, C-13), 25.03 (CH₃, C-12), 16.36 (CH₃, C-15); HRMS (ESI): calcd for C₁₅H₂₃O $[\text{M} - 2\text{H}_2\text{O} + \text{H}]^+$ 219.1749, found 219.1748.

7 α ,10 β ,14-Triacetoxycameroonane (19a). Colourless oil, $^1\text{H NMR}$ (500 MHz, CDCl_3) δ , 5.11 (1H, s, H-7), 4.52 (1H, ddd, $J = 10.4, 9.7, 6.0$ Hz, H-10), 3.79 (1H, d, $J = 11$ Hz, H-14), 3.96 (1H, d, $J = 11$ Hz, H-14'), 2.13 (1H, m, H-1), 2.10 (3H, s, CH₃-CO), 2.04 (3H, s, CH₃-CO), 2.01 (3H, s, CH₃-CO), 1.90 (1H, m, H-2), 1.89 (1H, dd 12.3, 6.0 Hz, H-11) 1.80 (1H, d, $J = 14.4$ Hz, H-5), 1.64 (1H, dd, $J = 12.3, 10.4$ Hz, H-11'), 1.54 (2H, m, H-3, H-3'),

1.49 (1H, m, H-9) 1.37 (1H, m, H-2') 1.36 (1H, d, $J = 14.4$ Hz, H-5'), 1.20 (3H, s, H-13), 1.08 (3H, s, H-12), 0.98 (3H, d, $J = 6.5$ Hz, H-15). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ , 171.2 (C, CH₃CO), 170.75 (C, CH₃CO), 170.4 (C, CH₃CO), 89.12 (CH, C-7), 78.7 (CH, C-10), 68.93 (CH₂, C-14), 62.9 (C, C-8), 47.8 (CH₂, C-5), 49.25 (C, C-4), 49.15 (CH, C-1), 46.80 (CH, C-9), 42.35 (C, C-6), 40.1 (CH₂, C-11), 38.91 (CH₂, C-3), 28.85 (CH₂, C-2), 26.73 (CH₃, C-13), 24.93 (CH₃, C-12), 21.13 (CH₃CO), 21.04 (CH₃CO), 20.89 (CH₃CO), 16.70 (CH₃, C-15). HRMS (APGC⁺): calcd for C₂₁H₃₃O₆ $[\text{M} + \text{H}]^+$ 381.2277, found 381.2256.

Cameroonan-2 α ,3 β ,7 α -triol (20). Amorphous solid, $[\alpha]_D^{22} = -2.68$ (c 1.32 in CHCl_3); IR (film) ν_{max} 3381, 2949, 2858, 1456, 1378, 1040, 756 cm^{-1} ; $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 3.81 (1H, dbr, $J = 9.1$ Hz, H-3), 3.49 (1H, dd, $J = 8.6, 9.1$ Hz, H-2), 3.33 (1H, s, H-7), 2.10 (1H, dd, $J = 8.6, 3.7$ Hz, H-1), 1.94 (1H, m, H-9), 1.90 (1H, m, H-11 α), 1.83 (1H, d, $J = 13.5$ Hz, H-5 α), 1.73 (1H, m, H-10 β), 1.55 (1H, m, H-11 β), 1.34 (1H, m, H-10 α), 1.29 (1H, d, $J = 13.5$ Hz, H-5 β), 1.03 (3H, s, H-13), 1.03 (3H, s, H-14), 1.01 (1H, d, $J = 6.9$ Hz, H-15), 0.95 (3H, s, H-12). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 90.4 (CH, C-7), 86.5 (CH, C-3), 82.9 (CH, C-2), 64.10 (C, C-8), 54.8 (CH, C-1) 52.85 (CH₂, C-5), 49.8 (C, C-4), 43.7 (C, C-6), 38.61 (CH, C-9), 35.7 (CH₂, C-11), 34.9 (CH₂, C-10), 29.4 (CH₃, C-13), 24.3 (CH₃, C-14), 20.3 (CH₃, C-15), 20.0 (CH₃, C-12). HRMS (ESI⁺): calcd for C₁₅H₂₆O₃ $[\text{M} + \text{H}]^+$ 255.1960, found 255.1932.

2 α ,3 β ,7 α -Triacetoxycameroonane (20a). Colourless oil, $^1\text{H NMR}$ (500 MHz, CDCl_3) δ , 5.18 (1H, d, $J = 6.7$ Hz, H-3), 4.97 (1H, dd, $J = 6.7$ Hz, H-2), 4.87 (1H, s, H-7), 2.12 (1H, d, $J = 13.9$ Hz, H-5 α), 2.14 (3H, s, CH₃-CO), 2.08 (1H, m, H-1), 2.05 (3H, s, CH₃-CO), 2.05 (3H, s, CH₃-CO), 1.99 (1H, m superimposed, H-9), 1.95 (1H, m, superimposed, H-10 α), 1.80 (1H, dddd, $J = 12.7, 6.5, 4.3$ Hz, H-10 β), 1.64 (1H, m, H-11), 1.39 (1H, d, $J = 13.9$ Hz, H-5 β), 1.10 (3H, s, H-14), 1.22 (1H, m, H-11'), 1.02 (3H, s, H-12), 1.0 (3H, s, H-13), 0.96 (3H, d, $J = 6.8$ Hz, H-15). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 170.4 (CH₃CO), 170.4 (CH₃CO), 170.3 (CH₃CO), 89.9 (CH, C-7), 85.3 (CH, C-3), 83.2 (CH, C-2), 65.7 (C, C-8), 55.9 (CH, C-1), 52.4 (CH₂, C-5), 50.6 (C, C-4), 42.1 (C, C-6), 40.3 (CH, C-9), 35.6 (CH₂, C-10), 35.1, 30.3 (CH₃, C-14), 25.0 (CH₃, C-12), 21.1 (CH₃CO), 21.2 (CH₃CO), 20.9 (CH₃CO), 20.5 (CH₃, C-15), 19.8 (CH₃, C-13). HRMS (APGC⁺): calcd for C₂₁H₃₃O₆ $[\text{M} + \text{H}]^+$ 381.2277, found 381.2264.

7 α -Hydroxycameroonane-14,15-dioic acid 14-7-lactone (21). Colourless solid; mp 114 °C; $[\alpha]_D^{20} = -28.97$ (c 0.58 in CHCl_3); IR (film) ν_{max} , 3423, 2959, 1811, 1698, 1456, 1239, 913, 846 cm^{-1} ; $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 4.27 (1H, s, H-7), 3.01 (1H, dt, J 6.7, 8.3 Hz, H-1), 2.52 (1H, dt, J 6.7, 8.3 Hz, H-9), 2.28 (1H, d, J 14.4 Hz, H-5), 2.14 (2H, overlap, H-2', H-10), 2.04 (1H, m, H-10'), 1.90 (1H, m, H-3), 1.80 (1H, m, H-11 β), 1.64 (1H, dd, J 12.7, 6.7 Hz, H-3 β), 1.52 (1H, m, H-2), 1.44 (1H, dd, J 13.2, 6.4 Hz, H-11 α), 1.40 (3H, s, H-13), 1.37 (1H, d, J 14.4 Hz, H-5 β), 1.07 (3H, s, H-12); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 180.6 (C, C-15), 175.7 (C, C-14), 93.6 (CH, C-7), 65.3 (C, C-8), 62.3 (C, C-6), 54.8 (C, C-4), 51.7 (CH, C-9), 50.7 (CH, C-1), 45.1 (CH₂, C-5), 42.6 (CH₂, C-3), 33.0 (CH₂, C-10), 32.5 (CH₂, C-2), 31.1 (CH₂, C-11), 25.1 (CH₃, C-12), 18.0 (CH₃, C-13); HRMS (ESI): calcd for C₁₄H₂₁O₂ $[\text{M} - \text{CO}_2 + \text{H}]^+$ 221.1542, found 221.1541.



Acknowledgements

This research was supported by a grant from Ministerio de Economía y Competitividad (AGL2015-65684-C2-1-R) (MINECO-FEDER). The use of NMR facilities at the Servicio Centralizado de Ciencia y Tecnología (SCCYT) of the University of Cádiz is acknowledged.

Notes and references

- 1 Y. Elad, B. Williamson, P. Tudzynski and N. Delen, *Botrytis: Biology, pathology and control*, 2007.
- 2 *Botrytis-the fungus, the pathogen and its management in agricultural systems*, ed. S. Fillinger and Y. Elad, Springer International Publishing, 2016.
- 3 E. M. Govrin and A. Levine, *J. Nat.*, 2000, **10**, 751–757.
- 4 I. Kars and J. Van Kan, in *Botrytis: biology, pathology and control*, ed. Y. Elad, B. Williamson, P. Tudzynski and N. Delen, Kluwer Academic Publisher, 2007, pp. 99–118.
- 5 I. G. Collado and M. Viaud, in *Botrytis-the fungus, the pathogen and its management in agricultural systems*, ed. S. Fillinger and Y. Elad, Springer International Publishing, 2016, pp. 291–313.
- 6 J. L. Reino, R. Durán-Patrón, I. Segura, R. Hernández-Galán, H. H. Riese and I. G. Collado, *J. Nat. Prod.*, 2003, **66**, 344–349.
- 7 N. Deighton, I. Muckenschnabel, A. J. Colmenares, I. G. Collado and B. Williamson, *Phytochemistry*, 2001, **57**, 689–692.
- 8 A. J. Colmenares, J. Aleu, R. Durán-Patrón, I. G. Collado and R. Hernández-Galán, *J. Chem. Ecol.*, 2002, **28**, 997–1005.
- 9 I. G. Collado, A. J. M. Sánchez and J. R. Hanson, *Nat. Prod. Rep.*, 2007, **24**, 674–686.
- 10 H. Tani, H. Koshino, E. Sakuno and H. Nakajima, *J. Nat. Prod.*, 2005, **68**, 1768–1772.
- 11 H. Tani, H. Koshino, E. Sakuno, H. G. Cutler and H. Nakajima, *J. Nat. Prod.*, 2006, **69**, 722–725.
- 12 H. G. Cutler, S. R. Parker, S. a. Ross, F. G. Crumley and P. R. Schreiner, *Biosci., Biotechnol., Biochem.*, 1996, **60**, 656–658.
- 13 F. R. Rossi, A. Gárriz, M. Marina, F. M. Romero, M. E. Gonzalez, I. G. Collado and F. L. Pieckenstein, *Mol. Plant-Microbe Interact.*, 2011, **24**, 888–896.
- 14 V. Siewers, M. Viaud, D. Jimenez-Teja, I. G. Collado, C. S. Gronover, J.-M. Pradier, B. Tudzynski and P. Tudzynski, *Mol. Plant-Microbe Interact.*, 2005, **18**, 602–612.
- 15 C. Pinedo, C. M. Wang, J. M. Pradier, B. Dalmais, M. Choquer, P. Le Pêcheur, G. Morgant, I. G. Collado, D. E. Cane and M. Viaud, *ACS Chem. Biol.*, 2008, **3**, 791–801.
- 16 S. Fillinger, J. Amselem, F. Artiguenave, A. Billaut, M. Choquer, A. Couloux, C. Cuomo, M. B. Dickman, E. Fournier, A. Gioti, C. Giraud and C. Kodira, *Macromol. Grape Wines*, 2007, 125–133.
- 17 J. Amselem, C. A. Cuomo, J. A. L. van Kan, M. Viaud, E. P. Benito, A. Couloux, P. M. Coutinho, R. P. de Vries, P. S. Dyer, S. Fillinger, E. Fournier, L. Gout, M. Hahn, L. Kohn, N. Lapalu, K. M. Plummer, J. M. Pradier, E. Quévillon, A. Sharon, A. Simon, A. Have, B. Tudzynski, P. Tudzynski, P. Wincker, M. Andrew, V. Anthouard, R. E. Beever, R. Beffa, I. Benoit, O. Bouzid, B. Brault, Z. Chen, M. Choquer, J. Collémare, P. Cotton, E. G. Danchin, C. Da Silva, A. Gautier, C. Giraud, T. Giraud, C. Gonzalez, S. Grossetete, U. Güldener, B. Henrissat, B. J. Howlett, C. Kodira, M. Kretschmer, A. Lappartient, M. Leroch, C. Levis, E. Mauceli, C. Neuvéglise, B. Oeser, M. Pearson, J. Poulain, N. Poussereau, H. Quesneville, C. Rasclé, J. Schumacher, B. Ségurens, A. Sexton, E. Silva, C. Sirven, D. M. Soanes, N. J. Talbot, M. Templeton, C. Yandava, O. Yarden, Q. Zeng, J. A. Rollins, M. H. Lebrun and M. Dickman, *PLoS Genet.*, 2011, **7**, e1002230.
- 18 A. Porquier, G. Morgant, J. Moraga, B. Dalmais, I. Luyten, A. Simon, J. M. Pradier, J. Amselem, I. G. Collado and M. Viaud, *Fungal Genet. Biol.*, 2016, **96**, 33–46.
- 19 J. Moraga, B. Dalmais, I. Izquierdo-Bueno, J. Aleu, J. R. Hanson, R. Hernández-Galán, M. Viaud and I. G. Collado, *ACS Chem. Biol.*, 2016, **11**, 2838–2846.
- 20 R. Duran-Patron, J. M. Cantoral, R. Hernandez-Galan, J. R. Hanson and I. G. Collado, *J. Chem. Res.*, 2004, 441–443.
- 21 P. Weyerstahl, P. Weyerstahl, H. Marschall, H. Marschall, I. Seelmann, I. Seelmann, J. Jakupovic and J. Jakupovic, *Eur. J. Org. Chem.*, 1998, 1205–1212.
- 22 R. M. Coates, Z. Ho, M. Klobus and S. R. Wilson, *J. Am. Chem. Soc.*, 1996, **118**, 9249–9254.
- 23 T. Quidde, A. E. Osbourn and P. Tudzynski, *Physiol. Mol. Plant Pathol.*, 1998, **52**, 151–165.
- 24 J. H. Yu, Z. Hamari, K. H. Han, J. A. Seo, Y. Reyes-Domínguez and C. Scazzocchio, *Fungal Genet. Biol.*, 2004, **41**, 973–981.
- 25 Z. Antal, C. Rasclé, A. Cimerman, M. Viaud, G. Billon-Grand, M. Choquer and C. Bruel, *PLoS One*, 2012, **7**, e48134.
- 26 A. Simon, B. Dalmais, G. Morgant and M. Viaud, *Fungal Genet. Biol.*, 2013, **52**, 9–19.

