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Genetic and molecular basis of botrydial biosynthesis. Connecting cytochrome P450-encoding genes to biosynthetic intermediates.

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Abstract:

Over two hundred species of plants can be infected by the phytopathogenic fungus *Botrytis cinerea* under a range of different environmental conditions. In response to these the fungus produces unique terpenoid and polyketide metabolites. Parts of the plants may be killed by the phytotoxin, botrydial enabling the fungus to feed on the dead cells. In this paper we describe the genetic and molecular basis of botrydial biosynthesis and the function of the five genes of the genome of *B. cinerea* which together constitute the botrydial biosynthetic gene cluster. Genes *BcBOT*3 and *BcBOT*4, encoding two cytochrome P450 monooxygenases, were inactivated by homologous recombination and were shown to catalyse regio- and stereospecific hydroxylations, at the carbons C-10 and C-4 respectively, of the presilphiperfolan-8*β*-ol skeleton. The null mutants i.e. *bcbot3*Δ and *bcbot4*Δ accumulated key intermediates in the botrydial biosynthesis enabling the complete genetic and molecular basis of the botrydial biosynthetic pathway to be established. Furthermore, the *bcbot4*Δ mutant overproduced a significant number of polyketides, which included, in addition to known botcinins, botrylactones and cinbotolide A, two new botrylactones and two new cinbotolides, cinbotolides B and C.

INTRODUCTION

Fungal secondary metabolites have a wide impact on human health, medicine and agriculture. Whilst many secondary metabolites including antibiotics, immunosuppressants and phytohormones are beneficial, other such as phytotoxins and mycotoxins are harmful to palnts, humans and animals.[1] In order to manage or modify the biosynthesis of secondary metabolites, we need to identify the location of the genes encoding the relevant biosynthetic enzymes. The genes which encode the biosynthesis of structurally related secondary metabolites are often clustered. The horizontal gene transfer (HGT)[2] between distant fungi may be facilitated by such clustering, conferring an evolutionary advantage.[3-6]

The Ascomycete *Botrytis cinerea* is the cause of a "grey mould" disease of a wide range of dicoyledons under a variety of environmental conditions in which its secondary metabolites (SM) confer a selective advantage. *B. cinerea* has the characteristics of a necrotroph in which the infection may be mediated by the production of phytotoxins and reactive oxygen species as well as by inducing an oxidative burst in the plant.[7,8] A number of unique terpenoids and polyketides have been obtained from the mycelium. These include two groups of non-specific phytotoxins. The first are the sesquiterpenoid botryanes,[9] botrydial (**1**) and dihydrobotrydial (**2**). The second are the polyketide lactones, botrylactone (**3**)[10] together with botcinic (**4**) and botcineric acid (**5**)[11] and their cyclic relatives, the botcinins (**6**, **7**).[12] Cinbotolide (**8**) is a new polyketide that has been reported and which contains a carbon chain that possesses structural features and a stereochemistry reminiscent of the botcinins and botrylactone (Figure 1).[13]

Botrydial (**1**) is the major phytotoxin[14,15] of *B. cinerea* and it has been shown to reproduce the symptoms of the infection.[16,17] Spectroscopic, chemical and X-ray methods[18-20] were used to establish the irregular sesquiterpenoid structures of these botryane metabolites. Their biosynthesis via farnesyl diphosphate (FPP, Figure 2) has been elucidated.[21-24]

The genome of *B. cinerea* has been sequenced leading to the identification of the genes that are responsible for the synthesis of its secondary metabolites. Forty-four genes which encode key enzymes (KE) that are responsible for the committed biosynthetic steps, were

identified from sequences in two strains, BO5.10 and T4.[25,26] Typical of the secondary metabolism of filamentous fungi, the majority of the genes that encode these key enzymes are part of biosynthetic gene clusters. Among them, the botrydial biosynthetic gene cluster has been identified (Figure 3).[27,28] The cluster that consists of five genes (*BcBOT1* - *BcBOT5*) (for *Botrytis cinerea* BOTrydial) involves one gene coding for a sesquiterpene cyclase (*BcBOT2*), three mono-oxygenases (*BcBOT1*, *BcBOT3* and *BcBOT4*) and an acetyl transferase (*BcBOT5*).

Gene de-activation studies, which had been carried out previously, had shown that the *BcBOT1* gene, encoding a P450 mono-oxygenase is responsible for one of the final steps in botrydial biosynthesis,[27] whilst *BcBOT2* codes for a sesquiterpene cyclase (presilphiperfolan-8β-ol (PSP) synthase),[28] which is an early step in botrydial biosynthesis (Figure 2).

The P450 monooxygenases, under mild reaction conditions, catalyze regio- and stereospecific oxidations of non-activated hydrocarbons, to yield interesting chemical transformations in biosynthetic chemistry. [29]

This paper describes the molecular sequence of botrydial biosynthesis and the functions of the three cytochrome P450 mono-oxygenases involved in botrydial biosynthesis in terms of the hydroxylations at C-4, C-10 and C-15 on the PSP carbon skeleton. The structures of four new polyketide metabolites which were isolated from the *bcbot4*Δ mutant are also reported.

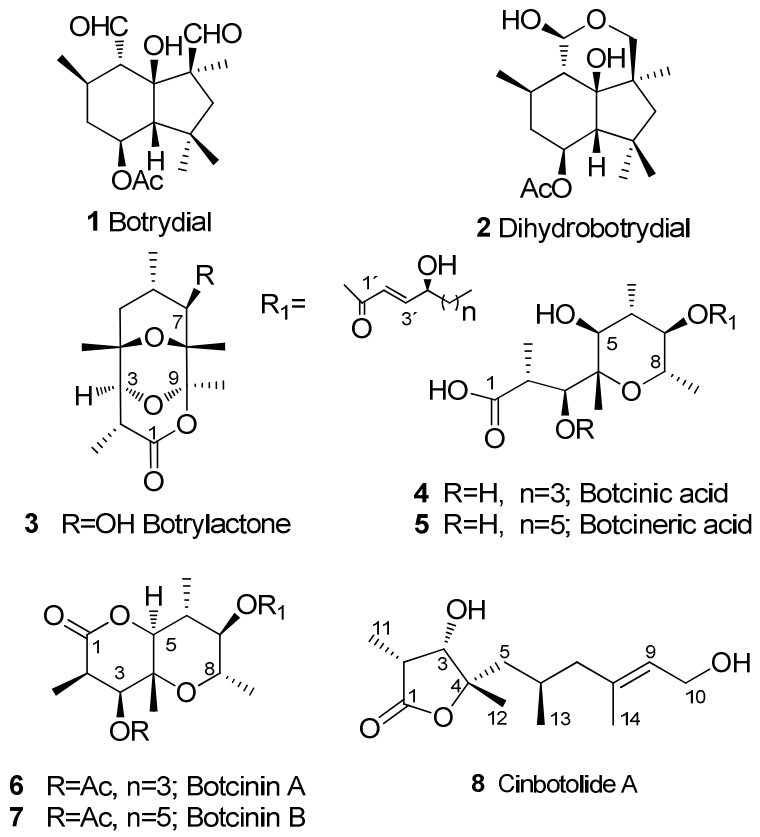


Figure 1. Some metabolites isolated from *Botrytis cinerea*

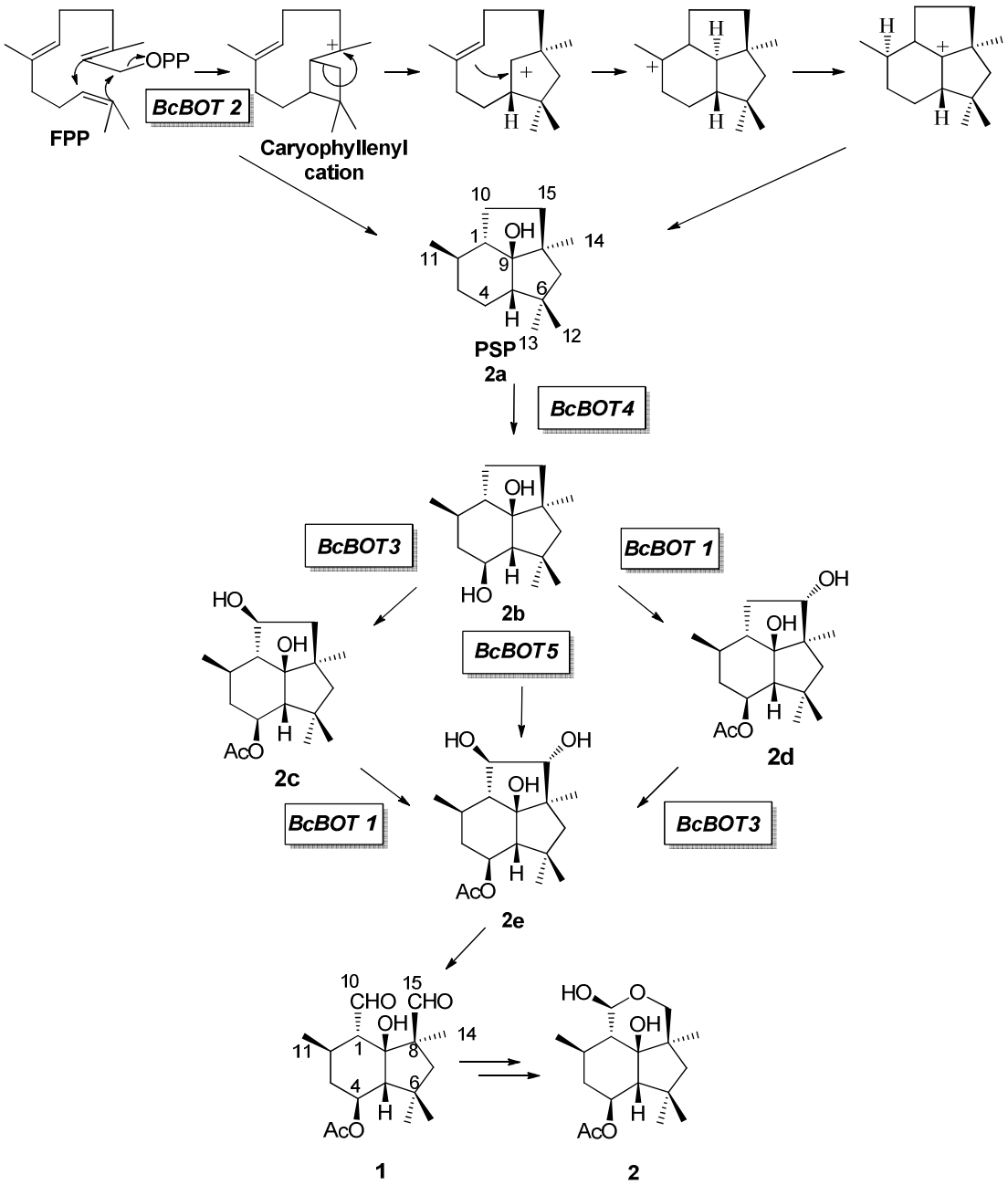


Figure 2. Biosynthetic pathway to Botrydial (**1**)

RESULTS and DISCUSSION

Previous experiments using isotopically labeled acetate and mevalonate established the origin of the carbon skeleton of botrydial (**1**) and the occurrence of a backbone rearrangement and 1,3-hydride shift during the cyclization of FPP.[9,24,30] The original mechanistic proposal (Figure 2) has been corroborated by the incubation of labeled FPP with a recombinant PSP synthase (BcBOT2) to afford the labeled intermediate tricyclic alcohol, presilphiperfolan-8β-ol (**2a,** PSP, probotryane).[31] This intermediate **2a** is converted to botrydial (**1**) by the action of the three cytochrome P450s encoded by the co-regulated genes *BcBOT1*, *BcBOT3* and *BcBOT4* together with the acetyltransferase (BcBOT5) which function in an as yet unknown sequence.

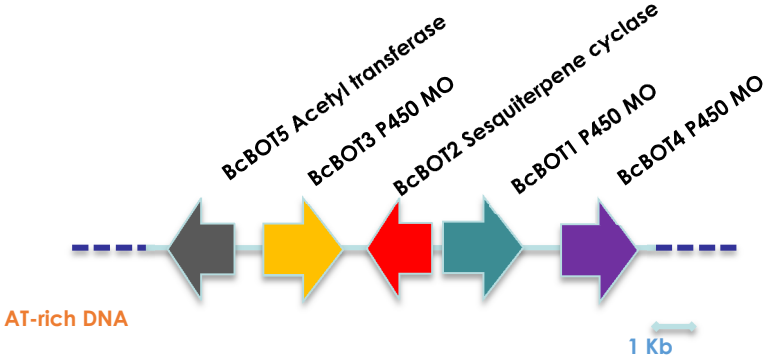


Figure 3. Scheme of the genes cluster involved in the biosynthesis of botrydial. Dash line indicates AT-rich DNA (more than 80% of A and T content).

Deletion of the genes encoding BcBOT3 and BcBOT4 *monooxygenases*

Previous studies of the amino acid sequences of the BcBOT3 and BcBOT4 proteins have revealed the presence of conserved P450 mono-oxygenase domains which suggested that they could be involved in botrydial biosynthesis.[27,28] A gene knock-out (KO) approach was used to confirm this hypothesis. Two KO DNA cassettes containing a nourseothricin resistance gene in place of the *BcBOT* genes, were constructed by PCR fusion and employed to transform protoplasts of the BO5.10 wild-type strains (for details, see Methods). The transformation

resulted in two independent *bcbot3*Δ and two independent *bcbot4*Δ mutants. PCR analyses verified integration of the replacement fragment at the targeted loci the genome and the absence of wild-type nuclei. Both mutants grow and produced conidia similarly to the parent strain B05.10 on a standard rich medium (data not shown).

Chemical characterization of the *bcbot3*Δ and *bcbot4*Δ mutants.

The KO *bcbot3*Δ and *bcbot4*Δ mutants which had been derived from the B05.10 strain, were grown on a solid malt agar medium. The metabolites were recovered in ethyl acetate and purified by column chromatography on silica gel. The products were finally purified by HPLC to yield the metabolites shown in figure 4. The results indicated that none of the mutants produced botrydial (**1**), confirming that these mutations were involved in botrydial biosynthesis.

The chromatographic study, characterization and quantification of the metabolites biosynthesized by the *bcbot3*Δ mutant showed, in addition to small amounts of botcinin A (**6**), high levels, (35 mg/L), of the biosynthetic intermediate 4β-acetoxy-15α-hydroxyprobotryane (**2d**), Figure 2, which was identified by extensive NMR studies, and comparison with authentic samples. In addition, the new metabolite **9** was isolated and characterized.

Compound **9** was obtained as colourless oil. It showed a molecular ion in its HRMS (ESI+) at m/z= 253.1807 [($C_{15}H_{25}O_3$), M+H]$^+$ in agreement with the ^{13}C NMR and DEPT spectra. The ^{1}H- and ^{13}C NMR spectra of compound **9** contained a similar pattern of signals to the probotryane intermediate (**2d**). However the signal for the C-4 CH(OH) appeared at ð 4.04, whilst the signals for the acetate group and the C-15 CH(OH) were missing. There was a signal in the ^{13}C NMR spectrum at ð 211.0 characteristic of a cyclopentanone whilst the H-10 protons resonances were shifted. These spectroscopic data indicated that compound **9** was the oxidation product of the biosynthetic intermediate **2d** possessing a carbonyl group at C-15. Acetylation of compound **9** yielded a mono-acetylated derivative **9a** which exhibited the characteristic ^{1}H NMR signals of a methyl group at ð 2.03 and a signal, ddd, ð 5.11 for the proton geminal to the acetate. The proton and carbon-13 NMR signals of both compounds **9** and **9a** were assigned by detailed analysis of their COSY, NOESY, HSQC and HMBC spectra (Supplementary Table S1). These confirmed their structures and relative stereochemistry.

The fermentation broth of mutant *bcbot4*Δ was chromatographed following the same procedure used for the KO mutant *bcbot3*Δ. Final purification by HPLC and GC-MS yielded the metabolites **2a**, **3**-**8,** Figures 1 and 2, and **10**-**18** showed in Figure 4. Interestingly, in addition to the key probotryane intermediate **2a**, from the cyclization of FPP by the sesquiterpene cyclase BcBOT2, an overproduction of polyketides was observed yielding the new polyketides **15**-**18**, Figure 4.

The probotryane **2a** which was accumulated by the *bcbot4*Δ mutant, was identified in the hexane fraction by comparison with an authentic sample. Although it had not been found in the wild type (Figure 5A), it was readily identified in the *bcbot4*Δ mutant fermentation after 4, 8 and 10 days (Figure 5B).

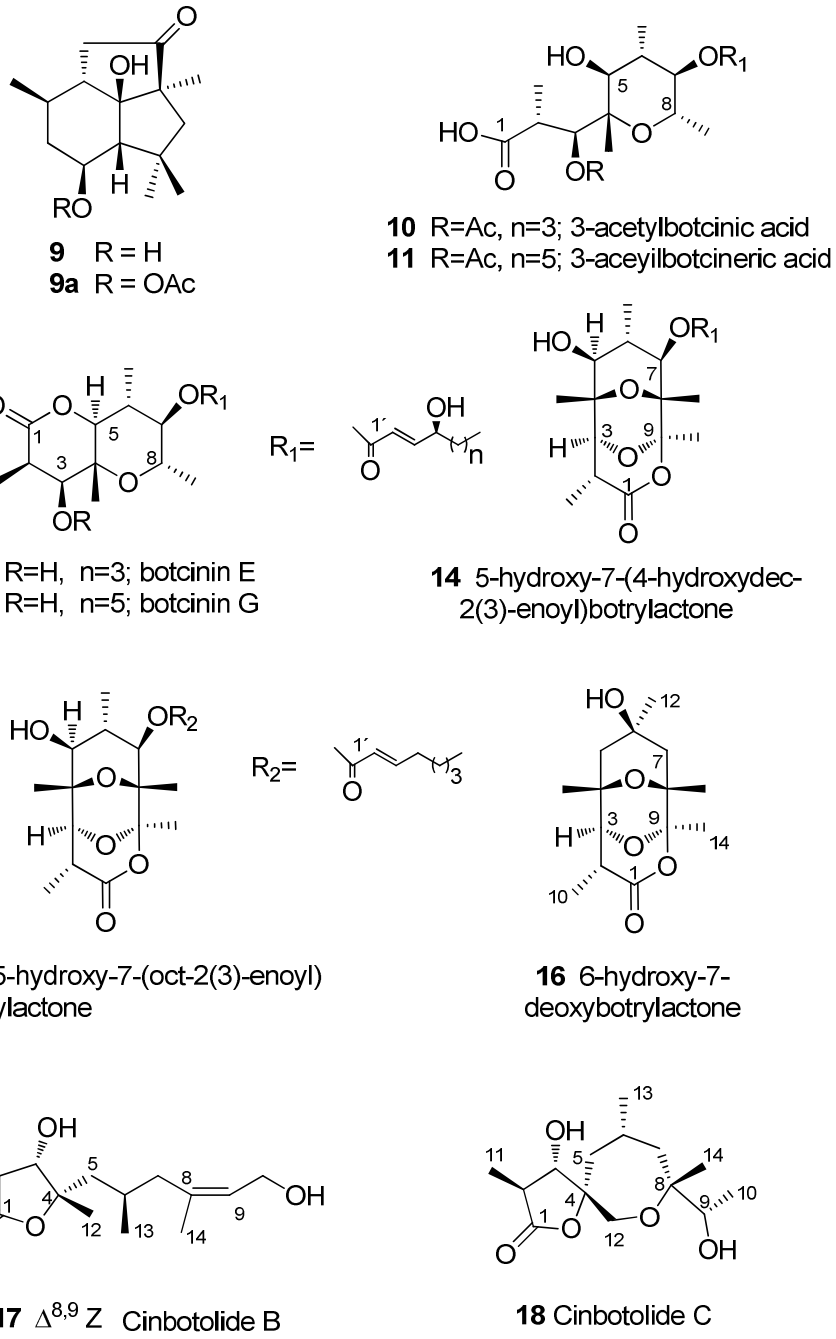
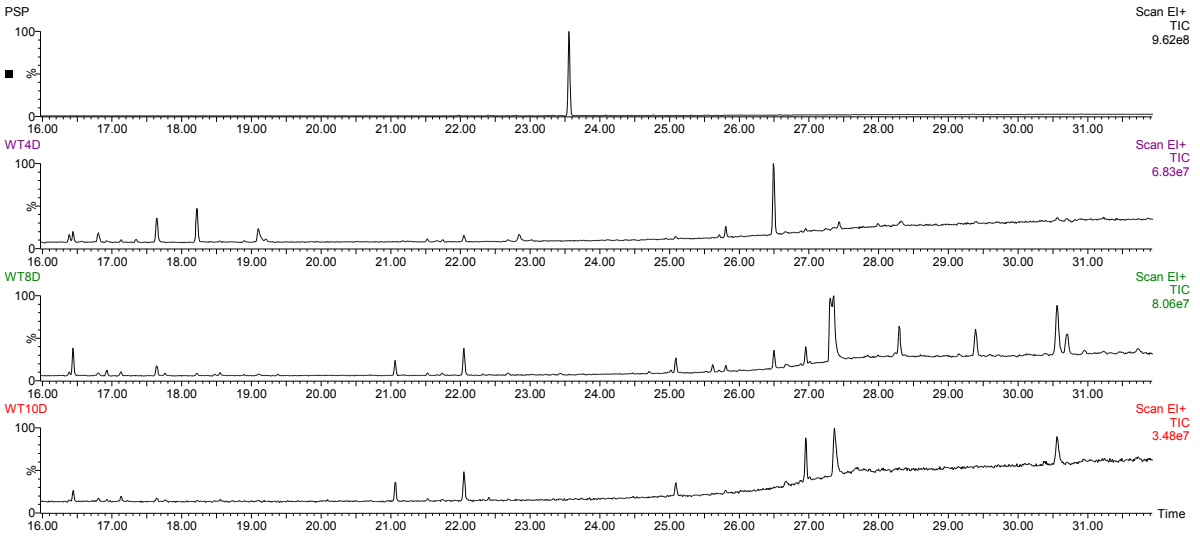


Figure 4. Some metabolites isolated from mutants *bcbot3*Δ and *bcbot4*Δ

5A



5B

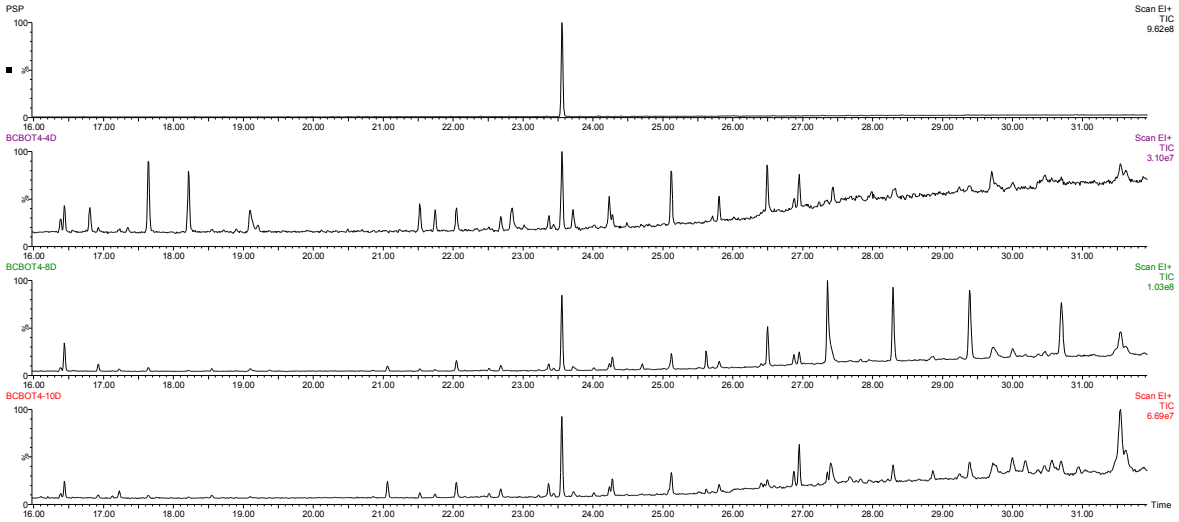


Figure 5. Comparative metabolites profiling by GC-MS analysis, for determination of probotryane **2a** in the strain broths. **5A)** Chromatograms of **2a** and of the extract obtained from the wild type, B05.10, to 4, 8 and 10 days. **5B)** Chromatograms of **2a** and of the extract obtained from the mutant *bcbot4*Δ to 4, 8 and 10 days.

Chemical characterization of polyketides isolated from *Bcbot4*Δ mutant.

The metabolites **15** and **16** showed a pattern of NMR signals which were characteristic of the botrylactones.[11] The ^{13}C NMR contained 22 signals indicating that the side chain contained eight carbon atoms. The ^{1}H NMR of compound **15** resembled that of **14.** The most significant differences were the multiplicity of the signals corresponding to the double bond and the absence of the signal of a geminal proton to a hydroxyl group in the side chain. Thus the vinylic proton signals appeared as doublet of triplets at ð 7.04, H-3´ and a doublet corresponding to H-2´, ð 5.84 indicating that the compound **15** possessed a side chain which was similar to that of compound **14** but with eight carbon atoms and without a hydroxyl group at C-4´.

The HRMS which was supported by the ^{13}C NMR spectrum, showed that compound **15** possessed a molecular formula of $C_{22}H_{34}O_7$. The COSY, HSQC and HMBC spectra and nOe experiments (Supplementary Table S2) led to the structure 5-hydroxy-7-(oct-2-enoyl)botrylactone for compound **15**.

Compound **16** showed ^{1}H NMR signals that were comparable to botrylactone (**3**).[11] The main differences were the lack of the signal typical of the C-7,CH(OH) whilst the signal assigned to the C-6 methyl group was a singlet rather than a doublet. The HRMS and ^{13}C NMR spectra indicated that the compound **16** had a molecular formula $C_{14}H_{22}O_5$. All the carbon and their associated proton signals were assigned from an examination of the COSY, HSQC, HMBC and NOESY spectra (Table S3), in accord with the structure and relative stereochemistry of compound **16**. The relative stereochemistry of the chiral centre at C-6 followed from the nOe experiments. The rigid structure of the compound meant that a beta configuration of the C-6 methyl group would produce nOe interactions with the C-4 and C-8 methyl groups (Supplementary Figure S1) which would not be observed with its epimer. However irradiation of the C-4 methyl group gave nOe interactions with H-2 and H-5β whilst irradiation of the C-8 methyl signals revealed interactions with H-7β and H-14. Furthermore irradiation of the C-6 methyl signal only gave nOe interactions with H-5α and H-7α indicating an α rather than a β configuration for this methyl group. These data led to the structure and stereochemistry of 6-β-hydroxy-7-deoxybotrylactone for **16** (Table S3, Figure S1).

The ^{1}H NMR signals of compound **17** resembled those of cinbotolide A (**8**).[13] There was one primary and one secondary hydroxyl group and one double bond in its structure. The main differences were the multiplicities of the -CH_2OH signal and the C-9 vinylic proton resonance. In addition to the signals assigned to the methyl groups at C-2, C-4, C-6 and C-8, there was a double-doublet at ∂ 5.39 (H-9) and signals at ∂ 4.15 (dd, J = 12.0, 6.9 Hz) and 4.05 (d, J = 5.4 Hz) attributed to H-10 and H-3 respectively.

The HRMS together with the ^{13}C NMR spectrum was consistent with a molecular formula $C_{14}H_{24}O_4$. Examination of the HSQC, HMBC and COSY spectra enabled all the carbon and their associated ^{1}H NMR signals to be assigned (Table S4). Thus compound **17**, which was named cinbotolide B, was the "Z" isomer of cinbotolide A (**8**).

The molecular formula of compound **18** was shown by HRMS to be $C_{14}H_{24}O_5$. The ^{13}C-NMR spectrum contained signals corresponding to four methyl, three methylene and four methine groups together with three quaternary carbons. The latter included a signal at δ 177.3 ppm attributed to a lactone. The four methyl groups signals in the ^{1}H NMR spectrum were three doublets (δ 0.98, 1.14 and 1.26) and a singlet (δ 1.21). The pattern of signals was typical of a cinbotolide lactone containing two hydroxyl groups. The proposed structure **18** was consistent with the HSQC, HMBC and COSY spectra (Table S5). The stereochemistry was deduced from nOe experiments. When H-3 was irradiated, there were enhancements to the signals corresponding to H-2, H-11 and H-5β and when H-2 was irradiated there were enhancements to the signals corresponding to H-3, H-11 and in particular to H-5α. Consequently compound **18** was a C-2 epimer (H-2α) of the cinbotolides A and B (**8** and **17**). This epimerization at C-2 has been observed in other polyketides biosynthesized by this fungus[11] and it was supported by conformational study carried out with molecular mechanic calculation (Figures S1 and S2). When H-6 was irradiated nOe effects were observed at H-14, H-12, H-5 and H-7. A nOe effect on H-2 and H-5β was observed on irradiation of H-5α whilst effects on H-12, H-9, H-6 and H-10 were produced by irradiation of H-14. These effects were consistent with the structure and stereochemistry of **18**, named cinbotolide C. In this cinbotolide, epimerization has taken place at C-2 and the C-4 methyl group (C-12) has been oxidized and an ether bridge has been formed to C-8.

Genetic and molecular basis of botrydial biosynthesis

Although the biochemical function of the enzymes *BcBOT1* and *BcBOT2* is known, the functional role of the proteins *BcBOT3, BcBOT4* and *BcBOT5* are still unknown. In order to understand the molecular basis and to complete the biosynthetic pathway to botrydial (**1**), the KO mutants for the cytochrome P450 monooxygenases BcBOT3 and BcBOT4 encoding genes were constructed and their secondary metabolism studied from the chemical point of view to identify the potential biosynthetic intermediate which were produced and to characterize both the P450 monooxygenases.

Previous studies showed that BcBOT2 protein catalyzes the cyclization of FPP to the sesquiterpene alcohol PSP, **2a**, and is required for the production of botryane and probotryane sesquiterpenes, which firmly established that BcBOT2 is responsible for the initial step of the botrydial biosynthesis (**1**).[28,31] On the other hand, inactivation of the *BcBOT1* gene demonstrated that those mutants were unable to produce 10β,15α-dihydroxyprobotryane (**2e**), botrydial (**1**) or some of its relatives. Instead the intermediate **2c** was accumulated, indicating that the monooxygenase BcBOT1 was responsible for the regiospecific hydroxylation at C-15, in intermediate **2b**, to give **2d** in botrydial biosynthesis, Figure 2.[27]

In this context, the knock out *bcbot3*Δ and *bcbot4*Δ mutants were fermented and their broths were examined to identify the secondary metabolites which were produced. Compounds **2d** and **9** (Figures 2 and 4) were isolated from the broth of the mutant *bcbot3*Δ and characterized. The large amount of the metabolite **2d** which was isolated clearly indicated that the monooxygenase BcBOT3 is involved in the regio- and stereospecific β hydroxylation at C-10, on the skeleton of intermediate **2b**, yielding intermediate **2c**, and thus the hydroxylated derivative at C-15, **2d**, accumulated.

It was observed that the *bcbot3*Δ mutant did not produce botrydial (**1**) and only produces small amounts of botcinin A (**6**). This result was unexpected, since we had previously established that the suppression or inhibition of the biosynthetic pathway leading to botrydial (**1**) induced the overproduction of the second family of toxins, botcinic and botcineric acids (**4, 5**) and botcinins A and B (**6**, **7**).[32] These results seem to suggest that the metabolic system of the

fungus behaved as though the production of botrydial (**1**) was not inhibited, keeping the gene expression and the production of botcinins low. This result leads us to propose that this P450 monooxygenase as a good target for controlling the fungus and its pathogenicity by its inhibition.

The mutant *bcbot4*Δ, accumulated the probotryane **2a** in the less polar fractions. This was established by GC-MS using an authentic sample of **2a** as reference, Figure 5. These results showed that the monooxygenase BCBOT4 catalyzes the first regio- and stereospecific hydroxylation of the probotryane skeleton **2a,** at C-4.

The previously reported isolation of intermediates **2b**, and acetylated derivatives **2c** and **2d**[23] led us to propose that the acetyl transferase BcBOT5 brought about the acetylation of the hydroxyl group at C-4 of probotryane **2b**.

These results lead us to propose the complete biosynthetic pathway to botrydial (**1**), Figure 2. Thus, direct and indirect evidence for the cyclisation of FPP to the parent tricyclic alcohol **2a**, by the sesquiterpene cyclase BcBOT2, has been reported.[28,31] In addition, the accumulation of intermediate probotryanes **2a**, **2c** and **2d** confirmed the proposed biosynthetic sequence in which the BcBOT4 monooxygenase produced the hydroxylation at C-4 to give intermediate **2b.** Acetylation of the hydroxyl at C-4 was carried out by the acetyl transferase BcBOT5, followed by the combined action of the P450 monooxygenases BcBOT3 and BcBOT1, to yield finally the glycol **2e**, *via* the regio- and stereospecific hydroxylations at C-10 and C-15 of the intermediates **2c** and **2d**, respectively.

The cleavage of the C-10-C-15 bond of probotryane skeleton is an intriguing and chemically important reaction which could be mediated by some of the monooxygenases or by a combination of them. Many P450 monooxygenases carry out the oxidative cleavage of vicinal diols, such as the side chain cleavage (scc) enzyme in testosterone biosynthesis that cleaves the 20,22-diol.[33] Although the mechanism of this cleavage has never been settled in detail, in this case it is possible that in the formation of **2e**, either BcBOT3 or BcBOT1 would oxidize either the 10- or the 15-hydroxy group to the hydroperoxide derivative which would then undergoes heterolytic fragmentation to give the dialdehyde botrydial (**1**).

The products isolated from these mutants shed an interesting light on a possible mechanism for the cleavage of the C-10:C-15 bond in botrydial biosynthesis. Earlier studies had shown that this takes place with the formation of botrydial rather than dihydrobotrydial and the use of stereospecifically labelled mevalonates implicated a trans C-10:C-15 glycol.[21,22,24] The formation of a C-15 ketone (**9**) when *BcBOT3* is knocked-out but *BcBOT1* remains, suggests that the oxidation of the C-15 alcohol to a ketone can be mediated by this enzyme. Since BcBOT1 is a mono-oxygenase rather than a dehydrogenase, the oxidation may occur by the reaction indicated in Figure 6 (A), where ketone (**9**) is obtained, via hydroperoxide, from the alcohol intermediate **2d**.

A corollary to this suggestion is that the cleavage of the C-10:C-15 bond is also mediated by this enzyme. This would involve the fragmentation of a C-15 hydroperoxide derived from **2e** (Figure 6 (B)). This would accord with the labelling pattern from the mevalonates and in particular the trans relationship between the functional groups.[24]

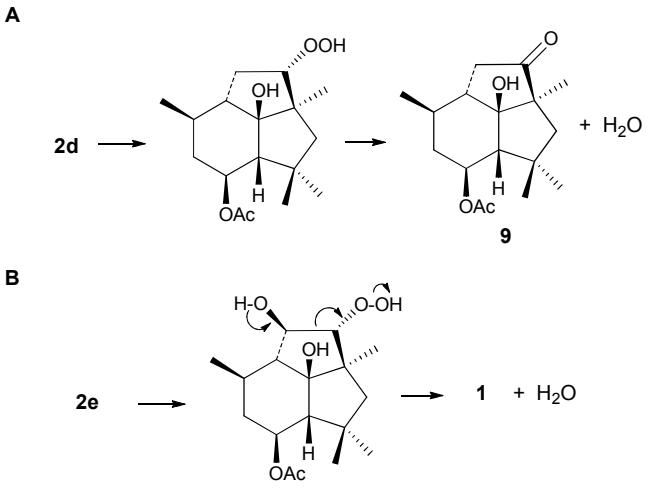


Figure 6. **6A**) Formation of compound **9** from intermediate **2d**. **6B**) Formation of botrydial (**1**) from intermediate **2e**

Here we have reported the genetic and molecular basis of botrydial biosynthesis and the assignment of the biochemical function to the five genes that are co-localized in the genome of *B. cinerea* and constitute the botrydial biosynthesis gene cluster. Genes *BcBOT*3 and *BcBOT*4, encoding two cytochrome P450 monooxygenases, catalysed regio- and stereospecific

hydroxylations, at the carbons C-10 and C-4 respectively, of the presilphiperfolan-8*β*-ol skeleton. Interestingly, the *bcbot4*Δ mutant overproduced a significant number of polyketides, which included the four new metabolites **15**-**18**.

METHODS

Fungal strains. The strain of *Botrytis cinerea* Pers. Fr. employed in this work, B05.10, was isolated from a *Vitis* field.[34]

Media and culture conditions. *B. cinerea* strains and mutants were cultivated at 23°C with 12 h daylight per day. Cultures were grown on malt agar medium (MA; 20g /l. malt extract, 5g/l. yeast extract, 15g/l. agar), and minimal medium (MMII; 20g/l. glucose, 2g/l. $NaNO_3$, 1g/l KH_2PO_4, 500 mg/l $MgSO_4.7H_2O$, 500mg/l KCl, 10 mg/l $FeSO_4.7H_2O$, 15g/l. agar). The transformation media was a minimal medium supplemented with saccharose (MMV; 20g/l. glucose, 200g/l saccharose, 2g/l. NaNO3, 1g/l KH2PO4, 500 mg/l MgSO4.7H_2O, 500mg/l KCl, 10 mg/l FeSO4.7H_2O, 15g/l. agar). The bcbot3 and bcbot4 mutants were selected with nourseothricin fungicide (Werner Bioagent, Germany- 70 μg/ml).

Standard molecular methods.

Gene deletion of the cytochrome P450-encoding genes *bcbot3* and *bcbot4*

The *B. cinerea* genes *bcbot3* and *bcbot4* (GenBank: AY277723.2) were deleted by homologous recombination. The 5' genomic non-coding regions of *bcbot3* and *bcbot4* (882 pb and 1442 pb respectively) were amplified by PCR. In the same way, we obtained the 3' flanking sequences of *bcbot3* and *bcbot4* (1027pb and 866 pb respectively) and the nourseothricin resistance gene (*nat*). For each gene, a Knock-Out (KO) cassette made of the 5' flanking sequence of *bcbot*, the nat resistance gene and the 3' flanking region of *bcbot* was obtained by double-joint PCR fusion[35] as previously described for *B. cinerea*.[36] All the PCR primers are listed in Table 1.

Protoplasts of the B05.10 strain were transformed as previously reported.[37] Transformants were selected and purified on medium agar containing 70 µg/ml nourseothricin. Gene inactivation were verified by PCR using primers designed outside the K.O. cassette and primers designed in the resistance gene *nat* (Primers verif integration, Table 1). In addition, the absence of the *bcbot3* or *bcbot4* WT alleles in the transformant was also checked by PCR. *B. cinerea* mutants used in this study are described at http://botbioger.versailles.inra.fr/botmut/.

Chemical methods// *Analysis of the Bcbot3Δ and Bcbot4Δ metabolite profile:*

General procedure. *B. cinerea* WT strain and mutants were grown on malt agar medium[32] at 25 °C to produce mycelium plugs (0.8 cm) that were further used to inoculate the medium for metabolite production. All strains were fermented in solid malt agar medium following the reported protocol.[32] Extraction of the solid agar malt medium was carried out with an ultrasonic bath, using ethyl acetate (3 x 0.5 vol.). The solvents were dried over Na_2SO_4 and concentrated to dryness.

Table 1: PCR primers used for *bcbot3* and *bcbot4* genes deletion

Primers Name pair	Sequence Fwd	Sequence Rev	Usage
BD48/BD51	tccattaagtctgtttggataaagt	GGGAATGCGGCTCTAGTCGGGTTGCTCTTCTCCAT	BcBot3 homologuous 5' region
BD49/BD50	gggcttgaaaagaatttgtattg	**cctgagcggcctgca**aaatggatcgcaacttcgtc	BcBot3 homologuous 3' region
BD44/BD46	**gcttcttgtccttccgctac**	GGGAATGCGGCTCTA**GTTCACTTTCTCCGATTCATG**	BcBot4 homologuous 5' region
BD43/BD45	**TATGACCGGAGAGCTCGAAA**	cctgagcggcctgca**cttaaagccctatgacagga**	BcBot4 homologuous 3' region
K7Nat1 Fwd/Rev	TAGAGCCGCATTCCCGATTCGG	TGCAGGCCGCTCAGGGGCAGGG	Nourseothricin K7
BD52/BD12	gctacaggcggtggtatctc	CTAAGGGCTCGCGGAGTTG	BcBot3 purity
BD52/Nat1Fverif	gctacaggcggtggtatctc	GACACCGCCCTGTACGAC	BcBot3 verif integration
BD2/BD13	cgcgggagaagtctctaagga	gcttgaattcccagggatctg	BcBot4 purity
BD47/Nat1Fverif	TCATTCACTCGAGCATACTTCC	GACACCGCCCTGTACGAC	BcBot4 verif integration

Purification of the metabolites was carried out by column chromatography (CC) and semipreparative and analytical HPLC.[28,32] Optical rotation was recorded with a digital polarimeter. IR spectra were determined on a FT-IR spectrophotometer and reported as wave number (cm^{-1}).[13]

^{1}H and ^{13}C NMR spectra were obtained on Agilent 400 and 500 MHz spectrometers with $SiMe_4$ as the internal reference at 25°C.[13] Mass Spectroscopy (MS) and High Resolution Mass Spectroscopy (HRMS) were performed on Thermoquest Voyager spectrometer and QTOF mass spectrometer, respectively.[13]

Bcbot3Δ metabolite analysis

10 days after inoculation, the solid agar malt medium was extracted[32] yielding a dense oil from *bcbot3Δ* culture (350 mg) that were separated by column chromatography on silica gel, eluted with mixtures containing increasing percentages of ethyl acetate/hexane (10–100%) and methanol as solvent.

Final purification of selected fractions was carried out by HPLC (Hexane (Hex)-Ethyl Accetate (EtAc) 40:60; flow 1 mL min^{-1}). Three compounds were isolated and characterized: 4β-acetoxy-15α-hydroxyprobotryane (**2d**) (39 mg), 4β-hydroxyprobotryan-15-ona (**9**) (1.1 mg) and botcinin A (**6**) (1.5 mg).

4β-hydroxyprobotryan-15-one (**9**). Colourless oil; $[\alpha]_D^{25}$ = -9.62 (c=0.106mg, $CHCl_3$); **IR** υ_{max} (film) 3425.38, 2961.33, 1729.85, 1454.75, 913.80, 771.28 cm^{-1}. **^{1}H NMR** (600 MHz, $CDCl_3$), δ 4.04 (1H, ddd, *J*=11.2, 9.6, 4.0 Hz, H-4), 2.69 (1H, dd, *J*= 18.1, 8.6 Hz, H-10), 2.63 (1H, dd, *J*= 18.1, 3.3 Hz, H-10´), 2.13 (1H, d (br), H-7), 1.91 (1H, ddd, J= 12.8, 4.0, 2.9 Hz, H-3), 1.82 (1H, m, H-2), 1.64 (1H, ddd, J= 11.6, 8.6, 3.3 Hz, H-1), 1.46 (1H, d, *J*=9.6 Hz, H-5), 1.37 (3H, s, H-14), 1.34 (3H, s, H-13), 1.32 (3H, s, H-12), 0.97 (3H, d, *J*=6.4 Hz, H-11). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 211.0 (s, C-15), 94.4 (s, C-9), 70.0 (d, C-4), 63.2 (s, C-8), 60.6 (d, C-5), 48.7 (d, C-1), 47.2 (s, C-6), 44.2 (t,

C-7), 44.1 (t, C-3), 42.6 (t, C-10), 36.2 (q, C-12), 33.4 (d, C-2), 27.8 (q, C-13), 23.9 (q, C-14), 21.0 (q, C-11). **HRMS (ES$^+$) m/z** calcd for $C_{15}H_{25}O_3$ $[M + H]^+$ 253.1804, found 253.1807.

Acetylation of 4β-hydroxyprobotryan-15-one (9).

Compound **9** (5 mg) was dissolved in the minimum quantity of dry pyridine (0.5 ml), and acetic anhydride (30 mg) was added to the solution at 25 ºC. The mixture was kept at room temperature for 24 h and the solvent evaporated. The crude was chromatographed over sílica gel (hexane-ethyl acetate 8:2) and HPLC (Hex-EtAc) 80:20; flow 1 mL min^{-1}; tR=19.5 min) to give derivative **9a** (4.5 mg).

4β-acetoxyprobotryan-15-one (**9a**). Colourless oil; $[\alpha]_D^{25}$ = -9.40 (c=0.1mg, CHCl3); **IR υmax (film)** 2350.4, 1727.5, 1248, 776.61, 669.12 470.26 cm^{-1}. **^{1}H NMR** (600 MHz, benzene) δ 5.11 (1H, td, J=10.9, 4.0 Hz), 2.70 (1H, dd, J= 18.1, 8.6, Hz, H-10), 2.64 (1H, dd, J= 18.1, 3.4, Hz, H-10´), 2.17 – 2.08 (2H, m), 2.03 (3H, s, CH_3COO-), 2.02 (1H, dt, J=7.0, 5.4 Hz, H-3), 1.95 (1H, m, H-2), 1.37 (3H, d, J=0.9 Hz), 1.29 (3H, s), 1.24 (2H, t, J=3.6 Hz), 1.16 (3H, s), 1.23 – 1.01 (2H, m), 0.95 (3H, d, J=6.4 Hz, H-11). **^{13}C NMR** (151 MHz, $CHCl_3$) δ 210.5 (s, C-15), 170.4 (s, CH_3COO-), 94.0 (s, C-9), 72.2 (d, C-4), 63.1 (s, C-8), 56.2 (d, C-5), 48.6 (d, C-1), 47.15 (s, C-6), 44.1 (t, C-7), 42.7 (t, C-10), 39.6 (t, C-3), 35.8 (q, C-12), 33.2 (d, C-2), 27.5 (q, C-13), 24.1 (q, C-14), 21.4 (CH_3COO-), 20.9 (d, C-11). **HRMS (ES$^-$) m/z** calcd for $C_{17}H_{25}O_4$ $[M - H]^-$ 293.1753, found 293.1779.

Bcbot4Δ metabolite analysis

The analysis was carried out as described for *bcbot3Δ*. Evaporation of the solvent under reduced pressure afforded a dense oil from the *bcbot4Δ* culture (360 mg) that was chromatographed by CC on silica gel. Final purification by HPLC and GC-MS yielded the metabolite probotryane **2a** and a significant number (fifteen) of metabolites with a polyketide skeleta. In addition to the known polyketides: botrylactone (**3**) (21.7 mg), botcinic and botcineric acids (**4**, **5**) (6.9, 4.5 mg), botcinins A, B, E and G (**6**, **7**, **12**, **13**), (63.6, 37.6, 10.1, 7.9 mg) cinbotolide (**8**) (12 mg), acetylbotcinic and acetylbotcineric acids (**10**, **11**) (8.3, 7.0 mg), and 5-hydroxy-7-(4-hydroxydec-2(3)-enoyl)botrylactone (**14**) (12.2 mg); the new botrylactones, 5-

hydroxy-7-(oct-2(3)-enoyl) botrylactone (**15**) (11.5 mg) (Semi-preparative (Semprep) HPLC: Hex-EtAc-acetone(Act) 70:20:10; flow 2.5 mL min^{-1}; tR=27 min) and 6-hydroxy-7-deoxybotrylactone (**16**) (15 mg) (Semprep. HPLC: Hex-EtAc-Act 70:20:10; flow 2.5 mL min^{-1}; tR=38.5 min), and the new cinbotolides B (**17**) (2.4 mg) (Semprep. HPLC: Hex-EtAc-Act 50:40:10; flow 2.5 mL min^{-1}; tR=40 min) and C (**18**) (11.8 mg) (Semprep HPLC: Hex-EtAc 75:25; flow 3 mL min^{-1}; tR=45 min) were isolated and characterized.

Presilphiperfolan-8β-ol (Probotriane) (2a).

The less polar fraction obtained from the *bcbot4*Δ extract with hexane was studied by GC-MS. Fermentations, for four, eight and ten days, of wild type B05.10 and of the mutant *bcbot4Δ* were carried out and the less polar fractions obtained by extraction with hexane studied by GC-MS. An authentic sample of the presilphiperfolan-8β-ol (**2a**) was used as reference to detect the compound in the extracts. Compound **2a** was not detected in the hexane fractions from the wild type to 4, 8 and 10 days. However, the presence of compound **2a** was clearly revealed in all fractions from the fermentations of the mutant *bcbot4*Δ on different days (see Figure 5).

***2R, 3S, 4S, 6S, 8R, 9R*-5-hydroxy-7-(oct-2(3)-enoyl) botrylactone (15)** Yellow oil; $[\alpha]_D^{25}$ 4.7 (c=0.1mg, $CHCl_3$); **IR υ_{max} (film)** 3348, 2929.48, 1721.36, 1456.89, 1260.51, 1105.94, 1015.96, 719.49 cm^{-1}. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.04 (1H, dt, *J*=15.6, 6.9 Hz, H-3´), 5.84 (1H, dt, *J*=15.6, 1.6 Hz, H-2´), 4.93 (1H, d, *J*=10.9 Hz, H-7), 3.68 (1H, d(br), *J*=11.1 Hz, H-5), 3.52 (1H, d, *J*=0.5 Hz, H-3), 2.76 (1H, qd, *J*=7.4, 0.5 Hz, H-2), 2.21 (2H, m, H-4´), 1.97 (1H, m, H-6), 1.55 (3H, s, C_9-CH_3), 1.47 (3H, d, *J*=7.4 Hz, C_2-CH_3), 1.38-1.24 (m, H-5´, H-6´,H-7´), 1.15 (3H, s, C_4-CH_3), 1.10 (3H, s, C_8-CH_3), 1.04 (3H, d, *J*=6.5 Hz, C_6-CH_3), 0.90 (3H, t, *J*=6.9 Hz, H-8´). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 171.0 (s, C-1), 165.5 (s, C-1´), 151.4 (d, C-3´), 120.2 (d, C-2´), 104.2(s, C-9), 80.8 (d, C-3), 79.3 (d, C-8), 76.2 (s, C-4), 75.2 (d, C-5), 73.8 (d, C-7), 36.5 (d, C-6), 34.5 (d, C-2), 32.3(t, C-4´), 31.3 (t, C-5´), 27.6 (t, C-6`), 22.4 (t, C-7´), 21.7 (q, C_9-CH_3), 18.5 (q, C_2-CH_3), 18.2 (q, C_4-CH_3), 18.1 (q, C_8-CH_3), 13.9 (q, C-8´), 13.8 (q, C_6-CH_3). **HRMS (ES^+) m/z** calcd for $C_{22}H_{35}O_7$ $[M + H]^+$ 411.2383, found 411.2370.

6-hydroxy-7-deoxybotrylactone (16). White amorphous powder; $[\alpha]_D^{25}$ 1.16 (c=1.146mg, $CHCl_3$); **IR υ_{max} (film)** 3447.87, 2938.54, 1740.30, 1258.90, 1065.66, 976.23, 691.56 cm^{-1}; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 3.33 (1H, d, *J*=0.7 Hz, H-3), 3.27 (1H, s(br), -OH), 2.74 (1H, qd, *J*=7.3, 0.7 Hz, H-2), 1.91 – 1.69 (4H, m, H-5, H-7), 1.44 (3H, d, *J*=7.3 Hz, C_2-CH_3), 1.44 (3H, s, C_9-CH_3), 1.30 (3H, s, C_8-CH_3), 1.27 (3H, s, C_4-CH_3), 1.24 (3H, s, C_6-CH_3). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.1 (s, C-1), 104.4 (s, C-9), 81.6 (d, C-3), 76.5 (s,C-8), 72.8 (s, C-4), 70.3 (s, C-6), 45.0 (t, C-5), 43.1 (t,C-7), 34.4 (d, C-2), 28.2 (q, C_6-CH_3), 27.5 (q, C_4-CH_3), 26.4 (q, C_8-CH_3), 2206 (C_9-CH_3), 18.6 (q, C_2-CH_3). **HRMS (ES^-)** ***m/z*** calcd for $C_{14}H_{21}O_5$ $[M-H]^-$ 269.1389, found 269.1385.

Cinbotolide B (17). Yellow oil; $[\alpha]_D^{25}$ -16.03 (c=0.131mg, $CHCl_3$); **IR υ_{max} (film)** 3417.83, 2925.61, 1747.89, 1455.50, 1383.58, 1217.32, 996.51, 934.66, 748.70 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.39 (1H, q, *J*=6.9 Hz, H-9), 4.15 (2H, dd, *J*= 12.0, 6.9 Hz, H-10), 4.05 (1H, d, *J*=5.5 Hz, H-3), 2.94 (1H, dq, *J*= 7.3, 5.5 Hz, H-2), 2.26 (1H, dd(br), *J*=13.1, 6.4 Hz, H-5), 2.02 – 1.75 (3H, m, H-5´, H-6, H-7), 1.68 (3H, d, *J*=6.9 Hz, C_8-CH_3), 1.71 – 1.6 (1H, m, H-7´), 1.36 (3H, s, C_4-CH_3), 1.24 (3H, d, *J*=7.3 Hz, C_2-CH_3), 0.95 (3H, d, *J*= 6.2 Hz, C_6-CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 177.6 (s, C-1), 137.3 (s, C-8), 125.2 (d, C-9), 87.8 (s, C-4), 76.5 (d, C-3), 60.0 (t, C-10), 44.5 (t, C-7), 40.7 (t, C-5), 40.0 (d, C-2), 27.2 (d, C-6), 23.2 (q, C_4-CH_3), 21.4 (q, C_6-CH_3), 13.2 (q, C_8-CH_3), 8.3 (q, C_2-CH_3). **HRMS (ES^+) m/z** calcd for $C_{14}H_{25}O_4$ $[M + H]^+$ 257.1747, found 257.1758.

Cinbotolide C (18). Colourless oil; ; $[\alpha]_D^{25}$ 52.5 (c=0.12mg, $CHCl_3$); **IR υ_{max} (film)** 3426.45, 2926.73, 1752.33, 1643.35, 1456.46, 1046.65, 771.09 cm^{-1}. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 4.18 (1H, d, *J*=5.5 Hz, H-3), 4.01 (2H, d, *J*=1.3 Hz, H-12), 3.65 (1H, q, *J*=6.5 Hz, H-9), 2.86 (1H, dq, *J*=7.3, 5.5 Hz, H-2), 2.26 (1H, m, H-6), 1.78 (1H, ddd, *J*=14.5, 2.8, 1.6 Hz, H-5), 1.66 (1H,dd, *J*= 14.2, 11.4 Hz, H-7´), 1.57 (1H, dd, *J*= 14.5, 10.9 Hz, H-5´), 1.47 (1H, dt, *J*=14.2, 1.7 Hz, H-7), 1.26 (3H, d, *J*=7.3 Hz, C_2-CH_3), 1.21 (3H, s, C_8-CH_3), 1.14 (3H, d, *J*=6.5 Hz, C_9-CH_3), 0.98 (3H, d, *J*=6.7 Hz, C_6-CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 177.3 (s, C-1), 87.7 (s, C-8), 79.8 (s, C-4), 76.6 (d, C-3), 74.4 (d, C-9), 63.1 (t, C-12), 45.7 (t, C-5), 42.7(t, C-7), 39.7 (d, C-2), 24.6 (q, C_6-CH_3), 24.6 (d, C-6), 18.9 (q,

C_8-CH_3), 17.3 (q, C_{10}-CH_3), 8.3 (q, C_2-CH_3). **HRMS (ES^+) m/z** calcd for $C_{14}H_{25}O_5$ $[M + H]^+$ 273.1702, found 273.1701.

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Supporting Information Available: Details of spectroscopic and characterization data, and reproductions of ^{1}H- and ^{13}C-NMR spectra. This material is available free of charge via Internet.

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