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A Shared Biosynthetic Pathway for Botcinins and Botrylactones Revealed through Gene Deletions

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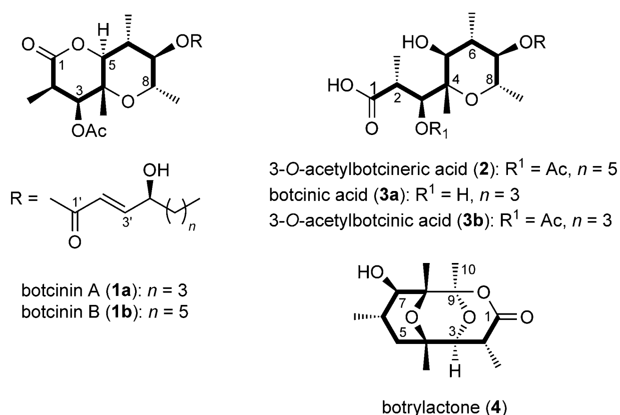
Isotopic labelling experiments and the study of mutants with disrupted genes encoding botcinic acid have revealed a common link in the biosynthesis of the polyketide toxins excreted by *Botrytis cinerea*: botcinins and botrylactones. Furthermore, the results reported here shed light on the origin of the starter unit, thereby solving a long-standing mystery in the biosynthesis of botcinins.

Introduction

Botrytis cinerea is the causal agent of grey mould disease, which affects more than 200 agriculturally important and ornamental plant species. This necrotrophic fungus is able to kill host cells through the production of toxins and reactive oxygen species and by inducing a plant-produced oxidative burst.[1,2] Two groups of nonspecific phytotoxins have been identified: the botryanes[3] and the botcinins (**1**–**3**).[4] In addition, a new polyketide metabolite family, the botrylactones (**4**), whose phytotoxic activity has not yet been evaluated, has been identified.[5] Botcinins (**1**–**3**) and botrylactones (**4**) are related polyketides sharing an identical C1–C8 fragment (Scheme 1). Currently, metabolites of the botcinin family are of particular interest because of their phytotoxicity and antifungal activity against the fungus *Magnaporthe grisea*, the causal agent of rice blast.[4] Furthermore, a synergistic behaviour of botcinin and botryane toxins in the phytotoxicity and infection mechanism of the phytopathogen *B. cinerea* has been reported,[6] and two new potential biological targets for the control of this phytopathogen have been revealed. Consequently, there is great interest in ascertaining the mode of action of these molecules and their biosynthetic pathways.

Preliminary studies in which sodium [1-^{13}C]- and [1,2-$^{13}C_2$]-acetates, [1,2,3-$^{13}C_3$]propionate and L-[$^2H_3$-methyl]methionine were fed to cultures of *B. cinerea* clearly demonstrated 3-*O*-acetylbotcineric acid (**2**) to be an acetate-derived polyketide; it is assembled from C-8 to C-1, with its four pendent methyl groups originating from the methyl group of methionine (Scheme 2).[7,8] Incorporation of methyl groups from methionine by a methyltransferase domain is often observed in fungal iterative type I polyketide synthases (PKSs). Unusually though, in this system we observed an apparent three-carbon starter unit derived from acetate and methionine. To the best of our knowledge, an acetate/methionine-derived C_3 starter unit has only been observed in a small handful of metabolites.[9–12]

We recently published a revised structure and absolute configuration of botrylactone, thereby demonstrating that derivatives of botcinin (**1**–**3**) and botrylactone (**4**) share the same functional groups and stereochemistry, and differ only by one acetate unit. This finding led us to propose a common biosynthetic origin.[5c] In this paper, we confirm this on the basis of



Scheme 1. Polyketides from *Botrytis cinerea*.

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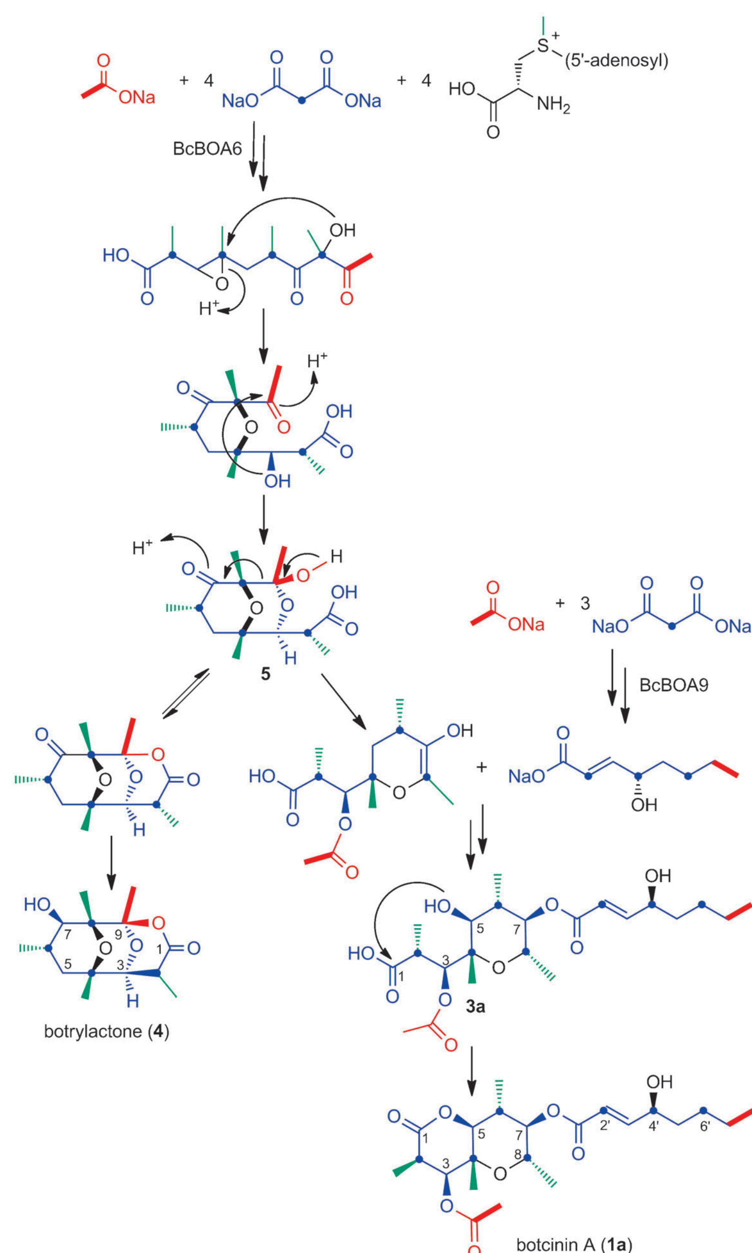

Scheme 2. Proposed common biosynthetic route to botcinins (**1**–**3**) and botrylactones (**4**). Labelling pattern resulting from feeding experiments with sodium [1,2-$^{13}C_2$]acetate*, [2-^{13}C]malonate and L-[$^2H_3$-methyl]methionine. *Coupling was also observed between carbons C1-C2, C3-C4, C5-C6 and C7-C8 in both families of polyketides because of acetate↔malonate interconversion.

isotopic labelling experiments and the functional analysis of *B. cinerea* mutants that are impaired in the production of some compounds and overproduce others.[13]

Results and Discussion

The PKS genes responsible for botcinic acid (**3a**) production have recently been investigated by using transcriptomic and reverse genetic approaches. Inactivation of the genes *BcBOA6* and *BcBOA9* (formerly named *BcPKS6* and *BcPKS9*) indicated that they act in concert to synthesise **3a**.[13] Indeed, the study of mycelium cultures by the previously described methodology[6,7] indicated that the *bcboa6*Δ and *bcboa9*Δ null mutants do not produce **3a** or its derivatives. Additionally, no botrylactone (**4**) was detected in the growth filtrate of the *bcboa6*Δ mutant, whereas this compound was shown to be overproduced by the *bcboa9*Δ mutant.[13] Taken together, these data suggested that *BcBOA6* is required for the production of both **3a** and **4**. Bioinformatics study of both BcBOA6 and BcBOA9 enzymes predicted the three essential PKS domains, that is, the ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains. Interestingly, additional domains were also predicted: dehydratase (DH), ketoreductase (KR), methyl transferase (MT) and enoyl reductase (ER) domains. Although DH and KR domains were found in both PKS enzymes, the MT domain is present in BcBOA6 only, and the ER domain is present in BcBOA9.[13] The MT domain could be responsible for the incorporation four methyl groups.

It is likely then that BcBOA6 mediates the formation of the per-methylated tetraketide core, common to both **4** and the botcinins (**1**), whereas BcBOA9 is likely to be responsible for the 7-*O*-acyl tetraketide side chain of botcinic acid and its derivatives (**1**–**3**). This assembly would be analogous to that by the two PKSs in lovastatin biosynthesis, in which one PKS makes the carbon skeleton and a second is involved in furnishing the *O*-acyl side chain.[14] The pathways to botrylactone (**4**) and botcinins (**1**) are likely to diverge, with a botrylactone-like precursor (**5**) opening up through a retro-Claisen mechanism to afford botcinins and derivatives (**1**–**3**; Scheme 2).

In order to investigate this hypothesis, feeding experiments with sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate, [2-^{13}C]malonate and L-[$^2H_3$-methyl]methionine were carried out with the botrylactone-overproducing strain *bcboa9*Δ.

The resultant labelled botrylactones (**4**) were extracted, purified and analysed by ^{13}C or ^{2}H NMR (Table 1, Scheme 2). When sodium [1-^{13}C]acetate was fed to a *bcboa9*Δ culture, significant enrichment was observed at C-1, C-3, C-5, C-7 and C-9. Feeding experiments with sodium [2-^{13}C]acetate and with a mixture of sodium [2-^{13}C]malonate and unlabelled sodium acetate led to enhanced signals that corresponded to C-2, C-4, C-6 and C-8 in both experiments. Further signal enhancement corresponding to the C-10 methyl group was observed in the experiment with [2-^{13}C]acetate. The ^{13}C NMR spectrum of **4** derived from sodium [1,2-$^{13}C_2$]acetate exhibited ^{13}C,^{13}C spin–spin coupling for all the carbons with the exception of the methyl groups at C-2, C-4, C-6 and C-8. The measured J_{CC} values are given in Table 1 and prove the presence of the following intact acetate units: C-1/C-2, C-3/C-4, C-5/C-6, C-7/C-8 and C-9/C-10.

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Table 1. Isotopic enrichment of carbons in botrylactone (**4**) after feeding sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetates and sodium [2-^{13}C]malonate to *bcboa9Δ*.

Carbon atom (N)	δ_C ($CDCl_3$)	Atom % ^{13}C excess[a] [1-^{13}C]acetate	[2-^{13}C]acetate	[2-^{13}C]malonate	J [Hz] [1,2-$^{13}C_2$]acetate
1	171.3	1.94	−0.30	−0.15	53.1
2	34.6	−0.22	3.32	4.52	53.1
2-CH_3	18.7	0	0	0	
3	81.7	1.51	−0.11	−0.06	39.7
4	71.2	−0.02	4.00	2.59	39.7
4-CH_3	26.1	0.08	−0.02	−0.06	
5	40.9	1.74	−0.19	−0.11	32.9
6	30.4	−0.16	3.78	5.44	32.9
6-CH_3	17.4	0.03	0.18	−0.07	
7	76.1	1.89	−0.12	−0.08	39.7
8	80.4	−0.15	3.17	3.28	39.7
8-CH_3	17.2	0.02	−0.07	−0.09	
9	104.1	0.90	−0.50	−0.41	49.1
10	21.4	−0.18	4.26	0.20	49.1

[a] Atom % ^{13}C excess = {($R_N/R_{N(UL)}$ × 1.1}−1.1, where R_N is the ratio of the peak intensity at the N-position in labelled compound calculated on the basis of the peak intensity at the 2-CH_3-position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at position N in unlabelled compound. Value 1.1 is the theoretical ^{13}C natural abundance (atom %).

These results clearly reveal the origin of 10 of the 14 carbon atoms in botrylactone (**4**): units C-1/C-2, C-3/C-4, C-5/C-6, and C-7/C-8 come from acetate units via malonate, whereas the C-9/C-10 fragment is derived directly from acetate.

The next step was to establish the origins of the remaining four carbons of **4**. If botrylactones and botcinins have a common biosynthetic origin, these four carbon atoms would be derived from the methyl group of methionine. The ^{2}H NMR of **4** isolated from a feeding experiment with L-[$^2H_3$-methyl]methionine showed four singlets at δ = 1.40, 1.12, 1.12 and 1.03 ppm, attributable to methyl groups at C-2, C-4, C-6 and C-8, respectively. This confirms that the methyl group at C-9 arises from the C-2 of acetate and that the extra methyl groups come from the methyl group of methionine.

All these data are consistent with our hypothesis. *B. cinerea* biosynthesises a C_{10}-polyketide by condensation of four units of malonyl-SCoA with one unit of acetyl-SCoA, which would be methylated in activated methylene groups to yield **5**. This putative bicyclic acid intermediate (**5**) could then either be converted to botrylactone (**4**) derivatives or lose the starter acetate unit through a retro-Claisen type C–C bond cleavage to yield botcinin derivatives (**1**–**3**; Scheme 2).

^{13}C-labelled malonate can be used to detect lack of polyketide acetate starter, because a significantly lower degree of labelling of acetate starter unit carbon atoms would be observed when this unit is present. Feeding of sodium [2-^{13}C]-malonate, administered in a mixture with unlabelled sodium acetate, was used to demonstrate the loss of starter unit in the biosynthesis of aurovertins.[15]

In order to determine whether the acetate starter unit is present in botcinins and derivatives (**1**–**3**), a feeding experiment with sodium [2-^{13}C]malonate and unlabelled sodium acetate was performed with the strain *bcbot2Δ*. This mutant, obtained by inactivation of the botryanes biosynthesis gene *BcBOT2* (encoding a sesquiterpene cyclase) is unable to produce botryanes (major metabolites of *B. cinerea*) but is capable of producing elevated amounts of botcinins (**1**–**3**).[16] The ^{13}C NMR spectra of the resulting isolated botcinins (**1**) showed clear enhancement of the signals corresponding to C-2, C-4, C-6, C-8, C-2′, C-4′ and C-6′ for both compounds (Table 2, Scheme 2) and further enhancement of the signal corresponding to C-8′ for botcinin B (**1 b**). All carbons were labelled to the same degree. The signals corresponding to the acetyl group (CH_3CO) and the starter unit of the side chain (C-8′ for botcinin A (**1 a**) and C-10′ for botcinin B (**1 b**)) did not exhibit any enhancement. Thus a clear "acetate starter" effect is observed, thus indicating that the side chain on C-7 is formed from a single polyketide chain.

Table 2. Isotopic enrichment of carbons in botcinins A and B after feeding sodium [2-^{13}C]malonate to *B. cinerea* mutant *bcbot2Δ*.

Carbon atom (N)	Botcinin A (**1 a**) δ_C ($CDCl_3$)	Atom % ^{13}C excess[a]	Botcinin B (**1 b**) δ_C ($CDCl_3$)	Atom % ^{13}C excess[a]
1	173.8	−0.24	173.2	0.61
2	37.3	3.72	37.3	4.11
2-CH_3	10.2	0	10.2	0
3	74.2	0.07	74.3	−0.08
4	75.2	1.89	75.2	2.82
4-CH_3	11.9	0.10	11.9	−0.07
5	78.5	0.11	78.6	0.09
6	35.4	3.61	35.4	4.95
6-CH_3	13.7	0.20	13.7	−0.18
7	76.0	0.01	76.1	−0.25
8	68.4	4.28	68.4	4.48
8-CH_3	18.2	0.09	18.2	0.14
1′	165.9	−0.02	165.7	1.21
2′	119.0	4.30	119.1	4.51
3′	151.8	0.02	151.8	0.07
4′	71.1	4.28	71.1	4.28
5′	36.3	0.04	36.7	0.25
6′	27.3	4.15	25.2	4.38
7′	22.5	0.09	29.1	0.04
8′	13.9	0.39	31.7	4.56
9′	–	–	22.5	−0.10
10′	–	–	14.0	0.31
CH_3CO	20.6	0.54	20.6	0.67
CH_3CO	170.1	0.06	170.1	1.53

[a] Atom % ^{13}C excess = {($R_N/R_{N(UL)}$ × 1.1}−1.1, where R_N is the ratio of the peak intensity at the N-position in labelled compound calculated on the basis of the peak intensity at the 2-CH_3 position. Similarly, $R_{N(UL)}$ is the ratio of the peak intensity at position N in unlabelled compound. Value 1.1 is the theoretical ^{13}C natural abundance (atom %).

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The enhancement observed at C-8, similar to that for the carbons proceeding from malonate, and the absence of label in the 3-*O*-acetyl group point to the biosynthetic pathway outlined in Scheme 2, where the botrylactone precursor **5** is turned into 3-*O*-acetylbotcineric acid (**2**) through a retro-Claisen type reaction.

A further feeding experiment with sodium [$^2H_3$]acetate was conducted to obtain additional evidence in support of this hypothesis. The incorporation of this labelled precursor into botcinins through the postulated bicyclic acid intermediate **5** (Scheme 2) should result in the total loss of deuterium atoms at C-8.

Sodium [$^2H_3$]acetate was pulse fed twice to the *bcbot2*Δ mutant, and the resultant botcinins (**1**) were analysed by ^{2}H NMR. Although a low degree of incorporation was observed, signals assigned to ^{2}H atoms on C-2′, C-2, C-6′ and C-8′, and on the methyl acetate group at C-3 were unambiguously identified for both botcinins. A further signal corresponding to ^{2}H atoms on C-10′ was also identified for botcinin B (**1 b**). No signal was observed between 3.4 and 4.0 ppm where ^{2}H on C-8 should have appeared. This result is consistent with the biosynthetic pathway outlined in Scheme 2.

Conclusions

The isotopic labelling experiments reported here, together with the results obtained with the *B. cinerea* mutants *bcbot2*Δ and *bcboa9*Δ, enable us to propose and confirm the biosynthetic route outlined in Scheme 2: sequential assembly of a pentaketide (C_{10}) based on an acetate primer unit leading to botrylactones (**4**) or to botcinins (**1**–**3**). This biosynthetic route clarifies the origin of the starter unit in botcinins (**1**–**3**); this unit arises from acetate and it is lost through a retro-Claisen type C–C bond cleavage of a botrylactone-like precursor (**5**). It is highly likely that the 3-*O*-acetyl group in botcinins (**1**, **2**, **3 b**) is derived from the acetate starter unit of the botrylactone-like precursor (**5**).

Two polyketide synthases BcBOA6 and BcBOA9[13] are involved in the biosynthesis of botcinins. BcBOA6 mediates the formation of the per-methylated tetraketide core, common to both botrylactone (**4**) and the botcinin derivatives (**1**–**3**), whilst BcBOA9 is probably required for the biosynthesis of the tetraketide side chain of botcinic acid (**3 a**). Our scenario has similarities to the well-described biosynthesis of lovastatin in *Aspergillus terreus*.[14]

The *BcBOA6 and BcBOA9* genes are two of 17 *BcBOA* genes, separated into two different gene clusters: cluster A (*BcBOA1* to *BcBOA6*) and cluster B (*BcBOA7 to BcBOA17*); all are putatively involved in botcinic acid (**3 a**) biosynthesis.[13] *BcBOA5* encodes an ER that might take over the ER function of BcBOA6, as described for other fungal polyketides.[14,17,18] *BcBOA2, BcBOA3, BcBOA4* and *BcBOA7* encode P450 monooxygenases. *BcBOA2, BcBOA3* and *BcBOA4* might be involved in further hydroxylations at C4, C5 and C8, whereas *BcBOA7*, close to *BcBOA9*, could potentially be involved in the hydroxylation at C4 in the side chain of botcinins (**1**–**3**).

Experimental Section

General procedures: The NMR experiments were carried out on a Unity 400 MHz and Inova 600 MHz spectrometers (Varian) with $CDCl_3$ as solvent (Merck). TLC was performed on Kieselgel 60 F_{254} plates (0.25 mm; Merck). Silica gel 60PF_{254} (60–100 mesh; Merck) was used for column chromatography. HPLC was performed with an L-6270 apparatus (Hitachi/Merck) equipped with an UV/Vis detector (L-4250) and a differential refractometer detector (L-7490). LiChrospher Si 60 (5 μm) LiChroCART (250 mm×4 mm) and LiChrospher Si 60 (10 μm) LiChroCART (250 mm×10 mm) columns were used for isolation experiments.

Labelled precursors: [2-^{13}C]Malonic acid (99%), L-[$^2H_3$-methyl]methionine (98%) and sodium [1-^{13}C]- (99%), [2-^{13}C]- (99%), [1,2-$^{13}C_2$]- (99%) and [$^2H_3$]acetates (99%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). All samples were dissolved in water and sterilised through 0.22 μm Millex GP filters (Millipore) before being added to the fermentation bottles. Malonic acid was dissolved in water, neutralised with NaOH (1 N) and filter-sterilised.

Microorganisms: *B. cinerea* mutants, *bcbot2*Δ and *bcboa9*Δ, were previously obtained by inactivation of the genes encoding the sesquiterpene synthase BcBOT2[15] and the polyketide synthase BcBOA9 (BcPKS9),[13] respectively, and are maintained in the BIOGER strain collection, INRA (Grignon, France). Conidial stock suspensions of these strains were maintained in glycerol (80%) at −40 °C.

Feeding experiments of labelled precursors; extraction and isolation of polyketides

General methods. B. cinerea was grown in Roux bottles, each containing Czapek-Dox medium (150 mL: glucose (50.0 g), yeast extract (1.0 g), KH_2PO_4 (5.0 g), $NaNO_3$ (2.0 g), $MgSO_4 \times 7H_2O$ (0.5 g), $FeSO_4 \times 7H_2O$ (0.01 g) and distilled water to 1.0 L). The pH of the medium was adjusted to pH 7.0 with aqueous NaOH (4 M). Each Roux bottle was inoculated with 2×10^6 fresh conidia or six uniform discs of 0.9 cm diameter mycelial of a five-day-old culture on malt agar. A filter-sterilised aqueous solution of the labelled precursor was fed as a pulse at the precisely determined optimum time. Roux bottles were incubated at 25±2 °C in daylight under static conditions for the optimum period of time to isolate the polyketide toxins. After this, the culture medium and mycelia were separated by filtration. The broth was saturated with NaCl and extracted with ethyl acetate (3×). The organic extract was washed with distilled water (3×) and dried over anhydrous Na_2SO_4. The organic extract obtained was evaporated at reduced pressure to dryness.

*Isolation of botcinins A (**1 a**) and B (**1 b**).* The extract was dissolved in $CHCl_3$ and stirred with drops of HCl (35%) for 5–7 days to achieve lactonisation of 3-*O*-acetylbotcinic acid (**3 b**) and 3-*O*-acetylbotcineric acid (**2**) to botcinins A (**1 a**) and B (**1 b**), respectively. The crude extract was passed through silica gel partition column chromatography with *n*-hexane/ethyl acetate (65:35; 300 mL) as eluent. This fraction was purified by normal phase HPLC with a LiChrospher Si 60 (10 μm) LiChroCART (250 mm×10 mm) column and *n*-hexane/ethyl acetate (70:30) as mobile phase, to yield fractions 1 and 2 ($t_r \approx 29$ and 35 min, respectively). Fractions 1 and 2 were purified with a LiChrospher Si 60 (5 μm) LiChroCART (250 mm×4 mm) and *n*-hexane/ethyl acetate (80:20) as mobile phase, to yield compounds **1** ($t_r \approx 42$ and 55 min, respectively).

*Isolation of botrylactone (**4**).* The extracts were subjected to column chromatography on silica gel with an increasing gradient of ethyl acetate in *n*-hexane as eluent. Final purification was carried out by normal phase HPLC in a LiChrospher Si 60 (10 μm) LiChroCART

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(250 mm × 10 mm) column with *n*-hexane/ethyl acetate (60:40) as mobile phase, to yield **4** ($t_r \approx 32$ min).

Feeding of sodium [1-^{13}C]acetate to *bcboa9*Δ: Ten subcultures in Roux bottles with discs of the *bcboa9*Δ mutant (described in ref. [13]) were fed with a filter-sterilised solution of sodium [1-^{13}C]-acetate in H_2O on day 2 to a final concentration of 533 ppm. Extraction of the broth seven days post-inoculation yielded a crude extract (85.0 mg, 8.5 mg per bottle), which was purified as described above to afford **4** (2.0 mg).

Feeding of sodium [2-^{13}C]acetate to *bcboa9*Δ: Twenty cultures in Roux bottles, each subcultured with 2×10^6 fresh conidia, were pulse fed on day 3 with an aseptic aqueous solution of sodium [2-^{13}C]acetate to a final concentration of 533 ppm. Extraction of the broth five days post-inoculation yielded a crude extract (446.3 mg, 22.3 mg per bottle), which was purified as described in the general method to afford **4** (4.7 mg).

Feeding of sodium [1,2-$^{13}C_2$]acetate to *bcboa9*Δ: A sterile aqueous solution of sodium [1,2-$^{13}C_2$]acetate was pulse fed on day 3 into 20 Roux bottles, each containing subcultures with 2×10^6 fresh conidia, to a final concentration of 667 ppm in the medium. Extraction of the broth five days post-inoculation yielded a crude extract (575.9 mg, 28.8 mg per bottle), which was purified as described above to afford **4** (0.5 mg).

Feeding of sodium [2-^{13}C]malonate to *bcboa9*Δ: In accordance with the above procedure, twenty Roux bottles, each subcultured with 2×10^6 fresh conidia were fed on day 3 with a filter-sterilised solution of sodium [2-^{13}C]malonate/sodium acetate (1:3) in H_2O to a final concentration of 533 and 920 ppm, respectively. Extraction of the broth 5 days post-inoculation yielded a crude extract (647.5 mg, 32.4 mg per bottle), which was purified as described in the general method to afford **4** (10.6 mg).

Feeding of sodium L-[$^2H_3$-methyl]methionine to *bcboa9*Δ: Fifteen cultures in Roux bottles with discs of *bcboa9*Δ were fed on day 2 with an aseptic aqueous solution of L-[$^2H_3$-methyl]methionine to a final concentration of 440 ppm. Extraction of the broth seven days post-inoculation yielded a crude extract (101.0 mg, 6.7 mg per bottle), which was purified as described above to afford **4** (1.9 mg).

Feeding of sodium [2-^{13}C]malonate to *bcbot2*Δ: Seven cultures in Roux bottles with discs of *bcbot2*Δ mutant (described in ref. [15]) were fed with an aseptic aqueous solution of sodium [2-^{13}C]-malonate/sodium acetate (1:1.5) to a final concentration of 378 and 533 ppm, respectively (day 0). Extraction of the broth after seven days yielded a crude extract (303.8 mg, 43.4 mg per bottle), which was purified as described in the general method to afford compounds **1a** (3.6 mg) and **1b** (5.6 mg).

Feeding of sodium [$^2H_3$]acetate to *bcbot2*Δ: In accordance with the above procedure, 20 subcultures in Roux bottles with discs of *bcbot2*Δ were pulse fed with filter-sterilised sodium [$^2H_3$]acetate (200 μL, 375 mg mL^{-1}) in H_2O on days 0 and 2. After 7 days, the culture medium and mycelia were separated by filtration. Extraction of the broth yielded a crude polyketide extract (388.0 mg, 19.4 mg per bottle), which was purified as described in the general method to afford **1a** (18.7 mg) and **1b** (15.2 mg).

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