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Studies on the biosynthesis of secobotryane skeleton

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Abstract—The labelling and coupling patterns of secobotrytrienediol, biosynthesised from [1-^{13}C] and [1,2-$^{13}C_2$]-acetate by the fungus *Botrytis cinerea*, have been used to define the mode of formation and the biogenetic origin of secobotrytrienediol. [10-^{2}H]-Botrydiol was not incorporated into the secobotryane skeleton. In addition, this feeding experiment led to the isolation of three new unlabelled derivatives possessing a secobotryane skeleton, secobotrydiene-3,10,15-triol, secobotrydiene-3,4,10,15-tetraol, and secobotrytriene-10,12,15-triol.

1. Introduction

The major characteristic metabolites of the plant pathogen *Botrytis cinerea* possess the botryane skeleton. In addition there are some other minor phytotoxic metabolites.[1] Recently we have reported[2] the isolation of secobotrytrienediol (**1**), a new slightly phytotoxic compound which possesses a novel carbon skeleton. From the biosynthetic studies reported,[3] the structure of compound **1** suggests that it might be a biodegradation product arising from botryanes. However in our studies[4] on the stages at which the metabolites are formed during the growth of *B. cinerea*, secobotrytrienediol (**1**) had disappeared when botrydial (**2**) reached the maximum concentration. Furthermore, when (4*E*,8*R*)-caryophyll-4(5)-en-8-ol[5] was fed to *B. cinerea*, compound **1** was isolated in good yield while botrydial (**2**) could not be detected.

These observations are intriguing and place some constraints on proposals for the formation of this new skeleton. Consequently, we are investigating the biosynthesis of secobotrytrienediol (**1**) in *B. cinerea*. In this paper, we describe the incorporation of sodium [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate into secobotrytrienediol (**1**), the results of feeding [10-^{2}H]-botrydiol (**3**) and botrydial (**2**) to *B. cinerea* and the conclusions that may be drawn from this work.

Keywords: *Botrytis cinerea*; secobotryane; biosynthesis; labelling; isolation.
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2. Results and discussion

The structure of compound **1** and its derivatives do not obey the simple isoprene rule. However, if we assumed a relationship to the botryane skeleton, then the biosynthetic evidence for the origin of the latter from farnesyl diphosphate[3] may be extended to these compounds. In order to confirm this relationship we have carried out feeding experiments with sodium [1-^{13}C]- and [1,2-$^{13}C_2$]-acetates.

The optimum time for feeding acetate to resting cell cultures of *B. cinerea* and for isolating the metabolites was previously established. As a result of the time:course study for the production of secobotrytrienediol (**1**), 48 h old resting cell cultures of *B. cinerea* were fed with ^{13}C labelled sodium acetates. Purification of the fermentation broths led the isolation of compound **1**, which was analysed by ^{13}C NMR spectroscopy. The ^{13}C–^{13}C couplings which were observed are given in Table 1. Specific incorporations were calculated by HREIMS (Table 2).

When sodium [1-^{13}C]-acetate was fed to *B. cinerea* as a pulse at the carefully determined time of maximum metabolite production, significant enrichments were observed at C-2, C-4 and C-10. In addition to the enrichments, couplings were observed in the ^{13}C NMR spectrum between C-6 and C-7 and between C-7 and C-8 (Scheme 1). A second feeding experiment was carried out with a 1:2 mixture of sodium [1,2-$^{13}C_2$]-acetate and unlabelled sodium acetate. The incorporation of intact ^{13}C–^{13}C units was established by analysis of the satellite signals arising from the ^{13}C–^{13}C coupling. Thus, the signals for the carbons pairs C-1:C-10, C-2:C-11, C-4:C-5,

Table 1. 100 MHz ^{13}C NMR data, and enrichment and coupling patterns of compound **1** after feeding of ^{13}C-labelled sodium acetate

Carbon	δ_C (ppm)	Coupling constant (Hz)[a]	
		[1-^{13}C]-Acetate	[1,2-$^{13}C_2$]-Acetate
1	136.1 (s)		45.4
2	126.6 (d)	en	43.5
3	115.3 (t)		en
4	131.1 (d)	en	55.7
5	148.6 (s)		55.7
6	45.2 (s)	34.3	35.1
7	52.3 (t)	34.3;[b]	en
8	52.5 (s)	[b]	35.9
9	140.0 (s)		en
10	68.3 (t)	en	45.4
11	14.7 (q)		43.5
12	30.7 (q)[c]		en
13	29.2 (q)[c]		35.1
14	24.0 (q)		35.9
15	70.3 (t)		en

[a] en=enriched.
[b] J_{7-8} not calculated (C-7 is superimposed on C-8).
[c] Interchangeable signals.

quently, it is possible that compound **1** might be biosynthesised from botrydial (**2**) by means of a biodegradation or detoxification mechanism, as is shown in Schemes 1 and 2. In order to explore this possibility we examined the incorporation of [10-^2H]-botrydiol (**3**) into **1** and carried out a feeding experiment with botrydial (**2**).

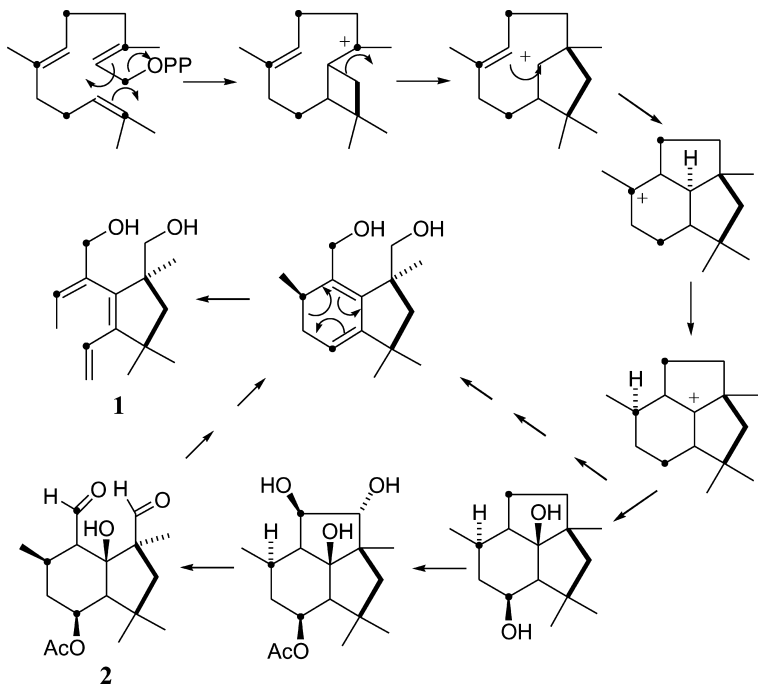
Labelled **3** was synthesised in 53% yield by the reduction of dihydrobotrydial (**4**) with sodium borodeuteride.[6] The triol was fed to resting cell cultures of *B. cinerea* at a final concentration of 0.5 mM. The level of incorporation was determined by the incorporation of deuterium into 10-dehydroxydihydrobotrydialone[7] (**5**) after 5 days of fermentation. The isotopic composition of **5**, determined by integration of the resultant ^1H NMR spectrum, was 88.1%. Nevertheless, no incorporation of the isotope was apparent in secobotrytrienediol (**1**).

However, a detailed study of this fermentation led to the isolation of three new metabolites with the secobotryane skeleton. These were secobotrydiene-3,10,15-triol (**6**), secobotrydiene-3,4,10,15-tetraol (**7**) and secobotrytriene-10,12,15-triol (**8**). Some of these metabolites have now been detected in other fermentation experiments. Their structures were established by mass spectroscopy and NMR studies. Their spectra showed no incorporation of deuterium from [10-^2H]-botridiol (**3**).

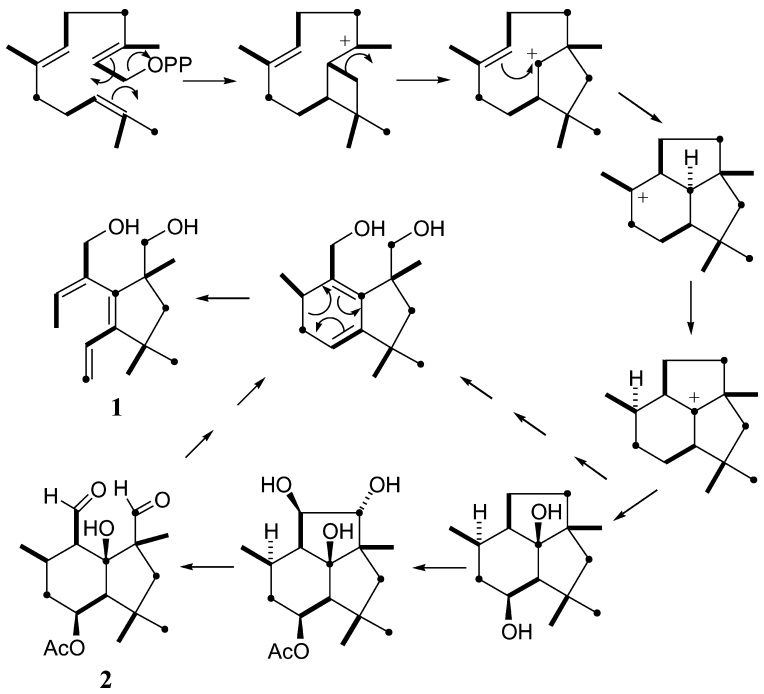
Table 2. Incorporation of label determined by HREIMS from labelled sodium acetate into **1**

Labelled precursor	Incorporation into **1** (%)							
	M	M+1	M+2	M+3	M+4	M+5	M+6	M+7
Sodium [1-^{13}C]-acetate	25.6	31.9	18.2	13.1	7.0	3.5	<1	–
Sodium [1,2-$^{13}C_2$]-acetate	46.9	16.4	19.3	7.6	5.6	2.7	1.5	<1

C-6:C-13 and C-8:C-14 showed the expected incorporation of an intact $^{13}C_2$ unit (Scheme 2). These results together with the couplings that were generated between C-6, C-7 and C-8 in the experiment with the [1-^{13}C]-acetate suggests that secobotrytrienediol (**1**) is biosynthesised from farnesyl diphosphate by the same folding as that utilised in the biosynthesis of botryanes (see Schemes 1 and 2).[3]

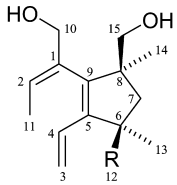


Scheme 1. Pattern of folding of farnesyl diphosphate obtained from sodium [1-^{13}C]-acetate.

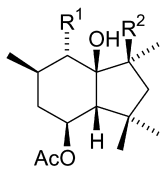


Scheme 2. Pattern of folding of farnesyl diphosphate obtained from sodium [1,2-$^{13}C_2$]-acetate.

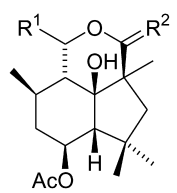
Compounds **6**, **7** and **8** were colourless oils. Their IR, ^{1}H and ^{13}C NMR spectra showed that they possessed structures which were similar to that of secobotrytrienediol[2] (**1**). The principal difference between the ^{1}H NMR spectra of **6** and **7** and that of **1** was the absence of signals characteristic of the C-3:C-4 double bond. The presence in the ^{13}C NMR spectrum of two methylene signals at δ_C 61.9 and 28.7 in the spectrum of **6** and a methylene (δ_C 66.0) and a methyne (δ_C 69.8) in **7** indicated that these compounds were mono-hydroxylated at C-3 and dihydroxylated at C-3 and C-4, respectively. Because of the small amount of compound **7** which was isolated, we were unable to determinate its configuration at C-4.



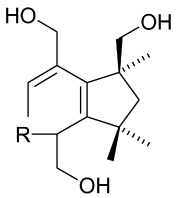
1: R = CH_3
8: R = CH_2OH



2: $R^1 = R^2$ = CHO
3: R^1 = CDHOH; $R^2 = CH_2OH$



4: R^1 = β-OH; R^2 = H, H
5: R^1 = α-D; R^2 = O



6: R = H, H
7: R = OH

The absence of a methyl signal in the ^{1}H and ^{13}C NMR spectra of compound **8** together with the presence of a new hydroxymethyl resonance indicated that compound **8** was hydroxymethyl derivative of **1**. The new hydroxymethyl group was assigned to C-12 by comparing the ^{13}C NMR spectrum with that of compound **1**. The stereochemistry at C-6 was shown to be *R* based on the deshielding of the H-7β resonance from δ_H 2.08 in **1** to δ_H 2.28 in **8**. This signal had been assigned to H-7β because a doublet, which was correlated with it in the COSY spectrum, was enhanced in an NOE experiment on irradiation of H-14.

The formation of a hydroxymethyl group at this position has been found previously in the aromatic botryanes,[2] and is characteristic of the biotransformation of fungitoxic compounds by *B. cinerea*. These oxidised derivatives possess a much reduced biological activity when compared to the parent compounds.[8]

In order to determine the phytotoxic activity of compounds with the secobotryane skeleton, in vivo phytotoxicity assays were carried out with *Phaseolus vulgaris* using previously described methodology.[9] Although the phytotoxicity of secobotrytrienediol (**1**) has already been reported in vitro on tobacco leaves,[2] we reassayed this compound in this investigation to obtained comparable results. Interestingly, compounds **1** and **6**–**8** were inactive at the maximum concentration tested (1000 ppm).

When botrydial (**2**) (22 mg) was fed to a culture of *B. cinerea*, dihydrobotrydial (**4**) and a higher amount (33.2 mg) of botrydial (**2**) were obtained. However, secobotrytrienediol (**1**) was not detected in a careful study of the fermentation broth.

These data together with the fact that the secobotryanes did not display phytotoxicity, suggests that these compounds are biodegradation products arising from a branch in the biosynthetic pathway that leads to botrydial (**2**). The triene

could arise by an electrocyclic ring opening of a cyclohexadiene reminiscent of the relatioship between vitamin D and dehydrocholesterol. The dienes could also be the precursors of the aromatic botryanes.[2] Work is in progress to establish the stage in the botryane pathway at which these secobotryanes emerge.

3. Experimental

3.1. General experimental procedures

Melting points were measured with a Reichert–Jung Kofler block and are uncorrected. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FTIR™. ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini 200 MHz (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz) and Varian Unity 400 MHz (^{1}H at 399.952 MHz, ^{13}C at 100.570 MHz) spectrometers. ^{2}H NMR Spectra were recorded on a Varian Unity 400 MHz (^{2}H at 61.395 MHz) spectrometer. Chemical shifts are quoted relative to TMS (Me_4Si) in $CDCl_3$. Mass spectra were recorded on GC-MS Thermoquest, model Voyager, and VG-Autospec spectrometers. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV-VIS detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F_{254}, 0.25 mm thick. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was carried out with a Si gel column (LiChrospher Si-60, 10 μm, 1 cm wide, 25 cm long or 5 μm, 0.4 cm wide, 25 cm long).

3.2. Labelled precursors

Sodium [1-^{13}C]-acetate (99 at.% ^{13}C) and sodium [1,2-$^{13}C_2$]-acetate (99 at.% ^{13}C) were obtained from the Aldrich Chemical Company. [10-^{2}H]-Botrydiol (**3**)[6] was synthesised by treatment of dihydrobotrydial (**4**, 116 mg, 0.37 mmol) in MeOH (6 ml) with $NaBD_4$ (3 mg, 0.07 mmol) (≥99 at.% D, purchased from the Fluka Chemical Company). After stirring for 2 h at room temperature, the reaction mixture was poured onto ice, acidified with 2N HCl (10 ml) and stirred for 10 min. The solution was then diluted with H_2O (45 ml) and extracted with $CHCl_3$ (30 ml, ×3). The solvent was evaporated and the crude extract subjected to column chromatography to afford [10-^{2}H]-botrydiol (**3**, 62 mg, 0.20 mmol, 53%, mp 170–171°C). The isotopic composition, calculated by integration of the resultant ^{1}H NMR spectrum, was 98.9%.

3.3. Organism

B. cinerea 2100 was obtained from the Colección Española de Cultivos Tipo (CECT), Facultad de Biología, Universidad de Valencia, Spain. Conidial stock suspensions of this strain were maintained viable in 80% glycerol at −20°C.

3.4. Feeding experiments of labelled precursors: extraction and isolation of metabolites

General method. *B. cinerea* 2100 was grown at 24–26°C on shake culture at 250 rpm in 500 ml Erlenmeyer flasks, each containing 200 ml of Czapek–Dox medium.[2] The pH of the medium was adjusted to 7.0 and each flask was inoculated with 3×10^{6} fresh conidia. After 48 or 72 h of incubation (see detailed experiments below) the mycelia was transferred into the same number of 500 ml Erlenmeyer flasks, each containing 200 ml of Czapek–Dox medium (without glucose) and an aseptic solution of the labelled precursor in H_2O or EtOH. After 48 h from the administration of the precursor, the culture medium and mycelia were separated by filtration. The broth was saturated with NaCl, and extracted with EtOAc (×4). The EtOAc extract was washed with H_2O (×3) and then dried over anhydrous Na_2SO_4. Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by means of column chromatography, with an increasing gradient of ethyl acetate in petroleum ether. Final purification was carried out with HPLC.

3.5. Feeding of sodium [1-^{13}C]-acetate to *B. cinerea*

Eleven cultured flasks with *B. cinerea* were fed with 360 μl of an aseptic aqueous solution of sodium [1-^{13}C]-acetate to give a final concentration of 450 ppm per flask on day 2. Extraction of the broth yielded a crude extract (61 mg), which was purified as described in the general method to afford secobotrytrienediol[2] (**1**, 2.3 mg). The isotopic composition of **1** was determined by HREIMS and is shown in the Table 2.

3.6. Feeding of sodium [1,2-$^{13}C_2$]-acetate to *B. cinerea*

Six cultured flasks with *B. cinerea* were fed with 400 μl of an aseptic aqueous solution of sodium [1,2-$^{13}C_2$]-acetate: sodium acetate (1:2) to give a final concentration of 450 ppm per flask on day 2. Extraction of the broth yielded a crude extract (51 mg), which was purified as described in the general method to afford **1**[2] (2.1 mg). The isotopic composition of **1** was determined by HREIMS and is shown in the Table 2.

3.7. Feeding of [10-^{2}H]-botrydiol to *B. cinerea*

Eleven cultured flasks with *B. cinerea* were fed with 375 μl of an aseptic solution of [10-^{2}H]-botrydiol (**3**) in EtOH to give a final concentration of 150 ppm per flask on day 3. Extraction of the broth yielded a crude extract (590 mg), which was purified as described in the general method to afford secobotrytrienediol[2] (**1**, 2.8 mg), secobotrydiene-3,10,15-triol (**6**, 1.2 mg), secobotrydiene-3,4,10,15-tetraol (**7**, 0.5 mg), secobotrytriene-10,12,15-triol (**8**, 0.7 mg) and [10-^{2}H]-dehydroxydihydrobotrydialone[7] (**5**, 14.5 mg). The isotopic composition of **5**, calculated by integration of the resultant ^{1}H NMR spectrum, was 88.1%.

3.7.1. Secobotrydiene-3,10,15-triol (6). Colourless oil; $[\alpha]_D^{25}=+57$ (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3354, 2925, 2862, 1739, 1451, 1373, 1261, 1033, 801 cm^{-1}; EIMS *m/z* (%) 236 (1) $[M-H_2O]^+$, 223 (10) $[M-CH_2OH]^+$, 206 (90) $[M-CH_2OH-OH]^+$, 175 (82) $[M-2\times CH_2OH-OH]^+$, 161 (58) $[M-3\times CH_2OH]^+$, 145 (71), 133 (88), 119 (100); HREIMS calcd. for $C_{15}H_{26}O_3$ $[M]^+$ 254.1882, found 254.1904. See Tables 3 and 4 for ^{1}H and ^{13}C NMR spectroscopic data.

Table 3. 400 MHz ^{1}H NMR data of compounds **6**–**8** in $CDCl_3$

Proton	δ_H (ppm)		
	6	**7**	**8**
2	5.60 (qdd)	5.54 (q)	5.71 (q)
3	3.69 (ddd)	3.55 (dd)	5.07 (dd)
3′	3.80 (ddd)	3.72 (dd)	5.40 (dd)
4	2.07 (ddd)	4.21 (dd)	6.30 (dd)
4′	2.32 (ddd)		
7α	1.55(d)	1.60 (d)	1.71(d)
7β	2.13 (d)	2.11 (d)	2.28 (d)
10	4.13 (d)	4.17 (d)	4.08 (d)
10′	4.33 (dt)	4.26 (dt)	4.34 (dt)
11	1.53 (dd)	1.57 (dd)	1.53 (dd)
12	1.16 (s)[a]	1.25 (s)[a]	3.45 (d)
12′			4.02 (d)
13	1.15 (s)[a]	1.12 (s)[a]	1.27 (s)
14	0.92(s)	0.94 (s)	0.99 (s)
15	3.17 (d)	3.17 (d)	3.44 (d)
15′	3.58 (d)	3.51 (d)	3.60 (d)

J (Hz): **6**: J_{2-10}=0.9; $J_{2-10'}$=2.0; J_{2-11}=6.7; $J_{3-3'}$=10.4; J_{3-4}=2.3; $J_{3-4'}$=10.7; $J_{3'-4}$=4.5; $J_{3'-4'}$=4.2; $J_{4-4'}$=14.6; $J_{7\alpha-7\beta}$=12.8; $J_{10-10'}$=13.7; $J_{10'-11}$=2.1; $J_{15-15'}$=11.7. **7**: J_{2-11}=6.7; $J_{3-3'}$=10.8; J_{3-4}=3.0, $J_{3'-4}$=9.8, $J_{7\alpha-7\beta}$=13.3; $J_{10-10'}$=13.8; $J_{10'-11}$=2.3; $J_{15-15'}$=11.5. **8**: J_{2-11}=6.7; $J_{3-3'}$=1.4; J_{3-4}=11.9; $J_{3'-4}$=18.4; $J_{7\alpha-7\beta}$=14.3; $J_{10-10'}$=13.3; $J_{10'-11}$=1.6; $J_{12-12'}$=10.6; $J_{15-15'}$=11.5.

[a] Interchangeable signals.

Table 4. 100 MHz ^{13}C NMR data of compounds **6**–**8** in $CDCl_3$

Carbon	δ_C (ppm)		
	6	**7**	**8**
1	137.1 (s)	137.0 (s)	
2	123.1 (d)	122.9 (d)	126.2 (d)
3	61.9 (t)	66.0 (t)	114.9 (t)
4	28.7 (t)	69.8 (d)	131.2 (d)
5		148.8 (s)	
6	46.4 (s)	46.0 (s)	
7	49.2 (t)	50.1 (t)	49.3 (t)
8	52.8 (s)	54.2 (s)	
9		137.7 (s)	
10	67.4 (t)	66.5 (t)	68.0 (t)
11	14.3 (q)	16.3 (q)	14.4 (q)
12	30.3 (q)[a]	31.1 (q)[a]	69.3 (t)
13	29.2 (q)[a]	28.3 (q)[a]	26.5 (q)
14	23.2 (q)	24.4 (q)	25.1 (q)
15	70.0 (t)	70.4 (t)	71.9 (t)

[a] Interchangeable signals.

3.7.2. Secobotrydiene-3,4,10,15-tetraol (7). Colourless oil; $[\alpha]_D^{25}$=+14 (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3350, 2832, 1460, 1378, 1723, 824 cm^{-1}; EIMS *m/z* (%) 271 (6) $[M+1]^+$, 221 (99) $[M-CH_2OH-H_2O]^+$, 204 (61) $[M+1-CH_2OH-2\times H_2O]^+$, 191 (65) $[M+1-2\times CH_2OH-H_2O]^+$, 173 (45) $[M+1-2\times CH_2OH-2\times H_2O]$, 119 (100); HREIMS calcd. for $C_{15}H_{27}O_4$ $[M+1]^+$ 271.1909, found 271.1898. See Tables 3 and 4 for ^{1}H and ^{13}C NMR spectroscopic data.

3.7.3. Secobotrytriene-10,12,15-triol (8). Colourless oil; $[\alpha]_D^{25}$=+23 (*c* 0.07, $CHCl_3$); IR (KBr) ν_{max} 3409, 2925, 2855, 1733, 1457, 1375, 1260, 1037 cm^{-1}; EIMS *m/z* (%) 234 (2) $[M-H_2O]^+$, 222 (43) $[M-2\times CH_3]^+$, 221 (20) $[M-CH_2OH]^+$, 204 (24) $[M-2\times CH_3-H_2O]^+$, 191 (61) $[M-CH_2OH-2\times CH_3]^+$, 173 (100) $[M-CH_2OH-H_2O-2\times CH_3]^+$. See Tables 3 and 4 for ^{1}H and ^{13}C NMR spectroscopic data.

3.8. Feeding of botrydial (2)

B. cinerea 2100 was grown in two flasks containing Czapek–Dox medium under the conditions described above. Each flask was inoculated with 1.1×10^7 fresh conidia. An aseptic solution of botrydial (22 mg) (**2**), obtained from previous fermentation experiments,[9] in EtOH (450 μl) was fed to the fermentation in one pulse to give a final concentration of 55 ppm per flask 3 days after inoculation. The broth was harvested after a further 2 days as above to afford botrydial[6] (**2**, 33.2 mg) and dihydrobotrydial[6] (**4**, 0.5 mg).

Acknowledgements

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