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Biosynthetic Studies on the Botcinolide Skeleton: New Hydroxylated Lactones from *Botrytis cinerea*†

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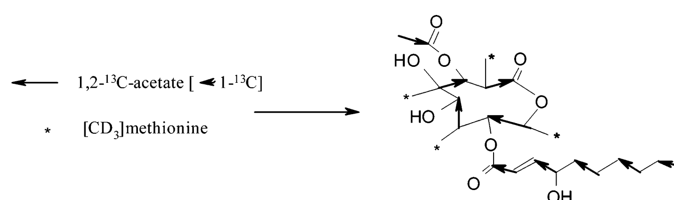

The biosynthetic origin of the botcinolide skeleton was investigated by means of feeding ^{13}C- and ^{2}H-labeled precursors to *Botrytis cinerea*. Three new compounds, two homobotcinolide derivatives, 3-*O*-acetylhomobotcinolide (**5**) and 8-methylhomobotcinolide (**6**), and a new 11-membered lactone (**7**), were isolated. Their structures were elucidated on the basis of spectroscopic data, including one-bond and long-range ^{1}H−^{13}C correlations. The relative stereochemistries were determined by combined analyses of NOE data and ^{1}H−^{1}H coupling constants. According to the results of feeding experiments with ^{13}C- and ^{2}H-labeled acetate and L-*S*-methylmethionine, **5** is an acetate-derived polyketide whose methyl groups originate from L-*S*-methylmethionine. This is a rare example of the incorporation of a methyl from methionine into a supposed C_3 starter unit of the polyketide synthesis.

Introduction

The botcinolide family represents a small group of compounds that to date comprises nine secondary metabolites,[1] all of which are derived from the phytopathogen *Botrytis cinerea*, a fungus responsible for both the so-called noble and gray rot. The term botcinolide refers to the group's first reported member, botcinolide (**1**), which was reported by Cutler et al. in 1993.[2,3]

The metabolites that comprise the botcinolide group contain two carbon chains linked through an ester function: a polyoxygenated nonalactone and a fatty acid side chain. The distinctive structural variation between botcinolide and homobotcinolide[4] is found in the fatty acid side chain and involves the chain length.

At present, the metabolites of the botcinolide family are of particular interest due to their phytotoxic activity and relatively low acute toxicity, characteristics which make them interesting candidates for development as biodegradable contact herbicides.[5−7] On the other hand, a synergistic action of botcinolide and botrydial toxins, in the phytotoxicity and infection mechanism of the phytopathogen *B. cinerea*, has been reported.[8,9] Consequently, there is great interest in ascertaining the mode of action of this molecule. Much remains to be done, as the biosynthetic origin of these compounds has not yet been investigated.

In the course of our studies on the relationship between virulence and toxin production, we detected a hyperproduction of compounds with the botcinolide skeleton in the fermentation

† Dedicated to Professor Joaquin Plumet on the occasion of his 60th birthday.

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broth of the mutant strain *B. cinerea* cat 2. This mutant is an infective extracellular catalase-deficient strain.[10]

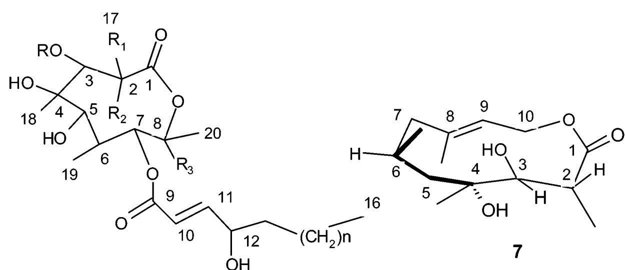
As part of the program under development at Cádiz University aimed at understanding the role of botcinolide toxins in the metabolism and infection mechanism of *B. cinerea*, we have investigated the biosynthesis of the botcinolide skeleton. In the course of this investigation we isolated three new compounds: two homobotcinolide derivatives and a related 11-membered lactone from the fermentation broth of *B. cinerea* cat 2.

Results and Discussion

The botcinolide group of metabolites show structural similarities to erythromycins, which are biosynthesized by modular polyketide synthases through repetitive condensations of simple monomers such as acetic or propionic acid derivatives. With the aim of confirming the polyketide origin of the botcinolide skeleton, we fed *B. cinerea* with sodium [1,2,3-$^{13}C_3$]propionate, sodium [1-^{13}C]- and [1,2-$^{13}C_2$]acetates, and L-[*S-methyl*-$^2H_3$]-methionine.

The optimum times for feeding the precursor to resting cell cultures of *B. cinerea* and for isolating the produced metabolites had been previously established, so *B. cinerea* cat 2 was cultured by being shaken in a Czapek-Dox medium for 5 days. The fermentation broth, after filtration and extraction as described previously,[11] was studied by TLC and GC−MS. These methods revealed the presence of a large number of metabolites containing the botryane skeleton.[1] The GC−MS experiment confirmed that this strain produces botrydial, dihydrobotrydial, botryenedial, and botrydienal in addition to botcinolide derivatives. Nevertheless, the GC analysis showed the presence of new compounds which were isolated using column chromatography and HPLC.

In addition to the known compounds mentioned above, four new compounds containing the botcinolide skeleton, 3-*O*-acetyl-2-epihomobotcinolide (**3**), 2-epihomobotcinolide (**4**), 3-*O*-acetylhomobotcinolide (**5**), and 8-methylhomobotcinolide (**6**), and the new metabolite 2,4,6,8-tetramethyl-3,4-dihydroxydec-8(9)-enolide (**7**) were isolated. Botrylactone (**8**), a strong antibiotically active C-9 polyhydroxylated lactone which has only been reported by Tschesche et al.,[12] was also isolated. **8** was later synthesized and the originally published structure revised.[13]



1 R_1=Me, R_2=H, R_3=H, R=H, n=2
2 R_1=Me, R_2=H, R_3=H, R=H, n=4
3 R_1=H, R_2=Me, R_3=H, R=Ac, n=4
4 R_1=H, R_2=Me, R_3=H, R=H, n=4
5 R_1=Me, R_2=H, R_3=H, R=Ac, n=4
6 R_1=Me, R_2=H, R_3=Me, R=H, n=4
9 R_1=H, R_2=Me, R_3=H, R=Ac, n=2
10 R_1=H, R_2=Me, R_3=H, R=H, n=2

7

8

Comparison of the spectroscopic data of compounds **3** and **4** with those described for other compounds isolated previously from *B. cinerea* indicated that both compounds were derivatives from the homo series of 3-*O*-acetyl-2-epibotcinolide (**9**) and 2-epibotcinolide (**10**), respectively.[14] The ^{1}H NMR spectra of **3** and **4** were almost identical to those described for **9** and **10**. In contrast, the ^{13}C NMR spectra showed the presence of new methylene signals at δ_c 31.6 and 22.5 ppm and δ_c 26.4 and 23.6 ppm, respectively. This fact together with differences observed in high-resolution mass spectrometry indicated that **3** and **4** contain two additional methylene groups in the side chain at C-7. The coupling constant $J_{2-3} = 9.6$ and 9.4 Hz for **3** and **4**, respectively, confirmed that these compounds belong to the 2-epi series.

The ^{1}H and ^{13}C NMR spectra of **5** (Table 1) were consistent with the molecular formula $C_{24}H_{40}O_9$, which corresponds to an acetylhomobotcinolide. Signals at δ_H 2.13 (3H, s), δ_C 20.8 (CH$_3$-CO), and δ_C 172.7 (CH$_3$$C$O) ppm indicated the presence of an acetyl group located at C-3 on the basis of the chemical shift of H-3. The coupling constant observed between H-2 and H-3 ($J = 3.9$ Hz) indicates a β disposition for the methyl group on C-2. All these data confirmed the structure of 3-*O*-acetylhomobotcinolide for compound **5**.

TABLE 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data in CD$_3$OD and Isotopic Enrichment and Coupling Constants of 5 after Feeding of [1-^{13}C]Acetate (A), [1,2,3-$^{13}C_3$]Propionate (B), and [1,2-$^{13}C_2$]Acetate and L-[*S-methyl*-$^2H_3$]methionine (C)

			^{13}C enrichment			
C	δ_H, ppm (*m*, *J* (Hz))	δ_C, ppm (*m*)	A	B	C[c]	$J_{^{13}C-^{13}C}$[d] (Hz)
1		177.7 (s)	2	12	36	54.5
2	2.98 (dq, 3.9, 7.1)	40.7 (d)	1	23	52	54.5
3	5.15 (d, 3.9)	78.1 (d)	5	*b*	*b*	*b*
4		79.5 (s)	1	26	*a*	44.8
5	3.25 (d, 10.6)	72.9 (d)	8	20	43	36.6
6	1.88 (m, 10.6, 6.1)	39.4 (d)	1	20	47	36.6
7	4.34 (dd, 10.6, 9.7)	78.1 (d)	7	*b*	*b*	
8	3.65 (dq, 9.7, 6.0)	69.4 (d)	1	21	57	42.6
9		167.7 (s)	5	14	35	75.4
10	6.02 (dd, 15.7,1.8)	120.1 (d)	1	20	32	75.4
11	6.98 (dd, 15.7, 4.8)	153.7 (d)	7	21	31	45.8
12	4.24 (m)	71.6 (d)	1	23	35	45.8
13	1.53 (m)	37.5 (t)	6	18	32	35.4
14	1.31[e] (m)	32.9 (t)	1	24	34	35.4
15	1.31[e] (m)	30.3[f] (t)	5	21	34	34.7
15′	1.31[e] (m)	26.4[f] (t)	1	20	36	34.7
15″	1.31[e] (m)	23.6[f] (t)	6	20	36	35.0
16	0.89 (t, 5.0)	14.4 (q)	1	25	37	
17	1.19 (d, 7.1)	16.5 (q)	1			
18	1.32 (s)	14.6 (q)	1			
19	0.95 (d, 6.1)	14.8 (q)	1			
20	1.02 (d, 6.0)	18.1 (q)	1			
CH$_3$*C*O		172.7 (s)	1	18	22	60.0
*C*H$_3$CO	2.13 (s)	20.8 (q)	3	19	26	60.0

[a] Not detected. [b] ^{13}C enrichment could not be calculated due to overlapping of the signals. [c] ^{2}H enrichment H-17/46, H-18, H-19/51, H-20/46. [d] Calculated after feeding with [1,2,3-$^{13}C_3$]propionate. [e] Overlapping signals. [f] Interchangeable signals.

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5 was unstable in $CHCl_3$ solution, and was converted to **3**. This result leads to the conclusion that the botcinolide epimers are formed during the isolation, purification, and identification processes. Hence, it appears that compounds **3** and **4** are artifacts which arise from **5** and homobotcinolide (**2**), respectively.

The two main differences in the ^{1}H NMR spectrum of compound **6**, with respect to that of compound **2**, were the absence of one of the characteristic methyl doublets and of the multiplet corresponding to H-8. Two methyl singlets at δ_H 1.05 and 1.08 ppm were also present. In addition, a change in multiplicity of the H-7 methine was observed. The signals corresponding to the methyl groups at δ_H 1.05 (s) and 1.08 (s) ppm were assigned to H-20 and H-21, respectively, with both methyl groups being situated on C-8. These data, together with the low coupling constant observed between H-2 and H-3 and the presence of two additional methylene groups (26.3 and 23.6 ppm) in the ^{13}C NMR spectrum, were consistent with a structure of 8-methylhomobotcinolide for compound **6**.

Compound **7** displayed a molecular formula of $C_{14}H_{24}O_4$, as deduced from high-resolution mass spectral data. The ^{13}C NMR spectrum showed 14 signals arising from four methyls, three methylenes, four methines, and three quaternary carbon atoms. IR absorption bands at 3418 and 1748 cm^{-1}, along with the presence, in the ^{13}C NMR spectrum, of three singlet signals at δ_C 177.5, 141.8, and 88.0 ppm and two doublets at δ_C 76.5 and 125.4 ppm, indicated that **7** was an unsaturated dihydroxylactone. The ^{1}H NMR spectrum showed the presence of four methyl groups: two on a methyne carbon (d), one on an oxygenated quaternary carbon (s), and one on a vinylic carbon (s). The carbonyl group signal at δ_C 177.5 ppm presented correlations in the HMBC spectrum with signals at δ_H 2.94 and 1.24 ppm, which correlated with the CH carbon signal at δ_C 76.5 ppm. Correlations between the quaternary carbon signal at δ_C 88.0 ppm and the proton signal at δ_H 4.03 ppm correlated in the HSQC spectrum with the carbon at δ_C 76.5 ppm, confirming the presence of a C-1−C-4 fragment equivalent to that in the botcinolide skeleton. On the other hand, the 2D ^{1}H−^{13}C shift correlations helped to determine the sequence $CH_2C(CH_3)$═$CHCH_2O$ for the fragment C-7−C-10. In this way, correlations observed in the HMBC spectrum between the quaternary vinylic carbon and two protons of a CH_2 group and the methyl group at δ_H 1.67 ppm confirmed the fragment $CH_2C(CH_3)$═, while the correlations in the COSY and HMBC spectra between a CH_2 at δ_H 4.10−4.19 ppm and the vinylic CH confirmed the fragment ═$CHCH_2O$−. Both fragments C-1−C-4 and C-7−C-10 were connected through the sequence $CH_2(CH_3)CH$− as deduced by the correlations observed in the HMBC spectrum between a doublet methyl group (δ_H 0.95 ppm) and C-7 and between the carbon of the CH_2 group and the quaternary carbon on OH, C-4.

The relative stereochemistry of **7** was determined by taking into account the correlations found in its NOE experiments in combination with molecular models. The *E* stereochemistry of the double bond was defined upon observation of the vinylic methyl signal at δ_C 16.1 (q) ppm and the NOE enhancement produced on the C-7 methylene proton signal upon irradiation of the olefinic proton signal at δ_H 5.41 ppm. An NOE effect was observed between the proton signals at C-2 and C-3 and the methyl group on C-4, indicating a trans disposition between the methyl group on C-2 and the hydroxyl groups at C-3 and a trans relationship between both hydroxyl groups. Mutual NOE enhancements observed upon irradiation of H-3 and the methyl group on C-6 established a trans disposition between them.

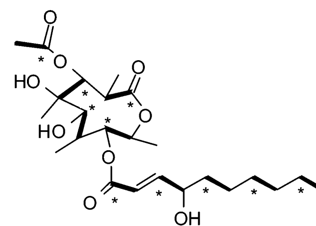

FIGURE 1. Arrangement of intact acetate units in **5**. An asterisk corresponds to carbon derived from C-1 of acetate, and a bold line represents an intact acetate unit.

Botcinolides and compounds **7** and **8** are related polyketides derived from *B. cinerea* with a common structural feature; the fragment C-1−C-4 has identical connectivity in all of them.

Once the fermentation conditions had been determined under which the metabolites were produced, a 48 h old resting cell culture of *B. cinerea* was fed with sodium [1-^{13}C]acetate. Purification of the fermentation broth led to the isolation of labeled **5**, which was analyzed by means of ^{1}H NMR and ^{13}C NMR spectroscopy. The significant enrichment[15] observed in the ^{13}C spectrum at carbons C-1, C-3, C-5, C-7, C-9, C-11, C-13, C-15, and C-15″ and the carbonyl carbon of the acetoxyl group on C-3 (see Figure 1) confirmed the polyketide origin of the botcinolide derivatives.

The production of **5**−**7** by *B. cinerea* seems to indicate that different units are being used as starters in polyketide biosynthesis. An interesting aspect of the botcinolide structure is that it can be dissected into four propionate units. Therefore, to determine whether propionate could, in fact, serve as the starter and/or as an elongation unit in botcinolide biosynthesis, we carried out a feeding experiment with sodium [1,2,3-$^{13}C_3$]-propionate. The ^{13}C NMR spectrum of **5** derived from [1,2,3-$^{13}C_3$]propionate showed the incorporation of 10 intact C_2 units:[16] four in the lactone ring (C-1/C-2, C-3/C-4, C-5/C-6, and C-7/C-8), five at the side chain, and one in the acetoxyl group on C-3 (Figure 1, Table 1). No coupling was observed for the pendant methyl groups of the lactone ring. These results corroborate a putative doubly labeled acetate incorporation that probably occurs as a result of propionate incorporation via pyruvate. Consequently, the methyl groups at C-17 to C-20 of the botcinolide skeleton do not arise from the methyl group of the propionate units.

Finally, a feeding experiment was carried out in which sodium [1,2-$^{13}C_2$]acetate and L-[*S-methyl*-$^2H_3$]methionine were incorporated to confirm the results of the experiment described above as well as to determine the source of the methyl groups. The ^{13}C NMR spectrum of the labeled **5** showed a pattern of coupling pairs identical to that observed in the previous experiment (Figure 2). Moreover, the decrease of the signals corresponding to the methyl groups at positions C-2, C-4, C-6, and C-8 in the ^{1}H NMR spectrum as well as changes in the coupling pattern for protons H-2, H-6, and H-8 indicated that these positions were deuterated. This conclusion was confirmed by data from

(15) The ^{13}C enrichment was calculated from the intensity of labeled carbons divided by the intensity of the corresponding signal in the unlabeled compound, normalized (C-6) to give a ratio of 1 for the unenriched peak.

(16) The ^{13}C enrichment was calculated from the intensity of the double signal of the labeled carbon versus the intensity of the enclosed singlet signal of the unlabeled carbon.

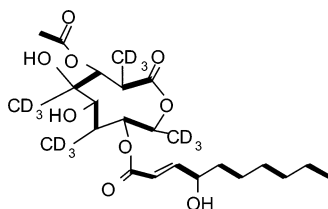

FIGURE 2. Labeling pattern of **5** resulting from the feeding experiment with [1,2-$^{13}C_2$]acetate and L-[*S-methyl*-$^2H_3$]methionine.

the ^{2}H NMR spectrum, which contained signals at δ_D 1.11 (C-17), 1.24 (C-18), 0.89 (C-19), and 0.94 (C-20) ppm.

Feeding experiments thus demonstrated that **5** is an acetate-derived polyketide whose methyl groups originate from the *S*-methyl of methionine. The explicit incorporation of intact acetate units also indicates the direction of the polyketide synthesis, which starts at carbon atom C-8 and ends at C-1. An important question is the origin of the methyl group on the C-8 carbon (C-20) in the molecule. The high level of enrichment of C-20 (46%) in **5** after feeding L-[*S-methyl*-$^2H_3$]methionine showed clearly its common origin with the rest of the methyl groups. Nevertheless, the most satisfying proposal for incorporation of methyl branches in polyketides is that methyl transferase activities are present within the polyketide synthase, which catalyzes the formation of carbon−carbon bonds at activated methylene groups.[17] This is a rare example of the incorporation of a methyl from methionine into a supposed C_3 starter unit of the polyketide synthesis. To our knowledge, an acetate/methionine-derived C_3 starter unit has been reported only for myxovirescin A_1,[18] borophycin,[19] and apicularens[20] as well as asteltoxin[21] and aurovertins,[22] where the alternative starter propionate was incorporated equally well.

In conclusion, we have been able to demonstrate the origin of all the carbon atoms in 3-*O*-acetylhomobotcinolide. Presumably, acetate acts as the starter unit of the polyketide chain, which is then alkylated at methylene carbon atoms. At what stage the methyl group is incorporated in the starter unit is not yet known. A C_3 starter unit arising from propionate is discounted.

Experimental Section

Organism. *B. cinerea* cat 2 is a mutant strain provided by Dr. Jan A. L. van Kan of the Wageningen Agriculture University (The Netherlands). Conidial stock of this strain was maintained viable in 80% glycerol at −40 °C.

Culture Conditions. *B. cinerea* cat 2 was grown in 20 flasks (500 mL) containing 200 mL of Czapek-Dox medium.[5] The pH of the medium was adjusted to 7.0 using aqueous NaOH. Each flask was inoculated with 2×10^7 fresh conidia and then incubated for 5 days at 24−26 °C on an orbital shaker at 250 rpm (revolutions per minute).

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Extraction and Isolation of Metabolites. The broth (4 L) was saturated with NaCl and extracted with ethyl acetate (4×). The organic 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 (250 mg) 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** (4.3 mg), **4** (5.1 mg), **9** (6.8 mg),[14] **10** (6.4 mg),[14] **5** (10.6 mg), **6** (3.3 mg), **7** (1 mg), and **8**[12,13] were obtained.

Labeled Precursors. Sodium [1-^{13}C]acetate (isotopic purity 99 atom % ^{13}C), sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C), sodium [1,2,3-$^{13}C_3$]propionate (99 atom % ^{13}C), and l-[*S-methyl*-$^2H_3$]-methionine (98 atom % D) were used for the feeding experiments.

Feeding Experiments of Labeled Precursors: Extraction and Isolation of Metabolites. 1. General Method. *B. cinerea* cat 2 was grown in Erlenmeyer flasks (500 mL), each containing 200 mL of Czapek-Dox medium. The pH of the medium was adjusted to 7.0, and then each flask was inoculated with 2×10^7 fresh conidia and incubated at 24−26 °C on an orbital shaker at 250 rpm. After 48 h of incubation, the mycelia were transferred into the same number of Erlenmeyer flasks (500 mL), each containing 200 mL of Czapek-Dox medium (without glucose) and an aseptic solution of the labeled precursor in H_2O. Seventy-two hours after administration of the precursor, the culture medium and mycelia were filtered and separated. The broth was extracted and purified as described before to afford labeled **5**, which was analyzed by NMR spectroscopy.

2. Feeding of Sodium [1,2,3-$^{13}C_3$]Propionate. Thirteen cultured flasks with *B. cinerea* were fed with 750 μL of an aseptic aqueous solution of sodium [1,2,3-$^{13}C_3$]propionate and 1900 μL of sodium propionate (final ratio of labeled to nonlabeled compound, 1:3) to give a final concentration of 450 ppm per flask on day 2. Extraction of the broth yielded a crude extract (150 mg), which was purified as described above to afford **5** (3 mg). The relative incorporation is given in Table 1.

3. Feeding of Sodium [1-^{13}C]Acetate. Twenty 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 (190 mg), which was purified as described above to afford **5** (6 mg). The relative incorporation is given in Table 1.

4. Feeding of Sodium [1,2-$^{13}C_2$]Acetate and L-[*S-methyl*-$^2H_3$]-methionine. Twenty culture flasks with *B. cinerea* were fed with 1250 μL of an aseptic aqueous solution of sodium [1,2-$^{13}C_2$]acetate/sodium acetate (1:8) to give a final concentration of 450 ppm per flask on day 2, and with 2500 μL of an aseptic aqueous solution of L-[*S-methyl*-$^2H_3$]methionine to give a final concentration of 365 ppm per flask, also on day 2. Extraction of the broth yielded a crude extract (110.5 mg), which was purified as described above to afford **5** (3.0 mg). The relative incorporation is given in Table 1.

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Supporting Information Available: General experimental procedures, experimental details (HPLC methods) for new compounds, spectroscopic data for compounds **3**−**7**, copies of ^{1}H and ^{13}C NMR spectra of compounds **3**, **4**, and **6**, copies of ^{1}H and 2D NMR spectra of compound **7**, and copies of ^{1}H, ^{13}C, and ^{2}H NMR spectra of labeled **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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