Botrylactone: new interest in an old molecule—review of its absolute configuration and related compounds

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1. Introduction

Botrytis cinerea is a well-known pathogen affecting a number of commercial crops, which produces several structurally unique metabolites. Botrytis produces two series of phytotoxic metabolites: a family of characteristic sesquiterpene metabolites with the basic botryane skeleton, principally botrydial and dihydrobotrydial and a family of polyketide lactones. The first isolated polyketide was reported by Cutler in 1993 who called it botcinolide (1), proposing a highly hydroxylated nonanolactone structure. Based on botcinolide structures and in addition to other botcinolide metabolites, we described new metabolites and investigated the biosynthesis of the botcinolide skeleton.

In 2005, Nakajima’s group reported the isolation of a group of antifungal metabolites, which they designated as botcinins. The absolute configuration of botcinin A (2) was determined through modified Mosher method. A careful reinvestigation of the spectroscopic data reported for botcinolide analogues allowed them to revise the structures of botcinolide derivatives to botcinin (3) and botcineric (4) acids and their cyclized derivatives, botcinins A–F. Ultimately, the revised structures of this group of natural products were unequivocally determined by total synthesis and a revision comparing the botcinolides with their corresponding botcinin structures has been reported.

Recently, a genetically modified strain of B. cinerea, Δbcbot2, which is unable to produce botryanes but significantly overproduces botcinic acid (3) and its derivatives was constructed. The higher production capacities of this strain prompted us to reinvestigate the metabolites produced by it. Additionally, in the course of our investigation on biotransformation with a wild strain of B. cinerea (UCA 992), a new metabolite with a botrylactone skeleton was isolated. This paper focuses on the characterization and biosynthetic route proposal of an intriguing new family of compounds, botrylactones, which could be an intermediate in the biosynthesis to botcinins. Additionally, the absolute configuration of botrylactone (6) and the structure of 2-epihomobotcinolide (5) are revised.

2. Results and discussion

Botrylactone is a unique C–9 polyhydroxylated lactone described as a powerful antibiotically active compound reported by Welmar et al. Several unsuccessful attempts to isolate this intriguing compound have been made and some of them reported. Recently we isolated it from the B. cinerea cat 2 strain. Isolation of botrylactone together with botcinins, and the similarity of their spectroscopic data, led us to believe that they are closely related metabolites. However, comparison of the stereochemistry proposed for botrylactone (6) and that proposed for botcinin A (2) turned out to be diametrically opposed. These data, together with the overproducer mutant Δbcbot2, prompted us to conduct a study of focusing on botcinic acid derivatives.

The Botrytis cinerea mutant, Δbcbot2, was cultured on malt agar medium at 24–26 °C for 7 days. The fermentation broth, after filtration and extraction was purified following the methodology described in the Experimental section. In addition to botcinin A (2) and B (7), (+)-botrylactone (11) and 3-acetylbocineric acid (8), a metabolite whose spectroscopic and physical constants were identical to those described for 2-epihomobotcinolide (5) and a new compound with a botrylactone skeleton, 5-hydroxy-7-(4-hydroxydec-2(3)-enoyl)botrylactone (12), were isolated.

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A careful spectroscopic study of the physical constants of S and its comparison with those of botcinin derivatives described in the literature showed that the $^1$H NMR and $^{13}$C NMR of S were very similar to those of botcinin E (9,10). The $^{13}$C NMR, including DEPT data, showed that S has two more methylenes than 9. The fragment ion assignable to the fatty acyl portion was detected at $m/z$ 169, which is characteristic of a C$_{10}$H$_{15}$O. Thus, S differs from 9 in the length of the acyl portion. The key NOE correlations were identical to those of 9 indicating that S and 9 share the same relative stereochemistry. Therefore, the structure of 2-epihomobotcinolide (5) should be revised to 10 and renamed as botcinin G.

Botrylactone was reported for the first time by Welmar et al. (9) who initially proposed structure 6a based on spectroscopic data. Although they used the term, ‘absolute configuration’ in the text of the paper, they did not determine the absolute configuration but its relative configuration by X-ray diffraction of its acetate derivative (6b). Later, Redlich et al. synthesized it and revised the originally published structure to 7-OH3-botrylactone (6). The optical rotation of natural botrylactone had not been previously reported. Welmar et al. prepared acetyl botrylactone and reported its optical rotation as $+88$ (c 1, CHCl$_3$), while Redlich et al. described it for synthetic botrylactone (6) as $-31$ (c 0.14, MeOH). The isolation of a sufficient amount of natural botrylactone from $\Delta$botcbot2 has enabled us to measure its optical rotation determined as $+19$ (c 2.6, CHCl$_3$), the opposite sign of that described for the synthetic compound. Acetylation of it led us to a product whose spectroscopic data were identical to those described for natural acetyl botrylactone whose optical rotation proved to be $+73$ (c 2.4 mg, CHCl$_3$), coinciding with the sign described by Welmar et al. As a result, it can be inferred that the structure synthesized by Redlich et al. should be the enantiomeric to the natural compound, (−)-botrylactone (6). In order to confirm this hypothesis we determined the absolute configuration of natural (−)-botrylactone using the NMR method. (−)-Botrylactone was treated with $R$(-) and $S$ (+)-MPA acid yielding the corresponding $R$ and S-MPA esters 11a and 11b, respectively. Comparison of the chemical shifts in $^1$H NMR spectra of the two compounds showed a negative $\Delta \delta^{R}$ value for C$_8$-CH$_3$ and C$_9$-CH$_3$ (−0.57 and −0.26 ppm, respectively), while the $\Delta \delta^{S}$ for H-6 and C$_6$-CH$_3$ were positive (0.11 and 0.47 ppm). Application of the Mosher rule showed a 7R configuration for esterificated products 11a and hence for (−)-botrylactone (11).

The stereochemistry of the rest of the chiral carbons was confirmed on the basis of NOE data (Fig. 1). Irradiation of the H-7 signal of compound 11a produced NOE enhancement of H-5β, C$_6$-CH$_3$ and C$_9$-CH$_3$, establishing a boat conformation for this tetrahydropyran ring, which was confirmed by irradiation of H-5β that enhanced the signals of H-6, C$_6$-Me, C$_4$-Me and H-5α. A quasi-boat conformation was established for pentanolide ring on the basis of NOE enhancement of C$_2$-Me, C$_4$-Me and H-3 observed when H-2 was irradiated. These data coincide with a configuration where H-2, C$_4$-Me and C$_6$-Me were on the β face and H-3, H-7, C$_6$-Me and C$_9$-CH$_3$ were on the α face. The absolute stereochemistry of natural botrylactone, (+)-11, was definitively established as 2R, 3S, 4S, 6S, 7R, 8R, 9R.

Compound 12 was isolated as an oil whose molecular formula was established as C$_{24}$H$_{38}$O$_8$ by HRMS and $^{13}$C NMR data requiring 6 unsaturation. Its $^1$H NMR spectrum was very similar to that of botcinins however the presence of a signal in $^{13}$C NMR at δ 104.2 ppm, attributable to a ketal group plus an additional methyl group, should correspond to an additional C$_2$ unit resulting in a structure similar to that of botrylactone (11). However, the $^1$H NMR showed signals at δ 7.00, 6.07 and 4.33 ppm characteristic of the fatty acyl portion on C-7 of botcinins. The fragment ion detected at $m/z$ 169 was characteristic of a fatty acyl chain with molecular formula C$_{10}$H$_{15}$O. The HMBC experiment performed on 12 showed correlations between the quaternary ketalic carbon signal (δ$_{C}$ 104.2 ppm), two methyl singlet groups (δ$_{H}$ 1.53 and 1.10 ppm), which were further correlated with a quaternary oxygenated carbon at δ$_{C}$ 79.2 ppm and two signals at δ$_{H}$ 4.93 and 3.52 ppm attributable to H-7 and H-3, respectively (Fig. 2). H-3 was further correlated with signal at δ$_{C}$ 171.0 ppm corresponding to carbon C-1 while H-7 exhibited correlation with the carbon signal at δ$_{C}$ 165.4 ppm corresponding to the C-1 of the fatty acyl portion. Additional correlations were observed between carbon C-3 (δ$_{C}$ 80.9 ppm) and two methyl groups at δ$_{H}$ 1.46 (d) and 1.14 (s) ppm. The COSY experiment showed correlations between the signal doublet at δ$_{H}$ 4.93 ppm (H-7) and a multiplet at δ$_{H}$ 3.52 ppm, which was further correlated with two doublets at δ$_{H}$ 3.67 ppm and 1.02 ppm, pointing to the presence of a fragment O-CH-CH(CH$_3$)-CH=$\equiv$O in the molecule. All these data are consistent with a structure of 5-hydroxybotrylactone bearing a fatty acyl chain on C-7 for this compound. The stereochemistry was determined by N.O.E experiments and was consistent with the stereochemistry assigned to compound 11 confirming the structure of 2R, 3S, 4S, 6S, 7R, 8R-5-hydroxy-7-(4-hydroxydec-2(3)-enoyl) botrylactone for compound 12.

![Fig. 1. NOEs interactions in 11a.](Image)

![Fig. 2. Selected HMBC-COSY correlations for 12 and 13.](Image)

Furthermore, during the course of our biotransformation experiments with B. cinerea UCA 99213 we found a new compound 13 with an NMR pattern very similar to that of (−)-botrylactone (11), the principal difference being the absence of the characteristic H-7 signal in its $^1$H NMR spectrum and one of the carbon bearing to oxygen and the presence of a signal corresponding to one more methylene in the $^{13}$C NMR spectrum. Therefore, 13 differ from 11 in the absence of the hydroxyl group on C-7. HRMS confirms this showing an ion assignable to C$_{13}$H$_{22}$O$_3$ [M−CO] at $m/z$ 226.1573. The botrylactone skeleton was confirmed by the 2D NMR data where correlation in the HMBC experiment between the characteristic
quaternary carbon signal (δc 104.8 ppm) and signals corresponding to H-3, H2-7 and two methyl singlet groups, which were further correlated with C-8 were observed. The key NOE correlations were identical to those of 11a, indicating that 11 and 13 share the same relative stereochemistry. On the basis of these data the structure of 13 was suggested to be 2R, 3S, 4S, 6S, 8R, 9R-7-dehydroxybotrylolactone.

The occurrence of botrylolactone derivatives and botcinins in the same strain, their structural homology, the resulting absolute configuration of natural botrylolactone (11) therefore being identical to that assigned to botcinins by Nakajima except in C-8, seems to indicate that both compounds may be biogenetically related.

Incorporation studies with 13C and 2H-labelled precursors conducted by our group indicated that both compounds may be biogenetically related.

3. Conclusions

A plausible explanation for this, which has been previously reported for other natural products, such as aurovertin, is that B. cinerea biosynthesis involves a C10-polyketide, which is methylated at activated methylene groups, followed by the loss of the starter-acetate unit through a retro-Claisen type C–C bond cleavage with inversion of configuration at C-8 (Scheme 1).

This hypothesis is in agreement with the common biosynthetic origin of botrylolactone (11) and botcinins, where a hypothetical bicyclic acid intermediate 15 could be the branching point to give botrylolactone or botcinin derivatives as shown in Scheme 1. This proposal explains the presence of the methyl group on C-8 of botcinins and the different stereochemistry in this carbon.

4. Experimental section

4.1. General experimental procedures

1H and 13C NMR measurements were recorded on Varian Unity 400 MHz and Varian Inova 600 MHz spectrometers with SiMe4 as the internal reference. Chemical shifts were referenced to CDCl3 (δH 7.25, δc 77.0). HPLC was performed with a Hitachi/Lichrosolv L-5270 apparatus equipped with a differential refractometer detector (RI-7490). A LichroSpher Si 60 (5 μm) LichroCart (250 mm × 4 mm) column and a LichroSpher Si 60 (10 μm) LichroCart (250 mm × 10 mm) were used in isolation experiments. Silica gel (Merck) was used for column chromatography. TLC was performed on Merck Kieselgel 60 F254, 0.25 mm thick.

4.2. Microorganism

B. cinerea mutant strain, bcbot2Δ, was supplied by Dr. Muriel Viaud of the UMR BIOGER, INRA (Versailles, France). The strain was maintained viable on mycelia discs of 0.5 cm diameter submerged in 80% glycerol at –40 °C.

B. cinerea (UCA 992) was obtained from grapes from Domecq vineyard, Jerez de la Frontera, Cadiz, Spain. This culture is deposited at the Universidad de Cadiz, Facultad de Ciencias Mycological Herbarium Collection (UCA).

4.3. Culture conditions

bcbot2Δ was grown on malt agar medium (20 g of d-glucose, 10 g of malt extract, 20 g of agar, pH 6.57 per liter of water) at 25 °C and used to inoculate Roux bottles or Erlenmeyer flasks. For surface culture, mycelium was grown in 1 L Roux bottles containing 150 mL of modified Czapek-Dox medium (50 mg of d-glucose, 1 g of yeast extract, 5 g of KH2PO4, 2 g of Na2SO4, 0.5 g of MgSO4·7H2O and 0.01 g of FeSO4·7H2O, pH 6.57) per liter of water) at room temperature. For shaken cultures, mycelium was grown in Erlenmeyer flasks containing 200 mL of the same medium agitated on an orbital shaker at 140 rpm at 25 °C. Each Roux bottle or Erlenmeyer flask was inoculated with mycelium with six small slices of agar (1 cm).

4.4. Extraction and isolation of metabolites

After 7 days of incubation under fluorescent light, the culture media were filtered, saturated with NaCl, extracted with ethyl acetate (3 × 0.5 vol) and washed with water (3 × 0.25 vol). The organic extracts were dried over Na2SO4 and concentrated to dryness.

Preliminary fractionation of the extracts was achieved by column chromatography eluting with petroleum ether/ethyl acetate (100%) to give 10 fractions F1–F10. The final purification of each fraction was carried out by means of semi-preparative or analytical HPLC. Botcinin A (2), botcinin B (2a), botcinin G (10), 3-O-acetylbottyolactone (8), (+)-bottyolactone (11) and 5-hydroxy-7-(4-hydroxydec-2(3)-enoyl)botrylolactone (12) were obtained.

4.4.1. Botcinin A (2) and B (2a)

Compounds 2 and 2a were obtained from purification of fractions F4 and F5 as a colourless oil. Semi-preparative HPLC: hexane–ethyl acetate 70:30; flow 3 mL min−1; tR around 29 and 35 min, respectively.

4.4.2. Botcinin G (10)

Compound 10 was obtained from purification of fractions F7 as a colourless oil. Semi-preparative HPLC: hexane–ethyl acetate 75:25; flow 3 mL min−1; tR = 70 min (2a)35–34° (c 2.0, CHCl3); IR vmax (film) 3452, 2933, 2870, 1723, 1652, 1545, 1566 cm−1; 1H NMR (400 MHz, CD3OD) δ 7.02 (dd, 1H, J=15.5, 4.9 Hz, H-3), 6.05 (dd, 1H, J=15.5, 1.7 Hz, H-5), 4.50 (dd, 1H, J=10.5, 9.7 Hz, H-7), 4.25 (m, 1H, H-4), 4.10 (d, 1H, J=9.4 Hz, H-5), 3.77 (dq, 1H, J=9.7, 5.9 Hz, H-8), 3.20 (dq, 1H, J=9.4, 7.3 Hz, H-2), 2.21 (m, 1H, J=10.5, 6.4 Hz, H-6), 1.49 (m, 2H, H-5), 1.31 (m, 2H, H-5), 1.17 (m, 6H, H-7, H-8, H-9), 1.17 (t, 3H, C4-CH3), 1.11 (d, 3H, J=5.9 Hz, C6-CH3), 1.13 (t, 3H, J=7.3 Hz, C7-CH3); 13C NMR (100 MHz, CD3OD) 177.4 (s, C-1), 167.6 (s, C-1′), 154.0 (d, C-3′), 137.8 (s, C-2), 119.9 (d, C-8), 118.9 (d, C-9), 97.1 (d, C-7), 77.9 (d, C-5), 78.1 (d, C-7), 78.1 (d, C-5), 75.0 (s, C-4), 75.0 (s, C-4), 73.1 (d, C-3), 71.6 (d, C-2), 69.6 (d, C-8), 39.6 (d, C-2), 37.5 (t, C-5), 36.8 (d, C-6), 36.9 (d, C-6), 32.9 (t, C-8), 30.2 (t, C-7), 26.4 (t, C-6), 23.6 (t, C-9), 18.6 (q, C6-CH3), 14.3 (q, C-10), 13.9 (q, C7-CH3), 11.5 (q, C8-CH3), 10.4 (q, C9-CH3); EIMS m/z (rel int.) 394 [M-H2O]+ (9), 226 (26), 169 (9).
4.4. Acetylation of botrylactone

Botrylactone (11, 10 mg) was dissolved in dry pyridine (1 mL, 0.01 mol) and acetic anhydride (2.4 mL, 24.6 mmol) was added dropwise. The reaction mixture was stirred for 24 h. Then the solvent was removed and the crude reaction product chromatographed to give botrylactone acetate (11c). Colourless oil; [α]D25 +67.3 (c 2.4 mg, CHCl3).15

4.6. α-Methoxyphenylacetyl ester of botrylactone

A solution of the botrylactone (11, 10 mg, 0.037 mmol) in dry dichloromethane (CH2Cl2) (1.5 mL) was treated with DMAP (9.05 mg, 0.02 equiv) and (+)–25- or (–)–2R)-2-methoxy-2-phenylacetic acid MPA (13.85 mg, 0.22 equiv). After 15 min stirring at room temperature, EDC (14.91 mg, 0.21 equiv) was added. Stirring was maintained for 24 h. The solvent was stirred under reduced pressure. Residue purification was achieved by flash column chromatography on silica gel (elution with 60:40 hexane:ethyl acetate).

4.6.1. (R)-α-Methoxyphenylacetyl ester of botrylactone (11a).131 H NMR (400 MHz, CDCl3) δ 7.40 and 7.33 (m, 2H and 3H, C8–CH3), 4.87 (d, 1H, J = 10.8 Hz, H-7), 4.75 (s, 1H, H-2), 3.39 (s, 3H, C2–OMe), 3.27 (br s, 1H, H-3), 2.63 (q, 1H, J = 7.3 Hz, H-2), 1.90 (m, 1H, H-6), 1.71 (dd, J = 4.7, 13.5 Hz, H-5), 1.55 (dt, J = 12.3, 13.5 Hz, H-5x), 1.39 (d, 3H, J = 7.3 Hz, C2–CH3), 1.26 (s, 3H, C9–CH3), 1.12 (s, 3H, C8–CH3), 0.91 (d, 3H, J = 6.4 Hz, C6–CH3), 0.86 (s, 3H, J = 7.2 Hz, H-10).142 (d, 3H, J = 7.3 Hz, C2–CH3), 1.12 (s, 3H, C4–CH3), 1.04 (s, 3H, C8–CH3), 0.44 (d, 3H, J = 6.4 Hz, C6–CH3).

4.7. Biotransformation of 2-benzylideneindan-1-one

Botrytis cinerea UCA 992 was grown at 25°C on a Czapek-Dox medium (200 mL per flask). The shaken culture was incubated in an orbital shaker at 140 rpm under fluorescent light. 2-Benzylidenean-1-one was dissolved in ethanol and then distributed over 12 flasks (150 ppm per flask) and the fermentation continued for 5 days in six flasks and 10 days in the others. The mycelium was then filtered and the broth was extracted as described below. The solvent was then evaporated and the residue was purified first on a silica gel column and then with HPLC with an increasing gradient of ethyl acetate to petroleum ether.

Chromatography of the extract fermented for 5 days produced 2-benzylidenean-1-one (4 mg), O-methylidihydrobotrydial (3.2 mg), botrydial (4 mg), dihydrobotrydial (3 mg), 7-deoxybotrydial (13.2 mg) and 2-(p-hydroxyphenylmethyl)-1-indanone (50 mg) [α]D25 +6° (c 0.1, MeOH), 36% ee.

Chromatography of the extract fermented for 10 days produced 2-benzylidenean-1-one (4 mg), botrydial (2 mg), dihydrobotrydial (3 mg), 7-deoxybotrydial (13, 15.3 mg), botricin acid (3, 60 mg) and 2-(p-hydroxyphenylmethyl)-7-hydroxyindan-1-one (17 mg) [α]D25 −3.7° (c 0.1, MeOH, 7% ee).

4.7.1. 7-Deoxybotrydial (13). Compound 13 obtained from fraction F3. Column chromatography: hexane–ethyl acetate 80:20. Amorphous solid; [α]D25 +2.245° (c 0.3 CHCl3); IR νmax (film) 2925, 1735, 1459, 1103, 950 cm−1; 1H NMR (400 MHz, CDCl3) δ 3.28 (s, 1H, H-6), 2.70 (q, 1H, J = 7.3 Hz, H-2), 1.75 (m, 1H, H-6), 1.63 (m, 1H, H-5), 1.57 (m, 2H, H-7) 1.44 (s, 3H, C9–CH3), 1.40 (d, 3H, J = 7.3 Hz, C2–CH3), 1.37 (m, 1H, H-5), 1.26 (m, 1H, H-5x), 1.14 (3H, J = 6.4 Hz, C6–CH3), 1.18 (3H, J = 6.7 Hz, C6–CH3); 13C NMR (100 MHz, CDCl3) 171.0 (s, C-1), 165.4 (s, C-1), 151.0 (C-3), 119.0 (d, C-2), 104.2 (s, C-9), 80.9 (d, C-3), 79.2 (s, C-8), 76.2 (s, C-4), 75.1 (d, C-5), 74.2 (d, C-7), 71.1 (d, C-4), 36.7 (d, C-5), 36.5 (t, C-6), 34.5 (d, C-2), 31.6 (t, C-8), 29.1 (t, C-7), 25.2 (t, C-6), 22.5 (t, C-9), 21.7 (q, C8–CH3), 18.5 (q, C6–CH3), 18.1 (q, C2–CH3), 14.0 (q, C-10), 13.8 (q, C9–CH3); EIMS m/z (rel.int.) 454 [M]+ (1), 426 [M–CO]+ (6); HR EIMS calc for C21H38O7 [M–CO]+ 426.2618, found 426.2641.

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References and notes