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Structure and Solution-State Conformation of Botcinolide, a New Biologically Active Metabolite from the Fungus *Botrytis cinerea*

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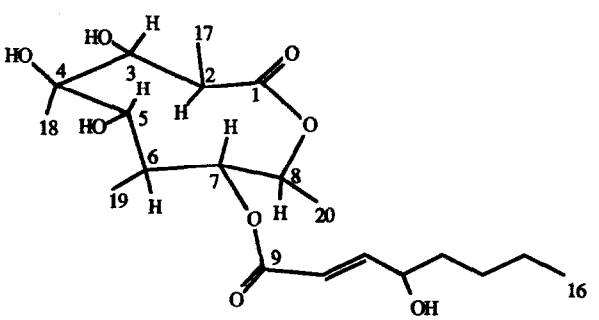
Abstract: The structure of botcinolide, a new, phytotoxic, trihydroxylated nona-lactone, esterified with 4-hydroxy-2-octenoic acid, has been elucidated using both spectroscopic and chemical techniques. Botcinolide is a metabolite of the mould *Botrytis cinerea* (UK185RRC).

INTRODUCTION

In the course of a screening program to discover natural products with novel biological activities from microorganisms,[1] a strain of the fungus, Botrytis cinerea (Moniliaceae) was found to produce



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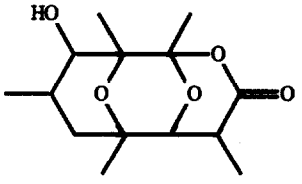
a new, highly-substituted lactone, which we have trivially named botcinolide(**1**).[2] This compound inhibits the growth of etiolated wheat coleoptiles, and causes marked phytotoxicity in greenhouse-grown bean, corn and tobacco plants.[2] However, botcinolide was inactive in limited antibacterial and antifungal tests.[2] Preliminary toxicological studies with mice indicate that the acute toxicity of botcinolide is relatively low (>350 mg/kg, i.p.).[3] Different strains of *Botrytis cinerea* (best known as the "noble rot" fungus which attacks grapes),[4] have previously yielded the metabolites botrylactone (**2**)[5] and cinereain (**3**),[6] *inter alia*. A comparison of the structures of botrylactone and



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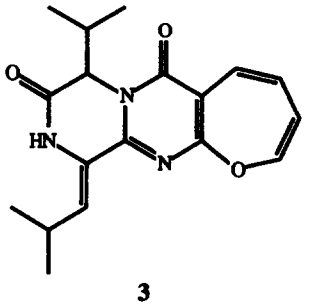

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botcinolide suggests a possible biogenetic relationship between them. Botcinolide has a unique structure incorporating a nine-membered ring lactone, a feature which is very rare amongst natural products.[7] In this paper, we report the details of the structure elucidation of botcinolide, and the assignment of its solution-state conformation.

RESULTS AND DISCUSSION

The *Botrytis cinerea* (Accession No. UK185RRC), isolated from raspberry fruit, was cultured on potato-dextrose broth.[2] Bioassay-directed extraction and fractionation were carried out using an etiolated wheat coleoptile assay.[8] Botcinolide was obtained as a chromatographically homogeneous, amorphous solid, which underwent partial decomposition on standing at rt for several hours, but which could be stored indefinitely at -8°C without substantial deterioration. Low-resolution EI mass spectrometry did not show a molecular ion; the low-resolution FAB mass spectrum gave a molecular weight of 403 amu. The high resolution FAB mass spectrum of this material indicated a molecular formula of $C_{20}H_{34}O_8$, hence four degrees of unsaturation. This formula was supported by one-dimensional ^{13}C and DEPT[9] NMR spectra, which showed the presence of two carbonyls, one oxygen-bearing quaternary carbon, nine methines, three methylenes, and five methyls. Of the nine methines, the ^{13}C chemical shifts suggested that two were olefinic, five were oxygen-substituted, and the remaining three were aliphatic. Since the two carbonyls and one double bond account for three degrees of unsaturation, the presence of one ring

in the carbon skeleton was indicated. The IR spectrum showed a strong carbonyl absorption, consistent with an ester or a lactone. The UV spectrum was consistent with an α,β -unsaturated ester.

The structure and chemical shift assignments of botcinolide were determined primarily through the use of two-dimensional COSY[9], TOCSY[10], HETCORR[9], and HMBC[11] NMR spectra. The bulk of the structure determination proceeded in a straightforward fashion, initially using data from the COSY and HETCORR experiments to determine the skeletal fragments C2-C3, C5-C8 and C10-C16. The connections between these fragments then were discerned using information from the TOCSY and HMBC spectra, such that the entire backbone structure was obtained.

The only structural feature of botcinolide not readily determined is the location of the requisite ring closure. This requirement is met only if the carbonyl carbon resonating at 180.1 ppm (C1) is part of a lactone linkage with one of the oxygenated carbons C3, C4, C5, C8 or C12. Linkage through C3 or C4 would lead to δ- or γ-lactones, respectively, which are unlikely due to the absence of a (sufficiently) high frequency carbonyl absorption in the IR spectrum. A thirteen-membered lactone, resulting from ring closure at C12, may also be excluded on the grounds that the proton chemical shifts and the nOe/rOe data (discussed below) obtained for the C10-C16 portion of the molecule indicate that the carbon-carbon bonds along this chain are undergoing free rotation, and so cannot be part of a ring structure.

The remaining two possible structures, a six- or a nine-membered lactone, cannot be differentiated by either chemical shift or nOe/rOe data. We had expected that the six- or nine-membered ring structures would show long-range (three-bond) coupling interactions between C1 and either H5 or H8 (respectively) that would be detectable in the HMBC spectrum and would thus allow unequivocal determination of the ring size. However, we were unable to find any hint of such correlations in the HMBC spectrum, which showed, with excellent sensitivity, all of the other possible long-range correlations for C1 (to H2, H3 and H17), and all of the analogous two- and three-bond correlations for the other carbonyl carbon, C9.

We then attempted to elucidate the ring closure location using selective INEPT[12] spectra. We eventually obtained a matrix of nine selective INEPT spectra, with protons H8, H5 and H3 selectively irradiated and with long-range coupling optimizations of 10, 6 and 4.5 Hz. Since the H3 and H8 protons are very close to each other (ca. 12 Hz apart at 400 MHz), we obtained spectra with H3 irradiated in order to confirm that results obtained from irradiating H8 (if the nine-membered ring structure were present) were in fact due to coupling of H8 to C1 and were not a result of accidental partial irradiation of the H3 proton, since H3 had already shown long-range coupling to C1 in the HMBC spectrum.

The selective INEPT spectra we obtained confirm that the botcinolide structure contains a nine-membered lactone. All of the correlations obtained supported the nine-membered ring structure, but the key correlation was H8 to C1, obtained with 10 Hz optimization. H8 showed no correlations with 6 or 4.5 Hz optimization, while H3 correlated with C1 at 10 Hz optimization and with both C1 and C4 with 6 Hz optimization, thus showing that correlations from H8 and from H3 can be differentiated reliably. H5 showed correlations only to C4, C18 and C19, depending upon optimization.

In parallel with our NMR work, we also investigated the use of chemical methods for solving the ring size problem in botcinolide. The first method we attempted was the use of the deuterium-induced differential isotope shift (DIS).[13] In this technique, the ^{13}C chemical shifts of the hydroxylated carbons are obtained with and without deuterium exchange of the hydroxyls, and the values compared. However, our results (Table 1) were inconclusive, suggesting that neither C4 nor C8 were hydroxyl-bearing, i.e., both were lactonic, a result which is inconsistent with the molecular formula. An attempt to identify the ring closure location by acetylation also was unsatisfactory. After reaction of our sample with acetic anhydride/pyridine, we observed the H3 and H12 resonances shifted downfield approximately 1.8 and 1.2 ppm, respectively, while the H5 resonance moved downfield by 0.31 ppm, and H8 was displaced downfield by 0.16 ppm. This result indicated that neither C5 nor C8 was acetylated, suggesting that both were lactone-linked, again inconsistent with the molecular formula. However, incomplete acetylation is not unexpected in sterically congested poly-hydroxylated compounds, and this possibility was supported by the integration of the ^{1}H spectrum of the acetylated botcinolide, which corresponded to the introduction of only three acetyl groups into the molecule.

The final chemical technique which we applied to the problem at hand was derivatization using trichloroacetyl isocyanate, a reagent which has been used to effect in situ conversion of hydroxy groups to the corresponding urethanes in an NMR sample solution.[14] This method also produces large downfield shifts of the protons on the derivatized carbons. Upon reaction of botcinolide with trichloroacetyl isocyanate in CD_3COCD_3, the H3, H5 and H12 resonances shifted downfield by approximately 1.5, 1.3 and 1.3 ppm, respectively, whereas the H8 resonance moved downfield by only 0.2 ppm. These changes in chemical shift also determine C8 as the location of the lactone ring closure, consistent with our results from the selective INEPT NMR experiments. We note that this conclusion does not contradict the results of our DIS and acetylation experiments, both of which indicated that C8 was part of a lactone linkage, and that in our hands the trichloroacetyl isocyanate derivitization method provided more reliable results than did the acetylation method. The chemical shift assignments of botcinolide and of its acetyl and trichloroacetyl urethane derivatives are summarized in Table 1, below.

Table 1. NMR Data for Botcinolide and Derivatives in CD_3OD.

C#	$\delta^{13}C$[a]	$\delta^{1}H$			^{13}C DIS value[c]
	Native	Native	Isocyanate[b]	Acetate	Native, H - D
1	180.1 (s)	-	-	-	+1.9
2	39.7 (d)	2.74 (dq, 2.3, 7.1)	3.12	3.44	+0.3
3	77.6 (d)	3.57 (d, 2.3)	5.08	≈5.4	+0.8
4	79.9 (s)	-	-	-	-
5	72.4 (d)	3.78 (d, 10.8)	5.11	4.09	+0.3
6	39.3 (d)	1.87 (m, 4.9, ≈10, 10.8)	2.23	2.23	+0.1
7	78.4 (d)	4.33 (bt, ≈10, 10.8)	4.45	4.53	+0.1
8	69.3 (d)	3.60 (m)	3.79	3.76	-0.1
9	167.7 (s)	-	-	-	-
10	120.1 (d)	6.04 (dd, 1.6, 15.6)	6.12	5.98	-
11	153.6 (d)	6.98 (dd, 4.8, 15.6)	7.00	6.95	-
12	71.5 (d)	4.24 (m)	5.53	≈5.4	+0.1
13	37.2 (t)	1.54 (m)	1.78	1.69	-
14	28.6 (t)	≈1.4 (m)	≈1.4	≈1.3	-
15	23.6 (t)	≈1.4 (m)	≈1.4	≈1.3	-
16	14.3 (q)	0.92 (bt, 7.1)	0.88	0.90	-
17	17.4 (q)	1.32 (d, 7.2)	1.26	≈1.0	+0.7
18	14.9 (q)	1.23 (s)	1.51	1.24	-
19	14.7 (q)	0.97 (d, 4.9)	0.89	≈1.0	-
20	18.1 (q)	0.99 (d, 4.7)	1.05	≈1.0	+0.1

[a] ^{13}C multiplicities are from the DEPT spectrum.
[b] in CD_3COCD_3.
[c] DIS = Deuterium induced Isotope Shift, when measurable; see text for details.

The approximate solution state conformation of botcinolide was discerned from a qualitative analysis of two-dimensional NOESY[15] and ROESY[16] spectra,[17] the relevant proton-proton coupling constants, and inspection of molecular models. The most significant interactions observed in the spectra were the strong (and roughly equally intense) correlations between the H18 methyl singlet (1.23 ppm) and H2, H6 and H8. These correlations impose upon the nine-membered ring the relative configuration shown in the structure (1), such that the three protons H2, H6 and H8 are on the same side of the approximate plane of the ring, and are oriented so that they are all sufficiently close to the H18 methyl to show a strong nOe interaction. After thus determining the ring geometry, the arrangement of groups on the C5 to C8 section of the ring was established by means of proton coupling constants and nOe data. The coupling constants measured for the protons along the C5-6-7-8 fragment are as follows: H5-H6: 10.8 Hz; H6-H7: ~ 10 Hz; H7-H8: ~ 10 Hz. These values suggest that the protons on adjacent carbons are anti to one another.[18a] In addition, we observed a strong nOe interaction between H5 and H7, and a moderately strong nOe between H6 and H8 (consistent with the nOe's between these protons and the H18 methyl group noted above). This pair of nOe interactions is indicative of an anti arrange-

ment of adjacent protons, since the protons two carbons apart are then approximately syn, and so close enough to exhibit nOe's. After establishing the orientation of the protons on the C5 to C8 region, and arranging the linkages to fulfill the geometric requirements imposed by the nOe's involving the H18 methyl group, the C19 and C20 were determined, by default, to be on the outside of the ring. No nOe's were observed between the C20 and C18 methyl groups, as expected on the basis of the foregoing conformational assignments. The H17 methyl group also was determined to lie on the outside of the ring, using similar reasoning. The orientation of H3 was determined as follows. The coupling constant for H2-H3 was found to be 2.3 Hz, suggesting a fairly small dihedral angle between these protons.[18a] Applying the equation of Colucci et al.[19] gave a dihedral angle for this H-C-C-H fragment of approximately 46°. The resulting location of H3 on the inside of the ring, as shown, was suggested by the of nOe interactions between H3 and both H5 and H7.

The C9-C16 portion of botcinolide does not have a rigid, well defined structure. Evidence for this is derived from the fact that the two protons of each methylene group in this chain have (accidentally) equivalent chemical shifts, suggesting that free rotation is occurring about the C-C bonds along the chain. Indeed, we observed no nOe's between any part of the C9-C16 fragment and the rest of the molecule, although some nOe's within the C10-C16 chain were observed. Finally, the H10-H11 coupling constant of 15.6 Hz indicates a *trans*- orientation of the C10-C11 double bond.[18b]

EXPERIMENTAL

NMR Spectroscopy. NMR spectra were recorded at 400.13 MHz for ^{1}H and 100.63 MHz for ^{13}C using Bruker AM- or AMX-400 spectrometers. The sample consisted of approximately 20 mg of botcinolide in 0.5 mL of CD_3OD. All spectra were recorded at 25°C and the chemical shifts are referenced to the residual solvent signals (^{1}H: 3.3 ppm, ^{13}C: 49.0 ppm, relative to TMS). DIS experiments were conducted with samples of 10 mg botcinolide in 0.5 mL of CD_3OH and 10 mg of deuterium-exchanged botcinolide in CD_3OD. The deuterium exchange was performed by dissolving botcinolide in CD_3OD and evaporating to dryness under vacuum; this was repeated three times. Since some of the chemical shift changes were not assignable based upon 1D spectra alone, the assignments for the sample in CD_3OH, and hence the DIS values, were confirmed with HETCORR and HMBC spectra.

IR Spectroscopy. The IR spectrum was determined on the sample as a thin film, using a Beckman IR 4210 spectrometer.

UV Spectroscopy. The UV spectrum was obtained on a methanol solution of the sample, using a Shimadzu UV-160U spectrophotometer.

Mass Spectroscopy. Low resolution EI mass spectra were obtained from a Hewlett-Packard 5985B mass spectrometer, using a direct probe. Low resolution FAB mass spectra were acquired on a VG Analytical VG7070EHF mass spectrometer operated at an accelerating potential of 6kV. The sample was dissolved in methanol and ionized from a glycerol matrix with xenon. Spectra were obtained in either positive or negative ion mode. High resolution FAB mass spectra were obtained with a Kratos MS50 Triple Analyzer, operated at an accelerating potential of 6 kV. The sample was ionized from a matrix of 3-nitrobenzyl alcohol, using argon.

Culture and Isolation. Botrytis cinerea, Accession No. UK185RRC, was isolated from raspberry fruit growing in Watkinsville, Georgia, USA. Culture conditions for this organism, and details of the bioassay-directed isolation of botcinolide are reported elsewhere.[2]

Botcinolide (1). Amorphous solid; UV lmax (MeOH) 212 nm (log e 4.18); IR (film), 3400, 1728 cm-1; ^{1}H and ^{13}C NMR, Table I; LREIMS, m/z 366 (1), 226 (9), 141 (44), 124 (56), 109 (89), 97 (100), 85 (60); LRFABMS, m/z 403 (20) (M+H)+, 385 (25) (M+H -H_2O)+, 367 (13) (M+H-$2H_2O$)+, 245 (19), 227 (97), 209 (100). HRFABMS, m/z 403.2328 (M + H)+, (calcd. for $C_{20}H_{35}O_8$ 403.2323, 0.4 mmu).

Derivatization. Acetylation of botcinolide was carried out by dissolving botcinolide (5.0 mg) in dry pyridine (1 mL) and acetic anhydride (0.5 mL), and allowing the mixture to stand overnight. After evaporation of all volatiles under high vacuum, the resulting gum was examined by NMR without further purification. The trichloroacetyl isocyanate derivative of botcinolide was prepared in situ by adding trichloroacetyl isocyanate (2 drops) to botcinolide (5 mg) in CD_3COCD_3 (0.5 mL) in a 5 mm NMR tube. Reaction was allowed to proceed for several minutes at ca. 20°C before spectra were acquired.

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