Dihydroramulosin from *Botrytis* sp.

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Botrytis sp., isolated from the inner bark of the Pacific yew, *Taxus brevifolia*, was shown to produce ramulosin (**1**), 6-hydroxyramulosin (**2**), and the new compound 8-dihydroramulosin (**3**). The structure of dihydroramulosin was deduced from the NMR spectra and confirmed by chemical conversion from ramulosin.

For the past five years we have been looking at the endosymbionts found in the inner bark of the Pacific yew, *Taxus brevifolia*.[1] In the course of these investigations we have isolated a variety of fungi. The chloroform extract of one of these fungal growths showed strong antibiotic activity against *Staphylococcus aureus*, *Candida albicans*, and *Vibrio harveyii*, so we decided to look at the bioactive components. The fungus, *Botrytis* sp. was grown in mycological broth for 21 days and extracted with CH_2Cl_2. This extract had strong antibiotic activity. The extract was fractionated on LH-20 and then by counter current column chromatography (CCCC). The antibiotic activity against *S. aureus* was followed during these fractionations to give three antimicrobial compounds. These compounds showed moderate antimicrobial activity in the disk assay.

The two less polar antimicrobial compounds were identified as ramulosin (**1**) and 6-hydroxyramulosin (**2**) by comparison of their NMR spectra to those of the known compounds.[2,3] These two fungal metabolites have previously been isolated from *Pestalosia ramulosa*,[2,3] and ramulosin has been more recently reported from *Truncatella hartigii*[4] and *Hypoxylon howeianum*.[5] The absolute configuration of ramulosin has also been established as shown in **1.**[6] Because the ^{13}C NMR data have not been reported for these compounds, they are shown in Table 2.

The more polar antimicrobial metabolite, **3**, gave a very weak parent ion in the EIMS at *m/z* 184, and larger fragment ions at *m/z* 169 (M^+-CH_3) and 166 (M^+-H_2O). The molecular formula of $C_{10}H_{16}O_3$ was deduced from the HRCIMS, which gave a strong [$M^+ + 1$] peak at *m/z* 185. Alcohol **3** readily formed a monoacetate when treated with Ac_2O/pyridine. These data suggested that **3** was a dihydroramulosin. Detailed examination of the 500 MHz ^1H NMR and COSY spectra of **3** suggested the alcohol functionality was at C-8. Reduction of the enol group in ramulosin should then give **3**.

Reduction of the enol of ramulosin proved to be not as simple as originally thought. When ramulosin was treated with sodium borohydride for 10 min at 20 °C and acetylated with Ac_2O−pyridine the product was found to be a mixture of diacetates. These diacetates were formed from the reduction of both the enol and ester groups. The diacetates were separated by HPLC on Si gel. One of the acetoxy protons for the more polar major isomer, **6**, appeared as a doublet (J = 8.5 Hz) at δ 5.54. This proton was assigned to C-1 and given the relative stereochemistry shown based on the large diaxial proton coupling. The second acetoxy proton appeared as a doublet of triplets (J = 10.2, 4.6 Hz) at δ 4.72 and was assigned at C-8 and given the relative stereochemistry shown, again based on the coupling. Reduction of the ester group with sodium borohydride gave a surprising result. When ramulosin was treated with sodium borohydride at 0 °C, the reaction gave only one product that had an identical ^1H NMR spectrum to that of dihydroramulosin (**3**).

The relative stereochemistry of **3** was established from difference NOE studies. Irradiation of the proton at δ 4.39 (H-3) gave a strong enhancement at δ 2.95 (H-8a), which indicated that these protons had a cis arrangement. Irradiation at δ 2.95 gave the expected enhancement at δ 4.39 and also enhanced the other bridgehead proton δ 2.13 (H-4a). The other irradiations and enhancements are shown in Figure 1.

Table 1. ^1H NMR Data for Compounds **1**−**4** (500 MHz, $CDCl_3$)

C no.	1	2	3	4
3	4.43 (m)	4.50 (m)	4.39 (m)	4.41 (m)
4	1.90 (m)	1.89 (dm, 13.4)	2.23 (ddd, 14.1, 6.4, 3.8)	2.08 (m)
	1.28 (m)	1.29 (m)	1.23 (m)	1.32 (m)
4a	2.48 (tm, 11.2)	2.89 (br t, 11.1)	2.13 (m)	2.26 (m)
5	2.34 (m)	1.97 (dm, 13.1)	1.61 (br d, 12.7)	1.63 (m)
	1.12 (m)	1.35 (m)	0.92 (qd, 13.0, 3.2)	1.20 (m)
6	1.88 (m)	4.34 (br s)	1.7 (m)	2.08 (m)
	1.85 (m)		1.23 (m)	1.36 (m)
7	1.62 (m)	2.63 (br d, 19.3)	1.87 (dt, 13.0, 4.0)	2.01 (m)
	1.90 (m)	2.39 (d, 19.3)	1.72 (m)	1.70 (m)
8			3.61 (dt, 11.3, 5.2)	4.92 (br s)
8a			2.95 (br t, 5.3)	3.03 (br t, 4.8)
CH_3	1.35 (d, 6.2)	1.38 (d, 6.2)	1.31 (d, 6.2)	1.32 (d, 6.2)
Ac				2.05 (s, 3H)

Table 2. ^{13}C NMR Data for Compounds **1**−**3**

C no.	1	2	3
1	174.7 (s)	171.9	174.9 (s)
3	76.5 (d)	76.8	73.4 (d)
4	37.5 (t)	37.5	36.5 (t)
4a	32.9 (d)	26.4	31.9 (d)
5	29.5 (t)	36.9	30.9 (t)
6	29.0 (t)	63.8	23.4 (t)
7	20.9 (t)	35.8	32.8 (t)
8	171.8 (s)	171.5	70.1 (d)
8a	96.8 (s)	96.5	44.2 (d)
CH_3	21.7 (q)	21.7	20.6 (q)

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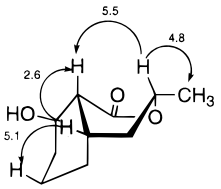
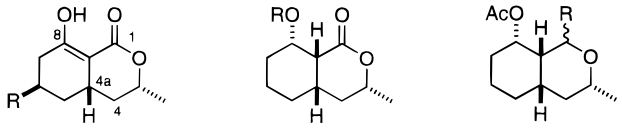


Figure 1. Difference NOE experiments on **3** (numbers on the arrows indicate the % NOE enhancements).



1 R = H
2 R = OH

3 R = H
4 R = Ac

5 R = α-OAc
6 R = β-OAc

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on Bruker DRX-500 and DRX-250 spectrometers. ^{1}H NMR spectra were recorded at 500 MHz, and the ^{13}C NMR spectra were recorded at 67.5 MHz. All of the chemical shifts were recorded with respect to the deuterated solvent shift. The IR spectrum was recorded on a Perkin−Elmer 1310 spectrometer. The optical rotation was recorded on a Perkin−Elmer 241 MC polarimeter using a 1-mL cell. The melting points were recorded on a Mel-TempII melting apparatus. The MS were provided by the Montana State Mass Spectrometer facility at Montana State University. All solvents used were spectral grade.

Extraction and Isolation. The isolation of this fungus has been described previously.[1] The identification of this isolate as *Botrytis* sp. was confirmed by fatty acid analysis through Microbial ID Inc. (ID 2377). *Botrytis* sp. was grown in mycological broth (2 × 2 L) in still culture. After 21 days, the mycelia was filtered off and the broth extracted with CH_2Cl_2 (3 X with 1 L of CH_2Cl_2) to give 0.3076 g of a brown oil. This crude extract was fractionated on LH-20 ($CHCl_3$−MeOH 1:1). The fractions that showed antibiotic activity against *S. aureus* were further fractionated by CCCC using hexane−EtOAc−MeOH−H_2O (50:70:80:65) to give three major bioactive fractions.

Ramulosin (1): white crystals; mp 115−117 °C, $[\alpha]^{23}_D$ +14.5° (*c* 0.5 MeOH), which exhibited comparable spectral (^{1}H NMR) data to published values.[2]

6-Hydroxyramulosin (2): white solid; mp 122−125 °C, $[\alpha]^{23}_D$ +48.9° (*c* 0.47 MeOH), which exhibited comparable spectral (^{1}H NMR) data to published values.[2]

Dihydroramulosin (3): white solid; mp 142−144 °C, $[\alpha]^{23}_D$ +18.6° (*c* 0.59 MeOH); ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS (70 eV) *m*/*z* 184 (1), 169 (2), 166 (3), 156 (71), 113 (100), 95 (50), 80 (54); HRCIMS *m*/*z* 185.1174 (calcd for $C_{10}H_{17}O_3$, 185.1178).

Table 3. ^{1}H NMR Data for **5**, and **6**, Selected Peaks

C no.	5	6
1	6.15 (d, 3.0)	5.54 (d, 8.5)
3	3.91 (m)	3.68 (pd, 6.2, 4.2)
8	4.61 (td, 10.7, 4.6)	4.72 (dt, 10.2, 4.6)
CH3	1.12 (d, 6.2)	1.18 (d, 6.2)
OAc's	2.03, 1.97	2.05, 1.98

Acetylation of 3. Compound **3** (3 mg) was dissolved in Ac_2O (1.0 mL) and pyridine (1.0 mL) and the solution stirred. After 24 h the volatiles were removed in vacuo and the acetate purified by flash Si gel chromatography, giving acetoxydihydroramulosin **(4)**: oil; ^{1}H NMR data, see Table 1; EIMS (70 eV) *m*/*z* M^+ not observed, 166 [M^+ − AcOH] (4), 80 (100), 79 (44).

Reduction and Acetylation of Ramulosin. Ramulosin (5 mg) was dissolved in 3 mL of MeOH, $NaBH_4$ (5 mg) was added, and the reaction was stirred for 10 min at 20 °C. The MeOH was removed from the reaction solution in vacuo and H_2O (3 mL) and CH_2Cl_2 (3 mL) added. The organic layer was separated, dried (Na_2SO_4), and the crude products acetylated by placing them in Ac_2O (1.0 mL) and pyridine (1.0 mL). After 24 h, the volatiles were removed in vacuo and the diacetates separated by HPLC on Si gel (Rainin), to give the less polar diacetate (**5**): (^{1}H NMR data, see Table 3) and the more polar diacetate (**6**): (^{1}H NMR data, see Table 3).

Reduction of Ramulosin at 0 °C. Ramulosin (5 mg) was dissolved in 3 mL of MeOH, $NaBH_4$ (5 mg) was added, and the reaction was stirred for 5 min at 0 °C. The MeOH was removed from the reaction solution in vacuo, and H_2O (3 mL) and CH_2Cl_2 (3 mL) were added. The organic layer was separated and dried (Na_2SO_4). The major product was purified by HPLC on Si gel (Rainin) to give a compound that exhibited a ^{1}H NMR spectrum identical to that of dihydroramulosin (**3**).

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