Pseudomonic acid derivatives from a marine bacterium

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Abstract. The sponge *Darwinella rosacea* was found to harbor a host of marine microorganisms. One of these bacteria was identified as a marine *Alteromonas* species. The organic extract of this bacterium was antimicrobial, and the activity was traced to two novel pseudomonic acid derivatives **1** and **7**. The structures of these compounds were elucidated by interpretation of the spectral data and extensive use of 2-dimensional NMR techniques and NOE studies on the parent compounds and the acetate derivatives **2** and **8**.
Key words. Pseudomonic acid derivatives; marine bacterium; antimicrobial activity; *Darwinella rosacea*; *Alteromonas*.

Marine microorganisms, including bacteria, cyanobacteria, dinoflagellates, fungi, and diatoms, represent a virtually untapped resource in the search for new biologically active metabolites. Many of the commonly prescribed antibiotics, including the penicillins, cephalosporins, and tetracyclines [1], are the metabolic products of terrestrial microorganisms. Many other compounds such as calicheamicin [2] and esperamicin [3] exhibit potent antineoplastic activity. Marine microorganisms should be no less adept at producing compounds with potentially useful biological activity.

We initiated a research program to examine culturable microorganisms isolated from marine sponges as potential sources of new pharmacologically active compounds. Sponges have been shown to produce a wide variety of biologically interesting compounds [4]. We have found their bacterial flora to be an equally rich source of new compounds. To date we have cultured over 100 organisms isolated from a variety of sponges. More than 50% of the extracts from these organisms have shown antimicrobial, cytotoxic or genotoxic activities. We describe here the antimicrobial components isolated from the liquid culture of one of these bacteria.

The rose colored sponge, *Darwinella rosacea* was collected in Bermuda. A small portion of this sponge was placed on marine agar and the microbial flora was allowed to grow. The microorganisms were repeatedly transferred to new plates until clean cultures were obtained. These cultures were grown in small liquid cultures (100 ml) and the methanol extracts tested for antimicrobial activity against a variety of bacteria. The most antimicrobial culture was a strictly marine, pink colored facultative anaerobe identified as an *Alteromonas* species by Microbial ID, Inc. This bacterium was grown in a large culture (36 l) and the antimicrobial components were isolated.

The *Staphylococcus aureus* toxic components of the pink bacterium isolated from *D. rosacea* were purified by a combination of size exclusion, flash silica gel and centrifugal countercurrent chromatographies. The more po-

Table 1. ^{1}H NMR Data of compounds **1**, **7**, **2** and **8** (chem. shift, mult, coupling const.)

Proton	**1**	**7**	**2**	**8**
H-2	6.02, bs	5.76, bs	5.84, bs	5.72, bs
H-4	4.31, bs	2.62 and 2.40*	5.29, bs	2.20*
H-5	3.83, m	3.71, dt, 7.5, 2.6	3.99, dd, 9.7, 2.4	3.89, dt, 9.4, 4.0
H-6	3.68, bd, 9.6	3.44, dd, 7.5, 2.4	5.01, dd, 9.7, 3.7	4.80, m
H-7	3.96, bs	3.89, bt	5.29, bs	5.24, bt, 2.7
H-8	1.82*	1.90*	1.90*	1.90*
H-9	2.20*	2.20*	2.20*	2.20*
H-10	5.46, dt, 15.6, 5.8	5.49, dt, 16.0, 4.5	5.46, m	5.44, m
H-11	5.40, dd, 15.6, 7.8	5.41, dd, 16.0, 6.0	5.46, m	5.44, m
H-12	2.10*	2.10*	2.10*	2.10*
H-13	3.52, m	3.54, m	4.82, m, 6.2	4.80, m
H-14	1.16, d, 6.4	1.16, d, 6.4	1.15, d, 6.2	1.14, d, 6.5
H-15	0.99, d, 7.3	0.99, d, 7.2	1.00, d, 7.3	0.99, d, 7.2
H-16	3.52, m	3.51, bd, 11.5	3.68, bd, 11.5	3.61, bd, 11.5
	3.83, m	3.80, dd, 11.5, 2.5	3.75, dd, 11.5, 2.5	3.78, dd, 11.5, 3.6
H-17	2.15, bs	2.21, bs	2.21, bs	2.20, bs
H-1′	4.12, m	4.07, t, 6.6	4.08, t, 6.6	4.07, t, 6.6
H-1″	6.50, bd	6.48, bd	6.35, bd	6.36, bd
H-2″	4.28, m	4.26, m, 6.4	4.27, m, 6.3	4.27, m
H-3″	2.52 and 1.52*	2.50 and 1.52*	2.50 and 1.50*	2.60 and 1.50*
H-4″	1.90*	1.90*	1.90*	1.90*
H-5″	3.32, bt	3.34, bt, 5.8	3.35, bt	3.35, bt
H-6″	6.13, bs	5.76, bs	5.74, bs	5.77, bs

* estimated from the 2-d COSY experiments.

lar component **1** (31 mg from a 36-l culture) gave a molecular ion at 583 (M + 1) in the FAB mass spectrum. A high resolution spectrum run on this ion established the molecular formula of **1** as $C_{30}H_{50}O_9N_2$. Acetylation of **1** gave the tetraacetate **2**. The IR spectrum of **1** exhibited OH stretch and C=O stretch at 3350 and 1712 cm^{-1}, respectively. The 500 MHz proton (table 1), carbon and 2-dimensional XHCORR spectra (table 2) on compound **1** established the following: three carboxylic acid derivatives, a trisubstituted double bond of the type $O-CO-CH=C-CH_3$, a disubstituted double bond $CH_2-CH=CH-CH$, seven carbons attached to oxygen, two aliphatic carbons attached to nitrogen, two methines, nine methylenes and three methyls. Even at 500 MHz the proton region from 3.1–4.2 ppm was hard to fully interpret due to a large number of overlapping and coupled peaks. The tetraacetate, **2**, was sufficiently resolved and could be analyzed by decoupling and 2-D COSY spectra to assign all protons on carbons 2 to 17. The second spin system indicated by these spectra was a cycloornithyl moiety. In order to assign all of the proton and carbon resonances for this, α-N-acetyl-anhydroornithine, **3**, was synthesized. L-ornithine was dehydrated using the procedure of Blade-Font[5] and then acetylated with acetic anhydride. At this point all carbons and protons could be assigned within these two fragments. The rest of the carbon resonances were methylene and could be placed between these two end fragments. The relative configuration of the tetrahydropyran ring substituents could be assigned by consideration of the proton-proton coupling constants around the ring. Comparison of this proposed structure for compound **1** with that of the known antibiotic, pseudomonic acid C (**4**), indicated structural similarities.

Table 2. ^{13}C NMR, DEPT and XHCORR data on compound **1**

Carbon No.	Chem. shift	Mult	Attached protons
1	168.6	s	
2	116.1	d	6.02
3	161.2	s	
4	74.3	d	4.31
5	77.5	d	3.83
6	65.7	d	3.68
7	71.8	d	3.96
8	43.9	d	
9	33.4	t	
10	129.8	d	5.46
11	135.7	d	5.40
12	45.3	d	
13	72.1	d	3.52
14	20.3	q	1.16
15	16.7	q	0.99
16	65.9	t	3.82, 3.52
17	16.3	q	2.15
1′	64.8	t	4.12
2′	28.8	t	
3′	29.7	t	
4′	30.1	t	
5′	30.0	t	
6′	27.0	t	
7′	36.9	t	
8′	176.0	s	
2″	50.7	d	4.28
3″	26.7	t	
4″	22.3	t	
5″	42.8	t	3.32
7″	173.0	s	

Pseudomonic acid C was isolated from the terrestrial bacterium *Pseudomonas fluorescens*[6]. Comparison of spectroscopic data of **1** and pseudomonic acid C showed three major differences: the additional hydroxyl substituent at C4 in **1**, an unbranched eight carbon chain instead of the nine carbon chain and a terminal amide portion containing an anhydroornithyl group. Hydrolysis of the ester function of **1** with NaOH/MeOH followed by acetylation gave a neutral fragment, **5**, which proved to be anhydroornithine, α-N-acylated with 8-acetoxy octanoic acid. The major acids formed during the described hydrolysis could be purified by HPLC. EIMS on the major isomer, **6**, did not give a parent ion. CIMS of **6** with methane as the gas gave a M + 1 ion and with ammonia gave the expected M + 18 peak and indicated a molecular formula of $C_{23}H_{34}O_{10}$. The proton NMR of **6** lacked the olefinic proton and vinylic methyl group, and contained a methyl singlet at δ1.44 ppm and an AB pattern at δ2.78 and δ2.45 ppm. This product formed a triacetate. Consideration of these data and inspection of the 500 MHz proton spectrum of **6** indicated that the product was bicyclic and had undergone a 1,4-addition of the 6-hydroxy group to the unsaturated carbonyl system. When the proton spectrum was recorded in benzene-d_6 (500 MHz) all protons could be sufficiently resolved for unequivocal assignment based on decoupling data.

NOEDS experiments indicated the relative stereochemistry around the five-membered ring. Nuclear Overhauser enhancements were seen between the proton at C4 (δ5.89 ppm) and the methylene at C2 (δ2.71 and δ2.92 ppm, 5%), and between the proton at C4 and the C6 methine (δ4.15 ppm, 5%) indicating a mutually cis arrangement. A large enhancement was also seen between the C5 methine (δ4.08 ppm) and the C3 methyl group (δ1.59 ppm, 12%). These studies correlated the relative C4 stereochemistry to that of the six-membered ring, and allowed the stereochemical assignment of the C4 hydroxyl in the parent compound. Comparison of the proton and carbon chemical shifts in compound **1** to those of pseudomonic acid C **4** indicated the same relative stereochemistries at C12 and C13.

The second antimicrobial compound, **7**, isolated from the marine bacterium gave a molecular ion at 567 (M + 1) in the FABMS. High resolution studies on this ion indicated a molecular formula of $C_{30}H_{50}O_8N_2$. This compound formed a triacetate **8**. Consideration of the formula for the parent compound and the acetylation data indicated one less hydroxyl in **7** than in **1**. The obvious difference in the proton spectrum was the loss of the broad singlet at δ4.31 ppm which had been assigned to the C4 methine in **1**. A loss of one of the oxygenated carbons was also evident from the carbon spectrum.

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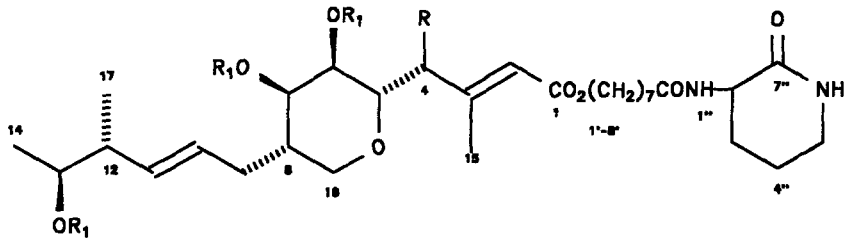

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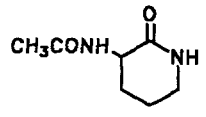
7 R=H R_1=H
8 R=H R_1=Ac



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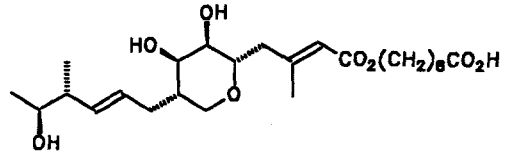

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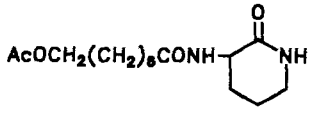

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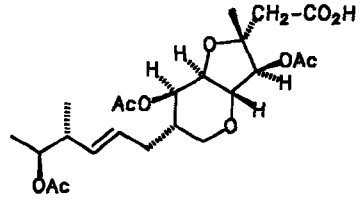

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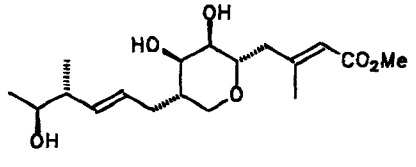

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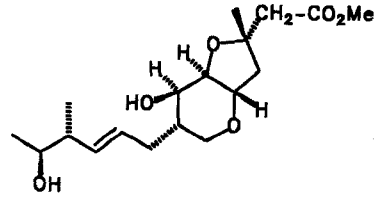

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Compound **7** must then be the 4-deoxy compound of **1**. Hydrolysis of **7** under basic conditions followed by acetylation gave a neutral fragment identical to **5** and a mixture of acids. The methyl esters of the acids could be separated by HPLC. Consideration of the mass spectral and NMR spectral data on these acids lead to the structure **9** and the cyclized product **10**.

These two pseudomonic acid derivatives described here are some of the first antibiotic compounds reported from a marine bacterium. Although they are similar to compounds isolated from terrestrial sources they contain the unusual anhydroornithyl end group. These compounds show potent antimicrobial activity against *S. aureus*, comparable to commonly used antibiotics. We compared the antibacterial activity of our compounds with the activities of tetracycline (30 μg/disk), penicillin G (10 units/disk), and streptomycin (10 μg/disk), provided by DIFCO as sensitivity test disks. Using standard disk assays we compared zones of inhibition induced by these antibiotics and our compounds. At 40 μg/disk, both **1** and **7** produced zones of 26 mm, compared to a zone of 28 mm induced by tetracycline. At these rates of applications, all three compounds were tested at $\approx 6.8 \times 10^{-2}$ μM. At an application concentration of 1.72×10^{-2} μM, both **1** and **7** induced zones of 15 mm, compared with a 13 mm zone for streptomycin at the same test concentration. A 10-unit test disk of penicillin G induced a zone of 24 mm.

Materials and methods

Instrumentation and general procedures. NMR spectra were recorded on a Bruker AC-300 (300 MHz for ^1H and 75.5 MHz for ^{13}C) and a Bruker AM-500 (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometers. Spectra were obtained in $CDCl_3$ unless otherwise noted and reported as ppm from TMS. For ^1H NMR spectra, the $CHCl_3$ resonance was set at 7.24 ppm and the ^{13}C NMR resonance set at 77.0 ppm. The reported multiplicities for the ^{13}C NMR spectra are from DEPT experiments. IR spectra were run on a Perkin-Elmer 1310 spectrophotometer in $CHCl_3$. UV spectra were obtained on a Cary 14 UV-VIS spectrophotometer. Low resolution EIMS were recorded on a VG Instruments MM16F spectrometer. High resolution EIMS and FABMS were run on a VG 7070EHF spectrometer. Optical rotations were measured on a Perkin-Elmer 241MC polarimeter. HPLC was carried out with a Perkin-Elmer Series 3 liquid chromatograph. The centrifugal countercurrent chromatography was manufactured by P.C. Inc. Chromatographic separations utilized either the ISCO V-4 or the Perkin-Elmer LC-55B variable wavelength detectors.

Isolation and growth of the bacterium. Darwinella rosacea was collected in Harrington Sound, Bermuda, at a depth of -1 m in the summer of 1986. The sponge was returned to the laboratory where a small piece of tissue was excised and placed on DIFCO marine agar. The resulting microorganisms were allowed to grow for three days, at which time they were carefully streaked for isolation using standard methodology. Once pure cultures were obtained (6 cultures isolated), the cultures were grown up in small cultures (125 ml) in DIFCO marine broth for about 2 weeks. After that time the cultures were freeze-dried. This dried material was extracted with MeOH, the MeOH removed and the residue partitioned between water and CH_2Cl_2. The water and CH_2Cl_2 extracts were tested for antibiotic activity against several common pathogens using the standard disc assay. The CH_2Cl_2 extract of the bacterium, *Alteromonas* sp. was found to be the most antimicrobial. This bacterium was grown in 12 3-l batches.

Extraction and compound isolation. The bacterium from the above large culture was killed with CH_2Cl_2 after 3 weeks. The CH_2Cl_2 was removed and the culture processed as described above. The CH_2Cl_2 extract (2.59 g of a red oil) was chromatographed on LH-20 with 1 : 1 MeOH/$CHCl_3$. Approximately 10-ml fractions were taken in test tubes and each fraction tested against *Staphylococcus aureus* in the standard disk assay. The active fractions (tubes 8–11, 270 mg) were combined and rechromatographed on LH-20 with MeOH. Each fraction was again tested for antimicrobial activity. The active fractions were combined (tubes 5–11, 233 mg) and fractionated on flash silica gel using a gradient of CH_2Cl_2 with increasing MeOH. The antimicrobial fractions eluted with 25% CH_2Cl_2/MeOH. The active material was combined (30.9 mg) and subjected to countercurrent chromatography with 25 : 34 : 20 $CHCl_3$/MeOH/H_2O, mobile phase-lower to give **1** as an oil (20.8 mg) and the less polar **7** as an oil (6.9 mg).

1: oil $[\alpha]_D - 1.8$ (c 0.003, MeOH); UV (MeOH) λ_{max} 220 nm (ε 14000); IR 3350, 2930, 2865, 1712, 1650, 1528, 1448, 1215, 1165, 1110, 1042 cm^{-1} (NaCl plate); ^1H NMR (500 MHz, see table 1); ^{13}C NMR (75.5 MHz, see table 2); HRFABMS 583 (M + 1) 583.3621, $C_{30}H_{51}O_9N_2$ requires 583.3594.

Acetylation of **1.** Compound **1** (10 mg) was dissolved in 500 µl of pyridine and 500 µl of acetic anhydride and stirred for 24 h. After that time the solvents were removed under vacuum. The acetate **2**, was an oil (11 mg). ^1H NMR (see table 1); EIMS 750(1), 690(2), 576(1), 396(7), 257(18), 175(19), 115(49), 43(100).

Preparation of α-N-acetyl-anhydroornithine **(3).** L-ornithine (2.0 g) was placed into toluene (50 ml) and silica gel (6.0 g) added. The mixture was refluxed for 3 hours with removal of the water by a Dean-Stark trap. The mixture was cooled the solid filtered and the solid washed with 9 : 1 CH_2Cl_2/MeOH. Evaporation of the solvents yielded a gummy oil (2.2 g). This oil was treated with Et_2O to dissolve the lactam, filtered and the solvent removed to give a white solid (1.8 g). The crude lactam was acetylated with pyridine/acetic anhydride to give **3** as a white solid (1.9 g).

3: MP 179–180 C; IR 3300, 2955, 1652, 1548, 1109, 972, 674 cm^{-1} (NaCl plate); ^1H NMR (300 MHz, MeOH-d4) 1.95 (s, 3H), 3.30 (bt, 2H), 4.25 (m, 1H), 7.55 (bs, NH), 8.20 (bs, NH); ^{13}C NMR (75.5 MHz, 1 : 1 $CDCl_3$/MeOH-d4) 21.7 (t), 22.7 (q), 28.1 (t), 42.2 (t), 50.3 (d), 172.2 (s), 172.4 (s); EIMS 156(100), 114(98), 113(95), 100(85), 98(90), 85(85), 69(98), 57(95), 43(85).

Hydrolysis of **1.** **1** (4.5 mg) was dissolved in MeOH (1 ml), and 3 drops of 0.1 M KOH was added. The mixture was stirred for 24 h. After that time the MeOH was removed, the residue dissolved in pyridine (500 µl) and acetic anhydride (500 µl) and the mixture stirred. After 24 h the volatiles were removed and the residue dissolved in 0.1 M KOH/CH_2Cl_2. The organic layer was separated, dried over Na_2SO_4 and the solvent removed to give **5** (1.5 mg) as an oil. The water layer from above was acidified with 0.1 M HCl and extracted twice with CH_2Cl_2. The organic layers were combined, dried and evaporated. HPLC of the oil on an analytical Partisil PXS 5/25 column with 50 : 50 ETOAc/hexane gave **6** (1.1 mg) as an oil.

5: IR 3300, 2930, 2855, 1738, 1642, 1240, 1060 cm^{-1} (NaCl plate); ^1H NMR (300 MHz) 2.01 (s, 3H), 3.28 (bt, 2H), 4.01 (t, 2H, J = 6.8 Hz), 4.22 (m, 1H); EIMS 298(58), 239(28), 202(24), 156(93), 113(88), 43(100).

6: ^1H NMR (500 MHz, Bz-d6). 0.89 (d, 3H, J = 6.9 Hz), 1.05 (d, 3H, J = 6.2 Hz), 1.59 (s, 3H), 1.70 (s, 3H), 1.71 (s, 3H), 2.02 (s, 3H), 2.71 (d, 1H, J = 16.2 Hz), 2.92 (d, 1H, J = 16.2 Hz), 3.52 (bd, 1H, J = 12.2 Hz), 3.60 (dd, 1H, J = 12.2, 2.9 Hz), 4.08 (t, 1H, J = 10.0 Hz), 4.15

(dd, 1H, J = 10.4, 2.3 Hz), 4.95 (m, 1H, J = 6.0 Hz), 5.24 (dt, 1H, J = 15.1, 7.5 Hz), 5.47 (bt, 1H), 5.50 (dd, 1H, J = 15.1, 6.6 Hz), 5.98 (d, 1H, J = 8.9 Hz); CIMS(CH_4) 471(M + 1), CIMS(NH_3) 488(M + 18); EIMS 427(M − 43)(10), 411(15), 367(37), 308(42), 133(80), 95(100), 55(95).

7: oil $[\alpha]_D$ − 3.1 (c 0.005, MeOH); UV(MeOH) λ_{max} 220 nm (ε 14000); IR 3300, 2930, 2860, 1715, 1650, 1542, 1452, 1222, 1150, 1105, 1048 cm^{-1} (NaCl plate); ^1H NMR (500 MHz, see table 1); ^{13}C NMR (75.5 MHz) 16.7 (q), 19.2 (q), 20.4 (q), 21.0 (t), 25.4 (t), 25.9 (t), 27.2 (t), 28.4 (t), 28.8 (t), 28.9 (t), 32.3 (t), 36.5 (t), 41.7 (2C, t), 43.0 (t), 44.7 (d), 50.4 (d), 63.7 (t), 64.8 (t), 68.8 (d), 70.3 (d), 71.1 (d), 74.8 (d), 117.4 (d), 129.4 (d), 134.3 (d), 157.1 (s), 166.9 (s), 171.9 (s), 173.6 (s); HRFABMS 567 (M + 1) 567.3610, $C_{30}H_{51}O_8N_2$ requires 567.3645, 257.1838, $C_{13}H_{25}O_3N_2$ requires 257.1865.

Acetylation of **7.** Compound **7** (3 mg) was acetylated as above. The acetate **8**, was isolated as an oil (3.5 mg). ^1H NMR (300 MHz, see table 1), EIMS 692(1), 632(1), 512(1), 257(15), 156(15), 115(46), 43(100).

Hydrolysis of **7.** **7** (2.0 mg) was dissolved in MeOH (1 ml), and 3 drops of 0.1 M KOH was added. The mixture was stirred for 24 h, the MeOH removed and the residue dissolved in water /EtOAc. The organic layer was removed, dried and the solvent removed to give an oil. This oil was acetylated (Py/Ac_2O) to give a product identical to **5** isolated from above. The water layer from above was acidified and extracted with EtOAc (3 ×). These organic extracts were treated with an ethereal solution of CH_2N_2 and the resulting oil subjected to HPLC on Partisil PXS 5/25 analytical column to give the methyl esters **9** (0.6 mg) and **10** (0.3 mg) as oils.

9: ^1H NMR (300 MHz) 1.00 (d, 3H, J = 7.3 Hz), 1.18 (d, 3H, J = 6.8 Hz), 2.04 (bs, 3H), 2.64 (db, 1H, J = 15.3 Hz), 3.42–3.55 (m, 3H), 3.69 (s, 3H), 3.70 (m, 1H), 3.80 (dd, 1H, J = 12.0, 3.0 Hz), 3.92 (bs, 1H), 5.42 (dd, 1H, J = 15.3, 7.4 Hz), 5.49 (dd, 1H, J = 15.3, 6.7 Hz), 5.70 (bs, 1H).

10: ^1H NMR (300 MHz) 0.97 (d, 3H, J = 7.0 Hz), 1.14 (d, 3H, J = 6.5 Hz), 1.37 (s, 3H), 2.65 (s, 2H), 3.50 (m, 2H), 3.64 (m, 2H), 3.66 (s, 3H), 3.82 (m, 1H), 4.11 (bs, 1H), 5.42 (m, 2H); EIMS 342(10), 324(22), 298(95), 211(65), 172(68), 110(95), 88(93), 43(100).

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Identification and phytotoxicity of 3-nitropropanoic acid produced in vitro by *Melanconis thelebola*

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Abstract. A phytotoxic metabolite was found to accumulate in culture of a strain of *Melanconis thelebola*, isolated from canker on red alder (*Alnus rubra* Bong.). The metabolite was identified by elemental analysis, ^1H- and ^{13}C-NMR and IR spectroscopy as 3-nitropropanoic acid.
Key words. Melanconis thelebola; phytotoxins; aliphatic carboxylic acid; 3-nitropropanoic acid.

Melanconis thelebola (Fr.) Sacc. is well known in Europe and North America as the causal agent of cankers on the trunk and branches of many alder species (*Alnus cordata, A. glutinosa, A. incana* and *A. rubra*)[1,2]. The fungus causes extensive dieback of the crown in both forest broadleafs and spontaneous broadleafs such as *A. rubra* Bong.; this species can be an important impediment to successful reforestation programmes, because of its rapid and unwanted growth. For this reason a study in British Columbia, Canada, has suggested that *M. thelebola* from cankerous *A. rubra* should be investigated for possible use in the biological control of broadleaf pests invading