Pseudotrienic Acids A and B, Two Bioactive Metabolites from *Pseudomonas* sp. MF381-IODS

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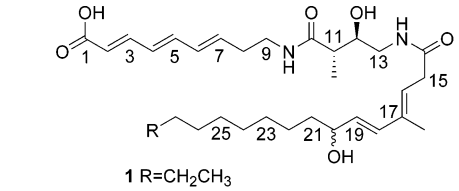
Received July 6, 2005

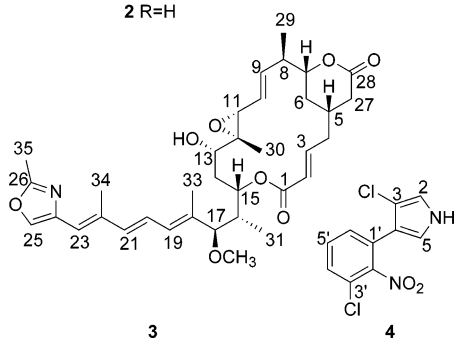
Bioassay-guided fractionation of the liquid culture broth of *Pseudomonas* sp. MF381-IODS yielded two new antimicrobial substances, identified as (2*E*,4*E*,6*E*)-9-[((2*S*,3*R*)-3-hydroxy-4-{[(3*E*,5*E*,7*RS*)-7-hydroxy-4-methylhexadeca-3,5-dienoyl]amino}-2-methylbutanoyl)amino]nona-2,4,6-trienoic acid and the tetradeca equivalent, named pseudotrienic acids A (**1**) and B (**2**), respectively. The compounds are prone to lactone formation, and their structures suggest them to be derived from ring opening of a macrolide. Pseudotrienic acids A and B inhibited growth of *Staphylococcus aureus* (MIC 70 μg/mL) and *Pseudomonas syringae* pv. *syringae* (MIC 70 μg/mL). Two known antimicrobial compounds, the polyketide 2,3-deepoxy-2,3-didehydrorhizoxin (**3**) and the tryptophan-derived pyrrolnitrin (**4**), were also identified.

Microorganisms such as fungi and bacteria cause considerable damage to crop production, both in the field and in storage. During the last decades, these microorganisms have been controlled by the use of various synthetic antimicrobial compounds. This method may, however, be a hazardous choice due to the development of resistance to these substances or due to accumulation of these compounds in the ecosystem. One way of replacing synthetic antimicrobial compounds in agriculture is by using biodegradable antimicrobial agents, which could include the use of whole-organism biocontrol agents as well as chemicals based on natural products. A potential source for new compounds active against pathogens is microorganisms. Although many organisms have been investigated, the vast majority of species have not been screened for antimicrobial secondary metabolites.[1] One group of bacteria that have been extensively investigated is the pseudomonads, which have been found to produce a variety of bioactive secondary metabolites with a wide range of activities.[2−4] The presence of diverse metabolic pathways in this group of bacteria makes it an interesting source for novel compounds, and some *Pseudomonas* strains have been shown to produce combinations of antimicrobial metabolites.[5,6] An interesting type of bioactive natural products is the structurally diverse group called polyenes.[7] To date, only a few polyenes produced by pseudomonads have been described, with 2,3-deepoxy-2,3-didehydrorhizoxin[8] (DDR), first isolated from the fungus *Rhizopus chinensis*,[9] and a 19-membered macrolide named FR252921 and its homologues as examples.[10] In contrast, different *Streptomyces* species have been the source for most polyenes described from bacteria.[7] Most of these are polyene or oxopolyene macrolides[11] including the important antifungal compounds nystatin and amphotericin B, but nonmacrolide antifungal polyenes such as the linearmycines[12] and oxazolomycines[13,14] have also been described. In the case of the migrastatin/dorrigocin group,[15,16] both the macrolides and their open chain equivalents have been isolated.

The rhizosphere bacterium *Pseudomonas* sp. isolate MF381-IODS (Pseudomonadaceae) is part of a collection with activity in vivo against the pathogenic fungi *Fusarium culmorum* and *Microdochium nivale,*[17] both of which cause diseases in wheat seedlings. The isolate MF381-IODS showed strong disease-suppressive effects in greenhouse trials, and whole-organism bioassays indicated that bioactive metabolites play a key role in its antimicrobial activity.[18,19] This investigation was aimed at studies of bioactive metabolites from the isolate, which resulted in the isolation and characterization of two new antimicrobial metabolites of polyene nature (**1** and **2**), as well as two previously described antifungal substances (**3** and **4**).



1 R=CH_2CH_3
2 R=H



3 **4**

Results and Discussion

When the cell-free supernatant of *Pseudomonas* sp. MF381-IODS liquid culture was fractionated by SPE, antimicrobial activity was found in the 95% CH_3CN extract. This fraction was subjected to gradient preparative HPLC, where several fractions showed strong inhibition of *F. culmorum*, *Staphylococcus aureus*, and *Drechslera sorokiniana*. The active fractions were subjected to two con-

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10.1021/np050243a CCC: $30.25
Published on Web 09/08/2005

Table 1. NMR Data (CD_3OD, 150/600 MHz) for Pseudotrienic Acids A (**1**) and B (**2**) (data for **1** and **2** are identical except where indicated)

					correlations to atoms in	
position	carbon δ (ppm)	proton δ (ppm)	mult.	J (Hz)	^{1}H−^{1}H COSY	HMBC
1	170.3					
2	121.9	5.85	d	$^3J_{H2,H3}$ = 15.4 Hz	3	1, 4
3	145.7	7.26	dd	$^3J_{H3,H4}$ = 11.1 Hz	2, 4	1, 4, 5
4	129.6	6.31	dd	$^3J_{H4,H5}$ = 14.8 Hz	3, 5	2, 3, 6
5	141.6	6.59	dd	$^3J_{H5,H6}$ = 11.4 Hz	4, 6	3, 6, 7
6	132.9	6.26	dd	$^3J_{H6,H7}$ = 16.0 Hz	5, 7	4, 8
7	137.1	5.94	m	$^3J_{H7,H8}$ = 11.4 Hz	6, 8	5, 8, 9
8	33.7	2.36	m	$^3J_{H8,H9}$ = 12 Hz	7, 9	5, 6, 7, 9
9	39.3	3.28	dt		8, NH	7, 8, 10
NH		7.79[a]	t		9	
10	177.3					
11	45.4	2.34	m	$^3J_{H11,H12}$ = 15.4 Hz	CH_3-11, 12	10, 12, 30
CH_3-11	14.7	1.14	d		11	10, 11, 12
12	73.1	3.70	dt	$^3J_{H12,H13a}$ = 6.0 Hz	11, 13ab	10, 11, 30
13a	44.4	3.16	ddd	$^3J_{H13a,H13b}$ = 14.4 Hz	12, 13b, NH	11, 12, 14
13b		3.41	ddd	$^3J_{H12,H13b}$ = 6.0 Hz	12, 13a, NH	14
NH		7.72[a]	d		13ab	
14	174.2					
15	36.3	3.12	d	$^3J_{H15,H16}$ = 7.0 Hz	16	14, 16, 17, 18
16	125.0	5.62	t		15	14, 15, 17, 18, 31
17	137.8					
CH_3-17	12.6	1.80	s			14, 16, 17, 18
18	135.0	6.25	d	$^3J_{H18,H19}$ = 15.8 Hz	19	16, 17, 20, 31
19	132.1	5.65	dd	$^3J_{H19,H20}$ = 13.8 Hz	18, 20	17, 20, 21
20	73.4	4.07	dt		19, 21ab	18, 19, 21, 22
21a	38.4	1.48		$^3J_{H21a,H21b}$ = 15 Hz	20	23
21b		1.55			20	
22a	26.3	1.32				
22b		1.38				23
23[b]	30.5	1.31				
24[b]	30.5	1.31				
25(23)[c]	30.5	1.31				
26(24)[c]	30.5	1.31				27(25)[c]
27(25)[c]	33.0	1.28				28(26)[c]
28(26)[c]	23.1	1.31				29(27)[c]
29(27)[c]	14.3	0.90	t		28(26)[c]	27(25),[c] 28(26)[c]

[a] Measured in DMSO-d_6. [b] Positions missing in **2**. [c] Number in parentheses refers to position in **2**.

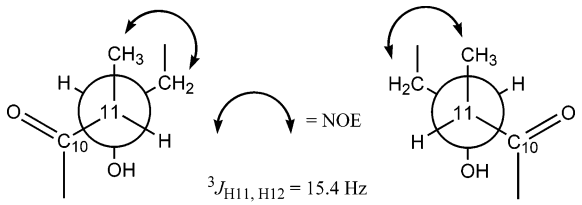
secutive isocratic chromatographic runs, which led to the isolation of compounds **1**−**4**.

ESIMS of compound **1** showed pseudomolecular ions at *m/z* 569.2 $[M + Na]^+$ and 545.2 $[M - H]^-$ corresponding to a molecular mass of 546.2. HRFABMS determined the $[M + Na]^+$ to be 569.3541, indicating an elemental composition of $C_{31}H_{50}O_6N_2Na$ (calcd 569.3567). NMR data (Table 1) indicated signals of nine protons attached to double bonds at δ_H 5.62−7.26. Signals for CH_2 at δ_H 1.28−1.55 and a terminal CH_3 at δ_H 0.90, corresponding to a linear alkyl chain with an integral equivalent to ca. 19 protons, were also present, as well as two additional CH_3 signals, a doublet at δ_H 1.14 and a singlet at δ_H 1.80. Two CH resonances at δ_H 3.70 and 4.07 with cross-peaks in the HSQC spectrum at δ_C 73.1 and 73.4, respectively, suggested two hydroxyl groups. By analysis of COSY and TOCSY spectra the spin systems H-2 to H-9, H-11 to H-13 including CH_3-11, H-15 to H-16, and H-18 to H-22 were assigned. The length of the alkyl chain could be determined as nine carbons by correlating ^{1}H NMR integrals and the elemental composition obtained by HRFABMS. A cross-peak at δ_C 170.3 (C-1) from H-2 and H-3 in the HMBC spectrum suggested a carboxylic acid. Treating a crude sample of **1** with a cation-exchange resin (H^+) moved the H-2 resonance upfield 0.05 ppm and the H-3 resonance downfield 0.2 ppm, which supported the presence of a carboxylic acid.

Further, analysis of the HMBC spectrum showed the presence of two additional carbonyls, one at δ_C 177.3 (C-10), giving diagnostic correlations to H-9 (δ_H 3.28), H-11 (δ_H 2.34), and CH_3-11 (δ_H 1.14), and the other at δ_C 174.2 (C-14), with diagnostic correlations to H-13a (δ_H 3.16), H-13b (δ_H 3.41), and H-15 (δ_H 3.12). Changing the solvent to DMSO-d_6 revealed two resonances of exchangeable protons at δ_H 7.79 (cross-peak in COSY to H-9) and δ_H 7.72 (cross-peak in COSY to H-13), suggesting the last two carbonyls to be part of two amide bonds. The CH_3-17 singlet gave cross-peaks in the HMBC spectrum to C-16, C-18, and the quaternary C-17, affording the missing connection of the spin systems. The combined MS and NMR data thus gave a complete skeleton for compound **1**, identifying it as 9-({3-hydroxy-4-[(7-hydroxy-4-methylhexadeca-3,5-dienoyl)amino]-2-methylbutanoyl}amino)nona-2,4,6-trienoic acid.

Compound **2** showed NMR data identical to **1** except for the alkyl tail, which had an integrated area in the ^{1}H NMR spectrum corresponding to seven carbons. HRFABMS exhibited a $[M + Na]^+$ peak at *m/z* 541.3208, indicating an elemental composition of $C_{29}H_{46}O_6N_2Na$ (calcd 541.3254). The production of **2** was consistent over the majority of all batches, but production of **1** varied significantly.

The double bonds at C-2, C-4, C-6, and C-18 were all determined to have *trans* configurations since couplings over the double bonds gave $^3J_{HH}$ values in the range 14.8−16.0 Hz. The C-16 double bond was also determined as *trans* by NOESY experiments, which showed NOE correlations between CH_3-17 and H-15, while H-16 showed correlations only with H-18 and H-15. The relative stereochemistry of C-11 and C-12 was proposed as *R*,*S* or *S*,*R* from the three-bond coupling constants and the NOESY



11*R*,12*S* 11*S*,12*R*

Figure 1. Relative configuration at C-11−C-12 in **1** and **2** was proposed to be *R*,*S* or *S*,*R* by NOE and $^3J_{H,H}$ values.

spectrum. The coupling constant between H-11 and H-12 was 15.4 Hz, and assuming a staggered conformation around the C-11−C-12 bond, this suggested an anti-periplanar relationship of the protons. There were strong NOEs between H-13ab and CH_3-11, which could be accounted for with C-13 and CH_3-11 in the *gauche* position (Figure 1).

In acidic environment compounds **1** and **2**, each with a hydroxy group at C-20, were found to form the lactones **1a** and **2a**, possibly by an intramolecular substitution of H_2O (Scheme 1). When 0.1% TFA was used in the final chromatographic step, the evaporated fractions originally containing **1** and **2** were found to contain 50% of the corresponding lactones. Treatment of **2** with TFA monitored by HPLC, MS, and NMR verified lactone formation of the material. When using nonacidic conditions in the last chromatographic step, the lactones were not isolated, nor were they detected by LC-ESIMS in the untreated supernatant. This suggested that in the investigated bacterial strain, **1** and **2** mainly exist in the C-20 hydroxy form and that the lactones isolated are secondary products. However, the structures of **1a** and **2a** may be precursors of **1** and **2**. Since no lactones were detected by LC-ESIMS directly in untreated supernatant, this may indicate that the lactones, if at all present, are opened nonenzymatically inside the bacterial cells or shortly after excretion to the medium. The lactones have the same bonding pattern as the previously isolated macrolactones FR252922 and FR252921,[10,20] which were isolated from a *Pseudomonas fluorescens* strain. The proposed transformation of the compounds in this group is similar to the transformations in the migrastatin/dorrigocin compound group.[15]

To arrive at the absolute configuration of **1** and **2**, the configuration of the chiral centers C-11, C-12, and C-20 needed to be determined. As NMR data for **1** and **2** were identical in all critical parts, the determination of absolute configuration was done exclusively on **2**. Determination of absolute configuration of **2** with the Mosher method[21] was not applicable due to inconsistent chemical shift differences. Instead, to determine the configuration at C-11 and C-12, compound **2** was hydrolyzed, releasing the C-10 to C-13 γ-amino acid moiety. This amino acid, 4-amino-3-hydroxy-2-methylbutanoic acid, is also present in the bistramide/bistratene group,[22−24] with a reported 2*S*,3*R* configuration for bistramides A[25] and C.[26] The crude hydrolysate of **2** was esterified with (*S*)-2-BuOH and racemic 2-BuOH to yield the (*S*)- and (*R*)-2-butyl esters of 4-amino-3-hydroxy-2-methylbutanoic acid, the latter ester being chromatographically equivalent to the (*S*)-2-butyl ester of the enantiomeric amino acid. Furthermore, during hydrolysis and/or esterification ca. 10% of each sample underwent epimerization at C-2 and/or C-3. Thus, the derivatization procedure led to compounds chromatographically equivalent (on a nonchiral column) to all possible diastereomers of (*S*)-2-butyl esters of the amino acid. The 2-butyl esters were derivatized for compatibility with GC-MS, and subsequent GC analysis separated all diastereomers. When compared with a hydrolyzed and (*S*)-2-BuOH-derivatized sample of bistratene A, the amino acid derived from **2** was found to behave chromatographically as the amino acid derived from bistratene A. Thus, the central amino acid in **1** and **2** has the same absolute configuration as in bistratene A, i.e., 2*S*,3*R*, which corresponds to 11*S*,12*R* in **1** and **2**.

Determination of the configuration at C-20 was carried out by ozonolysis of **2** followed by oxidative workup, resulting in a 2-hydroxynonanoic acid with C-2 originating from C-20 of **2**. The 2-hydroxycarboxylic acid was then analyzed as its 2-methoxy (*S*)-phenylethyl amide derivative on GC-MS, for which the elution order of diastereomers is known to be (*R*)-2-methoxy prior to the (*S*)-2-methoxy.[27] When compared with standards, both forms of C-20 were found. Compounds **1** and **2** were thus identified as (2*E*,4*E*,6*E*)-9-[((2*S*,3*R*)-3-hydroxy-4-{[(3*E*,5*E*,7*RS*)-7-hydroxy-4-methylhexadeca-3,5-dienoyl]amino}-2-methylbutanoyl)-amino]nona-2,4,6-trienoic acid and its tetradeca equivalent, respectively. The presence of both configurations at C-20 could be due to the fact that **1** and **2** are the products of conjugated ring opening of FR252922 and FR252921, as discussed above. Conjugate addition of H_2O at C-20 of the macrolide would result in ring opening of the lactone and a racemization at C-20 (Scheme 1).

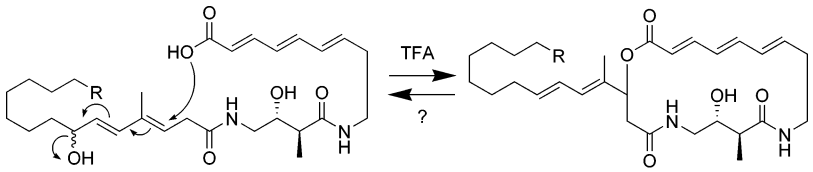
LC-ESIMS of the crude 95% CH_3CN SPE fraction showed diagnostic pseudomolecular ions of 2,3-deepoxy-2,3-didehydrorhizoxin (DDR, **3**) at *m/z* 632.3 $[M + Na]^+$, but MS analysis of the isolated compound gave a pseudomolecular ion at *m/z* 664.3 $[M + Na]^+$ almost exclusively, i.e., a mass difference of 32 amu compared to DDR. The NMR data of this compound, after MS analysis, corresponded well with literature values for DDR[9] except on two points: an extra CH_3 singlet at δ_H 3.71 with an HMBC cross-peak to the C-28 carbonyl at δ_C 173.9 and a chemical shift of the H-7 signal at δ_H 3.18 compared to δ_H 3.75 in DDR. The mass difference could then be attributed to the addition of CH_3OH. As sample preparation of the purified compound before NMR analysis included contact with CH_3OH, it is suggested that the six-membered lactone of DDR (**3**) opened by formation of a methyl ester. This strongly indicated that the DDR methyl ester was a sample-handling artifact. Compound **4** was identified as pyrrolnitrin by ESIMS, by comparison of NMR data with literature values, and by HPLC with a pyrrolnitrin standard.

Minimum inhibitory concentrations (MICs) of **1** and **2** were determined for organisms representing human and agricultural fungal pathogens as well as Gram-positive and Gram-negative bacteria. Compounds **1** and **2** showed activity directed against Gram-positive and Gram-negative bacteria and were found to inhibit growth of *Pseudomonas syringae* pv. *syringae* and *S. aureus*, both at a MIC of 70 μg/mL. They did not inhibit *Aspergillus fumigatus*, *Candida albicans*, *D. sorokiniana*, *F. culmorum*, *Fusarium oxysporum*, *Heterobasidion annosum*, *Microsporum canis*, or *Pseudomonas sevastanoi* at concentrations up to 100 μg/mL. The activity against *F. culmorum* and *D. sorokiniana* during the bioassay-guided fractionation was attributed to trace amounts of pyrrolnitrin and/or DDR in the pseudotrienic acid fractions.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR data were acquired on 600 and 400 MHz NMR spectrometers equipped with a 2.5-mm SEI microprobe ($^1H/^{13}C$) and a 5-mm QNP probe ($^1H/^{13}C/^{31}P/^{15}N$), respectively. All NMR experiments were recorded at 30 °C. Standard pulse sequences were used

Scheme 1. Lactone Formation of **1** and **2** under Acidic Conditions



for determination of ^{1}H and ^{13}C frequencies and connectivities. For complete structure elucidation, 1D ^{1}H NMR, COSY, TOCSY, NOESY, DEPT-HSQC, and HMBC were applied. Chemical shifts were determined relative to internal $CHCl_3$ (δ_C 77.23; δ_H 7.27), DMSO-d_5 (δ_H 2.50), or CH_3OH-d_3 (δ_C 49.15; δ_H 3.31). Positive and negative mode ESIMS were obtained on an ion-trap instrument with CH_3OH as solvent. LC-MS was done with the same mass spectrometer coupled to an analytical HPLC system. HRFABMS was performed on a four-sector tandem mass spectrometer with glycerol as matrix and PEG as internal standard. SPE was done with 1 or 10 g prepacked columns or columns packed in-house with bulk C_{18} material. Preparative HPLC was run at a flow of 10 mL/min with UV monitoring at 210 or 254 nm. Fractions were collected in polypropylene 2-mL square well plates. For the mobile phase CH_3CN of HPLC gradient grade and deionized filtered H_2O were used.

Isolate Origin and Identity. The psychrotrophic *Pseudomonas* sp. strain MF 381-IODS was isolated, as previously described,[19] from the roots of a field-grown oilseed rape specimen [*Brassica napus* ssp. *oleifera* (DC.) Metzger (Cruciferae)] collected in Lipperswil, Switzerland, in March 1997. The strain was identified as belonging to the genus *Pseudomonas*, RNA group I, by the German Collection of Microorganisms and Cell Cultures (DSMZ) by means of physiological tests, cellular fatty acid analysis, and partial sequencing of the 16S rDNA.

Production of Bacterial Cultures. Cultures of the isolate MF381-IODS were produced on Mineral Medium (MM) for *Pseudomonas*[28] modified by adding 125 mg of citric acid, 12.5 mg of $Na_2MoO_4 \times 2\ H_2O$, and 250 mg of trichloroacetic acid per 1000 mL of the medium, with 0.1% glycerol as the sole carbon source. The MM cultures were started by transferring 10 + 10 mL of 24 h old inocula, which were grown in half-strength vegetable peptone broth [15 g of VPB in 1000 mL of deionized H_2O] and in nutrient broth [8 g of NB in 1000 mL of deionized H_2O], respectively. The cultures were incubated on a rotary shaker (120 rpm) for 68−72 h at 20−25 °C. Cells were removed by centrifugation (11 000 rpm, 15 min, 4 °C), and cell-free supernatants were immediately fractionated by SPE or stored at −70 °C before being processed.

Sample Workup and Isolation Procedures. Five liters of cell-free supernatant was fractionated on a 150-g SPE column. The column was packed and activated with 500 mL of CH_3CN and equilibrated with 500 mL of H_2O before sample loading. Hydrophilic components were washed out with 500 mL of aqueous 5% CH_3CN followed by 500 mL of 20% CH_3CN in H_2O before the lipophilic fraction was eluted with 700 mL of aqueous 95% CH_3CN. Fractionation was done in three HPLC steps with in vitro bioassay verification of antimicrobial activity in between. The first run was a gradient from 20% to 100% CH_3CN in H_2O in 10 min with an 8 min hold at 100%. The second run was isocratic at aqueous 63% CH_3CN, yielding **3** (2.0 mg) and **4** (1.5 mg). The final isocratic step was aqueous 53% CH_3CN, which yielded **1** (1.7 mg) and **2** (14 mg). The column used in steps 1 and 3 was a Reprosil-pur C_{18} (100 × 20 mm with guard column 30 × 20 mm, 5 μm) and in step 2 a Kromasil C_{18} (150 × 21.2 mm, 5 μm). CH_3OH was used for sample transfer prior to spectroscopic analysis.

In Vitro Bioassay and MIC Determination. Antimicrobial fractions were identified during isolation using a previously developed protocol, based on inhibition of spore germination or cell growth in microtiter plates.[29,30] *Fusarium culmorum, Drechslera sorokiniana*, and *Staphylococcus aureus* were used as test organisms. Aliquots from the HPLC fractions were transferred into 96-well microtiter plates, and the solvent was evaporated. Spore suspensions (*F. culmorum* and *D. sorokiniana*) or cell suspensions (*S. aureus*), 100 μL at a concentration of 10^4 spores or cells/mL, were added to the wells, resulting in sample concentrations 200−400 times higher than in the culture supernatant. Spore/cell suspensions were used as positive controls, and sterile medium was used as negative control. The MIC values of **1** and **2** were determined using a microtiter plate bioassay with the organisms *Aspergillus fumigatus*, *Candida albicans*, *D. sorokiniana*, *F. culmorum*, *F. oxysporum*, *Heterobasidion annosum*, *Microsporum canis*, *Pseudomonas sevastanoi*, *P. syringae* pv. *syringae*, and *S. aureus*. All MIC tests were performed in triplicate and repeated once.

(2*E*,4*E*,6*E*)-9-[((2*S*,3*R*)-3-Hydroxy-4-{[(3*E*,5*E*,7*RS*)-7-hydroxy-4-methylhexadeca-3,5-dienoyl]amino}-2-methylbutanoyl)amino]nona-2,4,6-trienoic acid (pseudotrienic acid A, 1): colorless oil; ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m/z* 569.2 $[M + Na]^+$; ESIMS *m/z* 545.2 $[M - H]^-$; HRFABMS 569.3541 (calcd for $C_{31}H_{50}O_6N_2Na$, 569.3567).

(2*E*,4*E*,6*E*)-9-[((2*S*,3*R*)-3-Hydroxy-4-{[(3*E*,5*E*,7*RS*)-7-hydroxy-4-methyltetradeca-3,5-dienoyl]amino}-2-methylbutanoyl)amino]nona-2,4,6-trienoic acid (pseudotrienic acid B, 2): colorless oil; $[\alpha]^{20}_D$ +1.9° (*c* 0.4, MeOH); ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m/z* 541.4 $[M + Na]^+$; HRFABMS 541.3208 (calcd for $C_{29}H_{46}O_6N_2Na$, 541.3254).

Pseudotrienic acid A lactone (1a): ^{1}H NMR (CD_3OD, 600 MHz) δ 7.26 (1H, dd, H-3), 6.59 (1H, dd, H-5), 6.31 (1H, dd, H-4), 6.26 (1H, dd, H-6), 6.26 (1H, dd, H-19), 6.06 (1H, d, H-18), 5.94 (1H, m, H-7), 5.85 (1H, d, H-2), 5.66 (1H, dt, H-20), 4.42 (1H, dd, H-16), 3.70 (1H, dt, H-12), 3.41 (1H, ddd, H-13b), 3.28 (2H, dt, H-9), 3.16 (1H, ddd, H-13a), 2.43 (2H, dd, H-15), 2.36 (2H, m, H-8), 2.34 (1H, m, H-11), 2.10 (2H, m, H-21), 1.75 (3H, s, C*H*$_3$-17), 1.38 (1H, m, H-22b), 1.32 (1H, m, H-22a), 1.31 (10H, m, H-23 to H-26 and H-28), 1.28 (2H, m, H-27), 1.14 (3H, d, C*H*$_3$-11), 0.90 (3H, t, H-29); ^{13}C NMR (CD_3OD, 150 MHz) δ 177.3 (C, C-10), 174.5 (C, C-14), 170.3 (C, C-1), 145.7 (CH, C-3), 141.6 (CH, C-5), 137.3 (C, C-17), 137.1 (CH, C-7), 136.2 (CH, C-20), 132.9 (CH, C-6), 129.6 (CH, C-4), 127.1 (CH, C-19), 126.7 (CH, C-18), 121.9 (CH, C-2), 75.2 (CH, C-16), 73.1 (CH, C-12), 45.4 (CH, C-11), 44.4 (CH_2, C-13), 43.4 (CH_2, C-15), 39.3 (CH_2, C-9), 34.1 (CH_2, C-21), 33.7 (CH_2, C-8), 33.0 (CH_2, C-27), 30.5 (4CH_2, C-23 to C-26), 26.3 (CH_2, C-22), 23.1 (CH_2, C-28), 14.7 (CH_3, CH_3-11), 14.3 (CH_3, C-29), 12.4 (CH_3, CH_3-17); diagnostic HMBC connectivities, H-15→C-14; C*H*$_3$-17→C-16, 17, 18; ESIMS *m/z* 527.5 $[M - H]^-$.

Pseudotrienic acid B lactone (2a): ^{1}H NMR (CD_3OD, 600 MHz) δ 7.26 (1H, dd, H-3), 6.59 (1H, dd, H-5), 6.31 (1H, dd, H-4), 6.26 (1H, dd, H-6), 6.26 (1H, dd, H-19), 6.06 (1H, d, H-18), 5.94 (1H, m, H-7), 5.85 (1H, d, H-2), 5.66 (1H, dt, H-20), 4.42 (1H, dd, H-16), 3.70 (1H, dt, H-12), 3.41 (1H, ddd, H-13b), 3.28 (2H, dt, H-9), 3.16 (1H, ddd, H-13a), 2.43 (2H, dd, H-15), 2.36 (2H, m, H-8), 2.34 (1H, m, H-11), 2.10 (2H, m, H-21), 1.75 (3H, s, C*H*$_3$-17), 1.38 (1H, m, H-22b), 1.32 (1H, m, H-22a), 1.31 (6H, m, H-23, H-24 and H-26), 1.28 (2H, m, H-25), 1.14 (3H, d, C*H*$_3$-11), 0.90 (3H, t, H-27); ^{13}C NMR (CD_3OD, 150 MHz) δ 177.3 (C, C-10), 174.5 (C, C-14), 170.3 (C, C-1), 145.7 (CH, C-3), 141.6 (CH, C-5), 137.3 (C, C-17), 137.1 (CH, C-7), 136.2 (CH, C-20), 132.9 (CH, C-6), 129.6 (CH, C-4), 127.1 (CH, C-19), 126.7

(CH, C-18), 121.9 (CH, C-2), 75.2 (CH, C-16), 73.1 (CH, C-12), 45.4 (CH, C-11), 44.4 (CH_2, C-13), 43.4 (CH_2, C-15), 39.3 (CH_2, C-9), 34.1 (CH_2, C-21), 33.7 (CH_2, C-8), 33.0 (CH_2, C-27), 30.5 ($2CH_2$, C-23 and C-24), 26.3 (CH_2, C-22), 23.1 (CH_2, C-26), 14.7 (CH_3, CH_3-11), 14.3 (CH_3, C-27), 12.4 (CH_3, CH_3-17); diagnostic HMBC connectivities, H-15→C-14; C*H*$_3$-17→C-16, 17, 18; ESIMS *m/z* 499.5 $[M - H]^-$; ^1H NMR spectra of **2a** in CD_3CN-D_2O and experimental data for lactone formation of **2** to **2a** are included in the Supporting Information.

Configuration at C-11 and C-12 of 1 and 2. A sample of **2** (50 μg) in 0.5 mL of 6 M HCl was heated at 110 °C in an evacuated glass ampule for 20 h, after which the solvent was evaporated under a stream of N_2. The residue was dissolved in 0.5 mL of H_2O and evaporated under reduced pressure. A sample of 25 μg of bistratene A (Sigma-Aldrich, St Louis, MO) was treated in the same way. The hydrolysate of **2** was split in half and treated with 200 μL of (*S*)-2-BuOH−AcCl (10:1) and 2-BuOH−AcCl (10:1), respectively, and allowed to stand at 100 °C for 40 min, yielding the 2-butyl ester of the released amino acid. Each sample was evaporated under N_2 and heated at 100 °C in 200 μL of pentafluoropropionic anhydride for 40 min. After evaporation with N_2, the sample was dissolved in 100 μL of EtOAc and subjected to GC-MS analysis. The bistratene A sample was derivatized as above with (*S*)-2-BuOH. The samples were analyzed on a fused silica column (BP5; 0.25 μm, 30 m × 0.25 mm, SGE Ltd., Ringwood, Australia) with a temperature gradient (60−119 °C at 10 °C/min, 119−121 °C at 0.1 °C/min, and 121−240 °C at 10 °C/min). The injector was held at 240 °C and the GC-MS interface at 240 °C. Samples (1 μL) were injected in split mode (50:1), and He was used as a carrier gas at 1 mL/min. The (*R,S*)-2-butyl esters of the hydrolysate of **2** yielded peaks at t_R 19.05 and 19.21 min (1:1 ratio, major peaks) and t_R 18.60 and 18.80 min (1:1 ratio, minor peaks), all with identical mass spectra. The two main peaks correspond to the (*S*)-2-butyl ester and the (*R*)-2-butyl ester of the 4-amino-3-hydroxy-2-methylbutanoic acid. The (*R*)-2-butyl ester is chromatographically equivalent to the (*S*)-2-butyl ester of the enantiomeric amino acid. The two minor peaks represent diastereomeric esters formed by epimerization at C-2 and/or C-3 during the hydrolysis and/or esterification step. The hydrolysate of **2**, esterified with (*S*)-2-BuOH, showed two peaks, at t_R 18.79 (minor peak) and 19.06 min (major peak). Smaller peaks were also observed at t_R 18.60 and 19.19 min. Analysis of the hydrolyzed bistratene A sample resulted in two peaks, at t_R 18.79 (minor peak) and 19.07 min (major peak), as well as smaller peaks at t_R 18.60 and 19.20 min, all with mass spectra identical to the hydrolyzed **2**.

Configuration at C-20 of 1 and 2. Approximately 1.8 mg of **2** was dissolved in 2 mL of dry CH_3OH and treated with excess ozone for 10 min at −20 °C. The CH_3OH was evaporated, the residue was dissolved in 1 mL of glacial AcOH, and 0.2 mL of 30% H_2O_2 was added. The mixture was stirred for 18 h at 50 °C, after which the solvent was evaporated under reduced pressure. The residue was dissolved in 5 mL of H_2O, which was evaporated, a procedure repeated once. After ozonolysis no signals of olefinic protons were visible in the ^1H NMR spectrum.

One-tenth of the ozonized material was derivatized as previously described.[27] The sample was transferred to a screw cap test tube and dissolved in 1 mL of 1.3 M methanolic HCl and kept at 80 °C for 30 min. Water (1 mL) was added after cooling, the hydroxy acid methyl ester was extracted with 2 × 1 mL of diethyl ether, and the ether was subsequently evaporated. Diethyl ether (100 μL) was added, followed by 50 μL of trimethylsilyldiazomethane at 0 °C. After 5 min 8 μL of BF_3 etherate was added and the mixture allowed to stand for 15 min. The reaction was quenched with 50 μL of CH_3OH, and 2 mL of diethyl ether was added. The mixture was washed with 0.5 mL of aqueous 0.5 M HCl, 0.5 mL of saturated aqueous $NaHCO_3$, and 1 mL of H_2O, after which the organic phase was evaporated. To regain the free acid function, the material was heated in 1 mL of 0.1 M KOH in MeOH−H_2O (9:1) at 60 °C for 120 min, followed by concentration to about half the volume under N_2. After dilution with 1 mL of H_2O, the sample was washed with 2 mL of diethyl ether, acidified with 0.25 mL of 0.5 M HCl, extracted with 2 × 1 mL of diethyl ether, washed with 1 mL of H_2O, and dried. The methoxy acid was dissolved in 70 μL of benzene with 0.5 mg of Na_2CO_3, after which 5 μL of $SOCl_2$ was added, and the mixture was kept at 60 °C for 30 min. After evaporation, the residue was redissolved in 70 μL of benzene, and 5 μL of (*S*)-phenylethylamine was added. The mixture was allowed to stand at 25 °C for 30 min and then diluted with 2 mL of diethyl ether and washed with 0.5 mL of 0.5 M HCl and 0.5 mL of H_2O before evaporation. A standard of (±)-2-hydroxydodecanoic acid (Larodan Fine Chemicals, Malmö, Sweden) was derivatized in the same way. Methyl (±)-2-methoxynonanoate (synthesized as described below), functioning as 2-hydroxynonanoic acid standard, was derivatized as above, starting from the alkaline hydrolysis step.

The derivatized samples were analyzed by GC-MS with selective ion-monitoring (SIM) 100 ms/ion, on a fused silica column (BP5; 0.25 μm, 30 m × 0.25 mm, SGE Ltd., Ringwood, Australia) using a temperature gradient (120 °C for 5 min, 20 °C/min to 255 °C, 255 °C for 15 min). The injector was held at 255 °C and the GC-MS interface at 260 °C. Samples (1 μL) were injected in splitless mode, and He was used as a carrier gas at 1 mL/min. The diastereomers of the derivatized (±)-2-hydroxydodecanoic acid standard eluted at t_R 14.99 (*R*) and 15.36 min (*S*), respectively, both with diagnostic ions at *m/z* 333 $[M]^+$, 303, 301 (loss of CH_3OH), 193 (McLafferty), and 185. For the derivatized (±)-2-hydroxynonanoic acid standard, t_R were 12.61 (*R*) and 12.80 min (*S*), respectively, both with SIM on ions *m/z* 291 $[M]^+$, 261, 259 (loss of MeOH), 193 (McLafferty), and 143. The ozonolysis sample exhibited two peaks with equal intensity at t_R 12.61 and 12.79 min, respectively, with diagnostic ions in all parts comparable to the 2-OH nonanoic acid standard.

(±)-Methyl 2-methoxynonanoate.[31] Nonanoic acid (1.75 mL, 10 mmol), $SOCl_2$ (2.91 mL, 40 mmol), and 1 mL of $CHCl_3$ were stirred at 65 °C for 30 min. The mixture was evaporated under reduced pressure and the residue dissolved in 3 mL of toluene, which was subsequently evaporated. The nonanoic acid chloride was dissolved in 2.5 mL of $CHCl_3$, and 0.31 mL of Br_2 (6 mmol) was added. The mixture was stirred at 65 °C for 190 min when 300 μL (3 mmol) of cyclohexene was added to destroy excess Br_2. A part of the mixture was evaporated under a stream of N_2, and the resulting oily residue was transferred to a beaker with 45 mL of 0.1 M sodium methoxide in methanol. The mixture was stirred at 25 °C for 130 min and then neutralized with 6 M HCl. The racemic methyl 2-methoxynonanoate was extracted with 2 × 15 mL of $CHCl_3$ and then washed with 15 mL of H_2O before evaporation. The product was purified by gradient HPLC with 60% CH_3CN in H_2O for 10 min, then to 90% in 3 min with an 8 min hold at 90% (column Reprosil-pur C_{18} [100 × 20 mm with guard column 30 × 20 mm, 5 μm]). The identity and purity of the compounds were checked by GC-MS.

Acknowledgment. We are grateful to Mr. S. Gohil at the Department of Chemistry, SLU, for help in acquiring the HRFABMS data, Mr. C. Hedberg at the Department of Organic Chemistry, Uppsala University, for ozonolysis, and Dr. R. Hedman and Mr. S. Daugherty at Agrivir AB for providing the pyrrolnitrin standard and cooperation with HPLC method development. Agrivir AB, Uppsala, Sweden, also provided all microbial spore/cell suspensions. This work was supported by the Foundation for Strategic Environmental Research (MISTRA).

Supporting Information Available: NMR and MS data for DDR and pyrrolnitrin, NMR spectra for **1** (DQFCOSY, TOCSY, NOESY, DEPT-HSQC, and HMBC), and experimental data for and ^1H NMR spectra of lactone formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP050243A