Dihydromaltophilin; A Novel Fungicidal Tetramic Acid Containing Metabolite from *Streptomyces* sp.

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(Received for publication June 16, 1997)

A new tetramic acid containing metabolite, A90931a has been isolated from *Streptomyces* sp., along with a second factor (A90931b) recently described and known as maltophilin. The structures were determined from spectroscopic data of the isolates and their acetylated products. A90931a was spectroscopically identical to the previously described antibiotic TAN-883b whose structure was not reported. A90931a and A90931b exhibit fungicidal activity against the grape pathogen *Plasmopara viticola*. Due to its similarity to maltophilin, A90931a has been called dihydromaltophilin.

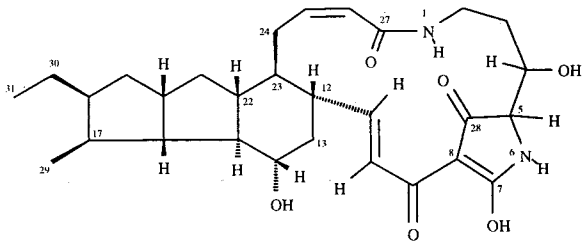
In the course of searching for novel fungicidal antibiotics, we have discovered two macrocyclic compounds A90931a (**1**) and b (**2**) containing a tetramic acid residue, and a tricyclic fused ring system, similar to the previously described discodermide[1]. A90931a appears to be identical spectroscopically to the molecule TAN-883b[2] whose structure has remained undetermined in the literature, and very similar to catacandin B[3], another compound whose structure remains undetermined. A90931b, which was present in smaller quantities, has recently been described arising from *Stenotrophomonas maltophilia*[4], and called maltophilin. In the present paper, we describe the isolation and structural characterization of A90931a which we have called dihydromaltophilin (Figure 1). Activity has been measured against *Plasmopara viticola*, the causal fungus of grape downy mildew, one of the most destructive diseases afflicting commercial viticulture worldwide.

Materials and Methods

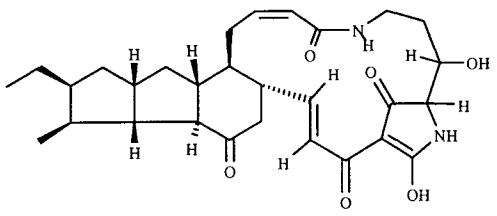
Producing Organism

The culture was obtained from a soil sample collected in Carmel, Indiana as part of a science project undertaken

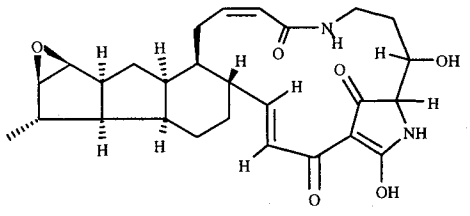
Fig. 1. Structures of A90931a, b and discodermide.



1: A90931a (Dihydromaltophilin)

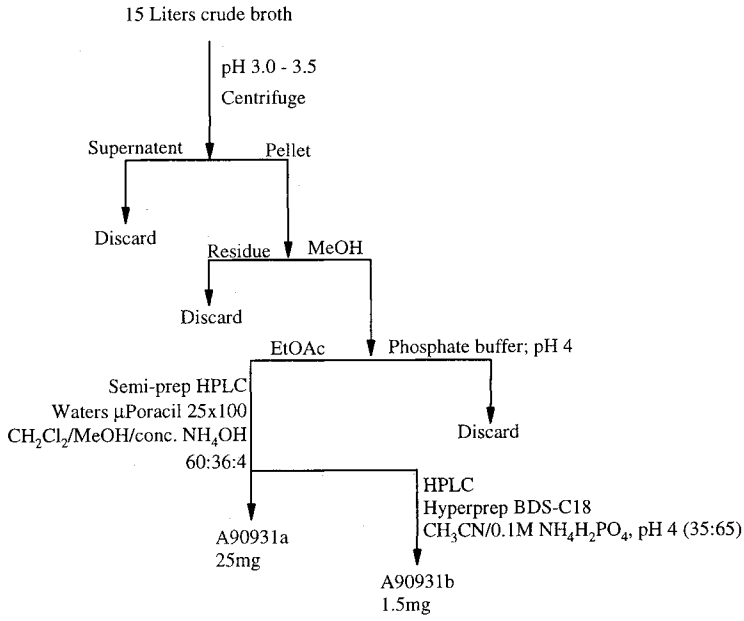


2: A90931b (Maltophilin)



3: Discodermide

Scheme 1. Isolation of active factors A90931a and A90931b



at Woodbrook Elementary School, and characterized at the American Type Culture Collection.

Fermentation

The culture was fermented for 72 hours in inoculum production medium (50 ml) containing tryptic soy broth (30 g), yeast extract (Difco, 3 g), magnesium sulfate (2 g), glucose (5 g), maltose (Difco, 4 g) per liter of deionized water. Incubation was maintained at 28~30°C with shaking at 150 rpm for 3 days. 10 ml of this inoculum was then added to 600 ml of production medium containing anhydrous D-glucose (12.5 g), blackstrap molasses (15 g), Bacto-peptone (Difco, 3.75 g), calcium carbonate (2 g) per liter of deionized water; and incubated for 5 days at 28~30°C with shaking at 150 rpm.

Isolation of Actives

The isolation procedure is summarized in Scheme 1. The isolation of compounds was followed by bioassay. After centrifugation of 15 liters of crude broth, the solid material was extracted 3 times with methanol to give a total of 2.5 liters of methanol solution. This was dried, suspended in 7.5 liters of phosphate buffer (pH 4), and extracted 3 times with ethyl acetate to give a total of 7.5 liters of EtOAc extract. After drying and evaporation, the 1.37 g of solid material was purified in 150 mg aliquots by HPLC on a Waters μPorasil column to yield 25 mg of A90931a. Fractions containing factor B were further purified on a Shandon Hyperprep BDS-C18 column to yield 1.5 mg of factor B.

Spectral Analysis

NMR spectra were run at 400.13 MHz on a Bruker AM400 spectrometer, or 599.91 MHz on a Varian Unityplus 600 Spectrometer. Carbon NMR spectra were acquired at 100.13 MHz on the Bruker AM400 spectrometer. The 2D spectra were all run at 600 MHz. For the COSY, 512 experiments consisting of 2048 data points were acquired utilizing a Z gradient; Zero filling was used in F1 to give a final data set of 2 K × 1 K. For the HMQC, 256 experiments were acquired with 1 K data points at 16 scans per increment. Zero-filling in both dimensions gave a final data set of 2 K × 1 K; for processing, a sine-bell window was used in F2, and a Gaussian window (GB = 0.2) used in F1. The HMBC was optimized with a delay of 60 ms, 512 experiments were acquired at 64 scans per increment, each consisting of 2 K data points. Zero filling in F1 gave a final dataset of 2 K × 1 K. Mass spectroscopy was performed on a Finnigan MAT-95 magnetic sector mass spectrometer.

Fungicidal Assays

Test plants were raised from grape seed (*Vitis vinifera*) cv. Ugni Blanc and inoculated with a freshly sporulated metalaxyl sensitive isolate of *Plasmopara viticola* (Berk. & Curt.). Plants were maintained in a glasshouse at 30°C. The test compounds were applied as 20% methanol solutions containing 100 ppm triton X-100 to the target

Table 1. Comparison of A90931 with *S. atroolivaceus* and *S. roseus*.

	A90931	*S. atroolivaceus*	*S. roseus*
Spore mass color	Gray	Gray	Red, yellow
Aerial mycelium color	Light gray, light grayish reddish brown	Light gray, light grayish reddish brown	Pale yellow
Substrate mycelium color	Colorless, light olive, dark olive brown	Colorless, light olive, dark olive brown	Colorless, light brown
Utilization of carbon source			
Glucose	+	+	+
Arabinose	−	+	Trace
D-Fructose	+,−	+	+,−
i-Inositol	−	+,−	Trace
D-Mannitol	−	+,−	Trace
Raffinose	−	+,−	Trace
Rhamnose	+	+	Trace
Sucrose	−	+,−	Trace
Xylose	+	+	+

plant foliage, with complete foliar coverage to run-off always obtained. The compounds were assessed in 24 hour protectant studies. Disease assessments were carried out by visual estimation of the percentage of target plant foliage showing sporulating or necrotic tissue. This value is then expressed on the Barrett-Horsfall scale[5] which assigns a value from 1 ~ 9 depending on the perceived percentage disease control.

Table 2. Physical properties of A90931a.

Appearance	Colorless solid
UV λ_{max}^{Meoh} nm	318
HR-MS Found	535.281[M+Na]
Calc.	535.278
Mol Formula	$C_{29}H_{40}N_2O_6$

Results

Taxonomy of the Producing Organism

The organism has been identified by fatty acid analysis, nutrient utilization and morphology as a *Streptomyces* species closely resembling *S. atroolivaceus* (ATCC #19725) and *S. roseus* (ATCC #19808). It differs from *S. roseus* in the production of gray aerial mycelium, and dark olive brown substrate mycelium, and from *S. atroolivaceus* by carbon source utilization only (Table 1).

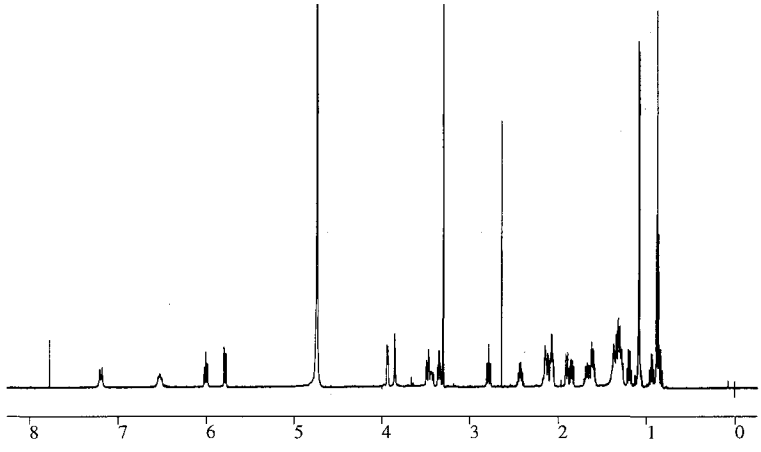
Structural Determination of A90931a

The physical properties are summarized in Table 2. A90931a is insoluble in virtually all organic solvents, being sparingly soluble in methanol, and slightly more soluble in pyridine. The compound is acidic, and readily forms salts with amines and other cations, thus mass spectroscopy was performed on a sodium salt. A search of the literature revealed two closely related if not identical molecules whose structure had not been determined. One of these–catacandin B–had been shown to contain a tetramic acid moiety. The second related compound was TAN 883B, which had an identical ^1H spectrum to A90931a, yet little structural evidence had been published.

The structure of A90931a was determined by NMR spectroscopy, and is shown in Figure 1. Assignments of the NMR spectra (Figure 2) and determination of the structure of A90931a were plagued with difficulties caused by the poor solubility of the free base, and the apparent existence of an equilibrating mixture of conformers in solution. Data collected for the free base in pyridine-d_5 showed fewer peaks than expected in the carbon and 2D spectra. This absence, coupled to line broadening of one of the olefinic groups and the quaternary carbons, meant that many key cross peaks were missing from the HMBC experiment, crucial for structural identification purposes. Eventually, data was collected from a solution of *ca.* 8 mg dissolved in a mixture of $CDCl_3$ and MeOD-d_3 (1 : 3), although even in this solvent mixture, some peaks in the proton spectrum exhibited broadening due to exchange (Figure 2). These data and parts of the data previously acquired were used in the structural determination of A90931a, and assignments are shown in Table 3.

The structure of A90931a was determined by analysis of the HMQC, HMBC and COSY spectra of the free

Fig. 2. ^{1}H NMR spectrum of A90931a.



base acquired at 600 MHz, and fine tuned using data from a COSY spectrum in pyridine-d_5 at 400 MHz. The assignments are listed in Table 3, along with HMBC correlations that were unambiguously derived from the spectra. No correlations are indicated for 13 and 20a as these have identical chemical shifts in the carbon spectrum, and so at the resolution of the 2D experiments, it was not possible to identify exactly from which carbon the cross-peak arose. HMBC and COSY data allowed the building up of the tricyclic portion of the structure into the macrocycle, with ring closures being determined by correlation of the HMBC data for carbons 14, 15, and 16, which unambiguously indicated a 5-5-6 ring system similar to that assigned for discodermide (**3**), a member of the macrocyclic tetramic acid metabolites most closely related to A90931a (Figure 3). With the tetramic acid residue being a ring, the degrees of unsaturation indicated that the remainder of the molecule consisted of a macrocyclic lactam. This ring was built up by further analysis of COSY data, a key correlation being a cross-peak between the amide proton and H-2, and relevant HMBC data. No relevant cross-peaks were determined for two of the quaternary carbon atoms, thus at this point it was not possible to determine the absolute nature of the tetramic acid group.

The secondary structure of the tricyclic fused section of the molecule was deduced by a series of 1D nOe experiments, irradiating available protons in the tricycle, which gave few enhancements. However, irradiation of H-16 showed enhancements at H-20 and H-29 (Figure 4). Additionally irradiation of H-29 showed enhancement of H-16, H-30a, and one or both of H-18 and H-17. These data suggest that the methyl and the ethyl groups

Table 3. NMR assignments of A90931a.

C	H	C13	HMBC connections
2a	3.48 (ddd)	38.0	3a, 4
2b	2.78 (ddd)		
3a	1.6 (m)	32.3	5, 2b
3b	1.4 (m)		
4	3.93 (d, bd)	72.4	2a, 2b, 5
5	3.85 (s, br)	69.0	
7			
8		103	
9			
10	7.19 (d, br)	126.8	
11	6.52 (dd, br)	148.9	
12	2.1 (m)	46.7	13a, 24b, 22
13a	1.90 (ddd)	43.3	
13b	1.3 (m)		
14	3.34 (ddd)	75.2	13a, 13b, 15, 16, 22
15	1.19 (ddd)	60.6	13a, 16, 21a, 22
16	1.85 (ddd)	59.7	14, 15, (19a or 21a), 20
17	1.3 (m)	48.1	15, 19a, 29
18	1.3 (m)	55.4	19a, 29, 30a, 31
19a	2.07 (m)	41.7	16, 21b, 30a
19b	0.85 (m)		
20	2.43 (dddd)	43.3	
21a	2.07 (m)	38.7	19b, 22
21b	0.94 (ddd)		
22	1.67 (ddd)	49.3	15, 21a, 21b, 24a
23	1.3 (m)	45.4	13a, 15, 21a, 22, 24b
24a	3.44 (ddd, bd)	29.6	26
24b	2.15 (m)		
25	6.00 (ddd)	142.0	24a, 24b
26	5.78 (dd)	124.6	24b
27		168.7	2b, 25, 26
28		195.2	5
29	1.08 (d)	19.0	16
30a	1.60 (m)	27.4	31
30b	1.07 (m)		
31	0.88 (t)	13.2	30a

Unassigned ^{13}C resonances; 168, 178 ppm.

Fig. 3. HMBC (single headed) and COSY (double headed arrows) correlations.

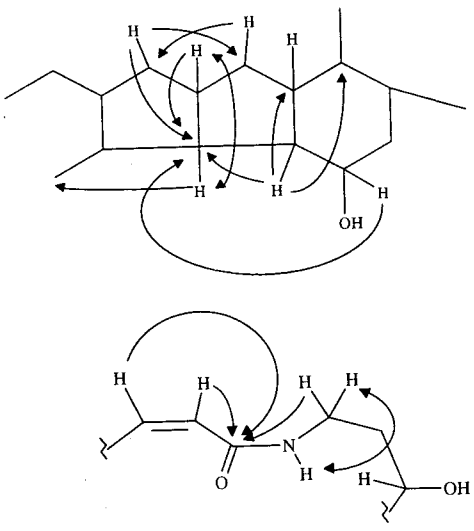
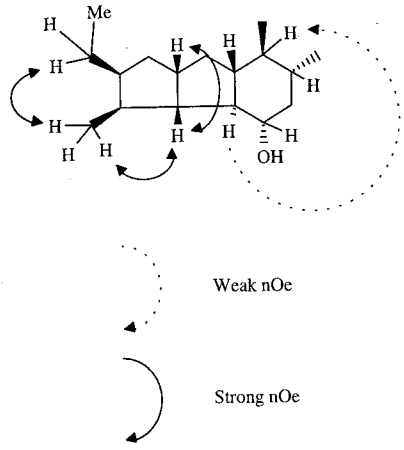


Fig. 4. Secondary structure elucidation of A90931a.



are both on the same side of the ring plane as the two protons on the 5/5 ring junction as indicated in Figure 4.

There were no nOe signals indicated on irradiation of H-22, and irradiation of H-14 gave very little responses. In this experiment, there was a possible enhancement between H-14 and H-13a, and H-14 and H-12. Careful irradiation of H-15 also gave a very weak signal within the envelope of signals at 1.3 ppm. Close examination of the shape of the multiplet, and comparison with cross peaks extracted from the HMQC experiment suggested that this may indicate an enhancement of the H-23 signal. This is consistent with an axial-axial relationship between these protons in a "chair" conformation for the six member ring. Further evidence for this conformation was deduced by examination of the coupling constants arising for protons 13a, 14, 15, 16, 21a, and 22. Some of these indicated axial/axial coupling constants for protons 15 ∧ 16, 15 ∧ 22, 14 ∧ 15, 22 ∧ 23; thus confirming the chair conformation.

Acetylation of the molecule with acetic anhydride/pyridine at room temperature gave a molecule with MW 638 implying addition of three acetyl groups. Large downfield shifts were noted for H-4, H-5, and H-14. Two of these were consistent with the existence of two hydroxy groups, however the shift of H-5 indicates acetylation of the tetramic acid function. This result is seemingly inconsistent with the molecule arising from acetylation of discodermide[1], the most closely related reported molecule, in which only one acetyl group is added at the equivalent of H-4, resulting in the slight upfield shift for H-5. The proton spectrum for A90931a triacetate indicates that the tricarbonyl function has been "frozen" into a single conformer as indicated by the enhanced resolution now apparent for the olefin protons (H-10 and H-11). Interestingly, the assignment of discodermide was undertaken as the acetate as this conferred some solubility onto the molecule, and made it possible to record the NMR spectra in pyridine-d_5. In our hands the solubility of the triacetate of A90931a was much enhanced over the natural product, and spectra were able to be recorded in chloroform-*d*. The exact nature of the third acetyl group was not determined due to lack of sample. However the third methyl signal was shifted downfield to 2.6 ppm (wrt 2.0 and 2.1) possibly suggesting an acetamide group which would infer a downfield shift to H-5. Why this would force the tricarbonyl functionality into a single conformer remains unresolved, but the presence of a very broad proton resonance at 13.0 ppm suggests the presence of a hydrogen bond now present in the molecule possibly between 7-OH and the carbonyl group on the acetamide. Further evidence for this interaction has not been obtained.

Structural Determination of A90931b

A90931b differed from A90931a by the lack of 2 mass units. The proton spectrum indicated the loss of 14-H, thus indicating a ketone function at C-14. This molecule is thus identical to maltophilin[4], and thus we have called A90931a dihydromaltophilin.

Biological Activity of Dihydromaltophilin

The biological activity of the isolated compounds is summarized in Table 4. Dihydromaltophilin exhibited

Table 4. Disease control by A90931a and b in a 24 hours residual protectant test *vs. Plasmopora viticola.*

Compound	(ppm)	Disease score[a]
A90931a	400	9
	100	8
	25	6
	6.2	5
A90931b	400	9
	100	8
	25	8
	6.2	6

[a] Barrett-Horsfall scale: 9=100%, 8=97~99%, 7=90~96%, 6=75~89%, 5=60~74%, 4=40~59%, 3=30~39%, 2=20~29%, 1=0~19% control.

good residual protectant activity against the pathogen, but did not perform well in curative or translaminar assays.

Discussion

Dihydromaltophilin belongs to an emerging class of polyketide antibiotics containing a macrocyclic lactam structural unit as well as the tetramic acid[6]. It is structurally very similar to discodermide, although there is some difference in the relative stereochemistry of the tricyclic portion of the molecule. In this study, we have not determined the stereochemistry of the macrocycle, although it seems likely that ornithine is a contender for the biosynthetic source of C-2 to C5, as was found in alteramide[7].

Acknowledgments

The authors would like to thank ERICA BRUNS for providing the soil sample, and FRED MERTZ, Eli Lilly and company, for providing the original fermentation samples, and the strain. The authors acknowledge the assistance of DEAN CARLSON and PATRICK TISHMACK from Purdue University for their help in acquiring the 600 MHz NMR data, and STEVE BROWN for providing the mass spectral analyses.

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