STIGMATELLIN, A NEW ANTIBIOTIC FROM *STIGMATELLA AURANTIACA* (MYXOBACTERALES)†

I. PRODUCTION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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An antibiotic activity was extracted from the cell mass of the myxobacterium, *Stigmatella aurantiaca* strain Sg a15. The antibiotic was toxic for yeasts and filamentous fungi, but not for most bacteria. The compound had the molecular formula $C_{30}H_{42}O_7$, appears to be a new antibiotic, and was named stigmatellin. In addition to stigmatellin, the strain produced relatively large quantities of a second, structurally unrelated antibiotic, a mixture of three myxalamid homologues.

During a screening of gliding bacteria for new antibiotics, the myxobacterium *Stigmatella aurantiaca* strain Sg a15 was found to produce activity against yeasts, filamentous fungi and several Gram-positive bacteria. Part of the activity appeared in the culture supernatant, and part within the cell mass. By thin-layer chromatography it could be shown, that the antibiotic activity was due to at least two different substances. One of the activities was identified as a mixture of three myxalamids, compounds which were recently isolated in our laboratory from the myxobacterium, *Myxococcus xanthus* strain Mx x12[1,2]. The second antibiotic had a completely different chemical structure, proved to be new, and was named stigmatellin. In some fermentation experiments traces (up to 2%) of a stereoisomer of stigmatellin were also isolated. As it is not yet unequivocally proven whether this stereoisomer is a natural compound, we assume that we are dealing with one antibiotic only. In this paper we wish to report on the production and isolation of the different antibiotics of Sg a15, and on some physico-chemical and biological properties of stigmatellin. The structure elucidation of stigmatellin[3] and its mode of action[4] will be published elsewhere.

Organism

Stigmatella aurantiaca strain Sg a15 (Dawid Stamm 1) was isolated in 1977 by Dr. W. DAWID, Bonn, from rotting wood collected in the Siebengebirge mountains near Bonn, FRG. Stock cultures (cell suspensions in peptone liquid medium) were stored in a deep freezer at −80°C or in liquid nitrogen. The organism was grown in standard peptone liquid medium (1% peptone from casein, tryptically digested, from Merck, Darmstadt; 0.1% $MgSO_4 \cdot 7H_2O$; pH 6.8). Batch cultures of 100 ml or of 500 ml in 250-ml or 1,000-ml Erlenmeyers flasks, respectively, were incubated at 30°C on a rotary shaker at 160 rpm for 3~5 days. Addition of 0.05~0.2% yeast extract to the medium enhanced growth up to the four-fold cell mass yield. This stimulation proved to be due to vitamin B_{12} and thiamine. Strain Sg a15 was able to use various sugars, polysaccharides and acids of the citrate cycle as substrates. In a basal medium composed of Casamino Acids (Difco) 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $(NH_4)_2SO_4$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%,

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phosphate buffer 1 mM pH 7.0, and standard vitamin and trace element solutions (0.1 ml/liter each) the following carbohydrates (tested at 0.5% each) stimulated growth appreciably: glucose, fructose, maltose, starch, and Dextrin 10 (from Serva, Heidelberg). The strain could thus be cultivated in defined media with one of the listed sugars as the main carbon source.

The strain grew also well in technical media on the basis of Probion (single cell protein prepared from *Methylomonas clarae*: Hoechst Comp., Frankfurt), Zein (maize gluten from Maizena, Hamburg) and corn steep powder (from Roquette Frères, Lille, France).

Assay for the Antibiotics

The development of antibiotic activity in the culture was followed by the agar diffusion assay. The test organism, *Saccharomyces cerevisiae*, was suspended in N3 agar (Bacto peptone (Difco) 1%, yeast extract (Difco) 1%, glycerol 2%, 50 mM phosphate buffer, pH 6.3, agar 1.5%). The samples were first separated into cell mass and supernatant by centrifugation. Then the wet cell mass was extracted with acetone, and the culture supernatant with ethyl acetate. Aliquots of the extracts were applied to paper disks of 6 mm diameter.

The quantitative determination of the antibiotics was done in the following way. Depending on the stage of the culture, samples of 100 to 25 ml were taken and processed as described above. *N*-Propionanilide, synthesized from propionic anhydride and anilide, was added (0.5 mg/ml) as an internal standard to the solvent used for extraction. The compound was chosen, because it absorbs in the proper UV range, has a convenient Rf in the system used for separation of the antibiotics, and is stable under our experimental conditions. Aliquots of the extracts (5~10 μl) were chromatographed on precoated thin-layer plates (silica gel 60 F_{254}, Merck 5553) with dichloromethane - acetone - methanol (71 : 27 : 2) as the solvent. The Rf value of the myxalamids was 0.30, that of stigmatellin 0.46, and that of the standard 0.57. The chromatograms were scanned at 266 nm using a Shimadzu CS 920 high speed scanner. The concentrations of stigmatellin and of the myxalamids were calculated from the peak areas of the antibiotics by comparison with the standard and after calibration of the system with pure antibiotic substance. In addition, a factor was needed which related the peak areas with the absolute amounts. This factor differed for each antibiotic and each peak area, and could be read from a calibration curve obtained with pure antibiotic substance. To give an example: From a late log-phase shake culture in peptone - starch medium (Table 1), a 35-ml sample was taken, the cells harvested and extracted with 2 ml of acetone containing 1 mg of the standard. After chromatography of an aliquot (the accurate amount of which is irrelevant in certain limits), the following peak areas were determined: for stigmatellin 9019, for the myxalamids 7084, and for the standard 2139. The factor for stigmatellin at this specific peak area was 0.1026, that for the myxalamids 0.6954. From these data the absolute amounts were calculated with the following formula:

$$\text{Amount (antibiotic, in mg)} = \text{f (antibiotic)} \times \frac{\text{I (antibiotic)}}{\text{I (standard)}} \times \text{amount (standard, in mg)}$$

where f stands for factor, I for the integrated peak area, and amount standard for the amount originally added (here, 1 mg). If we give the antibiotics per liter, we obtain: 12.3 mg of stigmatellin and 65.8 mg of myxalamids.

Production of the Antibiotics

In shake cultures with 100 ml of peptone liquid medium in 250-ml Erlenmeyer flasks the average yield of the antibiotics was 0.2~0.8 mg/liter of stigmatellin, and up to 70 mg/liter of myxalamids. The

Table 1. Effect of various carbohydrates and organic acids on antibiotic production by *S. aurantiaca* Sg a15 in shake culture.

Carbon source*	Dry weight of cell mass (mg/liter)	Antibiotic titer**	
		Myxalamids (mg/liter)	Stigmatellin (mg/liter)
Glucose	520	69.3	11.2
Fructose	470	35.1	1.9
Lactose	160	24.4	1.0
Maltose	660	70.7	10.5
Starch	580	68.0	12.3
Inulin	170	25.5	1.0
Dextrin 10	450	62.5	11.1
Pyruvate	260	60.6	4.45
Succinate	140	33.8	1.6

* The basal medium was: peptone from casein, tryptically digested (Merck Darmstadt) 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%, phosphate buffer 0.5 mM (pH 7.1), standard vitamin and trace element solutions, 1 ml/liter each. The carbohydrates were added at a concentration of 0.5%. Harvest was at the end of the growth phase after 4 days.

** Only the antibiotics contained in the cell mass were determined.

Table 2. Antibiotic production by *S. aurantiaca* strain Sg a15 in technical media in shake culture.

Substrate*	Antibiotic titer**	
	Myxalamids (mg/liter)	Stigmatellin (mg/liter)
Corn steep powder	46.5	3.0
Probion PF	55.0	4.3
Probion PS	73.0	5.7
Zein	120	10.7

* The medium consisted of 1% of the technical substrate+0.1% $MgSO_4 \cdot 7H_2O$, pH 7.3. The cells were harvested, when pH of the culture was 7.8 to 8.0.

** Only the antibiotics in the cell mass were determined.

addition of yeast extract or of vitamins allowed better growth, but had little effect on antibiotic production. Cultivation in a lean peptone medium with additions of various carbohydrates as carbon and energy source resulted in a 10-fold increase in the yield of stigmatellin, while the myxalamids remained more or less constant (Table 1). Among the technical substrates tested for antibiotic production, Zein gave the best results (Table 2).

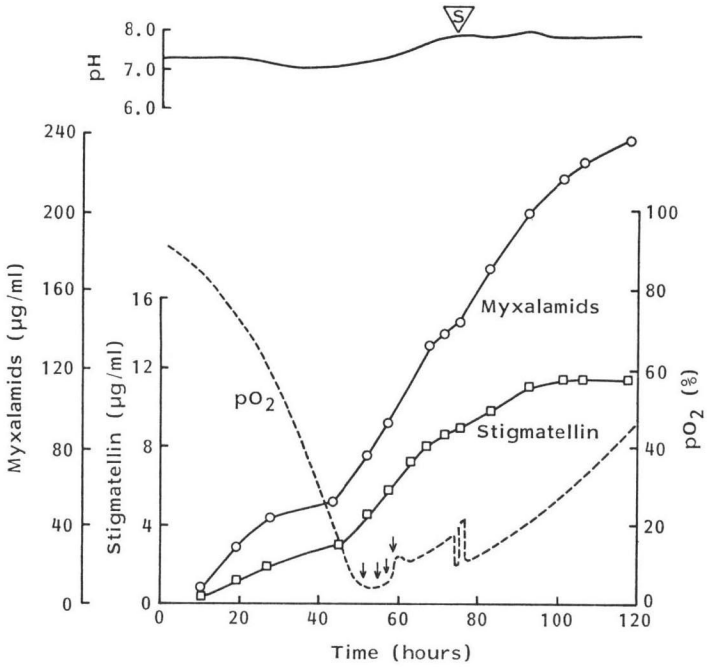
Antibiotic production on a larger scale was performed in Zein liquid medium (Zein 1%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.3). Ten liters of shake culture grown for 3 days in peptone liquid medium were inoculated into 55 liters of Zein liquid medium in a type b 50 bioreactor (from Giovanola Frères, Manthey, Switzerland). Silicon antifoam agent (Merck, Darmstadt) was added to the medium at a concentration of 0.008%. The seed fermentor was maintained at 30°C and was agitated with a turbine stirrer at 200 rpm. The aeration rate was 0.025~0.09 v/v/minute. The pH, which initially drifted into the acid range, was left to rise to 7.8 and was then kept constant by titration with 30% acetic acid. To start the culture in the production fermentor, the content of the seed fermentor was pumped after 3~5 days into 265 liters of Zein medium+0.003% Silicon antifoam agent in a type b 200 bioreactor. The fermentor was kept at 30°C and was agitated by a circulating pump stirrer system at 600 rpm. The aeration rate was between 0.04 and 0.065 v/v/minute. The pH was regulated as described above. The pO_2 in the culture broth was continuously recorded using a polarographic oxygen electrode. At the beginning of the fermentation, the pO_2 was at about 90%. It fell within 50 hours to about 5%, and was then maintained at about 10% by periodically increasing the aeration rate. Under these conditions the antibiotics accumulated essentially during the growth phase (Fig. 1). The fermentation was stopped, when the cells began to form spheroplasts (between the 70th and 120th hour). At this time the average yield of the antibiotics was: 240 mg/liter of myxalamids, and 11 mg/liter of stigmatellin (Fig. 1). About 90% of the antibiotics were found in the cell mass, and 10% in the supernatant.

Isolation of the Antibiotics

Biomass and culture broth were separated by centrifugation. As the latter contained only minor

Fig. 1. Time course of antibiotic production by *S. aurantiaca* strain Sg a15 during fermentation in 320 liters of Zein medium.

S indicates the point at which the pH was corrected for the first time by addition of acetic acid. The arrows on the pO_2 curve show points at which the aeration rate was manually adjusted.



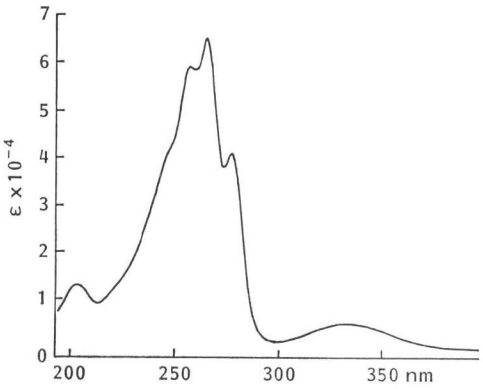
quantities of the antibiotics, it was discarded. The biomass was extracted twice with acetone. The extracts were combined, and the acetone was removed at 40°C under reduced pressure. The remaining water phase was extracted twice with ethyl acetate. The ethyl acetate extract was concentrated and purified by three consecutive chromatographic steps. The myxalamids were detected under the UV lamp at 366 nm (intense yellow fluorescence, dark spot at higher concentrations), and stigmatellin by agar diffusion assay with *S. cerevisiae* as indicator organism. After chromatography on Sephadex LH-20 with methanol - water (9: 1) to remove contaminating material, the myxalamids were separated from stigmatellin by preparative HPLC on reversed phase silica gel RP18 (Merck, Darmstadt) with methanol - water (8: 2). The myxalamids eluted first and were further purified as described elsewhere[2]. The yield of the crude myxalamids was 12 g from 320 liters of culture. Stigmatellin was then chromatographed on silica gel (HPLC) with chloroform - methanol - acetic acid (998: 1: 1). Separation problems and instability of the compound caused considerable loss of substance. The antibiotic was obtained as a yellow oil.

Physico-chemical Properties of Stigmatellin

Stigmatellin crystallized from toluene - hexane, yielding colorless crystalls with a melting point of 128~130°C. The compound was soluble in methanol, acetone, ethyl acetate and chloroform, sparingly soluble in ether and water, and almost insoluble in hexane. In aqueous solution stigmatellin was stable at neutral and slightly alkaline pH, but slowly decomposed below pH 5. On TLC on silica gel 60 F_{254} (Merck) the following Rf values were observed: with ethyl acetate as the solvent, 0.4; with dichloromethane - methanol (96: 4), 0.46; with 2-propanol - heptane (4: 1), 0.44. Elemental analysis

and mass spectra established the molecular formula $C_{30}H_{42}O_7$. The electronic absorption spectrum of stigmatellin (dissolved in methanol) had characteristic maxima at 266 and 330 nm (Fig. 2). The IR spectrum and the ^{13}C NMR spectrum are shown in Figs. 3 and 4. Fig. 5 shows the result of the structure elucidation, which is published elsewhere[3].

Fig. 2. Electronic absorption spectrum of stigmatellin in methanol.



Biological Properties of Stigmatellin

The minimal inhibitory concentration (MIC) of stigmatellin for a number of bacteria and yeasts as determined by the serial dilution assay (broth), and the activity of the antibiotic against

Fig. 3. IR spectrum of stigmatellin in chloroform.

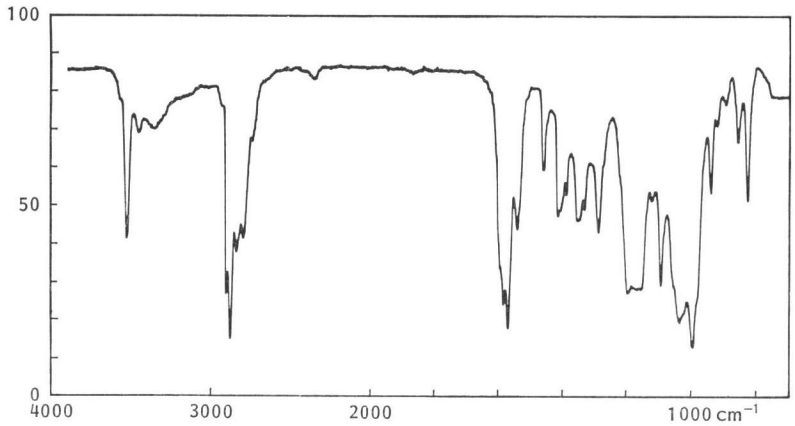


Fig. 4. ^{13}C NMR spectrum of stigmatellin in $CDCl_3$ recorded with a Bruker WM400 spectrometer.

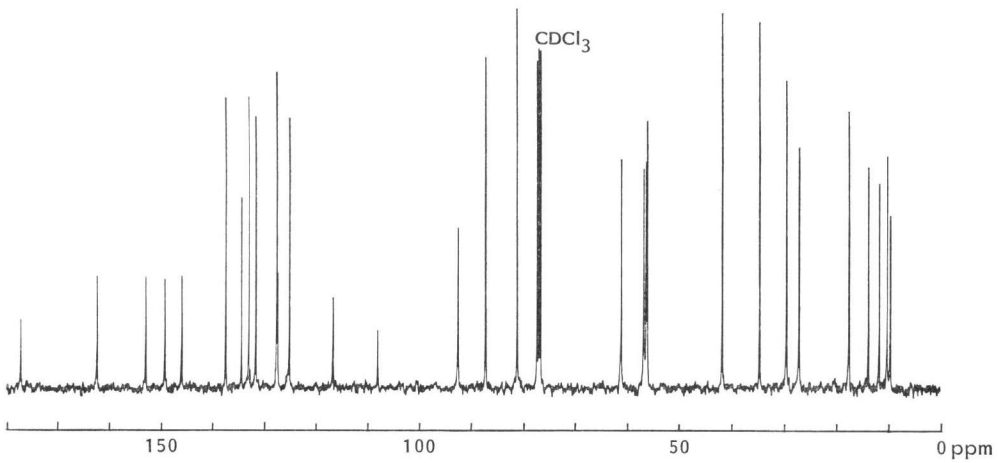


Table 3. The antimicrobial spectrum of stigmatellin.

Test organism	MIC* (μg/ml)
Stigmatella aurantiaca Sg a15	>50
Acinetobacter calcoaceticus DSM 30006	>100
Escherichia coli	>100
Pseudomonas aeruginosa	>100
Salmonella typhimurium DSM 50912	>100
Serratia marcescens	>100
Klebsiella pneumoniae	>100
Brevibacterium ammoniagenes DSM 20305	2.5
Bacillus subtilis ATCC 6051	>100
Micrococcus luteus	5
Rhodococcus sp. ATCC 13258	2.5
Staphylococcus aureus	>100
Candida albicans	2.5
Debaryomyces hansenii DSM 70238	2.5
Hansenula anomala Tü 279	2.5
Nadsonia fulvescens Tü 661	2.5
Saccharomyces cerevisiae	0.1
Torulopsis glabrata DSM 70614	0.5
	Diameter of inhibition zone** (mm)
Paecilomyces varioti	16
Penicillium digitatum CBS 31948	8
Polyporus sp.	7
Polystictus sp.	7
Rhizoctonia solani	20

* Determined by the serial dilution assay. The size of the inoculum was 10^5 cells/ml. The bacteria were assayed in EBS medium: peptone from casein, tryptically digested (Merck) 0.5%, Proteose - peptone (Difco) 0.5%, beef extract (Oxoid) 0.1%, yeast extract (Difco) 0.1%, pH 7.0. The yeasts were tested in a medium containing Bacto - peptone (Difco) 1.0%, yeast extract (Difco) 1.0%, glycerol 2.0%, pH 6.3.

** Determined by agar diffusion assay with paper disks of 6 mm diameter. The fungi were tested on Mycophil agar: Phytone peptone (BBL) 1.0%, glucose 1.0%, agar 1.6%, pH 7.2. The antibiotic was dissolved in methanol and applied at 40 μg/disk.

Fig. 5. Structure of stigmatellin as elucidated by HÖFLE *et al.*[5].

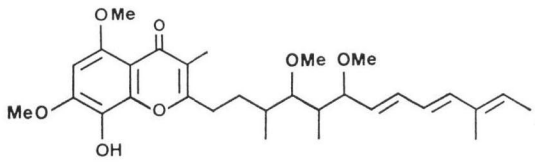
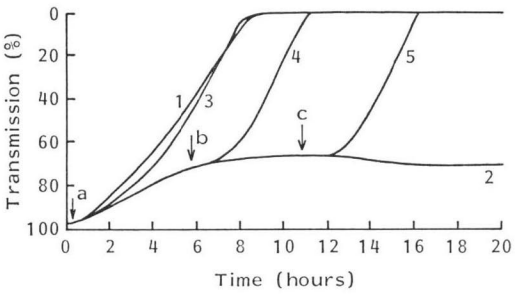


Fig. 6. The antagonistic effect of glucose on the growth inhibition by stigmatellin in *S. cerevisiae*.
The growth curves represent the following culture conditions: 1) Control without stigmatellin and glucose; 2)~5) with 3.3 μg/ml of stigmatellin and either without glucose (2) or with 1% glucose added at time a (3), b (4), or c (5).



filamentous fungi as evaluated by the agar diffusion assay is shown in Table 3. Stigmatellin was strongly inhibitory for yeasts and most filamentous fungi. Gram-negative bacteria were not affected by concentrations up to 100 μg/ml, but a few Gram-positives were sensitive.

The antibiotic proved highly toxic for animals. In the mouse the LD_{50} was 2 mg/kg sc, and 30 mg/kg po.

The effect of stigmatellin on *S. cerevisiae* was fungistatic. When the antibiotic was added to a log-phase culture at concentrations of up to 10 μg/ml, the cells stopped to multiply, but the number of cells able to form colonies upon plating remained constant during several hours.

On the Mechanism of Action

In *S. cerevisiae* RNA and protein synthesis, measured as incorporation of [*U*-^{14}C]uracil and of [*U*-^{14}C]-leucine into trichloroacetic acid insoluble material, stopped immediately upon the addition of stigmatellin to the culture. Several sensitive organisms became resistant to stigmatellin, when grown in presence of glucose. This glucose effect was studied in detail with *S. cerevisiae* using a biophotometer (model Bonet-Maury, from Jobin Yvon, Longjumeau, France). When glucose was added to the culture

at the same time as stigmatellin, there was no inhibitory effect at all. When glucose was added to a culture which had been blocked before with stigmatellin, growth resumed after a delay of a few hours (Fig. 6).

Discussion

The myxobacterium, *S. aurantiaca* strain Sg a15 produced at least two kinds of structurally unrelated antibiotics. One was a mixture of myxalamids, antibiotics recently isolated in our laboratory from cultures of the myxobacterium, *M. xanthus* strain Mx x12[1,2]. However, while Mx x12 synthesizes four homologues, *viz.* myxalamids A to D with B and A as predominant components, we found in Sg a15 mainly myxalamid B, small quantities of C and D, and no A. The myxalamids were produced by Sg a15 with remarkably high yields of up to 250 mg/liter (with the original wild strain but after optimization of the culture conditions). This is far beyond the 0.2 to 20 mg/liter usually observed with myxobacterial antibiotics. The myxalamids act on complex I of the respiratory chain and were shown to specifically inhibit NADH: ubiquinone oxidoreductase in beef heart submitochondrial particles[1].

The second compound found in Sg a15, stigmatellin, appears to be a new antibiotic. Initially, stigmatellin was produced in very small quantities, below 1 mg/liter, but by changes in the composition of the medium and optimization of the culture conditions the yields could be somewhat improved to about 10 mg/liter. During these studies it turned out that Sg a15 was much more versatile metabolically than is generally assumed for myxobacteria. Thus with this strain there is a much better chance to identify and to overcome supposed catabolite repressions in antibiotic synthesis than with many other myxobacteria. This and the fact that the organism produces at least two chemically entirely different antibiotics makes Sg a15 particularly useful for studies on the regulation of secondary metabolism, about which very little is known in myxobacteria. As the two antibiotics can easily be quantitated by our analytical system, all experiments can be analyzed for effects on two different biosynthetic systems.

Stigmatellin as well as the myxalamids seem to be synthesized by Sg a15 during the growth phase. It must be emphasized, however, that the strain was growing relatively slowly at all times, and that the fermenter culture was kept under oxygen limitation.

Like the myxalamids, stigmatellin, too, seems to interfere with respiration. This was suggested: a) by the immediate and complete shut-off of RNA and protein synthesis upon addition of the antibiotic; and b) by the antagonistic effect of glucose in organisms which are able to metabolize sugars by fermentation. Such a glucose effect has been described for several respiration inhibitors, *e.g.* antimycin[5], mucidin[6] and myxothiazol[7]. In the meantime it has been proved by *in vitro* studies that stigmatellin is indeed an inhibitor of the respiratory chain acting on the bc_1-segment[4]. The high toxicity of the compound for animals is thus understandable. It is curious that one and the same organism produces two different types of respiration inhibitors, and it remains to be seen whether this is merely a matter of chance.

While there seem to be many myxobacterial strains of different genera and species that produce myxalamids[1], stigmatellin is a rare antibiotic. So far no other organism appeared in our screening of about 800 strains that produced this substance.

Acknowledgments

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