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Aurafuron A and B, New Bioactive Polyketides from *Stigmatella aurantiaca* and *Archangium gephyra* (Myxobacteria)

Fermentation, Isolation, Physico-chemical Properties, Structure and Biological Activity†

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Abstract New antibiotic polyketides, named aurafuron A (**1**) and B (**2**) were isolated from culture extracts of myxobacteria of the species *Stigmatella aurantiaca* and *Archangium gephyra*, strain Ar 10844. By multi-step chromatography **1** and **2** were separated from a variety of other non-related co-metabolites, and their structures elucidated by spectroscopic methods as new 5-alkenyl-3 3(2*H*)-furanones. Aurafurons inhibited the growth of some filamentous fungi and additionally, aurafuron B was weakly active against few Gram-positive bacteria. Both compounds also showed cytotoxic activity against the mouse fibroblast cell line L929.

Keywords Myxobacteria, bioactive polyketides, 5-alkenyl-3 3(2*H*)-furanones, aurafurons

Introduction

In the course of our continuous screening for new bioactive metabolites, strains of the genus *Stigmatella* emerged to be secondary metabolite multiproducing myxobacteria. For example *S. aurantiaca* strain DW4/3-1 simultaneously produces the electron transport inhibitor myxothiazol [1], the dawenols [2], the myxochromides [3], and the iron chelators myxochelin A and B [4, 5]. In culture extracts of *S. aurantiaca*, strain Sg a15 even five structurally entirely different groups of metabolites have been discovered, namely the inhibitors of the eukaryotic electron transport, the aurachins [6], stigmatellins [7, 8], and myxalamids [7, 9, 10], and as in strain DW4/3-1 also the myxochelins [5] and minor amounts of myxochromides components [3]. Recently by genetic studies a variety of typical secondary metabolic gene clusters encoding multimodular polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) have been identified in both *S. aurantiaca*, DW4/3-1 [3, 11, 12], and *S. aurantiaca*, Sg a15 [5, 13～16]. Some of those gene clusters were shown to be responsible for the formation of the known secondary metabolites, others may be used for the biosynthesis of novel natural products whose structures are yet to be determined.

As a result of both detailed HPLC-DAD analysis of culture extracts and molecular biosynthetic studies with *S. aurantiaca* we here describe the production, isolation,

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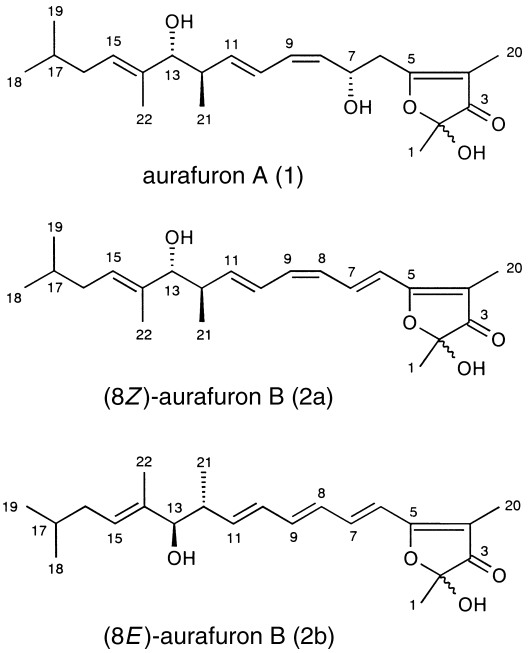

Fig. 1 Structures of aurafuron A (**1**) and B (**2a**, **2b**). For C-12 and C-13 only the relative configuration is shown.

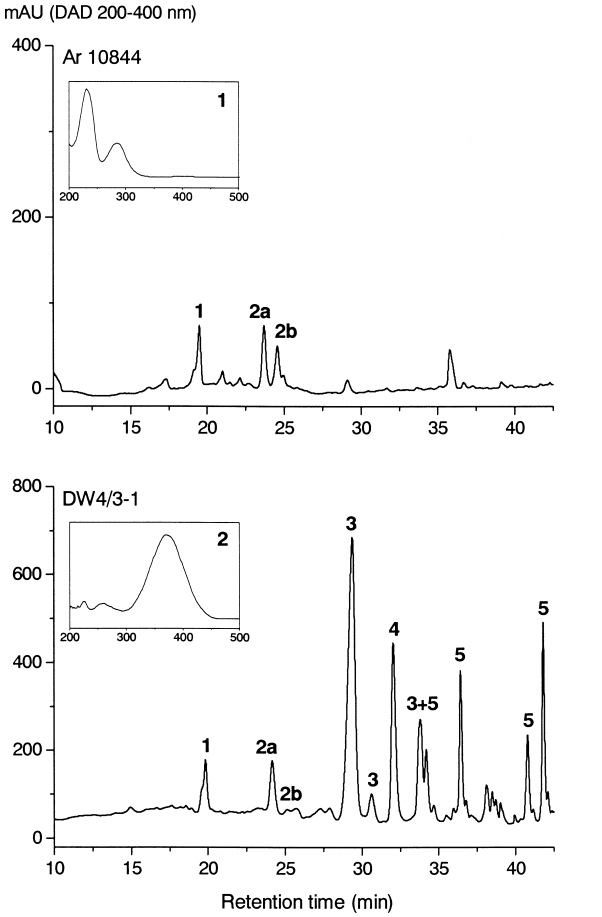

Fig. 2 HPLC analysis of crude extracts from the fermentation of *Archangium gephyra* Ar 10844 and *Stigmatella aurantiaca* DW4/3-1, and overlaid UV spectra from aurafuron A (**1**) and B (**2**). **1** aurafuron A, **2a**, **2b** aurafuron B, **3** myxochromides, **4** myxothiazol A, **5** dawenols.

physico-chemical properties, structure elucidation as well as the biological activity of the new aurafurons A (**1**) and B (**2**) (Fig. 1). In addition to strains of the genus *Stigmatella*, the new compounds have also been isolated from a strain of the genus *Archangium*. Both genera belong to the family *Cystobacteraceae*, and strains of the genus *Archangium* are also known to produce a variety of secondary metabolites with biological activity [17]. Among these, due to their potential as anticancer drugs, particularly the tubulysins [18, 19], have attracted great attention.

Results

Fermentation and Isolation

Because of the fact that aurafuron A and aurafuron B showed completely different UV spectra during examination of crude extracts with diode-array-detected (DAD) RP-HPLC they were isolated and characterized from different species on different occasions. An analytical HPLC/DAD profile of fermenter culture extracts of *A. gephyra* Ar 10844 and *S. aurantiaca* DW4/3-1 is given in Fig. 2.

For the production of aurafuron A (**1**), strain Ar 10844 was selected which, compared to strain DW4/3-1, produced by far less and smaller amounts of other non-related metabolites (Fig. 2). Large-scale fermentations were performed in M7 liquid medium with the addition of 1% of the adsorber resin Amberlite XAD-16. In a 280-liter fermentation batch described in the experimental section, **1** accumulated till the end of the fermentation at 68 hours up to about 2.4 mg/liter as analyzed by HPLC. Isolation and purification was achieved by extraction, solvent partition and multi-step chromatography yielding 260 mg of pure aurafuron A.

For the isolation of aurafuron B (**2**) fermentations of strain DW4/3-1 were used, which were originally run for the production of other metabolites, *e.g.,* the dawenols [2] and the myxochromides [3]. These were performed in 150 liters bioreactors containing 85 liters Zein liquid medium with 1% of the adsorber resin Amberlite XAD-16 as described in detail previously [2, 3]. A 90-liter fermentation was harvested 70 hours after inoculation and aurafuron B (**2**) was isolated from the fermenter crude

Table 1 Physico-chemical properties of aurafuron A (**1**) and (8*Z*)-aurafuron B (**2a**)

	Aurafuron A	(8*Z*)-Aurafuron B
Appearance	Yellow oil	Yellow oil
$[\alpha]_D$ (MeOH)	−40.5	−72.9
Molecular weight	378	360
Molecular formula	$C_{22}H_{34}O_5$	$C_{22}H_{32}O_4$
ESI-MS $(M-H)^-$	377	359
HRDCI-MS(NH_3) *m/z*		
Calcd. for $C_{22}H_{38}NO_5$ $(M+NH_4+)$	396.2750	
Found	369.2794	
HREI-MS *m/z*		
Calcd. for $C_{22}H_{32}O_4$		360.2301
Found		360.2290
UV λ_{max} (MeOH), nm (ε)	231 (14960), 281 (6850)	259 (5900), 369 (33500)
IR (KBr) cm^{-1}	1696, 1616	1682, 1601
TLC[a] Rf-value	0.4	0.59
HPLC[b] retention time	19.9 minutes	24.1 minutes

[a] Silica gel Si 60 F_{254} aluminium sheets (Merck). Solvent: CH_2Cl_2/acetone/MeOH (75 : 20 : 5). Detection: UV absorption at 254 nm and staining with cerium (IV) sulfate/phosphomolybdic acid in sulphuric acid and heating to 120°C, giving blue spots; The Rf-value for (8*E*)-aurafuron B (**2b**) was 0.64.

[b] See experimental section; the retention time for (8*E*)-aurafuron B (**2b**) was 25.1 minutes.

extract as described below yielding 220 mg of crude aurafurons from which pure 16 mg (8*Z*) isomer **2a** and 2 mg (8*E*)-isomer **2b** were obtained by RP chromatography.

Physico-chemical Properties and Structure

The physico-chemical properties of aurafuron A (**1**) and (8*Z*)-aurafuron B (**2a**) are summarized in Table 1. Structure elucidation is best started with **2a** which, in contrast to **1**, shows simple NMR signal patterns (Tables 2 and 3). From NMR data and HREI mass spectra the elemental composition $C_{22}H_{32}O_4$ is deduced accounting for 7 double bond equivalents. Analysis of proton and carbon spectra including 2D correlated spectra (not shown) straightforward revealed the structure of an unsaturated hydroxy and methyl substituted alkyl chain. Its 6*E*, 8*Z*, 10*E* configuration follows from vicinal proton coupling constants, whereas 14*E* configuration is indicated by a strong NOEs between 22-H_3 and 16-H_2, and 13-H and 15-H. The remaining molecular fragment $C_6H_7O_3$ consists of two methyl groups appearing as singlets, a quaternary acetal carbon at 103.9 ppm and three carbonyl/olefinic signals at 206.0, 178.7 and 109.5 ppm. From the various combinations of these building blocks only a 3(2*H*)-furanone is consistent with the C, H-correlated NMR spectra. In addition, the IR spectrum with bands for carbonyl and double bonds at 1682 and 1601 cm^{-1} and UV bands for a β-oxyenenone and an extended double bond system from C-3 to C-11 at 259 and 369 nm are in full accordance with the proposed structure. The minor isomer **2b** differs only by an 8*E* double bond resulting in a 15 Hz vicinal coupling constant between 8-H and 9-H. In sharp contrast, aurafuron A (**1**) differs significantly from B (**2**) by a shift of IR and UV bands to shorter wavelengths indicating interruption of the conjugated double bond system. In addition, most NMR signals appear doubled. From the elemental composition a double bond was replaced by a hydroxyethylene group, which has to be placed in 6,7-position according to the proton NMR spectrum. All three aurafurons, **1**, **2a** and **2b** obviously exist as 1:1 mixtures of stereoisomers at the C-2 hemiacetal centre. However, only in aurafuron A the next stereocenter, C-7, is close enough to cause signal doubling for the diastereomers present. The absolute (*S*) configuration of C-7 was determined by ozonolysis. Only L-malic acid was detected by chiral GC analysis. The relative *anti* configuration at C-12 and C-13 follows from the vicinal coupling of the respective protons by 8.3 Hz as it was observed with myxalamids [10].

Biological Activity

As shown in Table 4, aurafuron A (**1**) and B (**2**) showed moderate activity against some filamentous fungi. Additionally, (8*Z*)-aurafuron B (**2a**) showed weak activity against a few Gram-positive bacteria. In individual cases

Table 2 ^{1}H data of aurafuron A (**1**) and (8*Z*)-aurafuron B (**2a**) and (8*E*)-aurafuron B (**2b**) in CD_3OD

1				**2a**				**2b**		
Atom	δ (ppm)	Multipl.	$J_{H,H}$ (Hz)	Atom	δ (ppm)	Multipl.	$J_{H,H}$ (Hz)	δ (ppm)	Multipl.	$J_{H,H}$ (Hz)
1-H_3	1.44			1-H_3	1.53	s		1.53	s	
	1.47									
6-Ha	2.65	dd	13.6; 6.5	6-H	6.61	d	14.7	6.58	d	15.3
6-Hb	2.94	dd	13.6; 7.2							
6-Ha	2.77	dd	13.7; 6.7							
6-Hb	2.86	dd	13.7; 6.7							
7-H	5.02	m		7-H	7.74	dd	14.7; 12.1	7.28	dd	15.1; 10.3
	5.03	m								
8-H	6.36	t	10.2	8-H	6.21	t	11.3	6.31	dd	15.3; 10.7
	6.38	t	10.2							
9-H	6.08	t	11.0	9-H	6.44	t	11.1	6.46	dd	14.9; 11.3
	6.10	t	11.0							
10-H	6.41	dd	15.0; 11.4	10-H	6.78	dd	14.4; 12.1	6.71	dd	14.8; 10.7
	6.44	dd	15.0; 11.4							
11-H	5.79	dd	15.1; 8.0	11-H	6.04	dd	14.9; 8.1	6.08	dd	15.1; 8.0
	5.80	dd	15.1; 8.0							
12-H	2.42	sextett	7.3	12-H	2.54	sextett	7.2	2.54	sextett	7.2
13-H	3.72	d	8.4	13-H	3.79	d	8.3	3.79	d	8.3
15-H	5.44	t	7.4	15-H	5.46	t	7.2	5.46	t	7.2
16-H	1.98	t	7.0	16-H_2	2.00	t	7.0	2.00	t	7.0
17-H	1.67	m		17-H	1.68	m		1.68	m	
18-H	0.95	d	6.6	18-H_3	0.95	d	6.8	0.95	d	6.8
19-H	0.96	d	6.6	19-H_3	0.96	d	6.8	0.96	d	6.8
20-H	1.70	s		20-H_3	1.79	s		1.79	s	
	1.72	s								
21-H	0.91	d	6.9	21-H_3	1.00	d	7.2	1.00	d	7.2
	0.92	d	6.9							
22-H_3	1.63	sbr		22-H_3	1.65	sbr		1.65	sbr	

the inhibitory activity of aurafurons was stronger when the test organisms were cultivated in minimal media, as demonstrated for the representative test organism *Mucor hiemalis*. When grown in minimal medium, the minimum inhibitory concentrations (MIC) of aurafuron A and B for *M. hiemalis* were 6.3 μg/ml. The compounds also proved to be cytotoxic. The IC_{50} for cultivated L929 mouse fibroblasts was 4 μg/ml for **1** and 0.35 μg/ml for **2a**.

Discussion

Our screening of more than 60 strains of *Stigmatella aurantiaca* showed that 11 of these strains synthesize aurafurons, as identified by HPLC-DAD analysis. All of those strains produce simultaneously myxothiazol A, myxochromides and dawenols. Compared to these co-metabolites, the aurafurons were produced in each case only in minor amounts as described for strain DW 4/3-1 (Fig. 2). Within the genus *Archangium*, from approximately 240 strains exclusively strain Ar 10844 produced aurafurons. The structures of aurafurons have as characteristic features a 3(2*H*)-furanone ring system as 2-hemiacetal and an unsaturated C-5 side-chain, and thus show a close relationship to polypropionates isolated from the marine molluscs of the genus Siphonaria [20~25]. Further structural relationships exist to the natural compound AS-183, which was isolated from the fungus *Scedosporium* sp. strain SPC-15549 and was shown to inhibit acyl-CoA: cholesterol acyltransferase (ACAT) of rabbit liver microsomes [26].

Table 3 ^{13}C NMR chemical shifts of aurafuron A (**1**) and (8*Z*)-aurafuron B (**2a**)

Atom	1	2a
	* (ppm)	
C-1	22.2, 22.3	22.4
C-2	104.1, 104.2	103.9
C-3	205.1, 205.2	206.0
C-4	110.1, 110.2	109.5
C-5	185.6, 185.7	178.7
C-6	38.3, 38.5	118.6
C-7	66.0, 66.1	135.3
C-8	126.2, 126.3	126.8
C-9	131.0, 131.4	139.2
C-10	131.6, 131.9	126.7
C-11	141.5, 141.6	145.0
C-12	41.8, 41.9	42.2
C-13	83.3	83.1
C-14	137.6	137.6
C-15	128.0, 128.1	127.9
C-16	37.8	37.8
C-17	30.0, 30.7	30.0
C-18	22.8	22.9
C-19	22.9	22.8
C-20	5.8	5.5
C-21	17.7, 17.8	17.8
C-22	11.5	11.7

Experimental

General

Optical rotation was determined on a Perkin Elmer 241 MC polarimeter. UV and IR spectra were recorded on a Shimadzu UV/Vis-2102 PC spectrometer and Nicolet 20DXB FT-IR spectrometer respectively. NMR spectra were recorded with a Bruker ARX 400 and AM 300 spectrometer (^1H: 400.1 MHz, ^{13}C: 100.6 and 75.5 MHz) using the solvent signal as internal standard. EI- and DCI-MS spectra were recorded with a Finnigan spectrometer MAT 95 (resolution $M/\Delta M=1000$; high-resolution data from peak matching, $M/\Delta M=10000$), ESI-MS spectra with a Sciex Api 2000 LC/MS/MS.

Producing Organisms and Culture Conditions

Archangium gephyra strain Ar10844 was isolated at the GBF from a soil sample collected in 1998 near Ubud on Bali, Indonesia. It belongs to those myxobacteria that live by degrading proteins and even whole cells of other microorganisms. Thus *Archangium* strains could best be obtained by inoculating streaks of living *Escherichia coli* on plain water agar with small bits of a soil sample. Details of this and other isolation procedures have been described by H. Reichenbach and M. Dworkin [27]. *Stigmatella aurantiaca* DW4/3-1 was kindly provided by Prof. Dr. David White (Bloomington, Indiana). For maintenance both strains were grown on VY/2 agar plates (bakers' yeast 0.5%, $CaCl_2 \cdot 2H_2O$ 0.1%, cyanocobalamine 0.5 µg/ml, agar 1.5%, pH 7.2). In liquid cultures strain Ar 10844 was grown in M7 liquid medium consisting of Probion (single cell protein prepared from *Methylomonas clarae*; Hoechst A.G.) 0.5%, starch 0.5%, glucose 0.2%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.1%, cyanocobalamine 0.1 mg/liter, HEPES buffer 1%, pH 7.4. Strain DW 4/3-1 was cultured in tryptone liquid medium containing tryptone 1%, $MgSO_4 \cdot 7H_2O$ 0.2%, pH 7.2, and in media based on technical substrates, *e.g.*, Probion or Zein [2]. Batch cultures of 100 ml or of 400 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a gyratory shaker at 160 rpm for 3～5 days. Stock cultures were stored in a deep freezer at −80°C or in liquid nitrogen.

Fermentation and Isolation of Aurafuron A from *A. gephyra*, Ar 10844

Large-scale fermentations were performed in M7 liquid medium (modification 10 mM HEPES, pH 7.0) to which 1% (v/v) of the adsorber resin Amberlite XAD-16 (Rohm & Haas, Frankfurt, Germany) had been added before sterilization. A 350-liter bioreactor (Giovanola Frères, Monthey, Switzerland; periphery modified by GBF) containing 280-liter of modified M7 medium (see above) was inoculated with 5.2 liters of shaking cultures grown for 3 days in M7 liquid medium. The bioreactor was kept at 30°C, aerated at 0.05 vvm per minute and agitated with a flat-blade turbine stirrer at 100 rpm. The pH value rose till the end of the fermentation after 68 hours from 7.0 to 7.5. The pO2, recorded with a polarographic oxygen electrode was around 95% saturation at the beginning of the fermentation and dropped to about 5% after 32 hours; it then fluctuated between 5% and 0% till the end of the fermentation. Foam formation could be suppressed by addition of 100 ml of a 30% antifoam emulsion (Dow Corning, USA) to the medium before sterilization.

At the end of the fermentation the adsorber resin and the cell mass were collected with a sieve and by centrifugation, respectively. Both were extracted separately with 5 and 8.5 liters of acetone, respectively. The content of aurafuron A in the XAD and cell extracts was estimated by HPLC to be 0.6 g and 0.2 g, respectively. The combined extracts were concentrated *in vacuo* to 3.5 liters of an aqueous phase.

Table 4 Aurafurons: biological activity

Test organisms[a]	Diameter of inhibition zone (mm)[b]	
	Aurafuron A	Aurafuron B
Escherichia coli DSM 423	0	0
Bacillus subtilis DSM 10	0	0
Corynebacterium fascians DSM 20131	0	tr
Corynebacterium fascians[c] DSM 20131	0	12
Micrococcus luteus[c] GBF 26	0	tr
Staphylococcus aureus GBF 16	0	tr
Candida albicans GBF 129	0	0
Saccharomyces cerevisiae[d] GBF 36	0	0
Aspergillus fumigatus	(tr)	(9)
Botrytis cinerea DSM 877	0	11
Botrytis cinerea DSM 877[c]	0	tr
Gibberella fujikuroi DSM 893	0	0
Mucor hiemalis DSM 2655	(17)	(20)
Mucor hiemalis DSM 2655[c]	22	25
Trichoderma koningii DSM 63060	(17)	(24)
Ustilago maydis	0	(9)

[a] The organism were tested on standard complex media. [b] Determined by the agar diffusion test with 20 μg aurafurons per 6-mm paper disc. Figures in parentheses indicate an incomplete inhibition. [c] The organism was cultivated in minimal media. [d] The organism was cultivated in a medium containing 2% glycerol instead of glucose.

After pH phase adjustment to 5.5 with KH_2PO_4 the liquid was extracted 3 times with 1 liter of ethyl acetate. The organic phase was evaporated *in vacuo* and the residue distributed between heptane (0.5 liter) and methanol (1 liter). From the methanol phase 18 g of crude extract was obtained and further separated by chromatography on a silica gel column (300 g, 63～200 μm) with a dichloromethane/methanol gradient from 99 : 1 to 90 : 10 (5.5 liters). Aurafuron containing fractions were identified by TLC (Table 1, footnote a), combined and evaporated to give 2.18 g of oil. This enriched material was further separated in 2 runs by RP chromatography (HD-Sil18-20-60; 7×50 cm, methanol/10 mM ammonium acetate buffer 8 : 2, detection 227 nm). A first fraction of 165 mg of impure and a second fraction of 260 mg of pure aurafuron A were obtained as yellow oil.

Fermentation and Isolation of Aurafuron B from *S. aurantiaca*, DW 4/3-1

Batch fermentations of strain DW4/3-1 were carried out in a 150-liter bioreactor (Bioengineering, Wald, Switzerland) containing 85 liters Zein liquid medium (Zein 0.8%, peptone from casein tryptically digested 0.1%, $MgSO_4 \cdot 7H_2O$ 1, HEPES 10 mM, XAD-16 1%, pH 7.2) as described [2]. The fermenter was inoculated with 5 liters of well grown shaking cultures, and the fermentation was carried out for 70 hours at 30°C with an aeration rate of 7.4 1iters air per minute and an agitation of 110～180 rpm with a flat-blade stirrer.

At the end of the fermentation the cell mass and the Amberlite XAD 16 adsorber resin from the culture broth were eluted with acetone and methanol, respectively. The combined eluates were concentrated *in vacuo* resulting in an aqueous layer, which was extracted with ethyl acetate. Evaporation of the solvent yielded a dark oily residue (32 g) that was partitioned between methanol (1.5 liter) and heptane (1 liter). The concentrated methanol layer (29 g) was fractionated on a LH-20 column (10×100 cm, methanol, 15 fractions) and according to TLC (Table 1, footnote a), fractions 7 and 8 (5.4 g) contained myxochromides and aurafurons. Silica gel chromatography of 3.3 g of this mixture (70 g silica gel 25～40 μm, dichloromethane/methanol gradient 100 : 0 to 90 : 10) gave 220 mg of crude aurafurons which were further purified by RP chromatography (Nucleodur C-18, methanol/water 78 : 22, detection 340 nm) to give 16 mg of (8*Z*)-aurafuron B (**2A**) and 2 mg (7*E*)-aurafuron B (**2B**).

Degradation of Aurafuron A

A solution of 1 mg of **1** in 1 ml of methanol was ozonized

at −60 degrees, 0.1 ml of 0.1 mM NaOH and 20 μl of 30% H_2O_2 were added and boiled shortly. Excess peroxides were destroyed with Pt/C, and the methanol evaporated. After acidification it was extracted with butanol, the butanol was evaporated and the residue stirred with diethyl ether, 20 μl of methyl iodide and 100 mg of silver oxide over night. GC analysis on a Hepatakis-(2,6-di-*o*-methyl-3-*o*-pentyl)-β-cyclodextrin column and co-chromatography with authentic samples indicated the presence of permethylated L-malic acid.

Analysis of Secondary Metabolites

The spectrum of secondary metabolites produced by the different strains was determined in aliquots of concentrated acetone extracts by diode-array-detected HPLC analysis using a Hewlett Packard series 1100 instrument. Chromatographic conditions were as follows: Column ET 125×2 mm and precolumn, Nucleosil 120-5-C18. The solvent was methanol/water 45 : 55 (A)/methanol (B); isocratic conditions were 0% B for the first 7 minutes, then a gradient up to 45% B at 20 minutes, followed by isocratic conditions of 45% B for 6 minutes (the time point was 26 minutes in the HPLC run), followed by a gradient up to 81.8% B at 40 minutes; the flow rate was 0.5 ml/minute and detection was at 200~400 nm.

Biological Assays

Standard strains for testing the biological activity spectrum were obtained from the Deutsche Sammlung von Mikroorganismen (DMSZ) and the stock collection of our laboratory at the GBF.

The antimicrobial spectrum of aurafuron A (**1**) and B (**2a**) was determined by an agar-plate diffusion assay. From a sample of 1 mg/ml 20 μl aliquots were applied to filter disks of 6 mm diameter. The test plates were incubated for 24~48 hours at the temperature that permitted optimal growth of the test organisms. To determine the minimal inhibition concentrations of **1** and **2**, the conventional serial two-fold dilution method was used. 1.6×10^5 spores/ml were used as inoculum, and the antibiotics were dissolved in methanol, giving MeOH concentrations in the cultures of not more than 3%. Growth inhibition was evaluated after incubation 48 hours. Cytotoxicity against L929 cells (mouse, connective tissue, ATCC CCL 1) was determined as reported [28].

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