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Pluraflavins, Potent Antitumor Antibiotics from *Saccharothrix* sp. DSM 12931

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The new pluramycin-type antibiotics pluraflavin A, $C_{43}H_{54}N_2O_{14}$, pluraflavin B, $C_{43}H_{56}N_2O_{15}$, and pluraflavin E, $C_{36}H_{41}NO_{14}$ were isolated from cultures of the *Saccharothrix* species DSM 12931. The structures of the novel compounds were elucidated with the aid of 2D NMR and mass spectrometric investigations. The characteristic structural element of pluraflavins A and B is an additional 4-epi-vancosamine unit at position 13 of the anthraquinone-γ-pyrone ring system. Pluraflavin E has a carboxyl group in this position. Pluraflavin A has a reactive dimethyl epoxide side chain at position 2 of the anthraquinone-γ-pyrone aglycon, which may explain the high activity of the antibiotic. The outstanding biological characteristic of pluraflavin A is its powerful, organ-dependent cytostatic action: the IC_{50} in the colon carcinoma proliferation assay is in the subnanomolar range.

Pluramycin A[1] and related[2] natural products belong to the anthraquinone class of antibiotics, which contain an anthraquinone-γ-pyrone nucleus (11-hydroxy-4*H*-anthraceno[1,2-b]pyran-4,7,12-trione chromophore) as a characteristic structural element. They undergo intercalation into the DNA helix and are active antitumor agents. Certain pluramycin-type antibiotics contain substituents in the 2-position bearing one or more epoxide rings. These compounds, kapurimycin[3], altromycin B[4,5], and hedamycin[6] for example, merit particular scrutiny on account of their ability to act as specific DNA markers by 'threading' between the DNA base pairs and alkylating guanine at the N7 position through electrophilic attack of the epoxide[7,8]. Intercalation predominantly occurs close to nucleotide bulges, resulting in specific alkylation of guanine, which is located opposite the bulges. Altromycin B and related DNA-cleaving antibiotics thus exhibit extremely high selectivity and can be employed as chemical probes for bulges in DNA and RNA[5].

We have succeeded in isolating a series of new pluramycin-type antibiotics from cultures of *Saccharothrix* sp. DSM 12931, which we termed pluraflavins. One of these compounds, pluraflavin A, is an epoxide with a 11-hydroxy-4*H*-anthraceno[1,2-b]pyran-4,7,12-trione aglycon. Since it was expected to show antineoplastic activity, we investigated the new antibiotic and found it to exhibit extraordinarily strong cytostatic activity and notable organ selectivity. Reported herein are details of the microbiological culturing, isolation, and structural characterization of the pluraflavins, and some of their biological properties.

Experimental

General

Strain Maintenance and Fermentation

For the preparation of a glycerol culture of *Saccharothrix* sp.[9] DSM 12931, 100 ml of nutrient solution (malt extract 2.0%, yeast extract 0.2%, glucose 1.0%, $(NH_4)_2HPO_4$ 0.05%, pH 6.0) in a sterile 300 ml conical flask was inoculated with the strain *Saccharothrix* sp. DSM 12931 and incubated for 7 days at 28°C on a rotating shaking machine at 180 rpm. 1.5 ml of this culture was then diluted

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with 1.5 ml of 99% glycerol and stored at −20°C.

Precultures or cultures of *Saccharothrix* sp. DSM 12931 were prepared as follows: A sterile 300 ml conical flask containing 100 ml of a nutrient solution comprising 15 g/liter glucose, 15 g/liter soy meal, 5 g/liter cornsteep solid, 5 g/liter NaCl, and 2 g/liter $CaCO_3$ was inoculated with a slant culture (same nutrient solution, but with 2% agar) or with 1 ml of a glycerol culture and incubated at 30°C on a shaking machine at 180 rpm. Maximum production of at least one of the pluraflavins was reached after about 120 hours. For inoculation of the 9-liter fermenter, a 48~96-hours old submerged culture (inoculation volume: approx. 10%) in the same nutrient solution was sufficient.

9-liter fermenter (Biostat V, B. Braun, Melsungen, Germany) were operated using a nutrient medium containing 15 g/liter glucose, 15 g/liter soy meal, 5 g/liter cornsteep solid, 5 g/liter NaCl, and 2 g/liter $CaCO_3$ at pH 7.0 (prior to sterilization), with a fermentation time of 92 hours, an incubation temperature of 28°C, a stirrer speed of 300 rpm, and an aeration rate of 5 liters/minute. Foam formation could be suppressed by repeated addition of an ethanolic polyol solution. Maximum production was reached after about 70~96 hours.

Isolation of the Pluraflavins

At the end of the fermentation of *Saccharothrix* sp. DSM 12931, the culture broth in the fermenter (80 liters) was filtered with addition of about 2% filter aid (Celite®) and the cell material (0.6 liters) extracted with 3 liters of methanol. The methanolic solution containing the active compounds was filtered from the mycelium and concentrated under reduced pressure. The extract was pooled with the concentrated culture filtrate and loaded onto a prepared 0.4 liters MCI® gel, CHP20P (Mitsubishi Chemical Corporation, Tokyo) column, which was eluted with a gradient of 0.1% acetic acid in water to 0.1% acetic acid in 2-propanol at a rate of 1.2 liters/hour. The eluate was collected in 0.25 liters fractions and the pluraflavin-containing fractions (20~23) were pooled. Concentration under reduced pressure gave, after freeze-drying, 1.4 g of a brown powder. This was loaded onto a column with a capacity of 3.9 liters (10×50 cm) packed with Fractogel® TSK HW-40 s (E. Merck, Darmstadt, Germany). The mobile phase (water/acetonitrile 1 : 1) was pumped through the column at a rate of 50 ml/minute, and the eluate collected in 65 ml fractions. The pluraflavins were contained mainly in fractions 13~16. These were pooled and freed from solvent under reduced pressure, yielding 130 mg of a pluraflavin mixture.

130 mg of the pluraflavin mixture was injected onto a preparative HPLC column (122 ml, 2.5×25 cm) packed with Nucleosil® 100-7 C18 HD. This was eluted with 10% acetonitrile in 0.1 M aqueous ammonium acetate solution at a flow rate of 50 ml/minute and the eluate collected in 50 ml fractions. Pluraflavin E was contained in fraction 6, pluraflavin C in fractions 12~17, pluraflavin B in fractions 25~27, and pluraflavin A in fractions 35~37. After concentration under reduced pressure and freeze-drying, the separated pluraflavins were dissolved in 10% aqueous acetonitrile, adjusted to pH 2.8 with trifluoroacetic acid, and purified on a 250/10 LiChrospher® RP-18e (5 μm, E. Merck) column. The column was eluted with a gradient of 10~50% acetonitrile containing 0.05% aqueous trifluoroacetic acid. Freeze-drying afforded the following amounts: pluraflavin A: 18 mg, ESI+ MS: 823 Da $(M+H)^+$; pluraflavin B: 18 mg, ESI+ MS: 841 Da $(M+H)^+$, pluraflavin C: 11 mg, ESI+ MS: 974.8 Da $(M+H)^+$, 844.7 $(M-\text{oliose})^+$, 831.8 $(M-\text{vancosamine})^+$, 701.8 $(M-\text{vancosamine}-\text{oliose})^+$. UV λ_{max} (pH 7.0 phosphate buffer/acetonitrile 1 : 1): 215, 243, 271 (sh), 426 nm. HPLC (see Table 1), retention time: 7.2 minutes, elution with 52% acetonitrile. IR ν_{max} (KBr): 3424, 1648, 1465, 1378, 1065, 1014 cm^{-1}; pluraflavin E: 5 mg, ESI+ MS: 712.7 Da $(M+H)^+$, 582.5 $(M-\text{oliose})^+$; ESI-MS: 711 $(M-H)^-$, 667 $(M-CO_2)^-$.

NMR Spectroscopy

NMR samples of pluraflavin A were prepared by dissolving 2 mg of compound in 0.5 ml methanol-d_4 and 4 mg in 0.5 ml DMSO-d_6. For pluraflavin B 8 mg of compound, and for pluraflavin E 2.5 mg, was dissolved in 0.5 ml methanol-d_4. Wilmad 5 mm NMR tubes (grade 528) were used.

All spectra were recorded either on a BRUKER DRX600 spectrometer operating at 600.13 MHz (^{1}H) and 150.92 MHz (^{13}C) or on a BRUKER DRX500 spectrometer operating at 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C). The temperature was set at 300 K for all samples. All experiments were performed in phase-sensitive mode using standard pulse sequences employing time-proportional phase incrementation (TPPI) for quadrature detection in F_1[10].

For the homonuclear experiments (double quantum-filtered [DQF]-COSY[11~13], ^{1}H-^{1}H total correlation spectroscopy (TOCSY)[14], ROESY[15]) the spectral width was set at 10 ppm (methanol-d_4) or 14 ppm (DMSO-d_6) in both dimensions (F_1 and F_2). 512 increments in t_1 were recorded with 2048 complex data points in t_2. 8, 16, 32, or 64 transients were averaged for each t_1 value, depending on the sample concentration. In the TOCSY experiments an

MLEV17 spin-lock sequence was applied for 80 ms at a B_1 field strength of 10 kHz. ROESY spectra were recorded with a mixing time of 150 ms and a B_1 field strength of 3 kHz.

For the heteronuclear multiple quantum correlation (HMQC) spectra[16] 512 increments with 8, 16, 32, or 64 scans each in t_1 and 2048 complex data points in t_2 were collected using a sweep width of 10 ppm in the proton dimension and 160 ppm in the carbon dimension. A bilinear rotation-decoupling (BIRD) pulse was applied to suppress magnetization of protons bound to ^{12}C. A delay of 3.45 ms, corresponding to 145 Hz, was used for the evolution of ^1H-^{13}C one-bond couplings. The heteronuclear multiple bond correlation (HMBC) spectra[17] were acquired with a sweep width of 10 ppm (methanol-d_4) or 14 ppm (DMSO-d_6) in the proton dimension and 220 ppm in the carbon dimension. 512 increments in t_1 and 2048 complex data points in t_2 were recorded. The delay for the evolution of long-range correlations was set to correspond to a $^nJ_{CH}$ value of 7 Hz and 32, 64, or 96 scans per increment were collected.

Before undergoing Fourier transformation, all 2D time domain data were subjected to apodization using adjusted sine and squared sine bell window functions. The software packages XWINNMR and AURELIA (Bruker, Rheinstetten, Germany) were used for data processing.

Mass Spectrometry

Mass spectrometric studies were performed on Finnigan/MAT LCQ and TSQ 700 instruments. MS and MS/MS spectra were obtained by electrospray ionization (ESI) in positive and negative mode.

Biological Properties

The glucose-6-phosphatase gene transcription assay was carried out as described[18].

Cell line: A 605 bp oligonucleotide from the promoter region of the human glucose 6 phosphatase gene[18] has been inserted into a firefly luciferase reporter vector (pGL3-Basic/Neo, *Promega*) and transfected into H-4-II-E cells (ATCC CRL-1548) to form a stable construct. A monoclonal line has been isolated and expanded. The cells were grown in suspension.

Assay: Cells were seeded in a white 96-well plate at a density of 30,000 cells/well in 140 μl medium (MEM, GIBCO BRL/Life Technologies) supplemented with 10% FBS. Dosing of cells with test compounds was done 24 hours after seeding. Test compounds were diluted in the medium without FBS. 20 μl of the solutions of the test compounds were added followed by a subsequent 16~24 hours incubation period (37°C, 5% CO_2).

Determination of luciferase activity was carried out using the 'Steady-Glo™ Luciferase Assay System' from *Promega* [Steady-Glo™ Luciferase Assay System (Cat. No. E2510)]. After removing the medium, 100 μl luciferase-reagent was added to each well. The relative light units were determined using a Microplate Scintillation Counter.

Cell Proliferation Assay

All cell lines were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum, penicillin/streptomycin and supplemented with L-glutamine (Life Technologies). Cells were plated into 96-well tissue culture plates at the following densities (MDA-MB-435 −2500 cells/well, PC-3 −2500 cells/well, Colo 205 −1000 cells/well, NCI-H460 −1000 cells/well, HL-60 −2500) and allowed to adhere overnight at 37°C and 5% CO_2. Suspension cells (*e.g.* HL-60) were plated the same day as the assay. The seeding numbers of cells/well had been optimized previously for each cell line. The exposure time for compounds in the assay had also been optimized. Flavopiridol was typically diluted at a concentration range of 2.0 to 0.001 μM. Compounds were tested in triplicate at all concentrations. The cells were washed with fresh media and the compounds added to a final volume of 100 μl. The cells were then incubated with the compound at 37°C and 5% CO_2 for approximately 72 houres.

At the desired time point, 25 μl of a solution 10 mg of thiazolyl blue (MTT, 1-[4,5 dimethylthiazol-2-yl]-3,5-diphenylformazan, Calbiochem-Novabiochem, San Diego, USA) per ml HBSS (Hank's balanced salt solution, Sigma-Aldrich, St. Louis, USA) was added to each well. Plates were incubated at 37°C for 2~4 hours.

The MTT and media were aspirated from the wells and 200 μl of DMSO was added to each well. The plates were then read at 570 nm on a Spectra Max Plate Reader (Molecular Devices).

Results and Discussion

The strain *Saccharothrix* sp. DSM 12931 was isolated from a Turkish soil sample. It possesses a brownish red substrate mycelium and ivory colored aerial mycelium.

Analysis of the cell wall fatty acid composition revealed a predominance of 15:0 *anteiso*, 15:0 *iso*, 16:0 *iso*, 17:0 *anteiso*, and 17:0 *iso* structural units, a composition characteristic of the genus *Saccharothrix*[9].

The pluraflavins were obtained by fermenting the strain

Table 1. Physico-chemical properties of pluraflavin A, B, and C.

	Pluraflavin A	B	E
Appearance	dark orange solid	dark orange solid	dark orange solid
Molecular formula	$C_{43}H_{54}N_2O_{14}$	$C_{43}H_{56}N_2O_{15}$	$C_{36}H_{41}NO_{14}$
Molecular weight	822.91	840.93	711.73
ESI-MS (*m/z*) found:	823 $(M+H)^+$, 821 $(M-H)^-$	841.8 $(M+H)^+$	712.7 $(M+H)^+$
HRFAB-MS (*m/z*) found:	823.3706 $(M+H)^+$	841.3754 $(M+H)^+$	712.2591 $(M+H)^+$
calcd.:	823.3653	841.3759	712.2600
UV λ_{max}, nm			
0.1% H_3PO_4/MeCN	214, 243, 426	212, 242, 426	214, 245, 426
$NaHPO_4$/MeCN, pH 7	213, 243, 426	212, 242, 426	213, 245, 426
IR μ_{max} (KBr) cm^{-1}	3424, 1680, 1650 1464, 1203, 1131	3440, 2981, 2934 1652, 1464, 1065	3427, 2981, 1653 1462, 1277
Solubility: soluble in	MeOH, DMSO, EtOAc, dilute acid	MeOH, DMSO, EtOAc, dilute acid	MeOH, DMSO, EtOAc
HPLC[a] retention time:	10.3 min	7.9 min	4.5 min
at % MeCN:	62%	56.2%	38.5%

[a] Column, Superspher® 100, RP-18 endcapped, 250-4 (E. Merck), gradient elution of 15% acetonitrile (MeCN) in 25 mM pH 7.0 sodium phosphate buffer to 62% MeCN in 25 mM pH 7 sodium phosphate buffer in 9 min, followed by isocratic elution with 62% MeCN in 25 mM NaH_2PO_4 buffer. The flow rate was 1 ml/minute at 40°C. Detection: UV absorbance at 210 and 425 nm.

Saccharothrix sp. DSM 12931 for 94 hours at 28°C in a nutrient medium containing 1.5% glucose, 1.5% soy meal, and 0.5% cornsteep solid. The course of the fermentation was monitored by means of the glucose-6-phosphatase gene transcription assay[18]. Strong inhibitory activity was observed from 40 hours onwards, the inhibitory compounds being present mainly in the culture filtrate.

The 80 liter of culture medium was subjected to solid-phase extraction, size exclusion chromatography and reversed-phase preparative HPLC, yielding 18 mg of pluraflavin A, 18 mg of pluraflavin B, 11 mg of pluraflavin C, and 5 mg of pluraflavin E. Some physicochemical properties of the series of related compounds isolated are summarized in Table 1. Of the four compounds, pluraflavin C was the most abundant in the culture medium, but isolated yields were less satisfactory on account of its tendency to decompose in solution, which prevented us from establishing its structure unambiguously.

The UV spectra of the four isolated compounds all show characteristic UV absorption maxima at 215, 243, and 426 nm in neutral media and at pH 2. In alkaline or more strongly acidic conditions the pluraflavins undergo chemical transformations, so no details are given of these UV absorptions. The listed UV maxima correspond to those reported for the altromycins[4], the diagnostically characteristic bands in the IR spectrum at 1650 cm^{-1} likewise pointing to the presence of a hydroxyanthraquinone-γ-pyrone subunit. Molecular weights, determined by electrospray ionization mass spectrometry operating in both positive and negative modes, were 822 Da for pluraflavin A, 840 Da for pluraflavin B, 974 Da for pluraflavin C, and 711 Da for pluraflavin E.

Structure Elucidation

Pluraflavin A, B and E were investigated by a combination of 1D and 2D NMR techniques, which enabled us to establish their structure on the basis of ^1H, ^{13}C, DQF-COSY[11~13], TOCSY[14], ROESY[15], ^1H-^{13}C HMQC[16], and ^1H-^{13}C HMBC spectra[17]. Assignment of the proton and carbon resonances in methanol-d_4 was possible for all four compounds and in the case of pluraflavin A, also in DMSO-d_6 (Tables 1 and 2).

Pluraflavin A (Fig. 1) consists of four structural units: a 1-hydroxyanthraquinone-γ-pyrone aglycon (indomycinone),

Table 2. ^{13}C NMR chemical shift assignments for pluraflavins A, B, and E in methanol-d_4 and DMSO-d_6 (pluraflavin A only) at 300 K.

Position	Pluraflavin A DMSO	Pluraflavin A methanol	Pluraflavin B methanol	Pluraflavin E methanol
2	165.62	168.39	176.38	176.48
3	110.39	111.95	111.10	110.30
4	177.56	180.25	181.26	178.39
4a	124.33	126.08	125.96	124.69
5	148.22	150.44	150.43	149.56
6	119.05	121.34	121.03	121.17
6a	136.28	138.34	138.12	138.95
7	181.12	182.68	182.66	182.40
7a	131.18	133.23	133.16	133.21
8	118.29	120.05	120.04	119.82
9	134.41	135.63	135.52	135.61
10	135.79	137.35	137.44	137.04
11	159.39	161.06	161.02	161.33
11a	116.43	118.14	118.01	118.04
12	187.39	189.12	189.24	189.21
12a	120.35	122.00	121.89	121.59
12b	155.50	157.66	157.41	156.86
13	68.48	70.83	70.83	175.28
14	59.35	61.17	77.67	77.69
15	61.61	63.69	72.61	72.61
16	19.32	20.24	23.90	23.78
17	12.93	13.69	17.00	17.09
1'	96.57	98.90	98.87	-
2'	35.50	37.24	37.20	-
3'	56.33	58.32	58.39	-
3'Me	23.69	24.57	24.45	-
4'	68.65	71.15	71.08	-
5'	68.19	70.44	70.42	-
6'	16.75	17.21	17.19	-
1"	68.21	70.23	70.13	70.34
2"	24.79	28.00 br	27.74	27.35
3"	62.19	65.19	65.14	64.88
3"NMe	42.14, 39.25	43.24 br, 41.65 br	43.30, 41.40	42.5 br
4"	71.88	75.13	75.02	75.22
5"	70.22	72.55	72.61	72.29
6"	17.54	18.09	18.15	18.25
1"'	98.89	101.39	101.38	101.40
2"'	32.07	33.40	33.42	33.43
3"'	64.17	66.61	66.59	66.61
4"'	70.13	71.98	71.97	72.03
5"'	67.05	69.52	69.52	69.50
6"'	16.94	17.51	17.49	17.52

Table 3. ^{1}H NMR chemical shift assignments for pluraflavins A, B, and E in methanol-d_4 and DMSO-d_6 (pluraflavin A only) at 300 K.

Position	Pluraflavin A DMSO	Pluraflavin A Methanol	Pluraflavin B Methanol	Pluraflavin E Methanol
3	6.27 s	6.37 s	6.73 s	6.69 s
6	8.43 s	8.61 s	8.52 s	7.92 s
8	7.78 d	7.86 d	7.81 d	7.60 d
9	7.87 d	7.90 d	7.83 d	7.74 d
11-OH	13.38 s, br	-	-	-
13	5.30 d, 5.51 d	5.38 d, 5.63 d	5.36 d, 5.60 d	-
15	3.48 q	3.41 q	4.40 q	4.35 q
16	1.86 s	1.92 s	1.67 s	1.62 s
17	1.22 d	1.27 d	1.35 d	1.31 d
1'	5.06 d, br	5.04 dd	5.05 dd	-
2'	1.87, 1.95	2.05, 2.12	2.09 m	-
3'Me	1.35 s	1.50 s	1.51 s	-
3'NH_2	8.16 s, br	-	-	-
4'	3.17 d	3.27 s, br	3.29 s, br	-
4'-OH	5.47 d	-	-	-
5'	4.01 q	4.00 q	4.01 q	-
6'	1.18 d	1.34 d	1.33 d	-
1"	5.44 br	5.51 t	5.48 t	5.46 dd
2"	2.35 br, 2.78 br	2.48 m, 2.91 m,br	2.50 m, 2.88 m	2.86 m, 2.44 m
3"	3.43 br	3.45 m, br	3.46 m	3.50 m
3"NMe	2.89, 2.97	3.05 br, 3.05 br	3.01 s, 3.12 s	3.08
3"NH^+	8.78 br	-	-	-
4"	4.20 s, br	4.32 s, br	4.32 s, br	4.30 s, br
5"	3.75 q, br	3.97 q, br	3.97 q, br	3.87 q, br
6"	1.28 d	1.40 d	1.39 d	1.35 d
1"'	5.11 br	5.24 m, br	5.24 m, br	5.23 m
2"'	1.75, 1.92	2.05, 2.10	2.07 m	2.08 m, 2.06 m
3"'	3.96 m, br	4.12 m	4.13 m	4.12 m
3"'-OH	4.70 s, br	-	-	-
4"'	3.45 br	3.67	3.67 br	3.67 s, br
4"'-OH	4.44 s, br	-	-	-
5"'	4.10 q, br	4.16 q, br	4.16 q, br	4.17 q, br
6"'	1.10 d	1.28 d	1.27 d	1.28 d

a 2,6-dideoxyhexopyranose (oliose), and two 2,6-dideoxy-3-aminohexopyranoses (3'-*epi*-vancosamine, rhodosamine). For the indomycinone aglycon characteristic singlets are seen in the ^{1}H NMR spectrum at 6.37 ppm for proton H3 of the pyrone ring and 8.61 ppm for the isolated proton H6 of the anthraquinone system. As was reported for the altromycins, and in contrast to pluramycin, hedamycin and kidamycin[4,19~23], the anthraquinone nucleus is not substituted with an angolosamine unit at the C8 position. This is demonstrated by the observation of a proton signal for H8 that is coupled to H9 in an AB spin pattern. Comparison of the observed carbon chemical shifts with

Fig. 1. Structures of pluraflavins A, B, and E (see text for stereochemistry).

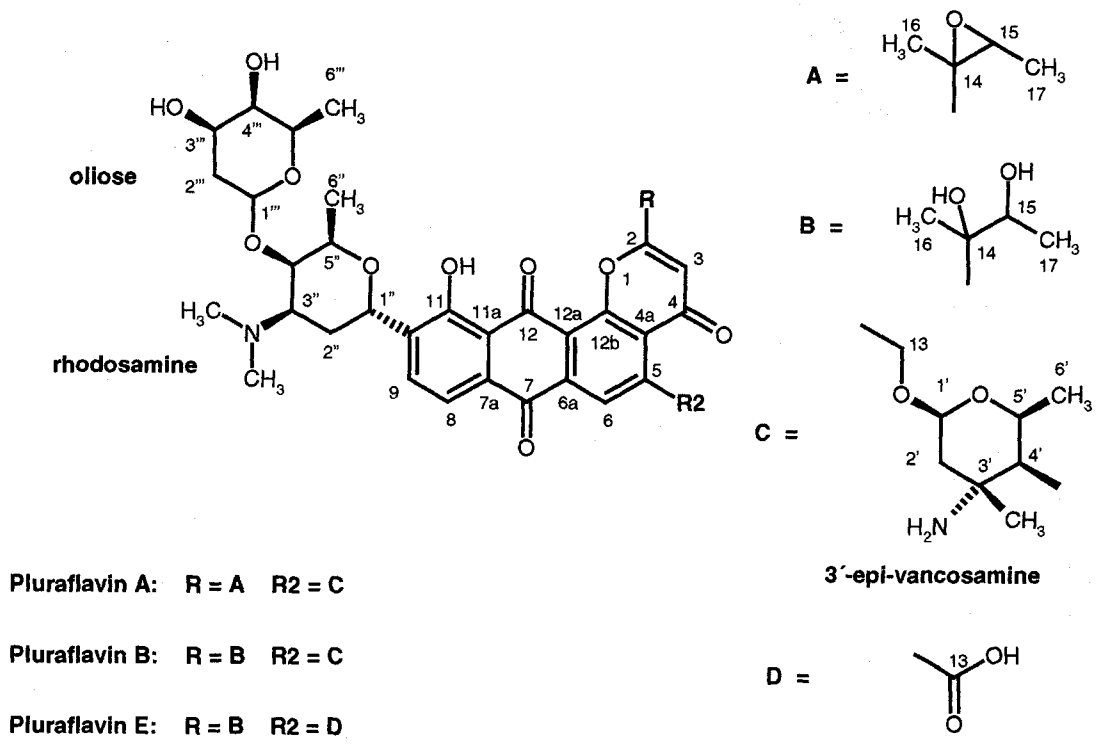
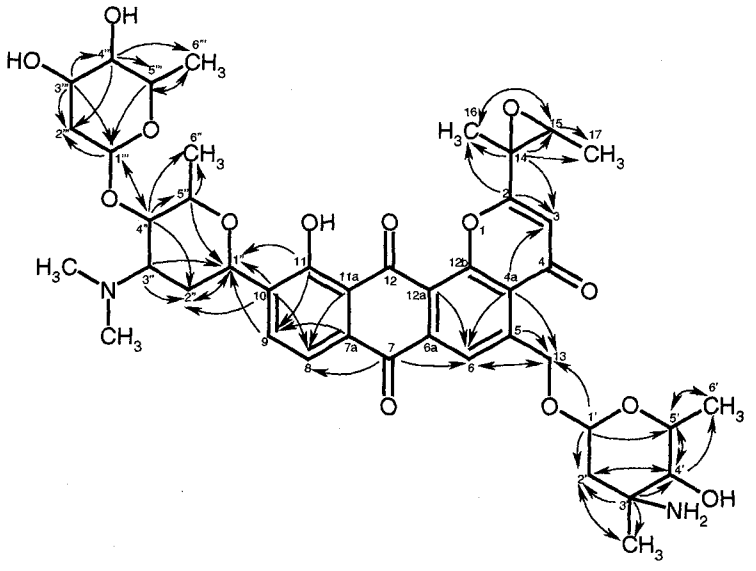


Fig. 2. ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum of pluraflavin A.

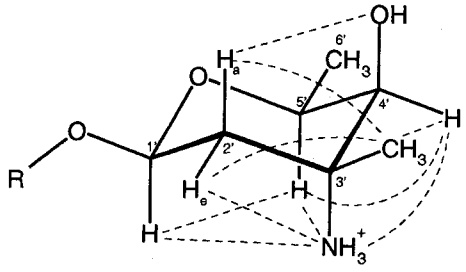


The arrows from the carbon atoms to the correlated proton indicate the correlations detected. Double-headed arrows indicate observation of both possible correlations.

published data and analysis of the one-bond and long-range ^{1}H-^{13}C heteronuclear correlation data (Fig. 2) showed the aglycon to have the same structure as that reported for altromycin D, thereby permitting assignment of all the carbon and proton resonances of the chromophore, including the epoxide moiety. For the dimethyl epoxide side chain a strong NOE correlation between H15 and 16-CH_3 and the absence of a cross peak between the protons of the two methyl groups 16-CH_3 and 17-CH_3 in the ROESY spectrum strongly suggests that these two methyl groups are oriented *trans* to one another.

Pluraflavin A shows a major structural variation from known pluramycins and altromycins in its substitution pattern at C13. Whereas this position is occupied by a methyl group in the case of the pluramycins and by a quaternary carbon substituted with a carboxymethyl group, a hydroxyl group, and a neutral *C*-glycosidic sugar in altromycins A~D and G, in pluraflavin A it is a methylene group substituted with a basic *O*-glycosidic sugar. This is evidenced by two geminally coupled protons at 5.38 and 5.63 ppm ($^{2}J_{HH}$=18 Hz) bound to a carbon atom resonating at 70.83 ppm. In the HMBC spectrum ^{1}H-^{13}C long-range correlations are observed between carbons C4a, C5, and C6 of the indomycinone aglycon and both methylene protons, confirming the connection to carbon C5. In addition, a correlation between carbon C1′ and 13-CH_2 establishes the presence of an *O*-glycosidic linkage of C13 to a basic sugar, which was identified as 3′-*epi*-vancosamine. In the DQF-COSY and TOCSY spectra two proton spin systems (H1′/H2′) and (H4′/H5′/H6′) are observed for this sugar residue, which are linked to the quaternary carbon C3′ by ^{1}H-^{13}C long-range correlations. C3′ is further substituted with a methyl and an amino group, as demonstrated by the HMBC spectrum and by its ^{13}C chemical shift (58.32 ppm). In DMSO the 3′-amino function is protonated, resulting in a ^{1}H NMR signal at 8.16 ppm with an intensity of 3H. The relative stereochemistry of this moiety was determined from the NOE cross peaks observed in the ROESY spectrum (Fig. 3) and from the coupling constant data. A 1-3-5 triaxial series of NOE correlations is seen between H1′, H5′, and the 3′-amino protons. In addition, there is a 1~3 diaxial cross peak between H2″$_a$ and 4′-OH and an NOE between H2′$_e$ and 3′-NH_3^+. These data, together with the vicinal coupling constants $^{3}J_{H1'/H2'e}$=2.0 Hz, $^{3}J_{H1'/H2'a}$=9.8 Hz and $^{3}J_{H4'/H5'}$=1.0 Hz, lead to the configuration depicted in Fig. 3 which is identical to that of the 3′ epimer of vancosamine. Although the structure shows the sugar in the absolute stereochemistry (C1′, C3′, C4′, C5′=*S*, *R*, *S*, *S*) expected for vancosamine, there are no data in support of this supposition, nor are any data available on the absolute stereochemistry of the dimethyl epoxide moiety described earlier.

Fig. 3. NOE correlations and derived relative stereochemistry for the 3′-*epi*-vancosamine fragment.

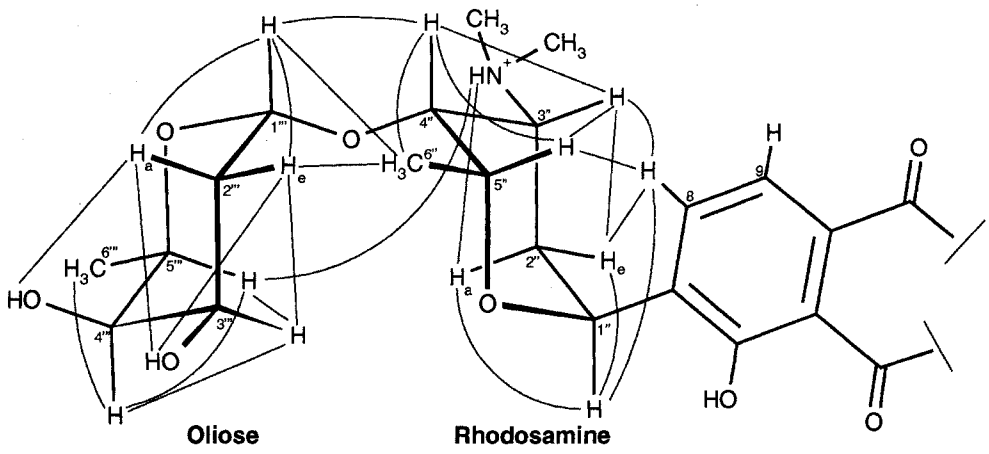


Observed NOEs are indicated by dotted lines. For reasons of clarity only those correlations relevant to the relative stereochemistry are shown.

As is the case for the altromycins, the anthraquinone aglycon is further substituted with a disaccharide moiety at carbon C-10, through a *C*-glycosidic linkage. From the DQF-COSY, TOCSY and ROESY spectra and ^{1}H-^{13}C one-bond (HMQC) and long-range (HMBC) correlation spectra, the two sugars were identified as rhodosamine and oliose. Rhodosamine has a pseudoanomeric carbon at 70.23 ppm and is connected to the anomeric carbon (101.39 ppm) of oliose by an (1-4)-*O*-glycosidic bond. Evidence for this linkage is provided by a strong NOE between protons H4″ and H1‴ and by $^{3}J_{CH}$ correlations in the HMBC spectrum. As with the 3′-*epi*-vancosamine residue, the relative stereochemistry of the disaccharide was determined by the observed NOE (Fig. 4) and *J*-coupling data. For rhodosamine a 1~3 diaxial cross peak between H3″ and H5″ is seen in the ROESY spectrum. In addition, both H3″ and H5″ exhibit NOE correlations to H4″, indicating an all-*cis* configuration for these three protons. H1″ shows cross peaks to H2″$_e$ and H2″$_a$ of almost equally intensity, whereas H2″$_e$ shows an NOE connectivity only with H3″, and H2″$_a$ only with 3″-NH^+. This data, in combination with the coupling constants $^{3}J_{H1''/H2''e}$=4.0 Hz, $^{3}J_{H1''/H2''a}$=6.0 Hz, $^{3}J_{H2''a/H3''}$=12.0 Hz, $^{3}J_{H3''/H4''}$<1.5, and $^{3}J_{H4''/H5''}$<1.5, allowed us to assign the relative configuration, as shown.

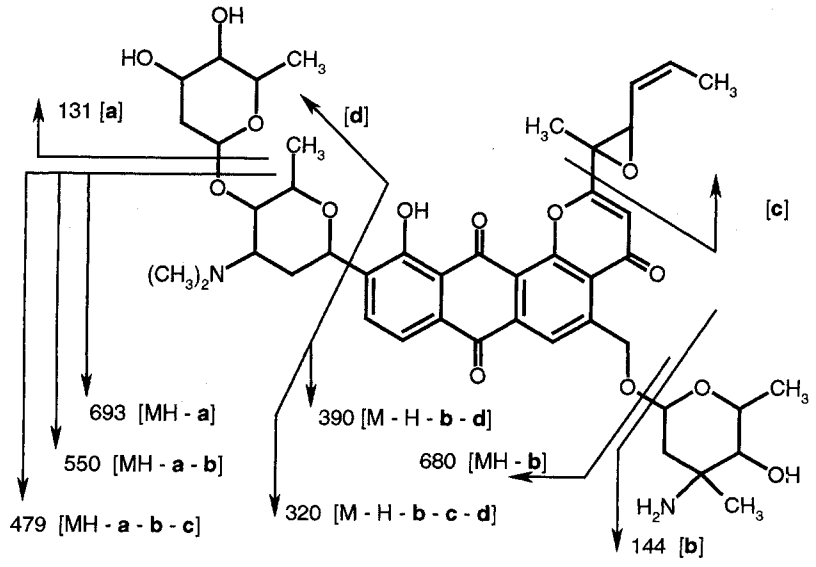
Almost the same set of NOE correlations is observed for the third sugar residue: a 1~3-diaxial cross peak between H3‴ and H5‴ and a correlation of both protons to H4‴, cross peaks from H1‴ to H2‴$_e$ and H2‴$_a$ of almost equally intensity, and connectivities only between H2‴$_e$/H3‴ and H2‴$_a$/3‴-OH. Taking into account the observed *J* coupling

Fig. 4 NOE correlations and derived relative stereochemistry for the disaccharide moiety.



Observed NOE are indicated by dotted lines. For reasons of clarity only those correlations relevant to the relative stereochemistry are shown.

Fig. 5. MS fragmentation pattern of pluraflavin A under ESI+ conditions.



The fragments *m/z* 390 and 320 were obtained in negative mode.

constants $^{3}J_{H1'''/H2'''}$=4 Hz and 1.5 Hz, $^{3}J_{H2'''a/H3'''}$=11 Hz, $^{3}J_{H2'''e/H3'''}$=5 Hz, $^{3}J_{H3'''/H4'''}$=3 Hz, and $^{3}J_{H4'''/H5'''}$<1.5 Hz, we were able to identify this residue as oliose. Assuming that both sugar residues of the disaccharide have a D-configuration, the absolute stereochemistry is shown in Fig. 4. Although this contention is not backed up by any firm evidence as such, from the observed interresidual NOE correlations the two residues clearly both have either a D- or L-configuration.

The chemical structure of pluraflavin A deduced from the NMR analyses was confirmed by mass spectrometric studies. High-resolution positive electrospray ionization

(ESI$^+$) mass spectra showed a signal at m/z=823.370631 $(M+H)^+$, which is in good agreement with the chemical formula $C_{44}H_{55}N_2O_{14}$ (deviation 5.0 milli-atomic mass units [milli-amu]). The number of double bond equivalents in the neutral molecule is 18, which is consistent with the determined chemical structure. ESI$^+$ daughter ion scan spectra revealed signals at m/z=693, 680, 550, 479, 144, and 131, which correspond to the fragments depicted in Fig. 5. In the negative mode fragments with m/z=390 and 320 were observed.

The structure of pluraflavin B was established through comparison of the ^{1}H and ^{13}C NMR data with those obtained for pluraflavin A, major chemical shift differences being observed only for carbon C2 and the epoxide side chain. In particular, the carbon resonances C2, C14, and C15 are shifted significantly downfield, showing signals at 176.38, 77.67, and 72.61 ppm respectively. A downfield shift is likewise observed for the ^{1}H NMR signal of H15 (3.41 to 4.40 ppm). In the high-resolution ESI$^+$ spectrum a signal was detected at m/z=841.375440 $(M+H)^+$, which is in good agreement with the molecular formula $C_{44}H_{57}N_2O_{15}$ (deviation 0.5 milli-amu). This finding is consistent with a formal addition of H_2O and, considered alongside the NMR observations, we arrived at the diol structure depicted in Fig. 1 for pluraflavin B. Since almost identical ^{1}H and ^{13}C chemical shifts were observed for the rest of the molecule, the relative stereochemistry of the sugar moieties must be the same as that determined for pluraflavin A.

In the MS-MS studies pluraflavin B likewise shows a characteristic fragmentation pattern analogous to that of pluraflavin A, providing further confirmation of the structure postulated on the basis of the NMR data. The following intense ESI+ fragments were detected: m/z=711 [M−oliose]$^+$, 568 [M−oliose−*epi*-vancosamine]$^+$, 144 [*epi*-vancosamine]$^+$, 131 [oliose]$^+$ and in negative mode: m/z=[M−butanediol]$^-$, and 539 [M−oliose−methyl-*epi*-vancosamine]$^-$.

Pluraflavin E contains the same diol side chain as pluraflavin B, but is lacking the 3′-*epi*-vancosamine residue (Fig. 1). In addition, the CH_2–O moiety (70.83 ppm) at position C13 is replaced by a carboxylic acid moiety (175.28 ppm). This result was confirmed by the mass spectrometric determination of a molecular weight of 712. The relative configuration of the remaining disaccharide unit is identical to that of pluraflavins A and B, as is evident from the chemical shift data.

Pluraflavins A, B, and E are novel compounds that can be classified as members of the pluramycin class. Structurally, they are closely related to the altromycins, but with an aminosugar in the 13 position instead of a hydroxy-anthraquinone-γ-pyrone subunit. Pluraflavins A and B were found to contain a 4-*epi*-vancosamine unit[24] in this position, an aminosugar seen up to now only in glycopeptide antibiotics. Also observed for the first time, in pluraflavin E, is a carboxyl group at position 5 of the indomycinone aglycon. As yet unclarified in these studies is the absolute configuration of the pluraflavins; further studies are likewise necessary to establish the structure of the unstable pluraflavin C.

A key structural characteristic of pluraflavin A is the dimethyl epoxide side chain at position 2 of the anthraquinone-γ-pyrone subunit, which is responsible for the reactivity of the compound and forms the basis for its expected high activity against double-strand DNA and RNA.

Biological Properties

Pluraflavin A is an inhibitor of glucose-6-phosphatase gene transcription with an IC_{50} of <50 nM, its cytostatic activity being more pronounced.

Pluraflavin A was tested on a number of tumor cell lines and demonstrated excellent activity in the cell anti-proliferation assay. The following results were obtained:

Cell line	IC_{50} (nM)
MDA-MB 435 (breast)	<23
PC-3 (prostate)	10
Colo 205 (colon)	0.35
NCI-H460 (lung)	1
HL-60 (leukemia)	0.08

Flavopiridol[25] was in each case tested as the reference compound, with results in the 50~100 nM range. The results for pluraflavin A demonstrate anti-proliferative activity against all of the cell lines tested. Several of these cell lines including Colo205 and HL60 appear to be more sensitive to this compound. Pluraflavin A had been tested under the conditions outlined in the Materials and Methods section, however, it is possible that encouraging results could be obtained with shorter compound-cell incubation times.

The antiproliferation assay is a common method to evaluate potential chemotherapeutic agents. Pluraflavin A was tested against a number of tumor cell lines and demonstrated significant activity in this assay. Flavopiridol, a known cyclin-dependent kinase inhibitor[25], was also tested as the reference compound in these yielding IC_{50} values of 50~100 nM for all cell lines tested. Pluraflavin A demonstrated potent activity against these tumor cell lines with the Colo-205 and HL-60 cells showing an apparent sensitivity to this compound. Further analysis of pluraflavin

A against an expanded set of tumor cell lines will be necessary to confirm the sensitivity of a specific tumor cell type. Clearly, this compound is showing activity that is significant relative to flavopiridol. In addition, preliminary data also suggests that pluraflavin A is active against a number of cell lines in a soft agar assay (data not shown). This compound may have considerable potential as chemotherapeutic agent. The activity of pluraflavin A in combination with other chemotherapeutic agents has yet to be addressed both *in vitro* and *in vivo*. This may be a promising compound for anti-tumor therapy.

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