AEROCAVIN, A NEW ANTIBIOTIC PRODUCED BY *CHROMOBACTERIUM VIOLACEUM*

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A new antibiotic, aerocavin, has been isolated from fermentation broths of a non-pigmented strain of *Chromobacterium violaceum*. The structure **1** was deduced from its spectroscopic properties and X-ray diffraction analysis. Aerocavin exhibits activity *in vitro* against Gram-positive and Gram-negative bacteria.

In the course of screening for antibiotics with activity against *Staphylococcus epidermidis*, we found a new natural product, aerocavin. In this paper we describe the taxonomy of the producing organism, fermentation, isolation, structure and physico-chemical properties of the compound including its biological properties.

Taxonomy

The organism producing aerocavin was isolated from a water sample collected in Allamuchy Mountain State Park, New Jersey. It is a Gram-negative rod that is motile by means of monotrichous polar flagella. Lateral to sub-polar flagella are occasionally seen. The organism is cytochrome oxidase-positive, metabolizes glucose fermentatively without the production of gas, and is resistant to the vibriostat, 2,4-diamino-6,7-diisopropylpteridine. These characteristics are shared by members of both *Aeromonas* and *Chromobacterium*.

Our organism is non-pigmented and was found to share key differentiating properties with both genera making proper generic assignment by conventional microbiological tests difficult. Non-pigmented strains of *Chromobacterium* have been reported and are frequently mistaken for aeromonads[1].

The DNA dot blot test was used to resolve this impasse. DNA was isolated from the aerocavin-producing strain and was labeled with ^{32}P by nick translation by the method of FEINBERG and VOGELSTEIN[2]. This was used as the probe against DNA from reference cultures of *Aeromonas* and *Chromobacterium* that had been immobilized on nitrocellulose filters. Hybridization was carried out in 3×SSC with formamide at 37°C overnight[3]. Upon completion of hybridization, the unbound probe was removed and the filters were washed to remove any residual unbound DNA. The filters were dried and assayed by autoradiography. The probe was found to bind only to DNA from pigmented and non-pigmented strains of *Chromobacterium*. Based on this evidence our culture is considered to be a non-pigmented strain of *Chromobacterium violaceum*. It has been deposited with the American Type Culture Collection under accession number ATCC 53434.

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Fermentation

Seed cultures of *C. violaceum* ATCC 53434 were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: Yeast extract 0.4%, malt extract 1.0% and glucose 0.4% in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm, 5.1-cm stroke) for approximately 24 hours.

A 1-% transfer was made from the seed culture flasks to 500-ml Erlenmeyer flasks containing 100 ml portions of the following medium: Yeast extract 1.0%, malt extract 1.0%, peptone 0.1% and glucose 2.0% in distilled water. The flasks were incubated at 25°C for approximately 24 hours, with the same operating conditions as described for the germinator flasks. At the end of the incubation period, the contents of the flasks were pooled and the pooled broth centrifuged to remove the bacterial cells.

Isolation

Aerocavin was isolated from the fermentation broths as outlined in Fig. 1. The antibiotic from the broth supernate was extracted into ethyl acetate at pH 5.5. Further purification was effected by a combination of chromatography on silicic acid, Sephadex LH-20 and cellulose followed by crystallization from ethyl acetate - heptane.

Physico-chemical Properties and Structure

Aerocavin (**1**) is a colorless acidic antibiotic. It is substantially soluble in methanol, acetone, ethyl acetate, less soluble in heptane and insoluble in water. It has an Rf of 0.2 on silica gel eluting with ether - ethyl acetate (7 : 3) and gives positive color reaction to phosphomolybdic acid spray.

Fig. 1. Isolation of aerocavin.

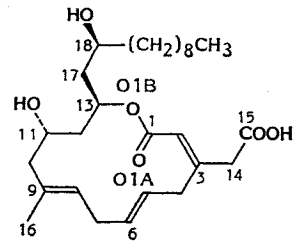
Broth supernate
1) extraction with EtOAc at pH 5.5
2) chromatography on silicic acid eluting with $CHCl_3$
3) chromatography on Sephadex LH-20 in MeOH - $CHCl_3$ - heptane (1 : 3 : 6)
4) chromatography on cellulose eluting with heptane followed by heptane - ether (1 : 1)
5) chromatography on cellulose eluting with heptane - ether (1 : 1)
6) recrystallization from EtOAc - heptane
Aerocavin

Table 1. MS data for aerocavin.

Solvent	Positive-ion mode	Negative-ion mode
Thioglycerol	573 $(M+H+T)^+$, 555 $(M+H+T-H_2O)^+$, 465 $(M+H)^+$, 447 $(M+H-H_2O)^+$, 429 $(M+H-2H_2O)^+$	571 $(M-H+T)^-$, 527 $(M-H+T-CO_2)^-$, 463 $(M-H)^-$, 419 $(M-H-CO_2)^-$
Dithiothreitol and dithioerythritol	619 $(M+H+D)^+$, 601 $(M+H+D-H_2O)^+$, 465 $(M+H)^+$, 447 $(M+H-H_2O)^+$, 429 $(M+H-2H_2O)^+$	617 $(M-H+D)^-$, 573 $(M-H+D-CO_2)^-$, 463 $(M-H)^-$, 419 $(M-H-CO_2)^-$

T; Thioglycerol, D; dithiothreitol+dithioerythritol.

The molecular weight and empirical formula were determined by fast atom bombardment (FAB)[4] mass spectrometry; the mass spectral data are given in Table 1. In thioglycerol as solvent matrix, a weak peak at *m/z* 465 $(M+H)^+$ was observed. The major fragment ion was at *m/z* 447 $(M+H-H_2O)^+$ in the positive-ion mass spectrum. The ions at *m/z* 573 and 555 corresponded to the solvent adduct and to the adduct minus water, respectively. Similarly, in the negative-ion mass spectrum, a weak peak at *m/z* 463 $(M-H)^-$ was observed; the major ions were at *m/z* 571 $(M-H+solvent)^-$, 527 $(M-H+solvent-CO_2)^-$ and 419 $(M-H-CO_2)^-$.



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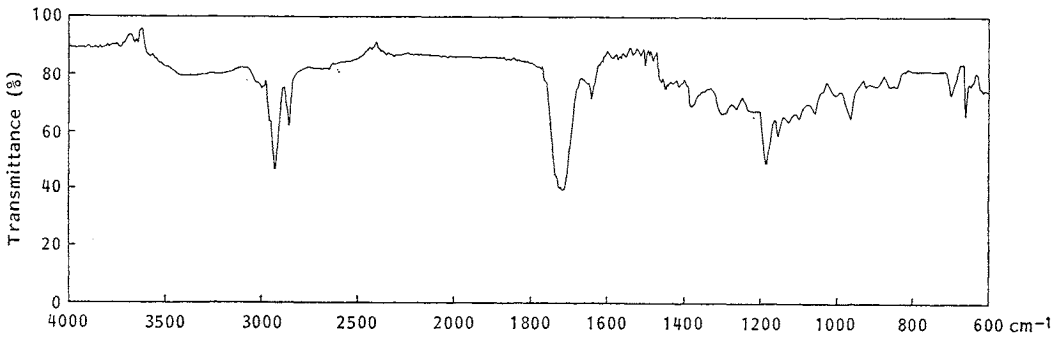
Similar results were obtained with dithiothreitol - dithioerythritol as solvent matrix. Accurate mass measurement for the $(M+H-H_2O)^+$ and $(M-H-CO_2)^-$ ions gave values of 447.312 and 419.316 corresponding to $C_{27}H_{43}O_5$ (calcd 447.311) and $C_{26}H_{43}O_4$ (calcd 419.316), respectively. This led to the empirical formula $C_{27}H_{44}O_6$ for aerocavin. This formula was supported by elemental analysis.

The UV spectrum in methanol showed an absorption maximum at 220 nm (ε 11,600) and no change in the absorption was observed with the addition of acid and base. The IR spectrum in $CHCl_3$, Fig. 2, exhibited bands at 1716 (carboxylic acid and α,β-unsaturated ester) and 1643 cm^{-1} (double bond). Aerocavin consumed 3 mol of hydrogen (5% Pd-C, EtOAc, 26 hours), indicating the presence of 3 double bonds. The presence of a carboxylic acid was demonstrated by the conversion of aerocavin to a monomethyl ester with diazomethane. The molecular formula, $C_{27}H_{44}O_6$, requires 6 double bond equivalents (DBE), 5 of which have been accounted for, suggesting that the remaining 1 DBE is from a cyclic system. The disposition of these functional groups in the macrocyclic structure was demonstrated through single-crystal X-ray analysis.

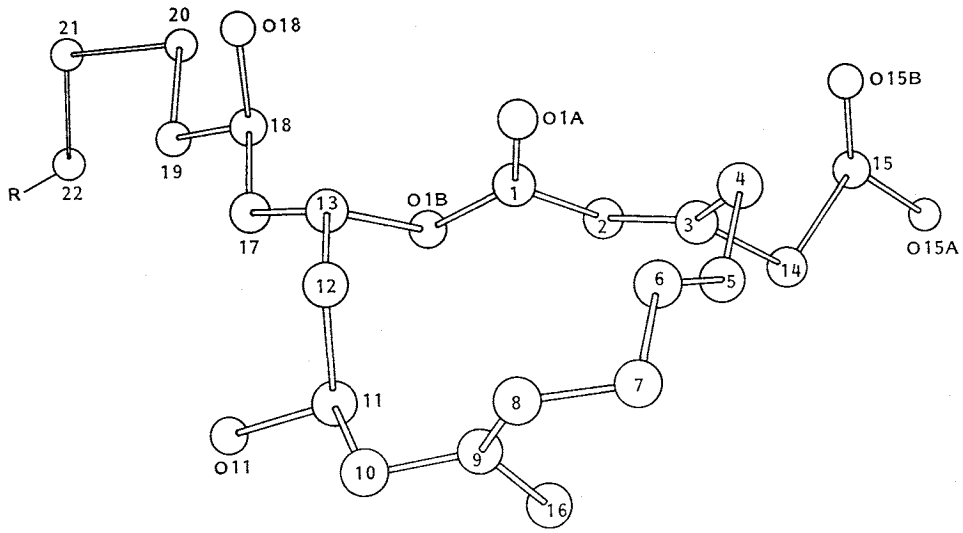
Aerocavin crystallizes from moist heptane as a monoclinic hemihydrate in which two independent molecules of the antibiotic are present in the asymmetric unit. *E*-Maps from direct methods clearly revealed the similar macrocyclic conformations of the two independent molecules, Fig. 3, as well as the position of the water of crystallization†. The positions of the three double bonds could be as-

Fig. 2. IR spectrum of aerocavin ($CHCl_3$).



† The measured crystal density suggested the additional presence of some heptane in freshly crystallized samples. The probable sites for the heptane within the crystal were identified, but the positions of the individual atoms were not resolved.

Fig. 3. X-Ray structure of aerocavin (absolute configuration unknown).



R=n-C_5H_{11} (not resolved in the analysis)

signed within the macrocycle on the basis of bond lengths and the expected coplanarity of double bonded atoms and their immediate substituents.

The α,β-unsaturated carbonyl moiety adopts an *S-cis* conformation with an approximately planar arrangement of the atoms C1-C4, O1A, O1B, and C14. The isolated disubstituted double bond and its substituents, atoms C4-C7, and the trisubstituted double bond with its substituent atoms, C7-C10 and C16, form two other relatively rigid planar links in the 14-membered macrolide ring. Two other substituents were also evident at chiral centers: A secondary hydroxyl group, O11, on the ring and another secondary hydroxyl group, O18, on the pendant carbon chain. The oxygen atoms were readily identified since all hydroxyl oxygen atoms (O11, O18 and O15A and the corresponding atoms of the second molecule, O11′, O18′ and O15A′) participate in an extensive hydrogen bonding network with the water molecule of crystallization, Ow, and the carbonyl oxygen atoms, O1A, O15B, O1A′ and O15B′.

Portions of the carbon chain beyond C22 were ill-defined in both molecules of the asymmetric unit. The chains extend into disordered hydrophobic regions of the crystal packing environment which probably also contain some ill-defined heptane molecules of crystallization.

The structure from the X-ray analysis (Fig. 3) includes all oxygen atoms, double bonds and rings, and accounts for all but five of the carbon atoms (C_5H_{11}) of aerocavin. Corroboration of this structure and the identification of the five missing atoms as an n-pentyl chain attached to C22 followed from an examination of the ^{13}C and ^{1}H NMR data.

The ^{13}C NMR data are given in Table 2. Multiplicities were determined using the INEPT[5] technique. The peaks at δ 165.2 and 174.0 are assigned to the carbons at positions 1 and 15, respectively. The two tertiary olefinic carbons at δ 152.1 and 133.7 are assigned to the carbons at positions 3 and 9, respectively. The three methine carbons at δ 69.1, 70.9, and 65.6 are assigned to the carbons at positions 11, 13, and 18; the assignments are interchangeable.

The proton chemical shifts, proton multiplet appearance, ^{1}H-^{1}H coupling constants and ^{1}H-^{1}H

Table 2. ^{1}H and ^{13}C NMR data for aerocavin.

Position No.	^{1}H NMR		^{13}C NMR	
	Chemical shifts (δ)[a]	Multiplicity	Chemical shifts (δ)[b]	Carbon type[c]
1	—	—	165.2	C
2	5.76	d	129.2	CH
3	—	—	152.1	C
4	2.50, 4.01	m, dd	39.5[d]	CH_2
5	5.26	ddd	120.6[e]	CH
6	5.37	ddd	124.9[e]	CH
7	2.50, 2.58	m, ddd	44.8[d]	CH_2
8	5.26	m	125.6[e]	CH
9	—	—	133.7	C
10	2.02, 2.25	dd, d	31.8[f]	CH_2
11	3.88	ddd	69.1[g]	CH
12	1.60, 2.22	ddd, dd	34.6[f]	CH_2
13	5.11	m	70.9[g]	CH
14	3.13, 3.20	d, dd	49.4[d]	CH_2
15	—	—	174.0	C
16	1.49	s	16.4	CH_3
17	1.88	m	37.1[f]	CH_2
18	3.66	m	65.6[g]	CH
19	1.45	m	38.4[f]	CH_2
20	1.26	m	22.6[h]	CH_2
21	1.26	m	25.4[h]	CH_2
22	1.26	m	29.2[h]	CH_2
23	1.26	m	29.5[h,i]	CH_2
24	1.26	m	29.5[h,i]	CH_2
25	1.26	m	29.5[h,i]	CH_2
26	1.26	m	30.9[h]	CH_2
27	0.87	t	14.0	CH_3

[a] ppm downfield from TMS using $CHCl_3$ (7.24 ppm) as internal standard.
[b] ppm downfield from TMS using $CHCl_3$ (77.0 ppm) as internal standard.
[c] Assignments are made by the INEPT technique.
[d~h] Assignments are interchangeable.
[i] The peak at δ 29.5 integrated for 3 carbons.

two-dimensional (2D) NMR connectivities are shown in Tables 2 and 3. The protons at C14 (δ 3.13 and 3.20) appear as an AB quartet, the A portion of which shows allylic coupling to the C2 proton (δ 5.76). This correlation is also seen in the 2D NMR spectrum. The geminal protons at C4 (δ 2.50 and 4.01) show a large difference in chemical shifts (1.5 ppm) and are coupled to the olefinic proton at C5 (δ 5.26). The olefinic protons at C5 and C6 (δ 5.26 and 5.37) appear as an AB quartet, both A and B parts of which are further split by protons at C4 (δ 2.50 and 4.01) and C7 (δ 2.50 and 2.58). The connectivity 4-5-6-7 is determined by 2D NMR data (Table 3). The protons at C7 (δ 2.50 and 2.58) are further coupled to the proton at C8 (δ 5.26). The proton at C11 (δ 3.88) is coupled to the protons at C10 (δ 2.02 and 2.25) and C12 (δ 1.60 and 2.22). The methine proton at C13 (δ 5.11) appears as a multiplet due to the splitting from the protons at C12 (δ 1.60 and 2.22) and C17 (δ 1.88). Similarly, the methine proton at C18 (δ 3.66) appears as a multiplet due to the splitting from the protons at C17 (δ 1.88) and C19 (δ 1.45). The triplet at C27 (δ 0.87) is coupled to the methylene at C26 (δ 1.26). The methylene protons at C20 through C26 appear as a broad singlet at δ 1.26. The singlet at δ 1.49 is assigned to the methyl group at C16.

Table 3. ^{1}H-^{1}H coupling constants and 2D connectivities for aerocavin.

Connection[a]	J (Hz)	2D connectivity
4a,5	4.7	+
4b,5	8.0	+
4a,4b	13.2	+
5,6	15.0	+
6,7a	5.2	+
6,7b	8.7	+
7a,7b	14.0	+
7a,8	9.6	+
7b,8	Not clear	+
10a,11	2.6	+
10b,11	11.0	+
10a,10b	11.3	+
11,12a	2.6[b]	+
11,12b	11.0[c]	+
12a,12b	12.0	+
12a,13	3.4[b]	+
12b,13	Not clear[c]	+
13,17a	8.6	+
13,17b	7.3	+
17a,17b	14.8	Not clear
17a,18	8.6	+
17b,18	7.3	+

[a] a,b: Geminal protons.
[b,c] Interchangeable.

Table 4. Antibacterial activity of aerocavin.

Organism	MIC (μg/ml)[a]
Staphylococcus aureus SC1276	6.3
S. aureus SC2399	6.3
S. aureus SC10016 (tetracycline^{R})[b]	3.1
S. aureus SC9593 (penicillin^{R})	6.3
S. aureus SC10820 (erythromycin^{R})	3.1
S. epidermidis SC9052	6.3
S. epidermidis SC10547 (penicillin^{R})	3.1
Escherichia coli SC8294	100.0
E. coli SC10857	12.5
Pseudomonas aeruginosa SC9545	25.0
Acinetobacter calcoaceticus SC8333	12.0

SC No. is the number in the microorganism collection of E.R. Squibb & Sons, Inc., Princeton, New Jersey.
[a] MICs were determined at 37°C by agar dilution; 10^4 cfu.
[b] (^{R}) indicates that the organism is resistant to the antibiotic named.

The 2D nuclear Overhauser effect spectroscopy (NOESY) spectrum indicates that the methyl group at C16 is close to the protons at C7 and C10, strengthening the assignment.

All double bonds of aerocavin have the *E* configuration. With respect to the macrocyclic ring, substituents C14, C16, and C17 are *syn* to one another and *anti* to O11 and O1A. The optical rotation of aerocavin is $[\alpha]_D^{22}$ +25.1° (*c* 0.9, MeOH); however, the absolute configuration has not been determined. The configuration at chiral centers C11, C13, and C18 is therefore either *R,S,R* or *S,R,S*, respectively.

The formal chemical name of aerocavin is [3*E*,6*E*,9*E*,12α,14β(*R**)]-12-hydroxy-14-(2-hydroxyundecyl)-10-methyl-2-oxooxacyclotetradeca-3,6,9-triene-4-acetic acid.

Biological Properties

The antibacterial activity of aerocavin is given in Table 4. Aerocavin has moderate activity against Gram-positive bacteria. No activity against *Candida albicans* or *Saccharomyces cerevisiae* was observed in an agar diffusion plate assay at 100 μg of aerocavin per 6.3 mm paper disc. When tested in mice by intraperitoneal administration, aerocavin was found to have an LD_{50} of 80 mg/kg.

Experimental

^{1}H and ^{13}C NMR spectra were obtained on a Jeol GX 400 and FX 270 NMR spectrometer respectively, using a 5-mm probe. Quantitative ^{13}C spectra were collected with the decoupler gated off during the delay between acquisitions and with an appropriate delay between pulses. Correlation spectroscopy (COSY) spectra were obtained using a standard two pulse (90-τ_1-90) sequence. NOESY spectra were obtained with a three pulse sequence (90-τ_1-90-τ_2-90) for several values of τ_2. The IR

spectrum was recorded on a Perkin-Elmer model 1420 spectrometer. The UV spectrum was recorded on a Shimadzu UV-260 spectrometer. MS were determined on a VG Analytical Model ZAB 1F spectrometer. The optical rotation was measured on a Perkin-Elmer Model 241 polarimeter. Isolation was monitored by agar diffusion assay on *Staphylococcus epidermidis* or *Staphylococcus aureus* and by TLC on silica gel [Et_2O - EtOAc (7 : 3), Rf 0.2].

Isolation of Aerocavin

At harvest, the cells from a 160-liter fermentation of *C. violaceum* were separated by centrifugation. The supernate, adjusted to pH 5.5 with 6 N HCl, was extracted with two 80-liter portions of EtOAc. The organic extract was concentrated to dryness *in vacuo* to give 15 g of a syrup. This was chromatographed on a silicic acid column (Fisher, 2.5×54 cm) packed in hexane - $CHCl_3$ (1 : 1). The column was eluted with hexane - $CHCl_3$ (1 : 1, 500 ml) followed by hexane - $CHCl_3$ (1 : 2, 500 ml) and $CHCl_3$ (3 liters). The active fractions were pooled and concentrated *in vacuo* giving 4 g of a residue. This residue, dissolved in 20 ml of MeOH - $CHCl_3$ - heptane (1 : 3 : 6), was then chromatographed on a Sephadex LH-20 column (2.5×50 cm) packed in the same solvent, eluting with the same solvent. Active fractions were combined and concentrated to give 1 g of a residue. The residue was dissolved in 20 ml of heptane and chromatographed on a Whatman CF 11 cellulose column (2.5×28 cm) packed in heptane. The column was eluted with heptane (500 ml) followed by heptane - ether (1 : 1, 500 ml). The concentrate of the pooled, active fractions was rechromatographed on a Whatman CF 11 cellulose column (2.5×25 cm) packed in petroleum ether (35~60°C). The column was eluted with petroleum ether (500 ml) followed by petroleum ether - heptane (1 : 1, 500 ml), heptane (500 ml), heptane - ether (1 : 1, 500 ml) and ether (500 ml). The active fractions were combined and concentrated *in vacuo* to give 200 mg of a crystalline residue. This was recrystallized from EtOAc - heptane (1 : 9) to give pure crystalline aerocavin (100 mg) mp 127°C.

X-Ray Diffraction Studies

The unit cell parameters were obtained through a least squares analysis of the experimental diffractometer settings of 15 reflections with $2\theta \geq 45°$, using CuK_α monochromatic radiation (λ=1.5418 Å): a=23.21(3), b=16.832(5), c=16.66(1) Å, β=97.14(9)°, V=6456(17) Å^3. Space group C2 was assigned on the basis of systematic absences hkl (h+k=2n+1) on Weissenberg and precession photographs. The measured crystal density, D_{OBS}=1.08(3) gcm^{-3}, measured by flotation in hexane - carbon tetrachloride mixtures, is consistent with an asymmetric unit containing $C_{61}H_{106}O_{18}$ as two molecules of **1**, 2×($C_{27}H_{44}O_6$), one molecule of heptane (C_7H_{16}), and a molecule of H_2O. For Z=4, D_{calc}=1.08 gcm^{-3}. A total of 3385 reflections were measured on a Syntex $P2_1$ diffractometer at 23°C with the θ-2θ variable scan technique and were corrected only for Lorentz-polarization factors. Some crystal decomposition, presumably due to partial desolvation, was observed during data collection.

Initial phase angles were obtained by direct methods[6]. The positions of atoms C1-C22, O1A, O1B, O11, O15A, O18, the corresponding atoms of the other independent molecule, and the water of crystallization, Ow, were obtained from E-maps and difference Fourier maps and refined together with isotropic temperature factors. The five remaining carbon atoms of the side chain were ill-defined for both molecules. These ten atoms were not included in structure factor calculations or least squares refinements. The full matrix least squares refinements were based on 1673 "observed" reflections with $I \geq 3\sigma(I)$. The least squares weights $w=\sigma^{-2}(F_o)$ were calculated with the assumption that $\sigma^2(I)=\varepsilon^2+(pI)^2$ where ε is a statistical counting error and p=0.04. The final R-factor is 0.12.

Tables of atomic coordinates, thermal parameters, bond distances, and bond angles have been sent to the Crystallographic Data Centre, Cambridge, England.

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