AEROCYANIDIN, A NEW ANTIBIOTIC PRODUCED BY *CHROMOBACTERIUM VIOLACEUM*

WILLIAM L. PARKER, MARLENE L. RATHNUM, JANICE H. JOHNSON, J. SCOTT WELLS, PACIFICO A. PRINCIPE and RICHARD B. SYKES†

The Squibb Institute for Medical Research, P.O. Box 4000, Princeton, New Jersey 08543-4000, U.S.A.

(Received for publication July 13, 1987)

Aerocyanidin, a new antibiotic containing an isonitrile group, has been isolated from fermentations of *Chromobacterium violaceum* ATCC 53434. Structure **1** was assigned on the basis of spectroscopic characterization of the antibiotic and of a degradation product that results from treatment with base. The antibiotic is primarily active against Gram-positive bacteria.

In the course of screening for new antibiotics with activity against Gram-positive bacteria, it was found that *Chromobacterium violaceum* ATCC 53434 produces two novel antibiotics. One of these, aerocavin, is the subject of the preceding paper[1] in which the producing organism is described. The other antibiotic, aerocyanidin, is the subject of this paper.

Fermentation

Seed culture was prepared by transferring loopfuls of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100-ml portions of Nutrient medium composed of Tryptone 0.5%, malt extract 0.3%, glucose 1.0% and yeast extract 0.3% in distilled water. The flasks were incubated for 24 hours at 25°C on a rotary shaker (300 rpm, 5.1-cm stroke) and the contents used to inoculate additional 100-ml portions of the same medium that were incubated under the same conditions. This growth was used as inoculum (1.5%) for 250 liters of medium consisting of Tryptone 0.5%, malt extract 0.3%, cerelose hydrate 1.1%, yeast extract 0.3% and Ucon-LB 625 0.05% in distilled water. The fermentation was carried out in a 380-liter stainless steel vessel for 24 hours at 25°C with an agitation rate of 130 rpm and an airflow of 285 liters/minute.

Isolation

The isolation of aerocyanidin was monitored by conventional paper-disc agar diffusion assay using *Staphylococcus aureus* FDA 209P as the test organism and also colorimetrically with a modified Sievert-Hermsdorf method[2]. The lability of this antibiotic caused considerable difficulty in devising an efficient isolation procedure. Silica gel chromatography was particularly unsuitable because of extensive decomposition on this adsorbent. However, a combination of countercurrent chromatography using a high-speed countercurrent chromatograph followed by reversed-phase chromatography on a macroreticular polystyrene resin proved highly effective in giving essentially pure aerocyanidin. The procedure outlined in Fig. 1 gave an overall recovery of activity from the crude ethyl acetate extract to the final crystalline antibiotic of 33% based on the colorimetric Sievert-Hermsdorf assay.

† Present address: Glaxo Group Research Ltd., Greenford, Middlesex, UK.

Fig. 1. Isolation of aerocyanidin.

Fermentation broth (260 liters)

↓ centrifugation, extraction of the supernate at pH 6 with EtOAc and concentration of the extract

Extract concentrate (87 g)

↓ distribution in hexane - toluene - MeOH - water (3 : 3 : 4 : 2) and concentration of the lower phase

Lower phase concentrate (4.1 g)

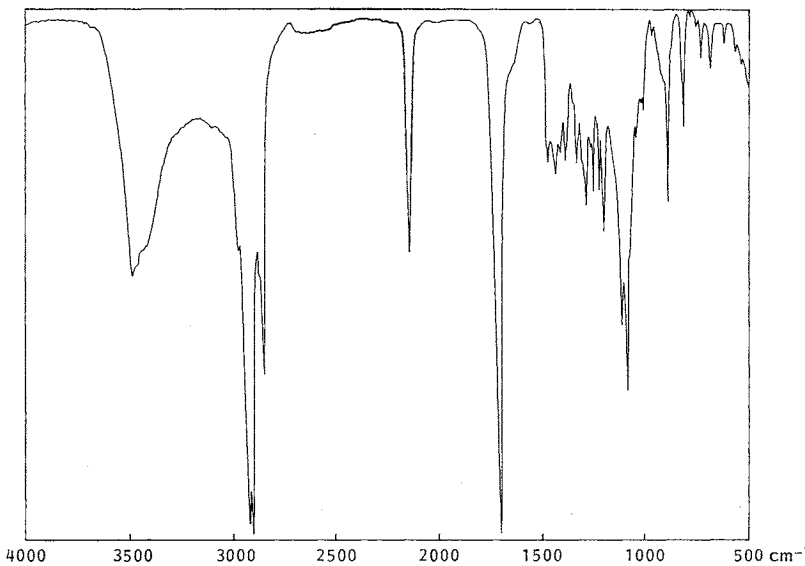
↓ countercurrent chromatography in hexane - EtOAc - MeOH - water (1 : 1 : 1 : 1)

Crude aerocyanidin (669 mg)

↓ chromatography on MCI gel CHP20P resin, eluting with an acetonitrile - water gradient

Aerocyanidin (138 mg)

Fig. 2. IR spectrum of aerocyanidin in KBr.



Characterization and Structure

Aerocyanidin is a colorless crystalline compound that is very labile to base and somewhat acid labile. Its properties are summarized in Table 1. Paper electrophoresis in simple aqueous buffers was unsuccessful due to the nonpolar nature of the antibiotic. However, using propane-1,2-diol (which is compatible with bioautography on *S. aureus*) in 1 : 1 combination with 0.05 M sodium phosphate buffers (pH 2.3 ~ 7.0) and 0.05 M carbonate - bicarbonate buffer (pH 9.2) gave mobilities relative to *p*-nitrobenzenesulfonate anion (+1.00) and vitamin B_{12} (0.00) of −0.12 at pH 2.3, +0.08 at pH 4.5, +0.37 at pH 7.0 and +0.35 at pH 9.2.

Table 1. Properties of aerocyanidin.

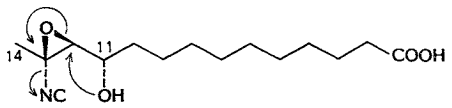
Empirical formula	$C_{15}H_{25}NO_4$
MW	283.37
MP (°C)	63.5 to 65.5
$[\alpha]_D^{23}$ (*c* 0.5, MeOH)	−20°
IR (KBr, cm^{-1})	3490, 2142, 1712, 1199, 1114, 1086
UV (MeOH)	End absorption
Rf[a]	0.28

[a] Silica gel ($CHCl_3$ - MeOH, 9 : 1).

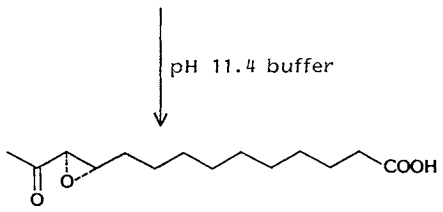
The IR spectrum, Fig. 2, has prominent peaks at 2142 and 1712 cm^{-1}. In conjunction with the

acidic nature of the antibiotic and the character of the OH absorption, the 1712 cm^{-1} peak was attributed to a carboxylic acid group. The 2142 cm^{-1} peak is in a range indicative of a triple bond or cumulated double bond system. Its specific origin was established by a positive Sievert-Hermsdorf reaction[2] which is characteristic of isonitriles. This function is also responsible for the weakly basic nature of aerocyanidin that is revealed by electrophoresis at pH 2.3.

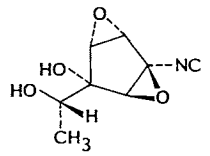
An accurate mass measurement of the protonated molecular ion produced by fast atom bombardment in a dithiothreitol - dithioerythritol - dimethyl sulfoxide - glycerol matrix gave a value of 284.1866 amu. Of the various empirical formulae consistent with this value, the only one also consistent with the degree of oxidation and functionality present is $C_{15}H_{26}NO_4$ (calcd 284.1862), leading to empirical



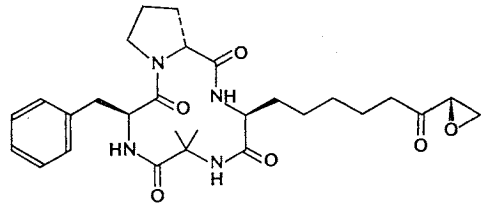
1



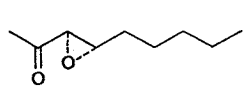
3



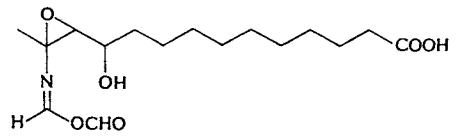
2



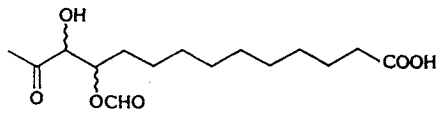
4



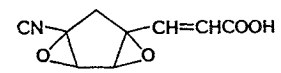
5



7



6



8

Table 2. NMR data[a] for aerocyanidin (**1**) and degradation products **3** and **6**.

Position	^{1}H data for **1**		^{13}C data for **1**[b]		^{1}H data for **3**		^{1}H data for **6**	
	δ	J	δ	Type	δ	J	δ	J
1			179.6	C				
2	2.34	7.3, 7.3	34.0[c]	CH_2	2.35	7.5, 7.5	2.35	7.5, 7.5
3			24.6	CH_2				
4			29.0	CH_2				
5			29.1	CH_2				
6	1.2~		29.2	CH_2				
7	1.75		29.3	CH_2				
8			29.3	CH_2				
9			24.6	CH_2				
10			34.6[c]	CH_2				
11	3.66	8.0, 8.0, 4.5	69.6	CH	3.07	6.0, 5.0, 2.1	5.30	9.5, 2.5, 2.5
12	2.84	8.1	64.8	CH	3.18	1.8	4.43	2.9
13			65.2 (br)	C				
14	1.76		22.1	CH_3	2.06		2.31	
NC			161.0	C				
OH/COOH	*ca.* 6.8				*ca.* 10.1		9.6, 3.6	
OCHO							8.15	

[a] NMR spectra were taken in $CDCl_3$. Chemical shifts are in ppm downfield from internal TMS; coupling constants are in Hz.

[b] The number of attached protons was determined by the INEPT technique.

[c,d] These assignments are interchangeable.

formula $C_{15}H_{25}NO_4$ for aerocyanidin. From this and the NMR data listed in Table 2, structure **1** (not including stereochemistry) was deduced. The number of double bond equivalents dictated by the empirical formula is four, and this is accounted for by the isonitrile and carboxylic acid functions plus one ring. The peak at 2.84 ppm in the ^{1}H NMR spectrum is indicative of an epoxide proton and a vicinal relationship of this proton to an oxymethine proton at 3.66 ppm was established by decoupling. The other position on the epoxide is quaternary and thus is substituted by both the isonitrile function and the methyl group that gives the singlet at 1.76 ppm in the ^{1}H NMR spectrum and a peak at 22.1 ppm in the ^{13}C NMR spectrum. The chemical shift assigned for this quaternary epoxide carbon (65.2 ppm) is similar to that reported for the corresponding carbon (68.4 ppm) in trichoviridin **2**[3]. An unbranched carbon chain is apparent from the ^{1}H NMR spectrum. Preliminary experiments implied the presence of a ketone (yellow color with 2,4-dinitrophenylhydrazine - H_3PO_4 reagent) and structure **1** contains a masked ketone that would be revealed with either acid or base.

The stereochemistry about the epoxide ring was evident from a nuclear Overhauser effect (NOE) enhancement of the 2.84 ppm ^{1}H resonance upon irradiation of the 1.76 ppm methyl singlet, placing the methyl group and the epoxide proton on the same side of the ring. The stereochemistry at position 11 was deduced from the product of base-catalyzed decomposition of aerocyanidin. Dissolution of the antibiotic in 0.05 M sodium phosphate buffer, pH 11.4, at room temperature results in its rapid destruction. Extraction of the product into ethyl acetate at pH 4.5 and purification by recrystallization from hexane gave a crystalline solid. The MS of the product indicated a MW of 256, corresponding to a loss of HCN from aerocyanidin. Structure **3**, which results from a Payne rearrangement with elimination of HCN, was apparent from the ^{1}H NMR spectrum (Table 2). Since the coupling between the epoxide protons is *ca.* 2 Hz, the ring is *trans* substituted[4].

The stereochemistry of the Payne rearrangement is well known[5] and involves inversion at the central carbon. Thus the formation of a *trans* epoxide indicates that the stereochemical relationship between positions 11 and 12 in aerocyanidin is *erythro*, assuming that the configuration at position 12 in **3** is stable under the conditions used. To test this assumption, the reaction was also carried out in D_2O and the product (extracted into ethyl acetate at pD *ca.* 4.5 but otherwise unpurified) examined by ^1H NMR spectroscopy. Exchange at position 12 was negligible (<0.2 H loss) although substantial exchange (*ca.* 1.2 H loss) was evident at position 14. The relative stereochemistry of aerocyanidin is therefore as shown in structure **1**.

Table 3. Antibacterial activity of aerocyanidin[a].

Organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	<0.05
S. aureus SC2399	<0.05
S. aureus SC2400	<0.05
Streptococcus faecalis SC9011	0.2
S. agalactiae SC9287	<0.05
Micrococcus luteus SC2495	0.4
Escherichia coli SC8294	>50.0
E. coli SC10896	25.0
E. coli SC10909	1.6
Klebsiella aerogenes SC10440	>50.0
K. pneumoniae SC9527	>50.0
Proteus mirabilis SC3855	1.6
Salmonella typhosa SC1195	25.0
Shigella sonnei SC8449	25.0
Enterobacter cloacae SC8236	50.0
Pseudomonas aeruginosa SC8329	>50.0

[a] MICs were determined by a 2-fold dilution method on DST agar (Oxoid). The final inoculum level was 10^4 cfu and the incubation time was 24 hours.

The circular dichroism (CD) spectrum of **3** shows a positive $n\rightarrow\pi^*$ transition at 295 nm, $[\theta]=+4600$ deg·cm^2 dmol^{-1}. The best currently available model for assigning the absolute configuration from the CD spectrum is chlamydocin, **4**, for which the shown absolute configuration was determined by X-ray crystallography[6]. In a recent synthetic study of antibiotics of this type[7] the ellipticity in the 300 nm region was shown to be due entirely to the epoxyketone, and a molar ellipticity of −830 at 288 nm was reported for synthetic chlamydocin. This leads to the tentative assignment of 11*S*,12*R* stereochemistry for **3** and thus 11*S*,12*S*,13*S* stereochemistry for **1**. The assignment of this absolute configuration is based on the assumption that the conformation of the epoxyketone is similar for **3** and **4** and that the substituent on the epoxide in **3** that is remote from the carbonyl group does not contribute as strongly to the ellipticity as the closer atoms. However, the large difference in molar ellipticity for **3** and **4** make these assumptions somewhat questionable[†].

Treatment of aerocyanidin with 9.6% HCOOH at room temperature for 4 minutes also results in complete destruction of the antibiotic. The product was assigned structure **6** on the basis of the ^1H NMR spectrum (Table 2) and the mass spectrum. It is presumably formed *via* intermediate **7** which results from the 1,1-addition of formic acid to the isonitrile.

Biological Properties

The activity of aerocyanidin against aerobic bacteria is listed in Table 3. It is highly active against Gram-positive bacteria and moderately to weakly active against some Gram-negative bacteria. *Candida albicans* and most anaerobic bacteria are unaffected at 50 μg/ml. Aerocyanidin was not efficacious in mice against a *S. aureus* infection at 100 mg/kg by subcutaneous administration and had an LD_{50} between 100 and 150 mg/kg by intraperitoneal injection.

[†] An essentially ideal model, **5**, was recently reported but with no chiroptical data[8].

Discussion

The structure of aerocyanidin places this antibiotic in the rather small group characterized by an isonitrile function. Approximately fifteen fermentation products and several sponge metabolites containing an isonitrile have been reported but none are closely related to aerocyanidin in their carbon skeletons. The closest relatives by virtue of containing an epoxide substituted with an isonitrile are trichoviridin **2**[8,9] and antibiotic compound **8**[10]. Trichoviridin is also similar in having a hydroxyl group adjacent to the epoxide with the same relative stereochemistry as that in **1** and thus might be expected to undergo a Payne rearrangement with elimination of HCN although this has not been reported.

The toxicity of aerocyanidin is comparable to that of NaCN (*per os*) on a molar basis which is not surprising, considering the ease of elimination of HCN. However, the qualitative activity of aerocyanidin against *S. aureus* in an agar diffusion assay is much greater than the activity of inorganic cyanide so it is unlikely that the activity results from release of HCN by decomposition in the assay medium. The mode of action is currently unknown.

Experimental

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on Jeol Model GX 400 (400 MHz) and Jeol Model FX 270 (67.5 MHz) spectrometers, respectively. The solvent was purified by washing with water and filtering through basic alumina. MS were determined on VG Analytical Model ZAB 1F and Finnigan TSQ spectrometers. Optical rotations were determined on a Perkin-Elmer Model 241 polarimeter. IR spectra were recorded on a Matson Model Sirius 100 FT-IR spectrometer and UV spectra on a Hewlett-Packard Model 8450 spectrometer. The CD spectrum was determined on an instrument built in house. MP were determined in borosilicate glass capillaries. TLC was done on Merck Silica gel 60 F plates.

The isolation was monitored colorimetrically with a modified Sievert-Hermsdorf method[2] in which a 0.1-ml portion of the test sample was added to 0.9 ml of reagent prepared from equal volumes of a 1-mg/ml solution of 3,3′,5,5′-tetramethylbenzidine in MeOH - CH_3COOH (9 : 1) and a 3-mg/ml solution of cupric acetate monohydrate in water. The increase in absorption at 370 nm relative to a blank prepared from ethanol and the reagent was proportional to the quantity of aerocyanidin present. The increase in absorption at 660 nm was also suitable for this assay.

Isolation of Aerocyanidin (**1**)

At harvest, the whole broth, 260 liters, pH 5.9, was cooled to 10°C, solids were removed by centrifugation and the supernate was extracted with 125 liters of EtOAc. The extract was concentrated to 2 liters, filtered, washed with 0.1 M sodium phosphate buffer (pH 6.0) and further concentrated to give 87 g of nonvolatile residue. This was subjected to a 2-funnel countercurrent distribution in hexane - toluene - MeOH - water (3 : 3 : 4 : 2), 850 ml per phase, to remove nonpolar impurities (mostly antifoam agent). Concentration of the combined lower phase followed by extraction with EtOAc and concentration of the extract gave 4.1 g of crude antibiotic. This was subjected to countercurrent chromatography in hexane - EtOAc - MeOH - water (1 : 1 : 1 : 1) in a high-speed countercurrent chromatograph (P.C. Inc., Potomac, MD, U.S.A.) operated at 800 rpm using a multilayer Teflon tubing (1.6 mm i.d.) coil with a volume of 330 ml. The system was eluted with the upper phase at 4 ml/minute. Aerocyanidin emerged in a volume corresponding to a partition coefficient of 1.1. Active fractions were combined to give 669 mg of the crude antibiotic. Chromatography of this material on a 2.5 × 20-cm column of 75 to 150 μ MCI gel CHP20P resin (Mitsubishi Chemical Industries Limited), eluting at 2 ml/minute with a linear gradient of 30 to 70% CH_3CN in water over a volume of 2.2 liters, gave an active band eluting between 700 and 760 ml. This effluent was diluted with water and the antibiotic was extracted into EtOAc. The resulting extract, containing 138 mg of nonvolatile residue, was stored at 4°C. Concentration of a small sample gave a crystalline residue, mp 59~62°C, that appeared to be homogeneous by TLC. The highest mp observed in similar isolations was 63.5~65.5°C.

(*trans*)-11,12-Epoxy-13-oxomyristic Acid (3)

Aerocyanidin, 6.1 mg, was dissolved in 2.5 ml of 0.05 M sodium phosphate buffer, pH 11.4. After 20 minutes at room temperature, 188 μl of 1 M H_3PO_4 was added and the resulting solution (pH *ca.* 4.5) was extracted with EtOAc. The extract was concentrated and the residue, 5.7 mg, was recrystallized three times from hexane, giving 3.3 mg of **3** as fine needles: MP 60~61°C; ^{1}H NMR, Table 2; $[\alpha]_D^{21}$ +11° (*c* 0.3, MeOH); UV in MeOH, λ_{max} 285 nm (ε 37); IR ($CHCl_3$) 1709 cm^{-1}; MS (CI with CH_4 - N_2O) *m/z* 255 $(M-H)^-$ and 274 $(M+NH_4)^+$ (from adventitious NH_3 in the spectrometer).

11-(Formyloxy)-12-hydroxy-13-oxomyristic Acid (6)

A 10-mg sample of aerocyanidin was dissolved in 0.5 ml of 9.6% HCOOH. After 4 minutes, the solution was extracted with EtOAc. Chromatography of the residue from the EtOAc extract on a MCI gel CHP20P resin column (10 ml) eluting with a 30 to 100% gradient of acetonitrile in water gave 2.5 mg of the major product as an oil that was homogeneous by TLC ($CHCl_3$ - MeOH, 9:1) with Rf 0.29; ^{1}H NMR, Table 2; MS (CI, CH_4 - N_2O) *m/z* 301 $(M-H)^-$ and 320 $(M+NH_4)^+$ (from adventitious NH_3 in the spectrometer).

Acknowledgments

The authors wish to thank R. M. HUGILL and R. A. SMITH for fermentations, A. D. KAHLE and S. P. DRUCKMAN for NMR spectra, B. M. WARRACK and T. J. MCCORMICK for mass spectra, H. G. BRITTAIN for measuring the circular dichroism, S. K. TANAKA for determination of antimicrobial activity *in vitro*, and J. M. CLARK for efficacy and toxicity studies.

References

1) SINGH, P. D.; W.-C. LIU, J. Z. GOUGOUTAS, M. F. MALLEY, M. A. PORUBCAN, W. H. TREJO, J. S. WELLS & R. B. SYKES: Aerocavin, a new antibiotic produced by *Chromobacterium violaceum*. J. Antibiotics 41: 446~453, 1988
2) SMITH, P. A. S.: The Chemistry of Open-Chain Organic Nitrogen Compounds. Vol. 1. p. 225, Benjamin, New York, 1965
3) OLLIS, W. D.; M. REY, W. O. GODTFREDSEN, N. RASTRUP-ANDERSEN, S. VANGEDAL & T. J. KING: The constitution of the antibiotic trichoviridin. Tetrahedron 36: 515~520, 1980
4) JACKMAN, L. M. & S. STERNHELL: Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry. 2nd Ed. p. 287, Pergamon Press, New York, 1969
5) BEHRENS, C. H.; S. Y. KO, K. B. SHARPLESS & F. J. WALKER: Selective transformation of 2,3-epoxy alcohols and related derivatives. Strategies for nucleophilic attack at carbon-1. J. Org. Chem. 50: 5687~5696, 1985, and references contained therein
6) FLIPPEN, J. L. & I. L. KARLE: Conformation of the cyclic tetrapeptide dihydrochlamydocin. Iabu-L-Phe-D-Pro-LX, and experimental values for 3→1 intramolecular hydrogen bonds by X-ray diffraction. Biopolymers 15: 1081~1092, 1976
7) KAWAI, M.; J. H. GARDNER & D. H. RICH: Stereoselective synthesis and absolute configuration of epoxyketones in chlamydocin and related cyclic tetrapeptides. Tetrahedron Lett. 27: 1877~1880, 1986
8) MOLANDER, G. A. & G. HAHN: Lanthanides in organic synthesis. 4. Reduction of α,β-epoxy ketones with samarium diiodide. A route to chiral, nonracemic aldols. J. Org. Chem. 51: 2596~2599, 1986
9) NOBUHARA, M.; H. TAZIMA, K. SHUDO, A. ITAI, T. OKAMOTO & Y. IITAKA: A fungal metabolite, novel isocyano epoxide. Chem. Pharm. Bull. 24: 832~834, 1976
10) OHSUGI, K.; J. ICHIDA & E. TAKAHASHI (Kureha Chemical): Antibiotic compound. Ger. Offen. 3,326,518, Jan. 26, 1984