Communications to the editor

A NEW ANTIBIOTIC, 3,6-DIHYDROXYINDOXAZENE

Sir:

A new antibiotic 3,6-dihydroxyindoxazene which is active against Gram-negative bacteria has been found in the cultured broth of a bacterial strain BMF34-EG2. This strain was isolated from a soil sample collected at Gosen City, Niigata Prefecture, Japan and classified as *Chromobacterium violaceum*.[1] In this communication, the isolation, characterization and structural study of the antibiotic are reported.

Chromobacterium violaceum BMF34-EG2 was cultured at 27°C for 22 hours on a reciprocal shaker (180 rpm) in a Sakaguchi flask containing 125 ml of a medium (2% glycerol, 1% glucose, 0.5% meat extract, 0.5% peptone and 0.5% NaCl, pH 7.0). The seed culture (2.5 ml) thus prepared was inoculated into 125 ml of the same medium in each flask and cultured in 182 flasks for 23 hours. Concentrations of the antibiotic were determined by the cylinder plate method against *Escherichia coli* K-12 on a 0.5% peptone agar plate (pH 7.0), using the crystalline antibiotic (1,000 μg/ml) as the standard.

The antibiotic in the cultured broth (pH 7.2, 20.9 liters, 13.6 μg/ml) was extracted with 20.9 liters of ethyl acetate at pH 2.0 (adjusted with diluted HCl) and re-transferred into 10.3 liters of water at pH 8.0 (adjusted with 2 N NaOH). The antibiotic in the aqueous layer was re-extracted with 4.6 liters of ethyl acetate at pH 3.0 and the extract was concentrated to give a crude solid (1.55 g, 161 μg/mg). The antibiotic in the solid was purified by column chromatography on silica gel (Mallinckrodt, AR, 130 g) developed with a mixture (20: 1) of chloroform and methanol to yield crude crystals of the antibiotic (260 mg, 908 μg/mg). Recrystallization from hot ethyl acetate gave colorless crystals (mp 248~250°C (decomp.)) of the optically inactive antibiotic (157 mg, 1,000 μg/mg). *Anal.* Calcd. for $C_7H_5NO_3$: C 55.63, H 3.34, N 9.27, O 31.76. Found: C 55.71, H 3.40, N 9.07, O 32.19. The molecular formula was determined by high-resolution MS (*m/z* 151.0254, calcd. mol. wt.: 151.0269). Potentiometric titration in 67% methyl cellosolve gave *pKa'* values of 6.2 and 10.7. In the UV spectra maxima were at 219 (ε 18,420), 252 (6,950), 260 (sh.), 280 (sh.), 283 (6,490) and 290 nm (6,040) in methanol; at 206 (ε 21,140), 252 (8,230), 260 (sh.), 280 (sh.), 283 (6,340) and 290 nm (6,340) in 0.1 N HCl - 90% methanol; and at 220 (sh.), 237 (ε 18,120), 264 (4,380), 273 (sh.) and 296 nm (5,590) in 0.1 N NaOH - 90% methanol. The IR spectrum is shown in Fig. 1. The ^1H and ^{13}C NMR chemical shifts are shown in Table 1.

The antibiotic is readily soluble in methanol, ethanol, acetone, dioxane, tetrahydrofuran, dimethyl sulfoxide and alkaline water, soluble in ethyl acetate and butyl acetate, but almost insoluble in water. It gives positive Fast Blue B (for phenols), pentacyanoamine ferroate (for nitroso compounds), ferric chloride (orange, for phenols or hydroxamic acids) and cupric chloride (dark green, for oximes) reactions, but negative ninhydrin, red tetrazolium and Brady reactions. The Rf value of TLC on a Silica Gel G plate (Merck, Art. 5721) developed with a mixture (4 : 1) of chloroform and methanol is 0.40.

Acetylation of the antibiotic with acetic anhydride in pyridine at room temperature gave the

Fig. 1. Infrared spectrum of 3,6-dihydroxyindoxazene (KBr).

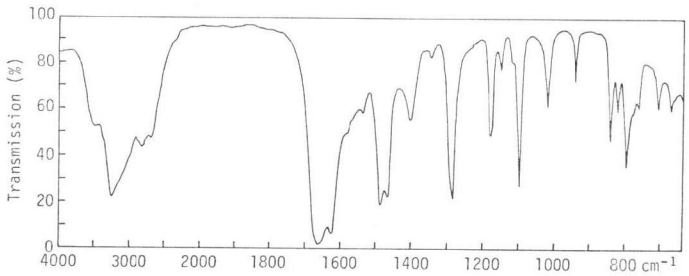


Table 1. NMR chemical shifts of 3,6-dihydroxyindoxazene.

Position	^{13}C NMR		^{1}H NMR	
	δ, ppm	$J_{C,H}$, Hz	δ, ppm	J, Hz
3	167.4 s			
7a	166.8 s	9.2, 3.1		
6	163.1 s	9.2, 3.1		
4	123.6 d	164.8	7.52	0.5, 8.5
5	114.5 d	161.7, 4.3	6.80	2.0, 8.5
3a	108.1 s	9.2, 4.3		
7	96.2 d	164.8, 4.9	6.72	0.5, 2.0

Spectra were taken with a JEOL FX200 spectrometer in a methanol-d_4 solution using TMS as the internal reference. Assignments, s and d show multiplicity of off-resonance experiment.

diacetate, in 81% yield, which was crystallized from hot ethyl acetate, mp 141~143°C (on a hot-stage microscope). High-resolution MS: m/z 235.0490, calcd. for $C_{11}H_9NO_5$: m/z 235.0480.

The diacetate was suitable for determination of the crystal structure by X-ray diffraction methods. A crystal of approximate dimensions 0.2×0.05×0.7 mm was cut from a larger plate grown in an ethyl acetate solution and was used for diffraction study. The lattice constants and intensity data were obtained on a Philips PW1100 diffractometer using CuKα radiation monochromated by a graphite plate. Of the total of 4861 reflections within the 2θ range of 6° through 156°, 2920 were measured as above the 2σ(I) level. The crystal data are shown in Table 2. The asymmetric unit contains two independent molecules. The $hk0$ reflections showed systematically weak reflections of the type $h+k=2n+1$ in addition to the systematically absent reflections for $P2_1/c$: $h0l$, $l=2n+1$ and $0k0$, $k=2n+1$. The locations of all 34 heavier atoms were determined by direct methods using MULTAN and the remaining 18 hydrogen atoms were found on the difference electron-density map. The refinement was carried out by the block-diagonal least-squares methods to a final R value of 0.067 including the hydrogen atoms with isotopic temperature factors.* The two crystallographically independent molecules have almost the same conformation and the differences in the corresponding bond lengths and angles were almost within their mean standard deviations.

* Tables of atomic parameters, bond lengths and angles are deposited with the Cambridge Crystallographic Data Centre. Those of Fo and Fc may be obtained from one of the authors (H. N.) upon request.

Table 2. Crystal data of 6-acetoxy-2-acetyl-1,2-benzisoxazolin-3-one.

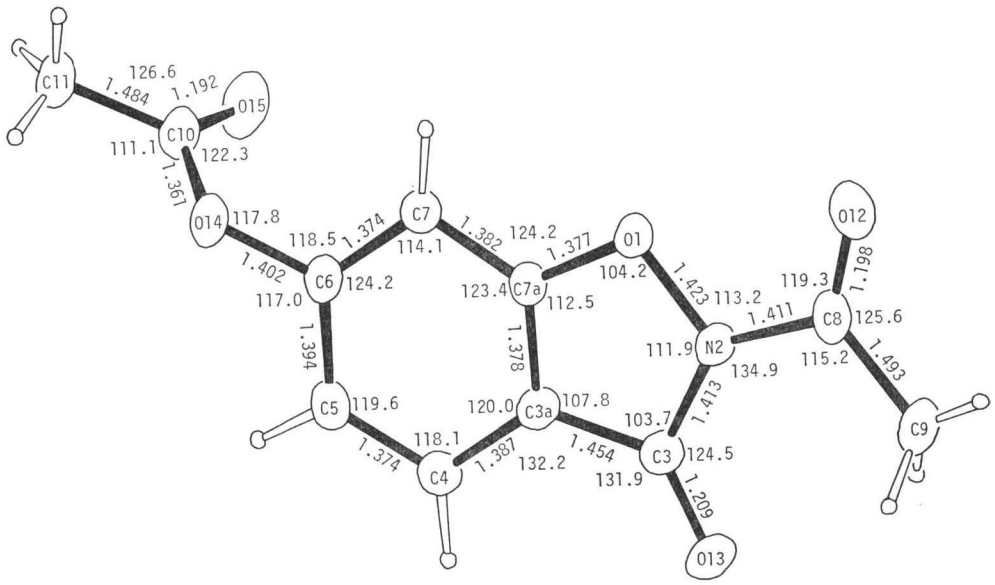
$C_{11}H_9NO_5$, MW = 235.2, monoclinic, space group $P2_1/c$, a = 23.954(12), b = 5.476(3), c = 17.419(9) Å, β = 110.40(6)°, U = 2141.6 Å^3, Z = 8, D_{cal} = 1.459 Mgm^{-3}, μ for CuKα radiation = 9.57 cm^{-1}

The skeleton of the molecule is approximately planar. In each molecule, the deviations of atoms from the least-squares plane through O1–C7a are less than 0.048Å and the acetyl group is also in the same plane but the acetoxy group is twisted out from the plane. The torsional angles O1–N2–C8–O12 are 0.8° and 1.4° in molecule A and B respectively, while those of C7–C6–O14–C10 are 83.1° and 83.0°. The molecular structure with the mean bond lengths and angles for two molecules is shown in Fig. 2. The values are consistent with the chemical structure of 6-acetoxy-2-acetyl-1,2-benzisoxazolin-3-one.

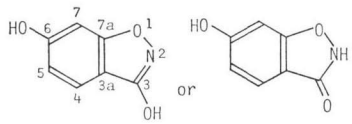
Table 3. The minimum inhibitory concentrations on a nutrient agar plate.

Test organism	μg/ml
Staphylococcus aureus FDA209P	>100
Staphylococcus aureus Smith	>100
Micrococcus flavus FDA16	>100
Micrococcus luteus PCI1001	>100
Bacillus anthracis	>100
Bacillus subtilis NRRL B-558	>100
Corynebacterium bovis 1810	>100
Mycobacterium smegmatis ATCC607	>100
Escherichia coli NIHJ	>100
Escherichia coli K-12	>100
Escherichia coli K-12 ML1629	>100
Escherichia coli JR66/W677	100
Escherichia freundii GN346	6.25
Shigella dysenteriae JS11910	1.56
Shigella flexneri 4b JS11811	3.13
Shigella sonnei JS11746	12.5
Salmonella typhi T-63	6.25
Salmonella enteritidis 1891	12.5
Proteus vulgaris OX19	0.78
Proteus mirabilis IFM OM-9	>100
Proteus rettgeri GN311	6.25
Proteus rettgeri GN466	6.25
Proteus rettgeri 549	25
Pseudomonas aeruginosa A3	>100
Pseudomonas aeruginosa No. 12	>100
Klebsiella pneumoniae PCI602	6.25

Fig. 2. A perspective view of 6-acetoxy-2-acetyl-1,2-benzisoxazolin-3-one (molecule A) with the mean bond lengths and angles for two molecules.



Therefore, the structure of the parent antibiotic was determined to be 3,6-dihydroxy-1,2-benzisoxazole (3,6-dihydroxyindoxazene) or 6-hydroxy-1,2-benzisoxazolin-3-one as shown in the following structure:



The ^{1}H and ^{13}C NMR chemical shifts were assigned by gated and selective proton decoupling techniques and ^{2}H isotope effect measurements as shown in Table 1.

3,6-Dihydroxyindoxazene inhibits the growth of some Gram-negative bacteria as shown in Table 3. Intravenous injection of 200 mg/kg of the sodium salt caused no deaths in mice.

Masa Hamada
Shinichi Kondo
Hikaru Nakamura
Takako Ikeda
Daishiro Ikeda
Katsuharu Iinuma
Shuichi Gomi
Yoko Ikeda
Tomio Takeuchi
Hamao Umezawa
Institute of Microbial Chemistry
14-23 Kamiosaki 3-Chome,
Shinagawa-ku, Tokyo 141
Japan

Yoichi Iitaka
Faculty of Pharmaceutical Sciences,
University of Tokyo, Hongo, Bunkyo-ku,
Tokyo 115, Japan

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Reference

1) Buchanan, R. E. & N. E. Gibbons (*ed.*): Bergey's Mannual of Determinative Bacteriology. 8th ed. pp. 355~356, The Williams & Wilkins Company, Baltimore, 1974