G1499-2, A NEW QUINOLINE COMPOUND ISOLATED FROM THE FERMENTATION BROTH OF *CYTOPHAGA JOHNSONII*

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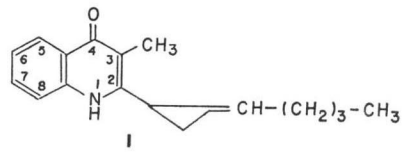
A new quinoline compound, G1499-2 [$C_{18}H_{21}NO$(I)] is produced by *Cytophaga johnsonii*. G1499-2 has an unusual structure containing a cyclopropylidene radical. The compound has limited antibiotic activity against a few bacteria. It is not toxic to mice.

A compound called G1499-2, with antibiotic activity against a narrow range of organisms, was isolated from the culture broth of *Cytophaga johnsonii* ATCC 21123, an organism that has been reported to produce a number of lytic enzymes[1]. Physical and chemical measurements of crystalline G1499-2 suggest it to have the novel structure 3-methyl-2-(2-pentylidenecyclopropyl)-4-quinolinone (I).



I

This paper describes the production, isolation and properties of this compound.

Experimental

Antibiotic detection and assay.

To follow the production of G1499-2 during fermentation, samples of broth were centrifuged and supernatant liquids assayed by the agar diffusion cup-plate method described by NOBLE *et al.*[2] The test organism was a *Flavobacterium* species (Glaxo strain 1980).

Isolation of G1499-2 was followed by an agar diffusion method in which samples were applied to thin layers of cellulose (20×20 cm; 13254 containing fluorescent indicator; Eastman-Kodak Co., Rochester, N.Y., U.S.A.). To detect active material the air-dried thin-layer plates were sprayed with a solution of agar (0.7%, no. 3, Oxoid Ltd., London, England), then overlayered with nutrient agar inoculated with *Flavobacterium* 1980 and containing 0.0002% 2,3,5-triphenyl tetrazolium chloride[3]. The overlayered plates were incubated at 37°C for 16 hours and the diameter (mm) of zones of growth inhibition recorded. Samples were also applied to thin layers of silica and subjected to chromatography (see later) before overlayering the plates with nutrient agar and test organism.

Purified G1499-2 was assayed by the cup-plate method[2] both with and without 2,3,5-triphenyl tetrazolium chloride in the nutrient agar. Test organisms were *Flavobacterium* 1980 and *Staphylococcus aureus* Oxford H strain VI.

Fermentation.

Cytophaga johnsonii ATCC 21123, was grown for 48 hours at 28°C on slopes containing bacteriological peptone (L34, Oxoid Ltd.), 0.5%; glucose, 2% and agar (no. 3, Oxoid Ltd.), 2%, made up in distilled water (final pH 7.0~7.2).

Growth from one slope was suspended in 5 ml sterile distilled water and 0.5 ml of the suspension was transferred to 50 ml of sterilised medium containing bacteriological peptone, 1%; Lab-Lemco beef extract (L29, Oxoid Ltd.), 1%; and NaCl, 0.5%. The inoculated medium was shaken (220 rev/minute on a rotary shaker with a 2-inch throw) in a 250-ml conical flask for 24 hours at 28°C.

A portion (40 ml) of the shake flask fermentation broth was transferred to a 5-litre fermenter containing 4 litres of sterilised medium (bacteriological peptone, 0.5% and malt extract (L39, Oxoid Ltd.), 0.5%, made up in tap water and adjusted to pH 7 with NaOH). The mixture was stirred (550 rev/minute) and aerated (1.5 litres air/minute) for 24 hours at 28°C.

A portion (3 litres) of the 5-litre fermentation broth was transferred to 150 litres of the same medium in a 230-litre fermenter. The mixture was stirred (250 rev/minute) and aerated (5 cu.ft. air/minute) for 22 hours at 28°C.

Antibiotic isolation.

Broth (135 litres) at harvest was adjusted to pH 3 with sulphuric acid and centrifuged for 45 minutes (K6 chamber bowl centrifuge, Westfalia Separator A.G., 4740, Oelde, Westfalia, Germany). The supernatant liquid was applied to a column of XAD-4 resin (10 litres; Rohm and Haas, U.K. Ltd., Croydon, Surrey, England) and after it had percolated into the resin the column was washed sequentially with water (33 litres) and with methanol – water (1: 1 by volume, 58 litres). Active material was eluted with methanol (50 litres).

The eluate was evaporated, the residue suspended in water (3 litres) and the mixture extracted twice with 2 litres of ethyl acetate. The combined ethyl acetate extracts were dried over $MgSO_4$ and evaporated to give a residue.

The residue was dissolved in a small volume of chloroform and the solution was applied to a column of dry silica (80×2.5 cm; Woelm silica gel activity III, ICN Pharmaceuticals GmbH & Co., 3440 Eschwege, West Germany). The silica was eluted with chloroform; active fractions were combined and evaporated to a yellow oil.

The oil was applied to a column of silica (Kieselgel 60, E. Merck, Darmstadt, West Germany) packed in toluene – ethyl acetate (1: 1 by volume) to give a bed of 50×4.5 cm. The silica was eluted with the same solvent, active fractions were combined and evaporated to a pale yellow solid.

The solid was dissolved in a small volume of methanol and the solution was applied to a column of Sephadex LH20 (160×4.5 cm; Pharmacia Fine Chemicals AB., Uppsala, Sweden) packed in methanol. Elution was with the same solvent; active fractions were combined and evaporated. The residue was dissolved in water and freeze-dried to yield 200 mg of white solid.

The solid was crystallised from acetone.

Thin-layer chromatography.

Samples were applied by means of micropipettes (Drummond Scientific Co., Broomall, Pennsylvania, U.S.A.) to thin layers of silica (20×20 cm; 5735; E. Merck, Darmstadt, West Germany). Samples from the isolation of the antibiotic were applied to thin-layers and developed with ethyl acetate. Other solvents as well as ethyl acetate (Table 2) were used for chromatography of the pure antibiotic. After development at 24°C the plates were air dried, examined under U.V. light (254 nm) and overlayered with *Flavobacterium* 1980 or with *Staphylococcus aureus* Oxford H strain VI. After incubation at 37°C for 16 hours Rf values of zones of growth inhibition were recorded.

Spectroscopy.

The mass spectra (electron ionisation and field desorption) were recorded on a Varian MAT 311A spectrometer, the 200 MHz proton n.m.r. spectrum on a Varian HR220 instrument and the ^{13}C n.m.r. spectrum on a Jeol FX100 instrument.

Evaporation.

Evaporation of solvent was under reduced pressure, large volumes in a pot still, volumes of 20 litres or less in a rotary evaporator.

Acute mouse toxicity.

Test compound was dissolved in methanol – water (1: 1 by volume) and dilutions made with the same solvent. Each concentration of compound was administered i.p. to a group of 4 albino female

mice (Charles River, Harefield strain). Each mouse weighed 20 g and received 0.2 ml of the appropriate test solution. Solvent (0.2 ml) not containing test compound was administered to each of a group of 10 mice. Survivors were counted daily for seven days.

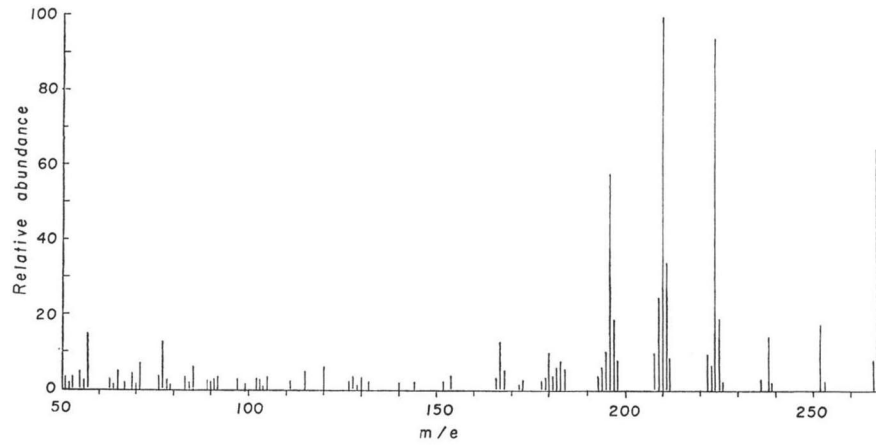
Results

Physical and Chemical Properties

G1499-2 is a white crystalline (rosettes) compound soluble in methanol, acetone, ethyl acetate and chloroform but insoluble in water and hydrocarbon solvents. Crystals of G1499-2 melt at 192~193°C; $[\alpha]_D^{25}$ +172° (*c* 0.5, chloroform).

Ions of G1499-2 at *m/e* 267.2, 268.2 and 269.1 (M^+, $M+1^+$, $M+2^+$) were detected by field desorption mass spectrometry. An electron impact mass spectrum (Fig. 1) of the compound confirmed the molecular weight. Fragmentation ions at *m/e* 252, 238, 224 and 210 also were detected, presumably due to loss of methyl, ethyl, propyl and butyl radicals. An accurate mass match of the molecular ion gave *m/e* 267.1621 corresponding with a molecular formula of $C_{18}H_{21}NO$ (*m/e* 267.1623). Elementary analysis gave C, 81.1; H, 7.9; N, 5.1; $C_{18}H_{21}NO$ requires C, 80.9; H, 7.9; N, 5.2%.

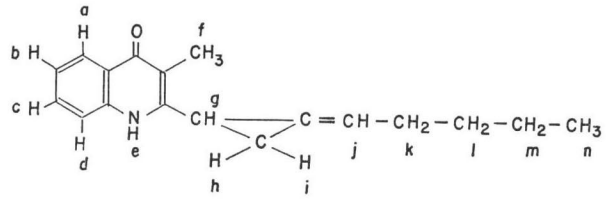
Fig. 1. Electron impact mass spectrum of G1499-2



An ultraviolet spectrum of an ethanol solution containing 0.0016% G1499-2 had maxima (nm with $E_{1cm}^{1\%}$ within brackets) at 244 (1190), 248 (1210), 323 (450) and 337 (500). Maxima were obtained in methanol at 244 (1260), 322 (420) and 336 (445) and in methanol containing aqueous HCl at 236 (1840) and 310 (410).

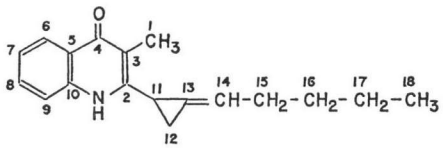
The infrared spectrum of a bromoform solution of G1499-2 had absorptions (cm^{-1}) at 3350 (NH), 1630, 1610 and 1570 (quinolinoid system) and 758 (*o*-disubstituted benzene).

A 100 MHz proton n.m.r. spectrum of a deuterochloroform solution of G1499-2 had peaks [assignments (**II**) given as italic letters] at τ 1.57 (s, 1H, *e*); 1.65 [d(8 Hz); 1H, *a*], 2.4~2.8 (m, 3H, *b, c* and *d*), 3.73 (m, 1H, *j*), 7.14 (m, 1H, *g*), 7.66 [q (6 Hz), 2H, *k*], 7.72 (s, 3H, *f*), 8.13 [t (9 Hz), 1H, *h*], 8.4~8.6 (m, 5H, *i, l* and *m*) and 9.06 [t (6 Hz), 3H, *n*]. The peak at τ 1.57 disappeared on addition of D_2O.



II

Table 1. ^{13}C n.m.r. spectrum of G1499–2



δ (p.p.m.)	Multiplicity	Assignment	$^{13}C-^{1}H$ J (Hz)
178.7	s	4	
150.6	s	2	
139.9	s	10	
132.3	d	8	
125.7	d	6*	
124.3	d	7*	
124.3	s	5	
123.0	d	14*	
122.5	s	13	
118.7	d	9	
117.6	s	3	
32.3	t	15**	
32.2	t	16**	
23.3	t	17	
18.1	d	11	≃170
14.2	q	18	
11.4	t	12	≃170
11.2	q	1	

* These assignments may be interchanged.
** These assignments may be reversed.

Table 2. T.l.c. of G1499–2 on silica

Solvent	Rf
Methanol	0.85
Acetone	0.70
Dioxane	0.75
Ethyl acetate	0.60
Chloroform	0.12
Benzene	0
Toluene – ethyl acetate (2: 1)	0.11
Toluene – ethyl acetate (3: 1)	0.08
Hexane – ethyl acetate (1: 1)	0.15
Hexane – ethyl acetate (2: 1)	0.06

A 220 MHz n.m.r. spectrum of a deuterochloroform solution of G1499–2 is shown in Fig. 2. Spin decoupling experiments indicated that the olefinic proton giving the multiplet at τ 3.73 was coupled (J, 6 Hz) to the low-field methylene protons at τ 7.66.

Addition of $Eu(fod)_3$ to a deuterochloroform solution of G1499–2 induced a down-field shift of all the signals in the spectrum but the induced shift of the doublet at τ 1.65 (**II**, *a*) was the largest followed by that of the singlet at τ 7.72 (**II**, *f*).

Table 1 shows the results obtained from a ^{13}C n.m.r. spectrum of G1499–2, along with an assignment of the signals.[4]

Rf values for G1499–2 obtained after thin-layer chromatography on silica are given in Table 2.

Biological Properties

G1499–2, assayed by an agar diffusion (cup-plate) method (see Experimental section), inhibited the growth of *Flavobacterium* 1980 and of *Staphylococcus aureus* Oxford H strain VI (Table 3) and inhibition of growth was more pronounced in the presence of tetrazolium chloride than in its absence. G1499–2 also inhibited the growth of a few other strains of *Staphylococcus aureus* and of *Bacillus subtilis*, but did not inhibit the growth of the majority of Gram-positive and Gram-negative bacteria tested, nor of yeasts and fungi.

Fig. 2. 220 MHz ^{1}H n.m.r. spectrum of G1499–2 in deuterochloroform; high field signals.

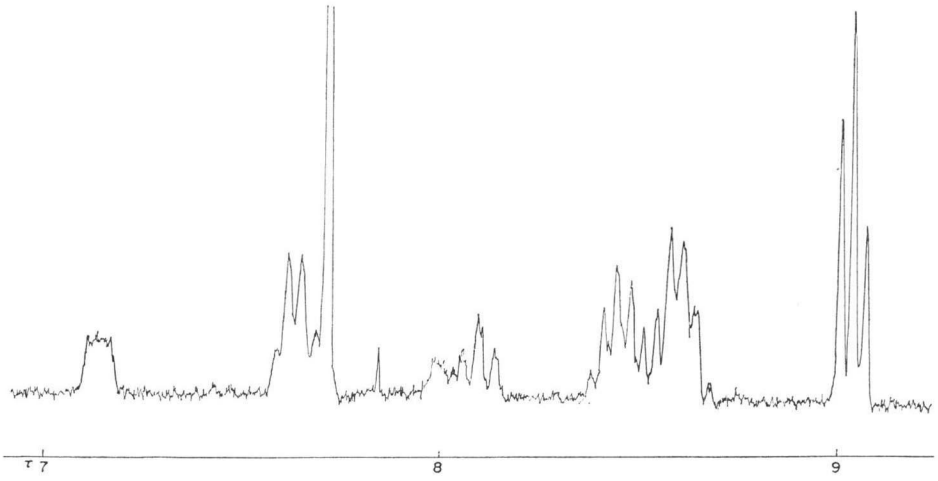
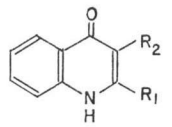


Table 3. Effect of triphenyl tetrazolium chloride on the antibiotic activity of G1499–2 measured by agar diffusion cup-plate assay

Concentration G1499–2 (μg/ml)	Inhibitory zone diameter (mm)			
	Flavobacterium 1980		*Staphylococcus aureus* Oxford H strain VI	
	+tetrazolium	−tetrazolium	+tetrazolium	−tetrazolium
1,500			29.2	21.2
375			23.6	18.0
94			21.5	0
24	48.8	44.2	19.8	0
6	43.9	41.2	0	0
1.5	35.7	30.3	0	0
0.38	32.4	28.7	0	0
0.1	26.8	19.3	0	0
0.025	19.0	0	0	0



		R_1	R_2
III	Pyo Ib	$-(CH_2)_6CH_3$	H
IV	Pyo Ic	$-(CH_2)_8CH_3$	H
V	Pyo III	$-CH=CH(CH_2)_4CH_3$	H
VI		$-(CH_2)_4CH_3$	H
VII		$-CH_2-CH=CH(CH_2)_3CH_3$	$-CH_3$
VIII		$-(CH_2)_2(CH=CHCH_2)_2CH_3$	H
IX		$-(CH_2)_8CH_2OH$	H
X		$-(CH_2)_9COCH_3$	H
XI		$-(CH_2)_3CH=CHCH_2CH_3$	H
XII		$-(CH_2)_{10}CH_3$	H

The LD_{50} of G1499–2 administered i.p. to mice was >500 mg/kg body weight.

Discussion

The physical and chemical properties of G1499–2 are consistent with structure **I**.

The ultraviolet absorption spectrum of G1499–2 is similar to that of alkaloids containing the 4-quinolinone nucleus and the absorption maximum on addition of acid is characteristic of this chromophore[5]. The n.m.r. spectrum is also consistent with the presence of this heterocyclic system, the doublet at τ 1.65 (**II**, *a*) would accord with the C_5-proton of such a system.

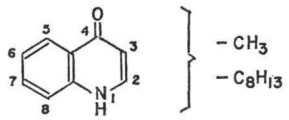
From the n.m.r. spectrum and the mass spectral fragmentation pattern of G1499–2 the presence of a short aliphatic chain can be deduced.

Several antibiotics produced by fermentation of *Pseudomonas* strains are known that contain the 4-quinolinone nucleus substituted with an aliphatic chain; Pyo compounds **III**, **IV**, **V**[6,7], structures **VI**[8] (from a marine pseudomonad) and **VII**[9]. Other examples of this series of alkaloids are found in

higher plants (**VIII**, **IX**, **X**[10]; **XI**[11]; **XII**[12]). However, on the basis of its molecular formula and n.m.r. spectrum, G1499–2 is not identical with any of these compounds.

The molecular structure of G1499–2 was deduced from n.m.r. and from mass spectral data.

The singlet at τ 1.57 (**II**, *e*), which disappears on addition of D_2O, and that at τ 7.72 (**II**, *f*) indicate the presence of a N–H proton and of a methyl group substituted on a double bond, respectively. There are four aromatic protons with signals at τ 1.65 (**II**, *a*) and τ 2.4~2.8 (**II**, *b*, *c*, *d*); thus the partial structure (**XIII**) can be written.



XIII

The positions of the substituents around the ring were elucidated by the application of a lanthanide shift reagent. The large down-field shift of the signal at τ 1.65 (**II**, *a*) noted on addition of Eu(fod)$_3$ to a solution of G1499–2 indicates that the europium atom has complexed to the 4-keto group. The 3-proton singlet at τ 7.72 (**II**, *f*) showed the second largest shift induced by europium and thus the methyl group was assigned to the 3-position. Each of the signals of the four aromatic protons show *ortho* coupling, and the $-C_8H_{13}$ sidechain was therefore assigned to the 2-position.

The electron impact mass spectrum of G1499–2 (Fig. 1) indicates the presence of a *n*-butyl group and this attribution is supported by the high resolution n.m.r. spectrum (Fig. 2). The multiplets given by two methylene groups (**II**, *l* and *m*) show that they are coupling vicinally and that the methylene group at lower field (τ 7.66) is also coupling to one of them. Further, the $-C_8H_{13}$ sidechain contains only one olefinic proton (τ 3.73) and thus a ring must be present. Spin decoupling revealed vicinal coupling between the olefinic proton (**II**, *j*) and the low-field methylene protons at τ 7.66 (**II**, *k*). Only structure **I** is compatible with these conclusions.

The ^{13}C n.m.r. spectrum (Table 1) confirms the presence of a cyclopropane ring. The spectrum exhibited resonances at δ 11.4 and 18.1 for a methylene and a methine group, respectively; these resonances had one-bond ^1H–^{13}C couplings of about 170 Hz, characteristic of cyclopropane rings.[13]

The stereochemistry about the exocyclic double bond is not known.

Acknowledgement

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