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# Autotoxic Antibiotic Production by a Marine *Chromobacterium*

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*Abstract*

An antibiotic-producing *Chromobacter* was isolated from a sea-water sample from the North Pacific Gyre. The bacterium produces 3 bromine-containing metabolites: tetrabromopyrrole, 2-(2'-hydroxy-3', 5'-dibromophenyl)-3,4,5-tribromopyrrole, and hexabromo-2,2'-bipyrrole. It also synthesizes n-propyl 4-hydroxybenzoate and 4-hydroxybenzaldehyde. Some of these compounds were shown to be responsible for autoinhibition of the *Chromobacterium* itself, as well as for antibiotic action against other kinds of bacteria.

*Introduction*

Microbiologists have long known that marine bacteria produce antimicrobial substances which are believed to be partly responsible for the natural antibiotic activity of seawater, as well as playing a major role in the population dynamics of microorganisms (Rosenfeld and Zobell, 1947; Buck *et al.*, 1962; Burkholder, 1973). Despite the potential importance of such substances, almost nothing is known of their chemical structures. Burkholder and co-workers provided the first structure with the discovery that *Pseudomonas bromoutilis* produced the novel halogenated antibiotic 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (Burkholder *et al.*, 1966; Hanessian and Kaltenbronn, 1966; Lovell, 1966). We report herein a second example of halogenated metabolite production by a marine bacterium. Our studies have shown that a marine species of *Chromobacterium* produces a series of highly brominated metabolites which can inhibit the growth of selected human pathogens, as well as certain marine bacteria. We have also demonstrated autoinhibition by the bacterial metabolites, which may account for the tendency of certain strains to die out unless frequently subcultured in fresh media.

*Materials and Methods*

Description and Culturing of the Bacteria

The strain we studied was isolated from a sea-water sample taken in the North Pacific Gyre region (28°N; 155°W) by Dr. R.A. Lewin, who found that it had antibiotic activity. It has been provisionally designated I-L-33.

The maintenance medium consisted of Bacto-Tryptone (10 g/l), yeast extract (1 g/l), and agar (10 g/l) in filtered seawater. All nutrient media were sterilized by autoclaving at 120°C for 15 min. Transfers were necessary at least every 4 days to keep the culture viable. Human pathogens used as test organisms were obtained from Abbott Laboratories and cultured on Tryptic Soy Agar. Marine bacteria, other than I-L-33, were kindly supplied by Dr. K. Nealson of this Institution.

Ultraviolet and visible spectra of the pigment were obtained from 95% ethanol extracts of cells scraped from agar plates and were recorded on a Perkin Elmer 124 double-beam spectrophotometer. Chemical tests performed on the pigment were those described by Sneath (1956).

Procedures for the indole, methyl red, Voges-Proskauer, nitrate reduction, sucrose, catalase, and oxidase tests were those listed in Collins (1967). The tests for hydrogen cyanide production and starch hydrolyses followed the procedures of Gibbs and Skinner (1966).

Mass-culturing Techniques and Extraction Procedures

To obtain sufficient cellular extracts for chemical studies, the bacteria were mass-cultured by two methods.

The first method consisted of 18-l broth cultures. Filtered seawater, to which was added Bacto-Peptone (5 g/l), yeast extract (1 g/l), and NaBr (500 mg/l), was autoclaved in pyrex culture bottles fitted with fritted glass bubblers for aeration and agitation. 500-ml inocula of a 24-h sea-water broth culture of I-L-33 were added, and the cultures were incubated with aeration at 25°C for 2 weeks. At the end of this time, ethyl acetate (3 l) and sodium chloride (2400 g) were added, and the mixture was thoroughly stirred for several hours. After settling had occurred, the ethyl acetate layer was siphoned off and the extract concentrated *in vacuo*. The extraction procedure was repeated three times, and the ethyl acetate residues were combined.

The second method consisted of growing heavy lawns of bacteria on trays (2 x 30 x 60 cm) of sterilized agar medium containing Bacto-Tryptone (10 g/l), yeast extract (1 g/l), and NaBr (500 mg/l). Twelve-hour broth inocula were spread uniformly over the agar surfaces, which were then

covered with heavy aluminium foil and incubated at 25°C for 4 days. Scraping the agar surfaces yielded a dark purple paste of wet cells which was freeze-dried, homogenized in methanol (3 ml/g dry cells), and extracted with ethyl acetate (17 ml/g dry cells). The cellular extracts were obtained by filtering the solid cell residue from the ethyl acetate and evaporating the filtrate *in vacuo*.

### Chemical Analysis of Compounds Produced by *Chromobacterium* I-L-33

NMR spectra of pure compounds were run on a Varian HR220 spectrometer. Infra-red spectra were recorded on a Perkin Elmer Model 700 infrared spectrophotometer. Mass spectra were recorded on a Hewlett Packard Model 5930A mass spectrometer, and ultra-violet spectra on a Perkin Elmer 124 double-beam spectrophotometer. Melting points were determined on a Fisher John's melting point apparatus and are uncorrected.

Crude extracts obtained from both methods of mass-culturing were triturated with benzene (100 ml/g crude extract) to precipitate the purple pigment. The benzene extracts obtained by filtration were evaporated *in vacuo*, and the residue was chromatographed on Florisil, eluting with hexane, benzene, and ethyl acetate gradients to obtain the following pure compounds:

*Tetrabromopyrrole (1)*: yield 10 mg (0.2% of the crude benzene triturate from broth cultures); NMR ($CDCl_3$) none; mass spectrum (70 ev) m/e $M^+$ $C_4HNBr_4$ 378.7, 380.7, 382.7, 384.7, 386.7 (1:4:6:4:1); 300, 302, 304, 306 (1:3:3:1) M-Br; 273, 275, 277, 279 (1:3:3:1) M-HCNBr; 221, 223, 224 (1:2:1) M-$Br_2$; 194, 196, 198 (1:2:1) M-$HCNBr_2$; 142, 144 (1:1) M-$Br_3$; 115, 117 (1:1) M-$HCNBr_3$; 63 M-$Br_4$; 62 M-$HBr_4$. The mass spectrum was identical to that of synthetic tetrabromopyrrole, prepared by adding four equivalents of bromine to pyrrole in acetic acid (Palmer, 1967). Tetrabromopyrrole is unstable and extremely light sensitive: we were unable to record an ultra-violet spectrum or a melting point.

*2-(2'-Hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (2)*: yield 15 mg (0.3% of crude); NMR ($CDCl_3$) $\delta$ 6.07 (s,1H), 7.60 (d,1H,J=2.5Hz), 8.13 (d,1H,J=2.5Hz), 9.5 (broad, 1H); ultra-violet $\lambda_{max}$ 286, 308 mμ (MeOH), 284, 293, 355 (MeOH/NaOH); mass spectrum (70 eV) m/e $M^+$ $C_{10}H_4NOBr_5$ 548.5, 550.5, 552.5, 554.5, 556.5, 558.5 (1:5:10:10:5:1); 470.5, 472.5, 474.5, 476.5, 478.5 (1:4:6:4:1) M-Br; 442, 445, 447, 449, 450 (1:4:6:4:1) M-HCNBr; 391, 393, 395, 397 (1:3:3:1) M-$Br_2$; 364, 366, 368, 370 (1:3:3:1) M-$HCNBr_2$; 312, 314, 316 (1:2:1) M-$Br_3$; 285, 287, 289 (1:2:1) M-$HCNBr_3$; 80, 82 (1:1) HBr (base peak). This material was identical to a synthetic sample of 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (Hannessian and Kaltenbronn, 1966) kindly supplied to us by Dr. J.S. Kaltenbronn.

*Hexabromo-2,2'-bipyrrole (3)*: yield 10 mg (0.2% of crude); NMR ($D_6$ benzene) none; ultra-violet $\lambda_{max}$ 258 mμ (MeOH), 272 mμ (MeOH/NaOH); mass spectrum (70 eV) m/e $M^+$ $C_8H_2N_2Br_6$ 559.6, 601.6, 603.6, 605.6, 607.6, 609.6, 611.6 (1:6:15:20:15:6:1); 520.5, 522.5, 524.5, 526.5, 528.5, 530.5 (1:5:10:10:5:1) M-Br; 494, 496, 498, 500, 502, 504 (1:5:10:10:5:1) M-HCNBr; 442, 444, 446, 448, 450 (1:4:6:4:1) M-$Br_2$; 415, 417, 419, 421, 423 (1:4:6:4:1) M-$HCNBr_2$; 363, 365, 367, 369 (1:3:3:1) M-$Br_3$; 336, 338, 340, 342 (1:3:3:1) M-$HCNBr_3$; 126 M-$Br_6$; 125 M-$Br_6$-H; 124 M-$Br_6$-$H_2$. This material was identical to synthetic hexabromo-2,2'-bipyrrole obtained by treating 2,2'-bipyrrole (Rapoport and Castagnoli, 1962) with 6 equivalents of pyridinium hydrobromide perbromide (Feiser and Feiser, 1968) in acetic acid at 16°C. The instability of this compound prevented us from obtaining acceptable analytical data.

*n-Propyl 4-hydroxybenzoate (4)*: yield 10 mg (0.2% of crude); crystallized from ether; melting point 96°C (lit. 96.2°C); mass spectrum (70 eV) m/e $M^+$ $C_{10}H_{12}O_3$ 180; 163 M-OH; 121 M-$OCH_2CH_2CH_3$ (base peak). The natural material was identical to authentic n-propyl 4-hydroxybenzoate (Aldrich Chemical Chemical Company).

*4-Hydroxybenzaldehyde (5)*: yield 50 mg (1% of crude); crystals from chloroform; melting point 116°C (lit. 117° to 119°C); NMR ($CDCl_3$ $\delta$ 7.02 (d,2H,J=8Hz), 7.80 (d,2H,J=8Hz), 9.00 (b,1H), 9.90 (s,1H). The natural material was identical to authentic 4-hydroxybenzaldehyde (Aldrich Chemical Company).

### Antibiotic Screening

All antibiotic activity was tested by use of the disc assay method. Pure compounds were applied as dilute solutions (i.e., 100 μl of 50 mg/ml solutions) to sterilized 1.2 cm paper discs, which were then placed on agar plates freshly inoculated with the test organisms. Positive inhibition was indicated by the appearance of a bacteria-free zone more than 1 mm wide around the assay disc after the proper period of incubation (15 h at 37°C for human pathogens; 2 to 3 days at 25°C for marine bacteria). Chromotographic fractions were also monitored by screening against *Staphylococcus aureus* to determine which contained active antibiotic compounds.

## *Results*

### Description and Identification of the Bacteria

On agar plates, I-L-33 produces smooth, convex colonies which, in 2 to 3 days, grow to about 2 mm in diameter. During the first 12 h of growth the colonies are an opaque beige color; they begin to develop purple pigmentation only during the next 12 h. The pigment appears first in the center of

the colonies, spreading slowly to the edges and eventually coloring the entire colony a deep purple.

Cultures tend to lose viability after 3 to 4 days, depending on how heavily the original plate is inoculated. Inoculating with only a few cells or with one small colony generally results in longer viability. Incubation of the bacteria at 4°C produces no growth, while incubation at 37°C produces slight growth. The optimum incubation temperature was found to be close to room temperature (22° to 27°C).

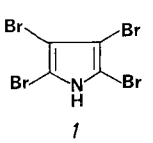
Spectral analysis of the purple pigment in 95% ethanol reveals an absorption maximum at 578 mμ, shifting to 698 mμ in 10% $H_2SO_4$/ethanol. Treating ethanolic solutions of the pigment with equal volumes of the following aqueous reagents resulted in the corresponding colors: 10% KOH, green; 50% $HNO_3$, yellowish orange; 50% $H_2SO_4$, emerald green; glacial acetic acid, blue; bromine water, decolorized; 5M $NH_4OH$, sapphire blue. The spectral analysis, considered along with these color tests, indicated conclusively that the pigment is violacein (Sneath, 1956).

I-L-33 is a Gram-negative, motile, purple-pigmented, rod-shaped bacterium. The following biochemical tests were negative: indole production, methyl red, Voges-Proskauer, and catalase. I-L-33 was found to hydrolyze starch, produce hydrogen cyanide, and exhibit strong oxidase activity.

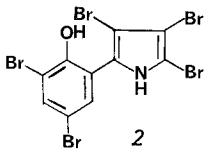
## Compounds Produced by I-L-33

Extraction of 108 l of bacterial broth culture resulted in 4.5 g of crude benzene triturate. Chromatography of the benzene triturate on Florisil yielded 5 pure compounds. Analyses of the mass spectrum and of ultraviolet, infrared, and NMR spectra of the pure compounds led to the structures proposed. All the proposed structures were confirmed by comparison with authentic synthetic material.

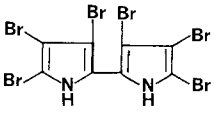
The 5 compounds produced by the bacteria in broth culture are tetrabromopyrrole (*1*), 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), hexabromo-2,2'-bipyrrole (*3*), n-propyl 4-hydroxybenzoate (*4*), and 4-hydroxybenzaldehyde (*5*).




*1*




*2*

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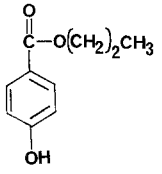
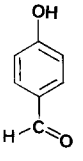



*4*




*5*

Chromatography of the benzene triturate of the extracts from cells grown on the solid agar trays resulted in the isolation of only hexabromo-2,2'-bipyrrole (*3*) and 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), the latter in very small quantities. Examination of ethyl acetate extracts of the agar from which the cells had been scraped revealed the presence of 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), 4-hydroxybenzaldehyde (*5*), and traces of n-propyl 4-hydroxybenzoate (*4*). Failure to find tetrabromopyrrole (*1*) in either the solid agar or cell extracts is undoubtedly due to its great instability, especially when exposed to light and oxygen.

The results of the agar-tray extractions suggest that hexabromo-2,2'-bipyrrole (*3*) is exclusively an intracellular component, that 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*) is mainly extracellular, and that 4-hydroxybenzaldehyde (*5*) and n-propyl 4-hydroxybenzoate (*4*) are exclusively extracellular metabolites.

## Antibiotic Screening

Crude benzene triturates inhibited the growth of a variety of test organisms, as well as I-L-33 itself. The pure compounds responsible for the inhibition of the human pathogens (Table 1) were tetrabromopyrrole (*1*), the tribromopyrrole (*2*), and 4-hydroxybenzaldehyde (*5*). All the pure compounds from the benzene triturate showed some activity against the selected marine test organisms, while only 3, tetrabromopyrrole (*1*), 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), and 4-hydroxybenzaldehyde (*5*), possess the ability to inhibit I-L-33.

## *Discussion*

The definite sodium ion requirement for growth of strain I-L-33 indicates that it is probably a true marine species, and its production of the purple pigment violacein indicates that it is in the genus *Chromobacterium* (Sneath, 1956). Furthermore, its physiological, cultural, and morphological characteristics are essentially identical to those of *C. marinum*, as described by Hamilton and Austin (1967). (We obtained a freeze-dried culture of *C. marinum*, ATCC No. 19699, and attempted to determine whether it also produced brominated antibiotics like those of I-L-33, but the culture was apparently not viable.)

Our results demonstrating the production of brominated metabolites are the first documentation of halogenation by a marine species of *Chromobacterium*. *Pseudomonas bromoutilis* is the only other marine bacterium which has been shown to halogenate organic compounds. 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), a highly active antibiotic, is a metabolite of both species of bacteria, which may indicate a phylogenetic relationship between the two. I-L-33 also produces tetrabromopyrrole (*1*) and hexabromo-2,2'-bipyrrole

Table 1. *Chromobacterium* metabolites. Results of disc-assay antibiotic screening. All compounds were tested at concentrations of 5 mg/disc except 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole, which was tested at 0.03 mg/disc. Zones of inhibition greater than 6 mm are designated by ++, 1-6 mm by +, and no inhibition by -. NT: not tested

| Test organisms | Test compounds | | | | |
|---|---|---|---|---|---|
| | Tetrabromopyrrole | 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole | Hexabromo-2,2'-bipyrrole | n-propyl 4-hydroxybenzoate | 4-hydroxybenzaldehyde |
| Human pathogens | | | | | |
| *Staphylococcus aureus* (ATCC 6538P) | ++ | ++ | - | - | + |
| *Escherichia coli* (ATCC 11775) | + | NT | - | - | - |
| *Pseudomonas aeruginosa* (ATCC 10145) | + | NT | - | - | - |
| *Candida albicans* (ATCC 10231) | + | NT | - | - | + |
| *Enterobacter aerogenes* (ATCC 13048) | - | NT | - | - | - |
| Marine bacteria | | | | | |
| *Photobacterium fisheri* | ++ | - | + | - | + |
| *P. mandapamensis* | ++ | - | + | - | + |
| *P. phosphoreum* | + | + | + | + | ++ |
| *Beneckea harvevi* | + | - | - | - | + |
| *Chromobacter* I-L-33 | + | + | - | - | + |

(*3*), previously unreported as natural products. Hexabromo-2,2'-bipyrrole is the first example of a naturally occurring compound with a bipyrrole skeleton which does not bear other carbon substituents. It may be related biosynthetically to the prodigiosenes (Williams and Hearn, 1967).

Hamilton and Austin, when describing *Chromobacterium marinum*, attributed the short life of laboratory cultures of this bacterium to the accumulation of toxic by-products in the media (Hamilton and Austin, 1967). A similar situation appears to prevail with I-L-33. The autotoxic substances may, in fact, be tetrabromopyrrole (*1*), 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), and 4-hydroxybenzaldehyde (*5*). The results of our agar extraction experiments show that the pentabromophenylpyrrole *2* and the benzaldehyde *5* are excreted into the medium, and we have also shown that these compounds inhibit growth of *Chromobacterium*. We were unable to determine whether tetrabromopyrrole (*1*) is also excreted into the medium, but since it is a minor component it seems reasonable to suppose that 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*) and 4-hydroxybenzaldehyde (*5*) are the most effective autoinhibitors. Autoinhibition by metabolites would not be expected to be an important phenomenon in the ocean, where dilution would keep the concentrations of such extracellular metabolites low. However, it may be the limiting factor in agar-plate culture of these organisms.

The studies of Burkholder and his co-worker on *Pseudomonas bromoutilis* (Burkholder *et al.*, 1966; Lovell, 1966), and now our own studies on I-L-33, demonstrate that there is much to be learned from chemical investigations of the secondary metabolites of marine bacteria. New compounds formed by novel biosynthetic pathways may be discovered, providing chemical bases for microbiological phenomena hitherto unexplained. Such studies also raise questions concerning the ecological significance of these compounds. One wonders whether they could eventually accumulate in concentrations sufficient to be responsible for the natural antibiotic activity of seawater, and whether they might really aid the bacteria which produce them in the competition for nutrients.

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Dr. J.S. Kaltenbronn for the gift of an authentic sample of 2(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole (*2*), and Abbott Laboratories for supplying cultures of human pathogens. We also thank Drs. Lewin and Nealson for their constant encouragement and advice. This research was supported by grants from the National Science Foundation (GB-37227) and the Department of Commerce, and by gifts from Abbott Laboratories and Hoffmann-LaRoche. R.J.A. was supported by a predoctoral fellowship from the National Research Council of Canada.

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