treated cellular lysates at 37°C with plasmin for measured time periods and then used immunoprecipitation and SDS-PAGE analysis. The 180,000-dalton pro-C3 was cleaved to peptides with mobilities identical to those of the α and β chains (115,000 and 75,000 daltons) of the C3 secreted by the hepatoma as well as to those of purified C3 protein from human plasma. Continued incubation resulted in further cleavage of the α chain to an *a'* chain (100,000 daltons) (Fig. 1B). Neither urokinase alone, in an amount present in the plasmin preparation (lane 1 in Fig. 1B), nor incubation of the lysate alone at 37°C (lane 8 in Fig. 1B) resulted in cleavage of pro-C3.

Previous studies had established, on the basis of the identity of partial amino acid sequences, kinetics of synthesis in cell culture, cell-free synthesis, and conversion in vitro of procomplement to native protein, that pro-C4 is the precursor of C4. We have now demonstrated that the third component of human complement is synthesized as a procomplement protein (pro-C3), and we have defined the precursor-product relationship between the single-chain pro-C3 and disulfide-linked two-chain native C3. Hence, in addition to their similar structural (*27*) and functional features, there are similarities in the biosynthesis and postsynthetic processing of C3 and C4. Moreover, our data indicate the potential utility of the Hep G2 cell line in delineating the synthesis, processing, and control of secretion of other human plasma proteins.

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Isolation of Chlorine-Containing Antibiotic from the Freshwater Cyanobacterium *Scytonema hofmanni*

Abstract. Scytonema hofmanni, *a filamentous freshwater cyanobacterium (blue-green alga), produces secondary metabolites which inhibit the growth of other cyanobacteria and green algae. A rapid, qualitative assay for this inhibition has been developed with* Synechococcus *as the test organism. This assay procedure has led to the isolation and characterization of an antibiotic (named cyanobacterin) from* Scytonema. *The antibiotic has a molecular weight of 430 and an empirical formula of* $C_{23}H_{23}O_6Cl$ *and contains a γ-lactone and a chlorinated aromatic nucleus. It inhibits the growth of various algae but has limited effect on nonphotosynthetic bacteria or protozoans and thus may have potential use as a specific algicide.*

The production and excretion of antimicrobial compounds by algae have been reported. Most of these are produced by marine flora, principally the red and brown algae (*1–3*). Some antibiotic substances have been reported in freshwater chlorophytes (*4*) and may also occur in the cyanobacteria (*5*). It has been suggested that some of these may have allelopathic effects and thus play a role in the species succession noted in freshwater (*6, 7*) and marine (*8*) ecosystems.

Except for the mammalian neurotoxin, anatoxin A from *Anabaena flos-aquae* (*9*), antibiotic substances from freshwater cyanobacteria have not been isolated or characterized. We now report the occurrence of an antibiotic in the freshwater cyanobacterium *Scytonema hofmanni* (UTEX 1581). We have isolated a pure compound from this alga and have partially characterized it with respect to chemical structure and biological activity.

The antimicrobial properties of *Scytonema hofmanni* were first observed in laboratory cultures while attempting to grow this organism with other species of algae (*10*). The effects were most obvious in petri plates where filaments of *S. hofmanni* and another alga were initially streaked at right angles to each other on the agar. Within 7 to 10 days, *S. hofmanni*, which grows rather slowly under our conditions, had cleared the area in its vicinity of almost any other species with which it was paired. To eliminate the possibility that this clearing was due to competition for nutrients, cell-free extracts of *S. hofmanni* were prepared and tested against *Synechococcus* sp. (ATCC 27146), a unicellular cyanobacterium, growing in liquid culture. Growth of *Synechococcus* was monitored by measuring the optical density of the cultures over a period of 2 weeks. Growth of *Synechococcus* was inhibited by crude extract protein (as little as 30 μg/ml), an indication of the presence of potent antibiotic activity (*11*). Similar results were obtained with medium from a *S. hofmanni* culture as the antibiotic source. After 2 weeks of growth, the *S. hofmanni* filaments were removed by filtration. The spent medium was then lyophilized to dryness, and the residue was resuspended in a small volume of distilled water. After filter-sterilization, the medium was added to *Synechococcus* cultures and it inhibited the growth of these organisms as did the cell extracts. Thus under the conditions of laboratory culture, it is probable that *S. hofmanni* actively excretes the antibiotic.

Scytonema hofmanni filaments or cell-

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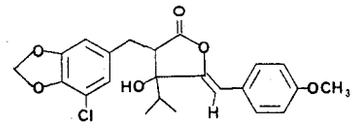
free extract inhibited the growth of various microorganisms (Table 1). The antibiotic from *S. hofmanni* is particularly active in inhibiting the growth of other cyanobacteria; it is effective against some eukaryotic algae, but appears to be relatively ineffective against nonphotosynthetic bacteria or the protozoan *Tetrahymena pyriformis*. Electron micrographs of inhibited *Synechococcus* cultures show a deterioration of the thylakoid membranes and the cell walls (Fig. 1).

Cell fractions were tested for antibiotic activity by dropping a known volume of filter-sterilized material onto a previously prepared lawn of *Synechococcus* sp. plated on Bg 11. The plates were incubated at 20°C at 250 μEinstein/m^2-sec for 12 to 15 hours. Active antibiotics will create a clear zone in the *Synechococcus* lawn. This procedure proved to be a reliable and rapid method of detecting the antibiotic and was used for subsequent purification. Using this assay, we determined that the antibiotic could be efficiently extracted from disrupted cells with organic solvents. Although the antibiotic could also be obtained from the medium, extraction was more cumbersome and total yields were low.

Cell-free extract (protein concentration, ~ 15 mg/ml) was lyophilized; the dried material was extracted with ethyl ether, and the extract taken to dryness (*12*). The residue was dissolved in ethyl ether and further purified by preparative thin-layer chromatography on silica gel and finally by high-performance liquid chromatography (*13*). A final chromatography on silica gel showed one ultraviolet-quenching band. From 100 g (wet weight) of cells, approximately 40 mg of highly purified antibiotic was obtained.

The purified antibiotic was characterized by several analytical techniques. The empirical formula of the antibiotic is $C_{23}H_{23}O_6Cl$ by high-resolution mass spectrometry. Further structural analysis by ^{13}C and proton nuclear magnetic resonance and by infrared spectroscopy indicate that the active component is a diaryl substituted γ-lactone. The spectral analysis has been partially confirmed by x-ray diffraction (*14*). We now suggest the name cyanobacterin for this compound.



Purified cyanobacterin is readily soluble in organic solvents such as ether, ethanol, and dimethyl sulfoxide, but relatively insoluble in water. A suspension of cyanobacterin was effective at 1.5 μg/ml (minimum effective dose) in inhibiting the growth of *Synechococcus* cultures in

Table 1. Organisms tested for ability to grow in the presence of *Scytonema hofmanni*; +, inhibition by *S. hofmanni*; −, no effect observed.

Test organism	Source*	Inhibition by		Test organism	Source*	Inhibition by	
		Intact filaments	Cell-free extract			Intact filaments	Cell-free extract
	Cyanobacteria†				Chlorophytes§		
Synechococcus sp.	ATCC 27146	+	+	*Ankistrodesmus angustus*	ATCC 30448	+	
Anacystis nidulans	UTEX 625	+		*Chlamydomonas reinhardtii*	UTEX 89	−	
Microcystis aeruginosa	UTEX 1939	+		*Characium californicum*	UTEX 2097	+	
Aphanocapsa sp. 6308	A. J. Smith	+	+	*Chlorella pyrenoidosa*	UTEX 251	−	
Gloeocapsa alpicola	CCAP 1430/1	+		*Chlorococcum macrostigmatum*	UTEX 109	−	
Agmenellum quadruplicatum	C. van Baalen	+		*Coelastrum probos-cideum* var. *gracile*	ATCC 30410	+	
Anabaena cylindrica	IVL	+		*Cosmarium botrytis*	UTEX 175	+	
Anabaena sp. 7119	IVL	+	+	*Golenkinia minutissima*	UTEX 929	+	
Anabaena flos-aquae	UTEX 1444	+		*Haematococcus lacustris*	ATCC 30402	+	
Nostoc muscorum	UTEX 486	+		*Pandorina morum*	UTEX 873	−	
Nostoc commune	UTEX 584	+		*Pediastrum biradiatum*	UTEX 27	+	
Cylindrospermum maius	CCAP 1415/2	+		*Scenedesmus quadricauda*	ATCC 11460	−	
Hapalosiphon welwitschii	UTEX 1830	−		*Selenastrum capri-cornutum*	ATCC 22662	+	
Scytonema hofmanni	UTEX 1581		−	*Staurastrum* sp.	UTEX 173	+	
Tolypothrix tenuis	CCAP 1482/3	+		*Stichococcus bacillaris*	UTEX 176	−	
Fremyella diplosiphon	UTEX 481	+		*Stigeoclonium pascheri*	UTEX 320	+	
Plectonema boryanum	UTEX 581	+		*Ulothrix acuminata*	UTEX 1178	+	
Plectonema calothricoides	UTEX 598	+			Rhodophytes§		
Oscillatoria prolifera	UTEX 1270	+		*Porphyridium aerugineum*	UTEX 755	+	
Phormidium autumnale	UTEX 1580	+			Euglenophytes‖		
Lyngbya kuetzingii	UTEX 1547	+		*Euglena gracilis* Z	UTEX 753	+	+
Chlorogloea fritschii	A. J. Smith	−			Protozoa¶		
	Bacteria‡			*Tetrahymena pyriformis* ST	Y. Suyama		−
Escherichia coli B	B. Ames		−				
Salmonella typhimurium	B. Ames		−				
Bacillus brevis	R. Crawford		+				
Nocardia asteroides	CDC		−				
Streptomyces viridosporus	R. Crawford		−				

*Sources: ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852; UTEX, University of Texas at Austin Collection, R. C. Starr, Department of Botany, Austin 48712; A. J. Smith, Department of Biochemistry, University College of Wales, Aberystwyth, Dyfed, Great Britain; CCAP, Culture Centre of Algae and Protozoa, 26 Storey's Way, Cambridge, England; C. van Baalen, University of Texas Marine Science Institute, Port Aransas 78373; IVL, Institut för Vatten och Luftvards forskning, A. Neilson, Box 21060, S-10031 Stockholm, Sweden; R. Crawford, Gray Freshwater Biological Institute, University of Minnesota, Navarre 55392; CDC, Centers for Disease Control, Atlanta, Georgia; Y. Suyama, Department of Biology, University of Pennsylvania, Philadelphia 19104. †Cyanobacteria were grown in Bg 11 (*19*), liquid or solidified with 1.5 percent agar, at 20°C and constant illumination from Sylvania Coolwhite lamps (250 μE/m^2-sec), except for *Agmenellum quadruplicatum*, which was grown in ASP-2 (*20*). ‡Bacteria were grown in liquid culture on Bacto-Nutrient Broth at 30°C. §Chlorophytes and rhodophytes were grown in MBL medium (*21*), liquid or solidified with 1.5 percent agar, under the same light conditions as for cyanobacteria. ‖*Euglena gracilis* was grown on liquid medium (*22*) under the same light conditions as for cyanobacteria. ¶*Tetrahymena pyriformis* was grown in liquid medium (2 g of proteose peptone, 0.1 g of yeast extract, 10 mg of Geigy iron chelate) at 20°C.

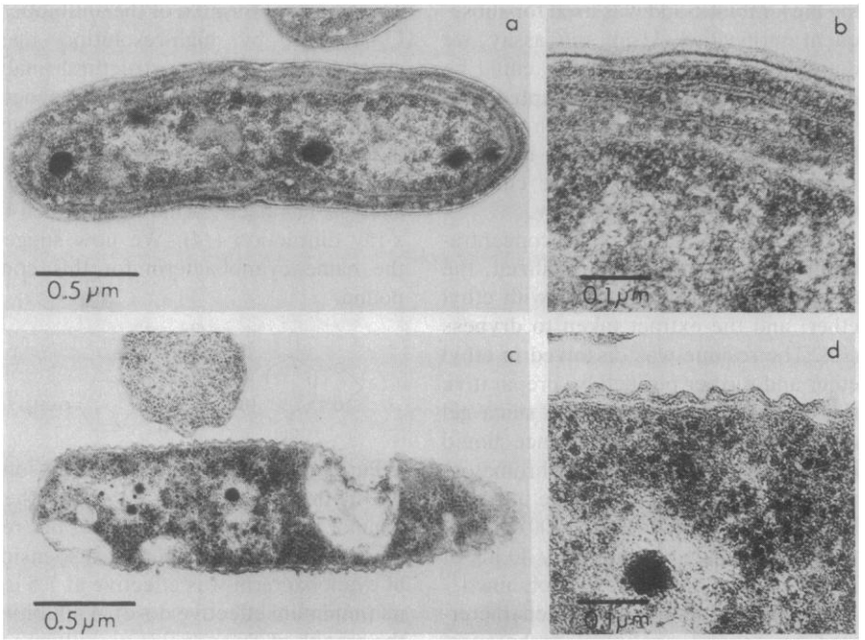


Fig. 1. Effect of cell-free extract of *Scytonema hofmanni* on structure of *Synechococcus* sp. *Scytonema hofmanni* was grown and cell-free extract prepared as described in (*11*). The extract was added to *Synechococcus* cultures (1.6 mg of protein per milliliter) and the cultures allowed to grow for 2 days. The control culture received an equivalent volume of 50 m*M* tris-HCl buffer, *p*H 7.5. Cells were then harvested by centrifugation and prepared for electron microscopy (*23*). (a) An untreated *Synechococcus* cell at 30,000-fold magnification. (b) A section of the cell wall in (a) at a 100,000-fold magnification. The cell wall appears as a multilayered structure typical of cyanobacteria (*23*). Two parallel arrays of thylakoid membranes are clearly visible in the cytoplasm at the periphery of the cell. (c) Morphology of a *Synechococcus* cell after treatment with *Scytonema* extract. Clear areas in the cytoplasm indicate a loss of cellular components, presumably due to increased permeability of the cell wall and membrane. A 100,000-fold magnified section of the cell wall in (d) shows that the wall, although still multilayered, is grossly distorted. No thylakoid membranes are evident in the treated cells.

tests similar to those described (*11*). The hydrophobic nature of cyanobacterin further supports the evidence from electron microscopy, which shows that the cell membrane and thylakoids are the major target of the antibiotic.

Perhaps the most unusual aspect of the structure of cyanobacterin is the presence of a chlorinated aromatic substituent. Halogenated compounds are well known in marine algae, principally the rhodophytes (*15*). Many of these have been shown to have toxic or antibiotic properties (or both). Halogenated toxins have also been found in marine cyanobacteria (*5, 16, 17*). However, these halogenated compounds are not present in freshwater species. Our isolation of a chlorinated antibiotic from a freshwater cyanobacterium confirms that these organisms also have the metabolic capability of producing halogenated compounds (*18*).

Our data (Table 1) demonstrate that cyanobacterin is principally an algicide, its major effect being on cyanobacteria and green algae. Although the study to date has been done only on laboratory cultures, the evidence suggests that cyanobacterin may be an allelopathic substance, allowing a slow-growing species such as *Scytonema* to survive in the presence of the more prolific species. A search for halogenated aromatics in freshwater ecosystems where blue-green algae predominate could provide valuable information on species succession and naturally occurring algicides.

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11. *Scytonema hofmanni* was grown in liquid medium Bg 11 (*19*) for 2 weeks at 20°C and a constant light intensity of approximately 50 μE/m²-sec photosynthetic active radiation from a Sylvania Coolwhite fluorescent lamp. Cells were harvested by filtration through Whatman 1 filter paper. The clumps of filaments are retained on the paper. The filaments were resuspended in 10 m*M* tris-HCl buffer, *p*H 7.5, and the cells were broken by sonic oscillation (Heat Systems model W350 sonicator). The suspension was then centrifuged at 10,000*g* for 15 minutes (Sorvall RC-5 centrifuge set at 4°C) and the supernatant was assayed for antibiotic activity. The chemical nature of the toxin was unknown in these experiments. Therefore, the amount of extract added to test cultures was quantified by measuring the protein concentration. Protein was determined by the biuret method [A. G. Gornall, C. J. Bardawill, M. M. David, *J. Biol. Chem.* **177**, 751 (1949)]. *Synechococcus* sp. was inoculated into culture tubes containing 10 ml of liquid Bg 11 on day 1; the tubes were incubated at 20°C (constant light intensity of 250 μE/m²-sec). Extract of *S. hofmanni* (3.2 mg of protein per milliliter) was filter-sterilized and added to the *Synechococcus* cultures on day 3. The control received 1 ml of 10 m*M* tris-HCl buffer, *p*H 7.5. Growth was monitored by absorbancy at 650 nm.
12. Batch cultures were grown in 10-liter carboys, containing 9 liters of Bg 11 medium (*19*) supplemented with 1 g of Na_2CO_3 per liter. The carboys were inoculated with 300 ml of 3-week-old *Scytonema* culture, and aerated with a mixture of 5 percent CO_2 in air. The initial light intensity was 260 μE/m²-sec and was increased to 650 μE/m²-sec after 1 week of growth. Cells were harvested after approximately 3 weeks. The cells were resuspended in 60 m*M* tris-HCl buffer, *p*H 7.5, and sonicated and centrifuged (*11*).
13. Lyophilized cell extracts were suspended in ethyl ether (Mallinckrodt, Anhydrous AR). After thorough mixing, the ether layer was decanted and taken to dryness on a rotary evaporator. The residue was dissolved in ethyl ether and applied to silica gel GF plates (Analtech, 1000-μm plates) and the plates were developed with chloroform (certified ACS grade, Fisher Scientific). Bands that quenched fluorescence of the gel at 250 nm were scraped off the plate and extracted with chloroform. The extracts were taken to dryness on a rotary evaporator. The resulting residues were resuspended in ether, filter-sterilized, and tested for activity in the *Synechococcus* plate assay. One band with an R_F of approximately 0.5 was active and was purified further. The bioactive material was dissolved in methanol (HPLC grade, Fisher Scientific), and various components were separated by high-performance liquid chromatography (Hewlett-Packard, model 1084 BLC; Whatman Partisil M90D52 column). The mobile phase was 75 percent methanol and 25 percent H_2O. The peaks absorbing at 254 nm were collected and assayed for antibiotic activity. An active component eluted at 24.5 minutes. This material was again dried and redissolved in ether.
14. The exact conformation (because of the two chiral centers) remains undetermined.
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