that shedding occurs as a free-running circadian rhythm in the rat [M. M. LaVail, *Science* **194**, 1071 (1976)]. Light exposure for 2 hours after dark adaptation of 1 to 7 days results in shedding of a greater number of larger phagosomes in larval frogs than does exposure to cyclic light (*16*).

5. C. D. B. Bridges, J. G. Hollyfield, J. C. Besharse, M. E. Rayborn, *Exp. Eye Res.* **23**, 637 (1976).
6. Taylor and Kollros stages XIV to XVII, [A. C. Taylor and J. J. Kollros, *Anat. Rec.* **94**, 7 (1946)].
7. Eyes of *R. pipiens* tadpoles were fixed by the same procedure used previously (*16*). Eyes of *X. laevis* tadpoles were fixed with 1.5 percent glutaraldehyde in 0.067*M* cacodylate buffer at *p*H 7.4 containing 0.5 percent $CaCl_2$. All eyes were postfixed in 1 percent OsO_4 and processed for sectioning. Procedures for preparation of autoradiographs have been reported [J. G. Hollyfield, L. S. Mottow, A. Ward, *Exp. Eye Res.* **20**, 383 (1973)] (*17*).
8. The light source was a 25-watt tungsten bulb which yielded illumination of about 200 lu/m^2 at the level of the animal containers. In previous studies of the effect of light on ROS renewal, the illumination was much higher (about 6400 lu/m^2), sufficient to quickly damage albino rat photoreceptors (*2, 3*).
9. Throughout this report the rate of radioactive band displacement is assumed to bear a direct relationship to the rate of ROS disc addition (renewal) (*10*).
10. In two separate experiments the rate of radioactive band displacement declined during the second 6-day period. The meaning of this observation is not immediately obvious, and is not directly pertinent to the major points of this report. However, it does not necessarily mean that the rate of ROS disk addition changed with increasing postinjection time. For example, if ROS discs were packed together to a greater extent as they were displaced, this would produce an apparent slowing of band displacement.
11. M. S. Kinney and S. K. Fisher, paper presented at the annual meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Fla., April 1976. We thank Kinney for providing us with her fixation technique for eyes of *X. laevis* (*7*).
12. Tadpoles were at stages 54 to 55 at the time of injection [P. D. Nieuwkoop and J. Faber, Eds. *Normal Table of Xenopus laevis (Daudin)* (North-Holland, Amsterdam, 1967), pp. 181–182].
13. R. W. Young, in *The Retina*, B. R. Straatsma, M. O. Hall, R. A. Allen, F. Crescitelli, Eds. (Univ. of California Press, Berkeley, 1969), p. 177. Disk addition to frog ROS was approximately doubled with a 10°C rise in temperature. In larval Ozark cave salamanders, constant light at about 250 lu/m^2 results in increased ROS length [J. C. Besharse and R. A. Brandon, *J. Morphol.* **149**, 527 (1976)]. In addition, light exposure for 12 hours daily results in an increase in the ROS renewal rate (*17*).
14. With knowledge of the Q_{10} of ROS renewal it is possible to predict the temperature increase due to light absorption necessary to account for a given increase in the renewal rate. Thus, if a Q_{10} of 2 is assumed (*13*), the 54 percent increase in the rate in constant light would require a 5.4°C rise in local temperature whereas the 24 percent increase in animals receiving 2 hours of light per day would require a 29°C rise in temperature during the time of light exposure. This would correspond to an average increase of 2.4°C over the entire 24-hour period. Likewise, the 300 percent increase in constant light after 6 days of dark adaptation would require a rise in temperature in excess of 20°C. The latter two values would place the temperature in the photoreceptor microenvironment outside the range compatible with cell viability.
15. Since this paper was submitted for publication, we have found that light influences renewal in adult *R. pipiens* as well. Over a period of 5 days, the renewal rate was 0.74 μm/day in darkness, 0.84 μm/day in cyclic light, and 1.14 μm/day in constant light. During 24 hours in light after 5 days in darkness, the radioactive band was displaced 1.5 μm.
16. J. G. Hollyfield, J. C. Besharse, M. E. Rayborn, *Exp. Eye Res.* **23**, 623 (1976).
17. J. C. Besharse and J. G. Hollyfield, *J. Exp. Zool.* **198**, 287 (1976).
18. We thank C. D. B. Bridges for critical reading and helpful suggestions on the original manuscript. Supported by NIH research grants EY-00624 and EY-01632, postdoctoral fellowship 1 F32 EY-05119, and research career development award 1 K04 EY-00023, and by a grant from Fight For Sight, Inc.

7 September 1976; revised 23 November 1976.

Antileukemia Activity in the Oscillatoriaceae: Isolation of Debromoaplysiatoxin from *Lyngbya*

Abstract. *Chloroform extracts of several seaweeds, of the family Oscillatoriaceae, from Enewetak Atoll, Marshall Islands, display activity against P-388 lymphocytic mouse leukemia. A P-388 active compound, debromoaplysiatoxin, has been isolated from* Lyngbya gracilis *and characterized. This compound also has dermonecrotic activity and may be the dermatitis-producing substance in* L. majuscula, *the causative agent of "swimmers' itch" outbreaks in Hawaiian waters.*

Over the past two decades a few reports of biological and pharmacological activities of extracts of marine blue-green algae have appeared in the literature, but little progress has been made on the isolation and identification of the active principles. Lipid extracts of *Lyngbya majuscula* Gomont, the causative organism in sporadic outbreaks of a contact dermatitis (swimmers' itch) among swimmers in Hawaiian waters, show dermonecrotic activity (*1*). *Schizothrix calcicola* (Ag.) Gomont, an alga suspected of being associated with the appearance of toxic fish on the atoll of Marakei in the Gilbert Islands (*2*), contains two lipid-soluble toxins (*3*). Extracts of several species of *Hydrocoleum* (*4*) and *L. majuscula* (*5*) have demonstrated antibiotic properties. Antiviral activity has also been reported for extracts of *L. majuscula* (*5*).

We have found that marine blue-green algae are potential sources of anticancer compounds. In the fall of 1975, specimens of several blue-green algae were collected at Enewetak Atoll in the Marshall Islands. Chloroform extracts of these algae were tested for activity against P-388 lymphocytic leukemia in mice. Extracts of seaweeds belonging to the family Oscillatoriaceae consistently displayed activity in the P-388 assay, three extracts, those of *Lyngbya*, the *Oscillatoria-Schizothrix* mixture, and *Symploca*, being particularly active (Table 1).

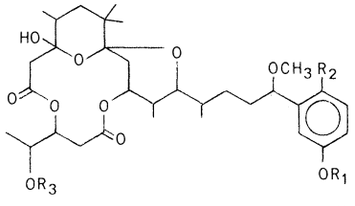
The availability of a large amount of a *Lyngbya* from a single location prompted us to select this alga for initial study. Frozen *L. gracilis* Gomont (*6*) (3 kg wet weight) collected from Reefer 8 Pinnacle, Enewetak lagoon, was homogenized and extracted with a mixture of chloroform and methanol (1 : 2 by volume). Water was added to the filtrate and

Table 1. Activity of chloroform extracts of blue-green algae collected at Enewetak Atoll against P-388 lymphocytic leukemia in mice. The activity is expressed as ratio of the mean survival time of the diseased treated (T) mice to the mean survival time of the diseased control (C) mice ×100. The dose indicates the amount of extract injected intraperitoneally twice a day for 10 days commencing 24 hours after injection of the cancer cells. Dosages were not optimized.

Alga	Collection site	Activity (T/C × 100)	Dose (mg)
	Family Oscillatoriaceae		
Lyngbya gracilis	Reefer 8 pinnacle	144	0.011
Lyngbya gracilis	South Elmer pinnacle	137	0.013
Oscillatoria nigroviridis and *Schizothrix calcicola* (1:1)*	Enewetak, seaward side	140	0.0047
Oscillatoria nigroviridis and *Schizothrix calcicola* (1:1)*	Enewetak, lagoon side	122	0.74
Symploca muscorum	Enewetak, lagoon side	142	0.15
Microcoleus tenerrimus	Ananij, seaward side	120	0.27
Schizothrix calcicola and *Calothrix crustacea* (10:1)*	Enewetak, seaward side	125	0.44
	Family Rivulariaceae		
Calothrix crustacea	Enewetak, seaward side	100	0.98
Calothrix crustacea	Reef flat near Mike and Koa Craters	117	0.40
	Family Nostocaceae		
Nostoc muscorum	Enewetak, terrestrial	104	0.31

*Inseparable mixture. Relative amounts of algae as indicated.

the chloroform layer was washed repeatedly with water, dried over anhydrous sodium sulfate, and evaporated to give the crude extract (22 g). Column chromatography of this extract on Florisil yielded a toxic fraction (4.2 g) that was eluted with chloroform and methanol (9 : 1). Gel permeation chromatography of this fraction on Sephadex LH-20, with chloroform and methanol (1 : 1) being used as the eluant, rendered the P-388 active component nearly pure. Final purification was achieved by column chromatography on silica gel (thin-layer chromatography grade) with chloroform and methanol (9 : 1) as the eluant. The pure anticancer compound (400 mg; 0.013 percent yield) crystallized as a microcrystalline powder from diethyl ether and pentane. Recrystallization from aqueous methanol yielded colorless needles of the drug, melting point (m.p.) 105.5° to 107.0°C. The similarities of the proton magnetic resonance spectrum of this compound to those reported for the monoacetate and diacetate derivatives of debromoaplysiatoxin (*7*) suggested to us that the antileukemia compound in the *Lyngbya* was debromoaplysiatoxin (**1**).



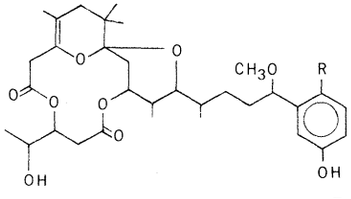
1 $R_1 = R_2 = R_3 = H$

2 $R_1 = R_3 = H$ $R_2 = Br$

3 $R_1 = R_3 = Ac$ $R_2 = H$

4 $R_1 = R_3 = Ac$ $R_2 = Br$

Field desorption (FD) mass spectrometry indicated a molecular weight of 592, as required for compound **1**. In addition to the parent ion peak the FD mass spectrum showed a strong fragment ion peak at 574 due to loss of water from the molecular ion. This facile loss of water has been reported to be a diagnostic feature of the aplysiatoxin (*7*). We found that our debromoaplysiatoxin slowly dehydrated in chloroform solution to form anhydrodebromoaplysiatoxin (**5**), m.p. 116.0° to 117.5°C after high-pressure liquid chromatography and crystallization from diethyl ether and pentane.



5 R = H

6 R = Br

Table 2. The response of mice with P-388 lymphocytic leukemia to injections of debromoaplysiatoxin. Ten mice, each weighing approximately 20 g, were used in all the experiments. The dose indicates the amount of toxin injected intraperitoneally twice a day for 10 days commencing 24 hours after intraperitoneal injection of 10^6 cancer cells. The activity is expressed as the ratio of the mean survival time of the diseased treated (T) mice (animals dying from chronic toxicity not included) to the mean survival time of the diseased control (C) mice × 100.

Dose (μg/mouse)	Mice surviving toxicity (No.)	Average survival 8days)	Activity (T/C × 100)
0.0	10	9.4	
0.1	10	11.1	118
0.2	10	12.0	133
0.4	9	12.1	129
0.6	10	13.2	140
0.8	7	13.9	148
1.0	9	11.8	126
1.2	7	15.1	161
1.4	9	15.7	167
1.6	3	15.7	167
1.8	4	17.5	186

Kato and Scheuer, in their study of the toxic constituents of the digestive gland of the sea hare *Stylocheilus longicauda*, isolated aplysiatoxin and debromoaplysiatoxin and elucidated their structures as **1** and **2** (*8*, *9*). In *Stylocheilus* these two toxins, along with the corresponding anhydro derivatives **5** and **6** and a pigment, formed a chromatographically unresolvable mixture. Acetylation of the mixture, however, allowed the isolation of the pure diacetates **3** and **4**. Because of the sensitivity of the toxins to acid and base, these investigators were only able to deacetylate enough of compound **3** to confirm the authenticity of debromoaplysiatoxin (**1**) by thin-layer chromatography. No physical data, therefore, could be obtained for debromoaplysiatoxin from *Stylocheilus*.

Since neither physical data nor authentic debromoaplysiatoxin were available for comparative purposes, the identity of our P-388 active component was secured by converting it to the diacetate derivative. This derivative proved to be spectrally identical with debromoaplysiatoxin diacetate (**3**) from the sea hare.

Using the same procedure we have shown that the active substance in the inseparable mixture of *Oscillatoria nigroviridis* Gomont and *Schizothrix calcicola* is also debromoaplysiatoxin. It is interesting that an algal sample collected from the seaward side of Enewetak was much more toxic than a specimen collected on the lagoon side of the island.

Data on the response of mice with P-388 lymphocytic leukemia to injections of debromoaplysiatoxin are summarized in Table 2. Mice infected intraperitoneally with the leukemia were treated intraperitoneally with the drug, and good antileukemia activity was exhibited by the compound when used in amounts that resulted in nearly 50 percent of the mice dying from toxicity at the end of the 10-day treatment (LD_{50}). When mice infected subcutaneously with the P-388 leukemia were treated with debromoaplysiatoxin, only marginal activity [ratio of treated (T) to control (C) mice × 100 = 125] was found for the drug near the LD_{50}.

Two derivatives of debromoaplysiatoxin were also assayed for P-388 activity. At doses of 2 μg per injection, compounds **3** and **5** exhibited activities of 130 and 119, respectively. At ten times the dose of compound **1**, the activity of compound **3** was essentially equivalent to that of debromoaplysiatoxin. Unfortunately, compound **3** also showed marked chronic toxicity.

Kato and Scheuer (*9*) reported that accidental contact with the aplysiatoxins caused inflammation and swelling of the mucous membranes of the eyes and nose and induced redness and pus on the skin. We have also observed these effects, which are comparable to the effects reported for contact with dermatitis-producing *L. majuscula* (*10*) and its extracts (*11*). Debromoaplysiatoxin must be considered a prime suspect of the swimmers' itch malady in Hawaii (*12*).

The alga *Lyngbya* is a preferential food source of *Stylocheilus longicauda*. Many of the sea hares were found in our algal collection and were carefully removed prior to our experiments. There can be little doubt that the debromoaplysiatoxin of *Stylocheilus* is obtained from its diet. Sea hares raised from larvae in aquaria on an abnormal diet lack toxicity (*13*). Aplysiatoxin is not a constituent of *Lyngbya* from Enewetak. Whether this compound is a metabolite of compound **1** in *Stylocheilus* or a constituent of another blue-green alga is not known.

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12. *Note added in proof*: In the first week of September 1976, an outbreak of dermatitis-producing *L. majuscula* was reported in Laie Bay, Oahu. We collected a specimen of the alga and thin-layer and PMR spectral analyses of the crude extract showed that debromoaplysiatoxin was indeed present.
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14. We thank M. D. Hoyle, Department of Botany, University of Hawaii, and R. Tsuda, Marine Laboratory, University of Guam, for identifying the algae and D. Brent of Burroughs-Wellcome for determining the field desorption mass spectrum. This work was supported by NSF and PHS grants and in part by ERDA contract AT(26-1) 628.

30 August 1976; revised 2 November 1976

Denervated Skeletal Muscle Fibers Develop Discrete Patches of High Acetylcholine Receptor Density

Abstract. *Denervated skeletal muscle fibers of mice develop discrete patches of high acetylcholine receptor density. The patches vary in size from less than 1 micrometer up to 30 micrometers, depending on the muscle and the period of denervation. Within the patches the acetylcholine receptor density is some 20 times greater than elsewhere along the muscle fiber and probably approaches that in the subsynaptic membrane.*

A current problem in the study of synapse formation concerns the manner in which receptors become preferentially localized at sites of synaptic contact during the course of development. Recent observations on the distribution of acetylcholine (ACh) receptors in cultures of embryonic chick muscle cells are particularly interesting in this respect. During the first few days in culture, the cells display a relatively uniform distribution of receptors along their entire length, but after about 1 week they develop "ACh hot spots," discrete patches of membrane where the number of receptors is considerably greater than elsewhere on the cell (*1–3*). Estimates based on the uptake of α-bungarotoxin, which binds specifically and with high affinity to the receptors, indicate that the receptor density in these patches approaches that in the subsynaptic membrane of normal adult skeletal muscle (*1*). These findings have therefore raised the possibility that the patches might be targets for growing nerve fibers (*1, 2*). Consistent with this suggestion is the finding that the development of the patches is not dependent upon previous innervation of the cells (*4*).

So far, the occurrence of such discrete patches of ACh receptors has been demonstrated only on cultured embryonic muscle cells. The question therefore arises whether their development is somehow related to the artificial conditions of the culture situation or whether they also develop in vivo. It is also pertinent to know if their development is restricted to embryonic muscle. To answer these questions we have examined the distribution of ACh receptors on adult denervated muscle fibers. It is well known that after denervation, muscle fibers acquire ACh receptors along their entire length (*5*). Estimates, based on the uptake of α-bungarotoxin, indicate that these extrajunctional receptors attain a mean density which is about one-tenth of that in the subsynaptic membrane (*6*). There is evidence, however, that the distribution of the receptors is not uniform (*7*). This conclusion is extended by the present study which indicates that many denervated muscle fibers develop discrete patches of high ACh receptor density analogous to those which have previously been observed in cultures of embryonic muscle.

The experiments were carried out mainly on the plantaris muscle of adult CBA and Swiss mice weighing 20 to 25 g and 30 to 35 g, respectively. The mice were anesthetized and the muscles were denervated by removing a 4- to 8-mm segment of the appropriate nerve remote from its entry into the muscle. The animals were then maintained for various periods of time up to 3 weeks. In order to examine receptor distribution the receptors were stained with fluorescent conjugates of α-bungarotoxin as previously described (*8*). This technique is especially suitable for visualizing regions of high receptor density. In brief, muscles

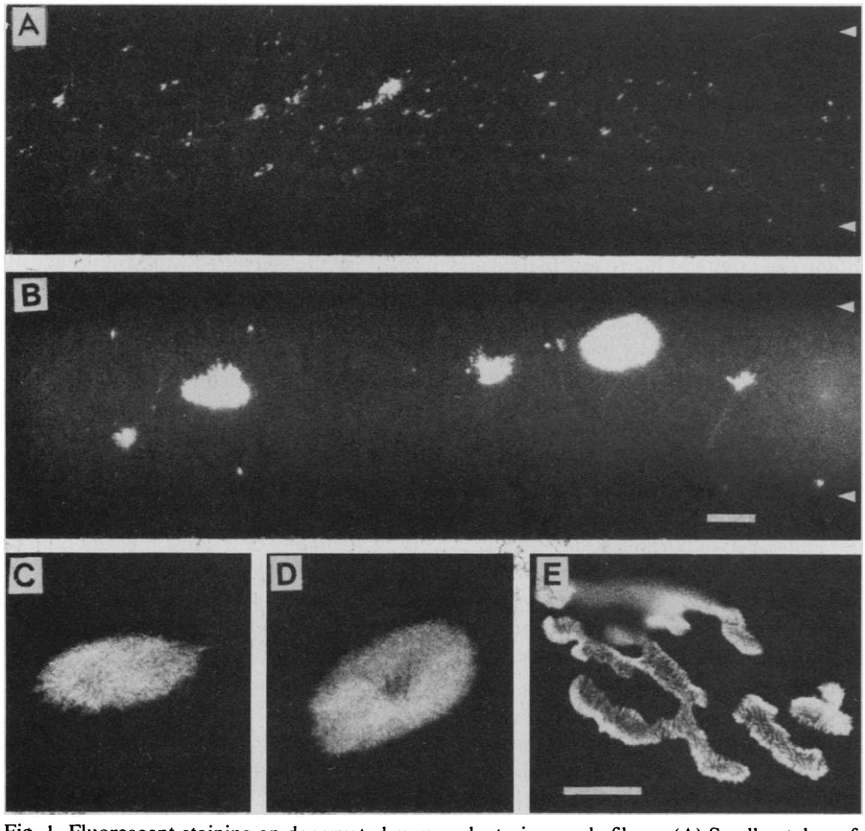

Fig. 1. Fluorescent staining on denervated mouse plantaris muscle fibers. (A) Small patches of stain on a muscle fiber denervated for 4 days. Arrowheads indicate edges of fiber. (B) Larger patches on a fiber 10 days after denervation. (C and D) Higher magnification views of single patches 9 and 14 days after denervation. (E) Fluorescent staining of an endplate on a fiber denervated for 9 days. Scale bars, 10 μm. The endplate shows bright outlining and fine bright lines whereas the patches show a relatively uniform staining intensity. In all examples except B the muscles were stained while alive. In B, the muscle was fixed with 4 percent paraformaldehyde for 90 minutes at 37°C before being stained. Such fixation did not affect the staining patterns.