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Pharmaceuticals from cultured algae

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SUMMARY

An algae screening program, including cultured macroalgae, cultured cyanobacteria and cultured eukaryotic microalgae has been undertaken. Methods for the isolation, purification, preservation and cultivation of axenic cyanobacteria and eukaryotic cultures have been developed. Screening of these groups for biologically active components has lead to the isolation of pachydictyol and caulerpenyne from cultured macroalgae, while a series of hapalindoles and an antifungal depsipeptide have been isolated from cyanobacteria.

INTRODUCTION

Natural products are an important source of new pharmaceuticals and pharmaceutical 'lead' compounds. The overwhelming majority of active compounds have been derived from streptomycetes and fungi, although additional significant sources include other bacteria and plants. A major problem with focusing on these sources in the search for novel, biologically active molecules, is the rediscovery of previously known natural products. One way to minimize this problem is to develop selective bioassays and investigate new therapeutic areas. Another approach is to look at new and different sources of natural products.

The algae represent such a source, but have been largely ignored by the pharmaceutical industry. This probably reflects a reluctance to deal with the technical problems involved in collecting algae in nature and then culturing them in the laboratory. Clearly the algae are producers of bioactive materials as evidenced by public health concerns over algal toxins involved in 'red tides' and paralytic shellfish poisoning produced by dinoflagellates [15], as well as fish and cattle kills resulting from blooms of cyanobacteria and other algae [6,2]. While such a history may discourage some from investigating algal natural products, it is also indicative of a rich

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source of novel secondary metabolites which may be applicable to the development of new pharmaceuticals.

MATERIALS AND METHODS

Growth media

BG-12 medium consisted of $NaNO_3$ 1.5 g, K_2HPO_4 0.031 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, Na_2CO_3 0.02 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, ethylenediaminetetraacetic acid (disodium magnesium salt) 0.001 g, H_3BO_3 0.00286 g, $MnCl_2 \cdot 4H_2O$ 0.00181 g, $ZnSO_4 \cdot 7H_2O$ 0.00022 g, $Na_2MoO_4 \cdot 2H_2O$ 0.00039 g, $CuSO_4 \cdot 5H_2O$ 0.00008 g and $CoCl_2 \cdot 6H_2O$ 0.00004 g in 1 liter of 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.5. BG-13 medium was identical in composition to BG-12 medium except that no HEPES buffer was used and $NaHCO_3$ (1.7 g/l) was added. BG-13 medium used to grow eukaryotic microalgae was supplemented with yeast extract (0.1 g/l). Algae grown in BG-12 or BG-13 medium were incubated under an atmosphere of 5% (v/v) CO_2 in air. A completely organic medium consisting of nutrient broth (Difco) containing 1% (w/v) glucose also was used to grow some eukaryotic microalgae. Algae grown in this medium were incubated under an air atmosphere. All media were prepared using water which was purified to 18 Mohm resistance and Difco Agar Noble (15 g/l) was used to prepare all agar media.

Cultures of macroalgae were grown in sterile filtered natural seawater (SNSW) or SNSW supplemented with Provasoli's Enriched Seawater [5,9,10] to a level of 82 μM nitrate and penicillin (10 mg/l) and streptomycin (5 mg/l).

Isolation

Cyanobacteria and eukaryotic microalgae are ubiquitous in nature, occurring almost anywhere that light is available [12,13]. Enrichment culture techniques were used to isolate cyanobacteria and eukaryotic microalgae from soils and direct plating procedures were utilized for isolations from marine and fresh water sources.

Enrichment cultures were established by inoculating 2.5–3.0 ml of BG-13 medium in the well of a 24-well tissue culture plate with 5–10 mg of soil. The enrichments were incubated at 25°C under an atmosphere of 5% (v/v) CO_2 in air and illuminated (ca. 5 000 lux, cool-white fluorescent lamps) continuously. After 3–4 weeks incubation, small algal 'blooms' were observed in the wells of the tissue culture plate. The material from a bloom was then plated onto BG-12 agar medium and, following incubation, morphologically distinct isolates were picked and transferred to BG-12 agar. In cases where the bloom consisted of a mat of filamentous cyanobacteria, it was necessary to disperse the growth using a tissue grinder before plating. The direct plating of aquatic samples was done by first concentrating the algal cells present in the sample by membrane filtration. Volumes of 0.1 ml and 1.0 ml of the sample were filtered onto separate 1 um pore diameter polycarbonate membrane filters (47 mm dia., Nuclepore Corp.). The filters were then washed with 25–30 ml of distilled water, drained and transferred, inoculated side up, to plates of BG-12 agar. The filters were incubated as described above for enrichment cultures and after 2–3 weeks, algal colonies were observed growing on the filter surface. Colony morphology was observed using a dissecting microscope (10–50 × magnification) and different appearing colonies were picked and transferred to BG-12 agar.

Cultures of macroalgae were obtained from Ocean Genetics Inc., in Santa Cruz, California. Table 1 presents data describing cultures which were studied. Those isolates which were collected in the field were prepared for laboratory culture by clipping tips of the meristimatic apices of the plant and placing them in SNSW. The tips then were incubated at 23°C under a continuous light irradiance of 50 $\mu E/m^2/s$ (cool-white fluorescent lamps). After these cultures had grown (2–3 g fresh weight), they were used as inocula for scaled-up cultures.

Purification

One of our primary requirements for a program to find new pharmaceuticals from cyanobacteria and eukaryotic microalgae was to work strictly us-

Table 1

Identity and sources of macroalgae

Code number	Species	Collection site[a]	Collection date
M90	*Caulerpa vonbosseae*	UCB (#656) Puerto Penasco, Sonora, Mexico	6/69
M108	*Dictyota dichotoma*	UCB (#3085) Naples, Italy	1960
M125	*Dictyota flabellata*	San Diego, CA	4/3/86
M179	*Dictyota flabellata*	San Diego, CA	4/3/86
M190	*Dictyota flabellata*	Isla Smith, Baja, CA	5/4/86
M227	*Dictyota* sp.	Punta La Gringa, Baja, CA	5/6/86
M649	*Dictyota flabellata*	Bird Rock, La Jolla, CA	9/9/87
M715	*Caulerpa taxifolia*	Aquarium Shop, Santa Cruz, CA	?

[a] UCB = University of California at Berkeley. Numbers refer to UCB collection.

ing pure culture techniques. This required the development of methods to free algal isolates from non-algal prokaryotic and eukaryotic microorganisms and to ascertain that these isolates were indeed unialgal. A simple method worked well for the purification of unicellular eukaryotic microalgae and consisted of growing the alga, along with its non-algal contaminants, in BG-12 medium under photolithotropic conditions and then plating appropriate dilutions of the culture onto BG-12 agar medium containing the antibacterial antibiotic imipenem (100 μg/ml). After incubation, isolated pure colonies of algae were observed, picked and transferred. The growth of bacteria on the plates was controlled by the antibiotic and, while fungal colonies were observed growing on the plates, their growth was usually restricted on the nutrient-poor medium. In the case of cyanobacteria, the antibiotics nystatin (100 μg/ml) and cycloheximide (100 μg/ml) were used to eliminate fungal contaminants; however, a novel procedure was developed for the elimination of all non-algal bacteria (manuscript in preparation). Briefly, the procedure involved adding low concentrations of organic nutrients and imipenem to a contaminated cyanobacterial culture which had been grown in BG-12 medium under photolithotrophic conditions. After the additions were made, the culture was incubated in the dark, during which time growth of the cyanobacteria essentially ceased, while the growth of the chemoorganotrophic bacteria was stimulated by the organic nutrients and the cells were killed by the antibiotic which inhibits bacterial cell wall biosynthesis. After imipenem treatment, the cyanobacteria were plated onto BG-12 medium and, following incubation, isolated pure colonies were picked and transferred.

Following purification, each algal culture was certified as axenic by inoculating it into an organic nutrient medium and, after 2–4 days incubation in the dark, observing for the absence of any microbial growth. The determination that axenic isolates were unialgal was made on the basis of colony characteristics and microscopic morphology according to established criteria [4, 11]. Any isolates which obviously consisted of mixed algal populations were separated into pure cultures.

Cultures of macroalgae were treated with penicillin and streptomycin to decrease the bacterial population present, however, no further attempts to purify the macroalgal cultures were made.

Preservation

To preserve cyanobacteria, a very simple and effective procedure was developed which involved cutting plugs aseptically from agar plates containing confluent growth or colonies of these bacteria. After removal from the plate, the plugs were placed in a sterile vial and then frozen at −80°C; no cryoprotectant was used with the plugs. When plugs were used as inocula, they were thawed at ambient temperature and then placed in broth or on agar medium and incubated. This method has been used to preserve more than 400 isolates of axenic unicellular and filamentous cyanobacteria for as long as 4 years, without any problem in establishing viable and productive cultures from the frozen plugs. The same procedure has been used to preserve cultures of eukaryotic microalgae and although we have less experience using the method with these microorganisms, it appears that it will be useful.

Broth cultivation

The culture vessel that was used to grow broth cultures of algae consisted of a three-neck 500 ml CELSTIR bottle (Wheaton Glass Co., Inc.) fitted with a cap and an inlet and outlet for continuous gassing of the headspace with CO_2 (5% v/v) enriched air or normal air. Cells were kept suspended in the culture vessel by a magnetic bar operating at 100–200 rpm. The lamps used to illuminate the cultures were 4 ft, 40 watt cool-white fluorescent bulbs or 2 ft., 20 watt cool-white fluorescent bulbs (General Electric Co., Inc.). The light intensity of the lamps was regulated using a voltage controller (model SCR, Cole-Parmer Inst. Co.) to provide an intensity of 3 500–5 000 lux at the wall of the culture vessel. This level of illumination was appropriate to grow most isolates of cyanobacteria and eukaryotic microalgae, however, occasionally much lower (ca. 1000 lux) light intensities were used because the normal illumination level was found to be inhibitory to the growth of some cells. Light intensity was measured using an illuminance meter (model T-1M, Minolta Camera Co., Ltd.) The incubation temperature used for growing cultures of both cyanobacteria and eukaryotic microalgae was 25°C.

Large scale (16 l) cultures of macroalgae were grown in SNSW with penicillin (10 mg/l) and streptomycin (5 mg/l) in 5 gal. polycarbonate carboys. The cultures were sparged with air and supplemented with sodium nitrate/potassium phosphate/ferric chloride in a molar ratio of 1000:100:1. The concentration of nitrate in the medium was monitored using a continuous-flow analyzer and maintained above 40 μM. When algal biomass reached 0.5 g/l, the light irradiance was increased from 50 to 200 $\mu E/m^2/s$ (cool-white fluorescent lamps) and kept on a 16 h light – 8 h dark photoperiod. Carboy cultures were harvested 2–4 times per month by removing excess algal biomass such that a level of about 2 g fresh weight per liter remained in the carboy. The harvested algal biomass was placed in nylon bags and immediately frozen in liquid nitrogen. The frozen biomass then was lyophilized and stored at 0°C until sufficient biomass had accumulated to yield 10–50 g dry weight.

Nutrient analysis

In an attempt to correlate nutrient depletion with the production of a bioactivity of interest by a cyanobacterium or eukaryotic microalga, the concentrations of phosphate, nitrate and sulfate in culture medium from which cells had been removed, were monitored. This was done via HPLC using a Dionex system, equipped with an HPIC-AS4A separator column and a conductivity detector. The analysis was performed using a 1.80 mM Na_2CO_3/1.70 mM $NaHCO_3$ buffering system, a flow rate of 1.7 ml/min and a conductivity range of 10 μS.

Chemicals

Nystatin was purchased from Sigma Chemical Co. and cycloheximide was obtained from Calbiochem Co. Imipenem was obtained from Merck and Co., Inc. Silica gel was purchased from E.M. Science; Sephadex LH-20 was obtained from Pharmacia. All other chemicals were of the highest purity available.

Spectroscopic methods

^{1}H-NMR and ^{13}C-NMR spectra were obtained at 400 MHz and 100 MHz, respectively, on a Varian XL-400 spectrometer at ambient room temperature. Chemical shifts are expressed in ppm relative to TMS at 0 ppm, and are referenced to $CDCl_3$ at 7.24 ppm or C_6D_6 at 7.15 ppm or CD_3OD at 3.30 ppm; coupling constants are reported in hertz. EI-MS data were obtained on a Finnigan MAT-212 mass spectrometer; FAB data were obtained on a Finnigan MAT-731 mass spectrometer. IR spectra were obtained on a Perkin-Elmer 1750 Fourier Transform Infrared Spectrophotometer equipped with a Multiple Internal Reflectance Accessory.

Pachydictyol A ***1*** *[3]*

Twenty-one grams of *Dictyota dichotomata* was extracted overnight with 1 l CH_2Cl_2/MeOH/H_2O (1:2:1), filtered and 200 ml H_2O added to partition this extract into two layers. The organic layer (1.5 g) was chromatographed using a silica gel step gradient (CH_2Cl_2, CH_2Cl_2/EtOAc 1:1, EtOAc, MeOH), which yielded 24 mg of partially purified ma-

terial in the CH_2Cl_2 solvent step. The final purification was performed via reverse phase HPLC using a DuPont Zorbax ODS column, with MeOH/H_2O (9:1) as the solvent system, to yield 11 mg of **1**.

The HREIMS of **1** indicated $C_{20}H_{34}O$ (found *m/z* 362.3038, calculated 362.3005, for M^+ + TMS). ^{13}C-NMR in $CDCl_3$: 152.56, 141.40, 131.56, 124.70, 107.11, 75.09, 60.44, 47.78, 46.03, 40.63, 35.01, 34.73, 33.83, 25.73, 25.60, 23.52, 17.71, 17.52, 15.90. ^{1}H-NMR in $CDCl_3$ 5.33 1H m (H-3), 5.12 1H t J = 6.6 (H-14), 4.74 2H d J = 2.1 (H-18) 3.91 1H bs (H-6), 1.79 3H d J = 2.7 (H-17), 1.69 3H s (H-20), 1.61 3H s (H-16), 0.99 3H d J = 5.7 (H-19). IR: 3500 cm^{-1} (OH), 1640 cm^{-1} (C = C).

*Caulerpenyne **2** and related compounds **3** and **4** [1]*

Ten grams of *Caulerpa vanbosseae* was extracted with 250 ml CH_2Cl_2/MeOH/H_2O (1:2:1) for 3 h and with an additional 250 ml of the same solvent system overnight. These extracts were filtered, combined and 150 ml of H_2O added. The resulting lower, organic layer was concentrated and chromatographed on 200 ml LH-20 using CH_2Cl_2/hexane/MeOH (10:10:1) as the eluting solvent. Final purification was accomplished via chromatography on 100 ml silica gel, EtOAc/hexane (25:75) to yield 27 mg of **2**. Compound **2** was identical to caulerpenyne via ^{1} H NMR and MS.

Caulerpenyne (5.9 mg) in 1 ml MeOH was stirred with ~5 mg of $NaBH_4$ for 5 min at room temperature. Two ml of CH_2Cl_2 and 0.5 ml of H_2O were added and the resulting lower, organic layer removed and concentrated to yield 15.6 mg. Silica gel chromatography (EtOAc/hexane, 1:1) resulted in 3.3 mg of the diol **3**.

The diol **3** (1.5 mg) was stirred for 30 min at room temperature with 5 drops acetic anhydride in 10 drops pyridine. The reaction mixture was concentrated to dryness and partitioned between CH_2Cl_2 and H_2O (1 ml each). Concentration of the CH_2Cl_2 layer resulted in 1.2 mg of compound **4**.

*Hapalindolinone A (**5**) and B (**6**) [14]*

The isolation and structure determinations of these compounds were detailed in the reference indicated.

*Hapalindoles **7** and **8***

One liter of a broth culture of *Fischerella* sp. isolated in our laboratory (ATCC 53558) was centrifuged, the supernatant decanted and the resulting cell pellet extracted with 100 ml MeOH. This MeOH solution was extracted with 100 ml CH_2Cl_2 (2×), concentrated to dryness and chromatographed on 40 ml silica gel (CH_2Cl_2) to yield 21 mg of **7**, as well as components **5** and **6** described previously.

The isonitrile, as well as the indole N-H were evident in the IR spectrum with absorbances at 2140 cm^{-1} and 3465^{-1}, respectively. The MS indicated the presence of a single Cl and a molecular formula of $C_{21}H_{23}N_2Cl$ (found 338.1548, calculated 338.1550). ^{13}C-NMR in $CDCl_3$: 158.96 s (C-23), 145.39 s (C-16), 137.88 d (C-20), 135.61 s (C-8), 126.52 s (C-9), 123.40 d (C-6), 122.17 d (C-5), 119.68 d (C-2), 117.36 t (C-21), 117.24 d (C-4), 113.41 t (C-17), 112.61 s (C-3), 111.51 d (C-7), 67.48 d (C-11), 63.02 d (C-13), 44.80 s (C-12), 44.14 d (C-15), 39.16 t (C-14), 35.48 d (C-10), 25.59 q (C-18), 18.64 q (C-19). ^{1}H-NMR in $CDCl_3$: 8.12 1H bs (H-1), 7.41 1H bs (H-2), 7.10–7.38 4H m (H-4, H-5, H-6, H-7), 6.46 1H dd J = 11.2, 17.7 (H-20), 5.60 1H d J = 11.2 (H-21), 5.45 1H d J = 17.7 (H-21), 4.87 1H bs (H-17), 4.72 1H bs (H-17), 4.34 1H dd J = 4.8, 12.1 (H-13), 4.03 1H bs (H-11), 3.64 1H bd J = 12.1 (H-10), 3.05 1H ddd J = 4.1, 12.1, 12.1 (H-15), 2.23 1H m H-14, 2.15 1H m (H-14), 1.53 3H s (H-18), 1.46 3H s (H-19).

Ten mg of **7** was stirred for 45 min at room temperature in 1 ml MeOH containing 2 drops in 1 N H_2SO_4. The reaction mixture was concentrated to dryness and partitioned between EtOAc and H_2O (1 ml of each). The EtOAc layer was concentrated and chromatographed on a 5 ml silica column, using EtOAc/hexane (1:1) as the eluting solvent, to yield 2.8 mg of the *N*-formyl derivative **8**.

HREIMS of **8** indicated $C_{21}H_{25}N_2OCl$ (found 356.1648, calculated 356.1655). Compound **8** was not stable in $CDCl_3$, therefore, the ^{1}H-NMR was obtained in C_6D_6. ^{1}H-NMR: 6.50 1H m (H-20), 5.28 1H d J = 10.5 (H-21), 5.20 1H d J = 18.0 (H-21), 4-80 1H s H-17, 4.52 1H s H-17, 4.07 1H dd J = 9.7, 6.8 (H-13), 3.60 1H dd J = 2.6, 12.8 (H-11), 3.35 1H

dd J = 2.6, 10.2 (H-10), 2.69 1H m (H-15), 2.13 2H m (H-14), 0.5 1H m (H-22).

Cryptophycin **9**

Cells from a *Nostoc* sp. isolated in our laboratory (ATCC 53789) were cultivated in 14-l fermentors (New Brunswick Scientific, New Brunswick, NJ) containing modified BG-13 medium (0.062 g K_2HPO_4 instead of 0.031 g). The fermentors were illuminated by 4 sets of 2 ft. fluorescent tubes delivering an average of 5000 lux on the inside surface of the glass vessel. The fermentors were operated at 300 rpm, 25°C and were sparged with 5% CO_2 (v/v) in air (Union Carbide, Linden, NJ) at a rate of 100 ml/min. Packed cell volume was determined on daily samples by centrifuging 10 ml of the culture at 4000 rpm for 10 min. The supernatant was assayed by HPLC for sulfate, phosphate and nitrate concentrations. The cell pellet was mixed with 1 ml of MeOH, centrifuged at 10 000 rpm for 1 min and the concentration of **9** in the supernatant was assayed by HPLC (DuPont Zorbax ODS, 21.2 mm × 25 cm, MeOH/H_2O 70:30, UV 210 nm).

The cells from this culture were removed by filtration and extracted with 1 l MeOH. After filtration, 200 ml of H_2O was added and the active constituent partioned into CH_2C1_2 (500 ml, 2×). The CH_2Cl_2 layers were combined, concentrated and chromatographed via MPLC (LiChroprep RP-18 25–40 mm, MeOH/H_2O, 75:25). The rich cut from this step was then chromatographed via prep HPLC (DuPont Zorbax ODS, 21.2 mm × 25 cm, CH_3CN/H_2O, 60/40) to yield 27 mg of **9**.

HREIMS indicated $C_{35}H_{43}N_2O_8Cl$ for **9** (found 654.2697, calculated 654.2708). The trimethylsilyl derivative of the total acid hydrolysate was analyzed via GC-MS to yield (1) bis-silyl-beta-amino-isobutyric acid, (2) bis-silyl-alpha-hydroxyisovaleric acid, (3) bis-silyl-(chloro, methoxy) phenylalanine (found 358.1062, calculated 358.1062 for $C_{10}H_{12}ClNO_3$ + $(SiC_3H_8)_2$-CH_3) and (4) bis-silyl-7,8-epoxy-5-hydroxy-6-methyl-8-phenyloctene-2-oic acid (found 391.1775, calculated 391.1761 for $C_{15}H_{18}O_4$ + $(SiC_3H_8)_2$-CH_3). The IR spectrum of **9** showed peaks at 3600–3000 cm^{-1}, 1750 cm^{-1}, 1726 cm^{-1}, 890 cm^{-1}, 755 cm^{-1}, 699 cm^{-1}. ^{13}C-NMR in CD_3OD: 177.48 s, 174.04 s, 172.22 s, 168.26 s, 155.33 s, 143.25 d, 138.63 s, 132.19 s, 131.49 s, 129.75(2C) d, 129.51 d, 129.31 d, 126.91 (2C) d, 125.56 d, 123.22 s, 113.47 d, 77.73 d, 72.60 d, 64.45 d, 60.11 d, 57.45 q, 56.59 d, 41.74 d, 41.11 t, 40.63 t, 38.99 d, 38.56 t, 36.35 t, 25.61 d, 23.29 q, 21.65 q, 15.12 q, 14.03 q. ^{1} H-NMR in CD_3OD: 7.33 1H m, 7.17 1H dd J = 8.5, 2.3, 6.98 1H d J = 8.5, 6.70 1H m, 5.87 1H dd J = 15.2, 1.7, 5.19 1H m, 4.90 1H m, 4.50 1H dd J = 10.7, 3.7, 3.85 1H s, 3.81 1H d J = 2.0, 3.60 1H dd J = 12.7, 3.7, 3.23 1H m, 3.17 1H dd J = 14.4, 3.0, 2.98 1H dd J = 7.6, 2.0, 2.73 1H m 2.42 1H m, 1.86 m J = 6.9, 1.7, 1.70 1H m, 1.60 1H m, 1.46 1H m, 1.18 1H d J = 7.5, 1.14 1H d J = 6.9, 0.84 1H d J = 6.5, 0.82 1H d J = 6.5.

RESULTS AND DISCUSSION

Sample preparation for screening

In order to prepare samples for biological evaluation, cyanobacteria and eukaryotic microalgae cultures were centrifuged and the resulting cell pellets extracted with one-tenth the culture volume of MeOH. This procedure resulted in a very clean preparation with few assay interferences from media constituents. The supernatant fluid from the culture was neutralized, lyophilized and later reconstituted for testing. This preparation interferred with some assays due to high concentrations of salts present in the reconstituted material.

Macroalgae were received from Ocean Genetics as a lyophilized solid. These samples were steeped overnight in the single phase solvent mixture of CH_2Cl_2/MeOH/H_2O (1:2:1), forced to partition into two layers upon the addition of H_2O and the two layers separated and submitted for assays. This solvent mixture was chosen because single solvents such as MeOH, acetone, CH_2Cl_2 or EtOAc could not penetrate and efficiently extract these dried samples. Additionally, the liquid culture medium from these cultures was submitted to selected assays.

Actively pursued lead compounds

The decision to actively pursue the isolation and

structure determination of any given active compound was based solely on the biological activity of the culture extract in the assay of interest and the reproducibility of that biological activity in subsequent culture extracts. Interesting and unusual structural types, without supporting biological activity, were not pursued. These criteria were very selective and were utilized to focus resources on a small number of cultures most likely to produce biologically useful compounds.

Macroalgae

Two active compounds from macroalgae supplied by Ocean Genetics have been studied. The first compound was isolated from a brown alga *Dictyota dichotoma* via extraction and silica gel chromatography. The purified compound inhibits the binding of arginine vasopressin to kidney tissue with an IC_{50} of 33 mM. A combination of MS and ^{13}C- and ^{1}H-NMR spectroscopy was used to identify the active constituent as pachydictyol A (**1**), a compound originally isolated from a sample of *Pachydictyon coriaceum* collected in the wild [3].

Pachydictyol A (Fig. 1) has subsequently been identified as the active constituent in a number of macroalgae identified as *Dictyota* sp. This lead was not pursued because of the lack of novelty and because of its low potency in the assay of interest.

An algal extract from the green alga *Caulerpa vanbosseae* was active in inhibiting prenyl transferase, 5-lipoxygenase and contained an antibacterial agent with good activity against a strain of methicillin-resistant *Staphylococcus aureus*. The isolation involved extraction and partition, followed by Sephadex LH-20 and silica gel chromatographies. The conclusion of the bioassay-directed isolation was that all three activities were due to the same compound, identified as caulerpenyne (**2**), an acetylenic sesquiterpenoid previously isolated from a wild sample of *Caulerpa prolifera* [1] (Fig. 2). The IC_{50} against prenyl transferase was 10 mM, the IC_{50} against 5-lipoxygenase in human polymorphonuclear leukocytes was 13 mM and 3 mM in a broken cell preparation. In view of its low potency in the assays, it seems likely that this compound was detected in the original culture extract because it was present in such large quantities.

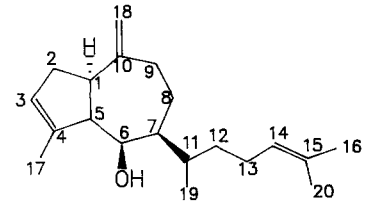
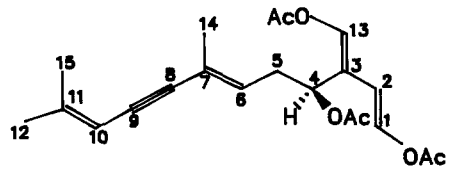


Fig. 1. Structure of pachydictyol (**1**).

Examination of the structure suggested that the source of the activity was either the dienyne system or the diacetoxy butadiene system. $NaBH_4$ reduction of the diacetoxy butadiene system yielded **3** and subsequent acetylation yielded **4** [1]. It was felt that **4** most closely resembled caulerpenyne (**2**) without the presence of the reactive diacetoxy butadiene group. Both **3** and **4** were inactive in all three therapeutic areas, strongly suggesting that the diacetoxy butadiene was responsible for the observed activities. Caulerpenyne has subsequently been identified as the active principal in a number of other extracts from cultures of *Caulerpa* sp.

Cyanobacteria

A number of interesting compounds have also been isolated from cultured cyanobacteria. An ex-



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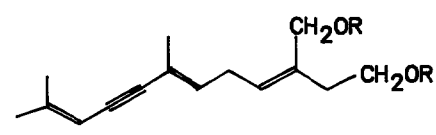
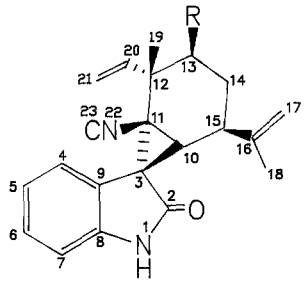


3 R=H
4 R=OAc

Fig. 2. Structures of caulerpenyne (**2**) and derivatives **3** and **4**.



5 R=Cl
6 R=H

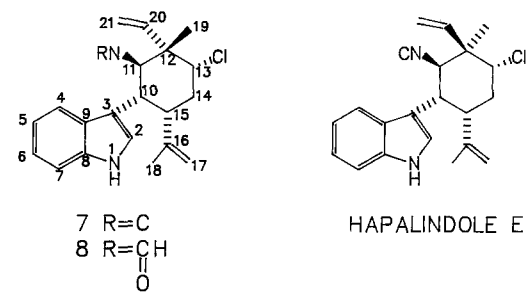
Fig. 3. Structures of hapalindolinone A (**5**) and B (**6**).

tract of the cell pellet of a cultured cyanobacterium belonging to the genus *Fischerella* (ATCC 53558) inhibited the binding of arginine vasopressin. Two novel active components were identified and the results published [14]. Hapalindolinone A (**5**) inhibited the binding of arginine vasopressin with an IC_{50} of 37.5 mM. The potency of the biological activity was disappointing, but the structure was intriguing (Fig. 3). An indolinone with an isonitrile at the ring juncture between the spiro-fused cyclopropane and a six-membered chlorine-containing ring confirmed that cyanobacteria were capable of synthesizing structurally unusual natural products.

Table 2

Comparison of the ^{13}C-NMR spectra in $CDCl_3$ of compound **7** and Hapalindole E [7,8]

Carbon	Compound **7**	Hapalindole E	$\Delta\,\delta$ (ppm)
10	35.48	34.71	−0.77
11	67.48	67.04	−0.44
12	44.80	44.54	−0.26
13	63.02	60.77	−2.25
14	39.16	38.10	−1.06
15	44.14	43.90	−0.25
16	145.39	145.10	−0.29
17	113.41	113.36	−0.05
18	18.64	18.55	−0.09
19	25.59	16.04	−9.55
20	137.88	141.70	3.82
21	117.36	116.06	−1.3
23	158.96	158.45	−0.51



7 R=C
8 R=CH
O

HAPALINDOLE E

Fig. 4. Structures of compound **7**, **8** and hapalindole E.

A third major component (**7**), which was structurally similar to hapalindole E [7,8], was isolated from this culture (Fig. 4); however, chemical shift differences in the ^{13}C-NMR (Table 2) and ^{1}H-NMR (Table 3) spectra suggested a difference in the stereochemistry of the six-membered ring.

Since the couplings of the ring protons were virtually identical in both compounds (Table 4), it seems likely that the conformation of the ring is the same and that the difference lies in the stereochemistry of the quartenary center at C-12. Large differences in the ^{13}C-NMR chemical shifts of C-19,

Table 3

Comparison of the ^{1}H-NMR spectra in $CDCl_3$ of compound **7** and Hapalindole E [7,8]

Carbon	Compound **7**	Hapalindole E
10	3.64 1Hbd J = 12.1	3.61 1Hbdd J = 12.1, 2.9
11	4.03 1Hbs	3.81 1Hbd J = 2.9
13	4.34 1Hdd J = 12.1, 4.8	4.46 1Hdd J = 12.1, 5.0
14	2.23 1Hm	2.25 1Hddd J = -13.7, 5.0, 4.3
14	2.15 1Hm	2.15 1Hdt J = -13.7, 12.1, 4.3
15	3.05 1H ddd J = 12.1, 12.1, 4.1	3.06 1Hdt J = 12.1, 12.1, 4.3
17	4.87 1Hbs	4.85 1 H dq J = 1.5, 0.8
17	4.72 1Hbs	4.72 1Hpent J = 1.5, 1.5
18	1.53 3Hs	1.55 3Hs
19	1.43 3Hs	1.48 3Hs
20	6.46 1Hdd J = 17.7, 11.2	6.05 1Hdd J = 17.5 10.9
21	5.60 1Hd J = 11.7	5.30 1Hdd J = 10.9, 0.3
21	5.45 1Hd J = 17.7	5.25 1Hdd J = 17.5, 0.3

Table 4

Comparison of the coupling constants of the ring protons in the ^{1}H-NMR spectra ($CDCL_3$) of compound **7** and Hapalindole E [7,8]

Hydrogens	Component **7**	Hapalindole E
10–11	J = 2.4 (C_6D_6)	J = 2.9
10–15	J = 12.1	J = 12.1
15–14	J = 12.1	J = 12.1
15–14	J = 4.1	J = 4.3
13–14	J = 12.1	J = 12.1
13–14	J = 4.8	J = 5.0

C-20 and C-21, as well as large changes in the ^{1}H-NMR chemical shifts at C-20 and C-21 support this structure.

It was suspected that the activity of these compounds was due to the highly reactive isonitrile functionality. Hydrolysis of **7** yielded **8**. The IC_{50} of **7** was 30 mM, while the IC_{50} of **8** was 100 mM, or about three-fold less potent. Also, randomly selected isonitriles did not inhibit binding of vasopressin. This data suggests that the activity of these compounds was not solely due to the reactive isonitrile, however, the potency of the N-formyl derivative was so low as to be of no further interest.

A number of other isonitrile-containing hapalindoles have been detected and abandoned due to presence of the isonitrile functionality.

An antifungal agent was detected in the cell extract of another cyanobacterium culture. The structure of the active antifungal agent was determined via MS, and ^{13}C- and ^{1}H-NMR spectroscopy, as reported in the Materials and Methods (full details and interpretation will be published elsewhere). The active compound **9**, is a cyclic didepsipeptide and contains an unusual chlorine-containing *O*-methyl tyrosine residue, as well as a number of other unusual residues (Fig. 5).

This compound was active against filamentous fungi and yeast of the genus *Cryptococcus*, but not *Candida* sp. In fact, **9** was so potent against *Cryptococcus* sp. that we would like to suggest the name cryptophycin for this cyclic peptide. The MICs

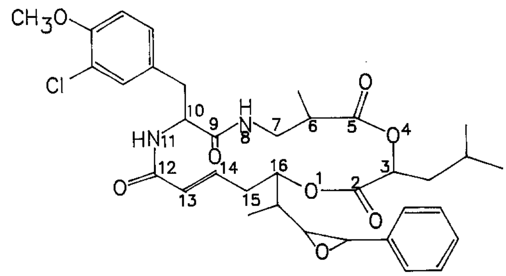


Fig. 5. Structure of cryptophycin (**9**).

against 84 *Cryptococcus* isolates were tested and are summarized in Table 5.

Kinetics of formation of cryptophycin ***9***

Fig. 6 shows a typical time course for *Nostoc* sp. (ATCC 53789) growth and cryptophycin production in a fermentor. Active growth occured between 20 and 300 h with a doubling time of 100 h. Growth cessation at 300 h (Fig. 7) correlated with the depletion of phosphate in the medium. The other major nutrients, nitrate and sulfate were not limiting to growth of the organism. Cryptophycin synthesis occurred between 200 and 480 h with a rate of 18 mg/l/h. Most of the synthesis was associated with the stationary phase of the organism, suggesting that it is not a growth associated phenomenon.

An in vivo mouse test showed no acute 24 h toxicity; however, TD_{50}s of 6.25 mg/kg and 3.13 mg/kg at days 7 and 14, respectively, were recorded, indicating that **9** was a potent, toxic agent. Postmortem examination of the mice revealed that the small

Table 5

Summary of activity of **9** against 84 strains of *Cryptococcus neoformans*

	Cryptophycin (**9**)	Amphotericin B
G-MIC[a]	0.03 mg/ml	≤0.16 mg/ml
Range	≤0.008 – >16.0 mg/ml	≤0.063 – 0.25 mg/ml
MIC_{50}[b]	0.031 mg/ml	0.125 mg/ml
MIC_{90}[c]	0.031 mg/ml	0.25 mg/ml

[a] geometric mean of MIC; [b] concentration at which 50% of the strains were inhibited; [c] concentration at which 90% of the strains were inhibited.

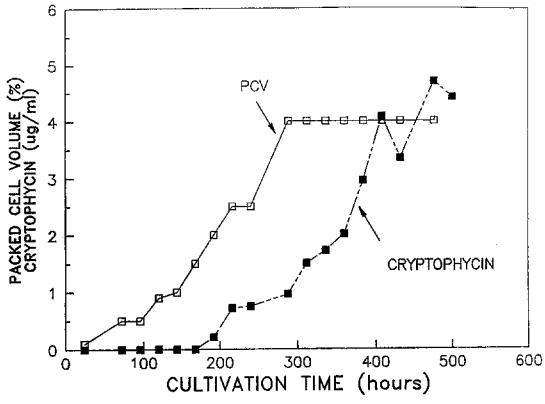


Fig. 6. Time course for *Nostoc* sp. growth and cryptophycin production in a fermentor.

intestine was enlarged and hemorrhagic, while the caecum and large intestine, as well as other organs were unaffected.

CONCLUSION

Based on our limited experience with cultured macroalgae, it appears that macroalgae make many of the same compounds in culture that they produce in the wild. While it seems likely that more known compounds will be isolated from this group, since wild macroalgae have been fairly well investigated in the past, one cannot rule out that novel compounds will be found, nor that novel activities for the previously isolated compounds will not be discovered.

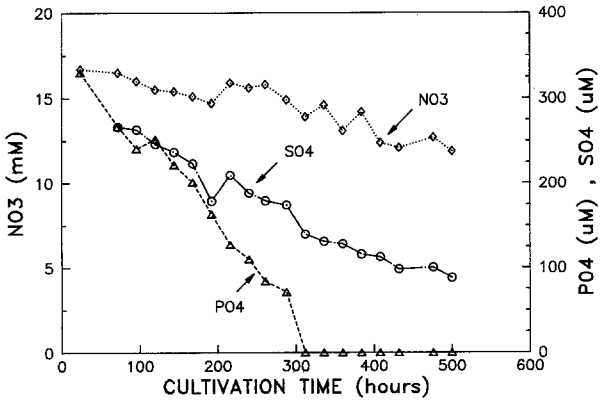


Fig. 7. Time course of nutrient utilization in *Nostoc*. sp.

Screening of cyanobacteria at Merck & Co., Inc., thus far, has led to the isolation of isonitrile-containing indoles and toxic peptides. The literature indicates that other investigators are finding similar types of natural products from these bacteria. Although the hapalindoles are fascinating structurally, it seems unlikely that they will lead to any viable therapeutic agents, and measures (such as IR spectroscopy) should be taken at an early stage to identify and eliminate them. Peptides, on the other hand, may be useful therapeutic agents as in the cases of cyclosporins and echinocandins. However, peptides from cyanobacteria have a propensity for being potent toxins and should be evaluated for toxic effects at an early stage.

'Drugs from the Sea' is a concept that has been promoted for nearly two decades, with little in the way of a practical payoff for the originally advertised new pharmaceuticals. Some past major efforts, directed specifically at the discovery and development of marine-derived drugs, have been unusually prolific in their discovery and publication of novel and biologically active marine natural products, however, they were unable to generate a new pharmaceutical agent.

These past efforts took a 'shotgun' approach with regard to organisms for screening, and a more focused approach with regard to screening assays. The screening organisms covered the whole range of marine plants and invertebrates from cyanobacteria to tunicates, and were collected via 'beachcombing', SCUBA and minisubmersibles. The collection of such a wide variety of organisms from a number of exotic locations was probably a fairly expensive portion of these operations. On the other hand, the screening assays focused on a fairly limited number of therapeutic areas. The development and maintenance of assays can be a very expensive proposition, and focusing on a few therapeutic areas with the greatest chances of success, was probably a very cost-effective means of proceeding.

The algae screening program at Merck & Co., Inc. has taken a different approach, that is, samples are being screened in a broad variety of therapeutic

areas, while screening samples are derived from laboratory culture of a few groups of macro and microalgae. This is seen as a very efficient method of screening since a wide range of existing bioassays are available and additional material can be obtained and reproduced through the laboratory culture of the alga of interest.

This approach has the advantage that it can be incorporated into existing screening programs and in an efficient manner generate information on the secondary metabolites being produced by a given group of algae. However, as in any natural products screening program, the identification of the most promising compounds requires a critical evaluation of their biological activity and efficacy, and a little bit of luck.

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REFERENCES

1 Amico, V., G. Oriente, M. Piatelli, C. Tringali, E. Fattorusso, S. Magno and L. Mayol. 1978. Caulerpenyne, and unusual sesquiterpenoid from the green alga *Caulerpa prolifera*. Tetrahedron Lett. 3593–3596.

2 Gorham, P.R. and W.W. Carmichael. 1979. Phycotoxins from blue-green algae. Pure Appl. Chem. 52: 165–174.

3 Hirschfeld, D.R., W. Fenical, G.H.Y. Lin, R.M. Wing, P. Radlick and J.J. Sims. 1973. Marine natural products. VIII. Pachydictyol A, an exceptional diterpene from the brown alga, *Pachycityon coriaceum*. J. Am. Chem. Soc. 95: 4049–4050.

4 Irvine, D.E.G. and D.M. John (ed.). 1984. Systematics of the green algae. Academic Press, New York, New York.

5 McLachlan, J. 1973. Growth media-marine. In: (Stein, J.R., ed.), Handbook of Phycological Methods, pp. 25–51, Cambridge University Press. New York.

6 Moore, R.E. 1977. Toxins from blue-green algae. Bioscience. 27: 797-802.

7 Moore, R.E., C. Cheuk, X.G. Yang, G.M.L. Patterson, R. Bonjouklian, T.A. Smitka, J.S. Mynderse, R.S. Foster, N.D. Jones, J.K. Swartzendruber and J.B. Deeter. 1987. Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. J. Org. Chem. 52: 1036–1043.

8 Moore, R.E. and G.M.L. Patterson. 1985. Hapalindoles. Eur. Patent 0 171 283 A2.

9 Provasoli, L. 1963. Growing marine seaweeds. Proc. Intern. Seaweed Symp. 4: 9–17. Pergamon Press. New York.

10 Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. In: (A. Watanabe and A. Hattori, eds.), Cultures and collections of algae. Proc. U.S. – Japan Conf. Hakone. Sept. 1966. Jap. Soc. Pl. Physiol., pp. 63–75.

11 Rippka, R., J. Deruelles, J.B. Waterbury, M. Herdman and R.Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111: 1–61.

12 Rippka, R., J.B. Waterbury and R.Y. Stanier. 1981. Isolation and purification of cyanobacteria: some general principles. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. Starr, H. Stolp, H.G. Truper, A. Balows and H.G. Schlegel, eds.), pp. 212–220, Springer-Verlag, New York.

13 Round, F.E. 1981. In: The ecology of algae. Cambridge University Press, New York.

14 Schwartz, R.E., C.F. Hirsch, J.P. Springer, D.J. Pettibone and D.L. Zink. 1987. Unusual cyclopropane-containing hapalindolinones from a cultured cyanobacteria. J. Org. Chem. 52: 3704:3706.

15 Shimizu, Y. 1978. Dinoflagellate toxins. In: Marine natural products, chemical and biological perspectives, Vol. I (Scheuer, P.J. ed.), pp. 1–42.