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# Toxins from cyanophytes belonging to the scytonemataceae

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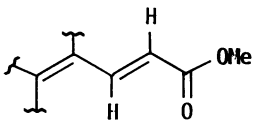
Abstract -- Two highly cytotoxic substances, scytophycins A and B, have been isolated from cultured Scytonema pseudohofmanni. Gross structures are proposed for scytophycins A and B, mostly on the basis of nuclear magnetic resonance spectral studies. The scytophycins are structurally related to tolytoxin, a toxic lipid found in field-collected Tolypothrix conglutinata var. colorata, another blue-green alga belonging to the family Scytonemataceae. At sublethal doses the scytophycins display moderate activity against P-388 lymphocytic leukemia and Lewis lung carcinoma in mice.

## INTRODUCTION

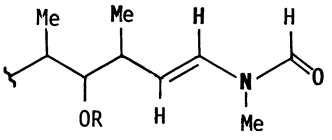
Potent toxins are present in some blue-green algae. The best known are the water-soluble toxins associated with the freshwater cyanophytes Anabaena flos-aquae, Aphanizomenon flos-aquae, and Microcystis aeruginosa (ref. 1). A bicyclic alkaloid, anatoxin-a, is the toxic principle of Anabaena flos-aquae (ref. 2) and the paralytic shellfish poisons saxitoxin and neosaxitoxin are two toxic substances that have been found in Aphanizomenon flos-aquae (ref. 3). Several unusual cyclic peptides account for the toxicity of M. aeruginosa and the gross structure of one of these toxins, cyanoginosin-LA, has recently been reported (ref. 4). Only lipophilic toxins, however, have been isolated and identified to date from marine cyanophytes (ref. 5). One of these is the indole alkaloid lyngbyatoxin A which is responsible for the toxicity of one variety of Lyngbya majuscula (ref. 6). Two others are the acetogenic bis-lactones debromoaplysiatoxin and aplysiatoxin (ref. 7) which are responsible for the toxicity of another strain of L. majuscula (ref. 8).

In screening extracts of field-collected blue-green algae for cytotoxicity and antineoplastic activity, we discovered that the crude extract of a terrestrial scytonematacean alga, viz. a strain of Tolypothrix conglutinata var. colorata which was found on the moist wall of a shed near the Cable Station on Fanning Island, was fairly toxic to mice (ref. 9). At sublethal levels, however, this extract showed good activity in the P-388 lymphocytic mouse leukemia screen (ref. 10). Using solvent partitioning, gel filtration, and reverse-phase HPLC, the major active compound, tolytoxin (1), was isolated in about 0.03% yield from the dried alga (ref. 9).

The field desorption mass spectrum of tolytoxin suggested that the molecular weight was 1275. Its 220 MHz $^1$H NMR spectrum in $CDCl_3$ showed the presence of five O-methyl, one N-methyl, and six C-methyl groups. One of the methoxyl groups was proposed to be in a methyl ester functionality of a $\alpha,\beta,\gamma,\delta$-dienoate system (1a). The presence of a dienoate chromophore was supported by the ultraviolet spectrum of tolytoxin. The proton NMR spectrum also showed that tolytoxin possessed partial structure 1b. Unfortunately more extensive $^1$H NMR studies could not be carried out on the original tolytoxin sample to determine the total gross structure. Essentially all of the compound had decomposed during the initial NMR work, apparently due to contact with traces of hydrochloric acid in the $CDCl_3$ solvent.




1a




1b

To our disappointment tolytoxin-producing alga could not be cultured from the voucher specimen of the dried cyanophyte. Furthermore no attempt was made to recollect T. conglutinata var. colorata from the original collecting site on Fanning Island (ref. 9). Last year, however, we were able to find a very cytotoxic species, identified as Scytonema pseudohofmanni Bharadwaja, in a soil sample collected at Camp Paumalu near Pupukea, Oahu (Hawaii) in September, 1983 and to mass culture this organism in the laboratory. The chemical, spectral, and pharmacological properties of the two major cytotoxins in this cyanophyte were very similar with those of tolytoxin. In this paper we describe the isolation, gross structure determination, and anticancer properties of these two substances, scytophycins A and B.

## ISOLATION

The alga, which was collected in a moist, heavily forested region of the Koolauloa District on the island of Oahu, Hawaii, was identified as an epidaphic variety of Scytonema pseudohofmanni Bharadwaja (Scytonemataceae (Fig. 1) (ref. 11). By repeated subculture on solidified media (ref. 12), a clonal culture of the cyanophyte, designated strain number BC-1-2, was isolated. Unialgal, non-axenic cultures of BC-1-2 were grown in 25 L glass bottles containing an aqueous inorganic medium (ref. 13) adjusted to pH 7. The cultures were illuminated continuously at an incident intensity of 330 microEinsteins $m^{-2}$ $sec^{-1}$ from banks of cool-white fluorescent tubes and were vigorously aerated with 0.5% carbon dioxide in air. Following incubation at 24 ± 1°C for periods ranging from 29 to 33 days, the alga was harvested by filtration or centrifugation at 5000 x g in a refrigerated continuous flow centrifuge and freeze-dried. Yields of the lyophilized algal cells were typically 0.3 to 0.4 g per liter of culture.

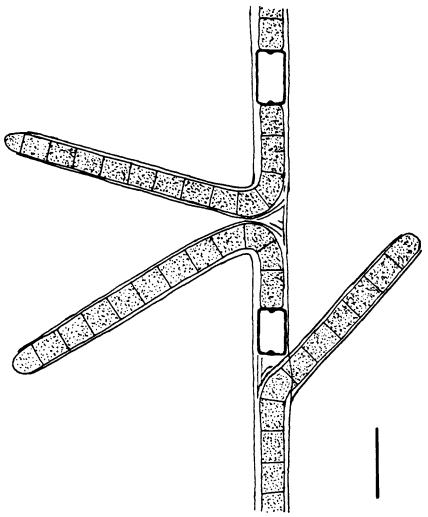


Fig. 1. Scytonema pseudohofmanni Bharadwaja (Nostocales, Scytonemataceae). Mature filaments with single and geminate false branches. Note single intercalary heterocysts, somewhat broader than the trichome. The bar represents 20 μm.

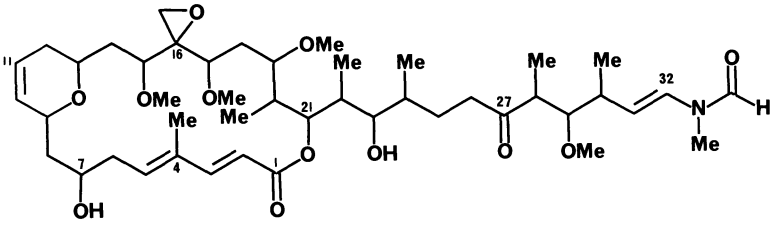
Twenty-three grams of the dried alga was homogenized in a Waring blender with one liter of 40% ethanol-water for ten minutes. The mixture was shaken overnight and then centrifuged at 12,000 x g for twenty minutes. The supernate was concentrated under reduced pressure at 40°C to a volume of 300 mL and 100 mL of 95% ethanol was added to give 400 mL of a 25% ethanol-water solution which was then subjected to sonication for two minutes to disperse a fine floc.

The 25% ethanol-water solution was passed through a 1 mL C-18 column (Sep-pak, Millipore Corporation) in 10 mL portions at a flow rate of 5-10 mL/min. After the passage of each 10 mL aliquot, the C-18 column was washed with 10 mL of 25% ethanol-water and the crude cytotoxins were eluted from the column with 15 mL of 50% ethanol-water. The column was then regenerated for the next run by a reversed wash with 95% ethanol (to remove accumulated precipitate) followed by a normal wash with 5 mL of water. The combined 50% ethanol-water effluent (600 mL) was concentrated *in vacuo* to remove the ethanol and the aqueous concentrate (300 mL) was lyophilized.

The residual semisolid was dissolved in 70% ethanol-water and the filtered solution (4.25 mL) was subjected to reverse-phase HPLC in 200 µL portions on a Beckman 1 cm x 25 cm column of ODS bonded to 5 µm spherical silica-based packing, employing 65% acetonitrile and 35% 0.10 M triethylamine and 0.122 M acetic acid in water as the eluant. Using a flow rate of 4 mL/min, a 200 µL aliquot could be introduced onto the column every 10 minutes. Ultraviolet absorption at 300 nm was monitored and two fractions having retention times of 6'20" (A) and 7'50" (B) were collected. Fractions A and B showed single peaks at 9'20" and 12'46" when each one was rechromatographed on the same column using 57.5% $CH_3CN$ - 42.5% $Et_3N$/HOAc buffer. Water (40 mL) was added to each fraction and the acetonitrile was evaporated under reduced pressure. The turbid aqueous mixture was then lyophilized twice to remove the volatile buffer. Fraction A provided 14.3 mg of scytophycin A (0.062%) and fraction B yielded 34 mg of scytophycin B (0.15%).

## GROSS STRUCTURE DETERMINATION

The major cytotoxin, scytophycin B (2), was found to have a molecular weight of 819 by fast-atom bombardment mass spectrometry. Its positive ion FABMS showed a small MH ion at m/z 820 along with M+Na and M+K ions at m/z 842 and 858, respectively. The most intense peak in the FABMS was at m/z 802 ($MH-H_2O$) and a high resolution measurement of this ion, m/z 802.5123, coupled with $^1H$ and $^{13}C$ NMR spectral data (Table 1) established that its composition was $C_{45}H_{72}NO_{11}$ (calcd. 802.5105). The molecular formula of scytophycin B was therefore $C_{45}H_{73}NO_{12}$.




2

Scytophycin B appeared to be structurally related to tolytoxin. Its $^1H$ NMR spectrum indicated the presence of four O-methyl groups, six C-methyl groups, and an ene-N-methylformamide unit, similar to 1b in tolytoxin (ref. 9), which exists in two slowly interconverting conformations. The signals for several of the protons in this enamide unit were doubled due to restricted rotation around the C(32)-N(33) and N(33)-C(34) bonds. The formyl (H-34) and N-methyl signals for the major conformer were singlets resonating at δ 8.360 and 2.990, respectively, and difference NOE experiments strongly suggested that the major conformer had the enamide functionality arranged as shown in 2a. Irradiation of the N-methyl group produced a NOE in the H-31 signal (δ 5.118) whereas irradiation of the formyl proton resulted in a NOE in the H-32 signal (δ 6.786). The formyl and N-methyl signals for the minor conformer, on the other hand, were singlets at δ 8.113 and 3.108, respectively, and difference NOE experiments indicated that the enamide conformation was probably as shown in 2b. Irradiation of the N-methyl group caused a NOE in the formyl signal, but a NOE was not observed in either the


2a 2b

TABLE 1. NMR data for scytophycin B (2) in acetone-$d_6$

| $\delta_{^{13}C}$ [a,b,c] | Position | $\delta_{^{1}H}$ [d,e] | $\delta_{^{13}C}$ [a,b,c] | Position | $\delta_{^{1}H}$ [c,d] |
|---|---|---|---|---|---|
| 169.03 s | 1 | | 57.18 q | MeO on 19 | 3.198 s |
| 114.98 d | 2 | 5.780 d | 37.55 d | 20 | 2.089 m |
| 151.25 d | 3 | 7.657 d | 8.63 q | Me on 20 | 0.859 d |
| 134.16 s | 4 | | 75.91 d | 21 | 5.217 br d |
| 11.69 q | Me on 4 | 1.853 br s | 37.55 d | 22 | 1.988 m |
| 139.35 d | 5 | 6.017 br dd | 8.63 q | Me on 22 | 0.875 d |
| 41.48 t | 6 | 2.483 ddd | 75.91 d | 23 | 3.030 dd |
| | 6' | 2.567 br ddd | 33.18 d | 24 | 1.690 m |
| 68.26 d | 7 | 4.063 br td | 17.67 q | Me on 24 | 0.986 d |
| 40.91 t | 8 | 1.262 ddd | 22.11 t | 25 | 1.37 m |
| | 8' | 1.768 ddd | | 25' | 1.75 m |
| 70.41 d | 9 | 4.582 br d | 38.76 t | 26 | 2.54 m |
| 130.91 d | 10 | 5.655 ddt | 213.47 s | 27 | |
| 124.40 d | 11 | 5.807 ddt | 48.88 d | 28 | 2.776 dq |
| 31.24 t | 12 | 1.910 m | | | (2.740 dq) |
| 66.27 d | 13 | 3.39 m | 13.05 q | Me on 28 | 0.919 d |
| 35.03 t | 14 | 1.455 | 87.66 d | 29 | 3.284 dd |
| | 14' | 1.545 ddd | 60.52 q | Me on 29 | 3.312 s |
| 77.84 d | 15 | 3.942 dd | 37.38 d | 30 | 2.46 m |
| 56.86 q | MeO on 15 | 3.372 s | (37.75) | | |
| 60.52 s | 16 | | 19.02 q | Me on 30 | 1.149 d |
| 44.96 t | $CH_2$ on 16 | 2.629 d | 110.58 d | 31 | 5.118 dd |
| | | 2.719 d | (112.65) | | (5.180 dd) |
| 74.60 d | 17 | 3.875 dd | 129.55 d | 32 | 6.786 d |
| 52.23 q | Me on 17 | 3.243 s | (124.84) | | (7.107 d) |
| 26.85 t | 18 | 1.456 ddd | 26.62 q | Me on N-33 | 2.990 s |
| | 18' | 1.955 ddd | (32.43) | | (3.108 s) |
| 76.89 d | 19 | 3.308 ddd | 162.29 d | 34 | 8.360 s |
| | | | (161.0) | | (8.113 s) |

$J_{H,H}$ in Hz: 2,3 = 15.8; Me(4),5 = 1.5; Me(4),6' = 1.3; 5,6 = 9.3; 5,6' = 4.3; 6,6' = -16.2; 6,7 = 10.2; 6',7 = 3.1; 7,8 = 10.2; 7,8' = 1.2; 8,8' = -14.7; 8,9 = 1.8; 8',9 = 9.8; 9,10 = 2.9; 9,11 = 1.6; 9,12 = 1.8; 10,11 = 10.4; 10,12 = 1.8; 11,12 = 4; 13,14 = 2.0; 13,14' = 8.4; 14,14' = -14.5; 14,15 = 7.4; 14',15 = 3.1; gem $CH_2$(16) = 4.5; 17,18 = 4.0; 17,18' = 11.4; 18,18' = -13.6; 18,19 = 9.7; 18',19 = 4.0; 19,20 = 1.0; 20,Me(20) = 7.0; 20,21 = 10.3; 21,22 = 1; 22,Me(22) = 6.8; 22,23 = 9.7; 23,24 = 2.0; 24,Me(24) = 6.7; 28,Me(28) = 7.0; 28,29 = 9.5; 29,30 = 2.2; 30,Me(30) = 7.0; 30,31 = 9.2 (9.0); 31,32 = 14.1 (14.8).

a 75 MHz; $CDCl_3$ as internal reference = 76.90 ppm.
b $^{1}H$-$^{13}C$ connectivities determined using a phase-cycled 16-step heteronuclear chemical shift correlation map (CSCM) experiment as described by Bax (ref. 14).
c Concentration = 0.1 mM.
d 300 MHz; residual $CHCl_3$ as internal reference = 7.25 ppm.

H-31 or H-32 signal. The geometry of the enamide double bond was E since the coupling constant was 14.1 and 14.8 Hz for $J_{31,32}$ in 2a and 2b, respectively. Interestingly the H-32 signal was a doublet of 1:1:1 triplets, the triplet pattern due to coupling of H-32 to N-33 (0.6 Hz for 2b and roughly 0.3 Hz for 2a). Proton-proton decoupling experiments, aided by difference spectroscopy, showed that H-31 was coupled to H-30 (δ 2.46) by 9.2 Hz, H-30 was coupled to H-29 (δ 3.284) by 2.2 Hz, and H-29 was coupled to H-28 (δ 2.776 for 2a and δ 2.740 for 2b) by 9.5 Hz. They also indicated that methyl groups were on C-28 and C-30. An appreciable NOE was seen in the methoxyl signal at δ 3.312 when the methyl group on C-28 (δ 0.919) was irradiated; conversely the C-28 methyl signal showed a NOE when the methoxyl signal at δ 3.312 was irradiated. The NOEs suggested that a methoxyl group was attached to the C-29 methine (δ 3.284). The H-28 signals were at relatively low field (δ 2.776 for 2a and δ 2.740 for 2b), strongly suggesting that C-28 was attached to a carbonyl (C-27). The $^{13}C$ NMR spectrum showed two carbonyl peaks, one at δ 213.47 for a ketone carbonyl and another at δ 169.03 for an ester carbonyl. C-27 had to be a ketone carbon, however, since the ultraviolet spectrum of scytophycin B in ethanol, $\lambda_{max}$ 264 nm (ε 28,500), which was similar to that of tolytoxin, indicated that the chromophore was either a dienoic acid or ester (ref. 15). The carboxylic acid possibility, however, could be ruled out since scytophycin B did not form a methyl ester with diazomethane.

The 300 MHz $^1$H NMR spectrum showed that scytophycin B was an ester of an E,E-γ-methyl-α,β,γ,δ-dienoic acid. The chemical shifts for H-2, H-3, and H-5 and the methyl group on C-4 were very close to those reported for similar protons in streptolic acid (ref. 16) and tirandamycic acid (ref. 17). The geometry of the dienoate was clearly E,E, since the coupling constant between H-2 and H-3 was 15.8 Hz and strong NOEs were observed by difference spectroscopy for the H-2 and H-3 signals when the C-4 methyl and H-5 signals were irradiated, respectively.

Two-dimensional COSY (ref. 18) and relayed coherence transfer (RCT) spectra (ref. 19 and ref. 20) were very useful in determining the structure of the C(1)-C(15) segment. In the conventional COSY spectrum (Fig. 2) the signal for H-5 (δ 6.017) showed two off-diagonal peaks, one reflecting vicinal coupling to the two protons on C-6 (δ 2.483 and 2.567) and the other allylic coupling to the methyl group on C-4 (δ 1.853). It was not possible to decide from the COSY spectrum what the C-6 protons were further coupled to, since other proton signals were present in the 2.4-2.6 ppm multiplet. The RCT spectrum (Fig. 3), however, readily indicated that the C-6 protons were coupled to H-7 (δ 4.063) since the signal for H-5 showed a cross peak to H-7. This was corroborated by decoupling experiments whereby H-5 and H-7 where irradiated in separate experiments and the decoupled C-6 proton signals were observed by difference spectroscopy. Irradiation of H-7 indicated that H-7 was also coupled to two protons of another non-equivalent methylene (δ 1.262 and 1.768). In the normal COSY spectrum H-7 showed a cross peak for only one of these protons, viz. the C-8 proton that was strongly coupled to H-7 (δ 1.262); in the long range COSY spectrum (ref. 18), however, the relative intensity of the cross peak for the

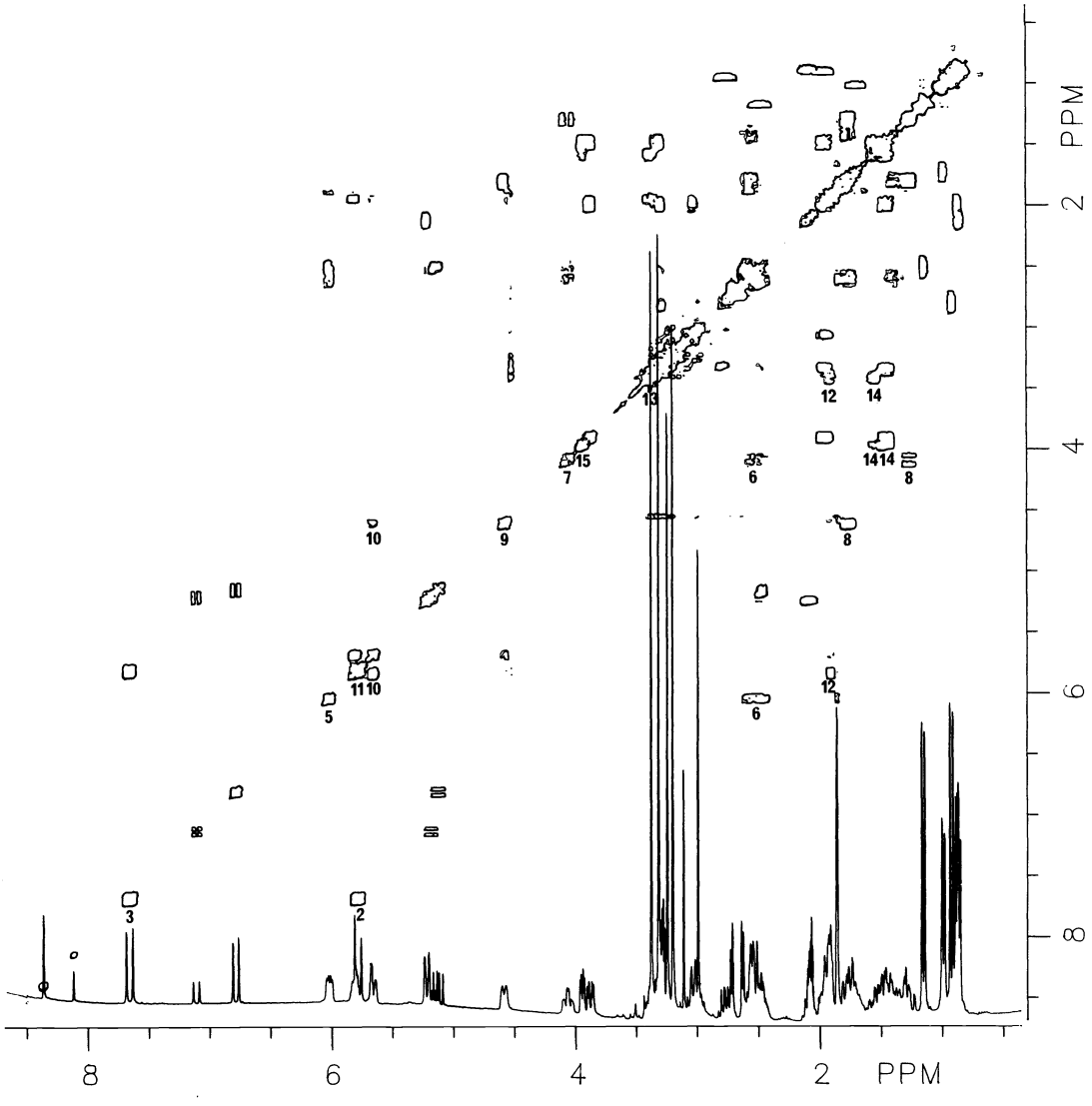



Fig. 2 The 300 MHz $^1$H NMR spectrum and two-dimensional COSY projection of scytophycin B in acetone-$d_6$.

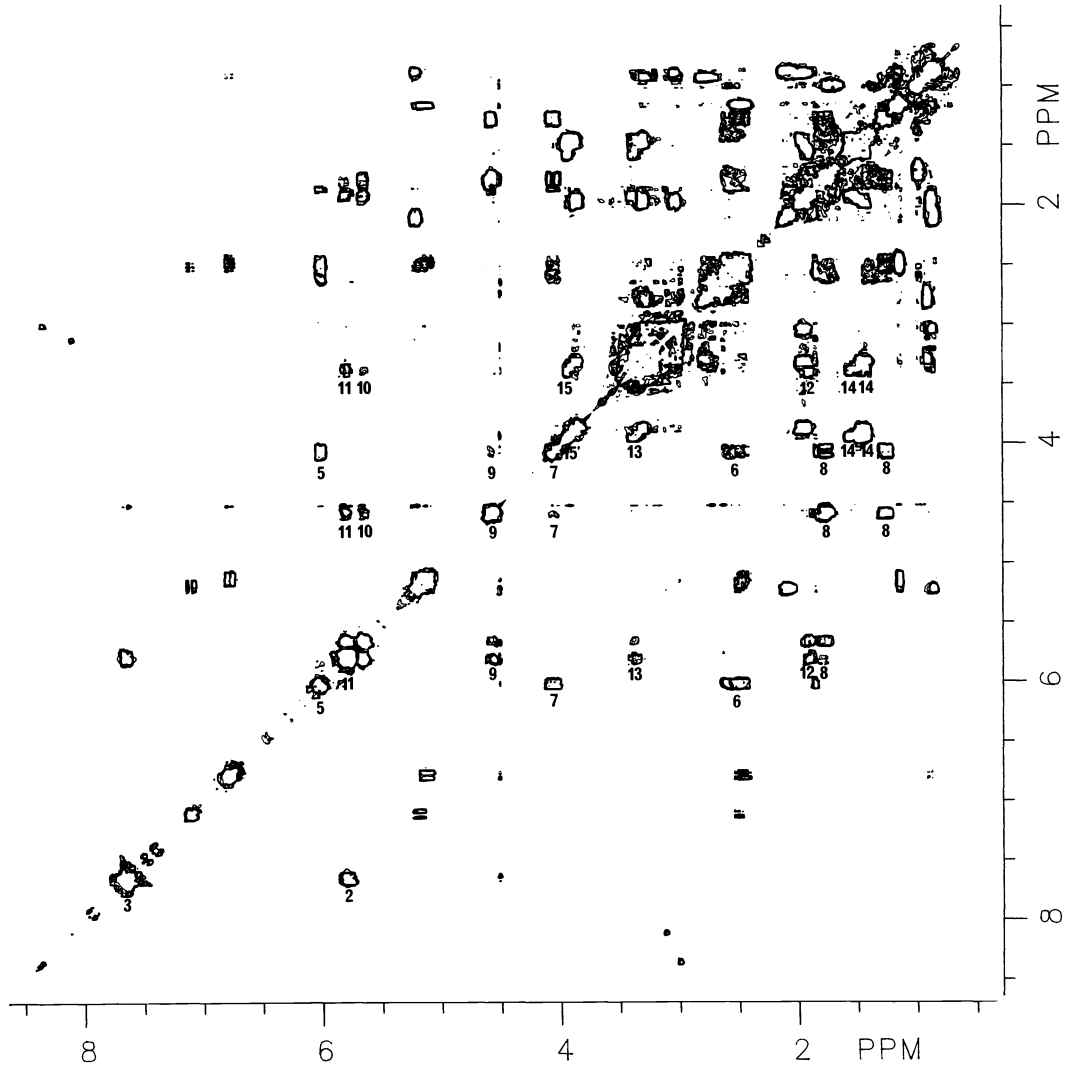



Fig. 3 The two-dimensional relayed coherence transfer spectrum of scytophycin B in acetone-$d_6$.

C-8 proton that was weakly coupled to H-7 was now clearly visible. Inspection of the RCT spectrum (Fig. 3) revealed off-diagonal peaks from H-7 to both C-8 protons and also to H-9 (δ 4.582). Decoupling experiments showed that H-9 was vicinally coupled to H-10 (δ 5.655), allylically coupled to H-11 (δ 5.807), and homoallylically coupled to two protons on C-12 (δ 1.910). The COSY and RCT spectra supported these findings. Analysis of the COSY spectrum indicated that H-11 was coupled to H-9, H-10, and the two protons on C-12 and the RCT spectrum showed that the C-12 methylene was coupled to H-13 (δ 3.39). In the normal COSY spectrum H-13 showed a cross peak to only one proton on the C-14 methylene, viz. the one that was strongly coupled to H-13 (δ 1.545); the cross peak for the C-14 proton that was weakly coupled to H-13 (δ 1.455), however, could be seen in the long range COSY spectrum. As expected in the RCT spectrum, H-13 showed intense off-diagonal peaks to both protons on C-14 and to H-15 (δ 3.942). Further examination of the COSY and RCT spectra revealed that H-15 was coupled to only the two protons on C-14, strongly suggesting that C-16 was quaternary.

The ester oxygen on C-1 was concluded to be connected to a methine carbon (C-21). The chemical shift of the methine proton resonating at δ 5.217 (H-21) suggested that this proton was attached to the carbon bearing the ester oxygen. Decoupling experiments showed that H-21 was vicinally coupled to two methine protons, to H-20 (δ 2.089) by 10.3 Hz and to H-22 (δ 1.988) by 1 Hz. Off-diagonal peaks in the COSY and RCT spectra indicated that methyl group were on C-20 (δ 0.859) and C-22 (δ 0.875). Additional decoupling experiments, with the aid of difference spectroscopy, showed that H-20 was coupled to H-19 (δ 3.308) by 1.0 Hz and that H-19 was in turn coupled to two protons of a methylene, specifically to H-18 (δ 1.456) by 9.7 Hz and to H-18' (δ 1.955) by 4.0 Hz. Both of

these protons, H-18 and H-18', were further coupled to a methine proton at δ 3.875 (H-17) by 4.0 and 11.4 Hz, respectively. H-17 was not coupled to any other proton, however, suggesting that C-17 was connected to a quaternary carbon (C-16).

Difference decoupling experiments established that H-22 was coupled to H-23 (δ 3.030) by 9.7 Hz which in turn was coupled to H-24 (δ 1.690) by 2.0 Hz. The COSY and RCT spectra revealed that H-24 was further coupled to a methyl group on C-24 (δ 0.986) and to two protons of a methylene (δ 1.37, H-25; δ 1.75, H-25') and that H-25 and H-25' were coupled to two protons on C-26 (complex multiplet centered at δ 2.54). Since H-26 and H-26' were found to resonate at δ 2.54, it was concluded that C-26 was attached to the ketone group (C-27).

The chemical shifts for H-7, H-9, H-13, H-15, H-17, H-19, and H-23 indicated that oxygens were attached to the carbons bearing these protons. A hydroxyl group was clearly on C-7. Acetylation of scytophycin B with 1.2 equivalents of acetic anhydride, 1.2 equivalents of triethylamine, and a catalytic amount of 4-dimethylaminopyridine in methylene chloride at 25° for 5 hours led to a monoacetate (3H singlet at δ 2.05) which exhibited a $^1$H NMR spectrum that was essentially the same as the one for scytophycin B, except that the H-7 signal had shifted downfield to δ 5.20 (spectrum determined in acetone-$d_6$). In the spectrum of this 7-acetate, a doublet (J = 3.8 Hz) could also be seen at δ 4.06 for an OH proton. Irradiation at δ 3.038 (H-23) caused this OH signal to collapse to a singlet. A hydroxyl group was therefore on C-23, but apparently this secondary alcohol group was highly hindered, since acetylation of scytophycin B with acetic anhydride in pyridine for 24 hours at 25° led only to a monoacetate, viz. the 7-acetate.

The coupling constants associated with the protons in the C(9)-C(13) segment suggested that these carbons were in a dihydropyran ring. First of all the coupling between H-10 and H-11 was 10.4 Hz, indicating that the C(10)-C(11) double bond was cis. Secondly the coupling between H-11 and both protons on C-12 was about 4 Hz, suggesting that in the Newman projection H-C(11) was bisecting H-C(12)-H, i.e. the dihedral angles between H-11 and H-12 and between H-11 and H-12' were about 60°. Eventhough the exact couplings between each C-12 proton and H-13 were not determined, both were estimated to be roughly 2-5 Hz. None of these couplings were consistent with an acyclic system, but rather with a dihydropyran ring where C-8 was attached equatorially to C-9 and C-14 was attached axially to C-13.

Difference NOE experiments suggested that methoxyl groups were on C-15, C-17, and C-19. Irradiation of the methoxyl group resonating at δ 3.372 resulted in an appreciable NOE in the H-15 signal and conversely irradiation of H-15 produced a significant NOE in the methoxyl signal at δ 3.372. Irradiation of the methoxyl group resonating at δ 3.243, however, caused only a very small NOE in the H-17 signal, but irradiation of H-17 gave an appreciable NOE in the methoxyl signal at δ 3.243 and also the one at δ 3.372. Finally irradiation of the methoxyl group resonating at δ 3.198 showed an appreciable NOE in the H-21 signal, but a NOE could not be observed in the H-19 signal. On the basis of these experiments, the 3H singlets at δ 3.372, 3.243, and 3.198 were tentatively assigned to methoxyl groups on C-15, C-17, and C-19, respectively.

All that remained now for total assemblance of the gross structure was the placement of a methylene and an oxygen. Both of these had to be on the quaternary carbon (C-16) and this meant that scytophycin B possessed an oxirane ring. Both the $^1$H and $^{13}$C NMR spectra supported this conclusion. The $^1$H NMR spectrum showed doublets at δ 2.629 and 2.719 for the non-equivalent methylene protons and the positive geminal coupling of 4.5 Hz proved that this methylene was in an oxirane ring (ref. 21). In the $^{13}$C NMR spectrum the oxirane methylene and C-16 resonated at δ 44.96 and 60.52 (ref. 22), respectively, and one-bond $^{13}$C-H coupling for the oxirane methylene was found to be 175 Hz (ref. 23). The $^{13}$C NMR data compared nicely with values reported in the literature. Finally irradiation of H-17 produced a significant NOE in the epoxide proton signal at δ 2.719, providing additional evidence that C-17 was attached to C-16. Scytophycin B therefore was concluded to have the gross structure depicted in 2.

Scytophycin A (3) was found to be closely related to 2. The positive ion FAB mass spectrum indicated that its molecular weight was 821, two mass units higher than of scytophycin B's. A strong MH ion was observed at m/z 822, accompanied by significant M+Na and M+K ions at m/z 844 and 860, respectively. A high resolution measurement of the MH ion (obs. 822.5407; calcd. for $C_{45}H_{76}NO_{12}$, 822.5368) suggested that the molecular formula of scytophycin A was $C_{45}H_{75}NO_{12}$. The UV spectrum of scytophycin A in ethanol, $\lambda_{max}$ 264 nm (ε 34,800), was essentially identical with that of scytophycin B and a close inspection of the $^1$H NMR spectrum, in particular the COSY spectrum, suggested that scytophycin A differed in structure from 2 only at C-27. A secondary alcohol group was on C-27 instead of a keto group.

Since the $^1$H NMR spectrum of tolytoxin is very similar to that of scytophycin B, it is very difficult to rationalize a molecular weight of 1275 for tolytoxin. A reexamination

of the original field desorption mass spectral data, however, shows the presence of an intense peak at m/z 873, a much more reasonable value for tolytoxin's molecular weight. Furthermore tolytoxin is probably not a methyl ester as previously proposed (ref. 9), but rather a macrocyclic lactone like scytophycin B. Again a reexamination of the $^1$H NMR spectral data for tolytoxin in chloroform-d (ref. 9) suggests that a methoxyl group, possibly the one (δ 3.63) assigned earlier to a methyl ester, is probably attached to C-6. For tolytoxin the H-5 signal is a broad doublet at δ 5.87 (obscured by the H-2 and H-11 signals in chloroform-d but seen more clearly in acetone-$d_6$ or benzene-$d_6$), showing vicinal coupling to only one proton on C-6 (triplet at δ 4.29). Since the chemical shift of H-6 is δ 4.29, an oxygen has to be attached to C-6, but whether this is a methoxyl oxygen could not be rigorously deduced. Tolytoxin appears to possess the same epoxide ring as scytophycin B, since the same signals for the oxirane methylene protons are seen in its $^1$H NMR spectrum. If tolytoxin has the same gross structure as scytophycin B with an additional methoxyl group on C-6, the only problem that remains is how to assign 24 mass units to complete the structure. Since no tolytoxin is in our hands at the present time, we are not able to deduce the nature of this last piece. The complete gross structure of tolytoxin will have to await recollection of Tolypothrix conglutinata var. colorata from Fanning Island.

## PHARMACOLOGY

The minimum lethal dose of scytophycin B is about 650 μg/kg in mice. Its toxicity is comparable to that shown by curare and strychnine, but is almost two orders of magnitude less than that shown by saxitoxin and tetrodotoxin (ref. 24).

Scytophycins A and B are potent cytotoxins. The minimum toxic doses against KB human epidermoid carcinoma (ref. 25) and NIH/3T3 mouse fibroblast cell lines were determined to be 1 ng/mL and 0.65 ng/mL, respectively, using methods described in ref. 26. Both compounds exhibited moderate activity against intraperitoneally implanted P388 lymphocytic leukemia (ref. 27) and Lewis lung carcinoma (ref. 28), but no activity against intraperitoneally implanted B16 melanoma (ref. 27) (Table 2).

Female hybrid BDF1 (DBA/2 x C57BL/6) mice were used for the evaluation of the scytophycins against the three tumors. The mice were first inoculated intraperitoneally with $10^6$ cells of P388 ascites leukemia, 2 to $6x10^5$ cells of Lewis lung carcinoma homogenate, or 0.3 mL of 20% homogenate of B16 tumor masses. Drug treatment was started the next day (day 1) and continued daily for 6-9 days. Five mice were used for each dosage.

TABLE 2. Effect of the scytophycins on intraperitoneally implanted P388 lymphocytic leukemia, Lewis lung carcinoma, and B16 melanoma in BDF1 mice

| Drug | Tumor | Dose μg/mouse | Days of Treatment | Mean survival time, days Treated | Controls | % T/C |
|---|---|---|---|---|---|---|
| A | P388 | 1 | 8 | 10.0 | 8.0 | 125 |
| B | P388 | 4 | 8 | 12.0 | 9.4 | 128 |
| | | 3.25 | 6 | 11.4 | 8.4 | 136 |
| | | 2 | 8 | 12.2 | 9.4 | 130 |
| | | 1 | 8 | 10.6 | 9.4 | 113 |
| | | 0.65 | 8 | 10.0 | 8.0 | 125 |
| | | 0.5 | 8 | 9.4 | 9.4 | 100 |
| B | LLC | 4 | 9 | 12.6 | 9.8 | 129 |
| | | 2 | 9 | 12.2 | 9.8 | 124 |
| | | 1 | 9 | 11.2 | 9.8 | 114 |
| | | 0.5 | 9 | 10.4 | 9.8 | 106 |
| B | B16 | 8 | 8 | 12.0 | 19.0 | 63(toxic) |
| | | 4 | 8 | 19.8 | 19.0 | 104 |
| | | 2 | 8 | 19.4 | 19.0 | 102 |

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