C. Song, *Biochem. Biophys. Res. Commun.* **91**, 982 (1979).
7. O. R. Brown and C. Song, *ibid.* **93**, 172 (1980).
8. M. A. Heitkamp, thesis, University of Missouri, Columbia (1980).
9. H. J. Forman, J. Nelson, A. B. Fisher, *J. Biol. Chem.* **255**, 9879 (1980).
10. Y. Kobayashi, S. Okahala, T. Usui, *Biochem. Biophys. Res. Commun.* **91**, 1288 (1979); J. S. Bus, S. D. Aust, J. E. Gibson, *ibid.* **58**, 749 (1974); A. P. Autor, *Life Sci.* **14**, 1309 (1974); R. C. Baldwin, A. Pasi, J. T. MacGregor, C. H. Hine, *Toxicol. Appl. Pharmacol.* **32**, 298 (1975).
11. J. W. Foster and A. G. Moat, *Microbiol. Rev.* **44**, 83 (1980); R. K. Gholson *et al.*, *J. Biol. Chem.* **239**, 1208 (1968); R. K. Gholson, *Nature (London)* **212**, 933 (1966).
12. P. Pinder, J. B. Clark, A. L. Greenbaum, *Methods Enzymol.* **18B**, 20 (1971).
13. M. Klingenberg, in *Methods of Enzymatic Analysis*, H. V. Bergmeyer, Ed. (Academic Press, New York, 1963), p. 528.
14. E. E. Snell and L. D. Wright, *J. Biol. Chem.* **139**, 675 (1941).

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Lophotoxin: A Novel Neuromuscular Toxin from Pacific Sea Whips of the Genus *Lophogorgia*

Abstract. *A new neuromuscular toxin, lophotoxin, has been isolated from several Pacific gorgonians of the genus* Lophogorgia. *The structure of lophotoxin was deduced by combined spectrochemical methods, and belongs to the well-known cembrene class of diterpenoid molecules. Lophotoxin contains furanoaldehyde and α,β-epoxy-γ-lactone functional groups, in sharp contrast to the cationic ammonium functional groups of the established neurotoxins.*

As part of a program to explore the chemical defense adaptations of marine organisms, we have investigated numerous representatives of the horny corals or gorgonians (sea fans and whips; phylum Cnidaria, order Gorgonaceae), found mainly in tropical and subtropical waters. In contrast to the hard corals, gorgonians possess no skeletal protection, and hence their survival appears dependent on alternative strategies based certainly, in part, on the production of noxious and toxic chemicals (*1*).

During May and June 1978, a collaborative chemical and pharmacological investigation of the abundant gorgonian fauna of Pacific Mexico was undertaken onboard the research vessel *Alpha Helix*. On-site bioassays indicated that alcohol extracts of several gorgonians from this locale possessed considerable cytotoxic, ichthyotoxic, and antibacterial activity (*2*). In particular, extracts of the colorful sea whips of the genus *Lophogorgia* showed consistent ichthyotoxicity, and in nature these animals showed little evidence of predation. Subsequent chemical and pharmacological studies have now resulted in the isolation and structure assignment of a potent new neuromuscular toxin, lophotoxin (**1**), from four species of *Lophogorgia*. Reported herein are the structural chemistry and fundamental pharmocological properties of this toxin.

Chloroform extracts of three Mexican *Lophogorgia* species, *L. alba* (Duch. and Mich.), *L. cuspidata* (Verrill), and *L. rigida* (Verrill), were each recognized to contain various concentrations of lophotoxin. The California gorgonian, *Lophogorgia chilensis* Verrill (*L. panamensis*), has subsequently also been found to produce lophotoxin. Therefore, the distribution of toxin-producing gorgonians extends from Panama Bay northward to Point Conception, California (*3*).

To isolate the toxin for structure analysis, an extract of *L. rigida* was chromatographed over Florisil and eluted with increasing amounts of ethyl acetate in isooctane. Pure ethyl acetate eluted the toxin in a mixture which was further purified by high performance liquid chromatography (μ-Porasil column; with 60 percent ethyl acetate and isooctane) (*4*). Lophotoxin crystallized from one liquid-chromatography fraction as white needles [melting point, 164° to 166°C; $[\alpha]_D^{27}$ +14.2°C (c = 1.7, $CHCl_3$) (0.2 percent dry weight)].

The structure of lophotoxin was determined by spectral and chemical methods. Along with lophotoxin, we isolated a minor amount of the cembrenolide, pukalide (**2**), a metabolite earlier described by Scheuer and co-workers from the Hawaiian alcyonacean soft coral *Sinularia abrupta* (*5*). The similarity in structure and in spectral features of lophotoxin and pukalide strongly supported our initial structural assignment. A molecular formula of $C_{22}H_{24}O_8$ was established for **1** by mass spectral analysis and by interpretation of ^{13}C nuclear magnetic resonance (NMR) data (Table 1), and this composition indicated 11 degrees of unsaturation. Infrared absorptions at 2850 and 1689 cm^{-1}, coupled with ^{13}C NMR bands at 184.5 (d), 161.8 (s), 149.8 (s), 123.0 (s), and 105.6 (d) allowed the formulation of a furanoaldehyde group. An additional absorption at 1792 cm^{-1} initially suggested the presence of a saturated γ-lactone functional group. However, after recognizing that two trisubstituted epoxides were present by analysis of ^{13}C NMR data [64.1 (d), 61.3 (s), 56.1 (s), and 55.3 (d)] the γ-

Table 1. ^1H and ^{13}C nuclear magnetic resonance data for lophotoxin (**1**); recorded in $CDCl_3$ solution at 220 MHz (^1H) and 20 MHz (^{13}C), with internal trimethylsilyl as standards. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet; gem, geminal; and J, coupling constant.

Assignments*	^{13}C chemical shift	J_r+	Proton chemical shift
18	184.5 (d)	38.0	9.87 (s)
20	170.2 (s)		
21 (CH_3COO)	168.1 (s)		
3	161.8 (s)		
6	149.8 (s)		
15	148.3 (s)		
4	123.0 (s)		
16	111.0 (t)	23.9	4.93 (bs) 4.91 (bs)
5	105.6 (d)	31.6	6.57 (s)
10	76.7 (d)	23.4	4.81 (dd,$J_{10,9\beta}$=4, $J_{10,9\alpha}$=3)
13	70.3 (d)	22.2	4.99 (d,$J_{13,14\beta}$=7)
11	64.1 (d)	28.1	4.17 (s)
12	61.3 (s)		
8	56.1 (s)		
7	55.3 (d)	26.1	4.09 (s)
9	39.0 (t)	15.4	2.49 (dd,J_{gem}=−16, $J_{9\beta,10}$=4) 2.05 (dd,J_{gem}=−16, $J_{9\alpha,10}$=3)
1	36.5 (d)	16.4	3.99 (ddd,$J_{1,2\beta}$=11, $J_{1,14\beta}$=11,$J_{1,2\alpha}$=4)
2	32.9 (t)	16.6	3.07 (dd,J_{gem}=−18, $J_{2\alpha,1}$=4) 2.95 (dd,J_{gem}=−18, $J_{2\beta,1}$=11)
14	31.7 (t)	14.3	2.50 (m,J_{gem}=−15, $J_{14\beta,1}$=11, $J_{14\beta,13}$=7) 1.70 (d,J_{gem}=−15)
17	21.1 (q)	14.4	1.90 (s)
22 (CH_3COO)	20.4 (q)	14.9	2.05 (s)
19	20.2 (q)	12.4	1.14 (s)

*Assignments are made based on spin-decoupling measurements (^1H) and proton single frequency decoupling experiments (^{13}C). Residual coupling constants (J_r) (*13*) became useful in interrelating proton and carbon resonances.

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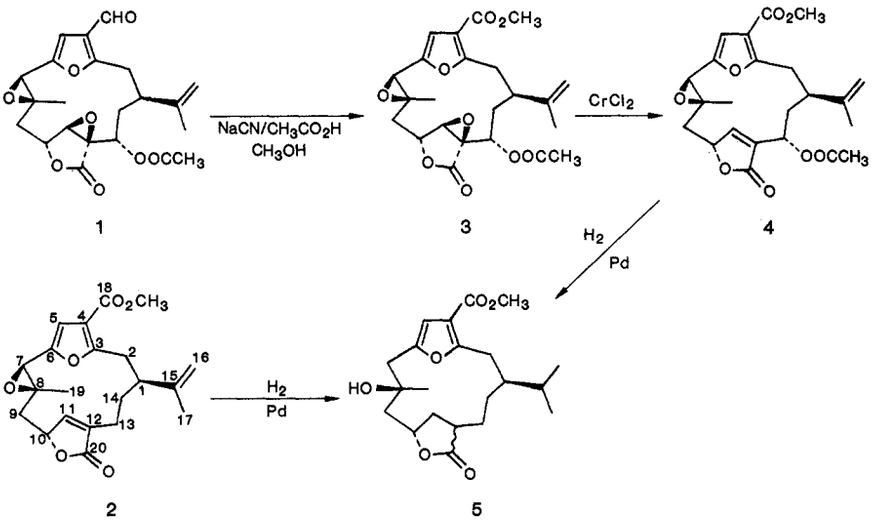
lactone was revised to an α,β-epoxy-γ-lactone group. This rare functional group has been observed in several terrestrial natural products, including micromelin (*6*) and linderane (*7*). A further infrared absorption at 1735 cm^{-1}, coupled with ^{13}C NMR bands at 168.1 (s), 70.3 (d), and 20.4 (q), confirmed the presence of the secondary acetate ester.

Treatment of lophotoxin with manganese dioxide and sodium cyanide in methanol (*8*) smoothly converted the furanoaldehyde to the corresponding methyl ester, **3**. This compound was also isolated as a natural product from *L. rigida*.

To confirm the existence of the epoxylactone in **1** and **3**, compound **3** was deoxygenated with chromous chloride in a mixture of acetone and acetic acid to yield the α,β-unsaturated lactone **4**. The unsaturated γ-lactone carbonyl group produced gave rise to an infrared absorption at 1760 cm^{-1}, reflecting the 30 cm^{-1} shift also observed in the deoxygenation of linderane (*7*). Final confirmation of the basic cembrenolide structure of lophotoxin, including several centers of stereochemistry, was obtained via catalytic hydrogenation of **4** (Pd and ethyl acetate) to yield the hydrogenolysis product **5**, $[\alpha]_D$ +33.5°C (c = 0.3, $CHCl_3$). Under similar conditions, pukalide was reported to yield **5** as the major product (*5*). Hydrogenation of pukalide under our conditions indeed gave **5**, $[\alpha]_D$ +34.0°C (c = 0.9, $CHCl_3$), as a major product which was identical to the product from **4**. Since hydrogenation would not influence the stereochemistry of substituents at C-1, C-8, and C-10, both lophotoxin and pukalide must have the same absolute stereochemistry at these centers. Only the placement of the acetoxyl group and the stereochemistry of the epoxide at C-11, C-12 remained to fully define lophotoxin. Fortunately, proton decoupling experiments at 220 MHz established these structural features. Two sets of mutually coupled protons were observed, the C-9, C-10 set and the C-13, C-14, C-1, C-2 array. As in pukalide, the C-9 methylene protons appeared at different shifts (δ 2.49 and 2.05), and each was coupled to the C-10 lactone methine proton which appeared at δ 4.81. This proton was not coupled to the epoxide proton at C-11, suggesting a dihedral angle between these atoms approaching 90°. Recent ^1H NMR data for some synthetic 1,3-dialkylated α,β-epoxy-γ-lactones showed less than 1-Hz couplings in the *trans* arrangement of the β-epoxy and γ-lactone methine protons, and 2- to 3-Hz couplings in the *cis* orientation (*9*). Our assignment of the substituents at C-10, C-11 as *trans* in lophotoxin is based on these observations and on conformational analysis of both epoxide isomers (*10*).



The readily resolved allylic methine proton at C-1, which is observed as an identical multiplet in both lophotoxin and pukalide, served as a cornerstone to establish the four-carbon unit C-13, C-14, C-1, and C-2. As in pukalide the C-1 methine proton was coupled only to three of the four proximate protons. Both C-2 protons were coupled to C-1 (J = 11 Hz, J = 4 Hz), and only one of the C-14 methylene protons showed coupling to C-1 (J = 11 Hz). The C-14 methylene proton, which was coupled to the C-1 proton, was also coupled (J = 11 Hz) to an acetoxyl-bearing methine, therefore placed at C-13. The geminal coupling of the C-14 protons was easily established since their chemical shifts were widely separated (see Table 1).

Subcutaneous injection of lophotoxin in mice was lethal (the 50 percent lethal dose being 8.0 μg/g). Death was preceded by ataxia, paralysis, and severe respiratory depression. Experiments in the in vitro rat phrenic nerve-hemidiaphram preparation demonstrated that lophotoxin irreversibly inhibits nerve-stimulated contraction without affecting contraction evoked by direct electrical stimulation of the muscle. Such findings are usually indicative of an action at the neuromuscular junction. Neuromuscular blockade was observed with bath concentrations of lophotoxin as low as 8×10^{-8} M. Both the kinetics and irreversibility of lophotoxin resemble those of α-bungarotoxin, a paralytic component of the venom of the Southeast Asian snake *Bungarus multicinctus*.

The epoxylactone and furanoaldehyde functionalities of lophotoxin are found separately only in a few other natural products, and these groups have not previously been observed in marine natural products. Both functional groups may be important for the pharmacological activity of lophotoxin. The carbomethoxy derivative **3** shows only weak to moderate toxicity, and pukalide (**2**), which lacks the epoxylactone functionality, shows almost no activity. Other α,β-epoxy-γ-lactone–containing natural products possess important biological activities. The previously mentioned lactone micromelin, isolated from the Asian evergreens *Micromelum integerrium* and *M. minutum*, for example, demonstrates significant activity against P-388 lymphocytic leukemia and Lewis lung carcinoma. The corresponding synthetic α,β-unsaturated γ-lactone derivative of micromelin showed no such activity (*6*). Picrotoxinin, an ichthyotoxin from the poisonous plant genus *Menispermum*, also possesses the epoxylactone functionality, and apparently acts by blocking γ-aminobutyric acid to produce central nervous system stimulation and convulsions (*11*).

The inference that epoxylactone and furanoaldehyde groups may be responsible for the potent biological properties of lophotoxin leads to speculation concerning their respective modes of action. It is clear that both functionalities could react with biological nucleophiles providing a site for highly selective alkylation.

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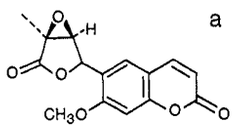
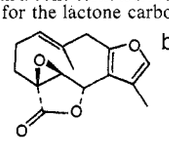
References and Notes

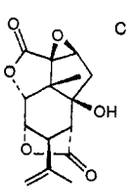
1. The toxicity of sea fans and whips, as it may relate to chemical defense, has recently been reported [G. J. Bakus, *Science* **211**, 497 (1981)]. For a complete description of the chemical components from gorgonian corals, see B. Tursch *et al.*, in *Marine Natural Products*, P. J. Scheuer, Ed. (Academic Press, New York, 1978), chap. 4, pp. 247–296.
2. Cytotoxicity was assayed against the fertilized egg of the Pacific Mexico–endemic urchin *Toxopneustes roseus* (Agassiz). The pharmacological aspects of this assay have been described [R. S. Jacobs, S. White, L. Wilson, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 26 (1981)]. Fish toxicity was tested with the commercially available damselfish *Eupomacentrus leucostictus*, and antibacterial assays were performed with the standard assay disc-agar plate method. Details of the fish toxicity assay have been described [V. J. Paul and W. Fenical, *J. Org. Chem.* **45**, 3401 (1981)].
3. We thank Dr. David Hardin for taxonomic assignments of Pacific gorgonians. *Lophogorgia* species were collected near Cabo San Lucas and along the offshore islands Isla Soccoro, Isla Clarion, and Islas Tres Marias, Mexico. The morphologically related gorgonian *L. chilensis* was collected in several locales near La Jolla, Calif.
4. A group of less toxic and structurally related compounds were also isolated from the various *Lophogorgia* species studied here (M. M. Bandurraga, R. K. Okuda, W. Fenical, in preparation).
5. M. G. Missakian, B. J. Burreson, P. J. Scheuer, *Tetrahedron* **31**, 2513 (1975). Since the preliminary publication of the structure of pukalide, an x-ray study has confirmed the proposed structure and provided the relative stereochemistry as shown in **2** (P. J. Scheuer and J. Clardy, personal communication).
6. J. M. Cassady, N. Ojima, C. J. Chang, J. L. McLaughlin, *Lloydia* **42**, 274 (1979). The α,β-epoxy-γ-lactone functional group in micromelin (**a**) gives rise to a carbonyl absorption at 1785 cm^{-1}.



7. B. S. Joshi, V. N. Kamat, T. R. Govindachari, *Tetrahedron* **23**, 261 (1967). Chromous chloride reduction of linderane (**b**) yielded an unsaturated lactone with a consistent infrared shift of 1770 to 1747 cm^{-1} for the lactone carbonyl.



8. E. J. Corey, N. W. Gilman, B. E. Ganem, *J. Am. Chem. Soc.* **90**, 5616 (1968).
9. R. K. Boeckman, Jr., and E. W. Thomas, *ibid.* **101**, 987 (1979).
10. On the basis of molecular models, both pukalide and lophotoxin appear to be highly conformationally rigid molecules. The proposed "up" epoxide isomer appears to be free of steric interactions, while the "down" isomer would experience severe steric crowding from the methyl group at C-8.
11. The structure of picrotoxinin has been described as (**c**). See L. A. Porter, *Chem. Rev.* **67**, 441 (1967).



12. N. S. Bhacca, F. W. Wehrli, N. K. Fischer, *J. Org. Chem.* **38**, 3618 (1973).

13. The R.V. *Alpha Helix* was supported by NSF grant OCE-750-93824 to W.F. The structural chemical and pharmacological studies of lophotoxin were supported by NOAA, Office of Sea Grant, Department of Commerce, under grant 04-8-MOI-189 to the University of California (projects R/MP-15 and R/MP-18).

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Metastatic Potential Is Positively Correlated with Cell Surface Sialylation of Cultured Murine Tumor Cell Lines

Abstract. *The ability of murine tumor cells to metastasize spontaneously from subcutaneous sites is positively correlated with the total sialic acid content of the cells in culture, the degree to which the sialic acid is exposed on the tumor cell surface, and, most strongly, with the degree of sialylation of galactosyl and N-acetylgalactosaminyl residues in cell surface glycoconjugates. These findings suggest that sialic acid on the cell surface may play a role in tumor cell metastasis.*

Much attention has been devoted to studying alterations in cell surface carbohydrates in neoplastic transformation (*1*). Particular interest has been focused on changes in the composition and metabolism of sialic acid (*N*-acetylneuraminic acid) in transformed cells (*1–8*). No consistent patterns have so far emerged (*1*); sialic acid levels (*2–4*), sialylated membrane components (*5*, *6*), and sialyl transferase enzymes (*4*, *5*, *7*, *8*) in transformed cells have been found to be increased in some studies (*2*, *5–8*) and decreased in others (*3*, *4*).

Several studies have suggested, however, that cell surface sialic acid might play a role in determining the metastatic properties of neoplastic cells. Sialic acid has been implicated in determining cellular adhesiveness (*9*), the ability of intravenously injected cells to implant in various organs (*10*), cellular motility and invasiveness in vitro (*11*), and immunogenicity (*12*). Each of these properties has been linked to the metastatic behavior of cells (*13*). Previous studies with variants of the B16 mouse melanoma that differ in lung implantation properties after intravenous injection showed higher levels of neuraminidase-releasable cell surface sialic acid on cells from the cell line with enhanced ability to implant in lungs (*14*, *15*). Prompted by these findings, we examined the sialic acid content and cell surface sialylation of a variety of murine tumor cell lines of diverse types which vary in their spontaneous metastatic properties (*16–19*).

The cultured cell lines we used include spontaneously transformed, DNA and RNA virus–transformed, and chemically transformed cells derived from (or producing in vivo) carcinomas, sarcomas, and melanomas (Fig. 1, legend). The metastatic properties of the cell lines were defined by determining the percentage of animals developing spontaneous metastases to any site after the excision of a primary subcutaneous tumor (*18*, *20*). Tumors were excised at sizes ranging from 2 to 6 g for rat tumors and 1 to 3 g for mouse tumors; animals were killed either when obvious metastases developed or after prolonged observation (5 to 17 months after excision in rats, 4 months in mice). One to five experiments were performed for each cell line, with 5 to 65 animals tested per line and with reproducible results from experiment to experiment (*18*, *20*). The cell lines exhibited a wide range of metastatic behavior, with the overall incidence of metastasis ranging from 0 to 100 percent (Fig. 1). Three nontumorigenic cell lines were also studied.

Using cells that were within several tissue culture passages of those used in the in vivo experiments, we examined three aspects of the sialylation of cultured cells: (i) the total sialic acid content of the cells (Fig. 1A), (ii) the degree to which metabolically labeled sialic acid was exposed to neuraminidase on the cell surface (Fig. 1B), and (iii) the extent to which galactosyl (Gal) and *N*-acetylgalactosaminyl (GalNAc) residues on cell surface glycoconjugates were substituted with sialic acid (Fig. 1C). All three of these parameters correlated positively with the spontaneous metastatic potential of the cells. The best correlate of metastatic potential was the degree of sialylation of cell surface Gal and GalNAc.

The sialic acid contents of the ganglioside and glycoprotein fractions of cultured cells from 23 of the cell lines were determined separately by resorcinol and thiobarbituric acid assays (*17*), and the values were combined to give the total cellular content of sialic acid. The total sialic acid content of the cultured cells correlated significantly with their metastatic potential (Fig. 1A). The average sialic acid content of high metastatic cells (> 50 percent of incidence of metastasis) was 1.4-fold greater than the average sialic acid content of low metastatic cells (< 50 percent incidence of metastasis).

The exposure of sialic acid on the surface of cultured cells from 29 of the cell lines was determined by metabolically labeling cells for 48 hours in medium containing *N*-acetyl-D-[6-^{3}H]mannosamine (1 μCi/ml) (29 Ci/mmole; New England Nuclear), which specifically labels sialic acid residues (*21*), and then incu-

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