

# Brasilenyne and *cis*-dihydrorhodophytin: Antifeedant medium-ring haloethers from a sea hare (*Aplysia brasiliana*)

(chemical ecology/plant secondary metabolites/acquired distastefulness/Mollusca)

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**ABSTRACT** Two straight-chain C<sub>15</sub> fish antifeedants have been isolated from the sea hare *Aplysia brasiliana*. Chemical, spectral, and x-ray diffraction studies led to the characterization of these medium-ring ethers as brasilenyne (2) and *cis*-dihydrorhodophytin (3). The oxonin ring system of 2 is novel in nature. Biosynthetic considerations permit the postulation that a third compound, a noncrystalline congener of these compounds, is *cis*-isodihydrorhodophytin (4).

*Aplysia brasiliana* is a large slug-like gastropod mollusk that lives in the Atlantic Ocean adjacent to the North American continent. Known as a sea hare, it is an algal browser and occasional swimmer that makes its way slowly and gracefully by rhythmic undulations of its wing-like parapodia. Incapable of evasive maneuvering, and seemingly defenseless, the animal gives every indication of being vulnerable to predation. Tests done by one of us (T.E.) with large sharks at the Mote Marine Laboratory (Sarasota, FL) showed *Aplysia* to be ignored by these predators. "Fooled" into taking morsels consisting of *Aplysia* pieces sandwiched between fish filets (*Euthynnus alletteratus*), the sharks retained the filets but invariably spat out the *Aplysia* chunks. Smaller teleost fish (*Cyprinodon variegatus*, *Floridichthys carpio*, and *Fundulus grandis*) also rejected *Aplysia* portions when tested in aquaria. All sections of the body wall proved to be unacceptable (including pieces of the mantle and parapodia), as did the so-called digestive gland. Other internal organs were variably palatable, and one, the muscular buccal mass, was consistently eaten. Subsequent efforts were aimed at isolating and identifying the unpalatable factors in *A. brasiliana*. Three compounds had previously been characterized, including a bromoallene (1) and two brominated eudesmanes (2). We here report on the structures of three halogenated cyclic ethers that we isolated from the digestive gland of *A. brasiliana*.

## OBSERVATIONS AND DISCUSSION

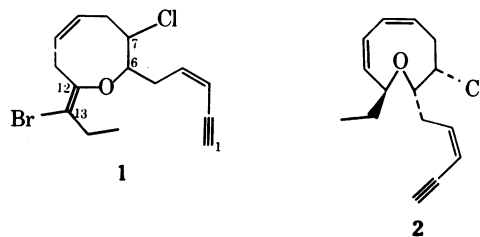
Two *Aplysia* digestive glands (15.4 g, wet weight) were ground in a Waring Blendor under ether/methylene chloride 1:1 (vol/vol). Fractionation of the crude extract by gradient column chromatography [silica gel, hexane to 50% (vol/vol) hexane/benzene] afforded two major fractions. Further purification by either reverse-phase high-pressure liquid chromatography or multiple development thin-layer chromatography provided pure samples of three compounds (94, 130, and 40 mg, respectively).

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## Brasilenyne

The first of these, brasilenyne, could be crystallized from pentane at low temperatures [mp 37–38°C;  $[\alpha]_D^{21} + 216^\circ$  ( $c = 0.017$  in CHCl<sub>3</sub>)]. Combustion analysis (*Anal.* Calcd for C<sub>15</sub>H<sub>19</sub>ClO: C, 71.71; H, 7.57; Cl, 14.34. Found: C, 71.75; H, 7.89; Cl, 14.42), indicated a molecular formula of C<sub>15</sub>H<sub>19</sub>ClO. Support for this molecular formula was provided by the low-resolution mass spectrum, which exhibited a weak molecular ion at *m/e* 250 and an M<sup>+</sup> – Cl peak at *m/e* 215 shown to have the composition C<sub>15</sub>H<sub>19</sub>O by high-resolution mass spectrometry. Neither carbonyl nor hydroxyl absorptions were visible in its infrared spectrum. Intense absorptions at 1080 and 1108 and at 3300 cm<sup>-1</sup> suggested the presence of an ether and a terminal acetylene function. An ultraviolet maximum at 224 nm ( $\epsilon = 14,000$ ) with a shoulder at 233 nm indicated the presence of a terminal enyne chromophore (3).

Carbon-13 magnetic resonance spectroscopy (CMR) (using broad-band and single-frequency off-resonance proton decoupling experiments) showed 15 resonances corresponding to six olefinic carbon atoms [141.2 (d), 134.2 (d), 133.3 (d), 129.5 (d), 128.6 (d), and 110.4 (d) ppm], a pair of terminal acetylenic carbon atoms [82.0 (d), and 80.1 (d) ppm], two ether carbon atoms [76.0 (d), and 75.6 (d) ppm], a carbon atom bearing chlorine [62.9 (d) ppm], three methylene carbon atoms [36.3 (t), 35.9 (t), and 28.5 (t) ppm], and a single methyl carbon atom [9.94 (q) ppm]. From these data, brasilenyne appeared to be a C<sub>15</sub> fatty acid-derived cyclic ether related to the recently characterized (4)<sup>§</sup> algal metabolite rhodophytin (1).



Catalytic hydrogenation (Pt, diethyl ether) of brasilenyne yielded a single decahydro derivative, decahydrobrasilenyne (M<sup>+</sup>, *m/e* 260, 262; C<sub>15</sub>H<sub>29</sub>ClO). The observation of significant M<sup>+</sup> – C<sub>2</sub>H<sub>5</sub> peaks in the mass spectra of both brasilenyne and its decahydro derivative suggests C-13 (rather than C-12, as in

Abbreviations: CMR, <sup>13</sup>C magnetic resonance spectroscopy; PMR, proton magnetic resonance.

<sup>§</sup> For revised structure, see Howard, B. M., Fenical, W., Hirotsu, K., Solheim, B. & Clardy, J. (1979) *Tetrahedron*, in press.

Table 1. 220-MHz PMR data obtained for brasilenyne in three solvents

Carbon	CCl <sub>4</sub>	C <sup>2</sup> HCl <sub>3</sub>	C <sub>6</sub> <sup>2</sup> H <sub>6</sub>	Coupling, Hz
1	3.00	3.14	≈2.7	d, J = 2
3	≈6.0	≈6.0	≈5.8	dd, J = 11, 2
4	5.74	5.82	5.66	ddd, J = 11, 8, 8
5a	≈2.5	≈2.6	2.38	ddd, J = 14, 8, 8
5b	2.36	2.50	2.06	ddd, J = 14, 8, 3
6	4.05	4.09	3.80	ddd, J = 8, 3, 1
7	3.76	3.91	3.65	ddd, J = 7, 7, 1
8a, 8b	≈2.5	≈2.7	≈2.7	m
9	≈6.04	≈6.09	≈5.8	m
10	≈5.5	≈5.5	5.41	dd, J = 11, 2
11	6.16	6.24	≈5.8	ddd, J = 10, 2, 1
12	≈5.5	≈5.5	5.27	dd, J = 10, 8
13	4.27	4.29	4.42	dddd, J = 8, 7, 7, 1
14a	1.61	1.73	1.65	dddd, J = 14, 7, 7, 7
14b	1.44	1.55	1.41	dddd, J = 14, 7, 7, 7
15	0.87	0.91	0.87	dd, J = 7, 7

All resonances are reported in ppm relative to internal tetramethylsilane and are listed sequentially along the carbon chain, beginning at the terminal acetylenic carbon atom.

1) as one terminus of the ether link. Facile loss of C<sub>5</sub>H<sub>5</sub> from brasilenyne and of C<sub>5</sub>H<sub>11</sub> from decahydrobrasilenyne corroborates the attachment of a C<sub>5</sub> substituent to the C-6 ether carbon, as in 1. These mass spectral data and the six degrees of unsaturation require the presence of an unusual oxonin ring skeleton.

Brasilenyne could be assigned structure 2 on the basis of a detailed analysis of its proton magnetic resonance (PMR) spectrum. Although certain bands overlap in chloroform, the use of two other solvents resulted in their resolution (Table 1). Spin decoupling experiments performed on sequential resonances permitted complete analysis of the spectrum. These decoupling experiments revealed the presence of a homoannular diene system from C-9 through C-12. This structure was confirmed, and its relative and absolute stereochemistry was established, on the basis of an x-ray diffraction study.

Brasilenyne crystallized in the orthorhombic crystal system with  $a = 17.083(6)$ ,  $b = 5.223(3)$ , and  $c = 16.111(6)$  Å. Systematic extinctions uniquely indicated space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, and the density indicated one molecule of C<sub>15</sub>H<sub>19</sub>ClO per asymmetric unit. All unique diffraction maxima with  $2\theta \leq 50^\circ$  were collected on a fully automated four-circle diffractometer using graphite monochromated Mo K $\alpha$  (0.71069 Å) radiation and a variable-speed  $\omega$  scan. After correction for Lorentz, polarization, and background effects, 1612 (66%) of the 2445 reflections surveyed were judged observed [ $F_0^2 \geq 3\sigma(F_0^2)$ ].

The structure was solved routinely by using a multiresolution weighted tangent formula approach.<sup>†</sup> Full-matrix least-squares refinement with anisotropic nonhydrogen atoms, isotropic hydrogens, and anomalous dispersion corrections for Cl have converged to a standard crystallographic residual of 0.036 for the structure and 0.037 for the enantiomer (5). A computer-

<sup>†</sup> All crystallographic calculations were done on a Prime 400 computer operated by the Materials Science Center and the Department of Chemistry, Cornell University. The principal programs used were: REDUCE and UNIQUE (data reduction programs, M. E. Leonowicz, Cornell University, 1978); BLS (block-diagonal least-squares refinement, K. Hirotsu, Cornell University, 1978); ORFLS (modified) (full-matrix least-squares, W. R. Busing, K. O. Martin, and H. S. Levy, Oak Ridge, ORNL-TM-305); ORTEP (crystallographic illustration program, C. Johnson, Oak Ridge, ORNL-3794); BOND (structural parameters and errors, K. Hirotsu, Cornell University, 1978); Multan-76 (direct methods and fast Fourier transform, G. Germain, P. Main, and M. Woolfson, University of York).

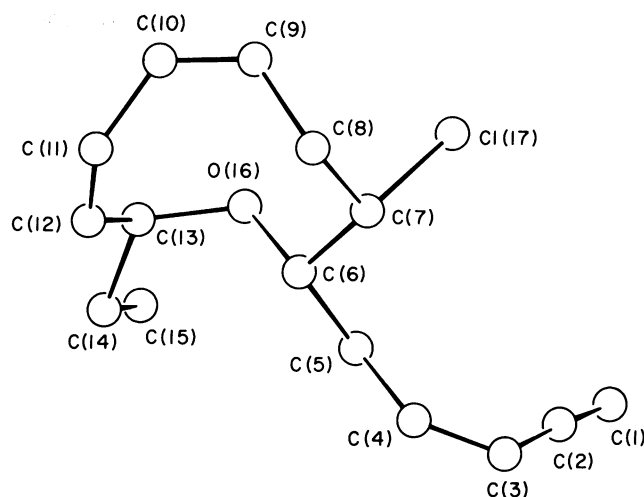


FIG. 1. Computer-generated perspective drawing from the crystal structure of brasilenyne (2); the hydrogen atoms are omitted for clarity. The absolute configuration was determined by anomalous dispersion corrections for the chlorine atom.

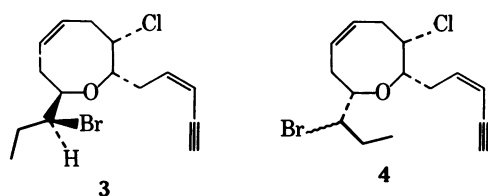
generated perspective drawing of the final x-ray model is given in Fig. 1. Bond distances and angles agreed well with generally accepted values, and there were no abnormally short intermolecular contacts.<sup>‡</sup>

### *cis*-Dihydrorhodophytin

The most abundant of the digestive gland components (mp 37–39°C;  $[\alpha]_D^{25} +74.8^\circ$ ) was shown to have a molecular formula of C<sub>15</sub>H<sub>20</sub>BrClO by high-resolution mass spectrometry ( $m/e$  330.0367, 332.0363, and 334.0323; calcd: 330.0368, 332.0367, and 334.0337). Facile loss of a C<sub>5</sub>H<sub>5</sub> unit ( $m/e$  265.0032 and 267.0005; calcd for C<sub>10</sub>H<sub>15</sub>BrClO: 264.9995 and 266.9976) revealed the presence of a pentenyl group attached at an ether carbon atom. The loss of a bromopropyl group ( $m/e$  209.0729 and 211.0693; calcd for C<sub>12</sub>H<sub>14</sub>ClO: 209.0733 and 211.0704), five degrees of unsaturation, and the presence of a pentenyl side chain require an oxocin skeleton reminiscent of rhodophytin (1). The infrared [3280 (s) and 2080 (w) cm<sup>-1</sup>] and ultraviolet [ $\lambda_{max}^{MeOH}$  224 nm ( $\epsilon$  11,600), sh at 233 nm] spectra of this compound confirmed the presence of a terminal enyne system. The CMR spectrum showed 15 resonances consisting of four olefinic carbon atoms [140.2 (d), 130.1 (d), 127.4 (d), and 111.3 (d) ppm], two acetylenic carbon atoms [82.6 (d) and 80.0 (s) ppm], two ether carbon atoms [79.3 (d) and 73.2 (d) ppm], two carbon atoms bearing halogen [64.1 (d) and 60.3 (d) ppm], four methylene carbon atoms [35.2 (t), 33.4 (t), 30.1 (t), and 30.0 (t) ppm], and a methyl carbon atom [12.5 (q) ppm]. The PMR spectrum\*\* of this major compound was not as easily resolved as that of brasilenyne, but identifiable patterns could be discerned at 1.15 (t, J = 7 Hz, 3H), 3.20 (d, J = 2 Hz, 1H), 4.24 (dd, J = 7 Hz, 1H), and 5.6 (dd, J = 10, 2 Hz, 1H) ppm. The spectroscopic data suggested a *cis*-dihydrorhodophytin structure (3) saturated at carbons 12 and 13. The structure and stereochemistry of *cis*-dihydrorhodophytin was established unambiguously by x-ray crystallography.

<sup>‡</sup> Tables of fractional coordinates, bond distances, bond angles, and observed and calculated structure factors are available from J. Clardy.

\*\* Spectral data for *cis*-dihydrorhodophytin: NMR (C<sup>2</sup>HCl<sub>3</sub>, 270 MHz)  $\delta$  1.15 (t, J = 7 Hz, 3H), 1.89 (m, 1H), 2.25 (m, 1H), 2.44–2.81 (m, 6H), 3.20 (d, J = 2 Hz, 1H), 3.93 (m, 1H), 4.06–4.16 (m, 2H), 4.24 (dd, J = 7, 7 Hz, 1H), 5.60 (dd, J = 10, 2 Hz, 1H), 5.77–5.95 (m, 2H), and 6.05 (dt, J = 10, 7 Hz, 1H).



*cis*-Dihydrorhodophytin crystallized in the orthorhombic crystal system with  $a = 10.307(4)$ ,  $b = 30.984(6)$ , and  $c = 4.997(1)$  Å. Systematic extinctions, the known chirality, and density considerations uniquely indicated  $P2_12_12_1$  as the space group with one molecule of  $C_{15}H_{20}BrClO$  per asymmetric unit.

All unique diffraction maxima with  $2\theta \leq 46.5^\circ$  were collected on a Syntex  $P2_1$  diffractometer with  $Mo\ K\alpha$  radiation (0.71069 Å) by using an  $\omega$  scan technique at a minimum scan rate of  $2^\circ/\text{min}$ . A total of 1846 reflections were measured and, after correction for Lorentz, polarization, and background effects, 1530 (83%) were considered observed [ $F_0^2 \geq 3\sigma(F_0^2)$ ]. Periodic examination of the standard reflections indicated a decrease in intensities to 60% of the original value by the end of data collection. The decay in intensity was linear during data collection and the data were scaled to the zero time intensities by a linear correction.

The bromine atom was located by means of a Patterson synthesis.<sup>†</sup> A subsequent bromine phased  $F_0$ -synthesis served to reveal the remaining nonhydrogen atoms. Theoretical positions for the hydrogens were computed and included for full-matrix least-squares refinement of the structure. All nonhydrogen atoms were assigned anisotropic thermal parameters; the hydrogens were assigned isotropic parameters. Anomalous dispersion corrections for the bromine atom brought the standard crystallographic residual to 0.058 for the structure and 0.077 for its enantiomer, a statistically significant difference (5).

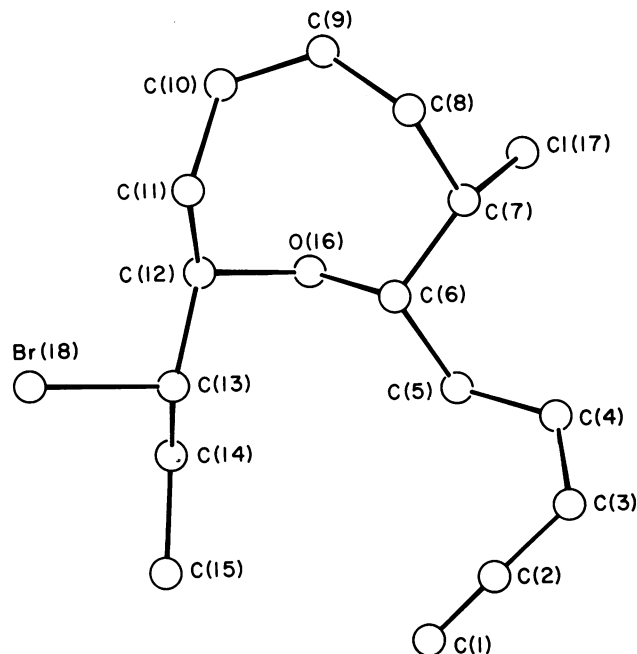


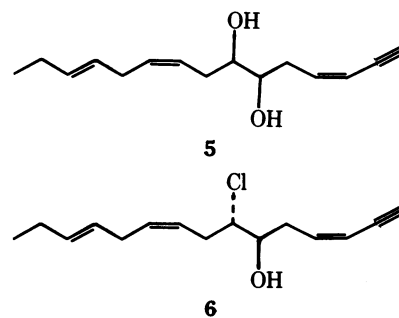
FIG. 2. Computer-generated perspective drawing from the crystal structure of *cis*-dihydrorhodophytin (3); the hydrogen atoms are omitted for clarity. The absolute configuration was determined by anomalous dispersion corrections for the bromine and chlorine atoms.

Fig. 2 is a computer-generated perspective drawing of *cis*-dihydrorhodophytin.<sup>††</sup> The stereochemistry is C(6) (*S*), C(7) (*S*), C(12) (*R*), and C(13) (*S*), which is consistent at C(6) and C(7) with that found for brasilenyne and for obtusenyne (12-bromo-11,12-dihydrobrasilenyne), recently isolated from *Laurencia obtusa* (6).

### *cis*-Isodihydrorhodophytin

The third compound was an oil,  $[\alpha]_D^{25} = 71.4^\circ$  ( $c = 0.0042$  in  $CHCl_3$ ); it was shown to have a molecular formula of  $C_{15}H_{20}BrClO$  by high-resolution mass spectrometry ( $m/e$  330.0391 and 332.0355; calcd: 330.0368 and 332.0367). The third peak of the isotopic cluster at  $m/e$  334 was too weak for mass measurement. The loss of  $C_5H_5$  ( $m/e$  265.0026, 266.9976, and 268.9946) and  $C_3H_6Br$  ( $m/e$  209.0731; calcd for  $C_{12}H_{14}^{35}ClO$ : 209.0733) moieties was again observed and suggested an oxocin ring skeleton.

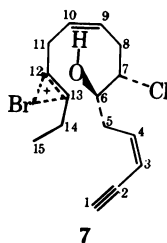
This minor compound, which we have named *cis*-isodihydrorhodophytin (4), appeared to be a stereoisomer of 3. Hydrogenation of 3 and 4 yielded two octahydro derivatives that differed in their physical (thin-layer-chromatography) and spectroscopic (infrared, NMR, CMR) properties. That 3 and 4 were not double bond isomers was evident from the PMR spectrum of 4.<sup>††</sup> Decoupling experiments permitted assignment of the protons of the cyclic double bond [5.50 (ddd,  $J = 7, 10, 10$  Hz, 1H) and 5.86 (ddd,  $J = 7, 10, 10$  Hz, 1H) ppm] and required the presence of adjacent methylene groups. In addition, the absorptions at 5.55 (dd,  $J = 10, 2$  Hz, 1H,  $-HC=CH-C\equiv CH$ ) and 3.21 (d,  $J = 2$  Hz, 1H,  $-C\equiv CH$ ) ppm indicated a *cis* double bond which was also evident from the CMR data.<sup>††</sup> The CMR spectra of 3 and 4 had common absorptions that were assigned to carbons 1–5 and 7–10 by using *cis*-rhodophytin and *cis*-chondriol as model systems (7). *cis*-Isodihydrorhodophytin therefore differed from *cis*-dihydrorhodophytin in its CMR spectrum only at carbons 6 and 12–15. The major differences lie in the bromopropyl side chain and are consistent with the upfield shift of the methyl carbon in 4 [10.4 (q) ppm], whose assignment is unequivocal, relative to that in 1 [12.8 (q) ppm] and 3 [12.5 (q) ppm]. These spectral data suggest that 3 and 4 are diastereomers differing in configuration at C-12 and possibly at C-13.



The formulation of *cis*-isodihydrorhodophytin as 4 is consistent with a plausible biosynthetic origin. Laurediol 5 (which

<sup>††</sup> Spectral data for *cis*-isodihydrorhodophytin: NMR ( $C^2HCl_3$ , 270 MHz)  $\delta$  1.06 (t,  $J = 7$  Hz, 3H), 1.97 (m, 1H), 2.19 (m, 1H), 2.43 (m, 1H), 2.68–3.29 (m, 5H), 3.21 (d,  $J = 2$  Hz, 1H), 3.73–3.92 (m, 4H), 5.50 (ddd,  $J = 10, 10, 7$  Hz, 1H), 5.57 (dd,  $J = 10, 2$  Hz, 1H), 5.86 (ddd,  $J = 10, 10, 7$  Hz, 1H), and 6.00 (dt,  $J = 10, 7$  Hz, 1H); CMR ( $C^2HCl_3$ , 20 MHz)  $\delta$  10.4 (q), 24.8 (t), 32.1 (t), 34.3 (t), 34.5 (t), 52.2 (d), 61.6 (d), 71.9 (d), 80.2 (s), 82.6 (d), 84.1 (d), 111.5 (d), 126.3 (d), 130.4 (d), and 140.1 (d).

occurs in nature as various stereoisomers) has been postulated as an intermediate in the biosynthesis of several halogenated derivatives (8). Bromonium ion-induced cyclization of the hypothetical C<sub>15</sub> precursor 6, a chlorine-containing analog of laurediol, could afford 2–4. Thus, addition of bromine to either face of the C-12 bond of 6 might produce two diastereomeric bromonium ions (7). An intramolecular nucleophilic displacement at C-13 in one of these (by the C-6 hydroxyl group), affords obtusenyne and, after dehydrobromination, brasilenyne. Similar displacements at C-12 would afford diastereomers 3 and 4 directly. Although speculative, this postulate accounts for the formation of the observed compounds and is strengthened by the finding that brasilenyne, *cis*-dihydrorhodophytin, and obtusenyne have the same stereochemistry at C-6 and C-7.



The two compounds available in sufficient amounts for testing, brasilenyne and *cis*-dihydrorhodophytin, were shown to be antifeedants in bioassays with swordtail fish (*Xiphophorus helleri*). Samples of the compounds were applied topically in methylene chloride solution to small beetle larvae (*Tribolium confusum*; mean weight = 0.8 mg) and these were offered, together with controls (larvae treated with methylene chloride only), to groups of swordtails in aquaria. Whereas the controls were taken and promptly swallowed, treated items were either rejected outright or were “mouthed” for some seconds before being swallowed or rejected. “Mouthing” is a behavior that we have found to be typical of many fish, who take distasteful items into the buccal cavity and subject them to an apparent intra-oral rinsing action, effected by pumping water in and out through mouth and gills. The response can be timed and provides a measure of the antifeedant potency of a given dosage applied to a morsel. In swordtail tests, mouthing was elicited in significant durations (1–2 sec) by as little as 0.1 μg of brasilenyne and 0.3 μg of *cis*-dihydrorhodophytin.

If the halogenated cyclic ethers were indeed real contributors to the distastefulness of *Aplysia*, they would be expected to be present not only in the digestive gland of the animal but also in its body wall, and absent from the buccal mass. This was

confirmed for several specimens of *Aplysia* by gas chromatographic analysis. Only the buccal mass consistently lacked these compounds. Their concentration in parapodia and other portions of the body wall (that is, in those parts of *Aplysia* first tasted by a predator) was found to be variable but within limits that ranged upward (0.3 μg/mg wet weight of tissue) to encompass levels of proven detergency in the swordtail tests. Of course, overall distastefulness of an *Aplysia* tissue is likely to be attributable not only to these cyclic ethers but also to a whole complement of substances, including the other known halogenated compounds (1, 2).

There appears to be little doubt that the halogenated compounds of *A. brasiliensis*, and presumably those of other species of *Aplysia*, are sequestered by the animals from the algae that they eat (9). We have obtained gas chromatographic evidence for the presence of compounds 2, 3, and 4 in extracts of an alga known to coexist with *Aplysia* along the Florida Gulf coast and readily consumed by the animal in captivity (John A. Paige, personal communication).

The predation tests with sharks as well as the initial bioassays were done at the Mote Marine Laboratory (Sarasota, FL); we thank its director, Dr. Perry Gilbert, and Ms. Patricia Bird for hospitality and help. We thank John A. Paige for providing several *Aplysia*. We are indebted to the National Institutes of Health [Grants AI-12020 and PR 00798 (Southern New England High Field NMR Facility) to J.M. and CA-24487 to J.C.], the National Science Foundation (Oceanography Section, Grant OCE 78-17202 to W.F.), and the American Cancer Society (Fellowship PF-1549 to R.K.D.) for partial support of this research. A Faculty Fellowship granted to R.B.K. by Hamilton College is also acknowledged with gratitude. T.E. acknowledges the support of the National Institutes of Health (Grant AI-02908) and the National Science Foundation (Grant PCM-15914).

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