source of biogenic volatile sulfur compounds to the marine atmosphere. Its quantitative importance becomes even more impressive when the DMS emissions from continental sources are added to our estimate of emissions from the sea surface. Adams *et al.* (*30*) have measured the flux of various reduced volatile sulfur compounds from a large variety of ecozones in the United States. From these data, they have extrapolated a global flux estimate of 64×10^{12} g of sulfur per year, of which the average DMS contribution is 21 percent. The extrapolation of the data from the temperate into the tropical zone introduces a substantial amount of uncertainty; further research on sulfur gas emissions in the biologically highly active tropical regions is needed to improve the terrestrial flux estimate. When the estimate of Adams *et al.* (*30*) is added to the marine flux, a DMS flux of 52×10^{12} g year^{-1} and a total biogenic sulfur flux of 103×10^{12} g year^{-1} is predicted, about equal to the anthropogenic SO_2 flux of 104×10^{12} g of sulfur per year (*2*).

MEINRAT O. ANDREAE
Department of Oceanography,
Florida State University,
Tallahassee 32306

HANS RAEMDONCK
Instituut voor Nucleaire
Wetenschappen, Rijksuniversiteit,
9000 Gent, Belgium

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31. We thank the captain and crew of the R.V. *Conrad* and the chief scientists (P. N. Froelich and W. C. Burnett) for their cooperation. We thank T. Engstrom, who performed the chlorophyll determinations; R. Ferek, who collected some of the atmospheric samples; and M. Dancy, who helped with the preparation of the manuscript and figures. One of us (H.R.) is indebted to the Belgian "Nationaal Fonds voor Wetenschappelijk Onderzoek" for travel and research support. This work was supported in part by grants from the National Science Foundation (ATM-8017574) and the North Atlantic Treaty Organization [SA.9-6-02(SRG.20)-83(81)CS].

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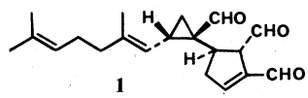
Isolation of Halimedatrial: Chemical Defense Adaptation in the Calcareous Reef-Building Alga *Halimeda*

Abstract. *Halimedatrial, a structurally unprecedented diterpenoid trialdehyde, has been identified as the major secondary metabolite in six species of the calcareous reef-building alga* Halimeda. *In laboratory bioassays, halimedatrial is toxic toward reef fishes, significantly reduces feeding in herbivorous fishes, and has cytotoxic and antimicrobial activities. The widespread occurrence of halimedatrial and its potent biological activities suggest that this metabolite represents a chemical defense adaptation in this pantropical marine alga.*

Calcareous algae of the genus *Halimeda* Lamouroux (Chlorophyta, Udoteaceae) are abundant and widely distributed in tropical marine habitats (*1*). Because of their high calcium carbonate composition (between 50 to 90 percent, dry weight), primary productivity, substrate stabilization, and provision for microhabitats, *Halimeda* species are considered major contributors to the structure of coral reefs (*1–3*).

In many reef systems, *Halimeda* species are most abundant in biomass among the macroalgae exposed to herbivory. The generally low overall algal abundance on tropical reefs has been attributed to the intense grazing activities of herbivorous fishes and sea urchins (*4–8*). Two reports indicate that *Halimeda incrassata* was consumed but not preferred by fishes under certain experimental conditions (*8, 9*). However, our own observations, feeding preference studies, and stomach content analyses indicate that *Halimeda* species are largely avoided by generalist herbivores (*4, 7, 10–16*).

While the basis for the successful adaptation of *Halimeda* species has not been defined, it has been generally accepted that calcification provides a physical deterrent against predation (*4, 5, 10, 17–19*). We here propose and provide evidence that the successful adaptation of *Halimeda* species involves multicomponent strategies of defense, including the protective function of naturally occurring chemical substances. Investigations of the secondary metabolites of numerous Caribbean *Halimeda* species have illustrated the production of a diterpenoid trialdehyde with high biological activity. This compound, halimedatrial (**1**), produces a wide spectrum of delete-



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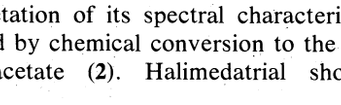
rious biological effects, and is structurally similar to numerous insect antifeedants such as warburganal (*20*) and the iridoid aldehydes (*21, 22*).

Collections made in the Bahama Islands of the widespread Caribbean *Halimeda* species—*H. tuna* (Ellis and Solander) Lamouroux, *H. opuntia* (Linnaeus) Lamouroux, *H. incrassata* (Ellis and Solander) Lamouroux, *H. simulans* Howe, *H. scabra* Howe, and *H. copiosa* Goreau and Graham—were found to contain significant amounts (~ 15 percent of the dichloromethane extracts), of halimedatrial (*23, 24*). The structure of this compound was determined by interpretation of its spectral characteristics and by chemical conversion to the triol triacetate (**2**). Halimedatrial showed



2

$[\alpha]_D^{27}$ of −59° (c = 0.9, $CHCl_3$), and could readily be assigned as a bicyclic diterpene trialdehyde by interpretation of its combined spectral features (*25*), particularly its mass spectral and high-resolution nuclear magnetic resonance characteristics. A molecular formula of $C_{20}H_{26}O_3$, reflecting eight degrees of unsaturation, was determined by high reso-

Table 1. ^{1}H and ^{13}C NMR spectral data for halimedatrial (**1**), recorded in the indicated solvents with tetramethylsilane as internal standard. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; α, protons below the plane of the molecule; β, protons above the plane; *J*, coupling constants in hertz; nOe, nuclear Overhauser enhancements; δ, chemical shift, M, multiplicity.

Carbon	^{1}H-NMR*				^{13}C-NMR†		
	δ	M	*J* (Hz)	nOe‡	δ	M	J_{C-H} (Hz)§
C-1	10.0	d	1	2	201.2‖	d	174
C-2	4.18	m		1, 6, 8α, 19	60.2	dd	135,25
C-3					131.3	s	
C-4	6.96	d	2	5, 16	155.7	d	166
C-5	2.70	m		4, 6	37.4	t	135
C-6	2.46	m	5, 5, 8, 2, 1	2, 5, 8α, 10	39.1	d	130
C-7					40.2	s	
C-8β	1.55	dd	9, 6	9	20.5	t	163
C-8α	1.23	dd	6, 5	2, 6, 10			
C-9	2.22	ddd	9, 8, 5	8β, 19	24.5	d	161
C-10	4.99	d	8	6, 8α	124.7¶	d	153
C-11					145.0#	s	
C-12	2.08	m			39.9	t	125
C-13	2.08	m			26.9	t	125
C-14	5.04	m			120.8¶	d	156
C-15					141.3#	s	
C-16	1.72‖	s			17.7	q	126
C-17	1.65‖	s			25.8	q	125
C-18	1.60‖	s			16.9	q	130
C-19	8.57	d	1.5		200.0‖	d	178
C-20	9.77	s			189.6	d	177

*Recorded at 360 MHz in $CDCl_3$ solution. †Recorded at 50 MHz in $(CH_3)_2CO$-d_6 solution; multiplicities were determined by off-resonance decoupling techniques. ‡Indicates proton enhancements observed when protons were irradiated at that carbon atom. §Natural proton coupling constants as determined by gated decoupling techniques. ‖,¶,#Assignments may be reversed.

lution mass measurement of the molecular ion at 314.1864 amu. Mass spectral fragmentation for the loss of a $C_{10}H_{15}$ unit, in combination with the appropriate ^{13}C-NMR (nuclear magnetic resonance) signals, showed that halimedatrial possessed a linear terpene component structurally analogous to geraniol. The remaining ten carbons were then assigned to the aldehyde functional groups, and to the cyclopropane and cyclopentene rings. The presence of a trisubstituted cyclopropane ring was indicated in the ^{13}C-NMR features of **1** by two high-field multiplets [20.5 (t) and 24.5 (d)], which showed characteristic large natural proton couplings (Table 1).

The complete assignment of halimedatrial was accomplished by proton-decoupling experiments, by ^{13}C-NMR measurements, and by proton nuclear Overhauser enhancement difference spectroscopy (nOeds) (Table 1). Assignments of all protons and their mutual couplings, and the majority of the carbon shift assignments were made on the basis of these studies. The relative stereochemistry of halimedatrial was assigned based on the nuclear Overhauser enhancement results in conjunction with appropriate molecular models. Sodium borohydride reduction of **1**, followed by acetylation, yielded triacetate **2** which, through extensive spectral analysis, confirmed these latter structural assignments (*26*).

Like other molecules with multiple aldehyde functionalities, halimedatrial shows diverse and potent biological effects which were measured with the use of known algal predators and potential pathogenic epibionts in laboratory bioassays. At standard bioassay concentrations of 100 μg per disk (agar plate method), halimedatrial showed antimicrobial activity toward a variety of marine microorganisms, including the common bacteria *Serratia marinorubra*, *Vibrio splendida*, *V. leiognathi*, and *V. harveyi*. Halimedatrial was also highly inhibitory toward a marine bacterium (VJP Cal 8101) and a gray fungus (VJP Cal 8104), isolated from the surfaces of Caribbean marine algae. At concentrations of 1 μg/ml in seawater, halimedatrial completely inhibited cell division for the first cleavage of fertilized sea urchin eggs (*27*). At 1 μg/ml, halimedatrial completely inhibited the motility of sea urchin sperm (*Lytechinus pictus* Verril). To assess the effects of halimedatrial on herbivorous fishes, both its toxic and feeding deterrent effects were measured. The damselfishes *Eupomacentrus planifrons* and *Dascyllus aruanus* died in 1 hour at a dose of 5 μg/ml (N = 5). Halimedatrial also produced significant feeding inhibition in herbivorous damselfishes. Halimedatrial, at concentrations found in nature (*28*) was added to suitable food pellets and the pellets were offered to *E. planifrons*. The number of bites taken of treated and control samples were compared by the Mann-Whitney test, [N (treated), 33; N (control), 36; P = 0.01].

The diverse biological properties of halimedatrial, and its chemical similarities to other biologically active aldehydes suggest a role for this compound in chemical defense. The calcareous nature of *Halimeda* may be an effective deterrent to some predators, and also lower its nutritive value, digestibility, and accessibility. However, many tropical reef herbivores are well adapted for the consumption of calcareous material (*7, 29, 30*). Fishes of the family Scaridae (parrotfishes), for example, are an abundant group of tropical herbivores that have a highly evolved pharyngeal mill requiring 50 to 90 percent calcareous material to aid in grinding algae (*7, 29, 30*). The lack of stomach acid in these fishes (*p*H, 7 to 8) further indicates their specialization. Given the abundance of *Halimeda* species along with the intense grazing pressures noted, and especially considering the capacities of many predators to utilize calcareous prey, the successful survival of *Halimeda* species would appear to depend at least in part on chemical measures of defense, including a significant function for the biologically active terpenoid, halimedatrial.

VALERIE J. PAUL
WILLIAM FENICAL
Institute of Marine Resources, Scripps Institution of Oceanography, La Jolla, California 92093

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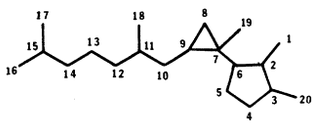
23. These research activities were performed from the University of Miami's R.V. *Calanus* in numerous habitats of the Bahama Islands. *Calanus* operations were supported by the National Science Foundation.
24. Extracts of two *Halimeda* species, less common in our study sites in the Bahamas, *H. goreauii* Taylor and *H. lacrimosa* var. *globosa* Dawes, contained little if any halimedatrial but did contain new, as yet unknown, terpenoids.
25. Halimedatrial showed infrared absorptions at 1720, 1690, and 1657 cm^{-1} for the saturated aldehyde, cyclopropyl aldehyde, and unsaturated aldehyde carbonyl groups. Ultraviolet absorbance at 244 nm (ϵ = 11,400) confirmed the latter infrared band to correspond to an α,β-unsaturated aldehyde. We suggest the name "halimedane" to describe this new diterpene ring system, and the numbering sequence provided in **3**.



26. The spectral features of triacetate **2** were recorded as follows: $[\alpha]_D^{27} = +13.3°$ (c = 0.9, $CHCl_3$); mass spectrum: M^+ −HOAc m/z 386.2447, M^+ −2 HOAc m/z 326.2235, M^+ −3 HOAc. m/z 266.2028, IR: 2990, 1720, 1450, 1390, 1240 cm^{-1}, ^1H NMR (360 MHz, benzene-d_6): δ 5.55 (1 H, bs), 5.18 (1 H, t, J = 7), 4.99 (1 H, d, J = 8), 4.64 (2 H, s), 4.10 (1 H, dd, J = 12, 6), 4.0 (1 H, dd, J = 12, 6), 4.0 (1 H, d, J = 15), 3.85 (1 H, d, J = 15), 3.08 (1 H, m), 2.42 (1 H, dd, J = 15, 9), 2.23 (1 H, dd, J = 15, 2), 2.13 (1 H, t, J = 7), 2.07 (1 H, m), 1.78 (1 H, dd, J = 9, 4), 1.76 (3 H, s), 1.70 (3 H, s), 1.67 (3 H, s), 1.67 (3 H, s), 1.61 (3 H, s), 1.55 (3 H, s), 1.50 (1 H, m), 0.83 (1 H, dd, J = 9), 0.44 (1 H, dd, J = 5), ^{13}C-NMR (50 MHz, $CDCl_3$): δ 171.0 (s), 170.9 (s), 170.7 (s), 138.7 (s), 138.0 (s), 131.4 (s), 131.0 (d), 124.0 (d), 122.4 (d), 69.0 (t), 66.0 (t), 62.0 (t), 48.9 (d), 45.0 (d), 39.5 (t), 34.9 (t), 28.7 (s), 26.5 (t), 25.9 (q), 21.8 (d), 3x 21.0 (q), 19.7 (t), 17.7 (q), 16.6 (q).
27. Pharmacological aspects of the fertilized sea urchin egg assay have recently appeared [R. S. Jacobs, S. White, L. Wilson, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 26 (1981)].
28. The concentration used, 2000 ppm, represents the average concentration of halimedatrial on a dry weight basis. Details of similar toxicity and feeding avoidance bioassays have been described [V. J. Paul, O. J. McConnell, W. Fenical, *J. Org. Chem.* **45**, 3401 (1980)].
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Free-Metal Ion Depletion by "Good's" Buffers

Abstract. *Metal-ion affinity (formation) constants were determined for two "Good's" buffers,* N-*tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and* N,N-*bis(2-hydroxyethyl)glycine (bicine). The metal chelates formed undergo loss of an internal ligand (alcohol) proton (bicine) and undergo hydrolysis (bicine and TES) and dimerization reactions (TES). Bicine and TES buffer not only hydrogen ions but also metal ions. The metal complexes of "Good's" buffers also buffer hydrogen ions by secondary reactions. The consequences of these reactions are considered in relation to biomedical research.*

In 1966, Good *et al.* reported (*1*) a new series of buffers for use in biological research. The buffers had several important properties including a pK_a (the negative logarithm of the acid dissociation constant) between 6 and 8, maximum water solubility with minimum solubility in other solvents, minimum salt effects, low ability to cross biological membranes, and supposedly low affinities for biologically important metal ions. It is the last of these properties that is of concern here.

The widespread use of "Good's" buffers in biological studies involving metal ions, for example, the calculation of protein-metal affinity constants, probably is in part responsible for the many conflicting data and conclusions derived by investigators studying identical metal cation–protein systems at the same pH in carefully executed experiments. For example, if one is studying the binding of Co^{2+} to an apoenzyme at pH 7.3 in an ADA {[(carbamoylmethyl)imino]diacetic acid} buffer, the actual reaction under consideration is

$$Co(ADA)_2^{2-} + \text{apoenzyme} \rightleftharpoons 2ADA^{2-} + \text{M-enzyme complex} \quad (1)$$

The same reaction studied in a BES {2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid} buffer is

$$Co(BES)^{+} + \text{apoenzyme} \rightleftharpoons BES^{-} + \text{M-enzyme complex} \quad (2)$$

Equations 1 and 2 will yield very different metal cation–protein affinity constants. This conclusion is based on metal-buffer stability constants and ligand–metal ion stoichiometries that have been determined in this laboratory. The metal cation–protein binding constants will be dependent on the buffer used. Correct affinity constant values for Eq. 3

$$M^{x+} + \text{apoenzyme} \overset{K_f}{\rightleftharpoons} \text{M-enzyme complex} \quad (3)$$

could be obtained in the presence of various buffers if the appropriate M-buffer formation constants (K_B) were known. From Eqs. 4 and 5,

$$M^{x+} + \text{buffer}^{y-} \overset{K_B}{\rightleftharpoons} M(\text{buffer})^{(x-y)+} \quad (4)$$

$$M(\text{buffer})^{(x-y)+} + \text{apoenzyme} \overset{K'_f}{\rightleftharpoons} \text{M-enzyme complex} + \text{buffer}^{y-} \quad (5)$$

(where K_B is the equilibrium constant of the buffer), it is obvious that

$$K_f = K'_f K_B \quad (6)$$

Furthermore, the metal ion interaction of various "Good's" buffers can be quite complex, and Eq. 6 represents only the most simple case, that is, no hydroxo complex formation (Eq. 7)

$$\text{M-buffer}^{x+} + OH^{-} \rightleftharpoons \text{M-buffer}\,(OH)^{(x-1)+} \quad (7)$$

no 2:1 or higher complex formation of buffer to metal ion (Eq. 8),

$$M(\text{buffer})^{x+} + \text{buffer}^{y-} \rightleftharpoons M(\text{buffer})_2^{(x-y)+} \quad (8)$$

and no ionization of "internal" buffer protons (Eq. 9)

$$M(\text{buffer})^{x+} \rightleftharpoons M(H_{-1}\ \text{buffer})^{(x-1)+} + H^{+} \quad (9)$$

We have obtained data (*2*) that reactions such as those in Eqs. 7, 8, and 9 do occur with "Good's" buffer-metal complexes in the pH range 6 to 9 commonly used in biological and medical research.

Table 1 presents metal ion affinity (formation) constants (*3*) for bicine [*N,N*-bis(2-hydroxyethyl)glycine] and TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], two of the "Good's" buffers. These buffers were chosen to emphasize that "Good's" compounds buffer H^+ concentration as well as metal ion concentrations and also that the resultant metal complexes buffer H^+ (Eqs. 7 and 9). Bicine forms 1:1 and 2:1 buffer–metal ion complexes with Cu(II), Co(II), and Zn(II), and only 1:1 complexes with Mn(II) and Ca(II). The bis(bicinato)copper(II) chelate undergoes a most remarkable reaction, dechelation of an aminoacidate group upon alcohol proton ionization (Eq. 10) (*4*)

$$Cu(\text{bicine})_2 \overset{K_{1a}}{\rightleftharpoons} Cu(H_{-1}\ \text{bicine})H_2O + \text{bicine}^{-1} + H^{+} \quad (10)$$

This reaction is also followed by deprotonation of the coordinated H_2O to produce the corresponding hydroxo complex (K_{OH}) (Eq. 11)

$$Cu(H_{-1}\ \text{bicine})H_2O \overset{K_{OH}}{\rightleftharpoons} Cu(H_{-1}\ \text{bicine})OH^{-} + H^{+} \quad (11)$$