brook); P. K. Freeman (Oregon State University); P. von. R. Schleyer (University of Erlangen); H.-D. Scharf (University of Bonn); R. Sauers (Rutgers University); G. Brieger (Oakland University, Michigan); T. Katz (Columbia University); M. Jones, Jr. (Princeton University); P. Lansbury (State University of New York at Buffalo); N. A. LeBel (Wayne State University); R. M. Magid (University of Tennessee); T. Sorensen (University of Calgary, Alberta); D. Farnum (Michigan State University); R. Malherbe (University of Lausanne); J. E. Lyons (Suntech Inc., Pennsylvania); D. Fărcaşiu (Exxon Research Laboratories, Linden, N.J.); J. M. Harris (University of Alabama); E. Kyba and M. J. S. Dewar (University of Texas at Austin); T. Sasaki (Nagoya University).

(7) The fourth letter of the Greek alphabet led to the convenient name *deltacyclane* for this key tetracyclic hydrocarbon, whose full IUPAC name is given in the Experimental Section. We avoided prefixes like *quad* or *tetra* to prevent confusion with the tetracyclic hydrocarbon, quadricyclane, and with the "tetracycline" class of antibiotics.
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Marine Natural Products: Halitoxin, Toxic Complex of Several Marine Sponges of the Genus *Haliclona*[1]

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A complex mixture of high molecular weight toxic pyridinium salts designated halitoxin has been isolated from the sponges *Haliclona rubens, H. viridis,* and *H. erina.* The toxin has been separated into molecular weight range fractions of 500–1000, 1000–25 000, and > 25 000, each of which shows the same spectral and biological properties. A general structure for halitoxin has been proposed based on ^{1}H and ^{13}C NMR analyses and identification of a group of 3-alkenylpyridines obtained in good yield upon pyrolysis of the toxin. The oligomeric/polymeric toxin consists of 3-alkylpyridine units connected by the nitrogen of one ring and the terminus of the 3-alkyl chain of the next. No functionality other than the pyridinium ring has been detected. Halitoxin is cytotoxic, haemolytic, and toxic to fish and mice.

Sponges from several species of the genus *Haliclona* have been reported to give extracts toxic to fish.[2] Baslow and Turlapaty[3] found that a crude aqueous extract of *H. viridis* was toxic to mice (LD_{50} ~ 275 mg/kg) and also inhibited the growth of Ehrlich ascites tumors. These authors coined the name halitoxin for this crude toxic extract but did not report any effort to isolate a pure toxin. In our ongoing search[4] for pharmacologically active compounds from marine organisms, we found that extracts of *H. rubens* are toxic to mice (LD_{50} ~ 7 mg/kg) and fish and cytotoxic in the National Cancer Institute's KB cell culture bioassay.[5] We also have found that other species of the genus *Haliclona* contain what appears to be the same toxin. However, not all of the *Haliclona* sp. examined yielded the toxin. In this paper we report the partial purification, spectral characterization, and chemical degradation which have lead to a proposed gross structure for halitoxin from four different *Haliclona* species.

The sponge we have studied most extensively is *Haliclona rubens,* a red tubular sponge commonly found in shallow (15 ft or less) reef waters of the Caribbean. Samples of the sponge for chemical work have been preserved in various ways: air-dried, freeze-dried shortly after collection, and preserved in alcohol. The method of preservation appears to have little effect on the character of the toxin isolated as judged by biological activity and spectral analysis. The toxin is obtained easily from the sponge preserved by any of the above methods.

Isolation and Purification of Halitoxin. Toxin was obtained from air-dried *H. rubens* by first defatting the ground specimens with chloroform and then extracting them continuously with methanol. After removal of most of the solvent, the methanol extract was dissolved in water and extracted several times with 1-butanol. The 1-butanol fractions contained virtually all of the toxin as determined by spectral

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analysis and bioassay.

Halitoxin was obtained from freeze-dried and alcohol-preserved specimens using a slightly different extraction sequence. Freeze-dried sponges were extracted with methanol continuously to give a crude extract. In the case of alcohol-preserved sponges, the crude extract was obtained by filtration and evaporation of the solvent. The alcohol extracts, diluted with water, were defatted by extraction with dichloromethane and then extracted several times with 1-butanol. The butanol fraction contained the toxin.

Further purification of the crude toxin has been accomplished most effectively and efficiently by membrane ultrafiltration of aqueous solutions. Low molecular weight materials were removed by the use of a 500 nominal molecular weight cutoff membrane. The <500 molecular weight materials were neither cytotoxic nor toxic to mice, and they did not show any of the spectral characteristics observed for the toxic fractions. The retentate was divided into molecular weight range fractions of 500–1000, 1000–25 000, and greater than 25 000 by successive ultrafiltrations using membranes with a larger pore size at each step. After lyophilization, these fractions were obtained as hygroscopic brown foams or glasses. The ^{1}H NMR spectra of the three different molecular weight range samples were virtually identical. The crude toxin in the 1-butanol extract, and all of the different molecular weight range fractions, exhibited the same cytotoxicity against the KB line of cancer cells (ED_{50} = 5–7 μg/mL). The LD_{50} in mice of the 1-butanol extract and of the 500–1000 molecular weight range fraction was ~5 mg/kg.

The crude toxin, a quaternary ammonium salt, also was purified via picrate formation. The picrate could not be recrystallized but was separated into acetone-soluble and -insoluble portions by repeated triturations with hot acetone. Halitoxin was recovered by dissolving the acetone-soluble picrate in an aqueous hydrochloric acid–acetone mixture and then extracting with ether to remove picric acid. This gave halitoxin in which the counterion was assumed to be exclusively chloride.

Some of the 500–1000 molecular weight range material was chromatographed on ion exchange columns in order to effect further purification and convert the toxin exclusively to the chloride form. The toxin was strongly retarded on a carboxylic acid resin and was eluted with 1 M sodium chloride. Anion exchange was also achieved by chromatography in water over the chloride form of a quaternary ammonium ion column. Comparison of the analytical data on crude, ultrafiltered, and ion exchange purified halitoxin indicated that chloride ion is the predominant, if not exclusive, counterion in the native toxin.

Of the numerous methods applied to attempt to purify halitoxin, ultrafiltration was found to be the most effective and efficient. Little additional purification was effected by chromatography subsequent to ultrafiltration. The alternate and supplementary means of purification consisted of chromatography using a variety of the common inorganic adsorbents, ion exchange resins, Sephadex gels, polypropylene powder,[6] 10% acetylated cellulose, and Carbowax-treated controlled pore glass beads.[7]

Characterization and Structure Determination of Halitoxin. Combustion analysis, spectral characterization, and chemical degradation of halitoxin have been carried out on toxin that has been purified in various ways: chromatography on silica gel, membrane filtration, ion exchange chromatography, and regeneration from the picrate complex. All of these materials exhibited virtually identical ^{1}H NMR spectra and the same degree of biological activity.

Combustion analysis of different preparations of halitoxin gave fairly consistent ratios for C, H, N, Cl and showed that small but variable amounts of phosphorus and sulfur were present. An empirical formula of $(C_{15}H_{24}NCl{\cdot}2H_2O)_n$ was reasonably consistent with the analytical data (see below) if sulfur and phosphorus are assumed to be due to impurities. That the samples contained water was corroborated by ^{1}H NMR analysis (see Figure 1). If the maximum sulfur and phosphorus values are included in the calculation, a minimum molecular weight of ~2300 is calculated. This is considerably greater than the 500–1000 molecular weight range assigned to one of the fractions on the basis of ultrafiltration behavior. While the inclusion of sulfur and/or phosphorus cannot rigorously be ruled out on the basis of the analytical data, these elements do appear to be, at the most, very minor components in halitoxin, and we believe they are due to impurities.

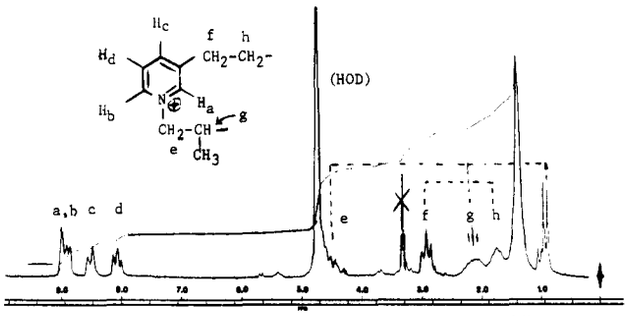


Figure 1. NMR spectrum of halitoxin (100 MHz, CD_3OD).

All of the spectral data characterized halitoxin as an alkylpyridinium salt without any other functionality. Halitoxin exhibited UV absorption characteristic[8] of an alkylpyridinium salt [λ_{max} 267 nm, 273 (sh), and 212], while the infrared spectrum showed absorption at 1640 cm^{-1}, typical[9] of such salts. The possibility that the IR absorption at 1640 cm^{-1} was due to an amide or any other type of carbonyl group was ruled out by ^{13}C NMR data; i.e., no peaks were observed below 146 ppm. Broad absorption was observed in the hydroxyl region of the infrared spectrum (centered at 3400 cm^{-1}), but this was attributed to water of hydration in the samples. The possibility that halitoxin possesses hydroxyl groups was eliminated by virtue of the fact that the toxin did not undergo acetylation or formylation under a variety of conditions and that the ^{1}H NMR and ^{13}C NMR spectra were devoid of the signals characteristic of alcohols.

The ^{1}H NMR spectrum (see Figure 1) provided convincing evidence for a 3-substituted pyridinium ring in halitoxin and also revealed many of the remaining structural features. Both the chemical shift and multiplicity of the aromatic protons, labeled a–d, match closely those of the model compounds, 1-isobutyl-3-methylpyridinium bromide (**2**) and 1-hexadecyl-3-methylpyridinium bromide (**3**) (see Experimental Section). The signal at δ 4.5, labeled e, largely obscured by the HOD peak, is attributed to the methylene group bonded to the quaternary nitrogen. Addition of a few drops of pyridine-d_5 shifted the HOD peak downfield slightly and revealed a complex multiplet at δ 4.5 corresponding to approximately two protons. Decoupling showed that these protons were coupled to the proton(s) absorbing at approximately δ 2.22 (protons g), which in turn were coupled to the methyl doublet at δ 0.92, thus demonstrating that a large percentage of the alkyl groups attached to nitrogen are methyl substituted β to nitrogen. The broad signal at δ 2.22 is resolved into two one-proton multiplets at 360 MHz (δ 2.06 and 2.22). Only one of these, δ 2.22, is coupled to both the methyl group at δ 0.92 and the methylene protons adjacent to the pyridinium nitrogen, again confirming that the predominant type of alkyl residue bonded to nitrogen is methyl branched, as indicated in Figure 1.

At 360 MHz, the protons labeled e are observed as two signals, δ 4.42 (~1 H) and 4.68 (~2 H). The δ 4.42 signal is a dd,

Table I. GC/MS Data[a] for 3-Alkenylpyridines from Halitoxin from *H. rubens*

GC fraction[b]	EI, *m/e* M^+ (%)	CI, *m/e* $(M+1)^+$ (%)	CI, *m/e* $(M+29)^+$ (%)	Formula/structure
A	189 (9)	190 (100)	218 (13)	$C_{13}H_{19}N$/**4**
B	203 (7)	204 (100)	232 (10)	$C_{14}H_{21}N$/**8**
C	203 (5)	204 (100)	232 (20)	$C_{14}H_{21}N$/**5**
D	217 (4)	218 (100)	246 (28)	$C_{15}H_{23}N$/**6**
E	231 (3)	232 (100)	260 (20)	$C_{16}H_{25}N$/**9**
F	231 (5)	232 (100)	260 (18)	$C_{16}H_{25}N$/**7**
G	243 (1)	244 (93)	272 (3)	$C_{17}H_{23}N$/**10**
		230 (100)	258 (13)	$C_{17}H_{21}N$/?
H	238 (2)	240 (56)	268 (12)	$C_{14}H_{22}HCl$/**11**
	240 (0.5)	242 (20)	270 (3)	
		204 (100)		
I	252 (0.5)	254 (60)	282 (7)	$C_{15}H_{24}NCl$/**12**
		256 (13)	284 (3)	
		218 (100)		
J	232 (8) ($M^+ - HCl$)	268 (100)	297 (1.5)	$C_{16}H_{28}NCl$/?
		270 (22)	298 (1.5)	
		232 (100)		

[a] A 6 ft × 1/8 in 10% Carbowax 20M/2% KOH column; programmed at 150–230 °C (2 °C/min). [b] See Table III.

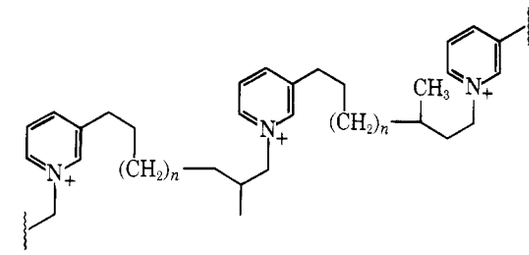
J = 8 and 13 Hz, in which the small coupling arises from interaction with the proton resonating at δ 2.22 and the large coupling is due to geminal coupling as expected for one of the diastereotopic protons at e. The larger signal at δ 4.68, a complex multiplet, appeared to be altered by irradiation at δ 2.22 and also at δ 2.06. Thus, the δ 4.68 signal is considered to arise from the remaining diastereotropic proton at e plus the methylene protons of some alkyl chains that are not branched at the β position (see degradation products below).

The small doublet at δ 1.04 was not collapsed by irradiation of any of the peaks downfield from the methylene envelope at δ 1.4, and hence it is presumably coupled to a proton in that envelope. The small difference in chemical shift between the δ 1.4 and 1.04 peaks precluded definitive verification of this point. By integration the small methyl doublet corresponds to slightly less than one-third the area of the larger doublet at δ 0.92.

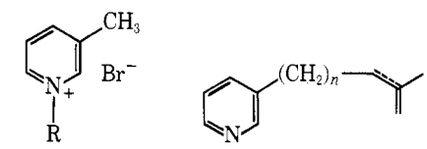
The multiplicity (triplet) of the two-proton, benzylic-type signal at δ 2.92 (coupled to δ 1.76) showed that the initial segment of the alkyl chain at C-3 of the pyridinium ring consists of two contiguous methylene groups. Combination of the foregoing data leads to the partial structure shown on Figure 1.

The absence of terminal methyl groups in halitoxin was indicated by the absence of the typical triplet in the region above 1.0 ppm in the 100 MHz spectrum, and this was confirmed by analysis at 360 MHz where the methyl doublet signals are well separated and no other absorptions are observed in this region. The ^1H NMR spectra also clearly show that there are no pyridine rings in which the nitrogen is not quaternized since even in the presence of base no peaks occur in the region where the aromatic protons of a free 3-alkylpyridine typically resonate. These facts suggest that the terminus of the alkyl chain at C-3 of one pyridinium ring is bonded to the nitrogen of another to give an oligomeric or polymeric structure as shown in formula **1**.

The ^{13}C NMR data are in agreement with the proposed structure. Five low-field carbon resonances are observed (δ 149.5, 144.6, 144.0, 142.4, and 128.5), and these coincide with those of the model salts **2** and **3**. The signal for the methylene carbon bonded to the quaternary nitrogen in halitoxin and **2** occurs at nearly the same position (δ 67.8 and 69.0), while the analogous carbon in **3** absorbs at slightly higher field (δ 6.19), as expected for a branched vs. straight chain structure.[10] The remainder of the ^{13}C NMR signals occurs at less than 36 ppm,

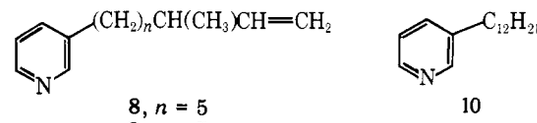


1 (n = 2, 3, 4, 5)



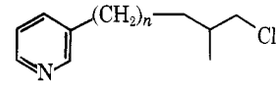
2, R = $CH_2CH(CH_3)_2$
3, R = $(CH_2)_{15}CH_3$

4, n = 4
5, n = 5
6, n = 6
7, n = 7



8, n = 5
9, n = 7

10



11, n = 5
12, n = 6

confirming that there are no carbons bearing hydroxyl groups in halitoxin.

Halitoxin was treated under a variety of conditions to probe for chemical functionality. Treatment with acetic anhydride in pyridine or triethylamine, with acetic anhydride/boron trifluoride etherate,[11] or with acetic–formic anhydride[12] did not cause any acetylation or formylation, and so provided chemical evidence for the lack of hydroxyl groups. Reaction with aqueous methanolic base (NaOH or K_2CO_3) produced intractable brownish gums, whereas the toxin was recovered unchanged after heating with aqueous methanolic hydrochloric acid. Halitoxin reacted with sodium borohydride or sodium dithionite, but gave unmanageable reduction products. Halitoxin readily formed precipitates with picric acid,

Table II. ^{1}H NMR Data for 3-Alkenylpyridines from Halitoxin from *H. rubens* Collected by GC

	GC fraction (Table III)								
	A	B	C	D	E	F	G	H	I
	Compound								
^{1}H NMR, δ	4/8	8	5	6	9	7	10/?	11	12
8.21, 7.36, 7.04 (pyr ring protons)	+	+	+	+	+	+	+	+	+
4.96 (t) ($-CH_2CH=C(CH_3)_2$)	+		+	+		+			
4.50 ($-C(CH_3)=CH_2$)									
4.86 (dd, J = 10, 1)	+	+			+		+		
4.89 (dd, J = 18, 1)									
5.6 (m) ($-CH_nCH=CH_2$)									
1.56 ($-C(CH_3)=CH_2$)	+		+	+		+			
1.46, 1.56 ($-CH=C(CH_3)_2$)									
0.96 ($-CH(CH_3)-$)	+	+			+			+	+
3.4 (dd, 6.1) ($-CH(CH_3)CH_2Cl$)								+	+

sodium tetraphenylborate,[13] or chloroplatinic acid. Of these salts, only the picrate was purified by redissolution, but that with considerable loss of material.

Dealkylation of halitoxin by heating with ethanolamine[14] was attempted but was unsuccessful under conditions which smoothly dealkylated a model compound, 3-carbamoyl-1-hexadecylpyridinium bromide. Attempts to break the quaternary ammonium structure via elimination were unsuccessful using alcoholic base, potassium *tert*-butoxide in dimethyl sulfoxide, diazabicyclooctane, and a conventional Hofmann elimination procedure.

Halitoxin was successfully degraded by pyrolysis. Heating at 140–160 °C decomposed the toxin and yielded a mixture of 3-alkenylpyridines and 3-(ω-chloroalkyl)pyridines, Tables I–III. The pyrolysis products were analyzed by combined gas chromatography–mass spectrometry (electron impact and chemical ionization) and also by ^{1}H NMR and MS analyses of fractions isolated by preparative gas chromatography. These results are summarized in Tables I and II.

The ^{1}H NMR spectrum of the residue from the pyrolysis is comparable to that of the toxin itself, except that the peaks are all very broad and the aromatic proton signals are shifted upfield slightly.

In the electron impact mass spectra, intense peaks were observed for all of the pyrolysis products at *m/e* 92, 93, and 106, indicative of a pyridine ring plus one and two methylene groups, respectively. One of these peaks was always the base peak. The electron impact spectra showed molecular ions for all of the components except those containing chlorine (peaks I and J). The chemical ionization spectra showed strong $(M + 1)^+$ and $(M + 29)^+$ peaks for all components including those containing chlorine.

The ^{1}H NMR spectra of fractions C, D, and F (Table II) clearly show that these samples are mixtures containing double-bond isomers, the difference occurring in the chain-terminating feature: isopropenyl vs. isopropylidene. Decoupling experiments on component C confirmed the olefinic methyl chemical shift assignments. This ^{1}H NMR data in conjunction with mass spectral analysis permits the assignment of the structures **5, 6,** and **7** to these fractions.

Fraction A contains the signals for the isopropenyl/isopropylidene mixture, and in addition it contains signals appropriate for a terminal vinyl group (δ 4.86, 4.89, and 5.6). Since the mass spectrum shows a predominant molecular ion at 189 and a minor one at 203, this fraction is judged to contain predominantly alkenylpyridine **4** with a little of compound **8.**

Components B and E show ^{1}H NMR signals for a secondary methyl and a terminal vinyl group. The pattern for the nonterminal olefinic proton closely resembles that of 3-methyl-1-butene and indicates that the methyl group is on the allylic carbon. With molecular weights of 203 and 231, components B and E are assigned structures **8** and **9,** respectively. It was not possible to assign the position of the methyl group conclusively on the basis of the mass fragmentation pattern.

Fraction G has not been identified conclusively. It is an alkenylpyridine with two degrees of unsaturation in its side chain.

The chemical ionization mass spectra show that peaks H, I, and J, contain chlorine. The ^{1}H NMR spectra of peaks H and I lack olefinic proton absorption, but they do have signals for a secondary methyl group and a methylene group deshielded by chlorine. These data and the molecular weights established by mass spectrometry suggest structures **11** and **12** for the components H and I. Only mass spectral data is available for fraction J, and this indicates a molecular formula corresponding to the hydrochloride of either **7** or **9.**

Since there were no olefinic proton or vinyl methyl signals in the ^{1}H NMR spectrum of halitoxin, the unsaturation sites in the 3-alkenylpyridines clearly mark the sites of the nitrogen–alkyl links in the toxin. Since halitoxin also lacks quaternary methyl and isopropyl groups (^{1}H NMR), all of the alkyl chains must be joined through a terminal methylene group to the nitrogen. The isopropylidene groups in the pyrolysis products probably arise by acid-catalyzed isomerization of the initially formed isopropenyl group, the acid coming from the elimination. This supposition was corroborated when it was observed that the product from pyrolysis of halitoxin in the presence of powdered potassium carbonate contained signals only for an isopropenyl group.

The chemical and spectral data indicate an oligomeric or polymeric structure for halitoxin as shown in **1.** We propose that the toxin is a complex mixture containing molecules of different sizes, and with random variation in the length and structure of the alkyl chains linking the pyridinium rings.

We feel that at least for the 500–1000 molecular weight range materials the evidence indicates an overall macrocyclic structure containing 4–6 alkylpyridine units. This conclusion is based on the following arguments. First, there is no evidence for any nonquaternized pyridine rings in the ^{1}H NMR spec-

Table III. % Composition of Halitoxin Pyrolysis Products by GC Analysis

Halitoxin sample	Fractions, % A	B	C	D	E	F	G	H	I	J	All others
H. rubens[a] (crude)	4 (15.8)[b]	6 (17.5)	26 (21)	18 (25.4)	9 (26.5)	7 (29.8)	~1 (38)	10 (40.5)	9 (47.5)	6 (55.5)	
H. rubens[a] (crude + K_2CO_3)	6	0.7	23	19	3	0.8	1	23	16	5	3
H. rubens[c] (500–1000)	0.7	1	15	13	3	1.6	2.6	27	14	10	13
H. rubens[c] (1000–25 000)	0.4	0.7	20	15	3	1	2.5	20	13	10	14
H. rubens[c] (>25 000)	0.4	1	18	15	3	2	2	22	16	10	11
H. erina[d]	10	10	33	24	1	1	1.3	5	14	3	

[a] A 6 ft × 1/8 in 10% FFAP on Chromosorb W, AW-DMCS, 60–80 mesh column; programmed at 170–230 °C (2 °C/min); initial flow rate, 20 mL/min. [b] Representative retention times in minutes. [c] A 6 ft × 1/8 in 3% OV-225 on Gas Chrom Q, 100–120 mesh, column; programmed at 100–240 °C (2 °C/min). [d] A 6 ft × 1/8 in 10% Carbowax 20M/2% KOH column; programmed at 150–230 °C (2 °C/min).

trum, even when it is measured in the presence of base. Secondly, there appear to be no alkyl chains terminating in methyl or isopropyl groups judging from a ^1H NMR spectrum (360 MHz) of the toxin itself and the structures of the pyrolysis products. In the absence of the saturated C terminal chains and nonquaternized pyridine rings, a macrocyclic structure is inferred. Phosphate or sulfate links between the pyridine nitrogen and the alkyl groups to form macrocyclic rings are possible, but we think that such structures are contraindicated by ^1H and ^{13}C NMR data on the 500–1000 molecular weight range fraction and by the low and variable sulfur and phosphorus contents observed.

As expected for an alkylpyridinium salt, halitoxin undergoes complete reduction upon hydrogenation with platinum catalyst; the NMR spectrum of the product is completely free of aromatic absorption. Field desorption mass spectral analysis of the reduction product from the 500–1000 molecular weight fraction showed a number of ions in the region *m/e* 811–963, consistent with a tetrameric structure, but it was not possible to see conclusively if there was a series of ions corresponding to higher oligomers.

In order to determine if halitoxin has the same composition in different species and in the different molecular weight range fractions, crude samples from *H. rubens* and *H. erina*, as well as the three different molecular weight range fractions from *H. rubens*, were pyrolyzed and the product compositions compared. One pyrolysis of crude halitoxin from *H. rubens* was carried out in the presence of powdered potassium carbonate to minimize any acid-catalyzed rearrangements. The composition of these pyrolysis products is summarized in Table III. The same major degradation products are observed in each case, but with some variation in the relative amounts of individual products. Minor variation in product composition was also noted on duplicate pyrolyses of a given fraction. The results indicate that halitoxins from the two species and all of the different molecular weight range fractions have essentially the same composition. The predominant monomer units in halitoxin are those corresponding to the alkenylpyridines **5** and **6**. These two products and their corresponding halides **11** and **12** account for nearly 50% or more of all the pyrolysis products in each case.

Table IV lists the different species of *Haliclona* that we have examined for halitoxin content and summarizes the toxicity and cytotoxicity data for the crude aqueous alcohol extracts of these sponges. The presence of halitoxin was ascertained by ^1H NMR analysis of the 1-butanol-soluble fraction from workup of the sponge extracts. In addition to cytotoxicity and toxicity to mice, halitoxin caused haemolysis

Table IV. Occurrence of Halitoxin in *Haliclona* Species and Bioactivity of Crude Aqueous Alcoholic Extracts

Species	Halitoxin	KB	LD_{50}, mg/kg
H. rubens	+	7.0	5
H. viridis	+	2.8	2–3
H. erina	+	2.8	3
Haliclona sp.	+	26	~3
H. permallis (?)	−	100	Not toxic

at a threshold concentration of 1 μg/mL. Water containing 100 μg/mL of crude halitoxin (1-butanol soluble) or halitoxin purified by cation exchange chromatography was toxic to goldfish (survival times, 40 and 25 min, respectively). Out of 15 microorganisms tested, halitoxin showed significant antibiotic activity against only two, *Bacillus subtillus* and *Streptococcus pyogenes*, both gram-positive organisms.

We have thus far been unable to detect in the sponge extracts any simple alkylpyridine derivatives that might be likely precursors of halitoxin.

Two N-methylated pyridine salts, homarine[15] and trigonelline,[15,16] have been isolated from sponges, but halitoxin appears to be the first marine pyridinium salt in which a long chain alkyl group alkylates the nitrogen. Other pyridine alkaloids of marine origin with 3-alkenyl substituents include anabasine[17] from a nemertine worm and navenone-A,[18] one component of a trail-breaking alarm pheromone from an ophistobranch mollusc. The side chain length in navenone-A resembles those found in halitoxin. The predominant methyl-branched 3-alkyl substituents linking the pyridinium rings in halitoxin are identical in structure with the alkyl portion of muscopyridine.[19]

Experimental Section[20]

Isolation of Halitoxin from Freeze-Dried Sponge. *Haliclona rubens* from near Isla Maguayez, P.R., Dec 1974, was freeze-dried shortly after collection to give 83.5 g of dry sponge. This was extracted continuously with methanol for 14 h. Evaporation of the methanol yielded 29.3 of residue, which was suspended in water and extracted with three 200-mL portions of dichloromethane. The aqueous solution then was extracted with three portions of 1-butanol (400 mL and 2 × 200 mL). Evaporation of the combined 1-butanol solution gave crude halitoxin (3.09 g).

Isolation of Halitoxin from Air-Dried Sponge. *H. rubens* collected at Isla Maguayez, P.R., May 1972, was air-dired. A 1.03-kg batch was powdered in a blender and defatted by continuous extraction with chloroform for 2 days in a Ciereszko[21] apparatus to yield 45.6 g of chloroform extract. The marc was dried in a current of air and then extracted continuously with methanol for 3 days. The methanol extract was concentrated at reduced pressure, water was

added to give 1400 mL, and the aqueous solution was extracted with three portions (500 mL and 2 × 300 mL) of 1-butanol. Evaporation of the combined 1-butanol solution afforded crude halitoxin (51.1 g).

Ultrafiltration. A 3.18-g sample of crude halitoxin from *H. rubens* was dissolved in water and filtered under nitrogen pressure through a 500 nominal molecular weight limit membrane (Diaflo UM05, Amicon Corp., Lexington, Mass.). Lyophilization of the filtrate gave 0.472 g of residue (<500 molecular weight material). The retentate was diluted with water and similarly filtered through a 1000 molecular weight limit membrane (Pellicon PSAC, Millipore Corp., Bedford, Mass.). The filtrate was lyophilized to yield 0.762 g of residue which constitutes the 500–1000 molecular weight range fraction. The retentate was lyophilized, and a 0.700-g portion of it was redissolved in water and filtered through a 25 000 molecular weight limit membrane (Pellicon PSED). Lyophilization of both filtrate and retentate afforded residues weighing, respectively, 0.100 (1000–25 000 molecular weight range fraction) and 0.600 g (>25 000 molecular weight range fraction). At each stage of the ultrafiltration, the retentate was diluted and the ultrafiltration was repeated two times using the same membrane to insure that all materials of molecular weight less than the membrane's nominal molecular weight cutoff range had passed into the filtrate.

Purification of Halitoxin via Picrate Formation. An aqueous solution of the toxin (500–1000 molecular weight range, 14.6 g) was passed through a column (3.8 × 40 cm) of anion exchange resin (Bio-Rad AG-21K) which had been prewashed with a picric acid solution.[22] The cloudy eluate was lyophilized to give 16.5 g of picrate. The picrate could not be recrystallized, but it was purified somewhat by repeated trituration (total volume 800 mL) with hot acetone. The acetone solubles were treated with charcoal and the solvent evaporated to give 5.81 g of picrate. Picrate complex was also formed by addition of halitoxin to a saturated solution of picric acid in ethanol.

Acetone and then 1 M hydrochloric acid were added to 5.8 g of picrate, and the mixture was stirred while being heated on a steam bath. The solution was decanted from the insoluble picrate, which was washed twice more in like manner. The combined washings were extracted three times with ether, and the aqueous solution was lyophilized to give 3.21 g of halitoxin (chloride form).

Halitoxin Chloride via Anion Exchange. A 1.00-g sample of halitoxin, 500–1000 molecular weight, was dissolved in water (20 mL), and the solution was divided into two equal volumes. One of these was passed through a column of 13.5 g of AG-21K chloride form resin (Bio-Rad Laboratories, Richmond, Calif.). The column was washed with water, and the combined aqueous solution was lyophilized to yield a light brown powder.

Anal. Calcd for $C_{15}H_{24}NCl{\cdot}2H_2O$ (av): C, 62.3; H, 9.6; N, 4.8; Cl, 12.1. Found: C, 61.90; H, 8.12; N, 6.45; Cl, 11.14.

The remaining half of the original solution was lyophilized to yield a similar substance.

Anal. Calcd: see above. Found: C, 61.94; H, 8.16; N, 6.39; Cl, 9.24.

Halitoxin (brown powder): IR (thin film) 3495 (H_2O), 3040, 1630 (pyridinium) cm^{-1}; UV λ_{max} (95% ethanol) 267, inflection at 273 nm; ^1H NMR (CD_3OD) δ 0.92 (3 H, d, J = 7 Hz), 1.04 (ca. one-third the area of δ 0.92 peak, d, J = 7 Hz), 1.4 (~10 H, brd s), 1.76 (brd m), 2.22 (brd m, coupled to δ 0.92 and ~4.5), 2.93 (2 H, t, J = 7 Hz), 4.3–4.8 (m, partially obscured by HOD peak), 8.11 (1 H, t, J = 7 Hz, pyr C-5), 8.56 (1 H, d, J = 7 Hz, pyr C-4), 8.93 (1 H, d, $J \cong 6$ Hz, pyr C-6), 9.05 (1 H, brd s, pyr C-2); ^1H NMR (CD_3OD/C_5D_5N) δ 4.4–5.0, HOD peak at δ 5.1); ^1H NMR ($CD_3OD/C_5D_5N/D_2O/Na_2CO_3$) aromatic region at δ 8.12 (t), 8.56 (d), 8.92 (brd s overlapping d); ^1H NMR (360 MHz, CD_3OD) δ 0.92 (3 H, d, J = 7 Hz), 1.04 (d, J = 7 Hz, one-third the area of δ 0.92 peak), 1.4 (~15 H, brd s, $-CH_2-$), 1.76 (~3 H, brd s coupled to δ 2.92), 2.06 (1 H, brd s coupled to δ 4.68), 2.22 (1 H, brd s coupled to δ 0.92, 4.42 and 4.68), 2.92 (2 H, t, $J \cong 7$ Hz), 4.42 (1 H, dd, $J \cong 8$ and 12 Hz), 4.68 (~2 H, m), 8.08 (1 H, pyr C-5), 8.52 (1 H, pyr C-4), 8.9 (1 H, pyr C-6), 9.0 (1 H, brd s, pyr C-2); ^{13}C NMR (Me_2SO-d_6/D_2O) δ 145.9, 144.6, 144.0, 142.4, 128.5, 67.8, 35.8, 33.6, 32.9, 30.5, 29.3, 28.9, 26.5, 17.0.

Anal. Calcd: see above. Found: (*sample purified by cation exchange*) C, 64.80; H, 8.70; N, 5.12; Cl, 11.08; others (S, 1.33, 0.9; P, 0.3, 1.23).

Pyrolysis of Halitoxin. (a) From *H. rubens*. A 2.16-g sample of halitoxin was placed in a 25 mL round-bottom flask fitted with a Kugelrohr receiver. The apparatus was evacuated to 0.005 mmHg, and the flask was heated at 160–170 °C for 4 h. A yellow oily pyrolysate (1.33 g, 62%) was obtained. For ^1H NMR, MS, and GC analyses, see Tables I–III. ^1H NMR analysis of the pyrolysis residue: (CD_3OD) δ 8.40, 7.66, 7.36, 4.88, 2.64, 1.34–3.1 (several humps), 1.34 (large brd s), 0.96.

(b) From *H. erina*. A 530-mg sample of the 500–1000 nominal molecular weight range fraction of halitoxin from *H. erina* was heated in a Kugelrohr apparatus at 150–165 °C and 0.02 mmHg for 90 min. A yellow oily distillate (325 mg, 61%) was obtained, leaving a black pot residue (135 mg, 25%). Loss of water probably accounts for the 14% lost. For MS and GC analyses, see Tables I and III.

Pyrolysis in the Presence of Base. A mixture of halitoxin (500 mg) from *H. rubens* and potassium carbonate (5.0 g) was ground to a fine powder and then heated at 168 °C and 0.01 mmHg for 4 h. A brown distillate (157 mg, 31%) was obtained. For GC analysis, see Table III.

Hydrogenation of Halitoxin. A suspension of 150 mg of platinum oxide in 2 mL of methanol was stirred under hydrogen for 15 min, and then a solution of 99 mg of halitoxin (purified via picrate formation, 500–1000 molecular weight, from *H. rubens*) in 3 mL of methanol was added. After being stirred at room temperature and atmospheric pressure for 18 h, the suspension was filtered and the filtrate was evaporated. The residue was dissolved in dichloromethane/diethylamine (9:1) and passed through a short column of Silicar CC-7 (fine mesh). The column was washed with the same solvent, and the combined eluate was evaporated to give a colorless viscous residue (28.5 mg): ^1H NMR (100 MHz, $CDCl_3/CD_3OD$) (integral based on assumption of 6 H at δ 3.02) δ 1.00 (~2 H, d), 1.28 (brd s), 1.38 (brd s) (total area δ 1.20–1.60, ~16 H), 1.80 (brd s, ~4 H), 3.02 (brd d, 6 H), 4.58 (s, HOD); field desorption mass spectrum (prominent ions) m/e 364, 484, 575, 595, 629, 643, 811, 825, 839, 853, 864, 867, 880, 881, 963.

1-Isobutyl-3-methylpyridinium Bromide (2). The salt **2** was prepared by the reaction of isobutyl bromide with β-picoline in methanol. The crude product was dissolved in water and washed with benzene to remove unreacted material. Lyophilization of the aqueous solution gave 1-isobutyl-3-methylpyridinium bromide as a hygroscopic crystalline mass: IR (KBr) 3010, 2940, 1640, 1510, 1475 cm^{-1}; ^1H NMR [$CDCl_3/CD_3OH$ (1:1)] δ 9.08–8.76 (2 H, m), 8.46 (1 H, d, J = 8 Hz), 8.10 (1 H, m), 4.59 (d, J = 7 Hz; also HOD), 2.68 (3 H, s), 2.41 (1 H, heptet, J = 7 Hz), 1.05 (6 H, d, J = 7 Hz); ^{13}C NMR (Me_2SO/D_2O) δ 146.3 (d), 144.0 (d), 141.6 (d), 140.0 (s), 127.7 (d), 69.0 (t), 31.3 (d), 19.6 (q, $2CH_3$), 19.0 (q).

A picrate prepared in the usual manner and recrystallized from aqueous methanol melted at 118–120 °C.

Anal. Calcd for $C_{16}H_{18}O_7N_4$: C, 50.79; H, 4.80; N, 14.81. Found: C, 50.61; H, 4.80; N, 14.73.

1-Hexadecyl-3-methylpyridinium Bromide (3). Reaction of cetyl bromide with β-picoline followed by purification as described for **2** gave **3** as a white powder, mp 44–48 °C, after one recrystallization from benzene/hexane: IR (KBr) 2920, 2840, 1640, 1505, 1475, cm^{-1}; ^1H NMR [$CDCl_3/CD_3OD$ (1:1)] δ 9.02–8.72 (2 H, m), 8.43 (1 H, d, J = 8 Hz), 8.05 (1 H, m), 4.70 (m; also HOD), 2.65 (3 H, s), 2.08 (2 H, brd m), 1.28 (26 H, brd), 0.90 (3 H, t, J = 3 Hz); ^{13}C NMR (Me_2SO/D_2O) δ 146.3 (d), 144.0 (d), 142.0 (d), 139.8 (s), 128.1 (d), 61.9 (t), 32.9, 32.3, 30.9 (several CH_2), 26.9 (t), 23.3 (t), 19.0 (q), 14.6 (q).

Picrate mp 68–69 °C, after one recrystallization from methanol.

Anal. Calcd for $C_{28}H_{42}O_7N_4$: C, 61.52; H, 7.56; N, 10.25. Found: C, 61.53; H, 7.56; N, 10.20.

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Registry No.—**2**, 66902-16-7; **2** picrate, 66902-18-9; **3**, 2315-39-1; **3** picrate, 66902-19-0; **4**, 66902-08-7; **5**, 66902-10-1; **6**, 66902-11-2; **7**, 66902-13-4; **8**, 66902-09-8; **9**, 66902-12-3; **11**, 66902-14-5; **12**, 66902-15-6; halitoxin-R, 54990-72-6; isobutyl bromide, 78-77-3; β-picoline, 108-99-6; cetyl bromide, 112-82-3.

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Biosynthetic Studies of Secondary Plant Metabolites with $^{13}CO_2$. *Nicotiana* Alkaloids. 2.[1] New Synthesis of Nornicotine and Nicotine. Quantitative Carbon-13 NMR Spectroscopic Analysis of [2′,3′,*N*-CH_3-$^{13}C_3$]Nicotine[2]

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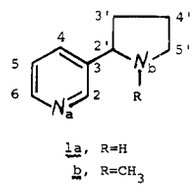
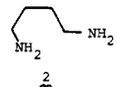
An efficient synthesis of the tobacco alkaloids, nornicotine (**1a**) and nicotine (**1b**), is achieved by Michael condensation of the α-lithiomethoxime of 3-acetylpyridine (**11**) with a ketene thioacetal monoxide (**4**) to give **12a**, thus providing all the pyrrolidine ring atoms of **1a** in masked form. Subsequent reduction with diborane and reductive cyclization in refluxing 97% formic acid are used to produce *N*-formyl-**1a** in high yield. The latter is converted to **1a** or **1b** by literature procedures in 60% overall yield from 3-acetylpyridine. The chemistry of several alternative, but inefficient, synthetic approaches to **1a** also is discussed, in particular a route from 3-acetylpyridine and mesylaziridine (**16**). The synthesis of [2′,3′,*N*-CH_3-$^{13}C_3$]-**1b** is achieved by this route from [1,2-$^{13}C_2$]acetic acid and [^{13}C]formaldehyde via [1′,2′-$^{13}C_2$]-3-acetylpyridine. Analysis of the proton-decoupled ^{13}C NMR spectrum of the triply ^{13}C-labeled **1b** is done to certify the accuracy of the quantitative determination of the relative ^{13}C enrichment in **1b** biosynthetically labeled by $^{13}CO_2$. Thereby an earlier conclusion about the symmetry of ^{13}C labeling of carbons 2′ and 5′ of **1b** is circumstantially validated, i.e., that these four carbons are unequally ^{13}C labeled by $^{13}CO_2$ within experimental error.

We are studying the applicability of highly enriched $^{13}CO_2$ as a biosynthetic probe of secondary plant metabolites, particularly alkaloids. In our first paper concerning the tobacco alkaloids[1] the results of some initial feeding experiments using 97 atom % $^{13}CO_2$, in which we studied the biosynthesis of nicotine (**1b**), the major alkaloid of *N. tabacum* and *N. glutinosa*, were described and *tentatively* interpreted as corroborating some of Rapoport's earlier observations obtained with $^{14}CO_2$:[4] that the *N*-methylpyrrolidine ring of **1b** could become unsymmetrically labeled by incorporation of isotopically labeled CO_2. Since such conclusions are in variance with all of the other data concerning nicotine's biosynthesis,[1,5] i.e., that the *N*-methylpyrrolidine ring of **1b** is formed in vivo via putrescine (**2**) and thereby should be symmetrically labeled by isotopic carbon labeled precursors, it is very important to certify the experimental error of our technique of ^{13}C label distribution analysis (^{13}C NMR spectroscopy). This is especially important since the intramolecular ^{13}C labeling inequality of **1b** that we reported was C(2′) (62%), C(3′) (65%), C(4′) (58%), and C(5′) (49%),[1] such values perhaps being equivalent within experimental error, although Matwiyoff and Burnham had certified that the technique we used was accurate to within ±1.4% for uniformly and nonuniformly ^{13}C-labeled acetate.[6] For this reason we developed a new synthesis of nornicotine (**1a**) and **1b** designed to meet our special needs for the synthesis of [2′,3′,*N*-CH_3-$^{13}C_3$]-**1b**. The chemistry that was encountered during the development of our most efficient synthetic route to **1a** and **1b** is reported here as well as the synthesis and quantitative ^{13}C NMR spectroscopic analysis of the triply ^{13}C-labeled **1b**. From our new results the earlier



1a, R=H
b, R=CH_3



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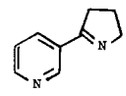

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