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I. INVESTIGATION OF A NEW METHOD FOR SYNTHESIZING α-METHYLENEBUTYROLACTONES. II. HALITOXIN-R: ISOLATION AND PARTIAL STRUCTURE DETERMINATION OF A TOXIC MATERIAL FROM THE SPONGE HALICLONA RUBENS. III. STUDIES ON THE RED PIGMENTS FROM THE SPONGE HALICLONA RUBENS. IV. ELISTANOL: A NOVEL MARINE STEROL. V. DACTYLYNE: A NOVEL MARINE ACETYLENIC OXETANE.

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GRADUATE COLLEGE

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V. DACTYLYNE: A NOVEL MARINE ACETYLENIC OXETANE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

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DAVID CLARK CAMPBELL

Norman, Oklahoma

1974

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APPROVED BY

Francis J. Schmitz

Leon S. Ciereszko

Stanley C. Kuehl

DISSERTATION COMMITTEE

Dedication

To Martha and Davy

ACKNOWLEDGEMENTS

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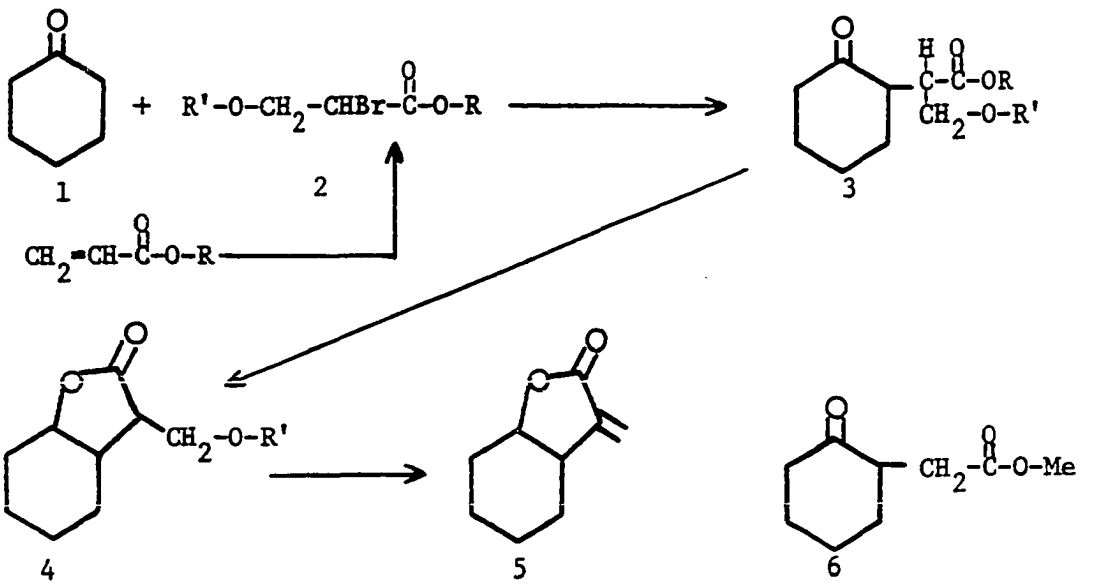
I. INVESTIGATION OF A NEW METHOD FOR SYNTHESIZING α-METHYLENEBUTYROLACTONES

INTRODUCTION

The α-methylenebutyrolactone functionally fused to another carbocyclic ring is present in a large number of natural products[1]. The fact that some of these compounds have shown biological activity[1] has stimulated interest in finding efficient synthetic methods for adding the α-methylenebutyrolactone moiety to a carbocyclic ring. Prior to the work described here, all of the methods[2-6] reported in the literature for building this structural feature gave low yields and/or entailed long sequences of reactions. Since completion of the work in this thesis, a number of efficient methods have appeared in the literature which overcome these faults. These newer methods will be discussed later.

The purpose of the work presented here was to develop a sequence of reactions that would lead to the easy addition of an α-methylenebutyrolactone functionality to a carbocyclic ring in high overall yield. The cyclohexyl ring system was chosen as the model carbocyclic ring to which it was planned to add the lactone ring. It was envisioned that the way to attack the problem would be to add to the cyclohexyl ring a unit possessing the necessary carbon atoms and functionality for facile subsequent conversion to the α-methylenebutyrolactone system. In order to

minimize the number of steps required for conversion to the lactone after addition of the appropriate carbon fragment, it was planned to include in the moiety being added to the cyclohexyl ring system a group that could be easily removed in the last step to give the α-methylene portion of the system. In view of these considerations the general approach that evolved and was evaluated is shown in Scheme 1.



SCHEME 1

The results of the evaluation of Scheme 1 are discussed for the cases where R = R' = Me and R = R' = Et. The use of the pyrrolidine enamine of cyclohexanone and the cyclohexyl imine of cyclohexanone in attempts to add the necessary group to the cyclohexyl ring (1→3) are discussed. In addition, the synthesis of keto ester 6 using published procedure[7] is discussed.

It was assumed that stereoisomers would result from most of the reactions. Gas chromatography of the reaction products confirmed that

this was the case, but no attempt was made to resolve these mixtures. In one case it was possible to assign stereochemistry to a major product on the basis of the unusual chemical shift of a proton absorption seen in the proton magnetic resonance spectrum.

RESULTS AND DISCUSSION

It was known[8] that sodium ethoxide reacted with the dibromide of ethyl acrylate to give ethyl 3-ethoxy-2-bromopropionate in good yields. It was also known[8] that the analogous reaction between the dibromide of methyl acrylate and sodium methoxide did not give good yields. However, because of the simplification of proton magnetic resonance (pmr) spectra interpretation, methyl bromo ester 8 was prepared and used in the preliminary study of the reaction sequence.

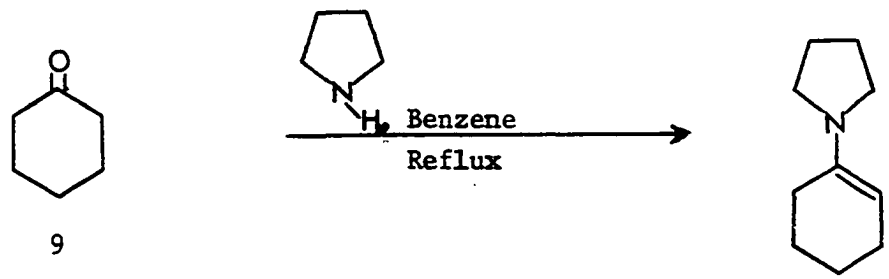
Using the procedure of Wood and du Vigneaud[8] methyl 2-bromo-3-methoxypropionate was prepared from methyl acrylate (7) by addition of bromine to the double bond followed by nucleophilic displacement of the terminal bromine by sodium methoxide to give the bromo ester 8 in 32.2% yield.

$$\underset{7}{CH_2{=}CH{-}\overset{O}{\overset{\|}{C}}{-}O{-}Me} \xrightarrow[2)\ NaOMe,\ MeOH]{1)\ Br_2,MeOH} \underset{8\ (32.2\%)}{Me{-}O{-}CH_2{-}CHBr{-}\overset{O}{\overset{\|}{C}}{-}O{-}Me}$$

The bromo ester 8 was to be used to alkylate cyclohexanone, 9 There are several ways to alkylate active methylene compounds[9], but many of these reactions are accomplished in the presence of strong base through an enolate ion. In the present case the use of a strong base could lead to undesirable side reactions (elimination of methanol or

dehydrobromination). An alternate method to effect alkylation which avoids the use of a strong base is the reaction of a ketone enamine with an alkyl halide.

The pyrrolidine enamine of cyclohexanone 10 was prepared using the procedure of Stork and coworkers[10]. Equimolar amounts of cyclohexanone (9) and pyrrolidine were refluxed in benzene with azeotropic removal of the water was formed to give the enamine 10. It was found

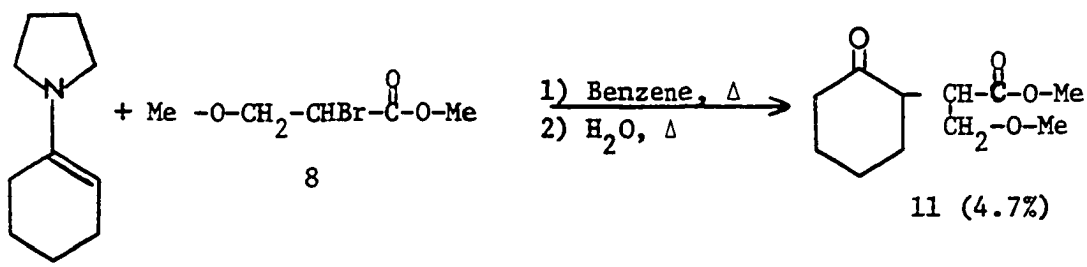


9

10 (75.3%)

that the enamine 10 was unstable if stored at room temperature for long periods. Therefore, it was necessary to store the enamine in the cold under nitrogen if it was not to be used immediately. Under these conditions it did not show appreciable decomposition over a two month period.

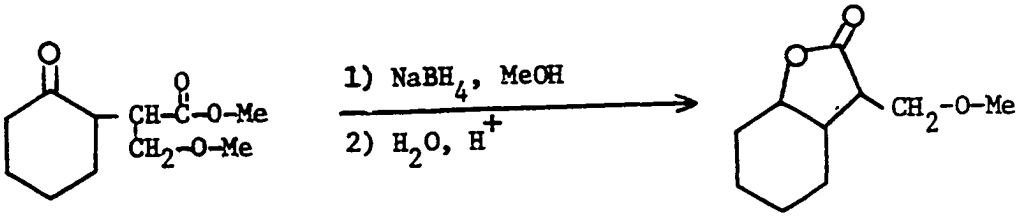
The alkylation was performed by refluxing the enamine 10 with the bromo ester 8 in dioxane. Hydrolysis of the alkylated enamine, which was not isolated, gave the keto ester 11. The keto ester 11 was then reduced with sodium borohydride and lactonized in aqueous acid to give the lactone 12 in 56.2% yield.



8

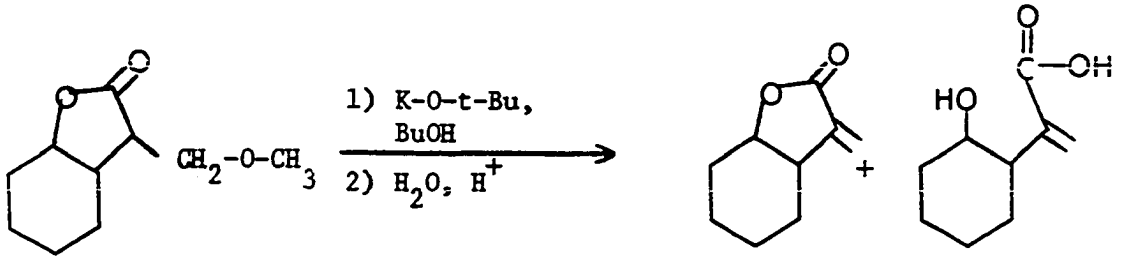
10

11 (4.7%)



12 (56.2%)

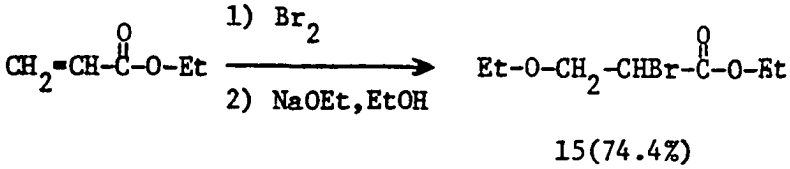
Reaction of lactone 12 with potassium tert-butoxide afforded a mixture of the unsaturated lactone 5 (55%), the hydroxy acid 13 (12%) and the starting material 12 (33%) as determined by infrared (ir) and pmr analysis.



12 5 (55%) 13 (12%)

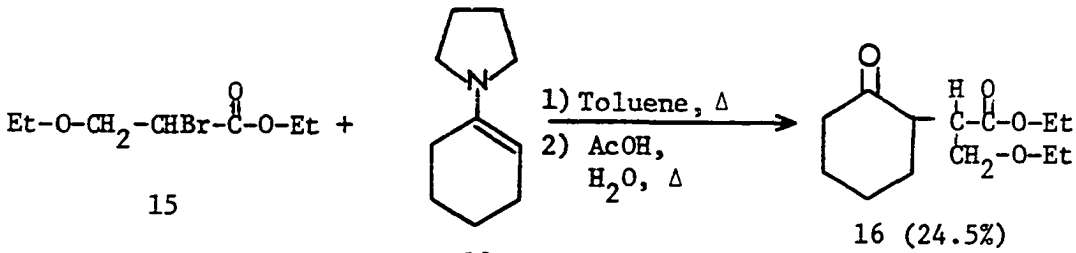
Based on the results of this preliminary study, it was concluded that yields of the reduction/lactonization and the elimination reactions were satisfactory, but that the yields of the methyl bromo ester synthesis and the enamine alkylation were poor.

Because of the good yields reported in the literature[8], ethyl 2-bromo-3-ethoxypropionate 15 was prepared using the procedure previously discussed for methyl 2-bromo-3-methoxypropionate 8. The reaction afforded ethyl 2-bromo-3-ethoxypropionate (15) in 74.4% yield.



15(74.4%)

The ethyl bromo ester 15 was refluxed with enamine 10 in toluene for 72 hr. The alkylated enamine was not isolated, but was immediately subjected to hydrolysis with acetic acid and water to give keto ester 16.



15

10

16 (24.5%)

In an attempt to improve the yields of enamine alkylation, the reaction was run in several different solvents. The results are shown in Table I.

TABLE I

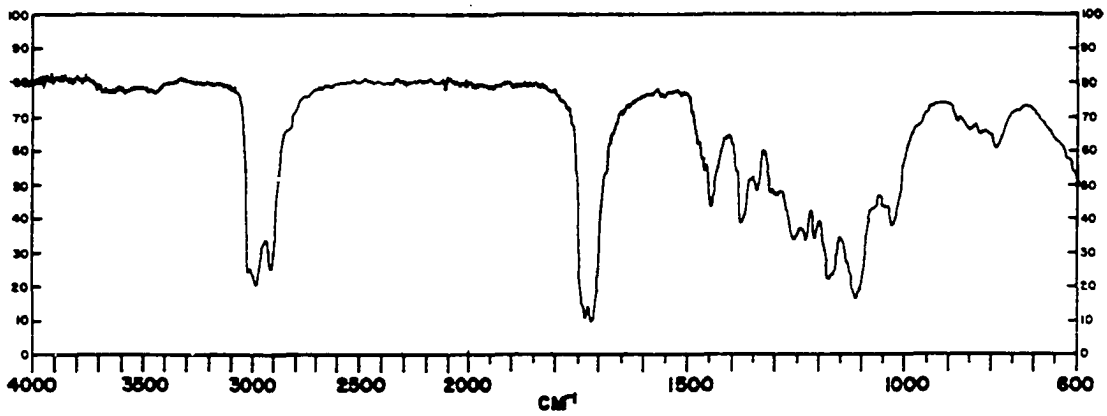
Compound	Solvent	Temperature	Time	% Yield
8	MeOH	Reflux	2.25hr	4.7
15	EtOH	Reflux	12 hr	15.5
15	DMF	Reflux	18 hr	0.0
15	Toluene	Reflux	72 hr	24.5

The proton magnetic resonance spectrum, infrared spectrum and the mass spectrum of keto ester 16 are shown in Figure I. The multiplet centered at 3.4 ppm in the pmr spectrum is a four proton signal caused by the two methylene groups which flank the ether oxygen. The apparent quartet centered at 1.15 ppm is due to the overlapping triplets of the two ethyl groups. The infrared spectrum shows an ester absorption at 1732 cm^{-1}

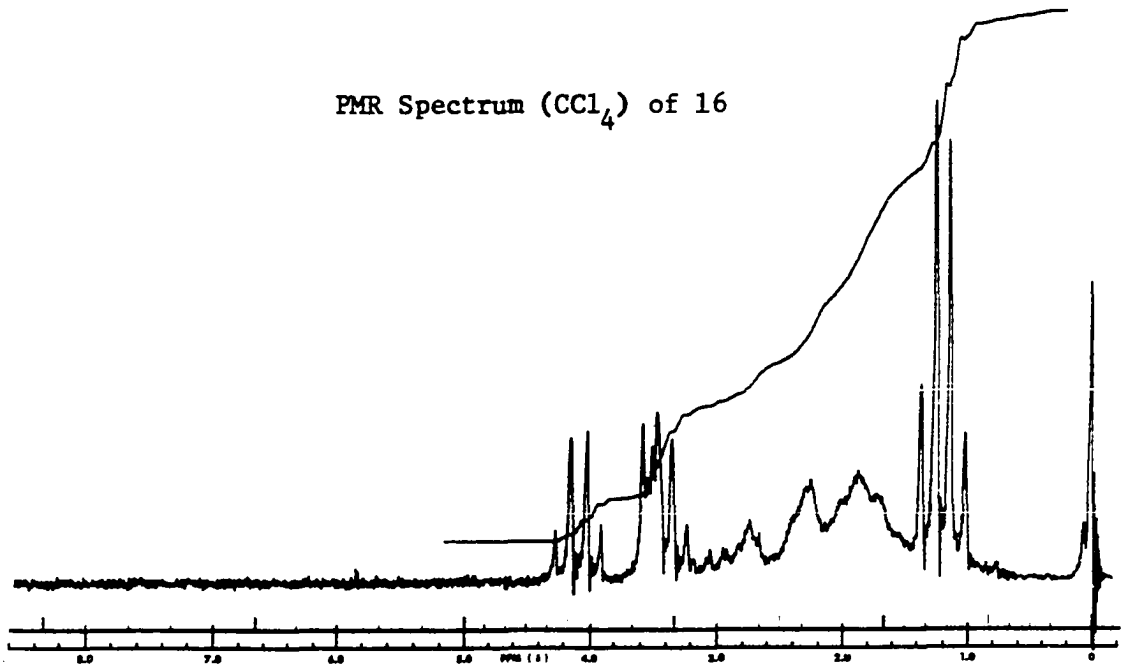
Figure I

Spectra of Ethyl 2-(2-Oxocyclohexyl)-3-ethoxypropionate (16)

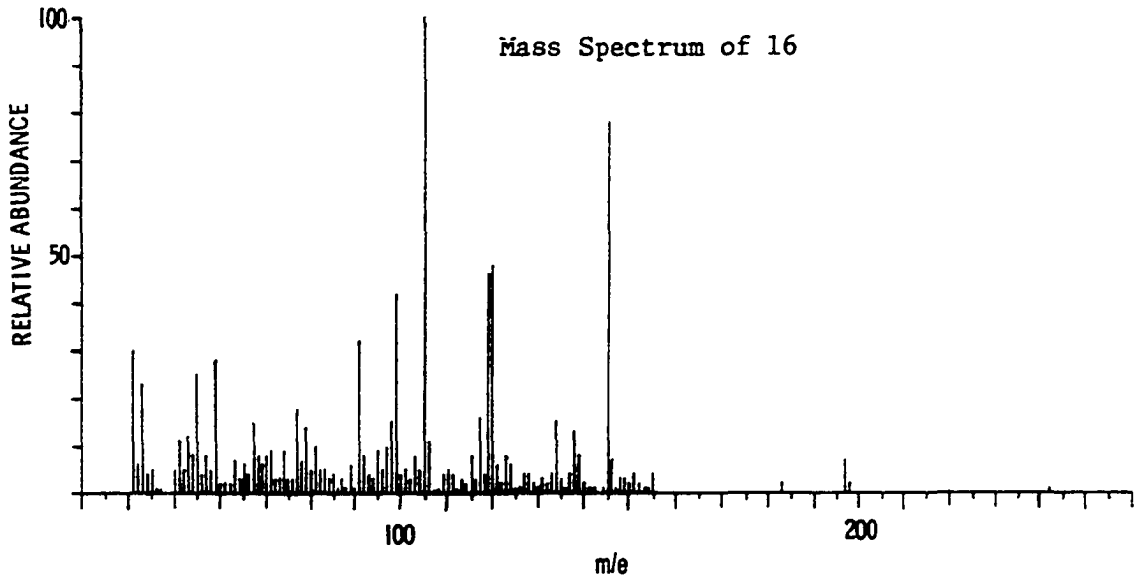
IR Spectrum (Thin Film) of 16



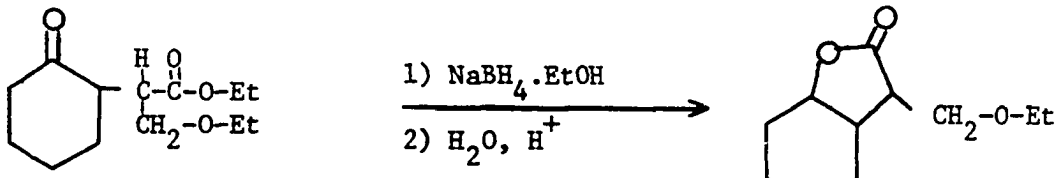
PMR Spectrum (CCl_4) of 16



Mass Spectrum of 16



and a six-membered ring ketone absorption at 1710 cm^{-1}. The mass spectrum shows peaks at 242 (M^+), 197 ($M^+ - CH_3-CH_2-O$), and 145 (M^+ - cyclo hexanone). All of these spectral data are consistent with structure 16. The keto ester 16 was reduced with sodium borohydride in ethanol and the resulting hydroxy ester was lactonized with aqueous acid to give 3-ethoxymethyl-(3H)-hexahydrobenzofuran-2-one (17).



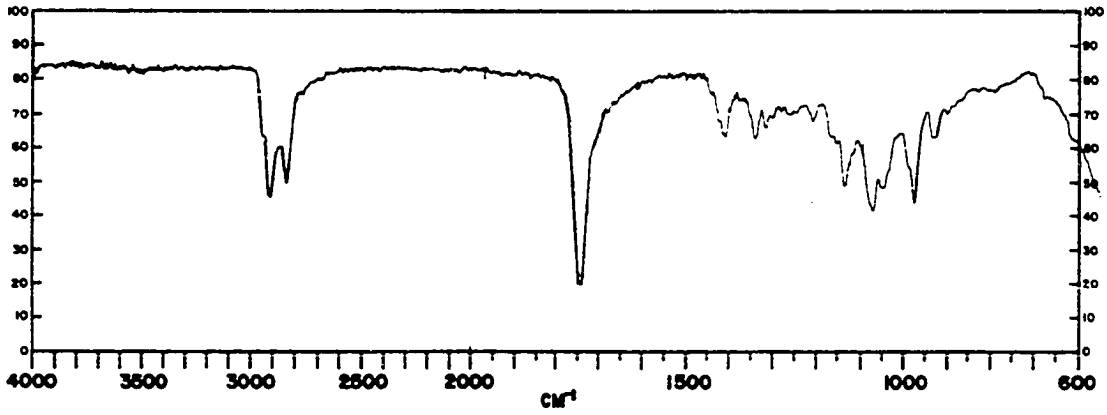
16

17 (56.6%)

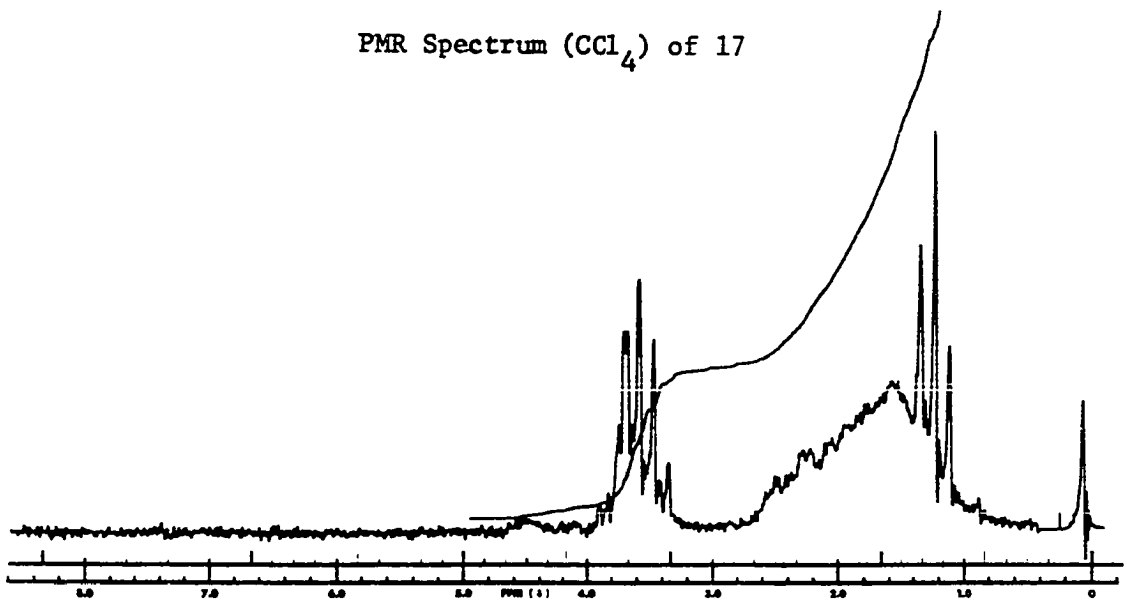
The spectra of lactone 17 are shown in Figure II. The multiplet centered at 3.56 ppm in the pmr spectrum is a five proton signal caused by the four methylene protons flanking the ether oxygen and the proton under the lactone oxygen. The triplet at 1.26 ppm is due to the three protons of the terminal methyl of the ether. The infrared spectrum indicates the presence of a five membered ring lactone by the strong band at 1780 cm^{-1}. The mass spectrum shows peaks at 198 (M^+), 154 ($M^+ - O-CH_2-CH_3$ or M^+-CO_2) and 138 (M^+ CH_2-O-Et). The spectral data are all consistent with the structure 17.

The unusual upfield chemical shift exhibited by the proton under the lactone oxygen in 17 can be explained by ether oxygen shielding. If the lactone is trans fused the protons at the ring junction are then trans diaxial. If the ether moiety is on the same side of the molecule as the axial proton under the lactone oxygen, then that proton

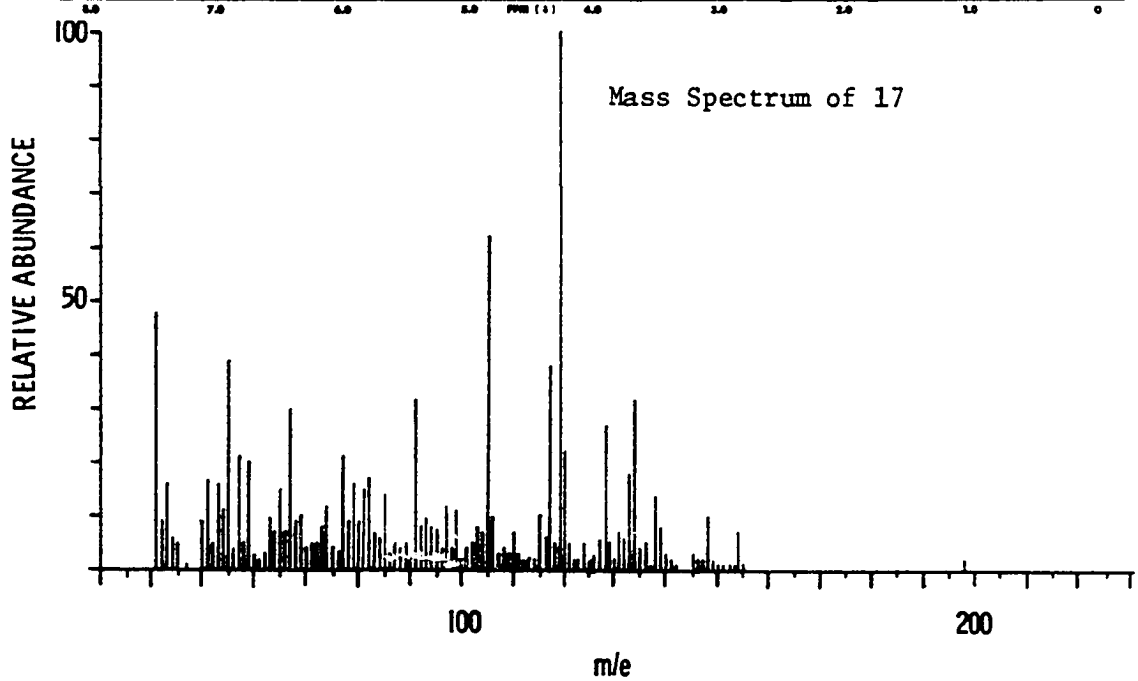
Figure II
Spectra of 3-ethoxymethyl-(3H)-hexahydrobenzofuran-2-one (17)
IR Spectrum (Thin Film) of 17



PMR Spectrum (CCl_4) of 17



Mass Spectrum of 17



will be in close proximity to the ether oxygen. This would result in shielding and consequent upfield shifting of the proton absorption. Figure III shows the isomer that has the proper stereochemistry to do this. Models confirm that the ether oxygen and the lactone proton are in close proximity. Models also show that none of the cis ring fused isomers would show this effect.

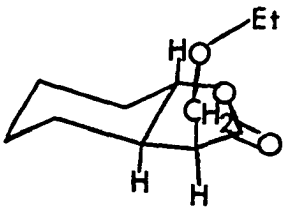
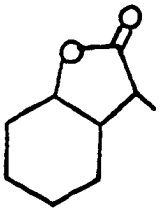


Figure III

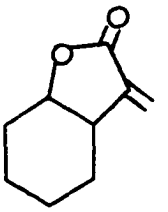
As can be seen from the pmr of lactone 17 (the multiplet at 4.5 ppm) other stereoisomers in which the proton under the lactone oxygen is not shielded are also present and exhibit the expected chemical shift. However, based on gas chromatography and the pmr spectrum they are present in minor concentration.

To carry out the final step of the reaction sequence lactone 17 was reacted with potassium tert-butoxide. Since the crude product contained both the lactone 5 and some of the corresponding hydroxy acid the product was stirred with aqueous acid to complete lactonization and give all lactone 5.



17

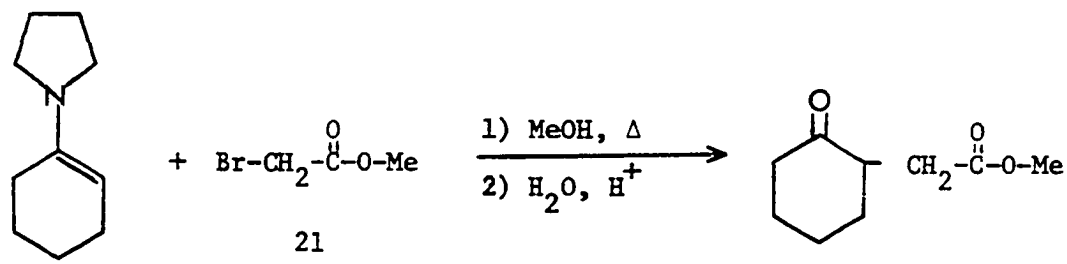
1) t-BuOK, t-BuOH
2) H_2O, H^+



5 (44.8%)

The spectra of 3-methylenehexahydrobenzofuran-2-one (5) are shown in Figure IV. The two doublets at 6.18 and 5.42 ppm in the pmr are very characteristic of the two exo-methylene protons which are in different magnetic environments. The coupling constants (2 Hz) are the expected values for allylic coupling. The signal of the proton under the lactone oxygen appears as a multiplet centered at 4.47 ppm. The proton allylic to the exo-methylene and at the ring juncture gives rise to a broad multiplet at 2.97 ppm. The lactone absorption in the infrared spectrum falls at 1765 cm^{-1} which is consistent with that of an α-methylenebutyrolactone. The mass spectrum exhibits peaks at 152 (M^+), 124 ($M^+ - C_2H_4$ or CO), 96 ($M^+ - C_4H_8$) and 95 ($M^+ - C_4H_9$).

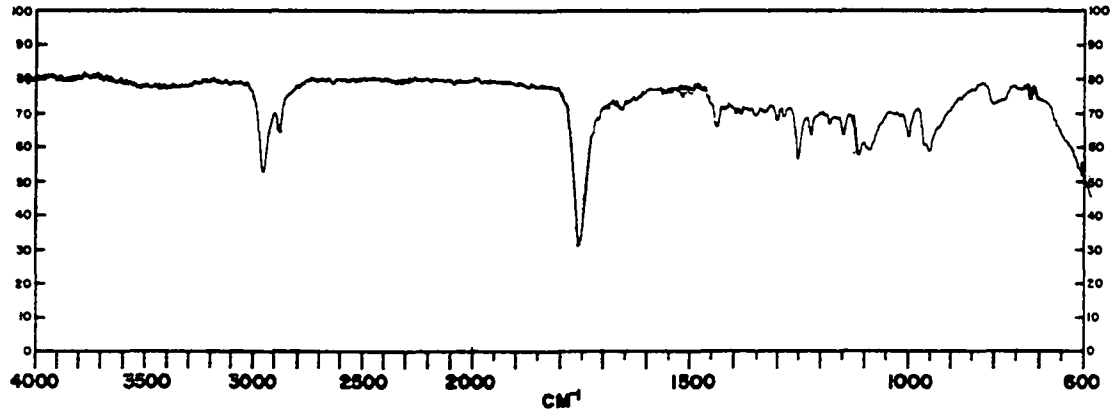
Because of the low yields encountered in the alkylation reactions of the enamine the technique and reagents were checked by attempting the alkylation reaction using a published procedure[7]. The enamine 10 was reacted with methyl bromoacetate (21) to give methyl 2-(2-oxocyclohexyl)-acetate (22) in 57.5% yield by gas chromatography and in 49% isolated yield. The published yield for ethyl 2-(2-oxocyclohexyl)-acetate is 58%[7].



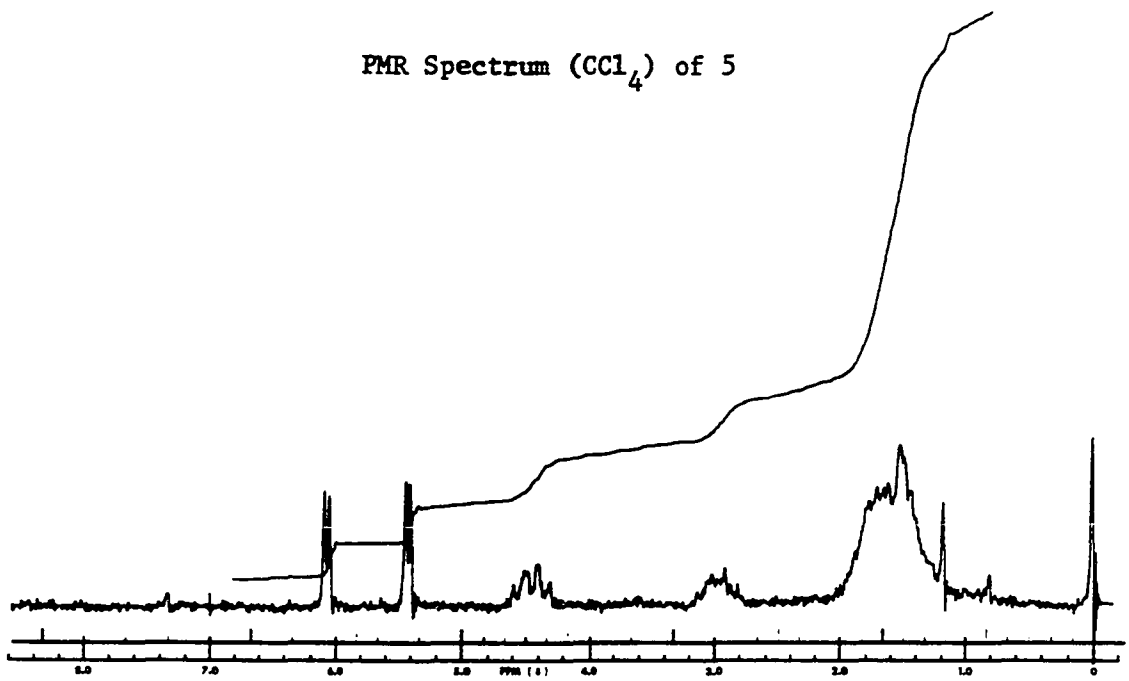
10 21 22 (49%)

The spectra for compound 22 are shown in Figure V. The only clearly resolved peak in the pmr is the methyl ester singlet at 3.63 ppm. The infrared spectrum shows ester carbonyl absorption at 1740 cm^{-1} and

Figure IV
Spectra of 3-Methylenehexahydrobenzofuran-2-one (5)
IR Spectrum (Thin Film) of 5



PMR Spectrum (CCl_4) of 5



Mass Spectrum of 5

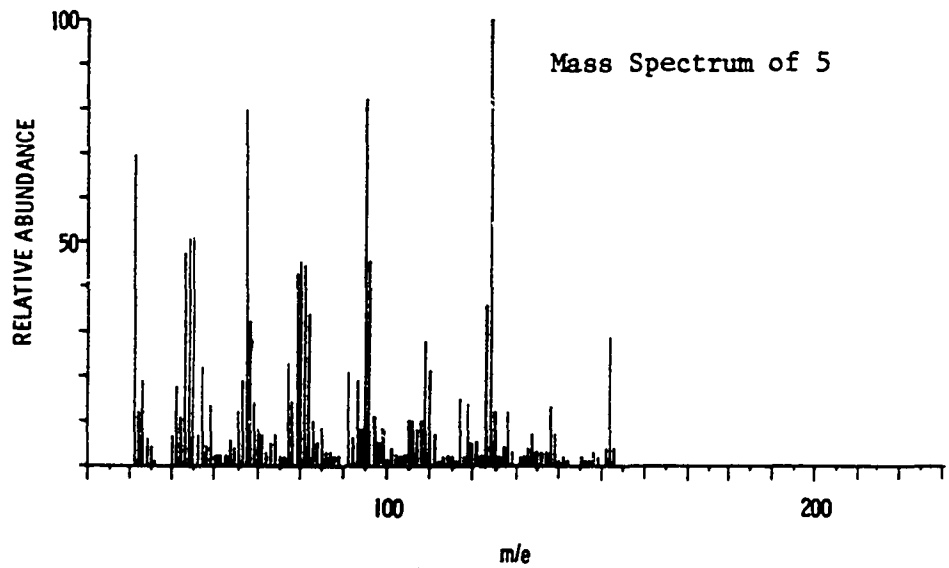
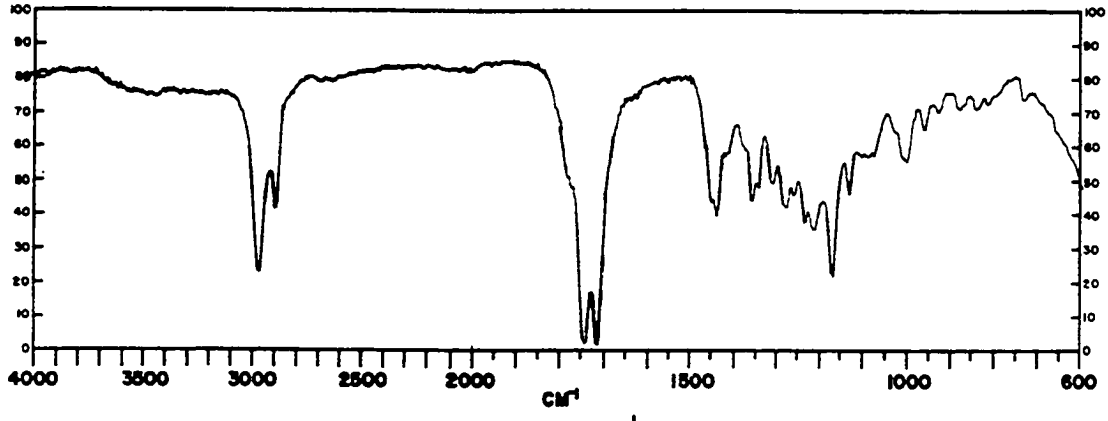
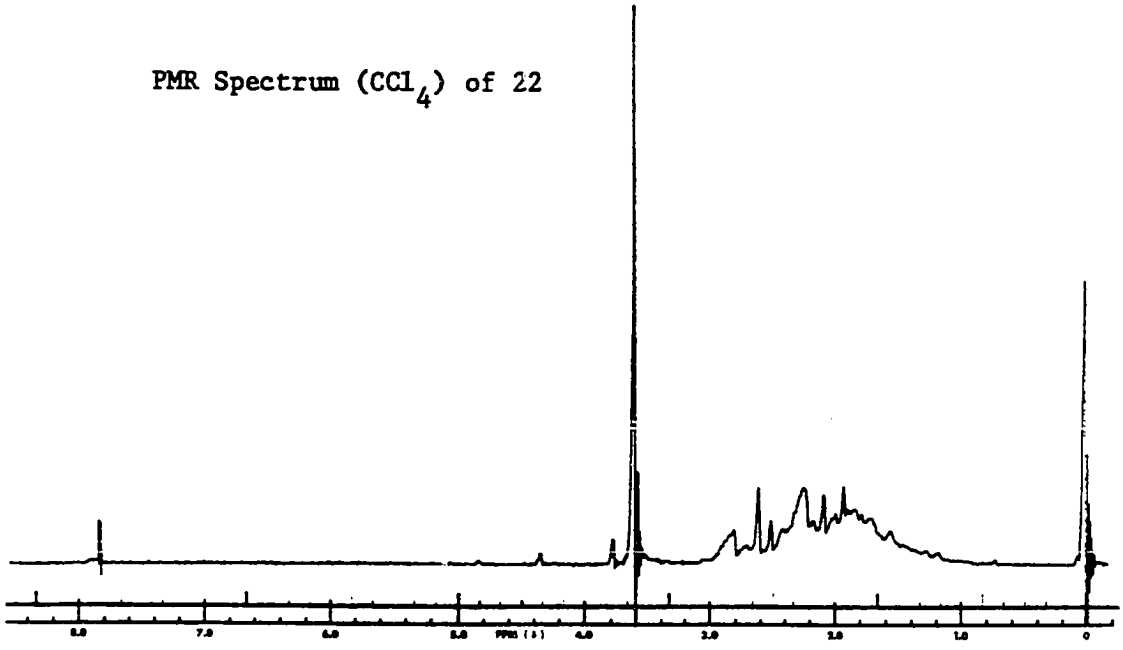


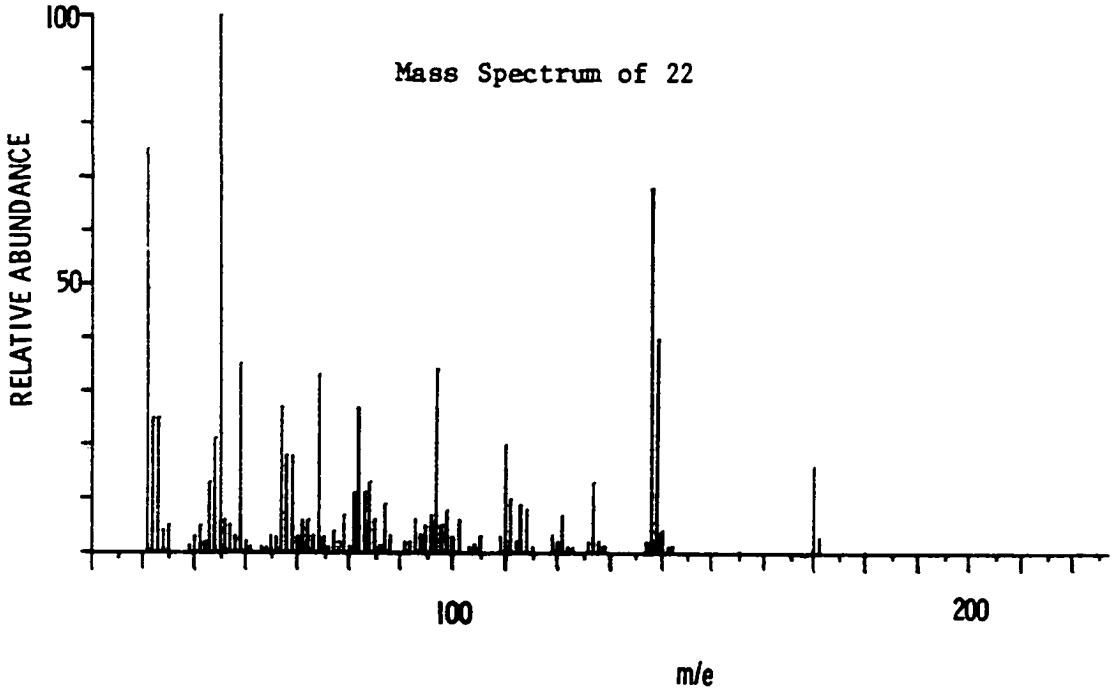
Figure V
Spectra of Methyl 2-(2-Oxocyclohexyl)-acetate (22)
IR Spectrum (Thin Film) of 22



PMR Spectrum (CCl_4) of 22



Mass Spectrum of 22

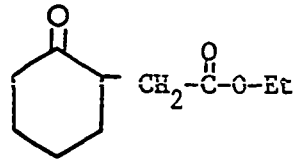


six membered ring ketone absorption at 1714 cm^{-1}. The mass spectrum shows peaks at 170 (M^+), 139 ($M^+ - CH_3O$), 138 (M^+ - methanol), and 97 (M^+ - the side chain). All of the spectral data are consistent with structure 22.

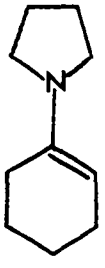
The low yields encountered in the attempts to alkylate the enamine can be attributed to two factors. However, the contribution of each one individually would be hard to assess on the basis of the limited amount of work done here.

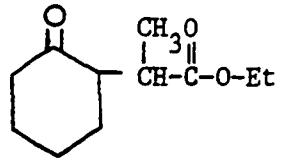
The first factor is steric. This can best be illustrated by the following reactions.



9 + $BrCH_2-\overset{O}{\overset{\|}{C}}-O-Et$ $\xrightarrow[2)\ H_2O,\ H^+]{1)\ MeOH,\ \Delta}$ 

23 (58%) (Ref. 11).



9 + $CH_3-CHBr-\overset{O}{\overset{\|}{C}}-O-Et$ $\xrightarrow[2)\ H_2O,\ H^+]{1)\ MeOH,\ \Delta}$ 

24 (44%) (Ref. 11)

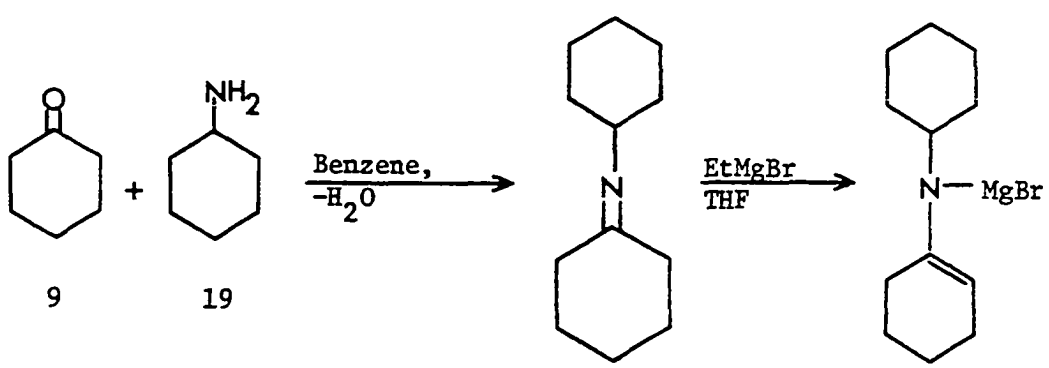
As the bulk of the group attached to the α-carbon increases the yields of enamine alkylation decrease. The group attached to the α-carbon of bromo esters 8 and 15 was even bulkier than the illustrated compounds.

The second factor is the possibility of N-alkylation instead of C-alkylation. This has been shown to be a very major factor in some

reactions[12,13]. The result of this reaction would be the recovery of a large amount of the starting ketone upon hydrolysis.

Because of the poor yields encountered in the previous attempts to alkylate cyclohexanone via the enamine an alternate means of accomplishing this step was sought. A reaction which appeared attractive was that of an N-alkyl imine magnesium bromide with an alkyl halide. This reaction had proved very useful for alkylations of unactivated halides[14].

The cyclohexyl imine magnesium bromide 18 was prepared according to the procedure of Stork and Dowd[14] as shown in Scheme II. The alkylation of the imine magnesium bromide 18 was attempted using the bromo ester 15 and methyl bromoacetate (21). In each case only the bromo ester and cyclohexanone could be isolated.



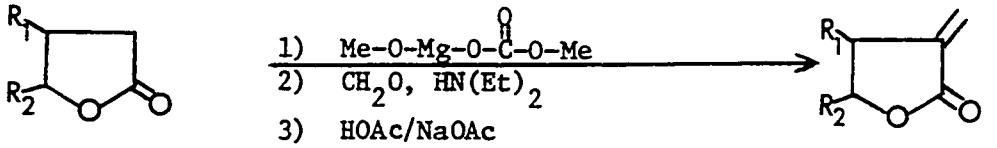
9 19 20 (69.8%) 18

Scheme II

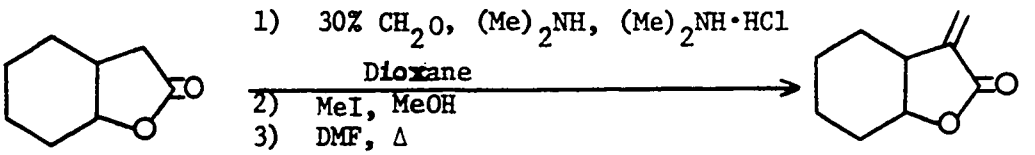
Except for the alkylation step no attempt was made to maximize the yields of the reactions in the overall scheme for preparing α-methylenebutyrolactones. Adjustment of the conditions and the reagents of the reduction/lactonization and elimination reactions might lead to better yields.

Since the completion of this work feverish activity has erupted in attempts to synthesize α-methylenebutyrolactones. This has

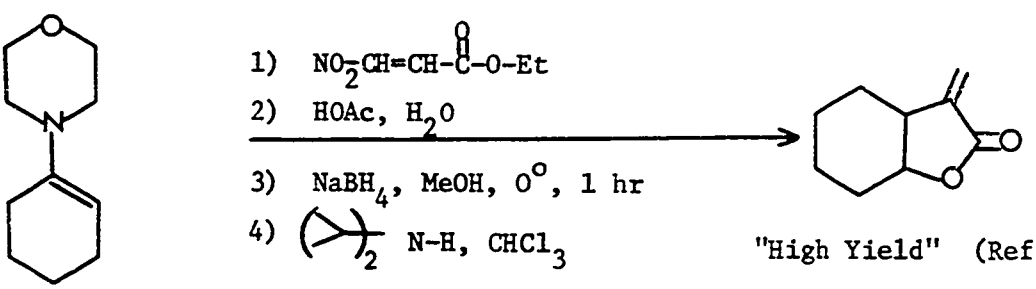
been spurred by the large number of compounds containing the α-methylene butyrolactone moiety which have shown biological activity[1]. The approaches found in the literature are outlined in the following equations. Yields shown are overall yields from the indicated starting compound.



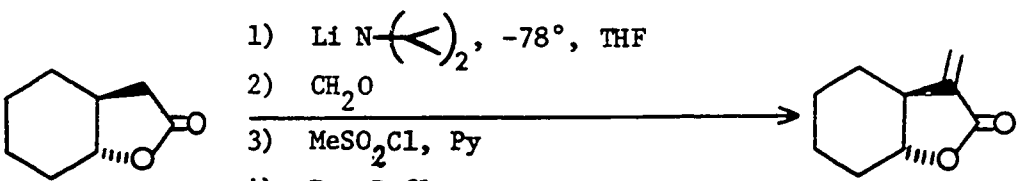
36% (Ref. 15)



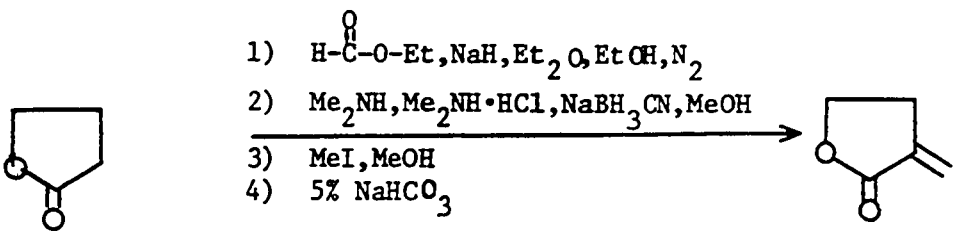
67% (Ref. 16)



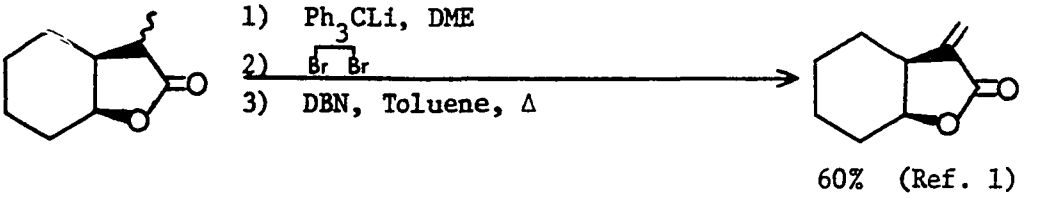
"High Yield" (Ref. 17)



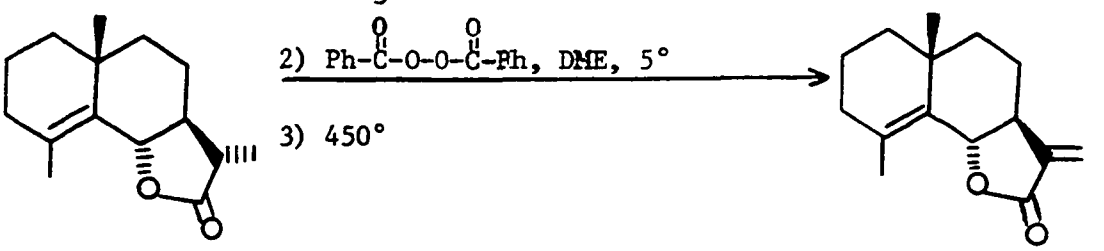
76% (Ref. 18)



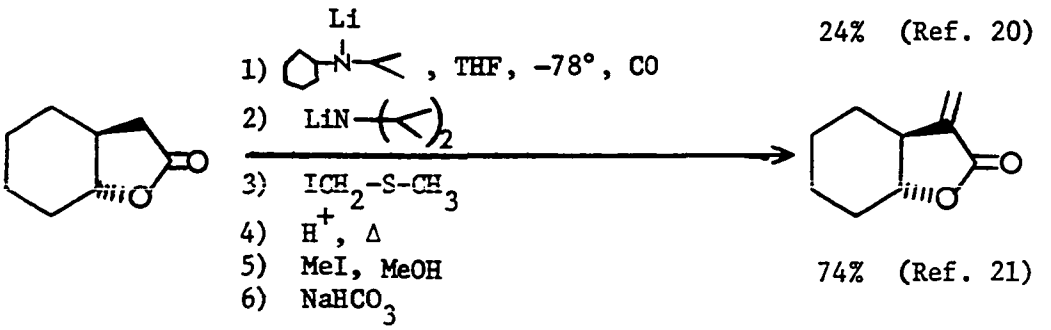
60% (Ref. 19)



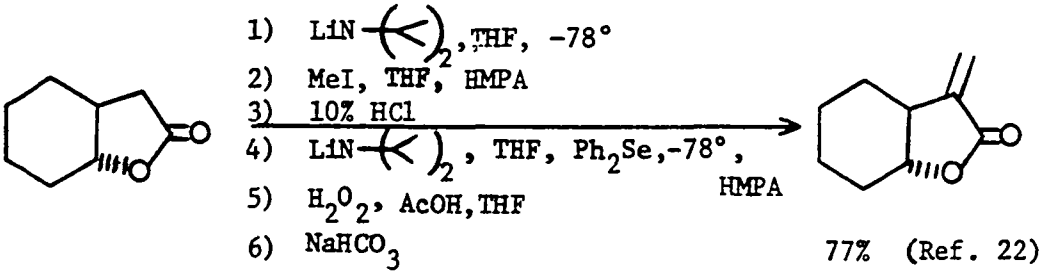
60% (Ref. 1)



24% (Ref. 20)



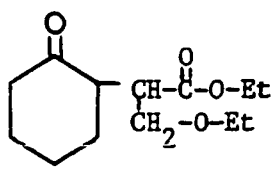
74% (Ref. 21)



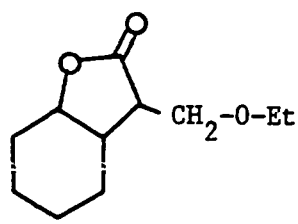
77% (Ref. 22)

SUMMARY

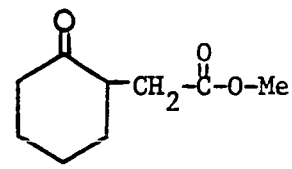
A synthetic route to 3-methylenehexahydrobenzofuran-2-one was explored. Starting with cyclohexanone the following new compounds were synthesized as outlined in Scheme I: Ethyl 2-(2-oxocyclohexyl)-3-ethoxy-propionate (16), 3-ethoxymethyl-(3H)-hexahydrobenzofuran-2-one (17), and methyl 2-(2-oxocyclohexyl)-acetate (22).



16



17



22

Yields of the reduction/lactonization and elimination reactions were satisfactory. However, yields of the alkylation reaction of the pyrrolidine enamine of cyclohexanone were poor. In an attempt to improve the alkylation yields, the enamine of cyclohexanone was replaced by the N-cyclohexyl imine magnesium bromide of cyclohexanone. This proved unsuccessful since none of the alkylated product could be isoleted.

EXPERIMENTAL

All melting points and boiling points are uncorrected. All solvents were redistilled prior to use. Anhydrous ethanol was prepared by distillation from magnesium. Other anhydrous solvents were prepared by distillation from calcium hydride. Anhydrous tetrahydrofuran was purchased (Fisher Scientific). Column chromatography supports were silicAR CC-7 (Mallinckrodt, 100/200 mesh) and silica Gel H (Merck AG, Darmstadt). High speed liquid chromatography was carried out on Chromasep S (Chromatec, Ashland, Mass.).

Thin layer chromatography was performed on 5 x 20 cm glass plates coated with silica gel H (Merck AG, Darmstadt) or on prepared plates of Sil Gel G/UV_{254} (Macherey-Nagel & Co. Duren). The developed plates were placed under ultraviolet light and/or exposed to iodine vapors for visualization of the chromatogram.

Gas chromatographic analyses were performed on Varian Aerograph Model 1220-1 or Aerograph Model 1740-1 gas chromatograph. The infrared (ir) spectra were recorded on a Beckman IR-8 spectrophotometer. The infrared spectra were run as a thin film between two sodium chloride discs.

Proton magnetic resonance (pmr) spectra were taken on Varian A-60 or T-60 spectrometers using tetramethylsilane (TMS) as an internal reference. Samples were run in varying concentrations in carbon

tetrachloride. Chemical shifts are reported in δ-units (parts per million from TMS) and are followed by the multiplicity of the signal, the number of protons, the corresponding coupling constant(s) and the assignment. The multiplicities are denoted by the symbols: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; and m, multiplet.

The mass spectra were taken on a Hitachi Perkin-Elmer RMU-7E using perfluorokerosene-H as an internal reference. Major peaks and molecular ions are reported followed by the percentage of the base peak.

Combustion analyses were carried out by Mr. E. Meier, Chemistry Department, Stanford University, Stanford, California.

Methyl 2-Bromo-3-methoxypropionate (8). The procedure of Wood and du Vigneaud[8] was used except that methyl 2,3-dibromopropionate was not isolated. Bromine (159.8 g, 1 mol) in 100 ml of dry methanol was added dropwise over a one hour period to vigorously stirred methyl acrylate (7) (86.1 g, 1 mol) previously cooled to 0°. After addition of the bromine solution was complete the reaction mixture was stirred for 2.5 hr at 0°.

Sodium methoxide, prepared by adding sodium (23 g, 1 mol) to 300 ml of dry methanol, was added dropwise to the cold stirred reaction mixture over a 30 minute period. Sodium bromide began to precipitate 5 minutes after the addition was started. After all of the sodium methoxide solution had been added the reaction mixture was allowed to come to room temperature and then was stirred for one hour. Carbon dioxide, generated from dry ice, was bubbled through the reaction mixture until the solution was neutral to pH paper. Ether (200 ml) was added to the

reaction mixture and the solids were removed by filtration. The salt cake was washed with ether and the solvent was removed under reduced pressure. Ether (200 ml) was added to the residue and the resulting solution extracted with 0.1 N hydrochloric acid (2 x 100 ml), saturated calcium chloride (100 ml), and finally with distilled water (100 ml). The ether solution was dried over anhydrous sodium sulfate and the ether was removed under reduced pressure. The resulting yellow oil was distilled to yield methyl 2-bromo-3-methoxypropionate as a clear liquid (63.64 g, 32.2%), b.p. 78-83° (10 mm) [lit.[23] b.p. 70-80° (6 mm)].

Cyclohexanone Pyrrolidine Enamine (10). The procedure of Stork and coworkers[10] was used. To 117.8 g (1.2 mol) of cyclohexanone (9) in 300 ml of benzene was added, in one portion, 93.8 g, (1.32 mol) of pyrrolidine. The mixture was refluxed with azeotropic removal of water until no more water was produced (approximately 6 hours). The benzene was removed under reduced pressure. The resulting yellow oil was distilled to yield the enamine as a clear liquid (136.4 g, 75.3%) b.p. 106-109° (13 mm) [lit.[10] b.p. 105-107°(13 mm)].

Methyl 2-(2-oxocyclohexyl)-3-methoxypropionate (11). To a solution of methyl 2-bromo-3-methoxypropionate(8) (21.6 g, 0.11 mol) in 500 ml of dioxane was added the cyclohexanone pyrrolidine enamine (10) (16.6 gm, 0.11 mol). The mixture was refluxed under nitrogen for 72 hours. Water (25 ml) was added and the mixture was refluxed for 2.25 hr. The reaction mixture was allowed to cool and a large excess of water was added.

The mixture was extracted with ether (3 x 200 ml) and the ether solution was extracted with 5% hydrochloric acid (2 x 50 ml) and 5% sodium bicarbonate (50 ml). The ether solution was dried over anhydrous magnesium sulfate and the ether was removed under reduced pressure. The resulting yellow oil was distilled to yield a pale yellow liquid (1.506 g, 6.7%) b.p. 143-147°.

The pale yellow liquid was chromatographed on 30 g of silicAR CC-7 beginning with benzene as the eluent and progressing through 6% ethyl acetate-benzene mixture to yield methyl 2-(2-oxocyclohexyl)-3-methoxypropionate (1.055 g, 4.7%): ir (thin film) 1740 cm^{-1} (ester) and 1714 cm^{-1} (ketone); pmr (CCl_4) δ 3.3-3.8 (m, 3, 2 methylene protons next to the ether oxygen and one proton α to the carbonyl), 3.6 (s, 3, methyl ester), 3.2 (s, 3, methyl ether) and 2.5-1.0 (m, 9, cyclohexanone).

3-Methoxymethyl-(3H)-hexahydrobenzofuran-2-one (12). To a solution of methyl 2-(2-oxocyclohexyl)-3-methoxypropionate (11) (1.055 g, 5 mmol) in methanol (20 ml) was added sodium borohydride (0.265 g, 7 mmol) and the mixture was allowed to stir at room temperature for 2.5 hr. The pH was adjusted to 2 (pH paper) with concentrated hydrochloric acid and the mixture was allowed to stir at room temperature for 14.5 hr.

The solvent was removed under reduced pressure and the residue was taken up in ether. The ether solution was washed with 5% sodium bicarbonate (2 x 25 ml) and water (2 x 25 ml), then dried over sodium sulfate and the ether was removed under reduced pressure. The resulting

yellow liquid was chromatographed on 15 g of silicAR CC-7 using 3% ethyl acetate-benzene as eluent to yield 3-methoxymethyl-(3H)-hexahydrobenzofuran-2-one (12) (0.5167 g, 56.2%): ir (thin film) 1775 cm^{-1} (lactone carbonyl), 1170 and 1070 cm^{-1} (ester carbon-oxygen stretch) and 1105 cm^{-1} (ether carbon-oxygen stretch); pmr (CCl_4) δ 3.36-3.7 (m, 3, 2 methylene protons adjacent to the oxygen and one proton α to the carbonyl), 3.3 (s, 3, methyl ether), 2.5-1.0 (m, 10, cyclohexane) and 4.4 (m, 1, proton under lactone oxygen).

3-Methylenehexahydrobenzofuran-2-one (5). Potassium-tert-butoxide (0.3136 g, 28 mmol) was dissolved in 25 ml of tert-butyl alcohol and 25 ml of ether. To this stirred solution under nitrogen was added dropwise over a 30 minute period 3-methoxymethyl-(3H)-hexahydrobenzofuran-2-one (12) (0.5167 g, 2.8 mmol) in 50 ml of ether. The mixture was allowed to stir one hr at room temperature after the addition was complete.

The pH of the reaction mixture was adjusted to 1 (pH paper) with concentrated hydrochloric acid and the mixture was allowed to stir for 72 hr. Additional ether (100 ml) was added and the organic phase was separated. The ether solution was washed with 5% sodium bicarbonate (50 ml) and water (50 ml). The aqueous solutions were combined, saturated with sodium chloride and extracted with ether. The ether solutions were combined, dried over sodium sulfate and the ether was removed on the rotary evaporator. Infrared (thin film) and pmr (CCl_4) spectra indicated the reaction mixture contained starting material, 33%, (2-hydroxycyclohexyl)-acrylic acid (13), 12% and

3-methylenehexahydrobenzofuran-2-one (5), 55%. No further purification was attempted with this mixture.

Ethyl 2-bromo-3-ethoxypropionate (15). The procedure of Wood and du Vigneaud[8] was used except that ethyl 2,3-dibromopropionate was not isolated. Bromine (159.8 g, was added dropwise to cold (0°) stirred ethyl acrylate (14) (100.12 g, 1 mol) over a one hour period. After the addition of bromine was complete the temperature was maintained at 0° and the solution was allowed to stir for 3 more hr.

Sodium ethoxide, prepared by adding sodium (23 g, 1 mol) to 500 ml of absolute ethanol, was added dropwise to the cold, stirred mixture over a one hr period. NaBr began to precipitate immediately. When the addition was complete the reaction mixture was allowed to come to room temperature. Carbon dioxide, generated from dry ice, was bubbled through the reaction mixture until the mixture was neutral to pH paper, about one hr. Ether (500 ml) was added to the reaction mixture and the solids were removed by filtration. The salt cake was washed with ether and the ether and ethanol were removed under reduced pressure. The residue was taken up in ether (200 ml) and washed with 5% hydrochloric acid (3 x 100 ml), saturated calcium chloride (100 ml), 5% sodium bicarbonate (3 x 50 ml) and water. The ether solution was dried over sodium sulfate and the ether was removed under reduced pressure. The resulting yellow oil was distilled to yield ethyl 2-bromo-3-ethoxypropionate (15) (167.42 g, 74.4%), b.p. 88-93° (10 mm) [lit.[8] b.p. 84-88° (10-12 mm)].

Ethyl 2-(2-oxocyclohexyl)-3-ethoxypropionate (16).A.-- To ethyl 2-bromo-3-ethoxypropionate (15) (67.5 g, 0.3 mol) in toluene (300 ml) was added cyclohexanone pyrrolidine enamine (10) (44 g, 0.29 mol). The mixture was refluxed under nitrogen for 72 hours. Acetic acid (50 ml) and water (50 ml) were added to the reaction mixture and it was allowed to reflux for 17 hr.

After the reaction mixture had cooled, ether (300 ml) was added and the two layers were separated. The organic phase was washed with 5% hydrochloric acid (3 x 100 ml), 5% sodium bicarbonate (3 x 100 ml), and water (2 x 50 ml), then dried over sodium sulfate and the solvent was removed under reduced pressure. The resulting yellowish brown oil was distilled to yield ethyl 2-(2-oxocyclohexyl)-3-ethoxypropionate (16) (15.36 g, 24.5%) b.p. 147-152° at 5.5 mm. The distillate was determined to be 84% 16 by gas chromatographic analysis.

An analytical sample was prepared by chromatography of distilled 16 on 40 gm of silica gel H beginning with benzene and progressing through 5% ethyl acetate-benzene. Fractions 4 and 5 (benzene) yielded a pale yellow oil which was chromatographed on high speed liquid chromatography using Chromasep S as the support and 1% 2-propanol/hexane as the eluent to yield a clear, colorless oil: n_D^{25} 1.4590; ir (thin film) 1735 cm^{-1} (ester carbonyl), 1712 cm^{-1} (ketone carbonyl), 1175 cm^{-1} (ester carbon-oxygen stretch), 1110 cm^{-1} (ether carbon-oxygen stretch); pmr (CCl_4) δ 4.08 (q, 2, methylene of ethyl ester), 3.46 (m, 4, methylene on each side of the ether oxygen), 3.2-1.4 (m, 10, cyclohexanone ring protons) and 1.2 (q, 6, two overlapping triplets from the terminal methyl groups). Mass spectrum 242 (1), 197 (7), 145 (78),

134 (15), 120 (48), 119 (46), 105 (100), 99 (42), 91 (32), 59 (28), 55 (25), 43 (23) and 41 (30).

Anal. Calcd for $C_{13}H_{22}O_4$: C, 64.44; H, 9.15. Found C, 64.65; H, 9.11.

B.-- To ethanol (250 ml), dried by refluxing over magnesium and distilling, was added ethyl 2-bromo-3-ethoxypropionate (15) (22.5 g, 0.1 mol) and cyclohexanone pyrrolidine enamine (15.1 g, 0.1 mol). The mixture was refluxed under nitrogen for 10 hr. Glacial acetic acid (30 ml) and water (70 ml) were added and the mixture was allowed to reflux for an additional 12 hr.

The reaction mixture was concentrated to 100 ml under reduced pressure. Ether (100 ml) and water (50 ml) were added to the concentrate and the layers were separated. The ether layer was washed with 5% hydrochloric acid (3 x 25 ml), 5% sodium bicarbonate (2 x 25 ml) and water (25 ml), and then dried over sodium sulfate. The aqueous layers were combined, saturated with sodium chloride and extracted with ether (200 ml). The ether solution was washed with 5% sodium bicarbonate (2 x 50 ml) and water (25 ml), then dried over sodium sulfate. The ether solutions were combined and the ether removed under reduced pressure to yield a yellow oil (13.85 g). The oil was distilled to yield 16 (4.745 g, 15.5%) a colorless oil, b.p. 148-153° (5.5 mm).

C.-- The alkylation of the enamine was attempted under the conditions described above except that dimethyl formamide was used as the solvent. None of the desired product could be isolated.

3-Ethoxymethyl-(3H)-hexahydrobenzofuran-2-one (17). To a

stirred solution of ethyl 2-(2-oxocyclohexyl)-3-ethoxypropionate (16) (13 g, 60 mmol) in 5 ml of 95% ethanol was added dropwise over a 30 minute period sodium borohydride (0.629 g, 16 mmol) in 20 ml of 95% ethanol. The mixture was allowed to stir for one hr at room temperature. The pH was adjusted to 6 (pH paper) with glacial acetic acid and the resulting mixture was allowed to stir for one hour. The ethanol was removed under reduced pressure and ether was added to the residue. The ether solution was washed with water (2 x 30 ml), 5% sodium bicarbonate (2 x 20 ml), and again with water (20 ml), then dried over sodium sulfate and finally the ether was removed on a rotary evaporator. The last traces of solvent were removed under vacuum (0.5 mm) to yield a yellow oil (9.04 gm, 85.4%). Infrared analysis indicated that reduction was complete but lactonization was not.

The yellow oil was taken up in 10 ml of 95% ethanol. To this solution was added 10% hydrochloric acid (10 ml), and water (10 ml) and the final mixture was refluxed for 18 hr. The solution was concentrated and ether (50 ml) was added to the remaining aqueous solution. The layers were separated and the ether solution was washed with 5% sodium bicarbonate (2 x 20 ml) and water (2 x 20 ml), then dried over sodium sulfate and concentrated under reduced pressure. The residue was distilled to yield 3-ethoxymethyl-(3H)-hexahydrobenzofuran-2-one (17) (6.73 g, 56.6%) b.p. 104-108° (0.1 mm). The distilled product was found to be 98.5% pure by gas chromatographic analysis.

An analytical sample was prepared by high speed liquid chromatography using Chromasep S as the support and 1% 2-butanol/hexane as the eluent to yield a clear, colorless oil: n_D^{25} 1.4687; ir (thin film)

1780 cm^{-1} (γ lactone), 1170 and 1105 cm^{-1} (carbon-oxygen stretch of the lactone), 1010 cm^{-1} (carbon-oxygen stretch of the ether); pmr (CCl_4) δ 3.9-3.3 (m, 5, 4 protons next to the ether oxygen and one proton under the lactone oxygen), 1.23 (t, 3, methyl of ether) and 2.6-0.8 (m, 10, cyclohexane). Mass spectrum 198 (2), 154 (7), 148 (10), 134 (32, 128 (27), 120 (22), 119 (100), 117 (38), 105 (62), 91 (32), 67 (30), 59 (20), 55 (39) and 41 (48).

Anal. Calcd. for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15. Found: C, 66.47; H, 9.08.

3-Methylenehexahydrobenzofuran-2-one (5). To tert-butyl alcohol (125 ml) was added potassium tert-butoxide (3.22 g, 28.6 mmol) and the mixture was placed under a nitrogen atmosphere. To the stirred, basic, solution was added dropwise over a 30 minute period 3-ethoxy-(3H)-hexahydrobenzofuran-2-one (17) (5.03 g, 25.4 mmol) in 50 ml of ether. The mixture was allowed to stir under nitrogen for 2 hr after addition of the lactone was complete. The pH was adjusted to 3 (pH paper) with concentrated hydrochloric acid and the mixture was allowed to stir for 15 minutes.

The ether and tert-butyl alcohol were removed on the rotary evaporator and 50 ml of ether and 50 ml of water were added to the residue. The aqueous and organic layers were separated and the ether solution was washed with 5% sodium bicarbonate (2 x 25 ml) and water (25 ml), then dried over sodium sulfate and evaporated. The residue was distilled to yield 3-methylenehexahydrobenzofuran-2-one (1.886 g, 47.6%) b.p. 114-118° (1.5 mm) [lit.[5] 70° (0.1 mm)] as a pale yellow

oil. This yellow oil was chromatographed on silicAR CC-7 (60 gm) using benzene as the eluent to yield 3-methylenehexahydrobenzofuran-2-one (5) (1.776 g, 44.8%) as a colorless oil.

A spectral sample was prepared by high speed liquid chromatography using Chromasep S as the support and 1% 2-butanol/hexane as the eluent: n_D^{25} 1.4974; ir (thin film) 3020 cm^{-1} (double bond), 1765 cm^{-1} (exo-methylene lactone), 1664 cm^{-1} (exo-methylene double bond); pmr (CCl_4) δ 6.07 (d, 1, J=3, exo-methylene), 5.25 (d, 1, J=3, exo-methylene), 4.45 (m, 1, proton under lactone oxygen), 2.96 (m, 1, allylic proton), 2.0-1.0 (m, 8, cyclohexyl protons). Mass spectrum 152 (29) 138 (13), 124 (100) 110 (21), 109 (28) 96 (46) 95 (82), 91 (21), 82 (35), 81 (45), 79 (43), 77 (23), 68 (32) 67 (80), 57 (22), 55 (51) 54 (51) 54 (51), 53 (48), and 41 (70).

Methyl 2-(2-oxocyclohexyl)-acetate (22). The procedure used was that developed by Baumgarten[7] for the ethyl ester. To anhydrous methanol (200 ml) was added methyl bromoacetate (21) (61.2 g, 0.2 mol) and the mixture placed under a nitrogen atmosphere. To this refluxing, stirred solution was added cyclohexanone pyrrolidine enamine (10) (30.4 g, 0.2 mol) dropwise over a 1 hr period. The reaction mixture was allowed to reflux an additional 2 hr. Water (25 ml) was then added and the mixture refluxed for an additional 2 hr. The reaction mixture was cooled and the methanol removed on the rotary evaporator. Water (100 ml) was added to the residue and the solution was extracted with ether (3 x 100 ml). The ether solution was dried over sodium sulfate and the ether was removed under reduced pressure to yield a light orange

oil. Gas chromatography indicated a 57.5% yield of methyl 2-(2-oxocyclohexyl)-acetate. The light orange oil was distilled to yield (22) (16.64 g, 49%) a colorless oil b.p. 90-92° (1 mm) which was shown to be 99% pure by gas chromatographic analysis.

An analytical sample was prepared by high speed liquid chromatography using Chromasep S as the support and 1% 2-butanol-hexane as the eluent: n_D^{25} 1.4619; ir (thin film) 1740 cm^{-1} (ester) and 1715 cm^{-1} (six-membered ring ketone); pmr (CCl_4) δ 3.63 (s, 3, methyl of ester), 3.0-1.0 (m, 11, rest of protons); Mass spectrum 170 (16), 139 (40), 138 (68), 110 (20), 97 (34), 82 (27), 74 (33), 67 (27), 59 (35), 55 (100), 54 (21), 43 (25), 42 (25), and 41 (76).

Anal. Calcd. for $C_9H_{14}O_3$: C, 63.51; H, 8.29. Found: C, 59.36, 59.07; H, 7.66, 7.77.

N-cyclohexylidenecyclohexylamine (19). The procedure of Stork and Dowd[14] was used. To a solution of cyclohexanone (9) (98.14 g, 1 mol) in benzene (300 ml) was added cyclohexylamine (19) (99.14 g, 1 mol). The mixture was refluxed with azeotropic removal of water until water ceased to come over (about 6 hours). The benzene was evaporated and the residue distilled to yield the imine 19 as a colorless oil (124.96 g, 69.8%), b.p. 71-73° (0.04 mm) [lit.[24] b.p. 92-94° (2 mm)].

Ethyl Magnesium Bromide. To magnesium ribbon (5.45 g, 0.224 mol) in 300 ml of anhydrous tetrahydrofuran under nitrogen was added ethyl bromide (24.42 g, 0.224 mol) in one portion. The temperature was maintained at 25°. Ethyl bromide was added as necessary during the

course of the reaction to consume all of the magnesium ribbon. The ethyl magnesium bromide was not isolated but used immediately.

Attempted Synthesis of ethyl 2-(2-oxocyclohexyl)-3-ethoxy-propionate via Schiff Base Salt Reactions. (16). The procedure of Stork and Dowd[14] was used. To the ethyl magnesium bromide reaction mixture, under nitrogen, was added N-cyclohexylidenecyclohexylamine (19) (40.1 g, 0.224 mol). The mixture was refluxed until the evolution of ethane had stopped (about 2 hours) and then allowed to cool to room temperature. To the cooled reaction mixture was added ethyl 2-bromo-3-ethoxypropionate (15) (54.9 g, 0.265 mol) and the mixture refluxed under nitrogen for 10.5 hr. 10% hydrochloric acid (200 ml) was added and the mixture was refluxed for 1.75 hr.

The reaction mixture was cooled and 200 ml of ether was added. The phases were separated and the ether solution was washed with 5% hydrochloric acid (2 x 50 ml), 5% sodium bicarbonate (2 x 50 ml), and water (50 ml) then dried over sodium sulfate. The aqueous solutions were saturated with sodium chloride and extracted with 200 ml of ether. The ether solution was washed with 5% hydrochloric acid (2 x 50 ml), 5% sodium bicarbonate (2 x 5 ml) and water (50 ml), then dried over sodium sulfate. The two ether solutions were combined and the ether removed on the rotary evaporator. The resulting yellowish brown oil was distilled but none of compound 16 was found.

Attempted synthesis of methyl 2-(2-oxocyclohexyl)-acetate (22). An attempt was made to alkylate the cyclohexyl imine magnesium bromide

of cyclohexanone with methyl bromoacetate using the procedure described above. However, none of the desired product was isolated.

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II. HALITOXIN-R: ISOLATION AND PARTIAL STRUCTURE DETERMINATION OF A TOXIC MATERIAL FROM THE SPONGE *HALICLONA RUBENS*

INTRODUCTION

Haliclona rubens is a red tubular sponge commonly found in shallow (15 feet or less) coastal or reef waters of the Caribbean. The sponge grows as a single tubular stalk or as a sparsely branched, fleshy, treelike structure.

It has been reported[1,2] that the alcoholic extract of *Haliclona rubens* is toxic to many species of fish but that at least two species of angelfish feed upon the sponge with no apparent ill effect. It was the purpose of the work presented here to attempt to isolate and characterize the toxin produced by this sponge. The fact that the sponge contained a toxic substance was discovered when the extracts were submitted for cancer screening. The two systems used to test for anticancer activity were the cell culture human epidermoid carcinoma of the nasopharynx [KB, reported as the effective dose (ED_{50}) in micrograms/ml needed to inhibit the growth of cells to 50% of that of a control culture] and *in vivo* testing in mice of lymphoid leukemia (LE, reported as milligrams of sample/kilogram of mouse body weight and the survival time of test animals to control animals; expressed as percent). For KB, a report of any number less than 10 indicated a promising level of activity while for

LE a result of 125% indicated activity.

A toxic fraction (active by KB, toxic by LE) was isolated from the sponge and the major constituent of that fraction (> 80% as judged by proton magnetic resonance analysis) which was presumed to be the toxic material was named halitoxin-R. A partial structure for this compound based on spectral data and what little chemical evidence could be obtained on the impure material is presented.

An aqueous extract of a related sponge Haliclona viridis, has previously been reported to have toxicity in mice[3] and this crude extract was named halitoxin. The toxicity of the aqueous extract from Haliclona viridis is much less than that of the aqueous ethanolic extract of Haliclona rubens (270 mg/kg of body weight for halitoxin versus 5 mg/kg of body weight for halitoxin-R; both injections intraperitoneal in mice). It has been reported[3] that at 270 mg/kg dosage level of halitoxin acute toxic effects occur within one hour, while with halitoxin-R at 25 mg/kg death occurs within thirty seconds and at 9 mg/kg usually occurs within three minutes.

In several ways halitoxin and halitoxin-R are similar. The symptoms exhibited by a mouse injected with halitoxin and halitoxin-R are very similar (tremors, convulsions, and paralysis preceding death). The solubility of halitoxin and halitoxin-R are also similar (soluble in water and alcohols but not in other organic solvents).

There are, however, some very important differences between halitoxin and halitoxin-R. Halitoxin loses its potency when stored in solution in the cold for several days[3]. On the other hand halitoxin-R retains its potency when stored in solution at room temperature over

extended periods of time. Halitoxin is of relatively high molecular weight (nondialyzable)[3], while halitoxin-R has an apparent molecular weight of less than 1,000 (membrane filtration). Because of the differences between halitoxin and halitoxin-R it is likely that they are quite different molecules.

RESULTS AND DISCUSSION

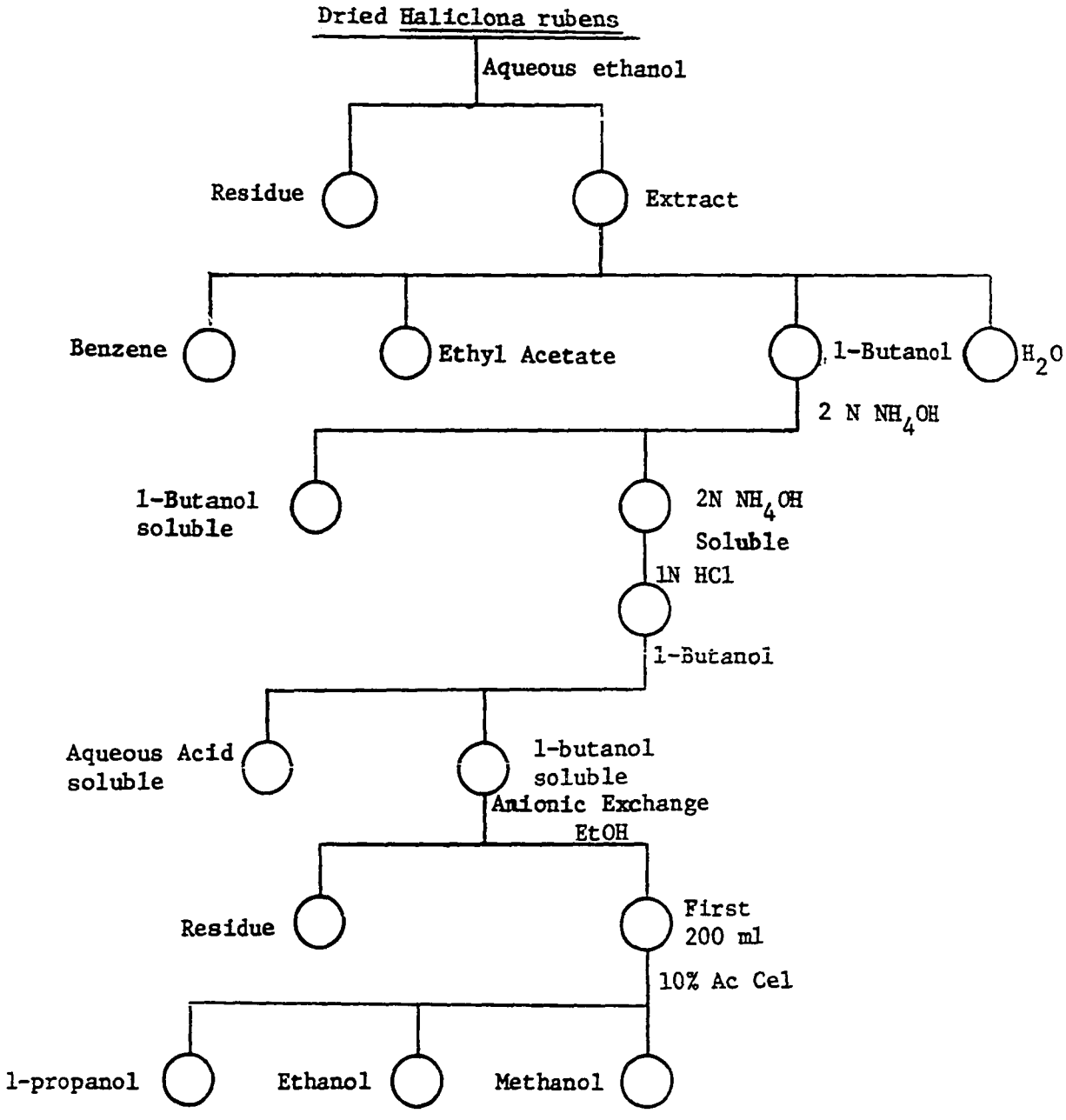
The sponge Haliclona rubens was collected from reefs near Parguera, Puerto Rico. The sponge material was air dried and then broken into small pieces for shipment and extraction.

All extractions of the sponge were performed at room temperature. Samples from most of the fractions resulting from the separation schemes were subjected to KB testing to determine which fraction(s) retained anticancer activity and hence should be carried on. Three different extraction and fractionation schemes were used to obtain the active material.

The first extraction and fractionation method (Scheme I) closely followed the general procedure of Burkholder and co-workers[4]. Each succeeding separation method (Schemes II and III) was designed to try to obtain crude halitoxin-R with fewer contaminants.

In the first method (Scheme I) the dried sponge was exhaustively extracted with aqueous ethanol (KB, 7.0; LE toxic to 5 mg/kg dose). The concentrated aqueous ethanolic extract was sequentially extracted with benzene (KB, 54.0), ethyl acetate (KB, 49.0), and 1-butanol (KB, 4.8; LE toxic to 5 mg/kg). The 1-butanol fraction was extracted with 1 N ammonium hydroxide; the ammonium hydroxide extract was acidified and the acidic solution was extracted with 1-butanol (KB, 5.3). This 1-butanol extract was then chromatographed on 10% acetylated cellulose. The

Scheme I

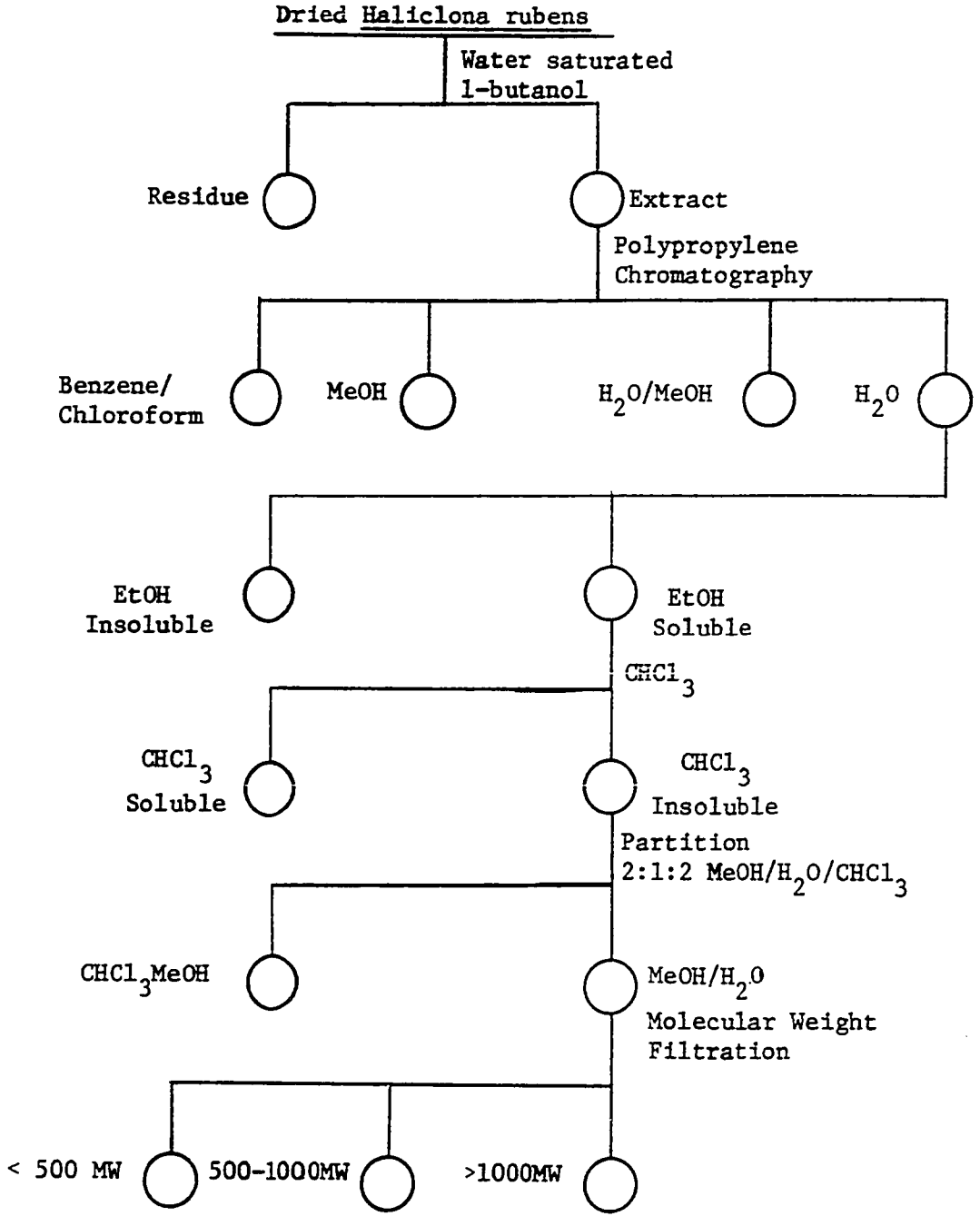

Dried Haliclona rubens
Aqueous ethanol
Residue
Extract
Benzene
Ethyl Acetate
1-Butanol
H_2O
2 N NH_4OH
1-Butanol soluble
2N NH_4OH Soluble
1N HCl
1-Butanol
Aqueous Acid soluble
1-butanol soluble
Anionic Exchange
EtOH
Residue
First 200 ml
10% Ac Cel
1-propanol
Ethanol
Methanol

cellulose was eluted with three solvents, 2-propanol (KB, 4.7), ethanol (KB, 5.2) and methanol (KB, 7.1).

In the second method (Scheme II) the dried sponge was exhaustively extracted with 1-butanol saturated with water (KB, 50). The concentrated 1-butanol extract was chromatographed on polypropylene. The use of polyethylene to desalt an extract has been reported by Scheuer and Moore[5] in the isolation of palytoxin. It was hoped that the use of polypropylene would accomplish the same objective. The polypropylene column was eluted sequentially with water (KB, 5.3), water:ethanol (KB, 55.0), methanol (KB, > 100) and chloroform/benzene (KB, > 100). The water fraction was evaporated to dryness and the residue was extracted with ethanol. The ethanol solution was in turn evaporated to dryness and the residue was extracted with chloroform. The chloroform insoluble material was partitioned between methanol/water and chloroform/water. The methanol/water fraction was subjected to a molecular weight separation by membrane filtration through 500 and 1000 MW cut off membranes. Three fractions were collected < 500 MW (KB, > 100), 500-1000 MW (KB, 5.8) and > 1000 MW (KB, 5.7).

Each time a fraction had shown activity, a proton magnetic resonance (pmr) spectrum was taken of the material to see if there was a common spectral feature in the active fractions. It developed that there was such a feature. Each active fraction exhibited a series of three absorptions in the region from δ 9.1-7.9. As each successive purification step was performed and the same pattern kept appearing it became more probable that this feature was associated with the activity. Assuming that such a correlation does exist, pmr analysis was used instead of

Scheme II


Dried Haliclona rubens
Water saturated 1-butanol
Residue
Extract
Polypropylene Chromatography
Benzene/ Chloroform
MeOH
H_2O/MeOH
H_2O
EtOH Insoluble
EtOH Soluble
$CHCl_3$
$CHCl_3$ Soluble
$CHCl_3$ Insoluble
Partition 2:1:2 MeOH/H_2O/$CHCl_3$
$CHCl_3$MeOH
MeOH/H_2O
Molecular Weight Filtration
< 500 MW
500-1000MW
>1000MW

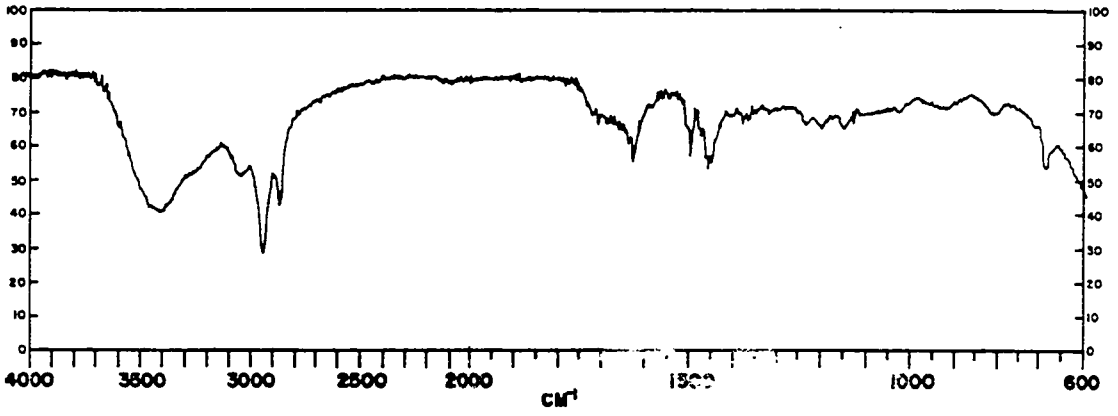
KB testing to locate the active fractions in the various stages of the third extraction method.

In the third extraction and fractionation method (Scheme III) the dried sponge was extracted with 1-propanol. The 1-propanol extract was then extracted with acetone and the acetone insoluble material was soaked with 2-butanol. Both the 2-butanol soluble and insoluble fractions contained halitoxin-R as judged by pmr analysis. Since both fractions contained the desired material, both were processed identically, but separately. Each fraction was chromatographed on a silicAR CC-4 column which was eluted first with ethanol, then with methanol/acetonitrile, and finally with methanol. By pmr analysis both the methanol/acetonitrile and the pure methanol fractions contained halitoxin-R, and therefore, these fractions were combined.

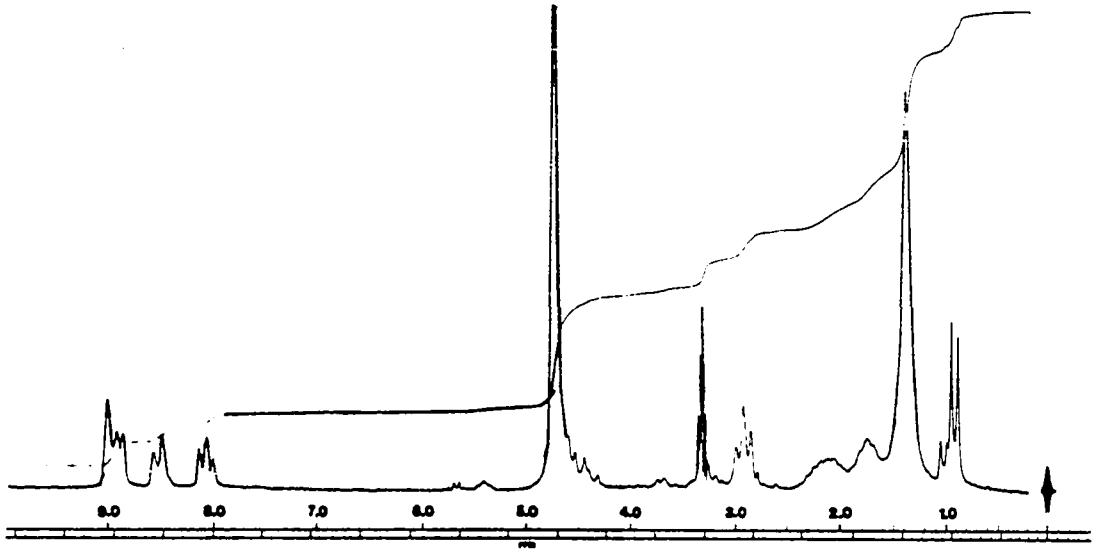
Because of the small amount of material obtained in the extraction schemes I and III, the > 500 MW material from the membrane filtration in Scheme II was carried on for further purification. The > 500 MW material was chromatographed on carboxymethyl Sephadex. A solution of 1 M sodium chloride was required to elute halitoxin-R from this column. The 1 M sodium chloride fraction was extracted with 1-butanol to dissolve halitoxin-R and leave (most of) the salt behind. The 1-butanol soluble material was twice chromatographed on silicAR CC-4 using ethanol and then methanol as eluents to give a yellow glass. That yellow glass gave the spectra shown in Figure VI. The proposed tentative structure for halitoxin-R (4 or 5) is based in large measure on these spectra.

The infrared spectrum of halitoxin-R (Figure VI) shows a major

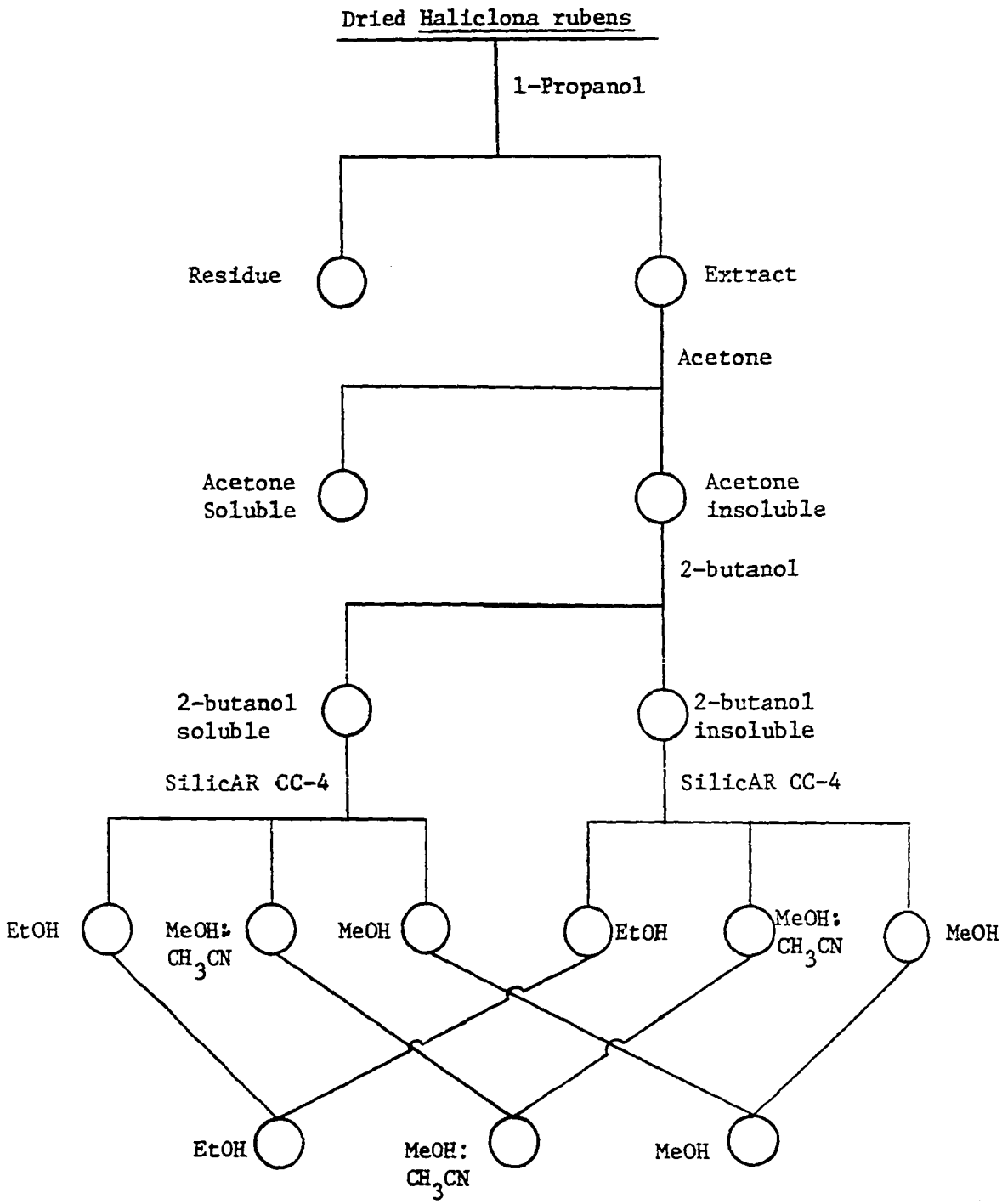
Figure VI
Spectra of Halitoxin-R
IR Spectrum (Thin Film) of Halitoxin-R



PMR Spectrum (methanol-d_4) of Halitoxin-R



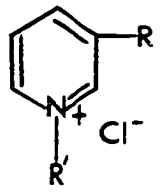
Scheme III


Dried Haliclona rubens
1-Propanol
Residue
Extract
Acetone
Acetone Soluble
Acetone insoluble
2-butanol
2-butanol soluble
2-butanol insoluble
SilicAR CC-4
SilicAR CC-4
EtOH
MeOH: CH_3CN
MeOH
EtOH
MeOH: CH_3CN
MeOH
EtOH
MeOH: CH_3CN
MeOH

absorption at 3495 cm^{-1} which would be appropriate for either hydroxyl, amine or water absorption. Chemical evidence (attempted acetylation and formylation) indicates that the absorption is probably caused by water rather than by hydroxyl or amine. At 3015 and 1630 cm^{-1} there are two absorptions which are attributable to a pyridinium ring. The remainder of the infrared bands are of the type expected for alkyl groups.

The pmr spectrum of halitoxin-R (Figure VI) is much more informative than the infrared spectrum. The singlet at δ 9.01 integrates for one proton and its chemical shift position is in the range expected for a proton between the positive nitrogen and α meta substituent in a pyridinium ring. The doublet at δ 8.9 also integrates for one proton and its chemical shift is appropriate for a proton ortho to the positive nitrogen in a pyridinium ring flanked by an ortho substitutent. The 5 Hz coupling apparent in the δ 8.9 signal is typical of ortho coupling in an aromatic ring. The one proton doublet at δ 8.53 is attributable to a proton para to the positive nitrogen of a pyridinium ring. The 7 Hz coupling is in the range expected for ortho protons on an aromatic ring. The chemical shift and multiplicity of the signal at δ 8.07 (double doublet) are suitable for a proton meta to the positive nitrogen of a pyridinium ring and flanked by two other protons. The coupling constants of the signal at δ 8.07 (J=7 and 5 Hz) are of the size expected for a proton on an aromatic ring with two ortho neighbors. This pmr data is all strongly indicative of a pyridinium ring substituted in the meta position. In addition, the downfield portion of the pmr spectrum of halitoxin-R is almost identical in chemical shift and splitting pattern with the pmr spectrum of 1-(2-carbamoylethyl)-3-methylpyridinium

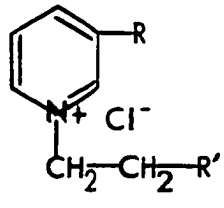
chloride[6]. Because of the method of isolation (cationic ion exchange chromatography and elution with sodium chloride) the counter ion for the positive nitrogen must be chloride ion. Therefore, the partial structure shown in 1 can be drawn.



1

Upfield from the pyridinium ring absorptions is a multiplet at δ 4.7 which integrates for 2 protons. This absorption is somewhat obscured because the hydroxyl peak of methanol-d_4 and the exchangeable protons in the sample fall almost on top of it. However, if 50 µl of pyridine-d_5 are added to the pmr solution, the hydroxyl absorption shifts downfield to δ 5.22 and it no longer obscures the absorption at δ 4.7. The position of the latter absorption is appropriate for two protons on a methylene carbon bonded to the positive nitrogen of a pyridinium ring.

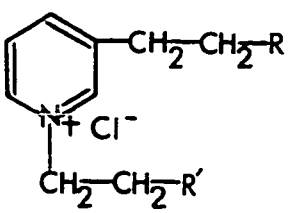
When the sample of halitoxin-K is irradiated at δ 4.7 the broad two proton multiplet centered at δ 2.12 is perturbed. Conversely irradiation at δ 2.12 perturbs the multiplet at δ4.7. Therefore, the partial structure shown in 2 can be drawn. However, this portion of the



2

spectrum is not readily interpretable because the splitting pattern of the protons on the methylene adjacent to the positive nitrogen appears to be poorly resolved. A possible explanation is that there is an impurity in the sample which also absorbs at δ 4.7.

Upfield from the absorption at δ 4.7 is a two proton triplet (J=7 Hz) at δ 2.93 which can be assigned to two protons on a methylene attached to one of the carbons of a pyridinium ring. When the protons absorbing at δ 2.93 are irradiated the broad multiplet at δ 1.76 is perturbed. Irradiation of the sample at δ 1.76 collapses the triplet at δ 2.93 to a singlet. From this data the partial structure of 2 can be expanded to that shown in 3.



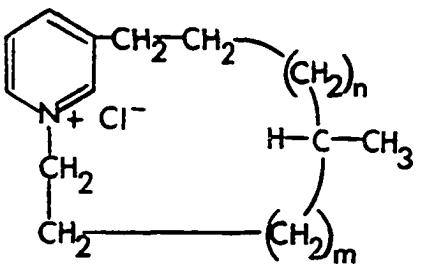
3

The broad multiplets at δ 2.12 and 1.76 have been assigned to methylene groups adjacent to the protons absorbing at δ 4.7 and 2.93, respectively, on the basis of decoupling evidence. The broad singlet at δ 1.41 integrates for 10 protons. The chemical shift of this absorption is in the range expected for methylene protons, either acyclic or possibly in a ring system of some sort.

The final absorption in the pmr spectrum is the doublet at δ 0.92 which integrates for 3 protons and has a J value of 7 Hz. The

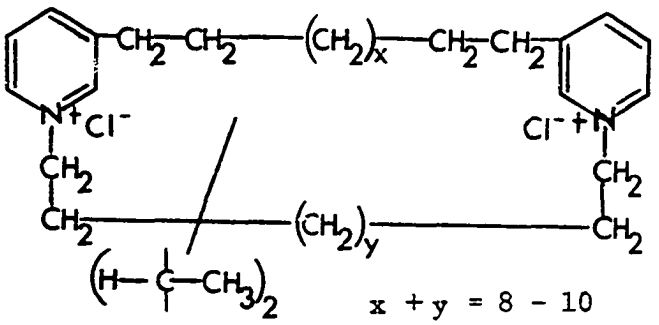
chemical shift, the splitting pattern, and the J value are all consistent with the signal of a secondary methyl group situated somewhere along a chain of methylene units.

Since there are no terminal methyl group absorptions in the pmr spectrum, the conclusion can be drawn that the methylene carbons are probably part of a ring system. The structures that are consistent with the infrared and proton magnetic resonance spectra are shown as 4 and 5.



m + n = 4 or 5

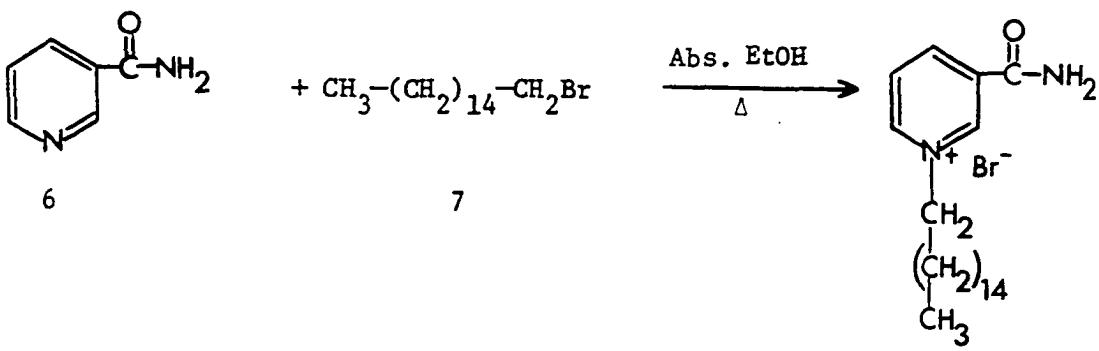
4



x + y = 8 - 10

5

In order to have a model system somewhat similar to the toxic material for comparison of pmr data and for evaluation of chemical degradation reactions bromide 8 was synthesized by condensing 1-hexadecyl bromide (7) with nicotinamide (6)[7].

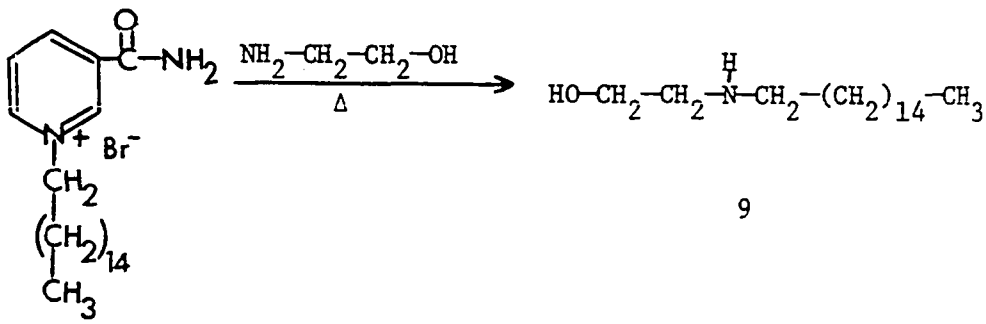


6 7

8 (63.4%)

The pmr and infrared spectra of the pyridinium salt 8 are shown in Figure VII. As can be seen there are similarities between the salt 8 and halitoxin-R. In particular the downfield (δ 9.1-7.9) absorptions in the pmr spectrum are similar to those present in the spectrum of halitoxin-R.

The pyridinium salt 8 was used to check reported[8-10] conditions for removal of the alkyl group from a quaternized nitrogen. Hunig and co-workers[8-10] have shown that if a quaternary amine salt is reacted with ethanolamine, one of the alkyl groups bonded to the positive nitrogen is transferred to the nitrogen of ethanolamine. In the case of a pyridinium salt the result of this reaction would be the transfer of the alkyl group bonded to the nitrogen of the pyridinium ring. The reaction of the pyridinium salt 8 with ethanolamine produced the hydroxy amine 9 (identified by pmr analysis).



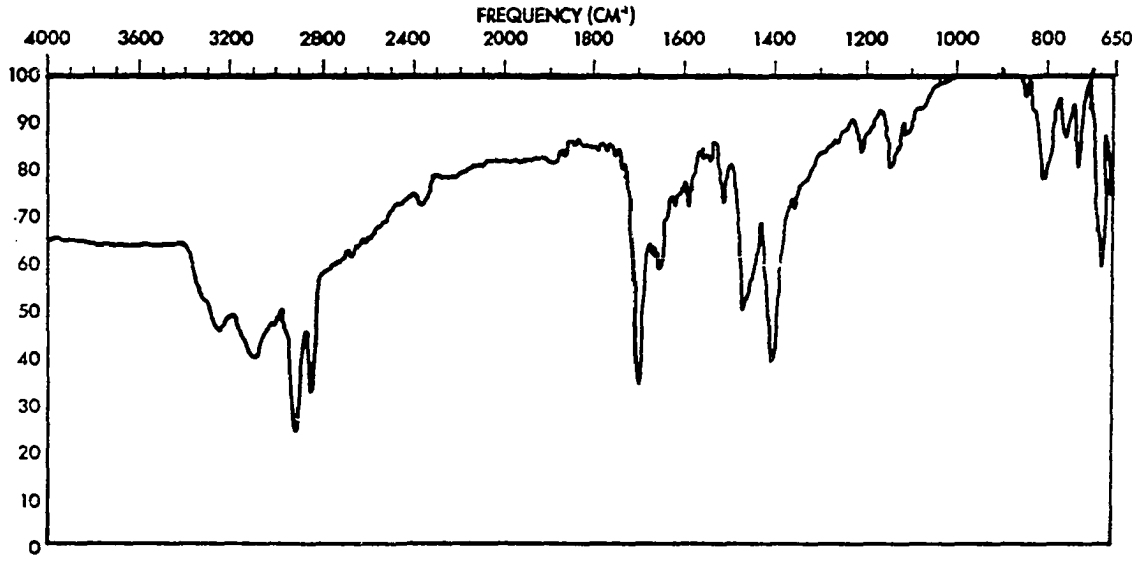
8

9

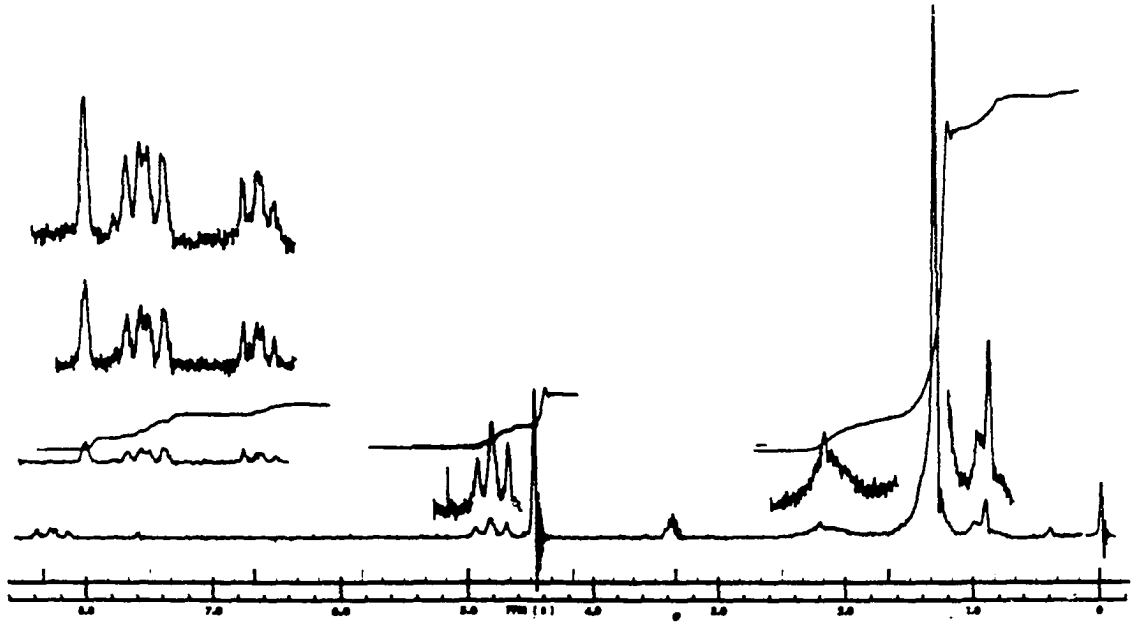
Since the reaction between ethanolamine and the pyridinium salt 8 was successful, it was next attempted on impure halitoxin-R from Scheme II. Even after prolonged reaction times no recognizable products could be isolated.

In an attempt to obtain a product that would be more amenable

Figure VII
Spectra of 3-Carbamoyl-1-hexadecylpyridinium bromide (8)
IR Spectrum (KBr) of 8



PMR Spectrum (chloroform-3/methanol-d_4) of 8



to isolation and purification a reduction of the pyridinium ring was attempted. It is well known[11] that pyridinium salts reduce readily over platinum oxide in absolute ethanol. The reduction reaction was attempted on halitoxin-R from Scheme II. The pmr spectrum of the crude reaction mixture indicated that the pyridinium ring had been reduced (no absorption at δ 9.1-7.9). This result is supportive evidence for the presence of a pyridinium ring. However no recognizable product could be isolated.

Because the infrared spectrum indicated the possible presence of a hydroxyl or amine functionality, attempts were made to derivatize any such moieties. Halitoxin-R from Scheme II was reacted with acetic anhydride in pyridine, acetic-formic anhydride, acetic anhydride in triethyl amine and acetic anhydride with boron trifluoride etherate. In each case the halitoxin-R was recovered unaltered.

In an attempt to isolate a pure derivative or a major piece of halitoxin-R, the following chemical conversions were tried:

1. Oxidation.
    a. Sodium dichromate and sulfuric acid
    b. Concentrated nitric acid
2. Reduction.
    a. Sodium dithionite
    b. Sodium borohydride
3. Hydrolysis.
    a. Sodium hydroxide, water, methanol
    b. Potassium carbonate, water, methanol
    c. Hydrochloric acid, water, methanol

4. Complex Formation and Anion Exchange.

    a. Ferric chloride

    b. Picric acid

    c. Sodium tetraphenylborate

    d. Chloroplatinic Acid

In the oxidation and reduction reactions the product was a brownish yellow gum from which nothing recognizable could be isolated. The basic hydrolysis gave polymer like material, while halitoxin-R was recovered unchanged from acidic hydrolysis. Complex formation and anion exchange appeared to occur, but the resulting precipitates were completely unmanageable.

Numerous attempts were made to purify halitoxin-R by chromatography. The following is a list of the various types of chromatographic methods and materials that were used.

1. TLC

    a. Silica Gel H.

    b. Silica Gel G.

    c. Cellulose.

    d. Cellulose, 10% acetylated

    e. Kieselguhr

    f. Polyamide

    g. Cellulose, DEAE

    h. Neutral alumina

    i. Acid washed alumina

    j. Acid washed silica gel

2. Column packings

    a. SilicAR CC-4

    b. Cellulose, 10% acetylated

    c. Kieselguhr

    d. Polyamide

    e. Cellulose, DEAE

    f. Acid alumina

    g. Sephadex G-10 and G-15

    h. Sephadex LH-20

    i. Sephadex CM-25

    j. Strong anionic and cationic ion exchange resins

    k. Celite

Various solvent systems were tried with each of the TLC absorbents. Most of the chromatographic columns were eluted with solvent gradients. SilicAR CC-4 and Sephadex CM-25 were the only column packings to give any measurable purification of the toxic material.

In spite of the prodigious amount of effort expended in trying to obtain the toxin or one of its derivatives in a pure state this was not accomplished. In nearly every reaction or purification method tried the resulting product was a brownish yellow glass or gum. Because of this fact one is tempted to speculate that something is complexed with the pyridinium ring and interfered with whatever was attempted.

SUMMARY

The results of efforts to isolate a toxic substance designated halitoxin-R from the sponge Haliclona rubens are discussed. Two possible structures for halitoxin-R are presented which are consistent with all of the data now available. All of the spectral data is consistent with the conclusion that halitoxin-R contains a pyridinium ring which in turn is an integral part of a larger ring system.

This section represents four years of extremely frustrating work, and in no way represents the amount of work expended in attempts to purify and characterize halitoxin-R.

EXPERIMENTAL

All melting points are uncorrected. All solvents except 95% ethanol were distilled prior to use. Absolute ethanol was purchased (U.S. Industrial Chemical Co.). Column chromatography packings were silicAR CC-4 (Mallinckrodt, 100/200 mesh), 10% acetylated cellulose (Macherey-Nagel and Co., Duren), kieselguhr G (E. Merck AG, Darmstadt), polyamide (M. Woelm, Eschwege), DEAE cellulose (Whatman), acidic alumina (E. Merck AG, Darmstadt), Sephadex G-10 and G-15 (Pharmacia Fine Chemicals, Inc.), Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.), Amberlite IRA-900 (Rohm and Haas), Dowex 50W (J. T. Baker), Bio-Rex 70 (Bio-Rad), Celite (Johns Manville), polypropylene MX 3574.02 (Dow Chemical), and Sephadex CM-25 (Pharmacia Fine Chemicals, Inc.).

Thin layer chromatography was performed on 5 x 20 cm glass plates with silica gel H (E. Merck AG, Darmstadt) or on prepared plates of Sil Gel G/UV_{254} (Machery-Nagel, Duren), MN Cel 300 (Machery-Nagel, Duren), MN Cel 300 AC 10 (Machery-Nagel, Duren), Kieselguhr F-254 (E. Merck AG, Darmstadt), Polyamide-6 UV_{254} (Machery-Nagel, Duren), MN Cel 300 DEAE (Machery-Nagel, Duren), neutral alumina (E. Merck AG, Darmstadt), acid washed alumina (M. Woelm, Eschwege), acid washed silica gel (Mallinckrodt). The developed plates were placed under ultraviolet light and/or exposed to iodine vapors or sprayed with Dragendorff's reagent for visualization of the chromatogram.

Proton magnetic resonance (pmr) spectra were taken on Varian A-60, T-60 or XL-100 spectrometers using tetramethylsilane (TMS) as an internal reference. Samples were run in varying concentrations of methanol-d_4 or methanol-d_4/chloroform-d mixtures. Chemical shifts are reported in δ-units (parts per million from TMS) and are followed by the multiplicity of the signal, the number of protons, the corresponding coupling constant and the assignment. The multiplicities are denoted by the symbols: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; and m, multiplet.

Ultraviolet (uv) spectra were taken on a 95% ethanol solution with a Hitachi Perkin-Elmer, Model 124 spectrometer. Infrared spectra were taken on a Beckman IR-8 or a Perkin-Elmer Model 700 spectrometer as thin films or as potassium bromide pellets.

KB activity for each extract or partition step tested, is reported as an ED_{50} value followed by the LE result in mice. KB testing was performed in the Biochemistry Department of the University of Miami, Miami, Florida, under the direction of Mr. William Lichter, and the LE screening was performed by the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Me. Toxicity tests in healthy mice were performed by Dr. P. R. Pabrai in Dr. P.N. Kaul's Laboratory of Pharmacology, School of Pharmacy, University of Oklahoma. These results are reported as toxicity in healthy mice (THM) followed by the dosage level in mg/kg of mouse body weight.

Collection of Haliclona rubens. The sponge Haliclona rubens was collected in 3-5 feet of water on the reefs near Parguera, Puerto Rico. The sponge was air dried for 48 hours or air dried for 8 hours

and then dried in an oven at 80° for 12 hours. The dried sponge was broken into smaller pieces for shipment.

Extraction of Haliclona rubens. A. (Scheme I)--The dried sponge (300 g) was extracted at room temperature with 1:1 95% ethanol/water (2 1) and the extract was concentrated to 1 liter (KB 7.0, LE toxic to 5 mg/kg). The concentrate was extracted sequentially with benzene (1 1; KB, 54.0), ethyl acetate (1 1; KB, 49.0) and 1-butanol (1 1; KB, 4.8). The 1-butanol solution was extracted with 1 1 of 2 N ammonium hydroxide. The basic solution was acidified with 1 N hydrochloric acid and then extracted with 1-butanol (2 x 100 ml; KB, 5.3, LE, toxic to 5 mg/kg). This latter 1-butanol extract was concentrated and chromatographed on Amberlite IRA-900 anionic exchange resin using 95% ethanol as the eluent. The first 200 ml of eluent were collected as one fraction and upon evaporation of the solvent afforded 2.2 g of a dark brown glass. This glass was chromatographed on 50 g of 10% acetylated cellulose packed for development according to dry column procedure. The sample was applied to the top of the column absorbed on a small amount of the column packing. The column was eluted with 2-propanol (250 ml), 95% ethanol (250 ml) and methanol (250 ml). The 2-propanol fraction gave 0.75 g of a brownish yellow glass (KB, 4.7), the ethanol fraction gave 0.1 g of a yellowish glass (KB, 5.2) and the methanol fraction gave 0.44 g of a yellowish glass (KB, 7.1). Pmr analyses indicated that each fraction contained halitoxin-R, but the majority of it was in the 2-propanol fraction.

B. (Scheme II)--The dried sponge (1 kg) was ground in a blender then extracted at room temperature with 1-butanol saturated with water

(5 x 2.5 l). The 1-butanol extracts were concentrated to 250 ml and the concentrate chromatographed on polypropylene. The polypropylene column was prepared by slurrying the polypropylene powder in methanol and packing the column using the same solvent. The packed column was then eluted with water until all of the methanol had been washed off. The 1-butanol concentrate was placed on the column and eluted with water to give (61.96 g; KB 5.3), 1:1 95% ethanol/water (13.6 g; KB 55.0), methanol (14.9 g; KB > 100) and 1:1 chloroform/benzene (10.7 g; KB > 100). Pmr analysis confirmed that halitoxin-R was present only in the water fraction. The water fraction was evaporated to dryness and the residue was soaked in 95% ethanol (1 l) to give 52 g of ethanol soluble material (THM, 12.5 mg/kg). The ethanol soluble material was soaked with 500 ml of chloroform to give 11.5 g of chloroform soluble material and 30.5 g of chloroform insoluble material. By pmr analysis the chloroform insoluble residue contained halitoxin-R.

C. (Scheme III)--The dried sponge (198 g) was soaked in 1.2 l of 1-propanol for 2 weeks. The solvent was drained off and the soaking repeated. The two extracts were combined and concentrated *in vacuo* to yield 15.3 g of a dark brown gum. This gum was treated with acetone (2 x 300 ml) and there remained after this treatment 11.3 g of a dark brown, acetone insoluble gum. The acetone insoluble material was extracted with 300 ml of 2-butanol to give 6.23 g of soluble material and 5.05 g of insoluble material. By pmr analysis both the 2-butanol soluble fraction and the insoluble fraction contained halitoxin-R. Each fraction was dissolved in 95% ethanol and chromatographed on silicAR CC-4. The columns were eluted with 95% ethanol, then 1:1 methanol/

acetonitrile and finally methanol. The like fractions from each chromatography were combined and evaporated to give three fractions with the following weights: ethanol fraction (4.64 g), methanol/acetonitrile fraction (3.13 g) and methanol fraction (0.72 g). Pmr analysis revealed that both the methanol/acetonitrile and the methanol fractions contained halitoxin-R. Hence those fractions were combined.

Solvent Partition (Scheme II). A pair of solvent mixtures for use in solvent partition was made by mixing methanol (2 l), water (1 l) and chloroform (2 l) in a separatory funnel and allowing the layers to separate. The chloroform insoluble residue from Scheme II was dissolved in 500 ml of the water/methanol solution and extracted (4 x 500 ml) with the chloroform/methanol solution. The chloroform/methanol solutions were combined and extracted (5 x 500 ml) with the aqueous methanol solution. Upon evaporation the chloroform/methanol solution yielded 5.5 g of material and the aqueous/methanol solution yielded 19.7 g of material. By pmr analysis only the aqueous/methanol solution contained halitoxin-R.

Molecular Weight Filtration (Scheme II). The aqueous methanol soluble material (19.7 g) from the above solvent partition fraction (19.7 g) was dissolved in 300 ml of water and placed in a Dia-Flo (Amicon Corp., Lexington, Mass.) membrane filtration unit over a 500 MW cutoff semipermeable membrane. The unit was pressurized to 60 psi with nitrogen and the filtrate collected. When the volume in the unit had decreased to 50 ml, 250 ml of water was added to the concentrate and the filtration repeated to yield 7.28 g of material of 500 MW or less (KB > 100). This material, upon pmr analysis, exhibited no absorption

between δ 9.1-7.9.

The retentate from the above procedure was processed in a similar manner using a 1000 MW cutoff semipermeable membrane. Upon evaporation of the filtrate to dryness a brownish yellow glass weighing 9.8 g was obtained (KB, 5.8). The material that did not come through the membrane weighed 2.62 g (KB, 5.7). Pmr analysis revealed that the larger percentage of halitoxin-R was in the filtrate but that the retentate still contained a considerable amount of halitoxin-R.

Chromatography on Sephadex CM-25. The material that came through the 1000 MW exclusion membrane (9.5 g) was chromatographed on 50 g of Sephadex CM-25 in the salt (Na^+) form. The column was eluted with 500 ml each of water, 0.1 M sodium chloride, 0.2 M sodium chloride, 0.5 M sodium chloride, 1 M sodium chloride and 2 M sodium chloride. The organic material was recovered from the aqueous sodium chloride solution by extraction of the aqueous solutions with 1-butanol, concentration of the resulting 1-butanol solution, and filtration of the 1-butanol concentrate. If necessary this process was repeated until all of the sodium chloride was removed. The 1 M sodium chloride fraction yielded 5.95 g of a brownish yellow glass (THM, 9.0 mg/kg) which contained halitoxin-R by pmr analysis. None of the other fractions contained halitoxin-R.

Chromatography on SilicAR CC-4. A column of silicAR CC-4 was prepared by slurrying 50 g of the packing material in methanol and decanting the fines 5 times. The column was packed in methanol and then eluted with 300 ml of methanol. The column was then washed with 300 ml of 95% ethanol.

Two grams of the material that came from the 1 M sodium chloride fraction of the Sephadex CM-25 chromatography was dissolved in 5 ml of 95% ethanol and placed on top of the column. The column was eluted with 500 ml of 95% ethanol and then 500 ml of methanol; 0.65 g of material was obtained from the ethanol fraction and 0.94 g from the methanol fraction. By pmr halitoxin-R was present only in the methanol fraction.

The 0.94 g from the methanol fraction was chromatographed again on silicAR CC-4 using the procedure described above except that the solvent elution sequence was 95% ethanol, 1:1 ethanol/methanol and finally pure methanol. The collected fractions yielded the following weights of material: 95% ethanol, 25 mg, 1:1 95% ethanol/methanol, 36 mg; and methanol, 91 mg. The methanol fraction was the only one that contained halitoxin-R by pmr analysis. According to pmr spectrum the halitoxin-R was approximately 95% pure judged by the relative heights of the peaks of halitoxin-R and what appears to be impurities. The sample exhibited the following spectral characteristics: uv λ max 267.5 nm, inflection at 273 nm; ir (thin film) 3495 cm^{-1} (water), 3040 cm^{-1} (aromatic carbon-hydrogen stretch), 1630 cm^{-1} (pyridinium carbon-carbon stretch); pmr (CD_3OD) δ 9.01 (s, 1, proton on pyridinium ring between positive nitrogen and alkyl substituent), 8.9 (d, 1, J=5 Hz, proton on pyridinium ring next to positive nitrogen), 8.53 (d, 1, J=7 Hz proton para to positive nitrogen), 8.07 (dd, 1, J=7 and 5 Hz, proton meta to positive nitrogen), 4.7 (m, 2, protons on methylene group attached to the nitrogen of the pyridinium ring), 2.93 (t, 2, J=7 Hz, protons of methylene group attached to the pyridinium ring meta to the positive nitrogen), 2.12 (m, 2, methylene protons adjacent to methylene

group absorbing at 4.7), 1.76 (m, 2, methylene protons adjacent to methylene group absorbing at 2.93), 1.41 (bs, 10, methylene protons), 0.92 (d, 3, J=7 Hz, secondary methyl group).

Chromatography on silicAR CC-4 was attempted a third time to complete clean-up of halitoxin-R, but no material could be recovered from the column.

3-Carbamoyl-1-hexadecylpyridinium bromide (8). The procedure of Harris and co-workers was used[6]. To 100 ml of absolute ethanol was added 12.2 g (0.1 mol) of nicotinamide. The mixture was stirred until all of the nicotinamide had dissolved and then 30.54 g (0.1 mol) of cetyl bromide was added in one portion. The mixture was refluxed, protected by a drying tube, for 48 hr. When the reaction mixture was cooled to room temperature a white solid precipitated. The solid was removed by suction filtration and the reaction mixture was concentrated. A second crop of solid was collected and combined with the first crop. The solid was recrystallized from methyl ethyl ketone to yield the pyridinium salt 3 (27.05 g; 63.4%) as small white plates m.p. 211.5-212° with decomposition (lit[5]. 213-216°); ir (KBr) 3250 and 3100 cm^{-1} (N-H stretch), 1695 cm^{-1} (amide carbonyl absorption), 1640 cm^{-1} (pyridinium carbon-carbon stretch); pmr (1:1 $CDCl_3/CD_3OD$) δ 9.3 (s, 1, proton between amide and positive nitrogen), 8.86 (m, 2, protons ortho and para to the positive nitrogen of pyridinium ring), 8.28 (dd, 1, proton meta to the positive nitrogen of the pyridinium ring), 4.82 (t, 2, protons of methylene group attached to the positive nitrogen), 2.2 (m, 2, protons of methylene group adjacent to the methylene group attached to the positive nitrogen), 1.3 (bs, 26, protons on alkyl side chain), 0.9 (m, 3,

terminal methyl absorption).

N-hexadecyl-2-aminoethanol (9). To 10 ml of ethanolamine was added 0.53 g (1.2 mmol) of 3-carbamoyl-1-hexadecylpyridinium bromide (8). The mixture was heated on a steam bath for 30 minutes. Water (25 ml) was added to the cooled reaction mixture and then it was extracted sequentially with benzene (50 ml) and chloroform (50 ml). The organic solutions were combined and the solvents were evaporated to yield the hydroxy amine 9; pmr ($CDCl_3$) δ 3.58 (t, 2, HO-$\underline{CH_2}$-CH_2-N<), 2.75 (t, 4, HO-CH_2-$\underline{CH_2}$-N<$\underline{CH_2}$), 2.48 (s, 2, -OH and -NH), 1.3 (bs, 28, methylene protons), 0.9 (m, 3, terminal methyl group).

Reduction of Halitoxin-R. Platinum oxide (0.38 g) was suspended in absolute ethanol (10 ml) and prehydrogenated for 2 hr at atmospheric pressure and room temperature. To the stirred solution of the catalyst was added 0.28 g of the material that came from Sephadex CM-25 chromatography. The mixture was hydrogenated at room temperature and atmospheric pressure for 18 hr. The catalyst was removed by filtration and the ethanol evaporated at reduced pressure. The pmr spectrum of the residue was devoid of any absorptions in the δ 7.9-9.1 region and hence it could be concluded that the pyridinium ring had been reduced. TLC indicated a very complex mixutre which could not be resolved by chromatography.

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III. STUDIES ON THE RED PIGMENTS FROM THE SPONGE HALICLONA RUBENS

INTRODUCTION

In addition to the toxin discussed in the previous section, a series of red pigments were isolated from the sponge Haliclona rubens. These pigments comprise the majority of the natural color of the sponge.

The facile extraction of these pigments was discovered somewhat by accident. It had been noted that upon collection a slight red color bled from the sponge. However, there was no indication that these pigments would extract easily after the animal was dried. A day's collection of the sponge had been spread on the flat roof of one of the buildings at The University of Puerto Rico, Mayaguez, Marine Sciences Laboratory at Parguera, Puerto Rico. An afternoon rain shower came up before the sponge could be removed from the roof. A cascade of red water was noticed issuing from the roof. When the sponges were rescued from the roof, they had a decided bleached appearance.

The purpose of the work presented here was to isolate and characterize the red pigments. The data available at this time will be presented. The pigments were tested for biological activity but were found to be inactive.

RESULTS AND DISCUSSION

Batches of Haliclona rubens which had already been extracted with aqueous ethanol to remove the KB active materials discussed in the previous section were used as source material for the isolation of the red pigments. Repeated extraction of the aqueous ethanol processed sponge specimens at room temperature with distilled water gave a deep red extract. This extract was lyophilized, and the residue was dissolved in a minimum amount of water and filtered. Addition of methanol to give a 60/40% mixture by volume of methanol, caused precipitation of the red pigment material. The precipitated red pigments were filtered from the solution and chromatographed twice on Sephadex G-15.

The red fraction from the Sephadex chromatography was hydrolyzed in 6 N hydrochloric acid using the procedure of Moore and Stein[1]. Thin layer chromatography (TLC) of the hydrolysate on cellulose revealed a large number of positive spots when the plate was sprayed with ninhydrin. When the plate was sprayed first with sodium periodate and then benzidine a yellow spot attributable to a sugar was found. In view of these preliminary findings the hydrolysate was subjected to amino acid analysis. The results of that analysis are shown in Table II where the relative amounts of amino acids were calculated using the quantity of phenylalanine as the unit integral molar quantity.

TABLE II

Amino Acid Analysis of Red Pigments.

	Amino Acid	Micro moles/ total sample	Minimum residues/mole	Nearest Integer
1.	Lysine	0.0412	2.4	2
2.	Histidine	0.0237	1.4	1
3.	Arginine	0.0264	1.5	2
4.	Aspartic Acid	0.0790	4.5	5
5.	Threonine	0.0258	1.5	2
6.	Serine	0.0505	2.9	3
7.	Glutamic Acid	0.0624	3.6	4
8.	Proline	0.0310	1.8	2
9.	Glycine	0.0439	2.6	3
10.	Alanine	0.0165	2.2	2
11.	Valine	0.0352	2.0	2
12.	Isoleucine	0.0195	1.1	1
13.	Leucine	0.0462	2.7	3
14.	Tyrosine	0.0041	0.2	0
15.	Phenylalanine	0.0172	1.0	1
16.	Glucosamine	0.02+	1.2	1
17.	Hydroxy Proline	0.0495	2.6	3
18.	Cysteic Acid	0.0447	2.6	3
			residues	40

From the data in Table II it would appear that the pigment contains amino acids, amino sugars, and probably a separate chromophore. Assuming that the minimum residues/mole rounded off to the nearest integer is correct an average molecular weight for the peptide and glucosamine portion of the pigment would be about 4,500. The hydrolysis procedure used minimizes the loss of tyrosine by oxidation. Therefore, the trace of tyrosine reported would seem to indicate that oxidation of tyrosine had occurred in the isolation procedure or that the pigment material is a mixture of pigments with tyrosine present in perhaps only one of these.

In an effort to further purify the pigment after the two Sephadex G-15 chromatographies an anodic polyacrylamide disc gel electrophoresis was performed. The electrophoresis effected the separation of six bands. The leading band was yellow and the five remaining bands were red. Judged on the basis of color intensity there were two major red bands and three minor red bands.

The two major red bands were each subjected to amino acid analysis. The analysis of the band that moved faster is shown in Table III with the quantity of proline observed taken as the unit micromole quantity. If rounding off the minimum residues/mole is correct then the molecular weight for the peptide and glucosamine portion of the pigment is about 6,200. At this writing the results of the amino acid analysis of the slower migrating band have not been received.

In an attempt to break the pigment into more manageable parts it was subjected to trial degradation with trypsin and chymotrypsin[2]. As judged by gel electrophoresis the pigment mixture was not affected

TABLE III

Amino Acid Analysis of Red Pigment From

Faster Moving Electrophoresis Band

	Amino Acids	Micro moles/ total sample	Minimum residues/mole	Nearest Integer
1.	Lysine	0.0027	1.1	1
2.	Histidine	0.0031	1.2	1
3.	Arginine	0.0026	1.0	1
4.	Aspartic Acid	0.0162	6.5	7
5.	Threonine	0.0096	3.8	4
6.	Serine	0.0104	4.2	4
7.	Glutamic Acid	0.0148	6.0	6
8.	Proline	0.0025	1.0	1
9.	Glycine	0.0156	6.2	6
10.	Alanine	0.0165	6.6	7
11.	Valine	0.0107	4.3	4
12.	Isoleucine	0.0065	2.5	3
13.	Leucine	0.0100	4.0	4
14.	Tyrosine	0.0044	1.5	2
15.	Phenylalanine	0.0061	2.3	2
16.	Glucosamine	0.0101	4.0	4
			residues	57

by treatment with either of these enzymes. When the pigment mixture was subjected to basic hydrolysis in 6 N sodium hydroxide the color changed but nothing could be isolated that would give any indication of the nature of the chromophore. According to Goodwin[3] and Nicol[4] carotenoid pigments are very common in sponges and the visible spectrum of the red pigment isolated from Haliclona rubens is consistent[3] with a carotenoid chromophore (λ max 505). Therefore, the chromophore is possibly a carotenoid which would make the pigment a carotenoprotein.

It would seem likely that the five red bands obtained on polyacrylamide gel electrophoresis each contain the same chromophore and that they differ by way of molecular weight, amino acid composition and sequence of the peptide units. This would seem to be borne out by the results of the amino acid analysis. For example, there are two amino acids found in the pigment mixture from Sephadex chromatography which are not found in the single band isolated by disc gel electrophoresis. Furthermore, the relative concentrations of some of the individual amino acids vary widely between the mixture analysis and the individual band analysis.

Further work on the pigments should focus on isolating the chromophore and characterizing it. A possible approach to this problem would be to first enzymatically cleave the peptide chain without disturbing the chromophore and then to isolate and characterize the chromophore containing portion of the molecule under conditions which would avoid further hydrolysis. In addition, to complete the structure of the pigment it would be necessary to sequence the peptide.

SUMMARY

Several red pigments have been isolated from the sponge Haliclona rubens. The amino acid content of the mixture of pigments and the amino acids present in one of the major bands from polyacrylamide gel electrophoresis are presented. Based on the evidence available at this time some speculations were made concerning the general identity of the chromophore in the pigment. It seems likely that the chromophore is carotenoid in nature because of the similarities in visible absorption spectrum of the pigment from Haliclona rubens to that of other reported carotenoproteins.

EXPERIMENTAL

The column chromatography packing used was Sephadex G-15 (Pharmacia Fine Chemicals, Inc.). Thin layer chromatography was performed on prepared plates of MN Cel 300 (Machery-Nagel, Duren).

Amino acid analyses were performed on a Biochrom amino-acid-analyser (Bio Cal Instrument Co. Richmond, Calif.) or a Beckman Model 120 C Automatic Acid Analyzer.

Ultraviolet (uv) spectra were obtained on a Beckman DK spectrophotometer using distilled water as solvent.

Polyacrylamide gel electrophoresis was performed on a Buchler Polyanalyst Disc Electrophoresis Apparatus (Buchler Instrument Div., Nuclear-Chicago, Corp.). The gel electrophoresis was run in tubes 75 x 5 mm. The lower gel (running gel) was 45 mm long and the upper gel (stacking gel) was 10 mm long.

Extraction of Haliclona rubens. The dried sponge (400 g) was extracted with aqueous ethanol to remove the toxic material (Section II). The sponge residue was then extracted with distilled water (5 x 1 l) to give a deep red cloudy solution. The extract was filtered and then concentrated to 500 ml. To the clear concentrate was added 750 ml of methanol and the precipitated crude red pigment was collected by filtration. The solid pigment was taken up in 100 ml of water and the resulting clear red solution was concentrated to 15 ml. This slightly

cloudy concentrate was chromatographed twice on 280 g of Sephadex G-15 using water as the eluent to yield 2.3 g of the red pigment; uv λ max 505 and 265 nm.

Hydrolysis of the Pigment. The pigment mixture was hydrolyzed according to the procedure of Moore and Stein[1]. The red pigment mixture (1.3 mg) obtained by two sequential chromatographies over Sephadex G-15 was placed in a 16 x 150 mm pyrex test tube and 1 ml of 6 N hydrochloric acid was added. The tube was constricted and the acidic solution was frozen in a dry ice 2-propanol bath. To remove dissolved oxygen the frozen solution was evacuated and the pressure was maintained at 1 mm for 4 hr. The tube was sealed under vacuum and after first allowing the solution to thaw, the sealed tube was placed in an oven and heated at 110° for 20 hr. After the tube had cooled it was opened and the resulting brown solution was filtered to remove the suspended black particles. The water was removed under reduced pressure. Water (1 ml) was added to the residue and then evaporated in vacuo. The addition of water and subsequent removal of it under reduced pressure was repeated until the odor of hydrochloric acid could no longer be detected. This sample was submitted for amino acid analysis. The results of this analysis are shown in Table II.

TLC of Red Pigment Hydrolysate. The red pigments (115.5 mg) were hydrolyzed as described above. (TLC analysis of the hydrolysate was performed on MN Cel 300 using a mixture of 2-propanol (40), formic acid (2) and water (10) as the developing solvent. Upon spraying the plate with ninhydrin many positive spots appeared. These positive spots are indicative of amino acids.

Alternately when the developed plate was sprayed first with a sodium periodate solution, then allowed to dry and sprayed with a benzidine solution, a yellow spot on a blue background appeared. This spot is indicative of a sugar.

Polyacrylamide Disc Gel Electrophoresis of Red Pigments. The gel mixtures, buffer composition and concentrations, and the procedure was that of Orstein and Davis[5]. No tracking dye was used because the desired bands are colored. To 24 mg of the crude precipitated mixture was added 180 µl of distilled water. Small samples of this pigment solution (15 µl containing 2 mg of pigment) were carefully layered onto the top of the gel in each of 12 electrophoresis tubes. The electrophoresis apparatus was run at 1.25 ma per tube (15 ma total) until the first band had migrated through the stacking gel and then the current was increased to 2.5 ma per tube (30 ma total). The electrophoresis was allowed to run until the leading yellow band had advanced to within approximately 2 mm from the exit end of the tube (about 2 hr). The sample separated into six bands, the leading band being yellow and the five remaining bands being red. Judged on the basis of color intensity two of the red bands were major and three were minor. The two major bands were cut from the gel and extracted into distilled water to yield approximately 2 mg of red pigment per band. The two major bands were submitted for amino acid analysis. The results of the amino acid analysis on the faster moving red band are shown in Table III. The amino acid analysis of the slower moving major band is not available at this writing.

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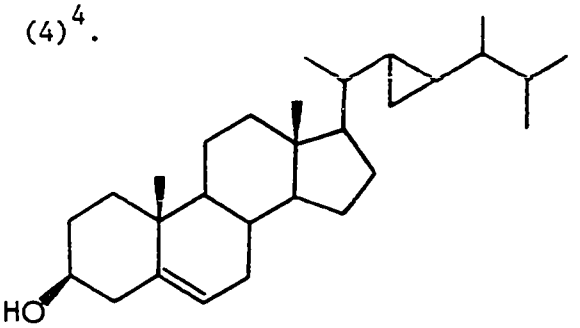
IV. ELISTANOL: A NOVEL MARINE STEROL

INTRODUCTION

The isolation and structure elucidation of several novel marine sterols from gorgonians of the genus Pseudopterogrogia have been reported by investigators from The Department of Chemistry, University of Oklahoma. The sterols which have been reported are 23-demethylgorgosterol (1)[1], Δ^5-3,11-dihydroxy-9,11-seco-gorgosten-9-one (2)[2], 3,11-dihydroxy-5α, 6α-epoxy-9,11-seco-gorgostan-9-one (3)[2,3], and gorgost-5-en-3,9,11,-triol (4)[4].



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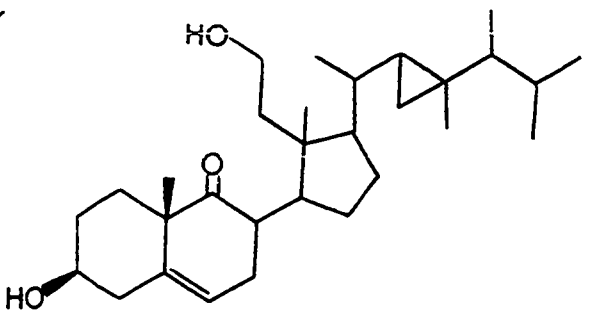

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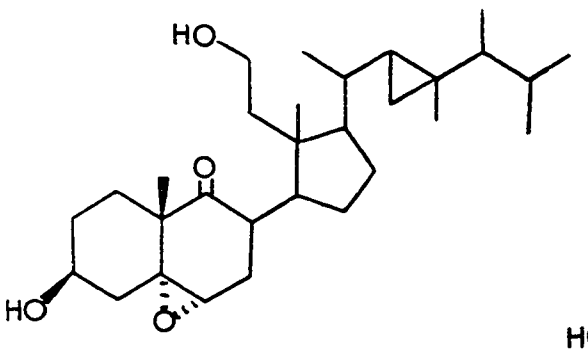

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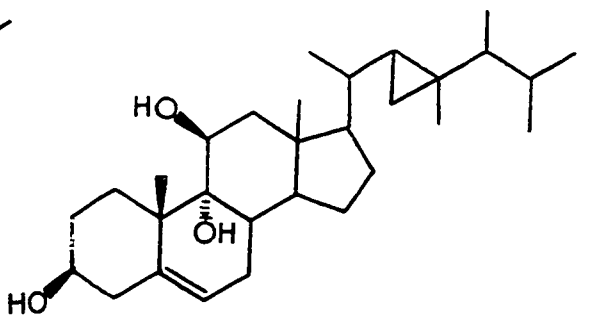


4

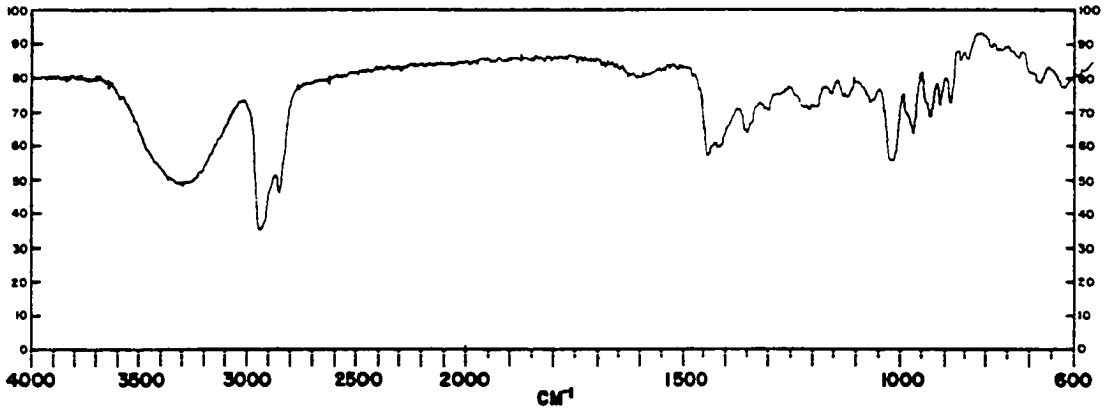
The gorgonian Pseudopterogorgia elisabethae Bayer is found throughout much of the West Indian Region[5]. The colonies are usually 4-10 inches in height, are highly branched and are a bright yellow color with slight tinges of purple. The purpose of the work presented here was to determine the structure of a novel marine sterol named elistanol isolated from Pseudopterogoriga elisabethae Bayer.

RESULTS AND DISCUSSION

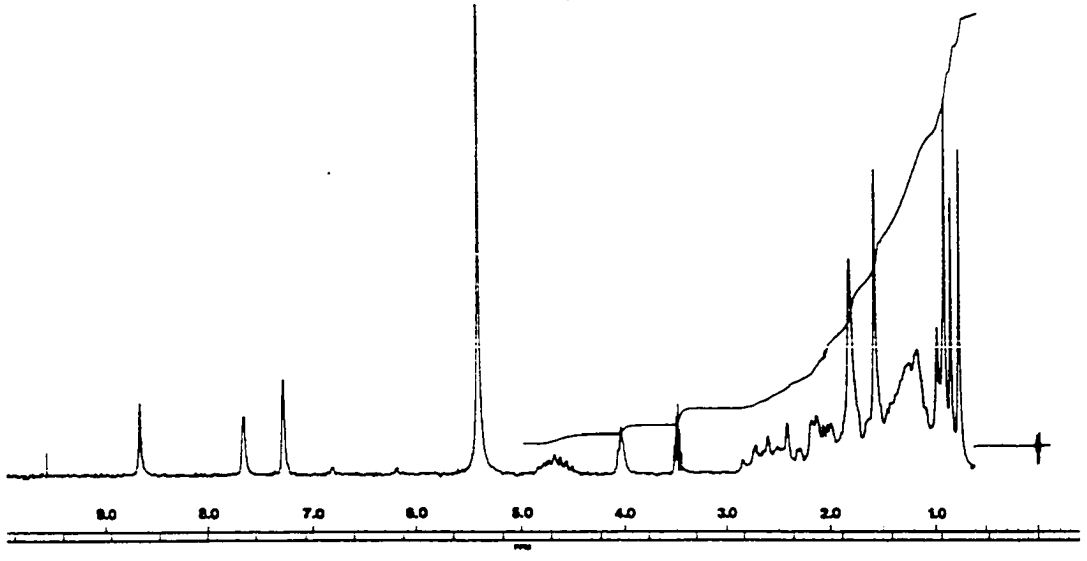
Elistanol, (5), was isolated from both the aqueous ethanolic and cold hexane extracts of dried Pseudopterogorgia elisabethae Bayer collected from the north shore of Puerto Rico. The sterol was obtained from both the hexane and aqueous ethanolic extracts of the gorgonian by chromatography over silica gel. Ivariably the sterol was found in the 5% methanol/chloroform fractions of the chromatographies. Purification was effected by repeated chromatography using either silica gel or neutral alumina as the column packing.

Elistanol, 5, was obtained, after sublimation, as a white, amorphous solid with a melting point of 288-289° with decomposition, $[\alpha]_D^{25}$ -17° (c 0.2 MeOH). Low resolution mass spectrometry (Figure VIII) indicated a molecular wight of 418 (M^+ 418) which corresponds to the formula $C_{27}H_{46}O_3$. This formula was confirmed by high resolution mass spectrometry. The infrared spectrum (Figure VIII) of elistanol indicated the presence of hydroxyl functionality (3340 cm^{-1}) but was devoid of any carbonyl absorption. The 100 MHz proton magnetic resonance spectrum (Figure VIII) exhibited two quaternary methyl group singlets (δ 1.61 and 0.80) and three secondary methyl group doublets (one at δ 0.98 and two coincident ones at δ 0.90). The carbon-13 magnetic resonance spectrum (Table IV) revealed absorptions for a total of twenty-seven carbons, four of which are appropriate for carbons attached to

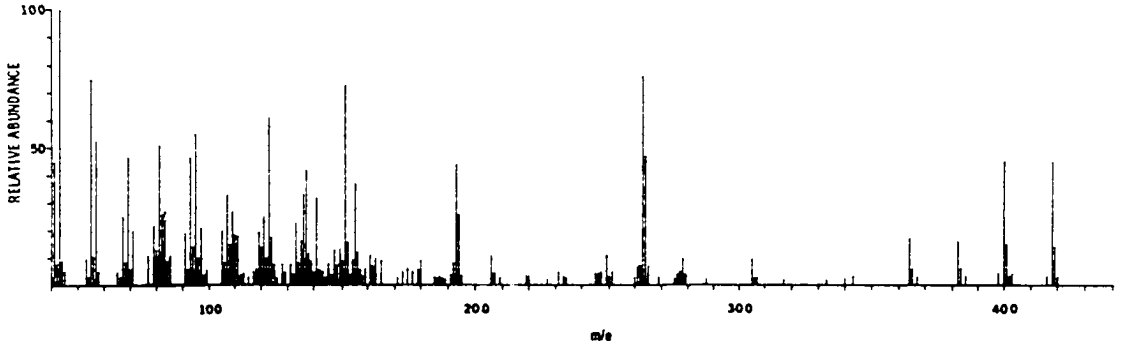
Figure VIII
Spectra of Elistanol (5)
IR Spectrum (KBr) of 5



PMR Spectrum (pyridine-d_5 + methanol-d_4) of 5



Mass Spectrum of 5



oxygen and no absorptions for olefinic carbons. The formula ($C_{27}H_{46}O_3$), the carbon-13 magnetic resonance spectrum and the number and type of methyl group absorptions in the proton magnetic resonance spectrum are all indicative of a cholestane derivative.

From the molecular formula ($C_{27}H_{46}O_3$) it is apparent that the molecule contains five degrees of unsaturation. If the cholestane nucleus is assumed, then the rings of the system account for four of the degrees of unsaturation leaving only one degree of unsaturation to account for. Since the carbon-13 magnetic resonance spectrum exhibited no vinyl carbon signals and since the infrared spectrum indicated that no carbonyl groups were present, the other degree of unsaturation must be due to another ring.

The pmr spectrum reveals that there are two protons attached to carbons bearing oxygen (absorptions at δ 4.04 and 4.68). Since the molecular formula indicates there are three oxygens present in the molecule, the third oxygen atom must be bound to one (if a hydroxyl) or two (if an ether) tertiary carbon center(s). Since the carbon-13 magnetic resonance spectrum displays signals for four carbons attached to oxygen and the pmr spectrum exhibits absorptions for two protons on carbons bearing oxygen, the third oxygen must be attached to two tertiary carbons to form a cyclic ether. The cyclic ether linkage then accounts for the fifth degree of unsaturation.

In light of the facts presented to this point it appears likely that the sterol is a cholestane derviative, that it contains two secondary alcohols, that it has a tetrasubstituted cyclic ether linkage and that no other funtionality is present. There are still several unanswered

questions to deal with.

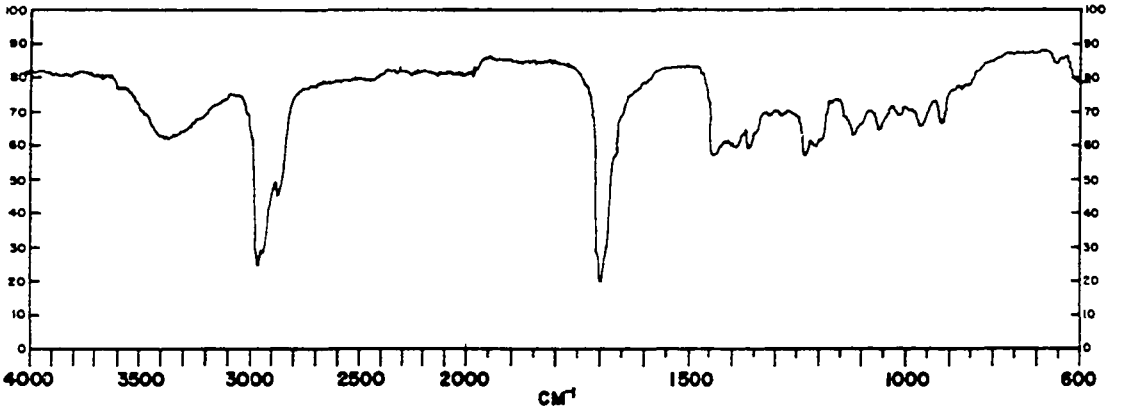
1. Where are the hydroxyl groups located?
2. What is the stereochemistry of the alcohol groups?
3. Where is the ether located?
4. How large is the ether ring?

To try to answer these questions chemical and spectral investigations were undertaken.

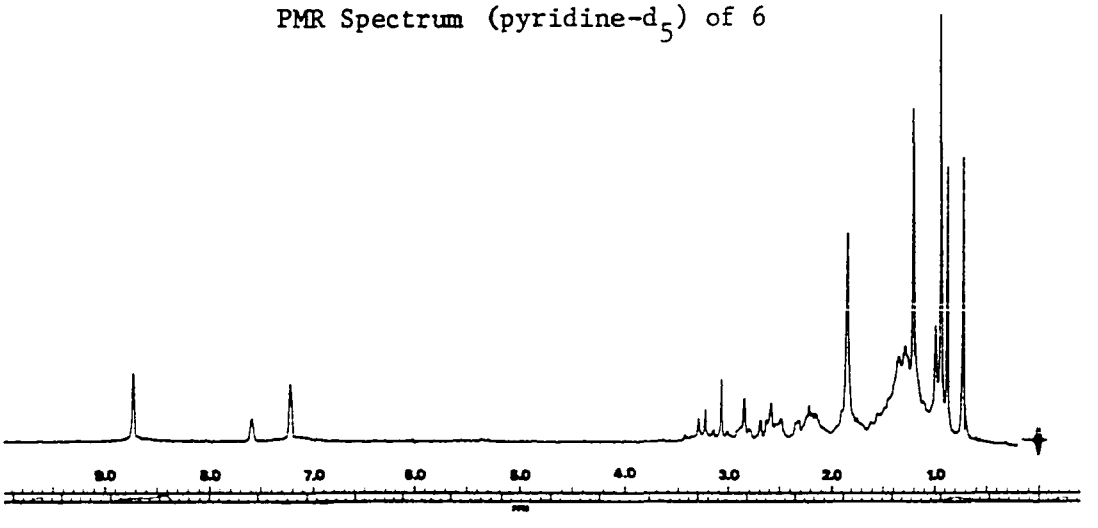
The first reaction performed in an attempt to answer the questions posed above was Jones[6] oxidation of the alcohol groups. The product of this reaction was a white crystalline solid, 6. The low resolution mass spectrum of 6 (Figure IX) displayed an M^+ ion of 414 which corresponds to a molecular formula of $C_{27}H_{42}O_3$. High resolution mass spectrometry confirmed a molecular weight of 414 and a molecular formula of $C_{27}H_{42}O_3$. The infrared (ir) spectrum (Figure IX) exhibited hydroxyl or water absorption (3380 cm^{-1}) and six membered ring ketone absorption (1714 cm^{-1}). The absorption at 3380 cm^{-1} is most likely due to water because the intensity of this absorption varies quite widely in relation to the intensities of the other peaks in a spectrum from one time to the next. The pmr spectrum (Figure IX) showed no proton signals downfield of δ 3.0 indicating that both secondary alcohol groups had been oxidized to the corresponding ketones.

Analysis of the mass spectrum of 6 and the original sterol 5 yielded considerable information regarding the possible location of the hydroxyl groups in the sterol. Sterol 5 showed a fragment ion of m/e 57 ($C_3H_5O^+$), 26% which is common for sterols having the conventional 6-membered ring A with the usual hydroxyl group at C-3[7,8]. The mass

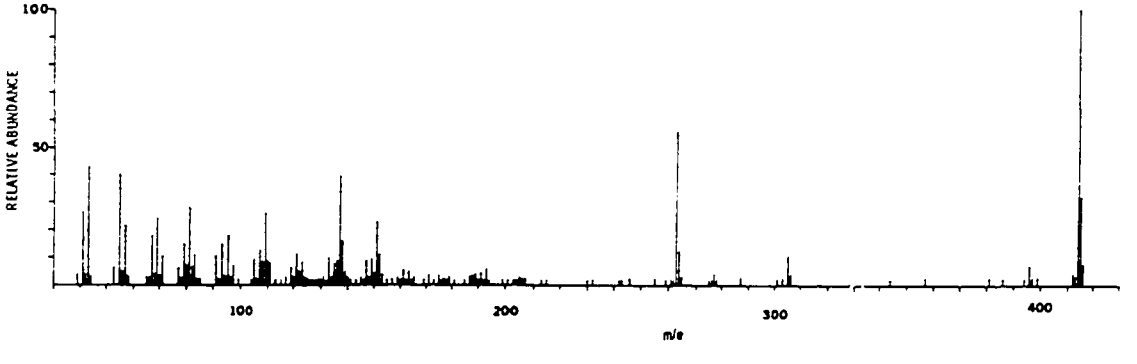
Figure IX
Spectra of Elistanol-3,-6-dione (6)
IR Spectrum (CHl_3) of 6



PMR Spectrum (pyridine-d_5) of 6



Mass Spectrum of 6



spectrum of the ketone 6 should contain a $C_3H_3O^+$ (m/e 55) fragment ion resulting from the same type of cleavage and indeed this fragment ion is present with an intensity of 59% of the base peak. Hence, this mass spectral data supports the conclusion that one of the hydroxyl groups is at C-3.

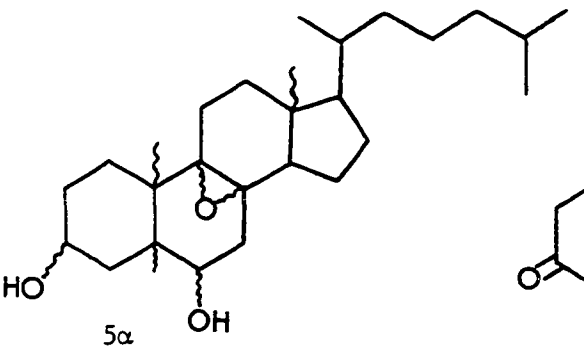
Further analysis of the mass spectra leads to a conclusion regarding the position of the other secondary alcohol. In both the original sterol and its dione there are peaks at m/e 278 and 277 with the formulas $C_{19}H_{33}O$ and $C_{19}H_{32}O$. These fragments could correspond to ions generated by cleavage between C-5, 7 and between C-9, 10. If this is the case then the fragments corresponding to the remainder of the molecule should be seen. In fact the fragments $C_8H_{13}O_2$ and $C_8H_{12}O_2$ (m/e 141 and 142) are evident in the spectrum of the hydroxy sterol and $C_8H_9O_2$ and $C_8H_8O_2$ (m/e 137 and 136) are present in the dione spectrum. This indicates that the other hydroxyl group is affixed to ring A or C-6. There is, furthermore, a peak at m/e 307 ($C_{20}H_{35}O_2$) in the spectrum of the hydroxyl sterol and a peak at m/e 305 ($C_{20}H_{33}O_2$) in the spectrum of the dione which correspond to ions generated by cleavage of the C-9, 10 and C-5, 6 bonds with charge retention on the large fragment. The complementary fragment ions corresponding to the remainder of the molecule, ring A plus the C-10 methyl group, m/e 111 ($C_7H_{11}O$) and m/e 109 (C_7H_9O), are also present in the respective mass spectra. The conclusion that can be drawn is that the second hydroxyl group is at C-6.

A prominent peak in the mass spectrum of both the sterol 5 and the dione 6 is found at m/e 263 ($C_{18}H_{31}O$). Since this ion is common to both the sterol 5 and the dione 6, it appears that the oxygen which is

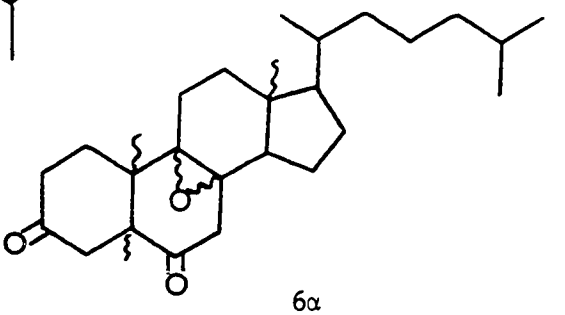
not oxidized in the conversion of 5→6 must be included in this fragment. The number of hydrogens contained in the mass of the 263 fragment make it necessary to include the side chain as part of this fragment. The proper number of carbon atoms (18) for this ion can be obtained from the side chain and rings D and C of a cholestane skeleton. The mass spectra of both 5 and 6 show peaks at m/e 305 and 301 (M^+ -113) respectively and both show a peak at m/e 113 (C_8H_{17}). It can be concluded from this data that the sterol 5 and dione 6 most probably contain a conventional cholestane side chain.

The mass spectra of 5 and 6 also contain peaks at m/e 194 and 193 ($C_{14}H_{26}$ and $C_{14}H_{25}$) which could arise from cleavage between C-12, 13 and between C-8, 14 with charge retention on the ring D plus side chain fragment and the remainder of the molecule existing as a neutral fragment (no M^+ -194 or 193 fragment ions). If the 193, 194 ions originate from such a cleavage then all of the oxygen atoms must be in rings A, B and/or C of the sterol nucleus. If the m/e 263 ion contains all the carbon atoms of the steroid rings C and D plus the conventional C_8H_{17} side chain and if the fragments m/e 193 and 194 correctly indicate that there are no oxygen atoms in ring D or the side chain, then the oxygen atom of the 263 fragment ion must be attached to ring C. Since the oxygen in ring C is the ether oxygen and since the ether carbons were shown earlier to be tetrasubstituted, then there are only three carbon atoms available (C-8,9 and 14) to which the ether oxygen can be attached. The ring C cleavage just discussed seems to eliminate C-14 from consideration. Therefore the third oxygen must be attached to C-8 and -9 to form an epoxide. Correlation of all of the data presented to this

point allows one to postulate structures 5α and 6α for the original sterol and the corresponding dione, respectively.



5α



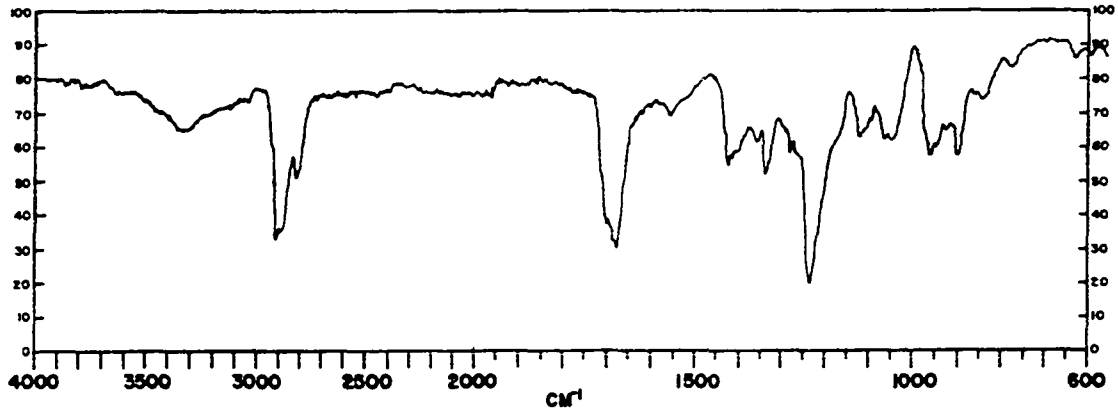
6α

The pmr spectrum of sterol 5 indicates that the proton absorbing at δ4.68 is attached to a carbon bearing an equatorial hydroxyl group (broad multi lined absorption) and that the proton absorbing at δ 4.04 is attached to a carbon bearing an axial hydroxyl group (relatively narrow absorption). In order to determine the sterochemistry of the two hydroxyl groups at C-3 and C-6, elistanol, 5, was reacted with ethyl chlorocarbonate in pyridine. The carbonate esters which result from this reaction are called cathylates and in sterols only equatorial hydroxyl groups react to form these esters[9-13]. Elistanol formed only a monocathylate (7), and hence it may be concluded that one of the hydroxyl groups is equatorial and the other is axial. This conclusion is corroborated by the fact that the broad signal at δ, 4.68 in the pmr spectrum of 5, which is indicative of an equatorial hydroxyl group, is shifted downfield to δ 5.08 in the pmr spectrum of 7. Therefore, the proton signal at δ 4.04 in the sterol pmr spectrum (δ 3.62 in chloroform-d in the cathylate spectrum) is the signal associated with the axial hydroxyl group. The splitting pattern and the width of the absorption (46 Hz) of

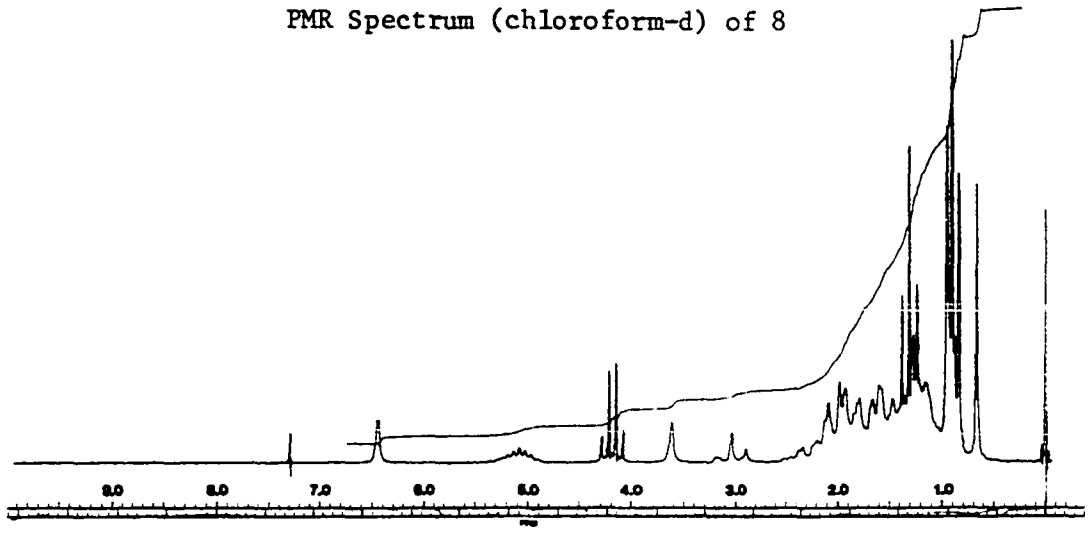
the proton on the carbon bearing the equatorial hydroxyl group is nearly identical with that of the proton on the carbon bearing the equatorial hydroxyl group of cholesterol. Therefore, the absorption at δ 4.68 in elistanol, 5, is due to the proton on the carbon bearing the equatorial hydroxyl group at C-3 and the hydroxyl group at C-6 is axial. Additional evidence for the 6 β hydroxyl assignment is provided by the large upfield shift (50 Hz) of the 19-methyl group absorption in the pmr spectrum of 6 compared to that of 5. The value for Δ δ is very close to that reported by Page[15] for this transition. The fact that the 19-methyl group absorption exhibits this dramatic upfield shift provides strong evidence that the 19-methyl group is cis to the 6 β hydroxyl group.

In an attempt to gain more information about the position and stereochemistry of the two secondary hydroxyl groups and the A/B ring juncture the cathylate was subjected to Jones[6] oxidation. The product of that oxidation was a white crystalline solid, 8. The ir spectrum of 8 (Figure X) indicated the presence of a carbonate ester and a six membered ring ketone (absorptions at 1738 and 1714 cm^{-1}). The ir spectrum exhibits an absorption at 3390 cm^{-1} and the pmr spectrum (Figure X) exhibits two absorptions which disappear upon addition of methanol-d_4 (δ 3.62 and 6.46). The combustion analysis of 8 is consistent with two additional water molecules. Therefore, the absorption in the ir spectrum (3390 cm^{-1}) and the two absorptions in the pmr spectrum (δ 3.62 and 6.46) are likely due to water. The pmr spectrum of 8 (Figure X) displayed an absorption at δ 5.08 caused by the proton on the carbon being the cathylate but was devoid of any signal in the δ 3.62 region (except for the exchangeable proton absorption) where the proton on the carbon

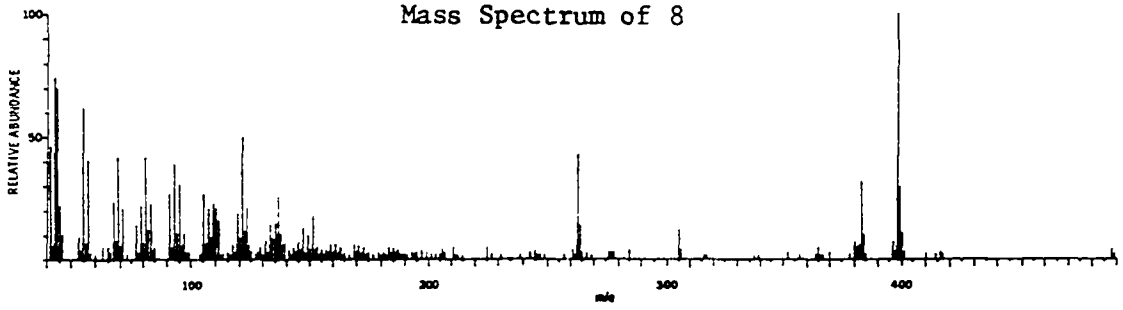
Figure X
Spectra of Elistanol-3-cathvlate-6-one (8)
IR Spectrum ($CHCl_3$) of 8



PMR Spectrum (chloroform-d) of 8



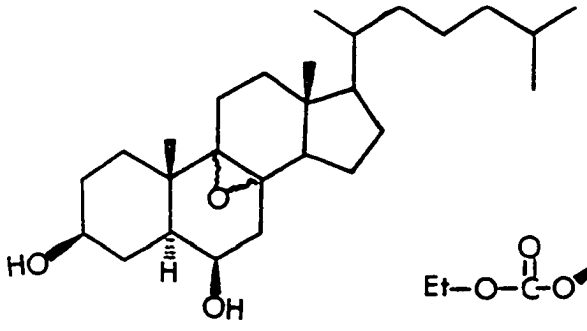
Mass Spectrum of 8



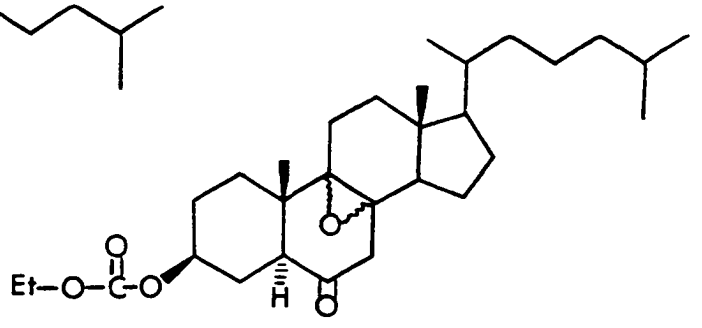
bearing the axial hydroxyl group resonates.

The mass spectrum of the cathylate-one 8 provides support for the conclusions drawn in connection with the mass spectra of 5 and 6. All of the peaks discussed for 5 and 6 are present except the M^+-194 or 193 which are absent in the dione 6 also.

In order to establish the location of the ketone with more surety the circular dichroism (CD) curve for the cathylate-one 8 was obtained. Steroidal 3-ketones with A/B trans ring junctures exhibit positive Cotton effects[14] and steroidal 3-ketones with A/B cis ring junctures display weakly negative ($\sim$ -1000) Cotton effects[14]. On the other hand steroidal 6-ketones with A/B trans ring junctures exhibit moderately negative ($\sim$ -4,000) Cotton effects[14] and steroidal 6-ketones with A/B cis ring junctures display strongly negative ($\sim$ -10,000) Cotton effects[14]. The CD curve of the cathylate-one of elisterol was -3,000 indicating that the ketone in 8 is probably at C-6 and hence the cathylate must be at C-3 and the stereochemistry of the ring juncture is most likely A/B trans. Since the stereochemistry of the A/B ring is trans and the sterochemistry of the C-3 hydroxyl group is β and the general pmr spectral characteristics seem to be very close to those of cholestanol it is not unreasonable to assume that the sterochemistry of the C/D ring juncture and the side chain of the molecule is the same as cholestanol. Therefore, structures 5*b* and 8*a* can be drawn.



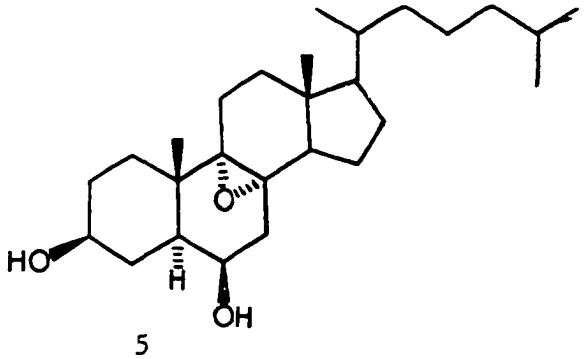
5 *b*



8 *a*

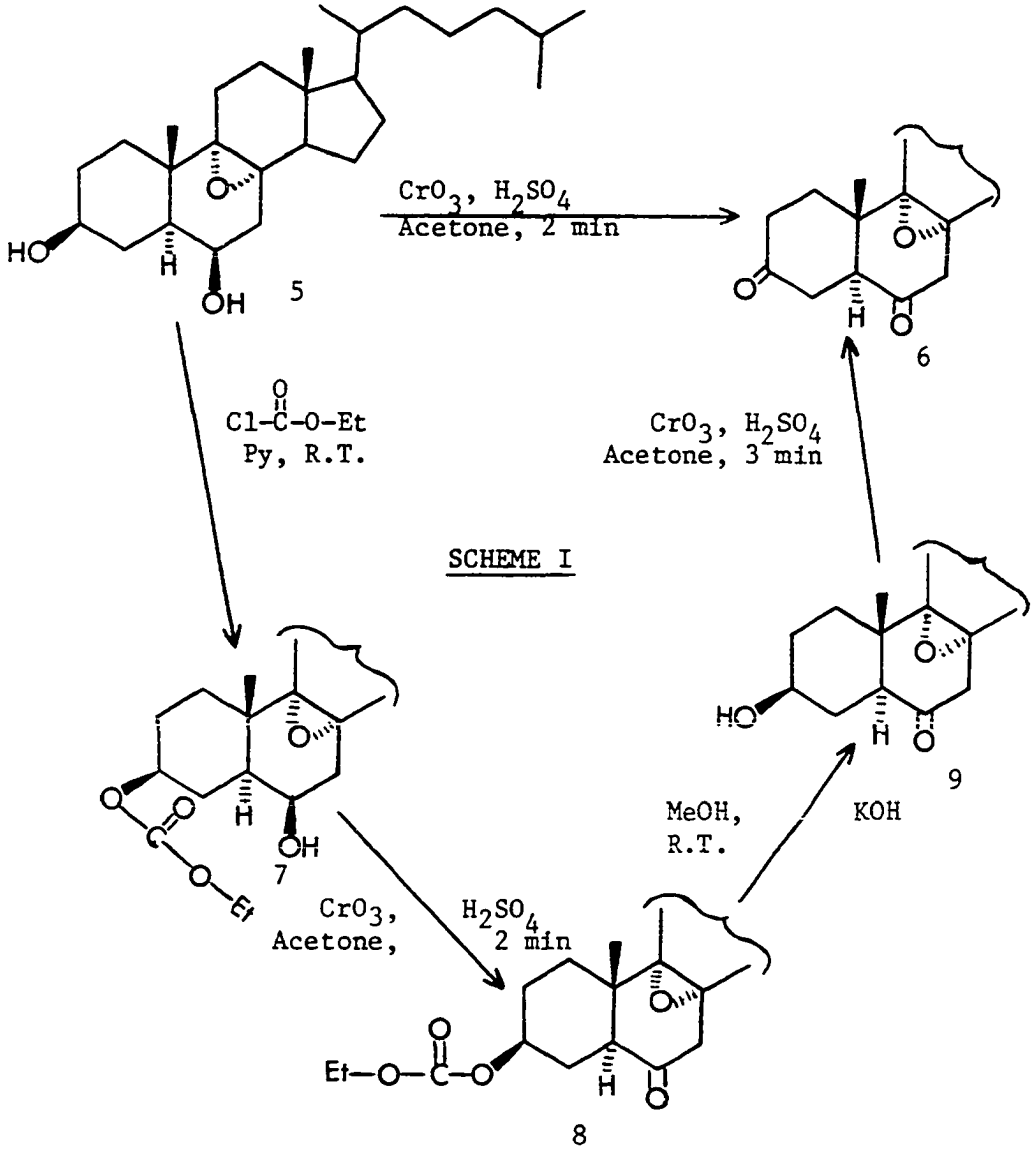
In order to insure that no rearrangements or other reactions had taken place it was decided to convert the cathylate-one 8 to the dione 6. The cathylate-one 8 was subjected to hydrolysis in alcoholic potassium hydroxide. The product was an amorphous white solid whose ir spectrum (3400 and 1714 cm^{-1}) indicated that hydrolysis of the ester to give the 3-hydroxy-6-one 9 had occurred. Oxidation of 9 with Jones reagent[6] yielded a white crystalline solid which was identical with dione 6 in all respects.

The remaining question to be answered is what is the stereochemistry of the C-8, 9 epoxide. If the epoxide were β then the 18-methyl group absorption in the pmr spectrum of 5 would be expected to occur approximately 50 Hz downfield from its normal position[16] but this is not observed. Therefore, the epoxide is most likely 8*a*, 9*a*. On the basis of all of the foregoing data and discussion, structure 5 is proposed for elistanol.



5

The various chemical conversions of elistanol, 5, discussed en-route to the proposed structure are shown in Scheme I.



SCHEME I

The carbon-13 nuclear magnetic spectrum was very helpful in postulating a structure for elisterol, 5. Because of the solubility properties of 5 it was necessary to take the spectrum in pyridine-d_5. The spectrum of 5 was then correlated with those of cholestane, cholestanol and cholesterol reported in the literature[17]. Since the spectra reported in the literature were taken in carbon tetrachloride, a spectrum of

TABLE IV

CHOLESTEROL AND ELISTANOL C^{13} CHEMICAL SHIFT ASSIGNMENTS

Cholesterol			Elistanol	
Carbon No.	Chemical Shift CCl_4 [17]	Pyridine-d_5	Carbon No.	Chemical Shift Pyridine-d_5
1	37.8	37.83	1	30.92
2	31.9	32.16	2	31.51
3	71.6	71.11	3	79.42
4	42.7	43.40	4	36.67
5	141.5	141.73	5	43.91
6	121.6	120.95	6	79.13
7	32.3	32.16	7	43.91
8	32.3	32.55	8	67.92
9	50.8	50.47	9	76.75
10	36.8	36.86	10	32.70
11	21.5	21.36	11	29.23
12	28.6	28.48	12	29.03
13	42.7	42.48	13	42.62
14	57.2	56.86	14	57.20
15	24.6	24.51	15	25.06
16	40.3	40.01	16	40.24
17	56.8	56.38	17	49.46
18	12.3	12.06	18	12.06
19	19.7	19.61	19	21.19
20	36.1	36.03	20	34.19
21	19.1	18.94	21	19.23
22	36.7	36.47	22	36.07
23	24.4	24.17	23	24.76
24	39.9	39.72	24	37.06
25	28.3	28.19	25	29.03
26	22.8	22.67	26	23.17
27	23.0	22.91	27	23.47

cholesterol was taken in pyridine-d_5 and a correlation was made between cholesterol in pyridine-d_5 and cholesterol in carbon tetrachloride. The assignments of cholestero in carbon tetrachloride[17] and pyridine-d_5 and elistanol in pyridine-d_5 are shown in Table IV. As can be seen from the chemical shift data there are no vinyl carbon absorptions and four absorptions for carbons bearing oxygen. The assignment of the epoxide carbons is consistent with reported values[18], however, the unequivocal assignment of a specific carbon number to each of the four chemical shift values is not possible. The carbon-13 spectral data strongly support the structure proposed for elistanol 5.

In one attempt to try to purify some elistanol 5 it was sublimed. The product from that sublimation was reacted with a silylating reagent in pyridine in order to prepare a sample for gas chromatography-mass spectral analysis. It seems likely that the epoxide partially opened to give the allylic alcohol because both the tri-silylated and the di-silated derivative were found in the mass spectrum. The most likely place for this to occur was during sublimation of the elistanol.

The di-acetate of elistanol was also made (acetic anhydride/pyridine) but a very waxy solid was all that could be obtained even after repeated attempts to crystallize the derivative in various solvents.

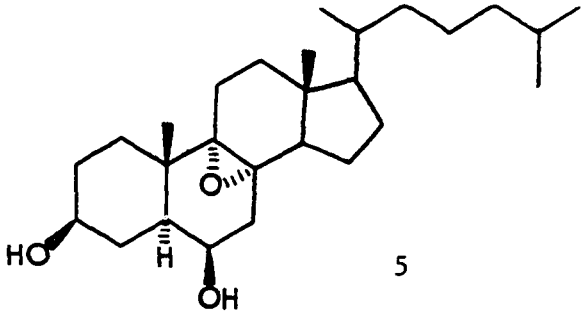
There is another possible interpretation of the data presented. The combustion analyses obtained and the exchangeable protons observed in the pmr spectra would be better explained by a tetraol than the epoxy diol proposed. The 3β, 6β, 8α, 9α tetraol would seem to be the most attractive possibility. However, in the opinion of the author, there are fewer inconsistencies between the available data and the proposed epoxy diol structure than there are between the data and the tetraol

structure.

In order to substantiate the proposed structure of elistanol it would be preferable to have a more concrete confirmation of the 8α, 9α epoxide. The ideal way to accomplish this would be to synthesize by an unambiguous route a compound having the proposed structure 5. However, because of the lack of an appropriate starting material the route to elistanol would be long and tortuous. An alternate method would be to convert elistanol to a known compound. However, because of the scarcity of staring material in hand this was not possible.

SUMMARY

Chemical and spectral evidence is presented which form the basis for proposing the structure 5 for a novel sterol called elistanol which was isolated from Pseudopterogorgia elisabethae Bayer.



5

The conversion of the sterol 5 to its 3,6-dione 6, 3-cathylate-6-ol 7, 3-cathylate-6-one 8 and the 6-one-3-ol 9 are discussed. Additional evidence for the proposed structure was obtained from the carbon-13 nuclear magnetic resonance spectrum. A discussion of the method used to make the assignments is included.

EXPERIMENTAL

All melting points are uncorrected. All solvents except 95% ethanol were distilled prior to use. Pyridine (reagent grade, Fisher Scientific) was kept over molecular sieves (Linde, 4A).

Column chromatographic packings used were silicAR CC-7 (Mallinkrodt, 100-200 and 200-325 mesh), silica gel (Grace 60-200 mesh), and neutral alumina (Fisher Scientific). Thin layer chromatography was performed on 5 x 20 cm glass plates coated with silica gel H (E. Merck AG, Darmstadt) or on prepared plates of Sil Gel G/UV_{254} (Machery-Nagel, Duren), silica gel F-254 (E. Merck AG, Darmstadt), Sil Gel N-HR/UV_{254} (Machery-Nagel, Duren) or aluminum oxide F-254, Type T (E. Merck AG, Darmstadt). The developed plates were placed under ultraviolet light and/or exposed to iodine vapors or sprayed with 10% sulfuric acid solution for visualization of the chromatogram.

Proton magnetic resonance (pmr) spectra were taken on Varian T-60 or XL-100 spectrometers using tetramethylsilane (TMS) as an internal reference. Samples were run in varying concentrations of pyridine-d_5, pyridine-d_5/methanol-d_4 mixtures, chloroform-d or chloroform-d/methanol-d_4 mixtures. Chemical shifts are reported in δ-units (parts per million from TMS) and are followed by the multiplicity of the signal, the number of protons, the corresponding coupling constant(s) and the assignment. The multiplicities are denoted by the symbols: s, singlet; bs, broad

singlet; d, doublet; dd, double doublet; t, triplet; q, quartet and m, multiplet.

Infrared (ir) spectra were taken as potassium bromide pellets or chloroform solutions on a Beckman IR-8 spectrometer. The carbon-13 nuclear magnetic resonance spectra were taken on a Varian XL-100 spectrometer using a Nicolet/TTI Fourier Transform with the carbon-13 resonance of TMS used as an internal reference. Samples were run in varying concentrations of pyridine-d_5 or a carbon tetrachloride/methanol-d_4 mixture. Chemical shifts were printed-out by the Nicolet computer.

Low resolution mass spectra were taken on a Hitachi Perkin-Elmer RMU-7E mass spectrometer using perfluorokerosene-H as an internal reference. The spectra are reported as the mass of the ion followed by the percentage of the base peak. High resolution mass spectra were provided by personnel in Dr. Klaus Bieman's laboratory, Massachusetts Institute of Technology, Cambridge, Mass.

The circular dichroism spectrum was taken on a Model J-20 Spectrometer (Japan Spectroscopic Co. Ltd., Tokyo). Combustion analyses were carried out by Mr. Eric Meier, Chemistry Department, Stanford University, Stanford, California.

Isolation of elistanol 5. The dried gorgonian Pseudopterogorgia elisabethae Bayer (3.02 kg) was extracted with a mixture of 60% ethanol/water (7 x 2 l) at room temperature. The ethanol/water extract was concentrated to 1 l and 1.5 l of methanol was added to the aqueous solution. The aqueous methanolic solution was extracted with hexane (4 x 1 l). The hexane extract was concentrated in vacuo to yield 43.5 g of a dark brown oil. The aqueous methanolic extract was concentrated to

200 ml and extracted with chloroform. The chloroform extract was evaporated under reduced pressure to give 33.9 g of a brown gum. The animal residue from the aqueous ethanolic extract was extracted with hexane (2 x 3 l) and the solvent removed under reduced pressure to yield 35.7 g of a dark brown glass. The two hexane extracts and the chloroform extract were combined and chromatographed on 240 g of silica gel. The column was eluted with 5 l of chloroform (48.7 g of material came off), 6 l of 1% methanol/chloroform (14.0 g), 6 l of 5% methanol/chloroform (8.5 g), 4 l of 10% methanol/chloroform (7.5 g) and 3 l of pure methanol (19.3 g). TLC indicated that only the 5% methanol/chloroform fraction contained elistanol. The 5% methanol/chloroform fraction was chromatographed again on a 4.2 x 47 cm column of silica gel using the identical elution sequence described above to yield 2.27 g of impure elisterol from the 5% methanol/chloroform fraction. This fraction was chromatographed again on 200 g of silica gel using the same elution sequence to yield 0.86 g of purer elistanol from the 5% methanol/chloroform fraction. This fraction was chromatographed on a 3.5 x 32 cm column of neutral alumina to yield 0.37 g of almost pure elistanol from the 5% methanol/chloroform fraction. The almost pure elistanol was precipitated three times from pyridine/ether to give 279.9 mg (0.009% of the dry animal weight) of pure (by TLC) elistanol, 5. However, combustion analysis indicated a 0.58% residue. Therefore, 190 mg of elistanol was sublimed at 215° (0.35 mm). Mp (Unsublimed) 293-295° d, mp (Unsublimed in evacuated sealed tube) 304-305°, mp (sublimed) 288-289° d; $[\alpha]_D^{25}$ -17° (c 0.2 MeOH); ir (KBr) 3340 cm^{-1} (hydroxyl groups), and 1050 cm^{-1} (carbon-oxygen stretch); pmr (pyridine-d_5 and pyridine-d_5/methanol-d_4)

δ 6.9 and 6.44 (bs, 1 each, hydroxyl protons), 6.12 and 5.95 (m, 9 total, water, addition of methanol-d_4 caused the peaks at 6.9, 6.44, 6.12 and 5.95 to all disappear and coalesce to one peak at δ 5.2), 4.86 (m, 1, proton at C-3), 4.17 (bs, 1, proton at C-6), 1.61 (s, 3, 19-methyl group), 0.98 (d, 3, 21-methyl group), 0.90 (d, 6, terminal isopropyl methyl groups of the side chain), 0.80 (s, 3, 18-methyl group); mass spectrum 418 (45), 400 (45), 382 (16), 364 (17), 264 (47), 263 (77), 194 (26), 193 (44), 155 (37), 151 (72), 141 (32), 137 (42), 136 (34), 133 (23), 123 (61), 121 (25), 109 (27), 107 (33), 105 (20), 97 (21), 95 (55), 93 (46), 83 (27), 82 (26), 81 (51), 79 (21), 71 (20), 69 (46), 67 (25), 57 (52), 55 (75), 43 (100), and 41 (45).

Molecular Weight. Calcd for $C_{27}H_{46}O_3$; 418.34470. Found: 418.34570.

Anal. Calcd for $2C_{27}H_{46}O_3 \cdot 3H_2O$: C, 72.81; H, 11.01. Found: C, 72.89; H, 10.77.

Oxidation of elistanol. To 10 ml of cold (ice bath) stirred acetone was added 21.7 mg of elistanol, 5. When the sterol had dissolved, 0.2 ml of Jones Reagent[6] was added dropwise over a one minute period and the solution was allowed to stir for two minutes. Water (25 ml) and dichloromethane (25 ml) were added to the reaction mixture and the layers separated. The aqueous layer was extracted with dichloromethane (2 x 50 ml) and the organic solutions were combined and washed with water (2 x 50 ml). The dichloromethane solution was dried over sodium sulfate and the solvent removed under reduced pressure to give a white solid. This white solid was chromatographed on silicAR CC-7 using chloroform as the eluent to give (in fractions 12-26) 15.2 mg of dione 6: mp 220-221°; ir ($CHCl_3$) 3380 cm^{-1} (water) and 1714 cm^{-1} (six membered

ring ketone); pmr (pyridine-d_5) δ 1.21 (s, 3, 19-methyl group), 0.98 (d, 3, 21-methyl group). 0.91 (d, 6, terminal isopropyl methyl groups of the side chain), and 0.73 (18-methyl group); mass spectrum 414 (100), 396 (7), 263 (56), 137 (40), 109 (26), 81 (28), 69 (24), 57 (21), 55 (40), 43 (43) and 41 (26).

Molecular weight: Calcd for $C_{27}H_{42}O_3$: 414.31340. Found: 414.31347.

Cathylation of elistanol. To 5 ml of anhydrous pyridine was added 33.2 mg of elistanol. When all of the sterol had dissolved, 1 ml of ethyl chlorocarbonate was added dropwise over a one minute period. The reaction mixture was allowed to stir at room temperature protected by a drying tube for 7 hr. Water (25 ml) was added to the reaction mixture and a white solid precipitated immediately. The precipitate was removed by filtration then washed with water and then taken up in acetone and chloroform. The organic solution was dried over sodium sulfate and then the solvent removed in vacuo to yield a pale yellow solid. This solid was chromatographed on 7 g of silicAR CC-7 using chloroform as the eluent to give (in fractions 15-39) 21.2 mg of the mono-cathylate 7: mp 189-190°; $[\alpha]_D^{25}$ -30° (c 0.25 $CHCl_3$); ir ($CHCl_3$) 3410 cm^{-1} (hydroxyl group), 1735 cm^{-1} (cathylate carbonyl), 1270 cm^{-1} (carbon-oxygen stretch of a carbonate ester); pmr (chloroform-d) δ 5.2 (m, 1, proton at C-3 deshielded by cathylate), 4.2 (q, 2, methylene protons of cathylate ester), 3.65 (bs, 1, proton at C-6), 1.28 (s, 3, 19-methyl group), 0.87 (d, 6, terminal isopropyl methyl groups of the side chain), and 0.70 (18-methyl group). Anal. Calcd for $C_{30}H_{50}O_5 \cdot H_2O$; C, 70.87; H, 10.02. Found: C, 70.51; H, 10.14.

Oxidation of cathylate. To 10 ml of cold (ice bath) stirred

acetone was added 51.1 mg of the cathylate 7. When all of the cathylate had dissolved 0.3 ml of Jones Reagent[6] was added dropwise over a one minute period. The reaction mixture was allowed to stir for two minutes and then water (30 ml) and dichloromethane (30 ml) were added to the reaction mixture and the layers were separated. The aqueous layer was extracted with dichloromethane (2 x 50 ml). The dichloromethane solutions were combined then washed with water (2 x 50 ml), dried over sodium sulfate and the solvent was removed under reduced pressure. The resulting solid was chromatographed on 9 g of silicAR CC-7 using chloroform as the eluent to yield (in fractions 7-13), after recrystallization (chloroform/hexane), 48.4 mg of the cathylate-one 8: mp 229.5-230.5°; ir ($CHCl_3$) 3390 cm^{-1} (water) 1735 cm^{-1} (carbonyl of the carbonate), 1714 cm^{-1} (six membered ring ketone), and 1270 cm^{-1} (carbon-oxygen stretch of a carbonate ester); pmr (chloroform-d) δ 6.45 and 3.62 (water, disappears upon addition of methanol d_4), 5.18 (m, 1, proton at C-3 deshielded by cathylate ester), 4.19 (q, 2, methylene protons of the cathylate), 3.04 (bt, 1, proton at C-5), 1.32 (t, 3, methyl group of ethyl ester), 0.97 (s, 3, 19-methyl group), 0.93 and 0.86 (2d, 6, terminal isopropy methyl groups of side chain), and 0.68 (s, 3, 18-methyl group); mass spectrum 488 (2), 399 (30), 398 (100), 383 (32), 305 (12), 263 (43), 136 (27), 123 (20), 121 (50), 109 (23), 107 (21), 105 (27), 95 (31), 93 (39), 91 (27), 83 (23), 81 (42), 79 (22), 71 (21), 69 (42), 67 (24), 57 (41), 55 (62), 45 (22), 44 (70), 43 (74) and 41 (46); CD (c 0.052 dioxane) 26°, $[\theta]_{350}$ 0; $[\theta]_{319}$-3010; $[\theta]_{250}$0.

Anal. Calcd for $C_{30}H_{46}O_5 \cdot 2H_2O$; C, 68.67; H, 9.99. Found: C, 68.84; H, 9.84.

Hydrolysis of Cathylate-one 8. To 5 ml of methanol was added 43.1 mg of the cathylate-one 8. The solution was placed under a nitrogen atmosphere and 1 ml of 5% methanolic potassium hydroxide was added and the mixture allowed to stir under nitrogen for 48 hr. The basic solution was made acidic to pH paper with 0.5 N hydrochloric acid and 50 ml of water was added. A white solid precipitated immediately. The precipitate was removed by filtration, then washed with water, and then taken up in acetone and chloroform and the solution was dried over sodium sulfate. The solvent was removed in vacuo and the resulting solid reprecipitated twice from chloroform/hexane to give the mono-one 9 (35.2 mg) as a white amorphous solid: mp 255-256° d; ir (KBr) 3250 cm^{-1} (hydroxyl group) and 1710 cm^{-1} (six membered ring ketone).

Oxidation of the mono-one. To 20 ml of cold (ice bath) stirred acetone was added 35.0 mg of the mono-one 9. The solution was placed under a nitrogen atmosphere and when the mono-one had dissolved 0.3 ml of Jones Reagent[6] was added dropwise over a one minute period. The solution was allowed to stir for three minutes and then water (30 ml) and dichloromethane (30 ml) were added and the organic layer was separated. The aqueous solution was extracted with dichloromethane (2 x 50 ml) and the organic solutions were combined and then washed with water (2 x 50 ml). The organic solutions were dried over sodium sulfate and the solvent removed under reduced pressure to yield a white solid. The white solid was recrystallized twice from chloroform/hexane to yield 20.5 mg of the dione; mp 220.5-221°, mixed mp (mixed with 6) 220-221°. All spectra were identical with those of the dione 6 made by direct oxidation of elistanol.

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V. DACTYLYNE: A NOVEL MARINE ACETYLENIC OXETANE

INTRODUCTION

Aplysiids or sea hares have a world-wide distribution and number about thirty-five species[1]. Sea hares are found in all sizes ranging from small ones not much larger than a human thumb nail to species that are about 12-18 inches in length. The term "sea hare" is a Roman appellation stemming from the fact that aplysiids resemble a sitting rabbit[2].

Sea hares are normally found in coastal waters ranging from the tidal zones down to depths of about 200 feet. They are found primarily in areas where algae and eel grass are plentiful. The major staples of their diet are algae[2] and seaweed[3] but they have been known to devour animal substances[2] also.

One of the larger sea hares, Aplysia dactylomela Rang (approximately 10 inches in length) is a common subtropical species found in the West Indian region. This sea hare is yellow in color with brown spots randomly located on the mantle. The purpose of the work presented here was to elucidate the structure of a novel halogen containing acetylenic compound called dactylyne isolated from Aplysia dactylomela Rang. In addition to dactylyne, three novel sesquiterpene ethers have been isolated from this sea hare[4].

Several compounds have been isolated from marine algae which

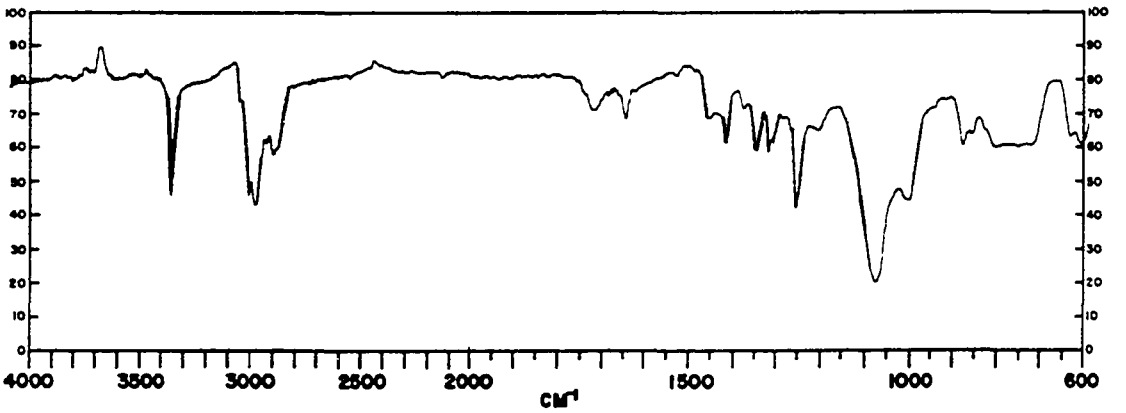
have similarities to dactylyne[5-13]. In addition a halogen containing compound has been isolated from Aplysia californica[14].

RESULTS AND DISCUSSION

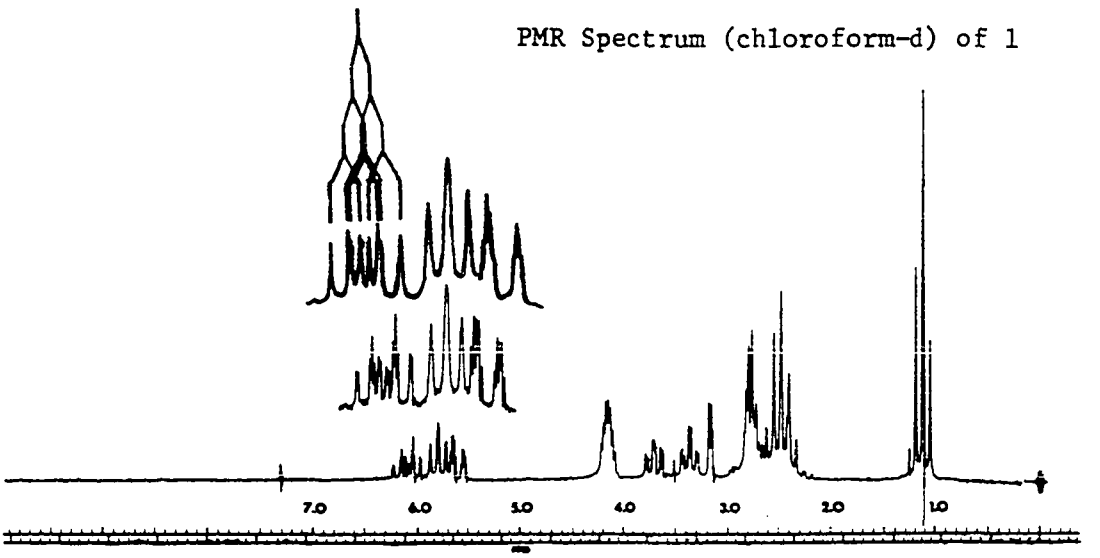
The Aplysia dactylomela Rang used in the study presented here were collected in the environs of Bimini, Bahamas Islands in the latter part of May of 1972. The freshly sacrificed whole animals were preserved in 2-propanol for shipment to Oklahoma. Soon after the specimens arrived sufficient water was added to bring the preservative liquid to a 40/60 (v/v) water/alcohol mixture and the animals were allowed to soak in this mixture for 1-2 days. The aqueous alcoholic solution was removed from the specimens by decantation and filtration. Further processing of this extract will not be discussed in this thesis. The recovered aplysia bodies were air-dried and then extracted in a Soxhlet apparatus with hexane for 2-4 days. This hexane extract after multiple chromatographies yielded dactylyne, 1, as a clear, colorless oil $[\alpha]_D^{25}$ + 33° (c 6.5 $CHCl_3$). The mass spectrum (Figure XI) indicated a molecular weight of 408 and the molecular ion showed the isotopic pattern expected for a compound containing two bromine atoms and one chlorine atom (408, 1.5%; 419, 3% and 412, 2%). Combustion analysis confirmed the ratio of halogen atoms indicated by the mass spectrum. From these data a molecular formula of $C_{15}H_{19}Br_2ClO$ is calculated for dactylyne. This formula reveals that dactylyne, 1, has five degrees of unsaturation.

The infrared (ir) spectrum (Figure XI) gives considerable information regarding the type of unsaturation present in dactylyne. The

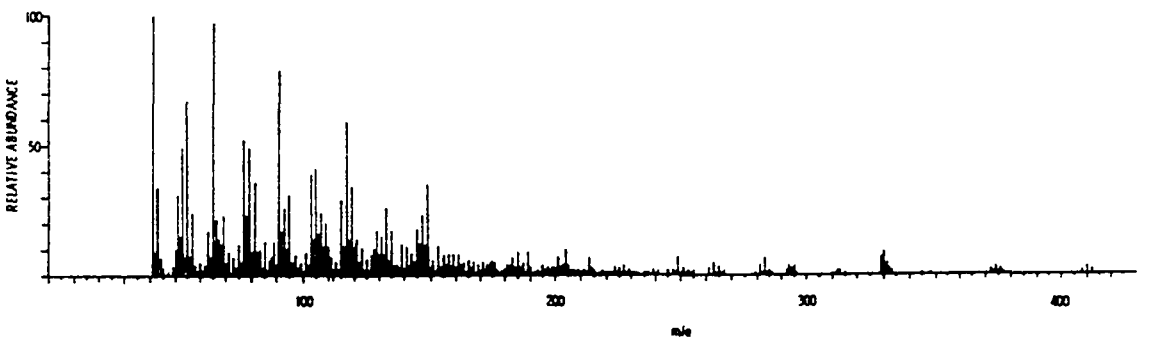
Figure XI
Spectra of Dactylyne (1)
IR Spectrum ($CHCl_3$) of 1



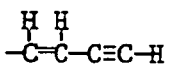
PMR Spectrum (chloroform-d) of 1



Mass Spectrum of 1



sharp strong absorption at 3305 cm^{-1} and the very weak absorption at 2100 cm^{-1} correspond to the carbon-hydrogen stretch and the carbon-carbon stretch respectively of a terminal acetylenic moiety. The ultraviolet spectrum (λ 222.5 nm with an inflection at 230 nm, ε = 12,000) is consistent with that of a molecule having a double bond in conjugation with a terminal acetylene. From these data the partial structure shown as 1*a* can be drawn. An enyne accounts for three degrees of unsaturation leaving only two other degrees of unsaturation to be accounted for.



1 *a*

The proton magnetic resonance (pmr) spectrum (Figure XI) and the decoupling data (Table V) confirm the conclusions drawn from the infrared and ultraviolet spectra. One informative feature of the pmr spectrum that should be noted at the outset is that except for the triplet at δ 1.14 all of the protons in the molecule absorb below δ 2.1. Therefore, all of the other protons are allylic, bonded to an unsaturated carbon, deshielded by a heteroatom or influenced by some other unusual structural feature.

There are three sets of absorptions observable in the olefinic region of the pmr spectrum. The first one is a fourteen line signal centered at δ 6.12 which integrated for one proton. There are three large couplings visible (J = 11, 8, and 6.5 Hz) to give four sets of doublets. Each member of each doublet is split again by a small coupling (J = 2 Hz) to give a maximum of sixteen lines, but because of the overlap

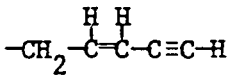
of some of the signals only fourteen lines are visible. The coupling constants, chemical shift and splitting pattern of this multiplet are consistent with expectations for the signal of an olefinic proton that is coupled to three vicinal protons and coupled long range to one more. The set of absorptions farthest upfield in the olefinic region is a doublet signal centered at δ 5.6. This signal exhibits a large coupling (J = 11 Hz) with one adjacent proton and three small couplings (J = 2, 1.5 and 1 Hz) to other protons. This absorption pattern is in agreement with that anticipated for an olefinic proton which is coupled to a vicinal olefinic proton (the large J value) and then coupled long range with three more protons (the small J values). As will be noted from the double irradiation data (Table V) the protons whose signals appear at δ 6.12 and 5.6 are coupled with each other. Since the two protons at δ 6.12 and 5.6 are coupled to each other they should be the two olefinic protons of the *1a* moiety. Irradiation of the protons at δ 6.12 or 5.6 collapsed the signal at δ 3.18 to a doublet. The absorption at δ 3.18 integrates for one proton and the chemical shift is appropriate for a terminal acetylenic proton signal. Irradiation of the acetylenic proton (δ 3.18) eliminated a 2 Hz splitting from both the δ 6.12 and 5.6 signals, and hence it is evident that these two olefinic protons are both coupled to the terminal acetylenic proton with a J of 2 Hz. In addition to being coupled to the olefinic proton and the acetylenic proton, the proton whose signal appears at δ 6.12 indicates by its splitting pattern (total of four different J's) that it is coupled to two more protons on another adjacent carbon. Therefore, the partial structure *1a* can be expanded to the partial structure shown in *1b*.

TABLE V

DOUBLE IRRADIATION OF DACTYLYNE 1.[a]

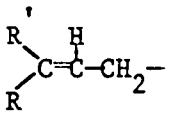
Proton Irr.	Signal Obs.	Change Obs.	Remarks
1.14	2.48	Collapsed to s[b,c]	
2.48	1.14	Collapsed to s	
6.12	2.7	Disturbed[c]	
	5.6	Collapsed to bs	
	3.18	Collapsed to d	J = 2 Hz
2.7	6.12	Collapsed to dd	J = 11,2 Hz
	3.71	Collapsed to d	J = 2 Hz
	5.6	Disturbed	J = 11 Hz
5.6	6.12	Collapsed to dd	
	2.7	Disturbed	
	3.18	Collapsed to s	
5.8	2.4	Disturbed[c]	
2.4	5.8	Collapsed to s	
	3.37	Collapsed to d	J = 2 Hz
4.16	3.71	Collapsed to dd	J = 7,8 Hz
	3.37	Collapsed to t	J = 7 Hz
	2.78	Disturbed	
3.71	4.16	Disturbed	
	2.7	Disturbed	
3.37	4.16	Disturbed	
	2.4	Disturbed	
3.18	6.12	Collapsed to 8 lines	
	5.6	Collapsed to bd	J = 11 Hz
2.78	4.16	Collapsed to d	J = 6 Hz

[a]Double irradiation experiments were performed on the Varian XL-100 or 220 MHz instruments in chloroform-d. [b]Abreviations used in this table: s, singlet; bs, board singlet; d, doublet; dd, double doublet; t, triplet; bd, broad doublet. [c]These spectral changes are unambigously observable only in the 220 MHz decoupling experiments.



1 *b*

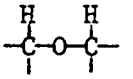
The remaining olefinic proton absorption appears at δ 5.81 and is a triplet which integrates for one proton. Since this absorption is a simple triplet the proton producing this signal must have only two protons on the adjacent allylic carbon and none on the neighboring olefinic carbon. Because of this fact the partial structure 1*c* can be drawn.



R and R' ≠ H

1 *c*

Upfield from the olefinic region is a two proton multiplet centered at δ 4.16. The chemical shift of this signal is typical of protons on a carbon bearing oxygen. Since the ir spectum (Figure XI) does not show a hydroxyl absorption these two protons must be on carbon(s) bearing an ether oxygen. Because of the multiplicity, at least a septet, and symmetrical shape of the peak it would seem likely that these protons are on opposite sides of the ether oxygen leading to the conclusion that partial structure 1*d* can be drawn.



1 *d*

Upfield from the two proton absorption at δ 4.16 is a one proton double double doublet signal at δ 3.71. The chemical shift of this

absorption is appropriate for a proton on a carbon bearing halogen. The multiplicity of this signal, a double double doublet, J = 8, 7, and 2 Hz, indicates that the proton absorbing at δ 3.71 is coupled to two protons on one adjacent carbon and one proton on the other neighboring carbon.

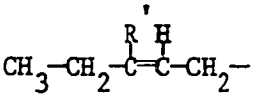
At δ 3.37 is a one proton doubled triplet. The chemical shift of this signal is also appropriate for a proton on a carbon bearing halogen. Since the two protons attached to carbons bearing halogen absorb at different chemical shift positions, it is likely that the particular halogens associated with each of these absorptions is different, i.e., chlorine vs bromine. The splitting pattern of the δ 3.37 absorption indicates that the proton producing this signal also has two protons on one neighboring carbon and one on the other adjacent carbon. These data indicate there are two 1*e* units in dactylyne.



1 *e*

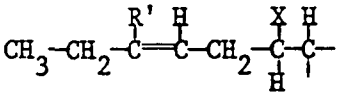
The δ 3.03-2.1 region in the pmr spectrum of dactylyne integrates for eight protons and except for the apparent quartet signal centered at δ 2.52 it is difficult to tell much about this area. The quartet centered at δ 2.52 is attributable to the absorption of methylene protons that are flanked on one side by a methyl group and on the other side by an olefinic carbon bearing no proton. If this is the case then irradiation at δ 2.52 should collapse the three proton triplet at δ 1.41 to a singlet and conversely irradiation at δ 1.41 should collapse the

quartet at δ 2.52 to a singlet. As can be seen from Table V this is exactly what happens. Therefore, one of the R groups shown in partial structure 1*c* must be an ethyl group and partial structure 1*c* can be expanded to partial structure 1*f*.



1 *f*

The sequence in which the isolated structural fragments that have been discussed thus far are joined together can be determined by examination of the remainder of the coupling data in Table V. As shown in Table V, when the sample is irradiated at δ 5.8, the position of the triplet absorption corresponding to the vinyl proton in partial structure 1*f*, the absorption at δ 2.4 is altered slightly. Conversely when the δ 2.4 region is irradiated not only does the signal at δ 5.8 collapse to a singlet, but the signal at δ 3.37 for one of the protons on a carbon bearing halogen collapses to a doublet (J = 2 Hz). Therefore, the allylic methylene of partial structure 1*f* is the same as the methylene of one of the 1*e* halogen containing groupings and 1*f* can be expanded to the partial structure 1*g* by combining 1*f* and one 1*e* unit.

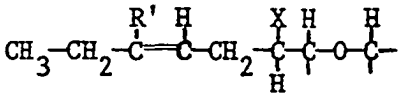


X = Br or Cl

1 *g*

Table V also reveals that when the proton on the carbon bonded to the halogen in 1*g* (δ 3.37) is irradiated the absorption of the proton on carbon bearing oxygen (δ 4.16) is disturbed. Therefore, the

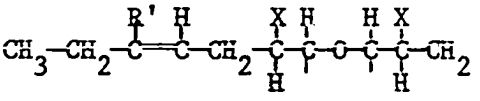
single proton on the carbon adjacent to the —CHX— unit in 1*g* is the same as one of the protons on the ether carbons in 1*d*; hence 1*d* and 1*g* can be combined to give partial structure 1*h*.



X = Br or Cl

1 *h*

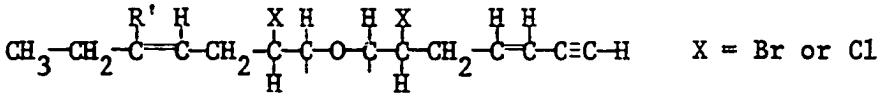
When the δ 4.16 position is irradiated, not only does the absorption at δ 3.37 collapse to a triplet but, as can be seen from Table V the signal at δ 3.71 collapses to a double doublet. This indicates that the proton on the other ether carbon in 1*h* must be the proton on the more substituted end carbon of the second 1*e* unit and hence partial structure 1*h* can be expanded to partial structure 1*i*.



X = Br or Cl

1 *i*

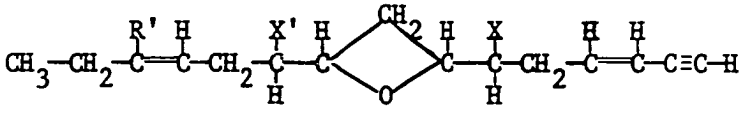
If the proton absorbing at δ 3.71 is irradiated, not only is the absorption at δ 4.16 disturbed, but a signal at δ 2.7 is disturbed. If the proton absorbing at δ 2.7 is irradiated not only does the signal at δ 3.71 collapse to a doublet (J = 2 Hz) but the absorption at δ 6.12 collapses to a double doublet and the signal at δ 5.6 is disturbed. This indicates that the methylene group adjacent to the carbon bearing halogen on the far right in partial structure 1*i* and the methylene group flanking the olefinic carbon of partial structure 1*b* must be the same. Therefore, partial structures 1*i* and 1*b* can be combined to give partial structure 1*j*.



X = Br or Cl

1 *j*

Counting the atoms of partial structure 1*j* gives the formula $C_{14}H_{17}X_2O$. This falls short of the formula established for dactylyne by only one carbon atom, two hydrogen atoms and a halogen atom. Since all of the downfield absorptions, i.e., δ 3.37, 3.71 and 4.16, caused by the deshielding of the heteroatoms have been accounted for, it would seem that the remaining carbon and hydrogen atoms are present as a methylene group which joins the two ether carbons of 1*i* together to form an oxetane. The data in Table V lends support to this conclusion because if the protons absorbing at δ 2.78 are irradiated the multiplet at δ 4.16 (two hydrogens on carbons bearing oxygen) collapses to a doublet. The chemical shift position of δ 2.78 is very close to that observed for the central methylene protons of oxetane itself[15] (δ 2.7) and a related naturally occurring oxetane (δ 2.9) isolated by Irie and co-workers[5,8]. Therefore partial structure 1*j* can be expanded to structure 1*k*.

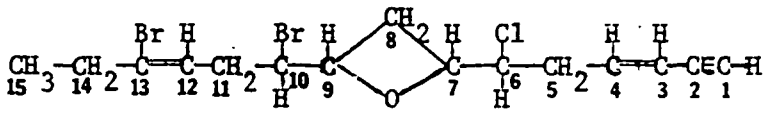


X and X' = Br or Cl

1 *k*

The structure 1*k* accounts for all of the carbon and hydrogen atoms and leaves only the relative placement of the halogen atoms to be determined. Mass spectral evidence indicates that a cleavage occurs that gives fragment ions consistent in isotopic ratio and weight for R' to be bromine ($M^+ - CH_3-CH_2-CBr=CH-CH_2$, m/e 261, and 263 and the

complementary ions m/e 147 and 149). Hence, R' must be bromine. Mass spectral evidence further indicates that both the bromine atoms are on the same side chain and that the chlorine atom is on the other side chain which contains the acetylenic moiety, because the cleavage between C-7, 8 and C-7, oxygen with a hydrogen transfer to the side chains were X = Cl (in 1*k*) gives a peak at m/e 127 and the corresponding peaks for the remainder of the molecule at m/e 281, 283 and 285. Although the peaks corresponding to this cleavage with X = Br are present (237, 239; 171, 173) the intensities are weaker than the sets mentioned above and hence the structure 1*k* with X = Cl is favored. Because of the greater electronegativity of chlorine as opposed to bromine, chlorine would be expected to deshield a proton more than bromine. Since the proton associated with the δ 3.71 absorption was shown by decoupling evidence to be homoallylic to the conjugated enyne moiety, the chlorine is then on the side chain bearing the acetylene. Therefore, the halogens can now be added to structure 1*k* to give structure 1*l*.



1 *l*

The stereochemistry of the double bonds and the relative stereochemistry of the substituents on the oxetane ring can be assigned from spectral data. Irie and co-workers have shown that for double bonds in conjugation with a terminal acetylene, a coupling constant of 11 Hz between vinyl protons indicates cis double bond geometry while a coupling constant between olefinic protons of 15 Hz correspond to a trans

configuration[5-8]. The coupling constant between the olefinic protons of dactylyne is 11 Hz. Therefore, the double bond conjugated with the terminal acetylene in dactylyne is cis.

The carbon-13 magnetic resonance spectrum (Table VI) gives some indication as to the stereochemistry of the isolated double bond in the side chain 17. The chemical shifts of carbons allylic to an isolated double bond containing bromine were calculated using chemical shift values of similar compounds found in reference 16. The calculated values were obtained in the following manner, The chemical shift values of C-2 and -5 of cis and trans 3-octene[16] were taken as the base values. It was assumed that the deshielding effect of bromine on a carbon β to it is the same whether bromine is bonded to a saturated or an olefinic carbon. It was further assumed that the shielding effect of bromine on a carbon γ to it is the same whether bromine is bonded to a saturated or an olefinic carbon. The reported chemical shift of the proper alkane carbon was then subtracted from the reported value of the same carbon in the corresponding bromo alkane to obtain a Δ ppm. The Δ ppm value calculated in this manner was then added to the values for the C-2 and -5 carbons of both cis and trans 3-octene keeping in mind that C-11 of dactylyne has a bromine both β and γ to it. The values calculated for 3-octene by this method were: cis C-2, $\approx$ 31, C-5, $\approx$ 34; trans C-2, $\approx$ 36, C-5, $\approx$ 39. The observed values for dactylyne are C-11, 35.36 and C-14, 34.05. Since the values

TABLE VI

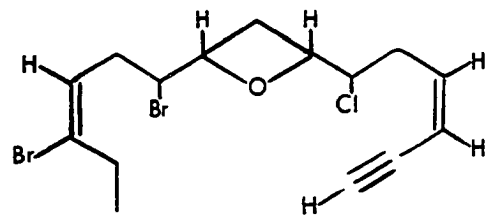
DACTYLYNE C^{13} CHEMICAL SHIFT[a] ASSIGNMENTS*

Carbon No. (12)	Chem. Shift	Mult[b]
1	79.25	--[c,d]
2	79.68	s
3	110.78	d
4	139.75	d
5	39.04	t
6	54.59	d
7	83.2	--[d]
8	29.04	t
9	79.45	--[d]
10	46.02	d
11	35.36	t
12	125.12	d
13	131.3	s
14	34.05	t
15	13.27	q

[a] Chemical shifts were determined using the C^{13} resonance of TMS as an internal standard. [b] Multiplicities were determined from the off-resonance decoupled spectrum of dactylyne. [c] Symbols used in this table are: s, singlet; d, doublet; t, triplet; and q, quartet. [d] Each of these peaks should be a doublet. However, one leg of the doublet is coincident with a solvent peak and cannot be seen.

*Except for C-5 and -11 the chemical shift assignments were made by comparison of observed chemical shift values with those reported in tables [16] together with multiplicity information. C-5 and -11 were assigned on the basis of the generalization gleaned from tables[16] that carbons γ to bromine are shielded. Therefore, C-11 should be farther upfield than C-5.

calculated* for the cis double bond more closely approximate those observed than do the trans values, structure 1ℓ can be drawn as shown in 1m.



1 *m*

The question of the sterochemistry of the oxetane ring still remains. In the pmr spectrum of dactylyne, the oxetane methylene proton signals fall in the same region as the allylic protons resonances and this makes it impossible to determine the multiplicity of the former absorption. Therefore, to remove the allylic proton absorptions dactylyne was hydrogenated over platinum oxide. A white crystalline solid, 2, was obtained. The mass spectrum (Figure XII) of 2 shows a molecular ion of 338 with an isotopic ratio expected of a compound that contains one bromine and one chlorine. This molecular ion corresponds to a molecular formula of $C_{15}H_{29}BrClO$. The pmr spectrum indicates that all of the carbon-carbon unsaturation has been reduced (absence of olefinic and the acetylenic proton absorptions). The signals of the

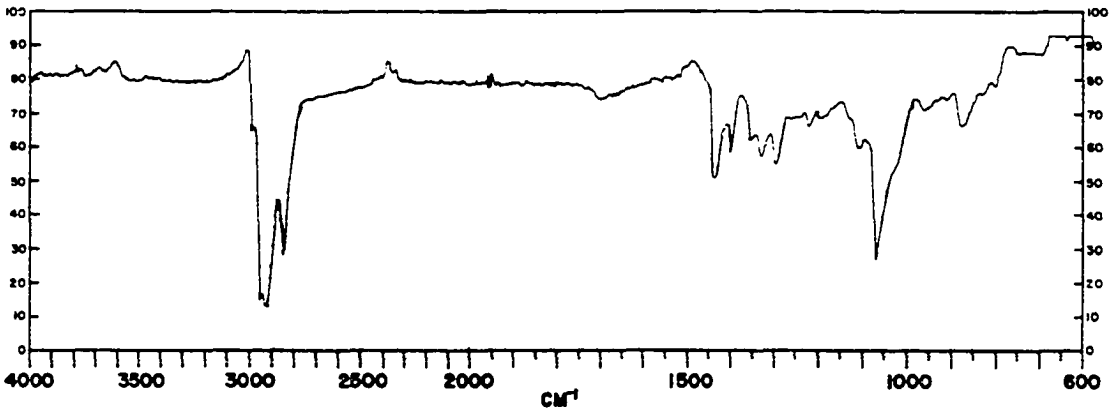
---

* 3-octene

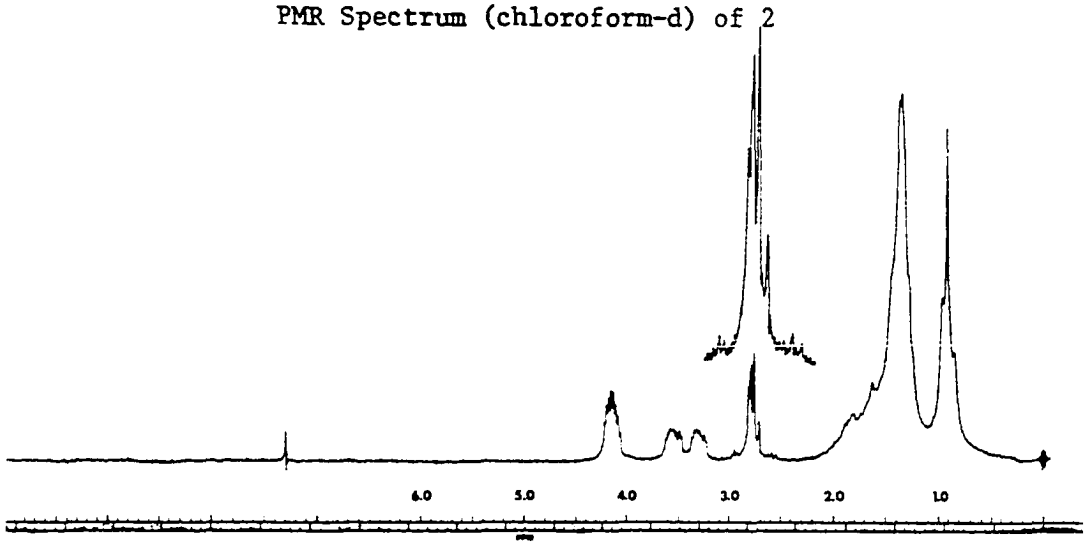
Cis			trans		
C-2, 20.7	C-5, 26.8		C-2, 25.4	C-5, 32.2	
+10.6	+10.6		+10.6	+10.6	
31.3	37.4		36.0	42.8	
	-3.7			-3.7	
	33.7			39.1	

1 - Bromo pentane	C-2	33.2	1 - Bromo pentane	C-3	30.8
Pentane	C-2	22.6	Pentane	C-3	34.5
		+10.6			- 3.7

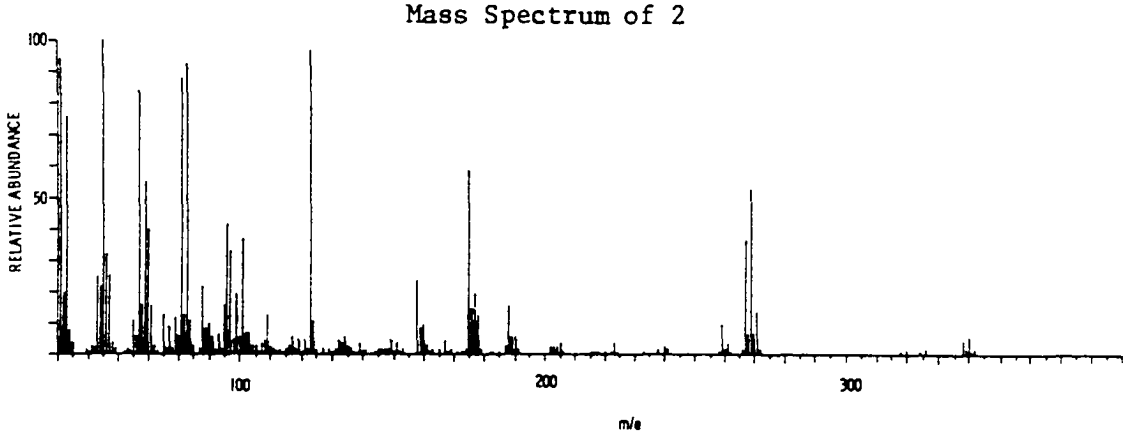
Figure XII
Spectra of octahydrodebromodactylyne (2)
IR Spectrum ($CHCl_3$) of 2



PMR Spectrum (chloroform-d) of 2

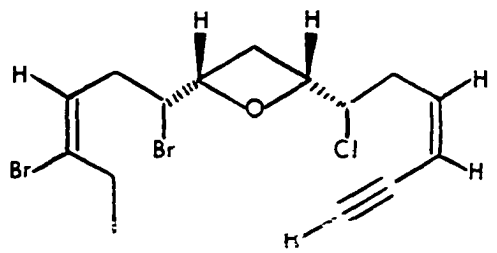


Mass Spectrum of 2

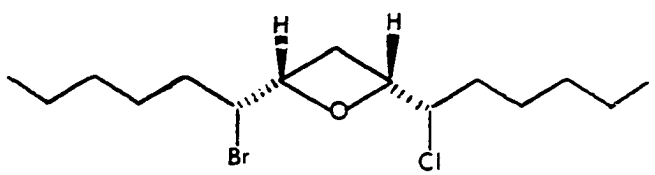


protons on carbon bearing haolgen are present (δ 3.32 and 3.58) and have shifted only slightly from those in dactylyne (δ 3.37 and 3.7). The absorption of the two protons on carbons bonded to oxygen is present at δ 4.15. As expected the signal for the methylene protons in the oxetane are now clearly visible at δ 2.78. When the proton absorbing at δ 2.78 is irradiated the multiplet at δ 4.15 collapses to a broad doublet. Irradiation of the protons absorbing at δ 4.15 simplifies the absorption at δ 2.78. This is strong evidence for the presence of an oxetane ring. The pmr spectrum of oxetane itself[15] shows a well resolved five line signal with equal coupling between each line for the two central methylene protons of the ring. As can be seen from the pmr spectrum of octahydrodebromodactylyne (Figure XII) the absorption of the methylene protons of the oxetane ring (δ 2.78) is quite different from that of the corresponding protons of oxetane. In octahydrodebromodactylyne the oxetane methylene absorption consists of two triplets (J = 4 and 2 Hz) at δ 2.76 and 2.79. If the substituent side chains on the oxetane ring were trans, the splitting pattern of each of the methylene protons would be a double doublet, corresponding to a large and a small coupling, with each of the protons on the carbon bearing oxygen to give a total of eight lines. For the trans case examination of models indicates the couplings would be approximately 6 and 2 Hz. If the substitutent side chains on the oxetane were cis oriented, the methylene proton cis to the alkyl substituents would experience a trans and equal coupling to the two neighboring protons and hence give rise to a triplet signal. At the same time the methylene proton trans to the alkyl substituents would experience a cis coupling to the two neighboring protons and also give rise to a

triplet. However, the magnitude of the coupling constants would be expected to be different for each triplet because of the difference in dihedral angles. For the cis case the coupling constants should be different but fairly small (from analysis of models). The observed pattern (two triplets) and the coupling constants (J = 4 and 2 Hz) more closely coincide with what is expected for the cis substituted oxetane ring. This conclusion leads to the proposed structure 1 for dactylyne and 2 for its octahydrodebromo derivative.



1

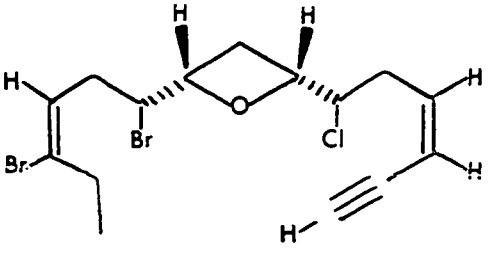


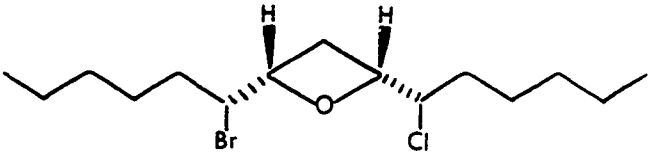
2

There are three items of the proposed structure of dactylyne, 1, that need further verification. These items are the stereochemistry of the isolated double bond in the side chain, the stereochemistry (relative and absolute if possible) of the substituents on the oxetane ring and the position of the secondary halides (which side chain each is in). Complete resolution of the stereochemical problems may ultimately require x-ray analysis.

SUMMARY

The spectral and chemical evidence which forms the basis for a proposed structure of a novel halo acetylenic compound called dactylyne, 1, isolated from the sea hare Aplysia dactylomela Rang is presented. The conversion of dactylyne, 1, to its octahydrodebromo derivative 2 is discussed along with the sterochemical conclusions that can be drawn from the pmr spectrum of 2.





2

EXPERIMENTAL

All melting points are uncorrected. All solvents were distilled prior to use. Column chromatographic packings used were silicAR CC-7 (Mallinckrodt, 100-200 and 200-325 mesh), florisil (Fisher Scientific), silica gel (Grace, 60-200 mesh) and silica gel H (E. Merck AG, Darmstadt). Thin layer chromatography was performed on 5 x 20 cm glass plates with silica gel H (E. Merck Ag, Darmstadt) or on prepared plates of Sil Gel G/UV_{254} (Machery-Nagel, Duren), silica gel F-254 (E. Merck AG, Darmstadt) or Sil Gel N-HR/UV_{254} (Machery-Nagel, Duren). The developed plates were placed under ultraviolet light and/or exposed to iodine vapors or sprayed with a 10% sulfuric acid solution for visualization of the chromatogram. Preparative layer chromatography was performed on 20 x 20 cm glass plates with a 2 mm layer of silica gel PF-254 + 366 (E. Merck AG, Darmstadt).

Proton magnetic resonance (pmr) spectra were taken on Varian T-60, XL-100 or 220 MHz spectrometers using tetramethylsilane (TMS) as an internal reference. Samples were run in varying concentrations of chloroform-d. Chemical shifts are reported in δ-units (parts per million from TMS) and are followed by the multiplicity of the signal, the number of protons, the corresponding coupling constant(s) and the assignment. The muliplicities are denoted by the symbols: s, singlet; bs, broad singlet; d, doublet; dd, doulbe doublet; ddd, double double doublet; t, triplet, q, quartet; and m, multiplet.

The carbon-13 nuclear magnetic resonance spectra were taken on a Varian XL-100 spectrometer using a Nicolet/TTI Fourier Transform with the carbon-13 resonance of TMS used as an internal reference. The sample was run in chloroform-d. Chemical shifts were printed-out by the Nicolet computer.

Infrared spectra were taken on a Beckman IR-8 spectrometer in chloroform solutions. The ultraviolet spectrum was taken on an isooctane solution on a Cary Model 118 (Varian) spectrometer. Mass spectra were taken on a Hitachi Perkin-Elmer RMU-7E mass spectrometer using perfluorokerosene-H as an internal reference. Mass spectra are reported by the mass of the ion follwed by the percentage of the base peak.

Combustion analyses were carried out by Mr. Eric Meier, Chemistry Department, Stanford University, Stanford, California.

Isolation of Dactylyne. The aplysia bodies recovered from a 1:1 2-propanol/water extraction were air-dried and then extracted in a Soxhlet apparatus with distilled hexane for 2-4 days. The hexane extract was filtered and the solvent was evaporated to yield 235 g of a dark brown, viscious oil. A portion of the crude extract (75 g) was chromatographed on florisil (1500 g) using the following solvent gradient elution:

A. Hexane (3 l)

B. 1:1 Hexane/benzene (3 l)

C. Benzene (3 l)

D. 1:1 Benzene/chloroform (3 l)

E. Chloroform (3 l)

F. 5% Methanol/chloroform (3 l)

G. Methanol (3 l).

Fractions B, C and D were combined to give 28 g of an orange oil. A protion of this orange oil (10 g) was chromatographed on silica gel H (120 g) using the following solvent gradient elution:

A. Hexane (500 ml)

B. 2.5% Ether/hexane (250 ml)

C. 5% Ether/hexane (one 500 ml and one 400 ml fraction)

D. 10% Ether/hexane (400 ml)

E. Chloroform (400 ml)

F. Methanol (400 ml).

The last 400 ml of the 5% ether/hexane and the 10% ether/hexane fractions were combined to yield 3.9 g of an orange oil. This orange oil (3.9 g) was cnromatographed on silica gel H (62 g) and eluted with 10% ether/hexane. Fractions were collected (100 ml) and the second and third fraction yielded 2.74 g of a pale orange oil. A portion of this pale orange oil (242 mg) was chromatographed on silicAR CC-7 (200-325 mesh) using 25% benzene/hexane as the eluent. Fractions (5 ml) were collected and fractions 48-72 were combined to yield 223 mg of a pale yellow oil. This pale yellow oil (223 mg) was chromatographed on a silica gel preparative layer plate that was eluted four times with 15% benzene/hexane to yield 130 mg of pure dactylyne 1 (5.5%): $[\alpha]_D^{25}$ + 33° (c 6.5 $CHCl_3$); uv 222.5 nm with an inflection at 230 nm (ε = 12,300); ir ($CHCl_3$) 3310 cm^{-1} (C-H of terminal acetylene), 1650 cm^{-1} (olefinic carbon-carbon stretch), 1090 cm^{-1} (carbon-oxygen stretch) and 790 cm^{-1} (carbon-chlorine stretch); pmr ($CDCl_3$) δ 6.12 (14 lines, 1, J = 11, 8, 6.5 and 2 Hz, olefinic proton), 5.8 (t, 1, J = 8 Hz, olefinic proton), 5.6 (10 lines,

1, J = 11, 2, 1.5 and 1 Hz, olefinic proton), 4.16 (m, 2, -CH-O-), 3.71 (ddd, 1, J = 8, 7 and 2, -CHCl-), 3.37 (dt, 1, J = 8 and 2, -CHBr-), 3.16 (dd, 1, J = 2 and 1, terminal acetylenic proton), 3.0-2.1 (m, 6, allylic and oxetane ring methylene protons), 2.48 (q, 2, J = 8, methylene protons of the ethyl group) and 1.14 (t, 3, J = 8 Hz, methyl of ethyl group); mass spectrum 412 (2), 410 (3), 408 (1.5), 376 (2), 374 (3), 372 (2), 332 (3), 331 (5), 330 (9), 329 (5), 328 (7), 296 (1), 295 (4), 294 (3), 293 (4), 292 (2), 285 (2), 283 (6.5), 281 (4), 265 (3.5), 263 (5), 261 (3), 149 (35), 147 (23), 145 (18), 133 (26), 119 (34), 117 (59), 115 (29), 109 (20), 107 (24), 105 (41), 103 (39), 93 (26), 91 (79), 81 (36), 79 (49), 78 (23), 77 (52), 69 (23), 66 (22), 65 (97), 57 (24), 55 (67, 53 (49), 51 (31), 43 (34), and 41 (100).

Anal. Calcd for $C_{15}H_{19}Br_2ClO$: C, 43.88; H, 4.66; Br, 38.92; Cl, 8.64. Found: C, 41.86; H, 4.60; Br, 41.55; Cl, 9.12.

Reduction of dactylyne. To 20 ml of ethyl acetate was added 600 mg of dactylyne. Platinum oxide was added and the mixture was hydrogenated at 45 psi and room temperature for 14 hr. The catalyst was filtered off and the solvent removed at reduced pressure to yield 509 mg of a light orange oil which crystallized upon standing in the cold. The solid was chromatographed on 25 g of silicAR CC-7 using 50% benzene/hexane as eluent. Fractions of 7 ml were collected and the hydrogenated product came off the column in fractions 8-16. The hydrogenated product was recrystallized from methanol to give 207 mg of a white crystalline solid, 2: mp 52-53°; $[\alpha]_D^{25}$ -7° (c. 1.4 $CHCl_3$); ir ($CHCl_3$) 1085 cm^{-1} (ether carbon-oxygen stretch); pmr ($CDCl_3$) δ 4.15 (m, 2, protons on carbon bearing oxygen), 3.54 (m, 1, -CHCl), 3.31 (m, 1 -CHBr-), 2.78

(t, 1, J = 2 Hz, one of oxetane methylene protons), 2.76 (t, 1, J= 4 Hz, one of oxetane methylene protons), 1.35 (m, 14, methylene protons of side chain), 0.91 (m, 6, terminal methyl group protons); mass spectrum 340 (6), 338 (5), 271 (14), 269 (53), 188 (16), 178 (13), 177 (20), 176 (15), 175 (59), 123 (97), 101 (37), 99 (19), 97 (33), 96 (42), 95 (16), 88 (22), 83 (93), 81 (88), 70 (40), 69 (55), 67 (84), 57 (25), 56 (32), 55 (100), 54 (22), 53 (25), 43 (76), 42 (20), 41 (94).

Anal. Calcd for $C_{15}H_{28}BrClO$: C, 52.87; H, 8.58; Br, 23.45; Cl, 10.40. Found: C, 53.47; H, 8.24; Br, 23.11; Cl, 10.09.

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