[CONTRIBUTION FROM THE STERLING CHEMISTRY LABORATORY, THE BINGHAM OCEANOGRAPHIC LABORATORY, AND THE BERMUDA BIOLOGICAL STATION FOR RESEARCH]

# CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS. XXIII.[1] STEROLS FROM SPONGES OF THE FAMILY *HALICLONIDAE*

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In the XIXth communication of this series (1), the isolation of chalinasterol from the sponge, *Chalina arbuscula*, has been described, and in a subsequent paper the suggestion has been made that this sterol is identical with ostreasterol (2). Since then, Dr. de Laubenfels,[3] the eminent sponge taxonomist, has informed the senior author that the name of this sponge should be changed to *Haliclona arbuscula*, because more recent taxonomic studies have shown the sponge to be a typical representative of the family *Haliclonidae*, genus *Haliclona*. Approximately one hundred species have so far been placed within this genus, the taxonomy of which is not always lucid. It appeared, therefore, of interest to ascertain whether the presence of chalinasterol is sufficiently characteristic for certain studies. For this reason the sterols of six other species of *Haliclona* have been investigated. The sponges have been collected by the senior author in the waters near Bermuda and Florida,[4] and they have been identified by Dr. M. W. de Laubenfels. The contents of the fatty material of these sponges are shown in Table I.

I. *Haliclona variabilis* (Dendy) de Laubenfels. This sponge is fairly common in the shallow waters of the Bermuda Archipelago, particularly in Harington Sound and Walsingham Pond. After only a few recrystallizations, the acetate of its sterol showed the constant melting point 147–148°; $[\alpha]_D^{26}$ −52°. Its identity with poriferasteryl acetate (3) was demonstrated by comparison with an authentic sample, and by its conversion to the high-melting tetrabromide, and to poriferasterol, m.p. 155.5–156°; $[\alpha]_D^{26}$ −50°. It is estimated that poriferasterol represents in excess of 80% of the original sterol mixture. This sponge therefore affords the richest source of poriferasterol which has so far been encountered.

II. *Haliclona permollis* (Bowerbank) de Laubenfels. This soft, compressible, purple sponge was collected in Hungry Bay, Bermuda. The acetate of the crude sterol obtained from this sponge melted at 133–135°; $[\alpha]_D^{26}$ −46°. Fractionation of the bromine addition products led to the isolation of a high-melting tetrabromide, which upon debromination yielded poriferasteryl acetate.

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Debromination of the more soluble bromides yielded an acetate, m.p. 135–136°; $[\alpha]_D^{26}$ −38°, which appeared to be clionasteryl acetate. Lack of material prevented a more detailed separation of the components of the sterol mixture, but there appears little doubt that poriferasterol and clionasterol are the major constituents.

III. *Haliclona coerulescens* (Topsent). This light blue sponge was collected on Featherbed Bank near Miami. The acetate of the crude sterol obtained from this sponge melted at 133°; $[\alpha]_D^{26}$ −40°. Fractionation by way of the bromides gave results similar to those described under *Haliclona permollis*. It is estimated that the original sterol mixture contained from 30–35% of poriferasterol and 65–70% of clionasterol.

IV. *Haliclona viridis* (Duchaissaing and Michelotti) de Laubenfels. This common, soft, green sponge was collected in the shallow waters near Miami and Bermuda. The sponges from the different localities were investigated separately, but no significant differences in their composition were observed.

TABLE I

COMPOSITION OF DRIED *Haliclona* SPECIES

| SPECIES | % OF TOTAL | | % OF ORGANIC FAT | % OF FAT UNSAPON. | % OF UNSAPON. STEROL |
|---|---|---|---|---|---|
| | Spicules | Organic | | | |
| *arbuscula* | 30 | 70 | 5 | 35 | 38 |
| *variabilis* | 8 | 92 | 7.2 | 24.5 | 63.5 |
| *coerulescens* | 7 | 93 | 2.6 | 48 | 67.5 |
| *permollis* | 20 | 80 | 13.5 | 30 | 44 |
| *viridis* | 25 | 75 | 5.8 | 30.5 | 68.5 |
| *rubens* | 20 | 80 | 3.7 | 76 | 14.5 |
| *longleyi* | 44 | 56 | 3.5 | 38.5 | 74 |

The acetate of the crude sterol obtained from this sponge melted at 120–130°; $[\alpha]_D^{25}$ −40°. Systematic fractionation of the more difficultly soluble bromides of the acetate mixture led to the isolation of a small amount of a tetrabromide of m.p. 200–205°. The high melting point of this product contradicts its identity with poriferasteryl acetate tetrabromide. Lack of material prevented further characterization of this product. The bulk of the material, the more difficultly soluble bromides, consisted of cholesteryl acetate dibromide, which was characterized by its conversion to cholesteryl acetate and cholesterol. Debromination of the more soluble bromides eventually lead to the isolation of an acetate of m.p. 136°; $[\alpha]_D^{27}$ −38°.

The sterol mixture of this sponge, therefore, consists of at least three components, of which cholesterol represents more than 50%. From 5–10% consists of a di-unsaturated sterol of the probable empirical formula, $C_{28}H_{46}O$. The remainder of the material shows similarity to clionasterol.

V. *Haliclona rubens* (Duchaissaing and Michelotti). This tall and thick red sponge was collected in the shallow waters of Biscayne Channel near Miami.

Its deep red pigment shows remarkable resistance against photo-oxidation, and it is only in part extracted by lipoid solvents. The acetate of the crude sterol obtained from this sponge melted at 130–135°; $[\alpha]_D^{25}$ −44°. Systematic fractionation gave results similar to those obtained with the sterol from the preceding sponge.

VI. *Haliclona longleyi* de Laubenfels. This ramose sponge was collected in Biscayne Bay, Miami, in 1945 and 1948. Investigated separately, the two lots gave practically identical results. The acetate of the sterol obtained from it melted at 140–141°; $[\alpha]_D^{27}$ −46.6°. Its unexpectedly high degree of uniformity was demonstrated by the fact that its properties did not change with numerous recrystallizations. Bromination of the acetate yielded a minute amount of a tetrabromide of m.p. 187–190°. The bulk of the material was a nicely crystalline dibromide of m.p. 125–126°. Addition of ethanol to a solution of the bromide in ether leads to the formation of a gelatinous precipitate which gradually changes into clear, thin prisms, up to one centimeter in length. This peculiar

TABLE II

PROPERTIES OF HALICLONASTEROL

| DERIVATIVE | M.P., °C. | $[\alpha]_D^\circ$ | $[M]_D^\circ$ |
|---|---|---|---|
| sterol | 140.5–141 | −42 | −168 |
| steryl acetate | 140–141 | −46 | −203 |
| steryl acetate dibromide | 125–126 | | |
| steryl benzoate | 146.5 | −15 | −76 |
| steryl *m*-dinitrobenzoate | 209 | | |
| stanol | 137.5 | +18 | +72 |
| stanyl acetate | 136.5 | +11 | +49 |
| stanyl benzoate | 133–134 | | |
| stanyl *m*-dinitrobenzoate | 218 | | |

behavior sets this dibromide apart from all other steryl acetate dibromides which have so far been described in the literature. Debromination of this product, and the various fractions obtained from the bromination mother liquors gave acetates with the same physical properties as the starting material.

The properties of the sterol obtained by way of the acetate, and those of a series of its derivatives are shown in Table II. They indicate the difference of this sterol from all other sterols which have so far been described. It is therefore proposed to name it *haliclonasterol.* The ready formation of an acetate dibromide, titration with perbenzoic acid, and quantitative catalytic hydrogenation prove the sterol to be mono-unsaturated. The differences between the molecular rotations of the sterol, its acetate and benzoate, and between those of the saturated and unsaturated derivatives (4) locate the double bond in 5,6-position. The results of the analyses of the steryl *m*-dinitrobenzoate, the stanyl *m*-dinitrobenzoate, and the acetate dibromide suggest the empirical formula $C_{28}H_{48}O$ for haliclonasterol. This sterol therefore appears to be an isomer of campesterol (5) and 22,23-dihydrobrassicasterol (6).

Unfortunately, lack of sufficient quantities of haliclonasterol has as yet prevented elucidation of the nature of this isomerism.

## DISCUSSION

The investigations described above have shown that chalina (ostrea) sterol is not the typical sterol of all sponges which have at present been placed within the genus *Haliclona*. On the contrary, the presence of this sterol has been demonstrated in only one of the seven species which have so far been studied. There exists a lack of uniformity in the nature of the sterols which have been isolated from the other six sponges. The sterol mixtures of two of these contain one sterol in excess of 80% of the total, such as the poriferasterol of *H. variabilis*, and the new haliclonasterol of *H. longleyi*. The sterols of the other sponges are rather complex mixtures. A distinct similarity is noticeable in the composition of the mixtures obtained from *H. permollis* and *H. coerulescens*, and those from *H. viridis* and *H. rubens*. In the former the two major components appear to be poriferasterol and clionasterol. In the latter the most characteristic feature is the presence of substantial amounts of cholesterol which may exceed 50% of the total.

It is at present difficult to evaluate the significance of these observations. In future publications it will be shown that there exists a remarkable uniformity in the sterols isolated from various species of other sponge genera. The corresponding lack of uniformity among the species of *Haliclona* suggests that many species have been grouped within this genus which show only superficial relations to each other.

## EXPERIMENTAL

All melting points are corrected. All optical rotations were taken in a 1-dm. tube, the sample being dissolved in 3.06 cc. of chloroform.

*Preparation of the sterols.* The air-dried sponges were ground and then thoroughly extracted with acetone in a large Soxhlet apparatus. After evaporation of the acetone, the residue was dissolved in benzene, and the water removed by co-distillation. In all instances certain amounts of smeary, brown, water-soluble material, remained undissolved in the benzene. The benzene extract was then evaporated to dryness, and the residue dried to constant weight at 80°. This acetone-benzene-soluble fraction is referred to as fat in Table I. The data in the table are all based on weights of crude sponge material from which the non-spicular ash has been subtracted as described in a previous communication (7). The saponification of the fat, and the isolation of the sterol was carried out as previously described. The sterol content of an aliquot part of the non-saponifiable fraction was determined by precipitation with digitonin. The crude sterol was at once acetylated by refluxing with acetic anhydride.

*Haliclona variabilis.* The crude acetate, (1.92 g.) m.p. 141–142°, after one recrystallization each from ether and chloroform-methanol gave poriferasteryl acetate (590 mg.), m.p. 147–148°;$[\alpha]_D^{27}$ −52° (36.5 mg.; $\alpha$, −0.62°). Saponification yielded poriferasterol, m.p, 155.5–156°; $[\alpha]_D^{27}$ −50° (37.2 mg.; $\alpha$, −0.61°). When mixed with authentic material these products did not show a depression of melting point.

A sample of acetate (840 mg.), obtained from the mother liquors was dissolved in ether (8 cc.) and treated with a 5% solution of bromine in glacial acetic acid (17 cc.). There was obtained a high-melting bromide (760 mg)., difficultly soluble in ether, which, after two

recrystallizations from ethyl acetate, afforded poriferasteryl acetate tetrabromide, m.p. 190–192°.

*Anal.* Calc'd for $C_{31}H_{50}Br_4O_2$: Br, 41.3. Found: Br, 42.0.

Debromination of the tetrabromide with zinc in glacial acetic acid gave poriferasteryl acetate, m.p. 146–147°; $[\alpha]_D$ −51°. Concentration of the bromination mother liquor gave further quantities of tetrabromide.

*Haliclona permollis and H. coerulescens.* The acetates of the sterols obtained from either sponge melted at 133–135°; $[\alpha]_D$ −40° to −44°. A sample of each of the acetates (350 mg.) was dissolved in ether (3 cc.) and treated with a 5% solution of bromine in glacial acetic acid (6 cc.). Both samples gave a tetrabromide difficulty soluble in ether, which after recrystallization from ethyl acetate melted at 189–192°.

*Anal.* Calc'd for $C_{31}H_{50}Br_4O_2$: Br, 41.3. Found: Br, 41.6.

Debromination of the tetrabromide with zinc in acetic acid gave poriferasteryl acetate, m.p. 146–147°; $[\alpha]_D^{27}$ −51°. Concentration of the bromination mother liquors gave a bromine-free acetate, which, after several recrystallizations from ethanol, melted at 135°.

*Haliclona viridis.* Recrystallization of the crude acetate gave two fractions: I, (650 mg.), m.p. 125–130°, and II, (780 mg.), m.p. 112–116°. Bromination of I (480 mg.), in ether (2.5 cc.) with 5% bromine-acetic acid solution (7.5 cc.) gave 465 mg. of precipitated bromides. Trituration of the bromide with ether gave an insoluble fraction (40 mg.) which after several recrystallizations from ether, by means of continuous extraction from a thimble, melted at 200–205°.

*Anal.* Calc'd for $C_{30}H_{48}Br_4O_2$: Br, 42.04. Found: Br, 41.86.

After several recrystallizations from ether-glacial acetic acid, the ether-soluble portion of the precipitated bromides afforded cholesteryl acetate dibromide, m.p. 115–116°.

*Anal.* Calc'd for $C_{29}H_{48}Br_2O_2$: Br, 27.16. Found: Br, 27.50.

Debromination with zinc in glacial acetic acid gave cholesteryl acetate, m.p. 114°; $[\alpha]_D^{27}$ −43°, and hydrolysis of the latter yielded cholesterol, m.p. 147°; $[\alpha]_D^{27}$ −38°. No depressions of melting point were observed when these derivatives were mixed with authentic material.

Fraction II (780 mg.) was brominated as before, and the precipitated bromide separated into an ether-soluble and an insoluble fraction. The former yielded cholesteryl acetate dibromide (360 mg.).

The mother liquors from the brominations of fractions I and II were debrominated with zinc. The acetate thus obtained was rebrominated, and the soluble fraction debrominated as before. The acetate thus obtained, after numerous recrystallizations from ethanol, melted at 137°; $[\alpha]_D^{27}$ −42°. It gave no depression of melting point when mixed with clionasteryl acetate.

*Haliclona rubens.* The unsaponifiable fraction of this sponge was not a wax-like solid like that of most other sponges, but an oil of a rather low viscosity. The presence of the oil and of an amorphous, white substance made difficult the usual extraction of the sterol with hot methanol. Many recrystallizations from methanol and several treatments with Norit were required before a colorless sterol was obtained.

The crude acetate (1.12 g.), m.p. 130–135°; $[\alpha]_D^{27}$ −43°, was brominated as before, and the precipitated fraction (485 mg.), separated on the basis of solubility in ether. After two crystallizations from ethyl acetate, the insoluble fraction (120 mg.) melted at 195–200°.

*Anal.* Calc'd for $C_{30}H_{4.}Br_4O_2$: Br, 42.0. Found: Br, 41.9.

The soluble fraction yielded cholesteryl acetate dibromide, m.p. 114–115°. The bromination mother liquors were debrominated with zinc, and the acetate thus obtained saponified. Bromination of the resulting sterol (320 mg.) in ether and glacial acetic acid gave cholesterol dibromide (90 mg.), m.p. 112–114°. Debromination of the soluble bromides, and acetylation of the resulting product gave an acetate, which after several recrystallizations from ethanol, melted at 135–136°; $[\alpha]_D^{27}$ −42°.

*Haliclona longleyi. Haliclonasteryl acetate.* The crude acetate melted at 139–140°. After

several recrystallizations from anhydrous ether in a Skau-tube, the melting point remained constant at 140–141°; $[\alpha]_D^{27}$ −46.5° (23.0 mg.; $\alpha$, −0.35°). The acetate, purified over the dibromide, melted at 140°; $[\alpha]_D^{27}$ −46° (43.9 mg.; $\alpha$, −0.66°).

*Anal.* Calc'd for $C_{30}H_{50}O_2$: C, 81.4; H, 11.4.

Found: C, 81.1; H, 11.3.

*Haliclonasteryl acetate dibromide.* A sample of the acetate (700 mg.) was dissolved in ether (7 cc.) and treated with a 2.5% solution of bromine in glacial acetic acid (20 cc.). After 24 hours there had formed 90 mg. of a precipitate of which 30 mg. was difficultly soluble in ether. After one recrystallization from ethyl acetate this tetrabromide melted at 187–190°.

*Anal.* Calc'd for $C_{30}H_{48}Br_4O_2$: Br, 42.0. $C_{31}H_{50}Br_4O_2$: Br, 41.3.

Found: Br, 42.2.

The ether-soluble fraction of the first precipitate was combined with the bromination mother liquor, and the mixture left standing in an open vessel at room temperature to permit gradual evaporation of the ether. After several days long, clear prisms began to separate. They were washed with glacial acetic acid and methanol, and dried *in vacuo;* (425 mg.), m.p. 125°. After recrystallization from ether-glacial acetic acid and ethyl acetate, the bromide melted at 125–126°. Fractions recovered from various mother liquors showed the same melting point. The peculiar behavior of the bromide upon recrystallization from ether-ethanol has been discussed in the introduction.

*Anal.* Calc'd for $C_{30}H_{50}Br_2O_2$: C, 59.80; H, 8.36; Br, 26.53.

$C_{31}H_{52}Br_2O_2$: C, 60.39; H, 8.50; Br, 25.92.

Found: C, 59.75; H, 8.43; Br, 26.52.

Debromination of various fractions of bromide and of the final bromination mother liquor gave haliclonasteryl acetate of m.p. 139–141°; $[\alpha]_D^{27}$ −46° to −47°.

*Haliclonasterol.* The sterol was obtained by saponification of the acetate. The crude product melted at 140–141°, and after two recrystallizations from ether, sharply at 140.5–141°; $[\alpha]_D^{27}$ −41.5° (40.6 mg.; $\alpha$, −0.55°). The sterol contained solvent of crystallization which could not be completely removed.

*Haliclonasteryl benzoate.* A sample of sterol (50 mg.) was dissolved in pyridine (1 cc.) and benzoyl chloride (0.2 cc.) was added to the solution. After 48 hours methanol was added to the solution until a copious precipitate had been obtained. This was washed thoroughly with methanol and recrystallized several times from acetone, m.p. 146.5°; $[\alpha]_D^{27}$ −14.7° (33.3 mg.; $\alpha$, −0.16°).

*Anal.* Calc'd for $C_{35}H_{52}O_2$: C, 83.3; H, 10.4.

Found: C, 83.1; H, 10.5.

*Haliclonasteryl m-dinitrobenzoate.* This derivative was prepared in a manner analogous to the one described above. The dinitrobenzoate was recrystallized from benzene-methanol and ethyl acetate, m.p. 209°.

*Anal.* Calc'd for $C_{35}H_{50}N_2O_6$: C, 70.67; H, 8.47.

$C_{36}H_{52}N_2O_6$: C, 71.02; H, 8.61.

Found: C, 70.7; H, 8.4.

*Haliclonastanyl acetate.* Haliclonasteryl acetate was hydrogenated in ethyl acetate at room temperature and atmospheric pressure with a platinum black catalyst (Willstätter). The hydrogenated material gave a negative Liebermann-Burchard reaction. It was recrystallized several times from ether in a Skau-tube, m.p. 136–136.5°; $[\alpha]_D^{27}$ +10.6° (23.0 mg.; $\alpha$, +0.08°).

*Anal.* Calc'd for $C_{30}H_{52}O_2$: C, 81.02; H, 11.79.

Found: C, 81.06; H, 12.31.

*Haliclonastanol.* It was obtained by saponification of the acetate. After recrystallization from chloroform-methanol and ether, the stanol was obtained in the form of small needles of m.p. 137–137.5°; $[\alpha]_D^{27}$ +18.2 (30.6 mg.; $\alpha$, +0.18°). The stanol contains solvent of crystallization which could not be removed completely.

*Haliclonastanyl m-dinitrobenzoate.* This derivative was prepared as described above. After several recrystallizations from benzene-ethanol and ethyl acetate it melted at 217.5–218°.

*Anal.* Calc'd for $C_{35}H_{52}N_2O_6$; C, 70.44; H, 8.78.
$C_{36}H_{54}N_2O_6$; C, 70.78; H, 8.91.
Found: C, 70.37; H, 8.85.

*Haliclonastanyl benzoate.* This derivative was prepared in the usual manner. It shows a peculiar behavior upon recrystallization from chloroform-methanol. There is first formed a light flocculent material which gradually changes into small needles, which upon heating turn turbid at 130° and clear at 133°. The same phenomenon is observed when the benzoate is recrystallized from ether. After several such crystallizations, the benzoate melted to a clear liquid at 133.5°, the first turbidity appearing at 131°.

## SUMMARY

1. The sterols of six species of sponges of the genus *Haliclona* have been isolated and investigated.

2. Poriferasterol has been shown to be the principal sterol of *Haliclona variabil s.*

3. The sterols from *H. permollis* and *H. coerulescens* have been shown to be mixtures containing poriferasterol and probably clionasterol.

4. The sterols from *H. viridis* and *H. rubens* have been shown to be mixtures containing more than 50% cholesterol.

5. *Haliclona longleyi* has been shown to contain a new sterol in a remarkably high degree of purity. The name of haliclonasterol has been proposed for this compound which is mono-unsaturated and of the probable formula $C_{28}H_{48}O$. A number of derivatives of haliclonasterol have been described.

6. The possible significance of these observations has been discussed.

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