afforded (N-4-antipyrinyl-N-methyl)aminoacetonitrile (VI) and 4-methylaminoantipyrine (I).

The condensation of 4-methylaminoantipyrine (I) with paraformaldehyde and potassium cyanide on being warmed in glacial acetic acid also furnished (VI).

The respective action of an excess of paraformaldehyde on (I) and (III) in dil. hydrochloric acid gave (II). However, in conc. hydrochloric acid, the respective action of an excess of paraformaldehyde on (I), (II), (III), and (VII) afforded (IV).

(Received October 11, 1957)

UDC 547.92:582.273

Kyosuke Tsuda, Saburo Akagi, and Yukichi Kishida: Steroid Studies. VIII. Cholesterol in some Red Algae.

(*Institute of Applied Microbiology,* University of Tokyo, and Takamine Research Laboratory,** Sankyo Co., Ltd.*)

We reported[1] before on the sterols isolated from some brown seaweed (Phaeophyceae) and determined the structure of the new sterol, sargasterol,[2] isolated from *Sargassum ringgoldianum* HARVEY. This paper describes the isolation of a cholesterol from some red seaweed (Rhodophyceae).

Dried powder of alga, which was carefully selected to avoid contamination of any traces of animal sources, was extracted several times with boiling benzene and the dark brown oil obtained was saponified with 4% methanolic alkali. Subsequent extraction with benzene gave an unsaponifiable substance. On standing its methanolic solution over night, a crude sterol was obtained. Yield from each process on several algae is summarized in Table I.

TABBLE I.

Algae	Japanese name	Crude oil (%)	Nonsapon. subst. (%)	Crude sterol (%)
Rhodoglossum pulcherum (KÜTZING) SETCHELL ET GARDNER	Akabaginnanso	1.1	12~13	55.0
Gelidium subcostatum OKAM.	Hirakusa	0.48	19.3	33.8
Gelidium amansii LAM.	Makusa	0.14	35.5	13.6
Pterocladia tenuis OKAM.	Obakusa	0.26	38.4	14.0
Gelidium japonicum OKAM.	Onikusa	0.26	38.4	12.0
Acanthopeltis japonica OKAM.	Tori-no-ashi	1.50	22.9	41.7

Several recrystallizations of a crude sterol of the *Rhodoglossum* from methanol gave a sample of m.p. 142~145°, which precipitated with digitonin and which was positive to Liebermann-Burchard's color test. Purification of the sterol (m.p. 142~145°) twice through its dibromoacetate, which was precipitable from solution in dry ether and glacial acetic acid, afforded a pure sample of m.p. 147~148°; $[\alpha]_D$ −40.0. Perbenzoic acid titration, bromination, and catalytic hydrogenation of its steryl acetate indicated that the sterol possesses just one double bond. Physical constants of the many derivatives of the sterol are summarized in Table II.

* Yayoi-cho, Bunkyo-ku Tokyo (津田恭介).
** Nishi-shinagawa, Shinagawa-ku, Tokyo (赤木三郎, 岸田有吉).

1) K. Tsuda, R. Hayatsu, Y. Kishida, S. Akagi: J. Am. Chem, Soc. (in press).
2) K. Tsuda, S. Akagi, Y. Kishida, R. Hayatsu: This Bulletin, **5**, 85(1957).

Table II.

Derivative	m.p.	$[\alpha]_D^{25}$	Light absorption
Sterol	147~148°	−40.0	
Dibromide	112~114	−44.3	
Acetate	114~115.5	−44.0	
Dibromoacetate	113~114	−47.8	ν_{max} 1236 and 1748 cm^{-1}
Benzoate	144~145.5	−14.0	
3-Stenone	84~86	+88	λ_{max}^{EtOH} 241 mμ (ε 17,800)
Stanol	142~143	+23.5	
Stanyl acetate	108~109	—	

All these compounds were identified with the corresponding derivatives prepared from an authentic cholesterol by mixed melting point and infrared spectra. Furthermore, the result of X-ray diffraction analysis of the algal stenone was the same in all respects with that of authentic cholestenone within an error of 1% including experimental errors. We are, therefore, convinced that the sterol is cholesterol. The analytical data of X-ray diffraction are summarized in Table III.

Table III.

Cholestenone (diameter of sample=0.55 mm.)				Algal stenone (diameter of sample=0.52 mm.)			
No. of lines	Calibrated θ	d	Intensity	No. of lines	Calibrated θ	d	Intensity
1	6.68	9.84	s	1	6.64	9.90	s
2	8.03	8.20	m	2	7.99	8.24	m
3	8.83	7.46	m	3	8.79	7.49	m
4	11.48	5.75	s	4	11.44	5.77	s
5	12.23	5.40	vw	5	12.14	5.44	vw
6	13.28	4.98	vs	6	13.29	4.98	vs
7	14.88	4.46	w	7	14.84	4.47	w
8	16.83	3.95	m	8	16.89	3.94	m
9′	17.93	3.72	vw	9′	17.74	3.76	vw
9	18.65	3.58	w	9	18.44	3.62	w
10	19.28	3.47	m	10	19.29	3.47	m
11	21.70	3.10	w	11	21.79	3.08	w

s, strong; m, medium; w, weak; vs, very strong; vw, very weak.

In addition, we also isolated cholesterol as its dibromoacetate from the easily-soluble fractions of the sterols obtained from all the red algae listed in Table I. Besides cholesterol, from all the algae except *Gelidium subcostatum*, we isolated unidentified sterols, one of which resembled chalinasterol,[3,4] whose appearance was reported before by Matsumoto, *et al.* from *Gelidium amansii* and *Pterocladia tenuis*. The identity was made by the characteristic infrared patterns[5] of three prominent bands between 800 and 900 cm^{-1} and of the band at 970 cm^{-1}.

The alga of *Acanthopeltis japonica* was a little contaminated with some sponges which could not be completely eliminated. Nevertheless, the isolation of chalinasterol and cholesterol might be due to the inherent algal herb from the overwhelming proportion of it to the amount of sponges.

Isolation of cholesterol from several red algae might be of some interest to biogenetical study of sterol because there has been no report on its isolation from the vegetable kingdom, although some derivatives of cholesterol, zymosterol ($\Delta^{8,24}$-chole-

3) W. Bergmann, H. P. Schedl, E. M. Low : J. Org. Chem., **10**, 587(1945).
4) S. Ito, K. Nagashima, T. Matsumoto : Nippon Kagaku Zasshi, **77**, 1119(1956).
5) H. J. Cahnmann; J. Org. Chem., **21**, 1412(1956).

stadiene)[6] and 22-α-hydroxycholesterol[7] were reported respectively from yeast and *Narthecium ossifragum* HUDS.

We wish to thank Dr. Y. Yamada, Dr. T. Uchida, Dr. Y. Nakamura, and Dr. M. Yamada of Hokkaido University for their kind advice which helped us to collect and identify the algae. We are also indebted to Dr. S. Sudo of Tokai Fishery Research Institute and the members of Ito Fishery Research Section in Shizuoka Prefecture for their kind cooperation. We thank the members at Gokyu Research Laboratory of the University of Tokyo for X-ray diffraction analysis.

Experimental[8]

General Procedure for Extraction of Sterol—Dry finely powdered alga was extracted three times with boiling benzene with stirring. Crude dark brown oil remained after the removal of the solvent, 100 parts each of the oil (wt) and MeOH (vol.), 20 parts of benzene (vol.) were mixed with 30 parts of 40% NaOH, and the mixture was refluxed in a large flask for 1~3 hrs. The cold reaction mixture was diluted with hydr. MeOH to 400 parts (vol.) of MeOH and to 60% (vol.) of MeOH concentration. Then the diluted soap solution was extracted with 1,000 parts of benzene (vol.) and successively 5 times with 400 parts of the same solvent. The combined solution was washed twice with 50% MeOH, and the solvent was removed. The residual dark brown unsaponifiable substance was boiled with an equivalent volume of MeOH and left to stand overnight in refrigerator to give yellow colored crude sterol.

Purification of the Sterol from *Rhodoglossum pulcherum*—The crude sterol was recrystallized three times from MeOH to give a sample of m.p. 142~145°, which was converted to its acetate, m.p. 112~117° (needles from 95% EtOH). To a solution of 11 g. of the acetate in 110 cc. of dry ether, 4.1 g. of bromine dissolved in 138 cc. of glacial AcOH was added. After a few hrs., white crystalline bromide precipitated. The bromide dissolved in EtOH was refluxed with 15 g. of NaI for 1 hr. After cool, the reaction mixture was poured into cold solution of $NaHSO_3$ and the precipitated white solid was washed with water. This was recrystallized three times from 99% EtOH to give white needles of m.p. 113.5~114.5. The sample was repurified by the same method and 5 g. of the purest acetate of m.p. 114~115.5°; $[\alpha]_D$ −44.0, was obtained. *Anal.* Calcd. for $C_{29}H_{48}O_2Br_2$ (dibromoacetate): C, 59.20; H, 8.24; Br, 27.15. Found: C, 59.35; H, 8.24; Br, 27.24. ν_{max} 1236 and 1740 cm^{-1} (in CS_2). By adding water to the mother liquor of the first bromination mixture, 1.5 g. of the white solid of m.p. 111~114° (dibromosterylacetate) was recovered. Sterol, benzoate, steryl dibromide, dibromosterylacetate (see Table II) were prepared from the acetate (m.p. 114~115.5°).

Stenone from the Sterol of *Rhodoglossum pulcherum*—A solution of 2.65 g. of the sterol (m.p. 147~148°; $[\alpha]_D$ −40.0) in 30 cc. of cyclohexanone and 90 cc. of toluene was refluxed until 30 cc. of toluene was azeotropically removed. 4 g. of aluminum isopropoxide dissolved in 24 cc. of toluene was added dropwise for 30 mins., and the reaction mixture was further refluxed for 30 mins. After cool, 30 cc. of saturated aqueous solution of Rochelle salt was added and the steam was introduced to remove the solvent. The residue which was extracted four times with ether, after removal of the solvent, afforded a pale yellow solid of m.p. 70~74° (1.9 g.). It was purified by chromatography on alumina and subsequent recrystallization from MeOH, m.p. 85~86° (1.6 g.). This sample was used for X-ray diffraction analysis. *Anal.* Calcd. for $C_{27}H_{44}O$: C, 84.31; H, 11.53. Found: C, 83.89; H, 11.58.

Hydrogenation of the Sterylacetate from *Rhodoglossum pulcherum*—The algal sterylacetate (0.96201 g.) was reduced with Adams' catalyst in EtOH and glacial AcOH (1 : 2, 30 cc.) and 52.8 cc. of hydrogen was absorbed. The stanylacetate of m.p. 105~107° resulted from the reaction mixture was recrystallized. m.p. 108~109°. The stanol melted at 142~143° when it was recrystallized several times and then dried at 25° *in vacuo* for 24 hrs.

Perbenzoic Acid Titration of the Sterylacetate from *Rhodoglossum pulcherum*—To $CHCl_3$ solution of 33.29 mg. of the algal sterylacetate, 4 cc. of the $CHCl_3$ solution of perbenzoic acid was added. $CHCl_3$ was added to it so that the whole volume became 25 cc., while the blank test was set up. After being kept for 21 hrs. at 23°, they were titrated with 0.1*N* $Na_2S_2O_4$ solution (f=1.1298) in the usual way. F=1.017. Another test with the sample of 221.60 mg. showed F=1.051.

All derivatives listed above were identified by analyses and with corresponding derivatives prepared from authentic cholesterol by mixed m.p. and infrared spectra.

Gelidium subcostatum—The crude sterol (15 g.) was divided into two fractions: 2 g. of (A), m.p.

6) I. Smedley-MacLean: Biochem. J., **22**, 22(1928); L. F. Fieser, M. Fieser: "Natural Products related to Phenanthrene," Reinhold Publishing Corp., N. Y., 239(1949).
7) A Stabursvik: Acta Chem. Scand., **7**, 1220(1953).
8) All melting points are uncorrected and optical rotations were mesured in $CHCl_3$ (c=1.0~2.3) at 25°.

108~125°, and 10 g. of (B), m.p. 130~137°, by fractional crystallization from a mixture of 95% EtOH and MeOH (1 : 1). After repeated chromatographic separation, the acetate of (A) afforded two substances, m.p. 100~104° and m.p. 60°, which were not identified but seemed to be paraffin. Chromatography on alumina of the fraction (B) afforded an alcohol, m.p. 138~141° (acetate, m.p. 115~116°). The acetate was thoroughly purified through its dibromoacetate as described above. The pure sample was identified with cholesterol on its dibromoacetate (m.p. 113~114°) by mixed m.p. (112.5~114°) and by infrared spectra. *Anal.* Calcd. for $C_{29}H_{48}O_2Br_2$: C, 59.20; H, 8.24; Br, 27.15. Found : C, 59.49; H, 8.32; Br, 27.17.

Gelidium amansii—The crude sterylacetate was fractionated into (A) and (B) by chromatography and repeated crystallization. (A) alcohol, m.p. 131~134°; acetate, m.p. 118~122°; benzoate, m.p. 143~144°. By infrared analysis, this sample seemed to be a mixture of chalinasterol and cholesterol. (B) alcohol, m.p. 130~135°; acetate, m.p. 110~116°. This was not identified. (A) and (B) were combined (total 310 mg.) and converted to bromoacetate, which melted at 115~120°(decomp.). The sample of m.p. 115~120° was recrystallized from hot benzene and MeOH to give dibromoacetate of m.p. 113~114°, which was identified with dibromocholesterylacetate by mixed m.p. From the mother liquor of bromination mixture, two alcohols of m.p. 115~123° and 110~124° were obtained but not identified.

Pterocladia tenuis—Fractional crystallization and alumina chromatography of the crude sterylacetate afforded two fractions of m.p. 110~117°(A) [alcohol, m.p. 134~136°; $[\alpha]_D$ −41.3; benzoate, m.p. 143~146°(clear at 158°)] and m.p. 124~127°(B) [alcohol, m.p. 134~136°; $[\alpha]_D$ −29.7; benzoate, m.p. 142~144°(clear at 150°)]. Dibromoacetate (m.p. 112~114°) of (A), which was prepared as above and recrystallized from benzene and MeOH, was identified with dibromocholesterylacetate by mixed m.p. (B) seemed to be a mixture of chalinasterol and cholesterol from its infrared analysis.

Gelidium japonicum—The crude sterols (3 g.) were divided into two parts; the less and easily soluble fractions of 1.3 g. of (A) and 1.3 g. of (B) as immediately above. (A) acetate, m.p. 124~127°; alcohol, m.p. 132~134°; benzoate, m.p. 142~144°. (B) acetate, m.p. 110~112°, alcohol, m.p. 140~141°; benzoate, m.p. 146°. Infrared spectrum of this fraction (B) was very similar to that of cholesterol. The acetate was converted to its dibromide of m.p. 112~114° by the method described above and was identified with dibromocholesterylacetate by mixed m.p.

Acanthopeltis japonica—(i) Repeated crystallization of 2 g. of the crude sterol finally afforded an alcohol of m.p. 134~135° (benzoate, m.p. 141~144°). The infrared pattern of the alcohol was very similar to that of chalinasterol.

(ii) The crude sterylacetate (52 g.) was decolorized through a column of alumina and fractionated into the less (A) and easily soluble (B) fractions. (A) m.p. 124~129°, 13.5 g. (B) m.p. 95~115°, 9.8 g. The fraction (A) was divided into three portions (A-1, A-2, A-3) by using bromination and usual fractional crystallization. A-1, 0.6 g. : alcohol, m.p. 140~144°, $[\alpha]_D$−48.5; acetate, m.p. 142°, $[\alpha]_D$ −51.1. The infrared analysis of this sample showed a *trans* double bond in the side-chain and Δ^5-double bond. A-2, 5 g. : alcohol, m.p. 138°; acetate, m.p. 126~128°; benzoate, m.p. 142~144°. This seemed to be a mixture of chalinasterol and cholesterol by the infrared spectra. A-3, 1.5 g. : alcohol, m.p. 138°; $[\alpha]_D$ −48.0; acetate, m.p. 128°; benzoate, m.p. 140~141°. This sample seemed to be chalinasterol by infrared analysis. The fraction (B) was fractionated into two portions: B-1 and B-2. B-1, 1.4 g. : alcohol, m.p. 143~146°, $[\alpha]_D$ −40.5; acetate, m.p. 112~113°, $[\alpha]_D$ −43.0; benzoate, m.p. 145~146°(clear at 178°), $[\alpha]_D$ −12.8. This sample was identified with cholesterol on its dibromoacetate by mixed m.p. and infrared spectra. B-2, 1.5 g. : alcohol, m.p. 142~144°, $[\alpha]_D$ −44.0; acetate, m.p. 130~132°, $[\alpha]_D$ −48.0; benzoate, m.p. 146~147°. By its physical constants and infrared spectra this sample was identified as chalinasterol.

Summary

The sterol isolated from *Rhodoglossum pulcherum* was identified as cholesterol by mixed melting point of various derivatives with the corresponding derivatives prepared from authentic specimen of cholesterol. Furthermore, the identity was confirmed by X-ray diffraction analyses of the algal stenone and cholestenone.

The authors also confirmed the existence of cholesterol in several red seaweed listed in Table I. Besides cholesterol, the authors isolated another sterol, chalinasterol from some algae. The discovery of cholesterol might be of some interest to biogenetic study of sterol, because cholesterol has been considered to exist only in animal sources.

(Received October 28, 1957)