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PHYCOCHEMICAL EXAMINATION OF *HYPNEA VALENTIAE* (GIGARTINALES, RHODOPHYTA)

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Abstract

Investigation of the methanolic extract of *Hypnea valentiae* (Turner) Montagne, collected from Karachi, Pakistan, afforded seven sterols, a fatty acid derivative and 12 fatty acids. Study of the sterol fraction through ^{1}H and ^{13}C NMR and mass spectrometry exhibited the occurrence of 22-dehydrocholesterol, desmosterol, 24-methylene cholesterol, 24-methyl chlolesterol, *Nor* 31-cycloartanol and cycloartanol as well as a fatty acid derivative, 7-hydroxy tetradec-4-enoic acid. In the lipid fraction 9 saturated fatty acid methyl esters *viz*., myristate, pentadecylate, palmitate, margarate, stearate, nonadecylate, arachidate, behenate and pentacosanoate and 3 unsaturated fatty acid methyl esters *viz*., tetradecatrienoate, oleate and hexacosenoate were determined through GC-MS.

Introduction

Phycochemistry is the study of the natural products and the chemical constituents occurring in algae from a biological point of view (Shameel, 1990 a). Various species of *Hypnea* have often been subjected to a broad phycochemical investigation, dealing with sterols (Tsuda *et al*., 1959; Fattorusso *et al*., 1979; Combaut *et al*., 1984), fatty acids (Kato & Ariga, 1982), carrageenans (Davanzo *et al*., 1970; Combaut *et al*., 1981; Furneaux & Miller, 1986), proteins (Bruni & Stancher, 1974) and carbohydrates (Shimizu, 1976; Laserna *et al*., 1981; Mahran *et al*., 1985). Apart from studies on phycocolloids (Rao & Krishnamurthy, 1978) and nucleosides (Kazlauskas, 1983) no other work has been carried out on *H. valentiae*. The present investigation reports a phycochemical study of the sterols and fatty acids in *Hypnea valentiae* (Turner) Montagne, a red alga commonly growing as epilithon on lower to sub-littoral rocks in Manora to Sonmiani seashore waters of Pakistan (Shameel, 1990 b).

Materials and Methods

Fresh thalli of *Hypnea valentiae* (1.5 kg) were collected from lower littoral rocks as well as drift material from the rocky ledges at Buleji, near Karachi during December 1988. Healthy specimens, free from epiphytes and animal castings were selected, thoroughly washed and dried in the shade.

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***Isolation of sterols*:** Dried thalli of *H. valentiae* were extracted with MeOH (1 L) under reflux for 8 h and the process was repeated 4 times. Combined methanolic extract was concentrated under reduced pressure to give the reddish residue weighing 3.5 g. The residue was mixed with water and repeatedly extracted with EtOAc to yield 2.0 g of the material after evaporation. The EtOAc extract was chromatographed on silica gel column, eluted with hexane-ether of increasing polarity to afford 256 mg of sterols containing fraction. Purification of the sterol fraction was brought about with preparative thin layer chromatography and the sterols were developed in hexane-ether. The sterols so obtained were scanned through ^{1}H and ^{13}C NMR and mass-spectrometry. The details of these techniques were the same as described previously (Hayee-Memon *et al.*, 1991).

***Isolation of a fatty acid derivative*:** The fatty acid derivative was obtained through column chromatography by eluting it with chloroform-methanol (1:1). Its purification was brought about through repeated crystallization in methanol which gave a positive test for fatty acid with iodine spray. The pure compound was identified by means of ^{1}H, ^{13}C NMR spectrometry and mass spectrometry.

***Isolation of fatty acids*:** Dried *H. valentiae* was extracted with hexane: chloroform (1:1) to give a total soluble extract, which on evaporation under reduced pressure afforded a reddish residue weighting 1.0 g. An aliquot of the extract (0.5 g) was saponified with KOH in ethanol and refluxed at boiling temperature for 4 hours. This aqueous mixture was acidified with 1N HCl (pH 4-5) and extracted with ethyl acetate. The EtOAc layer was then treated with diazomethane. The methylated fatty acid mixture was analysed by GC-mass spectrometry.

Results

The hexane: ether (4:6) yielded 22-dehydrocholesterol and desmosterol, hexane : ether (3:7) afforded 24-methylene cholesterol and 24-methyl cholesterol and hexane: ether (2:8) yielded fucosterol, *Nor* 31-cycloartanol and cycloartanol (Table 1). Mass spectral data, the fragmentation pattern and NMR data of these sterols are as follows:

22-Dehydrocholesterol [1]: Mass (EI) m/z 384 (M^+, $C_{27}H_{44}O$), 313 (H^+-C_5H_{11}), 271 (M^+-C_8H_{15}-2H), 255 (M^+-C_8H_{15}-H_2O), 217, 185, 157, 127 98, 69. ^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.67 (3H, s, 18-Me), 0.83 (3H, d, J = 6.0 Hz, 26-Me), 1.22 (3H, d, J = 7.0 Hz, 21-Me), 3.51 (3 a-H), 4.22 (1H, t, J = 6.0 Hz, 6-H).

Desmosterol [2]: Mass (EI) m/z 384 (M^+, $C_{27}H_{44}O$), 369 (M^+-CH_3), 366 (M^+-HOH), 271 (M^+-C_8H_{15}, side chain-2H), 255 (M^+-C_8H_{15}-H_2O), 253 (M^+-side chain-2H-$2H_2O$), 213, 199, 145, 111, 69. ^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.55 (3H, s, 18-Me), 1.00 (3H, s, 19,-Me), 1.24 (3H, d, J = 7.0 Hz, 21-Me), 1.57 (6H, s, 26, 27-Me), 3.56 (3 a-H), 4.23 (2H, m, 6, 24-H).

24-Methylene cholesterol [3]: Mass (EI) m/z 398 (M^+, $C_{29}H_{46}O$), 351, 314 (M^+-C_6H_{12}), 271 (M^+-C_9H_{17}-2H), 237, 215, 171, 135, 103, 69. ^{1}H-NMR (400 MH_z, $CDCl_3$), ppm: 0.65 (3H, s, 18-Me), 0.87 (3H, s, 19-Me), 1.24 (3H, d, J = 7.0 Hz, 21-Me), 1.57 (6H, m, 26, 27-Me), 3.52 (3β-H), 5.72 (1H, m, 5-H), 3.34 (2H, s, 28-H).

24-Methyl cholesterol [4]: Mass (EI) m/z 400 (M^+, $C_{28}H_{48}O$), 385 (M^+-CH_3), 382 (M^+-H_2O), 367 (M^+-CH_3-H_2O), 314, 297, 269, 227, 139, 69. ^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.66 (3H, s, 18-Me), 0.89 (3H, s, 19-Me), 1.24 (1H, d, J = 7.0 Hz, 21-Me), 1.60 (6H, m, 26, 27-Me), 3.48 (1H, m, 3β-H), 5.33 (1H, m, 5-H). ^{13}C-NMR 34.29 (C-1), 29.76 (C-2), 73.36 (C-3), 38.59 (C-4), 146.96 (C-5), 126.42 (C-6), 2.17 (C-7), 35.74 (C-8), 53.69 (C-9), 35.77 (C-10), 20.99 (C-11), 28.59 (C-12), 41.98 (C-13), 55.92 (C-14), 24.17 (C-15), 40.11 (C-16), 56.03 (C-17), 12.03 (C-18), 12.21 (C-19), 34.29 (C-20), 19.53 (C-21), 37.15 (C-22) 22.33 (C-23), 39.54 (C-24), 29.76 (C-25), 20.99 (C-26), 22.27 (C-27), 20.84 (C-28).

Fucosterol [5]: Mass (EI) m/z 412 (M^+, $C_{29}H_{48}O$), 397, (M^+-CH_3), 314 (M^+-C_7H_{14}), 299 (M^+-C_7H_{14}-CH_3), 271 (M^+-side chain-ring C + D cleavage), 255 (M^+-side chain-OH), 229 (M^+-side chain-ring D cleavage), 69, 55. ^{1}H-NMR (400 MH_z, $CDCl_3$), ppm: 0.67 (3H, s, 18-Me), 0.84 (3H, s, 19-Me), 0.91 (3H, d, J = 7.0 Hz, 21-Me), 1.47 (3H, J = 12.8 Hz, 29-Me), 3.51 (3β-H), 5.31 (2H, m, 5, 28-H).

***Nor* 31-cycloartanol [6]:** Mass (EI) m/z 414 (M^+, $C_{29}H_{50}O$), 399 (M^+-CH_3), 396 (M^+-H_2O), 381 (M^+-CH_3-H_2O), 341 (M^+-C_4H_7-H_2O), 301 (M^+-side chain) 283 (M^+-side chain-H_2O), 259, 245, 231, 175, 137, 109, 93, 81, 67. ^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.54 (3H, s, 18-Me), 0.83-0.91 (15H, m, Me, 18, 26, 27, 28, 29), 1.20-1.37 (24H, m, H-1, 2, 6, 7, 11, 12, 15, 16, 19, 22, 23, 24), 3.65 (1H, m, H-5), 4.19 (1H, dd, J = 6 Hz, H-3).

Cycloartanol [7]: Mass (EI) m/z 428 (M^+, $C_{30}H_{52}O$), 413 (M^+-CH_3), 410 (M^+-H_2O), 395 (M^+-CH_3-H_2O), 288 (M^+-$C_8H_{15}O$-H), 346, 312, 213, 185, 111, 69. ^{1}H-NMR (300 MHz, $CDCl_3$), ppm: 0.75 (3H, s, 21-Me), 0.82-0.86 (18H, s, 18, 26, 27, 28, 29, 30-Me), 1.21-1.31 (24H, m, H-1, 2, 6, 7, 11, 12, 15, 16, 19, 22, 23, 24), 4.13 (1H, dd, J = 9 Hz, H-3), 3.62 (1H, t, J = 8.8 Hz, H-5).

The fatty acid derivative was identified as 7-hydroxy-tetradec-4-enoic acid [8], with the following spectral data:

7-Hydroxy-tetradec-4-enoic acid [8]: $C_{14}H_{20}O_3$, mol. wt. 242, ^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.84 (3H, t, J = 6.5 Hz, H-14), 1.15-1.23 (16H, m, H-3, 6, 8, 9, 10, 11, 12, 13), 3.64 (1H, m, H-7), 5.84 (1H, dd, J = 16 Hz, H-5), 5.16 (1H, dd, J = 16 Hz, H-4), 4.05 (2H, br.d, H-2). ^{13}C-NMR (75 MHz, $CDCl_3$), ppm: 176.60 (C-1), 34.13 (C-2), 24.91 (C-3), 129.15 (C-4), 135.30 (C-5), 39.60 (C-6), 71.07 (C-7), 41.50 (C-8), 22.69-31.94 (C-9, 10, 11, 12, 13), 14.10 (C-14).

The saponified fatty acid fraction yielded 9 saturated and 3 unsaturated fatty acids (Table 2). The mass spectral data and fragmentation pattern of their methyl esters are as given below:

Methyl myristate: GC-MS m/z 242 (M^+, $C_{15}H_{30}O_2$, 80%), 211 (M^+-31, 30%), 199 (M^+-43, 28%), 185 (5%), 171 (8%), 157 (4%), 143 (13%), 129 (5%), 113 (8%), 99 (10%), 85 (39%), 71 (100%).

Methyl pentadecylate: GC-MS m/z 256 (M^+, $C_{16}H_{32}O_2$, 22%), 213 (M^+-43, 9%), 199 (17%), 185 (78%), 171 (25%), 157 (13%), 143 (33%), 129 (80%), 115 (41%), 101 (32%), 83 (83%), 73 (100%).

Methyl palmitate: GC-MS m/z 270 (M^+, $C_{17}H_{34}O_2$, 4%), 239 (M^+-31, 24%), 227 (M^+-43, 80%), 213 (70%), 199 (50%), 185 (60%), 171 (50%), 157 (70%), 143 (50%), 129 (60%), 115 (50%), 101 (60%), 87 (70%) (100%).

Methyl margarate: GC-MS m/z 284 (M^+, $C_{18}H_{30}O_2$, 10%), 227 (M^+-57, 19%), 213 (63%), 199 (16%), 185 (45%), 171 (46%), 157 (31%), 143 (17%), 129 (80%), 115 (42%), 101 (27%), 87 (44%), 73 (100%).

Methyl stearate: GC-MS m/z 298 (M^+, $C_{19}H_{38}O_2$, 80%), 267 (M^+-31, 23%), 255 (M^+-43, 38%), 241 (9%), 227 (15%), 213 (41%), 199 (30%), 185 (29%), 171 (25%), 157 (22%), 143 (71%), 129 (75%), 115 (30%), 101 (31%), 87 (80%), 73 (100%).

Methyl nonadecylate: GC-MS m/z 312 (M^+, $C_{20}H_{40}O_2$, 4%), 269 (M^+-43, 2%), 225 (1%), 241 (8%), 227 (80%), 213 (60%), 199 (70%), 185 (50%), 171 (60%), 157 (50%), 143 (50%), 129 (60%), 115 (50%), 101 (70%), 87 (80%), 71 (100%).

Methyl arachidate: GC-MS m/z 326 (M^+, $C_{21}H_{42}O_2$, 9%), 295 (M^+-31, 17%), 281 (31%), 267 (3%), 253 (2%), 239 (4%), 225 (5%), 211 (13%), 197 (5%), 183 (2%), 169 (10%), 155 (80%), 141 (8%), 127 (5%), 113 (54%), 99 (7%), 85 (32%), 71 (100%).

Methyl behenate: GC-MS m/z 354 (M^+, $C_{23}H_{46}O_2$, 27%), 323 (M^+-31, 1%), 309 (1%), 295 (11%), 281 (22%), 267 (5%), 241 (16%), 227 (28%), 213 (27%), 199 (18%), 185 (30%), 171 (25%), 157 (16%), 143 (80%), 129 (72%), 115 (45%), 101 (72%), 87 (82%), 73 (100%).

Methyl pentacosanoate: GC-MS m/z 396 (M^+, $C_{26}H_{52}O_2$, 20%), 363 (M^+-31, 2%), 350 (48%), 336 (1%), 322 (9%), 308 (2%), 294 (1%), 280 (4%), 266 (4%), 252 (7%), 238 (10%), 224 (7%), 210 (2%), 196 (7%), 182 (3%), 168 (5%), 154 (7%), 140 (6%), 126 (18%), 112 (30%), 98 (38%), 84 (27%), 70 (100%).

Methyl tetradecatrienoate: GC-MS m/z 236 (M^+, $C_{15}H_{24}O_2$, 12%), 204 (M^+-32, 16%), 162 (M^+-74, 15%), 148 (12%), 134 (7%), 120 (7%), 106 (5%), 92 (8%), 78 (7%), 64 (100%).

Methyl oleate: GC-MS m/z 296 (M^+, $C_{19}H_{36}O_2$, 12%), 264 (M^+-32, 3%), 222 (M^+-74, 38%), 208 (12%), 194 (7%), 180 (3%), 166 (4%), 152 (9%), 138 (23%), 124 (13%), 110 (25%), 96 (17%), 82 (33%), 68 (100%).

Methyl hexacosenoate: GC-MS m/z 408 (M^+, $C_{27}H_{52}O_2$, 10%), 351 (M^+-57, 5%), 337 (1%), 323 (3%), 309 (1%), 295 (11%), 281 (23%), 267 (4%), 241 (15%), 227 (28%), 213 (28%), 199 (18%) 185 (30%), 171 (25%), 157 (17%), 143 (80%), 129 (71%), 115 (36%), 101 (72%), 87 (100%).

Discussion

In *H. valentiae* altogether 7 sterols *viz.*, 22-dehydrocholesterol, desmosterol, 24-methylene cholesterol, 24-methyl cholesterol, fucosterol, *Nor* 31-cycloartanol and cycloartanol were found to be present (Table 1), of which 22-dehydrocholesterol was present in the largest quantity. Only C-27 sterols in substantial amount have been reported in red algae including *Hypnea* (Fattorusso *et al.*, 1975), whereas 22-dehydrocholesterol, 24-methylene cholesterol and fucosterols have been reported from various species of *Hypnea* (Tsuda *et al.*, 1959; Kato & Ariga, 1982). Although cholesterol is the most abundant sterol in *H. cervicornis* and *H. ceramioides* (Combaut *et al.*, 1984), it was not detected in *H. valentiae*. However, 22-Dehydrocholesterol and desmosterol appear to be of usual occurrence in Gigartinales as they have also been

Table.1. Sterol composition of *Hypnea valentiae*.

Systematic name	Common name	Molecular formula	Mol. wt.
22-dehydrocholest-5-en-3β-o1	22-dehydrocholesterol	$C_{27}H_{44}O$ [1]	384
Cholesta-5, 24-dien-3β-ol	Desmosterol	$C_{27}H_{44}O$ [2]	384
24-methylene-cholest-5-en-3β-o1	24-methylene cholesterol	$C_{28}H_{46}O$ [3]	398
24-methyl-cholest-5-en-3β-o1	24-methyl cholesterol	$C_{28}H_{48}O$ [4]	400
Stigmasta-5, 24 (28)-dien-3β-ol	Fucosterol	$C_{29}H_{48}O$ [5]	412
Nor 31, 9β, 19-cycloartanostan-3β-o1	*Nor* 31-cycloartanol	$C_{29}H_{50}O$ [6]	414
9β, 19-cycloartanostan-3β-o1	Cycloartanol	$C_{30}H_{52}O$ [7]	428

found in other seaweeds of this order like *Gracilaria foliifera* (Forssk.) BØrg. (Hayee-Memon *et al.*, 1991).

The occurrence of 7-hydroxy-tetradec-4-enoic acid in *H. valentiae* is not unusual. Both short and long chain hydrocarbons and their derivatives are produced as secondary metabolites in the members of Gigartinales. Certain polyunsaturated acids have been found in *Gracilaria verrucosa* (Huds.) Papenf. (Takagi *et al.*, 1985), *cis* and *trans*-phytol in *G. andersonii* (Grun.) Kylin (Sims & Pettus Jr., 1978) and an unsaturated fatty alcohol in *G. foliifera* (Hayee-Memon *et al.*, 1991).

Nine saturated and 3 unsaturated fatty acid methyl esters have been identified from lipid fraction of *H. valentiae* (Table 2). Both the quantity and variety of saturated fatty acids were appreciably greater than those of unsaturated fatty acids, similar observations have also been made in other red seaweeds of Karachi (Shameel, 1990 a). Kato & Ariga (1982) made an opposite observation in the red algae of Japan, which indicates geographical differences in the phycochemistry of seaweeds. Methyl palmitate was detected in small quantity, while in other red algae it is the major fatty acid (Shameel, 1990 a; Hayee-Memon *et al.*, 1991). Although various saturated and unsaturated fatty acids were found in almost equal amount, methyl hexacosenoate was in relatively largest amount. Methyl hexacosenoate and methyl pentacosanoate are long chain fatty acids, not previously known from other investigated seaweeds of Karachi (Qasim, 1986).

Table 2. Fatty acids of *Hypnea valentiae* analysed as methyl esters.

Systematic name	Common name	Molecular formula	Mol. wt.	Retention time (Min.)	Relative % age
Saturated fatty acid methyl esters:					
Methyl-*n*-tetradecanoate	Methyl myristate	$C_{15}H_{30}O_2$	242	15'43"	6.58
Methyl-*n*-pentadecanoate	Methyl pentadecylate	$C_{16}H_{32}O_2$	256	16'52"	6.88
Methyl-*n*-hexadecanoate	Methyl palmitate	$C_{17}H_{34}O_2$	270	19'33"	7.08
Methyl-*n*-heptadecanoate	Methyl margarate	$C_{18}H_{36}O_2$	284	20'19"	7.50
Methyl-*n*-octadecanoate	Methyl stearate	$C_{19}H_{38}O_2$	298	21'05"	8.25
Methyl-*n*-nonadecanoate	Methyl nonadecylate	$C_{20}H_{40}O_2$	312	19'57"	8.96
Methyl-*n*-eicosanoate	Methyl arachidate	$C_{21}H_{42}O_2$	326	26'04"	9.17
Methyl-*n*-docosanoate	Methyl behenate	$C_{23}H_{46}O_2$	354	18'47"	9.92
Methyl-*n*-pentacosanoate	Methyl pentacosanoate	$C_{26}H_{52}O_2$	396	20'19"	10.75
Unsaturated fatty acid methyl esters:					
Methyl-tetradecatrienoate	Methyl tetradecatrienoate	$C_{15}H_{24}O_2$	236	12'16"	5.21
Methyl-9,10-octadecenoate	Methyl oleate	$C_{19}H_{36}O_2$	282	13'20"	8.13
Methyl-17,18-hexacosenoate	Methyl hexacosenoate	$C_{27}H_{52}O_2$	408	19'08"	11.57

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