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Clionasterol, a Triterpenoid from the Kenyan Marine Green Macroalga *Halimeda macroloba*

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***Abstract*—**The triterpenoid clionasterol **(1)**, a 29 carbon structure compound was isolated from the less polar extract (20% EtOAc in hexanes) of the green alga *Halimeda macroloba* collected at Shimoni near Mombasa, Kenya. The structure and relative stereochemistry of this compound was elucidated by spectroscopic data, mainly NMR and mass spectrometry. This metabolite was inactive against DLD-1 cells on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. Further experiments on mosquito larvae and brine shrimp lethality confirmed this result.

INTRODUCTION

In our continued interest in prospecting for novel pharmaceuticals from Kenyan marine organisms and on the basis of potent antibacterial and antifungal activities of its 'spore-like' green epiphytes, *Halimeda macroloba*, was selected for investigation. In this study, it is established that the potent activity exhibited by the epiphytes is not derived from the host. The epiphytes had a 2.2 cm inhibition zone against the fungus *Aspergillus niger* and 1.9 cm against the gram-negative bacteria *Escherichia coli* on the 12.5 mm disc.

These antimicrobial activities of the epiphytes are in great contrast to the observations made on clionasterol (**1**), which did not exhibit any activity. Consequently, we report on the isolation and structure determination of clionasterol, a triterpenoid previously only isolated from the Indian marine red alga *Gracilaria edulis* (Das et al., 1992) and from an earlier synthesis via a Wittig reaction of its analogue extracted from the sponge *Verongia aerophoba* (De Luca et al., 1973).

MATERIALS AND METHODS

General experimental procedures

LRMS ESMS were obtained on a Finnigan Masslab Navigator Electrospray Mass Spectrometer. For details of NMR instruments and solvents, see Table 1. Silica gel $60F_{254}$ TLC sheets were purchased from Merck (Germany). Normal phase HPLC utilised a phenomenex sphereclone 5m column. The size exclusion sephadex LH-20 was from Amersham Pharmacia Biotech AB (Sweden).

Collection

Halimeda macroloba algal specimens were collected from the intertidal zone at Shimoni, 100 km south of Mombasa, Kenya in March 2000 and kept in cold conditions in methanol prior to extraction. A voucher specimen can be found at the University of Aberdeen where extraction and analysis took place.

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Extraction and isolation

A 200 g sample (80 g dry weight) was homogenised in MeOH. Sequential extraction with MeOH, CH_2Cl_2 and partitioning with 1:1 CH_2Cl_2/H_2O resulted into the aqueous and organic phases. The organic CH_2Cl_2 phase was then partitioned with hexane in 90% MeOH/H_2O. The hexane extract (98.5 mg) was applied to a sephadex LH-20 (Pharmacia) gel filtration column. Elution was with 1:1 CH_2Cl_2/MeOH. The normal (60:40 hexane/EtOAC) TLC chromatography of the Sephadex column collection afforded three sub-samples. The second sub-sample was subjected to normal phase HPLC (80:20 hexane/EtOAc) on a phenomenex sphereclone 5μ silica column resulting into 11 peaks. Clionasterol (**1**) was collected as a pale green liquid from the 9th peak at 14:09, 14:32 and 14:51 min on a Waters pump attached to a Perkin Elmer differential refractometer 40.

The hexane fraction was examined by ^{1}H NMR spectroscopy. This extract was fractionated on Sephadex LH-20. Final purification was achieved by normal-phase HPLC as outlined in the experimental section.

Brine shrimp toxicity assay

About 20 newly hatched brine shrimp (*Artemia salina*) in ca. 0.5 ml seawater were added to each well containing different concentrations of sample in 50 ml EtOH and 4.5 ml brine shrimp media (BSM). The experiment was run in triplicate. After 24 h at 25 °C, the brine shrimp were observed and counted under a dissecting light microscope. The percentage of live shrimp against total number of shrimp was used to determine LD_{50} values.

DLD-1 MTT assay

A solution of clionasterol was made in MeOH as it proved insoluble in DMSO. The cytotoxicity of clionasterol against DLD-1 cells was compared with that of a vehicle drug control and a control blank at concentrations ranging between 0 and 100 mM using a modification of the MTT-microtitre plate tetrazolium cytotoxicity assay as originally described by Mossman and cited elsewhere (Swaffar et al., 1994).

Mosquito larvae toxicity

A twelve-well system was used to determine the lethal dose of clionasterol against 5 mosquito larvae in ca. 0.5 ml saline media at different concentrations of the sample in MeOH. The LD_{50} was arrived at through a percentage of live against total larvae.

Antibacterial and antifungal activity

Disc diffusion assays used conventional methods (Chand et al., 1994). Briefly, a lawn of microorganisms was prepared by pipetting and spreading evenly overnight cultures of *E. coli* (IFO 3545) and *A. niger* [from the Hebrew University School of Pharmacy] (conc. 10^6–10^7 CFU/ml) onto agar and media in Petri dishes. The media for the *E. coli* was made up of neopeptone, agar, glucose and maltose whereas that for the fungi utilised sabaraud prepared in a similar fashion. A 12.5 mm sterile filter disc was used to which 50 ml of the test compound dissolved in solvent were added. The plates were inverted, stored in the refrigerator for 3 hours prior to overnight incubation at 35 °C after which the diameters of the zone of inhibition around the discs were measured. Control experiments were performed with equivalent volumes of solvents without the test compound.

RESULTS AND DISCUSSION

The molecular formula of clionasterol was deduced as $C_{29}H_{50}O$ from a combination of MS with ^{13}C NMR spectroscopy. Initially the compound was thought to be $C_{28}H_{49}OH$ but closer examination of the APT revealed an overlapped carbon on C4 at δ43.3. Negative electron impact mass spectrometry (EIMS) gave the $[M\text{-}H]^+$ at 413.3, $[M+ Na]^+$ at 437.4 and there was an evident $[M+ Na + MeOH]^+$ peak displayed at 469.5.

Clionasterol

Pale green liquid; $C_{29}H_{50}O$; EIMS m/z obs $[M - H]^+$ 413.3, $[M + Na]^+$ 437.4, $[M + Na + MeOH]^+$ 469.5 (calc'd for $C_{29}H_{50}O$, 414); elution time 14:09, 14:32, 14:51 min; for ^{1}H and ^{13}C NMR data see Table 1.

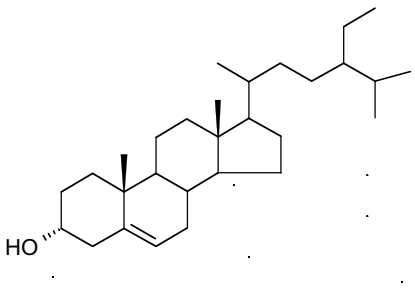

Fig. 1. Structure of clionasterol

This molecular composition required clionasterol to have 5 double bond equivalents with 1 olefinic bond at δ141.8 on the ^{13}C NMR spectra. The ^{1}H spectrum revealed the previously described olefinic bond at δ5.31 and a hydroxy moiety at δ3.47. Overall, APT and MF data describes the compound as being composed of 3 quarternary carbons, 9 methines, 11 methylenes and 6 methyl groups with a typical 4-ring steroid structure (Fig. 1). Interpretation of the ^{1}H and ^{13}C NMR (Table 1), ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY data along with HMBC information confirmed the substructures shown in Fig. 2.

The partial sterol parent substructure was determined effectively by HMBC couplings assigning two of the six methyl groups to C18 and C19 respectively. The two methyl carbons of these functionalities resonate at δ12.9 and 20.6. ^{1}H-^{1}H COSY correlated the olefinic hydrogen H6 at δ5.31 d with $H_2$7 at δ1.92 m and 1.46 m. It also correlated with $H_2$4/4' at δ2.24 m and 2.16 m.The correlation between $H_2$1 and H6, though evident,

Table 1. ^{1}H and ^{13}C NMR assignments for clionasterol (1)

C#	δC	DEPT (mult)	δH	COSY	HMBC
1	38.3	CH_2	1.80 m, 1.03 m		C3,C19
2	32.7	CH_2	1.78 m, 1.44 m		C3,C4/4'
3	72.8	CH	3.47 dddd; **3.52 m**		C1/1', C2/2', C4/4'
4/4'	43.3	CH_2 + C	2.24 m, 2.16 m		C4-C6,C2,C2'//C4'-C17,C12,C18
5	141.8	C	–		C1/1',C4/4',C7/7', C19
6	122.8	CH	5.31 d; **5.35 m**		C4/4',C1/C1',C7/7'
7	33.0	CH_2	1.92 m, 1.46 m		C6
8	33.0	CH	1.46 m		C7
9	51.2	CH	0.89 m		C1',C2',C7',C8,C11,C12,C19
10	37.5	C	–		C2,C4/4',C6
11	22.1	CH_2	1.46 m		C12
12	40.8	CH_2	1.96 m, 1.11 m		C17,C18
14	57.8	CH	0.96 m		C2,C7',C8,C11,C12,C17,C18
15	25.3	CH_2	1.55 m		C1,C2,C14,C16
16	29.3	CH_2	1.82 m, 1.22 m		C17,C20
17	57.1	CH	1.06 m		C16,C21
18	12.9	CH_3	0.64 s; **0.65 s**		C12
19	20.6	CH_3	0.97 s; **1.00 s**		C2
20	37.3	CH	1.32 m		C21
21	19.9	CH_3	0.88 d; **0.90 d**		
22	35.0	CH_2	1.30 m, 0.93 m		C21
23	27.4	CH_2	1.32 m		C28
24	47.1	CH	0.88 m		C28/28',C26,C27
25	30.0	CH	1.64 m		C26,C27
26	20.0	CH_3	0.77 d; **0.82 d**		
27	20.4	CH_3	0.79 d; **0.81 d**		
28	24.0	CH_2	1.28 m, 1.11 m		C29
29	13.4	CH_3	0.81 t; **0.85 t**		

All spectra in $CDCl_3$; ^{1}H at 400 MHz, ^{13}C at 100 MHz; assignments by ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMBC experiments. Acquired on a Varian-Unity INOVA spectrometer. Note: C4' is synonymous with C13 in the text. The assignments in bold refer to the original assignments of clionasterol (Das et al., 1992).

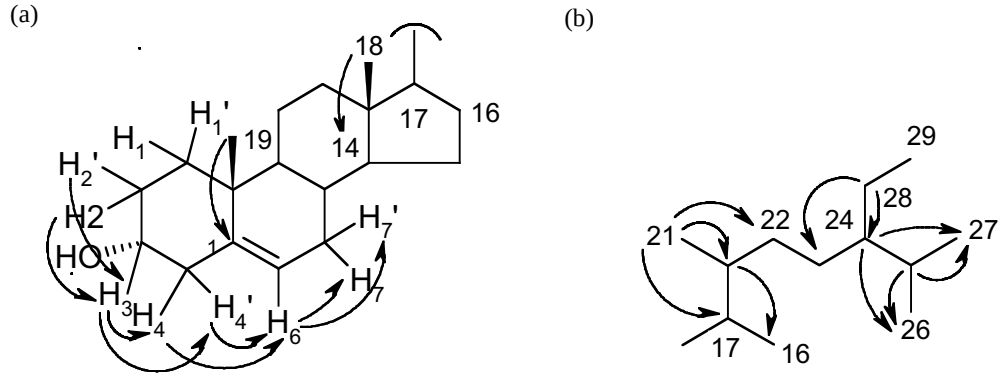

Fig. 2. Substructures for clionasterol showing the sterol moiety (a) and its side chain (b) with selected nOes

was weak at 4 bonds away. Other ^{1}H-^{1}H COSY correlations suggesting the parent sterol structure are those between $H_2$2/2' (δ1.78 m/1.44 m) and H3 at δ3.47 dddd and to $H_2$4/4' (δ2.24 m/ 2.16 m). The nOe between $H_2$2 and H3 and $H_2$4 require that the hydroxyl group on C3 should be down, suggesting that the protons at C2, C3, C4 and C7 are axial and oriented on the same side of the sterol ring structure.

The long range heteronuclear ^{1}H-^{13}C coupling (HMBC) between C5 at δ141.8 and the methyl hydrogens $H_3$19 at 0.97 s; that between C14 at δ57.8 and the $H_3$18 at δ0.64 and the 'solenoid'-like correlations between C3 at δ72.8 and $H_2$2 at δ1.78 m, 1.44 m; C5 at δ141.8 and $H_3$19 at 0.97 s; C14 at δ57.8 and $H_3$18 at δ0.64 confirms the parent sterol substructure (Fig. **2a**). Other correlations concluding substructure **2a** are shown in Table 2. Substructure **2a** was connected to substructure **2b** by the coupling between C17 and $H_3$21 at δ57.1 and δ0.88d respectively and to the coupling between C16 at δ29.3 to H20 at δ1.32 m on the HMBC. There were ^{1}H-^{13}C correlations between C22 m at δ35.0 and the $H_3$21 doublet protons at δ0.88; between C23 m at δ27.4 and the methylene protons $H_2$28 at δ1.28 m and δ1.11 m. The strong correlations between C24 at δ47.1 and the H_3 of C26 and C27 at δ0.77 d and δ0.79 d respectively as well as those of C24 and the $H_2$28 protons completely describes the structure of the side chain leaving an unassigned methylene group well defined by a tertiary C29 methyl signal at δ13.4.

Most HMBC were within 2 bonds ($^2J_{CH}$) away or 3 bonds ($^3J_{CH}$) away at most. Although the structure elucidation of clionasterol isolated from the Kenyan algae was largely afforded by a combination of mass spectrometry and 1D and 2D NMR experiments, most of the assignments of the clionasterol isolated from the Indian marine algae *G. edulis* were through the interpretation of the observed mass spectra (Das et al., 1992).

Antibacterial and antifungal activities

The epiphytes of *H. macroloba*, which had a deep green color, had potent antibacterial and antifungal activities based on the inhibition zones of the crude methanolic extracts (Table 3). The experiments were done on a 12.5 mm diffusion disc. The inhibition zone was 2.2 cm against the fungus *A. niger* strain and 1.9 cm against the gram-negative bacterium *E. coli*. The mass of these epiphytes (8 g), however, was inadequate for isolation of the active metabolites. This led to investigating the potency of the host (*H. macroloba*), which resulted in the isolation of compound **1**.

Table 2. Important 2D NMR HMBC correlations for substructure 2a

C1 ~ H3	$^3J_{CH}$
C2 ~ H3	$^2J_{CH}$
C3 ~ H1/1', H2/2', H4/4'	$^3J_{CH}$, $^2J_{CH}$, $^2J_{CH}$
C4 ~ H6	$^3J_{CH}$
C4' ~ H12, H17, $H_3$18	$^2J_{CH}$, $^2J_{CH}$, $^2J_{CH}$
C5 ~ H1/1', H4/4', H7/7',$H_3$19	$^3J_{CH}$, $^2J_{CH}$, $^3J_{CH}$, $^3J_{CH}$
C6 ~ H4/4', H7/7'	$^3J_{CH}$, $^2J_{CH}$
C7 ~ H6	$^2J_{CH}$
C9 ~ H1, H12, $H_3$19	$^3J_{CH}$, $^3J_{CH}$, $^3J_{CH}$
C10 ~ H6	$^3J_{CH}$
C14 ~ H12, H17, $H_3$18	$^3J_{CH}$, $^3J_{CH}$, $^3J_{CH}$
C17 ~ H21	$^3J_{CH}$

Table 3. Activity of clionasterol and on the epiphytes of *Halimeda macroloba*

	Halimeda macroloba epiphytes	Clionasterol
1. Antimicrobial activity		
a. *Aspergillus niger*	2.2 cm	Nil
b. *Escherichia coli*	1.9 cm	Nil
2. DLD-1 cancer cell line	N/d	>100 μm/ml
3. Brine shrimp toxicity	N/d	Nil
4. Mosquito larvae	N/d	Nil

Antimicrobial activity was measured as zones of inhibition on a 12.5 mm sterile filter disc (Chand et al., 1994). DLD-1 cancer cell line was measured as the LC_{50} (Swaffar et al., 1994) whereas the brine shrimp toxicity and the mosquito larvae bioassays were both determined as the LD_{50}. N/d implies that the experiments were not done. Nil represents lack of activity.

Mosquito larvae, brine shrimp and DLD-1 MTT toxicity

Clionasterol had no activity on mosquito larvae even at elevated concentrations; neither did it have any toxicity against brine shrimp and DLD-1 cancer cell line (LC-50 >100 mM/Ml) (Table 3).

In conclusion, although C29 compounds have been reported in marine organisms (Faulkner, 1996; Schmitz, 1978), this is the first time that clionasterol has been isolated from a Kenyan marine green macroalgae.

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