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Jaspars, Marcel, Dzeha, Thomas and Tabudravu, Jioji ORCID: 0000-0002-6930-6572 (2003) Clionasterol, a Triterpenoid from the Kenyan Marine Green Macroalga Halimeda macroloba. Western Indian Ocean Journal of Marine Science, 2 (2). pp. 157-161. ISSN 0856-860X

It is advisable to refer to the publisher's version if you intend to cite from the work.
<http://dx.doi.org/10.4314/wiojms.v2i2.28436>

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

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Key words: clionasterol, macroalga, *Halimeda macroloba*, spectroscopy, bioprospecting, Kenya

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 60F₂₅₄ 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.

Extraction and isolation

A 200 g sample (80 g dry weight) was homogenised in MeOH. Sequential extraction with MeOH, CH₂Cl₂ and partitioning with 1:1 CH₂Cl₂/H₂O resulted into the aqueous and organic phases. The organic CH₂Cl₂ phase was then partitioned with hexane in 90% MeOH/H₂O. The hexane extract (98.5 mg) was applied to a sephadex LH-20 (Pharmacia) gel filtration column. Elution was with 1:1 CH₂Cl₂/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 5 μ 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 ¹H 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 LD₅₀ 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 LD₅₀ 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. 10⁶–10⁷ 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 C₂₉H₅₀O from a combination of MS with ¹³C NMR spectroscopy. Initially the compound was thought to be C₂₈H₄₉OH but closer examination of the APT revealed an overlapped carbon on C4 at δ 43.3. Negative electron impact mass spectrometry (EIMS) gave the [M-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; C₂₉H₅₀O; EIMS m/z obs [M - H]⁺ 413.3, [M + Na]⁺ 437.4, [M + Na + MeOH]⁺ 469.5 (calc'd for C₂₉H₅₀O, 414); elution time 14:09, 14:32, 14:51 min; for ¹H and ¹³C NMR data see Table 1.

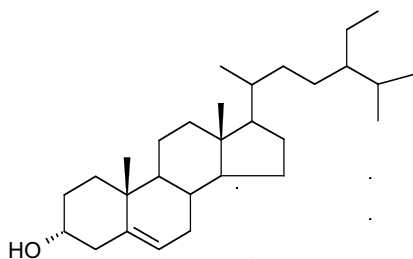


Fig. 1. Structure of clionasterol

This molecular composition required clionasterol to have 5 double bond equivalents with 1 olefinic bond at $\delta 141.8$ on the ^{13}C NMR spectra. The ^1H spectrum revealed the previously described olefinic bond at $\delta 5.31$ and a hydroxy

moiety at $\delta 3.47$. 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 ^1H and ^{13}C NMR (Table 1), ^1H - ^1H COSY and ^1H - ^{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 $\delta 12.9$ and 20.6 . ^1H - ^1H COSY correlated the olefinic hydrogen H6 at $\delta 5.31$ d with H₇ at $\delta 1.92$ m and 1.46 m. It also correlated with H_{2,4/4'} at $\delta 2.24$ m and 2.16 m. The correlation between H₂1 and H6, though evident,

Table 1. ^1H 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 ; ^1H at 400 MHz, ^{13}C at 100 MHz; assignments by ^1H - ^1H COSY, ^1H - ^{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).

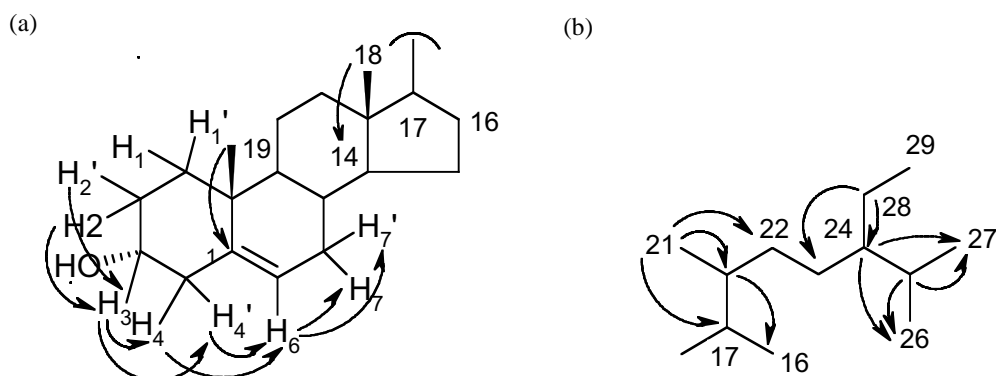


Fig. 2. Substructures for clionasterol showing the sterol moiety (a) and its side chain (b) with selected nOes

was weak at 4 bonds away. Other ^1H - ^1H COSY correlations suggesting the parent sterol structure are those between $\text{H}_{2,2'}$ (δ 1.78 m/1.44 m) and H_3 at δ 3.47 dddd and to $\text{H}_{2,4/4'}$ (δ 2.24 m/ 2.16 m). The nOe between $\text{H}_{2,2}$ and H_3 and $\text{H}_{2,4}$ 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 ^1H - ^{13}C coupling (HMBC) between C5 at δ 141.8 and the methyl hydrogens $\text{H}_3,19$ at 0.97 s; that between C14 at δ 57.8 and the $\text{H}_3,18$ at δ 0.64 and the 'solenoid'-like correlations between C3 at δ 72.8 and $\text{H}_{2,2}$ at δ 1.78 m, 1.44 m; C5 at δ 141.8 and $\text{H}_3,19$ at 0.97 s; C14 at δ 57.8 and $\text{H}_3,18$ at δ 0.64 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 $\text{H}_3,21$ at δ 57.1 and δ 0.88d respectively and to the coupling between C16 at δ 29.3 to H_{20} at δ 1.32 m on the HMBC. There were ^1H - ^{13}C correlations between C22 m at δ 35.0 and the $\text{H}_3,21$ doublet protons at δ 0.88; between C23 m at δ 27.4 and the methylene protons $\text{H}_2,28$ at δ 1.28 m and δ 1.11 m. The strong correlations between C24 at δ 47.1 and the H_3 of C26 and C27 at δ 0.77 d and δ 0.79 d respectively as well as those of C24 and the $\text{H}_2,28$ protons completely describes the structure of the side chain leaving an unassigned methylene group well defined by a tertiary C29 methyl signal at δ 13.4.

Most HMBC were within 2 bonds ($^2\text{J}_{\text{CH}}$) away or 3 bonds ($^3\text{J}_{\text{CH}}$) 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	$^3\text{J}_{\text{CH}}$
C2 ~ H3	$^2\text{J}_{\text{CH}}$
C3 ~ H1/1', H2/2', H4/4'	$^3\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}$
C4 ~ H6	$^3\text{J}_{\text{CH}}$
C4' ~ H12, H17, H ₃ 18	$^2\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}$
C5 ~ H1/1', H4/4', H7/7', H ₃ 19	$^3\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}$
C6 ~ H4/4', H7/7'	$^3\text{J}_{\text{CH}}, ^2\text{J}_{\text{CH}}$
C7 ~ H6	$^2\text{J}_{\text{CH}}$
C9 ~ H1, H12, H ₃ 19	$^3\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}$
C10 ~ H6	$^3\text{J}_{\text{CH}}$
C14 ~ H12, H17, H ₃ 18	$^3\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}, ^3\text{J}_{\text{CH}}$
C17 ~ H21	$^3\text{J}_{\text{CH}}$

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

	<i>Halimeda macroloba</i> epiphytes	Clionasterol
1. Antimicrobial activity		
a. <i>Aspergillus niger</i>	2.2 cm	Nil
b. <i>Escherichia coli</i>	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 LC₅₀ (Swaffar et al., 1994) whereas the brine shrimp toxicity and the mosquito larvae bioassays were both determined as the LD₅₀. 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/MI) (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.

Acknowledgement—We are grateful to Shadrack Tunje of the Kenya Marine Fisheries Research Institute (KMFRI) for his assistance in the collection of the algal material and to Jacqueline Uku of the same institute for identifying the taxa. From Aberdeen University, we acknowledge Drs Berg [Botany department] for confirming the taxonomic identification, Heather Wallace [Department of Medicine and Therapeutics] for the DLD-1 MTT assay and Peter Billingsley [Department of Zoology] for the mosquito larvae lethality experiments. We thank Dr Linda Lawton [Robert Gordon University], in whose laboratory the brine shrimp toxicity tests were performed. We cannot forget the invaluable contribution of emeritus Professor Shmuel Halevy of the School of Pharmacy, Hebrew University, Jerusalem, who also provided the microbial strains used in the

antimicrobial bioassays. This work was supported by a grant ICB/34896/00 8D from the Organisation for the Prohibition of Chemical Weapons (OPCW) of the Hague, the Netherlands and the Faculty of Science and Engineering of the University of Aberdeen in Scotland.

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