

Isolation of Stigmast-5,24-dien-3-ol from marine brown algae *Sargassum tenerrimum* and its antipredatory activity

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Abstract:

In recent years many sterols with potent biological activity have been identified from marine sources. Here we report the isolation of stigmast-5,24-dien-3-ol (fucosterol) as a major metabolite from bioactive hexane-fraction of *Sargassum tenerrimum* using different chromatographic techniques. Moreover, the chemical defense of brown algae *Sargassum tenerrimum* was investigated using feeding assay performed in aquarium and in field.

Introduction:

Macroalgae are classified into three higher taxa, brown (Phylum: Phaeophyta), red (Phylum: Rhodophyta) and green (Phylum: Chlorophyta), based on their pigmentation. Macroalgae, also known as seaweeds or sea vegetables, have been traditionally incorporated into Pacific and Asian foods for last several of years. These have recently become a popular addition to some Western diets and could be used as functional ingredients. Owing to the harsh environments (for example, changes of salinity, temperature, nutrients, UV-Vis irradiation, etc.) in which many macroalgae exist, they have developed effective defense mechanisms producing a great variety of secondary (biologically active) metabolites, which cannot be found in other organisms.¹ Macroalgae are harvested and used globally for many different applications, such as emulsifying agents, cosmetic formulations, medicine etc.² Despite their many desirable commercial aspects, high growth rates can cause ecological problems. Moreover, the

ecological significance of macroalgae is highlighted by the fact that they assist in supplying oxygen to the sea, acting as one of the primary producers in the marine food chain and also addressing various environmental pressures *i.e.*, defense against predators, tissue repair, holdfast adhesion, and protection against reactive species generated by oxidative processes.³ Therefore, macroalgae need to adapt quickly to different biotic and abiotic stresses in ecosystem by means of chemical protection mechanisms such as creation of antioxidants to limit oxidation⁴ and production of feeding-deterrents to prevent grazing.⁵

In the Phaeophyta (brown algae), *Dictyota* species have been extensively studied and are known to produce interesting natural products. Nevertheless, many of these metabolites had shown to possess broad-spectrum feeding deterrents against herbivores or show other biological activities.⁶ For example, the few diterpenes such as pachydictyol A, dictyol E, dictyol B, dictyol B acetate, dictyol H, and an isolinerol/linearol mixture have been shown to be active anti-feedant metabolites.⁷ Another class of metabolite widely found in Phaeophyta is terpenoid/steroids. It is always exciting to understand the ecological significance of these major constituents (in particular sterols) of seaweeds. In general, sterols are widely distributed throughout the majority of living cells. However, the principal sterols are synthesized differently depending on the organisms, *i.e.* cholesterol in animal; ergosterol in fungal cells, whereas fucosterol is predominantly found in brown seaweeds which constitute 83–97% of the sterol content.⁸ Interestingly, stigmast-5,24-dien-3-ol (fucosterol) is widely utilized in biological activity studies, including antioxidant, hepatoprotective,⁹ anti-inflammatory,¹⁰ anticancer,¹¹ anti-fungal,¹² anti-diabetic,¹³ antihistaminic, and anticholinergic.¹⁴ Additionally, Vahouny *et al.* reported that fucosterol reduces the gastrointestinal absorption of cholesterol¹⁵ and Hagiwara *et al.* observed that, fucosterol decreases angiotensin converting enzyme levels through the reduction of glucocorticoid receptors in endothelial cells.¹⁶ The ecological significance of this sterol activity remains to be tested. Yet, this does not rule out possible grazer deterrence or chemical defense function of sterols in brown algae. In continuation of our research in marine natural product chemistry, herein we have reported the isolation of major metabolite from brown algae *Sargassum tenerrimum* and effect of their organic fractions on fish feeding. The main goal of present study was to examine the role of metabolites of *Sargassum tenerrimum*, as prospective anti-feedants.

Result and Discussion:

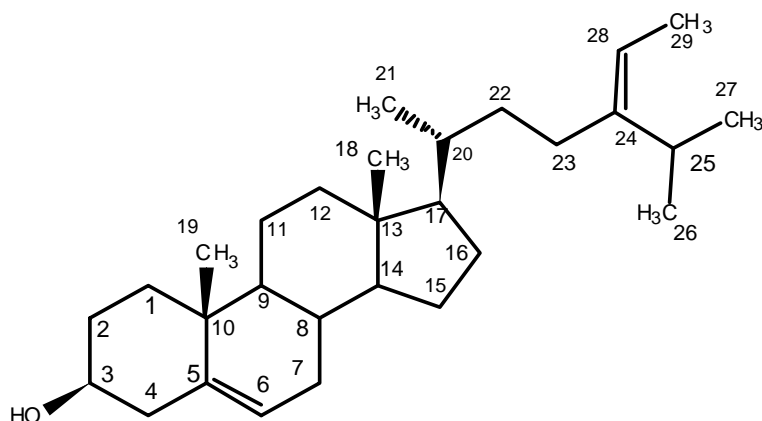


Figure 1: Structure of isolated stigmaster-5,24-dien-3-ol (**1**) from *S. tenerrimum*

A wide array of marine natural products has been identified from tropical algae and many of these metabolites deter herbivore from feeding on those algae in field as well as in laboratory aquarium. However, many non-polar compounds from algae have no effect on fish but it could have effect on other predators. Example, Teixeira *et al.* investigated¹⁷ the ecological role of nonpolar metabolite i.e. cytotoxic hydroperoxysterol from Brazillian brown seaweeds *D. justii* & *S. schroederi* as defensive strategy against herbivory crabs. Later, Fenical *et al.* identified secosterol from octocoral *Pseudopterogorgia americana* which provide chemical defense to both coral and alga using feeding assay experiment.¹⁸ Moreover, feeding deterrence of brown algae has received the most attention and phlorotannins were considered as defensive function in it. In present study, we had investigated *S. tenerrimum* for its metabolite contents and fish feeding. Crude extract, hexane and ethyl acetate fraction elicited a significant rejection of food pellets compared to control. In contrast, butanol fraction did not deter fish feeding in laboratory as well as in field experiment. Chemical analyses of this fraction indicated the presence of sterol as major metabolite. The positive test for sterol in crude extract and fractions was confirmed by the development of silica gel TLC. The plate showed purple color spot after being sprayed with 10% H₂SO₄ solution followed by heating in oven. The BuOH fraction contains either amino acids or peptides (positive ninhydrin test). As consequence, we assumed that the deterrence of *S. tenerrimum* may be due to the effects of major metabolite i.e. sterol. Purification of hexane and ethyl acetate fraction by repeated gel permeation and column chromatography using sephadex and silica gel respectively yielded major compound identified as stigmaster-5,24-dien-3-ol (**1**) as shown in Figure 1.

Compound **1** was isolated as a white solid that showed a molecular ion $[M+H]^+$ peak at m/z 413 in ESI-MS spectrum. The compound showed the IR absorption peaks at 3400 & 1600 cm^{-1} which were attributed to hydroxyl and olefinic group respectively. The ^1H NMR spectrum showed signals at δ 1.56 (d, $J = 6.8$ Hz, 3H), 1.02 (s, 3H), 1.01 (d, $J = 6.4$ Hz, 3H), 1.00 (d, $J = 6.8$ Hz, 3H), 0.96 (d, $J = 6.4$ Hz, 3H) and 0.76 (s, 3H) for 6 methyl groups indicating compound **1** as sterol core with an allyl methyl. The characteristic peak at δ 3.46 was attributed to oxygenated methine proton. Moreover, 2-olefinic proton signals at δ 5.34 ($J = 6.8$ Hz, 1H) and 5.17 (q , $J = 6.8$ Hz, 1H) indicated that this compound could be a stigmast-diene type sterol. The ^{13}C NMR showed signals for 29 carbons. The peaks at δ 146.9 and 140.7 were attributed to 2 olefinic quaternary carbons, whereas 2 methine olefinic carbons appeared at δ 121.6 and 115.5. One oxygenated methine carbon appeared at δ 71.8 and 6 methyl carbon signals were seen at δ 22.2, 21.1, 19.4, 18.7, 13.2, 11.8. Finally, the isolated compound was identified as stigmast-5,24-dien-3-ol (**1**) and confirmed through the matching of physical and spectroscopic data with literature data.¹⁹

Table 1: ^1H and ^{13}C NMR spectroscopic data of compound **1** in CDCl_3 .

Position	δC , mult.	δH , mult.
1	37.25	
2	31.65	
3	71.8	3.52,m
4	42.29	
5	140.75	
6	121.7	5.36 d
7	31.9	
8	31.9	
9	50.12	
10	36.50	
11	21.07	
12	39.75	
13	42.34	
14	56.75	
15	24.32	
16	28.23	
17	55.77	
18	11.84	0.76,s
19	19.39	1.02,s
20	36.41	
21	18.74	1.01,d
22	35.21	
23	25.69	
24	146.99	
25	34.77	2.2,sep
26	22.13	0.96,d
27	22.24	1.0,d
28	115.5	5.19,q
29	13.16	1.56,d

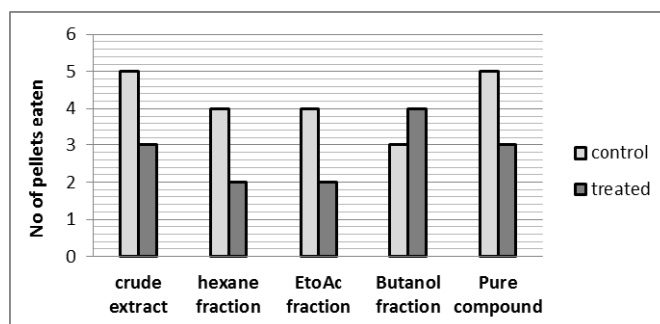


Figure 2: Laboratory assay: Consumption of food pellets containing crude organic extract, fractions & pure stigmast-5,24-dien-3-ol from *S. tenerrimum* by fishes. Extracts were considered deterrent if the number of pellets eaten were ≤ 3 .

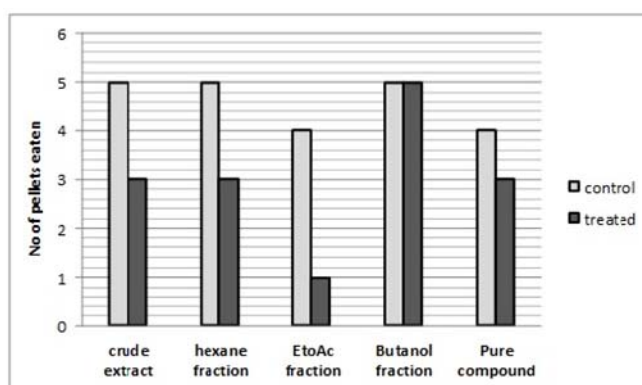


Figure 3: Field assay: Consumption of food pellets containing crude organic extract, fractions & pure fucosterol from *S. tenerrimum* by fishes. Extracts were considered deterrent if the number of pellets eaten were ≤ 3 .

Recent studies documented that fucosterol exhibits cytotoxicity against breast and colon carcinoma cell lines i.e. proliferation of T47D cells, T47D and HT-29 cell.¹¹ Additionally, it is also known for its antidiabetic and antioxidant activity.¹³ The antioxidant capacity of compounds has been related to the prevention of several diseases including cancer, coronary heart disorders, inflammatory disorders, ageing, and neurological degeneration in medicinal chemistry.²⁰ However, ecological significance of this reported activity remains to be subject of further studies. In general, the ecological roles of steroids remains unclear, although invertebrates produces this sterol for their reproduction, chemical signaling, defense or some time acts as a biosynthetic precursors to generate other toxic derivatives. Interestingly, crude extract, two fractions, and pure compound at natural concentration elicited significant rejection of artificial food pellets compared to control pellets in laboratory experiment (Figure 2). Whereas, field experiment²¹⁻²³ showed almost similar results except ethyl acetate fraction, which showed more

significance rejection compared to other fractions (Figure 3). In fact, these fractions of *S. tenerrimum* extract seemed to some extent inhibit grazing by rock pool fishes. The feeding effect could be due to two possible reasons either distasteful or toxic nature of stigmast-5,24-dien-3-ol (**1**). In first assay we verified that the crude extract inhibit activity of the rock pool fishes *Istigobio ornatus* and *Istiblennius dussumieri*. Additionally, fish feeding experiment with pure stigmast-5,24-dien-3-ol confirm the effect of deterrence towards feeding by *Istigobio ornatus* and *Istiblennius dussumieri* fishes. Thus we believe that, these metabolites produced by brown algae *S. tenerrimum* produces chemical defense and play ecological roles to protect themselves.

Conclusions

We have reported the isolation of the major metabolite fucosterol from *Sargassum tenerrimum* as a new source. According to the result reported herein, we proposed the ecological significance for the sterol found in this species of brown algae as a defensive chemical against fish feeding in particular *Istigobio ornatus* and *Istiblennius dussumieri* fishes. The probable reason for this observed deterrence could be the cytotoxicity or distasteful effect of fucosterol. Overall, fucosterol- a bioactive compound was found as an anti-feedant and seems to be involved in chemical defense mechanism of producing organism against rock pool fishes.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Isolation of Stigmast-5,24-dien-3-ol from marine brown algae *Sargassum tenerrimum* and its Antipredatory effect

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1. Experimental

General Remarks. The ¹H and ¹³C NMR were recorded in the deuterated solvents (CDCl₃) on Bruker 300 MHz spectrometer. The mass spectra were recorded on electrospray ionization-quadrupole time of flight Mass spectrometry (ESI- QTOF MS) QSTAR XL, Applied Biosystem, Canada. Column chromatography was performed with Sephadex LH20 and silica gel (60-120 mesh) as packing material. Thin-layer chromatography was performed with Kieselgel 60 F254 (Merck aluminum support plates).

Collection, extraction, and isolation. The marine algae *Sargassum tenerrimum* was collected from the intertidal zone at Anjuna Goa, central western coast of India. Sample was frozen as soon as possible and transferred to the laboratory. Identification was carried out by emeritus scientist Dr. P. A. Thomas of the Vizhingam Research Center of the Central Marine Fisheries Research Institute, Kerala, India. The sample is deposited at the CSIR-National Institute of Oceanography, Dona-Paula, Goa, India (Vocher specimen no. NIO1001). The freshly collected brown alga was initially freeze dried. The sample was homogenized in a blender with little water and exhaustively extracted at room temperature with MeOH (1 Lit. x 3). The combined extracts were filtered and concentrated under vacuum on a rotary evaporator at low temperature to give crude methanolic extracts. Methanolic extract (~5 g) of the algae *S. tenerrimum* was fractionated with hexane (100 ml x 3), ethyl acetate (100 ml x 3) followed by butanol (100 ml x 3). The hexane fraction (200 mg) was chromatographed initially on series of sephadex column eluting with chloroform in methanol. Subsequently, purification was carried out on silica gel column

(60-120 mesh) using different proportion of hexanes and ethyl acetate mixture as eluting solvents. These resulted in the purification of Fucosterol (**1**) as white solid.

Feeding experiment

Laboratory palatability assays. Pawlik protocol (Pawlik et al. 1995) was used to perform aquarium feeding assays using rock pool fishes, which were captured on the same site from which brown algae were collected. The artificial food (treated) pellets were prepared by adding agar (1.5 g) to 60 mL of distilled water and 3.0 g of commercially available artificial fish food followed by heating in microwave oven until boiling. Test solution (crude extracts or pure compound) was added to 15 ml above melt. The concentration used for test compounds was calculated on the basis of the natural concentration found in fresh seaweed tissues. The control food pellets were treated with the same amount of solvent used in treated pellets. After hardening gel solution by cooling at 5 °C, the pellets were cut and offered in control-treated pairs, to fish at the top of the aquarium (1 pellet at a time). Five replicates were made of each test solution, using 5 different aquaria with 3 fishes each. A significant difference between the number of treated and control pellets eaten was determined for an individual 5 replicate assay (1-tailed test, Pawlik et al. 1995). The consumption of treated food and control food was assumed to be dependent on each other, since consumption of one food item means less consumption of the other in same container (Peterson et al. 1989). Differences were considered significant only when $p < 0.05$ i.e. 5%.

Field palatability assays. Field assay was performed at Anjuna Goa, Central western coast of India in the month of November 2013. Field strip were prepared by using previously reported protocol by (Pawlik and Fenical 1992). For each experiment, 5 treated and 5 control strips were arranged in pairs and attached to 2 ropes. The ropes were anchored slightly above the bottom near to the same site where seaweeds were collected. After 3 h, the ropes were recovered and the numbers of strips eaten were measured. During the experiments several fishes were observed feeding on the test strips.

2. Characterization data of Fucosterol

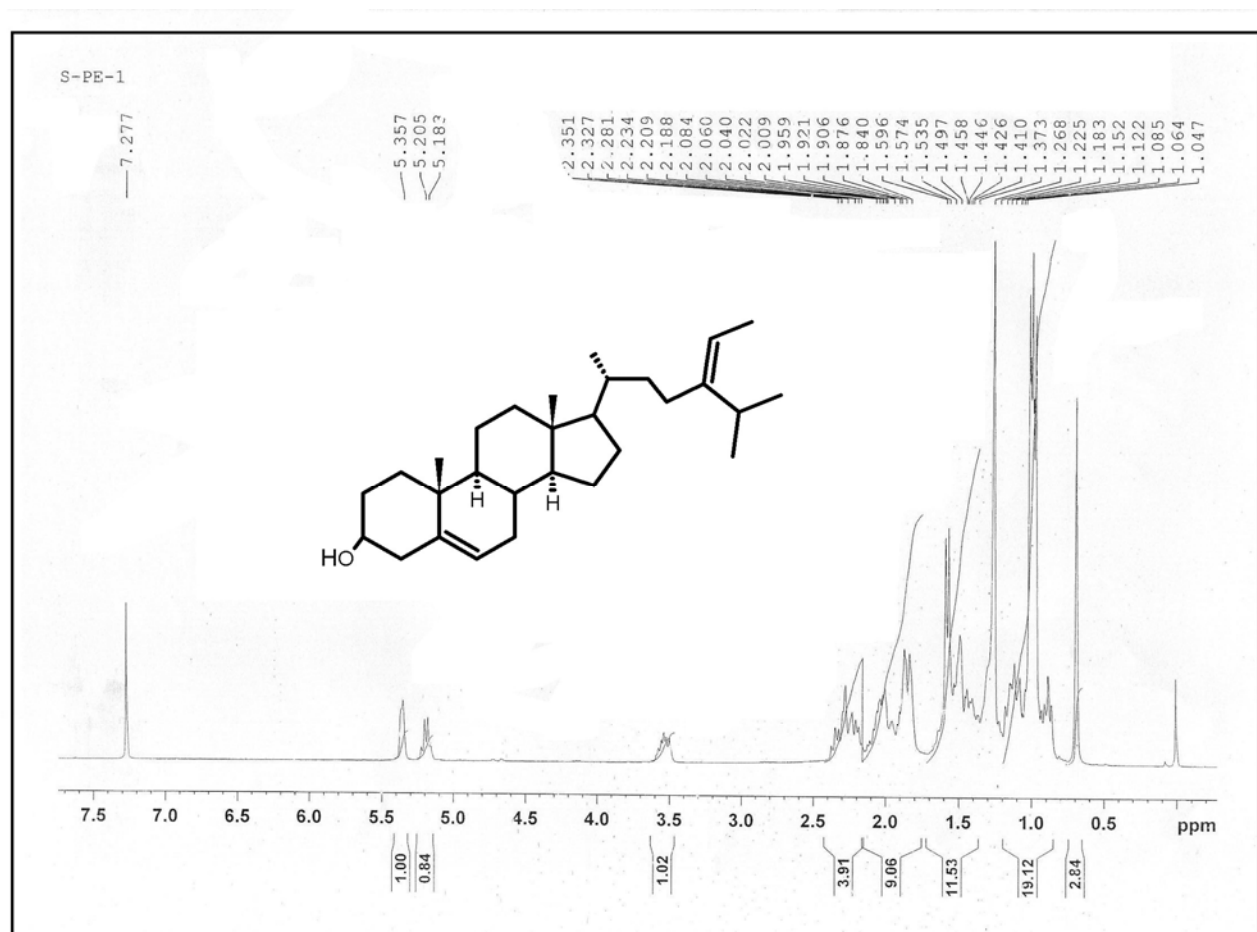


Figure S1. ^1H NMR (CDCl_3) spectrum of fucosterol 1

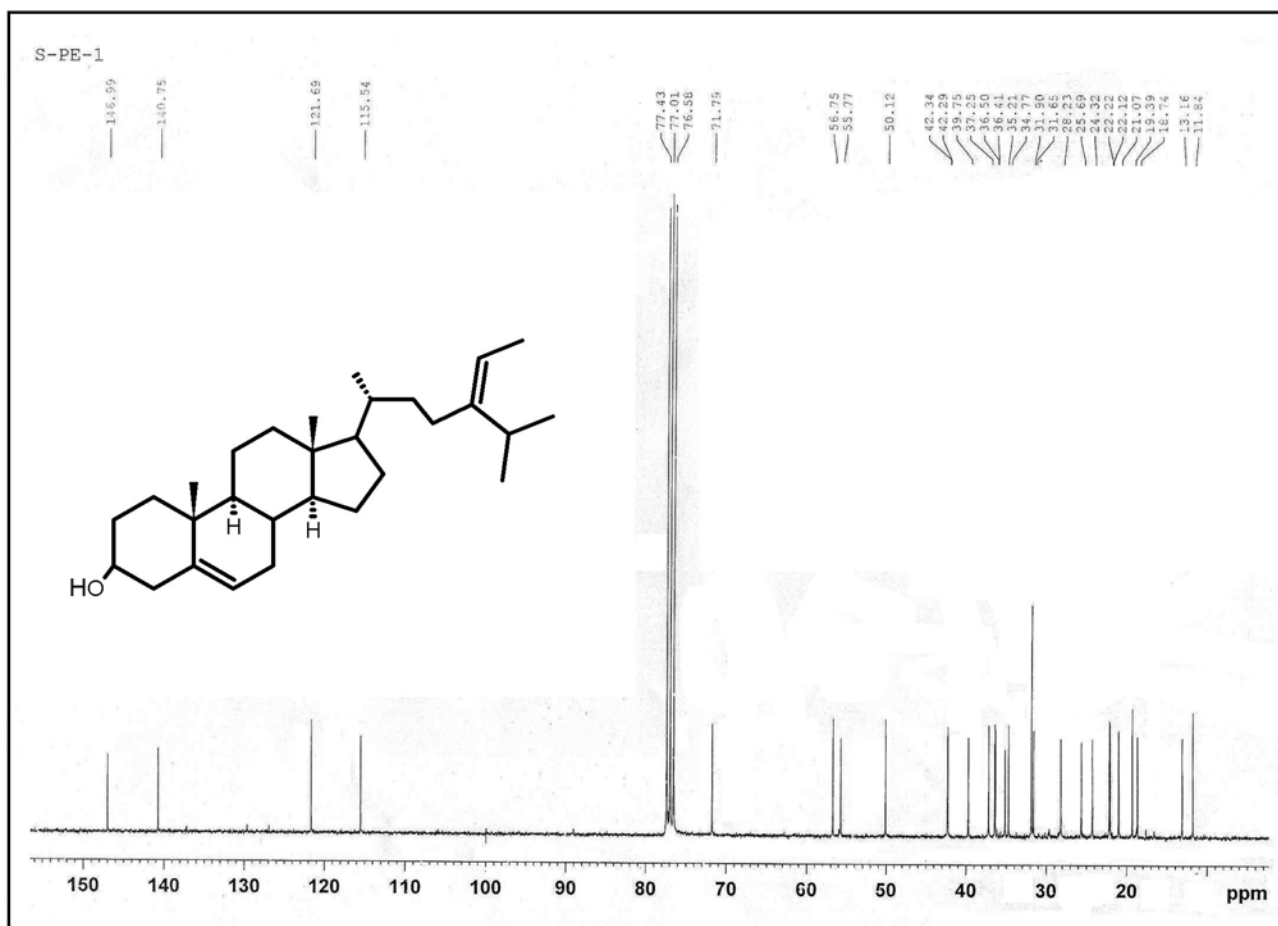


Figure S2. ^{13}C NMR (CDCl_3) spectrum of fucosterol 1

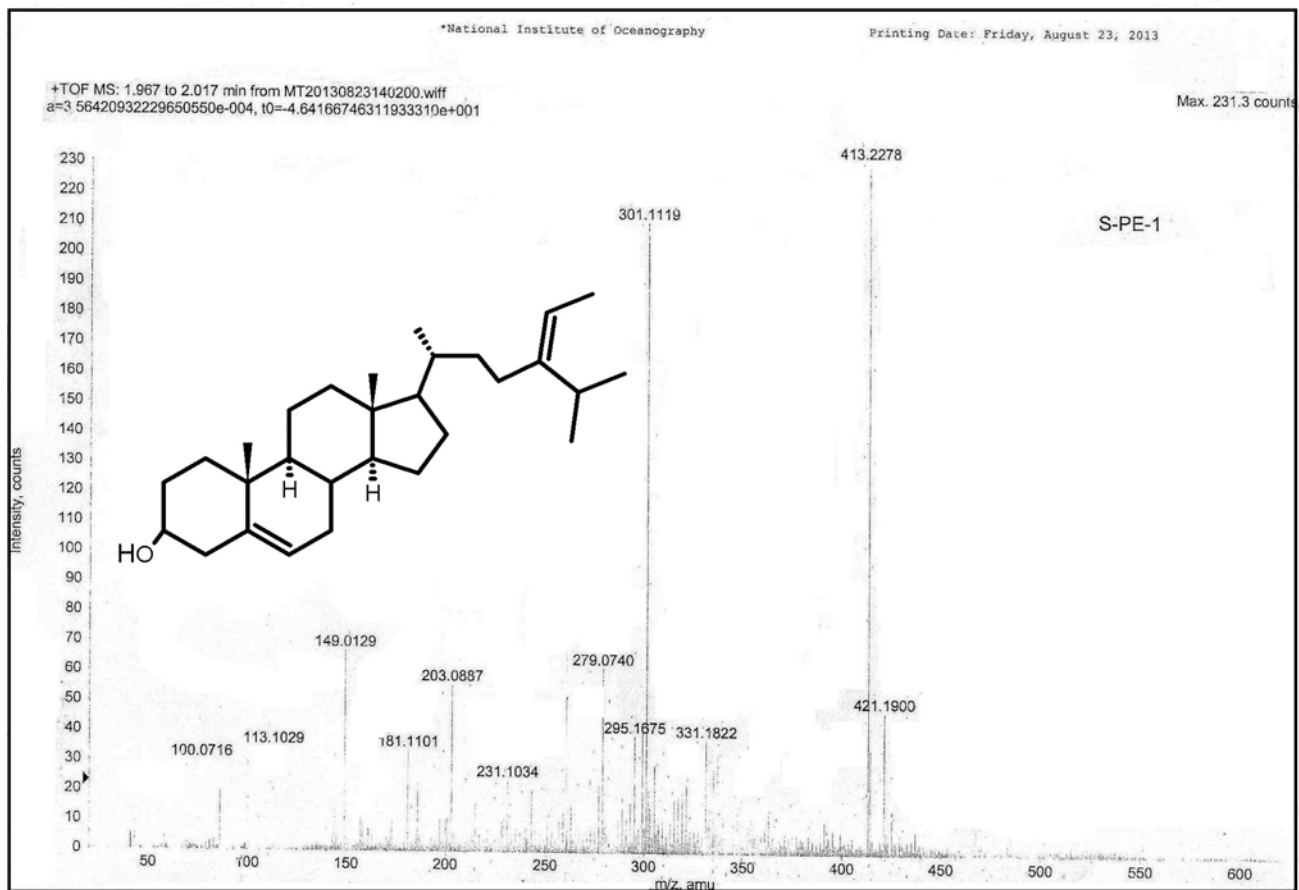


Figure S3. ESI-MS of fucosterol 1

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