Version of Record: https://www.sciencedirect.com/science/article/pii/S1878535214002524
Manuscript_b19e31472719932d5cf70b0f5f50817e

Sterols from the brown alga *Cystoseira foeniculacea*: Degradation of fucosterol into saringosterol epimers

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Abstract

The present study was carried out in order to determine the phytochemical composition of the marine brown alga *Cystoseira foeniculacea* collected off the coasts of Algeria. After a preliminary fractionation of its organic crude extracts by column chromatography, the resulting fractions were further analysed by ^{1}H NMR. Even though algal species of the genus *Cystoseira* are commonly known to produce a wide variety of meroditerpenoids, in the case of *C. foeniculacea* none of the fractions were found to contain such compounds: most of the fractions showed typical ^{1}H NMR signals of fatty acids and derivatives (mainly glycerolipids and glycolipids). Nevertheless, the thorough analysis of a sterol-enriched fraction by RP-C_8

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HPLC led to the isolation, for the first time from this species, of fucosterol (**1**) and a mixture of saringosterols (**2** and **3**). The NMR data of compounds **1**-**3** were fully determined with the help of 1D and 2D experiments which allowed the reassignment of some attributions in comparison to those reported in literature. This work also confirms evidence of the oxidative degradation of fucosterol into a C-24 epimeric mixture of saringosterols.

Keywords: *Cystoseira foeniculacea*, Sargassaceae, Fucosterol, Saringosterols, Oxidative degradation.

1. Introduction

Brown seaweeds constitute an important part of the marine biomass. However, if the exploitation of hydrocolloids for the food industry is flourishing, the valorization of algal metabolites remains under-exploited. Indeed, more than 1300 chemical compounds have been isolated from brown algae and this chemical group represents more than 5% of all natural products described to date from marine sources (MarinLit, 2013). In this phylum, the phytochemical composition of species belonging to the Sargassaceae and Dictyotaceae families has been widely studied: more than 1100 secondary metabolites have been described from such brown algae. More particularly, among the species of Sargassaceae, a number of them are thus recognized for their ability to produce high amounts of terpenoids, especially acyclic diterpenoids and meroditerpenoids.

Cystoseira foeniculacea (Linnaeus) Greville is a marine brown alga (Fucales, Sargassaceae) which is distributed extensively, as most of the *Cystoseira* species, in the Mediterranean Sea and to a lesser extent, along the northwestern coasts of the Atlantic Ocean (Guiry et al., 2013). According to the literature review, there had been no specific study conducted on the chemical composition of this species.

As part of our ongoing research on phytochemical investigation of brown algae (Bouzidi et al., 2008; El Hattab et al., 2008, 2009; Mokrini et al., 2008; Viano et al., 2011; Othmani et al., 2014), the present paper describes the chemical analysis of organic extracts obtained from Mediterranean samples of *C. foeniculacea* collected off the Algerian coasts. This work highlights the absence of terpenoids and allows the isolation and characterization of three known sterols, fucosterol (**1**) and saringosterols (**2** and **3**) (Fig. 1), for the first time from this species. In this study, a simple chromatographic method for the analysis of algal sterols by RP-C_8 HPLC is described. The oxidative degradation of fucosterol (**1**) into a C-24 epimeric mixture of saringosterols (**2** and **3**) is as well revealed.

2. Material and methods

2.1. General

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer. Compounds were solubilized in $CDCl_3$ (Sigma-Aldrich, France): chemical shifts were given in ppm and were referenced to the residual solvent peaks (δ_H 7.26 and δ_C 77.16). Fractions were solubilized in $CDCl_3$, CD_3OD or D_2O (Sigma-Aldrich, France) according to their polarity. Silica gel (Si60, 40-63 μm, Merck) was used for column chromatography.

HPLC analyses and purification of compounds **1**-**3** were performed on a Biotek Instrument chromatographic chain composed of a degasser (Model 3493), a ternary pump (Model 525), a column oven (Model 582), and an autosampler (Model 560). This chromatographic system was equipped with a LDC Analytical UV single-wavelength detector.

The chemicals and solvents used in this study were all of analytical grade, in exception of acetonitrile (MeCN) which was of HPLC gradient grade, all of them were provided by Sigma Aldrich (France).

2.2. Plant material

Whole thalli of *C. foeniculacea* were collected off the Mediterranean coasts of Algeria at Cherchell (36°36'40" N, 2°12'19" E). A voucher specimen (n° HS-17) was deposited in the Herbarium of the Laboratory of Biological Oceanography and Marine Environment (University of Sciences and Technology Houari Boumediene, Algiers, Algeria).

*2.3. Extraction and isolation of compounds **1**-**3***

After collection, algae were carefully inspected in order to remove biological contaminants and shade-dried at room temperature. The algal material (537 g) was extracted with $CHCl_3$/MeOH (1:1, v/v) at room temperature yielding a dark greenish residue (9.1 g). Following the partition of this crude extract in a mixture of $CHCl_3$/MeOH/H_2O (1/1/1; v/v/v),

an organic phase ($CHCl_3$/MeOH) and an aqueous phase (MeOH/H_2O) were then obtained. After removal of the solvents under reduced pressure, the organic extract (7 g) was fractionated by silica gel column chromatography with mixtures of *n*-hexane/EtOAc of increasing polarity. A ^1H NMR spectrum was then recorded for each fraction. Fraction eluted with *n*-hexane/AcOEt (3:2, v/v), which was previously treated with Na_2CO_3 (5%, w/v) in order to remove fatty acids, was subjected to analytical RP-C_8 HPLC (Merck, Purospher STAR; 4.6 × 125 mm, 5 μm) with pure MeCN as eluent (flow rate: 1 mL.min^{-1}; UV detection: 205 nm) to give a mixture (1:1) of compounds **2** and **3** (t_R = 3.5 min.; 4 mg) and pure compound **1** (t_R = 5.8 min.; 9 mg). Both compounds which were isolated as white crystalline solids, were analyzed by 1D and 2D NMR.

3. Results

The extraction step allowed the obtaining of a lipidic extract from *C. foeniculacea* air-dried material in a yield of 1.7% (weight of extract/dry algal weight) which ranged in typical values found for algae of the genus *Cystoseira* (Valls et al., 1993). After partition of the crude extract, its organic part was fractionated by column chromatography. Then, each fraction was analysed by ^1H NMR spectroscopy in order to carry out a large scale chemical screening and to determine the overall chemical composition of *C. foeniculacea*. Most of the fractions were found to be constituted by simple lipids, e.g. fatty acids and their derivatives, but one of them showed typical ^1H NMR signals of steroids. This fraction was further investigated by reversed-phase HPLC on a C_8 column (RP-C_8) in order to characterize its main chemical components. The chromatographic profile of this sterol fraction showed two main peaks which were separated and obtained in sufficient amounts, by repetitive chromatographic runs, for further NMR study.

The complete analysis of the NMR data of the two sub-fractions along with a comparison with literature data allowed their structure to be elucidate as fucosterol (**1**) (Hwang et al.,

2012) and an epimeric mixture of saringosterols (**2** and **3**) (Ayyad et al., 2003; Catalan et al., 1983; Huh et al., 2012). The duplicate signals (C-17, C-20, C-22 to C-24, C-27 to C-29) in the ^{13}C NMR spectrum of **2/3** were in accordance with the occurrence of the two C-24 epimers. Further analysis of 2D NMR data, in particular HMBC and ^{1}H-^{1}H COSY spectra allowed corrections of some attributions for the ^{13}C NMR data of these compounds (Table1) in comparison with those reported in the literature (Ayyad et a*l.*, 2003; Huh et al., 2012). Moreover, after its storage during 24 h at room temperature in an unsealed vial, a further NMR analysis of fucosterol (**1**) was performed and additional signals were then observed on its ^{1}H and ^{13}C NMR spectra. A thorough examination revealed that these new signals were the same as those reported for saringosterols (Fig. 2). This sample was also analyzed by RP-C_8 HPLC under the same conditions as above; the chromatogram revealed the existence of two peaks eluted at retention times which were exactly the same as those of **1** and **2/3**. These data therefore confirmed the spontaneous oxidative degradation of fucosterol (**1**) into saringosterols (**2** and **3**). Thus, saringosterols (**2/3**) isolated in this study from the crude extracts of *C. foeniculacea* could be considered as artifacts formed throughout the various stages of the process that led to the obtaining of pure compounds from these algal samples.

4. Discussion

From a chemotaxonomic aspect, this work states that, as several other *Cystoseira* species (e.g. *Cystoseira humilis* and *Cystoseira compressa*), *C. foeniculacea* could be classified into a "chemical group" holding none lipophilic secondary metabolites (*e.g.* diterpenoids or meroditerpenoids) (Amico, 1995; Jégou et al., 2010; Valls and Piovetti, 1995). These results were in accordance with a recent study which described *Cystoseira* as a polyphyletic genus where all these terpenoids non-producing species were gathered into the same clade (Draisma et al., 2010).

Concerning the chromatographic method developed here for the analysis of the sterol fraction of *C. foeniculacea*, it may be pointed out that algal sterols, and more generally plant sterols, could be detected and quantified through a variety of analytical techniques (Abidi, 2001). The final isolation of sterols from algal lipophilic extracts was mainly conducted by RP-C_{18} HPLC (Hwang et al., 2012, Terasaki et al., 2009). In this work, a chromatographic method based on the use of a stationary phase with a smaller hydrophobic selectivity (RP-C_8 instead of RP-C_{18}) was developed: it allowed a quick and easy separation of the main steroidal components from algal extracts. In the case of sterols with few polar groups, which were highly retained on a C_{18} stationary phase, this method led to a similar separation with a significant economy of time and mobile phase.

Finally, with such a methodology it was then possible to characterize easily the main components of the sterol fraction of *C. foeniculacea* by NMR as fucosterol (**1**) and saringosterols (**2** and **3**). Fucosterol occurs as the predominant sterol in brown algae (Terasaki et al., 2009) and this characteristic component displays a wide variety of biological activities (Balboa et al, 2013). In Sargassaceae, fucosterol has been described in several species, particularly, in some representatives of the genus *Cystoseira*, such as *C. usneoides*, *C. nodicaulis*, *C. tamariscifolia* (Lopes et al., 2011), *C. hakodatensis* (Abidi, 2001), *C. amentacea* var. *stricta* (Piovetti et al., 1991), *C. barbata* and *C. crinita* (Milkova et al., 1997). Saringosterols were noted in a number of Phaeophyta (Ikekawa et al., 1966; Knights, 1970; Liu et al., 2009; Okano et al., 1985; Wachter et al., 2001) and in rare cases, they were described in both red and green algae (Huang et al., 2007; Shi et al., 2011). Saringosterols have been previously shown to exhibit wide-ranging biological activities (Chen et al., 2014; Hoet et al., 2007; Huh et al., 2012; Kim et al., 2014; Wachter et al., 2001).

Fucosterol is highly reactive and its degradation was already mentioned. Thereby, it has been shown that the presence of saringosterols in the brown algae *Ascophyllum nodosum* (Knights,

1970) or *Cystoseira crinita* (Milkova et al., 1997) were in fact artefacts arising from the auto-oxidation during the air-drying of the alga, while, the fresh material would contains only fucosterol. Thus, chemical analysis of fucosterol, and more generally of sterol fractions and/or crude extracts containing fucosterol, might absolutely take into account the chemical conversions of this compound, in particular through degradation process, they necessarily lead to biased results otherwise.

5. Conclusion

The present study investigated the separation and the characterization of fucosterol (**1**) and a mixture of 24*R*- (**2**) and 24*S*- (**3**) saringosterols from the brown alga *C. foeniculacea* by using a simple and easy method based on RP-C_8 chromatography. The analysis by 1D and 2D NMR experiments allowed the reassignment of some attributions of chemical shift of the isolated sterols **2** and **3** by comparison with those reported in the literature. We have also confirmed the oxidative degradation by HPLC analysis of a degraded sample of fucosterol. Indeed, the NMR analysis of pure **1** stored for few times revealed the presence of new signals corresponding to those of the **2/3** mixture. Importantly, we highlighted in this work the spontaneous oxidation of fucosterol (**1**) into saringosterols (**2** and **3**).

Acknowledgments

This work was supported financially by grants of CMEP-PHC Tassili (N° 06 MDU 680) and Arcus-Ceres MAEE/PACA research programs. One of the researchers (Y. Viano) was funded by the French "Provence-Alpes-Côte d'Azur (PACA)" regional council (PhD grant). The authors are indebted to Pr. Louis Piovetti (Université de Toulon, MAPIEM, France) for many valuable and helpful discussions.

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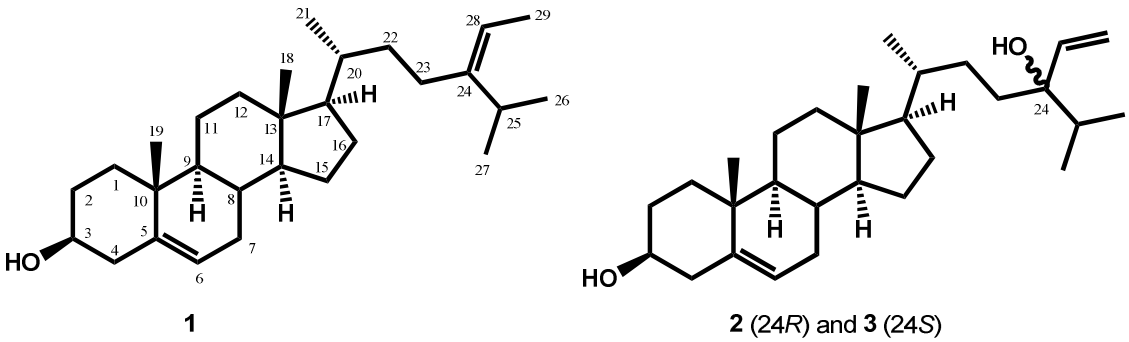
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1 **2** (24*R*) and **3** (24*S*)

Figure 1 Chemical structures of compounds 1-3.

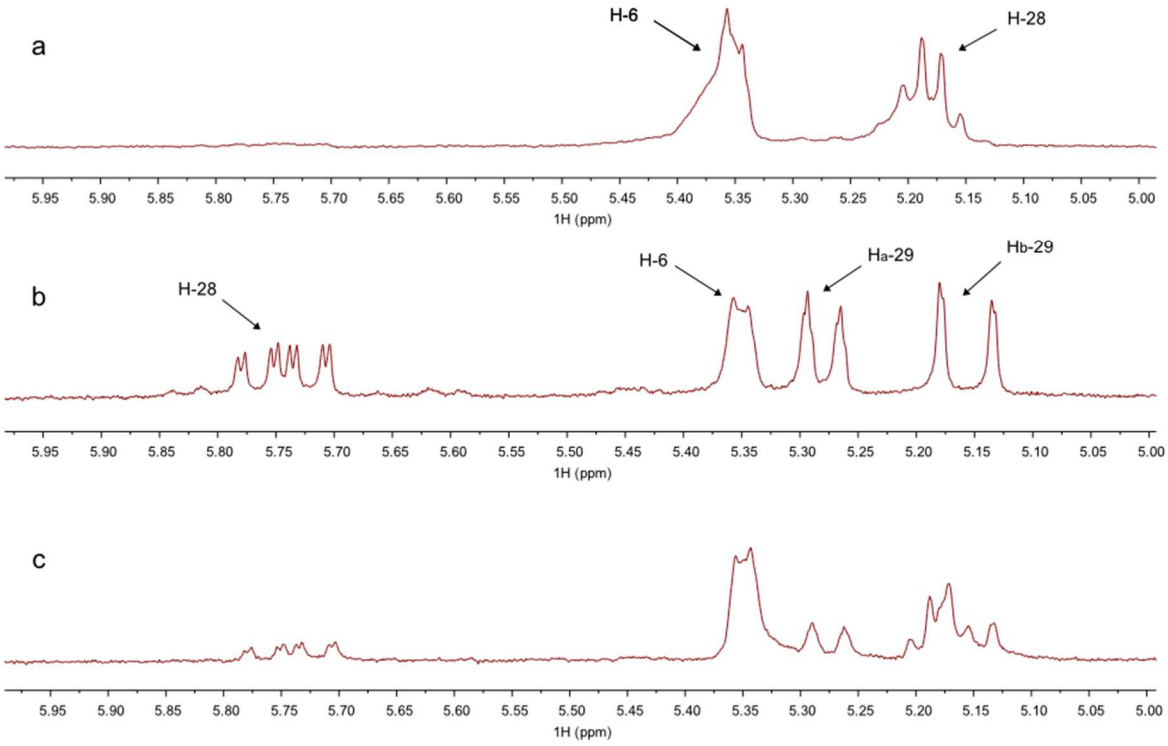


Figure 2 Details of ^{1}H NMR spectra of: a) fucosterol (**1**), b) mixture of saringosterol C-24 epimers (**2**-**3**), and c) naturally oxidized fucosterol.

Table 1 ^{13}C NMR data of compounds **2-3** ($CDCl_3$, 100 MHz)

N°	Compounds **2/3**δ_C[a]	Ayyad et al. (2003) δ_C	Huh et al. (2012) δ_C[b]
1	37.35	37.15	36.47
2	31.76	32.55	31.87
3	71.94	72.47	71.77
4	42.40[c]	37.91	31.87
5	140.86	141.41	140.71
6	121.87	122.38	121.67
7	32.00[d]	32.33	31.62
8	32.00[d]	36.86	36.17
9	50.19	50.76	50.09
10	36.62	37.91	37.22
11	21.19	21.73	21.04
12	39.84	40.40	39.72
13	42.45[c]	42.99	42.26
14	56.85	57.40	56.71
15	24.42	24.96	24.26
16	28.32[e]	29.04	28.44
17	55.75[f]	56.54	55.88
18	11.99	12.51	11.83
19	19.54	17.33	16.65
20	36.31[f]	36.51	35.91
21	19.02	18.37	17.67
22	28.53[e,f]	32.32	31.62
23	28.42[e,f]	28.92	28.25
24	89.26[f]	89.83	89.10
25	31.76[d]	29.16	29.67
26	17.84	20.05	19.36
27	16.74[f]	19.54	18.86
28	137.29[f]	137.84	137.17
29	116.58[f]	117.00	116.31

[a] Chemical shifts were referenced to the residual solvent peak (δ_C 77.16 ppm); [b] Data for 24*R*-saringosterol; [c,d,e] Signal assignments may be exchanged; [f] Duplicated signals.