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Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia*

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Abstract

A meroditerpenoid metabolite has been isolated from the brown alga *Cystoseira tamariscifolia* and characterized as methoxybifurcarenone, by spectral analysis. Methoxybifurcarenone possesses antifungal activity against three tomato pathogenic fungi: *Botrytis cinerea*, *Fusarium oxysporum* sp. *mycopersici* and *Verticillium alboatrum* and antibacterial activity against *Agrobacterium tumefaciens* and *Escherichia coli*.

Keywords: Cystoseira tamariscifolia; Brown alga; Antifungal; Antibacterial agent; Meroditerpenoid

1. Introduction

Several species of the brown algal genus *Cystoseira* (Cystoseiraceae, Pheophyta) are widespread in the Atlantic and Mediterranean sea, but very few of them have been reported to contain biologically active compounds (Faulkner, 1984; Francisco, Banaigs, Teste, & Cave, 1986; Amico, Cunsolo, Neri, Piatelli, & Ruberto, 1988; Valls, Piovetti, & Praud, 1993).

In the course of our continuing investigation of brown algae for the presence of antifungal and antibacterial metabolites, we observed that the crude ether extract of the brown alga *Cystoseira tamariscifolia* possesses antifungal activity against some tomato pathogenic fungi: *Botrytis cinerea*, *Fusarium oxysporum sp. mycopersici* and *Verticillium alboatrum*.

Since preliminary experiments indicated that most of the activity was associated with fractions containing diterpenoid derivatives, these were examined in detail and an antifungal compound methoxybifurcarenone was isolated.

2. Results and discussion

The MS spectrum of methoxybifurcarenone show a molecular ion at $m/z = 456$ compatible with a molecular formula $C_{28}H_{40}O_5$. The infrared spectrum of methoxybifurcarenone showed absorptions for hydroxyl ($\nu_{OH} = 3400$ cm^{-1}), α,β-unsaturated ketone ($\nu_{C=O} = 1680$ cm^{-1}, $\nu_{C=C} = 1620$ cm^{-1}) and benzenoid functionalities ($\nu_{C=C} = 1600$ cm^{-1}). UV absorptions at 214 and 289 nm ($\varepsilon = 10500$ and 2200) indicated a phenol moiety and an absorption at 224 nm ($\varepsilon = 11800$) characterized the α,β-unsaturated ketone. ^{1}H NMR spectra of methoxybifurcarenone in $CDCl_3$ at 500 MHz resembled that of bifurcarenone (Sun, Ferrara, McConnel, & Fenical, 1980), the main difference consisted of presence of a singlet (3H) at 3.72 ppm corresponding to methoxy function (Table 1).

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Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data of methoxybifurcarenone

Position No.	^1H NMR			^{13}C NMR
	δ (ppm)	multiplicity	J (Hz)	δ (ppm)
1	3.32	–	–	30.65
2	5.3	t	7.3	127.85
3				130.71
4	3.01	s	–	47.35
5				209.32
6	2.4	d	16	55.72
	2.3	d	16	
7	–	–	–	59.95
8	–	–	–	34.25
9	–	–	–	20.01
10	–	–	–	36.84
11				46.81
12				204.82
13	6.6	d	15.5	122.59
14	6.8	d	15.5	152.87
15				71.06
16	1.70	s	–	16.71
17	1.13	s	–	20.17
18	1.12	s	–	21.19
19	1.32	s	–	29.34
20	1.30	s	–	29.34
1′				146.58
2′				128.05
3′	6.55	d	2.6	113.03
4′				153.13
5′	6.48	d	2.6	114.06
6′				126.23
7′	2.25	s	–	16.54
OCH_3	3.72	s	–	55.61

The presence of OCH_3 in position 4′ was confirmed by NOE, COSY data and by MS spectrum:

Cosy: 6.48 (H-5′) correlated to 6.55 (H-3′).
NOE: irradiation of 3.72 enhances 6.55 and 6.48.
MS spectrum showed a peak at $m/z = 189$ corresponding to oxonium ion **A**.

2.1. Antifungal activity of the methoxybifurcarenone

The in vitro inhibition assays on solid media by incorporating the methoxybifurcarenone in the medium with mould isolates showed a zone of inhibition around the spot inoculation. This zone was measured after 2 weeks incubation on five different sides.

Results reported in Table 2 showed an inhibitory action on the mycelium development of the strains. This action is related to the concentration of the methoxybifurcarenone.

Differences in the action on the three mould isolates were observed. The activity of the three concentration 0.1, 0.5 and 1 mg/ml on the mycelium of *Botrytis cinerea* were significantly higher than those observed with the other strains. This assay may suggest that the methoxybifurcarenone had a similar antifungal activity on *Fusarium oxysporum f.* sp *lycopersici* and *Verticillium alboatrum*. All the concentrations tested had an effect on the mould growth and the inhibition zone diameters expressed in mm are observed with a concentration of 0.1 mg/ml for all the moulds. These diameters were more developed with higher concentrations. The most sensitive strains was *Botrytis cinerea* which showed higher zones than the other strains.

2.2. Antibacterial test

Methoxybifurcarenone was assayed twice at a concentration of 15 μg/disc. Antibacterial activity of methoxybifurcarenone was measured as radius of zone of inhibition around the disc (Table 3).

Table 3
Antibacterial activity of methoxybifurcarénone

Bacteria	Zone of inhibition (mm)
Agrobacterium tumefaciens	17
Escherichia coli	15

Table 2
Zone of inhibition (mm) after 15 days

	Amount of methoxybifurcarenone used	*Botrytis cinerea*	*Fusarium oxysporum f.* sp.*lycopersici*	*Verticillium albo atrum*
	1 μg	24	15	17
	5 μg	29	26	25
	10 μg	33	30	31
	15 μg	37	35	38
	20 μg	39	36	38
Control	solvent	0	0	0
	distilled water	0	0	0

In the same way, we have evaluated tetracycline activity against these bacteria. Inhibition zones are, 21 and 13 mm, respectively, against *Agrobacterium tumefaciens* and *Escherichia coli*.

Antibacterial activity of methoxybifurcarenone is comparable to tetracycline activity.

Antibacterial activity has been previously reported from extracts of some algae. Overall, algae extracts caused growth inhibition in gram-positive and gram-negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganisms (Faulkner, 1990).

3. Experimental

3.1. General experimental procedures

^1H and ^{13}C NMR spectra: 500 and 125 MHz, respectively, $CDCl_3$, TMS as int. stand. Carbon multiplicities were determined by a DEPT spectrum. Mass spectra were measured on a JEOL SX-102A mass spectrometer (low resolution MS: 70 eV). Final purification of methoxybifurcarenone was achieved by HPLC (Waters) on μm porasil. TLC was carried out using glass-packed precoated silica gel F_{254} plates (Merck). All solvents were spectral grade or distilled prior to use.

3.2. Collection

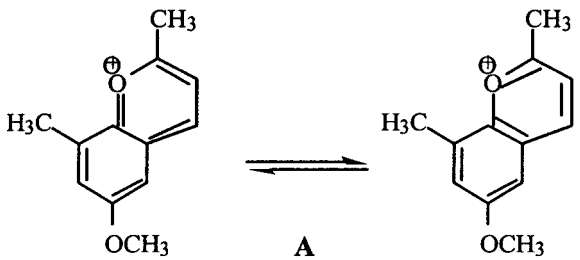
C. tamariscifolia was collected on rocks at about 3 m depth in November 1991 at Rose Marie, near Rabat, Morocco.

3.3. Extraction and purification

The alga was freeze-dried and subsequently extracted with $CHCl_3$/EtOH (5/5) at room temperature with continuous stirring. The combined extracts were concentrated by evaporation under reduced pressure, to yield the final crude extract as a dark green oil. The crude extract was then extracted successively with hexane, ether, dichloromethane and butanol. Ether extract (3.5 g) was chromatographed on silica gel (100–200 mesh, 150 g) with hexane containing increasing amounts of EtOAc. Fractions of 50 ml were collected and those exhibiting similar TLC profiles were combined. Fraction number 11 eluted with hexane/ethyl acetate 515 contained methoxybifurcarenone which was purified by HPLC on μ Porasil silica gel column with Isooctane/EtOAc (6/4). We obtained 82 mg of pure methoxybifurcarenone as a light yellow oil, 2.3% of Ether extract.

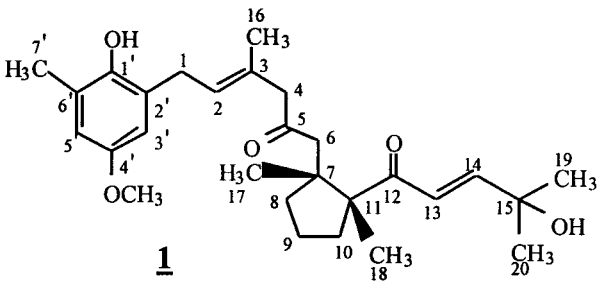
Methoxybifurcarenone **1**: UV λ_{max}^{EtOH} nm (log ε): 214 (4.02), 224 (4.07), 289 (3.34); IR ν_{max}^{film} cm^{-1}: 3400, 2800–3000, 1680, 1620, 1600.

EIMS 70 eV *m/z* (rel. Int.): 456 $[M^+]$ (2), 454 $[M-2]^+$ (3), 438 $[M-H_2O]^+$ (7), 420 $[M-36]^+$ (10), 251 $[M-205]^+$ (8), 233 $[M-223]^+$ (41), 215 $[M-241]^+$ (9), 205 $[M-251]^+$ (29), 191 $[M-265]^+$ (30), 189 $[M-267]^+$ (100), 175 $[M-281]^+$ (17), 163 $[M-293]^+$ (11), 150 $[M-306]^+$ (34), 137 $[M-319]^+$ (27), 123 $[M-333]^+$ (16), 113 $[M-343]^+$ (83), 95 $[M-361]^+$ (84), 81 $[M-375]^+$ (20), 69 $[M-387]^+$ (25), 55 $[M-401]^+$ (18).



^1H NMR (500 MHz, $CDCl_3$): 6.8 (1H, d, J = 15.5), 6.6 (1H, d, J = 15.5), 6.55 (1H, d, J = 2.6), 6.48 (1H, d, J = 2.6), 5.3 (1H, t, J = 7.3), 3.72 (3H, s), 3.32 (2H), 3.01 (2H, s), 2.4 (1H, d, J = 16), 2.3 (1H, d, J = 16), 2.25 (3H, s), 1.70 (3H, s), 1.32 (3H, s), 1.30 (3H, s), 1. 13 (3H, s), 1.12 (3H, s).

^{13}C NMR (125 MHz, $CDCl_3$): 209.32, 204.82, 153.13, 152.87, 146.58, 130.71, 128.05, 127.85, 126.23, 122.59, 114.06, 113.03, 71.06, 59.95, 55.72, 55.61, 47.35, 46.81, 36.84, 34.25, 30.65, 29.34, 29.34, 21.19, 20.17, 20.01, 16.71, 16.54.



3.3.1. Antifungal test

3.3.1.1. Mould species. The strain *Botrytis cinerea* was isolated from infected stalk attaked plants (tomato) harvested from the region of Casablanca (Morocco). *Fusarium* and *Verticillium* strains were isolated from soil collected around the roots of attaked plants. All the isolates were grown on PDA or on Czapeck-Dox broth with nitrate. Incubation was done at 25° in dark for 29 days.

3.3.1.2. In vitro inhibition assays. The in vitro assays to study the inhibition were carried out according to the method described by Benhamou (1992). The methoxybifurcarenone was incorporated into the culture medium in aliquots of 10 μl of solvent, incorporated in the culture media and poured in petri plates. Concentrations of 0.1, 0.5, 1, 1.5 and 2 mg/ml were used. The poured plates were allowed to solidify and spot inoculated with the mould strains to be tested. The inhibition was evaluated by the mecylium reduction around the spot inoculation.

3.3.2. Antibacterial test

Methoxybifurcarenone was tested against two terrestrial bacteria: *E. coli* and *A. tumefaciens*. Antibacterial assays were conducted using the standard disc-diffusion assay (McCaffrey & Erdean, 1985). Product was applied to 6 mm sterile discs in aliquots of 15 μl of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37° for 24 h. Zones of growth inhibition, if any, were measured following incubation.

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