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NOVEL MEROTERPENOIDS FROM *CYSTOSEIRA MEDITERRANEA*: USE OF THE CROWN-GALL BIOASSAY AS A PRIMARY SCREEN FOR LIPOPHILIC ANTINEOPLASTIC AGENTS

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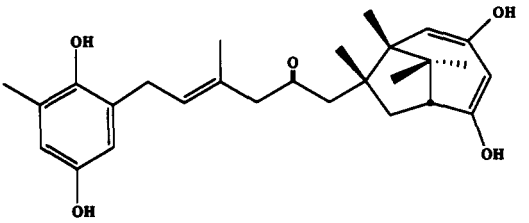
ABSTRACT.—Using a slight modification of the crown-gall potato disc bioassay, we were able to apply this test for two previously described antineoplastic lipophilic metabolites, didemnin B and mediterraneol A [**1**], and to use it as a guide for chromatographic separations of meroterpenoids from *Cystoseira mediterranea*. An active compound, mediterraneone [**3**], was isolated, and its structure was found to be a novel norsesquiterpenoid by chemical and spectral methods.

Several species of the genus *Cystoseira* (Cystoseiraceae, Pheophyta) are widespread in the Mediterranean Sea, but very few of them have been reported to contain biologically active compounds (1). Previous studies in our laboratory showed the Et_2O extract of *Cystoseira mediterranea* (J. Ag.) Sauv. (2) to possess antineoplastic activities attributable to mediterraneol A [**1**], one of its major components. In the present paper, we reinvestigated this alga using the crown-gall potato disc bioassay (3–5) to guide the chromatographic separations, as a high correlation between this test and the mouse P-388 leukemia protocol has been demonstrated (6). These experiments led to the isolation of two novel rearranged meroterpenoids (i.e., terpenoids of mixed biogenesis).



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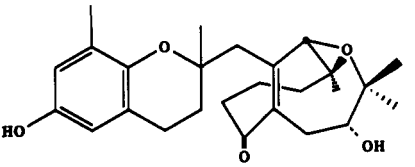

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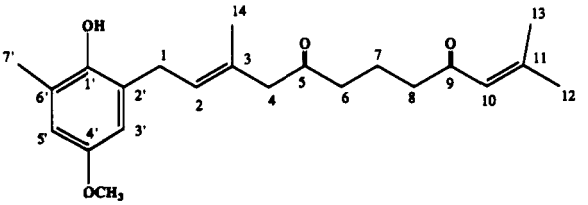

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Crown gall is a neoplastic disease of plants due to the transfer of part of a bacterial tumor-inducing plasmid to the plant genome. The Gram negative bacterium, *Agrobacterium tumefaciens*, strain B6, was used to induce tumors. We have adapted this procedure (see Experimental for details) to the hydrophobic compounds extracted from *C. mediterranea*. Didemnin B, a potent antitumor cyclic depsipeptide (7,8) and mediterraneol A [**1**], a lesser active product already described as possessing antineoplastic activities (9), were used as lipophilic references during development of our protocol. Neither compound shows antibacterial activities against *A. tumefaciens*; such inactivity is a prerequisite for this test.

RESULTS AND DISCUSSION

C. mediterranea was collected near Banyuls-sur-Mer (France) during June 1986. The alga was freeze-dried and subsequently extracted from $CHCl_3$-MeOH (1:1). Terpenoids were purified by standard Si gel chromatography of the crude extract. The various chromatographic fractions were screened on the crown-gall tumor growth assay on potato discs (3,4). Active fractions were further purified by hplc on μ Porasil (EtOAc/isooctane) and tested again. The controls and inactive fractions were found to develop crown gall tumors after 8–10 days. Active compounds (Table 1) needed at least 15–20 days to allow the counting of the tumors, obliging us to compare the results of cystoseirol [**2**] (10) and of novel unknown products with those obtained from mediterraneol A [**1**] (because, at that time, the counting from controls was very difficult due to the high number of tumors). It is obvious from Table 1 that all the compounds except didemnin B displayed the same level of activity at 0.01 mg/ml and would be worthy of testing in vivo in the P-388 leukemia test.

The novel terpenoid, mediterraneone [**3**], was isolated from this bioassay-directed fractionation as an oily substance. It had the molecular formula $C_{22}H_{30}O_4$ by eims and ^{13}C-nmr analysis; ir absorption established the presence of alcohol functionalities ($\nu_{OH} = 3400\ cm^{-1}$), an unstrained ketone ($\nu_{c=o} = 1705\ cm^{-1}$), a conjugated ketone ($\nu_{c=o} = 1675\ cm^{-1}$), and an aromatic ring ($\nu = 1605\ cm^{-1}$). In the uv spectrum, absorptions at 211 and 289 nm ($\epsilon = 11000$ and 2390) indicated a hydroquinone chromophore, and a conjugated ketone was observed to produce a band at 248 nm ($\epsilon = 12700$). The presence of important ions in the mass spectra at $C_{12}H_{15}O_2$ (7%), $C_{12}H_{13}O_2$ (28%), $C_9H_{11}O_2$ (12%), and $C_9H_{10}O_2$ (11%), in conjunction with ^{13}C-nmr and ^1H-nmr analysis, was in agreement with the structure proposed for the phenol moiety and the first isoprene unit with *E* olefin geometry, confidently proving that **3** is an acyclic nor-sesquiterpenoid. The final

TABLE 1. Activity of Test Compounds on the Crown-gall Potato Bioassay.

Compound tested[a]	Tumors (Mean number per disc)[b]	Inhibition (%)
Control	47 ±6	0
Cystoseirol [**2**]	12.6 ± 3.5	73
Mediterraneol A [**1**]	9.6 ± 2.2	88
EB432[c]	5.3 ± 2.2	88
Mediterraneone [**3**]	11 ±3	76
Didemnin B	0	100

[a]All compounds were tested at 10^{-2} M except didemnin B at 10^{-3} M.
[b]Five discs per plate and three plates were used per determination.
[c]An unresolved mixture of meroditerpenoids.

structural assignment for compound **3** was accomplished by extensive spin decoupling ^{1}H-nmr analysis in connection with ^{13}C-nmr spectra (Table 2). The unstrained ketone was positioned at C-5 (and not at C-7) after the reduction of **3** with $LiAlH_4$, giving a secondary alcohol ($\nu_{OH} = 3450$ cm^{-1}, $\delta = 4.05$ ppm) which shifted the C-4 methylene signal from 2.87 ppm (bs) to 2.20 ppm (bt, $J = 6.8$ Hz). Mediterraneone [**3**] is thus (2*E*,10*E*)-1-(1′-hydroxy-4′-methoxy-6′-methylphenyl)-5,9-dione-3,11-dimethyldodeca-2,10-diene.

TABLE 2. ^{1}H- and ^{13}C-nmr Data of Mediterraneone [**3**].

Position	δ ppm in C_6D_6	m	^{1}H nmr			^{13}C nmr		
			J (Hz)	δ ppm in $CDCl_3$	m	*J* (Hz)	δ ppm in $CDCl_3$	m
1	3.26	d	8.3	3.37	d	8.3	30.2	t
2	5.29	bt	8.3	5.39	bt	9.0	126.2	d
3							131.0	s
4	2.83	bs		3.10	bs		55.2	t
5							210.4	s
6	2.06	t	7.3	2.47	t	7.8	40.2	t
7	1.67	m		2.47	m		30.7	t
8	2.53	bt	8.3	2.56	bt	7.8	43.3	t
9							203.1	s
10	5.82	bs		6.09	bs		127.4	d
11							157.9	s
12	1.56	bs		1.77	s		21.6	q
13	1.63	s		1.87	s		27.2	q
14	1.66	s		2.13	s		16.8	q
1′							161.9	s
2′							122.7	s
3′	6.44	d	2.5	6.53	d	2.6	113.1	d
4′							157.5	s
5′	6.70	d	2.5	6.57	d	2.6	114.1	d
6′							123.6	s
7′	2.17	s		2.24	s		16.3	q
OMe	3.45	s		3.74	s		55.7	q

The rearranged meroterpenoids exhibited good activity against the crown-gall bioassay without antibacterial activities against *A. tumefaciens*. We are currently attempting to extend this test to other marine compounds (mainly to cyclic peptides), and we will try in the future to correlate these results with the in vivo P-388 leukemia protocol.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir and uv spectra were recorded on Perkin-Elmer Model spectrophotometers. ^{1}H-nmr and ^{13}C-nmr spectra (at 360 and 90.5 MHz, respectively) were recorded on a Bruker instrument. Eims was obtained on a ZAB HS (VG-analytical, Manchester) operating at a 70 eV accelerating voltage. All chemical shifts are reported relative to TMS (δ 0), and coupling constants are in Hz. Purifications of all metabolites and reaction products were achieved by hplc on preparative Si gel column using various proportions of EtOAc and isooctane.

EXTRACTION AND ISOLATION.—The alga (960 g dry wt) was collected in June 1986 at Banyuls-sur-Mer, France. The freeze-dried material was ground and extracted with $CHCl_3$-MeOH (1:1). After filtration and evaporation, the extract was partitioned between H_2O and Et_2O. The Et_2O-soluble material was dried over $MgSO_4$ and filtered, and the filtrate was evaporated to yield 14 g of a crude organic extract (62% inhibition). Compounds were eluted from a Si gel column with a mixture of hexane in Et_2O. The fraction eluted with hexane-Et_2O (70:30) (60% inhibition) yielded compound **3**, which was obtained in a pure form (4 mg) by hplc in EtOAc-isooctane (15:85). The other novel meroditerpenoids (15 mg, EB 432, Table 1) (80% inhibition) were eluted with hexane-Et_2O (60:40) and incompletely purified by hplc with EtOAc-isooctane (25:75).

Mediterraneone [**3**].—Oil (12 mg): uv (MeOH)

λ max nm (ε) 211 (11000), 248 (12700), 289 (2390); ir (film) ν max cm^{-1} 3400, 1705, 1675, 1605; eims *m/z* (%) 358 (7), 205 (5), 191 (7), 189 (28), 151 (12), 150 (11), 125 (9), 83 (4), 55 (5), 43 (100); ^1H and ^{13}C nmr see Table 2.

REDUCTION OF **3**.—A cold solution of **3** (3 mg) in dry Et_2O (1 ml) containing $LiAlH_4$ (9 mg) was stirred at 0° for 1.5 h. Excess reagent was destroyed by slow addition of EtOAc. Addition of a saturated $MgSO_4$ solution and extraction with Et_2O yielded 2 mg of an oil that was chromatographed on hplc (7% EtOAc in isooactane) to give 1 mg of the corresponding reduction product: ir (film) ν max cm^{-1} 3450, 1605; selected values ^1H nmr (360 MHz, $CDCl_3$) δ 5.20 (1H, d, $J = 8$ Hz, H-10), 4.40 (1H, m, H-9), 4.05 (1H, m, H-5), 2.20 (2H, bt, $J = 6.8$ Hz, H-4).

CROWN-GALL POTATO DISC BIOASSAY.—*A. tumefaciens*, strain B6, was obtained from the Institut Pasteur. The whole protocol was performed under a laminar flow hood as previously described (3,4,6). The bacteria were grown in 0.8% nutrient broth (Difco) supplemented with 0.5% sucrose and 0.1% yeast extract. This medium was solidified as required with 1.5% agar (Difco). Tubers from potato were surface-sterilized by immersion in Clorox for 20 min. A core of the tissue was then extracted from each tuber, and a 2-cm piece was removed from each end. The remainder of the cylinder was cut into 0.5-cm-thick discs, and the discs were transferred to 1.5% H_2O agar plates (5 discs per plate).

Several attempts were made to adapt the method to lipophilic substances. The best results were obtained by dissolving the samples with H_2O-MeOH (1:1) at concentrations of $0.25\ 10^{-4}$ M, except didemnin B, which was prepared at 10^{-5} and 10^{-7} M. This solution (5 μl) was spread over the surface of a disc, and the solvent was allowed to evaporate during 30 min. Then the discs were inoculated by spreading 0.1 ml of bacterial suspension (10^9 cells) over the surface. The plates were incubated at 27°. Controls were the discs not receiving the samples and with or without 5 μl of H_2O-MeOH (1:1). All the compounds that were active during these tests were examined for their ability to affect bacterial growth of *A. tumefaciens* by the standard agar plate-assay disc method and were found to be inactive.

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