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Antibiotic long-chain and α,β-unsaturated aldehydes from the culture of the marine fungus *Cladosporium* sp.

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Abstract

Long-chain and α,β-unsaturated aldehydes with antibiotic activity were isolated from the culture of the fungus *Cladosporium* sp., obtained from an intertidal marine sediment. The structures were determined by spectroscopical methods.
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1. Introduction

Marine fungi have proved to be a source of bioactive natural products (Schiehser et al., 1986; Fenical and Jensen, 1993; Kobayashi and Ishibashi, 1993; Davidson, 1995). Since these microorganisms grow in a unique and extreme habitat, they may have the capability to produce unique and unusual metabolites. Generally, the reason why they produce such metabolites is not known, but is believed that many of these metabolites may act as chemical defense as an adaptation of fungi competing for substrates (Fenical and Jensen, 1993).

In the course of our screening program for antibiotic fungal metabolites from terrestrial (Levy et al., 2000; Cabrera et al., 2002) or marine sources (Cabrera and

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Seldes, 1997), either of real marine origin or adapted to the marine environment, we investigated a microbial extract with antibiotic activity, obtained from the fungal culture of *Cladosporium* sp., isolated from a marine sediment. We report herein the isolation and identification of the antibiotic metabolites, most of them not described previously.

2. Materials and methods

2.1. General

GC analysis was performed on a Hewlett-Packard 5890 using N_2 as carrier gas. GCEIMS was carried out on a Hewlett-Packard 5890 gas chromatograph interfaced with a mass spectrometer (Trio-2 VG Masslab, Manchester, UK), using He as carrier gas. NMR spectra were recorded on a Bruker AC 200 in $CDCl_3$ with TMS as internal standard.

2.2. Fungal strain

The fungus *Cladosporium* sp. was isolated from an intertidal marine sediment sample collected at San Antonio Oeste, Province of Río Negro, Argentina and classified by Dr. J.E. Wright (PRHIDEB-CONICET, Departamento de Biodiversidad y Biología Experimental, FCEN, UBA). The strain was deposited in the BAFC Culture Collection, No. 486 (FCEN-UBA, CONICET).

2.3. Fermentation

Fermentation was carried out using a medium consisting of 1% peptone, 0.5% yeast extract, 1% dextrose and 100% artificial sea water (Instant Ocean). Erlenmeyer flasks (500 ml) containing 100 ml of medium were inoculated with agar slants of the strain. The flasks were incubated statically at 25 °C for 2 weeks.

2.4. Extraction and isolation

The culture was filtered and the mycelium was extracted with EtAcO, yielding ca. 500 mg of extract per liter of culture. This organic extract was vacuum-chromatographed on silica gel using mixtures of cyclohexane/CH_2Cl_2 of increasing polarity. The active fraction eluted with cyclohexane/CH_2Cl_2 1:1 was subjected to preparative TLC employing toluene as eluent, yielding fraction A ($R_f = 0.7$, 8 mg/l) and fraction B ($R_f = 0.85$, 8 mg/l). Fraction A was analyzed by GC (HP-5 column, 25 m, 100–280 °C, 10 °C/min) and GC–MS (SPB5 column, 30 m, 200–290 °C, 10 °C/min). Fraction B was analyzed by GC and fractionated by HPLC (C18, 5 μm, 250 × 10 mm column, MeOH–H_2O 93:7, 3 ml/min) yielding subfractions 1–8 (fr 1–4, <0.5 mg; fr 5, 1 mg; fr 6, 1 mg; fr 7, 2 mg; fr 8, 2 mg). GC–MS of the ozonolysis products: Ultra 2 column, 50 m, 50 (1 min) to 280 °C, 10 °C/min, He as carrier.

2.5. Antibiotic assay

The antibiotic activity was determined by the agar diffusion method (Vanden and Vlietinck, 1991; Ericsson and Sherris, 1971). *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 18804 were used as test organisms. Gentamicin was used as the positive test compound.

2.6. Spectroscopic data of α,β-unsaturated aldehydes

Compound **1** (Subfraction 1). EI MS (70 eV): 434 ($M^{+\bullet}$, 7), 265 (4), 251 (7), 237 (9), 223 (2), 111 (16), 109 (15), 95 (28), 43 (100).

Compounds **2–4** (Subfraction 2). EI MS (70 eV): 458 ($M^{+\bullet}$, 3), 289 (4), 279 (1), 275 (3), 265 (2), 261 (5), 247 (3), 233 (2), 223 (1), 109 (22), 95 (43), 43 (100).

Compounds **5**, **6** (Subfraction 3). EI MS (70 eV): 460 ($M^{+\bullet}$, 5), 291 (4), 277 (7), 265 (2), 263 (9), 251 (2), 249 (5), 237 (2), 109 (25), 95 (47), 43 (100).

Compounds **7**, **8** (Subfraction 4). EI MS (70 eV): 462 ($M^{+\bullet}$, 8), 293 (2), 279 (4), 265 (6), 251 (4), 237 (6), 223 (2), 109 (13), 95 (25), 43 (100).

Compound **9** (Subfraction 5). EI MS (70 eV): 482 ($M^{+\bullet}$, 9), 289 (3), 275 (3), 261 (5), 247 (5), 109 (24), 95 (52), 67 (100). ^{1}H NMR ($CDCl_3$): 9.36 (s, 1H), 6.43 (t, $J = 7.3$ Hz, 1H), 5.35 (m, 8H), 2.77 (br t, 4H), 2.35 (m), 2.23 (m), 2.05 (m), 1.27 (m), 0.89 (br t, 6H).

Compounds **10**, **11** (Subfraction 6). EI MS (70 eV): 484 ($M^{+\bullet}$, 2), 291 (1), 289 (1), 277 (1), 275 (2), 263 (2), 261 (2), 249 (2), 247 (2), 109 (12), 95 (25), 43 (100). ^{1}H NMR ($CDCl_3$): 9.36 (s, 1H), 6.43 (t, $J = 7.2$ Hz, 1H), 5.35 (m, 6H), 2.77 (br t, 2H), 2.35 (m), 2.23 (m), 2.05 (m), 1.27 (m), 0.88 (br t, 6H).

Compounds **12**, **13** (Subfraction 7). EI MS (70 eV): 484 ($M^{+\bullet}$, 6), 291 (1), 289 (5), 277 (2), 275 (4), 263 (4), 261 (7), 249 (3), 247 (6), 109 (26), 95 (51), 43 (100). ^{1}H NMR ($CDCl_3$): 9.36 (s, 1H), 6.43 (t, $J = 7.3$ Hz, 1H), 5.34 (m, 6H), 2.77 (br t, 2H), 2.35 (m), 2.23 (m), 2.05 (m), 1.27 (m), 0.88 (br t, 6H).

Compound **14** (Subfraction 8). EI MS (70 eV): 486 ($M^{+\bullet}$, 4), 291 (2), 277 (3), 263 (8), 249 (7), 109 (22), 95 (44), 43 (100). ^{1}H NMR ($CDCl_3$): 9.36 (s, 1H), 6.43 (t, $J = 7.3$ Hz, 1H), 5.34 (m, 4H), 2.35 (m), 2.23 (m), 2.04 (m), 1.27 (m), 0.88 (br t, 6H).

3. Results and discussion

From an EtAcO extract of the mycelia of the fungus *Cladosporium* sp. and by bioassay-guided fractionation, two fractions A and B, with antibiotic activity, were isolated. Minimum inhibitory content values for fraction A were 4.3 and 9.2 μg/disk against *B. subtilis* and *E. coli*, while for fraction B were 17.1 and 7.5 μg/disk, respectively. All the compounds were inactive against *C. albicans*.

Fraction A was a mixture of linear aldehydes as shown by ^{1}H NMR. In this spectrum, a triplet signal ($J = 1.8$ Hz) for an aldehydic proton at 9.77 ppm, olefinic protons at δ 5.35 (m), methylene protons between double bonds at δ 2.77 (br t, $J = 5.8$ Hz) and methylene protons adjacent to a carbonyl carbon at 2.42

ppm (dt, $J = 1.8$ and 6.9 Hz) besides hydrocarbon aliphatic chain protons were observed. This fraction was analyzed by GC and GC–MS and the results are summarized in Table 1. The identification of the constituents was based on their mass spectra.

Fraction B was also a mixture. The ^{1}H NMR spectrum showed an α,β-unsaturated aldehyde (α,β-USA) proton at 9.36 ppm, a conjugated olefin proton at δ 6.43 (t, $J = 7.3$ Hz) and typical proton signals of linear fatty-acid-like compounds. These data suggested the presence of 2-alkenyl aldehydes in the fraction (Suzuki et al., 1987; De Rosa et al., 1995). The *E* configuration of the conjugated system for many of the components was evident by the analysis of the NOESY spectrum, where an intense correlation between the aldehyde proton at δ 9.36 and the conjugated double bond proton at δ 6.43 was observed. The ^{13}C NMR spectrum confirmed this configuration for all the components, since carbon signals for the conjugated system appeared at 195.2, 155.1 and 143.8 ppm (Metzger and Casadevall, 1989) and no other minor carbon signals came out in this field region.

Fraction B, which yielded poorly resolved chromatograms at different conditions when analyzed by GC, was fractionated by HPLC leading to the isolation of eight microheterogeneous components. These components were analyzed by ^{1}H NMR and MS indicating the composition showed in Table 2. The identification of the compounds was based on the main fragmentations observed in the MS spectra, the allylic fragmentation to each side of the conjugated double bond (De Rosa et al., 1995), and the ^{1}H NMR assignments.

These isolated compounds could be derived from a head-to-head condensation of aldehydes, and as observed for dehydration of aldols, the most highly favored products were the *E* isomers (Metzger and Casadevall, 1989). In this study, the *Z* isomers were not observed, even at trace level. It is not surprising that the composition of the fatty aldehydes obtained in fraction A is quite similar to the precursor aldehydes of fraction B.

Ozonolysis of the mixture, followed by reductive cleavage of the ozonides (Gupta et al., 1982) and GC–MS of the resulting products established the double bond position of at least the main compound. The main reaction product, 1,1-dimethoxynonane, indicated the double bond was located on positions 8, 9 for the

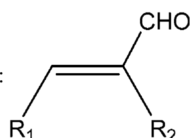
Table 1
Composition of fraction A, determined by GC and GC–MS

t_R	Aldehyde (C:*n*)[a]	Percentage[b]
6.12	15:0	9.2
6.99	15:2	3.2
7.80	17:2	38.5
7.88[c]	17:1	44.3
8.17	17:0	4.8

[a] Number of carbon atoms of fatty aldehydes:number of unsaturations.
[b] Percentage values (±0.1) were determined by GC, and are the average of three replications.
[c] This peak contains one minor isomeric compound overlapped with a main one, (8*Z*)-heptadecenal.

Table 2
Composition of fraction B

Subfraction	Compound	Molecular weight	R_1[a]	R_2[a]	Aldol condensation of $R_1CHO + R_2CH_2CHO$
1	**1**	434	$C_{14}H_{29}$	$C_{13}H_{27}$	C15 : 0 + C15 : 0
2	**2**	458	$C_{16}H_{29}$	$C_{13}H_{27}$	C17 : 2 + C15 : 0
	3		$C_{14}H_{29}$	$C_{15}H_{27}$	C15 : 0 + C17 : 2
	4		$C_{14}H_{25}$	$C_{15}H_{31}$	C15 : 2 + C17 : 0
3	**5**	460	$C_{16}H_{31}$	$C_{13}H_{27}$	C17 : 1 + C15 : 0
	6		$C_{14}H_{29}$	$C_{15}H_{29}$	C15 : 0 + C17 : 1
4	**7**	462	$C_{14}H_{29}$	$C_{15}H_{31}$	C15 : 0 + C17 : 0
	8		$C_{16}H_{33}$	$C_{13}H_{27}$	C17 : 0 + C15 : 0
5	**9**	482	$C_{16}H_{29}$	$C_{15}H_{27}$	C17 : 2 + C17 : 2
6	**10**	484	$C_{16}H_{31}$	$C_{15}H_{27}$	C17 : 1 + C17 : 2
	11		$C_{16}H_{29}$	$C_{15}H_{29}$	C17 : 2 + C17 : 1
7	**12**	484	$C_{16}H_{31}$	$C_{15}H_{27}$	C17 : 1 + C17 : 2
	13		$C_{16}H_{29}$	$C_{15}H_{29}$	C17 : 2 + C17 : 1
8	**14**	486	$C_{16}H_{31}$	$C_{15}H_{29}$	C17 : 1 + C17 : 1

[a] According to the structure: 

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principal C17:1 precursor aldehyde, (8*Z*)-heptadecenal. The presence of 1,1-dimethoxyhexane could be derived from (8*Z*,11*Z*)-heptadecenal, although the presence of other C17:1 isomer cannot be discarded.

The *Z* configuration of the chain double bonds was assumed based on the ^{1}H NMR data and the usual biosynthetic pathways.

Compounds **1**–**14** were very unstable and decomposed easily once isolated.

The long-chain aldehyde (LCA)-forming activity might occur widely in the plant kingdom, including marine brown, red and green algae and terrestrial plants (Kawasaki et al., 1998). LCA have been identified in cucumber (Kemp, 1975, 1977), several species of the Ulvaceae, as the characteristic volatile compounds of this genus (Kajiwara et al., 1987), the sea grass *Zostera marina* (Kawasaki et al., 1998) and the fresh water dinoflagellate *Peridinium gatunense* (Ginzburg et al., 1998).

The previously reported fatty aldehydes of C15 and C17 are pentadecanal (Kemp, 1975; Kajiwara et al., 1987), (8*Z*)-pentadecenal (Kemp, 1977), heptadecanal (Berdyshev et al., 1992) and (8*Z*)-heptadecenal, (8*Z*,11*Z*)-heptadecadienal and (8*Z*,11*Z*,14*Z*)-heptadecatrienal (Kawasaki et al., 1998; Kemp, 1975; Kajiwara et al., 1987). It was proposed that these compounds would result from the removal of a carbon from the carboxyl end of a fatty acid (Kemp, 1977). Most of the LCA obtained in this work were previously reported, although the presence of a minor C17:1 isomer and C15:2, not fully characterized was not described previously.

The presence of α,β-USA has been proved to occur naturally in many algae, although these type of compounds have been reported as artifacts in heated meat (Suyama et al., 1981). The mild conditions employed in this work during the work-up precluded this possibility.

Botryals, C_{52}–C_{64} α,β-USA, were isolated from the green alga *Botryococcus braunii* (Metzger and Casadevall, 1989), and a C_{28} and a C_{30} α,β-USA (compound **1**) were isolated from the red algae *Laurencia undulata*, *L. papillosa, L. spectabilis* (Suzuki et al., 1987) and *Corallina mediterranea* (De Rosa et al., 1995), but there is no report on their occurrence in fungi.

The α,β-USA accounted in this work, **2–14**, to the best of our knowledge, were not previously reported as natural products.

It is noteworthy that α,β-USA are typical products from marine algae and, the fungus cultured in this work was isolated from a marine sediment. The presence of producer microorganisms on the algae surface cannot be discarded, since many marine natural products are recognized today as produced by symbiotic microorganisms (Kobayashi and Ishibashi, 1993).

Regarding the antibiotic activity shown by the compounds, it is known that secondary metabolites possessing aldehyde groups and specially unsaturated aldehydes are bioactive, since they are potent electrofiles which can react with proteins (Fenical and Jensen, 1993).

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