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(+) Aeroplysinin-1: antibacterial and cytotoxic principle from the sponge *Pseudoceratina* (*Aiolocroia*) *crassa*

Moustapha E. Sesse Koker[1*]

Roger Adams Laboratory, University of Illinois, 505 South Mathews, Urbana, Illinois 61801. USA
[1]**Present Address:** Department of Chemistry, Fourah Bay College**,** University of Sierra Leone, Mount Aureol, Freetown. Sierra Leone.

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Abstract
Bioactivity- guided fractionation of methylene chloride, ethyl acetate fractions of the sponge *Pseudoceratina (Aiolocroia) crassa* has led to the isolation and characterisation of (+)-aeroplysinin-1 as the bioactive principle. The structure of the compound was determined by spectroscopic methods. (+)-Aeroplysinin-1 showed broad spectrum activity against a number of aerobic bacteria, Gram-positive and Gram-negative anaerobes, L1210, P 388 cells, weakly cytotoxic against KB cells in addition to the reported antibacterial activity.

Keywords: Bioactivity-guided isolation; (+) Aeroplysinin-1, Antibacterial; Cytotoxic

Introduction

Marine sponges have been found to be a rich source of bioactive compounds (Rinehart *et al*., 1981). Numerous studies of marine organisms have led to the isolation and characterization of compounds which have structures or structural units derivable from bromotyrosine.

The brominated dienone, 1, has been isolated from *Verongia cauliformis* and *Verongia fistularis* (Sharma *et al.*, 1967). The structure was determined by spectroscopic means and by conversion to a known compound. These workers also isolated the ketal, 2 (Sharma *et al.*, 1970). (-)-Aeroplysinin-1, 3, has been isolated from the sponge *Ianthella ardis* (Fulmore *et al.*, 1970). The (+)-isomer of this compound, 4 (Fattorusso *et al.*, 1970) has been obtained from an organism known as *Aplysina (Verongia) aerophoba* but has now been re-identified as *Verongia cavernicola* (Cimino *et al*., 1983). A closer examination of these two species yielded aeroplysinin-2, 5, a brominated γ-lactone (Minale *et al*., 1972). Both (+)-aeroplysinin-1 and (-)-aeroplysinin-1 were found to possess antibacterial activity.

In the quest for biologically useful compounds from marine organisms, a study of the sponge *Pseudoceratina (Aiolocroia) crassa* was undertaken to isolate and characterize biologically active principles from it. Isolation was by bioassay-guided isolation schemes, followed by evaluation of the biological activities of the pure isolated compounds.

* Corresponding author. *E-mail address*: mesessekoker@yahoo.co.uk *Tel:* +232- 76693810.
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Experimental

General. Melting points were determined on a Thomas-Hoover apparatus or Kofler hot stage, are uncorrected, and are given in degrees centigrade. Optical rotations were measured on an Autopol III automatic polarimeter. Infrared (IR) spectra were recorded on a Beckman IR 12 spectrophotometer, either in solution, in Nujol, in a potassium bromide pellet or neat. Ultraviolet (UV) spectra were obtained using a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer.

Proton magnetic resonance (^{1}H NMR) spectra were determined on Varian EM 390, Varian HR-220 and Nicolet NTC 360 spectrometers using tetramethylsilane (TMS) as an internal standard. Carbon magnetic resonance (^{13}C NMR) spectra were recorded on JEOL FX-60, Varian XLFT-100 and Nicolet NTC 360 instruments at 15 MHz, 25.2 MHz and 90.6 MHz respectively, using various deuterated solvents.

Low resolution electron impact (EI) spectra were determined on a Finnigan MAT CH-5 spectrometer. Field desorption (FD) and High Resolution Electron Impact (HREI) mass spectra were determined on a Finnigan MAT 731 spectrometer.

High pressure liquid chromatography (HPLC) was performed on Waters Associate instruments equipped with a variable wavelength detector and an Altex Ultra Sphere ODS column (25 cm in length, 1 cm internal diameter, 5µ mesh); or an Altex instrument equipped with a 254-nm filter and a Dupont Instruments column (25 cm in length, 8 cm internal diameter 5µ mesh).

Thin layer chromatography, TLC, was carried out on Brinkmann Polygram SIL G/UV_{253} (0.25 mm or 2 mm) and on Whatman $KC_{18}F$ reversed phase (200µ) TLC plates. Compounds were detected under a UV lamp or by the use of the appropriate spray reagent.

Column chromatography was carried out with silica gel (Brinkmann, 50-200µ), Sephadex LH-20 (Pharmacia, 25-100µ), with a stationary phase to compound ratio of at least 100:1. Glass wool was placed in the bottom of the column just above the stopcock and the column was partially filled with the appropriate solvent. A slurry of the stationary phase was allowed to settle while the column was tapped gently to ensure even packing as the solvent flowed out.

Antimicrobial, antiviral, and cytotoxicity assays were carried out using a 6.5mm filter disk-agar diffusion method (Schroeder *et al*., 1981). Zone of inhibition was measured in millimeters.

Bioautography: A TLC plate of a crude sample or relatively pure fraction was run in an appropriate solvent system. After allowing solvent to evaporate at room temperature, UV-active spots were marked under the UV lamp and the plate placed face down on microorganism-seeded agar in a Petri dish. The spots were also marked on the Petri dish and the Petri dish was placed in a refrigerator (4ºC) for an hour (so that all components on the TLC plate were absorbed onto the agar), after which the TLC plate was removed from the Petri dish and the latter was transferred to an incubator (35ºC) for 12-24 hr. A zone of inhibition was observed around any spot that had an antimicrobial component.

Sample Collection. Sponge samples were collected by SCUBA techniques during the *Alpha Helix* Caribbean Expedition in 1978 (AHCE 1978) at Turneffe Island, Belize (17º 10.8’ N latitude, 87º 55.3’W longitude, AHCE 6-III-78-4-7, #324) at -7 to -8 m, at Gallows Point Reef, Belize (17º 25.9’N latitude).

Extraction of sponge and isolation of bioactive compounds. Two grams (wet weight) of *Pseudoceratina crassa* was chopped into small pieces and placed in a Waring blender, 5 mL of 1:3 toluene:

methanol was added, and the sponge was then homogenized for 2 min. The extract was filtered and the solution obtained, which contained some suspended material, was centrifuged. Solvent was removed under reduced pressure from the clear solution to yield 30 mg an orange product. Biological activity of this sample was determined against the following organisms: *B subtilis, E. coli, S. cerevsiae* and *P. atrovenatum*. It was moderately active against *B. subtilis* and *E. coli*.

Ethanol-stored sponge (100g) was cut into small pieces and placed in Waring blender with 100 ml of its supernatant and 100 mL of methanol-toluene (3:1). The mixture was filtered and the residue obtained was extracted repeatedly with 100 ml portions of methanol-toluene (3:1) until the filtrate was colourless. The combined filtrate was diluted with 1 litre of 1 M sodium nitrate to give toluene and aqueous layers. The aqueous layer was extracted successively (9 x 150 mL per solvent) with methylene chloride, ethyl acetate and 1-butanol. Solvent was removed at reduced pressure to yield toluene (0.62 g), methylene chloride (0.49 g) and ethyl acetate (0.51 g) fractions, with a negligible amount of material in l-butanol layer.

Material from the methylene chloride fraction (0.260 g) was dissolved in a minimum amount of ethyl acetate-hexane (1:1) and applied to a silica gel column (500 g, 13.5 x 80 cm) prepared with the same solvent system. This was eluted with 50 % (1.2 L), 65 % (1.0 L), 80 % (1.0 L), 90 % (1.0 L) ethyl acetate in hexane, then ethyl acetate, and 20 – 200 mL fractions were collected. Fraction no. 17 was found to contain most of the biologically-active compound (80 mg). Purification by preparative TLC using ethyl acetate-hexane (2:3) was followed by recrystallisation from acetate-hexane to give bioactive compound (30 mg). This was purified further by reversed-phase HPLC using water-methanol (2:1) with UV detection at 215 nm.

Silica gel column chromatography (500 g, 13.5 x 85 cm) of material from the ethyl acetate fraction (0.42 g) was carried out in the same manner as for the methylene chloride fraction. The resulting fraction which contained mainly the biologically active compound (45 mg) was purified as above.

Ethanol-stored sponge (750 g) was extracted with methanol-toluene (4 L, 3:1) and diluted with 1 L of 1 M sodium nitrate. The aqueous layer was extracted successively (10 x 150 mL per solvent) with methylene chloride, ethyl acetate, and 1-butanol to give methylene chloride (6.5 g) and ethyl acetate (4.65 g) fractions with negligible amount of material in the 1-butanol layer. Silica gel chromatography of the methylene chloride and ethyl acetate fractions using gradients of methanol in chloroform (8 %, 15 %, 20 %, 25 % methanol) gave bioactive-containing fractions. Sephadex LH-20 chromatography of these fractions using methanol-chloroform (3:7) followed by reversed-phase HPLC using water-methanol (2:1) with UV detection at 215 nm gave bioactive compound from the methylene chloride layer and 25 mg from the ethyl acetate layer).,

Results

This sponge was collected by SCUBA techniques at Turneife Island and Gallows Point, Belize, Central America. Crude extracts of this organism was active against *B subtilis* (Zone of inhibition, 15mm), *E. coli* (Zone of inhibition 14mm). Cytotoxicity runs gave a value [13 (100)].

Isolation of the bioactive compound

Two isolation procedures were used to isolate the biologically active components. Extraction of *P. crassa* with methanol-toluene (3:1) gave an extract from which 100μg (1mg/ mL) gave 14.5-mm zone of inhibition against

B. subtilis. Partitioning of the extract with 1M sodium nitrate gave a toluene and an aqueous layer. The aqueous layer was extracted sequentially with methylene chloride, ethyl acetate, and 1-butanol. The toluene layer was inactive against *B. subtilis*. The methylene chloride extract (100µg, 1 mg/mL) showed a zone of inhibition of 29mm against *B. subtilis* and a zone of inhibition of 14 mm against *E. coli*. The ethyl acetate layer exhibited a zone of inhibition of 22 mm against *B. subtilis*.

Following bioautography, silica gel chromatography of the methylene chloride extract was carried out in a gradient of ethyl acetate in hexane, which gave fractions that were active against *B. subtilis*. The bioactive fraction obtained was subjected to prep TLC in ethyl acetate-hexane (2:3) followed by recrystallisation from acetone-hexane to give a pale yellow product. This was purified further by reversed-phase HPLC using water-methanol (2:1). (Scheme 1)

The ethyl acetate extract was worked up using the same procedure as above to give the bioactive component. It turned out the bioactive component obtained from the methylene chloride and the ethyl acetate extracts were the same. The same product was obtained by silica gel chromatography of the methylene chloride and ethyl acetate fractions, employing gradients of methanol in chloroform, followed by Sephadex LH-20 chromatography and reversed-phase HPLC of the product obtained from the water-methanol (2:1) effluent. (Scheme 2)

Properties of the bioactive compound

The bioactive compound had mp 128-128.5°;

$[\alpha]_D^{25}$ + 178.2° (*c* 1.57 in MeOH);

UV λ_{max} (MeOH) 284 nm, (ε .3867), 207 nm (ε 2325);

IR (neat) 3370, 2910, 2230, 1610, 1570 cm^{-1};

^1H NMR (CD_3CN) δ 6.20 (s, 1H, H-5), 4.10 (br, d, 2H, which on shaking with D_2O gave a peak at δ 4.05, (s, 3-OH, H-3), 3.7 (s, 3H, OCH_3), 2.77 (s, 2H, H-7), 2.27 (br s, 1H, 4-OH),

^{13}C NMR (CD_3OD – $CDCl_3$) (1:1) δ 148.3 (C-1), 132.7 (C-2) 121.1 (C-6), 117.9 (C-8), 113.9 (C-5), 78.0 (C-4), 74.1 (C-3), 60.1 (OCH_3) and 26.5 (C-7);

FDMS $^m/_z$ 337, 339, 341, (M^+); EIMS (70 eV) $^m/_z$ 337 (12), 319 (15), 304 (14), 240 (60), 226 (21), 218 (62), 189 (45), 173 (43), 161 (32), 83 (74), 28 (100).

Identification of bioactive compound

This compound gave mp 128-128.5^0, $[\alpha]_D^{25}$ +178.2^0 (c 1.57 in methanol) and UV maxima at 283.6 nm (ε 3900) and 207 nm (ε 2300). The IR spectrum showed peaks at 3370 cm^{-1}, 2910 cm^{-1} and 2230 cm^{-1}. The FD mass spectrum showed a molecular cluster at $^m/_z$ 337/379/341 with an intensity ratio of 1:2:1. The EI mass spectrum showed a molecular ion peak at $^m/_z$ 337, a peak at $^m/_z$ 319 and a peak at $^m/_z$ 161. The ^1H NMR spectrum showed a peak at δ 6.30, broad peak at δ 4.10 which collapsed to a singlet at δ 4.05 on shaking with deuterium oxide, a broad proton singlet at δ 2.27 which disappeared on shaking with deuterium oxide, a singlet at δ 3.70 and another singlet at δ 2.77.

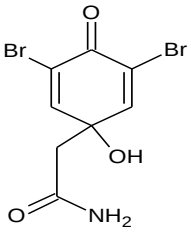
The ^{13}C NMR spectrum showed nine singlets between δ 150 and δ 25: δ 148.3, 132.7, 121.1, 117.9, 113.9, 78.0, 74.1, 60.1 and 26.5

Biological activity

This compound was found to be active at 100µg/disc against *B. subtilis* (zone of inhibition, 27mm) and *Escherichia coli* (15mm).

(+) Aeroplysinin-1 was found to be active against *B subtilis, E. coli* was found to have broad spectrum activity against the aerobic bacteria tested, was weakly active against gram-positive and gram-negative anaerobes. It was also active against L1210 leukaemia cells, had marginal activity against P388 leukaemia cells and was weakly cytotoxic against KB cells.

STRUCTURES



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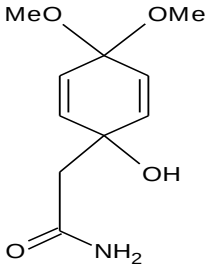

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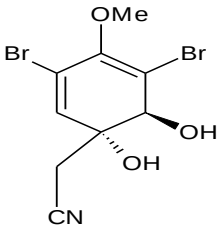

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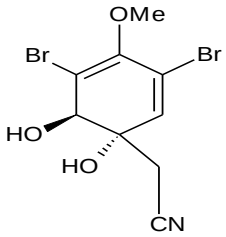

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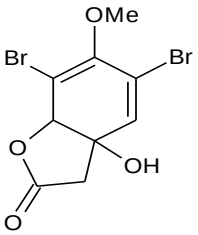

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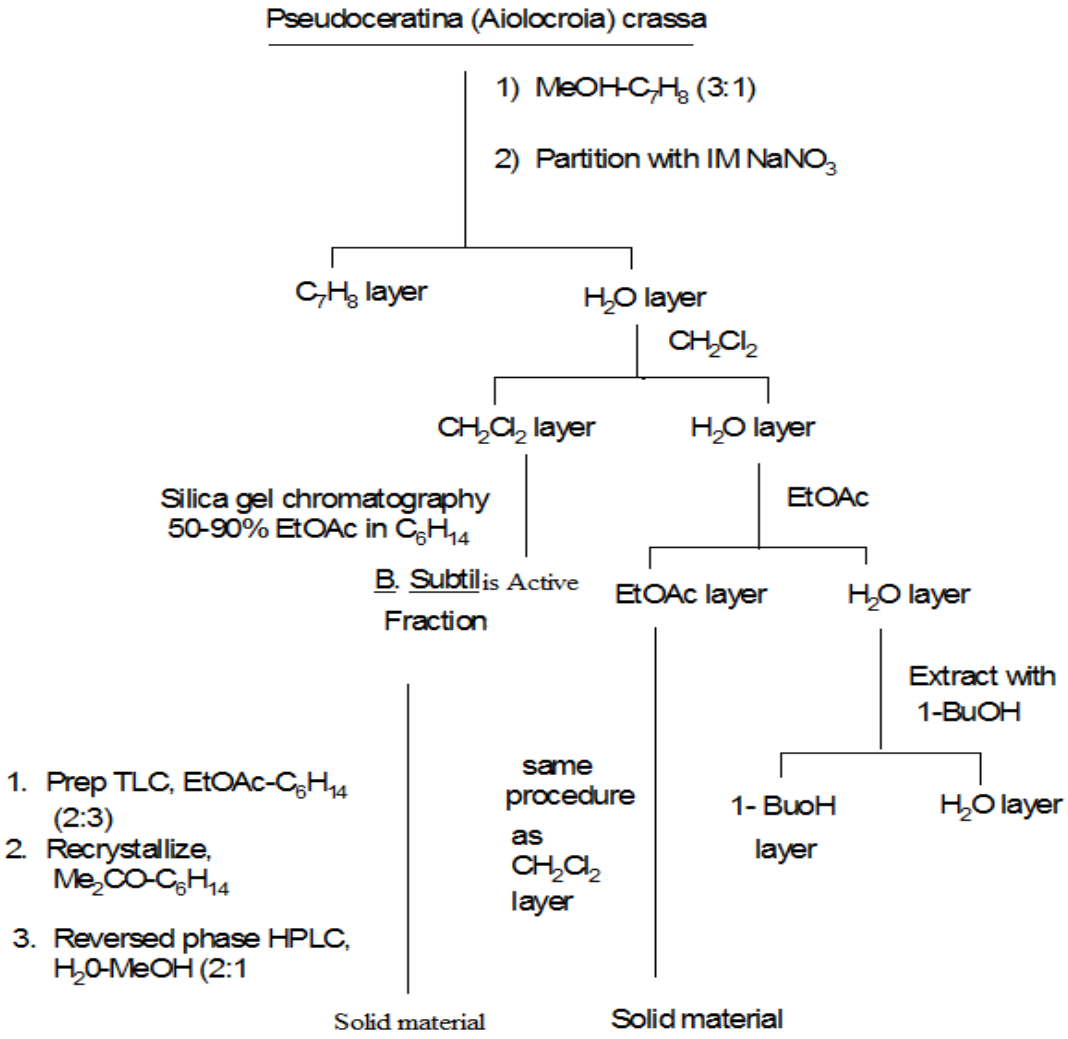
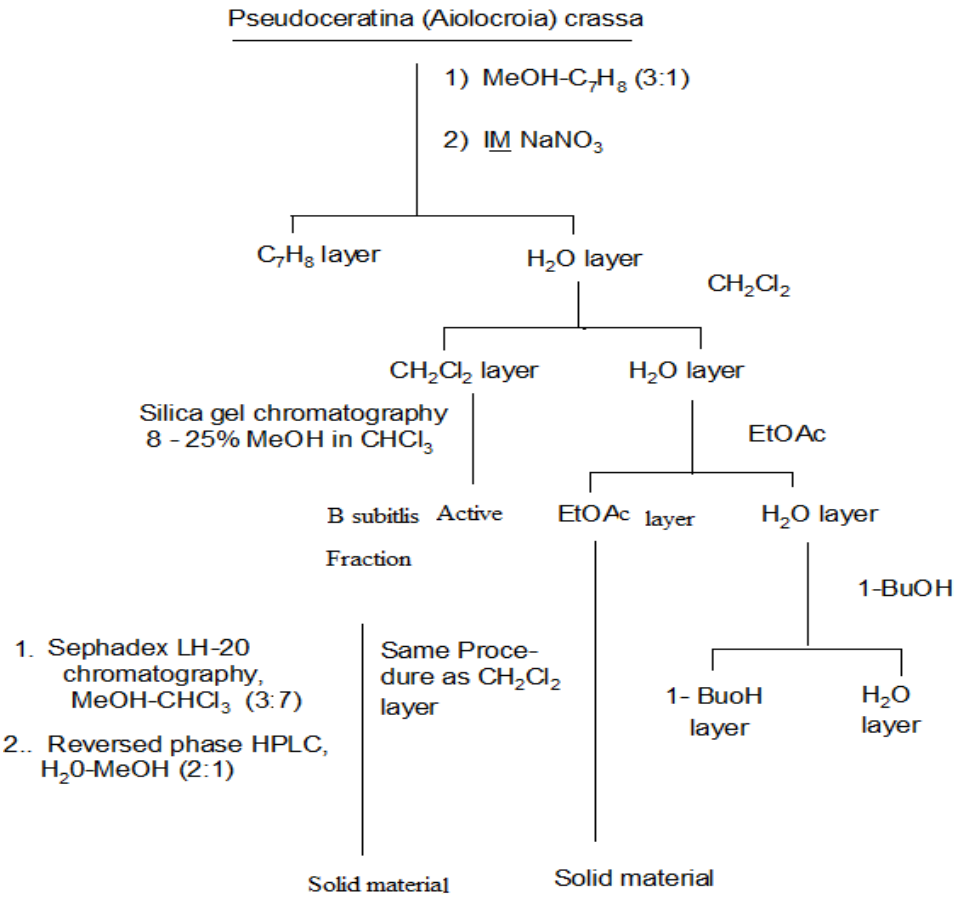


Scheme I



Scheme 2

Table 1. Biological activities for (+)-aeroplysinin-1

Microorganism	Minimum Inhibitory Concentration (µg/ml)
Staphylococcus aureus	125
Streptococcus pyogene	125
E. coli	125
Salmonella schottmuelleri	125
Klebsiella pneumonia	62
Streptococcus pneumonia	250
Streptococcus faecalis	500
Pseudomonas aeruginosa	500
Propionobacterium acnes	16
Bacteroides melaninogenius	31
Fusobacterium necrophorum	31
Eubacterium lentum	31
Bacteroides fragilis *(Cultures # 6513, 6428)*	62
Veillonella alcalescens	62
Clostridium perfringens	62
Clostridium sporogenes	62
Clostridium difficile	62

Discussion

Crude extracts of this sponge was marginally active against B. subtilis, *E. coli* and was cytotoxic. Bioassay-guided isolation of the bioactive principle from the methylene chloride and ethyl acetate fractions were found to be the same; as a light yellow solid.

The IR showed peaks at 3370cm^{-1} due to a hydroxyl, 2910cm^{-1} due to a cyano group. The FD mass spectrum showed a molecular cluster at m/z 337/339/341 with an intensity ratio of 1:2:1 indicating the compound contains two bromine atoms. The E.I mass spectrum showed a molecular ion peak at m/z 337 ($C_9H_9Br_2NO_3$), a peak at m/z 161 (M-[H_2O-2Br]). The proton NMR showed a peak at δ 6.30 due to an olefinic proton, a two – proton broad peak at δ 4.10 which collapsed to a singlet at δ 4.05 on shaking with D_2O due to a C<u>H</u>-O<u>H</u> group, a broad hydroxy proton singlet at 2.27 which disappeared on shaking with D_2O; a methoxy singlet at δ 3.70 and a two-proton singlet at 2.77. The ^{13}C NMR showed nine signals between δ 150 and 50 which have been assigned as follows (Levy *et al*., 1980): 148.3 (C-1), 132.7 (C-2), 121.1 (C-6), 117.9 (C-8 cyano group), 113.9 (C-5), 78 (C-4), 74.1 (C-3), 60.1 (C-9 methoxy group) and 26.5 (C-7).

The above data are very similar to values reported earlier (Fattorusso *et al*., 1970) for (+)-aeroplysinin-1. Hence, this compound is (+)-aeroplysinin-1.

Acknowledgements

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