Zarzissine, a New Cytotoxic Guanidine Alkaloid from the Mediterranean Sponge Anchinoe paupertas

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ZARZISSINE, A NEW CYTOTOXIC GUANIDINE ALKALOID FROM THE MEDITERRANEAN SPONGE *ANCHINOE PAUPERTAS*

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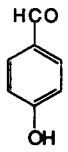
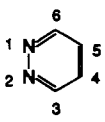
ABSTRACT.—From a CH_2Cl_2 extract of the Mediterranean sponge *Anchinoe paupertas* were isolated and characterized zarzissine [**2**], a new 4,5-guanidino-pyridazine compound and the known *p*-hydroxybenzaldehyde [**1**]. The structure of zarzissine [**2**] was elucidated by spectroscopic methods, including the application of a number of 2D nmr techniques. Biological activities of compounds **1** and **2** were also determined, with zarzissine exhibiting cytotoxicity against three human and murine tumor cell lines.

A wide variety of nitrogen-containing products have been isolated from marine organisms, many of which possess purine or pyrimidine skeletons (1–5). Among the nitrogen-containing compounds, those which contain a guanidine unit are particularly interesting and are often unique to marine organisms, as pointed out by Baker and Murphy (6). A large number of these compounds have potent biological activities (7). In our continuing studies on biologically active products from marine organisms (8–11), we have now identified from the Mediterranean sponge *Anchinoe pauperta* Topsent (Bowerbank) (Anchinoidae) a new 4,5-guanidino-pyridazine compound that was named zarzissine [**2**] and the known *p*-hydroxybenzaldehyde [**1**]. The antimicrobial activity of compounds **1** and **2** has been examined against the Gram-positive bacterium *Staphylococcus aureus* and two yeasts, *Candida albicans* and *C. tropicalis*. The cytotoxic activity of compound **2** has been examined against three strains of mammalian carcinoma and leukemia cells, namely, human nasopharyngeal carcinoma cells (KB), human lung carcinoma cells (NSCLC-N6), and murine leukemia cells (P-388).



1



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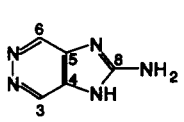

2

Compound **1** (0.0008% wet wt) was purified from the CH_2Cl_2 extract of *A. paupertas*. The molecular formula $C_7H_6O_2$ was established by hreims (observed *m/z* 122.03643, required *m/z* 122.03678). Referring to literature data, the spectral properties of **1** (ms, ir, ^{1}H-nmr, ^{13}C-nmr; see Experimental) led to the identification of *p*-hydroxybenzaldehyde [**1**]. This molecule is symmetrical in the ^{1}H-nmr but not in the ^{13}C-nmr spectrum, suggesting that the aldehyde group is not in free rotation but has the carbonyl group co-planar to the aromatic ring. *p*-Hydroxybenzaldehyde, previously isolated from a marine bacteria pseudomonad (12) and a *Chromobacter* sp. (13), is widely distributed in plants in very small amounts and is obtained as a by product from the Reimer-Tiemann reaction for salicylaldehyde from phenol (14).

Zarzissine [**2**] (0.0003% wet wt) was purified as a colorless powder, sublimation point 270–271°. The molecular formula $C_5H_5N_5$ was established by hreims (observed *m/z* 135.05500, required *m/z* 135.05449). The molecular formula indicated that **2** was an isomer of the purine base adenine, but its ^{1}H- and ^{13}C-nmr

data suggested that this new compound was not characterized by a purine skeleton. The ^{13}C-nmr spectrum of **2** exhibited only three carbon signal resonances (Table 1) which were assigned by a DEPT spectrum as one methine carbon and two quaternary carbon signals. These data suggested that the molecule was symmetrical. The ^{1}H-nmr spectrum of **2** showed the presence of a signal at 8.09 ppm (2H, br s) correlated (one-bond HETCOR spectrum) to the carbon signal at 152.4 ppm and two signals at 7.11 (2H, br s) and 12.80 ppm (1H, br s) which disappeared on addition of D_2O. The olefinic resonance at 8.09 ppm (H-3, H-6) showed a long-range correlation (long-range HETCOR spectrum) with the quaternary carbon signal at 139.4 ppm (C-4, C-5). The downfield signals at 152.4 and 139.4 ppm corresponded, respectively, to the two methine carbons (C-3, C-6) and to the two quaternary carbons (C-4, C-5) requiring that each of these carbon atoms must have one nitrogen attached to it. These data suggested the presence of a pyridazine ring ($C_4H_2N_2$) [**A**] bisubstituted in the positions C-4 and C-5. The downfield signal at 155.2 ppm (C-8), along with a positive reaction to Sakaguchi reagent, suggested that compound **2** contains a guanidino group. All these data led us to propose structure **2** for zarzissine.

Compounds **1** and **2** showed a slight antimicrobial activity against the Gram-positive bacterium *S. aureus* and the two yeasts *C. albicans* and *C. tropicalis*. Inhibition diameters (100 μg, purified product) were 8, 7, and 7 mm for **1** and 12, 10, and 11 mm for **2**. The cytotoxic activity of zarzissine [**2**], expressed by IC_{50} values, was determined in three tumor cell lines, with IC_{50} values of 12 μg/ml for murine leukemia cells (P-388), 5 μg/ml for human nasopharyngeal carcinoma cells (KB), and 10 μg/ml for human lung carcinoma cells (NSCLC-N6), respectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded at 200 MHz for ^{1}H and 50 MHz for ^{13}C on a Bruker AC-200 spectrometer and a Bruker AM-400 spectrometer for the long-range HETCOR spectrum. All chemical shifts are reported as ppm with respect to TMS (δ=0). The ir spectra (KBr) were recorded on a Nicolet 20SxC spectrophotometer. The mass spectra were determined on a Fisons MM305 spectrometer. The uv spectra were obtained on a Perkin-Elmer Lambda 19 uv/vis spectrophotometer. Melting and sublimation points were measured on an Electrothermal 900. The hplc system used in this work was a Varian model pump Vista 5000 with injector and CDS 401 control station, the chromatograph was monitored by a uv detector (Pye Unicam PU 4021 diode array) and by a refractive index detector (Varian RI-3).

ANIMAL MATERIAL.—The sponge *Anchinoe paupertas* was collected by scuba diving (−15 m) at Zarzis, Tunisia in September 1992. It was identified by Dr. Jean Vacelet and Dr. Nicole Bourry-Esnault, Station Marine d'Endoume, rue de la Batterie des Lions, 13007 Marseille, France. The two compounds described below were also identified from an *A. paupertas* specimen collected near Marseille, France in November 1993. The voucher specimen (Z1) was deposited at Station Marine d'Endoume, Marseille, France.

EXTRACTION AND ISOLATION.—The specimens of the fresh sponge (4.2 kg wet wt) were extracted in EtOH (15 liters). The EtOH extract was washed with CH_2Cl_2 (2×8 liters). The CH_2Cl_2 extract was taken to dryness, re-dissolved in MeOH-CH_2Cl_2 (1:1) and was subjected to sequential fractionation on a Sephadex LH-20 column (MeOH-CH_2Cl_2, 1:1), vlc on diol gel (CH_2Cl_2-MeOH, 10:0 to 5:5). The fraction eluted with CH_2Cl_2-MeOH (8:2) was further chromatographed using reversed-phase prep. hplc (300×25 mm, RP-18, 12–40 μm, C.E.D.I., MeOH-H_2O, 8:2). Complete purification of the active fraction was achieved by hplc (250×10 mm, Lichrosorb diol gel 7 μm, CH_2Cl_2-MeOH, 98:2) which gave compound **1** (34 mg, 0.0008% wet wt), and by additional hplc (100×8 mm, Radial Pak, Waters NH_2 gel 10 μm, CH_2Cl_2-MeOH, 9:1) which gave compound **2** (12.5 mg, 0.0003% wet wt).

ANTIBIOTIC ASSAY PROTOCOL.—The purified products were tested using the paper disk agar diffusion method against the Gram-positive bacterium *Staphylococcus aureus* and yeasts *Candida albicans* and *Candida tropicalis*, as test microorganisms, with Muller-Hinton medium (Pasteur Institute Production) for the bacterial species and Casitone (Pasteur Institute Production) for yeasts (15,16). The inhibition diameter around the paper disk (6-mm diameter) was measured after 18 h incubation at 37°.

EVALUATION OF CYTOTOXIC ACTIVITY.—The

cytotoxicity of zarzissine [**2**] was tested against three cloned cancer cells. KB cells were maintained in a suspension culture of BME medium supplemented with 5% calf serum containing 1% of glutamine at 200 mM and 1% of antibiotic solution (streptomycin: 10000 ui/ml). NSCLC-N6 (17) and P-388 cells were maintained in a suspension culture of RPMI-1640 supplemented with 5% calf serum containing 1% of a penicillin-streptomycin mixture (10000 ui/ml). A 50-μl aliquot of each cell culture (NSCLC-N6 cells 1×10^4, KB cells 1×10^3, P388 cells 5×10^3) was mixed with a 50-μl aliquot of serial dilution of compounds and the mixture was incubated in a microtiter well plate (96-well, Falcon) for 72 h at 37° in a humidified incubator containing 5% CO_2 in air. Cell proliferation was estimated by a colorimetric test (18): 10μl of MTT [3-(4,5 dimethylthiazool-2-yl)-2,5-diphenyltetrazolium bromide] was added. After 4 h the dark blue crystals, formed in mitochondria of living cells during the reduction of MTT, were solubilized with 100 μl of isopropanoic acid. Microplates were read by Elisa using a multiscan MK2 with a 570-nm filter. The optical density of the wells then enabled a dose/effect curve to be plotted and the IC_{50}, the cell growth inhibition for each product relative to the controls, to be determined.

p-*Hydroxybenzaldehyde* [**1**].—Yield 0.0008% wet wt, was obtained as a colorless powder: mp 115–116°; uv (MeOH) λ max (log ε) 216 (4.76), 282 (4.63) nm; ir (KBr) ν max 3212, 3074, 3045, 3028, 2886, 2752, 1667, 1651, 1594, 1517, 1453, 1283, 1219, 1164, 839, 698, 603, 508 cm^{-1}; ^1H nmr (CD_3OD, 200 MHz) δ 6.91 (d, J=8 Hz, H-2, -6), 7.78 (d, J=8 Hz, H-3, -5), 9.76 (s, H-7); ^{13}C nmr (CD_3OD, 50 MHz) δ 192.8 (d, C-7), 165.2 (s, C-4), 133.4 (d, C-2), 130.4 (d, C-6), 130.2 (s, C-1), 116.9 (d, C-3), 115.9 (d, C-5); hreims *m/z* 122.03643 (required for $C_7H_6O_2$, 122.03678); eims *m/z* $[M]^+$ 122 (96), 121 (100), 93 (28), 53 (12), 50 (6), 44 (8).

Zarzissine [**2**].—Yield 0.0003% wet wt, was obtained as a colorless powder: sublimation point 270–271°; uv (MeOH) λ max (log ε) 206 (4.61), 259 (3.91) nm; ir (KBr) ν max 3418, 3117, 3012, 1650, 1602, 1417, 1307, 1246, 1122, 1024, 910, 797, 721, 644, 540 cm^{-1}; ^1H nmr (DMSO-d_6, 400 MHz) δ 7.11 (2H, br s, NH_2), 8.09 (2H, br s, H-3, -6), 12.80 (1H, br s, NH); ^{13}C nmr (DMSO-d_6, 100 MHz) δ 139.4 (s, C-4, -5), 152.4 (d, C-3, -6), 155.2 (s, C-8); hreims *m/z* 135.05500 (required for $C_5H_5N_5$, 135.05449); eims *m/z* $[M]^+$ 135 (48), 114 (8), 108 (20), 81 (22), 69 (9), 54 (22), 53 (40), 52 (18), 45 (25), 44 (100), 43 (48), 41 (36), 40 (16).

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LITERATURE CITED

1. R. Kazlauskas, P.T. Murphy, J. Wells, J.A. Bairt-Lambert, and D.D. Jamieson, *Aust. J. Chem.*, **36**, 165 (1983).
2. Y.H. Kim, R.J. Nachman, L. Pavelka, H.S. Mosher, F.A. Fuhrman, and G.J. Fuhrman, *J. Nat. Prod.*, **44**, 206 (1981).
3. G. Cimino, A. Crispino, S. De Stefano, M. Gavagnin, and G. Sodano, *Experientia*, **42**, 1301 (1986).
4. G. Cimino, A. De Giulio, S. De Rosa, S. De Stefano, R. Puliti, C.A. Mattia, and L. Mazzarella, *J. Nat. Prod.*, **48**, 523 (1985).
5. S. De Rosa, S. De Stefano, R. Puliti, C.A. Mattia, and L. Mazzarella, *J. Nat. Prod.*, **50**, 876 (1987).
6. J.T. Baker and V. Murphy, "Handbook of Marine Science: Compounds from Marine Organisms." CRC Press, Cleveland, OH, 1976, Vol. I, p. 50.
7. L. Chevolot, in: "Marine National Products." Ed. by P.J. Scheuer, Academic Press, New York, 1981, Vol. 4, p. 53.
8. R.G.S. Berlinck, J.C. Braekman, D. Daloze, I. Bruno, R. Riccio, D. Rogeau, and P. Amade, *J. Nat. Prod.*, **55**, 528 (1992).
9. N. Bouaïcha, C. Tringali, D. Pesando, M. Malléa, C. Roussakis, and J.F. Verbist, *Planta Med.*, **59**, 256 (1993).
10. N. Bouaïcha, D. Pesando, D. Puel, and C. Tringali, *J. Nat. Prod.*, **56**, 1747 (1993).
11. P. Amade, M. Mallea, and N. Bouaicha, *J. Antibiot.*, **47**, 201 (1994).
12. S.J. Wratten, M.S. Wolfe, R.J. Andersen, and D.J. Faulkner, *Antimicrob. Agents Chemother.*, **11**, 411 (1977).
13. R.J. Andersen, M.S. Wolfe, and D.J. Faulkner, *Mar. Biol.*, **27**, 281 (1974).
14. L.F. Fieser, and M. Fieser, "Organic Chemistry." Reinhold, New York, 1956, 3rd Ed., p. 681.
15. P. Regli, H. Ferrari, M. Goudard, and Y. Buffard, *Bull. Soc. Franç. Myc. Méd.*, **11**, 359 (1982).
16. P. Courvalin, F. Goldstein, A. Philippon, and J. Sirot, Eds., "L'antibiogramme," MPC Paris, Bruxelles, 1985, p. 22.
17. C. Roussakis, C. Cratas, F. Audoin, I. Leboterff, G. Dabouis, M.J. Andre, E. Moyon, N.H. Vo, G. Pradal, and J.F. Verbist, *Anticancer Res.*, **11**, 2239 (1991).
18. T. Mosmann, *J. Immunol. Method*, **65**, 55 (1983).

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