Aulosirazole, a Novel Solid Tumor Selective Cytotoxin from the Blue-Green Alga *Aulosira fertilissima*

Klemens Stratmann, Jack Belli, Craig M. Jensen, Richard E. Moore,* and Gregory M. L. Patterson

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822

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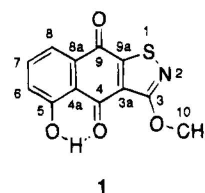
Aulosirazole, the major cytotoxin in the blue-green alga (cyanobacterium) *Aulosira fertilissima* Ghose (UH strain DO-8-1), shows solid tumor selective activity in the Corbett assay. Its structure has been determined to be 5-hydroxy-3-methoxynaphtho[2,3-*d*]-1,2-thiazole-4,9-dione by a combination of spectroscopic, notably NMR, and X-ray crystallographic methods. The carbon skeleton and the juglone nature of **1** was determined by two-dimensional $^{1}H-^{13}C$ NMR correlation spectral analysis and one-dimensional $^{13}C-^{13}C$ decoupling experiments, the latter on the ^{13}C-enriched cytotoxin. The presence of an isothiazole ring and the position of attachment for the juglone system was rigorously established by an X-ray crystallographic study.

Toward the goal of discovering new efficacious drugs for the treatment of solid tumors in the clinic, Corbett[1,2] has developed a rapid and relatively inexpensive disk-diffusion assay to find agents that exhibit greater cytotoxicity against solid tumor cells than leukemia cells (solid tumor selectivity) and/or greater cytotoxicity against tumor cells than normal cells (tumor selectivity). The assay is modeled after the disk diffusion assay that is commonly used in antifungal and antibacterial screens. In screening 1988 extracts of blue-green algae (cyanobacteria) in the Corbett assay, 16 of the extracts (0.8%) showed consistent solid tumor selectivity. Each of these extracts produced a zone of inhibition for one or more of the solid tumor cell lines (e.g. murine colon C38; murine pancreatic P03; human lung H125) that was at least 250 zone units (7.5 mm) larger than the zone of inhibition for the leukemia cell (L1210 or P388). The crude extract of *Aulosira fertilissima* Ghose (UH strain DO-8-1) displayed a differential of 280 zone units for $Z_{C38} - Z_{L1210}$. Using a bioassay-guided fractionation procedure, a novel yellow pigment, aulosirazole, was isolated which accounted for the solid tumor selective activity ($Z_{P03} - Z_{P388} = 250$ and $Z_{H125} - Z_{P388} = 360$).[3] We describe here the structure elucidation of aulosirazole (**1**, 5-hydroxy-3-methoxynaphtho[2,3-*d*]-1,2-thiazole-4,9-dione),[4] an example of a naturally-occurring isothiazole.[5]



1

Aulosira fertilissima Ghose (UH strain DO-8-1) was isolated from a soil sample near Moon Beach, Okinawa, in 1986 and mass cultured as previously described.[6] The lipophilic extract (1:1 CH_2Cl_2/2-propanol) of *A. fertilissima* was fractionated by size exclusion chromatography (LH20, MeOH) and the activity monitored by KB (a human nasopharyngeal carcinoma) cytotoxicity. Most of the cytotoxicity was found in a fraction which eluted as a slow-moving yellow band and consisted of 95% pure aulosirazole (**1**). Aulosirazole, which crystallized from chloroform, exhibited IC_{50} values against KB and LoVo (a human colorectal adenocarcinoma cell line) of 350 and 45 ng/mL, respectively.

The EIMS of **1** displayed a very intense molecular ion peak at *m/z* 261 and a high resolution measurement established its molecular composition as $C_{12}H_7NO_4S$. The formula was consistent with the ^1H- and ^{13}C-NMR data (Table 1). A refocused INEPT experiment established that the ^{13}C spectrum was comprised of 1 methyl, 3 methine, and 8 nonprotonated carbon signals, indicating that **1** contained 12 carbons and 3 hydrogens attached to carbon. The ^1H NMR spectrum, however, revealed the presence of a signal (12.4 ppm) which did not ^1J-correlate with any of the carbon signals in a HMQC experiment. Ausosirazole therefore possessed one exchangeable hydrogen. The four oxygens in the molecule appeared to be located in two carbonyl groups (δ_C 176.6, 183.3), a methoxyl (δ_H 4.25), and a strongly H-bonded hydroxyl (δ_H 12.4).

The ^1H and ^{13}C chemical shifts and $^1H-^1H$ coupling constants (Table 1) were consistent with a methoxy group connected to a multicyclic, aromatic heterocycle possess-

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(1) Corbett, T. H.; Valeriote, F. A.; Polin, L.; Panchapor, C.; Pugh, S.; White, K.; Lowichik, N.; Knight, J.; Bissery, M-C.; Wozniak, A.; LoRusso, P.; Biernat, L.; Polin, D.; Knight, L.; Biggar, S.; Looney, D.; Demchik, L.; Jones, J.; Jones, L.; Blair, S.; Palmer, K.; Essenmacher, S.; Lisow, L.; Mattes, K. C.; Cavanaugh, P. F.; Rake, J. B.; Baker, L. In *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development*; Valeriote, F. A., Corbett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Norwell, 1992; pp 35–87.

(2) Valeriote, F. A.; Moore, R. E.; Patterson, G. M. L.; Paul, V. J.; Scheuer, P. J.; Corbett, T. In *Discovery and Development of Anticancer Agents*; Valeriote, F. A., Corbett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Norwell, 1994; pp 1–25.

(3) The results of its evaluation *in vivo* against solid tumors implanted in mice will be published elsewhere.

(4) Naphthoquinones possessing *in vivo* antitumor activity are known [Wagner, H.; Kreher, B.; Lotter, H.; Hamburger, M. O.; Cordell, G. A. *Helv. Chim. Acta* **1989**, *72*, 659–67].

(5) For other examples of naturally-occurring aromatic heterocyclic compounds possessing a N–S bond, see brassilexin, an indolo[3,2-*d*]-1,2-thiazole [Devys, M.; Barbier, M.; Loiselet, I.; Rouxel, T.; Sarniguet, A.; Kollmann, A.; Bousquet, J.-L. *Tetrahedron Lett.* **1988**, *29*, 6447–8]; dendrodoine, a 1,2,4-thiadiazole [Heitz, S.; Durgeat, M.; Guyot, M.; Brassy, C.; Bachet, B. *Tetrahedron Lett.* **1980**, *21*, 1457–8]; neamphine, an imidazo[4,5-*e*]-1,2-thiazine [de Silva, E. D.; Racok, J. S.; Andersen, R. J.; Allen, T. M.; Brinen, L. S.; Clardy, J. *Tetrahedron Lett.* **1991**, *32*, 2707–10].

(6) Patterson, G. M. L.; Baldwin, C. L.; Bolis, C. M.; Caplan, F. R.; Karuso, H.; Larsen, L. K.; Levine, I. A.; Moore, R. E.; Nelson, C. S.; Tschappat, K. D.; Tuang, G. D.; Furusawa, E.; Furusawa, S.; Norton, T. R.; Raybourne, R. B. *J. Phycol.* **1991**, *27*, 530–6.

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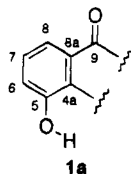
Table 1. ^1H- and ^{13}C-NMR Data for Aulosirazole in $CDCl_3$

position	^1H	^{13}C	^1H-HMBC
3		167.7 (s)	10
3a		122.1 (s)	
4		183.3 (s)	
4a		115.9 (s)	6,8
5		163.1 (s)	7
6	7.35 (d, 8.3)	126.3 (d)	8
7	7.65 (dd)	136.2 (d)	
8	7.80 (d, 7.5)	120.4 (d)	6
8a		133.0 (s)	7
9		176.6 (s)	8
9a		166.4 (s)	
10	4.25 (s)	57.4 (q)	
OH	12.40 (s)		

ing a benzenoid ring. The benzenoid ring had to be 1,2,3-trisubstituted since 1H doublets at 7.35 and 7.80 ppm and an unresolved 1H doublet of doublets at 7.65 ppm with ortho coupling constants of 7.5 and 8.3 Hz could be seen in the ^1H NMR spectrum. HMQC and HMBC experiments led to rigorous assignments of the three methine and four nonprotonated carbon signals to the benzenoid ring and an attached carbonyl group as depicted in **1a**. The lowfield position of the carbon signal



1a

at 163.1 ppm suggested that the hydroxy substituent was attached to the benzenoid group as also shown in **1a**. The UV spectrum further suggested that **1a** was part of a juglone system. The protons of the methoxy group only showed one HMBC cross peak, viz. a three-bond coupling to the carbon signal at 167.7 ppm; however, it was not possible to determine the position of this methoxyl-bearing carbon with respect to the proposed juglone system and the nitrogen and sulfur atoms.

Aulosirazole was therefore enriched with ^{13}C by cultivating *A. fertilissima* on $^{13}CO_2$ as the sole carbon source. The >60% ^{13}C-enriched **1** that was obtained was then analyzed by one-dimensional $^{13}C-^{13}C$ decoupling studies. When the C-4a signal was irradiated, the unresolved doublet of doublets at 183.3 ppm (C-4) collapsed to a doublet. Similarly when the C-9 signal was irradiated, the unresolved doublet of doublets at 166.4 ppm (C-9a) collapsed to a doublet. Both the C-4 and C-9a signals were coupled to an unresolved doublet of doublets of doublets (1:3:3:1 quartet) at 122.1 ppm (C-3a), thus confirming the presence of the juglone system and rigorously establishing the carbon assignments. The C-3a signal was also coupled to the doublet signal at 167.7 ppm, which meant that the methoxyl-bearing carbon (C-3) was connected to the juglone. Since the C-9a signal was an unresolved doublet of doublets (1:2:1 triplet), either the sulfur or the nitrogen that remained had to be attached to this carbon. This meant that **1** was an isothiazole, not a thiazole. The chemical shifts of the various carbon atoms, however, did not allow an unequivocal assignment of the nitrogen and sulfur positions within the isothiazole, nor did the coupling between C-3 and C-3a (72.6 Hz), which was significantly larger than the other $^1J_{CC}$ couplings (55.4–66.4 Hz). Rather than establish the position of the nitrogen in **1** unequivocally

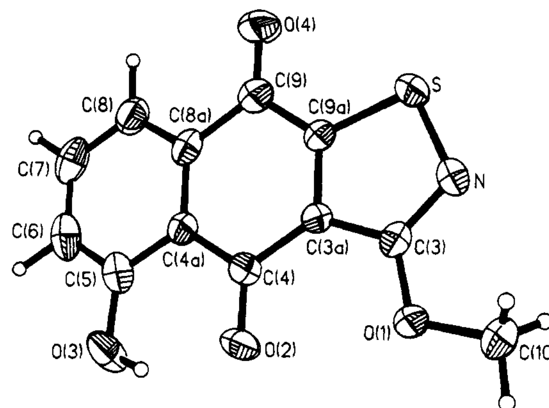


Figure 1. X-ray crystallographic structure of aulosirazole (**1**).

by a ^{15}N-labeling experiment, the alkaloid, which had crystallized from chloroform in the meantime, was subjected to single crystal X-ray diffraction. A computer-generated perspective drawing of the X-ray structure of **1**, which rigorously shows that the sulfur and nitrogen are located at positions 1 and 2, respectively, is depicted in Figure 1.

Experimental Section

Spectral Analysis. NMR spectra were determined on a 11.75-T instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C. ^1H chemical shifts are referenced in $CDCl_3$ to residual $CHCl_3$ (7.24 ppm); ^{13}C chemical shifts are referenced to the solvent ($CDCl_3$, 77.0 ppm). One bond heteronuclear $^1H-^{13}C$ connectivities were determined by HMQC; two and three bond $^1H-^{13}C$ connectivities were determined by HMBC using a mixing time to detect 7 Hz coupling. Homonuclear ^{13}C connectivities were determined by 1D decoupling experiments. Mass spectra, including high resolution mass measurements, were determined in the EI mode. UV spectra were measured in MeOH at 20 °C.

Isolation and Cultivation of Alga. An edaphic form of *Aulosira fertilissima* Ghose, designated UH strain DO-8–1, was isolated from a soil sample collected near the Moon Beach Hotel on Okinawa (Japan) (26° 26′ 39″ N, 127° 48′ 13″ E) and purified by repeated subculture on solidified media. The cyanophyte was cultured in autoclaved 20-L glass carboys or unsterilized 160-L fiberglass tanks. The vessels contained an inorganic medium (modified BG-11) adjusted to pH 7.0. The cultures were continuously illuminated at an incident intensity of 100 μmol photons m^{-2} s^{-1} (photosynthetically active radiation, PAR) from banks of cool-white fluorescent tubes and vigorously aerated at a rate of 5 L min^{-1} with a mixture of 1% CO_2 in air at a temperature of 24 ± 1 °C as previously described.[6] After incubation, the alga was harvested by filtration. The yield of lyophilized cells from 20-L carboys incubated for 25–29 days ranged from 0.24 to 0.29 g/L. The yield of 160-L tanks, following 36 days incubation, was 0.33 g/L.

Uniform ^{13}C Enrichment of Aulosirazole.[7] *A. fertilissima* was grown in a 6-L airlift bioreactor (LH Fermentation) containing 5 L of modified BG-11 medium from which the natural abundance Na_2CO_3 had been omitted. Following inoculation with the cell mass (without medium) from a single 500 mL culture of alga, the culture was incubated at 24 ± 0.1 °C, illuminated continuously at an incident intensity of 80 μmol of photons m^{-2} s^{-1} (PAR) for 14 days. The bioreactor was initially configured to sparge with 0.3 L min^{-1} air (without added CO_2). After 14 days, the bioreactor was reconfigured

(7) Aulosirazole could not be detected in an experiment where $NaHCO_3$ was employed as the carbon source, using a previously described general procedure [Moore, R. E.; Bornemann, V.; Niemczura, W. P.; Gregson, J. M.; Chen, J.-L.; Norton, T. R.; Patterson, G. M. L.; Helms, G. L. *J. Am. Chem. Soc.* **1989**, *111*, 6128–32].

to recirculate the sparging gas stream by means of a bellows-type metering pump attached to a 20-L glass vessel acting as a gas reservoir. The sparging gas within the system was supplemented with $^{13}CO_2$ (99%), which was added by a pH controller. Once each day, the system was purged with air. After an additional 14 days incubation, the culture was harvested by filtration and lyophilized. The cells (3.5 g) were extracted and processed to give 1 mg (0.03%) of ^{13}C-enriched **1**: ^{13}C NMR δ (mult,[8] $^{1}J_{C,C}$ in hertz) δ 183.4 (t, 60.4/55.4), 176.7 (t, 56.3/56.3), 167.8 (d, 72.6), 166.3 (t, 56.3/56.3), 163.1 (t, 66.4/62.0), 136.2 (t, 57.8/55.8), 133.0 (q, 61.4/60.0/56.3), 126.3 (t, 66.4/57.8), 122.1 (q, 72.6/60,4/63.1), 120.4 (t, 60.0/55.8), 115.9 (q, 62.0/61.4/55.4). The degree of enrichment was determined to be >60% by integration of the ^{1}H-NMR methoxy signals.

Isolation of Aulosirazole. Freeze-dried alga (40.2 g) was extracted twice with 2 L portions of 1:1 CH_2Cl_2/2-propanol overnight with stirring. The extracts were combined and evaporated under reduced pressure to give a green solid (1.9 g) which was dissolved in 20 mL of MeOH. The filtered solution was applied to a column of Sephadex LH20-120 (Fluka, 70 cm × 4.5 cm, flow rate 50 mL/h) equilibrated in MeOH. After approximately 400 mL of MeOH had passed through the column, green material began to be eluted. Fractions were collected between 400 to 1500 mL based on TLC (C-18, MeOH) analysis and assayed for KB cytotoxicity. Most of the cytotoxicity was found in the 1360−1460 mL fraction which eluted from the column as an intense yellow band. NMR and TLC analyses indicated that the fraction was essentially a single component, aulosirazole (**1**), having a purity of >95% (R_f 0.57, ODS, MeOH). To remove the small amount of green pigment, the aulosirazole was further chromatographed on an ODS column (500 mg) with 80% MeOH to give 7 mg (0.018% yield) of **1** as deep yellow crystals.

Aulosirazole (1): EIMS m/z (rel intensity) 261 (M^+, 100), 233 (23), 204 (30), 120 (20); HREIMS m/z 261.0094 ($C_{12}H_7NO_4S$, Δ0.2 mmu); UV (MeOH) λ_{max} nm (ϵ) 216 (20 500), 248 (11 200), 272 (8500), 424 (6300), sh 346 (2430);[9] unchanged in acid, but peaks shift to 226, 280, 312, and 536 nm on addition of dilute aqueous NaOH. ^{1}H and ^{13}C NMR: see Table 1.

(8) Geminal ($^{2}J_{CC}$) couplings can be clearly seen in the C-3a, C-4a, C-8a, C-9a signals ($^{2}J_{3a,4a}$ = 12.6 and $^{2}J_{8a,9a}$ = 15.8 Hz). The J-values are comparable in size to those between carbons attached to carbonyls in simple ketones [Kalinowski, H-O.; Berger, S.; Braun, S. In *Carbon-13 NMR Spectroscopy*; Wiley: Chichester, 1991; pp 549−67].

(9) Juglone: UV (MeOH) λ_{max} nm (ϵ) 249 (13 200), 262 (10 000), 339 (1500), 425 (4000), 495 (600). [Scott, A. I. In *Interpretation of the Ultraviolet Spectra of Natural Products*; Pergamon Press: Oxford, 1964; p 124].

Crystallographic Studies. Orange rhombohedral crystals of **1**, suitable for X-ray diffraction, were obtained from chloroform. A crystal was mounted onto a glass fiber with epoxy glue and centered on a Nicolet P3 automated diffractometer. The unit-cell parameters were obtained by least-squares refinement of the setting angles of 25 reflections. Reflections with 2θ in the range $4 \leq 2\theta < 40$ ($+h,+k,\pm l$) were measured by using the $2\theta-\theta$ scan technique. A Ψ-scan absorption correction was applied. Crystal and instrument stability were monitored with a set of three standard reflections measured every 97 reflections; no significant variations were found. Details of other crystal data and relevant information are summarized as follows:[10] Space group $P2_1/c$ with a = 11.48(1), b = 13.14(1), c = 7.171(6) Å and β = 99.44(7)°; v = 1068(1) Å^3; Z = 4; λ = 0.71073 Å (Mo Kα radiation); ϱ_{calc} = 1.625 g/cm^3; T = 294 K; m = 0.294 mm^{-1}; minimum/maximum transmission coeff = 0.3130/0.4090; $R = \sum|F_o| - |F_c|/\sum F_o$ = 4.65%; $R_w = [w\sum(|F_o| - |F_c|)^2/\sum wF_o^2)]^{1/2}$ = 5.30%; goodness of fit (GOF) = $[w\sum(|F_o| - |F_c|)^2/N_o - N_v)]^{1/2}$ = 1.38; residual density = −0.37,0.32 e/Å^3.

Following data reduction a unique set of 998 reflections was obtained, of which 812 reflections were observed ($F > 3.0\sigma(F)$). The structure was solved by direct methods using SHELX PLUS computer programs (Nicolet Instrument Corp.) and refined by full-matrix least-squares procedures. All non-hydrogen atoms were refined with anisotropic temperature coefficients. The hydrogen atoms were located and refined with isotropic temperature coefficients. In the final least squares cycle, 191 parameters were refined.

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(10) The authors have deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.