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Isolation and Structures of Axistatins 1-3 from the Republic of Palau Marine Sponge *Agelas axifera* Hentschel¹

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Abstract

An investigation begun in 1979 directed at the Republic of Palau marine sponge *Agelas axifera* Hentschel for cancer cell growth inhibitory constituents subsequently led to the isolation of three new pyrimidine diterpenes designated axistatins 1 (**1**), 2 (**2**), and 3 (**3**), together with the previously reported formamides **4**, **5** and agelasine F (**6**). The structures were elucidated by analysis of 2D-NMR spectra and by HRMS. All of the isolated compounds were found to be moderate inhibitors of cancer cell growth. Axistatins 1–3 (**1–3**), formamide **4**, and agelasine F (**6**) also exhibited antimicrobial activity.

The Demospongiae animal class contains most of the primitive sponges and is believed to represent the beginning of the animal evolutionary tree. Until recently, this phase of evolution was believed to have occurred very rapidly during the Cambrian “explosion” some 540 million years ago. New evidence² based on biochemical (the unique 24-isopropylcholestane component of Demospongiae) and geological dating indicates that the evolution was actually a slow process that began about 635 million years ago. Thus, animal evolution proceeded slowly from sponges, which is consistent with Darwin's early view of evolutionary progression. Addition of this new evidence to our modern knowledge increases our understanding of why the Porifera are such productive sources of most carefully designed constituents for development of new drugs against a broad spectrum of medical problems. Illustrative are various small-molecule sponge constituents with cancer cell growth inhibitory,^{3a–g} antifungal,^{3h} antimalarial,^{3i–l} antibacterial,^{3m} antiprotozoal,^{3n,o} antiinflammatory,^{3p,q} factor XIa (antithrombotic evaluation) inhibitory^{3r} and other enzyme inhibitory^{3s,t} activities. On further biological evaluation, other new sponge components such as the dimeric sesquiterpenoid quinones will probably reveal potentially useful properties.^{3u,v}

We have been evaluating species of marine Porifera from the onset of our investigation of such marine organisms for anticancer constituents, as initially reported in 1970.⁴ As part of these objectives, we collected from the Republic of Palau in 1979 a yellow-orange marine sponge specimen, later identified as *Agelas axifera* Hentschel (Agelasiidae family), which

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gave extracts with cancer cell growth inhibitory activity against the murine P388 lymphocytic leukemia cell line. Subsequently, the genus *Agelas* yielded the cancer cell inhibitory agelastatin series,⁵ agelasines (Na, K-ATPase inhibitory and antimicrobial),⁶ and a variety of bromopyrrole alkaloids,⁷ a number of which have been shown to be fish antifeedant metabolites.⁸ In 1984, Capon and Faulkner reported the isolation of the moderately antimicrobial and ichthyotoxic agelines A (agelasine F) and B from *Agelas dispar*, together with their formamide analogs.^{9a} Recently, three new cyclic peroxide members of the plakortin family designated gracilioethers AC from *Agelas gracilis* were found to have antimalarial activity; gracilioether B also exhibited antileishmanial activity.^{9b} We have continued (from 1995) the extensive investigation of an *Agelas axifera* Hentschel extract that led to the isolation of agelastatin A and assignment by X-ray of its absolute configuration.^{5c} Utilizing a bioassay- (P388 lymphocytic leukemia cell line) directed separation of certain other cancer cell growth inhibitory fractions, we have uncovered three new cancer cell line inhibitory constituents named axistatins 1 (**1**), 2 (**2**), and 3 (**3**), as well as two previously known formamides (**4** and **5**^{9a}) and agelasine F (**6**).^{6b,9a} Isolation and structure elucidation proceeded as follows.

Results and Discussion

A CHCl₃–2-propanol extract of *A. axifera* (51% life extension at 100 µg/kg against the P388 in vivo leukemia) was subjected to a solvent partition sequence (successive extraction of a CH₃OH–H₂O solution at 9:1, and 3:2 dilutions, with hexane and CH₂Cl₂, respectively). The final CH₂Cl₂–soluble P388 active fraction was carefully separated by an extensive series of Sephadex LH-20 gel permeation and partition chromatographic procedures, followed by final isolation on a reversed-phase HPLC column (Zorbax SB C18) with 2:3 CH₃CN–H₂O (0.1% CF₃COOH) as eluant. These separations afforded axistatins 1–3 (**1**, **2**, and **3**), along with the previously known compounds **4–6**, which were identified by comparison of their spectroscopic data (Tables 1 and 2 with the supplementary data) with those reported in the literature.^{6b,9a}

Axistatin 1 (**1**) was obtained as a colorless powder, and its molecular formula was established as C₂₆H₄₁N₅O by HRFABMS. The IR bands at 3500, 1660, and 1600 cm^{–1} and UV absorptions at 262 and 225 nm were in agreement with literature values^{9a,10} for the 6-amino-5-(formylamino)-4-(methylamino)-1,3-diazine unit. The ¹H NMR spectrum contained signals at δ 7.96 (1H, s) and 8.14 (1H, s), assigned to the formamide and C-2' protons. The signals at δ 2.98 (3H, d, *J* = 4.2 Hz) and 4.96 (1H, brq, *J* = 4.2 Hz), due to the –NHCH₃ group, and at 4.90 (2H, brs) for the NH₂ group correlated well with the reported^{9a} values for those groups. The nitrogen atom locations were thereby established, leaving a C₂₀H₃₃ alkyl residue to be identified. This diterpene portion was defined as a *cis*-clerodane by comparison of the NMR spectroscopic data with those of known clerodane diterpenes.^{6a,6d,9a} The ¹H NMR spectrum contained signals at δ 4.14 (2H, d, *J* = 7.6 Hz), 5.34 (1H, t, *J* = 7.6 Hz), and 1.58 (3H, s), which were assigned to a 3-methyl-2-butenyl entity attached to the *N*-formyl nitrogen atom.^{9a} The ¹H NMR signals at δ 0.74 (3H, d, *J* = 6.0 Hz), 0.79 (3H, s), 1.02 (3H, s), 1.67 (3H, s), and 5.26 (1H, brs) were compatible with a clerodane ring system. Comparison of the ¹³C NMR spectroscopic data of axistatin 1 (**1**) with those of agelasine A (**7**),^{6a,6d} formamide **5**,^{9a} and sagittariol acetate¹¹ confirmed the location of a trisubstituted olefin at C-3 and the relative configuration of the bicyclic ring system. The signals at δ 24.0 (CH₂, C-2) and 33.1 (CH₃, C-19) are characteristic of a *cis*-clerodane [**7**, δ 24.0 (CH₂, C-2) and 33.0 (CH₃, C-19); **5**, δ 23.9 (CH₂, C-2) and 34.7 (CH₃, C-19); sagittariol acetate, δ 23.7 (CH₂, C-2) and 34.4 (CH₃, C-19)] rather than a *trans*-clerodane [agelasine B (**8**),^{6a,6d} δ 27.5 (CH₂, C-2) and 19.9 (CH₃, C-19); hardwickiol,¹¹ δ 18.0 (CH₂, C-2) and 21.2 (CH₃, C-19)] ring system. Moreover, the ¹H NMR and ¹³C NMR signals of the bicyclic ring system of axistatin 1 (**1**) were essentially identical to those of

agelasine A (**7**).^{6a,6d} All of these considerations were used to assign structure **1** to axistatin **1**, and the overall assignments (Tables 1 and 2, supplementary data) of ¹H NMR and ¹³C NMR data were unambiguously made based on the DEPT, COSY, NOESY, TOCSY, HMQC, and HMBC spectra.

As with **1**, the molecular formula of axistatin **2** (**2**) was found to be C₂₆H₄₁N₅O by HRFABMS and the results of ¹H NMR and ¹³C NMR spectroscopic interpretations (Tables 1 and 2, supplementary data). The NMR spectra were almost identical to those of **1**, except for differences in the signals due to the bicyclic ring system of the diterpene moiety, and it was concluded that axistatin **2** (**2**) also contained a 6-amino-5-(formylamino)-4-(methylamino)-1,3-diazine unit as well as the 3-methyl-2-butenyl segment of the diterpene. The bicyclic diterpene portion of axistatin **2** (**2**) was defined as a *trans*-clerodane by comparison again of the NMR spectroscopic data with those of known clerodane diterpenes.^{6a,6d} The signals at δ 27.4 (CH₂, C-2) and 19.9 (CH₃, C-19) are characteristic of a *trans*-clerodane (see the diagnostic signals of **8**^{6a,6d} above) ring system, and the ¹H NMR and ¹³C NMR signals of the bicyclic ring system of axistatin **2** (**2**) were found to be nearly identical to those of **8**.^{6a,6d} Thus, structure **2** was determined to represent axistatin **2**.

The molecular formula of axistatin **3** (**3**) proved to be C₂₉H₄₇N₅O on the basis of HRFABMS data combined with ¹H NMR and ¹³C NMR spectroscopic results (Tables 1 and 2). The ¹H NMR and ¹³C NMR spectra were found to be almost identical to those of formamide **4**,^{9a} with the addition of signals corresponding to an isopropyl group. The HMBC experiment showed a long-range correlation between one hydrogen [-CH(CH₃)₂] at δ 3.35 (1H, m) and one unsaturated carbon (C-6') at δ 160.45, revealing the site of attachment of the isopropyl unit to be at the 6'-NH₂. Thus, the structure of axistatin **3** was assigned as **3**.

In the present investigation, the six *Agelas axifera* constituents (**1–6**) were first tested against the murine P388 lymphocytic leukemia cell line followed by a selection of human cancer cell lines. As expected from the isolation bioassay results, all were found to exhibit significant cancer cell growth inhibitory activities (Table 3), especially against the human cancer cell lines. Because agelasine B (**8**) isolated from *Agelas axifera* has been found to be protein kinase C (PKC) active,^{6f} future biological studies of the axistatins (**1–3**) and related *Agelas* heterocyclic systems will include evaluation of their PKC activities.

Compounds **1–6** were available in sufficient quantity for antimicrobial evaluation. Axistatins **1–3** (**1–3**) had identical antimicrobial profiles, inhibiting Gram-positive bacteria, the exquisitely sensitive Gram-negative pathogen *Neisseria gonorrhoeae*, and the opportunistic fungus *Cryptococcus neoformans* (Table 4). Agelasine F (**6**) had broad-spectrum activity, inhibiting all bacteria and fungi in the panel (Table 4). Activity against *Mycobacterium tuberculosis* has previously been reported for agelasine F.^{6h} Formamide **4** had the same antimicrobial profile as **1–3** except that **4** did not inhibit *C. neoformans*. Formamide **4** was previously shown to inhibit *Bacillus subtilis* at high concentrations (100 μ g/disk).^{9a}

Experimental Section

General Experimental Procedures

Melting points were determined with a Fisher Scientific electrothermal melting point apparatus and are uncorrected. Optical rotations were obtained with a Perkin-Elmer 241 polarimeter. UV and IR spectra were recorded using a Perkin-Elmer Lambda 3BUV/Vis spectrophotometer and a Thermo Nicolet Avatar 360 infrared spectrometer respectively. NMR spectra were obtained employing a Varian XL-300 or a Varian UNITY INOVA-500 spectrometer with tetramethylsilane (TMS) as an internal reference. High-resolution mass

spectra were obtained using a JEOL LCMate magnetic sector instrument in the FAB mode, with a glycerol matrix reference.

All chromatographic solvents were redistilled. Sephadex LH-20 used for partition column chromatography was obtained from Pharmacia Fine Chemicals AB. Analytical HPLC was conducted with a Hewlett-Packard Model 1050 HPLC coupled with a Hewlett-Packard diode-array detector. Semipreparative HPLC was performed on a Zorbax SB C₁₈ column with aqueous acetonitrile (40 %) as mobile phase.

Specimen Collection

In January 1979, during our marine organism specimen selection near Koror in the Republic of Palau, we collected on a coral reef a reddish-orange (yellow-orange underside, yellowish-orange interior) marine sponge presenting as an irregular mass with large excavations and crevices. The sponge *Agelas axifera* Hentschel (1911, Order Agelasida, Family Agelasidae.) was identified by one of us (J. N. A. H.), and a voucher specimen (Z0003590) is maintained in the Northern Territory Museum, Darwin, Australia.

Extraction and Initial Separation

After collection,^{5e} *Agelas axifera* was preserved and shipped in CHCl₃–2-propanol. Soon following arrival in our laboratory a portion of the 1979 collection was extracted with CH₃OH. The CH₃OH extract was dissolved in CH₃OH–H₂O (9:1) and the solution filtered to remove insoluble material. The filtrate was partitioned (4 ×) between hexane and 9:1 CH₃OH–H₂O. The hexane layer was removed and concentrated to yield 30 g (P388 ED₅₀ 0.5 μg/mL) of black-brown material. The CH₃OH–H₂O phase was diluted to give a ratio of 3:2 and extracted (4×) with CH₂Cl₂. The CH₂Cl₂ layer was concentrated to afford a black-brown P388-active (40 g, ED₅₀ 0.2 μg/mL) fraction. The remaining CH₃OH–H₂O solution was inactive and discarded.

Isolation of Compounds 1–6

The CH₂Cl₂ fraction (705 g) from scale-up procedures based on the preceding smaller scale methods, was passed through a Sephadex LH-20 column, with CH₃OH as eluent. Four fractions (A, B, C, and D) active against both P388 and human cancer cell lines were obtained. Fractions A–C were combined and rechromatographed on a Sephadex LH-20 column in 3:2 CH₃OH–CH₂Cl₂, resulting in a concentration of activity in three of the fractions. These were combined and fractionated on a Sephadex LH-20 column in hexane–CH₃OH–2-propanol (8:1:1) to afford three active fractions that were combined and further separated on a Sephadex LH-20 column in hexane–EtOH–toluene–CH₂Cl₂ (17:1:1:1). The resulting four active fractions were combined and chromatographed on a Sephadex LH-20 column in hexane–EtOAc–CH₃OH (4:5:1) to provide compound **5** (2.5 g) and another two active fractions (I and II). A 60-mg aliquot of active fraction I was separated on a semipreparative reversed-phase HPLC Zorbax SB C₁₈ column with 40:60 CH₃CN–H₂O (0.1% TFA, a flow rate of 4 mL/min and the UV detector at 210 nm). By that procedure, axistatin 1 (**1**, 11.3 mg) eluted at 30.7 min, and axistatin 2 (**2**, 10.7 mg) eluted at 34.9 min. A 100-mg sample of active fraction II was separated by the same procedure to yield axistatin 3 (**3**, 10.2 mg) at 47.8 min and formamide **4** (56.7 mg) at 50.4 min. Finally, active fraction D from the first gel filtration column was separated on a Sephadex LH-20 column in hexane–CH₃OH–2-propanol (8:1:1) to give pure agelasine F (**6**, 2.0 g).^{6b,9a}

Axistatin 1 (1)—colorless solid; mp 99–100 °C; [α]_D²³ –31.0 (c 0.10, CH₃OH); UV (CH₃OH) $X_{\max}$ (log ϵ) 262 (3.4), 225 (4.2), 208 (4.4) nm; IR (CH₂Cl₂) $\nu_{\max}$ 3500, 1660, 1600 cm^{–1}; for the ¹H and ¹³C NMR data see Tables 1 and 2 (supplementary data)

FABMS (positive) m/z 440.33797 $[M + H]^+$ (calcd for $C_{26}H_{42}N_5O$, 440.33893).

Axistatin 2 (2)—colorless solid; mp 96–97 °C; $[\alpha]_D^{23}$ –21.1 (c 0.60, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 262 (3.4), 225 (4.2), 207 (4.3) nm; IR (CH_2Cl_2) ν_{max} 3500, 1660, 1600 cm^{-1} ; for the 1H and ^{13}C NMR data see Tables 1 and 2 (supplementary data); FABMS (positive) m/z 440.33886 $[M + H]^+$ (calcd for $C_{26}H_{42}N_5O$, 440.33893).

Axistatin 3 (3)—colorless solid; mp 106–107 °C; $[\alpha]_D^{23}$ –8.5 (c 0.10, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 260 (3.5), 225 (4.3), 206 (4.3) nm; IR (CH_2Cl_2) ν_{max} 3501, 1660, 1600 cm^{-1} ; for the 1H and ^{13}C NMR data see Tables 1 and 2 (supplementary data); FABMS (positive) m/z 482.38602 $[M + H]^+$ (calcd for $C_{29}H_{48}N_5O$, 482.38589).

Formamide 4—colorless solid; mp 101–103 °C (lit.^{9a} mp 100–104 °C); $[\alpha]_D^{23}$ –8.3 (c 0.10, CH_3OH) [lit.^{9a} $[\alpha]_D$ –8.2 (c 2.4, $CHCl_3$)].

Formamide 5—colorless solid; mp 83–84 °C; $[\alpha]_D^{23}$ –9.3 (c 0.20, CH_3OH) [lit.^{9a} $[\alpha]_D$ –9.2 (c 0.73, $CHCl_3$)].

Agelasine F (6)—colorless crystalline solid; mp 175–176 °C (lit.^{6b} mp 176–180 °C); $[\alpha]_D^{23}$ –8.4 (c 0.10, CH_3OH) [lit.^{9a} $[\alpha]_D$ –8.4 (c 3.0, $CHCl_3$)].

Cancer Cell Line Procedures

The National Cancer Institute's standard sulforhodamine B assay was used to assess inhibition of human cancer cell growth as previously described.¹² In summary, cells in 5% fetal bovine serum/RPMI-1640 medium were inoculated in 96-well plates and incubated for 24 h. Serial dilutions of the compounds were then added. After 48 h, the plates were fixed with trichloroacetic acid, washed, stained with sulforhodamine B, and read with an automated microplate reader. Growth inhibition of 50% (GI_{50} , drug concentration causing a 50% reduction in the net protein increase) was calculated from optical density data with Immunosoft software. The murine P388 lymphocytic leukemia cell line results were obtained using 10% horse serum/Fisher medium, with incubation for 24 hours. Serial dilutions of the compounds were added, and after 48 hours, cell growth inhibition (ED_{50}) was calculated using a Z1 Coulter particle counter.

Antimicrobial Susceptibility Testing

The antibacterial and antifungal activities of compounds **1–6** were assessed using the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution assays.^{13,14} Compounds were reconstituted in a small volume of sterile DMSO and diluted in the appropriate media immediately prior to susceptibility experiments. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that inhibited all visible growth of the test organism (optically clear). Assays were repeated on separate days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of Axistatins **1** and **2** and tabulated ^1H and ^{13}C NMR data for Axistatins **1**, **2** and **3** and compounds **4–6**. Additional analytical data for compounds **4–6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Table 3

Murine and Human Cancer Cell Line Inhibition Values (ED₅₀ and GI₅₀ Expressed in $\mu\text{g/mL}$ or μM (in brackets)) for *Agelas axifera* Constituents **1–6**^a

cell line ^b	compound					
	1	2	3	4	5	6
P388	8.7(19.8)	>10(22.8)	4.3(8.9)	6.9(15.7)	>10(18.2)	>10(21.8)
BXPC-3	2.1(4.8)	2.4(5.5)	2.9(6.0)	2.6(5.9)	2.2(4.0)	1.6(3.5)
MCF-7	2.5(5.7)	3.0(6.8)	2.8(5.8)	2.6(5.9)	1.7(3.1)	1.8(3.9)
SF-268	1.6(3.6)	1.7(3.9)	1.7(3.5)	1.7(3.9)	1.7(3.1)	1.6(3.5)
NCI-H460	2.0(4.6)	1.9(4.3)	2.6(5.4)	3.0(6.8)	2.0(3.6)	1.7(3.7)
KML20L2	1.8(4.1)	1.8(4.1)	3.3(6.9)	3.1(7.1)	1.6(2.9)	1.6(3.5)
DU-145	2.1(4.8)	2.2(5.0)	3.6(7.5)	3.0(6.8)	2.5(4.6)	1.9(4.2)

^aIn DMSO.

^bCancer cell lines: Murine lymphocytic leukemia (P388); pancreatic adenocarcinoma (BXPC-3); breast adenocarcinoma (MCF-7); CNS glioblastoma (SF-268); lung large-cell carcinoma (NCI-H460); colon adenocarcinoma (KM20L2); prostate carcinoma (DU-145).

Table 4

Inhibition of Bacteria and Fungi by *Agelas axifera* Constituents (Minimum Inhibitory Concentration Range Expressed in $\mu\text{g/mL}$)^a

microorganism	ATCC or (Presque Isle) number	compound					
		1	2	3	4	5	6
<i>Cryptococcus neoformans</i>	90112	1-4	2	8-16	*	*	2
<i>Candida albicans</i>	90028	*	*	*	*	*	4
<i>Staphylococcus aureus</i>	29213	1-4	1	4-16	8-16	*	2
<i>Streptococcus pneumoniae</i>	6303	8-32	8	32	32-64	*	16
<i>Enterococcus faecalis</i>	29212	1-4	1-2	8-16	8	*	2-4
<i>Micrococcus luteus</i>	(456)	1-4	1	8	4	*	<0.5-0.5
<i>Escherichia coli</i>	25922	*	*	*	*	*	32
<i>Enterobacter cloacae</i>	13047	*	*	*	*	*	32
<i>Stenotrophomonas maltophilia</i>	13637	*	*	*	*	*	16-32
<i>Neisseria gonorrhoeae</i>	49226	1-8	2-4	8	16-32	*	2-4

^a_* = no inhibition at 64 $\mu\text{g/mL}$.