ISOLATION OF GEODIASTATINS 1 AND 2 FROM THE MARINE SPONGE *GEODIA MESOTRIAENA*[1]

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ABSTRACT.—An aqueous extract of the Gulf of California *Geodia mesotriaena* von Lendenfeld (Porifera) has been found to contain two chromoprotein antineoplastic agents designated geodiastatins 1 and 2. The active brownish-black proteins from this siliceous sponge were found to contain major (>1%) amounts of silicon and to inhibit growth of the murine P388 lymphocytic leukemia. A related protein (geodiatoxin 1) was found to be toxic at 6 mg/kg (LD_{100}, mouse).

Marine sponges of the genus *Geodia* (Demospongiae class, Tetractinellida order, Geodidae family) are essentially unexplored in respect to chemical constituents and do not appear to have received any prior study for biologically active components. Only the Mediterranean giant siliceous *Geodia gigas* (2) and Newfoundland *Geodia megostrella* (3) have received preliminary chemical investigation. And this has led to isolation of inositol, taurine, taurobetaine (2), agmatine, histamine (4) and herbipoline (5) from *G. gigas* and a series of cholestane derivatives from *G. megastrella*. Because of our long-term interest in evaluating various species of the Porifera phyla for potentially useful antineoplastic biosynthetic products (6–9) we collected the Eastern Pacific (ranges from the Gulf of California to southern Alaska) *G. mesotriaena* von Lendenfeld (black exterior, pale cream interior).

Specimens of *G. mesotriaena* for this study were first collected (at a depth of ~3 m.) from the Gulf of California near Bahia Kino, Sonora, Mexico, in 1971. A 2-propanol extract was found to produce a confirmed level of activity in the National Cancer Institute's (NCI) 9KB (cells from a human nasopharynx carcinoma) *in vitro* test system, and toxicity in the murine P388 lymphocytic leukemia (PS) screen *in vivo* at dose levels ranging from 400 mg/kg to 3 mg/kg (table 1). A large scale recollection in the same area in 1976 yielded 2-propanol and ethanol extracts which showed unacceptable 9KB *in vitro* and no PS *in vivo* activity (at the dose levels tested) but did show acceptable cytotoxicity in the NCI's PS *in vitro* evaluation. Consequently the alcohol extracts were concentrated by solvent partition methods (9) to cytotoxic chloroform and aqueous methanol fractions. Numerous attempts employing various chromatographic techniques (directed by bioassay) to isolate the cytotoxic component(s) proved unsuccessful but did serve to indicate that the main active material was water soluble and was probably a biopolymer. The capricious nature of the biological responses outlined in table 1 for the various collections continued during fractionation and necessitated the second recollection (1978).

The 1978 recollection gave solvent extracts that were inactive in both the 9 KB and PS *in vitro* systems, but two showed activity against the PS *in vivo* system. The 2-propanol extract produced life increases of up to 50% at 50 mg/kg, and one aqueous extract showed a 33% increase at 15 mg/kg. Both the ethanolic and aqueous extracts were toxic at 25 mg/kg and 50 mg/kg (and higher) dose levels, respectively. Study of the 1978 2-propanol extract led to the same general

[1]Antineoplastic Agents part 74; for part 73 see Ref. 1.

results experienced with the 1976 collection. But the 1978 aqueous extract did prove useful, and two new antineoplastic substances designated geodiastatins 1 and 2 were isolated, accompanied by a toxic companion termed geodiatoxin 1.

Extensive attempts to concentrate the anticancer components of the aqueous extract by various solvent partition and Sephadex gel permeation chromatographic techniques finally led to the following greatly simplified procedure for obtaining geodiastatins 1–2. The aqueous extract was first triturated with methanol, the insoluble residue was dissolved in water, and the solution was filtered. The cloudy filtrate was centrifuged at 37,000 g to remove additional colloidal-like insoluble material. The supernatant solution was chromatographed on Sephadex G–50 to remove salts and other relatively small water-soluble molecules. Three bands were eluted from the column. The most mobile band contained high and intermediate molecular weight material and was separated from two incompletely resolved bands (containing salts and other low molecular weight compounds). There was a noticeable formation of colloidal-like material in the first fraction during chromatography, which necessitated cleaning the top column filter during elution. Material from the two lower molecular weight bands was inactive in the PS *in vivo* system, but the first band was active and showed toxicity at dose levels down to 6 mg/kg. The first high molecular weight fraction was dissolved in 0.5 M ammonium acetate solution and centrifuged at 37,000 g. The insoluble material was collected and further separated as summarized below. Meanwhile the supernatant solution from the centrifuge step was chromatographed on Sepharose 2B; a series of very toxic but PS (*in vivo*) inactive fractions were obtained.

The insoluble fraction obtained by the centrifuge treatment of the G–50 high molecular weight band was further divided as follows. The material was resuspended in water and spun at 1000 g centrifugal force for 1 hr. The supernatant was recentrifuged at 15,000 g for a further 1 hr, and this procedure was repeated at 37,000 g. The three fractions which settled from suspension at 1000 g, 15,000 g and 37,000 g corresponded to, in order of sedimentation, geodiastatins 1 and 2 and geodiatoxin 1. Total yields of geodiastatins 1 and 2 and geodiatoxin 1 from 128 g of aqueous extract amounted to 738 mg, 813 mg and 152 mg, respectively. And geodiastatins 1 and 2 caused 26% (at 25 and 5 mg/kg, respectively) life extension in the PS *in vivo* system. Geodiatoxin was toxic (LD_{100}) at 6 mg/kg and gave evidence of toxicity down to 3 mg/kg.

The geodiastatins were obtained as shiny brownish-black solids that seemed to be novel silicon-containing chromoproteins. Amino acid analyses indicated substantial amounts of Asx, Gly and Glx. In general, the amino acid compositions resembled proteins as diverse as diphosphopyridine nucleosidase (porcine brain) and glutamate dehydrogenase (bovine liver). Based on the amino acid analyses, the percent of protein was calculated to be 34 and 28 for geodiastatins 1 and 2 and 21 for geodiatoxin 1. Trace metal analyses revealed that geodiastatins 1 and 2 contained a significant amount (>1.0%) of silicon, and geodiastatin 2 contained a high (>1.0%) magnesium content. Geodiatoxin 1 was found to contain only a minor amount of silicon. Since members of the *Geodia* genus represent some of the few animals that deposit silica rather than limestone skeletons, the geodiastatins may have a role in such structural formations and in protection of the sponge surface from predators.

Separation of the geodiastatins and geodiatoxin 1 from solution at relatively low centrifuge speeds suggests that they may be aggregates (formed during the G–50 gel permeation separation step) of smaller biopolymers. Due to the unexceptional antineoplastic activity in the PS *in vivo* system, the nature of the chromo-

phore and silicon-containing units, detailed molecular weight estimates, and other potentially interesting chemical aspects of the geodiastatins and geodiatoxin 1 were not further pursued. However, the geodiastatins most probably represent a new series of biopolymers of fundamental biological importance and/or interest and clearly warrant future detailed chemical and biological investigations.

EXPERIMENTAL[2]

ANIMAL COLLECTION.—The initial collection (~1 kg) of *Geodia mesotriaena* von Lendenfeld was made by GRP and Mr. W. E. Pettit in November, 1971, at Bahia Kino, Sonora, Mexico. A 2-propanol extract of the animals showed considerable PS *in vivo* toxicity and confirmed 9KB *in vitro* activity (see table 1). A large-scale recollection was made in the same area in March 1976 by GRP, Dr. R. H. Ode, and Messrs. G. C. Bryan and L. D. Vanell. The 2-propan 1 and ethanol extracts (see table 1) were PS *in vitro* active. A second large-scale recollection (~50 kg of wet sponge, table 1) was made in the same area in May 1978 by GRP, Drs. D. L. Doubek, and P. Brown, Miss R. K. Pettit, and Messrs. G. R. Pettit, III and F. Ward. The 2-propanol extracts from this collection were *in vivo* (PS) active, but one ethanol extract proved toxic at 100, 50 and 25 mg/kg dose levels, while a second was found active (PS *in vivo*) in the 15–60 mg/kg range. One aqueous extract was toxic at 100 and 50 mg/kg and was inactive at 25 mg/kg. A second aqueous extract was PS *in vivo* active (T/C 133) at 15 mg/kg. The main aqueous extract (128 g) from the 1978 recollection was used to isolate geodiastatins 1 and 2 and geodiatoxin 1.

EXTRACTION.—The general extraction procedure was as follows. The sponge was collected and shipped in 1 gallon sealed cans containing 2-propanol. The solvent was decanted and replaced with fresh 2-propanol (about 2 liters per can). After approximately one month, this second extract was decanted and combined with the shipping solution. Next, the animals were finely divided, wrapped in muslin cloth and placed in a modified (stainless steel) Soxhlet extraction apparatus. The animals were extracted (48 hr. each solvent) with refluxing ethanol followed by refluxing water. All extracts were evaporated and freeze-dried to remove any remaining solvent. Weights of the extracts and results of cytotoxicity and antineoplastic evaluations are given in table 1.

ISOLATION OF GEODIASTATINS 1–2.—The aqueous extract (256 g) from the 1978 collection was triturated with methanol (3 x 2 liters) for a total of 72 hr. The insoluble portion (~70 g) was dissolved in water (300 ml); the resulting aqueous solution was filtered (No. 1 filter paper) and centrifuged at 37,000 g for 1 hr to remove insoluble material. The supernatant solution (toxic down to 30 mg/kg) was chromatographed on a Sephadex K100/100 column (7 liter volume) packed with Sephadex G-50. The first fraction (high mol. wt. material) eluted contained some suspended precipitate and was recovered by evaporation of the water *in vacuo*. To the residue (5.0 g) was added a solution (50 ml) of 0.5 M ammonium acetate. After being vigorously stirred for 0.5 hr, the solution was centrifuged at 37,000 g for 1 hr. The insoluble material (2.5 g) was resuspended in water (200 ml) and centrifuged at 1000 g for 1 hr. The supernatant was carefully decanted and recentrifuged at 1000 g for 1 hr. The combined solids were washed once with distilled water (100 ml) and recovered by recentrifugation at 15,000 g. The latter fraction (738 mg after lyophilization) was designated geodiastatin 1. Similar procedures were followed at 15,000 g and 37,000 g with the above combined supernatant and wash solutions; after recovery by lyophilization, geodiastatin 2 (813 mg) and geodiatoxin 1 (152 mg), were obtained.

Geodiastatin 1 was obtained as a shiny brownish-black powder insoluble in 1% SDS, 10% acetic acid, 6 M guanidine hydrochloride and 0.2 M sodium hydroxide.[3] Upon hydrolysis with 6N HCl (1 ml), at 4.0 mg specimen exhibited the following amino acid composition: Ala 8.23, Arg 4.00, Asx 10.78, Cys 1.01, Glx 11.30, Gly 10.07, His 2.05, Ile 5.05, Leu 8.08, Lys 3.63, Met 1.86, Phe 4.62, Pro 5.16, Ser 6.98, Thr 6.90, Tyr 3.71, Val 6.45 in mol. % of amino acid corresponding to an approximate 34% protein content.

Anal. Found: C, 43.55; H, 6.16; N, 7.16; P, 0.81. A trace metal analysis showed (major

[2]Sephadex G-50 and Sepharose 2B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. A Beckman model L-2 ultracentrifuge was used for high speed centrifugation and an International portable refrigerated centrifuge for low speed centrifugation. Dr. Ann Yates determined amino-acid compositions using a model 121 Beckman-Spinco analyzer. Elemental microanalyses were performed by Dr. A. W. Spang of the Spang Microanalytical Laboratory, Eagle Harbor, Michigan. Dr. M. J. Parsons provided trace metal analyses employing a Jarrell-Ash 3-4 spectrograph.

[3]The same refractory solubility behavior was exhibited by geodiastatin 2 and geodiatoxin 1. Hence efforts to obtain some routine spectral data (uv and optical rotations) were not further pursued.

TABLE 1. *Geodia mesotriaena*—Collection and biological activity data.

Collection date (Wet Sponge Wt.)	2-Propanol extract				Ethanol extract				Aqueous extract			
		Biological Evaluation				Biological Evaluation				Biological Evaluation		
	Extract Weight (g)	PS *in vivo* T/C Dose (mg/kg)	PS *in vitro* ED_{50}	9KB ED_{50}	Extract Weight (g)	PS *in vivo* T/C Dose (mg/kg)	PS *in vitro* ED_{50}	9KB ED_{50}	Extract Weight (g)	PS *in vivo* T/C Dose (mg/kg)	PS *in vitro* ED_{50}	9KB ED_{50}
November 1971 (~1 kg)	41	Tox-400 Tox-200 Tox-100 Tox- 50 Tox- 25 Tox- 12 Tox- 6 Tox- 3	N.T.[a]	3.3	N.E.[b]	-	-	-	20	<110 400 <110 200 <110 100	N.T.[a]	100
March 1976 (~31 kg)	946	93 60 114- 30 88- 15	2.9	26	285	98- 60 96 - 30 95- 15	3.3	>100	129	N.T.	N.T.[a]	N.T.
May 1978 (~50 kg)	1882	113- 100 150- 50 128- 25	>100	>100	241	Tox-100 Tox- 50 Tox- 25	82	20	128	Tox 100 Tox 50 103- 25	85	47
May 1978 (~2 kg)	62	120- 60 122- 30 142- 15	>100	22	67	122- 60 125- 30 132- 15	70	>100	16	Tox 60 115- 30 133- 15	>100	>100

[a]Not tested.

[b]Not extracted with ethanol.

>1.0%, minor 0.1-1.0% and trace <0.01% each by weight) a major amount of silicon, minor amounts of Ag, Al, Ca, Cu, Fe, Mg, Na and Ti.

When analyzed by the same methods, geodiastatin 2 (shiny brownish-black solid) exhibited, respectively: Amino acid analysis: Ala 7.68, Arg, 3.16, Asx 11.20, Cys 0.62, Glx 10.57, Gly 10.83, His 1.84, Ile 6.00, Leu 8.62, Lys 2.36, Met 1.79, Phe 4.85, Pro 6.45, Ser 6.09, Thr 6.61, Tyr 3.51, Val 7.82 in mol. % of amino acid.

Anal. Found: C, 52.38; H, 7.69; N, 5.30; P, 0.69; S, 0.22; trace element analysis, silicon and magnesium (major), minor amounts of Al, Ca, Cu, Mn, Na, and trace quantities of Ag.

Geodiatoxin 1 (dark brown powder) gave the following analytical results. Amino acid analysis: Ala 7.35, Arg 4.27, Asx 11.47, Cys 0.91, Glx 10.25, Gly 10.63, His 2.35, Ile 5.48, Leu 8.69, Lys 4.17, Met 1.47, Phe 4.87, Pro 5.82, Ser 5.13, Thr 5.98, Tyr 3.65, Val 7.50 in mol. % of amino acid.

Anal. Found: C, 53.85; H, 7.85; N, 5.58; S, 0.25; P, 0.70; trace element analysis; minor amounts of Al, Ca, Fe, Mg, Mn, Si and trace amounts of Ag and Cu.

The apparent protein content of geodiastatin 2 and geodiatoxin 1 was calculated from the amino acid content to be 28% and 21%, respectively. Since these biopolymers proved to be relatively insoluble and may have resisted hydrolysis, the protein values may be lower than the true molecular composition. Calculation of percent protein values from the N content (under the assumption that proteins contain 16% N) resulted in values of 33% and 35%, respectively.

At present geodiastatins 1 (T/C 126 at 25 mg/kg and T/C 128 at 12.5 mg/kg) and 2 (T/C 126 at 5 mg/kg and 122 at 1.25 mg/kg) have been evaluated against the P388 lymphocytic leukemia (10). Geodiatoxin was found to exhibit toxicity at 3 mg/kg and to be lethal (LD_{100}) at 6 mg/kg in the CDF_1 mouse. From these results it is apparent that *G. mesotriaena* may contain other PS *in vivo* active constituents such as lipoproteins (that may have complicated the earlier lipid-type separations originating from the 2-propanol extracts) which may or may not account for some of the original cytotoxicity data.

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