Makaluvic Acids from the South African Latrunculid Sponge *Strongylodesma aliwaliensis*

Robert A. Keyzers,† Catherine E. Arendse,‡ Denver T. Hendricks,‡ Toufiek Samaai,§ and Michael T. Davies-Coleman*,†

Department of Chemistry, Rhodes University, Grahamstown, South Africa, Division of Medical Biochemistry, University of Cape Town, Rondebosch, South Africa, and School of Biological and Conservation Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa

Received December 21, 2004

Two new metabolites, makaluvic acid C (**1**) and *N*-1-*β*-D-ribofuranosylmakaluvic acid C (**2**), were isolated from the recently described sponge *Strongylodesma aliwaliensis* collected off the east coast of South Africa. Standard spectroscopic techniques provided the structures of both compounds. Chiral GC analysis of the peracetylated aldononitrile derivative of the acid hydrolysate of **2** confirmed the D-configuration of the ribose moiety in this compound. Compound **2** and four related pyrroloquinoline metabolites, isolated previously from *S. aliwaliensis*, exhibited *in vitro* cytotoxicity against esophageal cancer cells.

The marine sponge family Latrunculiidae has recently been the subject of substantial taxonomic revision, and five genera, *Cyclacanthia*, *Latrunculia*, *Strongylodesma, Tsitsikamma,* and *Sceptrella*, are presently assigned to this family.[1–4] *Sceptrella* species are deep-water latrunculid sponges predominantly distributed in the northern and western Atlantic Ocean, and there is no record of their occurrence in the Southern Hemisphere.[1] The former four latrunculid sponge genera, however, are all well represented in southern African waters, and the discovery of several new species from these genera over the past decade suggests that the southern African region is a latrunculid sponge biodiversity hotspot.[3] Not unexpectedly, associated with this unique sponge biodiversity is an equally diverse cornucopia of novel secondary metabolites, and our ongoing studies of the natural product chemistry of subtidal southern African latrunculid sponges have thus far yielded 21 new and nine known alkaloid metabolites possessing either tetra-, hexa-, or octa-hydrogenated variants of pyrrolo[4,3,2-*de*]quinoline, pyrrolo[4,3,2-*de*]pyrrolo[2,3-*h*]quinoline, and pyrido[2,3-*h*]pyrrolo[4,3,2-*de*]quinoline core skeletons.[5–8]

The genus *Strongylodesma* is currently represented in the coastal waters off southern Africa by three species: *S. tsitsikammaensis*, *S. algoaensis,* and *S. aliwaliensis*.[2,4] We report here further results from our examination of the polar aqueous eluent from HP-20 chromatography of an extract of *S. aliwaliensis* collected by scuba from the Aliwal Shoal, a large subtropical reef system off the east coast of South Africa.[4,8] Two new pyrroloquinoline metabolites, makaluvic acid C (**1**) and its glycosydated analogue, *N*-1-*β*-D-ribofuranosylmakaluvic acid C (**2**), were isolated from the aqueous HP-20 chromatography eluent. Our previous investigation of the pyrroloquinoline metabolites of *S. aliwaliensis* afforded two novel *N*-1-*β*-D-ribofuranosylpyrroloquinoloines, *N*-1-*β*-D-ribofuranosyldamirone C (**3**) and *N*-1-*β*-D-ribofuranosylmakaluvamine I (**4**), in addition to the three known metabolites: damirone C (**5**), makaluvamine I (**6**), and makaluvamine M (**7**).[8,9]

* To whom correspondence should be addressed. Tel: +27 46 603 8264. Fax: +27 46 6225109. E-mail: M.Davies-Coleman@ru.ac.za.
† Rhodes University.
‡ University of Cape Town.
§ University of KwaZulu-Natal (Westville Campus).

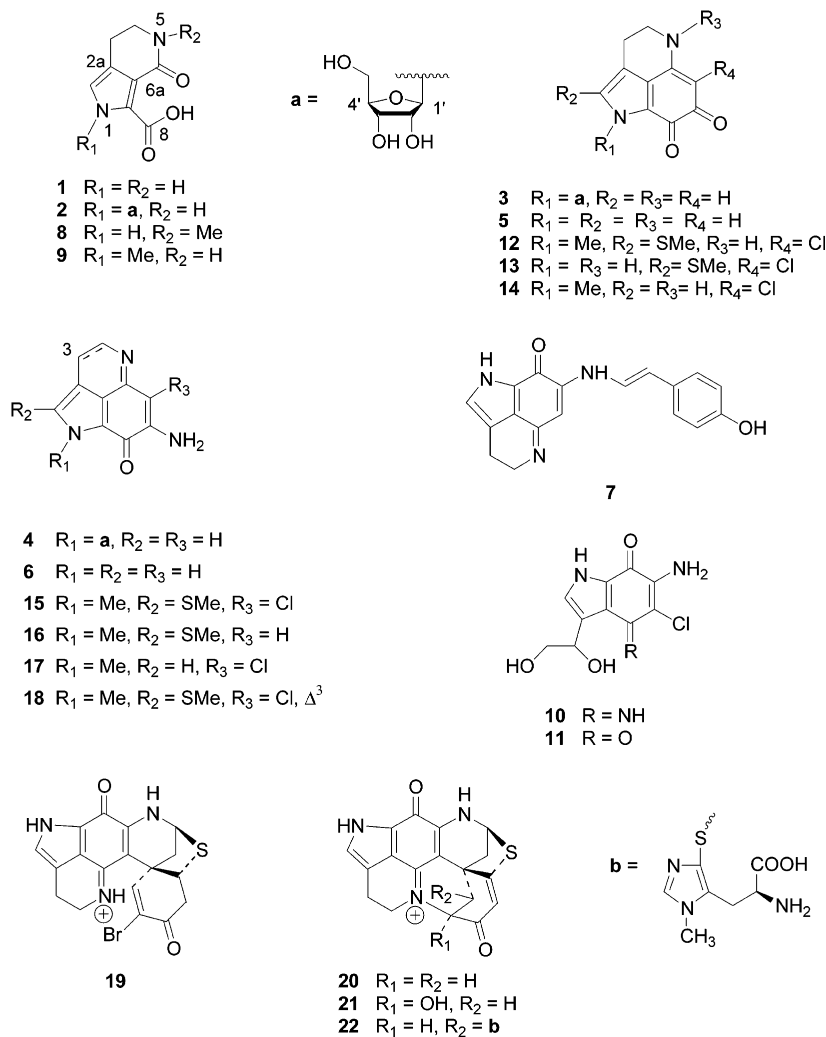
Results and Discussion

Lyophilized specimens of *S. aliwaliensis* (180 g dry weight) were extracted with MeOH, and the MeOH extract was partitioned between EtOAc and H_2O. Lyophilization of the aqueous partition fraction afforded a dark brown gum. Our previous isolation of compounds **3**–**7** from this gum required initial partitioning of a portion (2 g) of the gum on HP-20 resin.[8] During our earlier workup of the *S. aliwaliensis* extract[8] we established that both the ribofuranosylpyrroloquinolines **3** and **4** and the non-glycosydated pyrroloquinoline metabolites **5**–**7** were readily absorbed onto the HP-20 resin, and we initially paid little attention to the final aqueous HP-20 chromatography eluent, which we erroneously assumed contained very little, if any, organic material. Interestingly, this final aqueous eluent remained a deep red color, reminiscent of pyrroloquinoline pigments, and a cursory examination of the ^{1}H NMR spectrum (CD_3OD) of a lyophilized portion of this deep red aqueous eluent revealed resonances typical of a simple ribofuranosylpyrroloquinoline compound (δ_H 7.54, 6.73, 3.80–4.04, 3.42, 2.73). From the ^{1}H NMR spectrum it was evident that this compound not only was different from compounds **3** and **4**[8] but was also present in significant quantities. Consequently, the remainder of the aqueous HP-20 column eluent was lyophilized and subjected to solid-phase extraction (C18 Sep-Pak, MeOH/H_2O/0.05% TFA), which effectively removed most of the unwanted inorganic salts and provided initial access to the pyrroloquinoline metabolites. The Sep-Pak fraction eluted with 9:1 H_2O/MeOH/0.05% TFA was further purified using gradient reversed-phase HPLC (H_2O/MeOH/0.05% TFA) to yield makaluvic acid C (**1**) (2.3 mg), while the Sep-Pak fraction eluted with 4:1 H_2O/MeOH/0.05% TFA afforded *N*-1-*β*-D-ribofuranosylmakaluvic acid C (**2**) (31.6 mg). Our earlier reservations about the instability of ribofuranosylpyrroloquinoline metabolites in mildly acidic conditions[8] appeared to have been misplaced, as no noticeable hydrolysis in the mildly acidic chromatography solvents was observed from careful ^{1}H NMR monitoring of the fractions before and after solid-phase extraction.

High-resolution FABMS established the molecular formula of **1** as $C_8H_8N_2O_3$. Analysis of the ^{1}H, ^{13}C, and HSQC NMR spectra revealed the presence of two carbonyls (δ_C 166.8, 160.0), a protonated aromatic center (δ_C 118.6, δ_H

10.1021/np049589w CCC: $30.25 © 2005 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/22/2005

Chart 1



6.92), three quaternary aromatic carbons (δ_C 123.7, 122.6, 115.1), a deshielded aminomethylene (δ_C 41.3, δ_H 3.42), a methylene (δ_C 19.6, δ_H 2.73), and three exchangeable proton resonances (δ_H 16.09, 12.35, 8.62) (Table 1). These resonances accounted for four of the six degrees of unsaturation required by the molecular formula and implied a bicyclic structure for **1**. Bicyclic pyrroloquinoline metabolites are ring-opened analogues of the tricyclic pyrrolo[4,3,2-*de*]quinoline nucleus, and only four examples, makaluvic acids A and B (**8**, **9**),[10] secobatzelline A (**10**), and the potentially artifactual quinone secobatzelline B (**11**),[5,11] have been previously isolated from marine sponges. A putative biosynthesis of makaluvic acids, via oxidative cleavage and decarboxylation of a pyrroloquinoline precursor (accessible from phenylalanine), is presented in Scheme 1.[5,12,20]

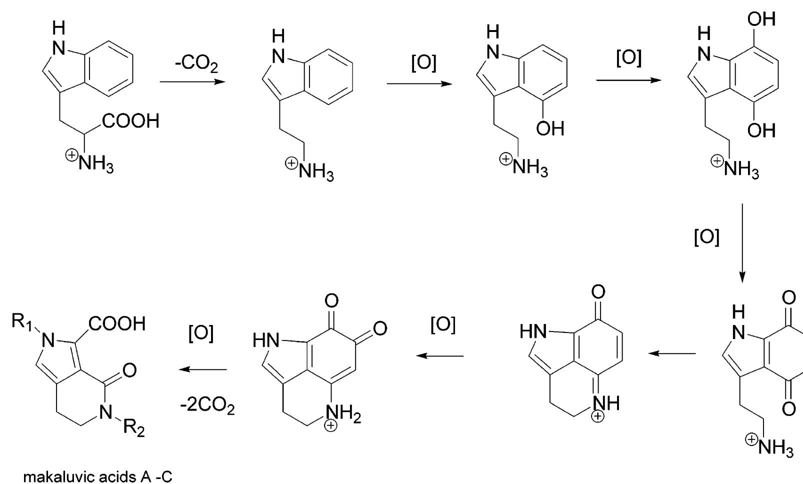
The COSY and HMBC data of **1** allowed facile assignment of the fused pyrrole and lactam rings of **1** and immediately confirmed that **1** was a third member of the makaluvic acid and not the secobatzelline series. HMBC correlations observed from both H-4 methylene protons (δ_H 3.42) and NH-5 (δ_H 8.62) to the lactam carbonyl C-6 (δ_C 166.8), from H-2 (δ_H 6.92), 2H-3 (δ_H 2.73), and 2H-4 to the ring junction carbon C-2a (δ_C 122.6), and from H-2, 2H-3, and NH-5 to the other ring junction carbon C-6a (δ_C 115.1) delineated the bicyclic structure of **1**. Further support for the bicyclic structure of **1** followed from the COSY spectrum, in which a long-range coupling was observed between H-2 and 2H-3. A two-bond HMBC correlation between the exchangeable proton 8-OH (δ_H δ 16.09) and the carboxylic acid carbonyl C-8 (δ_C 160.0) secured the carboxylic acid structure of the substituent on the bicyclic nucleus. Although there were no HMBC correlations observed between the 8-OH and any of the bicyclic ring carbons, the carboxy-

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of **1** and **2**

	1[a]			**2**[b]		
position	δ_C	δ_H	mult. (*J*), int.	δ_C	δ_H	mult. (*J*), int.
1		12.35	s, 1H			
2	118.6	6.92	br t (2.6), 1H	119.9	7.54	s, 1H
2a	122.6			123.8		
3	19.6	2.73	td (6.9, 0.9), 2H	22.0	2.73	t (6.5), 2H
4	41.3	3.42	tt (6.9, 2.47), 2H	42.3	3.42	t (6.5), 2H
5		8.62	br s, 1H			
6	166.8			167.6		
6a	115.1			118.1		
7	123.7			126.0		
8	160.0			164.6		
8-OH		16.09	s, 1H			
1′				93.3	6.73	d (2.4), 1H
2′				78.8	4.05	m, 1H
3′				70.2	4.14	dd (5.0, 7.2), 1H
4′				85.0	4.04	m, 1H
5′				61.9	3.94	dd (2.6, 12.4), 1H
					3.80	dd (3.7, 12.4), 1H

[a] D_6-DMSO. [b] CD_3OD.

Scheme 1. Possible Biosynthetic Pathway to the Makaluvic Acids from a Phenylalanine Precursor (adapted from Lill et al.[12] and Urban et al.[20])



lic acid substituent was placed on the deshielded quaternary carbon C-7 (δ_C 123.7) from comparison of the ^{13}C chemical shifts of **1** with those of **8** and **9**.[10]

High-resolution FABMS data provided a molecular formula of $C_{13}H_{16}N_2O_7$ for **2**, and although the enhanced structural complexity of **2** (cf. **1**) was immediately apparent from the ^1H and ^{13}C NMR spectra of this compound, there was sufficient congruency between the NMR data of **1** and **2** to suggest that these two compounds possessed the same pyrroloquinoline skeleton and differed only in substitution at N-1 in **2**. The unsaturated bicyclic pyrroloquinoline accounted for six of the seven degrees of unsaturation required by the molecular formula and, in the absence of any spectroscopic evidence in support of a further olefin, implied that **2** was tricyclic. The pentose structure of the third ring in **2** was supported by the COSY spectrum, in which a contiguous coupling sequence was observed from an anomeric proton (δ_H 6.73, d, J = 2.4 Hz), via three oxymethine protons (δ_H 4.05, m; 4.14 dd, J = 5.0, 7.2 Hz; 4.04, m) to a pair of diastereotopic oxymethylene protons (δ_H 3.80, dd, J = 3.7, 12.4; 3.94 dd, J = 2.6, 12.4). A further ^4J *W*-coupling from the anomeric proton H-1′ to H-4′ (δ_H 4.04) and a three-bond HMBC correlation between H-1′ and C-4′ (δ_C 85.0) unequivocally established that the sugar moiety was present in a furanose and not a pyranose form (Figure 1). The attachment of the sugar at N-1 in the makaluvic acid nucleus was determined from reciprocal HMBC correlations between H-1′ and C-2 (δ_C 119.9) and from H-2 (δ_H 7.54) to C-1′ (δ_C 93.3), and also from a ^4J coupling observed between H-1′ and H-2 in the COSY spectrum of **2** (Figure 1).

The chemical shifts and multiplicities of the ^1H and ^{13}C resonances assigned to the furanose ring of **2** were consistent with those observed for the ribofuranose moieties in *N*-1-β-D-ribofuranosyldamirone C (**3**) and *N*-1-β-D-ribofuranosylmakaluvamine I (**4**).[3] Chiral gas chromatographic analysis of the peracetylated aldononitrile derivatives[13] of pentose sugars secured the D-ribose structure of the pentose sugars hydrolyzed from **3** and **4**, and this technique was similarly used to confirm both the identity and configuration of the pentose moiety in **2**. Accordingly, the retention time (19.46 min) on a Chirasil-Val chiral GC column of the peracetylated aldononitrile derivative of the ribose present in the acid (1:1 MeOH/1 M TFA) hydrolysate of **2** was compared with the retention times of the peracetylated aldononitrile derivatives of authentic samples of D- and L-ribose (19.41 and 19.81 min, respectively) to unequivocally establish the D-ribose structure of the pentose substituent in **2**. Having secured the absolute configurations at C-2′−C-4′ in the pentose substituent in **2**, NOE and COSY data was used to determine the orientation of the glycosidic bond at C-1′. A NOESY correlation between H-1′ and H-4′, along with the *W*-coupling observed between these two protons, confirmed the *cis* relationship between these two protons and thus required a β-orientation of the bicyclic makaluvic acid moiety at the anomeric carbon in **2**.

The recently described *Strongylodesma* genus is proving to be both a taxonomic and chemotaxonomic enigma. Taxonomically, the genus *Strongylodesma* is the only genus within the family Latrunculiidae in which the constituent species do not contain discorhabd microscleres, the distinctive small spicules that have traditionally been one of the diagnostic characteristics linking the genera within this family.[4,5] The global distribution of species within the genus *Strongylodesma* is relatively unknown and possibly underestimated, leading to a number of sponges being misidentified in the past, usually as either *Prianos* or *Batzella* species.[4,5] For example three Caribbean deep-water *Batzella* species [sources of batzellines A−C (**12**−**14**),[14] secobatzellines A and B (**10**, **11**),[11] and isobatzellines A−D (**15**−**18**)[15]] and the Okinawan sponge *Prianos melanos* [the original source of prianosin/discorhabdin A (**19**) and discorhabdins D (**20**) and 2-hydroxydiscorhabdin D (**21**)[16−18]] have been recently reassigned to the genus *Strongylodesma*.[4,5,19] Our recent isolation of **3**−**7** from *S. aliwaliensis*[8] and discorhabdins A (**19**), D (**20**), and H (**22**) from *S. algoaensis*[7] suggests that this genus is a reservoir of most of the major classes of pyrroloquinoline metabolites, viz., bicyclic makaluvic acids and secobatzellines, tricyclic batzellines, isobatzellines and damirones, tri- and tetracyclic makaluvamines, and hexa- and heptacyclic discorhabdins.

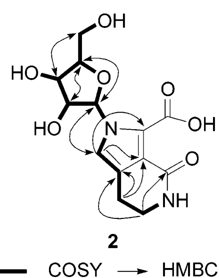


Figure 1. Key COSY and HMBC correlations observed for compound **2**.

In the past, chemotaxonomic trends within marine sponges producing pyrroloquinoline metabolites have suggested that discorhabdins and makaluvamines are definitive chemotaxonomic markers for *Latrunculia* (family Latrunculiidae) and *Zyzzya* (family Acarnidae) species, respectively.[5,20] With the likelihood of further new *Strongylodesma* species (possibly containing a cohort of new pyrroloquinoline metabolites) being discovered in the future, this broad chemotaxonomic generalization may have to be treated with some circumspection.

Esophageal cancer is a prevalent form of cancer among the rural populations in southern Africa resulting from a combination of several extraneous factors including poor diet, the inadvertent ingestion of carcinogenic fungal toxins from incorrectly stored grain, and the continuous exposure of many individuals to excessive wood and cigarette smoke.[21] With the incidence rates of esophageal cancer in southern Africa greater than those observed in most other parts of the world we have initiated a program in South Africa to search for potential antiesophageal cancer agents from marine organisms.[22] As part of this ongoing program, the cytotoxicity of the two pyrroloquinoline natural products isolated during this study, in addition to four of the five metabolites we isolated during our earlier investigation of *S. aliwaliensis* extracts,[8] were evaluated against the WHCO-1 (esophageal) cancer cell line. Regrettably a paucity of makaluvamine I (**6**) prevented its inclusion in the cytotoxicity assays. While makaluvic acid C (**1**) was inactive ($IC_{50} > 150\ \mu M$), *N*-1-β-D-ribosylfuranosylmakaluvic acid C (**2**), *N*-1-β-D-ribofuranosyldamirone C (**3**), and damirone C (**5**) exhibited moderate cytotoxicity against esophageal cancer cells (IC_{50} 61, 38, and 56 μM, respectively) when compared to the commonly used chemotherapeutic agent cisplatin, which has an IC_{50} against this cell line of 13 μM.[23] Conversely, makaluvamine M (**7**) and *N*-1-β-D-ribofuranosylmakaluvamine I (**4**) both exhibited good activity against esophageal cancer cells (IC_{50} 0.7 and 1.6 μM, respectively). The latter two results continue to support the generally accepted hypothesis that the presence of a pyrroloiminoquinone functionality is necessary for potent cytotoxicity in pyrroloquinoline metabolites.[5]

Experimental Section

General Experimental Procedures. The optical rotation was measured using a Perkin-Elmer 141 polarimeter at the sodium D line (598 nm), and UV spectra were recorded using a Varian Cary 500 UV–vis–NIR spectrophotometer. The NMR spectra were measured on a Bruker AVANCE 400 MHz spectrometer using standard pulse sequences. Chemical shifts are reported in ppm and referenced to residual solvent resonances (CD_2HOD δ_H 3.30, δ_C 49.00 and d_5-DMSO δ_H 2.50, δ_C 39.52),[24] and coupling constants are reported in Hz. HRFABMS data were obtained on a JEOL SX102 spectrometer by L. Fourie of the Mass Spectrometry Unit at the Potchefstroom campus of the North West University. Chiral GC analyses were performed on an HP-6890 series system using an Altech Chirasil-Val column (25 m × 0.25 mm) using helium carrier gas and a TCD detector. Diaion HP-20 polystyrene beads (supplied by Supelco) and Waters C18 Sep Pak cartridges were used for the initial chromatographic separations. High-performance liquid chromatography was performed using a Phenomenex Lunar C18 column (9 mm i.d., 25 cm) on an HP Agilent 1100 series gradient HPLC system equipped with diode array detection.

Animal Material. Specimens of *Strongylodesma aliwaliensis* Samaai, 2004 (Porifera; Demospongiae, Poecilosclerida; Latrunculiidae) were collected by scuba from depths of 15–18 m off the Aliwal Shoal, an offshore reef system running along much of the KwaZulu-Natal coast of South Africa, in the winter of 1994. The holotype of this species (SAM H-5083) is deposited in the South African Museum, while the paratype SAF94-023 is retained in the Rhodes University marine invertebrate collection.[4]

Extraction and Isolation. All sponge material was frozen immediately after collection. Freeze-dried specimens of *S. aliwaliensis* (180 g) were exhaustively extracted with MeOH. The MeOH extract was partitioned between H_2O and EtOAc. The aqueous phase was evaporated to yield a dark brown gum (20.1 g). A portion of this gum (2.01 g) was dissolved in MeOH (250 mL) and passed through a column of HP-20 resin (2.0 × 25.0 cm, 80 mL). The eluent was diluted with H_2O (250 mL) and passed through the column again. The resulting eluent was diluted with H_2O (500 mL) and passed through the column one final time. The final eluent from the loading of the HP-20 column was lyophilized to yield a red gum, which was redissolved in 0.05% $TFA_{(aq)}$ (10 mL), and the aqueous solution passed through a 35 mL C18 Sep-Pak cartridge. The cartridge was subsequently sequentially eluted with 100 mL volumes of (1) 0.05% $TFA_{(aq)}$, (2) 10% MeOH/0.05% $TFA_{(aq)}$, (3) 20% MeOH/0.05% $TFA_{(aq)}$, (4) 30% MeOH/0.05% $TFA_{(aq)}$, (5) 50% MeOH/0.05% $TFA_{(aq)}$, and (6) MeOH. Fraction 2 (160 mg) was further purified using gradient HPLC on a C18 column (eluting at 4 mL/min from 0 to 40% MeOH/0.05% $TFA_{(aq)}$ over 40 min). Under these conditions makaluvic acid C (**1**, 2.3 mg) eluted with a retention time of 28.7 min. Fraction 3 (120 mg) from the Sep-Pak separation was similarly purified using gradient C18 HPLC (eluting at 4 mL/min from 10 to 30% MeOH/0.05% $TFA_{(aq)}$ over 20 min, held at 30% MeOH/0.05% $TFA_{(aq)}$ for 10 min). Under these conditions *N*-1-β-D-ribosylfuranosylmakaluvic acid C (**2**, 31.6 mg) eluted with a retention time of 8.6 min.

Makaluvic acid C (1): brown solid; UV (MeOH) λ_{max} (log ϵ) 200 (4.30), 260 (4.02), 289 (3.94); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m/z* $[M + H]^+$ 181.0612 (calcd for $C_8H_9N_2O_3$, 181.0613).

***N*-1-β-D-Ribosylfuranosylmakaluvic acid C (2):** brown solid; $[\alpha]_D^{26}$ +41.2° (*c* 0.0003, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.93), 260 (3.70), 286 (3.61); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m/z* (M^+) 312.0958 (calcd for $C_{13}H_{16}N_2O_7$, 312.0957).

Preparation of the Peracetylated Aldononitrile Derivatives of Pentose Sugars.[13] A portion of **2** (5.0 mg) was hydrolyzed by refluxing in 1:1 MeOH/1 M $TFA_{(aq)}$ for 8.5 h, after which the solvents were evaporated *in vacuo*. D-Ribose (4.1 mg), L-ribose (5.9 mg), and the hydrolysis product of **2** were each separately dissolved in H_2O (0.2 mL), to which a solution of $NH_2OH.HCl$ (10 mg) dissolved in *N*-methylimidazole (0.4 mL) was added. All three resultant solutions were stirred at 80 °C for 10 min. Portions of Ac_2O were added slowly to each flask (4 × 0.25 mL), after which they were again heated at 80 °C for 2 h. Each reaction mixture was extracted with $CHCl_3$ (5 mL) and the $CHCl_3$ extract washed sequentially with H_2O (3 mL), saturated $NaHCO_{3(aq)}$ (3 mL), 10% $H_2SO_{4(aq)}$ (3 mL), and finally H_2O (2 × 3 mL). The $CHCl_3$ layer was dried over anhydrous $MgSO_4$, filtered, evaporated to dryness under N_2, and taken up in $CHCl_3$ (50 μL) for GC analysis.

Chiral-GC Analysis of the Peracetylated Aldononitrile Derivatives of Pentose Sugars. Chiral-GC analyses of the derivatized D- and L-ribose and hydrozylate prepared from **2** were performed on a Chirasil-Val capillary column.[8] The GC oven was held at 100 °C for 1 min, after which it was ramped at 4 °C/min to 180 °C, at which point it was held for a further 2 min. The peracetylated aldononitrile derivatives of the hydrolyzed natural product, D- and L-ribose, were eluted with retention times of 19.46, 19.41, and 19.81 min, respectively.

Cell Culture. Cells were routinely maintained at 37 °C and 5% CO_2. WHCO-1 cells were maintained in DMEM, supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

MTT Assay. IC_{50} determinations were carried out using the MTT kit from Roche (Cat #1465007), according to manufacturer's instructions. Briefly, 1500 cells were seeded per well in 96-well plates. Cells were incubated (24 h), after which aqueous DMSO solutions of each compound (10 μL, with a constant final concentration of DMSO = 0.1%) were plated at

various concentrations. After 48 h incubation, observations were made, and MTT (10 μL) solution was added to each well. After a further 4 h incubation, solubilization solution (100 μL) was added to each well, and plates were incubated overnight. Plates were read at 595 nm on an Anthos microplate reader.

Acknowledgment. B. Carte and C. Buxton formerly of SmithKline Beecham and Rhodes University, respectively, are thanked for the original scuba collection of *S. aliwaliensis* a decade ago. Financial assistance in the form of research funding (M.D-C, T.S.) and an NRF postdoctoral bursary (R.A.K.) and Ph.D. bursary (C.E.A.) from the South African National Research Foundation and the Department of Environmental Affairs and additional financial support from the University of Cape Town and Cancer South Africa (D.T.H, C.E.A.) and a DAAD fellowship (C.E.A.) is gratefully acknowledged. A. Sonemann (Rhodes University) and L. Fourie (North West University) are thanked for assistance with the chiral GC analyses and acquisition of HRFABMS data, respectively.

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NP049589W