Occurrence of the α-Glucosidase Inhibitor 1,4-Dideoxy-1,4-imino-D-arabinitol and Related Iminopentitols in Marine Sponges[⊥]

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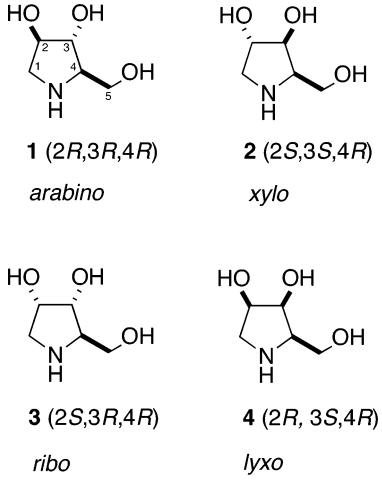
The α-glucosidase inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol (**1**) was isolated from two marine sponges collected in Western Australia and shown by LC-MS to be responsible for the α-glycosidase inhibitory activity in different sponge extracts collected over a wide geographic area. The configuration of **1** was determined by application of Marfey's method. The two most inhibitory extracts contained only **1**, while the less inhibitory extracts contained 1,4-dideoxy-1,4-imino-D-xylitol (**2**) or the putative diastereomeric imino pentitols **3** and **4**. The least active or inactive extracts showed no detectable imino pentitols. While both **1** and **2** are known from plants, this is the first report on the isolation and detection of **1** and **2** in marine invertebrates.

α-Glucosidases carry out hydrolytic cleavage of glucose from the nonreducing end of substrates bearing α-glucosidic linkage, such as disaccharides, oligosaccharides, and aryl glucosides.[1] This class of enzyme is involved in several important biological processes including digestion and maturation of glycoproteins. Inhibition of α-glucosidase activity blocks the trimming of glucose in the protein−$GlcNAc_2Man_9Glc_3$ complex and the cascade of downstream steps in the biosynthesis of glycoproteins.[2] In vitro studies have shown that α-glucosidase inhibitors prevent replication of viruses (e.g., HIV and hepatitis B) by disrupting the proper folding of mature viral glycoproteins that require the aid of the chaperone calnexin.[5] α-Glucosidase is also expressed in the microvilli of the small intestine. Since only monosaccharides can be absorbed and taken up through the small intestine, α-glucosidase is required to catalyze the breakdown of sugars in the final step of carbohydrate digestion.[6,7] Aza sugar mimics that act as inhibitors of this enzyme were found to be therapeutically relevant for the treatment of type II non-insulin-dependent diabetes mellitus by interfering with the enzymatic action in the bowel, slowing the breakdown of dietary polysaccharides and disaccharides to glucose. These assist in delaying glucose absorption, decreasing postprandial plasma glucose level, and avoiding the late complications associated with diabetes.[7] Small-molecule inhibitors of α-glucosidase may be useful for the development of treatments for AIDS and diabetes and as adjuvants for the opportunistic fungal diseases that often accompany these disease states.

In a search for α-glucosidase inhibitors, we screened our collection of marine invertebrates ($n = 543$). Preliminary studies[8] showed that the crude extract of 15 sponges possessed strong in vitro inhibitory activity against yeast α-glucosidase (E.C. 3.2.1.20, an amyloglucosidase), with IC_{50}'s ranging from 0.28 to 23.7 μg/mL. This finding guided the isolation of the active component and led to its identification as the known compound 1,4-dideoxy-1,4-imino-D-arabinitol (**1**). Iminopentitol **1** was first isolated as a natural product from the fruits of *Angylocalyx boutiqueanus*[4a] and subsequently from the leaves and roots of *Morus* spp.[4b] Polyhydroxylated pyrrolidines related to the diastereomeric iminopentitols **1**−**4** have been isolated from various terrestrial plants[4,5,9,10] and are known glycosidase inhibitors. There are currently only five reports on the isolation of α-glucosidase inhibitors from marine sponges,[11−15] but none of these describe polyhydroxylated pyrrolidines. This paper reports the isolation of **1** from *Haliclona* sp. and *Raispalia* sp. and the detection of 1,4-dideoxy-1,4-imino-D-xylitol (**2**) and isomeric polyhydroxylated pyrrolidines, tentatively identified as **3** and **4**, in other marine sponges.



1 (2*R*,3*R*,4*R*) *arabino*

2 (2*S*,3*S*,4*R*) *xylo*

3 (2*S*,3*R*,4*R*) *ribo*

4 (2*R*, 3*S*,4*R*) *lyxo*

The sponge *Haliclona* sp. (93−111) was collected in Exmouth Gulf, Western Australia, and kept frozen (−20 °C) until needed. Samples of the sponge were extracted three times with MeOH, the combined extracts were concentrated to dryness and triturated with anhydrous MeOH, and the MeOH extract was partitioned against hexane. The defatted MeOH extract was fractionated by size-exclusion chromatography (Sephadex LH-20, elution with MeOH) to yield seven fractions. The third fraction, exhibiting strong inhibition of α-glucosidase, was passed through a reversed-phase solid-phase extraction cartridge (SPE) and finally purified by reversed-phase (C_{18}) HPLC to give compound **1** as a white solid (1.3 mg, 0.0030% of wet weight). Compound **1** elicited a yellow color upon exposure to ninhydrin (TLC), which was suggestive of a secondary amine.[16,17] ESIMS analysis of **1** gave a pseudomolecular ion at *m*/*z* 134 $[M + H]^+$ consistent with known polyhydroxylated pyrrolidines.[4,16,17] The formula, $C_5H_{11}NO_3$, was established by HRTOFMS at *m*/*z* 134.08076 $[(M + H)^+$ (Δ −0.41 mmu)], which indicated one degree of unsaturation. The presence of one ring was inferred by the absence of olefinic signals in the ^1H and ^{13}C NMR spectra.

[⊥] Dedicated to the late Dr. Kenneth L. Rinehart of the University of Illinois at Urbana−Champaign for his pioneering work on bioactive natural products.

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Table 1. ^{1}H NMR Data for Compound **1** (400 MHz, D_2O)

position	δ, ppm [mult., J (Hz)] isolated **1**	authentic **1**
H_a-1	3.34 dd (2.8, 12.4)	3.38 dd (2.8,12.8)
H_b-1	3.56 dd (4.4, 12.4)	3.60 dd (4.8, 12.8)
2	4.34 ddd (2.6, 2.8, 4.8)	4.36 ddd (2.4, 2.8, 4.4)
3	4.09 dd (3.2, 3.6)	4.12 dd (3.2, 3.6)
4	3.59 ddd (4.4, 4.8, 8.0)	3.63 ddd (4.0, 4.2, 8.4)
H_a-5	3.84 dd (8.0, 12.0)	3.86 dd (8.0, 12.0)
H_b-5	3.96 dd (4.8, 12.0)	3.98 dd (4.8, 12.0)

Table 2. Paired HPLC-MS Analysis of D- and L-Marfey's Derivatives of Iminopentitols

entry no.	compd[a]	source	D-Marfey's derivative, t_R (min)	L-Marfey's derivative, t_R (min)
1	**1**	Sigma (St. Louis, MO)	8.5	10.2
2	**2**	IRL (New Zealand)	8.9	11.9
3	**1**	93-111 (*Haliclona* sp.)	8.7	9.9
4	**1**	93-111 (*Haliclona* sp.) + standard		10.1
5	**1** + **2**	93-111 (*Haliclona* sp.) + standard		10.2, 11.9
6	**1**	93-073 (*Raspailia* sp.)	8.6	10.3
7	**1**	93-073 (*Raspailia* sp.) + standard		10.3
8	**1** + **2**	93-073 (*Raspailia* sp.) + standard		10.2, 11.9

[a] For entry nos. 4, 5, 7, and 8, authentic **1** or **2** was co-mixed with the extract prior to derivatization.

A search of a proprietary database (MarinLit)[18] did not give a match for any known marine natural product. The ^{1}H NMR signals at δ 4.36 (H-2), 4.12 (H-3), 3.86 (H-5a), and 3.98 (H-5b) were shown from the COSY spectrum to constitute a linear spin sequence (Table 1), characteristic of a pyrrolidine-type 1,4-dideoxy-4-hydroxymethyliminopentitol,[4] and strongly suggested a structure corresponding to **1**. This was confirmed by comparisons (^{1}H NMR and ^{13}C NMR) with an authentic sample of 1,4-dideoxy-1,4-imino-D-arabinitol and from the LC-MS retention times of the corresponding Marfey's derivatives (see below).

Authentic **1**, **2**, and the sponge-derived compound were reacted separately with both L-2-(5-fluoro-2,4-dinitrophenylamino)propanamide (L-Marfey's reagent) and the D-enantiomer under standard conditions,[19] and the derivatives were analyzed using reversed-phase LC-MS coupled with single-ion monitoring (SIM) mass spectrometry (*m*/*z* 386). The retention times of the D- and L-Marfey's derivatives of isolated **1** both closely matched those of authentic **1** (8.5 and 10.2 min, respectively, Table 2). Spiking of the sponge-derived compound with authentic (2*R*,3*R*,4*R*)-**1** prior to Marfey's derivatization gave a single peak at t_R = 10.1 min (entry 4, Table 2), while co-addition of authentic **2** showed two peaks at 10.2 and 11.9 min (entry 5, Table 2). Thus, it is shown unequivocally that isolated **1** has the configuration 2*R*,3*R*,4*R*.

Extraction and purification of a second sponge with glycosidase inhibitory activity, *Raspailia* sp. (93-073), collected from the same location, was carried out using a protocol similar to that described above and also gave **1** (1.1 mg, 0.0014% of wet weight), of the same configuration.

In order to ascertain the distribution of iminopentitols in marine invertebrates, a selection of the 15 most active extracts from the total of 543 was assayed quantitatively for the presence of **1** and **2** by extraction and treatment of partially purified extracts with L-Marfey's reagent followed by LC-MS analysis as described above. Only two of the 15 samples with glycosidase activity showed the presence of **1** (entries 3 and 4, Table 3), while another sample of *Raspailia* sp. (entry 6) contained **2**. Two specimens (entries 5 and 8) showed LC-MS peaks with a mass (*m*/*z*) isomeric with the Marfey's derivatives of **1** and **2**, but at a different retention time of

Table 3. α-Glucosidase Inhibition and Iminopentitol Content of Sponge Extracts by LC-MS L-Marfey's Analysis

entry no.	sample	sponge ID	IC_{50}, μg/mL[a]	L-Marfey's derivative, t_R (min)[b]	mg imino-pentitol/g crude extract[c]
1	**1**		0.16	10.2	
2	**2**			11.9	
3	93-111	*Haliclona* sp.	0.28	10.2	12.6
4	93-073	*Raspailia* sp.	1.23	9.9	7.8
5	93-093	*Raspailia* sp.	4.11	15.3	0.2
6	93-027	*Raspailia* sp.	8.38	11.8	6.6
7	93-105	*Cymbastela* sp.	10.0	12.8	3.6
8	01-118	*Haliclona* sp.	12.0	15.3	2.1
9	95-024	U[d]	12.2	n.d.[e]	<0.026
10	01-162	U[d]	12.4	n.d.[e]	<0.026
11	93-134	U[d]	13.0	n.d.[e]	<0.026
12	93-058	U[d]	13.1	n.d.[e]	<0.026
13	93-066	U[d]	17.3	n.d.[e]	<0.026
14	99-016	U[d]	19.7	n.d.[e]	<0.026
15	93-077	U[d]	21.1	n.d.[e]	<0.026
16	95-064	U[d]	22.2	n.d.[e]	<0.026
17	01-129	U[d]	23.7	n.d.[e]	<0.026

[a] Sponge samples are ordered by decreasing activity against yeast α-glucosidase (E.C. 3.2.1.20); IC_{50} data from ref 8. [b]Observed t_R for SIM at *m*/*z* 386m. LC conditions are described in the Experimental Section. [c]Authentic **1** co-mixed with extracts prior to derivatization. [d]U = Unidentified sponge. [e]n.d. = concentration is below the LOD (314 ng/mL).

t_R = 15.3 min. A third sponge, *Cymbastela* sp. (entry 7), gave rise to a Marfey's derivative with retention time t_R = 12.8 min. These latter two peaks represent derivatives of iminopentitols that are isomeric with **1** and **2** and are most likely to be the *ribo* and *lyxo* diastereomers **3** and **4** or their enantiomers. The lack of authentic samples of **3** and **4** precluded verification of the identities of these putative diastereomers, and insufficient material was available for bioassay-guided purification of the active components. Thus, the presence of **3** and **4** in these sponges is presently only a tentative proposition. The remaining sponge extracts in the panel with α-glycosidase inhibitory activity showed no detectable **1**, **2**, or isomers thereof. We conclude that the α-glycosidase inhibitory activity of these specimens is likely associated with compounds of a different chemical class than iminopentitols and is worthy of further investigation. This is the subject of ongoing research in our laboratory.

The emergence of the same iminopentitols from sponges collected over widely different geographic regions leads to speculation that the compounds may actually be produced from symbiotic microorganisms. Production of alkaloid natural products by endophytic fungi within grasses and legumes has been demonstrated. For example, species in the genera *Astragalas* and *Oxytropis* (locoweed) from Western United States contain entomopathogenic species of the fungus *Embellisia* that are responsible for the production of swainsonine,[20] an α-mannosidase inhibitor that is the toxic agent responsible for some forms of "locoism" in cattle that consume the plants. Fungi have been isolated from marine sponges and shown to produce familiar classes of natural products under fermentative culture conditions,[21] but not in whole sponge tissue. It remains to be proven that symbiotic fungi in sponges are responsible for sponge-derived natural products.

In conclusion, we have identified the iminopentitols **1** and **2** as responsible for α-glycosidase inhibitory activity in a prescreened panel of extracts obtained from marine sponges in Exmouth Gulf, Western Australia. We estimate that the titers of **1** and **2** present in the most highly active extracts are sufficient to account for total α-glycosidase inhibitory activity within the sponges. Paired analysis, using D- and L-Marfey's reagents and LC-MS, is a sensitive and unambiguous method for both quantitation *and* configurational analysis of iminopentitols in crude extracts. α-Glycosidase inhibitory activity in the remaining nine extracts could not be accounted for by the presence of **1** or **2** and is likely attributable to

α-glucosidase inhibitors of different chemical structures. This is the first report on the isolation of **1** and the detection of **2**−**4** from marine sponges

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded in D_2O using a Varian INOVA NMR spectrometer at 400 and 100 MHz, respectively. Chemical shifts are referenced to internal dioxane (δ_H 3.76 ppm) added as a secondary standard and calibrated to external standard sodium 3-(trimethylsilyl)propionate (TSP) in D_2O (δ_H 0.00 ppm). HPLC-ESIMS data were recorded using an Agilent HPLC 1100 coupled to a Thermo Finnigan Surveyor MSQ mass spectrometer. Size-exclusion chromatography was done using Sephadex LH-20 (Amersham Biosciences) or Toyopearl HW-40F (Tosoh Bioscience). 1,4-Dideoxy-1,4-imino-D-arabinitol (**1**) (Sigma, St. Louis, MO) and 1,4-dideoxy-1,4-imino-D-xylitol (**2**) (Industrial Research Ltd, New Zealand) were obtained as HCl salts and used as received.

Animal Material. The sponges were collected by hand using scuba in Western Australia in 1993 (accession number prefix "93-"), Key Largo, Florida, in 1995 (accession number prefix "95-"), Bahamas in 1999 ("99-"), and Pohnpei, Federated States of Micronesia in 2001 ("01-"). The materials were frozen immediately after collection and stored at −20 °C until use. Voucher specimens are archived in the Department of Chemistry and Biochemistry, University of California at San Diego.

Extraction and Isolation. A frozen sample of *Haliclona* sp. (93-111; 44.0 g) was extracted exhaustively with MeOH overnight at room temperature (200 mL × 3), and the combined extracts were concentrated to dryness (2.0 g) under reduced pressure and triturated in anhydrous MeOH. The MeOH solution was filtered, concentrated, redissolved in MeOH, and partitioned against hexane. The MeOH-soluble portion (680 mg) was fractionated by elution through a column of Sephadex LH-20 (120 × 2 cm, MeOH) to yield seven fractions. The third bioactive fraction (61.0 mg) was passed through a reversed-phase SPE cartridge (Phenomenex Strata, C_{18} bonded phase) with stepwise gradient elution using aqueous MeOH (80−100% MeOH). The first fraction (24 mg) was purified by reversed-phase HPLC (Dynamax, 5 μm, C_{18} column, 10 × 250 mm, 9:1 H_2O/MeOH + 0.1% TFA) to afford **1** (1.3 mg, 0.0030% of wet weight). A frozen specimen of *Raspailia* sp. (93-073; 990.0 g) was extracted in the same manner as above (3.5 L MeOH × 2), and the crude extract (32.6 g) was triturated in anhydrous MeOH, partitioned in hexane, and concentrated to dryness. A portion of the dried MeOH extract (2.50 g) was fractionated using Toyopearl HW-40F (1:1 H_2O/MeOH). Fraction 7 (6.0 mg) was purified by reversed-phase HPLC (Dynamax, 5 μm, C_{18} column, 10 × 250 mm, 100% MeOH + 0.05% TFA) to afford **1** (1.1 mg, 0.0014% of wet weight).

1,4-Dideoxy-1,4-imino-D-arabinitol (1): white solid; ^{1}H NMR data (400 MHz, D_2O), see Table 1; ESIMS *m*/*z* 134 $[M + H]^+$; HRTOFMS *m*/*z* 134.08076 $[M + H]^+$, calcd for $C_5H_{12}NO_3$, 134.08117.

Configuration of Isolated 1,4-Dideoxy-1,4-imino-D-arabinitol (1). Marfey's Method.[19] The isolated **1** was dissolved in water, and an aliquot (25 μL, equivalent to ∼20 μg) was heated for 10 min at 80 °C with either a solution of 5-fluoro-2,4-dinitrophenyl-5-D-alaninamide (50 μL, 1% w/v in acetone) or the enantiomeric 5-fluoro-2,4-dinitrophenyl-5-D-alanine amide (50 μL) in the presence of 1.0 M $NaHCO_3$ (5 μL). The mixture was cooled to rt and quenched with 1 N HCl (5 μL) prior to analysis by reversed-phase HPLC-ESIMS (Phenomenex Luna, 3 μm, C_{18} column, 2.0 × 100 mm, 0.4 mL/min, SIM *m*/*z* 386, gradient of 90:10 H_2O/CH_3CN to 65:35 H_2O/CH_3CN + 0.05% TFA over 42 min). Aliquots of authentic **1** and **2** (25 μL, equivalent to 50 μg) were also treated in the same manner as above. Extracts were spiked with authentic standards as follows: to an aliquot of the isolate (∼20 μg) was added 8.5 μg of authentic **1** or **2**, and the mixture was diluted with water to 25 μL and treated with Marfey's reagent as above prior to HPLC-ESIMS analysis.

Screening of Extracts for 1,4-Dideoxy-1,4-imino-D-arabinitol (1). An aliquot (5 mL) of the crude MeOH extracts was concentrated under reduced pressure and triturated in 5 mL of anhydrous MeOH, and the MeOH-soluble fraction was partitioned with 5 mL of hexane. Three portions of the defatted crude extracts (1 mg each) were spiked with authentic **1** as follows: 0.0, 8.5, and 34 μg, respectively. The extracts were diluted with H_2O to 25 μL and heated for 10 min at 80 °C with a solution of 5-fluoro-2,4-dinitrophenyl-5-D-alanine amide (1% in acetone, 50 μL) in the presence of 1.0 M $NaHCO_3$ (5 μL). The mixture was cooled to rt, quenched with 1 N HCl (5 μL), and blown to dryness with a stream of N_2. The dried mixture was dissolved in 35:50 H_2O/acetone and analyzed by reversed-phase HPLC-ESIMS.

α-Glucosidase Inhibitory Activities. Extracts and isolates were assayed in DMSO in 96-well plates. Yeast α-glucosidase (E.C. 3.2.1.20) was purchased from Sigma Chemical Co. The α-glucosidase activity was determined using 4-nitrophenyl α-D-glucopyranoside as the substrate at the optimum pH of the enzyme according to the manufacturer's specifications. Compound **1** was used as a positive control (IC_{50} 0.16 μg/mL). The mixture was incubated at 37 °C for 15 min, the reaction was stopped using 0.2 M Na_2CO_3 (aq), and the absorbance of the liberated 4-nitrophenoxide ion was measured at λ = 400 nm using a Molecular Devices Spectramax Plus microplate reader.

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