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Kakelokelose, a Sulfated Mannose Polysaccharide with anti-HIV Activity from the Pacific Tunicate *Didemnum molle*

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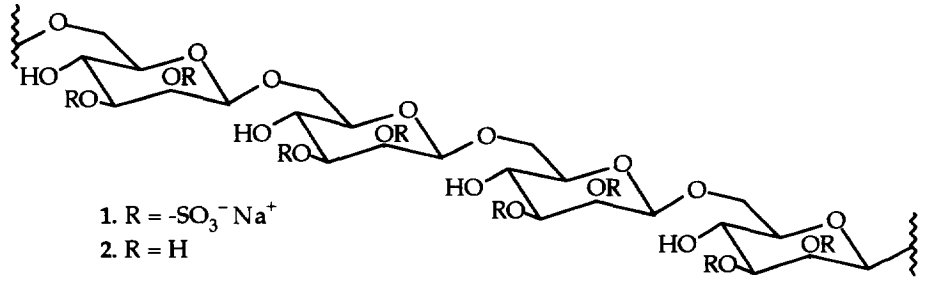
Abstract: An unusual sulfated mannose homopolysaccharide (1) showing *in vitro* anti-HIV activity has been isolated from the mucous secretion of the Pacific tunicate *Didemnum molle*. Analysis of ^{1}H and ^{13}C NMR data of the polysaccharide and its desulfated derivative 2 revealed that it consists of a sequence of 2,3-disulfated mannose units joined through β (1,6) glycosidic linkages.

The anti-viral activity of sulfated polysaccharides has been known since 1964, when the inhibitory effect of heparin on *Herpes simplex* virus was reported.[1] Several sulfated polysaccharides and other polyanionic substances were shown to be inhibitors of various enveloped viruses and of the replication of HIV-1 *in vitro*.[2-10] The activity of these polysaccharides has been related to the presence of polyanionic charges, probably independent of the structure of the polymeric backbone.[6-10] In the marine environment sulfated polysaccharides are common constituents of algae; they have also been isolated from the tunics of ascidians[11-18] and from mantles of holothurians.[11,19,20] The anti-HIV activity of sulfated polysaccharides from marine algae has been reported[21-24] and a sulfated polysaccharide of λ-carrageenan inhibits avian myeloblastosis and HIV reverse transcriptase.[23]

Extracts of the tunicate *Didemnum molle* collected in Pohnpei, Micronesia in June 1990 and again in Manado, Indonesia in October, 1992 showed excellent activity in preliminary anti-HIV screening. Subsequent observations revealed that the active material was in the seawater collected with the animals and was associated with the copious mucus exuded by the animals upon collection. Qualitative tests indicated that the active component was also anionic and of high molecular weight, which pointed to a sulfated polysaccharide.

The slimy seawater (800 mL) was decanted from approximately 1.5 kg of *D. molle* (wet wt.) and lyophilized. The insoluble residue, after extraction with 95% ethanol, was dried to give 60 g of a crude active fraction. This was separated batchwise by sequential ultrafiltration with decreasing nominal molecular weight cutoff (MWCO). In a typical run, a portion of the crude fraction (10 g) was dissolved in water (100 mL) in an ultrasonic bath. The insoluble portion was removed by centrifugation and filtration of the supernatant. The resulting viscous brown

solution was filtered through an Amicon XM300 ultrafiltration membrane(300,000 MWCO) in an Amicon stirred cell (50 mL). The retentate, a clear, very viscous solution, was thoroughly washed with water and lyophilized to give 295 mg of fluffy white solid. The filtrate from the XM300 membrane was filtered sequentially through membranes with lower MWCO to 500 and the retentates were recovered in the same manner. Anti-HIV tests showed that the fraction retained on the XM300 membrane was the most active. Decreasing activity was observed in retentates of medium molecular weights, and little activity resided in the lower molecular weight fractions (<3000 MWCO). Dissolution of the high MW fraction, followed by precipitation with EtOH, afforded 193 mg of a white solid that was reasonably pure sulfated polysaccharide (1), which we call kakelokelose.[25,26] It slowly dissolved in water to give a very viscous solution and could be stained with toluidine blue. Its FT-IR spectrum (KBr) showed an intense band at 1252 cm^{-1}, which was assigned to R-$OSO3^{-}$.[27,28]



^{1}H and ^{13}C NMR spectra of 1 (Table 1) showed signals for seven protons and six carbons. These were assigned by using DEPT, 2D NMR experiments (COSY and HMQC), and by comparison with reported data for methyl-α- and β-mannopyranosides.[29] The NMR spectral data clearly indicated that 1 is a homopolysaccharide, consisting of a disulfated hexose sequence. Polyacrylamide gel electrophoresis showed a single band that moved faster than the 500 kD marker, and the protein content was estimated to be <5%. Acid hydrolysis, then conversion to the methyl glycosides, led to the identification of mannose as the only sugar present by comparison to reference compounds. The ^{13}C data reported for the methyl mannopyranosides,[29] along with the known effects of glycosidation[30-32] and those from sulfate groups,[33] support a 2,3-disulfated 1-6-polymannan structure for 1. Sulfate content was determined to be 38.3% of the dry weight, and the sulfate/mannose ratio was estimated as 1.8.

The low-field signals assigned to C-3 and C-2 (78.9 and 77.3 ppm) and the CH_2 carbon signal at 70.3 ppm confirmed substitution at these positions, either by glycosidation or sulfate groups. The unusally low field proton signal at δ 5.47 ppm (H-2) supported the presence of a sulfate group at C-2. Since the H-3 resonance also was shifted to low field (δ 4.79 ppm), the second sulfate was likely to be located at C-3. Thus, C-6 had to be involved in the glycosidic linkage. Simple disulfated1,6 linked polymannans are uncommon among marine organisms, especially ascidians.

Table 1. NMR Data for **1**, **2** and Methyl-α- and β-mannopyranosides (**3** and **4**)

	H-1	H-2	H-3	H-4	H-5	H-6	C-1	C-2	C-3	C-4	C-5	C-6
1[a]	5.20	5.47	4.79	4.16	4.09	4.27-4.64	100.3	77.3	78.9	66.2	76.1	70.3
2[b]	4.89	4.22	3.78	3.78	3.69	3.99-4.35	101.4	71.0	73.5	67.3	75.8	69.4
3							101.9	71.2	71.8	68.0	73.7	62.1
4							101.3	70.6	73.3	67.1	76.6	61.4

a. Determined at 70°C. Proton assignments supported by COSY and decoupling experiments.

b. ^{1}H spectrum determined at 50°C, ^{13}C at 25°C

An alternative structure with sulfate groups at C-2 and C-6 and glycosidation at C-3 could be ruled out by analysis of the ^{1}H and ^{13}C NMR spectral data of the desulfated derivative **2**, obtained by solvolysis of the pyridine salt of **1**. A solution of **1** (40 mg) in 5 mL of water was stirred for several minutes with 1 g of Dowex 50W-X8 resin (H^+ form, 200-400 mesh); the resin was removed and the filtrate was neutralized with pyridine and lyophilized.[12] The reaction mixture was diluted with 50 mL of water and the desulfated derivative **2** (10 mg) was recovered by ultrafiltration on an Amicon YM100 membrane. Major changes observed in the ^{13}C NMR spectrum of **2** were the strong upfield shifts of the C-2 and C-3 signals ($\Delta\Delta$ 6.3 and 5.4 ppm, respectively). This located the sulfate groups at C-2 and C-3, which was supported by the upfield shift of the H-2 and H-3 signals to δ 4.22 and 3.78 ppm, respectively, in the ^{1}H NMR spectrum. The presence of the low field C-6 methylene resonance (69.4 ppm) in the spectrum of **2** was definitive evidence for the 1,6 glycosidic linkage. The axial hydroxyl group at C-2 in mannose makes identification of the configuration of the anomeric carbon by ^{1}H NMR unreliable. The stereochemistry can be assigned from ^{13}C NMR data since the resonances of C-3 and C-5 are shifted upfield in the α-anomer. Because of the presence of the sulfate groups at C-2 and C-3 in **1**, the only diagnostic resonance supporting a β-glycosidic linkage would be that of C-5 (76.1 ppm). However, once the glycosidation at C-6 has been established, the ^{13}C NMR resonances assigned to C-3 and C-5 (73.5 and 75.8 ppm, respectively) in the desulfated derivative **2** are in excellent agreement with the reported data for methyl-β-mannopyranoside and quite different from those of the α-anomer, thus establishing the β-configuration of the anomeric linkage in **1**. The negative value of $[\alpha]_D$ of **1** (-76.4°, H_2O) is also consistent with a β-D-mannopyranose (methyl-β-D-mannopyranoside: $[\alpha]_D$ -53.3°, H_2O).

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