Sulcatin, a Novel Antiproliferative *N*-Methylpyridinium Alkaloid from the Ascidian *Microcosmus vulgaris*

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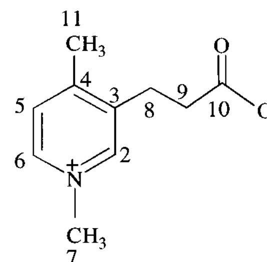
A new *N*-methylpyridinium alkaloid, with an interesting antiproliferative activity in vitro, has been isolated from the Mediterranean tunicate *Microcosmus vulgaris*. Its structure has been elucidated by spectroscopic analysis, including extensive 2D NMR experiments.

Low-molecular weight quaternary ammonium molecules are widely distributed in terrestrial plants, as well as in algae and marine invertebrates. Most of these are pyridinium derivatives with an amphoteric nature, collectively called betaines; examples are homarine and trigonelline, frequently found in marine sources,[1,2] baikiain betaine,[3] isolated from the red alga *Pterocladia capillacea*, and pyridinebetaines A and B, isolated from the sponge *Agelas dispar*.[4] The pyridine and pyridinium ring systems have also been found in several bioactive metabolites isolated from marine sponges; these compounds all are β-substituted pyridines with aliphatic side chains frequently carrying terminal amino, *N*-methylamino, methoxyamino, or methoxyiminoether groups, and they display diverse pharmacological properties, such as icthyotoxic, cytotoxic, and antimicrobial activities.[5–11]

As a part of our ongoing search for bioactive metabolites of Mediterranean ascidians, we have recently examined the species *Microcosmus vulgaris* Heller 1877 (= *M. sulcatus* Harant 1927; family Pyuridae), which yielded an EtOAc extract that was toxic to WEHI 164 (murine fibrosarcoma cell line) cells. The bioactivity-directed fractionation of the extract afforded a new alkyl sulfate with antiproliferative activity on four different tumor cell lines.[12] Following the same approach, we have now examined the hydrophilic fractions obtained from the methanolic extract of the organism. This resulted in the isolation of a novel antiproliferative low-molecular-weight alkaloid, named sulcatin, with a 3,4-disubstituted *N*-methyl pyridinium nucleus. The polar extract of *M. vulgaris* also contained larger amounts of the well-known compound homarine,[1] which proved to be inactive in the antiproliferative assay. To our knowledge, sulcatin is the first pyridine alkaloid isolated from a tunicate. The sulcatin molecule is contained in the structure of amphikuemin, a pyridinium compound previously isolated from the sea anemone *Amphiprion perideraion*.[13] Actually, this compound is the condensation product of sulcatin with lysine, and it is involved in the species-specific association between the sea anemone and anemone fish. Thus, the finding of sulcatin in *M. sulcatus* may have also a significant ecological importance.

The methanol extract of several specimens of *M. vulgaris* that had been dissected from their tunics was concentrated and partitioned between water and ethyl acetate and, subsequently, the polar layer was re-extracted with *n*-butanol. The latter organic phase was subjected to reversed-phase C_{18} chromatography using a linear gradient of MeOH (from 0 to 100%) in H_2O. The fast running water-soluble fractions, which showed antiproliferative activity, were further separated and purified by RP_{18} HPLC to give homarine[1] (50 mg) and sulcatin (5 mg) in the pure state.



The positive mode FABMS of sulcatin revealed a pseudomolecular $[M + H]^+$ ion at *m*/*z* 180 with its corresponding sodium adduct $[M + Na]^+$ at *m*/*z* 202. The high-resolution analysis gave a value of 180.1026 for the protonated molecular ion, showing only one likely elemental possibility for this measurement as $C_{10}H_{14}NO_2$.

The five degrees of unsaturation indicated by the molecular formula of sulcatin accounted for the pyridine ring and a carboxylate function, whose presence was suggested from the resonance present in the ^{13}C NMR spectrum at δ 180.0 (s). The remainder of the ^{13}C NMR spectrum contained, in addition to three aliphatic carbon signals (two methylenes and one methyl), five low-field carbon resonances and a methyl carbon signal at δ 47.9, indicating that sulcatin was a disubstituted *N*-methyl pyridinium derivative. All the ^{13}C NMR signals were associated with the directly bonded proton resonances in a HMQC experiment.

The ^1H NMR spectrum of sulcatin exhibited signals at δ 8.62 (br s, 1H), 8.55 (br d, 1H, $J = 6$ Hz), and 7.84 (d, 1H, $J = 6$ Hz), whose chemical shifts and coupling constants were reminiscent of a 1,3,4-trisubstituted pyridinium ring. An *N*-methyl signal at δ 4.32 (br s, 3H) was also present; this signal showed a strong NOE effect with the signals at δ 8.62 and 8.55, which were thus assigned to C-2 and C-6, respectively. This assignment, supported by the long-range couplings observed in the COSY spectrum and by the HMBC correlations (see Table 1), provided convincing evidence for the proposed *N*-methyl-3,4-disubstitution.

Intensive NMR work (COSY, HMQC, HMBC, NOE) revealed the remaining structural features of sulcatin. The

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Table 1. NMR Data of Sulcatin in CD_3OD

position	δ_H (mult, J)	δ_C	COSY	HMBC (^{1}H to C)	NOE
2	8.62 (br s)	145.1	6, 7, 8	C-3, -4, -6, -8, -11	7, 8
3		143.1			
4		160.0			
5	7.84 (br d, 6)	129.8	6,11	C-3, -6	11
6	8.55 (br d, 6)	143.1	2, 5, 7	C-2, -4, -11	7
7	4.32 (br s)	47.9	2, 6	C-2, -6	2, 6
8	3.10 (t, 6.5)	27.9	2, 9	C-2, -3, -4, -8, -10	2, 11
9	2.57 (t, 6.5)	37.1	8	C-3, -8, -10	
10		180.0			
11	2.68 (br s)	19.8	5	C-3, -4, -5	5, 8

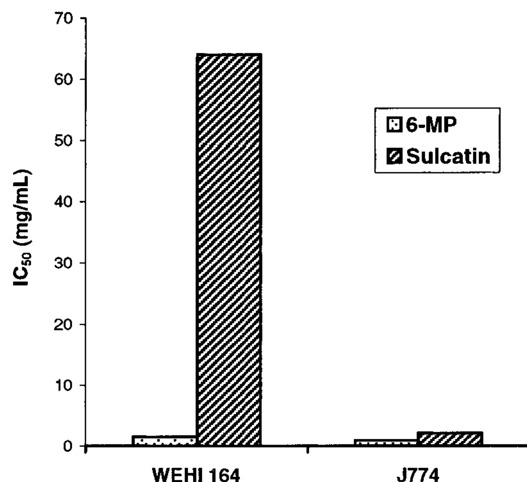

Figure 1. IC_{50} (μg/mL) of sulcatin. The results are expressed as mean ± SEM of three experiments in triplicate.

^{1}H NMR spectrum of sulcatin contained also two mutually coupled triplets (δ 3.10 and 2.57, 2H each), due to two contiguous methylene units, and a signal at δ 2.68 (br s, 3-H). This signal showed a HMQC correlation peak with a carbon resonating at δ 19.8 (CH_3); thus, it was ascribed to an additional methyl substituent on the pyridinium ring. The protons of this methyl group slowly exchange with CD_3OD, because methyl protons on a pyridinium ring can exchange in a protic solvent if linked at the α- or γ-position, and the NMR data of sulcatin excluded the α substitution, so the methyl resonating at δ 2.68 must be placed at C-4.

The $-CH_2CH_2COO^-$ structure of the last substituent, obviously linked at C-3, was inferred by the HMBC correlations of the carboxylate carbon (δ 180.0) with both the above-mentioned methylene signals. The dipolar couplings, measured by NOE difference experiments, between CH_3-11 (δ 2.68) and H-5 (δ 7.84) and between CH_2-8 (δ 3.1) and H-2 (δ 8.62), as well as the whole series of COSY and HMBC correlations (see Table 1), confirmed the substitution pattern on the pyridinium ring and, therefore, allowed the unambiguous definition of the structure of sulcatin.

The antiproliferative activity of sulcatin was evaluated on J774 (murine monocyte/macrophage) and WEHI 164 (murine fibrosarcoma) cell lines in vitro. Sulcatin inhibited the growth of both cell lines evaluated at 96 h, and this effect is reported in Figure 1 as IC_{50} (the concentration that inhibited the cell growth by 50%). These preliminary data show that sulcatin is about 40 times more potent on J774 cells than on WEHI 164 cells; monocyte/macrophage cells, thus, seem to be the target of its antiproliferative effect. On this basis, we can speculate that sulcatin is a potential therapeutic tool in some diseases involving an abnormal proliferation of the monocyte lineage, such as leukemia. Therefore, it should be of interest to investigate the mechanism of action of sulcatin on these cells.

Experimental Section

General Experimental Procedures. FAB analysis was carried out on M-Scan's VG Analytical ZAB 2SE high-field mass spectrometer. A cesium ion gun was used to generate ions for the acquired mass spectra, which were recorded with an instrument resolution of 8000 for the high-resolution measurement. ^{1}H (500.14 MHz) and ^{13}C (125.03 MHz) NMR spectra were determined on a Bruker AMX-500 spectrometer; chemical shifts were referred to the residual solvent signal (CD_3OD: δ_H = 3.34, δ_C = 49.0). Methyl, methylene, and methine carbons were distinguished by DEPT experiments. Homonuclear ^{1}H connectivities were determined by using COSY experiments. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with the Bax−Subramanian[6] HMQC pulse sequence using a BIRD pulse 0.50 s before each scan in order to suppress the signals originating from protons not directly bound to ^{13}C (interpulse delay set for $^1J_{CH}$ =140 Hz). Two- and three-bond ^{1}H−^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J_{CH}$ of 10 Hz. The NOEs were determined on a Bruker AMX-500 spectrometer with the aid of a Bruker microprogram. The sample used for NOE measurements was previously degassed by bubbling Ar through the solution for 40 min. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with an RP_{18} Si gel (particle size 40−63 μm) packed column. High performance liquid chromatography (HPLC) separations were achieved on a Waters 501 apparatus equipped with an RI detector and with a RP_{18} LiChrospher (250 × 4 mm) column.

Biological Material. WEHI 164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v:v) heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). J774 cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm, and incubated at 37 °C in DMEM supplemented with 10% FBS, 25 mM HEPES, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).

All reagents for cell cultures were from Celbio. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] and 6-mercaptopurine (6-MP) were from Sigma.

Animal Material. Specimens of *M. vulgaris* were collected in the Bay of Naples (Procida, Punta Pizzaco) at a depth of 40 m. A voucher specimen is deposited at the Dipartimento di Chimica delle Sostanze Naturali, Napoli, Italy.

Extraction and Isolation of Sulcatin. The tunic was removed from the animals, and the whole bodies (40 g dry wt after extraction) were extracted at room temperature with MeOH (4 × 300 mL). The concentrated aqueous combined residue was partitioned between EtOAc and H_2O, and subsequently between *n*-butanol and H_2O. Separation of the *n*-butanol soluble material (5 g) was achieved by a RP_{18} MPLC eluting with a linear gradient of MeOH in H_2O (0% → 100). The fast-running fractions (1 g), eluted with H_2O, were subjected to a desalting step on a Sephadex LH-20 column with MeOH as the eluent. This chromatography yielded pure homarine (50 mg) and a bioactive fraction (46 mg) that was further purified by HPLC on a RP_{18} column (250 × 4.6 mm, 3 μm) eluting with H_2O/MeOH, 95:5. This afforded 5 mg of sulcatin (**1**) in the pure state.

Sulcatin (1): obtained as amorphous solid; HRFABMS (positive mode) *m/z* 180.1026 (calcd for $C_{10}H_{14}NO_2$, 180.1024); ^{1}H and ^{13}C NMR data, see Table 1.

Antiproliferative Activity. WEHI 164 and J774 (3.5 × 10^3 cells) were plated on 96-well plates in 50 μL and allowed to adhere at 37 °C in 5% CO_2/95% air for 2 h. Thereafter 50 μL of 1:4 (v/v) serial dilution of the test sulcatin was added and then the cells incubated for 96 h. In some experiments 6-MP was added as a standard compound for antiproliferative activity. Cell viability was assessed through an MTT conversion assay.[14] Briefly, after 96 h, 25 μL of MTT (5 mg/mL) was

added, and the cells were incubated for additional 3 h. After this time, the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v/v) *N*,*N*-dimethylformamide, 20% (w/v) SDS with an adjusted pH of 4.5.[15] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620-nm filter. The viability of each cell line in response to treatment with sulcatin and 6-MP was calculated as: % dead cells = 100 − (OD treated/OD control) × 100. Figure 1 shows the results expressed as the IC_{50} value. Statistical analysis was made by paired two-tailed Student's *t*-test. The level of statistically significant difference was defined as $p < 0.01$.

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