Structural Elucidation of a New Cytotoxin Isolated from Mussels of the Adriatic Sea†

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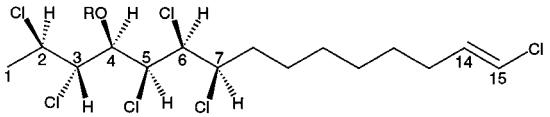
A detailed analysis of the toxic composition in the hepatopancreas of mussels from northern Adriatic sea has been performed. Along with some polyether toxins of DSP (diarrhetic shellfish poisoning) type, such as yessotoxin and its analogues, which are responsible for a variety of human seafood poisonings throughout the world, we have now isolated a new type of toxin, the chlorosulfolipid **1**, which is completely different in structure from the polyether DSP-toxins isolated so far. The structural determination of the new toxin, including its absolute stereochemistry, has been performed by extensive NMR analysis and molecular mechanics and dynamics calculations.

Introduction

Wild and cultured suspension-feeding bivalve molluscs are often a dominant component of the benthic macrofauna in nearshore coastal waters. Due to their ability to filter large volumes of water they are the primary vectors for the transfer of algal toxins, such as paralytic, diarrhetic, and amnesic shellfish poisoning toxins (PSP, DSP, and ASP, respectively) to humans.[1] Therefore, these food-borne marine toxins are responsible for a variety of human seafood poisonings throughout the world.

During the past decade, these types of poisonings have spread also to the northwestern Adriatic sea,[2] thus causing serious economic problems, since many shellfish farms are established there. Consequently, monitoring of natural banks and mussel-breeding areas was intensified. At the same time, a research program was initiated by our group in 1990 to carefully examine the toxin profiles in mussels from Northern Adriatic sea. Since then, a number of polyether toxins have been isolated and characterized by us;[3−8] some of them represent new additions to the DSP-class of biotoxins and seem to be unique to the northwestern Adriatic Sea, since they have never been reported in any other region.[6−8]



1 R = SO_3H
2 R = H
3 R = (*S*)-MTPA
4 R = (*R*)-MTPA

Figure 1. Structure of the novel chlorosulfolipid **1**.

During our investigation on toxic Adriatic mussels, we isolated a new type of toxin, which is completely different in structure from the polyether DSP-toxins isolated so far. In the present paper we report the isolation and the stereostructural determination of this novel chlorosulfolipid cytotoxin, **1** (Figure 1).

Structurally related chlorosulfolipids were previously isolated from some species of microalgae.[9,10] These compounds, whose structure has been so far assigned devoid

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† Dedicated to the memory of Prof. Romano Viviani, who was the pioneer of the study on marine toxins from the Adriatic Sea.

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Table 1. ^{13}C and ^{1}H NMR Data for Compound 1 (CD_3COCD_3)[a]

position	δ_C	δ_H, mult. J (Hz)
1	19.00	1.56 (d, 6.3)
2	57.23	5.05 (dq, 6.3; 1.5)
3	69.01	4.38 (dd, 9.9; 1.5)
4	75.42	4.66 (d, 9.9)
5	68.10	4.74 (d, 9.9)
6	69.22	4.68 (dd, 9.9; 1.1)
7	63.20	5.12 (ddd; 1.1; 4.4)
8	38.17	1.75; 1.82 (m)
9	27.00	1.51; 1.51 (m)
10	29.27	1.30; 1.30 (m)
11	31.68	1.82; 1.82 (m)
12	29.31	1.35; 1.35 (m)
13	29.42	2.05; 2.05 (dt)
14	117.89	5.89 (dt, 14.8)
15	135.27	6.08 (d, 14.8)

[a] Assignment based on DEPT, COSY, HMQC, and HMBC experiments.

of stereochemical details, are usually divided in two series: the polychlorodocosane 1,14-disulfates and the polychlorotetracosane 1,15-disulfates, with a number of chlorine atoms that ranges from zero to six in various combinations of positions on the aliphatic chain. They represent a unique class of products in that they are essentially polar at both ends of the molecule.

Results and Discussion

Compound **1** was isolated from the digestive glands (4.3 kg) of *Mytilus galloprovincialis*, collected in Autumn 1998 from one sampling site located along the Emilia Romagna coasts of Italy during a period of high toxicity.

Animals were found to be toxic by the official mouse bioassay method for DSP.[11] The isolation of **1** from the digestive glands was bioassay-guided as reported in the Experimental Section.

The negative ion FABMS spectrum of **1** showed a 4:7:6:3:1 quintet at *m/z* 509, 511, 513, 515, 517, respectively, for the pseudomolecular ion peak $[M - H]^-$, indicating the presence of six chlorine atoms in the molecule. The complete molecular formula of **1** was established to be $C_{15}H_{24}O_4SCl_6$ by the negative ion HRFABMS spectrum (measured mass *m/z* 510.9462; calculated 510.9419 for $C_{15}H_{23}O_4S^{35}Cl_5{}^{37}Cl$). The molecular formula was corroborated by NMR data.

The detailed analysis of NMR spectra allowed the structural elucidation of the novel toxic compound. ^1H and ^{13}C NMR, DEPT and HMQC experiments showed that all the 15 carbon atoms were protonated, 13 of which being of sp^3 type (1 methyl, 6 methylenes, and 6 methines). The remaining two sp^2 methine carbons evidenced a 1,2-disubstituted carbon−carbon double bond (see Table 1).

As indicated by the molecular formula, the above protonated carbons must constitute a linear chain to which six chlorine atoms and an OSO_3H group are linked, the presence of the last functionality being evidenced by the absorptions at ν_{max} 1240, 1220, and 820 cm^{-1} in the IR spectrum.

The ^1H NMR spectrum of **1** (Supporting Information) exhibited a quite good dispersion of the signals, both in high and low field region; this looked promising for an easy determination of the planar structure of **1** through the connectivities present in the $^1H-^1H$ COSY spectrum. Unfortunately, instead of a single spin system, the $^1H-^1H$ COSY spectrum, as well as the HOHAHA spectrum, individuated three spin systems, namely H_3-1/H-2, H-3/H-4, and H-5/H-15. The lack of coupling between H-2 and H-3 and between H-4 and H-5 prevented the above units to be connected. However, their assembly was allowed by some key correlations present in the HMBC and ROESY spectra. Particularly, the long-range coupling between C-1 and H-3, emerging from the HMBC spectrum, was useful to connect the partial structural units from H-1 to H-4. Furthermore, a ROESY cross-peak between H-4 and H-5 was diagnostic to complete the connectivity of the molecule.

To fully establish the gross structure **1**, it remained to assign the exact location of the seven functional groups on the carbon chain. They have to be positioned on the six sp^3 methines (from C-2 to C-7) and on the terminal sp^2 methine. This was accomplished by individuating the location of the sulfate group on the basis of the following evidence. An inspection of the chemical shift values of the sp^3 methine carbons suggested that this functionality was linked at C-4 (δ 75.42). This was further supported by solvolysis of **1** carried out with a mixture of pyridine:dioxane 1:1 for 3 h at 130 °C which afforded the corresponding alcohol **2**, identified on the basis of its NMR and mass spectral properties reported in the Experimental Section; in its ^1H NMR spectrum (CD_3OD), compared with that of **1** (CD_3OD, see Experimental Section), a remarkable upfield shift was observed for H-4, which resonated at δ 4.72 in **1** and at δ 4.03 in **2**.

At this point it remained to be assigned the stereochemistry of compound **1**, containing six centers and one stereogenic axis, namely the carbon−carbon double bond and six chiral carbons. The *E* geometry of the double bond was derived from the large coupling constant (*J* 14.8 Hz) between H-14 and H-15. Additional evidence was obtained from the ROESY cross-peaks between H-14 and the methylene protons at C-12, as well as between H-15 and the methylene protons at C-13.

The paucity of isolated compound **1** and its flexible structure resulted in the determination of the stereochemistry of the six chiral centers to be quite laborious. It was based on the assignment of their relative configurations taking advantage of the fact that all the chiral carbons were adjacent; this resulted in the existence of dominant conformers around the C−C bonds from C-2 to C-7. In fact, in this part structure for each couple of vicinal protons the ^1H NMR spectrum exhibited, instead of the usual *J* characteristic of acyclic structures (ca. 8 Hz), smaller or larger values, indicative of dominant gauche or anti orientation, respectively. In particular, the large value for $^3J_{H-3/H-4}$ (9.9 Hz) revealed the antiperiplanar relationship between H-3 and H-4, as well as between H-5 and H-6 ($^3J_{H-H}$ 9.9 Hz), while the small value for $^3J_{H-2/H-3}$ (1.5 Hz), $^3J_{H-4/H-5}$ (∼0 Hz), $^3J_{H-6/H-7}$ (1.1 Hz) indicated the gauche relationship of the pertinent protons.

From these data our molecule appeared to possess the right conformational requirements essential to apply the very recent *J-based configuration analysis* method proposed by Murata et al.[12] It is based on the combined

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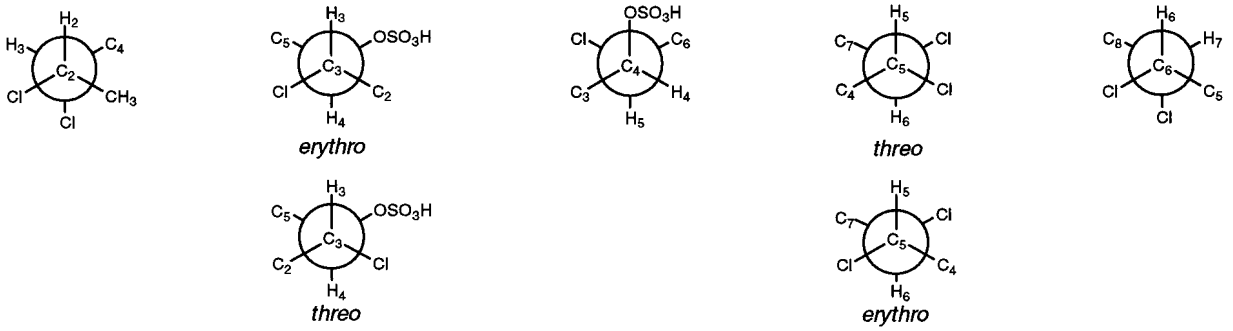
Table 2. $^3J_{H,H}$, $^2J_{C,H}$ and $^3J_{C,H}$ from C2 to C7 Portion of Compound 1

C2−C3 axis	C3−C4 axis	C4−C5 axis	C5−C6 axis	C6−C7 axis
H2−H3 small	H3−H4 large	H4−H5 small	H5−H6 large	H6−H7 small
H2−C4 small	H3−C5 small	H4−C6 small	H5−C7 small	H6−C8 small
C1−H3 large	C2−H4 small	C3−H5 small	C4−H6 small	C5−H7 small
C2−H3 large	C3−H4 large	C4−H5 small	C5−H6 large	C6−H7 small
C3−H2 small	C4−H3 large	C5−H4 small	C6−H5 large	C7−H6 small



analysis of $^3J_{H,H}$, $^3J_{C,H}$, and $^2J_{C,H}$ coupling constants and can be applied to a limited number of molecules. In addition to the evaluation of $^3J_{H,H}$ and $^3J_{C,H}$, whose values are both related to conformational parameters through Karplus rule, Murata's method considers also the geminal carbon−proton coupling constants ($^2J_{C,H}$). It is to be noted that the use of $^2J_{C,H}$ in conformational studies is quite limited because of the absence of clear dependence on structural parameters. However, when the α-carbon carries an electronegative substituent (X) such as an oxygen or halogen atom (^{1}H−C−^{13}C−X systems), and this is the case of our molecule, a clear relation has been reported between the magnitude of $^2J_{C,H}$ and the dihedral angle between the proton and the heteroatom.[13]

Following the above remarks, we focused our attention on the segment C1−C8, composed of the six chiral carbons, and through an analysis of the ^{1}H NMR spectrum and a phase sensitive PS-HMBC spectrum, we were able to determine the homonuclear ($^3J_{H,H}$) and heteronuclear ($^3J_{C,H}$ and $^2J_{C,H}$) coupling constants, respectively. The pertinent data are reported in Table 2.

The heteronuclear coupling constants have been only qualitatively ("*small*" or "*large*") determined simply by comparing intensities of the cross-peaks in the PS-HMBC spectrum (Supporting Information). According to Murata et al.,[12] this qualitative elaboration is allowed taking into account the significant differences in $^{2,3}J_{C,H}$ values between the anti and gauche rotamers. In the PS-HMBC spectrum of **1** we observed an intense cross-peak for C1−H3 correlation, indicating a large ^3J value, while those for H2−C4, C2−H4, H3−C5, C3−H5, H4−C6, C4−H6, H5−C7, C5−H7, and H6−C8 did not appear, thus indicating a small value of these $^3J_{C,H}$. Analogously, the same experiment showed large $^2J_{C,H}$ between C2−H3, C3−H4, C4−H3 and C5−H6, C6−H5, while no cross-peaks between C3−H2, C4−H5, C5−H4, C6−H7, and C7−H6 were observed, indicating their small $^2J_{C,H}$ values.

The obtained *J* data indicated the stereochemical relationship for each pair of vicinal asymmetric carbons reported in Table 2. Just for the C3−C4 and C5−C6 bonds which have an H/H-anti orientation, as underlined by Murata, the unassisted knowledge of the coupling constants does not allow discrimination between *erythro* and *threo* rotamers, characterized by a C/C-anti or a C/C-gauche orientation, respectively (see Table 2). In this case, NOE experiments are a practical way to assign the appropriate relationship. In fact, for C/C-gauche conformation, the hydrogens linked to the two gauche carbons should come within the range of NOE, while in the case of C/C-anti conformation, no NOE should be observed between them. In this way, by observing the presence of ROE between H4 and H7 and its absence between H2 and H5, respectively, we identify the right rotamers along C3−C4 and C5−C6 bonds (*erythro* and *threo*, respectively).

The whole of both *J* and NOE data was consistent to unambiguously assign the relative configuration of the six chiral carbons present in **1**.

The relative stereochemistry of **1** was further substantiated by molecular mechanics calculation, carried out using the CHARMm force field.

The molecular mechanics analysis of the 32 possible enantiomeric couples was performed in vacuo (dielectric constant = 1). Global minimum energy conformations were obtained by carrying out a high temperature molecular dynamics simulation followed by energy minimization. By means of this dynamic simulation, 500 conformations of each investigated couple were achieved and then subjected to an energy minimization (1000 steps, conjugated gradient algorithm). Inspection of all the minimized structures indicated that only one enantiomeric couple (2*S*,3*R*,4*S*,5*R*,6*R*,7*S* and its enantiomer) adopted the preferred conformation consistent with both *J* and NOE data. Indeed, for all the remaining stereoisomers, the difference of energy between the lowest energy conformation and the one responding to our experimental evidences was over 3.5 kcal/mol, thus allowing their exclusion.

Our final task was to establish the absolute configuration of the molecule. For this purpose we applied the modified Mosher method suggested by Kakisawa[14] to define the configuration of the chiral center at C-4 and hence of the entire molecule.

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Therefore, the desulfated compound **2** was treated with *R*(−) and *S*(+) α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride in pyridine solution at room temperature for 2 h to give the ester derivatives, **3** and **4**, respectively. The stereochemical determination was based on the chemical shift differences of the protons at C-2, C-3, C-5, and C-6 [$\Delta\delta$ (*S*-*R*): H-2−0.01; H-3−0.1; H-5 +0.05; H-6 +0.08]. The obtained results suggested the *R* configuration at C-4. The whole absolute configuration of the molecule was consequently assigned as 2*R*,3*S*,4*R*,5*S*,6*S*,7*R*.

Compound **1** was tested for its antiproliferative activity on J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma), and P388 (murine leukemia) cell line, and it was found to inhibit the growth of all cell lines evaluated at 72 h with an IC_{50} of 12.1, 16.3, and 10.4 μg/mL, respectively.

The presence of this toxic compound in edible shellfish, in addition to contamination of DSP toxins, extends the Adriatic sea toxin profile. To prevent the damages owing to pollution from harmful marine algae, both to the public health and to the shellfish industries, it is necessary to implement careful monitoring, both at markets and shellfish farms. Monitoring, in turn, cannot be run without good knowledge of the causative organisms and the nature of implicated toxins. Therefore, an accurate analysis of toxic mussels is indispensable in order to identify new toxins, even other than DSP polyether toxins, and isolate a larger amount for deeply clarifying their toxicological effects.

Experimental Section

General. ^{1}H (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured on a 500 MHz spectrometer; chemical shifts are referenced to the residual solvent signals (CD_3OD: δ_H = 3.34; CD_3COCD_3: δ_H = 2.05; δ_C = 205.7 and 29.8). Methyl, methylene, and methine carbons were distinguished by a DEPT experiment. Homonuclear ^{1}H connectivities were determined by using COSY experiment. One bond heteronuclear ^{1}H−^{13}C connectivities were determined with the Bax and Subramanian HMQC pulse sequence.[15] Two- and three-bond ^{1}H−^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 10 Hz. Low- and high-resolution FABMS (negative ion mode) were performed by M-Scan S. A. (12, chemin des Aulx, 1228 Plan-les-Ouates, Switzerland) on a high-field mass spectrometer. Positive ESI MS was carried out with an ion trap mass spectrometer. Medium-pressure liquid chromatographies (MPLC) were performed using SiO_2 (230−400 mesh) and RP-18 (40−63 μm) stationary phases.

Isolation of 1. The new cytotoxin was isolated from the digestive glands (4.3 kg) of mussels *Mytilus galloprovincialis*, collected in autumn 1998 from one sampling site located along the Emilia Romagna coasts of Italy. The hepatopancreas was found to be toxic by the official mouse bioassay method for DSP.[11] Digestive glands were extracted with acetone twice. After evaporating acetone, the aqueous concentrate was extracted thrice with EtOAc. The extract obtained after removal of the solvent was partitioned between 80% MeOH and hexane. Toxic residue obtained in the 80% MeOH layer was further partitioned between MeOH−H_2O (4:6) and CH_2Cl_2. The dichloromethane soluble material was then chromatographed on a Develosil ODS column washing stepwise with MeOH−H_2O (8:2 and 9:1) and MeOH in this order. Toxins eluted in the last fraction were passed through a Toyopearl HW-40 SF column with MeOH. The final HPLC purification was carried out on a RP 18 column with CH_3CN−H_2O 1:1 as eluent, thus obtaining 2.3 mg of pure compound **1**: $[\alpha]^{25}_D$ +20.4 (*c* = 0,0015, MeOH); ^{1}H and ^{13}C NMR (CD_3COCD_3) data are reported in Table 1; selected ^{1}H NMR chemical shifts (CD_3OD): δ 4.94 (H-2); 4.89 (H-7); 4.82 (H-5); 4.62 (H-6); 4.48 (H-3); 4.72 (H-4); 1.64 (H_3-1); IR (KBr) ν_{max} 1240, 1220, and 820 cm^{-1}; FABMS (negative ion mode) *m*/*z* 509, 511, 513, 515, and 517; HRFABMS *m*/*z* 510.9462, $C_{15}H_{23}O_4S^{35}Cl_5{}^{37}Cl$ requires 510.9419.

Solvolysis of 1. Compound **1** (2 mg) was dissolved in a dioxane−pyridine mixture 1:1 (1 mL) and heated at 130 °C (3 h). H_2O (2 mL) was added to the cooled solution before extraction with $CHCl_3$ (3 × 2 mL). The combined extracts were evaporated in vacuo to give the alcohol **2** (1.8 mg): ESIMS *m*/*z* 429, 431, 433, 435, and 437; selected ^{1}H NMR chemical shifts (CD_3COCD_3): δ 4.78 (H-2); 4.99 (H-7); 4.62 (H-5); 4.40 (H-6); 4.44 (H-3); 4.03 (H-4); 1.58 (H_3-1).

Synthesis of the (*R*)- and (*S*)-MTPA Esters of 2. To compound **2** (0.8 mg) in anhydrous pyridine (200 μL) was added 5 μL of (*R*)-MTPA chloride [MTPA = α-methoxy-α-(trifluoromethyl)phenylacetyl], and the mixture was allowed to react at room temperature. After 2 h, H_2O (5 mL) and solid K_2CO_3 were added, and the solution was extracted with $CHCl_3$ (5 mL). The organic phase, after evaporation of the solvent, yielded the (*S*)-MTPA triester **3**. The use of (*S*)-MTPA chloride in the same procedure led to the (*R*)-MTPA triester **4**.

Compound 3. Selected ^{1}H NMR chemical shifts (CD_3OD): H-2 δ 4.76; H-3 δ 4.02; H-5 δ 3.98; H-6 δ 4.81.

Compound 4. Selected ^{1}H NMR chemical shifts (CD_3OD): H-2 δ 4.75; H-3 δ 3.92; H-5 δ 4.03; H-6 δ 4.89.

Molecular Mechanics and Dynamics Calculations. Molecular modeling was carried out on SGI Personal Iris 35G computer using the CHARMm force field (QUANTA 4.0 software package). All the force-field calculations were carried out in vacuo (dielectric constant = 1). Global minimum energy conformations were obtained by performing a high-temperature molecular dynamics simulation (HTMDS) followed by energy minimization.[16] By means of a molecular dynamics simulation of 100-ps at 2000 K, 500 conformations for all the 32 possible enantiomeric couples of the new cytotoxin were achieved. All the conformations were then subjected to an energy minimization (1000 steps, conjugated gradient algorithm). Inspection of the minimized structures provided the enantiomeric couple with the lowest energy conformation consistent with NMR data of **1**.

Cytotoxicity Assay. WEHI 164 cells were maintained in adhesion with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/mL), and 2 mM L-glutamine. J774 and P388 cells were grown in adhesion on Petri dishes with DMEM medium supplemented with 10% FBS, 25mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/mL), and 2 mM L-glutamine. All reagents for cell culture were from Biowhittaker. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] was from Sigma.

WEHI 164, J774, and P388 (1×10^4 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 the medium was replaced with 50 μL of fresh medium, 50 μL of 1:4 v/v serial dilution of the test compound was added, and then the cells were incubated for 72 h. The cells viability was assessed through an MTT conversion assay.[17] Briefly, after 72 h of incubation, 25 μL of MTT (5 mg/mL) was added to each well, and the cells were incubated for additional 3 h. Following this time the cells were lysed, and the dark blue crystal was solubilized with 100 μL of a solution containing 50% (v:v) *N*,*N*-dimethylformamide, 20% (v:v) SDS with an adjusted pH of 4.5.[18] The optical density (OD) of each well was measured with a microplate spectrophotometer equipped with a 620 nm filter. The viability of each cell line in response to treatment with compound **1** was calculated as % dead cells = 100 − (OD treated/OD control) ×

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100. The results are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%).

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Supporting Information Available: ^{1}H NMR (CD_3COCD_3), ^{13}C NMR (CD_3COCD_3), DEPT, ^{1}H−^{1}H COSY, ^{1}H−^{1}H ROESY, HOHAHA, ^{1}H−^{13}C HMQC, ^{1}H−^{13}C HMBC, FABMS, and HRFABMS spectra for **1** and ^{1}H NMR spectrum for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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