Characterization of Anticholinesterase-Active 3-Alkylpyridinium Polymers from the Marine Sponge *Reniera sarai* in Aqueous Solutions

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From the marine sponge *Reniera sarai* 3-alkylpyridinium oligomers and polymers have been isolated. 3-Alkylpyridinium polymers are potent anticholinesterase agents; in addition, they show hemolytic and cytotoxic activities. Oligomers with a molecular weight lower than 3000 Da do not possess any significant activity. We report structural characterization of 3-alkylpyridinium polymers and their behavior in aqueous solutions. We found that biologically active polymers are composed of head-to-tail 3-alkylpyridinium units. According to MALDI-TOF spectrometry two species of polymers exist, the smaller with a molecular weight of 5520 Da and the larger with a molecular weight of 18 900 Da. Both polymers are soluble only in water, while low molecular oligomers are readily soluble in organic solvents. Polymers form large water-dissolved supramolecular structures with an average hydrodynamic radius of 23 ± 2 nm and, therefore, cannot be separated with size-exclusion chromatography.

Recently, several 3-alkylpyridinium compounds endowed with potent biological activities have been isolated from various marine sponges. These compounds have been described as constituted of 3-alkylpyridinium salt subunits (APS). The 3-alkyl chain is bound head-to-tail to the nitrogen of the adjacent subunit as in (a) epidermal growth factor (EFG)-active factors, constituted of oligomers built on straight C_8 alkyl chains (**1**);[1] (b) halitoxin, built on C_8−C_{11} methyl branched alkyl chains (**2**);[2] (c) cyclostellettamines, composed of straight C_{12}−C_{15} alkyl chains (**3**);[3] and (d) amphitoxin, which is composed of oligomeric chains of 3-alkylpyridinium and 3-alkenylpyridinium units (**4a, 4b**).[4] Related to these compounds are niphatoxins, non-ring oligomers with uncharged pyridine end groups,[5] and haliclamines, trihydropyridine cyclic compounds.[6] These have been proposed as models of biogenic precursors for many complex alkaloids, such as the manzamines[7] and the petrosins,[8] all isolated from marine sponges.

The isolated compounds exerted cytotoxicity towards tumor cell lines and hemolysis,[2] inhibition of the epidermal growth factor and toxicity to fish and mice,[1] inhibition of binding of methyl quinuclidinyl benzilate to muscarinic acetylcholine receptors,[3] and strong antifeeding activity.[4] 3-Alkylpyridinium oligomers mimicking the EGF-active factors, with an oxygen atom in place of a methylene group in the alkyl chain, have been recently synthesized.[9] Some of these studies revealed that the higher the polymerization degree the higher were both biological activity[1,9] and hydrophilicity.[9]

Despite the well-defined structure of a single 3-alkylpyridinium unit, little is known about the size of 3-alkylpyridinium polymers (poly-APS) and their possible formation of supramolecular structures in aqueous solutions that might also have an impact on their biological activities. We found that aqueous extracts from the marine sponge *Reniera* (=*Haliclona*) *sarai* Pulitzer-Finali (Haliclonidae) possess strong anticholinesterase and hemolytic activity as well as moderate antimicrobial and cytotoxic activities.[10] In the present paper we report isolation and some structural characteristics of poly-APS (**5**). Isolated poly-APS are structurally related to the EGF-active factors,[1] but of a significantly higher polymerization degree and solubility in H_2O. We present evidence that poly-APS form large non-covalently bound supramolecular structures in H_2O.

Results and Discussion

The aqueous extract of *R. sarai* contains a mixture of poly-APS and oligo-APS. Poly-APS were routinely isolated as summarized in Table 1 and were separated from oligo-APS by an ultrafiltration membrane with a 3000-Da mol wt cut-off. Almost all AChE-inhibitory activity was associated with the retained material containing poly-APS. Extracted poly-APS were eluted from Sephadex G-50 as well from Sephacryl S-200 at the void volume, indicating that their apparent molecular weight exceeded 100 kDa. Typically, the yield of poly-APS was about 20 mg from 100 g of sponge wet weight. Lyophilized poly-APS appeared as tiny white fibers that could be solubilized in H_2O but not in organic solvents.

In contrast to poly-APS, the ultrafiltrate that contained oligo-APS and exhibited only a slight AChE-inhibitory activity, gave two weakly active peaks with typical pyridinium UV spectra when applied to a reversed-phase HPLC C-18 column. Oligo-APS were not further characterized because of the lack of significant biological activities. Attempts to separate poly-

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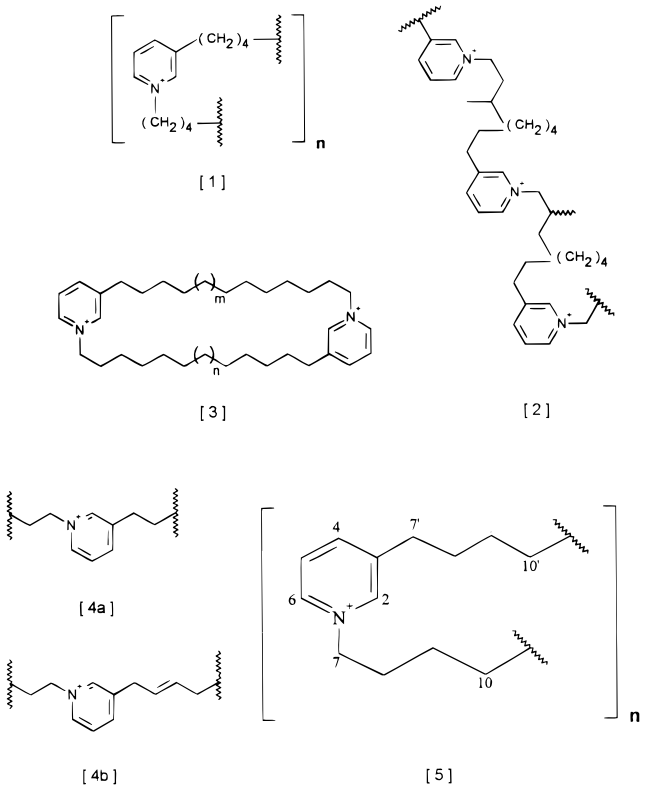
Table 1. Purification of Poly-APS

purification step	dry matter (mg/mL)	*V* (mL)	total activity (IU)[a]	spec inhib activity (IU/mg)	purification factor
homogenate in H_2O	40.5	38	12 700	8.26	1
extract in 60% EtOH	7.4	104	34 600	44.8	5.4
ultrafiltration retentate	115.0	1.5	15 000	87.0	10.5
ultrafiltration filtrate	58.7	12	1500	2.1	0.25
peak 1−Sephadex G-50	0.12	59	13 000	505	61.2
concentrated fractions	0.9	4.5	3000	705	85.3

[a] Inhibitory unit (IU) was defined as the amount of the inhibitor that reduced the rate of acetylthiocholine hydrolysis by 50% at 25 °C.



APS on a Suplex pKb column, suitable for low-molecular-weight quaternary ammonium compounds, were not successful because the polymers stuck tightly to the column bed. Poly-APS also failed to migrate on Si gel and amino TLC plates with any solvent. They were intensely stained with the Dragendorff reagent, characteristic for pyridinium compounds.

The incubation of poly-APS in 1M NaOH or 1M HCl caused the loss of inhibitory activity by 70% and 50%, respectively. The UV spectrum obtained after HCl treatment was unchanged, while in NaOH the typical pyridinium maximum at 266 nm disappeared.

The poly-APS showed a series of ^{1}H- and ^{13}C-NMR signals similar to those reported for the EGF-active factors.[1] However, while rather sharp ^{13}C signals ($W_{1/2}$ 3−5 Hz) were observed in fully decoupled carbon spectra, proton spectra consisted of non-resolved multiplets. This is in contrast with well-defined coupling patterns for the EGF-active factors.[1] The lack of signals for terminal pyridine groups and solubility in H_2O—but not in MeOH, DMSO, or pyridine—suggests a polymeric nature for poly-APS. The NMR and EIMS analyses show that poly-APS are constituted of the same repeating unit as already observed for the oligomeric EGF-active factors (**1**).[1] These structural assignments of poly-APS were also supported by COSY and ^{1}H−^{13}C correlation experiments. The highly polymeric nature of poly-APS is reflected in a relaxation time typical for

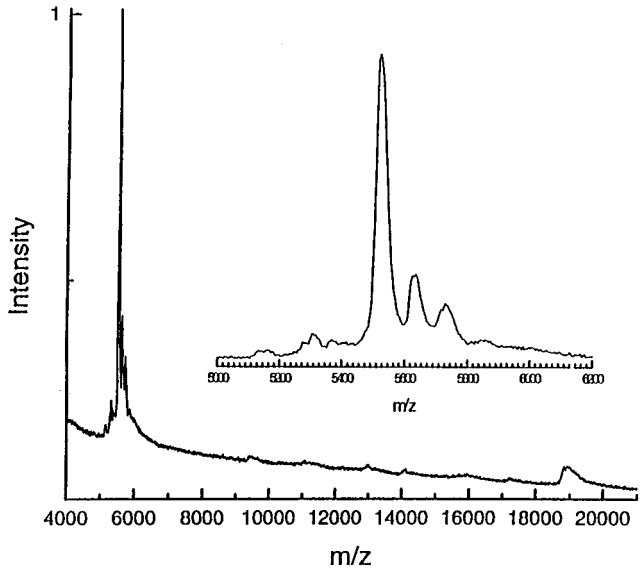


Figure 1. MALDI-TOF spectrum of poly-APS in sinapinic acid in the 4000−21 000 Da mass range. Signal intensity is reported in arbitrary units. Inset of spectrum shows expanded *m*/*z* scale for 5520 Da peak.

slow-tumbling spin rates. Thus, while pyridinium protons are characterized by T_1 values of 0.28−0.44 s, only slightly shorter than in small organic molecules, the T_2 values proved to be quite short, 20−40 ms.

On heating at 170−200 °C, the freeze-dried poly-APS gave EIMS with an intense peak at *m*/*z* 190, deriving from pyrolitic cleavage of N^+−C(7) of two adjacent subunits. HR EIMS on this ion gave the composition $C_{13}H_{20}N$ corresponding to the 3-octylpyridinium ion.

We determined the molecular weight of poly-APS by matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-TOF). The poly-APS's sample dispersed in a sinapinic acid matrix gave two ion clusters in the MALDI mass spectrum. The most intense peak was centered at 5520 Da and a less intense but broader one at 18 900 Da. Low intensity signals occurred at *m*/*z* 2760 and 9450, corresponding to doubly charged species of those responsible for the parent signal. No peaks indicative of higher-order structures or polymers were detected (Figure 1). Because of a limited knowledge and interpretation of MALDI spectra for charged organic polymers, only a tentative rationalization about the detailed nature of these ions can be given. Should these peak intensities represent singly charged species partially neutralized by electrons and not the trifluoroacetate counterion, the two main clusters correspond to the polymers of 29 and 99−100 3-octylpyridinium units, respectively. It is noteworthy that in the ion-spray mass spectra pyridinium oligomers retained the trifluoromethansulfonate counterions[1] and that all the oligomeric species showed mass peaks corresponding to parent-anion adducts. However, the

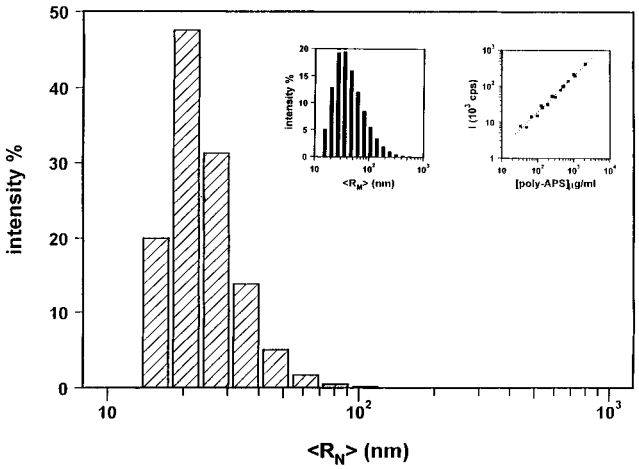


Figure 2. Distribution of the hydrodynamic radius weighted by the number of particles obtained with DLS. Poly-APS concentration was 133 μg/mL in H_2O at 25 °C. **Left inset:** Same experiment as before but the distribution of hydrodynamic radius weighted by the mass of particles is plotted. **Right inset:** Intensity of the scattered light vs. poly-APS's concentration (from the same experiments reported in Table 2).

laser ablation seen in MALDI is much stronger than electrospray nozzle atomization and, thus, probably fully dissociates the counterions. Regardless of the ambiguity in molecular mass assignments, the finding of only two kinds of poly-APS molecules is contrasted by their behavior in size-exclusion chromatography. Actually, MALDI-TOF spectrometry cannot exclude the existence of higher-ordered structures or non-covalent interactions between organic polymers, because these are usually not seen if ordinary MALDI techniques are used.[11] In fact, the elution profiles of purified poly-APS on Sephadex G-50, Sephadex G-100, and Sephadex G-200 columns indicate that poly-APS's size exceeds the gels' separation range inasmuch as they always elute with the void volume, irrespectively of the poly-APS's concentration and the gel used. This estimation suggests that in aqueous solutions poly-APS form aggregates composed of 5520 and 18 900 Da units. We were unable to separate poly-APS on Sephacryl or Sepharose gels and the HPLC size-exclusion column because poly-APS were strongly adsorbed on the chromatographic beds.

To obtain more information about the poly-APS and their organization in aqueous solutions, we applied a dynamic light scattering (DLS). This indicates a large degree of polydispersity (Figure 2). Particles with sizes ranging from 15 to 80 nm are present with a greater number of smaller ones. The average hydrodynamic radius (either H_2O or D_2O) is at 23 ± 2 nm, independent of poly-APS's concentration and ionic strength (Table 2). Furthermore, the intensity of the scattered light is directly proportional to the concentration of the centers of scattering on all the range reported (Figure 2, right inset). These data suggest that poly-APS in aqueous solutions are organized predominantly as non-covalently bound aggregates that behave primarily as hard spheres. In fact, our data exclude either a strong electrostatic repulsion within the particles (which should lead to a dependence of $<R_N>$ on the ionic strength) or a concentration-dependent state of aggregation.[12] To check the validity of the size estimation, we used a diluted solution of the cationic polymer polyethylenimine (avg mol wt 50 kDa). We found an average hydrodinamic radius of 6 ± 1 nm, in good agreement with Kuga[13] who reported about 8 nm for 50 kDa polyethylene oxide, and 5 nm for 50 kDa dextran. The estimated radius of poly-APS's particles is four times larger than that of the polyethylenimine standard, suggesting an average molecular weight of about 3×10^6 Da for the aggregates.

Table 2. DLS Size Dependence of Poly-APS on Concentration and Ionic Strength[a]

[poly-APS] (μg/mL)	$<R_N>$ nm	SD nm	[NaCl] (mM)	$<R_N>$ nm	SD nm
33	23	9	0	16	6
48	22	9	98	22	9
67	23	6	192	21	9
97	21	6	283	18	8
133	19	7	454	23	10
190	26	6	833	17	8
267	23	9	1154	20	9
310	27	10			
430	24	9			
532	27	8			
710	28	8			
1070	25	10			
2100	28	11			

[a] Same result even after storing the diluted sample in a refrigerator for one or ten days.

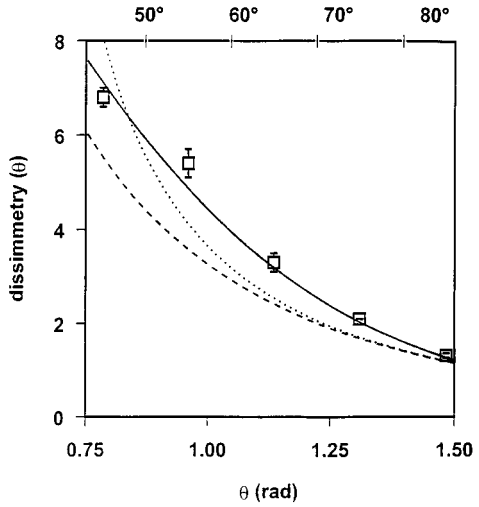


Figure 3. Dissymmetry of poly-APS structures was estimated at a concentration of 0.43 mg/mL in H_2O by changing angle between 45 and 135 degrees. Mean values (□) from three different determinations were fitted to theoretical functions (see Experimental Section and Discussion) pertinent to a spherical (—), thin rigid rod (···), and gaussian chain (- - -) shape of particles. Values for best fits were $<R_{sph}> \sim 127$ nm, $<R_{rod}> \sim 158$ nm and $<R_{ggc}> \sim 400$.

From the static light scattering[14] (SLS) we deduced that at least the largest structures formed by the poly-APS adopt a spherical configuration. In Figure 3 we report the experimental points and the theoretical fit with three alternative spatial arrangements: the spherical shape seemed to be the most reliable structure adopted by a polymer aggregate in solution. The best fit[15,16] of the dissymmetry as a function of q, supposing a spherical rearrangement, gave an estimated radius of about 127 nm.

Further experimental evidence that poly-APS form aggregates was obtained with the fluorescent marker Rhodamine 6G. This compound is a well-known and sensitive probe for determination of critical micelle concentration of cationic surfactants;[17] its fluorescence decreases when it becomes trapped into growing micelles. We found that the formation of non-covalently

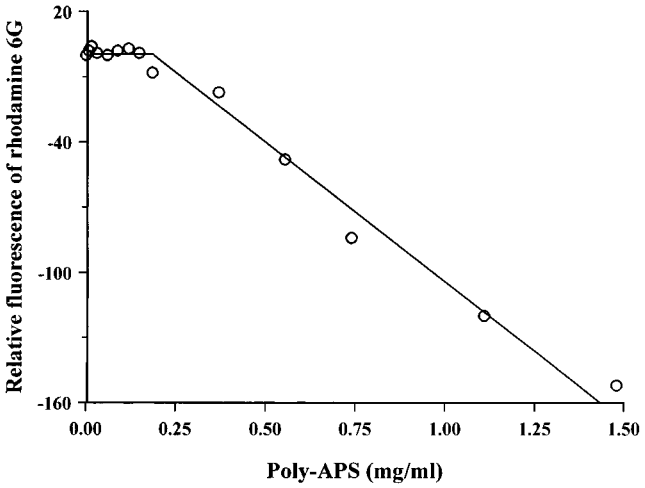


Figure 4. Determination of aggregation point with fluorescent probe Rhodamine 6G. To the each vial containing different concentrations of poly-APS a fluorescent probe Rhodamine 6G was added to achieve 10 μM final concentration. The vials were mixed and left in the dark for 48 h at the room temperature. The samples were excitated at 480 nm and their emission was recorded at 550 nm.

Table 3. Anticholinesterase Activity of Poly-APS (**5**)

	μg/mL poly-APS	
enzyme	50% inhib	100% inhib
recombinant insect AChE	0.06	0.37
electric eel AChE	0.08	0.41
human erythrocyte AChE	0.57	2.03
equine serum BuChE[a]	0.14	12.2

[a] Butyrylthiocholine as a substrate; Inhibitory activity was measured for 3 min.

bound aggregates occurs at about 0.2 mg/mL of poly-APS in H_2O (Figure 4), a concentration which is, however, about one order of magnitude larger than the lowest used in DLS and size-exclusion chromatography experiments. The observed difference could be due to the presence of positively charged Rhodamine 6G, which shifts the aggregation point to a somewhat higher concentration of positively charged polymers. Concentrations relevant for AChE inhibition and hemolysis are far below the concentrations used in either method. Therefore, it is possible that in concentrations relevant for their biological activities poly-APS actually exist as monomolecular polymers.

So far, few AChE inhibitors have been isolated from marine organisms,[18,19] in particular from marine sponges.[20] Poly-APS isolated from *R. sarai* act as very potent anti-AChE agents. They inhibit electric eel AChE, human erythrocyte AChE, insect recombinant AChE, and horse serum butyrylcholinesterase (BuChE) (Table 3). In contrast, inhibition of trypsin or alkaline phosphatase was not observed. The mechanism of AChE inhibition is rather complex with a predominant irreversible inhibition component. A detailed mechanism of inhibition kinetics is described by Sepčić *et al.*[21]

Poly-APS are lytic for erythrocytes and some other types of cells,[10] as previously reported for the halitoxin complex.[2] The hemolytic activity of oligo-APS, which readily passed a 3000-Da cut-off ultrafiltration membrane, was very low, which is consistent with their weak AChE-inhibitory activity. The hemolytic activity of poly-APS corroborates the described surfactant activity of alkylpyridinium compounds and could be compared to some cationic surfactants like cetylpyridinium salts.[22,23]

Very recently, Berlinck *et al.*[24] reported the isolation of alkylpyridinium compounds from the sponge *Amphimedon viridis.* The authors were able to separate 500-, 2000-(major peak), and 5000-Da 3-alkylpyridinium salts using size-exclusion chromatography; however, they used a less polar solvent system in the extraction procedure and chromatography. In our case, purified poly-APS were not soluble in MeOH. We also tried to repeat the extraction procedure described by Schmitz *et al.*[2] However, the yield of AChE-specific activity was about 5-fold lower compared to our extraction procedure. It is, therefore, possible that different extraction procedures result in the isolation of alkylpyridinium compounds that differ significantly in terms of molecular weight and their biological activities;[1,2,24] differences in biological activity have been observed in some previous studies.[1,9] We conclude that *R. sarai* possesses several oligo- and poly-APS, and that only poly-APS display anti-AChE and hemolytic activity. We present evidence that the isolated poly-APS, having molecular weights of 5520 and 18 900 Da by MALDI-TOF, form supramolecular non-covalently bound aggregates in aqueous solutions.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were performed on a Varian XL-300 spectrometer; ^{1}H-NMR spectra at 299.94 MHz and ^{13}C-NMR spectra at 75.43 MHz, with δ values in ppm, in D_2O using 3-(trimethylsilyl)-propionic acid-d_4 sodium salt (=0 ppm) as reference. C-multiplicity assignments were obtained by DEPT. Homonuclear ^{1}H connectivities were determined using COSY60, and assignments were confirmed by selective decoupling irradiations. ^{1}H−^{3}C connectivities were determined with Varian's standard pulse program HETCOR, optimized for an average C−H coupling costant of 140 Hz. Longitudinal relaxation times T_1 were obtained by inversion-recovery pulse sequence, while for transversal relaxation times a T_2 CPMG pulse[25] sequence was used. EIMS were obtained with a Kratos MS80 mass spectrometer equipped with home-built computerized acquisition data system. Portions of freeze-dried poly-APS were introduced and heated to 170−200 °C, and the mass spectra were recorded in both low- and high-resolution modes. MALDI-TOF measurements were performed on a linear time-of-flight spectrometer equipped with a frequency-tripled Continuum Surelite I Nd:YAG, 355 nm, laser. Acquired data were processed using commercially available software (LabCalc). All spectra were obtained in the positive ion mode. Mass assignments were made using external calibration equation generated by singly and doubly charged masses of bovine insulin at 5734.5 Da. Analyte/matrix preparations used in MALDI-TOF were prepared as follows: lyophilized poly-APS's sample was taken up in 0.1% TFA to a final concentration of 1 mg/mL, then serially diluted to 0.067 mg/mL. A saturated solution of matrix, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), was prepared by dissolving two parts of 5% aqueous TFA in one part HPLC grade MeCN. A solution of poly-APS (2 μL) was combined with an equal volume of matrix solution, applied to the surface of a stainless steel insertion probe tip, and allowed to air dry prior to insertion into the mass

spectrometer vacuum system. The size of poly-APS was estimated using size-exclusion chromatography. Lyophilized poly-APS (1 mg) were dissolved in 1 mL of H_2O and applied to a column (0.5 × 20 cm) filled with either Sephadex G-50, G-100, G-200, Sephacryl S-200, or Sepharose CL-6B and eluted with H_2O as a mobile phase. Attempts to estimate the dimensions of poly-APS by size-exclusion HPLC were also made using a Progel-TSK GMPW column (Supelco). DLS and SLS measurements were carried out with a Zetasizer3 particle sizer of Malvern with a 64-channel correlator, which can provide particle-size estimates in the range from 5 to 5000 nm. The observed autocorrelation functions were analyzed by the method of cumulants using Malvern Application Software. The first cumulant provides the mean intensity-weighted diffusion coefficient from which the mean hydrodynamic radius $<R>$ can be derived through the Stokes−Einstein relation.[16]

Because of the polydispersity of the distribution, however, we preferred to use a hydrodynamic radius weighted by the number of particles ($<R_N>$) under the Rayleigh−Gans−Debye approximation.[15,16] The shape of the polymer in solution was estimated from the angular dependence of the SLS. We used the dissymmetry function $d(q)$ defined as $d(q) = I(q)/I(p - q) = P(q)/P(p - q)$, which correlates $I(q)$, the experimentaly observable light scatter intensity at an angle q, with $P(q)$, the form factor. Three different shapes have been used for polymeric particles: hard spheres (sph), thin rigid rods (rod), and gaussian globular chains (ggc), that is, long flexible polymer chains, organized as balls.[15,16] Because only particles having dimensions comparable to the wavelength of the incident light present a dependence of $I(q)$ from the scattering angle,[15] $d(q)$ gives information only on the larger particles present in solution. Estimation of a poly-APS's aggregation point was made with a fluorometric method for evaluation of critical micellar concentrations of cationic surfactants as described by De Vendittis *et al.*[17] Lyophilized poly-APS were dissolved in H_2O to give different concentrations ranging from 7 μg/mL to 1.5 mg/mL, and the fluorescent probe Rhodamine 6G was added to achieve a 10-μM final concentration. After 48 h of equilibration in the dark at room temperature fluorescence intensity of the mixtures was recorded on a Perkin-Elmer LS50 spectrofluorimeter. Excitation and emission wavelengths were set at 480 and 550 nm, respectively.

Animal Material. Sponges were collected by scuba diving in the northern Adriatic Sea, mostly at the entrance of underwater caves at depths of 15−40 m. They were immediately put on ice and transferred to the laboratory, where they were kept at −20 °C until use. Dr. Jean Vacelet from Centre d'Oceanologie de Marseille, CNRS, Station Marine d'Endoume, Marseille, France, kindly identified the sponge as *Reniera* (= *Haliclona*) *sarai* Pulitzer-Finali. Voucher specimens are deposited as a part of the collection of Adriatic sponges (specimen no. 4) at the Department of Biology, University of Ljubljana, Slovenia.

Extraction and Isolation. Typically, 19 g of sponge were thawed, and 19 mL of deionized H_2O were added before homogenization with a blender. The homogenate was centrifuged twice at 15 000 rpm. Absolute EtOH was added to the supernatant to achieve a 60% final concentration. Precipitated materials were centrifuged at 15 000 rpm for 30 min. The obtained supernatant was concentrated about 10 times at a reduced pressure. Some of the precipitate that appeared was removed by centrifugation at 13 000 rpm. The clear, yellow supernatant was passed through an Amicon YM3 membrane (3000 Da cutoff). The retained material (1 mL 100−150 mg/mL dry wt), which possessed a strong AChE-inhibitory activity was applied to a Sephadex G-50 fine column (V_t = 344 mL) and eluted with deionized H_2O at a flow rate of 18 mL/h. Elution was monitored at 266 nm. Active fractions containing poly-APS eluted in the first peak with a void volume. They were pooled and lyophilized. The filtrate from the YM3 membrane was passed through Sephadex G-15 in deionized H_2O giving oligo-APS with a very weak anti-AChE activity. For comparison, either concentrated poly- and oligo-APS were applied to an ODS2 Spherisorb semipreparative reversed-phase HPLC column (Phase Separations) connected to the Waters HPLC system equipped with a Millenium software program (Millipore-Waters). Before separation, samples were passed through a Millex-GV 22-μm filter unit. Fractions were eluted with a linear 0−50% H_2O−MeCN gradient containing 0.1% TFA at a flow rate of 2.5 mL/min. We tried unsuccessfully to separate poly-APS eluted from Sephadex G-50 column by HPLC on a Suplex pKb-100 column (Supelco) using MeCN−10 mM sodium phosphate buffer with 5 mM SDS, pH 7.0, 30:70 (v/v). In order to check the purity and mobility in different solvent systems, aliquots of poly-APS obtained after gel filtration on Sephadex G-50 were applied onto Si gel TLC plates, which were developed with MeOH−CCl_3 3:1 (v/v). The samples were also applied on an amino TLC plate using MeOH−EtOAc 1:1 (v/v) or MeOH−H_2O 7:3 (v/v) as the mobile phase. Spots were detected with Dragendorff staining. All procedures were performed at room temperature, except gel chromatography and ultrafiltration, which were done at 4 °C.

Poly-APS (5): obtained as tiny white fibers; UV (H_2O) λ_{max} 266 nm, ϵ 2000 for every APS unit; UV (HCl) λ_{max} 266 nm; UV (NaOH) absorption at 266 nm disappeared; ^{1}H NMR (D_2O, 299.94 MHz) δ 8.76 (1H, br s, H-2), 8.73 (1H, m, H-6), 8.43 (1H, m, H-4), 8.03 (1H, m, H-5), 4.61 (2H, m, H_2-7), 2.89 (2H, m, H_2-7′), 2.04 (2H, m, H_2-8), 1.72 (2H, m, H_2-8′), 1.34 (8H, m, H_2-9, H_2-9′, H_2-10, H_2-10′); ^{13}C NMR (D_2O, 75.43 MHz) δ 148.13 (d, C-4), 147.03 (s, C-3), 146.11 (d, C-2), 144.31 (d, C-6), 130.44 (d, C-5), 64.45 (t, C-7), 34.63 (t, C-7′), 33.26 (t, C-8), 32.28 (t, C-8′), 30.92, 30.72, and 30.56 (t, C-10, C-9′ and C-10′), 27.91 (t, C-9); EIMS (70 eV, solid probe, 170−200 °C) 190 (32), 106 (76), 93(100), 92 (50); no other peak exceeded 10% rel int in the EIMS; MALDI-TOF sinapinic acid/poly-APS mass clusters m/z 18 900 (medium), 9450 (very weak), 5520 (strong), 2760 (weak); HREIMS m/z 190.1593 (calcd for $C_{13}H_{20}N$ 190.1596).

Determination of AChE-Inhibitory Activity. AChE-inhibitory activity was measured spectrophotometrically according to Ellman *et al.*[26] using a Shimadzu 2100 UV/vis spectrophotometer. Inhibitory activity was expressed in inhibitory units, 1 IU being defined as the amount of inhibitor that reduced the rate of acetylthiocholine (or butyrilthiocholine) hydrolysis by 50%. Electric eel AChE was used in most of experiments. Inhibitory activity was also assayed on human eryth-

rocyte AChE or horse serum BuChE and recombinant insect AChE using the same 5 units/mL stock solutions of the enzymes.

The ability of poly-APS to inhibit trypsin and alkaline phosphatase was also tested. The activity of trypsin was measured using casein as the substrate[27] and that of alkaline phosphatase with *p*-nitrophenylphosphate.[28] The poly-APS were used at a concentration that caused 50% inhibition of electric eel AChE.

Determination of Hemolytic Activity. Hemolytic activity was measured with a turbidimetric method[29,30] using an 0.05% suspension of bovine erythrocytes in 0.13 M NaCl−0.02 M Tris−HCl, pH 7.4. One hemolytic unit (1 HU) was defined as the amount of the sample that caused a 50% decrease of apparent absorbance at 700 nm in 2.5 min at 25 °C.

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References and Notes

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