Influence of polymeric 3-alkylpyridinium salts from the marine sponge *Reniera sarai* on the growth of algae and wood decay fungi

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Influence of polymeric 3-alkylpyridinium salts from the marine sponge Reniera sarai on the growth of algae and wood decay fungi

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Polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge Reniera sarai act as antifouling and anticholinesterase agents. They also show moderate haemolytic and cytotoxic activities against different cell lines. The haemolytic activity of poly-APS is due to their detergent-like structure and behaviour in aqueous solutions. In this work, the lytic activity of poly-APS against freshwater and marine algae, and inhibitory effects on wood decay fungi, were investigated. The results show that poly-APS inhibit the proliferation and movements of susceptible algae. Effects of poly-APS were time- and concentration-dependent and differed between various algal species. No growth inhibition effects were observed towards the examined wood fungi.

Keywords: 3-alkylpyridinium polymers; antialgal activity; antifouling; antifungal activity; marine sponge; Reniera sarai

Introduction

Marine sponges produce a plethora of different biologically active natural products, e.g. antiviral, antibacterial, cytotoxic, cytolytic, haemolytic, immunomodulatory and enzyme-inhibitory compounds. The production of bioactive compounds in sponges may serve as a natural repelling system, may help these organisms in territorial competition, or can act as a defense against fouling micro- and macroorganisms. Some of these compounds may find use as pharmaceuticals (Faulkner 2000), or as antifouling (AF) agents that prevent settlement of aquatic micro- and macroorganisms on submerged surfaces (Fusetani 2004).

Cytolytic agents, water-soluble polymeric 3-octylpyridinium salts (poly-APS) (Figure 1), were isolated from the marine sponge Reniera sarai (Sepčić et al. 1997a,b). These polymers are structurally and functionally related to cytotoxic halitoxins, 0.5–25 kDa 3-alkylpyridinium polymers isolated from several haplosclerid marine sponges (Schmitz et al. 1978; Berlinck et al. 1996; Scott et al. 2000). Poly-APS were determined to be a mixture of 29 (5520 Da) and 99 (18,900 Da) head-to-tail polymerized 3-octylpyridinium units (Sepčić et al. 1997b). In aqueous solution, they form larger structures with an average hydrodynamic radius of 23 nm ± 3 nm and have surfactant-like characteristics (Sepčić et al. 1997b). Experiments have shown that, besides a potent and complex antiacetylcholinesterase activity (Sepčić et al. 1998), poly-APS were antimicrobial towards marine bacteria (Garaventa et al. 2003; Chelossi et al. 2006), haemolytic (Malovrh et al. 1999), cytotoxic for different cell lines including transformed cells (Sepčić et al. 1997a, Paleari et al. 2006), and are potentially interesting as new tools for stable transfection of mammalian cells (Tucker et al. 2003). Poly-APS also were found to inhibit the settlement of Balanus amphitrite cypris larvae, acting via a reversible, non-toxic AF mechanism (Faimali et al. 2003), and to be slightly toxic towards the marine microalgae Tetraselmis suecica, with an IC$_{50}$ value of 10.66 µg ml$^{-1}$. In view of the potential use of poly-APS as new AF compounds, the work reported here broadened the study of toxic effects to another three ecologically relevant freshwater and marine algal species. Additionally, the inhibitory effect of poly-APS was assessed against two species of wood decay fungi. Poly-APS are chemically similar to alkyl ammonium compounds (AACs) that have been used in wood preservation (Eaton and Hale 1993), which consist of two main synthetic types: tertiary amine salts and quaternary ammonium compounds.
Materials and methods

Materials

Poly-APS

Poly-APS were purified from the marine sponge *Reniera sarai* as described previously (Sepčić et al. 1997b). Briefly, 50 g of the fresh sponge were homogenized with 50 ml of deionized water, and the homogenate was centrifuged twice for 30 min at 15,000 rpm. Absolute ethanol was added to the supernatant to achieve a 60% final concentration, and precipitated materials were centrifuged at 15,000 rpm for 30 min. The supernatant obtained was concentrated about 10 times at a reduced pressure, and the resulting precipitate was removed by centrifugation as described above. The supernatant was passed through an Amicon YM3 membrane (3000 Da cut-off). Three millilitres of the retained material were applied to a Sephadex G-50 fine column ($V_t = 344$ ml), and eluted with deionized water at a flow rate of 18 ml h$^{-1}$. Elution was monitored at 266 nm. Fractions corresponding to the first peak, which was eluted with the void volume, were pooled and lyophilized to obtain pure poly-APS. A series of poly-APS solutions was prepared by diluting the 50 mg ml$^{-1}$ stock solution in deionized water.

Selected algal species

Three different algal species were used as test organisms. A non-axenic culture of the freshwater alga *Scenedesmus subspicatus* (Chlorophyta) was obtained from the National Institute of Biology, Ljubljana, Slovenia and non-axenic unicultures of the marine species *Nitzschia* sp. (Bacillariophyta) and *Dunaliella* sp. (Chlorophyta) from the Marine Biological Station, Piran, Slovenia. The growth medium for freshwater algae was prepared in accordance with ISO (1989). For the two marine species, adjusted marine water was used. Marine water was collected from the Adriatic Sea (Gulf of Trieste, Slovenia) and filtered through Whatman GF/c. The initial pH value was ~7.5. Continuous illumination for the algal species was provided by Osram L 18 W/72 Biolux white fluorescent lamps together with Sylvania GrO-Lux F 18 W/GrO-T8 lamps. The experiments were carried out in Erlenmeyer flasks (50 ml of culture) in a temperature-controlled chamber at 20°C and an irradiance of 100 $\mu$mol m$^{-2}$ s$^{-1}$. The cultures were mixed by occasional manual shaking, usually twice a day. Selected algal cultures were exposed to different concentrations of poly-APS and the antialgal activity was monitored daily. The applied test procedures were modified versions of the test procedures described by ISO (1989). The duration of the test with *S. subspicatus* was 7 days, and with *Nitzschia* sp. and *Dunaliella* sp. 8 days. Inoculations were made with algal precultures set up 2 days before the experiments, and grown under the same conditions during the test.

Fungi and wood samples

The following wood decay basidiomycetes were used: a brown rot fungus *Coniophora puteana*, strain ZIM L008, and a white rot fungus *Trametes versicolor*, strain ZIM L057. Both strains were from the Culture collection at the Department of Wood Science and Technology, Biotechnical faculty, Ljubljana (Raspor et al. 1995). Antifungal tests with the brown rot fungus were carried out on wood samples made from Norway spruce (*Picea abies*), and those with the white rot fungus on beech wood (*Fagus sylvatica*). The dimensions of samples used for the mini block test (MBT) were $24.5 \times 9 \times 5$ mm (Norway spruce) and $31 \times 10 \times 5.5$ mm (beech). The dimensions of both wood samples for the EN 113 standard assay were $15 \times 25 \times 50$ mm. The orientation and quality of the wood as well as fungicidal tests met the requirements of the standard EN 113 (European Committee for Standardization 1996).

Methods

Antialgal activity

Algal cells were counted microscopically using a Bürker–Türk haemocytometer. When counting was inappropriate for cell quantification, as in the case of cell flocculation of *Nitzschia* sp., chlorophyll *a* from algal cells was extracted with hot methanol and measured as described elsewhere (Vollenweider 1974). The inhibition of algal movement was assessed by the addition of three different concentrations of poly-APS (2.5, 14, or 80 $\mu$g ml$^{-1}$) to non-axenic cultures of *Dunaliella* sp. (10$^6$ cells ml$^{-1}$). Cells then were counted...
at three different time intervals after the addition (A: 0–5, B: 20–30, C: 60–80 min). Moving, slow-moving (slow spinning and twitching) and immobile cells at time intervals A, B in C were counted separately. Each of the above-described experiments was repeated three times.

Statistical analyses
From the inhibition curves, the 50% effect concentration (EC50) was determined. With the suppositions that (i) every set of repetitions presents random samples from heterogeneous population, (ii) every parental population is normally distributed and (iii) every parental population has equal variance, testing the analysis of variance (ANOVA) between treated and control cultures was appropriate. Two-factor ANOVA with replication was used for testing the statistically significant differences of results from all growth experiments with algae. Every set, which represented one curve, was tested to all the other sets and to the control-set curve. Data from different bioassays were analyzed separately, because the set of poly-APS concentrations for different algal species was different. Single factor ANOVA was used for testing the data from experiments with cell movements of Dunaliella sp. Statistical significance, P value (P < 0.05) and degrees of freedom (df) are included in the Results section.

Antifungal activity
For rapid determination of fungicidal activity, screening tests in Petri dishes on potato dextrose agar medium (PDA, DIFCO) were performed with a broad range of poly-APS concentrations (1, 5, 10, 50, 100, 500, 1000, and 5000 μg ml⁻¹). Each concentration (0.1 ml) was pipetted into small pits (Φ = 5 mm) that were previously cut around the perimeter of PDA medium, which had been inoculated centrally with a small piece of mycelium. After 7 days, the mycelial growth of both fungi was estimated visually. CuSO4 was used as a positive control. Each concentration was assayed in five parallel trials.

Further studies of the fungicidal properties of poly-APS were performed on wood samples that were coated once with poly-APS in the concentration range 1 to 5000 μg ml⁻¹. Retention of the wood specimens resulted in an aqueous solution uptake of about 300 g m⁻². The MBT experiments were performed in Petri dishes. The EN 113 standard assays were performed in Kolle flasks. Wood specimens were placed on sterilized plastic grids on mycelium-overgrown PDA medium and exposed to fungal decay in the growth chamber (25°C, 75% relative humidity). The results were determined after 8 and 12 weeks for the MBT and EN 113 assays, respectively. Mass losses of exposed samples were determined gravimetrically on five parallel specimens (European Committee for Standardization 1996). Each test was performed in five parallel trials. The activity of poly-APS is not destroyed by elevated temperatures and the compound is stable for years as an aqueous solution kept at 4°C (K. Sepčić, personal observation). Therefore, it was assumed that the compound was stable during the 8- and 12-week exposures.

Results
Growth inhibition of algae – Calculated as EC50 values
Table 1 shows the toxicity of poly-APS for algae calculated as EC50 values (μg ml⁻¹) over the time interval of 0–8 d (for Dunaliella sp. and Nitzschia sp.) or 0–7 days (for S. subspicatus). The lowest biomass EC50 (b-EC50) of Dunaliella sp. is approximately two times lower compared with the b-EC50 of S. subspicatus, and nearly three times lower compared with the b-EC50 of Nitzschia sp. The lowest growth rate EC50 (r-EC50) of Dunaliella sp. is approximately two times lower compared to the r-EC50 of Nitzschia sp., and nearly five times lower compared with the r-EC50 of S. subspicatus.

<table>
<thead>
<tr>
<th>Algal species</th>
<th>Tested poly-APS concentrations (μg ml⁻¹)</th>
<th>b-EC50 (μg ml⁻¹)</th>
<th>r-EC50 (μg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. subspicatus</td>
<td>2.5–140.0</td>
<td>9.7</td>
<td>34.8</td>
</tr>
<tr>
<td>(0–7 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitzschia sp.</td>
<td>2.5–25.0</td>
<td>13.6</td>
<td>16.1</td>
</tr>
<tr>
<td>(0–8 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dunaliella sp.</td>
<td>2.5–8.0</td>
<td>5.3</td>
<td>6.8</td>
</tr>
<tr>
<td>(0–8 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EC50 values based on growth area (biomass) are denoted as b-EC50, and those based on growth rate as r-EC50 at different time intervals (0–7 or 0–8 days).
19 df) the chlorophyll $a$ content of *Nitzschia* sp. in comparison with the control. Effects induced by other tested concentrations (2.5, 8 and 14 $\mu$g ml$^{-1}$) were not significantly different ($P > 0.05$, 19 df) from the control (Figure 3). *Dunaliella* sp. was more susceptible than the other algal species tested, showing significant reduction ($P < 0.05$, 39 df) of growth at 2.5 and 8 $\mu$g ml$^{-1}$ of poly-APS (Figure 4).

**Inhibition of algal movement**

Inhibition of the movement of *Dunaliella* sp. was time- and concentration-dependent (Figure 5). Partially
inhibited movement resulted in slow-moving and spinning or twitching of *Dunaliella* sp. cells. The differences in the numbers of cells between time intervals A, B and C within the same concentration were not statistically significant ($P > 0.05$, 47 df). Similarly, no statistically significant differences were found between the control and 2.5 $\mu$g ml$^{-1}$ poly-APS for moving, slow-moving and immobile cells ($P > 0.05$, 47 df). All the other differences were statistically significant ($P < 0.05$, 47 df).

**Antifungal activity**

The results of all three fungicidal tests, carried out with two wood decay fungi, clearly showed that poly-APS was not able to inhibit the growth of mycelium. Slight retardation in mycelial growth was detected only with the screening test on agar plates at the highest concentration used (5000 $\mu$g ml$^{-1}$, Figure 6).

Similar results also were obtained by the MBT and EN 113 tests, in which the mass losses of treated and control wood samples were almost the same. Table 2 presents the results obtained by the EN 113 assay.

**Discussion**

To date, 3-alkylpyridinium oligomers and polymers have been reported to be cytotoxic, haemolytic, antibacterial, antifeeding, AF, toxic to animals, antimitotic, neurotoxic, inhibitory for acetylcholinesterase and histone deacetylase, epidermal growth factors, muscarinic receptors, and to cause irreversible membrane potential depolarization and stable cell transfection (reviewed in Sepčić and Turk 2006). The biological significance of 3-alkylpyridinium compounds from marine sponges is not yet well understood. However, there is emerging evidence that these natural surfactants possess a large variety of biological activities and may serve as unspecific antibiotics affecting micro- and macroorganisms, and as natural repelling agents. Poly-APS, forming a thin greasy layer at the surface of the sponge, may prevent the settlement of other organisms and clogging of the pores of the sponge.

The cytotoxic, cytolytic and haemolytic activities of poly-APS, at least in part, may be explained by their surfactant-like characteristics. These compounds bear several relatively delocalized positive charges and hydrophobic alkyl chains, which are known to be associated with toxic effects, especially those deriving from the disruption of membranes (Kaiser et al. 1998). At present, the exact mechanism of pore formation by poly-APS remains obscure. Despite their polymeric nature and a rather short alkyl chain flanked by pyridinium rings, poly-APS demonstrate properties very similar to those of some other cationic detergents such as cetylpyridinium chloride and cetyltrybutylammonium bromide (Malovrh et al. 1999). Electrophysiological recordings and experiments with osmotic protectants suggest that poly-APS produce discrete lesions in erythrocyte (Malovrh et al. 1999) and mammalian cell membranes (McCleeland et al. 2003), allowing stable transfection of the latter cells with heterologous DNA (Tucker et al. 2003).

Alkyl ammonium compounds often exert antifungal activity and are used in wood preservation (Eaton and Hale 1993). However, in the study reported here, the fungal decay pattern of poly-APS-treated wood was comparable with the control (Table 2), demonstrating that poly-APS neither prevented wood decay by the fungi, nor acted as fungal nutrient supplements (Humar and Pohleven 2005; Humar et al. 2006a). Humar et al. (2006b) also observed lower effectiveness of amine-containing wood preservatives. The slight inhibition of mycelial growth, observed in the screening test at the highest tested concentration (5000 $\mu$g ml$^{-1}$), could be caused by a high concentration of poly-APS in the nutrition medium, which spontaneously influenced physiological processes in the fungal cells (Figure 6). Based on these results, poly-APS are not recommended as biocides in wood preservatives against wood-rotting fungi. However, they can be effective against algae which cause the surface disfiguration of wood products, or as anti-foulants for wooden boats.

Poly-APS inhibited the growth of the tested freshwater and marine algal species. The observed lytic activity of poly-APS towards the marine alga *Tetraselmis suecica*, with a 72-h IC$_{50}$ value of about 10 $\mu$g ml$^{-1}$ (Faimali et al. 2003), is ~30-fold lower than that obtained on erythrocytes and other cell lines (Sepčić et al. 1997b; Malovrh et al. 1999). In the study reported here, the moderate toxicity of poly-APS towards marine and freshwater microalgae was confirmed, showing 7- or 8-day b-EC$_{50}$ values still in the

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**Figure 6.** Effect of poly-APS (A) and CuSO$_4$ (B) on mycelial growth of *T. vesicolor*. The fungicidal activity of both compounds was assessed on mycelium-inoculated potato dextrose agar medium, as described in the Materials and methods section. Numbers denote the tested concentrations of poly-APS and CuSO$_4$ in mg ml$^{-1}$. 

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![Image](image-url)
range of several micrograms per millilitre (Figures 2–4, Table 1). The lowest b-EC50 value of 5.3 \( \mu \text{g ml}^{-1} \) was observed with Dunaliella sp. (Table 1). Inhibitory effects on the algal movements were time- and concentration-dependent (Figure 5). However, higher doses of poly-APS can show direct and potent lytic effects on other selected algal species, as shown in experiments with Skeletonema costatum (Logar et al. 2005), and in the study reported here (Figures 2–4).

The toxicity of poly-APS towards freshwater and marine algae is approximately 100-fold lower compared to booster biocides such as zinc or copper pyrithione, compounds usually used in AF paints and acting by a toxic, irreversible AF mechanism (Faimali et al. 2003). The EC50 value of booster biocides against T. suecica is very similar to the concentration causing 50% settlement inhibition of the barnacle B. amphitrite. In contrast, poly-APS act as AF agents without damaging organisms belonging to the first ring of the trophic chain in aquatic ecosystems. These data, in concert with the inhibition of proliferation and movements of susceptible algal species presented in this paper, justify the efforts to chemically synthesize poly-APS analogues that would act by a non-toxic reversible antisettlement mechanism, and could be used as ecologically-friendly antifoulants. First monomeric, dimeric and tetrameric 3-alkylpyridiniums have already been synthesized (Mancini et al. 2004). These compounds showed almost no haemolytic activity, but relatively high antibacterial and anti-acetylcholinesterase activities that increased with higher degrees of oligomerization (Mancini et al. 2004). Their AF potential was evaluated in the B. amphitrite antisettlement test (Faimali et al. 2005), showing promising, albeit lower, AF potential than the natural compound with a higher degree of oligomerization. The results suggested that AF activity was favoured by increasing the length of the alkyl chain, or by the presence of uncharged pyridine units. The presence of single or multiple charges, however, did not influence their AF potential. Because all the tested compounds were less efficient than poly-APS, which also showed a higher toxicity towards the naupliar stage of B. amphitrite, it could be concluded that the high and reversible anti-macrofouling activity of poly-APS derive from their detergent-like properties and behaviour in aqueous solutions.

**Acknowledgements**

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**Abbreviations used**

- b-EC50: the lowest biomass EC50, e.g. EC50 in terms of biomass reduction
- EC50: concentration of poly-APS causing a 50% effect compared to the control
- Poly-APS: polymeric alkylpyridinium salts
- r-EC50: the lowest growth rate EC50, e.g. EC50 in terms of growth rate reduction
- PDA: potato dextrose agar
- MBT: mini block test

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