ARTICLE

OBC
www.rsc.org/obc

Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge *Reniera sarai*†

Ines Mancini,[a] Adriana Sicurelli,[a] Graziano Guella,[a] Tom Turk,[b] Peter Maček[b] and Kristina Sepčić*[b]

[a] *Laboratorio di Chimica Bioorganica, Università di Trento, via Sommarive 14, I-38050 Povo Trento, Italy*

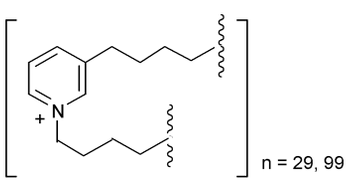
[b] *Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1111 Ljubljana, Slovenia. E-mail: kristina.sepcic@uni-lj.si; Fax: +38612573390; Tel: +38614233388*

Received 19th January 2004, Accepted 26th February 2004
First published as an Advance Article on the web 1st April 2004

Dimers and tetramers of linear 3-alkylpyridinium salts have been synthesized by an efficient synthetic pathway, which is also applicable to the preparation of higher oligomers. Mono-, di- and tetrameric compounds have been tested for antibacterial and hemolytic activities and for the inhibition of acetylcholinesterase and protein phosphatase 2A. Their activities were compared to those of the natural poly-3-octylpyridinium alkaloids isolated from the Mediterranean sponge *Reniera sarai.* Relatively high antibacterial and anti-acetylcholinesterase activities were observed that increase with higher degrees of oligomerization.

Introduction

The water soluble polymers **1** (Chart 1), composed of 3-octylpyridinium units linked head-to-tail, have recently been isolated from the Mediterranean sponge *Reniera sarai* (Haliclonidae).[1] These compounds showed a broad spectrum of biological activities, including potent anti-acetylcholinesterase (anti-AChE) activity with a rather unusual pattern of inhibition,[2] and both hemolytic[3] and cytotoxic[4] activities. MALDI-TOF spectrometric analysis[1] showed that the compounds comprised a mixture of two polymers with molecular weights centred at 5520 Da and 18900 Da and with a low degree of polydispersity. These polymers are related structurally to the epidermal growth factor (EGF)-active alkaloids from the sponge *Callyspongia fibrosa*[5] and to cytotoxic and hemolytic halitoxins isolated from several sponges of the genus *Haliclona*.[6]



1

Chart 1 Structure of polymeric alkylpyridinium metabolites **1** from *Reniera sarai*.

In addition, compounds **1** have recently shown promising antifouling activity, in terms of inhibiting reversible settlement of larvae of *Balanus amphitrite*[7a] and inhibition of natural marine biofilm formation.[7b]

Our previous study on the natural minor uncharacterized oligomeric alkylpyridinium salts from *R. sarai* showed their low anti-AChE and hemolytic effects, and indicated the importance of polymerization for their biological activity.[1] For this reason, we have prepared synthetic poly-alkylpyridinium compounds with a defined number of units, and assessed their activities. We report here an efficient synthetic pathway for preparing linear oligomers, of general application to higher oligo- and polymeric alkylpyridinium salts. The biological activities of some mono-, di- and tetrameric compounds related to the natural poly-alkylpyridinium salts (poly-APS, **1**) have been determined.

Results and discussion

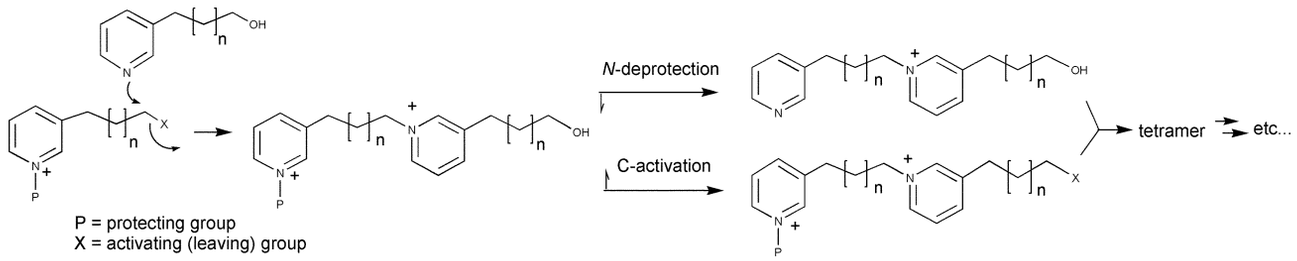
Synthesis

Much effort has been devoted to the synthesis of 3-alkylpyridinium compounds, among them the cyclic dimeric alkaloids cyclostellettamines A–F,[8] that could be biogenetically related to the more complex structure of manzamines. As an alternative to direct cyclodimerization of the relevant 3-alkylpyridine substrate bearing an iodide as leaving group,[9] a strategy, first demonstrated successfully in the synthesis of haliclamines A, B,[10] has been applied. It is based on the nucleophilic substitution of one pyridine subunit with a masked leaving group at the terminal alkyl position on another *N*-protected pyridinium subunit bearing a leaving group at the terminal position of the 3-alkyl chain. The assembly includes protection of pyridine as the *N*-oxide, and bromination of the alkyl hydroxyl group.[9] Another example involves *N*-protection as a *p*-methoxybenzyl (PMB) derivative, and its subsequent removal by refluxing in pyridine.[11]

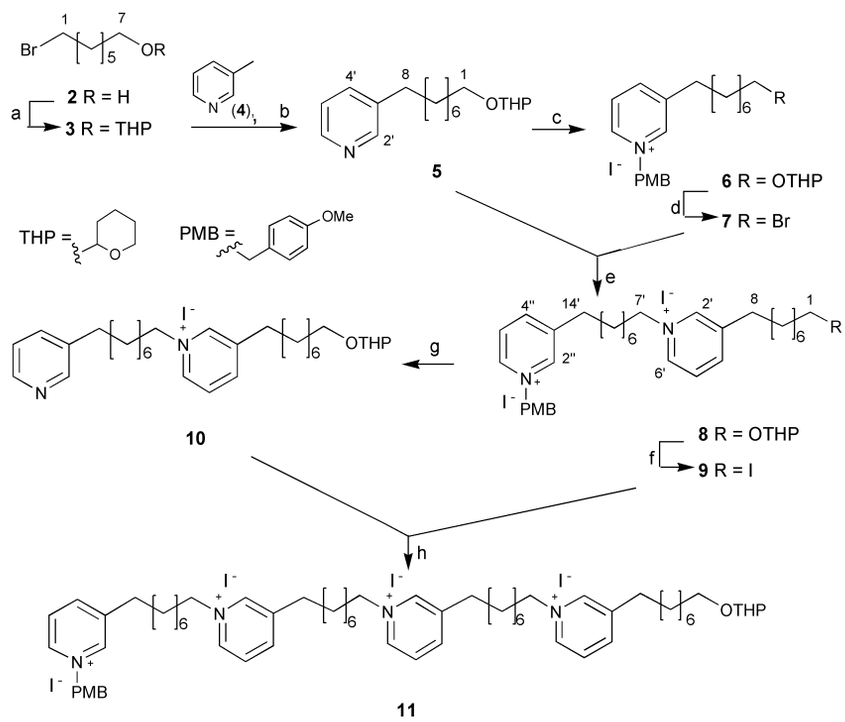
In the synthetic route to large linear oligomers analogues of the EGF-active pyridinium alkaloids from the sponge *Callyspongia fibrosa*,[5] the induction of head-to tail oligomerization was effected using the traditional approach. The reaction of a free *N*-pyridine monomer with different leaving groups gave a mixture of dimer, trimer, tetramer and higher order oligomers, of difficult separation because of their increasing polarity. Similarly, synthetic studies using the alkyl ether model compound 3-[3-(4-chloro-butoxy-propyl)]pyridine gave an inseparable mixture of water soluble polymers when a solution of substrate of unknown concentration was refluxed in acetonitrile.[12] Selective entry to linear oligomeric pyridinium compounds was later realised by the same authors using the *N*-protection/C-activation sequence involving the Zincke reaction, allowing the number of alkylpyridinium subunits in the polymers to be controlled by an iterative process.[13]

† Dedicated to the memory of Dr Sebastiano Geraci.

DOI: 10.1039/b400782d



Scheme 1 Synthetic strategy for the preparation of linear oligomeric analogues of polyAPS **1**.



Scheme 2 Synthesis of linear 3-octylpyridinium oligomers. *Reagents, conditions and yields:* (a) 3,4-dihydro-2*H*-pyran, PPTS, CH_2Cl_2, rt, 18 h, 92%; (b) $^tPrNH_2$, *n*BuLi, THF, DMPU, addition of **4**, from −78 °C to rt, 18 h, 90%; (c) PMB-Cl, KI, CH_3CN, rt, 20 h, 90%; (d) PBr_3, $CHCl_3/CH_3CN$ 10 : 1, 0 °C, 15 min, reflux 1 h, sat. aq. $NaHCO_3$, 82%; (e) KI, CH_3CN, reflux 20 h, 80%; (f) 1) PBr_3, $CHCl_3/CH_3CN$ 1 : 1, 0 °C, 15 min, reflux 1 h, sat. aq. $NaHCO_3$ 2) KI, CH_3CN, reflux 20 h, 74% over two steps; (g) pyridine or 3-methylpyridine (**4**) in excess, reflux 16 h, 75%; (h) CH_3CN, reflux 20 h, 75%.

Therefore, we have adopted the *N*-protection/C-activation sequence as the method of choice, in order to obtain linear oligomers, analogues of the natural compounds **1** (Scheme 1).

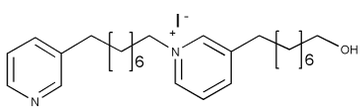
The optimized sequence of reactions that we have achieved is reported in Scheme 2. The monomeric 3-octylpyridine substrate **5** was obtained by standard methods from the commercially available 3-methylpyridine (**4**), which was lithiated with *in situ* generated lithium diisopropylamide (LDA) at −78 °C in tetrahydrofuran in the presence of dimethyltetrahydro-2(1*H*)pyrimidinone (DMPU), and then treated with purified tetrahydropyranyl (THP) ether **3**. The monomeric 3-octylpyridine unit was *N*-protected to avoid intramolecular cyclization in one case, and activated on the alkyl chain in the other case, to give a linear dimeric compound by nucleophilic substitution. An aliquot of compound **5** was so converted to its *N*-PMB pyridinium derivative **6** (Experimental) and then transformed into bromide **7**. The latter was obtained directly in good yield by treating the THP-ether with phosphorus tribromide,[8] and washing with an aqueous solution of $NaHCO_3$. The remaining portion of the *N*-free THP ether **5** was refluxed together with the *N*-PMB pyridinium bromide **7** in acetonitrile in the presence of potassium iodide to give the dimer **8**, and purified from the slight excess of **5** by washing with hexane/ethyl acetate, and from inorganic salt by flash chromatography on alumina. The basic washing of the organic phase from the reaction with phosphorus tribromide was essential to avoid traces of acidic materials, causing substantial deprotection of the THP-group by refluxing in acetonitrile, to give a mixture of dimer **8** and its corresponding alcohol. A portion of **8** was then converted to **9** by preparing the corresponding bromide, which was then treated with sodium iodide to avoid having to purify the water-soluble tetramer from the inorganic salt. Compound **10** was obtained by heating the *N*-PMB pyridinium dimer **8** in 3-methylpyridine with better results than in pyridine[11] (Experimental). By treatment with **9** under reflux in acetonitrile, it gave tetramer **11**, which was purified by flash chromatography on NH_2-stationary phase with water/acetonitrile. Applying this controlled and iterative process on the oligomeric compounds successively obtained, it is possible to increase the degree of polymerization.

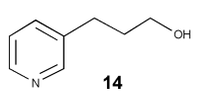
Biological activities

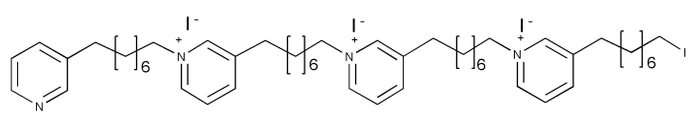
The synthetic compounds **5**–**7**, **11** (Scheme 2), **12** and **13**, together with commercial compounds **4** and **14** and their corresponding salts **4·HCl** and **14·HCl** obtained by treatment with an aqueous solution of HCl, were tested for their antibiotic and hemolytic activities, and for inhibition of AChE and protein phosphatase 2A (PP2A). Their activities were compared with those of the natural polymers **1**. While antibacterial and hemolytic activities and AChE inhibition are often attributed to alkylpyridinium compounds,[14] PP2A-inhibitory effects have not previously been reported for this family of compounds.

Table 1 Minimal inhibitory concentrations (MIC) of the tested compounds against selected bacteria. n.i. = no inhibition

Microorganism	MIC (μM)								
	4	**4·HCl**	**6**	**7**	**12**	**13**	**14**	**14·HCl**	Poly-APS (**1**)
Gram +									
Staphylococcus aureus	n.i.	n.i.	4638	482.6	95.4	7.89	n.i.	n.i.	73.8
Bacillus subtilis	n.i.	15503	1391	1.9	1.9	0.79	n.i.	n.i.	286.9
Streptococcus foecalis	n.i.	n.i.	n.i.	482.6	477.1	0.79	n.i.	n.i.	327.9
Micrococcus luteus	n.i.	n.i.	n.i.	289.5	477.1	39.4	n.i.	n.i.	245.9
Gram −									
Pseudomonas aeruginosa	n.i.	n.i.	n.i.	1737	n.i.	709.8	n.i.	n.i.	n.i.
Escherichia coli	n.i.	n.i.	n.i.	772.2	2290	7.9	n.i.	n.i.	n.i.
Salmonella typhimurium	n.i.	n.i.	n.i.	965.2	3816	709.8	n.i.	n.i.	n.i.
Klebsiella pneumoniae	n.i.	n.i.	n.i.	482.6	4770	591.5	n.i.	n.i.	n.i.
Proteus vulgaris	n.i.	n.i.	n.i.	1544	n.i.	709.8	n.i.	n.i.	n.i.







Pyridinium compounds often exhibit antibacterial activity, and this feature is nowadays used in the fields of industry and medicine. Substituted pyridinium derivatives, for example, can be attached to different surfaces and used for removing bacteria from water,[15] or can be incorporated in dental resins, thus protecting from infections with oral streptococci.[16]

The antibacterial activity against four Gram positive (*Staphylococcus aureus*, *Streptococcus foecalis*, *Micrococcus luteus*, *Bacillus subtilis*) and five Gram negative bacterial strains (*Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) was evaluated using the classical agar diffusion test (Experimental). The results are reported in Table 1 as minimal inhibitory concentrations (MIC).

Compounds **7**, **12** and **13** exhibited the highest antibacterial activity. Poly-APS **1** and compound **6** showed moderate effects only on Gram positive bacteria, while **4**, **14** and its pyridinium salt **14·HCl** did not inhibit bacterial growth. For the most active tetramer **13**, the MIC values were in the micromolar range against Gram positive and the millimolar range against Gram negative bacteria. Compounds **7** and **12** were approximately 10 fold less inhibitory as compared to compound **13**. These three compounds were all active against both Gram positive and Gram negative bacteria, while the natural poly-APS (**1**) only inhibited the growth of Gram positive strains. The pronounced activity against Gram positive bacteria exhibited by the synthetic alkylpyridinium analogues correlates with other data obtained on natural alkylpyridines[6,17] and is probably due to the presence of an additional outer membrane in Gram negative strains. Furthermore, activity increased with increasing number of positive charges (from 0 to 3) or pyridinium rings (from 1 to 4) (Fig. 1).

Monomeric analogues without a protective group, with or without charge, were not active, pointing to the importance of oligomerization for antibacterial activity. In addition, the antibacterial activity of alkylpyridinium oligomers appears to be correlated with the number of positive charges, which is probably due to the negative surface charge on bacteria under physiological conditions. Similar charge-dependent antibacterial effects have been reported for the 3-alkylpyridinium derivatives isolated from the sponge *Calyx podatypa*, xestamines,[17b] for which quaternary nitrogen compounds were

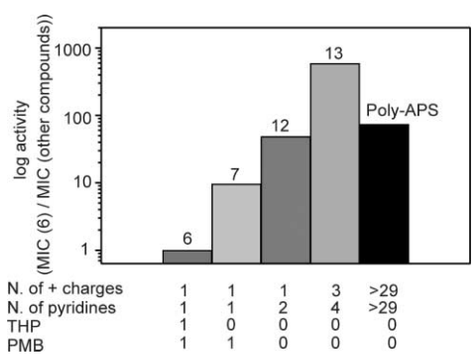


Fig. 1 Correlation between antibacterial activity against *Bacillus subtilis* and the structure of the tested compounds (number of positive charges, pyridinium rings and protective groups). Activity is expressed as the ratio between the lowest activity, corresponding to MIC (μM) of compound **6**, and the MIC (μM) values for other compounds.

40 fold more active against Gram positive bacteria than the corresponding tertiary amines. Finally, poly-APS (**1**), bearing more than 29 positive charges, had lower antibacterial activity, comparable to that of the dimer **12**. This effect could be the consequence of poly-APS micellization, as already shown for the antibacterial activity of quaternary ammonium salts which rapidly decreases during the formation of micelles and is due to the decreased number of free active monomers.[18]

Decrease of the antibacterial activity of quaternary ammonium salts is also correlated to the length of the alkyl chain;[19] its elongation lowering the critical micelle concentration.[18] In our case, all the compounds that showed bioactivity carried equal length octyl chains, so the above effect was not observed.

Only compounds **7** and **13** induced lysis of bovine erythrocytes, and were approximately 100 fold less active than poly-APS (**1**).[3] The time course of the hemolysis was very rapid, lacking a lag-phase and resembling that obtained with the cationic detergent cetylpyridinium bromide.[3] In Fig. 2, the reciprocal t_{50} values of hemolysis, induced by active compounds using a turbidimetric method (Experimental), are plotted against their concentration. The synthetic analogues exhibited a surprisingly low hemolytic activity, considering the pronounced cytotoxic effects of a number of natural alkylpyridinium compounds, including the monomeric ones.[4,6,20]

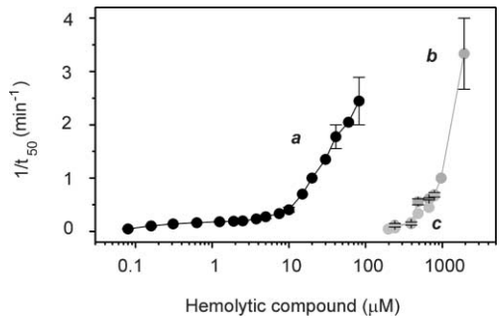


Fig. 2 Reciprocal values of the half-times (t_{50}) of hemolysis, caused by poly-APS **1** (*a*), by compound **13** (*b*) and **7** (*c*), as a function of their concentration. Each point represents the mean of three measurements with the corresponding standard error.

The reason for this weak activity could be reflected in the relatively high critical micelle concentrations (CMC) of the synthetic compounds. It is known that the detergents having a lower CMC induce hemolysis at much lower concentrations, probably because of the poor solubility of the monomers in water and their high affinity for the non-polar membrane.[21]

Acetylcholinesterase (AChE, EC 3.1.1.7) hydrolyses the neurotransmitter acetylcholine in nervous system synapses. This takes place at the bottom of a 20 Å deep enzyme gorge. The choline is bound by an anionic binding site and cleavage is performed *via* a serine, activated by a catalytic triad. At the rim of the gorge, another choline binding site exists—the peripheral anionic site.[22] Several irreversible AChE inhibitors are known as warfare agents and insecticides, and some reversible inhibitors are used for treating certain neurological diseases connected with AChE dysfunction, for example Alzheimer's disease and *myasthenia gravis*. The monomeric compounds **5**–**7**, the dimer **12** and the tetramers **13** and **11** all inhibited AChE, with the degree of inhibition increasing with the number of pyridine rings and positive charges (Fig. 3).

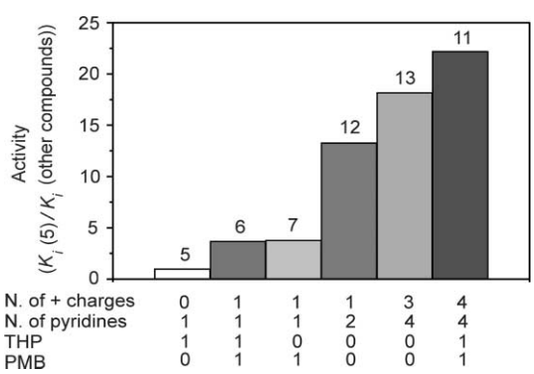


Fig. 3 Correlation between anti-AChE activity and the structure of the tested compounds (number of positive charges, pyridinium rings, and protective groups). Activity is expressed as the ratio between the lowest activity, corresponding to the K_i (μM) of compound **5**, and the K_i (μM) of other compounds.

The time-course of inhibition was always linear, in contrast to the non-linear inhibition observed with the natural polymeric alkylpyridinium salts.[2] The inhibitory constants (K_i) and the type of enzyme inhibition for each tested compound were determined from Dixon plots (Table 2). The non-linear inhibitory kinetics of poly-APS, reflecting their polymeric structure and ending in the irreversible inhibition of the enzyme,[23] cannot be compared to the kinetic patterns obtained with the synthetic analogues. Only the analysis of first 15 seconds of the AChE–poly-APS interaction showed a classical non-competitive inhibition, with an estimated of K_i of 11 ± 3 nM.[2] This value is 10 fold lower than that of the most inhibitory synthetic compound **11**.

Table 2 Inhibition constants and types of inhibition of electric eel acetylcholinesterase by the compounds tested. n.i. = no inhibition

Compound	K_i (μM)	Type of inhibition
4	n.i.	n.i.
4·HCl	n.i.	n.i.
5	14	competitive
6	2	non-competitive
7	2	competitive
11	0.12	non-competitive
12	0.6	competitive
13	0.18	non-competitive
14	n.i.	n.i.
14·HCl	n.i.	n.i.
Poly-APS (**1**)	/	irreversible

The synthetic analogues are either competitive or non-competitive reversible AChE inhibitors (Table 2). In the former case, they compete with the substrate for binding at the anionic site at the bottom of the enzyme gorge, while in the latter they bind to the aromatic and anionic residues at the peripheral anionic site, limiting access of the substrate to the active site gorge. The most active tetramers **11** and **13** were both non-competitive inhibitors, probably being too large to reach the active site.

Previous studies on AChE inhibition by small synthetic pyridinium derivatives, such as derivatives of 2-amino-4,6-dimethylpyridine,[24] or different alkylated[25] and halogenated[26] pyridinium salts, have also confirmed reversible (competitive, non-competitive or mixed) inhibition with constants being in the concentration range comparable to those of our monomers (1–20 μM).

The inhibition of protein phosphatase 2A (PP2A) was studied with both synthetic and natural compounds. Cellular phosphatases and kinases are involved in phosphorylation and dephosphorylation of a variety of proteins, inducing their activation or inactivation. Dysfunction of these enzymes is reflected in abnormal cell metabolism. Several metabolites isolated from marine sponges[27] inhibit phosphatases, but none of them bears the pyridinium ring. Only compounds **5**, **7**, **13** and natural poly-APS (**1**) were found to inhibit PP2A (Experimental). The time-course of the reaction was linear, suggesting reversible inhibition. While the synthetic compounds showed only weak inhibition, natural polymers **1** caused moderate inhibition of PP2A, with 50% inhibition at a concentration of 4 μg ml^{-1} (0.3 μM).

Conclusions

In this work, we have established an efficient approach to the synthesis of oligomeric analogues of the naturally occurring alkylpyridinium compounds. The degree of polymerization can be increased stepwise by the controlled and iterative process of oligomerization, providing a general and practical access to a wide range of linear polycationic compounds. The mono- and oligomeric compounds thus prepared have been tested for antibacterial and for hemolytic activity, and inhibition of both AChE and protein phosphatase 2A, and compared to the activities exhibited by the natural polymers **1**.

The synthetic alkylpyridinium analogues were found to exhibit relatively high antibacterial and anti-AChE activities. Both increase with the increasing number of pyridinium rings and positive charges. Furthermore, the type of AChE inhibition (competitive or non-competitive) depends on the dimensions of the molecule. In contrast, the hemolytic activity of all the synthetic compounds was either very low or zero. This is in agreement with previous findings, showing that the lytic activity increases with the degree of polymerization and consequent lowering of the CMC,[14,21] while the same parameters are associated with decreased antibacterial activity.[18] Based on the recently observed ability of the natural poly-APS compounds

to inhibit the formation of marine bacterial biofilms at relatively low concentrations,[7b] their low-molecular mass synthetic analogues could be applied as active agents incorporated in environment friendly antifouling paints. Their relatively high antibacterial activity, together with low cytotoxicity, makes these compounds potential candidates for this purpose, particularly in view of the recommended obligatory checking of all possible side-effects (especially cytotoxicity) of new antibacterial compounds.[28] Finally, recent findings emphasize the role of acetylcholine as a neurotransmitter or/and neuromodulator in the settlement process of *Balanus amphitrite* cypris larvae on submerged surfaces.[29] The marked anti-AChE activity of these synthetic compounds is thus an additional reason to focus attention on them as potential antifoulants.

Experimental

General

Anhydrous CH_2Cl_2 and 3-methylpyridine were obtained by distillation over calcium hydride under nitrogen. All other reagents were used as obtained from commercial sources. Starting materials: 7-bromo-1-heptanol, 3,4-dihydro-2*H*-pyran, PPTS, $^{i}Pr_2NH$, $^{n}BuLi$, DMPU, 3-methylpyridine, *p*-methoxybenzyl chloride, phosphorus tribromide and anhydrous THF were purchased from the Aldrich Chemical Company. Yields are given with respect to reacted substrates. Where necessary, all air- and moisture-sensitive reactions were performed in flame-dried glassware under nitrogen.

Thin layer chromatography (TLC): Merck silica gel F_{254}, Merck RP-18 (reversed phase) F_{254} and Merck NH_2 (amino) F_{254} plates, with visualization by either UV light or by treatment with an acid solution of cerium sulfate. Flash-chromatography (FC): Merck Si-60 15–25 μm, Merck Lichroprep RP-18, Merck Lichroprep NH_2 40–63 μm and Merck aluminium oxide 60H, type E, 60–230 mesh. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer operating at 299.94 and 75.43 MHz respectively in $CDCl_3$ if not otherwise specified. δ values are given in ppm, with respect to Me_4Si as an internal standard and to the solvent residual signal δ(H) 3.31 ppm for CD_3OD. *J* values are given in Hz. Multiplicities are from APT, ^{1}H, ^{1}H correlations from COSY60 and selective decoupling irradiations, ^{1}H, ^{13}C assignments from HMQC experiments on compounds **3** and **5**. For the description of the NMR data, a numbering system has been adopted (Scheme 2), which considers the priority of pyridinium subunits with respect to the THP protection. ESI-MS experiments were performed in the positive ion mode, on a Bruker Esquire-LC™ ion trap spectrometer *via* an electrospray interface as direct infusion. EI-MS experiments were performed with a Kratos MS-80 mass spectrometer, equipped with a home-built computerized data system.

Synthesis

1-Bromo-7-(tetrahydropyran-2-yloxy)heptane (3). A solution of 7-bromo-1-heptanol (**2**, 1.02 g, 5.22 mmol), 3,4-dihydro-2*H*-pyran (0.50 cm^3, 5.48 mmol) and PPTS (630 mg, 2.50 mmol) in anhydrous CH_2Cl_2 (20 cm^3) was stirred at room temperature for 18 hours. After washing with 2 M aq. Na_2CO_3 (10 cm^3), the organic phase was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was subjected to FC (silica gel; from hexane to hexane/EtOAc 7 : 3), to yield **3** (1.31 g, 90%) as a colourless oil; R_f 0.50 (TLC, silica gel; hexane/EtOAc 9 : 1). δ_H (300 MHz, $CDCl_3$): 4.56 (1H, m, 2′-H), 3.90–3.30 (4H, series of m, 7-H_2, 6′-H_2), 3.39 (2H, t, *J* 6.7, 1-H_2), 1.84 (2H, quintet, *J* 6.7, 2-H_2), 1.80–1.35 (14H, series of m); δ_C (75 MHz, $CDCl_3$): 98.87 (s, C-2′), 67.52 (t, C-6′), 62.38 (t, C-7), 34.00 (t, C-1), 32.73, 30.76, 29.62, 28.59, 28.10, 26.19, 26.07, 25.46 (all t, C-2–C-6, C-3′–C-5′); ESI-MS: *m*/*z* 301/303 ([M + Na]$^+$); MS/MS (301/303): 221 ([M − HBr + Na]$^+$).

3-(8-Tetrahydropyran-2-yloxyoctyl)pyridine (5). $^{n}BuLi$ 1.6 M in hexane (6.75 cm^3, 10.81 mmol) was added to a solution of $^{i}Pr_2NH$ (1.5 cm^3, 10.81 mmol) in dry THF (8 cm^3) at 0 °C in a two necked flask equipped with a magnetic stirrer and constant pressure addition funnel. The solution was stirred at 0 °C for 30 minutes, DMPU (1.30 cm^3, 10.81 mmol) added, maintained at 0 °C for 15 minutes and then treated over 10 minutes with a solution of 3-methylpyridine (**4**, 1.05 cm^3, 10.81 mmol) in THF (5 cm^3). After stirring for 30 minutes at 0 °C, the solution was cooled to −78 °C and treated over 10 minutes with compound **3** (1.15 g, 4.12 mmol) in THF (10 cm^3). The resulting mixture was stirred for 18 hours, being allowed to gradually warm to room temperature, and quenched with sat. aq. NH_4Cl (5 cm^3) and water (5 cm^3). The two-phase mixture was separated and the aq. phase extracted with EtOAc. The combined organic phases were dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a yellow oil. FC (silica gel, hexane/EtOAc/Et_3N 95 : 5 : 3) yielded **5** (1.08 g, 90%) as a pale yellow oil; TLC (silica gel, hexane/EtOAc 1 : 1 + drops of Et_3N) R_f 0.47; δ_H(300 MHz, $CDCl_3$): 8.41 (2H, m, 2′-H, 6′-H), 7.46 (1H br d, *J* 7.6, 4′-H), 7.18 (1H, dd, *J* 7.6, 4.8, 5′-H), 4.56 (1H, pseudo t, *J* 3.0, 2″-H), 3.90–3.68 (2H, m, 1-H_a, 6″-H_a), 3.50–3.33 (2H, m, 1-H_b, 6″-H_b), 2.58 (2H, t, *J* 7.5, 8-H_2), 1.80–1.30 (16 H, series of m, 2-H_2–7-H_2, 3″-H_2–5″-H_2); δ_C (75 MHz, $CDCl_3$): 149.53, 146.7 (d, C-2′ and C-6′), 137.58 (s, C-3′), 136.08 (d, C-4′), 122.8 (d, C-5′), 98.5 (d, C-2″), 67.23 (t, C-6″), 62.0 (t, C-1), 32.61, 30.76, 30.42, 29.35, 28.97, 28.70, 25.93, 25.84, 25.39, 25.13 (all t, C-2–C-8, C-3′–C-5′); ESI-MS: *m*/*z* 314 ([M + Na]$^+$).

3-(8-Tetrahydropyran-2-yloxyoctyl)pyridinium-*N*-*p*-methoxybenzyl iodide (6). A mixture of compound **5** (0.5 g, 1.71 mmol), potassium iodide (0.24 g, 1.43 mmol) and *p*-methoxybenzyl chloride (PMBCl, 1.16 cm^3, 1.77 mmol) in acetonitrile (5 cm^3) was stirred vigorously at room temperature for 20 hours. Dichloromethane (8 cm^3) was added and the salts removed by filtration. The residue from evaporation of the filtrate was subject to FC (silica gel, hexane/EtOAc 7 : 3 to remove PMBCl in excess, then CH_3OH for elution of product), to give **6** (0.83 g, 90%) as a pale yellow oil; δ_H (300 MHz, $CDCl_3$): 8.98 (1H, s, 2′-H), 8.93 (1H, d, *J* 8.0, 6′-H), 8.14 (1H, br d, *J* 8.0, 4′-H), 7.89 (1H, br t, *J* 8.0, 5′-H), 7.46 (2H, d, *J* 8.7, Ar), 6.87 (2H, d, *J* 8.7, Ar), 5.85 (2H, s, ArC*H*$_2$), 4.56 (1H, pseudo t, *J* 5, 2″-H), 3.73 (3H, s, OMe), 3.70–3.20 (4H, series of m, 1-H_2 and 6″-H_2), 2.75 (2H, br t, *J* 7.5, 8-H_2), 1.85–1.30 (18H, series of m, 2-H_2–7-H_2 and 2″-H_2–5″-H_2); δ_C (75 MHz, $CDCl_3$): 160.51 (s, Ar), 144.62, 143.18, 141.73 (all d, C-2′, C-4′and C-6′), 143.90 (s, C-3′), 131.05 (2C, d, Ar), 127.73 (d, C-5′), 123.97 (s, Ar), 114.87 (2 C, d, Ar), 98.84 (d, C-2″), 67.21, 62.16 (two t, C-1 and C-6″), 63.75 (t, Ar*C*H$_2$), 55.08 (q, OMe), 32.55, 30.80, 30.42, 29.34, 29.04, 29.00, 26.71, 25.83, 25.46, 25.10 (all t, C-2–C-8, C-3′–C-5′); ESI-MS: *m*/*z* 412 ([M − I]$^+$).

***N*-*p*-Methoxybenzyl-3-(8-bromooctyl)pyridinium iodide (7).** Phosphorus tribromide (0.52 cm^3, 5.58 mmol) was added dropwise to a solution of **6** (0.75 g, 1.39 mmol) in $CHCl_3$ (20 cm^3) with 10% CH_3CN at 0 °C under nitrogen. The solution was stirred for 15 minutes at 0 °C, heated under reflux for 1 hour, cooled to room temperature, filtered and sat. aq. $NaHCO_3$ added. The residue from evaporation was subjected to FC (silica gel, CH_3CN, CH_3OH) to give **7** (0.59 g, 82%); δ_H(300 MHz, $CDCl_3$): 9.13 (2H, br s, 2′-H and 6′-H), 8.11 (1H, d, *J* 7.9, 4′-H), 7.93 (1H, br t, *J* 7.9, 5′-H), 7.63 (2H, d, *J* 8.7, Ar), 6.84 (2H, d, *J* 8.7, Ar), 6.05 (2H, s, ArC*H*$_2$), 3.77 (3H, s, OMe), 3.38 (2H, t, *J* 6.8, 1-H_2), 2.79 (2H, br t, *J* 7.5, 8-H_2), 1.85–1.30 (12H, series of m, 2-H_2–7-H_2); δ_C (75 MHz, $CDCl_3$): 160.64 (s, Ar), 144.58, 143.75, 142.17 (all d, C-2′, C-4′and C-6′), 144.00 (s, C-3′), 131.36 (2C, d, Ar), 127.76 (d, C-5′), 124.84 (s, Ar), 114.82 (2 C, d, Ar), 63.80 (t, Ar*C*H$_2$), 55.35 (q, OMe), 34.05 (t, C-1), 32.72, 32.61, 30.90, 30.19, 28.85, 28.47, 27.94 (all t, C-2–C-8); ESI-MS: *m*/*z* 390/392 ([M − I]$^+$).

***N-p*-Methoxybenzyl tetrahydropyranyl dimeric pyridinium iodide 8.** To a solution of **5** (0.35 g, 1.20 mmol) and **7** (0.57 g, 1.10 mmol) in CH_3CN (10 cm^3), 0.18 g of potassium iodide were added. The mixture was heated at reflux for 20 hours, cooled to room temperature and evaporated. The residue was washed with hexane/EtOAc 9 : 1 to remove the excess monomer **5** and then submitted to FC (Al_2O_3, hexane/EtOAc 7 : 3, $CHCl_3$/CH_3OH 94 : 6, CH_3OH) to yield the dimer **8** (0.75 g, 80%); δ_H(300 MHz, $CDCl_3$ + CD_3OD): 8.90 (4H, m, 2′-H, 6′-H, 2″-H, 6″-H), 8.40 (2H, m, 4′-H, 4″-H), 8.05–7.95 (2H, m, 5′-H, 5″-H), 7.52 (2H, d, *J* 8.7, Ar), 6.91 (2H, d, *J* 8.7, Ar), 5.97 (2H, s, ArCH_2), 4.73 (3H, m, 2‴-H and 7′-H_2), 3.70 (3H, s, OMe), 3.80–3.20 (4H, series of m, 1-H_2 and 6‴-H_2), 2.80 (4H, m, 8-H_2, 14′-H_2), 1.90–1.30 (series of m, 2-H_2–7-H_2, 8′-H_2, 13′-H_2, and 3‴-H–5‴-H); δ_C (75 MHz, $CDCl_3$ + CD_3OD): 160.64 (s, Ar), 145.32, 144.91, 144.79, 144.29, 144.28, 144.24, 144.07, 143.70 (pyridinium), 131.12 (2C, d, Ar), 127.91 and 127.77 (all d, pyridinium), 124.46 (s, Ar), 114.79 (2 C, d, Ar), 98.83 (d, C-2‴) 63.80, 62.96, 62.36, 62.09 (all t, C-1, C-7′, C-6‴, Ar*C*H_2), 55.28 (q, OMe), 32.80–25.35 (all t, C-2–C-8, C-8′–C-14′, C-3″–C-5″); ESI-MS : *m*/*z* 729 ([M − I]$^+$).

***N-p*-Methoxybenzyl dimeric pyridinium iodide 9.** Phosphorus tribromide (0.15 cm^3, 1.65 mmol) was added dropwise to a solution of dimer **8** (0.35 g, 0.41 mmol) in $CHCl_3$/CH_3CN 1 : 1 (10 cm^3) at 0 °C under nitrogen. The solution was stirred for 15 minutes at 0 °C, heated under reflux for 1 hour, cooled to room temperature, filtered and quenched with sat. aq. $NaHCO_3$. The residue from evaporation was subjected to FC (silica gel, EtOAc/hexane 2 : 1, then CH_3OH), to give the corresponding pure bromide (0.28 g); δ_H(300 MHz, $CDCl_3$ + CD_3OD): 8.90 (4H, m, 2′-H, 6′-H, 2″-H, 6″-H), 8.40 (2H, m, 4′-H, 4″-H), 7.98 (2H, m, 5′-H, 5″-H), 7.58 (2H, d, *J* 8.7, Ar), 6.95 (2H, d, *J* 8.7, Ar), 5.94 (2H, s, ArCH_2), 4.75 (3H, m, 2‴-H and 7′-H_2), 3.81 (3H, s, OMe), 3.38 (2H, t, *J* 6.8, 1-H_2), 2.84 (4H, m, 8-H_2, 14′-H_2), 1.90–1.24 (series of m, 2-H_2–7-H_2, 8′-H_2–13′-H_2); ESI-MS : *m*/*z* 707/709 ([M − I]$^+$); MS/MS (707/709): 459/461.

Potassium iodide (0.06 g, 0.38 mmol) was added to a solution of bromide (0.27 g, 0.32 mmol) in CH_3CN (5 cm^3). The mixture was heated under reflux for 20 hours under stirring, and then cooled to room temperature. The residue from evaporation of the solvent was subjected to FC (CN stationary phase, from H_2O/CH_3CN 97 : 3, to CH_3CN) to give iodide **9** (0.25 g, 74% of overall yield from **8**); δ_H(300 MHz, $CDCl_3$ + CD_3OD): 8.90 (4H, m, 2′-H, 6′-H, 2‴-H, 6‴-H), 8.42 (2H, m, 4′-H, 4″-H), 7.96 (2H, m, 5′-H, 5″-H), 7.60 (2H, d, *J* 8.7, Ar), 6.95 (2H, d, *J* 8.7, Ar), 5.92 (2H, s, ArCH_2), 4.75 (3H, m, 2‴-H and 7′-H_2), 3.82 (3H, s, OMe), 3.17 (2H, t, *J* 6.8, 1-H_2), 2.85 (4H, m, 8-H_2, 14′-H_2), 1.95–1.24 (series of m, 2-H_2–7-H_2, 8′-H_2–13′-H_2); ESI-MS : *m*/*z* 755 ([M − I]$^+$).

Tetrahydropyranyl dimeric pyridinium iodide 10. A solution of dimer **8** (0.38 g, 0.44 mmol) and 3-methylpyridine (**4**, 1.0 cm^3, 10.6 mmol) was heated under reflux for 16 hours with stirring, then cooled to room temperature. The residue from evaporation was subjected to FC (-NH_2 stationary phase, from H_2O/CH_3CN 97 : 3 to CH_3CN), to give **10** (0.20 g, 75%); δ_H(300 MHz, $CDCl_3$): 8.90 (2H, m, 2′-H, 6′-H), 8.42 (3H, m), 8.00 (1H, m), 7.46 (1H, m), 7.19 (1H, m), 4.73 (3H, m, 2‴-H and 7′-H_2), 4.56 (1H, m, 2‴-H), 3.80–3.20 (4H, series of m, 1-H_2 and 6‴-H_2), 2.86 (2H, m, 8-H_2), 2.56 (2H, m, 14′-H_2), 1.90–1.30 (series of m, 2-H_2–7-H_2, 8′-H_2–13′-H_2 and 3‴-H_2–5‴-H_2); δ_C (75 MHz, $CDCl_3$ + CD_3OD): 146.96, 146.70,146.12, 145.80, 144.96, 144.79, 137.92, 137.50, 128.35, 126.61 (C-2′–C-6′ and C-2″–C-6″); 98.85 (d, C-2‴), 63.26, 62.36, 61.75 (all t, C-1, C-7′, C-6‴), 32.35–25.30 (all t, C-2–C-8, C-8′–C-14′, C-3″–C-5″); ESI-MS: *m*/*z* 481 ([M − I]$^+$).

***N-p*-Methoxybenzyl tetrahydropyranyl tetrameric pyridinium iodide 11.** A solution of **9** (0.23 g, 0.26 mmol) and **10** (0.18 g, 0.29 mmol) in CH_3CN (20 cm^3) was refluxed with stirring for 20 hours, and then cooled to room temperature. Evaporation of the solvent gave a crude mixture from which a slight excess of dimer **10** was taken away by washing with $CHCl_3$/MeOH 95 : 5. The remaining residue was a quite pure tetramer **11** (0.29 g, 75%). A small amount of **11** has been purified by FC (-NH_2 stationary phase, H_2O/CH_3CN gradient elution) for biological testing. δ_H (300 MHz, CD_3OD): 9.03 (4H, s), 8.87 (4H, m), 8.46 (4H, m), 8.00 (4H, m), 7.53 (2H, d, *J* 8.7, Ar), 6.99 (2H, d, *J* 8.7, Ar), 5.78 (2H, s, ArCH_2), 4.65 (7H, m, 3 × NCH_2, OC*H*O), 3.80 (3H, s, OMe), 3.80–3.30 (4H, series of m) 2.89 (8H, m, 4 × pyridinium-CH_2), 2.10–1.30 (series of m); ESI-MS: *m*/*z* 1513 ([M − I + NaI]$^+$), 1363 ([M − I]$^+$).

Hydroxyl dimeric pyridinium iodide 12. To a solution of **10** (0.018 g, 0.03 mmol) in MeOH (1 cm^3), *p*-toluenesulfonic acid monohydrate (0.008 g) was added. The resulting mixture was stirred at room temperature for 2 h and then evaporated. Potassium iodide in excess was added to the residue in acetonitrile (3 ml) and the mixture was stirred for 1 h. After that it was filtered and the filtrate evaporated, to leave a residue that was subjected to FC (-NH_2 stationary phase, H_2O/CH_3CN) to give **12** (0.013 g, 86%). δ_H (300 MHz, $CDCl_3$): 8.90 (2H, m), 8.42 (3H, m), 8.00 (1H, m), 7.48 (1H, m), 7.20 (1H, m), 4.69 (2H, t, *J* 7.6, CH_2N), 3.49 (2H, t, *J* 6.7, CH_2OH), 2.78 (2H, t, *J* 7.6, pyridinium-CH_2), 2.46 (2H, t, *J* 7.6, pyridine-CH_2), 1.90–1.30 (24H, series of m); ESI-MS: *m*/*z* 397 ([M − I]$^+$). MS/MS (397): 208, 190.

Tetrameric pyridinium iodide 13. During the conversion of the tetramer **11** to the corresponding iodide by the same treatment used to obtain dimer **9** from **8**, accidental evaporation of the reaction mixture to dryness caused a partial *N*-PMB deprotection, giving the minor compound **13** together with the expected iodide.

FC (-NH_2 stationary phase, H_2O/CH_3CN gradient elution) then furnished the major product in good yield and a small amount of the less polar tetramer **13** (10.8 mg), but sufficient to be tested for biological activities. δ_H (300 MHz, CD_3OD + $CDCl_3$): 9.0–8.05 (series of m, pyridinium and pyridine), 7.46 (1H, br d, *J* 7.6, pyridine), 7.18 (1H, m, pyridine), 4.78 (6H, m, N CH_2), 2.80 (6H, m, pyridinium-CH_2), 3.15 (2H, t, *J* 6.9, CH_2I), 2.56 (2H, m, pyridine-CH_2), 2.00–1.30 (series of m); ESI-MS: *m*/*z* 1141 ([M − I]$^+$).

Bioassays

Synthetic and natural alkylpyridinium compounds. For biological tests, the stock solutions (10 mg ml^{-1}) in deionized water (compounds **4·HCl** and **14·HCl**), methanol (compounds **4**–**7**, **11**, **12** and **14**), or ethanol (compound **13**) were prepared and kept at 4 °C prior to use. Poly-APS compounds (**1**) were isolated from the marine sponge *Reniera sarai* by a standard procedure.[1] For biological tests, a stock solution (10 mg ml^{-1}) was prepared in deionized water and kept at 4 °C until use. Since poly-APS compounds are a mixture of two polymers, the molar concentration calculations were estimated assuming a 1 : 1 ratio between them, giving a molecular weight of 12200 Da.

Estimation of biological activities. *Antimicrobial activity.* The following bacterial strains, obtained from the local collection at the Department of Biology, University of Ljubljana, were used: *Staphylococcus aureus*, *Streptococcus foecalis*, *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Activities were evaluated using the standard agar diffusion test. Bacteria were allowed to grow overnight and their concentration then determined. Bacterial culture was incorporated in Lauria Broth nutrient agar previously cooled to 42 °C. The final concentration of bacteria was approximately 5 × 10^5 CFU ml^{-1} (CFU: colony forming unit). Twenty milli-

litres of inoculated medium were poured into Petri dishes and kept at 4 °C until use. Circles of agar (Φ = 1 cm) were cut out from the cooled medium.

For estimating minimal inhibitory concentration (MIC), the compounds were progressively diluted in appropriate solvents. 100 millilitres of each dilution were poured into the holes cut in the inoculated medium, after which the system was kept at 37 °C for 24 h. Finally, the diameters of inhibition zones were measured. The inhibitory activity of pure solvents and of potassium iodide was also checked and found to be zero.

Hemolytic activity was measured by a turbidimetric method.[30] The compounds were diluted progressively in appropriate solvents. 20 µl of the resulting solution or pure solvents were added to 180 µl of bovine erythrocyte suspension with an apparent absorbance of 0.5 at 650 nm. The decrease of apparent absorbance was recorded for 20 minutes at 650 nm using a Kinetic Microplate Reader (Dynex Technologies, USA) to determine the time necessary for 50% hemolysis, t_{50}. All experiments were performed at 25 °C, repeating every measurement at least three times. Compounds **5** and **11** were not tested due to lack of material at the time of these bioassays. For those compounds testing active, the concentrations leading to t_{50} of 10 minutes were: 2 µg ml^{-1} (polyAPS, **1**); 100 µg ml^{-1} (**7**); 450 µg ml^{-1} (**13**).

Inhibition of acetylcholinesterase. AChE activity was measured by Ellman's method,[31] using acetylthiocholine iodide (2, 1 or 0.5 mM) as a substrate in 100 mM potassium phosphate buffer pH 7.4 at 25 °C, and electric eel AChE as a source of enzyme (Sigma, 6.25 U ml^{-1}). Hydrolysis of acetylthiocholine iodide was followed on a Kinetic Microplate Reader (Dynex Technologies, USA) at 412 nm. AChE inhibition was monitored for 5 minutes for each compound, which has been progressively diluted in the appropriate solvent. The effect of the pure solvents on enzyme inhibition was also checked. The organic solvents did not exceed 5% of the total volume of the reaction mixture. All readings were corrected for their appropriate blanks. Every measurement was repeated at least three times.

Sometimes, aromatic compounds can induce bleaching of the Ellman's reagent due to π–π interactions between their aromatic rings and thionitrobenzoic acid, leading to misinterpretation of the kinetic results. For this reason, before testing the AChE inhibition, the effect of the synthetic compounds on 5-thio-2-nitrobenzoic acid was checked as described previously.[2] It was found that in the concentration range used for the tests they did not induce any changes in the 5-thio-2-nitrobenzoate spectrum.

Inhibition of protein phosphatase 2A. The activity of erythrocyte PP2A (Upstate biotechnologies, USA) was assayed on a Kinetic Microplate Reader (Dynex Technologies, USA), using a colorimetric method described by Tubaro *et al.*[32] Each compound (**1**, **5**–**7**, **12**–**14**, and **4**,**14**, and their respective pyridinium salts) was diluted gradually in the appropriate solvents. Both the solutions thus obtained (25 µl) and the pure solvents were mixed with 150 µl of buffer pH 8.4 (40 mM TRIS·HCl, 34 mM $MgCl_2 \times 6H_2O$, 4 mM EDTA and 4 mM DL-DTT) and 50 µl of the substrate (141 mM *p*-nitrophenyl phosphate). 50 µl of the enzyme (0.25 U ml^{-1}) were then added and the reaction monitored at 405 nm for 1 hour. The effect of the pure solvents on enzyme inhibition was also checked. Every measurement was repeated at least three times.

For the compounds testing active, the IC_{50} values were: 4 µg ml^{-1} (polyAPS, **1**); 20 µg ml^{-1} (**5**); 30 µg ml^{-1} (**7**); 250 µg ml^{-1} (**13**).

Acknowledgements

We thank Mr. A. Sterni for technical assistance with mass spectra, and Miss Tamara Potočnik for her help in performing biological assays. This work has been financially supported in Italy by MIUR (Rome) and in Slovenia by the Ministry of Education, Science and Sport.

References

1 K. Sepčić, G. Guella, I. Mancini, F. Pietra, M. Dalla Serra, G. Menestrina, K. Tubbs, P. Maček and T. Turk, *J. Nat. Prod.*, 1997, **60**, 991–996.
2 K. Sepčić, V. Marcel, A. Klaebe, T. Turk, D. Šuput and D. Fournier, *Biochim. Biophys. Acta*, 1998, **1387**, 217–225.
3 P. Malovrh, K. Sepčić, T. Turk and P. Maček, *Comp. Biochem. Physiol. C*, 1999, **124**, 221–226.
4 K. Sepčić, U. Batista, J. Vacelet, P. Maček and T. Turk, *Comp. Biochem. Physiol. C*, 1997, **117**, 47–53.
5 M. T. Davies-Coleman, D. J. Faulkner, G. M. Dubowchik, G. P. Roth, C. Polson and C. Fairchild, *J. Org. Chem.*, 1993, **58**, 5925–5930.
6 F. J. Schmitz, K. H. Hollenbeak and D. C. Campbell, *J. Org. Chem.*, 1978, **43**, 3916–3922.
7 (*a*) M. Faimali, K. Sepčić, T. Turk and G. Geraci, *Biofouling*, 2003, **19**, 47–56; (*b*) F. Garaventa, M. Faimali, K. Sepčić and S. Geraci, *Biol. Mar. Medit.*, 2003, **10**, 565–567.
8 J. E. Baldwin, D. R. Spring, C. E. Atkinson and V. Lee, *Tetrahedron*, 1998, **54**, 13655–13680.
9 J. E. Baldwin, T. D. W. Claridge, A. J. Culshaw, F. A. Heupel, V. Lee, D. R. Spring and R. C. Whitehead, *Chem. Eur. J.*, 1999, **5**, 3154–3161.
10 Y. Morimoto and C. Yokoe, *Tetrahedron Lett.*, 1997, **38**, 8981–8984; C. Morimoto, C. Yokoe, H. Kurihara and T. Kinoshita, *Tetrahedron*, 1998, **54**, 12197–12214.
11 M. J. Wanner and G-J. Koomen, *Eur. J. Org. Chem.*, 1998, 889–895.
12 L. Gil, A. Gateau-Olesker, Y.-S. Wong, L. Chernatova, C. Marazano and B. C. Das, *Tetrahedron Lett.*, 1995, **36**, 2059–2062.
13 A. Kaiser, X. Billot, A. Gateau-Olesker, C. Marazano and B. C. Das, *J. Am. Chem. Soc.*, 1998, **120**, 8026–8034.
14 K. Sepčić, *J. Toxicol., Toxin Rev.*, 2000, **19**, 139–160.
15 (*a*) T. Seçkin, Y. Önal, Ö. Yesilada and A. Gültek, *J. Mat. Sci.*, 1997, **32**, 5993–5999; (*b*) G. Li, J. Shen and Y. Zhu, *J. Appl. Polym. Sci.*, 2000, **78**, 668–675; (*c*) G. Li and J. Shen, *J. Appl. Polym. Sci.*, 2000, **78**, 676–684.
16 S. Imazato, R. R. B. Russell and J. F. McCabe, *J. Dent.*, 1995, **23**, 177–181.
17 (*a*) D. B. Stierle and D. J. Faulkner, *J. Nat. Prod.*, 1991, **54**, 1134–1136; (*b*) K. Hirano, T. Kubota, M. Tsuda, Y. Mikami and J. Kobayashi, *Chem. Pharm. Bull.*, 2000, **48**, 974–977.
18 F. Kopecky, *Pharmazie*, 1996, **51**, 135–143.
19 M. Makino, S. Ohta and H. Zenda, *Yakugaku Zasshi*, 1994, **114**, 73–79.
20 (*a*) E. Quinoa and P. Crews, *Tetrahedron Lett.*, 1987, **28**, 2467–2468; (*b*) C. Bialojan and A. Takai, *Biochem. J.*, 1988, **256**, 283–290; (*c*) N. Fusetani, K. Yasumuro, S. Matsunaga and H. Hirota, *Tetrahedron Lett.*, 1989, **30**, 6891–6894; (*d*) J. Kobayashi, T. Murayama, Y. Ohizumi, T. Sasaki, T. Ohta and S. Nozoe, *Tetrahedron Lett.*, 1989, **30**, 4833–4836; (*e*) A. R. Carroll and P. J. Scheuer, *Tetrahedron*, 1990, **46**, 6637–6644; (*f*) J. Kobayashi, T. Murayama, S. Kosuge, F. Kanda, M. Ishibashi, H. Kobayashi, Y. Ohizumi, T. Ohta, S. Nozoe and T. Sasaki, *J. Chem. Soc., Perkin Trans. 1*, 1990, 3301–3303; (*g*) S. Matsunaga, H. Fujiki, D. Sakata and N. Fusetani, *Tetrahedron*, 1991, **47**, 2999–3006; (*h*) J. Kobayashi, C. Zeng, M. Ishibashi, H. Shigemori, T. Sasaki and Y. Mikami, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1291–1294; (*i*) R. Talpir, A. Rudi, M. Ilan and Y. Kashman, *Tetrahedron Lett.*, 1992, **33**, 3033–3034; (*j*) R. G. S. Berlinck, C. A. Ogawa, A. M. P. Almeida, M. A. A. Sanchez, E. L. A. Malpezzi, L. V. Costa, E. Hajdu and J. C. Freitas, *Comp. Biochem. Physiol. C*, 1996, **115**, 155–163; (*k*) S. Matsunaga, T. Wakimoto, N. Fusetani and M. Suganuna, *Tetrahedron Lett.*, 1997, **38**, 3763–3764.
21 R. W. Moni, P. G. Parsons, R. J. Quinn and R. J. Willis, *Biochem. Biophys. Res. Commun.*, 1992, **182**, 115–120.
22 D. M. Quinn, *Chem. Rev.*, 1987, **87**, 955–979.
23 K. Sepčić, N. Poklar, G. Vesnaver, D. Fournier, T. Turk and P. Maček, *J. Protein Chem.*, 1999, **18**, 251–257.
24 J. Debord, P. N'diaye, J. C. Bollinger, K. Fikri, B. Benicaut, J. M. Robert, S. Robert-Piessard and G. Le Baut, *J. Enzyme Inhib.*, 1997, **12**, 13–26.
25 C. G. Whiteley and D. S. Ngwenya, *Biochem. Byophys. Res. Commun.*, 1995, **211**, 1083–1090.
26 C. G. Whiteley and D. S. Ngwenya, *Biochem. Byophys. Res. Commun.*, 1995, **36**, 1107–1116.
27 (*a*) H. K. Tachibana, P. J. Scheuer, Y. Tsukitani, H. Kikuchi, D. Van Engen, J. Clardy, Y. Gopichand and F. J. Schmitz, *J. Am. Chem.*

Soc., 1981, **103**, 939–945; (*b*) S. Matsunaga, H. Fujiki and D. Sakata, *Tetrahedron*, 1991, **47**, 2999–3006; (*c*) E. D. De Silva, D. E. Williams and R. J. Andersen, *Tetrahedron Lett.*, 1992, **33**, 1561–1564; (*d*) S. R. Gunasekera, P. J. McCarthy, R. E. Longley, S. A. Pomponi, A. E. Wright, E. Lobkovsky and J. Clardy, *J. Nat. Prod.*, 1999, **62**, 173–175; (*e*) T. L. McCready, B. F. Islam, F. J. Schmitz, H. A. Luu, J. F. Dawson and C. F. B. Holmes, *J. Biol. Chem.*, 2000, **275**, 4192–4198; (*f*) A. Loukaci, I. Le Saout, M. Samadi, S. Leclerc, E. Damiens, L. Meijer, C. Debitus and M. Guyot, *Bioorg. Med. Chem.*, 2001, **9**, 3049–3054.

28 H. Nagamune, T. Maeda, K. Ohkura, K. Yamamoto, M. Nakajima and H. Kourai, *Toxicol. in Vitro*, 2000, **14**, 139–147.

29 M. Faimali, C. Falugi, L. Gallus, V. Piazza and G. Tagliafierro, *Biofouling*, 2003, **19**(Suppl.), 213–220.

30 G. Belmonte, C. Pederzolli, P. Maček and G. Menestrina, *J. Membr. Biol.*, 1993, **131**, 11–22.

31 G. L. Ellman, D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmacol.*, 1961, **7**, 88–95.

32 A. Tubaro, C. Florio, E. Luxic S. Sosa, R. Della Loggia and T. Yasumoto, *Toxicon*, 1996, **34**, 743–752.