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Original Research

Biological and Antioxidative Efficiency of Some Pyrrolidinium Salts

H. Kleszczyńska [1]*, J. Sarapuk [1], K. Bielecki [2], D. Bonarska–Kujawa [1], H. Pruchnik [1]

[1] Department of Physics and Biophysics, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland
[2] Department of Plant Nutrition, Wrocław University of Environmental and Life Sciences, Grunwaldzka 53, 50-357 Wrocław, Poland

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Abstract

A series of new pyrrolidinium salts (PYE-n) was synthesized for potential application in agrochemistry. They differed in the number of carbon atoms in the alkyl chain (n = 7, 9, 11, 13 and 15). These salts may also be used as effective antioxidants since they include a substituted free radical scavenging group.

The aim of these studies was to determine the concentration level at which PYE-n can be possibly used as biocides and to examine their antioxidative capacities. Biocidal activity was determined by measuring PYE abilities to hemolyze pig erythrocytes, to influence cucumber (*Cucumis sativus*) growth and its membrane properties, and to influence the main transition temperature of DPPC liposomes. Measurements of fluidity changes in the ghost erythrocyte membrane induced by PYE compounds were also done. Three probes: DPH, TMA-DPH and laurdan were used in the measurements. The results of those experiments showed that the pyrrolidinium salts studied exhibit fairly good membrane activity, which makes them potentially useful agrochemical agents.

Antioxidative capacity of PYE-n was determined by measuring the inhibition of the autooxidation of linoleic acid, tested in radical chromogen $ABTS^{\bullet+}$ cation decolorization assay, and by measuring the protection of erythrocyte membrane lipids against UV irradiation. All the methods used to check the potential antioxidative properties of PYEs showed that they can be used as very good free radical scavengers.

Keywords: pyrrolidinium salts, biological activity, antioxidative activity

Introduction

Protection of cell components against free radicals is a reason for wide use of natural and synthetic antioxidants [1-6]. A series of new pyrrolidinium chlorides was synthesized. They belong to a class of substances that combine two different features. They can be used in agrochemistry as common pesticides or as efficient antioxidative agents, since they include a substituted free radical scavenging group in their polar part. When incorporated into biological membranes at low concentration the compounds do not damage these membranes but protect them against free radicals. However, at high enough concentration the compounds can destroy membranes, acting as common biocides. Particular PYEs differed in the number of carbon atoms in their alkyl chains (n = 7, 9, 11, 13 and 15). A similar group of bifunctional compounds (pyrrolidinium and piperidinium bromides; n = 8, 10, 11, 12, 13, 14 and 16 and n = 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16, respectively) was studied earlier, and it was shown that they may be potentially used as effective antioxidants or pesticides depending

*e-mail: halina@ozi.ar.wroc.pl

on the concentrations applied [7-9]. In each case the efficiencies of the salts were found to be dependent on their lipophilicity.

The aim of this investigation was to find concentrations at which pyrrolidinium salts destroy erythrocytes, and to determine their influence on properties of the model membranes used, i.e. fluidity of ghost erythrocyte membranes and the main phase transition of DPPC liposomes. Antioxidative properties were studied by determining the capacity of particular compounds to protect erythrocyte membrane lipids against peroxidation induced by UV irradiation, by the inhibition of autooxidation of linoleic acid [10] and in chromogen experiments where PYE-n compounds reduced the radical $ABTS^{\bullet+}$ [ABTS, 2,2`-azinobis-(3-ethylbenziothiazoline-6-sulfonic acid)] to a nonreactive form, with the results compared to the radical reduction by Trolox - an analogue of the natural antioxidant vitamin E [11].

Materials and Methods

The compounds studied of general formulae shown in Fig. 1 were synthesized at the Department of Chemical Technology, Wrocław University of Technology, Poland. The structure and purity were checked by NMR spectra and elemental analysis.

Freshly heparinized pig erythrocytes were used in the hemolytic experiments. The blood was centrifuged for 3 min at 3000 x g, plasma removed and the cells washed three times with isotonic phosphate buffer, pH 7.4. The cells were then incubated for half an hour at 37ºC in the same buffer containing various concentrations of pyrrolidinium salts. After incubation samples were taken, centrifuged, and the supernatant assayed for hemoglobin content using a spectrophotometer (Spekol 11, Carl Zeiss, Jena) at 540 nm wavelength. Hematocrit was 2%.

Erythrocyte membranes were prepared from fresh heparinized pig blood [12]. Erythrocyte ghosts were suspended in a phosphate solution of pH 7.4 at protein concentration ca. 1 mg/ml. Two kinds of suspensions were prepared. One contained erythrocyte ghosts only, and the other erythrocyte ghosts plus chosen amounts of the antioxidants studied. Lipid peroxidation in the erythrocyte membrane

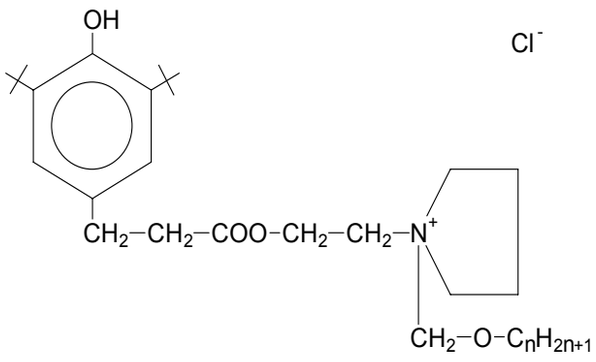

Fig. 1. The structure of pyrrolidinium salts (PYE-n) studied; n = 7, 9, 11, 13, 15.

was induced by UV radiation (bactericidal lamp intensity was 4 mW/cm^2). The degree of lipid peroxidation was determined measuring the concentration of malonic dialdehyde released in the samples by using its color reaction with thiobarbituric acid. Supernatant absorption was determined spectrophotometrically at 535 nm.

Antioxidant activity was determined by measuring the inhibition of the autooxidation of linoleic acid. One ml of 7.5×10^{-3} mM linoleic acid was incubated at 37°C for 24 h in phosphate buffer of pH 7.0 in the presence of different amounts of pyrrolidinium salts. 25-100 µl samples were taken for a lipid hydroperoxide assay with the ferric-xylenol orange complex. Solutions containing the hydroperoxide were mixed with appropriate volumes of reagents to give final concentrations of 110 mM perchloric acid, 100 µM xylenol orange, and 250 µM ferrous ammonium sulfate in a volume of 2 ml. The final pH was 1.8. After 30 min in the dark, the absorbance was read at 560 nm [13]. Thereafter, the concentration of particular PYE inhibiting 50% linoleic acid autooxidation was determined.

The standard TEAC (Trolox Equivalent Antioxidant Capacity) assay has also been used. It assessed the total radical scavenging capacity of a compound to scavenge blue-green chromogen radical $ABTS^{\bullet+}$ in 20 min. Transition of chromogen to a colorless form was monitored spectrophotometrically at 414 nm. (The procedure was described in detail earlier [14-16]). The results obtained were compared with the effect caused by the standard antioxidant Trolox. The TEAC index of the compound was calculated as the concentration of Trolox showing an antioxidant capacity equivalent to 1 M of the tested compound. The results obtained in these experiments are a direct indication of a compound's antioxidative activity.

Multilamellar vesicles for differential scanning calorimetry (DSC) were prepared from appropriate amounts of PYE and dipalmitoylphosphatidyl choline (DPPC) purchased from Sigma Aldrich. PYE and DPPC were dissolved in chloroform and the mixture was evaporated to form a thin film on the flask wall. Traces of chloroform were removed with a stream of dry nitrogen under vacuum for 2 h. Then distilled water was added and the flask heated to 60 °C in a water bath. The lipid film was dispersed by agitating the flask on a vortex mixer to give a milky suspension of liposomes. The final lipid concentration was 25 mg/cm^3. Lipid suspension was loaded into the sample cell of a DSC microcalorimeter of the Mettler Toledo Thermal Analysis System D.S.C. 821®. Scan rates were 2ºC/min, and incubation performed at 4ºC lasted 3 days.

Fluidity experiments were done on erythrocyte ghosts, which were subjected to the action of the compounds studied. Three fluorescent probes were used; 1,6-diphenyl-1,3,5-hexatriene (DPH), 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan), and 1-(4-trimethylammoniumphenyl) -6-phenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH), all at 1 µM concentration. They were expected to give more detailed information about the membrane fluidity since they incorporate into different membrane regions. Erythrocyte ghosts (protein concentrations in the samples

was ca. 100 µg/ml) were incubated with probes and PYE-n for 15 min at 25°C. The measurements were performed with an SFM 25 spectrofluorimeter (KONTRON, Switzerland) at 25°C. The excitation and emission wavelengths were 354 nm and 429 nm for TMA-DPH and 360 nm and 425 nm for DPH. The excitation wavelength for laurdan was 360 nm and the emission wavelengths were 440 and 490 nm. The fluorescence anisotropy (A) was calculated according to the formula [17]:

$$A = (I_{II} - GI_{\perp})/(I_{II} + 2GI_{\perp})$$

...where I_{II} is the intensity of fluorescence emitted in a direction parallel to the polarization plane of the exciting light, $I_{\perp}$ is the intensity of fluorescence emitted in the perpendicular direction, and G is the grating correction factor.

The GP (generalized fluorescence polarization) values of laurdan probe were calculated according to the formula [18]:

$$GP = (I_b - I_r)/(I_b + I_r)$$

...where I_b and I_r are the corresponding emission intensities at 440 and 490 nm, the excitation wavelength being 360 nm.

Each sample was measured five times. All reagents used were of analytical grade. The fluorescent probes were purchased from Molecular Probes Inc. (Eugene, Oregon, USA).

The growth tests with individual compounds were conducted on cucumber *(Cucumis sativus* cv 'Krak F1') in a SANYO® growth chamber at 25°C with 9:15 h light: dark cycle, photosynthetic photon flux density (PPFD) – 150 µmol m^{-2} s^{-1}. Concentration of 1 mM of each substance was used utilizing three replicates. Seeds were germinated at 25°C for two days in darkness. Fifteen uniform seedlings were transferred to Petri dishes (9 cm) with two discs of Whatman No. 1 filter paper wetted with distilled water (control) or solutions of the test compounds. The length of the cucumber hypocotyls and roots were measured after 72 h.

Potassium leakage was measured by a modification of the procedure described previously [19]. Cucumber was grown with 16:8 h light:dark cycle, at 25/20ºC, PPFD - 150 µmol m^{-2} s^{-1}). Cotyledons 7-d-old seedlings were used for the experiments. Discs of 10 mm diameter were cut from cotyledons, avoiding the midrib. They were rinsed in double distilled water and floated for 24 h under constant light on 1 mM compound solution. Effusate was diluted 20 times before analysis. Potassium release was measured by atomic absorption spectrophotometry with a hollow-cathode lamp (spectrophotometer SpectrAA-200, Varian) at 760 nm wavelength. Air/acetylene flame, wavelength setting 766.5 nm.

Chlorophylls in cotyledon discs were extracted in 80% acetone as described previously [20].

Results and Discussion

It was found that PYE compounds hemolyzed erythrocytes when used in small (micromolar) concentrations, which makes them moderately good potential pesticides (Table 1). No significant differences in hemolytic activity were found for individual compounds, although the longest alkyl chain compound (PYE-15) was the weakest in causing hemolysis. This compound's influence on the main transition temperature was significantly greater than that of other PYEs. The influence increased with alkyl chain length of PYE (Fig. 2), meaning greater membrane structure disorder.

Similarly, lipophilicity-dependent behavior was found in the physiological experiments where the two longest alkyl chain compounds caused the appearance of highest concentrations of potassium ions in the effusate (Table 1). This means ca. 70% destruction of membranes in cucumber tissues. Chlorophyll content in cotyledons was similar for PYE-7, 9, 11 and 13. In the presence of PYE-15 chlorophyll content was lowest. It must be underlined that pyrrolidinium salts at 1 mM concentrations, used in physiological experiments, did not influence cucumber hypocotyl growth but strongly inhibited root length.

Table 1. Concentrations of compounds that induced 50% hemolysis (C_{50}) of erythrocytes, erythrocyte oxidation inhibition by 50% (IC_{50}), Trolox equivalents that induce the same effect (1 M of compound is equivalent to X M of Trolox), inhibit autooxidation of linoleic acid by 50% (L_{50}), influence chlorophyll content in cotyledons discs (CC), induce potassium leakage from cucumber (K), and cucumber root length (RL).

Compounds	C_{50} [mM]	IC_{50} [mM]	X Trolox [mM]	L_{50} [mM]	CC [% control]	K [ppm]	RL [% control]
PYE-7	0.70	8.5	1.62	0.296	79.7	25.7	45,6
PYE-9	0.78	8.0	1.45	0.391	78.2	25.9	44,1
PYE-11	0.80	6.8	1.60	0.253	84.0	25.8	43,6
PYE-13	0.65	4.6	1.52	0.354	80.0	28.8	44,2
PYE-15	0.90	6.2	1.64	0.287	72.0	31.9	41,3
Control					100	1.05	100

Means of 3 replicates. Deviation ca. 8%

The results of studies on a possible antioxidative capacity of PYE are collected in Table 1. It can be seen that all the pyrrolidinium salts studied can be regarded as good antioxidants. Comparison with the well-known 3,5-di-tert--butyl-4-hydroxytoluene (BHT) revealed that PYE-n are better antioxidants than BHT [3]. They inhibited 50% peroxidation of erythrocytes when used in concentrations two orders of magnitude lower than those inducing 50% hemolysis. Their antioxidant efficacy depended on the length of the hydrophobic parts of the compounds and there was a maximum effect for PYE-13.

Moreover, the two chemical tests performed, i.e. chromogen radicals experiments and inhibition of linoleic acid autooxidation, confirmed that PYE-n compounds could be used as quite efficient free radical scavengers. Their antioxidative capacities are much better then that of the known antioxidant Trolox.

The membrane fluidity experiments, for all the fluorescent probes used, showed lipophilicity-dependent behaviour of PYE-n.

DPH and TMA-DPH also demonstrated concentration-dependent behaviour, especially for the highest concentration of the pyrrolidinium salts studied (Figs. 3 and 4). The DPH probe showed increased rigidity in the hydrophobic region of the ghost erythrocyte membrane upon incorporation of PYE with maximum effect for PYE-13.

No difference in the effect on the generalized fluorescence polarization (laurdan) was found for 10 μM PYE-n (Fig. 5). At higher concentrations pyrrolidinium salts enhanced polarization but the more lipophilic compounds gave weaker effects. It seems that the salts with shorter chain made the hydrophilic region of membrane more rigid than the longer-chain compounds.

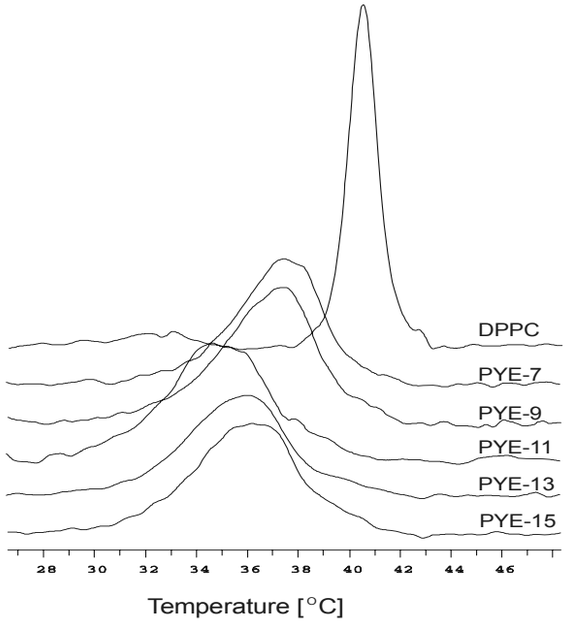

Fig. 2. Shift of the main phase transition temperature for DPPC liposomes by pyrrolidinium compounds; compound to DPPC molar ratio 0.1.

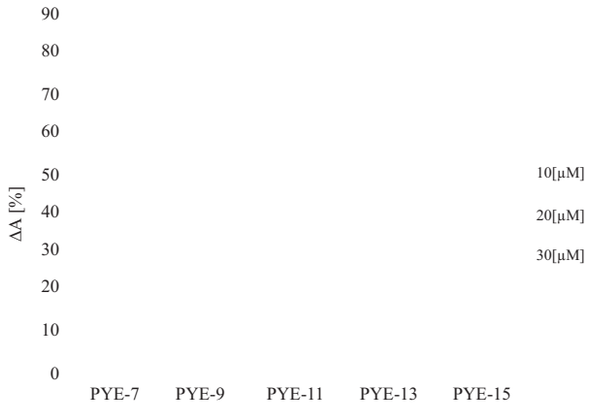

Fig. 3. Influence of PYE-n on fluorescence anisotropy in erthrocyte ghost membranes (measured with 1,6-diphenyl-1,3,5-hexatriene probe (DPH)).

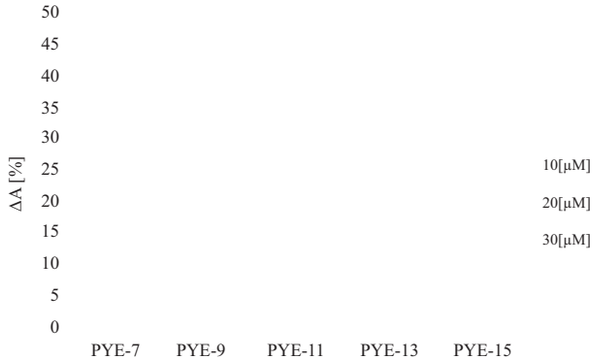

Fig. 4. Influence of PYE-n on fluorescence anisotropy in erythrocyte ghost membranes (measured with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, p-toluene-sulfonate (TMA-DPH)).

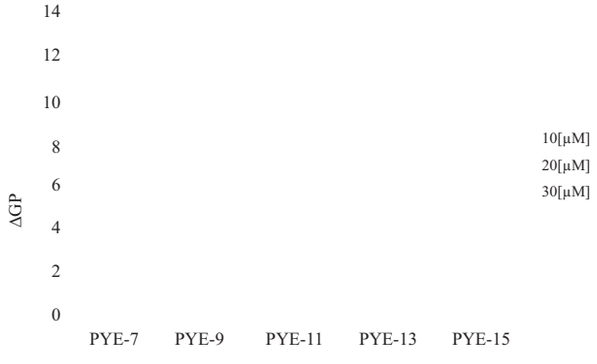

Fig. 5. Influence of PYE-n on * general fluorescence polarization in erythrocyte ghost membranes, measured with dodecanoyl-2-dimethylaminonaphthalene (laurdan).

The results of the measurements of erythrocyte peroxidation and membrane fluidity with DPH suggest that there may be formed interdigitated structure in the membranes for incorporating compounds of long enough hydrophobic parts. It can happen when a PYE compound extends its hydrocarbon chain into the opposite monolayer to that it incorporated, thus stabilizing the bilayer. At the same time it causes significant shifting of the main phase transition peak as seen in the case of PYE-15. Such so-called cut-off phenomenon was found for some surfactants interacting with biological and model membranes, whose activities diminished when their alkyl chain reached a certain length [21-24].

Acknowledgements

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