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THE ANTIOXIDANT ACTIVITY OF BHT AND NEW PHENOLIC COMPOUNDS PYA AND PPA MEASURED BY CHEMILUMINESCENCE

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Abstract: The antioxidative properties of two series of new phenolic, amphiphilic compounds were evaluated using the chemiluminescence (CL) method. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was used as a source of free radicals, to obtain high and prolonged CL. Three different kinds of buffers (organic and inorganic) were tested. The CL level varied only slightly depending on the buffer but increased significantly with the pH. Twelve newly synthesised compounds were compared with butylated hydroxytoluene (BHT), a commercially used antioxidant. The new antioxidants included two classes of quaternary ammonium salts with a phenol substituent functioning as an antioxidant. The salts were synthesised by quaternarization of pyrrolidine ethyl esters of dihydrocinnamic acid by n-alkoxymethyl bromides (PYA-n) or quaternarization of 2-dimethylaminoethyl esters by n-alkyl bromides (PPA-n). All the tested compounds quenched CL proportionally to their concentrations. In our experimental conditions 8.5 μM BHT quenched 50% of the CL. The PYA and PPA compounds had IC50 two to six times lower than BHT. CL inhibition was proportional to the pH for all antioxidants. The relationships between the structure and activity of the tested compounds are discussed.

Key Words: Chemiluminescence, Free Radicals, Phenolic Antioxidant, AAPH, BHT

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INTRODUCTION

To minimise the negative effects of oxidative damage synthetic and/or natural compounds with antioxidant functions have been used commercially. A lot of these compounds were found to be carcinogenic or toxic, which excluded them from use in the food industry. The formerly used phenolic antioxidant butylated hydroxytoluene (BHT) is being withdrawn from use because in cells it is modified to carcinogenic chinone derivatives [1]. New, non-toxic antioxidants are very extensively sough after, on account of their potential application in the food industry or human health management.

Quaternary ammonium salts with a phenol substituent functioning as an antioxidant may play an important role in this respect. These compounds which have varying length of alkyl chain can be incorporated into lipid membranes, playing a protective free-radical scavenging role. In the case of yeast cells or erythrocytes, the protective action of these antioxidants is strongly dependent on the length of the alkyl chain [2, 3]. Since peroxyl radical scavenging seems to be an important function of phenolic antioxidants with varying hydrophobicity, these kinds of compound can be employed as potential stoppers of the lipid peroxidation process.

The determination of the formation, propagation and action of free radicals in cells is technically complicated. Therefore an *in vitro* study in simplified model systems is often required. A variety of methods have been developed to assess the oxidation or autoxidation of lipids [4, 5]. Chemiluminescence (CL) is a potentially sensitive method, and permits the observation of the reaction kinetics. The lipid peroxidation process causes the CL that is coincided with the decomposition of hydroperoxides, rather than the formation of secondary products [6]. However, this CL is extremely weak at photon fluxes below 10^4 photons cm^{-2} s^{-1}. Therefore, it is important to use an appropriate CL enhancer. Most CL methods use only a few chemical components which are excited by the reaction with free radicals, and have a high quantum yield of photon emission. The most frequently used compounds are lucigenin, isoluminol and luminol. Lucigenin and isoluminol cannot penetrate the plasma membrane, and thus react with superoxide anions outside the cells. For this reason they applied in studies of macrophage activity [7, 8]. Luminol can cross biological membranes and becomes a light source after excitation by different kinds of free radicals, including peroxyl radicals. Hence this compound can be used for the determination of the activity of different antioxidants inside cells.

Because of their simplicity and accuracy, thermally decomposed peroxides, hyponitrites and azo compounds are used as free radical initiators. Azo compounds undergo thermal decomposition without enzymes or biotransformation. This yields molecular nitrogen and two carbon radicals $R^{\bullet}$ [9]. The carbon radicals may form pairs, or recombine to more stable products, but a lot of them react rapidly with oxygen and give peroxyl radicals $RO_2^{\bullet}$

(Fig. 1). 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) is often used as a source of hydrophilic radicals [10-13]. At 37°C in neutral aqueous solutions, the half-life of AAPH is about 175 h, and the generation rate of radicals is constant for the first few hours. The rate of free radical generation (R_i) from AAPH at 37°C equals 1.36 x 10^{-6} mol l^{-1} s^{-1} [9].

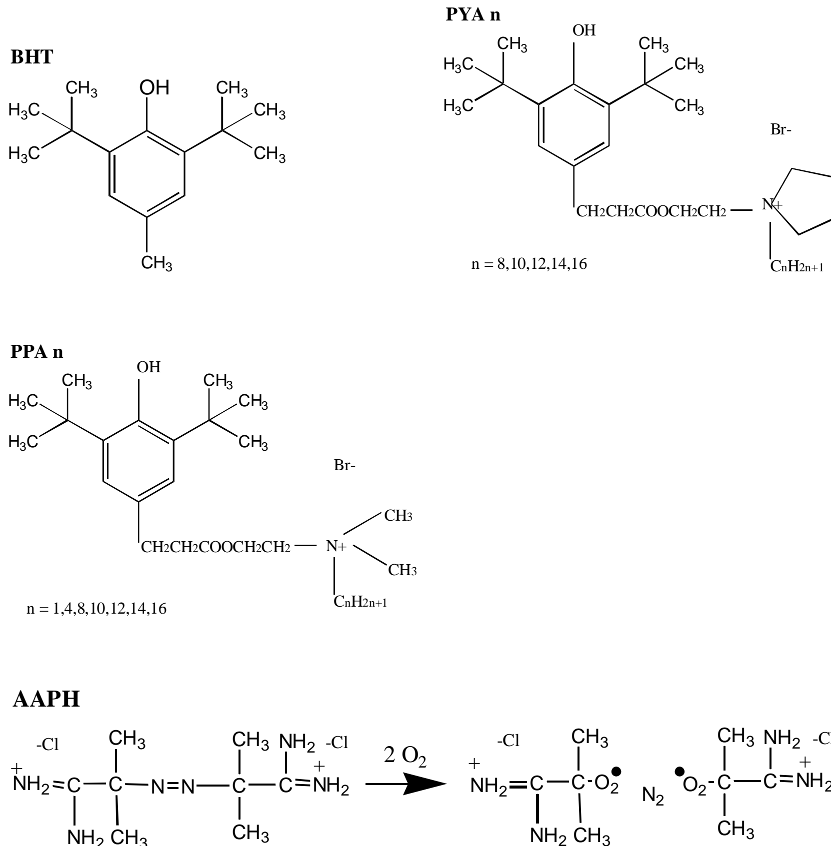

Fig. 1. The chemical structures of BHT, PYA-n, PPA-n and AAPH.
BHT - Butylated hydroxytoluene, **PYA-n -** quaternary ammonium salt pyrrolidinethyl esters of 3,5-di-t-butyl-4-hydroxy-dihydrocinnamic acid with varying numbers of carbons (n) in the alkyl chain, **PPA-n** - quaternary ammonium salt of dimethylaminoethyl esters of 3,5-di-t-butyl-4-hydroxy-dihydrocinnamic acid with varying numbers of carbons (n) in the alkyl chain, **AAPH** - (2,2'-azobis (2-amidinopropane) dihydrochloride) undergoes thermolysis, generating nitrogen and 2 alkyl radicals, which react with oxygen forming peroxyl radicals.

The present study aims to examine the AAPH-luminol system as a fast and sensitive method to compare the antioxidant potential of new compounds with a commercially used lipid protector BHT (butylated hydroxytoluene).

MATERIALS AND METHODS

Photons were counted in an EG&G Berthold LB96P microplate luminometer at 30ºC. The experiments were performed in a final volume of 250 µl on white microplates in 0.1 M Tris pH 9.0 buffer and borax/boric acid buffer (4 mM/4 mM) pH 9.0. The determination of the pH dependence of luminescence intensity was done in the Britton and Robinson buffer (0.04 M acetic acid, 0.04 M phosphoric acid, 0.04 M boric acid and 0.2 M NaOH). 25 µl of freshly prepared AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) was pipetted into a microplate well. 1 mM stock solution of luminol was diluted four times with distilled water. 100 µl of the diluted solution was automatically injected into the sample at the beginning of the measurement and the tested compound - 60 s later.
AAPH (Polyscience USA) 40 mM stock solution was prepared in distilled water. Luminol (Aldrich Poland) 1 mM stock solution was obtained by solubilisation in 10 mM NaOH. BHT, butylated hydroxytoluene (Aldrich Poland) 20 mM stock solution was prepared in ethanol. Solutions of the PYA-n and PPA-n compounds (Institute of Organic and Polymer Technology, Technical University, Wroc³aw, Poland) were prepared in distilled water (PPA with n = 1, 4 and PYA with n = 8, 10, 12, 14 and 16 carbon atoms in the alkyl chain) or in ethanol (PPA with n = 8, 10, 12, 14, 16). The final concentrations of ethanol were 0.095-0.0095%. The structure and purity of the PYA and PPA compounds were checked by ^{1}H-NMR spectra (Bruker Advance DRX_{300} instrument, in deuteriochloroform, TMS as the internal standard) [10, 11].

RESULTS AND DISSCUSION

AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) with luminol generates strong and prolonged luminescence. The luminol-AAPH system can be used to test various antioxidants. Lissi and co-workers [12, 13, 14] attempted to explain the mechanism of luminol chemiluminescence induced by ABAP (2,2'-azobis (2-amidinopropane) hydrochloride) decomposition to peroxyl radicals. In this particular system, the luminescence intensity rapidly reaches a maximum value and then remains almost constant for several minutes [12]. Similar results were obtained here, using the same luminol concentrations, and AAPH instead of ABAP.
The CL intensity increased with the pH (Fig. 2). At pH 7.0, CL was 5-10 times higher than the background level (empty plate about 20 RLU/s, luminol alone 40 RLU/s). At pH from 7.0 to 10.5, the light intensity increased linearly

thereafter growing more rapidly. It is known that the CL of luminol is remarkably high under alkaline conditions; pH 10-11 [15]. The decision was taken to perform these experiments at pH 9.0, which is still in the range of the buffering power of Tris. The luminescence in these conditions was approximately 2500 - 3000 RLU (Fig. 2), about 100 times higher than the background, thus giving highly reproducible results. The organic buffer could react with free radicals, hence the CL in Tris and in inorganic buffer (Borax/Boric Acid) were compared. Slightly lower mean luminescence was observed in the organic buffer (2757 versus 2873). The calculated p value with the ANOVA test is 0.634. As the two means are not significantly different, it is probably that in these experimental conditions, Tris was not trapping free radicals.

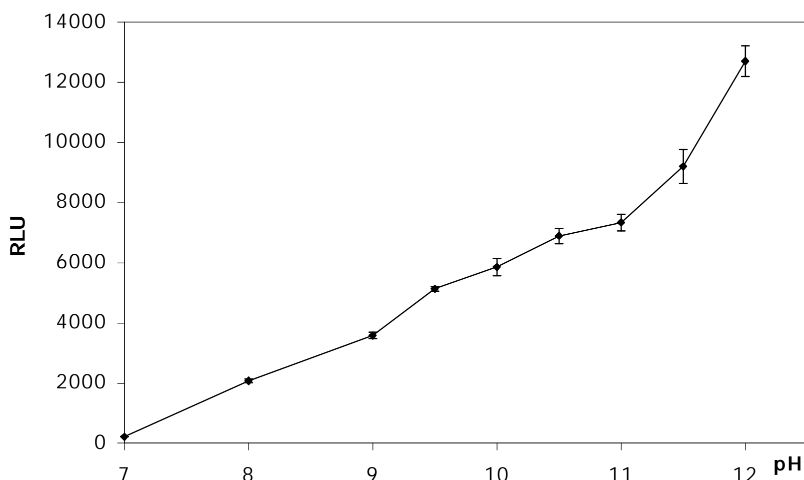

Fig. 2. Dependence of the luminol CL intensity on pH. Luminol – 100 μM, AAPH – 4 mM, ± SD (n = 2)

The addition of the tested compounds (BHT, PYA and PPA) quenched CL (Fig. 3A, B). This CL inhibition was stable and long lasting. Similar profiles of CL inhibition were observed in both organic and inorganic buffers.

Compounds from the PPA group with a chain longer than 8 carbon atoms were soluble in ethanol (the final concentration of ethanol was 0.095%-0.0095%). This ethanol concentration did not affect luminescence (data not shown). The luminescence inhibition by 4 μM of PPA1 compound solubilised in ethanol was 64.3 ±SD = 0.9 and in water was 66.0 ± SD = 1.7. Thus, at least in these particular experimental conditions, the solvent did not significantly influence the observed luminescence inhibition.

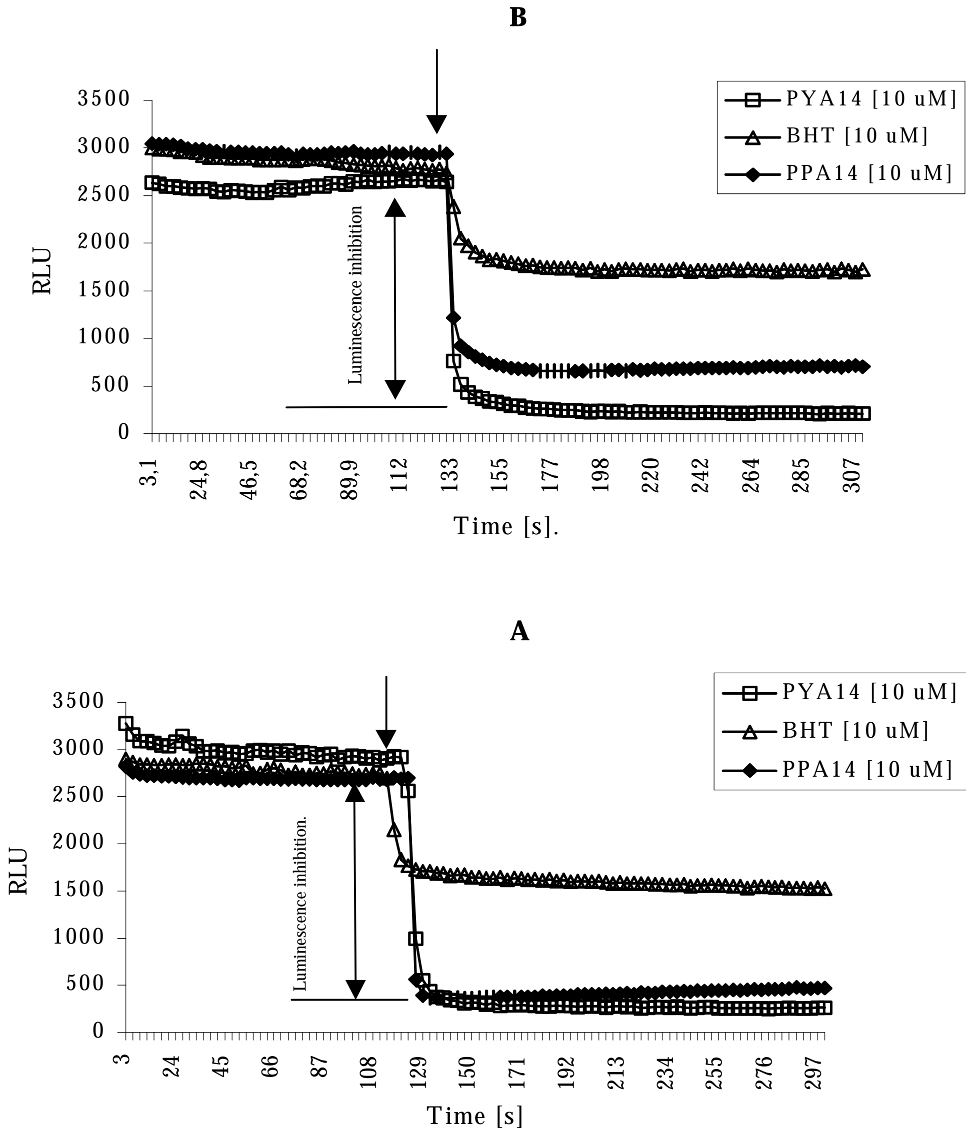

Fig. 3. Luminol luminescence after BHT, PYA 14 and PPA 14 addition (arrows) in **A.** Tris buffer**, B.** Borax/Boric Acid buffer.

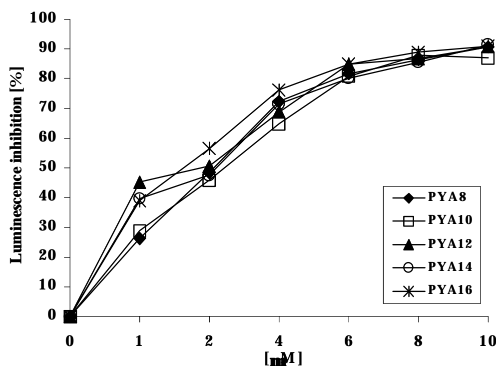

Fig. 4. Concentration dependent relative luminescence inhibition for PYA 8-16 compounds.

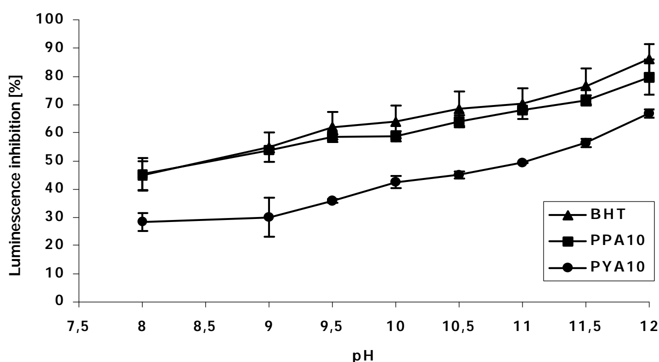

Fig. 5. Dependence of CL inhibition by BHT, PYA 10 and PPA 10 on pH in the Britton and Robinson buffer. Luminol – 100 μM, AAPH – 4 mM, BHT – 10 μM, PYA 10, PPA 10 – 1 μM, ± confidence interval (n = 4-6)

Luminescence inhibition was evaluated for all the tested compounds at various concentrations, as shown for PYA 8-16 (Fig. 4). Independently of the concentration, alkyl chain length seems to have only a slight impact on the antioxidant activity of PYA compounds. A good parameter to compare the antioxidant activity of the various compounds could be the concentration inhibiting 50% of CL (IC50). Table 1 compares IC50 for BHT, and the PYA and PPA classes of antioxidants. Table 2 compares the IC50 for BHT and selected PPA and PYA antioxidants in organic and inorganic buffers. For BHT, PYA10 and PPA10 the observed difference was statistically significant

($p<0.05$). However, as seen for BHT, for which there is the highest replicate number, the observed difference is only minor.

The absolute values of IC50 were also pH dependent, and decreased with pH increase (Fig. 5). This relationship was similar for selected compounds from all the tested groups (BHT, PYA and PPA) and suggested a similar mechanism of free radical trapping.

In all the compounds the OH group on the phenolic ring was responsible for antioxidant properties. The homolytic decay of AAPH generates two positively charged peroxyl radicals. At low pH the majority of the OH groups of the tested molecules are not charged. Putatively, with the increase of pH, the OH group starts to ionise, thus facilitating the reaction with positively charged, AAPH derived free radicals.

Tab. 1. IC50 values for the BHT, PYA and PPA classes of antioxidants in Tris buffer, pH = 9.0

Compound	IC50	Number of replicates	Confidence interval*
BHT	8.54	8	0.08
PPA1	3.22	3	1.44
PPA4	1.96	5	0.46
PPA8	4.90	3	3.04
PPA10	2.68	3	0.14
PPA12	5.30	5	0.95
PPA14	4.33	5	0.66
PPA16	4.23	4	1.63
PYA8	4.37	3	2.25
PYA10	2.49	7	0.3
PYA12	1.92	2	0.03
PYA14	3.67	3	1.54
PYA16	1.68	5	0.20

* p=0.95

Tab. 2. IC50 values for BHT and selected PYA and PPA antioxidants in Borax/Boric Acid buffer and Tris buffer, pH = 9.0

Compound	Borax/Boric Acid buffer		Tris buffer		P*
	IC50	Number of replicates	IC50	Number of replicates	
BHT	8.6	5	8.5	8	0.014
PPA4	1.25	2	1.96	5	0.132
PPA10	1.55	2	2.68	3	0.002
PYA10	1.50	2	2.49	7	0.020
PYA16	1.80	2	1.68	5	0.532

*p was calculated using the ANOVA test.

The IC50 [μM] values for the tested compounds (Tab. 1) were between 1.68 for PYA 16 and 5.30 for PPA 12. Thus, all the new synthetic antioxidants were more efficient than BHT (8.54 μM). BHT antioxidant activity seems to be weaker that of PYA and PPA compounds. This could be due to the higher pKa value (>10) that of PYA and PPA compounds (for example: PYA 12 pKa = 8.6, PPA 12 pKa = 8.3). In our experimental conditions (pH = 9.0) the newly synthesised antioxidants are more ionised than BHT, hence they could react more efficiently with free radicals. The slight differences between the IC50 of the new compounds suggest that the length of the alkyl chains in PYA and PPA had no major impact on their antioxidants properties, but could have a great importance in biological systems. Alkyl chain length of tested compounds could affect their incorporation and its depth in biological membranes, thus modifying antioxidant status in various specific locations. In fact, the influence of PYA and PPA compounds on living cells is known to vary [2, 3, 16].

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