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Evaluation of Antioxidant Properties of New Functionally Substituted Aryl Carbamates

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Abstract. The antioxidative activity of new carbamate derivatives has been compared by studying their influence on the generation of superoxide anion radical $O_2^{-\bullet}$ in the enzyme/substrate system xanthine/xanthine oxidase, on adrenaline autoxidation in an alkaline medium and on the level of lipid peroxidation in bester liver. Investigated aryl carbamates showed the inhibition of the nitroblue tetrazolium reduction by $O_2^{-\bullet}$ generated in the xanthine/xanthine oxidase system, the accumulation of adrenaline oxidation reaction products both in the absence and in the presence of tilapia liver cytosol and the level of carbonyl oxidation by-products, which can react with thiobarbituric acid. These results showed that the new functionally substituted aryl carbamates possess a potent protective effect both preventing the development of oxidative stress and inhibiting oxidative processes under conditions of the development of oxidative stress.

INTRODUCTION

Carbamate derivatives play an important role in modern drug discovery and medicinal chemistry. Introduction of the carbamate fragment into a known molecule contributes to the improvement of its pharmacodynamic and pharmacokinetic properties [1]. Thus, it is known that carbamate was successfully used to protect phenolic drugs [2]. A number of organic carbamates were found to exhibit different types of bioactivities: anti-HIV [3], antifungal [4], anticancer [5,6], antimicrobial [7] and antituberculosis [8]. However, much less is known about the antioxidant activity of these compounds [9, 10], which is one of the most important characteristics of a substance, which often plays a key role in the mechanisms of bioactivity of a compound. The antioxidant properties of compounds are of particular importance in the regulation of oxidative processes in animals and human beings.

An important role in maintaining the oxidative balance of cells and tissues belongs to catecholamines (adrenaline, norepinephrine and dopamine). It is known that adrenaline may undergo autoxidation to form quinone and semiquinone that enter in redox cycling which results in excess level of reactive oxygen species (ROS) including superoxide anion radical ($O_2^{-\bullet}$) and lead to oxidative stress [11]. Superoxide anion radical is the initial ROS in aerobic organism which plays an important role in the regulation of biological and physiological processes, being a crucial biological messenger and an essential antibacterial agent [12]. Also $O_2^{-\bullet}$ can initiate different pathologic processes [13]. Under normal circumstances, the biological system releases antioxidant enzymes, superoxide dismutases (SODs) [EC 1.15.1.1], which specifically maintains the $O_2^{-\bullet}$ concentration at an optimal level [14]. Low molecular weight compounds with antioxidative activity also contribute to maintaining the pro-/antioxidant balance, the violation of which due to an excessive accumulation of reactive oxygen, nitrogen or sulfur species can lead to oxidative damage of biomolecules: nucleic acids, proteins, carbohydrates and lipids.

In this regard, the antiradical activity and redox properties of new carbamate derivatives **1-5** (Fig. 1) have been studied. The antioxidative activity of these compounds was estimated by their influence on the generation of superoxide anion radical in the enzyme/substrate system xanthine/xanthine oxidase, on adrenaline autoxidation in an

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alkaline medium, including in the presence of tilapia liver cytosol and on lipid peroxidation the bester liver homogenate (*Huso huso* x *Acipenser ruthenus*).

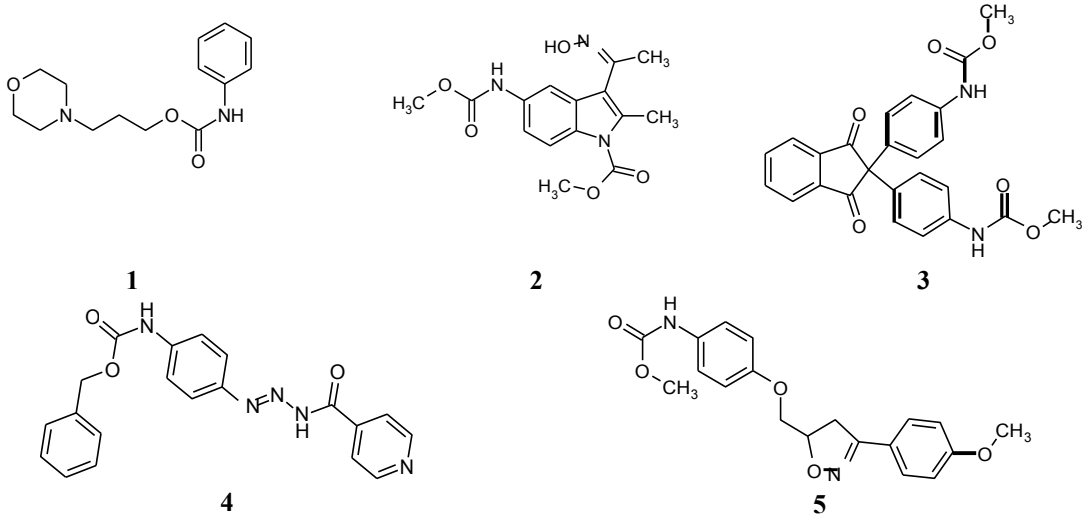

FIGURE 1. Chemical structures of new carbamate derivatives **1-5**

MATERIALS AND METHODS

New functionally substituted aryl carbamates **1-5** were synthesized by methodic that is published earlier [15] and were dissolved in the ethanol. The reagents included 0.1% (5.46 mM adrenaline (epinephrine, a pharmaceutical form), all other reagents, including xanthine oxidase (25 MU), bovine serum albumin were purchased from SigmaAldrich Chemical Co.

Determination of superoxide anion radical generation in the enzyme/substrate system xanthine/xanthine oxidase (NBT assay)

In this work, the superoxide anion was generated enzymatically by the enzyme/substrate system xanthine/xanthine. $O_2^{-\bullet}$ was identified by nitroblue tetrazolium (NBT). $O^{-\bullet}$ reduces NBT into formazan, which detected spectrophotometrically at 560 nm [16]. The reaction mixture consisted of 2.70 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine, 0.03 ml of 0.5% bovine serum albumin, 0.03 ml of 2.5 mM NBT and 0.06 ml of the sample solution in ethanol. To the mixture, 0.12 ml of xanthine oxidase (0.04 units) was added at 25 °C and the absorbance at 560 nm (by formation of blue formazan) was recorded by microplate spectrophotometer Zenyth 200rt Anthos for 60 s. A control experiment was carried out by replacing the sample solution with the same amount of ethanol. Antioxidant compounds compete with NBT for $O_2^{-\bullet}$ and thus decrease the rate of NBT reduction [17, 18]. The percentage inhibition of $O^{-\bullet}$ scavenging activity (*I*) was calculated as follows: $I\ (\%) = [(1 - A_i/A_0) \times 100\%]$, where A_i is the absorbance in the presence of the testing compound and A_0 is the absorbance of the blank solution. All experiments were performed three times.

Determination of adrenochrome level in the reaction of adrenaline autoxidation in alkaline medium

Adrenaline autoxidation in an alkaline medium (0.2 M bicarbonate buffer, pH 10.65) has been assessed spectrophotometrically by measuring the accumulation of the reaction product, adrenochrome, at wavelength of 347 nm [19]. The reaction was started by adding 10 µl of 0.1 % adrenaline hydrochloride to a 0.2 ml buffer with continuous stirring. The ethanol solutions of carbamates were added 5 µl to the buffer prior to adding an adrenaline. The reaction mixture contained 230 µM of adrenaline hydrochloride, 0.43 mM of carbonate buffer (pH 10.65) and

25 μM of carbamate. The percentage inhibition of adrenochrome accumulation (*I*) was calculated as follows: $I\ (\%) = [(1 - A_i/A_0) \times 100\%]$, where A_i is the absorbance in the presence of the testing compound and A_0 is the absorbance of the blank solution. In the case when the level of adrenochrome was determined in the presence of biopreparation, 1 μl of the tilapia liver cytosol was added to the buffer *prior* to adding a testing compound. In this case, the blank solution contained a biopreparation. The optical density was recorded for 4 min by microplate spectrophotometer Zenyth 200rt Anthos and measurement was repeated in triplicate.

Determination of level of bester liver lipid peroxidation

The intensity of bester liver has been assessed by the accumulation of carbonyl oxidation by-products, which reacted with thiobarbituric acid (TBARS) using the methodic that was published earlier [20]. The content of TBARS was expressed as nano-moles per 1 ml supernatant of bester liver homogenate.

RESULTS AND DISCUSSION

Superoxide anion radical is produced by the one-electron reduction of molecular oxygen and can be generated both enzymatically and non-enzymatically. In the xanthine/xanthine oxidase enzyme/substrate system, enzyme xanthine oxidase (EC 1.2.3.2) (XO) catalyzes the oxidation of xanthine to uric acid, with generates ROS such as superoxide radicals and hydrogen peroxide. XO is an enzyme widely distributed in mammalian tissues and it is the most thoroughly examined biological source of $O_2^{-\bullet}$ [21]. According to the obtained results (Table 1), compounds **1-3** at 25 μM concentration inhibit NBT reduction into formazan, which indicates the $O_2^{-\bullet}$ scavenging activity of these compounds (10–88%) in the used enzyme system, since NBT is known as an indicator of superoxide anion [22].

TABLE 1. Influence of new carbamate derivatives **1-5** on the generation of superoxide anion radical in the model system xanthine/xanthine oxidase and in reaction of adrenaline autoxidation in an alkaline medium

Compound	NBT assay (% inhibition)	Model system of adrenaline autoxidation in an alkaline medium (% inhibition)	
		In the absence of biopreparation	In the presence of biopreparation
1	10.4 ± 0.07	2.3 ± 0.01	-10.1 ± 0.09
2	87.5 ± 0.01	1.8 ± 0.01	8.56 ± 0.07
3	29.1 ± 0.05	3.3 ± 0.01	6.51 ± 0.08
4	-37.3 ± 0.11	2.2 ± 0.01	57.73 ± 0.05
5	-22.3 ± 0.10	2.3 ± 0.01	-21.9 ± 0.10

The strongest inhibitory activity was observed in the presence of compound **2** (88%), which contains a fragment of indole. On the contrary, the addition of compounds **4** and **5** increased the $O_2^{-\bullet}$ generation in the xanthine/xanthine oxidase system compared to the control experiment. However, for all studied compounds the slight inhibition of the adrenochrome formation in the reaction of adrenaline autoxidation in alkaline media was observed. In particular, adrenochrome, relatively stable intermediate of adrenaline autoxidation, has been shown to be cardio- and neurotoxic [23, 24]. Production of $O_2^{-\bullet}$ in oxidation of adrenaline in bicarbonate buffer (pH 10.65) is known to be accompanied with formation of hydrogen peroxide [25], carbon dioxide anion radical and bicarbonate anion radical [26]. Both model systems used in this work are systems capable of generating ROS. However, the revealed inhibitory activity of compounds in model system xanthine/xanthine oxidase indicates the $O_2^{-\bullet}$ scavenging activity of these compounds, in adrenaline autoxidation in an alkaline medium, namely about gross inhibitory activity.

It has been shown that the addition of compounds **2-4** in an incubation medium with cytosolic fraction of fish (tilapia) liver leads to inhibition of adrenaline oxidation compared to the control experiment in the presence of biopreparation. Interestingly, the addition of the biopreparation in an incubation medium leads to increase of adrenochrome accumulation compared to the control experiment in the absence of added supernatant of liver homogenate. It is known that the liver cytosol contains copper, zinc dependent superoxide dismutase (Cu, Zn-SOD), whose activity is independent of pH [27]. This result of experiments with tilapia liver homogenate requires further investigations. A likely explanation is that the Cu, Zn-SOD of the cytosolic fraction of the liver homogenate can catalyze the formation of the most active form of oxygen from H_2O_2, the hydroxyl radical [28], which oxidizes adrenaline to adrenochrome. Hereby, this most active oxygen specie could oxidatively attack adrenaline to afford

adrenochrome. Compounds **1** and **5** exhibited the promoting activity in adrenaline autoxidation reaction in the presence of fish liver cytosol.

It is well known that overproduction of ROS can lead to oxidative stress, one of its biomarker is a level of lipid peroxidation (LPO). The effect of compounds **1-5** on the level of TBARS accumulation in the bester liver was determined at different stages of long-term *in vitro* oxidation (1, 24, 48 and 72 h, see Table 2).

TABLE 2. TBARS level (nmol/ml) in the bester liver lipids in the presence of compounds **1-5** during long-term *in vitro* oxidation

	Control	**1**	**2**	**3**	**4**	**5**
1 h	1.22±0.07	1.20±0.06	1.18±0.09	1.18±0.03	1.18±0.09	1.18±0.13
24 h	1.50±0.10	1.35±0.07	1.29±0.05	1.33±0.06	1.33±0.05	1.34±0.06
48 h	1.92±0.11	1.60±0.04	1.61±0.03	1.55±0.11	1.76±0.13	1.58±0.12
72 h	2.56±0.04	1.97±0.07	1.97±0.05	1.95±0.10	2.36±0.04	2.25±0.09

In a control experiment the level of TBARS after 72 h increased for 100% in comparison with 1 h ($p < 0.0001$). The addition of all studied compounds to supernatant of bester liver homogenate led to the reduction of the level of accumulation of TBARS at all stages of oxidation. It can be concluded from the results obtained in the presence of compounds **1-3** (Table 2), that a gradual increase in the efficiency of the antioxidant action was observed. The highest inhibitory activity of compounds **1-3** was achieved at the late stage of LPO after 3 days of lipid oxidation of bester liver. The inhibitory activity of compounds under study on the level of LPO seems to be important because most of the lipid peroxidation derived carbonyl by-products are toxic since they can easily diffuse through membranes and can covalently modify important biomolecules far from their unmodified state [29].

CONCLUSIONS

Thus, this study showed that the series of new carbamate derivatives possesses a potent superoxide anion radical scavenging ability, the inhibitory activity on the toxic adrenochrome formation in adrenaline autoxidation reaction in alkaline media, including in the presence of fish liver cytosol and protective activity in lipid peroxidation reaction. The results obtained *in vitro* experiments indicate a number of new functionally substituted aryl carbamates as promising antioxidants capable both of preventing the development of oxidative stress and inhibiting oxidative processes under conditions of the development of oxidative stress.

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