

Research on Biological Activity of Products Synthesized by Tetraketones and Arylidene-Arylamines

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Abstract: Based on the reaction of 1,6-diaryl-3, 4-dihydroxy-2, 4-hexadiene-1, 6-dione and arylidene-arylamines various 6-aryl-substituted-4-benzoylacetyl-4-hydroxy-5, 6-dihydro-4H-1, 3-oxazines were synthesized. Due to the relevance of search of new biologically active compounds with antioxidant activity, there was analyzed antiradical activity of derived oxazines. Antiradical properties of these compounds were studied through the binding reaction of stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Trolox was used as a standard. Six compounds with moderate antioxidant activity were detected. Antioxidant activity was studied on the *Escherichia coli* BW 25113 strain grown aerobically. When constructing a model of oxidative stress with solution of hydrogen peroxide on cells of *Escherichia coli* BW 25113 strain there was observed specific growth of cells per 1 hour with 6 substances of oxazine group. Two compounds have pro-oxidant activity and one compound demonstrated antioxidant activity. The outcomes of spectrophotometry with DPPH and on cells of *Escherichia coli* BW 25113 experiment are well correlated.

Key words: Schiff bases • Tetraketones • Research of antioxidant activity • Oxidative stress • Resveratrol • Free radical oxidation • DPPH • *Escherichia coli* BW 25113

INTRODUCTION

In recent years, most studies proved that the majority of pathologic processes progress due to common toxic condition - oxidative stress. Its formation is initiated by free radicals (FR) – extraordinary active chemicals with one valence electron. At the heart of FR formation mechanism are chain reactions, which were analyzed and described by the Nobel Prize winner N.Semenov. Free radicals pool in the organism causes active forms of oxygen (AFO), which are formed moderately during normal vital activity of cell and proliferate in case of hypoxia or excessive mitochondria load. AFO are free radicals themselves and differ in stability and oxidation ability. Most free radicals are distinguished by high chemical activity and ability to provide massive deleterious effect on membrane lipids, proteins and DNA. The oxidative stress is mainly manifested through lipid peroxide oxidation (LPO) [1]. Compounds with different chemical structure and general functionality, called “antioxidants”, are the natural “protection” against aggression of AFO. In recent years, more and more

scientists pay attention to finding biologically active substances with antioxidant action.

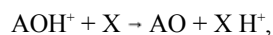
The major part of commonly used medicines with antioxidant effect is direct antioxidants. While seeking new antioxidants which are perspective to be clinically used, the direct antioxidants are being paid special attention.

Earlier it was proved that antioxidant activity is determined by several mechanisms: direct binding of free radicals, chelation, i.e. iron chelation in Fenton reaction [1, 2].

The available literature analysis allows to classify direct antioxidants into 5 main groups:

- Proton donors;
- Polyenes;
- Catalysts;
- Radical traps;
- Complexing agents.

Proton donors are compounds with easy movable hydrogen atom. They intercept free radicals in reaction:



where AO H^+ - antioxidant with movable hydrogen atom, X - radical initiator or intermediate radical product of free radical oxidation.

The proton donors group contains the following compounds: phenols, nitrogenous heterocycles, thiols, α , β -dienols, porphyrins.

Antiradical activity of antioxidants-proton donors may not correlate with inhibition efficiency. The proton donors are the biggest antioxidant group which is used in medicine.

Polyenes are compounds with a few unsaturated bonds, which are easily oxidized, rival for AFO and radicals with biomolecules, thereby preventing them from oxidation. They are able to interact with different free radicals, attaching them covalently with double bond. The main of them are: retinoids (retinal, retinoic acid, retinol and its ethers) and carotenoids (carotenes, lycopene, spirilloxanthin, astacin, astaxanthin etc.).

Catalysts are compounds which are able to catalyze elimination of free radicals and intermediate products of FRO without new formation. They are also known as enzyme mimetics. In contrast to direct antioxidants, catalytic antioxidants are effective in much lower concentrations and are not wasted in elimination reaction FR. It means that they can be used in much lower doses, the organism keeps their effect much longer and their probability of adverse reaction is lower.

Radical traps: typical representatives are nitrons, in particular, phenyl-tert-butyl nitron, which effectively binds superoxide and hydroxyl radicals. The experiments on animals showed protective effect of nitrons during oxidative damage of central nervous system. All the components of free radicals can inhibit due to elimination of primary generative free radical oxidation.

Complexing agents (chelators) - the main are: EDTA and its salts (Na_2EDTA , versene, complexone III), despheroxamine, 1, 10-batophenanthroline, carnosine, isonicotinoyl compounds, some flavonoids, carvedilol. Only metal-dependent reactions of FRO inhibit due to binding of metal cations with variable valence, which catalyze AFO formation reactions.

This classification is not considered as completely full, as far as it takes into account only basic elements of molecule, which are responsible for antioxidant properties of a substance. However, this classification is appropriate to be used in searching and initial screening for new direct antioxidants with determined mechanism of action.

According to some authors nowadays there are 5 main ways in which antioxidants influence cell metabolism and peroxide oxidation.

- Interaction with free radicals.
- Interaction with receptors ("own" vs. "alien").
- Influence on enzymes' activity (interaction with catalytic and allosteric centres).
- Membrane fusion and alteration of membrane structure and function.
- Interaction with genetic apparatus of cell.

All these factors stipulate variety of biological activity of antioxidants and allow to use them not only to prevent various pathologies, but also to actively affect normal metabolism of cell.

MATERIALS AND METHODS

In recent years, most studies point at the key role of free-radical oxidation (FRO), which is developed mainly through active forms of oxygen (AFO) - free radicals with high oxidizing power [3]. AFO accumulation in cell leads to toxic condition called oxidative stress. Antioxidants play specific role in binding and minimization of AFO effect. One of the main applications in pharmacology and pharmaceutical chemistry is a search for compounds with antioxidant activity.

In order to analyze synthesized compounds the screening of antioxidant activity was conducted. Common methods to study antioxidants as individual compounds and polycomponent plant extracts are direct methods of antioxidant activity (AOA) evaluation, which are based on the study of kinetics of model reactions, illustrating mechanisms of proteins and lipids oxidation [3].

Nowadays one of the important applications in chemistry is formation of new biologically active compounds. Most of the drugs are biologically active agents in the form of specific chemicals. In this way there was identified Resveratrol - a chemical polyphenolic compound with antioxidant properties, which was isolated from the seeds and leaves of red grapes.

Structural analysis of this antioxidant bears some structural similarity to oxazines, which were synthesized by reaction of tetraketones and arylidene-arylamines.

As a substrate for the synthesis of oxazines was chosen 1,6-diaryl-3,4-dihydroxy-2,4-hexadiene-1,6-dione, as reagents - various arylidene-arylamines - Schiff bases.

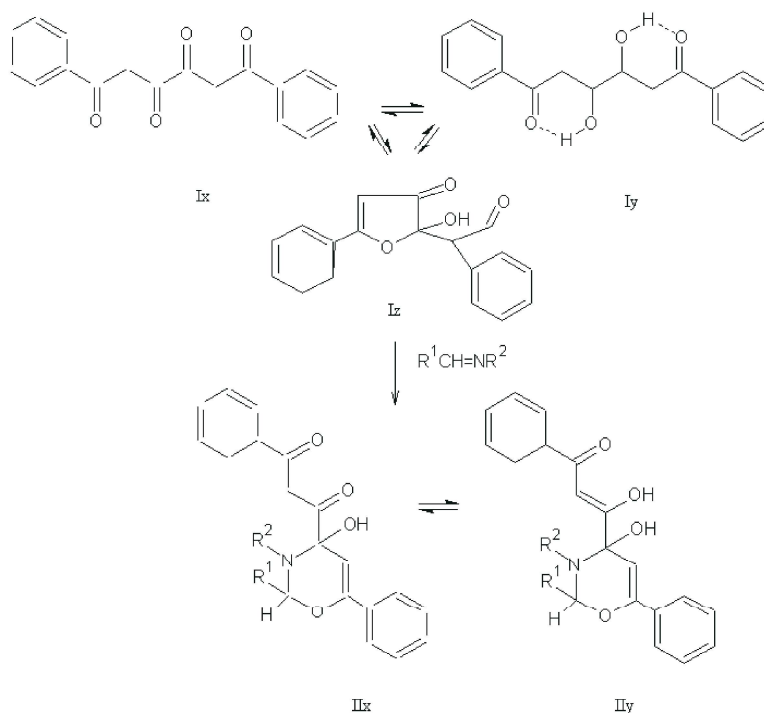


Fig. 1: The general scheme for 6-aryl-substituted-4-benzoylacetyl-4-hydroxy-5,6-dihydro-4H-1, 3-oxazines (II a-f)

The features of Schiff bases are the nucleophilic component of amines and the electron deficient bond in aldehyde fragment of the molecule [4]. Earlier there were performed quantum chemical calculations with account for formation heat value (H_f) and coplanarity of the 1,6-diaryl-3,4-dihydroxy-2,4-hexadiene-1,6-dione molecule, which proved that in solutions the keto form of 1,6-diaryl-3,4-dihydroxy-2,4-hexadiene-1,6-dione has the highest stability [5].

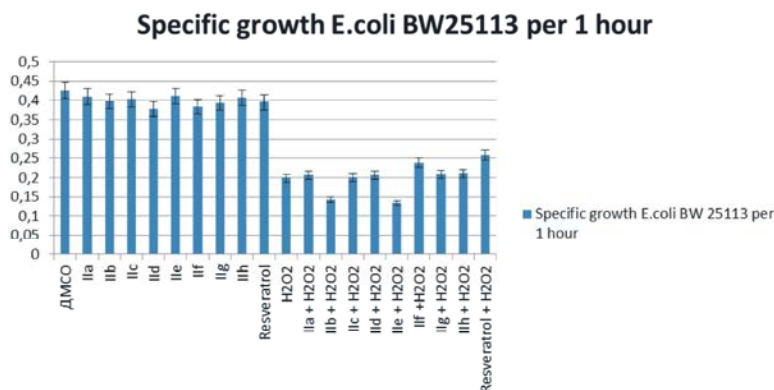
It is determined that 1,6-diaryl-3,4-dihydroxy-2,4-hexadiene-1,6-dione (Ix, Iy, Iz) interreacts with arylidene-arylamines forming 6-aryl-4-benzoylacetyl-4-hydroxy-2-phenyl-5,6-dihydro-2-phenyl-4H-1,3-oxazines (III-f), which have a crystalline structure, are yellow, soluble in dimethylsulphoxide, dimethylformamide, benzol, toluol, sparingly soluble in ethanol, chloroform, water-insoluble (Fig. 1).

The compounds (II a-f) are formed with preparative yields. Apparently, in solutions oxazines are represented both as predominant β -dioxo form for benzoylacetyl fragment (IIx) and minor β -keto-enol tautomer (IIy). The structure of prototropic forms was defined through spectroscopy data $^1\text{H-NMR}$: the marker signals are a singlet of two methylene ($-\text{CH}_2-$) protons (IIx) 3,99-4,22 ppm and a singlet of methine (CH) proton 5,82 – 6,55 ppm [6, 7].

Spectral characteristics of interaction products of 1,6-diaryl-3,4-dihydroxy-2,4-hexadiene-1,6-dione with arylidene-arylamines testify in favor of the proposed structure [6, 7].

When selecting a screening technique for research on antioxidant activity there was assumed high lipophilicity of synthesized oxazines. The screening was implemented through spectrophotometric determination of antiradical activity by 2,2-di (4-tert-oktyl phenyl) -1-picrylhydrazyl (DPPH) at 517 nm wavelength [3, 8]. DPPH solution concentration was $3 \times 10^{-4} \text{M}$. A medium with pH close to the plasma was reproduced using tris-hydrochloride buffer solution with pH 7.4. The total antioxidant activity was compared with ethanol solution with antiradical activity – trolox.

One of the indirect methods is the one based on interaction of antioxidants and stable chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich manufacture) [8]. In visible spectrum zone DPPH in organic solvents has absorption maximum at 515÷520 nm wavelength, which vanishes when radical interacts with proton donors or free radicals with another structure [8, 9]. Trolox - water-soluble form of tocopherol - was chosen as a standard of antiradical activity. The reaction with stable radical diphenylpicrylhydrazyl (DPPH) was used in order to determine antiradical activity.

Fig. 2: Specific growth of *E. coli* BW 25113 per 1 hour

This is a rate determining step of chemical reaction, because the formed free radical interacts with other molecule DPPH.



The reaction mixture consists of 3 ml 0,3mM DPPH solution, 1 ml THAM hydrochloride buffer solution with pH 7,4 and 0,01mM substances dissolved in dimethyl sulfoxide (DMSO). It is incubated in standard temperature conditions ($T = 293 \text{ K}$) within 30 minutes. Afterwards, optical density of reaction mixture is measured on photoelectrocolorimeter KФK-3-01 at 517 nm wavelength in 0,5 cm cuvette. Inhibiting effect is calculated with the formula:

$$\text{IC}_{50} = 100(D_0 - D_x)/D_0,$$

where D_0 - optical density of DPPH control solution, D_x - optical density of solution with observable substance or trolox solution.

While using the method of antiradical activity with DPPH synthesized compounds showed conspicuous activity (Table 1).

Antioxidant activity was studied on the *Escherichia coli* BW 25113 strain grown aerobically in M9 minimal liquid medium with glucose. The research was conducted in Microbial physiology laboratory of the Institute of Ecology and Genetics of Microorganisms, Ural Division of Russian Academy of Sciences (Oktyabrsky O., Smirnova G.). When constructing a model of oxidative stress with 0,6 mm hydrogen peroxide solution on cells of *E. coli* BW 25113 strain there was observed specific growth of cells per 1 hour with 6 substances of oxazine group [10].

Table 1: The results of research into antiradical activity of oxazines

Compounds	Radicals		
	R ¹	R ²	Radicals binding, %
Ila	p-CH ₃ C ₆ H ₄	p-CH ₃ C ₆ H ₄	42,74±0,20□
Iib	p-CH ₃ C ₆ H ₄	C ₆ H ₅	50,06±0,53□
Iic	p-CH ₃ OC ₆ H ₄	p-NO ₂ C ₆ H ₄	38,95±1,44
Iid	p-NO ₂ C ₆ H ₄	p-BrC ₆ H ₄	38,65±0,95
Iie	p-NO ₂ C ₆ H ₄	p-CH ₃ C ₆ H ₄	52,56±0,87
IIf	p-CH ₃ OC ₆ H ₄	p-CH ₃ C ₆ H ₄	53,17±1,26□
Trolox	-	-	72,17±1,14

* p = 0,05 in comparison with Trolox

The research results revealed that these compounds are biologically active (Fig. 2).

RESULTS

Reactions of 1, 3, 4, 6-tetrakarbonyl compounds with Schiff bases led to derivation of 6-aryl-substituted-4-benzoylacetyl-4-hydroxy-5, 6-dihydro-4H-1, 3-oxazines, which are quite promising to be studied as antioxidants.

The research proves that compounds *Iib* and *Iie* have pro-oxidant activity and compound *IIf* has antioxidant activity on the cells *Escherichia coli* BW 25113.

The research revealed that, apparently, the p-CH₃C₆H₄ radical presence in the structure increases antiradical and antioxidant activity.

As shown by the studies received 6-aryl-substituted-4-benzoylacetyl-4-hydroxy-5, 6-dihydro-4H-1, 3-oxazines not have a cytotoxic effect on the cells of *Escherichia coli* BW 25113.

CONCLUSIONS

At the time of experiment on the *Escherichia coli* BW 25113 strain oxazines *Iib* and *Iie* have pro-oxidant activity

and compound *Iif* demonstrated antioxidant activity. The outcomes of spectrophotometry with DPPH and *E. coli* experiment are well correlated. In order to identify possible mechanisms of antioxidant action of synthesized compounds, in the perspective interest are analysis of chelation capability of oxazines and research on how these substances with pro-oxidant activity influence expression of regulon gene, hydroperoxidase (catalase), HPI and superoxiddismutase Mn-SOD, as the earlier studies contain information that pro-oxidant effects of substances relate to their ability to augment hydrogen peroxide production. Earlier it was shown that pro-oxidant effect in a certain way protects *E. coli* cells from sizeable hydrogen peroxide concentration.

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