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Antioxidant properties of terpene-substituted phenols

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Antioxidant activity of terpene-substituted phenols and their aminomethyl derivatives as compared to the activity of standard antioxidants, ionol and trolox, has been studied by spectrophotometry (by the ability to react with the stable radical of 2,2-diphenyl-1-picrylhydrazyl) and coulometry (by the ability to react with a bromine radical).

Key words: terpenoids, phenols, amines, antioxidants, spectrophotometry, coulometry.

At present, a free-radical theory of aging and development of age diseases (cardio-vascular, oncological, neurodegenerative) is widely spread.[1,2] According to this theory, an antioxidant system, which provides protection of human organism from damage by free radicals (FR), weakens with age, resulting in FR to damage proteins, nucleic acids, and lipid membranes. Accumulation of damages of biological macromolecules leads in the end to acceleration of aging process and appearing age diseases. Human antioxidant system is a complex multilevel system, with such components as enzymes (superoxide dismutase, catalase, *etc*.), transition metal chelators (albumin, ferritin, hepatocuprein), and low-molecular-weight antioxidants (AO). Antioxidant protection in living systems is represented by various compounds and systems. Some AO are synthesized in human organism (melatonin, amino acids, glutation, some vitamins).[3] Important sources of AO are vegetable food, food additives, and synthetic AO.

Taking into account significance of exogenic AO for maintaining human health, a search for new water- and liposoluble inhibitors of peroxide oxidation of lipids is an actual problem. There are found natural compounds capable of protecting living cells from damage by the light, oxygen, and photosensitizers. Synthetic compounds can also serve as sources of new promising bioantioxidants. Drugs of antioxidant action are a new pharmacological group of medicines, possessing a wide range of biological activity. Low- and high-molecular-weight phenolic compounds are an important class of exogenic AO.[4,5] In addition, in the last decades plenty attention is paid to chiral compounds, which are important for medicine. That is why the study of chiral phenolic antioxidants in racemic and enantiomeric forms grows very fast. Several chiral drugs of a wide range of action (for example, tasophelon and its analogs) have been developed based on phenolic AO. Recently,[6—9] there has been also a report on the development of antioxidant methods of protection from cigarette smoke.

A wide variety of antioxidants, synergism and antagonism of their action, as well as specificity with respect to certain forms of activated oxygen metabolites, create difficulties in their determination both *in vitro* and *in vivo* systems. Attempts are made to find integral indices characterizing activity of antioxidant systems as a whole.[10] For the study of antioxidant properties of drugs *in vitro*, various experimental systems with the use of biochemiluminescence, ESA, determination of oxygen absorption, and so on, have been developed.[11] Antioxidant properties of promising medicines are also studied *in vivo* on animal models of free-radical pathologies or oxidative stress. Complications in determination of activity of antioxidant systems consist in the fact that activity of each AO depends on the medium and conditions in which it acts. Antioxidant properties revealed in different experimental models depend on the type of oxidative reactions and their conditions, and, therefore, can be different.[10]

Since FR formed in organisms differ in chemical properties (lipo-, water-soluble) and time of life, efficient protection of organisms can be provided by AO differing in chemical properties and affinity to different classes of FR. At present, certain rules of testing food and individual compounds on the presence in them antioxidant properties are developed. One of these rules is testing AO by several methods.[11]

The present work is devoted to the comparative study of antioxidant activity (AOA) of new synthetic compounds, *viz*., terpene-substituted phenols and their derivatives. Such phenols are prepared by C-alkylation of hydroxy arenes with terpenes. Representatives of this family of com-

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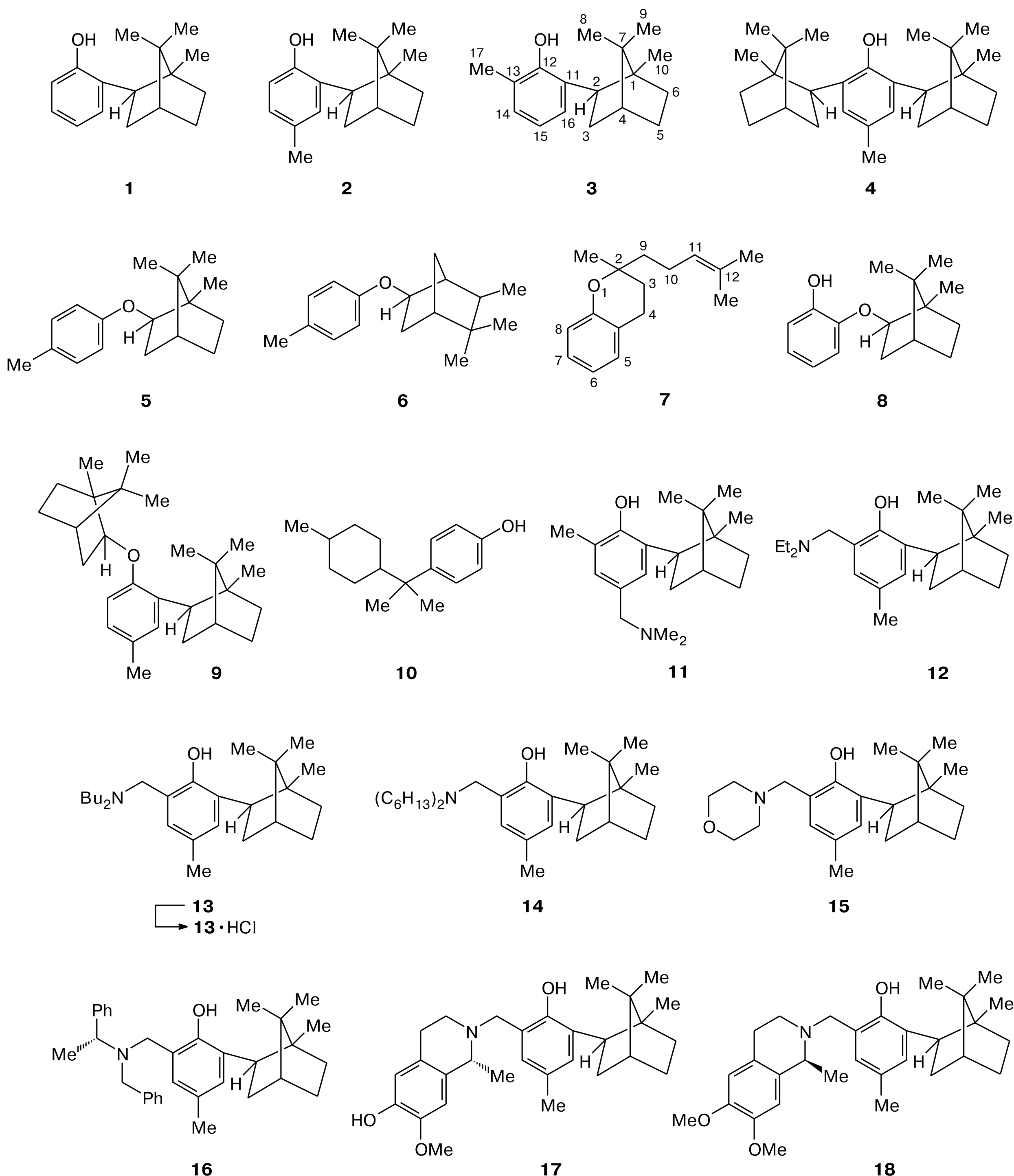
pounds are found in natural sources; synthetic versions for their preparation are also suggested.

To evaluate AOA of terpene-substituted phenols and their analogs **1—18**, we used two methodical approaches: spectrophotometric determination of the reaction of tested compound with stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH)[12] and coulometric measurement of the reaction of tested compound with bromine radical.[13]

Experimental

Compounds **1**, **2**, **4—6**, **8—10** were obtained according to the described procedures.[14—17] Derivatives **11—18** were obtained by aminomethylation reaction.[18—20] Ionol and trolox were used as comparison standards.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM spectrometer (400.13 and 100.61 MHz, respectively) in $CDCl_3$.



Assignment of signals in the ^{1}H NMR spectrum were made using the COSY, NOESY, and HETCORR experiments. IR spectra were recorded on a Specord M-80 spectrophotometer for neat samples and in KBr pellets. Monitoring of purity of starting compounds and analysis of reaction products were performed by GLC on a Schimadzu GC-2010AF instrument with a flame-ionizing detector (helium as a carrier gas) on a HP-1 capillary column (Agilent, 60 m × 0.25 mm × 0.25 μm, the temperature regime was 100—240 °C, heating at 6 deg min^{-1}); TLC was performed on Sorbfil and Silufol UV-254 plates using hexane—Et_2O as an eluent.

2-*exo*-(2-Hydroxy-3-methylphenyl)-1,7,7-trimethylbicyclo-[2.2.1]heptane (3). *o*-Cresol (10.8 g, 0.1 mol) was heated to 180 °C in a round-bottom flask equipped with a thermometer and a reflux condenser followed by addition of aluminum foil (0.06 g, 2.2 mmol) in small portions. After aluminum was completely dissolved, the solution was cooled to 40 °C and camphene (13.6 g, 0.1 mol) was added to it. The reaction was carried out at 160 °C until camphene was almost completely consumed (GLC monitoring). The mixture was then cooled, diluted with Et_2O followed by addition of 18% aqueous HCl to decompose the catalyst. To remove the residual unreacted *o*-cresol, the ethereal solution was washed with 5% KOH in aq. ethanol and water until neutrality. The organic layer was dried with $CaCl_2$, the solvent was evaporated at reduced pressure, and the residue was purified by distillation *in vacuo* (the fraction with b.p. 140—146 °C (5 Torr)). Compound **3** (16.7 g, 68%) was obtained by crystallization from hot hexane as light yellow crystals, m.p. 85 °C. Found (%): C, 83.62; H, 9.80. $C_{17}H_{24}O$. Calculated (%): C, 83.55; H, 9.90. IR (KBr), ν/cm^{-1}: 3600, 3000—2800 (OH), 1170 (C—O), 830, 770 (Ar). ^{1}H NMR, δ: 0.78, 0.84, 0.89 (all s, 3 H each, $C(10)H_3$,$C(9)H_3$, $C(8)H_3$); 2.24 (s, 3 H, $C(17)H_3$); 1.33—1.38 (m, 1 H, H(5)); 1.41—1.45 (ddd, 1 H, H(6), J = 9.0 Hz, J = 3.0 Hz, J = 3.0 Hz); 1.55—1.65 (m, 2 H, H(3), H(6)); 1.84—1.87 (m, 2 H, H(4), H(5)); 2.18—2.23 (m, 1 H, H(3)); 3.07 (t, 1 H, H(2), J = 8.8 Hz); 4.65 (s, 1 H, OH); 6.80 (t, 1 H, H(15), J = 7.6 Hz); 6.94 (d, 1 H, H(14), J = 7.3 Hz); 7.15 (d, 1 H, H(16), J = 7.8 Hz).

2-Methyl-2-(4-methylpent-3-enyl)chromane (7). Aluminum phenolate (3.98 g, 13 mmol) and geraniol (2.0 g, 13 mmol) were heated at 120 °C in a 100-mL two-neck flask equipped with a thermometer and a reflux condenser until geraniol was completely converted (GLC and TLC monitoring). The mixture was then cooled, diluted with Et_2O, washed with 0.1 M aq. HCl to decompose the catalyst, then with 5% aq. NaOH to remove the residue of the starting phenol, and with water until neutrality. The organic layer was dried with anhydrous Na_2SO_4, the solvent was evaporated at reduced pressure. The reaction mixture was purified by column chromatography on silica gel (Silica gel 70/230 μ using the light petroleum—Et_2O solvent mixture with the increase of the content of the latter in it as an eluent). Product **7** (1.28 g, 43%) was obtained as a light yellow oil. Found (%): C, 83.38; H, 9.70. $C_{16}H_{22}O$. Calculated (%): C, 83.43; H, 9.63. IR (neat), ν/cm^{-1}: 1676 (C=C, geranyl fragment), 1616—1492 (C=C, Ar), 1460, 1380 (=$C(CH_3)_2$), 1244—1232, 1112 (C—O), 888 (CH_2 of chromane), 756 (CH, Ar). ^{1}H NMR, δ: 1.31 (s, 3 H, $C(2(1))H_3$); 1.61 (s, 3 H, $C(12(1))H_3$); 1.70 (s, 3 H, $C(12(2))H_3$); 2.08—2.14 (m, 2 H, H(10)); 2.76 (t, 2 H, H(9), J = 7.8 Hz); 5.10 (t, 1 H, H(11), J = 2.8 Hz); 6.89—6.90 (m, 4 H, 2 H(3), 2 H(4)); 7.05 (d, 1 H, H(5), J = 7.6 Hz); 6.75—6.89 (m, 3 H, H(6), H(7), H(8)). ^{13}C NMR, δ: 17.57 (C(12(2)); 25.65 (C(12(1))); 30.95 (C(2(1))); 22.28 (C(10)); 40.11 (C(9)); 75.92 (C(2)); 117.28 (C(6)); 118.93 (C(3)); 120.51 (<u>C</u>(Ar)—CH_2); 122.83 (C(13)); 124.41 (C(11)); 126.97 (C(7)); 127.20 (C(5)); 131.26 (C(2)); 131.58 (C(4)); 153.98 (<u>C</u>(Ar)—O).

Spectrophotometry. The antioxidant activity of terpene-substituted phenols and their derivatives was determined by the ability of their samples to react with the stable radical DPPH under the *in vitro* conditions.[21] The reaction was carried out in plates. Each sample was analyzed three times. A mixture containing all the components, except the analyzing compound, served as a control. A solution of DPPH (150 μL, 0.6 mM) in ethanol was added to 50 μL of 1, 0.1, 0.01, or 0.001% ethanolic solution of the sample. Simultaneously, the reactions with ethanolic solutions of trolox and ionol in the same concentrations were carried out. Optical density was measured on a PowerWave 200TM tablet spectrophotometer (Bio-Tek Instruments, USA) at λ = 517 nm, first, immediately after addition of DPPH and vigorous stirring (t_0), then, after 30 min of incubation in the dark under polyethylene film (t_1). The antioxidant activity (in %) was calculated by the formula (1)[12]

$$AOA = (OD_{517,bl(0)} - OD_{517,sm(1)})/OD_{517,bl(0)} \cdot 100, \quad (1)$$

where $OD_{517,bl(0)}$ is the optical density of the blank, measured immediately after addition of DPPH; $OD_{517,sm(1)}$ is the optical density of the sample, measured after 30 min of incubation.

Then, the AOA was plotted against concentration of the analyzing compound and the concentration of the compound, which corresponded to 50% loss of color of DPPH ($DPPH_{50}$), was found.

Coulometry. Evaluation of the antioxidant activity was performed by coulometric titration with electrogenerated bromine-containing compounds[22] An EKSPERT-006 coulometric analyzer (Ekoniks-Ekspert, Moscow) with glassy carbon electrodes was used for the studies. The reference (anode of 2.3 cm^2 in area) and auxiliary (cathode) electrodes were glassy carbon rods of 3 mm in diameter; needle-like platinum electrodes were an indicator; 0.2 M aq. potassium bromide in 0.1 M sulfuric acid was a supporting electrolyte; an operating current was 5.27 mA, auxiliary current was 0.79 mA; the level of measurement was 300 mV, the level of reduction was 500 mV. The cathode and anode compartments were divided by a semipermeable membrane, platinum electrodes were used as measurement electrodes.

Supporting electrolyte (100 mL) was placed into a coulometric cell, then reference, auxiliary, and indicatory electrodes were dipped into the electrolyte. The cell was placed on a magnetic stirrer and the instrument was turned on with constant stirring of the electrolyte. According to the built-in program, the instrument first passed an "auxiliary" current through the generator electrodes until the potential difference of the indicatory system reached the measurement level. In this case, a certain amount of active forms of bromine is formed and maintained on the same level in the supporting electrolyte. An analyzed sample was placed into the anode compartment of the coulometric cell. The antioxidants present in the analyzed sample reacted with active forms of bromine, that results in the rise in the potential difference in the indicatory system. When the level of decrease was reached, the analyzer began to pass the "main" current through the electrolyte and started to count out the time (titration) until the potential difference in the indicatory system reached the measurement level. Simultaneously, the starting bal-

ance of electrogenerated bromine radicals, disturbed by the introduction of an analyzed sample, was restored.

Quantity of electricity (in C) consumed in the titration was calculated according to the formula (2)[13]

$$Q = (100 \cdot I \cdot t)/V_{al}, \quad (2)$$

where I is the current intensity; t is the time to reach the end point of titration; V_{al} is the volume of aliquot (in mL). Measurement units of AOA: cC (100 g of tested compound)$^{-1}$.

Each sample was analyzed five times with calculation of arithmetical mean and standard deviation.

Results and Discussion

Advantages of the DPPH method for determination of AOA are a simplicity of registration of reaction, a possibility to perform mass screening of compounds, as well as a large body of publications accumulated by now, in which this method was used for the analysis of AO of phenolic nature. The disadvantage of the method consists in the dependence of the reaction rate of tested compound from the size of its molecule, since the unpaired electron in the DPPH molecule is poorly available. A considerable disadvantage of the method consists in the relatively small range of linear dependence between the concentration of tested compound and optical density. To overcome this problem, the tested compound is studied in several dilutions. Antioxidant activity of compound is expressed in the concentration units, which corresponds to a 50% discoloration of the DPPH radical occurred over a certain period of time.[23]

Trolox reacts with DPPH the most actively among the tested compounds, which causes a 50% discoloration of the radical at the concentration of 0.004% (Table 1); ionol has the $DPPH_{50}$ value equal to 0.09%. The study of AOA of compounds **1—18** by spectrophotometric method showed that compounds **3**, **4**, **6**, **8**, **12—14**, **17**, and **18** are between the standard AO in the ability to react with DPPH. Terpene-substituted phenols **2**, **9**, **11**, **13 · HCl**, and **16** have lower value of $DPPH_{50}$ than ionol, however, they belong to the range of analyzed concentrations. Compounds **1**, **5**, **7**, **10**, **12**, and **15** possess low ability to react with DPPH.

Spectrophotometry revealed maximum activity of trolox. In AOA, it is approached by medicines **4** and **17**. According to the spectrophotometric analysis data, antioxidant activity of ionol is lower than that of trolox, the coulometric analysis results showed that two standard compounds have approximately the same activity. Results obtained by coulometry do not agree with the results of testing compounds by spectrophotometry. The absence of correlation between results of two analyses shows the same picture. Apparently, this is due to the fact that the methods used are based on different principles.

Coulometry has been developed relatively not long ago.[12] The method is based on titration of antioxidants with electrogenerated active forms of bromine (Br_3^-, Br_2, $Br^\bullet$), which can be involved into different reactions: radical, oxidation-reduction, electrophilic substitution, and addition at the multiple bonds.[24] The method is simple, has high reproducibility, and allows one to involve a wide range of biologically active compounds of various structure and possessing antioxidant properties; for all this, it requires no complex equipment and reagents. This method found its application in the study of antioxidant properties of drinks and biological liquids.[25—27]

The study of terpene-substituted phenols and their aminomethyl derivatives by coulometric titration showed that medicines **1** and **17** have the maximum AOA (~2 times as high as that of trolox and ionol). Antioxidant activity of compounds **7** and **10** is somewhat higher than that of the standard samples. Activity of medicines **2**, **3**, **12**, **15**, and **16** is approximately the same as that of the standard samples, whereas activity of other compounds is lower than that of the comparison samples. Ether **9** has the minimum activity (see Table 1). The results on determination of AOA obtained by two methods show that the mechanism of the reaction of terpene-substituted phenols with free radicals of different nature (stable sterically hindered hydrazyl radical and inorganic short-lived bromine radical) is different.

Table 1. Antioxidant activity (AOA) of terpene-substituted phenols **1—18** determined by spectrophotometry and coulometry

Compound	DPPH-binding activity (%)	AOA /cC (100 g)$^{-1}$
Trolox	0.004	66.61±3.98
Ionol	0.09	68.86±7.89
1	—*	122.98±1.96
2	0.25	68.75±1.45
3	0.06	63.77±6.99
4	0.017	10.96±1.41
5	—*	46.33±10.69
6	0.085	34.90±7.37
7	—*	77.21±10.04
8	0.085	52.43±6.97
9	0.095	12.02±1.86
10	—*	73.89±6.97
11	0.1	23.81±0.77
12	0.06	61.79±7.81
13	0.074	35.87±6.28
13 · HCl	1.0	45.98±6.22
14	0.08	42.60±12.44
15	—*	58.28±4.69
16	0.5	58.03±7.79
17	0.04	144.62±21.45
18	0.055	44.91±3.21

* The value is beyond the range of linear dependence of optical density from concentration.

The data represented showed that terpene-substituted phenols and their derivatives are promising synthetic AO. It should be emphasized that the studies performed are only the first step in evaluation of antioxidant properties of terpene-substituted phenols. In further studies, we plan to find how these compounds behave *in vivo*.

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