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Antioxidant Properties of Conjugates of Triterpenic Acids with Amido Derivatives of Trolox

V. R. Khairullina[a], A. Ya. Gerchikov[a,*], A. B. Safarova[a], R. R. Khalitova[b], A. Yu. Spivak[b], E. R. Shakurova[b], and V. N. Odinokov[b]

[a] *Bashkortostan State University, Ufa, 450074 Bashkortostan, Russia*

**e-mail: gerchikov@inbox.ru*

[b] *Institute of Petrochemistry and Catalysis, Russian Academy of Sciences, Ufa, 450075 Bashkortostan, Russia*

e-mail: ink@anrb.ru

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Abstract—The antioxidant properties of conjugates of betulonic and betulinic acids with amido derivatives of the acid Trolox were studied for initiated 1,4-dioxane oxidation as a model reaction. The antioxidant activity of the compounds was characterized in terms of the apparent inhibition rate constant fk_{In}.

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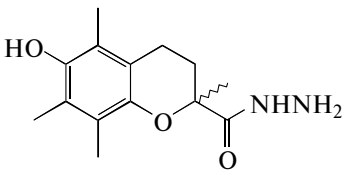
Lipid peroxidation in the cell membranes of living organisms yields labile intermediate species (superoxide radical anion $O^{\bullet-}$, hydroperoxyl radical $HO_2^{\bullet}$, and nitrogen oxide NO), which often cause various pathologies [1]. Natural and synthetic antioxidants (AOs) acting at the physiological level allow the risk of disease to be reduced [2–4].

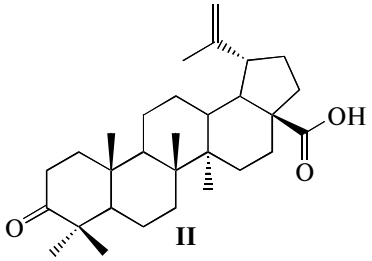
When creating new drugs for prophylactics and complex medical treatment of diseases associated with the intensification of lipid peroxidation, researchers pay great attention to polyfunctional drugs, such as synthetic analogues of α-tocopherol and their conjugates with pharmacophores of various biologically active substances [5–12]. We have recently reported [13, 14] the synthesis of the first representatives of the conjugates of synthetic analogues of α-tocopherol with triterpenic (betulonic and betulinic) acids, compounds executing a wide variety of biological actions [15–17]. It turned out that the antioxidant activity of these compounds is an important characteristic of their biological activity.

The purpose of the present work is to quantitatively study the antioxidant effect of the conjugates of betulonic and betulinic acids with amido derivatives of 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-chromene-2-carboxylic acid (Trolox).

The structural formulas of the compounds examined here (**I**–**IX**) are given below.



I



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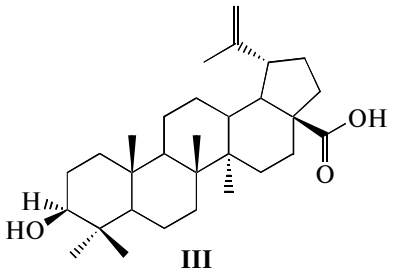

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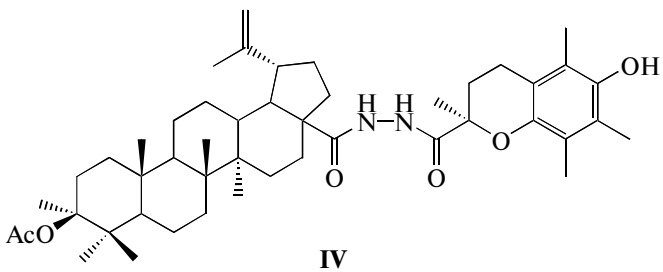
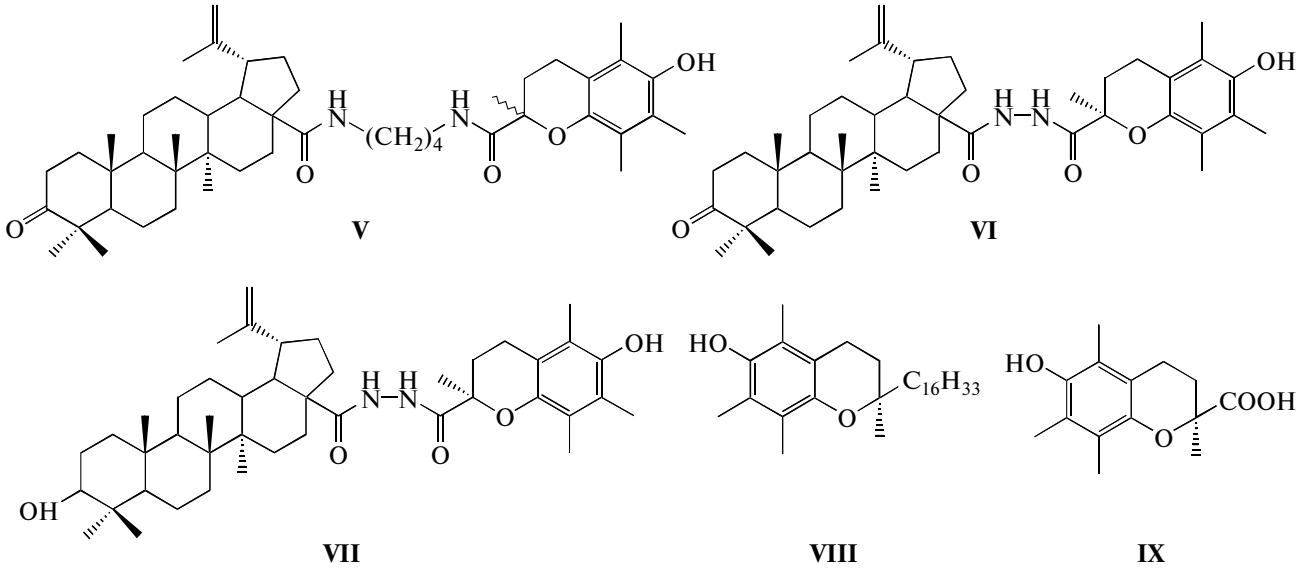


IV



EXPERIMENTAL

The hydrazide of 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-chromene-2-carboxylic acid was characterized by IR and ^{1}H NMR spectroscopy. For conjugates **IV–VII**, optical rotation angle data are presented along with their spectral characteristics.

Compound I. UV (1,4-dioxane): λ_{max} = 291 nm (ε = 3000). ^{1}H NMR ($CDCl_3$), δ, ppm: 1.39 (s), 1.67–1.77 (m), 1.99 (s), 2.07 (s), 2.09 (s), 2.19–2.25 (m), 2.38–2.56 (m), 4.34 (s) 7.46 (s), 8.48 (s).

Compound IV. $[\alpha]_D^{20}$ = +7.14° (c = 1.47, $CHCl_3$). UV (1,4-dioxane): λ_{max} = 295 nm (ε = 2400). IR, ν, cm^{-1}: 1640 (CONH), 1740 (O–C=O), 3390 (CONH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.84 (s), 0.87 (s), 0.93 (s), 0.95 (s), 0.96 (s), 1.17–2.50 (m), 1.68 (s), 2.04 (s), 2.09 (s), 2.18 (s), 2.21 (s), 2.62 (m), 3.05 (m), 4.47 (t, J = 6.8 Hz), 4.60 (s), 4.73 (s), 8.08 (d, J = 5.2 Hz), 8.66 (d, J = 5.2 Hz), 8.17 (d, J = 5.2 Hz), 8.77 (d, J = 5.2 Hz).

Compound V. $[\alpha]_D^{20}$ = +17.20° (c = 0.29, $CHCl_3$). UV (1,4-dioxane): λ_{max} = 288 nm (ε = 2000). IR, ν, cm^{-1}: 1740 (CONH), 1710 (C=O), 3390 (CONH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.91 (s), 0.95 (s), 0.97 (s), 1.01 (s), 1.06 (s), 1.17–2.30 (m), 1.68 (s), 1.51 (s), 2.10 (s), 2.18 (s), 2.30–2.70 (m), 3.10–3.40 (m), 4.61 (s), 4.74 (s), 5.72 (s), 6.46 (s).

Compound VI. $[\alpha]_D^{20}$ = +12.70° (c = 0.45, $CHCl_3$). UV (1,4-dioxane): λ_{max} = 286 nm (ε = 2500). IR, ν, cm^{-1}: 1740 (CONH), 1700 (C=O), 3390 (CONH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.92 (s), 0.96 (s), 0.98 (s), 1.02 (s), 1.07 (s), 1.17–2.50 (m), 1.68 (s), 2.11 (s), 2.19 (s), 2.23 (s), 2.64 (m), 3.06 (m), 4.61 (s), 4.74 (s), 7.91 (d, J = 4.8 Hz), 8.60 (d, J = 5.6 Hz), 8.00 (d, J = 4.8 Hz), 8.72 (d, J = 5.2 Hz).

Compound VII. $[\alpha]_D^{20}$ = +5.26° (c = 0.76, $CHCl_3$). UV (1,4-dioxane): λ_{max} = 291 nm (ε = 3000). IR, ν, cm^{-1}: 1740 (CONH), 3390 (CONH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.75 (s), 0.85 (s), 0.89 (s), 0.93 (s), 0.96 (s), 1.18–2.50 (m), 1.68 (s), 2.11 (s), 2.18 (s), 2.22 (s), 2.63 (m), 3.06 (m), 3.17 (dd, J = 8.8 Hz, J = 4.5 Hz), 4.60 (s), 4.74 (s), 7.94 (d, J = 5.2 Hz), 8.63 (d, J = 5.2 Hz), 8.00 (d, J = 5.6 Hz), 8.73 (d, J = 5.6 Hz).

The antioxidant activity of chromanol (**I**), betulonic acid (**II**), betulinic acid (**III**), and hybrid molecules (**IV–VII**) was studied in initiated 1,4-dioxane oxidation at 348 K as a model reaction using the manometric method (atmospheric oxygen uptake measurements) and kinetic spectrophotometry (time variation of the antioxidant concentration) [18]. Azobisisobutyronitrile (AIBN) was used as the initiator of oxidation.

The antioxidant activity was measured by the manometric method at a constant rate of initiation of the oxidation process, $w_i = 1 \times 10^{-7}$ mol l^{-1} s^{-1}, in a temperature-controlled glass reactor. The reactor was charged with solutions of the initiator and an antioxidant in 1,4-dioxane. The inhibition efficiency of the conjugates of betulonic and betulinic acids with the amido derivatives of Trolox was estimated as the decrease in the initial rate of oxygen uptake. The numerical values of the latter were derived from the initial portions of oxygen uptake curves by the least-squares method.

The antioxidant activity of the conjugates of betulonic and betulinic acids with the amido derivatives of Trolox were also studied by kinetic spectrophotometry at an initiation rate of $w_i = 2 \times 10^{-7}$ mol l^{-1} s^{-1}. A temperature-controlled cell was placed in the cell compartment of a Shimadzu UV-2401 PC spectrophotometer. The consumption of compounds was mea-

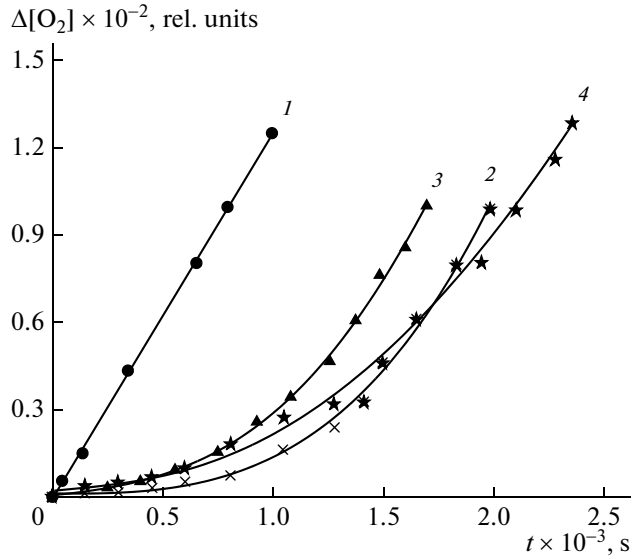


Fig. 1. Typical oxygen uptake kinetics for 1,4-dioxane oxidation ($w_i = 1 \times 10^{-7}$ mol l^{-1} s^{-1}, 348 K) in the (*1*) absence and (*2–4*) presence of compounds **I**, **VI**, and **VII**. Concentrations: (*2*) **[I]** = 7.0×10^{-5} mol/l, (*3*) **[VII]** = 7.0×10^{-5} mol/l, and (*4*) **[VI]** = 8.1×10^{-5} mol/l.

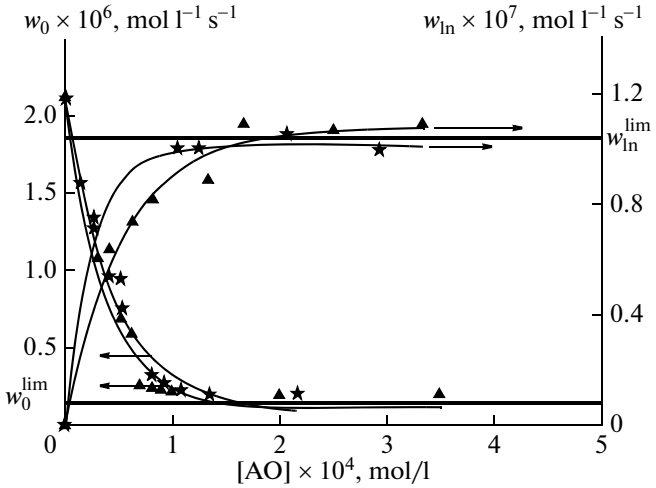


Fig. 2. Initial rate of 1,4-dioxane oxidation ($w_i = 1 \times 10^{-7}$ mol l^{-1} s^{-1}, 348 K) and the antioxidant consumption rate ($w_i = 2 \times 10^{-7}$ mol l^{-1} s^{-1}) as a function of the concentration of (★) **VI** and (▲) **VII**.

sured as the decrease in the absorbance at the absorption maximum. As was indicated above, λ_{max} for compounds **I**, **IV**, **V**, **VI**, and **VII** is 291, 295, 288, 286, and 291 nm, respectively. The numerical values of the disappearance rate for compounds **I–VII** (w_{In}) were calculated by least squares applied to the initial portions of the corresponding kinetic curves. 1,4-Dioxane was purified using a standard procedure [19]. The initiation rate was determined as $w_i = k_i$[AIBN], where k_i is the initiation rate constant, under the assumption that $k_i = 2ek_d$ (k_d is the AIBN decomposition rate constant, and e is the probability of a radical escaping to the bulk). The k_d value measured in cyclohexanol was accepted here:

$$\log k_d = 17.70 - 35/(4.575T \times 10^{-3}).$$

The probability of a radical escaping to the bulk was taken to be $e = 0.5$ [4, 20–22].

The antioxidant activity of the compounds was characterized by the apparent inhibition rate constant fk_{In}, where f is the stoichiometric inhibition coefficient [4, 20–22].

RESULTS AND DISCUSSION

All of the compounds exert a pronounced inhibition effect on the initiated oxidation of 1,4-dioxane. The typical oxygen uptake curves for 1,4-dioxane oxidation in the presence of compounds **I**, **VI**, and **VII** taken at similar concentrations are shown in Fig. 1. The initial oxidation rate of the model substrate, w_0, was derived from the slope of the initial portion of the curve for each antioxidant. The plots of w_0 versus the concentration of compounds **VI** and **VII** are shown in Fig. 2.

To determine the apparent inhibition rate constants fk_{In}, the experimental data were represented in the coordinates of Eqs. (1) and (2) [4, 20–22]:

$$F = \frac{w_0^O}{w_0} - \frac{w_0}{w_0^O} = fk_{In}[AO]/\sqrt{2k_6 w_i}, \quad (1)$$

$$\Delta[O_2] = \frac{k_2}{fk_{In}}[RH]\ln\left(1 - \frac{t}{\tau}\right), \quad (2)$$

where w_0^O and w_0 are the initial oxygen uptake rates in 1,4-dioxane oxidation in the absence and in the presence of AO, respectively; [AO] is the AO concentration; k_{In} and $2k_6$ are the rate constants of chain termination on the AO and of quadratic-law chain termination on the peroxyl radicals of the substrate, respectively [4, 20–22]; $\Delta[O_2]$ is the amount of oxygen absorbed at the moment t (Fig. 1); τ is the induction period in the oxygen uptake curves; [RH] is the concentration of 1,4-dioxane ([RH] = 11.75 mol/l); and k_2 is the rate constant of oxidation chain propagation for the model substrate ($k_2 = 7.9$ l mol^{-1} s^{-1} [4]). These values were calculated using the rate of quadratic-law chain termination known from the literature: $2k_6 = 6.67 \times 10^7$ l mol^{-1} s^{-1} [4].

The anamorphoses of the oxygen uptake curves in the coordinates of Eq. (2) for compounds **I**, **VI**, and **VII** are presented in Fig. 3. Equations (1) and (2) satisfactorily describe the antioxidant effect of all of the compounds examined (the correlation factor is above 0.96). As can be seen from the data presented in the table, the fk_{In} values calculated via formulas (1) and (2) are fairly similar. The apparent inhibition rate constants are also given in the table. The antioxidant

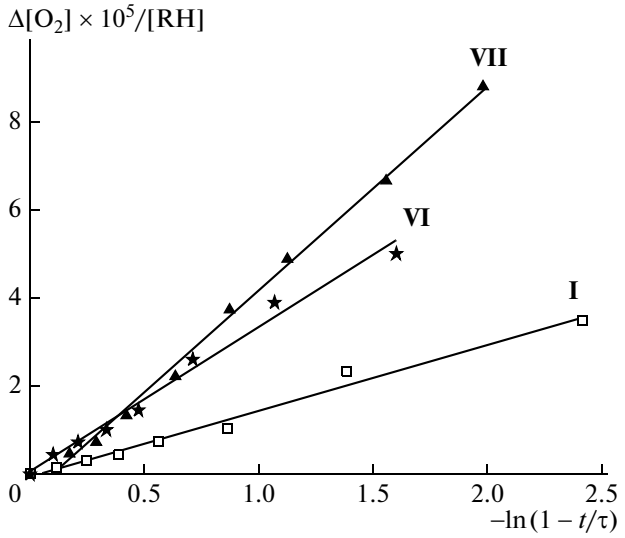


Fig. 3. Anamorphoses of the oxygen uptake curves in the coordinates of Eq. (2) for inhibited 1,4-dioxane oxidation at 348 K in the presence of compounds **I**, **VI**, and **VII**. Concentrations (mol/l): **[I]** = 7.00×10^{-5}, **[VI]** = 8.1×10^{-5}, and **[VII]** = 7.0×10^{-5}.

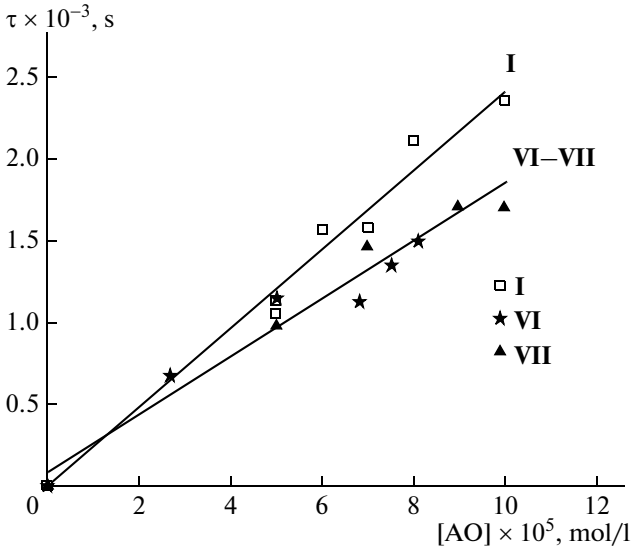


Fig. 4. Dependence of the induction period on the concentration of antioxidants **I**, **VI**, and **VII** at 348 K.

effects of the compounds can be compared in terms of the tocopherol equivalent (TE), which was calculated as the ratio of fk_{In} for a given conjugate to the same characteristic of α-tocopherol.

The rate of inhibited 1,4-dioxane oxidation as a function of the antioxidant concentration tends to a limit (Fig. 2): at concentrations of **I** and **IV**–**VII** higher than 9.0×10^{-5} mol/l, the rate of inhibited 1,4-dioxane oxidation and the consumption rate of these compounds stop to depend on their concentrations. The dependences of w_0 and w_{In} on the initial AO concentration are antibatic. This is further evidence that the main cause of inhibition is the termination of the oxidation chain due to the interaction of the peroxyl radical with the inhibitor.

By fitting the experimental w_{In}^{lim} and w_0^{lim} data for inhibited substrate oxidation in the presence of **I** and **IV**–**VII** to Eqs. (3) and (4) [4], the stoichiometric coefficient for the inhibitors was estimated at $f \approx 2$.

$$fw_i = w_0^{lim}, \quad (3)$$

$$w_i = fw_{In}^{lim}. \quad (4)$$

The same result was obtained by representing the experimental dependences in the coordinates of the equation

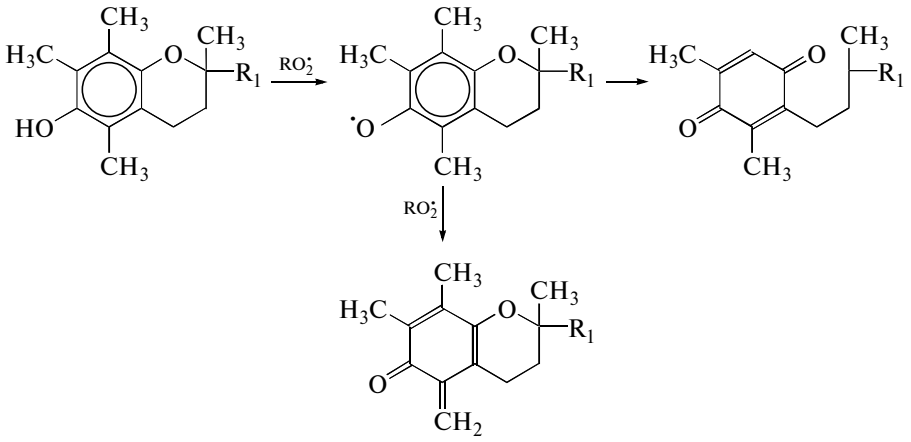
$$\tau = \frac{f[AO]}{w_i}, \quad (5)$$

where τ is the induction period in the oxygen uptake curves for AO-inhibited 1,4-dioxane oxidation. As can be seen from Fig. 4, τ shows a classical linear dependence on the AO concentration.

The above results suggest that the hybrid molecules examined here are considerably inferior in antioxidant

Quantitative characteristics of the antioxidant activity of the conjugates of betulonic and betulinic acids with the amido derivatives of 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-chromene-2-carboxylic acid at 348 K

Compound	$fk_{In} \times 10^{-4}$, l mol^{-1} s^{-1}		$k_{In} \times 10^{-4}$, l mol^{-1} s^{-1}	TE
	calculated via Eq. (1) [4]	calculated via Eq. (2) [22]		
I	20.0 ± 3.0	54 ± 5	10.0 ± 2.0	0.80
II (betulonic acid)	–	–	–	–
III (betulinic acid)	0.27 ± 0.03	–	0.27 ± 0.03	0.01
IV	5.0 ± 0.5	17 ± 0.2	2.5 ± 0.3	0.2
V	5.0 ± 0.5	20 ± 0.2	2.5 ± 0.3	0.2
VI	10.0 ± 1.0	24 ± 2	5.0 ± 0.5	0.4
VII	15.0 ± 2.0	20 ± 2	7.5 ± 1.0	0.6
VIII (α-tocopherol)	25.0 ± 4.0	26 ± 0.3	12.5 ± 2.0	1.00
IX (Trolox)	8.0 ± 1.0	10 ± 0.1	4.0 ± 0.5	0.32



Interaction of the peroxyl radicals of 1,4-dioxane with the chromane-family antioxidants.

activity to α-tocopherol (**VIII**) and are nearly as active as the well-known chromane-family antioxidant Trolox (**IX**) (table). Based on the structure of inhibitors **I** and **IV–VII**, we may assume that the mechanism of their antioxidant effect is similar to that of Trolox and α-tocopherol. The likely mechanism of the interaction of the peroxyl radicals of 1,4-dioxane with the molecules of antioxidants **I** and **IV–VII** is shown in the scheme.

Betulinic acid (**III**) also possesses weak antioxidant properties. As can be seen from the table, k_{In} for this compound is two orders of magnitude smaller than the same characteristic of α-tocopherol. Betulinic acid contains no phenol moieties and, hence, should differ in the antioxidant effect mechanism from compounds **I** and **IV–VII**. It can be assumed that the decrease in the rate of 1,4-dioxane oxidation in the presence of betulinic acid is due to the replacement of the active peroxyl radicals of 1,4-dioxane by the radical intermediates formed from this terpenoid, which are less reactive in chain propagation events. This phenomenon will be the subject of our forthcoming studies.

Thus, the antioxidant properties of the conjugates of betulonic and betulinic acids with the amido derivatives of Trolox were studied using the initiated oxidation of 1,4-dioxane as a model reaction. The apparent rate constants of inhibition of the reaction by the conjugates of betulonic and betulinic acids with the amido derivatives of Trolox were measured: at 348 K, $k_{In} = 10.0 \times 10^{-4}$, 2.5×10^{-4}, 2.5×10^{-4}, 5.0×10^{-4}, and 7.5×10^{-4} l mol^{-1} s^{-1} for compounds **I**, **IV**, **V**, **VI**, and **VII**, respectively.

It was established using kinetic spectrophotometry and manometric methods (oxygen uptake measurements) that the stoichiometric coefficient of inhibition for all of the antioxidants is 2.

The antioxidant properties of the tocopherol derivatives are mainly determined by the inhibition effect of the chromanyl moiety, and the introduction of betulinic and betulonic substituents into the molecular structure reduces the inhibition effect.

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