Xymedon conjugate with biogenic acids. Antioxidant properties of a conjugate of Xymedon with L-ascorbic acid *

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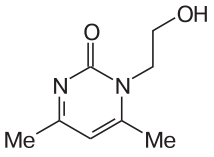
Antiradical activity and antioxidant properties of a conjugate of Xymedon with L-ascorbic acid were studied. In contrast to Xymedon, a conjugate of Xymedon with L-ascorbic acid was found to react with free radicals. Pro-oxidant activity of the conjugate of Xymedon with L-ascorbic acid in a chemiluminescence system is weaker as compared to individual L-ascorbic acid. This is the evidence of the increase in the stability of ascorbic acid upon conjugation with a molecule of Xymedon. Conjugate of Xymedon with L-ascorbic acid facilitates the decrease in the concentration of a lipid peroxidation product (malondialdehyde) and the activity of superoxide dismutase in liver and serum of laboratory animals exposed to intoxication with a known hepatotropic toxin CCl_4. This observation shows the contribution of the antioxidant action to the hepatoprotective effect of the studied Xymedon derivative.

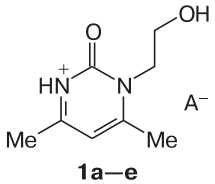
Key words: pyrimidines, antiradical activity, toxic liver injury, ascorbic acid, antioxidant activity.

Pyrimidine derivatives attract attention due to their ability to stimulate tissue regeneration. Among them, Xymedon (1-(2-hydroxyethyl)-4,6-dimethyl-1,2-dihydropyrimidine-2-one) is a known medicine with considerable regenerative and reparative properties,[1] which is successfully used in clinical practice for treatment of burn wounds and other pathological states, as well as in the post-operative period.[2—5] In our earlier studies,[6,7] we revealed hepatoprotective activity of Xymedon, and, in particular, the decrease in pathomorphological changes in liver and normalization of biochemical markers of liver disease.

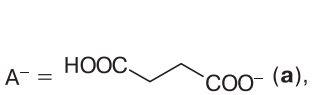


Xymedon

In recent publications,[8—10] chemical modification of therapeutic drugs, and conjugation in particular, is regarded as a way to increase their bioavailability and efficiency. Earlier, in our publication[11] we reported the synthesis of Xymedon complexes **1a—e** with biogenic acids, namely, succinic, ascorbic, *p*-aminobenzoic, nicotinic, and L-2-amino-4-(methylthio)butanoic (L-methionine) acids.



1a—e



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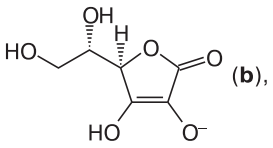

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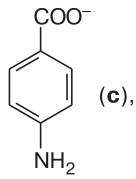

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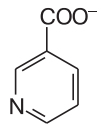

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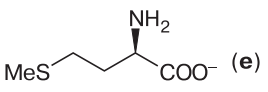

(**e**)

These biogenic acids were chosen for preparation of Xymedon complexes **1a—e** since they are common me-

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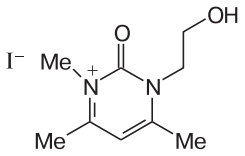
tabolites, which serve a number of useful biological functions. Some of them are used in combination therapy of liver diseases and for detoxification therapy. For example, a common Russian drug Remaxol,[12] which comprises succinic acid, nicotinamide, methionine, and inosine, shows antioxidant, antihypoxant, and hepatotropic activities. Hepatoprotective properties of *p*-aminobenzoic acid, which is one of the vitamins belonging to the class of B complex vitamins (vitamin B_{10}), are still waiting to be explored; however, this compound is necessary for the synthesis of folic acid, it plays an important role in metabolism, cell protection, and it is also a fragment of molecules of pharmacologically active compounds.[13]

In the work,[11] the data are presented on the primary evaluation of the hepatoprotective and antitoxic activities of Xymedon conjugates with biogenic acids **1a**—**e** under conditions of intoxication of liver with CCl_4, and specific features of their biological activities are considered in relationship with their composition. The set of the test compounds was extended with 3-(2-hydroxyethyl)-1,4,6-trimethyl-2-oxo-2,3-dihydropyrimidinium iodide (**1f**),[14] which was obtained by alkylation of the nitrogen atom in pyrimidine cycle of Xymedon with iodomethane.



1f

In accordance with the obtained data,[11] Xymedon derivatives **1a**—**e** enhance viability of laboratory animals (rats) under conditions of the toxic damage with hepatotropic toxin CCl_4 and improve their clinical state, in particular, the weight loss is decreased, and the hypothermia is milder. Histological analysis of liver sections showed that administration of Xymedon conjugate with L-ascorbic acid (compound **1b**) at the dose of 20 mg kg^{-1} resulted in the least liver patterns with necrosis and steatosis (15.9±2.2%) as compared to other studied groups. The area of damaged tissue in controls was evaluated as 36.3±2.7%, upon administration of Xymedon at the dose of 20 mg kg^{-1} it was 19.1±5.1%, for compound **1a** at the dose of 3 mg kg^{-1} it was 22.1±2.5%, for compound **1c** at the dose of 6 mg kg^{-1} it was 31.4±7.1%, for compound **1d** at the dose of 6 mg kg^{-1} it was 24.0±3.2%, for compound **1e** at the dose of 20 mg kg^{-1} it was 38.1±2.3%, and for compound **1f** at the dose of 6 mg kg^{-1} it was 35.9±6.8%. The doses for all test compounds were calculated based on the ratio 1/300 of LD_{50}. Statistically significant decrease in the area of injured liver was observed under administration of Xymedon and its derivatives **1a**,**b**,**d**. Besides, the efficiency of test compounds was evaluated using 20 biochemical markers of toxic damage of liver and other organs. Thus, we observed statistically significant decrease in concentration of bilirubin and elevation of concentration of total serum protein under administration of conjugates **1a**,**c**; besides, triglyceride level was normalized under administration of compound **1b**.

Xymedon conjugate with L-ascorbic acid **1b** was found to enhance the adaptive reserves of the organism exposed to the stress conditions of elevated physical activity in an exhaustive swimming test,[15,16] and it also demonstrated considerable hepatoprotective activity.[17] Compound **1b** and conjugate of Xymedon with *p*-aminobenzoic acid (**1c**) were found to stimulate regeneration of the nerve tissue in the spinal cord after contusion injury.[18,19]

Since one of the routes of induction of liver cell damage under intoxication with CCl_4 includes the increase in concentration of free radicals, malfunction of endogenous antioxidant defense system, and intensification of lipid peroxidation,[20] the aim of the present work was to study the antiradical and antioxidant properties of compound **1b**, which was found to restore the liver morphology most efficiently.

Results and Discussion

Antiradical properties of Xymedon and its conjugate with L-ascorbic acid (1b). Antiradical properties of compound **1b** were investigated with respect to Xymedon, L-ascorbic acid, and their mixture. Figures 1—3 show the results of the study of antiradical properties of test compounds.

The data presented in Fig. 1 evidence that addition of 10 μL of 100 m*M* solution of Xymedon to the luminol—2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) chemiluminescent system exerted no changes on the chemiluminescence intensity. Noteworthy, the con-

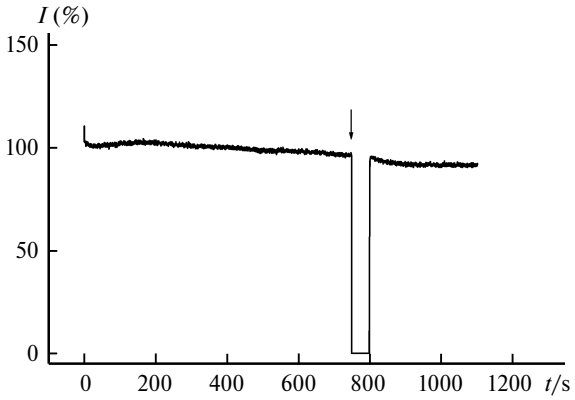


Fig. 1. Effect of Xymedon on the intensity of chemiluminescence of luminol—AAPH system in a buffer (pH 8.6) (see Experimental). The moment of adding of Xymedon is indicated by an arrow. Addition of Xymedon induces the decrease in the intensity line to 0 followed by the abrupt regeneration of the line almost to the initial level. This type of reaction of the system to the addition of Xymedon shows the absence of its reaction with radicals. Chemiluminescence intensity (*I*) is given as % of the basic level.

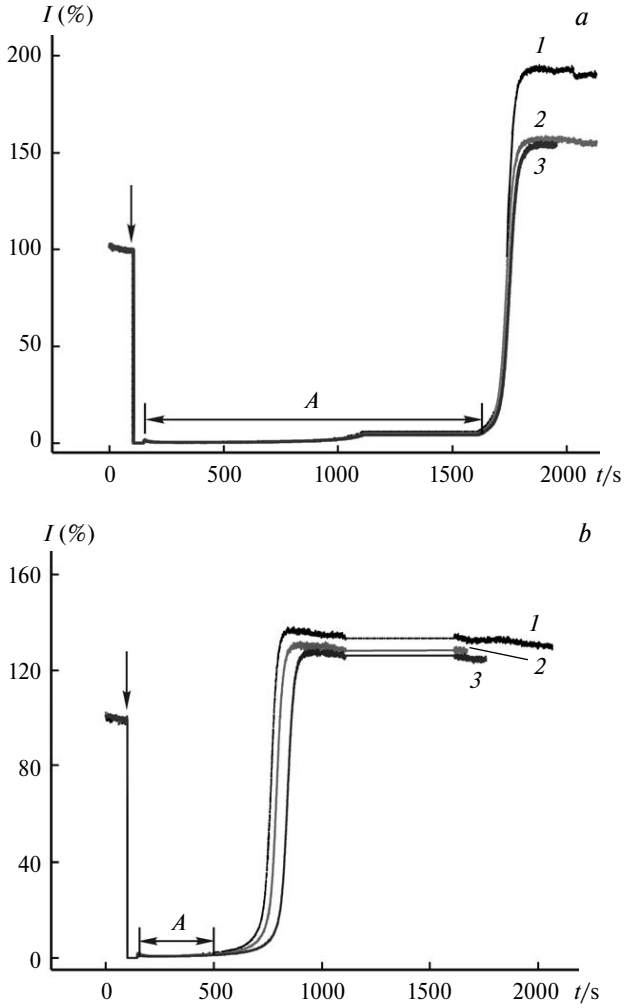


Fig. 2. Effect of L-ascorbic acid (*1*), derivative **1b** (*2*), and equimolar Xymedon—L-ascorbic acid mixture (*3*) on the chemiluminescence in luminol—AAPH system. Concentrations of the added compounds were 0.5 (*a*) and 0.25 mmol L^{-1} (*b*) (see Experimental). The moment of adding of the solutions of the test compounds is indicated by the arrows. Addition of solutions induces the decrease in the intensity line to 0 followed by a latent period (line segment *A*; reaction of the compound with radicals) and regeneration to the basic level (the reaction of the compound with radicals ceased). The increase above the basic level (see Fig. 2, *a*, curve *1*) is characteristic of the pro-oxidant action. Chemiluminescence intensity (*I*) is given as % of the basic level.

centration of the added Xymedon solution was more than 100 higher than the concentration of the antioxidant (ascorbic acid, 0.25—1.00 M*M*), which considerably decreased chemiluminescence intensity upon the addition to the chemiluminescent system (Fig. 2). In conclusion, Xymedon did not interact with free radicals.

The study of L-ascorbic acid, derivative **1b**, and equimolar mixture of Xymedon with L-ascorbic acid revealed the pronounced antiradical activity. The curves of chemiluminescence intensity were different for different concentrations of the examined solutions. Upon addition of 10 μL of 1 M*M* solution of compound **1b** or L-ascorbic acid to luminol—AAPH chemiluminescent system, chemiluminescence intensity dropped to zero, and this effect was observed for both compounds. According to the work,[21] addition of antioxidant to the luminol—AAPH assay system resulted in either partial decrease in the signal intensity or its drop to zero. The rationale is that the radicals are involved in the reaction with an antioxidant. The time interval when the signal intensity is close to zero is called a latent period (see Fig. 2).

At the moment, when antioxidant stops reacting with radicals, radicals start to react with luminol, and the intensity of chemiluminescence increases. In the case the intensity rises higher than the initial level, the assumption about its pro-oxidant properties can be made. Upon addition of 10 μL of 1 M*M* solutions of L-ascorbic acid and compound **1b** to a luminol—AAPH system, the difference was observed both in the duration of the latent period and the maximum chemiluminescence intensity. For example, after addition of 1 M*M* solution of L-ascorbic acid the duration of the latent period was 35 min (Fig. 3), and the following maximum chemiluminescence level was estimated to be 3.2 times higher than the starting level. After the addition of 10 μL of 1 M*M* solution of compound **1b** the latent period was as long as 26 min, and the following maximum chemiluminescence intensity exceeded the basic level by 1.4 times. It can be concluded that L-ascorbic acid showed somewhat stronger antioxidant activity than Xymedon derivative **1b**, as in the case of L-ascorbic acid the duration of the reaction with free radicals was longer. However, the chemiluminescence intensity enhancement in the luminol—AAPH system upon addition

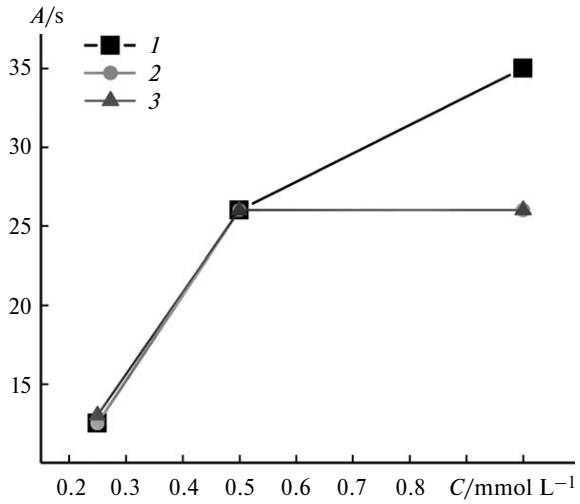


Fig. 3. Plot of the duration of the latent period (*A*) of chemiluminescence intensity *vs* concentration (*C*) of L-ascorbic acid (*1*), derivative **1b** (*2*), and components of equimolar Xymedon—L-ascorbic acid mixture (*3*) (see Experimental).

of L-ascorbic acid was more pronounced as compared to compound **1b** or a Xymedon—L-ascorbic acid mixture. The obtained results indicate pro-oxidant activity is caused by products of L-ascorbic acid degradation. Note that pro-oxidant properties of pure L-ascorbic acid were more pronounced than that of **1b** and Xymedon—L-ascorbic acid mixture.

Antiradical activities of L-ascorbic acid, derivative **1b**, and Xymedon—L-ascorbic acid mixture were studied in detail at concentrations of 0.5 and 0.25 mmol L^{-1} (see Fig. 2). Curves of chemiluminescence intensity for compound **1b** and Xymedon—L-ascorbic acid mixture at both concentrations were identical, and they differed from those plotted for L-ascorbic acid. At concentration of 0.5 mmol L^{-1}, the latent periods for three test compounds were similar and lasted for 26 min (see Fig. 3). Maximum chemiluminescence intensity observed after the latent period in the case of L-ascorbic acid was twice as high as the basic level, and for compound **1b** and Xymedon—L-ascorbic acid mixture it exceeded the basic level by 50% (see Fig. 2, *a*). Hence, compound **1b** and Xymedon—L-ascorbic acid mixture showed antioxidant properties comparable to those of ascorbic acid; however, the increase in chemiluminescence intensity registered after the latent period, which is indicative of pro-oxidant action, was less pronounced upon addition of compound **1b** and Xymedon—L-ascorbic acid mixture as compared to L-ascorbic acid.

At concentration of 0.25 mmol L^{-1}, the latent periods in all three cases were also similar and lasted for 12.5—13 min (see Figs. 2, *b* and 3), and for Xymedon—L-ascorbic acid mixture the trend to elongation of the latent period was observed in contrast to pure L-ascorbic acid and compound **1b**. Upon addition of L-ascorbic acid at concentration of 0.25 mmol L^{-1}, the elevation of the chemiluminescence intensity over the basic level was less pronounced than at higher concentrations and reached 36%. For compound **1b** and Xymedon—L-ascorbic acid mixture, elevation of the chemiluminescence level over the basic value was as high as 28—30%. Consequently, at the smallest concentration, L-ascorbic acid, compound **1b**, and Xymedon—L-ascorbic acid mixture showed similar reactivity towards free radicals.

The effect of Xymedon and its conjugate with ascorbic acid 1b on antioxidant system parameters of the organism. We studied the effect of Xymedon and its derivative **1b** on parameters of the oxidant protection system of the body, in particular, the concentration of malondialdehyde (MDA) that helps to estimate the rate of lipid peroxidation (LP) in the organism, and the activity of the superoxide dismutase (SOD), which is produced in the body and plays a key role in defense of living cells from the damage caused by superoxide radical ($O_2^{\bullet}$). Malondialdehyde concentration and SOD activity were measured in blood serum and liver homogenates after intoxication with CCl_4, which is a hepatotropic toxin, followed by a 5-day treatment course with test compounds. Measurement of blood serum values allows estimating systemic changes in the parameters of antioxidant defense, and analysis of liver homogenates helps to evaluate the changes occurring in the liver as the target organ. The obtained data are shown in Figs 4 and 5.

After intoxication with CCl_4, the level of MDA in the blood serum of rats in the control group increased and attained maximum values 6 days after administration of CCl_4 (Fig. 4, *a*). In liver homogenates, MDA level was also elevated, however, this result was not statistically significant. In contrast to the control, MDA value in the serum of the group, which received Xymedon and com-

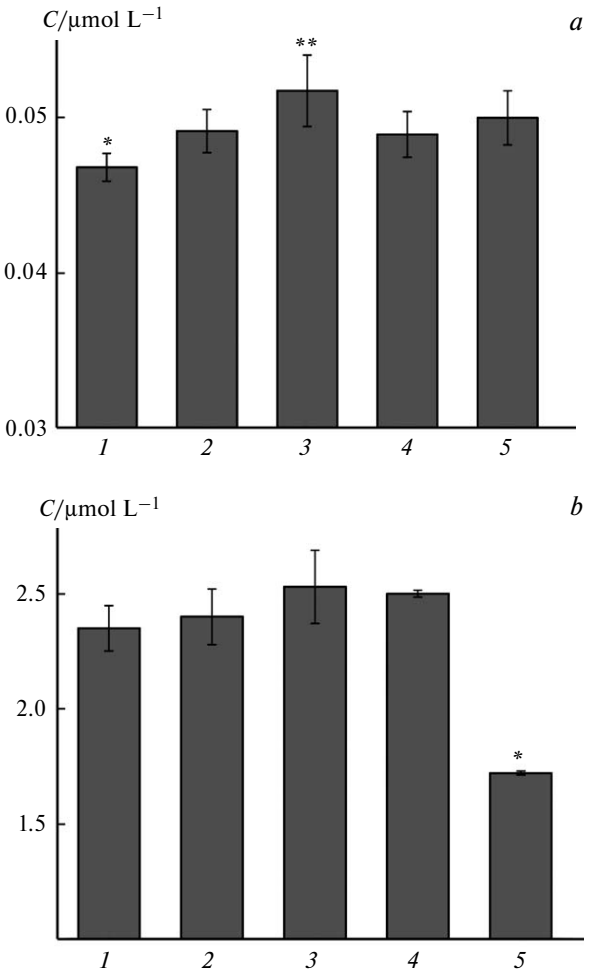


Fig. 4. Concentration of malondialdehyde (*C*) in blood serum (*a*) and liver homogenates (*b*): *1*, the intact control group (no compounds administered); *2*, the measurement was carried out 24 h after the last portion of CCl_4 was administered; *3*, the control group; *4*, the group of the animals, which received Xymedon; *5*, the group of the animals, which received compound **1b** (see Experimental).

* Statistically significant difference with the control group at $p < 0.05$.

** Statistically significant difference between the control group and the group of the intact control.

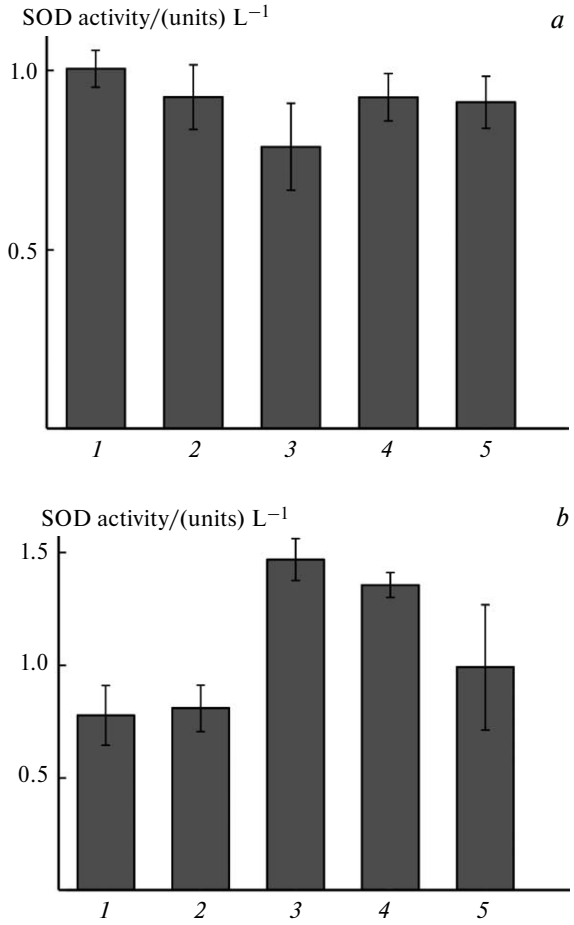


Fig. 5. Superoxide dismutase activity in the blood serum (*a*) and in rat liver homogenates (*b*): *1*, intact control group (no compounds administered); *2*, the measurement was carried out 24 h after the last portion of CCl_4 was administered; *3*, the control group; *4*, the group of the animals, which received Xymedon; *5*, the group of the animals, which received compound **1b** (see Experimental).

pound **1b**, slightly changed in comparison to the intact control. In liver homogenates of animals administered with Xymedon, MDA level was similar to the corresponding value for the control. In the group administered with compound **1b**, MDA concentration was significantly lower (the difference from the control group is statistically significant at $p < 0.05$). The obtained results show that test pyrimidine derivatives slow down the rate of lipid peroxidation. It is notable that the conjugate of Xymedon with L-ascorbic acid **1b** demonstrated a more pronounced effect.

As a consequence of intoxication with CCl_4, SOD activity in the liver increased almost twice, however, this parameter tended to decrease in the blood serum (Fig. 5). This phenomenon is associated with adaptation processes in the organism in response to enhancement of LP and elevated concentration of free radicals.

Superoxide dismutase activity in groups, which received Xymedon, and compound **1b**, was similar to that found for the intact control group (see Fig. 5, *a*). In liver homogenates of the animals administered with Xymedon, SOD activity was similar to this value in the control group. In the group, which received compound **1b**, SOD activity was similar to this parameter found for intact control group (see Fig. 5, *b*). The decrease in SOD activity in liver homogenates found in animals, which received compound **1b**, to the level of the intact control group is due to antioxidant activity of this compound, which helped to decrease the concentration of free radicals, and consequently, attenuated expression of proteins of liver antioxidant defense system.

In conclusion, in this work, antiradical and antioxidant properties of Xymedon and its conjugate with L-ascorbic acid were studied. The study of antioxidant activity showed that, in contrast to Xymedon, conjugate of Xymedon with L-ascorbic acid is able to react with free radicals similar to that of L-ascorbic acid. In the case of the derivative of Xymedon with L-ascorbic acid, we observed weaker prooxidant properties of products of conversion of ascorbic acid, which are formed in the chemiluminescent system, as compared to individual L-ascorbic acid. This phenomenon indicates stabilization of ascorbic acid upon conjugation with a molecule of Xymedon. Administration of Xymedon—L-ascorbic acid conjugate in rats resulted in the suppression of the formation of LP product (malondialdehyde) in liver. This is the evidence of the role of antioxidant action in the hepatoprotective effect of this compound.

Experimental

1-(2-Hydroxyethyl)-4,6-dimethyl-1,2-dihydropyrimidine-2-one (Xymedon) was synthesized from 4,6-dimethyl-1,2-dihydropyrimidine-2-one and 2-chloroethanol following the described procedure.[22] Synthesis of conjugate **1b** was described earlier.[11]

Determination of antiradical activity of compounds using chemiluminescence method. Antiradical properties of compounds were examined using a known[21,23] chemiluminescence method adapted to Lum-100 chemiluminescence analyzer (DISoft, Russia). The data were processed on a PC using PowerGraph (http://www.powergraph.ru) and Origin Lab software.

Luminol (Alfa Aesar) solution (1 mmol L^{-1}) was prepared using a described procedure[23] by dissolution of luminol in aqueous 0.1 *M* NaOH. Immediately before the analysis, the stock solution of luminol was diluted 4-fold with distilled water.

Chemiluminescence analysis was carried out as follows. Thermostated at 30 °C cell of Lum-100 apparatus was charged with 1 mL of the reaction mixture containing 400 μL of 250 μ*M* luminol solution, 500 μL of 0.5 *M* of Tris buffer (Fisher Chemical), pH 8.6, and 100 μL of 40 м*M* aqueous AAPH solution (Acros Organics). The basic chemiluminescence signal was measured for 10 min, then the cell was opened and 10 μL of the solution of the test compound was added, and the chemiluminescence signal was acquired for 20—30 min. Xymedon was examined as a 100 mmol L^{-1} solution, compound **1b** and L-ascorbic acid were examined as 1, 0.5, and 0.25 mmol L^{-1}

solutions. At first, stock solutions with higher concentrations were prepared, and the analytes were prepared by dilution of stock solutions with distilled water. Xymedon—L-ascorbic acid mixture was prepared immediately before the experiment. Xymedon and L-ascorbic acid were used in equimolar amounts. At first, solutions of test compounds with higher concentration were prepared, which were then diluted to concentrations of 1, 0.5, and 0.25 mmol L^{-1} of the tested component with distilled water.

The obtained results were given in % of the initial basic chemiluminescence level, which was taken as 100%.

Determination of antioxidant activity. In order to evaluate the effect of Xymedon and its derivative **1b** on the antioxidant defense system of the organism, MDA concentration and SOD activity were assessed in the blood serum and liver homogenates of rats.

Preparation of biological samples. The experiments were conducted on male Sprague Dawley rats of 6 months old obtained from the Center for Laboratory Animal Breeding (Branch of the Shemyakin—Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Pushchino). The indicators of antioxidant defense system of the organism, namely, MDA concentration and SOD activity, were assessed in blood sera and liver homogenates of laboratory animals, involved in the following experiment. Intact group did not receive any medications, was not subjected to any manipulations and was used as an intact control. Other animals were exposed to intoxication with CCl_4 over 5 days (once daily) by oral administration of oil 35% CCl_4 solution.[24] Subsequently, the animals were randomized into three groups 4 rats in each. Then over a period of 5 days, the control group received 0.1 mL of normal saline per 100 g of live weight intraperitoneally, animals in experimental groups received solutions of compound **1b** at a dose of 0.5 mg kg^{-1}, and Xymedon at a dose of 0.24 mg kg^{-1}, which is equimolar to the dose of **1b**, *via* the same delivery route. Before administration, the tested compounds were dissolved in a sterile normal saline for injections in such a way, that the volume of injection was 0.1 mL/100 g of live weight.

The animals were euthanized by exsanguination *via* the external carotid artery under anesthesia with chloral hydrate. The blood samples were taken during euthanasia, and the liver samples were taken after euthanasia. Sera were prepared by a double centrifugation of the whole blood at 4 °C. Prior to the examination, the sera were stored at −25 °C. Liver samples were frozen in liquid nitrogen and stored at −80 °C. Liver homogenates were prepared as follows. Frozen pieces of the tissue were placed into 2 mL microcentrifuge tubes, 0.1 *M* phosphate buffer (pH 7.8) was added in ratio 1 : 200, a stainless steel bead was added to each tube, and microtubes were shaken for 5 min at medium speed using a TissueLyser LT bead mill (Qiagen, Germany). Subsequently, the samples were centrifuged at 4 °C at 3000 rpm and supernatant was drawn for examination.

Malondialdehyde concentration was assessed using a known procedure[25] adapted to BioTek Epoch microplate reader. This method is based on the formation of colored adduct of MDA and thiobarbituric acid. A sample of serum (15μL) or homogenate was diluted with 200 μL of distilled water, 100 μL of aqueous 17% trichloroacetic acid and 100 μL of aqueous 0.8% thiobarbituric acid were added. The sample was kept at 100 °C for 10 min, sedimented protein was removed by 10 min centrifugation at 3000 rpm. The absorbance was registered at λ = 540 nm. Malondialdehyde concentration (*C*) was calculated using the equation

$$C\ (\mu\text{mol L}^{-1}) = \frac{0.415A \cdot 10^6}{0.150\varepsilon},$$

where *A* is absorbance at λ = 540 nm, 0.415 is the volume of aqueous phase (mL), 0.150 is the volume of the homogenated sample (mL), 10^6 is a coefficient for transformation of mol L^{-1} into μmol L^{-1}, ε is a coefficient of molar extinction, $156 \cdot 10^5$ L mol^{-1} cm^{-1}.

Superoxide dismutase activity was assesses using an indirect assay. Sensitive and specific spectrophotometric assay for SOD is based on inhibition of quercetin autooxidation in the presence of SOD.[25] This method was adapted to a BioTek Epoch microlplate reader.

The experimental samples were prepared as follows. A microcentrifuge tube was charged with 50 μL of 0.1 *M* phosphate buffer (pH 7.8), 50 μL of 5.0 м*M* of TMEDA solution (prepared by dissolution of TMEDA in 0.5 *M* EDTA solution) and 10 μL of liver homogenate or blood serum were placed. The mixture was transferred to a microplate well, 10 μL of 0.5 *M* quercetin solution in DMSO was added, and absorption was measured at λ = 406 nm immediately after the introduction of quercetin and 20 min later, and the difference (ΔA_{exp}) between these two values was calculated.

The control sample was prepared similarly with only exclusion that 0.9% aqueous solution of NaCl (10 μL) was used instead of liver homogenate. The absorption $\Delta A_{control}$ was measured as described for experimental samples.

The percentage inhibition of quercetin autooxidation was calculated using the following equation:

$$c(\text{units}) = \frac{\Delta A_{exp}}{\Delta A_{control}}.$$

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