Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ
(PHARMACY & PHARMACOLOGY)

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2026-14-2-175-188

Prediction, *in silico* antioxidant activity, and targeted synthesis of sterically hindered phenol azomethine derivatives

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Received 27 June 2025 | After peer review 09 Jan 2026 | Accepted 15 March 2026

Molecular design and synthesis of a new series of biologically active azomethines containing a sterically hindered phenolic fragment were carried out. Within the scope of the study, 8 compounds were synthesized, and their antioxidant activity was evaluated under *in vitro* conditions. To establish the mechanism of action, molecular docking was used to model the interaction of the synthesized ligands with the active site of glutathione peroxidase-4 (GPx-4). The conducted analysis revealed key structural features determining antioxidant efficacy and established a correlation between molecular structure and biological activity.

The aim. Synthesis, computer screening, and investigation of the antioxidant properties of new azomethines based on sterically hindered phenol, as well as establishing structure–activity relationships.

Materials and methods. A new series of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(N-phenyl)-azomethine]phenols was synthesized by the condensation of corresponding ketones with aromatic amines in the presence of catalytic amounts of p-toluenesulfonic acid. The structure and purity of the obtained compounds were confirmed by a complex of physicochemical methods, including IR spectroscopy, H NMR spectroscopy, and elemental analysis. For the initial assessment of the biological potency of the synthesized compounds, computer prediction (*in silico*) of their antioxidant, antiradical, and cardiotonic properties was performed using the online platform PASS Online. Molecular modeling of potential inhibitory activity against human glutathione peroxidase-4 (GPx-4) was carried out using the Autodock 4.0 program. The conformational mobility of the ligands was taken into account, for which optimal torsion angles were previously determined and set. Experimental study of antioxidant activity (AOA) was conducted in two model systems: induction of lipid peroxidation (LPO) in a complex of corn oil fatty acids under UV irradiation; and the Fenton system (H_2O_2/Fe^{2+}). To compare efficacy, ubiquinone and bottled hydroxytoluene (BHT, the active substance of the drug dibulin), representing the class of sterically hindered phenols, were used as reference standards.

Results. The spectrum of biological activity of the studied compounds was predicted *in silico* using the PASS Online service. As it was expected, all substances have cardiotonic, membrane-stimulating, and antioxidant potential. The presence of AOA and the ability to scavenge free radicals allows these molecules to be classified as antiradical agents. Experimental verification of AOA was carried out in two model systems: based on photooxidation (UV irradiation) of a complex of fatty acids from corn oil (system No. 1) and on the Fenton system (H_2O_2/Fe^{2+}, system No. 2). In all the cases, the studied compounds demonstrated high efficacy, inhibiting lipid peroxidation LPO by 42–48%. This result significantly exceeds the activity of standard antioxidants — ubiquinone (11%) and BHT (39%) — in the same conditions.

Conclusion. The results of molecular docking indicate a high affinity of the new ligands to the GP-4 protein, with the calculated binding energy for the most promising structures being comparable to that of known standards—ubiquinone, dibulin (hydroxybutylated toluene), and mexidol. *In vitro* experimental data confirmed the pronounced antioxidant activity of the synthesized compounds. “Lead” structures were identified that surpass classical antioxidants—ubiquinone and dibulin — in efficacy.

Keywords: azomethines; azomethine phenols; sterically hindered phenols; antioxidant activity; lipid peroxidation; PASS Online

Abbreviations: SHPs — sterically hindered phenols; TLC — thin-layer chromatography; LPO — lipid peroxidation; AOA — antioxidant activity; UV irradiation — ultraviolet irradiation; PhAs — phenolic antioxidants; WSOM — water, alcohol and oil mixture; TCA — trichloroacetic acid; Pa — probability of activity manifestation.

For citation: O. Tsakulova, I.P. Kodonidi, A.S. Chiriapkin, F.N. Bidarova, M.T. Kisieva, L.A. Usmanova. Prediction, *in silico* antioxidant activity, and targeted synthesis of sterically hindered phenol azomethine derivatives. *Pharmacy & Pharmacology.* 2026;14(2):175-188. **DOI:** 10.19163/2307-9266-2026-14-2-175-188

Для цитирования: Т.В. Цакулова, И.П. Кодониди, А.С. Чиряпкин, Ф.Н. Бидарова, М.Т. Кисиева, Л.А. Усманова. Прогноз, антиоксидантная активность *in silico* и целенаправленный синтез азометиновых производных пространственно-затруднённого фенола. *Фармация и фармакология.* 2026;14(2):175-188. **DOI**: 10.19163/2307-9266-2026-14-2-175-188

RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY
(ФАРМАЦИЯ И ФАРМАКОЛОГИЯ)

Прогноз, антиоксидантная активность *in silico* и целенаправленный синтез азометиновых производных пространственно-затруднённого фенола

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Получена 27.06.2025 После рецензирования 09.01.2026 Принята к печати 15.03.2026

Проведено молекулярное конструирование и синтез нового ряда биологически активных азометинов, содержащих пространственно-затруднённый фенольный фрагмент. В рамках исследования 8 соединений, для которых оценена антиоксидантная активность в условиях *in vitro*. Для установления механизма действия методом молекулярного докинга выполнено моделирование взаимодействия синтезированных лигандов с активным центром глутатионпероксидазы-4 (GPx-4). Проведённый анализ позволил выявить ключевые структурные особенности, определяющие антиоксидантную эффективность, и установить корреляционную связь между строением молекул и их биологической активностью.

Цель. Синтез, компьютерный скрининг и исследование антиоксидантных свойств новых азометинов на основе пространственно-затруднённого фенола, а также установление корреляций «структура–активность».

Материалы и методы. Методом конденсации соответствующих кетонов с ароматическими аминами в присутствии каталитических количеств *п*-толуолсульфокислоты был осуществлен синтез нового ряда 2,6-*ди*-*трет*-бутил-4-[С-алкил-(арил)-(N-фенил)-азометино]фенолов. Структура и чистота полученных соединений подтверждены комплексом физико-химических методов, включая ИК-спектроскопию, ^{1}Н-ЯМР-спектроскопию и элементный анализ. Для первичной оценки биологической потенции синтезированных соединений проведено компьютерное прогнозирование (*in silico*) их антиоксидантных, антирадикальных и кардиотонических свойств с использованием онлайн-платформы PASS Online. Молекулярное моделирование потенциальной ингибирующей активности в отношении глутатионпероксидазы-4 (GPx-4) человека выполнялось в программе Autodock 4.0. При этом учитывалась конформационная подвижность лигандов, для которых были предварительно определены и заданы оптимальные торсионные углы. Экспериментальное изучение антиоксидантной активности (АОА) проводилось в двух модельных системах: индуцирование перекисного окисления липидов (ПОЛ) в комплексе жирных кислот кукурузного масла под действием УФ-облучения; система Фентона (H_2O_2/Fe^{2+}). Для сопоставления эффективности в качестве референтных стандартов были использованы убихинон и бутилированный гидрокситолуол (БГТ, действующее вещество препарата дибулин), представляющий класс экранированных фенолов.

Результаты. Спектр биологической активности исследованных соединений предсказан *in silico* с помощью сервиса PASS Online. Согласно прогнозу, все вещества обладают кардиотоническим, мембраностимулирующим и антиоксидантным потенциалом. Наличие АОА и способности захватывать свободные радикалы позволяет отнести данные молекулы к классу антирадикальных агентов. Экспериментальная проверка АОА была проведена в двух модельных системах: на основе фотоокисления (УФ-облучение) комплекса жирных кислот кукурузного масла (система № 1) и на системе Фентона (H_2O_2/Fe^{2+}, система № 2). Во всех случаях исследуемые соединения продемонстрировали высокую эффективность, ингибируя ПОЛ на 42–48%. Данный результат существенно превышает активность стандартных антиоксидантов — убихинона (11%) и БГТ (39%) — в аналогичных условиях.

Заключение. Результаты молекулярного докинга свидетельствуют о высоком сродстве новых лигандов к белку ГП-4, причем расчётная энергия связывания для наиболее перспективных структур сопоставима с таковой для известных эталонов — убихинона, дибулина (гидроксибутилированного толуола) и мексидола. Экспериментальные данные *in vitro* подтвердили выраженную антиоксидантную активность синтезированных соединений. Выделены «лидерные» структуры, превосходящие по эффективности классические антиоксиданты — убихинон и дибулин.

Ключевые слова: азометины; азометинофенолы; пространственно-затруднённые фенолы; антиоксидантная активность; перекисное окисление липидов; PASS Online

Список сокращений: ПЗФ — пространственно-затруднённые фенолы; ТСХ — тонкослойная хроматография; ПОЛ — перекисное окисление липидов; АОА — антиоксидантная активность; УФО — ультрафиолетовое облучение; ФАО — фенольные антиоксиданты; ВСМ — водно-спиртово-масляная смесь; ТХУК — трихлоруксусная кислота; Ра — вероятность проявления активности.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2026-14-2-175-188

INTRODUCTION

The modern view on the role of lipid peroxidation (LPO) indicates that an imbalance in this process is a key pathogenetic link in numerous pathologies, especially cardiovascular diseases. In particular, recent studies have led to the concept that LPO significantly contributes to the development of venous thrombosis and thromboembolism in patients with heart failure. This is based on oxidative stress, characterized by excessive generation of reactive oxygen species, which ultimately increases the thrombogenic potential of blood [1–3].

In the context of developing new drugs, one of the current strategies in pharmaceutical science is the creation of molecules with combined action, capable of simultaneously affecting multiple biological targets. Such polypharmacological effects are achieved through the rational design of "hybrid" main compounds and the pharmacophore fragments of so-called "privileged molecules" are integrated into their structure [4–6]. Azomethines, considered as a basis for antiplatelet drugs targeting thrombosis [7–9], are a promising chemical class for such design. The synergistic effect, combining antioxidant and antiradical action, can be enhanced by incorporating a free radical scavenger—a structural element of a sterically hindered phenol (SHP)—into the azomethine molecular scaffold [10, 11].

Among polyfunctional phenolic antioxidants (PhAs), derivatives of 4-methyl-2,6-diisobornylphenol, which exhibit antiplatelet and antithrombotic activity [12], are the most thoroughly studied and used. However, the range of such polyfunctional compounds on the market is extremely limited, which is due to the following main problems:

The multi-step nature of known synthetic compound preparation methodologies, economic costs, and poor adaptability for industrial scaling.

The lack of systematized knowledge and data on the relationship between the chemical structure of polyfunctional PhAs and their antioxidant efficacy ("structure–activity") hinders the targeted synthesis of compounds superior to existing analogs.

Thus, up-to-date task is the targeted search and study of new highly effective and safe compounds that combine the properties of antioxidants, antiradical, and antithrombotic agents. A promising direction in this field is the investigation of a series of azomethines containing a sterically hindered phenol fragment, followed by an assessment of their antioxidant potential *in vitro.*

THE AIM. Synthesis of new sterically hindered phenol azomethine derivatives, *in silico* prediction of compounds with optimal pharmacokinetic parameters, *in vitro* study of antioxidant activity, and identification of structure-activity relationship patterns.

MATERIALS AND METHODS

Prediction

The structural formulas of the modeled compounds were constructed using BIOVIA Draw 17.2. Computer analysis of the biological activity of virtual compounds was performed using the PASS program, based on the analysis of structural descriptors of multilevel atomic neighborhoods of known substances. The obtained results were presented as a list of biological activities with calculated probabilities of activity manifestation (Pa) for each constructed substance [13].

Molecular docking was performed using the freely distributed program Autodock 4.0 [14]. Molecular modeling was carried out considering the conformational mobility of ligands, whose torsion angles were set and defined in this program. The charges of all atoms in the modeled system were calculated using the Gasteiger algorithm. The program was set to search for 200 energetically favorable conformations of molecular complex formation between the studied compounds and the protein target using the Lamarckian genetic algorithm scoring function for calculating interaction energy (Lamarckian GA 4.2). The grid spacing was 0.364 Å. A three-dimensional model of the enzyme for computational experiments was selected from the RCSB Protein Data Bank (www.rcsb.org) [15].

Virtual structures of the modeled compounds were built in HyperChem 8.0.4 and then geometrically optimized using the AbInitio method with the STO-3G basis set [16].

The conversion of the .hin format to .pdb, required for molecular modeling, was performed in Open Babel 2.4.1 [17].

As a target for predicting the inhibitory activity of compounds against glutathione peroxidase-4 (GP-4), a virtual model of the human enzyme with identification number 6HKQ was used. This structure contains an inhibitor of this enzyme—ML162 ((2S)-2-[2-chloroanilino(3-chloro-4-methoxyphenyl)amino]-*N*-(2-phenylethyl)-2-thiophenylacetamide) [18–20]. The computational experiment region is a cube centered at the following coordinates: x = -22.487, y = 9.200,

z = 2.438. The number of points along the x and y axes is 40, and along the z axis is 26.

Analysis

Melting point determination was performed on a PTP (M) TU 92–891 (Russia) instrument. IR spectra were recorded on an FSM 1201 FT-IR spectrometer (InfraSpec LLC, Russia) in a KBr pellet. 1H NMR spectra were recorded on a Bruker instrument (Germany) at a working frequency of 400 MHz in DMSO-d6 or deuterated chloroform (CDCl3) solutions, using the solvent as an internal standard. Reaction progress was monitored by thin-layer chromatography using "Sorbfil" plates. The mobile phase used was a chromatographic system of n-butanol–acetic acid–water (4 : 1 : 2). Substance spots were detected under UV light. Elemental analysis was performed on a Flash EA 1112 CHNSO analyzer (Thermo Scientific, USA).

Synthesis

General procedure for the preparation of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenol derivatives. To a solution containing equimolar amounts (0.01 mol) of the corresponding 1-(3,5-di-tert-butyl-4-hydroxyphenyl)-alkyl-(aryl)-ketone and a primary aromatic amine in 50 mL of anhydrous toluene, a catalytic amount of *p*-toluenesulfonic acid was added. The mixture was boiled for 4 hours, cooled, and the precipitated solid was washed with petroleum ether, dried, and recrystallized from aqueous methanol.

4-[*N*-(4-bromophenyl)-C-methyl-azomethine]-2,6-di-tert-butyl-phenol (3a). The reaction product is a fine crystalline, odorless beige substance. Yield—76 %. M.p. = 145–147 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3560 (OH), 3052 (CH_{arom}), 2851 (t-Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.35, 1.46 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.91); 5.72 (s, 1H, C(4) OH, J = 4.83); 7.61 (s, 2H, Ar, J = 10.05); 7.40, 7.43 (2d, 1H each, H(5, 6), J = 5.14, J = 5.17). Elemental analysis data (%) for $C_{22}H_{28}BrNO$ (402.34): Calculated: C, 65.67; H, 7.01; Br, 19.86; N, 3.48; O, 3.98. Found: C, 65.81; H, 7.06; Br, 20.10; N, 3.53; O, 4.05.

4-[*N*-(2-aminophenyl)-C-ethyl-azomethine]-2,6-di-tert-butyl-phenol (3b). Yield—74 %. M.p. = 132–134 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3562 (OH), 3044 (CHarom), 2847 (t-Bu), 1662 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.38, 1.48 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.92); 5.78 (s, 1H, C(4) OH, J = 4.90); 7.58 (s, 2H, Ar, J = 10.02); 7.42, 7.44 (2d, 1H each, H(5, 6), J = 5.10, J = 5.16). Elemental analysis data (%) for $C_{23}H_{32}N_2O$ (352.48): Calculated: C, 78.36; H, 9.15; N, 7.95; O, 4.54. Found: C, 78.81; H, 9.22; N, 8.02; O, 4.05.

2,6-di-tert-butyl-4-[C-methyl-*N*-(p-tolyl)-azomethine]-phenol (3c). Yield—80 %. M.p. 142–144 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t-Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.36, 1.42 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.88); 5.80 (s, 1H, C(4) OH, J = 4.91); 7.65 (s, 2H, Ar, J = 10.02); 7.40, 7.48 (2d, 1H each, H(5, 6), J = 5.14, J = 5.16). Elemental analysis data (%) for $C_{23}H_{31}NO$ (337.47): Calculated: C, 81.85; H, 9.26; N, 4.15; O, 4.74. Found: C, 81.74; H, 9.18; N, 3.98; O, 4.05.

***N*-[4-[1-(3,5-di-tert-butyl-4-hydroxy-phenyl)-ethylideneamino]-phenyl]-acetamide (3d).** Yield—80 %. M.p. = 147–149 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t-Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.40, 1.46 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.90); 5.72 (s, 1H); (s, 1H, C(4) OH, J = 4.82); 7.62 (s, 2H, Ar, J = 10.05); 7.44, 7.49 (2d, 1H each, H(5, 6), J = 5.17, J = 5.12). Elemental analysis data (%) for $C_{24}H_{32}N_2O_2$ (380.49): Calculated: C, 75.75; H, 8.48; N, 7.36; O, 8.41. Found: C, 78.81; H, 8.18; N, 7.88; O, 8.05.

1-[4-[1-(3,5-di-tert-butyl-4-hydroxy-phenyl)-ethylideneamino]-phenyl]-ethanone (3e). Yield—78 %. M.p. = 100–102 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t–Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.38, 1.48 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.94); 5.74 (s, 1H, C(4) OH, J = 4.88); 7.63 (s, 2H, Ar, J = 10.02); 7.38, 7.42 (2d, 1H each, H(5, 6), J = 5.15, J = 5.10). Elemental analysis data (%) for $C_{24}H_{31}NO_2$ (365.48): Calculated: C, 78.86; H, 8.55; N, 3.83; O, 8.76. Found: C, 78.92; H, 8.18; N, 3.72; O, 8.05.

2,6-di-tert-butyl-4-[C-(4-chlorophenyl)-*N*-phenylazomethine]-phenol (3f). Yield—72 %. M.p. = 185–187 °C (recrystallization from methanol). IR spectrum (in potassium bromide pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t-Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.34, 1.42 (2s, 2H, C(3,5)-tert-Bu, J = 4.90); 5.80 (s, 1H, C(4) OH, J = 4.92); 7.61 (s, 2H, Ar, J = 10.00); 7.40, 7.48 (2d, 1H each, H(5, 6), J = 5.14, J = 5.09). Elemental analysis data (%) for $C_{27}H_{30}ClNO_2$ (419.95): Calculated: C, 77.21; H, 7.20; Cl, 8.55; N, 3.34; O, 3.81. Found: C, 77.92; H, 7.55; Cl, 8.19; N, 3.72; O, 8.014.

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DOI: 10.19163/2307-9266-2026-14-2-175-188

***N*-[4-[1-(3,5-di-tert-butyl-4-hydroxy-phenyl)-propylideneamino]-phenyl]-acetamide (3g).** Yield—76 %. M.p. = 128–130 °C (recrystallization from methanol). IR spectrum (in KBr pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t–Bu), 1663 (C = N) cm^{-1}. 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.40, 1.52 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.94); 5.74 (s, 1H, C(4) OH, J = 4.86); 7.60 (s, 2H, Ar, J = 10.04); 7.42, 7.48 (2d, 1H each, H(5, 6), J = 5.18, J = 5.14). Elemental analysis data (%) for $C_{25}H_{34}N_2O_2$ (394.52): Calculated: C, 76.21; H, 8.69; N, 7.10; O, 8.11. Found: C, 76.92; H, 8.55; N, 7.21; O, 8.17.

2,6-di-tert-butyl-4-[C-methyl-*N*-(4-nitrophenyl)-azomethine]-phenol (3h). Yield—80 %. M.p. = 132–134 °C (recrystallization from methanol). IR spectrum (in potassium bromide pellet): 3600 (OH), 3050 (CH_{arom}), 2865 (t-Bu), 1663 (C = N) cm^{-1}, 1520 (vas NO_2), 1360 (vs NO_2). 1H NMR spectrum (400 MHz, CDCl3), δ, ppm: 1.38, 1.45 (2s, 1H each, C(3) tert-Bu, C(5) tert-Bu, J = 4.91); 5.78 (s, 1H, C(4) OH, J = 4.80); 7.63 (s, 2H, Ar, J = 10.02); 7.40, 7.48 (2d, 1H each, H(5, 6), J = 5.15, J = 5.10). Elemental analysis data (%) for $C_{23}H_{31}N_2O_3$ (383.47): Calculated: C, 72.03; H, 8.15; N, 7.30; O, 12.52. Found: C, 72.44; H, 8.05; N, 7.52; O, 12.36.

In vitro study of the antioxidant activity of compounds 3a–3h

The antioxidant properties of the synthesized azomethine phenols were evaluated in model systems based on corn oil containing a complex of saturated and unsaturated fatty acids. Lipid oxidation was initiated both physically (ultraviolet irradiation, UVI) and chemically, using the Fenton system (H_2O_2/Fe^{2+}) as a free radical generator. This protocol was previously successfully tested in our studies of benzimidazole derivatives containing a sterically hindered phenolic fragment [21]. To quantitatively assess the obtained results, the antiradical activity of the compounds was compared with the action of reference antioxidants: ubiquinone (Biologische Heilmittel Hee, Germany) and butylated hydroxytoluene (BHT, 99.0 %, CDH, India)—a representative of the sterically hindered phenol class used in the drug “Dibulin”. The antioxidant activity of ubiquinone under all experimental conditions was taken as the reference (100 %, or 1.0 ubiquinone unit).

General method for determining the *in vitro* antioxidant activity of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols 3a–3h

To study the antioxidant activity (AOA) of compounds (3a–3h), a Fenton system-induced oxidation model was used. Each compound under investigation was pre-dissolved to a concentration of 10 %. The model lipophilic medium was prepared as a water–alcohol–oil emulsion: 800 µL of oil was added to 3 mL of ethanol, vigorously shaken, and brought to the mark in a 100 mL volumetric flask with distilled water. The incubation mixture was formed in centrifuge tubes by combining 2 mL of the prepared emulsion, 100 µL of the test substance solution, 200 µL of a 10 % solution of iron (II) sulfate ($FeSO_4$), and 10 µL of 3 % hydrogen peroxide (H_2O_2) to generate hydroxyl radicals. The reaction was carried out in a thermostat at 37 °C for 60 min. In the control experiment, the test substance was replaced by an equivalent volume of distilled water. After incubation, 1 mL of 28 % trichloroacetic acid (TCA) was added, and the mixture was centrifuged for 10 min at 600 rpm. The content of LPO products was assessed by reaction with thiobarbituric acid. 1 mL of a 1 % TBA solution was added to 2 mL of the supernatant, and the mixture was heated for 15 min in a boiling water bath. After cooling, the optical density was recorded on a SF-46 spectrophotometer (JSC “LOMO”, Russia). For differential assessment of LPO stages, measurements were taken at 450 nm (diene conjugates) and 532 nm (malondialdehyde). The incubation medium without the addition of the test substances served as the control. The percentage of LPO inhibition was calculated by the formula:

$$ING\% = 100 - \left(\frac{I_0}{I_k} \times 100 \right),$$

where I_o is optical density of the test sample; I_k is optical density of the positive control sample.

AOA was expressed in ubiquinone units (Q – ed) by the formula:

$$AOA(Q - ed) = \%\Delta ING_i - \%\Delta ING_Q,$$

where $\%\Delta ING_i$ is a percentage decrease in the formation of TBA-reactive products in the test system in the presence of the tested synthetic sample; $\%\Delta ING_Q$ is a percentage decrease in the formation of TBA-reactive products in the test system in the presence of ubiquinone.

For comparison, the AOA of typical antioxidants—ubiquinone and butylated hydroxytoluene—was studied. A 1 % solution of butylated hydroxytoluene substance was prepared, 100 µL was taken, added to the test system described above, and incubated under standard conditions; for ubiquinone, 100 µL of a working solution of the preparation, prepared by

dissolving 1 capsule of ubiquinone in 10 mL of distilled water, was added to the test system described above and incubated under standard conditions.

Statistical analysis

Statistical analysis of the results was performed using computer software packages: "Microsoft Excel 2010" (Microsoft Office, USA) and "Statistica 10" (Statsoft, USA) using the paired Student's *t*-test. The Pearson method was used to assess correlational relationships between individual parameters studied.

RESULTS AND DISCUSSION

2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols 3a–3h were synthesized by boiling equimolar amounts of the corresponding substituted 1-(3,5-di-tert-butyl-4-hydroxyphenyl)-alkyl-(aryl)-ketones and primary aromatic amines in anhydrous toluene in the presence of catalytic amounts of p-toluenesulfonic acid for 4 h (Fig. 1).

The structure of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols was confirmed by IR spectroscopy, ^{1}H NMR, and elemental analysis. All compounds exhibit characteristic absorption bands for the valence vibrations of the unassociated (ν 3600 cm^{-1}) and associated (ν 3400 cm^{-1}) O-H groups, bands for the valence vibrations of aromatic ring C-H bonds (ν 3000–3050 cm^{-1}), bands for the asymmetric and symmetric valence vibrations of tert-butyl group C-H bonds (ν 3000–2850 cm^{-1}), and bands for the valence vibrations of the >C=N- bond (ν 1663 cm^{-1}). In the IR spectrum of compound 3h, intense absorption bands in the region of 1520–1526 cm^{-1} and 1330–1360 cm^{-1}, attributed to asymmetric ν^{as}_{NO2} and symmetric ν^{s}_{NO2} valence vibrations of the nitro group, are indicative.

In the ^{1}H NMR spectra of the obtained compounds 3a–3h, there are two singlets for the protons of the tert-butyl groups, which exhibit magnetic inequivalence; the chemical shifts for these protons are observed in the range of 1.34–1.42 ppm and 1.45–1.52 ppm.

A singlet (s) with chemical shifts in the range of 5.72–5.80 ppm corresponds to one proton of the hydroxyl group.

The signal of the aromatic protons of the phenolic fragment of the molecule (positions 3,5) has a chemical shift of 7.61–7.65 ppm and an intensity in the range of 10.01–10.05 ppm, which corresponds to two protons.

The aromatic protons of the aromatic ring (positions 5, 6) give two doublets due to spin-spin coupling with chemical shifts in the range of 7.38–7.40 ppm and 7.43–7.49 ppm.

Information on some physicochemical parameters of compounds **3a–3h** is presented in Table 1.

To assess the prospects of synthesizing new derivatives of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols 3a–3h, an in silico prediction of the spectrum of their probable pharmacological properties was performed using the online service PASS Online. A list of probable biological activities with the probability of their presence (Pa) and absence (Pi) in fractions of a unit is presented in Table 2.

Comparative analysis of predicted data for aromatic Schiff base derivatives containing a shielded phenol fragment indicates their potentially high pharmacological activity. Representatives of this series, according to predictions, may exhibit a cardiotonic effect and the ability to stabilize cell membranes. Furthermore, expected increased expression of the CYP2J2 enzyme may mediate cardioprotection through the activation of mitochondrial ATP-dependent potassium channels (mitoKATP) [22, 23], providing physiological benefits by altering reactive oxygen species production.

The results of computational modeling indicate that a series of studied azomethine derivatives demonstrate potential activity against the GP-4 target. The molecular complex with compound 3h exhibits the greatest stability, with a formation energy of -6.60 kcal/mol, indicating its high affinity (Table 3). Compounds 3f and 3a may also exhibit a pronounced inhibitory effect. A key structural feature common to the active ligands (3a, 3f, 3h) is the presence of strong electron-withdrawing groups—bromine, chlorine, and nitro groups.

Thus, it can be concluded that the introduction of electron-withdrawing substituents into the azomethine structure significantly increases their ability to bind to the active site of GP-4 [24]. Ubiquinone, hydroxybutylated toluene, and mexidol have significantly lower affinity for the GP-4 binding site. It is known [25] that mexidol exhibits pronounced antioxidant activity, including through the activation of endogenous antioxidant enzymes. The performed computer modeling suggests that the binding energy of the studied ligands with the GP-4 enzyme will be comparable in effectiveness to the reference

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compounds: ubiquinone, hydroxybutylated toluene, and mexidol itself. Molecular docking revealed key amino acid residues of the enzyme's active center involved in interaction with virtual ligands: Gln 45, Sec 46, Gly 47, Lys 48, Gln 81, Trp 136, Asn 137, and Phe 138. Analysis of the bond types showed that: compounds 3d and 3g form hydrogen bonds through carbonyl groups with the Asn 137 residue. Ligand 3e forms a similar hydrogen bond with the Gly 47 residue. Compounds 3f and 3h are characterized by the formation of a hydrogen bond between the nitrogen of the azomethine group and the amino acid Trp 136 (see Table 3).

The preliminary prediction stage allows for an assessment of the expediency of both further molecular design and preparative research for the synthesis of highly effective and safe drug substances among derivatives of this series.

Investigation of the antioxidant activity of derivatives (3a–h) in a test system with ultraviolet irradiation

During the first stage of the work, a screening of the AOA of synthesized azomethines containing a sterically hindered phenol fragment was carried out. The study was performed in a model test system based on a complex of saturated and unsaturated fatty acids. LPO was initiated by ultraviolet irradiation (UVI) in the presence of Fe^{2+} ions at an optimal biological concentration (1.0×10^{-3} mol/L). Compounds 3a, 3c, 3e, and 3h demonstrated the greatest antioxidant effect, expressed in ubiquinone units (Q-units). Statistically processed data are presented in Table 4.

Further detailed analysis showed that derivatives 3a, 3c, and 3h inhibit free radical processes by 44–48 %, surpassing the reference compound—hydroxybutylated toluene (39 % inhibition)—in activity. For the remaining compounds in the series (3b, 3d, 3e, 3f, 3g), the inhibition level was 28–37 %. The lowest AOA was characteristic of samples 3d and 3g, which is likely due to steric hindrance created by the bulky acetamide group in the phenyl fragment conjugated with the azomethine bond.

During the final stage, the dependence of AOA on initiator concentration was studied. Comparative analysis with hydroxybutylated toluene confirmed that the maximum antioxidant activity of the studied compounds is observed at a concentration of iron (II) ions (1.0×10^{-3} mol/L).

Investigation of the antioxidant activity of compounds 3a–3h in a test system with hydrogen peroxide

During the study of the AOA of the investigated compounds in a test system using chemical (hydrogen peroxide and iron (II) sulfate—Fenton system H_2O_2/Fe^{2+}) inducers of free radical processes, it was found that the maximum inhibitory effect was also observed in the presence of compounds 3a, 3c, and 3h—42–45 %, exceeding the antioxidant effect in the same system of the reference substance—hydroxybutylated toluene (36.5 %) (Table 5).

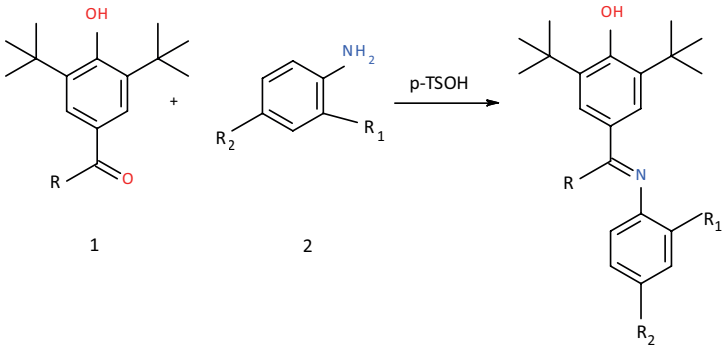
However, it should be noted that compounds **3a, 3c**, and **3h** showed a less pronounced effect in this test system, indicating lower stability of the described compounds towards chemical inducers of free radical processes.

Other tested samples in this series, **3b, 3d, 3e, 3f**, and **3g**, inhibit free radical processes by 29–38 %. Compounds **3b, 3f,** and **3g** exhibited the least pronounced antioxidant properties.

The main structural element determining the polypharmacological profile of this series of hybrid compounds is the 2,6-di-tert-butylphenol fragment. Its incorporation into the structure of *N*-substituted 3-(benzimidazol-2-yl)-chromones and derivatives of 1,3-dimethyl-8-(chromon-3-yl)-xanthine provides not only a pronounced antioxidant effect, comparable to the activity of the standard trolox, but also cytotoxic action against human colorectal cancer cell lines HCT116 and breast cancer MCF7 [10, 26].

The ability of this phenolic fragment to induce cerebroprotective properties is of particular interest. It has been experimentally shown that 4-hydroxy-3,5-di-tert-butylcinnamic acid (at a dose of 100 mg/kg) reduces the degree of neurological deficit in animals, promotes the restoration of mitochondrial membrane potential, normalizes the ratio of aerobic and anaerobic metabolism, and suppresses the activity of caspase-3—a key effector of apoptosis [27].

Furthermore, compounds containing the 4-hydroxy-3,5-di-tert-butylphenyl substituent demonstrate a complex neuroprotective effect. This manifests as the restoration of mitochondrial enzyme activity (aconitase, citrate synthase, and α-ketoglutarate dehydrogenase), as well as a reduction in the pathological accumulation of tau protein in hippocampal tissue [28].



R = Me, R_2 = Br (3a); R = Et, R_1 = NH_2 (3b); R = Me, R_2 = Me (3c); R = Me, R_3 = NHAc (3d); R = Me, R_2 = Ac (3e); R = p-ClPh, R_1 = R_2 = H (3f); R = Et, R_3 = NHAc (3g); R = Me, R_2 = NO_2 (3h).

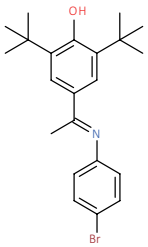
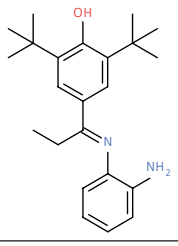
Figure 1 – Synthesis of 2,6-di-tert-butyl-4-[(Z)-C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols (3a–3h).
Note: p-TsOH, p-toluenesulfonic acid.

Table 1 — Physicochemical characteristics of compounds 3a–3h

Compound Code	Yield, %	(THF:DMF)	Mol. mass	Gross formula*
3a	76	145–147	402,34	$C_{22}H_{28}BrNO$
3b	74	132–134	352,48	$C_{23}H_{32}N_2O$
3c	80	147–149	337,47	$C_{23}H_{31}NO$
3d	80	142–144	380.49	$C_{24}H_{32}N_2O_2$
3e	78	100–102	365.48	$C_{23}H_{31}NO_2$
3f	72	185–187	419.95	$C_{27}H_{30}ClNO$
3g	76	128–130	394.52	$C_{25}H_{34}N_2O_2$
3h	80	132–134	383.47	$C_{23}H_{31}N_2O_3$

Note: * according to elemental analysis data (obtained values correspond to calculated values). THF, tetrahydrofuran; DMF, dimethylformamide.

Table 2 — Predicted biological activities of azomethine derivatives of sterically hindered phenol (3a–3h) using the PASS Online Web Resource

Compound Code	Structural Formula of Compound	Biological Activity								
		Ubiquinone cytochrome-c inhibitor	Mucous membrane protector	Aspulvinone dim. inhibitor	Treatment of heart failure	Membrane integrity agonist	Glutathionethiolest. inhibitor	M Inhib. decarbox. dehydrog.	NADP + inhibitor	CYP2J substrate
3a		72	71	65	65	–	55	–	57	–
3b		84	78	79	95	–	64	60	67	71

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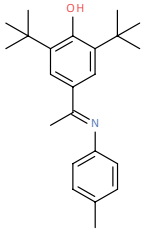
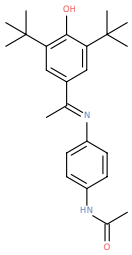
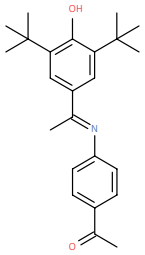
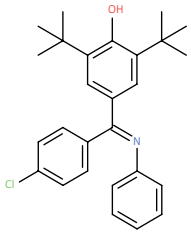
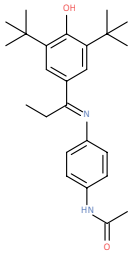
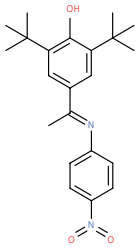
Compound Code	Structural Formula of Compound	Biological Activity								
		Ubiquinone cytochrome-c inhibitor	Mucous membrane protector	Aspulvinone dim. inhibitor	Treatment of heart failure	Membrane integrity agonist	Glutathionethiolest. inhibitor	И Inhib. decarbox. dehydrog.	NADP + inhibitor	CYP2J substrate
3c		73	63	–	59	73	–	–	–	–
3d		71	85	68	69	86	57	58	63	65
3e		83	77	81	78	–	71	73	77	73
3f		73	66	–	69	71	–	–	–	–
3g		75	65	–	89	–	–	–	–	–
3h		89	76	65	56	–	–	–	–	–

Note: the probability of biological activity is characterized by the Pa value in %.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY
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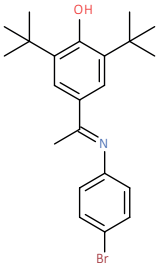
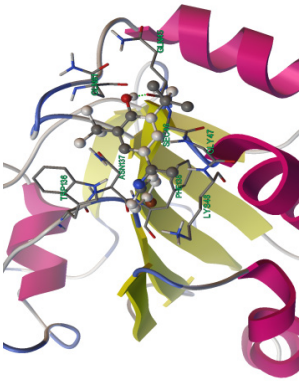
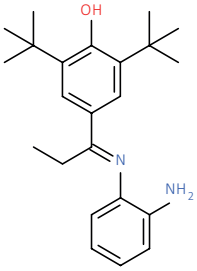
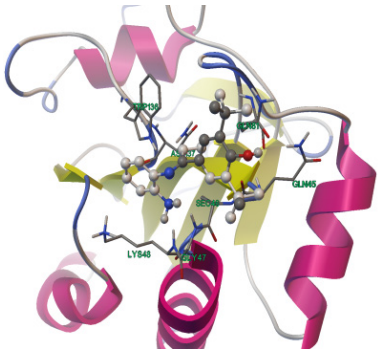
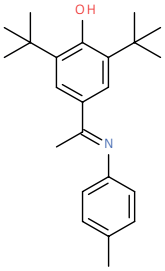
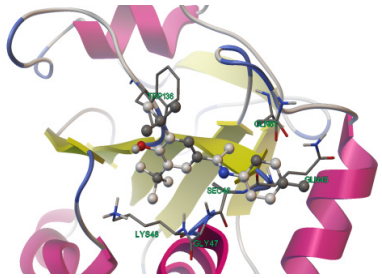
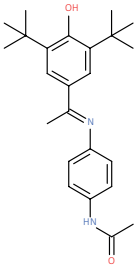
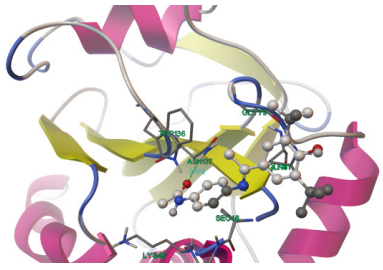
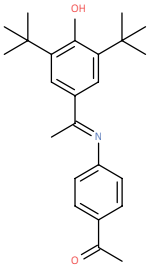
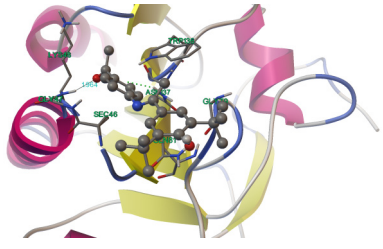
Table 3 – Ligand-enzyme complex formation energies of ligands with glutathione peroxidase-4

Compound Code	Docking Energy, kcal/mol	Ligand Chemical Formula	Ligand Location
3a	-6,02		
3b	-5.51		
3c	-4.49		
3d	-4.54		
3e	-4.67		

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DOI: 10.19163/2307-9266-2026-14-2-175-188

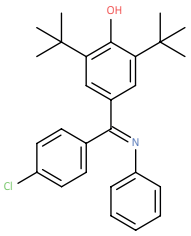
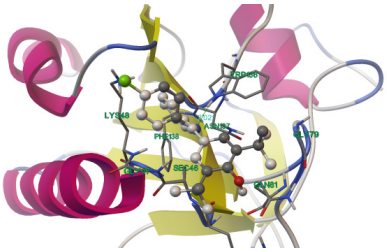
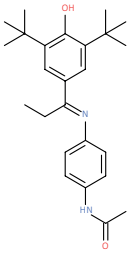
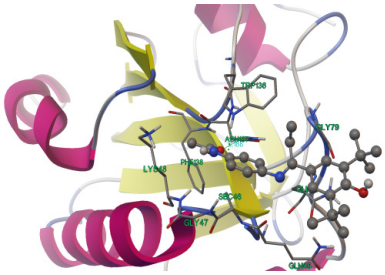
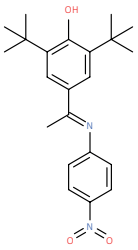
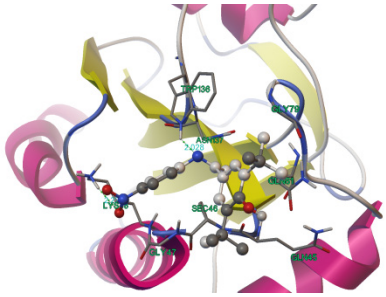
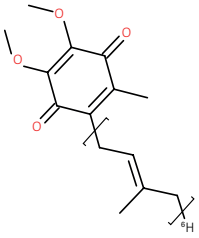
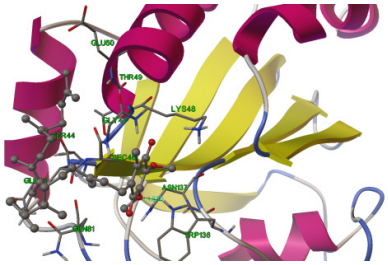
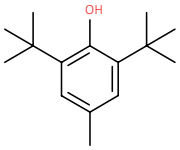
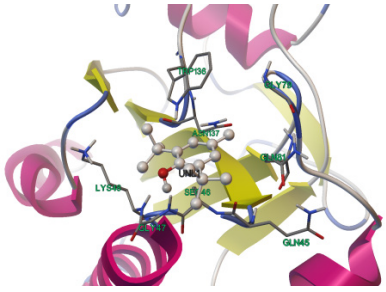
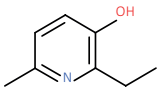
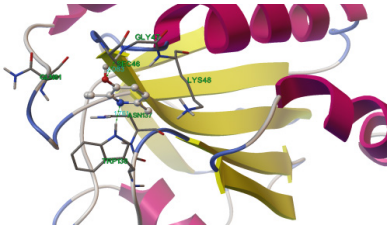
Compound Code	Docking Energy, kcal/mol	Ligand Chemical Formula	Ligand Location
3f	-6.10		
3g	-4.53		
3h	-6.60		
Ubiquinone	-4.23		
Hydroxybutylated toluene	-5.12		
Mexidol	-4.39		

Table 4 — Antioxidant activity of compounds 3a-h and reference substances in the UVI/Fe^{2+} test system

Compound Code	Concentration, mol/L	Optical Density		AOA, Q-ed
		λ= 450 nm	λ=532 nm	
3a	1.0×10^{-3}	0.325 ± 0.004	0.125 ± 0.002	55.0 (5.0)
3b	1.0×10^{-3}	0.352 ± 0.005	0.168 ± 0.003	48.0 (4.4)
3c	1.0×10^{-3}	0.328 ± 0.004	0.152 ± 0.001	52.0 (4.7)
3d	1.0×10^{-3}	0.402 ± 0.006	0.278 ± 0.003	32.0 (2.9)
3e	1.0×10^{-3}	0.431 ± 0.02	0.369 ± 0.01	20.0 (1.8)
3f	1.0×10^{-3}	0.445 ± 0.01	0.375 ± 0.03	18.0 (1.6)
3g	1.0×10^{-3}	0.354 ± 0.005	0.196 ± 0.002	45.0 (4.1)
3h	1.0×10^{-3}	0.454 ± 0.01	0.396 ± 0.02	15.0 (1.4)
Ubiquinone	1.0×10^{-3}	0.354 ± 0.005	0.196 ± 0.002	45.0 (4.1)
Hydroxybutylated toluene	1.0×10^{-3}	0.460 ± 0.003	0.430 ± 0.001	11.0 (1.0)

Note: AOA — antioxidant activity; UVI — ultraviolet irradiation.

Table 5 — Antioxidant activity of compounds 3a-h and reference substances in the Fe^{2+}/H_2O_2 test system

Compound Code	Concentration, mol/L	Optical Density		AOA, Q-ed
		λ= 450 nm	λ=532 nm	
3a	1.0×10^{-3}	0.330 ± 0.004	0.160 ± 0.002	51.0 (3.2)
3b	1.0×10^{-3}	0.355 ± 0.005	0.185 ± 0.003	45.0 (2.8)
3c	1.0×10^{-3}	0.332 ± 0.004	0.168 ± 0.001	50.0 (3.1)
3d	1.0×10^{-3}	0.412 ± 0.006	0.298 ± 0.003	29.0 (1.8)
3e	1.0×10^{-3}	0.448 ± 0.02	0.382 ± 0.01	17.0 (1.1)
3f	1.0×10^{-3}	0.451 ± 0.01	0.389 ± 0.03	16.0 (1.0)
3g	1.0×10^{-3}	0.361 ± 0.005	0.219 ± 0.002	42.0 (2.6)
3h	1.0×10^{-3}	0.460 ± 0.01	0.430 ± 0.02	12.0 (0.7)
Ubiquinone	1.0×10^{-3}	0.450 ± 0.003	0.390 ± 0.001	16.0 (1.0)
Hydroxybutylated toluene	1.0×10^{-3}	0.398 ± 0.005	0.237 ± 0.002	36.5 (2.3)

Note: AOA, antioxidant activity.

Thus, the 2,6-di-tert-butylphenol fragment serves as an effective pharmacophore module, critically important for realizing the potent antioxidant potential of molecules. The obtained experimental data convincingly confirm this role and are in good agreement with known literature findings on the mechanisms of action of sterically hindered phenols.

Study Limitations

Limitations of the characterization method for synthesized azomethine phenols: additional methods (e.g., X-ray diffraction analysis for crystals) might be required to confirm the configuration of the azomethine bond (E/Z isomerism).

Limitations of *in silico* prediction: molecular docking was performed on only one target (HT-4), although biological effects, especially antioxidant effects, may be mediated through other receptors or mechanisms.

The study represents a preliminary screening of a new class of compounds with antioxidant potential. Computer predictions regarding cardiotonic activity and membrane-stabilizing activity require targeted verification on appropriate biological models.

CONCLUSION

An optimized synthesis method allowed for the preparation of 8 derivatives of 2,6-di-tert-butyl-4-[C-alkyl-(aryl)-(*N*-phenyl)-azomethine]-phenols, the structures of which were confirmed by nuclear magnetic resonance, elemental analysis, and IR spectroscopy. According to *in silico* predictions, the studied compounds may possess significant cardiotonic properties, stabilize cell membranes, and increased expression of the CYP2J2 enzyme enhances mitoKATP activation, which is believed to provide physiological benefits by altering reactive oxygen species production. In accordance with the results of molecular docking calculations, it can be assumed that the optimal ligand-receptor interaction energy with HT-4 will be comparable to the values exhibited by ubiquinone, hydroxybutylated toluene, and mexidol.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2026-14-2-175-188

During *in vitro* pharmacological screening, the substances under investigation demonstrated pronounced antioxidant activity. The lead compounds are 3a, 3c, and 3h, which surpass the reference compounds – ubiquinone and hydroxybutylated toluene.

FUNDING

This study did not have financial support from third-party organizations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Tamara V. Tsakulova — data curation and formal analysis, investigation, validation, writing—original draft; Ivan P. Kodonidi — conceptualization, validation, data analysis, revision and editing of the manuscript; Alexey S. Chiriapkin — formal analysis; Fatima N. Bidarova — writing—review & editing; Manana T. Kisieva — data curation, validation; Luisa A. Usmanova — data curation. All authors confirm that their authorship meets the international ICMJE criteria (all authors have made significant contributions to the development of the concept, research and preparation of the article, read and approved the final version before publication).

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