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Synthesis and Pharmacological Activity of 3-Phenoxybenzoic Acid Derivatives

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Abstract—New esters of *N*-benzoyl-3-phenoxyphenylcarboxamide acid and *N*-benzoyl-*N*'-4-bromophenyl-3-phenoxybenzamidine were synthesized. Some of the synthesized compounds were shown to inhibit the activity of dipeptidyl peptidase-4 and nonenzymatic glycosylation of proteins and manifested antiplatelet and antioxidant properties. The compounds tested did not display the antagonistic effect toward angiotensin II type 1 receptor, did not influence the activity of glycogen phosphorylase and had very little ability to break cross-links of the glycated proteins. The derivatives with the biological activity of two types were found, which can serve as basic molecules in the search for new drug products.

Keywords: biologically active compounds, *N*-benzoyl-3-phenoxyphenylcarboxamide acid, diphenyl oxide, ethers, synthesis

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INTRODUCTION

Many of the diphenyl oxide derivatives are of interest as important synthetic objects and potential biologically active compounds, which are characterized by certain pharmacological properties. Particularly, nimesulide [*N*-(4-nitro-2-phenoxyphenyl)methanesulfonamide] displays anti-inflammatory, analgesic, antipyretic, and antiplatelet effects and serves as a starting compound for the synthesis of triazole derivatives, effective agents against colon cancer [1]. Permethrin [3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] and phenothrin [(3-phenoxyphenyl)methyl 2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylate] are used as drug products displaying antiparasitic properties [2]. Diphenyl oxide derivatives are bioisosteric analogues of diphenyl derivatives [3]. The latter are characterized by antihypertensive, antimicrobial, antidiabetic, diuretic, and antiplatelet activities; also, they demonstrate psychotropic effects [4]. In addition, diphenyl derivatives pertain to the so-called privileged substructures [5], which can interact with biotargets of several families. Specific properties of these substructures come from their spatial and electron characteristics, which support the binding to various targets depending on the substituents introduced into the basic structure and, thereby, support their polyfunctional effects and a wide spectrum of pharmacological activity [6]. Taking this into account, we believe that the synthesis and studies of the pharmacological activity of new diphenyl oxide derivatives are promising.

RESULTS AND DISCUSSION

In the Pinner reaction, aromatic nitriles interact with alcohols and hydrogen chloride to give imidate hydrochlorides.

The reaction of imidate hydrochlorides (**IIa**–**e**) with benzoyl chloride resulted in *N*-(benzoyl)-3-phenoxyphenylcarboximidates (**IVa**–**e**) (Scheme 1). As an acceptor of hydrogen chloride TEA was used. The reactions were performed at equimolar ratios of the reagents and a twofold excess of TEA in absolute 1,4-dioxane at 60–65°C for 2 h. The reactions proceeded selectively in 85 to 96% yields.

The presence of a –C=NH · HCl functional group in the imidate hydrochlorides synthesized makes it possible to synthesize free imidates (**IIIa, b**). To this end, imidate hydrochloride was treated with TEA.

With the goal of obtaining the target amidine (**V**) containing a diphenyl oxide fragment, butyl *N*-(ben-

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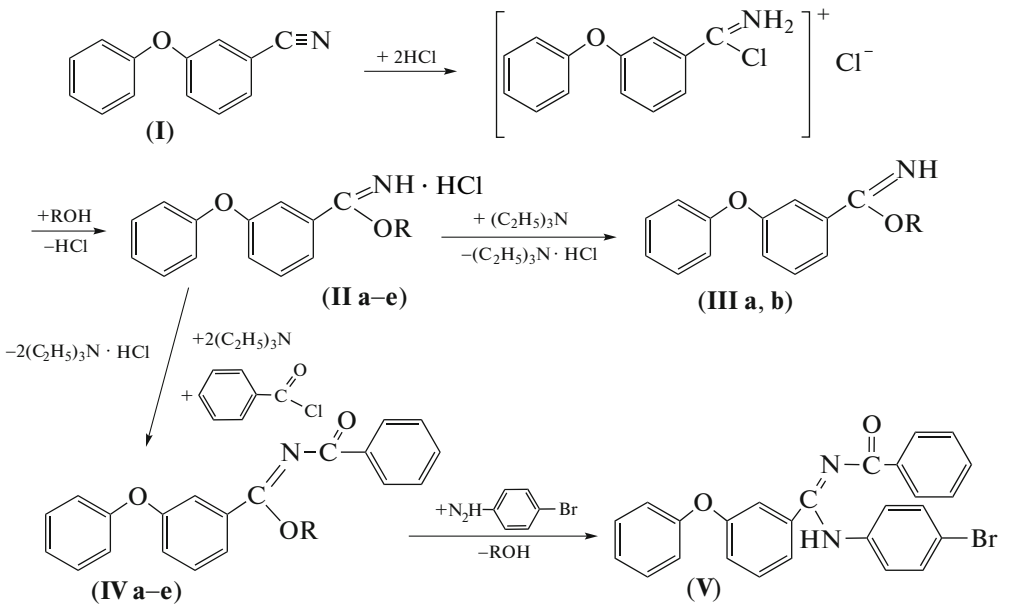
Abbreviations: AT_1, angiotensin type 1 receptor; DPP-4, dipeptidyl peptidase-4; GP, glycogen phosphorylase; LPO, lipid peroxidation; TBA, thiobarbituric acid; TEA, triethylamine.

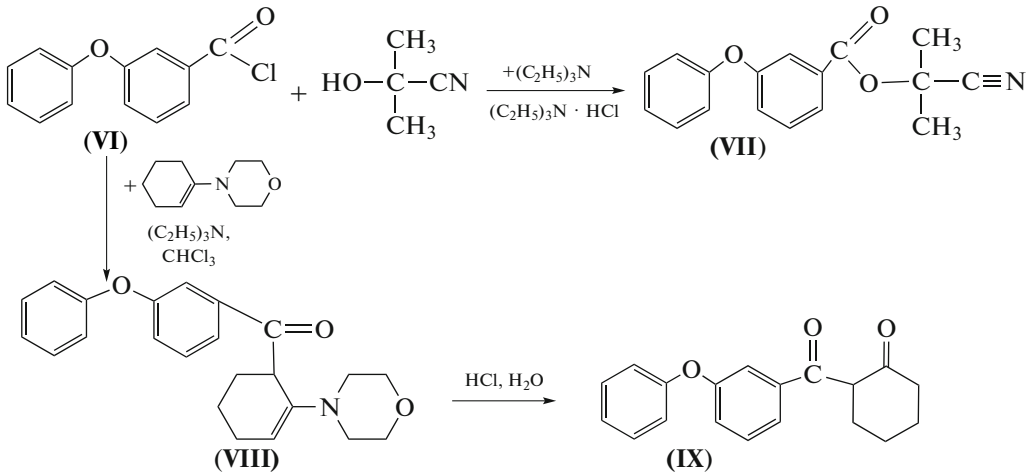
zoyl)-3-phenoxyphenylcarboximidate **(IVa)** was treated with *p*-bromoaniline in benzene at 65–70°C for 2 h (Scheme 1). *N*-(Benzoyl)-*N*'-4-bromophenyl-3-phenoxybenzamidine **(V)** was purified by recrystallization from a 1 : 1 ethanol–water mixture in 60% yield.



where R = *n*-C_4H_9 **(IIa**, **IVa)**; *sec*-C_4H_9 **(IIb**, **IVb)**; *iso*-C_4H_9 **(IIc**, **IVc)**; C_6H_5 **(IId**, **IVd)**; C_6H_5-CH_2-C_6H_4 **(IIe**, **IVe)**; C_2H_5 **(IIIa)**, C_3H_7 **(IIIb)**.

Scheme 1. Preparation of 3-phenoxyphenylcarboximidates **(IVa–e)**, **(IIIa, b)** and *N*-benzoyl-*N*'-4-bromophenyl-3-phenoxybenzamidine **(V)**.

For the synthesis of 2-cyanopropyl 3-phenoxybenzoate **(VII)** 3-phenoxybenzoyl chloride **(VI)** was treated with 2-hydroxy-2-methylpropionitrile under stirring in absolute diethyl ether in the presence of TEA for 1 h at 20–25°C (Scheme 2). The yield of nitrile **(VII)** was 95%.

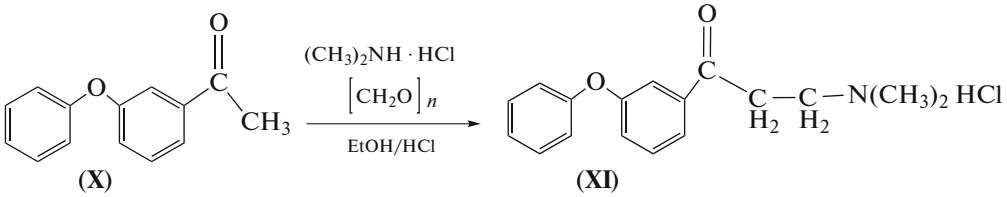


Scheme 2. Preparation of 2-cyanoprop-2-yl 3-phenoxybenzoate **(VII)** and 2-(3-phenoxybenzoyl)cyclohexanone **(IX)**.

Using acylation of enamines with acyl chlorides as a reference [7] we performed acylation of 4-(cyclohexen-1-yl)morpholine with 3-phenoxybenzoyl chloride **(VI)** at a reagent molar ratio of 1 : 1 in chloroform at 50–60°C for 7–8 h in the presence of TEA. The treatment with hydrochloric acid of the

product obtained (**VIII**) led to 1,3-diketone (**IX**) (Scheme 2).

The Mannich bases were obtained in accordance with the published procedure for the synthesis of β-dimethylaminopropiophenone hydrochloride [8]. The interaction of 1-(3-phenoxyphenyl)-1-ethanone (**X**) with dimethyl amine hydrochloride and paraformaldehyde in ethanol in the presence of concentrated hydrochloric acid gave 40% *N*,*N*-dimethyl-2-(3-phenoxybenzoyl)ethylamine hydrochloride (**XI**) (Scheme 3).



Scheme 3. Preparation of *N*,*N*-dimethyl-2-(3-phenoxybenzoyl)ethylamine hydrochloride (**XI**).

The structures and composition of the compounds synthesized were confirmed by element analysis, IR and ^{1}H NMR spectroscopy, and chromato-mass-spectrometry.

Pharmacological Properties of Diphenyl Oxide Derivatives

AT_1-antagonistic, antiplatelet, antioxidant, antiglycation, and inhibitory activities toward DPP-4 and GP, as well as the capacity to break cross-links of the glycated proteins, were studied for the 11 new derivatives of 3-phenoxybenzoic acid (**IIIa, b**), (**IVa–e**), (**V**), (**VII**), (**IX**), and (**XI**).

The most potent antiplatelet activity was found for *N*-benzoyl substituted 3-phenoxyphenyl imidate (**IVe**), which only slightly exceeded that of the reference acetyl salicylic acid. Other derivatives of 3-phenoxybenzoic acid, *N*-benzoyl substituted phenyl imidate (**IVd**), propyl imidate (**IIIb**), and 2-methyl-2-(3-phenoxybenzoate)propionitrile (**VII**), manifested moderate antiplatelet properties, which did not statistically differ from the activity of the reference compound (table). In total, five active compounds were found, whose antiplatelet effect statistically significantly exceeded that of the control preparation.

The experiments demonstrated that eight of the tested derivatives of 3-phenoxybenzoic acid displayed the antiglycation activity (table). Compounds (**IVb, e**), (**IIIa, b**), (**V**), (**VII**), (**X**), and (**XI**) reliably inhibited specific fluorescence of glycated BSA in the range of 11.9 to 27.1%. The compounds studied displayed moderate and low antiglycation activity and were inferior in this parameter to the reference aminoguanidine.

The study of biological properties showed that the derivatives of 3-phenoxybenzoic acid, *N*-benzoyl substituted *n*-butyl imidate (**IVa**) and *N*-benzoyl substituted imidates (**IVb,c**), inhibited DPP-4 although with efficacy inferior to the reference vildagliptin (table). For compound (**IVb**), the statistically significant moderate level of the DDP-4 inhibitory activity was observed, which can be explained by the presence of a rather bulky substituent. The efficacy of DPP-4 inhibition statistically differed from the control only for this derivative.

Compounds (**IVe**), (**IIIb**), and (**V**) were shown to have moderate antioxidant activity, which was 3.5 times lower than that of the reference dibunol (table). In addition, compound (**XI**) demonstrated a low level of antioxidant activity.

All of the compounds tested were weak GP inhibitors and low effective AT_1 antagonists (table).

When studying the capacity to break cross-links of glycated proteins, we found five compounds, whose activity was statistically significantly different from the control. However, the level of their activity did not exceed that of the reference alagebrium.

To summarize, among the new derivatives of 3-phenoxybenzoic acid we found the compounds displaying antioxidant, antiplatelet, and antiglycation properties, as well as the compounds with the DPP-4 inhibitory activity. Although the new derivatives did not show the marked activity, even the low activity makes them promising for further modifications. The results demonstrated that 3-phenoxybenzoic acid derivatives (**IVb, e**), (**IIIb**), (**V**), (**VII**), and (**XI**) can be used as basic molecules for the directed design and search for highly active polyfunctional compounds with a combined activity of two types.

EXPERIMENTAL

IR spectra (ν, cm^{-1}) were registered on SPECORD M 82 (Germany) and PERKIN-ELMER (United States) in a film for liquid compounds and in vaseline oil for solid forms.

^{1}H NMR spectra (δ, ppm, *J*, Hz) were registered on VarianMercury 300BB spectrometer (United States) in DMSO-d_6 with hexamethyldisiloxane as the internal standard.

Chromatography mass spectra were obtained on a Varian MAT-11 (United States) at an ionization volt-

Pharmacological activity of diphenyl oxide derivatives

Compound	AT_1-antagonistic activity/ $\Delta\%$ ($M \pm m$)	Antiplatelet activity/ $\Delta\%$ ($M \pm m$)	Break of cross-links/ $\Delta\%$ ($M \pm m$)	Antiglycation activity/ $\Delta\%$ ($M \pm m$)	Inhibitory activity/$\Delta\%$ ($M \pm m$)		Antioxidant activity/ $\Delta\%$ ($M \pm m$)
					DPP-4	GP	
(IVa)	3.54 ± 3.54#	8.27 ± 8.27#	0.90 ± 0.15*#	3.21 ± 0.81#	39.77 ± 18.01#	−14.58 ± 4.43#	−0.55 ± 2.26#
(IVb)	6.86 ± 3.35#	3.08 ± 1.65#	0.76 ± 0.61#	16.35 ± 2.53*#	56.08 ± 15.93*#	−0.16 ± 5.20#	−2.81 ± 2.12#
(IVc)	17.49 ± 10.65#	4.02 ± 2.27#	0.59 ± 0.63#	27.08 ± 11.76#	43.21 ± 19.96#	−15.81 ± 2.31*#	−6.70 ± 2.73#
(IVd)	7.70 ± 7.70#	29.41 ± 1.03*	3.35 ± 0.71*#	8.27 ± 3.00#	3.28 ± 2.79#	−14.36 ± 16.69#	7.39 ± 6.23#
(IVe)	1.17 ± 0.60#	35.22 ± 3.29*	3.80 ± 2.14*#	12.80 ± 0.78*#	15.18 ± 8.54#	11.52 ± 30.32#	20.36 ± 1.69*#
(IIIa)	2.90 ± 0.86#	2.08 ± 1.10#	0.06 ± 0.09#	13.40 ± 1.86*#	21.02 ± 13.29#	−19.29 ± 4.42#	−3.12 ± 1.49#
(IIIb)	2.56 ± 1.77#	26.12 ± 2.70*	−0.15 ± 0.17#	11.91 ± 2.99*#	9.13 ± 5.16#	−19.28 ± 5.63#	24.27 ± 4.23*#
(V)	1.90 ± 0.95#	2.69 ± 1.59#	1.54 ± 0.34*#	23.90 ± 0.63*#	13.68 ± 7.90#	−21.02 ± 3.42#	26.22 ± 1.13*#
(VII)	9.25 ± 9.25#	24.82 ± 6.94*	1.79 ± 0.01*#	15.91 ± 3.04*#	30.56 ± 32.31#	−9.65 ± 6.33#	−7.00 ± 2.37#
(IX)	1.27 ± 0.82#	2.52 ± 1.36#	0.13 ± 0.96#	11.90 ± 3.16*#	14.19 ± 4.98#	10.00 ± 10.30#	−2.34 ± 0.64#
(XI)	4.10 ± 2.52#	4.28 ± 0.62*#	−1.48 ± 0.34*#	20.00 ± 2.29*#	0.80 ± 0.80#	−11.94 ± 4.21#	7.90 ± 0.71*#
Valsartan	98.32 ± 0.68*	–	–	–	–	–	–
Acetylsalicilic acid	–	30.83 ± 5.34*	–	–	–	–	–
Alagebrium	–	–	8.06 ± 0.88*	–	–	–	–
Aminoguanidine	–	–	–	57.83 ± 0.58*	–	–	–
Vildagliptin	–	–	–	–	97.84 ± 1.82*	–	–
CP-316819	–	–	–	–	–	94.10 ± 4.76*	–
Dibunol	–	–	–	–	–	–	92.95 ± 0.78*

Concentrations of the tested compounds were 0.1 mM when assessing inhibitory activities toward GP and DPP-4 and antiplatelet activity; 1 mM for antiglycation activity and capacity to break cross-links of glycated proteins; and 10 μM for AT_1-antagonistic and antioxidant activities. *The data are significant with respect to the control (the Mann–Whitney U-criterion $p < 0.05$). #The data are significant with respect to the reference compound (the Mann–Whitney U-criterion $p < 0.05$).

age of 70 eV and cathode emission current of 240 μA. The samples were directly injected into the ionization chamber.

Butyl *N*-(benzoyl)-3-phenoxyphenylcarboximidate (IVa). A solution of butyl phenoxyphenylcarboximidate hydrochloride (**IIa**) (6.4 g, 0.021 mol) in absolute dioxane (10 mL) was placed in a four-neck reactor equipped with a mechanical stirrer, thermometer, and a refluxing system. Solutions of TEA (4.25 g, 0.042 mol) in dioxane (10 mL) and benzoyl chloride (3 g, 0.021 mol) in dioxane (5 mL) were successively added dropwise under cooling on an ice bath at 5–10°C. The reaction mixture was stirred for 30 min at room temperature and kept at 60–65°C for 2 h. The precipitate of TEA hydrochloride was separated and the solvent was removed. During concentrating the reaction mixture was crystallized. The target compound was purified by recrystallization from anhydrous CCl_4 to give 6.8 g (94%) of the product. IR: 1702 (C=O); 1648 (C=N); 1246–1072 (C–O–C). ^{1}H NMR: 1.08–1.13 (t, 3H, CH_3); 2.38–2.51 (m, 2H, CH_2); 3.01–3.05 (m, 2H, CH_2); 3.23–3.37 (t, 2H, CH_2); 6.96–7.97 (m, 9H, $C_6H_5OC_6H_4$). Found, %: C 77.10; H 5.85; N 3.46. ($C_{24}H_{23}O_3N$). Calc., %: C 77.21; H 6.17; N 3.75.

***N*-(Benzoyl)phenoxyphenylcarboximidates (IVb, c)** were prepared similarly.

(IVb): Yield 85%. IR: 1702 (C=O); 1650 (C=N); 1243–1072 (C–O–C). Found, %: C 77.20; H 5.79; N 3.36. ($C_{24}H_{23}O_3N$). Calc., %: C 77.21; H 6.17; N 3.75.

(IVc): Yield 89%. IR: 1702 (C=O); 1650 (C=N); 1240–1072 (C–O–C). Found, %: C 77.05; H 5.72; N 3.58. ($C_{24}H_{23}O_3N$). Calc., %: C 77.21; H 6.17; N 3.75.

Phenyl *N*-(benzoyl)phenoxyphenylcarboximidate (IVd). A solution of phenyl phenoxyphenylcarboximidate hydrochloride (**IId**) (2 g, 6.1 mmol) in absolute dioxane (5 mL) was placed in a four-neck reactor equipped with a mechanical stirrer, thermometer, and a refluxing system. Solutions of TEA (1.245 g, 12.3 mmol) in dioxane (5 mL) and benzoyl chloride (0.857 g, 6.1 mmol) in dioxane (3 mL) were successively added dropwise under cooling on an ice bath at 5–10°C. The reaction mixture was treated as described for (**IVa**). The target compound was purified by recrystallization from absolute CCl_4 to give 2.21 g (92%) of (**IVd**). ^{1}H NMR: 6.97–8.00 (m, 19H, Ar). Found, %: C 79.68; H 4.37; N 4.09. ($C_{26}H_{19}O_3N$). Calc., % C 79.37; H 4.87; N 3.56.

3-Phenoxyphenyl *N*-(benzoyl)phenoxyphenylcarboximidate (IVe) was obtained similarly in a yield of 85%. ^{1}H NMR: 6.89–8.11 (m, 23H, Ar). Found, %: C 79.05; H 4.32; N 2.08. ($C_{32}H_{23}O_4N$). Calc., %: C 79.18; H 4.74; N 2.89.

Ethyl 3-phenoxyphenylcarboximidate (IIIa). Ethyl 3-phenoxyphenylcarboximidate hydrochloride (**IIa**) (5 g, 0.018 mol) was placed in a reactor and a solution of TEA (2.02 g, 0.02 mol) in dioxane (10 mL) was added dropwise under cooling on an ice bath at 5–10°C. The mixture was stirred for 1.5–2 h at room temperature. The precipitated TEA hydrochloride was separated and the solvent was removed. While concentrating, the reaction mixture was crystallized. The target compound was purified by recrystallization from absolute C_6H_6 to give 3.6 g (83%) of compound (**IIIa**); mp 130–131°C. IR: 3434, 3398, 1780-1690 (N–H); 1648 (C=N); 1240–1072 (C–O–C). ^{1}H NMR: 1.14–1.19 (t, 3H, CH_3); 3.59–3.65 (m, 2H, CH_2); 6.19 (s, H, NH); 6.93–7.45 (m, 9H, $C_6H_5OC_6H4$). Found, %: C 74.58; H 5.93; N 5.31. ($C_{15}H_{15}O_2N$). Calc., %: C 74.69; H 6.22; N 5.81.

Propyl 3-phenoxyphenylcarboximidate (IIIb) was prepared similarly in a yield of 85%. ^{1}H NMR: 0.81–0.90 (m, 3H, CH_3); 1.64–1.87 (m, 2H, CH_2); 3.58–3.73 (t, 2H, CH_2); 6.20 (s, H, NH); 6.92–7.45 (m, 9H, Ar). Found, %: C 75.57; H 6.31; N 5.08. ($C_{16}H_{17}O_2N$). Calc., %: C 75.29; H 6.67; N 5.49.

***N*-Benzoyl-*N*'-4-bromophenyl-3-phenoxybenzamidine (V).** A solution of butyl 3-phenoxyphenylcarboximidate hydrochloride (**IVa**) (2.1 g, 5.9 mmol) in absolute benzene (10 mL) was placed in a four-neck reactor equipped with a mechanical stirrer, thermometer, and a refluxing system. *p*-Bromoaniline (1.22 g, 7.08 mmol) was added dropwise under cooling on an ice bath at 5–10°C. The reaction mixture was kept for 55–60°C for 2 h and the solvent was removed. While concentrating, the reaction mixture was crystallized. The target compound was purified by recrystallization from a 1 : 1 ethanol water–mixture to give 60% of the product. IR: 3430–3394 (C–N); 1702 (C=O); 1648 (C=N); 1204 (N–H). ^{1}H NMR: 6.89–7.76 (m, 18H, Ar). Found, %: C 66.57; H 4.67; N 5.29; Br 16.38. ($C_{26}H_{19}O_2N_2Br$). Calc., %: C 66.25; H 4.06; N 5.94; Br 16.95.

2-Cyanoprop-2-yl-3-phenoxybenzoate (VII) was obtained as described in [9].

2-(3-Phenoxybenzoyl)cyclohexanone (IX). A solution of 4-(cyclohexen-1-yl)morpholine (10.4 g, 62.5 mmol) and anhydrous TEA (6.3 g, 4.7 mL, 62.5 mmol) in anhydrous chloroform (32 mL) was placed in a round-bottomed three-neck flask equipped with a dropping funnel, a refluxing system, and a mechanical stirrer. The reaction mixture was heated on a glycerol bath at 35°C and a solution of 3-phenoxybenzoate chloride (7.3 g. 31.25 mmol) (**VI**) in anhydrous chloroform (12.5 mL) was added dropwise. The mixture was stirred at 35°C for 3 h and diluted with 20% HCl (32 mL). The mixture was refluxed under intensive stirring for 3 h, cooled to room temperature, and the aqueous layer was separated. The organic layer was washed with water (6 × 10 mL), dried with magnesium sulfate, filtered, and evaporated. The residue was concentrated in vacuum at 10–15 mm Hg. The resulting oil was slowly crystallized under cooling to give 2-(3-phenoxybenzoyl)cyclohexanone (**IX**) (6.4 g, 21 mmol, 70%); mp 141–143°C/10 mm Hg. Mass (*m/z*): 197 (82%,

$[C_6H_5OC_6H_4C(O)]^+$), 294 (100%, $[M]^+$), 295 (25%, $[M + 1]^+$). Calc.: *M* 294 ($C_{19}H_{18}O_3$). ^{1}H NMR: 1.81–2.27 (s, 8H, CH_2); 3.73 (m, H, CH); 7.14–7.79 (m, 9H, $C_6H_5OC_6H_4$).

***N,N*-Dimethyl-2-(3-phenoxybenzoyl)ethylamine hydrochloride (XI).** Morpholine hydrochloride (2.84 g, 23 mmol) and paraform (1.065 g, 35.5 mmol) were added to a solution of 3-phenoxyacetophenone (5.19 g, 23 mmol) in isopropanol (30 mL). The reaction mixture was saturated with dry hydrogen chloride to pH 0–1.0 and refluxed for 20 h. The solvent was removed at reduced pressure and the residue was dissolved in diluted hydrochloric acid and washed with ether. The aqueous layer was alkalized with potassium carbonate to pH 8.0–9.0 and extracted with ether. The combined ether extracts were washed with water (100 mL) and dried. After the drying agent was removed a solution of dry hydrogen chloride in anhydrous isopropanol was added and kept at 0°C for 18 h. The precipitate was filtered off, washed with an isopropanol–ether mixture, air-dried to a constant weight, and recrystallized from isopropanol to give 25% of the product; mp 165–166°C. IR: 936–688, 1520–1496 (Ar); 1700 (C=O); 1496 (CH_3, CH_2); 1364–1340 (–C–N–); 1252–1192 (–C–O–C–). ^{1}H NMR: 2.39 (s, 6H, CH_3); 2.65–2.76 (m, 4H, CH_2); 6.84–8.0 (m, 9H, $C_6H_5OC_6H_4$).

Pharmacological Tests

The antagonistic activity toward angiotensin AT_1 receptors was *in vitro* tested on isolated portal veins of outbred rats of both sexes as described in [10] using a modification with the Krebs buffer solution (pH 7.4) at the constant oxygenation with 95% O_2–5% CO_2 and thermostating at 24°C [11].

The activity of the compounds was assessed by the inhibition of the spasmogenic effect of the isolated portal vein induced by 0.01 μM angiotensin II. The contractions were registered with an isotonic 7006 transducer and a four-channel digital recorder at the isotonic load of 0.5 g in a single-chamber organ bath (UgoBasile, S.R.L., Italy) and a LabScribe3.0™ software (iWorx Systems, Inc., United States). The compounds were tested at a concentration of 10 μM. As a reference, the valsartan preparation, an AT_1 antagonist (Sigma, United States), was used.

The effects of the compounds at a concentration of 100 μM on the platelet aggregation of Chinchilla rabbits were determined in vitro as described in [12]. The platelet aggregation was induced with 5 μM ADP (Reanal, Hungary). The study was performed on a two-channel laser 230 LA analyzer of platelet aggregation (NPF Biola, Russia). The activity was calculated as a ratio of the reduction of ADP-induced platelet aggregation in the presence of the compound tested to that of the control compounds (as Δ%). Acetylsalicylic acid was used as a reference compound (Sigma, United States).

The properties of the compounds to break cross-links of glycated proteins in vitro were determined as described in [13]. The protein glycation reaction was modeled in the reaction mixture containing glucose (400 mM) and BSA (0.8 mg/mL) dissolved in 50 mM phosphate buffer (pH 7.4). The mixture was incubated at 60°C for 40 h and a mixture of BSA and glucose (200 μL of each) was added into each of the Eppendorf tubes followed by the addition of 100% TCA (20 μL). The mixture was centrifuged at 15000 rpm for 4 min and 50 mM phosphate buffer (pH 7.4) was added to the precipitated glycated BSA to the volume of 300 μL. A solution of the tested compound (30 μL) was added (the volume of the reaction mixture was 330 μL) and the mixture was incubated at 60°C for 40 h. In addition, a similar number of samples were prepared with nonglycated nonprecipitated BSA (300 μL in each sample), in which the tested compound was added, and the mixture was incubated. All the compounds were dissolved in DMSO (Fisher Scientific, United States). The tested compounds at a final concentration of 1 mM were added to the samples and the proper solvent volume, into the control solutions. After the incubation was completed the precipitated glycated and nonglycated BSA were dissolved in the phosphate buffer (1 mL, pH 10.0) and specific fluorescence of the samples was determined on a microplate reader (Infinite M200, Tecan, Austria) at λ_{ex} 370 nm and λ_{em} 440 nm.

The capacity of the tested compounds to break cross-links of glycated proteins was calculated by formula (1):

$$Ac\ (\%) = ((Fc - Fb) - (Fs - Fsb)/(Fc - Fb)) \times 10, \quad (1)$$

where Fc is the fluorescence of incubated BSA, glucose, and DMSO (control); Fb, the fluorescence of incubated BSA (nonglycated); Fs, is the fluorescence of incubated BSA, glucose, and the tested compound; Fsb, the fluorescence of incubated BSA (nonglycated) and the tested compound. Alagebrium was used as a reference compound.

The capacity of the tested compounds to inhibit DPP-4 was assessed as described in [14]. A solution of the tested compound (0.1 mM, 10 μL), 0.1 M Tris-HCl (pH 8.0, 50 μL), and human plasma (40 μL) were preincubated for 5 min at 37°C. The reaction substrate *p*-nitroanilide Gly-Pro (Sigma, United States) (1 mM, 100 μL) was added and the mixture was incubated for 15 min at 37°C. Intensification of yellow color due to the release of 4-nitroaniline was determined at 405 nm using a microplate reader (Infinite M200, Tecan, Austria). Vildagliptin (Sigma, United States) was used as a reference compound.

For the evaluation of the inhibitory activity toward GP, a mixture of 50 mM HEPES buffer (pH 7.2, 100 μL) containing 100 mM potassium chloride,

2.5 mM magnesium chloride, 0.5 mM glucose 1-phosphate (Sigma, United States), and 1 mg/mL glycogen was preincubated for 30 min with muscular rabbit α-GP (Sigma, United States) and the tested compounds at 30°C. After preincubation 1 M HCl (150 μL) containing 10 mg/mL ammonium molybdate and 0.38 mg/mL malachite green were added and the released inorganic phosphate was measured in 20 min. The intensification of the color was determined at 620 nm with a microplate reader (Infinite M200, Tecan, Austria) [15]. As a reference compound, CP-316819 (Sigma, United States) was taken. For assaying the activity of the compounds under study and CP-316819 their solutions in 14% DMSO were used, which were added to the reaction mixture at a concentration of 0.1 mM. Into the control mixture, only a solvent was added.

The antiglycation activity of the compounds was determined in vitro as described in [16]. The glycation reaction was modeled in the reaction mixture containing 500 mM glucose and 1 mg/mL BSA dissolved in a phosphate buffer (pH 7.4). The compounds were dissolved in DMSO. The compounds under study at a final concentration of 1 mM were added to the samples and the solvent in a proper volume was added to the control solutions. All the solutions were incubated for 24 h at 60°C. Specific fluorescence of glycated BSA was measured on an F-7000 spectrofluorimeter (Hitachi, Japan) at λ_{ex} 370 nm and λ_{em} 440 nm. The antiglycation activity was calculated relative to the fluorescence of the controls. As a reference compound, aminoguanidine, a known inhibitor of nonenzymatic glycosylation, was used [17].

Antioxidant activity was studied in vitro at a 10 μM concentration of the compounds using a model of ascorbate-dependent LPO [18]. As a substrate, 4% homogenate of rat liver was used. The LPO was initiated by the addition of 50 mM ascorbic acid (Chemapol, Czech Republic). The oxidation rate was evaluated by the accumulation of TBA-positive products in the reaction with TBA (Fluka, Switzerland).

The optical density of the colored product was measured at a wavelength of 532 nm on a PD-303UV spectrophotometer (APEL, Japan) in a cuvette with an optical path length of 10 mm. The activity of the compounds was expressed in percent relative to the control. As a reference compound, butyloxytoluene (dibunol) (Merck, United States) was used. Statistical analysis was performed with the Mann–Whitney nonparametric test, Microsoft Excel 2007, and the GraphPad Prism 5.0. program.

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