published by Ural Federal University
eISSN 2411-1414
chimicatechnoacta.ru

LETTER
2025, vol. 12(2), No. 12215
DOI: 10.15826/chimtech.2025.12.2.15

Synthesis of 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazines and study of antiglycation activity

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Abstract

A novel method for the synthesis of a series of 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazine derivatives was developed using ethyl-3-morpholinoacrylate as a key azo component. The synthesized compounds were evaluated for their antiglycation activity. It was found that the obtained compounds demonstrated significantly higher efficacy compared to aminoguanidine as a well-known reference drug. Among the derivatives, compound 9g exhibited the most potent activity, surpassing aminoguanidine by 2.1 times with IC50 values of 999.0 μM and 2134.5 μM, respectively. The use of the MTT assay allowed investigating the cytotoxicity of the lead compound, which showed the absence of cytotoxic properties and the presence of a cytovitalizing effect at a concentration of 10 μM, indicating its potential as a promising candidate for the development of new antidiabetic agents. These findings highlight the potential of azolotriazine derivatives as a foundation for further pharmacological exploration and drug design in the treatment of diabetes and its complications.

Accompanying information

Article history
Received: 21.03.25
Revised: 13.04.25
Accepted: 22.04.25
Available online: 25.04.25

Keywords
azolotriazines, antidiabetic activity, antiglycation activity, synthesis, azo-coupling, cytotoxicity

Funding
The work was carried out under the agreement with The Russian Science Foundation No. 23-73-01099.

Supplementary information
Supplementary materials: READ
Transparent peer review: READ

Sustainable Development Goals

1. Introduction

Diabetes mellitus (DM) and diabetic nephropathy (DN) have reached epidemic proportions worldwide, affecting millions of people. Today, more than 536.6 million individuals suffer from DM [1], leading many clinicians to classify it as a non-communicable epidemic. DN is a major contributor to disability and mortality among diabetic patients.

A key factor in DN progression is the accumulation of Advanced Glycation End Products (AGEs). Hyperglycemia promotes excessive AGE formation through non-enzymatic glycation of proteins, lipids, and nucleic acids. In DN, declining glomerular filtration rate (GFR) further impairs AGE excretion, while oxidative stress accelerates their accumulation. Both direct AGE toxicity and their interaction with the Receptor for Advanced Glycation End Products (RAGE) activate inflammatory and fibrotic pathways, worsening DN progression [2].

Given the growing impact of DM, reducing its medical and social impact is a key priority in healthcare. AGE inhibitors have demonstrated potential in mitigating diabetic complications, including DN [3]. Therefore, the search for novel inhibitors of non-enzymatic protein glycation remains an active and promising area of research.

Numerous compounds with antiglycation properties have been identified, including aspirin [4], aminoguanidine [5], *N*-phenacylthiazolium bromide [6], quercetin, and various flavonoids [7]. However, existing AGE inhibitors have several limitations, such as poor pharmacokinetics, adverse side effects and insufficient efficacy. Aminoguanidine, one of the most promising candidates, was discontinued in clinical trials due to toxicity concerns. Thus, developing new

strategies to regulate AGE levels is a promising approach for treating the DM complications.

Heterocyclic compounds have demonstrated antiglycation activity in the micromolar range. Their structural flexibility and improved metabolic stability make them attractive candidates for further development [8].

Recent studies have shown that azoloazines are promising heterocyclic compounds with potential antiglycation and antidiabetic properties [9,10]. These compounds exhibit a wide range of biological activities, including antiviral [11–15] and antimicrobial effects [16,17], and ace as adenosine receptor agonists [18,19]. In addition to these effects, azolotriazine derivatives possess notable antioxidant properties [20,21] and antiglycation activity [9, 10, 22]. The most pronounced antiglycation and antidiabetic effects have been observed in azolo[5,1-c][1,2,4]triazines containing electron-withdrawing substituents, such as nitrile, ethoxycarbonyl, or nitro groups, at the third position of the triazine ring [10].

Our previous research demonstrated that 3-cyano-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazines and 3-nitro-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazines exhibit significant antiglycation activity [22]. To refine the structure of promising antidiabetic molecules and discover new heterocyclic compounds with comparable activity, in this study we synthesized a new series of 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazines and evaluated their antiglycation activity.

2. Experimental

Commercial reagents were procured from Sigma-Aldrich, Acros Organics, or Alfa Aesar and utilized without any preliminary processing. All workup and purification procedures employed analytical-grade solvents. One-dimensional ^{1}H and ^{13}C NMR spectra were acquired using a Bruker DRX-400 instrument (Karlsruhe, Germany) at 400 and 101 MHz, respectively, with DMSO-d_6 and $CDCl_3$ serving as solvents and external references. Elemental analysis was carried out on a PerkinElmer 2400 CHN analyzer (USA). IR spectra were recorded on a Bruker Alpha spectrometer with an ATR module equipped with a ZnSe crystal. Melting points were determined using a Staffordshire ST150SA instrument. Reaction progress was monitored via TLC on Silufol UV254 plates. Mass spectra were obtained using a Shimadzu GCMS-QP 2010 Ultra mass spectrometer with electron impact ionization (EI, 70 eV, 40–200 °C).

2.1. Method for the synthesis of ethyl-3-morpholinoacrylate (3)

A mixture of morpholine **2** (0.2 mol), triethyl orthoformate **4** (0.2 mol), and monoethyl malonate **6** (0.2 mol) was heated under reflux at 140 °C (oil bath) with stirring for 3 hours. Upon completion, volatile components were removed under reduced pressure. The crude residue was dissolved in 200 mL of dichloromethane, washed with 1M $NaHCO_3$ (100 mL) and 100 mL of water. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography on silica gel using dichloromethane as the eluent. Yield 29.64 g (80%), Yellow thick liquid. ^{1}H NMR (DMSO-d_6 , δ, ppm) 7.20 (d, H, *J* = 13.2 Hz, CH), 4.54 (d., H, *J* = 13.2 Hz, CH), 3.97 (q., 2H, *J* = 7.1 Hz, CH_2), 3.63 – 3.43 (m, 4H, 2*CH_2), 3.06 (t., 4H, *J* = 5.0 Hz, 2*CH_2), 1.10 (t., 3H, *J* = 7.1 Hz, CH_3); ^{13}C NMR (Chloroform-*d*, δ, ppm): 169.25, 160.76, 151.62, 85.83, 66.05, 59.97 – 57.71 (m), 48.49, 40.46, 14.50. IR spectrum, ν, cm^{-1} 2972, 2900, 2855, 1675, 1604, 1144, 1108, 1019, 788. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 185 $[M]^+$ (47.3).

2.2. General synthetic procedure for 4-Hydroxy-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazin-3-ethoxycarbonyl Derivatives (9a–g)

A solution of $NaNO_2$ (0.936 g, 0.011 mol) in water (3 mL) was added dropwise under stirring to a cooled (−7 to −10 °C) mixture of the corresponding 3-amino-1,2,4-triazole (0.01 mol), water (5 mL), acetonitrile (5 mL), and concentrated HCl (10 mL, 0.12 mol). The reaction mixture was maintained at this temperature for 10 min. Subsequently, a solution of ethyl 3-morpholinoacrylate (1.58 g, 0.01 mol) in acetonitrile (60 mL) was added, and the resulting mixture was stirred at 0 °C for 1 h, then allowed to warm to room temperature and stirred for an additional 6 h. The resulting precipitate was collected by filtration, washed with the acetonitrile–water mixture (1:1, v/v), and dried in air.

2.2.1. 4-Hydroxy-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazine-3-ethoxycarbonyl (9a)

Yield 1.69 g (80%), White powder. mp=180–182 °C; ^{1}H NMR (DMSO-d_6, δ, ppm): 12.78 (s, H, NH), 7.92 (s., H, H7), 7.61 (d., H, *J* = 7.7 Hz, OH), 6.61 (d., H, *J* = 7.7 Hz, H4), 4.26 (q., 2H, *J* = 7.1 Hz, CH_2), 1.29 (t., 3H, *J* = 7.1 Hz, CH_3);^{13} C NMR (DMSO-d_6, δ, ppm): 162.44, 150.38, 146.41, 131.23, 71.5, 60.99, 14.05. Found: C 39.83; H 4.29; N 33.04. Calculated: C 39.81; H 4.30; N 33.16. IR spectrum, ν, cm^{-1} 1101, 1682, 3116. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 193 $[M\text{-}H_2O]^+$ (1.75), 149, 121.

2.2.2. 7-Methyl-3-ethoxycarbonyl-4-hydroxy-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazine (9b)

Yield 1.96 g (77%), White powder. mp=200-202 °C; 1H NMR (DMSO-d6, δ, ppm): 12.59 (s., H, NH), 7.42 (d., H, J = 7.1 Hz, OH), 6.48 (d, H, J = 7.2 Hz, H4), 4.28 (q., 2H, J = 7.2 Hz, CH2), 2.26 (s., 3H, CH3), 1.34 (t., 3H, J = 7.1 Hz, CH3); 13C NMR (DMSO-d6, δ, ppm): 163.02, 159.41, 147.15, 131.60, 71.57, 61.45, 14.55, 14.46. Found: C 42.68; H 4.94; N 31.27. Calculated: C 42.67; H 4.92; N 31.10. IR spectrum, ν, cm^{-1} 1060, 1724, 3179. Mass spectrum (EI, 70 eV), m/z (Irel (%)): 207 $[M\text{-}H_2O]^+$ (2.44), 163, 135.

2.2.3. 3-Ethoxycarbonyl-4-hydroxy-7-methylthio-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazine (9c)

Yield 1.77 g (69%), White powder. mp=124–126 °C; ^{1} H NMR (DMSO-d_6 , δ, ppm): 12.71 (s., H, NH), 7.52 (d., H, *J* = 7.7

 DOI: 10.15826/chimtech.2025.12.2.15

Hz, OH), 6.51 (d., H, *J* = 7.7 Hz, H4), 4.30 (q., 2H *J* = 7.1 Hz, CH_2), 2.54 (s., 3H, CH_3), 1.35 (t., 3H, *J* = 7.1 Hz, CH_3); ^{13}C NMR (DMSO-d_6, δ, ppm): 162.84, 160.57, 147.71, 132.16, 71.80, 61.55, 14.53, 14.03. Found: C 37.47; H 4.33; N 27.36. Calculated: C 37.35; H 4.33; N 27.36. IR spectrum, ν, cm^{-1} 1013, 1236, 1556, 1682, 3112. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 239 $[M\text{-}H_2O]^+$ (42.06), 195, 167.

2.2.4. 3,7-Diethoxycarbonyl-4-hydroxy-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazine (9d)

Yield 2.28 g (81%), White powder. mp=200–201 °C (deg.). ^{1} H NMR (DMSO-d_6, δ, ppm): 12.94 (s, H, NH), 7.92 (s, H, OH), 6.65 (s, H, H4), 4.26-4.38 (m., 4H, 2*CH_2), 1.27–1.33 (m., 6H, 2*CH_3); ^{13}C NMR (DMSO-d_6, δ, ppm): 162.67, 159.89, 159.50, 152.96, 147.60, 132.50, 72.77, 14.51, 14.47. Found, %: C 42.57; H 4.69; N 24.72. Calculated, %: C 42.41; H 4.63; N 24.73. IR spectrum, ν, cm^{-1}: 1095, 1191, 1569, 1720, 3261. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 265 $[M\text{-}H_2O]^+$ (3.11), 221, 193, 68.

2.2.5. 3-Ethoxycarbonyl-4-hydroxy-7-trifluoromethyl-1,4-dihydro-1,2,4-triazolo[5,*1*-c][1,2,4]triazine (9e)

Yield 1.36 g (49%), Yellow powder. mp=169–171 °C; ^{1}H NMR (DMSO–d_6, δ, ppm): 13.11 (s., H, NH), 7.97 (d., H, *J* = 7.8 Hz, OH), 6.68 (d., H, *J* = 7.8 Hz, H4), 4.30 (q., 2H, *J* = 6.9 Hz, CH_2), 1.30 (t., 3H, *J* = 6.9 Hz, CH_3); ^{13}C NMR (DMSO-d_6, δ, ppm): 162.03, 150.67 (q, J = 38.9), 147.52, 132.49, 119.20 (q, J = 269.8), 72.62, 61.25, 13.98. Found: C 34.68; H 2.79; N 25.10. Calculated: C 34.42; H 2.89; N 25.09. IR spectrum, ν, cm^{-1} 1150, 1688, 3238. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 261 $[M\text{-}H_2O]^+$ (2.42), 217, 189.

2.2.6. 3-Ethoxycarbonyl-4-hydroxy-7-ethylthio-1,4-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazine (9f)

Yield 1.76 g (65%), White powder. mp=110–112 °C;^{1} H NMR spectrum (DMSO-d_6, δ, ppm): 12.80 (s., H, NH), 7.63 (d., H, *J* = 7.6 Hz, OH), 6.53 (d., H, *J* = 7.6 Hz, H4), 4.28 (q., 2H, *J* = 7.0 Hz, CH_2), 3.07 (q., 2H, *J* = 7.2 Hz, CH_2), 1.26–1.35 (m., 6H, 2*CH_3); ^{13}C NMR spectrum (DMSO-d_6, δ, ppm): 162.33, 159.19, 147.10, 131.64, 71.29, 61.03, 25.10, 15.05, 14.03. Found: C 39.80; H 4.74; N 25.79. Calculated: C 39.84; H 4.83; N 25.81. IR spectrum, ν, cm^{-1} 1093, 1295, 1566, 1710, 3275. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 253 $[M\text{-}H_2O]^+$ (100), 181, 85.

2.2.7. 4-Hydroxy-3,8-diethoxycarbonyl-1,4-dihydropyrazolo[5,*1*-c][1,2,4]triazine (9g)

Yield 2.53 g (90%), Yellow powder. mp=158–160 °C; ^{1}H NMR (DMSO-d_6, δ, ppm): 12.21 (s., H, NN), 7.89 (s., H, H7), 7.58 (s., H, OH), 6.49 (s., H, H4), 4.33 – 4.21 (m., 4H, 2*CH_2), 1.25–1.33 (m., 6H, 2*CH_3); ^{13}C NMR (DMSO-d_6, δ, ppm): 163.05, 162.10, 141.27, 138.75, 131.95, 95.56, 70.27, 61.51, 60.09, 14.85, 14.52. Found: C 42.36; H 4.57; N 24.75. Calculated: C 42.41; H 4.63; N 24.73. IR spectrum, ν, cm^{-1} 1139, 1688, 3268. Mass spectrum (EI, 70 eV), *m/z* (I_{rel} (%)): 264 $[M\text{-}H_2O]^+$ (16.32), 219, 192.

2.3. Bioassays

2.3.1. Calculation of energies of frontier molecular orbitals

The calculations were executed through the sequential optimization of three-dimensional molecular geometries and the computation of frontier molecular orbital energies for the ultimately formed structures. Initial two-dimensional models were generated using ChemDraw 19.0. Preliminary optimization of the 3D structures involved rotating torsionally flexible bonds via the Dihedral Driver function in Chem3D 19.0. Subsequently, the resulting conformations were refined using the MM+ molecular mechanics method and the PM3 semiempirical method in HyperChem 8.0.8. Final optimization and molecular orbital energy calculations were conducted using the *ab initio* method with the 6-31G(d,p) basis set in HyperChem 8.0.8.

2.3.2. Study of anti-glycation activity

The model for investigating anti-glycation properties was based on the glycation of bovine serum albumin (BSA) with glucose. The reaction medium comprised glucose (0.36 M) and BSA (1 mg/ml) dissolved in phosphate buffer solution (PBS, pH 7.4, 0.05 M). The compounds under study were dissolved in 99% dimethyl sulfoxide (DMSO). Aminoguanidine served as a reference anti-glycation agent. The final concentration of all compounds in the reaction medium was 1000 or 100 μM, whereas for aminoguanidine it was 3333 and 1000 μM due to its anticipated lower activity. An equivalent volume of solvent was added to the control samples. The samples were incubated at 60 °C for 24 h. After the incubation, the protein was precipitated with trichloroacetic acid and centrifuged (15000 rpm, 4 min, 4 °C). The supernatant was discarded, and the protein precipitate was washed with phosphate buffer solution. The dissolution was repeated in phosphate buffer solution (pH 10.5). [27]

The fluorescence of AGEs was quantified in the samples at excitation wavelengths of λ_{ex} = 370 nm and emission wavelengths of 440 nm using a spectrofluorimeter M 200 PRO (TECAN). Logarithmic normalization, as shown in Equation 1, was employed to mitigate false positives potentially arising from compounds that suppress the fluorescence of AGEs, independent of the inhibition of AGEs formation:

$$Flu(lg) = 10^{(lg(Exp)-lg(Blank))}-1, \quad (1)$$

where Flu(lg) denotes the normalized fluorescence intensity of AGEs, while lg(Exp) and lg(Blank) represent the decimal logarithms of the actual fluorescence levels of glycated and corresponding unglycated samples, respectively.

The activity of compounds that neither inhibited the intrinsic fluorescence of AGEs nor exhibited fluorescence at the specified wavelengths was quantified using Equation 2:

$$Flu(lin) = Exp - Blank, \quad (2)$$

where Flu(lin) represents the fluorescence intensity of AGEs, while Exp and Blank denote the actual fluorescence

 DOI: 10.15826/chimtech.2025.12.2.15

levels of glycated and corresponding unglycated samples, respectively.

The activity, defined as the percentage suppression of AGEs fluorescence, was calculated using the following equation:

$$\% = 100-(Flu(Exp)\cdot 100/Flu(Contr)), \quad (3)$$

where Flu(Exp) and Flu(Contr) are the fluorescence intensity of AGEs in experimental and control samples, respectively (lg-normalised or non-lg-normalised).

2.3.3. Cytotoxicity study on peritoneal macrophages

The cytotoxicity of the lead compound 9g was analyzed at concentrations of 10 μM using the MTT assay. Peritoneal macrophages (PM) were isolated from the peritoneal exudate of albino mice. All experiments were conducted in accordance with the requirements of GOST ISO/ IEC 17025-2009 and the Animal Research: Reporting In Vivo Experiments (ARRIVE) 2.0 guidelines. Ethical approval was granted by the local ethics committee of the Volgograd State Medical University. IRB registration number 00005839 IORG 0004900 (OHRP). The method of anesthesia in mice includes premedication with xylazine at a concentration of 20 mg/ml at a dose of 0.05 ml/kg body weight; the substance is administered intraperitoneally [28]. To facilitate the accumulation and activation of PM, the mice received an intraperitoneal injection of 1 ml of a 3% peptone solution. After a period of three days, the mice were humanely euthanized via cervical dislocation. Peritoneal exudate cells were subsequently harvested through aseptic lavage of the abdominal cavity using 5 ml of sterile Hanks' solution, maintained at 4–6 °C, devoid of calcium and magnesium ions. The lavage was subjected to centrifugation at 250 g, the supernatant was carefully removed, and the resultant pellet was resuspended to yield a cell suspension. The total cell count was determined using a Goryaev counting chamber, and cell viability was assessed via 0.4% trypan blue exclusion. The cell concentration was meticulously adjusted to $2.0\cdot10^6$ cells/ml in complete DMEM medium, supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were then seeded at a density of 200 μl/well into 96-well plates and incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by washing the wells with Hanks' solution. Following a 24-hour incubation period, 20 μL of the supernatant was extracted, and 20 μM of the test substance was introduced with a final concentration in the wells of 10 μM, followed by an additional 24-hour incubation at 37 °C in a 5% CO_2 atmosphere. Subsequently, the MTT assay was conducted in accordance with the established protocol.

2.3.4. Determination of cell viability by MTT test

The impact of the of the synthesized lead compound on cellular viability was evaluated utilizing the colorimetric MTT assay. Following a 24-hour incubation period with the test substance, the cells were exposed to an MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS) at a 1:10 ratio and incubated for an additional 2.5 hours. Upon completion of the incubation, the MTT medium was carefully aspirated, and 150 μl of DMSO was introduced to solubilize the resultant formazan crystals. The plates were subsequently agitated for 10 min. Optical density was then measured at a wavelength of 565 nm.

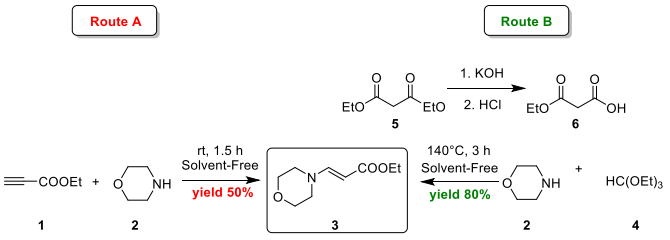
3. Results and discussion

3.1. Chemistry

Ethyl-3-morpholinoacrylate was selected as a key precursor for the synthesis of the target 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-c][1,2,4]triazines. The literature reports its preparation through the reaction of ethyl propionate (**1**) and morpholine (**2**), followed by chromatographic purification [23]. Although literature sources suggest a nearly quantitative yield (~100%), our attempts to replicate this synthesis resulted in a yield of approximately 50% (Scheme 1, Route A).

To improve the efficiency and scalability of the process, we developed an alternative method for synthesizing ethyl-3-morpholinoacrylate (**3**) *via* a three-component condensation reaction. This reaction involved morpholine (**2**) triethyl orthoformate (**4**), and monoethyl malonate (**6**). Monoethyl malonate was prepared according to a previously established method [24]. The condensation was carried out under reflux for four hours, followed by chromatographic purification, yielding the product in 80% yield (Scheme 1, Route B).

Ethyl-3-morpholinoacrylate (**3**) was employed as a coupling agent in the azo-coupling reaction with azolyl diazonium chlorides (**8a-g**). The reaction was carried out using our previously developed methodology, which involves conducting the azo-coupling reaction in an excess of hydrochloric acid [15]. This approach allowed the reaction to proceed efficiently, without the need to isolate intermediate hydrazines, as shown in Scheme 2. The process successfully yielded the target 3-ethoxycarbonyl-4-hydroxy-1,4-dihydro-azolo[5,1-c][1,2,4]triazines (**9a-g**), with yields ranging from 49% to 90%, depending on the structure and solubility of the starting aminoazoles. The low yield of compound **9e** is likely attributed to its increased solubility, which complicates isolation. This behavior can be rationalized by the presence of a trifluoromethyl (CF_3) group in the molecule, as such substituents are known to enhance solubility in water and organic solvents [25].



Scheme 1 Preparation of 3-ethylmorpholinacrylate **3**.

 DOI: 10.15826/chimtech.2025.12.2.15

In the ^{1}H NMR spectra of compounds (**9a-g**), two characteristic signals are observed: a doublet for the proton H4 of the triazine ring at δ = 6.3–6.5 ppm and a doublet for the hydroxyl group at δ = 8.0–8.2 ppm, alongside signals corresponding to the substituents in the azole ring. Similarly, the ^{13}C NMR spectrum exhibits a characteristic signal at δ = 72–74 ppm (C4). The IR spectrum exhibits two distinct absorption bands in the regions of 1740–1685 cm^{-1} and 1100–1200 cm^{-1}, which are characteristic of the ethoxycarbonyl functional group. In the spectrum of compound (**9e**), an absorption band characteristic of the trifluoromethyl group is observed at 1150 cm^{-1}. Additionally, it was found that all compounds in the series undergo dehydration during mass spectrometric analysis (see Supporting file).

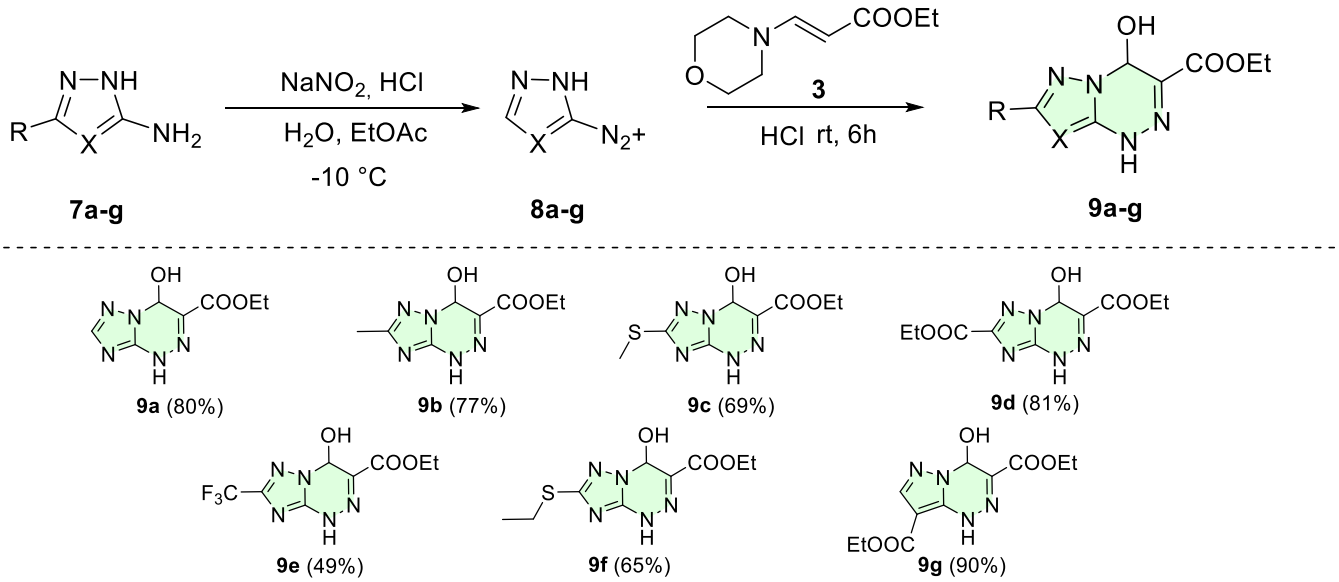
3.2. Calculation of energies of frontier molecular orbitals

The calculations show that the energy values of the α and ß frontier molecular orbitals are nearly identical, suggesting their potential for similarly effective use in predicting pharmacological activity. The detailed results are presented in Table 1.

Correlation analysis of the energies of the frontier molecular orbitals and antiglycation activity reveals no statistically significant rank correlation between the calculated values and the antiglycation activity. However, a trend is observed in the correlation between antiglycation activity at 1000 μM concentration and both ELUMO α and ELUMO ß (*rs* = -0.9, *ip* = 0.083). This trend was also observed in earlier analyses at 100 μM concentration with ELUMO α, which can be attributed to the small sample size. Previous studies have identified a relationship between antiglycation activity and orbital energies, including the energy gap (Δ*E*HOMO - LUMO), and established a dependence of activity on these parameters using artificial neural networks [22].

3.3. Study of anti-glycation activity

The *in vitro* antiglycation activity of compounds (**9a–c, f, g**) was assessed, with the results summarized in Table 2. Among the tested compounds, **9g** exhibited the highest activity, demonstrating more than a twofold increase in antiglycation efficacy compared to the reference drug aminoguanidine. This enhanced activity can be attributed to the presence of ethoxycarbonyl groups at the 3rd and 7th positions of the azolotriazine ring. These structural features appear to play a crucial role in enhancing antiglycation activity, as supported by the previous studies [10]. Such investigations showed that similar molecular motifs significantly inhibit the formation of advanced glycation end products (AGEs), suggesting that this structural fragment is key to the observed biological effect. While compounds **9a–c** and **9f** also demonstrated notable antiglycation activity, they were less effective than **9g**.



Scheme 2 Synthesis of 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolotriazines **9a-g.**

Table 1 Results of calculations of energies of frontier molecular orbitals.

Compound	E_{HOMO}α, eV	E_{HOMO}ß, eV	E_{LUMO}α, eV	E_{LUMO}ß, eV	ΔE_{HOMO}α – $_{LUMO}$α, eV	ΔE_{HOMO}ß – $_{LUMO}$ß, eV
9a	−9.541584	−9.54159	2.479632	2.479662	−12.02120	−12.02130
9b	−9.312506	−9.312487	2.62302	2.623003	−11.93550	−11.93550
9c	−8.749205	−8.765175	2.460634	2.247517	−11.20980	−11.01270
9f	−8.624901	−8.642008	2.470538	2.255285	−11.09540	−10.89730
9g	−8.814218	−8.814298	2.310554	2.311109	−11.12480	−11.12540
Aminoguanidine	−9.582258	−9.582258	5.765419	5.765419	−15.34767	−15.34767

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Compound **9g** exhibited high antiglycation activity compared to the reference aminoguanidine, with an IC_{50} value 2.1 times lower (Table 2). These findings underscore the potential of compound **9g** as a promising candidate for further development as an antiglycation agent.

The table presents the antiglycation activity (%) and the half-maximal inhibitory concentration (IC50, μM) for the studied compounds. These compounds were examined at concentrations of 1000 and 100 μM and compared based on their IC50 values. (The detailed description of the methodology is provided in section 3.3.2. Study of anti-glycation activity).

3.4. Cytotoxicity study on peritoneal macrophages

The cytotoxicity assessment on peritoneal macrphages utilizing the MTT assay revealed that the lead compound **9g** does not inhibit cell viability at the specified concentration (10 μM). The extent of viability suppression exhibited negative values (–30.0±0.6%), which, assuming no interference, indicates a moderate cytovitalizing effect. This outcome necessitates comprehensive evaluation and further investigation.

4. Limitations

The antiglycation activity of the synthesized compounds was evaluated only *in vitro* using a bovine serum albumin (BSA) glycation model. While this model is widely accepted, it may not fully replicate the complex biological environment in vivo. Further studies using cell-based assays or animal models are necessary to confirm the efficacy and mechanism of action of these compounds under physiological conditions.

The study compared the activity of the synthesized compounds only with aminoguanidine. Including comparisons with other known antiglycation agents, such as pyridoxamine or ALT-711, would provide a more comprehensive evaluation of their relative efficacy.

5. Conclusion

In this study, we developed a novel and efficient method for synthesizing a series of 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-*c*][1,2,4]triazine derivatives using ethyl-3-morpholinoacrylate as a key azo component.

Table 2 The antiglycation activity of compounds and a reference in a reaction medium containing a phosphate buffer solution using a model of glycation of bovine serum albumin with glucose.

Compound	Activity, 1000 μM, %	Activity, 100 μM, %	IC_{50}, μM
9a	28.8±1.7	7.8±1.7	–
9b	27.7±2.6	4.5±2.5	–
9c	35.4±3.5	10.0±3.1	–
9f	39±1.7	8.9±3.1	–
9g	50.0±1.5	9.6±1.0	999.0
Aminoguanidine	62.2±2.0	32.7±1.5	2134.5

The proposed approach is characterized by high efficiency (yields up to 90%) and simplicity, making it suitable for scale-up and further applications in synthetic chemistry. The synthesized compounds demonstrated significant antiglycation activity *in vitro*, greater that the aminoguanidine, which was used as a standard reference drug. Notably, compound **9g**, bearing ethoxycarbonyl groups at the 3rd and 7th positions, exhibited more than twice the activity of aminoguanidine. Cytotoxicity studies (MTT assay, 10 μM) confirmed the absence of cytotoxicity of the newly synthesized lead compound and the presence of cytovitalizing effect, supporting its potential as antiglycation agents. These findings open new avenues for the development of therapeutics aimed at preventing diabetes-related complications, such as diabetic nephropathy.

To further validate their therapeutic potential, *in vivo* studies are recommended to assess efficacy, safety, pharmacokinetics, and molecular mechanisms of action. A better understanding of these aspects will provide prospects for their clinical application.

In conclusion, 3-ethoxycarbonyl-4-hydroxy-1,4-dihydroazolo[5,1-*c*][1,2,4]triazines are promising candidates for antiglycation drug development. They have potential to improve the quality of life for patients with diabetes and its complications.

Supplementary materials

This manuscript contains supplementary materials (spectra of compounds), which are available on the corresponding online page.

Data availability statement

The data that supports the findings of this study (e.g. spectra of given compounds) are available in the supplementary materials of this article.

Acknowledgments

Spectral data were obtained in the Laboratory of Complex Research and Expert Evaluation of Organic Materials, Center for Collective Use of unique equipment of the Ural Federal University, https://ckp.urfu.ru.

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 DOI: 10.15826/chimtech.2025.12.2.15

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Conflict of interest

The authors declare no conflict of interests.

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 DOI: 10.15826/chimtech.2025.12.2.15