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DF-5 COMPOUND DELAYS DEVELOPMENT OF DIABETIC NEPHROPATHY IN RATS

A.A. Spasov[1,2], O.N. Zhukovskaya[3], A.I. Rashchenko[1], A.A. Brigadirova[1,2], R.A. Litvinov[1,2], N.A. Gurova[1], A.V. Smirnov[1,2], N.G. Pan'shin[1], H.S.A. Abbas[3], A.S. Morkovnik[3]

[1] Volgograd State Medical University,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[2] Volgograd Medical Research Center,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[3] Institute of Physical and Organic Chemistry, Southern Federal University,
Bldg 2, 194, Stachki Ave., Rostov-on-Don, Russia, 344090

E-mail: litvinov.volggmu@mail.ru

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Advanced glycation end-products play an important role in the development of diabetic complications, so slowing down of glycated proteins' cross-links formation have been suggested as a potential therapeutic option for the treatment of vascular diabetic complications and preventing their progression.

The aim of the work was to assess the influence of novel anticrosslinking agent DF-5 on the renal advanced glycation end-products and collagen contents, body weight, blood glucose and glycated hemoglobin levels and the development of early renal disease in streptozotocin-induced diabetic rats.

Materials and methods. 40 male Sprague-Dawley rats were used in the study. Two months after inducing diabetes, the study substance was administered intragastrically once a day for 28 days (12.5 mg/kg). Measurements included the assessment of blood glucose and HbA1c levels, the evaluation of the renal function, and the results of histology and immunohistochemical staining of kidneys.

Results. A repeated intragastric administration of DF-5 for 30 days significantly reduced the level of HbA1c in the blood, but did not affect the level of fasting blood glucose. DF-5 compound significantly reduced proteinuria and prevented kidney damage in experimental animals by limiting damage of the glomeruli and tubules. It was found that DF-5 inhibits the progression of an early renal dysfunction in rats with streptozotocin-induced diabetic nephropathy. This was associated with a decreased accumulation of advanced glycation end-products in the kidney, accompanied by the improvement of both renal morphology and function.

Conclusion. The results obtained provide investigators with additional therapeutic options for the treatment of diabetic nephropathy and possibly other complications of diabetes.

Keywords: advanced glycation endproducts; cross-links; ALT-711 (alagebrium); diabetes mellitus; diabetic kidney disease (nephropathy); streptozotocin-induced diabetes.

Abbreviations: BSA – bovine serum albumin; ECM – extracellular matrix; GBM – glomerular basement membrane; DMSO – dimethyl sulfoxide; IR – infrared spectroscopy; ELISA – enzyme-linked immunosorbent assay; AGEs – advanced glycation end products; m.p. – melting point; NMR – nuclear magnetic resonance; IDF – International Diabetes Federation; HbA1c – glycated hemoglobin.

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СОЕДИНЕНИЕ ДФ-5 ЗАМЕДЛЯЕТ РАЗВИТИЕ ДИАБЕТИЧЕСКОЙ НЕФРОПАТИИ У КРЫС

А.А. Спасов[1,2], О.Н. Жуковская[3], А.И. Ращенко[1], А.А. Бригадирова[1,2], Р.А. Литвинов[1,2], Н.А. Гурова[1], А.В. Смирнов[1,2], Н.Г. Паньшин[1], Х.С.А. Аббас[3], А.С. Морковник[3]

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1

[2] Государственное бюджетное учреждение «Волгоградский медицинский научный центр», 400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1

[3] Научно-исследовательский институт физической и органической химии федеральное государственное автономное образовательное учреждение высшего профессионального образования «Южный федеральный университет», 344090, Россия, г. Ростов-на-Дону, пр-т Стачки, д. 194/2

E-mail: litvinov.volggmu@mail.ru

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Конечные продукты гликирования играют важную роль в развитии осложнений сахарного диабета. По этой причине замедление образования поперечных сшивок гликированных белков, как предполагается, является потенциальным терапевтическим подходом к лечению и профилактике осложнений заболевания, связанных с поражением сосудов.

Цель. Оценка способности нового антисшивающего соединения ДФ-5 влиять на количество конечных продуктов гликирования и коллагена в почках, на массу тела, уровни глюкозы и гликированного гемоглобина, а также на развитие ранних проявлений поражения почек у крыс со стрептозотоциновым сахарным диабетом.

Материалы и методы. Работа проведена на 40 самцах крыс Sprague-Dawley. Через 2 месяца после индукции диабета исследуемое вещество вводили внутрижелудочно (12,5 мг/кг) 1 р/сут в течение 28 дней с помощью зонда. Определяли уровень глюкозы и гликированного гемоглобина в крови, оценивали функцию почек, а также проводили гистологическое и иммуногистохимическое исследования тканей почек.

Результаты. Регулярное внутрижелудочное введение ДФ-5 в течение 30 сут статистически значимо снижало уровень HbA1c в крови, но не влияло на уровень глюкозы в крови натощак. Соединение ДФ-5 существенно уменьшало протеинурию и предотвращало повреждение почек у экспериментальных животных за счет ограничения повреждений клубочков и канальцев. Было установлено, что соединение ДФ-5 замедляет повреждение почек на ранней стадии диабетической нефропатии, что сопровождается снижением количества конечных продуктов гликирования в ткани почек, улучшением их морфологической картины и функции.

Заключение. Полученные результаты открывают возможность для разработки дополнительного терапевтического подхода к лечению диабетической нефропатии и, возможно, других осложнений сахарного диабета.

Ключевые слова: конечные продукты гликирования; поперечные сшивки; ALT-711 (алагебриум); сахарный диабет; диабетическая болезнь почек (нефропатия); стрептозотоцин-индуцированный диабет

Список сокращений: БСА – бычий сывороточный альбумин; ВКМ – внеклеточный матрикс; ГБМ – гломерулярная базальная мембрана; ДМСО – диметилсульфоксид; ИК – инфракрасная спектроскопия; ИФА – иммуноферментный анализ; КПГ – конечные продукты гликирования; $T_{пл}$ – температура плавления; ЯМР – ядерный магнитный резонанс; IDF – Международная диабетическая федерация; HbA1c – гликированный гемоглобин.

INTRODUCTION

Diabetes is a serious chronic disease affecting an increasing number of people worldwide because of its prevalence, costs, and health effects [1]. According to estimates from the World Health Organization (WHO)[1] [2] in 2014, a total of 422 million adults had diabetes and in 2019, an estimated 1.5 million deaths were directly caused by this illness. The International Diabetes Federation (IDF) estimates that there were about 537 million people with diabetes worldwide in 2021, which indicates a rapid spread of the disease.

Diabetes and its complications are rapidly becoming the world's most significant cause of morbidity and mortality. In the case of chronic hyperglycemia, glucose and other reducing sugars react with proteins by a series of non-enzymatic reactions to form a class of

[1] World Health Organization (WHO). Diabetes. Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes

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heterogeneous compounds that are called advanced glycation end products (AGEs) [2, 3]. Some of these AGEs form irreversible cross-links throughout the lifetime of many large proteins (such as collagen and elastin), covalently modifying their structure and damaging their function. AGEs formation in the long-lived connective tissue and extracellular matrix components is a causal factor in the development of long-term diabetic complications and ageing-related diseases, according to many studies [4–10]. AGEs are important in the pathogenesis of such diabetes complications as nephropathy, neuropathy, retinopathy, cataract, cardiomyopathy. AGEs are also involved in the formation of immunopathologies, neoplastic diseases and atherosclerosis. [2, 11, 12].

In case of a diabetic kidney disease (nephropathy), AGEs could accumulate in glomerular basement membrane (GBM), mesangial cells, endothelial cells and podocytes in patients with diabetes. Therefore, AGEs play an important role in the development and progression of nephropathy leading to the formation of glomerulosclerosis and tubulointerstitial fibrosis in the renal tissues [7]. In addition, heavily glycated proteins are more resistant to digestion by the proteasomal – as well as the lysosomal proteolytic systems [13]. The ability of anti-AGE agents to decrease tissue AGEs is believed to be a potential effective therapeutic approach to restore the elasticity of vascular extracellular matrix (ECM) as well as to treat vascular diabetic complications and prevent their progression.

In the present study, the improved procedure of the synthesis of a new representative of anti-crosslinking agents, 9-benzyl-2-biphenylimidazo[1,2-a] benzimidazole (DF-5 compound, **I**) is described, its *in vivo* activity, namely, the influence on glycated hemoglobin (HbA1c) levels, body weight, blood glucose and the development of early renal disease in streptozotocin-induced diabetic rats, is evaluated. The *in vivo* activity of this novel anti-crosslinking agent was compared with that of well-known AGE cross-link breaker ALT-711 (alagebrium) [14].

THE AIM of the work was to assess the influence of novel anti-crosslinking agent DF-5 on the renal advanced glycation end-products and collagen contents, body weight, blood glucose and glycated hemoglobin levels and the development of early renal disease in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Materials

All the chemicals used in the present experiments were of analytical grade and commercially available. Streptozotocin and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma-Aldrich (USA); glucose was purchased from Agat-Med (Russia); bovine serum albumin (BSA), fraction V was purchased from Biowest (France); dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (USA); rabbit polyclonal anti-BSA antibody and goat anti-rabbit IgG secondary antibody were purchased from Cloud-Clone (USA); 10% (w/v) neutral buffered formalin, Masson's trichrome stain and hematoxylin were purchased from Moditech (Russia); homogenized paraffin media was purchased from Bio Vitrum (Russia); rabbit polyclonal anti-AGE antibody was purchased from Abcam (USA); ALT-711 was purchased from Kailu Xingli Pharmaceutical Co., Ltd. (China).

Synthesis of Benzyl-2-biphenylimidazo[1,2-a] benzimidazole (DF-5)

The synthesis of DF-5 compound (**I·HCl**) was carried out in the Institute of Physical and Organic Chemistry at Southern Federal University (Rostov-on-Don, Russia) from 2-amino-1-benzylbenzimidazole (**II**).

IR spectra (v/cm^{-1}) of new compounds were recorded on a Varian Excalibur 3100 FT-IR spectrophotometer (Varian, USA) for powdered samples using the method of attenuated total reflection; ^{1}HNMR spectra were recorded on a Bruker Avance 600 (Germany) spectrometer. Chemical shifts for ^{1}H were given as δ values in parts per million (ppm) relative to the signals of residual protons of a deuterated solvent DMSO-d_6 and $CDCl_3$ (δ 2.49 and 7.24, respectively) and coupling constants were in hertz (Hz).

Melting points were measured on a Fisher-Johns Melting Point Apparatus (Fisher Scientific, USA). The elemental analysis was carried out using a classical method [15]. The reaction progress and purity of the obtained compounds were monitored by thin layer chromatography (TLC) (plates with Al_2O_3 III degree of activity, eluent $CHCl_3$, visualization with iodine vapors in a moist chamber).

2-Imino-3-[(2-biphenyl-4-yl)-2-oxoethyl)]-1-benzylbenzimidazoline hydrobromide (III). 4-(bromoacetyl)biphenyl (2.75 g,10 mmol) was added to a hot solution of 2.23 g (10 mmol) of 2-aminobenzimidazole **II** in 65 mL of MeCN. The mixture was stirred until dissolving of the quaternizating reagent, then heated until the completion of the precipitate formation and kept for 6–8 h at room temperature. After that, the precipitate of salt **III** was filtered off and thoroughly washed with acetone. The yield was 94%, the m.p. was 254–256°C. The IR, v/cm^{-1}, was: 3207, 3240 (NH_2), 1687 (C=O). The following data were established: C, 67.47; H, 4.85; Br, 16.03; N, 8.43%. The ultimate

analysis for $C_{28}H_{24}BrN_3O$ was as follows: C, 67.45; H, 4.88; Br, 16.00; N, 8.39%. ^{1}HNMR (600 MHz, DMSO-d_6), δ: 5.57 (s, 2H, CH_2CO); 6.07 (s, 2H, $N_{Bzm}CH_2$); 7.30–7.36 (m, 5H, 5,6-H_{Bzm} + 3H_{Ph}); 7.40–7.48 (m, 3H, 2H_{Ph} +1H_{Biph}); 7.51–7.55 (m, 3H, 7-H_{Bzm} +2H_{Biph}); 7.67–7.68 (m, 1H, 4-H_{Bzm}); 7.81 (d, 2H, 2H_{Biph}, *J* = 7.2 Hz); 7.97 (d, 2H, H_{Biph}, *J* = 8.4 Hz); 8.19 (d, 2H, H_{Biph}, *J* = 8.4 Hz); 9.06 (s, 2H, N^+H_2).

9-Benzyl-2-(biphenyl-4-yl)-9*H*-imidazo[1,2-*a*] benzimidazole (I). A mixture of bromide **II** (2 g, 4.0 mmol), finely powdered Na_2CO_3 (0.89 g), EtOH (40 mL) and water (9 mL) was refluxed until the reaction completion (20–25 h). Then, the alcohol was distilled off from the reaction mixture, the water (25–30 mL) was added and the product was filtered off and dried in air. The obtained base was purified by column chromatography on Al_2O_3, $CHCl_3$ was used as eluent collecting a fraction with R_f 0.9. After the solvent evaporation, the residue was recrystallized from DMF to obtain 1.3 g (81%) of **I.**

A mixture of 2.0 g (4 mmol) of amine **III** and freshly melted sodium acetate 0.33 g (4 mmol) was boiled in glacial acetic acid (5 hr), controlling the completeness of the reaction by thin layer chromatography. Then the reaction mass was cooled to room temperature, the precipitate of imidazo[1,2-a]benzimidazole **I** was filtered off, washed with water and dried at 85°C. The yield was 1.5 g (92%), the m.p. was 228–229 °C. The IR, ν/cm^{-1}, was 1513, 1617 (C=C), 1664 (C=N). The following data were established: C, 84.21; H, 5.38; N, 10.46%. The ultimate analysis for $C_{28}H_{21}N_3$• HCl was as follows: C, 84.10; H, 5.30; N, 10.52%. ^{1}HNMR (600 MHz), δ: 5.76 (s, 2H, CH_2); 7.29–7.53 (m, 10H, 5H_{Ph}, 6,7-H_{Bzm} +3H_{Biph}), 7.76 (d, 2H, H_{Biph}, *J* = 7.9 Hz), 7.84 (d, 2H, 5,8-H_{Bzm}, *J* = 8.3 Hz), 7.97 (d, 1H, H_{Ar}, *J* = 7.8 Hz), 8.03 (d, 2H, H_{Ar}, *J* = 8.3 Hz); 8.64 (s, 1H, 3-H).

9-Benzyl-2-(biphenyl-4-yl)-9*H*-imidazo[1,2-*a*] benzimidazole hydrochloride (DF-5). This compound was prepared by treatment of imidazo[1,2-*a*]benzimidazole **I** with conc. HCl. The yield was 93%, the m.p. was 237–238°C. The IR, ν/cm^{-1}, was 1512, 1614 (C=C), 1663 (C=N). The following data were established: C, 77.11; H, 5.13; Cl, 8.08; N, 9.58%. The ultimate analysis for $C_{28}H_{21}N_3$• HCl was as follows: C, 77.14; H, 5.09; Cl, 8.13; N, 9.64%.

Cleavage of AGE-BSA-collagen complexes pre-formed *in vitro*

The ability of DF-5 to cause cleavage of pre-formed AGE-BSA-collagen complex was tested by an enzyme-linked immunosorbent assay (ELISA) technique [16] with minor modifications detalized in Patent RU 2627769 C1, Russia (2017) [17].

Collagen was extracted from rat tails by a 0.1% (w/v) solution of acetic acid for seven days, centrifuged (8000 g, 10 min) to precipitate the debris, and used to coat plate wells. The BSA solution (50 mg/mL) was incubated with glucose (0.5 M) in phosphate-buffered saline for three months. The AGE-BSA was added to plate wells and the incubation was carried out at 37°C for 4 h to form cross-links (AGE-BSA-collagen complexes). A solution of DF-5 or ALT-711 was added to the plate wells after the incubation and the plate was incubated at 37°C for 16 h. After washing, 80 µL/well of rabbit anti-BSA antibodies (1:500) were added to the plate wells and the plate was incubated for 50 min at 37°C. After the next washing, 80 µL/well of horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibodies were added (1:1000) to the plate wells and the plate was incubated for 50 min at 37°C. Then 100 µL/well of substrate 3,3′,5,5′-tetramethylbenzidine was added. After the 20-min incubation in darkness at room temperature, the reaction was stopped with 2M H_2SO_4. The absorbance of plate wells was measured at 450 nm with an Infinite M200 Pro microplate reader (Tecan, Austria).

When evaluating the activity of each compound, 4 groups of data were formed: 1) glycated BSA + collagen, 2) glycated BSA + collagen + test compound, 3) non-glycated BSA + collagen, 4) non-glycated BSA + collagen + test compound. To minimize the interference due to the non-specific adhesion of BSA to the collagen matrix, the results of the samples containing glycated BSA, were subtracted from the samples results of the appropriate composition containing non-glycated BSA.

Animals

Animal experiments were conducted in accordance with animal research standards defined by the Russian Federation law (GOST R 33044-2014 and GOST R 33647-2015) and EASC technical standards for Good Laboratory Practice. The study was performed after approval by the Local Research Ethics Committee, Volgograd, Russia [registration number: IRB 00005839 IORG 0004900 (OHRP)] dated June 5, 2015 (Protocol No. 2016-2015).

Male Sprague-Dawleyrats (5–6 weeks old, weighing 200–230 g) were purchased from LLC “NIC BMT”, Moscow, Russia. The animals were housed at Volgograd State Medical UniversityAnimal Care Unit. The animals were acclimated to the housing environment in a room with a 12/12-hour light/dark cycle at an ambient temperature of 25°C for 2 weeks. The animals had a free access to food and water before the study.

Diabetic model of rats

Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (65 mg/kg in citrate buffer, pH 4.5) after an overnight fast. The

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procedure was performed in 40 animals. Additionally, non-diabetic animals were injected with a citrate buffer only (n=10). Three days after the streptozotocin injection, only rats with blood glucose levels exceeding 15 mmol/L measured in fasting conditions were classified as diabetic and included in the study (n=30). The animals that had failed to reach this criterion were excluded from the current experiment (n=10). Five diabetic animals died during the next two months after the streptozotocin injection (before the treatment and groups formation).

The choice of the effective dose (12.5 mg/kg) for ALT-711 and DF-5 was based on the experimental studies results of ALT-711 in various animal models of diabetic complications described in the scientific literature [18–20]. Two months after inducing diabetes, the animals with diabetes (n=24) were randomly divided into three groups: the untreated diabetic control group (distilled water, 5 mL/kg) and two diabetic treatment groups, receiving either DF-5 or ALT-711 (12.5 mg/kg, dissolved in distilled water, 5 mL/kg). Three animals were excluded from the experiment at the beginning of the treatment: for achieving equal groups' volume – 8 animals per group – 2 non-diabetic rats and 1 diabetic rat. During a 4-week treatment period, the administration was performed *via* intragastral administration once a day in the morning (1 h after the lights were on). The untreated non-diabetic (ND) group of 8 healthy animals habituated to the same regiment and administration (distilled water, 5 mL/kg intragastric) was also included in this study. The animals' blood glucose level and body weight were monitored periodically. The study was carried out for over 12 weeks.

Biochemical analysis

The blood glucose level was measured using a blood glucose meter (Glucocard, Arkray, Japan) after the collection of blood samples from the tail vein. A HbA_{1c} concentration was quantified at the end of the study using a Diabet-Test Assay Kit (HbA_{1c}) (LLC Fosfosorb, Russia) according to the manufacturer's instruction).

Renal function

The renal function was assessed at the end of the study by measuring a daily urine output, a urinary total protein concentration and excretion. During the material collection, the rats were housed in the metabolic cages (Open Science, Russia) for a 24 h urine collection. Total urinary protein was measured using an assay kit (Total Protein-PK-Vital Cat. No. B 06.03, Vital Development Corporation, Russia) according to the manufacturer's instruction.

Kidney histology and immunohistochemistry

The animals were sacrificed *via* decapitation at the end of the experiment (the animals had been anesthetized with chloral hydrate, 400 mg/kg i.p.). The kidneys were removed and the renal cortex and medulla specimens were fixed for 24 h in 10% (w/v) neutral buffered formalin and embedded in paraffin. For the assessment of the injury, 3–5 µm thickness sections were stained with Masson's trichrome to evaluate glomerulosclerotic changes and a connective tissue deposition. Other formalin-fixed kidney sections were mounted on slides and stained with rabbit polyclonal anti-AGE antibodies according to the manufacturer's instructions. After the immunological reaction, the tissues were stained with hematoxylin. Imaging was performed with the Axiostar Plusmicroscope (Carl Zeiss Microscopy GmbH, Germany) and a digital camera Axiocam 105 color (Carl Zeiss Microscopy GmbH, Germany). The images were analyzed with Zeiss Zen Pro 2012 software (Carl Zeiss Microscopy GmbH, Germany). The results were expressed as the percentage of area with positive staining.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., USA). The data were analyzed by ANOVA followed by a Tukey post hoc test used for multiple comparisons versus the control group. The data normality test was carried out using Shapiro-Wilk test. A $p \leq 0.05$ was considered statistically significant. The data are presented as M ±SD.

RESULTS

Chemical synthesis

Compound **I** was synthesized from 2-amino-1-benzylbenzimidazole (**III**) by the reaction sequence depicted in Fig. 1 as described before [21] but with the additionally improved Stage 2. In the first stage, amine **III** was quaternized by an equimolar amount of 4-(bromoacetyl)biphenyl (MeCN, room temperature, 6–8 h) and a nearly quantitative yield of salt **III** was obtained. Then, in the final stage, this salt was cyclized in MeCOOH (reflux, 5 h) in the presence of sodium acetate. The yield of compound **I** was 92%, which was much more than for cyclization of salt **III** in aq. EtOH [21], while the reaction time was much shorter in this case. It has also been notified that in refluxing DMF, the cyclization proceeds was even faster (15–20 min), but with a low yield.

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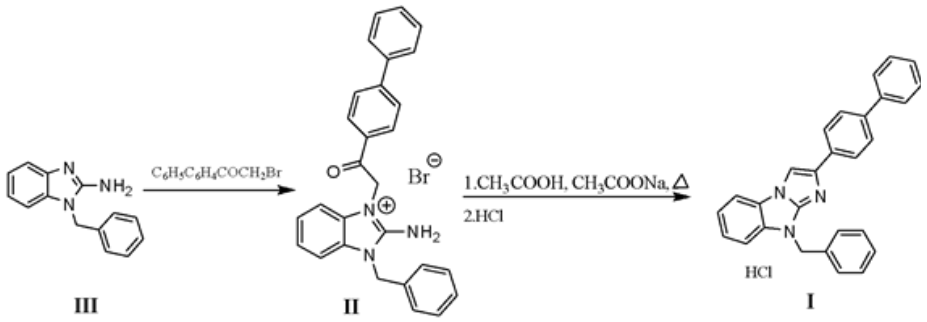

Figure 1 – Synthesis scheme for preparation of 9-benzyl-2-biphenylimidazo[1,2-a]benzimidazole hydrochloride (DF-5)

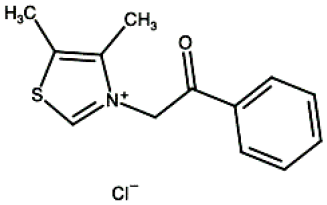

Figure 2 – Chemical structure of ALT-711 (alagebrium)

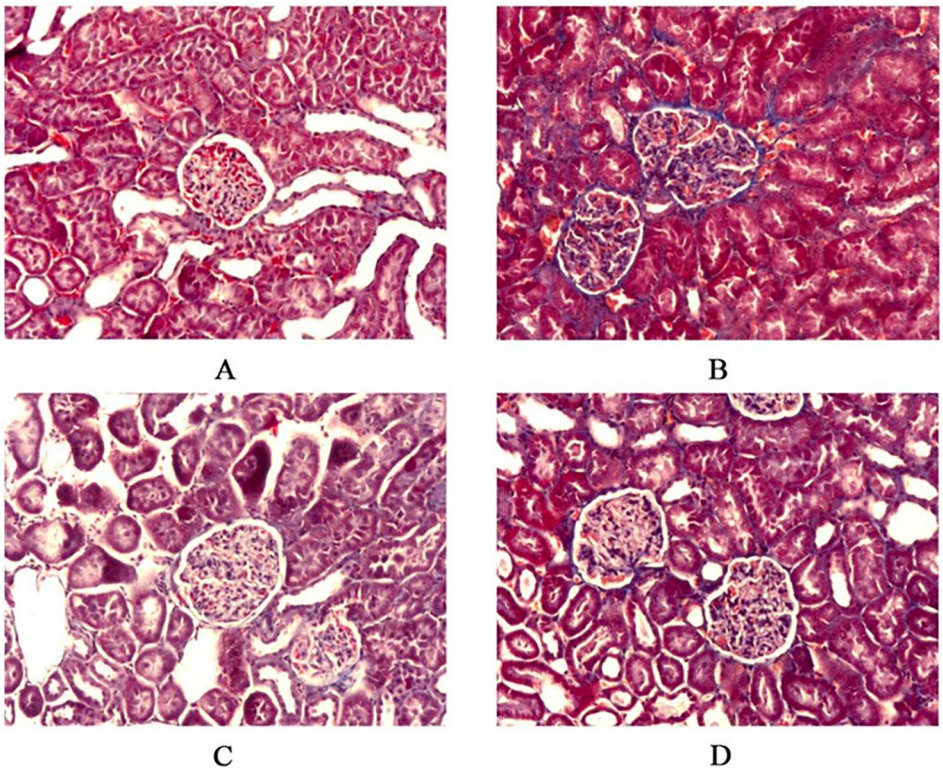

Figure 3 – Glomerular histopathology. Effect of diabetes and either DF-5 or ALT-711 on kidney tissues
Note: Masson trichrome staining: (A) non-diabetic control, (B) diabetic control, (C) diabetic + ALT-711 and (D) diabetic + DF-5. Magnification ×100.

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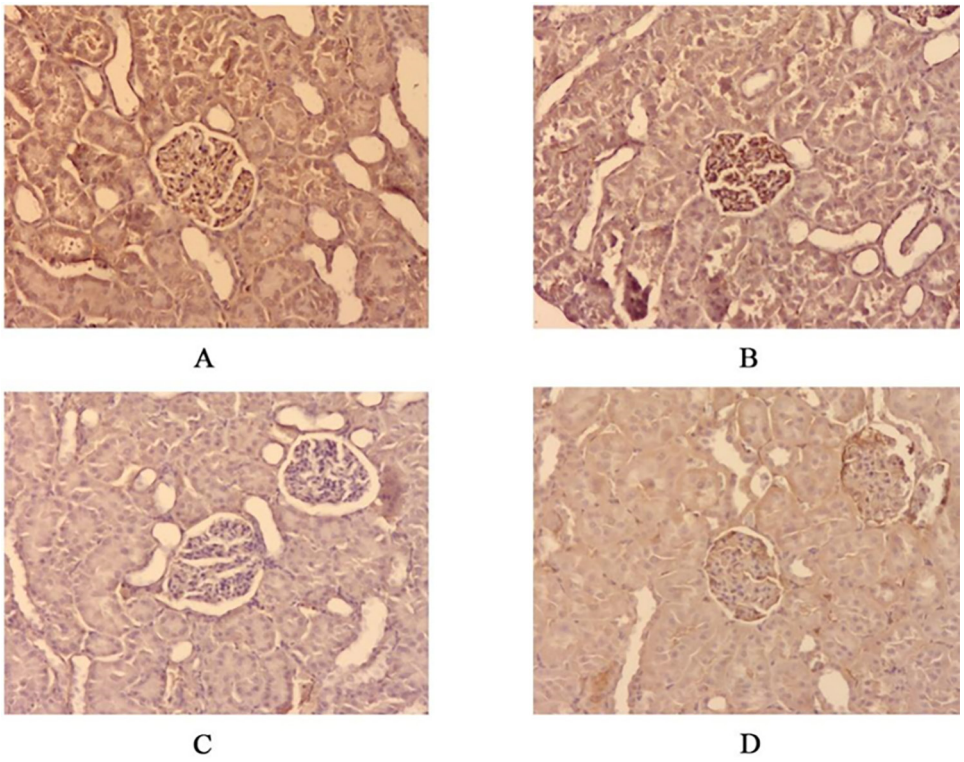

Figure 4 – Effect of diabetes and either DF-5 or ALT-711 on renal AGEs accumulation
Note: Immunohistochemical staining for AGEs in kidneys: (A) non-diabetic control, (B) diabetic control, (C) diabetic + ALT-711 and (D) diabetic + DF-5. Magnification ×100.

Table 1 – Body weight, blood glucose, HbA1c levels and mortality levels in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Final body weight (g)	Blood glucose (mmol/L)	HbA_{1c} (%)	Mortality
ND + vehicle	455.25±17.82	5.37±0.18	6.42±0.49	0/8
D + vehicle	323.80±18.75a	26.83±1.14[a]	19.77±0.08[a]	3/8
D + DF-5 (12.5 mg/kg)	358.00±32.35	27.93±0.95[a]	11.46±1.39[ab]	2/8
D + ALT-711 (12.5 mg/kg)	335.83±20.80	25.78±0.43a	12.30±1.88ab	2/8

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 2 – Renal function and urinary protein excretion in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Urine output (mL/24 h)	Urinary total protein concentration (g/L)	Urinary total protein excretion (mg/24 h)
ND + vehicle	15.00 ± 1.54	0.017 ± 0.004	0.26 ± 0.08
D + vehicle	23.20 ± 1.62[a]	0.058 ± 0.005[a]	1.32 ± 0.08[a]
D + DF-5 (12.5 mg/kg)	21.83 ± 1.58[a]	0.025 ± 0.007[ab]	0.59 ± 0.21[ab]
D + ALT-711 (12.5 mg/kg)	22.83 ± 4.15[a]	0.033 ± 0.005[ab]	0.72 ± 0.13[ab]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 3 – Morphometric analysis of glomerular damage in Masson trichrome-stained kidney sections in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Total glomerular area (μm²)	Glomerular connective tissue area (μm²)	Relative area of glomerular connective tissue (%)
ND + vehicle	14885.17±1932.23	695.92±195.25	4.6
D + vehicle	12572.63±1015.25	3356.46±1871.64[a]	26.7[a]
D + DF-5 (12.5 mg/kg)	14753.21±975.23	2397.29±832.43	16.2[b]
D + ALT-711 (12.5 mg/kg)	13968.94±856.26	1640.38±543.19	11.7[b]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 4 – AGEs staining in kidneys of non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Total glomerular area (μm2)	Positive AGE-stained glomerular area (μm2)	Relative AGE-stained glomerular area (%)
ND + vehicle	15287.35 ±1759.13	1634.04 ± 759.17	10.68
D + vehicle	12121.19 ±1234.57	3746.13 ±1098.73	30.90[a]
D + DF-5 (12.5 mg/kg)	12982.17 ±971.56	2471.09± 658.81	17.48[b]
D + ALT-711 (12.5 mg/kg)	13106.82 ± 1346.49	2117.85 ± 732.43	19.03[b]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Cleavage of AGE-BSA-collagen cross-links pre-formed *in vitro*

It was found that DF-5 exhibits a significant anti-crosslinking activity on AGE-BSA-collagen pre-formed cross-links (half-maximal inhibitory concentration, IC_{50}=0.31 mM), which is 6-fold more potent than the activity of ALT-711 (the structure is shown in Fig. 2), a well-known cross-link breaker (IC_{50}=1.89 mM). The ability of the ALT-711 compound to break cross-links has been described, although the mechanism of action has not yet been established. A more detailed description is given in Patent RU 2627769 C1, Russia (2017) [17]. It has been notified that based on the present experiment, the exact mechanism of the decrease in crosslinking cannot be assumed and that is why it is called "an anti-crosslinking agent" instead of "a cross-link breaker".

The design of the experiment takes into account the correction for interference associated with non-specific adhesion of BSA to the surface of the collagen matrix and the plate plastic. For this reason, compounds DF-5 and ALT-711 are believed to act on a certain product formed as a result of the interaction of albumin glycation products with amino acid residues of collagen, and attach albumin to the collagen matrix.

Cyclic beta-keto iminium cations are well suited for the role of products that react on the albumin side and quickly form a cross-link. They are the result of the cyclization of Lederer's glucosone or pentosone. Reacting with the free guanidine group of arginine, they are able to form glucosepane or pentosidine in several stages. It is possible that Lederer's glucosone and pentosone, as well as Amadori products that precede them, are also important for the formation of complexes that fix BSA on the collagen matrix, but their contribution remains to be established.

Animal body weight

At the end of the study, the mean final body weights of the diabetic animals were significantly lower than those of non-diabetic rats (Table 1; $p \leq 0.05$). DF-5 and ALT-711-treated diabetic rats showed a slight increase in weight compared to their untreated counterparts, but the difference was statistically significant only in case of DF-5-treated diabetic rats (Table 1; $p \leq 0.05$).

Glycemic control

Diabetic rats had significantly increased blood glucose and HbA_{1c} levels compared with the non-diabetic rats (Table 1; $p \leq 0.05$). The elevated HbA_{1c} contents observed in diabetic animals, were significantly decreased by almost 40% with the treatment of either DF-5 or ALT-711 (Table 1; $p \leq 0.05$). The treatment of diabetic rats with DF-5 or ALT-711 only slightly, not significantly, reduced a blood glucose concentration.

Renal function and histology

Diabetes was associated with an increased urinary volume output and a urinary total protein excretion ($p \leq 0.05$ vs. non-diabetic control; Table 2). At the end of the study, the diabetic rats had an elevated proteinuria

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(Table 2). The treatment of diabetic rats with DF-5 or ALT-711 significantly prevented an increase in a urinary total protein concentration (≈50%, $p \leq 0.05$), but not in a daily urine output, compared to the untreated diabetic rats (Table 2).

A histopathological examination showed an increased kidney tissue damage in the diabetes group. Diabetes is typically accompanied by a progressive glomerular and tubular damage. At the end of the study, the kidneys of rats in the diabetes group showed significant morphological changes compared with the non-diabetic control group (Fig. 3). The GBM thickening and mesangial matrix expansion in the glomeruli were observed in the diabetic rats. GBM thickening and a range of mild to moderate mesangial matrix expansion causing capillary luminal narrowing were consistently observed. Compared to the non-diabetic control animals, the mean glomerular area was slightly decreased, but the difference was not statistically significant, and the mean glomerular connective tissue area and the relative area of the glomerular connective tissue were significantly ($p \leq 0.05$) 4.8-fold and 5.8-fold increased, respectively, in the diabetes group (Table 3). The treatment of diabetic animals with either DF-5 or ALT-711 significantly reduced the extent of the glomerular damage (Fig. 3). The mean relative areas of the glomerular connective tissue observed in the streptozotocin-diabetic rats treated with either DF-5 or ALT-711, were significantly ($p \leq 0.05$) 1.6-fold and 2.3-fold lower, respectively, than in the vehicle-treated diabetic animals (Table 3).

Kidney AGEs level

Immunohistochemical staining for AGEs in the rats' kidneys demonstrated that there was a widespread staining for AGEs in the diabetic rats glomeruli compared to the non-diabetic control rats. This increased staining was attenuated by treatment of diabetic rats with either DF-5 or ALT-711 (Fig. 4). The mean relative AGE-stained glomerular areas observed in the streptozotocin-diabetic animals treated with either DF-5 or ALT-711, were significantly ($p \leq 0.05$) 1.6-fold and 1.8-fold lower, respectively, than in the diabetic vehicle-treated group (Table 4).

DISCUSSION

Protein glycation and formation of AGEs play an important role in the pathogenesis of long-term diabetes mellitus complications like retinopathy, nephropathy, neuropathy, cardiomyopathy along with some other diseases such as rheumatoid arthritis, osteoporosis and ageing [2]. Glycation and cross-linking of proteins not only lead to a decrease in the elasticity of blood vessels, but also affect the structural integrity and physiological functions of internal organs.

Glycation is a stepwise process involving the formation of hemiaminal, Schiff base, enaminol, Amadori product, enediol, Lederer's glucosone (or pentosone). Then it undergoes an intramolecular condensation between the amino group and the terminal aldehyde function, which affords the positively charged ring compound named a cyclic beta-keto iminium ion (6 or 7 membered) [2, 22]. The latter, reacting with the guanidine group of arginine, forms complex and stable cross-links, such as glucosepane or pentosidine. Early products also decompose into carbonyl compounds (methylglyoxal, glyoxal, etc.), which are capable of forming other types of crosslinking AGEs, such as MOLD and GOLD [22]. All AGEs are characterized by a high stability.

Diabetic nephropathy is defined as a diabetes-associated progressive decline in the glomerular filtration rate, accompanied by proteinuria and other kidneys complications [2]. In relation to the kidneys, glycation and activation of the complement cascade *via* recognition of glycated proteins by mannan-binding lectin and/or dysfunction of glycated complement regulatory proteins [23], activation of signaling cascades of receptors for AGEs [24] etc. are noted. Key events in GBM, mesangial and tubulointerstitial matrix, such as an increase in collagen IV and VI, an increased glycation of collagen IV, an increased cross-linking of collagen IV, an increase in laminin and fibronectin, an increase in collagen type I etc., are directly related or indirectly associated with glycation [25]. The expansion of the mesangial matrix and thickening of the GBM, podocyte injury, glomerulosclerosis and renal fibrosis are observed [26–28].

Cleavage of pre-formed AGEs within the kidney by a cross-link breaker, such as ALT-711, is believed to confer renoprotection in diabetes. Forbes J.M. et al. (2001) believed that ALT-711 might provide a new kind of therapy for the treatment of diabetic nephropathy. The intervention with ALT-711 from weeks 16–32 of the study had the capacity to not only improve functional parameters, such as albuminuria, but also markers of structural injury, including glomerulosclerosis, a tubulointerstitial injury, and an oxidative stress [29].

The mechanism of a cross-link breaking activity is not yet entirely understood. According to the data originally proposed for the ALT-711 analogue, phenylthiazolium bromide [16], it involves the cleavage of the C–C bond between two adjacent carbonyl groups. AGEs of this 1,2-dicarbonyl structural type, however, have not yet

been experimentally identified, although the cross-links' rupture has been immunologically proved by experiments with antibodies to one or another of the two cross-linked proteins [30-33]. It should be noted that such a C–C bond between two adjacent carbonyl groups is presented in some early glycation products [22]. In addition, there is compelling evidence from *in vivo* experiments that demonstrate the positive effect of ALT-711 on diabetic nephropathy [34, 35]. The concept of a direct destruction of AGE cross-links in the whole organism is controversial, [36] despite convincing evidence of the activity of AGE cross-link breakers in diabetic nephropathy. The anti-crosslinking mechanism can theoretically be realized by hydrolysis, through the cleavage of immature cross-links (an anti-crosslinking activity), formed on early stages of the cross-linking process, and through the inactivation of early products of glycation.

It can be assumed that in the whole organism, the inactivation of intermediate pre-crosslinked electrophilic products, glucose-derived adducts or free derivates of glucose, are possible mechanisms of action. Their structures are similar to the structure of a theoretical dicarbonyl cross-link which has never been found out [14, 16]. Such products may include Schiff bases, carbonyl compounds, α-oxoaldehyde protein adducts etc. As for α-oxoaldehydes, their participation in the formation of glycation cross-links has been proved for 2-ammonio-6-({2-[(4-ammo-nio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene}amino) hexanoate (GODIC), 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino) hexanoate (MODIC), glucosepane [37, 38], and many more. The next important product, cyclic b-keto iminium ion, possesses electrophilic properties [22] and, when interacting with the guanidine group, forms pentosinane at one stage, pentosidine at two stages (if it is a 6-member ring) or glucosepane (if it is a 7-member ring).

The possibility of the early α-oxoaldehyde adducts destruction by cross-link breakers is described in [39], using glycation of apolipoprotein A-I by methylglyoxal in the presence of ALT-711. This is discussed as a possible action mechanism for cross-link breakers in [40], and a direct possibility of methylglyoxal binding (representative of α-oxoaldehydes) by ALT-711 was considered in [40].

Thus, the hypothesis of the action mechanism assumes the ability of cross-link breakers to produce nucleophilic attacks on electrophilic glycation intermediates and early products of cross-linking (an anti-crosslinking activity). A detailed molecular mechanism of the likely reaction requires clarification, but considering nucleophilicity of DF-5 and ALT-711, this mechanism is possible.

An *in vivo* study was conducted to confirm whether the DF-5 compound could prevent the development of an early diabetic kidney disease in streptozotocin-induced diabetic rats. The test compound or the reference compound ALT-711, were administered intragastrically to the experimental animals for 30 days. Throughout the whole duration of the experiment, the blood glucose level of diabetic rats was not less than 15 mmol/L, which contributed to the development of diabetic vascular complications. At the end of the study, the animals' body weight, blood glucose and HbA_{1c} levels as well as renal function and histology in the groups of non-diabetic, diabetic rats treated with vehicle, DF-5 or ALT-711, were assessed and compared.

Being administered intragastrically on a regular basis for 30 days, DF-5 significantly reduced HbA_{1c} levels in blood, but demonstrated the absence of significant influence on fasting blood glucose levels. This fact likely points to the ability of DF-5 to act as assumed above – a breaker of early-stages products, including the so called Amadori products (HbA_{1c} has a structure of products of such a type) [41]. According to the above discussed hypothesis on the mechanism of the AGE cross-link breakers action, they could detach reversible glucose from its reversible adducts with proteins (Schiff bases and/or Amadori products) [42].

The current study has demonstrated the possibility of preventing diabetic nephropathy in streptozotocin-induced diabetic rats by using DF-5. As expected, diabetes induced an increase in proteinuria over time. The test compound DF-5 significantly reduced proteinuria and prevented a kidney damage in the experimental animals by limiting glomerular and tubular injuries. A similar effect has also been described for ALT-711 [43]. The observed phenomena, such as a reduced increase in the amount of the connective tissue and a reduced content of AGEs in the kidneys when compared with a control group of animals, are probably characteristic properties of the anti-crosslinking agents activity.

The accumulation of AGEs is associated with a renal production of extracellular matrix components in diabetes. An early intervention in this process can ameliorate long-term functional and structural features of diabetic nephropathy. In this study, the AGEs accumulation within the kidney was increased by diabetes and attenuated by the treatment with DF-5 compound.

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CONCLUSION

To sum up, the novel anti-crosslinking agent DF-5 has been identified. However, the elucidation of the exact action mechanism of the compound requires further research. DF-5 (12.5 mg/kg) inhibits the progression of an early renal dysfunction in streptozotocin-induced diabetic rats. This compound improves both functional and morphologic damages of experimental diabetic nephropathy. These changes were associated with a decreased accumulation of AGEs in the kidney. These findings provide investigators with additional therapeutic options for the treatment of diabetic nephropathy and possibly other diabetes complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Alexander A. Spasov – supervision, conceptualization; Olga N. Zhukovskaya, Haider S.A. Abbas, Anatoliy S. Morkovnik – DF-5 synthesis and structure elucidation; Andrey I. Rashchenko, Anasthasia A. Brigadirova, Roman A. Litvinov, Natalia A. Gurova – methodology, investigation, software, article writing; Alexey V. Smirnov, Nikolay G. Pan'shin – investigation, visualization; Andrey I. Rashchenko, Anasthasia A. Brigadirova, Natalia A. Gurova – data processing; Roman A. Litvinov – hypothesis development of the action mechanism not associated with a direct breaking of mature glycated proteins' cross-links. All the authors have read and approved of the final manuscript version.

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AUTHORS

Alexander A. Spasov – Doctor of Sciences (Medicine), Academician of RAS, Head of the Pharmacology and Bioinformatics Department, Volgograd State Medical University; Head of the Laboratory of Experimental Pharmacology, Volgograd Medical Research Center. ORCID ID: 0000-0002-7185-4826. E-mail: aspasov@mail.ru

Olga N. Zhukovskaya – Candidate of Sciences (Chemistry), Senior researcher of the Organic Synthesis Laboratory, Institute of Physical and Organic Chemistry, Southern Federal University. ORCID ID: 0000-0003-2485-2139. E-mail: zhukowskaia.ol@yandex.ru

Andrey I. Rashchenko – Candidate of Sciences (Pharmacy), Senior researcher of the Metabotropic Drugs Laboratory, Volgograd State Medical University; Volgograd Medical Research Center. ORCID ID: 0000-0002-0162-0653. E-mail: a.rashencko@yandex.ru

Anasthasia A. Brigadirova – Candidate of Sciences (Medicine), Associate Professor, Pharmacology and Bioinformatics Department, Volgograd State Medical University; Volgograd Medical Research Center. ORCID ID: 0000-0003-0957-7087. E-mail: a.brigadirova@gmail.com

Roman A. Litvinov – Candidate of Sciences (Medicine), Senior researcher of the Metabotropic drugs Laboratory, Volgograd State Medical University; Researcher of the Laboratory of Experimental Pharmacology, Volgograd Medical Research Center. ORCID ID: 0000-0002-0162-0653. E-mail: litvinov.volggmu@mail.ru

Natalia A. Gurova – Doctor of Sciences (Medicine), Professor of the Pharmacology and Bioinformatics Department, Volgograd State Medical University. ORCID ID: 0000-0002-0670-1444. E-mail: gurova.vlgmed@mail.ru

Alexey V. Smirnov – Doctor of Sciences (Medicine), Head of the Pathological Anatomy Department, Volgograd State Medical University. ORCID ID: 0000-0001-5351-6105. E-mail: alexey-smirnov@rambler.ru

Nicolay G. Pan'shin – Candidate of Sciences (Medicine), Associate Professor, Pathological Anatomy Department, Volgograd State Medical University. ORCID ID: 0000-0002-4035-4108. E-mail: panshin.nickolay@gmail.com

Haider S.A. Abbas – Master's student of the Organic Synthesis Laboratory, Institute of Physical and Organic Chemistry, Southern Federal University. E-mail: vip.haider89@gmail.ru

Anatoliy S. Morkovnik – Doctor of Sciences (Chemistry), Chief Researcher, Organic Synthesis Laboratory, Institute of Physical and Organic Chemistry, Southern Federal University. ORCID ID: 0000-0002-9182-6101. E-mail: asmork@mail.ru