Nucleophilic substitution of nitro group in nitrotriazolo-triazines as a model of potential interaction with cysteine-containing proteins

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HS-R^1 = L-Cysteine, L-Gluthatione
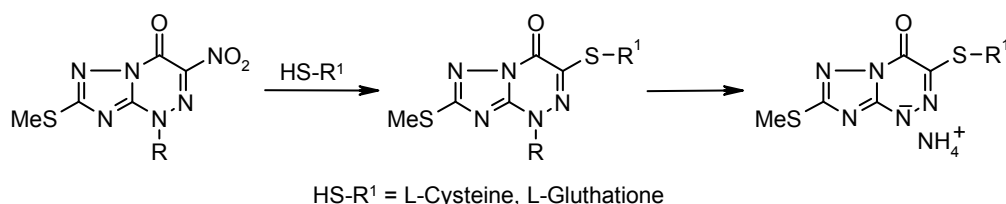
The nucleophilic susbstitution of nitro group in [1,2,4]triazolo[5,1-*c*][1,2,4]triazinones upon treatment with cysteine and glutathione was studied as a model for the interaction with thiol groups of virus proteins, which mimics the metabolic transformations of antiviral drug Triazavirin® and its derivatives.

Etioptropic drugs acting on different stages of virus replication currently enjoy a steadily growing use for the treatment of influenza. Among the main classes of such substances are the M2 ion channel blockers (rimantadine and amantadine) and neuraminidase inhibitors (oseltamivir and zanamivir).[1,2] Due to mutations, however, viruses appear that are resistant to the known drugs.[2] The ubiquity of the influenza virus and the appearing of highly pathogenic strains in combination with the variability of the viral genome determine the urgency of development of new effective means for the prevention and treatment of this disease. Through the joint efforts of scientists from Ural Federal University, Institute of Organic Synthesis, Ural Branch, Russian Academy of Sciences, and Research Institute of Influenza, as well as Ural Center for Biopharma Technologies and Medsintez Pharmaceutical Plant, a new antiviral drug Triazavirin® (**1**) (2-methylsulfanyl-6-nitro [1,2,4]triazolo[5,1-*c*][1,2,4]triazin-7(4*H*)-one sodium salt dihydrate, Fig. 1)[3] has been created. This representative of

azoloazines, a new class of non-nucleoside antiviral substances, has completed a full cycle of clinical trials for the treatment of influenza infection in 2011–2014.[4–9]

Trizavirin and its derivatives are nitro-substituted [1,2,4]-triazolo[5,1-*c*][1,2,4]triazines. They have demonstrated a high efficiency *in vivo* and, interestingly, a significantly lower activity in cell cultures. This prompts a suggestion that the mechanism of action of this drug and its analogs depends on the generation of its active metabolites in the body.

Considering the molecular structure of Triazavirin and chemical properties of other nitroazolo[5,1-*c*][1,2,4]tri-
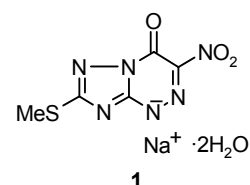

Figure 1. Structure of Triazavirin (**1**).

azines[10] it can be assumed that the drug interacts with the virus surface proteins through substitution reactions of the nitro group by *N*- and *S*-nucleophilic moieties of amino acids like lysine, arginine, or cysteine. However, in experiments performed *in vitro* using an isotopic label we have not observed an ability of Triazavirin to covalently bind with peptides containing the residues of these amino acids. Apparently, such interaction is a property of Triazavirin metabolites. Since the exact structure of these metabolites remains unknown, a computer modeling of the interaction of Triazavirin molecule with hemagglutinin of the pandemic influenza virus A/California/04/2009 (H1N1) was carried out in order to determine the accessibility of amino acid residues for the reaction with Triazavirin. The structure of the potential interaction sites was determined, and model experiments to study the interaction of the drug with the respective biogenic *S*-nucleophiles were carried out.

Such approach allows, on one hand, to predict the chemical transformations of the drug in the body, and on the other, to model the process of covalent bond formation of the potential metabolites (6-nitro[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazines) with the corresponding amino acid residues of the key virus proteins by means of synthesizing the appropriate compounds. The synthesis of potential metabolites can also be of interest as a means of search for more active Triazavirin derivatives for biological testing.

The results of computer modeling of hemagglutinin A/California/04/2009 (H1N1) molecule revealed five potential interaction sites with Triazavirin. The three-dimensional structure of the protein is shown in Figure 2 with highlighted amino acid residues which could potentially coordinate Triazavirin. The analysis of the spatial distribution of the interaction sites in the hemagglutinin molecule shows that the sites containing cysteine are most favored in respect to the energy of electrostatic and Van-der-Vaals interactions between the protein and the drug. Such arrangement of the sites suggests that they will be accessible also to the potential metabolites of Triazavirin derivatives, which would be able to interact covalently with protein amino acid residues by means of the nucleophilic substitution of the nitro group by the thiol groups in the protein. In this work, therefore, L-cysteine (**2**) and L-glutathione (**3**) were used as the nucleophilic reagents that can serve both as models of cysteine-containing protein fragment and as biogenic fragments to be incorporated in the molecule of [1,2,4]-triazolo[5,1-*c*][1,2,4]triazine.

It was established that Triazavirin does not directly react with L-cysteine (**2**) or L-glutathione (**3**). This agrees with our earlier investigations[10,11] where it was shown that in 4-unstubstituted triazolotriazines, a nitro group is virtually inert towards nucleophilic substitution because of the formation of anions that are stable towards a nucleophilic attack. Conversely, triazolotriazines with substituents in position 4 easily undergo substitution reactions with *N*- and *S*-nucleophiles. It is important to note that alkylation or glycosylation of Triazavirin at the nitrogen atom possible also metabolically, since Triazavirin
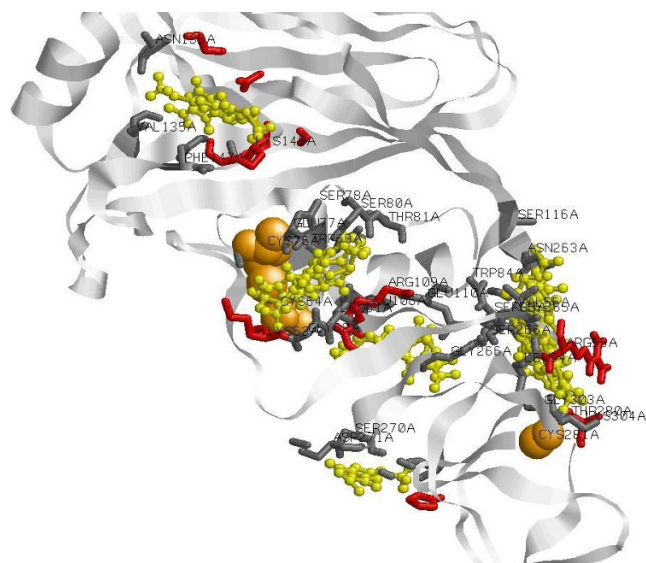
Figure 2. Results of a multiple docking of Triazavirin to hemagglutinin H1 of influenza virus. The energetically most favored interacting positions of the molecules. The principal amino acid residues near (less than 4 Å) the Triazavirin molecule are shown in red, cysteine residues are shown in orange, Triazavirin molecules are shown in yellow color.

is an isostere of purine bases for which are known for such transformations.[12] Besides, drugs in the body can undergo methylation and addition of glucuronic acid residue.[13]
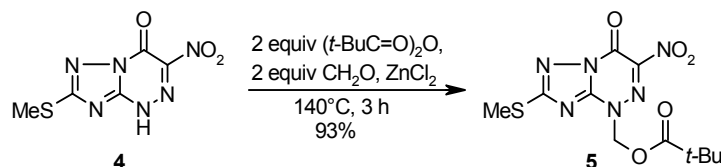
Triazavirin derivatives containing a substituent at the nitrogen atom, 2-methylsulfanyl-6-nitro[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazin-7-ones **5**, **6a–f**, were synthesized by methods developed by us earlier.[11,14,15] For the synthesis of compound **5**, a new effective method was found for introducing pivaloyloxymethyl moiety into position 4 of triazolo[5,1-*c*][1,2,4]triazin-7-one. For this purpose, sodium salt **1** was transformed into the NH-acid form **4** which was then fused with pivaloyl anhydride, paraformaldehyde, and zinc chloride at 140°C (Scheme 1).

Compounds **5** and **6a–f** reacted with cysteine **2** at room temperate in absolute ethanol both in the presence of 1 equiv sodium bicarbonate and in its absence producing 3-(2-amino-2-carboxyethylsulfanyl)-7-methylsulfanyl[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-ones **7** and **8a–f**, respectively (Scheme 2, Table 1). The products containing cysteine moiety were obtained either in the acid form or as sodium salts.
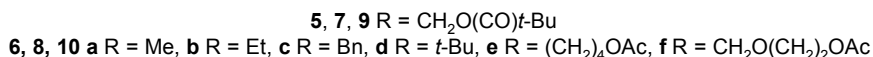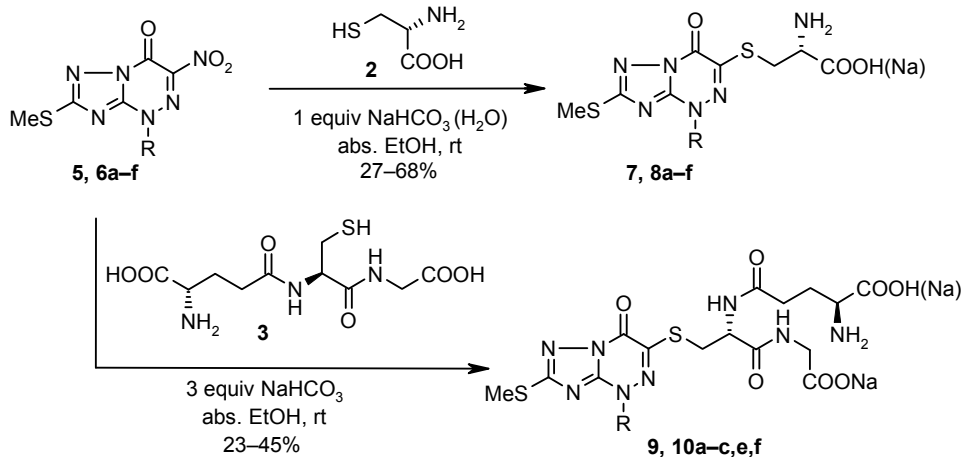
In the ¹H NMR spectra of the obtained compounds **7** and **8a–f** there is a singlet of the methylsulfanyl group, proton signals of the alkyl and cysteine moieties. The signals of the SCH₂ protons are split into one-proton double doublets (δ 3.88–3.98 ppm, J = 14.7–15.0 Hz, J = 4.0–4.2 Hz and δ 4.13–4.18 ppm, J = 8.0–9.4 Hz, J = 4.0–4.2 Hz) (Table 2).

The nitro group was easily substituted also upon the reaction of compounds **5** and **6a–f** with glutathione **3**, a tripeptide more closely resembling cysteine-containing proteins. As a result, 3-(glutathion-*S*-yl)-7-methylsulfa-nyl[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-ones **9** and

Scheme 1

Scheme 2

5, **7**, **9** R = CH₂O(CO)*t*-Bu
6, **8**, **10** **a** R = Me, **b** R = Et, **c** R = Bn, **d** R = *t*-Bu, **e** R = (CH₂)₄OAc, **f** R = CH₂O(CH₂)₂OAc

10a–c,**e**,**f** was formed (Scheme 2, Table 1, Fig. 3). Product **10d** could not be obtained through the nitro group substitution, most likely due to a steric hindrance caused by the bulky *tert*-butyl group. As optically pure L-cysteine and L-glutathione were used in the reactions, also the products were optically active. The optical rotation values were measured and presented in Table 1.

The pivaloyloxymethyl group is widely used as a protecting group that can be removed under basic conditions.[16,17] The removal of the pivaloyloxymethyl group in compounds **7** and **9** produced derivatives of cysteine and glutathione **11**, **12** with unsubstituted position 4 (Scheme 3, Table 1). The structures of the synthesized compounds were confirmed by ¹H and ¹³C NMR spectra (Table 2).

Scheme 3

7, **11** R = Cys, **9**, **12** R = SG
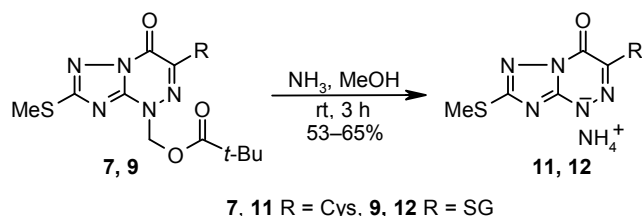
3-(Glutathion-*S*-yl)-1-(2-hydroxyethyloxymethyl)-7-methyl-sulfanyl[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-one **13**, like antiviral drug aciclovir, contains a substituent mimicking the furanosyl moiety. 2-Hydroxyethyloxy-methyl derivative **13** was obtained by hydrolysis of the acetoxyethyloxymethyl group of compound **10f** by 1 N hydrochloric acid solution (Scheme 4).

Thus, the model reactions of Triazavirin derivatives and biogenic S-nucleophiles that were carried out let conclude that metabolic processes involving nitro group substitution and covalent bonding by cysteine residues of hemagglutinin are likely taking place. The biological activity of the new compounds will be considered in a separate report.
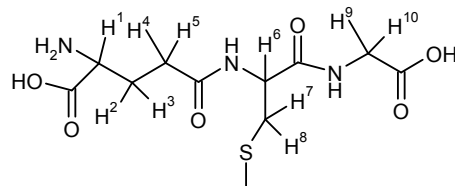

Figure 3. Atom numbering in the glutathione moiety.

Experimental

¹H and ¹³C NMR spectra were registered on a Bruker DRX-400 instrument (400 and 100 MHz, respectively), chemical shifts were given in the δ-scale against TMS as internal standard (for the ¹H and ¹³C NMR spectra). Elemental analysis was performed on a Perkin Elmer 2400-II CHNS/O instrument. Melting points of the synthesized compounds were determined on a Boetius apparatus. The completion of the reactions and the purity of the obtained products were determined by TLC on Sorbfil plates (ZAO Sorbpolimer) in ethyl acetate or butanol–acetic acid–water, 4:1:1. Column chromatography was carried on silica gel (Alfa Aesar). Optical rotation was measured on a Perkin Elmer 343 Plus spectrophotopolarimeter.

Triazavirin (**1**) and 1-alkyl-7-methylsulfanyl-3-nitro-[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-ones **6a–f** were obtained following published methods.[3,11,14,15] The interaction of Triazavirin with hemagglutinin was simulated using protein structures from the PDB 3LZG

Scheme 4

10f → (1N HCl, H₂O, rt, 72 h, 41%) → **13**
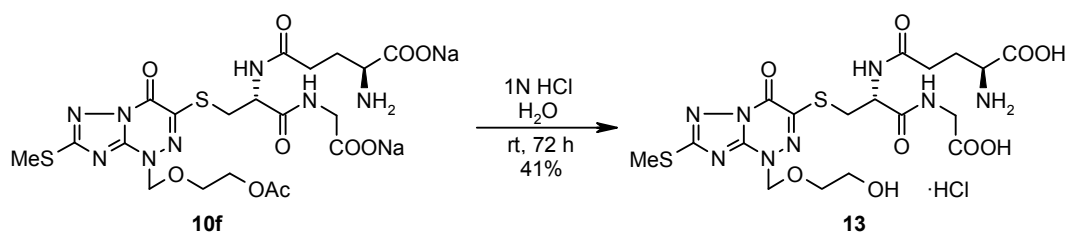
(H1) database and a Triazavirin structure optimized with the HyperChem 8.0 software. Heteroatoms and water molecules were eliminated from the coordinate files. The atom coordinates of the monomeric protein were used as initial data. The docking was performed using the server version of the Hex software (http://hexserver.loria.fr/) with the standard parameters. The simulation was carried out with accounting for electrostatic interactions; the 500 best positions of the ligand were considered. Following the method used in the Hex software, the initial relative positions of the ligand in respect to the receptor were selected on a grid superimposed on the receptor. The size of the cells in the grid corresponded to the recommendations by the authors of the software for the docking of small molecules. For comparison of the interaction energies of the ligand in different orientations, the energy values were calculated in the opls force filed using the corresponding option of the Hex software. The value of the scoring function of binding energy for all the described positions of the ligand relative to the receptor was approximately $-300 \div -500$ units used in the Hex software.

The analysis of the contacts between the molecules of the ligand and the protein was carried out using the RasMol software. The distance of the ligand molecule to the amino acid residues of the receptor was used as the criterion for the selection of amino acid residues in contact with the ligand. If the distance between the atoms of an amino acid residue and those of the ligand was less than 5 Å it was assumed that this amino acid residue is in contact with the ligand. The value of 5 Å was selected by trial using the experience from the previous simulations of enzyme–inhibitor interactions.

Table 1. Physicochemical charcteristics of compounds **7–13**

Com-pound	Empirical formula	Found, % Calculated, %			Mp,°C	Yield, %	$[\alpha]_D$ (Concentration (mol/ml), solvent)
		C	H	N			
7	$C_{14}H_{19}N_6NaO_5S_2 \cdot H_2O$	36.53 / 36.84	4.51 / 4.64	18.48 / 18.41	162–164	35	−20.40 (1.75, 10% TFA)
8a	$C_9H_{11}N_6NaO_3S_2 \cdot H_2O$	30.58 / 30.33	3.99 / 3.68	23.75 / 23.58	184–186	62	−5.86 (2.18, 50% TFA)
8b	$C_{10}H_{14}N_6O_3S_2 \cdot H_2O$	34.17 / 34.47	4.57 / 4.63	23.84 / 24.12	209–211	68	−6.28 (2.17, 50% TFA)
8c	$C_{15}H_{16}N_6O_3S_2 \cdot H_2O$	43.62 / 43.89	4.30 / 4.42	20.25 / 20.47	153–155	56	−12.21 (1.33, 50% TFA)
8d	$C_{12}H_{17}N_6NaO_3S_2 \cdot H_2O$	36.34 / 36.17	4.82 / 4.81	21.14 / 21.09	167–169	27	−20.40 (1.93, 10% TFA)
8e	$C_{14}H_{19}N_6NaO_5S_2 \cdot H_2O$	37.17 / 36.84	4.89 / 4.64	18.72 / 18.41	175–177	46	−13.70 (2.07, 50% TFA)
8f	$C_{13}H_{18}N_6O_6S_2 \cdot H_2O$	35.22 / 35.77	4.70 / 4.62	19.05 / 19.25	158–160	51	−4.48 (1.34, H_2O)
9	$C_{21}H_{28}N_8Na_2O_9S_2 \cdot 3H_2O$	36.28 / 36.00	5.07 / 4.89	15.74 / 15.99	223–225	38	−28.80 (1.00, H_2O)
10a	$C_{16}H_{21}N_8NaO_7S_2 \cdot 3H_2O$	33.29 / 33.22	4.80 / 4.70	19.12 / 19.37	241–243	34	−35.65 (2.09, H_2O)
10b	$C_{17}H_{23}N_8NaO_7S_2 \cdot 3H_2O$	34.41 / 34.46	4.87 / 4.93	18.63 / 18.91	209–211	35	−25.66 (2.29, H_2O)
10c	$C_{22}H_{24}N_8Na_2O_7S_2 \cdot 3H_2O$	39.24 / 39.05	4.71 / 4.47	16.41 / 16.56	230–232	23	−43.82 (2.00, H_2O)
10e	$C_{21}H_{28}N_8Na_2O_9S_2 \cdot 3H_2O$	36.21 / 36.00	4.73 / 4.89	15.73 / 15.99	171–173	42	−27.81 (2.48, H_2O)
10f	$C_{20}H_{27}N_8NaO_{10}S_2 \cdot 3H_2O$	35.47 / 35.29	4.91 / 4.89	16.63 / 16.46	233–235	45	−27.21 (1.09, 10% TFA)
11	$C_8H_{12}N_7NaO_3S_2 \cdot H_2O$	26.76 / 26.74	4.28 / 3.93	27.01 / 27.28	189–191	53	−50.80 (1.93, H_2O)
12	$C_{15}H_{21}N_9Na_2O_7S_2 \cdot 3H_2O$	29.99 / 29.85	4.48 / 4.51	20.68 / 20.89	255–157	65	−1.71 (2.00, H_2O)
13	$C_{18}H_{26}N_8O_9S_2 \cdot HCl \cdot 2H_2O$	33.89 / 34.04	4.84 / 4.92	17.91 / 17.64	227–229	41	–

Table 2. Spectral characteristics of compounds **7**–**13**

Com-pound	^{1}H NMR spectrum, δ, ppm (*J*, Hz)*	^{13}C NMR spectrum, δ, ppm**
7	1.18 (9H, s, C(CH₃)₃); 2.67 (3H, s, SCH₃); 3.55 (1H, dd, *J* = 15.0, *J* = 8.1, CH); 3.90 (1H, dd, *J* = 15.0, *J* = 4.2) and 4.21 (1H, dd, *J* = 8.1, *J* = 4.2, SCH₂); 6.28 (2H, s, CH₂)	13.2; 25.9; 29.4; 38.6; 51.7; 74.7; 143.2; 146.6; 151.2; 168.9; 169.3; 179.0
8a	2.68 (3H, s, SCH₃); 3.57 (1H, dd, *J* = 15.0, *J* = 8.0, CH); 3.88 (1H, dd, *J* = 15.0, *J* = 4.2) and 4.18 (1H, dd, *J* = 8.0, *J* = 4.2, SCH₂); 4.07 (3H, s, CH₃)	13.2; 29.3; 41.5; 51.5; 141.3; 146.9; 151.4; 168.7; 169.7
8b	1.48 (3H, t, *J* = 7.1, CH₃); 2.68 (3H, s, SCH₃); 3.53 (1H, dd, *J* = 15.0, *J* = 8.4, SCH₂) and 3.93 (1H, dd, *J* = 15.0, *J* = 4.1); 4.17 (1H, dd, *J* = 8.4, *J* = 4.1, CH); 4.45 (2H, q, *J* = 7.1, CH₂)	12.2; 13.2; 29.3; 50.3; 51.6; 141.3; 147.2; 151.1; 168.6; 169.6
8c	2.64 (3H, s, SCH₃); 3.23 (1H, dd, *J* = 14.3, *J* = 8.7, CH); 3.52 (1H, dd, *J* = 8.7, *J* = 3.3) and 3.75 (1H, dd, *J* = 14.3, *J* = 3.3, SCH₂); 5.48 (2H, s, CH₂); 7.27–7.39 (3H, m, H Ph); 7.52 (2H, d, *J* = 6.7, H Ph)	13.8; 29.5; 52.3; 56.0; 128.1; 128.2; 128.5; 129.0; 136.8; 149.2; 152.5; 165.1; 168.4
8d	1.77 (9H, s, C(CH₃)₃); 2.70 (3H, s, SCH₃); 3.47 (1H, dd, *J* = 14.7, *J* = 9.4, CH); 3.98 (1H, dd, *J* = 14.7, *J* = 4.0) and 4.13 (1H, dd, *J* = 9.4, *J* = 4.0, SCH₂)	12.9; 26.6; 28.8; 51.1; 68.6; 137.8; 146.9; 150.3; 167.9; 169.2
8e	1.75 (2H, m, CH₂); 2.04 (2H, m, CH₂); 2.08 (3H, s, COCH₃); 2.66 (3H, s, SCH₃); 3.52 (1H, dd, *J* = 8.8, *J* = 15.0, CH); 3.93 (1H, dd, *J* = 4.2, *J* = 15.0, SCH₂); 4.10–4.19 (3H, m, OCH₂, SCH₂); 4.45 (2H, t, *J* = 6.5, NCH₂)	13.3; 13.4; 20.7; 24.3; 24.8; 25.1; 51.7; 63.3; 124.2; 148.8; 151.7; 162.9; 164.5; 170.3
8f	2.00 (3H, s, CH₃); 2.63 (3H, s, SCH₃); 3.54 (1H, br. s, CH); 3.83–4.05 (3H, m, CH₂, SCH₂); 4.09–4.29 (3H, m, CH₂, SCH₂); 5.77 (2H, s, NCH₂)	13.7; 20.5; 30.7; 53.7; 63.9; 68.6; 83.4; 143.6; 147.6; 152.1; 169.1; 171.9; 174.3
9	1.17 (9H, s, C(CH₃)₃); 2.09–2.18 (2H, m, H^{2,3}); 2.52 (2H, dd, *J* = 15.4, *J* = 7.1, H^{4,5}); 2.68 (3H, s, SCH₃); 3.27 (1H, dd, *J* = 14.4, *J* = 9.9, H^1); 3.72–3.92 (4H, m, H^{7,8,9,10}); 4.95 (1H, dd, *J* = 10.0, *J* = 4.5, H^6); 6.30 (2H, dd, *J* = 11.0, *J* = 1.0, CH₂)	13.5; 26.2; 26.4; 31.7; 31.7; 38.8; 43.5; 51.0; 54.5; 74.6; 144.6; 147.4; 151.6; 168.7; 171.4; 173.9; 175.0; 176.0; 179.7
10a	2.10–2.21 (2H, m, H^{2,3}); 2.48–2.61 (2H, m, H^{4,5}); 2.67 (3H, s, SCH₃); 3.36–3.43 (1H, m, H^1); 3.68–3.84 (4H, m, H^{7,8,9,10}); 4.06 (3H, s, CH₃); 4.88 (1H, dd, *J* = 8.8, *J* = 4.9, H^6)	13.4; 26.3; 31.3; 31.5; 41.6; 43.5; 51.8; 54.2; 142.5; 147.4; 151.6; 168.6; 171.3; 174.0; 175.0; 176.1
10b	1.50 (3H, t, *J* = 7.2, CH₃); 2.09–2.14 (2H, m, H^{2,3}); 2.50–2.55 (2H, m, H^{4,5}); 2.67 (3H, s, SCH₃); 3.39 (1H, dd, *J* = 14.3, *J* = 9.0, H^1); 3.63–3.89 (4H, m, H^{7,8,9,10}); 4.43 (2H, q, *J* = 7.2, CH₂); 4.90 (1H, dd, *J* = 8.9, *J* = 5.1, H^6)	12.4; 13.2; 26.4; 31.1; 31.4; 43.3; 50.2; 51.5; 54.1; 142.5; 147.4; 151.1; 168.2; 171.3; 174.4; 175.1; 176.1
10c	1.98–2.19 (2H, m, H^{2,3}); 2.41–2.51 (2H, m, H^{4,5}); 2.69 (3H, s, SCH₃); 3.19 (1H, dd, *J* = 14.4, *J* = 9.8, H^6); 3.58–3.87 (4H, m, H^{7,8,9,10}); 4.62 (1H, dd, *J* = 9.7, *J* = 4.6, H^1); 5.45 (1H, d, *J* = 14.6) and 5.64 (1H, d, *J* = 14.6, CH₂); 7.32–7.48 (5H, m, H Ph)	13.7; 26.7; 31.5; 31.8; 43.6; 51.3; 54.4; 57.8; 128.8; 128.9; 129.1; 133.9; 143.1; 147.0; 151.4; 168.5; 171.2; 174.4; 175.0; 176.1
10e	1.68–1.81 (2H, m, CH₂); 1.97–2.15 (7H, m, COCH₃, CH₂, H^{2,3}); 2.41–2.58 (2H, m, H^{4,5}); 2.68 (3H, s, SCH₃); 3.29–3.39 (1H, m, H^1); 3.51–3.92 (4H, m, H^{7,8,9,10}); 4.07–4.16 (2H, m, CH₂); 4.40–4.50 (2H, m, CH₂); 4.92 (1H, dd, *J* = 9.4, *J* = 4.9, H^6)	13.3; 20.4; 23.9; 24.7; 27.3; 31.3; 31.6; 43.4; 51.4; 54.2; 54.4; 64.6; 142.7; 147.3; 151.4; 166.2; 168.5; 171.3; 174.5; 175.3; 176.0
10f	1.94 (3H, s, COCH₃); 2.09–2.20 (2H, dd, *J* = 14.2, *J* = 7.7, H^{2,3}); 2.47–2.60 (2H, m, H^{4,5}); 2.60 (3H, s, SCH₃); 3.28 (1H, dd, *J* = 14.3, *J* = 9.4, H^1); 3.72–3.82 (4H, m, H^{7,8,9,10}); 3.92 (2H, dd, *J* = 5.3, *J* = 3.7) and 4.16 (2H, dd, *J* = 5.3, *J* = 3.7, CH₂); 4.80 (1H, dd, *J* = 10.0, *J* = 5.0, H^6); 5.72 (2H, s, NCH₂)	13.7; 20.5; 26.6; 31.7; 31.9; 43.5; 52.0; 54.6; 64.0; 68.6; 83.4; 144.2; 147.7; 152.1; 169.0; 171.5; 174.0; 174.2; 175.3; 175.7
11	2.66 (3H, s, SCH₃); 3.50 (1H, dd, *J* = 15.1, *J* = 7.8, CH); 3.73 (1H, dd, *J* = 15.1, *J* = 3.7) and 4.09 (1H, dd, *J* = 7.8, *J* = 3.7, SCH₂)	13.9; 29.4; 51.3; 141.6; 147.5; 152.6; 166.9; 169.6
12	2.06–2.21 (2H, m, H^{2,3}); 2.44–2.61 (2H, m, H^{4,5}); 2.67 (3H, s, SCH₃); 3.39 (1H, dd, *J* = 14.4, *J* = 8.6, H^1); 3.58–3.82 (4H, m, H^{7,8,9,10}); 4.67 (1H, dd, *J* = 8.6, *J* = 4.5, H^6)	13.1; 26.1; 31.3; 33.0; 43.3; 53.3; 54.1; 138.3; 149.4; 158.6; 167.7; 171.6; 174.1; 174.8; 176.2
13	2.10–2.18 (2H, m, H^{2,3}); 2.46–2.60 (2H, m, H^{4,5}); 2.68 (3H, s, SCH₃); 3.36 (1H, dd, *J* = 14.4, *J* = 9.4, H^1); 3.67–3.92 (8H, m, H^{7,8,9,10}, 2CH₂); 4.89 (1H, dd, *J* = 9.2, *J* = 4.7, H^6); 5.79 (2H, s, NCH₂)	13.3; 26.2; 31.2; 31.4; 43.1; 51.6; 54.1; 60.2; 71.4; 83.1; 143.8; 147.5; 151.8; 168.6; 171.5; 173.9; 175.0; 175.6

* ^{1}H NMR spectra were registered in D₂O (compounds **7**, **8a**,**b**,**d**–**f**, **9**, **10a**–**c**,**e**,**f**, **11**–**13**) or DMSO-*d*₆ (compound **8c**).

** The ^{13}C NMR spectra were registered in D₂O with addition of CF₃COOD (compounds **7**, **8a**,**b**,**d**),
  in D₂O (compounds **8f**, **9**, **10a**–**c**,**e**,**f**, **12**, **13**), or DMSO-*d*₆ (compounds **8c**,**e**, **11**).

7-Methylsulfanyl-1-pivaloyloxymethyl-3-nitro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-one (5). A mixture of 7-methylsulfanyl-3-nitro[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4(1*H*)-one (**4**) (1.13 g, 5 mmol), pivaloic anhydride (2 ml, 10 mmol), paraformaldehyde (0.30 g, 10 mmol), and a catalytic amount of ZnCl₂ (0.07 g, 0.5 mmol) was heated for 3 h at 140°C. The reaction mixture was cooled to room temperature. The product was extracted with CHCl₃ (20 ml). The extract was washed with water (2×10 ml), dried over Na₂SO₄, and evaporated. Hexane (30 ml) was added to the residue and kept overnight. The precipitate

and was filtered off. Yield 1.40 g (82%), yellow powder, mp 79–81°C. Found, %: C 38.69; H 3.99; N 24.42. C₁₁H₁₄N₆O₅S. Calculated, %: C 38.59; H 4.12; N 24.55.

Nucleophilic substitution in 1-alkyl-7-methylsulfanyl-3-nitro[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4-ones: synthesis of compounds 7, 8a–f, 9, 10a–c,f (General method). A solution of starting triazolotrazine **5** or **6** (1 mmol) in abs. EtOH (40 ml) was added under stirring to a freshly prepared solution of cysteine (**2**) or glutathione (**3**) (1 mmol) in 1 M NaHCO₃ solution (1 equiv (for cysteine) or 3 equiv (for gluta-thione)) at room temperature. The product was filtered off and recrystallized from 50% EtOH (compounds **7**, **8a–f**) or 65%

EtOH (compounds **10a–c**,**e**,**f**). 3-(Glutathion-*S*-yl)-7-methyl-sulfanyl-1-pivaloyloxymethyl[1,2,4]triazolo[5,1-*c*][1,2,4]-triazin-4(1*H*)-one (**9**) was isolated by column chromato-graphy, eluent BuOH–AcOH–H$_2$O, 4:1:1.

Removal of pivaloyl protecting group (compounds 11, 12) (General method). Compound **7** or **9** (0.4 mmol) was dissolved in a mixture NH$_3$–MeOH, 1:3 (5 ml) and stirred at room temperature for 3 h. The reaction mixture was evaporated, and the residue was recrystallized from 65% EtOH.

1-(2-Hydroxyethyloxymethyl)-3-(glutathion-*S*-yl)-7-methylsulfanyl[1,2,4]triazolo[5,1-*c*][1,2,4]triazin-4-(1*H*)-one (13). A solution of 1-(2-acetoxymethyl)-3-(glutathion-*S*-yl)-7-methylsulfanyl[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazin-4(1*H*)-one (**10f**) (0.3 g, 0.4 mmol) in 1 N HCl (10 ml) was stirred at room temperature for 72 h. The reaction mixture was evaporated. The product was isolated by column chromatography, eluent BuOH–AcOH–H$_2$O, 4:1:1.

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