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Synthesis, physicochemical properties and *in vitro* cytotoxic activity of aziridine-containing derivatives of 1,3,5-triazine

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1. Materials and methods

1.1 General synthetic procedures and substance identification

^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were obtained on a Bruker Avance III 400 spectrometer (Germany) (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C) in $CDCl_3$ and D_2O at 298.15 K. Mass spectral analysis was performed on a Bruker GmbH "MaXis" mass-spectrometer (Germany). Single crystals of compound **7** were selected using an optical microscope, encased in an oil-based cryoprotectant, and mounted on cryoloops. The measurement was carried out using a Rigaku Oxford Diffraction XtaLAB SuperNova diffractometer with an HyPix3000 CCD area detector operated with monochromated microfocused CuKα radiation (λ[CuKα] = 1.54184 Å). Experimental data processing was carried out using CrysAlisPro software. The structures were solved by direct methods and refined using the SHELX [S1,S2] program incorporated in the OLEX2 [S3] software package.

Methyl 6-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]hexanoate (**2**) and methyl 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate (**3**) were obtained in accordance with the reported method.[S4]

Sodium 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate (4)

To a solution of methyl 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate **3** (0.23 g, 0.68 mmol) in methanol (15 ml) was added a solution of NaOH (0.04 g, 1.0 mmol) in water (5 ml). The mixture was stirred at ambient temperature for 24 h. After partial spraying of the solution, the precipitate that formed was filtered off, washed with acetone, and dried in a stream of air.

Yield 0.6 g (86%). White crystals. ^{1}H NMR (400 MHz, D_2O) δ, ppm: 8.41 (s, 1H, NH), 3.84 (dd, *J* = 17.1, 10.6 Hz, 2H), 3.40 – 3.05 (m, 2H), 2.12 (d, *J* = 7.5 Hz, 2H),1.67 – 1.42 (m, 4H), 1.38 – 1.19 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ, ppm: 183.9 (C=O), 175.8 ($C_{triazine}$), 171.3 ($C_{triazine}$), 164.8 ($C_{triazine}$), 40.54 (CH_2), 37.53 (CH_2), 28.18 (CH_2 aziridine), 27.22 (CH_2 aziridine), 26.06 (CH_2), 25.9 (CH_2), 25.6 (CH_2).

7,8-Dihydroimidazo[1,2-*a*][1,3,5]triazine-2,4(3*H*,6*H*)-dione (7)

A solution of 6-aminohexanoic acid (1.07 g, 8.2 mmol) in water (10 ml) was added to a solution of cyanuric chloride (1.5 g, 8.2 mmol) in acetone (40 ml) while cooling to 0°C. To the resulting mixture was added Et_3N (1.65 g, 16.3 mmol). The mixture was stirred for 40 min at 0-5°C and 2 h at rt, and acetone was then evaporated under reduced pressure. To the resulting aqueous solution was added a solution of aziridine (8.2 mmol) and Na_2CO_3 (8.2 mmol) in water (20 ml) and chloroform (50 ml). The reaction was carried out for 3 h at the same temperature.

The organic layer was separated, dried over anhydrous $CaCl_2$, filtered, and evaporated under reduced pressure. The product was purified by silica gel column chromatography using a chloroform-methanol (9.5/0.5) eluting system.

Yield 0.69 g (54%). White crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm: 6.72 (s, CH), 3.31–3.26 (m, CH_2), 2.86–2.78 (m, CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ, ppm: 151.9 (C=O), 98.6 (CH), 42.8 (CH_2), 38.7 (CH_2).

Crystal Data for **7** $C_5H_6N_4O_2$ (M =154.14 g/mol): triclinic, space group P-1 (no. 2), a = 4.2285(3) Å, b = 8.2532(8) Å, c = 9.5635(5) Å, α = 105.974(7), β = 97.567(6), γ = 104.502(7)°, V = 303.34(4) Å^3, Z = 2, T = 100(2) K, μ(Cu Kα) = 1.156 mm^{-1}, D_{calc} = 1.688 g/cm^3, 2855 reflections measured (9.852° ≤ 2Θ ≤ 144.984°), 1195 unique (R_{int} = 0.0340, R_{sigma} = 0.0396). The final R_1 was 0.0495 (I > 2σ(I)) and wR_2 was 0.1485 (all data). CCDC 2243309.

1.2 Study of physicochemical properties of substances 3 and 4 aqueous solutions

The temperature and concentration dependencies of physicochemical properties in the binary system substance **3** (**4**)–water was studied using following apparatus: (*i*) Anton Paar DSA 5000 (Austria) for measuring density; (*ii*) Lovis 2000 M Anton Paar microviscometer (Austria) for measuring viscosity. The description of experimental techniques, as well as metrological characteristics are presented elsewhere.[S5,S6].

1.3 Solubility of substances 3 and 4 in aqueous solutions

The measurements of the solubility of substance **3** (**4**) in water at atmospheric pressure were carried out in the temperature range T = 293.15–318.15 K with isothermal saturation method for 12 h using a thermostatically controlled shaker LAUDA ET 20 with shaking frequency of 80 Hz. The temperature was measured with an error of 0.1 K. The required volume (about 5 cm^3) was taken from the heterogeneous system at atmospheric pressure for spectrophotometric analysis. Then the liquid phase was separated by vacuum filtration for spectrophotometric analysis.

The concentration of substance **3** (**4**) in a saturated solution was determined spectrophotometrically using an SF-2000 spectrophotometer (Russia). Fig. S4 presents the electronic spectrum of substance (a) at λ = 200–400 nm. Fig. S5 demonstrates the agreement with Beer–Lambert–Bouguer law (R^2 = 0.989) at λ = 220 nm. For substance **3** (**4**) concentration determination in a saturated solution, the following equation was applied (at optical wavelength equal to 1 cm):

$$C = 0.049 \cdot A\ (220\ \text{nm}), \qquad (1)$$

where C is the volume concentration of substance (a) ($g \cdot dm^{-3}$), A (220 nm) is the absorbance at λ = 220 nm.

1.4 Stability of compound 4 aqueous solutions

The hydrolysis reaction of compound **4** in D_2O was studied at 25 °C by recording the ^1H NMR spectra of the reaction mixture on Bruker Avance III 400 (400.13 MHz) for 7 days in automatic mode with one spectrum recording every 12 h.

1.5 Antioxidant activity

1.5.1 Photoinduced hemolysis

The measurements were carried out on an SF-2000 spectrophotometer (OKB SPEKTR, Russia) in a cuvette with an optical path length of 5 mm. The study of the photokinetic activity of substance **3** was carried out by recording a decrease in the optical density of erythrocyte suspension at wavelength of 800 nm at five-second intervals until complete hemolysis. The measurements were carried out in a thermostated cuvette of a spectrophotometer at 310.15 K; concentration of the studied blood sample varied in the range C = 10–100 μM. Antioxidant properties were evaluated using a device for studying photoinduced cell lysis according to the technique published earlier.[S5]

1.5.2 Interaction with NO radical

For the experiment, the reaction mixture containing 1 ml of sodium nitroprusside (C = 15 μM) and 0.5 ml of an aqueous solution of substance **3** (C = 10–100 μM) was incubated for 150 min in a thermostatic shaker at 333.15 K. Then, 0.5 ml of phosphate buffer (PBS pH = 7.4) and 0.5 ml of 1% Griess reagent solution were added to 0.25 ml of the resulting solution. The obtained mixture was incubated for 30 min at room temperature. The resulting diazo compound was determined spectrophotometrically at λ = 540 nm. Sodium azide at similar concentrations was used as a control.[S5]

1.6 Studying of the binding of the compounds 3, 4 with DNA by spectral methods

UV spectra were recorded in the 200–400 nm range on a Beckman Coulter DU 800 spectrometer (USA) using quartz cuvettes (l = 1.0 cm). Circular dichroism (CD) spectra were recorded on a Jasco J-1500 spectropolarimeter (Japan) using quartz cuvettes (l = 0.2 cm). For the compound **3** (**4**) and working solutions of DNA were checked the observance of the absorption law in the concentration range. A stock solution of DNA in NaCl 0.9% and 3.5 mM Tris–HCl/50 mM NaCl buffer solution (pH = 7.2) gave a ratio of UV absorbance of 1.0–1.1 at 260 and 280 nm, indicating that the DNA was sufficiently free of protein, and the concentration of DNA was determined by measuring the absorbance at 260 nm using ε = 6600 $M^{-1}cm^{-1}$.[S7] The synthesized

compounds **3** (**4**) used in DNA-binding experiments was directly dissolved in Tris–HCl buffer (pH = 7.2).

1.7 Cytotoxicity

MTT analysis (colorimetric test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed on PANC-1 and A549 cell lines. Cells at a concentration of $5 \cdot 10^4$ per well were seeded in a 96-well plate and incubated for 12 h in DMEM-F12 medium supplemented with 10% thermally inactivated fetal bovine serum, 1% L-glutamine, 50 $U \cdot ml^{-1}$ penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. After cultivation, fresh DMEM - F12 medium containing various concentrations of compounds **3**, **4** and **7** were added to the wells. Next, the plate was then incubated at 37°C in a humidified atmosphere CO_2 - incubator in the presence of 20% O_2, 5% CO_2. After 48 h, 0.1 ml of DMEM-F12 and 0.03 ml of the MTT reagent (0.5 $mg \cdot ml^{-1}$) were added to the wells and the incubation continued for 1 h, after which the supernatant was removed. The formazan crystals formed during MTT reduction by viable cells were dissolved in 0.1 ml of DMSO and the optical density was measured on a BioRad x Marx plate spectrophotometer (USA) at λ = 540 nm, subtracting the background optical density at λ = 690 nm. For each cell line, the half-maximal inhibition concentration (IC_{50}) was determined.

1.8 Computational approach

At the first stage we have calculated electronic structure and the most preferable geometry of substances **3** and **4** by Density Functional Theory (DFT) method realized in DMol^3 module from Materials Studio package. We applied PBE functional with DNP (4.4) electronic basis and COSMO model for simulation of water environment.[S8–S10] At the second stage we applied classical molecular dynamics (MD) to determine the interaction of various atoms of molecules **3** and **4** with water. Charges determined with Mulliken's scheme from DFT calculations for substances **3** and **4**, was used with short-range Universal Force Field parameters. One molecule of each substance **3** or **4** was surrounded by 1000 molecules of water in NVT ensemble at 300 K with a step of 1 fs in the NVT ensemble at 298 K.

2. Figures

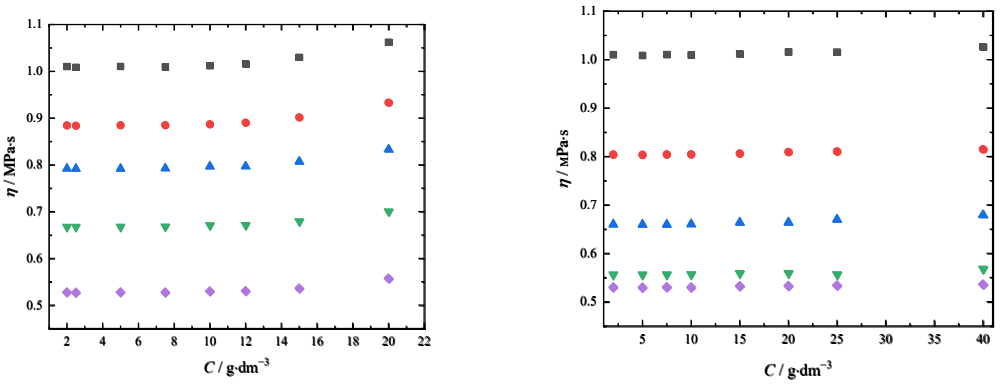

Fig. S1. The viscosity of substances **3** (*left*) and **4** (*right*) in water (T = 293.15–318.15 K)

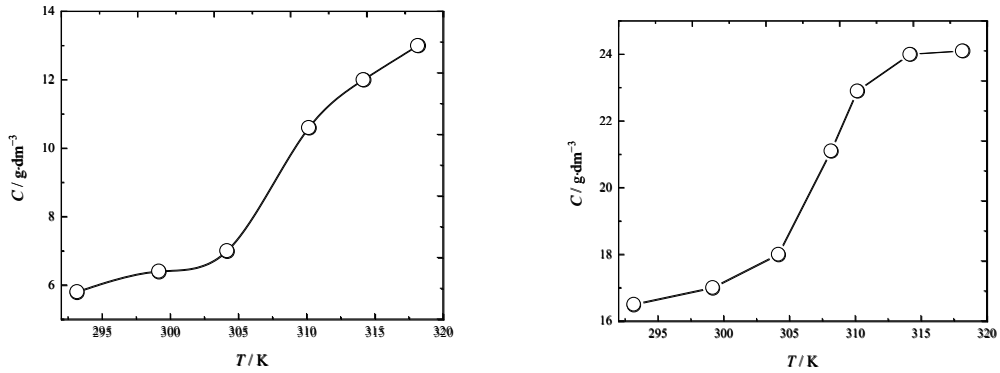

Fig. S2. The solubility of substances **3** (*left*) and **4** (*right*) in water (T = 293.15–318.15 K)

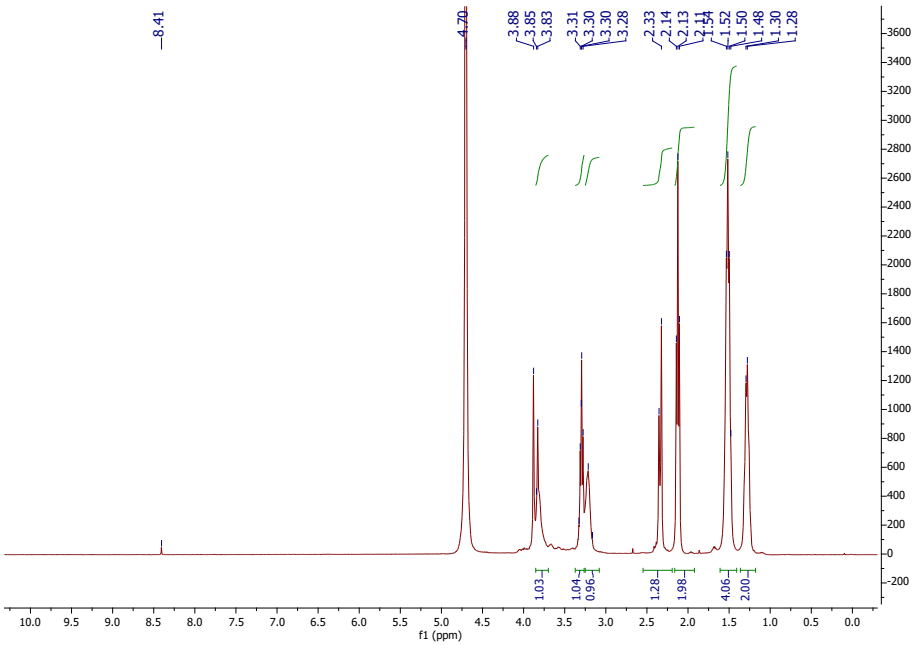

Fig. S3. ^{1}H NMR spectrum of sodium 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate (**4**) after 7 days.

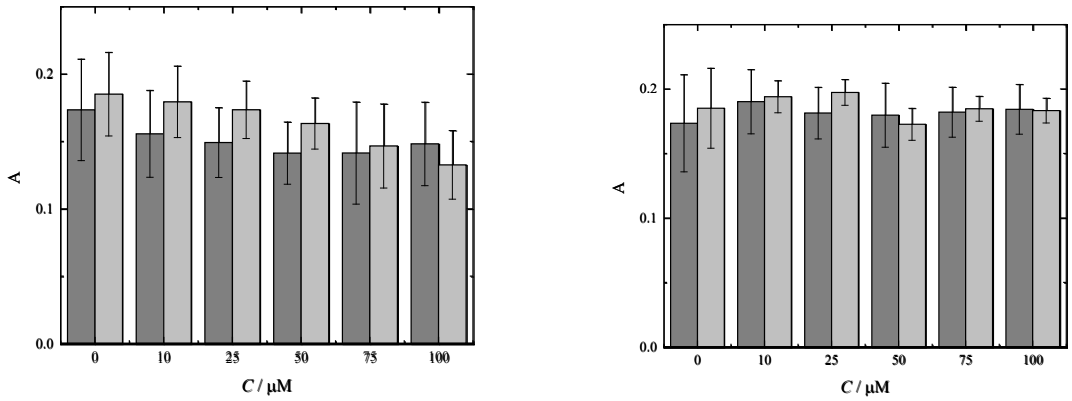

Fig. S4. Effect of compound **3** (*left*) and **4** (*right*) on the scavenging of NO radicals (light gray) and sodium azide (dark gray).

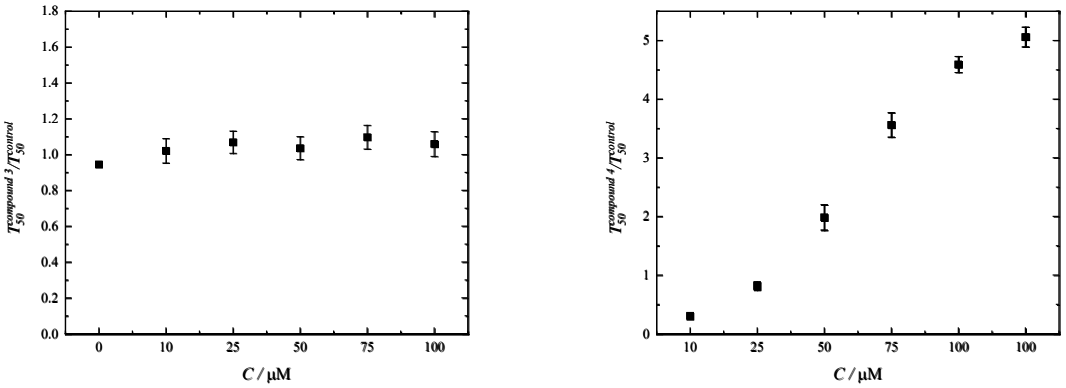

Fig. S5. Concentration dependence of the degree of photohemolysis in the presence of substance **3** (*left*) and **4** (*right*). C is the molar concentration of substance **3** (*left*) and **4** (*right*), T_{50}^{samp} is the time of photohemolysis of 50% of erythrocytes in the presence of substance **3** (*left*) and **4** (*right*), $T_{50}^{control}$ is the same time in the presence of saline solution.

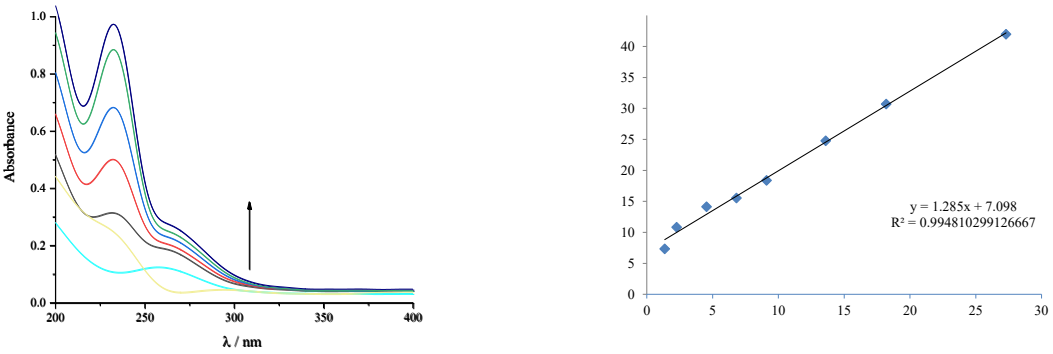

Fig. S6. *(left)* UV spectra of aqueous solutions (0.9% NaCl) DNA (*C* 5.7 μM) in the presence of compound **4** at various concentrations (*C* = 0; 0.57–14.25 μM); *(right)* Dependence in Wollfe-Shimmer coordinates for compound **4**.

3. Tables

Table S1. The values of density of compound **3** and **4** at the temperature range from 25°C to 50°C

$C_{\text{compound 3}}$ / g·dm^{-3}	ρ				
	25°C	30°C	40°C	45 °C	50 °C
2	0.99824	0.99569	0.99226	0.98806	0.98323
2.5	0.99827	0.99572	0.99228	0.98811	0.98319
5	0.9983	0.99574	0.99231	0.98812	0.98329
7.5	0.9984	0.99584	0.99241	0.98824	0.98335
10	0.99849	0.99593	0.99249	0.98834	0.98344
12	0.99875	0.99618	0.99274	0.98854	0.9837
15	0.99978	0.99719	0.99373	0.98951	0.98466
20	1.00093	0.99832	0.99484	0.99062	0.98517
$C_{\text{compound 4}}$ / g·dm^{-3}					
2	0.99609	0.98684	0.98233	0.97609	0.9734
5	0.99613	0.98687	0.98235	0.97615	0.97335
7.5	0.99616	0.9869	0.98238	0.97615	0.97346
10	0.99626	0.98699	0.98247	0.97627	0.97351
15	0.99634	0.98708	0.98255	0.97637	0.9736
20	0.9966	0.98733	0.9828	0.97656	0.97386
25	0.99763	0.98833	0.98378	0.97753	0.97481
40	0.99878	0.98945	0.98488	0.97863	0.97531

Table S2. The charges on the atoms and the values of the optimal distance between water molecules and the nitrogen, chlorine, sodium and oxygen atoms in compounds **3** and **4**

The charges on the atoms (e)									
Compound	N1	N2	N3	N4	N5	O1	O2	Cl	Na
3	-0.301	-0.301	-0.335	-0.201	-0.229	-0.493	-0.437	-0.154	
4	-0.310	-0.306	-0.359	-0.193	-0.222	-0.668	-0.662	-0.156	0.878
The optimal distance (Å)									
Compound	N1	N2	N3	N4	N5	O1	O2	Cl	Na
3	3.45	3.33	3.31	3.55	3.65	3.33	3.33	3.67	
4	3.43	3.67	3.75	3.39	3.43	3.29	3.25	3.43	2.35

Table S3. XRD data for compound **7** fractional Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Displacement Parameters ($Å^2 \times 10^3$) for compound 7. U_{eq} is defined as 1/3 of of the trace of the orthogonalised U_{IJ} tensor.

Atom	*x*	*y*	*z*	U(eq)
O1	-941(3)	2953.2(16)	5380.0(13)	22.1(4)
O2	7867(3)	7868.6(17)	7863.1(15)	23.9(4)
N3	2948(4)	2906.9(19)	7242.6(16)	18.4(4)
N2	7604(4)	5436.6(19)	8583.9(16)	19.0(4)
N1	3517(4)	5423(2)	6606.2(16)	19.6(4)
N4	6588(4)	2693.1(19)	9000.8(17)	19.4(4)
C1	1660(5)	3706(2)	6332.0(19)	18.3(4)
C3	5848(4)	3777(2)	8307.0(19)	17.7(4)
C2	6448(4)	6325(2)	7707.9(19)	19.1(4)
C5	3859(4)	1018(2)	8559(2)	20.0(4)
C4	1840(5)	1020(2)	7102.3(19)	20.1(4)

Table S4 Anisotropic Displacement Parameters ($Å^2 \times 10^3$) for compound **7**. The Anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2}U_{11}+2hka^*b^*U_{12}+\ldots]$.

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O1	20.2(7)	19.8(7)	18.6(6)	4.9(5)	-6.8(5)	-0.3(5)
O2	22.5(7)	17.4(7)	25.8(7)	7.1(5)	-2.0(5)	-0.8(5)
N3	15.7(8)	16.5(7)	17.2(8)	3.5(6)	-3.5(6)	0.0(6)
N2	17.1(7)	17.5(8)	17.9(8)	4.2(6)	-1.6(6)	1.4(6)
N1	18.9(8)	18.2(8)	17.8(8)	6.2(6)	-2.6(6)	2.0(6)
N4	16.4(7)	18.8(8)	17.9(7)	6.0(6)	-4.1(5)	0.5(6)
C1	18.6(8)	18.9(8)	15.1(8)	4.3(6)	1.8(6)	4.0(7)
C3	15.0(8)	18.7(9)	15.4(8)	2.3(6)	0.8(6)	2.7(7)
C2	17.3(8)	20.1(9)	17.3(8)	4.4(7)	2.1(7)	3.6(7)
C5	18.2(8)	17.0(8)	19.2(8)	4.7(6)	-3.0(7)	0.4(7)
C4	20.0(8)	15.6(8)	19.8(9)	5.0(6)	-2.3(7)	1.1(6)

Table S5 Bond Lengths for compound **7**

Atom	Atom	Length/Å	Atom	Atom	Length/Å
O1	C1	1.228(2)	N2	C2	1.374(2)
O2	C2	1.223(2)	N1	C1	1.370(2)
N3	C1	1.364(2)	N1	C2	1.401(2)
N3	C3	1.372(2)	N4	C3	1.323(2)
N3	C4	1.471(2)	N4	C5	1.474(2)
N2	C3	1.318(2)	C5	C4	1.536(2)

Table S6 Bond Angles for compound **7.**

Atom	Atom	Atom	Angle/˚	Atom	Atom	Atom	Angle/˚
C1	N3	C3	121.73(16)	N2	C3	N3	124.75(17)
C1	N3	C4	126.78(15)	N2	C3	N4	125.76(16)
C3	N3	C4	110.89(15)	N4	C3	N3	109.49(16)
C3	N2	C2	116.64(15)	O2	C2	N2	122.45(17)
C1	N1	C2	125.03(16)	O2	C2	N1	119.14(17)
C3	N4	C5	111.39(14)	N2	C2	N1	118.42(16)
O1	C1	N3	122.66(16)	N4	C5	C4	102.44(13)
O1	C1	N1	123.92(16)	N3	C4	C5	101.38(13)
N3	C1	N1	113.41(16)				

Table S7 Torsion Angles for compound **7.**

A	B	C	D	Angle/˚	A	B	C	D	Angle/˚
N4	C5	C4	N3	-19.96(17)	C3	N4	C5	C4	18.18(18)
C1	N3	C3	N2	1.7(3)	C2	N2	C3	N3	-1.6(3)
C1	N3	C3	N4	-178.20(15)	C2	N2	C3	N4	178.34(15)
C1	N3	C4	C5	-171.82(16)	C2	N1	C1	O1	178.43(16)
C1	N1	C2	O2	-178.91(15)	C2	N1	C1	N3	-0.5(2)
C1	N1	C2	N2	0.6(3)	C5	N4	C3	N3	-8.0(2)
C3	N3	C1	O1	-179.53(16)	C5	N4	C3	N2	172.08(16)
C3	N3	C1	N1	-0.6(2)	C4	N3	C1	O1	10.3(3)
C3	N3	C4	C5	17.10(18)	C4	N3	C1	N1	-170.80(15)
C3	N2	C2	O2	179.92(16)	C4	N3	C3	N2	173.35(16)
C3	N2	C2	N1	0.4(2)	C4	N3	C3	N4	-6.6(2)

Table S8 Hydrogen Atom Coordinates ($Å \times 10^4$) and Isotropic Displacement Parameters ($Å^2 \times 10^3$) for compound **7.**

Atom	***x***	***y***	***z***	**U(eq)**
H4	8471.19	2940.63	9644.04	23
H5A	2486.46	1002.61	9320.68	24
H5B	4738.89	-7.58	8391.88	24
H4A	2407.27	287.2	6220.49	24
H4B	-597.67	595.69	7040.45	24
H1	2840(70)	6040(30)	6050(30)	29(6)

4. NMR Spectra

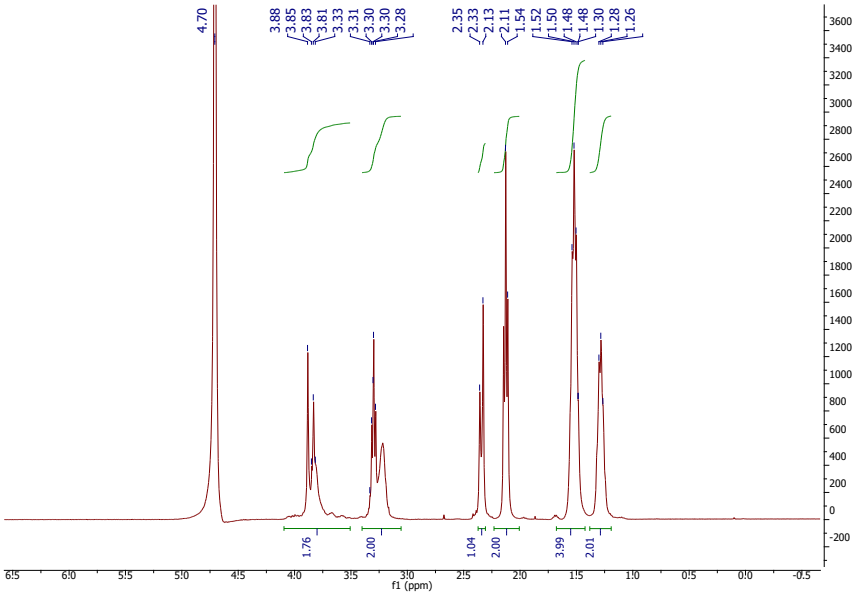

Fig S7. ^{1}H NMR spectrum of spectra of sodium 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate (**4**) D_2O.

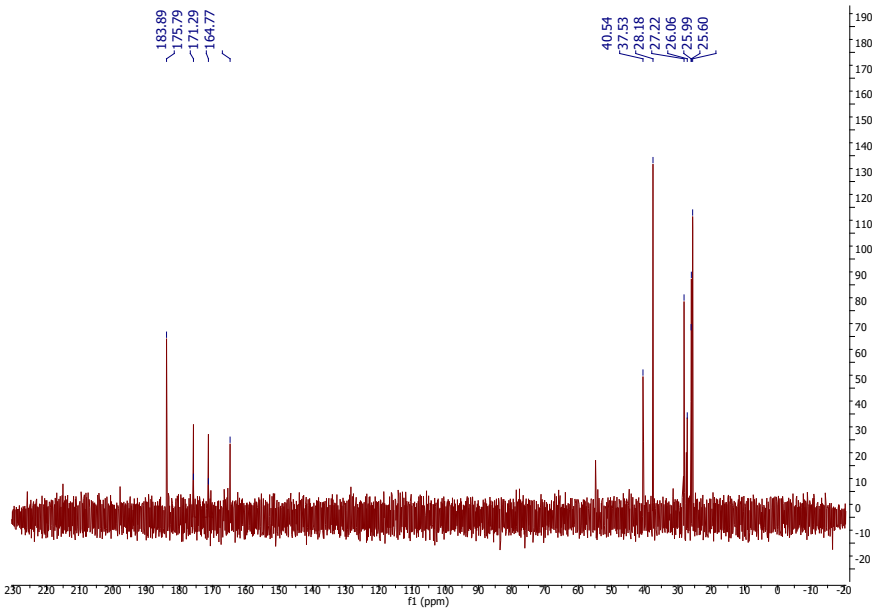

Fig S8. ^{13}C NMR spectrum of spectra of sodium 6-[(4-(aziridin-1-yl)-6-chloro-1,3,5-triazin-2-yl)amino]hexanoate (**4**) D_2O.

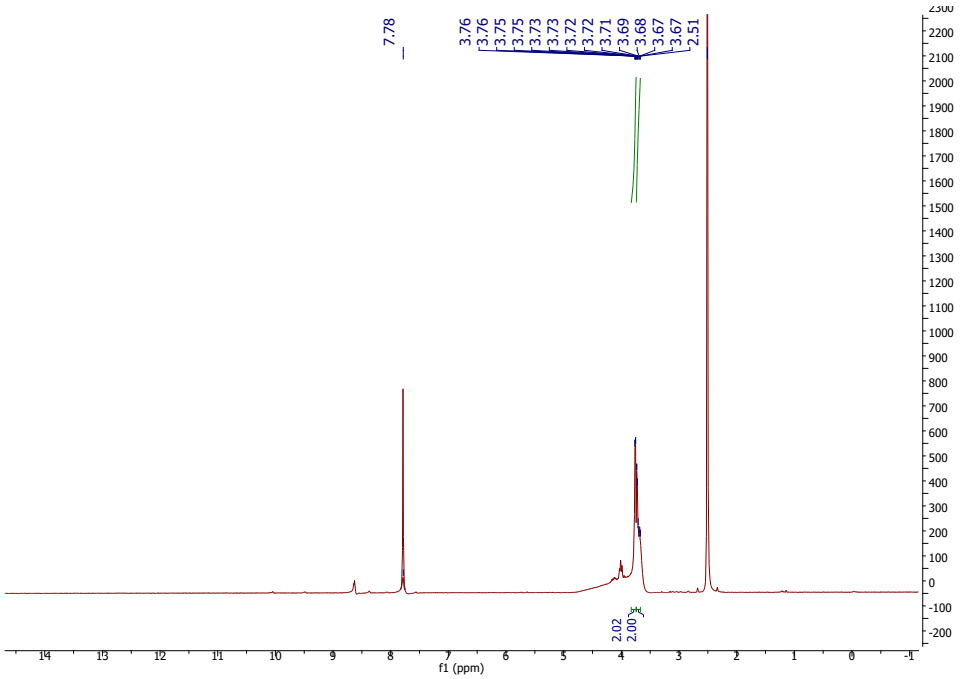

Fig S9. ^{1}H NMR spectrum of 7,8-dihydroimidazo[1,2-*a*][1,3,5]triazine-2,4(3*H*,6*H*)-dione **(7)** DMSO-d_6.

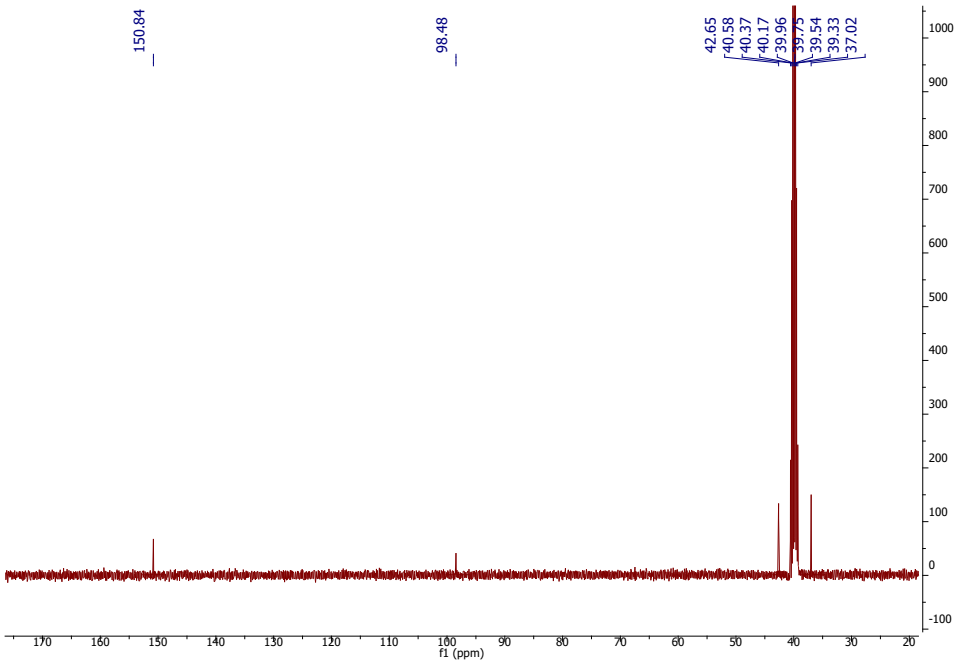

Fig S10. ^{13}C NMR spectrum of 7,8-dihydroimidazo[1,2-*a*][1,3,5]triazine-2,4(3*H*,6*H*)-dione **(7)** DMSO-d_6.

References

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