Synthesis of the [^{2}H,^{15}N]-labeled antiviral drug "triazavirine"*

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Selective methods for the incorporation of stable isotopes ^{15}N and ^{2}H into the structure of antiviral medicine "triazavirine" **1** were developed. The synthesized isotopically modified "triazavirine" **1-^{2}H$_3$,^{15}N$_3$** contained the labeled atoms in both the azole and the azine rings. ^{13}C and ^{15}N NMR spectra of the isotope-containing sample **1-^{2}H$_3$,^{15}N$_3$** were thoroughly analyzed.

Key words: diazonium salt, azocoupling, [^{2}H,^{15}N]-labeled "triazavirine", NMR spectra.

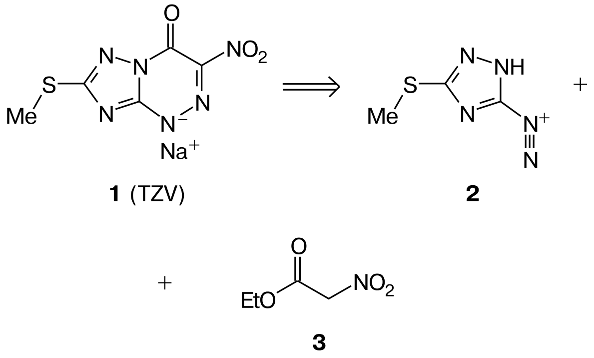
Incorporation of stable isotopes (^{2}H, ^{13}C, ^{15}N) into the structures of biologically active compounds is used as an efficient approach to the development of methods for their detection by mass spectrometry and NMR spectroscopy. The use of this method allows one to study pharmacokinetics and metabolic ways of medicinal drugs.[1—5]

6-Nitro-1,2,4-triazolo[5,1-*c*][1,2,4]triazin-7-one derivatives belong to the new class of compounds possessing antiviral activity.[6,7]A special role in this series plays the drug "triazavirine" **1** (TZV) , which is now in the second phase of clinic testing and proved efficient against different strains of the flu virus, including the H5N1 strains (bird flu virus).[8—10]

The purpose of the present study is to develop a method for the synthesis of compound **1** containing stable isotopes ^{15}N and ^{2}H in the azole and the azine rings.

A scheme for the synthesis of sodium salt **1** is based on the azocoupling of diazonium salt **2** with ethyl nitroacetate **3** (Scheme 1).[8] Compound **2** was obtained from 3-amino-5-methylthio-1,2,4-triazole and nitrous acid. The diazonium salt is not isolated in the pure form, rather it is used in the solution. Thus, incorporation of the ^{15}N and ^{2}H atoms into the structure of compounds **2** and **3** would allow one to obtain the labeled TZV.

Scheme 1



Enriched sodium nitrite (^{15}N, 98%) has been chosen as a donor of the ^{15}N isotope for the synthesis of labeled ethyl nitroacetate **3**, the former reacted with ethyl acetoacetate in acetic acid (Scheme 2) to yield nitroso derivative **5-^{15}N** existing in DMSO-d$_6$ solution as an oxime **5B-^{15}N**.

It should be noted that compound **5B-^{15}N** exists as a mixture of *E*- and *Z*-isomers in the ratio 10 : 1. This conclusion on the structure of oxime **5B-^{15}N** was inferred from the ^{1}H and ^{15}N NMR spectroscopic data. The ^{15}N NMR spectrum of compound **5B-^{15}N** exhibits two signals for the ^{15}N atoms at δ 401.31 and δ 396.86, whereas the ^{1}H NMR spectrum shows two singlets at δ 13.25 and δ 12.99 corresponding to the OH groups. However, we did not fully assign all the signals in the ^{15}N and ^{1}H NMR spectra of compound **5B-^{15}N** to the isomeric *E* and *Z* forms. Subsequent treatment of compound **5-^{15}N** with sodium dichromate allowed us to synthesize labeled

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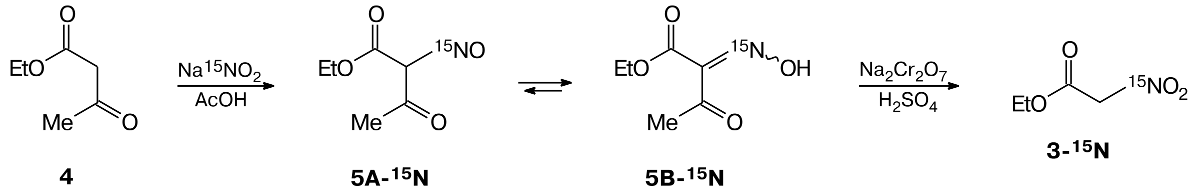
* Dedicated to Academician of RAS V. N. Charushin in honor of his 60th birthday.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 4, pp. 714—717, April, 2011.

Scheme 2



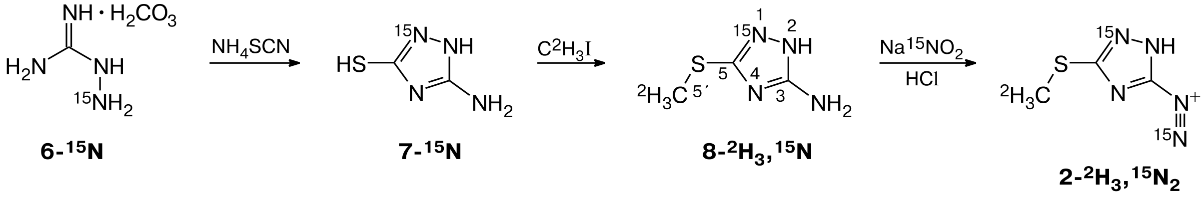
ethyl nitroacetate **3-^{15}N**. Analysis by ^{1}H NMR spectroscopy showed that this oxidation reaction resulted in a mixture of compounds **3-^{15}N** and **5B-^{15}N** in the ratio 3 : 1, respectively. This mixture was used in subsequent azocoupling without additional purification. The formation of compound **3-^{15}N** was confirmed by the presence of additional splitting of the signal for the methylene group (CH_2) in the ^{1}H NMR spectrum, which results from the spin-spin interaction with the ^{15}N atom ($^2J_{H,N}$ = 2.0 Hz).

To introduce the ^{15}N isotope into the azole ring of diazonium salt **2**, we developed a synthetic scheme based on the use of aminoguanidine **6-^{15}N** (enriched with ^{15}N by 86%) (Scheme 3). Treatment of compound **6-^{15}N** with ammonium isothiocyanate led to thiourazole **7-^{15}N**. Position of the isotope in compound **7-^{15}N** was determined by the synthetic scheme. The following step in the preparation of the isotopically modified diazonium salts **2** consisted in the selective incorporation of the ^{2}H atoms using the alkylation reaction of triazole **7-^{15}N** with deuterated methyl iodide (^{2}H, 98%), which resulted in obtaining compound **8-^{2}H$_3$,^{15}N** in 63% yield. The presence of the isotopic labels in this case was confirmed by the mass spectrometric data ($[M + H]^+$ 135.05). In addition, the ^{15}N NMR spectrum of compound **8-^{2}H$_3$,^{15}N** exhibited signal for the N(1) atom in the form of a singlet (δ 262.70). Further transformation of hetarylamine **8-^{2}H$_3$,^{15}N** to the diazonium salt **2-^{2}H$_3$,^{15}N$_2$** was performed upon the action of ^{15}N-nitrous acid generated from Na^{15}NO$_2$ (enriched with ^{15}N by 98%) in acidic medium. The use of ^{15}N-labeled nitrous acid in the diazotization of amine **8-^{2}H$_3$,^{15}N** led to the introduction of additional ^{15}N label into the structure **2**.

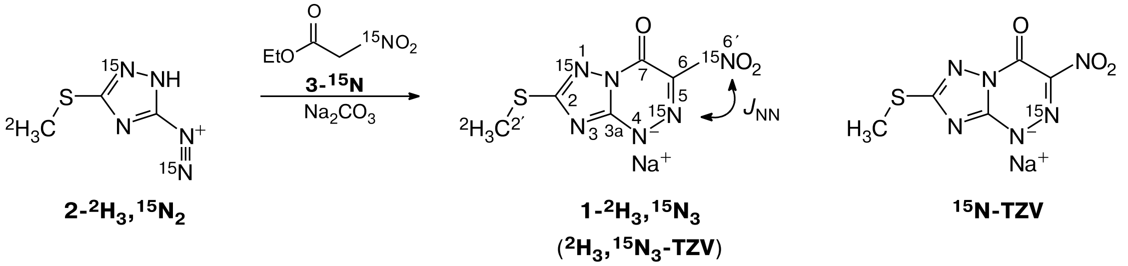
Such an approach to the introduction of isotope ^{15}N into the 1,2,4-triazole diazonium salts, important synthons for the preparation of various 1,2,4-triazolo[5,1-*c*]-[1,2,4]triazine derivatives, has been described earlier.^{11} The very approach to the incorporation of isotope ^{15}N at position 5 of fused 1,2,4-triazolo[5,1-*c*][1,2,4]triazines has been used in the synthesis of isotope-labeled TZV containing a single label ^{15}N(5) ([5-^{15}N]-2-methylthio-6-nitro-1,2,4-triazolo[5,1-*c*][1,2,4]triazin-7-one sodium salt (^{15}N-TZV)) (Scheme 4).

The coupling reaction between the diazonium salt **2-^{2}H$_3$,^{15}N$_2$** and ethyl nitroacetate **3-^{15}N** in the presence of the sodium carbonate solution allowed us to synthesize the antiviral drug "triazavirine" **1-^{2}H$_3$,^{15}N$_3$** enriched with isotopes ^{15}N and ^{2}H (see Scheme 4).

Scheme 3



Scheme 4



The ^{15}N NMR spectrum of compound **1-$^2H_3$,$^{15}N_3$** exhibits signals for the N(5) and N(6′) atoms as doublets with the spin-spin coupling constant $^2J_{N,N}$ = 6.3 Hz, whereas the signal for the N(1) atom has the form of a singlet (Table 1). Such a spectral picture confirms the presence of three isotopic labels ^{15}N in compound **1-$^2H_3$,$^{15}N_3$**. Assignment of the signals for the nitrogen atoms in the isotope-containing "triazavirine" (**$^2H_3$,$^{15}N_3$-TZV**) was made with allowance for the splitting of the signals, as well as based on the chemical shift for the $^{15}N(5)$ (δ 397.20) in the labeled **^{15}N-TZV** (see Ref. 11). The presence of the ^{15}N atom in the structure of compound **1-$^2H_3$,$^{15}N_3$** leads to the appearance of ^{13}C-^{15}N heteronuclear spin-spin coupling constants for the signals of the carbon atoms C(2), C(3a), C(6), and C(7) (see Table 1). In addition, the carbon spectrum exhibits additional splitting of the signals for the atoms C(2′) and C(2), which is due to the presence of the ^2H-^{13}C spin-spin interaction. The ^{13}C-^{15}N spin-spin coupling constant were measured based on the 1D ^{13}C NMR experiments recorded with the selective ^{15}N decoupling. The results obtained confirm selectivity of incorporation of three ^{15}N isotopic labels into the structure of TZV. In addition, the presence of the spin-spin interaction of the C(6) and C(7) atoms with the N(1) atom confirms the [5,1-*c*] type of fusion between the azole and azine fragments in heterocycle **1**.

In conclusion, we developed a method for the selective incorporation of isotopes ^{15}N and ^2H at different positions of sodium salt **1**, which resulted in obtaining isotope-containing antiviral drug "triazavirine" **1-$^2H_3$,$^{15}N_3$** with high content of the ^{15}N and ^2H atoms. It can be used as an internal standard in the study of pharmacokinetic of drug "triazavirine" by mass spectrometry. Characteristics of ^{13}C and ^{15}N NMR spectra of compound **1-$^2H_3$,$^{15}N_3$** were also studied.

Table 1. The ^{13}C and ^{15}N NMR spectral data of compound **1-$^2H_3$,$^{15}N_3$**

^{13}C		^{15}N	
Atom	δ, *J*/Hz	Atom	δ, *J*/Hz
C(2′)	13.40* ($^1J_{C,D}$ = 21)	N(1)	259.91
C(2)	166.33* ($^2J_{C,N(1)}$ = 5.1, $^3J_{C,D}$ = 0.7)	N(5)	397.18 ($^2J_{N,N}$ = 6.3)
C(3a)	160.70 ($^2J_{C,N(5)}$ = 2.1, $^2J_{C,N(1)}$ = 0.4)	N(6′)	368.42 ($^2J_{N,N}$ = 6.3)
C(6)	145.15 ($^1J_{C,N(5)}$ = 1.8, $^1J_{C,N(6′)}$ = 23.4, $^3J_{C,N(1)}$ = 1.4)		
C(7)	143.43 ($^2J_{C,N(5)}$ = 1.3, $^2J_{C,N(6′)}$ = 5.3, $^2J_{C,N(1)}$ = 3.3)		

* The signals contain additional splitting as a result of the ^2H—^{13}C spin-spin coupling.

Experimental

^1H, ^{13}C, and ^{15}N NMR spectra of compounds **5B-^{15}N**, **3-^{15}N**, **7-^{15}N** and **8-$^2H_3$,^{15}N** were recorded on a Bruker Avance II 400 spectrometer in DMSO-d_6 with $SiMe_4$ as an internal standard (^{13}C, ^1H) and liquid ammonia as an external standard (^{15}N). The study of compound **1-$^2H_3$,$^{15}N_3$** by NMR was performed on a Bruker Avance 700 spectrometer equipped with a triple resonance probe (^1H,^{13}C,^{15}N).

The ^{13}C—^{15}N spin-spin coupling constants were measured using a method for the nonlinear approximation of the line shapes in the ^{13}C 1D spectra recorded with and without selective ^{15}N decoupling.**12** Adiabatic impulses (WURST-20) of 10—20 ms in length and the width of inversion range ~1 kHz (14 ppm for ^{15}N) were used for the selective ^{15}N decoupling.

Mass spectrometric study was performed on a Bruker Daltonics series MicrOTOF-Q II mass spectrometer (Bremen, Germany) in the electrospray-ionization mode (ESI).

Sodium ^{15}N-nitrite (enriched with ^{15}N by 98%) was purchased from ISOTEC. ^{15}N-Aminoguanidine **6-^{15}N** (enriched with ^{15}N by 86%) was synthesized according to the procedure described earlier.**13**

Ethyl [^{15}N]-isonitrosoacetoacetate (5B-^{15}N). A solution of $Na^{15}NO_2$ (1.8 g, 25.71 mmol) in water (2.5 mL) was added to a mixture of ethyl acetoacetate (3 mL, 23.72 mmol) and AcOH (4 mL) at 5—15 °C. The solution obtained was kept at 15 °C for 0.5 h and poured into the ice water (60 mL). The suspension obtained was kept for 16 h and extracted with several portions of ethyl acetate (4×15 mL). The extracts were combined and the organic solvent was evaporated to obtain an oily residue (1.9 g), which was further used without purification for the synthesis of ethyl nitroacetate (**3-^{15}N**). The yield (**5B-^{15}N**) was 51%. The NMR spectral data are given with allowance for existence of oxime **5B-^{15}N** in DMSO-d_6 in two isomeric forms (*E*/*Z*). ^1H NMR (400 MHz), δ: 1.22 (t, 3 H, $OCH_2C\underline{H}_3$, *J* = 7.0 Hz); 1.23 (t, 0.3 H, $OCH_2C\underline{H}_3$, *J* = 7.0 Hz); 2.33 (s, 0.3 H, $C\underline{H}_3$); 2.34 (s, 3 H, $C\underline{H}_3$); 4.23 (q, 0.2 H, $OC\underline{H}_2CH_3$, *J* = 7.3 Hz); 4.24 (q, 2 H, $OC\underline{H}_2CH_3$, *J* = 7.0 Hz); 12.99 (br.s, 0.1 H, N—OH); 13.25 (br.s, 1 H, N—OH). ^{15}N NMR (40.5 MHz), δ: 401.31; 396.86. MS, *m*/*z* (I_{rel} (%)): 161.05 $[M + H]^+$ (100).

Ethyl [^{15}N]-nitroacetate (3-^{15}N). A solution of sodium dichromate dihydrate (3.1 g, 10.40 mmol) in a mixture of water (7 mL) and concentrated H_2SO_4 (2.5 mL) was added to a suspension of compound **5B-^{15}N** (1.9 g) in water (6 mL). Then the reaction mixture was stirred for 1.5 h, poured into ice water (10 mL), then heated to room temperature. The suspension obtained was treated with several portions of ethyl acetate (4×15 mL). The extracts were combined, dried with anhydrous Na_2SO_4 and concentrated to obtain an oily residue (1 mL), which was further used for the synthesis of sodium salt **1-$^2H_3$,$^{15}N_3$** without additional purification. According to the ^1H NMR spectroscopic data, the residue was a mixture of compounds **5B-^{15}N** and **3-^{15}N** in the ratio 1 : 3. ^1H NMR of **3-^{15}N** (400 MHz), δ: 1.27 (t, 3 H, $OCH_2C\underline{H}_3$, *J* = 7.0 Hz); 4.24 (q, 2 H, $OC\underline{H}_2CH_3$, *J* = 7.0 Hz); 5.68 (d, 2 H, $C\underline{H}_2$, $^2J_{N,H}$ = 2.0 Hz).

[^{15}N]-3-Amino-1,2,4-triazole-5-thione (7-^{15}N). Aminoguanidine carbonate **6-^{15}N** (1.986 g, 14.5 mmol) was added to a solution of ammonium isothiocyanate (1.108 g, 14.6 mmol) in water (0.73 mL). The reaction mixture was stirred for 1 h at 90—100 °C, followed by a dropwise addition of conc. hydrochloric acid (6.9 mL) over 2 h. Then the reaction mixture was heated for 0.5 h at 100 °C, followed by addition of a solution of KOH (3.68 g, 64.6 mmol) in water (3.7 mL) and heating for 2 h at 110—120 °C. Then the mixture was cooled and filtered. The solution obtained was acidified with hydrochloric acid to pH = 3—4, a precipitate was filtered off, washed with cold water (3—5 mL), and dried. The yield was 1.026 g (60%), m.p. >300 °C. MS, m/z (I_{rel} (%)): 118.02 $[\text{M} + \text{H}]^+$ (100).

[^{2}H$_3$,^{15}N]-3-Amino-5-methylthio-1,2,4-triazole (8-^{2}H$_3$,^{15}N). [^{15}N]-3-Amino-1,2,4-triazole-5-thione (**7-^{15}N**) (1.026 g, 8.77 mmol) was added to a solution of NaOH (0.380 g, 9.50 mmol) in water (9 mL). The mixture was cooled to 5 °C, followed by addition of C^2H_3I (0.55 mL, 8.83 mmol, d 2.329) with stirring. Then the reaction mixture was stirred for 3—5 h at 10—15 °C and kept for 16 h at room temperature. The solution was filtered and two thirds concentrated *in vacuo*. After cooling to 0—5 °C, a precipitate that formed was filtered off and dried. The yield was 0.764 g (63%), m.p. 136—137 °C. ^{13}C NMR (100 MHz), δ: 13.12 C(5´); 156.88 C(5); 157.52 C(3). ^{15}N NMR (40.5 MHz), δ: 262.70. MS, m/z (I_{rel} (%)): 135.05 $[\text{M} + \text{H}]^+$ (100).

[^{2}H$_3$,^{15}N$_3$]-2-Methylthio-1,2,4-triazolo[5,1-*c*][1,2,4]triazin-7-one sodium salt (1-^{2}H$_3$,^{15}N$_3$). Hydrochloric acid (11 *M*, 0.5 mL) was added to a suspension of amino-1,2,4-triazole **8-^{2}H$_3$,^{15}N** (0.4 g, 2.98 mmol) in water (3 mL). The mixture was cooled to 0 °C, followed by a dropwise addition of a solution of $Na^{15}NO_2$ (210 mg, 3 mmol) in water (3 mL). Then the reaction mixture was stirred for 10 min and added to a cooled to 0 °C suspension of 17% aqueous Na_2CO_3 (7 mL) and a mixture of compounds **3-^{15}N** and **5B-^{15}N** (0.6 mL), which was obtained earlier. The formed reaction mixture was stirred for 2 h at room temperature, a precipitate that formed was filtered off and crystallized from 50% aq. acetic acid to obtain sodium salt **1-^{2}H$_3$,^{15}N$_3$** (0.45 g) as a dihydrate. The yield was 51%, m.p. > 300 °C. Found (%): C, 20.85; H, 2.93. $C_5H_4{}^2H_3N_3{}^{15}N_3O_5SNa \cdot 2\,H_2O$. Calculated (%): C, 20.55; H, 3.45. MS, m/z (I_{rel} (%)): 233.00 $[\text{M} - \text{Na}]^-$ (100). The ^{13}C and ^{15}N NMR spectral data are given in Table 1.

This work was financially supported by the Russian Foundation for Basic Research (Project No. 10-03-01007-a), the Ministry of Education and Science of Russian Federation (State Contracts P2444 dated 19.11.2009 and 402.740.11.0260), the Council on Grants at the President of the Russian Federation (Program of State Support for Leading Scientific Schools of the Russian Federation, Grant NSh-65261.2010.3).

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Received 3, February 2011