2-THIOZEBULARINE: BASE MODIFIED NUCLEOSIDE FULLY CONSTRAINED IN C3′-*endo* CONFORMATION IN SOLUTION

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This article is dedicated to Professor Antonín Holý on the occasion of his 75th birthday in recognition of his outstanding contribution to the area of nucleic acids chemistry.

2-Thiopyrimidinone ribofuranoside (2-thiozebularine, s^2zeb) was synthesized by the adaptation of silyl method of N-glycosidic bond formation and using thionation of protected 2-oxonucleoside derivative (zebularine, zeb) with Lawesson reagent. The X-ray crystal structure of s^2zeb and NMR determined conformations of s^2zeb and zeb in solution were compared with structures of 2-thiouridine (s^2U) and uridine (U). In the solid state s^2zeb molecule adopts conformation typical for ribonucleosides: C3′-*endo* C2′-*exo* twist type of ribofuranose pucker, *anti* of N-glycosidic bond and *trans* around C4′–C5′ bond. In aqueous solution, however, almost 100% population of s^2zeb exhibits C3′-*endo* ribofuranose pucker. The population of N-conformer of s^2zeb is about 20% higher than for zeb (analogously to pair of s^2U and U nucleosides) indicating similar influence of steric effect of bulky sulfur atom on stabilization of N-type ribose conformation. Interestingly, the absence of 4-carbonyl function in zeb and s^2zeb raises the population of C3′-*endo* conformation by about 30% in comparison to U and s^2U as a result of significant anomeric effect. Additive action of both effects makes the 2-thiozebularine almost fully constrained in C3′-*endo* conformation in aqueous solution. Cytotoxic properties of s^2zeb are less pronounced in comparison to zebularine, with IC_{50} > 100 mM for HeLa and K562 cancer cells and for HUVEC non-cancerous cells.

Keywords: 2-Thiozebularine; Zebularine; Modified nucleosides; Conformation of nucleosides; X-ray; NMR; Cytotoxity of nucleosides.

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2-Thiozebularine (s^2zeb) is one of the 2-pyrimidinone nucleoside analogues in which carbonyl group at C-2 position of the heterobase moiety was replaced by thiocarbonyl function. First synthesis of 2-thiozebularine was described by Wightman and Holý in 1973 [1]. The family of 2-pyrimidinone nucleosides plays an important role among biologically active analogues of natural pyrimidine nucleosides and was intensively investigated. Parent 1-β-D-ribofuranosylpyrimidin-2-one (zebularine, zeb) exhibits an antibacterial activity connected with its *in vivo* transformation into 1-(2′-deoxy-β-D-ribofuranosyl)pyrimidin-2-one 5′-phosphate, a strong inhibitor of thymidylate synthetase[2,3]. Zebularine is also a strong cytidine deaminase inhibitor[4,5] and effectively inhibits DNA methylation[6,7].

Besides of the wide spectrum of biological activity, 2-pyrimidinone nucleosides are important tools as model compounds in structural studies involving RNA, and nucleic acid-binding proteins. This is mainly because 2-pyrimidinone nucleosides lacking N3-amide hydrogen and carbonyl function at C4 position in heterobase moiety, offer entirely different possibilities for base pairing within RNA duplexes[8,9].

As the sulfur modification of pyrimidine nucleosides by the replacement of the C2-carbonyl group with C2-thiocarbonyl is known to have a great impact on their structural features, we are interested in 2-thiozebularine in the context of our structural studies on modified nucleosides with non-natural bases[10].

In the present work we describe the results of our conformational studies of 2-thiozebularine (s^2zeb, **1**, Fig. 1) in the solid state and in aqueous solution. The resolved crystal structure of **1** is compared to the published structures of zebularine, (zeb, **2**)[11], 2-thiouridine (s^2U, **3**)[12] and uridine (U, **4**)[13]. Conformational studies in aqueous solution were performed for **1** and also for not so far precisely determined conformation of **2** by means of high-

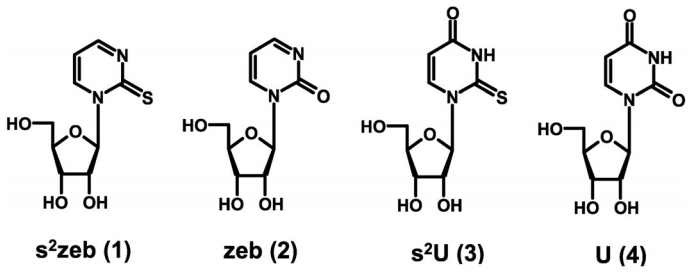


FIG. 1
Structures of nucleosides

resolution NMR methods and the results were compared with the known data for s^2U [14] and U [15]. Moreover, cytotoxic properties of s^2zeb and zeb as well as their 2′-deoxyribo analogues were determined by MTT assay in HeLa and K542 cancer cells and in HUVEC non-cancerous cells.

EXPERIMENTAL

Materials

All reagents were commercially available. In particular, CH_3CN was distilled from P_2O_5; MeOH from Mg and benzene from Na. $SnCl_4$ was distilled from P_2O_5 under reduced pressure. All other reagents were carefully dried before use. Silylation of heterobase and the reaction of N-glycoside bond formation were performed under anhydrous conditions.

For column chromatography Merck silica gel 60 (230–400 mesh) was used. TLC was performed on analytical silica plates (Kieselgel 60 F_{254}/0.2 mm thickness).

NMR spectra were obtained on Bruker DPX 250 MHz and Bruker Advance II Plus 700 MHz. ^1H and ^{13}C NMR chemical shifts are given in ppm (δ-scale), coupling constants (*J*) in Hz. Mass spectra were obtained on Finnigan MAT 95 spectrometer. UV-data were obtained on HITACHI UV Vis U-2800A.

2-Trimethylsilylmercaptopyrimidine (**5**)

To a suspension of 2-mercaptopyrimidine (6.2 g, 55 mmol) in anhydrous benzene (550 ml), trimethylsilyl chloride (9.6 ml, 68.8 mmol) and triethylamine (9.6 ml, 68.8 mmol) dissolved in 12 ml of anhydrous benzene were added dropwise and the mixture was stirred at room temperature for 7 days. After this time the resulting precipitate ($Et_3N{\cdot}HCl$) was filtered off with careful exclusion of moisture. The filtrate was evaporated to a small volume (15 ml) and the residue was distilled under reduced pressure (0.1 mm Hg, 105 °C) to afford silylated 2-mercaptopyrimidine **5** (7 g, 38 mmol; 69% yield).

2′,3′,5′-Tri-*O*-benzoyl-1-(β-D-ribofuranosyl)-1,2-dihydropyrimidine-2-thione (**7**) and 2*S*-(2′,3′,5′-Tri-*O*-benzoyl-β-D-ribofuranosyl)-2-thiopyrimidine (**8**)

Trimethylsilyl derivative **5** (1.84 g, 10 mmol) in anhydrous acetonitrile (25 ml) was added to the solution of 1-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranoside (**6**; 3.53 g, 7 mmol) in anhydrous acetonitrile (50 ml). Then a solution of $SnCl_4$ (1.3 ml, 11 mmol) in 80 ml of acetonitrile was dropped with stirring under exclusion of moisture. After 40 min at room temperature, when the reaction was judged to be complete by TLC (chloroform–methanol 98:2), the mixture was concentrated to 2/3 of its volume, diluted with CH_2Cl_2 (100 ml) and neutralized by addition of saturated aqueous solution of $NaHCO_3$ (100 ml). After filtration through a layer of Celite to remove the tin salts and repeated washings of the Celite with CH_2Cl_2, the layers were separated and the aqueous phase was extracted with 2 × 100 ml of CH_2Cl_2. The combined organic phase was washed with water (50 ml) and dried over $MgSO_4$. The final solution was filtered off, evaporated in vacuo and the residue was purified by silica gel column chromatography (hexane/ethyl acetate gradient from 2:1 to 1:2 v/v) to give N-glycoside **7** (3.19 g, 5.74 mmol; isolated yield 82%) and S-glycoside **8** (0.39 g, 0.70 mmol; isolated yield 10%).

N-Glycoside **7**: R_F 0.11 $CHCl_3$–MeOH 98:2; 0.51 $CH_3COOC_2H_5$. ^{1}H NMR (250 MHz, $CDCl_3$): 8.46 dd, 1 H, ^3J(5,4) = 4.0, ^4J(6,4) = 2.3 (H4); 8.42 dd, 1 H, ^3J(6,5) = 6.7, ^4J(6,4) = 2.3 (H6); 7.82–8.09 m, 6 H (Ar-H); 7.26–7.68 m, 9 H (Ar-H); 7.03 d, 1 H, ^3J(1′,2′) = 2.0 (H1′); 6.46 dd, 1 H, ^3J(5,4) = 4.0, ^3J(5,6) = 6.7 (H5); 6.01 dd, 1 H, ^3J(2′,1′) = 2.0, ^3J(2′,3′) = 5.0 (H2′); 5.77 dd, 1 H, ^3J(3′,2′) = 5.0, ^3J(3′,4′) = 7.8 (H3′); 4.87–4.97 m, 2 H (H4′,H5′); 4.65–4.72 m, 1 H (H5″).

S-Glycoside **8**: R_F 0.47 $CHCl_3$–MeOH 98:2; 0.64 $CH_3COOC_2H_5$. ^{1}H NMR (250 MHz, $CDCl_3$): 8.56 d, 2 H, ^3J(6,5) = ^3J(4,5) = 4.9 (H4, H6); 7.30–8.13 m, 15 H (Ar-H); 7.03 t, 1 H, ^3J(5,6) = ^3J(5,4) = 4.9 (H5); 6.51 d, 1 H, ^3J(1′,2′) = 3.8 (H1′); 6.04 dd, 1 H, ^3J(2′,3′) = 5.0, ^3J(2′,1′) = 3.8 (H2′); 5.96 dd, 1 H, ^3J(3′,2′) = 5.0, ^3J(3′,4′) = 5.9 (H-3′); 4.80 m, 1 H (H4′); 4.71 dd, 1 H, ^2J(5′,5″) = 12.1, ^3J(5′,4′) = 4.0 (H5′); 4.60 dd, 1 H, ^2J(5″,5′) = 12.1, ^3J(5″,4′) = 4.1 (H5″).

1-(β-D-Ribofuranosyl)-1,2-dihydropyrimidine-2-thione (s^2zeb, **1**)

The benzoylated derivative **7** (800 mg, 1.44 mmol) was suspended in anhydrous MeOH (57 ml), slightly warmed and then the solution was allowed to cool down to the room temperature. Afterwards 1 M solution of MeONa in MeOH was added (1.4 ml). The mixture was stirred at room temperature for 30 min. After this time TLC analysis (15% MeOH in $CHCl_3$) showed that the starting material was completely consumed. The mixture was worked up with Dowex (pyridine salt form) and after filtered off the resin, the remaining solution was evaporated in vacuo and coevaporated with toluene. The oily residue was dissolved in water (15 ml) and washed with diethyl ether (3 × 10 ml). The aqueous layer was frozen and lyophilized to give N-glycoside **1** (0.25 g, 1.02 mmol; yield 71%). ^{1}H NMR (700 MHz, D_2O): 8.82 dd, 1 H, ^3J(6,5) = 6.8, ^4J(6,4) = 2.2 (H6); 8.49 dd, 1 H, ^3J(5,4) = 4.4, ^4J(6,4) = 2.2 (H4); 7.09 dd, 1 H, ^3J(5,4) = 4.4, ^3J(5,6) = 6.8 (H5); 6.38 s, 1 H (H1′); 4.37 d, 1 H, ^3J(2′,3′) = 4.9 (H2′); 4.22 ddd, 1 H, ^3J(4′,5′) = 2.4, ^3J(4′,5′) = 3.5, ^3J(4′,3′) = 9.4 (H4′); 4.10 dd, 1 H, ^3J(3′,2′) = 4.9, ^3J(3′,4′) = 9.4 (H3′); 4.05 dd, 1 H, ^3J(4′,5′) = 2.4, ^2J(5′,5″) = 13.2 (H5′); 3.85 dd, 1 H, ^3J(4′,5′) = 3.5, ^2J(5′,5″) = 13.2 (H5″). ^{13}C NMR (175 MHz, D_2O): 179.76 (C2), 160.24 (C4), 145.69 (C6), 111.50 (C5), 95.93 (C1′), 83.55 (C4′), 74.54 (C2′), 67.26 (C3′), 59.23 (C5′). MS-FAB (*m/z*, %): 245.1 (100) $[M + H]^+$, calculated for $C_9H_{12}O_4N_2S$: 244. UV-Spectrum (H_2O): λ_{max} 281 nm (ε 15739), 216 nm (ε 6630), 351 nm (ε 2662).

2S-(β-D-Ribofuranosyl)-2-thiopyrimidine (**9**)

The benzoylated derivative **8** (100 mg, 0.18 mmol) was dissolved in saturated methanolic ammonia (10 ml) and the mixture was stirred at room temperature for 48 h. After this time the reaction was judged to be complete by TLC (20% MeOH in $CHCl_3$) and the mixture was evaporated in vacuo. The residue was dissolved in water (10 ml) and washed with diethyl ether (3 × 10 ml) to remove the methyl benzoate and benzamide. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH gradient from 100 to 90%). The fraction containing product was evaporated, dissolved in water, frozen and lyophilized to give S-glycoside **9** (36 mg, 0.15 mmol; 82%). ^{1}H NMR (250 MHz, D_2O): 8.55 d, 2 H, ^3J(4,5) = ^3J(6,5) = 4.9 (H4, H6); 7.23 t, 1 H, ^3J(5,6) = ^3J(5,4) = 4.9 (H5); 5.89 d, 1 H, ^3J(1′,2′) = 5.0 (H1′); 4.20–4.31 m, 2 H, ^3J(3′,4′) = 9.4, ^3J(2′,1′) = 5.0 (H3′, H2′); 4.02–4.08 m, 1 H, ^3J(4′,5′) = 3.7, ^3J(4′,5″) = 4.9, ^3J(4′,3′) = 9.4 (H4′); 3.70 dd, 1 H, ^2J(5′,5″) = 12.5, ^3J(5′,4′) = 3.7 (H5′); 3.61 dd, 1 H, ^2J(5′,5″) = 12.5, ^3J(5″,4′) = 4.9 (H5″). ^{13}C NMR (62 MHz, D_2O): 168.91 (C2), 158.15 (C6, C4), 118.32 (C5), 86.29 (C1′), 85.05 (C4′), 74.43 (C2′), 70.74 (C3′), 61.44 (C5′).

MS-CI (*m/z*, %): 245.1 (100) $[M + H]^+$, calculated for $C_9H_{12}O_4N_2S$: 244. UV-Spectrum (H_2O): λ_{max} 241.5 nm (ε 10049).

Transformation of 2′,3′,5′-Tri-*O*-benzoylzebularine to 2′,3′,5′-Tri-*O*-benzoyl-2-thiozebularine (**7**) with Lawesson Reagent

To a solution of 0.25 g (0.46 mmol) 2′,3′,5′-tri-*O*-benzoyl-1-(β-D-ribofuranosyl)-1,2-dihydropyrimidine-2-one in anhydrous dioxane (11.5 ml) Lawesson reagent (0.25 g, 0.51 mmol) was added and the mixture was stirred at boiling point for 1.5 h. After this time the reaction was judged to be complete by TLC (2% MeOH in $CHCl_3$) and 4.7 ml of water was added to the reaction mixture. After 30 min the mixture was evaporated and the residue was dissolved in 10 ml of $CHCl_3$ and then washed with 2 × 20 ml of 5% aqueous solution of $NaHCO_3$ and 2 × 20 ml of water. The combined organic phase was dried over $MgSO_4$. The final solution was filtered off, evaporated in vacuo and the residue was purified by silica gel column chromatography (CH_2Cl_2/$(CH_3)_2CO$ gradient from 100 to 95%) to give 89 mg of N-glycoside **7** (0.16 mmol; yield 35%).

X-ray Structure Analysis

Crystal data for 2-thiozebularine: $C_9H_{12}N_2O_4S$, M = 244.28, monoclinic, space group $P2_1$, a = 4.9452(1) Å, b = 7.6077(2) Å, c = 13.7185(4) Å, β = 92.783(4)°, V = 515.50(2) Å^3, Z = 2, D_x = 1.574 g cm^{-3}, T = 293 K, μ = 0.315 mm^{-1}, λ = 0.71073 Å, data/parameters = 1779/147; flack x = 0.06(5), final R_1 = 0.0181.

Crystals of 2-thiozebularine were obtained by slow evaporation from their methanolic solutions. The measurements of the crystals were performed on a SMART diffractometer with graphite-monochromated MoKα radiation (λ = 0.71073 Å) at room temperature. The structures were solved by direct method and refine with SHELXTL [16]. E-maps provided positions for all non-H-atoms. The full-matrix least-squares refinement was carried out on F^2′s using anisotropic temperature factors for all non-H-atoms. All C-bound H atoms were placed in idealized locations and refined using a riding model, with C–H = 0.93 Å and U_{iso}(H) = 1.2 U_{eq}(C).

CCDC 818090 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge, CB2 1EZ, UK; fax: +44 1223 336033; or deposit@ccdc.cam.ac.uk).

NMR Studies of Modified Nucleoside

The one- and two-dimensional NMR experiments were recorded on 700.2 MHz spectrometer at 25 °C in D_2O with DSS as the internal standards. The samples for the NMR measurements were prepared by dissolving 6–8 mg of the nucleosides in 0.6 ml of D_2O. Spectra were processed by means of TopSpin 2.1 software (Bruker BioSpin). In the case of overlapping signals in ^{1}H 1D-NMR spectra the DAISY (Bruker BioSpin) deconvolution procedure was applied. ^{1}H-^{13}C vicinal coupling constants were derived from the coupled ^{13}C NMR spectra and verified by J-HMBC experiments[17]. NOESY spectra were recorded with 0.5, 1 and 2 s mixing-times.

Cells and MTT Cytotoxicity Assay

The cytotoxicity experiments were carried out with two cancer cell lines HeLa (human cervix carcinoma) and K562 (human chronic myelogenous leukemia).

The HeLa and K562 cells were cultured in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum in a 5% CO_2–95% air atmosphere. 7×10^3 cells were seeded on each well on 96-well plate (Nunc). 24 h later cells were exposed to the test compounds for another 24 or 48 h. Stock solutions (100 mM) of test compounds were freshly prepared in water (distilled, MiliQ). The final concentrations of the compounds tested in the cell cultures were 1, 1×10^{-2}, 1×10^{-4} and 1×10^{-6} mM. The values of IC_{50} (the concentration of test compound required to reduce the cell survival fraction to 50% of the control) were calculated from dose-response curves and used as a measure of cellular sensitivity to a given treatment.

The cytotoxicity of all compounds was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) assay as described previously[18,19]. Briefly, after 24 or 48 h of incubation with drugs, cells were treated with the MTT reagent and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7 and absorbance was read at 570 and 650 nm on an microplate reader FLUOstar Omega (BMG LABTECH). As a control (100% viability), cells grown in the presence of vehicle (1% DMSO) only were used.

RESULT AND DISCUSSION

Chemical Synthesis

The general methodology of nucleosides synthesis by N-glycosidic bond formation was successfully applied for 2-thiopyrimidinone nucleoside. Using this methodology, 2-thiozebularine was obtained for the first time by Wightman and Holý, in the reaction of metal salts of 2-mercaptopyrimidine and 2,3,5-tri-*O*-benzoyl ribofuranosyl chloride[1] to give a mixture of S- and N-glycosides. Further efficient conversion of S-glycoside to the N-isomer on treatment with tin tetrachloride raised the yield of desired per-benzoylated 2-thiozebularine to 68%. Final removal of benzoyl groups from N-riboside sugar moiety by alkaline methanolysis afforded the deprotected 2-thionucleoside in good overall yield.

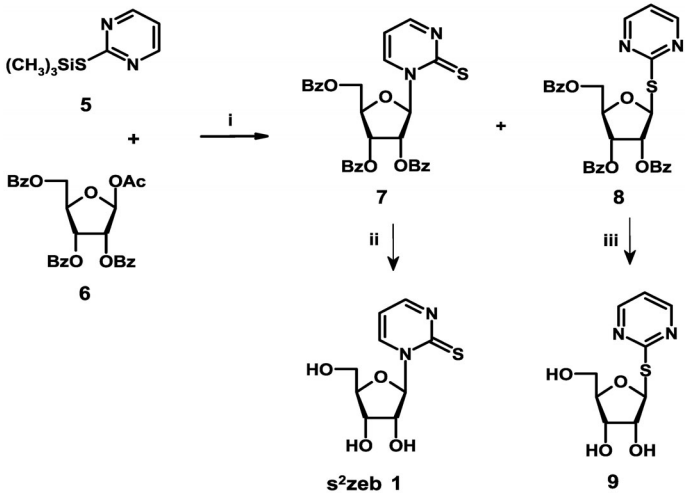
Exclusive formation of 2-mercaptopyrimidine N-riboside was reported by Niedballa and Vorbruggen using the silyl method of nucleoside synthesis[20]. Reaction of the silylated 2-mercaptopyrimidine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in the presence of $SnCl_4$ afforded per-benzoylated 2-thiozebularine in very good yield, but the sugar deprotected nucleoside was not characterized[20].

For our conformational study 2-thiozebularine (s^2zeb, **1**) was synthesized using the silyl method, according to Scheme 1.

Condensation of silyl derivative of 2-mercaptopyrimidine **5** with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in acetonitrile, catalyzed with $SnCl_4$, gave the protected N-glycoside **7** and S-glycoside **8** in 82 and 10% of isolated yield, respectively.

It is worth to note that deprotection of benzoyl groups of N-glycoside **7** with standard methanolic ammonia treatment led to nucleoside decomposition while the S-isomer **8** was stable under these conditions. Removal of the sugar protecting groups of **7** by transestrification with 0.1 M NaOMe in MeOH, following work-up with Dowex (in pyridinium salt form), quantitatively gave the title nucleoside **1**. The purity and structural identity of s^2zeb and its S-isomer **9** were fully confirmed by TLC chromatography and spectral data (UV, MS and NMR).

Additionally, the new approach for the synthesis of s^2zeb using transformation of zebularine derivative with Lawesson thionation reagent[21] was elaborated. The starting 2′,3′,5′-tri-*O*-benzoyl-zebularine obtained according Vorbruggen procedure[20] was treated with 1.1 molar excess of Lawesson reagent in dioxane at 101 °C for 1.5 h. Purification of the product by column chromatography afforded per-benzoylated 2-thiozebularine in 35% yield.



SCHEME 1

Synthetic route for the preparation of 2-thiozebularine **1**. Reaction conditions are as follows: (i) $SnCl_4$, acetonitrile, r.t., 40 min; (ii) 0.1 M NaOMe/MeOH, r.t., 30 min; (iii) NH_3/MeOH, r.t., 48 h

Structural Analysis

Conformational analysis of modified nucleosides was carried out based on parameters defined by Altona and Sundaralingam[22,23]. Three parameters describe the primary features of the conformation of a pyrimidine nucleoside: the glycosidic bond torsion angle χ (O4′–C1′–N1–C2) describes the orientation of the base relative to the furanose ring; the C4′–C5′ torsion angle γ determines the orientation of the 5′-hydroxyl group relative to the furanose ring (angle O5′–C5′–C4′–C3′); and the furanose ring puckering is specified by the pseudorotational phase angle *P*. Two major furanose ring conformations are strongly preferred for the nucleosides: C3′-*endo* (North, N-type conformation) and C2′-*endo* (South, S-type conformation)[22,23].

X-ray Structure Analysis

2-Thiozebularine was crystallized from methanol solution to give yellow needles. The detailed structure of **1** was established by X-ray crystallography and required data were deposited at the CCDC database. Figure 2a presents the ORTEP drawings of 2-thiozebularine molecule in projections perpendicular to the plane passing through atoms C1′, C4′ and O4′, showing conformational details of the molecule. Selected geometrical parameters of s^2zeb and related nucleosides: zeb, s^2U and U, indispensable for conformations comparison and discussion are listed in Table I.

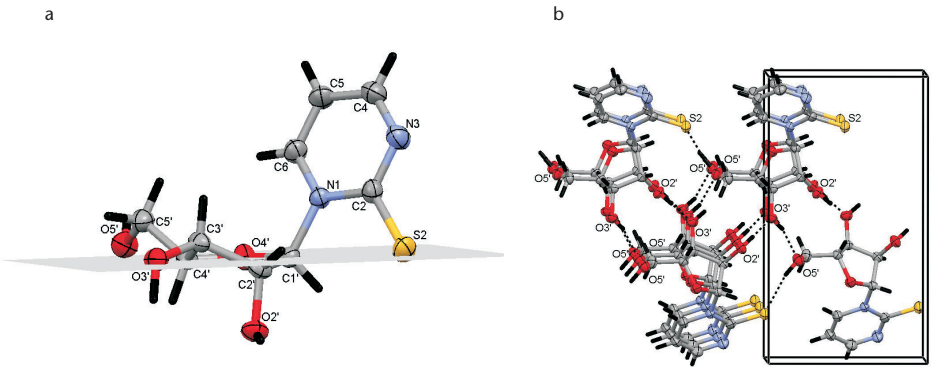


FIG. 2
The ORTEP drawings of 2-thiozebularine (a); hydrogen bonds pattern in the crystal of s^2zeb down *a*-axis (b)

The geometry of the 2-thiopyrimidinone heterobase moiety of s^2zeb is not remarkably changed in comparison to those of 2-pyrimidinone, 2-thiouracil or uracil residues in zeb [11], s^2U [12], and U [13] structures. However, the presence of the bulky sulfur atom at C-2 position of heterobases influences the conformation of the modified nucleoside treated as a whole (Fig. 2a, Table I), as well as the hydrogen bonds pattern in the crystals (Fig. 2b, Table II).

In the crystal structure of s^2zeb the sugar residue adopts C3′-*endo*/C2′-*exo* twist type N-conformation ($^3_2$T), with phase angle of pseudorotation P = 6.7° and pseudorotation amplitude 40.4°. It is worth to note that in the structure of s^2zeb the sugar twist conformation is less symmetrical (more

TABLE I
Selected torsion angles and conformational parameters from X-ray crystal structure of 2-thiozebularine (s^2zeb) in comparison to zebularine (zeb)[11], 2-thiouridine (s^2U)[12] and uridine (U)[13]

	s^2zeb	zeb[11]	s^2U[12]	U[13]	
				A	B
Torsion angels, °					
χ	–168.3	–178.5	–163.0	–161.7	–155.7
ν_0	8.2	12.6	6.0	10.5	3.4
ν_1	–30.1	–31.9	–27.0	–31.4	–27.9
ν_2	39.4	38.3	36.0	39.5	40.4
ν_3	–35.5	–31.8	–34.0	–34.6	–39.5
ν_4	17.3	12.1	18.0	15.3	22.8
γ	–169.8	52.3	–169.0	45.9	39.6
Conformational parameters					
P, °	6.7 (N)	–0.3 (N)	9.7 (N)	3.8 (N)	13.9 (N)
ψ_m, °	40.4	38.3	36.5	39.6	41.6
Sugar moiety	C3′-*endo* C2′-*exo*	C3′-*endo* C2′-*exo*	C3′-*endo* C2′-*exo*	C3′-*endo* C2′-*exo*	C3′-*endo*
C4′–C5′ bond	*trans*	*gauche* (+)	*trans*	*gauche* (+)	*gauche* (+)
C1′–N1 bond	*anti*	*anti*	*anti*	*anti*	*anti*

χ = O4′–C1′–N1–C2, ν_0 = C4′–O4′–C1′–C2′, ν_1 = O4′–C1′–C2′–C3′, ν_2 = C1′–C2′–C3′–C4′, ν_3 = C2′–C3′–C4′–O4′, ν_4 = C3′–C4′–O4′–C1′, γ = O5′–C5′–C4′–C3′

C3′-*endo*) than in zebularine molecule, for which nearly ideal twist conformation occurs. For s^2zeb, the C3′ atom is displaced by 0.44 Å from the plane C1′–O1′–C4′ on the same side as C5′, while by 0.31 Å for zeb, whereas C2′ is at a distance of 0.21 Å from this plane on the opposite side for s^2zeb and 0.32 Å for zeb.

The orientation of the heterocyclic base relative to the sugar moiety in s^2zeb molecules (χ = –168.3°) is in the typical *anti* range, similar to zeb, s^2U and U structures (Table I).

The conformation around the C4′–C5′ ribose bond of s^2zeb is in *trans* arrangement, observed less frequently than *gauche*(+) in pyrimidine nucleosides having N-type sugar ring pucker[23]. The preference of **1** to exist as the C4′–C5′ *trans* conformer comes from the intermolecular hydrogen bonding between 5′O-H sugar group and 2-thiocarbonyl function of another molecule (Table II), similarly to hydrogen bonds pattern in the crystals of s^2U molecules[12].

It is important to underline that the absence of N-3 hydrogen donor and 4-carbonyl acceptor functions in the heterobase influences the molecular packing of s^2zeb in crystals. The observed net of intermolecular hydrogen bonds varies from those ones observed for pyrimidine nucleosides: s^2U [12], and U [13]. Comprehensive description of the hydrogen bonds in s^2zeb crystal structure is summarized in Table II. The architecture of crystals of s^2zeb (in the $P2_1$ space group) is based on helical molecular arrangements by only the "sugar-to-sugar" junctions, with the strongest O2′–H2′···O3′ hydrogen bonds (Fig. 2b). Two remaining H-bonds, one strong (O3′–H3′···O5′) and one weak (5′O –H5′···S2), are responsible for joining helices present in the crystal (Table II). It is interesting that the identified three hydrogen bonds are managed to form 11-membered rings with graph-set notation of $[R^3_3(11)]$ [24], the same as in the structure of s^2U.

TABLE II
Hydrogen bond geometry in s^2zeb structure

D–H···A	Sym. code	D–H, Å	H···A, Å	D···A, Å	D–H–A, °
O2′–H2′···O3′	2–*x*, 1/2+*y*, 1–*z*	0.94	1.76	2.697(1)	171
O3′–H3′···O5′	1–*x*, 1/2+*y*, 1–*z*	0.92	1.90	2.784(2)	162
O5′–H5′···S2	–1+*x*, *y*, *z*	0.93	2.42	3.296(1)	157

NMR Conformational Analysis

1D and 2D NMR techniques were used to determine the solution conformations of 2-thiozebularine (s^2zeb) and zebularine (zeb). In general, nucleoside conformation in solution is characterized by the dominant sugar pucker and preferred glycosidic bond arrangement[25–28].

Conformation of the Sugar Moiety

It was assumed that the sugar ring of nucleoside in solution exists as an equilibrium mixture of the two puckered forms: C2′-*endo* (S conformer) or C3′-*endo* (N conformer). The percentage of S and N conformers can be estimated based on the values $^3J_{H1'\text{-}H2'}$ and $^3J_{H3'\text{-}H4'}$ coupling constants according to the following equations: % C2′-*endo* = 100 $J_{H1'\text{-}H2'}/(J_{H1'\text{-}H2'} + J_{H3'\text{-}H4'})$ and % C3′-*endo* = 100 – % C2′-*endo* [25,26]. The experimental $^3J_{H\text{-}H}$ coupling constants and the calculated populations of C2′-*endo* and C3′-*endo* conformers of s^2zeb and zeb in comparison to s^2U and U are listed in Table III.

Resonance signal of H1′ proton for s^2zeb was observed as a single line with 1.85 Hz half-width. Assuming similar puckering for s^2zeb and zeb sugar rings and the same sum of values of *J*(H1′,H2′) and *J*(H3′,H4′) coupling constants are equal to 10 Hz [25,26], the coupling constant *J*(H1′,H2′) for

TABLE III
Proton-proton vicinal (n = 3) and geminal (n = 2) coupling constants for s^2-zeb and zeb (25 °C in D_2O) in comparison to data for s^2U and U. Population of sugar S and N conformers is calculated on the basis of $^3J_{H\text{-}H}$ coupling constants

^nJ (H,H), Hz	s^2zeb	zeb	s^2U[14]	U[15]
4-5	4.4	4.5	–	–
5-6	6.8	6.7	8.3	8.1
4-6	2.2	2.7	–	–
1′-2′	0[a]	2.0	2.5	4.8
2′-3′	4.9	5.0	4.0	5.2
3′-4′	9.5	8.0	6.0	5.4
4′-5′	2.2	2.6	1.6	2.9
4′-5″	3.6	4.3	3.0	4.4
5′-5″	13.2	13.0	13.5	12.7
Population N/S, %	100/0	80/20	71/29	53/47

[a] Signal for H1′ proton was observed as a singlet.

s^2zeb was estimated at 0.5 Hz. A lack of splitting of H1′ line was confirmed by simulation of the line shape for different coupling constants by means of DAISY software. Splitting of the 1.85 Hz half-width line was observed when coupling constant was higher than 1 Hz. On the basis of *J*(H1′,H2′) = 0.5 Hz the population of C3′-*endo* conformer was estimated as at least 95%.

Complete pseudorotation analysis of s^2zeb and zeb was performed using the PSEUROT software (version 6.2)[29]. In this program, minimization of the differences between the experimental and calculated values of couplings is accomplished by non-linear Newton–Raphson minimization, while the quality of the fit is expressed by root-mean-square (rms) differences. Calculations for s^2zeb, based on three coupling constants *J*(H1′,H2′), *J*(H2′,H3′) and *J*(H3′,H4′) were performed by means of the PSEUROT automated procedure which led to 2800 results. For further analysis were taken only data with rms below 0.55 and puckering amplitude 30–40°. For different values of *J*(H1′,H2′) (0.0, 0.5 and 1.0 Hz) the dominating was N conformer (99%) and the best results were achieved for *J*(H1′,H2′) equal to 1 Hz. The lowest rms 0.17 corresponds to N conformer with the angle of pseudorotation P_N = 31° and puckering amplitude Ψ_{mN} = 41°, when for the minor S conformer P_S = 169° and Ψ_{mS} = 31°. Analogous PSEUROT analysis of zebularine led to 89% population of N conformer (P_N = 29°, Ψ_{mN} = 36°, P_S = 172°, Ψ_{mS} = 38°, rms 0.00).

Our results evidently confirm that heterobase modification strongly affects conformational characteristics of the ribose moiety (Table III). Structure of pyrimidine nucleobase influences the pentafuranose conformation mainly through the steric effects and steroelectronic interactions within O4′–C1′–N1 fragment (anomeric effect)[30]. The replacement of C2 oxygen atom in U by more sterically demanding sulfur atom causes an increase of N conformer population of s^2U by about 20% (Table III). This feature is attributed to steric effects between the 2-tiocarbonyl group of nucleobase and 2′-hydroxyl group of the ribose ring[31–33], although recent hybrid DFT and MP2 calculations showed that the distant electrostatic effects between 2′-OH and the 2-tiocarbonyl function may enhance the selectivity of the C3′-*endo* conformation of ribose in the 2-thiouridine molecule[34]. The same extent of increase of the C3′-*endo* population was observed when 2-carbonyl function of zeb was replaced by bulky 2-tiocarbonyl group in s^2zeb (Table III; 80% of N conformer for zeb, 100% for s^2zeb). Interestingly, an absence of the carbonyl oxygen at C4 in zeb and s^2zeb raises the population of the C3′-*endo* conformers by about 30% in comparison to U and s^2U, respectively (Table III). This feature can be attributed to the presence of the strong anomeric effect caused by electron-deficient 2-pyrimidinone or

2-thiopyrimidinone heterobase. In the case of s^2zeb, strong steric and stereoelectronic effects are additive and therefore the N conformer population increases to almost 100%. It is an unique case where the structure of nucleobase forces the ribose conformation to almost exclusive C3′-*endo* pucker, without any sugar modification.

Conformation Around the Glycosidic Bond

The *syn/anti* conformation around the N-glycosidic bond was probed by means of vicinal carbon-proton couplings and NOE effects. Proton-coupled ^{13}C spectra reveal carbon-proton couplings that indicate the *syn/anti* conformation according to the following relationships $J_{C6\text{-}H1'} > J_{C2\text{-}H1'}$ = *anti*; $J_{C2\text{-}H1'} > J_{C6\text{-}H1'}$ = *syn* [27,28,35,36].

For example, the magnitudes of the corresponding *J* values $J_{C6\text{-}H1'}$ = 3.6 Hz and $J_{C2\text{-}H1'}$ = 2.4 Hz for uridine clearly confirm the preference of *anti* conformation for this nucleoside in solution[36].

In the case of 2-thiozebularine and zebularine the measured smaller values of $J_{C2\text{-}H1'}$ than $J_{C6\text{-}H1}$ (1.8 vs 2.1 Hz for s^2zeb and 1.1 vs 2.6 Hz for zeb) evidently indicate domination of the *anti* conformation around their N-glycoside bond. The population of the *anti* conformer can be estimated from the equation % *anti* = $[10 - (J_{C2\text{-}H1'} + J_{C6\text{-}H1'})/6.4]$ [31] and for s^2zeb and zeb nucleosides the contents of *anti* conformers are similar and equal to 95 and 98%, respectively.

To confirm domination of anti conformation around N-glycosidic bond of s^2zeb and zeb the NOE measurements were performed. Results of 1D NOE experiments were inconclusive because resonance of heterobase H6 and H4 protons were to close for selective irradiation of diagnostic H6 one. Thus the NOESY experiments were performed. The cross-peaks were integrated and interproton distances were calculated from the relation $\eta_A/\eta_B = r^6_B/r^6_A$ [27,37], where η_A and η_B are NOEs measured for two pairs of protons A and B, and r_A and r_B are distances for these two pairs of protons. As reference the NOE for H6 and H5 protons was used as the distance between these protons is known and equal to 2.45 Å. For both nucleosides the relationship H6-H1′ > H6-H3′ was observed (3.2 > 2.7 Å and 3.2 > 2.6 Å for s^2zeb and zeb, respectively), characteristic for *anti* conformer domination.

Conformation Around the C4′–C5′ Bond

The local conformation around the C4′–C5′ bond was examined by analysis with three staggered forms: *gauche*(+), *trans*, and *gauche*(–), using coupling

constants H5′ and H5″ protons with H4′ [27,38]. The stereospecific assignment of the H5′ and H5″ proton in ^{1}H NMR spectra was based on the deshielding effect of the phosphate group on H5′ and H5″ in 3′-monophosphates of uridines[39]. It was shown that the H5′ and H5″ spectral region shows a similar characteristic spectral pattern: δ(H5′) > δ(H5″) and $J_{4'\text{-}5'} < J_{4'\text{-}5''}$ in a number of other nucleosides and nucleotides[25]. Therefore, it appears reasonable to assume that for all these nucleosides where this pattern is observed the more shielded proton is assigned as H5″. The stereospecific assignments of H5′ and H5″ methylene protons in the ^{1}H NMR spectra of s^{2}zeb and zeb and measurements of the $J_{4'\text{-}5'}$ and $J_{4'\text{-}5''}$ proton-proton coupling constants enabled the determination of the population of three exocyclic C4′–C5′ rotamers *gauche*(+), *trans* and *gauche*(–). The results are given in Table IV. All of analyzed nucleosides exhibit preferentially *gauche*(+) conformation with the higher population of this rotamer for 2-thionucleosides.

TABLE IV
Population (in %) of *gauche*(+), *trans* and *gauche*(–) conformers at 25 °C in D_2O of s^{2}zeb and zeb in comparison with s^{2}U and U

Nucleoside	$^3J_{H4'\text{-}H5'}$, Hz	$^3J_{H4'\text{-}H5''}$, Hz	*gauche*(+)	*trans*	*gauche*(–)
s^2-zeb (**1**)	2.2	3.6	74	26	0
zeb (**2**)	2.6	4.3	68	31	1
s^{2}U (**3**)[28]	1.6	3.0	94	5	1
U (**4**)[29]	3.1	4.4	61	32	7

Cytotoxicity of Zebularine and 2-Thiozebularine and Their 2′-Deoxyanalogues

A series of compounds: zebularine **2**, 2-thiozebularine **1**, 2′-deoxyzebularine **10** and 2-thio-2′-deoxyzebularine **11** was screened for cytotoxic properties against K562 (leukemia), HeLa (human cervix carcinoma) and HUVEC (normal) cells. The viability of cells was determined in the MTT assay at four different compound concentrations: 1, 1×10^{-2}, 1×10^{-4} and 1×10^{-6} mM. Compounds **1**, **10** and **11** were not toxic toward cells used in the study both after 24 and 48 h incubation time (IC_{50} > 1 mM). However, zebularine **2** showed limited toxicity toward K562 leukemia cancer cells only. Cytotoxicity of **2** seems to be time-dependent, as its concentration

needed to induce cell death after 48 h (100 μM) is six-fold lower comparing to IC_{50} at 24 h (600 μM).

CONCLUSIONS

The s^2zeb nucleoside was synthesized in good overall yield by coupling of silyl derivative of 2-mercaptopyrimidine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in acetonitrile, catalyzed with $SnCl_4$, and removal of the sugar protecting groups with NaOMe in MeOH, or alternatively, by the thionation of appropriate zebularine derivative with Lawesson reagent. X-ray analysis revealed that in solid state s^2zeb molecules adopt C3′-*endo*/C2′-*exo* twist puckering of ribofuranose ring and *anti* of N-glycosidic bond conformation, generally consistent with those of zeb, s^2U and U crystal structures. NMR spectroscopy analysis of s^2zeb and zeb conformations in solution and their comparison to reference s^2U and U compounds evidently confirms that the sugar pucker can be steered by heterobase structure (from 53% of N population in uridine to almost 100% in 2-thiozebularine). The replacement of oxygen atom at C2 either of zeb or U by more sterrically demanding sulfur atom, leads to 20% increase of C3′-*endo* conformer population, as in s^2zeb and s^2U. The lack of carbonyl oxygen at position 4, as in s^2zeb or zeb, gives the 30% increase of the C3′-*endo* conformer population due to the electron-withdrawing effect of modified heterobase on sugar moiety. In the case of s^2zeb both effects operate simultaneously and, therefore, the population of N conformer increases to almost 100%. 2-Thiozebularine is unique example of pyrimidine ribonucleoside which in aqueous solution is fully constrained in the C3′-*endo* conformation and this feature is exerted exclusively through nucleobase steric and stereoelectronic interactions with ribose ring. The cytotoxicity studies indicate that replacement of oxygen by sulfur at position 2 of the nucleobase of zebularine, which is known anticancer agent, completely abolishes cytotoxic properties of the resulting s^2zeb (IC_{50} > 100 mM).

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