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Synthesis and Biological Activity of Nucleosides and Nucleotides Related to the Antitumor Agent 2-β-D-Ribofuranosylthiazole-4-carboxamide

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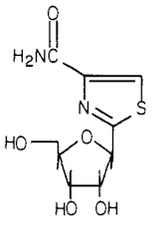
Phosphorylation of 2-β-D-ribofuranosylthiazole-4-carboxamide (1) provided the 5′-phosphate derivative **2**, which was converted to the corresponding 5′-triphosphate **4** and the cyclic 3′,5′-phosphate **5**. Treatment of 2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-carbonitrile (**6**) with NH_3–NH_4Cl provided 2-β-D-ribofuranosylthiazole-4-carboxamidine hydrochloride (**7**), and treatment with H_2S–pyridine provided the corresponding 4-thiocarboxamide **9**. Compound **9** was treated with ethyl bromopyruvate, followed by treatment with methanolic ammonia, to yield 2′-(2-β-D-ribofuranosylthiazol-4-yl)thiazole-4′-carboxamide (**11**). 5′-Phosphate **2** was cytotoxic to L1210 cells in culture and significantly effective against the intraperitoneally implanted murine leukemias in mice. Amidine **7** was slightly toxic to L1210 in culture and inhibitory to purine nucleoside phosphorolysis. The cyclic 3′,5′-phosphate **5** was less effective than the corresponding 5′-phosphate **2** or the parent nucleoside **1** as an antitumor agent.

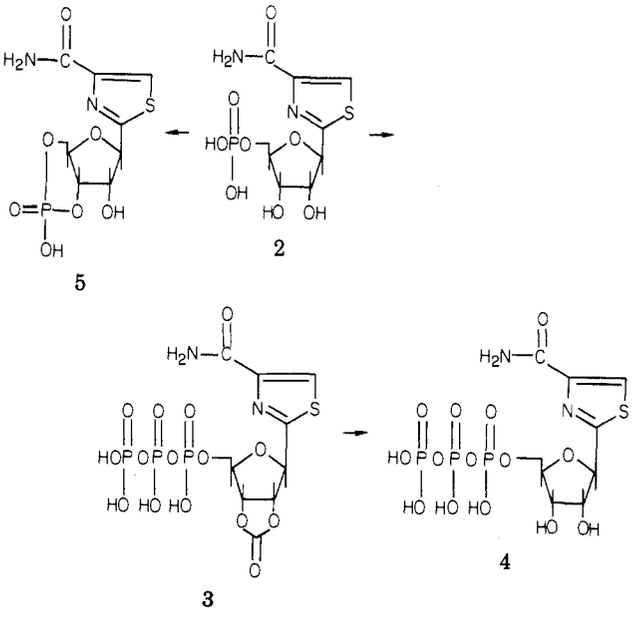
Tiazofurin,[1] 2-β-D-ribofuranosylthiazole-4-carboxamide (**1**), is a C-nucleoside previously reported by us.[2] Tiazofurin has shown potent antitumor activity in animals and is currently being pursued by the National Cancer Institute as a high-priority candidate for clinical trials with potential importance for treatment of lung tumors and metastases.[3]



1

Studies of the mechanism of action and metabolism of this new oncolytic nucleoside were subsequently initiated and have recently been published.[4] The nucleoside **1** would be expected to be metabolized in vivo via the 5′-monophosphate to the corresponding 5′-triphosphate. The syntheses of these and other analogues of **1** were therefore initiated to make these compounds available for biological evaluation. The metabolic formation of 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-phosphate (**2**) was first observed[4] when the parent nucleoside **1** was incubated with P-388 cells in culture and the nucleoside pools were examined by HPLC. The 5′-phosphate **2**, isolated by HPLC, was found to be identical with an authentic sample[4] of **2**, first synthesized by us and supplied to Dr. David G. Johns for comparison and to Dr. Ven L. Narayanan for antitumor screening. Compound **2** was synthesized in our laboratory by phosphorylation of **1** with trichloropyrophosphopyridinium chloride[5] generated in situ via the treatment of phosphoryl chloride with pyridine and water in acetonitrile. This method of 5′-phosphorylation of carboxamide-bearing nucleosides was found superior[5] to the method utilizing trimethyl phosphate–phosphoryl chloride.

Scheme I



5 **2** **3** **4**

The latter reagent also reacts with the carboxamide group, resulting in the formation of unidentified side products. The synthesis of **2** has also been recently reported by Johns and co-workers.[6]

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(1) Generic name given to compound **1**.
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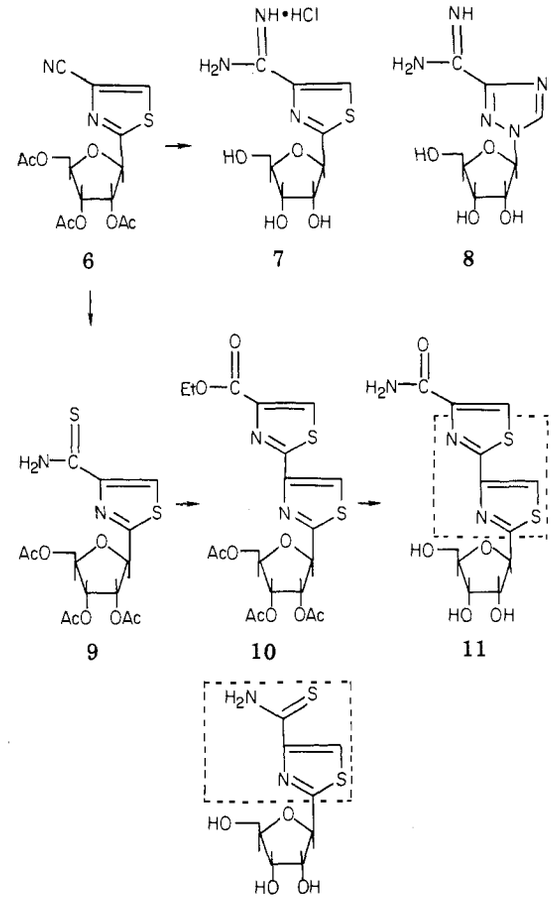
The possibility of anabolism of nucleoside 1 to the corresponding triphosphate (4) was also considered, and nucleotide 4 was subsequently synthesized. Nucleoside 5′-monophosphates react with *N,N*-carbonyldiimidazole under mild conditions to provide phosphorimidazolidate intermediates, which readily react with inorganic pyrophosphate to provide the nucleoside 5′-triphosphates. The procedure was first developed by Hoard and Ott[7] and was utilized by us for the synthesis of 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-triphosphate[8] (4). However, the procedure described by Hoard and Ott required certain modifications. Our HPLC results indicated that during the reaction of nucleoside 5′-monophosphate 2 with carbonyldiimidazole, an intermediate product was formed. This intermediate product was probably the cyclic 2′,3′-carbonate 3, which could be formed[9] by the reaction of carbonyldiimidazole with cis 2′,3′-hydroxyl groups of 2. Treatment of 3 with sodium hydroxide (pH 8.5) for 30 min then gave the desired nucleotide 4. Finally, the 5′-phosphate 2 was cyclized to 2-β-D-ribofuranosylthiazole-4-carboxamide cyclic 3′,5′-phosphate via the treatment of a dilute solution of 2 in pyridine with dicyclohexylcarbodiimide as described by Khorona and co-workers.[10] (Scheme I).

The synthetic nucleoside 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) is a broad-spectrum antiviral agent.[11] The corresponding amidine derivative,[12] 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine (8), is a competitive, reversible inhibitor of inosine phosphorolysis by human lymphoblast purine nucleoside phosphorylase. The amidine 8 is not, however, a substrate for this enzyme.[13] Since tiazofurin (1) bears structural similarity to ribavirin and has an aglycon–sugar linkage (C–C bond) that is stable to enzymatic hydrolysis, the amidine derivative 7 would be expected to be an even more powerful inhibitor of the phosphorylase enzyme. 2-β-D-Ribofuranosylthiazole-4-carboxamidine (7) was therefore synthesized[8] in 40% yield by heating 2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-carbonitrile[2] (6) with excess liquid ammonia and 1 molar equiv of ammonium chloride[12,14] in a bomb.

The remarkable antitumor activity observed with 1 appeared to result from the presence of the thiazole ring. The antibiotic bleomycin contains two thiazole rings linked together and also exhibits antitumor activity. The potent antitumor activity associated with these thiazole compounds prompted us to synthesize and evaluate 2′-(2-β-D-ribofuranosylthiazol-4-yl)thiazole-4′-carboxamide (11). Treatment of nitrile 6 with hydrogen sulfide in pyridine provided 2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-thiocarboxamide (9). Compound 9 was treated with ethyl bromopyruvate to give the intermediate compound, ethyl 2′-[2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-yl]thiazole-4′-carboxylate (10). Subsequent ammonolysis and deacetylation of 10 with methanolic ammonia yielded compound 11,[15] which gave satisfactory elemental analysis for C, H, N, and S. In the ^{1}H NMR spectrum, the signals observed at δ 8.28 and 8.32 for H_5 and $H_{5'}$ (of the two thiazole rings), at 7.72 for carboxamide, and at 5.07 for the anomeric protons were consistent with the structure of 11 (Scheme II).

Scheme II



Antitumor Evaluation. The tiazofurin derivatives 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-phosphate (2), amidine (7), and thiazolylthiazole nucleoside 11 were evaluated for antitumor activity. Tiazofurin phosphate 2 was found to by cytotoxic (ID_{50} = 3.2 × 10^{-6} M) to L1210 cells in culture at ~1.6-fold higher concentrations than the parent nucleoside 1 (ID_{50} = 1.9 × 10^{-6} M). Tiazofurin 5′-phosphate (2) was also significantly effective against the intraperitoneally (ip) implanted L1210 murine leukemias[16] in groups of six mice (36 mice in untreated control groups). An increased life span (ILS) of 96, 83, 159, 86, and 36%, respectively, at 800, 400, 200, 100, and 50 mg/kg was observed following intraperitoneal administration of 2 on days 1–5 (Table I). An ILS ranging from 14 to 55% was observed when the cyclic 3′,5′-phosphate 5 was similarly tested against leukemia P-388 at the dose levels ranging from 50 to 400 mg/(kg day). The activity of 1 against leukemia L1210 is reported for comparison (Table I). The amidine 7 at 10^{-4} M concentration inhibited the growth of L1210 cells in culture to the extent of 19%, indicating

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Table I. Effect of Tiazofurin (1) (Experiment 1), Tiazofurin 5'-Phosphate (2) (Experiment 2), and Tiazofurin Cyclic 3',5'-Phosphate (5) (Experiment 3) on the Life Span of Mice[a]

drug	dose, mg/kg	median life span, days	body wt change,[b] g	ILS, %
		Experiment 1		
untreated controls		8.1	1.9	
1	800	15	−4.4	85
	400	16.2	−3.9	100
	200	16.5	−2.9	103
	100	14.8	−2.5	82
	50	13.5	−2.1	66
	25	12.8	−2.6	58
		Experiment 2		
untreated controls		8.3	0.3	
2	800	16.3	−3.6	96
	400	15.2	−2.0	83
	200	21.5	−1.4	159
	100	15.5	−1.0	86
	50	11.3	−0.6	36
		Experiment 3		
untreated controls		11.6	1.3	
5	400	18.0	−3.1	55
	200	17.7	−1.9	52
	100	14.3	−0.6	23
	50	13.3	−1.1	14

[a] Mice were inoculated intraperitoneally with leukemia L1210 for experiments 1 and 2 and with leukemia P-388 for experiment 3. [b] Average body weight, in grams, on day 5 minus average body weight on day 1.

only slight activity. Compound **7** also was found by a standard test[17] to be selectively cytotoxic ($ED_{50} = 77 \times 10^{-6}$ M) for T cells in the presence of 2'-deoxyguanosine. Compound **7** as the hydrochloride at 300×10^{-6} M concentration completely inhibited inosine phosphorolysis by human lymphoblast purine nucleoside phosphorylase when studied as described in a previous report.[13] The thiazolylthiazole nucleoside **11** was not cytotoxic to L1210 cells in culture when tested at a concentration of 10^{-4} M. The inactivity of **11** is similar to that observed with the structurally related compound, 2-β-D-ribofuranosylthiazole-4-thiocarboxamide[2] (**12**), which was also found to be devoid of any antiviral or antitumor activity.[2,4]

Experimental Section

The physical properties were determined with the following instruments: melting point, Thomas-Hoover apparatus (uncorrected); ^{1}H NMR, JEOL FX-900 Fourier transform spectrometer (resonances reported downfield from the internal Me_4Si standard). The presence of exchangeable protons was confirmed by ^{1}H NMR spectroscopy in absolute Me_2SO-d_6 by exchange with D_2O, followed by reintegration. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Where analyses are indicated by only samples of the elements, the analytical results for those elements were within ±0.4% of the theoretical values. Baker analyzed silica gel powder (60–200 mesh) was used for column chromatography, and E. Merck silica gel 60 F-254 precoated TLC sheets (0.20 mm) were used to determine the purity of the compounds. The purity of nucleotides was examined on PEI cellulose TLC sheets (Schleicher and Schull, Inc.) using a 0.5 N ammonium bicarbonate (NH_4HCO_3) solvent system. The spots on TLC were detected by visualization under a UV lamp (UVS-11 mineralight, 254 nm). The UV spectra were performed on a Cary 15 spectrophotometer. A Beckman high-pressure liquid chromatography (HPLC, Model 332) equipped with a Hitachi variable-wavelength spectrophotometer and Model C-RIA peak integrator was used to determine the retention time and purity of the nucleoside 5'-triphosphate. High liquid ion-pairing chromatography was performed on a reversed-phase Ultrasphere C-18 ODS column (Beckman, 4.6 × 25 cm) with an attached RP-18 Lichrosorb precolumn (Rheodyne, 10 μm, 3 cm size). A parabolic gradient consisting of solvent A (0.1 M KH_2PO_4, 5 mM tetrabutylammonium phosphate from Waters, pH 5.0) and solvent B (methanol from Burdick and Jackson) at 1 mL/min and ambient temperature was used. Acid-washed AU-4 charcoal (Barneby-Cheney, Columbus, OH) was used for column chromatography.

2-β-D-Ribofuranosylthiazole-4-carboxamide 5'-Phosphate (2). Water (151 mg, 8.4 mmol) was added carefully to a solution (maintained at 0 °C by stirring) of freshly distilled phosphoryl chloride (2.0 g, 13.2 mmol), pyridine (1.21 g, 14.4 mmol), and acetonitrile (2.3 g, 56.7 mmol). 2-β-D-Ribofuranosylthiazole-4-carboxamide[2] (powdered and dried over P_2O_5; 800 mg, 3.0 mmol) was added to the solution, and the reaction mixture was stirred continuously for 4 h at 0 °C. The reaction mixture was poured into ice–water (ca. 50 mL), and the pH was adjusted to 2.0 with 2 N sodium hydroxide. The solution was applied to a column of activated charcoal (20 g, acid–washed Au-4), and the column was washed thoroughly with water until the eluate was salt free. The column was eluted with a solution of ethanol–water–concentrated ammonium hydroxide (10:10:1), and the fractions (25 mL each) were collected. The fractions containing pure [TLC, silica gel, acetonitrile–0.1 N ammonium chloride (7:3)] nucleotide **2** were collected and evaporated to dryness under vacuum. The anhydrous residue was dissolved in water and passed through a column of Dowex 50W-X8 (20–50 mesh, H^+ form, 15 mL). The column was washed with water, and the fraction containing the nucleotide was collected. The solution was concentrated to a small volume (5 mL) and passed through a column of Dowex 50W-X8 (20–50 mesh, Na^+ form, 15 mL). The column was washed with water, and the fraction containing the nucleotide sodium salt was lyophilized. The residue was triturated with ethanol, collected by filtration, and dried (P_2O_5), to provide 560 mg (47%) of **2** as the monosodium dihydrate in the crystalline form: ^{1}H NMR (Me_2SO-d_6) δ 8.25 (s, 1, H_5), 7.59–7.81 [s (pair), 2, $CONH_2$]. Anal. ($C_9H_{12}N_2O_8PSNa{\cdot}2H_2O$) C, H, N, P, S.

2-β-D-Ribofuranosylthiazole-4-carboxamide 5'-Triphosphate (4). To a solution of tri-*n*-butylamine (0.24 mL, 1 mmol) and dimethylformamide (10 mL) was added 1 mmol of nucleoside 5'-monophosphate (**2**, free acid form). The suspension was warmed to 35 °C and dissolved. The solution was evaporated at 35 °C in vacuo to a syrup, which was coevaporated twice with dimethylformamide and finally dissolved in dimethylformamide (15 mL). *N,N'*-Carbonyldiimidazole (0.81 g, 5 mmol) was added and swirled until dissolved. The reaction mixture was kept under anhydrous conditions (P_2O_5) and partial vacuum in a desiccator at room temperature for 12 h. Anhydrous methanol (0.16 mL, 4 mmol) was added to the reaction mixture to decompose excess *N,N'*-carbonyldiimidazole. The reaction mixture was kept under anhydrous conditions for 30 min. A solution of dry dimethylformamide (10 mL) and the tri-*n*-butylammonium salt of pyrophosphoric acid (5 mmol) was added, and the reaction mixture was maintained under anhydrous conditions for 20 h. Solid material was filtered, and the reaction mixture was evaporated in vacuo at 35 °C. The syrup was cooled to 0 °C and then dissolved in a minimum of cold water, and the pH was adjusted to 8.5 with cold 0.5 N NaOH. The solution was allowed to stand at 20 ± 5 °C for 30 min and then extracted with ether, and the aqueous portion was applied to a bicarbonate-form DEAE-Sephadex column (2.5 × 15 cm). A linear gradient of water to 0.8 M triethylammonium bicarbonate (1 L each) at 4 °C separated the 5'-triphosphate **4** from salts and the corresponding 5'-di- and 'monophosphates. The proper fractions were identified by comparison with 5'-monophosphate **2**, 5'-AMP, 5'-ADP, and 5'-ATP on TLC (PEI cellulose) and HPLC. The pure fractions of triphosphate were collected, evaporated in vacuo at 30 °C, and coevaporated 10 times with methanol (or until the odor of triethylamine was not detected). The resulting syrup was dissolved in a minimum of methanol and poured into a solution of sodium iodide (0.60 g, 4 mmol) in acetone (20 mL). The precipitate was separated by centrifugation and thoroughly washed 5 times with

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acetone and twice with ether. The resulting crystalline product was dried at room temperature over P_2O_5 in vacuo for 2 h to yield (30%) 5'-triphosphate 4 as the tetrasodium salt, which was stored in the freezer with exclusion of moisture: UV λ_{max} (pH 1) 236 nm; UV λ_{max} (pH 7) 235 nm; UV λ_{max} (pH 11) 236 nm. The HPLC analysis[18] of an aliquot of 4·Na_4 stored at room temperature for ~24 h showed only 82% of triphosphate [TLC (PEI cellulose, 0.5 N NH_4HCO_3) R_f 0.30] and also the presence of the corresponding diphosphate (R_f 0.43) and monophosphate 2 (R_f 0.54), indicating gradual decomposition of 4.

2-β-D-Ribofuranosylthiazole-4-carboxamide Cyclic 3',5'-Phosphate (5). To 2-β-D-ribofuranosylthiazole-4-carboxamide 5'-phosphate (2; 3.40 g, 10 mmol) in anhydrous pyridine (150 mL) was added 4-morpholino-*N*,*N*-dicyclohexylcarboxamidine[19] (2.93 g, 10 mmol), and the resulting solution was evaporated several times with dry pyridine to an anhydrous syrup. The residual syrup was dissolved in pyridine (750 mL) and added dropwise (over 2-h period), through a reflux condenser, into a refluxing anhydrous solution of dicyclohexylcarbodiimide (DCC; 10.30 g, 50 mmol) in pyridine (2000 mL). The solution was refluxed for an additional 5 h before water (125 mL) was added dropwise. After standing at room temperature for another 15 h, the solution was evaporated to dryness. To the residue were added water (125 mL) and ethyl ether (100 mL). The suspension was stirred vigorously and then filtered. The aqueous phase was evaporated and extracted with ethyl ether (3 × 100 mL). The aqueous layer was passed through a Dowex 50-X8 (Na^+ form, 100–200 mesh, 2.5 × 25 cm) resin column, and the eluent was evaporated to a syrup. Ethanol (100 mL) was added to the syrup, and the resulting mixture was kept at room temperature for 24 h. A small amount of impurity was filtered out and discarded. The clear filtrate was allowed to stand at 0–5 °C for 2 days. The resulting precipitate (1.5 g) was collected and dissolved in water (~5 mL), and the product was precipitated by adding ethanol (50 mL). The light brown product was found to be ~95% pure. The impure product was dissolved in water (~10 mL), adjusted to pH 2 with cold 0.1 N HCl, and purified by HPLC using the reverse-phase column (C-18) technique and 0.01% acetic acid in MeOH as the solvent. The fraction containing the pure product was evaporated to dryness, and the residue was dissolved in water and passed through a Dowex 50-X8 (Na^+ form, 100–200 mesh, 2.5 × 10 cm) resin column. The eluent was again concentrated to ~100 mL and lyophilized. The residue (solid) was triturated with anhydrous MeOH, filtered, and dried to yield 0.90 g (26.2%) of homogeneous product 5 as the monosodium salt: mp >200 °C dec; UV λ_{max} (pH 7) 235 nm (ϵ 6200); UV λ_{max} (pH 1 and 11) 235 nm (ϵ 5850); ^{1}H NMR (Me_2SO-d_6) δ 5.14 (s, 1, $C_{1'}$H), 7.56 and 7.74 (2 br s, 2, $CONH_2$), 8.24 (s, 1, C_5H), and other sugar protons. Anal. ($C_9H_{10}N_2O_7PS$·Na) C, H, N, P, S, Na.

2-β-D-Ribofuranosylthiazole-4-carboxamidine Hydrochloride (7). A mixture of 2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-carbonitrile (6; 1.9 g, 5 mmol), ammonium chloride (dry; 270 mg, 5 mmol), and liquid ammonia (10 mL) was sealed with a stirring bar in a bomb and stirred at 80–85 °C (bath temperature) for 16 h. The mixture was cooled, and the solvent was evaporated under vacuum. The residue was dissolved in methanol, adsorbed on silica gel (ca. 2.0 g), and passed through a column packed with silica gel slurry (70 mL) in chloroform. The column was eluted with 25% methanol in chloroform. The fractions containing the major product were collected and evaporated under vacuum. The residue was dissolved in boiling acetonitrile–ethanol. White rosettes crystallized from the solution on standing at room temperature for a few days. The product was filtered and dried under vacuum over P_2O_5 to provide 675 mg (~40%) of 7: mp 171–172 °C; ^{1}H NMR (Me_2SO-d_6) δ 9.36 (br s, 3, amidine); ^{1}H NMR (Me_2SO-d_6-D_2O) δ 8.86 (s, 1, H_5), 5.02 (d, 1, J = 4.5 Hz, H_1), and other protons. Anal. ($C_9H_{13}N_3O_4S$·HCl) C, H, N, S.

2-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-thiocarboxamide (9). Hydrogen sulfide was bubbled through a stirred solution of 2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)thiazol-4-carbonitrile (6; 3.68 g, 10 mmol) in anhydrous pyridine (50 mL) for 16 h. The solvent was evaporated under vacuum, and the residue was dissolved in chloroform and washed with water. The chloroform portion was dried (Na_2SO_4) and evaporated. The residue (syrup) was passed through a column of silica gel packed in chloroform. The column was eluted with 5% ethyl acetate in chloroform. The fractions were analyzed by silica gel TLC using 25% ethyl acetate in chloroform as the solvent. The pure fractions showed a single spot when observed under UV light and a purple color when the chromatogram was developed by spraying with a dilute ethanolic solution of 2,3-dichloronaphthoquinone and exposed to ammonia.[2] The pure fractions were collected and evaporated under vacuum. The residue was crystallized from ethanol–water to yield (3.1 g, 72%) of 9, mp 106–108 °C. Anal. ($C_{15}H_{18}N_2O_7S_2$) C, H, N, S.

2'-(2-β-D-Ribofuranosylthiazol-4-yl)thiazole-4'-carboxamide (11). Ethyl bromopyruvate (Aldrich Chemical Co., 90%, tech grade, 1.1 g) was added to a stirred solution of 9 (804 mg, 2 mmol) in acetonitrile (10 mL). The reaction solution was stirred for 30 min at room temperature and then evaporated under vacuum. The residue was dissolved in chloroform and washed with a saturated solution of sodium bicarbonate, followed by water. The chloroform portion was dried (Na_2SO_4) and evaporated under vacuum to provide the crude intermediate 10 as a syrup, which was purified by silica gel column chromatography. The column was packed in chloroform and eluted with 10% ethyl acetate in chloroform. The appropriate fractions [TLC, silica gel, $CHCl_3$/EtOAc (1:1)] were collected and evaporated under vacuum to provide 10 (syrup), which was identified by ^{1}H NMR ($CDCl_3$): δ 8.20 and 8.22 (2 s, 1 and 1, H_5 and $H_{5'}$ of thiazoles), 2.1–2.2 (3 × 3, 9, triacetyls), and other protons. Intermediate 10 was dissolved in methanol (50 mL) saturated with ammonia (0 °C) and stirred in a pressure bottle at room temperature for 2 days. The solvent was evaporated, the residue was adsorbed on silica gel (5 g) with methanol and applied on a silica gel column (2.8 × 35 cm) packed in ethyl acetate. The column was eluted with a solvent system [ethyl acetate/1-propanol/H_2O, 4:1:2 (top layer)], and the homogeneous fractions containing the major product were collected. The solvent was evaporated in vacuo, and the residue was crystallized from ethanol–water to provide 305 mg (overall 30%) of 11: mp 243–244 °C; ^{1}H NMR (Me_2SO-d_6) δ 8.28 and 8.32 (2 s, 1 and 1, H_5 and $H_{5'}$ of thiazoles), 7.72 (d, 2, $CONH_2$), 5.07 (d, 1, J = 4.5 Hz, $H_{1'}$ anomeric). Anal. ($C_{12}H_{13}N_3O_5S_2$) C, H, N, S.

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