Synthesis of Nonhydrolyzable Analogues of Thiazole-4-carboxamide and Benzamide Adenine Dinucleotide Containing Fluorine Atom at the C2′ of Adenine Nucleoside: Induction of K562 Differentiation and Inosine Monophosphate Dehydrogenase Inhibitory Activity

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Thiazole-4-carboxamide adenine dinucleotide (TAD) analogue **7** containing a fluorine atom at the C2′ *arabino* configuration of the adenine nucleoside moiety was found to be a potent inducer of differentiation of K562 erythroid leukemia cells. This finding prompted us to synthesize its hydrolysis-resistant methylenebis(phosphonate) and difluoromethylenebis(phosphonate) analogues **8** and **9**, respectively. Since both TAD and benzamide adenine dinucleotide (BAD) are potent inhibitors of inosine monophosphate dehydrogenase (IMPDH), the corresponding fluorine-substituted methylenebis(phosphonate) analogue **12** of BAD was also synthesized. Thus, 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (**13**) was converted in five steps into the corresponding methylenebis(phosphonate) analogue **18**. Dehydration of **18** with DCC led to the formation of the bicyclic trisanhydride intermediate **19a**, which upon reaction with 2′,3′-*O*-isopropylidenetiazofurin (**20**) or -benzamide riboside (**21**) followed by hydrolysis and deprotection afforded the desired methylene-bridged dinucleotides **8** and **12**, respectively. The similar displacement of the 5′-mesyl function of 2′,3′-*O*-isopropylidene-5′-*O*-mesyltiazofurin (**24**) with the difluoromethylenebis(phosphonic acid) derivative gave the phosphonate **25** which was coupled with **13** to afford **26**. The desired difluoromethylenebis(phosphonate) analogue **9** was obtained by deprotection with Dowex 50/H^+. This compound as well as β-CF_2-TAD (**4**) showed improved differentiation-inducing activity over β-CH_2-TAD (**3**), whereas analogues containing the $-CH_2-$ linkage (**8** and **12**) were inactive.

Introduction

Inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides. Suppression of IMPDH activity results in a reduction of the level of guanine nucleotides, with marked consequences for the regulation of cell growth and differentiation. Recently, induction of cell differentiation in human leukemic cells has been demonstrated by an antisense oligonucleotide targeting IMPDH messenger RNA.[2] The known IMPDH inhibitors, such as tiazofurin and mycophenolic acid, induce differentiation in a variety of human leukemia cells, including HL-60 myeloid leukemia cells and K562 erythroid leukemia cells, as well as human breast cancer cells and melanoma cells.[3] Tiazofurin (**1**, TF) and its active metabolite TAD (**2**), as well as the nonhydrolyzable, methylenebis(phosphonate) analogue β-CH_2-TAD (**3**), were found to be potent inducers of HL-60 cell differentiation.[4] Analogue **3** was most effective in TF-resistant leukemias. Although, **2** and **3** bind to IMPDH with K_is 4 orders of magnitude lower than that of TF, concentrations of **2** and **3** 1−1.5 orders of magnitude higher than that of TF are needed to induce a similar level of differentiation in HL-60 cells. This has been attributed to less efficient cell membrane penetration by the dinucleotides **2** and **3** as well to the hydrolytic instability of pyrophosphates **2**.[4]

Recently, we have synthesized three analogues of TAD containing a fluorine atom at the C2′ of the adenine nucleoside in both the *ribo* and *arabino* configurations (**5** and **7**, respectively) and at the C3′ in the *ribo* configuration (**6**).[5] Each of these compounds showed good inhibitory activity against IMPDH, generally binding in the 10^{-7} M range. Among them, compound **7** was found to be a potent inducer of differentiation in K562 cells. In the experiments reported here we have compared the ability of these fluorinated analogues to induce differentiation in K562 cells with that of the previously described compounds **1**−**3**.

Induction of cell differentiation requires the transport of exogenous analogue across the cell membrane. TAD analogues resistant to hydrolysis must be transported intact. The advantage of such analogues is that they are active in tiazofurin-resistant cells,[6] as resistance is due in part to an increase in the activity of the phosphodiesterase that hydrolyzes TAD. This consideration prompted the synthesis of β-CH_2-TAD (**3**), a hydrolysis-resistant analogue with a methylene substitution for the pyrophosphate bridge.[6] In an effort to improve hydrolytic stability and enhance membrane permeability of the fluorinated pyrophosphate analogue **7**, we have synthesized the corresponding nonhydrolyzable analogues **8** and **9** in which the pyrophosphate oxygen is replaced by $-CH_2-$ and $-CF_2-$ groups, respectively.

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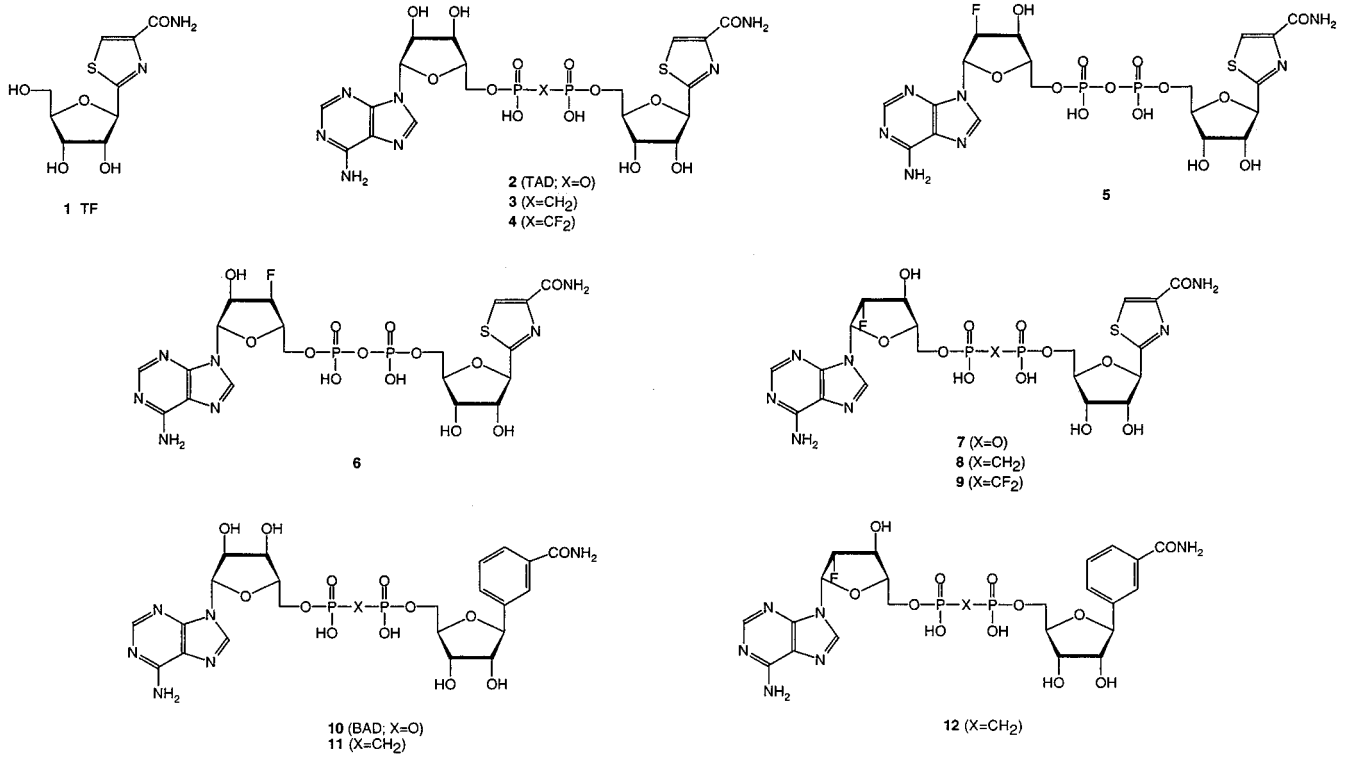
Chart 1



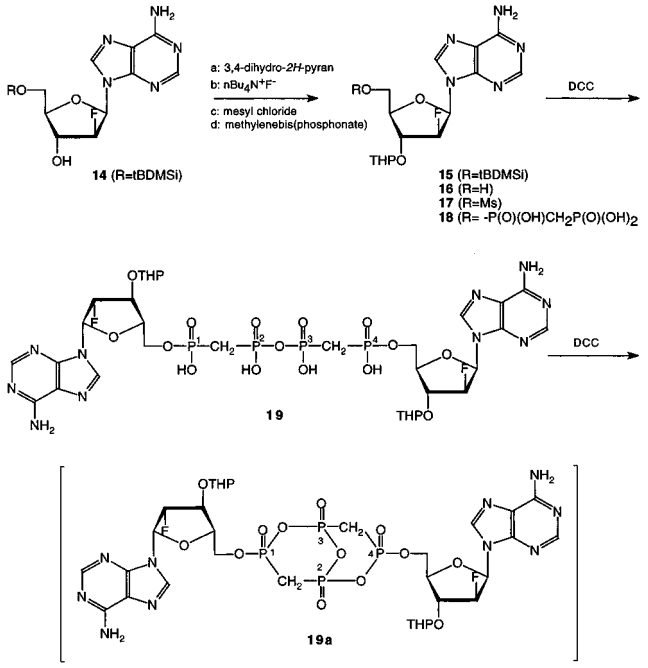
In addition to the preparation and characterization of the tiazofurin conjugates, we have in previous work also studied a nonhydrolyzable analogue (β-CH_2-BAD, **11**)[7] of benzamide adenine dinucleotide (BAD, **10**)[8] and found that **11** was a potent inhibitor of IMPDH.[7] Prompted by the activity of several of the fluorinated derivatives of TAD, we synthesized the 2′-fluoro *ara* analogue of the benzamide derivative (**12**). We have determined the activity of all these compounds as inhibitors of IMPDH and as inducers of K562 cell differentiation.

Chemical Synthesis

9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)adenine[9] (**13**) was treated with *tert*-butyldimethylsilyl chloride to give nucleoside **14** which was then further protected as 3′-*O*-tetrahydropyranyl derivative **15** (Scheme 1). Removal of the silyl protecting group from **15** with Et_3NHF afforded **16** which was mesylated to give **17**. The 5′-mesylate **17** was treated with the tris(tetrabutylammonium) salt of methylenebis(phosphonic acid).[10] Displacement of the mesyl function occurred smoothly to give the methylenebis(phosphonate) analogue **18** which could be used for DCC-promoted coupling with 2′,3′-*O*-isopropylidenetiazofurin (**20**) or 3-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)benzamide (**21**).

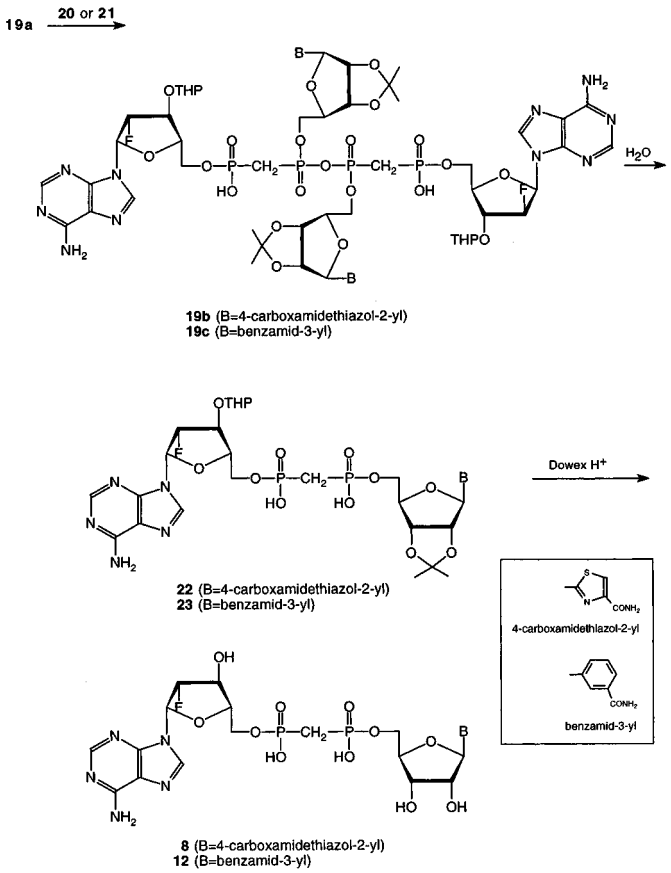
Recently, we developed a new procedure for DCC-catalyzed coupling of nucleoside 5′-methylenebis(phosphonate)s with nucleosides, sugars, and alcohols.[11] We found that the first step in this reaction was the formation of a P^1,P^4-dinucleoside-P^1:P^2,P^3:P^4-bismethylene tetraphosphonate analogue (Np_4N) such as **19**. Np_4A analogues were further dehydrated by the action of DCC forming active bicyclic trisanhydride intermediates[11] such as **19a**. Addition of the 2′,3′-*O*-isopropylidene-protected nucleoside at this stage of the reaction caused the nucleophilic attack of its 5′-hydroxyl group

Scheme 1

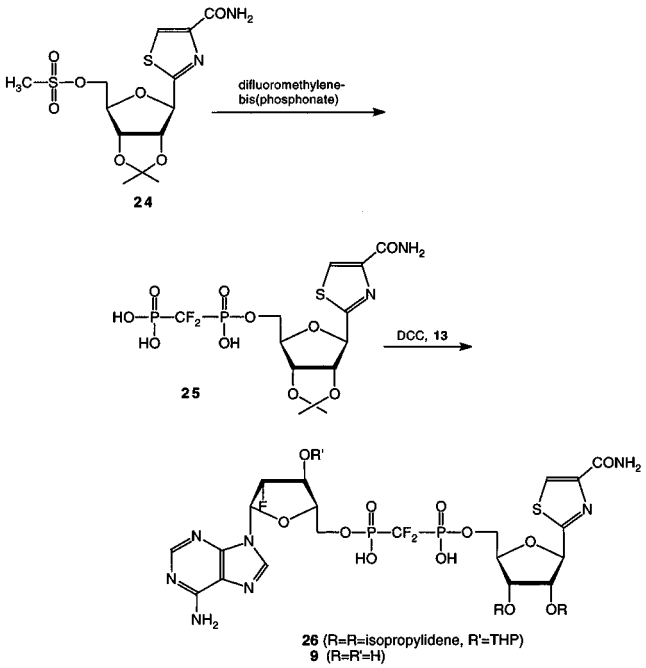


on the phosphorus atoms P^2 and P^3 with formation of the corresponding tetrakis-substituted Np_4N intermediate[11] **19b**. This intermediate was then hydrolyzed with water to the desired P^1,P^2-disubstituted methylenebis(phosphonate)s. Thus, treatment of the methylenebis(phosphonate) analogue **18** with DCC to give **19a** followed by addition of 2′,3′-*O*-isopropylidenetiazofurin (**20**) afforded the desired product **22** in 69% yield. Addition of 2′,3′-*O*-isopropylidenebenzamide riboside (**21**) to the same intermediate **19a** gave the corresponding analogue **23** in 79% yield. After deprotection with Dowex50/H^+ the desired products **8** and **12** were obtained in high yields.

Scheme 2



Scheme 3



We found that displacement of the mesyl group of 2′,3′-*O*-isopropylidene-5′-*O*-mesyltiazofurin (**24**; Scheme 2) with the tris(tetrabutylammonium) salt of methylenebis(phosphonic acid) did not work. The formation of unidentified products that the ^{1}H NMR spectrum showed two doublets (J = 3.5 Hz) at δ 6.64 and 7.04, characteristic for furan derivative, was observed. Aromatization of the tiazofurin sugar was reported to occur easily under basic condition.[12] However, similar reaction of **24** with the more acidic tris(tetrabutylammonium) salt of difluoromethylenebis(phosphonic acid) afforded the desired product **25**. We reported earlier that the coupling of **25** with 2′-deoxy-2′-fluoroadenosine gave, after deprotection, the desired β-CF_2-TAD analogue in 4.5% yield.[13] The similar coupling of **25** with **16**, performed according to our new procedure,[11] afforded after deprotection the desired product **9** in 35% overall yield.

Biological Effects: Results and Discussion

The new NAD analogues were assayed for both inhibitory activity against human IMPDH type II and antiproliferative activity against K562 erythroleukemic cells. IC_{50} values were measured in the presence of 100 μM NAD, 50 μM IMP, 100 mM Tris-HCl, 100 mM KCl, 3 mM EDTA, and 25 nM enzyme at pH 8.0. The formation of NADH was monitored by increase in absorbance at 340 nm as described previously.[8] IMPDH IC_{50} values of the new compounds as well as of some members of the series published previously[5,7,8] are given in Table 1. The ability to induce differentiation in K562 cells was also estimated by determining the fraction of benzidine positive cells converted following incubation with each analogue. In order to more readily compare these data, in each case the percentage of benzidine positive cells is divided by the concentration of analogue used. These results are also reported in Table 1.

Results for the parent nucleoside TF (**1**), the active anabolite TAD (**2**), and its nonhydrolyzable analogue **3** are comparable to those observed previously for HL-60 cells.[4] Although IMPDH inhibition by TAD and β-CH_2-TAD is similar, the β-methylene analogue is a less potent inhibitor of growth and inducer of differentiation. This has been attributed to the requirement of **3** to be

Table 1. Growth Inhibition and Differentiation of K562 Cells

	inhibition IC_{50} (μM)		differentiation		
compound	IMPDH	growth	conctn (μM)	positive cells (%)	%/ conctn
TF (**1**)		3.0	15.0	80	5.3
TAD (**2**)	0.09	3.7	15.0	70	7.7
β-CH_2TAD (**3**)	0.11	18.0	14.3	35	2.4
β-CF_2TAD (**4**)	0.3	11.0	14.0	48	3.4
3′-F-TAD (**6**)	0.7	3.2	2.5	26	10.4
2′-F-*ara*-TAD (**7**)	2.6	3.8	13.0	56	4.3
2′-F-*ara*-β-CH_2TAD (**8**)	6.0	70	14.0	9.5	0.7
2′-F-*ara*-β-CF_2TAD (**9**)	0.8	13.0	13.0	40	3.1
β-CH_2-BAD (**11**)	0.8	68	15.0	9.5	0.6
			60.0	45	0.8
2′-F-*ara*-β-CH_2BAD (**12**)	175	no effect	75.0	9.0	0.1

transported intact across the cell membrane.[4] In contrast, extracellular TAD can be hydrolyzed to the corresponding nucleosides and TF transported readily across the membrane to be reanabolized to the active TAD. Despite the weaker performance of β-CH_2-TAD, it has been suggested that compound **3** may be of particular value in TF-resistant cells. In these cells the expression of phosphodiesterase ("TADase") causes the breakdown of TAD. The β-CH_2-TAD is resistant to such hydrolysis[6] and is more cytotoxic than TAD in TF-resistant cells. Our data showed, however, that with the exception of β-CH_2-TAD other compounds containing a methylene bridge (**8**, **11**, and **12**) were poor inhibitors of cells growth and they did not induce differentiation. Interestingly, even a highly potent inhibitor of IMPDH such as β-CH_2-BAD (**11**) is not effective.

On the other hand, NAD analogues **4** and **9** containing the $-CF_2-$ linkage showed improved inhibitory and differentiation-inducing activity compared to β-CH_2-TAD, despite being less effective inhibitors of IMPDH. Replacement of the pyrophosphate oxygen with the isosteric and isopolar $-CF_2-$ group[14] retains better the pyrophosphate character of the construct than insertion of the $-CH_2-$ bridge. This suggests that either compounds **4** and **9** could be hydrolyzed in a similar fashion as pyrophosphates releasing TF or fluorine substitution of the β-methylene bridge does improve transport of the intact dinucleotide. If the last is the case this may provide a useful strategy in the design of compounds with potent differentiation and antiproliferation activity.

The hydrolyzable (pyrophosphate) analogues **6** and **7** containing fluorine at the adenine sugar show comparable growth inhibitory activity to TAD. The large percentage of differentiated cells/μM observed for compound **6** is likely an artifact of the high toxicity observed for 3′-deoxy-3′-fluoroadenosine[15] released during hydrolysis of **6**. The small percent of naturally occurring benzidine positive cells is inflated by cytotoxicity among the majority of undifferentiated cells. The differentiation-inducing activity of the 2′-fluoro *ara* derivative **7** is comparable to that of TAD, despite a significantly higher IC_{50} against IMPDH. However, the efficacy of this compound may be due to its extracellular hydrolysis, transport, and intracellular reanabolism to the parent TAD.

Experimental Section

General Methods. HPLC was performed on a Dynamax-60A C18-83-221-C column with flow rate of 5 mL/min or Dynamax-300A C18-83-243-C column with a flow rate of 20 mL/min of 0.1 M Et_3N H_2CO_3 (TEAB) followed by a linear gradient of 0.1 M TEAB−aqueous MeCN (70%). Elemental analyses were performed by Gaibraith Laboratories, Knoxville, TN. Nuclear magnetic resonance spectra were recorded on a Jeol Eclipse 270 spectrometer with Me_4Si or DDS as the internal standard for ^1H and external H_3PO_4 for ^{31}P. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), and dd (double doublet). Values given for coupling constants are first order.

9-[2-Deoxy-2-fluoro-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]adenine (14). A mixture of **13** (810 mg, 3 mmol) and *tert*-butyldimethylsilyl chloride (510 mg, 3.39 mmol) in pyridine (15 mL) was kept at room temperature overnight. Water (5 mL) was added, and the reaction mixture was concentrated *in vacuo*. The residue was chromatographed on a silica gel column with $CHCl_3$−EtOH (5%) followed by $CHCl_3$−EtOH (10%) to give **14** (900 mg, 78%): ^1H NMR ($CDCl_3$) δ 0.093 (3H, s, Me-Si), 0. 099 (3H, s, Me-Si), 0.90 (9H, s, tBu-Si), 3.84 (1H, dd, H5′, $J_{4',5'}$ = 6.0 Hz, $J_{5',5''}$ = 11.1 Hz), 3.91 (1H, dd, H5″, $J_{4',5''}$ = 4.4 Hz), 4.01−4.07 (1H, m, H4′), 4.64 (1H, two dd, H3′, $J_{3',F}$ = 17.4 Hz, $J_{2',3'}$ = 2.5 Hz, $J_{3',4'}$ = 4.0 Hz), 5.10 (1H, two dd, $J_{2',F}$ = 52.5 Hz, $J_{1',2'}$ = 3.6 Hz), 6.51 (1H, dd, H1′, $J_{1',F}$ = 18.1 Hz), 8.09 (1H, d, H8, $J_{8,F}$ = 2.5 Hz), 8.33 (1H, s, H2). Anal. ($C_{16}H_{26}FN_5O_3Si$) C, H, N.

9-[2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]adenine (15). To a mixture of compound **14** (780 mg, 2.03 mmol) and 3,4-dihydro-2*H*-pyran (3 mL, 18 equiv) in dioxane (10 mL) was added *p*-toluenesulfonic acid (600 mg, 3.1 mmol), and the mixture was kept at room temperature for 30 min. The excess of *p*-toluenesulfonic acid was neutralized with 1 M $NaHCO_3$, and the reaction mixture was concentrated *in vacuo*. The residue was partitioned between CH_2Cl_2 and water; the organic layer was separated and concentrated. The residue was chromatographed on a column of silica gel with CH_2Cl_2−EtOH (2%) followed by CH_2Cl_2−EtOH (4%) to give **15** (790 mg, 83%) as a mixture of two diastereomers: ^1H NMR ($CDCl_3$) δ 0.094 (12H, s, Me_2-Si of two isomers), 0.911 and 0.916 (two s, 9H each, tBu-Si), 1.52−1.60 (8H, m, THP-H4,4′,5,5′ of two isomers), 1.70 (4H, m, THP-H6,6′ of two isomers), 3.51−3.57 (2H, m, THP-H3 of both isomers), 3.80−3.90 (6H, m, H5′,5″, THP-H3′ of two isomers), 4.03−4.07 (1H, m, H4′), 4.12−4.15 (1H, m, H4′), 4.50 (1H, two dd, H3′, $J_{3',F}$ = 16.1 Hz, $J_{2',3'}$ = 2.8 Hz, $J_{3',4'}$ = 5.2 Hz), 4.64 (1H, two dd, H3′, $J_{3',F}$ = 16.8 Hz, $J_{2',3'}$ = 2.5 Hz, $J_{3',4'}$ = 4.9 Hz), 4.71−4.74 (1H, m, THP-H2), 4.82−4.84 (1H, m, THP-H2), 5.05 (1H, two dd, H2′, $J_{2',F}$ = 51.3 Hz, $J_{1',2'}$ = 2.9 Hz), 5.24 (1H, two dd, H2′, $J_{2',F}$ = 51.3 Hz, $J_{1',2'}$ = 2.9 Hz), 6.46 (2H, two dd, H1′, $J_{1',F}$ = 19.0 Hz), 8.07 (1H, d, H8, $J_{8,F}$ = 2.5 Hz), 8.09 (1H, d, H8, $J_{8,F}$ = 2.5 Hz), 8.34 (1H, s, H2), 8.35 (1H, s, H2). Anal. ($C_{21}H_{34}FN_5O_4Si$) C, H, N.

9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranosyl)adenine (16). Compound **15** (936 mg, 2 mmol) was dissolved in 1 M Et_3NHF/THF (5 mL) and left at room temperature overnight. Excess of Et_3NHF was neutralized with 1 M $NaHCO_3$ and concentrated *in vacuo*. The residue was coevaporated with pyridine (3 × 100 mL), suspended in pyridine, filtrated, and concentrated *in vacuo*. The residue was chromatographed on a silica gel column with $CHCl_3$−EtOH (5%) followed by $CHCl_3$−EtOH (10%) to give **16** (560 mg, 79%) as a foam: ^1H NMR ($CDCl_3$) δ 1.52−1.80 (12H, m, THP-H4,4′,5,5′,6,6′ of both isomers), 3.55−3.59 (2H, m, THP-H3 of two isomers), 3.86−3.96 (6H, m, H5′,5″, THP-H3′ of both isomers), 4.15−4.21 (2H, m, H4′), 4.50−4.66 (2H, two m, H3′), 4.74−4.75 (1H, m, THP-H2), 4.81−4.82 (1H, m, THP-H2), 5.11 (1H, two dd, H2′, $J_{2',F}$ = 52.3 Hz, $J_{1',2'}$ = 3.6 Hz, $J_{2',3'}$ = 2.0 Hz), 5.33 (1H, two dd, $J_{2',F}$ = 52.3 Hz, $J_{1',2'}$ = 3.6 Hz, $J_{2',3'}$ = 2.0 Hz), 6.40 (2H, two dd, H1′, $J_{1',F}$ = 18.0 Hz), 7.99 (1H, d, H8, $J_{8,F}$ = 2.0 Hz), 8.02 (1H, d, H8, $J_{8,F}$ = 2.0 Hz), 8.34 (2H, s, H2). Anal. ($C_{15}H_{20}FN_5O_4$) C, H, N.

9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-5-*O*-mesyl-β-D-arabinofuranosyl)adenine (17). To a solution of compound **16** (608 mg, 2 mmol) in pyridine (10 mL) was added mesyl chloride (200 μL); the reaction mixture was stirred for 30 min, diluted with EtOH (10 mL), and concentrated *in vacuo*. The residue was chromatographed on a silica gel column using CH_2Cl_2 as the eluent to give **17** (838 mg, 97%): ^1H NMR ($CDCl_3$) δ 1.52−1.87 (12H, m, THP-H4,4′,5,5′,6,6′ of both isomers), 3.04 (3H, s, Ms), 3.05 (3H, s, Ms), 3.55−3.59 (2H, m, THP-H3 of two isomers), 3.78−3.90 (2H, m, THP-H3′ of both isomers), 4.34−4.58 (8H, m, H3′,4′,5′,5″), 4.74−4.77 (2H, m, THP-H2), 4.81−4.82 (1H, m, THP-H2), 5.10 (1H, two dd, H2′, $J_{2',F}$ = 50.7 Hz, $J_{1',2'}$ = 3.0 Hz, $J_{2',3'}$ = 2.0 Hz), 5.29 (1H, two dd, $J_{2',F}$ = 50.5 Hz, $J_{1',2'}$ = 2.6 Hz, $J_{2',3'}$ = 2.0 Hz), 6.48 (1H, dd, H1′, $J_{1',F}$ = 21.2 Hz, $J_{1',2'}$ = 3.0), 6.52 (1H, dd, H1′, $J_{1',F}$ = 22.8 Hz, $J_{1',2'}$ = 2.6 Hz), 8.07 and 8.08 (two 1H singlets, H8), 8.34 and 8.35 (two 1H singlets, H2). Anal. ($C_{16}H_{22}FN_5O_6S$) C, H, N.

9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranos-5-yl)adenyl Methylenebis(phosphonate) (18). 9-(5-Mesyl-2-deoxy-2-fluoro-3-tetrahydropyranyl-β-D-arabinofuranosyl)adenine (**17**) (353 mg, 1 mmol) was added in one

portion to a solution of tris(tetrabutylammonium) methylenebis(phosphonate) (1.5 mmol, pH 9.0) in 1.5 mL of DMSO. The mixture was kept at room temperature for two h, diluted to 80 mL with water, and applied to a column of DEAE-Sephadex A-25 (30 mL, HCO_3^- form). The column was eluted with 0.05 M TEAB (50 mL) and then with 0.6 M TEAB until all UV-absorbing material was eluted. The buffer was removed by repeated coevaporation with water, and the residue was purified by HPLC to give **18** (314 mg, 44%) as the bis(triethylammonium) salt: ^{1}H NMR (D_2O) δ 1.24 (t, 18H, $^3J_{HH}$ = 7.2 Hz, CH_3CH_2N), 1.57 (m, 4H, H-4,5 THP), 1.82 (m, 2H, H-3 THP), 2.12 (t, 2H, $^2J_{PH}$ = 19.8 Hz, PCH_2P), 3.16 (q, 12H, $^3J_{HH}$ = 7.2 Hz, CH_3CH_2N), 3.64 (m, 1H, H-6 THP), 3.94 (m, 1H, H-6 THP), 4.18 (m, 2H, H-5′, H-5″), 4.37 (m, 1H, H-4′), 4.64−4.80 (m, 1H, H-3′ overlapped with H_2O), 4.97 (m, 1H, H-2 THP), 5.40 (ddd, 0.3H, $^2J_{HF}$ = 51.1 Hz, H-2′), 5.44 (ddd, 0.7H, $^2J_{HF}$ = 51.4 Hz, $^3J_{HH}$ = 3.9, 3.4 Hz, H-2′), 6.49 (dd, 0.3H, $^3J_{HF}$ = 17.6 Hz, $^3J_{HH}$ = 3.6 Hz, H-1′), 6.51 (dd, 0.7H, $^3J_{HF}$ = 17.6 Hz, $^3J_{HH}$ = 3.6 Hz, H-1′), 8.21 (s, 1H, H-2), 8.42 and 8.43 (s, total 1H, H-8); ^{31}P NMR (D_2O) δ 14.34 (d, 0.7P, $^2J_{PP}$ = 9.2 Hz) and 20.36 (d, 0.7P, $^2J_{PP}$ = 9.2 Hz, T-p), 14.48 (d, 0.3P, $^2J_{PP}$ = 9.2 Hz) and 20.18 (d, 0.3P, $^2J_{PP}$ = 9.2 Hz, T-p).

***P*^{1}-[9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranos-5-yl)adenyl] *P*^{2}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl) Methylenebis(phosphonate) (22).** 9-(2′-Deoxy-2′-fluoro-3′-*O*-tetrahydropyranyl-β-D-arabinofuranos-5′-yl)-adenyl methylenebis(phosphonate) (**18**; 143 mg, 0.2 mmol, bis(triethylammonium) salt) was dissolved in anhydrous pyridine (2 mL), DCC (103 mg, 0.5 mmol) was added, and the mixture was left overnight at room temperature. Dicyclohexylurea began to precipitate after approximately 1 h. Part of the reaction mixture (0.5 mL, pyridine solution only) was transferred to an NMR tube, and the ^{31}P NMR spectrum was measured to check for the formation of intermediate **19a**. 2′,3′-Isopropylidenetiazofurin was then added (16.5 mg, 0.055 mmol, to the NMR tube and 50 mg, 0.165 mmol, to the reaction flask), and both mixtures were kept for 5.5 h at 55−60 °C and overnight at 40 °C. At that point ^{31}P NMR showed that the reaction was complete. After addition of water (20%, v/v) the mixtures were combined and left overnight in a refrigerator. Pyridine was evaporated; the residue was dissolved in water (10 mL) and extracted with ethyl ether to remove unreacted DCC. The water solution was then filtered, and compound **22** (137 mg, 69%) was separated as the bis(triethylammonium) salt by HPLC: ^{1}H NMR (D_2O) δ 1.21 (t, 18H, $^3J_{HH}$ = 7.4 Hz, CH_3CH_2N), 1.32 (s, 3H, CH_3-C), 1.47−1.62 (m, 4H, H-4,5 THP), 1.54 (s, 3H, CH_3-C), 1.75 (m, 2H, H-3 THP), 2.13 (t, 2H, PCH_2P), 3.13 (q, 12H, $^3J_{HH}$ = 7.4 Hz, CH_3CH_2N), 3.50−3.65 (m, 1H, H-6 THP), 3.8−3.9 (m, 1H, H-6 THP), 3.9−4.0 (m, 2H, H-5′,5″ T), 4.10−4.20 (m, 2H, H-5′,5″ fA), 4.22−4.38 (m, 2H, H-4′ A, H-4′ T), 4.52−4.7 (m, 1H, H-3′ A), 4.9 −5.0 (m, 3H, H-2′, H-3′ T, H-2 THP), 5.00 (d, 0.4H, $^3J_{HH}$ = 4.0 Hz, H-1′ T), 5.02 (d, 0.6H, $^3J_{HH}$ = 4.0 Hz, H-1′ T), 5.32 (ddd, 0.4H, $^2J_{HF}$ = 49 Hz, H-2′ A), 5.39 (ddd, 0.6H, $^2J_{HF}$ = 48 Hz, H-2′ A), 6.33 (dd, 0.4H, $^3J_{HF}$ = 18.1 Hz, $^3J_{HH}$ = 4.0 Hz, H-1′ A), 6.34 (dd, 0.6H, $^3J_{HF}$ = 17.3 Hz, $^3J_{HH}$ = 4.2 Hz, H-1′ A), 8.02 and 8.03 (s, total 1H, H-5 T), 8.06 and 8.07 (s, total 1H, H-2 A), 8.32 and 8.33 (s, total 1H, H-8 A); ^{31}P NMR (D_2O) δ 17.68 and 17.95 (AB system, 1.2P, $^2J_{PP}$ = 10.0 Hz), 17.77 and 17.88 (AB system, 0.8 P, $^2J_{PP}$ = 9.5 Hz).

***P*^{1}-[9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranos-5-yl)adenyl] *P*^{2}-[1-(2,3-*O*-Isopropylidene-β-D-ribofuranos-5-yl)benzene-3-carboxamido] Methylenebis(phosphonate) (23).** Compound **18** (71 mg, 0.1 mmol, bis(triethylammonium) salt) was dissolved in anhydrous pyridine (1 mL), DCC (52 mg, 0.25 mmol) was added, and the mixture was kept overnight at room temperature. The precipitation of dicyclohexylurea started after approximately 1 h and continued during the next several hours. The mixture (pyridine solution only) was transferred to an NMR tube and checked by ^{31}P NMR for the formation of intermediate **19a**. 2′,3′-*O*-Isopropylidenebenzamide riboside (**21**) (35 mg, 0.12 mmol) was then added, and the mixture was kept for 6 h at 55−60 °C and overnight at 40 °C. At that point ^{31}P NMR showed that the reaction was complete. Water (200 μL) was added, and the mixture was left at room temperature for 4 h. Pyridine was evaporated in vacuo; the residue was resuspended in water (5 mL), extracted with ethyl ether to remove unreacted DCC, and filtered to remove dicyclohexylurea. After HPLC purification, compound **23** (78 mg, 79%) was obtained as the bis(triethylammonium) salt: ^{1}H NMR (D_2O) δ 1.21 (t, 18H, $^3J_{HH}$ = 7.3 Hz, CH_3CH_2N), 1.29 (s, 1.2H, CH_3-C), 1.31 (s, 1.8H, CH_3-C), 1.50−1.56 (m, 4H, H-4,5 THP), 1.56 (s, 3H, CH_3-C), 1.65−1.85 (m, 2H, H-3 THP), 2.18 (t, 2H, $^2J_{PH}$ = 19.6 Hz, PCH_2P), 3.12 (q, 12H, $^3J_{HH}$ = 7.3 Hz, CH_3CH_2N), 3.6 (m, 1H, H-6 THP), 3.9 (m, 1H, H-6 THP), 4.0−4.3 (m, 6H, H-4′, H-5′, H-5″), 4.51 (dd, 1H, $^3J_{HH}$ = 3.6, 6.2 Hz, H-3′ B), 4.67 (dd, 1H, $^3J_{HH}$ = 5.3, 4.5 Hz, H-2′ B), 4.6−4.9 (m, 3H, H-1′ B, H-3′ fA, H-2 THP), 5.26 (ddd, 0.4H, $^2J_{HF}$ = 52.1 Hz, H-2′ A), 5.35 (ddd, 0.6H, $^2J_{HF}$ = 50.7 Hz, H-2′ A), 6.22 (dd, 0.4H, $^3J_{HF}$ = 18.5 Hz, $^3J_{HH}$ = 3.8 Hz, H-1′ A), 6.23 (dd, 0.6H, $^3J_{HF}$ = 17.2 Hz, $^3J_{HH}$ = 4.0 Hz, H-1′ A), 7.3−7.4 (m, 2H, aromatic), 7.55−7.60 (m, 2H, aromatic), 8.03 (s, 1H, H-2 A), 8.26 (s, 1H, H-8 A); ^{31}P NMR (D_2O) δ 17.86, 17.96, 18.00, 18.04, 18.13 (s, 1:4:6:4:1 ratio, mixture of 2 diastereoisomers).

***P*^{1}-(2′-Deoxy-2′-fluoro-β-D-adenos-5′-yl) *P*^{2}-(Tiazofurin-5′-yl) Methylenebis(phosphonate) (8).** Compound **22** (137 mg, 0.14 mmol, bis(triethylammonium) salt) was deprotected by treatment with Dowex 50WX8/H^+ (1 mL of the resin) in water solution (3 mL) for 2 h at 50 °C and then for 24 h at room temperature. The mixture (together with the resin) was applied to a Dowex 50WX8/Na^+ column (1 × 9 cm). The column was eluted with water. UV-absorbing fractions were combined, evaporated to dryness, and purified by HPLC to give **8** (82 mg, 84%) as the disodium salt: ^{1}H NMR (D_2O) δ 2.24 (t, 2H, $^2J_{HP}$ = 20.1 Hz, PCH_2P), 4.06 (dd, 2H, $^3J_{HH}$ = 4.0, 5.4 Hz, H-5′,5″ T), 4.20 (m, 6H, H-2′, H-3′, H-4′ T, H-4′, H-5′, H-5″ A), 4.62 (ddd, 1H, $^3J_{HF}$ = 18.3 Hz, $^3J_{HH}$ = 4.5, 5.1 Hz, H-3′ A), 4.98 (d, 1H, $^3J_{HH}$ = 3.8 Hz, H-1′ T), 5.29 (ddd, 1H, $^2J_{HF}$ = 49.0 Hz, $^3J_{HH}$ = 4.4, 3.9 Hz, H-2′ A), 6.37 (dd, 1H, $^3J_{HF}$ = 12.7 Hz, $^3J_{HH}$ = 4.4 Hz, H-1′ A), 7.87 (s, 1H, H-5 T), 8.06 (s, 1H, H-2 A), 8.32 (d, 1H, $^5J_{HF}$ = 1.8 Hz, H-8 A); ^{31}P NMR (D_2O) δ 17.92 (d, 1P, $^2J_{PP}$ = 10.0 Hz, T), 18.30 (d, 1P, $^2J_{PP}$ = 10.0 Hz, A).

***P*^{1}-[9-(2-Deoxy-2-fluoro-β-D-arabinofuranos-5-yl)adenyl] *P*^{2}-[1-(β-D-Ribofuranos-5-yl)benzene-3-carboxamido] Methylenebis(phosphonate) (12).** Compound **23** (78 mg, 0.08 mmol, bis(triethylammonium) salt) was deprotected by treatment with Dowex 50WX8 resin (H^+ form, 0.5 mL of the resin) in water solution (2 mL) for 2 h at 50 °C and then for 24 h at room temperature. The mixture (together with the resin) was applied to a Dowex 50WX8/Na^+ column (1 × 6 cm) and eluted with water. UV-absorbing fractions were combined, evaporated to dryness, and purified by HPLC to give **12** (40 mg, 72%) as the disodium salt: ^{1}H NMR (D_2O) δ 2.24 (t, 2H, $^2J_{HP}$ = 20.1 Hz, PCH_2P), 4.01 (dd, 1H, $^3J_{HH}$ = 6.7, 5.3 Hz, H-2′ B), 4.09−4.22 (m, 6H, H-4′, H-5′, H-5″), 4.26 (dd, 1H, $^3J_{HH}$ = 5.1, 3.6 Hz, H-3′ B), 4.62 (dt, 1H, $^3J_{HF}$ = 19.0 Hz, $^3J_{HH}$ = 4.3 Hz, H-3′ A), 4.71 (d, 1H, $^3J_{HH}$ = 6.7 Hz, H-1′ B), 5.26 (dt, 1H, $^2J_{HF}$ = 51.5 Hz, $^3J_{HH}$ = 4.2 Hz, H-2′ A), 6.33 (dd, 1H, $^3J_{HF}$ = 14.3 Hz, $^3J_{HH}$ = 4.3 Hz, H-1′ A), 7.31 (t, 1H, $^3J_{HH}$ = 7.8 Hz, H-5 aromatic), 7.46 (d, 1H, $^3J_{HH}$ = 7.8 Hz, H-4 aromatic), 7.55 (d, 1H, $^3J_{HH}$ = 7.8 Hz, H-6 aromatic), 7.68 (s, 1H, H-2 aromatic), 8.09 (s, 1H, H-2 A), 8.29 (d, 1H, $^5J_{HF}$ = 2.2 Hz, H-8 A); ^{31}P NMR (D_2O) δ 17.93 and 18.33 (AB system, $^2J_{PP}$ = 10.8 Hz).

***P*^{1}-[9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranos-5-yl)adenyl] *P*^{2}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl) Difluoromethylenebis(phosphonate) (26).** 2′,3′-*O*-Isopropylidenetiazofurin-5′-yl difluoromethylenebis(phosphonate)[13] (**25**; 100 mg, 0.14 mmol, bis(triethylammonium) salt) was dissolved in anhydrous pyridine (1 mL, in a NMR tube) and treated with DCC (80 mg, 0.4 mmol). The mixture was left for 3 days at room temperature. 9-(2-Deoxy-2-fluoro-3-*O*-tetrahydropyranyl-β-D-arabinofuranosyl)adenine (**16**; 50 mg, 0.14 mmol) was then added, and the mixture was kept at 55−60 °C for 20 h. At that time the reaction was terminated by addition of water (0.2 mL), pyridine was evaporated, and the residue was dissolved in water (5 mL). The water solution was extracted with ethyl ether to remove unreacted DCC and filtered to remove dicyclohexylurea. After purification by HPLC compound **26** was obtained (43 mg, 30%) as the bis(triethylammonium) salt: ^{1}H NMR (D_2O) δ 1.21 (t,

23H, $^3J_{HH}$ = 7.4, CH_3CH_2N), 1.31 (s, 1.8H, CH_3-C), 1.32 (s, 1.2H, CH_3-C), 1.48−1.60 (m, 4H, H-4,5 THP), 1.55 (s, 3H, CH_3-C), 1.70−1.84 (m, 2H, H-3 THP), 3.10 (q, 15H, $^3J_{HH}$ = 7.4 Hz, CH_3CH_2N), 3.50−3.65 (m, 1H, H-6 THP), 3.80−3.94 (m, 1H, H-6 THP), 4.06−4.44 (m, 6H, H-4′, H-5′, H-5″), 4.60 (m, 0.6H, H-3′ A), 4.68 (m, 0.4H, H-3′ A), 4.88−4.96 (m, 3H, H-2′, H-3′ T, H-2 THP), 5.09 (d, 0.4H, $^3J_{HH}$ = 3.5 Hz, H-1′ T), 5.11 (d, 0.6H, $^3J_{HH}$ = 3.5 Hz, H-1′ T), 5.33 (ddd, 0.4H, $^2J_{HF}$ = 51.3 Hz, $^3J_{HH}$ = 3 Hz, H-2′ A), 5.41 (ddd, 0.6H, $^2J_{HF}$ = 51.3 Hz, $^3J_{HH}$ = 3.4, 3.9 Hz, H-2′ A), 6.38 (dd, 0.4H, J_{HF} = 17.9 Hz, $^3J_{HH}$ = 3.4 Hz, H-1′ A), 6.39 (dd, 0.6H, $^3J_{HF}$ = 15.9 Hz, $^3J_{HH}$ = 4.1 Hz), 8.03 and 8.04 (s, total 1H, H-5 T), 8.09 and 8.10 (s, 1H, H-2 A), 8.33 and 8.34 (s, 1H, H-8 A); ^{31}P NMR (D_2O) δ 4.20 and 4.22 (ABX_2 system, $^2J_{PF}$ = 83.4 Hz, $^2J_{PP}$ = 53.4 Hz), 4.20 and 4.28 (ABX_2 system, $^2J_{PF}$ = 83.3 Hz, $^2J_{PP}$ = 53.4 Hz).

***P*^{1}-[9-(2-Deoxy-2-fluoro-*β*-D-arabinofuranosyl)adenyl] *P*^{2}-Tiazofurin-5′-yl Difluoromethylenebis(phosphonate) (9).** Compound **26** (43 mg, 0.042 mmol) was deprotected by treatment with Dowex 50WX8/H^+ (1 mL) in water solution (2 mL) for 30 min at 50 °C and then for 24 h at room temperature. The mixture (together with the resin) was applied to a Dowex 50WX8/Na^+ column (1 × 7 cm) and eluted with water. The UV-absorbing fractions were combined and evaporated to dryness to give **9** (20 mg, 67%) as the disodium salt: ^{1}H NMR (D_2O) δ 4.18−4.40 (m, 8H, H-2′, H-3′, H-4′, H-5′, H-5″ T, H-4′, H-5′, H-5″ A), 4.67 (ddd, 1H, $^3J_{HF}$ = 18.1 Hz, $^3J_{HH}$ = 4.5 and 3.4 Hz, H-3′ A), 5.05 (d, 1H, $^3J_{HH}$ = 4.5 Hz, H-1′ T), 5.32 (ddd, 1H, $^2J_{HF}$ = 51.7 Hz, $^3J_{HH}$ = 4.0, 4.4 Hz, H-2′ A), 6.45 (dd, 1H, $^3J_{HF}$ = 13.4 Hz, $^3J_{HH}$ = 4.4 Hz, H-1′ A), 7.95 (s, 1H, H-5 T), 8.14 (s, 1H, H-2 A), 8.36 (d, 1H, $^5J_{HF}$ = 1.4 Hz, H-8 A); ^{31}P NMR (D_2O) δ 4.46 and 4.53 (ABX_2 system, $^2J_{PF}$ = 82.9 Hz, $^2J_{PP}$ = 58 Hz).

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