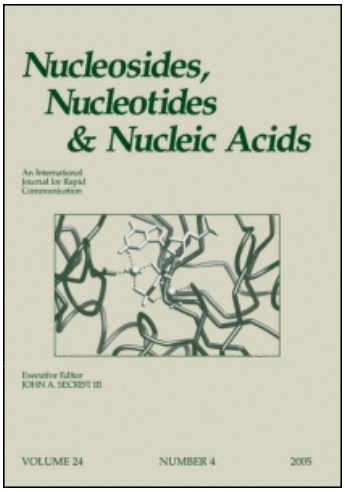

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

A New Synthesis of Some 4'-Thio-D-ribonucleosides and Preliminary Enzymatic Evaluation

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To cite this Article Leydier, C. , Bellon, L. , Barascut, J. -L. , Deydier, J. , Maury, G. , Pelicano, H. , Alaoui, M. A. El and Imbach, J. -L.(1994) 'A New Synthesis of Some 4'-Thio-D-ribonucleosides and Preliminary Enzymatic Evaluation', Nucleosides, Nucleotides and Nucleic Acids, 13: 10, 2035 — 2050
To link to this Article: DOI: 10.1080/15257779408013205
URL: http://dx.doi.org/10.1080/15257779408013205

NUCLEOSIDES & NUCLEOTIDES, 13(10), 2035-2050 (1994)

A NEW SYNTHESIS OF SOME 4'-THIO-D-RIBONUCLEOSIDES AND PRELIMINARY ENZYMATIC EVALUATION.

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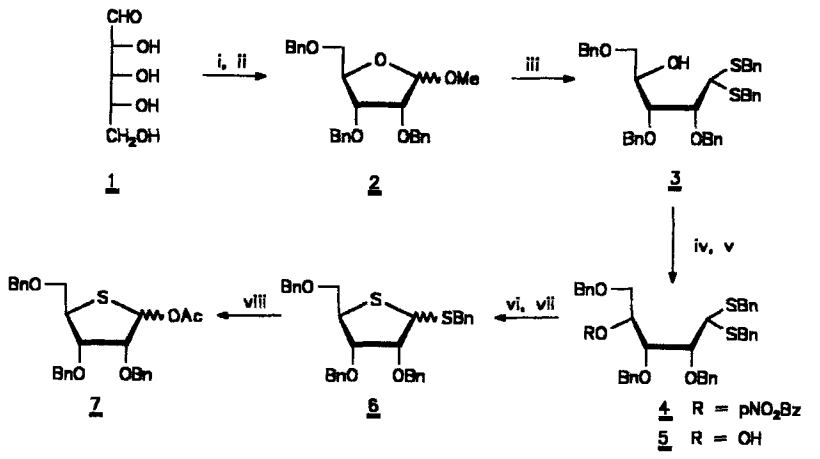
ABSTRACT :

A new synthesis of 4'-thioribonucleosides is presented together with the enzymatic evaluation of the adenosine analogues with respect to adenosine kinase. The 4-thio-D-ribofuranosyl carbohydrate precursor 7 was obtained in good yield from D-ribose and further reacted with adenine and cytosine to give the α and β anomers of 4'-thioadenosine and 4'-thiocytidine, respectively. 4'-thio-ß-D-adenosine, **12β**, is a poor substrate for bovine liver adenosine kinase but does not show substrate inhibition of the enzyme.

INTRODUCTION.

As part of our program to develop stable antisense RNA analogues, we recently studied the physico-chemical properties of oligoribonucleotides in which the furanose annular oxygen atom is replaced by a sulfur atom (4'-thiooligoribonucleotides) [1,2]. In order to prepare 4'-thiooligoribonucleotides of defined sequences complementary to biological targets, we designed a reliable method to obtain the necessary 4'-thioribonucleosides synthons. Herein, we report a markedly improved synthesis of the 4-thio-carbohydrate precursor 7.

We have previously shown [2] that 4'-thiooligoribonucleotides are more resistant to nuclease catalyzed hydrolysis than the corresponding natural oligoribonucleotides. However, once inside the cell, slow degradation of 4'-thiooligoribonucleotides could lead to the progressive liberation of 4'-thionucleotides with subsequent metabolization to the triphosphate, and then incorporation into nascent RNA[3]. In fact, reported antiviral



i = MeOH, HCl, ii = BnBr, KOH, THF, iii = BnSH, BF_3, Et_2O, iv = Ph_3P, DEAD, p-NO_2PhCO_2H, THF, v = K_2CO_3, MeOH, vi = MesCl, Pyr., vii = NBu_4I, $BaCO_3$, Pyr., viii = $Hg(OAc)_2$, AcOH

Figure 1.

properties [4, 5] of some 4'-thionucleosides analogues suggest that these compounds, although resistant to catabolism [6, 7], presumably undergo enzymatic phosphorylation [8]. To assess the magnitude of these potential pathways for 4'-thioadenosine, we have studied the substrate properties of the α- and ß-anomers of 4'-thioadenosine, **12α** and **12β** respectively, with respect to purified hepatic adenosine kinase.

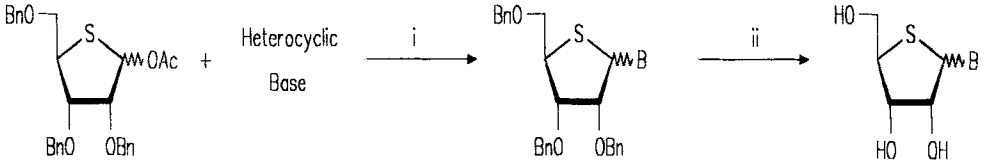
RESULTS.

Chemistry. In a previous paper [9], we reported a new synthetic pathway to prepare 4-thio-D-ribofuranoside from L-lyxose. Due to the high cost of the starting sugar, this methodology is not convenient for large scale preparation. Consequently, we have designed another strategy based on two consecutive S_N2 reactions to obtain the 4-thio-D-ribofuranoside derivative **7** starting from D-ribose, **1** (Figure 1). This work was in progress when Dyson and coworkers [10] published a similar procedure in the 4-thio-2-deoxyribofuranose series. The first S_N2 process in our synthesis is a Mitsunobu reaction [11] on the D-ribose dithiobenzylacetal derivative **3** with inversion the configuration of the C_4 atom, thus providing the L-lyxose dithiobenzyl acetal derivative **5**. This key intermediate posseses the L-lyxose structure needed to obtain the 4-thio-D-ribofuranoside

derivative **6** by another iodide-mediated S_N2 cyclisation. Using the Barker and Fletcher procedure [12], methyl-2,3,5-tri-O-benzyl-D-ribofuranoside, **2**, was prepared in 80% yield. Dithioacetalisation performed with benzyl mercaptan and boron trifluoride etherate [13, 14] led to the D-ribose dithiobenzylacetal **3** in 75% yield. This compound was further treated with triphenylphosphine, diethylazodicarboxylate and *p*-nitrobenzoic acid in dry tetrahydrofuran to give the corresponding 4-*p*-nitrobenzoyl-L-lyxose dithiobenzylacetal derivative **4** in 80% yield. Removal of the *p*-nitrobenzoyl group of compound **4** was achieved using potassium carbonate in dry methanol to give 2,3,5-tri-O-benzyl-L-lyxose dithiobenzylacetal, **5**, in 91% yield. As previously described [9], the treatment of **5** with mesyl chloride in pyridine followed by addition of barium carbonate and tetrabutylammonium iodide gave a 94% yield of benzyl-2,3,5-tri-O-benzyl-1,4-dithio-D-ribofuranoside, **6**, which was subsequently acetylated to **7**. The use of *p*-nitrobenzoic acid [15] instead of benzoic acid as in the 4-thio-2-deoxy-ribofuranose series by Dyson and co-workers [12] increased the yields of the inversion product and the cyclisation step (80% and 94%, respectively). This improved synthetic pathway furnished 1-O-acetyl-2,3,5-tri-O-benzyl-1,4-dithio-D-ribofuranoside, **7**, in a 35% overall yield by an 8-step synthesis from commercially available D-ribose as compared with 11% overall yield obtained in the 4-thio-2-deoxyribofuranose synthesis [10]. Although this strategy is more time-consuming than the original pathway which we previously described [9], the overall yield is markedly improved (35% *versus* 21%).

Starting from the 4-thio-D-ribofuranoside derivative **7**, the synthesis of the already known 4'-thiocytidine derivatives **9α** and **9β** [16, 17] and the 4'-thioadenosine derivatives **12α** and **12β** [18-20] were performed using appropriate glycosylation reactions. For example, trimethylsilyltriflate-catalyzed coupling [21, 22] of the thiosugar **7** with silylated N_4-benzoylcytosine provided a 1 / 1 anomeric mixture of N_4-benzoyl-2',3',5'-tri-O-benzyl-4'-thiocytidine **8α** and **8β** in 60% yield (Figure 2). After HPLC separation, deprotection of the ß-anomer **8β** using boron tribromide in methylene chloride gave the corresponding N_4-benzoyl-4'-thio-ß-D-cytidine, **9β**, in 69% yield. Complete deprotection of **9β** and **9α** was quantitatively achieved with a saturated methanolic solution of ammonia yielding **10β** and **10α**, respectively . 4'-Thio-ß-D-cytidine, **10β**, was thus obtained in 21% overall yield starting from the thiosugar **7**.

Using Lawesson's reagent [23], we also converted authentic 2',3',5'-tri-O-benzyl-4'-thio-ß-D-uridine, **13**, [9] to its thioamide **14** (Figure 3) which, on treatment with methanolic ammonia, gave 2',3',5'-tri-O-benzyl-4'-thio-ß-D-cytidine, **15**. On deprotection with boron tribromide, 4'-thio-ß-D-cytidine, **10β**, was obtained univocally in 70 % overall yield. All physico-chemical properties of the prepared nucleoside analogues unambigously confirm

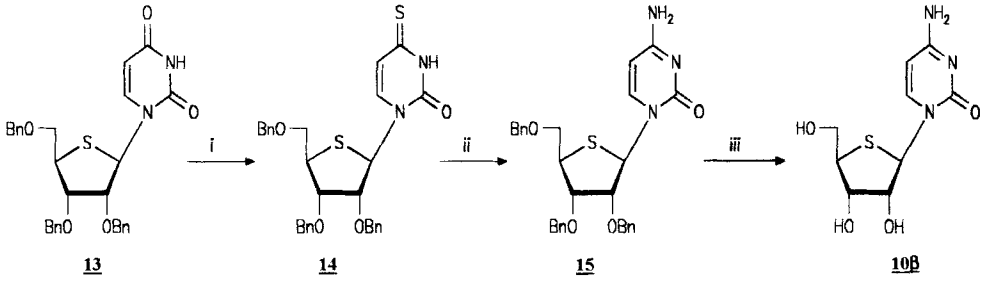


7 B = N4-benzoylcytidine 8α, 8β 9α, 9β

B = Adenine 11α, 11β 12α, 12β

Figure 2. (i = BSA, TMSTf, CH_3CN; ii = BBr_3, CH_2Cl_2)

(for Adenine : i = TMSTf, CH_3CN, MS 4Å)



13 14 15 10β

Figure 3 : i = Lawesson reagent, THF, ii = MeOH, NH_3, iii = BBr_3, CH_2Cl_2

our anomeric determinations. For example, the chemical shift of $H_{1'}$ of the protected α-anomers is markedly downfield from the signal of the corresponding $H_{1'}$ proton of the protected ß-anomers. These results contradict the conclusions of recent studies already mentionned [6, 24].

Enzymatic studies. 4'-thio-β-D-adenosine **12β** is known to be phosphorylated in cells to the 5'-monophosphate derivative but enzymatic studies of this compound using pure samples of adenosine kinase have been seldom performed. The phosphorylation of **12β** has already been studied in the presence of a crude preparation of rabbit liver kinase, but the results are unreliable because of the existence of other phosphorylating agents in the enzymatic sample [25]. Having purified and extensively studied bovine liver adenosine kinase [26], we have determined the substrate properties of **12α** and **12β** and the corresponding kinetic parameters with respect to this enzyme. Bovine liver adenosine kinase displays the properties of adenosine kinases from other sources including substrate inhibition by excess of adenosine under appropriate conditions [27, 28]. Adenosine

concentrations higher than 0.5μM in the presence of a large excess of ATP and Mg^{2+} induced substrate inhibition of bovine liver adenosine kinase, and the same phenomenom was observed for adenosine analogues [26]. It was therefore important to determine the concentration range in which a substrate of the enzyme might exhibit inhibiting properties. Under standard conditions defined as 1mM ATP, 0.5mM $MgCl_2$ at 37° C and pH 7.5, steady state kinetic data obtained with the β-anomer of 4'-thio-adenosine **12β** apparently agree with the Michaelis-Menten equation, and no inhibition by excess of substrate was observed up to a 170μM concentration. This allowed us to use a spectroscopic method based on a coupled enzyme reaction [29] to study the substrate properties of **12β**. A double reciprocal plot of enzyme activity against 4'-thio-β-adenosine concentration yielded a Km value of 22μM when adenosine was the substrate [26]. The maximum reaction rate of **12β** was about one third of the rate with adenosine, and the ratio Vmax/Km is only 0.2% of the corresponding value for adenosine. Compared to adenosine, 4'-thio-β-D-adenosine is therefore a weak substrate for bovine liver adenosine kinase. The comparison of kinetic parameters suggests that is mainly the consequence of a reduced affinity of **12β** for the enzyme.

In contrast to the β-anomer, 4'-thio-α-adenosine, **12α**, did not show any substrate activity even in the presence of large concentrations of enzyme. Previous studies of adenosine kinases from other sources suggest that the presence and a *trans* stereochemistry of the 2'-hydroxyl group in relation to the adenine nucleus may be critical for substrate activity [30, 31]. The unability of bovine liver adenosine kinase to catalyse the phosphorylation of **12α** might thus be explained by the relative stereo orientation of the 2'-OH and adenine moities.

Another important enzyme in the metabolism of adenine nucleosides is adenosine deaminase which catalyses the deamination of adenosine to inosine and 4'-thioadenosine to 4'-thioinosine [32, 33] and may thus deactivate adenosine analogues with potential biological activity [34, 35]. We examinated the substrate properties of **12α** and **12β** with respect to calf intestinal adenosine deaminase. Compared to an early study of **12β** conducted with a highly deactivated sample of the same enzyme [36], we found a lowered affinity for the enzyme (Km = 74μM) and a smaller catalytic efficiency (Vmax/Km = 60% compared to adenosine). The α-anomer **12α** is not a substrate for calf intestinal adenosine deaminase.

In conclusion, we present an improved synthesis of 4'-thio-D-ribofuranose derivative from D-ribose and some 4'-thio-D-ribonucleosides. The structure of these 4'-

thio-D-ribonucleosides was unambigously determined. Enzymatic studies show that β-4'-thio-adenosine is a weak substrate of bovine liver adenosine kinase but that is does not display substrate inhibition properties up to a 170 μM concentration. In contrast, 4'-thio-ß-D-adenosine is easily deaminated in the presence of adenosine deaminase suggesting that deamination may be an effective metabolic pathway compared to phosphorylation.

EXPERIMENTAL SECTION.

GENERAL METHODS.

^{1}H NMR and ^{13}C NMR spectra were determined with a BRUCKER AM 300 MHz or a BRUKER AC 250 MHz with tetramethylsilane as internal standard and chemical shifts are quoted in ppm (s = singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet, br = broad signal, td = double triplet, q = quadruplet). UltraViolet spectra were recorded on a UVIKON 810 ((KONTRON) spectrophotometer. Electron mass spectra (70 eV) were recorded on a JEOL JMS DX 300 mass spectrometer. Elemental analyses were carried out at the Service de Microanalyses du CNRS (Vernaison, France). Optical rotation were performed with a Perkin Elmer 241 polarimeter. Precoated MERCK Silica gel F_{254} plates were used for TLC. Column Chromatography was perfomed on MERCK silica gel (0.040-0.063 mm). HPLC analysis were performed with a Waters Associates apparatus (510 pumps, U6K injector, 680 solvent programmer).

Chemical reagents and enzymes used in assays [pyruvate kinase (PK), lactate dehydrogenase (LD) and calf intestinal mucosa adenosine deaminase type VIII] were from Sigma. 8-[^{14}C]adenosine (49 mCi / mmole) was purchased from New England Nuclear. Purified samples of bovine liver adenosine kinase (specific activity : 3 units per mg of protein) [25] were used in the kinetic experiments. One unit of adenosine kinase is defined as the amount of enzyme which catalyzes the phosphorylation of 1 μmole of adenosine per min at 37 °C.

2,3,5-tri-O-benzyl-D-ribose dithiobenzylacetal **3**.

The methyl-2,3,5-tri-O-benzyl-D-ribofuranoside **2**, [12] (4.34 g, 1.00 mmol) was stirred at 0° C with benzyl mercaptan (4.8 ml, 4.50 mmol) and boron trifluoride etherate (0.22 ml, 0.2 mmol). After 48 h of continuous stirring, the reaction mixture was neutralized with a 5 % aqueous $NaHCO_3$ solution, diluted with methylene chloride, and the organic compound extracted. The organic layer was dried over sodium sulfate and concentrated. The crude product was applied on a silica gel column chromatography and eluted with CH_2Cl_2-

MeOH, 99 : 1. The appropriate fractions were combined and evaporated to give **3** (487 mg, 75 %). Rf 0.46 (CH_2Cl_2-MeOH, 99 : 1) ; MS FAB>0 NBA m/z 651 $[M+H]^+$; ^1H NMR ($CDCl_3$) δ 3.61 (m, 3H, H_4, H_5, $H_{5'}$), 3.70 (m, 6H, -O-C<u>H</u>$_2$-Ph), 4.05 (m, 3H, H_1, H_2, H_3), 4.42-4.93 (m, 4H, -S-C<u>H</u>$_2$-Ph), 5.19 (m, 1H, OH), 7.11-7.72 (m, 25H, -O-CH_2-<u>Ph</u>). Anal. ($C_{40}H_{42}O_4S_2$) C 73.92, H 6.41, found C 73.84, H 6.46.

2,3,5-tri-<u>O</u>-benzyl-4-<u>O</u>-*p*nitrobenzoyl-<u>L</u>-lyxose dithiobenzylacetal **4**.

To a solution containing **3** (1.159 g, 1.78 mmol), p-nitrobenzoic acid (446 mg, 2.67 mmol) and triphenylphosphine (702 mg, 2.67 mmol) in dry THF (15 ml) diethylazodicarboxylate (DEAD) (393 mg, 2.67 mmol) was added under an argon atmosphere. After stirring the reactants at room temperature for 3 h, the solvent was evaporated under reduced pressure and the resultant mixture was purified by chromatography on silica gel (Dichloromethane-Hexane, 1 : 1) to give **4** (1.13 g, 80 %). MS FAB>0 NBA m/z 800 $[M+H]^+$, 676 $[M+H-Ph-CH_2-SH]^+$; ^1H NMR ($CDCl_3$) δ 3.64-3.70 (m, 2H, H_5, $H_{5'}$), 3.75 (s, 2H, -S-C<u>H</u>$_2$-Ph), 3.77 (s, 2H, -S-C<u>H</u>$_2$Ph), 4.28-3.92 (m, 5H, H_1, H_2, H_3,-O-C<u>H</u>$_2$-Ph), 4.45-5.01, (m, 4H, -O-C<u>H</u>$_2$-Ph), 5.64 (td, 1H, H_4, $J_{4,5}$ =5.7, $J_{4,5'}$ =5.7, $J_{4,3}$ = 2.4), 7.16-7.37 (m, 25H,-O-CH_2<u>Ph</u>), 8.06-8.22 (m, 4H, *p*-NO_2-<u>Ph</u>-CO). Anal. ($C_{47}H_{45}O_7S_2N$) C 70.67, H 5.68, found C 70.58, H 5.63.

2,3,5-tri-<u>O</u>-benzyl-<u>L</u>-lyxose dithiobenzylacetal **5**.

To a solution of **4** (1.02 g,1.27 mmol.) in dichloromethane (7 ml) and methanol (3 ml) was added potassium carbonate (25 mg, 0.19 mmol) at room temperature. The reaction mixture was stirred for 1 h, then neutralized to pH 7 with hydrochloric acid 2N, diluted with methylene chloride (100 ml), and washed with water (50 ml). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by chromatography on silica gel (elution with CH_2Cl_2) to give pure **5** (750 mg, 91 %). MS FAB>0 GT m/z 651 $[M+H]^+$, 633 $[M+H-H_2O]^+$, 527 $[M+H-Ph-CH_2-SH]^+$; ^1H NMR ($CDCl_3$) δ 3.61 (m, 3H, H_4, H_5, $H_{5'}$), 3.82 (m, 4H, -S-C<u>H</u>$_2$-Ph), 4.06-4.35 (m, 3H, H_1, H_2, H_3), 4.43-4.91 (m, 6H, -O-C<u>H</u>$_2$-Ph), 4.82 (br, 1H, OH), 7.30, (m, 25H, -O-CH_2-<u>Ph</u>, -S-CH_2-<u>Ph</u>).

Benzyl-2,3,5-tri-<u>O</u>-benzyl-1,4-dithio-<u>D</u>-ribofuranoside **6**.

This compound was already described in reference 9. ^{13}C NMR ($CDCl_3$) δ 37.07 (s, 1C, C_5), 47.61 (s, 1C, C_4), 50.95 (s, 1C, C_1), 72.30 (m, 4C,-C<u>H</u>$_2$-Ph), 80.96 (s, 1C, C_3), 83.42 (s, 1C, C_2), 127.23-128.95 (m, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$ aromatic), 137.64-138.08 (q, $C_{1'}$ aromatic). Anal. ($C_{33}H_{34}O_3S_2$) C 72.95, H 6.33, found C 73.06, H 6.27.

1-[2,3,5-tri-O-benzyl-4-thio-D-ribofuranosyl]-N_4-benzoyl-cytosine **8**.

A suspension of N_4-benzoylcytosine (880 mg, 4.09 mmol) in dry acetonitrile (17.5 ml) was refluxed with N,O-bis(trimethylsilyl)acetamide (BSA) (3.32 g, 16.35 mmol) until a clear solution was obtained. This mixture was then cooled at room temperature, and the acetyl-2,3,5-tri-O-benzyl-4-thio-D-ribofuranose **7**, [9] (1.95 g, 4.09 mmol) dissolved in dry acetonitrile (17.5 ml) was added followed by the addition of trimethylsilyl trifluoromethanesulfonate (TMSTf) (1.09 g, 4.90 mmol). The reaction mixture was stirred for 7 h before being quenched by the addition of aqueous sodium hydrogen carbonate, stirred for 30 min, diluted with dichloromethane (100 ml) and washed with aqueous sodium hydrogen carbonate. The organic layer was concentrated, dried over Na_2SO_4, and the crude products were purified by chromatography on silica gel (Hexane-CH_2Cl_2, 1 : 2). The ß-anomer was eluted first (77 mg, 30%) followed by the α-anomer (77 mg, 30%). **8α** : MS FAB>0 NBA m/z 634 $[M+H]^+$, 526 $[M+H\text{-}PhCH_2OH]^+$, 91 $[Ph\text{-}CH_2]^+$; ^1H NMR ($CDCl_3$) δ 3.47 (dd, 1H, $H_{5''}$, $J_{5'',5'} = 9,6$, $J_{5'',4'} = 6.4$), 3.66 (dd, 1H, $H_{5'}$, $J_{5',5''} = 9.6$, $J_{5',4'} = 4.5$), 3.96 (dd, 1H, $H_{3'}$, $J_{3',4'} = 6.9$, $J_{3',2'} = 3.1$), 4.01 (m, 1H, $H_{4'}$), 4.39 (dd, 1H, $H_{2'}$, $J_{2',1'} = 5.21$, $J_{2',3'} = 3.1$), 4.52 (m, 6H, -O-CH_2-Ph), 6.51 (d, 1H, $H_{1'}$, $J_{1',2'} = 5.2$), 7.11-7.36 (m, 16H, -O-CH_2-Ph, -O-CO-Ph), 7.51 (d, 1H, H_5, $J_{5,6} = 7.5$), 7.55 (m, 2H,-O-CO-Ph), 7.90 (m, 2H, -O-CO-Ph), 8.52 (d, 1H, H_6, $J_{6,5} = 7.5$), 8.91 (s, 1H, -NH). **8β** : MS FAB>0 NBA m/z 634 $[M+H]^+$, 526 $[M+H\text{- }Ph\text{-}CH_2OH]^+$, 91 $[Ph\text{-}CH_2]^+$; ^1H NMR ($CDCl_3$) δ 3.71-3.85 (m, 4H, $H_{5''}$, $H_{5'}$, $H_{4'}$, $H_{2'}$), 4.03 (d, 2H, -O-CH_2-Ph), 4.23 (d, 1H, $H_{3'}$), 4.50 (m, 2H, -O-CH_2-Ph), 4.90 (m, 2H, -O-CH_2-Ph), 6.13 (d, 1H, $H_{1'}$, $J_{1',2'} = 1.6$), 7.06-7.61 (m, 19H, -O-CH_2-Ph, -O-CO-Ph, H_5), 7.87 (m, 2H, -O-CO-Ph), 8.64 (s, 1H, -NH), 8.77 (d, 1H, H_6, $J_{6,5} = 7.5$).

1-(4-thio-α-and-ß-D-ribofuranosyl)-N_4-benzoyl-cytosine **9α** and **9β**.

To a solution of compound **8α** or **8β** (630 mg, 0.99 mmol) in dry dichloromethane (7 ml) cooled to -78°C, boron tribromide (1M) in dichloromethane (4.15 ml, 4.15 mmol) was added. After 15 min stirring at -78°C, the reaction mixture was quenched with methanol (6 ml) then neutralized with aqueous sodium hydrogen bicarbonate solution. The solvents were removed under vacuo and the residue was chromatographed on silica gel with Dichloromethane-Methanol, 80 : 20 to give **9α** or **9β** (252 mg, 69 %). **9α** : MS FAB>0 GT m/z 364 $[M+H]^+$; **9β** : MS FAB>0 GT m/z 364 $[M+H]^+$; ^1H NMR ($DMSOd_6$) δ 3.27 (m, 1H, $H_{4'}$), 3.61 (dd, 1H, $H_{5''}$; $J_{5'',4'} = 5.3$, $J_{5'',5'} = 11.4$), 3.73 (dd, 1H, $H_{5'}$, $J_{5',4'} = 5.9$, $J_{5',5''} = 11.3$), 4.04 (m, 1H, $H_{3'}$, $J_{3',2'} = 3.5$, $J_{3',4'} = 3.4$), 4.20 (m, 1H, $H_{2'}$, $J_{2',1'} = 6.2$, $J_{2',3'} = 3.4$), 5.23 (t, 1H, $OH_{5'}$, $J_{OH,5'} = 5.2$, $J_{OH,5''} = 5.2$), 5.28 (d, 1H, $OH_{3'}$, $J_{OH,3'} = 4.8$), 5.57 (d, 1H, $OH_{2'}$, $J_{OH,2'} = 5.8$), 5.96 (d, 1H, $H_{1'}$, $J_{1',2'} = 6.2$), 7.50 (m, 3H, -O-CO-Ph), 7.61 (d, 1H, H_5, $J_{5,6} = 6.9$), 7.99 (d, 2H, -O-CO-Ph), 8.57 (d, 1H, H_6, $J_{6,5} = 7.4$), 11.28 (s, 1H, -NH).

1-(4-thio-α-and-ß-D-ribofuranosyl)-cytosine **10α** and **10β**.

Compound **9α** or **9β** (90 mg, 0.24 mmol) was stirred at room temperature with methanolic ammonia (8 ml) for 24 h. The mixture was then concentrated under vacuo and the residue was chromatographed on a silica gel column (Dichloromethane-Methanol, 70 : 30) to give **10α** or **10β** (45 mg, 72 %).

Other methodology. To a solution of compound **15** (529 mg, 1.00 mmol) in dry dichloromethane (7 ml) cooled to -78°C, boron tribromide (1M) in dichloromethane (4.15 ml, 4.15 mmol) was added. After 15 min stirring at -78°C, the reaction mixture was quenched with methanol (6 ml) then neutralized with aqueous sodium hydrogen bicarbonate solution. The solvents were removed under vacuo and the residue was chromatographed on silica gel with Dichloromethane-Methanol, 80 : 20 to give **10β** (182 mg, 70 %). **10α** : MS FAB>0 NBA m/z 260 $[M+H]^+$; ^1H MNR ($DMSOd_6$) δ 3.43 (m, 1H, $H_{5''}$), 3.63 (m, 1H, $H_{4'}$, $J_{4',3'}$ = 3.7, $J_{4',5'} = J_{4',5''}$ = 8.0), 3.83 (m, 2H, $H_{3'}$, $H_{5'}$), 4.07 (m, 1H, $H_{2'}$), 4.97 (m, 1H, $OH_{5'}$), 5.28 (m, 1H, $OH_{3'}$), 5.60 (d, 1H, $OH_{2'}$, $J_{OH,2'}$ = 3.7), 5.73 (d, 1H, H_5, $J_{5,6}$ = 7.5), 6.23 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 4.6), 7.19 (d, 2H, NH_2), 8.00 (d, 1H, H_6, $J_{6,5}$ = 7.5). ^{13} C NMR (DMSO d6) δ 53.12 (s, 1C, $C_{4'}$), 58.80 (s, 1C, $C_{1'}$), 62.56 (s, 1C, $C_{5'}$), 73.29 (s, 1C, $C_{2'}$), 75.07 (s, 1C, $C_{3'}$), 92.59 (s, 1C, C_5), 144.86 (s, 1C, C_4), 155.75 (s, 1C, C_2), 165.15 (s, 1C, C_6). **10β** : $[\alpha]_D^{20}$ -4,1° (c 3.11 H_2O) [litt.[17] $[\alpha]_D^{25}$ -5.5° (c 3.0 H_2O)]. U.V. (EtOH 95) pH 7 λ_{max} 273 nm, ε 12735, λ_{min} 227 nm ; MS FAB>0 NBA m/z 260 $[M+H]^+$; ^1H MNR ($DMSOd_6$) δ 3.19 (m, 1H, $H_{4'}$), 3.51-3.69 (m, 2H, $H_{5'}$, $H_{5''}$), 3.99 (m, 1H, $H_{3'}$), 4.05 (m, 1H, $H_{2'}$), 5.19 (m, 2H, $OH_{3'}$, $OH_{5'}$), 5.35 (m, 1H, $OH_{2'}$), 5.77 (d, 1H, H_5, $J_{5,6}$ = 7.5), 5.95 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 6.6), 7.18 (d, 1H, $-NH_2$, J = 12.6), 7.96 (d, 1H, H_6, $J_{6,5}$ = 7.5). ^{13} C NMR (DMSO d_6) δ 45.59 (s, 1C, $C_{5'}$), 52.59 (s, 1C, $C_{4'}$), 63.18 (s, 1C, $C_{1'}$), 73.09 (s, 1C, $C_{3'}$), 76.83 (s, 1C, $C_{2'}$), 94.51 (s, 1C, C_5), 142.28 (s, 1C, C_6), 155.82 (s, 1C, C_2), 165.28 (s, 1C, C_4); Anal. ($C_9H_{13}O_4N_3S$) C 41.65, H 4.97, N 16.22, found : C 41.69, H 5.01, N 16.21.

9-(2,3,5-tri-O-benzyl-4-thio-α-and-ß-D-ribofuranosyl)-adenine **11α** and **11β**.

Compound **7** (489 mg, 1.02 mmol) was dissolved in acetonitrile (7 ml) under argon. To this solution was added adenine (261 mg, 1.92 mmol), 4Å molecular sieve (897 mg) and trimethylsilyltrifluoromethanesulfonate (TMSTf) (0.69 ml, 3.84 mmol). The solution was stirred for 48 hr at room temperature. The reaction was quenched by the addition of aqueous sodium hydrogen carbonate, stirred for 30 min, diluted with dichloromethane, washed with aqueous sodium hydrogen carbonate, dried, and concentrated to give an anomeric mixture of the protected nucleoside, which was further purified by chromatography over silica gel (Ether-Methanol, 95 : 5). Appropriate fractions were combined and evaporated to give the α-anomer **11α** (305 mg, 54%) and the β-anomer

11β (85 mg, 15%). **11α** : $[\alpha]_D^{20}$ +55.5° (c 1.098 $CHCl_3$) ; U.V. (EtOH 95) pH 7, λ_{max} 260 nm, λ_{min} 232 nm ; MS FAB>0 GT m/z 554 $[M+H]^+$, 136 $[BH]^+$, 91 $[Ph\text{-}CH_2]^+$; ^1H NMR ($CDCl_3$) δ 3.52, (dd, 1H, $H_{5''}$, $J_{5'',5'}$ = 10.0, $J_{5'',4'}$ = 5.9), 3.62 (dd, 1H, $H_{5'}$, $J_{5',5''}$ = 10.0, $J_{5',4'}$ = 5.5), 4.06 (m, 1H, $H_{4'}$), 4.13 (dd, 1H, $H_{3'}$, $J_{3',2'}$ = 3.3, $J_{3',4'}$ = 5.5), 4.32 (dd, 1H, $H_{2'}$, $J_{2',3'}$ = 3.3, $J_{2',1'}$ = 5.5), 4.38-4.66 (m, 6H, -O-C$\underline{H}_2$-Ph), 5.92 (s, 2H, -N$\underline{H}_2$), 6.34 (s, 1H, $H_{1'}$, $J_{1',2'}$ = 5.5), 6.93-7.38 (m, 15H, -O-CH_2-$\underline{Ph}$), 8.30 (s, 1H, H_2), 8.50 (s, 1H, H_8). **11β** : $[\alpha]_D^{20}$ +9,7° (c 1.135 $CHCl_3$) ; U.V. (EtOH 95) pH7, λ_{max} 260 nm, λ_{min} 232 nm ; MS FAB>0 GT, m/z 554 $[M+H]^+$, 136 $[BH]^+$, 91 $[Ph\text{-}CH_2]^+$; ^1H NMR ($CDCl_3$) δ 3.78 (m, 2H, $H_{5''}$, $H_{5'}$), 3.85 (m, 1H, $H_{4'}$), 4.11 (dd, 1H, $H_{3'}$, $J_{3',2'}$ = 3.7, $J_{3',4'}$ = 6.1), 4.36 (m, 2H, -O-C$\underline{H}_2$-Ph), 4.42 (pt, 1H, $H_{2'}$, $J_{2',3'}$ = 3.6, $J_{2',1'}$ = 3.9), 4.58 (s, 2H, -O-C$\underline{H}_2$-Ph), 4.68 (d, 2H, -O-C$\underline{H}_2$-Ph), 5.93 (s, 2H, -N$\underline{H}_2$), 6.10 (s, 1H, $H_{1'}$, $J_{1',2'}$ = 4.2), 7.16-7.40 (m, 15H, -O-CH_2-$\underline{Ph}$), 8.23 (s, 1H, H_2), 8.34 (s, 1H, H_8).

1-(4-thio-α- and -ß-D-ribofuranosyl)-adenine **12α** and **12β**.

Compounds **11α** (261 mg, 0.47 mmol) and **11β** (100 mg, 0.18 mmol) were independently deprotected using the same procedure as described for **9β** and gave respectively compounds **12α** (93.5 mg, 70 %) and **12β** (36 mg, 70%). **12α** : $[\alpha]_D^{20}$ +20.4° (c 0.88 H_2O-Pyridine, 1 : 1); U.V. (H_2O) pH 7 λ_{max} = 259 nm, λ_{min} = 230 nm ; mp = 248-249°C (H_2O-Methanol, 1 : 1) ; MS FAB>0 NBA m/z 284 $[M+H]^+$; ^1H NMR ($DMSOd_6$) δ 3.47 (m, 1H, $H_{5''}$, $J_{5'',5'}$ = 9.1, $J_{5'',4'}$ = 6.4), 3.75 (m, 2H, $H_{4'}$, $H_{5'}$), 4.09 (dd, 1H, $H_{3'}$, $J_{3',4'}$ = 5.3, $J_{3',2'}$ = 3.7), 4.25 (dd, 1H, $H_{2'}$, $J_{2',1'}$ = 4.9, $J_{2',3'}$ = 3.8), 5.05 (t, 1H, $OH_{5'}$, $J_{OH,5'}$ = 5.2, $J_{OH,5''}$ = 5.2), 5.50 (d, 1H, $OH_{3'}$, $J_{OH,3'}$ = 6.0), 5.68 (d, 1H, $OH_{2'}$, $J_{OH,2'}$ = 5.5), 6.19 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 5.0), 7.27 (s, 2H, -N$\underline{H}_2$), 8.14 (s, 1H, H_2), 8.40 (s, 1H, H_8); ^{13} C NMR (DMSO d6) δ 53.60 (s, 1C, $C_{4'}$), 57,00 (s, 1C, $C_{1'}$), 63.40 (s, 1C, $C_{5'}$), 73,85 (s, 1C, $C_{2'}$), 74,87 (s, 1C, $C_{3'}$), 117.93 (s, 1C, C_5), 141.50 (s, 1C, C_8), 149.57 (s, 1C, C_4), 152.08 (s, 1C, C_2), 155.65 (s, 1C, C_6); Anal. ($C_{10}H_{13}O_3SN_5$) C 41.48, H 4.63, N 24.76, require C 42.40, H 4.59, N 24.73. **12β** : $[\alpha]_D^{20}$ -36.3° (c 0.91 H_2O-Pyridine, 1 : 1) ; mp = 246-247°C (H_2O-Methanol, 1 : 1) [litt. [19] $[\alpha]_D^{20}$ -41° (c 0.54 H_2O- Pyridine, 1 : 1) ; mp = 246-248°C] ; U.V. (H_2O) pH 7 λ_{max} 259 nm, ε 10230, λ_{min} 230 nm ; MS FAB>0 NBA m/z 284 $[M+H]^+$; ^1H NMR ($DMSOd_6$) δ 3.43 (m, 1H, $H_{4'}$), 3.62 (dd, 1H, $H_{5''}$, $J_{5'',5'}$ = 11.4, $J_{5'',4'}$ = 5.8), 3.78 (dd, 1H, $H_{5'}$, $J_{5',5''}$ = 11.4, $J_{5',4'}$ = 5.4), 4.17 (dd, 1H, $H_{3'}$, $J_{3',4'}$ = 3.4, $J_{3',2'}$ = 3.4), 4.63 (dd, 1H, $H_{2'}$, $J_{2',1'}$ = 6.4, $J_{2',3'}$ = 3.4), 5.23 (t, 1H, $OH_{5'}$, $J_{OH,5'}$ = 5.5, $J_{OH,5''}$ = 5.5), 5.38 (d, 1H, $OH_{3'}$, $J_{OH,3'}$ = 6.0), 5.59 (d, 1H, $OH_{2'}$, $J_{OH,2'}$ = 5.5), 5.86 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 6.7), 7.31 (s, 2H, -N$\underline{H}_2$), 8.15 (s, 1H, H_2),

8.46 (s, 1H, H_8); ^{13}C NMR (DMSO d6) δ 53.05 (s, 1C, $C_{4'}$), 61.07 (s, 1C, $C_{1'}$), 63.02 (s, 1C, $C_{5'}$), 73.09 (s, 1C, $C_{3'}$), 76.79 (s, 1C, $C_{2'}$), 118.84 (s, 1C, C_5), 139.63 (s, 1C, C_8), 149.47 (s, 1C, C_4), 152.19 (s, 1C, C_2), 155.76 (s, 1C, C_6); Anal. ($C_{10}H_{13}O_3SN_5$) C 42.55, H 4.46, N 24.79; found : C 42.40, H 4.59, N 24.73.

1-(2,3,5-tri-O-benzyl-4-thio-ß-D-ribofuranosyl)-4-thio-uracil **14**.

To a solution of 2',3',5'-tri-O-benzyl-4'-thio-ß-uridine, **13** (2.80 g, 5.28 mmol) in dry 1,2-dichloroethane (120 ml), Lawesson's reagent (1.07 g, 2.64 mmol) was added. The mixture was refluxed under nitrogen for 4 hrs. The mixture was concentrated and the crude product was purified by column chromatography on silica gel to give coumpound **14** (2.50 g, 87 %). **14** : MS FAB>0 NBA m/z 547 $[M+H]^+$, 419 $[M-BH]^+$, 91 $[PhCH_2]^+$; ^{1}H MNR ($CDCl_3$) δ 3.76-3.86 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$), 4.02-4.29 (m, 3H, $H_{2'}$, -O-CH_2-Ph), 4.47 (q, 2H, -O-CH_2-Ph), 4.90 (q, 2H, -O-CH_2-Ph), 5.09 (d, 1H, H_5, $J_{5,6}$ = 7.4), 6.10 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 1.5), 7.15-7.50 (m, 16H, -O-CH$_2$-Ph, -NH), 8.36 (d, 1H, H_6, $J_{6,5}$ = 7,5).

1-(2,3,5-tri-O-benzyl-4-thio-ß-D-ribofuranosyl)-cytosine **15**.

To a solution of compound **14** in dry dichloromethane (6 ml), was added methanolic ammonia (28 ml), and the reaction mixture was warmed at 100°C for 4 h before being allowed to reach room temperature and then concentrated. The crude compound was purified by column chromatography on silica gel (CH_2Cl_2-MeOH, 99 : 1) to give **15** (2.17g, 90%).**15** : MS FAB>0 NBA m/z 530 $[M+H]^+$, 419 $[M-B]^+$, 112 $[B+H]^+$, 91 $[CH_2-Ph]^+$; ^{1}H NMR ($CDCl_3$) δ 3.68 (dd, 1H, $H_{5''}$, $J_{5'',4'}$ = 2.6), 3.76 (m, 1H, $H_{4'}$), 3.83 (dd,1H, $H_{5'}$, $J_{5',5''}$ = 9.8, $J_{5',4'}$ = 2.3), 3.88 (dd, 1H, $H_{3'}$, $J_{3',4'}$ = 6.9, $J_{3',2'}$ =3.5), 3.97 (t, 1H, $H_{2'}$, $J_{2',3'}$ = 3.4, $J_{2',1'}$ = 3.4), 4.40 (m, 4H, -O-CH_2-Ph), 4.70 (q, 2H, -O-CH_2-Ph), 5.80 (d, 1H, H_5, $J_{5,6}$ = 7.6), 6.00 (d, 1H, $H_{1'}$, $J_{1',2'}$ = 3.3), 7.23-7.41 (m, 15H, -O-CH_2-Ph), 9.62 (s, 2H, -NH_2).

Adenosine kinase assays and kinetic studies:

i) Spectrometric method :

The enzymatic activity was determined at 37° C in 100 mM Tris HCl buffer pH 7.4 containing 100 mM KCl, 2.4 mg/ml bovine serum albumin (BSA), 0.5 mM $MgCl_2$, 1 mM ATP, 0.25 mM phosphoenol pyruvate, 0.1 mM NADH, 30 µM 6-methylmercaptopurine riboside (6-MMPR), 5 units of PK, 13.8 units of LD and concentrations of adenosine kinase in the range of 100 to 300 nM depending on the measurement, in a final volume of 1 ml. A linear variation of optical density at 340 nm was obtained for at least 1 min. In kinetic studies, 6-MMPR was replaced by **12α** or **12β** in increasing concentrations up to 170 µM.

ii) Activity determination with labelled adenosine :

The measurements were carried out at 37° C in 50 mM Tris HCl buffer pH 7.4 containing 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM ATP, 1.2 mg/ml BSA, 1 mM DTT, 8-[^{14}C]adenosine (0.002 µCi), 0.5 µM adenosine and enzyme (final concentration : 0.3 nM) in a final volume of 1 ml. After 6 min, the reaction was stopped by pouring the reaction mixture to a Watman DE 81 filter. The filter was washed successively by 2 mM ammonium formate pH 7 (3 x 4 ml), water (2 x 4 ml) and ethanol (2 x 4 ml). After drying, the filter was immersed in scintillation fluid and the radioactivity counted in a Packard TriCarb 460 CD scintillation spectrometer. Under the conditions used, reactions velocities remained constant for at least 10 min. All measurements were made in duplicate.

Kinetics studies with 4'-thio-ß-D-adenosine, **12β**, using the coupled enzyme method yielded double reciprocal plots of initial velocity versus substrate concentration linear in the 0-170 µM range of concentrations. With the second method, using labelled adenosine, the double reciprocal plot was linear only between 0 and 0.5 µM with adenosine as substrate. Kinetic data were fitted to the simple Michaelis-Menten equation by a non-linear regression procedure. In each case, an HPLC analysis was performed to verify the formation of the monophosphate derivative of the substrate. Reaction conditions were the same as in the assay (spectrometric method). After one hour, the reaction was stopped by heating and the mixture was analyzed on a Radial-Pack Waters C-18 column : 10 cm, diam. 8 mm, granularity 5 µm, with an isocratic elution for 5 min with buffer A (phosphate pH 6, 200mM), a 5 min gradient from A to B (80% A + 20% methanol), then an isocratic elution with B. Under these conditions, the chromatogram corresponding to the reaction mixture from **12β** showed the presence of ATP, ADP and the 5'-monophosphate derivative of **12β** at retention time : 12.3 min (λ_{max} = 260 nm). With **12α**, there was no change in the chromatogram.

Adenosine deaminase assay and kinetic studies [37]:

The enzymatic activity was determined at 37° C in buffer phosphate 50 mM pH 7.5, with substrate concentrations in the range of 10 to 70 µM. The reaction was started by introduction of the enzyme to a total quantity of 2.10^{-2} unit/ml. The reaction mixture (1 ml) was rapidly stirred and the variation of optical density was recorded at 265 nm. The rate variation was constant for at least 30 sec and measurements were made in duplicate. Kinetic parameters were obtained from the Michaelis-Menten treatment as in the case of adenosine kinase. The product analysis by HPLC was performed on the reaction mixture obtained under the same conditions of the adenosine kinase assay except that a larger concentration of the enzyme was used (0.12 unit / ml). HPLC analysis of the reaction mixture from **12β**, with the same column and gradient program as in the same case of

adenosine kinase, showed the appearance of a new compound (ß-D-4'-thioinosine) at retention time 11.9 min (λmax = 248 nm); inosine was eluted at retention time 11.1 min (λmax = 247 nm). With **12α**, no change occurred.

ACKNOWLEDGEMENT:

We gratefully acknowledge the MRT (Ministère de la Recherche et de la Technologie) and the ANRS (Agence Nationale de Recherche sur le Sida) for financial support of this work, and one of us (C.L.) thanks also the MRT for the award of a research studentship.

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Received October 21, 1993
Accepted June 27, 1994