Synthesis of [1′,2′,5′,2-$^{13}C_4$]-2′-Deoxy-D-adenosine by a Chemoenzymatic Strategy to Enable Labelling of Any of the 2^{15} Carbon-13 and Nitrogen-15 Isotopomers

Niels Ouwerkerk,[a] Jacques van Boom,[a] Johan Lugtenburg,[a] and Jan Raap*[a]

Keywords: Biocatalysis / Isotopic labelling / Nucleosides

Enzymatic *trans-N*-glycosylation has been selected as the method of choice by which to couple [$^{13}C_1$]-adenine to [$^{13}C_3$]-2-deoxy-D-ribose. The enzymatic pentosylation of the labelled adenine base was achieved in a two-step/one-pot reaction, starting from thymidine labelled in the sugar ring, but not at the thymine base. The efficiency of this thymidine phosphorylase catalysed (TP-catalysed) and purine nucleoside phosphorylase catalysed (PNP-catalysed) transamination reaction was demonstrated by a high yield (91%) and stereochemical purity of the obtained [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine. To verify that all carbon and nitrogen positions and combination of positions in both the adenine and the sugar could be substituted by ^{13}C and ^{15}N at a minimum of cost, each of the steps was optimised to convert the commercially available and isotopically highly enriched (99%) synthons (acetaldehyde, acetic acid, ammonia, benzylamine, formic acid, methylamine, potassium cyanide, potassium thiocyanate and sodium nitrite) as quantitatively as possible.

Introduction

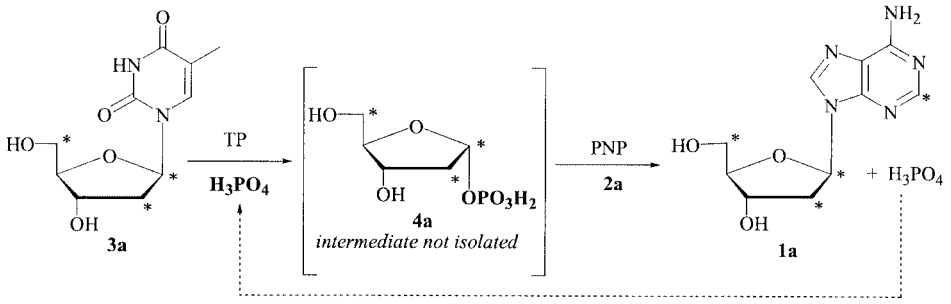
As part of a program to investigate the high-resolution structures of DNA/protein complexes by solid-state NMR spectroscopy we aim to develop synthetic routes for the preparation of all ^{13}C- and ^{15}N-isotopomers of the four monodeoxynucleosides. Previously, we described a synthetic route for the preparation of isotopically substituted pyrimidine 2′-deoxy-D-nucleosides, based on a chemical coupling of the sugar to thymine through 2-deoxy-3,5-di-*O*-*p*-tolyl-α-D-*erythro*-pentafuranosyl chloride.[1,2] In line with our continuous effort to develop regio- and stereocontrolled methods without the need for protection and deprotection steps, the procedure was improved by an one-pot, two-step enzymatically catalysed coupling between α-2-deoxy-D-ribose-1-phosphate and thymine.[3] Deoxyribose-1-phosphate was obtained, in situ, from 2-deoxy-D-ribose-5-phosphate by means of a migration of the phosphate group from the C5- to the C1-position of the sugar ring. This method, which allows substitution of all carbon and nitrogen positions of *pyrimidine* 2′-deoxynucleosides by stable isotopes, shortened the previous classical scheme by six steps. We have now extended this work to enable the synthesis of all 2^{15} different isotopomers of the *purine* nucleoside 2′-deoxyadenosine. However, it was found that the overall equilibrium of the two combined thermodynamically controlled reactions − the generation of the phosphate leaving group at the anomeric centre of the sugar ring and its coupling with the sparingly soluble adenine − was biased towards the reactants. In this paper we describe a procedure based on the combination of two methods: 1) to generate the 1-phosphate **4** by enzymatic phosphorylation of thymidine (Scheme 1)[4] and 2) to synthesise all labelled isotopomers of the sugar ring by an enzymatic aldol condensation of acetaldehyde and 2-deoxyribose-5-phosphate.[1] In addition, we describe here a synthetic route to all isotopomers of adenine. In spite of the fact that literature methods to incorporate isotopes both in five- and in six-membered rings are known, schemes that allow synthesis of all the 2^{10} isotopomers are still lacking.[5a,5b] A new reagent for the efficient incorporation of ^{13}C at position 8 has been developed. To allow specific labelling, asymmetric C- and N-synthons were used. All steps were optimised to incorporate the ^{13}C- and ^{15}N-enriched starting materials (acetic acid, potassium cyanide, formic acid, thiocyanate, benzylamine, sodium nitrite and ammonia) as quantitatively as possible. The applied procedure was proved to be successful by the synthesis of [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine (**1a**), starting from the triply ^{13}C-labelled thymidine **3a** and the monolabelled adenine **2a**. The structural solid-state NMR analysis of **1a** incorporated in macromolecular DNA/protein complexes will be published elsewhere.

Synthesis

The approach to the enzymatic coupling of a nucleobase **2a** with the 2-deoxy-D-ribose sugar to produce the purine

[a] Gorlaeus Laboratories, Leiden Institute of Chemistry, Leiden University
P. O. Box 9502, 2300 RA Leiden, The Netherlands

  1434−193X/02/0714−2356 $ 20.00+.50/0 *Eur. J. Org. Chem.* **2002**, 2356−2362

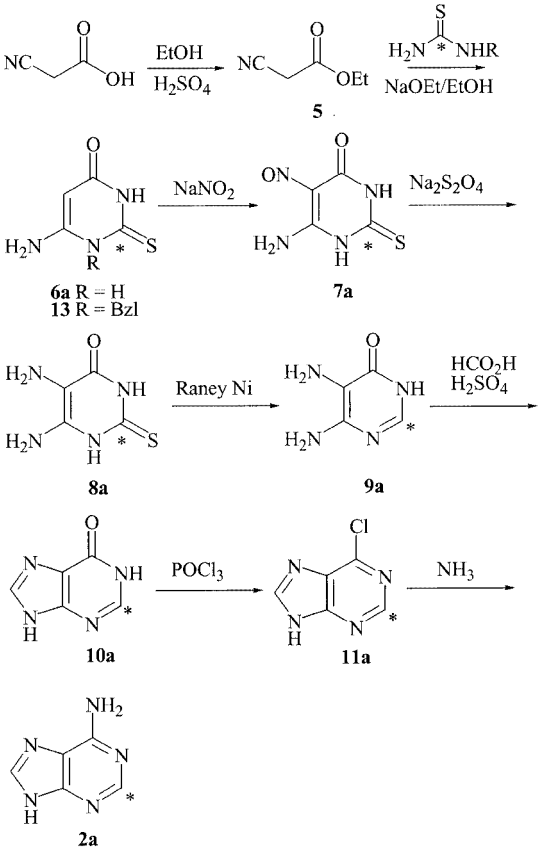


Scheme 1. The scheme shows the use of two different catalysts, thymidine phosphorylase (TP) to generate **4a** from **3a** and phosphate, followed by the action of purine nucleoside phosphorylase (PNP) in the presence of [2-^{13}C]-adenine (**2a**) on **4a**; these two reactions were carried out in a one-pot fashion and afforded very high yields (91%) of the mononucleoside **1a**

nucleoside **1a** involves the labile intermediate 2-deoxy-α-1-D-ribose-phosphate **4a** (Scheme 1). The nucleoside is produced when a nucleobase attacks at the anomeric position of **4a**, thereby displacing the phosphate. This step is catalysed by thymidine phosphorylase (TP) when thymine or uracil is used, and by purine nucleoside phosphorylase (PNP) in case of a purine nucleobase coupling. Compound **4a** is generated from [1′,2′,5′-$^{13}C_3$]-thymidine (**3a**), which is an attractive starting material, since its 2′-deoxyfuranoside ring is labelled efficiently.[1] The addition of a catalytic amount of phosphate and TP ensure the formation of **4a**. In this reaction the phosphate displaces the thymine and the α-anomeric phosphate ester arises (see Scheme 1). The subsequent attack of the purine nucleobase [2-^{13}C]-adenine (**2a**), which is catalysed by PNP, affords the desired nucleoside **1a**, while the phosphate serves as a leaving group. This reaction is conveniently carried out in a one-pot fashion, which reduces the losses that would occur if the labile compound **4a** were isolated.

Thus, in a two-step, one-pot reaction, catalysed by the enzymes TP and PNP, the mono-^{13}C-enriched adenine **2a** was efficiently coupled with the triply labelled [1,2,5-$^{13}C_3$]-2′-deoxy-D-ribose. In this way, the quadruply labelled product [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine (**1a**) was obtained in 91% yield. Other advantages of this enzymatic transamination were perfect control over the stereochemistry at the anomeric centre and total regioselectivity with respect to the N9 atom of adenine, without the need for any protection or deprotection step.

Scheme 2 depicts the route for the synthesis of **2a**. One of the key steps in the synthesis of 6-amino-1,2-dihydro-2-thioxo-4(3*H*)-pyrimidinone (**6**) and its isotopomer **6a** is the base-induced condensation of ethyl cyanoacetate and thiourea (Scheme 2).[6] The high-yielding preparation of the ethyl ester was achieved through bromination of acetic acid, followed by substitution of the bromine atom under aqueous, basic conditions.[7] The obtained cyanoacetic acid was then esterified in dichloromethane and 3 equiv. of ethanol in the presence of a trace of sulfuric acid. The yield of ethyl cyanoacetate based on acetic acid was 92% (three steps).



Scheme 2. The synthesis of adenine starting from ethyl cyanoacetate, *N*-benzylthiourea (urea), sodium nitrite, formic acid and ammonia; the critical conversion of the diamine **9a** to hypoxanthine **10a** was catalysed by sulfuric acid and water; all remaining steps routinely allowed for a very high yield, the conversions of **10a** to **2a** affording an 80% overall yield

[^{13}C]-Thiourea was subsequently added to a suspension of deprotonated ethyl cyanoacetate in ethanol and the mixture was heated under reflux for 2 h. Workup afforded 86% of compound **6a**. The use of *N*-benzylthiourea (**12**), prepared from potassium thiocyanate and benzylamine in 70%

yield, allowed the selective enrichment of N1 and N3 of compound **6**, contrary to the procedure with thiourea, which was incapable of distinguishing between these two nitrogen atoms. In the subsequent condensation reaction, a nucleophilic attack with the unsubstituted nitrogen atom of *N*-benzylthiourea took place, followed by ring-closure through the remaining substituted nitrogen atom.[8a−8c] The position of the benzyl group was checked by NOE experiments, which confirmed its position close to the exocyclic NH_2 group. Moreover, TLC analysis and highfield NMR spectroscopy revealed the presence of only one product. Hence, this reaction appeared to be regiospecific with respect to the benzylic group being specifically attached to the N1 atom, this permitting either the N1 or the N3 atom to be specifically enriched with ^{15}N. Removal of the benzyl function was achieved with Na(s) in liquid ammonia and yielded 87% of the desired product **6**.

At this point **6** could be isotopically enriched in any desired pattern. Traube has presented steps to convert this compound into a number of purine bases,[6] while Abad et al. have described schemes in which this compound was converted into (deoxy)adenosine and (deoxy)guanosine, and those steps resulting in 2′-deoxyadenosine were used here with minor modifications.[9] Nitrosylation of **6a** and the subsequent selective reduction of the nitroso function to sulfur compound **8a** were performed as described, affording in this case an overall yield of 86%.[5a] In a separate reductive step, the sulfur function was removed with Raney nickel to arrive at diamine **9a** in 92% yield. However, the closure of the ensuing diamine to give [2-^{13}C]-hypoxanthine (**10a**) was carried out in a different manner than in previously published papers.

Diamine **9a** was suspended in water and acidified with 1 equiv. each of formic acid and sulfuric acid. This mixture was subsequently heated under reflux to afford **10a** after a reaction time of 16 h. This method was suitable for the introduction of isotopes through the use of [^{13}C]-formic acid with no chance of isotope loss to the solvent and/or reagents. Exposure of **10a** to $POCl_3$ provided [2-^{13}C]-6-chloropurine (**11a**) in 94% yield, and heating of this product in ethanolic ammonia quantitatively replaced the halogen atom with NH_2 to afford [2-^{13}C]-adenine (**2a**).

Analysis

The ^1H NMR spectra (D_2O, 600 MHz) for both the $^{13}C_4$-labelled **1a** and the unlabelled 2′-deoxyadenosine **1** are shown in Figure 1. The presence and positions of the four ^{13}C atoms were derived from the large $^1J_{C-H}$ couplings, indicated in Figure 1, and the isotopic level of enrichment was determined from the integrals of the unsplit, residual signals. It was found that all designated positions were 99% ^{13}C-enriched, with the exception of position 5′. The slightly lower enrichment of this position (96%) was due to scrambling of 3% of the ^{13}C isotope to the 3′-position.[1]

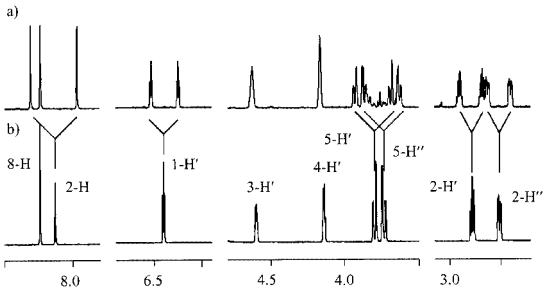


Figure 1. The ^1H NMR spectrum (600 MHz, D_2O) of unlabelled 2′-deoxy-D-adenosine (b) and the ^1H NMR spectrum (600 MHz, D_2O) of [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine (a); the signals arising from carbon atoms attached to proton nuclei, labelled in (a), are split, which is indicated in the spectra; the position of the ^{13}C isotopes is verified in this manner; in addition, analysis of the intensities in conjunction with mass spectrometric techniques was used to determine the level of enrichment

The ^{13}C NMR spectra (151 MHz, D2O) of both the unlabelled (a) and labelled (b) samples are depicted in Figure 2. Clearly the four isotopically substituted carbon atoms caused a large increase in their corresponding signal-to-noise ratios at the chemical shifts expected for the individual positions. This indicates that during the synthesis neither isotope dilution nor scrambling to other label positions had taken place.

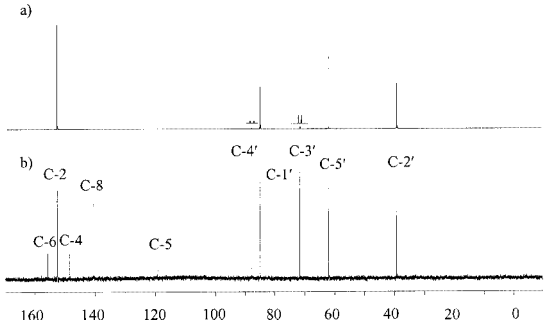


Figure 2. a) ^{13}C NMR spectrum (150 MHz, D_2O) of [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine; the large increase in signal-to-noise ratio of the isotopically substituted carbon atoms is apparent; b) a natural-abundance ^{13}C NMR spectrum (150 MHz, D_2O) of 2′-deoxy-D-adenosine

The NMR results were independently confirmed by the high-resolution mass spectra of the final products **1a** and **2a**.

Discussion

The classical organic chemistry used in coupling of purine bases to D-2-deoxyribose moieties is hampered by its multistep nature and the lack of both stereo- and regiospecificity, which makes it difficult to prepare the β-anomers of the *N*9-linked purine nucleosides selectively. After extensive purification, the final product can be obtained only in low yields.[10]

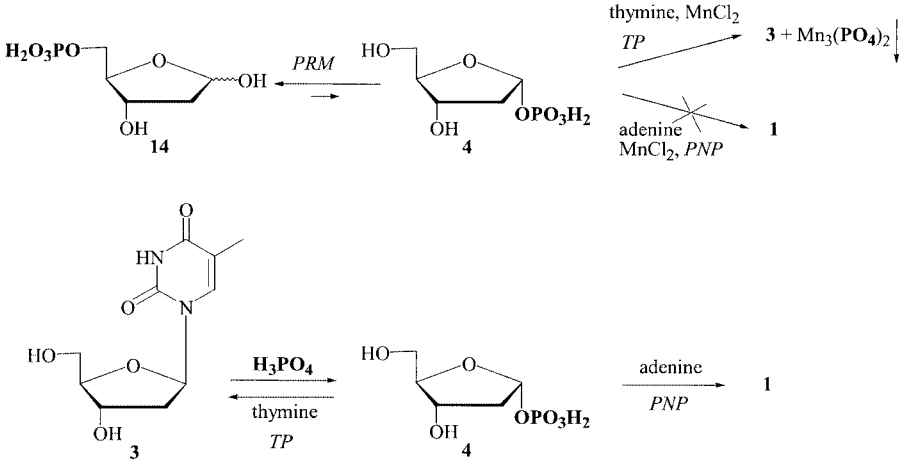
In an attempt to improve upon this method for the preparation of purine-2′-deoxynucleosides, the procedure originally developed for the synthesis of pyrimidine analogues was applied.[3] In this route, depicted at the top of Scheme 3, intermediate **4** is formed by migration of the phosphate group from the 5- to the 1-position in 2-deoxyribose. Subsequently, coupling of a nucleobase results in liberation of inorganic phosphate.[11] The key to this method is the precipitation of this phosphate group with manganese chloride, which drives the reaction towards the product. However, when 2′-deoxyadenosine was used, only 5−10% of the coupled product was obtained, and even less when guanosine was the reactant.[12] The addition of manganese phosphate did not result in a higher yield. In contrast, when **4** was generated from **3** the yield of **1** was nearly quantitative and only a small excess of **2** was necessary for high conversion.

The yield of **1** thus depended on the method of generation of intermediate **4**, which is interpreted in terms of a difference in the positions of the respective equilibria: between **14** and **4**, and between **3** and **4** (Scheme 3). ^{31}P NMR spectroscopy of a solution of **14** in the presence of a catalytic amount of PRM indicated no signal from **4**. Therefore, in the top reaction in Scheme 3, the equilibrium between the 5-phosphate and the 1-phosphate was clearly biased towards the former. However, TLC analysis of a TP-catalysed reaction between **3** and phosphate showed a substantial amount of free thymine, indicating that a considerable amount (at least 5−10%) of thymidine was in equilibrium with both **4** and thymine.

The success of the coupling of adenine to 2-deoxyribose-1-phosphate was based on first coupling an unlabelled thymine base to the labelled sugar moiety, followed by a transglycosylation with the labelled purine nucleobase. Note that the success of the coupling of thymine with **4** (the upper reaction shown in Scheme 3), compared to the corresponding coupling with adenine, has its cause in the solubility of thymine, which is ten times greater than that of adenine. Therefore, the use of more soluble purine compounds should afford similar results to the pyrimidine bases. Suitable candidates might be compounds such as 6-chloropurine and 2,6-diaminopurine, which are precursors for 2′-deoxyadenosine and 2′-deoxyguanosine, respectively. Unfortunately, in our hands these compounds have never shown any activity with PNP, despite literature claims.[5b,13]

The overall yield of the conversion of 2-deoxy-D-ribose-5-phosphate to 2′-deoxy-D-adenosine was very high, over 50% (based on the former compound). Compared to the multistep approach that is involved when chemical methods are used to couple the purine nucleobases to 2-deoxyribose derivatives, and the lengthy purification that is necessary, this is about double the previously obtained yield.[14a−14c]

This synthesis of purine nucleobases is adapted to permit maximum flexibility in positioning the isotopes. In general, the most important conversion in this synthesis is the transformation of the pyrimidinediamine into hypoxanthine, which in fact consists of two reaction steps. The first is the formation of the 5-formido derivative of the diamine, and that compound is prepared by heating the diamine **9** in the presence of formic acid. The subsequent ring-closure of this 5-formido derivative to afford the hypoxanthine is the critical step in the synthesis and produces usually only modest yields. In a recent article, the unusual reagent $TiCl_2(iPr)_2$ was deployed to close *N*-[(4-[^{15}N]-amino-6-chloro-5-pyrimidinyl)][^{13}C]-formamide in excellent yield.[15] However, attempts to close the *N*-[(4-amino-6-hydroxy-5-pyrimidinyl)]-formamide with this reagent failed, probably because of solubility difficulties. Attempts to apply a method using DMF, formic acid and diethoxymethyl acetate (DEMA) were unsuccessful because of the observed loss of isotope content at C-8 of the purine ring.[5b] Another investigated reagent, a mixture of formic acid and diethylene glycol, gave



Scheme 3. The two manners according to which 2-deoxy-α-D-ribose 1-phosphate (**4**) was prepared in situ; in the top reaction, phosphoribomutase (PRM) caused the phosphate group to migrate from position 5 to position 1; the thermodynamic equilibrium of this reaction was positioned towards the starting material; the desired thymidine could be obtained in high yield only by shifting the equilibrium by precipitation of the inorganic phosphate salt; however, adenine cannot be coupled to **4** in this way, most probably because of the lower solubility of adenine than of thymine, which is shown in the reaction below; in the bottom reaction the equilibrium of the conversion of thymidine to **4** was biased more towards the product side; this explains why in the next reaction 2′-deoxyadenosine was obtained in high yield in spite of the low concentration of adenine

erratic and modest yields.[16] When, however, the compound was heated in water with 1 equiv. of sulfuric acid and 1.05 equiv. of formic acid, [2-^{13}C]-hypoxanthine (**10a**) was obtained in a one-pot fashion and in 80−90% yield. The sulfuric acid was essential, because without it only the formylated product was obtained, albeit in excellent yield. After 18 h of heating, **10a** was isolated by precipitation, to provide the purine pure enough for synthetic purposes. This procedure is very simple and workup is reduced to a minimum.

Although not actively pursued in this work, 2′-deoxy-D-riboseguanosine and some derivatives can be prepared from the compounds synthesised here. This implies that our schemes might be exploited to acquire a number of different purine derivatives in any isotopically substituted form.

Conclusion

[2-^{13}C]-Adenine has been coupled to [1,2,5-$^{13}C_3$]-2-deoxy-D-ribose in an efficient transglycosylation reaction with [1′,2′,5′-$^{13}C_3$]-thymidine to produce [1′,2′,5′,2-$^{13}C_4$]-2′-deoxy-D-adenosine. The method was not only very quick, but required no protecting groups and provided very high yields. This makes all isotopic derivatives of 2′-D-adenosine available, over 30000 compounds in total (see Figure 3).

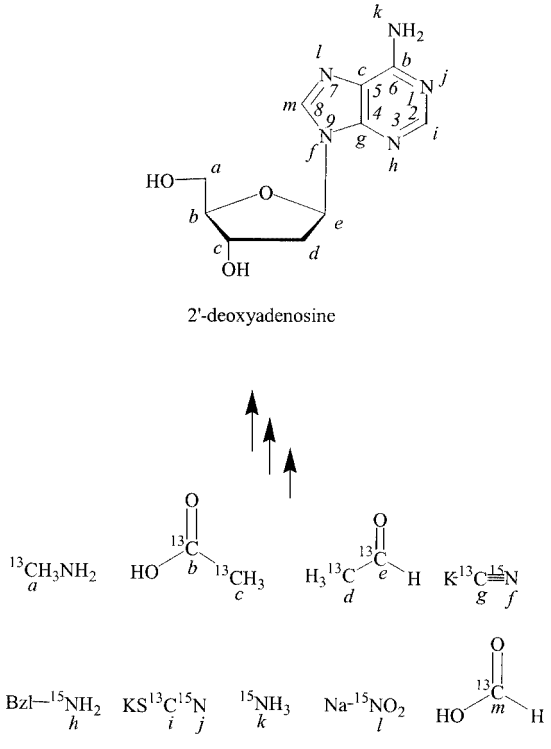


Figure 3. Correlation between the individual labelled atoms of 2′-deoxyadenosine and those in commercially available ^{13}C- and ^{15}N-enriched starting materials

Experimental Section

General: Labelled compounds are indicated by the numbers **1a**, **2a**, **3a** etc. and were synthesised by schemes optimised with unlabelled material; those compounds are numbered **1** through **14**. The route was subsequently carried out with the labelled compounds. The isotopically labelled starting material, [^{13}C]thiourea (99% ^{13}C), was purchased from Cambridge Isotope Laboratories, U.S.A. The synthesis of $^{13}C_3$-labelled thymidine **3a** has been described previously.[3] Unlabelled reagents and starting materials were acquired from Aldrich and Acros Chimica. Thymidine phosphorylase (TP, EC 2.4.2.4 from *E. coli*, in 0.5 M phosphate solution) and purine nucleoside phosphorylase (PNP, EC 2.4.2.1, bacterial as lyophilised powder) were obtained from Sigma. Enzymatic reactions were carried out in Millipore water. Organic solvents were used as follows. Ethanol (p.a. quality) was used throughout without additional purification. Diethyl ether was distilled immediately prior to use. Dichloromethane was used as received. "Saturated $NaHCO_3$ solution" refers to solution in water. Reactions were monitored by thin layer chromatography (TLC, on Merck$_{254}$ silica gel 60 aluminium sheets, 0.2 mm); spots were viewed under UV light (254 nm). Enzymatic reactions were monitored by ^{1}H NMR spectroscopy with lyophilised samples of the reaction mixtures. Precipitated products were isolated with fibreglass filters (GF/A). Column chromatography was performed on Merck silica gel 60 (0.040−0.063 mm, 230−400 mesh). Ion-exchange resin (Dowex 1 × 2, 400 mesh) was purchased from Aldrich and was converted into the OH^- form by rinsing with 1000 mL of 1 M NH_4OH prior to use. ^{1}H NMR spectra were recorded with a Bruker WM 300 or a Bruker AM 600 spectrometer as indicated, with tetramethylsilane in $CDCl_3$ (TMS; δ = 0.00 ppm) as internal standard and, for aqueous samples, with TMS as external standard. ^{1}H-noise-decoupled ^{13}C NMR spectra were recorded with a Bruker WM 300 at 75 MHz or a Bruker AM 600 spectrometer at 151 MHz, with chloroform (δ = 77.0 ppm) as internal standard and, for aqueous samples, with TMS (δ = 0.00 ppm) as external standard. Mass spectra were recorded with a Finnigan MAT 900 equipped with a direct insertion probe (DIP), EI-MS 70 eV or a Finnigan MAT 700-TSQ equipped with a custom made electron-spray interface (ESI). Melting points were measured with a Büchi apparatus and are given uncorrected.

[1′,2′,5′,2-$^{13}C_4$]-2′-Deoxy-D-adenosine (1a): [1′,2′,5′-$^{13}C_3$]-Thymidine (20 mg, 82 μmol) and **2a** (11 mg, 54 μmol) were suspended in water (400 μL). Inorganic phosphate (final concentration 1 μM) was added, and the pH was adjusted to 7.4 with some 1 M NaOH. Thymidine phosphorylase (25 units) and purine nucleoside phosphorylase (25 units) were added and the mixture was subsequently heated to 43 °C and kept at that temperature overnight, with gentle stirring. ^{1}H NMR was used to check the conversion after this period. The reaction mixture was loaded onto a Dowex 1X2 400-mesh ion exchange column (2 × 20 cm) in the OH^- form and eluted with water (200 mL), followed by 30% methanol/water (300 mL). Appropriate fractions were collected, and after evaporation of the solvent the residue was taken up in water (5 mL) and lyophilised. This yielded 13 mg of compound **1a** (49 μmol, 91%, based on [2-^{13}C]-adenine). Unchanged [1′,2′,5′-$^{13}C_3$]-thymidine (7.0 mg, 19 μmol) could be recovered after rinsing the column with TEAB (0−0.5 M gradient), which means that the yield based on [1′,2′,5′-$^{13}C_3$]-thymidine is 91%. ^{1}H NMR (600 MHz, D_2O): δ = 2.52 (dm, $^1J_{C-H}$ = 136.7, 1 H, 2-H′), 2.78 (ddm, $^1J_{C-H}$ = 132.7, $^2J_{C-H}$ = 5.7 Hz, 1 H, 2-H′), 3.74 (ddd, $^1J_{C-H}$ = 142.7, $^2J_{H-H}$ = 12.6, $^3J_{H-H}$ = 4.2 Hz, 1 H, 5-H″), 3.80 (ddd, $^1J_{C-H}$ = 143.8, $^2J_{H-H}$ = 12.6, $^3J_{H-H}$ = 3.0 Hz, 1 H, 5-H′), 4.61 (m, 1 H, 3-H′), 4.15 (m, 1 H, 4-H′), 6.42 (dt, $^1J_{C-H}$ = 167.4, $^3J_{H-H}$ = 6.9 Hz, 1 H, 1-H′), 8.16 (d, $^1J_{C-H}$ = 202.7 Hz, 1 H, 2-H), 8.26 (s, 1 H, 8-H) ppm. ^{13}C NMR (151 MHz, D_2O): δ = 39.3 (strong d, $^1J_{C-C}$ = 36.8 Hz, C-2′), 62.0 (strong signal, C-5′), 71.6 (d, $^1J_{C-C}$ = 35.3 Hz, C-3′), 85.0 (strong d, $^1J_{C-C}$ = 36.8 Hz, C-1′), 87.8 (d, $^1J_{C-C}$ =

39.6 Hz, C-4′), 119.2 (C-5), 140.5 (C-8), 148.6 (C-3), 152.6 (strong signal, C-2), 155.8 (C-6) ppm. HRMS (ESI): *m*/*z* calcd. for $C_6{}^{13}C_4H_{13}O_4N_5$ 255.1153; found 255.1168.

[2-^{13}C]-Adenine (2a): Compound **11a** (100 mg, 0.64 mmol) was dissolved in ethanol (3 mL), containing concentrated aqueous NH_3 (1.4 mmol NH_3). The solution was transferred into a Carius tube and frozen by application of liquid nitrogen. The tube was sealed and heated at 140 °C, and left to cool overnight. The sealed tube was frozen with liquid nitrogen and opened. Evaporation of the solvent afforded a white residue, which contained, in addition to [2-^{13}C]-adenine, ammonium chloride (135 mg, quantitative). This solid was used without further purification. ^{1}H NMR (300 MHz, D_2O): δ = 7.90 (s, 1 H, 8-H), 8.26 (s, 1 H, 8-H), 8.26 (s, 1 H, 8-H) ppm. ^{13}C NMR (75 MHz, D_2O): 138.9 (C-8), 150.4 (strong signal, C-2) ppm. HRMS (ESI): *m*/*z* calcd. for $C_4{}^{13}CH_6N_5$ 137.0720; found 137.0757.

Ethyl Cyanoacetate (5): Cyanoacetic acid (3.41 g, 40 mmol) was dissolved in dichloromethane (150 mL), after which ethanol (12 mL, 200 mmol) and H_2SO_4 (65 μL, 1.2 mmol) were added. This solution was heated under reflux for 18 h, using a reversed Dean−Stark apparatus to trap the liberated water. After the reaction period, the solution was poured into 100 mL of 10% $NaHCO_3$ solution, and after the separation of the ensuing layers the aqueous layer was extracted twice with dichloromethane (100 mL). The solution was dried with $MgSO_4$, followed by filtration and removal of the solvent in vacuo. The residual colourless oil weighed 4.3 g (38 mmol, 95%) and consisted of ethyl cyanoacetate. ^{1}H NMR (300 MHz, $CDCl_3$): 1.32 (t, $^3J_{H-H}$ = 7.2 Hz, 3 H, Et), 3.49 (s, 2 H, 2-H), 4.27 (q, $^3J_{H-H}$ = 7.2 Hz, 2 H, Et) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): 13.8 (Et), 24.6 (C-2), 62.8 (Et), 113.1 (C-3),162.9 (C-1) ppm. MS (ESI): *m*/*z* = 114 [M + H], 136 [M + Na].

6-Amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (6): Compound **13** (400 mg, 1.72 mmol) was placed in a 100-mL three-necked flask, containing a propylene (black) magnetic stirring bar. The flask and contents were cooled to −70 °C and liquid NH_3 (50 mL) was introduced. Na(s) (43 mg, 1.89 mmol) was added to this colourless solution in small portions. Once the reaction mixture remained blue, indicating the presence of solvated electrons in NH_3, NH_4Cl (1 g) was added to quench the reaction. While stirring, the flask was opened and the NH_3 was allowed to evaporate. The white residue was dissolved in water (4 mL) with the aid of the minimum amount of 1 M NaOH required for complete dissolution and precipitated with a small amount of acetic acid. Filtration and washing with water, ethanol and acetone (3 mL of each) afforded, after drying, 200 mg (1.4 mmol, 81%) of a white powder. ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 4.75 (s, 1 H, 5-H), 7−5 (br. s, 4 H, NH and SH) ppm. ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 78.6 (C-5), 155.2 (C-6), 162.7 (C-4), 174.8 (C-2) ppm. MS (ESI): *m*/*z* = 143 [M + H].

[2-^{13}C]-6-Amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (6a): Ethyl cyanoacetate (1.47 g, 13.0 mmol) was added to a solution of sodium ethoxide, prepared by dissolving sodium (298 mg, 13.0 mmol) in dry ethanol (10 mL). After about 1 min, a white suspension developed and [^{13}C]-thiourea (1.0 g, 12.96 mmol) was added. This suspension was heated under reflux for 2 h, during which the suspension became a clear, yellowish solution and then a white suspension again. The ethanol was removed in vacuo and the solid residue was dissolved in water (15 mL). After precipitation with acetic acid, the solid was collected by filtration and washed with water, ethanol and acetone (10 mL each). Drying in a vacuum oven (100 °C) overnight afforded 1.60 g (11.1 mmol, 86%) of the title compound. ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 4.75 (s, 1 H, 5-H), 7−5 (br. s, 4 H, NH and SH) ppm. ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 78.6 (C-5), 155.2 (C-6), 162.7 (C-4), 174.8 (strong signal, C-2) ppm. MS (ESI): *m*/*z* = 144 [M + H].

[2-^{13}C]-6-Amino-5-nitroso-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (7a): Compound **6a** (1.55 g, 10.7 mmol) was suspended in HCl (1 M, 40 mL) and cooled in ice. To this mixture was added a solution of sodium nitrite (785 mg, 11.3 mmol) in water (10 mL). Stirring and cooling was continued for 7 h, after which time the red suspension was filtered and washed with water and ethanol (10 mL). Drying in a vacuum desiccator (100 °C) yielded 1.71 g (9.9 mmol, 92%) of a red solid matter. ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 7.7 (br. s), 11.2, 12.6 ppm. ^{13}C NMR (75 MHz, [D_6]DMSO): 148.7 (C-5), 159.4 (C-6), 174.6 (C-4), 176.4 (strong signal, C-2) ppm. MS (ESI): *m*/*z* = 174 [M + H], 196 [M + Na].

[2-^{13}C]-5,6-Diamino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (8a): Compound **7a** (1.6 g, 9.3 mmol) was suspended in saturated $NaHCO_3$ (38 mL) and cooled in ice. Sodium dithionite (4.25 g, 24.3 mmol) was added in four portions and the reaction mixture was stirred for 6 h. To the now yellow suspension was (slowly) added acetic acid, and the solid was collected by filtration. Washing with water and ethanol and drying in a vacuum desiccator afforded 1.5 g (9.3 mmol, 94%) of a slightly yellow solid. ^{1}H NMR (200 MHz, [D_6]DMSO): δ = 8−5 (br. s) ppm. ^{13}C NMR (50 MHz, [D_6]DMSO): δ = 102.4 (C-5), 140.8 (C-6), 157.9 (C-4), 167.6 (strong signal, C-2) ppm. MS (ESI): *m*/*z* = 160 [M + H], 182 [M + Na].

[2-^{13}C]-5,6-Diamino-4(3*H*)-pyrimidinone (9a): Compound **8a** (1.5 g, 9.3 mmol) was dissolved in NH_3 (5%, 40 mL). Raney nickel (5 mL of a 50% slurry in water) was added to this solution, and the reaction mixture was heated under reflux for 90 min. The black mixture was filtered hot through two fibreglass filter papers, which were washed with boiling water (2 × 15 mL). The filtrate was concentrated in vacuo to afford 1.10 g of a yellow solid (8.7 mmol, 92%). ^{1}H NMR (200 MHz, [D_6]DMSO): δ = 5.4 (br. s, 5 H), 7.63 (d, $^1J_{C-H}$ = 200.0 Hz, 1 H, 2-H) ppm. ^{13}C NMR (200 MHz, [D_6]DMSO): δ = 110.5 (C-5), 138.8 (strong signal, C-2), 147.7 (C-6), 156.7 (C-4) ppm. MS (ESI): *m*/*z* = 128 [M + H].

[2-^{13}C]-Hypoxanthine (10a): Compound **9a** (800 mg, 6.3 mmol) was suspended in water (2 mL) to which were added H_2SO_4 (617 mg, 6.3 mmol) and formic acid (290 mg, 6.3 mmol). The mixture was heated under reflux overnight and was subsequently allowed to cool to room temperature. Additional water (1 mL) was added to keep the mixture fluid and the mixture was neutralised with ammonium hydroxide (25% in water) and acetic acid. The precipitate was cooled on ice, filtered and washed with water (twice, 4 mL), ethanol (96%, twice, 4 mL), and acetone (twice, 4 mL). The solid was dried in vacuo to give 802 mg (5.9 mmol, 94%) of a slightly coloured solid. ^{1}H NMR (200 MHz, [D_6]DMSO): δ = 8.0 (d, $^1J_{C-H}$ = 204.5 Hz, 1 H, 2-H), 8.10 (s, 8-H, 1 H) ppm. ^{13}C NMR (50 MHz, [D_6]DMSO): δ = 119.2 (C-5), 140.2 (C-8), 144.7 (strong signal, C-2), 153.3 (C-4), 155.4 (C-6) ppm. HRMS (ESI): *m*/*z* calcd. for $C_4{}^{13}CH_4N_4O$ 138.04969; found 138.04973.

[2-^{13}C]-6-Chloropurine (11a): $POCl_3$ (22 mL) was added to *N*,*N*-dimethylaniline (1.8 mL), followed by **10a** (800 mg, 5.9 mmol). The mixture was heated under reflux for 30 min, during which time it slowly turned brown. Excess $POCl_3$ was removed with a water aspirator and the last traces were removed with an oil pump. The

residue was dissolved in NH_3 (25%, 7 mL) and silica gel (1 g) was added. The solvent was evaporated in vacuo and the silica gel was loaded on top of a silica gel column and eluted with 30% MeOH in dichloromethane. This procedure afforded 730 mg (4.7 mmol, 80%) of **11a**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.6 (s), 8.7 (d, $^1J_{C-H}$ = 209.0 Hz, 1 H, 2-H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 129.3 (C-5), 146.4 (C-8), 147.7 (C-4), 151.4 (strong signal, C-2), 154.3 (C-6) ppm. HRMS (ESI): calcd. for $C_4{}^{13}CH_3N_4Cl$ 155.00798; found 155.00619.

***N*-Benzylthiourea (12):** Benzylamine (10 g, 93.5 mmol) was dissolved in water (50 mL) and acidified with HCl (6 M, 15.7 mL, 94 mmol). To this was added potassium thiocyanate (9.07 g, 93.5 mmol) in water (50 mL). This solution was concentrated to dryness and heated for 20 min at 140 °C in an oil bath. The resulting oil, containing some solid matter, was allowed to cool. Water (100 mL) was then added and the suspension was heated to 100 °C. Large lumps were broken up manually. After 20 min, the suspension was cooled and kept at 4 °C overnight. The cold liquid was filtered and the solid thus collected was washed with some water (10 mL) and dried in vacuo (100 °C). The white solid weighted 9.8 g (63.2 mmol, 68%). M.p. > 200 °C. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 70 °C, two rotamers): δ = 4.73 and 4.62 (two d, 2 H, *J* = 5.6, 5.3 Hz, CH_2), 6.95 (br. s, 2 H, NH_2), 7.32 (m, 5 H, ArH), 7.94 (br. s, 1 H, NH) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO, 70 °C, two rotamers): δ = 47.2 (CH_2) and 47.0 (CH_2), 126.5 (Ar-C), 127.0 (Ar-C), 138.6 and 138.7 (Ar-C), 127.9 (Ar-C), 183.2 (CS) ppm. MS (DIP): *m*/*z* = 166.

6-Amino-1-benzyl-2-thioxo-4(3*H*)-pyrimidinone (13): Ethyl cyanoacetate (3.2 g, 28.3 mmol) was added to a sodium ethoxide solution, freshly prepared by dissolving sodium (650 mg, 28.3 mmol) in dry ethanol (25 mL). *N*-Benzylthiourea (5.0 g, 32.5 mmol) was added to the suspension and this mixture was heated under reflux overnight. Water (20 mL) was added and acetic acid until the mixture tested acidic. Filtration, washing of the solid with water and drying afforded the pyrimidine compound as a white solid (5.6 g, 24 mmol, 84%), m.p. > 200 °C. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 70 °C): δ = 5.0 (s, 1 H, 5-H), 5.74 (s, 2 H, CH_2), 6.71 (s, 2 H, NH_2), 7.25 (m, 5 H, ArH), 11.7 (s, 1 H, 3-H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO, 70 °C): δ = 49.2 (CH_2), 80.5 (C-5), 125.8 (Ar-C), 126.7 (Ar-C), 128.0 (Ar-C), 135.0 (Ar-C), 155.2 (C-6), 159.1 (C-4), 177.1 (C-2) ppm. MS (ESI): *m*/*z* = 234 [M + H].

Acknowledgments

The authors are indebted to Cees Erkelens and Fons Lefeber for their part in acquiring high-resolution NMR spectra and to Bertil Hofte and Barry Karabatak for obtaining the mass spectra.

[1] N. Ouwerkerk, J. H. Van Boom, J. Lugtenburg, J. Raap, *Eur. J. Org. Chem.* **2000**, *26*, 861−866.
[2] M. Hoffer, *Chem. Ber.* **1960**, *93*, 2777−2780.
[3] N. Ouwerkerk, M. Steenweg, J. H. Van Boom, J. Lugtenburg, J. Raap, *J. Org. Chem.,* in press.
[4] T. A. Krenitsky, G. W. Koszalka, J. V. Tuttle, *Biochemistry* **1981**, *20*, 3615−3621.
[5] [5a] J.-L. Abad, B. L. Gaffney, R. A. Jones, *J. Org. Chem.* **1999**, *64*, 6575−6582. [5b] A. R. Pagano, W. M. Lajewski, R. A. Jones, *J. Am. Chem. Soc.* **1995**, *117*, 11669−11672.
[6] W. Traube, *Ber. Dtsch. Chem. Ges.* **1903**, *36*, 64.
[7] J. L. Roberts, C. D. Poultier, *J. Org. Chem.* **1978**, *43*, 1547−1550.
[8] [8a] V. Papesch, E. F. Schroeder, *J. Org. Chem.* **1951**, *26*, 1879. [8b] P. W. K. Woo, C. R. Kostlan, J. C. Sircar, M. K. Dong, R. B. Gilbertson, *J. Med. Chem.* **1992**, *35*, 1451. [8c] L. Fraisse, J.-B. Verlhac, B. Roche, M. C. Rascle, A. Rabion, J. L. Seris, *J. Med. Chem.* **1993**, *36*, 1465.
[9] J. L. Abad, A.J. Shallop, B. L. Gaffney, R. A. Jones, *Biopolymers* **1998**, *48*, 57−63.
[10] R. P. Hodge, C. K. Brush, C. M. Harris, T. M. Harris, *J. Org. Chem.* **1991**, *56*, 1553−1564.
[11] S. Pal, V. Nair, *Biotechnol. Lett.* **1997**, *19*, 349−351.
[12] S. Roy, Y. Hiyama, D. A. Torchia, J. S. Cohen, *J. Am. Chem. Soc.* **1986**, *108*, 1675−1678.
[13] A. Bzowska, E. Kulikowska, D. Z. Shugar, *Z. Naturforsch., Teil C* **1990**, *45*, 59−70.
[14] [14a] F. Seela, H. Winter, *Nucleosides Nucleotides* **1995**, *14*, 129−142. [14b] T. Patthak, J. Chattopadhyaya, *Tetrahedron* **1987**, *43*, 4227−4234. [14c] N. B. Hanna, K. Ramasamy, R. K. Robins, G. R. Revankar, *J. Heterocycl. Chem.* **1988**, *25*, 1899−1903.
[15] C. C. Orji, R. Michalczyk, L. A. "Pete" Silks III, *J. Org. Chem.* **1999**, *64*, 4685−4689.
[16] [16a] C. E. Carter, *J. Am. Chem. Soc.* **1950**, *72*, 1468. [16b] H. Brandenberger, *Biochim. Biophys. Acta* **1954**, *15*, 108. [16c] F. Weyland, H. Klebe, A. Trebst, H. Simon, *Z. Naturforsch., Teil B* **1954**, *9*, 450.
[17] H. Zao, A. R. Pagano, W. Wang, A. Shallop, B. L. Gaffney, R. A. Jones, *J. Org. Chem.* **1997**, *62*, 7832−7835.

Received January 9, 2002
[O02011]