ARTICLE

OBC
www.rsc.org/obc

The economical synthesis of [2′-^{13}C, 1,3-$^{15}N_2$]uridine; preliminary conformational studies by solid state NMR

Simon G. Patching,[a] David A. Middleton,[b] Peter J. F. Henderson[a] and Richard B. Herbert*[a]

[a] *Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, UK LS2 9JT. E-mail: r.b.herbert@chem.leeds.ac.uk*

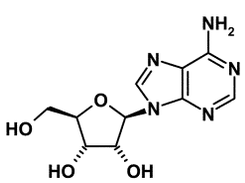
[b] *Department of Biomolecular Sciences, UMIST, PO Box 88, Manchester, UK M60 1QD*

Received 13th February 2003, Accepted 23rd April 2003
First published as an Advance Article on the web 9th May 2003

The synthesis of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** was achieved as follows. An epimeric mixture of D-[1-^{13}C]ribose **3** and D-[1-^{13}C]arabinose **4** was obtained in excellent yield by condensation of K^{13}CN with D-erythrose **2** using a modification of the Kiliani–Fischer synthesis. Efficient separation of the two aldose epimers was pivotally achieved by a novel ion-exchange (Sm^{3+}) chromatography method. D-[2-^{13}C]Ribose **5** was obtained from D-[1-^{13}C]arabinose **4** using a Ni(II) diamine complex (nickel chloride plus TEMED). Combination of these procedures in a general cycling manner can lead to the very efficient preparation of specifically labelled ^{13}C-monosaccharides of particular chirality. ^{15}N-labelling was introduced in the preparation of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** *via* [$^{15}N_2$]urea. Cross polarisation magic angle spinning (CP-MAS) solid-state NMR experiments using rotational echo double resonance (REDOR) were carried out on crystals of the labelled uridine to show that the inter-atomic distance between C-2′ and N-1 is closely similar to that calculated from X-ray crystallographic data. The REDOR method will be used now to determine the conformation of bound substrates in the bacterial nucleoside transporters NupC and NupG.

Introduction

All living cells utilise membrane transport proteins to assimilate nutrients and to expel waste products through cell membranes.[1] The concentrative (H^+-driven) nucleoside transport protein NupC from *Escherichia coli* is one such, important, transport protein.[2] *E. coli* also synthesises another concentrative nucleoside transporter, NupG,[3] the gene sequence of which is, curiously, unrelated to that of NupC. It has been deduced tentatively from structure–activity relationships with the substrate adenosine **1** and other nucleosides that the conformation of compounds on binding to NupC is required to be *anti* whereas on binding to NupG the more inherently crowded *syn*-conformation can be accommodated (Fig. 1).[4] This appears to be an important distinction between the two transport proteins relevant to the structures of the binding sites in the proteins and worthy of further exploration. Uridine and adenosine have very similar binding and transport characteristics. Complexities with the (NMR) analysis of protein-bound adenosine has directed us first to synthesise [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11**, in order to study substrate binding conformations in NupC and NupG using the solid state NMR method of cross polarisation magic-angle spinning (CP-MAS) NMR. This method enables study of the transporters in their natural states within native membranes.[4,5] In collaboration with Professor Watts, Oxford and others we are pioneering the application of solid state NMR spectroscopy to the solution of structure and mechanism of action of membrane transport proteins.[4–6] Implementation of such studies is critically dependent on the availability of appropriately labelled nucleoside derivatives. We demonstrate here innovative and efficient procedures for accomplishing dual-isotope labelling of a nucleoside substrate uridine **11**. We report also on the application of CP-MAS NMR spectroscopy to confirm an inter-atomic distance in uridine **11** in the crystalline state and thereby to validate the REDOR method used.



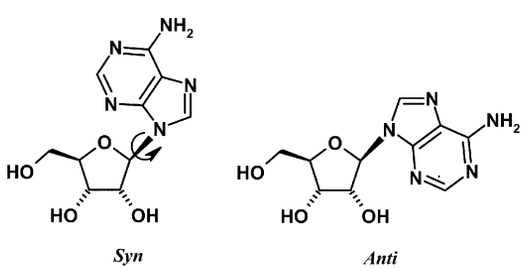
1

Results and discussion

Synthesis of [2′-^{13}C, 1,3-$^{15}N_2$]uridine

Model building on uridine with reference to known X-ray crystallographic data[7] led to the conclusion that the appropriate NMR experiments (below) were best carried out with a ^{13}C-label at C-2′ and a ^{15}N-label at N-3 (Fig. 2). It was simpler to synthesise a compound with a ^{15}N-label also at N-1; this label could provide a harmless stowaway or internal standard in the NMR experiments, since the distance between N-1 and C-2′ in the sugar, unlike C-2′/N-3, does not change on rotation about the glycosidic bond in uridine (Fig. 2).

The Kiliani–Fischer synthesis is traditionally used to convert an aldose into the next highest homologue, but the yields can be



Syn *Anti*

Fig. 1 Conformations of adenosine **1**.

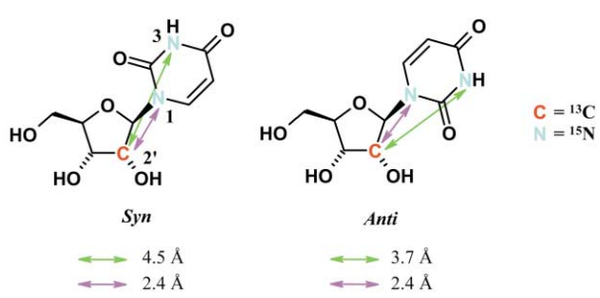
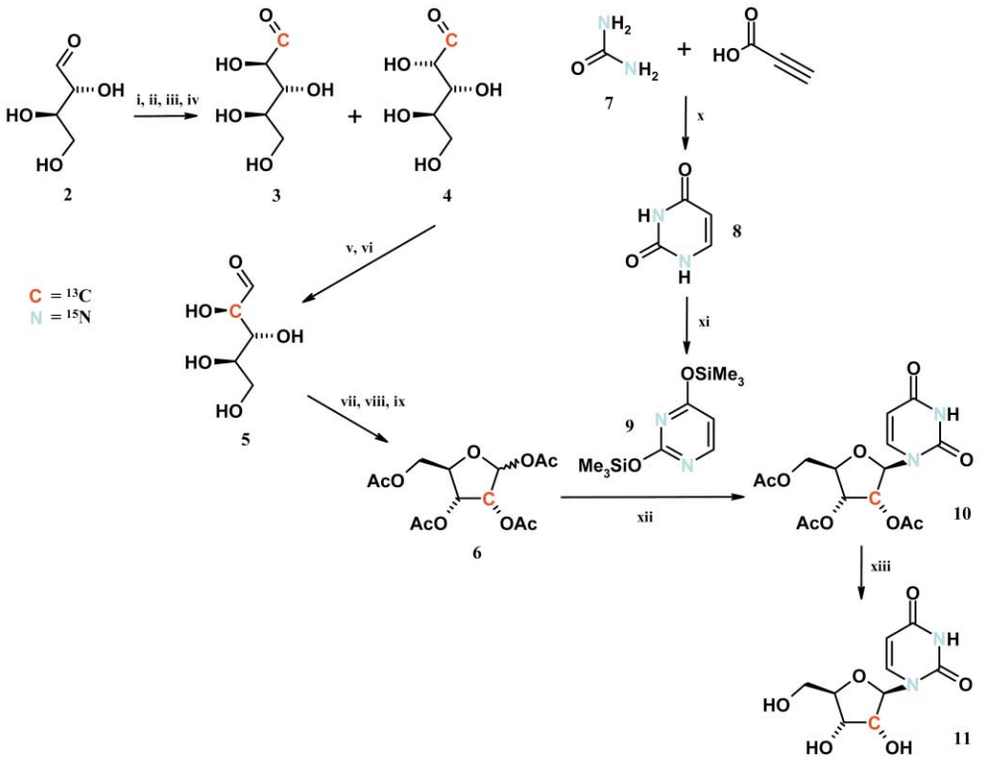


Fig. 2 Through-space internuclear distances between isotopic labels in extreme conformations of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11**.

DOI: 10.1039/b301275a



Scheme 1 i. $K^{13}CN$ (aq)/pH 7.2–7.5; ii. H_2–5% Pd–$BaSO_4$ (aq)/pH 4.2; iii. Dowex-H^+ (aq); iv. Separation on a cation-exchange column in the Sm^{3+} form; v. $NiCl_2 \cdot 6H_2O$–TEMED–MeOH/60 °C; vi. Separation on a cation-exchange column in the Sm^{3+} form; vii. MeOH–Dowex-H^+; viii. Ac_2O–Pyridine; ix. Ac_2O–AcOH–H_2SO_4; x. Polyphosphoric acid/85 °C; xi. *N,O*-Bis(trimethylsilyl)acetamide–MeCN; xii. $SnCl_4$–MeCN; xiii. NaOMe–MeOH.

low.[8] An improved version has as its key the stabilisation of the intermediate aldonitrile in moderately acidic solution prior to palladium-catalysed hydrogenation to an intermediate imine.[8] This method, starting with D-erythrose and $K^{13}CN$, was adapted to synthesise a mixture of D-[1-^{13}C]ribose **3** and its C-2 epimer D-[1-^{13}C]arabinose **4** (Scheme 1).

Separation of the two sugars using established published procedures for aldoses that involve ion exchange columns in Ca^{2+} or Ba^{2+} forms was problematic and indeed unacceptably inefficient in terms of recovery for costly labelled compounds. However, the effective analytical separation of D-ribose and D-arabinose has been observed on cation-exchange TLC plates in the La^{3+} or Sm^{3+} forms.[9] We adapted this procedure to preparative chromatography and immediately found that D-ribose and D-arabinose were efficiently and completely separated on an ion-exchange column in the Sm^{3+} form. High loading was possible, with D-arabinose eluting essentially in the void volume and D-ribose showing complex formation but still keeping to a practical elution time and volume. The Sm^{3+} column can be used repeatedly without recharging or cleaning and appears to be indefinitely stable. We recommend this type of column for wider application in the separation of isomeric carbohydrates and polyols.

The epimeric products D-[1-^{13}C]ribose **3** and D-[1-^{13}C]arabinose **4** were obtained pure in a 0.8 : 1 ratio, respectively with an acceptable overall yield of 49% from $K^{13}CN$; solution NMR analysis confirmed the expected exclusive labelling of C-1.

The proposed solid state NMR experiments require D-[2-^{13}C]-ribose **5** and not D-[1-^{13}C]ribose **3**. We achieved labelling of C-2, however, as planned by a rearrangement involving a switch of labelling from C-1 to C-2: there are a number of reactions of aldoses that involve transition metal complexes, which result in C-2 epimerisation and those involving molybdenate[10] and nickel(II)[11] have, importantly, been shown to proceed with a stereospecific 1,2-rearrangement in the carbon skeleton of the aldose. This we exploited, in a novel labelling strategy, using the C-1,2-rearrangement of D-[1-^{13}C]arabinose **4** to give the desired D-[2-^{13}C]ribose **5**. Reaction of the D-[1-^{13}C]arabinose **4** with molybdenyl acetylacetonate gave an unacceptable equilibrium mixture of **4** and **5** in 2 : 1 ratio (as in ref. 10) and 71% recovery after Sm^{3+} chromatography (*i.e.* 24% yield of D-ribose). On the other hand a nickel diamine complex (nickel(II) chloride plus TEMED) in methanol gave a rapid and favourable rearrangement and equilibration of the two epimers. After silica (partially removes nickel) and Sm^{3+} chromatography, a ratio of 28 : 72 (as in ref. 11) in favour of D-[2-^{13}C]ribose **5** was obtained (recovery after chromatography: 87%; D-ribose epimerisation and after chromatography: an acceptable 63% yield).

Overall, the route to D-[1-^{13}C]ribose **3** and also D-[1-^{13}C]-arabinose **4** permits the economical and efficient preparation of monosaccharides labelled with ^{13}C at C-1, or alternatively at C-2 by rearrangement and equilibration with nickel (or molybdenate). The advantage of our approach if used in a cyclical way is high efficiency and thereby the consumption of an unwanted epimer from the initial synthetic step. The rapid and clean separation on the Sm^{3+} column is an essential component of this approach.

[1,3-$^{15}N_2$]Uracil **8** was prepared from [$^{15}N_2$]urea **7** by adaptation of published methods[12] and protected by silylation (Scheme 1). The D-[2-^{13}C]ribose **5** was converted *via* its methyl glycoside into the tetraacetate **6** (Scheme 1). The sugar and basic moieties were fused together through $SnCl_4$ catalysis[13] to give exclusively the β-anomer **10**; deprotection gave [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** (Scheme 1) in 6.1% and 35.2% overall yield from $K^{13}CN$ and [$^{15}N_2$]urea, respectively. Solution ^{1}H, ^{13}C and ^{15}N NMR confirmed the labelling pattern in **11**.

A distance measurement on crystals of uridine by using CP-MAS solid state NMR with the application of REDOR

In a simple CP-MAS NMR experiment, dipolar (through space) coupling information between nuclei, *e.g.* heteronuclei ^{13}C and ^{15}N, is inevitably lost. This is a significant loss since it is from these couplings that crucial internuclear distances within

a substrate–protein complex may be measured. In the case of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** the distance between the labels on C-2′ and N-3 define the conformational relationship in the nucleoside between the sugar moiety and the base (Fig. 2). Ultimately, the goal of this work is to solve nucleoside structures within a protein. Initially, however, it was important to establish the accuracy of the measurements of inter-atomic distances in the nucleoside. Rotational echo double resonance (REDOR) is an effective technique whereby dipolar coupling information between heteronuclei may be restored and accurate distances measured. This involves two experiments. First, the intensity (S) is measured from a "full-echo" spectrum of one nucleus (*e.g.* ^{13}C) in the absence of dipolar coupling to the other nucleus or nuclei (*e.g.* ^{15}N). The experiment is then repeated in the presence of dipolar coupling, and the intensity (S_0) is measured from the resulting "dephased-echo" spectrum. In practice, a series of S and S_0 values are obtained after allowing the dipolar interaction to evolve for different time intervals. Numerical simulations of the dephasing profile of S/S_0 values at the different dephasing intervals yield the internuclear distance or distances.[14,15]

Crystals of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** were analysed by both ^{13}C and ^{15}N CP-MAS NMR to reveal two overlapping resonances for each of the labelled positions (Fig. 3). This is consistent with the known crystal structure of uridine where there are two molecules in the asymmetric unit.[7] A ^{13}C-observed, ^{15}N-dephased REDOR experiment was performed to measure the distance between C-2′ in the ribose unit and N-1 in the base moiety of **11**. The distance measured was 2.5 Å (Fig. 4, *cf.* Fig. 2). This matches the 2.4 Å that was calculated[16] from the X-ray crystal structure coordinates for uridine.[7] A ^{15}N-observed, ^{13}C-dephased REDOR experiment on the same material assisted the assignment of the two distinctly separate doublet peaks in the ^{15}N spectrum to the two ^{15}N labels (Fig. 5). Dephasing of the doublet at 149 ppm was greater than dephasing of the doublet at 158 ppm. Hence, the upfield doublet is ascribed to N-1, which is only 2.4 Å from C-2′, and, crucially, the downfield doublet to N-3, which is further away from C-2′. This latter is expected to provide the inter-atomic distance from which the uridine conformation in the protein can be determined.

These results establish, in our hands, the REDOR method and, supported by a good supply of labelled uridine **11**, give us the necessary confidence in further NMR experiments to determine the conformation of bound substrates in the bacterial nucleoside transporters NupC and NupG and thus to provide important information on the substrate binding sites of these two membrane transport proteins. Further importance is attached to these bacterial transporters in that NupC shares 27% amino acid sequence identity to, and the same natural substrate specificity with, the human nucleoside transporter hCNT1.[17] Information gained in the accessible bacterial system is of relevance to the human case, which is a much more difficult one to study.

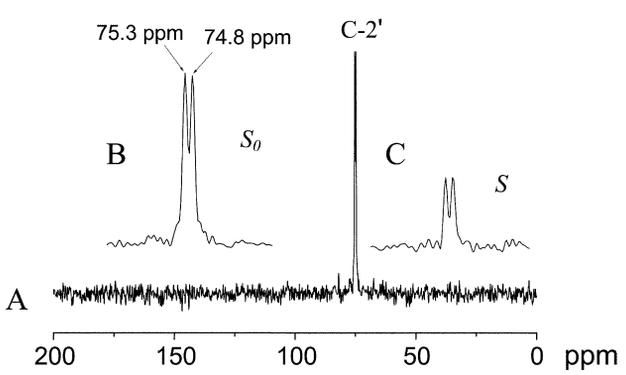


Fig. 3 Proton decoupled ^{13}C CP-MAS NMR spectra obtained in a ^{13}C-observed, ^{15}N-dephased REDOR experiment to measure the intramolecular distance between C-2′ and N-1 in crystals of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11**. The full-echo spectrum (**A**) was obtained for *ca.* 4 mg crystals of **11** in a normal cross polarisation (CP) experiment over 2951 acquisitions at 100.62 MHz for ^{13}C (400.14 MHz for ^{1}H) with a MAS rate of 4228 Hz and using a proton field strength of 65 kHz for CP and for decoupling and a CP contact time of 2 ms. An expansion of the signal for C-2′ is shown in **B**. An expansion of the dephased-echo spectrum is shown in **C**; this was obtained as for **A**/**B** but with rotationally synchronous dephasing π-pulses applied at the resonance frequency of ^{15}N (at 40.54 MHz) for an echo time of 4.5 ms.

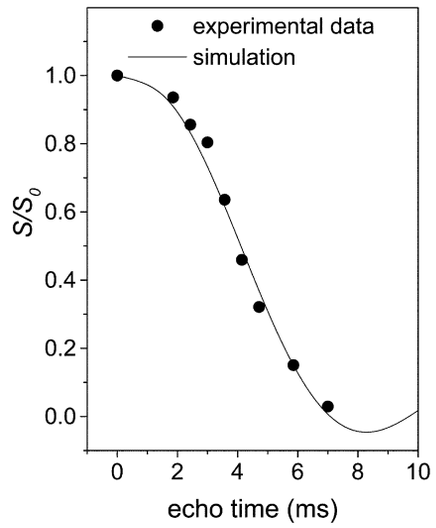


Fig. 4 The dephasing profile for a ^{13}C-observed, ^{15}N-dephased REDOR experiment to measure the intramolecular distance between C-2′ and N-1 in crystals of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11**. REDOR solid state NMR spectra were obtained for crystals of **11** as shown in Fig. 3 using a range of echo times of up to 7 ms. The intensity of the signal for C-2′ in each dephased-echo spectrum (S) was divided by that in the full-echo spectrum (S_0) to give the dephasing profile that was produced by recovering the dipolar coupling between C-2′ and N-1 in **11** (●). The computer-simulated dephasing profile for an internuclear distance of 2.5 Å between a ^{13}C–^{15}N spin pair is also shown (—).

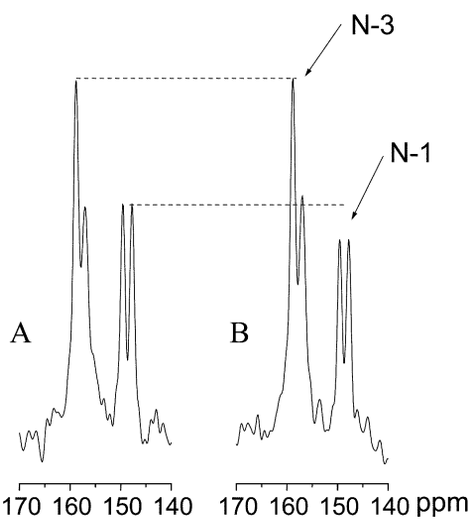


Fig. 5 Proton decoupled ^{15}N CP-MAS NMR spectra obtained with crystals of [2′-^{13}C, 1,3-$^{15}N_2$]uridine **11** in a ^{15}N-observed, ^{13}C-dephased REDOR experiment. The full-echo spectrum (**A**) and dephased-echo spectrum (**B**) were both obtained over 2048 acquisitions for an echo time of 2.2 ms. All other conditions were as stated in Fig. 3.

Experimental

General

Starting materials, reagents and reaction solvents were purchased from Aldrich (Gillingham, UK) or Lancaster synthesis

Ltd (Morecambe, UK). Ion-exchange resin was purchased from Aldrich. ^{13}C- and ^{15}N-labelled starting materials and deuterated DMSO were purchased from Cambridge Isotopes, Inc. (Andover, MA, USA). Deuterated chloroform was purchased from Apollo Scientific Ltd (Whaley Bridge, Derbyshire, UK) and deuterium oxide was purchased from Fluorochem Ltd (Old Glossop, Derbyshire, UK). General solvents were purchased from Fisher Scientific (Loughborough, UK), and solvents were dried using standard procedures. Water-sensitive materials were stored under vacuum over P_2O_5. Column chromatography was performed using Flash 60 (230–400 mesh) silica gel and normal phase TLC was performed on Merck silica gel 60 F_{254} coated aluminium sheets. Compounds were visualised on TLC plates under UV light (254 nm) or by treating with a 5% solution of sulfuric acid in ethanol followed by charring in an oven. Organic phases were dried using anhydrous Na_2SO_4 and freeze-drying was performed using a Chemlab apparatus. Solution state NMR spectra were recorded using a Bruker Avance 250 MHz instrument at the field strength indicated. Chemical shifts were reported as δ values downfield from the internal standard tetramethylsilane, which had a δ value of zero, and coupling constants (J) were reported in Hz. Electron ionisation (EI) mass spectra were recorded using a VT Autospec instrument and electrospray (ES) mass spectra were recorded using a Micromass quadrupole time-of-flight instrument. Solid state NMR spectra were recorded using a Bruker Avance 400 MHz instrument with a Bruker three channel probe using the conditions as indicated. Samples were contained within a 4 mm zirconia rotor.

In the following ^{13}C NMR data, a resonance with ^{13}C enrichment is indicated by *.

Synthesis of D-[1-^{13}C]ribose 3 and D-[1-^{13}C]arabinose 4 (adapted with essential modifications from ref. 8)

With rapid stirring at room temperature, the pH of an aqueous solution (50 cm^3) of potassium [^{13}C]cyanide (0.55 g, 8.33 mmol) was lowered to 7.2 by dropwise addition of aqueous acetic acid (3 M). An aqueous solution (30 cm^3) of D-erythrose **2** (1.00 g, 8.33 mmol) was slowly added, with addition of acetic acid (3 M) or sodium hydroxide (1 M) as appropriate to maintain a pH of 7.2–7.5 during addition of D-erythrose and subsequent reaction, which was allowed to proceed for 1.5 h. The reaction mixture was analysed by TLC (90 : 10 acetone–water) to reveal a complete conversion of D-erythrose (R_f 0.67) into the aldonitriles (R_f 0.81). The pH of the aldonitrile solution was lowered to 4.7 by dropwise addition of acetic acid (3 M) and the solution was stored at 4 °C awaiting reduction (*ca.* 1 h later). The pH of the aldonitrile solution was lowered to 4.2 by dropwise addition of acetic acid (3 M). Following addition of pre-reduced 5% palladium on barium sulfate (516 mg = 25.8 mg, 0.24 mmol Pd), the flask was evacuated three times and flushed with hydrogen. Vigorous stirring under an atmosphere of hydrogen was then allowed to proceed at room temperature for *ca.* 15 h. The reaction mixture was analysed by TLC (90 : 10 acetone–water) to reveal a complete conversion of the aldonitriles (R_f 0.80) into the imines (R_f 0.46). The catalyst was removed over Celite and the pH of the resultant filtrate was lowered from 4.4 to *ca.* 3.0 by repeated batch-wise treatment with Dowex 8 × 50W, 200–400, H^+ cation-exchange resin (4 × 10 g). The final filtrate was analysed by TLC (90 : 10 acetone–water) alongside the authentic compounds to reveal D-ribose (R_f 0.50) and D-arabinose (R_f 0.38) with no imine detected. The water was removed by freeze-drying to leave the epimeric mixture of **3** and **4** as a pale yellow oil (1.07 g) which was stored at 4 °C. The epimeric sugars were separated in two portions on a cation-exchange column prepared in the samarium (Sm^{3+}) form (see below). The oil was dissolved in distilled water (*ca.* 2 cm^3) and half of the resultant syrup was applied to the column, which was eluted under gravity with distilled water. Fifty fractions were collected (1–10, *ca.* 3 cm^3 each and 11–50, 8–10 cm^3 each), and these were analysed by TLC (90 : 10 acetone–water) to reveal nothing in fractions 1–3, unreacted imine in fraction 4, exclusively D-arabinose in fractions 5–10, D-ribose and a trace of D-arabinose in fraction 11 and exclusively D-ribose from fraction 12 onwards. Nothing was detected in the effluent collected after fraction 50. A similar separation was achieved with the second half of the product mixture. The fractions that were shown to contain exclusively D-ribose or D-arabinose were independently combined. The water was removed initially by evaporation under vacuum and then by freeze-drying and finally under vacuum over P_2O_5 to leave both **3** (280 mg, 22%) and **4** (336 mg, 27%) as colourless oils with partial crystallisation; in the following, f = furanose and p = pyranose forms; D-[1-^{13}C]ribose **3**: δ_C(62.9 MHz; D_2O) 101.2 (C-1βf, 11.4%)*, 96.5 (C-1αf, 5.8%)*, 94.1 (C-1βp, 62.4%)*, 93.8 (C-1αp, 20.4%)*, 88.2 (C-4αf), 82.7 (C-4βf), 75.5 (C-2βf), 71.3 (C-2βp), 70.7 (C-3βf), 70.3 (C-2αp and C-3αf), 69.5 (C-3αp), 69.3 (C-3βp), 67.6 (C-4αp), 67.4 (C-4βp), 63.2 (C-5αp and C-5βp) and 62.8 (C-5αf and C-5βf); D-[1-^{13}C]-arabinose **4**: δ_C(62.9 MHz; D_2O) 101.3 (C-1αf, 3.6%)*, 97.1 (C-1αp, 61.3%)*, 95.4 (C-1βf, 1.4%)*, 92.9 (C-1βp, 32.9%)*, 72.7 (C-4αp), 72.1 (C-2αp), 70.0 (C-4βp), 68.9 (C-2βp), 68.8 (C-3αp), 68.7 (C-3βp), 66.7 (C-5αp) and 62.7 (C-5βp).

Preparation of a samarium (Sm^{3+}) cation-exchange column and separation of D-ribose and D-arabinose

Dowex 8 × 50W, 200–400, H^+ cation-exchange resin (20 g) was rinsed three times with distilled water (120 cm^3, *ca.* 0.6 vol). The resin was stirred for *ca.* 20 minutes at room temperature with an aqueous solution of samarium(III) chloride hexahydrate (12 cm^3, 25%, *ca.* 0.6 vol) and then poured into a chromatographic column (42 cm × 2.2 cm). The resultant resin bed (6.5 × 2.0 cm) was washed under gravity elution with an aqueous solution of samarium(III) chloride hexahydrate (28 cm^3, 25%, *ca.* 1.4 vol) and then distilled water (800 cm^3). A test mixture of unlabelled D-ribose (200 mg) and D-arabinose (200 mg) was separated on the column as described above leading to the recovery of D-arabinose (200 mg, 100%) and D-ribose (197 mg, 98.5%).

Molybdate catalysed epimerisation of D-arabinose (adapted from ref. 10)

A solution of D-arabinose (250 mg, 1.67 mmol) and molybdenyl acetylacetonate (27 mg, 0.083 mmol) in DMF (16.8 cm^3) was stirred at 50 °C for 24 h. TLC analysis (90 : 10 acetone–water) alongside the authentic compounds revealed both D-arabinose (R_f 0.49) and D-ribose (R_f 0.41) with the former epimer predominating. The reaction mixture was cooled (ice-bath), diluted with water (40 cm^3) and then extracted with dichloromethane (2 × 80 cm^3). The aqueous layer was stirred for 1 h at room temperature with an excess of Dowex 8 × 50W, 200–400, H^+ cation-exchange resin (4 g). The resin was then removed by filtration and washed with water. The combined filtrate and washings were stirred for 1 h at room temperature with an excess of Dowex 1 × 8–50, 20–50 anion-exchange resin (4 g) prepared in the HCO_3^- form. The resin was removed by filtration, washed with water and the combined filtrate and washings were evaporated to leave a pale yellow oil (197 mg). TLC analysis alongside the authentic compounds revealed that the proportion of product epimers was unchanged. A portion of the oil (60 mg) was passed down a cation-exchange column prepared in the samarium form (see above) to give a clean separation of the two epimers. The water was removed from the combined D-arabinose and D-ribose-containing fractions by evaporation and then by freeze-drying to leave D-arabinose (36 mg, 47.3%) and D-ribose (18 mg, 23.6%) as a colourless oil with partial crystallisation; δ_C(62.9 MHz; D_2O) as for authentic D-ribose.

Synthesis of D-[2-^{13}C]ribose 5 (adapted from ref. 11)

A solution of D-[1-^{13}C]arabinose **4** (300 mg, 2.00 mmol) in methanol (25 cm^3) was stirred at 60 °C. Nickel(II) chloride hexahydrate (474 mg, 2.00 mmol) and TEMED (0.6 cm^3, 4.00 mmol) were sequentially added, to form pale green and dark green solutions, respectively. TLC analysis (90 : 10 acetone–water) after 5 min alongside the authentic compounds revealed D-arabinose (R_f 0.37) and D-ribose (R_f 0.47), with the latter epimer predominating, and a faint area of green material at the baseline. After allowing it to cool to room temperature the reaction mixture was diluted with distilled water (90 cm^3) and then maintained at a pH of 6.5 for 1 h, by addition of dilute sulfuric acid (0.5 M) as appropriate, whilst stirring at room temperature. The solution was then stirred for 30 minutes at room temperature with an excess of Dowex 8 × 50W, 200–400, H^+ cation-exchange resin (2.5 g). The resin was removed by filtration and washed with water. The combined filtrate and washings were stirred for 1½ h at room temperature with an excess of Dowex 1 × 8–50, 20–50 anion-exchange resin (2.5 g) prepared in the HCO_3^- form. The resin was removed by filtration, washed with water, and the combined filtrate and washings were evaporated to leave a pale green oil. Most of the remaining nickel-containing material (green) was removed on a silica column (100% acetone) to give the epimeric mixture as a cloudy oil. The sugars were separated in three portions on a cation-exchange column prepared in the samarium form (see above), which also removes any remaining nickel, followed by removal of the water by evaporation and then by freeze drying to give **4** (73 mg, 24%) and **5** (189 mg, 63%) as a colourless oil with partial crystallisation; δ_C(ribose) (250 MHz; D_2O) 75.5 (C-2βf, 11.8%)*, 71.3 (C-2βp, 64.4%)*, 71.2 (C-2αf, 6.0%)* and 70.3 (C-2αp, 17.8%)*; *m*/*z* (EI) 152 (M^+ + H), 120 (5%, $M^+ - CH_2OH$), 74 (90), 61.0 (100); *m*/*z* (ES) 174.0418 (M^+ + Na, required for $C_4{}^{13}CH_{10}O_5Na$ 174.0426).

Synthesis of 1,2,3,5-tetra-*O*-acetyl-D-[2–^{13}C]ribofuranoside 6

This compound was prepared from **5** over three steps *via* 1-*O*-methyl-D-[2-^{13}C]ribofuranoside and 1-*O*-methyl-2,3,5-tri-*O*-acetyl-D-[2-^{13}C]ribofuranoside using the Guthrie–Smith[18] method to give **6** (306 mg, 59.5%) as a pale yellow oil; δ_H(250 MHz; $CDCl_3$) 6.43 (dd, $J_{1\alpha,2\alpha}$ 3.8, $J_{1\alpha,C\text{-}2\alpha}$ 2.6, H-1α), 6.17 (br dd, $J_{1\beta,2\beta} < 1$, $J_{1\beta,C\text{-}2\beta}$ *ca.* 2, H-1β), 5.36 (ddd, $J_{2\beta,3\beta} = J_{3\beta,4\beta}$ 4.7, $J_{3\beta,C\text{-}2\beta}$ 1.9, H-3β), 5.34 (ddd, $J_{1\beta,2\beta} < 1$, $J_{2\beta,3\beta}$ 4.7, $J_{2\beta,C\text{-}2\beta}$ 162.7, H-2β), 5.28 (ddd, $J_{2\alpha,3\alpha}$ 6.8, $J_{3\alpha,4\alpha}$ 3.0, $J_{3\alpha,C\text{-}2\alpha}$ 1.6, H-3α) and 5.24 (ddd, $J_{1\alpha,2\alpha}$ 3.8, $J_{2\alpha,3\alpha}$ 6.8, $J_{2\alpha,C\text{-}2\alpha}$152.3, H-2α), 4.45 (dd, $J_{4\beta,5\beta a}$ 3.5, *J* 6.1, H-4β), 4.34 (dd, $J_{4\beta,5\beta a}$ 3.5, $J_{5\beta a,5\beta b}$ 11.6, H-5βa) (H-4α overlapping), 4.15 (dd, $J_{4\beta,5\beta b}$ 5.0, $J_{5\beta a,5\beta b}$ 11.6, H-5βb) (H-5α a and b overlapping), 2.14, 2.13, 2.12, 2.09 and 2.14, 2.11, 2.10, 2.08 (12H, 8 × s, 4 × CH_3, α and β, respectively); δ_C(62.9 MHz; $CDCl_3$) 170.9, 170.1, 169.8 (d, $J < 5$), 169.4 (d, $J < 5$) (4 × *C*=O, β), 98.5 (C-1β, t, $J_{1\beta,2\beta}$ 23.7), 94.4 (C-1α, t, $J_{1\alpha,2\alpha}$ 22.8), 82.0 (C-4α), 79.6 (C-4β), 74.5 (C-2β)*, 70.6 (C-3β, d, $J_{2\beta,3\beta}$ 20.4), 70.4 (C-2α)*, 70.1 (C-3α), 64.0 (C-5β, d, $J_{2\beta,5\beta} < 2$), 63.7 (C-5α) and 21.4, 21.1, 20.9, 20.8 (4 × *C*H$_3$, β) (4 × *C*H$_3$, α, overlapping); *m*/*z* (ES) 342 (100%, M^+ + Na), 661 (90, $2M^+$ + Na).

Synthesis of [1,3-^{15}N$_2$]uracil 8[12]

A mixture of [^{15}N$_2$]urea **7** (500 mg, 8.06 mmol), propiolic acid (496 µl, 564 mg, 8.06 mmol) and polyphosphoric acid (12.0 g, 5.8 cm^3) was heated at 85 °C (air condenser) with gentle stirring for 4 h to give a yellow syrup. The mixture was cooled (ice-bath) and water (24 cm^3) was slowly added. The flask was swirled to achieve complete dissolution of the syrup, on which a pale solid began to form. The flask was kept at 4 °C for *ca.* 24 h, then the solid was collected by filtration, washed with a little water and dried under vacuum over P_2O_5 to give **8** (532 mg, 57.9%) as a cream coloured solid; δ_H(250 MHz; DMSO-d_6) 11.06 (1H, d, $J_{NH\text{-}3,N\text{-}3}$ 89.8, N*H*-3), 10.90 (1H, dd, $J_{NH\text{-}1,N\text{-}1}$ 92.5, $J_{NH\text{-}1,N\text{-}3/6}$ 5.3, N*H*-1), 7.40 (1H, m, H-6) and 5.46 (1H, m, H-5); (unlabelled: 11.06 (1H, br s, N*H*-3), 10.86 (1H, br s, N*H*-1), 7.41 (1H, dd, $J_{5,6}$ 7.6, $J_{6,N\text{-}1}$ 5.6, H-6) and 5.46 (1H, dd, $J_{5,6}$ 7.6, $J_{5,N\text{-}1/3}$ 1.6, H-5)); δ_C(62.9 MHz; DMSO-d_6) 164.7 (C-4, d, $J_{4,N\text{-}3}$ 11.4), 151.9 (C-2, dd, $J_{2,N\text{-}1}$ 18.6, $J_{2,N\text{-}3}$ 16.3), 142.6 (C-6, d, $J_{6,N\text{-}1}$ 11.0) and 100.6 (C-5, d, $J_{5,N\text{-}3/1}$ 6.2); *m*/*z* (EI) 114 (100%, M^+).

Synthesis of 2′,3′,5′-tri-*O*-acetyl-[2′-^{13}C, 1,3-^{15}N$_2$]uridine 10 (adapted from ref. 13)

A suspension of [1,3-^{15}N$_2$]uracil **8** (196 mg, 1.72 mmol) in anhydrous acetonitrile (3.7 cm^3) was stirred at room temperature with *N*,*O*-bis(trimethylsilyl)acetamide (937 µl, 769 mg, 3.78 mmol). After 0.5 h the uracil had not completely dissolved, so further *N*,*O*-bis(trimethylsilyl)acetamide (500 µl) was added. After *ca.* 1 h the uracil had completely dissolved to give a pale yellow solution. A solution of 1,2,3,5-tetra-*O*-acetyl-D-[2-^{13}C]-ribofuranoside **6** (306 mg, 0.96 mmol) in anhydrous acetonitrile (9.1 cm^3) was added to the solution of silylated uracil. The combined solution was cooled (ice-bath) and tin(IV) chloride (244 µl, 537 mg, 2.06 mmol) was added dropwise with stirring, which was continued at room temperature. After 18.5 h, further tin(IV) chloride (300 µl) was added. After a total reaction time of 22.5 h the reaction mixture was cooled (ice-bath) and a solution of sodium hydrogen carbonate (1.0 g) in water (3.4 cm^3) was added dropwise with continued stirring. The resultant white precipitate was removed by filtration and washed with acetonitrile. The combined filtrates were evaporated to leave a pale yellow oil. The protected nucleoside was separated from unreacted starting materials by gradient elution on a silica column (99 : 1–90 : 10 chloroform–methanol) and then further purified on a second silica column (99 : 1 chloroform–methanol) to give **10** (222 mg, 62.0%) as a pale yellow oil; δ_H(250 MHz; DMSO-d_6) 9.55 (1H, d, $J_{NH\text{-}3,N\text{-}3}$ 91.1, N*H*-3), 7.44 (1H, dd, $J_{5,6}$ 8.2, $J_{5,NH\text{-}1/3}$ 2.0, H-5), 6.06 (1H, t, *J* 4.3, H-1′), 5.82 (1H, m, *J* 2.2, 2.3, 2.6, 2.7, H-6), 5.35 (1H, dt, $J_{1',2'} = J_{2',3'}$ 5.7, $J_{2',C\text{-}2'}$ 156.5, H-2′), 5.35 (1H, m, H-3′), 4.36 (3H, m, overlapping H-4′, H-5′a and H-5′b) and 2.16, 2.14, 2.11 (9H, 3 × s, 3 × CH_3); (unlabelled: 9.55 (1H, br s, N*H*-3), 7.42 (1H, d, $J_{5,6}$ 8.2, H-5), 6.06 (1H, d, $J_{1',2'}$ 4.9, H-1′), 5.82 (1H, d, $J_{5,6}$ 8.1, H-6), 5.36 (2H, m, overlapping H-2′ and H-3′), 4.36 (3H, m, overlapping H-4′, H-5′a and H-5′b) and 2.16, 2.14, 2.11 (9H, 3 × s, 3 × CH_3); δ_C(62.9 MHz; DMSO-d_6) 170.2, 169.7 (× 2) (3 × *C*=O), 162.9 (C-4, d, $J_{4,N\text{-}3}$ 9.5), 150.3 (C-2, t, $J_{2,N\text{-}1/3}$ 18.7), 139.3 (C-6, d, $J_{6,N\text{-}1}$ 12.6), 103.5 (C-5, d, $J_{5,N\text{-}3/1}$ 6.9), 87.4 (C-1′, td, $J_{1',2'}$ 21.9, $J_{1',N\text{-}1}$ 13.6), 79.5 (C-4′, d, $J_{2',4'}$ 1.1), 72.7 (C-2′)*, 70.7 (C-3′, d, $J_{2',3'}$ 19.3), 63.2 (C-5′) and 20.8, 20.5, 20.4 (3 × CH_3); R_f (90 : 10 chloroform–methanol) 0.57.

Synthesis of [2′-^{13}C, 1,3-^{15}N$_2$]uridine 11

Sodium methoxide anhydrous powder (13 mg, 0.24 mmol) was added to a stirred solution of 2′,3′,5′-tri-*O*-acetyl-[2′-^{13}C, 1,3-^{15}N$_2$]uridine **10** (150 mg, 0.40 mmol) in anhydrous methanol (5.3 cm^3); stirring was continued at room temperature for *ca.* 30 h. The reaction mixture was neutralised by stirring at room temperature with Amberlite IR-120 (strongly acidic) cation-exchange resin. The resin was removed by filtration, washed with anhydrous methanol and the combined filtrates were evaporated to leave the deprotected nucleoside as a pale yellow oil (97 mg, 98%). Crystallisation was achieved by cooling the oil in an ice bath and adding a little ice-cold methanol. Scratching with a glass rod induced the formation of pale crystals; these were collected by filtration to give **11** (1st crop 16.3 mg, 2nd crop 14.3 mg, total 31.0%) as pale cream plates; δ_H(250 MHz; DMSO-d_6) 11.30 (1H, br undefined d, N*H*-3), 7.87 (1H, dd, $J_{5,6}$ 8.1, $J_{5,NH\text{-}1/3}$ 2.1, H-5), 5.76 (1H, m, H-1′), 5.63 (1H, ddd, $J_{5,6}$ 8.1, $J_{6,N\text{-}1}$ 4.7, $J_{6,N\text{-}3}$ 2.4, H-6), 5.38 (1H, unresolved m, OH-2′), 5.10 (2H, m, overlapping OH-3′ and OH-5′), 4.00 (1H, dm, $J_{2',C\text{-}2'}$ 147.8, H-2′), 3.94 (1H, m, H-3′), 3.82 (1H,

m, H-4′) and 3.56 (2H, m, H-5′a and H-5′b); (unlabelled: 11.33 (1H, br s, N*H*-3), 7.92 (1H, d, $J_{5,6}$ 8.1, H-5), 5.78 (1H, d, $J_{1',2'}$ 5.3, H-1′), 5.66 (1H, d, $J_{5,6}$ 8.1, H-6), 5.42 (1H, d, $J_{2',2'\text{-OH}}$ 5.6, OH-2′), 5.14 (2H, m, overlapping OH-3′ and OH-5′), 4.03 (1H, q, $J_{1',2'} = J_{2',3'}$ 5.3, H-2′), 3.98 (1H, q, *J* 4.6 and 4.3, H-3′), 3.85 (1H, q, *J* 3.1 and 3.5, H-4′) and 3.60 (2H, m, H-5′a and H-5′b); δ_C(62.9 MHz; DMSO-d_6) 163.5 (C-4), 151.1 (C-2), 141.1 (C-6), 102.1 (C-5), 88.0 (C-1′), 85.2 (C-4′), 73.9 (C-2′)*, 70.2 (C-3′) and 61.2 (C-5′); *m/z* (EI) 247 (19%, M^+), 229 (39, $M^+ - OH + H$), 134 (5, $M^+ -$ base + H), 114 (100, $M^+ -$ sugar + H); *m/z* (ES) 270.0568 ($M^+ + Na$, required for $C_8{}^{13}CH_{12}{}^{15}N_2O_6Na$ 270.0567); R_f (90 : 10 chloroform–methanol) 0.05.

Acknowledgements

We are grateful to the Engineering and Physical Sciences Research Council (EPSRC) for a studentship (S. G. P.) and to the Wellcome Trust, the Biotechnology and Biological Sciences Research Council (BBSRC), SmithKline Beecham Pharmaceuticals and Glaxo Wellcome (now GlaxoSmithKline), the Royal Society and the University of Leeds for financial support of this research. This work was carried out as part of the BBSRC-funded North of England Structural Biology Centre. We thank Dr A. E. Ashcroft for mass spectra, Dr K. Seshadri for assistance in the calculation of distances from crystal structure coordinates and Professor S. A. Baldwin for discussions on nucleoside transporters.

References

1 P. J. F. Henderson, Function and Structure of Membrane Transport Proteins in *The Transporter Factsbook*, ed. J. K. Griffith and C. E. Sansom, Academic Press, London, 1998.
2 J. E. Craig, Y. Zhang and M. P. Gallagher, *Mol. Microbiol.*, 1994, **11**, 1159.
3 S. E. Westh-Hansen, N. Jensen and A. Munch-Peterson, *Eur. J. Biochem.*, 1987, **168**, 385.
4 S. G. Patching, PhD thesis, Chemical and Solid State NMR Approaches to Determine Structure-Activity Relationships for Substrates Bound to the Nucleoside Transport Proteins, NupC and NupG, of *Escherichia coli*, University of Leeds, 2002.
5 P. J. R. Spooner, N. G. Rutherford, A. Watts and P. J. F. Henderson, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 3877.
6 (*a*) P. J. R. Spooner, W. J. O' Reilly, S. W. Homans, N. G. Rutherford, P. J. F. Henderson and A. Watts, *Biophys. J.*, 1998, **75**, 2794; (*b*) A. N. Appleyard, R. B. Herbert, P. J. F. Henderson, A. Watts and P. J. R. Spooner, *Biochim. Biophys. Acta*, 2000, **1509**, 55; (*c*) H. Venter, A. E. Ashcroft, J. N. Keen, P. J. F. Henderson and R. B. Herbert, *Biochem. J.*, 2002, **363**, 243; (*d*) J. A. Watts, A. Watts and D. A. Middleton, *J. Biol. Chem.*, 2001, **276**, 43197; (*e*) D. A. Middleton, Z. Ahmed, C. Glaubitz and A. Watts, *J. Magn. Reson.*, 2000, **147**, 366; (*f*) D. A. Middleton, S Rankin, M. Esmann and A. Watts, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 13602.
7 E. A. Green, R. D. Rosenstein, R. Shiono, D. J. Abraham, B. L. Trus and R. E. Marsh, *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.*, 1975, **31**, 102.
8 A. S. Serianni, H. A. Nunez and R. Barker, *Carbohydr. Res.*, 1979, **72**, 71.
9 Y. Israeli, C. Lhermet, J. Morel and N. Morel-Desrosiers, *Carbohydr. Res.*, 1996, **289**, 1.
10 M. L. Hayes, N. J. Pennings, A. S. Serianni and R. Barker, *J. Am. Chem. Soc.*, 1982, **104**, 6764.
11 T. Tanase, F. Shimizu, M. Kuse, S. Yano, M. Hiddai and S. Yoshikawa, *Inorg. Chem.*, 1988, **27**, 4085.
12 K. L. Anderson-Altmann, C. G. Phung, S. Marromoustakos, Z. W. Zheng, J. C. Facelli, C. D. Poulter and D. M. Grant, *J. Phys. Chem.*, 1995, **99**, 10454.
13 (*a*) K. L. Anderson-Altmann, C. G. Phung, S. Marromoustakos, Z. Zhang, J. C. Facelli, C. D. H. Vorbrüggen and G. Höfle, *Chem. Ber.*, 1981, **114**, 1256; (*b*) M. Saneyoshi and E. Satoh, *Chem. Pharm. Bull.*, 1979, **27**, 2518; (*c*) U. Niedballa and H. Vorbrüggen, *J. Org. Chem.*, 1976, **41**, 2084.
14 T. Gullion and J. Schaefer, *J. Magn. Reson.*, 1989, **81**, 196.
15 J. M. Goetz and J. Schaefer, *J. Magn. Reson.*, 1997, **127**, 147.
16 The fractional cell coordinates were transformed into orthogonal cartesian coordinates by means of a suitable transformation matrix using the cell parameters *a*, *b*, *c* and α, β, γ. A Fortran code was used to accomplish this.
17 M. W. L. Ritzel, S. Y. M. Yao, M-Y. Huang, J. F. Elliot, C. E. Cass and J. D. Young, *Am. J. Physiol.*, 1997, **272**, C707.
18 R. D. Guthrie and S. C. Smith, *Chem. Ind. (London)*, 1968, 547.