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Research Article

Synthesis of multiply labelled ribonucleosides for sequence-specific labelling of oligo-RNA

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Summary

The synthesis of ribonucleotide blocks multiply labelled with ^{2}H, ^{13}C and ^{15}N for solid support synthesis of sequence specifically labelled RNA is described. Labels were introduced in the ribose ring (^{13}C), C5 position of pyrimidine nucleobases (^{2}H) and exocyclic amino groups (^{15}N) and serve as multiple probes for studying the various physicochemical consequences of physiologically important RNA folding by high-resolution multi-nuclear NMR spectroscopy. Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: ribonucleoside; multiple labelling; stable isotopes; ^{2}H; ^{13}C; ^{15}N; oligo-RNA

Introduction

Labelling of biologically important macromolecules like proteins and nucleic acids with stable NMR active isotopes has contributed immensely to our understanding of the influence of the biopolymer structure and function.[1–4] The initial approach was to label these

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molecules *via* enzymatic means, which gives mainly uniformly labelled molecules.[5–9] Although such uniform labelling has led to many important results, this method has its intrinsic limitations in that no segmental labelling can be easily achieved, and the problems of overcrowding of resonances become acute (specially for large RNA with repeating sugar and aglycon units) as the molecular size increases. Such labelling, although very productive,[5–9] has serious limitations.

Specifically labelled oligonucleotides[10–18] appear to be the best means for studies of this kind as problems associated with crowding of spectral lines are overcome and the potential of modern NMR methods can be fully exploited.[19–21]

Since the preparation of labelled oligonucleotides involves considerable expense and labor,[22–26] it would be of great value to collect as many label-related data as possible from one synthesized oligomer.

Multiple labelling of nucleosides has already been used with great success.[22,27–33] Usually multiple labelling was limited to one part of the nucleoside moiety, either the ribose or base. The nucleoside with the label introduced into both of these parts will serve as an even more versatile component for producing multilabelled RNA for NMR studies. The label arrangements we report here are as follows: ^{13}C labelling of all ribose carbon atoms, deuteriation of the C5 of pyrimidines and ^{15}N labelling of the exocyclic amino groups of A, C and G residues. All of these labels should be introduced at a level close of 100% isotopic abundance in order to avoid interference from the stray signals.

Experimental

All reagents were from Aldrich and, unless otherwise stated, were used without further purification. Labelled $^{15}NH_4Cl$ (99% isotopic purity) was from Martek Biosciences Corp. (USA), labelled $KC^{15}N$ (98% isotopic purity) was from Aldrich. Adenosine deaminase was purchased from Sigma (cat. no. A1030). The 1-O-acetyl-2,3,5-tri-O-toluoyl-β-D-[1,2,3,4,5-$^{13}C_5$]-ribofuranose for the synthesis of labelled nucleosides was synthesized according to our earlier procedure.[34] Methylene chloride, ethyl acetate, cyclohexane, hexane, acetone, methanol and ethanol were distilled prior to use. Triethylamine and *N*,*N*-diisopropylethylamine (DIPEA) were distilled from CaH_2 and stored over molecular sieves (4 Å). Toluene and pyridine were refluxed over CaH_2

for 6 h, distilled with exclusion of moisture and stored over molecular sieves (4 Å). THF was refluxed over CaH_2 under nitrogen and distilled immediately before use with rigorous exclusion of moisture. NMR spectra were recorded on a Jeol GX 270 spectrometer operating at 270.2 MHz for ^{1}H, 67.9 MHz for ^{13}C, 27.0 MHz for ^{15}N and 109.4 MHz for ^{31}P. Chemical shifts (δ) are reported in ppm relative to TMS (internal standard) for ^{1}H and ^{13}C, to [^{15}N]-nitromethane for ^{15}N and H_3PO_4 (external) for ^{31}P spectra.

Mass spectra were recorded with AMD 604 spectrometer, using FAB technique with nitrobenzyl alcohol matrix.

*6-Chloro-N^9-(2-tetrahydropyranyl)-purine (**2**).* Anhydrous ethyl acetate (40 ml) was warmed to 50°C and 6-chloropurine (**1**) (1.75 g, 11.31 mmol) and *p*-toluenesulfonic acid (68 mg, 0.026 mmol) were added. The mixture was stirred and 2,3-dihydro-2*H*-pyran (1.35 ml, 1.24 g, 14.76 mmol) was added dropwise over 30 min, maintaining the reaction temperature between 55 and 60°C. The solution was stirred for an additional hour during which time it was allowed to cool to room temperature. Concentrated aqueous ammonia (2.3 ml) was added and the solution stirred for 5 min. It was then extracted with water (2×20 ml). The ethyl acetate solution was dried over anhydrous $MgSO_4$ and the solvent removed in a rotary evaporator. The remaining syrupy residue was purified by chromatography on SiO_2 using a gradient of methanol (0–4%) in methylene chloride giving **2** (2.43 g, 10.18 mmol, 90%). ^{1}H-NMR ($CDCl_3$): 8.76 (s, 1H), 8.35 (s, 1H), 5.81 (m, 1H), 3.81–4.20 (m, 2H), 1.50–2.24 (m, 6H); MS (FAB) 239.1; 241.0 (MH^+).

*N^9-(2-Tetrahydropyranyl)-[6-^{15}N]-adenine (**3**).* A mixture of **2** (1.06 g, 4.44 mmol), $^{15}NH_4Cl$ (0.475 g, 8.89 mmol), and $NaHCO_3$ (1.116 g, 13.29 mmol) in DMSO (6.75 ml) was sealed in a 15 ml vial which was kept in an oven at 80°C for 6 days. The cooled (0°C) reaction vial was opened carefully and the resulting suspension was poured into a big Petri dish. The major part of the DMSO was removed by hot air flushing. The residue was suspended in methylene chloride, transferred onto a silica column and 0–4% gradient of methanol in methylene chloride was applied to elute the pure product. Appropriate fractions were collected and after removing solvents **3** was obtained (0.921 g, 4.20 mmol, 95%). ^{1}H-NMR ($CDCl_3$): 8.37 (s, 1H), 8.05 (s, 1H), 6.36 (d, $J_{N-H} = 89.8$ Hz, 2H) NH_2, 5.72 (m, 1H), 3.78 (m, 2H), 1.63–2.14 (m, 6H). ^{13}C-NMR ($CDCl_3$): 155.4 (d, $J_{N-C} = 20.1$ Hz, C-6), 152.7, 149.2, 138.2, 119.4 (d, $J_{N-C} = 4.0$ Hz, C-5), 81.8, 68.7, 31.8, 24.8, 22.7.

^{15}N-NMR ($CDCl_3$): −298.5. HRMS (FAB^+) found 221.11590, calculated for MH^+ ($C_{10}H_{14}ON_4{}^{15}N$) 221.11678.

*[6-^{15}N]-Adenine (**4**).* To **3** (0.921 g, 4.18 mmol) dissolved in 1,4-dioxane (15 ml) 0.01 M HCl (30 ml) was added and the pH was adjusted to 1.5 with 1 M HCl solution. After 24 h the reaction mixture was diluted with water (100 ml) and concentrated to 30 ml to remove dioxane (repeated three times). It was neutralized with Dowex-1 (HCO_3^- form) (100 ml). The product was eluted from the resin with water (2 l) and concentrated to **4** as yellowish solid that was not further purified. ^{1}H-NMR (DMSO-d_6): 12.62 (br. s, 1H) NH-9, 8.13, 8.14 (2 × s, 2H) H-2 & H-8, 7.16 (d, J_{N-H} = 89.7 Hz, 2H), NH_2. ^{13}C-NMR (DMSO-d_6): 155.2 (d, J_{N-C}= 20.1 Hz, C-6), 152.4, 151.4, 139.4, 117.4. ^{15}N-NMR (DMSO-d_6): −299.1. HRMS (FAB^+) found 137.05843, calculated for MH^+ ($C_5H_6N_4{}^{15}N$) 137.05931.

*N^6-Benzoyl-[6-^{15}N]-adenine (**5**).* A mixture of **4** (4.18 mmol) and benzoic anhydride (1.91 g 8.46 mmol) was heated at 160°C for 2 h, then cooled, dissolved in ethanol (20 ml) and heated under reflux for 30 min. The reaction mixture was concentrated to dryness and then purified by short column chromatography on silica gel (gradient 0–10% of methanol in methylene chloride) giving **5** (0.776 g, 3.24 mmol, 77% from **3**). ^{1}H-NMR (DMSO-d_6): 12.42 (br. s, 1H) NH-9, 11.55 (br. d, J_{N-H}= 72 Hz, 1H) NH-6, 8.76, (s, 1H), 8.53, (s, 1H), 8.14 (d, 2H), 7.68 (m, 1H), 7.58 (m, 2H). ^{13}C-NMR (DMSO-d_6): 166.5 (d, J_{N-C}= 12.4 Hz, C-6), 151.0, 145.9, 144.7 (br., C-4), 132.6, 128.4, 128.4, 114.5 (br., C-5). ^{15}N-NMR (DMSO-d_6): −243.5. HRMS (FAB^+) found 241.08349, calculated for MH^+ ($C_{12}H_{10}ON_4{}^{15}N$) 241.08557.

*9-(β-D-Ribfuranosyl)-[2-amino-6-(N-methoxy)-amino]-[2-^{15}N]-purine (**8**).* Potassium ^{15}N-cyanide (2 g, 30.23 mmol) was dissolved in anhydrous methanol (500 ml), cooled to 0°C and bromine (1.55 ml, 4.84 g, 30.26 mmol) was added with stirring. Stirring and cooling was continued for 3 h, when solid **6**[27] (5.62 g, 19.86 mmol) was added. The suspension was stirred at 0°C for 1 h and at room temperature for an additional 8 h, and the solid became gradually dissolved. Volatiles were evaporated and the residue was dried in vacuo. The crude **7** (12.5 g) was dissolved in dry DMF (75 ml), and triethylamine (7 ml, 5.08 g, 50.2 mmol) was added. The flask was flushed with argon and the mixture was left for 45 min in darkness. Then iodomethane (3 ml, 6.84 g, 48.2 mmol) was added and the mixture was left in darkness for 5 h. The solvent was evaporated and the dry residue was dissolved in 0.25 N NaOH (300 ml). After 1 h the base was neutralized with 1 M HCl

(pH 7.5). An equal volume of ethanol was added, and the solution was heated at 60°C for 5 h. The solution was concentrated to 30 ml and was applied onto column of Dowex 50 (H^+) resin (6 × 20 cm). The column was washed with 0.05 M HCl (1 l), water (1 l) and then the product was eluted with 3% aqueous ammonia (1 l). Evaporation of the ammonia fraction gave **8** (4.5 g, 14.36 mmol, 72%) as a light brown foam. ^{1}H-NMR (DMSO-d_6): 7.73 (s, 1H), 6.43 (br. d, J_{N-H}= 78 Hz, 2H), 5.62 (d, J= 5.7 Hz, 1H), 4.33 (m, 1H), 4.07 (m, 1H), 3.81 (m, 1H), 3.72 (s, 3H), 3.54 (m, 2H). ^{15}N-NMR (DMSO-d_6): –247.7 ppm. HRMS (FAB^+) found 314.13754, calculated for MH^+ ($C_{11}H_{17}N_5{}^{15}NO_5$) 314.12295.

*[2-^{15}N]-Guanosine (**9**)*. The ^{15}N labelled 9-(β-D-ribfuranosyl)purine derivative **8** (4.1 g, 13.1 mmol) was dissolved in 0.1 M phosphate buffer at pH 7.4 (300 ml) and adenosine deaminase (720 units) was added. The reaction mixture was gently shaken at 37°C for 90 h. After keeping at 0°C overnight, the precipitate was filtered and washed with small amount of water to afford guanosine **9** (2.61 g, 9.21 mmol, 69%). ^{1}H-NMR (D_2O): 8.06 (s, 1H), 6.04 (br. d, 1H), 4.83 (t, 1H), 4.49 (t, 1H), 4.35 (m, 1H), 3.83 (m, 1H), 3.54 (m, 1H). ^{15}N-NMR (D_2O): −228.7. MS (FAB^+) 285.1 (MH^+).

*[2-^{15}N]-Guanine (**10**)*. [2-^{15}N]-Guanosine **9** (2.61 g, 9.1 mmol) was suspended in 1 M HCl and was stirred at 100°C for 90 min. The reaction mixture was cooled, neutralized with 1 M $NaHCO_3$ (pH 7.5) and left overnight at 4°C. The precipitate was filtered, washed with cold water and dried to give guanine **10** (1.05 g, 6.90 mmol, 76%). MS (FAB^+) found 153.05504, calculated for MH^+ ($C_5H_6N_4{}^{15}NO$) 153.05422.

*N^2-Isobutyryl-[2-^{15}N]-guanine (**11**)*. Thoroughly dried (100°C, 1 torr) labelled guanine **10** (1.05 g, 6.90 mmol) was suspended in dimethylacetamide (15 ml), isobutyric anhydride (3.5 ml, 3.34 g, 21.1 mmol) was added and the mixture was heated at 150°C for 2.5 h. The solution was cooled and evaporated to dryness. The residue was co-evaporated with methanol and recrystallized from ethanol-water. The product **11** (1.15 g, 5.17 mmol, 75%) was separated as buff-coloured crystals. ^{1}H-NMR (DMSO-d_6): 13.14 (br. s, 1H), 12.08 (s. 1H), 11.56 (br. d, J_{N-H}= 79 Hz, 1H), 8.04 (s, 1H), 2.76 (sept, J= 6.8 Hz, 1H), 1.12 (d, 6, J= 6.8 Hz, 6H). ^{13}C-NMR (DMSO-d_6): 180.0 (d, J_{N-C}= 10.4 Hz, *C*(O)iBu), 147.3 (d, J_{N-C}= 22 Hz, *C*-2), 34.7; 18.9. ^{15}N-NMR (DMSO-d_6): −244.7. HRMS (FAB^+) found 223.09620, calculated for MH^+ ($C_9H_{12}N_4{}^{15}NO_2$) 223.09613.

*N^2-Isobutyryl-O^6-diphenylcarbamoyl-[2-^{15}N]-guanine (**12**)*. Dried **11** (1.15 g, 5.17 mmol) was suspended in dry DMF (6 ml), acetic anhydride

(1.1 ml, 1.19 g, 11.65 mmol) was added and the mixture was heated at 100°C for 45 min. The clear solution was evaporated to dryness and the solid residue was suspended in dioxane (15 ml) and stirred overnight. Crystals were filtered and dried (1.16 g). The acetyl derivative was co-evaporated with pyridine (3×10 ml), suspended in pyridine (16 ml), DIPEA (1.55 ml, 1.15 g, 8.8 mmol) and diphenylcarbamoyl chloride (1.12 g, 4.8 mmol) were added and stirring was maintained at room temperature for 1.5 h. Water (1.5 ml) was added, and after 10 min the solution was evaporated. The residue was boiled with ethanol-water (16 ml) and after cooling crystalline product **12** (1.72 g, 4.12 mmol, 79%) was collected. ^{1}H-NMR (DMSO-d_6): 13.53 (br. s, 1H), 10.56 (d, J_{N-H}= 89 Hz, 1H), 8.47 (s, 1H), 7.25–7.51 (m, 10H), 2.78 (sept, J= 6.8 Hz, 1H), 1.09 (d, J= 6.8 Hz, 6H). ^{13}C-NMR (DMSO-d_6): 174.7 (d, J_{N-C}= 10 Hz, *C*(O)iBu), 152.2 (d, J_{N-C}= 21.5 Hz, C-2), 150.3 (6-*C*(O)O), 141.9; 129.6, 129.3, 127.5, 34.6, 19.2. ^{15}N-NMR (DMSO-d_6): −235.7. HRMS (FAB$^+$) found 418.16335, calculated for MH$^+$ ($C_{22}H_{21}N_5{}^{15}NO_3$) 418.16455.

Synthesis of 2′,3′,5′-tri-O-(4-toluoyl) [1′,2′,3′,4′,5′-$^{13}C_5$]-nucleosides **13a–d** were carried out according to the reported procedure[34].

*2′,3′,5′-Tri-O-toluoyl-N^6- benzoyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(6-^{15}N)]-adenosine (**13a**).* Labelled acylated ribose (2.34 g, 4.25 mmol) and compound **5** (1.22 g, 5.1 mmol) gave **13a** (2.35 g, 3.14 mmol, 74% on the basis of labelled sugar) as a foam. ^{1}H-NMR ($CDCl_3$): 8.97 (d, J_{N-H}= 89 Hz, 1H), 8.73 (s, 1H), 8.17 (s, 1H), 8.02–7.15 (m, 17H), 6.55 (br. d, J_{C-H}= 166 Hz, 1H), 6.37 (br. d, J_{C-H}= 156 Hz, 1H), 6.22 (br. d, J_{C-H}= 158 Hz, 1H), 4.83 (br. d, J_{C-H}= 151 Hz, 1H), 4.90 (br. d, J_{C-H}= 153 Hz, 1H), 4.68 (br. d, J_{C-H}= 148 Hz, 1H). ^{13}C-NMR ($CDCl_3$): 165.3 (d, J_{C-N}= 16.5 Hz, *C*(O)Bz), 149.6 (d, J_{C-N}= 19.8 Hz, C-6), 86.6 (d, J_{C-C}= 43.6 Hz, C-1′); 80.9 (dd, J_{C-C}= 38.9 Hz, J_{C-C}= 43.1 Hz, C-4′), 73.6 (dd, J_{C-C}= 37.5 Hz, J_{C-C}= 43.6 Hz, C-2′); 71.2 (dd, J_{C-C}= 37.5 Hz, J_{C-C}= 38.9 Hz, C-3′), 63.2, (d, J_{C-C}= 43.1 *Hz*, C-5′). ^{15}N-NMR ($CDCl_3$): −248.0. HRMS (FAB$^+$) found 732.26871, calculated for MH$^+$ ($C_{36}{}^{15}C_5H_{36}N_4{}^{15}NO_8$) 732.26994.

*2′,3′,5′-Tri-O-(4-toluoyl)-N^2-isobutyryl-O^6-(diphenylcarbamoyl)-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(2-^{15}N)]-guanosine (**13b**).* Labelled acylated ribose (1.98 g, 3.58 mmol) and compound **12** gave **13b** (1.95 g, 2.15 mmol, 60%) as a foam. ^{1}H-NMR ($CDCl_3$): 8.20 (d, J_{H-N}= 89 Hz, 1H), 8.07 (s, 1 H, H-8), 7.95–7.13 (m, 22H), 6.34 (br. d, J_{C-H}= 151 Hz, 1H), 6.30 (br. d, J_{C-H}= 158 Hz, 2H) 4.86 (br. d, J_{C-H}= 149 Hz, 2H), 4.68 (br. d,

J_{C-H}= 147 Hz, 1H), 2.47, 2.41, 2.37 (3 × s, 3 × 3 H, toluoyl). ^{1}C-NMR (DMSO-d_6): 174.7 (d, J_{N-C}= 10 Hz, *C*(O)iBu), 152.2 (d, J_{N-C}= 21.5 Hz, C-2), 150.3 (6-*C*(O)O), 141.9, 129.6, 129.3, 127.5, 87.0 (d, J_{C-C}= 43.6 Hz, C-1′), 80.9 (dd, J_{C-C}= 37.1 Hz,J_{C-C}= 43.7 Hz, C-4′), 74.1 (dd, J_{C-C}= 43.6 Hz, J_{C-C}= 39.5 Hz, C-2′), 71.4 (dd, J_{C-C}= 39.5 Hz, J_{C-C}= 37.1 Hz C-3′), 63.6 (d, J_{C-C}= 43.7 Hz, C-5′), 34.6, 19.2, ^{15}N-NMR ($CDCl_3$): −238.7.

*2′,3′,5′-Tri-O-(4-toluoyl)-[1′,2′,3′,4′,5′-$^{13}C_5$]-uridine (**13c**)*. Labelled acylated ribose (8.32 g, 15.10 mmol) and uracil (2.03 g, 18.20 mmol) gave **13c** (7.28 g, 12.07 mmol, 80%) as white foam. ^{1}H NMR($CDCl_3$): 8.79 (s, 1H), 7.95–7.13 (m, 13 H, touoyl, H-6), 6.30 (br. d, J_{C-H}= 167 Hz, 1H) H-l′, 5.89 (br. d, J_{C-H}= 163 Hz, 1H) H-3′, 5.66 (m, 1H) H-2′, 5.58 (d, J_{H6-H5}= 6 Hz, 1H) H-5, 4.74 (br. d, J_{C-H}= 150 Hz, 1H) H-5′, 4.62 (m, 1H) H-4′, 4.55 (br. d, J_{C-H}= 153 Hz, 1H) H-5″, 2.41, 1.39, 2.36 (3 × s, 3 × 3 H, toluoyl). ^{13}C-NMR ($CDC1_3$): 87.6 (d, J_{C-C} =43.7 Hz, C-l′), 80.8, (dd, J_{C-C}= 39.5 Hz, J_{C-C} = 43.1 Hz, C-4′), 73.6 (dd, J_{C-C}= 43.7 Hz, J_{C-C}= 40.5 Hz, C-2′), 71.1 (dd, J_{C-C}= 40.5 Hz, J_{C-C}= 39.5 Hz, C-3′), 63.6, (d, J_{C-C}= 43.1 Hz, C-5′).

*2′,3′,5′-Tri-O-(4-toluoyl)-N^2-isobutyryl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(2-^{15}N)]-guanosine (**13d**)*. The diphenylcarbamoyl derivative **13b** (3.63 g, 4.00 mmol) was dissolved in 90% CF_3COOH and left at room temperature for 15 min. Volatiles were evaporated, the remaining oil was dissolved in CH_2Cl_2 (20 ml) and washed with $NaHCO_3$, water, dried ($MgSO_4$) and evaporated to a foam, which was purified by column chromatography (methylene chloride with 0–2% methanol) to yield **13d** (2.37 g, 3.32 mmo1, 83%). ^{1}H-NMR ($CDCl_3$): 9.3 (d, J_{N-H}= 91 Hz, 1H), 8.0–7.14 (m, 13 H, toluoyl, H-8), 6.55 (br. d, J_{C-H}= 157 Hz, 1H), 6.33 (br. d, J_{C-H}= 160 Hz, 1H), 6.16 (br. d, J_{C-H}= 167 Hz, 1H), 4.85 (m, 3H), 2.67 (sept, J= 6.9 Hz, 1H), 2.41, 2.39 (2 × s, 3 + 6 H, toluoyl), 1.31 (d, J= 6.9 Hz, 6H). ^{13}C-NMR ($CDCl_3$): 165.2 (d, J_{N-C}= 16.5 Hz, *C*(O)iBu), 155.3, 149.6 (d, J_{N-C}= 19.7 Hz, C-2), 147.8, 138.4, 122.6, 87.7 (d, J_{C-C}= 44.1 Hz, C-l′), 80.2 (dd, J_{C-C}= 35.9 Hz, J_{C-C}= 39.2 Hz, C-4′), 73.1 (dd, J_{C-C}= 43.6 Hz, J_{C-C}= 39.5 Hz, C-2′), 70.8 (dd, J_{C-C}= 39.5 Hz, J_{C-C}= 37.1 Hz, C-3′), 62.9 (d, J_{C-C}= 43.7 Hz, C-5′. ^{15}N-NMR ($CDCl_3$): −245.3. HRMS (FAB^+) found 714.28223, calculated for MH^+ ($C_{33}{}^{13}C_5H_{38}N_4{}^{15}NO_9$) 714.28049.

*N^6-Benzoyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(6-^{15}N)]-adenosine (**14a**)*. Compound **13a** (4.96 g, 6.63 mmol) was dissolved in ethanol-pyridine (20 + 20 ml), 2 M NaOH and ethanol (24 + 24 ml) were added and the mixture stirred for 6 min at room temperature. Dowex 50 resin

(pyridinium form) was added to neutralize the base (approx. 80 ml). The suspension was filtered, the resin was washed with ethanol and pyridine (100 + 100 ml) and the filtrate was evaporated. The solid residue was triturated with diethyl ether (2 × 30 ml) and methylene chloride (2 × 30 ml) and filtered. The product **14a** (2.49 g, 99%) was obtained as a white solid and it was used without further purification. ^{1}H-NMR (DMSO-d_6): 11.26 (br, 1H), 8.78, 8.74 (2 × s, 2 × 1H), 6.06 (br. d, J_{C-H}= l65 Hz), 4.68 (br. d, J_{C-H}= 142 Hz), 4.20 (br. d, J_{C-H}= 144 Hz), 4.00 (br. d, J_{C-H}= 149 Hz), 3.70 (2 × m, J_{C-H}= 152 Hz), 3.60 (2 × M, J_{C-H}= 145 Hz); ^{13}C-NMR (DMSO-d_6): 165.5 (d, J_{C-N}= 12.9 Hz, *C*(O) benzoyl), 152.4, 151.8, 150.6 (d, J_{C-N}= 19.6 Hz, C6), 143.3, 133.6, 132.6, 128.5, 126.0, 87.7 (d, J_{C-C}= 42.0 Hz, Cl′), 85.8 (dd, J_{C-C}= 40.1 Hz, J_{C-C}= 37.0 Hz, C4′), 73.7 (dd, J_{C-C}= 41.7 Hz, J_{C-C}= 37.6 Hz, C2′), 70.4 (J_{C-C}= 37.4 Hz, 41.0 Hz, C3′), 61.3 (d, J_{C-C}= 40.1 Hz, C5′) ^{15}N-NMR (DMSO-d_6): −242.1. HRMS (FAB$^+$) found 378.14430, calculated for MH$^+$ ($C_{12}{}^{13}C_5H_{18}N_4{}^{15}NO_5$) 378.14445.

N^2-Isobutyryl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(2-$^{15}N_1$)]-guanosine (***14d***). The title compound **14d** (0.898 g, 2.49 mmol, 74%) was obtained from **13d** (2.40 g, 3.37 mmol) as described for **14a**. It was used without further purification. ^{1}H NMR (DMSO-d_6): 12.2 (br. s, 1H), 11.7 (d, J_{N-H}= 91 Hz), 8.28 (s, 1H), 5.82 (br. d, J_{C-H}= l69 Hz, 1H), 4.45 (br. d, J_{C-H}= 152 Hz, 1H), 4.16 (br. d, J_{C-H}= 151 Hz, 1H), 3.93 (br. d, J_{C-H}= 150 Hz, 1H), 3.60 (2 × m, 2H), 2.78 (sept, J= 6.9 Hz, 1H), 1.13 (d, J= 6.9 Hz, 6H); ^{13}C NMR (DMSO-d_6): 180.5 (d, J_{N-C}= 11.5 Hz, *C*(O)Bz), 155.2, 149.1, 148.4 (d, J_{N-C}= 21.8 Hz, C2), 138.0, 120.2, 86.8 (d, J_{C-C}= 42.7 Hz, Cl′), 85.5 (dd, J_{C-C}= 38.1 Hz, J_{C-C}= 43.6 Hz, C4′), 74.2 (dd, J_{C-C}= 43.3 Hz, J_{C-C}= 39.7 Hz, C2′), 70.4 (dd, J_{C-C}= 39.7 Hz, J_{C-C}= 38.1 Hz, C3′), 61.3 (d, J_{C-C}= 43.7 Hz, C5′); ^{15}N-NMR (DMSO-d_6): −245.0. HRMS (FAB$^+$) found 360.15612, calculated for MH$^+$ ($C_9{}^{13}C_5H_{20}N_4{}^{15}NO_6$) 360.15500.

[(1′,2′,3′,4′,5′-$^{13}C_5$)-(5-^{2}H)]-Uridine (***15***). Compound **13c** (7.24 g, 11.99 mmol) was dissolved in methanolic ammonia (23% w/w, 190 ml) and was left for 60 h at ambient temperature. The solvent was evaporated and the residue was partitioned between dichloromethane and water. The water phase was washed once with dichloromethane and evaporated to dryness. The residue (**14c**) was dissolved in D_2O (15 ml), evaporated to dryness, and the procedure was repeated. After dissolving the solid in D_2O (20 ml), anhydrous potassium carbonate (1.88 g, 13.60 mmol) was added and the solution was heated in an oil bath

(95°C) with protection from atmospheric moisture. Deuteriation was followed by NMR. After 34 h the dark solution was cooled, water (50 ml) was added and the base was neutralized with Dowex 50 (pyridinium form). The resin was washed with water, methanol and pyridine (100 ml each) and the eluates were evaporated to give **15** (2.93 g, 11.72 mmol, 97%) as a brown foam which was used for the next steps without further purification. ^{1}H-NMR (D_2O): 7.42 (s, 1H), 5.91 (br. d, J_{C-H}= 167 Hz, 1H), 5.72 (trace, 0.04H), 4.34 (br. d, J_{C-H}= 156 Hz, 1H), 4.27 (br. d, J_{C-H}= 146 Hz, 1H), 4.12 (br. d, J_{C-H}= 154 Hz, IH), 3.94 (br. d, J_{C-H}= 141 Hz, 1H), 3.79 (br. d, J_{C-H}= 141 Hz, 1H). ^{13}C-NMR (D_2O): 89.5 (d, J_{C-C}= 43.1 Hz, C-1′), 84.4 (dd, J_{C-C}= 38.8 Hz, J_{C-C}= 38.8 Hz, C-4′), 73.8 (dd, J_{C-C}= 37.8 Hz, J_{C-C}= 42.5 Hz, C-2′), 69.5 (dd, J_{C-C}= 38.3 Hz, J_{C-C}= 42.0 Hz, C-3′), 60.9 (d, J_{C-C}= 42.0 Hz, C-5′). MS (FAB$^+$) found 251.00985, calculated for MH$^+$ ($C_4{}^{13}C_5H_{12}DN_2O_6$) 251.10029.

*2′,3′,5′-Tri-O-acetyl-O^4-(2-nitrophenyl)-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(5-^{2}H)]-uridine (**16**)*. Uridine **15** (2.1 g, 8.39 mmol) was acetylated with acetic anhydride (6 ml, 6.49 g, 63 mmol), to give tri-O-acetyl derivative[35] (2.60 g, 6.91 mmol), which was dissolved in dry dichloromethane (60 ml). Triethylamine (8.45 ml, 6.13 g, 60.2 mmol), mesitylenesulfonyl chloride (4.0 g, 18.29 mmol) and DMAP (0.125 g, 1.02 mmol) were added to the solution. After stirring at room temp. for 1 h, 2-nitrophenol (3.95 g, 28.39 mmol) and DABCO (0.14 g) were added and stirring was continued for 2 h. The reaction mixture was quenched with satd. $NaHCO_3$, the organic layer was separated, the water phase was extracted with methylene chloride (2 × 50 ml). The combined extracts were dried ($MgSO_4$) and evaporated. Chromatography (silica gel, methylene chloride −3% methanol) gave **16** (3.08 g, 6.19 mmol, 74%) as light yellow foam. ^{1}H-NMR ($CDCl_3$): 8.14–7.31 (m, 5H), 6.28 (d, J = 7.4 Hz, ~0.04H), 6.10 (br. d, J_{C-H} = 172 Hz, 1H), 5.35 (2 × m, J_{C-H} = 149 Hz, 2H), 4.39 (2 × m, J_{C-H} = 148 Hz, 3H), 2.16, 2.10 (2 × s, 6 + 3H). ^{13}C-NMR ($CDCl_3$): 89.2 (d, J_{C-C} = 44.0 Hz, C-1′), 80.1 (dd, J_{C-C} = 39.9 Hz, J_{C-C} = 42.5 Hz, C-4′), 73.8 (dd, J_{C-C} = 39.7 Hz, J_{C-C} = 39.1 Hz, C-2′), 69.9 (dd, J_{C-C} = 42.6 Hz, J_{C-C} = 39.9 Hz, C-3′), 62.9 (d, J_{C-C} = 43.6 Hz, C-5′). HRMS (FAB$^+$) found 498.14911, calcd. for MH$^+$ ($C_{16}{}^{13}C_5H_{21}DN_3O_{11}$) 498.14829.

*N^4-Acetyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(4-^{15}N)-(5-^{2}H)]-cytidine (**18**)*. Labelled $^{15}NH_4Cl$ (3.0 g, 55 mmol) and K_2CO_3 (7.62 g, 55.13 mmol) were suspended in DMSO (45 ml) and the mixture heated in a pressure flask at 80°C for 30 min. After cooling in ice, the flask was opened and 4-*O*-

(2-nitrophenyl)-uridine **16** (2.44 g, 4.89 mmol) was added. The closed flask was heated at 80°C for 45 h. After cooling the content of the flask was purged with nitrogen, which was trapped in 10% HCl. (After evaporating this solution 1 g of labelled $^{15}NH_4Cl$ was recovered). The DMSO solution was filtered, the solid was washed with pyridine (150 ml) and the filtrates were concentrated to approx. 70 ml and evaporated three times with water (150 ml). The remaining solution was applied onto a column of Dowex 50 (H^+) (130 ml). The column was washed with 0.05 M HCl (500 ml), water (500 ml) and 3% NH_3 (750 ml). The ammonia solution was evaporated, leaving cytidine **17** (1.3 g) as a yellowish foam. The foam was dissolved in DMF (25 ml), acetic anhydride (0.51 ml, 0.55 g, 53.6 mmoles) was added and the whole was left for 24 h. Solvent was evaporated, the residue boiled with methanol 40 ml) and cooled. Crystals were filtered and dried to furnish acetylated cytidine **18** (1.18 g, 82%). ^{1}H-NMR (DMSO-d_6): 8.36 (s, 1H); 5.91 (d, J_{C-H}= 175 Hz, 1H), 4.29 (d, J_{C-H}= 140 Hz, 1H), 4.20 (2 × m, J_{C-H}= 146 Hz, 2H), 3.94 (2 × m, J_{C-H}= 144 Hz, 2H); 2.25 (s, 3H). ^{13}C NMR (DMSO-d_6): 90.5 (d, J_{C-C}= 42.5 Hz, C-5′), 84.0 (dd, J_{C-C}= 39.4 Hz, J_{C-C}= 41.1 Hz, C-4′), 74.1 (dd, J_{C-C}= 37.9 Hz, J_{C-C}= 39.1 Hz, C-2′), 69.4 (dd J_{C-C}= 38.4 Hz, J_{C-C}= 41.1 Hz, C-3′), 60.9 (d, J_{C-C}= 42.1 Hz, C-5′) ^{15}N-NMR: −233.5. HRMS (FAB^+) found 293.12307 calculated for MH^+ ($C_6{}^{13}C_5H_{15}DN_2{}^{15}NO_6$) 293.12386.

*5′-O-Dimethoxytrityl-N^6-benzoyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(6-^{15}N)]-adenosine (**19a**).* Adenosine **14a** (2.46 g, 6.52 mmol) was evaporated with pyridine (3 × 15 ml), dissolved in pyridine (30 ml) and dimethoxytrityl chloride (2.54 g, 7.5 mmol) was added. After 18 h the solvent was evaporated, the residue was dissolved in methylene chloride, washed with sat. $NaHCO_3$, then water and dried with $MgSO_4$. Evaporation of solvent and chromatography (methylene chloride-3% methanol) gave the product **19a** (3.37 g, 4.96 mmol, 76%) as a yellow foam. ^{1}H-NMR ($CDCl_3$): 9.15 (d, J_{N-H}= 90 Hz, 1H), 8.67 (s, 1H), 8.24 (s, 1H), 8.01–6.71 (m, 18H), 6.08 (br. d, J_{C-H}= 167 Hz, 1H), 4.93 (m, 1H), 4.48 (br. d, J_{C-H}= 154 Hz, 1H), 4.40 (br. d, J_{C-H}= 146 Hz, 1H), 3.74 (s, 6H), 3.46 (m, 1H), 3.33 (m, 1H). ^{13}C-NMR ($CDCl_3$): 165.9 (d, J_{N-C}= 13 Hz, $C(O)^{15}NH$), 150.6 (d, J_{N-C} = 19.5 Hz, C-6), 90.4 (d, J_{C-C}= 41.7 Hz, C-l′), 85.8 (dd, J_{C-C}= 37.1 Hz, J_{C-C}= 43.1 Hz, C-4′), 75.6 (dd, J_{C-C}= 41.6 Hz, J_{C-C}= 37.6 Hz, C-2′), 72.2 (dd, J_{C-C}= 37.6 Hz, J_{C-C}= 37.2 Hz, C-3′), 63.5 (d, J_{C-C}= 43.2 Hz, C-5′); ^{15}N-NMR ($CDC1_3$): −248.8; MS (FAB) 680.1 (MH^+).

*5′-O-Dimethoxytrityl-N⁴-acetyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(4-^{15}N)-(5-^{2}H)]-cytidine (**19b**)*. Compound **18** (1.175 g, 4.03 mmol) was dimethoxytritylated as described for adenosine **14a** to yield **19b** (1.70 g, 2.86 mmol, 71%). ^{1}H-NMR($CDCl_3$): 9.01 (br. d, J_{H-N}= 89 Hz, 1H), 8.21 (s, 1H), 5.87 (br. d, J_{C-H}= 173 Hz, 1H), 4.42 (br. d, J_{C-H}= 147 Hz, 3H), 3.42 (m, 2H). ^{13}C-NMR ($CDCl_3$): 93.6 (d, J_{C-C}= 40.0 Hz, C-1′), 86.0 (dd, J_{C-C}= 39.9 Hz, J_{C-C}= 42.6 Hz, C-4′), 77.1 (dd, J_{C-C}= 40.0 Hz, J_{C-C}= 37.4 Hz, C-2′), 71.8 (dd, J_{C-C}= 37.4 Hz, J_{C-C}= 39.9 Hz, C-3′), 62.9 (d, J_{C-C}= 42.6 Hz, C-5′); ^{15}N-NMR ($CDCl_3$): −233.0. HRMS (FAB^+) found 595.25532, calculated for MH^+ ($C_{27}{}^{13}C_5H_{33}DN_2{}^{15}NO_8$) 595.25444.

*5′-O-Dimethoxytrityl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(5-^{2}H)]-uridine (**19c**)*. Compound **15** (0.90 g, 3.60 mmol) was dimethoxytritylated as described for **14a**, giving **19c** (1.65 g, 2.98 mmol, 83%) as yellow foam. ^{1}H-NMR ($CDCl_3$): 7.96 (s, 1H), 5.88 (br. d, J_{C-H}= 172 Hz, 1H), 5.33 (trace, ~0.04H), 4.42 (br. d, J_{C-H}= 151 Hz, 1H), 4.34 (br. d, J_{C-H}= 152 Hz, 1H), 4.19 (br. d, J_{C-H}= 150 Hz, 1H), 3.50 (2 × m, J_{C-H}= 150 Hz, 2H). ^{13}C-NMR ($CDCl_3$): 90.7 (d, J_{C-C}= 41.1 Hz, C-l′), 84.0 (dd, J_{C-C}= 37.9 Hz, J_{C-C}= 43.1 Hz, C-4′), 75.6 (dd, J_{C-C}= 41.1 Hz, J_{C-C}= 39.4 Hz, C-2′), 69.9 (dd, J_{C-C}= 39.4 Hz, J_{C-C}= 37.9 Hz, C-3′), 62.0 (d, J_{C-C}= 43.1 Hz, C-5′). HRMS (FAB^+) found 553.23192, calculated for MH^+ ($C_{25}{}^{13}C_5H_{29}DN_2O_8$) 553.23087.

*5′-O-Dimethoxytrityl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(2-^{15}N)]-N²-isobutyrylguanosine (**19d**)*. Compound **14d** (1.37 g, 3.82 mmol) was dimethoxytritylated as described for **14a**, giving **19c** (2.31 g, 2.75 mmol, 72%) as yellow foam. ^{1}H-NMR ($CDCl_3$): 7.70 (s, 1H), 5.84 (br. d, J_{C-H}= 171 Hz, 1H), 5.28 (br. d, J_{C-H}= 151 Hz, 1H), 4.56 (br. d, J_{C-H}= 152 Hz, 1H), 4.29 (br. d, J_{C-H}= 151 Hz, 1H), 3.49 (m, 1H), 3.09 (br. d, J_{C-H}= 155 Hz, 1H), 1.90 (m, 1H), 0.93, 0.65 (2 × d, J= 7 Hz, 2 × 3H). ^{13}C-NMR ($CDCl_3$): 147.3 (d, J_{N-C}= 18 Hz, C-2), 90.1 (d, J_{C-C}= 43.5 Hz, C-l′) 85.2 (dd, J_{C-C}= 38.0 Hz, J_{C-C}= 43.6 Hz, C-4′), 72.6 (dd, J_{C-C}= 43.5 Hz, J_{C-C}= 39.6 Hz, C-2′), 71.3 (dd, J_{C-C}= 39.6 Hz, J_{C-C}= 38.1 Hz, C-3′), 63.7 (d, J_{C-C}= 43.7 Hz, C-5′);. ^{15}N-NMR ($CDCl_3$): −245.3. HRMS (FAB^+) found 662.27886, calculated for MH^+ ($C_{30}{}^{13}C_5H_{37}N_4{}^{15}NO_8$) 662.28558.

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-N⁶-benzoyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(6-^{15}N)]-adenosine (**20a**)*. Dried **19a** (3.52 g, 5.17 mmol) was dissolved in THF (40 ml), and pyridine (0.95 ml) was added, followed by silver nitrate (1.475 g, 8.68 mmol). The mixture was stirred for 20 min and *t*-butyldimethylchlorosilane (1.36 g, 9.02 mmol) was added. The

reaction mixture was stirred in darkness for an additional 6 h. The resulting suspension was filtered through a pad of Celite, washed with methylene chloride and the filtrates were evaporated. The oily residue was dissolved in methylene chloride, the solution was washed with $NaHCO_3$, dried with $MgSO_4$ and evaporated. Chromatography (silica gel, benzene-ethyl acetate, gradient 12–18%) gave the desired compound **20a** (2.16 g, 2.73 mmol, 53%) together with the 3′-OSi isomer (0.45 g, 11%). ^{1}H-NMR ($CDCl_3$): 8.98 (d, J_{N-H}= 89 Hz, 1H), 8.74 (s, 1H), 8.24 (s, 1H), 8.04–6.80 (m, 18H), 6.11 (br. d, J_{C-H}= 166 Hz, 1H), 5.02 (br. d, J_{C-H}= 149 Hz, 1H), 4.37 (br. d, J_{C-H}= 151 Hz, 1H), 4.28 (br. d, J_{C-H}= 150 Hz, 1H), 3.78 (s, 6H), 3.55 (2 × m, 1H), 3.42 (2 × m, 1H), 0.85 (s, 9H), 0.147, −0.005 (2 × s, 2 × 3H). ^{13}C-NMR ($CDCl_3$): 164.6 (d, J_{N-C}= 13.2 Hz, *C*(O)Bz), 149.7 (d, J_{N-C}= 20 Hz, C-6), 88.5 (d, J_{C-C}= 43.1 Hz, C-l′), 84.3 (dd, J_{C-C}= 36.9 Hz, J_{C-C}= 43.1 Hz, C-4′), 75.8 (dd, J_{C-C}= 43.1 Hz, J_{C-C}= 37.4 Hz, C-2′), 71.6 (dd, J_{C-C}= 37.4 Hz, J_{C-C}= 36.9 Hz, C-3′), 63.3 (d, J_{C-C}= 43.1 Hz, C-5′); ^{15}N-NMR ($CDCl_3$): −252.8. MS (FAB) 793.1 (MH^+).

*5′-O-Dimethoxytrity1-2′-O-t-butyldimethylsilyl-N^4-acety1-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(4-^{15}N)-(5-^{2}H)]-cytidine (**20b**)*. Compound **19b** (2.13 g, 3.58 mmol) was silylated as described for **20a**, giving **20b** (1.32 g, 1.86 mmol, 52%) as a colorless foam. ^{1}H-NMR ($CDCl_3$): 9.62 (d, J_{N-H} = 89 Hz, 1H, NH-4), 8.45 (s, 1H, H6), 7.45–6.85 (m, 13H), 5.95 (br. d, J_{C-H}= 176 Hz, 1H), 4.40 (br. d, J_{C-H}= 149 Hz, 1H), 4.32 (br. d, J_{C-H}= 156 Hz, 1H), 4.12 (2 × m, 1H) 3.81 (s, 6H), 3.61 (2 × m, 1H), 3.51 (m, 1H), 0.95 (s, 9H), 0.30 (s, 3H), 0.19 (s, 3H); ^{13}C-NMR ($CDCl_3$): 170.8 (d, J_{N-C}= 11.3 Hz, *C*OCH_3), 162.9 (d, J_{N-C}= 18.3 Hz, C-4), 155.4 (C-2), 97.5 (br., low intensity, C-5), 90.8 (d, J_{C-C}= 42.0 Hz, C-1′), 83.3 (dd, J_{C-C}= 39.7 Hz, J_{C-C}= 43.3 Hz, C-4′), 76.7 (dd, J_{C-C}= 42.0 Hz, J_{C-C}= 37.9 Hz, C-2′), 69.2 (dd, J_{C-C}= 37.9 Hz, J_{C-C}= 39.7 Hz, C-3′), 61.5 (d, J_{C-C}= 43.3 Hz, C-5′). ^{15}N-NMR ($CDCl_3$): −233.6. MS (FAB) 709 (MH^+).

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(5-^{2}H)]-uridine (**20c**)*. Compound **19c** (1.68 g, 3.05 mmol) was silylated as described for **20a**, giving **20c** (1.36 g, 2.04 mmol, 67%) as colorless foam. ^{1}H-NMR ($CDCl_3$): 7.91 (s, 1H, H6), 7.40–6.83 (m, 13H), 5.95 (br. d, J_{C-H}= 173 Hz, 1H), 4.35 (br. d, J_{C-H}= 151 Hz, 2H), 4.07 (m, 1H), 3.80 (s, 6H), 3.50 (2 × m, J_{C-H} = 146 Hz, 2H), 0.93 (s, 9H), 0.19, 0.16 (2 × s, 2 × 3H). ^{13}C-NMR ($CDCl_3$): 102 (br., low intensity, C-5), 88.8 (d, J_{C-C}= 43.1 Hz, C-l′), 76.4 (dd, J_{C-C}= 43.1 Hz, J_{C-C}= 36.9 Hz, C-2′), 70.5 (dd, J_{C-C}= 36.9 Hz, J_{C-C}= 41.0 Hz, C-3′), 83.6 (dd, J_{C-C}=

41.0 Hz, J_{C-C}= 43.1 Hz, C-4′), 62.4 (d, J_{C-C}= 43.1 Hz, C-5′). MS (FAB) 667.1 (MH^+).

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-N^2-isobutyryl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(2-^{15}N)]-guanosine (**20d**)*. Compound **19d** (2.29 g, 3.47 mmol) was silylated as described for **20a,** giving **20d** (1.54 g, 1.98 mmol, 57%) as a colorless foam. ^{1}H-NMR ($CDCl_3$):7.70 (s, 1H), 7.55–6.76 (m, 13H), 5.72 (br. d, J_{C-H}= 165 Hz, 1H), 5.30 (br. d, J_{C-H}= 144 Hz, 1H), 4.37 (br. d, J_{C-H}= 151 Hz, 1H), 4.22 (br. d, J_{C-H} = 150, 1H), 3.74 (s, 6H), 3.55 (m, 1H), 3.00 (2 × m, J_{C-H}= 142 Hz, 1H), 1.29–1.15 (m, 1H), 0.83 (s, 9H), 0.78, 0.50 (2 × d, J = 7 Hz, 2 × 3H), 0.017, −0.19 (2 × s, 2 × 3H). ^{13}C-NMR ($CDCl_3$): 160.9 (d, J_{N-C}= 16 Hz, *C*(O)iBu), 149.8 (d, J_{N-C}= 19 Hz, C-2), 90.5 (d, J_{C-C}= 43.6 Hz, C-l′), 86.1 (dd, J_{C-C}= 39.9 Hz, J_{C-C}= 43.6 Hz, C-4′), 74.2 (dd, J_{C-C}= 43.6 Hz, J_{C-C}= 39.4 Hz, C-2′), 72.3 (dd, J_{C-C}= 39.4 Hz, J_{C-C}= 39.9 Hz, C-3′), 63.2 (d, J_{C-C}= 43.6 Hz, C-5′). ^{15}N-NMR ($CDCl_3$): −245.0. MS (FAB) 776.2 (MH^+).

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-[1′,2′,3′,4′,5′-$^{13}C_5$]-nuclcoside phosphoramidites (**21a–d**)*. *General procedure*: The nucleoside **20a–d** (1 mmol) was dissolved in dry THF (5 ml) under nitrogen, DIPEA (0.35 ml, 0.259 g, 2 mmol) was added, followed by dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.90 ml, 0.945 g, 4 mmol). The reaction proceeded at room temperature for 2–4 h. After complete disappearance of substrate **20a–d** (TLC), methanol (1 ml) was added and after 10 min the reaction mixture was diluted with methylene chloride (35 ml), washed with $NaHCO_3$, sat. NaCl and dried with $MgSO_4$. Solvents were evaporated and the crude phosphoramidite was chromatographed on silica gel. Appropriate fractions were combined, evaporated, dissolved in minimal amount of methylene chloride containing 1% Et_3N and added dropwise to vigorously stirred anhydrous hexane at −70°C. The precipitated solid was centrifuged, the supernatant was decanted and the solid dried at 0.5 torr for 48 h.

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-N^6-benzoyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(6-^{15}N)]-adenosine 3′-O-(2cyanoethyl N,N-diisopropylphosphohramidite) (**21a**)*. Nucleoside **20a** (1.12 g, 1.41 mmol) was converted into phosphoramidite **21a** (0.91 g, 0.92 mmol, 65%). Chromatography: cyclohexane-3% Et_3N with gradient of methylene chloride from 20% to 35%. ^{31}P-NMR($CDC1_3$): 151.8, 151.6, 149.9, 149.6.

*5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-N^4-acetyl-[(1′,2′,3′,4′,5′-$^{13}C_5$)-(4-^{15}N)-(5-^2H)]-cytidine 3′-O-(2-cyanoethyl N,N-diisopropylphosphohramidite) (**21b**)*. Nucleoside **20b** (0.98 g, 1.38 mmol) was

converted into phosphoramidite **21b** (0.78 g, 0.85 mmol, 62%). Chromatography: cyclohexane-3% Et_3N with gradient of methylene chloride from 10% to 35%. ^{31}P-NMR ($CDCl_3$): 150.9, 150.8, 149.9, 149.7.

*5′- O - Dimethoxytrityl - 2′-O-t-butyldimethylsilyl -[(1′,2′,3′,4′,5′-$^{13}C_5$)- (5-^{2}H)]-uridine 3′-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) (**21c**)*. Nucleoside **20c** (0.83 g, 1.24 mmol) was converted into phosphoramidite **21c** (0.82 g, 0.95 mmol, 76%). Chromatography: cyclohexane-3% Et_3N with gradient of methylene chloride from 20% to 35%. ^{31}P-NMR ($CDCl_3$): 150.8, 150.4, 150.2.

*5′- O - Dimethoxytrityl -2′-O-t-butyldimethylsilyl-N^2-isobutyryl - [(1′,2′ 3′,4′,5′-$^{13}C_5$)-(2-^{15}N)]-guanosine 3′-O-(2-cyanoethyl N,N-diisopropyl-phosphohramidite) (**21d**)*. Nucleoside **20d** (1.21 g, 1.56 mmol) was converted into phosphoramidite **21d** (0.99 g, 1.01 mmol, 65%). Chromatography: cyclohexane-3% Et_3N with gradient of methylene chloride from 20 to 50%. ^{31}P-NMR ($CDCl_3$): 151.6, 149.5, 149.3.

Results and discussion

Recently we have reported[34] the synthesis of ribonucleosides with [$^{13}C_5$]-labelled ribose residues and introduced them into the TAR–RNA oligonucleotide at specifically chosen sites. The aim of the present study was to extend this approach to construct nucleoside building blocks, which are labelled with different isotopes in different residues of the molecule. Considering the fact that the cost and availability of the label is the limiting factor for the synthesis, we chose the following synthetic procedures.

Since in the case of purine nucleosides the introduction of the ^{15}N label is a multi-step process with moderate yields,[29,36–38] performing these reactions on ^{13}C labelled nucleoside leads to the loss of the valuable ^{13}C label. This prompted us to choose an optimal convergent approach in which the ^{15}N-labelled aglycon is prepared first followed by coupling to the [$^{13}C_5$]-labelled ribose moiety.

In the case of pyrimidine nucleosides the introduction of deuterium into the C5 position of the uridine is very simple and efficient.[39–41] Moreover, the ^{13}C labelled, deuteriated uridine is the starting material for the 4-[$^{15}NH_2$]-labelled cytidine. The conversion of the uridine into cytidine and ^{15}N labelling occur in the same reaction. This means that one coupling reaction of ^{13}C-ribose and uracil is the source of two

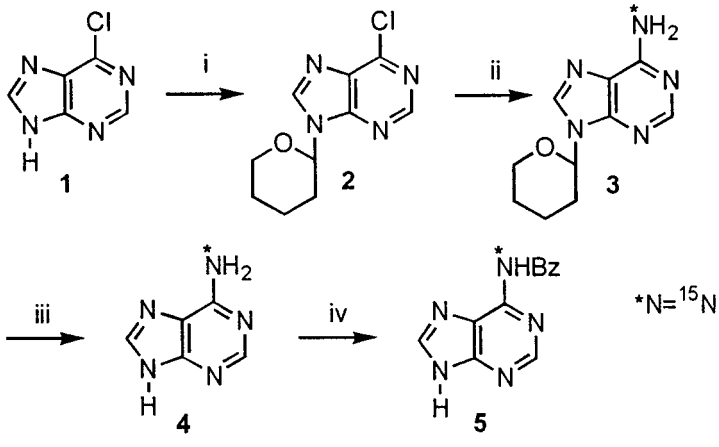
appropriately labelled nucleosides: uridine and cytidine. Hence we have chosen the linear approach.

The [$^{13}C_5$]-labelled acylated ribose was prepared in 42% total yield starting from commercially available [$^{13}C_6$]-D-glucose via our earlier reported procedure.[34]

The synthesis of the [6-^{15}N]-adenine aglycon was performed according to Scheme 1.

Amination of **1** gave in our hands inconsistent results of complicated reaction mixtures and thereby low yields of the desired [6-^{15}N]-adenine (**4**). Hence the 6-chloropurine **1** was first converted to its 9-tetrahydropyranyl (α/β) derivative **2**[42] (mimic of the nucleoside). After blocking of the N9 nitrogen atom, the reaction of **2** in the system $^{15}NH_4Cl/KHCO_3/DMSO$[29] smoothly afforded the labelled adenine derivative **3**. From this compound the tetrahydropyranyl group was removed with acidic treatment and the resulting labelled nucleobase **4** was benzoylated to give derivative **5**. The N^6-[6-^{15}N]-benzoyladenine (**5**) was used in the coupling reaction with the appropriate uniformly [$^{13}C_5$]-labelled ribose derivative.[34]

The [2-^{15}N]-labelled guanine (**10**) was synthesized according to Scheme 2, by modification of the procedure reported by Jones.[27] We have found that laborious and costly preparative HPLC for the



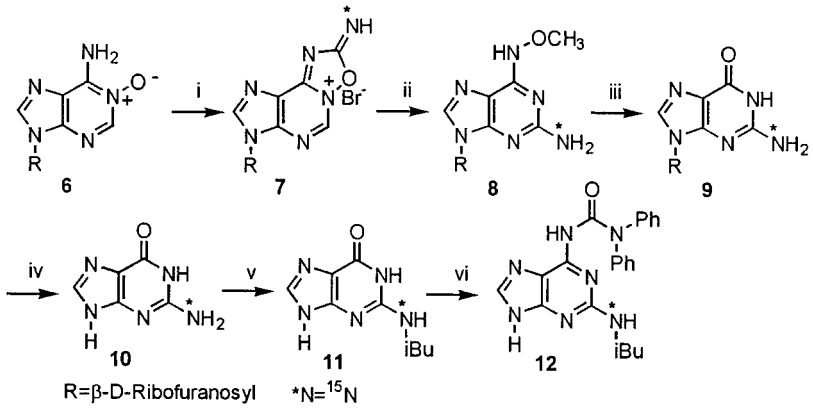
Reagents and conditions:

i. 3,4-Dihydro-2-H-pyran, TsOH,

ii. $^{15}NH_4Cl$, $NaHCO_3$, DMSO, 80°C,

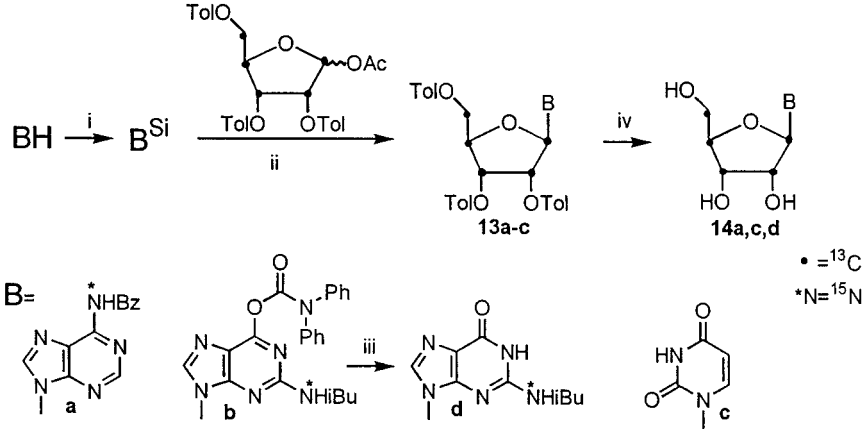
iii. 0.1 M HCl/dioxane, then Dowex (HCO_3^-), iv. Bz_2O

Scheme 1.



Reagents and conditions: i. 1.KC^{15}N, Br_2, methanol, 3hrs 2. Et_3N, DMF, 45 min, ii 1.CH_3I, 4 hrs, 2. NaOH r.t./45 min., then neutralization and heating 5 hrs, iii. Adenosine deaminase, pH 7.4, 37°C/90 hrs, iv. 1M HCl, 100°C/1 hr, v. Isobutyric anhydride, DMA, 150°C/2.5 hrs, vi 1. Acetic anhydride, DMF, 100°C/45 min. 2. Diphenylcarbamoyl chloride, DIPEA, pyridine, r.t./1.5 hrs

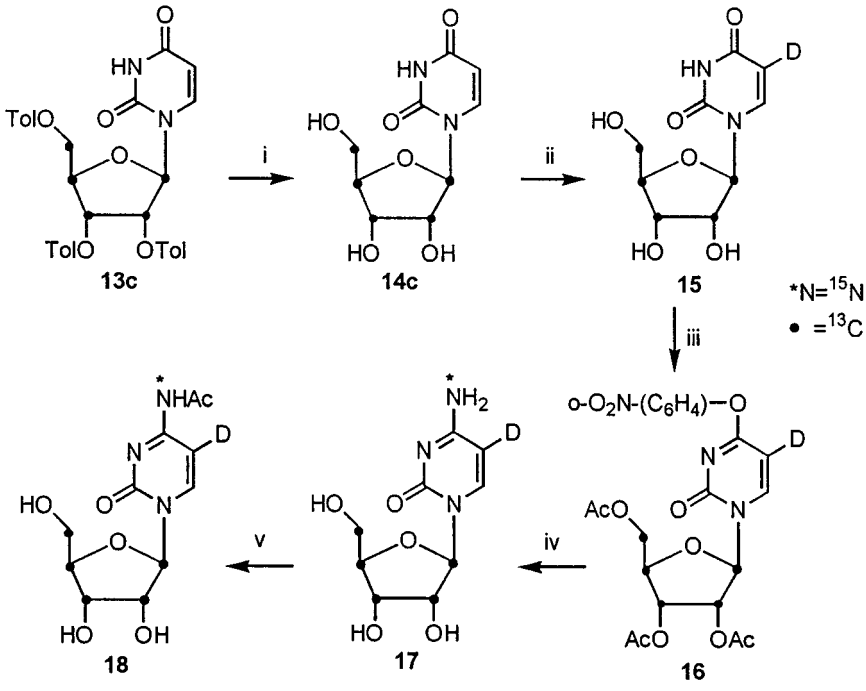
Scheme 2.



Reagents and conditions:
i. HMDS, TMS-Cl, reflux/3-6 hrs,
ii. TfOTMS, 1,2-dichloroethane or toluene (**12b**), 40-80°C/4-10 hrs,
iii. 90% CF_3COOH, 15 min,
iv. Methanol/NH_3, 2 days (**12c**) or NaOH/pyridine/ethanol, r.t./6min.

Scheme 3.

purification of intermediates could be omitted without reducing yields. Enzymatic deamination of intermediate **8** was carried out on crude reaction product without affecting the enzyme efficiency. Thus,

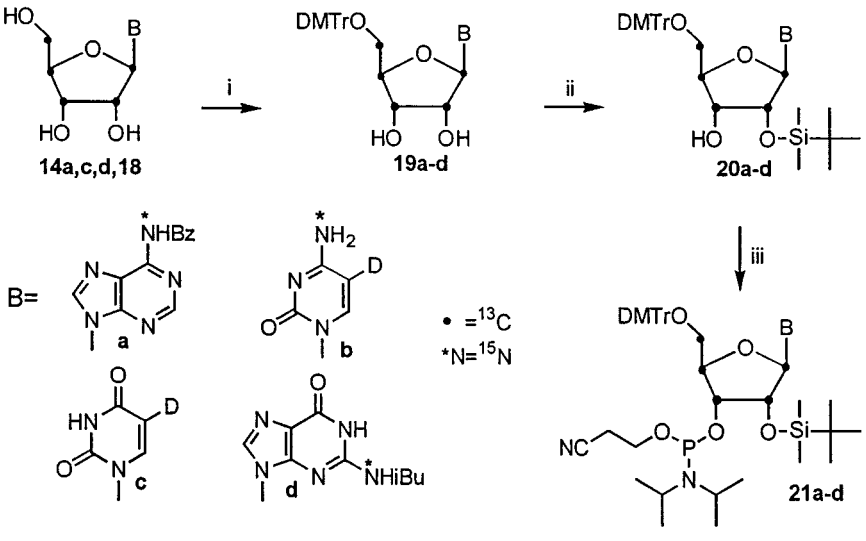


Reagents and conditions:
i. Methanol/NH_3, 2 days,
ii. D_2O, K_2CO_3, 95°C/34 hrs,
iii. 1. Acetic anhydride, pyridine, 2. Mesitylene sulfochloride, Et_3N, DMAP, 1 hr, then 2-nitrophenol, DABCO, r.t./2 hrs,
iv. $^{15}NH_4Cl$, K_2CO_3, DMSO, 80°C/45hrs,
v. Acetic anhydride, DMF, r.t./24 hrs.

Scheme 4.

adenosine 1-*N* oxide **6**[27] upon treatment with ^{15}N labelled cyanogen bromide (prepared *in situ* from the labelled $KC^{15}N$ and bromine) gave intermediate **7**. Further reaction with triethylamine followed by treatment with iodomethane and heating brought about a Dimroth rearrangement and led to 2-amino-6-methoxyamino-[2-^{15}N]-purine riboside **8**. After enzymatic deamination of the C6 position with adenosine deaminase, [2-^{15}N]-guanosine (**9**) was obtained. This nucleoside was cleaved with hot 1 M HCl giving the desired aglycon **10**. This was subsequently reacted with isobutyric anhydride to give **11** and further converted into O^6-diphenylcarbamoyl-N^2-isobutyryl-[2-^{15}N]-guanine (**12**), which was used in the coupling reaction.

The coupling reactions of ^{15}N labelled purine aglycons and uracil with uniformly ^{13}C labelled ribose were performed with the well-known



Reagents and conditions:
i. Dimethoxytrityl chloride, pyridine,
ii. $AgNO_3$, tBDMSiCl, cat. pyridine, THF,
iii. (2-cyanoethoxy)(*N,N*-diisopropylamino)chlorophosphine, DIPEA, THF, r.t./2-4 hrs

Scheme 5.

Vorbrüggen procedure[43], giving the multilabelled nucleosides **13a–c**. The diphenylcarbamoyl protection was selectively removed from guanosine **13b** *via* our earlier published procedure.[44]

The ^{13}C-labelled uridine **13c** was deprotected with methanolic ammonia to the free nucleoside **14c**. It was first evaporated with excess of D_2O to exchange the hydroxyl hydrogen atoms for deuterium, then it was heated in D_2O with potassium carbonate at 95°C for 34 h which resulted in a >96 at% exchange of the H5 for deuterium. Negligible (<7%) exchange of the H6 also occurred. Part of this deuterated uridine **15** was acetylated and converted into the O^4-(2-nitrophenyl) derivative **16**. A subsequent treatment of **16** with $^{15}NH_4Cl/K_2CO_3$/DMSO at 80°C for 45 h yielded cytidine **17**, a nucleoside labelled with ^{13}C at ribose ring, deuterium at C5 position of the pyrimidine ring and ^{15}N at the 4-exocyclic amino group.

The labelled cytidine was acetylated at [4-$^{15}NH_2$], giving derivative **18**, which was used for the synthesis of the oligonucleotide synthesis block.

The oligo-RNA synthesis blocks **21a–d** were obtained according to Scheme 5, using earlier published procedures.[34]

Conclusions

The above procedures enabled the efficient synthesis of multiply labelled oligoribonucleotide building blocks with different labels in the ribose (^{13}C) and base (^{15}N, ^{2}H) moieties of the molecule. To the best of our knowledge it is the first report on specific labelling with multiple isotopes both in base and ribose units by chemical means. The labelled blocks enable the construction of oligomers carrying selectively positioned labels which can serve as objects of simultaneous study of different aspects of RNA structure,[45–48] e.g. signal assignment, sugar-phosphate backbone geometry, sugar pucker, as well as base hydrogen bonding.

The deuteriation of the C5 position of pyrimidines contributes to the reduction of line overcrowding in the diagnostically important H1′ region.

In non-helical regions (bulges and loops) of an oligonucleotide, an exocyclic NH_2 becomes one of the primary targets of interactions with metal ions and other molecules.[49–53] All three bases (A, C, G) bearing such group are $^{15}NH_2$ labelled. The presence of the ^{15}N labelled exocyclic amino group gives the potential to study the interactions involving specific sites of the oligonucleotide by placing labelled units in such sites. Although this synthetic work was aimed mainly at RNA oligomers for NMR studies, ^{15}N labelled exocyclic NH_2 groups are also advantageous in FTIR[54,55] and Raman[56,57] spectroscopy studies of RNA association and hydrogen bonding.

Acknowledgements

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