Chemo-Enzymatic Synthesis of Thymidine ^{13}C-Labelled in the 2'-Deoxyribose Moiety

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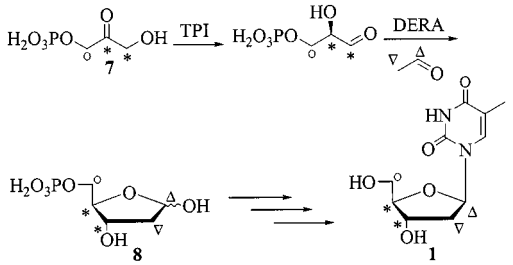
A synthesis of [3′,4′-$^{13}C_2$]thymidine (**1**) is described in which [$^{13}C_2$]acetic acid (**2**) is converted into the nucleoside in twelve steps with 9% overall yield. D-2-Deoxyribose-5-phosphate aldolase (DERA, EC 4.1.2.4) and triosephosphate isomerase (TPI, EC 5.3.1.1) are used for the stereocontrolled formation of D-[3,4-$^{13}C_2$]-2-deoxyribose-5-phosphate (**8**) from [2,3-$^{13}C_2$]dihydroxyacetone monophosphate (DHAP, **7**) and acetaldehyde in 80% yield. The route permits the introduction of isotopically enriched carbon atoms at any position or combination of positions in the furanose ring and the product can be coupled with any of the four naturally occurring base moieties.

Introduction

The site-directed incorporation of stable isotopes into biomolecules such as amino acids[1] and cofactors[2] as well as biologically active macromolecular complexes, and their subsequent detection by means of non-invasive isotope-sensitive techniques (e.g. solid-state NMR,[3] EPR,[4] FTIR,[5] and UV Laser Resonance Raman[6] spectroscopy) provides valuable information concerning the structure and function of the isotopically enriched site at an atomic level. In our research programme, this strategy has been successfully used to probe light-driven processes in the membrane proteins bacteriorhodopsin (a 26 kDa proton pump) and bovine rhodopsin (a 40 kDa visual pigment), as well as those occurring at photosynthetic reaction centres (100 kDa). We now extend the methodology to specifically labelled DNA in order to investigate the structure and function of globular protein–DNA complexes by heteronuclear NMR techniques.[7] For this, the availability of specifically labelled DNA was, or course, essential; it can be assembled from labelled and unlabelled monomers by means of solid-phase synthesis.

In this paper, we present a convenient synthesis of [3',4'-$^{13}C_2$]thymidine (**1**) and its achiral precursor [1,2-$^{13}C_2$]dihydroxyacetone monophosphate (DHAP, **7**). The compounds have been characterized by ^1H- and ^{13}C-NMR spectroscopy (Scheme 1).

The development of a synthetic scheme allowing the introduction of stable isotopes at any position or combination of positions is subject to unique restrictions: only a few isotopically enriched C_1 building blocks, such as formaldehyde and methyl iodide, are commercially available; C_2 and C_3 compounds, which can be purchased in three and seven isotopic substitution patterns, respectively, are even more rare. Moreover, isotopes must be incorporated in an efficient and specific way. Therefore, only asymmetric isotopomers and enantioselective syntheses are suitable. Since biocatalysts have become popular and powerful tools[8] in organic synthesis, an enzymatic aldol condensation process was exploited to construct the furanose ring from **7** and acetaldehyde. For this reaction, only the DHAP needs to be prepared in labelled form as acetaldehyde is commercially available in three differently labelled forms.



Scheme 1. The key enzymatic aldol condensation of **7** and acetaldehyde yields **8**, which is subsequently converted to **1**; we have selectively ^{13}C-labelled the positions marked with an asterisk (*); other positions that may be isotopically substituted are marked with ° or with $^{\Delta}$ and $^{\nabla}$

We chose to label **7** at its 3-position in order to check if any scrambling to the 1-position occurs through a possible transesterification reaction. The introduction of a second ^{13}C label at the 2-position allowed us to prepare thymidine with two adjacent ^{13}C labels in the furanose ring. It is our intention to use this compound to accurately determine torsion angles of nucleotide residues in DNA.[9]

Results

Our synthesis started with a bromination of [$^{13}C_2$]acetic acid (**2**) using bromine and trifluoroacetic anhydride (TFAA), which gave [$^{13}C_2$]bromoacetic acid (**3**) in excellent yield (98%).[10] Nucleophilic displacement of the bromine by the anion of benzyl alcohol then afforded [1,2-$^{13}C_2$]benzyloxyacetic acid (**4**) in 97% yield. To extend the two-carbon fragment to the desired three-carbon skeleton we used diazomethane (Scheme 2).[11]

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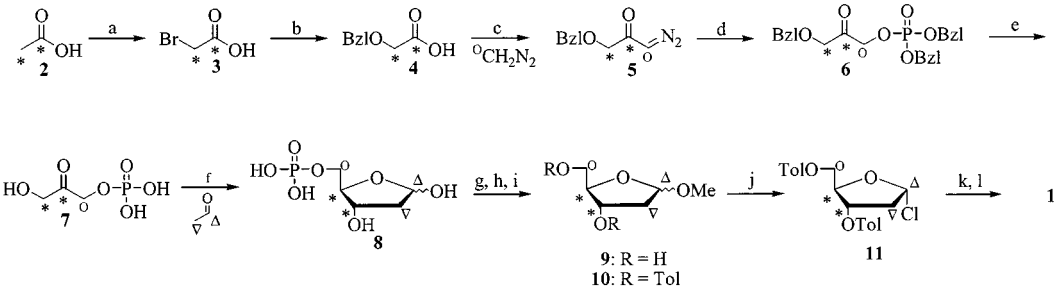

(a) TFAA/Br_2 (98%); (b) BzlOH/NaH/THF (97%); (c) 1. $SOCl_2$ 2. CH_2N_2 (75%); (d) $(BzlO)_2P(O)OH$ (93%); (e) Pd/C H_2 (95%); (f) acetaldehyde, TPI, DERA (80%); (g) alkaline phosphatase; (h) MeOH/HCl (65%, two steps); (i) p-toluoyl chloride/pyr (quant.); (j) AcOH/HCl (39%); (k) silylated thymine (80%); (l) MeOH/NH_3 (85%)

Scheme 2. Synthesis of [3',4'-$^{13}C_2$]thymidine (**1**); positions that are isotopically enriched are marked with an asterisk (*); other positions that may be isotopically enriched following this synthetic scheme are indicated with ° when derived from diazomethane (available from a labelled precursor that can be obtained commercially) or with $^{\Delta}$ and $^{\nabla}$ when derived from acetaldehyde

After reaction of **4** with thionyl chloride, the freshly prepared acetyl chloride was reacted with diazomethane to afford a 75% yield of [1,2-$^{13}C_2$]3-benzyloxy-1-diazopropanone (**5**). Heating of **5** with dibenzyl phosphate in toluene gave [2,3-$^{13}C_2$]dibenzyl-3-benzyloxypropanone phosphate (**6**) in 93% yield, which could subsequently be deprotected with Pd/C and H_2 to yield [2,3-$^{13}C_2$]dihydroxyacetone monophosphate (**7**). This proved to be the most critical transformation in the scheme (see Discussion). To the best of our knowledge, this is the first reported synthesis of specifically labelled DHAP and, since this compound is used in many enzyme-catalysed aldol condensations, its availability in labelled form should allow access to a variety of specifically labelled adducts.[12] The enzyme-catalysed aldol condensation furnished D-[3,4-$^{13}C_2$]2-deoxyribose-5-phosphate (**8**), which was obtained as an α,β anomeric mixture; an assay indicated the presence of 80% of **8**.[13] Dephosphorylation could be accomplished using alkaline phosphatase at pH 8.7–9.0 to give D-[3,4-$^{13}C_2$]2-deoxyribose. Since an acidic proton is generated upon hydrolysis of the phosphate ester and no buffer is used, the reaction medium has a tendency to become less alkaline. Therefore, the pH was checked at regular intervals and was adjusted as necessary. It was found that purification of the free sugar either by silica gel column chromatography or by crystallization afforded only around 45% of the purified product. However, chromatography after formation of the D-[3,4-$^{13}C_2$]methyl-2-deoxyriboside (**9**) afforded the desired product in 65% yield. Deoxynucleosides could be prepared by nucleophilic attack of the appropriate base moiety on the anomeric centre of [3,4-$^{13}C_2$]1-chloro-3,5-toluoyl-2-deoxyribose (**11**).[14] To obtain the required intermediate, the hydroxyl functions were protected with *p*-toluoyl groups. Subsequent activation of the anomeric centre as the α-chloro derivative **11** in 39% yield (two steps) rendered the compound sufficiently reactive to afford the corresponding 3',5'-protected nucleoside on reaction with silylated thymine (80% yield). Deprotection with methanolic ammonia gave [3',4'-$^{13}C_2$]thymidine (**1**) in 85% yield.

Characterisation of 1 by NMR Spectroscopy

Figure 1 depicts the salient regions of the ^{1}H-NMR spectrum (600 MHz, D_2O) of the labelled thymidine (top) aswell as the spectrum of the same sample recorded with ^{13}C de-

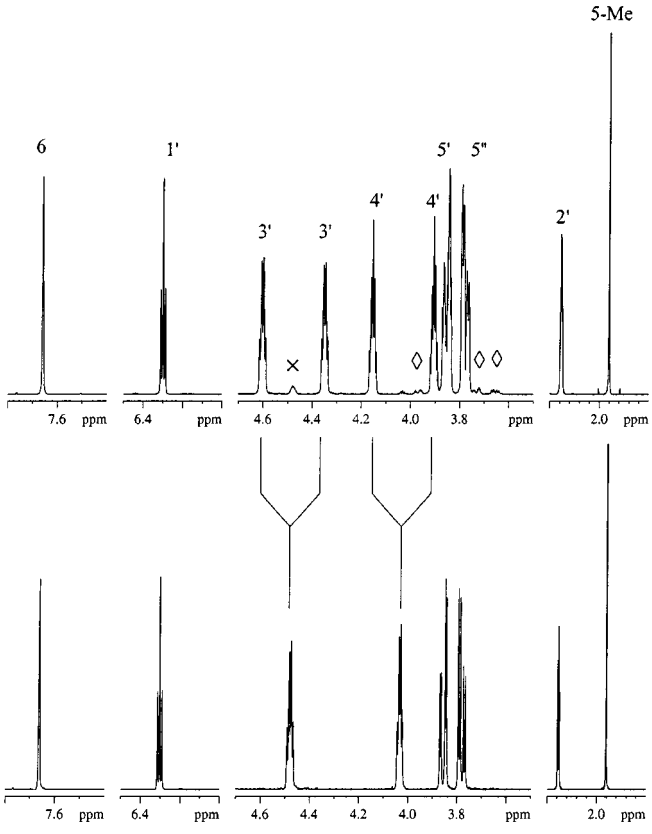

Figure 1. Selected parts of the ^{1}H-NMR spectrum (600 MHz, D_2O) of [3,4-$^{13}C_2$]thymidine (top) and the same sample measured with ^{13}C decoupling (bottom); the large splitting of the signals arising from 3'-H and 4'-H (δ = 4.48, respectively) due to an adjacent ^{13}C atom is indicated, clearly showing high isotope enrichment; a residual signal can be observed in the top spectrum at δ ≈ 4.5 marked with × and occurs as a result of isotope dilution, whereas the intricate pattern (referred to as ◇) s caused by isotope scrambling

coupling (bottom). As is apparent from Figure 1, the signals due to 3'-H and 4'-H are split in the top spectrum but lose this feature in the ^{13}C-decoupled spectrum, indicating the presence of the isotopic label adjacent to the respective hydrogen atoms. Some residual signals can be seen around $\delta = 4.5$ and between $\delta = 4.0$ and 3.7, which are marked with $\times$ and $\diamond$, respectively. These signals arise from a transesterification reaction of **7** labelled at the 2- and 3-positions, which leads to a trace amount of product labelled at the 1- and 2-positions. In other words, the phosphate group has migrated to the other side of the ketone function. This means that the 3-position becomes ^{13}C-diluted since some of the label ends up in the 1-position and thus implies that the 1-position becomes slightly ^{13}C-enriched. This can be seen in the NMR spectrum of **1**, bearing in mind that positions 1, 2, and 3 in **7** become 5', 4', and 3', respectively, in **1**. The small broad peak at ca. $\delta = 4.5$ (marked with $\times$) is due to isotope dilution. The occurrence of some scrambling is apparent from the intricate signals attributable to 5'-H and 5''-H, since these are flanked by low intensity patterns (indicated by $\diamond$). Analysis of these peaks shows that their intensities amount to around 4%. Corrected for a 99% isotopically enriched starting compound, this corresponds to an isotope dilution/scrambling of around 3%. Thus, 96% of the ^{13}C was present in the 3'-position and 3% was present in the 5'-position. Further analysis of the ^{1}H-NMR spectrum showed that, as expected, the 4'-position was still 99% ^{13}C substituted, which was the enrichment originally obtained from Cambridge Isotope Laboratories. The high degree of isotope enrichment made it possible to measure a large number of coupling constants between the isotopically enriched positions and the rest of the molecule. The observed coupling constants involving the ^{13}C-substituted positions are summarized in Table 1. These values are interesting because they can be used to assess structural features of the molecule.[15]

Table 1. Observed coupling constants (Hz) of the isotopes (either ^{13}C-3' or ^{13}C-4') with the other atoms in the sugar moiety; the values are given in Hz

Atom	C3'	C4'	Atom	C3'	C4'
1'-H	_[a]	_[a]	C1'	_[a]	_[a]
2'- and 2''-H	_[b]	_[b]	C2'	35.5	_[a]
3'-H	151.2	1.2	C3'	–	37.4
4'-H	4.8	148.8	C4'	37.4	–
5'-H	2.1	1.1	C5'	3.2	41.7
5''-H	3.3	1.2			

[a] No additional coupling was observed. – [b] Due to significant overlap of the signals, these values could not be determined.

Discussion

As can be seen from Scheme 2, the synthons used to obtain the furanose ring were acetic acid, acetaldehyde, and diazomethane. The first two are commercially available in any labelled form, whereas the latter has to be synthesized from *N*-methyl, *N*-nitroso-*p*-toluenesulfonamide, which is available in isotopically enriched form. Better still, from an economic point of view, is the preparation of the nitroso compound from cheap methylamine in high yield using known chemistry.[16] Thus, because all the synthons are available, we are able to selectively enrich any position or combination of positions in the furanose ring with ^{13}C.

One of the most important transformations along this route was the elongation of the two-carbon skeleton to a three-carbon chain using diazomethane. In a classical manner, the acetyl chloride (freshly prepared from **4** and thionyl chloride) was reacted with an excess of diazomethane, thereby leading to a high yield of diazo ketone **5** (70–80% based on **4**), although this required at least 4.5 equiv. of *N*-methyl, *N*-nitroso-*p*-toluenesulfonamide. In our hands, the use of 5 equivalents proved to be preferable in order to obtain a consistently high yield. Two problems associated with this procedure became apparent. Firstly, only 18% incorporation of diazomethane was achieved. Since it is our aim to develop a synthetic scheme that can, in principle, be used to prepare deoxynucleosides labelled in any position, the efficiency of diazomethane incorporation is very important and low incorporations need to be avoided. Secondly, the need for distillation and the use of a concentrated ethereal solution of diazomethane presented a considerable hazard owing to the toxic and potentially explosive nature of such a solution. We found that when the bubbler method, hitherto used only for the esterification of acids,[17] was modified to allow the synthesis of diazo ketones, yields as high as 75% (based on **4**) could be obtained. In this method, diazomethane is bubbled through a freshly prepared solution of [1,2-$^{13}C_2$]benzyloxyacetyl chloride in diethyl ether. Only 2.1 equivalents of the nitrosoamide precursor are required for complete reaction, corresponding to 35% diazomethane incorporation as opposed to 18% using the classical method. Addition of an excess of K_2CO_3 to the reaction mixture largely suppresses the formation of the chloromethyl ketone, which evolves when the diazo ketone reacts with liberated HCl. Attempts to use other bases, such as pyridine or triethylamine, led to complex reaction mixtures, probably due to ketene formation. Because the bubbler method does not require distillation of diazomethane and there is no need for large quantities of a concentrated diazomethane solution, the method is safe. To sum up, this method leads to more efficient diazomethane incorporation into the diazo ketone, is safe, and affords the same yield of the product as the classical method for diazo ketone preparation.

Another important step in this synthesis was the deprotection of **6** to afford **7**. Catalytic hydrogenation using Pd/C in various solvents (methanol/water, 4:1; methanol, and THF/water, 4:1) for various reaction times (4–18 h) at 500 kPa invariably led to the formation of side products. On the other hand, at atmospheric pressure the reaction did not reach completion within 24 h and prolonged stirring also led to the formation of some side-products. However, we found that use of a slightly elevated pressure (104 kPa),

achieved using a balloon, with dioxane/2-propanol/water (4:2:1) as the reaction medium, led overnight to a clean removal of the protecting groups in almost quantitative yield (95%), as determined by an enzymatic assay using NADH and glycerol-3-phosphate dehydrogenase.[18] It was anticipated that it would be possible for the phosphate group to migrate from the 1-position to the 3-position in DHAP during removal of the protecting groups. This would leave the same molecule but with a different isotope substitution pattern. As it turned out, this effect was almost negligible under the conditions used, as is evident from the ^{1}H-NMR spectrum of the final product (Figure 2). The isotopically enriched DHAP, prepared in five steps from **2** in 62% overall yield, may be used to monitor biochemical pathways followed by the compound *in vivo*, as a diagnostic tool (e.g. in NMR micro-imaging or mass spectrometry), and as a starting material in enzymatic syntheses of a variety of labelled carbohydrate-like products.

Coupling of bases to deoxyriboses is another critical issue. Intermediate **11** was specifically chosen because it is one of the best and most widely used intermediates for the coupling of bases to deoxyriboses and provides almost pure β-coupled products. In this respect, it is important to note that **11** can be used to produce all four D-2-deoxynucleosides.[19]

We also note that **1** can be easily converted into compounds showing anti-viral activity, e.g. AZT, where the availability of such compounds in isotopically substituted form might potentially be helpful in understanding the mode of action of such medicines.[20]

Conclusion

We have prepared [3',4'-$^{13}C_2$]thymidine and its achiral precursor [2,3-$^{13}C_2$]dihydroxyacetone monophosphate on a mmol scale in a stereospecific manner. The overall yield of **1** based on [$^{13}C_2$]acetic acid (**2**) was 9% (12 steps), which corresponds to an average yield of 82% per step. By applying this scheme using differently labelled precursors, such as acetaldehyde or *N*-methyl, *N*-nitroso-*p*-toluenesulfonamide, incorporation of the label at other carbon atoms of the furanose ring may be achieved. In principle, any carbon atom or any combination of carbon atoms can be isotopically enriched. Yields based on acetaldehyde and *N*-methyl, *N*-nitroso-*p*-toluenesulfonamide were 5.5% and 4%, respectively. Isotopically enriched DHAP has been synthesized in five steps from **2** in 62% overall yield.

Experimental Section

General: Organic solvents were distilled prior to use and dry solvents were obtained either by distillation from drying agents (methanol from Mg, $CHCl_3$ and toluene from CaH_2) or by storing over 4 Å molecular sieves (THF). Methanol was stored over molecular sieves (3 Å); other dry solvents were freshly distilled and used as promptly as possible.

Reactions were routinely monitored by thin-layer chromatography (TLC, on Merck F_{254} silica gel 60 aluminium-backed sheets, 0.2 mm); spots were visualized with UV light (254 nm). Ketones and aldehydes were sprayed with 3,4-dinitrophenylhydrazine solution and were made visible by heating. Column chromatography was performed on Merck silica gel 60 (0.040–0.063 mm, 230–400 mesh). Melting points were measured on a Büchi apparatus and are uncorrected. Dowex 50 W-X2 (400 mesh, H^+ form) was obtained from Acros Chimica.

^{1}H-NMR spectra were recorded on a Bruker WM-300 or a Bruker AM-600 spectrometer with tetramethylsilane (TMS; δ = 0.00) as an internal ($CDCl_3$) or external (D_2O) standard. ^{1}H noise-decoupled ^{13}C spectra were recorded on a Bruker WM-300 at 75 MHz or on a Bruker AM-600 spectrometer at 151 MHz with chloroform (δ = 77.0) as an internal standard or TMS (δ = 0.00) as an external standard. ^{31}P-NMR spectra were recorded on a Bruker WM-300 spectrometer operating at 121 MHz with 85% H_3PO_4 as an external standard.

Mass spectra were recorded on a Finnigan MAT 900 equipped with a direct insertion probe (DIP) or on a Finnigan MAT 700-TSQ equipped with a custom-made electron-spray interface (ESI).

Chemicals were obtained from Aldrich or Acros Chimica. Enzymes were purchased from Sigma, except for D-2-deoxyribose-5-phosphate aldolase (DERA), which was isolated from overproducing the *E. coli* strain DH5α, obtained from ATCC (ATCC 86963). Growing and harvesting of the enzyme was performed according to literature procedures,[5] except that no lysozyme was used; the cells were sonicated instead. The ammonium sulfate precipitate was found to effectively catalyse aldol condensation and this crude solution was used in the enzymatic transformations. Extracts and reaction mixtures were assayed as described previously;[13] a 2 L culture routinely afforded 15000 units. Labelled compounds, 99% isotopically enriched, were purchased from Cambridge Isotope Laboratories, U.S.A.

[$^{13}C_2$]Bromoacetic Acid (3): To [$^{13}C_2$]acetic acid (2.0 g, 32.3 mmol) was slowly added 7.4 mL (96 mmol) of trifluoroacetic acid anhydride (TFAA), followed by 5.16 g (1.66 mL, 32.3 mmol) of Br_2. The solution was stirred for 16 h, in the course of which it became colourless, and then 1.8 mL (100 mmol) of water was added. Trifluoroacetic acid (TFA) was removed by distillation. The distillate was found to contain a small additional amount of **3**, which was obtained by removing the TFA with a nitrogen stream. Total yield (residue and the small second crop) 4.45 g (98%, 31.6 mmol) of a white solid; m.p. 49 °C.[10] – ^{1}H NMR ($CDCl_3$): δ = 3.92 (dd, $^1J_{C\text{-}H}$ = 153.29, $^2J_{C\text{-}H}$ = 4.66 Hz, 2 H). – ^{13}C NMR ($CDCl_3$): δ = 173.1 (d, $^1J_{C\text{-}C}$ = 62.7 Hz), 25.2 (d, $^1J_{C\text{-}C}$ = 62.7 Hz). – HRMS (ESI): calcd. for $^{13}C_2H_3BrO_2$ 139.9383; found 139. 9387.

[1,2-$^{13}C_2$]2-Benzyloxyacetic Acid (4): Sodium hydride (3.0 g, 75 mmol) was washed three times with THF and then suspended in 80 mL of THF. Benzyl alcohol (8.1 mL, 79 mmol) in 50 mL THF was slowly added at room temperature and the mixture was stirred until no more hydrogen was evolved (ca. 30 min.). A solution of **3** (4.4 g, 31.2 mmol) in 20 mL of THF was then slowly added and the resulting mixture was stirred overnight. The solvent was evaporated in vacuo and the solid white residue was treated with 50 mL of water. Once all the solid had dissolved, the aqueous solution was adjusted to pH 8 and extracted with diethyl ether (3 × 50 mL). The aqueous layer was then adjusted to pH 1 and again extracted with diethyl ether (3 × 50 mL). The latter extracts were dried, filtered, and the solvent was evaporated in vacuo to yield **4**, 5.1 g (30.3 mmol, 97%) as a yellow oil. – ^{1}H NMR ($CDCl_3$): δ = 7.36

(m, 5 H), 4.65 (d, J = 4.25 Hz, 2 H), 4.15 (dd, $^1J_{C-H}$ = 144.02, $^2J_{C-H}$ = 4.5 Hz, 2 H). – ^{13}C NMR ($CDCl_3$): δ = 174.4 (d, $^1J_{C-C}$ = 60.5 Hz), 66.54 (d, $^1J_{C-C}$ = 60.5 Hz). – HRMS (ESI): calcd. for $^{13}C_2C_7H_{10}O_3$ 168.0697; found 168.0704.

[2,3-$^{13}C_2$]3-Benzyloxy-1-diazopropanone (5): **4** (2.4 g, 14.3 mmol) was converted to its acetyl chloride by refluxing with 9 mL of thionyl chloride for 1 h and then removing the excess thionyl chloride in vacuo. This was then reacted with diazomethane in one of two ways:

Method A: A concentrated diazomethane solution was prepared by conventional distillation starting from 15 g (5 equiv.) of *N*-methyl, *N*-nitroso-*p*-toluenesulfonamide in 50 mL of diethyl ether and 10 mL of 40% KOH in a mixture of 35 mL diethyleneglycol monoethyl ether and 10 mL diethyl ether. Once the yellow solution had been obtained, a solution of the freshly prepared acetyl chloride of **4** in 15 mL of dry diethyl ether was slowly added. The reaction mixture was stirred at room temp. for 3 h and then heated on a water bath for 1 h to remove the excess diazomethane. After evaporation of the remaining solvent in vacuo, the residue was purified by chromatography on silica gel (diethyl ether/40–60 light petroleum ether, 1:1) to afford 2.2 g (11.4 mmol, 80%) of diazo ketone **5** as a yellow liquid.

Method B: The bubbler method employed involved the use of two flasks. The first was charged with the nitrosoamide (6.1 g, 28.6 mmol) in a mixture of diethyleneglycol diethyl ether (80 mL) and diethyl ether (80 mL), the second with a solution of the freshly prepared acetyl chloride of **4** in 150 mL of dry ether and 15 g of powdered K_2CO_3. The flasks were connected by means of glass tubing. It was of the utmost importance that the nitrogen outlet of the first flask was kept well *above* the liquid surface, and that this glass tube, which provided the nitrogen inlet for flask two, was well *immersed* in the solution in the latter. This prevented any pumping over of the liquid in flask one. Flask two was protected from moisture with a calcium chloride guard tube. Nitrogen was passed through the flasks at a moderate rate and 2 mL of 40% KOH solution was injected into flask one, thus initiating the formation of diazomethane. After 1 h and again after 2 h, further 2 mL aliquots of the KOH solution were injected to maintain the reaction rate. After ca. 4 h, the reaction mixture in the first flask had become colourless. The contents of the second flask were then filtered, the solvent was evaporated, and the residue was purified by chromatography on silica gel (diethyl ether/PE, 1:1) to give 2.1 g (10.7 mmol, 75%) of **5**. – ^1H NMR ($CDCl_3$): δ = 7.35 (m, 5 H), 5.79 (d, 1 H, $^2J_{C-H}$ = 3.5 Hz), 4.57 (d, $^3J_{C-H}$ = 4.0 Hz), 4.04 (dd, $^1J_{C-H}$ = 144.0, $^2J_{C-H}$ = 4.2 Hz). – ^{13}C NMR ($CDCl_3$): δ = 193.44 (d, $^1J_{C-C}$ = 49.8 Hz), 73.71 (d, $^1J_{C-C}$ = 49.8 Hz).

[2,3-$^{13}C_2$]Dibenzyl-3-benzyloxypropanone Phosphate (6): To a solution of 1.66 g (8.6 mmol) of **5** in 40 mL of toluene was added dibenzyl phosphate (2.7 g, 9.9 mmol) and the mixture was heated at 70–75 °C for 8 h. The toluene was then removed in vacuo and the residue was purified by chromatography on silica gel (hexane/EtOAc/chloroform, starting from 2:1:1 to 1:1:1) to give 4.0 g (9.2 mmol, 93%) of **6** as a slightly yellow oil.[11] – ^1H NMR: δ = 7.34 (m, 5 H), 5.09 (d, $^2J_{P-H}$ = 9.6 Hz, 4 H), 4.71 (dd, $^2J_{P-H}$ = 9.7, $^2J_{C-H}$ = 3.9 Hz, 2 H), 4.52 (d, $^2J_{C-H}$ = 4.2 Hz, 2 H), 4.10 (dd, $^1J_{C-H}$ = 142.6, $^2J_{C-H}$ = 4.4 Hz, 2 H). – ^{13}C NMR ($CDCl_3$): δ = 201.88 (dd, $^1J_{C-C}$ = 45.9, $^2J_{P-C}$ = 5.6 Hz), 73.31 (d, $^1J_{C-C}$ = 45.9 Hz). – ^{31}P NMR ($CDCl_3$): δ = –0.518 (d, $^2J_{P-C}$ = 5.6 Hz). – MS (ESI): m/z = 465 [M + Na].

[2,3-$^{13}C_2$]Dihydroxyacetone Monophosphate Monosodium Salt (7): To a stirred suspension of Pd/C (670 mg) in dioxane/2-propanol/water (4:2:1; 56 mL) was added 1.6 g (3.62 mmol) of **6**. An atmosphere of H_2 was then introduced by means of a balloon (104 kPa) and the reaction mixture was stirred at approximately 500 rpm overnight, after which TLC (*n*-butanol/acetone/water, 5:3:2) indicated a single spot (R_f = 0.10). The Pd/C was then removed by filtration through a glass microfibre filter paper (GF/A) and the filtrate was treated with 1 equiv. of 1 M NaOH. The mixture was concentrated in vacuo and filtered once more to remove all traces of Pd/C. The solution thus obtained (pH 4.5) could be kept frozen for months without decomposition. Enzymatic analysis showed the presence of 3.4 mmol (95%) of **7**, which could be isolated as a white solid. In aqueous solution, **7** exists in both ketone and hydrate forms. – ^1H NMR (D_2O): δ (ketone) = 4.50 (dd, $^1J_{C-H}$ = 143.7, $^2J_{C-H}$ = 3.5 Hz, 2 H), 4.58 (dd, $^2J_{P-C}$ = 7.7, $^2J_{C-H}$ = 4.1 Hz, 2 H); δ (hydrate) = 3.83 (m, 2 H), 3.82 (dd, $^1J_{C-H}$ = 143.9, $^2J_{C-H}$ = 3.0 Hz, 2 H). – ^{13}C NMR (D_2O): δ (ketone) = 210.2 (dd, $^1J_{C-C}$ = 42.3, $^3J_{P-C}$ = 8.2 Hz), 66.0 (d, $^1J_{C-C}$ = 42.3 Hz); δ (hydrate) = 95.3 (dd, $^1J_{C-C}$ = 49.1, $^3J_{P-C}$ = 8.2 Hz), 64.5 (d, $^1J_{C-C}$ = 49.0 Hz). – ^{31}P NMR (D_2O, pH 5.5): δ (ketone and hydrate) = 1.14 (d, $^3J_{C-P}$ = 8.82 Hz), 0.68 (d, $^3J_{C-P}$ = 6.54 Hz). – MS (ESI): m/z = 171 [M – Na].

D-[3,4-$^{13}C_2$]2-Deoxyribose 5-Phosphate (8): A 35 mL solution containing 0.1 M **7**, 0.1 M TEA, 2 mM EDTA, 400 u DERA, 700 u TPI, and 0.25 M acetaldehyde (pH 7.5) was stirred at ambient temperature for 6 h. The reaction was monitored by enzymatic methods and by ^{13}C-NMR. When the reaction was complete, Dowex H^+ was added to lower the pH to 1.5. The resulting slurry was stirred for 30 min., filtered, and the filtrate was adjusted to neutral pH and lyophilized. The residue was taken up in water (10 mL) and the solution was centrifuged to remove insoluble material. An enzymatic assay of the resulting solution showed it to contain 2.8 mmol (80%) of **8**. – ^{13}C NMR (D_2O): δ = 85.6 (m, α and β anomer), 72.2 (m, α and β anomer). – ^{31}P NMR (D_2O, pH 7.0): δ = 4.0.

Methyl [3,4-$^{13}C_2$]2-Deoxyriboside (9): The solution obtained as described above was adjusted to pH 9, whereupon 500 u of alkaline phosphatase was added. After stirring for 18 h, TLC indicated a single spot showing the same R_f value as commercial D-2-deoxyribose (*n*-butanol/acetone/water, 5:3:2). After neutralization with 1 M HCl, the solution was concentrated to dryness. The white solid thus obtained was extracted with methanol (2 × 15 mL) and the combined extracts were concentrated in vacuo to give 0.8 g of a white solid. This crude material was used directly in the subsequent step. The solid was dried by co-evaporation of any moisture with dry dioxane and the dried material was redissolved in dry methanol (22 mL). To this solution, 2.5 mL of 1% HCl in dry methanol was added and the resulting mixture was stirred until TLC (15% MeOH/dichloromethane) showed complete consumption of the starting material (1 h). Addition of $AgCO_3$ and filtration through Celite gave a colourless solution, which was concentrated in vacuo. Chromatography of the residue on silica gel (15% MeOH/dichloromethane) yielded 0.27 g (1.8 mmol, 65%) of **9** as a pale-yellow liquid.[18] – ^{13}C NMR ($CDCl_3$): δ (α,β anomers) = 87.4 and 86.9 (2 d, $^1J_{C-C}$ = 38.1 and 37.0 Hz), 72.6 and 71.8 (2 d, $^1J_{C-C}$ = 43.0 and 42.1 Hz).

Methyl [3,4-$^{13}C_2$]3,5-Toluoyl-2-deoxyriboside (10): To a solution of **9** (0.27 g, 1.8 mmol) in dry pyridine was added *p*-toluoyl chloride (0.61 g, 3.96 mmol), which led to the almost immediate deposition of a white precipitate. The resulting slurry was stirred for 2 h at

40 °C. Work-up involved the addition of 25 mL of water and 30 mL of diethyl ether to give two layers. The aqueous layer was separated from the organic layer and the latter was washed sequentially with water, dilute aq. sulfuric acid, and 1 M $NaHCO_3$ solution. Drying with $MgSO_4$, filtration, and evaporation of the solvent gave **10** as a slightly yellow oil (0.8 g, quant).[14] – ^{13}C NMR ($CDCl_3$): δ (α,β anomers) = 81.8 and 80.9 (2 d, $^1J_{C\text{-}C}$ = 38.8 and 38.9 Hz), 75.4 and 74.6 (2 d, $^1J_{C\text{-}C}$ = 38.9 and 38.9 Hz).

[3,4-$^{13}C_2$]1-Chloro-3,5-toluoyl-2-deoxyribose (11): Glacial acetic acid (6 mL) was saturated with HCl at 0 °C and then a solution of **10** (1.9 g, 4.9 mmol) in glacial acetic acid (3 mL) was added. A stream of HCl was continuously passed through this mixture and after 10 min. white crystals separated. This solid was collected by filtration to give **11** (720 mg, 1.8 mmol, 39%) as a white powder; m.p. 110 °C (ref.[14] 109 °C). – ^1H NMR ($CDCl_3$): δ = 7.95 (m, 4 H, arom. H), 7.25 (m, 4 H, arom. H), 6.47 (ddd, *J* = 5.0, *J* = 7.5 Hz, 1 H, 1-H), 5.56 (m, $^1J_{C\text{-}H}$ = 148.5 Hz, 1 H, 3-H), 4.86 (m, $^1J_{C\text{-}H}$ = 148.8 Hz, 1 H, 4-H), 4.68 (m, 1 H, 5'-H), 4.57 (m, 1 H, 5''-H), 2.86 (m, 1 H, 2-H), 2.74 (m, 1 H, 2'-H), 2.42 (s, 3 H, CH_3), 2.41 (s, 3 H, CH_3). – ^{13}C NMR ($CDCl_3$): δ = 84.7 (d, $^1J_{C\text{-}C}$ = 38.6 Hz), 73.5 (d, $^1J_{C\text{-}C}$ = 38.6 Hz).

[3',4'-$^{13}C_2$]3',5'-Di-*O*-toluoylthymidine (12): To a suspension of thymine (0.45 g, 3.59 mmol) in 20 mL of hexamethyldisilazane (HMDS) was added one drop of TMSCl and the mixture was refluxed for 6 h. The HMDS was then distilled off from the clear, colourless solution under reduced pressure to leave a colourless residue. This silylated thymine was then redissolved in 30 mL of dry, freshly distilled $CHCl_3$ and the solid α-chloro sugar **11** was added in a single portion. The resulting solution turned a little cloudy in the course of 2 h, after which 0.2 mL of methanol was added and the excess thymine was filtered off. The solvent was removed in vacuo and the residue was recrystallized from EtOAc to yield 700 mg (1.45 mmol, 80%) of **12** as a white solid; m.p. 196 °C.[18] – ^1H NMR ($CDCl_3$): δ = 8.82 (s, 1 H, 3-H), 7.94 (m, 4 H, arom. H), 7.28 (m, 5 H, arom. H and 6-H), 6.47 (dd, $^3J_{C\text{-}H}$ = 8.9, $^3J_{C\text{-}H}$ = 5.5 Hz, 1 H, 1'-H), 5.63 (dd, $^1J_{C\text{-}H}$ = 158.8, $^3J_{C\text{-}H}$ = 5.6 Hz, 1 H, 3'-H), 4.78 (m, 1 H, 5'-H), 4.65 (m, 1 H, 5''-H), 4.53 (m, 1 H, 4'-H), 2.70 (m, 1 H, 2'-H), 2.44 (s, 3 H, CH_3), 2.43 (s, 3 H, CH_3). 2.31 (m, 1 H, 2''-H), 1.61 (d, $^3J_{C\text{-}H}$ = 1.2 Hz, 3 H, 5-H). – ^{13}C NMR ($CDCl_3$): δ = 82.8 (d, $^1J_{C\text{-}C}$ = 37.2 Hz), 74.8 (d, $^1J_{C\text{-}C}$ = 37.2 Hz). – MS (ESI): *m/z* = 503 [M + Na], 498 [M + NH_4], 481 [M + H].

[3',4'-$^{13}C_2$]Thymidine (1): The white solid obtained as described above was added to 30 mL of methanol saturated with NH_3 at 0 °C and the suspension was stirred for 2 days. The methanol was then removed in vacuo, the residue was taken up in water, and this solution was washed with diethyl ether. Removal of the water gave thymidine (300 mg, 85% yield) as a white solid. Recrystallization from water gave a white solid melting at 184 °C.[18] Analysis of the NMR spectra revealed a ^{13}C isotope enrichment of 96% at the 3'-position and of 99% at the 4'-position. The remaining ^{13}C atoms were found to be present in the 5'-position. – ^1H NMR (600 MHz, D_2O): δ = 7.66 (m, 1 H, 6-H), 6.30 (dd, $^3J_{C\text{-}H}$ = 6.7 Hz, 1 H, 1'-H), 4.48 (dm, $^1J_{C\text{-}H}$ = 151.2 Hz, 1 H, 3'-H), 4.03 (dm, $^1J_{C\text{-}H}$ = 148.8 Hz, 1 H, 4'-H), 3.85 (m, 1 H, 5'-H), 3.78 (m, 1 H, 5''-H), 2.38 (m, 2 H, 2'- and 2''-H), 1.90 (d, $^3J_{C\text{-}H}$ = 1.1 Hz, 3 H, 5-H). – ^{13}C NMR (600 MHz, D_2O): δ = 87.3 (d, $^1J_{C\text{-}C}$ = 37.4 Hz), 71.2 (d, $^1J_{C\text{-}C}$ = 37.4 Hz). – MS (ESI): *m/z* = 267 [M + Na]. – HRMS (DIP): calcd. for $^{13}C_2C_8H_{14}N_2O_5$ 244.09698; found 244.101553.

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