One-Pot Two-Step Enzymatic Coupling of Pyrimidine Bases to 2-Deoxy-D-ribose-5-phosphate. A New Strategy in the Synthesis of Stable Isotope Labeled Deoxynucleosides

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The enzymatic synthesis of thymidine from 2-deoxy-D-ribose-5-phosphate is achieved, in a one-pot two-step reaction using phosphoribomutase (PRM) and commercially available thymidine phosphorylase (TP). In the first step the sugar-5-phosphate is enzymatically rearranged to α-2-deoxy-D-ribose-1-phosphate. Highly active PRM is easily obtained from genetically modified overproducing *E. coli* cells (12000 units/84 mg protein) and is used without further purification. In the second step thymine is coupled to the sugar-1-phosphate. The thermodynamically unfavorable equilibrium is shifted to the product by addition of $MnCl_2$ to precipitate inorganic phosphate. In this way the overall yield of the *β*-anomeric pure nucleoside increases from 14 to 60%. In contrast to uracil, cytosine is not accepted by TP as a substrate. Therefore, 2′-deoxy-cytidine is obtained by functional group transformations of the enzymatically prepared 2′-deoxy-uridine. The method has been demonstrated by the synthesis of [2′,5′-$^{13}C_2$]- and [1′,2′,5′-$^{13}C_3$]thymidine as well as [1′,2′,5′-$^{13}C_3$]-2′-deoxyuridine and [3′,4′-$^{13}C_2$]2′-deoxycytidine. In addition the nucleoside bases thymine and uracil are tetralabeled at the (1,3-$^{15}N_2$,2,4-$^{13}C_2$)-atomic positions. All compounds are prepared without any scrambling or dilution of the labeled material and are thus obtained with a very high isotope enrichment (96−99%). In combination with the methods that have been developed earlier it is concluded that each of the ^{13}C- and ^{15}N-positions and combination of positions of the pyrimidine deoxynucleosides can be efficiently labeled starting from commercially available and highly ^{13}C- or ^{15}N-enriched formaldehyde, acetaldehyde, acetic acid, potassium cyanide, methylamine hydrochloride, and ammonia.

Introduction

CP/MAS NMR spectroscopy can be used to acquire detailed structural information about biomacromolecules up to 100 kDa, when used in conjunction with site directed ^{13}C(^{15}N)-isotope labeling.[1] For example, with 1-D rotational resonance MAS NMR spectroscopy distances of about 3 Å can be determined with a very high accuracy (up to ±0.2 Å) in the retinylidene ligand in bovine rhodopsin.[2]

This powerful technique is, in general, applicable to any macromolecular complex, for instance to the study of macromolecular DNA−protein interactions. Oligomers of DNA can be selectively ^{13}C-substituted by incorporating specifically substituted deoxymononucleosides either by solid-phase synthesis of the appropriately protected deoxymononucleotides or by the action of DNA polymerase on deoxynucleotide triphosphates (dNTP), which is known as a template-primer synthesis.[3]

Since the number of different ^{13}C- and ^{15}N-substitution patterns for nucleosides increases exponentially with the number of carbon atoms, e.g., thymidine has $2^{12} - 1$ differently labeled forms, most of them are not commercially available.

Recently, we published the synthesis of thymidine wherein the D-2-deoxyribose-5-phosphate can be labeled at any position and any combination of positions.[4] The critical coupling of thymine to the sugar moiety, which was preceded by selective functional-group-protection of the carbohydrate, was carried out in the classic organic synthetic manner.[5] In contrast, the synthesis of D-2-deoxyribose-5-phosphate made use of biocatalysts, which ensured correct stereochemistry. In addition, the yields were high, and another advantage was that two steps could be carried out in one pot, i.e., the isomerization of dihydroxyacetone monophosphate to D-glyceraldehyde-3-phosphate, which was followed by an aldol condensation. In this light it is not surprising that we now attempt to exploit biocatalysts to further reduce the number of steps and try to perform two transformations in one pot.

In the present paper thymine is enzymatically coupled to the sugar moiety using phosphoribomutase (PRM, EC 2.7.5.6) and thymidine phosphorylase (TP, EC 2.4.2.4) as the catalysts. The first step is the stereospecific phosphate migration from position 5 to the α-1 position of

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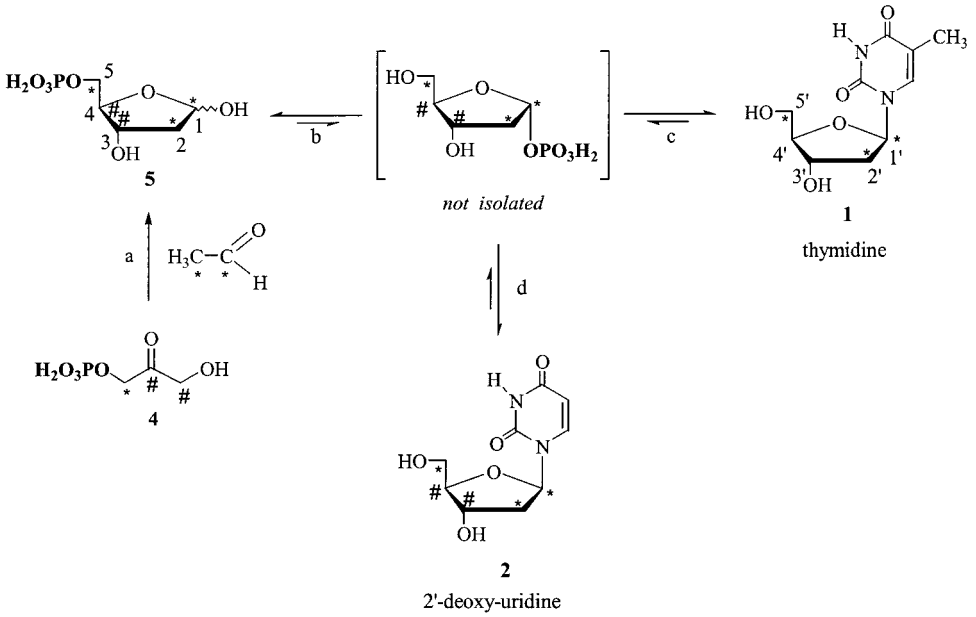
Scheme 1[a]



[a] Reagents and conditions: (a) D-2-deoxyribose-5-phosphate aldolase (DERA), triosephosphate isomerase (TPI);[4] (b) phosphoribomutase (PRM); (c) thymidine phosphorylase **(**TP), thymine (60%); (d) TP, uracil (52%); the different ^{13}C-label positions of compounds **1a** ([2′,5′-$^{13}C_2$]thymidine) and **1b** ([1′,2′,5′-$^{13}C_3$]thymidine) are shown by asterisks. **1a** is prepared starting from [2-^{13}C]acetaldehyde and (via **4a**, see Scheme 3) from [^{13}C]diazomethane and its precursor [^{13}C]methylamine·HCl. Compound **1b** is synthesized in the same way, but starting from [1,2-$^{13}C_2$]acetaldehyde instead of the monolabeled reagent. Compound **2a** ([1′,2′,5′-$^{13}C_3$]-2′-deoxyuridine) is prepared from [1,2-$^{13}C_2$]acetaldehyde, [^{13}C]methylamine·HCl (label positions are indicated by *) and (via **4b**) from [1,2-$^{13}C_2$]acetic acid (#).[4]

2-deoxy-D-ribose catalyzed by PRM. The ensuing compound is a substrate for TP and can thus be coupled, in an efficient and stereospecific manner to afford thymidine and 2′-deoxyuridine. The latter compound is subsequently converted to 2′-deoxycytidine by a functional group transformation.

For the conformational analysis of individual 2′-deoxynucleotide residues in macromolecular DNA/protein complexes, to be published elsewhere, the following compounds are prepared: [2′,5′-$^{13}C_2$]thymidine (**1a**), [1′,2′,5′-$^{13}C_3$]thymidine (**1b**), [1′,2′,5′-$^{13}C_2$]2′-deoxyuridine (**2a**), and [3′,4′-$^{13}C_2$]2′-deoxycytidine (**3a**). Finally, the nucleoside bases are labeled, i.e. [1,3-$^{15}N_2$,2,4-$^{13}C_2$]uracil (**12**) and [1,3-$^{15}N_2$,2,4-$^{13}C_2$]thymine (**13**).[6]

Results

Synthesis of Deoxynucleosides from 2-Deoxy-D-ribose-5-phosphate (5). The coupling of a pyrimidine base to the 2-deoxy-furanose ring is achieved in only two steps (Scheme 1). First, a leaving group is created on the α-anomeric position of the sugar ring. Then the nucleobase is coupled by a nucleophilic substitution reaction with inversion of the configuration. The leaving group is generated by migration of the phosphate of 2-deoxy-D-ribose-5-phosphate (**5**) from position 5 to position α-1.[7,8,9] This step is catalyzed by phosphoribomutase (PRM, EC 2.7.5.6) that is obtained from overproducing genetically modified *E. coli.* cells (see Experimental Section). Optimal activity of this enzyme is reached by addition of Mn^{2+} ions and D-glucose-α-1,6-biphosphate.

The second step, i.e., attack of thymine on the anomeric center, is catalyzed by thymidine phosphorylase (TP, EC 2.4.2.4), which concomitantly liberates inorganic phosphate. These two steps are conveniently carried out in one pot.

Reactions are performed at 10 mM concentration of sugar and 40 mM thymine at 37 °C, in the following stepwise approach. First, the starting compounds are mixed and the reaction is started by addition of freshly Mn^{2+}-activated glucose-α-1,6-biphosphate PRM (100 units) and thymidine phosphorylase (25 units). After the reaction has run overnight the solution is adjusted to pH 7.0 and a quantity of $MnCl_2$, to a concentration of 2 mM, is added to precipitate free phosphate. The precipitate is removed by a centrifuging step, and the pH of the solution is adjusted to 7.4 by addition of small amounts of 1 M NaOH followed by incubation of the supernatant with the mixture of PRM and TP enzymes. This process is repeated to give the highest conversion. The reaction is monitored with ^1H NMR and stopped when the reaction is complete. From the mixture a 57% yield of stereochemically pure thymidine (**1**), based on **5**, is secured.

Although TP exhibits high activity with both thymine and uracil, it does not show any activity with cytosine as a substrate. Consequently, cytosine cannot be coupled directly. Therefore, to prepare isotopically enriched 2′-deoxycytidines, uracil is first coupled, followed by conversion to the desired 2′-deoxycytidine. For example, uracil can be coupled to **5** to afford 2′-deoxyuridine (**2**) in 52% yield. To convert the double-bonded oxygen on position 4 to an amine, the following method is used (see Scheme 2). After acylation in pyridine, a tetrazole function is introduced at position 4.[10,11] This reactive tetrazole

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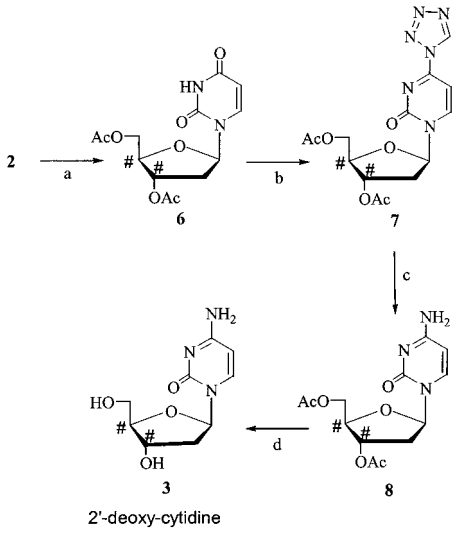
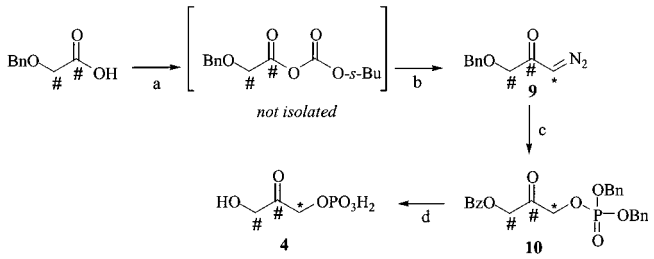
Scheme 2[a]



[a] Reagents and conditions: (a) acetic acid anhydride, pyridine (90%); (b) tetrazole, diphenyl phosphate, and tosyl chloride (92%); (c) 1.1 equiv of NH_3 in dioxane (62%); (d) MeOH/$BnNH_2$ (96%).

Scheme 3[a]

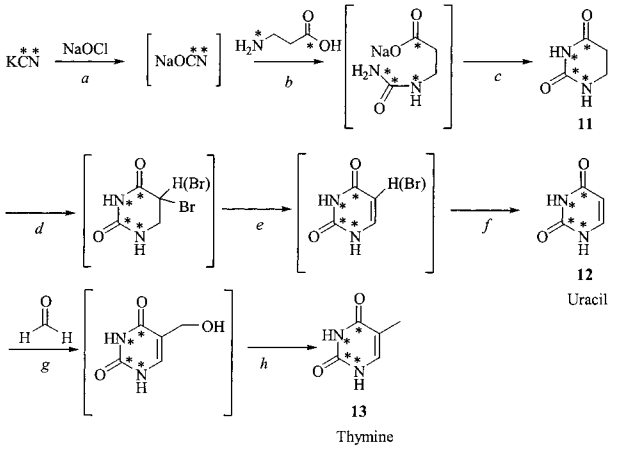


[a] Reagents and conditions: (a) *s*-butyl chloroformate, triethylamine; (b) [^{13}C]diazomethane (68%, based on *N*-[^{13}C]methyl-*N*-nitroso-*p*-toluenesulfonamide); (c) dibenzyl phosphate (81%); (d) Pd/C, H_2 (95%).

function is selectively substituted by NH_2 by using the reagent NH_3/dioxane. In this procedure the ester functions remain intact and are separately removed by benzylamine, see Scheme 1. This is a very useful feature because it means that, when ^{15}N-labeled ammonia is used, all the reagent is used to substitute the heterocyclic ring and no label is lost in the deprotection of the alcohol functions.

Synthesis of 2-Deoxy-D-ribose-5-phosphate (5). Dihydroxyacetone monophosphate (**4**) is the key precursor of compound **5**, and its synthesis is outlined in Scheme 3. Benzyloxyacetic acid, which can be easily prepared from acetic acid, is activated by reaction with *s*-butyl chloroformate at −30 °C and produces a labile intermediate, which is immediately subjected to a steady flow of the [^{13}C]diazomethane gas (see Scheme 3).[4,12] No acid is evolved, and this condition brings about less side reactions than in previous experiments. With equimolar amounts of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide and benzyloxyacetic acid, diazoketone **9** is obtained in 68% yield and 20% unreacted benzyloxyacetic acid is recovered from the aqueous layer in the workup procedure. So, when labels are present in the benzyloxyacetic acid, they can be almost quantitatively recovered and used again as starting material.

Scheme 4[a]



[a] Reagents and conditions: (a) aq NaOCl; (b) 100 °C; (c) 6 M HCl, 130 °C (96%); (d) Br_2 in acetic acid; (e) 170 °C; (f) Pd/$BaSO_4$/stream of H_2 gas, acetic acid/H_2O (75%); (g) paraformaldehyde, aq KOH, 65 °C; (h) Pd/$BaSO_4$/H_2 (50 psi), acetic acid/H_2O (75%); the compounds shown between square brackets are not isolated.

Dihydroxyacetone monophosphate (**4**) is obtained in excellent yield in two steps from **9**. First, the dibenzyl phosphate group is introduced via an addition−substitution reaction (81% yield), followed by a deprotection of the benzyl groups (95% yield) and conversion to **5**, see Scheme 2.[4,12]

In this manner enantiomerically pure [2′,5′-$^{13}C_2$]-thymidine (**1a**), [1′,2′,5′-$^{13}C_3$]thymidine (**1b**), [1′,2′,5′-$^{13}C_3$]-2′-deoxyuridine (**2a**), and [3′,4′-$^{13}C_2$]2′-deoxycytidine (**3a**) have been prepared at the gram scale.

Synthesis of Labeled Pyrimidine Bases. To demonstrate that our method permits the introduction of stable isotopes at the pyrimidine bases the 2′-deoxynucleosides, [1,3-$^{15}N_2$,2,4-$^{13}C_2$]uracil (**12**) and [1,3-$^{15}N_2$,2,4-$^{13}C_2$]thymine (**13**), are prepared. The critical steps are the oxidation of the commercially available isotopically labeled K^{13}C^{15}N to generate the crucial reagent NaO^{13}C^{15}N and the subsequent coupling with [1-^{13}C,^{15}N]*β*-alanine (the procedure of the ring closure has been described elsewhere).[6,14] In this way we succeeded to introduce labels at the first, second, third, and fourth positions for the first time (see Scheme 4).

Spectroscopic Analysis. The compounds are analyzed with ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy,

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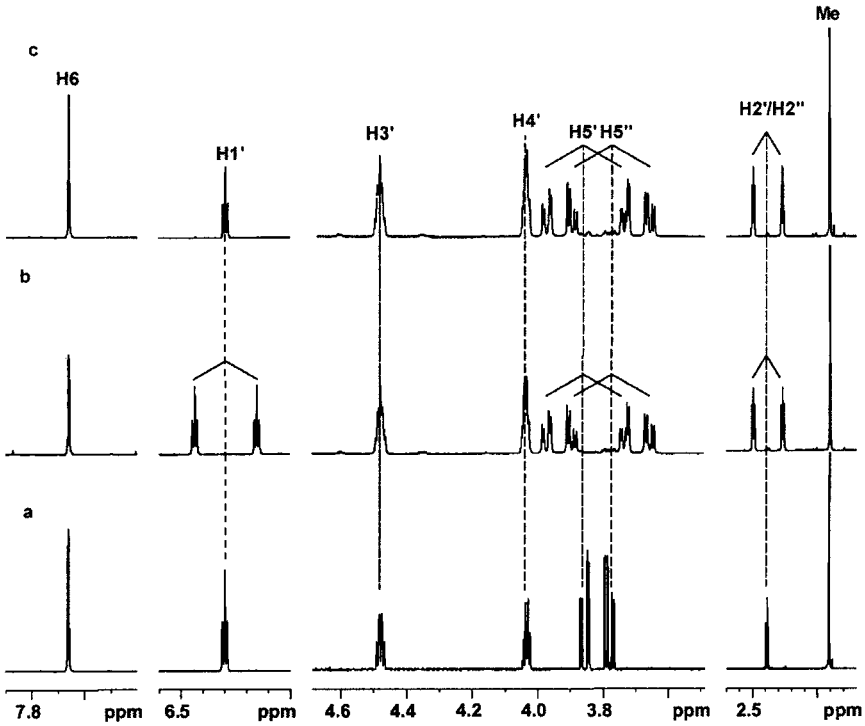

Figure 1. ^{1}H NMR spectra (600 MHz) of (a) natural abundance thymidine, (b) [1′,2′,5′-$^{13}C_3$]- and (c) [2′,5′-$^{13}C_2$]thymidine. Signals that are split due to the enrichment with ^{13}C-nuclei are indicated; the magnitudes of the $^{1}J_{C-H}$,$^{2}J_{C-H}$, and $^{3}J_{C-H}$ couplings are given in Table 1.

and mass spectrometry. Spectra acquired by the latter technique indicate the level of incorporation of stable isotopes in the molecules. For example, by comparing the spectra of natural abundance thymidine and [1,2,5-$^{13}C_3$]-thymidine isotopic incorporation can be calculated from peak intensities.

Information about the position of the ^{13}C-isotopes is derived from the ^{1}H NMR spectra. These spectra are essentially the same as those for commercially available unlabeled compounds, but exhibit additional splitting of some signals, which is due to the large $^{1}J_{C-H}$ coupling, see Figure 1. The position of the carbon isotopes is inferred from this additional splitting. It should be noted that the H1′-chemical shift of the α-nucleoside is absent. Therefore, the enzymatic coupling procedure only affords the desired β-nucleoside. The position of each label is in accordance with the predesignated position. Observation of the ratio of the intensities of the spit signal and the unsplit residual peak gives information about the degree of enrichment. All labeled positions are 99% enriched except for instances where position 5′ is enriched. This position is 96% isotopically substituted.[4]

Direct information about the position of each ^{13}C isotope is acquired from ^{13}C NMR spectra, see Figure 2. The strong signals arise from the isotopically enriched positions, and hence the place of each isotopes can be determined. A small unsplitted peak can usually be observed in the ^{13}C spectra, which indicates that labeling is 99%.

From the ^{1}H NMR spectra, the ^{1}H−^{13}C (Table 1) and from the ^{13}C NMR spectra the ^{13}C−^{13}C (Table 2) coupling constants are extracted. The values that are measured are in agreement with the expected values. For example, $^{2}J_{C-H}$ couplings are smaller than $^{3}J_{C-H}$ couplings, and the magnitudes of the $^{1}J_{C-H}$ and $^{1}J_{C-C}$ couplings are in accordance with previous observations from related compounds.[6]

Discussion

Synthesis of 2′-Deoxynucleosides from 2-Deoxy-D-ribose-5-phosphate (5). The thermodynamic equilibrium of the phosphate migration step is biased mainly on the side of the deoxyribose-5-phosphate. However, the second enzymatically catalyzed step, i.e., the nucleophilic attack of the nucleobase on deoxyribose-1-phosphate, is favored toward the product. In preliminary trial reactions, using equimolar concentrations of reactants, these competing forces result in approximately 10−20% conversion. Multiple additions of the enzyme preparation and prolonged incubation showed no further conversion, indicating that the thermodynamic equilibrium is reached. Improved results can in principle be accomplished by tampering with the concentrations of either starting materials or products. Easiest in this respect is increasing the concentration of one of the reactants, i.e., nucleobase or sugar. The pyrimidine nucleobases thymine and uracil have similar (low) solubilities (35 mM).[7,8] The pyrimidine nucleobases' poor solubility hampers the use of excesses to drive the reaction toward the product. On the other hand, increasing the amount of sugar does not suffer from poor solubility, but the phosphate-sugar could not be efficiently recovered from the reaction mixture utilizing ion-exchange chromatography (a DEAE Sephadex A25 column in the bicarbonate form). This thwarts the possibility to use an excess of sugar instead of an excess of nucleobase to shift the equilibrium.

Alternatively, one (or both) of the products, i.e., phosphate or nucleoside, can be separated from the reaction mixture. The nucleoside can be removed from the equilibrium for example through phosphorylation to the ensuing nucleotide. However, commercially unavailable

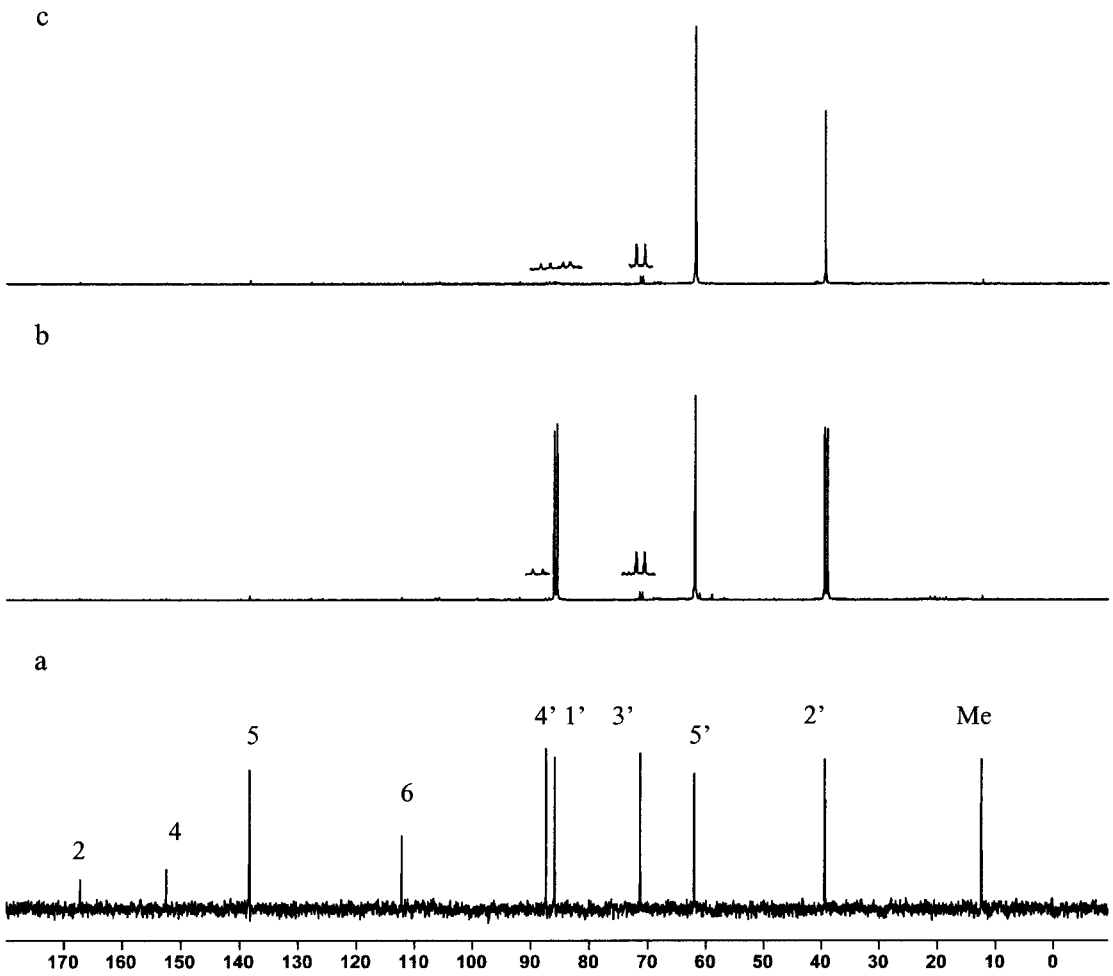

Figure 2. ^{13}C NMR (150 MHz) spectra of thymidine and its isotopomers: (a) unlabeled thymidine, (b) [1′,2′,5′-$^{13}C_3$]thymidine, and (c) [2′,5′-$^{13}C_2$]thymidine. Isotopic enrichments are apparent from the large increase in signal intensities.

Table 1. $^{1}H-^{13}C$ Coupling Constants (Hz) of Thymidine (1), 2′-Deoxy-D-uridine (2), and 2′-Deoxy-D-cytidine (3) Obtained from the ^{1}H NMR Spectra of the ^{13}C-Labeled Compounds 1a, 1b, 2a, and 3a

	C1′			C2′			C3′			C4′			C5′		
atom	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
H1′	170.2	170.7	–	–	–	–	*a*	–	*a*	–	–	–	–	–	–
H2′	–	–	–	134.6	134.1	–	–	–	2.9	*b*	–	4.3	–	–	–
H2″	–	–	–	134.6	135.2	–	*b*	–	2.5	*b*	–	2.5	–	–	–
H3′	–	–	–	*b*	–	*b*	151.2	*b*	151.2	1.2	*b*	1.2	3.5	–	–
H4′	–	–	–	0.7	–	–	4.8	*b*	4.4	148.8	*b*	148.9	1.0	–	–
H5′	–	–	–	–	–	–	2.1	–	3.6	1.1	–	2.1	142.6	143.3	–
H5″	–	–	–	–	–	–	3.3	–	2.1	1.2	–	1.4	143.3	142.8	–
H6	2.3	2.6	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] No additional coupling was observed. [b]Small but overlapping splitted signals were detected.

Table 2. Depicted Are the $^{13}C-^{13}C$ Coupling Constants (Hz) of Thymidine (1), 2′-Deoxy-D-uridine (2), and 2′-Deoxy-D-cytidine (3) Determined from the ^{13}C NMR Spectra of 1a, 1b, 2a, and 3a

	C1′			C2′			C3′			C4′			C5′		
atom	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
C1′	–	–	–	36.5	37.1	–	–	–	–	–	–	–	–	–	–
C2′	36.5	37.1	–	–	–	–	35.5	35.5	35.5	–	1.9	–	–	1.2	–
C3′	–	–	–	35.5	35.5	35.5	–	–	–	37.4	–	37.5	3.2	1.3	–
C4′	–	–	–	–	1.9	–	37.4	–	37.5	–	–	–	41.7	41.3	41.9
C5′	–	–	–	–	1.2	–	3.2	1.3	–	41.7	41.3	41.9	–	–	–

enzymes and expensive cofactors are needed to catalyze this reaction.

The alternative option is the removal of inorganic phosphate from the mixture. In general, salts of phosphates with heavy metals are poorly soluble. $MnCl_2$ is successfully used to precipitate phosphate from the reaction mixture as the heavy metal−phosphate salt. The *selectivity* of the precipitation of inorganic manganese phosphate relative to that of the organic manganese phosphate **5** was tested by addition of $MnCl_2$ solution (10

mM) to a series of 2-deoxy-D-ribose-5-phosphate (**5**) solutions (the range of final concentrations varied from 10 to 50 mM). It appeared that under these conditions no precipitate of organic manganese phosphate salt was formed.

Cytosine cannot be coupled, because the enzyme does not show any activity toward this substrate.[18] Therefore, [3′,4′-$^{13}C_2$]2′-deoxyuridine (**2c**) is efficiently elaborated to the ensuing [3′,4′-$^{13}C_2$]2′-deoxycytidine (**3a**). In our hands the use of tetrazole proved superior to both triazole and nitrotriazole in terms of yield and reliability.[19] The acetyl esters are removed by benzylamine, giving rise to the final product and benzyl acetamide. This latter compound can be easily extracted from the reaction mixture, thereby ensuring a higher yield. Because the tetrazole function is selectively removed in the presence of the esters, only 1.1 equiv of NH_3 is needed. In this manner the exocyclic NH_2 is efficiently isotopically labeled by a substitution reaction with $^{15}NH_3$.

Synthesis of 2-Deoxy-D-ribose-5-phosphate (5). Our premise for the development of a synthetic route to ^{13}C-enriched biomolecules is that any position and any combination of positions can be labeled. For the synthesis of the deoxyribose moiety this means that in addition to labeled acetaldehyde, which can be obtained commercially, the synthesis for both diazomethane and the acetic acid derivative must be suitable for incorporation of isotopes. Moreover, the coupling of these two fragments must proceed in an efficient manner, where both reactants are used in equimolar amounts (see Scheme 3).

The key compound in this synthesis is DHAP (**4**). The C_3-skeleton is prepared from an acetic acid derivative and diazomethane. Previously, the acetic acid derivative was activated as the acyl chloride.[4] However, when diazomethane reacts with an acyl chloride, HCl is liberated. This gas reacts with diazomethane (thus lowering the efficiency of the reagent) to chloromethane, and it also reacts with the product providing about 10% of chloromethyl ketone. Hence, it seemed that circumventing the occurrence of HCl altogether would greatly reduce the necessary amount of precursor resulting in a significantlytly improved yield. To this end *i*-butylchloroformate is used as an activation reagent. The absence of liberated acid increases the incorporation of diazomethane to 68%, which is almost twice the amount obtained by the previous method.[4]

Different amounts of benzyloxyacetic acid were used in the reaction. In addition, several reagents such as methyl chloroformate and ethyl chloroformate, various solvents such as diethyl ether, dichloromethane, and tetrahydrofuran, and temperatures in the range of −50 °C up to room temperature were tested. Performing the reaction at low temperature, in tetrahydrofuran, with *s*-butylchloroformate and starting with 1 equiv of precursor provided optimal results. The yield of diazomethane liberated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide is around 70−75%. Employing 1 equiv of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide therefore results in approximately 0.73 equiv of diazomethane, which gives rise to 68% yield of **9**. In this manner an excess of the unlabeled mixed anhydride is generated, which makes sure that the labeled diazomethane is completely trapped by the reagent.

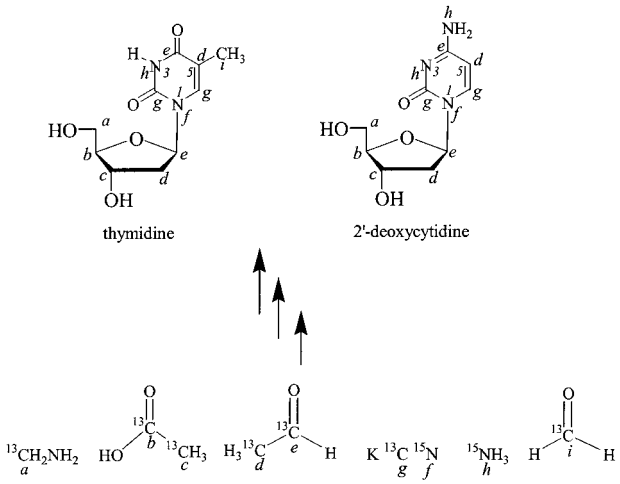

Figure 3. Correlation between the individual labeled atoms of the commercially available starting materials and the ^{13}C-enriched positions of the final product. By choosing the appropriate starting compounds, any ^{13}C- and ^{15}N-position and any combination of positions (up to $2^{12} - 1$) of the pyrimidine nucleoside can be labeled.

Conclusion

This work facilitates the use of enzymes in coupling nucleobases to deoxynucleosides, thereby combining the strengths of chemical methods to synthesize achiral phosphorylated precursors and the prime features of enzymatic transformations to govern stereochemically pure compounds. The important role of the phosphate in the subsequent reactions is nicely illustrated. First the phosphate is chemically incorporated into 2-deoxy-D-ribose-5-phosphate followed by a migration to α-2-deoxy-D-ribose-1-phosphate. This α configuration of the phosphate group facilitates nucleophilic attack of the pyrimidine base on the sugar from the top. This leads to exclusive formation of the *β*-anomer.

The enzymatic coupling of thymine to the sugar moiety reduces the number of steps that are needed for coupling by six (from thirteen to seven) and concomitantly increases the yield for the conversion of 2-deoxy-D-ribose-5-phosphate (**5**) to thymidine (**1**) and 2′-deoxy-D-uridine (**2**) from 14% to 52−63%. The overall yield based on methylamine hydrochloride is 24% (seven steps), whereas the overall yield based on acetaldehyde is 23% (two steps). In addition, the other pyrimidine deoxynucleosides, 2′-deoxyuridine and 2′-deoxycytidine, are prepared by the same scheme. Finally it is important to remark that all schemes are optimized to label ^{13}C- and ^{15}N-atoms of pyrimidine 2′-deoxynucleosides by using reagents (that can be obtained in the specifically isotopically labeled forms) in stoichiometric quantities. It is concluded that all positions and combination of ^{13}C- and ^{15}N-positions can be labeled. Figure 3 depicts the starting materials that can be employed and the positions they end up at in the final product.

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Experimental Section

General. Syntheses of the products, numbered by integers **1**, **2**,...**N**, were optimized using unlabeled synthons first. Labeled isotopomers of compound **N** are distinguished by **Na**, **Nb**, **Nc**. etc. [^{13}C]-Methylamine hydrochloride (99% ^{13}C) and [$^{13}C_2$]acetaldehyde (99% ^{13}C) and K^{13}C^{15}N (99% ^{13}C, 99% ^{15}N), were purchased from Cambridge Isotope Laboratories (Cambridge, MA). [2-^{13}C]acetaldehyde (99% enriched) was purchased from Eurisotope, France. Acetaldehyde was distilled immediately prior to use. Isotopically enriched [^{13}C]-diazomethane is prepared according to a slightly modified procedure that has been described earlier for unlabeled diazomethane.[4,12] Sigma provided 2-deoxy-D-ribose-1-phosphate as the cyclohexylammonium salt. Other chemicals were obtained from Aldrich or Acros Chimica.

Organic solvents were distilled prior to use, and dry solvents were obtained either by distillation from drying agents (toluene from CaH_2) or by storing over 4 Å molecular sieves (THF). Reactions were routinely monitored with thin-layer chromatography (TLC, on Merck F_{254} silica gel 60 aluminum 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 Buchi apparatus and are given uncorrected. Dowex 50W−X2 (400 mesh, H^+ form) was obtained from Acros Chimica.

^{1}H NMR spectra were recorded on a Bruker WM-300 or on a Bruker AM-600 spectrometer as indicated with tetramethylsilane in $CDCl_3$ (TMS; δ 0.00 ppm) as internal standard and in D_2O with TMS as external 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 ppm) as internal standard or for aqueous samples with TMS (δ 0.00 ppm) as external standard. ^{31}P NMR spectra were taken on a Bruker WM-300 spectrometer operated at 121 MHz with 85% H_3PO_4 as 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).

Enzyme reactions were carried out in Millipore water. Centrifugation steps were performed on a Sorvall SS3 automatic apparatus.

Triose phosphate isomerase from bakers' yeast (TPI, EC 5.3.1.1) and thymidine phosphorylase from *E. coli* (TP, EC 2.4.2.4) were purchased from Sigma. Phosphoribomutase was prepared as described below, and 2-deoxy-D-ribose-5-phosphate aldolase (DERA, EC 4.1.2.4), which was isolated from overproducing *E. coli* strain DH5α, was obtained from ATCC (ATCC 86963). Growing of the cells and harvesting of DERA was performed following literature procedures,[13] except that no lysozyme was used; the cells were sonicated instead. The ammonium sulfate precipitate was found to effectively catalyze aldol condensation, and this crude solution was used in enzymatic transformations. Assaying of the extracts and reaction mixtures was carried out as previously described;[4,13] a 2 L culture routinely afforded 15000 units, where one unit is sufficient to convert 1 μmol of 2-deoxy-D-ribose-5-phosphate to D-glyceraldehyde-3-phosphate and acetaldehyde min^{-1}. For PRM, one unit is sufficient to convert 1 μmol of 2-deoxy-D-ribose-5-phosphate to 2-deoxy-D-ribose-α-1-phosphate min^{-1}.

Expression of Recombinant Phosphoribomutase in *E. coli*. To have easy access to large amounts of phosphoribomutase, *E. coli* strains overproducing the enzymes were constructed. A 2 kb NdeI/Sal I fragment from pVH 19 with the *E. coli deoB ORF* was ligated into pET9 vector derivatives either with or without a N-terminal 6 × His-tag for one-step purification of the protein. The resulting constructs, pETdepBH and pETdeoB, respectively, were transformed into *E. coli* BL21(DE3)/pLysS for T7 RNA-polymerase expression.[20]

It was found that the clones without the $(His)_6$-tags gave 50% more overproduction than did clones with $(His)_6$-tags; 18000 units per 250 mL broth versus 11000 units per 250 mL. Because the purity of the preparation did not affect the results of the synthetic step, clones without $(His)_6$-tags were used throughout this paper.

Growing of the genetically modified microorganisms and harvesting of phosphoribomutase, was performed in the following manner. A flask with 25 mL 1% glucose, 50 μg/mL kanamycin, and 12.5 μg/mL chloramphenicol LC medium was inoculated with the desired strain and grown overnight at 37 °C. The cells were diluted to 250 mL with LC medium containing 50 μg/mL kanamycin and grown for 2 h. Subsequently, production of the protein was induced with isopropyl β-thiogalactopyranoside (IPTG) (0.250 mM), and after 2 h at 37 °C the cells were centrifuged (30 min, 8000 rpm). The pellet (about 0.8−1.0 g) was washed with physiologic salt solution (3 mL/g cells) and centrifuged. Cells were suspended in TRIS (100 mM) and EDTA (4 mM) pH 7.6 (3 mL/g wet cells) and sonicated (5 × 20s). Centrifugation (30 min, 8000 rpm) gave a pellet, which was discarded. The crude supernatant, which harbors 18000 units/250 mg protein, could be used for reactions; however, some additional purification was routinely carried out. The supernatant was brought to 1% streptomycin and centrifuged (30 min, 8000 rpm). Again the pellet was discarded and to the supernatant 226 mg/mL ammonium sulfate was added. Centrifugation (30 min, 8000 rpm) afforded a supernatant to which 187 mg/mL ammonium sulfate was added. Centrifugation (30 min, 8000 rpm) gave rise to a pellet, which was suspended in 2 mL TRIS (10 mM) and EDTA (1 mM) pH 7.6. The solution was dialyzed against 2 × 500 mL TRIS (10 mM), EDTA (1 mM) pH 7.6. This solution can be stored at 0 °C for at least two weeks and contains 12000 units/84 mg protein. Because the crude desalted solution provides satisfactory synthetic results, no further purification is routinely carried out.

To determine phosphoribomutase activity the reversed reaction catalyzed by that enzyme was coupled to the DERA-catalyzed reversed aldol condensation of 2-deoxy-D-ribose-5-phosphate. The occurring acetaldehyde is used by alcohol dehydrogenase (ADH) to oxidize $NADH^+$ to NAD^+, which can be spectroscopically monitored at 340 nm. A TRIS (100 mM) solution pH 8.0 was adjusted to contain 1 μM $MnCl_2$, 4 μM glucose-1,6-diphosphate, and 1.5 μM NADH. To this solution were added 4 units of ADH and a PRM enzyme aliquot. After incubation for 3 min, the assay was started by addition of 2 mM 2-deoxy-D-ribose-α-1-phosphate. The decrease in absorbance at 340 nm was measured with a millimolar absorptivity of 6.22 (mM·cm)$^{-1}$. The assay was calibrated at room temperature with commercially available 2-deoxy-D-ribose-α-1-phosphate (as the dicyclohexylammonium salt), and this compound was used without further purification. To ensure that the limiting step was the amount of mutase, assays were carried out with different aliquots of enzyme.

[2′,5′-$^{13}C_2$]Thymidine (1a). Thymine (50 mg, 0.4 mmol) was dissolved in water (10 mL), and **5a** (0.1 mmol, 24 mg) was added. After adjustment of the pH to 7.2, the mixture was heated to 43 °C, and thymidine phosphorylase (25 units) was added. Prior to addition of phosporibomutase (100 units) the enzyme was activated as described before. The reaction was run for 1.5 h, the pH was lowered to 7.0, and 0.02 mmol $MnCl_2$ was added. The mixture was adjusted to pH 7.2, and fresh enzymes were added. The reaction was again allowed to proceed for another 1.5 h. The process was repeated until proton NMR of a lyophilized sample indicated absence of 2-deoxy-D-ribose-5-phosphate (five times). After the mixture was lyophilized, the residue was dissolved in MeOH and absorbed on 500 mg of silica gel. Subsequently, the powder was loaded onto a silica gel column and eluted with dichloromethane/MeOH (90/10). This procedure yielded 15 mg (61

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μmol, 61%) of the title compound as a white solid. Mp 183 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.66 (m, H6, 1H), 6.30 (dd, $^2J_{H-H}$ 6.7, H1′, 1H), 4.48 (m, H3′, 1H), 4.03 (dd, $^2J_{C-H}$ 1.0, $^3J_{C-H}$ 0.7, H4′, 1H), 3.85 (d, $^1J_{C-H}$ 142.6, H5′, 1H), 3.78 (d, $^1J_{C-H}$ 143.3, H5″, 1H), 2.38 (d, $^1J_{C-H}$ 134.6, H2′ and H2″, 2H), 1.90 (d,$^4J_{H-H}$ 1.1, H5, 3H). ^{13}C NMR (600 MHz, D_2O): δ 167.1 (C4), 152.3 (C2), 138.2 (C6), 112.1 (C5), 87.3 (d, $^1J_{C-C}$ 41.7, C4′), 85.8 (d, $^1J_{C-C}$ 36.5, C1′), 71.2 (dd, $^1J_{C-C}$ 35.5, $^2J_{C-C}$ 3.2, C3′), 62.0 (strong s, C5′), 39.4 (strong s, C2′), 12.4 (5-Me). HRMS (ESI): calcd for $C_8{}^{13}C_2H_{14}N_2O_5$ 267.0868 (M + Na); found 267.0867 (M + Na).

[1′,2′,5′-$^{13}C_3$]Thymidine (1b). According to the above procedure, **5b** (72 mg, 0.3 mmol) and thymine (150 mg, 1.2 mmol) were reacted to yield 42 mg (0.17 mmol, 57%) of the title compound. The ^{1}H NMR and the ^{13}C NMR spectra of this labeled compound were identical to the corresponding spectra of the unlabeled compound except for the additional signal due to the presence of ^{13}C isotopes: ^{1}H NMR (600 MHz, D_2O): δ 7.66 (d, $^3J_{C-H}$ 2.3, H6, 1H), 6.30 (d, $^1J_{C-H}$ 170.2, H1′, 1H), 4.03 (dd, $^2J_{C-H}$ 1.0, $^3J_{C-H}$ 0.7, H4′, 1H), 3.85 (d, $^1J_{C-H}$ 142.6, H5′, 1H), 3.78 (d, $^1J_{C-H}$ 143.3, H5″, 1H). 2.38 (d, $^1J_{C-H}$ 134.6, H2′ and H2″, 2H). ^{13}C NMR (600 MHz, D_2O): δ 87.3 (d, $^1J_{C-C}$ 41.7, C4′), 85.8 (strong d, $^1J_{C-C}$ 36.5, C1′), 71.2 (dd, $^1J_{C-C}$ 35.5, $^2J_{C-C}$ 3.2, C3′), 62.0 (strong s, C5′), 39.4 (strong d, $^1J_{C-C}$ 36.5, C2′). HRMS (ESI): calcd for $C_7{}^{13}C_3H_{14}N_2O_5$ 268.0899 (M + Na); found 268.0879 (M + Na).

[1′,2′,5′-$^{13}C_3$]D-2′-Deoxyuridine (2a). According to the above procedure, **5a** (72 mg, 0.3 mmol) and uracil (134 mg, 1.2 mmol) were converted to the title compound in 52% yield (36 mg, 0.16 mmol). ^{1}H NMR (600 MHz, D_2O): δ 7.87 (dd, $^3J_{H-H}$ 8.1 Hz, $^3J_{H-H}$ 2.6 Hz, 1H, H6), 6.29 (dt, $^1J_{C-H}$ 170.7, 3J_{H-H} 6.3 Hz, 1H, H1′), 5.90 (d, $^3J_{H-H}$ 8.1, 1H, H5), 4.48 (dt, $^3J_{H-H}$ 6.4 Hz, $^3J_{H-H}$ 4.0 Hz, 1H, H3′), 4.06 (dt, $^3J_{H-H}$ 5.0 Hz, $^3J_{H-H}$ 3.8 Hz, 1H, H4′), 3.86 (dd, $^3J_{H-H}$ 12.6 Hz, $^1J_{C-H}$ 143.3 Hz, $^3J_{H-H}$ 3.6 Hz, 1H, H5′), 3.78 (dd,$^1J_{C-H}$ 142.8 Hz, $^3J_{H-H}$ 12.6 Hz, $^3J_{H-H}$ 5.0 Hz, 1H, H5″), 2.44 (ddd, $^3J_{H-H}$ 14.3 Hz, $^3J_{H-H}$ 6.7 Hz, $^1J_{C-H}$ 135.2 Hz, $^3J_{H-H}$ 4.0 Hz, 1H, H2″), 2.38 (dt, $^3J_{H-H}$ 14.3 Hz,$^1J_{C-H}$ 134.1 Hz, $^3J_{H-H}$ 6.7 Hz, 1H, H2′). ^{13}C NMR (151 MHz, D_2O): δ 167.1 (C4), 152,4 (C2), 142.8 (C6), 103.2 (C5), 87.6 (C4′), 86.3 (strong d, C1′), 71.3 (C3′), 62.0 (strong s, C5′), 39,6 (C2′, strong d). ESI MS: *m*/*z* = 254 (M + Na).

[3′,4′-$^{13}C_2$]D-2′-Deoxyuridine (2b) was prepared from **5b** (166 mg, 0.7 mmol) and uracil (336 mg, 3 mmol) according to the procedure as described above. The crude product was used to synthesize **6a.** ESI MS: *m*/*z* = 253 (M + Na).

[3′,4′-$^{13}C_2$]2′-Deoxycytidine (3a). Compound **8a** (58.0 mg, 0.185 mmol) was dissolved in methanol (4 mL). Benzylamine (0.534 mmol, 59 μL) was added, and the reaction mixture was stirred at room temperature for 3 days. Methanol was removed in vacuo, water (15 mL) was added, and the solution was extracted three times with dichloromethane (15 mL). The water layer was lyophilized, leaving a white powder (44 mg, 0.178 mmol, 96% in the monohydrate form). ^{1}H NMR (600 MHz, D_2O): δ 7.79 (d, 1H, $^3J_{H-H}$7.6 Hz, H6), 6.23 (t, 1H, $^3J_{H-H}$ 6.7 Hz, H1′), 6.00 (d, 1H, $^3J_{H-H}$ 7.6 Hz, H5), 4.39 (dm, 1H, $^1J_{C-H}$ 151.2 Hz, H3′), 4.01 (dm, 1H, $^1J_{C-H}$ 148.9 Hz, $^2J_{C-H}$ 4.7 Hz, H4′), 3.82−3.78 (dm, 1H, $^2J_{H-H}$ 12.5 Hz, H5′), 3.73−3.70 (dm, 1H, $^2J_{H-H}$ 12.5 Hz, H5″), 2.41−2.36 (m, 1H, H2″), 2.28−2.23 (m, 1H, H2′). ^{13}C NMR (150 MHz, D_2O): δ 166.9 (C4), 158.32 (C2), 142.3 (C6), 97.0 (C5), 89.3 (C1′), 87.3 (strong d, $^1J_{C-C}$ 37.5 Hz, C4′), 71.3 (strong d, $^1J_{C-C}$ 37.5 Hz, C3′), 62.0 (d, $^1J_{C-C}$ 41.8 Hz, C5′), 40.0 (d, $^1J_{C-C}$ 35.5 Hz, C2′). ESI MS: *m*/*z*=252 (M + Na^+).

[1-^{13}C]Dihydroxyacetone monophosphate (4a). [1-^{13}C]-Dibenzyl-3-benzyloxypropanone phosphate (**10a**) (1.2 g, 2.7 mmol) was dissolved in dioxane (32 mL), 2-propanol (16 mL), and water (8 mL). After addition of Pd/C (500 mg), hydrogen pressure was applied with a balloon, and the mixture was stirred (300 rpm) overnight. Conversion was checked with TLC (*n*-BuOH/acetone/water 5/3/2), and the mixture was filtered to give a clear, colorless solution. One equivalent of NaOH as a 1 M solution was added, and the solvent was evaporated in vacuo. The residue was used without further purification. This residue contained 2.5 mmol of the title compound (94%; based on enzymatic assay). ESI MS: *m*/*z* = 170 (M − H).

[2,5-$^{13}C_2$]2-Deoxy-D-ribose-5-phosphate (5a). [1-^{13}C]Dihydroxyacetone monophosphate (**4a**) (2.5 mmol) was dissolved in 25 mL water, that further contained 0.1 M TEA, 2 mM EDTA, 300 units of DERA, 700 units of TPI, and 0.2 M [2-^{13}C]-acetaldehyde. The solution was stirred for 2 h, after which time ^{13}C NMR indicated complete conversion. $BaCl_2$ dihydrate (1.22 g, 5 mmol) was added, and the suspension was stirred 40 min, followed by addition of ethanol (50 mL). The mixture was cooled for 18 h (0 °C), and the precipitate that was obtained after centrifugation was washed with ethanol and ether. After treatment with DOWEX H^+, the clear colorless solution was adjusted to neutral pH with 1 M NaOH and lyophilized. The yield at this point was 2.0 mmol, based on enzymatic assay. MS (ESI): *m*/*z* = 215 (M − Na).

[1,2,5-$^{13}C_3$]2-Deoxy-D-ribose-5-phosphate (5b) was prepared as described above by using **4a** (1 mmol) and [1,2-$^{13}C_2$]-acetaldehyde (92 mg, 2 mmol) as starting materials.

[3′,4′-$^{13}C_2$]3′,5′-Di-*O*-acetyl-2′-deoxyuridine (6a). Crude [3′,4′-$^{13}C_2$]-D-2′-deoxyuridine (**2b**, according to analytical data the crude compound contains 82 mg, 0.358 mmol of pure **2b**) was dissolved in 5.0 mL of dry pyridine, and acetic anhydride (3.2 mmol, 0.3 mL) was added. The suspension was stirred for 2 days at room temperature. The pyridine was removed under reduced pressure, and the resulting oil was purified by column chromatography (CH_3OH/CH_2Cl_2 5/95), giving a slightly yellow oil (102 mg, 0.323 mmol, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.51 (d, 1H, $^3J_{H-H}$ 8.2 Hz, H6), 6.32−6.27 (dd, 1H, $^3J_{H-H}$ 8.2 Hz, $^3J_{H-H}$ 5.8 Hz, H1′), 5.80 (d, 1H, $^3J_{H-H}$ 8.2 Hz, H5), 5.22 (dm, 1H, $^1J_{C-H}$ 158.2 Hz, H3′), 4.40−4.29 (m, 2H, 5′-H, H5″), 4.27 (dm, 1H, $^1J_{C-H}$ 151.9 Hz, H4′), 2.48−2.59 (m, 1H, H2″), 2.22−2.13 (m, 1H, H2′), 2.11 (s, 3H, Me), 2.12 (s, 3H, Me). ^{13}C NMR (75 MHz, CDCl3): δ 170.2 (CO), 163.3 (C4), 150.3 (C2), 138.8 (C6), 102. 9 (C5), 84.0 (C1′), 82.2 (strong d, $^1J_{C-C}$ 37.5 Hz, C4′), 74.0 (strong d, $^1J_{C-C}$ 37.5 Hz, C3′), 63.7 (d, $^1J_{C-C}$ 42.8 Hz, C5′), 37.7 (d, $^1J_{C-C}$ 36.6 Hz, C2′), 20.8 (Me), 20.70 (Me). ESI MS: *m*/*z* = 315 (M + H^+), 337 (M + Na^+).

4-(Tetrazol-1-yl)-1-(3′,5′-di-*O*-acetyl-2′-[3′,4′-$^{13}C_2$]deoxy-β-D-ribofuranosyl)pyrimidin-2-one (7a). Compound **6a** (102 mg, 0.323 mmol) was dissolved in 0.80 mL of dry pyridine. Tetrazole (0.45 mmol, 32 mg), diphenyl phosphate (0.27 mmol, 68 mg), and tosyl chloride (0.45 mmol, 86 mg) were added, and the reaction mixture was stirred at room temperature for 1.5 h. Pyridine was removed under reduced pressure, and the resulting oil was purified by column chromatography (CH_3OH/CH_2Cl_2 3/97), giving 110 mg (0.297 mmol, 92%) of the title compound. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.64 (s, 1H, tetrazole-H), 8.51 (d, 1H, $^3J_{H-H}$ 7.2 Hz, 6-H), 7.27 (d, 1H, $^3J_{H-H}$ 7.2 Hz, H5), 6.26 (t, 1H, $^3J_{H-H}$ 6.5 Hz, H 1′), 5.27 (dm, 1H, $^1J_{C-H}$ 157.7 Hz, $^2J_{CH}$=2.5 Hz, H3′), 4.48 (dm, 1H, $^1J_{C-H}$ 152.6 Hz, H4′), 4.48−4.36 (m, 2H, 5′-H, H5″), 3.02−2.92 (m, 1H, H2″), 2.30−2.17 (m, 1H, H2′), 2.15 (s, 3H, Me). 2.11 (s, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.2 (CO), 170.0 (CO), 157.2 (C4), 153.5 (C2), 147.0 (tetrazole-C), 140.5 (C6), 94.8 (C5), 83.5 (strong d, $^1J_{C-C}$ 37.9 Hz, C4′), 88.3 (C1′), 73.7 (strong d, $^1J_{C-C}$ 37.9 Hz, C3′), 63.3 (d, $^1J_{C-C}$ 42.5 Hz, C5′), 38.9 (d, $^1J_{C-C}$ 36.4 Hz, C2′), 20.7 (Me).

Di-*O*-acetyl-[3′,4′-$^{13}C_2$]2′-deoxycytidine (8a). Compound **7a** (68.0 mg, 0.192 mmol) was dissolved in 2.0 mL of dioxane. Ammonium hydroxide (0.22 mmol, 0.034 mL) was added, and the solution was stirred at room temperature for 3 days. The solvent was evaporated, and the residue was absorbed onto silica and purified by column chromatography (CH_3OH/CH_2Cl_2 10/90). The yield of the title compound was 58.0 mg (0.185 mmol, 96%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.71 (d, 1H, $^3J_{H-H}$ 7.5 Hz, H6), 6.24−6.20 (dd, 1H, $^3J_{H-H}$ 8.1 Hz, $^3J_{H-H}$ 5.8 Hz, H1′), 5.93 (d, 1H, $^3J_{H-H}$ 7.7 Hz, H5), 5.23 (dm, 1H, $^1J_{C-H}$ 160.7 Hz, H3′), 4.38−4.25 (m, 2H, 5′-H, H5″), 4.27 (dm, 1H, $^1J_{C-H}$ 152.0 Hz, H4′), 2.58−2.47 (m, 1H, H2″), 2.29−2.17 (m, 1H, H2′), 2.08 (s, 3H, Me), 2.06 (s, 3H, Me). ^{13}C NMR (75 MHz, CD_3OD): δ 172.2 (CO), 172.1 (CO), 167.6 (C4), 158.0 (C2), 141.7 (C6), 96.4 (C5): 86.2 (C1′), 83.7 (strong d, $^1J_{C-C}$ 36.9 Hz, C4′), 76.0 (strong d, $^1J_{C-C}$ 37.1 Hz, C3′), 65.1 (d, $^1J_{C-C}$ 42.6 Hz, C5′), 38.8 (d, $^1J_{C-C}$ 36.5 Hz, C2′), 20.8 (Me), 20.70 (Me). ESI MS: *m*/*z* = 314 (M + H^+), 336 (M + Na^+).

3-Benzyloxy-[1-^{13}C]1-diazopropanone (9a). Benzyloxyacetic acid (3.44 g, 20.7 mmol) was coevaporated with dioxane (3 × 40 mL) and subsequently dissolved in dry THF (100 mL). The reaction mixture was cooled to −30 °C, and triethylamine (2.12 g, 20.9 mmol) was added followed by chloroisobutyl carbonate (2.82 g, 20.6 mmol). The resulting suspension was stirred for 30 min and then cooled to −50 °C. In a separate flask *N*-[^{13}C]methyl-*N*-nitroso-*p*-toluenesulfonamide (4.5 g, 20.7 mmol) was dissolved in diethylene glycol monoethyl ether (75 mL) and diethyl ether (75 mL). Diazomethane formation was started through injection of 40% KOH solution (2 mL), which was repeated three times, every 40 min to keep the rate of diazomethane formation and the reaction rate high. A nitrogen stream facilitated entry of the ^{13}C-labeled diazomethane gas into the reaction vessel. The nitrogen stream containing the ^{13}C-labeled diazomethane was dried over KOH pellets (4−5 g) before entering the second reaction vessel. After the final addition of 40% KOH solution, the mixture was allowed to warm to room temperature, while maintaining the nitrogen stream, and subsequently the solvent was evaporated. The residue was taken up in diethyl ether (75 mL) and poured in 10% aqueous $NaHCO_3$ solution (75 mL). The layers were separated, and the ethereal layer was washed with water (75 mL) twice. Drying over $MgSO_4$, evaporation of the solvent and column chromatography (diethyl ether/PE 1/1) afforded **9a** (2.70 g, 14.1 mmol, 68%). ^{1}H NMR ($CDCl_3$): δ 7.35 (m, 5H), 5.79 (d, $^1J_{C-H}$ 203.6 Hz, 1H), 4.57 (s, 2H), 4.04 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 193.44 (d, $^1J_{C-C}$ 66.6, C2), 136.3 (Ar−C), 128.3 (Ar−C), 127.9 (Ar−C), 127.6 (Ar−C), 76.3 (CH_2) 73.71 (strong s, C3), 53.2 (C1). HRMS (ESI): calcd for $C_9{}^{13}CH_{11}N_2O_2$ 192.0854; found 192.08871.

Dibenzyl-3-benzyloxy-[1-^{13}C]propanone Phosphate (10a). Diazoketone **9a** (2.70 g, 14.1 mmol) was mixed with dibenzyl phosphate (4.52 g, 16.25 mmol) in toluene (30 mL) and heated for 8 h at 75 °C. After purification on silica gel (hexane/chloroform/ethyl acetate 1/1/1), **7** (5.06 g, 11.5 mmol, 81%) was obtained. ^{1}H NMR: δ 7.34 (m, 5H, Ar−H), 5.09 (d, $^3J_{P-H}$ 9.6 Hz, Bn-CH_2, 4H), 4.71 (dd, $^3J_{P-H}$ 9.7, $^1J_{C-H}$ 148.9 Hz, H1, 2H), 4.52 (s, Bn-CH_2, 2H), 4.10 (s, H3, 2H). ^{13}C NMR ($CDCl_3$): δ 201.9 (dd, $^1J_{C-C}$ 43.9 Hz , $^2J_{P-C}$ 5.6 Hz, CO), 73.3 (C1). ^{31}P NMR ($CDCl_3$): δ −0.518 (d, $^2J_{C-P}$ 5.5 Hz). MS (ESI): *m*/*z* 464 (M + Na^+)

[1,3-^{15}N$_2$,2,4-^{13}C$_2$]5,6-Dihydrouracil (11). $KO^{13}C^{15}N$ was prepared by addition of 13% aqueous NaOCl solution (5.75 mL, 9.8 mmol) to $K^{13}C^{15}N$ (0.6 g, 8.9 mmol) in 40 mL of water. The solution was stirred for 20 min. The excess NaOCl was destroyed with NaS_2O_5 (about 200−500 mg). Subsequently, [1-^{13}C,^{15}N]β-alanine (580 mg, 6.37 mmol), prepared as described earlier,[6] was added, and the solution was evaporated to dryness (100 °C, oil bath) in about 4 h. Once the mixture cooled to room temperature, 15 mL of 6 M HCl was added, and the ensuing solution was evaporated to dryness in vacuo, followed by heating on an oil bath at 150 °C for 25 min. Column chromatography (CH_2Cl_2/MeOH 9/1) afforded labeled dihydrouracil (700 mg, 6.14 mmol, 96%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.87 (br dd, $^1J_{N-H}$ 90.2 Hz, $^2J_{C-H}$ 1.6 Hz, 1H, H3), 7.72 (dd, $^1J_{N-H}$ 93.1 Hz, $^2J_{C-H}$ 1.4 Hz, 1H, H1), 3.46 (m, $^3J_{H-H}$ 7.2 Hz, $^3J_{H-H}$ 2.5 Hz, 2H, H6), 2.69 (ddt, $^3J_{H-H}$ 7.2 Hz,$^2J_{C-H}$ 6.7 Hz, $^3J_{N-H}$ 2.9 Hz, 2H, H5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 175.4 (strong signal, dd, $^1J_{N-C}$ 10.6 Hz, $^2J_{C-C}$ 1 Hz, C4), 156.6 (strong signal, ddd, $^1J_{N-C}$ 23 Hz, $^1J_{N-C}$ 17 Hz, $^2J_{C-C}$ 1 Hz, C2), 35.4 (dd, $^1J_{N-C}$ 9 Hz, $^2J_{C-C}$ 2 Hz, C6), 30.5 (dd, $^1J_{C-C}$ 47.9 Hz, $^2J_{N-C}$ 6 Hz, C5). ^{15}N NMR (330 MHz, DMSO-d_6): δ 152.5 (dd, $^1J_{C-N}$ 12 Hz, N1), 85.3 (d, $^1J_{C-N}$ 11 Hz, N3).

[1,3-^{15}N$_2$,2,4-^{13}C$_2$]Uracil (12). [1,3-^{15}N$_2$,2,4-^{13}C$_2$]Dihydrouracil (600 mg, 5.1 mmol **11**) was suspended in 9 mL of acetic acid. The solid did not dissolve, and this mixture was heated to 100 °C and Br_2 (0.393 mL, 7.63 mmol) in acetic acid (12 mL) added to the reaction mixture in about 30 min. The mixture was stirred while being heated for 6 h after which time it had lost the bromine color. The solvent was removed in vacuo to yield a white residue, which is a mixture of [1,3-^{15}N$_2$,2,4-^{13}C$_2$]5-bromo-6-hydropyrimidine and [1,3-^{15}N$_2$,2,4-^{13}C$_2$]5,5-dibromo-6-hydropyrimidine. The mixture of compounds obtained was subjected to 170 °C on an oil bath for 20 min. A hydrogen stream directed into the flask helped to remove liberated HBr. After the reaction period, the residue (a mixture of [1,3-^{15}N$_2$,2,4-^{13}C$_2$]5-bromo-6-hydropyrimidine and [1,3-^{15}N$_2$,2,4-^{13}C$_2$]5,5-dibromo-6-hydropyrimidine) was hydrogenated over $Pd/BaSO_4$ (500 mg) with H_2 and yielded, after column chromatography (CH_2Cl_2/MeOH 9/1), uracil (430 mg, 3.8 mmol, 75%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.88 (br s, 2H, H1 and H3), 7.37 (m, $^3J_{H-H}$ 7.6 Hz, 1H, H6), 5.43 (m, $^3J_{H-H}$ 7.6 Hz, 1H, H5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.4 (strong signal, d, $^1J_{N-C}$ 8.9 Hz, C4), 151.6 (strong signal dd, $^1J_{N-C}$ 17.8 Hz, $^1J_{N-C}$ 15.1 Hz, C2), 142.2 (C6), 100.3 (C5). ^{15}N NMR (30 MHz, DMSO-d_6): δ 161.2 (dd, $^1J_{C-N}$ 15 Hz, $^1J_{C-N}$ 9 Hz, N3), 133.7 (d, $^1J_{C-N}$ 18 Hz, N1). HRMS (ESI): *m*/*z* calcd for $C_2{}^{13}C_2H_4{}^{15}N_2O_2$ 117.0378; found 117.0359.

[1,3-^{15}N$_2$,2,4-^{13}C$_2$]Thymine (13). Compound **12** (300 mg, 2.59 mmol) was suspended in 4 mL of water with paraformaldehyde (93 mg, 3.1 mmol) and KOH (94 mg, 1.68 mmol) and was heated for 24 h at 65 °C. After the reaction was complete, which can be monitored with TLC using *n*-BuOH, HOAc, and H_2O 5/3/2 as the mobile phase, the mixture was acidified with Dowex H^+. The slurry was filtered and thoroughly washed with water, followed by evaporation of the solvent in vacuo. Lyophilization yielded [1,3-^{15}N$_2$,2,4-^{13}C$_2$]5-hydroxymethyluracil (360 mg, 2.5 mmol, 96%). This compound (340 mg, 2.9 mmol) was dissolved in 100 mL of acetic acid/H_2O 1/1 and hydrogenated (50 psi) over $Pd/BaSO_4$ (500 mg) for 18 h. The $Pd/BaSO_4$ was removed by filtration, and the residue was lyophilized to (320 mg, 2.9 mmol, 100%) crude [1,3-^{15}N$_2$,2,4-^{13}C$_2$]thymine. Purification on a silica gel column (CH_2Cl_2/MeOH 9/1) afforded the title compound (275 mg, 2.2 mmol, 75%) as a white solid. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.96 (d, $^1J_{N-H}$ 89.9 Hz, 1H, H3), 10.55 (d, $^1J_{N-H}$ 93.2 Hz, 1H, H1), 7.20 (ddd, 9.5 Hz, 3.6 Hz, 1.2 Hz, 1H, H6), 1.69 (dd, 3.9 Hz, 1.1 Hz, 3H, 5-Me) ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.7 (strong signal, d, $^1J_{N-C}$ 9.3 Hz, C4), 151.3 (strong signal, dd, $^1J_{N-C}$ 18.5 Hz, $^1J_{N-C}$ 16.0 Hz, C2), 137.5 (d, $^2J_{N-C}$ 9.9 Hz, C6), 107.5 (C5), 11.6 (5-Me). ^{15}N NMR (DMSO-d_6, 30 MHz): δ 159.2 (dd, $^1J_{C-N}$ 16 Hz, $^1J_{C-N}$ 10 Hz, N3), 130.9 (d, $^1J_{C-N}$ 19 Hz, N1). HRMS (DIP): *m*/*z* calcd for $C_3{}^{13}C_2H_6{}^{15}N_2O_2$ 130.0437; found 130.0430.

Preparation of ^{13}C-Labeled Diazomethane. *N*-[^{13}C]-Methyl-*p*-toluenesulfonamide. [^{13}C]Methylamine·HCl (3.00 g, 43.8 mmol) and tosyl chloride (8.35 g, 43.9 mmol) were added to a vigorously stirred two-phase system of water and dichloromethane (200 mL, v/v 1/1). A 50% solution of KOH in water is added in four portions in about 90 min for a total of 12 mL of solution (107 mmol of KOH). After acidification with 6 M HCl, the layers were separated, and the aqueous layer was washed twice with dichloromethane. The combined organic layers were then washed with saturated $NaHCO_3$ solution and then dried on Na_2SO_4 and evaporated to yield 7.9 g (42.3 mmol, 97%) of a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, $^3J_{H-H}$ 8.5 Hz, 2H, Ar−H), 7.31 (d, $^3J_{H-H}$ 7.9 Hz, 2H, Ar−H), 5.12 (br q, $^3J_{H-H}$ 5.2 Hz, 1H, N−H), 2.63 (dd, $^1J_{C-H}$ 139.7, $^3J_{H-H}$ 5.2 Hz, 3H, Me), 2.43 (s, 3H, Ar-Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 143.3 (Ar−C), 135.4 (Ar−C), 129.6 (Ar−C), 127.1 (Ar−C), 29.1 (strong $^{13}CH_3$-signal), 21.3 (Ar−Me). HRMS (ESI): calcd for $C_7{}^{13}CH_{11}NO_2S$ 186.05440; found 186.05297.

***N*-[^{13}C]Methyl-*N*-nitroso-*p*-toluenesulfonamide.** *N*-[^{13}C]-Methyl-*p*-toluenesulfonamide (3.95 g, 21.2 mmol) was dissolved in acetic acid (40 mL), and the solution was cooled in ice. $NaNO_2$ (2.18 g, 31.6 mmol) was dissolved in water (10 mL) and slowly added through a dropping funnel. The reaction solution was stirred for 20 min and subsequently diluted with water (100 mL). This solution was extracted with ether (three times 100 mL), and the organic layers were washed with saturated $NaHCO_3$ solution to remove acid. Drying of the ethereal extract on $MgSO_4$ and evaporation of the solvent afforded the title compound (4.55 g, 21.1 mmol, 99%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.87 (d, $^3J_{H-H}$ 8.4 Hz, Ar−H), 7.39 (d, $^3J_{H-H}$ 8.4 Hz, Ar−H), 3.12 (d, $^1J_{C-H}$ 143.8 Hz, 3H, Me), 2.46 (s, 3H, Ar-Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 146.1 (Ar−C), 133.9 (Ar−C), 130.3 (Ar−C), 127.8 (Ar−C), 28.8 (strong $^{13}CH_3$

signal), 21.6 (Ar−Me). HRMS (ESI): calcd For $C_7{}^{13}CH_{10}N_2O_3S$ 215.04456; found 215.04237.

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