Synthesis of Stable-isotope (^{13}C and ^{15}N) Labeled Nucleosides and Their Applications

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Abstract: NMR studies of stable-isotope labeled oligonucleotides have provided useful information regarding nucleic acid structures, nucleic acid - drug binding, and nucleic acid - protein interactions. For the conformational elucidation of the sugar-phosphate backbone and/or the sugar moieties in nucleic acids, ^{13}C-labeled oligonucleotides are very useful, and for the transformation of nucleic acid - drug binding sight are ^{15}N-labeled oligonucleotides.

The present paper has introduced the recent synthetic methods of nucleosides containing either a ^{13}C labeled sugar moiety or a ^{15}N-labeled exocyclic amine, and applications including our research work.

I. INTRODUCTION

The structural elucidation of mechanisms concerning the interaction of genes with proteins or drugs has emerged in recent years [1]. NMR and X-ray crystallography have been generally used to obtain such information. Crystallographic analysis is useful in the structural resolution of large biomolecules [2]. On the other hand, NMR methods can provide information relating to structures present in solution under quasi-physiological conditions. Furthermore, NMR studies using stable-isotope labeled oligonucleotides can provide precise information regarding nucleic acid structures, nucleic acid-drug binding and nucleic acid-protein interactions [3].

One early approach utilizing nucleosides possessing a ^{13}C labeled sugar moiety was the use of a uniformly labeled [1',2',3',4',5'-$^{13}C_5$]nucleoside derived through biosynthetic techniques. There are many excellent structural studies using labeled nucleic acids of this type [4]. More detailed studies, however, require site-specific labeling of nucleic acids, and this task cannot be achieved biosynthetically.

The chemical synthesis of site-specific labeled nucleosides has been successfully undertaken, and the resulting site-specific labeled oligonucleotides have provided precise information on local interactions such as hydrogen bonding [5], protonation [6], hydration [7], ligand interactions [8], stacking [9] and drug- and protein-nucleic acid structures [3a,10]. ^{2}H- and ^{13}C-labeled oligonucleotides have been used for the conformational elucidation of sugar-phosphodiester backbones [3d], the puckering of sugar moieties [3e] and for the determination of drug- and protein-nucleic acid binding sites [3f], while ^{15}N-labeled oligonucleotides have been used in studies concerning the transformation of nucleic acid-drug binding sites [3a,g, 10].

We developed novel and efficient methods for the highly diastereoselective synthesis of the (2'*R*)-[11] and (2'*S*)-[2'-$^{2}H_1$]nucleosides [12], the (5'*R*)- and (5'*S*)-[5'-$^{2}H_1$]-nucleosides [13], the synthesis of [5'-^{13}C]nucleosides [14] and the ^{15}N-labeling of the exocyclic amino group of nucleobases [15]. The synthesis of deuteronucleosides was reviewed by Chattopadhyaya and coworker [16]. The published methods for the chemical synthesis of ^{13}C and ^{15}N-labeled nucleosides have been reviewed by Serianni and coworker [17], Lagoja and Herdewijn [18], Milecki [19]. Kojima and coworker [20], and Ono and coworker [21]. The purpose of the present paper is to introduce our research work concerning the synthesis of a nucleosides containing either a ^{13}C site-specific labeled sugar moiety or a ^{15}N-labeled exocyclic amine, and applications combined with a review of the recent literatures.

II. ^{13}C-LABELED NUCLEOSIDES

II-1. Synthesis of ^{13}C-Labeled Nucleosides

The nucleosides incorporated site-specifically ^{13}C-labels into the ribose are extremely significant for use in investigations concerning conformational analyses of the sugar-phosphodiester backbone, puckering and the *N*-glycoside bond of C1'-N (nucleobase).

The torsion angle of the sugar-phosphodiester backbone is defined as follow; α ($_{(n-1)}O_{3'}$-P-$O_{5'}$-$C_{5'}$), β (P-$O_{5'}$-$C_{5'}$-$C_{4'}$), γ ($O_{5'}$-$C_{5'}$-$C_{4'}$-$C_{3'}$), σ($C_{5'}$-$C_{4'}$-$C_{3'}$-O_3), ε ($C_{4'}$-$C_{3'}$-$O_{3'}$-P), and ζ ($C_{3'}$-$O_{3'}$-P-$C_{5'(n+1)}$) (Fig. **1**) [22].

Conformation of sugar moiety is displayed as 2'-*endo* or 3'-*endo* (Fig. **2**) [22] while the conformation of the *N*-glycoside bond is defined as *anti* and *syn* (Fig. **3**) [22].

II-1-1. Site-Specific Carbon $^{13}C_1$-Labeling of the Ribose Moiety in Nucleosides

Labeling at C5' Carbon Atom of Nucleosides

The development of an efficient synthetic method for 2'-deoxy[5'-^{2}H]ribonucleosides (5'*S*:5'*R* = *ca*. 2:1) [13] has

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Torsion angle	Atoms involved
α	$_{n-1)}O_{3'}$-P-$O_{5'}$-$C_{5'}$
β	P-$O_{5'}$-$C_{5'}$-$C_{4'}$
γ	$O_{5'}$-$C_{5'}$-$C_{4'}$-$C_{3'}$
δ	$C_{5'}$-$C_{4'}$-$C_{3'}$-$O_{3'}$
ε	$C_{4'}$-$C_{3'}$-$O_{3'}$-P
ζ	$C_{3'}$-$O_{3'}$-P-$C_{5'(n+1)}$

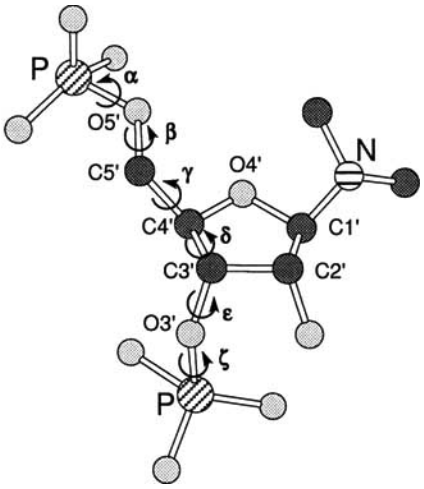


Fig. (1). Definition of Torsion Angles in Nucleotides.

fueled expectations that they would facilitate the unambiguous assignment of both the H5' and H5" signals of an oligodeoxyribonucleotide and the analysis of sugar-phosphodiester backbone conformations by NMR spectroscopy. Through the use of 2D ^{1}H-^{31}P HSQC spectroscopy on deuterium-labeled nucleotides, Ono and co-workers [24] achieved the assignment of the methylene protons at C5' of a DNA dodecamer. On the other hand, Kojima and coworkers [25] obtained the 15 ^{3}J coupling constants between H4' and H5'/H5" due to the simplified spin systems through the use of NOESY and DQF-COSY NMR analyses. We assumed that more precise analyses of the DNA backbone structure including distance information should be possible by using nucleotides labeled with carbon-13 at the 5' position in terms of heteronuclear multidimensional NMR spectroscopy.

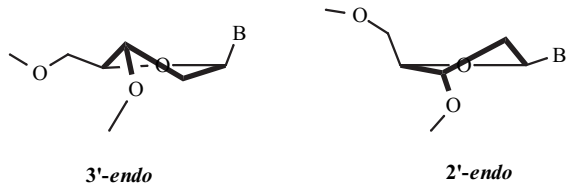


Fig.(2). Puckering; Conformation of sugar.

On the other hand, it was reported that the torsion angle γ (O5'-C5'-C4'-C3') could be specified, allowing for the determination of β (P5'-O5'-C5'-C4') through the use of 30% enriched RNA [26]. The application and use of ^{13}C-labeling at the C5' position is invaluable in the conformational analysis of the sugar-phosphdiester backbone, which is considered to be important in the overall elucidation of drug- and protein-nucleic acid recognition processes [27].

The chemical syntheses of [5'-^{13}C]adenosine and D-[5-^{13}C]ribose have been reported by Matwiyoff *et al.* [28] and Serianni *et al.,* [17c] respectively. [5'-^{13}C]Adenosine was prepared by the coupling reaction of an adenine derivative with a D-[5-^{13}C]ribose derivative, which was synthesized by way of L-[1-^{13}C]ribonic acid obtained by the reaction of L-erythrose with [^{13}C]potassium cyanide followed by hydrolysis under acidic conditions (Scheme **1**).

The synthesis of D-[5-^{13}C]ribose [17c] by Serianni and coworker, on the other hand, was carried out by the transformation from D-[6-^{13}C]glucose (**6**) prepared by the reaction of 1,2-*O*-isopropylidene-α-D-*xylo*-pentdialdo-1,4-furanoside (**5**) with [^{13}C]potassium cyanide. In this case, the 5-epimeric [6-^{13}C]cyanohydrins are reduced (H_2/Pd-$BaSO_4$) to the protected C5 epimeric [6-^{13}C]dialdoses, which were

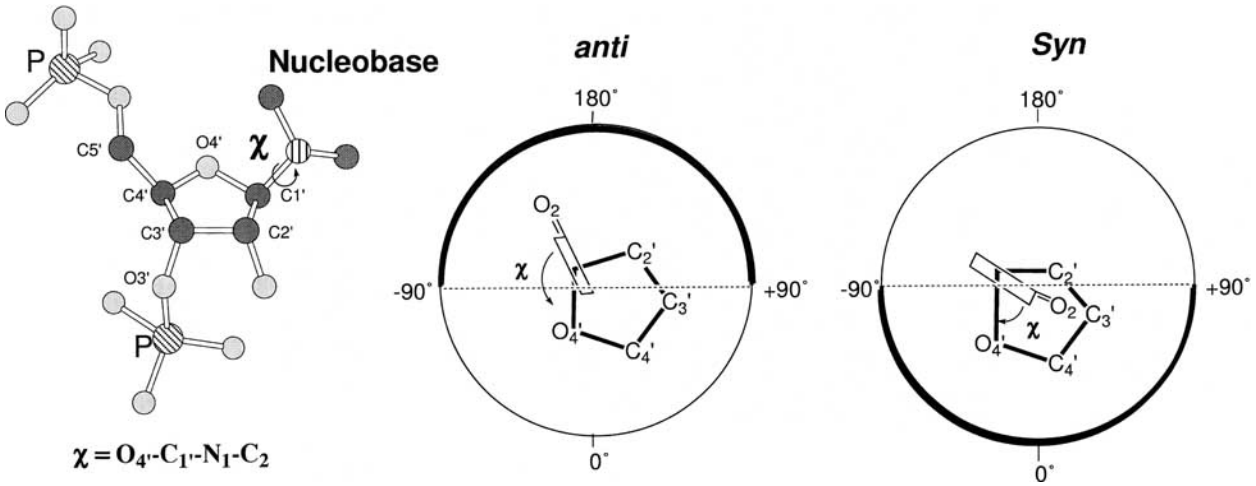
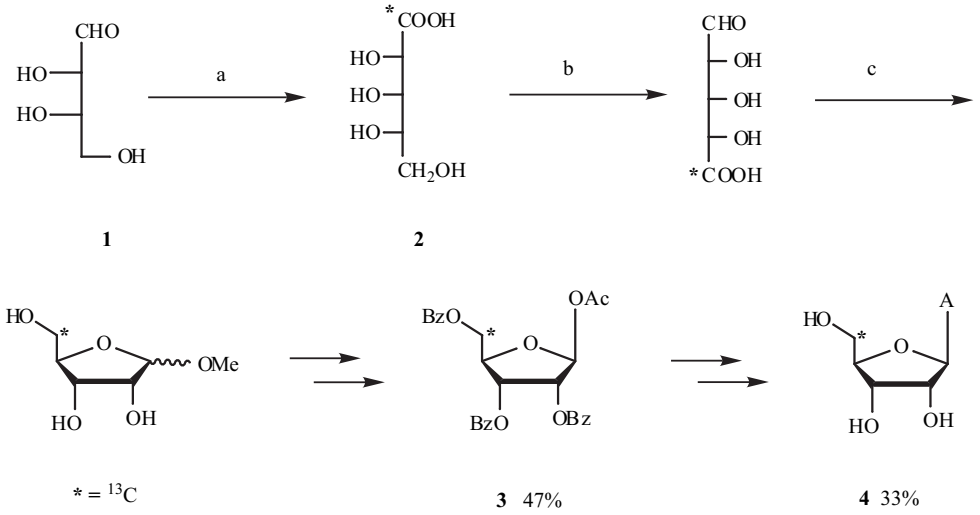


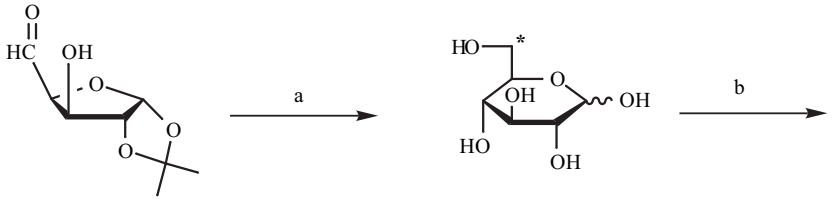
Fig (3). Definition of *anti* and *syn* conformational ranges according to IUPAC-IUB Joint Commission on Biochemical Nomenclature (1983) [23] shown for pyrimidine nucleoside, x is defined as torsion angle $O_{4'}$-$C_{1'}$-N_1-C_2. The pyrimidine base is toward the viewer; the base is rotated relative to sugar [22].

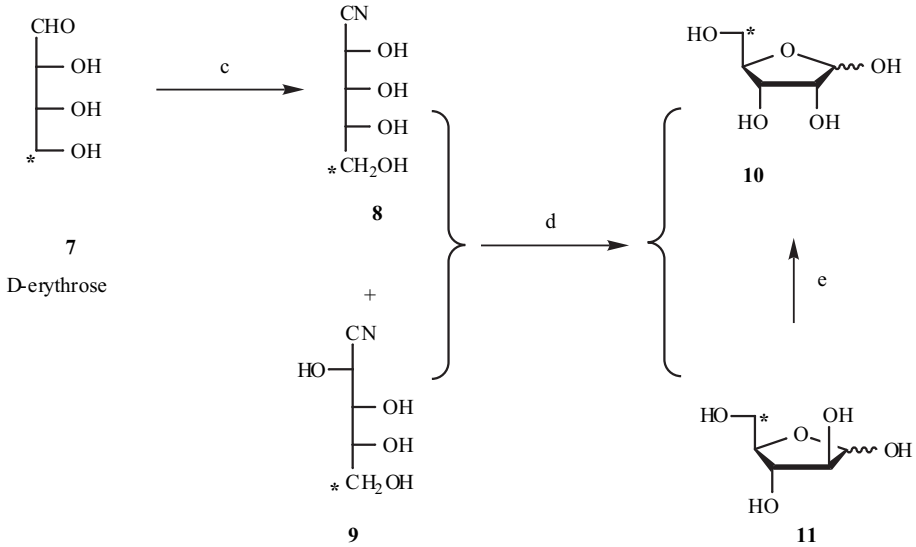


Scheme 1. *Reagents and Conditions:* (a) $K^{13}CN$ / OH^- (27%); (b) 1. H^+ / acetone 2. DMSO, DCC; 3. H^+ (54%); (c) 1. H^+ / MeOH; 2. $(MeOCH_2CH_2O)_2AlH$ (58%).

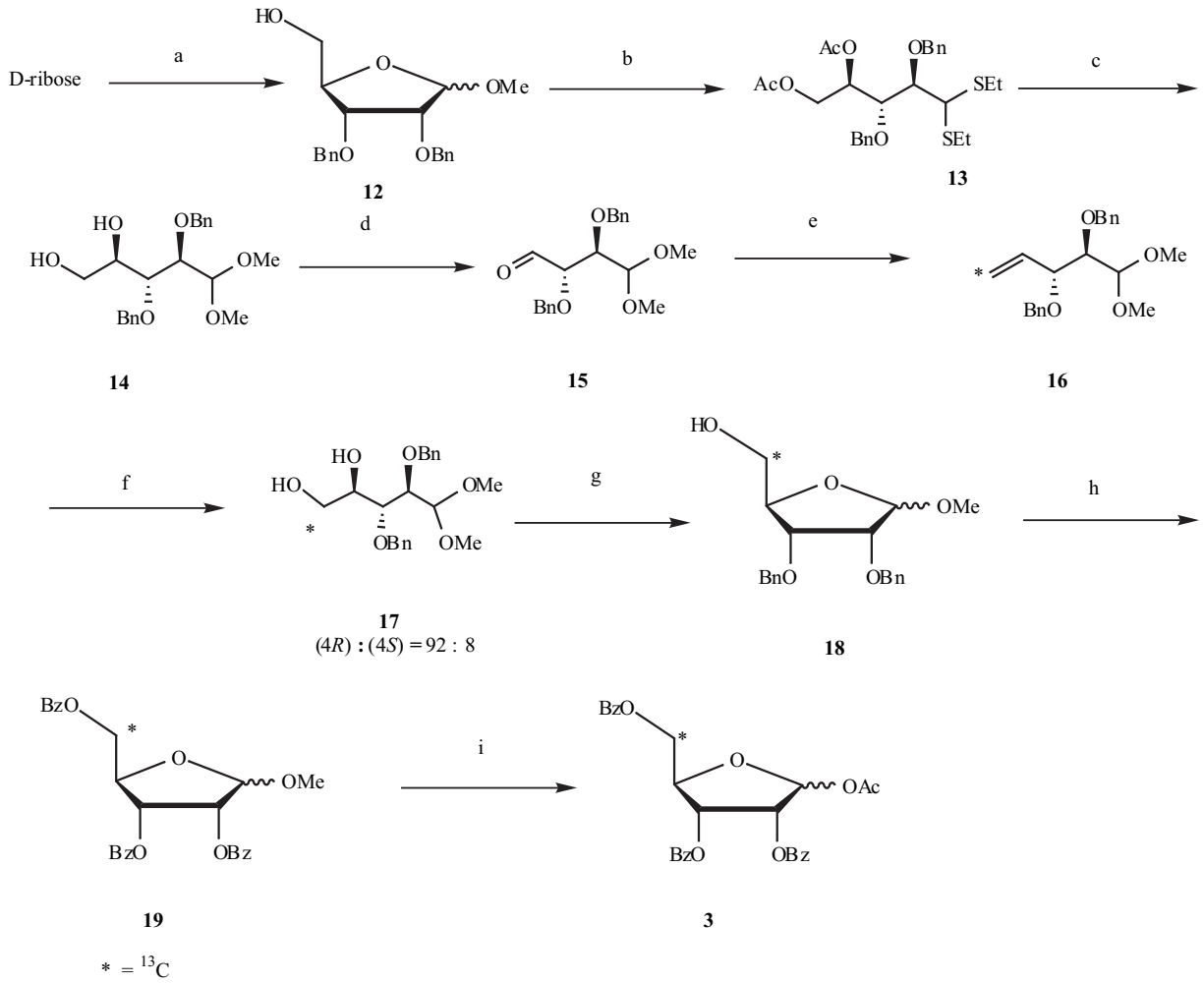
treated *in situ* with sodium borohydride and deprotected to yield D-[6-^{13}C]glucose (**6**) and L-[6-^{13}C]idose.

Treatment with potassium cyanide or [^{13}C]potassium cyanide yielded a C2 50:50 epimeric mixture (from **1** to **2**, in Scheme **1**, from **5** to **6**, and from **7** to **8** in Scheme **2**). These two synthetic methods involved further multiple steps after the introduction of the ^{13}C label to attain the final product, and were therefore judged impractical. Thus, more efficient processes were needed to prepare [5'-^{13}C]nucleosides.





Scheme 2. *Reagents and Conditions*: (a) 1. $H^{13}CN$ / pH 7.3; 2. H_2 / Pd-$BaSO_4$; 3. $NaBH_4$; 4. H_3O^+ (55%); (b) 1. $Pd(OAc)_4$; 2. H_3O^+ (80%); (c) HCN / pH 7.3; (d) H_2 / Pd-$BaSO_4$; (e) Na_2MnO_4 (70%).



Scheme 3. *Reagents and Conditions*: (a) 1. MeOH, c. H_2SO_4, 0 ˚C; 2. DMTrCl, pyridine, rt; 3. BnBr, NaH, DMF, rt; 4. 3% CCl_3COOH in $CHCl_3$, rt (88%); (b) 1. EtSH, c. HCl, 0 ˚C; 2. Ac_2O, DMAP, CH_2Cl_2, rt (93%); (c) 1. HgO, $HgCl_2$, $CaSO_4$, BnOH, 70 ˚C; 2. 2.0 M NaOH aq., MeOH, rt (85%); (d) $NaIO_4$, 5 mM H_2SO_4 in H_2O-EtOH (1:9) rt (99%); (e) $Ph_3P^{13}CH_3I$, BuLi, THF, 0 ˚C (93%); (f) OsO_4, 4-methylmorpholine *N*-oxide, H_2O, acetone, rt (96%); (g) $LiBF_4$, 2 % H_2O-CH_3CN, rt (98%); (h) 1. H_2, Pd/C, MeOH, rt; 2. BzCl, pyridine (92%); (i) Ac_2O, c. H_2SO_4, AcOH, rt (98%).

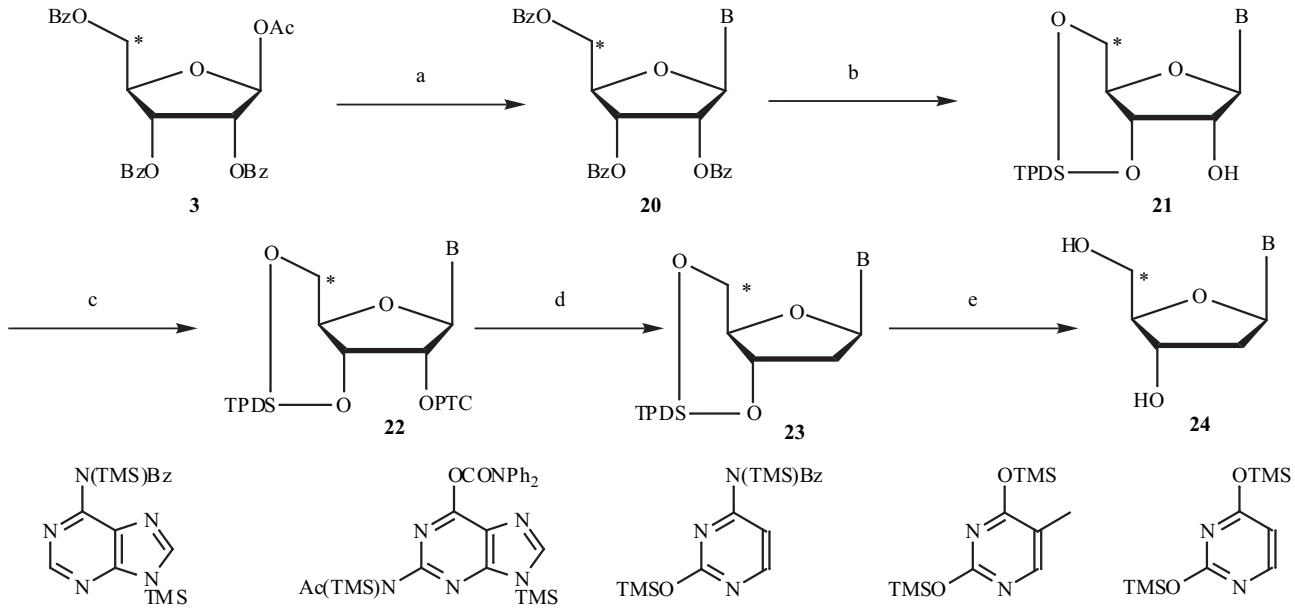
Consequently, we undertook the development of an efficient method for the synthesis of [5'-^{13}C]ribonucleosides and 2'-deoxy[5'-^{13}C]ribonucleosides [14]. The synthesis of [5'-^{13}C]ribonucleosides and 2'-deoxy[5'-^{13}C]ribonucleosides was achieved by the synthesis of the D-[5-^{13}C]ribose derivative as an intermediate *via* the Wittig reaction of 4-*aldehydo*-D-erythrose dialkyl acetals with $Ph_3P^{13}CH_3I$-BuLi to introduce the ^{13}C label at the 5-position of the pentose. This was followed by highly diastereoselective osmium dihydroxylation for the preparation of 2,3-di-*O*-benzyl-D-[5-^{13}C]ribose dialkyl acetal and the cyclization from D-[5-^{13}C]ribose dialkyl acetal derivatives to the alkyl D-[5-^{13}C]ribofuranoside derivative using lithium tetrafluoroborate. The methyl D-[5-^{13}C]ribofuranoside derivative obtained was converted into 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-[5-^{13}C]ribofuranose (**3**).

The efficiency of this approach was achieved with 58% overall yield of **3** from $Ph_3P^{13}CH_3I$, *i.e.*, after the introduction of the ^{13}C label by the Witting reaction, in contrast with 4% overall yield of **3** from [^{13}C]potassium cyanide by Matwiyoff and coworker [28] and 25% overall yield of **10** from [^{13}C]potassium cyanide reported by Serianni and coworker [17c]after introducing the ^{13}C label.

Subsequently, **3** was converted into [5'-^{13}C]ribonucleosides (**20**) using the Vorbrüggen method [29] and subsequently converted into the corresponding 2'-deoxynucleosides (**24**) (Scheme **4**).

It is perhaps the best to avoid using mercury compounds as reagents and to decrease the reaction process. For these reasons we developed an improved method that obviated the need for diethyldithioacetal, mercuric oxide and mercuric chloride, in addition to shortening the reaction process.

The improved method [30] utilized 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**25**) as the starting material. The key point of this approach is the conversion of (6*R*)-3-*O*-benzyl-1,2-*O*-isopropylidene-6-*O*-pivaloyl-α-D-[6-$^{13}C_1$] allofuranose (**29**) to 2-*O*-benzyl-5-*O*-pivaroyl- D-[5-$^{13}C_1$] ribofuranose (**31**). Compound **29** was prepared by ^{13}C Wittig reaction using $Ph_3P^{13}CH_3I$-BuLi and 5-oxo-ribose derivative (**26**), transformation into the D-[6-^{13}C]hexose (**28**) derivative by a diastereoselective hydroxylation reaction from **27**, selective acylation (**29**), and then a one-pot reaction

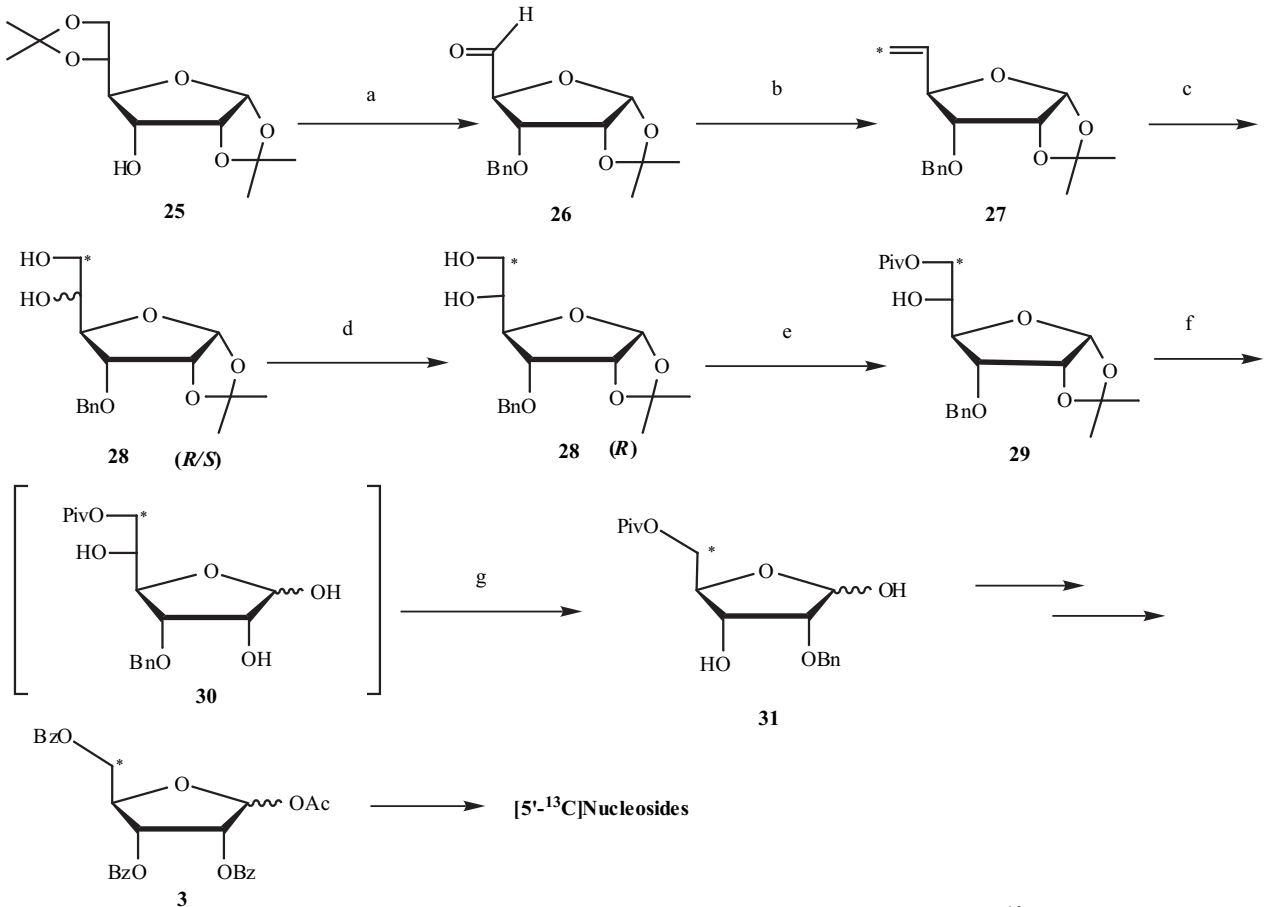


Scheme 4. *Reagents and Conditions*: (a) persilylated nucleobase (1.3 equiv.), TMSOTf, toluene, 80 ˚C or $ClCH_2CH_2Cl$, 70 ˚C; (b) 1. NaOH aq., MeOH, pyridine; 2. $TPDSCl_2$, pyridine; (c) PTCCl, pyridine, rt; (d) Bu_3SnH, AIBN, toluene,100 ˚C; (e) Et_4NF, THF, rt.

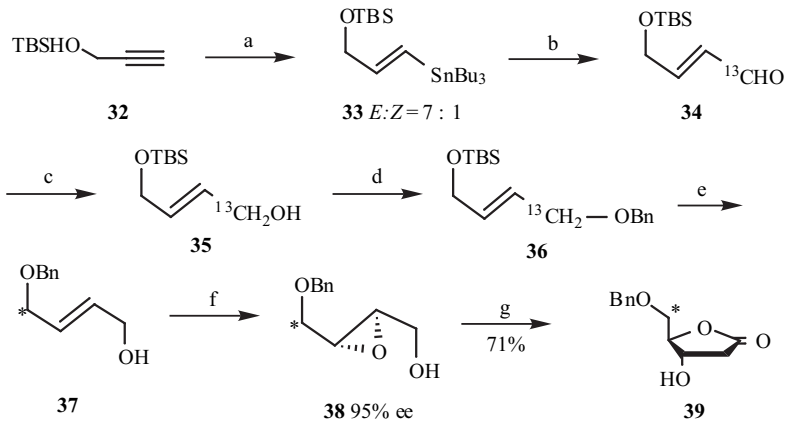
by treatment of **29** with trifluoroacetic acid and subsequently oxidized with sodium periodate. [5'-^{13}C]nucleosides were synthesized in the established manner from this [5-^{13}C]ribose derivative (**3**).

Synthesis of 2-deoxy-D-[5-^{13}C]ribonolactone [31]

The synthesis of the [5-$^{13}C_1$]lactone relied on the use of a ^{13}C-labeled glycosil donor, which can allow the formation of the [5'-$^{13}C_1$]nucleoside. The synthesis of 2'-deoxy[5-



Scheme 5. *Reagents and Conditions*: (a) 1. BnCl, NaH, DMF, rt; 2. 80% AcOH, $NaIO_4$, H_2O, rt; (b) $Ph_3P^{13}CH_3I$, BuLi, THF, -78 ˚C; (c) AD reaction; (d) Lipase PS "Amano", vinyl acetate, THF/Hexane (4 / 1); (e) PivCl, pyridine, chloroform, 0˚C → rt; (f) 90% CF_3COOH; (g) $NaIO_4$, H_2O, rt.

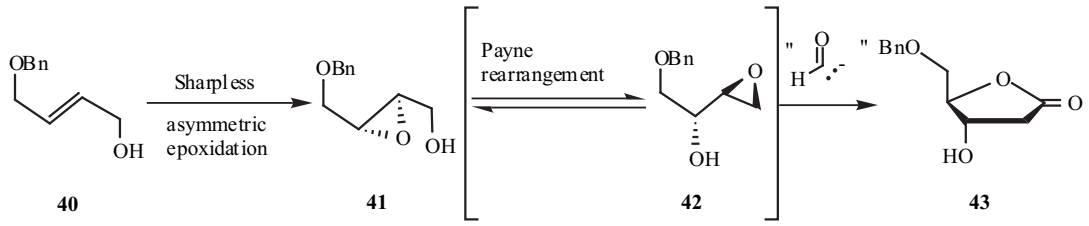


Scheme 6. *Reagents and Conditions*: (a) Bu_3SnH (1.3 equiv.) AIBN, 80 ˚C (72%); (b) BuLi, THF, -78 ˚C, $H^{13}C(O)NMe_2$ (69% from ^{13}C-DMF); (c) DIBAL, Et_2O, -78 ˚C (90%); (d) KH, BnBr, DME; (e) TBAF, H_2O (63% from **35**); (f) TBHP, $Ti(OiPr)_4$, D-(-)-DIPT, -20 ˚C; (g) KCN, KI, EtOH-H_2O, Δ then HCl, H_2O (71%).

$^{13}C_1$]ribonolactone [31] relied on the conversion of 2,3-epoxy[4-^{13}C]butanediols *via* a modified Payne rearrangement [32].

(2*R*,3*R*)-2,3-Epoxy-4-*O*-benzyl[4-$^{13}C_1$]butanediol (**38**) was prepared in 95% enantiomeric excess by the Sharpless epoxidation [33] of *O*-benzyl-1,4-[3-$^{13}C_1$]butendiol (**37**). This was then treated with potassium cyanide using Ganem's general protocol to effect a Payne rearrangement and subsequent incorporation of the cyano group. Hydrolysis of the resulting hydroxy nitrile under acidic conditions was accompanid by lactonization to yield the 2-deoxy[5-$^{13}C_1$]ribonolactone (**39**) in 71% yield from **38**. [1-^{13}C](*E*)-4-(*tert*-butyldimethylsilanyloxy)but-2-en-1-ol (**35**) was afforded by the diisobutylalminium hydride (DIBAL) reduction of [1-$^{13}C_1$]aldehyde (**34**), which was prepared by the addition of the anion obtained from 3-*O*-*tert*-butyldimethylsilyl-1-tributylstanylpropene (**33**) to [$^{13}C_1$]dimethylformaldehyde ([$^{13}C_1$]DMF) (Scheme **6**).

Similarly, the syntheses of the other four site-specific $^{13}C_1$-labeled 5-*O*-benzyl-2-deoxyribonolactones [31] were achieved *via* the $^{13}C_1$-labeled epoxide (**41**) by the Sharpless epoxidation of *O*-benzyl-1,4-butendiol (**40**), followed by the addition of a suitable formate anion equivalent (potassium cyanide) to the epoxides and subsequent lactonization of the resulting nitriles (Scheme **7**).

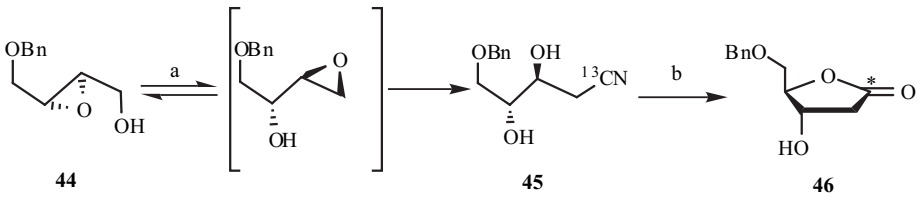


Scheme 7. The syntheses of 2-deoxyribonolactone **43** from **40** by the Sharpless epoxidation and Payne rearrangement followed by the addition of a suitable formate anion equivalent.

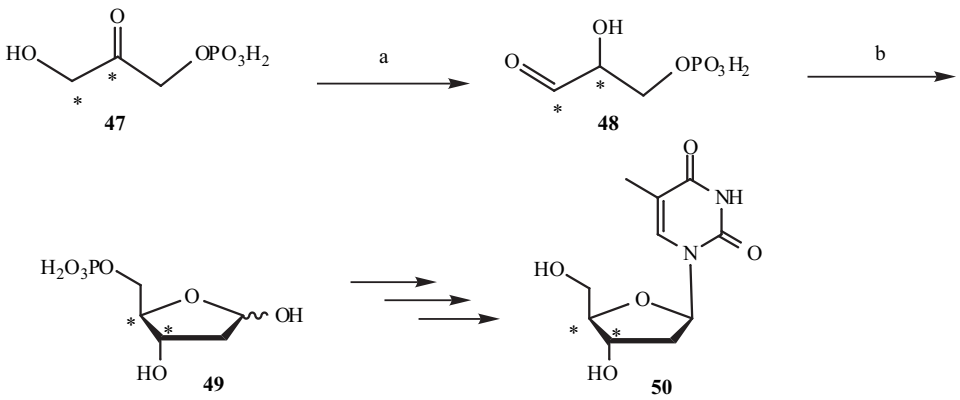
Synthesis of 2-deoxy-D-[1-$^{13}C_1$]ribonolactone

Nucleosides labeled at the C-1' position are required to investigate sugar and *N*-glycoside conformations [34], ^{13}C-^1H and ^{13}C-^{13}C spin coupling constants [35] and for the determination of ^{13}C and ^2H spin-lattice relaxation times [36].

The efficacy of Scheme **7** was demonstrated by the synthesis of C1-labeled ribonolactone [30]. Epoxide (2*R*,3*R*) **44** prepared in 94% enantiomeric excess by the Sharpless epoxidation of *O*-benzyl-1,4-butendiol was treated with [^{13}C]potassium cyanide with subsequent incorporation of the cyano group. Hydrolysis of the resulting hydroxy nitrile **45** under acidic conditions was accompanied by lactonization to yield the C1-labeled deoxynucleoside **46** in 71% yield from **44** (Scheme **8**). An enantiomeric excess of 92% was



Scheme 8. *Reagents and Conditions*: (a) K^{13}CN, KI, EtOH-H_2O, Δ; (b) HCl, H_2O.



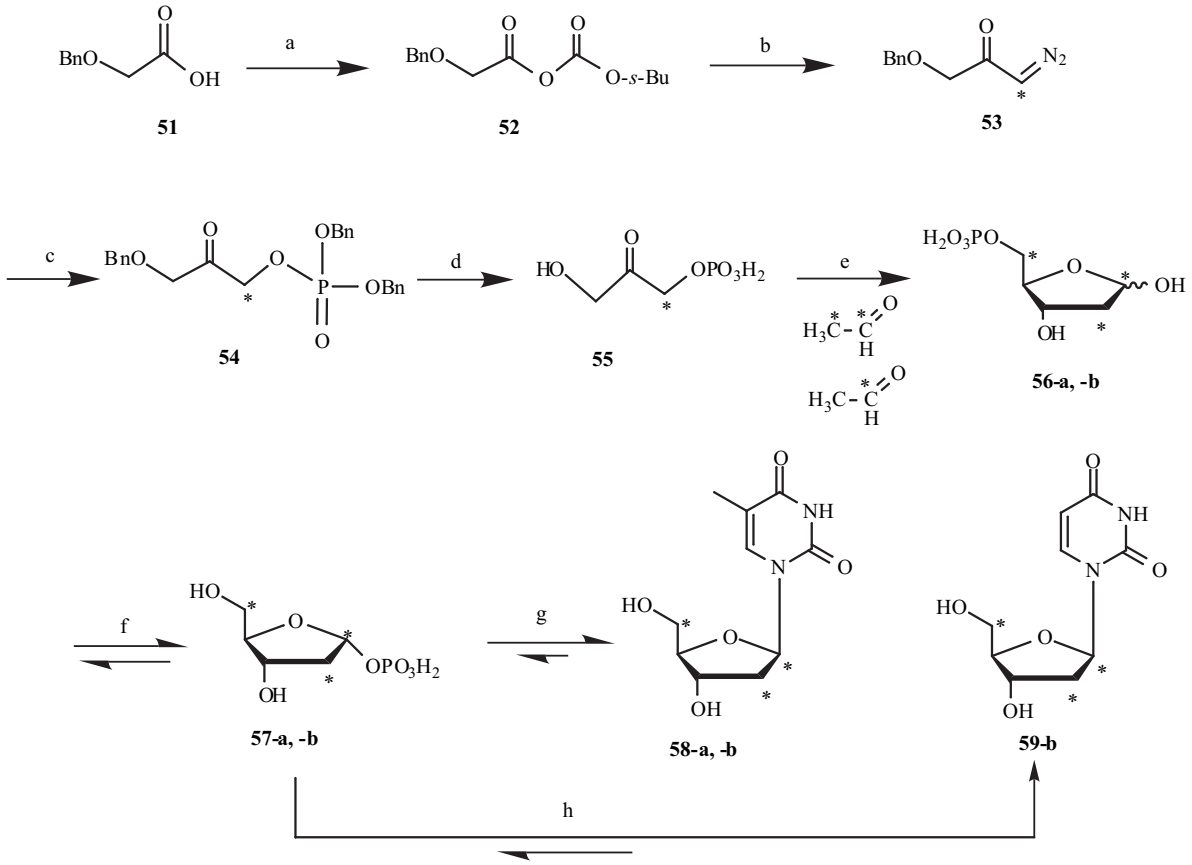
Scheme 9. *Reagents and Conditions*: (a) triosephosphate isomerase (TPI); (b) acetaldehyde, D-2-deoxyribose-5-phosphate aldolase (DERA).

achieved. The synthesis of 2-deoxy[2'-$^{13}C_1$]ribonolactone, 2-deoxy[3'-$^{13}C_1$]ribonolactone and 2-deoxy[4'-$^{13}C_1$]ribonolactone were achieved using the same method.

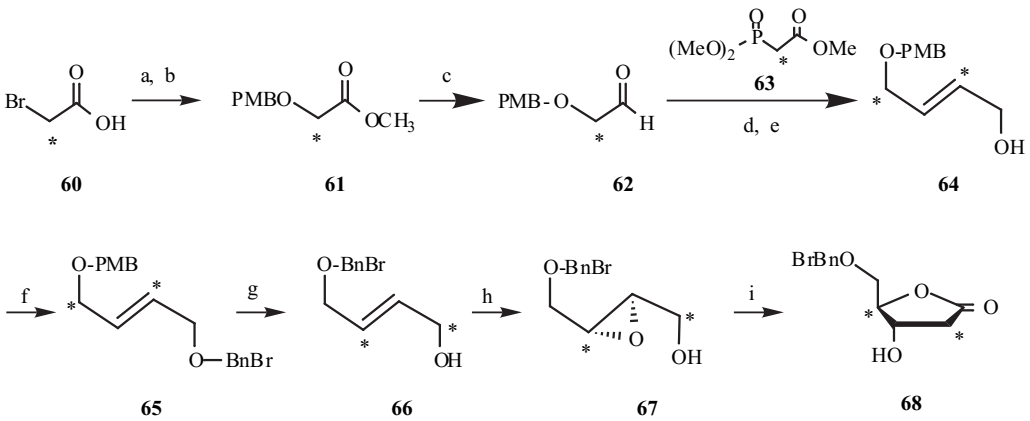
II-1-2. Multiple-Site-Specific Incorporation of ^{13}C-Label into Sugar Residues

The synthesis of [3',4'-$^{13}C_2$]thymidine [37] was achieved by conversion from [$^{13}C_2$]acetic acid in twelve-steps with 9% overall yield. D-2-Deoxyribose-5-phosphate aldolase (DERA) and triosephosphate isomerase (TPI) were used for the stereocontrolled formation of D-2-deoxy[3,4-$^{13}C_2$]ribose-5-phosphate (**49**) from [2,3-$^{13}C_2$]dihydroxyacetone monophosphate (DHAP) (**47**) 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.

The enzymic synthesis [38] of [2',5'-$^{13}C_2$]- and [1',2',5'-$^{13}C_3$]thymidine from 2-deoxy-D-ribose-5-phosphate was 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 was enzymically rearranged to α-2-deoxy-D-ribose-1-phosphate. Highly active PRM was easily obtained from genetically modified over-producing *E. coli* cells and



Scheme 10. *Reagents and Conditions*: (a) *s*-butyl chloroformate, triethy1amine; (b) [^{13}C]diazomethane (68%, based on *N*-[^{13}C]methy1-*N*-nitroso-*p*-toluenesulfonamide); (c) dibenzyl phosphate (81%); (d) H_2, Pd/C (95%); (e) D-2-deoxyribose-5-phosphate aldolase (DERA), triosephosphate isomerase (TPI); (f) phosphoribomutase (PRM); (g) thymidine phosphorylase (TP), thymine (60%); (h) TP, uracil (52%).

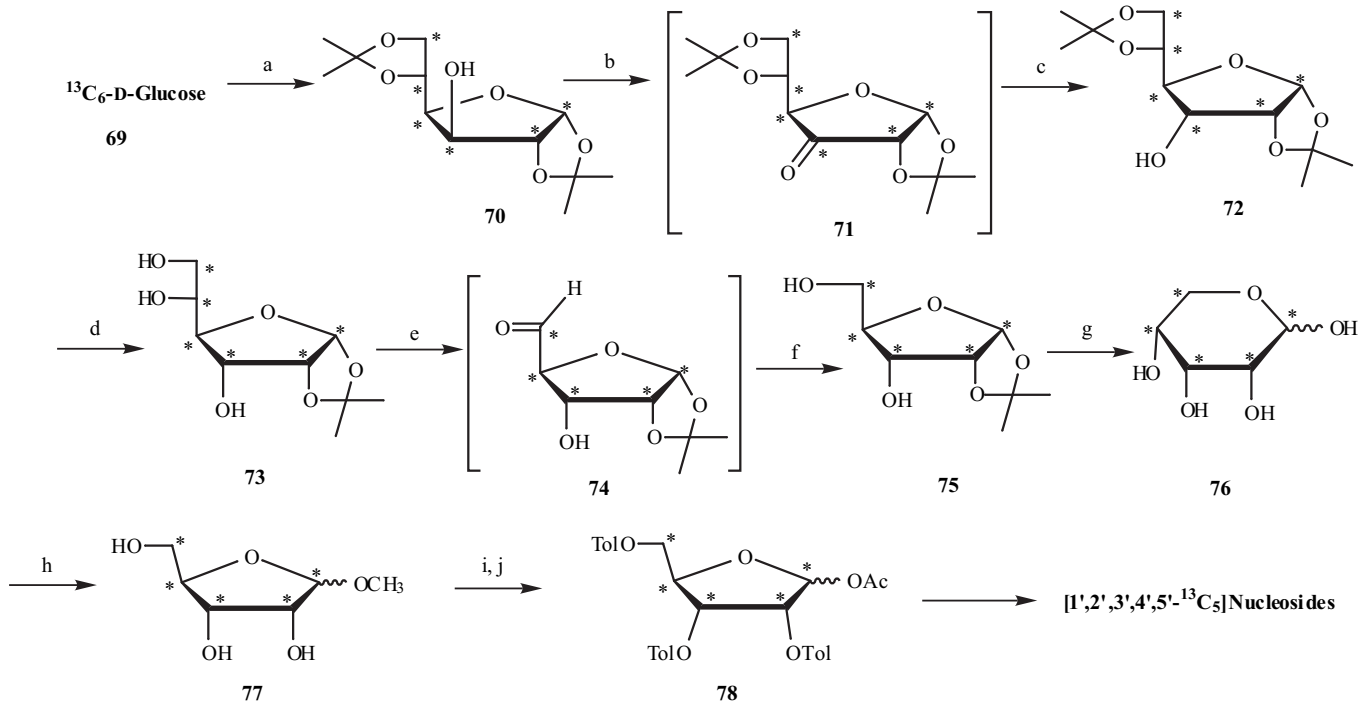


Scheme 11. *Reagents and Conditions:* (a) KH, PMBOH, THF, Δ; (b) CH_2N_2, Et_2O, 0 ˚C; (c) DIBAL, THF, -78 ˚C; (d) NaH, DME, 0 ˚C; (e) DIBAL, THF, -78 ˚C; (f) NaH, THF, BnBr, 0 ˚C; (g) DDQ, 9:1 DCM-H_2O, 0 ˚C; (h)TBHP, Ti(*O*-iPr)$_4$, D-(-)-DIPT, DMC –20 ˚C; (i) KCN, KI, EtOH-H_2O, Δ then HCl, H_2O.

was used without further purification. In the second step thymine was coupled to the sugar-1-phosphate. The thermodynamically unfavorable equilibrium was shifted to the product by addition of manganese dichloride to precipitate inorganic phosphate. In this way, the overall yield of the β-anomeric pure nucleoside increased from 14 to 60%. The sugar-5-phosphate **56-a,** precursor of [2',5'-$^{13}C_2$]thymidine (**58-a**), was prepared from [2-^{13}C]acetaldehyde and **55** which was prepared from [^{13}C]diazomethane. [1',2',5'-$^{13}C_3$]thymidine (**58-b**) was synthesized from [1,2-$^{13}C_2$]acetaldehyde and **55**. Compound **59-b** (2'-deoxy[1',2',5'-$^{13}C_3$]uridine) was also prepared using some method (Scheme **10**) (label positions are indicated by ∗).

In contrast to uracil, cytosine could not be used by TP as a substrate. Therefore, 2'-deoxycytidine was obtained by functional group transformations of the enzymically generated 2'-deoxyuridine. The method was successful in synthesizing [2',5'-$^{13}C_2$]- and [1',2',5'-$^{13}C_3$]thymidine in addition to 2'-deoxy[1',2',5'-$^{13}C_3$]uridine and 2'-deoxy[3',4'-$^{13}C_2$]cytidine. All compounds were prepared without any scrambling or dilution of the labeled material and were thus obtained with a very high isotope enrichment (96-99%).

1,3,5-$^{13}C_3$- and 2,4-$^{13}C_2$-labeled 2-deoxyribonolactones (**68**) [39], precursors to ^{13}C-enriched nucleoside, were similarly prepared making use of site-specific $^{13}C_1$-labeled 2-deoxyribonolactones [33] *via* an epoxide intermediate



Scheme 12. *Reagents and Conditions*: (a) acetone, $ZnCl_2$: H_3PO_4; (b) PDC, acetic anhydride, methylene chloride; (c) $NaBH_4$, ethanol; (d) 80% acetic acid, rt; (e) $NaIO_4$, ethanol-water, rt; (f) $NaBH_4$, ethanol, rt; (g) 60% acetic acid, 80 ˚C; (h) methanol. H_2SO_4, 4 ˚C; (i) toluoyl chloride, pyridine; (j) acetic acid, acetic anhydride, H_2SO_4, methylene chloride, 0 ˚C.

generated by Sharpless epoxidation of *O*-benzyl-1,4-butendiol and subsequent addition of potassium cyanide to yield the resultant nitriles (Scheme **11**).

II-1-3. Full Labeled [1',2',3',4',5'-$^{13}C_5$]Nucleoside at Ribose Moiety

[1',2',3',4',5'-$^{13}C_5$]Nucleoside

[1',2',3',4',5'-$^{13}C_5$]nucleoside [40] was synthesized from D-[$^{13}C_6$]glucose *via* a D-[$^{13}C_5$]ribose derivative. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucoforanose (**70**) converted from $^{13}C_6$-D-glucose (99 atoms ^{13}C) was transformed into the *allo*-diastereomer **72** by an oxidation/reduction reaction sequence. Treatment by selective hydrolysis of the 5,6-*O*-isopropyhdene group of **72**, subsequent oxidation with pyridinium dichromate and reduction with sodium borohydride yielded the D-ribose derivative **75**. Acidic hydrolysis of the remaining isopropylidene group of **75** gave the D-ribose **76**, which was converted into it's α/β-methyl furanosides **77** in a quantitative manner. The mixture of α and β-methyl furanosides **77** was converted to 1-*O*-acety1-2,3,5-tri-*O*-(4-toluoy1)-α/β-D-ribofuranose (**78**) (~3: 10 α/β mixture in 42% yield from $^{13}C_6$-D-glucose). Condensation of **78** under Vorbrüggen's conditions yielded the protected nucleosides in 66-81% yields [40a] (Scheme **12**).

Synthesis of [$^{13}C_5$]Labeled Anti-HIV Nucleosides [41]

Synthesis of [$^{13}C_5$]labeled anti-HIV nucleosides [41], e.g. d4T, ddI, ddA, were prepared from the [1',2',3',4',5'-$^{13}C_5$]nucleoside. The key step of this approach was the stereoselective dehomologation of 1,2:5,6-di-*O*-isopropylidene-3-oxo-α-D-glucofuranose with periodic acid and sodium borohydride, which gave the optically pure ribose derivative as the exclusive product. Nucleoside derivatives were obtained through the ribosylation of 1,2,-di-*O*-acetyl-3,5-di-*O*-benzyl-β-D-ribofuranose with persilylated nucleobases under Vorbrüggen conditions. Deoxygenation of intermediates under Corey-Winter conditions [42] afforded the desired labeled nucleoside analogs.

II-2. Applications of ^{13}C-Labeled Nucleosides

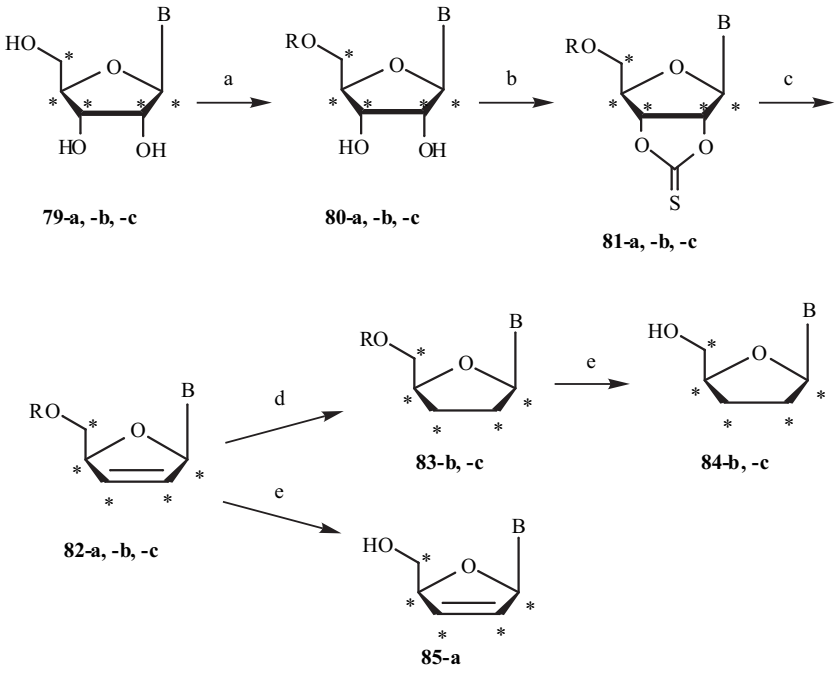
II-2-1. NMR

There are a great number of excellent structural studies dealing with stable isotope-labeled nucleic acid, which have provided important structural results [10a,43].

In this chapter we introduce applications utilizing 2'-deoxy[5'-^{13}C]nucleosides [14] combined with several recent applications of oligonucleotides possessing a ^{13}C-labeled d-ribose.

Applications of 2'-Deoxy[5'-^{13}C]nucleosides

New sequential assignment routes using 5'-^{13}C signals, C5'-H1' and C5'- H2' of a ^{13}C-labeled DNA dodecamer constructed with 2'-deoxy[5'-^{13}C]ribonucleotides [14], 5'-d(*^{1}C*^{2}G*^{3}C*^{4}G*^{5}A*^{6}A*^{7}T*^{8}T*^{9}C*^{10}G*^{11}C^{12}G)-3'(*N = [5'-^{13}C]nucleotide), were found in the 2D [^{1}H-^{13}C]-HMQC-NOESY spectrum [44]. Application of this sequential-walk enabled all of the 5'-^{13}C signals to be unambiguously assigned: 64.0 (C1), 66.7 (T7), 67.2 (C9), 67.3 (T8 and C11, overlap), 67.5 (C3), 68.1 (A6), 68.4 (G4), 68.5 (G2 and G10, overlap) and 68.6 (A5), ppm (residue name) (Fig. **4**). The assignment of each H1' was also achieved using the sequential-walk line between the inter-residue H1'(i-1)-C5'(i) (strong) and intra-residue H1'(i)-C5'(i) cross-peaks (weak) (Fig. **5**). A similar sequential assignment route was found between C5' and H2'.



a: B = thymine, **b:** B = adenine, **c:** B = hypoxanthine

Scheme 13. *Reagents and Conditions*: (a) TBDMSCl, imidazole, DMF; (b) TCDI, DEC; (c) triethylphosphite; (d) H_2, Pd/C (10%), TEA (0.5%)-MeOH; (e) TBAF, THF.

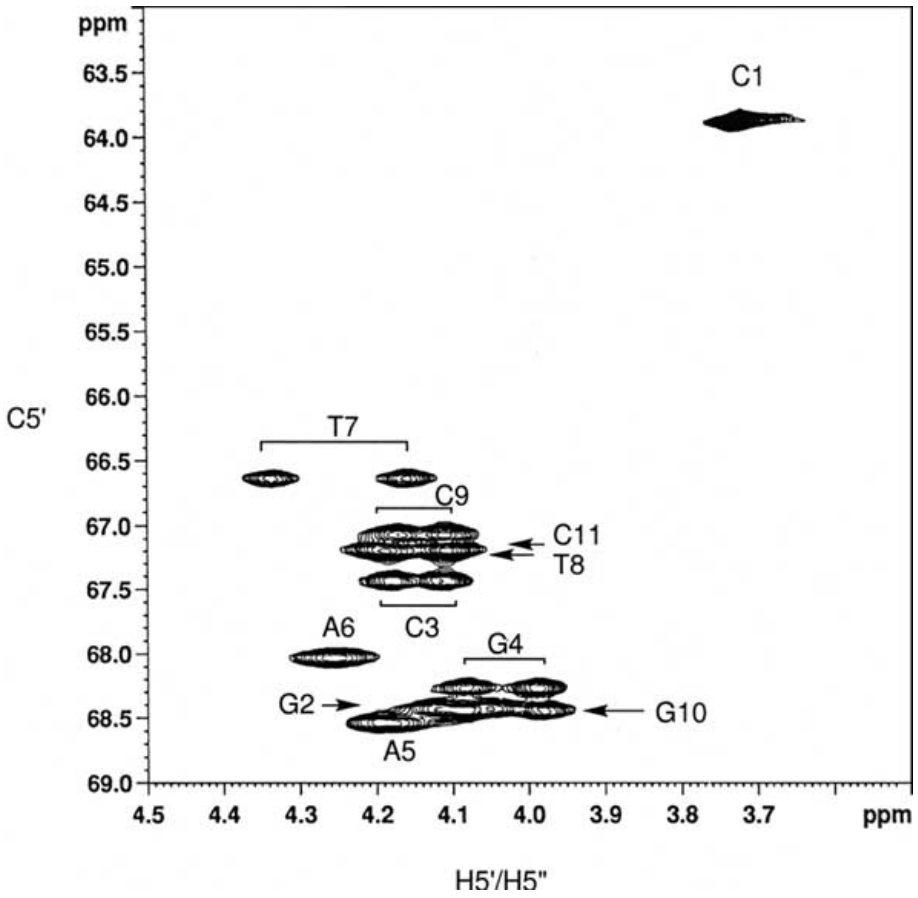


Fig. (4). C5'-H5' spectral region of the ^{1}H-^{13}C HSQC spectrum of the [5'-^{13}C]-labeled DNA dodecamer [5'-d(*^{1}C*^{2}G*^{3}C*^{4}G*^{5}A*^{6}A*^{7}T*^{8}T*^{9}C*^{10}G*^{11}C^{12}G)-3']. Resonance assignments are given by residue names.

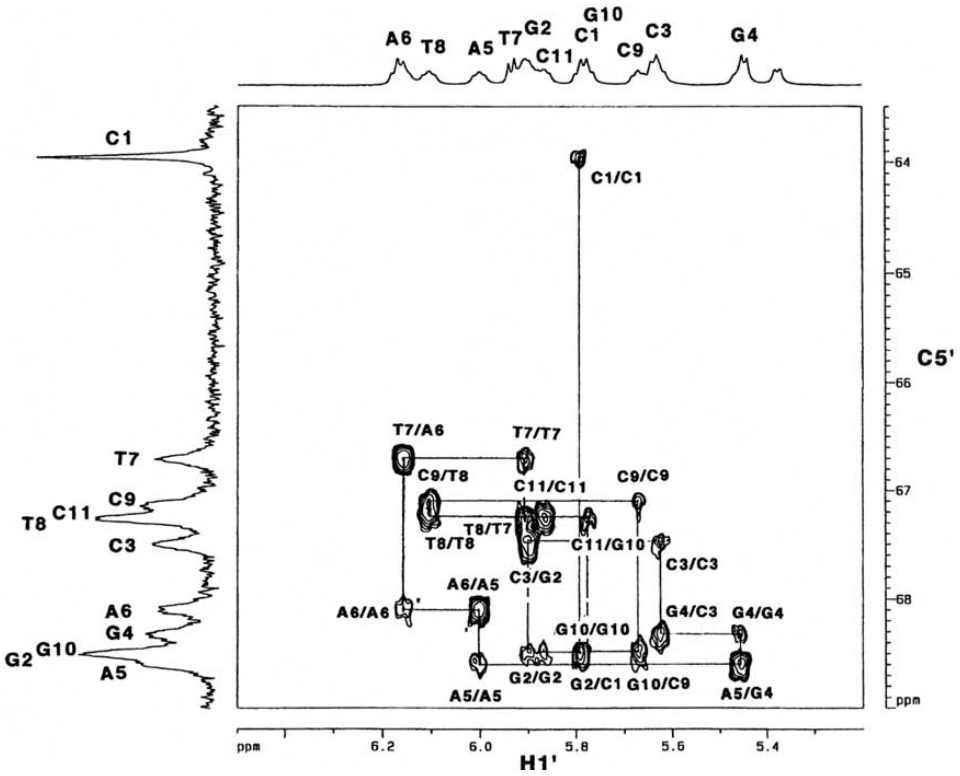


Fig. (5). C5'- H1' spectral region of the 2D [^{1}H-^{13}C]-HMQC-NOESY spectrum of the [5'-^{13}C]-labeled DNA dodecamer. Resonance assignments are given by the residue names. Lines in the spectrum indicate the sequential assignment routes.

Proton pair distances evaluated from the canonical B-DNA as well as A-DNA data indicated that these sequential-assignment routes on a 2D [^{1}H-^{13}C]-HMQC-NOESY spectrum work for most nucleic acid stem regions.

The salient feature of these assignment routes was the use of the 5'-^{13}C signals, rather than H5' and H5", to reduce the complexity of the NOESY spectrum. These sequential assignment routes will become a powerful tool in the second or third sequential-walk routes for most nucleic acids and in the assignment of the H4'/ H5'(5'*pro-S*) / H5"(5'*pro-R*) signals.

Application of ^{13}C-Full-Labeled [1',2',3',4',5'-$^{13}C_5$]DNA

a. Rotational Diffusion Properties [45]

Rotational diffusion properties have been determined for the DNA dodecamers d(**C**G**C**G**A**A**T**T**C**G**C**G)$_2$ and d(CG**C**G**A**A**T**T**C**G**C**G)$_2$, where bold-faced nucleotides are uniformly labeled with ^{13}C, from ^{13}C $R_{1\rho}$ and R_1 measurements on the $C_{1'}$, $C_{3'}$ and $C_{4'}$ carbons (methyne carbon) in samples uniformly enriched in ^{13}C. The narrow range of C-H bond vector orientations relative to the DNA axis make the analysis particularly sensitive to small structural deviations. As a result, it was found that the $R_{1\rho}/R_1$ ratios fitted poorly to the crystal structures of the dodecamer, but fitted well to a recent solution NMR structure, determined in liquid crystalline media, notwithstanding the fact that the global structures are quite similar. A fit of the $R_{1\rho}/R_1$ ratios to the solution structure is optimal for an axially symmetric rotational diffusion model, with a diffusion anisotropy and an overall rotational correlation time at 35 ˚C in D_2O, and was in excellent agreement with values obtained from hydrodynamic modeling.

b. The Glycosidic Torsion Angles [46]

The glycosidic torsion angles in uniformly ^{13}C-labeled nucleic acids was determined from the vicinal coupling constants $^3J_{C2/4\text{-}H1'}$ and $^3J_{C6/8\text{-}H1'}$. The precise measurements of the proton-carbon vicinal coupling constants $^3J_{C2/4\text{-}H1'}$ and $^3J_{C6/8\text{-}H1'}$ in uniformly ^{13}C-labeled nucleic acids were achieved by a two-dimensional, quantative *J*-correlation NMR experiment. In an effort to reduce the loss of signal due to ^{1}H-^{13}C dipole-dipole relaxation, a multiple-quantum constant time experiment with appropriately incorporated band selective ^{1}H and ^{13}C pulses was applied. The experiment was used to obtain the $^3J_{C2/4\text{-}H1'}$ and $^3J_{C6/8\text{-}H1'}$ coupling constants in a uniformly ^{13}C, ^{15}N-labeled [d($G_4T_4G_4$)]$_2$ quadruplex. The measured values and glycosidic torsion angles in the G-quadruplex, obtained by restrained molecular dynamics with explicit solvent using previously published restraints along with selected data from the literature, were used to check and modify existing parameters of the Karplus equations. The parameters were obtained using glycosidic torsion angles derived from the original solution.

c. Structural Integrity of Stem (27A and 43G), Bulge (24C) and Loop (31U) Regions of the 29mer HIV-1 TAR RNA Hairpin [47]

The structural integrity of the specifically ^{13}C-labeled (99 atom % ^{13}C) stem (27A and 43G), bulge (24C) and loop (31U) regions of the 29mer HIV-1 TAR RNA hairpin was demonstrated by detailed 2D-NMR studies using the NMR Relaxation Window. The expanded regions of the Ar-Hl' crosspeaks in the DQF-COSY (Panel A in Fig. **7**) and conventional NOESY (Panel B in Fig. **7**) spectra of specifically ^{13}C-labeled (99 atom % 13C) stem (27A and 43G), bulge (24C) and loop (31U) regions of the 29mer HIV-1 TAR RNA hairpin was compared. As expected, all 15 crosspeaks of H5-H6 at natural abundance according to the sequence of the 29mer TAR RNA was observed (Panel A in Fig. **7**), consistent with the target sequence (as shown in Fig. **6**). The cytosine and uracil (labeled by capital C and U in Panel A of Fig. **7**) residues could readily be discriminated based on differences in the H5-H6 coupling constants (7.6 and 8.8 Hz, respectively). The same crosspeak could be identified in NOESY spectra as shown in the boxes of Panel B in Fig. (**7**). The occurrence of a single set of resonances from the $^{13}C_5$-ribose labeled 29mer TAR RNA confirms that it possesses a single and unique structure.

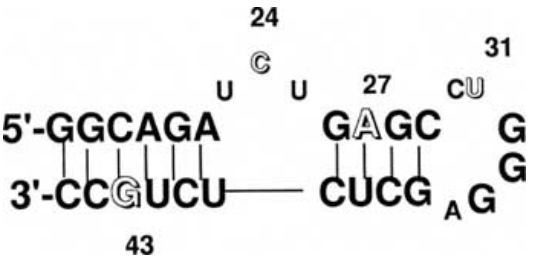


Fig. (6). Sequence and secondary structure of the 29mer HIV-1TAR RNA (open letters and numbering indicate $^{13}C_5$-ribofuranose labeled nucleotides).

All five regions of the ^{1}H-^{13}C crosspeaks, [1H(1')-^{13}C (1'), 1H(2')-^{13}C (2'), 1H(3')-^{13}C (3'), 1H(4)-^{13}C (4') and 1H(5')-^{13}C (5)], corresponding to four $^{13}C_5$-ribose labeled residues in the 29 mer TAR RNA by HSQC-CT spectra were clearly observed (Fig. **8**).

It has thus been demonstrated by heteronuclear 2D NMR that the non-uniform labeling of the HIV-1 TAR 29mer RNA achieved by chemical synthesis provides an optimal opportunity to perform full T1 and T2 relaxation measurements (the NMR Relaxation Window) for each type of sugar-carbons of all four strategically placed ^{13}C-labeled residues in a unique and unprecedented manner. This is due to the minimal overlap of ^{13}C resonances compared to uniformly labeled oligomer-RNA.

d. Measurement of Both Longitudinal and Transverse Relaxation Interference [48]

Measurement of both longitudinal and transverse relaxation interference (cross-correlation) between ^{13}C chemical shift anisotropy and ^{13}C-^{1}H dipolar interactions was performed at three temperatures using a DNA decamer duplex with purines randomly enriched to 15% in ^{13}C. The ratio of the transverse to longitudinal cross-correlation rates readily yields the ratio of spectral densities $J(0)/J(\omega_C)$, independent of any structural attributes such as internuclear distance or chemical shift tensor. The spectral density at zero frequency $J(0)$ is also independent of chemical exchange effects. With limited internal motions, the ratio also provides for an accurate evaluation of the correlation time for overall molecular tumbling.

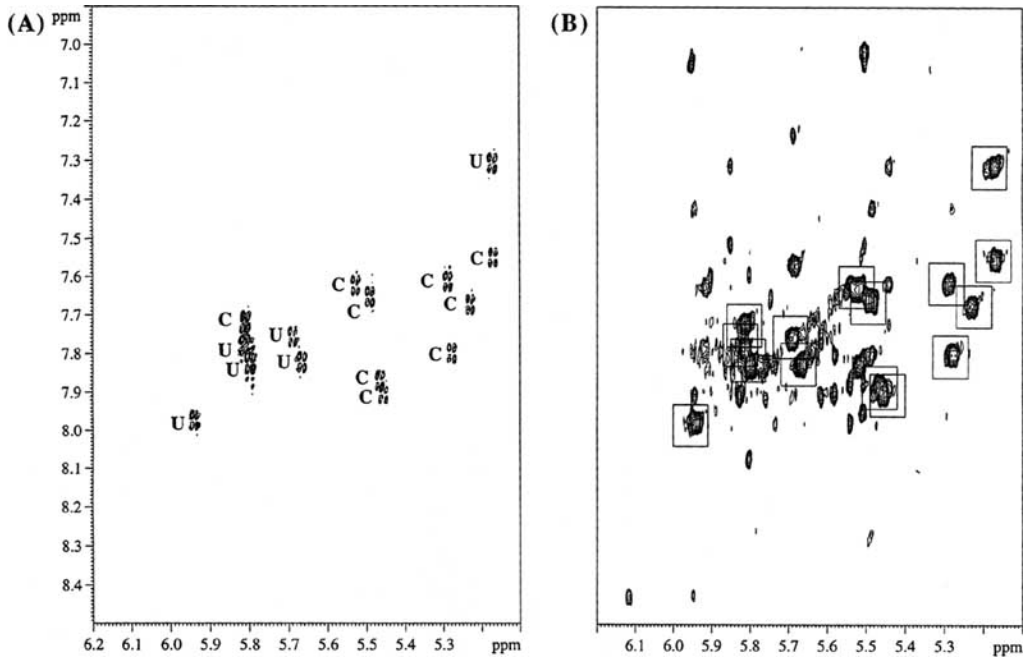


Fig. (7). The expanded region of H5-H6 crosspeaks in DQF-COSY spectrum (Panel A) and corresponding region in conventional NOESY (Panel B) spectrum of the selectively ^{13}C-ribose labeled (i.e. residues ^{24}C, ^{27}A, ^{31}U and ^{43}G) 29mer TAR RNA are compared. In Panel A, 15 H5-H6 crosspeaks for cytosine (labeled by capital C) and uridine (labeled by capital U) are found as expected, consistent with the TAR RNA sequence shown in Fig. **6**. The corresponding crosspeaks in the NOESY spectrum (Panel B) are surrounded in boxes.

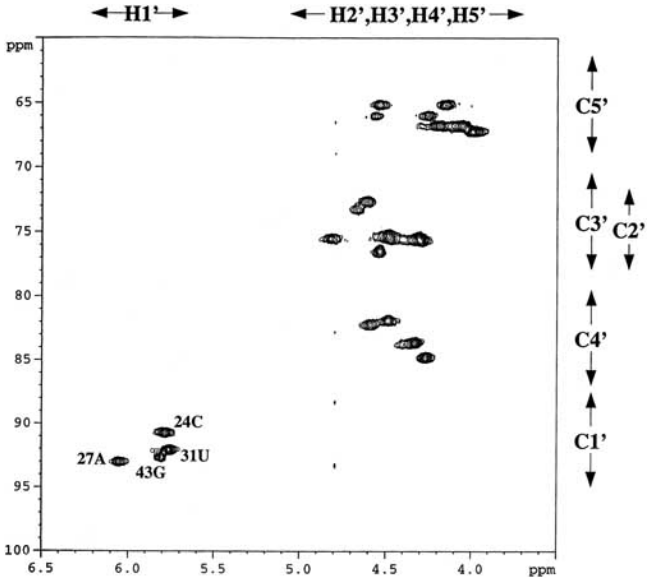


Fig. (8). HSQC-CT spectra of specifically $^{13}C_5$-ribose labeled (i.e. ^{24}C, ^{27}A, ^{31}U and ^{43}G residues) 29mer TAR RNA are shown. On the right (δ ^{13}C) and top (δ ^{1}H) of the spectra, the chemical shifts of different types of sugar-carbon or –proton regions are defined by arrows. For five carbon-proton regions, four sets of ^{1}H-^{13}C crosspeaks corresponding to four different specifically labeled residues are clearly observed.

II-2-2. Raman Spectrum

Vibrational Raman Spectra of [5'-^{13}C]Thymidine [49]

^{13}C-labeled nucleosides can also be utilized for vibrational studies by Raman spectroscopy. Vibrational Raman spectra of [5'-^{13}C]thymidine have been examined and compared to that of non-labeled thymidine. While many of the Raman bands remained relatively unchanged, several Raman bands shifted prominently towards lower frequency in the 5'-^{13}C species relative to the non-labeled species. For reference, the Raman spectrum of $CH_3{}^{13}CH_2OH$ was also determined and analyzed on the basis of *ab initio* MO calculations. From these results, probable assignments were deduced concerning the O-5'C-4'C bending (396 cm^{-1}), O-5'C-4'C symmetric stretching (852 cm^{-1}), O-5'C-4'C antisymmetric stretching (1016 cm^{-1}), 5'CH_2 rocking (1029 cm^{-1}), 4'C-H bending (1066 cm^{-1}), 5'CH_2 scissoring (1481 cm^{-1}) and 5'CH_2 symmetric stretching (2934 cm^{-1}) vibrations of the thymidine molecule.

III. ^{15}N-LABELED NUCLEOSIDES

III-1. Synthesis of Nucleosides Bearing a ^{15}N-Labeled Exocyclic Amino Group

The exocyclic amino group of nucleosides (Fig. **9**) is a good candidate for the ^{15}N-labeling of nucleic acids since this functional group plays important roles in forming hydrogen bonds with suitable acceptors such as those found in nucleic acids, proteins or drugs.

A variety of methods for the site-specific ^{15}N-labeling of nucleosides have been developed and there are a great number of excellent structural studies on such labeled nucleic acid fragments, which have provided important structural information.

Three types of approaches to the chemical synthesis of ^{15}N-labeled nucleosides have been reported. One involves the synthesis of an appropriately ^{15}N-labeled heterocycle, followed by its glycosylation with an appropriately functionalized D-ribofuranosyl or 2-deoxy-D-ribofuranosyl derivative to yield the desired ^{15}N-labeled nucleoside

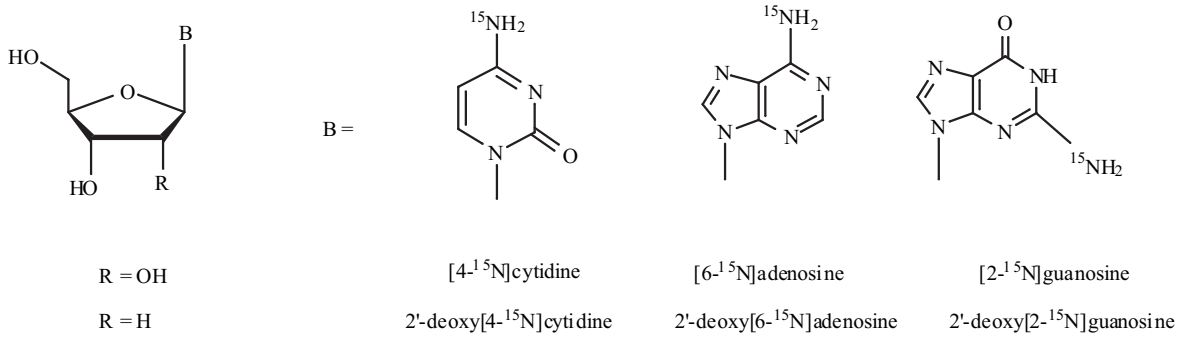


Fig. (9). Nucleosides bearing a ^{15}N-labeled exocyclic amino group.

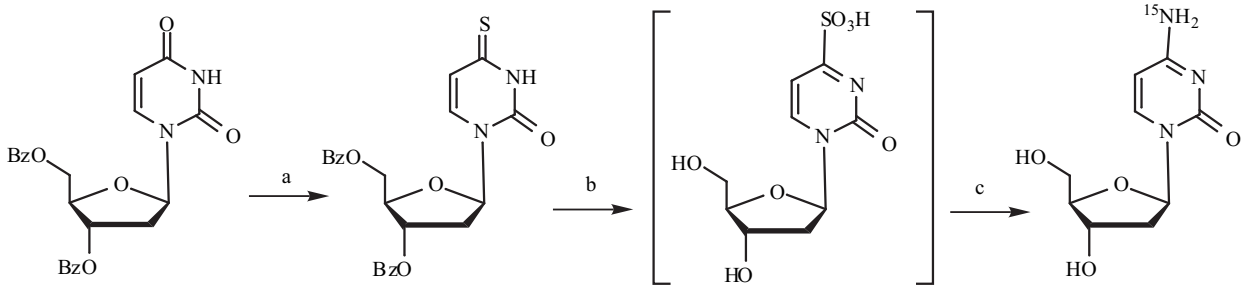
[50,71a]. An alternative approach involves the synthesis of an appropriately ^{15}N-labeled nucleoside intermediate, followed by its conversion into the ^{15}N-labeled nucleoside. While another approach, the chemical derivatization of an intact nucleoside to the corresponding ^{15}N-labeled nucleoside through the reaction of its activated intermediates with [^{15}N]ammonia or [^{15}N]benzylamine.

The latter approach might be much more promising than the others from a synthetic viewpoint. It would be further advantageous if it was possible to perform this approach using a small excess of solid nucleophile such as [^{15}N]phthalimide or potassium [^{15}N]phthalimide to achieve the introduction of the ^{15}N-label into the exocyclic amino group of the nucleosides.

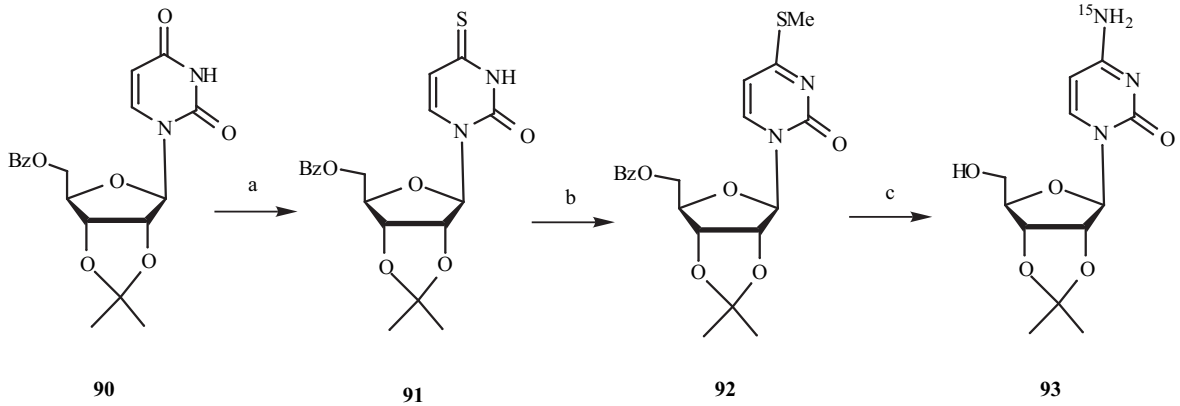
We recently developed efficient methods for the synthesis of [4-^{15}N]cytidine [11c,15a-c], 2'-deoxy[4-^{15}N]cytidine [11c,15a-c], [6-^{15}N]adenosine [11c,15a-c], 2'-deoxy[6-^{15}N]adenosine [11c,15a-c], [2-^{15}N]guanosine [15d], and 2'-deoxy[2-^{15}N]guanosine [15d]derivatives using [^{15}N]phthalimide, potassium [^{15}N]phthalimide or *N-tert*-butyldimethlysilyl[^{15}N]phthalimide [51] as a label reagent.

III-1-1. [4-^{15}N]cytidine and 2'-Deoxy[4-^{15}N]cytidine Derivatives

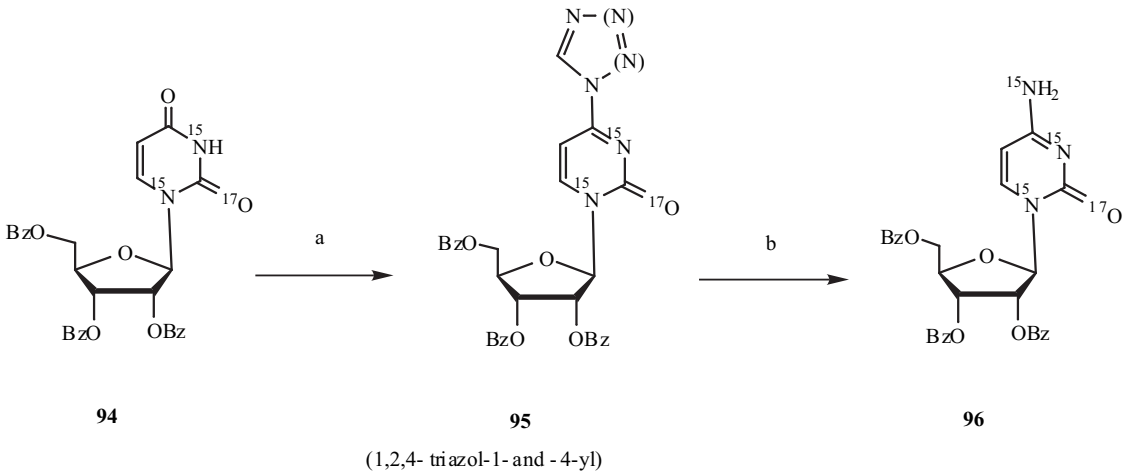
Cytidine and 2'-deoxycytidine derivatives can be prepared by glycosylation of cytosine with appropriately functionalized D-ribofuranosyl and 2-deoxy-D-ribofuranosyl derivatives, respectively [29]. In general the preparation of 2'-deoxynucleoside derivatives is followed with the



Scheme 14. *Reagents and Conditions*: (a) Lawesson's reagent, benzene, reflux, 6 h (83.2%); (b) 1. KOH, 0 ˚C, 1 h; 2. $KMnO_4$, 0 ˚C, 2 h; (c) $(^{15}NH_4)_2SO_4$, KOH (pH 8.5), 37 ˚C, 4 h (59.7%).



Scheme 15. *Reagents and Conditions*: (a) Lawesson's reagent, toluene, 80-100 ˚C, 5 h (89%); (b) 1. NaOH; 2. MeI, H_20 (95%); (c) $^{15}NH_3$/EtOH, 110-115 ˚C, 27 h (100%).



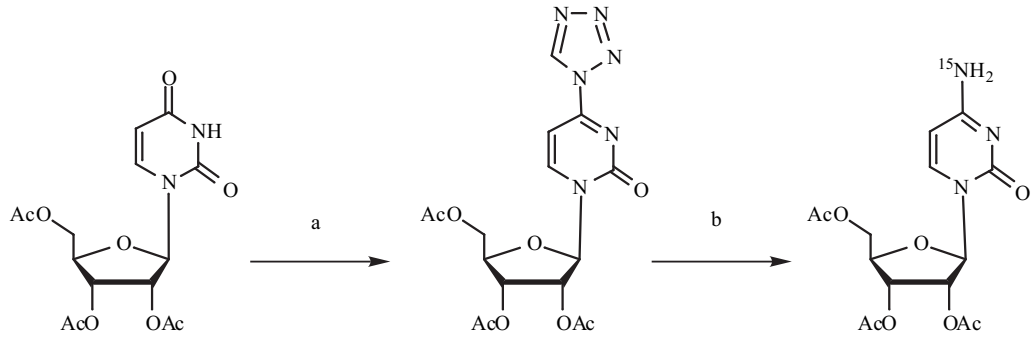
94 **95** **96**

Scheme 16. *Reagents and Conditions*: (a) tris(1,2,4-triazolyl)phosphine, CH_3CN, rt, overnight (94%); (b) $^{15}NH_4OH$/THF, rt, 5 h (71%).

undesirable formation of α-glycoside as a by-product [29]. Therefore, stable isotope (^2H, ^{13}C and/or ^{15}N) labeled 2'-deoxycytidine derivatives were prepared by 2'-deoxygenation of the corresponding cytidine derivatives, which were obtained by glycosylation of labeled cytosine with labeled D-ribofuranose [14a,52]. Cytosine can be obtained by the condensation of urea with cyanoacetaldehyde diethyl acetal [53a] or β-ethoxyacrylonitrile [53b] and by conversion of uracil [53c], thiouracil [53d] and dithiouracil [53e]. The procedures and results obtained by the above methods did not seem suitable for the introduction of the ^{15}N-label into the exocyclic amino group since yields are low and the time-consuming processing of the necessary intermediates is involved. Therefore, it is more efficient to start from uridine or 2'-deoxyuridine and convert the uricil base into the cytosine base.

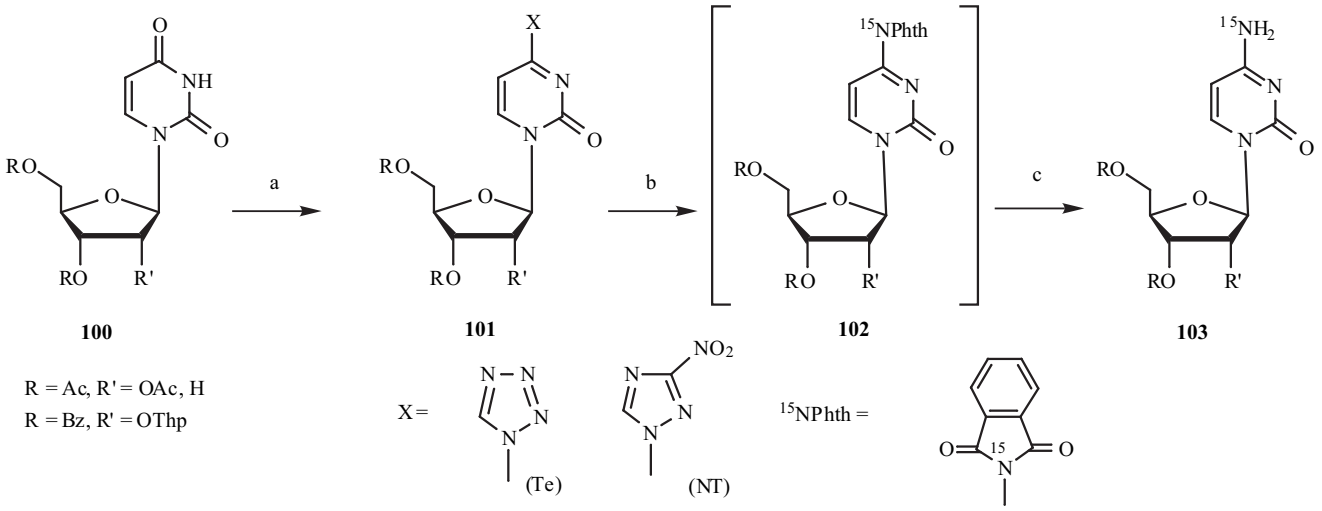
For the introduction of the exocyclic amino group, a leaving group such as –SMe [54], $-SO_3H$ [74a], triazolyl [55], 3-nitro-1,2,4-triazolyl [15] and tetrazolyl group [15,56] was introduced into the C4-position of the uracil derivatives, which was subsequently displaced by ^{15}N-label reagent. Generally the source of ^{15}N was labeled ammonia (in a water or alcoholic solution, or generated *in situ* from its salt) (Schemes **14-16**). The introduction of an amino group at the C4 generally requires a large excess of [^{15}N]ammonia, with one noted exception as described by Ariza *et al*., where the 4-tetrazolyl derivative (**98**) was used as a C4-activated derivative with $^{15}NH_4Cl$ as a label source in a 1.24 equivalent excess (Scheme **17**) [56]. Another possibility is the use of [^{15}N]phthalimide [11c,15a-c] or [^{15}N]benzylamine [57] as a label reagent.

We developed efficient methods for the synthesis of [4-^{15}N]cytidine and 2'-deoxy[4-^{15}N]cytidine derivatives (**103**) using [^{15}N]phthalimide as a label reagent, as shown in Scheme **18** [11c,15a-c]. An imide group can be introduced to a heterocyclic compound (cytidine or adenosine derivative) by reacting a 3-nitro-1,2,4-triazolyl- or tetrazolyl-linked heterocyclic compound with succinimide or phthalimide in the presence of base (triethylamine or diazabicyclo[5.4.0] undec-7-ene (DBU)) at room temperature. This method efficiently introduces the succinimido or phthalimido group, which is readily converted into an amino group, to the outside of a heterocyclic ring and is particularly suitable for the site-specific introduction of stable ^{15}N isotope to the amino group of a nucleoside heterocyclic ring. After treatment of the 4-tetrazolyl derivative (**101**, X = Te) with [^{15}N]phthalimide (1.5 equiv.) in the presence of DBU or triethylamine at room temperature, to the resulting solution was added H_2O-pyridine or hydrazine monohydrate to effect the deprotection of the N^4-phthaloyl group of the cytidine derivative (**102**). The corresponding [4-^{15}N]cytidine derivatives (**103**) were obtained in 96% and 92% yields, respectively. With this method, the [^{15}N]phthalimide label reagent is a solid at normal temperature, has excellent



97 **98** **99**

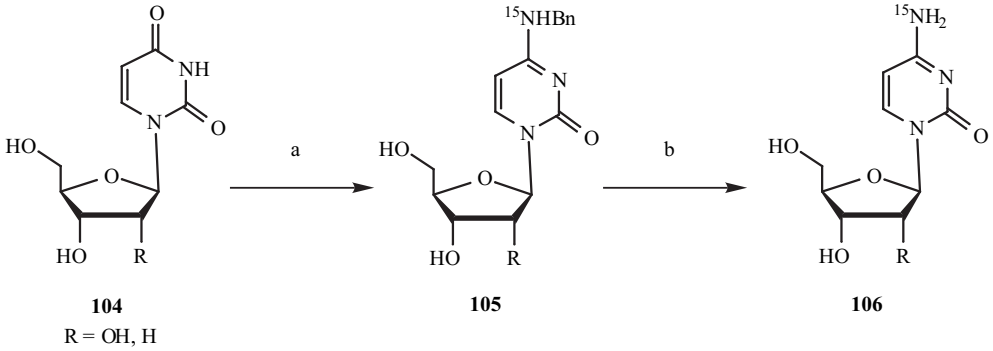
Scheme 17. *Reagents and Conditions*: (a) 1*H*-tetrazole, TsCl, $(PhO)_2P(O)OH$, pyridine, rt, 1.5 days (100%); (b) $^{15}NH_4Cl$,KOH, H_2O, CH_3CN, Et_3N, rt, 24 h (95%).



Scheme 18. *Reagents and Conditions*: (a) 1*H*-tetrazole or 3-nitro-1,2,4-triazole, TsCl, $(PhO)_2P(O)OH$, pyridine, rt, 1.5 days (84-94%); (b) [^{15}N]phthalimide, DBU or Et_3N, rt,1 h or 24 h ; (c) NH_2NH_2 H_2O, AcOH-pyridine, rt, 30 min or H_2O-pyridine, rt, 1 h (R = Ac, R' = H, 92% from **101** (X = Te); R = Bz, R' = OThp, 96% from **101** (X = Te)).

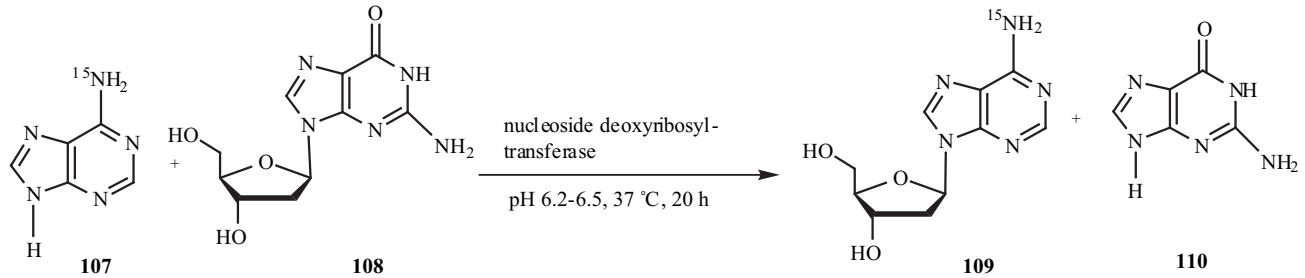
storage stability, provides excellent handling properties, can minimize the equivalent number of labeled compound required, and can be used efficiently to minimize the overall expense incurred. In addition, this method has the advantage in that it can be used to efficiently synthesize labeled an intermediate (**103**, R = Bz, R' = OThp) required for RNA oligonucleotide synthesis without damaging the benzoyl (Bz) and tetrahydropyranyl (Thp) protecting groups required for the hydroxy function, since the reaction is performed under mild conditions [15c].

Sako *et al.* reported on a method for the synthesis of [4-^{15}N]cytosine nucleosides (**106**) using [^{15}N]benzylamine (Scheme **19**) [57]. By direct treatment of uridine or 2'-deoxyuridine (**104**) with [^{15}N]benzylamine (2 equiv.) in refluxing hexamethyldisilazane (HMDS), the corresponding N^4-benzyl[4-^{15}N]cytosine derivatives (**105**) were prepared in one-step in 84% and 74% yields, respectively. Heating a solution of **105** with ammonium persulfate in a pH 7.0 buffer solution at 80 ˚C followed by chromatographic separation allowed for the isolation of [4-^{15}N]cytosine derivatives (**106**) in 79% and 53% yields, together with starting material (**105**), N^4-benzyl[4-^{15}N]cytosine and [4-^{15}N]cytosine.

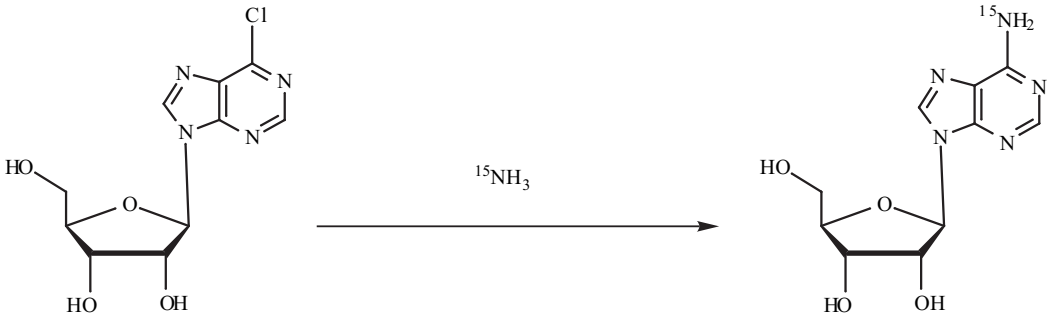


Scheme 19. *Reagents and Conditions*: (a) $Bn^{15}NH_2$, Me_3SiCl, α-picoline, HMDS, 140 ˚C, 2 days (R = OH, 84%; R = H, 74%); (c) $(NH_4)_2S_2O_8$, pH 7 phosphate buffer-CH_3CN, 80 ˚C, 30 min (R = OH, 53%; R = H, 79%).

Given that the conversion of uridine into cytidine is efficient (as described above), the synthetic approach often



Scheme 20. Reaction scheme of the conversion of [6-^{15}N]adenosine to 2'-deoxy[6-^{15}N]adenosine catalyzed by the nucleoside deoxyribosyltransferase.



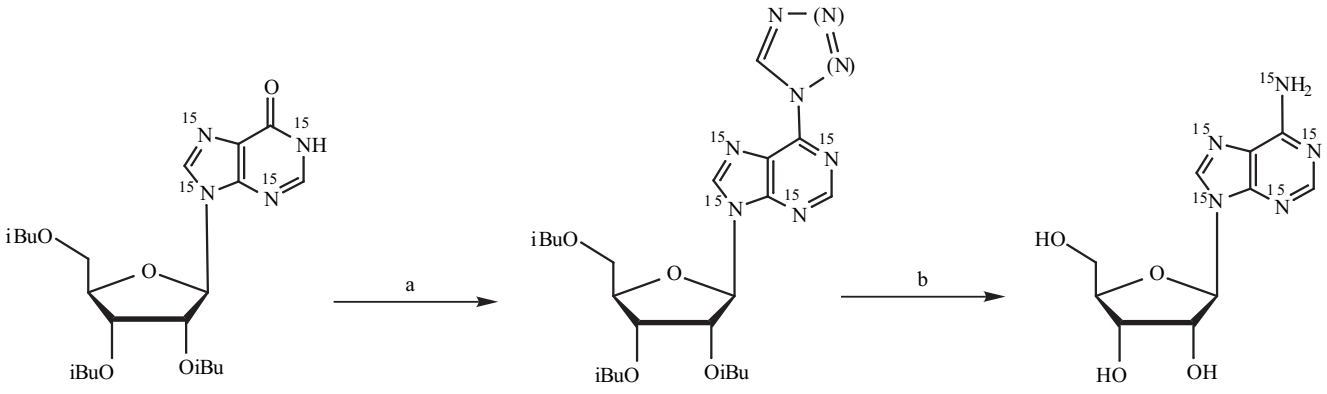
111 **112**

Scheme 21. *Reagents and Conditions*: $^{15}NH_3$ = $^{15}NH_3$/MeOH, rt, 16 h and then 40 ˚C, 2 h (96%) [59a], $^{15}NH_4Cl$, *N*-hydroxymethyl-1,3-propanediamine, *n*-BuOH, 120 ˚C, 24 h [59b], or $^{15}NH_4Cl$, KCO_3, DMSO, 80 ˚C, 2 days (84%) [59d].

taken to produce ^{2}H, ^{13}C and/or ^{15}N-labeled cytidine derivatives involves glycosylation of a labeled uracil base with labeled ribose, with the successive conversion of the uracil base into the cytosine base [14a,38,52,56].

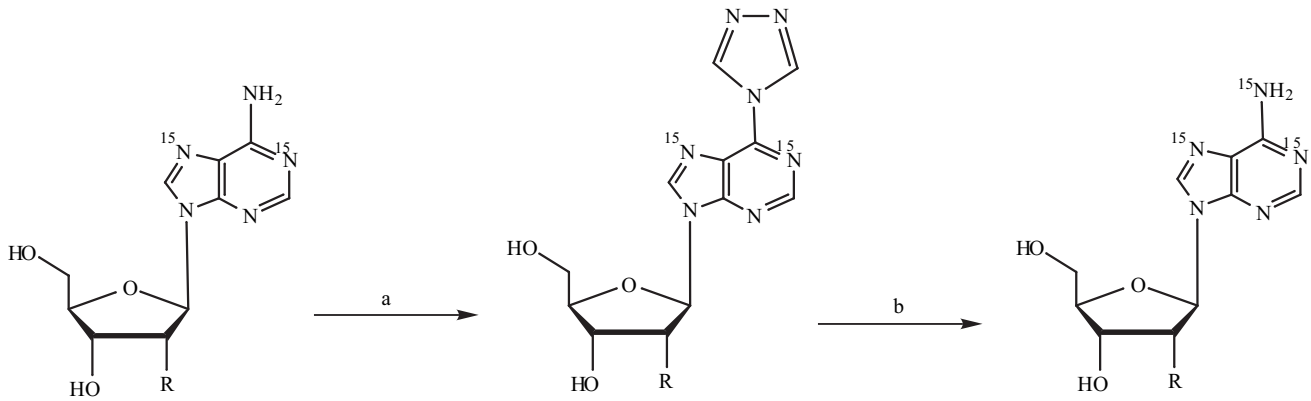
III-1-2. [6-^{15}N]Adenosine and 2'-Deoxy[6-^{15}N]adenosine Derivatives

Generally adenosine derivatives can be prepared by the glycosylation of adenine with an appropriately functionalized



113 **114** **115**

(1,2,4- triazol-1- and -4-yl)



116 **117** **118**

R = OH, H

Scheme 22. *Reagents and Conditions*: (a) tris(1,2,4-triazolyl)phosphine, Et_3N, CH_3CN, rt, 45 h (80% (6 : 1)); (b) $^{15}NH_4OH$/THF, rt, overnight, and then 55-60 ˚C, 3.5 h (90%); (c) *N,N*-dimethylformamideazine, Me_3SiCl, pyridine, 100 ˚C, 24 h (R = OH, 93%; R = H, 73%); (d) $^{15}NH_4Cl$, t-BuOK, DMSO, 80-85 ˚C, 2-5 days (R =OH, 76%; R = H, 82%).

D-ribofuranosyl derivative. On the other hand, 2'-deoxyadenosine derivatives [29] are prepared by 2'-deoxygenation of the corresponding ribonucleoside derivatives [14a,52], since the glycosylation of adenine with an appropriately functionalized 2-deoxy-D-ribofuranosyl derivative can generate an α-glycoside derivative as a by-product [29]. 2'-Deoxyadenosine derivatives can also be prepared by the enzymatic coupling of adenine with a 2-deoxy-D-ribose sugar [58,75a]. 2'-Deoxy[6-^{15}N]adenosine (**109**) was prepared by the enzymatic transglycosylation of [6-^{15}N]adenine (**107**), which is synthesized by treatment of chloropurine with [^{15}N]ammonia, in the presence of 2'-deoxyguanosine using nucleoside deoxyribosyltransferase (Scheme **20**) [75a].

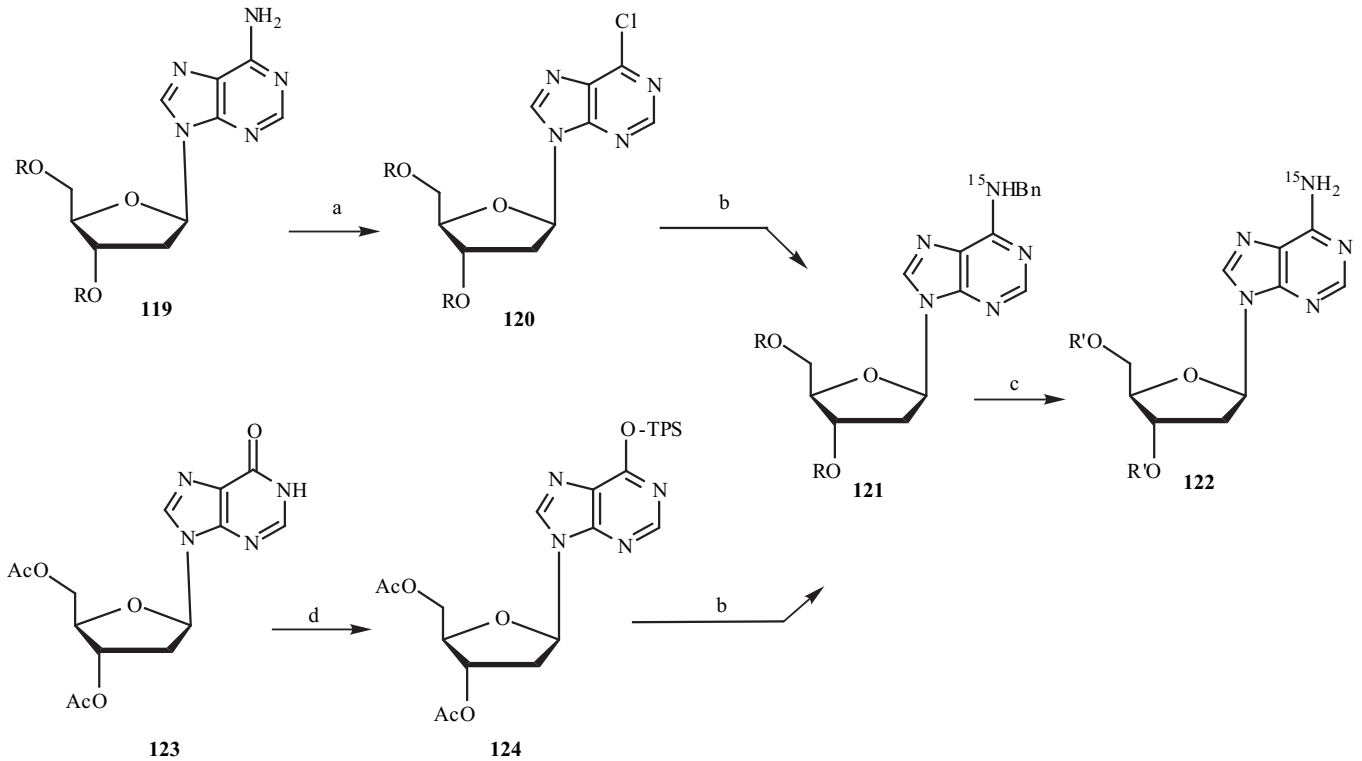
The introduction of the labeling at the nucleoside level has the advantage that no expensive isotope labeled material is lost during execution of the sugar-base glycosylation reaction. Therefore, it is much more efficient to start from an intact nucleoside such as adenosine, inosine or the corresponding 2'-deoxynucleosides and convert these to the corresponding [6-^{15}N]adenosine derivative through the reaction of the activated intermediates with a ^{15}N-labeled reagent.

To facilitate the introduction of the exocyclic amino group, a leaving group such as halogene [59], TPSO- (TPS: triisopropylbenezenesulfonyl) [59g,60], triazolyl [61], and 3-nitro-1,2,4-triazolyl group [11c,15a-c] is introduced into the C6-position of the purine derivatives, which is subsequently displaced by [^{15}N]ammonia [59a-f,61], [^{15}N]benzylamine [59g,h,60] or [^{15}N]phthalimide [11c,15a-c].

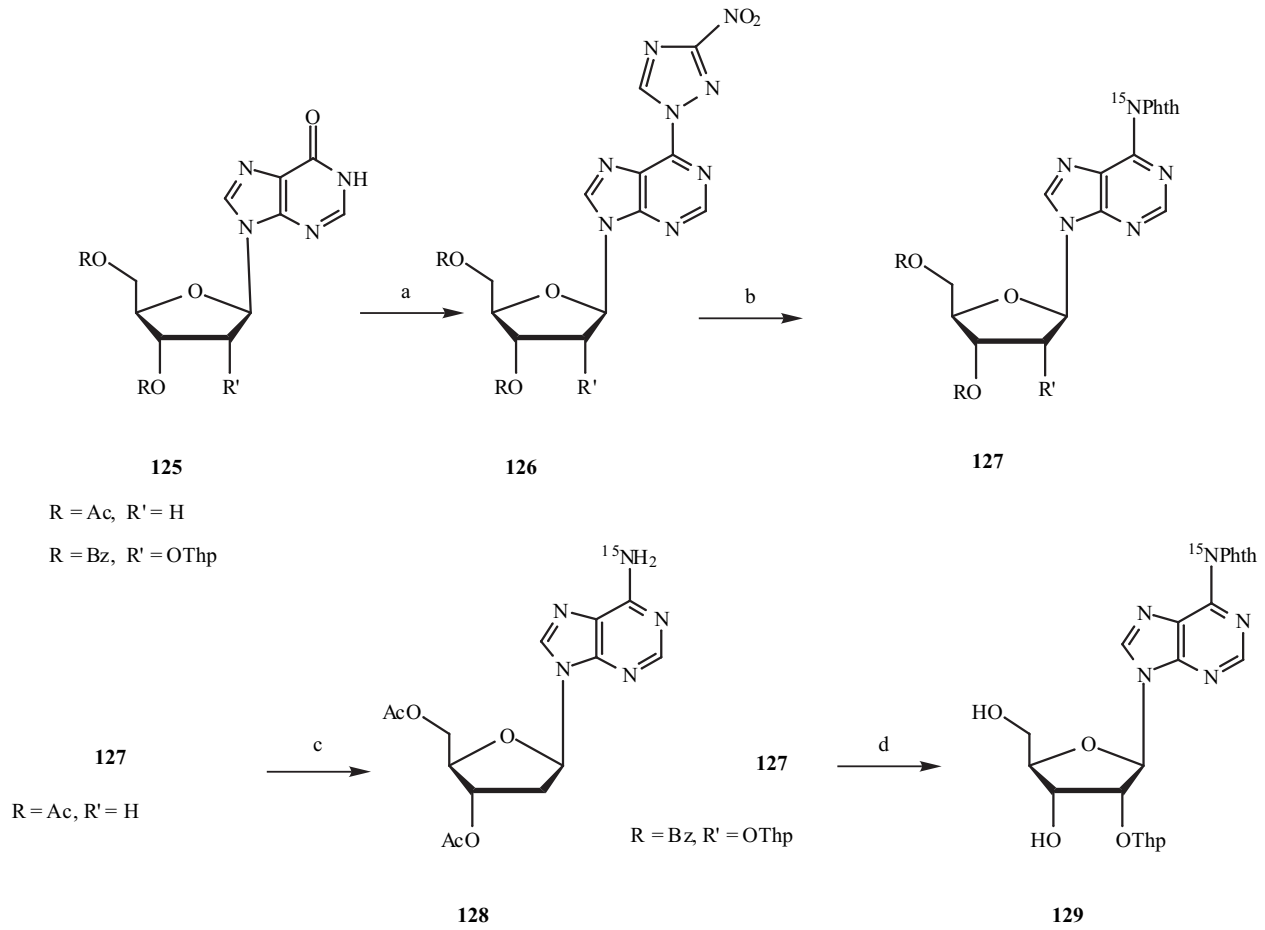
[6-^{15}N]Adenine nucleosides (**112**, **115**, and **118**) can be obtained by the direct amination of 6-chloro- or 6-triazolylpurine nucleosides (**111**, **114**, or **117**) with [^{15}N]ammonia at high temperature in a sealed tube (Schemes **21** and **22**) [59,61].

The nucleophilic substitution reaction of 6-Chloro- or 6-*O*-TPS-purine nucleoside (**120** or **124**) with [^{15}N]benzylamine occurs smoothly at room temperature to give N^6-benzyl-2'-deoxy[6-^{15}N]adenosine derivatives (**121**), respectively [59g,h,60]. Debenzylation of **121** was attempted under a variety of reductive conditions, none of which proved to be successful. Therefore, an alternative approach was to perform an oxidation of the N^6-benzyl group to the N^6-benzoyl group using a mixture of $NaIO_4$ and RuO_2 as an oxidant, and then to follow this with ammonolysis of the benzamide to give the corresponding [6-^{15}N]adenine nucleosides (**122**) (Scheme **23**).

We developed efficient synthetic methods for [6-^{15}N]adenosine and 2'-deoxy[6-^{15}N]adenosine derivatives (**127**), which is characterized by the nucleophilic substitution reaction of 6-(3-nitro-1,2,3-triazolyl)purine nucleoside (**126**) with [^{15}N]phthalimide, in addition to methods for the synthesis of [4-^{15}N]cytidine derivatives (Scheme **24**) [11c,15a-c]. The nucleophilic substitution reaction of **126** with [^{15}N]phthalimide took place in the presence of DBU at room temperature to give N^6-phthaloyl[6-^{15}N]adenosine derivatives (**127**) in high yield. The phthaloyl group of **127** was readily deprotected by treatment with hydrazine monohydrate in acetic acid-pyridine to give the coresponding [6-^{15}N]adenosine derivative (**128**) in 80% yield from **127**. In addition, the labeled intermediate (**129**) for the RNA oligonucleotide synthesis was synthesized without damaging the Bz and Thp protecting groups required for the hydroxy function [15c]. This method has clear advantages for the



Scheme 23. *Reagents and Conditions*: (a) *t*-$BuNO_2$, CCl_4 (R = TBDMS, 50-60%); (b) $Bn^{15}NH_3Cl$, DBU; (c) 1. $NaIO_4$, RuO_2, CH_2Cl_2-CH_3CN-H_2O; 2. NH_4OH (R' = H or TBDMS, 61-70% based on the $Bn^{15}NH_2$); (d) TPSCl (33%).



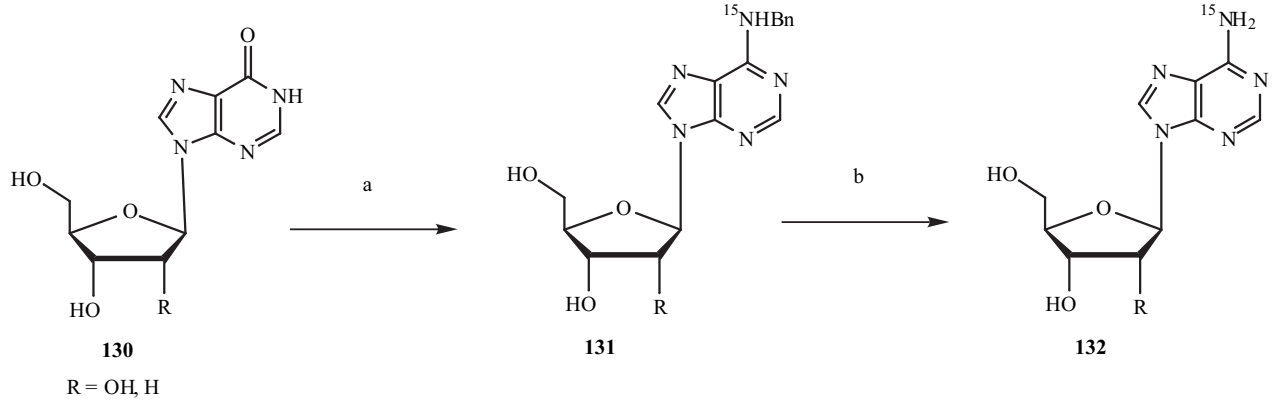
Scheme 24. *Reagents and Conditions*: (a) 3-nitro-1,2,4-triazole, TsCl, $(PhO)_2P(O)OH$, pyridine, 50 ˚C, 21.5-24.5 h (R = Ac, R' = H, 82%; R = Bz, R' = OThp, 80%); (b) [^{15}N]phthalimide, DBU,CH_2Cl_2, rt, 2-5 days (R = Bz, R' = OThp, 99%); (c) NH_2NH_2 H_2O, AcOH-pyridine, rt, 30 min (80% from **126**); (d) 1. NaOH, H_2O-pyridine, 0 ˚C, 1 h, 2. $(CF_3CO)_2O$, pyridine, rt, 30 min (59%).

large preparation of [6-^{15}N]adenosine derivatives compared to the other methods, particularly in view of the reaction conditions employed and of the use of a solid ^{15}N-source.

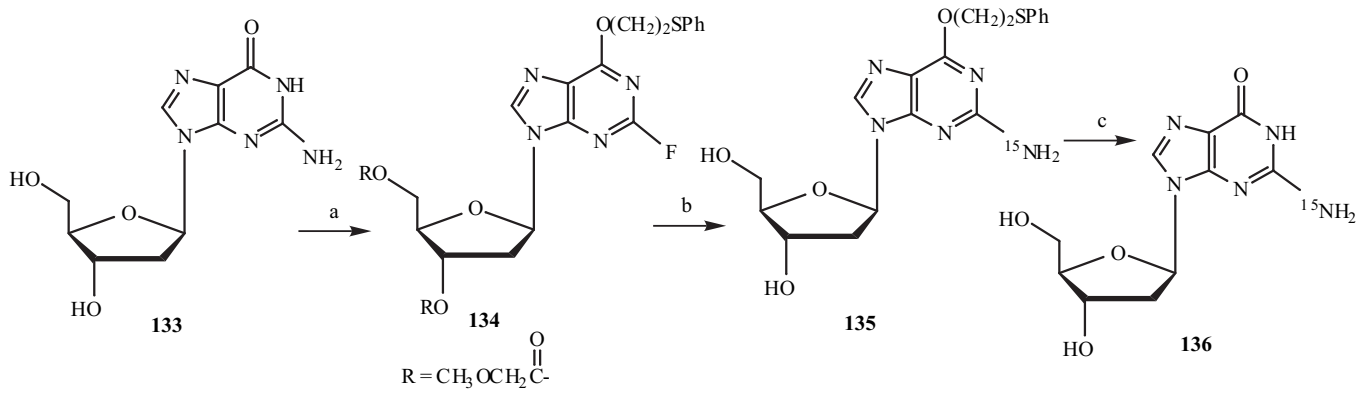
[4-^{15}N]Adenosine derivatives (**132**) can be also prepared by direct conversion of inosine (**130**) (Scheme **25**) [62] as well as the method for the synthesis of [4-^{15}N]cytidine derivatives (**106**) (Scheme **19**) [57]. This method is able to directly substitute oxygen in inosine (**130**). [6-^{15}N]Adenosine and 2'-deoxy[6-^{15}N]adenosine (**132**) were prepared by the silylation-benzylamination of **130** and subsequent oxidative debenzylation with ammonium persulfate under thermal conditions.

III-1-3. [2-^{15}N]Guanosine and 2'-Deoxy[2-^{15}N]guanosine Derivatives

Guanosine derivatives can be prepared by glycosylation of guanine with a D-ribofuranosyl derivative [29]. 2'-

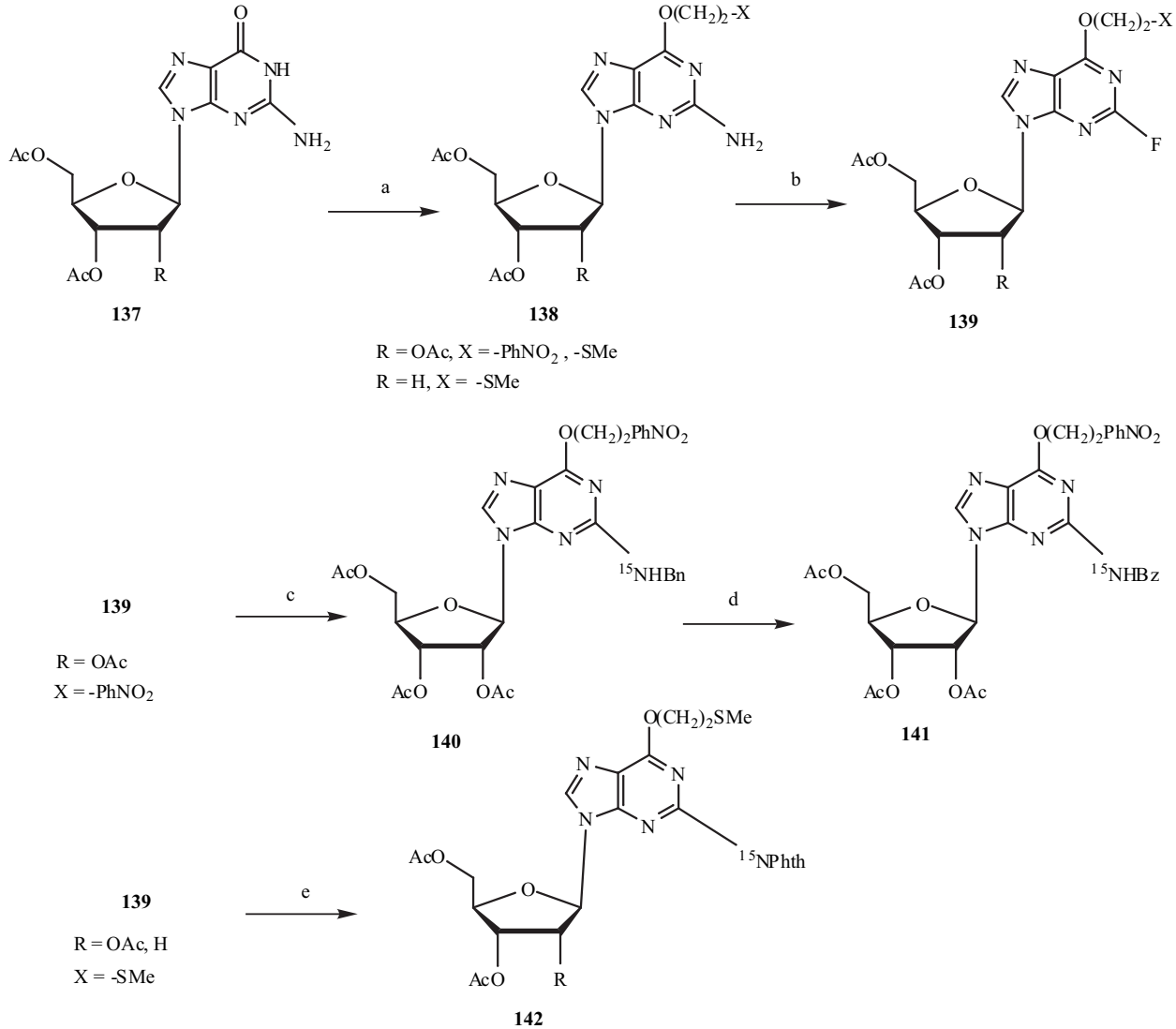


Scheme 25. *Reagents and Conditions*: (a) $Bn^{15}NH_2$, $(NH_4)_2SO_4$, HMDS, 140 ˚C, 36 h (R = OH, 95%; R = H, 92%); (b) $(NH_4)_2S_2O_8$, pH 7.2 1 M phosphate buffer-CH_3CN, 80 ˚C, 2 h (R = OH, 89%; R = H, 88%).

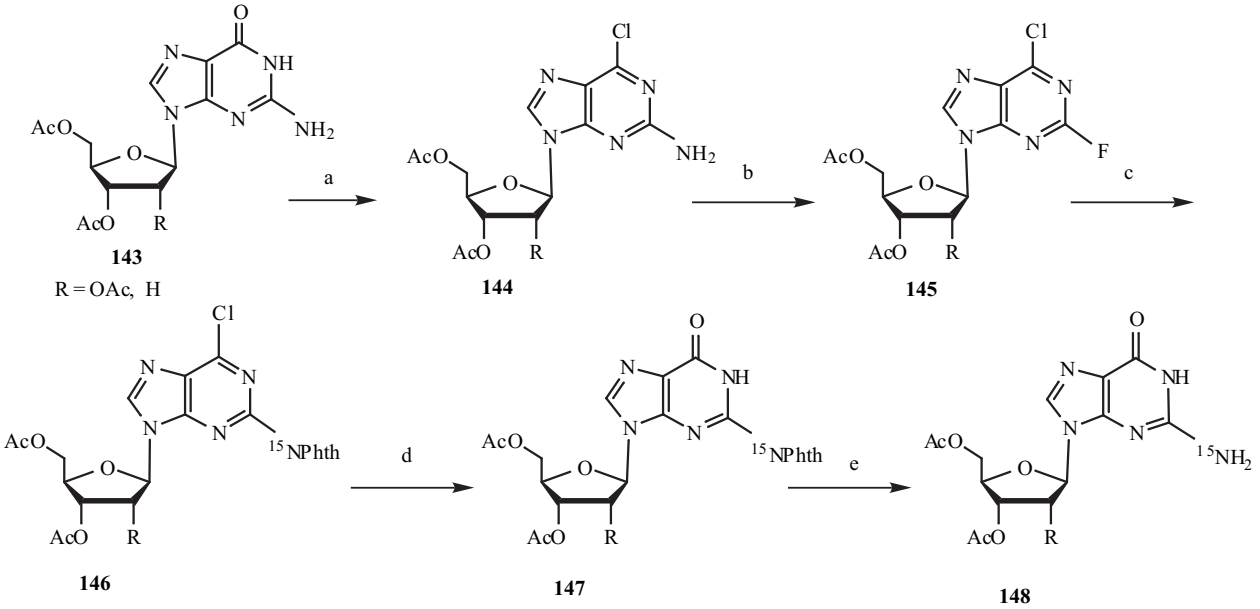


Scheme 26. *Reagents and Conditions*: (a) 1. $(MeOAc)_2O$, DMF-pyridine, rt; 2. $HO(CH_2)_2SPh$, Ph_3P, diethyl azodicarboxylate; 3. $NaNO_2$, HBF_4; (b) $(^{15}NH_4)_2SO_4$, NaOMe; (c) 1. $NaIO_4$; 2. DBU.

Deoxyguanosine derivatives are prepared by 2'-deoxygenation of guanosine derivatives [14a,52] and by the enzymatic coupling of guanine with a 2-deoxy-D-ribose suger [63].



Scheme 27. *Reagents and Conditions*: (a) $HO(CH_2)_2$-X (X = $-PhNO_2$ or -SMe), Ph_3P, diethyl azodicarboxylate, 1,4-dioxane, rt, 1.5-3 h (R = OAc, X = $-PhNO_2$ 90%; -SMe 84%) (R = H, X = -SMe, 65%); (b) 25% HF/pyridine, *t*-$BuNO_2$, in ice-bath, 1h and then rt, 4-9 h (R = OAc, X = $-PhNO_2$, 91%; -SMe, 84%) (R = H, X = -SMe, 87%); (c) $Bn^{15}NH_2$, Et_3N, DMF, rt, 2 h (93%); (d) $RuCl_3$, Bu_4NIO_4, CH_2Cl_2-CH_3CN-H_2O, rt, 2 h (71%); (e) 1. potassium [^{15}N]phthalimide, DMF, 90 °C, 1.5-2 days; 2. Ac_2O, pyridine, rt, 1 h (R = OAc, 62%; R = H, 56%).



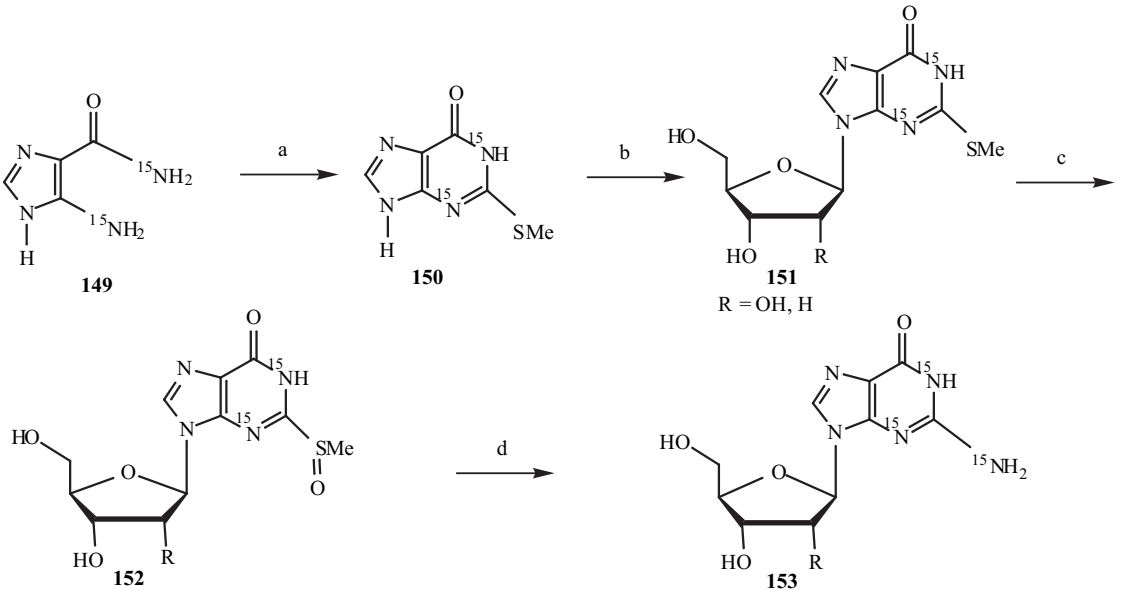
Scheme 28. *Reagents and Conditions*: (a) $OPCl_3$, Et_4NCl, CH_3CN, 80 ˚C, 5-40 min (R = OAc, 99%; R = H, 87%); (b) 45% HF/pyridine, *t*-$BuNO_2$, in ice-bath, 30 min (R = OAc, 85%; R = H, 90%); (c) potassium [^{15}N]phthalimide, DMF, rt, 30 h- 60 ˚C, 1.5 h (R = OAc, 61-64%; R = H, 37%); (d) 1. $NaO(CH_2)_2CN$, THF, 45 ˚C, 1 h; 2. Ac_2O, pyridine-DMF, rt, 2 h; (e) 1. NH_2NH_2 H_2O, AcOH-pyridine, rt, 30 min; 2. Ac_2O, pyridine, rt, 1 h (R = OAc, 71%; R = H, 76% from **146**).

For the synthesis of [2-^{15}N]guanine nucleosides, the introduction of the labeling at the nucleoside level has advantages, as well as for the synthesis of [4-^{15}N]cytosine and [6-^{15}N]adenine nucleosides.

The substitution at the 2-position of purine nucleosides with ^{15}N-label reagents lead to [2-^{15}N]guanine nucleosides, but the substituent in the 2-position of purine bases was less susceptible to nucleophilic attack. Therefore, activation of the 2-position of the purine base by a leaving group with strong electron-withdrawing capacity is necessary.

Kieper *et al.* reported the synthesis of 2'-deoxy[2-^{15}N]guanosine (**136**) using a 2-fluoro-2-deoxyinosine derivative (**134**), that was prepared from 2'-deoxyguanosine (**133**) in three steps (Scheme **26**) [64]. The introduction of the ^{15}N label into the amino group of 2'-deoxyguanosine was achieved through the nucleophilic addition-elimination reaction of **134** with [^{15}N]ammonia, which was generated *in situ* from [^{15}N]ammonium sulfate by the addition of sodium methoxide.

We developed synthetic methods for the synthesis of [2-^{15}N]guanosine and 2'-deoxy[2-^{15}N]guanosine derivatives



Scheme 29. *Reagents and Conditions*: (a) 1. NaSCSOEt, DMF, rt, 20 min and then reflux, 4 h; 2. MeI, H_2O, rt, 24 h (85%); (b) 7-methylguanosine or 2'-deoxyguanosine, purine nucleoside phosphorylase, K_2HPO_4, 43 ˚C, 2-7 days (R = OH, 83%; R = H, 71%); (c) Oxone ($2KHSO_5$-$KHSO_4$-K_2SO_4), H_2O, 0 ˚C, 4 h (R = OH, 96%); (d) $^{15}NH_4Cl$, $KHCO_3$, DMSO, 78 ˚C, 14 days (R = OH, 81%; R = H, 71% from **151**).

(**141**, **142**, and **147**), from guanosine and 2'-deoxyguanosine, respectively, through the nucleophilic addition–elimination reaction of their 2-fluoro derivatives (**139** and **145**) with [^{15}N]benzylamine [15d], potassium [^{15}N]phthalimide [15d] and *N*-*tert*-butyldimethlysilyl[^{15}N]-phthalimide [51], respectively (Schemes **27** and **28**).

The reaction of the 2-fluoroinosine derivative (**138**, R = OAc, X = -PhNO$_2$) with [^{15}N]benzylamine in the presence of triethylamine afforded the N^2-benzyl[2-^{15}N]guanosine derivative (**140**) in 93% yield, which was further converted into the N^2-benzoyl[2-^{15}N]guanosine derivative (**141**) by treatment with $RuCl_3$ and tetrabutylammonium periodate. A similar sequence of reactions of 2-fluoro-O^6-[2-(methylthio)ethyl]inosine (**139**, R = OAc, X = -SMe) and the 6-chloro-2-fluoro-9-(β-D-ribofuranosyl)-9*H*-purine derivative (**145**, R = OAc) with potassium [^{15}N]phthalimide afforded the N^2-phthaloyl[2-^{15}N]guanosine derivative (**142**, R = OAc; 62%) and 9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-6-chloro-2-[^{15}N]phthalimido-9*H*-purine (**146**, R = OAc; 64%), respectively. The introduction of an electron-withdrawing group at the 6-position of the guanine moiety enhanced the reactivity of the 2-fluoro functionality, and thus allowed for a lower reaction temperature, in contrast to the reaction of **139**, R = OAc, X = -SMe and **145**, R = OAc. Compounds **142** (R = OAc) and **146** (R = OAc) were then efficiently converted into 2',3',5'-tri-*O*-acetyl[2-^{15}N]guanosine. The corresponding 2'-deoxy derivatives **142** (R = H) and **146** (R = H) were also synthesized from 2-fluoro derivatives **139** (R = H, X = -SMe) and **145** (R = H) through similar procedures. The reactivity of the 2-fluoro function of **145** (R = H) was low in contrast to the reaction of **145** (R =OAc) and **145** (R = H), and the yield of the 6-chloro-2-[^{15}N]phthalimido-9*H*-purine derivative (**146**, R = H) was low and by-products (6-[^{15}N]phthalimido-9*H*-purine and 2,6-di[^{15}N]phthalimido-9*H*-purine derivatives) were formed. The reaction of 2-fluoroinosine derivatives (**145**) with *N*-*tert*-butyldimethlysilyl[^{15}N]phthalimide, rather than with potassium [^{15}N]phthalimide, in the presence of CsF as a catalyst can enhance the reactivity of the 2-fluoro function, affording 6-chloro-2-[^{15}N]phthalimido-9*H*-purine derivatives (**146**) with reasonable yield.

The methylsulfoxyl at the 2-position of the purine base proved to behave as a typical leaving group (Scheme **29**) [59e]. By treatment of 2-(methylsulfoxyl)inosine derivatives (**152**) with [^{15}N]ammonia generated from [^{15}N]ammonium chloride and $KHCO_3$ in DMSO at 78 ˚C for 14 days, the corresponding [2-^{15}N]guanine nucleosides (**153**) were prepared. The key intermediates (**152**) were prepared from 5-amino-4-imidazolecarboxamide (AICA) (**149**) in four steps. These included ring closure using sodium ethyl xanthate, methylation of mercaptohypoxanthine, enzymatic transglycosylation of 2-methylmercaptohypoxanthine with purine nucleoside phosphorylase (PNP) in the presence of either 7-methylguanosine or 2'-deoxyguanosine to yield the corresponding ribo- or 2'-deoxyribonucleosides (**152**), and oxidation of **152**.

The [2-^{15}N]guanine nucleosides can also be prepared by a synthetic pathway employing the use of a Dimroth rearrangement of the adenine nucleosides [65]. Another approach is to synthesize N^2-benzoyl[2-^{15}N]guanosine from AICA-riboside [66]. In both cases, ^{15}N-labeled cyanide is used as the primary source of the isotope.

III-2. Synthesis of ^{15}N-Labeled Oligonucleotides and Their Applications

The most general synthetic method in the preparation of oligonucleotides containing ^{15}N-labeled exocyclic amino groups is based on the preparation of ^{15}N-labeled nucleoside derivatives for use in enzymatic or chemical oligonucleotide synthesis. In enzymatic synthesis, DNA templates and enzyme polymerases have been used successfully to prepare labeled oligonucleotides (RNA [67] and DNA [4e,68]) from pre-labeled nucleoside triphosphates. One of the biggest disadvantages of the enzymatic approach is that the isotopic label is incorporated in a uniform and non-specific manner. In chemical synthesis, labeled monomers (**154**) are used as precursors in oligonucleotide synthesis by automated synthesizers (Fig. **10**) [69]. This method provides the opportunity of probing the specificity of the interaction between the labeled DNA and target molecule. Many efforts have been devoted to the development of synthetic methods dealing with the preparation of ^{15}N-labeled bases or nucleosides for their subsequent incorporation into nucleic acids.

On the other hand, the post-synthetic approach has been developed [70,74a]. This approach relied on the incorporation of 4-triazolyl- (U^{Tri}) [70a,b,74a,b], O^4-ethyl- (U^{Et}) [70b], O^6-pentafluorophenyl- (I^{PFPh}) [70b], 6-methylthio- (I^{MeS}) [70c], and 2-fluoro- (I^F) [70b] deoxyribonucleoside phosphoramidites (**156**) into oligonucleotides with subsequent treatment of the modified oligomer with concentrated [^{15}N]ammonium hydroxide to generate [4-^{15}N]cytosine, [6-^{15}N]adenine and [2-^{15}N]guanine

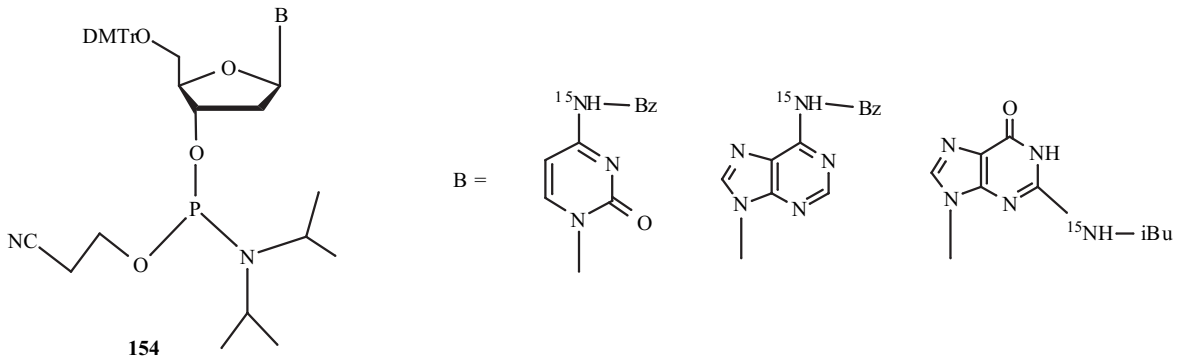
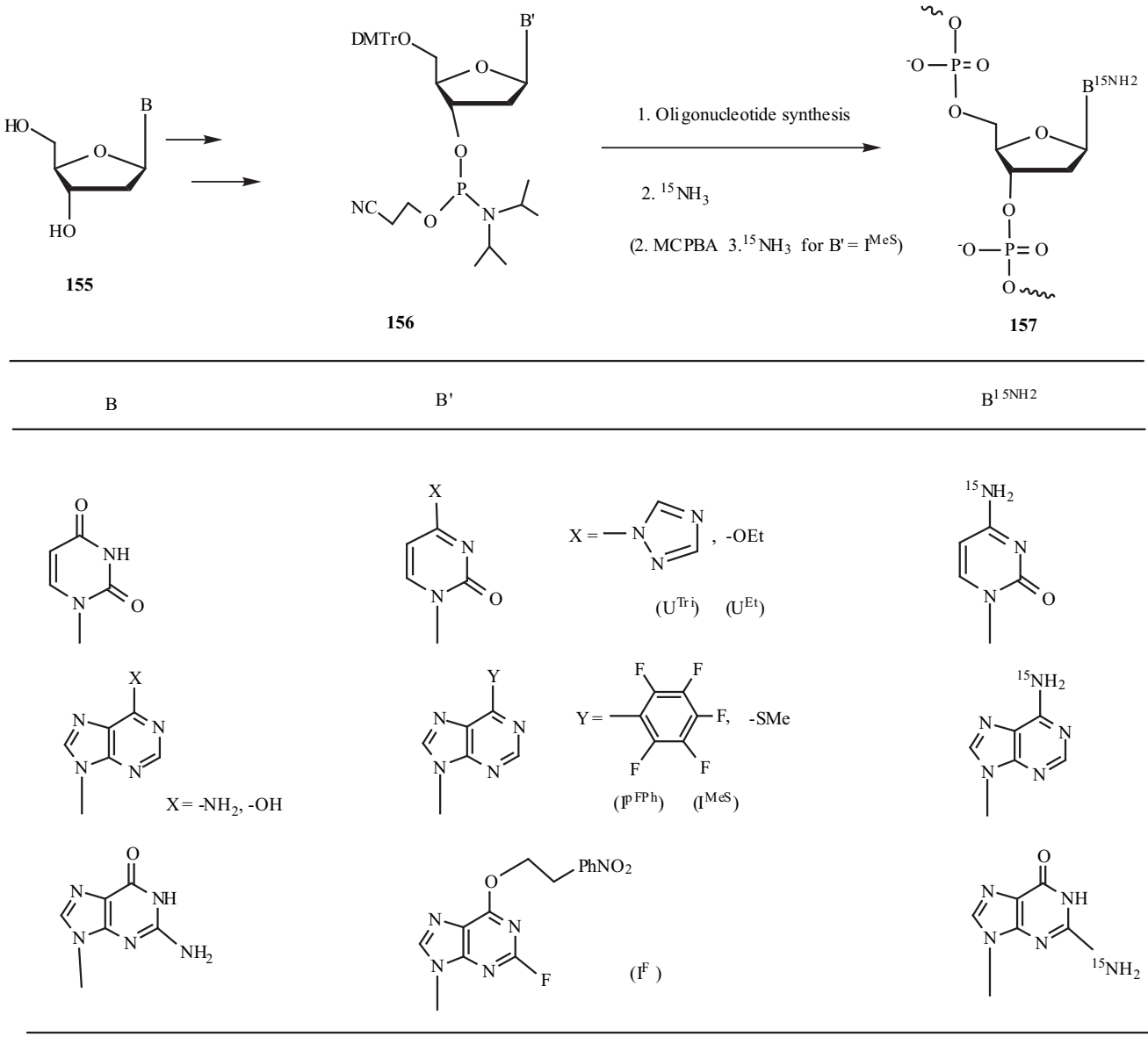


Fig. (10). ^{15}N-Labeled nucleoside phosphoramidites.



Scheme 30. Synthesis of ^{15}N-labeled oligonucleotides by the post-synthetic approach.

residues (Scheme **30**). Although this approach is convenient, one drawback is that depurination occurs during the removal of the DMTr group and hydrolysis resulted rather than ammonolysis [70c].

NMR studies of ^{15}N-labeled oligonucleotides have provided useful information regarding nucleic acid structures [71] (duplexes (hydrogen bonding [72], protonation [6b], stacking [9], triplexes [73], and tetraplexes [7]), nucleic acid-drug binding (ligand interactions) [8], and nucleic acid-protein interactions [74].

For example, ^{15}N-labeled phosphoramidites (**154**) (Fig. **10**) were prepared and incorporated at specific locations into oligonucleotides corresponding to the symmetric 18 base pairs of the lac operator. The advantage of applying ^{15}N-edited NOE spectroscopy to map the individual environment of the protons coupled to the labeled nuclei was demonstrated and sequence-specific ^{15}N-chemical shifts were reported [75].

^{15}N-NMR analysis of specifically labeled nucleic acids has been useful in probing local phenomena ranging from thermally induced local melting to the behavior of mismached base pairs, and to the structural changes triggered by enzyme recognition or drug binding. Duplex formation of the self-complementary oligonucleotides d(CGT[$^{15}N^1$-A]CG and d(CGT[$^{15}N^6$-A]CG), which were prepared using labeled phosphoramidites (Fig. **10**), was reflected by an upfield shift of ~ 2.6 ppm for the N^1-resonance and a downfield shift of ~ 1.2 ppm for the N^6-resonance [5a]. The T*m* and thermodynamic data obtained for the helix-to-coil transition from the ^{15}N-NMR chemical shifts agreed well with those values derived by other methods [5a].

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