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Heavy Atom Labeled Nucleotides for Measurement of Kinetic Isotope Effects

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Abstract

Experimental analysis of kinetic isotope effects represents an extremely powerful approach for gaining information about the transition state structure of complex reactions not available through other methodologies. Implementation of this approach to the study of nucleic acid chemistry requires the synthesis of nucleobases and nucleotides enriched for heavy isotopes at specific positions. In this review we highlight current approaches to the synthesis of nucleic acids site-specifically enriched for heavy oxygen and nitrogen and their application in heavy atom isotope effect studies.

Keywords

Kinetic Isotope Effect; Ribozyme; RNA Catalysis; Labeled Nucleotide; Transition State

Introduction

Kinetic isotope effects (KIEs) offer an extremely powerful method for interrogating enzymatic transition states and active sites. Substitution of a single atom represents the smallest possible perturbation to a chemical system, and replacement of an atom with a heavy isotope reports directly on changes to the bonding environment of that atom in the rate-limiting step of the reaction [1]. While this approach has been used frequently in the interrogation of many enzymatic reactions [2-5] the use of heavy atom isotope effects to

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measure enzymatic reactions involving nucleotides has been limited by several technical challenges.

First, the difference in mass between isotopes of heavy atoms is small, so detecting primary isotope effects for oxygen and nitrogen requires highly precise measurement of relative rates for isotopologues. This challenge can be met by determination of relative rates through internal competition rather than direct rate measurements [6] and by application of highly precise mass spectrometric methods [7]. Second, multi-step processes that involve reaction steps prior to chemistry can reduce the magnitude of observed isotope effects [8, 9]. This problem is especially pronounced in RNA catalysis where pre-chemical processes like folding or strand annealing can be rate-limiting or occur with rate constants on the same order as k_{cat} . Therefore, analysis of isotope effects requires a basic kinetic understanding of a given reaction, and potentially the development of an alternative system with rate-limiting chemistry. Additionally, interpretation of isotope effects can be complicated by a lack of knowledge of the starting ground state of the reaction and the influence of chemical entities not explicitly involved in bond formation like metal ions. Measured isotope effects can be interpreted with greater clarity when compared to measured isotope effects for reference reactions [10] and complemented with computational studies. Continued improvements in multiscale modeling of complex biochemical systems has greatly aided in the interpretation of measured isotope effects. Finally, measurement isotope effects nucleic acid chemistry requires the synthesis of nucleotides site-specifically enriched for heavy isotopes. While the literature is rife with strategies for the isotopic enrichment of nucleotides, very few are site-specific with respect to atomic position as most of these methodologies reported to date are for NMR studies [11-13].

Meeting these challenges enables the use of KIE analysis on reactions could not have been probed previously, which has the potential to greatly enhance and deepen our understanding of these processes. For example, in more than three decades of studies on RNA catalysis, our understanding of transition state interactions is well developed [14], however, our understanding of transition state structure remains in its infancy. Herein, we highlight a number of experiments that have employed nucleotides enriched site-specifically with heavy atoms for isotope effect measurements and describe the synthetic challenges involved in producing these compounds. Our motivation in this area stems from a long-standing interest in RNA catalysis and the fact that there has been no heavy atom isotope effect analysis for any nucleolytic ribozyme. Therefore, we frame this review around the measurements required to probe the transesterification reaction catalyzed by several small endonucleolytic ribozymes. In doing so, we hope to illustrate the complexity and subtlety involved in these studies and to motivate the need for a variety of site-specifically labeled nucleotides.

Most nucleolytic ribozymes catalyze phosphoryl transfer via an S_N2 -like mechanism, in which a 2' hydroxyl nucleophilically attacks its own 3' phosphodiester causing strand scission, displacing a 5' hydroxyl leaving group and producing a 2', 3' – cyclic phosphate (Scheme 1). Isotope effect analysis of this process would require, at a minimum, heavy atom enrichment of the 2' hydroxyl (to determine the extent of nucleophilic attack) and 5' hydroxyl (to determine the extent of leaving group departure). However, interpretation of these isotope effects would be complicated by the fact that the oxygen atoms are involved in

both bond cleavage and bond formation over the course of the reaction. The 5' oxygen, for example, loses its bond to the scissile phosphate and becomes protonated spontaneously. If strand scission and protonation both occur to some extent in the transition state, then it would be difficult to determine the relative contribution of each process to the measured isotope effect without additional information. Therefore, measurement of isotope effects for atoms implicated in proton transfer *to and from* the leaving group and nucleophile, respectively, greatly simplifies the interpretation of isotope effects for the nucleophile and leaving group.

In several nucleolytic ribozymes, including the hepatitis delta virus (HDV) [15], Varkud satellite (VS) [16], Hairpin [17] ribozymes and potentially the recently discovered Twister ribozyme [18], proton transfer is facilitated by one or two nucleobases positioned in the active site. For example, in the VS ribozyme, adenine and guanine nucleobases positioned in the active site have been implicated in general acid and general base catalysis, respectively, whereby the N1 atom of the catalytic guanine abstracts a proton from the 2' hydroxyl nucleophile while the N1 atom of the catalytic adenine donates a proton to the 5' hydroxyl leaving group [16]. Measurement of isotope effects for these two nitrogen atoms would serve two purposes: First, such measurements could further implicate the involvement of the putative general acid and general base in proton transfer during the rate-limiting step. Second, coupled with measurements of isotope effects for the 2' hydroxyl nucleophile and 5' oxygen leaving group, they could help to clarify the extent of nucleophilic attack and leaving group departure in the transition state.

In addition to the aforementioned isotope effects, the measurement of a number of secondary isotope effects would also be helpful in elucidating the precise mechanism employed by nucleolytic ribozymes. Of particular interest are the non-bridging phosphoryl oxygen atoms. While they are not directly involved in bond formation or cleavage, measurement of isotope effects for non-bridging oxygen atoms provides information on the structure of the transition state by reporting on the bonding environment around the scissile phosphate. Additionally, interrogation of deuterium or tritium isotope effects for the hydrogen atoms attached to the 2', 3' and 5' carbon atoms proximal to the scissile phosphate could provide information about changes to the bonding environment around those carbons.

In summary, an exhaustive interrogation of the mechanism of nucleolytic ribozyme with isotope effects would require (1) site-specific enrichment of heavy atoms at the 2' and 5' oxygens; for ribozymes outside of class II, like the group I intron, heavy atom at the 3' oxygen position would also be required; (2) the non-bridging phosphoryl oxygens; (3) the 2', 3', and 5' hydrogens; and (4) any nucleobase atom with suspected involvement in proton transfer – generally this would include N1 nitrogens for purines and N3 nitrogens for pyrimidines. In this review, we focus primarily on methods for the synthesis of nucleotides with heavy heteroatoms, since, unlike carbon and hydrogen, these atoms are directly involved in bond formation and scission and thus likely to exhibit primary isotope effects. Moreover, we highlight only state-of-the-art syntheses for these substrates with an emphasis on methodologies that generate substrates isotopically enriched at a single atomic position. Interested readers should also consult excellent reviews by Theodorou *et al.* [19] and Milecki [20], which contain a more complete account of isotopically labeled nucleotides.

Labeling Sugar Oxygens

Uridine containing 2'-¹⁸O has been synthesized by heating 2,2'-cyclohydridine in the presence of labeled benzoic acid, potassium hydride in dimethylformamide to give [2'-¹⁸O]benzoyluridine followed by treatment with methoxide to convert the benzoylated product into uridine. [21] (Scheme 2). The process is quite straightforward requiring only two steps with 88% yield and employs a relatively inexpensive label source, benzoic acid; however, because it utilizes a cyclic derivative of uridine as a starting material, it may be incompatible with the synthesis of other nucleosides labeled at the 2'-O position. However, synthesis of 2'-O labeled cytidine could be indirectly achieved through the known conversion of uridine to cytidine by activation at the C4 position with tetrazole followed by ammonolysis [22].

[3'-¹⁸O]Uridine has also been prepared by a related method that begins with a cyclic uridine derivative [23] (Scheme 3). Labeled benzoyl chloride was reacted with 2,3'-cyclohydridine at the 2' position in the presence of pyridine to give 2'-¹⁸O-benzoyl-2,3'-cyclohydridine, which underwent thermal Fox rearrangement revealing [2'-¹⁸O]2,2'-cyclohydridine. Subsequent ring opening with Me₃SiN₃ gave a 2'-azido product bearing a label at the 3' position in 11% yield from the starting material. However, ring opening in the presence of benzoic acid and potassium hydride followed by hydrolysis would give the 2'-hydroxyl product predominantly in the ribo conformation [21] Like the procedure for [2'-¹⁸O] uridine, this procedure employs a cyclic nucleoside as a starting material and therefore may not be directly used to synthesize other nucleosides labeled at the 3'O position. Moreover, this method employs benzoyl chloride as the label source, which is not commercially available. Labeled benzoyl chloride is generally prepared through the hydrolysis of unlabeled benzoyl chloride with labeled water to give labeled benzoic acid, which can be converted back to benzoyl chloride in a subsequent reaction with thionyl chloride [17].

Enrichment of nucleosides at the 5' oxygen has been achieved through a straightforward method (Scheme 4) [24]. First, the 5'-hydroxyl was activated for nucleophilic attack as a mesylate ester, which was subsequently treated with labeled benzoic acid to give heavy isotopologue enriched at the 5'-O position. The 5'-O-4,4-dimethoxytrityl (DMTr) protecting group can then be added back to give a nucleoside compatible with phosphoramidite chemistry. This method should be applicable to prepare all four 5'-¹⁸O labeled nucleosides with appropriate protection.

Labeling Non-Bridging Phosphate Oxygens

There are two useful methods for the incorporation of phosphates bearing heavy oxygen atoms in the non-bridging positions. First, labeled phosphoryl chloride can be added to unprotected hydroxyl groups to give phosphorodichloridates, which can be converted to phosphates by hydrolysis (Scheme 5A). This method has been employed to generate 5'-nucleotides [25]. Second, the oxidation of phosphite triesters generates phosphate esters containing labeled oxygen at a non-bridging position [26] (Scheme 5B). This method is useful for solid-state oligonucleotide synthesis whereby heavy non-bridging oxygens can be added during the oxidation step.

A drawback of both approaches is that they produce diastereomeric mixture of inseparable products, placing the heavy atom at either the pro-S_p and pro-R_p. A more desirable approach would be one that either is stereospecific or generates separable products. Notably, oxidation in the presence of a sulfurizing agent generates a mixture of diastereomeric phosphothioates, which can be separated by reversed-phase HPLC [27]. A diastereomerically pure phosphothioate could then be converted to a phosphate with an oxygen label by treatment with cyanogen bromide and labeled water [28] (Scheme 6).

Labeling Nucleobases

Vilarassa and co-workers have developed syntheses of nucleosides containing all four nucleobases and thymine and hypoxanthine enriched for ¹⁵N at positions likely to be involved in proton transfer [29-32]. Broadly, their method involves nitration of the nucleoside to form an N-nitro compound, followed by replacement of the nitroamine group with a nucleophilic labeled nitrogen source, (usually ¹⁵NH₃), which triggers two consecutive carbonyl addition/elimination steps that result in ring-opening and ring closing (Scheme 7). An optimized synthetic route to 2'-O-TBS-3-¹⁵N labeled uridine phosphoramidite (7 steps, 26% yield) was developed by Baral et al. (Scheme 8) [34]. This facile method utilizes commercially available ¹⁵NH₄Cl as a label source, and protecting groups that are compatible with solid-phase synthesis of oligonucleotides using phosphoramidite chemistry. However, N-nitration proceeds in high yields only under mild conditions when the target nitrogen is strongly activated by an adjacent carbonyl, which precludes direct preparation of labeled adenine by this method. Furthermore, for cytosine and guanine nucleosides the exocyclic amino groups provide a competitive nucleophile during ring closure, which allows formation of alternative products. Thus, labeling of A, G, and C nucleosides has proven more complex.

Cytidine labeled at N3 has been prepared from N-3 labeled uridine by activating the C4 carbonyl with tetrazole then treating it with ammonium chloride at low pH to reveal cytosine with the labeled position intact [29]. N1 labeled adenosine nucleosides have been prepared via an analogous method from inosine whereby the carbonyl is aminated after the labeling step [30, 31].

Labeled guanine nucleosides have also been prepared by a related, albeit significantly more complex method that makes use of costly palladium catalysts and involves several more steps than analogous procedures for the other nucleosides [32]. Briefly, 2,6-dichloropurine is prepared from N-nitroinosine by treatment with HO¹⁵NH₃⁺Cl⁻ and NaOAc to reveal N-hydroxyinosine followed by chlorination in the presence of POCl₃ and 2,6-lutidine. Sequential couplings in the presence of palladium catalysts replace the chloride ions, first with benzyl alcohol, which preferentially replaces Cl-6, and then with benzamide, which replaces Cl-2. Finally, removal of the benzyl group from O-6 using hydrogenation gave a 2-N protected [1-¹⁵N]guanosine in good yield (Scheme 9).

Labeling for Ribosome Studies

In an effort to interrogate the mechanism of peptidyl transfer in the ribosome using isotope effects, Strobel and co-workers recently synthesized a number of tRNA analogues enriched

for heavy atoms [35-38]. Peptidyl transfer involves nucleophilic attack of the α -amino group of the aminoacyl-tRNA in the A-site on the carbonyl carbon of the peptidyl-tRNA in the P-site to form a tetrahedral intermediate. As the intermediate resolves to form the new peptide bond, the bond connecting the peptidyl-tRNA through the 3'-oxygen breaks, releasing the P-site tRNA. The 2'-hydroxyl on the peptidyl tRNA had been proposed to participate in proton transfer from the nucleophilic amine to the 3'-oxygen leaving group. Therefore, investigation of this mechanism through isotope effects required synthesis of isotopologues with heavy atom enrichment at the α -amino nitrogen in the aminoacyl-tRNA and at the 2' and 3'-oxygen, carbonyl carbon and α -hydrogen in the peptidyl-tRNA (Scheme 10C)

Previous studies had revealed that tRNA docking, rather than chemistry, constituted the rate-limiting step of peptidyl transfer, so minimized A and P-site substrates were used that rendered the chemical step rate-limiting and allowed for a simplified synthesis of relevant isotopologues. The aminoacyl-tRNA analogue (CCA_N-Phe) was composed of a trinucleotide with a phenylalanine residue at its 3' terminus connected through a peptide, rather than ester, linkage (Scheme 10A) [35]. Synthesis began from commercially available ¹⁵N-enriched N-(9-fluorenylmethoxycarbonyl)-L-phenylalanine, which was coupled to puromycin aminonucleoside. Protection of the 5'-hydroxyl followed by coupling to a solid support via the 2'-hydroxyl allowed the addition of the remaining two nucleotides using standard phosphoramidite chemistry.

The peptidyl-tRNA analogue (CCAp_{cb}) (Scheme 10B) contained a trinucleotide linked through its 3' terminus to a short peptide followed by a long, flexible, biotinylated linker. Generally, the peptide portion and the adenosine were synthesized separately, coupled together and protected, allowing for addition of the remaining cytosines using standard phosphoramidite chemistry [38]. Isotopic enrichment at the 2'-oxygen position was achieved starting from arabinofuranosyladenine using cesium propionate as label source [39]. Nucleophilic attack at the 2' position results in inversion to the desired ribo-configuration and subsequent base hydrolysis reveals a 2'-hydroxyl. Enrichment of the 3'-oxygen was achieved through a related method using an adenosine derivative protected at the 2' and 5' positions. The compound was first activated for nucleophilic attack and then inverted to the arabino isomer with unlabeled cesium propionate. Following hydrolysis, the compound was again activated for nucleophilic attack with triflation and once again inverted to the ribo product with labeled cesium propionate (Scheme 11). In addition, phenylalanine derivatives bearing deuterium at the α position and ¹⁸O at the peptidyl position were also synthesized for measurement of isotope effects at those atoms [36].

With the relevant substrates in hand, Strobel and co-workers measured KIEs [40, 41] for the peptidyl transfer in the ribosome, providing unprecedented insight into the role of individual atoms in catalysis. An observed isotope effect near unity for the 3'-O leaving group suggested limited C-O bonding cleavage in the transition state. The significant normal primary effect observed for the carbonyl carbon combined with a large inverse secondary effect for the α -hydrogen (indicative of loss of hyperconjugation to the carbonyl π -bond) suggest a tetrahedral geometry for the carbon in the transition state. Perhaps most interestingly, a modest normal isotope effect for the nucleophilic nitrogen and a KIE near unity for the 2'-O suggested that deprotonating of the nucleophile and nucleophilic attack

both occurred in the rate-limiting step. They interpreted these data as a two-step mechanism that proceeds with a rate-limiting step involving coordinated deprotonation and nucleophilic attack to form a negatively charged tetrahedral intermediate that rapidly breaks down to form the products. This mechanism involving concerted proton transfer is supported by recent crystallographic evidence [42].

Nucleotide Labeling for Other Studies

Isotope effect studies on glycosidic bond cleavage require substrates enriched at either the N9 position for purines or the 1- position of pyrimidines. Adenine labeled at the 9-N position has been synthesized by treatment of 5-amino-4,6-dichloropyrimidine with labeled ammonia to give a precursor of 6-chloropurine (Scheme 12) [43]. Following formylation in the presence of diethoxymethyl acetate and amination with aqueous ammonia, [9-¹⁵N]adenine is formed. Subsequent glycosylation gave the corresponding nucleoside. The synthesis of [9-¹⁵N]guanine might be achieved from [9-¹⁵N]6-chloropurine by chlorination to form [9-¹⁵N]2,6-chloropurine then sequential coupling following Vilarassa's method to generate guanine [32]. Uracil labeled at both nitrogen positions has been synthesized in a single step from [¹⁵N₂]urea (Scheme 13B) [44]. Conversion to [¹⁵N₂]cytosine has been achieved through by the well established method [22] of treatment with tetrazole and subsequent amination [45]. [¹⁵N₂]pyrimidines are also commercially available (Cambridge Isotope Laboratories).

KIE studies of the hydrolysis of glycosidic bonds represent some of the earliest applications of isotope effects to the study of nucleic acid chemistry [46, 47]; however, these studies relied on measurements of isotope effects on the 1' carbon and hydrogen/deuterium atoms. More recently, [9-¹⁵N]adenine has been employed to glean information about the mechanism of glycosidic bond cleavage under a variety of conditions [48, 49]. For example, Schramm and co-workers interrogated the transition state of glycosidic bond cleavage by a number of purine nucleoside phosphorylases (PNPs) by measuring KIEs for 1'-H, 1'-C and 9-N atoms of purine ribonucleotides. Their study revealed that human, bovine and *P. falciparum* PNP-catalyzed reactions all proceed with similar extents of bond cleavage in the transition state.

In an effort to interrogate the mechanism of adenosine deamination, [6-¹⁵N]adenosine has been synthesized [50] (Scheme 13A). From 4-nitroimidazole, nitration and subsequent nucleophilic with potassium cyanide (bearing a label at either the carbon, nitrogen, or both) gave 5-nitroimidazole-4-carbonitriles. Reduction of the nitro group and cyclization yielded adenosine labeled at 6-N 6-C or both. Alternatively, [6-¹⁵N]adenosine can be readily synthesized through the amination of inosine in the presence of labeled ammonia. [6-¹⁵N] and [1-¹⁵N]adenosine were employed to measure isotope effects for orthologous of adenosine deaminase [50].

Conclusion and Outlook

We have assembled a collection of heavy atom labeled nucleosides currently available from known chemical syntheses. These molecules enable direct analysis of transition states using heavy atom isotope effects for my involving nucleic acids. It should be noted that we

presented this collection of nucleic acid analogs from a perspective of RNA catalysis. Consequently, we excluded methodologies for heavy atom nucleosides that have no obvious application for RNA catalysis related isotope effect studies including [7-¹⁵N] purines [51]. However, there exist many nucleic acid modifying enzymes that play essential roles in biology for which mechanistic enzymology has so far provided only limited understanding. Even fewer enzymes have had their transition state characterized by KIE analysis. Moreover, we lack, for most enzymes, quantitative information regarding how enzymatic transition states differ from the corresponding non-enzymatic transition state. This information, which is accessible only by isotope effect analysis, holds fundamental insights into how active sites work to facilitate chemical reactions.

Importantly, KIE analysis need not be restricted to enzymes and substrates in their natural form, but can be used in conjunction with site-specific modifications at the residue or atom level. On one hand, KIE analysis provides a means to characterize the transition state of chemically modified substrates frequently used in structure-function analysis such as substrates bearing sulfur substitutions in the non-bridging or leaving group positions. For example, we and others have used RNA substrates containing 3'-S and 5'-S-phosphorothiolate linkages to investigate the chemical mechanisms of ribozymes [15]. Understanding the intrinsic effect of the sulfur modification on the reaction transition state would empower deeper mechanistic analysis. On the other hand, analysis of KIEs can help determine whether an alteration to an enzyme or substrate affects catalysis directly by perturbing the transition state or indirectly by perturbing a non-chemical step. While classical site-directed mutagenesis studies are hampered by ambiguity in the interpretation of how alterations to the enzyme-substrate systems affect rate, isotope effect analysis can help to clarify whether such modifications impact catalysis directly, and if so, how they alter a reaction's transition state. In this vein, KIE analysis in conjunction with mutation provides a powerful means to test hypothesis about catalysis. For example, the effect of a mutation on the KIEs for the leaving group and nucleophile can provide insight as to whether a particular group acts as a general acid as a defective general acid would be expected to facilitate a more associative transition state [52].

In recent years, comparative enzymology has emerged as an important paradigm for deepening our understanding of biological catalysis and molecular evolution. Values for KIEs in various enzymes will provide fundamental traits for comparison of homologous and non-homologous enzymes catalyzing similar reactions on nucleic acids [48, 49]. We anticipate such comparisons between RNA and protein enzymes will be especially informative.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Application of isotopically labeled nucleotides for kinetic isotope effect analysis
- State-of-the-art synthetic methods for site-specifically labeled nucleotides
- Survey of recent isotope effect studies employing labeled nucleotides
- Potential applications for investigation of RNA catalysis with isotope effects

Scheme 1.

Putative mechanism nucleolytic cleavage by VS ribozyme [16]. Heavy atoms for which measured isotope effects would aid in the determination of the transition state structure are highlighted in red.

Scheme 2.

Conversion of 2,2'-cyclouridine to [2'- ^{18}O]uridine [19].

Scheme 3.

Synthesis of a [3'-¹⁸O]2'-azidouridine [21] and a potential synthesis of [3'-¹⁸O]uridine following the ring-opening methodology of Dai *et. al.* [19]. (TBDMS-Cl = *tert*-butyldimethylsilyl chloride; TMEDA = tetramethylethylenediamine, DMF = dimethylformamide)

Scheme 4.

Synthesis of $[5' - ^{18}\text{O}]$ guanosine [24]. (MsCl = methanesulfonyl chloride)

Scheme 5.

Two different methods for incorporating ^{18}O at the non-bridging phosphate positions. A) Addition of phosphoryl chloride to generate 5'-nucleotides [25]. B) Incorporation of ^{18}O during the oxidation step of solid-state oligonucleotide synthesis (DMTr = 4,4-dimethoxytrityl; TBDMS = *tert*-butyldimethylsilyl)

Scheme 6.

Stereospecific synthesis of $[\alpha\text{-}^{18}\text{O}]\text{-ADP}$ [28]. Reaction proceeds with inversion of the α -phosphorotioate to give the labeled α -phosphate with the opposite stereochemistry.

Scheme 7.

General scheme for imino labeling strategy employed by Vilarassa and co-workers [29].

Variations of this method have been used to generate all five nucleosides enriched for ^{15}N at the imino position.

Scheme 8.

Synthesis of [3- ^{15}N] Uridine phosphoramidite. [34]. General procedure developed by Vilarassa, but reagents and yields taken from the optimized synthesis developed by Hrdlicka. (DMTrCl = 4,4-dimethoxytrityl chloride; DMAP = 4-dimethylaminopyridine)

Scheme 9. Synthesis of [1-¹⁵N]-guanosine from inosine [32].

The A-Site and P-Site (A and B, respectively) substrates employed for isotope effect studies of ribosome catalyzed peptidyl transfer. C) Mechanism of peptidyl transfer catalyzed by the ribosome as indicated by kinetic isotope effects [40, 41]. Atoms for which isotope effects were measured are highlighted in red.

Scheme 11.

Synthesis of [3'-¹⁸O]-adenosine [38] for use in measuring isotope effects in peptidyl transfer. (TfCl = trifluoromethanesulfonyl chloride)

Scheme 12.
Synthesis of [9- ^{15}N]-deoxyadenosine [43].

Scheme 13.

A) Synthesis of [6-¹⁵NH₃]adenine [47] and B) synthesis of [¹⁵N₂]uracil [44]. (DHP = dihydropyran ; *p*-TsOH = *para*-toluenesulfonic acid ; TFA = trifluoroacetic acid; TEA = triethylamine)