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The “Speedy” Synthesis of Atom-Specific ^{15}N Imino/Amido-Labeled RNA

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Abstract

Although numerous reports on the synthesis of atom-specific ^{15}N -labeled nucleosides exist, fast and facile access to the corresponding phosphoramidites for RNA solid-phase synthesis is still lacking. This situation represents a severe bottleneck for NMR spectroscopic investigations on functional RNAs. Here, we present optimized procedures to speed up the synthesis of $^{15}\text{N}(1)$ adenosine and $^{15}\text{N}(1)$ guanosine amidites, which are the much needed counterparts of the more straightforward-to-achieve $^{15}\text{N}(3)$ uridine and $^{15}\text{N}(3)$ cytidine amidites in order to tap full potential of $^1\text{H}/^{15}\text{N}/^{15}\text{N}$ -COSY experiments for directly monitoring individual Watson–Crick base pairs in RNA. Demonstrated for two preQ₁ riboswitch systems, we exemplify a versatile concept for individual base-pair labeling in the analysis of conformationally flexible RNAs when competing structures and conformational dynamics are encountered.

Keywords

NMR spectroscopy; nucleosides; oligonucleotides; phosphoramidites

RNA Structure and Folding—NMR Spectroscopy

The complex, three-dimensional structure of folded RNA can be divided into three levels of organization: primary, secondary, and tertiary structure.[1] The primary structure refers to the line-up of the four canonical nucleotides and can be read from the gene encoding the RNA. The secondary structure is the two-dimensional pattern represented by all Watson–Crick and wobble base pairs as well as the unpaired regions. Common secondary structure motifs include duplexes, single-stranded regions, hairpins, bulges, internal loops, and junctions. The tertiary structure is made up of interactions between distinct secondary structure elements and defines the global architecture of the RNA. Prominent tertiary structure motifs comprise pseudoknots, triplexes, ribose zipper, loop-loop interactions, and many more.[1]

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The energies involved in the formation of secondary structures are larger than those involved in tertiary interactions. Tinoco et al. postulated a useful although oversimplified model of the hierarchical nature of RNA structure. The primary sequence determines the secondary structure which, in turn, determines its tertiary folding in a sequential manner, only minimally altering the secondary structure.[2] The hydrogen-bond interactions of Watson–Crick base pairs are—beside nucleobase stacking and metal-ion interactions—the main driving force for the formation of stable RNA structures. NMR spectroscopy offers the unique possibility to study these interactions in solution with simple one-dimensional ^1H NMR experiments, whereby the amido/imino proton region between 9 and 15 ppm directly reflects nucleobase pairing in the RNA.[3–6] More sophisticated experiments like $^1\text{H}/^{15}\text{N}/^{15}\text{N}$ (HNN) COSY experiments, which require ^{15}N -labeled RNA, allow for observing the hydrogen bonds in Watson–Crick base pairs directly by internucleotide $^2J_{\text{NN}}$ couplings.[7, 8] In general, NMR spectroscopy has become a powerful tool for studying nucleic acids in order to obtain information not only about their solution structure.[51] but additionally on their structural dynamics, their folding, and interactions with diverse binding partners that range from small organic molecules to large proteins.[6,9]

Why Atom-Specific Labeling of RNA?

Labeling of nucleic acids with stable isotopes (e.g., ^{13}C , ^{15}N) is crucial to elucidate the structure and dynamics of nucleic acids by NMR spectroscopic means. Usually, RNA labeling is accomplished by in vitro transcription of uniformly ^{13}C - and/or ^{15}N -containing nucleoside triphosphates, which are prepared from *Escherichia coli*[10] or *Methylophilus methylotrophus*[11] grown on ^{13}C - and/or ^{15}N -isotopically enriched media. Another approach to introduce stable isotopes into RNA is the incorporation of labeled nucleoside phosphoramidites into solid-phase synthesis.[12–17] Chemical synthesis offers the unique possibility to introduce a labeled nucleoside *anywhere* into the sequence. Out of this, the major strength of atom-specific labeling arises, namely to directly monitor the region of interest. This approach not only resolves the problem of signal assignment, but also offers a clear starting point for the experimental evaluation of a proposed molecular interaction. Utilizing selective-labeling concepts allows for spotting the proposed interaction without distraction and/or impairment by interfering signals of comparable intensities. This strategy is valuable for probing the RNA under various aspects, such as dynamic base pair and/or mismatch formation, secondary/tertiary structure folding and unfolding, small-molecule recognition, protein binding, and many more scenarios that all cover structure and structural dynamics. The length of RNA that can be achieved by solid-phase synthesis is currently limited to a maximum of about sixty nucleotides; however, many recently discovered functional RNAs, for instance, riboswitches and ribozymes (twister) fall into that size regime.[18,19] Therefore, efficient synthetic pathways to atom-specifically labeled nucleoside phosphoramidites become of increasing importance and will be key for meaningful NMR spectroscopic studies that pave the way towards a thorough understanding of these RNA's function and the underlying structural dynamics.

Synthetic Approaches to ^{15}N Imino/Amido-Labeled Ribonucleoside Phosphoramidites

A comprehensive earlier work by Pitsch and co-workers[20] describes selectively ^{15}N -labeled 2'-*O*-{[(triisopropylsilyl)oxy]methyl} (TOM)-protected ribonucleoside phosphoramidites. In their approach, the authors started from the fully protected 3'-OH precursors of 5'-*O*-DMT-2'-*O*-TOM ribonucleosides phosphoramidites (DMT = 4,4'-dimethoxytrityl).[21] This concept is elegant and cost-effective because the synthetically challenging and low-yielding 2'-*O*-protection is already present prior to ^{15}N introduction. Moreover, this concept has the advantage that the 2'-*O*-TOM group makes all follow-up intermediates lipophilic and convenient for standard workup procedures. However, a drawback is the limited commercial availability of the highly functionalized starting material. Another drawback arises for the purine nucleoside series because the appropriate precursors for the introduction of the ^{15}N isotope (from cost-effective sources) at the N(1) position are those with electron-withdrawing groups next to it. Therefore, most ^{15}N -labeling protocols for guanosine and adenosine start from inosine. Unfortunately, the pronounced reactivity of the lactam moiety of inosine and no satisfying protection of this functionality makes a direct introduction of the 5'-*O*-DMT and 2'-*O*-TOM protecting groups cumbersome. To solve this problem, Pitsch and co-workers transformed *N*-acetyl-5'-*O*-DMT-2'-*O*-TOM adenosine into 5',3'-*O*-acetyl-2'-*O*-TOM-inosine in a four-step reaction sequence. Starting from adenosine, the $^{15}\text{N}(1)$ -labeled adenosine phosphoramidite building block was prepared in a reaction sequence of 16 steps and $^{15}\text{N}(1)$ -labeled guanosine phosphoramidite in 21 steps.[20] For this reason, we aimed at a more step-economic preparation, in particular, for the purine nucleoside building blocks by introducing the required protecting groups for RNA solid-phase synthesis at late stage of the synthesis.

A convenient way to introduce the ^{15}N isotope proceeds through a ring-opening/ring-closing procedure that was first described by Vilarrasa and co-workers.[22] Thereby, a nitrogen in an imide-like substructure (e.g., uridine, inosine) is activated by a strong electron-withdrawing group to facilitate a nucleophilic attack by ^{15}N ammonia. The subsequent nucleobase transformations to the desired nucleobase are the most challenging aspect if this route is pursued.

Other approaches to prepare isotope-labeled nucleoside building blocks for RNA synthesis start with the assembly of the nucleobase and subsequent attachment of the sugar moiety. These have been extensively studied by Jones and co-workers,[14,23–27] thereby combining chemical and enzymatic methods. The isotope labels are introduced by means of a de novo synthesis of the nucleobases, which are then coupled to the respective ribose (or deoxyribose) through an enzymatic transglycosylation reaction.[28] Pyrimidine nucleosides are used as pentosyl donors and a combination of two enzymes (uridine phosphorylase and purine nucleoside phosphorylase) exchanges the nucleobase to yield the respective isotope-labeled purine ribonucleoside. Another chemical possibility to attach the sugar to the nucleobase can be achieved under Vorbrüggen conditions[29] coupling 1'-*O*-acetyl-(2',3',5'-*O*-tribenzoyl)- β -D-ribofuranose and the isotope-labeled nucleobase using Lewis acids like

trimethylsilyl trifluoromethanesulfonate as highly selective catalysts for the formation of 1'-*N*- β -glycosidic bonds.[17,30]

Recommended Synthetic Route for $^{15}\text{N}(1)$ -Adenosine Phosphoramidite

Our preferred synthetic route to $^{15}\text{N}(1)$ -labeled adenosine phosphoramidite **A7** starts from acetylated inosine **A1** that undergoes N(1) nitration (**A2**) and is subsequently substituted with in situ generated $^{15}\text{NH}_3$ according to Vilarrasa and coworkers to furnish **A3** (Scheme 1).[22] These key steps of nitration are performed with nitronium trifluoroacetate at -20°C ; however, the limited stability of 2',3',5'-*O*-triacetyl-1-nitro inosine **A2** precludes the purification by column chromatography for preparative scales exceeding 1 mmol. For this reason compound **A2** is crystallized from methanol[31] and subsequently reacted with $^{15}\text{NH}_4\text{Cl}$ and KOH in $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{NEt}_3$. This reaction proceeds much faster than with the corresponding uridine counterpart (see below; **U2**) and without concomitant loss of the acetyl protecting groups. The most challenging task of this synthesis is the base transformation from inosine to adenosine. Transformations either involve the preparation of 6-chloropurine[23,32] or 6-(3-nitro-1*H*-1,2,4-triazol-1-yl)purine[15,33] and subsequent substitution with NH_4Cl under harsh alkaline reaction conditions are incompatible with our preferred transient acetyl protection.

Therefore, we recommend to activate the carbonyl group in a different manner following a mild procedure originally developed by Wan and co-workers[34–36] for the activation of cyclic amides with (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP). Thereby, *O*-(benzotriazol-1-yl)inosine is formed which can be directly substituted through a nucleophilic displacement ($\text{S}_{\text{N}}\text{Ar}$) reaction (Scheme 2). These compounds can either be isolated[37] and used as convertible nucleosides after incorporation into DNA through solid-phase synthesis or directly derivatized with an appropriate electron-rich nucleophile.[34–36] For our route (Scheme 1), we use lithium azide to substitute the benzotriazolyl inosine derivative. In the presence of Cs_2CO_3 , the reaction proceeded smoothly in DMF in high yields.

It is worth noting that the reaction was also tested with 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) or *N,N*-diisopropylethylamine. Both base reagents worked well for unlabeled 2',3',5'-*O*-triacetyl inosine; however, when ^{15}N -labeled **A4** was used for the substitution reaction about 30% of the ^{15}N -label was substituted with ^{14}N , as witnessed by the $^2J_{\text{NH}}$ coupling with H(C2). Also of note, our attempts to transform 2',3',5'-*O*-*t*-BDMS-protected (*t*-BDMS = *tert*-butyldimethylsilyl) inosine into the corresponding 6-azido ribofuranosyl purine failed.

For the transformation of **A4** into **A5**, we developed a one-step reductive acylation path using thioacetic acid that was inspired by Rosen and co-workers.[38,39]

We observed that compound **A4**—when dissolved in four equivalents of thioacetic acid and stirred at room temperature overnight—was simultaneously reduced and acylated at *N*-C(6) (**A5**) in 50% yield. The major side-product was 2',3',5'-*O*-triacetyl adenosine (**A5 b**). Williams and co-workers previously found that the addition of 2,6-lutidine significantly accelerates the reaction rate for this type of reductive acetylation and postulated a reasonable

mechanism involving a thiatriazoline intermediate (Scheme 2).[40] Our own further optimization focused on different solvents for this transformation and indicated that the byproduct was repressed through decreased solvent polarity. When dichloromethane is applied, the reductive acetylation of **A4** into **A5** proceeds in a satisfying yield of 83 %. Of note, the byproduct **A5b** can be collected and acetylated at the exocyclic amino group in straightforward manner to further increase the yield of **A5**.

Subsequent deprotection of the ribose acetyl groups with NaOH in water/ethanol at 4 °C proceeds straightforward and furnishes *N*⁶-acetyl adenosine **A6**, which represents the starting point for the functionalization with the appropriate protecting groups required for RNA solid-phase synthesis. Compound **A6** is tritylated and *t*BDMS-protected according to Ogilvie[41] with silver nitrate in rather low stereoselectivity (about 3:2), but with base-induced equilibration the overall yield is increased to 65 %. Of note, applying the Beigelman 5',3'-silyl clamp[46] for introduction of the 2'-*t*BDMS group (see the synthesis of ¹⁵N-labeled cytidine phosphoramidite below) on already acetylated adenosine did not result in higher overall yields for **A6**. Phosphitylation of **A6** furnishes phosphoramidite **A7** in an overall yield of 15% over 10 steps. Alternatively, the corresponding 2'-*O*-TOM-protected phosphoramidite is prepared according to Pitsch and co-workers.[15]

Recommended Synthetic Route for ¹⁵N(1)-Guanosine Phosphoramidite

Synthetic routes to ¹⁵N-labeled guanosine phosphoramidite often make use of the Dimroth rearrangement of ¹⁵N(6)-labeled 1-alkoxy adenosines to prepare ¹⁵N(1)- as well as other ¹⁵N- and ¹⁵N, ¹³C-labeled guanosine derivatives.[20,23,24]

For ¹⁵N(1)-labeled guanosine phosphoramidite **G7** (Scheme 3), we prefer the synthesis in analogy to a procedure that was originally developed by Vilarrasa and co-workers.[43] This path makes use of the electrophilic reactivity of 2',3',5'-*O*-triacetyl-1-nitroinosine **A2** for the key step of incorporating the ¹⁵N isotope. Compound **A2** readily reacts with ¹⁵N-hydroxylamine under the formation of ¹⁵N-hydroxyinosine **G1**, which is a suitable precursor for the transformation into 2,6-dichloropurine ribonucleoside **G2**. [43,44] Then, instead of performing sequential palladium-catalyzed C—O and C—N couplings on the heteroaryl dichloride as conducted by Vilarrasa,[43] we recommend to prepare 2,6-diaminopurine ribonucleoside for the enzymatic deamination to guanosine using adenosine deaminase (ADA). Compound **G2** is quantitatively converted to 2,6-diazidopurine derivative **G3** using sodium azide in refluxing ethanol/water.[45] Subsequent reduction with palladium on charcoal in dimethylacetamide (DMA)/ethanol (**G4**) and deprotection of the ribose acetyl protecting groups with methylamine in ethanol furnished ¹⁵N(1)-diaminopurine **G5** in high yield. The enzymatic deamination of **G5** into the guanosine derivative **G6** proceeded in four days using ADA (type X from calf spleen 20 units mmol⁻¹) in phosphate buffer.

After simultaneous silylation of the 5'- and 3'-OH groups with di-*tert*-butylsilyl bis(trifluoromethanesulfonate), the remaining 2'-OH is silylated with *t*BDMS-Cl,[46] followed by introduction of the dimethylamidine protecting group at the exocyclic NH₂ group of the nucleobase using the reagent *N,N*-dimethylformamide dimethyl acetal. Removal of the strained cyclic silyl protection group proceeded straightforwardly upon

treatment with hydrogen fluoride in pyridine. Standard tritylation and phosphitylation gave the $^{15}\text{N}(1)$ -labeled guanosine phosphoramidite **G7** in an overall yield of 9% in 15 steps starting from inosine. Alternatively, the corresponding 2'-*O*-TOM-protected phosphoramidite can be prepared according to Pitsch and co-workers, starting from **G6**.^[15] Of note, the introduction of the TOM group to the 2'-*O* position of guanosine is highly selective, and hence, a very efficient synthetic alternative in combination with N(2)-acetyl protection.^[15,21]

Recommended Synthetic Route for $^{15}\text{N}(3)$ -Uridine Phosphoramidite

Our preferred synthetic route to $^{15}\text{N}(3)$ -labeled uridine phosphoramidite **U5** starts from the *t*BDMS-protected uridine **U1** (Scheme 4). Activation of the N(3)-nitrogen is achieved with in situ generated nitronium trifluoroacetate ($\text{NO}_2\text{OCOCF}_3$) to yield the *N*-nitro derivative **U2** under strictly anhydrous conditions. Treatment of **U2** with $^{15}\text{NH}_4\text{Cl}$ in the presence of KOH and Et_3N in acetonitrile/water for three weeks results in the incorporation of the ^{15}N label (**U3**). Subsequently, the silyl groups are conveniently cleaved with a polymer supported fluoride reagent (Amberlite IRA 900) in toluene to provide **U4** in excellent yield and purity without the requirement for chromatographic purification. After tritylation under standard conditions, selective silylation with *t*BDMS-Cl using silver nitrate according to Ogilvie^[41] yields the 2'-*O*-*t*BDMS-protected derivative in remarkable regioselectivity over the 3'-*O*-silylated product (5:1). To increase the overall yield even further, the undesired 3'-*O*-silylated regioisomer is treated with triethylamine in methanol, and equilibration generates again a favorable distribution between the 2'- and 3'-regioisomers. Phosphitylation was executed with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (CEP-Cl) in the presence of *N,N*-diisopropylethylamine in THF. Starting from uridine, phosphoramidite **U5** was isolated in an overall yield of 22% over seven steps. Alternatively, the corresponding 2'-*O*-TOM-protected phosphoramidite can be prepared according to Pitsch and co-workers, starting from **U4**.^[15]

Of note, we also investigated alternative hydroxyl protection to achieve the $^{15}\text{N}(3)$ -labeled uridine phosphoramidite **U5**. However, although 2',3',5'-*O*-triacetyl-3-nitro uridine derivative reacts faster with $^{15}\text{NH}_4\text{Cl}$ compared to the *t*BDMS-protected counterpart, partial loss of the acetyl groups during this reaction makes workup, purification, and isolation significantly more laborious in comparison to the above route.

We should also mention that Hrdlicka and co-workers recently published a route for incorporation of the ^{15}N label at a late step of the synthesis.^[47] Inspired by the route from Pitsch who transformed 3-nitrated, 5'-*O*-dimethoxytritylated, and 2'-*O*-TOM-protected uridine with $^{15}\text{NH}_4\text{Cl}$ under basic conditions, Hrdlicka and co-workers tested the applicability of the corresponding 2'-*O*-*t*BDMS-protected derivative for this transformation. Not surprisingly, they observed partial migration of the 2'-*O*-*t*BDMS group to the 3'-*O* position. In our hands, we additionally experienced partial loss of 5'-*O*-DMT group that limited the overall yields even more.

Recommended Synthetic Route for $^{15}\text{N}(3)$ -Cytidine Phosphoramidite

Our preferred route to $^{15}\text{N}(3)$ -labeled cytidine phosphoramidite **C3**^{N3} starts from *t*-BDMS-protected $^{15}\text{N}(3)$ uridine **U3** (Scheme 4, Scheme 5). This derivative was treated with 2,4,6-triisopropylbenzenesulfonyl chloride in the presence of triethylamine and DMAP in dichloromethane to give regioselective *O*⁴-trisylation. After workup, the trisylated intermediate (**U3**·**Tris**) can be used without further purification and is directly converted into the protected $^{15}\text{N}(3)$ cytidine derivative **C1**^{N3} by using aqueous ammonium hydroxide in THF. We point out that the corresponding double-labeled $^{15}\text{N}(3), ^{15}\text{N}(\text{C}4)$ cytidine **C**^{N3/N4} (see the Supporting Information) is conveniently accessible from the trisylated intermediate (**U3**·**Tris**) as well, when first substituted by 2-nitrophenol under the formation of *O*⁴-(2-nitrophenyl)uridine (**U3-o-NO₂Ph**) followed by treatment with $^{15}\text{NH}_4\text{Cl}$ and K_2CO_3 in DMSO in a pressure reactor, in analogy to a procedure by Chattopadhyaya and co-workers. [17] The *t*-BDMS protecting groups are conveniently cleaved with a polymer-supported fluoride reagent (Amberlite IRA 900) in boiling toluene to provide **C2** in excellent yield and purity without the requirement for chromatographic purification.

For the attachment of the necessary protecting groups for solid-phase synthesis, we recommend the Beigelman route.[46] Simultaneous protection of 3'- and 5'-hydroxyl with the di-*tert*-butylsilylene protecting group, subsequent silylation of the 2'-hydroxyl using *t*-BDMS-Cl and acetylation of the exocyclic amino group proceeded in excellent yields. Removal of the strained cyclic silyl protection group proceeded straightforwardly using hydrogen fluoride in pyridine. This procedure gives significantly higher yields for 2'-*O*-*t*-BDMS protection compared to the more widely used Ogilvie procedure,[41] which results in an unfavorable regioselectivity of 4:3 between 3'- and the desired 2'-*O*-*t*-BDMS protection. Standard tritylation and phosphitylation gave ^{15}N -labeled cytidine phosphoramidite building block **C3**^{N3} in an overall yield of 16% over 10 steps, and ^{15}N double-labeled **C3**^{N3/N4} in an overall yield of 10% over 12 steps. Alternatively, the corresponding 2'-*O*-TOM-protected phosphoramidites can be prepared according to Pitsch and coworkers, starting from **C2**. [15]

Concerning the ribose hydroxyl protection for the synthesis of the pyrimidine nucleosides **U5**, **C3**^{N1}, and **C3**^{N1/N4}, it is worth noting that the *t*-BDMS groups withstand the harsh basic conditions of in situ generated $^{15}\text{NH}_3$ very well. We prefer this protection to protection with acetyl groups that are partially cleaved during the extended time that is needed to complete ^{15}N insertions. Moreover, the protocol we developed for the deprotection of the *t*-BDMS groups based on the polymer supported fluoride reagent is much less labor-intensive compared to workup and purification protocols when acetyl protection is used.

Hydrogen-Bond Nucleobase Interactions Detected by NMR Spectroscopic Experiments

NMR spectroscopy allows the precise identification of base pairs in RNA based on the detection of hydrogen bonds involving amido protons (often coined "imino protons"), which are shielded from exchange with bulk water. Indirect evidence for hydrogen-bonds comes from NOE correlations of amido protons that establish base pairing in helical regions of

RNA,[4, 48] while direct evidence can be obtained from HNN-COSY experiments that were originally introduced by Dingley and Grzesiek.[7] The size of these cross hydrogen-bond scalar couplings ($^2J_{\text{NN}} = 6\text{--}7\text{ Hz}$) allows efficient magnetization transfer to correlate chemical shifts in base pairs.[7] To date, a large number of experiments are available to measure J -couplings across hydrogen bonds in nucleic acids.[49–58] A limitation, however, is that most of these approaches detect exchangeable protons. Hence, regions of stable secondary or tertiary structures are straightforward to determine, while weak base interactions are less readily detectable because of signal broadening due to proton exchange with bulk water and conformational dynamics, in particular at higher temperatures. Recently, the Sattler team introduced a new experiment termed BEST-sellr that provides a large sensitivity improvement for hydrogen-bond correlation experiments by the optimizations of magnetization transfers in combination with the detection of non-exchangeable protons.[59] This experiment enables the detection of weak and transient A–U base pairs. The novel developments in NMR spectroscopy of ribonucleic acids imply an increasing need for site-specifically (beside uniformly) ^{15}N -labeled RNA using optimized synthetic pathways as discussed above.

Conformational States of PreQ₁ Riboswitches Analyzed by Site-Specific ^{15}N -labeled Base Pairs

RNA secondary and tertiary structure predictions require experimental validation. Frequently, this is achieved by biochemical structure probing methods; however, the analysis becomes complicated if alternate structures and/or conformational dynamics are present, as for instance encountered by riboswitches.[60, 61] Riboswitches are important gene regulatory domains in the untranslated leader regions of mRNA that bind to small molecules in a highly selective manner. Binding of the ligand is accompanied by significant structural changes at the secondary and tertiary structure levels.[62–68] While the free riboswitch domains behave structurally flexible,[69] the ligand-bound complexes are rather rigid and often successfully crystallized for X-ray structure analysis.[66] Figure 1 depicts the secondary structure of a preQ₁ class-II riboswitch[70] (Figure 1 A, middle) and its X-ray structure (Figure 1 A, right) that was solved by Wedekind and co-workers.[71] Interestingly, a single-molecule-FRET study of this riboswitch revealed an uncoupled dynamic behavior of the stem P4 insertion and the pseudoknot interaction P3 (Figure 1 C).[72] Even in the ligand-bound form, two major populations were observed in solution and attributed to two conformers with different positioning of P4.[72] Likewise, the free RNA (with the same fluorophore labeling pattern) showed two major FRET-populations; however, it was less clear if the underlying two conformers possess both a tight pseudoknot P3 interaction. In this study, we therefore probed the P3 interaction with a single-labeled $^{15}\text{N}(3)\text{-U}:\text{}^{15}\text{N}(1)\text{-A}$ base pair and unequivocally observed base pair formation for *both* conformers based on the HNN-COSY experiments (Figure 1 B, right) suggesting that at low temperature (278 K) and at physiological Mg^{2+} concentrations P3 is closed on the NMR timescale for both conformers, even for the ligand-unbound RNA (Figure 1 B, middle).

We also point out that the hydrogen-bond readout of preQ₁ base occurs at its Watson–Crick face engaged in a *trans*-Watson–Crick/Watson–Crick base pair with the conserved base C30

located in J2-3.[71] Additionally, the conserved U21 recognizes with its Watson–Crick face the N2-N3-N9 edge of preQ₁ base. Application of ¹⁵N₃-(1,2,3) preQ₁ in the HSQC experiments confirmed binding, which was reflected by a new signal at 11.2 ppm arising from the solvent-shielded ¹⁵N(1)-H (Figure 1 B, right panel).

As a second example to demonstrate the value of individually labeled ¹⁵N base pairs in NMR experiments targeting flexible RNA folds, we investigated the preQ₁ class-I riboswitch from *Thermoanaerobacter tengcongensis* (Figure 2).[73–76] High-resolution structures exist of both, the free RNA and the preQ₁-bound RNA.[76] Strikingly, the major differences in the RNA between free and bound form concern the conformation of A14–C15.[76] In the free form A14 takes the position of the preQ₁ ligand and is stacked between the two base pairs of G11–C30 and G5–C16 (Figure 2 B).

This observation can be rationalized by a conformational rearrangement from a solvent-exposed base C15 to a flipped-in conformation of C15 that becomes the Watson–Crick pairing partner of the preQ₁ in the ligand-bound RNA complex. Importantly, in both X-ray structures, G11–C30 of the pseudoknot forms a Watson–Crick base pair.[69, 76] We therefore considered to probe the preformation of the pseudoknot in solution, using a single-labeled ¹⁵N(1)-G11:¹⁵N(3)-C30 pair (Figure 2A). In the ¹H-¹⁵N HSQC spectra of the free RNA, we observed three resonances for G11, two of them with a clear correlation to C30 in the HNN COSY spectra (Figure 2A). This observation suggests at least two major populations of conformers with a closed (pre-organized) pseudoknot in solution. It is tempting to propose that these two conformers in slow exchange represent on the one hand, the C15-exposed/A14 stacked conformer that is observed in the crystal structure of the free RNA, and on the other hand, a conformer that is structurally very close to the ligand-bound form.[69, 76] Interestingly, when lowering the temperature, one of the conformers was frozen out with a chemical shift value that exactly matched the chemical shift of the ligand-bound RNA at the same temperature (data not shown).

In an independent experiment, we probed the non-covalent ligand-RNA interaction in this preQ₁ class-I riboswitch using ¹⁵N-labeled preQ₁ base derivatives that we synthesized previously.[77, 78] PreQ₁ binds through formation of a Watson–Crick interaction to the highly conserved base C15; the preQ₁ base pairing to C15 is unequivocally confirmed by the corresponding correlation in the HNN COSY spectrum (Figure 2C). This basic set-up encourages mapping of binding sites[79–81] by using selectively ¹⁵N-labeled RNA in combination with ¹⁵N-labeled ligands to obtain precise structural information of their interactions in putative complexes, in particular when crystal structures are not available.

Concluding Statements

Isotope-labeled nucleoside phosphoramidites are an invaluable source to generate RNAs that comprise site-specific labeling patterns through solid-phase synthesis.[82] These RNAs are a prerequisite for thorough NMR spectroscopic investigations. They are not only required to resolve assignment problems due to the pronounced signal overlap encountered for large (uniformly labeled) RNA, but are also the basis for many recent NMR methodological advances in the determination of structural dynamics that underlie RNA function.

In this Concept article, we have focused on the four standard nucleosides with single ^{15}N -amido/imino labels at their Watson–Crick faces. In particular, the syntheses of the adenosine and guanosine phosphoramidites involve multiple steps, which has limited their widespread use so far. The routes presented here acknowledge previous work from numerous investigators and include our own synthetic developments to obtain highly optimized protocols with respect to time, practicability, robustness, and efficiency.

Finally, in a concept study, we have exemplarily demonstrated for two selected riboswitch classes how pursuing a single ^{15}N -labeled Watson-Crick base pair by NMR spectroscopic means contributes to analyzing the conformational flexibility of functional RNAs. In the light of constantly increasing numbers of newly discovered noncoding RNAs, the thorough understanding of their function has become a central theme in modern biochemical and biomedical research. The understanding of the underlying chemical principles requires multidisciplinary systemic approaches harnessing the power of organic synthesis, spectroscopy, and biophysics, as attempted here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Synthesis of $^{15}\text{N}(1)$ adenosine phosphoramidite building block **A7**: **a.** 2.25 equiv NH_4NO_3 , 4.5 equiv $(\text{CF}_3\text{CO})_2\text{O}$ in CH_2Cl_2 , -20°C , 60 min, 69%; **b.** 1.2 equiv $^{15}\text{NH}_4\text{Cl}$, 1.2 equiv KOH , 1.5 equiv NEt_3 in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, room temperature, 5 h, 91 %; **c.** i) 2 equiv benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), 2 equiv Cs_2CO_3 in DMF; ii) LiN_3 , 90%; **d.** 5 equiv thioacetic acid, 5 equiv 2,6-lutidine in in CH_2Cl_2 , reflux, 83%; **e.** i) 2M NaOH in $\text{EtOH}/\text{H}_2\text{O}$, 10 min, 4°C ; ii) DOWEX 50WX-8, 82%; **f.** i) 1.1 equiv 4,4'-dimethoxytrityl chloride in pyridine, room temperature, 8 h, 71%; ii) 1.6 equiv AgNO_3 , 1.7 equiv *tert*-butyldimethylsilyl chloride (*t*BDMS-Cl), 2.3 equiv pyridine in THF, room temperature, 6 h, 65% (after one round of 2',3' equilibration); iii) 3.5

equiv 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (CEP-Cl); 2 equiv *N,N*-diisopropylethylamine, room temperature, 3 h, 90%. ¹⁵N indicated in red.

Scheme 2.

Proposed mechanism for the activation of inosine with (benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate (BOP). Two independent studies[34,37] suggest that the reaction proceeds in a stepwise manner with in situ formation of the nucleoside phosphonium salt, which is readily displaced in an S_NAr -reaction by 1-hydroxybenzotriazole. The hydroxybenzotriazolyl oxy intermediate was directly transformed to 6-azidoribofuranosyl purine which exists in a dynamic azide–tetrazole equilibrium depending on the polarity of the solvent.[42] The reaction of azides with thioacetic acid most likely proceeds by means of [2+3] cycloaddition or a stepwise diazo transfer. Decomposition by a retro-[2+3] reaction leads to the desired amide, nitrogen and sulfur. [40] ^{15}N indicated in red.

Scheme 3.

Synthesis of $^{15}\text{N}(1)$ guanosine phosphoramidite building block G6: **a.** 1.4 equiv $^{15}\text{NH}_2\text{OH}\cdot\text{HCl}$, 2 equiv sodium acetate, in H_2O /acetonitrile, room temperature, 12 h, 93%; **b.** POCl_3 , 2,6-lutidine, 100 °C, 1 h, 48%; **c.** 2.5 equiv NaN_3 in $\text{EtOH}/\text{H}_2\text{O}$, 100 °C, 60 min, 84%; **d.** Pd/C in dimethylacetamide/ EtOH , room temperature, 17 h, 94%; **e.** CH_3NH_2 in EtOH , room temperature, overnight, quantitative; **f.** adenosine deaminase (ADA) in phosphate buffer/ DMSO , 4 d, 88%; **g.** i) 1.1 equiv di-*tert*-butylsilyl bis(trifluoromethanesulfonate) in DMF , 0 °C, 60 min; ii) 1.2 equiv *t*BDMS-Cl, 4.9 equiv imidazole, 60 °C, 2 h, 82%; iii) 2 equiv *N,N*-dimethylformamide dimethyl acetal in DMF , room temperature, overnight, 74%; iv) $\text{HF}\cdot\text{pyridine}$ in pyridine/ CH_2Cl_2 0 °C, 2 h, 84%; v) 1.1 equiv 4,4'-dimethoxytrityl chloride in pyridine, room temperature, overnight, 78%; vi)

2.5 equiv CEP-Cl; 10 equiv *sym*-collidine, 0.6 equiv *N*-methylimidazole in THF, room temperature, 1 h, 71 %. ¹⁵N indicated in red.

Scheme 4.

Synthesis of $^{15}\text{N}(3)$ uridine phosphoramidite building block U5: **a**. 2.3 equiv NH_4NO_3 , 4.5 equiv $(\text{CF}_3\text{CO})_2\text{O}$ in CH_2Cl_2 , 0°C , 90 min, 89%; **b**. 1.2 equiv $^{15}\text{NH}_4\text{Cl}$, 1.2 equiv KOH, 1.5 equiv NEt_3 in acetonitrile/ H_2O , room temperature, 3 weeks, 70%; **c**. Amberlite IRA 900 (fluoride form) in toluene, reflux, 4 h, 76%; **d**. i) 1.1 equiv 4,4'-dimethoxytrityl chloride in pyridine, room temperature, overnight, 71 %; ii) 1.6 equiv AgNO_3 , 1.7 equiv *t*BDMS-Cl, 2.3 equiv pyridine in THF, room temperature, 4 h, 75%; iii) 3.5 equiv CEP-Cl; 2 equiv *N,N*-diisopropylethylamine, room temperature, 3 h, 86%. ^{15}N indicated in red.

Scheme 5.

Syntheses of $^{15}\text{N}(3)$ cytidine phosphoramidite C3^{N3} and $^{15}\text{N}(3), ^{15}\text{N}(4)$ cytidine phosphoramidite C3^{N3N4} . Conditions for C3^{N3} : **a.** i) 2.5 equiv mesitylene sulfonylchloride in $\text{CH}_2\text{Cl}_2/\text{NEt}_3$, 3.5 h, ii) 32% aqueous NH_3 , in THF, room temperature, 16 h, 95%; for C3^{N3N4} : **a'** i) 2.5 equiv mesitylene sulfonylchloride in $\text{CH}_2\text{Cl}_2/\text{NEt}_3$, 1.5 h, ii) 4 equiv 2-nitrophenol, 1,4-diazabicyclo[2.2.2]octan (DABCO), DMAP, room temperature, overnight, 87%, iii) 4.5 equiv $^{15}\text{NH}_4\text{Cl}$, 4.5 equiv K_2CO_3 in DMSO, 80°C , 2 d, 67%. Conditions for both, C3^{N3} and C3^{N3N4} : **b.** Amberlite IRA 900 (fluoride form) in toluene, reflux, 4 h, 92%; **c.** i) 1.1 equiv di-*tert*-butylsilyl bis(trifluoromethanesulfonate) in DMF, 0°C , 40 min, ii) 1.1 equiv *t*BDMS-Cl, 4.2 equiv imidazole, 60°C , 2 h, iii) 2.4 equiv Ac_2O in DMF/pyridine; overnight, 60% (3 steps), iv) HF in pyridine/ CH_2Cl_2 , 2 h, v) 1.1 equiv 4,4'-dimethoxytrityl chloride in pyridine, room temperature, overnight, 66% (2 steps); vi) 3.5 equiv CEP-Cl, 2 equiv *N,N*-diisopropylethylamine, room temperature, 4 h, 73%. ^{15}N indicated in red.

Figure 1.

NMR spectroscopic probing of a single base pair in the pseudoknot stem P3 of the *L. rhamnosus* preQ₁ class-II riboswitch. Formation of P3 is critical for switching off translational gene expression. **A**) Secondary structure model (middle panel) with the ¹⁵N-labeled base pair (¹⁵N(3)-U:¹⁵N(1)-A) highlighted in red and X-ray structure[71] of the preQ₁ bound complex (right panel) by Liberman et al. in cartoon representation. **B**) ¹H-¹⁵N HSQC and *J*_{NN} HNN COSY spectra of the labeled RNA at 278 K. Conditions: *c*_{RNA} = 0.5 mM, 10 mM cacodylate buffer [Na-(CH₃)₂AsO₂·3H₂O], pH 6.5, temperatures as indicated;

$c_{\text{Mg}^{2+}} = 2.0 \text{ mM}$, $c_{\text{preQ1}} = 1 \text{ mM}$. C) Based on a recent single-molecule FRET study by Soulié et al.,[72] an uncoupled dynamic behavior of P4 positioning and pseudoknot formation was proposed for the preQ₁ class-II riboswitch (*B. subtilis*). The two populations were also found for the ligand-free RNA fold. NMR probing now shows that the two populations of the ligand-free RNA correlate with two conformers that both adapt a pseudoknot fold with a closed P3.

Figure 2.

NMR spectroscopic probing of the *T. tengcongensis* preQ₁ class-I riboswitch. Formation of P2 is critical for switching off translational gene expression. **A**) Secondary structure model (left) with the ¹⁵N-labeled base pair (¹⁵N(1)-G:¹⁵N(3)-C) highlighted in red. ¹H-¹⁵N HSQC and ¹J_{NN} HNN COSY spectra of the labeled RNA at 298 K. The free RNA adopts at least three conformers in slow exchange according to the three signals in the HSQC spectrum. Two of these conformers unequivocally comprise a closed pseudoknot P2 as evidenced by correlations observed in the HNN COSY spectra for the labeled Watson–Crick base pair. For

a detailed discussion see the main text. Conditions: $c_{\text{RNA}} = 0.5 \text{ mM}$, 10 mM cacodylate buffer $[\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O}]$, pH 6.5; $c_{\text{Mg}^{2+}} = 2.0 \text{ mM}$, $c_{\text{preQ1}} = 1 \text{ mM}$. **B)** Binding pockets of free (left) and preQ₁-bound RNA (right) as observed in the crystal structures. A14 “replaces” the ligand in the free form and makes a significant conformational change together with C15 while otherwise the overall folds of free and unbound RNA are very comparable. **C)** The non-covalent ligand-RNA interaction can be easily verified by HNN COSY experiments using ¹⁵N-labeled preQ₁ base derivatives (¹⁵N in red). This set-up encourages mapping of binding sites by using selectively labeled RNA in combination with labeled ligands to obtain precise structural information of their interactions in putative complexes when crystal structures are not yet available.