**Genetic Code Expansion Facilitates Position-Selective Labeling of RNA for Biophysical Studies**

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**Abstract:** Nature relies on reading and synthesizing the genetic code with high fidelity. Nucleic acid building blocks that are orthogonal to the canonical A-T and G-C base-pairs are therefore uniquely suitable to facilitate position-specific labeling of nucleic acids. Here, we employ the orthogonal kappa-xanthosine-base-pair for in vitro transcription of labeled RNA. We devised an improved synthetic route to obtain the phosphoramidite of the deoxy-version of the kappa nucleoside in solid phase synthesis. From this DNA template, we demonstrate the reliable incorporation of xanthosine during in vitro transcription. Using NMR spectroscopy, we show that xanthosine introduces only minor structural changes in an RNA helix. We furthermore synthesized a clickable 7-deaza-xanthosine, which allows to site-specifically modify transcribed RNA molecules with fluorophores or other labels.

**Introduction**

Over the last decade, an increasing number of functional roles has been identified for ribonucleic acids and their biophysical investigation is increasingly pursued. For a number of reasons, methods developed for proteins cannot easily be transferred for studies of RNAs. Thus, novel methods have to be developed, for example to attach labels that allow for detailed spectroscopic studies of RNA. Structural studies additionally require labels to be inserted in a site-selective manner to obtain position-specific readouts.

By chemical solid-phase synthesis, RNA with such position-specific label can be obtained. A variety of chemically modifiable building blocks has been made available over the last years. However, the use of solid phase synthesis brings along the well-known limitations and drawbacks of this technique; most importantly the limited product yield that decreases with the length of the RNA and the concomitant increase of by-products make purification of the target RNA difficult.

Several strategies to circumvent these limitations have been devised and successfully established in recent years. Many of these techniques use the standard approach of in vitro transcription to generate RNA, and employ an additional base pair that is orthogonal to the two Watson–Crick-like base-pairs that establish sequence specificity during transcription.[1–4]

Two main strategies have been previously reported: either larger hydrophobic moieties establish an entirely novel complementary system or the hydrogen bonding pattern of the standard purine and pyrimidine-based scaffolds are expanded. Both these strategies have generated additional, orthogonal base-pairs that can be employed to different degrees in in vitro applications or for genetic code expansion in vivo. Some of the designed bases have also been elegantly used for the incorporation of modifiers, that is, for nitroxide spin labels or fluorophores.[5–11]

Our focus for the current study was to use the capabilities of a labeling strategy employing one orthogonal DNA nucleotide together with the complementary ribonucleoside triphosphate to generate site-specifically modified RNA by in vitro transcription.

To introduce only minimal structural perturbation of the target RNA, we therefore opted to follow the strategy of modifying the hydrogen bond arrangement of the near canonical kappa nucleobase, which would also be sterically similar to the natural nucleobases and provide comparable stacking interfaces. Based on the work initiated by Piccirilli et al.,[4] we first developed a novel synthesis route for the kappa nucleoside, and incorporated the kappa nucleoside phosphoramidite into a DNA used as a template for RNA transcription. We successfully incorporated xanthosine at this position with high specificity...
and good efficiency. We introduced xanthosine into a sizeable RNA riboswitch and could site-specifically detect the introduced NMR-active label. We furthermore implemented novel covalent labeling options by attaching a modifier to the xanthosine-based nucleobase, focusing on a click chemistry compatible derivative.

Results and Discussion

The steps required here to obtain a site-specifically labeled RNA can be described as follows: (i) Synthesis of the kappa base (and DNA synthesis), (ii) transcription, (iii) quality control, and (iv) labeling.

Synthesis of kappa base phosphoramidite

Our synthetic strategy to obtain the kappa deoxynucleoside (Figure 1) incorporated a number of novel synthetic developments. Most importantly, we opted to synthesize the C–C glycosidic bond using a Heck coupling, which required generation of the glycal intermediate 4 (Figure 2).[12,13] In addition, formation of the protected amine ring substituents 9 was achieved using a palladium-catalyzed Buchwald–Hartwig coupling.[14]

To this end, we started with deoxythymidine 1, which was protected using tert-butyldimethylsilyl chloride (TBDMSCl) to yield 2. The elimination of the nucleobase to obtain the double silylated glycal 3 turned out to be experimentally demanding due to the instability of 3 and the required purification. The following deprotection was achieved with 1.0 M TBAF in THF solution. Here, both reaction monitoring and column purification had to be extensively optimized to reach a yield of 88 %. We assume that selectivity of the deprotection of C5 was limited, the yield of 4 therefore could not be optimized beyond 45 %; we could, however, not fully characterize the side products. As the double silylated glycal presumably cannot undergo the following Heck reaction,[15] it is necessary to block the bottom of the glycal 4 by the sterically demanding TBDMSCl-group to avoid the attack from this side which would result in the formation of the wrong α-anomer.

One of the key reactions of our Scheme was the palladium-catalyzed Heck reaction. The Heck reaction was applied to nucleoside chemistry by Holec et al. and Wagenknecht et al.[14–17] To perform this reaction, the commercially available 2,4-diaminopyrimidine phosphoramidite 12 was achieved using sodium triacetoxyborohydride (62 % yield) in THF solution. AcOH, 0 °C, 2 h, 37 % over two steps; i. sodium triacetoxyborohydride, AcOH, MeCN, 0 °C, 15 min, 70 %; g. imidazole, TBDMS-Cl, DMF, RT, 2 h, 76 %; h. Pd (PhF3)$_2$CO, C$_5$H$_5$N$_2$CO, C$_2$H$_5$OH, DMF, RT, 24 h, 37 %; i. N$_2$N-diisopropylethylamine, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DCM, RT, 2 h, 74 %.

![Figure 1. Examples of unnatural base pairs. Top: The isoC-isoG base pair which was the first developed unnatural base pair.[6] Z and P as well as S and B are two unnatural base pairs which were developed by Benner et al.[8,9] Middle: Hydrophobic base pairs (NaM and TPT3) which was developed by Romesberg et al.[12] and the TPT3[13] and NaM base pair which was developed by Kath-Schorr et al.[14] Bottom: The kappa (k) xanthosine (X) base pair used in this paper.](image1)

![Figure 2. Synthetic route to the benzoyl protected 2,4-diaminopyrimidine phosphoramidite.](image2)
Buchwald-Hartwig coupling to obtain the benzoyl-protected pyrimidine diamine, which was first applied by Wagenknecht et al.\textsuperscript{[14]} shows increased selectivity when the C5 of the ribose is silylated. We therefore coupled a TBDMS protection group yielding compound 8. Here the selectivity was high due to the slightly more reactive C5'-hydroxyl group, and the yield was 76%. The Buchwald-Hartwig coupling is catalyzed by palladium, and we increased the amount of benzamide to three equivalents to obtain the double protected compound 9 in sufficient yield (37%). However, purification of 9 at this point proved difficult due to the slow migration on the column and the poor separation from the starting benzamide. Removal of TBDMS group using trimethylamine trihydrofluoride yielded compound 10 in 62%. From here, the protocol for protection to form the phosphoramidite suitable for solid phase synthesis followed standard procedures: The DMTr protection of C5 to form 11 was achieved by DMAP-catalyzed coupling of 4,4'-dimethoxytritylchloride in dry pyridine. Formation of the phosphoramidite 12 was performed with 2-cyanoethyl N,N-disopropylethylamine in dry DCM.

As synthesis of the longer DNA especially for transcription of a riboswitch RNA with more than 80 nucleotides is demanding, we purchased DNA from IBA (Götingen), providing phosphoramidite 12 to be site-specifically incorporated into the desired transcription templates.

### Synthesis of functionalized 7-deazaxanthosine

Several options are available to provide a modifiable xanthosine derivative that maintains its Watson–Crick face pairing interactions. Introduction of chemical modifications at C8 would be the most immediate choice. However, introduction of a sterically demanding modification at C8 introduces steric clashes in the major groove of an A-form RNA helix.\textsuperscript{[18]} Thus, attachment of modifications at N7 promises to be structurally less perturbing, but their syntheses are difficult. However, several nucleoside derivatives have been reported that introduce a 7-deazapurine backbone and a number of synthetic strategies\textsuperscript{[19–22]} as well as suitable starting compounds are available. Major advantages of this approach are the conservation of the hydrogen bonding pattern at the Watson–Crick side, the advantageous substrate properties of such modified nucleotides for RNA polymerases\textsuperscript{[23]} as well as the possibility to specifically couple extended linkers using cross-coupling reactions.

The synthesis starts with the commercially available 4-chloro-7H-pyrrolo[2,3-d]pyrimidin-2-amine 13 (Figure 3). 13 was protected with pivaloyl chloride to obtain product 14 in excellent yield (90%). Without the pivaloyl group, the electrophilic substitution exclusively places the bromine or iodine at the undesired C8-position.\textsuperscript{[24]} It was also reported\textsuperscript{[21]} that without the pivaloyl protection glycosylation under Vorbrüggen conditions failed. Iodination to 15 was achieved with NIS (93%).\textsuperscript{[25]}

Initial attempts for glycosylation were made with HMDS as the silylating agent. As a result, the glycosylation reaction yielded one single product (16). NMR spectra, however, showed impurities and the yield was not satisfying (<40%). To improve both purity and yield, glycosylation was done with N,O-bis(trimethylsilyl)acetamide. Under these conditions, the glycosylation product was pure and the yields varied between 53–61%. After the following removal of the protecting groups with 0.5 M NaOMe at 80°C (17) and deamination using NaN\textsubscript{3}, the desired compound 18 was obtained.

In a previously published synthesis,\textsuperscript{[21]} the methyl ether at the C4-position was removed with trimethylsilyl (TMS) chloride and sodium iodide. Under these conditions, it was reported that a 3:2 ratio of the 7-deazaxanthosine without iodine and 7-deazaxanthosin with iodine was recovered and attempts to remove the methyl ether using sodium hydroxide were unsuccessful. In our case, the terminal alkyne linker is sufficiently stable during Sonogashira coupling conditions. We therefore directly conducted the Sonogashira coupling of the 1,7-octadiyne linker to obtain 19. We used 10 equivalents of 1,7-octadiyne and a 2:1 ratio of copper/palladium.\textsuperscript{[26]} The methyl protection group at O6 was only then removed using TMS chloride and sodium iodide at room temperature with a 61% yield of 20.

Triphosphorylation of the modified xanthosine derivative is necessary to make it amenable for T7 RNA polymerase-based transcription. Therefore, the final step is the coupling of the triphosphate specifically to C5 of the ribose. Here, protecting strategies have been used previously.\textsuperscript{[27]} We found that the conditions used by Yoshikawa and Ludwig\textsuperscript{[28–30]} were compatible with our modification, and provided the advantage that protection of the other two ribose hydroxyl moieties was not required to obtain the desired specificity for C5. We adapted the protocol and extended the hydrolysis step with 0.1 M TEAB.
buffer to eight hours. After purification by RP-HPLC, the final nucleoside triphosphate 21 was obtained in 16% yield. With this, both orthogonal base-pairing building blocks were ready to be tested in transcription and labeling.

Site-specific incorporation of xanthosine by in vitro transcription

We first tested incorporation of a xanthosine (X) to be decoded by a DNA nucleotide containing the kappa nucleobase by standard T7 run-off transcription. The kappa nucleobase was incorporated into two DNA-templates, encoding a 14mer RNA hairpin with a UUCG tetraloop motif, and the aptamer domain of the guanine-riboswitch-aptamer RNA (Gsw[31]) from Bacillus subtilis. Run-off transcription can utilize single-stranded DNA where only the T7 primer region of the template DNA is double stranded. Using this strategy, it is sufficient to synthesize the kappa DNA phosphoramidite.[31]

As a proof of concept, a G9X mutation was introduced into the 14mer UUCG-tetraloop hairpin RNA, as guanosine has high structural similarity to xanthosine. Transcription yielded a highly pure RNA. The yield upon switching from G to X dropped by 87% (Figure S1, Table S1). We further investigated the impact of introducing xanthosine on RNA structure using NMR spectroscopy. 1D $^1$H NMR showed that folding into the hairpin structure is retained and introduction of xanthosine leads to only small structural deviations (Figure S2). The UUCG tetraloop is usually highly ordered[30] and was destabilized by the mutation which is visible in both 1D $^1$H and $^1$H-$^1$H-NMR spectra (Figure 4 and Figure S2). These results show that preparative transcription using the modified constructs is feasible, and that replacing a guanosine with a xanthosine yields an interesting atomic mutagenesis approach to investigate local structural effects by NMR.

We then turned to a large structured RNA to test whether these findings also hold true for the analysis of functional/bio logically relevant RNA. Here, we used the guanine sensing riboswitch RNA from B. subtilis.[34–37] For this construct, we placed G79 forming a G·U wobble base pair in the P1 stem with X79 (Figure 5A). We optimized the transcription conditions with regard to the concentration of XTP (Figure S3). Under optimized conditions, the yield of the full-length G79X transcript was 71.4% in comparison with transcription of the unmodified Gsw[33]-transcript. Not surprisingly, we observed abortive products at the site of the mutation, with a ratio of 77:23 full-length RNA to aborting fragment. In absence of the xanthosine triphosphate, we also observed 22% full-length product (Figure 5B, Table S2), which presumably arises from misincorporation of adenosine.[6]

CD melting experiments showed a small difference of 0.25 K in the melting points ($\Delta T_m$) between the Gsw[33] and G79X riboswitch, indicating that the structure is retained (Figure 5C). We further purified the full-length G79X RNA and obtained about 22 nmol of RNA, which was sufficient for NMR studies. The NMR data fully supported our proposed strategy that introduction of xanthosine induces minimal structural perturbation. Furthermore, the previous G79 H1’ cross peaks are now visible as X79 H1’ cross peaks, indicating that the structure is retained (Figure 5D).

We prepared a reversed labeled riboswitch with $^{13}$C, $^{15}$N-labeled ATP, CTP, GTP and UTP together with unlabeled XTP. With this isotopically labeled RNA sample, we performed $^{13}$C and $^{15}$N-filtered 1D $^1$H-NMR experiments that suppress signals from hydrogen atoms bound to $^{13}$C and $^{15}$N. In this experimental setup, we detected the single $^{13}$C,$^{15}$N-”labeled” xanthosine, in particular the signals annotated XH1 and XH3 imino- and the XH8 aromatic signal in the background of the other 73 nucleotides of the riboswitch RNA (Figure 5E). Thus, transcribing RNA from a ssDNA template that contains the kappa nucleotide can yield large functional RNAs, as both selectivity and specificity are very high. Purification yields a highly homogeneous RNA sample suitable for NMR spectroscopy.

Post-transcriptional functionalization of RNA via click reaction

Site-specific introduction of a non-natural nucleobase can be exploited for labeling purposes.[6] We therefore used the 7-deazaxanthosine (7DX) derivative containing a terminal alkyne in the form of a triphosphate during in vitro transcription to introduce a position-selective spectroscopic label. Transcription of the kappa-modified Gsw[33] RNA-template with the alkyne-XTP 13 resulted in a 1:1 ratio of full-length RNA in 25 mM potassium phosphate buffer pH 6.2 and 10% D$_2$O, which was sufficient for NMR studies. The authors explored the impact of introducing xanthosine on RNA structure using NMR, and their findings show that the technique is feasible for larger, more complex RNA structures. They also demonstrated the potential for site-specific labeling using a click reaction, which is a versatile method for functionalizing RNA molecules with specific chemical modifications.
G79-7dX to abortion fragment (67 nt), with a total yield of 34.5 % full-length RNA compared to the unmodified riboswitch (Figure 6A, Figure S4, Table S3). As expected, the rate of misincorporation in absence of 7dXTP remains comparable to the misincorporation observed in absence of XTP under the respective optimized conditions for both transcripts. The modified 73mer RNA was purified by extraction from a polyacrylamide gel. For labeling, a click reaction with Cy3-Azide was performed, and the resulting RNA analyzed by PAGE. The signals shown in the gel lanes for RNA staining showed that the RNA remained intact during labeling, and no significant shift was observed. For RNA that contained the alkyne-modified xanthosine, a fluorescent signal could be observed comigrating with the unlabeled RNA, demonstrating a highly specific labeling (Figure 6B). The fluorescently labeled RNA was further excised from the gel, and analyzed using UV/Vis spectroscopy. Based on the intensities at the absorption maxima (DNA: 260 nm, Cy3: 555 nm), we calculated the labeling yield of the RNA to be 11% (Figure 6C).

Conclusion

We prepared two well-structured RNAs—a model 14mer as well as a 73 nt aptamer of a riboswitch—via run-off transcription and introduced a single guanosine-to-xanthosine mutation, in each case yielding a 100 mM NMR sample. We show that the structural perturbations introduced into two different RNA constructs by the unmodified xanthosine are minor. We furthermore devised a modifiable 7-deazaxanthosine triphosphate carrying a modifiable linker for posttranscriptional attachments of spectroscopic labels for example, for FRET-, EPR- or IR-measurements. Due to the demanding synthesis of this compound, the final triphosphorylation currently limits the obtainable yield, motivating the urgent need for novel synthetic routes for triphosphorylation.

We show that our approach facilitates labeling of biologically relevant RNAs (i.e. riboswitches) in a position-selective manner. With the synthesis of both the kappa DNA phosphoramidite and a modifiable RNA-xanthosinetriphosphate derivative, we provide a proof of concept to employ non-natural, Watson–Crick-like base pairs for site-specific bioorthogonal labeling of RNA.

Experimental Section

Solid-phase synthesis of the kappa (κ)-containing DNA template

The phosphoramidite containing the κ-nucleobase has been incorporated into the DNA via solid-phase synthesis by commercial DNA synthesis (IBA Lifesciences; Göttingen, Germany).
Expression and purification of P266L T7 RNA Polymerase

The protein was expressed in BL21(DE3) cells carrying a polyhistidine (His6-Tag) expression vector. Expression was induced at OD600 = 0.6~0.8 with 0.5 mM isopropyl-1-thiogalactopyranoside (IPTG). T7 RNA Polymerase was expressed over night at 37 °C and were purified via HiTrap HP (GE Healthcare) columns using 50 mM Tris/HCl (pH 8.1), 400 mM NaCl, 20 mM imidazole and 5 mM β-mercaptoethanol as lysis buffer. The protein was further purified via size exclusion chromatography with a buffer containing 20 mM sodium phosphate (pH 7.7) 150 mM NaCl, 1 mM EDTA and 5 mM DTT.

DNA templates

14mer-template (template 1): GCC ACC GAA GTG CCT ATA GTG AGT CGT ATT A
14mer: GCCACUUCGGUGCC
14mer2-template (template 2): GCC ACx GAA GTG CCT ATA GTG AGT CGT ATT A
14mer: GCCACUUCXGUGCC
Gsww3-template (template 3): GCC ACC CAT AGT CGG ACA TTT ACG GTG CCC GTG AGA AAC CTG CGT GCC ATA TCC ACG CAG TAT TAT GAG GCC CTG ATG CTG GGC ATA GGC CCG CAA GAA GCC GCC CGG UGC UAC UAC GGG CCA CCG UAA AUG UCC GAC UAU UGG GCC U C
Gsww7: GCC ACU CAU ACU GCG UGG AUA UGG CAC GCA GGU UUC UAC CGG GCA CCG UAA AUG UCC GAC UAU UGG GCC U C
G79X-template (template 4): GCC ACC CAT AGT CGG ACA TTT ACG GTG CCC GTG AGA AAC CTG CGT GCC ATA TCC ACG CAG TAT TAT GAG GCC CTG ATG CTG GGC ATA GGC CCG CAA GAA GCC GCC CGG UGC UAC UAC GGG CCA CCG UAA AUG UCC GAC UAU GXX GCC U C
G79-7dX: GCC ACU CAU ACU GCG UGG AUA UGG CAC GCA GGU UUC UAC CGG GCA CCG UAA AUG UCC GAC UAU G7dxG GCC U C
G79X-template: GCC ACC CAT AGT CGG ACA TTT ACG GTG CCC GTG AGA AAC CTG CGT GCC ATA TCC ACG CAG TAT TAT GAG GCC CTG ATG CTG GGC ATA GGC CCG CAA GAA GCC GCC CGG UGC UAC UAC GGG CCA CCG UAA AUG UCC GAC UAU G7dxG GCC U C

The underlined regions correspond to the inverse complementary T7 promoter sequence.

In vitro transcription

40 mM tris glutamate (pH 8.1), 2 mM spermidine, 20 mM DTT and 10 ng/μL P266L T7 RNA polymerase were used for all the transcription reactions. The other reaction components have been optimized for the 14mer to 45 mM Mg2+, 1 mM NTPs (each) and 3 mM Mg2+. The transcription reactions of Gsw3, G79X and G79-7dX were performed with the same concentration of tris glutamate, spermidine, DTT and RNA polymerase. Gsw3 was transcribed from 300 nM DNA template 3 in presence of 35 mM Mg2+, 6 mM NTPs (each) and 3 mM XTP (Jena Bioscience) were added. The transcription of G79-7dX was performed in presence of 200 nM DNA template 4, 35 mM Mg2+, 6 mM NTPs (each), 15% DMSO and 2.5 mM 7-deazaxanthosine derivative. The relative yield was determined with ImageJ or ImageLab software after polyacrylamide gel electrophoresis (PAGE).

RNA purification

The 14mer transcription reaction was purified using the protocol described by Helmling et al.[49] The G79X/-7dX transcription reaction was desalted with ddH2O using centrifuge concentrators (5 kDa MWCO cut-off, Satorius), subsequently gel purified with a 15% denaturing polyacrylamide gel. The desired fraction were extracted from gel by incubating in 0.3 M NaOAc over night at 37 °C. The supernatant was separated and the dissolved RNA was precipitated by addition of 2.5 V 99.5% EtOH (Carl Roth) and storage at –20 °C for 2 h. The RNA was collected by centrifugation at 8500 g for 30 min and the pellet was dried and dissolved in an appropriate volume of ddH2O. For removal of PAA impurities, the RNA was HPLC purified, lyophilized, desalted using centrifuge concentrators (5 kDa MWCO cut-off, Satorius) and first precipitated with ETOH (see above), then with 5 V 2% LiClO4 in Acetone (w/v) for 3 times to remove residual salt from HPLC-buffer. In the last step, the sample was desalted again and concentrated to 100–300 μM.

Click-reaction

2 μM RNA, 15X excess of Cy3-azide, 50% DMSO, 0.3 mM CuSO4, 2 mM TBT and 5 mM sodium ascorbate were mixed and incubated at 20 °C for 1 h. A sample was separated using PAGE and analysis of the fluorescence signal was carried out with a Typhoon9400. After further PAGE purification of the RNA, the click efficiency could be determined via UV absorption measurement using ε290 of 796.5 Lmmol−1 cm−1 for the RNA and ε555 of 150.0 Lmmol−1 cm−1 for Cy3.

CD melting analysis

16 μM RNA in 25 mM potassium phosphate buffer pH 6.2 and 50 mM KCl was provided. 400 μM Mg2+ was added. The melting curves were recorded between 5~95 °C and reverse at 264.2 nm. SigmaPlot 12.5 was used for the analysis. A sigmoidal fit was used to determine the melting points.

NMR-spectroscopy

14mer and 14mer2 were measured at 278 K and 600 MHz at 1 mM RNA concentration in 25 mM potassium phosphate buffer pH 6.2 and 10% D2O. The ¹H, ¹H-NMR spectra of G79X was recorded at 950 MHz in 25 mM potassium phosphate buffer pH 6.2 containing 50 mM KCl and 10% D2O. A sequence with jump-return-echo water suppression[41] was used. For the x-filter experiments,[42] a ¹C, ¹N-A, C, G, U labeled sample was prepared. For the imino region, jump-return-echo water suppression was used as well. The concentration was 160 μM in the same buffer as described above and 10% D2O. The aromatic region was recorded with transfer using DIPS12 sequence for mixing with ¹C- and ¹N-filter[43,44] and watergate sequences for water suppression.[45] The concentration was 60 μM in the same buffer as described above in 100% D2O. The spectra were recorded at 600 MHz at 308 K.

Chemical synthesis

Synthesis of 3',5'-bis-O-(tert-butylidimethylsilyl) thymidine (2): In a round flask tert-butylidimethylsilylchloride (TBDMS-Cl) (3 equiv, 65.1 g, 0.432 mol) and imidazole (6 equiv, 59.01 g, 0.867 mol) was dissolved in 1085 mL N,N-dimethylformamide (DMF). After that, thymidine (1 equiv, 35 g, 0.144 mol) was added. The reaction mixture was stirred overnight at room temperature. The mixture was quenched with 75 mL methanol (MeOH) and was diluted with 750 mL ethyl acetate (EtOAc). The organic phase was washed with distilled water (2×600 mL), with saturated sodium bicarbonate solution and dried over anhydrous magnesium sulfate. Evaporation of the solvent afforded 3',5'-bis-O-(tert-butylidimethylsilyl) thymidine (2) as a light yellow solid (57.74 g, 97%).
Synthesis of 1,4-anhydro-3,5-bis-O-(tert-butylidimethylsilyl)-2-deoxy-β-erythro-pent-1-enitol (3): Compound 2 (1 equiv, 67.07 g, 0.142 mol) was placed into a dried Schlenk flask under an argon atmosphere. Hexamethyldisilazane (HMDS) (744 mL, 3.60 mol) and ammonium sulfate (0.25 equiv, 4.7 g, 0.036 mol) were added, and the solution was stirred until dissolved. The solution was refluxed at 140 °C for 3.5 hours. HMDS was evaporated under reduced pressure. MeOH (600 mL) was slowly added at room temperature. Potassium carbonate (K2CO3) (11 equiv, 21.59 g, 0.156 mol) was added in portions and stirred for 45 min at 0 °C. This solution was filtered over a pad of celite and the solvent was evaporated under reduced pressure. The brown residue was purified by column chromatography on silica gel, elution with dichloromethane/ethyl acetate (EE) 1:2 afforded 2 a yellow oil. TLC (ethylacetate/dichloromethane:2 1) Rf = 0.91. Yield: 42.9 g (88%) (Figure S8–S10). 1H-NMR (500 MHz, CDCl3): δ = 6.64 (dd, J = 2.7, 1.8 Hz, H-1, H-1), 7.13 (s, 2H, CH3), 0.19 (2s, 2H, CH3), 0.09, 0.08, 0.07, 0.06 ppm (4s, 12H, Si(CH3)3). 13C-NMR (100 MHz, CDCl3): δ = 149.1 (C-1), 103.5 (C-2), 89.0 (C-3), 76.1 (C-4), 62.9 (C-5), 26.0, 25.9 (C(CH3)2), 18.5, 18.2 (C(CH3)), -4.2 (Si(CH3)), -4.3 (Si(CH3)), -5.2 ppm (Si(CH3)). MALDI-MS: m/z calcd for C12H22O3Si (M + H+): 277.16; found: 277.21.

Synthesis of 3-O-(tert-butylidimethylsilyl)-1,2-dideoxy-2,3-didehydro-α-D-ribofuranose (4): To a solution of compound 3 (1 equiv, 42.89 g, 0.124 mol) in THF (445 mL) at 0 °C was added in portions a solution of TBAF 1 M in THF (1 equiv, 124.45 mL, 0.124 mol), and the reaction mixture was stirred for 2 hours. After two hours the reaction mixture was diluted with distilled water (500 mL) and with dichloromethane (400 mL). The organic phase was washed with distilled water (200 mL), brine (200 mL) and dried over sodium sulfate (Na2SO4). The solvent was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel, elution with n-hexane/ethyl acetate 4:1 afforded 4 as a pale yellow oil. TLC (n-hexane/ethyl acetate:4 1) Rf = 0.39. Yield: 12.8 g (45%) (Figure S11–S13). 1H-NMR (500 MHz, D2DMSO): δ = 6.61 (dd, J = 2.7, 1.8 Hz, J2 = 0.8 Hz, 1H, H-1), 7.13 (s, 2H, CH3), 0.20 (2s, 2H, CH3), 0.19 (2s, 2H, CH3), 0.09 Hz, 1H, H-3), 4.11 (td, J = 6.7 Hz, 1H, H-4), 3.43 and 3.25 ppm (2x, 2x, J = 11.6 Hz, J2 = 6.7 Hz, 1H, H-2), 0.85 ppm (1H, CH3), 0.06 and 0.05 ppm (2x, 2x, 2x, Si(CH3)). 13C-NMR (125 MHz, D2DMSO): δ = 149.1 (C-1), 103.5 (C-2), 89.0 (C-3), 75.7 (C-4), 60.9 (C-5), 25.8 (C(CH3)), 17.7 (C(CH3)), -4.4 (Si(CH3)), -4.5 ppm (Si(CH3)). MALDI-MS: m/z calcd for C11H16O2Si (M + H+): 230.38; found: 230.98.

Synthesis of 1β-(2,4-dichloropyrimidin-5-yl)-1,2,3-trideoxy-3-oxo-D-ribofuranose (6): Palladium(II)acetate (0.01 equiv, 0.0056 mol) and tris(pentafluorophenyl)phosphine (0.02 equiv, 5.96 g, 0.011 mol) was dissolved in a dried Schlenk flask in dry chloroform (194 mL). The mixture was stirred for one hour at room temperature under an argon atmosphere. After 1 h compound 4 (1 equiv, 12.8 g, 0.056 mol) was added. 2,4-dichloro-pyrimidine-5-carboxylic acid (1 equiv, 18.4 g, 0.067 mol) and silver carbonate (1 equiv, 15.4 g, 0.056 mol) were added. The reaction mixture was stirred for 10 h at 70 °C. After being cooled to room temperature, the reaction mixture was filtered on pad of celite and eluted with chloroform. The solvent was removed under reduced pressure, and the remaining brown oil was dissolved in THF (357 mL). At 0 °C, 1.09 mol acetic acid and 4.35 mol of 1 M TBAF solution in THF were added. After 2 h, the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel, elution with n-hexane/ethyl acetate 1:1 afforded 6 as a yellow oil. TLC (n-hexane/ethyl acetate 1:1): Rf = 0.44. Yield: 5.5 g (37%) (Figure S14–S16). 1H-NMR (500 MHz, CDCl3): δ = 8.97 (d, J = 0.6 Hz, 1H, H-6), 5.46 (dd, J = 10.4, 6.3, 0.5 Hz, 1H, H-1), 4.12 (t, J = 3.1 Hz, 1H, H-4), 4.04 (dd, J = 12.1, 3.1 Hz, 1H, H-5), 3.17 (dd, J = 12.3, 3.1 Hz, 1H, H-6), 2.38 (m, 1H, H-2), 1.82, 10.6 Hz, 1H, H-2). 13C-NMR (125 MHz, CDCl3): δ = 211.5 (C-3), 160.1 (C-2/C-4), 159.9 (C-2/C-4), 158.5 (C-4), 158.4, 132.0 (C-5), 82.2 (C-4), 72.3 (C-1), 61.6 (C-5), 43.5 ppm (C-2). MALDI-MS: m/z calcd for C9H6Cl2N4O4 [M + 2H]+: 263.07; found: 264.92.

Synthesis of 1β-(2,4-dichloropyrimidin-5-yl)-1,2,3-dideoxy-5-O-(tert-butylidimethylsilyl)-ribofuranose (8): In a Schlenk flask compound 7 (1 equiv, 3.67 g, 0.014 mol) and imidazole (2.5 equiv, 2.36 g, 0.035 mol) were dissolved in dry DMF. Tert-butylidimethylsilyl chloride (1.2 equiv, 2.5 g, 0.017 mol) was slowly added to the reaction mixture. The mixture was stirred for 16 h at room temperature, and 150 mL of water were added. The aqueous phase was washed three times with 100 mL of ethyl acetate and dried over magnesium sulfate. The solvent was removed under reduced pressure. The remaining yellowish oil was purified by column chromatography on silica gel. Elution with n-hexane/ethyl acetate 3:1 afforded 8 as a colorless oil. TLC (n-hexane/ethyl acetate:3 1) Rf = 0.30. Yield: 3.99 g (76%) (Figure S20–S22). 1H-NMR (500 MHz, CDCl3): δ = 8.79 (d, J = 10.4 Hz, H-6), 5.35 (dd, J = 5.9 Hz, 1H, H-1), 1.44 (dd, J = 5.6, 2.2, 1.8 Hz, H-1), 4.03 (dd, J = 4.2, 3.1, 2.3 Hz, 1H, H-4), 3.79 (dd, J = 10.8, 3.4 Hz, 1H, H-5), 3.75 (dd, J = 10.8, 3.4 Hz, 1H, H-5).
In an argon purged dried Schlenk flask compound 10 (1 equiv, 2.31 g, 4.21 mmol) was dissolved in 59 mL THF. NET_{3} (3.26 equiv, 1.78 mL, 10.93 mmol) was added dropwise. After two hours, volatiles were removed under reduced pressure, and the remaining crude product was purified by column chromatography on silica gel. Elution with n-hexane/ethyl acetate 1:3 afforded 9 as a colorless solid. TLC (ethyl acetate/n-hexane 3:1): R_{f} = 0.08. Yield: 2.31 g (37%).

**Synthesis of 1-([2,4-bis(benzoylamo)pyrimidin-5-yl]-1,2-di-deoxy-S-(tert-butyldimethylsilyl)-O-ribofuranose (10):** Compound 9 (1 equiv, 2.31 g, 4.21 mmol) was dissolved in 59 mL THF. NET_{3} (3H F (2.6 equiv, 1.78 mL, 10.93 mmol) was added dropwise. After two hours, volatiles were removed under reduced pressure, and the remaining crude solid was purified by column chromatography on silica gel. Elution with dichloromethane/methanol 9:1 afforded 10 as a white solid. TLC (dichloromethane/methanol 9:1): R_{f} = 0.25. Yield: 1.2 g (62%).

**Synthesis of 1-([2,4-bis(benzoylamo)pyrimidin-5-yl]-1,2-di-deoxy-S-(tert-butyldimethylsilyl)-O-ribofuranose (11):** In an argon purged dried Schlenk flask compound 10 (1 equiv, 1.15 g, 2.65 mmol) and DMAP (0.05 equiv, 16 mg, 0.133 mmol) were dissolved in 32 dry pyridine. 4,4'-dimethoxytrityl chloride (1.5 equiv, 1.35 g, 4.0 mmol) was added slowly and the mixture was stirred at room temperature for 12 h. The reaction was stopped with 13 mL of MeOH and all solvents were removed under reduced pressure. The remaining crude product was purified by column chromatography on silica gel. Elution with n-hexane/acetone 2.3: +0.1% NEt_{3} afforded 11 as a white foam. TLC (n-hexane/acetone 2.3:): R_{f} = 0.23. Yield: 1.49 g (77%).

**Synthesis of 1-([2,4-bis(benzoylamo)pyrimidin-5-yl]-1,2-di-deoxy-S-(4,4'-dimethoxytriphenylmethyl)-O-ribofuranose-3-[2-cyanoethyl](N,N-diisopropyl)phosphoramidite (12):** In an argon purged dried Schlenk flask compound 11 (1 equiv, 253 mg, 0.343 mmol) and N,N-diisopropylethylamine (3 equiv, 0.18 mL, 1.03 mmol) were dissolved in 8 mL of dry dichloromethane. 2-cyanoethyl N,N-diisopropylethylamine (1.5 equiv, 0.115 mL, 0.515 mmol) was added and the reaction mixture was stirred for two hours at room temperature. All volatiles were removed under reduced pressure and the crude product was purified by column chromatography on silica gel. Elution with cyclohexane/acetonitrile 2.1: +0.1% triethylamine afforded 12 as a colorless solid. TLC (cyclohexane/acetonitrile 2.1): R_{f} = 0.31. Yield: 238 mg (74%).

**Synthesis of 1-([4-chloro-7-H]-pyrrolo[2,3-d]pyrimidin-2-yl]-2,2-dimethyl-propionamide (14):** Compound 13 (1 equiv., 14 g, 83 mmol) was dissolved in 196 mL of dry pyridine under an argon atmosphere. Pivaloyl chloride (1.8 equiv., 18.5 mL, 151 mmol) was then slowly added dropwise to this solution at room temperature. The reaction mixture was stirred for one hour at room temperature. During the reaction, a colorless solid precipitated (pyridine hydrochloride). By adding 100 mL of methanol, the reaction was stopped. The solvent was removed in an oil pump vacuum leaving a yellow solid. The yellow solid was taken up in 200 mL of ethyl acetate and washed three times with 100 mL of a 0.1 M hydrochloric acid solution. The organic phase was dried over magnesium sulfate (MgSO_{4}) and the solvent removed in vacuo. The crude product was purified by column chromatography. The eluent used was dichloromethane/methanol in a ratio of 10:1. Compound 14 was obtained as a yellowish solid. TLC (dichloromethane/methanol 98:2): R_{f} = 0.37. Yield: 19 g (90%) (Figure S35–S37).
stirred for one hour at room temperature. Then the solvent was removed in vacuo and the residue was purified by column chromatography. The eluent used was dichloromethane/methanol in the ratio 20:1. Compound 15 was isolated as a colorless solid. TLC (dichloromethane/methanol 20:1): Rf = 0.77. Yield: 25.1 g (93%) (Figure S38–540). 1H-NMR (600 MHz, [D6]DMSO): δ = 12.67 (s, 1H, NH), 10.09 (s, 1H, NH), 7.76 (d, J = 2.2 Hz, 1H, H-8), 1.22 ppm (s, 9H, tert-Butyl).

13C-NMR (151 MHz, [D6]DMSO): δ = 175.8, 152.5, 151.4, 150.7, 132.7, 112.3, 51.5, 26.8 ppm. ESI-MS: m/z calc for C9H10ClINO [M + Na]+ 378.60; found: 400.90.

Synthesis of 4-chloro-5-iodo-2-pivaloylamo-7-[(2,3,5-tri-O-benzoyl)-β-D-ribofuranosyl]-7H-pyrrolo[2,3-d]pyrimidin-16): Compound 15 (1 equiv., 15 g, 39.6 mmol) was suspended under an argon atmosphere in 350 mL of dry acetonitrile. To this was added dropwise N,N-diisopropylethylamine (1.24 equiv., 12 mL, 49.1 mmol) and the solid went completely into solution. The reaction mixture was stirred for ten minutes at room temperature. Then, trimethylsilyl trifluoro-methanesulfonate (1.4 equiv., 10 mL, 55.5 mmol) and 1’-acetate-2’,3’,5’-tribenzoate-β-D-ribofuranose (0.67 equiv., 13.4 g, 26.6 mmol) were added and heated to 50°C. 1’-acetate-2’,3’,5’-tribenzoate-β-D-ribofuranose (0.67 equiv., 13.4 g, 26.6 mmol) was then added again after six and twelve hours. After stirring for 24 hours, 800 mL of dichloromethane were added. The organic phase was washed with 150 mL of a saturated aqueous sodium bicarbonate solution, with 150 mL of a saturated sodium chloride solution (evolution of gas) and dried over magnesium sulfate. The solvent was removed in vacuo and the residue was purified by column chromatography. TLC (dichloromethane/methanol in the ratio 20:1). The product was isolated as an orange solid. TLC (dichloromethane/methanol in the ratio 5:1): Rf = 0.52. Yield: 1.5 g (61%) (Figure S47–549).

1H-NMR (600 MHz, [D6]DMSO): δ = 11.60 (s, 1H, NH), 7.48 (s, 1H, H-8), 5.94 (d, J = 6.1 Hz, 1H, H-1), 5.31 (s, 1H, OH-2), 5.10 (s, 2H, OH-3 + OH-5), 4.28 (dd, J = 5.6, 6.0 Hz, 1H, H-2), 4.04 (s, 1H, H-3), 3.97 (3H, CH3), 3.87 (m, 1H, H-4, H-5), 3.61–3.50 ppm (ddd, J = 3.5, 7.8, 11.2 Hz, 1H, H-5). 13C-NMR (151 MHz, [D6]DMSO): δ = 172.2, 163.9, 160.1, 126.0, 100.5, 86.5, 85.2, 73.8, 70.3, 61.5, 53.6, 52.1 ppm. ESI-MS: m/z calc for C18H10ClINO [(M + H)+] 423.16; found: 423.98.

Synthesis of 1,7-dihydro-5-iodo-4-methoxy-7-(β-D-ribofuranosyl)-2H-pyrrolo[2,3-d]pyrimidin-2-amine (19): Compound 18 (1 equiv., 1.2 g, 2.84 mmol) was first dried in vacuo at 65°C for one hour. Then, tetrakis(triphenylphosphine)palladium(0) (Pd[PPh3]4, 0.1 Eq., 328 mg, 284 μmol) and copper iodide (CuI, 0.2 Eq., 108 mg, 0.57 μmol) were added under argon atmosphere. The solids were again dried in vacuo for 20 minutes. To this was then added 10 mL of N,N-dimethylformamide and triethylamine (NEt3, 2.84 equiv., 1.12 mL, 8.05 mmol) and the reaction solution was then placed under argon and vacuum 20 times to remove any residual oxygen. Finally, 1.7-octadecyli (0.1 Eq., 3.76 mL, 28.4 mmol) was added. The reaction was stirred at room temperature overnight. Thereafter, the solvent was removed in an oil pump vacuum at 65°C to 90% subsequently. A column chromatographic purification was carried out. The eluent used was dichloromethane/ methanol in the ratio 9:1. The product 19 was isolated as an orange solid. Yield: 1.09 g (96%) (Figure S50–552). The product still contains residues of triethylamine, which could not be removed by the column chromatographic purification. TLC (dichloromethane/methanol 9:1): Rf = 0.39. 1H-NMR (600 MHz, [D6]DMSO): δ = 11.45 (s, 1H, NH), 7.53 (s, 1H, H-8), 5.97 (s, 1H, OH-2), 5.32 (d, J = 6.2 Hz, 1H, H-1), 5.10 (s, 1H, OH-2), 3.03 (3H, CH3), 4.29 (s, 1H, H-5), 4.05 (dd, J = 4.5, 7.6, 12.1 Hz, 1H, H-3), 3.97 (3H, CH3), 3.86 (1H, H-4), 3.61–3.59 (m, 1H, H-5), 3.54–3.51 (m, 1H, H-5), 2.79 (m, 1H = 2.6Hz, H-1a), 2.43 (t, J = 6.5 Hz, 2H, H-2), 2.25–2.22 (m, 2H, H-e), 1.68–1.60 ppm (m, 4H, H-c,d).

13C-NMR (126 MHz, [D6]DMSO): δ = 164.2, 134.9, 133.8, 133.7, 130.1, 130.0, 125.2, 90.3, 86.3, 85.1, 84.4, 73.8, 71.3, 70.5, 61.4, 53.6, 27.3, 27.0, 18.4, 17.3 ppm. ESI-MS: m/z calc for C17H17N3O3 [(M + Na)+] 410.41; found: 424.13.

Synthesis of 5-(octa-1,7-dienyl)-7-(β-D-ribofuranosyl)-1,3,7-trihydro-2H4-pyrrolo[2,3-d]pyrimidin-2,4-dione (20): Compound 19 (1 equiv., 750 mg, 1.87 mmol) and sodium iodide (1.97 equiv., 197 mg, 1.32 mmol) were suspended under an argon atmosphere in 28 mL of dry acetonitrile. To this suspension trimethylsilyl chloride (TMS-Cl, 2.28 equiv., 193 μL, 1.52 mmol) was added. A greenish discoloration of the reaction solution was observed. The reaction was stirred for one hour at room temperature. The reaction solution was filtered and washed with acetonitrile. The solvent was removed in vacuo and a column chromatographic purification followed. The eluent used was dichloromethane/ methanol in the ratio 5:1. The product 20 was isolated as a colorless solid. TLC (dichloromethane/methanol 5:1): Rf = 0.56. Yield: 453 mg (63%) (Fig-
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**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** fluorescence · genetic code expansion · NMR · RNA · site-specific labeling

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