Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians Universität München

Synthesis of natural and isotope-labeled tRNA nucleosides and their mass spectrometric quantification

Synthese natürlicher und isotopenmarkierten tRNA Nukleoside und deren massenspektrometrische Quantifizierung

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aus
Regensburg

2010

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung der LMU München vom 29. Januar 1998 von Prof. Dr. T. Carell betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, am 11.06.2010

Tobias Brückl

Dissertation eingereicht am: 14.06.2010

1. Gutachter: Prof. Dr. T. Carell
2. Gutachter: Prof. Dr. P. Knochel

Mündliche Prüfung am: 15.07.2010

Wish It, Dream It, Do It!

Parts of this thesis work were published or presented on conferences

Publications

M. Münzel, D. Globisch, T. Brückl, M. Wagner, V. Welzmiller, S. Michalakis, M. Müller, M. Biel, and T. Carell, Quantification of the Sixth DNA-Base Hydroxymethyl-dC in the Brain, *Angew. Chem.* **2010**, accepted (hot paper).

T. Brückl, D. Globisch, M. Wagner, M. Müller, and T. Carell, Parallel isotope-based quantification of modified tRNA nucleosides, *Angew. Chem. Int. Ed.* **2009**, *48*, 7932–7934 (hot paper).

T. Brückl, F. Klepper, K. Gutsmiedl, and T. Carell, A short and efficient synthesis of the tRNA nucleosides $PreQ_0$ and archaeosine, *Org. Biomol. Chem.* **2007**, *5*, 3821–3825.

Conference Presentations

Poster Presentation: 42nd IUPAC Congress, Chemistry Solutions, *Quantitative analysis of modified tRNA nucleosides*, Glasgow, United Kingdom, 2nd to 7th August 2009.

Poster Presentation: Bayer Ph.D. Student Course 2009, *Quantitation of modified tRNA nucleosides from E. coli, pork, HeLa, and HCT*, Cologne, Germany, 5th to 9th July 2009.

Poster Presentation: Synthesefest 2009 – A Celebration of Organic Chemistry in Munich, *Synthesis of the modified tRNA nucleosides queuosine, archaeosine, ms^2io^6A, and m^2A*, Munich, Germany, 17th and 18th March 2009.

Oral Presentation: 11th JCF-Frühjahrssymposium, *2-methylthio-N-6-hydroxyisopentenyl-adenosine (ms^2io^6A), 2-Methyladenosine (m^2A), queuosine (Q), and archaeosine (G*) – Total syntheses of modified tRNA nucleosides*, Essen, Germany, 11th to 14th March 2009.

Poster Presentation: ORCHEM 2008, *Making modifications – Total synthesis of modified tRNA nucleosides*, Weimar, Germany, 1st to 3rd September 2008.

Oral Presentation: 3rd Nucleic Acid Chemical Biology Ph.D. Summer School, *Total synthesis of queuosine (Q) and archaeosine (G*)*, Odense, Denmark, 24th to 28th June 2007.

Danksagung

Diese Arbeit wäre ohne die fachliche und persönliche Unterstützung zahlreicher Personen nicht entstanden.

An erster Stelle gilt mein Dank meinem Doktorvater *Prof. Thomas Carell*, der durch seinen unermüdlichen Einsatz die exzellenten Arbeitsbedingungen schafft, von denen diese Arbeit entscheidend geprägt war. Er ermöglichte mir ein spannendes Thema, dass mich in neue und interessante Gebiete der Chemie geführt hat, eine große wissenschaftliche Freiheit, die mir Raum ließ, mein Projekt zu entwickeln, und unterstütze mich sowohl beim Erreichen meiner arbeitskreisinternen als auch –externen Ziele.

Den Mitgliedern meiner Prüfungskommission danke ich für ihre Bemühungen bei der Evaluierung meiner Arbeit. Insbesondere bedanke ich mich bei Herrn *Prof. Paul Knochel* für die Übernahme der Zweitkorrektur und die unkomplizierte und effektive Unterstützung während meines Studiums und meiner Doktorarbeit. Herrn *Prof. Konstantin Karaghiosoff* danke ich für das Messen und Lösen der in dieser Arbeit diskutierten Kristallstrukturen. Unterstützung bei allen massenspektrometrischen Fragen habe ich von Herrn *Prof. Stephan Sieber* erhalten.

Frau *Slava Gärtner* danke ich für ihre Hilfe in allen organisatorischen Belangen. Außerdem geht mein Dank an Frau *Sabine Voß* für das Organisieren all der großen und kleinen Dingen, die man für die Chemie benötigt, und insbesondere für ihre Hilfe bei der Synthese. Bei den *Mitarbeitern der Zentralen Analytik* bedanke ich mich für das zuverlässige und schnelle Messen meiner Proben. *Herrn Pöller* von der Schweineschlachtung München GmbH und dem *Arbeitskreis Biel* sei für die Gewebeproben gedankt.

Mit der gesamten *Arbeitsgruppe Carell* verbindet mich eine gute Zusammenarbeit und zahlreiche gemeinsame Erlebnisse. Viele meiner Gefährten auf dem gelegentlich steinigen Weg zur Promotion haben sich als gute Freunde erwiesen. Ein großes Dankeschön für die tolle Atmosphäre und Hilfsbereitschaft richtet sich an alle meine ehemaligen und aktuellen Laborkollegen insbesondere an meine Benchnachbarn *Guido Clever*, *Volker Hickmann* und *Peter Thumbs* und an meine Kollegen in der in den letzten Jahren stark gewachsenen Modifications-Subgroup. Bei *Eva Jahn* danke ich mich für die hervorragende Vorarbeit auf dem Queuosine-Projekt. Tatkräftige Unterstützung in der Synthese und Biochemie habe ich von *Christina Hettstedt*, *Thomas Hofmann*, *Markus Müller*, *Ines Thoma*, und *Thomas*

Wildenhof erhalten. Hilfreiche Tipps und Diskussionen von und mit *Tim Gehrke*, *Andreas Glas*, *Markus Müller*, *Martin Münzel*, *Thomas Reissner*, *Sabine Schneider*, *Christian Trindler* und *Mirko Wagner* haben die Entwicklung dieser Arbeit begleitet. Den „Knochels“ *Christina Despotopoulou*, *Christoph Rohbogner* und *Andreas Wagner* sei für ihre Hilfe bei der Methodenentwicklung gedankt.

Tim Gehrke, *Daniel Globisch*, *Corinna Kaul*, *Martin Münzel*, *Markus Müller* und *Sabine Schneider* hatten das außerordentliche Vergnügen diese Arbeit Korrektur zu lesen, wofür ich mich an dieser Stelle herzlich bedanke.

Ein ganz besonderer Dank geht an *Daniel Globisch*, der in den letzten fast vier Jahren mein Projektpartner war und ein sehr guter Freund geworden ist. Eine bessere und produktivere Zusammenarbeit kann man sich nicht wünschen. Er war immer zur Stelle, wenn es einen Anlass zum Diskutieren, zum richtig Ackern, zum Aufmuntern, zum Lachen und zum Feiern gab und ganz neben bei hat er mir mehr über Fußball beigebracht als ich jemals wissen wollte.

An meine Freunde aus Neumarkt und aus dem Studium geht ein großes Dankeschön dafür, dass sie trotz meines stark arbeitslastigen Zeitmanagements zu mir halten.

Ganz herzlich bedanke ich mich bei dem besten Bruder und den besten Eltern der Welt: *Stefan*, *Elisabeth und Dieter Brückl*! Vielen Dank für eure uneingeschränkte Unterstützung über all die Jahre, die den Grundstein für diese Arbeit gelegt und sie ermöglicht hat. Ihr habt mich zu dem gemacht, was ich bin!

Mein letzter aber wohl wichtigster Dank geht an meine Frau *Sandra Brückl*, die im Moment nicht nur meine bessere Hälfte sondern sogar meine besseren 2/3 ist. Deine Unterstützung und Kraft haben diese Arbeit und unser Leben entscheidend geprägt. Ich wollte dir nur mal eben sagen, dass du das Größte für mich bist!

Vielen Dank auch an „*den kleinen Brückl*“, der uns aus dem Bauch heraus schon viel Freude bereitet hat.

Table of contents

1 Summary

In cellular nucleic acids there are more than 100 modified nucleosides, which fine-tune and regulate the activity of most RNAs and of genes in DNA. Due to these important assignments these modifications have stimulated research for decades and thousands of publications describe their occurrences, biosyntheses, and functions. Most knowledge has been obtained by the study of individual modifications, although it is known that they generally occur concertedly and bias each other.

Collective, quantitative investigation of biomolecules has led to tremendous advances in biochemistry during the current era of omics-research. For modified nucleosides such an approach is hampered by the lack of methodologies, which provide quantitative data of many modifications in parallel with the necessary sensitivity and accuracy. Therefore, a stable isotope dilution HPLC-MS method was established, which allows quantification of in principle all modified nucleosides from DNA and tRNA with high sensitivity and accuracy (Figure 1).

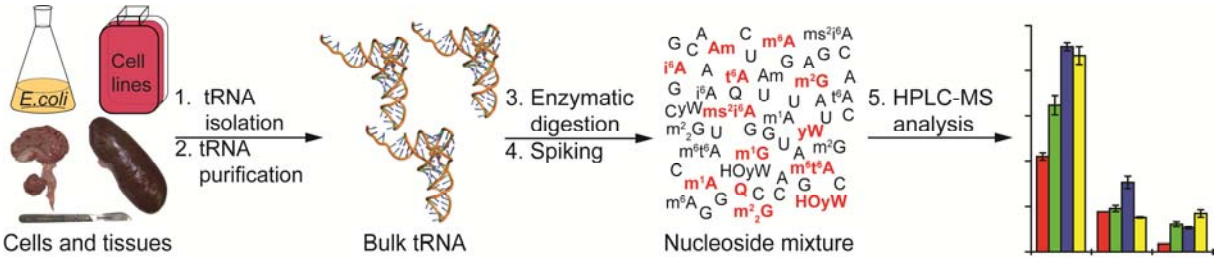


Figure 1: Schematic outline of the quantification method for modified tRNA nucleosides established during this thesis work. Modified DNA nucleosides were quantified accordingly.

It is composed of the following single steps:

1. Isolation of DNA or tRNA from biological material.
2. Purification of DNA or tRNA.
3. Enzymatic digestion of the nucleic acids to nucleosides.
4. Spiking of the nucleoside mixture with isotopically labeled standards.
5. Analysis of the mixture by HPLC-MS.

As a basis, the nucleosides of interest had to be synthesized in natural and isotopically labeled form. Thus, in the course of this thesis work nine modified nucleosides and their heavy atom labeled derivatives were synthesized (Figure 2).

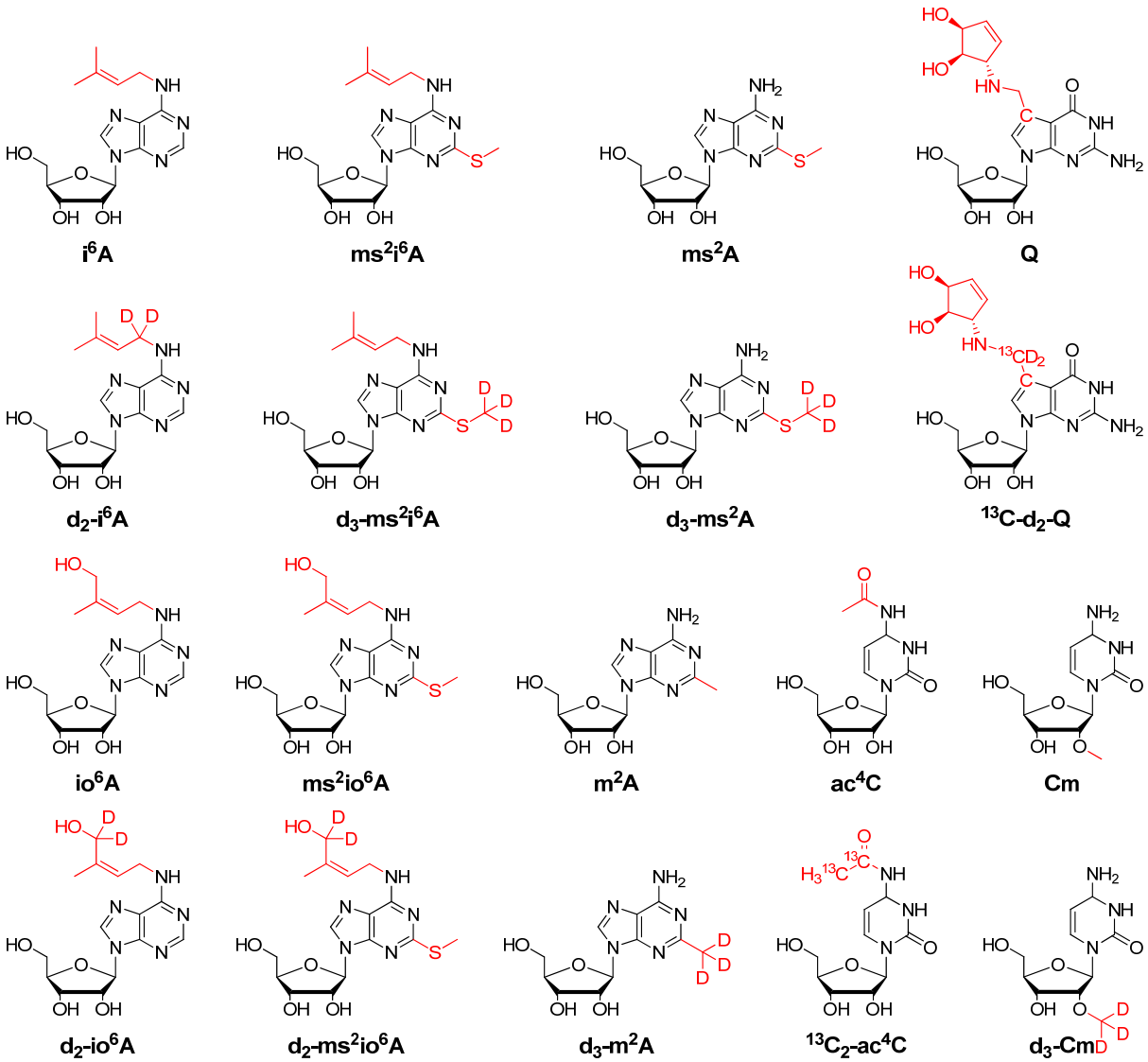
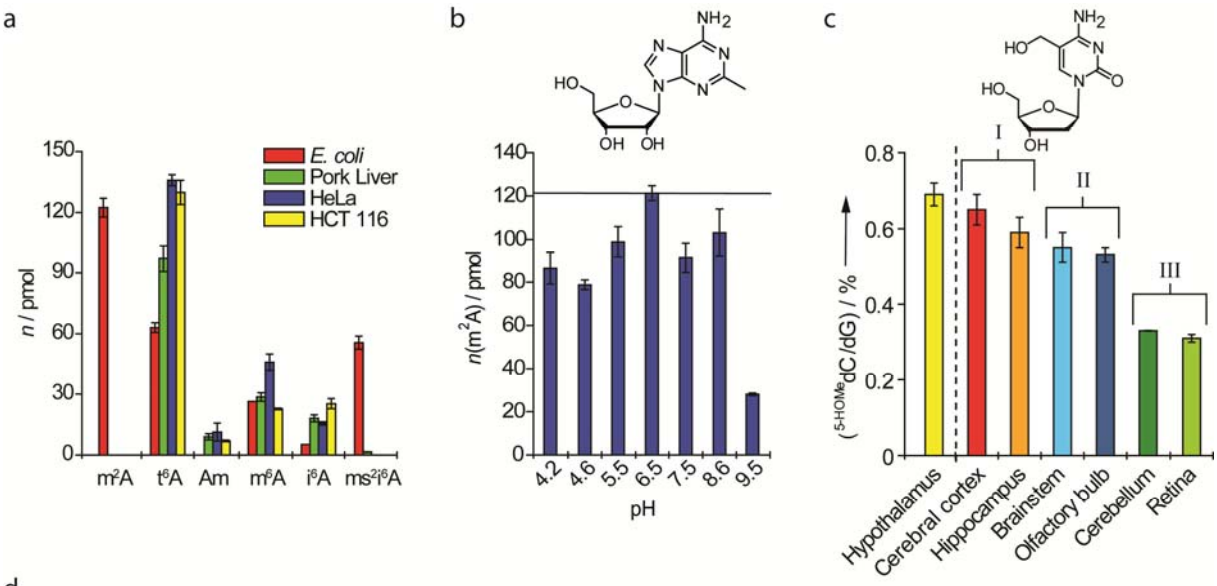


Figure 2: Compilation of the 18 nucleosides prepared for mass spectrometric quantification in the course of this thesis work.

Utilizing the prepared modified nucleosides genomic DNA and tRNA were investigated. The nucleosides $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC from DNA was studied, because recently the novel sixth DNA nucleoside $^{5\text{-HOMe}}$dC has been reported, which is of outstanding scientific interest. tRNA was selected as a representative for RNAs, as it contains the highest amount and density of modified nucleoside. These influence the translation of mRNA at the ribosome, one of the most important biological processes in a cell.

In an initial study of six modified nucleosides from *E. coli*, two cell lines (HeLa and HCT-116), and pork liver tRNA the feasibility of our method in conjunction with its high sensitivity and accuracy was proven (Figure 3 a). The initial measurements showed that the investigated cancer cell lines contain significantly different modification levels and that these

also vary from the levels in healthy tissue. In addition, the modified nucleoside ms^2i^6A, which is present in mitochondrial tRNA but not in the cytosolic tRNA, was not detected in cancer cell lines indicating an impaired mitochondrial activity in these cells in accordance with the Warburg hypothesis.



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Tissues	Modified nucleosides								
	t^6A	m^6t^6A	i^6A	OHyW	Q	m^1G+Gm	$m^2{}_2G$	m^2G	m^1A
Liver	215	21.9	39.6	33.6	55.2	1,060	563	1,176	1,010
Cerebellum	237	17.5	41.7	29.0	83.5	829	567	1,036	942
Spine marrow	199	11.8	36.0	26.0	50.8	800	457	971	911
Spleen	182	13.9	30.7	26.1	42.7	690	464	794	835
Kidney	178	15.8	30.7	21.1	32.3	816	451	896	872
Lung	184	13.9	33.6	21.7	33.9	794	427	879	876
Tongue	171	11.3	25.7	15.2	21.9	699	396	794	846
Cerebrum	166	10.5	26.1	20.3	57.2	587	406	682	733
Heart	175	11.0	29.0	12.0	29.6	638	373	673	797

Figure 3: Representative results of the four quantification studies of modified nucleosides performed in the course of this thesis work.
a) tRNA modification levels in *E. coli*, pork liver, and cell lines (HeLa and HCT-116). b) Amounts of m^2A measured in *E. coli* exposed to different environmental pH values. c) Ratio of $^{5\text{-HOMe}}dC$ to dG in the different tissues in percent. d) Color-coded tRNA modification levels in pork tissue. Error bars represent the determined standard deviations of at least two replicates and at least six HPLC-MS measurements.

Changes of the modification levels in *E. coli* upon external stimulation were investigated in a second study, which employed m^2A and m^6A, to evaluate whether modified nucleosides might be involved in the stress response of cells (Figure 3 b). For a number of stimulations no alteration could be detected but variations of the environmental pH and treatment with antibiotics induced alterations in the modification levels, e.g. a 75 % loss of m^2A at pH 9.5.

Quantification of the novel sixth nucleosides, $^{5\text{-HOMe}}$dC, in DNA samples isolated from seven different brain regions with our method showed that $^{5\text{-HOMe}}$dC is present in all of these confirming $^{5\text{-HOMe}}$dC as a general post-replicatively formed DNA modification mainly in neuronal tissue (Figure 3 c).

The largest screening was performed with pork tissue, which included ten tissues and twelve modified tRNA nucleosides (Figure 3 d). The levels of the mitochondrial modification ms^2i^6A were found to correlate with the mitochondrial activity in the investigated tissues. All other modified nucleosides exhibited a concordant, gradual variation of their amounts across tissues with only minor exceptions. They correlate with the protein synthesis rate of the tissues indicating a regulation of the protein synthesis capacity by tRNA modifications. Based on this correlation and the acceleration of the rate limiting step of translation by modified nucleosides, which is described in literature, the following general model was introduced:

In prokaryotes modified tRNA nucleosides establish a fitness and proliferation advantage by optimizing the translation efficiency. In mammals the tissue-specific modification levels are responsible for maintaining the ideal translation rate and protein synthesis capacity of each tissue.

Taken together, in the course of this thesis work it was possible to establish a quantification method for modified nucleosides, to utilize it for several studies, and to develop a novel, general concept for the functions of tRNA modifications from the obtained data.

2 Zusammenfassung

Natürliche Nukleinsäuren enthalten mehr als 100 modifizierte Nukleoside, die die Aktivität von RNAs und von Genen in der DNA regulieren. Wegen dieser wichtigen Aufgaben werden Modifikationen seit Jahrzehnten untersucht und tausende Publikationen beschreiben ihr Vorkommen, ihre Biosynthesen und ihre Funktionen. Die meisten Informationen über sie wurden durch die Untersuchung einzelner Modifikationen erhalten, obwohl sie in der Regel gemeinsam auftreten und sich auch gegenseitig beeinflussen.

In der aktuellen Ära der Omics-Forschung hat die kollektive, quantitative Untersuchung von Biomolekülen zu beachtlichen Fortschritten geführt. Bei modifizierten Nukleosiden wird dieser Ansatz durch das Fehlen passender Methoden, die parallel quantitative Daten zu vielen Modifikationen mit der nötigen Empfindlichkeit und Genauigkeit liefern, erschwert. Deshalb wurde eine HPLC-MS Isotopenverdünnungsmethode entwickelt, die die parallele Quantifizierung von prinzipiell allen modifizierten Nukleosiden aus DNA und tRNA mit hoher Empfindlichkeit und Genauigkeit ermöglicht (Abbildung 1).

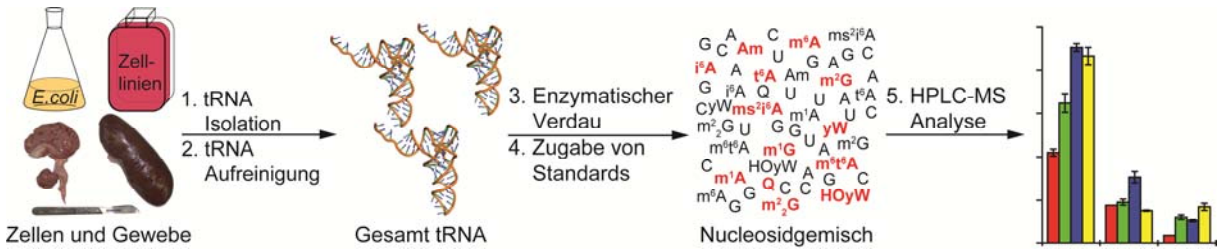


Abbildung 1: Skizze der Quantifizierungsmethode für modifizierte tRNA Nukleoside, die im Rahmen dieser Arbeit etabliert wurde. DNA Modifikationen wurden entsprechend quantifiziert.

Sie besteht aus den folgenden Einzelschritten:

1. Isolierung der DNA und tRNA aus biologischem Material.
2. Aufreinigung der DNA und tRNA.
3. Enzymatischer Verdau der Nukleinsäuren zu Nukleosiden.
4. Zugabe der Isotopenstandards zu dem Gemisch.
5. HPLC-MS Analyse des Gemisches.

Zudem mussten die zu untersuchenden Nukleoside in natürlicher und isotopenmarkierter Form synthetisiert werden. Deshalb wurden im Verlauf dieser Arbeit 18 modifizierte Nukleoside zusammen mit ihren schweratommarkierten Derivaten dargestellt (Abbildung 2).

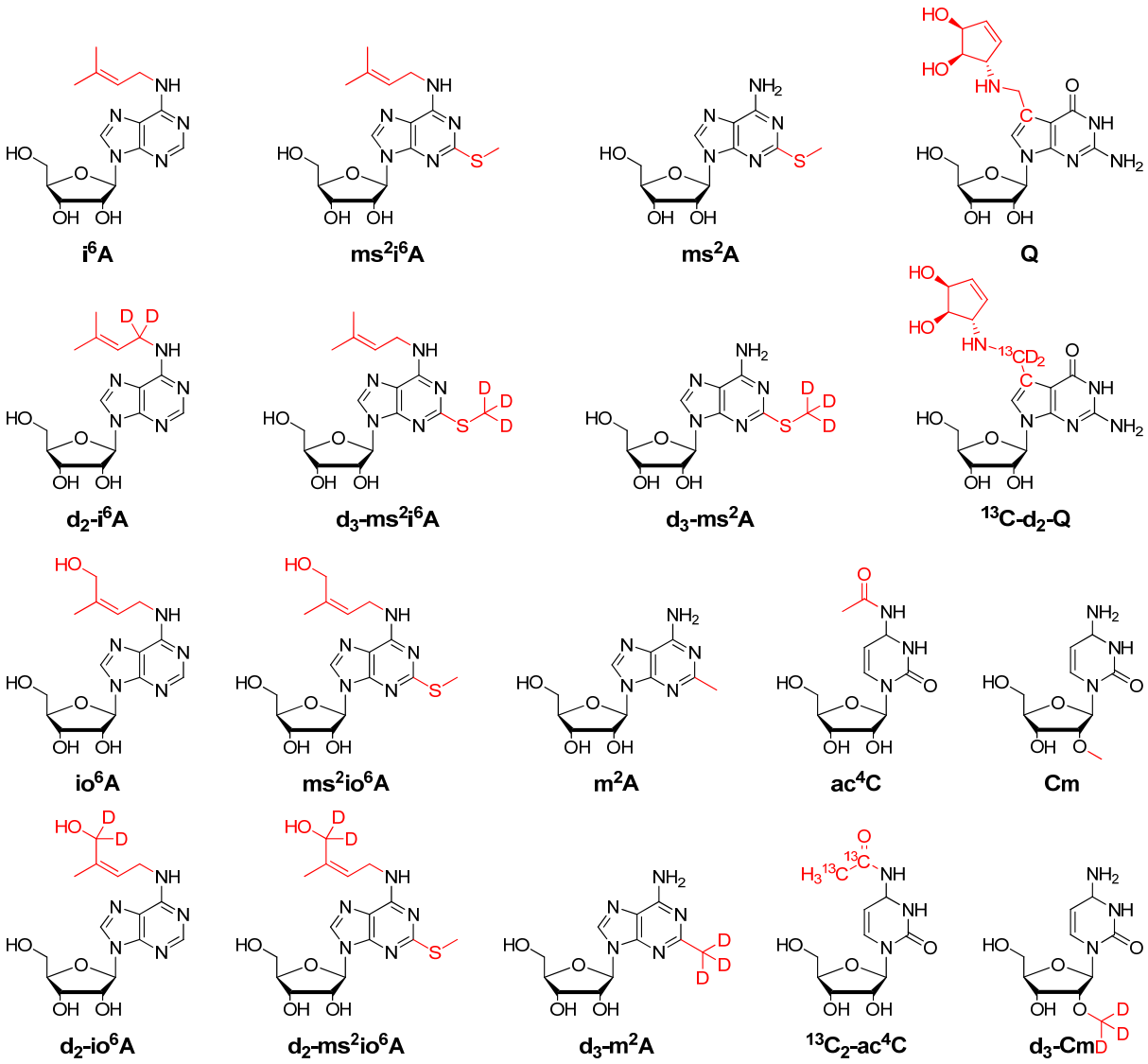
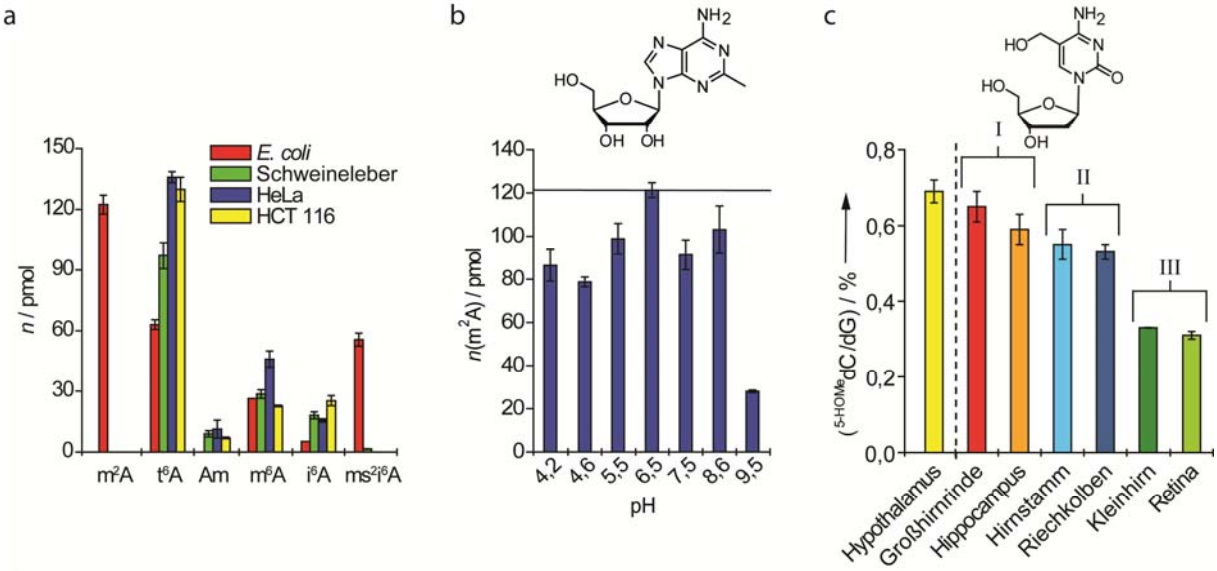


Abbildung 2: Übersicht der 18 Nukleoside, die im Rahmen dieser Arbeit für die massenspektrometrische Quantifizierung synthetisiert wurden.

Mit Hilfe der synthetisierten, modifizierten Nukleoside wurde genomische DNA und tRNA untersucht. In DNA wurden $^{5\text{-Me}}$dC und $^{5\text{-HOMe}}$dC quantifiziert, da vor kurzem von der Entdeckung des sechsten DNA Nukleosids $^{5\text{-HOMe}}$dC berichtet wurde, das von großem wissenschaftlichem Interesse ist. tRNA wurde als Repräsentant für RNAs gewählt, weil sie die größte Menge und Dichte an modifizierten Nukleosiden aufweist. Diese beeinflussen die Translation der mRNA am Ribosom, die einer der wichtigsten Prozesse in einer Zelle ist.

In einer ersten Studie wurde die Anwendbarkeit unserer Methode zusammen mit ihrer hohen Empfindlichkeit und Genauigkeit anhand von sechs modifizierten Nukleosiden aus tRNA von *E. coli*, zwei Zelllinien (HeLa und HCT-116) und Schweineleber bewiesen (Abbildung 3 a). Diese ersten Messungen zeigten zudem, dass die beiden untersuchten Zelllinien deutlich

unterschiedliche Modifikationsgrade besitzen und dass sich diese von den Modifikationsgraden in gesundem Gewebe unterscheiden. Das modifizierte Nukleosid ms^2i^6A, das in mitochondrialer aber nicht in zytosolischer tRNA vorkommt, wurde in den Zelllinien nicht gefunden, was in Übereinstimmung mit der Warburg Hypothese auf eine verminderte mitochondriale Aktivität in Krebszellen hinweist.



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Gewebe	Modifizierte Nukleoside								
	t^6A	m^6t^6A	i^6A	OHyW	Q	m^1G+Gm	$m^2{}_2G$	m^2G	m^1A
Leber	215	21,9	39,6	33,6	55,2	1.060	563	1.176	1.010
Kleinhirn	237	17,5	41,7	29,0	83,5	829	567	1.036	942
Rückenmark	199	11,8	36,0	26,0	50,8	800	457	971	911
Milz	182	13,9	30,7	26,1	42,7	690	464	794	835
Niere	178	15,8	30,7	21,1	32,3	816	451	896	872
Lunge	184	13,9	33,6	21,7	33,9	794	427	879	876
Zunge	171	11,3	25,7	15,2	21,9	699	396	794	846
Großhirn	166	10,5	26,1	20,3	57,2	587	406	682	733
Herz	175	11,0	29,0	12,0	29,6	638	373	673	797

Abbildung 3: Repräsentative Ergebnisse der vier Quantifizierungsstudien von modifizierten Nukleosiden, die im Rahmen dieser Doktorarbeit durchgeführt wurden.
a) Modifikationslevels in *E. coli*, Schweineleber und Zelllinien (HeLa und HCT-116) tRNA. b) m^2A Mengen in *E. coli* Zellen, die verschieden Umgebungs-pH-Werten ausgesetzt wurden. c) Farbcodierte tRNA Modifikationsgrade in Schweinegewebe. Fehlerbalken sind die Standardabweichungen von mindestens zwei Wiederholungen und mindestens sechs HPLC-MS Messungen.

In einer zweiten Studie wurden die Veränderungen des m^2A und m^6A Modifikationsgrads in *E. coli* durch externe Stimulation untersucht, um zu bestimmen, ob modifizierte Nukleoside an der Stressantwort von Zellen beteiligt sein könnten (Abbildung 3 b). Für eine Reihe an Stimuli wurden keine Veränderungen festgestellt. Dagegen führten die Veränderung des

Umgebungs-pH-Werts und die Behandlung mit Antibiotika zu einer Änderung des Modifikationsgrads, zum Beispiel einer 75 %igen Abnahme von m^2A bei pH 9.5.

Die Quantifizierung des neuen sechsten Nukleosids $^{5\text{-HOMe}}dC$ in DNA aus sieben verschiedenen Gehirnregionen mit unserer Methode zeigte, dass $^{5\text{-HOMe}}dC$ in allen untersuchten Proben vorkam und damit dass $^{5\text{-HOMe}}dC$ eine generelle post-replikativ gebildete DNA Modifikation vorrangig in neuronalem Gewebe ist (Abbildung 3 c).

Die größte Messreihe wurde mit zehn verschiedenen Schweinegeweben und zwölf modifizierten Nukleosiden durchgeführt (Abbildung 3 d). Die Menge der mitochondrialen Modifikation ms^2i^6A korreliert mit der mitochondrialen Aktivität der untersuchten Gewebe. Alle anderen Nukleoside weisen mit nur geringen Abweichungen eine übereinstimmende, graduelle Veränderung ihrer Mengen mit den Geweben auf. Diese korrelieren mit den Proteinsyntheseraten der Gewebe, was auf eine Regulation der Proteinsynthese durch tRNA Modifikationen hinweist. Basierend auf dieser Korrelation und der in der Literatur beschriebenen Beschleunigung des ratenbestimmenden Schritts der Translation durch modifizierte Nukleoside wurde das folgende, generelle Model aufgestellt:

In Prokaryoten bewirken modifizierte tRNA Nukleoside durch die Optimierung der Translationseffizienz einen Fitness- und Proliferationsvorteil. In Säugetieren sorgen die gewebespezifischen Modifikationsgrade für eine ideale Translationsrate und Proteinsynthesekapazität der einzelnen Gewebe.

Insgesamt wurde im Rahmen dieser Doktorarbeit eine Quantifizierungsmethode für modifizierte Nukleoside etabliert, diese für mehrere Studien eingesetzt und aus den erhaltenen Daten ein neues, allgemeines Konzept zur Funktion von tRNA Modifikationen erarbeitet.

3 Introduction

3.1 Natural modified nucleosides in DNA and RNA

Nucleic acids are the central "devices" in biological "information technology". Present textbooks usually content themselves with four nucleosides for deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) each: deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG), and deoxythymidine (dT) for DNA and adenosine (A), cytidine (C), guanosine (G), and uridine (U) for RNA. This general statement, although sufficient for an understanding of the biological functions and processes involving nucleic acids, is all but exhaustive. Both DNA and RNA contain so called modified nucleosides. These are derived from the canonical ones but feature further substituents. Their additional groups, which are attached either to the heterocycle or to the C2'OH, range from simple methyl groups to advanced ring systems. Modified nucleosides are involved in the most fundamental processes in cells: inheritance, transcription, and translation.

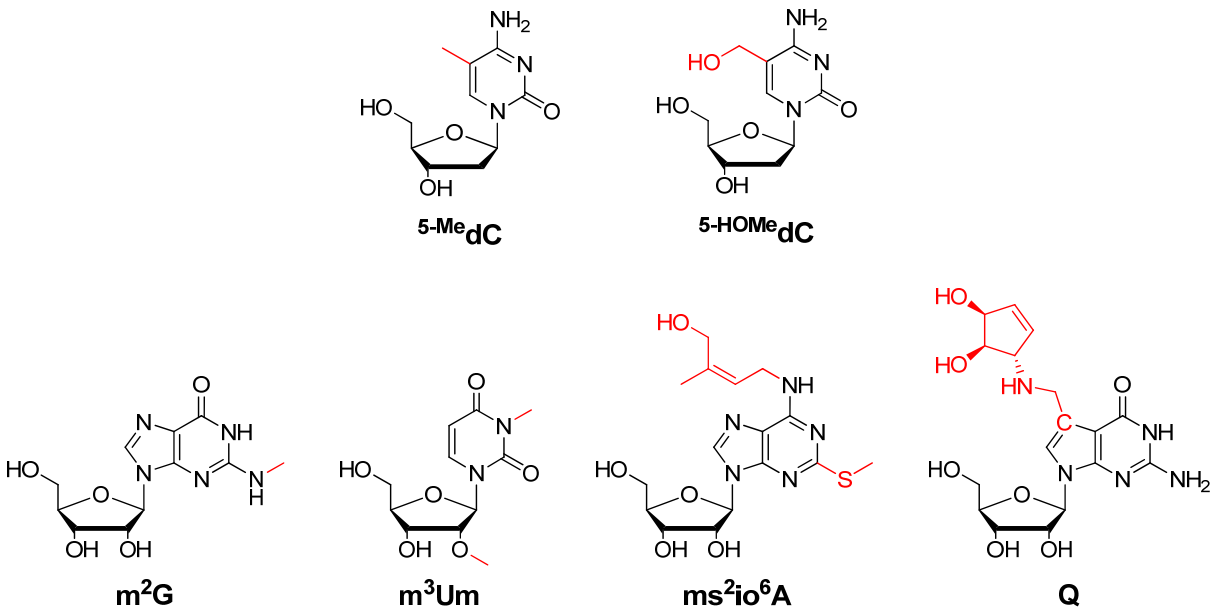


Figure 4: Structures of exemplary DNA and RNA modifications.
DNA modifications: $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC, upper row; RNA modifications: m^2G, m^3Um, ms^2io^6A, and queuosine (Q); bottom row. Alterations relative to the canonical nucleoside are indicated in red.

To date two modified nucleosides are known in DNA: 5-methyl deoxycytidine ($^{5\text{-Me}}$dC) and 5-hydroxymethyl deoxycytidine ($^{5\text{-HOMe}}$dC). The modification $^{5\text{-Me}}$dC is one of the key players in epigenetics and thus involved in the control of transcriptional activity.[1] Special

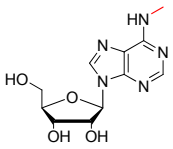
methyltransferases replace the H-atom at position C5 by a methyl group to form $^{5\text{-Me}}$dC.[2] Methylation occurs in CpG sequences and is mostly responsible for the silencing of genes.

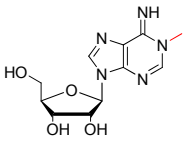
The modification $^{5\text{-HOMe}}$dC was established as a new post-replicatively formed DNA nucleoside in neuronal cells and stem cells in two recent back-to-back reports.[3] Nevertheless, the biosynthesis and the functions of $^{5\text{-HOMe}}$dC are still unknown.

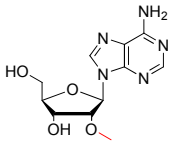
Modifications in RNA are by far more numerous. More than 100 different modified RNA nucleosides have been identified to date.[4] The highest modification level is found in tRNA. More than 80 tRNA modifications are presently known making this RNA class the first choice for the investigation of modified nucleosides. They form about 12 % of the tRNA nucleosides with a median of eight modifications per tRNA.[5] Correspondingly, numerous functional groups are used by nature to establish such a high number of different yet related molecules. To implement the various modifications in tRNA a broad range of chemistry has to be performed and numerous enzymes are involved. The structural diversity of tRNA modifications is also reflected by a myriad of functions they mediate. For instance they are involved in cellular processes such as mRNA decoding, cell development, translation control, development of diseases, occurrence of virulence, adaption to the environment, proliferation, and metabolism. An exhaustive discussion of all biological pathways and functions related to modified nucleosides thus by far exceeds the scope of this thesis. For the modifications synthesized during this thesis work an overview about their biosyntheses and functions is given as a short introduction preceding the discussion of their syntheses (chapter 7). In addition, they are discussed in numerous review articles.[5-6]

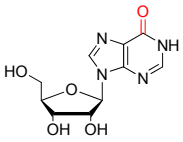
In general, all modified tRNA nucleosides effect the translation of genetic information from the messenger-RNA (mRNA) to proteins either by directly interacting with the mRNA and the ribosome or indirectly by influencing the three-dimensional structure of the tRNA. An impaired modification pattern of the tRNAs can thus severely impact the viability of the affected organism. In unicellular organisms this can lead to a reduced fitness or death.[7] In higher organisms it was shown that an altered tRNA modification pattern can cause severe diseases[8] and might be associated with progression and malignancy of cancer in humans.[9] As a foundation for the following discussion of this important topic all modified nucleosides in animals according to this work and Sprinzl and coworkers[4a, 4b] are summarized in Figure 5.

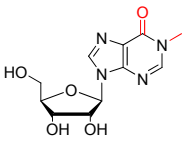
Adenosines

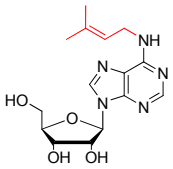

m^6A

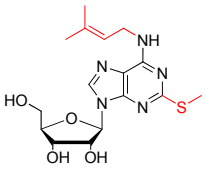

m^1A

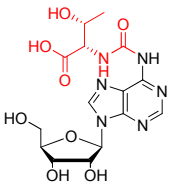

Am

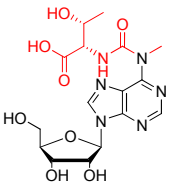

Inosine

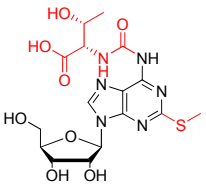

m^1I


i^6A

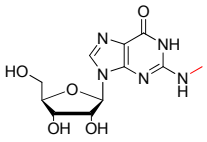

ms^2i^6A

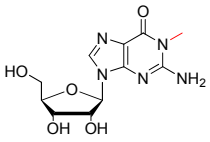

t^6A

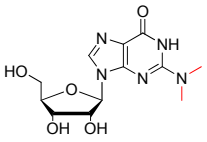

m^6t^6A

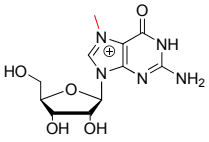

ms^2t^6A

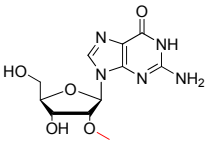
Guanosines

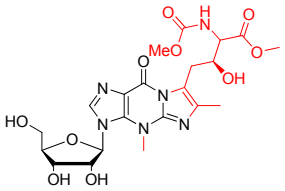

m^2G

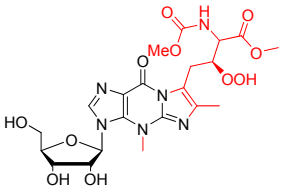

m^1G


m^2_2G

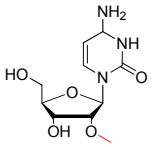

m^7G

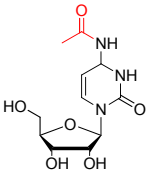

Gm

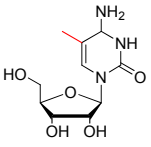

OHyW

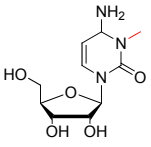

O_2yW

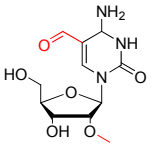
Cytidines


Cm

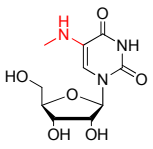

ac^4C

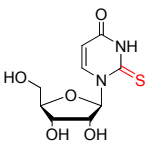

m^5C

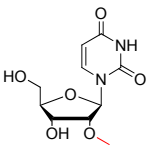

m^3C

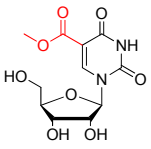

f^5Cm

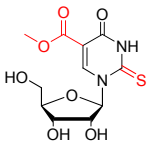
Uridines

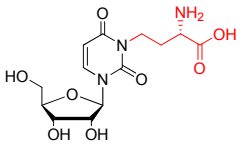

mnm^5U

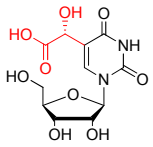

s^2U

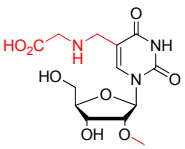

Um

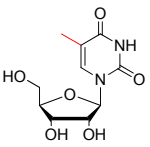

mcm^5U

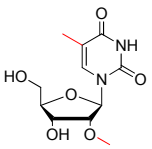

mcm^5s^2U

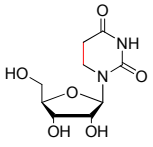

acp^3U

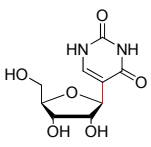

$mchm^5U$

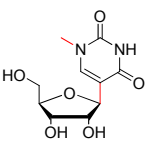

$cmnm^5Um$

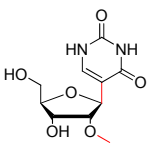

m^5U


m^5Um


Dihydrouridine


Ψ


$m^1Ψ$


Ψm

Deazaguanosines

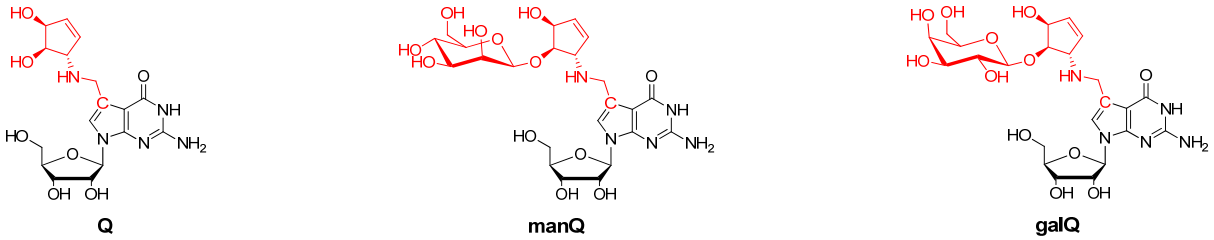


Figure 5: Summary of the modified nucleosides in animals according to this work and Sprinzl and coworkers.[4a, 4b] Alterations relative to the canonical nucleosides are indicated in red.

3.2 Modified tRNA nucleosides in cancer

3.2.1 tRNA methylation levels in tumor tissues

Neoplastic tissues exhibit an altered methylation pattern in comparison to their nearest healthy tissues.[10] Both hyper- and hypomodification have been observed, while the activity of tRNA methylating enzymes is generally increased in neoplastic tissues. The direction and the extent of the tRNA methylation level changes vary between tissues. Similar alterations have also been detected for other modified nucleosides like wybutosine.

When methylated bases were first observed in tRNA, they were suspected to control translation and thus to participate in differentiation including the development of cancer.[11] This assumption was supported by the finding that administration of carcinogenic dimethylnitrosamine to healthy animals induced development of tumors and, simultaneously, formation of methylated nucleosides not only in DNA but also in RNA.[12]

Inspired by these observations various groups investigated the activity of methyltransferases in healthy and in tumor tissues. The general approach included the isolation of tRNA from one species (generally bacteria or yeast) and collection of the methyltransferases from the tissue of interest. As different species contain different methylation patterns, methyltransferases from one species can further methylate tRNA from another species. In a large number of investigations preparations of methyltransferases from neoplastic tissues introduced two to ten times more methyl groups in tRNA samples than identical preparations from the closest healthy tissue.[10-11, 13] Similar results were obtained for studies with cell cultures. Comparison of mouse embryo and Syrian hamster kidney cell line BHK21 with their respective transformed cell lines revealed an increased methylation capacity of the transformed cells.[14] In addition, analysis of cells transformed by DNA viruses, which cause cancer, led to the same results.[15]

The first direct investigations of cancer-related tRNA alterations were based on the chromatographical separation of tRNA species, because changes in the tRNA methylation pattern are reflected in an altered chromatographical behavior.[10, 13] By now, numerous studies have shown, that usually one to five tRNA species exhibit an altered methylation pattern, when tRNA samples from healthy and neoplastic tissues are compared.[16] Nevertheless, no general rule could be deduced from these investigations but it is noteworthy that differences are commonly found for $tRNA^{Phe}$, $tRNA^{Tyr}$ and $tRNA^{Ser}$.[10, 17]

The most compelling proof for an increased methylation in tumor tissues comes from the direct determination of the methylation levels in tRNAs.[18] The most thorough investigation was performed by Viale and coworkers,[18c] who analyzed twelve different methylated nucleosides in eight distinct classes of brain cancer tissues and found significantly increased amounts of modifications in neoplastic tissues in comparison to normal brain (Table 1). Interestingly, a detailed look at the data in Table 1 (bold numbers) shows that higher grade tumors feature higher changes of the modified nucleoside levels. This was also found in several other investigations (chapter 3.2.2). Nevertheless, there are also examples from literature, where no changes of the modification contents in neoplastic tissues was observed.[9a, 13]

Nucleo-sides	Normal brain	Medullo-blastoma	Spongio-blastoma	Astrocytoma		Oligodendroglioma		Glio-blastoma	Neurinoma
				Grade		Grade			
				I	II – III	I	II – III		
m^5C	0.12	0.26	0.72	0.24	0.64	0.33	0.30	0.72	0.36
m^6A	0.18	0.45	0.54	0.52	0.86	0.38	0.62	1.14	0.28
$m^6{}_2A$	0.12	0.36	0.32	0.32	0.56	0.32	0.45	0.68	0.28
m^1G	0.02	0.46	0.88	0.48	0.84	0.57	0.52	1.58	0.42
m^2G	0.32	0.74	0.72	0.90	0.62	0.72	0.64	1.86	0.33
$m^2{}_2G$	0.54	0.52	1.04	0.76	0.98	0.66	0.73	1.13	0.48
T	0.32	0.08	0.36	0.64	0.42	0.51	0.36	0.62	0.21
m^1I	0.02	0.32	0.04	0.36	0.14	0.15	0.16	0.27	0.02
Cm	0.12	0.26	0.20	0.14	0.48	0.18	0.37	0.58	0.30
Am	0.04	0.06	0.08	0.08	0.35	0.12	0.16	0.42	0.17
Gm	0.25	0.30	0.38	0.26	0.84	0.44	0.60	0.90	0.64
Um	0.03	0.05	0.12	0.04	0.40	0.16	0.32	0.46	0.35
Sum	2.08	3.86	5.4	**4.74**	**7.13**	**4.54**	**5.23**	10.36	3.84

Table 1: Methylated nucleosides in tRNAs from human brain and brain tumors.[18c]

To allow the investigation of in principle all ribose-unmethylated modified nucleosides Randerath and coworkers developed a tritium derivative method.[9a] Using this approach no changes in the modification levels in healthy and in tumor brain tissues could initially be

detected.[19] Only after extension of the studies to liver and hepatomas differences were observed.[20] In contrast to the results discussed above an undermethylation and undermodification of tRNAs from neoplastic tissues was detected. Especially mitochondrial tRNAs were found to contain reduced amounts of modified nucleosides with higher reductions in a rapidly growing, highly malignant hepatoma than in a slowly growing one.[9a, 20b] Comparison of Morris hepatoma 5123D and normal liver cytoplasmic tRNA showed a 60 % reduction of m^2G in position 10 but not in position 6 of $tRNA^{Leu}$.[21] In the same neoplastic tissue a complete loss of Gm in $tRNA^{Ser}_{IGA}$ was detected.[22] Moreover, Nishimura and coworkers[23] uncovered an alteration of the methylation pattern in ascites hepatoma tRNAs in comparison to rat liver tRNAs. $tRNA^{Val}$ in hepatoma features Gm and C, whereas this tRNA from healthy tissue contains G and Cm in positions 18 and 32, respectively.

Similarly, a lack of the hypermodified nucleoside wybutosine has been detected in tumor tissues during several investigations.[24] Hyper- and hypomodifications for various modified nucleosides (e.g. m^7G, m^5C, and Q) in neoplastic tissues (e.g. Walker 256 carcinosarcoma, Morris Hepatoma 51231, and Ehrlich ascites cells) were observed in further studies.[17, 25] In non-small cell lung carcinomas increased expression levels of *hDUS2*, which is necessary for the biosynthesis of dihydrouridine in tRNA, were found and even could be associated with the course of disease.[26]

3.2.2 Hypomodification of tumor tissue tRNAs in respect to queuosine

The most intensively investigated tRNA modification in neoplastic tissues and in cancer cell lines is queuosine (Q, Figure 4). It is a hypermodified nucleoside, which occurs in the first position of the anticodon in $tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Asp}$, and $tRNA^{Tyr}$ of both eukaryotes and prokaryotes.[27] Mammals are not able to produce Q themselves but take up the corresponding base queuine from their nutrition or gut flora.[28] The base is then substituted for guanosine in tRNA at the wobble position by tRNA-guanine transglycosylases (TGTs).[29] Nucleoside Q is involved in numerous key processes, e.g. translation,[6c, 6n, 30] development,[28c, 31] cell proliferation,[32] metabolism,[32a] tyrosine biosynthesis,[33] and bacterial virulence.[34]

It has been found early on that tRNAs in neoplastic tissues and in cancer cell lines are often hypomodified in respect to Q or even completely lack this complex modification. For instance, it is less abundant in rat ascites hepatomas,[35] mouse lymphomas,[36] mouse xenografts,[37] human leukemias and lymphomas,[38] lung cancer tissues,[39] and ovarian malignant tumors.[40] Presence of guanosine for queuine was also found in tRNAs isolated from various cancer cell lines.[37, 41] A compilation of published Q-deficient neoplastic tissues

and cancer cell lines is shown in Table 2 and Table 3, respectively. Neoplastic tissues and cancer cell lines, which are fully modified with respect to Q but included in these publications, are also summarized.

tRNAs	Q-deficient neoplastic tissues	Fully Q-modified neoplastic tissues	Ref.
Bulk	AH7974 rat ascites hepatoma Morris hepatoma 7794A Ehrlich mouse ascites tumor SN36 mouse ascites tumor Sarcoma 180 mouse ascites tumor MM46 mouse ascites tumor	-	[35b]
Tyr, Asp, Asn, His	Ehrlich mouse ascites tumor	-	[35a]
His	Ehrlich mouse ascites tumor	-	[35c]
Bulk	rat ascites hepatoma AH7974	-	[35d]
Bulk	Dalton's lymphoma ascites tumour	-	[36]
His, Asp	Mouse colon adenocarcinoma Mouse xenografts rhabdomyosarcoma	-	[37]
Asp	Breast adenocarcinoma Breast adenocarcinoma Colon adenocarcinoma Kidney metastatic renal cell carcinoma Lung carcinoma, metastasis to liver Skin metastatic melanoma	Breast tumors Colon tumors Small bowel adenocarcinoma Kidney renal cell carcinoma Liver metastatic adenocarcinoma Lung adenocarcinoma Neck and head metastatic tumors Ovary metastatic tumors Pancreas metastatic tumors Prostate tumors Skin tumors Stomach tumors Chest wall recurrent adenocarcinoma	[37]
Bulk	Human leukemias and lymphomas	-	[38]
Bulk	Lung tumors	Lung tumors	[39]
Bulk	Dysgerminoma Granulosa cell tumor Teratoma malignisatum Serious adenocarcinoma Carcinoma anaplasticum Carcinoma endometriale	-	[40]
Bulk	Walker 256 carcinosarcoma	-	[25a]
Bulk	Astrocytomas Meningiomas	-	[42]

Table 2: Compilation of published Q-deficient tumor tissues.

tRNA	Q-deficient cancer cell lines	Fully Q-modified cancer cell lines	Ref.
Asp	GC 3/M GC 3/cl N-1000 T84 cultured in <5% FBS HL-60	Ei 28 T84 cultured in >9% FBS MDA-MB-436 MDA-MB-468 Y-79	[37]
His, Asp	TF-P10c FLc	-	[41a]
Bulk	$HxGC_3$ MCF-7	-	[41b]
Bulk	1548 Morris cell line	-	[41c]
Bulk	C3H10T1/2 RasC4	-	[41d]
Asp, Tyr, Asn, His	BALB/3T3	-	[43]
Bulk	HPB-ALL Jurkat Molt-4F PEER HUT78	-	[44]
Bulk	Friend murine erythroleukemia cells 745A and M18	-	[45]
Asp, Tyr, Asn, His	K562	-	[46]
Bulk	Friend murine erythroleukemia cells 745A, clone DS19	-	[47]
Asp, Tyr, Asn, His	Friend murine erythroleukemia cells 745A, clone GM86	-	[48]

Table 3: Compilation of published Q-deficient cancer cell lines.

In order to determine the Q-levels in tRNAs most studies listed here took advantage of the substrate specificity of *E. coli* TGT. The enzyme introduces queuine irreversibly into tRNA, but instead of queuine it also accepts guanine as a substrate for incorporation. Therefore, TGT can be used to introduce tritium labeled guanine in tRNAs, which are Q-deficient ((Q-)tRNA), but not in Q-containing tRNAs ((Q+)tRNA). Treatment of tRNA, which was extracted from the tissue of interest, with TGTs and radioactively labeled guanine and subsequent measurement of the radioactivity therefore allows determination of the amount of Q-deficiency in the tRNAs of the sample.[35b, 49]

3.2.2.1 Origins of queuosine hypomodification in neoplastic tissues

Several origins of the queuosine hypomodification have been proposed but a general description is still missing, mainly because the causes vary with the tissues examined. Based on the investigations presented hereafter the following origins for the hypomodification of tRNAs with respect to Q in neoplastic tissues have been proposed:[50]

- Decreased bioavailability of queuine and increased demand for queuine.
- Decreased activity of TGT.
- Presence of TGT inhibitors.
- Reduced queuine salvage.

The most likely cause is the reduced bioavailability of queuine in fast growing tissues.[9b, 50] tRNAs in rapidly dividing reticulocytes are hypomodified with respect to Q.[51] Similarly, regenerating liver tissue contains reduced levels of queuosine.[35b] In some cell lines addition of exogenous queuine causes repletion of (Q+)tRNA.[41d] Thus, the insufficient supply of rapidly growing tissues, like tumors, with queuine together with an increased turnover of tRNA in neoplastic tissues[52] most likely is responsible for the Q-hypomodification.

On the other hand, addition of large amounts of queuine to mice carrying Ehrlich tumors did not lead to normal Q-contents in their tRNA.[35c] This finding could be attributed to a reduced queuine salvage and decreased TGT activity, which was also detected in other mouse models.[37, 53] In contrast to these results several other investigations did not identify a similar absence of TGT activity in tumor tissues[35a, 35d] or even measured an increased expression of TGT in cancer cell lines.[44]

The increase of (Q-)tRNA might also be due to the production of substances, which interfere with queuosine formation in tumor tissues. Pteridines have been shown to inhibit TGTs and thus interfere with incorporation of queuine *in vivo*.[31b] Pteridine metabolism is modified in neoplastic tissues[54] and might therefore produce a TGT inhibitor in cancer cells. Parniak and coworkers[45] could show that treatment of murine erythroleukemia (MEL) cells with inducers of differentiation (e.g. DMSO) leads to an increase in tetrahydrobiopterin, a TGT inhibitor, and subsequently to higher levels of (Q-)tRNA. Alternatively, the uptake of queuine might be impaired in neoplastic tissues. For example, interferon has been shown to induce inhibition of queuine uptake in human fibroblast cells.[55]

3.2.2.2 Queuosine hypomodification and grade of malignancy

As illustrated in Table 2 and Table 3 lack of Q is common in neoplastic tissues and cancer cell lines but it is not a universal feature. Whether Q-hypomodification in cancerous tissues, if present, is somehow related to the development or propagation of cancer still remains an unanswered question. Such a relation is indicated by the involvement of queuosine in the phosphorylation of proteins and in the increase of the activity of proto oncogenes.[36b, 56] Furthermore, correlations between a decreased Q-content and grade of malignancy, tumor differentiation, and histopathological classification (Figure 6) imply an participation of Q-deficiency in the neoplastic process.[40]

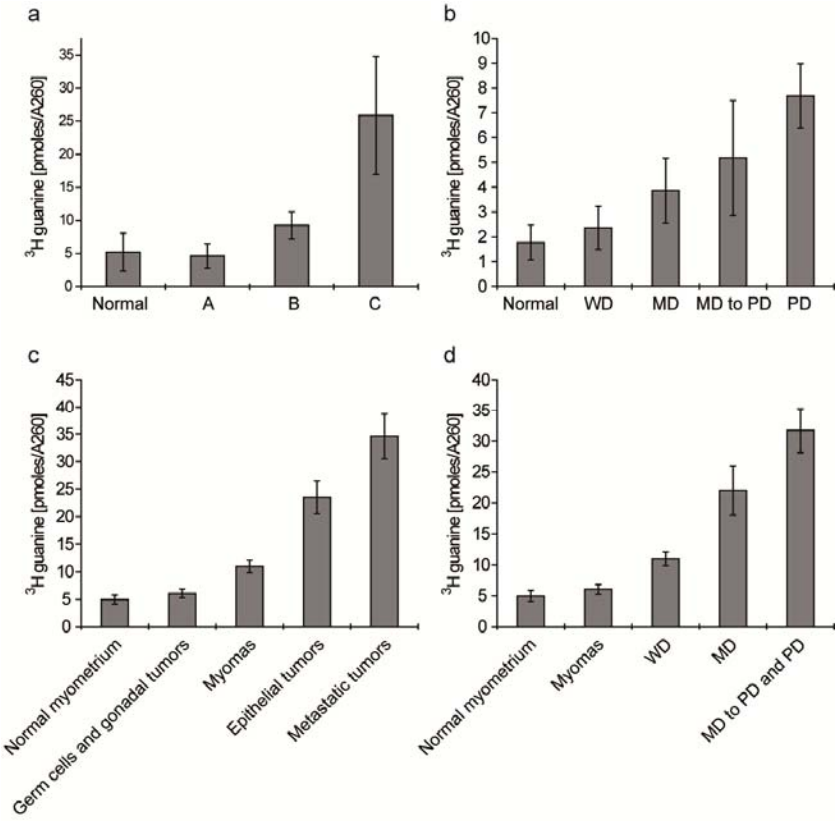


Figure 6: Correlations between (Q-)tRNA levels in tumor tissues and cancer classification.
a) Amounts of (Q-)tRNA in normal tissue and tumors grouped according to their prognostic stages A, B, and C (Binet classification); b) (Q-)tRNA levels in human lung cancer tissues and in the corresponding healthy tissues according to their different histopathological classifications; c) and d) Abundance of (Q-)tRNA in normal tissue and human ovarian tumors grouped according to their c) histopathological type and d) differentiation grade. (Q-)tRNA levels were measured by tritium guanine incorporation rates. WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated. Error bars indicate standard deviations.

Kersten and coworkers[38] detected similar amounts of (Q-)tRNA in lymphomas in favorable stages and in healthy tissues, while high grade lymphomas show higher levels of Q-deficient

tRNA. The amounts of (Q-)tRNA in lymphomas increased with the prognostic state of the disease (Binet classification, Figure 6 a).

Similarly, in tissues of lung cancer patients the amount of (Q-)tRNA was generally increased in tumors compared to samples from the respective healthy tissue.[39] In addition, a significant correlation between the state of the tumor differentiation and the (Q-)tRNA levels in these tumors was found (Figure 6 b).

Investigation of Q-deficiency in 16 malignant ovarian tumors, six myoma (benign tumors), and four normal myometrium (healthy tissue) revealed that the levels of (Q-)tRNA in healthy and in benign tumors did not differ significantly, whereas they were generally increased in malignant tumors.[40] Furthermore, the amounts of (Q-)tRNA correlated with the histopathological type of the tumor (Figure 6 c) as well as with its grade of differentiation (Figure 6 d).

In all three studies the detected correlations (Figure 6) were statistically significant. In addition, the level of Q-deficiency was also related to the course of disease and the survival rate.[38-40] Patients with high (Q-)tRNA levels died within the next one to four years and patients with medium Q-deficiency tumors suffered from recurrence of the disease. In contrast patients with the lowest (Q-)tRNA did not show any signs of recurrence until publication of the studies.

A similar correlation of (Q-)tRNA levels with grades of differentiation was measured in human K562 erythroleukemia cells.[46] Differentiation of the K562 cells was induced by different reagents (araC, sodium butyrate, hemin, and azaC). In parallel with the increase of differentiation the levels of (Q-)tRNAs declined. These results are in accord with the detection of an increase of (Q+)tRNA with differentiation in murine erythroleukemia cells.[47] As outlined above, there are also contradictory results showing a decrease of (Q+)tRNA with differentiation initiated by DMSO treatment of leukemia cells.[45, 48]

As Q-deficiency is expected to occur early during carcinogenesis, detection of Q-hypomodification in neoplastic tissues is a very promising candidate for straight-forward malignancy grading and prediction of the course of disease.[9b, 39-40]

3.2.3 Urinary nucleosides as tumor markers

Collection of information on the stage and progression of cancer as early as possible is highly important for an effective treatment. To allow this, tumor markers are necessary, which enable screenings and diagnosis at an very early stage ideally using noninvasive technologies.

As outlined above changes in the tRNA modification content occur early in carcinogenesis and are related to malignancy grading. These features make modified nucleosides outstanding tumor marker candidates. Urine samples are favored as they are easily available.

As early as 1960 modified nucleoside levels in the urine of cancer patients have been investigated and were shown to differ from levels of healthy volunteers.[57] Since that time numerous studies have shown an increased amount of modified nucleosides in urine samples from cancer patients in comparison to reference samples. Many of these investigations achieved an excellent diagnostic accuracy and showed the applicability of modified nucleosides as tumor markers.[58] These studies have also been summarized in several reviews.[59] Table 4 compiles investigations, which use modification levels as tumor markers and simultaneously correlate them with course and stage of disease. Although a few modified nucleosides, which do not originate from tRNA, were also studied in literature, here only modified tRNA nucleosides are discussed, because they are the central theme of this thesis.

Cancer type	Levels		Correlations			Ref.
	Stable	Increased	Stage of tumor	Disease activity	Course of disease	
Colon cancer	m^1A	Ψ, ac^4C, m^1I, m^2G, m^2_2G	Ψ, ac^4C, m^1I, m^2G, m^2_2G	-	-	[60]
Myelogenous leukemia	-	m^1I, Ψ, m^2_2G	m^1I, Ψ, m^2_2G	m^1I, Ψ, m^2_2G	m^1I, Ψ, m^2_2G	[61]
ALL	m^1A	m^1I, m^2_2G, Ψ, m^1G	-	m^1I, m^2_2G	m^1I, m^2_2G	[62]
Lymphomas	-	Ψ	Ψ	Ψ	Ψ	[63]
Lymphomas, leukemias	-	m^1A, Ψ	m^1A, Ψ	-	m^1A, Ψ	[64]
Breast cancer	-	m^2_2G, m^2G, m^1A, m^1I, m^1G	-	m^2_2G, m^2G, m^1A, m^1I, m^1G	-	[65]
Breast cancer	-	t^6A	-	t^6A	-	[66]
Breast cancer	m^7G	m^5C, ac^4C, m^1A, m^1I, Ψ	ac^4C, Ψ	m^1I, m^5C, ac^4C, Ψ	m^1A, m^1I	[67]
Small cell lung cancer	-	Ψ	Ψ	-	Ψ	[68]
Small cell lung cancer	-	m^1A, m^1I, m^2G, m^2_2G, I	-	-	m^1A, m^1I, m^2G, m^2_2G, I	[69]
Liver cancer	m^5U	m^1A, m^1I, m^1G, Ψ, ac^4C, m^2G	m^1A, m^1I, m^1G, Ψ, ac^4C, m^2G	-	-	[70]

Table 4: Modifications measured in urine samples of cancer patients and correlations with cancer. Levels: Stable or increased levels in comparison to urine samples from healthy humans. Correlations: Stage of tumor includes e.g. the tumor size and its differentiation state; disease activity for example refers to development of metastases and tumor growth rate; course of disease means the estimated or actual development of the disease. ALL: acute lymphoblastic leukemia.

Trewyn and coworkers[62a] showed in a study with 23 adults with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML) that all patients excreted increased amounts of m^1I, $m^2{}_2G$, Ψ, and m^1G in comparison to the normal population. Based on these levels patients with ALL can also be distinguished from patients with AML, because the obtained values for m^1I and $m^2{}_2G$ are significantly different. Furthermore, patients with ALL at initial diagnosis or in relapse excreted significantly more m^1I (2 times) and $m^2{}_2G$ (1.5 times) than patients in remission. Therefore, measurement of the excretion levels of m^1I and $m^2{}_2G$ allows to draw conclusions about disease activity. Interestingly, in a longitudinal study with one male ALL patient the initial values, which were obtained right after diagnosis, were significantly increased. In the course of treatment the modified nucleoside excretion declined with remission. When the patient relapsed after 17 months, he showed significantly elevated excretion levels similar to the ones measured initially.

Investigation of several modified nucleosides in parallel allows even more advanced conclusions about cancer progression in humans. Different combinations of the obtained data for m^5C, ac^4C, m^1A, m^1I, and Ψ levels of breast cancer patients correlated with different characteristics of the disease.[67] The excreted amounts of m^1A and m^1I were significantly increased in patients with a unfavorable prognosis and those who died within five years after sample collection. Patients with metastasis in bone marrow exhibited elevated levels of m^1I, m^5C, ac^4C, and Ψ excretion. Tumor size on the other hand correlated with the measured amounts of ac^4C and Ψ.

For leukemia,[61] breast cancer,[66] and nasopharyngeal cancer[71] modified nucleoside levels (e.g. m^1I, t^6A, Ψ, m^1A, m^1G, and m^2G) were found to be superior to common tumor markers. When both types of markers were used in parallel an increase of the modified nucleoside levels was detected considerably earlier than a change in the common tumor marker. Measurement of modified nucleoside excretion levels allowed the prediction of a relapse for breast cancer patients three to six months and for leukemia patients 42 days prior to its detection with standard tumor markers.

Kammerer and coworkers[72] analyzed the levels of 31 modified nucleosides in 212 urine samples (113 from breast cancer patients and 99 from healthy volunteers). Computational evaluation of the obtained data allowed breast cancer diagnosis with 88 % sensitivity and 90 % specificity. This is a considerable improvement compared to currently applied tumor markers, like the CA15-3 tumor marker, whose specificity is roughly the same but whose

sensitivity is significantly lower (50 %). Similar results were obtained, when the urine of bladder cancer patients (100 % sensitivity, 90 % specificity) was analyzed.[73]

3.3 Modified nucleosides in immunodeficiency diseases

3.3.1 Modified nucleoside levels in urine as biomarkers for AIDS

Modified nucleoside levels in urine have also been studied for a number of other diseases. For hepatitis, cirrhosis, acute pancreatitis, rheumatoid arthritis, and others modified nucleoside excretion levels did not differ significantly from those in reference samples, whereas patients with acute hepatitis, gout, and psoriasis show abnormal levels.[74] Similarly, bacterial infections lead to an increased excretion of modified nucleosides.[61-62] Generally, the differences are less common and reproducible across patients than those found for cancer.

Only for the important immunodeficiency diseases acquired immune deficiency syndrome (AIDS) and severe combined immunodeficiency (SCID) significant and reliable alterations have been detected.[59d] Children with adenosine deaminase-SCID were found to excrete elevated levels of the methylated adenosine derivatives m^1A, m^6A, Am, and m^6Am.[75] Similarly, patients with purine nucleoside phosphorylase-SCID exhibit elevated levels of I and G excretion, which follow the course of disease.[76]

Excretion of the nucleosides m^1I, m^2G, and ac^4C was elevated in adult male homosexuals with human immunodeficiency virus type 1 (HIV-1) antibodies compared to healthy males. Individuals with these antibodies have a 5-19 % likelihood to develop AIDS within the next five years. Measurement of modified nucleoside levels in urine might therefore help to identify individuals with a high risk of developing AIDS.[77] Individually elevated excretion levels were also measured for patients with lymphadenopathy syndrome, AIDS related complex and AIDS allowing approximation of the stage of disease.[77a] These studies again show that the parallel investigation of modified nucleosides gives reliable and meaningful data, which can be used as markers for high-impact diseases.

3.3.2 Fully modified $tRNA^{Lys,3}$ is the primer for HIV-1 reverse transcriptase

Apart from being a potential biomarker for AIDS modified nucleosides also play a key role in the initial stages of HIV replication. HIV-1 recruits $tRNA^{Lys,3}$ from its host during packaging, because it is the primer for reverse transcription of HIV-1 RNA in DNA. $tRNA^{Lys,3}$ anneals to the primer binding sequence (PBS) of the viral RNA and, starting from the $tRNA^{Lys,3}$ primer,

the reverse transcriptase copies the viral RNA into a single stranded DNA. Subsequently, the viral RNA is removed and a second primer binds to the DNA, from which the complementary DNA strand is produced. This way the viral RNA is faithfully translated into double stranded DNA, which can be introduced in the host DNA.[78]

Binding of $tRNA^{Lys,3}$ to the viral RNA occurs at different sequences (Figure 7 a). The actual primer is the 3'-end of the tRNA (red, nucleosides 59-76), which binds to the PBS region. The second sequence binding to the RNA of the virus is the 5'-end of the TΨC stem-loop (blue, nucleosides 48-55). It interacts with the primer activation sequence (PAS) in the viral nucleic acid. The HIV RNA also contains a loop region called HIV loop I, which pairs with the main part of the anticodon stem-loop (green, nucleosides 32-42). This duplex has been studies by NMR and is depicted in Figure 7 b.[79]

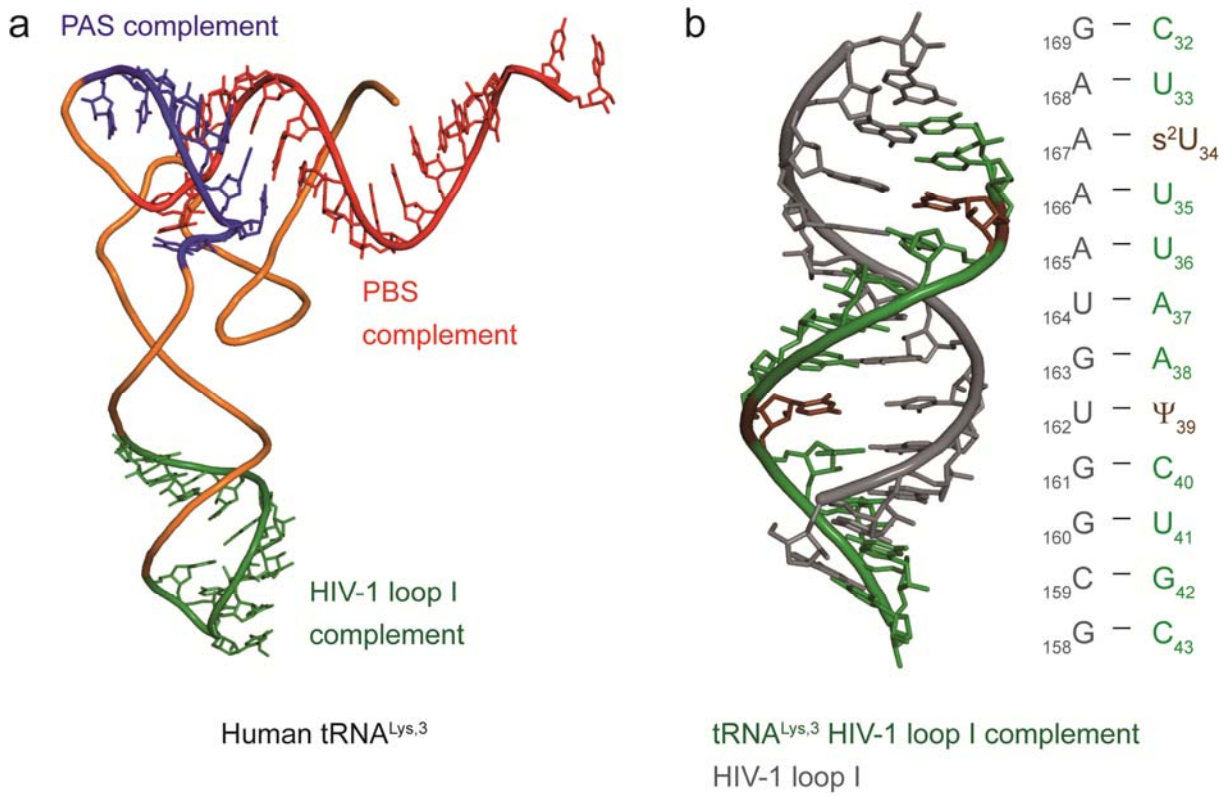


Figure 7: $tRNA^{Lys,3}$ and a duplex of the $tRNA^{Lys,3}$ HIV-1 loop I complement and HIV-1 loop I.
a) Color-coded crystal structure of human $tRNA^{Lys,3}$ with the distinct regions binding to the viral RNA.[80] red: complement to the viral primer binding sequence (PBS), blue: primer activation sequence (PAS) complement, green: HIV-1 loop I complement. b) Color-coded NMR structure of the synthetic $tRNA^{Lys,3}$ HIV-1 loop I complement (green) and HIV-1 loop I (gray) duplex.[79] The sequences of these strands are depicted at the right-hand side. Modification s^2U was incorporated instead of the naturally occurring mcm^5s^2U. The natural modification ms^2t^6A is replaced by unmodified adenosine. Modified nucleosides s^2U and Ψ are indicated in brown.

The modified nucleosides in the anticodon stem-loop region mcm^5s^2U, ms^2t^6A, and Ψ are important for the binding between $tRNA^{Lys,3}$ and the viral RNA. Unmodified $tRNA^{Lys,3}$ has almost no priming efficiency.[81] Modifications mcm^5s^2U and Ψ enhance the base stacking, which results in an increased stability of the RNA duplex between $tRNA^{Lys,3}$ and viral RNA. The sulfur moiety of mcm^5s^2U additionally decreases the flexibility of the free tRNA loop, further enhancing duplex formation.[79, 82] Davis and coworkers measured only a weakly stabilizing effect of the mcm^5 residue, whereas the methylthio substituent of ms^2t^6A significantly contributes to the stabilization of the binding between $tRNA^{Lys,3}$ and the RNA of the virus. The threonyl residue is suspected to destabilize the tRNA loop and thus to promote formation of an open tRNA conformation, which allows binding to the viral RNA.[83]

The modified nucleoside m^1A in the TΨC loop ensures efficacy and fidelity of the transcription.[78] The functions of the other modifications in $tRNA^{Lys,3}$ have not been investigated yet.

3.4 Lack of modified tRNA nucleosides induces mitochondrial diseases

The human mitochondrial (mt) genome contains 13 proteins, two ribosomal RNAs, and 22 tRNAs.[84] Mitochondrial genetics differs considerably from mendelian genetics. Mitochondria are polyploidy and they organize their mtDNAs in stable protein-DNA complexes called nucleoids, which may be exchanged between different mitochondria. Furthermore, they are only inherited *via* the maternal linage. Mitochondria in a cell constantly move, fuse, and divide. Turnover of mtDNA does not coincide with the cell cycle, though a connection has been suggested.[85]

The mutation rate in mtDNA is at least 10-fold higher than in nuclear DNA due to increased oxidative damage. This is caused by the proximity of the mtDNA to the inner mitochondrial membrane, where reactive oxygen species are produced during respiration, and by the lack of histones. Out of the 250 mutations known in mtDNA about 150 are correlated with diseases. The mutations are summarized in the MitoMAP database.[86] One in 10,000 people have a clinically manifested mtDNA disease and one in 6,000 people are at risk to develop one.[85] From the pathogenic mutations two-thirds are present in tRNA genes, although only a small part (10 %) of the mtDNA encodes tRNAs.[87] This clearly shows the importance of integrity of the mitochondrial tRNAs for the fitness of mitochondria.

Typical diseases caused by mtDNA point mutations are myoclonus epilepsy associated with ragged-red fibers (MERRF) and mitochondrial encephalomyopathies, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). An A to G transition occurs at position 8344 of $tRNA^{Lys}$ in most MERRF patients.[88] 80 % of the MELAS patients have a point mutation in position 3243 of the gene for $tRNA^{Leu}$ exchanging an A for a G. In addition, 10 % of these patients exhibit a T to C mutation in position 3271 of $tRNA^{Lys}$.[89]

Watanabe and coworkers[90] could show, that mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu}$ contain the two unusual modified nucleosides τm^5U and τm^5s^2U, respectively,[8] while MELAS and MERRF mutant cells do not feature these modifications(Figure 8).[91] Probably, the mutated tRNAs are no longer recognized by the modifying enzymes.[90b]

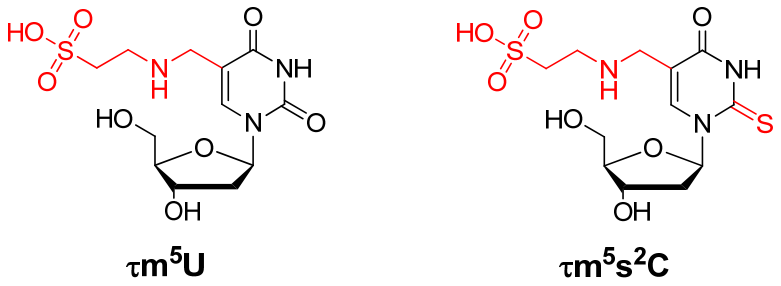


Figure 8: Modified nucleosides τm^5U and τm^5s^2U present in mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu}$, respectively.
Alterations from the canonical nucleoside uridine are indicated in red.

Fully τm^5U-modified $tRNA^{Lys}$ decodes the codons AAA and AAG. $tRNA^{Lys}$ isolated from MERFF-mutant cells, which lacks τm^5U, showed almost no ^{3}H-lysine incorporation in *in vitro* translation assays for both codons and a complete loss of protein synthesis activity.[92] *In vivo* MERRF-mutant cybrid cells show a severely reduced mitochondrial protein synthesis.[93] Therefore, one can conclude, that the MERFF phenotype is caused by a deficiency of τm^5U in mitochondrial $tRNA^{Lys}$.

Similarly, lack of τm^5s^2U in $tRNA^{Leu}$ of MELAS patients results in a severely reduced translation of the codon UUG. The second leucine codon UUA is still fully translated.[94] Nevertheless, lack of τm^5s^2U causes a severe phenotype, which is linked to a reduced expression of a respiratory chain complex I protein (ND6) in MELAS patients.[95] As this protein contains a large number of UUG codons, its expression is strongly impaired, when $tRNA^{Leu}$ lacks τm^5s^2U.[90b] In addition, Florentz and coworkers[96] measured a decreased level of m^2G in $tRNA^{Leu}$ isolated from the cell line 43B, which contains the MELAS specific

mtDNA mutation. It has been suggested that this deficiency is related to the expression of the MELAS phenotype by altering the binding of the tRNA to its partners during translation.

An impact of the pathogenic T7512C and G7497A point mutations in $tRNA^{Ser}$ on the decoration of this tRNA with modifications has been suggested as a reason for the reduction of its level in osteosarcoma cybrid cells to only 10 % of its amount in reference cells.[97] In two other studies the lack of activity of two tRNA modifying enzymes, TRMU and PUS1, due to mutations of their corresponding genes has been shown to lead to aminoglycoside-induced and nonsyndromic deafness[98] and mitochondrial myopathy and sideroblastic anemia (MLASA),[99] respectively.

3.5 Summary

The amounts of modified nucleosides in tRNAs, which were isolated from neoplastic tissues and cancer cell lines often differ to reference tRNAs collected from healthy tissues. Furthermore, correlations between the amount of modified tRNA nucleosides in tumors and their grade of malignancy and the course of disease were established.

The abundances of modified nucleosides in urine can be applied as tumor markers with exceptional sensitivity and specificity outranging tumor markers, which are currently applied. Moreover, modified nucleoside excretion levels of AIDS patients correlated with the stage of their disease.

The mitochondrial diseases MERFF and MELAS have been shown to originate from a lack of the modified nucleosides $\tau m^5 s^2 U$ and $\tau m^5 U$ in mitochondrial tRNA.

Taken together, these investigations clearly demonstrate that the detailed knowledge about modified nucleoside levels in tissues will provide penetrating insights into the origin of diseases like cancer, AIDS, and mitochondrial dysfunctions. Furthermore, they will help to establish modified nucleosides as improved biomarkers for cancer and AIDS. This can only be achieved, if a large number of modified nucleosides can be studied in parallel with outstanding accuracy.

4 Aims of the project

In living organisms RNAs have many functions. They are able to perform catalysis,[100] serve as guide structure for proteins,[101] are involved in protein synthesis (rRNA and tRNA) and cancer development,[102] and regulate gene expression,[103] proliferation,[102, 103c] and apoptosis.[104] The vast complexity of functions either performed or guided by the RNA led us to speculate that their chemical diversity may be of more fundamental importance than recognized today.

As outlined in the introduction, most likely, a thorough understanding of the biosyntheses and functions of modified nucleosides would tremendously advance medicinal analytics and our understanding of important diseases like cancer and AIDS, because the decoration of tRNAs with modified nucleosides differs significantly between neoplastic and healthy tissues.

Omics-research has led to great advances in biochemistry in recent years. Its central concept is the study of cellular components not isolated but in the context of whole biological systems and as a collective rather than individually.[105] For modified tRNA nucleosides the contexts are the investigated cells or tissues and the collective is the isolated bulk tRNA.

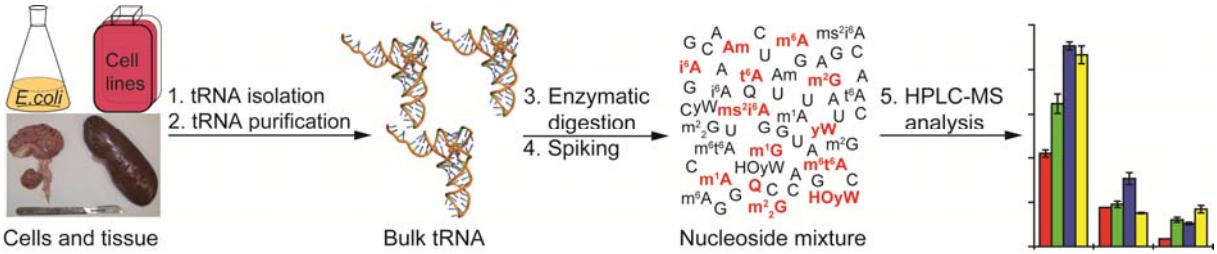


Figure 9: Sketch of the quantification method for modified tRNA nucleosides aspired to establish during this thesis work.

Therefore, the aim of this thesis was the establishment and evaluation of a method that allows the parallel quantification of in principle all modified nucleosides from tRNA with high accuracy and sensitivity. For this purpose the workflow depicted in Figure 9 was envisioned. After isolation and purification of bulk tRNA from the cells of interest its enzymatic digestion will yield a mixture of modified and unmodified nucleosides. To this solution isotopically labeled derivatives of the nucleosides of interest will be added. This spiked mixture will then be analyzed by HPLC-MS.

To establish this procedure several intermediate goals have to be reached:

- Establishment of a protocol for the isolation of bulk tRNA from cells.
- Creation of a method for its purification.
- Synthesis of the natural and heavy atom labeled modified nucleosides of interest.
- Creation of an HPLC approach for the separation of modified nucleosides.
- Establishment of a mass spectrometric method for their analysis.

This quantification method will be established and evaluated, by the measurement of modified nucleoside levels in *E. coli*, cancer cell lines, and healthy liver tissue.

With a reliable and sensitive method in hands modified nucleosides in *E. coli* exposed to different external stimulations will be quantified to determine their contribution to cellular stress response.

The most thorough investigation was envisioned for healthy pork tissues. These data will probably lead to novel insights in the functions of modified nucleosides in mammals.

5 Quantification of modified tRNA nucleosides

5.1 Establishment of a stable isotope dilution method for quantification of tRNA modifications

Quantitative studies of modified RNA nucleosides have already been achieved in the 60s and 70s using HPLC/UV approaches.[59a, 106] Other methods utilized for the quantification of modified nucleosides are tritium post-labeling,[107] ^{14}C-aminoacylation,[108] ^{3}H-aminoacylation,[109] and 2D-chromatography.[25d] Since these methods either have too high labor costs, do not provide the necessary sensitivity and accuracy, or do not allow non-invasive quantification, an HPLC-MS approach was selected. Its feasibility for the quantification of two uridine derivatives has been shown before.[110]

Omics research has evolved from a qualitative method, which facilitates comparison of data collected during a series of experiments, to a quantitative technique yielding absolute, generally comparable values. Analogous to proteomics, where the use of isotopically labeled media (e.g. SILAC) allows quantification,[111] recently, isotope-labeled HPLC-MS was used in metabolomics for a parallel determination of more than 100 absolute intracellular metabolite concentrations.[112] Thus, in this thesis work a stable isotope dilution HPLC-MS approach was selected as an ideal combination of high accuracy, outstanding sensitivity, and minimal work load once the method is established.

The general approach is depicted in Figure 10 and consists of five main steps:

1. tRNA isolation
2. tRNA purification
3. Enzymatic tRNA digestion
4. Spiking with stable isotope labeled nucleosides
5. Analysis by HPLC-ESI-MS

A detailed description of this procedure is given in chapter 10.4.2.

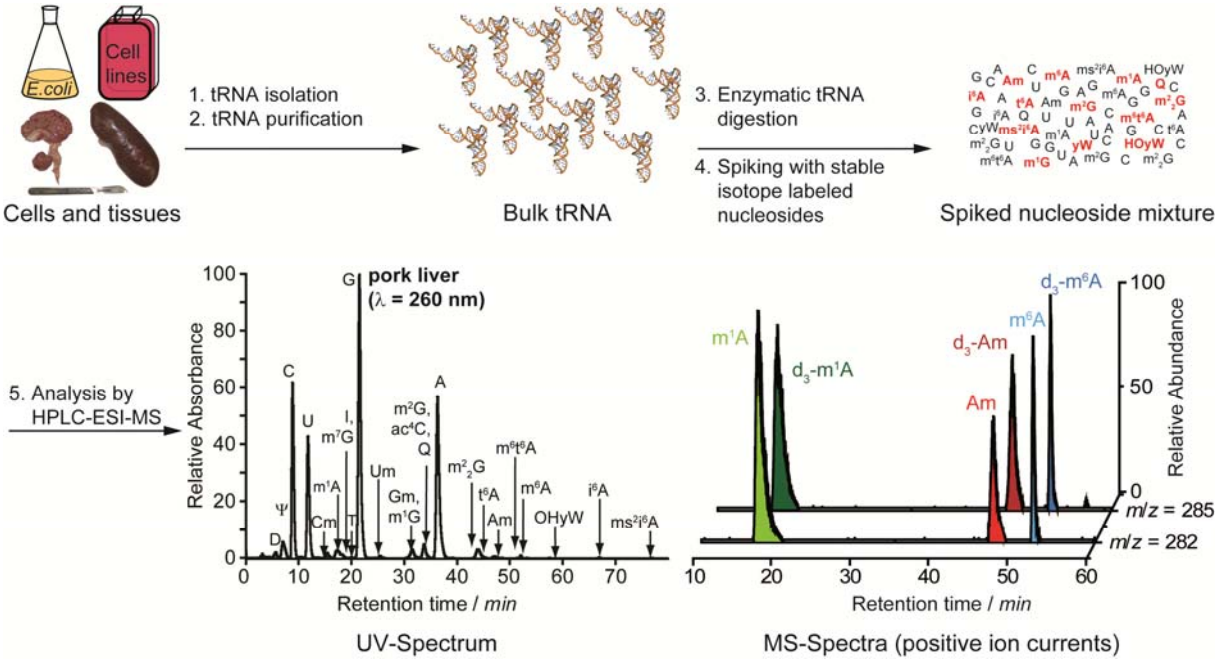


Figure 10: Schematic outline of the quantification method for modified tRNA nucleosides.
1. tRNA isolation: phenol extraction and precipitation of long chain nucleic acids; 2. tRNA purification: PD10 chromatography, anion exchange chromatography; 3. Enzymatic tRNA digestion: conversion of bulk tRNA mixture to nucleosides; 4. Spiking: addition of isotope labeled nucleosides; black symbols represent natural nucleosides; red symbols indicate added isotope labeled nucleosides; 5. HPLC-ESI-MS analysis: chromatographic separation and mass spectrometric analysis of the nucleoside mixture.

5.1.1 tRNA isolation

A thorough literature search showed that all methods for the isolation of tRNA from bacteria are similar. They use a phenol extraction and a precipitation step. A detailed discussion of tRNA extraction has been published,[106b] which was chosen as a starting point for establishing a tRNA extraction protocol. In order to disrupt the cell walls and to separate the nucleic acids from most other cell components, e.g. proteins, the first step is a phenol extraction. Nucleic acids are retained in the aqueous layer, while other cell material is mostly found in the phenol layer. This is followed by extraction steps with phenol, water, and chloroform to ensure minimal residual phenol and cell compounds in the aqueous layer and minimal remaining nucleic acids in the phenol layer.

Mature tRNAs have a length between 73 and 94 nucleotides and are therefore considerably shorter than other cellular nucleic acids, e.g. rRNA, mRNA, and DNA. This difference allows separation of tRNA from all other nucleic acids *via* a precipitation step. 2 M LiCl was found to be an ideal salt concentration to precipitate long nucleic acids but not shorter ones like tRNA.[106b] After centrifugation crude tRNA in the supernatant is isolated from most other nucleic acids in the pellet.

The method described here has been used for the isolation of tRNA not only from bacterial cells but also from yeast, plant, cancer, and pork tissue cells. For these only slight modifications of the procedure were necessary. Therefore, a scalable, straight forward method for the extraction of bulk tRNA from almost all kinds of cells has been established.

5.1.2 tRNA purification

The obtained, crude tRNA was subjected to purification by anion exchange chromatography. The crude mixture is applied to a column with a stationary phase containing positively charged residues. The negatively charged nucleic acids bind to the stationary phase and are therefore retained. Uncharged and positively charged material is washed from the column with a buffer containing a low salt concentration. Once the undesired material is removed the nucleic acids are eluted from the column with a buffer system containing an increased salt concentration. The salt concentration needed to break the binding of the nucleic acids to the stationary phase depends on the extent of their negative charge and is therefore directly related to their length. Thus, by gradually increasing the salt concentration in the eluant nucleic acids of different lengths can be separated. Remaining mRNA and most rRNAs are therefore efficiently removed. In addition, the obtained chromatogram allows to draw conclusions about the purity of the tRNA. A representative anion exchange chromatogram is shown in Figure 11.

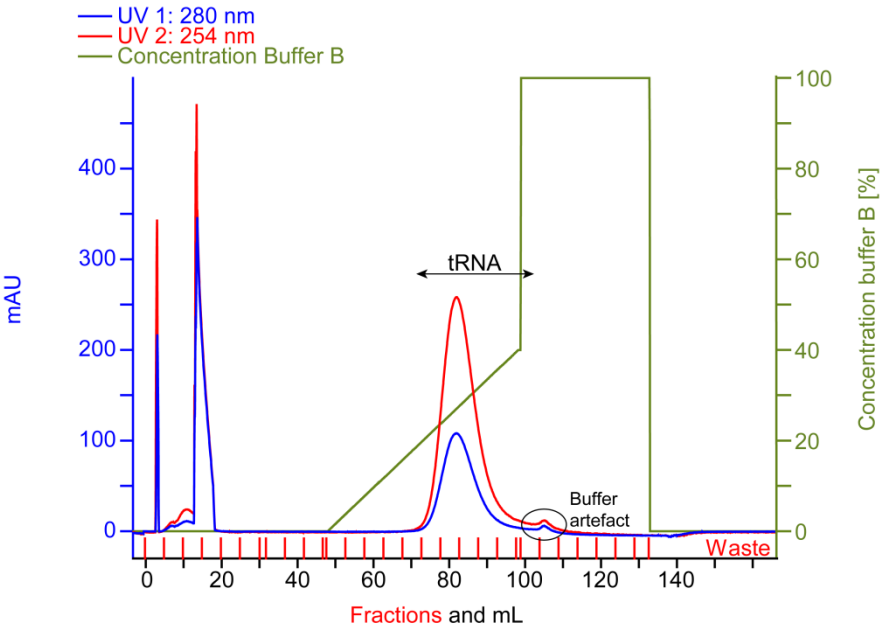


Figure 11: Representative anion exchange chromatogram.
The UV-chromatograms at 280 nm and 254 nm are shown as blue and red lines, respectively. On the y-axis the UV-absorption is plotted in mAU. The green line represents the salt concentration of the elution buffer B in % used during the chromatography with the highest value being 1 M NaCl.

A certain class of rRNAs, the so-called 5 S rRNA, has 120 nucleotides and can therefore not be removed by size-dependent methods. Nevertheless, 5 S rRNA does not contain any modified nucleosides and is present only in very low amounts. Hence, it does not influence the results.

Purification of tRNA as described above was successfully used for tRNA isolated from bacterial, yeast, plant, and cancer cells. When crude tRNA from tissue samples was directly applied to the anion exchange column it did not bind to the column, because the salt concentration of the sample itself was too high. For the necessary desalting of the sample a size exclusion chromatography step was introduced prior to anion exchange chromatography. A standard desalting column for biomolecules with a cross-linked dextran gel (Sephadex), namely a PD10 column, was used.

The purity of the obtained bulk tRNA was analyzed by gel electrophoresis either using 1 % TBE agarose gels or NaOAc gels with 7.5 % acrylamide. A representative, randomly picked TBE agarose gel is shown in Figure 12. Lanes b – j contain purified tRNA, while lane k shows crude RNA. Comparison of lanes b – j with lane k clearly shows that tRNA, which was purified with the method established in the course of this thesis, is highly enriched and purified. The slight bands visible in the middle of the gel in lanes b, c, e, and f are remaining rRNAs with an estimated content of less than one percent of the sample.

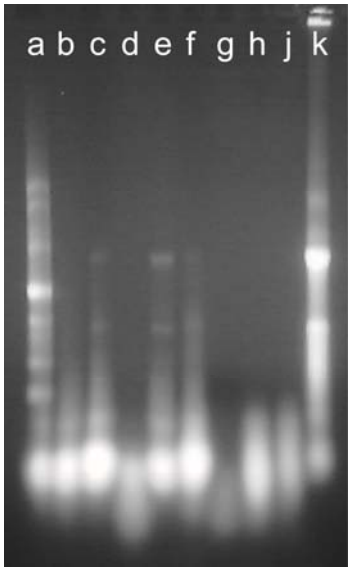


Figure 12: Gel electrophoresis (1 % TBE agarose) of tRNA samples from pork tissue.
High sample loadings (~5000 ng) were used to visualize low amounts of contamination. a) marker; b) cerebellum tRNA sample 1; c) cerebellum tRNA sample 2; d) cerebrum tRNA sample 1; e) cerebellum tRNA sample 3; f) spleen tRNA sample 1; g) spleen tRNA sample 2; h) thyroid gland tRNA sample 1; j) thyroid gland tRNA sample 2; k) crude RNA extract sample obtained from V. Welzmiller.

5.1.3 Enzymatic tRNA digestion

With pure tRNA in hands the enzymatic digestion of these polynucleotides to single nucleosides was optimized. As starting points the standard method established in the group for digestion of DNA oligonucleotides[113] and methods from literature for the digestion of RNA were chosen.[106f, 114] A thorough screening of digestion conditions led to the following procedure outlined in chapter 10.4.2.8. In brief a tRNA sample of 12 μg was treated for 3 h with nuclease S1 and for additional 3 h with antarctic phosphatase and snake venom phosphodiesterase I. Absence of nucleotides and dinucleotides proved complete digestion of the tRNA samples by mass-spectrometry.[114]

5.1.4 Spiking with stable isotope labeled standards

Standard analysis by mass spectrometry allows to draw conclusions about the presence of the analytes but it does not provide quantitative information on these. The number of detected analytes depends on a large set of parameters not exactly definable and most importantly not reproducible between experiments. To turn mass spectrometry quantitative an internal standard is needed for every analyte, which has the same behavior in the mass spectrometer as the analyte. Therefore, the physical properties of the standard and the analyte have to match as closely as possible. In addition, standard and analyte have to reach the mass spectrometer at the same time to be analyzed under the same conditions. But they still have to be distinguishable. For HPLC-MS quantification this means that they have to have the same retention time to reach the spectrometer simultaneously and a different mass to be specifiable. These prerequisites are best met by isotopically labeled derivatives of the analyte. Therefore, heavy atom labeled derivatives of the modified tRNA nucleosides in question had to be synthesized (chapter 7.1). Addition of these labeled standards to the nucleoside mixture obtained from tRNA digestion enabled quantification of the natural tRNA modifications by HPLC-MS.

After enzymatic digestion of the tRNA sample a mixture of different modified and canonical nucleosides was obtained. To allow quantification of the natural tRNA modifications (analytes) in this mixture by HPLC-MS the corresponding heavy atom labeled derivatives (standards) were added. To exclude systematic errors care was taken to add different amounts of the isotopically labeled standard to the nucleoside mixture when repeated measurements of the same bulk tRNA was performed.

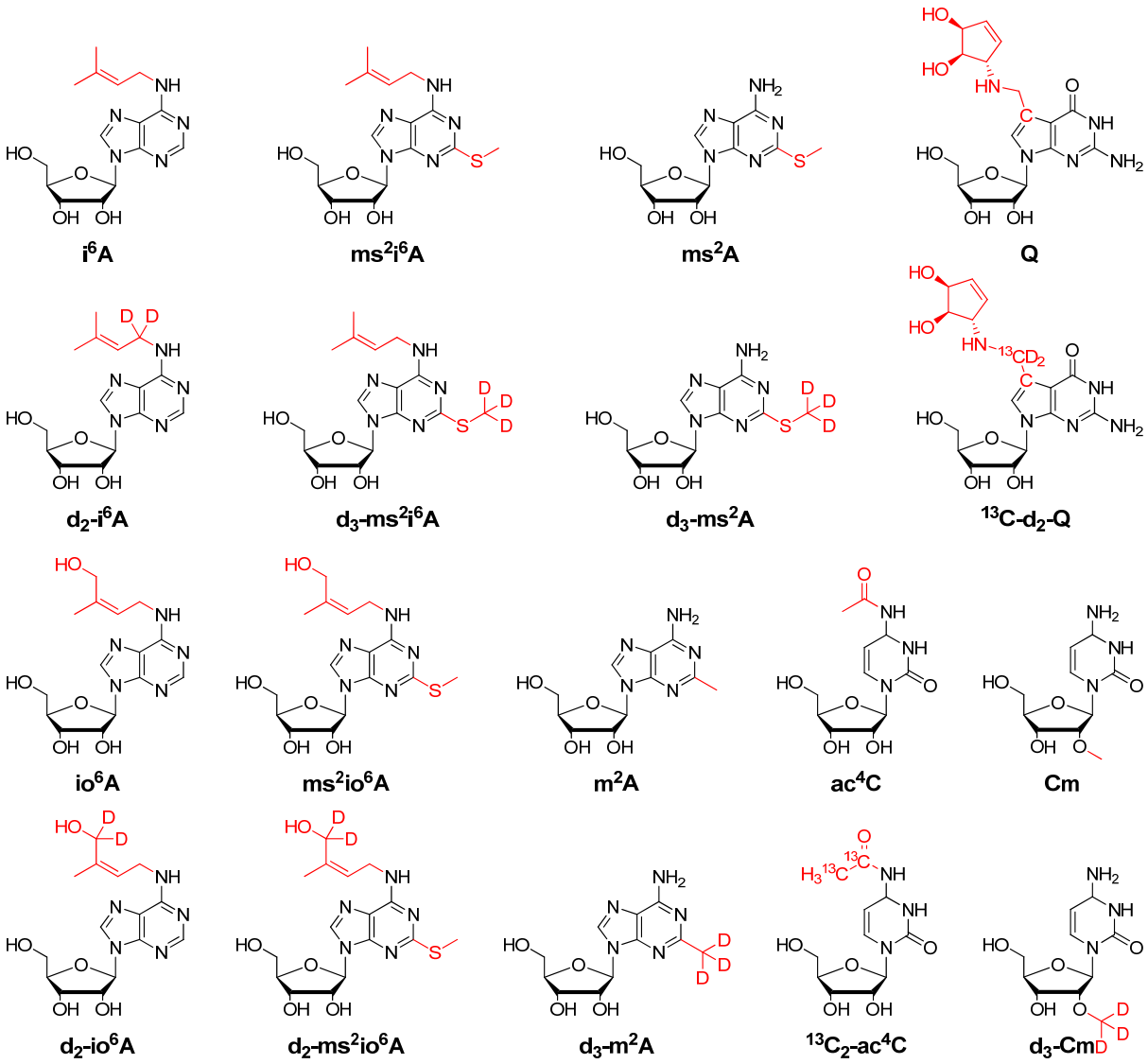


Figure 13: Compilation of the nucleosides and their isotopically labeled derivatives prepared for mass spectrometric quantification in the course of this thesis work.

During the course of this thesis nine modified nucleosides and their isotopically labeled derivatives were prepared (Figure 13). Their syntheses are discussed in chapter 7. All but the cytidine derivatives and ms^2A have been used for HPLC-MS analysis until now. Other nucleosides used for quantification experiments were prepared by D. Globisch, P. Thumbs, I. Thoma, and A. Hienzsch. All labeled nucleosides utilized for the study of modified nucleosides are presented in Figure 14 with their respective positions in tRNA. Modifications adjacent to the anticodon loop are m^6A, t^6A, m^6t^6A, OHyW, i^6A, m^1G, ms^2i^6A, yW, and m^2A. Q is found in the anticodon loop, while m^1A, Am, m^1G, m^2G, and $m^2{}_2G$ are present in other positions.

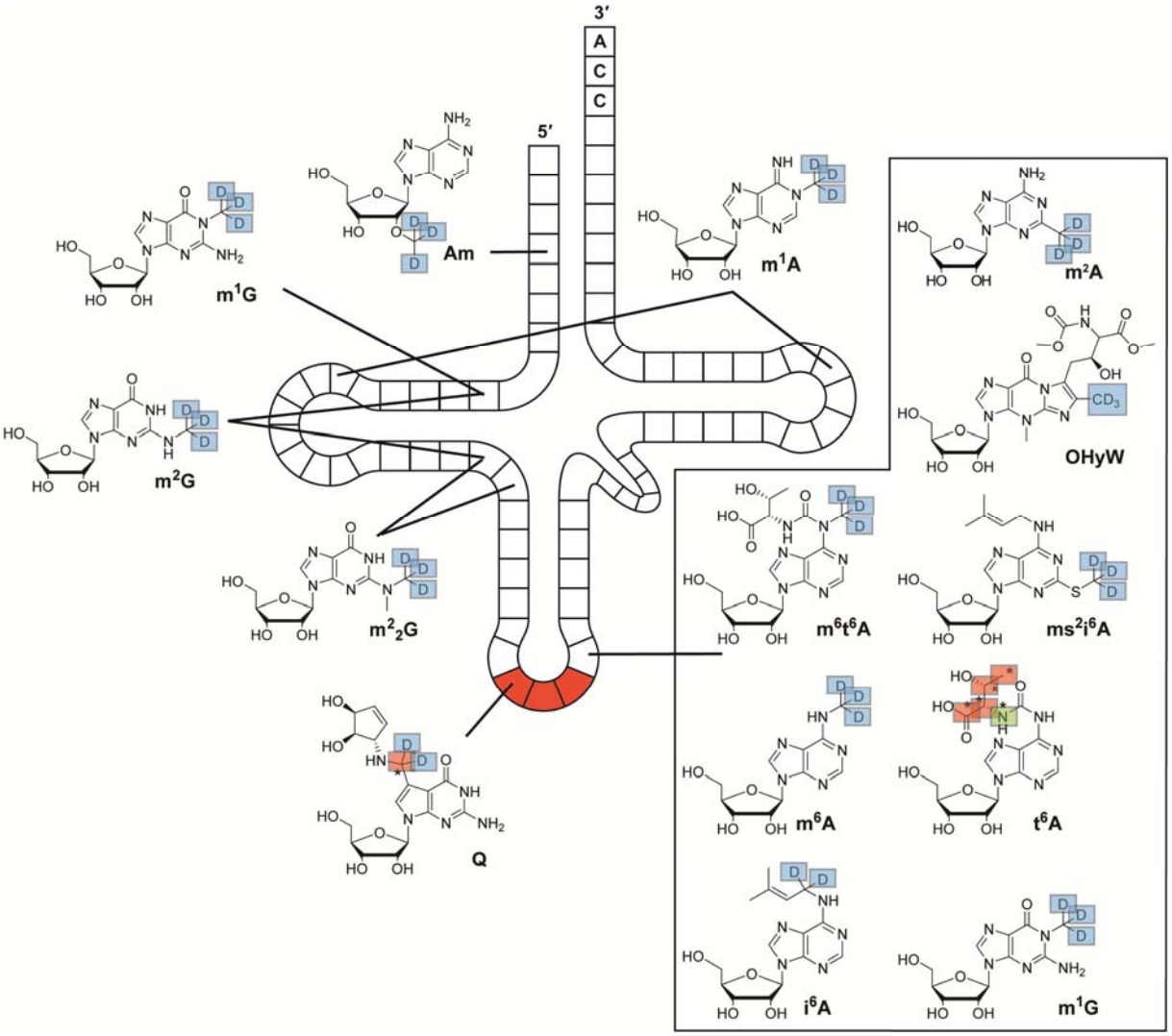


Figure 14: Modified nucleosides investigated during this thesis and their positions in tRNA.
The respective positions are indicated in a schematic tRNA. The isotope labels are marked in color. D = Deuterium, * = ^{13}C or * = ^{15}N.

5.1.5 Analysis by HPLC-ESI-MS

5.1.5.1 Calibration curves

For the calculation of the exact analyte levels from the mass spectrometric data calibration curve equations are necessary. Their determination was executed as follows: Solutions of the labeled and the unlabeled nucleoside with defined concentration were prepared. In this way defined amounts of substance can easily be handled. Mass spectrometric data were collected for five different concentration ratios of the labeled and corresponding unlabeled nucleoside (Figure 15). For each concentration ratio an average value of three independent measurements was determined using three mixtures of the labeled nucleoside solution with one out of three solutions with different concentrations of the corresponding unlabeled nucleosides. By

plotting the weight ratios against the experimentally determined ion count area ratios the calibration curve and its corresponding calibration equation (Figure 15) were obtained.

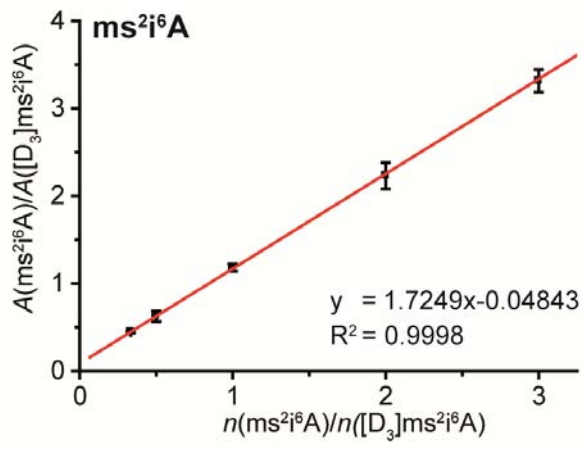


Figure 15: Representative example of a calibration curve and its equation.

These are necessary, because the reagents used to incorporate heavy atoms in the standard are never 100 % labeled themselves. Commercially available $LiAlD_4$ used here for example contains 98 % deuterium. Therefore, every standard solution also comprises a small amount of unlabeled material. In addition, measurement of three different concentrations of the unlabeled nucleoside against one concentration of the labeled nucleoside averages weighing errors. The use of calibration curves also compensates for systematic differences in the processing of labeled and unlabeled nucleosides, e.g. in the mass spectrometer. This is emphasized by Figure 15, where the slope is substantially higher than one. Calculation of the modification level without normalization using the calibration curve equation would thus result in a significant error. The calibration curves and equations for all other modifications studied in this work are compiled in chapter 10.6.2.

5.1.5.2 HPLC method

Advantageously, HPLC-MS quantification utilizes two levels of separation. First, the analytes are separated due to their different retention times during HPL chromatography (Figure 16) and, second, they are differentiated by their individual masses by mass spectrometry (Figure 17 a). On the other hand, molecules with the same mass and the same retention time are not quantifiable individually. For HPLC-MS quantification of modified tRNA nucleosides all modifications with the same mass have to reach the mass spectrometer at different times. Otherwise the mass signals overlap and an individual quantification of the overlapping nucleosides becomes impossible. Unfortunately, modified nucleosides with the same mass

tend to have similar retention times, because they are structurally related. For example methyladenosines m^2A and m^6A show this effect (Figure 17 b).

In addition, modified nucleosides have to be chromatographically separated from canonical nucleosides. The detector used here, a Thermo Finnigan LTQ Orbitrap XL, analyzes the ions reaching it in a two step process. First, a certain number of ions is collected. Then these accumulated ions are measured. The canonical nucleosides are present in the sample in high excess compared to the modified ones. Therefore, when a canonical nucleoside reaches the analyzer, it will be flooded by the ions of this nucleoside, which in turn leads to suppression of the ions of interest. The strength of suppression is proportional to the amount of the abundant nucleoside reaching the detector. Labeled and unlabeled nucleoside do not reach the mass spectrometer simultaneously due to a small difference of their retention times (e.g. 0.2 min for m^2A and d_3-m^2A, Figure 17). Therefore, their amounts, which reach the detector at the same time as the canonical nucleoside, are not identical. This leads to different ion suppressions of the nucleosides of interest and frustrates their reliable quantification.

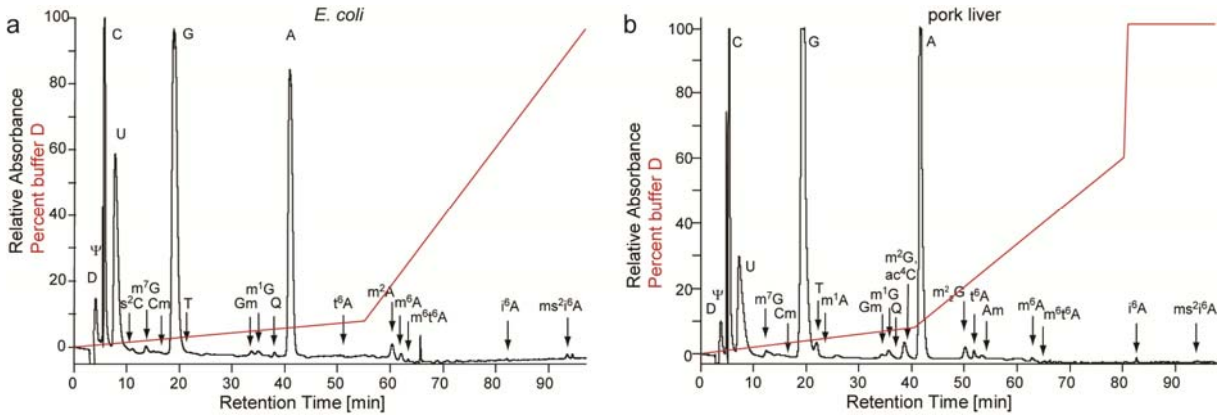


Figure 16: Representative HPL chromatograms and gradients.
HPL chromatogram and gradient of tRNA nucleoside mixtures from a) *E. coli*; b) pork liver (λ = 260 nm). The HPLC gradient is depicted in red as percentage of buffer D.

In order to circumvent these problems, a potent HPL chromatography method had to be established. There are numerous HPLC conditions known in literature for the separation and analysis of modified nucleosides,[59a, 106, 110] which either do not reach a sufficient level of separation or are not compatible with the equipment on site. For these reasons the HPLC method used in the Carell group for analysis of DNA digests was chosen as a starting point.[113] Additionally, the flow used for HPL chromatography (0.15 mL/min) lay within the range of the injection volume of the mass spectrometer and could therefore be adopted

without alterations and the buffer system was suitable for mass spectrometry (buffer C: 2 mM ammonium formiate, pH 5.5; buffer D: 2 mM ammonium formiate/MeCN 20/80, pH 5.5).

With the general HPLC conditions in place a screening of various gradients for maximum separation of modified nucleosides was conducted resulting in similar gradients for the various tRNA nucleoside mixtures from the cells investigated (Figure 16). In addition, coinjection studies were conducted to determine the retention times of the nucleosides in question. Detailed HPLC conditions are described in chapter 10.6.1. To our knowledge, our HPLC method for the first time allows separation of m^2A and m^6A making these nucleosides quantifiable (Figure 17 b).

Typical UV traces of the obtained *E. coli* and pork liver modification pattern are depicted in Figure 16 a and Figure 16 b, respectively. Next to the dominant signals for the canonical RNA nucleosides, the various modified nucleosides either appear as small signals or are hidden in the base line. Unambiguous assignment and quantification of the modifications is therefore only possible by MS analysis.

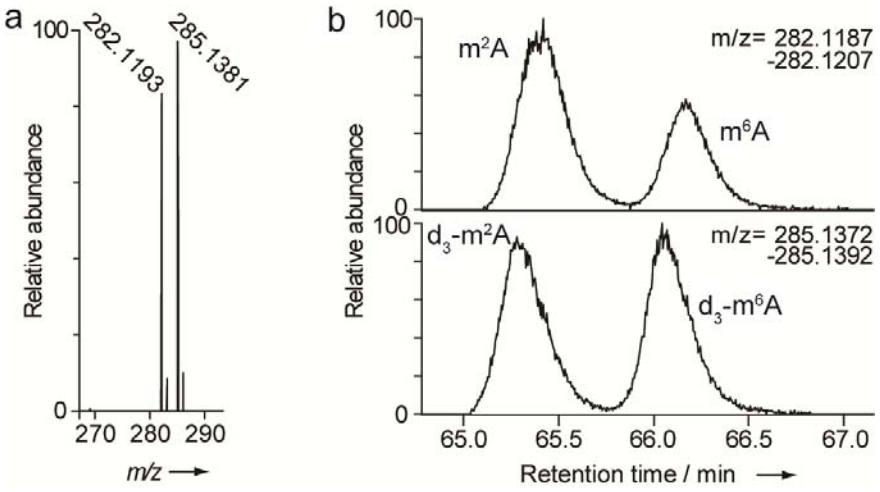


Figure 17: Mass spectrometric measurements of m^2A, m^6A, d_3-m^2A, and d_3-m^6A.
a) Relevant high resolution mass spectrometric data for unlabeled and labeled m^2A. b) Positive ion currents of the protonated nucleosides m^2A and m^6A and the corresponding synthetic isotope labeled derivatives.

5.1.5.3 Mass spectrometric analysis

After high resolution separation of the modified nucleosides by the optimized HPLC conditions they were quantified by mass spectrometry as described below using the example of m^2A and d_3-m^2A. The nucleoside mixture obtained from the digest is subjected to HPLC-

MS. The nucleosides m^2A and d_3-m^2A were separated from all other nucleosides. Due to their mass difference they appear in the mass spectra as two separate signals (Figure 17 a). After completion of the whole measurement the obtained data were analyzed with "Xcalibur". The measurement of high resolution mass spectrometry employed here allows isolation of those ions produced by methylated adenosines. By setting a defined mass range (for methyladenosine: 282.1187 – 282.1207; for d_3-methyladenosine: 285.1372 - 285.1392) the ion current of all methyladenosines and their labeled standards were extracted (Figure 16 b). Integration of the areas under the mass peaks yielded the ratio of m^2A and d_3-m^2A ions.

Substitution of this ratio into the corresponding calibration curve (chapters 5.1.5.1 and 10.6.2) gave the molar ratio of m^2A and d_3-m^2A present in the measured sample. This ratio was charged against the known amount of d_3-m^2A added to the sample prior to the measurement. This way an exact determination of the amount of m^2A in the sample was achieved.

It is important to not inject the first 3 minutes of an HPLC run into the mass spectrometer. All salts from the sample do not adhere to the reverse phase material of the column and therefore elute with the void volume. Injection of these in the mass spectrometer would clog its entrance inevitably leading to adulterated results.

As a control for the reliability and reproducibility of our approach intra- and inter-assay tests were performed for nucleosides m^1A, i^6A, ms^2i^6A, and m^1G. During the intra-assay tests the determined values of labeled to unlabeled nucleosides of a sample after enzymatic digestion showed excellent reproducibility for each nucleoside (N=5; 2.5 % for m^1A, 0.4 % for i^6A, 0.7 % for ms^2i^6A and 2.6 % for m^1G). Inter-assay tests gave good area ratio reproducibility on six subsequent days (N=6; of 6.3 % for m^1A, 1.0 % for i^6A, 1.8 % for ms^2i^6A and 4.3 % for m^1G). These tests attest the outstanding accuracy of the HPLC-MS quantification. The determined integrals for each labeled or unlabeled nucleoside not using the standards resulted in an average standard deviation of 34.5 % and showed insufficient reproducibility. Therefore, usage of the labeled nucleosides as standard is essential. No memory effect was observed during blank LC/MS experiments performed after several measurements of a sample excluding contamination by carry-over. Tuning of the spectrometer was performed with uridine. Further detailed description of the optimization of the digest, HPLC, and MS method will be discussed by D. Globisch in an upcoming Ph.D. thesis.

In summary, in this thesis work a stable isotope based method was established (Figure 10), that allows efficient, direct, and parallel quantification of theoretically all tRNA modifications from any cell or tissue with extremely high sensitivity.

5.2 Initial measurements of modified tRNA nucleosides in E. coli, HCT-116, HeLa, and pork liver

After establishment of the general method for quantification of modified tRNA nucleosides the applicability, accuracy, and sensitivity of our approach had to be demonstrated. Thus, tRNA modifications and cells of substantial biochemical interest were selected. As representative tRNA modifications m^6A, m^2A, Am, t^6A, i^6A, and ms^2i^6A were chosen. These nucleosides are known to be mostly present directly 3'-adjacent to the anticodon[4, 115] (Figure 14) and therefore likely to be directly involved in the decoding process.[116] While m^6A, t^6A, and i^6A are present in eukaryotes and prokaryotes, m^2A is found only in bacteria and Am only in eukaryotes.[4, 59a, 115, 117] These nucleosides are therefore ideal to verify the applicability of our method. Simultaneously, they are likely to provide initial insights concerning the functions of tRNA modifications adjacent to the anticodon. Moreover, the nucleoside ms^2i^6A is of particular interest, because it is present only in prokaryotes and in mitochondrial tRNAs.[4, 115] Its study may lead to insights into the mitochondrial tRNA modification levels. In addition, it is a good indicator for the sensitivity of our method, because mitochondrial tRNAs are only a minor constituent of the cellular tRNA, which is mainly composed of cytosolic tRNAs.

E. coli and pork were chosen as common representatives of prokaryotes and eukaryotes, respectively. Pork tissues are known to be very similar to human ones. In addition, cancer cell lines were selected for the initial screening, because literature indicates that tumor cells possess a significantly altered tRNA modification pattern compared to normal cells.[9b, 72a, 90b] Such a study will lead to a better characterization of tumor cells and may enable deeper insight in the metabolism of tumor cells.[72a]

Organism	Am	t^6A	m^2A	m^6A	i^6A	ms^2i^6A
	n/pmol					
E. coli	-	63.0 ±2.4	121 ±3.4	29.3 ±0.5	5.2 ±0.1	55.6 ±3.2
pork liver	9.00 ±1.5	97.1 ±6.5	-	28.6 ±2.1	18.1 ±1.7	1.50 ±0.1
HCT-116	7.00 ±0.4	130 ±6.0	-	22.7 ±0.4	25.3 ±2.5	-
HeLa	11.3 ±4.4	136 ±2.7	-	46.0 ±4.0	15.6 ±0.8	-

Table 5: Summary of the modified tRNA nucleoside levels from *E. coli*, pork liver, HCT-116, and HeLa cells.
Average values with standard deviations determined from two different preparations. For each experiment three independent digests and measurements were performed.

E. coli cells were harvested during exponential growth representing standard laboratory experimental conditions (chapter 10.4.1.1). Liver was chosen, because it is the most investigated tissue, easily manageable, available, and homogeneous (chapter 10.4.2.1). In addition, two epithelial cancer cell lines from different tissues HCT-116 (colonic epithelial cell line) and HeLa (cervical epithelial cell line) were investigated.

The tRNA modification levels in *E. coli*, pork liver, HCT-116, and HeLa cells are summarized in Table 5. Each value and its corresponding standard deviation are calculated from two independent experiments, which in this context required to conduct the whole process outlined above: tRNA extraction, tRNA purification, digestion, spiking, and measurement. For each experiment three independent digests, spikings, and measurements were performed.

In agreement with literature the modification m^2A was detected only in *E. coli* and not in eukaryotic cells. In contrast, the modification Am was found only in eukaryotic cells.[4, 59a, 115, 117] This confirmation of known data proves the general applicability of our quantification approach. Moreover, the low average error margin of only 5 % shows the outstanding accuracy of our method. Its high sensitivity is demonstrated by the reliable detection of as little as 1.5 pmol ms^2i^6A in 12 µg bulk tRNA from pork liver with a standard deviation of only 7 %.

The data summarized in Table 5 are presented graphically in Figure 18. The level of t^6A in healthy pork liver is significantly lower than in both tumor cell lines, which indicates that the biosynthesis of this modification may be upregulated in HeLa and HCT-116 cells. Further significant differences between the tumor cell lines were found for m^6A and i^6A. HeLa cells contain 100 % more m^6A than HCT-116 cells, which exhibit the same amount of m^6A as pork liver tissue. The level of i^6A is 30 % lower in HeLa cells compared to HCT-116 cells, whereas HeLa cells contain the same amount of i^6A as healthy liver tissue. These results support the idea that the analysis of modified tRNA nucleosides is able to differentiate between tumorogenic and non-tumorogenic cells and that even discrimination of different cancer cell lines may become possible once the analysis is extended to a broader variety of nucleoside modifications.

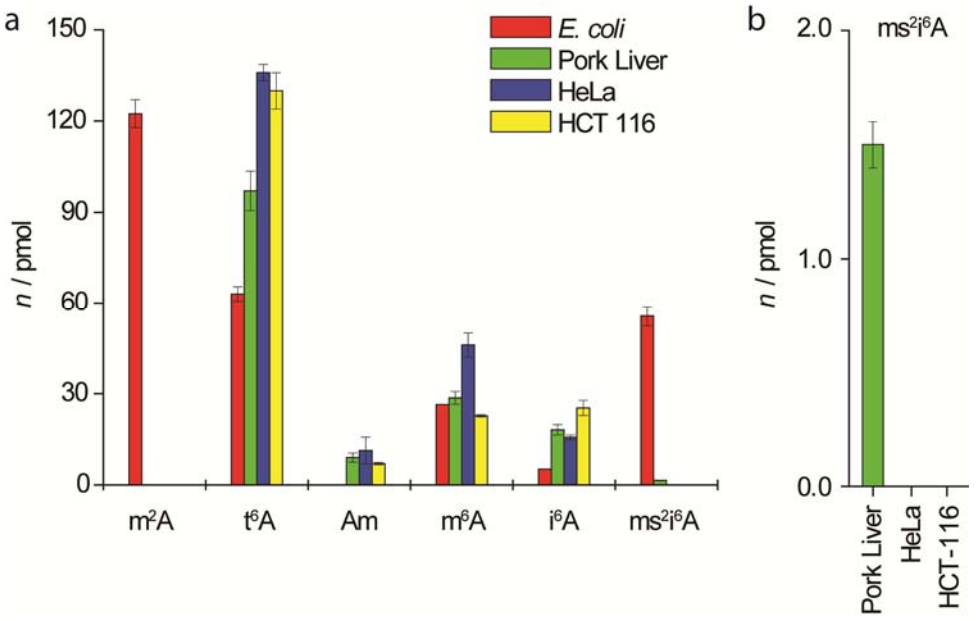


Figure 18: Comparison of the modified tRNA nucleoside level comparison of *E. coli*, pork liver, HeLa and HCT-116 cell lines.
a) m^2A, t^6A, Am, m^6A, i^6A, and ms^2i^6A levels of *E. coli*, pork liver, HeLa and HCT-116 tRNA. b) ms^2i^6A levels in pork liver, HeLa, and HCT-116 tRNA. Error bars represent the standard deviations calculated from multiple experiments.

The modification ms^2i^6A is present only in prokaryotes and mitochondria.[4, 115] Its quantification therefore provides an insight into how the mitochondrial tRNA modification level changes in tumor cells. It has been known for some time that tumors have a reduced oxidative phosphorylation activity and that many tumor cells derive most of their needed energy from glycolysis,[118] which induces a reduced pH-value in tumor tissue.[118a] In order to investigate this phenomenon the ms^2i^6A level of tRNA from healthy tissue and cancer cells was quantified. The result of this analysis is depicted in Figure 18. As expected, the modification ms^2i^6A is present in *E. coli* as well as in small but significant amounts in pork liver. Surprisingly, the analyzed tumor cells do not contain the modification ms^2i^6A at all (Figure 18 b). The mitochondrial tRNA nucleoside is totally absent in both tumor cell lines indicating that mitochondrial tRNAs in tumor cells and normal cells indeed differ substantially. The result provides a first indication that the impairment of mitochondria in tumor cells (Warburg hypothesis)[119] is detectable using our isotope based analysis method.

In summary, it could be shown that this quantification method possesses the applicability, accuracy, and sensitivity to allow direct and parallel quantification of in principle all tRNA modifications. Moreover, initial experiments revealed surprisingly large variations of the tRNA modification levels among tumor cell cultures as well as between tumor cell cultures

and non-tumorogenic tissue. Furthermore, it could be shown, that tRNA from the studied cancer cell lines lacks ms^2i^6A.

Assuming that different species, various mammalian tissues, and different tumor tissues possess substantially different modified tRNA nucleoside patterns,[16c, 106c] the here presented isotope method is a new tool for a detailed cell and tissue analysis and for a thorough analysis of the functions and biosyntheses of modified tRNA nucleosides.

5.3 tRNA modification levels in mammalian tissues

5.3.1 Measurement of the tRNA modification levels in tissues

Encouraged by the success of our initial study and the high scientific and medicinal interest of modified nucleosides (see chapter 3) a detailed analysis of the tRNA modification contents in different types of mammalian tissues was performed. The modified tRNA nucleoside content of pork heart, cerebellum, tongue, cerebrum, kidney, liver, lung, spine marrow, spleen, and thyroid gland was studied. As representative tRNA modifications ms^2i^6A, t^6A, m^6t^6A, i^6A, OHyW, Q, m^1G+Gm, $m^2{}_2G$, m^2G, m^1A, and Am were chosen. Varying from abundant to rare modifications and covering modifications present at various positions in tRNAs, these nucleosides constitute a representative cross-section of all tRNA modifications.

Modifications m^1G and Gm were analyzed together with d_3-m^1G used for spiking, because they could not be separated by HPLC. Nucleoside m^1A was observed to decompose to m^6A due to the increased waiting times associated with this large screening. This resulted in irreproducible results for m^6A, which were discarded. During the initial study (chapter 5.2) this did not pose a problem as the samples were measured quickly after processing. Quantification of m^1A was still possible, because the standard d_3-m^1A was added right after the digest and degraded to the same extent as unlabeled m^1A.

Preparations and measurements were conducted as discussed above. All data points presented are based on at least four sample preparations and twelve measurements. The sample and data collection is illustrated in Figure 19. First, for each tissue type at least two organs from different individuals were cleaned and surface areas were removed. From each organ at least two tissue samples were taken. Isolation and purification of the tRNA was performed as described in chapter 10.4.2. From the bulk tRNA at least three samples were taken and digested independently. Each digest mixture was equipped with different amounts of each isotopically labeled standard nucleosides and analyzed *via* HPLC-ESI-MS.

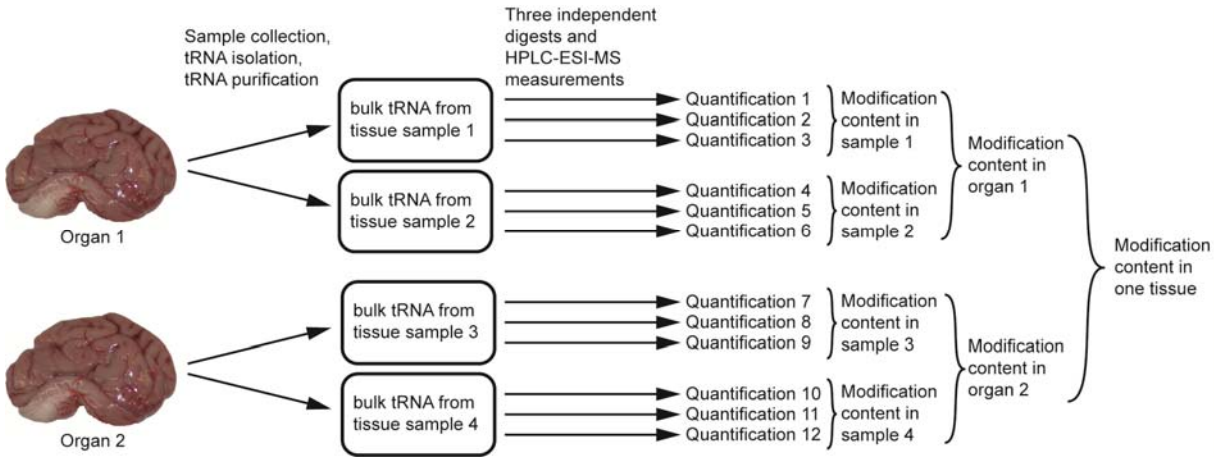


Figure 19: Schematic depiction of the pork tissue sample and data collection.

From the obtained quantification data average modification contents and the corresponding standard deviations for each sample were calculated (Figure 19). From these values average modification contents and the corresponding standard deviations for each organ were determined. These data finally yielded the average modification contents and the corresponding standard deviations for one tissue. This stepwise calculation process allows conclusions about the differences of the modification levels between tissue samples and between individuals. In all investigated cases the modification levels were stable between samples and individuals. The obtained data are summarized in Table 6 a and Table 7 and are given as the amount of each modification per 1000 tRNA molecules (‰) with descriptive, quantile-based color codes.

5.3.2 ms^2i^6A levels correlate with mitochondrial activity

As outlined in the previous chapter the nucleoside ms^2i^6A is found in bacterial tRNA and in mitochondrial tRNA of eukaryotes.[4, 115] Therefore, its amounts in tissues are expected to be associated with their mitochondrial activity. As a measure for this activity previously described ATP content,[120] mitochondrial protein abundance,[121] and activity[122] in the investigated tissues were collected. Comparison of these data with our measured modification levels showed an excellently correlation (Table 6; $r = 1.00$, sig. < 0.01 between ms^2i^6A values and mitochondrial MDH mRNA levels). Spleen, lung, and liver tissue feature low mitochondrial activity, thus correspondingly low ms^2i^6A levels were determined. As expected, for tissues with high mitochondrial activities, such as muscle (heart and tongue) and brain tissues (cerebellum and cerebrum), a high ms^2i^6A content was found. The obtained ms^2i^6A levels shown in Table 6 exhibit an even finer graduation as the accordant literature data for ATP content, mitochondrial protein abundance, and activity. The presented study

therefore allows a very close look at the mitochondrial activity of the investigated tissues from an unprecedented point of view.

a

Tissues	ms^2i^6A /1000 tRNAs	Relative ATP content[120] nM/µg protein	Mitochondrial MDH mRNA per total RNA[121] pg mRNA/µg total RNA	Citrate synthase activity[122] nmol/(min*mg) protein
Heart	18.4	40.0	4.0	12,500
Cerebellum	15.1	-	-	-
Tongue	12.7	-	-	-
Cerebrum	10.3	-	2.0	3,800
Kidney	8.41	15.0	-	1,500
Liver	4.72	20.0	0.8	800
Lung	4.27	-	0.7	-
Spine marrow	4.18	-	-	-
Spleen	2.78	3.0	0.6	-
Thyroid gland	< 1.00	-	-	-

Highest value
50 % Quantile
Lowest value

b

Mitochondrial measurement	r for correlation with ms^2i^6A level	significance	N
Relative ATP content	0.918	0.082	4
Mitochondrial MDH mRNA per total RNA	0.997**	0.000	5
Citrate synthase activity	0.975*	0.025	4

Table 6: Levels of ms^2i^6A in pork tissues, data about respective mitochondrial activity, and their correlation.
a) tRNA nucleoside values are given per 1000 tRNA molecules (‰). Repeated measurements of samples resulted in an average error of about 5 %. Relative ATP contents, mitochondrial MDH mRNA concentrations, and citrate synthase activity are obtained from literature. b) Correlations of ms^2i^6A level with mitochondrial measurements. * $P < 0.05$, ** $P < 0.01$. Correlations are calculated as Pearson's r coefficients with 2-tailed significance values.

5.3.3 Tissues exhibit individual modification levels

The quantitative data for all other modifications in different tissues are shown in Table 7 revealing that each tissue type uses a discrete level of tRNA modification for the translation of genetic information into peptide sequences. While liver and cerebellum tRNA contain a large number of modified nucleosides, tRNA isolated for example from lung and kidney tissue features fewer modifications. Significantly lower amounts were measured in the muscle tissue heart and tongue. The lowest value was measured for thyroid gland. At this point of the investigation no evident explanation can be provided for the steep drop of modification levels in thyroid gland in comparison to other tissues. One might speculate about a link to the high specialisation of this tissue, where synthesis of large amounts of certain proteins might necessitate a specialized translation apparatus. Except for ms^2i^6A the only nucleoside not following the general trend is Am. Am is surprisingly abundant in cerebrum and thyroid

gland, which are the tissues that feature the lowest global modification levels. The reason for this difference is at this point unclear but modified nucleoside are known to stabilize the tertiary structure of the tRNAs and due to its location in the amino acid acceptor stem Am might potentially have a general tRNA stabilizing function.[117] In this scenario its level is increased in order to maintain the tertiary structure of the tRNAs, which otherwise might be considerably destabilized or even lost due to the lack of other modifications.

Tissues	Modified nucleosides									
	t^6A	m^6t^6A	i^6A	OHyW	Q	m^1G+Gm	$m^2{}_2G$	m^2G	m^1A	Am
Liver	215	21.9	39.6	33.6	55.2	1,060	563	1,176	1,010	20.5
Cerebellum	237	17.5	41.7	29.0	83.5	829	567	1,036	942	67.8
Spine marrow	199	11.8	36.0	26.0	50.8	800	457	971	911	61.8
Spleen	182	13.9	30.7	26.1	42.7	690	464	794	835	80.4
Kidney	178	15.8	30.7	21.1	32.3	816	451	896	872	68.4
Lung	184	13.9	33.6	21.7	33.9	794	427	879	876	58.5
Tongue	171	11.3	25.7	15.2	21.9	699	396	794	846	45.4
Cerebrum	166	10.5	26.1	20.3	57.2	587	406	682	733	140
Heart	175	11.0	29.0	12.0	29.6	638	373	673	797	45.9
Thyroid gland	43.0	4.67	8.64	traces	8.84	240	123	204	272	200

Highest value
50 % Quantile
Lowest value

Table 7: Color-coded tRNA modification levels in pork tissue.
All tRNA nucleoside values are given per 1000 tRNA molecules (‰). Repeated measurements of samples resulted in an average error of about 5 %. Color code is based on quantile calculations; red: highest value, yellow: 50% quantile, green: lowest value, colorless: thyroid gland, for intermediate values appropriate shades of color were calculated. Traces: less than 1 modification per 1000 tRNAs.

In addition, the divergence between cerebrum and cerebellum is noteworthy, which indicates that the number of modifications does not simply reflect the tissue affiliation but has to be correlated with the particular function of the tissue. Moreover, relatively large amounts of the Q-nucleoside were detected in cerebellum and cerebrum. Currently, no explanation for these results is available due to the limited literature available on the functions of Q in the brain.

Nevertheless, these observations support the idea that Q might be of higher importance in brain than in other tissues. The base queuine is essential for the formation of Q in eukaryotes. As queuosine is present in brain tissues, queuine can obviously cross the blood brain barrier. Nevertheless, it has been observed that bone marrow cells do not contain Q. They do not transport queuine across their plasma membrane. Based on these observations Farkas and co-workers[123] suggested, that Q is more important in brain than in other tissues. They ascribe the increased importance to a reduced tolerance of the central nervous system to aberrant

polypeptides. Furthermore, the production of one superoxide dismutase, which is necessary in high amounts in brain, is induced by Q.[124]

5.3.4 tRNA modifications are conserved across mammalia

The significance of the detected correlation to a large extent depends on its general validity for mammals. To determine the conservation of modified nucleosides across mammals the available information from literature, tRNA sequences, and databases was evaluated.

Concerning modification levels in literature it was found, that the data obtained here for pork liver are in excellent agreement with a study of modified tRNA nucleosides in mouse liver by Hoffman and McCoy.[125] The percentages of the modifications m^6A+m^1A, $m^2{}_2G$, m^2G, and m^1G, which were measured both during this thesis and by Hoffman and McCoy, are summarized in Table 8. Although only a limited number of modifications has been measured in both surveys the concordance of modification levels implies their conversation across mammals. Even more so, if one considers that the studies are more than 30 years apart and different methods were used.

	Animal model	Modified nucleosides / %			
		$m^6A + m^1A$	$m^2{}_2G$	m^2G	m^1G
Carell group	Pork liver	28	16	32	24
Hoffman & McCoy	Mouse liver	27	15	29	29

Table 8: Relative amounts of m^6A+m^1A, $m^2{}_2G$, m^2G, and m^1G found in pig and mouse in percent.

In addition, a number of tRNA sequences ($tRNA^{Asn}$, $tRNA_i^{Met}$, $tRNA^{Phe}$, and $tRNA^{Val}$) are conserved in higher eukaryotes also implying a preservation of tRNA modifications in mammals.[126] An investigation of the modification content in liver from rat, beef, sheep, and rabbit clearly showed that the content does not vary significantly between these mammals.[106c] Similar observations were made by others.[107b]

Therefore, the tRNA sequence information from the Sprinzl database,[4a] which is today's biggest collection of tRNA sequences including modified nucleosides, was searched in regard to the modified tRNA nucleosides under investigation during this thesis. Amongst others the database information includes tRNA sequences from *homo sapiens*, *bos taurus*, *mus musculus*, and *rattus norvegicus*. A calculation showed that modifications in tRNAs, which code for the same amino acid, are generally conserved across mammalia (for details see

chapter 10.5). In only 6 % of all cases the number of a certain modified nucleoside varied, when the same tRNAs from various mammals were compared.

Based on this evidence for a conservation of tRNA modifications across mammals we expect the data concerning modification levels in pork tissues (Table 6 and Table 7) to be valid for a broad range of mammalian species including humans.

5.3.5 Potential origins of tissue-specific modification levels

5.3.5.1 Codon bias

Some codons are translated faster and more accurately than others. These efficient codons became more abundant to encode highly translated proteins in the course of evolution. The resulting, non-statistical distribution of codons in favour of more efficient ones is called codon bias.[127] It is only advantageous, if there is also an analogously high percentage of aa-tRNAs, which recognize the prefered codons. Consequently, the codon bias led to an adjustment of the tRNA composition.[127] It has indeed been found, that highly specialized tissues, like reticulocytes, which produce large quantities of a few proteins, have a specialized aminoacyl-tRNA (aa-tRNA) composition.[128] Therefore, a distinct codon bias in different tissues could explain the observed tissue-specific modification levels.

This could be a possible explanation for the unusually low modification content of thyroid gland. But at present no experimental support or literature precedence can be presented backing this suggestion.

Nevertheless, for all other tissues investigated here the protein demand is very diverse and a large fraction of the proteins, which are synthesized in these tissues, is identical. This in itself does not exclude the presence of codon bias and recently a tissue specific codon bias has been statistically proven in mammalia.[129] But its effect is so small that it cannot explain the differences in tRNA modification levels observed in this thesis work.[130]

5.3.5.2 General tRNA composition in tissues

Independent of the codon bias, the tRNA composition might still be tissue specific for some other reason. A microarray analysis performed by Dittmar *et al.*[131] indicates tissue-specific differences in the tRNA mixture. On the other hand chromatographic investigations of aa-tRNAs have shown that the numbers of isoaccepting tRNAs are stable between different organs.[6l, 132] In addition, in recent years tRNA gene copy numbers have been used fruitfully

as a measure for the cellular tRNA abundance.[133] As this number is the same in all tissues the applicability of this assumption implies that the tRNA composition is stable across tissues.

The data collected during this study complement evidences from literature against a tissue-specific tRNA composition as follows. Assuming a tissue-specific variation in tRNA composition, when different tissues are compared, the percentage of some tRNAs would go up while the percentage of others would necessarily go down. This would inevitably mean that the content of some modifications had to go up, while the content of others had to decline. In contrast the same trend of modification levels with tissues was observed here for all nucleosides but ms^2i^6A and Am (Table 7). The modifications OHyW and i^6A are rather scarce, present in a very limited number of tRNA species, and do not share a single common tRNA species, whereas the modifications m^2G and m^1A are present in almost all tRNAs. Nevertheless, they all show the same behavior in all investigated tissues.

These observations and data from literature indicate that the relative amounts of tRNAs do not change significantly across tissues. They rather hint at an additionally regulatory role of the modification levels in tRNAs.

5.3.5.3 Protein synthesis capacity

Roe & Tsen uncovered that an increased ribothymidine content in mammalian tRNAs leads to an increased maximum velocity for the overall synthesis of poly(Phe), while the apparent Michaelis constant (K_m) remained essentially unchanged. Based on this finding they postulated that "the modified nucleoside ribothymidine might be involved in the regulation of protein synthesis at the level of translation in mammalian liver."[134]

Taking the lead from this hypothesis our tissue-specific tRNA modification levels might be interpreted as a mechanism to regulate the protein synthesis capacity in mammalian tissues. It is not desirable for all tissues in one organism to produce the same amount of proteins. Some tissues will have to produce large quantities of proteins (e.g. liver), while others need only small amounts (e.g. heart). Nevertheless, all cells in a certain organism generally have the same translational apparatus. The numbers of single proteins are usually regulated on the transcriptional level by different copy numbers of their mRNA and on the translational level by sequence-specific RNA-binding proteins and microRNAs.[135] Global regulation of the translation efficiency is possible by modulation of initiation factor activites. Additionally, the different levels of tRNA modification in tissues might serve as a tool for the adjustment of tissue-specific protein synthesis rates.

a

Tissues	Modified Nucleosides				Protein synthesis rates			
	m^6t^6A	$m^2{}_2G$	m^1A	Normalized average	Shahbazian[136a] [%/h]	Johnson[136b] [%/d]	Suryawan[136c] [%/d]	Hayase[136d] [%/d]
Liver	21.9	563	1010	1.00	2.15	64.2	69.5	60.2
Spleen	13.9	464	835	0.75	-	-	36.8	-
Kidney	15.8	452	872	0.75	-	28.8	31.9	51.3
Lung	13.9	427	876	0.75	-	23.5	21.8	-
Cerebrum	10.5	406	733	0.68	0.62	12.4	-	12.2
Heart	11.0	373	811	0.62	0.66	14.8	17.4	-

Highest value
50 % Quantile
Lowest value

b

Protein synthesis rate	r	significance	N
Johnson	0.979**	0.004	5
Suryawan	0.961**	0.009	5
Hayase	0.804	0.406	3
Average (Johnson, Suryawan, Hayase)	0.944**	0.005	6

Table 9: Modification levels and protein synthesis capacity of tissues and their correlation.
Nucleosides m^6t^6A, $m^2{}_2G$, and m^1A are depicted as representative examples for rare and abundant modified nucleosides. For the normalized average all modification levels except Am and ms^2i^6A were normalized to the liver measurement and averaged. Color codes are based on quantile calculations; red: highest value, yellow: 50% quantile, green: lowest value, for intermediate values appropriate shades of color were calculated. b) Correlations of normalized nucleoside levels (excluding ms^2i^6A and Am) with protein synthesis rates. ** $P < 0.01$. Correlations are calculated as Pearson's r coefficients with 2-tailed significance values.

In this case the tissue-specific modification content should reflect the protein production of the individual tissues and thus correlate with the protein synthesis rates in tissues. In order to test this hypothesis, literature information about protein production yields was collected (Table 9 a).[136] Comparison of these data with the measured modification levels shows a surprisingly good correlation (Table 9 b) between average protein synthesis rate and average normalized modification levels. In all cases the observed modification levels compare well with the measured protein biosynthesis capacity, with liver having large quantities of modifications and a high protein synthesis capacity. Heart in contrast features fewer modifications and shows lower protein biosynthesis.

In addition, *in vitro* translation of globulin in rabbit reticulocyte lysates has been found to depend on the nature of the supplied rabbit tRNA.[137] *In vitro* translation with tRNA isolated from brain is significantly slower than with kidney, liver, or reticulocyte tRNA (Figure 20 a). Furthermore, liver tRNA facilitates a faster translation than tRNA isolated from kidney. The slopes of the graphs obtained for low tRNA concentrations, which are a measure for the activity of tRNAs during translation, correlate with the normalized average modification levels (Table 9) determined in pork tissue (Figure 20 b; r = 1.000, significance: 0.012, N = 3).

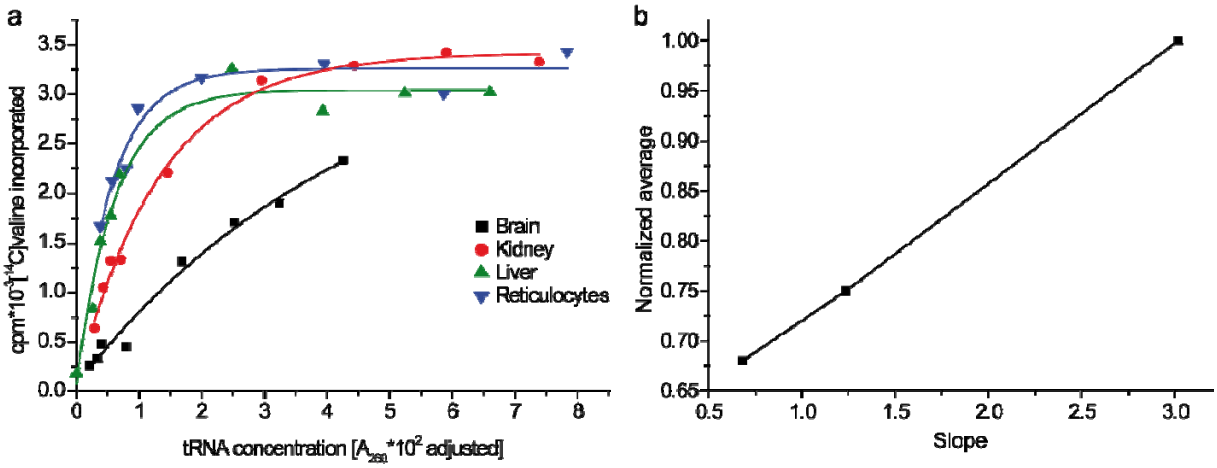


Figure 20: tRNA dependent *in vitro* translation rates and their correlation with modification levels. a) ^{14}C-valine incorporation during hemoglobin syntheses in reticulocyte lysates with its natural tRNA (blue), brain (black), kidney (red), and liver (green) tRNA. The tRNA concentrations were adjusted for the reduced amino acceptor activities of brain, kidney, and liver tRNAs in comparison to reticulocyte tRNA in the lysate. b) Plot of the slopes obtained from linear fits of the data for low tRNA concentrations (< 2 A_{260}*10^{-2}) against normalized average modification levels.

Taken together, these significant correlations suggest that the amount of tRNA modifications determines the protein manufacturing fitness in mammalian tissues. In this scenario, the tRNA modification level regulates the overall protein production capacity, which is indeed tissue specific in complex organisms.

5.3.6 High levels of modified tRNA nucleosides in cancer cell lines

In order to gain further support for this hypothesis the modification levels in cancer cell lines were determined. These were chosen, because they have an increased proliferation and protein synthesis rate and according to our theory should thus contain a modification content at least as high as the most abundantly modified tissues, liver and cerebellum.

In all investigated cancer cell lines a complete set of the modified nucleosides was detected (Table 10). They were found to generally possess modification levels close to or even above those detected for liver tissue, which is the highest modified tissue. The increase is specially high for t^6A, whose levels in cell lines exceed those in liver. SK-HEP-1, a cancer cell line derived from a liver tumor, shows an almost identical modification pattern as liver tissue.

The measured high modification levels in cancer cells compare well with reports from literature, which describe an increased modification of tumor tRNA and high excretion levels of modified nucleosides by cancer patients, although there are also reports of hypomodification in neoplastic tissues (chapter 3.2). The Q-hypomodification of MCF-7 stated in literature[41b] could not be confirmed.

Cancer cell lines	Modified nucleosides									
	t^6A	m^6t^6A	i^6A	OH-yW	Q	m^1G+Gm	$m^2{}_2G$	m^2G	m^1A	Am
MDA-MB-231	272	17.8	36.8	9.70	62.5	898	579	992	n.q.	14.0
MCF-7	278	19.2	50.3	28.4	65.3	879	608	1150	n.q.	15.2
T-47D	247	23.0	39.8	24.8	77.9	941	526	1154	n.q.	25.7
BT-549	247	22.5	34.9	tr	105	862	538	938	n.q.	14.6
SK-MEL-2	242	15.1	38.5	17.6	74.4	755	540	848	n.q.	25.7
SK-MEL-5	260	16.7	52.4	23.6	33.3	844	568	1003	n.q.	21.5
SK-MEL-28	264	18.6	46.2	11.8	51.0	870	586	1026	n.q.	25.4
IGR-1	276	21.1	44.9	12.0	88.0	839	549	909	n.q.	21.4
SK-HEP-1	228	22.3	37.8	24.9	53.6	843	556	901	n.q.	26.0
HeLa	261	21.4	33.0	17.2	79.1	720	469	771	n.q.	8.60

Table 10: Color-coded quantitative data for the investigated tRNA modifications in cancer cell lines. Color code is based on the absolute coloring in Table 7 for better visualization. n.q. = not quantifiable as m^1A LC-MS signals for cell lines overlap with guanosine LC-MS signals. Measurements were performed by M. Wagner and D. Globisch.

Generally reduced amounts were measured only for OHyW and Am. The latter has already been identified as a special case before and no explanation for its scarcity in cancer cell lines can be provided at present. For OHyW reduced levels were found probably due to its particularly complex biosynthesis that might be impaired in cell lines, e.g. by the lack of an enzyme or a cofactor. A similar reduction of OHyW has been found in tumors.[24]

At present, it cannot be ruled out, that the high modification content measured for cancer cell lines is rooted in their growth in cell cultures, rather than in their nature as cancer cells. Whatever the reason, the investigated cells have a high proliferation rate, which in turn necessitates a high protein production capacity. Therefore, their high modification levels coincide with high protein synthesis rates. This is a further evidence for our hypothesis that modified tRNA nucleosides regulate the overall protein production capacity and are thus coupled to efficient protein synthesis and cellular growth.

5.3.7 Modified tRNAs as regulators of protein synthesis?

In order to collect further information on the impact of tRNA modification on the translational process literature concerning this topic in prokaryotes was studied. Generally, the growth and protein synthesis rate of prokaryotes is reduced, when they are lacking one or more tRNA modifications. For instance, the loss of pseudouridine in *S. typhimurium* tRNA has been shown to lead to a reduction of protein synthesis *in vivo* by 20 to 25 %. The reduction occurred predominantly at the level of translation.[138] This finding is supported by a number of independent studies, which consistently show that hypomodification results in a diminished decoding efficiency,[139] a reduced aminoacylation rate,[140] and an impaired growth.[141] These investigation exemplify the impact of modified tRNA nucleosides on the translational process

and its impairment due to hypomodification supplying further hints at a regulation of protein synthesis capacity by tRNA modifications.

For an assessment of the feasibility of our hypothesis, its fit with the current pictures about the rate limiting step of translation and about the general functioning of modified tRNA nucleosides is evaluated. The rate-limiting step in the elongation of each codon is the stochastical search of the ribosome for a cognate tRNA in the A-site. The subsequent translation and translocation steps are much faster.[142] Therefore, the translation rate of mRNA into proteins depends on the availability of cognate aa-tRNAs and on the selection of these from the excess of near- and non-cognate ones.[143] This selection process is dominated by the competition between near-cognate and cognate aa-tRNAs for the binding to the ribosome. Therefore, the key factor that determines the protein synthesis capacity is the competitive, non-specific binding of the tRNAs to the ribosome.[144]

A broad range of functions are attributed to tRNA modifications, e.g. restriction of codon recognition, expansion of the base-pairing capacity of the anticodon (wobble), enabling of translocation, and maintenance of the mRNA reading frame. Recently, a unifying concept for all of these functions was introduced by Paul F. Agris that attributes them to a single effect of modified tRNA nucleosides in the anticodon domain.[6a] According to this theory modified tRNA nucleosides restrict the three-dimensional structure of the anticodon optimizing its fit to the ribosomal A-site. The reduction of the dynamics and of the fluctuation of the anticodon minimizes the need of the ribosome to constrain or remodel each tRNA for binding diminishing the entropic penalty for the interaction between ribosome and tRNA. Thus the translational process is optimized by tRNA modifications providing an evolutionary advantage and the physicochemical basis for their conservation.

It is generally accepted that most modified nucleosides influence the tRNA tertiary structure. While the modifications in the anticodon stem-loop contribute to its structural fine-tuning, modifications in other regions of the tRNA are often responsible for its general structural integrity and have thus even been termed “structural modifications”.[145] Therefore, it seems reasonable to extend Agris’ concept to all tRNA modifications regardless of their position in the tRNA.

The introduction of modified nucleosides increases the fit of the tRNAs to the ribosome. Simultaneously, the entropic penalty for their binding is reduced. Therefore, the presence of modified nucleosides decreases the time and energy cost of the interaction between tRNAs and the ribosome. As the competitive, non-specific binding of tRNAs to the ribosome is the

rate limiting step during translation, this in turn results in a faster screening of the available tRNAs for the cognate one decreasing the ribosomal step time. Conclusively and in line with our hypothesis, this results in an increased protein synthesis capacity.

For unicellular organisms an optimized translation of mRNA into proteins probably facilitates a high proliferation rate and provides the means to save energy and time.[6a] Modifications may have evolved as a growth advantage improving the translation efficiency in bacteria and unicellular organisms. Taking into account the species-specific codon bias, which is very pronounced in unicellular organisms, tRNA modification levels should be divergent across these, if this assumption is true. A look at the available tRNA sequences, e.g. from the Sprinzl database,[4a] confirms this divergence. It was also shown by a screening of the modification levels of various bacteria with our method. Furthermore, cluster analysis of these data furnished a phylogenetic tree identical with those obtained for phylogenetic analysis of rRNAs. This screening was mainly performed by D. Globisch (unpublished data).

Higher organisms must have different reasons for maintaining modified nucleosides, because an optimized protein production of each cell would burn huge amounts of energy regardless of the actual needs of the organism. In biological systems, which contain highly specialized cells, each compartment has individual task and therefore individual needs for proteins. This applies not only for types but also for amounts of proteins. Rather than making as much proteins as possible, it seems more appropriate for each part of an organism to adjust its protein synthesis capacity to its actual needs. This way efficient use is made of the available resources and thus the fitness of the whole organism is maximized. If the modification levels are a general tool to adjust protein synthesis rates, they should be stable across mammals, in contrast to their divergence across prokaryotes. And indeed, the modification content is highly conserved across mammals (chapter 5.3.4). As uncovered in the course of this thesis in mammalian tissues the tRNA modification level correlates with the corresponding protein synthesis capacity implying the use of specific modification levels for the regulation of protein production.

Based on the discussed concepts, literature data, and the insights gained during this thesis we would like to suggest the following general model:

In prokaryotes modified tRNA nucleosides establish a fitness and proliferation advantage by optimizing the translation efficiency. In mammals the tissue-specific modification levels are responsible for maintaining the ideal translation rate and protein synthesis capacity of each tissue.

5.4 Stress response of tRNA modification levels in E. coli

As discussed in the previous chapter tRNA modifications regulate the general protein synthesis capacity in cells by modulating their translational efficiency. Alternatively to this broad regulatory mechanism, they might also have a more specific regulatory function mediated by their presence or absence.

Numerous publications unanimously state that the chromatographic behavior of tRNAs is changed upon application of stress to the cells, from which they originate.[146] Amongst others the stress condition examined include growth of bacterial cells under thermal stress,[146e, 147] amino acid deprivation,[146a, 146b, 147a] antibiotic treatment,[146a, 146b, 148] exposure to various chemicals,[149] and starvation for essential ions.[146b-d, 150] These observations lead to the question, whether the changes of the tRNAs are induced by hypo- or hypermodification as a response mechanism to external stimulation.

Almost all known literature on changes of tRNAs with external stimuli relates to bacteria. Their main advantages in this respect are a high viability under hostile environmental conditions and a straight-forward managability. Of all prokaryotes *E. coli* was chosen, because it is the standard prokaryote for biochemical studies.

A stress-related alteration of modified tRNA nucleoside content is most likely to occur in connection with the translation of mRNA to proteins. Therefore, modifications m^2A and m^6A are of interest here, as they are present 3'-adjacent to the anticodon in position 37 and thus directly involved in the translational process. Other modifications present in the anticodon loop were not available, because the study was performed at an early stage of this thesis. Nucleosides m^2A and m^6A occur at the same position in different tRNAs, are both methylated, and have a similar biosynthesis. This way comparability was maximized. Importantly, they are synthesized by two different enzymes allowing the occurrence of independent responses to external stimuli. Nucleoside m^6A carries its methyl group at the exocyclic nitrogen, while m^2A is methylated at position 2 of the heterocycle and thus the only modified purine tRNA nucleoside assembled by a carbon-carbon bond formation.[151] Nucleoside m^6A is present in one tRNA species, while m^2A is present in six different tRNA species.[4a] The details of all experiments presented hereafter are outlined in chapter 10.4.1.

Growth conditions	n(m^2A) [pmol]	n(m^6A) [pmol]
Aerob (full medium)	**121 ±3.4**	**29.3 ±0.5**
Anaerob (full medium)	125 ±4.3	28.2 ±1.4
Aerob (minimal medium)	125 ±7.2	29.4 ±0.1
Anaerob (minimal medium)	125 ±5.4	30.9 ±1.9
Addition of desferal	110 ±5.2	31.2 ±0.5
Addition of 2-fluor-adenosine	106 ±4.1	29.9 ±1.3

Table 11: Content of m^2A and m^6A in *E. coli* tRNA under growth conditions.
Full medium: LB, minimal medium: MOPS. Amounts of m^2A and m^6A with standard deviations from 12 ng of total extracted tRNA. Reference values are depicted in bold letters.

As reference values, we detected 121.4 pmol m^2A and 29.3 pmol m^6A in 12 ng total tRNA from aerobically grown *E. coli* K12 at 37 °C using LB medium (Table 12). All presented values are average values of two independent experiments under the same conditions and six independent digests and HPLC-ESI-MS measurements.

A screening of the m^2A and m^6A content under various growth conditions was performed. *E. coli* cells were cultured aerobically in shaken cultures utilizing LB and in a fermenter using LB as well as Neidhardt's minimal MOPS medium[152] (Table 11). In contrast to what was expected from literature,[146a, 146b, 147a] growth of *E. coli* in a minimal medium lacking amino acids and all other non-essential nutrients did not lead to an alteration of its tRNAs with respect to the modifications m^2A and m^6A.

Identical experiments under anaerobic conditions yielded similar modification contents. Thus large differences of the *E. coli* metabolism between aerobic and anaerobic growth were not reflected by the levels of m^2A and m^6A in tRNA (Table 11).

It has been shown in literature, that deprivation of essential ions from the medium, especially of iron, leads to altered tRNAs.[146b-d, 150] Therefore, *E. coli* was grown in LB medium supplemented with desferal,[150c] which complexes iron and thus leads to a reduced concentration of these essential ions in the medium. This external stimulation did not lead to altered m^2A and m^6A levels in comparison to reference conditions. Similarly, addition of 2-fluoro-adenosine to the medium did not produce altered modification levels in *E. coli*.

Growth conditions	n(m^2A) [pmol]	n(m^6A) [pmol]
Reference	**121.4 ±3.4**	**29.3 ±0.5**
OD = 0.5, pH = 5.5	125.1 ±3.2	30.1 ±0.3
OD = 1.0, pH = 5.5	98.8 ±7.2	28.1 ±1.2
OD = 2.0, pH = 5.5	120.5 ±9.3	30.6 ±1.0

Table 12: Content of m^2A and m^6A in *E. coli* tRNA at different cell densities.
E. coli was grown under aerobic conditions in full medium to different cell densities (OD). Then the medium was adjusted to pH = 5.5. Amounts of m^2A and m^6A with standard deviations from 12 ng of total extracted tRNA.

When the proton concentration in the medium was increased, differences in the nucleosides levels were detected. During growth in a medium with pH 5.5 the amounts of m^2A and m^6A were both reduced by roughly 10 % (Table 12). In addition, dependence of this phenomenon on the optical density (OD), which reflects the cell density in the medium, was detected. The given ODs indicate the cell density at the initiation of the external stimulation. The stress was applied for different periods depending on the nature of the stimulation. When adjusting the pH of the medium to 5.5 at OD = 1.0 decreased nucleoside levels for both nucleosides m^2A and m^6A were found. For initiation of the pH stress at OD = 0.5 and OD = 2.0 no differences in regard to the reference values were detected (Table 12). At this point of the investigation an explanation for this observation is not possible. As a consequence stress initiation was strictly applied at OD = 1.0 for all following experiments.

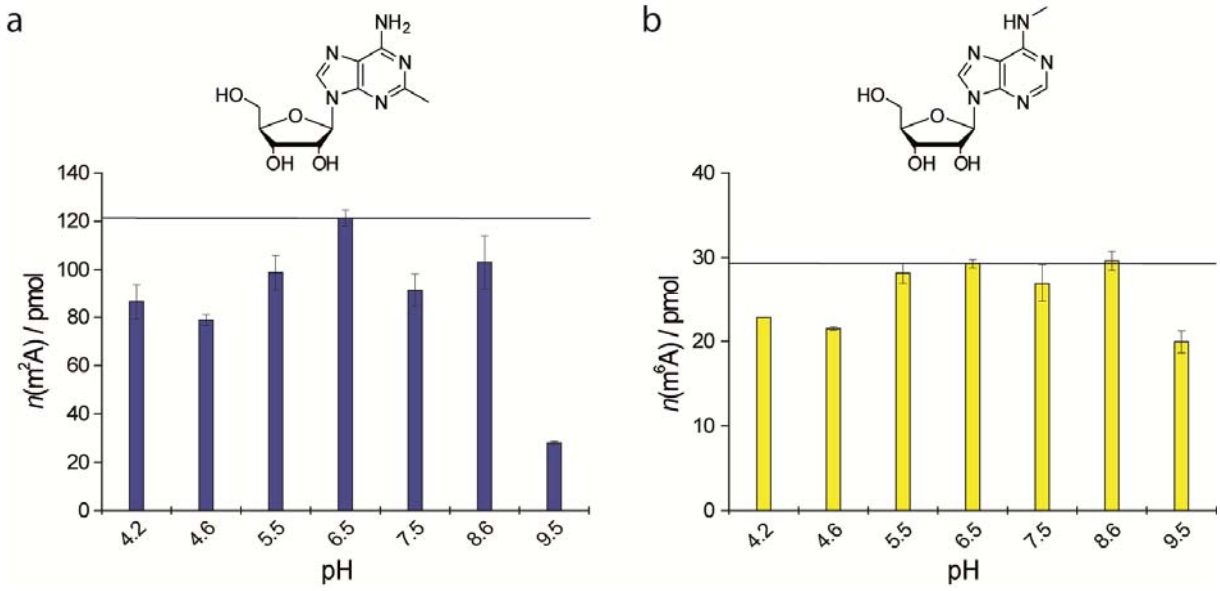


Figure 21: pH-dependent changes of m^2A and m^6A levels in *E. coli*.
Amounts of a) m^2A and b) m^6A with standard deviations from 12 ng of total extracted tRNA. Changes of the pH value of the medium were performed after *E. coli* K12 was grown under aerobic conditions in full medium to OD = 1.0.

A broad range of pH conditions was screened. The pH value of the medium during normal growth was determined to be 6.5. All experiments of this screening were performed as follows: *E. coli* was grown in full medium and under aerobic conditions to OD=1. At this point the pH of the medium was adjusted to the desired value with 4 M H_3PO_4 for pH values lower than 7.5 and 2 M NaOH for pH values higher than 7.5. The pH of the medium was changed to three different values of acidic stress (pH = 4.2, 4.6, and 5.5) and three different values of alkaline stress (pH = 7.5, 8.6, and 9.5).

For nucleoside m^2A the decrease in nucleoside level was significant for all variations from the normal pH value ranging from 20 % to 40 % with one exception (Figure 21 a). For pH = 9.5 a reduction of 75 % was detected. All changes of nucleoside levels were reproducible with high accuracy ruling out arbitrary alterations due to impaired cell growth or death. In the case of m^6A no or only moderate changes were detected for the pH range of 5.5 to 8.6 in contrast to more pronounced variations of m^2A under these conditions (Figure 21 b). For acidic conditions lower than 5.5 a reproducible decrease of 25 % was detected and for basic conditions the reduction was 30 %.

These results show a small dependence of the modified tRNA nucleoside level on outer stimuli and a distinct, individual reduction of the m^2A and m^6A amounts. Possible reasons for the drop of the modified nucleoside contents could be a reduced amount of the specific tRNA species in the cell or biosynthetic activity changes of the modifying enzymes.

The detection of this variation spurred further screenings of the impact of other external stimuli on the modified nucleoside levels in *E. coli* tRNA. In addition to the above mentioned stimulations salt (0.4 M NaCl),[153] oxidative (150 µl 30 % H_2O_2 in 1 L medium),[154] and heat stress (50 °C)[155] were applied to *E. coli* at OD=1 during exponential growth. In contrast to a constant m^2A nucleoside level under salt stress reduced nucleoside levels of approximately 10 % were detected for heat and oxidative stress (Figure 22 a). For the nucleoside m^6A moderate changes were found (Figure 22 b). Its amount stayed constant under heat stress and decreased during oxidative stress. In contrast, it increased, when elevated salt concentrations were applied. These incoherent results for both investigated nucleosides indicate an individual response of single tRNA modifications to stress conditions.

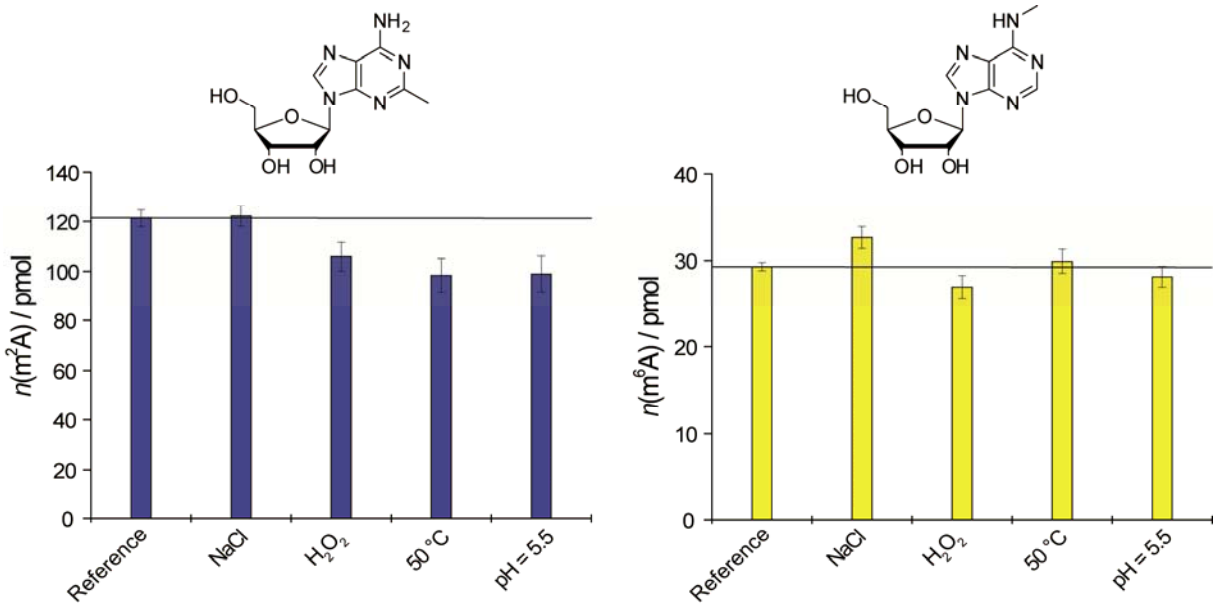


Figure 22: Stress-dependent changes of m^2A and m^6A levels in *E. coli*.
Amounts of a) m^2A and b) m^6A with standard deviations from 12 ng of total extracted tRNA. Stimulation by salt (0.4 M NaCl), oxidative (150 µl 30 % H_2O_2 in 1 L medium), heat stress (50 °C), and pH stress (2 M NaOH) after *E. coli* K12 was grown under aerobic conditions in full medium to OD = 1.0.

The impact of antibiotics on the tRNA nucleoside levels was also investigated. The content of m^2A and m^6A in *E. coli* bulk tRNA after application of five different ribosome-binding antibiotics was determined. These directly influence the bacterial translational process and are thus likely to induce changes in the translational apparatus. Furthermore, an impact of Chloramphenicol on bacterial tRNAs has been shown in literature before.[146a, 146b, 148] Antibiotics targeting the bacterial cell walls were excluded from the study, because they probably do not influence translation. Non-lethal concentrations of Streptomycin, Chloramphenicol, Spectinomycin, and Gentamycin were applied to exponentially growing *E. coli*.

The exposure of *E. coli* to antibiotics resulted in a decrease of the level of m^2A and variable changes in the m^6A nucleoside content in its tRNA. While for Chloramphenicol, Spectinomycin and Gentamycin the amount of m^2A was decreased by 5 % to 12 %, the impact of Streptomycin was more pronounced reducing the m^2A level by 25 % (Figure 23 a). The amount of the modification m^6A was only reduced after Streptomycin application, while its level was raised when Chloramphenicol, and Spectinomycin were applied. The changes for nucleoside m^6A ranged from 6 % to 12 % (Figure 23 b). For treatment with Gentamycin the amount of m^6A remained constant compared to reference data. Streptomycin, an aminoglycoside like Spectinomycin and Gentamycin, had a markedly different impact

compared to the other investigated antibiotics. It induced an at least two fold higher reduction of the m^2A amount then all other antibiotics and a unique decrease of the m^6A level. These results additionally show an individual alteration of the modified nucleoside level to external stimulation. Even specific changes to different antibiotics could be detected.

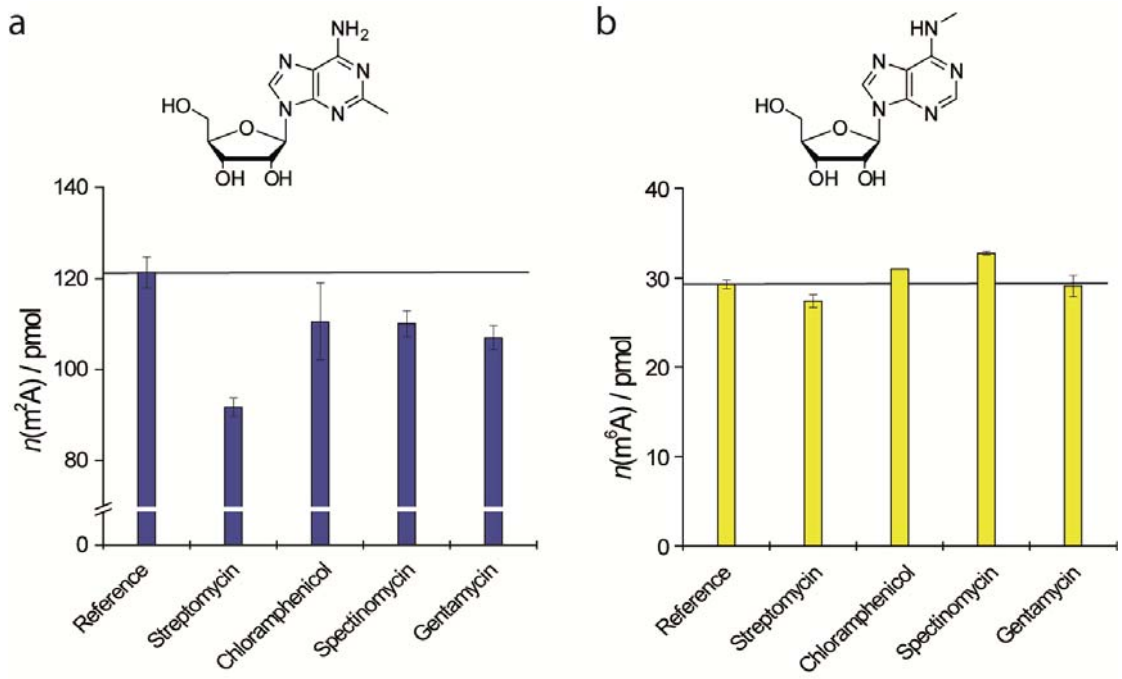


Figure 23: Antibiotic-dependent changes of m^2A and m^6A levels in *E. coli*.
Amounts of a) m^2A and b) m^6A with standard deviations from 12 ng of total extracted tRNA. *E. coli* K12 grown under aerobic conditions in full medium was treated with Streptomycin, Chloramphenicol, Spectinomycin, and Gentamycin.

In summary, an individual, stress-dependent response of the modified tRNA nucleosides m^2A and m^6A to different external stimulations was found. Next to a surprising OD-dependence, for many stresses applied considerable reductions of the m^2A and m^6A levels in total tRNA were observed. The main impacts on these translationally important nucleosides were detected during alkaline and antibiotic treatment yielding variations up to 75 % for m^2A and up to 25 % for m^6A. These reproducible changes imply directed nucleoside level variations as a reaction to external stimulation.

6 Isotope based quantification of the new sixth DNA-nucleoside hydroxymethyl-dC in brain

The genetic code is established by the sequence of the four canonical DNA nucleosides dA, dC, dG, and dT.[156] Of these four nucleosides dC can be chemically modified inside cells of higher organisms to control transcriptional activity (Figure 24).[1] Special methyltransferases replace the H-atom at position C5 by a methyl group to form $^{5\text{-Me}}$dC.[2] Methylation occurs in CpG sequences and is mostly responsible for the silencing of genes. In two recent back-to-back reports 5-hydroxymethyl-deoxycytidine ($^{5\text{-HOMe}}$dC) was established as a new post-replicatively formed DNA nucleoside.

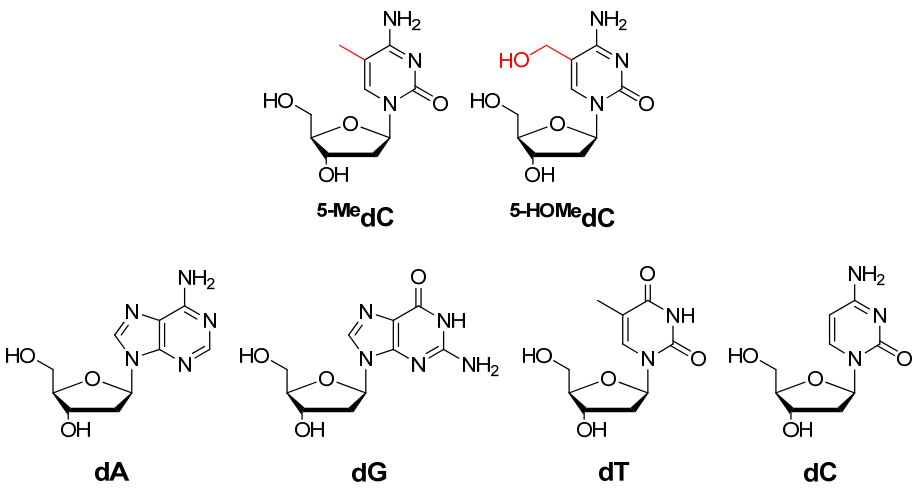


Figure 24: Structure of the post-replicatively formed DNA modifications $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC and of the four canonical nucleosides dA, dG, dT, and dC. Alterations to the canonical nucleosides are indicated in red.

Kriaucionis and Heintz[3a] detected $^{5\text{-HOMe}}$dC as a new sixth base in cerebrellar purkinje neurons. Rao, Aravind, Liu and co-workers[3b] reported the presence of $^{5\text{-HOMe}}$dC traces (~0.032 % of all nucleosides) in mouse embryonic stem cells and observed this nucleoside in CpG sequences in human embryonic kidney cells when they overexpressed the hydroxylating 2-oxoglutarate and Fe(II) dependent TET enzymes in these cells. It was additionally shown that these TET enzymes are able to oxidize the 5-methyl group of $^{5\text{-Me}}$dC to give the hydroxymethyl group *in vitro*. The new base was detected using thin layer chromatography and radioactive labeled [^{32}P]$^{5\text{-HOMe}}$dC. Its function is currently unclear but it is speculated that it may establish another level of transcriptional control.

DNA nucleoside $^{5\text{-HOMe}}$dC is an ideal candidate for the expansion of the here established quantification method. To this end $^{5\text{-HOMe}}$dC, $^{5\text{-Me}}$dC, and their heavy atom labeled derivatives had to be synthesized. In addition, the HPLC-ESI-MS and the digestion procedure had to be adjusted and an approach for the isolation of pure DNA from tissues had to be established. For method establishment cerebrum and cerebellum pork tissue samples were used, because these are available in large quantities and very likely to contain $^{5\text{-HOMe}}$dC.

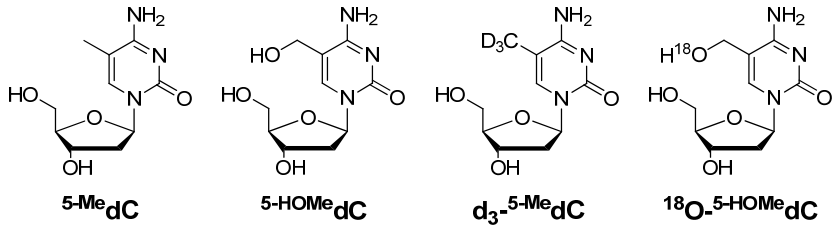


Figure 25: Isotope labeled nucleosides $^{5\text{-Me}}$dC, $^{5\text{-HOMe}}$dC, d_3-$^{5\text{-Me}}$dC, and ^{18}O-$^{5\text{-HOMe}}$dC.

Both nucleosides, $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC, were synthesized by M. Münzel in natural ($^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC) and isotope labeled form (d_3-$^{5\text{-Me}}$dC and ^{18}O-$^{5\text{-HOMe}}$dC, Figure 25).[157] In addition, the HPLC-ESI-MS procedure established for the quantification of modified tRNA nucleosides was adjusted to allow analysis of these modified DNA nucleosides by D. Globisch. With these DNA modifications and the adjusted HPLC-ESI-MS approach in hand the calibration curve equations were determined as discussed in chapters 5.1.5.1 and 10.6.2.[158] In both cases linear plots were obtained with R^2-values of 0.999. The protocol established for the digestion of tRNAs was found to be applicable for genomic DNA, too. Completeness of the digestion to single nucleosides was proven as described in chapter 5.1.3.

For the isolation and purification of genomic DNA, initially, the use of the standard DNA isolation kit "QIAamp DNA Mini Kit" was envisioned. This turned out to allow isolation of highly purified DNA from tissue samples within one day. Unfortunately, the amounts of DNA gained from most samples were not sufficient for the quantification of the rare nucleoside $^{5\text{-HOMe}}$dC. Therefore, an new DNA purification method had to be established, which allows collection of larger quantities. Based on the experiences from the isolation of tRNA and of DNA with the QIAGEN kit the following approach was established. For disruption of the cells and removal of the RNAs from the sample, the initial steps of the novel protocol, the QIAamp DNA Mini Kit was utilized. These steps were scaled up by a factor of four in comparison to the specifications from the kit to satisfy our need for increased amounts of DNA. In addition, the RNA digestion step was executed in duplicate to ensure full digestion

of the RNAs in the sample. Subsequently, a phenol/chloroform extraction, similar to the one used for tRNA isolation, was utilized to remove proteins and other cell components from the sample. After several precipitation steps pure DNA was obtained as analyzed by agarose gel electrophoresis (Figure 26). Comparison between the bands from the samples and the DNA ladder clearly shows that only very long nucleic acids, namely genomic DNA, are present in the samples. The details of this method are outlined in chapter 10.4.2.5.

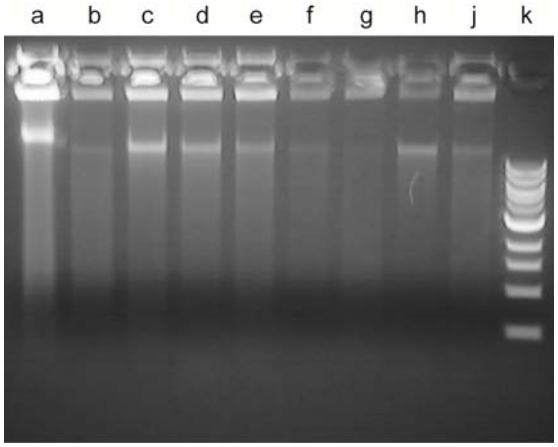


Figure 26: Representative analysis of DNA isolated from mouse brain samples *via* agarose gel electrophoresis.
a) retina DNA sample; b) hippocampus DNA sample from 1 d old mice; c) cerebellum DNA sample 1; d) cerebellum DNA sample 2; e) cortex DNA sample 1; f) cortex DNA sample 2; g) brainstem DNA sample 1; h) brainstem DNA sample 2; j) hippocampus DNA sample from 90 d old mice; k) 1 kd DNA ladder.

In agreement with literature analysis of brain cancer cell lines (Table 13) showed reduced $^{5\text{-Me}}$dC content (from typical 4.5 % to 3.1 %) proving the validity of our method.[159] In addition, it revealed that the new nucleoside $^{5\text{-HOMe}}$dC is absent in neuroblastal (Neuro-2a) and glial (U-87) cell lines.

Cell line	Origin	$^{5\text{-HOMe}}$dC/dG [%]	$^{5\text{-Me}}$dC/dG [%]
U-87 MG	Homo sapiens, astrocytoma	-	3.05
Neuro-2a	Mus musculus, neuroblastoma	-	3.09

Table 13: Ratio of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC to dG in a neuronal and a glial cell line in percent.

The modified nucleoside $^{5\text{-HOMe}}$dC was found predominantly in neuronal cells.[3a] Therefore, an initial quantification of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC was performed using various mouse brain

tissues. To this end hypothalamus, cortex, hippocampus, olfactory bulb, brainstem, cerebellum and retina of 90 days old mice were supplied by S. Michalakis from the Biel group. In addition, pork liver and kidney was investigated to evaluate the distribution of $^{5\text{-HOMe}}$dC in non-neuronal tissues. DNA isolation and digestion were executed as outlined above and in chapters 10.4.2.5 and 10.4.2.8. Afterwards, known amounts of the isotope labeled compounds d_3-$^{5\text{-Me}}$dC and ^{18}O-$^{5\text{-HOMe}}$dC were added and the nucleoside mixture was analyzed by LC-MS measurements on a *Thermo Finnigan LTQ Orbitrap XL*. In all experiments $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC eluted with retention times of 12.3 min and 18.5 min, respectively. Quantification was performed by comparing the areas of the ion currents of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC using the calibration curve equations. From repeated experiments an average error for the quantification data of ± 5.6 % for $^{5\text{-Me}}$dC and ± 7.2 % for $^{5\text{-HOMe}}$dC was determined (Table 14).

Mammal	Tissue	%($^{5\text{-Me}}$dC/dG)	%($^{5\text{-HOMe}}$dC/dG)
Pig	Cerebellum	6.18 ±0.39	0.72 ±0.04
Pig	Cerebrum	5.78 ±0.57	0.67 ±0.03
Pig	Liver	6.04 ±0.45	-
Pig	Kidney	5.89 ±0.39	0.14 ±0.04
Mouse	Hypothalamus	3.33 ±0.40	0.69 ±0.03
Mouse	Cortex	4.50 ±0.23	0.65 ±0.04
Mouse	Brain Stem	4.50 ±0.12	0.55 ±0.04
Mouse	Olfactory Bulb	4.54 ±0.13	0.53 ±0.02
Mouse	Cerebellum	4.39 ±0.12	0.33 ±0.00
Mouse	Retina	4.48 ±0.15	0.31 ±0.01
Mouse	Hippocampus	4.31 ±0.31	0.59 ±0.04
Mouse	Hippocampus (1 day old)	3.48 ±0.04	0.34 ±0.02

Table 14: Ratios of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC to dG in the DNA of pork and mouse tissues.
Standard deviations are given in absolute values. dG was chosen as a reference, because it forms base pairs with dC, $^{5\text{-HOMe}}$dC, and $^{5\text{-Me}}$dC in DNA.

Between 0.3 % and 0.7 % of all dC nucleosides are hydroxymethylated at position C5. Pork cerebellum and cerebrum contain the same amounts of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC. In the non-nervous tissues, liver and kidney, similar amounts of $^{5\text{-Me}}$dC as in brain tissues were measured but significantly less or no $^{5\text{-HOMe}}$dC was detected in accordance with literature.[3a] This is a further hint, that $^{5\text{-HOMe}}$dC is mainly a neuronal cell specific DNA modification.

Comparison of the data obtained for both investigated mammals reveals that pork has generally higher amounts of $^{5\text{-Me}}$dC and similar levels of $^{5\text{-HOMe}}$dC as mouse. Samples from

pork cerebrum were collected from the outer regions of the brain and are therefore considered as comparable with mouse cortex as a first approximation. Interestingly, this comparison shows that both mammals exhibit the same levels of $^{5\text{-HOMe}}$dC in the cortex, but significantly varying ones in cerebellum. In mouse cerebellum only half as much $^{5\text{-HOMe}}$dC as in pork cerebellum was detected. A possible explanation for the observed dissimilarities are the different preparations of the brain samples. For pork cerebellum samples parts of the cerebellar cortex were collected, whereas for mouse whole cerebellums were analyzed. Cerebellar cortex might contain higher levels of $^{5\text{-HOMe}}$dC than whole cerebellums. Alternatively, a connection with different functions of the cerebellum in mouse and pork is imaginable.

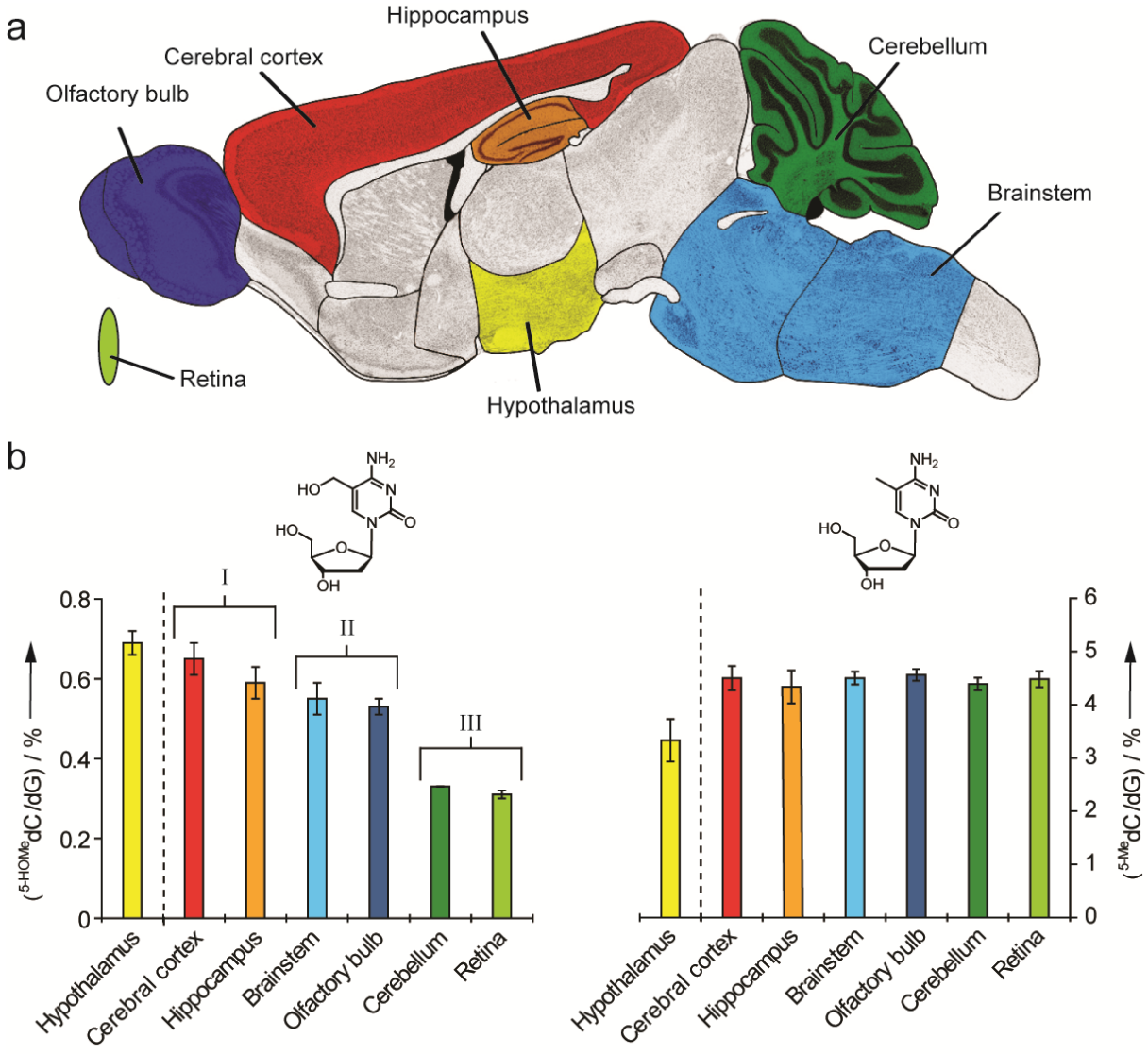


Figure 27: Brain regions investigated and the ratios of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC to dG in their DNA.
a) Depiction of a sagittal section of the mouse brain. Brain areas highlighted in color were studied here. b) Ratio of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC to dG in the different tissues in percent. Error bars represent the determined standard deviations. dG was chosen as a reference, because it forms base pairs with dC, $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC in DNA.

The new nucleoside $^{5\text{-HOMe}}$dC was clearly detected in all brain tissues (Table 14) establishing $^{5\text{-HOMe}}$dC as a new post-replicatively formed nucleoside in brain regions other than cerebellum. Figure 27 illustrates the different brain regions analyzed. The amount of $^{5\text{-HOMe}}$dC varied significantly in the investigated mouse brain tissues. The previous report observed $^{5\text{-HOMe}}$dC predominately in purkinje neurons,[3a] which are found in the cerebellum. Here, the amount of the nucleoside is even larger in cortex and hippocampus, where purkinje cells are not present indicating the presence of $^{5\text{-HOMe}}$dC also in brain cells, which are not purkinje neurons.

Following these data the possibility arises to roughly divide the mouse brain into three different areas (Figure 27 b). Most $^{5\text{-HOMe}}$dC is found in hippocampus and cortex (I), which are the brain areas that have higher cognitive functions. Brainstem, and the olfactory bulb form a second category, which possesses intermediate $^{5\text{-HOMe}}$dC levels (II). Cerebellum and retina finally contain significantly less $^{5\text{-HOMe}}$dC (III). Surprisingly, these initial data also show a relatively high level of $^{5\text{-HOMe}}$dC in the hypothalamus, which is part of the endocrine system that controls hormone based processes.

There is no correlation between the amount of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC in the investigated tissues. This would be expected, if $^{5\text{-HOMe}}$dC is exclusively generated from $^{5\text{-Me}}$dC as a precursor. The $^{5\text{-Me}}$dC values are significantly lower in hippocampus and hypothalamus, where high $^{5\text{-HOMe}}$dC values were detected. In accord with literature[160] in all other tissues the $^{5\text{-Me}}$dC values were stable at roughly 4.5 %, while the $^{5\text{-HOMe}}$dC levels are significantly varying. This hints at a function of $^{5\text{-HOMe}}$dC that does not go hand in hand with a reduced $^{5\text{-Me}}$dC value.

Finally, the impact of the animal's age on the values for $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC was investigated. Thus hippocampus tissue of only one day old mice (Figure 28) was analyzed. In contrast to a previous report by Heintz,[3a] who could not see an ageing effect, significant differences were detected here. In young mice the levels of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC were both significantly reduced. The $^{5\text{-Me}}$dC value increased with age from 3.5 ±0.1 % to 4.1 ±0.2 %. More significantly, the amount of $^{5\text{-HOMe}}$dC was raised in 90 days old mice in comparison to 1 day old mice by approximately 80 % from 0.34 ±0.02 % to 0.62 ±0.02 %. Again the amount of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC were not inversely correlated. In order to exclude that $^{5\text{-HOMe}}$dC accumulated due to oxidative stress in older animals the damaged base 8-oxodG, which is a typical oxidative stress marker, was analyzed. Here no difference could be detected, which shows that $^{5\text{-HOMe}}$dC in the brain is not the result of accumulation of oxidative DNA damage,[161] but a post-replicatively modified nucleoside.

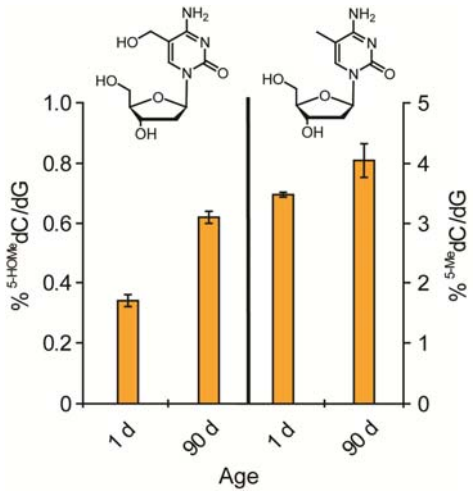


Figure 28: Ratio of $^{5\text{-HOMe}}$dC and $^{5\text{-Me}}$dC to dG in the hippocampus of 1 day and 90 days old mice in percent.

In summary, our method for the quantification of modified tRNA nucleosides has been expanded to modified DNA nucleosides allowing quantification of $^{5\text{-Me}}$dC and $^{5\text{-HOMe}}$dC in tissues with excellent accuracy. The successful broadening of the method is based on the synthesis of $^{5\text{-Me}}$dC, $^{5\text{-HOMe}}$dC, d_3-$^{5\text{-Me}}$dC, and ^{18}O-$^{5\text{-HOMe}}$dC, the adjustment of the HPLC-ESI-MS procedure for the quantification of these nucleosides, and the development a protocol, which allows high-yielding isolation of pure DNA from tissue samples.

Using this extended quantification method $^{5\text{-HOMe}}$dC was confirmed as a post-replicatively formed nucleoside. It was found that $^{5\text{-HOMe}}$dC is widely distributed in brain and that it is particularly prominent in those brain tissues, which are involved in higher cognitive functions. Finally, reduced levels of $^{5\text{-HOMe}}$dC in new born mice were uncovered. The established procedure will allow a detailed analysis of the distribution of $^{5\text{-HOMe}}$dC in response to different kinds of stimuli and may substantially contribute to the development of this brand new field in epigenetic research.

7 Syntheses

7.1 Syntheses of natural nucleosides and their heavy atom labeled derivatives

Apart from the mass spectrometry equipment the main tools for quantification *via* a stable isotope dilution approach are the molecules to be quantified and their heavy atom labeled derivatives. Both are needed to establish the calibration equations (chapter 10.6.2) essential for accurate quantification of the analyte. The natural nucleosides (analytes) and their heavy atom labeled derivatives (standards) of interest during this thesis work were not commercially available and thus had to be synthesized.

The main purpose of all syntheses presented in the following was to supply sufficient amounts for HPLC-MS quantification. Thus, as soon as this purpose was accomplished no second synthesis or further optimization of the reaction conditions were performed. For the nucleosides presented hereafter all analytical data are in good to excellent agreement with literature.

For each unlabeled nucleoside the discussions in this chapter will generally have the following layout:

1. General information on the nucleoside like biological activities and appearances in RNA
2. Functions
3. Biosynthesis
4. Known syntheses
5. Synthesis performed as part of this thesis

For the corresponding labeled nucleosides the realized syntheses will be discussed.

7.1.1 Syntheses of isopentenyl and methylthioadenosines

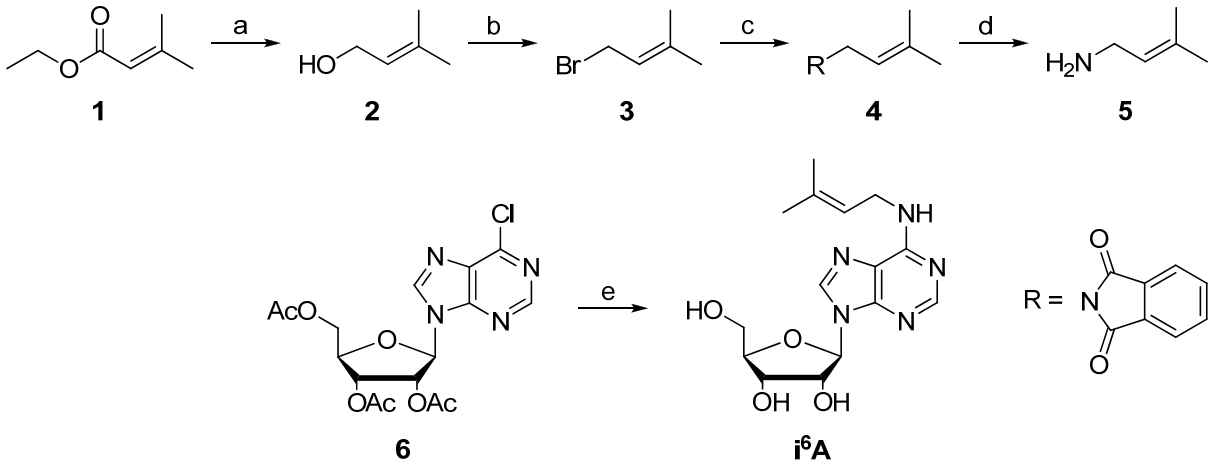
7.1.1.1 Synthesis of i^6A

Nucleoside i^6A has been shown to inhibit cell proliferation, induce apoptosis in a human colon cancer cell line DLD1,[162] and to be a potential therapeutic agent for a variety of epithelial cancers.[163] Furthermore, it is also a potent cytokinin and as such has stimulated research early on.[164]

The nucleoside i^6A has been found to be essential for the maturation of selenocystein tRNA[165] but its general functions are still unclear.

The biosynthesis of i^6A in tRNA has recently been characterized thoroughly. Different stages of the transformation of adenosine to i^6A in the anticodon loop of *E. coli* $tRNA^{Phe}_{GAA}$ by MiaA were crystallized.[166]

The first synthesis was published in 1966.[167] Apart from the preparation of the natural nucleoside a ^{14}C-labeled derivative was synthesized early on.[168] More recently it has also been incorporated into RNA strands.[169]



Scheme 1: Synthesis of i^6A.
Reagents and conditions: a) $LiAlH_4$, EtO_2, 0 °C to rt, 15 min; b) PBr_3, Et_2O, 0 °C, 30 min; c) KPhth, DMF, 80 °C, 30 min, 64 % (3 steps); d) hydrazine hydrate, 2 M HCl, MeOH, rt, 20 h, 99 %; e) **5**, EtOH, 60 °C, 18 h, 69 %.

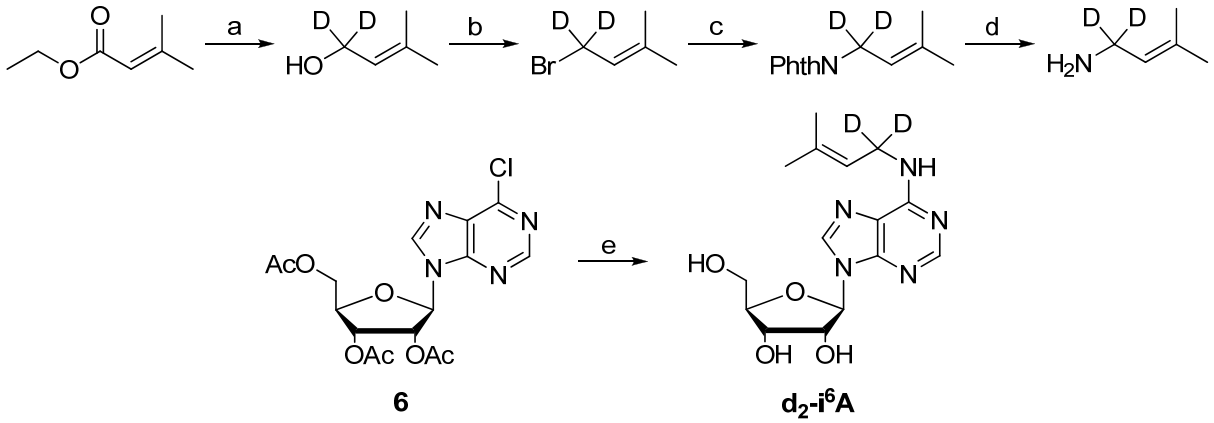
General features of the adenosine nucleoside syntheses presented hereafter are the use of acetyl protecting groups and the introduction of the allylamine moiety in position 4 by nucleophilic substitution of a chlorine substituent. This combination allows simultaneous attachment of the allylamine and removal of all protection groups in the last step minimizing the length of the syntheses.

As discussed above mass spectrometric quantification is based on the simultaneous, but individual measurement of the ions from the analyte and the standard. This is only possible for a mass deviation between analyte and standard of at least two. For a difference of only one significant amounts of the analyte have the same mass as the standard, because roughly 10 % of all carbons in natural compounds are ^{13}C. Therefore, all syntheses have to allow incorporation of at least two heavy atoms.

The most convenient approach usually is introduction of deuterium *via* a reduction. Attempts to synthesize i^6A by reduction of dimethylacrylonitrile with $LiAlH_4$ as described in the

original synthesis[167] failed. Therefore, a more reactive carbonyl compound, namely 3,3-dimethylacrylic acid ethyl ester **1**, was chosen for the reduction with $LiAlH_4$. The ester was reduced successfully to the corresponding alcohol **2**. This hydroxyl compound was converted into the bromo alkene **3**. Nitrogen was introduced by a *Gabriel synthesis*. Replacement of bromine with KPhth yielded compound **4**. Subsequent hydrazine induced deprotection furnished amine **5** in 63 % yield over 4 steps. This reaction sequence was optimized in a way that all four steps could be performed in 24 h yielding several grams of amine **5**. Nucleoside **6** was obtained from D. Globisch. Following the strategy outlined above treatment of chloro nucleoside **6** with allylamine **5** yielded i^6A in a straightforward manner. The synthesis of i^6A was accomplished in 7 steps and 33 % overall yield.

7.1.1.2 Synthesis of d_2-i^6A



Scheme 2: Synthesis of d_2-i^6A.
Reagents and conditions: a) $LiAlD_4$, EtO_2, rt, 5 min, 98 %; b) PBr_3, Et_2O, 0 °C, 30 min, 59 %; c) KPhth, DMF, 80 °C, 30 min, 80 %; d) hydrazine hydrate, 2 M HCl, MeOH, rt, 18 h, 95 %; e) 1-dideutero-3-methylbut-2-en-1-amine, EtOH, 60 - 80 °C, 21 h, 85 %.

The corresponding standard, d_2-i^6A, was synthesized as described for the natural nucleoside but using $LiAlD_4$ instead of $LiAlH_4$. The reaction sequence is illustrated in Scheme 2. Standard d_2-i^6A was obtained in 7 steps and 30 % overall yield.

7.1.1.3 Synthesis of ms^2i^6A

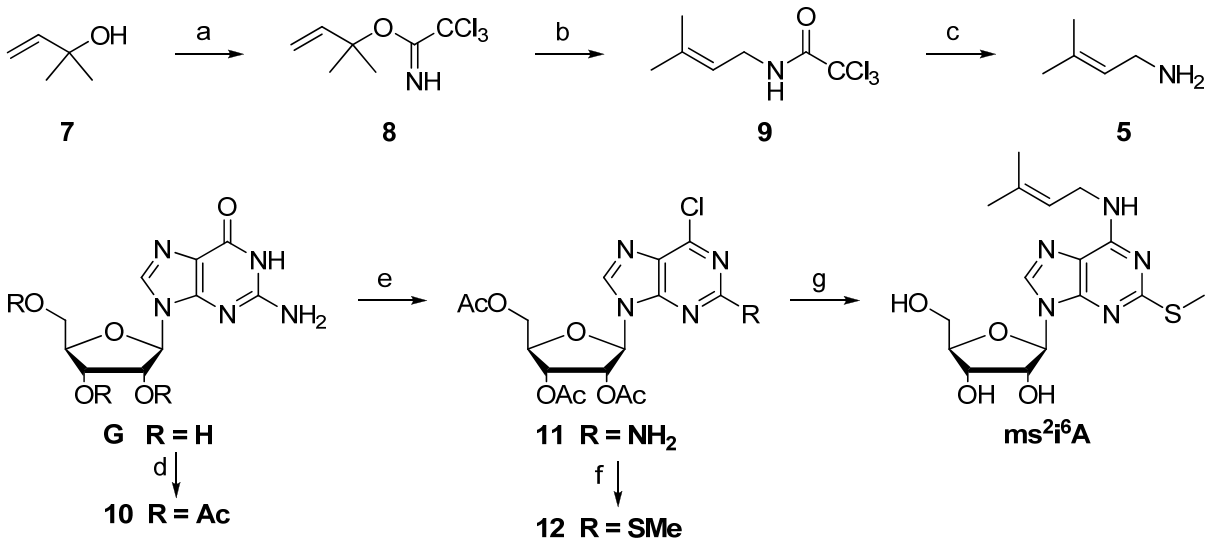
Nucleoside ms^2i^6A is a cytokinin and has been isolated from tRNA in 1966 for the first time.[170] It is present in bacterial, chloroplast, and mitochondrial tRNA.[4a, 4b, 158a]

Numerous functions of ms^2i^6A in RNA have been found:

- Prevention of codon misreading in *E. coli*.[171]
- Influence on the development of *B. subtilis*.[172]
- Influence on the structural stability of the anticodon loop.[173]
- Prerequisite for the virulence of *S. flexneri*.[116b, 174]
- Regulatory switch of the aromatic amino acid transport under iron deficiency.[146c, 175]

In addition to the incorporation of the allyl moiety by MiaA as described for i^6A introduction of the methyl thioether in position 2 is necessary. This is accomplished by MiaB, a radical-S-adenosylmethionine (SAM) enzyme[176] with two essential [4Fe-4S] clusters.[177]

Chemical studies of ms^2i^6A include its synthesis and incorporation in RNA.[169b] Isotopically labeled derivatives of ms^2i^6A have also been synthesized before.[168, 178]



Scheme 3: Synthesis of ms^2i^6A.
Reagents and conditions: a) 2-Methyl-3-buten-2-ol, NaH, trichloro acetonirile, THF, Et_2O, 0 °C to rt, 2 h; b) toluene, 115 °C, 2 h, 67 % (2 steps); c) 3 N KOH, DMF, rt, 4 h, 45 %; d) Ac_2O, DMF, pyridine, 75 °C, 1.5 h, 87 %; e) $POCl_3$, DMA, Et_4NCl, MeCN, 100 °C, 10 min, 51 %; f) Dimethyldisulfide, *N*-pentyl-nitrite, MeCN, 70 °C, 1.5 h, 65 %; g) **5** (neat), 50 °C, 1.5 h, 74 %.

Nucleoside ms^2i^6A was prepared as outlined in Scheme 3. Isopentenylamine **5** was synthesized from allylalcohol **7** on a multi-gram scale as described in literature.[179] This route was established when mass spectrometric quantification was not part of the project yet. As

discussed above this approach to allylamine **5** does not permit straightforward incorporation of heavy atoms. Therefore, for the synthesis of i^6A and d_2-i^6A an alternative route to amine **5** was chosen. An interesting feature of this reaction sequence is the *Overman Rearrangement*[180] of allyltrichloracetimidate **8** to allylamide **9**.

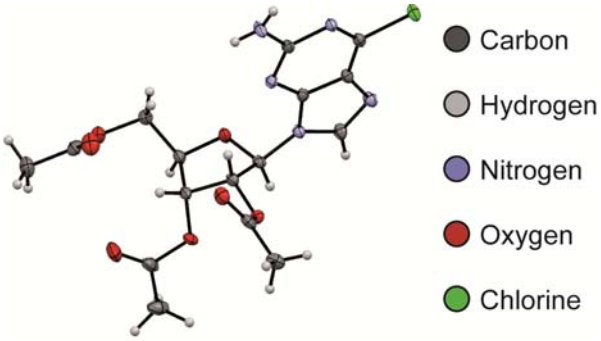


Figure 29: Crystal structure of nucleoside **11**.

For the synthesis of the second building block (Scheme 3) guanosine (G) was protected with acetic acid anhydride under standard conditions to give acetylated nucleoside **10**.[181] Conversion of the oxo to a chloro substituent yielded nucleoside **11**.[182] The crystal structure of this nucleoside is shown in Figure 29. The methylthio residue was introduced as described by Kierzek *et al*.[169b] Nucleoside **11** was diazotized with *N*-pentyl-nitrite. The diazo-intermediate was trapped with dimethyldisulfide to give nucleoside **12**.[183] Treatment of chloro nucleoside **12** with isopentenylamine **5** yielded ms^2i^6A in 7 steps and 6 % overall yield on a gram scale.

The crystal structure of nucleoside ms^2i^6A could be determined and is presented in Figure 30. Interestingly, in the crystal ms^2i^6A forms a hydrogen bond between C_5'OH and N1.

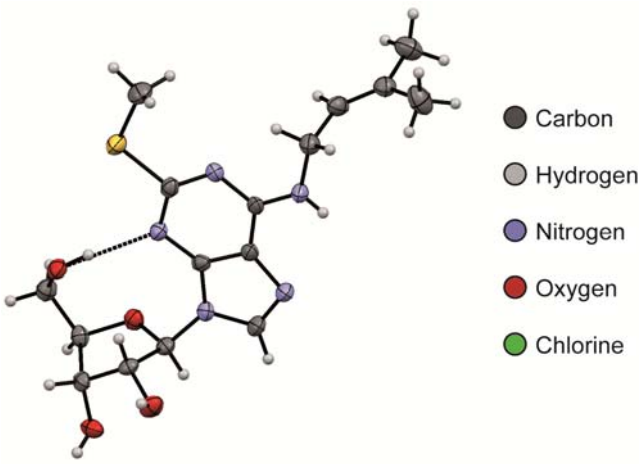
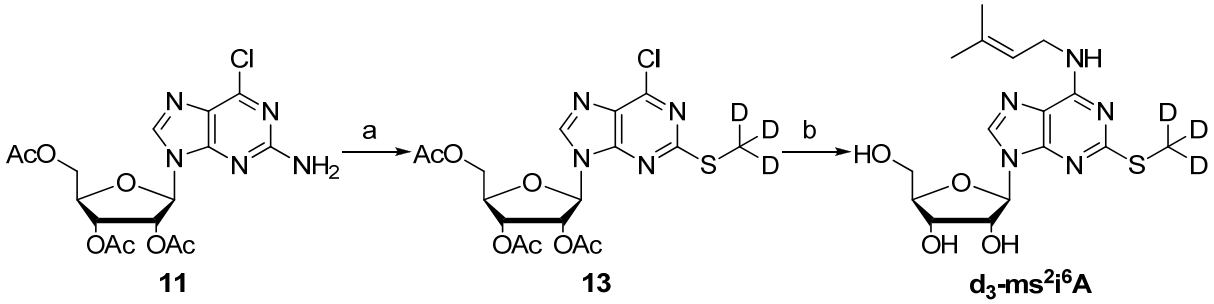


Figure 30: Crystal structure of ms^2i^6A.

7.1.1.4 Synthesis of d_3-ms^2i^6A



Scheme 4: Synthesis of d_3-ms^2i^6A.
Reagents and conditions: a) d_6-Dimethyldisulfide, *N*-pentyl-nitrite, MeCN, 70 °C, 5 min, 78 %; b) **5** (neat), 40 °C, 16 h, 39 %.

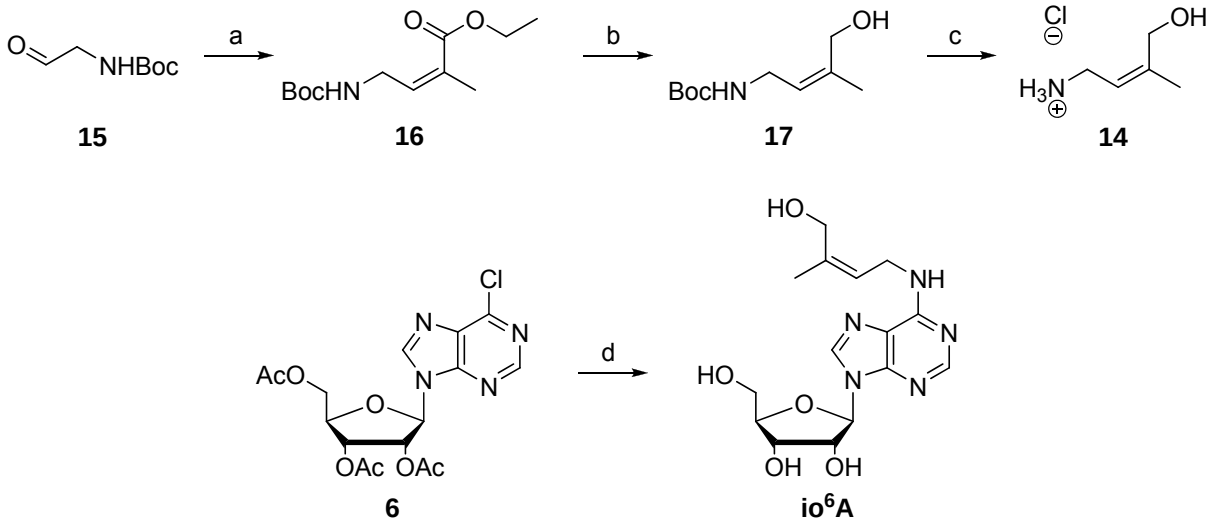
When the synthesis of labeled ms^2i^6A was planed, i^6A had not been a target molecule of this thesis work. Thus the route to deuterated allylamine *via* a reduction of 3,3-dimethylacrylic acid ethyl ester had not been established yet. At this time use of deuterated dimethyldisulfide was identified as the most convenient approach to isotopically label ms^2i^6A. Chloro nucleoside **11** is available on a multi-gram scale with little effort. Therefore, the reaction conditions for the conversion of the amine **11** to the d_3-methyl thioether **13** were optimized for minimal d_6-dimethyldisulfide consumption. All other reactions were performed as described for ms^2i^6A. Scheme 4 shows the reactions performed during the synthesis of d_3-ms^2i^6A, which have not already been discussed in chapter 7.1.1.3.

7.1.1.5 Synthesis of io^6A

In the course of this thesis work it could be shown that io^6A is both present in eukaryotes and bacteria. Functions and biosynthesis of io^6A in tRNA are still unidentified. One might speculate that it is a potential biosynthetic intermediate towards ms^2io^6A. But there is not enzyme know to catalyze the necessary methylthiolation, while the hydroxylation of ms^2i^6A has been studied.

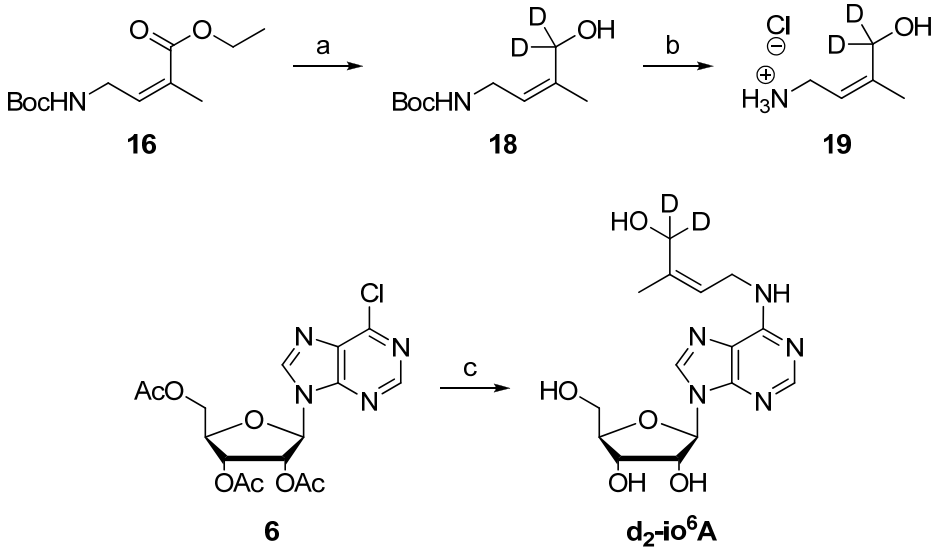
Synthesis of the allylammonium salt **14** was performed on the basics of a synthesis of 2-deoxy-ms^2io^6A by Evidente *et al.* (Scheme 5).[184] Commercially available aldehyde **15** was converted into the *Z*-allyl ester **16** under conditions derived from the Horner-Wadsworth-Emmons reaction. Subsequent reduction to allylalkohol **17** and deprotection yielded amine **14** in good yields and gram quantity. Reaction of chloro compound **6** with allylammonium

chloride **14** in Et_3N and MeOH gave io^6A in 6 steps and 9 % overall yield. Major losses were experienced in the last step due to the rigorous purification *via* HPL chromatography.



Scheme 5: Synthesis of io^6A.
Reagents and conditions: a) 2-(Diethoxy-phosphoryl)-propionic acid ethyl ester, ^{n}BuLi, THF, -78 °C to rt, 3 h, 55 %; b) $LiAlH_4$, Et_2O, -78 °C to rt, 2 h, 75 %; c) 2 M HCl, rt, 4 h, 94 %, d) **14**, Et_3N, MeOH, 60 °C, 48 h, 28 %.

7.1.1.6 Synthesis of d_2-io^6A



Scheme 6: Synthesis of d_2-io^6A.
Reagents and conditions: a) $LiAlD_4$, Et_2O, -78 °C to rt, 3 h, 87 %; b) 2 M HCl, 60 °C, 30 min, 73 %; c) **19**, Et_3N, MeOH, 60 °C, 16 h, 13 %.

Ester **16** was synthesized as described above. Reduction of its ester moiety with $LiAlD_4$ allowed introduction of two deuterium atoms (Scheme 6) and yielded alcohol **18**. Acidic deprotection gave ammonium salt **19**, which was used to convert chloro nucleoside **6** into d_2-io^{6}A.

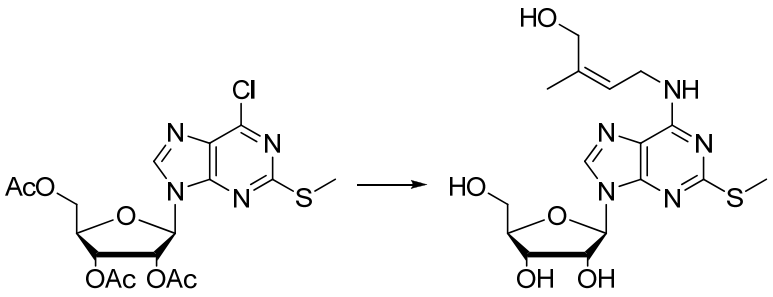
7.1.1.7 Synthesis of ms^{2}io^{6}A

Ribosyl-zeatin (ms^{2}io^{6}A) is the most important cytokinin of the isopentenyladenosine family.[185]

The functions of ms^{2}io^{6}A in tRNA have been studied thoroughly in bacteria.[186] It is present in *S. typhimorium* tRNA, where it is a possible regulator for aerobiosis.[187] It also regulates the growth on citric acid cycle intermediates *via* presence or absence of its side-chain hydroxyl group.[188]

Incorporation into tRNA takes place by conversion of ms^{2}i^{6}A to ms^{2}io^{6}A catalyzed by MiaE.[189]

There is literature precedence for the synthesis of ms^{2}io^{6}A[184, 190] and its heavy atom labeled derivative.[191]

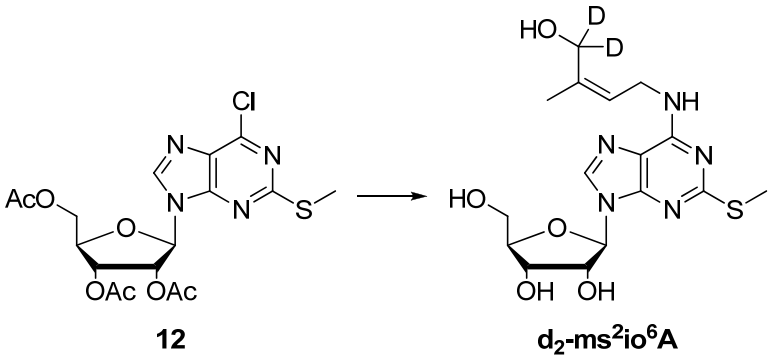


12 **ms^{2}io^{6}A**

Scheme 7: Synthesis of ms^{2}io^{6}A.
Reagents and conditions: **14**, Et_3N, MeOH, 60 °C, 24 h, 26 %.

Nucleoside **12** and ammonium salt **14** were prepared as described in chapter 7.1.1.3 and chapter 7.1.1.5, respectively. As depicted in Scheme 7 the reagents were stirred under slightly basic conditions to give ms^{2}io^{6}A with 26 % yield.

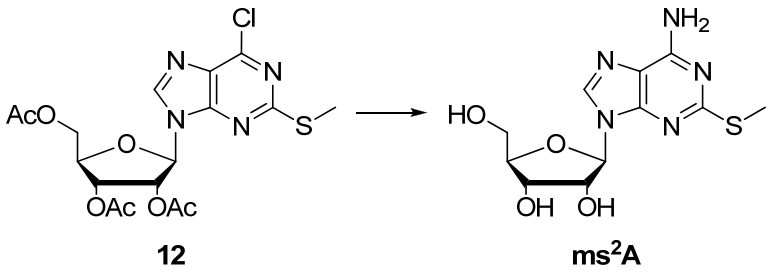
7.1.1.8 Synthesis of d_2-ms^2io^6A



Scheme 8: Synthesis of d_2-ms^2io^6A.
Reagents and conditions: **19**, Et_3N, MeOH, 60 °C, 16 h, 2 %.

The synthesis of d_2-ms^2io^6A is illustrated in Scheme 8. Reaction of nucleoside **12** (chapter 7.1.1.3) with the deuterated ammonium salt **19** (chapter 7.1.1.6) under slightly basic conditions yielded d_2-ms^2io^6A.

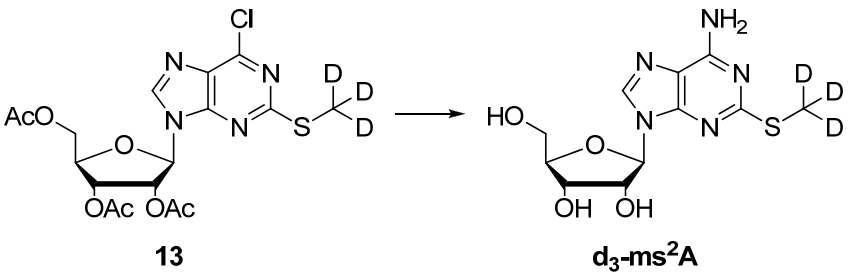
7.1.1.9 Synthesis of ms^2A



Scheme 9: Synthesis of ms^2A.
Reagents and conditions: NH_3, rt, 24 h, 81 %.

Nucleoside ms^2A has not been found in tRNA yet but it is a potential biosynthetic precursor of several natural methylthio nucleosides and might there be of interest at later stages of the larger project initiated with this thesis work. In addition, it is a convenient product obtained from treatment of chloro nucleoside **12** with ammonia. Thus, ammonia was condensed in a flask with nucleoside **12** and stirred for 24 h giving ms^2A in 81 % yield. The overall yield of this 4 step sequence was 23 %.

7.1.1.10 Synthesis of d_3-ms^2A



13 **d_3-ms^2A**

Scheme 10: Synthesis of d_3-ms^2A.
Reagents and conditions: NH_3, rt, 24 h, 17 %.

To allow quantification of ms^2A the corresponding heavy atom labeled nucleoside has been synthesized by treatment of trideutero nucleoside **13** with ammonia.

7.1.2 Syntheses of methyladenosines

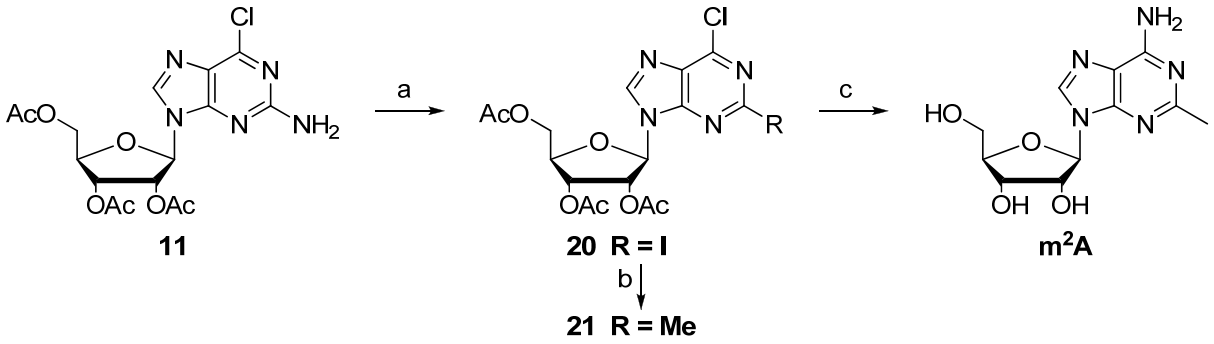
7.1.2.1 Synthesis of m^2A

Another class of modified nucleosides are methylated adenosines. Out of the eight known methyladenosines m^2A is the most striking one, because of its unusual methylation site. Introduction of a methylation in position 2 of the heterocycle necessitates activation of a C-H bond and formation of a novel C-C bond. Nevertheless, m^2A is a rather common and abundant modification. It was first found in 1960[192] and is present in bacterial and chloroplast tRNA[4b, 151, 193] and in rRNA.[194]

Its function in tRNA is not yet known.

In rRNA it is introduced *via* YfgB.[195] The corresponding enzyme for tRNA modification is still unidentified.

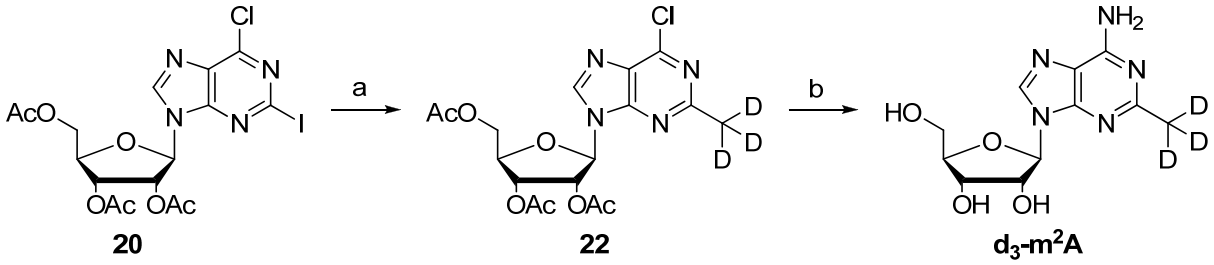
The first synthesis of m^2A was published in 1952 with the title "Some synthetic analogs of the natural purine nucleosides".[196] As it turned out later m^2A actually is a natural purine nucleoside, too. Later other syntheses of this interesting adenosine derivative followed.[197]



Scheme 11: Synthesis of m^2A.
Reagents and conditions: a) I_2, CH_2I_2, CuI, *N*-pentyl-nitrite, THF, 70 °C, 2 h, 61 %; b) $ZnCl_2$, MeMgCl, $Pd(PPh_3)_4$, THF, 0 °C to rt, 4 h, 86 %; c) NH_3, rt, 36 h, 71 %.

The first two steps of the route to m^2A are identical with those of the synthesis of ms^2i^6A (chapter 7.1.1.3). The common intermediate **11** is converted into iodo nucleosides **20** by use of a similar reaction as described for the introduction of the methylthio moiety. *N*-pentyl-nitrite, CuI, and CH_2I_2 are used for the functional group interconversion *via* a diazotation of amine **11**. Subsequent Negishi coupling yielded the 2-methylnucleoside **21**. The zinc organic compound used for this cross coupling reaction was prepared by reaction of MeMgCl with $ZnCl_2$. Treatment of nucleoside **21** with ammonia simultaneously led to deprotection and conversion of the chloro to the amino substituent. m^2A was obtained in 17 % overall yield in a 5 step linear sequence.

7.1.2.2 Synthesis of d_3-m^2A



Scheme 12: Synthesis of d_3-m^2A.
Reagents and conditions: a) $ZnCl_2$, d_3-MeMgCl, $Pd(PPh_3)_4$, THF, 0 °C to rt, 5 h, 25 %; e) NH_3, rt, 36 h, 6 %.

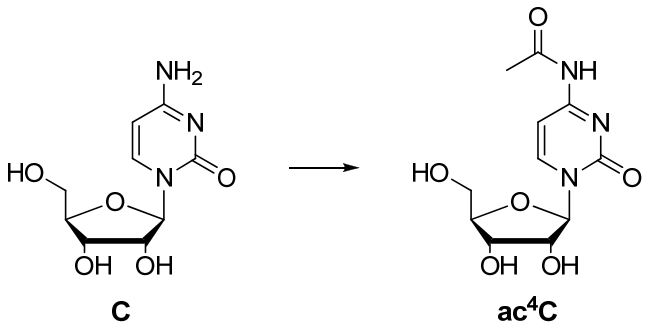
For the synthesis of trideuterated nucleoside **22** the heavy atoms were introduced by use of d_3-MeMgCl. It was utilized for preparation of the methyl zinc species of the Negishi coupling. Except for this all reactions were carried out as described above yielding d_3-m^2A.

7.1.3 Syntheses of cytidine derivatives

7.1.3.1 Synthesis of ac^{4}C

The modification ac^{4}C is present in position 12 in eukaryotic tRNA. There, it probably stabilizes the tRNA structure already in an early stage of the tRNA maturation.[198] In bacterial tRNA ac^{4}C occurs at the wobble position. It improves codon recognition and inhibits mistranslation of the isoleucine codon AUA.[199]

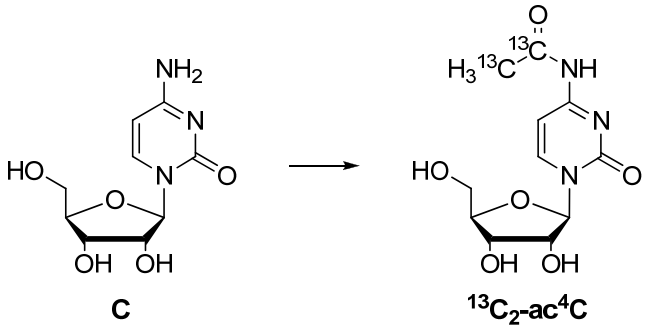
The enzyme Tan1p is involved in the biosynthesis of ac^{4}C in eukaryotes.[200] In prokaryotes the acetylation is catalyzed by an enzyme called TmcA, whose structure could be solved recently.[201]



Scheme 13: Synthesis of ac^{4}C.
Reagents and conditions: Ac_2O, EtOH, 80 °C, 6 h, 79 %.

The straight forward synthesis of ac^{4}C starting from cytidine (C) was performed similar to a synthesis published by Watanabe and Fox.[202] This approach yielded the acetylated nucleoside in one step, good yield and large quantity.

7.1.3.2 Synthesis of d$_3$-ac^{4}C



Scheme 14: Synthesis of $^{13}C_2$-ac^{4}C.
Reagents and conditions: $^{13}C_4$-Ac_2O, EtOH, 60 °C, 6.5 h, 13 %.

Initially, heavy atom labeled ac^4C was prepared by use of d_6-Ac_2O. Unfortunately, it turned out that the deuterium atoms in d_3-ac^4C exchange with proton from the HPLC buffer under the condition currently used for HPLC-MS analysis here. Therefore, significantly more expensive $^{13}C_4$-Ac_2O was utilized. In view of cost for $^{13}C_4$-Ac_2O a screening of reaction conditions with unlabeled Ac_2O was conducted, which led to a procedure requiring considerably reduced amounts of acetic acid anhydride in comparison to the literature approach. Reaction conditions were established, which employ only one equivalent of acetic acid anhydride for the formation of ac^4C.

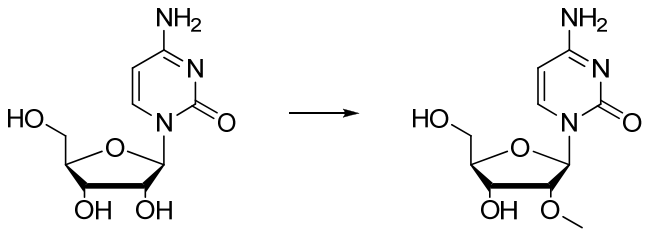
7.1.3.3 Synthesis of Cm

Cm is found in the anticodon stem-loop and in the amino acid acceptor stem of eukaryotic,[117, 203] archaeal,[204] and bacterial tRNA.[205]

In both positions it stabilizes the structure of the tRNA.[203] As part of the anticodon loop it also enhances the efficiency of the codon recognition[206] and prevents misreading of condons.[207]

Cm is incorporated into tRNA by enzymes with a *S*-Adenosyl-*L*-methionin (SAM) cofactor. Trm7p[203] and YfhQ[205] are enzymes synthesizing Cm in yeast and bacterial anticodon loops, respectively. In position 4 of eukaryotic tRNA Cm is introduced by Trm13.[117] In archaeal tRNA its biosynthesis is accomplished by Trm56.[204]

Cm has been introduced in RNA strands showing its stabilizing effect on RNA duplexes.[208] Cm has been synthesized by Quaedflieg *et al.*[209] in 1991 by a straight forward one pot methylation.



Scheme 15: Synthesis of Cm.
Reagents and conditions: NaH, MeI, DMF, 0 °C to rt, 4.5 h, 16 %.

This approach was chosen here, too. In terms of purification of the product it is quite disadvantageous as a mixture of numerous methylated cytidine derivatives was obtained. Cm

could be isolated from the mixture by subsequent column chromatography and recrystallisation in 16 % yield (Scheme 15). Attempts to purify the numerous side-products were partially successfully. The obtained nucleosides **23**, **24**, **25**, **26**, and **27**are summarized in Figure 31.

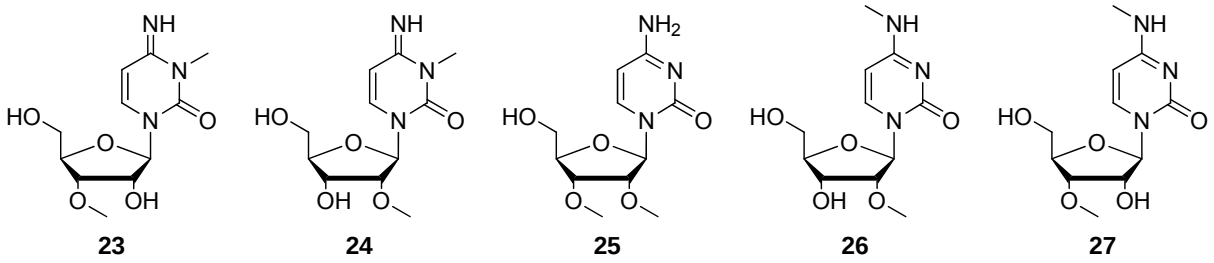
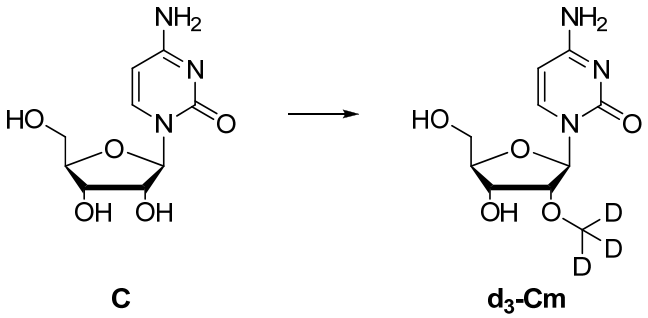


Figure 31: Nucleosides **23**, **24**, **25**, **26**, and **27** isolated from the reaction of cytidine with MeI.

7.1.3.4 Synthesis of d_3-Cm



Scheme 16: Synthesis of d_3-Cm.
Reagents and conditions: NaH, d_3-MeI, DMF, 0 °C to rt, 4.5 h, 19 %.

Nucleoside d_3-Cm was obtained in 19 % yield from the reaction of cytidine with NaH and d_3-MeI under identical conditions as used for the synthesis of its undeuterated analogue.

7.1.4 Synthesis of queuosine

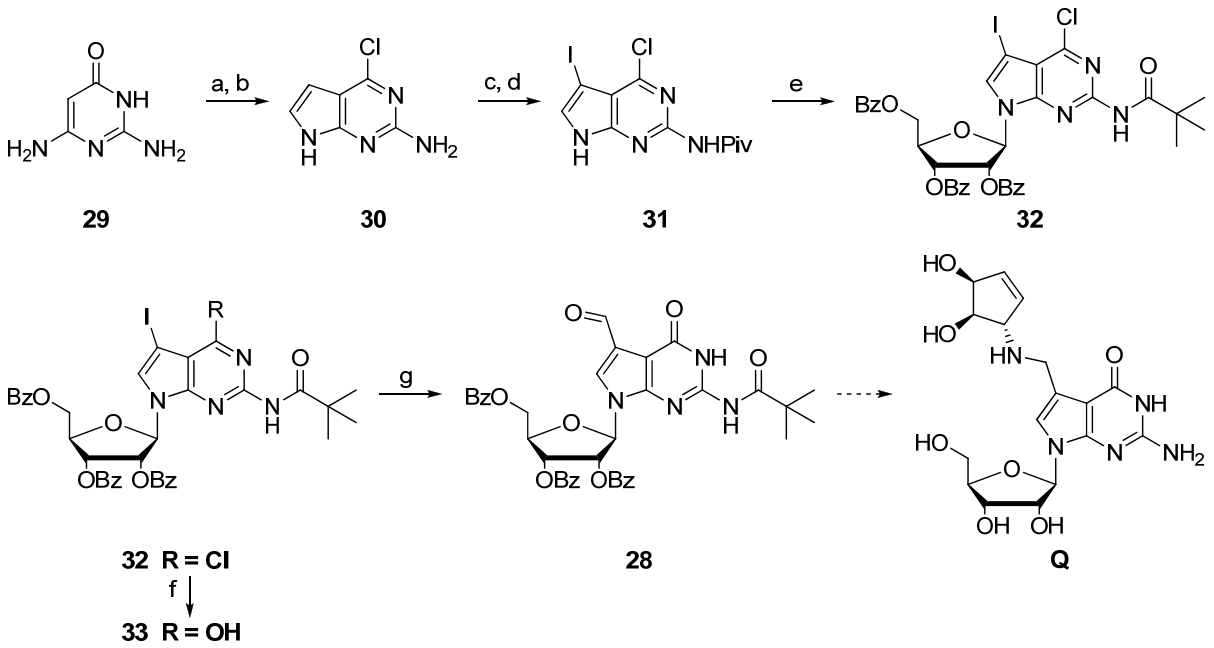
The hypermodified nucleoside queuosine is present in the wobble position of four tRNAs (Tyr, His, Asn, and Asp) in bacteria and eukaryotes.[210]

Numerous functions of queuosine have been discovered. It is suspected to influence development,[211] decoding,[212] biosynthesis of nitrite reductase,[213] and cancer cells.[9b, 35b, 42, 47-48]

Most of its complex biosynthesis has been disclosed. It features an uncommon class of enzymes the so-called tRNA guanine transglycosylases (TGTs).[29a-c]

A thorough discussion of the biosynthesis and the functions of queuosine is inappropriate here. Furthermore, there is a multitude of excellent reviews discussing the individual aspects of queuosine biosynthesis[6c, 6d, 6f, 29a, 29c, 29d, 214] and functions.[6c-j]

One route to Q was known,[215] when the queuosine project was started in the Carell group. As this synthesis is lengthy and costly and it was expected that large quantities of queuosine would be needed for biochemical studies, a novel approach towards queuosine has been established.



Scheme 17: Studies towards the synthesis of queuosine.
Reagents and conditions: a) Bromoacetaldehyde diethyl acetal, HCl, NaOAc, water, 90 °C and 80 °C, 4 h, 74 %; b) $POCl_3$, reflux, 2 h, 78 %; c) Pivaloyl chloride, pyridine, rt, 2 h, 64 %; d) NIS, THF, rt, 1 h, 81 %; e) 1-Acetyl-2,3,5-tribenzoyl-ribose, bistrimethylsilyl acetamide, trimethylsilyl triflate, MeCN, 50 °C, 24 h, 59 %, f) DABCO, CsOAc, NaOAc, DMF, rt, 45 h, 57 %; g) $Pd_2(dba)_3$, triphenylphosphine, CO, Bu_3SnH, toluene, 50 °C, 2 h, 93 %.

During this thesis the route towards queuosine was followed until formylnucleoside **28** was obtained. The final steps of the queuosine synthesis were performed by I. Thoma as part of a master thesis supervised during this thesis. The steps performed personally are depicted in Scheme 17 and will be discussed hereafter. Reactions performed by I. Thomas are discussed in detail in her master thesis.

Bromoacetaldehyde diethyl acetal was condensed with pyrimidinone **29**. Functional group interconversion yielded chloro compound **30**. Protection of the amine as pivaloylamide moiety and introduction of iodine with NIS yielded nucleobase **31**. For the glycosylation the *Silyl-Hilbert-Johnson Reaction* under *Vorbrüggen Conditions* was used to couple commercially available 1-acetyl-2,3,5-tribenzoyl-ribose with deazapurine base **31** resulting in nucleoside **32**.[216] After conversion of the chloro to the oxo substituent[217] nucleoside **33** was obtained. Palladium catalyzed formylation with CO and Bu_3SnH gave nucleoside **28** in excellent yield. The final steps to Q include a reductive amination to introduce the cyclopenten moiety and a two step deprotection.

Heavy atom labeled ^{13}C-d_2-Q was synthesized in collaboration with I. Thoma. As mentioned above nucleoside **28** was synthesized during this thesis and provided for the final steps of the synthesis of heavy atom labeled ^{13}C-d_2-Q. These were conducted as part of I. Thoma's master thesis work supervised during this thesis work. The incorporation of the heavy atoms was achieved by use of Bu_3SnD and ^{13}CO.

7.2 Syntheses of other nucleosides

In addition to the syntheses of the nucleosides and their heavy atom labeled derivatives presented in the last chapter several other unlabeled nucleosides were prepared. The methylated nucleosides discussed in this chapter are naturally occurring modified nucleosides or potential biosynthetic precursor. As such they are of general interest for mass spectrometric elucidation. But they have not been investigated further up to now, either because additional HPLC studies would be necessary to allow quantification or because they have not been found in the samples analyzed so far. $PreQ_0$ and archaeosine have been synthesized as part of a natural product synthesis project.

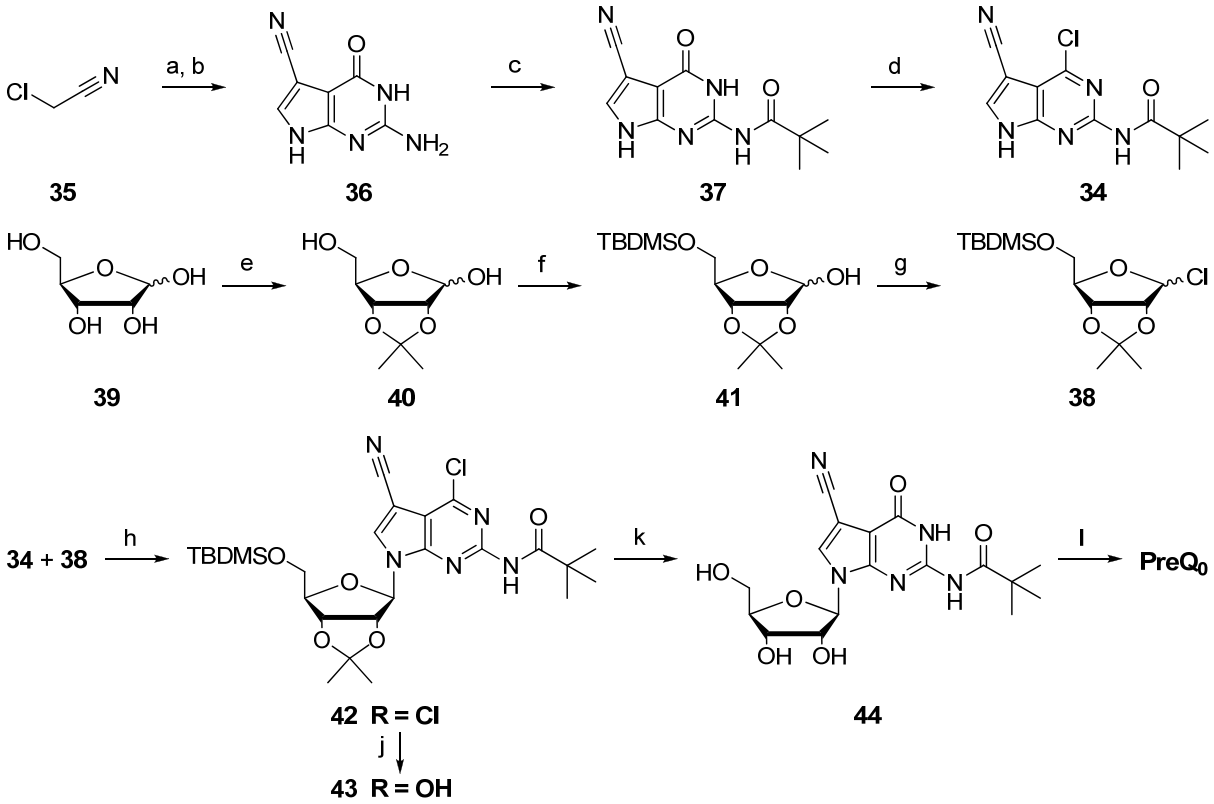
7.2.1 Synthesis of $PreQ_0$ and archaeosine

$PreQ_0$ is a biosynthetic precursor of archaeosine. Archaeosine is present in position 15 in the majority of archaeal tRNAs.[29a]

It was proposed that archaeosine has a highly stabilizing effect on the structural integrity of archaeal tRNAs.[218] Under physiological pH it carries a positive charge at its amidinium moiety. This positive charge is positioned in a cleft with a high negative electrostatic potential.[29a] In addition, loss of the structural element containing Q* (archaeosine) leads to denaturation of the tRNA.[219]

Biosynthetic precursor $PreQ_0$ has been synthesized before[220] while Q* was prepared for the first time in the course of this thesis work.[221]

$PreQ_0$ and archaeosine analysis in archaea has not been performed yet as no archaeal samples are available at the moment. In addition, quantification of the levels of archaeosine is not likely to lead to further insights. Due to our interest in deazaguanosines the routes to $PreQ_0$ and archaeosine (Scheme 18 and Scheme 19, respectively) were established as a natural product synthesis project.



Scheme 18: Synthesis of $PreQ_0$.
Reagents and conditions: a) HCOOMe, NaOMe, THF, 0 °C to 10 °C, 4 h; b) NaOAc, 2,6-diaminopyrimidine-4-one, water, THF, 50 °C to reflux, 3 h, 38 % (2 steps); c) Pivaloyl chloride, pyridine, 85 °C, 2.0 h, 75 %; d) $POCl_3$, Triethylbenzylammonium chloride (TEBA), DMA, acetonitrile, 90 °C, 1 h, 75 %; e) Acetone, conc. HCl, 0 °C, 2 h, 87 %; f) TBDMSCl, imidazole, DMF, rt, 16 h, 83 %; g) HMPA, tetrachloromethane, THF, -78 °C to rt, 4 h; h) NaH, acetonitrile, 0 °C to rt, 20 h, 20 %; j) Et_3N, DABCO, NaOAc, DMF, rt, 48 h, 81 %; k) Trifluoroacetic acid, water, 0 °C, 4 h, 77 %; l) 28 % Ammonia in water, 60 °C, 17 h, 99 %.

Synthesis of the heterocyclic building block **34** started from chloroacetonitrile **35**. Reaction of nitrile **35** with formic acid methylester and 2,6-diaminopyrimidine-4-one in a one pot procedure as established by Migawa *et al.*[222] gave nitrile nucleobase **36**. Pivaloyl protection of amine **36** yielded compound **37**. Subsequently, the heterocycle **37** was

activated for the subsequent glycosylation reaction by replacement of the hydroxyl group at the aromatic system for chlorine.[223] By that route nucleobase **34** was obtained in 21 % overall yield in only four steps.

The second building block **38** was synthesized starting from ribose **39**. Protection of the 2',3'-OH groups with acetone gave compound **40**.[224] Treatment of the crude product with TBDMSCl provided the 5'-*O*-protected sugar **41** in 72 % yield. Conversion of ribose derivative **41** into the glycosyl donor **38** was achieved with HMPA and CCl_4.[225] Due to the lability of compound **38**, it was used for the glycosylation reaction without further purification.

Glycosylation of nucleobase **34** was achieved by deprotonation of 1.4 equivalents of deazapurine base **34** with NaH and subsequent addition of chloro sugar **38** to the reaction mixture.[226] This reaction provided the nucleoside **42** in 20 % yield. Slow addition of sugar **38** at 0 °C and rigorously dried solvents and reagents are very important for the success of the reaction.

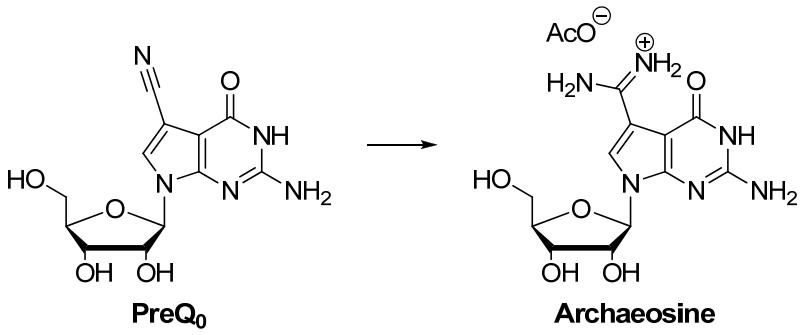
The configuration of the obtained product **42** was verified by HMBC and NOESY spectroscopy. Clear interactions could be observed between $C_{1'}H$ and C6, C5 and C7a in the HMBC spectrum. Furthermore, interactions between C6H and $C_{1'}$ were clearly detected.

The corresponding NOESY spectrum proved the *β*-configuration of the obtained nucleoside **42**. A strong interaction between C6H and $C_{2'}H$ was observed. Additional interactions (C6H – $C_{3'}H$ and C6H – $C_{5'}H$) usually observed for *β*-configured nucleosides and the absence of typical interactions (C6H – $C_{4'}H$) in *α*-nucleosides further supported the stereochemical assignment.

Conversion of the chloro to the needed oxo substituent in position 4 of the nucleoside **42** was achieved with DABCO, Et_3N and NaOAc yielding nucleoside **43** (Scheme 18).[227] The protecting groups at the sugar moiety of the nucleoside **43** were removed using a water / trifluoroacetic acid mixture to give triole **44**. The best method for the deprotection of the amine in position 2 of the nucleoside **44** proved to be 28 % ammonia in water. By this method the conversion of amide **44** to $PreQ_0$ was quantitative. The transformation of nucleoside **42** to $PreQ_0$ was therefore possible with 62 % yield in only three steps. The analytical data obtained from $PreQ_0$ compared very well with those published by Kondo *et al*.[228] and Cheng *et al*.[229]

Treatment of a solution of $PreQ_0$ in methanol with HCl (g) for 3 h, followed by the removal of the solvent and stirring of the resulting white solid in 7 N ammonia in

methanol following classical Pinner conditions[230] indeed gave archaeosine Q* as a white solid as depicted in Scheme 19.

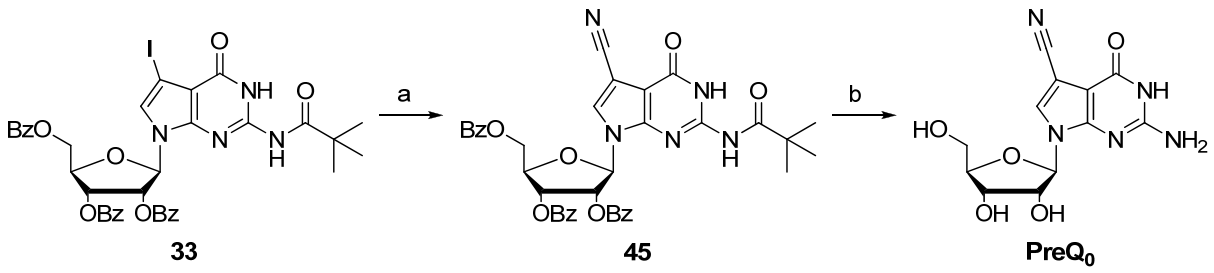


Scheme 19: Synthesis of archaeosine.
Reagents and conditions: HCl (g), MeOH, 7 M NH_3 in MeOH, rt, 19 h, 30 %.

Archaeosine was purified by reversed phase HPLC with a 0.1 M triethylammonium acetate buffer. The resulting white solid turned out to be archaeosine obtained as its acetate salt plus a small amount of additional triethylammonium acetate. Attempts to completely remove the excess triethylammonium acetate under high vacuum caused degradation of archaeosine partly back to $PreQ_0$. However, the analytical data obtained for the product were in excellent agreement with those presented by Gregson *et al*.[218]

In summary, a short and efficient synthesis of $PreQ_0$ (2 % overall yield, 11 steps) and of archaeosine (0.6 % overall yield, 12 steps) was established.

7.2.2 Synthesis of $PreQ_0$ and archaeosine *via* the queuosine route



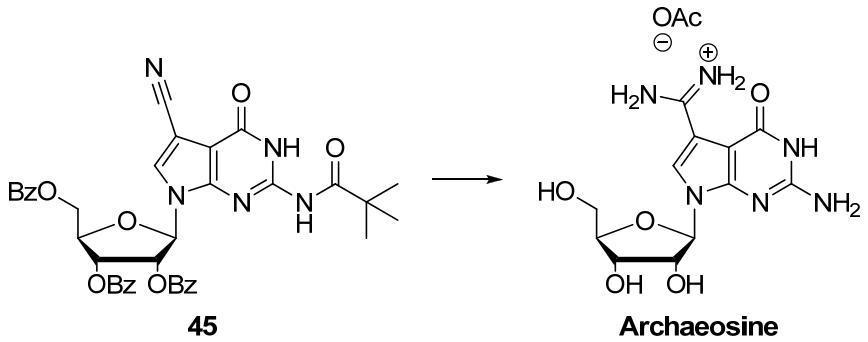
Scheme 20: Synthesis of $PreQ_0$ *via* the queuosine route.
Reagents and conditions: a) MeMgCl, ^{i}PrMgCl•LiCl, TosCN, toluene, -78 °C to rt, 18 h, 60 %; b) 28 % NH_3 in H_2O, 60 °C, 16 h, 78 %.

After the synthesis of $PreQ_0$ and Q* was performed as described above, the route to queuosine was established. An intermediate of the queuosine synthesis, nucleoside **33**, was identified as

an ideal precursor for $PreQ_0$ and Q*. Starting from nucleoside **33** $PreQ_0$ and archaeosine are available in a two step procedure.

For the synthesis of $PreQ_0$ a palladium catalyzed cross-coupling was envisioned to introduce the nitrile moiety. Unfortunately, cross-coupling of iodo nucleoside **33** with $Cu(CN)_2$ did not reliably give acetylated $PreQ_0$. Instead of a cross-coupling approach the iodine can also be converted into a metal-organic functionality. Treatment of iodo compound **33** with ^{i}PrMgCl•LiCl successfully created the corresponding grignard reagent, which could be trapped with water as an electrophile. By screening numerous reaction conditions the procedure for the iodine-magnesium-exchange was optimized. First, MeMgCl is used to deprotonate the amide groups of nucleoside **33**. The iodine-magnesium-exchange is most efficient, when diluted turbo-grignard[231] is added very slowly at -78 °C. As shown by TLC and HPLC studies the exchange is almost quantitative under the conditions described in chapter 10.11.2.1. Trapping of the magnesium species with an electrophile turns out to be the lossy process in this reaction. The obtained Grignard reagent does not seem to be potent enough to react at -78 °C, but increasing the temperature of the reaction mixture leads to decomposition of the nucleoside. Therefore, the best results were obtained, when the electrophile was added at -78 °C and the mixture was subsequently allowed to slowly warm to room temperature. When TosCN was used as an electrophile nitrile nucleoside **45** was obtained in 60 % yield. To our best knowledge this is the first Grignard reaction with a nucleoside tolerating labile functional groups like esters and amides.

Deprotection of nucleoside **45** with NH_3 in water readily gave $PreQ_0$ in 78 % yield. Establishment of this novel reaction and the new route to $PreQ_0$ allows its synthesis in only 8 steps and 5 % overall yield. This novel route reduces the number of steps and increases the overall yield of the $PreQ_0$ synthesis significantly compared to the procedure published before.



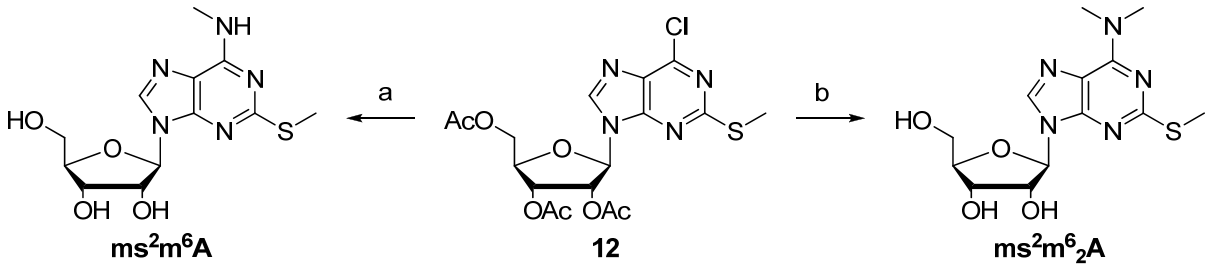
Scheme 21: Synthesis of archaeosine *via* the queuosine route.
Reagents and conditions: HCl (g), MeOH, 7 M NH_3 in MeOH, rt, 19 h, 40 %.

With nitrile nucleoside **45** in hands, the final step of the synthesis to archaeosine (Q*) is the conversion of this nucleoside to Q* under Pinner conditions. This approach not only converts the nitrile into the amidinium moiety but also removes all protecting groups in one step. Archaeosine was therefore obtained in 8 steps and 2 % overall yield. This novel approach to archaeosine improves the published routes towards this nucleosides in terms of number of steps and yield considerably.

Identification of nucleoside **33** as a versatile building block in deazaguanosine synthesis and establishment of a reliable, functional group tolerating Grignard reaction for the introduction of the nitrile functionality has turned the efficient route to Q into a divergent strategy for the preparation of almost all deazaguanosine nucleosides.

7.2.3 Synthesis of ms^2m^6A and $ms^2m^6{}_2A$

In addition to the adenosine nucleosides presented above one natural and one unnatural adenosine nucleoside were synthesized without their corresponding heavy atom labeled derivatives. ms^2m^6A is a naturally occurring modification in bacterial tRNA,[232] but nothing is known about its biological significance. Therefore, it has not been in our main focus and synthesized only in unlabeled form so far. The other adenosine nucleoside prepared without the corresponding heavy atom labeled derivative is $ms^2m^6{}_2A$. It has not been found in RNA so far.

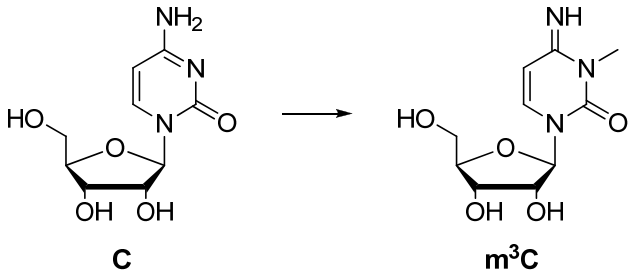


Scheme 22: Synthesis of ms^2m^6A and $ms^2m^6{}_2A$.
Reagents and conditions: a) $MeNH_2$, rt, 16 h, 73 %; b) Me_2NH, 60 °C, 16 h, 67 %.

Synthesis of ms^2m^6A and $ms^2m^6{}_2A$ were performed by treatment of nucleoside **12** with $MeNH_2$ and Me_2NH, respectively. Nucleoside ms^2m^6A was received in 73 % yield. Hence, a 3 step route to ms^2m^6A with 32 % overall yield was established. Similarly, $ms^2m^6{}_2A$ was isolated in 67 % yield resulting in a 3 step synthesis with 30 % overall yield.

7.2.4 Synthesis of m^3C

Methylated cytidine m^3C is present in eukaryotic tRNA in position 32 and is expected to influence the structure of the anticodon loop.[6b] Its biosynthesis is still unknown. Due to its presence in eukaryotes and in the anticodon loop its quantification would be of considerable interest.



Scheme 23: Synthesis of m^3C.
Reagents and conditions: Dimethylsulfate, DMF, 37 °C, 2 d, 7 %.

It was synthesized following a procedure of Brookes and Lawley.[233] Dimethylsulfate was used as methylating agent at 37 °C for 2 days. The resulting mixture was recrystallized twice from MeOH to yield m^3C in high purity.

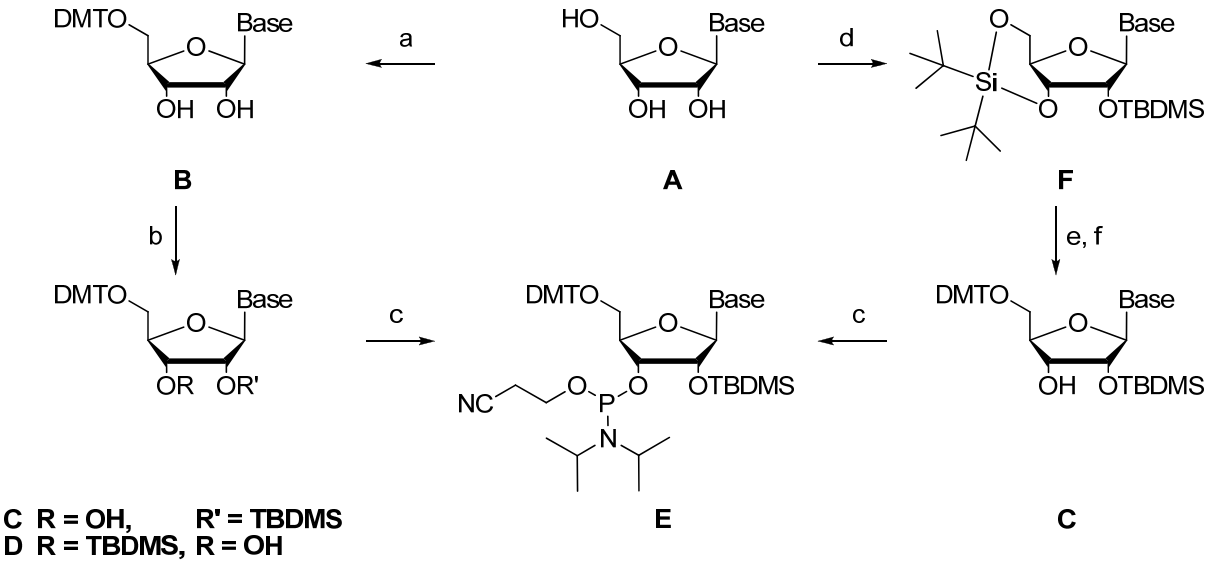
With an authentic sample in hands coinjection studies were conducted to determine the chromatographic properties of m^3C. Unfortunately, the HPLC conditions used during this thesis are not suitable for separating m^3C from all other methylcytidines. Therefore, an individual quantification of m^3C is not possible at present and the synthesis of its heavy atom labeled derivative is not expedient at present.

8 Studies towards the incorporation of ms^2i^6A, m^2A, and Q in RNA

The investigation of biosynthesis pathways, enzyme mechanisms, and functions of biomolecules is to the main part based on studies with the corresponding substrates. These usually are truncated intermediates or the biomolecules themselves. For the investigation of modified nucleosides in tRNA unmodified, partially modified, or fully modified tRNAs or tRNA sequences, e.g. an anticodon stem-loop, can be envisioned. Completely unmodified tRNAs are available as transcripts of the T7 RNA polymerase[234] but the tertiary structure of these unmodified tRNAs in many cases differs substantially from the three-dimensional structure of their fully or partially modified analogs. Therefore, they are often unsuitable for biochemical investigations. Fully modified tRNAs or tRNAs lacking only one or two modification are usually necessary to allow reliable and meaningful biochemical analysis. These are still hardly or not at all accessible. This shortage severely hampers investigation of modified nucleosides.[235] Therefore, it is imperative to establish a reliable, high yielding method for the preparation of fully or partially modified tRNAs or tRNA sections. Phosphoramidite solid phase synthesis is currently the most efficient and reliable method to achieve this goal.

Therefore, in the course of this thesis work the incorporation of the modified nucleosides ms^2i^6A, m^2A, and Q in tRNA or tRNA sequences by phosphoramidite chemistry was studied. With these RNA strands in hand a door will be opened for numerous biochemical studies, which in turn will hopefully advance our knowledge of the biosyntheses and functions of modified tRNA nucleosides significantly.

Preparation of RNA strands with modified nucleosides *via* phosphoramidite chemistry requires the availability of the modified nucleosides as phosphoramides with appropriate protecting groups. These are TBDMS at $C_{2'}OH$ and DMT at $C_{5'}OH$. Several other protecting groups are available for $C_{2'}OH$.[236] They have not been used here, because there is no consent in literature, which protecting group is superior and TBDMSCl is still the least expensive reagent in this context.



Scheme 24: Alternative routes to DMT-TBDMS-protected phosphoramidite **50**.
Reagents: a) DMTCl; b) TBDMSCl; c) $(N^iPr_2)_2P(OCH_2CH_2CN)$ d) $^tBu_2Si(OTf)_2$, TBDMSCl; e) HF-pyridine; f) DMTCl.

Scheme 24 shows the two usual routes used for the protection chemistry preceding solid phase oligonucleotide synthesis. On the left-hand side strategy the DMT-group is attached first.[237] DMTCl preferentially reacts with the primary alcohol at $C_{5'}$ of nucleoside **A** due to its high steric demand. Then TBDMSCl is used to protect the $C_{2'}$OH moiety of nucleoside **B**. The regioselectivity of this reaction is generally low and usually almost equimolar amounts of $C_{2'}$OH protected **C** and undesirable $C_{3'}$OH protected nucleoside **D** are isolated. These have to be separated before conversion of nucleoside **C** to phosphoramidite **D** with the *Bannwarth Reagent*.

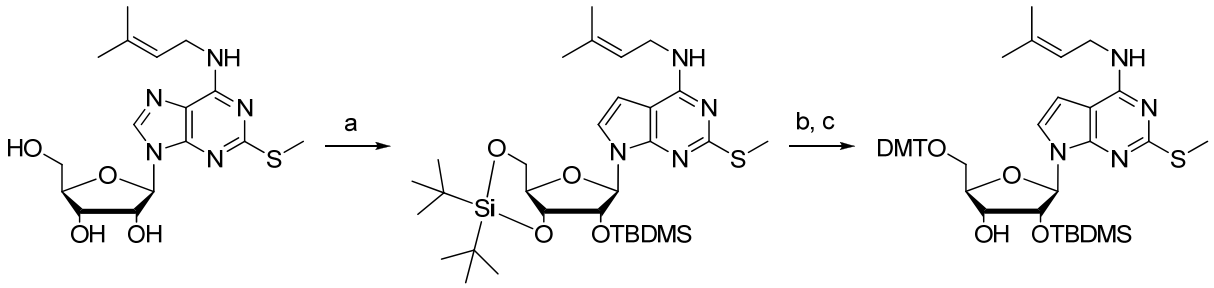
The second strategy is outlined in Scheme 24 on the right-hand side.[238] It features an additional protecting group. This silyl-clamp ($^tBu_2Si(OTf)_2$) is attached to nucleoside **A** and selectively masks its $C_{3'}$OH and $C_{5'}$OH. At this point the TBDMS group is regioselectively introduced at $C_{2'}$OH to yield fully protected nucleoside **F**. After removal of the silyl-clamp with HF-pyridine $C_{5'}$OH is selectively protected with DMTCl, which reacts with the less hindered primary alcohol, giving nucleoside **C**. Additionally, the secondary alcohol at $C_{3'}$ is shielded by the sterically demanding TBDMS group at $C_{2'}$OH. In summary, the first strategy is shorter by one step but most likely leads to higher losses during the synthesis due to its lower regioselectivity.

In consideration of the high amounts of protected nucleosides needed the second approach was chosen (Scheme 24 right-hand side). The reaction sequences described hereafter were

optimized in a way that only the final product of the sequence had to be purified by column chromatography. This is only possible with a highly regioselective reaction sequence and reduces labor time and material requisition considerably. This is especially important as it was anticipated that these nucleosides would be need in gram quantity and that the reactions would be performed repeatedly.

8.1 Studies towards the incorporation of ms^2i^6A in RNA

The tRNA nucleoside ms^2i^6A is highly modified carrying an isopentenyl and a methylthio substituent. Nevertheless, it was chosen as model compound to establish the protecting group chemistry necessary prior to attachment of the phosphoramidite moiety and solid phase synthesis. It was envisioned that neither the secondary amine nor the methylthio residue would be affected by the solid phase synthesis chemistry. Secondary amines are known to be compatible with phosphoramidite chemistry and Kierzek *et al.*[169b] have incorporated 2-methylthio-adenosines in RNA before. The accuracy of this assumption was verified by prolonged treatment of ms^2i^6A with all reagents used during RNA synthesis.

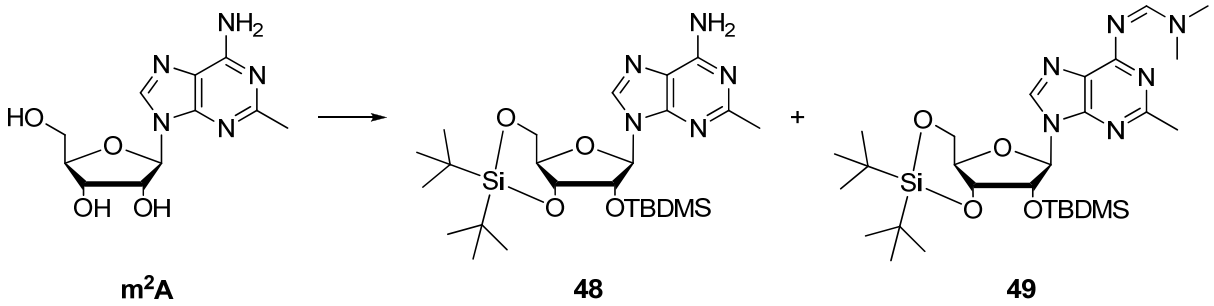


ms^2i^6A **47** **46**

Scheme 25: Synthesis of nucleoside **46**.
Reagents and conditions: a) $^tBu_2Si(OTf)_2$, imidazole, TBDMSCl, DMF, 0 °C to 60 °C, 3 h; b) HF-pyridine, pyridine, DCM, MeOTMS, 0 °C, 2 h; c) DMTCl, pyridine, MeOH, 0 °C, 16 h, 52 % (3 steps).

For the synthesis of nucleoside **46** the conditions originally published by Serebryany and Beigelman[238] were used. They proofed to give good results for the protection of ms^2i^6A (Scheme 25). Introduction of the silyl-clamp and the TBDMS group gave fully protected **47** in a one-pot reaction. Subsequently, the $C_{3'}OH$-$C_{5'}OH$-protecting group was removed with HF-pyridine and the DMT group was introduced. The crude product was purified by column chromatography to give nucleoside **46** in 52 % yield over 3 steps.

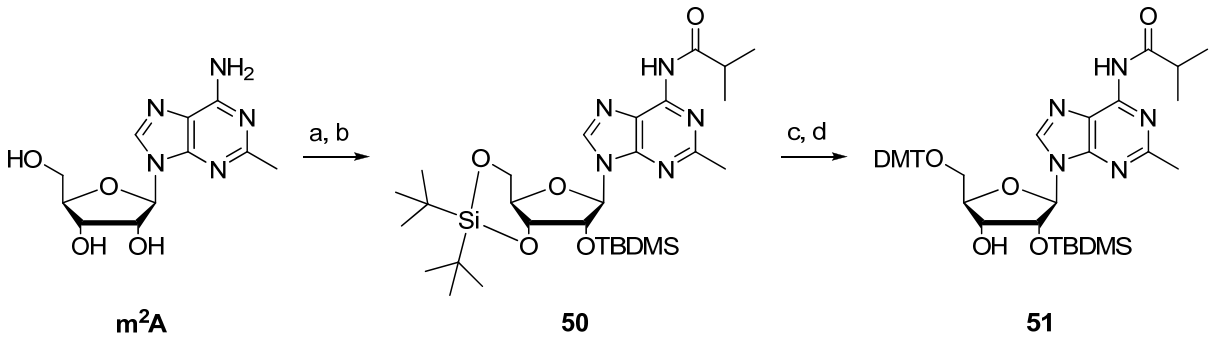
8.2 Studies towards the incorporation of m^2A in RNA



m^2A **48** **49**

Scheme 26: Synthesis of nucleoside **48** and DMF-protected nucleoside **49**.
Reagents and conditions: a) $^tBu_2Si(OTf)_2$, imidazole, TBDMSCl, DMF, 0 °C to 60 °C, 3 h.

For protection of m^2A with $^tBu_2Si(OTf)_2$ and TBDMSCl the conditions published in literature[238] and used for ms^2i^6A gave nucleoside **48** only in unsatisfactory yields. Under these conditions imidazole is used as base during the silyl protection. For m^2A it was found that imidazole catalyzes the formation of a dimethylformimide at the primary amine with DMF, which is used as solvent in the reaction to give side-product **49** (Scheme 26). This side-reaction could potentially be turned into a convenient method for the protection of the amine at the base. But no clean conversion to imide **49** was observed under various conditions. On the other hand reaction conditions omitting imidazole completely inhibited formation of the imide and were therefore preferred. A screening of reaction conditions revealed that reduction of the reaction temperature to -5 °C led to good yields (Scheme 27).



m^2A **50** **51**

Scheme 27: Synthesis of nucleoside **51**.
Reagents and conditions: a) $^tBu_2Si(OTf)_2$, TBDMSCl, DMF, -5 °C to rt, 21 h; b) *Iso*butyryl chloride, pyridine, DMF, 0 °C, 25 min; c) HF-pyridine, pyridine, DCM, MeOTMS, -13 °C to rt, 2 h; d) DMTCl, pyridine, MeOH, rt, 3.5 h, 37 % (4 steps).

In contrast to ms^2i^6A, for which all functional groups at the base are compatible with RNA solid phase synthesis, m^2A features a primary amine, which has to be masked for phosphoramidite chemistry. Several protecting groups are conceivable. The first one tested here, *iso*-butyryl chloride, was found to give good results and was thus utilized to prepare amine-protected nucleoside **50** (Scheme 27).

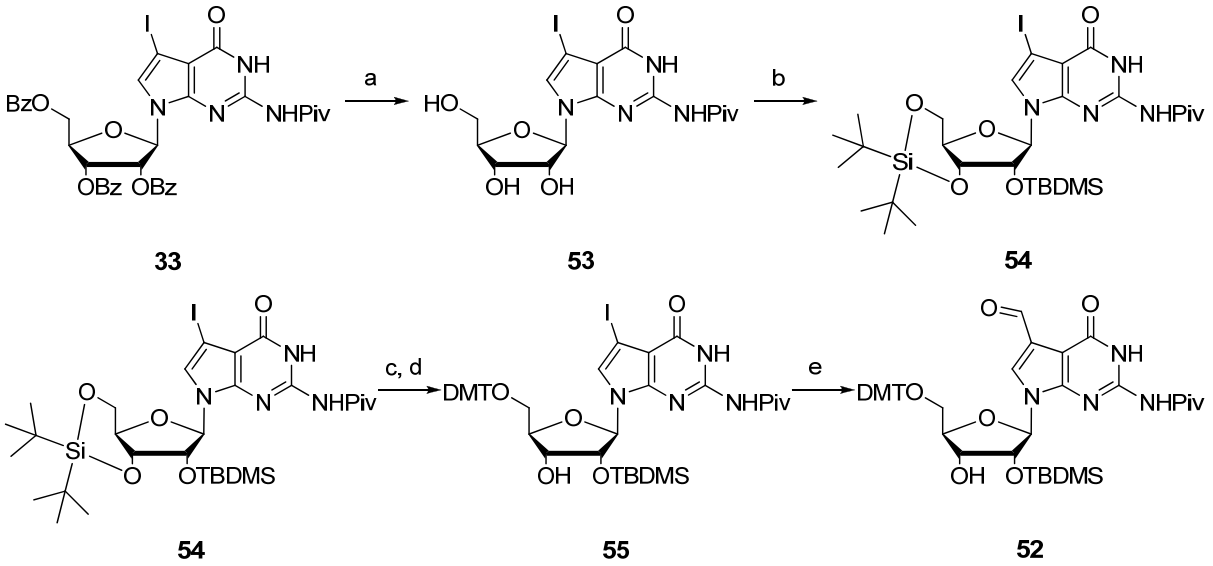
Nucleoside **50** turned out to be more reactive during HF deprotection than ms^2i^6A derivative **47**. To achieve satisfactory yields the reaction temperature was reduced in comparison to the conditions established for ms^2i^6A and in comparison to literature.[238] Subsequent DMT protection proofed to be most efficient when conducted at room temperature for 3.5 h. This reaction sequence provided protected nucleoside **51** in 4 steps and 37 % yield.

8.3 Studies towards the incorporation of Q in RNA

Studies towards the incorporation in RNA were conducted for three different nucleosides in this thesis. Of these queuosine is by far the most complex one. The final step of the current Q-synthesis is the acidic removal of an acetonide protecting group.[239] The highly acidic conditions necessary for this reaction would lead to decomposition of any RNA strand. The acetonide protecting group is not appropriate for incorporation of Q in RNA. Therefore, a post-synthetic approach was envisioned. It has been shown before, that aldehydes are compatible with phosphoramidite chemistry[240] and that reductive aminations can be performed in DNA and RNA strands.[241] Combination of these facts led to an auspicious strategy with two stages: First an aldehyde containing deazaguanosine nucleoside is incorporated in RNA. After successful RNA synthesis and deprotection of the RNA strand the unprotected cyclopenten moiety will be selectively attached to the aldehyde by reductive amination. Apart from being an ideal precursor for postsynthetic completion of the Q-synthesis the deazaguanosine aldehyde can also be used to attach any other amine to the RNA strand. The versatility of the aldehyde as linker to the RNA strand thus connects this project with biochemical research (e.g. by attachment of dyes or tags) and material science (e.g. by attachment of metals).[241b]

During the oligonucleotide synthesis the amine at C2 of the heterocycle has to be protected. The pivaloyl protecting group was used during solid phase oligonucleotide synthesis before and is cleavable under standard deprotection conditions afterwards.[242] It can either be reintroduced after complete deprotection of nucleoside **33** or the benzoyl groups can be

removed selectively. From these two possible approaches the second one was favored as it is shorter by one step.



Scheme 28: Synthesis of nucleoside **52**.
Reagents and conditions: a) 2 M NaOH, dioxane, rt, 50 min, 60 %; b) $^tBu_2Si(OTf)_2$, imidazole, TBDMSCl, DMF, 0 °C – rt, 22 h; c) HF-pyridine, pyridine, DCM, MeOTMS, -5 °C to rt, 3 h; d) DMTCl, pyridine, MeOH, rt, 16 h, 69 % (3 steps); e) $Pd_2(dba)_3$, PPh_3, CO, toluene, 55 °C, 1 h, 81 %.

The strategy outlined above makes nucleoside **52** the final product before introduction of the phosphoramidite group. Formylnucleoside **52** was expected to be available *via* a five step sequence starting from fully protected **33** an intermediate of the Q-synthesis (Scheme 28). For this route to nucleoside **52** the first step, a chemoselective deprotection, and the last step, a palladium catalyzed formylation, are the key reactions.

Full deprotection of nucleoside **33** was achieved in a straight forward manner under various conditions, but the chemoselective removal of all benzoyl groups, while leaving the pivaloyl amide untouched, proved to be challenging. A range of reaction conditions was tested. The only reagent giving amide **53** in more than 20 % yield was a mixture of 2 M NaOH and dioxane. Under these conditions nucleoside **53** was obtained in reasonable yield (60 %, Scheme 28).

Introduction of the silyl-clamp and the TBDMS group to give nucleoside **54** was accomplished under standard conditions. For the subsequent HF-pyridine deprotection a reaction temperature below 0 °C was found to increase the yield considerably. DMT protection was executed at rt for 16 h producing iodo nucleoside **55** in 3 steps and 69 % yield.

The final step of this sequence was introduction of the formyl moiety. This reaction was performed as described in chapter 7.1.4. Fortunately, these conditions proofed to be compatible with the altered protecting group pattern giving nucleoside **52** in 81 % yield.

In the course of this thesis work the nucleosides **46**, **51**, and **52**, the final stable intermediates before RNA solid phase synthesis were prepared. Once the phosphoramidite group is introduced the nucleosides are air-labile and ideally should be used for RNA synthesis at once. The studies towards the incorporation of ms^2i^6A, m^2A and Q in RNA strands have therefore been promoted to a stage, where everything is set up to start working on the actual RNA solid phase synthesis. At this point the project has been paused in favor of the quantification studies described in chapter 5, because these are currently more promising in terms of the scientific impact of the expected results.

9 Outlook

In the course of the last three years almost all natural modified adenosine and many guanosine derivatives in RNA have been synthesized. These cover rare and abundant nucleosides as well as different positions in tRNA enabling a representative investigation of tRNA modifications. Nevertheless, it is desirable to further extend our library to facilitate the investigation of a larger collective of modifications. Towards this goal some methylated cytidines have already been synthesized during this thesis work. Further interesting cytidine modifications are m^5C, m^5Cm, f^5C, and f^5Cm. Prior to an extension of our library to uridine derivatives a higher sensitivity of their detection *via* mass spectrometry has to be achieved. Switching from the positive to the negative detection mode in concert with a higher pH of the HPLC-MS buffer is likely to enhance the ionization and thus the sensitivity of their measurement considerably.

Some questions, which arose during this thesis work, like the high levels of Q in brain, remain unanswered and might lead to further interesting projects. A more detailed analysis of different brain regions and knock-outs of Q-incorporation in cell lines or mice might help to uncover the reasons for the high amounts of Q in the brain.

The hypothesis, that regulation of the translational capacity in tissues is procured by modified nucleoside levels, rests upon the correlation between protein synthesis rates and modification levels in pork tissues. Especially the correlation between different *in vitro* translation rates and modification levels is currently based on limited data. To verify this correlation for all tissues, *in vitro* translation rates with tRNAs from all pork tissues should be measured. Similarly, the mitochondrial activity in all tissues, which were studied here, should be determined. Measurement of modification levels in other mammals than pig, e.g. mouse, with our method would probably also help to further strengthen our hypothesis and the concept of a conservation of modification levels across mammals, which is currently based on comparison of tRNA sequences. In addition, determination of protein synthesis rates and modified nucleoside levels in prokaryotes with and without knockouts and under different growth conditions might also show a correlation between these and translational activity.

In more general terms it will be interesting to expand our quantification method to further natural nucleic acids, like mRNA, rRNA, mitochondrial tRNA, and viral RNA. In addition, and further samples, like plants, fungi, and especially tumors should be investigated. Application of our approach for the measurement of modified nucleosides as tumor markers would most likely also advance this field considerably.

10 Experimental

10.1 General chemical materials and methods

Chemicals and solvents were purchased from ABCR, Alfa Aesar, Acros, Fluka, Sigma-Aldrich or TCI in the qualities puriss., p.a. or purum, unless stated otherwise. For all solutions injected into the mass spectrometer MilliQ water and mass spectrometry grade solvents and reagents were used. Dry solvents (< 50 ppm H_2O) were obtained from Fluka and Acros. All reactions employing dry solvents were performed under inert atmosphere (N_2). Technical grade solvents were distilled prior to use for column chromatography and liquid-liquid extractions on a rotary evaporator (RV-05-ST, *Janke und Kunkel* or Heidolph Laborota 4000). Reaction products were dried at high vacuum (10 mbar). Aqueous solutions were dried on a SpeedVac plus CS110A or SPD 111V from Savant or lyophilized (Christ ALPHA 2-4).

Column chromatography was performed with silica gel 60 from Merck.

Thin layer chromatography (TLC) was performed with aluminum plates (silica gel 60 F_{254}, 10 × 5 cm). Substances were visualized by illumination with UV-light ($\lambda = 254$ nm) or by staining with subsequent heating. The staining was performed using potassium permanganate solution (1.0 g $KMnO_4$ in 100 mL H_2O), anisaldehyde solution (2.2 g anisaldehyde, 2.0 mL conc. H_2SO_4, in 100 mL acetic acid), ninhydrin solution (20 g ninhydrin in 600 mL ethanol), or molybdatophosphoric acid solution (10 g $Ce(SO_4)_2*H_2O$, 25 g molybdatophosphoric acid, and 60 mL H_2SO_4 in 940 mL H_2O).

HPLC was performed on a Merck-Hitachi system (L-7400 UV detector, L-7480 fluorescence detector, L-7100 pump), on a Waters system (alliance 2695 with PDA 2996 or 996 and fluorescence detector 2475; preparative HPLC: 1525EF with 2484 UV detector), and on a Dionex system (Ultimate 3000 HPLC). As columns VP 250/32 Nucleosil 100-7 C18, VP 250/10 Nucleosil 100-7 C18, CC 250/4 Nucleosil 120-3 C18, VP 250/10 Nucleodur 100-5 C18 ec, VP 250/4 Nucleodur 100-5 C18 ec, and CC 250/4 Nucleodur 120-3 C18 ec columns from Macherey-Nagel and Uptisphere120-3HDO columns from Interchim were used.

Mass spectrometry data for EI-MS were collected with a MAT CH 7A from Varian. ESI-MS was performed on a Finnigan LTQ FTICR and on a Thermo Finnigan LTQ Orbitrap XL. MALDI-TOF was performed on a Bruker Autoflex II spectrometer with 6-aza-2-thiothymine (ATT) as matrix (10 mg ATT in 1 mL H_2O).

Melting points were measured with a Büchi Melting Point B-540.

Infrared spectroscopy was performed on a Perkin Elmer Spectrum BX FT-IR-System. The detection ranged from 400 to 4000 cm^{-1}. The following abbreviations were used for the characterization of the bands: *s* (*strong*), *m* (*medium*), *w* (*weak*).

NMR spectra were recorded on the following spectrometers: *Varian Oxford 200, Bruker AC 300, Varian XL 400* and *Bruker AMX 600*. The chemical shifts (δ) are given in ppm, the coupling constants (*J*) in Hz.

10.2 Buffers

Name	**Composition**
Buffer A	0.10 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$
Buffer B	0.10 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 1.0 M NaCl
Buffer C	2 mM $HCOONH_4$ in H_2O, pH 5.5
Buffer D	2 mM $HCOONH_4$ in H_2O/MeCN 20/80, pH 5.5
Buffer E	0.1 M triethylamine/acetic acid in water
Buffer F	0.1 M triethylamine/acetic acid in 20 % water and 80 % MeCN
Buffer 1	0.01 M $Mg(OAc)_2$, 0.05 M NaOAc, 0.15 M NaCl, pH 4.5
Buffer 2	300 mM ammonium acetate, 100 mM $CaCl_2$, 1 mM $ZnSO_4$, pH 5.7
Buffer 3	500 mM Tris-HCl, 1 mM EDTA, pH 8.0
10 x NaOAc buffer	NaOAc (1 M)
2 x NaOAc-urea-sample buffer	0.5 M NaOAc, 6 % Ficoll™ Type 400, 0.005 % bromophenol blue, 0.025 % xylene cyanol, 3.5 M urea
Agarose gel buffer	tris base (89 mM), boronic acid (89 mM), EDTA (2 mM)
Agarose gel running buffer	tris base (45 mM), boronic acid (45 mM), EDTA (1 mM)

10.3 Biochemical materials

10.3.1 Equipment

Equipment	Supplier
Äkta purifier chromatography system	GE, Munich
Agarose gel electrophoresis chamber	Biorad, Munich
Autoclave Vakulab S3000	Systec, Gießen
Biofuge pico	Heraeus, Hanau
BioPhotometer 6131	Eppendorf, Hamburg
Blender, Waring, 37-110 mL	VWR, Darmstadt
Centrifuge 5810R	Eppendorf, Hamburg
Fermenter Minifors	Infors AG, Bottingen
French pressure cell press	Thermo, Dreieich
Elisa Reader, FP Spectrometer	Tecan, Crailsheim
Gel scanner IDA	Raytest, Straubenhardt
Gel documentation device LAS3000	Raytest, Straubenhardt
Inkubator 1S	Noctua, Wiesloch
Inkubator 44R	New Brunswick,
Mini Protean 3 Cell	Biorad, Munich
Multicaster	Biorad, Munich
Nanodrop UV-spectrometer	Peqlab, Erlangen
pH meter MP220	Mettler Toledo, Gießen
Sorvall centrifuge, Evolution RC	Kendro, Dreieich
Thermomixer Comfort	Eppendorf, Hamburg
Tissue grind tube, SZ 24	VWR, Darmstadt
TissueLyser	Qiagen, Hilden
Deep-freezer	Sanyo, Bad Nenndorf
Desktop centrifuge 5415R	Eppendorf, Hamburg
ultrasonic bath	Bandelin, Berlin
Vortexer	VWR, Darmstadt
Water bath	Labora, Mannheim
Waters Millipore System	Millipore, Schwalbach

10.3.2 Strains

Strain	Genotype	Supplier
E. coli AG1	F^- recA1 endA1 gyrA96 thi-1 hsdR17(r_k^- m_k^+) supE44 relA1	ME Collection
E. coli BW25113	rrnB DElacZ4787 HsdR514 DE(araBAD)567 DE(rhaBAD)568 rph-1	ME Collection
E. coli K12	wild type	DSMZ, Braunschweig
E. coli yfiF	BW25113; ΔyfiF kan	KEIO Collection
E. coli ygdE	BW25113; ΔygdE kan	KEIO Collection
Rosetta 2	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* pRARE2[3] (Cam^R)	Novagen, Schwalbach
S. cerevisiae	wild type	DSMZ, Braunschweig
HCT-116		Cell Lines Service, Eppelheim
HeLa		Cell Lines Service, Eppelheim
MDA-MB-231		Cell Lines Service, Eppelheim
Neuro-2a		Cell Lines Service, Eppelheim
SK-HEP-1		Cell Lines Service, Eppelheim
MCF 7		Cell Lines Service, Eppelheim
BT-549		Cell Lines Service, Eppelheim
SK-MEL-2		Cell Lines Service, Eppelheim
SK-MEL-5		Cell Lines Service, Eppelheim
SK-MEL-28		Cell Lines Service, Eppelheim
U-87 MG		Cell Lines Service, Eppelheim
T-47D		Cell Lines Service, Eppelheim
IGR-1		Cell Lines Service, Eppelheim

10.3.3 Specialized system

Name	Application	Supplier
QIAamp DNA Mini Kit (250)	Isolation of genomic DNA	Qiagen, Hilden

10.3.4 Tissue samples

Pork tissue samples were obtained from the local slaughterhouse (Schweineschlachtung München GmbH) right after sacrifice. All samples were processed within 4 hours after sacrifice.

Mouse brain tissue samples were obtained from the Biel group. All samples were stored in liquid nitrogen until usage.

10.3.5 Media

LB medium:	pH 7.5, 1.0 % (w/v) peptone, 0.5 % (w/v) yeast extract, and 0.5 % (w/v) sodium chloride
Medium agar:	respective medium containing 1.5% (w/v) agar
YPD medium:	pH 7.5, 2.0 % (w/v) peptone, 1.0 % (w/v) yeast extract, and 2.0 % (w/v) glucose

MOPS minimal medium was prepared according to literature with a final concentration of 0.2 % glucose.[152]

10.3.6 Enzymes

Nuclease S1 (A*spergillus oryzae*) was obtained from Sigma, snake venom phosphodiesterase I (C*rotalus adamanteus venom*) from USB corporation, antarctic phosphatase from New England Biolabs.

10.4 Biochemical methods

10.4.1 Microbiological methods

10.4.1.1 *E. coli* cultures

Bacteria were stored as glycerol stocks at -80 °C before usage. During the studies they were kept on LB agar at 4 °C and transferred to a new agar plate every second week. Inocula (50 mL) were grown over night at 37 °C shaking at 240 rpm in the medium, in which the later experiment was performed.

For shaken cultures LB medium (1 L) was inoculated with an overnight culture (5 to 8 mL) and shaken at 240 rpm at 37 °C until OD 1 (600 nm) was reached, unless stated otherwise. To gain reference modification levels samples were taken at this point. For stress response

studies the stress factor was introduced at this point and the culture was shaken at 240 rpm at 37 °C, unless stated otherwise, for the time indicated in Table 15.

Stress type	pH	salt	H_2O_2	antibiotics	anaerob	heat	desferal	2-fluoro-adenosine
Duration [min]	75	75	35	120	120	70	80	120

Table 15: Summary of durations of stress exposures.

For stress response studies the cultures were treated with the following chemicals:

pH: 4 M H_3PO_4 for pH values lower than 7.5, 2 M NaOH for pH values higher than 7.5

Salt: addition of 5 M NaCl to a 0.4 M final concentration in the medium[153]

Antibiotics: chloramphenicol (0.5 and 50 µg/L),[148a] streptomycin (0.5 and 5.0 µg/L),[243] gentamycin (0.5 µg/L) and spectinomycin (100 µg/L)

H_2O_2: addition of 150 µl 30 % H_2O_2 in 10 mL water to the medium[154]

Desferal: addition of desferal to 0.5 mM final concentration in the medium[150c]

2-Fluoro-adenosine: addition of 2-fluoro-adenosine to 0.1 µM final concentration in the medium

For heat stress response studies the cultures were shaken at 50 °C.[155]

For fermentation experiments *E. coli* was grown in a Minifors fermenter (Infors AG, Bottingen). LB broth (3.0 L) was inoculated with an overnight culture (50 mL) and stirred at 490 to 610 rpm and 37 °C. The oxygen concentration was kept at 20 % by introduction of air. The pH of the culture was kept at 7.5 by appropriate addition of 4 M NaOH or 4 M H_3PO_4. For aerobic growth samples (0.8 L) were taken during exponential growth at OD 2.8. Subsequently, medium (1.0 L) was added and the air was substituted for nitrogen. Nitrogen bubbled through the medium until all oxygen was removed. *E. coli* was fermented for another 120 min under anaerobic conditions before a sample (0.8 L) was taken.

For investigation of the impact of minimal medium on the modification content *E. coli* was grown in MOPS minimal medium[152] under the same conditions as stated above. Samples were also taken as stated in the previous paragraph.

Sample collection was performed as follows. After completion of growth and/or stress exposure the culture was quickly transferred to two precooled 500 mL centrifugal tubes equipped with ice. After centrifugation (8 min, 10816 g, 4 °C) the supernatants were discarded. The pellets were suspended in buffer 1 (10 mL, 0.01 M $Mg(OAc)_2$, 0.05 M NaOAc, 0.15 M NaCl, pH 4.5). The suspensions were combined in a 50 mL Falcon tube and centrifuged (30 min, 3220 g, 4 °C). The supernatant was discarded and the pellet was stored at -80 °C until further use.

10.4.1.2 *S. cerevisiae* cultures

S. cerevisiae was stored as a glycerol stock at -80 °C before usage. During the studies it was kept on YPD agar at 4 °C and transferred to a new agar plate every second week. Inocula (50 mL) were grown over night at 30 °C shaking at 240 rpm in YPD broth.

Typically, YPD broth (1 L) was inoculated with an overnight culture (5 to 8 mL) and shaken at 240 rpm at 37 °C until OD 1 (600 nm) was reached. After completion of growth the culture was quickly transferred to two precooled 500 mL centrifugal tubes equipped with ice. After centrifugation (8 min, 10816 g, 4 °C) the supernatants were discarded. The pellets were suspended in buffer 1 (10 mL). The suspensions were combined in a 50 mL Falcon tube and centrifuged (30 min, 3220 g, 4 °C). The supernatant was discarded and the pellet was frozen at -80 °C until further use.

10.4.1.3 Cell cultures

Cell culture experiments were performed by Mirko Wagner. All cell lines were grown to 80 to 90 % confluence at 37 °C and 5 % CO_2 in RPMI 1640 medium containing L-glutamine (Invitrogen GmbH, Karlsruhe, Germany). RPMI 1640 was supplemented with 10 % (v/v) fetal bovine serum and penicillin (10 mg/L) / streptomycin (0.025 mg/L).

10.4.2 Molecular biological methods

10.4.2.1 tRNA isolation from pork tissue I – phenol extraction and LiCl precipitation

Tissue samples were cut out from inside the organ omitting surface areas, inhomogeneous areas, and vessels. For brain samples meninges and surface blood vessels were removed before processing, because for these samples surface areas could not be omitted.

All extraction steps were performed on ice or at 4 °C. A Waring Blender was equipped with pork tissue (5 g), buffer 1 (15 mL) and ice. The mixture was blended until a homogenous suspension was obtained and transferred to a 50 mL Falcon tube. After addition of 80 % aq.

phenol (15 mL) the suspension was shaken vigorously for 30 min. The mixture was centrifuged (30 min, 3220 g). The aq. layer was collected and treated again with 80 % aq. phenol (20 mL). The suspension was shaken vigorously for 1 min, centrifuged (30 min, 3220 g) and the layers were separated. The second phenol layer was extracted with buffer 1 (5 mL). The aq. layer was collected, all aq. layers were combined and extracted with 80 % aq. phenol (5 mL). The aq. layer was collected and extracted with chloroform (5 mL) twice. The aq. layer was collected and 20 % KOAc, pH 4.5 (0.1 vol) and 12 M LiCl were added to a 2.0 M final LiCl concentration. DNA and long RNAs were precipitated on ice for 4 h and pelleted by centrifugation afterwards (20 min, 38724 g). The supernatant was added to abs. EtOH (3.0 vol) in a 500 mL centrifugal tube and kept at -20 °C over night. After centrifugation (60 min, 24336 g) the supernatant was discarded and the pellet was dried.

10.4.2.2 tRNA isolation II - desalting

A PD10 column (GE health care) was preequilibrated with buffer A (25 mL, 0.10 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$). The tRNA pellet obtained from pork tissue was dissolved in buffer A (2.5 mL) and applied on the PD10 column. The suspension containing the crude tRNA was allowed to enter the column and the flow-through was discarded. The crude tRNA was eluted with buffer A (10 to 15 mL) until the eluant showed no UV absorbance any more. The obtained solution containing crude tRNA was kept at -80 °C until subjection to anion exchange chromatography. The utilized PD10 column was reequilibrated with buffer A (25 mL) and reused up to ten times. For storage PD10 columns were equilibrated with 20/80 ethanol/water (25 mL).

10.4.2.3 tRNA isolation from bacteria, yeast, and cancer cell lines

The cell pellet was allowed to thaw on ice and suspended in buffer 1 (15 mL) and 80 % aq. phenol (15 mL) was added. Further tRNA isolation was performed as described in chapter 10.4.2.1 for pork liver tissue. Desalting was not necessary for tRNAs isolated from bacteria, yeast, and cancer cell lines. The obtained tRNA pellet was kept at -80 °C until anion exchange chromatography was performed.

Isolation of tRNA from cancer cell lines was performed by M. Wagner.

10.4.2.4 tRNA purification - anion exchange chromatography

The isolated tRNA was further purified by anion exchange chromatography. All associated steps were performed on ice or at 4 °C. The tRNA pellet from bacteria, yeast, and cell culture cells were dissolved in buffer A (10 mL). Crude tRNA from pork liver tissue entered this

purification phase already in solution. Remaining impurities were removed from the tRNA samples by weak anion exchange chromatography (DEAE Sepharose Fast Flow 5 mL, column volume (CV): 5 mL) utilizing an ÄKTA purifier. The gradient was 5 CV, 0 % buffer B (25 mL, 0.10 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 1.0 M NaCl); 10 CV, 0 % → 40 % buffer B; 5 CV, 100 % buffer B; 3 CV, 0 % buffer B. The fractions eluting at about 20 % to 40 % buffer B and showing approximately a 2:1 ration for the absorption at 254 nm and 280 nm were collected. To the combined fractions abs. EtOH (3.0 vol) was added and the mixture was kept at -20 °C over night. After centrifugation (60 min, 24366 g) the supernatant was discarded and the pellet was dissolved in MilliQ water (2 x 1.0 mL). In case the resulting tRNA concentration proved to be too low for the subsequent digestion (< 140 ng/µl) another EtOH precipitation step was conducted.

10.4.2.5 DNA isolation from tissue samples and cancer cell lines

Pork tissue samples were collected as described in chapter 10.4.2.1. Tissue samples from mouse brain were obtained from the Biel group.

Early DNA isolation steps were performed on the basis of the QIAamp DNA Mini Kit. All steps discussed in this paragraph (except RNase) were performed as described in the kit. For tissues samples with more than 25 mg weight all quantities were scaled up accordingly. First, the samples were weighed. Tissue samples (80 mg) or whole tissues (for tissue weights < 80 mg), PBS and a stainless steel bead (5 mm) were added to a 2 mL reaction tube. The sample was homogenized in a TissueLyser (Qiagen, 30 Hz, 2 min). ATL and proteinase K were added and the solution was mixed by vortexing. The sample was incubated and shaken (600 rpm) at 56 °C for 2 h. Afterwards it was transferred into a 2 mL reaction tube removing the steel bead in the process. The tube was centrifuged briefly to collect the sample at the bottom of the tube and DNase-free RNase A (4 µL per 25 mg tissue, 100 mg/mL) was added. After mixing the sample was incubated and shaken (600 rpm) at rt for 5 min. A second portion of DNase-free RNase A was added and the mixture was again incubated and shaken (600 rpm) at rt for 5 min. The tube was centrifuged briefly to remove drops from the lid and buffer AL was added. The sample was mixed by 15 s pulse-vortexing and incubated and shaken at 70 °C for 10 min. The tube was centrifuged briefly to remove drops from the lid. Following this step the sample was no longer processed on the basis of the QIAamp DNA Mini Kit.

The sample was distributed equally to two 2 mL reaction tubes, if necessary. A 1/1 mixture of Roti®Phenol/chloroform (1.0 vol) was added and the tube was shaken vigorously at rt for

5 min. The tube was centrifuged (12100 g, 15 min) and the aq. layer was collected. This procedure was repeated once. To the obtained aq. layer chloroform (1.0 vol) was added and the tube was shaken at rt for 1 min. After centrifugation (12100 g, 5 min) the aq. layer was collected. During collection of the aq. layers special care was taken to include the interphase. The sample was distributed equally to two 2 mL reaction tubes, if necessary. Ethanol (3 vol) was added. The sample was left to stand at rt for approximately 2 h. After precipitation of the DNA the tube was centrifuged (12100 g, 30 min). The supernatant was discarded and the pellet was dried. Subsequently, it was dissolved in water (200 µL). The solution was centrifuged (12100 g, 30 min) and the supernatant was collected.

Isolation of DNA from cancer cell lines was performed by M. Wagner.

10.4.2.6 Agarose gel electrophoresis

Isolated tRNAs were analyzed on 1 % TBE agarose gels. These were run with a horizontal cell (Sub-Cell Bio-Rad) at 100 V. The tRNA was stained with ethidium bromide. The bands were visualized using a Raytest IDA gelscanner.

Gel buffer: tris base (89 mM), boronic acid (89 mM), EDTA (2 mM)

Running buffer: tris base (45 mM), boronic acid (45 mM), EDTA (1 mM)

EtBr: 1 mg/mL

RNA-ladder: ssRNA ladder, New England BioLabs

Isolated DNAs were analyzed on 0.8 % TAE agarose gels. These were run with a horizontal cell (Sub-Cell Bio-Rad) at 100 V. The DNA was stained with ethidium bromide. The bands were visualized using a Raytest IDA gelscanner.

Gel buffer: tris base (40 mM, pH 8.3), EDTA (2 mM), pH adjusted with sodium acetate

Running buffer: tris base (20 mM, pH 8.3), EDTA (1 mM), pH adjusted with sodium acetate

EtBr: 1 mg/mL

RNA-ladder: 1 kb ladder, New England BioLabs

10.4.2.7 Polyacrylamide gel electrophoresis

Isolated tRNAs were analyzed by polyacrylamide gel electrophoresis. NaOAc gels with 7.5 % acrylamide (Rotiphorese sequencing gel concentrate, Roth) were used in a Mini-Protean 3-Chamber from Biorad. Gel thickness was 0.75 mm. The gels were run at 25 or 50 mA constant current with a maximum voltage of 200 V. The polyacrylamide gels were stained agitation in SYBR Green II solution (1:10000 dilution) for 10 min.

Diluter:	8.3 M Urea
10 x NaOAc buffer:	NaOAc (1 M)
2 x NaOAc-urea-sample buffer:	0.5 M NaOAc, 6 % Ficoll™ Type 400, 0.005 % bromophenol blue, 0.025 % xylene cyanol, 3.5 M urea
RNA ladder:	Low range ssRNA ladder, New England BioLabs

10.4.2.8 Enzymatic digestion

For the enzymatic digestion solutions of bulk tRNA from all samples in water (12 µg in 100 µL final volume) were heated to 100 °C for 3 min to denature tRNA and then rapidly cooled on ice. After addition of buffer 2 (10 µL, 300 mM ammonium acetate, 100 mM $CaCl_2$, 1 mM $ZnSO_4$, pH 5.7) and nuclease S1 (80 units, A*spergillus oryzae*) the mixture was incubated for 3 h at 37 °C. Addition of buffer 3 (12 µL, 500 mM Tris-HCl, 1 mM EDTA, pH 8.0), antarctic phosphatase (10 units), snake venom phosphodiesterase I (0.2 units, *Crotalus adamanteus venom*) and incubation for further 3 h at 37 °C completed the digestion. All labeled nucleosides of interest were added. Then the sample was centrifuged (12100 g, 15 min). The supernatant was removed and lyophilized to a total volume of 105 µL. Each digestion and HPLC-ESI-MS measurement was performed at least in triplicate with three independent concentrations of the appropriate labeled nucleosides. The concentrations of standard solutions were chosen to be in the expected range of the sample nucleoside concentration.

10.5 Calculation of conservation of modified tRNA nucleosides in mammals

All tRNA sequences from different mammals were collected and categorized according to the amino acid they carry. For each tRNA sequence the numbers of each modification of interest in this study were counted. The count was executed regardless of the positions of the modifications, because our method also does not differentiate positions in tRNA. An exemplary list of modification numbers for $tRNA^{Glu}$ is shown in Table 16. Then, the number of modifications in a certain tRNA were listed and compared with the number obtained for tRNA sequences of other mammals within this category. The differences were summed up. The number of theoretically possible differences was calculated. The number of actual differences was divided by the theoretical ones. This yielded the percentage of non-conserved modifications.

Organism	Anticodon	m1A	m2A	i6A	m6A	t6A	m6t6A	ms2t6A	Am	m1G	m2G	Gm	m22G	Q
Homo sapiens	CUC	0	0	0	0	0	0	0	0	0	1	0	0	0
Mus musculus	CUC	0	0	0	0	0	0	0	0	0	1	0	0	0
Rattus norvegicus	mcm^5s^2UUC	0	0	0	0	0	0	0	0	1	0	0	0	0

Table 16: Exemplary modification count for $tRNA^{Glu}$ sequences from the Sprinzl database.

For $tRNA^{Glu}$ the modification counts are presented in Table 16. An exemplary calculation using only these data is discussed in the following. First, these modification number comparisons were made: *H. sapiens – M. musculus* (0 differences), *H. sapiens – R. novegicus* (2 differences), *M. musculus – R. norvegicus* (2 differences). Out of the nucleosides presented in this table five occur in position 37 (Figure 14). This means, only one of these can be present in a certain tRNA at a time and these five count only for one theoretically possible difference. Q is present in position 34 and six modifications occur at various positions in tRNA. Hence, there are eight theoretically possible differences for each comparison. Three comparison thus yield 24 theoretically possible differences. This results in 17 % non-conserved modifications for tRNAGlu according to this calculation.

10.6 HPLC-ESI-MS Materials and Methods

10.6.1 HPLC-ESI-MS

The samples (100 µL injection volume) were analyzed by LC-ESI-MS on a *Thermo Finnigan LTQ Orbitrap XL* and were chromatographed by a *Dionex Ultimate 3000 HPLC* system with a flow of 0.15 mL/min over an Uptisphere120-3HDO column from *Interchim*. The column temperature was maintained at 30 °C. Eluting buffers were buffer C (2 mM $HCOONH_4$ in H_2O, pH 5.5) and buffer D (2 mM $HCOONH_4$ in H_2O/MeCN 20/80, pH 5.5). The gradient was 0 → 41.25 min; 0 % → 8 % buffer D; 41.25 → 80 min; 8 % → 60 % buffer D; 80 → 82 min; 60 % → 100 % buffer D; 82 → 100 min; 100 % buffer D; 100 → 105 min; 100 → 0 % buffer D; 105 → 115 min; 0 % buffer D or 0 → 55 min; 0% → 8 % buffer D; 55 → 100 min; 8% → 60% buffer D; 100 → 102 min; 60 % → 100% buffer D; 102 → 120 min; 100% buffer D; 120 → 125 min; 100 → 0% buffer D; 125 → 135 min; 0% buffer D. The elution was monitored at 260 nm (*Dionex Ultimate 3000 Diode Array Detector)*. The chromatographic eluant was directly injected into the ion source without prior splitting. Ions were scanned by use of a positive polarity mode over a full-scan range of *m/z* 200-1000 with a resolution of 30.000. Parameters of the mass spectrometer were tuned with a freshly mixed solution of adenosine (5 µM) in buffer C. The parameters used in this section were sheath gas

flow rate, 16 arb; auxiliary gas flow rate, 10 arb; sweep gas flow rate, 4 arb; spray voltage, 5.0 kV; capillary temperature, 200 °C; capillary voltage, 25 V, tube lens 60 V.

10.6.2 Calibration curves and equations

Calibration curves were measured by D. Globisch. Mass calibration curves were obtained at five different concentration ratios of the labeled and corresponding unlabeled synthesized nucleosides. For each concentration ratio an average value of three independent measurements was determined. Each labeled nucleoside solution was mixed with three solutions with different concentrations of the corresponding unlabeled nucleosides. The areas of labeled and unlabeled nucleosides from LC-MS measurements were determined using the *Qualbrowser* program by extraction of the accurate mass range with a mass filter (Table 17) from the total ion current (TIC).

Nucleoside	Nucleosides mass range *m/z*	Labeled nucleosides mass range *m/z*
Am, m^6A, m^1A, m^2A	282.1142-282.1262	285.1335-285.1435
t^6A	413.1315-413.1475	418.1420-418.1580
i^6A	336.1606-336.1716	338.1740-338.1840
ms^2i^6A	382.1484-382.1594	385.1679-385.1789
$m^2{}_2G$	312.1248-312.1368	315.1441-315.1561
m^2G, m^1G	298.1076-298.1196	301.1276-301.1396
Q	410.1640-410.1730	413.1778-413.1898
m^6t^6A	427.1532-427.1622	430.1707-430.1807
OHyW	525.1879-525.2029	528.2065-528.2195
yW	509.1937-509.2037	512.2138-512.2238
io^6A	352.0915-352.2315	354.1041-354.2441
ms^2io^6A	398.1423-398.1563	400.0918-400.2318
Cm	258.0384-258.1784	261.0573-261.1973

Table 17: High resolution mass ranges of natural and corresponding labeled nucleosides used for quantification.

From these data the area ratios unlabeled to corresponding labeled nucleoside were determined. The amount ratios unlabeled to corresponding labeled nucleoside were calculated from the employed amounts, which were known. The obtained area ratios were plotted against the amount ratios. The linear fits of these gave R^2-values of minimum 0.9990 (Figure 32).

The linear fit equations were used for calculation of the exact nucleoside contents in bulk tRNA samples. Synthetic labeled nucleosides were added to the digest solutions and the areas of labeled and unlabeled nucleosides were determined as described above. The amount of each nucleoside was calculated from the obtained area ratios and the linear fit equations of the calibration curves.

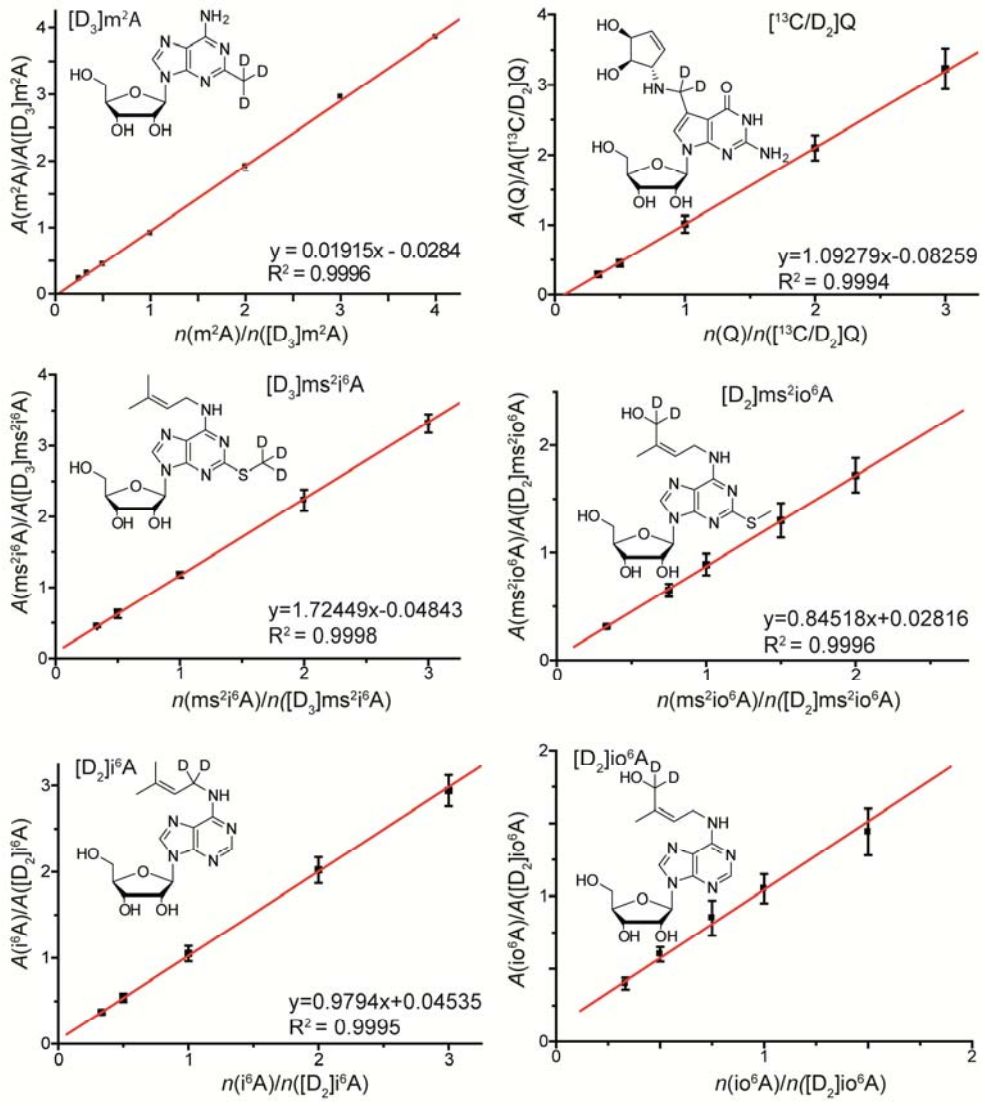


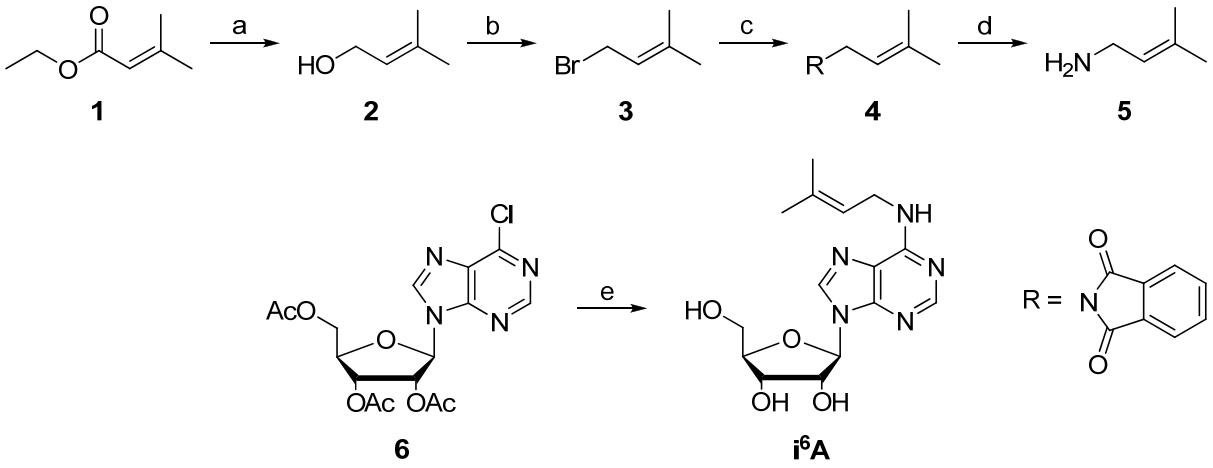
Figure 32: Calibration curves and equations for m^2A, Q, ms^2i^6A, ms^2io^6A, i^6A, and io^6A. Calibrations curves for all other modified nucleosides studied in the course of this thesis will be reported by D. Globisch, M. Münzel, V. Welzmiller, and A. Hienzsch.

10.6.3 Intra- and inter-assay test

The intra-assay test was performed by D. Globisch for nucleosides m^1A, i^6A, ms^2i^6A and m^1G. Detailed data will be reported by D. Globisch. The determined values of labeled to unlabeled nucleosides using the calibration curves of a sample after enzymatic digestion showed good reproducibility for each nucleoside (N=5); 2.5 % for m^1A, 0.4 % for i^6A, 0.7 % for ms^2i^6A and 2.6 % for m^1G. The single area values of each labeled or unlabeled nucleoside in the intra-assay test resulted in an average value of 4.1 %. The inter-assay test gave an area ratio reproducibility (N=6) of 6.3 % for m^1A, 1.0 % for i^6A, 1.8 % for ms^2i^6A and 4.3 % for m^1G on six subsequent days. The determined area values on their own for each labeled or unlabeled nucleoside resulted in an relative standard deviation of 34.5 %. This insufficient reproducibility proves the necessity of the labeled nucleosides as reference. No memory effect was observed during blank LC/MS experiments performed after several measurements of a sample. The blank analyses were not contaminated by carry-over.

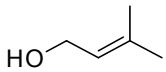
10.7 Syntheses of isopentenyl-adenosines

10.7.1 Synthesis of i^6A



Scheme 29: Synthesis of i^6A.
Reagents and conditions: a) $LiAlH_4$, EtO_2, 0 °C to rt, 15 min; b) PBr_3, Et_2O, 0 °C, 30 min; c) KPhth, DMF, 80 °C, 30 min, 64 % (3 steps); d) hydrazine hydrate, 2 M HCl, MeOH, rt, 20 h, 99 %; e) **5**, EtOH, 60 °C, 18 h, 69 %.

10.7.1.1 3-Methyl-but-2-en-1-ol (2).[244]



$LiAlH_4$ (7.20 g, 188 mmol, 1.10 eq) was added slowly to 3,3-dimethylacrylic acid ethyl ester **1** (24.0 mL, 171 mmol, 1.00 eq) in dry Et_2O (100 mL) at 0 °C. After stirring at rt for 15 min sat. NH_4Cl solution (100 mL) was added carefully. The organic layer was collected. The aq. layer was extracted with Et_2O (100 mL) twice. The organic layers were combined, dried over $MgSO_4$ and the solvent was removed carefully *in vacuo* to a final volume of 200 mL.

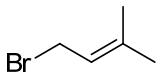
R_f = 0.46 (Isohexane/ethyl acetate 1/1);

^1H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.40 – 5.28 (m, 1H; CH), 4.13 – 4.00 (m, 2H; CH_2), 1.74 – 1.66 (m, 3H; CH_3), and 1.65 – 1.58 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 136.25 (C_q), 123.78 (CH), 59.33 (CH_2), 25.87 (CH_3), and 17.93 ppm (CH_3);

HR-MS (EI+) for $[C_5H_{10}O]^{\bullet+}$: calc.: 86.0726, found: 86.0713.

10.7.1.2 1-Bromo-3-methyl-but-2-ene (3).[245]



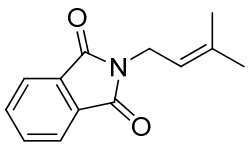
The equivalents given in this protocol refer to the amount of 3,3-dimethylacrylic acid ethyl ester **1** employed in this three step reaction sequence. The total solution obtained from the preceding reaction was cooled to 0 °C. PBr_3 (23.0 g, 85.5 mmol, 0.50 eq) was added dropwise at 0 °C. After stirring at 0 °C for 30 min sat. NH_4Cl solution (100 mL) was added. The organic layer was collected. The aq. layer was extracted with Et_2O (30.0 mL) twice. The organic layers were combined, dried over $MgSO_4$ and the solvent was removed carefully *in vacuo* (> 300 mbar) to yield crude bromide **3** (16.6 g).

R_f = 0.79 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.55 – 5.42 (m, 1H; CH), 4.01 – 3.93 (m, 2H; CH_2), 1.76 – 1.72 (m, 3H; CH_3), and 1.70 – 1.67 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 140.31 (C_q), 120.93 (CH), 29.91 (CH_2), 25.96 (CH_3), and 17.70 ppm (CH_3).

10.7.1.3 3-Methyl-1-phthalimido-but-2-ene (4).[246]



The total crude product **3** (16.6 g, 112 mmol, 1.00 eq) obtained from the previous reaction was dissolved in DMF (100 mL), treated with KPhth (22.6 g, 122 mmol, 1.10 eq) and stirred at 80 °C for 30 min. The suspension was allowed to cool to rt and treated with sludge (100 mL). The resulting white solid was collected by filtration and dried under high vacuum for 18 h yielding alkene **4** (15.2 g, 64 %, 3 steps).

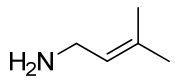
R_f = 0.55 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 7.84 – 7.76 (m, 2H; HC_{Ar}), 7.71 – 7.62 (m, 2H; HC_{Ar}), 5.29 – 5.18 (m, 1H; HC), 4.27 – 4.18 (m, 2H; CH_2), 1.83 – 1.76 (m, 3H; CH_3), and 1.68 – 1.66 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 168.38 (CO), 137.45 ($C_{q,Ar}$), 134.00 (HC_{Ar}), 132.47 (C_q), 123.33 (HC_{Ar}), 118.41 (CH), 36.02 (CH_2), 25.85 (CH_3), and 18.14 ppm (CH_3);

HR-MS (EI+) for $[C_{13}H_{13}NO_2]^{\bullet+}$: calc.: 215.0941, found: 215.0941.

10.7.1.4 3-Methyl-2-buten-1-amine (5).[179, 247]



Bromide **4** (2.00 g, 9.30 mmol, 1.00 eq) and hydrazine hydrate (2.20 mL, 28.0 mmol, 3.30 eq) were suspended in MeOH (50.0 mL). After stirring for 2 h at rt aq. HCl (2 M, 9.30 mL) was added and the solution was stirred for 18 h. The precipitate was removed by filtration. The solution was adjusted to pH = 2 with HCl (conc. and 2 M) and extracted with Et_2O (50.0 mL) three times. The resulting solution was adjusted to pH = 12 with NaOH (pellets and 2 M) and extracted with Et_2O (50.0 mL) twice. These organic layers were combined and dried over $MgSO_4$. The solvent was removed *in vacuo* (> 300 mbar) yielding amine **5** (0.78 g, 99 %) as colorless liquid.

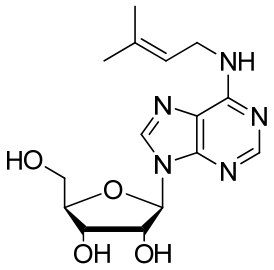
^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.28 – 5.19 (m, 1H; CH), 3.97 – 3.90 (m, 2H; CH_2), 1.78 – 1.73 (m, 3H; *E*-CH_3), and 1.73 – 1.68 ppm (m, 3H; *Z*-CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 132.8 (C_q), 126.1 (CH), 39.7 (CH_2), 25.6 (*E*-CH_3), and 17.6 ppm (*Z*-CH_3);

IR: 3362 *m* (N-H valence), 3290 *m* (N-H valence), 2969 *s* (C-H valence), 2915 *s* (C-H valence), 2861 *s* (C-H valence), 1664 *w* (C=C valence), 1592 *w* (N-H deform.), 1448 *m* (C-H deform.), and 1376 cm^{-1} *m* (CH_3 deform.);

HR-MS (EI+) for $[C_5H_{11}N]^{\bullet+}$: calc.: 85.0886, found: 85.0872.

10.7.1.5 6-*Iso*pentenylamino-9-(*β*-D-ribofuranosyl)-9*H*-purine (i^{6}A).[167, 248]



Chloro-nucleoside **6** was synthesized according to literature and provided by D. Globisch.

Nucleoside **6** (100 mg, 0.24 mmol) in EtOH (2 mL) was treated with amine **5** (0.2 mL) and stirred at 60 °C for 18 h. The suspension was allowed to cool to rt and the precipitate was collected by filtration. The solvent was removed *in vacuo* from the remaining solution. The yellow oil was treated with ^{i}PrOH and cooled to 0 °C. After standing at 0 °C for 1 h the precipitate was collected by filtration. The combined white solids were dried under high vacuum yielding i^{6}A (56 mg, 69 %).

Alternatively, the solvent was removed from the reaction mixture and the resulting mixture was recrystallized from MeOH or ^{i}PrOH.

R_f = 0.60 (DCM/MeOH 8/1);

M.p.: 130.5 – 131.2 °C;

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.27 (s, 1H; HC8), 8.25 (s, 1H; HC2), 5.98 (d, $^3J(HC_{1'},HC_{2'})$=6.5 Hz, 1H; $HC_{1'}$), 5.45 – 5.38 (m, 1H; $CH_2CHC(CH_3)_2$), 4.78 (dd, $^3J(HC_{2'},HC_{1'})$=6.5 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.36 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz,

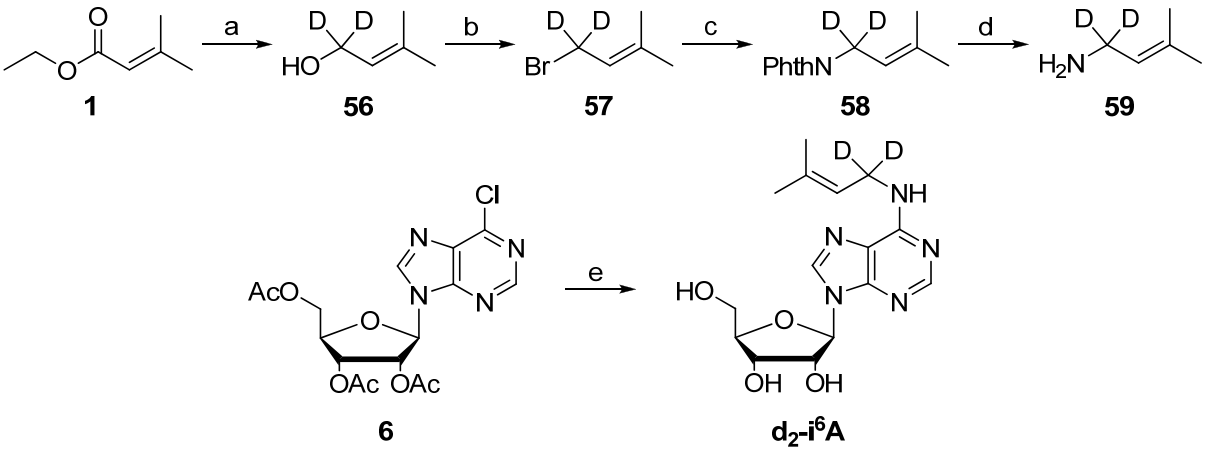
$^3J(HC_{3'},HC_{4'})$=2.5 Hz, 1H; $HC_{3'}$), 4.20 (m, 3H; $CH_2CHC(CH_3)_2$ and $HC_{4'}$), 3.92 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.5 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.5 Hz, 1H; $H_aC_{5'}$), 3.78 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.5 Hz, $^3J(H_bC_{5'},HC_{4'})$=2.7 Hz, 1H; $H_bC_{5'}$), and 1.84 – 1.76 ppm (s, 6H; 2x CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 156.19 (C6), 153.67 (C2), 149.26 (C4), 141.57 (C8), 137.34 ($CH_2CHC(CH_3)_2$), 121.62 (2C; $CH_2CHC(CH_3)_2$ and C5), 91.46 ($C_{1'}$), 88.37 ($C_{4'}$), 75.61 ($C_{2'}$), 72.86 ($C_{3'}$), 63.67 ($C_{5'}$), 39.71 ($CH_2CHC(CH_3)_2$), 25.97 (CH_3), and 25.40 ppm (CH_3);

IR: 3321.2 *m* (O-H valence, N-H valence), 3146.8 *m* (O-H valence, N-H valence), 2919.6 *m* (O-H valence, N-H valence), 1621.1 *s* (N-H deform.), 1589 *m* (N-H deform., aromatic vibration), and 1053.4 cm^{-1} *m* (C-O valence);

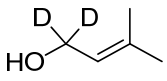
HR-MS (EI+) for $[C_{15}H_{21}N_5O_4]^{\bullet+}$: calc.: 335.1588, found: 335.1588.

10.7.2 Synthesis of d_2-i^{6}A.



Scheme 30: Synthesis of d_2-i^{6}A.
Reagents and conditions: a) $LiAlD_4$, EtO_2, rt, 5 min, 98 %; b) PBr_3, Et_2O, 0 °C, 30 min, 59 %; c) KPhth, DMF, 80 °C, 30 min, 80 %; d) hydrazine hydrate, 2 M HCl, MeOH, rt, 18 h, 95 %; e) **59**, EtOH, 60 - 80 °C, 21 h, 85 %.

10.7.2.1 1-Dideutero-3-methyl-but-2-en-1-ol (56).[244a]



$LiAlD_4$ (1.00 g, 25.0 mmol, 0.95 eq) was added slowly to 3,3-dimethylacrylic acid ethyl ester **1** (4.90 mL, 26.5 mmol, 1.00 eq) in dry Et_2O (50.0 mL) at rt. After stirring at rt for 5 min the solution was carefully treated with sat. NH_4Cl solution (50.0 mL). H_2O (30.0 mL) was added and the organic layer was collected. The aq. layer was extracted with Et_2O (50.0 mL). The organic layers were combined and the solvent was removed carefully *in vacuo* to yield alcohol **56** (2.30 g) as a colorless oil.

R_f = 0.46 (Isohexane/ethyl acetate 1/1);

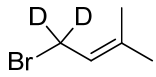
^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.41 – 5.32 (m, 1H; CH), 1.74 – 1.68 (m, 3H; CH_3), and 1.68 – 1.62 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 136.70 (C_q), 123.64 (CH), 58.83 (q, $^1J(CD_2,CD_2)$=22 Hz; CD_2), 25.95 (CH_3), and 18.01 ppm (CH_3);

^{2}D-NMR (46 MHz, $CDCl_3$, 27 °C): δ = 4.07 ppm (CD_2);

HR-MS (EI+) for $[C_5H_8D_2O]^{\bullet+}$: calc.: 88.0852, found: 88.0844.

10.7.2.2 1-Bromo-1-dideutero-3-methyl-but-2-ene (57).[244a, 245]



The total crude product (2.30 g, 26.1 mmol, 1.00 eq) obtained from the preceding reaction was cooled to 0 °C. PBr_3 (3.50 g, 13.0 mmol, 0.50 eq) was added dropwise at 0 °C. After stirring at 0 °C for 30 min sat. NH_4Cl solution (50.0 mL) was added carefully. The organic layer was collected. The aq. layer was extracted with Et_2O (20.0 mL) twice. The organic layers were combined, dried over $MgSO_4$ and the solvent was removed carefully *in vacuo* (> 300 mbar) to yield bromide **57** (2.28 g) as a colorless liquid.

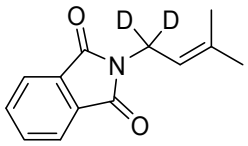
R_f = 0.79 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 5.51 – 5.47 (m, 1H; CH), 1.77 – 1.73 (m, 3H; CH_3), and 1.71 – 1.68 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 140.41 (C_q), 120.83 (CH), 29.57 (q, $^1J(CD_2,CD_2)$=23 Hz; CD_2), 25.99 (CH_3), and 17.76 ppm (CH_3);

^{2}D-NMR (46 MHz, $CDCl_3$, 27 °C): δ = 3.99 ppm (s; CD_2).

10.7.2.3 1-Dideutero-3-methyl-1-phthalimido-but-2-ene (58).



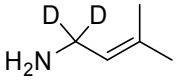
The total crude product (2.28 g, 15.2 mmol, 1.00 eq) obtained from the preceding reaction was dissolved in DMF (20.0 mL), treated with KPhth (3.12 g, 16.9 mmol, 1.11 eq) and stirred at 80 °C for 30 min. The suspension was allowed to cool to rt and treated with sludge (20.0 mL). The resulting white solid was collected by filtration and dried under high vacuum for 18 h yielding alkene **58** (2.65 g, 49 %, 3 steps).

R_f = 0.55 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 7.85 – 7.74 (m, 2H; HC_{Ar}), 7.72 – 7.62 (m, 2H; HC_{Ar}), 5.28 – 5.19 (m, 1H; CH), 1.82 – 1.77 (m, 3H; CH_3), and 1.70 – 1.66 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 168.35 (CO), 137.52 ($C_{q,Ar}$), 133.98 (CH_{Ar}), 132.49 (C_q), 123.31 (CH_{Ar}), 118.29 (CH), 35.55 (q, $^1J(CD_2,CD_2)$=21 Hz; CD_2), 25.85 (CH_3), 18.15 ppm (CH_3).

10.7.2.4 1-Dideutero-3-methyl-2-buten-1-amine (59).



Phthalimide **58** (2.65 g, 12.2 mmol, 1.0 eq) and hydrazine hydrate (2.9 mL, 36.4 mmol, 3.0 eq) were suspended in MeOH (50 mL). After stirring for 1.5 h at rt aq. HCl (2 M, 12.0 mL) was added and the solution was stirred for 18 h. The precipitated solid was removed by filtration. The solution was adjusted to pH = 2 using HCl (2 M and 37 %) and the precipitate was removed by filtration. The resulting solution was adjusted to pH = 12 (pellets and 2 M) and extracted twice with Et_2O (30 mL). The combined organic layers were dried over $MgSO_4$. The solvent was removed *in vacuo* (> 300 mbar) yielding a 3 to 1 mixture of MeOH and amine **59** (2.13 g, 95 %) as determined by $CDCl_3$-NMR) as colorless liquid.

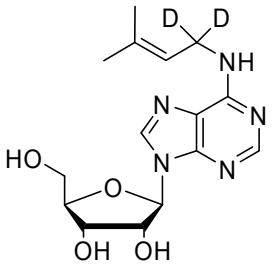
^{1}H NMR (300 MHz, CD_3OD, 27 °C): δ = 5.32 – 5.28 (m, 1H; CH), 1.77 – 1.75 (d, 3H; *E*-CH_3), and 1.71 – 1.68 ppm (d, 3H; *Z*-CH_3);

^{13}C NMR (75 MHz, CD_3OD, 27 °C): δ = 134.58 (C_q), 126.04 (CH), 39.7 (q, ^1J(*C*D_2, C*D*$_2$)=21 Hz; CD_2), 25.99 (*E*-CH_3), and 17.85 ppm (*Z*-CH_3);

^{2}D-NMR (61 MHz, CD_3OD, 27 °C): δ = 3.32 ppm (CD_2);

HR-MS (EI+) for $[C_5H_9D_2N]^{\bullet+}$: calc.: 87.1012, found: 87.1012.

10.7.2.5 6-(1-Dideutero-3-methyl-2-buten-1-amino)-9-(*β*-D-ribofuranosyl)-9*H*-purine (d_2-i^{6}A).



Nucleoside **6** (623 mg, 1.50 mmol, 1.00 eq) in EtOH (7.30 mL) was treated with amine **65** (1040 mg, 12.0 mmol, 8.00 eq) and stirred at 60 °C for 18 h and at 80 °C for 5 h. The solvent was removed *in vacuo* and the resulting mixture was recrystallized from ^{i}PrOH three times. The white solid was dried under high vacuum to yield d_2-i^{6}A (435 mg, 85 %) with less than 3 weight% ^{i}PrOH as determined by NMR.

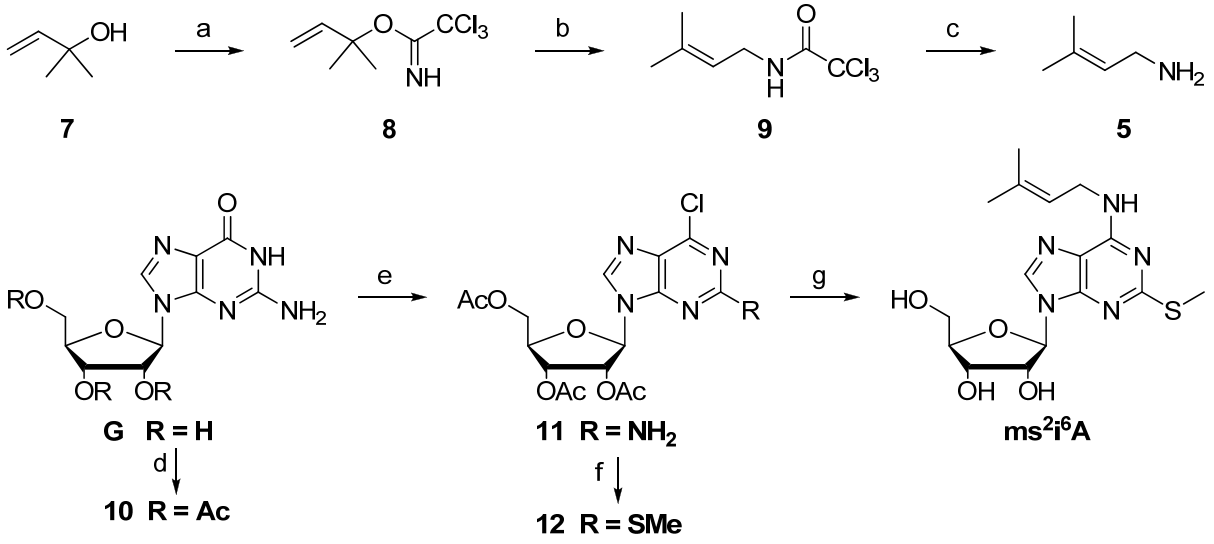
R_f = 0.60 (DCM/MeOH 8/1);

^{1}H NMR (600 MHz, CD_3OD, 27 °C): δ = 8.24 (s, 1H; HC8), 8.22 (s, 1H; HC2), 5.95 (d, $^3J(HC_{2'},HC_{1'})$=6.5 Hz, 1H; $HC_{1'}$), 5.41 – 5.34 (m, 1H; $CD_2CHC(CH_3)_2$), 4.74 (dd, $^3J(HC_{2'},HC_{1'})$=6.5 Hz, $^3J(HC_{2'},HC_{3'})$=5.1 Hz, 1H; $HC_{2'}$), 4.32 (dd, $^3J(HC_{3'},HC_{2'})$=5.1 Hz, $^3J(HC_{3'},HC_{4'})$=2.5 Hz, 1H; $C_{3'}H$), 4.17 (ddd, $^3J(HC_{4'},H_bC_{5'})$=2.6 Hz, $^3J(HC_{4'},HC_{3'})$=2.5 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.4 Hz, 1H; $HC_{4'}$), 3.89 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.6 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.4 Hz, 1H; $H_aC_{5'}$), 3.74 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.6 Hz, $^3J(H_bC_{5'},HC_{4'})$=2.6 Hz, 1H; $H_bC_{5'}$), and 1.18 ppm (m, 6H; 2x CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 156.21 (C6), 153.66 (C2), 149.09 (C4), 141.59(C8), 137.40 ($CH_2CHC(CH_3)_2$), 121.48 (2C; $CH_2CHC(CH_3)_2$ and C5), 91.46 ($C_{1'}$), 88.39 ($C_{4'}$), 75.59 ($C_{2'}$), 72.88 ($C_{3'}$), 63.68 ($C_{5'}$), 26.00 (CH_3), and 25.40 ppm (CH_3);

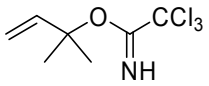
HR-MS (ESI+) for $[C_{15}H_{20}D_2N_5O_4]^+$: calc.: 338.1790, found: 338.1781.

10.7.3 Synthesis of ms^{2}i^{6}A



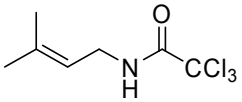
Scheme 31: Synthesis of ms^{2}i^{6}A.
Reagents and conditions: a) 2-Methyl-3-buten-2-ol, NaH, trichloro acetonirile, THF, Et_2O, 0 °C to rt, 2 h; b) toluene, 115 °C, 2 h, 67 % (2 steps); c) 3 N KOH, DMF, rt, 4 h, 45 %; d) Ac_2O, DMF, pyridine, 75 °C, 1.5 h, 87 %; e) $POCl_3$, DMA, Et_4NCl, MeCN, 100 °C, 10 min, 51 %; f) Dimethyldisulfide, *N*-pentyl-nitrite, MeCN, 70 °C, 1.5 h, 65 %; g) **5** (neat), 50 °C, 1.5 h, 74 %.

10.7.3.1 2,2,2-Trichloro-acetimidic acid 1,1-dimethyl-allyl ester (8).[179]



NaH (60 % in paraffin oil, 1.20 g, 25 mmol, 0.22 eq) was suspended in dry hexane (20.0 mL). The solids were allowed to settle and the solvent was removed by use of a syringe. This procedure was repeated once. The remaining hexane was removed *in vacuo*. The resulting solid was suspended in dry THF (5.00 mL) and cooled to 0 °C. 2-Methyl-3-buten-2-ol **7** (12.0 mL, 116 mmol, 1.00 eq) in dry THF (10.0 mL) and added dropwise to the NaH suspension. After stirring for 1 h at rt, the suspension was added slowly to trichloro acetonitrile (11.6 mL, 116 mmol, 1.00 eq) in ether (100 mL) at 0 °C. The resulting suspension was stirred for 1 h at 0 °C. The solvent was removed *in vacuo* (> 330 mbar, 40 °C).

10.7.3.2 *N*-(3-Methyl-2-butenyl)-2,2,2-trichloroacetamide (9).[179]



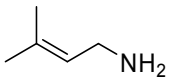
Crude **8** was diluted with toluene (100 mL) and heated to 115 °C for 2 h. The solvent was removed *in vacuo*. The resulting mixture was distilled at 90 °C – 140 °C and 30 – 14 Pa to give allyl amide **9** (17.8 g, 67 %, 2 steps) as white solid.

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.25 – 5.21 (m, 1H; CH), 3.95 – 3.91 (m, 2H; CH_2), 1.75 (s, 3H; *E*-CH_3), and 1.71 ppm (s, 3H; *Z*-CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 161.86 (CO), 138.85 (C_q), 118.31 (CH), 92.84 (CCl_3), 39.67 (CH_2), 25.89 (*E*-CH_3), and 18.22 ppm (*Z*-CH_3);

HR-MS (ESI+) for $[C_7H_{11}Cl_3NO]^+$: calc.: 229.9901, found: 229.9893.

10.7.3.3 3-Methyl-2-buten-1-amine (5).[179]



Molten **9** (17.8 g, 78.0 mmol) was added dropwise to 3 N KOH (200 mL) and DMF (4.00 mL). The mixture was stirred at rt for 4 h. The resulting solution was extracted with diethyl ether (50.0 mL) three times. The combined organic phases were washed with brine (50.0 mL). Careful removal of the solvent yielded a light yellow liquid, which was purified by distillation (95 – 100 °C, atmospheric pressure) to give amine **5** (3.00 g, 45 %) as colorless liquid.

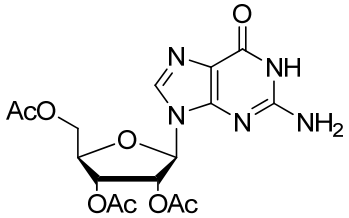
^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.28 – 5.19 (m, 1H; CH), 3.97 – 3.90 (m, 2H; CH_2), 1.78 – 1.73 (m, 3H; *E*-CH_3), and 1.73 – 1.68 ppm (m, 3H; *Z*-CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 132.8 (C_q), 126.1 (CH), 39.7 (CH_2), 25.6 (*E*-CH_3), and 17.6 ppm (*Z*-CH_3);

IR: 3362 *m* (N-H valence), 3290 *m* (N-H valence), 2969 *s* (C-H valence), 2915 *s* (C-H valence), 2861 *s* (C-H valence), 1664 *w* (C=C valence), 1592 *w* (N-H deform.), 1448 *m* (C-H deform.), and 1376 cm^{-1} *m* (C-H deform.);

HR-MS (EI+) for $[C_5H_{11}N]^{\bullet+}$: calc.: 85.0886, found: 85.0872.

10.7.3.4 6-Oxo-2-amino-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (10).[181]



Guanosine (5.00 g, 18.0 mmol, 1.00 eq) was suspended in pyridine (10.0 mL) and DMF (12.0 mL). Acetic acid anhydride (20.0 mL, 212 mmol, 12.0 eq) was added and the mixture was stirred at 75 °C until all solid material disappeared (approximately 1.5 h). Ethanol

(1.00 mL) was added and the solution was filtered. The solvent was removed *in vacuo* and the resulting gummy white solid was refluxed in isopropanol (20.0 mL). After cooling to 0 °C Nucleoside **10** (6.33 g, 87 %) precipitated as white solid and was collected.

R_f = 0.20 (DCM/MeOH 16/1);

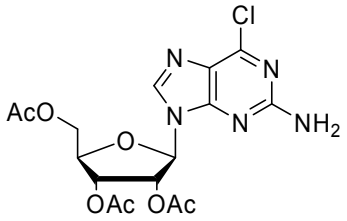
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.69 (s, 1H; HN), 7.92 (s, 1H; HC8), 6.52 (s, 2H; NH_2), 5.98 (d, $^3J(HC_{1'},HC_{2'})$=6.4 Hz, 1H; $HC_{1'}$), 5.79 (dd, $^3J(HC_{2'},HC_{1'})$=6.4 Hz, $^3J(HC_{2'},HC_{3'})$=6.0 Hz, 1H; $HC_{2'}$), 5.49 (dd, $^3J(HC_{3'},HC_{2'})$=6.0 Hz, $^3J(HC_{3'},HC_{4'})$=4.2 Hz, 1H; $HC_{3'}$), 4.40 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.8 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.0 Hz, 1H; $H_aC_{5'}$), 4.31 (ddd, $^3J(HC_{4'},H_bC_{5'})$=5.8 Hz, $^3J(HC_{4'},HC_{3'})$=4.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=4.0 Hz, 1H; $HC_{4'}$), 4.26 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.7 Hz, $^3J(H_bC_{5'},HC_{4'})$=5.8 Hz, 1H; $H_bC_{5'}$), 2.11 (s, 3H; H_3CCO), 2.04 (s, 3H; H_3CCO), and 2.04 ppm (s, 3H; H_3CCO);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 170.0 (CH_3CO), 169.3 (CH_3CO), 169.1 (CH_3CO), 156.5 (C4), 153.8 (C2), 151.0 (C6), 135.5 (C8), 116.7 (C5), 84.3 ($C_{1'}$), 79.4 ($C_{4'}$), 71.9 ($C_{2'}$), 70.2 ($C_{3'}$), 63.0 ($C_{5'}$), 20.4 (CH_3CO), 20.3 (CH_3CO), and 20.1 ppm (CH_3CO);

IR: 3408 *m* (N-H valence), 3164 *m* (N-H valence), 2864 *w* (C-H valence), 2735 *w* (C-H valence), 1755 *m* (C=O valence), 1740 *m* (C=O valence), 1692 *s* (C=O valence), 1669 *s* (C=O valence), 1600 *m* (N-H deform.), 1379 *m* (CH_3 deform.), 1224 *s* (C-O-C valence), and 1204 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{16}H_{20}N_5O_8]^+$: calc.: 410.1306, found: 410.1305.

10.7.3.5 6-Chloro-2-amino-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (11).[181]



Acetylated G **10** (1.00 g, 2.44 mmol, 1.00 eq), Et_4NCl (0.81 g, 4.88 mmol, 2.00 eq), DMA (0.30 mL, 2.44 mmol, 1.00 eq), and $POCl_3$ (1.40 mL, 16.6 mmol, 6.00 eq) were dissolved in MeCN (5.00 mL) and the mixture was stirred at 100 °C for a period of 10 min. The solvent

was removed *in vacuo* and the resulting oil was treated with water (30.0 mL). The solution was extracted with $CHCl_3$ (3 × 30.0 mL). The combined organic phases were washed with sat. $NaHCO_3$ (50.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography gave nucleoside **11** (0.54 g, 51 %) as white foam.

R_f = 0.20 (DCM/MeOH 16/1);

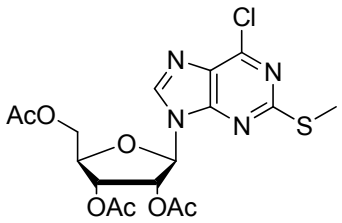
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 8.35 (s, 1H; HC8), 7.04 (s, 2H; NH_2), 6.10 (d, $^3J(HC_{1'},HC_{2'})$=6.0 Hz, 1H; $HC_{1'}$), 5.87 (dd, $^3J(HC_{2'},HC_{1'})$=6.0 Hz, $^3J(HC_{2'},HC_{3'})$=5.9 Hz, 1H; $HC_{2'}$), 5.54 (dd, $^3J(HC_{3'},HC_{2'})$=5.9 Hz, $^3J(HC_{3'},HC_{4'})$=4.2 Hz, 1H; $HC_{3'}$), 4.40 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.2 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.2 Hz, 1H; $H_aC_{5'}$), 4.43 (ddd, $^3J(HC_{4'},H_bC_{5'})$=5.3 Hz, $^3J(HC_{4'},HC_{3'})$=4.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=4.2 Hz, 1H; $HC_{4'}$), 4.28 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=11.2 Hz, $^3J(H_bC_{5'},HC_{4'})$=5.3 Hz, 1H; $H_bC_{5'}$), 2.12 (s, 3H; H_3CCO), 2.04 (s, 3H; H_3CCO), and 2.03 ppm (s, 3H; H_3CCO);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 170.0 (CH_3CO), 169.3 (CH_3CO), 169.1 (CH_3CO), 159.8 (C4), 153.6 (C6), 149.8 (C2), 141.1 (C8), 123.4 (C5), 84.8 ($C_{1'}$), 79.6 ($C_{4'}$), 71.8 ($C_{2'}$), 70.1 ($C_{3'}$), 62.8 ($C_{5'}$), 20.4 (CH_3CO), 20.3 (CH_3CO), and 20.1 ppm (CH_3CO);

IR: 3475 *w* (N-H valence), 3361 *w* (N-H valence), 3212 *w* (N-H valence), 2941 *w* (C-H valence), 1741 *s* (C=O valence), 1610 *s* (N-H deform.), 1561 *s* (N-H deform.), 1370 *w* (CH_3 deform.), and 1211 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{16}H_{19}ClN_5O_7]^+$: calc.: 428.0968, found: 428.0963.

10.7.3.6 6-Chloro-2-methylthio-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (12).[169b]



Nucleoside **11** (100 mg, 0.23 mmol, 1.00 eq) and dimethyldisulfide (0.21 mL, 2.30 mmol, 10.0 eq) were dissolved in dry MeCN (2.00 mL). *N*-pentyl-nitrite (0.16 mL, 1.15 mmol,

5.00 eq) was added and the mixture was stirred 1 h at rt and 1.5 h at 70 °C. The solvent was removed *in vacuo* and the resulting oil was purified *via* column chromatography yielding nucleoside **12** (70 mg, 65 %) as yellow foam.

R_f = 0.58 (DCM/MeOH 16/1);

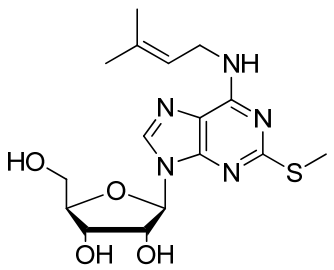
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 8.68 (s, 1H; HC8), 6.29 (d, $^3J(HC_{1'},HC_{2'})$=4.2 Hz, 1H; $HC_{1'}$), 6.04 (dd, $^3J(HC_{2'},HC_{1'})$=4.2 Hz, $^3J(HC_{2'},HC_{3'})$=6.0 Hz, 1H; $HC_{2'}$), 5.69 (dd, $^3J(HC_{3'},HC_{2'})$=6.0 Hz, 1H; $HC_{3'}$), 4.44 – 4.37 (m, 2H; $H_aC_{5'}$ and $HC_{4'}$), 4.26 - 4.18 (m, 1H; $H_bC_{5'}$), 2.62 (s, 3H; SCH_3), 2.11 (s, 3H; H_3CCO), 2.07 (s, 3H; H_3CCO), and 1.95 ppm (s, 3H; H_3CCO);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 169.93 (CH_3CO), 169.36 (CH_3CO), 169.23 (CH_3CO), 165.52 (C2), 151.97 and 149.54 (C6 and C4), 145.17 (C8), 128.58 (C5), 86.70 ($C_{1'}$), 79.07 ($C_{4'}$), 72.02 ($C_{2'}$), 69.51 ($C_{3'}$), 62.45 ($C_{5'}$), 20.34 (CH_3CO), 20.29 (CH_3CO), 20.21 (CH_3CO), and 14.19 ppm (SCH_3);

IR: 2933 *w* (C-H valence, O-H valence), 1742 *s* (C=O valence), 1595 *m* (N-H deform., aromatic vibration), 1548 *m* (N-H deform.), 1361 *m* (C-N valence), and 1205 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{17}H_{20}ClN_4O_7S]^+$: calc.: 459.0736, found: 459.0742.

10.7.3.7 6-(3-methyl-2-butenyl-1-amino)-2-methylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine (ms^{2}i^{6}A).[170b]



Nucleoside **12** (1.50 g, 3.38 mmol, 1.00 eq) was dissolved in amine **5** (3.00 mL) and stirred at 50 °C for 1.5 h. The solvent was removed *in vacuo*. The resulting oil was coevaporated with DCM (10.0 mL) three times. The obtained solid was refluxed in MeCN (5.00 mL) for 5 min.

After cooling to rt a white solid was collected by filtration and refluxed in water (5.00 mL) for 5 min. After cooling to rt the white solid was collected by filtration to give ms^2i^6A (0.95 g, 74 %) as white solid.

R_f = 0.49 (DCM/MeOH 8/1);

M.p.: 192.6 – 193.3;

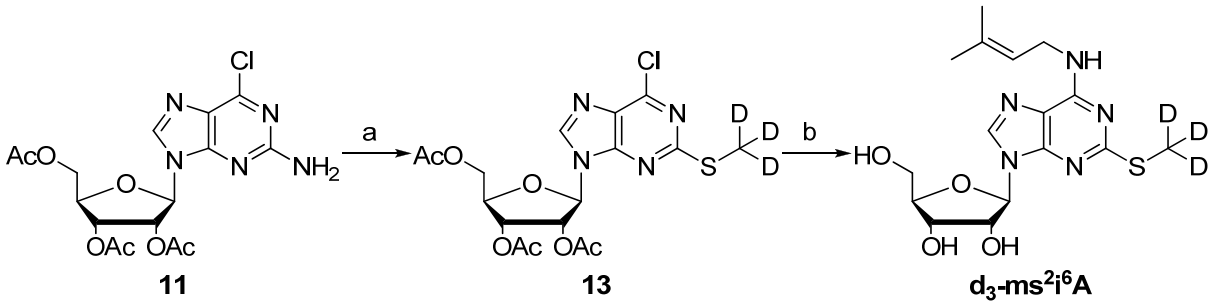
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 8.21 (s, 1H; HC8), 7.97 (s, 1H; HN), 5.83 (d, $^3J(HC_{1'},HC_{2'})$=5.8 Hz, 1H; $HC_{1'}$), 5.42 (d, $^3J(C_{2'}OH,HC_{2'})$=5.4 Hz, 1H; $C_{2'}OH$), 5.32 – 5.25 (m, 1H; $CH_2CHC(CH_3)_2$), 5.18 (d, $^3J(C_{3'}OH,HC_{3'})$=4.2, 1H; $C_{3'}OH$), 5.04 (t, $^3J(C_{5'}OH,H_2C_{5'})$=4.9 Hz, 1H; $C_{5'}OH$), 4.63 – 4.57 (m, 1H; $HC_{2'}$), 4.18 – 4.13 (m, 1H; $HC_{3'}$), 4.10 – 3.98 (m, 2H; $CH_2CHC(CH_3)_2$), 3.96 – 3.89 (m, 1H; $HC_{4'}$), 3.68 – 3.59 (m, 1H; $H_aC_{5'}$), 3.57 – 3.50 (m, 1H; $H_bC_{5'}$), 2.48 (s, 3H; SCH_3), 1.70 (s, 3H; CH_3), and 1.66 ppm (s, 3H; CH_3);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 164.15 (C2), 153.48 (C4), 149.39 (C6), 138.51 (C8), 133.29 ($CH_2CHC(CH_3)_2$), 121.92 ($CH_2CHC(CH_3)_2$), 117.25 (C5), 87.32 ($C_{1'}$), 85.48 ($C_{4'}$), 73.32 ($C_{2'}$), 70.50 ($C_{3'}$), 61.58 ($C_{5'}$), 37.62 ($CH_2CHC(CH_3)_2$), 25.42 (CH_3), 17.86 (CH_3), and 13.83 ppm (SCH_3);

IR: 3250 *m* (N-H valence), 2946 *w* (C-H valence), 1611 *s* (N-H deform.), 1579 *m* (N-H deform.), 1298 *m* (C-N valence), and 1079 cm^{-1} *m* (C-O valence);

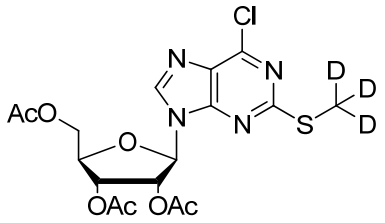
HR-MS (ESI+) for $[C_{16}H_{24}N_5O_4S]^+$: calc.: 382.1544, found: 382.1535.

10.7.4 Synthesis of d_3-ms^2i^6A



Scheme 32: Synthesis of d_3-ms^2i^6A.
Reagents and conditions: a) d_6-Dimethyldisulfide, *N*-pentyl-nitrite, MeCN, 70 °C, 5 min, 78 %; b) **5** (neat), 40 °C, 16 h, 39 %.

10.7.4.1 6-Chloro-2-trideuteromethylthio-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (13).



Nucleoside **11** (905 mg, 2.12 mmol, 2.00 eq) and d_6-dimethyldisulfide (100 mg, 1.06 mmol, 1.00 eq) were dissolved in dry MeCN (4.00 mL). *N*-pentyl-nitrite (0.56 mL, 4.24 mmol, 4.00 eq) was added and the mixture was stirred for 5 min at 70 °C. Silica gel was added and the solvent was removed *in vacuo*. Purification *via* column chromatography yielded nucleoside **13** (382 mg, 78 %) as yellow foam.

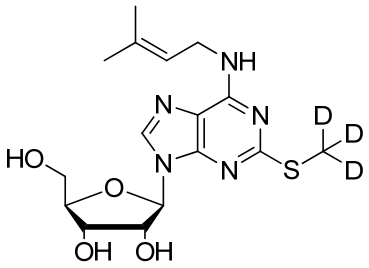
R_f = 0.58 (DCM/MeOH 16/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 8.08 (s, 1H; HC8), 6.09 (d, $^3J(HC_{1'},HC_{2'})$=4.5 Hz, 1H; $HC_{1'}$), 5.96 (dd, $^3J(HC_{2'},HC_{1'})$=4.5 Hz, $^3J(HC_{2'},HC_{3'})$=5.5 Hz, 1H; $HC_{2'}$), 5.62 (dd, $^3J(HC_{3'},HC_{2'})$=5.5 Hz, 1H; $HC_{3'}$), 4.43 – 4.39 (m, 2H; $H_aC_{5'}$ and $HC_{4'}$), 4.31 – 4.26 (m, 1H; $H_bC_{5'}$), 2.14 (s, 3H; H_3CCO), 2.12 (s, 3H; H_3CCO), and 2.08 ppm (s, 3H; H_3CCO);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 170.50 (CH_3CO), 169.70 (CH_3CO), 169.51 (CH_3CO), 167.60 (C2), 152.12 and 151.48 (C6 and C4), 142.39 (C8), 129.25 (C5), 87.27 ($C_{1'}$), 80.24 ($C_{4'}$), 73.14 ($C_{2'}$), 70.34 ($C_{3'}$), 62.91 ($C_{5'}$), 20.91 (CH_3CO), 20.70 (CH_3CO), 20.59 (CH_3CO), and 14.52 ppm (sept, $^1J(CD_3,CD_3)$=21 Hz; CD_3);

HR-MS (ESI+) for $[C_{17}H_{17}D_3ClN_4O_7S]^+$: calc.: 462.0921, found: 462.0932.

10.7.4.2 6-(3-methyl-2-butenyl-1-amino)-2-trideuteromethylthio-9-(β-D-ribofuranosyl)-9*H*-purine (d_3-ms^2i^6A).



Nucleoside **13** (50 mg, 0.11 mmol) was dissolved in amine **5** (0.5 mL) and stirred at 40 °C for 18 h. The solvent was removed *in vacuo*. The resulting oil was coevaporated with DCM (10 mL) three times. The obtained solid was purified by HPL chromatography to give d_3-ms^2i^6A (16 mg, 39 %) as white solid.

R_f = 0.49 (DCM/MeOH 8/1);

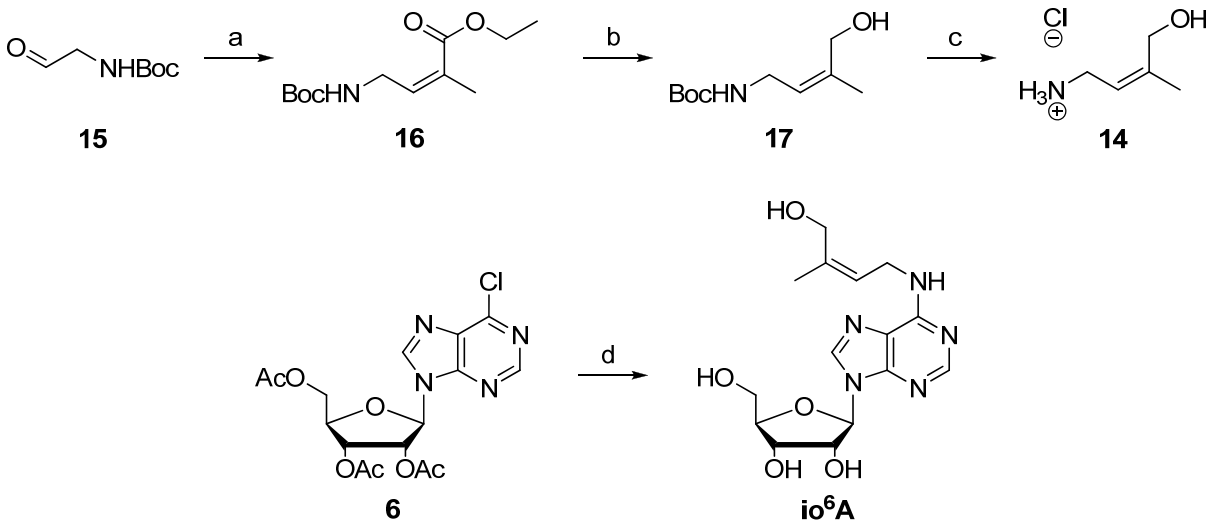
^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.32 (s, 1H; HC8), 6.16 (d, $^3J(HC_{1'},HC_{2'})$=5.8 Hz, 1H; $HC_{1'}$), 5.67 – 5.59 (m, 1H; $CH_2CHC(CH_3)_2$), 5.02 (dd, $^3J(HC_{2'},HC_{1'})$=5.8 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.60 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.7 Hz, 1H; $HC_{3'}$), 4.49 – 4.41 (m, 2H; $CH_2CHC(CH_3)_2$), 4.37 (ddd, $^3J(HC_{4'},HC_{3'})$=3.7 Hz, $^3J(HC_{4'},HC_{5b'})$=3.4 Hz, $^3J(HC_{4'},HC_{5a'})$=2.8 Hz, 1H; $HC_{4'}$), 4.11 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.8 Hz, 1H; $H_aC_{5'}$), 3.98 (dd, 1H; $^2J(H_bC_{5'},H_aC_{5'})$=12.3 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.4 Hz, $H_bC_{5'}$), 2.02 (s, 3H; CH_3) and 2.01 ppm (s, 3H; CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 140.29 (C8), 136.92 ($(CH_3)_2CCH_2$), 122.03 (CH), 118.74 (C5), 91.00 ($C_{1'}$), 87.72 ($C_{4'}$), 75.33 ($C_{2'}$), 72.55 ($C_{3'}$), 63.51 ($C_{5'}$), 39.49 (CH_2), 25.98 (CH_3), and 18.19 ppm (CH_3);

HR-MS (ESI+) for $[C_{16}H_{21}D_3N_5O_4S]^+$: calc.: 385.1729, found: 385.1736;

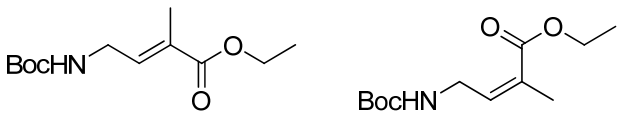
HPLC: gradient: 100 % H_2O, 0 % MeCN → 0 % H_2O, 100 % MeCN in 45 min; retention time = 26.6 min; flow 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.7.5 Synthesis of io^{6}A



Scheme 33: Synthesis of io^{6}A.
Reagents and conditions: a) 2-(Diethoxy-phosphoryl)-propionic acid ethyl ester, ^{n}BuLi, THF, -78 °C to rt, 3 h, 55 %; b) $LiAlH_4$, Et_2O, -78 °C to rt, 2 h, 75 %; c) 2 M HCl, rt, 4 h, 94 %, d) **14**, Et_3N, MeOH, 60 °C, 48 h, 28 %.

10.7.5.1 *Z*- and *E*-4-*tert*-butoxycarbonylamino-2-methyl-but-2-enoic acid ethyl ester (16).[249]



^{n}BuLi (2.5 M, 2.64 mL, 6.60 mmol, 1.05 eq) was added dropwise to 2-(diethoxy-phosphoryl)-propionic acid ethyl ester **15** (1.01 mL, 6.60 mmol, 1.05 eq) in dry THF (30.0 mL) at rt. The resulting solution was cooled to -78 °C and (2-Oxo-ethyl)-carbamic acid ^{t}butyl ester (1.00 g, 6.28 mmol, 1.00 eq) in dry THF (30.0 mL) was added dropwise at -78 °C. The mixture was stirred at -78 °C for 3 h. The reaction was allowed to warm to rt and H_2O (40.0 mL) and sat.

NH_4Cl solution (40.0 mL) was added carefully. The organic layer was collected. The aq. layer was extracted with DCM (50.0 mL) twice. The combined organic layers were extracted with brine (50.0 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* and the resulting oil was purified by column chromatography (Isohexane/ethyl acetate 3/2) to yield ester **16** (698 mg, 46 %) and its *E*-derivative (136 mg, 9 %).

Z-alkene (**16**):

R_f = 0.66 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 6.64 – 6.60 (m, 1H; $CH_2CHC(CH_3)CO_2Et$), 4.70 - 4.59 (m, 1H, HN), 4.17 (q, $^3J(CO_2CH_2CH_3,CO_2CH_2CH_3)$=7.1 Hz, 2H; $CO_2CH_2CH_3$), 3.94 – 3.82 (m, 2H; $CH_2CHC(CH_3)CO_2Et$), 1.88 – 1.80 (m, 3H; $CH_2CHC(CH_3)CO_2Et$), 1.43 (s, 9H; $C(CH_3)_3$), and 1.27 ppm (t, $^3J(CO_2CH_2CH_3,CO_2CH_2CH_3)$=7.1 Hz, 3H; $CO_2CH_2CH_3$);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 171.34 ($CO_2C(CH_3)_3$), 167.77 (CO_2Et), 155.92 ($CH_2CHC(CH_3)CO_2Et$), 138.03 ($CH_2CHC(CH_3)CO_2Et$), 129.80 ($CH_2CHC(CH_3)CO_2Et$), 79.91 ($C(CH_3)_3$), 60.95 ($CO_2CH_2CH_3$), 28.60 ($C(CH_3)_3$), 14.46 ($COOCH_2CH_3$), and 12.78 ppm ($CHCCH_3COOEt$);

HR-MS (ESI+) for $[C_{12}H_{25}N_2O_4]^+$: calc.: 261.1809, found: 261.1802.

E-alkene:

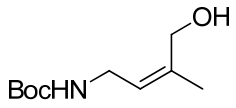
R_f = 0.61 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 5.98 – 5.84 (m, 1H; $CH_2CHC(CH_3)CO_2Et$), 5.13 - 4.96 (m, 1H, HN), 4.16 (q, $^3J(CO_2CH_2CH_3,CO_2CH_2CH_3)$=7.1 Hz, 2H; $CO_2CH_2CH_3$), 4.04 – 3.92 (m, 2H; $CH_2CHC(CH_3)CO_2Et$), 1.82 – 1.78 (m, 3H; $CHCCH_3CO_2Et$), 1.34 (s, 9H; $C(CH_3)_3$), and 1.25 ppm (t, $^3J(CO_2CH_2CH_3,CO_2CH_2CH_3)$=7.1 Hz, 3H; $CO_2CH_2CH_3$);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 167.66 ($CO_2C(CH_3)_3$), 167.48 (CO_2Et), 156.09 ($CH_2CHC(CH_3)CO_2Et$), 140.45 ($CH_2CHC(CH_3)CO_2Et$), 129.06 ($CH_2CHC(CH_3)CO_2Et$), 79.39 ($C(CH_3)_3$), 60.78 ($CO_2CH_2CH_3$), 28.49 ($C(CH_3)_3$), 20.19 ($CH_2CHC(CH_3)CO_2Et$), and 14.46 ppm ($CO_2CH_2CH_3$);

HR-MS (ESI+) for $[C_{12}H_{22}NO_4]^+$: calc.: 244.1543, found: 244.1536.

10.7.5.2 (*Z*)-(4-Hydroxy-3-methyl-but-2-enyl)-carbamic acid ^{t}butyl ester (17).[184, 249b, 250]

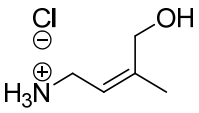


Alkene **16** (500 mg, 2.06 mmol, 1.00 eq) in dry Et_2O (2.50 mL) was cooled to -78 °C. $LiAlH_4$ (1 M, 2.26 mL, 2.26 mmol, 1.10 eq) was added dropwise. The mixture was stirred at -78 °C for 2 h, allowed to warm to rt and afterwards carefully treated with sat. NH_4Cl solution (10.0 mL) and water (10.0 mL). Et_2O (20.0 mL) was added and the layers were separated. The aq. layer was extracted with Et_2O (20.0 mL) twice. The combined organic layers were extracted with brine (30.0 mL) and dried over $MgSO_4$. Removal of the solvent *in vacuo* (> 300 mbar) yielded crude alcohol **17** (331 mg, 75 %) as colorless oil.

R_f = 0.21 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (200 MHz, $CDCl_3$, 27 °C): δ = 5.32 – 5.20 (m, 1H; CH), 4.14 – 4.07 (m, 2H, CH_2O), 3.77 – 3.67 (m, 2H; CH_2N), 1.84 – 1.77 (m, 3H, CH_3), and 1.41 ppm (s, 9H, $C(CH_3)_3$).

10.7.5.3 (*Z*)-4-Ammonium-2-methyl-but-2-en-1-ol chloride (14).[249b]



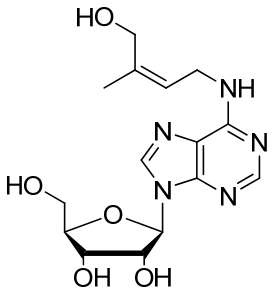
Crude **17** (310 mg, 1.55 mmol) in aq. HCl (2 M, 10.0 mL) was stirred at rt for 4 h. The solvent was removed *in vacuo*. The resulting oil was treated with MeOH (10.0 mL) and the solvent was removed *in vacuo*. This coevaporation was repeated once. The resulting red solid was dried under high vacuum for 16 h yielding crude **14** (206 mg, 94 %).

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.48 – 5.40 (m, 1H; CH), 4.18 – 4.15 (m, 2H; CH_2O), 3.69 – 3.64 (m, 2H; CH_2N), and 1.88 – 1.85 ppm (m, 3H; CH_3);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 145.38 (C_q), 119.02 (CH), 62.01 (CH_2O), 37.81 (CH_2N), and 21.95 ppm (CH_3);

HR-MS (EI+) for $[C_5H_9NO]^{\bullet+}$: calc.: 99.0679, found: 99.0673.

10.7.5.4 6-[(*Z*)-4-Amino-2-methyl-but-2-en-1-ol]-9-(*β*-D-ribofuranosyl)-9*H*-purine (io^{6}A).[184]



Nucleoside **6** (195 mg, 0.43 mmol, 1.00 eq) in MeOH (11.7 mL) was treated with amine **14** (332 mg, 3.29 mmol, 7.70 eq) and Et_3N (1.37 mL, 9.90 mmol, 23.0 eq). The mixture was stirred at 60 °C for 48 h and subsequently all volatile components were removed *in vacuo*. A portion (100 mg) of the crude product (723 mg) was purified *via* HPL chromatography to yield io^{6}A (6 mg, 28 %) as white solid.

R_f = 0.48 (DCM/MeOH 4/1);

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.25 (s, 1H; HC8), 8.22 (s, 1H; HC2), 5.95 (d, $^3J(HC_{1'},HC_{2'})$=6.5 Hz, 1H; $HC_{1'}$), 5.51 – 5.45 (m, 1H; $CH_2CHC(CH_3)CH_2OH$), 4.74 (dd, $^3J(HC_{2'},HC_{3'})$=5.0 Hz, $^3J(HC_{2'},HC_{1'})$=6.5 Hz, 1H; $HC_{2'}$), 4.32 (dd, $^3J(HC_{3'},HC_{2'})$=5.0 Hz, $^3J(HC_{3'},HC_{4'})$=2.4 Hz, 1H; $HC_{3'}$), 4.30 – 4.23 (m, 2H; $CH_2CHC(CH_3)CH_2OH$), 4.22 – 4.21 (m, 2H; $CH_2CHC(CH_3)CH_2OH$), 4.18 – 4.15 (m, 1H; $HC_{4'}$), 3.88 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.7 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.4 Hz, 1H; $H_aC_{5'}$), 3.74 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.7 Hz, $^3J(H_bC_{5'},HC_{4'})$=2.4 Hz, 1H; $H_bC_{5'}$), and 1.83 ppm (m, 3H; $CH_2CHC(CH_3)CH_2OH$);

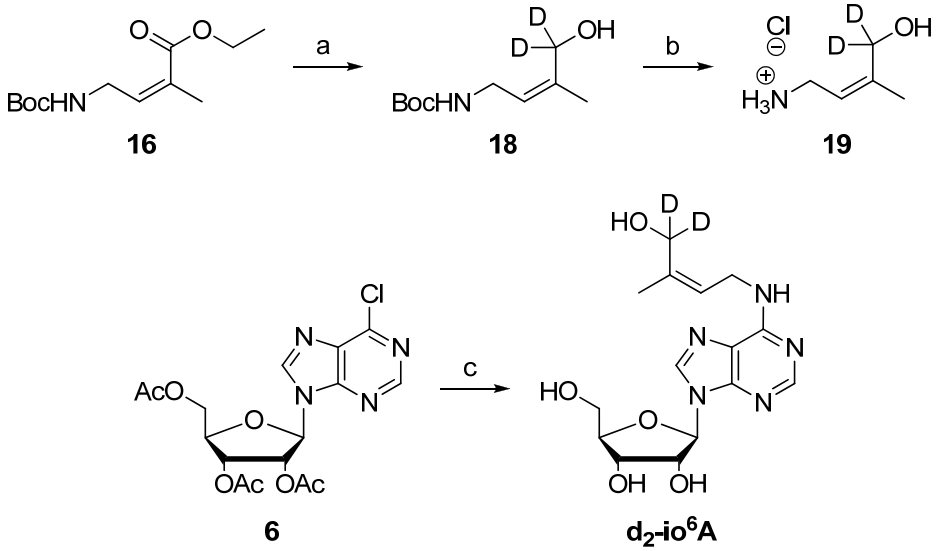
^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 156.13 (C6), 153.38 (C2), 143.77 ($CH_2CHCCH_3CH_2OH$), 141.55 (C8), 91.29 ($C_{1'}$), 88.22 ($C_{4'}$), 75.45 ($C_{2'}$), 72.71 ($C_{3'}$), 63.52 ($C_{5'}$), 61.60 ($CH_2CHCCH_3CH_2OH$), 30.83 ($CH_2CHC(CH_3)CH_2OH$), and 21.83 ppm

($CH_2CHC(CH_3)CH_2OH$); Quaternary carbons C4 and C5 cannot be assigned unambiguously due to the small amount of material available for NMR analysis.

HR-MS (ESI+) for $[C_{15}H_{21}N_5O_5Na]^+$: calc.: 374.1440, found: 374.1435;

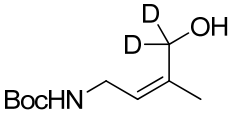
HPLC: gradient: 100 % H_2O, 0 % MeCN → 60 % H_2O, 40 % MeCN in 45 min; retention time = 27.6 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.7.6 Synthesis of d_2-io^{6}A



Scheme 34: Synthesis of d_2-io^{6}A.
Reagents and conditions: a) $LiAlD_4$, Et_2O, -78 °C to rt, 3 h, 87 %; b) 2 M HCl, 60 °C, 30 min, 73 %, c) **19**, Et_3N, MeOH, 60 °C, 16 h, 13 %.

10.7.6.1 (*Z*)-(4-Dideutero-4-hydroxy-3-methyl-but-2-enyl)-carbamic acid ^{t}butyl ester (18).



Ester **16** (2.50 g, 10.3 mmol, 1.00 eq) in dry Et_2O (30.0 mL) was cooled to -78 °C. $LiAlD_4$ (0.95 mg, 22.6 mmol, 2.20 eq) was added. The mixture was stirred at -78 °C for 3 h, allowed to warm to rt and treated with sat. NH_4Cl solution (30.0 mL) and H_2O (30.0 mL). Et_2O (40.0 mL) was added and the layers were separated. The aq. layer was extracted with Et_2O

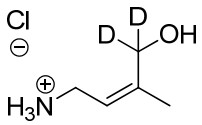
(30.0 mL) twice. The combined organic layers were extracted with brine (50.0 mL) and dried over $MgSO_4$. Removal of the solvent *in vacuo* (> 300 mbar) yielded crude **18** (1.79 g, 87 %) as colorless oil.

R_f = 0.21 (Isohexane/ethyl acetate 1/1);

^{1}H NMR (300 MHz, $CDCl_3$, 27 °C): δ = 5.28 – 5.18 (m, 1H; CH), 3.72 – 3.64 (m, 2H; CH_2N), 1.80 – 1.76 (m, 3H, CH_3), and 1.39 ppm (s, 9H, $C(CH_3)_3$);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 156.44 ($NHCOOC(CH_3)_3$), 139.06 (C_q), 123.88 (CH), 79.88 ($C(CH_3)_3$), 38.00 (CH_2), 28.58 ($C(CH_3)_3$), and 22.13 ppm (CH_3).

10.7.6.2 (*Z*)-4-Amino-1-dideutero-2-methyl-but-2-en-1-ol (19).



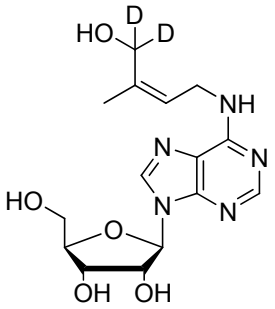
Crude **18** (1.80 g, 8.87 mmol) in aq. HCl (2 M, 50.0 mL) and MeOH (25.0 mL) was stirred at 60 °C for 30 min. The solvent was removed *in vacuo*. The resulting oil was treated with MeOH (10 mL) and the solvent was removed *in vacuo*. This procedure was repeated once. The resulting red solid was dried under high vacuum for 16 h yielding crude **19** (896 mg, 73 %).

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 5.40 – 5.34 (m, 1H; CH), 3.61 – 3.56 (m, 2H; CH_2N), and 1.81 – 1.77 ppm (m, 3H; CH_3);

^{2}D-NMR (61 MHz, CD_3OD, 27 °C): δ = 4.10 ppm (s; CD_2);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 145.30 (C_q), 119.11 (CH), 62.52 (q, $^1J(CD_2,CD_2)$=21 Hz; CD_2), 37.82 (CH_2N), and 21.92 ppm (CH_3).

10.7.6.3 6-[(*Z*)-4-Amino-1-dideutero-2-methyl-but-2-en-1-ol]-9-(*β*-D-ribofuranosyl)-9*H*-purine (d_2-io^{6}A).



Nucleoside **6** (100 mg, 0.24 mmol, 1.0 eq) in MeOH (6.0 mL) was treated with amine **19** (170 mg, 1.22 mmol, 5.1 eq) and Et_3N (0.70 mL, 6.9 mmol, 28.8 eq). The mixture was stirred at 60 °C for 16 h and subsequently all volatile components were removed *in vacuo*. The crude product was purified by HPL chromatography (100 % H_2O, 0 % MeCN → 60 % H_2O, 40 % MeCN in 45 min; retention time: 27.7 min, Nucleosil 120-3-C18) to yield d_2-io^{6}A (11 mg, 13 %) as white solid.

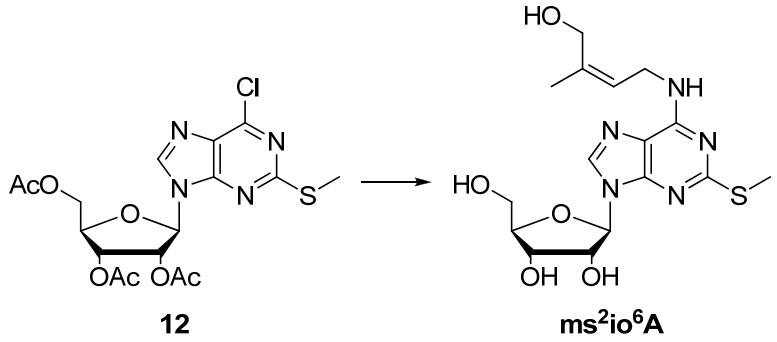
^{1}H-NMR (400 MHz, D_6-DMSO, 25°C): δ = 8.34 (s, 1H; HC8), 8.20 (s, 1H, HC2), 7.87 (s, 1H; HN), 5.88 (d, ^3J($HC_{1'}$,$HC_{2'}$)=6.2 Hz, 1H; $HC_{1'}$), 5.45 – 5.41 (m, 1H; $C_{2'}OH$), 5.40 – 5.31 (m, 2H; $C_{5'}OH$ and $CH_2CHCCH_3CD_2OH$), 5.16 (d, ^3J($C_{3'}OH$,$HC_{3'}$)=4.4 Hz, 1H; $C_{3'}OH$), 4.67 – 4.62 (s, 1H; $CH_2CHCCH_3CD_2OH$), 4.62 – 4.57 (m, 1H; $HC_{2'}$), 4.17 – 4.12 (m, 3H; $HC_{3'}$ and $CH_2CHCCH_3CD_2OH$), 3.99 – 3.93 (m, 1H; $HC_{4'}$), 3.71 - 3.63 (m, 1H; $H_aC_{5'}$), 3.59 – 3.51 (m, 1H; $H_bC_{5'}$), 1.72 – 1.67 ppm (m, 3H; $CH_2CHCCH_3CD_2OH$);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 154.25 (C6), 152.25 (C2), 148.29 (C4), 139.69 ($CH_2CHCCH_3CD_2OH$), 137.67 (C8), 123.12 ($CH_2CHCCH_3CH_2OH$), 119.76 (C5), 87.88 ($C_{1'}$), 85.84 ($C_{4'}$), 73.45 ($C_{2'}$), 70.59 ($C_{3'}$), 61.62 ($C_{5'}$), 59.02 (quint, ^1J(CD_2,CD_2)=21 Hz; CD_2), 36.69 ($CH_2CHCCH_3CD_2OH$), and 21.13 ppm ($CH_2CHC(CH_3)CD_2OH$);

HR-MS (ESI+) for $[C_{15}H_{20}D_2N_5O_5]^+$: calc.: 354.1741, found: 354.1741;

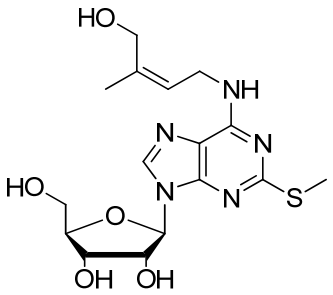
HPLC: gradient: 100 % H_2O, 0 % MeCN → 60 % H_2O, 40 % MeCN in 45 min; retention time = 27.7 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.7.7 Synthesis of ms^2io^6A



Scheme 35: Synthesis of ms^2io^6A.
Reagents and conditions: **14**, Et_3N, MeOH, 60 °C, 24 h, 26 %.

10.7.7.1 6-[(*Z*)-4-Amino-2-methyl-but-2-en-1-ol]-2-methylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine (ms^2io^6A).[190]



Nucleoside **12** (85 mg, 0.19 mmol, 1.00 eq) and amine **14** (150 mg, 1.49 mmol, 8.00 eq) were dissolved MeOH (5.00 mL) and triethylamine (0.50 mL). After stirring at 60 °C for 24 h the solvent was removed *in vacuo*. Recrystallisation from ^{i}PrOH gave the desired product as a white solid. Further purification was performed using HPL chromatography yielding ms^2io^6A (20 mg, 26 %) as white solid.

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.13 (s, 1H; HC8), 5.94 (d, $^3J(HC_{1'},HC_{2'})$=5.9 Hz, 1H; $HC_{1'}$), 5.56 – 5.49 (m, 1H; $CH_2CHC(CH_3)CH_2OH$), 4.73 (dd, $^3J(HC_{2'},HC_{1'})$=5.9 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.36 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.3 Hz, 1H; $HC_{3'}$), 4.34 – 4.26 (m, 2H; $CH_2CHC(CH_3)CH_2OH$), 4.25 – 4.22 (s, 2H; $CH_2CHC(CH_3)CH_2OH$), 4.15 (ddd, $^3J(HC_{4'},HC_{3'})$=3.3 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.8 Hz, 1H; $HC_{4'}$), 3.90 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.8 Hz,

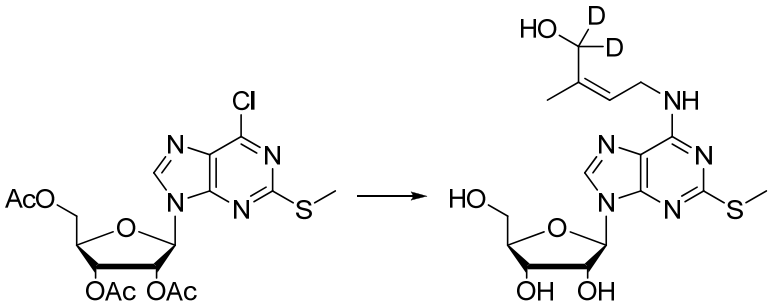
1H; $H_aC_{5'}$), 3.76 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_bC_{5'}$), 2.59 (s, 3H; SCH_3), and 1.89 - 1.84 ppm (m, 3H; $CH_2CHC(CH_3)CH_2OH$);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 167.47 (C2), 155.20 (C6), 150.67 (C4), 140.37 (C8), 139.87 ($CH_2CHC(CH_3)CH_2OH$), 124.75 ($CH_2CHC(CH_3)CH_2OH$), 118.79 (C5), 90.93 ($C_{1'}$), 87.66 ($C_{4'}$), 75.35 ($C_{2'}$), 72.55 and 72.55 ($C_{3'}$ and $CH_2CHC(CH_3)CH_2OH$), 63.48 ($C_{5'}$), 61.72 ($CH_2CHC(CH_3)CH_2OH$), 21.83 ($CH_2CHC(CH_3)CH_2OH$), and 14.74 ppm (SCH_3);

HR-MS (ESI+) for $[C_{16}H_{24}N_5O_5S]^+$: calc.: 398.1493, found: 398.1485;

HPLC: gradient: 100 % H_2O, 0 % MeCN → 60 % H_2O, 40 % MeCN in 5 min and 40 % H_2O, 60 % MeCN in 40 min; retention time = 39.4 min; flow: 0.5 mL/min; column: CC 250/4 Nucleodur 120-3-C18 ec.

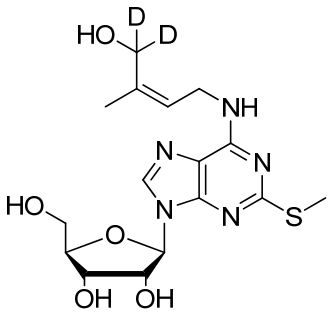
10.7.8 Synthesis of d_2-ms^2io^6A



12 **d_2-ms^2io^6A**

Scheme 36: Synthesis of d_2-ms^2io^6A.
Reagents and conditions: **19**, Et_3N, MeOH, 60 °C, 16 h, 2 %.

10.7.8.1 6-[(*Z*)-4-Amino-1-dideutero-2-methyl-but-2-en-1-ol]-2-methylthio-9-(*β*-D-ribofuranosyl)-9H-purine (d_2-ms^2io^6A).



Nucleoside **12** (100 mg, 0.24 mmol, 1.00 eq) and amine **19** (170 mg, 6.90 mmol, 28.8 eq) were dissolved in MeOH (6.00 mL) and triethylamine (0.70 mL). After stirring at 60 °C for 16 h the solvent was removed *in vacuo*. Purification was performed using HPL chromatography to yield d_2-ms^{2}io^{6}A (2 mg, 2 %) as white solid. ^{13}C NMR analysis could not be performed, because d_2-ms^{2}io^{6}A was obtained in insufficient amounts.

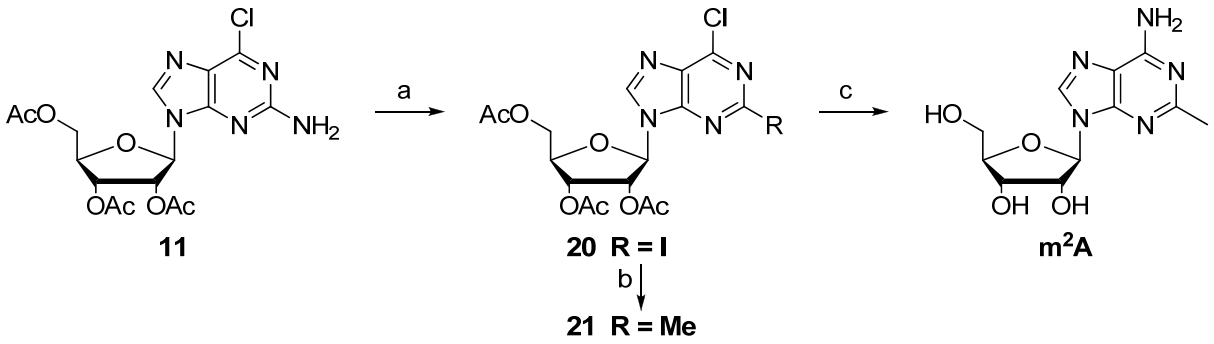
^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.08 (s, 1H; HC8), 6.09 – 5.79 (m, 1H; $HC_{1'}$), 5.56 – 5.47 (m, 1H; $CH_2CHC(CH_3)CH_2OH$), 4.74 – 4.75 (m, 1H; $HC_{2'}$), 4.42 – 4.21 (m, 3H; $HC_{3'}$ and $CH_2CHC(CH_3)CH_2OH$), 4.17 – 4.09 (m, 1H; $HC_{4'}$), 3.91 – 3.82 (m, 1H; $H_aC_{5'}$), 3.79 – 3.70 (m, 1H; $H_bC_{5'}$), 2.57 (s, 3H; SCH_3), and 1.85 – 1.77 ppm (m, 3H; $CH_2CHC(CH_3)CH_2OH$);

HR-MS (ESI+) for $[C_{16}H_{22}D_2N_5O_5S]^+$: calc.: 400.1618, found: 400.1618;

HPLC: gradient: 100 % H_2O, 0 % MeCN → 60 % H_2O, 40 % MeCN in 5 min and 40 % H_2O, 60 % MeCN in 40 min; retention time = 39.4 min; flow: 0.5 mL/min; column: CC 250/4 Nucleodur 120-3-C18 ec.

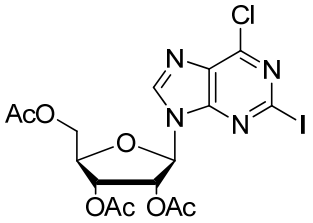
10.8 Syntheses of methyl-adenosines

10.8.1 Synthesis of m^{2}A



Scheme 37: Synthesis of m^{2}A.
Reagents and conditions: a) I_2, CH_2I_2, CuI, *N*-pentyl-nitrite, THF, 70 °C, 2 h, 61 %; b) $ZnCl_2$, MeMgCl, $Pd(PPh_3)_4$, THF, 0 °C to rt, 4 h, 86 %; c) NH_3, rt, 36 h, 71 %.

10.8.1.1 6-Chloro-2-iodo-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (20).[251]



Nucleoside **11** (430 mg, 1.00 mmol, 1.00 eq), I_2 (254 mg, 1.00 mmol, 1.00 eq), CH_2I_2 (0.81 mL, 10.0 mmol, 10.0 eq) and CuI (200 mg, 1.05 mmol, 1.05 eq) were dissolved in dry THF (5.00 mL). *N*-pentyl-nitrite (0.40 mL, 3.00 mmol, 3.00 eq) was added and the mixture was stirred at 70 °C for 2 h. The solvent was removed *in vacuo* and the resulting oil was purified *via* column chromatography (DCM/MeOH 200/1) yielding iodo nucleoside **20** (330 mg, 61 %) as yellow foam.

R_f = 0.56 (DCM/MeOH 16/1);

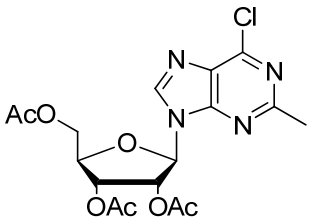
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 8.81 (s, 1H; HC8), 6.29 (d, $^3J(HC_{1'},HC_{2'})$=5.0 Hz, 1H; $HC_{1'}$), 6.04 (dd, $^3J(HC_{2'},HC_{3'})$=5.4 Hz, $^3J(HC_{2'},HC_{1'})$=5.0 Hz, 1H; $HC_{2'}$), 5.64 (dd, $^3J(HC_{3'},HC_{2'})$=5.4 Hz, $^3J(HC_{3'},HC_{4'})$=5.3 Hz, 1H; $HC_{3'}$), 4.43 (ddd, $^3J(HC_{4'},HC_{3'})$=5.3 Hz, $^3J(HC_{4'},H_bC_{5'})$=5.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.7 Hz, 1H; $HC_{4'}$), 4.40 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.0 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.7 Hz, 1H; $H_aC_{5'}$), 4.29 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.0 Hz, $^3J(H_bC_{5'},HC_{4'})$=5.2 Hz, 1H; $H_bC_{5'}$), 2.12 (s, 3H; H_3CCO), 2.06 (s, 3H; H_3CCO), and 2.01 ppm (s, 3H; H_3CCO);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 169.94 (CH_3CO), 169.28 (CH_3CO), 169.16 (CH_3CO), 152.15 and 148.96 (C6 and C4), 145.93 (C8), 131.63 (C5), 118.33 (C2), 86.21 ($C_{1'}$), 79.83 ($C_{4'}$), 72.45 ($C_{2'}$), 68.0 ($C_{3'}$), 62.59 ($C_{5'}$), 20.54 (CH_3CO), 20.33 (CH_3CO), and 20.20 ppm (CH_3CO);

IR: 2959 *w* (C-H valence), 1740 *s* (C=O valence), 1584 *m* (N-H deform.), 1551 *m* (N-H deform.), 1335 *m* (C-N valence), and 1205 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{16}H_{17}ClIN_4O_7]^+$: calc.: 538.9825, found: 538.9830.

10.8.1.2 6-Chloro-2-methyl-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (21).[252]



$ZnCl_2$ (1.0 M in diethyl ether, 0.28 mL, 0.28 mmol, 1.50 eq) was cooled to 0 °C and slowly treated with MeMgCl (2.56 M in THF, 0.22 mL, 0.56 mmol, 3.00 eq). The suspension was stirred for 2 h at 0 °C. Nucleoside **20** (100 mg, 0.19 mmol, 1.00 eq) and $Pd(PPh_3)_4$ (22 mg, 0.02 mmol, 0.10 eq) were dissolved in dry THF (1.00 mL) and cooled to 0 °C. The suspension was diluted with dry THF (1.00 mL) and added to the nucleoside solution. The resulting mixture was stirred at 0 °C for 10 min, then allowed to warm to rt and stirred at this temperature for 2 h. Saturated NaH_2PO_4 solution (10.0 mL) was added to the reaction mixture. The organic phase was collected and the aqueous phase was extracted with DCM (15.0 mL) three times. The combined organic phases were washed with brine (30.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography (DCM/MeOH 200/1) yielded nucleoside **21** (68 mg, 86 %) as white foam.

R_f = 0.39 (DCM/MeOH 18/1);

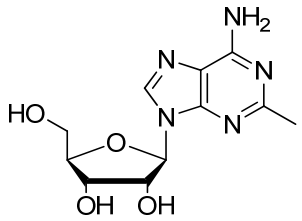
^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 8.15 (s, 1H; HC8), 6.15 (d, $^3J(HC_{1'},HC_{2'})$=5.0 Hz, 1H; $HC_{1'}$), 5.93 (dd, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, $^3J(HC_{2'},HC_{1'})$=5.0 Hz, 1H; $HC_{2'}$), 5.69 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=4.9 Hz, 1H; $HC_{3'}$), 4.44 (ddd, $^3J(HC_{4'},HC_{3'})$=4.9 Hz, $^3J(HC_{4'},H_bC_{5'})$=4.6 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.4 Hz, 1H; $HC_{4'}$), 4.42 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.2 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.4 Hz, 1H; $H_aC_{5'}$), 4.35 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.2 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.6 Hz, 1H; $H_bC_{5'}$), 2.78 (s, 3H; H_3C), 2.13 (s, 3H; H_3CCO), and 2.07 ppm (s, 6H; H_3CCO);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 170.43 (CH_3CO), 169.71 (CH_3CO), 169.51 (CH_3CO), 163.30 (C2), 151.92 (C6), 151.22 (C4), 143.09 (C8), 130.31 (C5), 86.99 ($C_{1'}$), 80.57 ($C_{4'}$), 73.28 ($C_{2'}$), 70.69 ($C_{3'}$), 63.17 ($C_{5'}$), 25.91 (CH_3), 20.91 (CH_3CO), 20.73 (CH_3CO), and 20.59 ppm (CH_3CO);

IR: 2957 *w* (C-H valence), 2925 *w* (C-H valence), 1744 *s* (C=O valence), 1399 *m* (CH_3 deform.), 1368 *m* (CH_3 deform.), and 1207 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{17}H_{20}ClN_4O_7]^+$: calc.: 427.1015, found:427.1009.

10.8.1.3 6-Amino-2-methyl-9-(*β*-D-ribofuranosyl)-9*H*-purine (m^2A).[196-197]



Ammonia (2.00 mL) was condensed into a pressurisable tube equipped with nucleoside **21** (1.50 g, 3.52 mmol). The tube was sealed and allowed to warm to rt. After stirring for 36 h the ammonia was removed. The resulting orange oil was dissolved in methanol and subsequently dried *in vacuo*. Addition of methanol and removal of the solvent were repeated twice. Purification *via* column chromatography (DCM/MeOH 10/1) yielded m^2A (704 mg, 71 %) as white solid.

R_f = 0.13 (DCM/MeOH 8/1);

M.p.: 128.0 – 128.6 °C;

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 8.24 (s, 1H; HC8), 7.28 (s, 2H; NH_2), 5.83 (d, $^3J(HC_{1'},HC_{2'})$=6.8, 1H; $HC_{1'}$), 5.79 (dd, $^3J(C_{5'}OH,H_bC_{5'})$=8.1 Hz, $^3J(C_{5'}OH,H_aC_{5'})$=3.3 Hz, 1H; $C_{5'}OH$), 5.41 (d, $^3J(C_{2'}OH,HC_{2'})$=6.1 Hz, 1H; $C_{2'}OH$), 5.19 (d, $^3J(C_{3'}OH,HC_{3'})$=3.4 Hz, 1H; $C_{3'}OH$), 4.63 (dd, $^3J(HC_{2'}, HC_{1'})$=6.8 Hz, $^3J(HC_{2'},C_{2'}OH)$=6.1 Hz, 1H; $HC_{2'}$), 4.14 – 4.09 (m, 1H; $HC_{3'}$), 4.00 – 3.95 (m, 1H; $HC_{4'}$), 3.70 – 3.64 (m, 1H; $H_aC_{5'}$), 3.60 – 3.51 (m, 1H; $H_bC_{5'}$), and 2.38 ppm (s, 3H; CH_3);

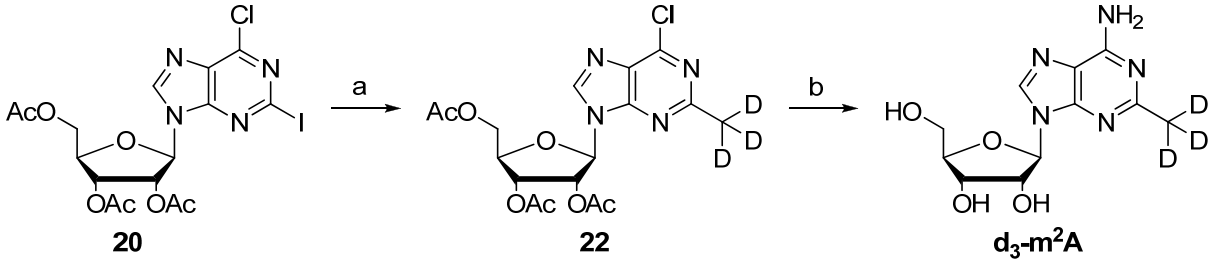
^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 161.02 (C2), 155.83 (C4), 149.57 (C6), 139.66 (C8), 117.68 (C5), 88.07 ($C_{1'}$), 86.29 ($C_{4'}$), 73.21 ($C_{2'}$), 70.99 ($C_{3'}$), 61.96 ($C_{5'}$), and 25.11 ppm (CH_3);

IR: 3326 *s* (N-H valence), 3182 *bs* (O-H valence, N-H valence), 2925 *m* (C-H valence), 1645 *s* (N-H deform.), 1599 *s* (N-H deform., aromatic vibration), 1584 *s* (N-H deform.), 1402 *s* (C-H deform.), 1333 *m* (C-N valence), and 1084 cm^{-1} *m* (C-O valence);

HR-MS (ESI+) for $[C_{11}H_{16}N_5O_4]^+$: calc.: 282.1196, found: 282.1195;

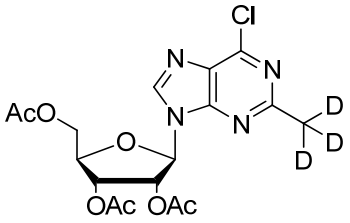
HPLC: gradient: 100 % H_2O, 0 % MeCN → 0 % H_2O, 100 % MeCN in 45 min; retention time = 13.7 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.8.2 Synthesis of d_3-m^2A



Scheme 38: Synthesis of d_3-m^2A.
Reagents and conditions: a) $ZnCl_2$, d_3-MeMgCl, $Pd(PPh_3)_4$, THF, 0 °C to rt, 5 h, 25 %; e) NH_3, rt, 36 h, 6 %.

10.8.2.1 6-Chloro-2-trideuteromethyl-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (22).



$ZnCl_2$ (1.0 M in diethyl ether, 2.80 mL, 2.80 mmol, 1.50 eq) was cooled to 0 °C and slowly treated with d_3-MeMgCl (1.0 M in THF, 5.58 mL, 5.58 mmol, 3.00 eq). The suspension was diluted with dry THF (10.0 mL) and stirred for 2 h at 0 °C. Nucleoside **20** (1.00 g, 1.86 mmol, 1.00 eq) and $Pd(PPh_3)_4$ (22 mg, 0.02 mmol, 0.01 eq) were dissolved in dry THF (15.0 mL) and cooled to 0 °C. The suspension was added to the nucleoside solution. The resulting mixture was allowed to warm to rt and stirred at this temperature for 3 h. Saturated NaH_2PO_4

solution (50.0 mL) was added to the reaction mixture. The organic phase was collected and the aqueous phase was extracted with DCM (30.0 mL) three times. The combined organic phases were washed with brine (50.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography (DCM/MeOH 200/1 and 150/1) yielded nucleoside **22** (199 mg, 25 %) as white foam.

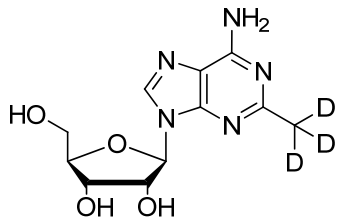
R_f = 0.38 (DCM/MeOH 18/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 8.16 (s, 1H; HC8), 6.16 (d, $^3J(HC_{1'},HC_{2'})$=5.0 Hz, 1H; $HC_{1'}$), 5.93 (dd, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, $^3J(HC_{2'},HC_{1'})$=5.0 Hz, 1H; $HC_{2'}$), 5.69 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=4.9 Hz, 1H; $HC_{3'}$), 4.43 (ddd, $^3J(HC_{4'},HC_{3'})$=4.9 Hz, $^3J(HC_{4'},H_bC_{5'})$=4.6 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.4 Hz, 1H; $HC_{4'}$), 4.42 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.4 Hz, 1H; $H_aC_{5'}$), 4.35 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.6 Hz, 1H; $H_bC_{5'}$), 2.13 (s, 3H; H_3CCO), and 2.07 ppm (s, 6H; H_3CCO);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 170.44 (CH_3CO), 169.73 (CH_3CO), 169.52 (CH_3CO), 163.25 (C2), 151.92 (C6), 151.22 (C4), 143.09 (C8), 130.32 (C5), 86.98 ($C_{1'}$), 80.56 ($C_{4'}$), 73.28 ($C_{2'}$), 70.68 ($C_{3'}$), 63.17 ($C_{5'}$), 25.02 (CD_3), 20.92 (CH_3CO), 20.74 (CH_3CO), and 20.60 ppm (CH_3CO);

HR-MS (ESI+) for $[C_{17}H_{17}D_3ClN_4O_7]^+$: calc.: 430.1203, found: 430.1213.

10.8.2.2 6-Amino-2-trideuteromethyl-9-(β-D-ribofuranosyl)-9*H*-purine (d_3-m^2A).



Ammonia (2.00 mL) was condensed into a pressurisable tube equipped with nucleoside **22** (200 mg, 0.47 mmol). The tube was sealed and allowed to warm to rt. After stirring for 72 h the ammonia was removed. The resulting orange oil was dissolved in methanol and subsequently dried *in vacuo*. Addition of methanol and removal of the solvent were repeated

twice. Purification *via* column chromatography (DCM/MeOH 10/1) yielded d_3-m^2A (8 mg, 6 %) as white foam.

R_f = 0.13 (DCM/MeOH 8/1);

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.21 (s, 1H; HC8), 5.94 (d, $^3J(HC_{1'},HC_{2'})$=6.8, 1H; $HC_{1'}$), 4.80 (dd, $^3J(HC_{2'},HC_{1'})$=6.8 Hz, $^3J(HC_{2'},HC_{3'})$=5.1 Hz, 1H; $HC_{2'}$), 4.35 (dd, $^3J(HC_{3'},HC_{2'})$=5.1 Hz, $^3J(HC_{3'},HC_{4'})$=2.0 Hz, 1H; $HC_{3'}$), 4.21 (dd, $^3J(HC_{4'},H_aC_{5'})$=2.3 Hz, $^3J(HC_{4'},H_bC_{5'})$=2.3 Hz, $^3J(HC_{4'},HC_{3'})$=2.0 Hz, 1H; $HC_{4'}$), 3.93 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.6 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.3 Hz, 1H; $H_aC_{5'}$), and 3.77 ppm (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.6 Hz, $^3J(H_bC_{5'},HC_{4'})$=2.3 Hz, 1H; $H_bC_{5'}$);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 163.50 (C2), 157.38 (C4), 150.68 (C6), 142.02 (C8), 119.43 (C5), 91.71 ($C_{1'}$), 88.67 ($C_{4'}$), 75.33 ($C_{2'}$), 73.12 ($C_{3'}$), and 63.88 ppm ($C_{5'}$);

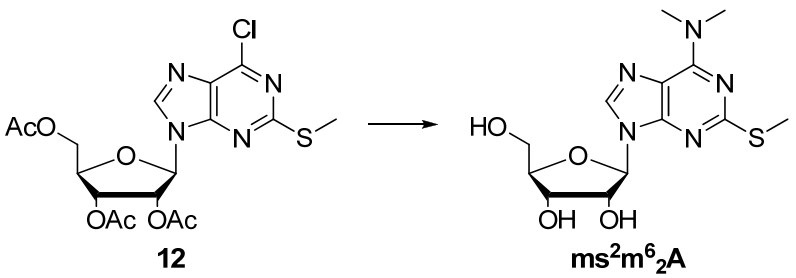
^{2}D-NMR (61 MHz, CD_3OD, 27 °C): δ = 2.45 ppm (CD_3);

HR-MS (ESI+) for $[C_{11}H_{13}D_3N_5O_4]^+$: calc.: 285.1385, found: 285.1388;

HPLC: gradient: 100 % H_2O, 0 % MeCN → 0 % H_2O, 100 % MeCN in 45 min; retention time = 13.7 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

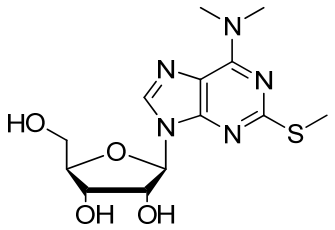
10.9 Syntheses of methylthio-adenosines

10.9.1 Synthesis of $ms^2m^6{}_2A$



Scheme 39: Synthesis of $ms^2m^6{}_2A$.
Reagents and conditions: Me_2NH, 60 °C, 16 h, 67 %.

10.9.1.1 6-Dimethylamino-2-methylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine ($ms^2m^6{}_2A$).[253]



Nucleoside **12** (0.50 g, 1.10 mmol, 1.00 eq) was dissolved in Me_2NH (33 % in EtOH, 2.50 mL, 14 mmol, 12.7 eq) and stirred at 60 °C for 16 h. The solvent was removed *in vacuo*. The resulting oil was dissolved in DCM (20.0 mL). The oil was applied to a silica plug. The silica plug was flushed with DCM (100 mL). The crude product was eluted with DCM/MeOH 20/1. After removal of the solvent *in vacuo* the yellow solid was recrystallized from ^{i}PrOH to yield $ms^2m^6{}_2A$ (251 mg, 67 %) as white solid.

R_f = 0.40 (DCM/MeOH 8/1);

M.p.: 173.3 – 174.3 °C;

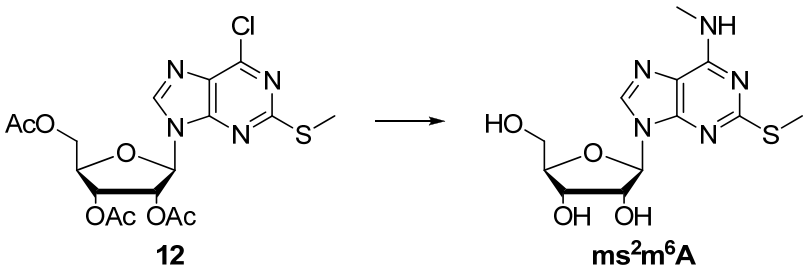
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 8.05 (s, 1H; HC8), 5.89 (d, $^3J(HC_{1'},HC_{2'})$=6.0 Hz, 1H; $HC_{1'}$), 4.72 (dd, $^3J(HC_{2'},HC_{1'})$=6.0 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.31 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.2 Hz, 1H; $HC_{3'}$), 4.15 (ddd, $^3J(HC_{4'},HC_{3'})$=3.2 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.0 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.6 Hz, 1H; $HC_{4'}$), 3.90 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.6 Hz, 1H; $H_aC_{5'}$), 3.76 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.0 Hz, 1H; $H_bC_{5'}$), 3.46 (s, 6H; $N(CH_3)_2$), and 2.53 ppm (s, 3H; SCH_3);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 166.23 (C2), 155.38 (C4), 152.22 (C6), 139.21 (C8), 139.21 ($N(CH_3)_2$), 119.40 (C5), 90.96 ($C_{1'}$), 87.66 ($C_{4'}$), 75.20 ($C_{2'}$), 72.53 ($C_{3'}$), 63.50 ($C_{5'}$), and 14.67 ppm (SCH_3);

IR: 3128 *m* (O-H valence, N-H valence), 2922 *m* (O-H valence, N-H valence), 1582 *s* (N-H deform., aromatic vibration), 1303 *m* (C-N valence), and 1029 cm^{-1} *m* (C-O valence);

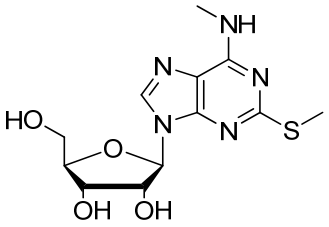
HR-MS (ESI+) for $[C_{13}H_{20}N_5O_4S]^+$: calc.: 342.1231, found: 342.1231.

10.9.2 Synthesis of ms^2m^6A



Scheme 40: Synthesis of ms^2m^6A.
Reagents and conditions: $MeNH_2$, rt, 16 h, 73 %.

10.9.2.1 6-Methylamino-2-methylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine (ms^2m^6A).[253b]



Nucleoside **12** (0.50 g, 1.10 mmol) was dissolved in $MeNH_2$ (33 % in EtOH, 2.50 mL, 20.0 mmol, 18.2 eq) and stirred at rt for 16 h. The solvent was removed *in vacuo*. The resulting oil was purified *via* column chromatography (DCM/MeOH 20/1). After removal of the solvent *in vacuo* the white solid was recrystallized from ^{i}PrOH to yield ms^2m^6A (261 mg, 73 %) as white solid.

R_f = 0.34 (DCM/MeOH 8/1);

M.p.: 110.2 – 111.9 °C;

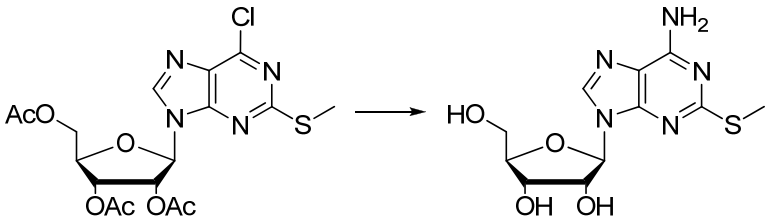
^{1}H NMR (600 MHz, CD_3OD, 25 °C): δ = 8.11 (s, 1H; HC8), 5.94 (d, $^3J(HC_{1'},HC_{2'})$=6.0 Hz, 1H; $HC_{1'}$), 4.78 (dd, $^3J(HC_{2'},HC_{1'})$=6.0 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.36 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{4'},HC_{3'})$=3.2 Hz, 1H; $HC_{3'}$), 4.16 (ddd, $^3J(HC_{4'},HC_{3'})$=3.2 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.8 Hz, 1H; $HC_{4'}$), 3.90 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.8 Hz, 1H; $H_aC_{5'}$), 3.77 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $^3J(H_bC_{5'}, HC_{4'})$=3.2 Hz, 1H; $H_bC_{5'}$), 3.38 (s, 3H; NCH_3), and 2.59 ppm (s, 3H; SCH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 167.48 (C2), 156.08 (C4), 150.44 (C6), 140.26 (C8), 139.21 ($N(CH_3)_2$), 118.88 (C5), 90.95 ($C_{1'}$), 87.68 ($C_{4'}$), 75.31 ($C_{2'}$), 72.54 ($C_{3'}$), 63.50 ($C_{5'}$), 50.00 ($NHCH_3$), and 14.67 ppm (SCH_3);

IR: 3315 *m* (O-H valence, N-H valence), 2925 *m* (O-H valence, N-H valence), 1616 *s* (N-H deform.), 1581 *m* (N-H deform., aromatic vibration), and 1297 cm^{-1} *m* (C-N valence);

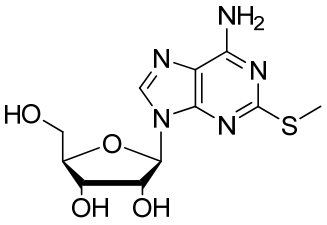
HR-MS (EI+) for $[C_{12}H_{17}N_5O_4S]^{\bullet+}$: calc.: 327.0996, found: 327.0994.

10.9.3 Synthesis of ms^{2}A



Scheme 41: Synthesis of ms^{2}A.
Reagents and conditions: NH_3, rt, 24 h, 81 %.

10.9.3.1 6-Amino-2-methylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine (ms^{2}A).[196, 254]



Nucleoside **12** (3.50 g, 11.2 mmol) was introduced in a pressurisable tube and cooled to -78 °C. Ammonia (5.00 mL) was condensed into the tube. The tube was close and allowed to warm to rt. The solution was stirred at rt for 24 h. The tube was cooled to -78 °C and opened. After removal of the ammonia the resulting oil was dissolved in MeOH. The solvent was removed *in vacuo*. The coevaporation step was repeated once. The resulting oil was dissolved in DCM (20.0 mL). The oil was applied to a silica plug. The silica plug was flushed with DCM (100 mL). The crude product was eluted with DCM/MeOH 10/1. After removal of the

solvent *in vacuo* the yellow solid was recrystallized from MeOH to yield ms^2A (1.95 g, 81 %) as white solid.

R_f = 0.23 (DCM/MeOH 4/1);

M.p.: 227.8 – 228.3 °C;

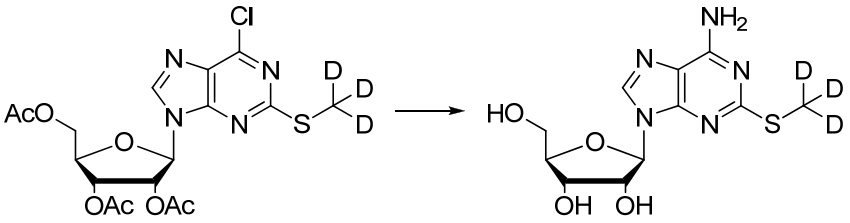
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 8.18 (s, 1H; HC8), 5.94 (d, $^3J(HC_{1'},HC_{2'})$=5.8 Hz, 1H; $HC_{1'}$), 4.75 (dd, $^3J(HC_{2'},HC_{1'})$=5.8 Hz, $^3J(HC_{2'},HC_{3'})$=5.1 Hz, 1H; $HC_{2'}$), 4.34 (dd, $^3J(HC_{3'},HC_{2'})$=5.1 Hz, $^3J(HC_{3'},HC_{4'})$=3.5 Hz, 1H; $HC_{3'}$), 4.12 (ddd, $^3J(HC_{4'},HC_{3'})$=3.5 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.4 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.9 Hz, 1H; $HC_{4'}$), 3.86 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.9 Hz, 1H; $H_aC_{5'}$), 3.74 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.3 Hz, $^3J(H_bC_{5'}, HC_{4'})$=3.4 Hz, 1H; $H_bC_{5'}$), and 2.54 ppm (s, 3H; SCH_3);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 167.56 (C2), 156.96 (C4), 151.53 (C6), 140.81 (C8), 118.33 (C5), 90.79 ($C_{1'}$), 87.50 ($C_{4'}$), 75.38 ($C_{2'}$), 72.42 ($C_{3'}$), 63.39 ($C_{5'}$), and 14.62 ppm (SCH_3);

IR: 3454 *w* (N-H valence), 3302 *m* (N-H valence), 3193 *m* (O-H valence, N-H valence), 3123 *m* (O-H valence, N-H valence), 2911 *m* (C-H valence, O-H valence), 2865 *m* (C-H valence, O-H valence), 2587 *m* (O-H valence), 2491 *m* (O-H valence), 2407 *m* (O-H valence), 1599 *s* (N-H deform., aromatic vibration), 1575 *s* (N-H deform., aromatic vibration), and 1459 cm^{-1} *m* (aromatic vibration);

HR-MS (ESI+) for $[C_{11}H_{16}N_5O_4S]^+$: calc.: 314.0918, found: 314.0919.

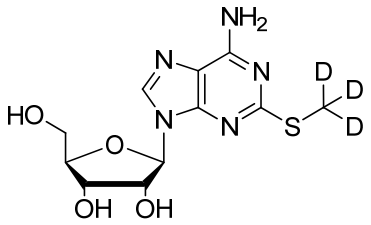
10.9.4 Synthesis of d_3-ms^2A



13 **d_3-ms^2A**

Scheme 42: Synthesis of d_3-ms^2A.
Reagents and conditions: NH_3, rt, 24 h, 17 %.

10.9.4.1 6-Amino-2-trideuteromethylthio-9-(*β*-D-ribofuranosyl)-9*H*-purine (d_3-ms^2A).



Nucleoside **12** (50 mg, 0.11 mmol) in a pressurisable tube was cooled to -78 °C. Ammonia (2.00 mL) was condensed into the tube. The tube was close and allowed to warm to rt. The solution was stirred at rt for 24 h. The tube was cooled to -78 °C and opened. After removal of the ammonia the resulting oil was dissolved in MeOH. The solvent was removed *in vacuo*. The MeOH step was repeated once. The resulting oil was purified by HPL chromatography to yield d_3-ms^2A (6 mg, 17 %) as white solid.

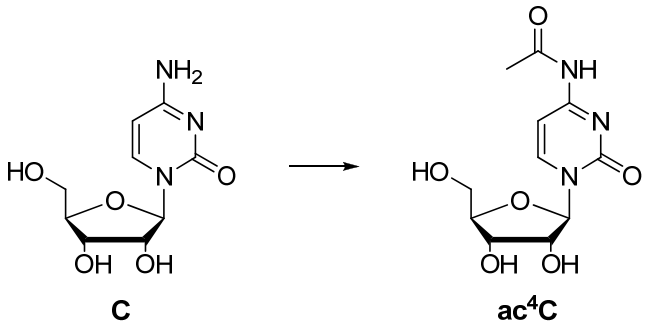
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 8.20 (s, 1H; HC8), 5.96 (d, $^3J(HC_{1'},HC_{2'})$=5.8 Hz, 1H; $HC_{1'}$), 4.77 (dd, $^3J(HC_{2'},HC_{1'})$=5.8 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.37 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.5 Hz, 1H; $HC_{3'}$), 4.17 (ddd, $^3J(HC_{4'},HC_{3'})$=3.4 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.4 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.0 Hz, 1H; $HC_{4'}$), 3.89 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.0 Hz, 1H; $H_aC_{5'}$), and 3.77 ppm (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.3 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.4 Hz, 1H; $H_bC_{5'}$);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 167.54 (C2), 156.95 (C4), 151.52 (C6), 140.81 (C8), 118.32 (C5), 90.78 ($C_{1'}$), 87.50 ($C_{4'}$), 75.37 ($C_{2'}$), 72.42 ($C_{3'}$), and 63.38 ppm ($C_{5'}$);

HR-MS (ESI+) for $[C_{11}H_{13}D_3N_5O_4S]^+$: calc.: 317.1106, found: 317.1109.

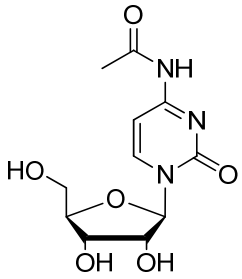
10.10 Syntheses of cytidines

10.10.1 Synthesis of ac^{4}C



Scheme 43: Synthesis of ac^{4}C.
Reagents and conditions: Ac_2O, EtOH, 80 °C, 6 h, 79 %.

10.10.1.1 *N*-[2-Oxo-1-(*β*-D-ribofuranosyl)-1,2-dihydropyrimidin-4-yl]-acetamide (ac^{4}C).[202]



Cytidine (C) (2.00 g, 8.40 mmol, 1.00 eq) and acetic acid anhydride (0.80 mL, 8.40 mmol, 1.00 eq) were refluxed in EtOH (20.0 mL) at 80 °C for 2 h. After this time acetic acid anhydride (0.80 mL, 8.40 mmol, 1.00 eq) was added and the white suspension was refluxed for an additional 4 h. The suspension was filtered hot and the resulting solid was washed with EtOH (10.0 mL). Crude ac^{4}C (1.9 g, 79 %) was obtained and 200 mg of which were further purified by HPL chromatography yielding highly purified ac^{4}C (153 mg, extrapolated: 61 %) as white solid.

R_f = 0.50 (DCM/MeOH 3/1);

M.p.: 194.4 – 195.8 °C;

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.88 (s, 1H; HN), 8.42 (d, ^{3}J(HC6,HC5)=7.5 Hz, 1H; HC6), 7.17 (d, ^{3}J(HC5,HC6)=7.5 Hz, 1H; HC5), 5.77 (d, $^{3}J(HC_{1'},HC_{2'})$=2.7 Hz, 1H; $HC_{1'}$), 5.65 – 5.06 (m, 3H; $C_{5'}OH$, $C_{3'}OH$ and $C_{2'}OH$), 3.99 – 3.93 (m, 2H; $HC_{2'}$ and $HC_{3'}$), 3.91 – 3.87 (m, 1H; $HC_{4'}$), 3.73 (dd, $^{2}J(H_aC_{5'},H_bC_{5'})$=12.3 Hz, $^{3}J(H_aC_{5'},HC_{4'})$=2.7 Hz, 1H; $H_aC_{5'}$), 3.59 (dd, $^{2}J(H_bC_{5'},H_aC_{5'})$=12.3 Hz, $^{3}J(H_bC_{5'},HC_{4'})$=3.0 Hz, 1H; $H_bC_{5'}$), and 2.09 ppm (s, 3H, $COCH_3$);

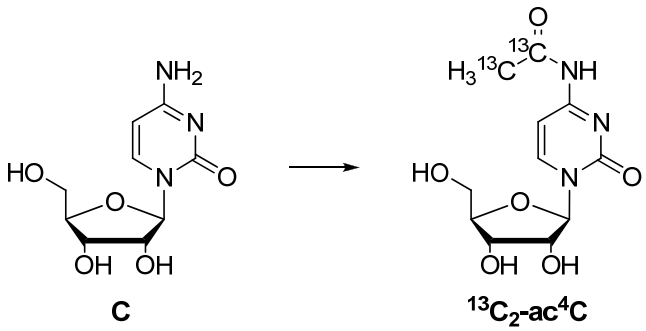
^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 171.06 ($COCH_3$), 162.32 (C4), 154.70 (C2), 145.41 (C6), 95.19 (C5), 90.16 ($C_{1'}$), 84.22 ($C_{4'}$), 74.56 ($C_{2'}$), 68.66 ($C_{3'}$), 59.93 ($C_{5'}$), and 24.40 ppm (CH_3);

IR: 3236 *bm* (O-H valence, N-H valence), 2936 *w* (C-H valence), 1651 *s* (C=O valence), 1536 *m* (N-H deform., C-N valence), 1494 *m* (C=O deform.), 1379 *m* (CH_3 deform.), 1306 *w* (C-N valence), 1101 *m* (C-O-C valence), and 788 cm^{-1} *m* (=C-H deform.);

HR-MS (ESI+) for $[C_{11}H_{15}N_3O_6Na]^+$: calc.: 308.0859, found: 308.0853;

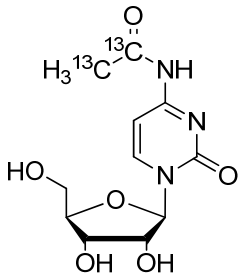
HPLC: gradient: 100 % H_2O, 0 % MeCN → 50 % H_2O, 50 % MeCN in 45 min; retention time = 15.0 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.2 Synthesis of $^{13}C_2$-ac^{4}C



Scheme 44: Synthesis of $^{13}C_2$-ac^{4}C.
Reagents and conditions: $^{13}C_4$-Ac_2O, EtOH, 60 °C, 6.5 h, 13 %.

10.10.2.1 *N*-[2-Oxo-1-(*β*-D-ribofuranosyl)-1,2-dihydropyrimidin-4-yl]-($^{13}C_2$)-acetamide ($^{13}C_2$-ac^{4}C).



Cytidine (559 mg, 2.30 mmol, 1.00 eq) and $^{13}C_4$-acetic acid anhydride (250 mg, 2.30 mmol, 1.00 eq, 99 atom% ^{13}C) were stirred in EtOH (5.70 mL) at 60 °C for 6.5 h. The suspension was filtered hot and dissolved in MeOH. Et_2O was added until a white precipitate appeared. The suspension was kept at 4 °C for 16 h. The solid was collected by filtration and dried *in vacuo*. The white solid was further purified by HPL chromatography yielding $^{13}C_2$-ac^{4}C (86 mg, 13 %) as white solid.

R_f = 0.53 (DCM/MeOH 3/1);

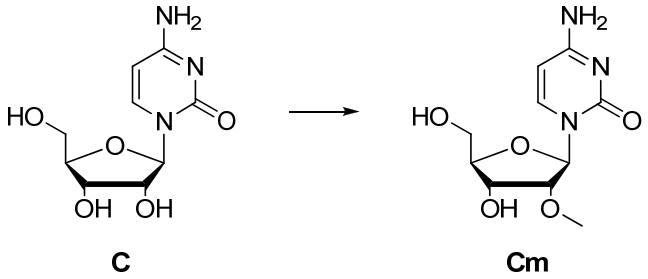
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.88 (s, 1H; HN), 8.42 (d, ^3J(HC6,HC5)=7.5 Hz, 1H; HC6), 7.18 (d, ^3J(HC5,HC6)=7.5 Hz, 1H; HC5), 5.77 (d, ^3J($HC_{1'}$,$HC_{2'}$)=2.8 Hz, 1H; $HC_{1'}$), 5.53 – 5.45 (m, 1H; $C_{2'}OH$), 5.21 – 5.12 (m, 1H; $HOC_{5'}OH$), 5.10 – 5.02 (m, 1H; $HOC_{3'}OH$), 4.00 – 3.86 (m, 3H; $HC_{2'}$, $HC_{3'}$, and $HC_{4'}$), 3.78 – 3.69 (m, ^2J($H_aC_{5'}$,$H_bC_{5'}$)=12.2 Hz, 1H; $H_aC_{5'}$), 3.62 – 3.55 (m, ^2J($H_bC_{5'}$,$H_aC_{5'}$)=12.2 Hz, 1H; $H_bC_{5'}$), and 2.01 ppm (dd, ^1J($^{13}C$$H_3$,$^{13}C$$H_3$)=128.7 Hz, ^2J(^{13}CO,$^{13}C$$H_3$)=6.4 Hz, 3H, $^{13}CO^{13}CH_3$);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 171.06 (d, ^1J(^{13}CO$^{13}CH_3$,$^{13}CO^{13}C$$H_3$)=51.7 Hz, $1^{13}C$; ^{13}CO$^{13}CH_3$), 162.33 (d, ^2J(C4,^{13}CO$^{13}CH_3$)=3.0 Hz, 1C; C4), 154.71 (C2), 145.41 (C6), 95.20 (C5), 90.15 ($C_{1'}$), 84.21 ($C_{4'}$), 74.54 ($C_{2'}$), 68.69 ($C_{3'}$), 59.94 ($C_{5'}$), and 24.36 ppm (d, ^1J($^{13}CO^{13}C$$H_3$,$^{13}CO^{13}CH_3$)=51.7 Hz, $1^{13}C$; $^{13}CH_3$);

HR-MS (ESI+) for $[C_9{}^{13}C_2H_{15}N_3O_6Na]^+$: calc.: 310.0926, found: 310.0920;

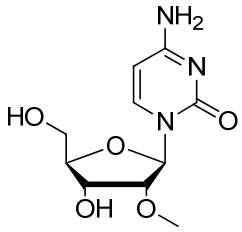
HPLC: gradient: 100 % H_2O, 0 % MeCN → 50 % H_2O, 50 % MeCN in 45 min; retention time = 14.9 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.3 Synthesis of Cm



Scheme 45: Synthesis of Cm.
Reagents and conditions: NaH, MeI, DMF, 0 °C to rt, 4.5 h, 16 %.

10.10.3.1 4-Amino-1-(2'-*O*-methyl-*β*-D-ribofuranosyl)-pyrimidin-2-1H-one (Cm).[209]



Cytidine (5.00 g, 20.6 mmol, 1.00 eq) was suspended in dry DMF (83 mL) at 0 °C. NaH (60 % in mineral oil, 1.00 g, 24.7 mmol, 1.20 eq) was added carefully. The mixture was stirred at 0 °C for 1 h. MeI (1.32 mL, 21.2 mmol, 1.03 eq) was added and the white suspension was stirred for 2.5 h at 0 °C. The suspension was allowed to warm to rt and stirred for 1 h. The solution was filtered and the solvent was removed *in vacuo*. The resulting oil was dissolved in MeOH (20.0 mL). The solvent was removed *in vacuo*. The MeOH treatment was repeated twice. Silica and MeOH (20.0 mL) were added and the solvent removed *in vacuo*. The resulting solid was treated with MeOH (20.0 mL) and dried *in vacuo* twice. The dried mixture was applied to column chromatography (DCM/MeOH 20/1 to 10/1). The fractions containing the desired product were dried *in vacuo*. The resulting solid was recrystallized from MeOH to yield Cm (846 mg, 16 %) as white solid.

R_f = 0.46 (DCM/MeOH 2/1);

M.p.: 255.3 – 257.2 °C;

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 7.90 (d, ^3J(HC6,HC5)=7.5 Hz, 1H; HC6), 7.20

(s, 2H; NH_2), 5.85 (d, $^3J(HC_{1'},HC_{2'})$=4.0 Hz, 1H; $HC_{1'}$), 5.71 (d, ^3J(HC5,HC6)=7.5 Hz, 1H; HC5), 5.10 (t, $^3J(C_{5'}OH,H_2C_{5'})$=5.2 Hz, 1H; $C_{5'}OH$), 5.06 (d, $^3J(C_{3'}OH,HC_{3'})$=6.3 Hz, 1H; $C_{3'}OH$), 4.05 (ddd, $^3J(HC_{3'},C_{3'}OH)$=6.3 Hz, $^3J(HC_{3'},HC_{4'})$=5.9 Hz, $^3J(HC_{3'},HC_{2'})$=5.1 Hz, 1H; $HC_{3'}$), 3.81 (ddd, $^3J(HC_{4'},HC_{3'})$=5.9 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.0 Hz 1H; $HC_{4'}$), 3.70 – 3.62 (m, 2H; $HC_{2'}$ and $H_aC_{5'}$), 3.55 (ddd, $^2J(H_bC_{5'},H_aC_{5'})$=12.1 Hz, $^3J(H_bC_{5'},C_{5'}OH)$=5.2 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_bC_{5'}$), and 3.38 ppm (s, 3H; $C_{2'}OCH_3$);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ=165.63 (C4), 155.13 (C2), 141.15 (C6), 93.96 (C5), 87.01 ($C_{1'}$), 84.19 ($C_{4'}$), 83.28 ($C_{2'}$), 68.08 ($C_{3'}$), 60.24 ($C_{5'}$), and 57.57 ppm (CH_3);

IR: 3322 *bs* (O-H valence), 3187 *bm* (N-H valence), 3063 *w* (C-H valence), 2903 *w* (C-H valence), 1642 *s* (C=O valence), 1611 *s* (C=O valence), 1496 *s* (N-H deform.), 1201 *m* (C-O-C valence), 1075 *m* (C-O-C valence), and 786 cm^{-1} *w* (C-H deform.);

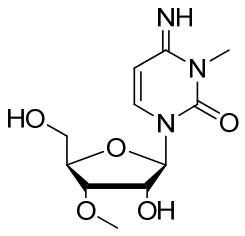
HR-MS (ESI-) for $[C_{10}H_{15}N_3O_5]^-$: calc.: 256.0939, found: 256.0936;

HPLC: gradient: 100 % H_2O, 0 % MeCN → 92 % H_2O, 8 % MeCN in 45 min; retention time = 22.1 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.4 Side products of the Synthesis of Cm

The side products of the reaction towards Cm (Chapter 10.10.3.1) were isolated *via* HPL chromatography (HPLC buffer E: 0.1 M triethylamine/acetic acid in water, HPLC buffer F: 0.1 M triethylamine/acetic acid in 20 % water and 80 % MeCN).

10.10.4.1 4-Imino-3-methyl-1-(2'-*O*-methyl-*β*-D-ribofuranosyl)-3,4-dihydropyrimidin-2(1*H*)-one (23).



R_f = 0.60 (DCM/MeOH 3/1);

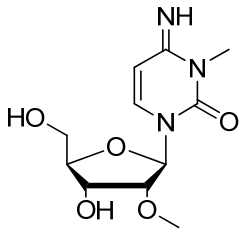
^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.25 (d, ^{3}J(HC6,HC5)=8.0 Hz, 1H; HC6), 6.09 (d, ^{3}J(HC5,HC6)=8.0 Hz, 1H; HC5), 5.86 (d, $^{3}J(HC_{1'},HC_{2'})$=3.4 Hz, 1H; $HC_{1'}$), 4.35 (dd, $^{3}J(HC_{2'},HC_{3'})$=4.8 Hz, $^{3}J(HC_{1'},HC_{2'})$=3.4 Hz, 1H; $HC_{2'}$), 4.13 (ddd, $^{3}J(HC_{4'},HC_{3'})$=6.2 Hz, $^{3}J(HC_{4'},H_aC_{5'})$=2.6 Hz, $^{3}J(HC_{4'},H_bC_{5'})$=2.6 Hz, 1H; $HC_{4'}$), 3.91 (dd, $^{2}J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $^{3}J(H_aC_{5'},HC_{4'})$=2.6 Hz, 1H; $H_aC_{5'}$), 3.83 (dd, $^{3}J(HC_{3'},HC_{4'})$=6.2 Hz, $^{3}J(HC_{3'},HC_{2'})$=4.8 Hz, 1H; $HC_{3'}$), 3.73 (dd, $^{2}J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $^{3}J(H_bC_{5'},HC_{4'})$=2.6 Hz, 1H; $H_bC_{5'}$), and 3.45 ppm (2 s, 6H; $C_{3'}OCH_3$, N3CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 161.02 (C4), 149.93 (C2), 141.03 (C6), 96.58 (C5), 92.77 ($C_{1'}$), 84.26 ($C_{4'}$), 79.39 ($C_{3'}$), 74.41 ($C_{2'}$), 61.51 ($C_{5'}$), 58.43 ($C_{3'}OCH_3$), and 30.68 ppm (N3-CH_3);

HR-MS (ESI+) for $[C_{11}H_{18}N_3O_5]^+$: calc.: 272.1246, found: 272.1241;

HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 92 % HPLC buffer E, 8 % HPLC buffer F in 45 min; retention time = 13.3 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.4.2 4-Imino-3-methyl-1-(3'-*O*-methyl-*β*-D-ribofuranosyl)-3,4-dihydropyrimidin-2(1*H*)-one (24).[255]



R_f = 0.60 (DCM/MeOH 3/1);

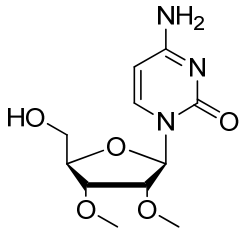
^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.34 (d, ^{3}J(HC6,HC5)=8.0 Hz, 1H; HC6), 6.09 (d, ^{3}J(HC5,HC6)=8.0 Hz, 1H; HC5), 5.91 (d, $^{3}J(HC_{1'},HC_{2'})$=2.2 Hz, 1H; $HC_{1'}$), 4.22 (dd, $^{3}J(HC_{3'},HC_{4'})$=7.5 Hz, $^{3}J(HC_{3'},HC_{2'})$=5.0 Hz, 1H; $HC_{3'}$), 4.01 (ddd, $^{3}J(HC_{4'},HC_{3'})$=7.5 Hz, $^{3}J(HC_{4'},H_bC_{5'})$=2.5 Hz, $^{3}J(HC_{4'},H_aC_{5'})$=2.4 Hz, 1H; $HC_{4'}$), 3.94 (dd, $^{2}J(H_aC_{5'},H_bC_{5'})$=12.5 Hz, $^{3}J(H_aC_{5'},HC_{4'})$=2.4 Hz, 1H; $H_aC_{5'}$), 3.85 (dd, $^{3}J(HC_{2'},HC_{3'})$=5.0 Hz, $^{3}J(HC_{2'},HC_{1'})$=2.2 Hz, 1H; $HC_{2'}$), 3.78 (dd, $^{2}J(H_bC_{5'},H_aC_{5'})$=12.5 Hz, $^{3}J(H_bC_{5'},HC_{4'})$=2.5 Hz, 1H; $H_bC_{5'}$), 3.58 (s, 3H; $C_{2'}OCH_3$), and 3.46 ppm (s, 3H; N3CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 161.05 (C4), 149.64 (C2), 141.17 (C6), 96.31 (C5), 90.33 ($C_{1'}$), 85.88 ($C_{4'}$), 85.07 ($C_{2'}$), 69.00 ($C_{3'}$), 60.72 ($C_{5'}$), 58.93 ($C_{2'}OCH_3$), and 30.69 ppm ($N3CH_3$);

HR-MS (ESI+) for $[C_{11}H_{18}N_3O_5]^+$: calc.: 272.1246, found: 272.1241;

HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 92 % HPLC buffer E, 8 % HPLC buffer F in 45 min; retention time = 18.6 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.4.3 4-Amino-1-(2',3'-di-*O*-methyl-*β*-D-ribofuranosyl)-pyrimidin-2(1*H*)-one (25).[256]



R_f = 0.60 (DCM/MeOH 3/1);

^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.13 (d, ^3J(HC6,HC5)=7.5 Hz, 1H; HC6), 5.95 (d, ^3J($HC_{1'}$,$HC_{2'}$)=2.7 Hz, 1H; $HC_{1'}$), 5.91 (d, ^3J(HC5,HC6)=7.5 Hz, 1H; HC5), 4.05 (ddd, ^3J($HC_{4'}$,$HC_{3'}$)=6.9 Hz, ^3J($HC_{4'}$,$H_bC_{5'}$)=2.7 Hz, ^3J($HC_{4'}$,$H_aC_{5'}$)=2.6 Hz, 1H; $HC_{4'}$), 3.98 (dd, ^3J($HC_{2'}$,$HC_{3'}$)=4.8 Hz, ^3J($HC_{2'}$,$HC_{1'}$)=2.7 Hz, 1H; $HC_{2'}$), 3.94 – 3.83 (m, 2H; $H_aC_{5'}$ and $HC_{3'}$), 3.73 (dd, ^2J($H_bC_{5'}$,$H_aC_{5'}$)=12.5 Hz, ^3J($H_bC_{5'}$,$HC_{4'}$)=2.7 Hz, 1H; $H_bC_{5'}$), 3.55 (s, 3H; $C_{2'}OCH_3$), and 3.41 ppm (s, 3H; $C_{3'}OCH_3$);

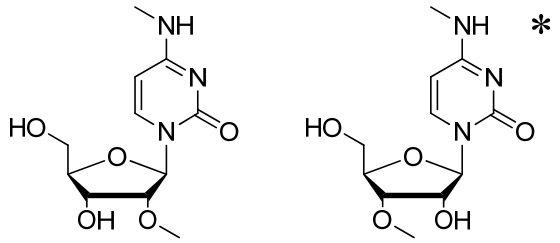
^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 167.62 (C4), 158.09 (C2), 142.68 (C6), 95.89 (C5), 89.93 ($C_{1'}$), 83.70 ($C_{4'}$), 83.36 ($C_{2'}$), 78.17 ($C_{3'}$), 61.37 ($C_{5'}$), 58.69 ($C_{2'}OCH_3$), and 58.31 ppm ($C_{3'}OCH_3$);

HR-MS (ESI-) for $[C_{11}H_{16}N_3O_5]^-$: calc.: 270.1090, found: 270.1092;

HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 92 % HPLC buffer E, 8 %

HPLC buffer F in 45 min; retention time = 26.0 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.4.4 4-Methylamino-1-(2'-*O*-methyl-*β*-D-ribofuranosyl)-pyrimidin-2(1H)-one (26), 4-Methylamino-1-(3'-*O*-methyl-*β*-D-ribofuranosyl)-pyrimidin-2(1H)-one* (27).[256]



R_f = 0.60 (DCM/MeOH 3/1);

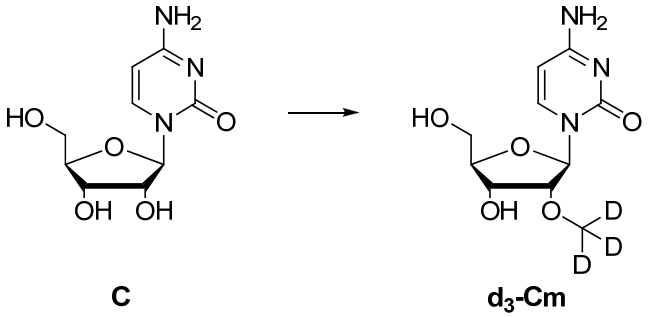
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 7.81 (d, ^3J(HC6,HC5)=7.5 Hz, 1H; HC6), 7.79 (d, ^3J(HC6*,HC5*)=7.5 Hz, 1H*; HC6*), 7.70 – 7.63 (m, 1H, 1H*; HN, HN*), 5.86 (d, ^3J($HC_{1'}$,$HC_{2'}$)=4.2 Hz, 1H; $HC_{1'}$), 5.74 (d, ^3J($HC_{1'}$*,$HC_{2'}$*)=4.4 Hz, 1H*; $HC_{1'}$*), 5.73 – 5.69 (m, 1H and 1H*; HC5 and HC5*), 5.32 (s, 1H*; $C_{2'}OH$*), 5.06 (s, 1H; $C_{3'}OH$), 4.13 – 4.09 (m, 1H*; $HC_{2'}$*), 4.08 – 4.04 (m, 1H; $HC_{3'}$), 3.92 – 3.89 (m, 1H*; $HC_{4'}$*), 3.80 (ddd, ^3J($HC_{4'}$,$HC_{3'}$)=5.9 Hz, ^3J($HC_{4'}$,$H_bC_{5'}$)=3.1 Hz, ^3J($HC_{4'}$,$H_aC_{5'}$)=2.8 Hz, 1H; $HC_{4'}$), 3.68 – 3.61 (m, 2H, 2H*; $HC_{3'}$, $H_aC_{5'}$, $HC_{2'}$*, $H_aC_{5'}$*), 3.57 – 3.50 (m, 1H, 1H*; $H_bC_{5'}$, $H_bC_{5'}$*), 3.37 (s, 3H; $C_{2'}OCH_3$), 3.33 (s, 3H*; $C_{3'}OCH_3$*), and 2.77 – 272 ppm (m, 1H, 1H*; N4CH_3, N4CH_3*);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 163.76 (C4*), 163.76 (C4), 155.30 (C2*), 155.04 (C2), 139.87 (C6*), 139.72 (C6), 94.56 (C5*), 94.39 (C5), 89.35 ($C_{1'}$*), 86.87($C_{1'}$), 86.87 ($C_{4'}$), 84.20 ($C_{2'}$), 81.80($C_{4'}$*), 78.50($C_{3'}$*), 72.55 ($C_{2'}$*), 68.13 ($C_{3'}$), 60.55 ($C_{5'}$*), 60.29 ($C_{5'}$), 57.54 ($C_{2'}OCH_3$), 57.39 ($C_{2'}OCH_3$*), 28.20 (N3CH_3*), and 26.87 ppm (N3CH_3);

HR-MS (ESI+) for $[C_{11}H_{17}N_3O_5Na]^+$: calc.: 294.1066, found: 294.1060;

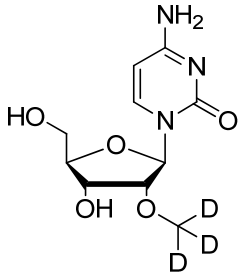
HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 92 % HPLC buffer E, 8 % HPLC buffer F in 45 min; retention time = 23.1 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.10.5 Synthesis of d_3-Cm



Scheme 46: Synthesis of d_3-Cm.
Reagents and conditions: NaH, d_3-MeI, DMF, 0 °C to rt, 4.5 h, 19 %.

10.10.5.1 4-Amino-1-(2'-*O*-trideuteromethyl-*β*-D-ribofuranosyl)-pyrimidin-2(1*H*)-one (d_3-Cm).



Cytidine (4.58 g, 18.8 mmol, 1.00 eq) was suspended in dry DMF (77.0 mL) at 0 °C. NaH (60 % in mineral oil, 0.90 mg, 22.6 mmol, 1.20 eq,) was added carefully. The mixture was stirred at 0 °C for 1 h. d_3-MeI (1.21 mL, 19.4 mmol, 1.03 eq) was added and the white suspension was stirred for 2.5 h at 0 °C. The suspension was allowed to warm to rt and stirred for an additional hour. The solution was filtered and the solvent was removed *in vacuo*. The resulting oil was dissolved in MeOH (20.0 mL). The solvent was removed *in vacuo*. The MeOH treatment was repeated twice. Silica and MeOH (20.0 mL) were added and the solvent removed *in vacuo*. The resulting solid was treated with MeOH (20.0 mL) twice. The dried mixture was applied to column chromatography (DCM/MeOH 20/1 to 10/1). The resulting solid was recrystallized from MeOH to yield d_3-Cm (922 mg, 19 %) as white solid.

R_f = 0.47 (DCM/MeOH 2/1);

M.p.: 251.8 – 253.7 °C;

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 7.90 (d, ^{3}J(HC6,HC5)=7.5 Hz, 1H; HC6), 7.20 (s, 2H; NH_2), 5.84 (d, ^{3}J($HC_{1'}$,$HC_{2'}$)=4.0 Hz, 1H; $HC_{1'}$), 5.71 (d, ^{3}J(HC5,HC6)=7.5 Hz, 1H; HC5), 5.10 (t, ^{3}J($C_{5'}OH$,$H_2C_{5'}$)=5.2 Hz, 1H; $C_{5'}OH$), 5.05 (d, ^{3}J($C_{3'}OH$,$HC_{3'}$)=6.3 Hz, 1H; $C_{3'}OH$), 4.05 (ddd, ^{3}J($HC_{3'}$,$C_{3'}OH$)=6.3 Hz, ^{3}J($HC_{3'}$,$HC_{4'}$)=5.9 Hz, ^{3}J($HC_{3'}$,$HC_{2'}$)=5.1 Hz, 1H; $HC_{3'}$), 3.81 (ddd, ^{3}J($HC_{4'}$,$HC_{3'}$)=5.9 Hz, ^{3}J($HC_{4'}$,$H_bC_{5'}$)=3.1 Hz, ^{3}J($HC_{4'}$,$H_aC_{5'}$)=2.9 Hz, 1H; $HC_{4'}$), 3.70 – 3.62 (m, 2H; $HC_{2'}$ and $H_aC_{5'}$), and 3.55 ppm (ddd, ^{2}J($H_aC_{5'}$,$H_bC_{5'}$)=12.1 Hz, ^{3}J($H_bC_{5'}$,$C_{5'}OH$)=5.2 Hz, ^{3}J($H_bC_{5'}$,$HC_{4'}$)=3.1 Hz, 1H; $H_bC_{5'}$);

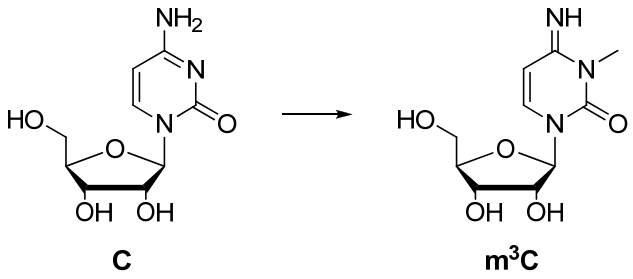
^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 165.62 (C4), 155.12 (C2), 141.14 (C6), 93.94 (C5), 87.03 ($C_{1'}$), 84.17 ($C_{4'}$), 83.19 ($C_{2'}$), 68.08 ($C_{3'}$), 60.23 ($C_{5'}$), and 57.67 ppm (sept, ^{1}J(CD_3, CD_3)=23 Hz; CD_3);

^{2}D-NMR (46 MHz, D_6-DMSO, 27 °C): δ = 3.33 (s; CD_3);

IR: 3326 *bs* (O-H valence), 3189 *bm* (N-H valence), 3063 *w* (C-H valence), 2904 *w* (C-H valence), 2063 *w* (C-D valence), 1643 *s* (C=O valence), 1611 *s* (C=O valence), 1496 *s* (N-H deform.), 1202 *m* (C-O-C valence), 1074 *m* (C-O-C valence), and 786 cm^{-1} *w* (C-H deform.);

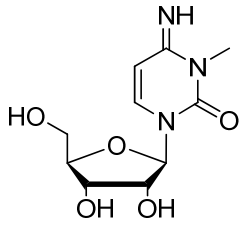
HR-MS (ESI-) for $[C_{10}H_{15}N_3O_5]^-$: calc.: 259.1122, found: 259.1124.

10.10.6 Synthesis of m^{3}C



Scheme 47: Synthesis of m^{3}C.
Reagents and conditions: Dimethylsulfate, DMF, 37 °C, 2 d, 7 %.

10.10.6.1 4-Imino-3-methyl-1-(*β*-D-ribofuranosyl)-3,4-dihydropyrimidin-2(1*H*)-one (m^3C).[233]



Cytidine (0.50 g, 2.06 mmol, 1.00 eq) was suspended in dry DMF (5.00 mL) at 0 °C. Dimethylsulfate (1.95 mL, 20.60 mmol, 10.0 eq) was added and the mixture was stirred at 37 °C for 2 d. MeOH was added to a total volume of 20.0 mL. Ethyl acetate was added until a white precipitate appeared, which was collected by filtration. The resulting solid was recrystallized from MeOH and ethyl acetate twice to yield m^3C (77 mg, 7 %) as white solid.[233]

R_f = 0.47 (DCM/MeOH 3/1);

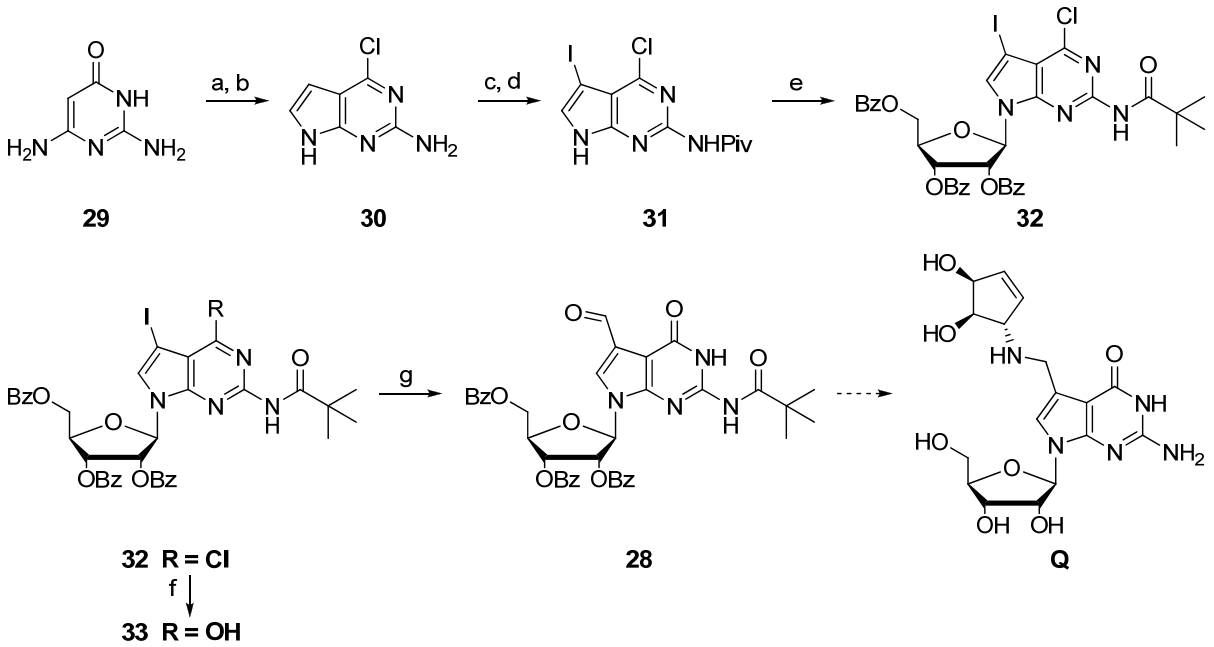
^{1}H NMR (400 MHz, CD_3OD, 27 °C): δ = 8.48 (d, ^3J(HC6,HC5)=7.9 Hz, 1H; HC6), 6.19 (d, ^3J(HC6,HC5)=7.9 Hz, 1H; HC5), 5.87 (d, ^3J($HC_{1'}$,$HC_{2'}$)=2.5 Hz, 1H; $HC_{1'}$), 4.22 – 4.12 (m, 2H; $HC_{2'}$,$HC_{3'}$), 4.10 – 4.03 (m, 1H; $HC_{4'}$), 3.93 (dd, ^2J($H_aC_{5'}$,$H_bC_{5'}$)=12.4 Hz, ^2J($H_aC_{5'}$,$HC_{4'}$)=2.2 Hz, 1H; $H_aC_{5'}$), 3.78 (dd, ^2J($H_aC_{5'}$,$H_bC_{5'}$)=12.4 Hz, ^2J($H_bC_{5'}$,$HC_{4'}$)=2.4 Hz, 1H; $H_bC_{5'}$), 3.51 ppm (s, 3H; CH_3);

^{13}C NMR (101 MHz, CD_3OD, 27 °C): δ = 161.26 (C4), 149.13 (C2), 143.51 (C6), 94.82 (C5), 93.02 ($C_{1'}$), 86.21($C_{4'}$), 76.25 ($C_{2'}$), 70.11 ($C_{3'}$), 61.19 ($C_{5'}$), 30.95 ppm (CH_3);

HR-MS (ESI+) for $[C_{10}H_{16}N_3O_5]^+$: calc.: 258.1090, found: 258.1084.

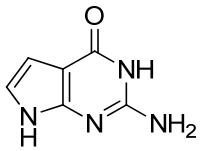
10.11 Synthesis of deazaguanosine nucleosides

10.11.1 Studies towards the synthesis of queuosine



Scheme 48: Studies towards the synthesis of queuosine.
Reagents and conditions: a) Bromoacetaldehyde diethyl acetal, HCl, NaOAc, water, 90 °C and 80 °C, 4 h, 74 %; b) $POCl_3$, reflux, 2 h, 78 %; c) Pivaloyl chloride, pyridine, rt, 2 h, 64 %; d) NIS, THF, rt, 1 h, 81 %; e) 1-acetyl-2,3,5-tribenzoyl-ribose, bistrimethylsilyl acetamide, trimethylsilyl triflate, MeCN, 50 °C, 24 h, 59 %; f) DABCO, CsOAc, NaOAc, DMF, rt, 45 h, 57 %; g) $Pd_2(dba)_3$, triphenylphosphine, CO, tributyltin hydride, toluene, 50 °C, 2 h, 93 %.

10.11.1.1 2-Amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (29a).[257]



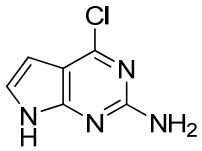
Bromoacetaldehyde diethyl acetal (21.4 mL, 27.3 g, 138 mmol, 1.00 eq) were suspended in water (70.0 mL) and conc. hydrochloric acid (3.00 mL) was added. The mixture was stirred at 90 °C until a clear solution was formed (approximately 30 min). The solution was cooled to rt and sodium acetate (13.6 g, 165 mmol, 1.20 eq) was added. This mixture was added to a suspension of 2,6-diamino-4-pyrimidinone **29** (20.0 g, 159 mmol, 1.15 eq) and sodium acetate (7.00 g, 85.4 mmol, 0.62 eq) in water (150 mL). The resulting mixture was stirred at 80 °C for

2 h. During this period the suspension first dissolved partially, before a white solid gradually precipitated. The obtained suspension was stirred at 0 °C for 90 min. The precipitate was collected and washed with little cold water and acetone. Drying *in vacuo* yielded nucleobase **29a** (15.4 g, 74 %) as white solid.

^{1}H NMR (200 MHz, D_6-DMSO, 27 °C): δ = 10.94 (s, 1H; HN), 10.35 (s, 1H; HN), 6.58 (d, ^3J(HC5,HC6)=3.2 Hz, 1H; HC5), 6.15 (d, ^3J(HC6,HC5)=3.2 Hz, 1H; HC6), and 6.09 ppm (s, 2H; NH_2);

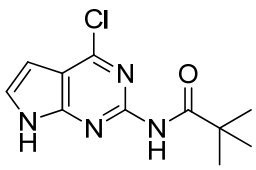
HR-MS (EI+) for $[C_6H_6N_4O]^+$: calc.: 150.0542, found: 150.0544.

10.11.1.2 2-Amino-4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (30).[258]



Nucleobase **29a** (10.0 g, 67.0 mmol, 1.00 eq) was suspended in $POCl_3$ (100 mL, 388 mmol, 5.80 eq). The mixture was refluxed for 2 h. The solvent was removed carefully *in vacuo*. The resulting oil was carefully added to ice cold water (120 mL). The precipitate was removed by filtration and the filtrate was adjusted to pH = 2 with conc. ammonia. After standing for 1 h at 0 °C the precipitate was collected by filtration and washed with little cold water and diethyl ether. Drying *in vacuo* yielded nucleobase **30** (8.70 g, 78 %) as yellow solid. Crude amine **30** was introduced in the next reaction without further purification.

10.11.1.3 4-Chloro-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine (30a).[216b]



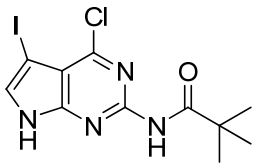
Amine **30** (8.50 g, 50.4 mmol, 1.00 eq) was suspended in dry pyridine (120 mL) and pivaloyl chloride (21.3 g, 176 mmol, 3.50 eq) was added. The mixture was stirred at rt for 2 h. The solvent was removed *in vacuo*. The remaining mixture was dissolved in DCM and extracted with 0.1 M HCl twice. The organic phase was dried over $MgSO_4$. Silica gel chromatography (DCM/MeOH 98/2) yielded amide **30a** (8.15 g, 64 %) as white solid.

R_f = 0.16 (DCM/MeOH 98/2);

^{1}H NMR (200 MHz, D_6-DMSO, 27 °C): δ = 12.32 (s, 1H; NHCO), 10.02 (s, 1H; HN), 7.15 (d, ^3J(HC5,HC6)=3.6 Hz, 1H; HC5), 6.50 (d, ^3J(HC6,HC5)=3.6 Hz, 1H; HC6), and 1.20 ppm (s, 9H; $COC(CH_3)_3$);

HR-MS (EI+) for $[C_{11}H_{13}ClN_4O]^+$: calc.: 252.0778, found: 252.0782.

10.11.1.4 4-Chloro-5-iodo-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine (31).[216b]



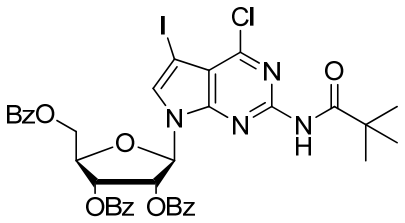
Nucleobase **30a** (3.10 g, 12.3 mmol, 1.00 eq) were dissolved in dry THF (60.0 mL) and the flask was wrapped with aluminum foil to exclude light. *N*-Iodosuccinimide (3.04 g, 13.51 mmol, 1.10 eq) was added. The mixture was stirred at rt for 1 h. The solvent was removed *in vacuo*. The remaining mixture was dissolved in DCM (500 mL) and extracted with water (200 mL). The organic phase was dried over $MgSO_4$. Silica gel chromatography (DCM/MeOH 99/1) yielded nucleobase **31** (3.76 g, 81 %) as white solid. Alternatively, the crude product was recrystallized from little MeOH.

R_f = 0.07 (DCM/MeOH 99/1);

^{1}H NMR (200 MHz, D_6-DMSO, 27 °C): δ = 12.67 (s, 1H; NHCO), 10.09 (s, 1H; HN), 7.74 (m, 1H; HC6), and 1.20 ppm (s, 9H; $COC(CH_3)_3$);

HR-MS (EI+) for $[C_{11}H_{12}ClIN_4O]^+$: calc.: 377.9744, found: 377.9756.

10.11.1.5 4-Chloro-5-iodo-2-pivaloylamino-7-[(2',3',5'-tri-*O*-benzoyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (32).[216d]



Nucleobase **31** (2.50 g, 6.60 mmol, 1.00 eq) was dissolved in dry MeCN (70.0 mL) and bistrimethylsilyl acetamide (2.00 mL, 1.66 g, 8.18 mmol, 1.24 eq) was added dropwise. The mixture was stirred at rt for 5 min. After addition of trimethylsilyl triflate (1.65 mL. 2.03 g, 9.13 mmol, 1.40 eq) and 1-acetyl-2,3,5-tribenzoyl-ribose (2.22 g, 4.40 mmol, 0.67 eq) the mixture was stirred at 50 °C. 1-Acetyl-2,3,5-tribenzoyl-ribose (2.22 g, 4.40 mmol, 0.67 eq) was added after 6 h and 12 h. After stirring for 24 h DCM (200 mL) was added and the organic layer was extracted with sat. $NaHCO_3$ solution (100 mL) and brine (100 mL) subsequently. The organic layer was dried over $MgSO_4$. Purification by column chromatography (isohexane/ethyl acetate 8/1) yielded nucleoside **32** (4.43 g, 59 %) as yellow foam.

As hardly separable side products open chained sugars (degraded starting material) were identified.

R_f = 0.30 (isohexane/ethyl acetate 3/1);

M.p.: 60.2 – 61.9 °C;

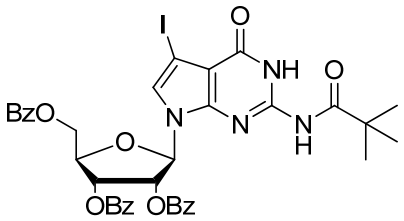
^{1}H NMR (400 MHz, $CDCl_3$, 27 °C): δ = 8.24 (s, 1H; HN), 8.01 – 7.95 (m, 6H; HAr), 7.59 - 7.52 (m, 3H; HAr), 7.44 – 7.32 (m, 7H; HAr and HC8), 6.47 (dd, $^3J(HC_{3'},HC_{4'})$=6.0 Hz, $^3J(HC_{3'},HC_{2'})$=5.8 Hz, 1H; $HC_{3'}$), 6.39 (d, $^3J(HC_{1'},HC_{2'})$=3.9 Hz, 1H; $HC_{1'}$), 6.23 (dd, $^3J(HC_{2'},HC_{3'})$=5.8 Hz, $^3J(HC_{2'},HC_{1'})$=3.9 Hz, 1H; $HC_{2'}$), 4.86 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.2 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.4 Hz, 1H; $H_aC_{5'}$), 4.8 – 4.76 (m, 1H; $HC_{4'}$), 4.67 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.2 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.4 Hz, 1H; $H_bC_{5'}$), and 1.29 ppm (s, 9H; $C(CH_3)_3$);

^{13}C NMR (101 MHz, $CDCl_3$, 27 °C): δ = 175.35 (COC$(CH_3)_3$), 166.31 (ArCO), 165.62 (ArCO), 165.45 (ArCO), 153.52 (C4), 151.77 (C7a), 151.68 (C2), 133.89 (Ar), 133.77 (Ar), 133.58 (Ar), 130.12 (2C; Ar), 130.05 (2C; Ar), 129.74 (2C; Ar), 129.55 (Ar), 129.07 (Ar),

128.81 (Ar), 128.80 (2C; Ar), 128.69 (2C; Ar), 128.68 (2C; Ar), 114.29 (C6), 98.61 (C4a), 88.50 ($C_{1'}$), 80.28 ($C_{4'}$), 74.75 ($C_{2'}$), 71.58 ($C_{3'}$), 63.64 ($C_{5'}$), 53.74 (C5), 40.47 ($CO\mathit{C}(CH_3)_3$), and 27.53 ppm (3C; $COC(\mathit{C}H_3)_3$);

HR-MS (ESI+) for $[C_{37}H_{33}ClIN_4O_8]^+$: calc.:823.1026, found: 823.1001.

10.11.1.6 5-Iodo-4-oxo-2-pivaloylamino-7-[(2',3',5'-tri-*O*-benzoyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (33).[216d]



Nucleoside **32** (1.01 g, 1.23 mmol, 1.00 eq), DABCO (137 mg, 1.23 mmol, 1.00 eq), and caesium acetate (705 mg, 3.67 mmol, 3.00 eq) in DMF (1.20 mL) were stirred at rt for 45 h. After addition of water (1.00 mL) the mixture was stirred for 30 min. Water (10.0 mL) was added and the mixture was extracted with ethyl acetate (10.0 mL) twice. The combined organic layers were washed with water (15.0 mL) and brine (15.0 mL) and dried over $MgSO_4$. Purification *via* silica gel column chromatography (isohexane/ethyl acetate 2/1) yielded nucleoside **33** (0.56 g, 57 %) as white solid.

R_f = 0.50 (isohexane/ethyl acetate 1/1);

M.p.: 92.3 – 93.8 °C;

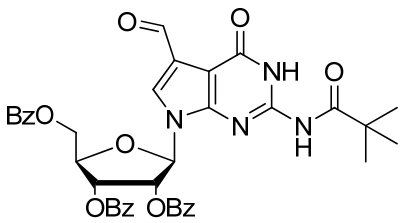
^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 11.53 (s, 1H; HN_{Ar}), 8.73 (s, 1H; NHPiv), 8.00 – 7.92 (m, 2H; HAr), 7.92 – 7.84 (m, 2H; HAr), 7.78 – 7.69 (m, 2H; HAr), 7.61 – 7.27 (m, 9H; HAr), 6.94 (s, 1H; HC6), 6.84 (dd, $^3J(HC_{3'},HC_{4'})$=8.5 Hz, $^3J(HC_{3'},HC_{2'})$=4.9 Hz, 1H; $HC_{3'}$), 6.45 (dd, $^3J(HC_{2'},HC_{3'})$=4.9 Hz, $^3J(HC_{2'},HC_{1'})$=1.7 Hz, 1H; $HC_{2'}$), 5.99 (d, $^3J(HC_{1'},HC_{2'})$=1.7 Hz, 1H; $HC_{1'}$), 4.80 – 4.54 (m, 3H, $HC_{4'}$, $H_aC_{5'}$, and $H_bC_{5'}$), and 1.33 ppm (s, 9H; $C(CH_3)_3$);

^{13}C NMR (75 MHz, $CDCl_3$, 27 °C): δ = 180.29 ($\mathit{C}OC(CH_3)_3$), 166.26 (ArCO), 166.17 (ArCO), 165.34 (ArCO), 156.85 (C4), 146.65 (C7a), 146.45 (C2), 133.97 (Ar), 133.94 (Ar), 133.71 (Ar), 129.97 (2C; Ar), 129.92 (2C; Ar), 129.41 (2C; Ar), 129.15 (Ar), 128.94 (Ar),

128.86 (Ar), 128.78 (2C; Ar), 128.71 (2C; Ar), 128.65 (2C; Ar), 127.24 (C6), 106.73 (C4a), 89.50 ($C_{1'}$), 78.71 ($C_{4'}$), 74.64 ($C_{2'}$), 70.78 ($C_{3'}$), 61.66 ($C_{5'}$), 56.18 (C5), 40.41 ($C(CH_3)_3$), and 27.04 ppm (3C; $C(CH_3)_3$);

HR-MS (ESI-) for $[C_{37}H_{32}IN_4O_9]^-$: calc.: 803.1219, found: 803.1210.

10.11.1.7 5-Formyl-4-oxo-2-pivaloylamino-7-[(2',3',5'-tri-*O*-benzoyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (28).



Under argon atmosphere nucleoside **33** (100 mg, 0.12 mmol, 1.00 eq), $Pd_2(dba)_3$ (11 mg, 0.01 mmol, 0.01 eq), and triphenylphosphine (20 mg, 0.08 mmol, 0.60 eq) were dissolved in dry toluene (1.60 mL). The solution was cooled to -78 °C. High vacuum was applied for 2 sec and the flask was ventilated with argon. This approach was repeated three times. Then after application of high vacuum the flask was ventilated with CO. Again this procedure was repeated three times. CO atmosphere was sustained in the flask afterwards using a CO balloon. The solution was heated to 55 °C and stirred. Tributyltin hydride (40.0 µL, 0.15 mmol, 1.20 eq) in dry toluene (0.95 mL) was added dropwise over a period of 2 h. Afterwards the reaction was allowed to cool to rt and the solvent was removed *in vacuo*. The resulting solid was purified *via* silica gel column chromatography (isohexane/ethyl acetate 2/1) yielding nucleoside **28** (81 mg, 93 %) as white solid.

R_f = 0.25 (isohexane/ethyl acetate 1/1);

M.p.: 105.4 – 106.7 °C;

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 10.35 (s, 1H; HCO), 8.83 (s, 1H; NHPiv), 7.97 – 7.94 (m, 2H; HAr), 7.92 – 7.87 (m, 2H; HAr), 7.77 – 7.72 (m, 2H; HAr), 7.62 – 7.50 (m, 3H; HAr), 7.59 (s, 1H; HC6), 7.45 – 7.40 (m, 2H; HAr), 7.39 – 7.35 (m, 2H; HAr), 7.33 – 7.29 (m, 2H; HAr), 6.87 (dd, $^3J(HC_{3'},HC_{4'})$=8.4 Hz, $^3J(HC_{3'},HC_{2'})$=5.0 Hz, 1H; $HC_{3'}$), 6.51 (dd,

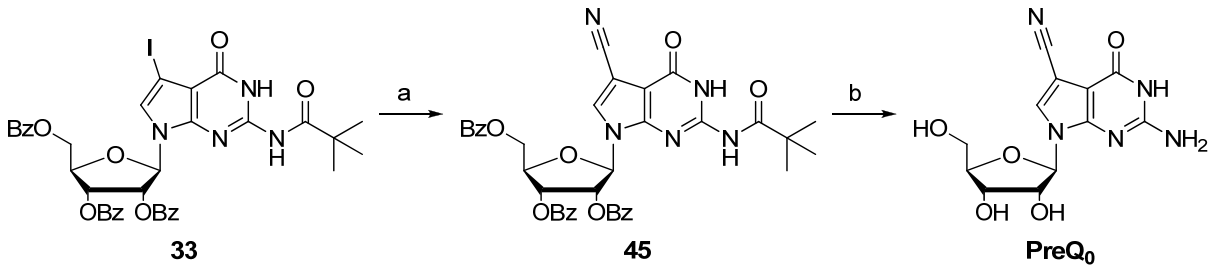
$^3J(HC_{2'},HC_{3'})$=5.0 Hz, $^3J(HC_{2'},HC_{1'})$=1.7 Hz, 1H; $HC_{2'}$), 6.05 (d, $^3J(HC_{1'},HC_{2'})$=1.7 Hz, 1H; $HC_{1'}$), 4.80 (ddd, $^3J(HC_{4'},HC_{3'})$=8.4 Hz, $(HC_{4'},H_bC_{5'})$=3.4 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.2 Hz, 1H; $HC_{4'}$), 4.74 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.4 Hz, $(H_aC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_aC_{5'}$), 4.65 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.4 Hz, $(H_bC_{5'},HC_{4'})$=3.4 Hz, 1H; $H_bC_{5'}$), and 1.36 ppm (s, 9H; $C(CH_3)_3$);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 186.29 (HCO), 180.53 ($COC(CH_3)_3$), 166.31 (ArCO), 166.24 (ArCO), 165.37 (ArCO), 156.96 (C4), 147.56 (C7a), 147.41 (C2), 134.12 (Ar), 134.06 (Ar), 133.83 (Ar), 130.04 (2C; Ar), 129.99 (2C; Ar), 129.46 (2C; Ar), 129.15 (Ar), 128.87 (2C; Ar), 128.84 (Ar), 128.82 (Ar), 128.78 (2C; Ar), 128.65 (2C; Ar), 126.81 (C6), 121.19 (C5), 104.89 (C4a), 90.68 ($C_{1'}$), 79.03 ($C_{4'}$), 74.59 ($C_{2'}$), 70.81 ($C_{3'}$), 61.61 ($C_{5'}$), 40.53 ($COC(CH_3)_3$), and 27.09 ppm (3C; $COC(CH_3)_3$);

HR-MS (ESI-) for $[C_{38}H_{35}IN_4O_{10}]^+$: calc.: 707.2348, found: 707.2348.

Further reactions leading to queuosine were performed by E. M. Jahn, F. Klepper,[239c] and I. Thoma.[239a] Further reactions leading to isotopically labeled queuosine were performed by I. Thoma.[239a]

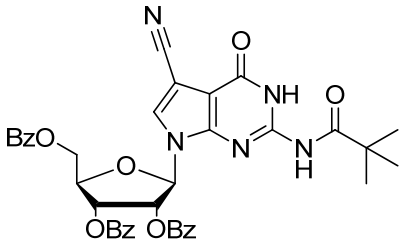
10.11.2 Synthesis of $PreQ_0$ *via* the queuosine route



33 **45** **$PreQ_0$**

Scheme 49: Synthesis of $PreQ_0$ *via* the queuosine route.
Reagents and conditions: a) MeMgCl, ^{i}PrMgCl•LiCl, TosCN, toluene, -78 °C to rt, 18 h, 60 %; b) 28 % NH_3 in H_2O, 60 °C, 16 h, 78 %.

10.11.2.1 5-Cyano-4-oxo-2-pivaloylamino-7-[2',3',5'-*O*-tribenzoyl-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (45).



Nucleoside **33** (100 mg, 0.12 mmol, 1.00 eq) was dissolved in dry toluene (1.00 mL) and cooled to -65 °C. MeMgCl (2.56 M in THF, 0.09 mL, 0.23 mmol, 2.00 eq) was added dropwise and the resulting mixture was stirred for 1 h. Over a period of 1 h ^{i}PrMgCl•LiCl (1.06 M in THF, 0.30 mL, 0.31 mmol, 2.50 eq) in dry toluene (0.70 mL) was added with a syringe pump. The solution was stirred for 4 h at -65 °C. TosCN (135 mg, 0.74 mmol, 6.00 eq) was added and the resulting mixture was allowed to warm to rt over a period of 16 h. Water (20.0 mL) and ethyl acetate (20.0 mL) were added to the solution. The organic layer was collected and the aqueous layer was extracted with ethyl acetate (3 × 20.0 mL). The combined organic layers were washed with brine (20.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography yielded nucleoside **45** (52 mg, 60 %) as white solid.

R_f = 0.64 (isohexane/ethyl acetate 1/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.73 (s, 1H; N*H*COC(CH$_3$)$_3$), 8.82 (s, 1H; HNAr), 7.98 (m, 2H; H$_{ortho}$), 7.91 (m, 2H; H$_{ortho}$), 7.75 (m, 2H; H$_{ortho}$), 7.59 (m, 3H; H$_{para}$), 7.45 (m, 2H; H$_{meta}$), 7.39 (m, 2H; H$_{meta}$), 7.38 (s, 1H; HC6), 7.35 (m, 2H; H$_{meta}$), 6.85 (dd, ^3J(HC$_{3'}$,HC$_{4'}$)=8.4 Hz, ^3J(HC$_{3'}$,HC$_{2'}$)=5.0 Hz, 1H; HC$_{3'}$), 6.48 (dd, ^3J(HC$_{2'}$,HC$_{3'}$)=5.0 Hz, ^3J(HC$_{2'}$,HC$_{1'}$)=1.6 Hz, 1H; HC$_{2'}$), 6.02 (d, ^3J(HC$_{1'}$,HC$_{2'}$)=1.6 Hz, 1H; HC$_{1'}$), 4.81 (ddd, ^3J(HC$_{4'}$,HC$_{3'}$)=8.4 Hz, ^3J(HC$_{4'}$,H$_b$C$_{5'}$)=3.3 Hz, ^3J(HC$_{4'}$,H$_a$C$_{5'}$)=3.0 Hz, 1H; HC$_{4'}$), 4.75 (dd, ^2J(H$_a$C$_{5'}$,H$_b$C$_{5'}$)=12.5 Hz, ^3J(H$_a$C$_{5'}$,HC$_{4'}$)=3.0 Hz, 1H; H$_a$C$_{5'}$), 4.68 (dd, ^2J(H$_b$C$_{5'}$,H$_a$C$_{5'}$)=12.5 Hz, ^3J(H$_b$C$_{5'}$,HC$_{4'}$)=3.3 Hz, 1H; H$_b$C$_{5'}$), and 1.37 ppm (s, 9H; COC(CH$_3$)$_3$);

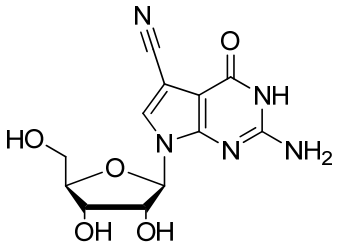
^{13}C NMR (151 MHz, $CDCl_3$, 25 °C): δ = 180.27 (*C*OC(CH$_3$)$_3$), 166.03 (PhCO), 166.02 (PhCO), 165.13 (PhCO), 155.32 (C4), 148.00 (C2), 146.41 (C7a), 133.94 (Ar), 133.88 (Ar), 133.75 (Ar), 130.27 (C6), 129.80 (Ar), 129.75 (Ar), 129.13 (Ar), 128.82 (Ar), 128.66 (Ar),

128.58 (Ar), 128.55 (Ar), 128.51 (Ar), 128.47 (Ar), 105.35 and 113.36 (C4a and CN), 90.12 and 90.14 (C5 and $C_{1'}$), 78.99 ($C_{4'}$), 74.26 ($C_{2'}$), 70.52 ($C_{3'}$), 61.18 ($C_{5'}$), 40.32 ($COC(CH_3)_3$), and 26.83 ppm (3C; $COC(CH_3)_3$),

IR: 3345 *w* (N-H valence), 3199 *w* (N-H valence), 2973 *m* (C-H valence), 2875 *w* (C-H valence), 2233 *m* (C≡N valence), 1724 *s* (C=O valence), 1673 *s* (C=C valence), and 1262 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI–) for $[C_{38}H_{32}N_5O_9]^-$: calc.: 702.2206, found: 702.2209.

10.11.2.2 2-Amino-5-cyano-4-oxo-7-*β*-D-ribofuranosyl-7*H*-pyrrolo-[2,3-*d*]-pyrimidine ($PreQ_0$).[228-229]



Nucleoside **45** (14.0 mg, 0.02 mmol) was dissolved in NH_3 in H_2O (28 %, 1.00 mL). The solution was stirred at 60 °C for 16 h. Subsequent removal of the solvent *in vacuo* resulted in a colorless oil, which was applied to column chromatography (ethyl acetate/MeOH 10/1). $PreQ_0$ (4 mg, 78 %) was obtained as white solid.

R_f = 0.30 (ethyl acetate/MeOH 5/1);

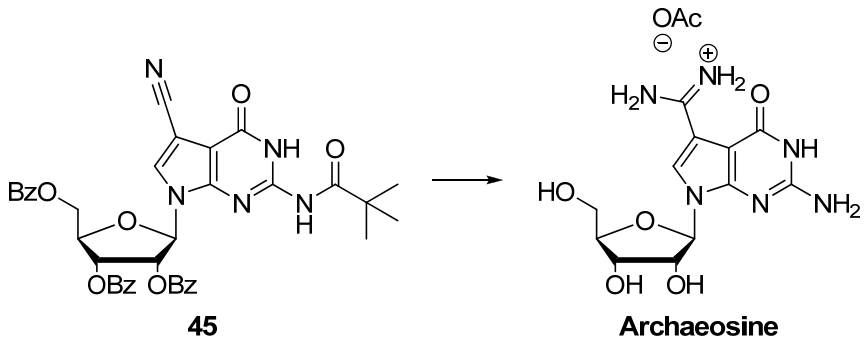
^1H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.86 (s, 1H; HNAr), 7.89 (s, 1H; HC6), 6.62 (s, 2H; NH_2), 6.05 (d, $^3J(HC_{1'},HC_{2'})$=5.9 Hz, 1H; $HC_{1'}$), 4.28 (dd, $^3J(HC_{2'},HC_{1'})$=5.9 Hz, $^3J(HC_{2'},HC_{3'})$=4.8 Hz, 1H; $HC_{2'}$), 4.03 (dd, $^3J(HC_{3'},HC_{2'})$=4.8 Hz, $^3J(HC_{3'},HC_{4'})$=3.7 Hz, 1H; $HC_{3'}$), 3.82 (ddd, $^3J(HC_{4'},H_aC_{5'})$=4.1 Hz, $^3J(HC_{4'},H_bC_{5'})$=4.0 Hz, $^3J(HC_{4'},HC_{3'})$=3.7 Hz, 1H; $HC_{4'}$), 3.57 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.9 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.1 Hz, 1H; $H_aC_{5'}$), and 3.50 ppm (dd, $^2J(H_bC_{5'},H_aC_{5'})$=11.9 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.0 Hz, 1H; $H_bC_{5'}$);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 157.21 (C4), 154.02 (C2), 151.38 (C7a), 127.48 (C6), 115.32 (CN), 98.62 (C4a), 86.48 and 86.34 ($C_{1'}$ and C5), 85.09 ($C_{4'}$), 74.07 ($C_{2'}$), 70.23 ($C_{3'}$), and 61.23 ppm ($C_{5'}$);

IR: 3305 *s*, 3219 *s* (N-H valence), 2935 *m* (C-H valence), 2229 *m* (C≡N valence), 1673 *s*, 1591 *m* (N-H deform.), 1561 *m* (N-H deform.), 1426 *w* (C-H deform.), 1104 *m* (C-O-C valence), and 1047 cm^{-1} *m* (C-O-C valence);

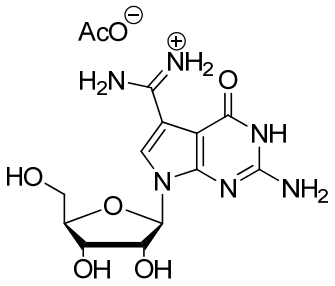
HR-MS (ESI–) for $[C_{12}H_{12}N_5O_5]^-$: calc.: 306.0844, found: 306.0837.

10.11.3 Synthesis of archaeosine *via* the queuosine route



Scheme 50: Synthesis of archaeosine *via* the queuosine route.
Reagents and conditions: HCl (g), MeOH, 7 M NH_3 in MeOH, rt, 19 h, 40 %.

10.11.3.1 2-Amino-5-amidinium-7-*β*-D-ribofuranosyl-7*H*-pyrrolo-[2,3-*d*]-pyrimidine-4-one acetate (archaeosine).[218]



Nucleoside **45** (30 mg, 0.04 mmol) was dissolved in MeOH (2.00 mL) under nitrogen and treated with gaseous HCl for 3 h. Subsequently, nitrogen was bubbled through the solution to remove the solvent. The resulting white solid was stirred in NH_3 in MeOH (7 N, 2.00 mL) for 16 h. Removal of the solvent *in vacuo* yielded a white solid, which was purified by HPL chromatography. The solvent was removed to give a 2 to 1 mixture of archaeosine and

triethylammonium acetate (8 mg, 40 %). Complete removal of the buffer lead to decomposition of the product yielding the corresponding nitrile compound.

R_f = 0.57 (isopropanol/water/glacial acid 5/1/1);

M.p.: decomposition > 215 °C;

^{1}H NMR (600 MHz, D_2O, 27 °C): δ = 8.03 (s, 1H; HC6), 6.06 (d, $^3J(HC_{1'},HC_{2'})$=5.9 Hz, 1H; $HC_{1'}$), 4.61 (dd, $^3J(HC_{2'},HC_{1'})$=5.9 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.38 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.9 Hz, 1H; $HC_{3'}$), 4.24 (ddd, $^3J(HC_{4'},H_bC_{5'})$=4.2 Hz, $^3J(HC_{4'},HC_{3'})$=3.9 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.1 Hz, 1H; $HC_{4'}$), 3.90 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.9 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.1 Hz, 1H; $H_aC_{5'}$), 3.84 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.9 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.2 Hz, 1H; $H_bC_{5'}$), and 1.93 ppm (s, 3H; H_3CCOO^-);

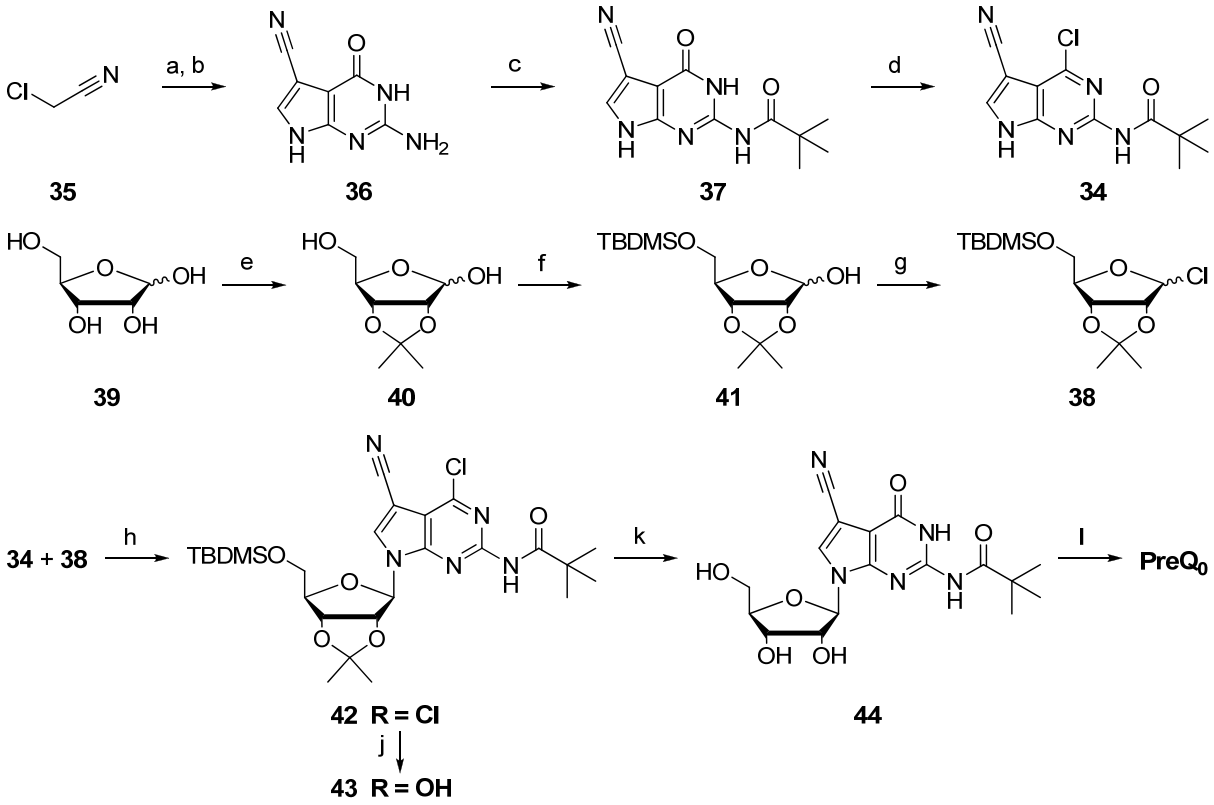
^{13}C NMR (151 MHz, D_2O, 27 °C): δ = 215.59 ($H_3C\mathit{C}OO^-$), 153.39, 154.75, 159.21, and 162.19 (C7a, $C_{amidine}$, C2, and C4), 125.32 (C6), 107.38 (C5), 98.16 (C4a), 87.94 ($C_{1'}$), 85.27 ($C_{4'}$), 74.18 ($C_{2'}$), 70.55 ($C_{3'}$), 61.50 ($C_{5'}$), and 23.43 ppm ($\mathit{C}H_3COO^-$);

IR: 3368 *s* (N-H and O-H valence), 3176 *s* (N-H and O-H valence), 2931 *s* (N-H and O-H valence), 1660 *s* (C=N valence), 1560 *m* (N-H deform.), 1058 *s* (C-O valence), and 1023 cm^{-1} *s* (C-O valence);

HR-MS (ESI–) for $[C_{12}H_{15}N_6O_5]^-$: calc.: 323.1109, found: 323.1116;

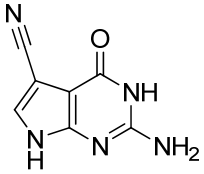
HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 80 % HPLC buffer E, 20 % HPLC buffer F in 45 min; retention time = 22.2 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.11.4 Synthesis of $PreQ_0$



Scheme 51: Synthesis of $PreQ_0$.
Reagents and conditions: a) HCOOMe, NaOMe, THF, 0 °C to 10 °C, 4 h, b) NaOAc, 2,6-diaminopyrimidine-4-one, water, THF, 50 °C to reflux, 3 h, 38 % (2 steps); c) pivaloyl chloride, pyridine, 85 °C, 2.0 h, 75 %; d) $POCl_3$, TEBA, DMA, acetonitrile, 90 °C, 1 h, 75 %; e) acetone, conc. HCl, 0 °C, 2 h, 87 %; f) TBDMSCl, imidazole, DMF, rt, 16 h, 83 %; g) HMPA, tetrachloromethane, THF, -78 °C to rt, 4 h; h) NaH, acetonitrile, 0 °C to rt, 20 h, 20 %; j) Et_3N, DABCO, NaOAc, DMF, rt, 48 h, 81 %; k) trifluoroacetic acid, water, 0 °C, 4 h, 77 %; l) 28 % ammonia in water, 60 °C, 17 h, 99 %.

10.11.4.1 2-Amino-5-cyano-3,4-dihydro-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (36).[222]



Nucleobase **36** was synthesised according to literature.

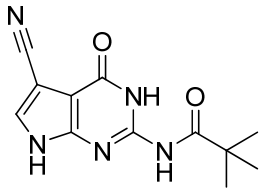
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 12.00 (s, 1H; HN3), 10.72 (s, 1H; HN7), 7.63 (s, 1H; HC6), and 6.40 ppm (s, 2H; NH_2);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 164.30 (C4), 158.24 (C2), 154.14 (C7a), 128.22 (C6), 116.18 (CN), 99.48 (C4a), and 85.21 ppm (C5);

IR: 3362 *s* (N-H valence), 3105 *s* (N-H valence), 2229 *s* (C≡N valence), 1627 *vs* (N-H_2 deform.), 1592 *vs* (aromatic vibration), and 1260 cm^{-1} *s* (C-N valence);

HR-MS (ESI–) for $[C_7H_4ON_5]^-$: calc.: 174.0421, found: 174.0426.

10.11.4.2 5-Cyano-3,4-dihydro-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (37).



Nucleobase **36** (1.50 g, 8.60 mmol, 1.00 eq) was dissolved in pyridine (10.0 mL) and slowly treated with pivaloyl chloride (3.10 g, 26.0 mmol, 3.00 eq). The resulting suspension was stirred for 2 h at 85 °C. After cooling to rt a white solid precipitated, which was removed by filtration. The resulting solution was neutralised with NH_3 in MeOH (7N) and left to stand at 4 °C for 16 h. The resulting precipitate was collected and washed with ethanol (20.0 mL) and diethyl ether (10.0 mL) to give the pivaloyl protected nucleobase **37** (1.70 g, 75 %) as brownish solid.

R_f = 0.21 (DCM/MeOH 20/1);

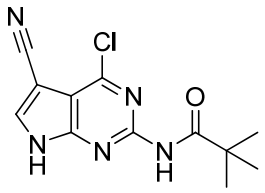
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 12.65 (s, 1H; NHPiv), 12.11 (s, 1H; HN3) 11.00 (s, 1H; HN7), 7.93 (s, 1H; HC6), and 1.25 ppm (s, 9H; CH_3);

^{13}C NMR (150 MHz, D_6-DMSO, 27 °C): δ = 181.73 (*C*OC(CH_3)$_3$), 155.67 (C4), 148.38 and 148.42 (C2 and C7a), 130.21 (C6), 115.17 (CN), 103.02 (C4a), 86.17 (C5), 39.96 (*C*(CH_3)$_3$), and 26.27 ppm (3C; C(*C*H_3)$_3$);

IR: 3125 *vs* (N-H valence), 2228 *s* (C≡N valence), 1684 *s* (C=O valence), 1646 *vs* (C=C valence), 1608 *vs* (aromatic vibration), 1579 *vs* (N-H deform.), 1409 *vs* (C-H deform.), 1239 *s* (C-N valence), 1176 *s* (C-N valence), and 782 cm^{-1} *s* (C-H deform.);

HR-MS (ESI+) for $[C_{12}H_{13}N_5O_2]^+$: calc.: 259.1069, found: 259.1088.

10.11.4.3 4-Chloro-5-cyano-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine (34).



Nucleobase **37** (200 mg, 0.77 mmol, 1.00 eq) was suspended in acetonitrile (1.20 mL) and dimethylaniline (0.41 mL, 3.28 mmol, 4.30 eq), triethylbenzylammonium chloride (89 mg, 0.39 mmol, 0.50 eq), and phosphorylchloride (0.72 mL, 7.70 mmol, 10.0 eq) were added. The suspension was heated to 90 °C for 1 h. The cooled down suspension was concentrated *in vacuo* and the resulting oil cautiously treated with ice. The acidic mixture was set to pH = 4 with conc. ammonia in methanol. The precipitate was collected by filtration and washed with water. Purification by column chromatography (isohexane/ethyl acetate 3/2) gave nucleobase **34** (160 mg, 75 %) as white solid.

R_f = 0.65 ($CHCl_3$/MeOH 9/1);

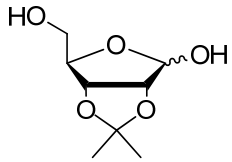
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 13.35 (s, 1H; NHPiv), 10.27 (s, 1H; HN7), 8.50 (s, 1H; HC6), and 1.22 ppm (s, 9H; $C(CH_3)_3$);

^{13}C NMR (150 MHz, D_6-DMSO, 27 °C): δ = 175.83 (COC$(CH_3)_3$), 153.08 (C2), 152.79 (C7a), 150.66 (C4), 137.51 (C6), 111.12 and 114.42 (C4a and CN), 83.37 (C5), 39.59 (CO$C$$(CH_3)_3$), and 26.72 (3C; COC$(CH_3)_3$);

IR: 3448 *w* (N-H valence), 3160 *vs* (N-H valence), 2231 *s* (C≡N valence), 1708 *s* (C=O valence), 1563 *vs* (N-H deform.), 1521 *s* (N-H deform.), 1445 *vs* (C-H deform.), 1419 *vs* (C-H deform.), 1271 *s* (C-N valence), 1164 *s* (C-N valence), and 785 cm^{-1} *s* (C-H deform.);

HR-MS (ESI+) for $[C_{12}H_{13}N_5OCl]^+$: calc.: 278.0806, found: 278.0796.

10.11.4.4 2',3'-*O*-Isopropylidene-*α*/*β*-D-ribofuranose (40).[224]



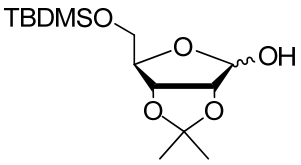
D-Ribose **39** (25.0 g, 150 mmol) was suspended in acetone. Conc. HCl (25.0 µL) was added at 0 °C and the mixture was stirred for 2 h. The resulting suspension was filtered through celite and activated charcoal. Concentration of the resulting solution gave the isopropylidene protected sugar **40** (33.0 g, 87 %) as white solid.

R_f = 0.29 (DCM/MeOH 20/1);

^{1}H NMR (200 MHz, D_6-DMSO, 27 °C): δ = 5.41 (s, 1H; $HC_{1'}$), 4.83 (d, $^3J(HC_{2'},HC_{3'})$=6.0 Hz, 1H; $HC_{2'}$), 4.57 (d, $^3J(HC_{3'},HC_{2'})$=6.0 Hz, 1H; $HC_{3'}$), 4.40 (m, 1H; $HC_{4'}$), 3.72 (m, 2H; $H_2C_{5'}$), 1.47 (s, 3H; CH_3), and 1.31 ppm (s, 3H; CH_3);

HR-MS (ESI+) for $[C_8H_{14}O_5]^+$: calc.: 190.0841, found: 190.0834.

10.11.4.5 5'-(*O*-^{t}butyl-dimethylsilyl)-2',3'-(*O*-isopropylidene)-*α*/*β*-D-ribofuranose (41).[259]



Sugar **40** (33.0 g, 174 mmol, 1.00 eq) and imidazole (29.0 g, 422 mmol, 2.42 eq) were dissolved in DMF (60.0 mL). TBDMSCl (27.4 g, 182 mmol, 1.05 eq) was added and the solution was stirred at rt for 16 h. The reaction mixture was treated with water (100 mL) and extracted with ethyl acetate (3 × 250 mL). The combined organic phases were

extracted with water (120 mL) twice and dried over $MgSO_4$. Removal of the solvent *in vacuo* yielded the protected sugar **41** (44.0 g, 83 %) as a white solid, which was used without purification in the next step. An analytically pure sample was obtained by column chromatography (isohexane/ethyl acetate 7/1).

R_f = 0.60 ($CHCl_3$/MeOH 10/1);

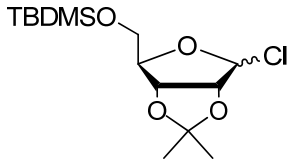
^{1}H NMR (600 MHz, D_6-DMSO, 27 °C): δ = 6.43 (d, $^3J(C_{1'}OH,HC_{1'})$=4.5 Hz, 1H; $C_{1'}OH$), 5.19 (d, $^3J(HC_{1'},C_{1'}OH)$=4.5 Hz, 1H; $HC_{1'}$), 4.63 (d, $^3J(HC_{3'},HC_{2'})$=6.0 Hz, 1H; $HC_{3'}$), 4.46 (d, $^3J(HC_{2'},HC_{3'})$=6.0 Hz, 1H; $HC_{2'}$), 3.95 (t, $^3J(HC_{4'},HC_{5'})$=6.4 Hz, 1H; $HC_{4'}$), 3.59 (d, $^3J(HC_{3'},HC_{2'})$=6.4 Hz, 2H; $H_2C_{5'}$), 1.37 (s, 3H; CH_3), 1.25 (s, 3H; CH_3), 0.88 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), and 0.06 ppm (s, 6H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (600 MHz, D_6-DMSO, 27 °C): δ = 111.17 ($C(CH_3)_2$), 101.82 ($C_{1'}$), 85.91 ($C_{4'}$), 85.56 ($C_{2'}$), 81.81 ($C_{3'}$), 64.29 ($C_{5'}$), 26.36 ($C(CH_3)_2$), 25.75 (3C; $Si(CH_3)_2C(CH_3)_3$), 24.74 ($C(CH_3)_2$), 17.95 ($Si(CH_3)_2C(CH_3)_3$), -5.34 and -5.44 ppm (2C; $Si(CH_3)_2C(CH_3)_3$);

IR: 3345 *s* (O-H valence), 2939 *s* (C-H valence), 2861 *s* (C-H valence), 1382 *w* (C-H deform.), 1258 *s* (C-O-C valence), 1084 *vs* (C-O-C valence), and 1062 cm^{-1} *vs* (C-O-C valence);

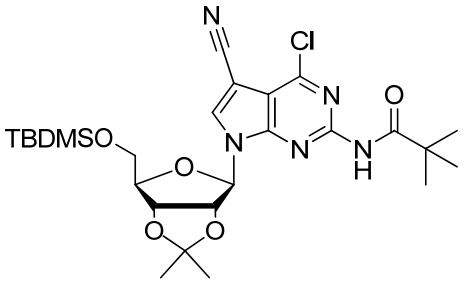
HR-MS (ESI–) for $[C_{14}H_{27}O_5Si]^-$: calc.: 303.1633, found: 303.1626.

10.11.4.6 1'-Chloro-5'-(*O*-^{t}butyl-dimethylsilyl)-2',3'-*O*-isopropylidene-*α*/*β*-D-ribofuranose (38).[259]



Sugar **41** (1.12 g, 3.70 mmol, 1.00 eq) and CCl_4 (0.50 mL, 5.92 mmol, 1.60 eq) were dissolved in dry THF (10.0 mL). HMPA (0.80 mL, 4.44 mmol, 1.20 eq) was added dropwise at -78 °C over a period of 5 min. After stirring at -78 °C for 2 h the mixture was allowed to warm to rt upon which a white solid precipitated. The obtained suspension containing chloro sugar **38** was used in the subsequent reaction *in situ*.

10.11.4.7 4-Chloro-5-cyano-7-[2',3'-(*O*-isopropylidene)-5'-(*O*-^{t}butyl-dimethylsilyl)-*β*-D-ribofuranosyl]-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine (42).



Acetonitrile (100 mL) was dried over 4 Å mole sieves for 1 h before addition of nucleobase **34** (1.10 g, 3.97 mmol, 1.00 eq) and NaH (95 %, 220 mg, 5.56 mmol, 1.40 eq). The solution was stirred at rt for 1 h. The suspension containing sugar **38** (1.80 g, 5.56 mmol, 1.40 eq) was added dropwise over a period of 20 min at 0 °C. The resulting solution was stirred at rt for 16 h. After removal of the solvent *in vacuo* the product was treated with water (50.0 mL) and ethyl acetate (50.0 mL). The layers were separated and the water layer extracted with ethyl acetate (100 mL) twice. The combined organic layers were washed with brine (200 mL) and dried over $MgSO_4$. The crude mixture was purified by column chromatography (isohexane/ethyl acetate 7/1) to yield nucleoside **42** (441 mg, 20 %) as white solid. An analytically pure sample was obtained *via* HPL chromatography.

R_f = 0.85 (isohexane/ethyl acetate 1/1);

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.51 (s, 1H; NHPiv), 8.64 (s, 1H; HC6), 6.25 (d, $^3J(HC_{1'},HC_{2'})$=1.3 Hz, 1H; $HC_{1'}$), 5.45 (dd, $^3J(HC_{2'},HC_{3'})$=6.3 Hz, $^3J(HC_{2'},HC_{1'})$=1.3 Hz, 1H; $HC_{2'}$), 5.21 (dd, $^3J(HC_{3'},HC_{2'})$=6.3 Hz, $^3J(HC_{3'},HC_{4'})$=3.7 Hz, 1H; $HC_{3'}$), 4.12 (ddd, $^3J(HC_{4'},H_aC_{5'})$=6.0 Hz, $^3J(HC_{4'},H_bC_{5'})$=5.2 Hz, $^3J(HC_{4'},HC_{3'})$=3.7 Hz, 1H; $HC_{4'}$), 3.67 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=11.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=6.0 Hz, 1H; $H_aC_{5'}$), 3.64 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=11.3 Hz, $^3J(H_bC_{5'},HC_{4'})$=5.2 Hz, 1H; $H_bC_{5'}$), 1.51 (s, 3H; *endo*-$C(CH_3)_2$), 1.32 (s, 3H; *exo*-$C(CH_3)_2$), 1.22 (s, 9H; $COC(CH_3)_3$), 0.73 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), -0.13 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and -0.16 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

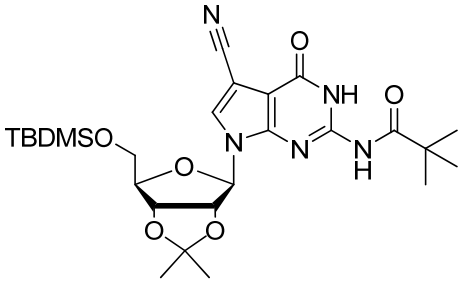
^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 175.87 ($COC(CH_3)_3$), 151.20 and 153.11 (C2 and C4), 150.94 (C7a), 138.22 (C6), 112.89 and 113.59 (C4a and CN), 112.89 ($C(CH_3)_2$),

90.59 ($C_{1'}$), 87.95 ($C_{4'}$), 84.19(C5), 83.68 ($C_{2'}$), 80.59 ($C_{3'}$), 63.36 ($C_{5'}$), 39.84 ($COC(CH_3)_3$), 26.86 (*endo*-$C(CH_3)_2$), 26.62 (3C; $COC(CH_3)_3$), 25.65 (3C; $Si(CH_3)_2C(CH_3)_3$), 25.16 (*exo*-$C(CH_3)_2$), 17.95 ($Si(CH_3)_2C(CH_3)_3$), -5.59 ($Si(CH_3)_2C(CH_3)_3$), and -5.66 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{26}H_{39}ClN_5O_5Si]^+$: calc.: 564.2404, found: 564.2368;

HPLC: gradient: 100 % hexane, 0 % ethyl acetate → 30 % isohexane, 70 % ethyl acetate in 45 min; retention time = 24.9 min; flow: 0.5 mL/min; column: CC 250/4 Nucleodur 100-5.

10.11.4.8 5-Cyano-3,4-dihydro-2-pivaloylamino-7-[2',3'-(*O*-isopropylidene)-5'-(*O*-^{t}butyl-dimethylsilyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (43).



Nucleoside **42** (74 mg, 0.13 mmol, 1.00 eq) was dissolved in DMF (2.00 mL). Et_3N (39 mg, 0.05 mL, 0.39 mmol, 3.00 eq), NaOAc (32 mg, 0.39 mmol, 3.00 eq) und DABCO (15 mg, 0.13 mmol, 1.00 eq) were added. The mixture was stirrred at rt for 48 h. Afterwards water (1.00 mL) was added and the solution was stirred for 1 h. The mixture was extracted with ethyl acetate (10.0 mL) three times. The combined organic phases were washed with brine (20.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography (isohexane/ethyl acetate 3/1) gave nucleoside **43** (58 mg, 81 %) as white foam.

R_f = 0.56 (isohexane/ethyl acetate 1/1);

^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 12.24 (s, 1H, NHCO), 11.03 (s, 1H; HNAr), 8.14 (s, 1H; HC6), 6.25 (d, $^3J(HC_{1'},HC_{2'})$=2.2 Hz, 1H; $HC_{1'}$), 5.13 (dd, $^3J(HC_{2'},HC_{3'})$=6.3 Hz, $^3J(HC_{2'},HC_{1'})$=2.2 Hz, 1H; $HC_{2'}$), 4.96 (dd, $^3J(HC_{3'},HC_{2'})$=6.3 Hz, $^3J(HC_{3'},HC_{4'})$=3.8 Hz, 1H;

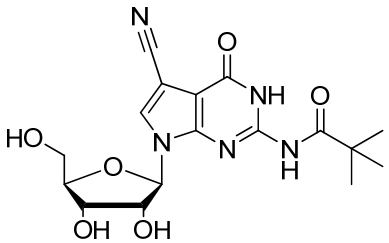
$HC_{3'}$), 4.14 (ddd, $^3J(HC_{4'},H_bC_{5'})$=4.9 Hz, $^3J(HC_{4'},H_aC_{5'})$=4.3 Hz, $^3J(HC_{4'},HC_{3'})$=3.8 Hz, 1H; $HC_{4'}$), 3.73 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=11.4 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.3 Hz, 1H; $H_aC_{5'}$), 3.69 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=11.4 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.9 Hz, 1H; $H_bC_{5'}$), 1.51 (s, 3H; *endo*-$C(CH_3)_2$), 1.32 (s, 3H; *exo*-$C(CH_3)_2$), 1.25 (s, 9H; $COC(CH_3)_3$), 0.85 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), and 0.02 ppm (s, 6H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 181.14 ($COC(CH_3)_3$), 155.35 (C4), 149.00 (C7a), 147.90 (C2), 129.73 (C6), 114.28 ($C(CH_3)_2$), 113.39 (CN), 103.10 (C4a), 88.29 ($C_{1'}$), 87.30 (C5), 86.42 ($C_{4'}$), 82.93 ($C_{2'}$), 80.44 ($C_{3'}$), 63.04 ($C_{5'}$), 40.05 ($COC(CH_3)_3$), 26.94 (*endo*-$C(CH_3)_2$), 26.25 (3C; $COC(CH_3)_3$), 25.75 (3C; $Si(CH_3)_2C(CH_3)_3$), 25.23 (*exo*-$C(CH_3)_2$), 18.03 ($Si(CH_3)_2C(CH_3)_3$), -5.51 ($Si(CH_3)_2C(CH_3)_3$), and -5.54 ppm ($Si(CH_3)_2C(CH_3)_3$);

IR: 3163 *bm* (N-H valence), 2933 *s* (C-H valence), 2232 *s* (C≡N valence), 1671 *s* (C=C valence), 1606 *s* (aromatic vibration), 1542 *m* (N-H deform.), 1250 *m* (C-O-C valence), 1077 *s* (C-O-C valence), 833 *s* (C-H deform.), and 780 cm^{-1} *s* (C-H deform.);

HR-MS (ESI+) for $[C_{26}H_{39}O_6N_5NaSi]^+$: calc.: 568.2562, found: 568.2572.

10.11.4.9 5-Cyano-3,4-dihydro-2-pivaloylamino-7-*β*-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (44).



Nucleoside **43** (18 mg, 0.03 mmol, 1.00 eq) was dissolved in water (0.15 mL) and trifluoroacetic acid (0.35 mL, 4.57 mmol, 152 eq) was added at 0 °C. The mixture was stirred at 0 °C for 4 h before removing the solvent *in vacuo* at 0 °C. The resulting solid was dissolved in water (2 mL) and stirred at rt over a period of 10 min. Concentration yielded a colorless oil, which was purified *via* column chromatography (DCM/MeOH 10/1). Nucleoside **44** (10 mg, 77 %) was obtained as a white solid.

R_f = 0.38 (DCM/MeOH 10/1);

M.p.: > 210 °C (decomposition);

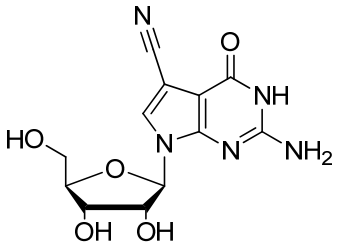
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 12.21 (s, 1H; NHCO), 11.17 (s, 1H; HNAr), 8.25 (s, 1H; HC6), 6.08 (d, $^3J(HC_{1'},HC_{2'})$=6.3 Hz, 1H; $HC_{1'}$), 4.32 (dd, $^3J(HC_{2'},HC_{1'})$=6.3 Hz, $^3J(HC_{2'},HC_{3'})$=4.5 Hz, 1H; $HC_{2'}$), 4.09 (dd, $^3J(HC_{3'},HC_{2'})$=4.5 Hz, $^3J(HC_{3'},HC_{4'})$=2.9 Hz, 1H; $HC_{3'}$), 3.89 (ddd, $^3J(HC_{4'},H_bC_{5'})$=4.4 Hz, $^3J(HC_{4'},H_aC_{5'})$=4.3 Hz, $^3J(HC_{4'},HC_{3'})$=2.9 Hz, 1H; $HC_{4'}$), 3.61 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.0 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.3 Hz, 1H; $H_aC_{5'}$), 3.54 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.0 Hz, $^3J(H_bC_{5'},HC_{4'})$= 4.4Hz, 1H; $H_bC_{5'}$), and 1.25 ppm (s, 9H; $COC(CH_3)_3$);

^{13}C NMR (101 MHz, D_6-DMSO, 27 °C): δ = 181.30 ($COC(CH_3)_3$), 155.38 (C4), 148.96 (C2), 148.61 (C7a), 129.62 (C6), 114.63 (CN), 102.92 (C4a), 87.05 (C5), 86.33 ($C_{1'}$), 85.43 ($C_{4'}$), 74.23 ($C_{2'}$), 70.35 ($C_{3'}$), 61.21 ($C_{5'}$), 40.04 ($COC(CH_3)_3$), and 26.23 (3C; $COC(CH_3)_3$);

IR: 3482 *bm* (O-H valence), 3417 *s* (O-H valence), 3152 *bm* (N-H valence), 2966 *m* (C-H valence), 2884 *m* (C-H valence), 2233 *m* (C≡N valence), 1693 *s* (C=C valence), 1659 *s* (C=C valence), 1598 *m* (N-H deform.), 1557 *m* (N-H deform.), 1538 *m* (N-H deform.), 1411 *m* (C-H deform.), 1091 *m* (C-O-C valence), and 775 cm^{-1} *m* (C-H deform.);

HR-MS (ESI+) for $[C_{17}H_{21}N_5O_6Na]^+$: calc.: 414.1384, found: 414.1363.

10.11.4.10 2-Amino-5-cyano-4-oxo-7-*β*-D-ribofuranosyl-7*H*-pyrrolo-[2,3-*d*]-pyrimidine ($PreQ_0$).[228-229]



Nucleoside **44** (18 mg, 0.05 mmol) was dissolved in NH_3 in H_2O (28 %, 4 mL). The solution was stirred at 60 °C for 17 h. Subsequent removal of the solvent *in vacuo* resulted in a colorless oil, which was purified *via* column chromatography (ethyl acetate/MeOH 7/1) yielding $PreQ_0$ (14 mg, 99 %) as white solid.

R_f = 0.30 (ethyl acetate/MeOH 5/1);

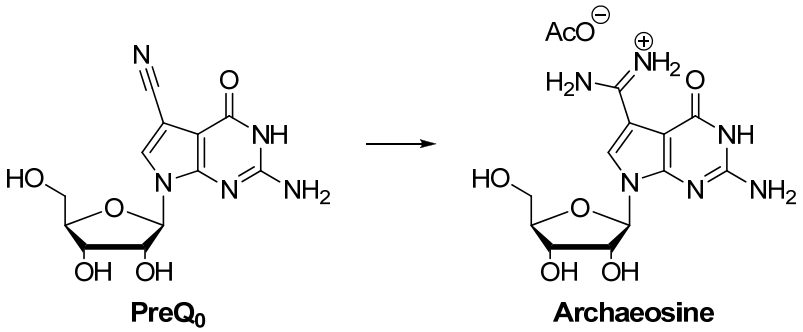
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 10.86 (s, 1H; HNAr), 7.89 (s, 1H; HC6), 6.62 (s, 2H; NH_2), 6.05 (d, $^3J(HC_{1'},HC_{2'})$=5.9 Hz, 1H; $HC_{1'}$), 4.28 (dd, $^3J(HC_{2'},HC_{1'})$=5.9 Hz, $^3J(HC_{2'},HC_{3'})$=4.8 Hz, 1H; $HC_{2'}$), 4.03 (dd, $^3J(HC_{3'},HC_{2'})$=4.8 Hz, $^3J(HC_{3'},HC_{4'})$=3.7 Hz, 1H; $HC_{3'}$), 3.82 (ddd, $^3J(HC_{4'},H_aC_{5'})$=4.1 Hz, $^3J(HC_{4'},H_bC_{5'})$=4.0 Hz, $^3J(HC_{4'},HC_{3'})$=3.7 Hz, 1H; $HC_{4'}$), 3.57 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.9 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.1 Hz, 1H; $H_aC_{5'}$), and 3.50 ppm (dd, $^2J(H_bC_{5'},H_aC_{5'})$=11.9 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.0 Hz, 1H; $H_bC_{5'}$);

^{13}C NMR (151 MHz, D_6-DMSO, 27 °C): δ = 157.21 (C4), 154.02 (C2), 151.38 (C7a), 127.48 (C6), 115.32 (CN), 98.62 (C4a), 86.48 and 86.34 ($C_{1'}$ and C5), 85.09 ($C_{4'}$), 74.07 ($C_{2'}$), 70.23 ($C_{3'}$), and 61.23 ppm ($C_{5'}$);

IR: 3305 *s* (N-H valence), 3219 *s* (N-H valence), 2935 *m* (C-H valence), 2229 *m* (C≡N valence), 1673 *s* (C=O valence), 1633 *s* (N-H deform.), 1591 *m* (N-H deform.), 1561 *m* (N-H deform.), 1426 *w* (C-H deform.), 1104 *m* (C-O-C valence), and 1047 cm^{-1} *m* (C-O-C valence);

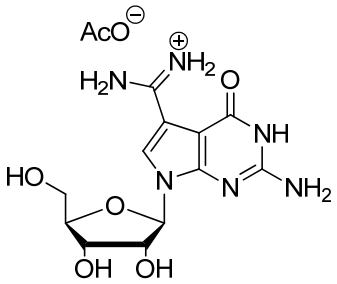
HR-MS (ESI–) for $[C_{12}H_{12}N_5O_5]^-$: calc.: 306.0844, found: 306.0837.

10.11.5 Synthesis of archaeosine



Scheme 52: Synthesis of archaeosine.
Reagents and conditions: HCl (g), MeOH, 7 M NH_3 in MeOH, rt, 19 h, 30 %.

10.11.5.1 2-Amino-5-amidinium-7-*β*-D-ribofuranosyl-7*H*-pyrrolo-[2,3-*d*]-pyrimidine-4-one acetate.[218]



$PreQ_0$ (10.0 mg, 0.03 mmol) was dissolved in MeOH (2.00 mL) and treated with gaseous HCl for 3 h. Subsequently, nitrogen was bubbled through the solution to remove the solvent. The resulting white solid was stirred in NH_3 in MeOH (7 N, 2.00 mL) for 16 h. Removal of the solvent *in vacuo* yielded a white solid, which was purified by HPL chromatography. The solvent was removed to give a 2 to 1 mixture of the desired product and triethylammonium acetate (4 mg, 30 %). Complete removal of the buffer lead to decomposition of the product yielding the corresponding nitrile compound.

R_f = 0.57 (isopropanol/water/glacial acid 5/1/1);

M.p.: decomposition > 215 °C;

^{1}H NMR (600 MHz, D_2O, 27 °C): δ = 8.03 (s, 1H; HC6), 6.06 (d, $^3J(HC_{1'},HC_{2'})$=5.9 Hz, 1H; $HC_{1'}$), 4.61 (dd, $^3J(HC_{2'},HC_{1'})$=5.9 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.38 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=3.9 Hz, 1H; $HC_{3'}$), 4.24 (ddd, $^3J(HC_{4'},H_bC_{5'})$=4.2 Hz, $^3J(HC_{4'},HC_{3'})$=3.9 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.1 Hz, 1H; $HC_{4'}$), 3.90 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.9 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.1 Hz, 1H; $H_aC_{5'}$), 3.84 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.9 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.2 Hz, 1H; $H_bC_{5'}$), and 1.93 ppm (s, 3H; H_3CCOO^-);

^{13}C NMR (151 MHz, D_2O, 27 °C): δ = 215.59 ($H_3C\mathit{C}OO^-$), 153.39, 154.75, 159.21, and 162.19 (C7a, $C_{amidine}$, C2, and C4), 125.32 (C6), 107.38 (C5), 98.16 (C4a), 87.94 ($C_{1'}$), 85.27 ($C_{4'}$), 74.18 ($C_{2'}$), 70.55 ($C_{3'}$), 61.50 ($C_{5'}$), and 23.43 ppm ($\mathit{C}H_3COO^-$);

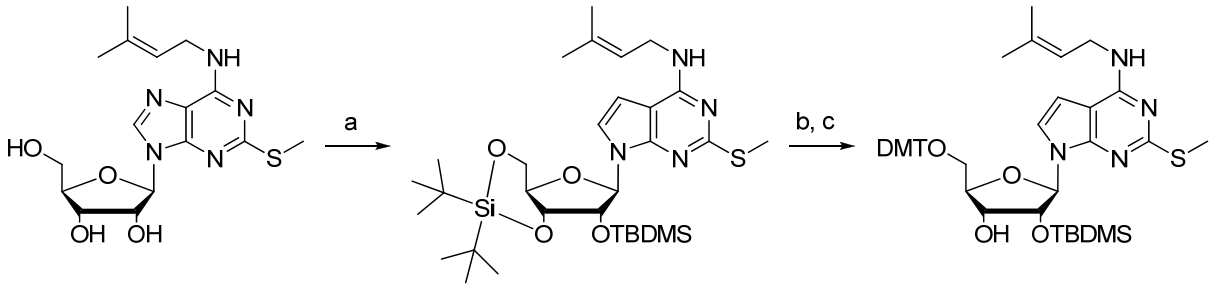
IR: 3368 *s* (N-H and O-H valence), 3176 *s* (N-H and O-H valence), 2931 *s* (N-H and O-H valence), 1660 *s* (C=N valence), 1560 *m* (N-H deform.), 1058 *s* (C-O valence), and 1023 cm^{-1} *s* (C-O valence);

HR-MS (ESI–) for $[C_{12}H_{15}N_6O_5]^-$: calc.: 323.1109, found: 323.1116;

HPLC: gradient: 100 % HPLC buffer E, 0 % HPLC buffer F → 80 % HPLC buffer E, 20 % HPLC buffer F in 45 min; retention time = 22.2 min; flow: 0.5 mL/min; column: CC 250/4 Nucleosil 120-3-C18.

10.12 Synthesis of DMT-TBDMS-protected RNA nucleosides

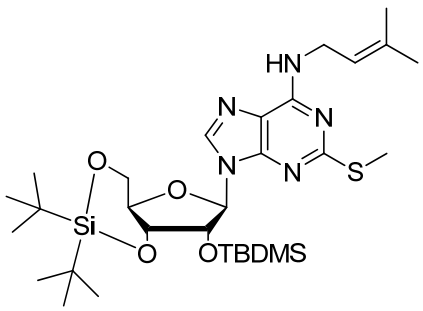
10.12.1 Synthesis of DMT-TBDMS-protected ms^{2}i^{6}A



ms^{2}i^{6}A **47** **46**

Scheme 53: Synthesis of nucleoside **46**.
Reagents and conditions: a) ^{t}Bu$_2$Si(OTf)$_2$, imidazole, TBDMSCl, DMF, 0 °C to 60 °C, 3 h; b) HF-pyridine, pyridine, DCM, MeOTMS, 0 °C, 2 h; c) DMTCl, pyridine, MeOH, 0 °C, 16 h, 52 % (3 steps).

10.12.1.1 6-(3-Methyl-2-butenyl-1-amino)-2-methylthio-9-[2'-(*O*-^{t}butyl-dimethylsilyl)-3',5'-(*O*-di-^{t}butylsilandiyl)-*β*-D-ribofuranosyl]-9*H*-purine (47).



Nucleoside ms^{2}i^{6}A (326 mg, 0.86 mmol, 1.00 eq) was dissolved in dry DMF (1.70 ml) and cooled to 0 °C. Di-^{t}butyl-silyl-ditriflate (306 µl, 0.95 mmol, 1.10 eq) was added dropwise. After stirring for 30 min at 0 °C imidazole (291 mg, 4.28 mmol, 5.00 eq) was added and the

reaction mixture was allowed to warm to rt. The mixture was stirred for 30 min and TBDMSCl (158 mg, 1.03 mmol, 1.20 eq) was added. The solution was heated to 60 °C for 2 h. The reaction was stopped by addition of H_2O (10.0 mL). The aq. layer was extracted three times with DCM (10.0 mL). The combined organic phases were extracted with brine (10 ml) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was applied in the next reaction without further purification. An analytical sample of nucleoside **47** was obtained *via* column chromatography (DCM/MeOH 100/1).

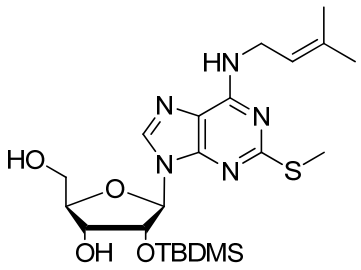
R_f = 0.64 (DCM/MeOH 40/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 7.50 (s, 1H; HC8), 5.75 (s, 1H; $HC_{1'}$), 5.53 (s, 1H; HN), 5.26 – 5.22 (m, 1H; $CH_2CHC(CH_3)_2$), 4.59 (d, $^3J(HC_{2'},HC_{3'})$=4.9 Hz, 1H; $HC_{2'}$), 4.37 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=9.2 Hz, $^3J(H_aC_{5'},HC_{4'})$=5.1 Hz, 1H; $H_aC_{5'}$), 4.32 (dd, $^3J(HC_{3'},HC_{4'})$=9.5 Hz, $^3J(HC_{3'},HC_{2'})$=4.9 Hz, 1H; $HC_{3'}$), 4.11 – 4.05 (m, 3H; $HC_{4'}$ and $CH_2CHC(CH_3)_2$), 3.92 (dd, $^3J(HC_{4'},H_bC_{5'})$=10.3 Hz, $^2J(H_bC_{5'},H_aC_{5'})$=9.3 Hz, 1H; $H_bC_{5'}$), 2.47 (s, 3H; SCH_3), 1.67 (s, 3H; $CH_2CHC(CH_3)_2$), 1.65 (s, 3H; $CH_2CHC(CH_3)_2$), 1.00 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 0.97 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 0.84 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.07 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and 0.06 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 166.47 (C2), 154.05 (C4), 149.23 (C6), 137.24 (C8), 136.94 ($CH_2CHC(CH_3)_2$), 120.44 ($CH_2CHC(CH_3)_2$), 118.08 (C5), 92.52 ($C_{1'}$), 76.33 ($C_{3'}$), 75.33 ($C_{2'}$), 74.65 ($C_{4'}$), 68.00 ($C_{5'}$), 38.61 ($CH_2CHC(CH_3)_2$), 27.76 (3C; $O_2Si(C(CH_3)_3)_2$), 27.27 (3C; $O_2Si(C(CH_3)_3)_2$), 26.12 (3C; $Si(CH_3)_2(C(CH_3)_3)$, 25.90 ($CH_2CHC(CH_3)_2$), 22.92 ($O_2Si(C(CH_3)_3)_2$), 20.60 ($O_2Si(C(CH_3)_3)_2$), 18.57 ($Si(CH_3)_2(C(CH_3)_3)$, 18.22 ($CH_2CHC(CH_3)_2$), 14.78 (SCH_3), -4.08 ($Si(CH_3)_2C(CH_3)_3$), and -4.74 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{30}H_{54}N_5O_4SSi_2]^+$: calc.: 636.3429, found: 636.3430.

10.12.1.2 6-(3-Methyl-2-butenyl-1-amino)-2-methylthio-9-(2'-(*O*-^{t}butyl-dimethylsilyl)-β-D-ribofuranosyl)-9*H*-purine (47a).



The equivalents given in this protocol refer to the amount of ms^2i^6A employed in this three step reaction sequence. HF-pyridine (86.0 µL, 0.85 mmol, 1.00 eq) was diluted in dry pyridine (534 mL, 6.62 mmol, 8.00 eq) at 0 °C. The total crude product of the preceding reaction was dissolved in dry DCM (4.30 mL) and cooled to 0 °C. The HF-pyridine solution was added dropwise to the nucleoside solution. The resulting mixture was stirred at 0 °C for 2 h. MeOTMS (594 µL, 4.34 mmol, 5.00 eq) was added and the reaction was allowed to warm to rt and stirred for 30 min. The solvent was removed *in vacuo* and the resulting oil was treated with H_2O (10.0 mL) and DCM (10.0 mL). The aq. layer was extracted with DCM (10.0 mL) three times. The combined organic phases were extracted with brine (10.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was applied in the next reaction without further purification. An analytical sample of nucleoside **47a** was obtained *via* column chromatography (DCM/MeOH 50/1).

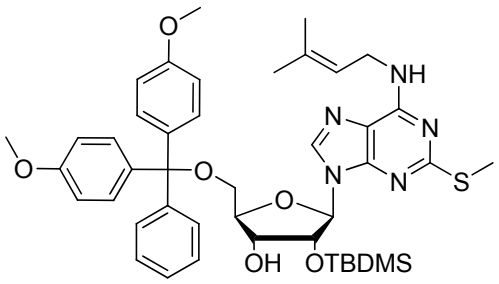
R_f = 0.34 (DCM/MeOH 40/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 7.56 (s, 1H; HC8), 5.72 (s, 1H; HN), 5.64 (d, $^3J(HC_{1'},HC_{2'})$=7.3 Hz, 1H; $HC_{1'}$), 5.34 (m, 1H; $CH_2CHC(CH_3)_2$), 5.16 (dd, $^3J(C_{2'}H,C_{1'}H)$=7.2 Hz, $^3J(HC_{2'},HC_{3'})$=4.9 Hz, 1H; $HC_{2'}$), 4.32 (d, $^3J(HC_{3'},HC_{2'})$=4.9 Hz, 1H; $HC_{3'}$), 4.30 (m, 1H; $HC_{4'}$), 4.20 (m, 2H; $CH_2CHC(CH_3)_2$), 3.94 (m, 1H; $H_aC_{5'}$), 3.72 (m, 1H; $H_bC_{5'}$), 2.57 (s, 3H; SCH_3), 1.74 (s, 3H; $CH_2CHC(CH_3)_2$), 1.60 (s, 3H; $CH_2CHC(CH_3)_2$), 0.79 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), -0.15 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and -0.36 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 166.50 (C2), 154.09 (C4), 148.90 (C6), 139.25 (C8), 137.20 ($CH_2CHC(CH_3)_2$), 120.16 ($CH_2CHC(CH_3)_2$), 119.15 (C5), 91.19 ($C_{1'}$), 87.60 ($C_{4'}$), 74.06 ($C_{2'}$), 73.07 ($C_{3'}$), 63.69 ($C_{5'}$), 38.63 ($CH_2CHC(CH_3)_2$), 25.91 ($CH_2CHC(CH_3)_2$), 25.77

($Si(CH_3)_2(C(CH_3)_3)$, 18.23 (3C; $CH_2CHC(CH_3)_2$), 18.05 ($Si(CH_3)_2(C(CH_3)_3)$, 14.80 (SCH_3), -5.06 ($Si(CH_3)_2C(CH_3)_3$), and -5.16 ppm ($Si(CH_3)_2C(CH_3)_3$).

10.12.1.3 6-(3-methyl-2-butenyl-1-amino)-2-methylthio-9-(5'-*O*-dimethoxytrityl-2'-(*O*-^{t}butyl-dimethylsilanyl)-*β*-D-ribofuranosyl)-9*H*-purine (46).



The equivalents given in this protocol refer to the amount of ms^2i^6A employed in this three step reaction sequence. The total crude product of the preceding reaction was dissolved in dry pyridine (1.70 mL) and cooled to 0 °C. Dimethoxytrityl chloride (320 mg, 0.95 mmol, 1.10 eq) was added the resulting mixture was stirred at 0 °C for 16 h. Methanol (38.0 µL) was added and the solution was stirred for 30 min at rt. DCM (15.0 mL) and water (10.0 mL) were added and the layers were separated. The DCM layer was extracted with sat. $NaHCO_3$ solution (10.0 mL) three times. The combined organic layers were dried over $MgSO_4$. The solvent was removed and the oil was applied to column chromatography (isohexane/ethyl acetate 10/1 to 2/1, 0.5 vol% Et_3N). Nucleoside **46** (352 mg, 52 %, 3 steps) was obtained as white foam.

R_f = 0.10 (DCM/MeOH 40/1);

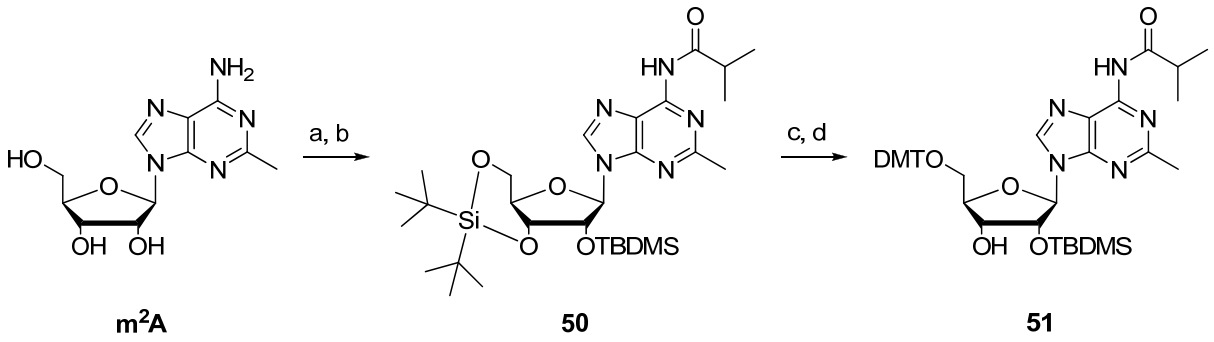
^{1}H NMR (600 MHz, $CDCl_3$, 27 °C) δ = 7.80 (s, 1H; HC8), 7.37 (d, $^3J(H_{ortho}Ph,H_{meta}Ph)$=7.5 Hz, 2H; $H_{ortho}Ph$), 7.27 – 7.23 (m, 4H; $H_{ortho}PhOMe$), 7.19 (dd, $^3J(H_{meta}Ph,H_{ortho}Ph)$=7.5 Hz, $^3J(H_{meta}Ph,H_{para}Ph)$=7.3 Hz, 2H; $H_{meta}Ph$), 7.18 (t, $^3J(H_{para}Ph,H_{meta}Ph)$=7.3 Hz, 1H; $H_{para}Ph$), 6.78 – 6.72 (m, 4H; $H_{meta}PhOMe$), 5.91 (d, $^3J(HC_{1'},HC_{2'})$=4.3 Hz, 1H; $HC_{1'}$), 5.72 (s, 1H; HN), 5.36 – 5.30 (m, 1H; $CH_2CHC(CH_3)_2$), 4.66 (dd, $^3J(HC_{2'},HC_{3'})$=4.5 Hz, $^3J(HC_{2'},HC_{1'})$=4.3 Hz, 1H; $HC_{2'}$), 4.62 (dd, $^3J(HC_{3'},HC_{4'})$=4.9 Hz, $^3J(HC_{3'},HC_{2'})$=4.5 Hz, 1H; $HC_{3'}$), 4.22 – 4.13 (m, 2H; $CH_2CHC(CH_3)_2$), 4.13 – 4.10 (m, 1H; $HC_{4'}$), 3.75 (s, 6H; $PhOCH_3$), 3.40 (dd,

$^{2}J(H_aC_{5'},H_bC_{5'})$=10.5 Hz, $^{3}J(H_aC_{5'},HC_{4'})$=3.5 Hz, 1H; $H_aC_{5'}$), 3.26 (dd, $^{2}J(H_bC_{5'},H_aC_{5'})$=10.6 Hz, $^{3}J(H_bC_{5'},HC_{4'})$=4.5 Hz, 1H; $H_bC_{5'}$), 2.48 (s, 3H; SCH_3), 1.73 (s, 3H; $CH_2CHC(CH_3)_2$), 1.71 (s, 3H; $CH_2CHC(CH_3)_2$), 0.85 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.10 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and 0.01 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C) δ = 166.08 (C2), 158.71 (C4), 158.69 (2C; C_{para}PhOMe), 154.02 (C6), 144.65 (C_{ipso}Ph), 137.91 (C8), 136.78 ($CH_2CHC(CH_3)_2$), 135.94 (2C; C_{ipso}PhOMe), 130.24 (4C; C_{ortho}PhOMe), 128.36 (2C; C_{ortho}Ph), 128.02 (2C; C_{meta}Ph), 127.04 (2C; C_{para}Ph), 120.54 ($CH_2CHC(CH_3)_2$), 118.02 (C5), 113.34 (4C; C_{meta}PhOMe), 89.56 ($C_{1'}$), 86.66 ($CPh(PhOMe)_2$), 84.37 ($C_{4'}$), 74.61 ($C_{2'}$), 72.41 ($C_{3'}$), 63.33 ($C_{5'}$), 55.40 (2C; OCH_3), 41.13 ($CH_2CHC(CH_3)_2$), 25.94 (3C; $Si(CH_3)_2(C(CH_3)_3$), 25.89 ($CH_2CHC(CH_3)_2$), 18.25 ($Si(CH_3)_2(C(CH_3)_3$), 18.21 ($CH_2CHC(CH_3)_2$), 14.77 (SCH_3), and -4.52 ppm (2C; $Si(CH_3)_2(C(CH_3)_3$);

HR-MS (ESI+) for $[C_{43}H_{56}N_5O_6SSi]^+$: calc.: 798.3714, found: 798.3745.

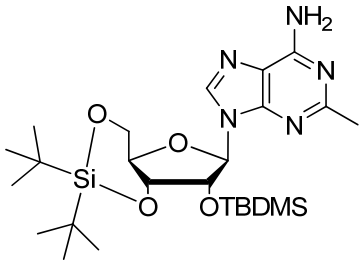
10.12.2 Synthesis of DMT-TBDMS-protected m^2A



m^2A **50** **51**

Scheme 54: Synthesis of nucleoside **51**.
Reagents and conditions: a) $^{t}Bu_2Si(OTf)_2$, TBDMSCl, DMF, -5 °C to rt, 21 h; b) Isobutyryl chloride, pyridine, DMF, 0 °C, 25 min; c) HF-pyridine, pyridine, DCM, MeOTMS, -13 °C to rt, 2 h; d) DMTCl, pyridine, MeOH, rt, 3.5 h, 37 % (4 steps).

10.12.2.1 6-Amino-2-methyl-9-(2'-(*O*-^{t}butyl-dimethylsilyl)-3',5'-(*O*-di-^{t}butylsilandiyl)-β-D-ribofuranosyl)-9*H*-purine (48).



Nucleoside m^2A (700 mg, 2.50 mmol, 1.00 eq) was dissolved in dry DMF (5.00 mL) and cooled to -5 °C. Di-^{t}butyl-silyl-ditriflate (1.62 ml, 5.00 mmol, 2.00 eq) was added dropwise. After stirring for 5 h at -5 °C TBDMSCl (755 mg, 5.00 mmol, 2.00 eq) was added. The cooling bath was removed and the reaction was stirred for 16 h at rt. The reaction was stopped by addition of water (20.0 mL). The aq. layer was extracted three times with DCM (20.0 mL). The combined organic phases were extracted with brine (20.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was applied in the next reaction without further purification. An analytical sample of nucleoside **48** was obtained *via* column chromatography (DCM/MeOH 100/1).

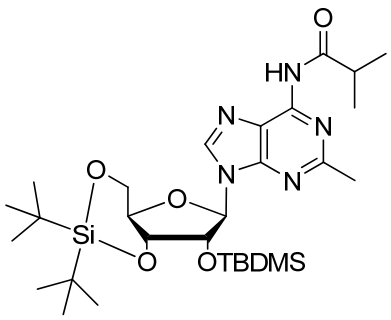
R_f = 0.50 (DCM/MeOH 8/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 7.72 (s, 1H; HC8), 5.84 (s, 1H; $HC_{1'}$), 4.56 (d, $^3J(HC_{2'},HC_{3'})$=4.7 Hz, 1H; $HC_{2'}$), 4.52 (dd, $^3J(HC_{3'},HC_{4'})$=9.5 Hz, $^3J(HC_{3'},HC_{2'})$=4.7 Hz, 1H; $HC_{3'}$), 4.47 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=9.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=5.1 Hz, 1H; $H_aC_{5'}$), 4.20 (ddd, $^3J(HC_{4'},H_bC_{5'})$=10.3 Hz, $^3J(HC_{4'},HC_{3'})$=9.5 Hz, $^3J(HC_{4'},H_aC_{5'})$=5.1 Hz, 1H; $HC_{4'}$), 4.02 (dd, $^3J(H_bC_{5'},HC_{4'})$=10.3 Hz, $^2J(H_bC_{5'},H_aC_{5'})$=9.3 Hz, 1H; $H_bC_{5'}$), 2.52 (s, 3H; CH_3), 1.03 (s, 18H; $O_2Si(C(CH_3)_3)_2$), 0.92 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.16 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and 0.13 ppm (s, 2H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 162.81 (C2), 155.18 (C4), 149.92 (C6), 138.30 (C8), 118.26 (C5), 92.84 ($C_{1'}$), 75.91 ($C_{3'}$), 75.75 ($C_{2'}$), 74.95 ($C_{4'}$), 68.07 ($C_{5'}$), 27.50 (3C; $O_2Si(C(CH_3)_3)_2$), 27.50 (3C; $O_2Si(C(CH_3)_3)_2$), 26.14 (3C; $Si(CH_3)_2C(CH_3)_3$), 25.69 (CH_3), 22.97 ($O_2Si(C(CH_3)_3)_2$), 20.57 ($O_2Si(C(CH_3)_3)_2$), 18.56 ($Si(CH_3)_2C(CH_3)_3$), -4.07 ($Si(CH_3)_2C(CH_3)_3$), and -4.76 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{25}H_{46}N_5O_4Si_2]^+$: calc.: 536.3083, found: 536.3086.

10.12.2.2 6-*Iso*butyrylamido-2-methyl-9-(2'-(*O*-^{t}butyl-dimethylsilyl)-3',5'-(*O*-di-^{t}butylsilandiyl)-β-D-ribofuranosyl)-9*H*-purine (50).



The equivalents given in this protocol refer to the amount of m^2A employed in this four step reaction sequence. The total crude product of the preceding reaction was dissolved in dry DMF (0.50 mL) and dry pyridine (0.16 mL) and cooled to 0 °C. *Iso*butyryl chloride (522 µl, 5.00 mmol, 2.00 eq) was added. After stirring for 25 min at 0 °C DCM (10.0 mL) and water (10.0 mL) were added. The layers were separated and the aq. layer was extracted with DCM (10.0 mL) three times. The combined organic phases were extracted with brine (20.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was applied in the next reaction without further purification. An analytical sample of nucleoside **50** was obtained *via* column chromatography (DCM/MeOH 200/1).

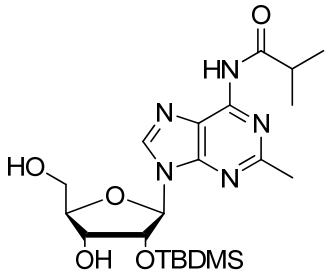
R_f = 0.83 (DCM/MeOH 8/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 7.94 (s, 1H; HC8), 5.91 (s, 1H; $HC_{1'}$), 4.54 (d, $^3J(HC_{2'},HC_{3'})$=4.9 Hz, 1H; $HC_{2'}$), 4.48 (dd, $^3J(HC_{3'},HC_{4'})$=9.2 Hz, $^3J(HC_{3'},HC_{2'})$=4.9 Hz, 1H; $HC_{3'}$), 4.44 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=9.5 Hz, $^3J(H_aC_{5'},HC_{4'})$=4.9 Hz, 1H; $H_aC_{5'}$), 4.22 (ddd, $^3J(HC_{4'},H_bC_{5'})$=10.4 Hz, $^3J(HC_{4'},HC_{3'})$=9.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=4.9 Hz, 1H; $HC_{4'}$), 4.01 (dd, $^3J(H_bC_{5'},HC_{4'})$=10.4 Hz, $^2J(H_bC_{5'},H_aC_{5'})$=9.5 Hz, 1H; $H_bC_{5'}$), 3.19 – 3.08 (m, 1H; $COCH(CH_3)_2$), 2.66 (s, 3H; CH_3), 1.27 (d, $^3J(COCH(CH_3)_2,COCH(CH_3)_2)$=3.6 Hz, 3H; $COCH(CH_3)_2$), 1.26 (d, $^3J(COCH(CH_3)_2,COCH(CH_3)_2)$=3.6 Hz, 3H; $COCH(CH_3)_2$), 1.05 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 1.02 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 0.92 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.16 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and 0.14 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 181.91 (*C*OCH(CH_3)$_2$), 162.67 (C2), 151.72 (C6), 149.36 (C4), 140.49 (C8), 121.27 (C5), 92.72 ($C_{1'}$), 76.00 ($C_{3'}$), 75.79 ($C_{2'}$), 74.96 ($C_{4'}$), 68.03 ($C_{5'}$), 36.10 (CO*C*H(CH_3)$_2$). 27.70 (3C; $O_2Si(C(CH_3)_3)_2$), 27.21 (3C; $O_2Si(C(CH_3)_3)_2$), 26.12 (3C; $Si(CH_3)_2C(CH_3)_3$), 25.79 (CH_3) 22.96 ($O_2Si(C(CH_3)_3)_2$), 20.55 ($O_2Si(C(CH_3)_3)_2$), 19.42 (COCH(CH_3)$_2$), 19.39 (COCH(CH_3)$_2$), 18.55 ($Si(CH_3)_2C(CH_3)_3$), -4.06 ($Si(CH_3)_2C(CH_3)_3$), and -4.78 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{29}H_{52}N_5O_5Si_2]^+$: calc.: 606.3502, found: 606.3512.

10.12.2.3 6-*Iso*butyrylamido-2-methyl-9-(2'-(*O*-^{t}butyl-dimethylsilyl)-*β*-D-ribofuranosyl)-9*H*-purine (50a).



The equivalents given in this protocol refer to the amount of m^2A employed in this four step reaction sequence. The total crude product of the preceding reaction was dissolved in dry DCM (12.0 mL) and the solution cooled to -13 °C. HF-pyridine (250 µL, 2.50 mmol, 1.00 eq) was diluted in dry pyridine (1.50 mL). The HF-pyridine solution was added dropwise to the nucleoside solution at -13 °C. The resulting mixture was stirred at -13 °C for 2 h. MeOTMS (1.40 mL, 10.0 mmol, 4.00 eq) was added and the reaction was allowed to warm to rt and stirred for 30 min. Water (10.0 mL), DCM (10.0 mL), and sat. $NaHCO_3$ (10.0 mL) were added. The layers were separated and the aq. layer was extracted three times with DCM (10.0 mL). The combined organic phases were extracted with brine (30.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was applied in the next reaction without further purification. An analytical sample of nucleoside **50a** was obtained *via* column chromatography (DCM/MeOH 100/1).

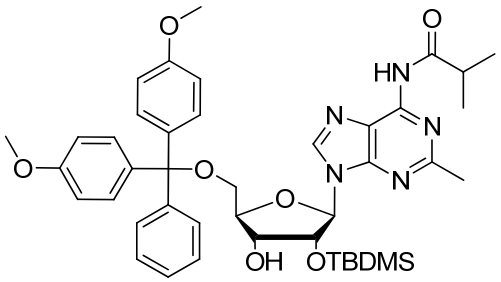
R_f = 0.81 (DCM/MeOH 8/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 7.87 (s, 1H; HC8), 5.75 (d, $^{3}J(HC_{1'},HC_{2'})$=7.4 Hz, 1H; $HC_{1'}$), 5.10 (dd, $^{3}J(HC_{2'},HC_{1'})$=7.4, $^{3}J(HC_{2'},HC_{3'})$=4.8 Hz, 1H; $HC_{2'}$), 4.38 – 4.35 (m, 1H; $HC_{4'}$), 4.34 (d, $^{3}J(HC_{3'},HC_{2'})$=4.8 Hz, 1H; $HC_{3'}$), 3.98 – 3.93 (m, 1H; $H_aC_{5'}$), 3.78 – 3.71 (m, 1H; $H_bC_{5'}$), 3.31 – 3.22 (m, 1H; $COCH(CH_3)_2$), 2.72 (s, 3H; CH_3), 1.30 (d, $^{3}J(COCH(CH_3)_2,COCH(CH_3)_2)$=4.1 Hz, 3H; $COCH(CH_3)_2$), 1.29 (d, $^{3}J(COCH(CH_3)_2,COCH(CH_3)_2)$=4.1 Hz, 3H; $COCH(CH_3)_2$), 0.78 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), -0.19 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and -0.41 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 176.39 ($COCH(CH_3)_2$), 162.78 (C2), 150.91 (C4), 149.89 (C6), 142.43 (C8), 121.79 (C5), 91.52 ($C_{1'}$), 87.88 ($C_{4'}$), 74.46 ($C_{2'}$), 73.09 ($C_{3'}$), 63.61 ($C_{5'}$), 36.31 ($COCH(CH_3)_2$), 25.81 (CH_3), 25.72 ($Si(CH_3)_2(C(CH_3)_3$), 19.36 ($COCH(CH_3)_2$), 19.30 (3C; $COCH(CH_3)_2$), 18.04 ($Si(CH_3)_2(C(CH_3)_3$), -5.06 ($Si(CH_3)_2C(CH_3)_3$), and -5.21 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{21}H_{36}N_5O_5Si]^+$: calc.: 466.2480, found: 466.2485.

10.12.2.4 6-*Iso*butyrylamido-2-methyl-9-(5'-*O*-dimethoxytrityl-2'-(*O*-^{t}butyl-dimethylsilanyl)-*β*-D-ribofuranosyl)-9*H*-purine (51).



The equivalents given in this protocol refer to the amount of m^2A employed in this four step reaction sequence. The total crude product of the preceding reaction was dissolved in dry pyridine (1.60 mL). Dimethoxytrityl chloride (72 mg, 0.21 mmol, 1.20 eq) was added and the resulting mixture was stirred at rt for 3 h. Water (1.00 mL) was added and the solution was stirred for additional 30 min at rt. DCM (10.0 mL) and water (10.0 mL) were added and the layers were separated. The aq. layer was extracted three times with DCM (10.0 mL). The combined organic layers were extracted with brine (20.0 mL) and dried over $MgSO_4$. The solvent was removed and the oil was purified *via* column chromatography (isohexane/ethyl

acetate 10/1 to 2/1, 1.0 vol% Et_3N). Nucleoside **51** (50 mg, 37 %, 4 steps) was obtained as white foam.

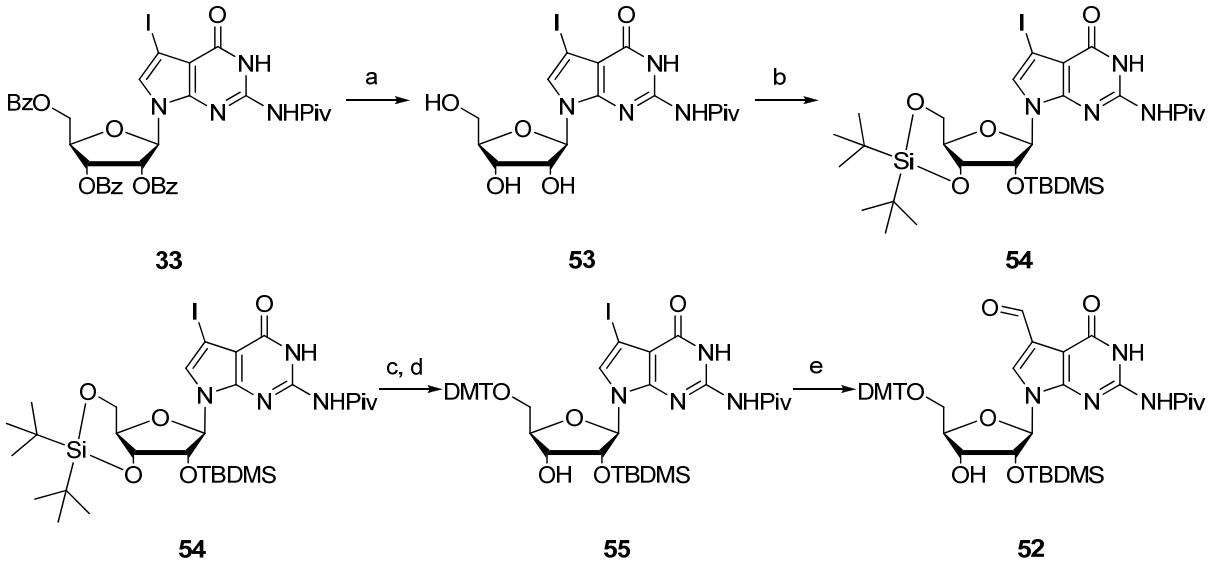
R_f = 0.90 (DCM/MeOH 18/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C) δ = 8.08 (s, 1H; HC8), 7.42 – 7.37 (m, 2H; H_{ortho}Ph), 7.31 – 7.26 (m, 4H; H_{ortho}PhOMe), 7.23 – 7.19 (m, 2H; H_{meta}Ph), 7.17 – 7.14 (m, 1H; H_{para}Ph), 6.78 – 6.73 (m, 4H; H_{meta}PhOMe), 6.02 (d, $^3J(HC_{1'},HC_{2'})$=4.8 Hz, 1H; $HC_{1'}$), 4.88 (dd, $^3J(HC_{2'},HC_{3'})$=4.9 Hz, $^3J(HC_{2'},HC_{1'})$=4.8 Hz, 1H; $HC_{2'}$), 4.32 (dd, $^3J(HC_{3'},HC_{2'})$=4.9 Hz, $^3J(HC_{3'},HC_{4'})$=4.2 Hz, 1H; $HC_{3'}$), 4.22 (ddd, $^3J(HC_{4'},HC_{3'})$=4.2 Hz, $^3J(HC_{4'},H_bC_{5'})$=4.1 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.2 Hz, 1H; $HC_{4'}$), 3.72 (s, 3H; PhOCH_3), 3.72 (s, 3H; PhOCH_3), 3.49 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=10.6 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_aC_{5'}$), 3.36 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=10.6 Hz, $^3J(H_bC_{5'},HC_{4'})$=4.1 Hz, 1H; $H_bC_{5'}$), 3.21 – 3.14 (m, 1H; COC$H(CH_3)_2$), 2.55 (s, 3H; CH_3), 1.26 (d, $^3J(COCHC(CH_3)_2,COCHC(CH_3)_2)$=0.6 Hz, 3H; $COCHC(CH_3)_2$), 1.24 (d, $^3J(COCHC(CH_3)_2,COCHC(CH_3)_2)$=0.6 Hz, 3H; $COCHC(CH_3)_2$), 0.81 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), -0.03 (s, 3H; $Si(CH_3)_2C(CH_3)_3$) and -0.13 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C) δ = 176.36 (COCH$(CH_3)_2$), 162.66 (C2), 158.69 (2C; C_{para}PhOMe), 152.11 (C6), 149.02 (C4), 144.66 (C_{ipso}Ph), 140.87 (C8), 135.74 (C_{ipso}PhOMe), 135.69 (C_{ipso}PhOMe), 130.20 (2C; C_{ortho}PhOMe), 130.19 (2C; C_{ortho}PhOMe), 128.24 (2C; C_{ortho}Ph), 128.01 (2C; C_{meta}Ph), 127.07 (C_{para}Ph), 120.74 (C5), 113.32 (2C; C_{meta}PhOMe), 113.32 (2C; C_{meta}PhOMe), 88.58 ($C_{1'}$), 86.72 (CPh$(PhOMe)_2$), 84.15 ($C_{4'}$), 75.87 ($C_{2'}$), 71.42 ($C_{3'}$), 63.47 ($C_{5'}$), 55.31 (2C; OCH_3), 36.10 (COCH$(CH_3)_2$), 26.02 (CH_3), 25.71 (3C; $Si(CH_3)_2(C(CH_3)_3$), 19.32 (COCH(CH_3)$_2$), 19.31 (COCH(CH_3)$_2$), 18.06 ($Si(CH_3)_2(C(CH_3)_3$), -4.81 ($Si(CH_3)_2(C(CH_3)_3$), and -4.52 ppm ($Si(CH_3)_2(C(CH_3)_3$);

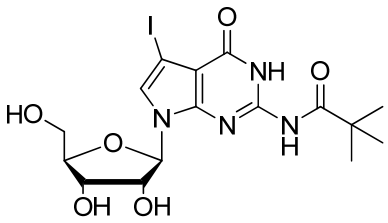
HR-MS (ESI+) for $[C_{42}H_{54}N_5O_7Si]^+$: calc.: 768.3787, found: 768.3797.

10.12.3 Synthesis of DMT-TBDMS-protected formyl-deazaguanosine



Scheme 55: Synthesis of nucleoside **52**.
Reagents and conditions: a) 2 M NaOH, dioxane, rt, 50 min, 60 %; b) $^{t}Bu_2Si(OTf)_2$, imidazole, TBDMSCl, DMF, 0 °C to rt, 22 h; c) HF-pyridine, pyridine, DCM, MeOTMS, -5 °C to rt, 3 h; d) DMTCl, pyridine, MeOH, rt, 16 h, 69 % (3 steps); e) $Pd_2(dba)_3$, PPh_3, CO, toluene, 55 °C, 1 h, 81 %.

10.12.3.1 3,4-Dihydro-5-iodo-2-pivaloylamino-7-*β*-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (53).



Nucleoside **33** (1.00 g, 2.03 mmol, 1.00 eq) was dissolved in dioxane (10.0 mL) and aq. NaOH (2 M, 5.00 mL) was added dropwise. The resulting suspension was stirred for 50 min at rt. The solution was neutralized with HCl (2 M). The solvent was removed *in vacuo* and the resulting oil was dissolved in water (10.0 mL). The pH of the mixture was adjusted to 3.0 with HCl (2 M). The suspension was extracted with diethyl ether (10.0 mL) twice. The combined organic layers were extracted with water (10.0 mL). The combined aqueous layers were neutralized with NaOH (2 M) and the solvent was removed *in vacuo*. Nucleoside **53** (0.37 g, 60 %) was obtained after column chromatography (DCM/MeOH 20/1) as almost colorless oil.

R_f = 0.52 (DCM/MeOH 8/1);

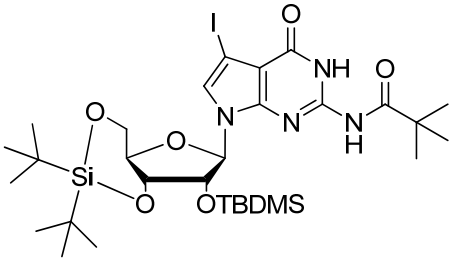
^{1}H NMR (400 MHz, D_6-DMSO, 27 °C): δ = 7.43 (s, 1H; HC6), 6.13 (d, $^3J(HC_{1'},HC_{2'})$=5.5 Hz, 1H; $HC_{1'}$), 4.34 (dd, $^3J(HC_{2'},HC_{1'})$=5.5 Hz, $^3J(HC_{2'},HC_{3'})$=5.4 Hz, 1H; $HC_{2'}$), 4.24 (dd, $^3J(HC_{3'},HC_{2'})$=5.4 Hz, $^3J(HC_{3'},HC_{4'})$=3.7 Hz, 1H; $HC_{3'}$), 4.02 (ddd, $^3J(HC_{4'},HC_{3'})$=3.7 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.6 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.2 Hz, 1H; $HC_{4'}$), 3.81 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.2 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_aC_{5'}$), 3.49 (dd, $^3J(H_bC_{5'},H_aC_{5'})$=12.2 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.6 Hz, 1H; $H_bC_{5'}$), and 1.31 ppm ($COC(CH_3)_3$);

^{13}C NMR: δ = 180.1, 156.5, 146.0, 127.2, 106.3, 91.3, 86.2, 73.8, 72.2, 63.3, 54.0, 40.3, and 27.1 ppm; E. M. Jahn, personal communication;

IR: 3319 *m* (O-H valence), 1668 *s* (C=O valence), 1605 *s* (aromatic vibration), 1539 *m* (N-H deform.), 1420 *m* (C-H deform.), 1050 *m* (C-O valence), 777 cm^{-1} *w* (C-Hal valence); E. M. Jahn, personal communication;

HR-MS (ESI+) for $[C_{16}H_{21}IN_4O_6]^+$: calc.: 493.0579, found: 493.0574; E. M. Jahn, personal communication.

10.12.3.2 3,4-Dihydro-5-iodo-2-pivaloylamino-7-[(2'-(*O*-^{t}butyl-dimethylsilyl)-3',5'-(*O*-di-^{t}butylsilandiyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (54).



Nucleoside **53** (367 mg, 0.75 mmol, 1.00 eq) was dissolved in dry DMF (5.00 mL) and cooled to 0 °C. Di-^{t}butyl-silyl-ditriflate (0.49 mL, 662 mg, 1.50 mmol, 2.00 eq) was added dropwise. After stirring for 15 min at -5 °C imidazole (254 mg, 3.73 mmol, 5.00 eq) was added and the solution was stirred at rt for 6 h. TBDMSCl (227 mg, 1.50 mmol, 2.00 eq) was added and the mixture was stirred at rt for 16 h. The aq. layer was extracted with DCM (20.0 mL) three times. The combined organic phases were extracted with brine (20.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was used in the next reaction

without further purification. An analytical sample of nucleoside **54** was obtained *via* column chromatography (DCM/MeOH 200/1).

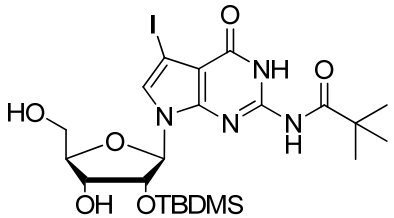
R_f = 0.24 (DCM/MeOH 50/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.65 (s, 1H; NHCO), 7.82 (s, 1H; HNAr), 6.79 (s, 1H; HC6), 5.92 (s, 1H; $HC_{1'}$), 4.46 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=9.3 Hz, $^3J(H_aC_{5'},HC_{4'})$=5.1 Hz, 1H; $H_aC_{5'}$), 4.25 (d, $^3J(HC_{2'},HC_{3'})$=4.8 Hz, 1H; $HC_{2'}$), 4.12 (ddd, $^3J(HC_{4'},HC_{5'})$=10.2 Hz, $^3J(HC_{4'},HC_{3'})$=10.1 Hz, $^3J(HC_{4'},H_aC_{5'})$=5.1 Hz, 1H; $HC_{4'}$), 4.01 (dd, $^3J(HC_{3'},HC_{2'})$=4.8 Hz, $^3J(HC_{3'},HC_{4'})$=10.1 Hz, 1H; $HC_{3'}$), 3.99 (dd, $^3J(H_bC_{5'},HC_{4'})$=10.2 Hz, $^3J(H_aC_{5'}, H_bC_{5'})$=9.3 Hz, 1H; $H_bC_{5'}$) 1.30 (s, 9H; $COC(CH_3)_3$), 1.05 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 1.01 (s, 9H; $O_2Si(C(CH_3)_3)_2$), 0.91 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.12 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and 0.11 ppm (s, 3H, $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 179.55 ($COC(CH_3)_3$), 156.88 (C4), 146.65 (C7a), 146.51 (C2), 123.80 (C6), 106.26 (C4a), 91.97 ($C_{1'}$), 76.73 ($C_{3'}$), 76.38 ($C_{2'}$), 74.44 ($C_{4'}$), 68.06 ($C_{5'}$), 56.09 (C5), 40.38 ($COC(CH_3)_3$), 27.71 (3C; $O_2Si(C(CH_3)_3)_2$), 27.26 (3C; $COC(CH_3)_3$), 27.23 (3C; $O_2Si(C(CH_3)_3)_2$), 26.14 (3C; $Si(CH_3)_2(C(CH_3)_3)$, 23.00 ($O_2Si(C(CH_3)_3)_2$), 20.58 ($O_2Si(C(CH_3)_3)_2$), 18.68 ($Si(CH_3)_2(C(CH_3)_3)$, -3.96 ($Si(CH_3)_2C(CH_3)_3$), and -4.76 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{30}H_{52}IN_4O_6Si_2]^+$: calc.: 747.2465, found: 747.2477.

10.12.3.3 3,4-Dihydro-5-iodo-2-pivaloylamino-7-[(2'-*O*-tert-butyl-dimethylsilyl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (54a).



The equivalents given in this protocol refer to the amount of nucleoside **54** employed in this three step reaction sequence. The total crude product of the preceding reaction was dissolved in dry DCM (3.75 mL) and cooled to -5 °C. HF-pyridine (0.08 mL, 2.77 mmol, 2.00 eq) in

dry pyridine (0.47 mL) was added dropwise. After stirring for 3 h at -5 °C excess MeOTMS (0.43 mL, 3.12 mmol, 4.16 eq) was added and the solution was stirred at rt for 30 min. Sat. $NaHCO_3$ (5.00 mL) and DCM (5.00 mL) were added. The layers were separated and the organic layer was extracted with DCM (20.0 mL) twice. The combined organic phases were extracted with brine (20.0 mL) and dried over $MgSO_4$. Removal of the solvent gave a white foam, which was used in the next reaction without further purification. An analytical sample of nucleoside **54a** was obtained *via* column chromatography (DCM/MeOH 100/1).

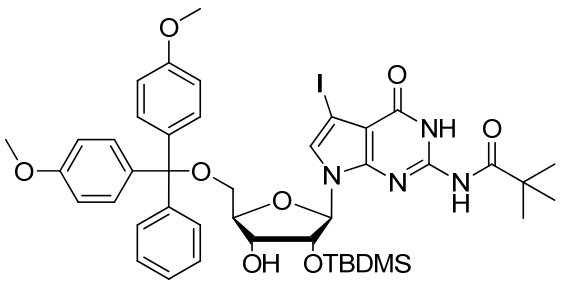
R_f = 0.61 (DCM/MeOH 8/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.68 (s, 1H; NHCO), 8.07 (s, 1H; HNAr), 6.86 (s, 1H; HC6), 5.52 (d, $^3J(HC_{1'},HC_{2'})$=7.1 Hz, 1H; $HC_{1'}$), 4.72 (dd, $^3J(HC_{2'},HC_{1'})$=7.1 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.28 (m, 1H; $HC_{4'}$), 4.23 (d, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, 1H; $HC_{3'}$), 3.92 (dd, $^3J(H_aC_{5'},H_bC_{5'})$=12.3 Hz, $^3J(H_aC_{5'}H,C_{4'})$=2.0 Hz, 1H; $H_aC_{5'}$), 3.74 (m, 1H; $H_bC_{5'}$), 1.31 (s, 9H; $COC(CH_3)_3$), 0.82 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), -0.13 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and -0.28 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 179.93 ($COC(CH_3)_3$), 156.53 (C4), 146.62 (C7a), 145.42 (C2), 127.98 (C6), 107.82 (C4a), 92.99 ($C_{1'}$), 86.86 ($C_{4'}$), 75.02 ($C_{2'}$), 72.61 ($C_{3'}$), 63.63 ($C_{5'}$), 54.57 (C5), 40.50 ($COC(CH_3)_3$), 27.17 (3C; $COC(CH_3)_3$), 25.74 (3C; $Si(CH_3)_2(C(CH_3)_3$), 18.12 ($Si(CH_3)_2(C(CH_3)_3$), -5.08 ($Si(CH_3)_2C(CH_3)_3$), and -5.13 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{22}H_{36}IN_4O_6Si]^+$: calc.: 607.1443, found: 607.1454.

10.12.3.4 3,4-Dihydro-5-iodo-2-pivaloylamino-7-[(2'-*O*-^{t}butyl-dimethylsilyl)-(5'-*O*-dimethoxytrityl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (55).



The equivalents given in this protocol refer to the amount of nucleoside **54** employed in this three step reaction sequence. The total crude product of the preceding reaction was dissolved in dry pyridine (4.00 mL) and DMTCl (304 mg, 0.90 mmol, 1.20 eq) was added. After stirring for 16 h at rt MeOH (4.00 mL) was added and the solution was concentrated to about 4 mL *in vacuo*. DCM (20.0 mL), water (10.0 mL), and sat. $NaHCO_3$ solution (10.0 mL) were added. The layers were separated and the aqueous layer was extracted with DCM (20.0 mL). The combined organic phases were extracted with brine (20.0 mL) and dried over $MgSO_4$. Nucleoside **55** (473 mg, 69 %, 3 steps) was obtained *via* column chromatography (isohexane/ethyl acetate 10/1 to 2/1) as yellow foam.

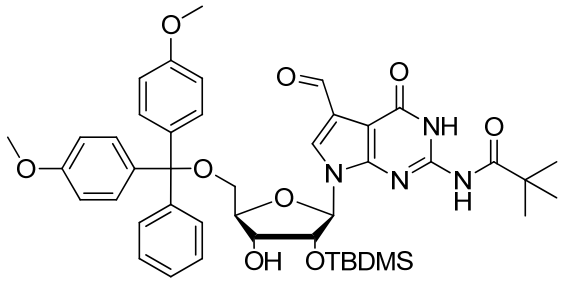
R_f = 0.32 (DCM/MeOH 50/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.84 (s, 1H; NHCO), 8.47 (s, 1H; HNAr), 7.53 - 7.49 (m, 2H; H_{ortho}Ph), 7.43 – 7.39 (m, 4H; H_{ortho}PhOMe), 7.38 – 7.34 (m, 2H; H_{meta}Ph), 7.35 (s, 1H; HC6), 7.31 – 7.27 (m, 1H; H_{para}Ph), 6.93 – 6.88 (m, 4H; H_{meta}PhOMe), 6.02 (d, $^3J(HC_{1'},HC_{2'})$=6.3 Hz, 1H; $HC_{1'}$), 4.66 (dd, $^3J(HC_{2'},HC_{1'})$=6.3 Hz, $^3J(HC_{2'},HC_{3'})$=5.2 Hz, 1H; $HC_{2'}$), 4.31 (dd, $^3J(HC_{3'},HC_{2'})$=5.2 Hz, $^3J(HC_{3'},HC_{4'})$=2.4 Hz, 1H; $HC_{3'}$), 4.27 (ddd, $^3J(HC_{4'},H_bC_{5'})$=3.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=2.4 Hz, $^3J(HC_{4'},HC_{3'})$=2.4 Hz, 1H; $HC_{4'}$), 3.86 – 3.83 (m, 6H; PhOCH_3), 3.50 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=10.7 Hz, $^3J(H_aC_{5'},HC_{4'})$=2.4 Hz, 1H; $H_aC_{5'}$), 3.41 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=10.7 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_bC_{5'}$), 1.32 (s, 9H; $COC(CH_3)_3$), 0.89 (s, 9H; $Si(CH_3)_2C(CH_3)_3$), 0.06 (s, 3H; $Si(CH_3)_2C(CH_3)_3$), and -0.12 ppm (s, 3H; $Si(CH_3)_2C(CH_3)_3$);

^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 179.95 ($COC(CH_3)_3$), 158.77 (C_{para}PhOMe), 158.76 (C_{para}PhOMe), 157.01 (C4), 147.89 (C7a), 146.64 (C2), 144.63 (C_{ipso}Ph), 135.70 (C_{ipso}PhOMe), 135.58 (C_{ipso}PhOMe), 130.26 (2C; C_{ortho}PhOMe), 130.16 (2C; C_{ortho}PhOMe), 128.22 (2C; C_{ortho}Ph), 128.16 (2C; C_{meta}Ph), 127.17 (C_{para}Ph), 124.64 (C6), 113.45 (4C; C_{meta}PhOMe), 105.90 (C4a), 87.17 ($C_{1'}$), 86.94 (CPh(PhOMe)$_2$), 84.05 ($C_{4'}$), 76.75 ($C_{2'}$), 72.01 ($C_{3'}$), 63.90 ($C_{5'}$), 55.86 (C5), 55.38 (OCH_3), 55.37 (OCH_3), 40.28 ($COC(CH_3)_3$), 27.00 (3C; $COC(CH_3)_3$), 25.63 (3C; $Si(CH_3)_2(C(CH_3)_3$), 18.04 ($Si(CH_3)_2(C(CH_3)_3$), -5.06 ($Si(CH_3)_2C(CH_3)_3$), and -5.09 ppm ($Si(CH_3)_2C(CH_3)_3$);

HR-MS (ESI+) for $[C_{43}H_{54}IN_4O_8Si]^+$: calc.: 909.2750, found: 909.2779.

10.12.3.5 3,4-Dihydro-5-formyl-2-pivaloylamino-7-[(2'-*O*-tert-butyl-dimethylsilyl)-(5'-*O*-dimethoxytrityl)-*β*-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (52).



Under argon atmosphere nucleoside **55** (473 mg, 0.52 mmol, 1.00 eq), $Pd_2(dba)_3$ (54 mg, 0.05 mmol, 0.10 eq), PPh_3 (81 mg, 0.31 mmol, 0.60 eq) was dissolved in dry toluene (6.40 mL). The solution was cooled to -78 °C. High vacuum was applied for 2 sec and the flask was ventilated with argon. This approach was repeated three times. After application of high vacuum the flask was ventilated with CO. Again this procedure was repeated three times. CO atmosphere was sustained in the flask afterwards using a CO balloon. The solution was heated to 55 °C and stirred. Tributyltin hydride (0.21 mL, 0.62 mmol, 1.20 eq) in toluene (3.00 mL) was added dropwise over a period of 1 h. Afterwards the reaction was stirred at 55 °C for 20 min and allowed to cool to rt. The solvent was removed *in vacuo*. The resulting solid was purified *via* column chromatography (isohexane/ethyl acetate 5/1 to 2/1) yielding nucleoside **52** (341 mg, 81 %) as white solid.

R_f = 0.14 (DCM/MeOH 50/1);

M.p.: 56.8 – 57.3 °C;

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.99 (s, 1H; NHCO), 10.15 (s, 1H; HCO) 8.58 (s, 1H; HNAr), 7.71 (s, 1H; HC6), 7.66 – 7.58 (m, 2H; HAr), 7.51 – 7.46 (m, 4H; HAr), 7.44 - 7.36 (m, 2H; HAr), 7.33 – 7.29 (m, 1H; HAr), 6.80 – 6.75 (m, 4H; H_{meta}PhOMe), 5.93 (d, ^3J($HC_{1'}$,$HC_{2'}$)=6.0 Hz, 1H; $HC_{1'}$), 4.52 (dd, ^3J($HC_{2'}$,$HC_{1'}$)=6.0 Hz, ^3J($HC_{2'}$,$HC_{3'}$)=4.7 Hz, 1H; $HC_{2'}$), 4.20 – 4.16 (m, 2H; $HC_{3'}$ and $HC_{4'}$), 3.74 – 3.71 (m, 6H; PhOCH_3), 3.41 – 3.35 (m, 1H; $H_aC_{5'}$), 3.34 – 3.29 (m, 1H; $H_bC_{5'}$), 1.19 (s, 9H; COC(CH_3)$_3$), 0.78 (s, 9H; Si(CH_3)$_2$C(CH_3)$_3$), -0.07 (s, 3H; Si(CH_3)$_2$C(CH_3)$_3$), and -0.25 ppm (s, 3H; Si(CH_3)$_2$C(CH_3)$_3$);

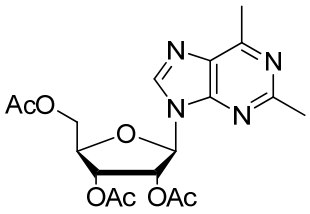
^{13}C NMR (151 MHz, $CDCl_3$, 27 °C): δ = 185.61 (HCO), 180.24 (*C*OC(CH$_3$)$_3$), 158.79 (2C; C$_{para}$PhOMe), 156.97 (C4), 147.48 and 147.51 (C7a and C2), 144.73 (C$_{ipso}$Ph), 132.99 (C$_{ipso}$PhOMe), 132.30 (C$_{ipso}$PhOMe), 130.20 (4C; C$_{ortho}$PhOMe), 128.13 (2C; C$_{ortho}$Ph), 127.17 (C$_{para}$Ph), 124.68 (C6), 113.43 (4C; C$_{meta}$PhOMe), 103.87 (C4a), 87.68 (C$_{1'}$), 86.87 (*C*Ph(PhOMe)$_2$), 84.28 (C$_{4'}$), 76.90 (C$_{2'}$), 71.77 (C$_{3'}$), 63.73 (C$_{5'}$), 55.36 (3C; C5 and OCH$_3$), 40.33 (CO*C*(CH$_3$)$_3$), 26.94 (3C; COC(*C*H$_3$)$_3$), 25.63 (3C; Si(CH$_3$)$_2$(C(*C*H$_3$)$_3$), 17.69 (Si(CH$_3$)$_2$(*C*(CH$_3$)$_3$), -5.01 (Si(*C*H$_3$)$_2$C(CH$_3$)$_3$), and -5.07 ppm (Si(*C*H$_3$)$_2$C(CH$_3$)$_3$);

IR: 3199 *bw* (O-H valence, N-H valence), 2956 *m* (-CH$_3$ and C-H valence), 2930 *m* (C-H valence), 2856 *w* (CH$_3$ valence), 1662 *s* (C=O valence), 1604 *s* (C=O valence), 1508 *s* (N-H deform.), 1248 *s* (C-N valence), and 1175 cm^{-1} *s* (C-O valence);

HR-MS (ESI-) for $[C_{44}H_{53}N_4O_9Si]^-$: calc.: 809.3587, found: 809.3579.

10.13 Syntheses of further nucleosides

10.13.1 2,4-Dimethyl-9-(2',3',5'-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (45a).



$ZnCl_2$ (1.0 M in diethyl ether, 0.28 mL, 0.28 mmol, 1.50 eq) was cooled to 0 °C and slowly treated with MeMgCl (2.56 M in THF, 0.22 mL, 0.56 mmol, 3.00 eq). The suspension was stirred for 2 h. Nucleoside **33** (100 mg, 0.19 mmol, 1.00 eq) and $Pd(PPh_3)_4$ (22 mg, 0.02 mmol, 0.10 eq) were dissolved in dry THF (1.00 mL) and cooled to 0 °C. The suspension was added to the nucleoside solution. The resulting mixture was stirred at rt for 18 h. Sat. NaH_2PO_4 solution (10.0 mL) was added to the reaction mixture. The aqueous phase was extracted with DCM (15.0 mL) three times. The combined organic phases were washed with brine (30.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography yielded 4-chloro-2-methyl-9-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine **45** (32 mg, 40 %) and

2,4-dimethyl-9-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine **45a** (33 mg, 43%) as white foams.

Nucleoside **45**: see chapter 10.8.1.2

Nucleoside **45a**:

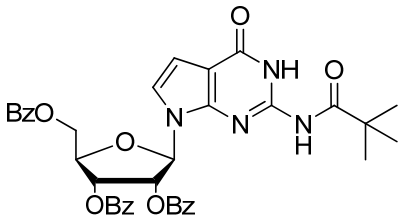
R_f = 0.32 (DCM/MeOH 18/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 8.07 (s, 1H; HC_6), 6.17 (d, $^3J(HC_{1'},HC_{2'})$=5.0 Hz, 1H; $HC_{1'}$), 5.98 (dd, $^3J(HC_{2'},HC_{1'})$=5.0 Hz, $^3J(HC_{2'},HC_{3'})$=5.0 Hz, 1H; $HC_{2'}$), 5.77 (dd, $^3J(HC_{3'},HC_{2'})$=5.0 Hz, 1H; $HC_{3'}$), 4.45 (m, 2H; $H_aC_{5'}$ and $HC_{4'}$), 4.37 (m, 1H; $H_bC_{5'}$), 2.81 (s, 3H; H_3C), 2.78 (s, 3H; H_3C), 2.15 (s, 3H; H_3CCO), 2.09 (s, 3H; H_3CCO), and 2.08 ppm (s, 3H; H_3CCO);

^{13}C NMR (150 MHz, $CDCl_3$, 27 °C): δ = 170.28 (CH_3CO), 169.52 (CH_3CO), 169.34 (CH_3CO), 162.43 (C6), 159.42 (C2), 150.61 (C4), 141.47 (C8), 131.45 (C5), 86.39 ($C_{1'}$), 80.16 ($C_{4'}$), 73.02 ($C_{2'}$), 70.59 ($C_{3'}$), 63.09 ($C_{5'}$), 25.96 (CH_3), 20.69 (CH_3CO), 20.53 (CH_3CO), 20.39 (CH_3CO), and 19.46 ppm (CH_3);

HR-MS (ESI+) for $[C_{18}H_{23}N_4O_7]^+$: calc.: 407.1561, found: 407.1548.

10.13.2 3,4-Dihydro-7-[2',3',5'-*O*-tribenzoyl-*β*-D-ribofuranosyl]-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one.



Side product during Grignard reaction of the 7-iodo-deazaG (Chapter 10.11.2.1).

R_f = 0.24 (isohexane/ethyl acetate 1/1);

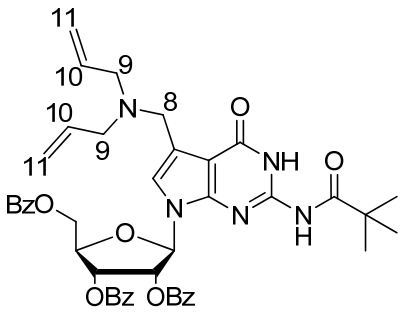
^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.62 (s, 1H; NHCO), 8.75 (s, 1H; HNAr), 8.00 (m, 2H; HBz_{ortho}), 7.91 (m, 2H; HBz_{ortho}), 7.76 (m, 2H; HBz_{ortho}), 7.57 (m, 3H; HBz_{para}), 7.44 (m,

2H; HBz_{meta}), 7.38 (m, 2H; HBz_{meta}), 7.32 (m, 2H; HBz_{meta}), 6.94 (dd, $^3J(HC_{3'},HC_{4'})$=8.3 Hz, $^3J(HC_{3'},HC_{2'})$=5.0 Hz, 1H; $HC_{3'}$), 6.87 (d, ^3J(HC5/6,HC5/6)=3.5 Hz, 1H; HC5/6), 6.68 (d, ^3J(HC5/6,HC5/6)=3.5 Hz, 1H; HC5/6), 6.52 (dd, $^3J(HC_{2'},HC_{3'})$=5.0 Hz, $^3J(HC_{2'},HC_{1'})$=1.6 Hz, 1H; $HC_{2'}$), 6.08 (d, $^3J(HC_{1'},HC_{2'})$=1.6 Hz, 1H; $HC_{1'}$), 4.78 (ddd, $^3J(HC_{4'},HC_{3'})$=8.3 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.2 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.0 Hz, 1H; $HC_{4'}$), 4.72 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=12.5 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.0 Hz, 1H; $H_aC_{5'}$), 4.65 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=12.5 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.2 Hz, 1H; $H_bC_{5'}$), and 1.36 (s, 9H; $COC(CH_3)_3$);

^{13}C NMR (125 MHz, $CDCl_3$, 25 C): δ = 179.91 (COC(CH$_3$)$_3$), 166.11 (PhCO), 165.95 (PhCO), 165.17 (PhCO), 157.52 (C4), 146.13 and 146.33 (C7a and C2), 133.72 (Bz_{para}), 133.68 (Bz_{para}), 133.46 (Bz_{para}), 129.80 (2C; Bz_{ortho}), 129.75 (2C; Bz_{ortho}), 129.26 (2C; Bz_{ortho}), 129.06 (Bz_{ipso}), 128.89 (Bz_{ipso}), 128.79(Bz_{ipso}), 128.58 (2C; Bz_{meta}), 128.49 (2C; Bz_{meta}), 128.36 (2C; Bz_{meta}), 104.39, 106.52, and 122.47 (C4a, C5, and C6), 89.56 ($C_{1'}$), 78.31 ($C_{4'}$), 74.63 ($C_{2'}$), 70.70 ($C_{3'}$), 61.62 ($C_{5'}$), 40.18 (3C; COC(CH$_3$)$_3$), and 26.92 ppm (COC(CH$_3$)$_3$);

HR-MS (ESI-) for $[C_{37}H_{33}N_4O_9]^-$: calc.: 677.2253, found: 677.2256.

10.13.3 5-(*N*,*N*-Diallylamino)methyl-3,4-dihydro-7-(2',3',5'-*O*-tribenzoyl-*β*-D-ribofuranosyl)-2-pivaloylamino-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (60).



Nucleoside **33** (50 mg, 0.06 mmol, 1.00 eq) was dissolved in dry toluene (0.50 mL) and cooled to -65 °C. MeMgCl (2.56 M in THF, 0.05 mL, 0.12 mmol, 2.00 eq) was added dropwise and the resulting mixture was stirred for 1 h. Over a period of 1 h ^{i}PrMgCl•LiCl (1.06 M in THF, 0.15 mL, 0.13 mmol, 2.5 eq) was added with a syringe pump. The solution was stirred for 4 h at -65 °C. A solution of N,N,N',N'-tetraallylmethanediamine (134 mg, 0.62 mmol, 10.0 eq) in dry DCM was cooled to 0 °C and trifluoroacetic acid anhydride

(0.09 mL, 0.62 mmol, 10.0 eq) was added dropwise. The resulting solution was stirred for 2 h at rt and then cooled to -20 °C. The Grignard solution prepared beforehand was added to the solution of the electrophile. The resulting mixture was left to warm to rt over 16 h. Water (20.0 mL) and ethyl acetate (20.0 mL) were added to the solution. The aq. layer was extracted with ethyl acetate (20.0 mL) three times. The combined organic layers were washed with brine (20.0 mL) and dried over $MgSO_4$. Purification *via* column chromatography yielded nucleoside **60** (25 mg, 51 %) as white solid.

R_f = 0.45 (isohexane/ethyl acetate 1/1);

^{1}H NMR (600 MHz, $CDCl_3$, 27 °C): δ = 11.61 (s, 1H; NHCO), 8.89 (s, 1H; HNAr), 8.00 (m, 2H; HBz_{ortho}), 7.89 (m, 2H; HBz_{ortho}), 7.70 (m, 2H; HBz_{ortho}), 7.61 (m, 1H; HBz_{para}), 7.55 (m, 1H; HBz_{para}), 7.51 (m, 1H; HBz_{para}), 7.46 (m, 2H; HBz_{meta}), 7.36 (m, 2H; HBz_{meta}), 7.28 (m, 2H; HBz_{meta}), 7.13 (dd, $^3J(HC_{3'},HC_{4'})$=9.0 Hz, $^3J(HC_{3'},HC_{2'})$=5.0 Hz, 1H; $HC_{3'}$), 6.78 (d, $^3J(HC_{1'},HC_{2'})$=0.8 Hz, 1H; $HC_{1'}$), 6.62 (dd, $^3J(HC_{2'},HC_{3'})$=5.0 Hz, $^3J(HC_{2'},HC_{1'})$=0.8 Hz, 1H; $HC_{2'}$), 6.52 (s, 1H; HC6), 5.77 (dddd, $^3J(C10H,C11H_{trans}H_{cis})_2$)=17.1 Hz, $^3J(C10H,C11H_{trans}H_{cis})_2$)=10.2 Hz, $^3J(C10H,C9H_aH_b)$=6.7 Hz, $^3J(C10H,C9H_aH_b)$=6.3 Hz, 2H; C10H), 5.14 (dd, $^3J(C11H_{trans}H_{cis},C10H)$=17.1 Hz, $^2J(C11H_{trans}H_{cis},C11H_{trans}H_{cis})_2$)=1.4 Hz, 2H; $C11H_{trans}H_{cis}$), 5.07 (dd, $^3J(C11H_{trans}H_{cis},C10H)$=10.2 Hz, $^2J(C11H_{trans}H_{cis},C11H_{trans}H_{cis})$=1.4 Hz, 2H; $C11H_{trans}H_{cis}$), 4.74 (ddd, $^3J(HC_{4'},HC_{3'})$=9.0 Hz, $^3J(HC_{4'},H_bC_{5'})$=3.1 Hz, $^3J(HC_{4'},H_aC_{5'})$=3.0 Hz, 1H; $HC_{4'}$), 4.72 (dd, $^2J(H_aC_{5'},H_bC_{5'})$=11.9 Hz, $^3J(H_aC_{5'},HC_{4'})$=3.0 Hz, 1H; $H_aC_{5'}$), 4.62 (dd, $^2J(H_bC_{5'},H_aC_{5'})$=11.9 Hz, $^3J(H_bC_{5'},HC_{4'})$=3.1 Hz, 1H; $H_bC_{5'}$), 3.87 (d, $^2J(C8H_aH_b,C8H_aH_b)$=13.9 Hz, 1H; $C8H_aH_b$), 3.51 (d, $^2J(C8H_aH_b,C8H_aH_b)$=13.9 Hz, 1H; $C8H_aH_b$), 3.16 (dd, $^2J(C9_aH_b,C9H_aH_b)$=14.0 Hz, $^3J(C9_aH_b,C10H)$=6.3 Hz, 2H; $C9_aH_b$), 2.97 (dd, $^2J(C9H_aH_b,C9H_aH_b)$=14.0 Hz, $^3J(C9_aH_b,C10H)$=6.7 Hz, 2H; $C9_aH_b$), and 1.35 ppm (s, 9H; $COC(CH_3)_3$);

^{13}C NMR (125 MHz, $CDCl_3$, 25 C): δ = 180.00 ($COC(CH_3)_3$), 166.17 (PhCO), 166.13 (PhCO), 165.18 (PhCO), 157.31 (C4), 147.56 (C7a), 145.84 (C2), 134.80 (2C; C10), 133.58 (C_{para}), 133.55 (C_{para}), 133.37 (C_{para}), 131.97 (C5), 129.73 (2C; CBz_{ortho}), 129.69 (2C; CBz_{ortho}), 129.26 (CBz_{ipso}), 129.21 (2C; CBz_{ortho}), 129.14 (CBz_{ipso}), 128.93 (CBz_{ipso}), 128.55 (2C; CBz_{meta}), 128.44 (2C; CBz_{meta}), 128.27 (2C; CBz_{meta}), 117.99 (2C; C11), 105.25 (C4a), 105.11 (C6), 87.30 ($C_{1'}$), 77.43 ($C_{4'}$), 74.95 ($C_{2'}$), 70.74 ($C_{3'}$), 61.43 ($C_{5'}$), 55.86 (2C; C9), 49.93 (C8), 40.16 ($COC(CH_3)_3$), and 26.87 ppm (3C; $COC(CH_3)_3$);

IR: 3349 *w* (N-H valence), 3216 *w* (N-H valence), 2958 *m* (C-H valence), 2924 *m* (C-H valence), 1722 *s* (C=O valence), 1669 *s* (C=O valence), 1603 *s* (C=O valence), and 1262 cm^{-1} *s* (C-O-C valence);

HR-MS (ESI+) for $[C_{44}H_{46}N_5O_9]^+$: calc.: 788.3290, found: 788.3282.

11 Appendix

11.1 Abbreviations

A	Adenosine
aa-tRNA	Aminoacyl-tRNA
ADA-SCID	Adenosine deaminase-severe combined immunodeficiency disease
AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATT	6-Aza-2-thiothymine
C	Cytidine
conc.	Concentrated
dA	Deoxyadenosine
DABCO	1,4-Diazabicyclo[2.2.2]octane
dba	Dibenzylideneacetone
dC	Deoxycytidine
DCM	Dichloromethane
dG	Deoxyguanosine
DMA	*N*,*N*-Dimethylaniline
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic acid
dT	Deoxythymidine
E. coli	Escherichia coli
EDTA	Ethylendiamine tetraacetate
EI	Electron ionization
ESI	Elektrospray ionisation
EtBr	Ethidium bromide
G	Guanosine
HIV-1	Human immunodeficiency virus type 1
HMPA	Hexamethylphosphoramide
HPLC	High performance liquid chromatography,
HR-MS	High resolution mass spectrometry
IR	Infra red
kb	Kilobase
KPhth	Potassium phthalimide
LB	Lysogeny broth
LC	Liquid chromatography
M.p.	Melting point
MELAS	Mitochondrial encephalomyopathies, encephalopathy, lactic acidosis, and stroke-like episodes
MeOTMS	Methoxytrimethylsilane
MERRF	Myoclonus epilepsy associated with ragged-red fibers
MLASA	Mitochondrial myopathy and sideroblastic anemia

mRNA	Messanger ribonucleic acid
MS	Mass spectrometry
mt	Mitochondrial
n	Amount of substance
NIS	*N*-Iodosuccinimide
NMR	Nuclear magnetic resonance
OD	Optical density
Pa	Pascal
PBS	Primer binding site
pH	Pondus hydrogenii
Ph.D.	Doctor of philosophy in arts and sciences
ppm	Parts per million
R_f	Rate of flow
RNA	Ribonucleic acid
rpm	Rotation per minute
rt	Room temperature
SAM	*S*-Adenosyl-*L*-methionin
sat.	Saturated
SCID	Severe combined immunodeficiency
SILAC	Stable isotope labeling with amino acids in cell culture
ss	Single stranded
TBDMS	*tert*-Butyldimethylsilyl
TEBA	Triethylbenzylammonium chloride
TGT	tRNA-guanine transglycosylase
THF	Tetrahydrofurane
TMS	Trimethylsilyl
TosCN	Tosylcyanide
tRNA	Transfer ribonucleic acid
U	Uridine
UV	Ultraviolet
vol.	Volume
w/v	Weight per volume
YPD	Yeast extract peptone dextrose

11.2 Detailed quantification data of the screening of modification levels in mammalian tissues

Tissues		Am	t^6A	i^6A	ms^2i^6A	$m^2{}_2G$	m^2G	Q	OHyW	m^1G+Gm	m^6t^6A	m^1A
Heart	Mean value	45.9	175.4	29.0	18.4	372.9	673.1	29.6	12.0	638.2	11.0	810.9
	Standard deviation	3.3	8.2	2.7	7.0	22.7	61.3	0.0	0.0	3.0	0.4	19.1
	Standard deviation in %	7.2	4.7	9.4	38.0	6.1	9.1	0.0	0.3	0.5	3.5	2.4
Liver	Mean value	20.5	214.6	39.6	4.7	562.7	1,176.5	55.2	33.6	1,059.6	21.9	941.6
	Standard deviation	4.8	6.4	2.7	0.8	26.1	54.7	4.6	2.9	43.6	1.8	23.6
	Standard deviation in %	23.2	3.0	6.8	17.9	4.6	4.7	8.4	8.7	4.1	8.0	2.5
Kidney	Mean value	68.4	177.8	30.7	8.4	451.5	896.0	32.3	21.1	816.3	15.8	871.6
	Standard deviation	19.1	7.7	0.5	0.3	25.4	55.0	13.8	4.7	40.3	1.5	35.5
	Standard deviation in %	28.0	4.3	1.8	3.7	5.6	6.1	42.8	22.3	4.9	9.5	4.1
Tongue	Mean value	45.4	170.6	25.7	12.7	395.9	794.0	21.9	15.2	698.7	11.3	846.5
	Standard deviation	10.5	4.9	2.9	4.7	0.6	7.5	0.1	1.3	5.8	0.3	6.6
	Standard deviation in %	23.1	2.9	11.4	36.7	0.2	0.9	0.7	8.5	0.8	2.3	0.8
Lung	Mean value	58.5	184.1	33.6	4.3	427.2	879.3	33.9	21.7	793.6	13.9	876.3
	Standard deviation	16.3	8.5	2.9	0.5	27.0	45.4	11.3	1.5	28.2	1.1	16.1
	Standard deviation in %	27.8	4.6	8.5	12.2	6.3	5.2	33.3	7.0	3.6	7.9	1.8
Spine marrow	Mean value	61.8	198.6	36.0	4.2	457.0	970.7	50.8	26.0	799.5	11.8	911.0
	Standard deviation	1.7	2.7	0.1	0.4	2.8	13.1	0.3	0.1	1.5	0.1	11.4
	Standard deviation in %	2.7	1.4	0.3	9.9	0.6	1.3	0.6	0.3	0.2	1.0	1.2

Tissues		Am	t^6A	i^6A	ms^2i^6A	$m^2{}_2G$	m^2G	Q	OHyW	m^1G+Gm	m^6t^6A	m^1A
Cerebellum	Mean value	67.8	237.3	41.7	15.1	566.8	1,036.4	83.5	29.0	829.4	17.5	1,102.8
	Standard deviation	6.1	0.6	0.3	0.1	1.2	1.2	4.8	1.9	13.8	0.0	6.7
	Standard deviation in %	9.0	0.2	0.6	0.4	0.2	0.1	5.7	6.5	1.7	0.2	0.6
Cerebrum	Mean value	139.9	165.8	26.1	10.3	406.2	682.2	57.2	20.3	586.8	10.5	732.6
	Standard deviation	15.9	2.8	1.5	0.1	7.3	34.6	3.8	1.2	0.1	0.6	26.3
	Standard deviation in %	11.3	1.7	5.9	0.5	1.8	5.1	6.6	5.7	0.0	5.9	3.6
Spleen	Mean value	80.4	182.1	30.7	2.8	464.3	793.6	42.7	26.1	690.5	13.9	833.6
	Standard deviation	12.6	8.9	1.0	0.0	19.6	29.5	5.9	2.2	13.4	0.4	45.2
	Standard deviation in %	15.6	4.9	3.2	1.5	4.2	3.7	13.8	8.4	1.9	2.9	5.4
Thyroid gland	Mean value	199.9	43.0	8.6	-	122.6	203.8	-	8.8	240.4	4.7	271.9
	Standard deviation	6.9	4.6	0.6	-	11.5	12.6	-	1.0	19.0	0.2	2.8
	Standard deviation in %	3.5	10.8	7.1	-	9.4	6.2	-	11.0	7.9	4.7	1.0

Table 18: Modification numbers per 1000 tRNAs in different pork tissues.
The table lists the average values calculated from the modification content of two different animals. From each animal at least two independent samples were investigated. For each sample at least three independent digests and measurements were performed. 5.9 % mean standard deviation was obtained for all nucleosides excluding Am. 14.7 % mean standard deviation was obtained for Am.

Tissues		Am	t^6A	i^6A	ms^2i^6A	$m^2{}_2G$	m^2G	Q	OHyW	m^1G+Gm	m^6t^6A	m^1A
Heart 1	Mean value	48.2	169.6	30.9	13.4	356.8	629.8	29.6	12.0	636.0	11.2	797.4
	Standard deviation	9.7	6.1	1.1	4.2	4.0	18.3	1.1	0.5	34.6	0.5	10.4
	Standard deviation in %	20.1	3.6	3.7	31.1	1.1	2.9	3.9	3.8	5.4	4.2	1.3
Heart 2	Mean value	43.5	181.3	27.1	23.3	389.0	716.5	29.6	12.1	640.3	10.7	824.5
	Standard deviation	12.1	7.1	3.0	3.4	40.7	85.2	0.9	1.1	38.8	1.1	28.5
	Standard deviation in %	27.8	3.9	11.0	14.4	10.5	11.9	3.1	9.0	6.1	10.5	3.5
Liver 1	Mean value	23.8	210.1	37.7	4.1	544.2	1,137.8	58.5	35.7	1,028.7	20.6	958.3
	Standard deviation	2.2	2.2	0.4	0.4	18.1	0.8	4.6	1.6	7.7	0.8	39.4
	Standard deviation in %	9.4	1.0	1.1	9.0	3.3	0.1	7.9	4.6	0.8	3.7	4.1
Liver 2	Mean value	17.1	219.1	41.5	5.3	581.1	1,215.2	51.9	31.5	1,090.4	23.1	925.0
	Standard deviation	1.2	12.1	1.9	0.0	39.5	86.9	1.9	3.4	39.5	0.9	30.0
	Standard deviation in %	6.8	5.5	4.6	0.7	6.8	7.2	3.7	10.9	3.6	3.9	3.2
Kidney 1	Mean value	81.9	172.4	30.3	8.6	433.5	857.1	22.5	17.8	787.9	14.8	846.5
	Standard deviation	23.7	19.1	2.8	1.7	30.1	89.1	2.1	2.1	43.0	2.0	25.5
	Standard deviation in %	28.9	11.1	9.4	19.7	6.9	10.4	9.4	11.6	5.5	13.3	3.0
Kidney 2	Mean value	54.8	183.3	31.1	8.2	469.5	934.9	42.1	24.5	844.8	16.9	896.7
	Standard deviation	15.8	2.9	2.9	4.0	3.7	10.7	0.1	3.8	3.8	0.4	40.4
	Standard deviation in %	28.8	1.6	9.2	48.3	0.8	1.1	0.2	15.4	0.4	2.5	4.5
Tongue 1	Mean value	52.8	167.2	23.6	9.4	395.5	799.4	22.0	14.3	694.6	11.1	841.8
	Standard deviation	8.2	12.4	1.9	3.6	18.8	66.5	1.6	1.7	23.0	1.1	37.2
	Standard deviation in %	15.6	7.4	8.2	38.2	4.8	8.3	7.2	12.2	3.3	9.8	4.4

Tissues		Am	t^6A	i^6A	ms^2i^6A	$m^2{}_2G$	m^2G	Q	OHyW	m^1G+Gm	m^6t^6A	m^1A
Tongue 2	Mean value	37.9	174.0	27.7	16.0	396.4	788.7	21.8	16.1	702.8	11.5	851.1
	Standard deviation	1.4	0.6	2.8	1.0	0.4	3.1	1.2	0.3	13.7	0.6	16.3
	Standard deviation in %	3.6	0.3	10.2	6.4	0.1	0.4	5.4	2.1	2.0	5.6	1.9
Lung 1	Mean value	47.0	178.1	31.6	3.9	408.0	847.2	25.9	20.7	773.7	13.2	864.9
	Standard deviation	1.2	4.3	0.1	0.2	5.3	3.2	4.6	0.7	0.1	1.2	3.7
	Standard deviation in %	2.5	2.4	0.3	5.2	1.3	0.4	17.6	3.5	0.0	8.8	0.4
Lung 2	Mean value	70.0	190.1	35.7	4.6	446.3	911.3	41.9	22.8	813.6	14.7	887.7
	Standard deviation	5.0	5.0	2.2	1.6	19.5	32.7	0.5	0.6	24.6	0.2	25.3
	Standard deviation in %	7.2	2.6	6.3	34.8	4.4	3.6	1.3	2.5	3.0	1.3	2.8
Spine marrow 1	Mean value	63.0	200.5	36.1	4.5	459.0	961.4	51.0	26.1	798.5	11.9	919.1
	Standard deviation	7.6	4.3	1.3	0.4	8.3	28.4	3.1	1.1	17.2	0.8	56.6
	Standard deviation in %	12.1	2.1	3.5	8.5	1.8	3.0	6.1	4.2	2.2	7.1	6.2
Spine marrow 2	Mean value	60.7	196.7	35.9	3.9	454.9	979.9	50.6	26.0	800.6	11.7	903.0
	Standard deviation	17.5	2.8	1.4	0.8	14.3	20.1	2.2	2.0	2.8	0.6	25.5
	Standard deviation in %	28.9	1.4	3.8	20.6	3.2	2.1	4.3	7.6	0.3	5.4	2.8
Cerebellum 1	Mean value	72.1	237.7	41.9	15.2	567.6	1,035.6	86.8	30.3	839.1	17.6	1,098.1
Cerebellum 2	Mean value	63.5	236.9	41.5	15.1	566.0	1,037.2	80.1	27.6	819.6	17.5	1,107.5
Cerebrum 1	Mean value	151.1	163.8	25.0	10.3	401.0	657.7	59.9	21.2	586.9	10.1	714.1
	Standard deviation	27.9	10.8	1.7	1.1	30.5	44.2	5.1	1.0	22.3	0.8	61.6
	Standard deviation in %	18.5	6.6	6.7	11.1	7.6	6.7	8.6	4.7	3.8	8.2	8.6

Tissues		Am	t^6A	i^6A	ms^2i^6A	$m^2{}_2G$	m^2G	Q	OHyW	m^1G+Gm	m^6t^6A	m^1A
Cerebrum 2	Mean value	128.7	167.8	27.2	10.3	411.3	706.7	54.6	19.5	586.7	11.0	751.2
	Standard deviation	0.1	7.8	0.7	1.0	6.1	19.0	1.5	0.7	18.7	0.4	38.1
	Standard deviation in %	0.1	4.6	2.5	9.3	1.5	2.7	2.8	3.8	3.2	3.8	5.1
Spleen 1	Mean value	89.3	175.8	30.0	2.7	450.4	772.8	38.6	24.5	681.0	13.6	801.7
	Standard deviation	6.7	2.6	0.0	0.9	14.4	16.6	0.7	2.2	1.6	0.4	6.6
	Standard deviation in %	7.5	1.5	0.1	31.1	3.2	2.1	1.7	9.1	0.2	3.0	0.8
Spleen 2	Mean value	71.5	188.4	31.4	2.8	478.2	814.5	46.9	27.6	699.9	14.2	865.5
	Standard deviation	11.2	9.7	1.9	1.0	30.7	76.0	0.7	0.3	87.3	1.5	102.8
	Standard deviation in %	15.6	5.1	5.9	35.3	6.4	9.3	1.5	1.0	12.5	10.5	11.9
Thyroid gland 1	Mean value	195.0	46.3	9.1	-	130.7	212.7	-	9.5	253.9	4.8	273.9
	Standard deviation	32.6	5.6	1.6	-	28.7	52.0	-	1.2	28.2	1.4	26.7
	Standard deviation in %	16.7	12.1	17.3	-	22.0	24.4	-	12.5	11.1	28.4	9.7
Thyroid gland 2	Mean value	204.8	39.7	8.2	-	114.4	194.9	-	8.2	227.0	4.5	269.9
	Standard deviation	63.5	4.5	0.5	-	8.6	11.6	-	1.6	5.9	0.4	23.3
	Standard deviation in %	31.0	11.4	6.3	-	7.5	6.0	-	20.2	2.6	8.6	8.6

Table 19: Modification numbers per 1000 tRNAs in different pork tissues.
The table lists the average values calculated from the modification content of at least two independent samples from one animal. For each sample at least three independent digests and measurements were performed. 6.7 % mean standard deviation was obtained for all nucleosides excluding Am. 17.0 % mean standard deviation was obtained for Am.

Detailed data on the modification levels of the cell lines presented in chapter 5.3.6 have been provided by M. Wagner (personal communication) and will be available from his upcoming thesis.

11.2.1 Stress response of tRNA modification levels in *E. coli*

External stimulation		m^2A / pmol	m^6A / pmol
Aerob (full medium)	Mean value	121.4	29.3
	Standard deviation	3.4	0.5
	Standard deviation in %	2.8	1.6
Anaerob (full medium)	Mean value	112.6	28.4
	Standard deviation	16.9	0.3
	Standard deviation in %	15.0	1.2
Aerob (minimal medium)	Mean value	125.0	29.4
	Standard deviation	7.2	0.1
	Standard deviation in %	5.8	0.3
Anaerob (minimal medium)	Mean value	125.0	30.6
	Standard deviation	5.4	1.9
	Standard deviation in %	4.3	6.2
pH = 5.5; OD = 0.5	Mean value	125.1	30.1
	Standard deviation	4.5	0.4
	Standard deviation in %	3.6	1.2
pH = 5.5; OD = 2.0	Mean value	120.5	30.6
	Standard deviation	13.2	1.5
	Standard deviation in %	10.9	4.8
Desferal	Mean value	109.7	31.2
	Standard deviation	5.4	0.5
	Standard deviation in %	4.9	1.5
2-Fluor-adenosine	Mean value	105.7	29.8
	Standard deviation	4.1	1.3
	Standard deviation in %	3.9	4.4
50 °C	Mean value	98.3	29.9
	Standard deviation	6.8	1.4
	Standard deviation in %	6.9	4.5
H_2O_2	Mean value	105.8	26.9
	Standard deviation	8.2	1.9
	Standard deviation in %	7.8	6.9
NaCl	Mean value	124.1	28.2
	Standard deviation	7.3	1.2
	Standard deviation in %	5.9	4.1

External stimulation		m^2A	m^6A
pH = 4.6	Mean value	78.7	21.5
	Standard deviation	3.1	0.3
	Standard deviation in %	4.0	1.3
pH = 5.5	Mean value	98.8	28.1
	Standard deviation	10.2	1.7
	Standard deviation in %	10.3	6.1
pH = 7.5	Mean value	91.3	26.9
	Standard deviation	9.8	3.1
	Standard deviation in %	10.7	11.6
pH = 8.6	Mean value	110.8	30.3
	Standard deviation	1.4	0.8
	Standard deviation in %	1.3	2.8
pH = 9.5	Mean value	28.0	19.9
	Standard deviation	0.9	1.8
	Standard deviation in %	3.0	9.1
Chloramphenicol	Mean value	110.6	31.0
	Standard deviation	12.0	0.0
	Standard deviation in %	10.9	0.0
Gentamycin	Mean value	107.0	29.1
	Standard deviation	3.9	1.7
	Standard deviation in %	3.6	5.9
Streptomycin	Mean value	91.7	27.4
	Standard deviation	2.9	1.0
	Standard deviation in %	3.1	3.6
Spectinomycin	Mean value	110.2	32.8
	Standard deviation	4.0	0.3
	Standard deviation in %	3.6	0.9

Table 20: Modification content in *E. coli* grown under different external stimulations.
The table lists the average values calculated from the modification content of at least two independent experiments. For each experiment at least three independent digests and measurements were performed.

11.2.2 Isotope based quantification of the new sixth DNA-nucleoside hydroxymethyl-dC in brain

Brain section		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Average mouse 1-4
Cerebral Cortex		4.45	4.68	4.72	4.15	4.50
	SD	0.45	0.46	0.11	0.12	0.23
	RSD / %	10.02	9.78	2.30	3.00	5.03
Brainstem		4.68	4.36	4.44	4.51	4.50
	SD	0.40	0.22	0.23	0.01	0.12
	RSD / %	8.47	4.98	5.25	0.19	2.59
Olfactory Bulb		4.37	4.42	4.66	4.68	4.54
	SD	0.05	0.49	0.12	0.19	0.13
	RSD / %	1.19	11.13	2.57	3.98	2.87
Cerebellum		4.29	4.57	4.41	4.28	4.39
	SD	0.26	0.25	0.10	0.33	0.12
	RSD / %	5.97	5.54	2.25	7.65	2.68
Retina		4.67	4.39	4.58	4.27	4.48
	SD	0.15	0.16	0.32	0.16	0.15
	RSD / %	3.25	3.76	7.05	3.74	3.43
Hippocampus		3.81	4.30	4.68	4.64	4.31
	SD	-	-	0.67	0.24	0.31
	RSD / %	-	-	14.31	5.07	7.27
Hypothalamus		3.73		2.93		3.33
	SD	-		-		0.40
	RSD / %	-		-		12.02
Hippocampus		3.44	3.53	-	-	3.48
(1 day old)	SD	-	-	-	-	0.04

Table 21: Nucleoside percentages of $^{5\text{-Me}}$dC to dG in different brain sections.
Each mouse brain section is listed with the determined values, the standard deviation (SD) and the relative standard deviation (RSD). The average mouse values are listed in the last column. For hypothalamus, the DNA of mouse 1 + 2 and 3+4 were mixed, respectively.

Brain section		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Average mouse 1-4
Cerebral Cortex		0.67	0.69	0.59	0.65	**0.65**
	SD	0.03	0.02	0.01	0.04	0.04
	RSD / %	3.73	2.19	1.23	5.91	5.82
Brainstem		0.52	0.51	0.56	0.62	**0.55**
	SD	0.02	0.05	0.03	0.02	0.04
	RSD / %	3.68	9.32	5.72	2.91	7.95
Olfactory Bulb		0.54	0.53	0.55	0.51	**0.53**
	SD	0.06	0.01	0.01	0.00	0.02
	RSD / %	10.24	1.16	1.29	0.87	3.05
Cerebellum		0.33	0.33	0.33	0.33	**0.33**
	SD	0.01	0.01	0.00	0.05	0.00
	RSD / %	4.09	4.09	0.70	13.75	0.49
Retina		0.32	0.31	0.31	0.29	**0.31**
	SD	0.01	0.02	0.00	0.02	0.01
	RSD / %	4.46	5.66	0.22	8.01	4.03
Hippocampus		0.63	0.60	0.52	0.58	**0.59**
	SD	-	-	0.00	0.04	0.04
	RSD / %	-	-	0.40	6.74	7.28
Hypothalamus		0.72		0.65		**0.69**
	SD	-		-		0.03
	RSD / %	-		-		5.08
Hippocampus		0.31	0.36	-	-	**0.34**
(1 day old)	SD	-	-	-	-	0.02
	RSD / %	-	-	-	-	7.38

Table 22: Nucleoside percentages of $^{5\text{-OHMe}}$dC to dG in different brain sections.
Each mouse brain section is listed with the determined values, the standard deviation (SD) and the relative standard deviation (RSD). The average mouse values are listed in the last column. For hypothalamus, the DNA of mouse 1 + 2 and 3+4 were mixed, respectively.

11.3 Crystallographic data

11.3.1 Crystallographic data for nucleoside 11

Crystal parameter and data collection details for nucleoside 11	
Chemical formula	$C_{16}H_{18}N_5O_{10}$
Molecular weight	475.80
Space group	C 1 2 1 (5) - monoclinic
a, b, c (Å)	21.5839(5), 7.7028(2), 13.3544(3)
α, β, γ (°)	90, 121.339 (2), 90
V (Å^3)	1896.33 (8)
Z	4
$D_{ber.}$ (g/cm^3)	1.667
$\mu_{MoK\alpha}$ (mm)	0.273
F(000)	984
Temperature (K)	373 (2)
Radiation λ (Å)	Mo*K*α, 0,71073
θ_{min}, θ_{max} (°)	3.67, 30.09
h	-30 → 30
k	-10 → 10
l	-18 → 18

Measured reflexes	12985
Independent reflexes	5534
R_{int}	0.0561
observed reflexes [I > 2σ(I)]	4145
$N_{Reflexes}$, $N_{Parameter}$	5534, 334
R, ωR_2, *S*	0.0309, 0.0554, 0.916
$\Omega = 1/[\sigma^2(F_0^2) + (0.247 \cdot P)^2 + 0.0 \cdot P]$	$P = (F_0^2 + 2\,F_c^2)/3$

Table 23: Crystal parameter and data collection details

Atom	X	Y	Z	U_{eq}
Cl1	0.10032(2)	0.86710(5)	0.43356(3)	0.02061(9)
O4	-0.36051(5)	1.23461(14)	0.20767(9)	0.0200(2)
O2	-0.22669(5)	1.20821(13)	0.40406(9)	0.0168(2)
O3	-0.18007(6)	1.47486(14)	0.46785(10)	0.0228(3)
N4	-0.07752(7)	1.26446(16)	0.32463(11)	0.0163(3)
N3	0.04795(7)	1.18098(17)	0.40295(11)	0.0162(3)
O1	-0.25304(6)	1.14057(13)	0.11886(9)	0.0188(2)
C1	0.03043(8)	1.0161(2)	0.39084(13)	0.0163(3)
C2	-0.04037(8)	0.96032(19)	0.34663(14)	0.0152(3)
N1	-0.15572(6)	1.01020(17)	0.28151(11)	0.0162(3)
C5	-0.00707(8)	1.29969(19)	0.36921(14)	0.0162(3)
C10	-0.19871(8)	1.3367(2)	0.48594(14)	0.0186(3)
C3	-0.09061(8)	1.0941(2)	0.31725(14)	0.0148(3)
C9	-0.23111(8)	1.2480(2)	0.29616(13)	0.0157(3)
C6	-0.22693(8)	1.0840(2)	0.23608(14)	0.0155(3)
N2	-0.07307(7)	0.79737(16)	0.32812(12)	0.0193(3)
C4	-0.14048(9)	0.83446(19)	0.29052(14)	0.0186(3)
N5	0.01336(8)	1.46668(19)	0.38138(14)	0.0224(3)
C8	-0.30344(8)	1.3241(2)	0.20240(14)	0.0171(3)
C11	-0.19528(10)	1.2839(2)	0.59630(16)	0.0240(4)
C12	-0.42343(9)	1.3252(2)	0.16660(15)	0.0255(4)
C7	-0.30684(8)	1.2739(2)	0.08889(14)	0.0184(3)
O6	-0.35443(6)	1.52727(15)	-0.03271(10)	0.0239(3)
O7	-0.30339(6)	1.72270(16)	0.11389(11)	0.0329(3)
O5	-0.42895(7)	1.47475(18)	0.13916(14)	0.0446(4)
C14	-0.29062(9)	1.4170(2)	0.02828(16)	0.0217(4)
C15	-0.35422(9)	1.6756(2)	0.02026(15)	0.0238(4)
C16	-0.42370(11)	1.7737(3)	-0.05037(19)	0.0313(4)
C13	-0.48181(10)	1.2122(3)	0.15852(17)	0.0314(4)

Table 24: Atom coordinates and equivalent anisotropic temperature factors for non-hydrogen atoms ($Å^2$).

Atom	X	Y	Z	U_{iso}
H141	-0.2484(8)	1.4787(18)	0.0841(12)	0.000(3)
H61	-0.2558(7)	0.999(2)	0.2365(12)	0.004(3)
H91	-0.1926(8)	1.327(2)	0.3122(13)	0.015(4)
H81	-0.3075(8)	1.448(2)	0.2078(12)	0.009(4)
H142	-0.2825(7)	1.367(2)	-0.0324(13)	0.013(4)
H4	-0.1783(8)	0.751(2)	0.2713(12)	0.005(4)
H162	-0.4252(9)	1.855(3)	0.0044(17)	0.041(5)
H71	-0.3551(9)	1.224(2)	0.0323(14)	0.016(4)
H163	-0.4184(9)	1.832(2)	-0.1074(16)	0.030(5)
H131	-0.4641(12)	1.127(3)	0.224(2)	0.057(7)
H132	-0.5196(12)	1.279(3)	0.1586(18)	0.051(6)

H113	-0.1610(12)	1.200(3)	0.6352(18)	0.051(6)
H51	-0.0154(12)	1.546(3)	0.3696(19)	0.046(7)
H112	-0.2411(12)	1.244(3)	0.5786(17)	0.046(6)
H52	0.0575(11)	1.482(3)	0.4187(18)	0.037(6)
H161	-0.4636(13)	1.704(3)	-0.078(2)	0.061(7)
H111	-0.1806(11)	1.382(3)	0.6497(18)	0.056(6)
H133	-0.4992(12)	1.147(3)	0.093(2)	0.056(7)

Table 25: Atom coordinates and equivalent isotropic temperature factors for hydrogen atoms (Å^2).

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Cl1	0.01430(17)	0.01689(18)	0.0261(2)	-0.00054(17)	0.00730(15)	0.00375(16)
O4	0.0130(5)	0.0225(6)	0.0273(6)	0.0028(5)	0.0123(5)	0.0017(4)
O2	0.0189(6)	0.0146(6)	0.0174(5)	-0.0021(4)	0.0097(5)	-0.0029(4)
O3	0.0211(6)	0.0151(6)	0.0296(7)	-0.0041(5)	0.0114(5)	-0.0018(4)
N4	0.0161(7)	0.0116(7)	0.0214(7)	0.0020(5)	0.0099(6)	0.0010(5)
N3	0.0138(7)	0.0139(7)	0.0207(7)	0.0020(5)	0.0088(6)	0.0022(5)
O1	0.0184(6)	0.0193(6)	0.0180(6)	0.0027(5)	0.0089(5)	0.0061(4)
C1	0.0149(7)	0.0180(8)	0.0144(8)	0.0000(6)	0.0066(6)	0.0033(6)
C2	0.0159(8)	0.0135(8)	0.0160(8)	-0.0018(6)	0.0081(7)	0.0003(6)
N1	0.0137(6)	0.0130(7)	0.0211(7)	0.0009(5)	0.0084(6)	0.0014(5)
C5	0.0175(8)	0.0149(8)	0.0174(8)	0.0008(6)	0.0098(7)	0.0018(6)
C10	0.0112(7)	0.0217(9)	0.0211(8)	-0.0048(6)	0.0071(6)	0.0022(6)
C3	0.0135(8)	0.0166(8)	0.0132(8)	0.0010(6)	0.0062(6)	0.0015(6)
C9	0.0138(8)	0.0142(8)	0.0190(8)	-0.0008(6)	0.0086(7)	-0.0001(6)
C6	0.0126(8)	0.0153(8)	0.0176(8)	-0.0007(6)	0.0072(7)	-0.0012(6)
N2	0.0175(7)	0.0165(7)	0.0229(8)	-0.0017(5)	0.0097(6)	0.0005(5)
C4	0.0183(8)	0.0130(9)	0.0221(8)	-0.0019(6)	0.0089(7)	-0.0003(6)
N5	0.0135(8)	0.0128(8)	0.0376(9)	0.0009(6)	0.0111(7)	0.0014(6)
C8	0.0142(8)	0.0135(8)	0.0248(9)	0.0024(6)	0.0109(7)	0.0012(6)
C11	0.0201(10)	0.0289(10)	0.0236(10)	-0.0056(8)	0.0118(8)	-0.0018(8)
C12	0.0156(8)	0.0403(12)	0.0203(9)	0.0028(8)	0.0092(7)	0.0052(7)
C7	0.0138(8)	0.0182(8)	0.0215(8)	0.0028(6)	0.0081(7)	0.0041(6)
O6	0.0235(6)	0.0228(6)	0.0238(6)	0.0050(5)	0.0112(5)	0.0047(5)
O7	0.0264(7)	0.0233(7)	0.0370(7)	-0.0035(6)	0.0081(6)	-0.0053(5)
O5	0.0280(7)	0.0414(9)	0.0668(10)	0.0248(7)	0.0264(7)	0.0183(6)
C14	0.0209(9)	0.0211(9)	0.0254(9)	0.0051(7)	0.0137(8)	0.0045(7)
C15	0.0257(9)	0.0162(8)	0.0311(10)	0.0040(7)	0.0160(8)	-0.0020(7)
C16	0.0297(11)	0.0249(11)	0.0345(12)	0.0058(9)	0.0132(9)	0.0092(8)
C13	0.0192(9)	0.0515(14)	0.0234(11)	-0.0007(10)	0.0108(8)	-0.0053(9)

Table 26: Anisotropic temperature factors for non-hydrogen atoms (Å^2).

Atom 1	Atom 2	Distance	Atom 1	Atom 2	Distance
Cl1	C1	1.7387(15)	C4	H4	0.962(15)
O4	C12	1.3631(19)	N5	H51	0.82(2)
O4	C8	1.4449(18)	N5	H52	0.82(2)
O2	C10	1.3614(18)	C8	C7	1.529(2)
O2	C9	1.4269(18)	C8	H81	0.966(15)
O3	C10	1.2056(19)	C11	H113	0.92(2)
N4	C3	1.3353(19)	C11	H112	0.94(2)
N4	C5	1.3412(19)	C11	H111	0.97(2)
N3	C1	1.311(2)	C12	O5	1.195(2)
N3	C5	1.3758(19)	C12	C13	1.489(3)
O1	C6	1.4282(19)	C7	C14	1.513(2)
O1	C7	1.4421(18)	C7	H71	0.994(16)
C1	C2	1.389(2)	O6	C15	1.342(2)
C2	C3	1.396(2)	O6	C14	1.4543(19)

C2	N2	1.3976(19)	O7	C15	1.213(2)
N1	C4	1.383(2)	C14	H141	0.952(14)
N1	C3	1.386(2)	C14	H142	0.993(15)
N1	C6	1.4436(19)	C15	C16	1.496(2)
C5	N5	1.342(2)	C16	H162	0.98(2)
C10	C11	1.493(2)	C16	H163	0.940(19)
C9	C8	1.522(2)	C16	H161	0.92(2)
C9	C6	1.524(2)	C13	H131	1.00(2)
C9	H91	0.960(16)	C13	H132	0.96(2)
C6	H61	0.909(15)	C13	H133	0.90(2)
N2	C4	1.3003(19)			

Table 27: Bond length (Å).

Atom 1	Atom 2	Atom 3	Angle	Atom 1	Atom 2	Atom 3	Angle
C12	O4	C8	115.60(12)	O4	C8	C7	108.96(12)
C10	O2	C9	115.06(12)	C9	C8	C7	102.59(12)
C3	N4	C5	112.28(13)	O4	C8	H81	110.9(9)
C1	N3	C5	117.32(13)	C9	C8	H81	114.4(9)
C6	O1	C7	108.86(11)	C7	C8	H81	111.4(9)
N3	C1	C2	122.35(14)	C10	C11	H113	111.3(13)
N3	C1	Cl1	116.98(11)	C10	C11	H112	109.6(12)
C2	C1	Cl1	120.67(12)	H113	C11	H112	110.3(19)
C1	C2	C3	114.41(14)	C10	C11	H111	110.0(13)
C1	C2	N2	134.08(14)	H113	C11	H111	106.0(17)
C3	C2	N2	111.47(13)	H112	C11	H111	109.6(17)
C4	N1	C3	105.87(12)	O5	C12	O4	122.43(16)
C4	N1	C6	125.10(13)	O5	C12	C13	126.55(17)
C3	N1	C6	128.90(13)	O4	C12	C13	111.00(16)
N4	C5	N5	118.22(13)	O1	C7	C14	106.89(12)
N4	C5	N3	126.67(14)	O1	C7	C8	106.77(12)
N5	C5	N3	115.10(14)	C14	C7	C8	116.17(13)
O3	C10	O2	122.39(15)	O1	C7	H71	108.0(9)
O3	C10	C11	126.01(15)	C14	C7	H71	108.6(9)
O2	C10	C11	111.60(14)	C8	C7	H71	110.1(9)
N4	C3	N1	128.39(14)	C15	O6	C14	117.34(13)
N4	C3	C2	126.94(14)	O6	C14	C7	108.05(12)
N1	C3	C2	104.66(13)	O6	C14	H141	112.9(8)
O2	C9	C8	114.51(12)	C7	C14	H141	109.8(8)
O2	C9	C6	111.25(13)	O6	C14	H142	106.8(8)
C8	C9	C6	101.69(12)	C7	C14	H142	109.9(9)
O2	C9	H91	108.4(9)	H141	C14	H142	109.4(11)
C8	C9	H91	109.7(9)	O7	C15	O6	123.44(15)
C6	C9	H91	111.2(9)	O7	C15	C16	124.83(17)
O1	C6	N1	107.93(12)	O6	C15	C16	111.72(15)
O1	C6	C9	103.11(12)	C15	C16	H162	105.4(11)
N1	C6	C9	116.55(13)	C15	C16	H163	104.4(11)
O1	C6	H61	110.3(9)	H162	C16	H163	111.4(17)
N1	C6	H61	107.2(9)	C15	C16	H161	112.3(15)
C9	C6	H61	111.6(10)	H162	C16	H161	106.6(17)
C4	N2	C2	103.40(12)	H163	C16	H161	116.4(17)
N2	C4	N1	114.60(14)	C12	C13	H131	113.4(13)
N2	C4	H4	125.5(9)	C12	C13	H132	111.9(12)
N1	C4	H4	119.8(9)	H131	C13	H132	106.8(17)
C5	N5	H51	121.1(15)	C12	C13	H133	107.7(15)
C5	N5	H52	114.5(14)	H131	C13	H133	105.0(19)
H51	N5	H52	122(2)	H132	C13	H133	111.8(18)
O4	C8	C9	108.16(12)				

Table 28: Bond angle (°).

Torsion atoms				Torsion angle	Torsion atoms				Torsion angle
1	2	3	4		1	2	3	4	
C5	N3	C1	C2	-0.4(2)	C3	N1	C6	C9	-41.5(2)
C5	N3	C1	Cl1	179.93(11)	O2	C9	C6	O1	163.65(11)
N3	C1	C2	C3	-0.4(2)	C8	C9	C6	O1	41.31(14)
Cl1	C1	C2	C3	179.28(12)	O2	C9	C6	N1	-78.34(16)
N3	C1	C2	N2	-177.76(17)	C8	C9	C6	N1	159.32(13)
Cl1	C1	C2	N2	1.9(2)	C1	C2	N2	C4	176.56(17)
C3	N4	C5	N5	-179.93(16)	C3	C2	N2	C4	-0.87(18)
C3	N4	C5	N3	1.3(2)	C2	N2	C4	N1	0.45(18)
C1	N3	C5	N4	-0.1(2)	C3	N1	C4	N2	0.12(19)
C1	N3	C5	N5	-178.89(14)	C6	N1	C4	N2	176.44(14)
C9	O2	C10	O3	1.1(2)	C12	O4	C8	C9	154.73(13)
C9	O2	C10	C11	-179.40(12)	C12	O4	C8	C7	-94.46(15)
C5	N4	C3	N1	176.39(15)	O2	C9	C8	O4	-39.20(17)
C5	N4	C3	C2	-2.2(2)	C6	C9	C8	O4	80.87(14)
C4	N1	C3	N4	-179.50(16)	O2	C9	C8	C7	-154.26(12)
C6	N1	C3	N4	4.4(3)	C6	C9	C8	C7	-34.19(14)
C4	N1	C3	C2	-0.64(17)	C8	O4	C12	O5	-7.4(2)
C6	N1	C3	C2	-176.77(14)	C8	O4	C12	C13	171.04(14)
C1	C2	C3	N4	1.9(2)	C6	O1	C7	C14	135.47(13)
N2	C2	C3	N4	179.83(15)	C6	O1	C7	C8	10.52(15)
C1	C2	C3	N1	-177.02(13)	O4	C8	C7	O1	-98.67(13)
N2	C2	C3	N1	0.95(18)	C9	C8	C7	O1	15.81(15)
C10	O2	C9	C8	-92.90(15)	O4	C8	C7	C14	142.24(13)
C10	O2	C9	C6	152.51(12)	C9	C8	C7	C14	-103.27(15)
C7	O1	C6	N1	-156.44(11)	C15	O6	C14	C7	99.14(16)
C7	O1	C6	C9	-32.55(15)	O1	C7	C14	O6	163.58(12)
C4	N1	C6	O1	-101.64(16)	C8	C7	C14	O6	-77.40(17)
C3	N1	C6	O1	73.81(18)	C14	O6	C15	O7	1.7(2)
C4	N1	C6	C9	143.02(15)	C14	O6	C15	C16	-178.48(15)

Table 29: Torsion angle (°).

11.3.2 Crystallographic data for ms^2i^6A

Crystal parameter and data collection details for ms^2i^6A	
Chemical formula	$C_{16}H_{23}N_5O_4S$
Molecular weight	381.45
Space group	P 21 21 21 (19) - orthorhombic
a, b, c (Å)	5.0993(2), 11.3043(4), 30.6517(11)
α, β, γ (°)	90, 90, 90
V ($Å^3$)	1766.89(11)
Z	4
$D_{ber.}$ (g/cm^3)	1.434
$\mu_{MoK\alpha}$ (mm)	0.217
F(000)	808
Temperature (K)	773 (2)
Radiation λ (Å)	Mo*K*α, 0.71073
θ_{min}, θ_{max} (°)	3.78, 30.10
h	-7 → 7
k	-15 → 15
l	-43 → 43
Measured reflexes	24115
Independent reflexes	5187

R_{int}	0,0775
observed reflexes [I > 2σ(I)]	2412
$N_{Reflexes}$, $N_{Parameter}$	5187, 2412
R, ωR_2, S	0.0351, 0.0495, 0.790
$\Omega = 1/[\sigma^2(F_0^2) + (0.204 \cdot P)^2 + 0.0 \cdot P]$	$P = (F_0^2 + 2\,F_c^2)/3$

Table 30: Crystal parameter and data collection details

Atom	X	Y	Z	U_{eq}
C1	0.8801(4)	0.16780(19)	0.92806(6)	0.0320(5)
C10	1.1534(4)	0.3191(2)	0.76126(7)	0.0324(5)
C11	0.0991(5)	0.5036(2)	0.97416(7)	0.0355(6)
C12	0.2137(4)	0.59759(19)	1.00337(6)	0.0349(5)
C13	0.1371(4)	0.62608(17)	1.04296(7)	0.0334(5)
C14	0.2674(6)	0.7224(2)	1.06882(9)	0.0501(7)
C15	-0.0814(4)	0.5670(2)	1.06697(7)	0.0514(7)
C16	0.2569(6)	0.6706(2)	0.84019(9)	0.0445(6)
C2	0.7622(4)	0.31309(16)	0.88521(6)	0.0239(4)
C3	0.6274(4)	0.31543(16)	0.92424(6)	0.0246(5)
C4	0.5568(4)	0.47494(16)	0.86218(6)	0.0265(5)
C5	0.4405(4)	0.40510(17)	0.92979(5)	0.0252(4)
C6	1.0785(4)	0.15882(17)	0.85408(6)	0.0261(5)
C7	0.9163(4)	0.10274(17)	0.81813(6)	0.0245(4)
C8	1.2565(4)	0.21522(17)	0.78699(6)	0.0269(5)
C9	1.1173(4)	0.09758(17)	0.78157(6)	0.0256(5)
N1	0.9272(3)	0.21630(13)	0.88792(5)	0.0272(4)
N2	0.7052(3)	0.22310(14)	0.95139(5)	0.0318(4)
N3	0.7349(3)	0.39202(13)	0.85218(4)	0.0256(4)
N4	0.4134(3)	0.48715(13)	0.89830(4)	0.0260(4)
N5	0.2853(3)	0.41034(16)	0.96500(5)	0.0322(5)
O2	1.2434(2)	0.24194(10)	0.83336(4)	0.0294(3)
O3	0.8045(3)	-0.00475(12)	0.83141(5)	0.0354(4)
O4	1.2999(3)	0.00436(13)	0.78949(4)	0.0321(4)
O5	0.8784(3)	0.33809(12)	0.76666(5)	0.0304(3)
S1	0.51997(11)	0.58146(4)	0.820730(16)	0.03771(15)

Table 31: Atom coordinates and equivalent anisotropic temperature factors for non-hydrogen atoms ($Å^2$).

Atom	X	Y	Z	U_{iso}
H1	0.974(3)	0.0989(15)	0.9367(5)	0.023(5)
H101	1.168(3)	0.3021(14)	0.7304(6)	0.023(5)
H102	1.262(4)	0.3869(18)	0.7684(6)	0.042(6)
H111	0.050(3)	0.5405(15)	0.9466(6)	0.026(5)
H112	-0.059(4)	0.4714(17)	0.9874(6)	0.048(7)
H12	0.368(4)	0.6396(15)	0.9907(5)	0.032(5)
H141	0.414(5)	0.760(2)	1.0530(7)	0.068(8)
H142	0.145(4)	0.7897(18)	1.0735(6)	0.043(6)
H143	0.324(4)	0.6932(18)	1.0983(7)	0.056(7)
H15A	-0.1160	0.4912	1.0541	0.062
H15B	-0.2361	0.6151	1.0653	0.062
H15C	-0.0325	0.5565	1.0970	0.062
H161	0.232(4)	0.7321(19)	0.8198(7)	0.056(7)
H162	0.305(4)	0.7094(19)	0.8675(8)	0.064(8)
H163	0.109(6)	0.632(2)	0.8446(8)	0.100(12)
H3	0.661(5)	-0.010(2)	0.8204(7)	0.063(9)
H4	1.260(4)	-0.0524(18)	0.7740(6)	0.045(7)
H5	0.297(4)	0.3613(16)	0.9827(5)	0.024(6)

H50	0.834(5)	0.361(2)	0.7932(8)	0.094(11)
H6	1.190(3)	0.1005(14)	0.8687(5)	0.018(4)
H7	0.791(3)	0.1569(15)	0.8101(5)	0.020(5)
H8	1.444(3)	0.2062(13)	0.7790(5)	0.017(4)
H9	1.044(3)	0.0885(13)	0.7527(5)	0.014(4)

Table 32: Atom coordinates and equivalent isotropic temperature factors for hydrogen atoms (Å^2).

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
S1	0.0472(4)	0.0331(3)	0.0328(3)	0.0090(2)	0.0052(3)	0.0071(3)
O2	0.0307(8)	0.0301(8)	0.0272(8)	-0.0001(6)	-0.0031(6)	-0.0037(7)
O3	0.0354(10)	0.0303(9)	0.0404(9)	0.0043(7)	-0.0018(8)	-0.0062(7)
O4	0.0328(9)	0.0279(9)	0.0355(9)	-0.0082(7)	-0.0069(7)	0.0068(8)
O5	0.0322(9)	0.0323(9)	0.0268(8)	0.0053(7)	-0.0012(7)	0.0045(7)
N1	0.0345(11)	0.0262(9)	0.0208(9)	0.0008(7)	-0.0025(8)	0.0020(8)
N2	0.0455(12)	0.0276(10)	0.0223(9)	0.0029(7)	-0.0009(9)	0.0094(9)
N3	0.0290(9)	0.0242(9)	0.0235(9)	0.0028(7)	-0.0020(7)	0.0011(8)
N4	0.0326(10)	0.0242(9)	0.0213(9)	-0.0015(7)	0.0022(7)	0.0012(8)
N5	0.0449(12)	0.0301(11)	0.0215(10)	0.0050(9)	0.0067(9)	0.0090(10)
C1	0.0422(14)	0.0297(12)	0.0240(11)	0.0045(10)	-0.0041(10)	0.0059(12)
C2	0.0275(12)	0.0198(11)	0.0243(11)	-0.0022(8)	-0.0054(9)	-0.0003(10)
C3	0.0316(12)	0.0212(11)	0.0210(11)	-0.0008(8)	-0.0015(9)	-0.0030(10)
C4	0.0302(12)	0.0259(11)	0.0235(10)	0.0015(8)	-0.0040(9)	-0.0037(10)
C5	0.0357(12)	0.0219(10)	0.0180(10)	-0.0017(8)	-0.0011(9)	-0.0028(11)
C6	0.0270(12)	0.0264(11)	0.0251(11)	0.0021(9)	-0.0037(10)	0.0056(10)
C7	0.0226(11)	0.0222(12)	0.0288(11)	0.0039(9)	-0.0031(10)	0.0034(10)
C8	0.0217(12)	0.0353(13)	0.0236(11)	-0.0008(9)	0.0015(10)	-0.0004(11)
C9	0.0271(11)	0.0298(12)	0.0199(11)	-0.0002(9)	-0.0062(9)	0.0045(11)
C10	0.0335(15)	0.0337(14)	0.0299(14)	0.0020(11)	-0.0022(10)	-0.0034(11)
C11	0.0405(15)	0.0354(14)	0.0305(13)	0.0028(11)	0.0038(11)	0.0060(13)
C12	0.0390(14)	0.0269(13)	0.0388(13)	0.0013(10)	0.0122(11)	0.0000(12)
C13	0.0337(13)	0.0320(13)	0.0344(12)	-0.0004(9)	0.0004(10)	0.0047(10)
C14	0.0551(19)	0.0396(16)	0.0556(19)	-0.0126(13)	0.0040(17)	0.0001(16)
C15	0.0465(16)	0.0630(17)	0.0448(14)	-0.0127(12)	0.0133(12)	-0.0089(14)
C16	0.0521(19)	0.0400(16)	0.0415(16)	0.0060(13)	-0.0040(14)	0.0128(15)

Table 33: Anisotropic temperature factors for non-hydrogen atoms (Å^2).

Atom 1	Atom 2	Distance	Atom 1	Atom 2	Distance
S1	C4	1.7605(17)	C6	H6	0.979(15)
S1	C16	1.781(3)	C7	C9	1.520(2)
O2	C6	1.412(2)	C7	H7	0.917(17)
O2	C8	1.455(2)	C8	C10	1.509(3)
O3	C7	1.403(2)	C8	C9	1.517(3)
O3	H3	0.81(2)	C8	H8	0.991(17)
O4	C9	1.427(2)	C9	H9	0.965(14)
O4	H4	0.82(2)	C10	H101	0.968(16)
O5	C10	1.428(2)	C10	H102	0.97(2)
O5	H50	0.88(3)	C11	C12	1.507(3)
N1	C1	1.368(2)	C11	H111	0.976(17)
N1	C2	1.383(2)	C11	H112	0.97(2)
N1	C6	1.447(2)	C12	C13	1.315(2)
N2	C1	1.303(2)	C12	H12	0.997(18)
N2	C3	1.393(2)	C13	C15	1.493(3)
N3	C4	1.341(2)	C13	C14	1.502(3)
N3	C2	1.357(2)	C14	H141	0.99(2)
N4	C4	1.334(2)	C14	H142	0.99(2)
N4	C5	1.346(2)	C14	H143	1.00(2)
N5	C5	1.340(2)	C15	H15A	0.9600

N5	C11	1.446(3)	C15	H15B	0.9600
N5	H5	0.778(17)	C15	H15C	0.9600
C1	H1	0.952(17)	C16	H161	0.94(2)
C2	C3	1.380(2)	C16	H162	0.98(2)
C3	C5	1.402(3)	C16	H163	0.89(3)
C6	C7	1.517(2)			

Table 34: Bond length (Å).

Atom 1	Atom 2	Atom 3	Angle	Atom 1	Atom 2	Atom 3	Angle
C4	S1	C16	103.05(11)	C10	C8	H8	106.7(9)
C6	O2	C8	109.20(14)	C9	C8	H8	109.5(9)
C7	O3	H3	108.4(17)	O4	C9	C8	108.87(15)
C9	O4	H4	108.3(15)	O4	C9	C7	110.05(15)
C10	O5	H50	113.6(18)	C8	C9	C7	101.62(15)
C1	N1	C2	105.33(16)	O4	C9	H9	109.2(9)
C1	N1	C6	123.93(16)	C8	C9	H9	112.0(9)
C2	N1	C6	129.56(15)	C7	C9	H9	114.8(9)
C1	N2	C3	103.09(17)	O5	C10	C8	113.48(18)
C4	N3	C2	111.03(15)	O5	C10	H101	102.6(10)
C4	N4	C5	117.87(15)	C8	C10	H101	109.2(10)
C5	N5	C11	125.20(18)	O5	C10	H102	114.5(12)
C5	N5	H5	119.1(14)	C8	C10	H102	107.4(11)
C11	N5	H5	115.7(14)	H101	C10	H102	109.5(14)
N2	C1	N1	114.93(19)	N5	C11	C12	112.01(18)
N2	C1	H1	125.8(9)	N5	C11	H111	108.2(10)
N1	C1	H1	119.3(9)	C12	C11	H111	108.3(10)
N3	C2	C3	125.67(17)	N5	C11	H112	110.5(12)
N3	C2	N1	128.89(16)	C12	C11	H112	109.7(11)
C3	C2	N1	105.43(15)	H111	C11	H112	108.0(15)
C2	C3	N2	111.22(17)	C13	C12	C11	127.3(2)
C2	C3	C5	117.24(17)	C13	C12	H12	118.6(10)
N2	C3	C5	131.54(17)	C11	C12	H12	114.1(10)
N4	C4	N3	129.31(16)	C12	C13	C15	124.5(2)
N4	C4	S1	118.01(14)	C12	C13	C14	122.2(2)
N3	C4	S1	112.68(13)	C15	C13	C14	113.25(19)
N5	C5	N4	119.12(18)	C13	C14	H141	112.8(13)
N5	C5	C3	122.11(17)	C13	C14	H142	110.8(12)
N4	C5	C3	118.77(16)	H141	C14	H142	102.5(17)
O2	C6	N1	109.95(15)	C13	C14	H143	111.3(12)
O2	C6	C7	106.04(14)	H141	C14	H143	111(2)
N1	C6	C7	114.68(16)	H142	C14	H143	107.5(16)
O2	C6	H6	108.0(9)	C13	C15	H15A	109.5
N1	C6	H6	106.5(9)	C13	C15	H15B	109.5
C7	C6	H6	111.6(9)	H15A	C15	H15B	109.5
O3	C7	C6	111.90(16)	C13	C15	H15C	109.5
O3	C7	C9	117.06(16)	H15A	C15	H15C	109.5
C6	C7	C9	100.58(15)	H15B	C15	H15C	109.5
O3	C7	H7	112.0(11)	S1	C16	H161	107.2(13)
C6	C7	H7	107.1(10)	S1	C16	H162	110.6(13)
C9	C7	H7	107.2(10)	H161	C16	H162	105.6(18)
O2	C8	C10	109.44(17)	S1	C16	H163	114.3(18)
O2	C8	C9	105.51(15)	H161	C16	H163	111(2)
C10	C8	C9	117.52(17)	H162	C16	H163	108(2)
O2	C8	H8	107.8(9)				

Table 35: Bond angle (°).

Torsion atoms				Torsion angle	Torsion atoms				Torsion angle
1	2	3	4		1	2	3	4	
C3	N2	C1	N1	0.7(2)	N2	C3	C5	N4	177.18(18)
C2	N1	C1	N2	-0.5(2)	C8	O2	C6	N1	-142.70(15)
C6	N1	C1	N2	-169.06(18)	C8	O2	C6	C7	-18.19(19)
C4	N3	C2	C3	0.1(3)	C1	N1	C6	O2	-138.63(17)
C4	N3	C2	N1	-178.98(17)	C2	N1	C6	O2	55.7(2)
C1	N1	C2	N3	179.19(18)	C1	N1	C6	C7	102.0(2)
C6	N1	C2	N3	-13.1(3)	C2	N1	C6	C7	-63.7(3)
C1	N1	C2	C3	0.00(19)	O2	C6	C7	O3	161.62(14)
C6	N1	C2	C3	167.72(17)	N1	C6	C7	O3	-76.9(2)
N3	C2	C3	N2	-178.81(18)	O2	C6	C7	C9	36.59(18)
N1	C2	C3	N2	0.4(2)	N1	C6	C7	C9	158.11(16)
N3	C2	C3	C5	2.0(3)	C6	O2	C8	C10	119.31(17)
N1	C2	C3	C5	-178.81(16)	C6	O2	C8	C9	-8.02(19)
C1	N2	C3	C2	-0.7(2)	O2	C8	C9	O4	-85.63(18)
C1	N2	C3	C5	178.4(2)	C10	C8	C9	O4	152.09(18)
C5	N4	C4	N3	-1.6(3)	O2	C8	C9	C7	30.49(18)
C5	N4	C4	S1	179.50(13)	C10	C8	C9	C7	-91.8(2)
C2	N3	C4	N4	-0.3(3)	O3	C7	C9	O4	-46.1(2)
C2	N3	C4	S1	178.65(12)	C6	C7	C9	O4	75.33(18)
C16	S1	C4	N4	-5.85(18)	O3	C7	C9	C8	-161.36(16)
C16	S1	C4	N3	175.07(16)	C6	C7	C9	C8	-39.93(17)
C11	N5	C5	N4	-5.0(3)	O2	C8	C10	O5	-68.1(2)
C11	N5	C5	C3	176.24(19)	C9	C8	C10	O5	52.1(3)
C4	N4	C5	N5	-175.19(16)	C5	N5	C11	C12	-94.9(2)
C4	N4	C5	C3	3.6(2)	N5	C11	C12	C13	-116.2(2)
C2	C3	C5	N5	174.97(17)	C11	C12	C13	C15	1.1(3)
N2	C3	C5	N5	-4.1(3)	C11	C12	C13	C14	-179.7(2)
C2	C3	C5	N4	-3.8(3)					

Table 36: Torsion angle (°).

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RESEARCH EXPERIENCE

09/2006 – Present Doctoral Thesis
Ludwig-Maximilians-University, Carell Group, Munich
Mass-Spectrometric quantification of modified nucleosides from bacteria and mammals.

12/2005 – 06/2006 Diploma Thesis
Ludwig-Maximilians-University, Carell Group, Munich
Natural product synthesis of the modified nucleosides $PreQ_0$ and *archaeosine*.

08/2004 – 10/2004 Research Internship
Osram Opto Semiconductors, Research and Development, Regensburg
Establishment of actinometry as basis for etch rate prediction.

01/2004 – 04/2004 Research Internship
Osram Opto Semiconductors, Research and Development, Regensburg
Investigation of actinometry in process development.

07/2003 – 12/2003 Research Internship
University of Oxford, Hodgson Group, Oxford
Investigation of catalytic enantioselective intermolecular 2,3-carbonyl-ylide-cycloaddition and its application in natural product synthesis of nemorensic acids.

04/2003 – 07/2003 Organic Chemistry Tutor, Ludwig-Maximilians-University, Munich

04/2001 – 10/2001 Research Assistant, GPC Biotech AG, Munich

EDUCATION

10/2000 – 07/2006	Chemistry Diploma *Ludwig-Maximilians-University, Munich*, grade: 1.5
08/1999 – 07/2000	Civil Service *Hospital Nuremberg, Microbiological Laboratory, Nuremberg*
07/1990 – 07/1999	High School *Willibald-Gluck-Gymnasium, Neumarkt*, grade: 2.2 Award for environmental science, "Jugend forscht", 1999.

FURTHER TRAININGS

12/2008	LTQ Orbitrap Biotech Operations Training Course *ThermoFisher Scientific Training Institute, Munich*
10/2008	Crash Course "Project Management" *Ludwig-Maximilians-University, Munich*
06/2008	Crash Course "Führung und Motivation sowie Förderung der Kommunikation, Zusammenarbeit und Leistung im Team" *LMU Center for Leadership and People Management, Munich*
06/2007	Nucleic Acid Chemical Biology (NACB) Ph.D. Summer School *University of Southern Denmark, Odense*

PERSONAL SKILLS AND ACTIVITIES

Languages	German	native speaker
	English	fluent
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Computer Skills	Microsoft: Word, Excel, Powerpoint Adobe: Photoshop, Illustrator PyMOL	
Activities	Member of the GDCh and the JCF (Jungchemikerforum) Munich Member of the organizing team for the JCF Alpenforum 2008-2010 Cooking, Reading, Rock Climbing, Snowboarding, Sailing	

PUBLICATIONS

6. M. Münzel, D. Globisch, T. Brückl, M. Wagner, V. Welzmiller, S. Michalakis, M. Müller, M. Biel, and T. Carell, Quantification of the Sixth DNA-Base Hydroxymethyl-dC in the Brain, *Angew. Chem.* **2010**, accepted (hot paper).

5. T. Brückl, D. Globisch, M. Wagner, M. Müller, and T. Carell, Parallel isotope-based quantification of modified tRNA nucleosides, *Angew. Chem. Int. Ed.* **2009**, *48*, 7932–7934 (hot paper).

4. T. Brückl, F. Klepper, K. Gutsmiedl & T. Carell, A short and efficient synthesis of the tRNA nucleosides $PreQ_0$ and archaeosine, *Org. Biomol. Chem.* **2007**, 5, 3821–3825.

3. T. Brückl & H. Zull, Actinometry of inductively coupled Cl_2/N_2 plasmas for dry etching of GaAs, *J. Appl. Phys.* **2005**, *98*, 023307.

2. D. M. Hodgson, F. Le Strat, T. D. Avery, A. C. Donohue & T. Brückl, Stereocontrolled synthesis of the nemorensic acids using 6-diazoheptane-2,5-dione in carbonyl ylide cycloadditions, *J. Org. Chem.* **2004**, *69*, 8796–8803.

1. D. M. Hodgson, T. Brückl, R. Glen, A. H. Labande, D. A. Selden, A. G. Dossetter & A. J. Redgrave, Catalytic enantioselective intermolecular cycloadditions of 2-diazo-3,6-diketoester-derived carbonyl ylides with alkene dipolarophiles, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5450–5454, Asymmetric Catalysis Special Feature.

CONFERENCE PRESENTATIONS

7. Poster Presentation: 42nd IUPAC Congress, Chemistry Solutions, *Quantitative analysis of modified tRNA nucleosides*, Glasgow, United Kingdom, 2nd to 7th August 2009.

6. Poster Presentation: Bayer Ph.D. Student Course 2009, *Quantitation of modified tRNA nucleosides from E. coli, pork, HeLa, and HCT*, Cologne, Germany, 5th to 9th July 2009.

5. Poster Presentation: Synthesefest 2009 – A Celebration of Organic Chemistry in Munich, *Synthesis of the modified tRNA nucleosides queuosine, archaeosine, ms^2io^6A, and m^2A*, Munich, Germany, 17th and 18th March 2009.

4. Oral Presentation: 11th JCF-Frühjahrssymposium, *2-methylthio-N-6-hydroxyisopentenyl-adenosine (ms^2io^6A), 2-Methyladenosine (m^2A), queuosine (Q), and archaeosine (G*) – Total syntheses of modified tRNA nucleosides*, Essen, Germany, 11th to 14th March 2009.

3. Poster Presentation: ORCHEM 2008, *Making modifications – Total synthesis of modified tRNA nucleosides*, Weimar, Germany, 1st to 3rd September 2008.

2. Oral Presentation: 3rd Nucleic Acid Chemical Biology Ph.D. Summer School, *Total synthesis of queuosine (Q) and archaeosine (G*)*, Odense, Denmark, 24th to 28th June 2007.

1. Invited Speaker: Plasma Processing Technologies for Compound Semiconductors Workshop, *Actinometry of Cl_2/N_2 plasmas and its extension to the first ever straightforward etch rate prediction*, Regensburg, Germany, 22nd and 23rd May 2006.