

**Synthetic Methods**

# The P(III)-Amidite Based Synthesis of Stable Isotope Labeled mRNA-Cap-Structures Enables their Sensitive Quantitation from Brain Tissue

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**Abstract:** The 5' cap structure is crucial to mRNA function, with its diverse methylation patterns depending on the cellular state. Sensitive analytical methods are sought after to quantify this cap variety also referred to as cap epitranscriptome. To address a bottleneck for accurate and precise quantitation, we report a facile and fast access to high-quality synthetic standards via a new route, involving P(III)-amidite chemistry. A range of cap nucleotides and their stable heavy isotopic labeled analogues were derived from nucleoside diphosphates, which themselves were directly prepared in a one-step reaction sequence starting from unprotected nucleosides using a triphosphorylating reagent in combination with ethylenediamine. Considering a wider scope, the route also enables direct access to magic spot nucleotides and diphosphates of isoprenyl-alcohols. Stable-isotope labeled cap nucleotides derived from this route paved the way for the development of a highly sensitive LC–MS/MS method, applied to the characterization of mouse brain cap epitranscriptomes, which turned out to be very different from those of cultured cell lines of widespread use in the life sciences.

the most ancient and most visible, but by far not the only species. More recent reports concern both long noncoding RNAs (lnc RNA) and various types of small noncoding RNAs.<sup>[2]</sup> Details of mRNA cap structures are important in regulation of different stages during the RNA life cycle, including splicing, polyadenylation, transport, stability and translation.<sup>[3]</sup> Especially the latter function is associated with a veritable battle of viral take-over strategies, and counter-measures of the innate immune system of the infected organism.<sup>[4]</sup>

The cap itself is structurally diverse, harboring different methylation patterns (Scheme 1A). To investigate this variety - the cap epitranscriptome - different LC–MS-based methods were recently developed (e.g. CapQuant, CAP-MAP).<sup>[5–8]</sup> These techniques allow detection and quantitation of cap structures present in cells and tissues. While details in protocols vary, they usually rely on an enzymatic digest of the RNAs followed by an LC–MS-based read-out of the cap nucleotides. Hence, an efficient (bio)synthetic access to cap nucleotides is crucial for the continued development of more sensitive methods for relative and absolute quantitation.

While nucleoside analytics of internal RNA modifications are routinely conducted in the low femtomole to attomole scale, the unfavorable ionization properties of nucleotides cause significantly higher limits of detection and quantitation of polyphosphate structures. Even more importantly, modern and increasingly diversified LC–MS instrumentation demands authentic sample compounds for calibration even of published protocols. In light of the recent success of industrial scale applications of cap compounds in medicinal mRNA, such as vaccines,<sup>[9]</sup> a future surge in

## Introduction

The 5' cap is a critical motive in RNA modification and processing.<sup>[1]</sup> Various steps of maturation and function in different RNA molecules depend on its presence and precise methylation pattern, with messenger RNA (mRNA) being

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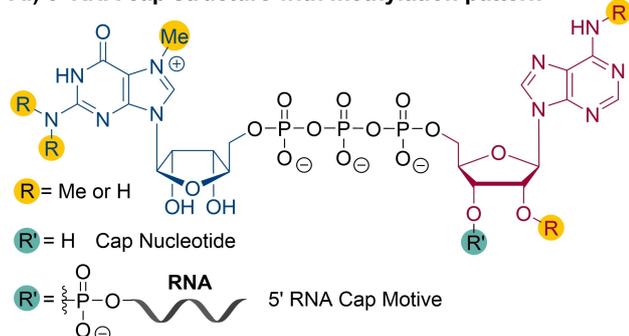
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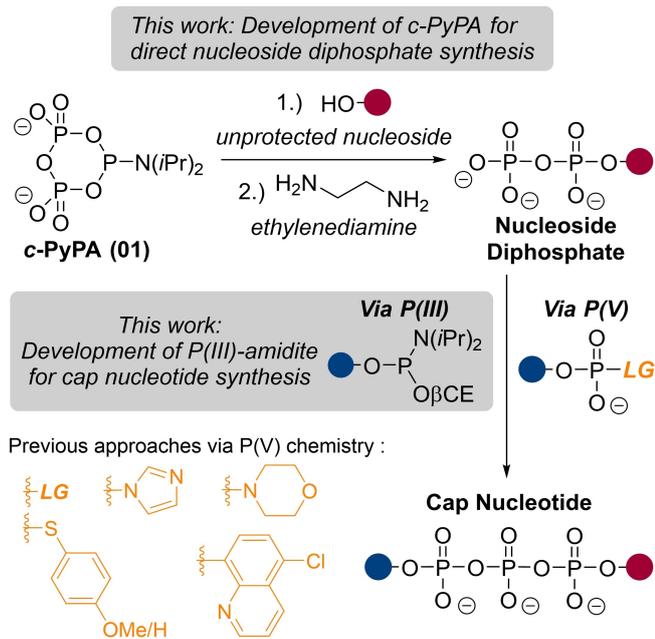
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## A.) 5' RNA cap structure with methylation pattern



## B.) Synthesis of cap nucleotide (prior and this work)



**Scheme 1.** A.) Prototypic 5' RNA cap structure (cap nucleotides) with variable methylation pattern. B.) Overview of published cap nucleotide syntheses and the P(III) based approach presented in this work. (LG = leaving-group).

demand of such compounds is anticipated.<sup>[6]</sup> The present study reports a versatile route to synthetic heavy isotope labeled cap nucleotides by phosphoramidite chemistry and demonstrates, how their application as references for LC–MS spectrometry studies improves detection and quantitation performance.

Cap nucleotides consist of two nucleosides linked together via a 5'–5' triphosphate bridge, a substructure also referred to as dinucleoside triphosphate.<sup>[10]</sup> Known synthetic approaches for dinucleoside triphosphates face a particular challenge in the asymmetric structure of caps.<sup>[11,12]</sup>

In general, available procedures rely on P(V)-chemistry in the P-anhydride generating step.<sup>[12]</sup> An electrophilic leaving-group (LG) activated phosphate is reacting with a nucleophilic phosphate group to form the phosphoanhydride bond. This activation and coupling was previously achieved using P(V)-imidazolides,<sup>[13]</sup> P(V)-morpholides,<sup>[14]</sup> P(V)-

phenylthio-<sup>[15]</sup> or P(V)-5-chloro-8-quinolyl-groups.<sup>[16]</sup> Activation can be achieved on the monophosphate (Scheme 1B) or on the diphosphate (not shown).<sup>[17]</sup> As a consequence, nucleoside diphosphates (NDPs) are required for cap nucleotide synthesis. Previously reported synthetic routes for NDPs are either based on protected nucleosides<sup>[18]</sup> or nucleoside monophosphates.<sup>[19,20]</sup>

A direct transformation of unprotected nucleosides into NDPs<sup>[21]</sup> is still a challenge and methods lag behind compared to nucleoside mono- or triphosphate synthesis.<sup>[22]</sup> In this context, we describe a robust one-step diphosphate synthesis starting from unprotected nucleosides by using the *c*-PyPA (**01**) reagent.<sup>[23,24]</sup> This P(III)-amidite reagent was already proven useful for the synthesis of  $\gamma$ -modified nucleoside triphosphates from unprotected nucleosides.<sup>[23]</sup> Notably, the use of P(III)-chemistry in cap-structure synthesis has not been explored previously. The benefits of such an approach<sup>[25]</sup> are short reaction times,<sup>[26]</sup> a facile protocol that tolerates moisture,<sup>[20]</sup> and a reduction of the overall step count, while enabling the synthesis of several cap nucleotides from unified precursor molecules.

## Results and Discussion

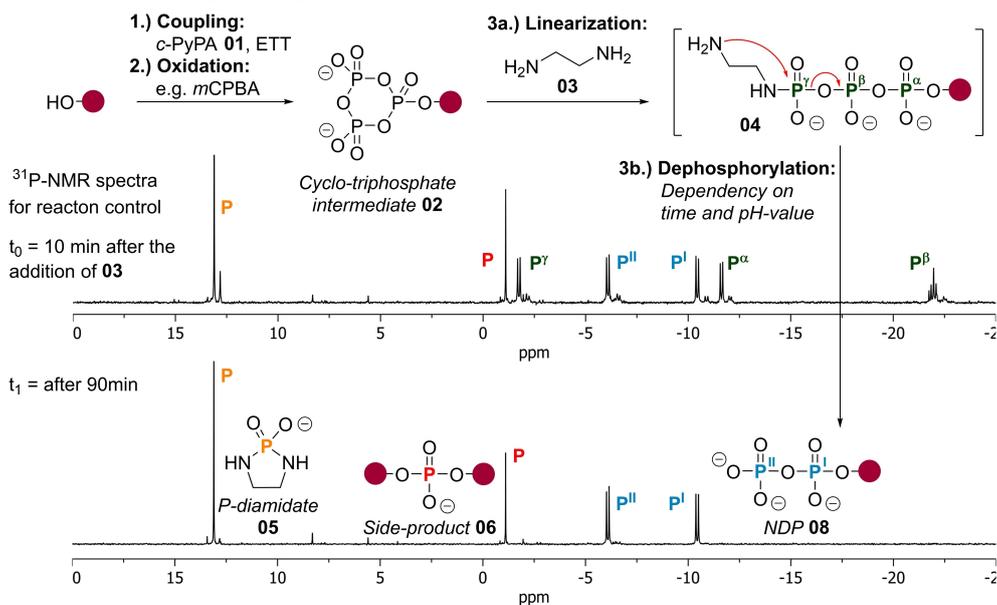
## Diphosphate Synthesis

For cap nucleotide synthesis, a convenient disconnection is in between the  $\beta$  and  $\gamma$ -phosphates, resulting in a diphosphate and a monophosphate that must then be connected in the forward sense. In our approach, the monophosphate is introduced in the P(III) oxidation state as a P-amidite and the diphosphate serves as nucleophile (Scheme 1B).

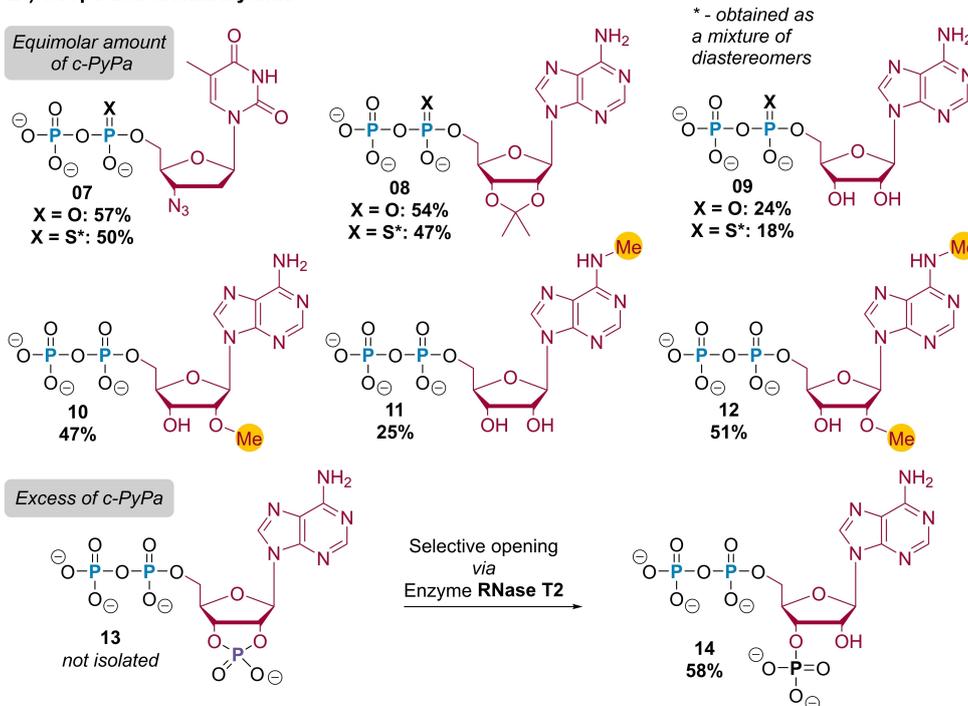
The direct synthesis of nucleoside diphosphates is underdeveloped and thus we explored a methodology to transform *cyclo*-triphosphate intermediates, such as (**02**) (Scheme 2) into diphosphates. Here, we followed a procedure initially reported by J. Ludwig and F. Eckstein in 1991.<sup>[27]</sup> This method was modified to obtain  $\alpha$ -borylated NDPs starting from protected nucleosides but was never scrutinized regarding scope.<sup>[28,29]</sup> Herein, we provide an analysis of scope by testing the diphosphorylation of several unprotected nucleosides, and also extend this to the direct synthesis of isoprenoid diphosphates (see Supporting Information). We find that this method can be broadly applied to access diphosphates of important metabolites in a straight-forward and modular fashion by combining it with the *c*-PyPA (**01**) cyclotriphosphorylating reagent.

As summarized in Scheme 2, we first investigated the direct transformation of cyclotriphosphate esters to the diphosphates, which encompasses linearization and terminal dephosphorylation (step 3a + b) simultaneously. This process is enabled by linearization with ethylenediamine (**03**) followed by cyclization and anhydride scission that removes the  $\gamma$ -phosphate as a phosphorous diamidate (**05**). Compared to literature precedence, which resulted in precipitation and yield losses,<sup>[28]</sup> we found it critical to reverse the sequence by introducing the cyclotriphosphate intermediate (**02**) into a solution of ethylenediamine and water. Fully

## A.) Mechanistic analysis of diphosphate synthesis



## B.) Scope and isolated yields



**Scheme 2.** A.) <sup>31</sup>P-<sup>1</sup>H NMR reaction monitoring of the nucleoside diphosphate synthesis by using c-PyPA (**01**) and ethylenediamine (**03**). Already at the first timepoint of measurement (t<sub>0</sub> = 10 min), the cyclo-triphosphate intermediate **02** cannot be seen anymore and was efficiently linearized. After 90 minutes, the NDP **08** is formed as main product. B.) An overview of the obtained molecules by the c-PyPA approach for dephosphorylation with isolated yields. Side-product **06** is shown in more detail in the SI, Scheme SI-1. The counter-ions for the phosphate moieties are also discussed in the experimental part of the SI.

dissolved reactants in the reaction mixture significantly facilitated the workflow, especially for scales above 100 mg. We also explored the suitability of different alkyl diamines for linearization and dephosphorylation.

Methanediamine did not undergo cyclization and 1,3-propyldiamine was significantly slower in diphosphate

formation, suggesting that ethylenediamine is currently the best choice for this transformation. 20 eq. of ethylenediamine gave optimal results: quantitative conversion into the diphosphate within ca. 90 min reaction time (Scheme 2A; for further details, see SI, Figure SI-4). The dephosphorylation step (3b, in Scheme 2A) was found to be pH dependent, and

pH values more acidic than 11 slowed the cyclization down. Usually, a mixture of ethylenediamine in water (1:3; pH value ca. 14) directly led to linearization and terminal dephosphorylation of the cyclotriphosphate (**02**) and was the optimal choice for diphosphate synthesis.

During the reaction optimization with 2',3'-*O*-isopropylidene-adenosine, we observed the formation of a mono-phosphate-diester (**06**) as a side-product. LC-MS analysis revealed that **06** was formed under aqueous alkaline conditions in approx. 20% (see SI, figure SI-6). This byproduct **06** was readily removed during strong anion exchange purification (SAX) or reversed phase chromatography and we could obtain the desired NDP **08** in a yield of about 50%.

After identifying the optimal conditions, we applied these to the synthesis of several other unprotected NDPs avoiding additional protection/deprotection steps (Scheme 2B). Nucleosides with only one or two reactive alcohols in the 5' and 3' position were transformed into their diphosphates in yields of around 50% (**07**, **08**, **10**, **12**). Nucleosides with free 2' and 3' alcohol moieties required a more complex chromatographic purification, which resulted in yields of ca. 25% (**09**, **12**). Nevertheless, the side products of an unwanted over-reaction on secondary alcohols are also of principal interest: they provide a direct entry into, for example, the synthesis of magic spot nucleotides.<sup>[30,31]</sup> By using excess *c*-PyPA and unprotected adenosine followed by ethylenediamine treatment, one obtains the 2'-3'-cyclophosphate (**13**) (Scheme 2). This can then be regioselectively ring-opened with RNase<sup>[30,31]</sup> giving ppAp (**14**) in a yield of 58%. To demonstrate the broad applicability of the approach, we also installed diphosphates onto isoprenyl-alcohols and we isolated the respective products after purification in a yield of up to 40% (see SI, **SI-06-07**).

Since P(III)-chemistry requires oxidation to the P(V) state, different oxidation reagents enable access to  $\alpha$ -P-modified nucleoside diphosphates. For example, application of the Beaucage reagent<sup>[32]</sup> led to  $\alpha$ -thiophosphate modified NDPs. These precursors allow access to  $\alpha$ -P-modified cap-structures, as described recently.<sup>[33]</sup>

### Synthesis of Cap Nucleotides

After developing the one-step synthesis of nucleoside diphosphates with the desired methylation pattern, we turned our attention towards the P(III)-amidite enabled anhydride synthesis to finalize the cap nucleotides. We decided to introduce the guanosine derived part of the cap nucleotide as its P(III)-amidite for solubility reasons. The required P-amidites (Scheme 3) were obtained on a 100 mg scale and could be stored at  $-20^{\circ}\text{C}$  for over 6 months.

Starting from commercially available guanosine (**15**), we first protected the 5' alcohol moiety with a TBS group on a 20 g scale. Afterwards, the remaining alcohols on the ribose subunit were protected as acetates. Fully protected guanosine derivative **16** was obtained quantitatively without further purification on a 5 g scale and served as a common intermediate to access all desired P(III)-amidites. A 5'-

deprotection by aq. HCl followed by phosphitylation led to guanosine P(III)-amidite **23**. There exists an interesting direct late-stage modification of P(III)-amidites to m<sup>1</sup>G or m<sup>2</sup>G derivatives as a potential alternative route.<sup>[34]</sup> However, the desired m<sup>7</sup>G derivatives required for this study were not accessible using this approach. Methylation by MeI or deuterated CD<sub>3</sub>I in dry THF, before 5'-deprotection and phosphitylation, provided access to m<sup>7</sup>G- or (CD<sub>3</sub>)<sup>7</sup>G P(III)-amidite (**24**, **25**). Reductive amination beforehand opened a route for trimethylated m<sup>2,2,7</sup>G P(III)-amidite (**26**) and the m<sup>7</sup> deuterium labeled derivative (**27**) was also prepared accordingly (Scheme 3).

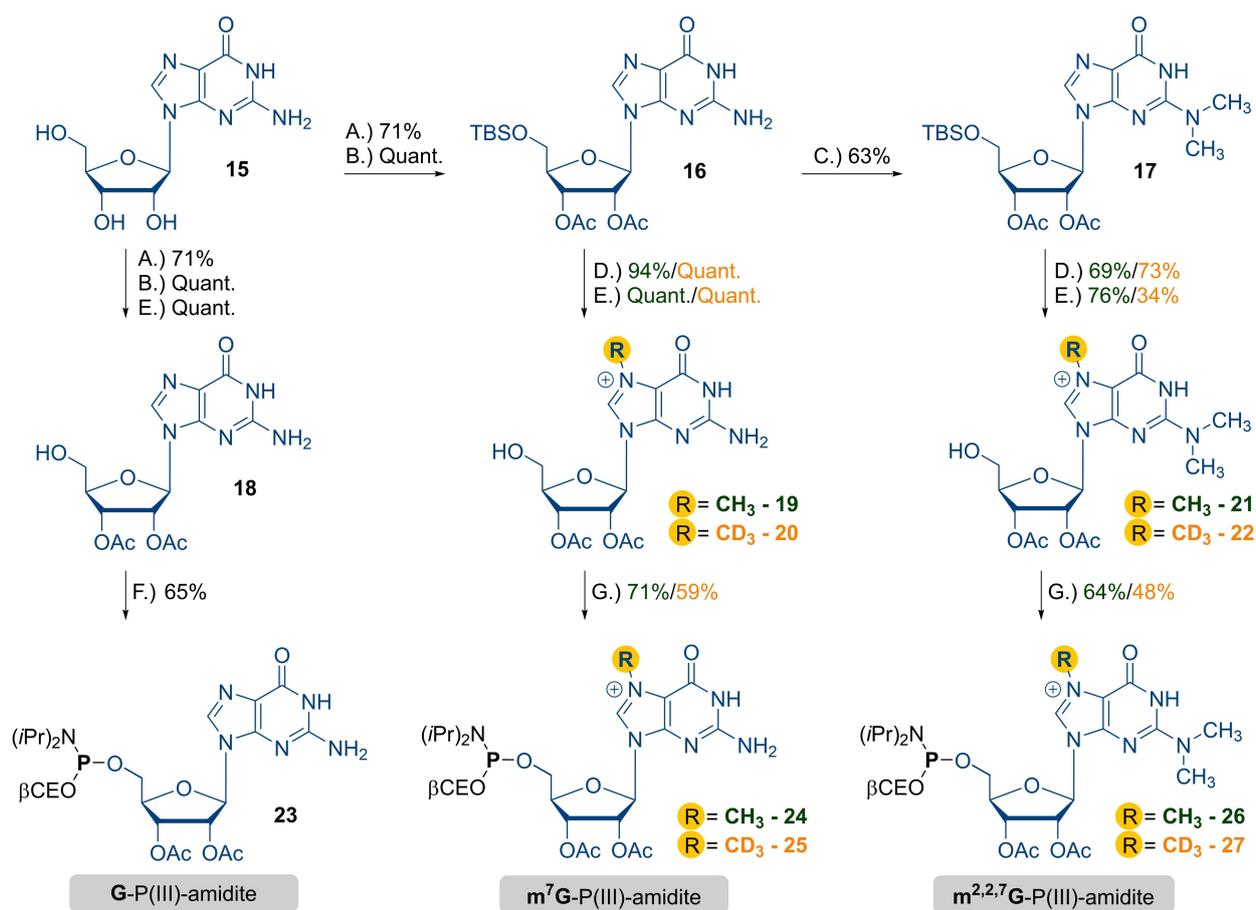
To finalize the cap nucleotides, we coupled the guanosine P(III)-amidites (**23-27**) with the corresponding adenosine diphosphates (Scheme 2, **10-12**) under slightly acidic conditions using ETT, as shown in Scheme 4. Since phosphate moieties react faster than alcohols, a protection OH functions on the NDPs was not required.<sup>[20]</sup> Clean oxidation was achieved with urea x H<sub>2</sub>O<sub>2</sub>.

We monitored the reaction progress by HPLC and found 1.7 eq. of P(III)-amidite suitable for a quantitative conversion followed by oxidation. An eluent mixture of 100 mM triethylammonium buffer at pH 7 against MeCN provided good separation on a C18 xbridge<sup>®</sup> column (Scheme 4A). These conditions were then applied for MPLC purification. During freeze-drying of the product, the  $\beta$ -cyanoethyl group on the phosphate was directly cleaved (Figure SI-13). Removal of the acetate protecting groups was not conducted with AMA-solution as the cap nucleotide decomposed quickly by nucleophilic attack of water on the 8-position.<sup>[35]</sup> To avoid this, we screened for a water-free deprotection conditions. A mixture of piperidine in dry MeOH was capable to remove the acetate groups within 60 min, without degrading the cap structure and resulting in almost quantitative conversion as seen by HPLC analysis (Scheme 4A).

To obtain cap-nucleotides in >99% purity for our analytical purposes, we performed a final preparative HPLC purification. Of note, an acidification of the mixture after deprotection with formic acid was necessary, to avoid decomposition during purification. In addition, capillary electrophoresis (CE)-MS analysis validated >99% isotopic enrichment of the deuterium labeled cap nucleotides (see SI).

### Cap Structure Analytics with Deuterium Labeled Standards

Synthetic cap nucleotides were applied to extensive LC-MS/MS method development. Intending to develop simultaneous quantitation of cap and other modified nucleosides, we started by using the MS parameters reported by Wang et al.,<sup>[5]</sup> the HPLC method of Muthmann et al.,<sup>[7]</sup> and our own source parameters<sup>[36]</sup> as starting point. No further optimization of MS parameters was necessary on our LC-QQQ (triple quadrupole) setup, as we achieved very similar sensitivity, which was not improved by variations of mass transitions, fragmentor voltage or collision energies. Chromatography optimization was conducted with an eye to clean separation of pairs of isobaric analytes with identical



**Scheme 3.** Unified synthetic steps to obtain guanosine derived P(III)-amidites with different methylation patterns. A.) TBS-Cl, imidazole, in DMF/DMSO. B.) Ac<sub>2</sub>O, NEt<sub>3</sub>, cat. DMAP, in MeCN. C.) *p*-formaldehyde, NaBH<sub>3</sub>CN, in AcOH. D.) MeI (or CD<sub>3</sub>I), in THF. E.) conc. aq. HCl, in EtOH. F.) βCEO(*i*Pr)<sub>2</sub>NPCL, DIPEA, in DCM. G.) βCEO[(*i*Pr)<sub>2</sub>N]<sub>2</sub>P, ETT, in DMF.

mass transitions, such as the analyte pairs <sup>m7</sup>GpppA–GpppAm and <sup>m7</sup>Gpppm<sup>6</sup>A–Gpppm<sup>6</sup>Am. We found significant impact of buffer ion strength, buffer pH and temperature on signal amplitude, peak width and symmetry, and separation, as detailed in the Supporting Information (Figure SI-20; Tables SI-1 to SI-4).

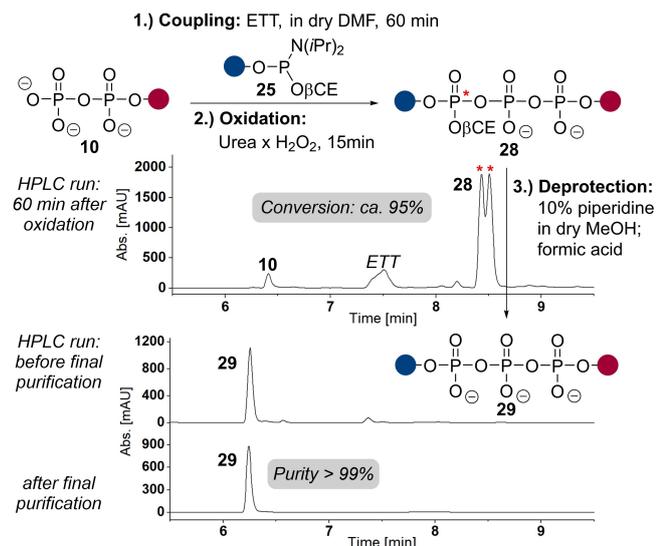
After optimization, an overall increase in peak abundance of ca. 2-fold and a robust separation were achieved. The subsequent optimization of ion source parameters, such as drying gas flow and temperature, capillary voltage, nozzle voltage, sheath gas flow and temperature, as well as nebulizer pressure, resulted in an additional increase in signal abundance by factors between 1.3 and 2.1. We determined instrument detection limits that varied only between 0.9 and 1.2 fmol for the different cap structures, with overall improvement factors of 2.1–3.4 relative to before optimization (Table SI-5).

The developed workflow for the quantitative analysis of 5' cap structures is depicted in Scheme 5A and starts with extraction of total RNA followed by polyA RNA pulldown with magnetic beads. After addition of labeled standard, enzymatical digestion leads to RNA on nucleoside level and cap nucleotides allowing the simultaneous detection of cap and internal nucleoside modifications by LC–MS/MS. For

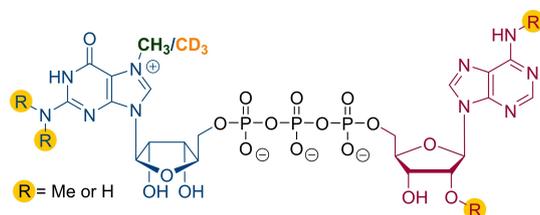
method validation, samples with known modification content were composed of a defined amount of cap nucleotides and HEK total RNA, and spiked with stable isotope-labeled internal standard (SILIS) of selected cap nucleotides, and measured against external calibration standards. Absolute quantitation that ignored the SILIS led to deviations from the true value up to 18 %, whereas taking SILIS corrections into account resulted in a considerable increase in quantitation accuracy with deviations from the true value of 0.3–7 %. As another useful feature, SILIS-based improvement was also achieved for analytes for which no structurally identical SILIS was available (see Table SI-6 and Figure SI-21). Scheme 5B shows the detection of five cap structures in a dilution series of the polyA fraction of HEK cell total RNA. Remarkably, the most abundant species <sup>m7</sup>Gpppm<sup>6</sup>Am was detectable in 5 ng polyA RNA, while <sup>m7</sup>GpppAm as the second most abundant was only measurable upwards of 100 ng, and comparative analysis of all five species only meaningful at 15 μg.

Since the above improvements pushed sensitivity for cap analysis to a level as previously available for nucleoside analysis, we used this same RNA preparation to demonstrate the feasibility of combining analysis of cap and internal modifications in a single run. As shown in

## A.) P(III)-amidite approach for cap nucleotide synthesis



## B.) Cap nucleotides and isolated yields after final purification



CH<sub>3</sub>: m<sup>7</sup>Gpppm<sup>6</sup>A: 52%, m<sup>7</sup>GpppAm: 26%, m<sup>7</sup>Gpppm<sup>6</sup>Am: 42%  
 CD<sub>3</sub>: m<sup>7</sup>Gpppm<sup>6</sup>A: 54%, m<sup>7</sup>GpppAm: 54%, m<sup>7</sup>Gpppm<sup>6</sup>Am: 54%  
 CH<sub>3</sub>: m<sup>2,2,7</sup>Gpppm<sup>6</sup>A: 55%, m<sup>2,2,7</sup>GpppAm: 60%  
 CD<sub>3</sub>: m<sup>2,2,7</sup>Gpppm<sup>6</sup>A: 58%, m<sup>2,2,7</sup>GpppAm: 50%, m<sup>2,2,7</sup>Gpppm<sup>6</sup>Am: 49%  
 Gpppm<sup>6</sup>A: 22%<sup>[a]</sup>, GpppAm: 30%<sup>[a]</sup>, Gpppm<sup>6</sup>Am: 36%<sup>[a]</sup>

**Scheme 4.** A.) Cap nucleotide synthesis by P(III)amidite chemistry, monitored by HPLC (254 nm). B.) Scope and isolated yields after final preparative HPLC purification of the cap nucleotides. [a] not optimized. The counter-ions for the phosphates are specified in the experimental part of the SI.

Scheme 5C, polyA RNA, which consists mainly of mRNA, lncRNA, and some ribosomal RNA (rRNA), contained N<sup>6</sup>-methyladenosine (m<sup>6</sup>A), 5-methylcytidine (m<sup>5</sup>C), pseudouridine (Ψ), and some N<sup>6</sup>,N<sup>6</sup>-dimethyladenosine (m<sup>6,6</sup>A), the latter presumably from polyadenylated rRNA.

We also quantified m<sup>7</sup>Gpppm<sup>6</sup>Am and m<sup>7</sup>GpppAm with SILIS in polyA RNA isolated from different mouse brain tissues, namely cerebellum, cortex and hippocampus. Here, the sensitivity of the method enabled the analysis of moderate amounts of polyA RNA ranging from 1 to 2.4 μg. Interestingly, we found that cortex tissue stood out, displaying fewer cap moieties per 1000 nucleotides (see Scheme 5D). Additionally, mouse brain tissues showed higher proportions of N<sup>6</sup>-methylation ranging from 0.96–0.98 compared to HEK cells with 0.90 (Scheme 5E). Of note, the RNA was isolated according to a protocol specifically optimized and validated to avoid RNA degradation.<sup>[37]</sup>

## Conclusions

The chemical synthesis of cap structures is important to access and alter this RNA modification. Recent approaches have relied on P(V) chemistry. Here, we explored the potential of a P(III) approach starting from nucleoside diphosphates and modified nucleoside P(III)-amidites. Through the intermediacy of a P(III)–P(V) anhydride, diverse structures were accessible and led to the synthesis of an array of different cap structures in excellent yields and quality. The synthesis was extended to access stable heavy isotope labeled internal standard cap structures on a multi-milligram scale for analytical applications. As an additional novelty, the required nucleoside diphosphates for cap synthesis were made in a one-step protecting-group free diphosphorylation reaction using the c-PyPA reagent and ethylenediamine. This reaction was further scrutinized regarding scope and also enables the direct synthesis of other important diphosphates from unprotected alcohol precursors.

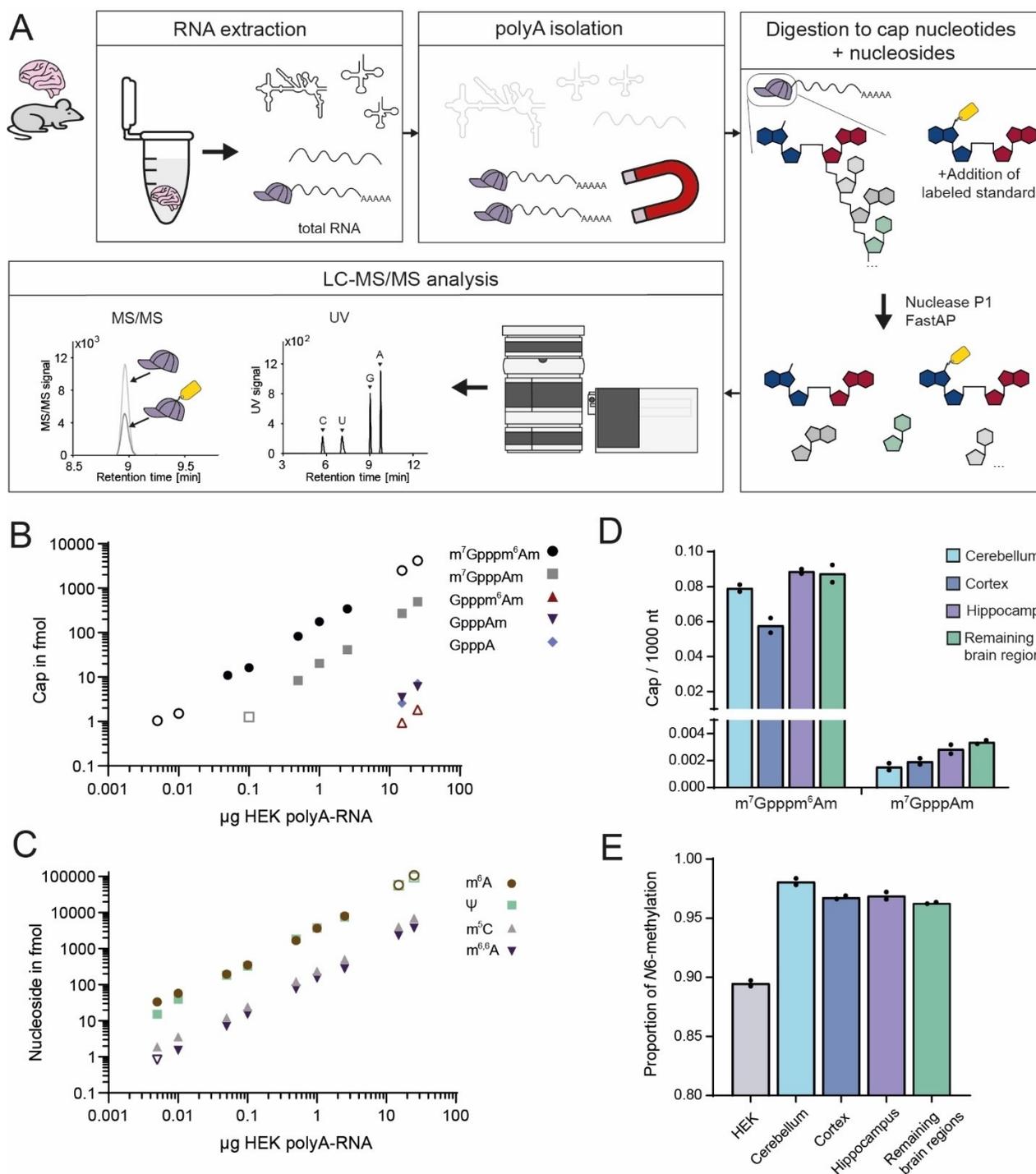
The utility of the new SILIS was demonstrated in the analysis of cap structures found in biological samples by HPLC-QQQ-MS. Of particular note are the excellent instrument detection limits in the attomole to femtomole range, as well as the possibility to detect, assign, and quantify cap structures as well as other modified nucleosides, such as m<sup>5</sup>C, in one single run. The straightforward synthesis and modification of high-quality reference cap structures with P(III) chemistry and the excellent analytical performance of the method will help to better understand the cap epitranscriptome in the future.

## Supporting Information

Supporting Information including experimental details, copies of LC-MS traces and NMR spectra. The authors have cited additional references within the Supporting Information.<sup>[38]</sup>

## Acknowledgements

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**Scheme 5.** A.) Schematic illustration of the workflow for the analysis of 5' cap structures in RNA. After organic extraction of total RNA and polyA RNA pulldown with magnetic beads, SILIS cap nucleotides are spiked and the whole sample is digested with nuclease P1 and fast alkaline phosphatase (FastAP) to nucleoside level while leaving the cap intact. The sample is subjected to LC-MS/MS, chromatographically separated (the peaks of the main nucleosides are assigned and abbreviated as C—cytidine, U—uridine, G—guanosine, and A—adenosine) and analyzed by MS. The cap structure is symbolized with a purple base cap. D<sub>3</sub>C-isotope labeled cap structures are marked with a yellow tag and an exemplary modified nucleoside is depicted in green. B.) Detection of five cap species in a dilution series of the polyA fraction of HEK cell total RNA. The empty markers are outside the calibrated range. C.) Detection of nucleosides in a dilution series of the polyA fraction of HEK cell total RNA. Empty markers are outside the calibrated range. D.) Quantitation of m<sup>7</sup>Gpppm<sup>6</sup>Am and m<sup>7</sup>GpppAm per 1000 nucleotides in polyA RNA isolated from different mouse brain tissues. E.) Proportion of N<sup>6</sup>-methylation calculated as the quotient of m<sup>7</sup>Gpppm<sup>6</sup>Am and the sum of m<sup>7</sup>Gpppm<sup>6</sup>Am and m<sup>7</sup>GpppAm in the polyA fraction of mouse brain regions and HEK cells.

**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** RNA cap · stable isotope labeling · phosphorylation · epitranscriptome · LC–MS

- [1] a) A. Ramanathan, G. B. Robb, S.-H. Chan, *Nucleic Acids Res.* **2016**, *44*, 7511; b) M. Helm, J. D. Alfonzo, *Chem. Biol. (Oxford, U. K.)* **2014**, *21*, 174.
- [2] a) A. G. Matera, R. M. Terns, M. P. Terns, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 209; b) N. Podvalnaya, A. W. Bronkhorst, R. Lichtenberger, S. Hellmann, E. Nischwitz, T. Falk, E. Karaulanov, F. Butter, S. Falk, R. F. Ketting, *Nature* **2023**, 622, 402.
- [3] J. Pelletier, T. M. Schmeing, N. Sonenberg, *Wiley Interdiscip. Rev.: RNA* **2021**, *12*, e1636.
- [4] a) P. Fechter, G. G. Brownlee, *J. Gen. Virol.* **2005**, *86*, 1239; b) H. Malet, H. M. Williams, S. Cusack, M. Rosenthal, *PLoS Pathog.* **2023**, *19*, e1011060; c) A. D. Gelinas, D. R. Davies, N. Janjic, *Curr. Opin. Struct. Biol.* **2016**, *36*, 122; d) L. Xie, C. Wartchow, S. Shia, K. Uehara, M. Steffek, R. Warne, J. Sutton, G. T. Muiru, V. H. J. Leonard, D. E. Bussiere, et al., *J. Biol. Chem.* **2016**, *291*, 363.
- [5] J. Wang, B. L. Alvin Chew, Y. Lai, H. Dong, L. Xu, S. Balamkundu, W. M. Cai, L. Cui, C. F. Liu, X.-Y. Fu, et al., *Nucleic Acids Res.* **2019**, *47*, e130.
- [6] J. Wang, B. L. A. Chew, Y. Lai, H. Dong, L. Xu, Y. Liu, X.-Y. Fu, Z. Lin, P.-Y. Shi, T. K. Lu, et al., *Nat. Protoc.* **2023**, *18*, 2671.
- [7] N. Muthmann, P. Špaček, D. Reichert, M. van Dülmen, A. Rentmeister, *Methods (Amsterdam, Neth.)* **2022**, *203*, 196.
- [8] A. Galloway, A. Atrih, R. Grzela, E. Darzynkiewicz, M. A. J. Ferguson, V. H. Cowling, *Open Biol.* **2020**, *10*, 190306.
- [9] a) U. Sahin, A. Muik, E. Derhovanessian, I. Vogler, L. M. Kranz, M. Vormehr, A. Baum, K. Pascal, J. Quandt, D. Maurus, et al., *Nature* **2020**, *586*, 594; b) J. M. Henderson, A. Ujita, E. Hill, S. Yousif-Rosales, C. Smith, N. Ko, T. McReynolds, C. R. Cabral, J. R. Escamilla-Powers, M. E. Houston, *Curr. Protoc.* **2021**, *1*, e39; c) M. Warminski, A. Mamot, A. Depaix, J. Kowalska, J. Jemielity, *Acc. Chem. Res.* **2023**, *56*, 2814.
- [10] a) L. Appy, C. Chardet, S. Peyrottes, B. Roy, *Molecules* **2019**, *24*; b) B. Roy, A. Depaix, C. Périgaud, S. Peyrottes, *Chem. Rev.* **2016**, *116*, 7854; c) N. Stern, D. T. Major, H. E. Gottlieb, D. Weizman, B. Fischer, *Org. Biomol. Chem.* **2010**, *8*, 4637; d) S. Mohamady, A. Desoky, S. D. Taylor, *Org. Lett.* **2012**, *14*, 402; e) A. Hofer, G. S. Cremonnik, A. C. Müller, R. Giambruno, C. Trefzer, G. Superti-Furga, K. L. Bennett, H. J. Jessen, *Chem. - Eur. J.* **2015**, *21*, 10116; f) Y. Ahmadibeni, K. Parang, *Org. Lett.* **2007**, *9*, 4483; g) S. M. Hacker, F. Mortensen, M. Scheffner, A. Marx, *Angew. Chem. Int. Ed.* **2014**, *53*, 10247.
- [11] a) F. Muttach, N. Muthmann, A. Rentmeister, *Beilstein J. Org. Chem.* **2017**, *13*, 2819; b) A. Kore, I. Charles, M. Shanmugasundaram, Z. Xiao, R. Conrad, *Mini-Rev. Org. Chem.* **2008**, *5*, 179; c) S. Mikkola, S. Salomaki, Z. Zhang, E. Maki, H. Lonnberg, *Curr. Org. Chem.* **2005**, *9*, 999.
- [12] M. Shanmugasundaram, A. Senthilvelan, A. R. Kore, *Chem. Rec.* **2022**, *22*, e202200005.
- [13] a) H. Sawai, H. Wakai, M. Shimazu, *Tetrahedron Lett.* **1991**, *32*, 6905; b) H. Sawai, M. Shimazu, H. Wakai, H. Wakabayashi, K. Shinozuka, *Nucleosides Nucleotides* **1992**, *11*, 773; c) A. R. Kore, M. Shanmugasundaram, I. Charles, A. V. Vlassov, T. J. Barta, *J. Am. Chem. Soc.* **2009**, *131*, 6364; d) J. Stepinski, C. Waddell, R. Stolarski, E. Darzynkiewicz, R. E. Rhoads, *RNA* **2001**, *7*, 1486; e) J. Jemielity, J. Stepinski, M. Jaremko, D. Haber, R. Stolarski, R. E. Rhoads, E. Darzynkiewicz, *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 691; f) M. Inagaki, N. Abe, Z. Li, Y. Nakashima, S. Acharyya, K. Ogawa, D. Kawaguchi, H. Hiraoka, A. Banno, Z. Meng, et al., *Nat. Commun.* **2023**, *14*, 2657.
- [14] Z.-H. Peng, V. Sharma, S. F. Singleton, P. D. Gershon, *Org. Lett.* **2002**, *4*, 161.
- [15] a) R. Iwase, M. Sekine, Y. Tokumoto, Y. Ohshima, T. Hata, *Nucleic Acids Res.* **1989**, *17*, 8979; b) T. Kamimura, Y. Osaki, M. Sekine, T. Hata, *Tetrahedron Lett.* **1984**, *25*, 2683; c) I. Nakagawa, S. Konya, S. Ohtani, T. Hata, *Synthesis* **1980**, 1980, 556; d) M. Sekine, S. Nishiyama, T. Kamimura, Y. Osaki, T. Hata, *Bull. Chem. Soc. Jpn.* **1985**, *58*, 850.
- [16] K. Fukuoka, F. Suda, R. Suzuki, H. Takaku, M. Ishikawa, T. Hata, *Tetrahedron Lett.* **1994**, *35*, 1063.
- [17] a) H. Sawai, H. Wakai, A. Nakamura-Ozaki, *J. Org. Chem.* **1999**, *64*, 5836; b) B. A. Wojtczak, M. Bednarczyk, P. J. Sikorski, A. Wojtczak, P. Surynt, J. Kowalska, J. Jemielity, *J. Org. Chem.* **2023**, *88*, 6827.
- [18] a) V. J. Davison, D. R. Davis, V. M. Dixit, C. D. Poulter, *J. Org. Chem.* **1987**, *52*, 1794; b) Q. Sun, S. Gong, J. Sun, S. Liu, Q. Xiao, S. Pu, *J. Org. Chem.* **2013**, *78*, 8417; c) S. Warnecke, C. Meier, *J. Org. Chem.* **2009**, *74*, 3024; d) M. Rachwalak, M. Rozniewska, J. Golebiewska, T. Jakubowski, M. Sobkowski, J. Romanowska, *Synth. Commun.* **2020**, *50*, 3836.
- [19] a) A. R. Kore, G. Parmar, *Synth. Commun.* **2006**, *36*, 3393; b) A. Depaix, S. Peyrottes, B. Roy, *Eur. J. Org. Chem.* **2017**, *2017*, 241; c) H. J. Jessen, N. Ahmed, A. Hofer, *Org. Biomol. Chem.* **2014**, *12*, 3526; d) E. Calleri, S. Ceruti, G. Cristalli, C. Martini, C. Temporini, C. Parravicini, R. Volpini, S. Daniele, G. Caccialanza, D. Lecca, et al., *J. Med. Chem.* **2010**, *53*, 3489.
- [20] G. S. Cremonnik, A. Hofer, H. J. Jessen, *Angew. Chem. Int. Ed.* **2014**, *53*, 286.
- [21] B. Fischer, J. L. Boyer, C. H. Hoyle, A. U. Ziganshin, A. L. Brizzolara, G. E. Knight, J. Zimmet, G. Burnstock, T. K. Harden, K. A. Jacobson, *J. Med. Chem.* **1993**, *36*, 3937.
- [22] S. M. Shepard, H. J. Jessen, C. C. Cummins, *J. Am. Chem. Soc.* **2022**, *144*, 7517.
- [23] J. Singh, A. Ripp, T. M. Haas, D. Qiu, M. Keller, P. A. Wender, J. S. Siegel, K. K. Baldrige, H. J. Jessen, *J. Am. Chem. Soc.* **2019**, *141*, 15013.
- [24] a) J. Singh, N. Steck, D. De, A. Hofer, A. Ripp, I. Captain, M. Keller, P. A. Wender, R. Bhandari, H. J. Jessen, *Angew. Chem. Int. Ed.* **2019**, *58*, 3928; b) A. Ripp, J. Singh, H. J. Jessen, *Curr. Protoc. Nucleic Acid Chem.* **2020**, *81*, e108.
- [25] a) M. Kukhanova, X. Li, S. H. Chen, I. King, T. Doyle, W. Prusoff, Y. C. Cheng, *Mol. Pharmacol.* **1998**, *53*, 801; b) H. J. Jessen, T. Dürr-Mayer, T. M. Haas, A. Ripp, C. C. Cummins, *Acc. Chem. Res.* **2021**, *54*, 4036.
- [26] H. Gold, P. van Delft, N. Meeuwenoord, J. D. C. Codée, D. V. Filippov, G. Eggink, H. S. Overkleeft, G. A. van der Marel, *J. Org. Chem.* **2008**, *73*, 9458.
- [27] J. Ludwig, F. Eckstein, *J. Org. Chem.* **1991**, *56*, 1777.
- [28] P. Li, Z. Xu, H. Liu, C. K. Wenefors, M. I. Dobrikov, J. Ludwig, B. R. Shaw, *J. Am. Chem. Soc.* **2005**, *127*, 16782.
- [29] a) T. Ginsburg-Shmuel, M. Haas, D. Grbic, G. Arguin, Y. Nadel, F.-P. Gendron, G. Reiser, B. Fischer, *Bioorg. Med. Chem.* **2012**, *20*, 5483; b) S. Yelovitch, J. Camden, G. A. Weisman, B. Fischer, *J. Med. Chem.* **2012**, *55*, 437.

- [30] T. M. Haas, D. Qiu, M. Häner, L. Angebauer, A. Ripp, J. Singh, H.-G. Koch, C. Jessen-Trefzer, H. J. Jessen, *J. Org. Chem.* **2020**, *85*, 14496.
- [31] T. M. Haas, P. Ebersperger, V. B. Eisenbeis, C. Nopper, T. Dürr, N. Jork, N. Steck, C. Jessen-Trefzer, H. J. Jessen, *Chem. Commun. (Camb.)* **2019**, *55*, 5339.
- [32] R. P. Iyer, W. Egan, J. B. Regan, S. L. Beaucage, *J. Am. Chem. Soc.* **1990**, *112*, 1253.
- [33] H.-J. Zhang, M. Ociepa, M. Nassir, B. Zheng, S. A. Lewicki, V. Salmaso, H. Baburi, J. Nagel, S. Mirza, B. Bueschbell, et al., *Nat. Chem.* **2024**, *16*, 249.
- [34] K. Ziemkiewicz, M. Warminski, R. Wojcik, J. Kowalska, J. Jemielity, *J. Org. Chem.* **2022**, *87*, 10333.
- [35] E. Darzynkiewicz, J. Stepinski, S. M. Tahara, R. Stolarski, I. Ekiel, D. Haber, K. Neuvonen, P. Lehikoinen, I. Labadi, H. Lönnberg, *Nucleosides Nucleotides* **1990**, *9*, 599.
- [36] J. Hertler, K. Slama, B. Schober, Z. Özrendeci, V. Marchand, Y. Motorin, M. Helm, *Nucleic Acids Res.* **2022**, *50*, e115.
- [37] F. Richter, J. E. Plehn, L. Bessler, J. Hertler, M. Jörg, C. Cirzi, F. Tuorto, K. Friedland, M. Helm, *Nucleic Acids Res.* **2022**, *50*, 4201.
- [38] a) G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176; b) I. B. Yanachkov, H. Chang, M. I. Yanachkova, E. J. Dix, M. A. Berny-Lang, T. Gremmel, A. D. Michelson, G. E. Wright, A. L. Frelinger, *Eur. J. Med. Chem.* **2016**, *107*, 204; c) S. Mikkola, N. Koissi, K. Ketomäki, S. Rauvala, K. Neuvonen, H. Lönnberg, *Eur. J. Org. Chem.* **2000**, *2000*, 2315; d) V. Tararov, S. Kolyachkina, C. Alexeev, S. Mikhailov, *Synthesis* **2011**, *2011*, 2483; e) Y. Ishido, N. Nakazaki, N. Sakairi, *J. Chem. Soc., Perkin Trans. 1 (1972–1999)* **1979**, 2088; f) X. Zhang, L.-H. Wei, Y. Wang, Y. Xiao, J. Liu, W. Zhang, N. Yan, G. Amu, X. Tang, L. Zhang, et al., *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 2919; g) J. H. Cho, S. J. Coats, R. F. Schinazi, *Org. Lett.* **2012**, *14*, 2488; h) D. Qiu, M. S. Wilson, V. B. Eisenbeis, R. K. Harmel, E. Riemer, T. M. Haas, C. Wittwer, N. Jork, C. Gu, S. B. Shears, et al., *Nat. Commun.* **2020**, *11*, 6035; i) *European pharmacopoeia 11.6, 2.2.46 Chromatographic Separation Techniques*, European Directorate for the Quality of Medicines: Council of Europe, Strasbourg, France, **2024**; j) N. P. Parra, L. Taylor, “Why Instrument Detection Limit (IDL) Is a Better Metric for Determining The Sensitivity of Triple Quadrupole LC/MS Systems Technical Note (Agilent)”, **2014**; k) T. L. Sheehan, R. A. Yost, *Spectroscopy* **2015**, *13*, 16; l) C. Brandscheid, F. Schuck, S. Reinhardt, K.-H. Schäfer, C. U. Pietrzik, M. Grimm, T. Hartmann, A. Schwierz, K. Endres, *Journal of Alzheimer's disease : JAD* **2017**, *56*, 775.

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