Synthesis of nucleotide analogs modified or functionalized in the oligophosphate chain and their application in the study of processes of biological or therapeutic significance

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Author's presentation for the application for the academic degree of habilitated doctor

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1. Name and surname.

Joanna Kowalska

2. Diplomas, degrees-with the name, place and year of obtaining them and the title of a doctoral dissertation.

Doctor of Physics (with distinction), University of Warsaw, Faculty of Physics, 13 December 2010, "Synthesis and properties of 5′ mRNA end (cap) analogs modified in the phosphate chain and their application in research on mechanisms of mRNA degradation and protein translation," supervisor dr hab. Edward Darżynkiewicz

Master's Degree in Chemistry (with distinction, uniform Master's studies within the MISMaP College), University of Warsaw, Faculty of Chemistry, 27 June 2006, "Synthesis and study of 5′ mRNA terminus analogs containing thiophosphate residue," supervisor Jacek Jemielity, PhD

Bachelor's degree in biotechnology in the field of molecular biology (first-degree studies within MISMaP), University of Warsaw, Faculty of Biology, 8 July 2005, "The role of RMA domains in RNA-protein interactions," supervisor Agnieszka Dzikowska, PhD

3. Information concerning previous employment in scientific institutions.

February 2011–still, scientific and didactic adjunct, Faculty of Physics, University of Warsaw

October 2010–January 2011 scientific and technical employee, Faculty of Physics, University of Warsaw (half time)

4. DISCUSSION OF THE MOST IMPORTANT ACHIEVEMENTS INCLUDED IN THE PAPERS PRESENTED FOR HABILITATION

An indication of the achievement resulting from Article 16(2) of the Act of 14 March 2003 on Academic Degrees and Academic Title and Degrees and Title in Art (Journal of Laws No. 65, item 595, as amended):

a) title of scientific/artistic achievement,

"Synthesis of nucleotide analogs modified or functionalized in the oligophosphate chain and their application in the study of processes of biological or therapeutic significance"

b) Original scientific creative works cited in the author's presentation, constituting the basis for the application for the initiation of habilitation proceedings: (author(s), title(s) of publication, year of publication, the name of the publishing house).

IF-impact factor from the year of publication

H1. Kowalska, J., Osowniak, A., Zuberek, J. and Jemielity, J.* (2012) Synthesis of nucleoside phosphosulfates. *Bioorganic & Medicinal Chemistry Letters*, 22, 3661–3664
IF 2.286 / citations: 10

H2. Dabrowski-Tumanski, P., Kowalska, J., Jemielity, J.* (**2013**) Efficient and Rapid Synthesis of Nucleoside Diphosphate Sugars from Nucleoside Phosphorimidazolides. *European Journal of Organic Chemistry*, **2013**, 2147–2154

IF 2.762 / citations: 12

H3. Strenkowska, M., Wanat, P., Ziemniak, M., Jemielity, J.,* Kowalska, J.* (**2012**) Preparation of Synthetically Challenging Nucleotides Using Cyanoethyl P-Imidazolides and Microwaves. *Organic Letters*, **14**, 4782–4785

IF 6.492 / citations: 34

H4. Baranowski, M.R., Nowicka, A., Rydzik, A.M., Warminski, M., Kasprzyk, R., Wojtczak, B.A., Wojcik, J., Claridge, T.D.W., Kowalska, J.*, Jemielity, J.* (**2015**) Synthesis of Fluorophosphate Nucleotide Analogues and Their Characterization as Tools for ^{19}F NMR Studies. *The Journal of Organic Chemistry*, **80**, 3982–3997

IF 4.425 / citations: 18

H5. Wanat, P., Walczak, S., Wojtczak, B.A., Nowakowska, M., Jemielity, J. and Kowalska, J.* (**2015**) Ethynyl, 2-Propynyl, and 3-Butynyl C-Phosphonate Analogues of Nucleoside Di-and Triphosphates: Synthesis and Reactivity in CuAAC. *Organic Letters*, **17**, 3062–3065.

IF 6.492 / citations: 14

H6. Baranowski, M.R.#, Nowicka, A.#, Jemielity, J., Kowalska, J.* (**2016**) A fluorescent HTS assay for phosphohydrolases based on nucleoside 5′-fluorophosphates: its application in screening for inhibitors of mRNA decapping scavenger and PDE-I. *Organic & biomolecular chemistry* 14 (20), 4595–4604

IF 3.235 / Cyt: 10

H7. Kasprzyk, R., Kowalska, J.,* Wieczorek, Z., Szabelski, M., Stolarski, R. Jemielity, J.* (**2016**) Acetylpyrene-labelled 7-methylguanine nucleotides: unusual fluorescence properties and application to decapping scavenger activity monitoring *Organic & biomolecular chemistry* 14 (16), 3863–3868

IF 3.235 / citations: 7

H8. Wanat P., Kasprzyk R., Kopcial M., Sikorski P.J., Strzelecka D., Jemielity J., Kowalska J.* (**2018**) ExciTides: NTP-derived probes for monitoring pyrophosphatase activity based on excimer-to-monomer transitions *Chemical Communications*, 54, 9773–9776

IF 6.290 / citations: 0

H9. Kozarski M., Kubacka D., Wojtczak B. A., Kasprzyk R., Baranowski M.R., Kowalska J* (**2018**) 7-Methylguanosine monophosphate analogues with 5′-(1, 2, 3-triazoyl) moiety: Synthesis and evaluation as the inhibitors of cNIIIB nucleotidase, *Bioorganic and Medicinal Chemistry*, 26(1), 191–199, 2018

IF 2.793 / citations: 0

H10. Mlynarska-Cieslak A., Depaix A., Grudzien-Nogalska E., Sikorski P.J., Warminski M., Kiledjian M., Jemielity J., Kowalska J.* (**2018**) Nicotinamide Containing Di-and Trinucleotides as Chemical Tools for Studies of NAD-Capped RNAs, *Organic Letters, 20, 23, 7650–7655*

IF 6.492 / citations: 1

H11. Strzelecka, D., Chmielinski, S., Bednarek, S., Jemielity, J., Kowalska, J.* (**2017**) Analysis of mononucleotides by tandem mass spectrometry: investigation of fragmentation

pathways for phosphate-and ribose-modified nucleotide analogues *Scientific Reports, 7,* article no. 8931
IF 4.847 / citations: 1

c) presentation of the scientific aim of the abovementioned papers and the results achieved, together with a discussion of their possible use.

Introduction

1. Low molecular nucleotides-a diverse group of natural compounds with important biological functions

Small-molecule nucleotides from the group of nucleoside and dinucleoside polyphosphates perform many key functions in living organisms (Fig. 1). Ribo- and deoxyribonucleoside triphosphates are used in the cell as **substrates for the synthesis of nucleic acids**, RNA and DNA, respectively. Some nucleotides, such as ATP or GTP, are a **source of free energy** needed for enzymatic reactions in the body. Nucleotide **enzymatic cofactors** NAD^+, $NADP^+$ and FAD^+ are necessary for the function of oxidoreductase enzymes, but also perform other important functions in the cell. Adenosine triphosphate (ATP) is a universal donor of the phosphate group for most cellular kinases, i.e., enzymes regulating the activity of small molecules and biopolymers through their phosphorylation. Similarly, 3'-phosphate-adenosine phosphosulfate (PAPS) is a universal donor of the sulfate group for sulfotransferases. Another group of nucleotides with a highly important role are **nucleotide-derived signaling molecules**. GTP is essential for the transmission of signals through G-protein-bound receptors. Cyclic nucleotides such as cAMP and cGMP are second-order signaling molecules which transfer information from a ligand-bound receptor to effectors. Nucleotides of Ap_nA, Ap_nU, Ap_n, Up_n (n=2–4) type and nucleotide sugars act as extracellular signaling molecules binding to metabotropic receptors, i.e., transmembrane receptors which receive signals from extracellular space and transmit them to the cell interior.(1) Nucleotide sugars are also involved in monosaccharide metabolism and transglycosylation reactions, serving as substrates for oligosaccharide synthesis. An example of this kind of compound is uridine diphosphate glucose (UDPG), which is a substrate for the glucosyltransferase enzyme and thus a source of active glucose form in metabolic reactions. (1) Finally, nucleoside oligophosphates are often **molecules that modify nucleic acids termini**, in particular, RNAs, allowing very strict regulation of gene expression processes. The best-known example of this type is the 7-methylguanine cap present at the 5' mRNA terminus in Eukaryotes, but recent studies indicate that other nucleotides such as NAD, FAD or adenine dinucleotides can also be found at the 5' RNA terminus. (2-4)

Due to the numerous and complex functions of nucleotides, their endogenous levels are strictly controlled, both at the biosynthesis and degradation stages. The disturbance of this control may lead both to the deficiency of key nucleotides and to their accumulation at toxic concentrations and, consequently, to the development of diseases. One of the important elements of regulation of nucleotide pool available for particular enzymatic or signaling processes is the control of their phosphorylation degree. Important classes of enzymes involved in these processes are phosphatases and pyrophosphatases catalyzing hydrolysis reactions of phosphoric and

pyrophosphate bonds, respectively, present in nucleotides. These enzymes are involved in key processes of biosynthesis, degradation, and repair of nucleotides and nucleic acids. Disorders of their activity has been associated with the development of cancer, metabolic or neuromuscular diseases. Consequently many of those enzymes have been identified as therapeutic targets. For many years now, research has been carried out around the world on the structures and mechanisms of pyrophosphatases important in the therapeutic context, and low molecular ligands modulating their activity are being sought, which may be helpful in understanding the mechanisms responsible for the regulation of pyrophosphatase activity and give rise to new therapies. (5)

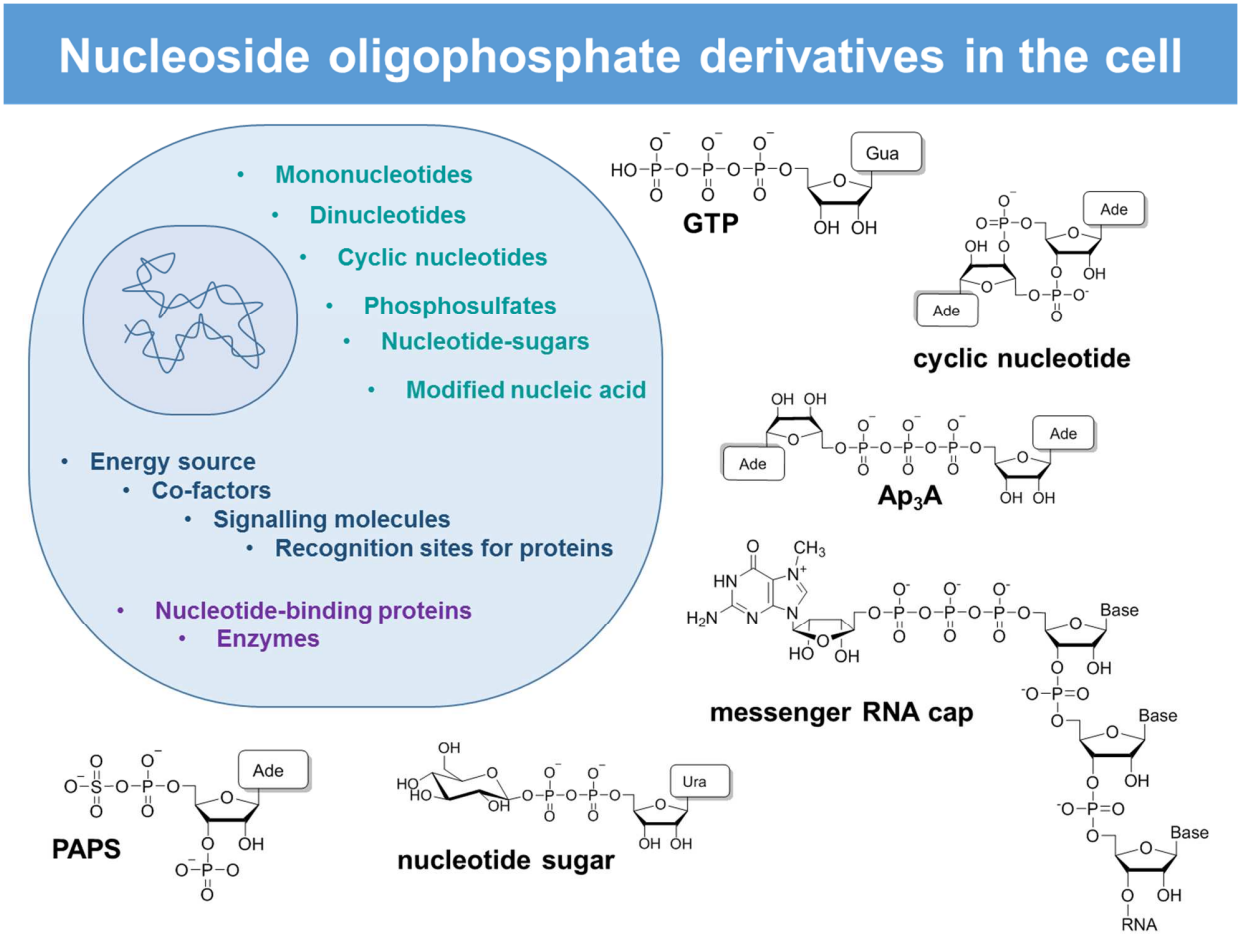


Fig. 1. Low molecular nucleotides in the cell-review of biological structures and functions.

Nucleotide analogs designed in a rational way can be helpful in understanding natural processes and disorders related to nucleotide metabolism, their diagnostics and proposing therapeutic solutions. Labeled analogs can be used to track biological processes related to nucleotides both *in vitro* ("in a tube") and *in vivo* (understood here as a living cell or organism) and to search for chemical compounds affecting these processes in the desired way (screening assays in search for potential drugs). However, analogs resistant to enzymatic degradation can be used as ligands in structural and biophysical studies as well as selective inhibitors of natural processes.

The physiological importance of nucleotides and their derivatives also implies an enormous therapeutic potential for synthetic nucleotide analogs. Nucleotide derivatives and nucleotide

analogs are used as anticancer and antiviral therapeutics. The active forms of these drugs are their phosphorylated metabolites, i.e., nucleotides. Unfortunately, small molecule nucleotides, due to their highly polar structure and negative resultant charge, are not capable of penetrating biological membranes on the basis of passive diffusion, which significantly limits their medicinal applications. However, thanks to the development of several cell delivery methods for polar nucleotides (the "pro-drug" concept, viral vehicles, nanogels, capsuling, liposome closure) there is an increasing number of experimental pharmaceuticals designed on the basis of nucleotide structure.(6)

2. Nucleotides modified in phosphate groups as molecular tools and potential therapeutics

When designing nucleotide analogs as therapeutics as well as research tools such as substrates for biochemical studies, ligands for structural and biophysical studies, or biologically active molecules for studies on cell culture models and living organisms, it is extremely important to choose the site and method of modification. Standard medicinal chemistry approaches focus on modifying nucleotides in the ribose moiety and nucleobases. Nonetheless, methods for the synthesis of nucleotides modified and functionalized in the phosphate moieties have also been intensively developed for many years. Among the first synthesized nucleotide analogs were methylene bis(phosphonate) and thiophosphate nucleotide derivatives. (7) For several decades since the publication of these pioneering works, many other methods of phosphate group modification have been developed and numerous applications have been proposed for them in order to develop biologically important nucleotides and nucleic acids. These modifications include the replacement of single atoms with other atoms or small substituents, as well as the introduction of large substituents and functional groups (Fig. 2). Small modifications of phosphate groups may affect the properties of the molecule, such as its geometry, acid-base properties, net charge under physiological conditions, polarizability and charge distribution in (oligo)phosphate residue, or the ability to coordinate metal and organic cations. This, in turn, may involve changes in related biological properties such as affinity, and thus selectivity towards the selected protein target, susceptibility to enzymatic degradation, or metabolic stability. Modification of phosphate residues with larger substituents may additionally influence their permeability through biological membranes and enable their subsequent functionalization, e.g. through fluorescent labeling or bioconjugation.

The studies presented in this author's presentation have contributed to the development of the chemistry of phosphate-modified nucleotides by (i) developing new methods for the synthesis of new analogs containing already known modifications, (ii) designing and synthesizing new types of phosphate modifications (selected examples are presented in Fig. 2C), as well as (iii) searching for new applications of those compounds in biophysical and biochemical studies and in solving biological problems.

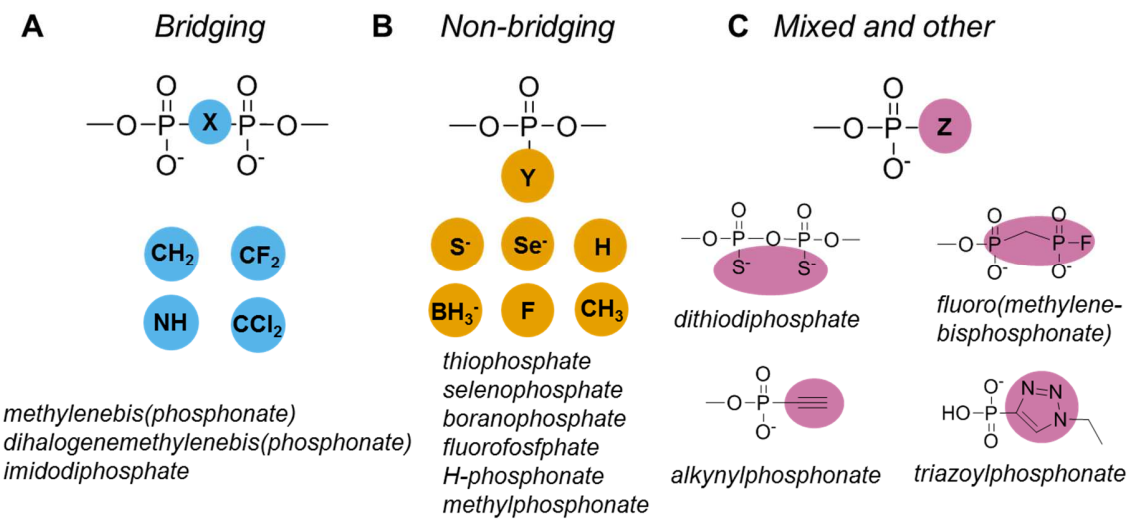


Fig. 2. Examples of phosphate group modifications in nucleotides.

3. Synthesis of new nucleotide analogs modified or functionalized in the phosphate chain

The synthesis of nucleotides is a challenging task due to their highly polar structure and the presence of numerous ionizable groups (acidic phosphate groups or phosphate salts) which make it difficult to apply reaction conditions and purification methods typical for organic chemistry. The key and often the most difficult step during the synthesis of nucleotides and their analogs is the formation of pyrophosphate bonds. Most often this step is carried out by reacting an appropriately activated, electrophilic nucleotide derivative with phosphate nucleophile. In the publication cycle H1-H5, together with our co-workers, we explored the possibilities of using phosphoroimidazolide chemistry to synthesize selected nucleotide synthetic targets (Fig. 3). The targets consisted of both natural nucleotides with modified or functionalized phosphate chain, such as nucleoside phosphosulfates (H1) and nucleotide sugars (H2), as well as synthetic nucleotide analogs modified in the phosphate chain (H3). These objectives were chosen because of their biological significance and the serious limitations of the then existing synthetic methods giving access to this type of compounds. In the publications H1, H2, H4 and H5, we demonstrated that, apart from typical nucleophiles such as nucleoside 5'-mono- and diphosphates, unusual nucleophiles such as phosphosugars, sulfate, fluoride, fluorophosphate anions or C-phosphonate anions functionalized with alkyne group can also be used in reactions activated with imidazole nucleotides (Fig. 3A). In each case, the key to efficient synthesis was the optimization of the reaction conditions, in particular the choice of solvent, the concentration of reagents, a suitable alkylammonium counter-ion for a negatively charged nucleophile, and the concentration and type of divalent metal chloride, which helps to solubilize other reagents and serves as the reaction mediator. In most cases, we used commercially available nucleophiles, which only required to be converted into an appropriate alkylammonium salt before the reaction. The exception was the paper H5, in which we designed and developed methods of synthesis for appropriate C-phosphonate subunits containing an alkyne function. In this study, we also optimized the conditions of azide-alkyne cycloaddition reactions between phosphonate nucleotide analogs and various compounds with functionalized azide moiety such as nucleosides, biotin or fluorescent labels. The method for the synthesis of "clickable"

phosphonate subunits and nucleotides containing them described in the paper H5 became the starting point for several subsequent projects, the results of which we published in the papers H8, H9 and (8).

However, the results of the studies mentioned above have made us realize that there are synthetic targets which cannot be obtained using the approach described above. In particular, the approach fails if obtaining an activated electrophilic nucleotide derivative in good yield is a synthetic challenge or if such derivative shows undesired reactivity. We encountered such difficulties mostly in the case of nucleotides containing more than two phosphate groups or modifications such as thiophosphate or boranophosphate. In order to overcome these challenges, in paper H3 we developed new electrophilic reagents, which enabled elongation of the oligophosphate chain in non-activated nucleotides (Fig. 3B). These reagents are electrophilic synthons protected with 2-cyanoethyl group and activated with imidazole, thereby allowing for phosphate chain elongation in various modified nucleotides without the need of their beforehand activation.

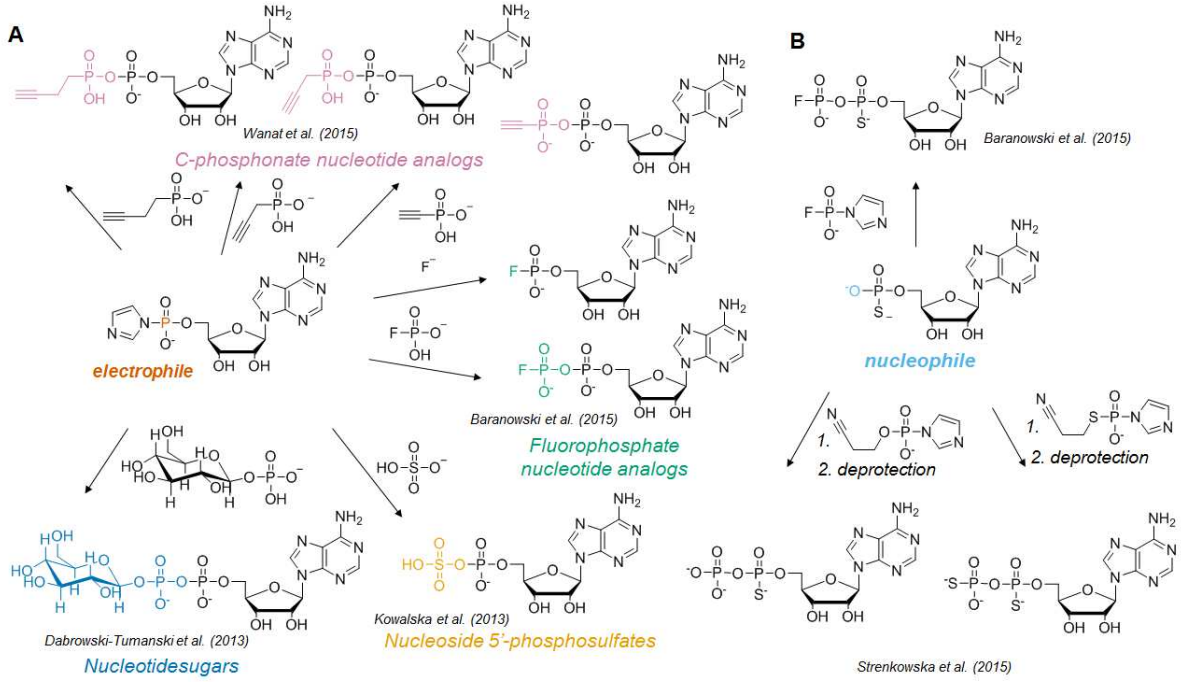


Fig. 3. Applications of imidazole derivatives in the synthesis of nucleotide analogs (based on papers H1-H5). A) Syntheses based on reactions of imidazole derivatives of nucleotides (represented here by adenosine 5′-phosphorimidazolide) with different nucleophiles; B) Syntheses using electrophilic phosphorylating reagents and non-activated nucleotides modified in the phosphate chain (represented here by adenosine 5'-thiophosphate). For simplicity, the reaction conditions are not included in the scheme.

The effectiveness of this approach, which can be referred to as "reverse activation," was demonstrated by obtaining a series of 23 nucleotides, the synthesis of which by known methods would be extremely difficult or even unattainable. This pool included nucleoside di-, tri- and tetraphosphates containing various modifications in the oligophosphate chain, including methylenebisphosphonate, imidodiphosphate, thiophosphate, boranophosphate, and their unique combinations previously unreported in the literature. One of the most demanding synthetic targets

were nucleotides having two adjacent thiophosphate moieties-the methodology of their synthesis developed in the paper H3 allowed to obtain a set of 5′ mRNA terminus dithiodiphosphate analogs (analogs 2S), which were characterized by high resistance to enzymatic degradation and gave mRNA exceptionally beneficial translational properties (9). The "reverse activation" was also used to obtain nucleotides and oligonucleotides containing the fluorophosphate residue, the obtaining of which was not efficiently using the standard method. This enabled efficient synthesis of nucleotides containing a combination of fluorophosphate modification with other modifications of the phosphate chain as well as 5′-fluorodiphosphorylated oligonucleotides.

4. Search for new applications for nucleotide analogs modified in phosphate groups

The development of efficient methods for the synthesis of different phosphate-modified nucleotides encouraged us to search for new research applications for these compounds. The publications constituting the basis for this author's presentation are related to several of such potential applications, listed below:

a) use of modified nucleotide analogs as ligands in biophysical studies on protein-nucleotide complexes

These studies concerned mainly nucleoside phosphosulfates and nucleoside fluorophosphates as ligands for eIF4E protein. The results of these studies were published in H1 and H4, but will not be discussed in detail in this author's presentation.

b) use of nucleotide and oligonucleotide analogs labeled with a fluorine atom in ^{19}F NMR studies

These studies concerned the use of nucleoside fluorophosphates as tools for the study of biologically important processes such as enzymatic degradation or oligonucleotide hybridization. The results were published mainly in the paper H5 and are briefly discussed in chapter 4.1.

c) use of modified or fluorescently labeled nucleotide analogs as alternative enzymatic substrates allowing monitoring the activity of pyrophosphatases.

These studies concerned the use of nucleoside fluorophosphates in combination with fluorogenic probes and fluorescently labeled nucleotides in studies on the activity of biologically important pyrophosphatases with particular emphasis on the DcpS enzyme. These results were published in H6, H7 and H8 and are briefly discussed in chapter 4.2.

Moreover, drawing inspiration from the latest reports on the biological functions of nucleotides and enzymes metabolizing them, we have undertaken the synthesis of two types of carefully designed nucleotide analogs as tools to study recently discovered biological phenomena.

These studies included the synthesis of degradation-resistant NAD analogs enabling the synthesis of stable NAD-RNA molecules and the synthesis of triazole m^7GMP analogs as potential inhibitors of the recently discovered nucleotidase cNIIIB. These results were published in H10 and H9, respectively, and are briefly discussed in chapters 4.3 and 4.4.

One of the key conditions for effective synthesis of nucleotide analogs is the ability to quickly and unambiguously identify reaction products. Although the standard method used in this context is ^{31}P

NMR, tandem mass spectrometry (MS) has also proved to be valuable in our studies. MS is much faster, less material-consuming and less demanding in the context of sample preparation. Thanks to a large number of nucleotide analogs obtained over the years, we have been able to examine the fragmentation pathways of nucleotides and their analogs in a systematic way and to draw general conclusions on this subject, which could also be useful for other scientists conducting similar studies.

These results were published in H11 and are briefly discussed in chapter 4.5.

4.1. Use of fluorophosphate nucleotide analogs for enzymatic activity studies.

The introduction of the fluorine atom into the nucleotide molecule enables its detection and analysis by ^{19}F NMR. Spectroscopic properties of ^{19}F nuclei such as spin equal to ½, high gyromagnetic ratio and 100% abundance make the sensitivity of ^{19}F spectroscopy almost comparable with ^{1}H NMR. However, unlike the proton, the physiological content of fluorine (understood here as the content in natural organic compounds) is very low. A molecule labeled with a fluorine atom can be selectively monitored by ^{19}F NMR even in complex chemical or biological mixtures. As a result, fluorine can be used as a molecular marker dedicated to studying both low molecular weight natural compounds and biomacromolecules by using ^{19}F NMR (10). Fluorine labeled molecular probes are used to track enzymatic processes, to observe conformational changes in biomolecules, and to look for ligands that bind therapeutic targets (10). In H4 we decided to verify whether fluorophosphate nucleotide analogs can be used in ^{19}F NMR studies. For this purpose, the analogs synthesized in this study were subjected to detailed characterization by NMR methods. We studied the relationships of chemical shifts of ^{19}F nuclei (δ_F) with nucleotide structure and external factors such as solution pH, the presence of magnesium ions and various buffering factors. It turned out that the fluorine atom in the fluorophosphate residue is very sensitive to changes in the structure of the compound, but relatively insensitive to other factors, which was a good premise in the context of potential applications. In the next stage of the study, we selected several model compounds and treated them with enzymes from the phosphohydrolase family. Time-dependent monitoring of reaction samples with ^{19}F NMR confirmed the possibility of using fluorophosphate analogs as versatile reporter molecules (Fig. 4).

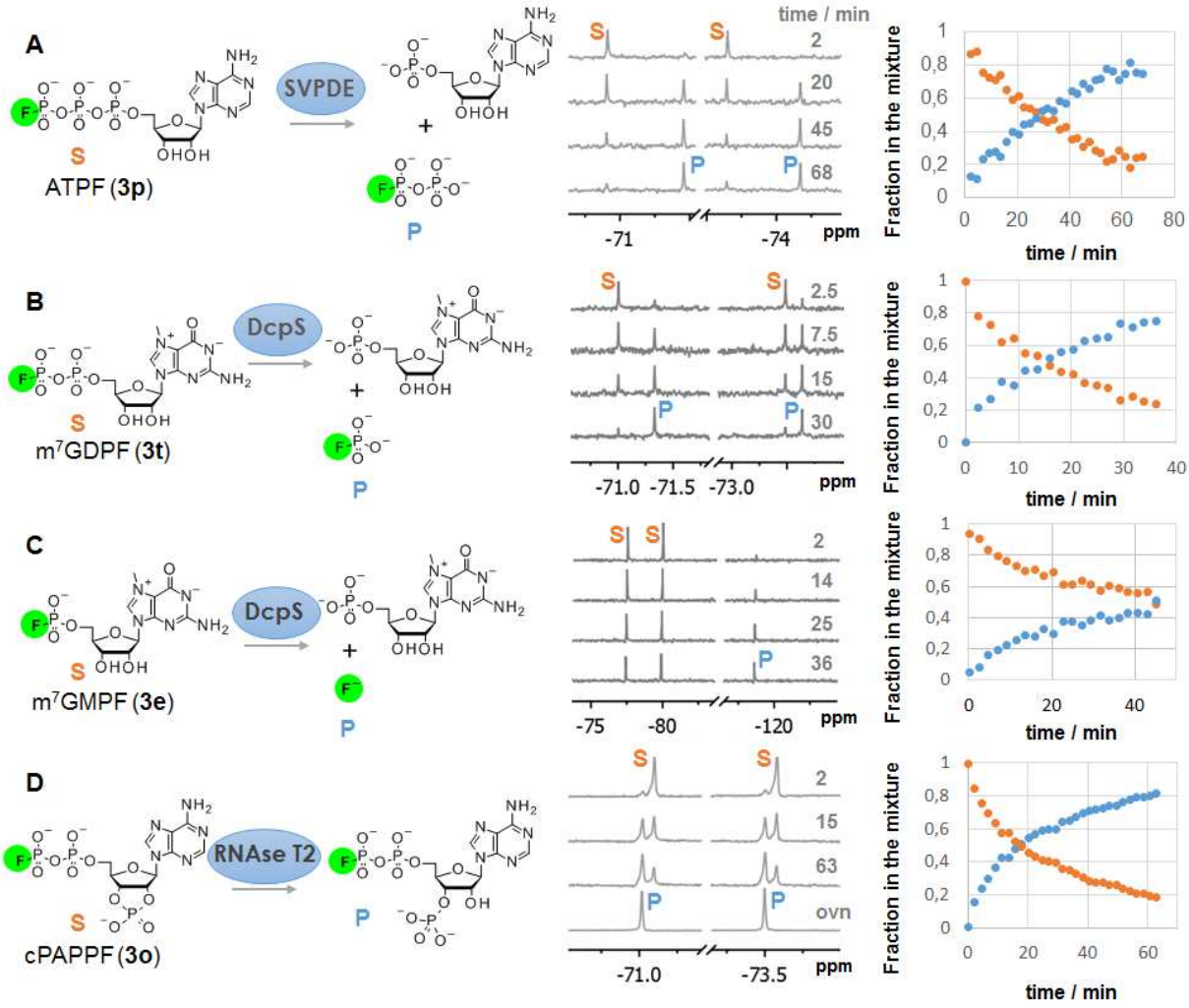


Fig. 4. The use of fluorophosphate nucleotide analogs to monitor the course of enzymatic reactions catalyzed by different enzymes. (A) Hydrolysis of ATPF by SVPDE phosphodiesterase; (B) Hydrolysis of m^{7}GTPF by DcpS enzyme; (D) Hydrolysis of cPAPPF by RNAse T2 (studies published in H4).

Another interesting example of the use of compounds obtained in H4 as molecular probes was the use of fluorodiphosphate oligonucleotides to monitor the formation of double-stranded DNA (Fig. 5). Finally, we also showed that the interaction of fluorophosphate nucleotide analogs with binding proteins can be observed by ^{19}F NMR. At present, we continue our research to extend the range of processes that can be observed through the use of fluorophosphate nucleotide and oligonucleotide analogs.

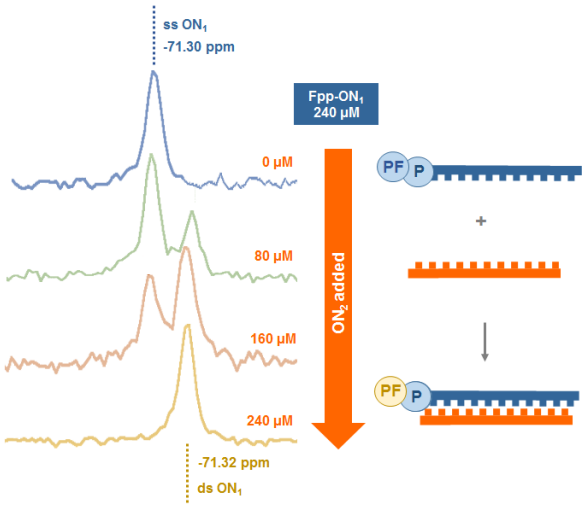


Fig. 5. Application of 5'-fluorodiphosphated oligonucleotide analog ($FppON_1$) for monitoring of duplex formation with a complementary unlabeled oligonucleotide (ON_2; studies published in H4).

4.2. Search for molecular probes for determination and monitoring of DcpS enzyme activity

DcpS (Decapping Scavenger) is an enzyme from the histidine triad superfamily (HIT) involved in mRNA degradation in eukaryotic organisms, including humans. In eukaryotic cells, there are two main mRNA degradation pathways, degradation in 5′→3′ direction and degradation in 3′→5′ direction. Both pathways are initiated by hydrolysis of polyA tail (deadenylation). In the 5′→3′ pathway the next step is the removal of the cap structure from the 5′ mRNA terminus. The cap is composed from 7-methylguanosine connected to the first transcribed nucleotide by a 5′,5′-triphosphate bridge (Fig. 1). mRNA decapping is performed through cleavage of the α,β-pyrophosphate bond by Dcp1-Dcp2 enzyme to release 7-methylguanosine 5′-diphosphate (m^7GDP) and mRNA 5′-monophosphate. Further degradation of mRNA is carried out by 5′-exonuclease.

In the course of degradation in 3′→ 5′ direction, mRNA is degraded by the exosome starting from the 3′ terminus. As a result of such degradation released are nucleoside 5′-monophosphates and 5′-terminus residuals in the form of dinucleotide caps or short capped oligonucleotides. These 5′ terminus residuals are then degraded by DcpS. DcpS hydrolyzes the cap between γ and β phosphates to release 7-methylguanosine 5′-monophosphate (m^7GMP) and a second product which is nucleoside or short oligonucleotide 5′-diphosphate, respectively (Fig. 6). Notably, full-length mRNAs terminated with cap are not substrates for DcpS. Moreover, m^7GDP, i.e. the product of mRNA degradation in the 5′→3′ direction, is not DcpS substrate either (11), but can be phosphorylated in the cell to m^7GTP to get cleaved by DcpS. DcpS activity is considered essential for cell homeostasis as excessive accumulation of cap residues released as a result of mRNA decay could adversely affect other key cap-dependent cellular processes, e.g. inhibit mRNA nucleocytoplasmic transport or translation. DcpS is localized in both cytoplasm and nucleus, where it is likely to be involved in the regulation of splicing. (12) It has been also suggested that the role of

DcpS in a cell may be more general and goes beyond its well-characterized function in 3′→5′ mRNA degradation, including participation in mRNA maturation. (13)

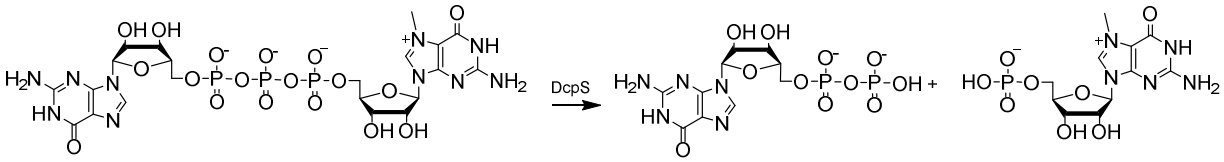


Fig. 6. Reaction catalyzed by DcpS enzyme exemplified on a dinucleotide substrate, m^7GpppG.

The biological importance of the DcpS is also reflected in its association with disease development. Mutations of the DcpS gene are very rare but have been associated with developmental disorders such as intellectual impairment called Al-Raqad syndrome.(14-16) This suggests an important role of DcpS in the nervous system development. In 2008, it was reported that inhibition of DcpS activity with low molecular weight inhibitors may have therapeutic effects in spinal muscular atrophy (SMA). SMA is a common neurodegenerative disease occurring on average once per 6000 births. (12) It is caused by the low level of SMN (Survival Motor Neuron) protein, which is coded by SMN genes. In humans, there are two SMN genes: *SMN1* and *SMN2*. The main difference between them is the change of sequence in exon 7, which affects pre-mRNA splicing. As a result, the expression of the *SMN1* gene leads to a stable and functional protein, while the protein expressed with *SMN2* is shortened. Mutations in both copies of the *SMN1* gene, including deletions, conversions to a *SMN2*-like gene and point mutations result in SMA disease. Individuals who have only one defective copy of *SMN1* are carriers of SMA but do not show disease symptoms.

The homologous *SMN2* gene cannot provide sufficient amounts of functional SMN protein, but it has been observed that more copies of the *SMN2* gene accompany a more benign course of the disease. Therefore, it is believed that compounds that increase the amount of protein encoded by the *SMN2* gene in a cell may be therapeutics against SMA. Using a high throughput screening assay, Signh et al. found out that some 5-substituted quinazolines increase the expression of the *SMN2* gene up to twofold. Trying to unravel the molecular mechanism underlying this activation DcpS has been identified as a C5-substituted quinazoline binding protein using radio-labeling.(17) These experiments initiated the still ongoing research into DcpS as a therapeutic target for the treatment of SMA. These studies have shown, inter alia, that the inhibitor potential is positively correlated with the level of *SMN2* gene promoter activation. The therapeutic potential of these compounds was then demonstrated *in vivo*, in a mouse model, and the best compounds have been clinically tested, but they have not been successful to date, probably due to the non-specific toxicity of compounds. This fact justifies the further search for compounds with the therapeutic potential to inhibit DcpS activity. Moreover, recent studies indicate that DcpS may also be a therapeutic target for the treatment of acute myeloid leukemia (AML). (18, 19)

Due to this important biological context, we decided to develop new methods for quantifying the activity of DcpS enzyme and screening for its inhibitors. This aim was an interesting research challenge, but the effects of its implementation could be of therapeutic significance. The first step

toward the development of the DcpS inhibitor screening assay was the discovery, described in H4, that the human enzyme DcpS accepts as substrates fluorophosphate analogs of 7-methylguanosine mono-, di-and triphosphates (m^7GMPF, m^7GDPF, m^7GTPF, respectively, Fig. 4). During the reaction with DcpS, these substrates are transformed into m^7GMP and the corresponding fluoro(oligo)phosphate or fluoride anion. We found that because the respective substrates and products of these reactions have different ^{19}F chemical shifts, the progress of each reaction can be monitored by ^{19}F NMR. In the case of m^7GMPF, hydrolysis by DcpS led to the release of fluoride ions, which made it possible to quantify their concentration using fluoride-selective fluorogenic probes.

Preliminary results confirming the usefulness of fluorophosphate analogs of substrates for determination of activity and search for DcpS enzyme inhibitors became the contents of a patent application (J. Kowalska, J. Jemielity, M. R. Baranowski, A. Nowicka, R. Kapsrzyk, "Fluorophosphate analogs of the 5′ mRNA terminus (cap), the way of obtaining and applications"), and further research, which resulted in the publication of the paper H6, was conducted within the LIDER project. The aim of the project was to develop and optimize new methods for high throughput screening (HTS) assays aimed at searching for nucleotide pyrophosphatase inhibitors of therapeutic importance based on fluorophosphate nucleotide analogs. The main focus of the project was on DcpS as a therapeutic target in SMA. In the course of the project, we tested the ability to monitor enzyme activity using both ^{19}F NMR and fluoride-detection-based fluorescent methods. The ^{19}F NMR method relied on a comparison of the substrate to product conversion degree in the presence of an inhibitor and in a control sample based on the intensity of the relevant signals in the ^{19}F NMR spectrum. In the case of the fluorescent method, the inhibitor potency was determined by comparing the amount of fluoride released in the reaction mixture containing the inhibitor with the reference mixture without the inhibitor (Fig. 7). The reaction substrate, m^7GMPF, is hydrolyzed to m^7GMP and fluoride anion by DcpS. The fluoride ion content is then determined by reacting with a fluorogenic probe protected with silyl groups (Fig. 7). The high affinity of fluoride ion to silicon is the driving force for the probe deprotection reaction and raise of a fluorescent signal. The development of this method required detailed optimization of many factors such as substrate, enzyme, and inhibitor concentrations, choice of fluorogenic probe, buffer composition, composition of the probe solution, composition of the fluorescence read-out buffer, temperature and reaction time, or the way of enzyme deactivation. Optimization provided good results, as evidenced, inter alia, by the Z′ factor higher than 0.6.(20)

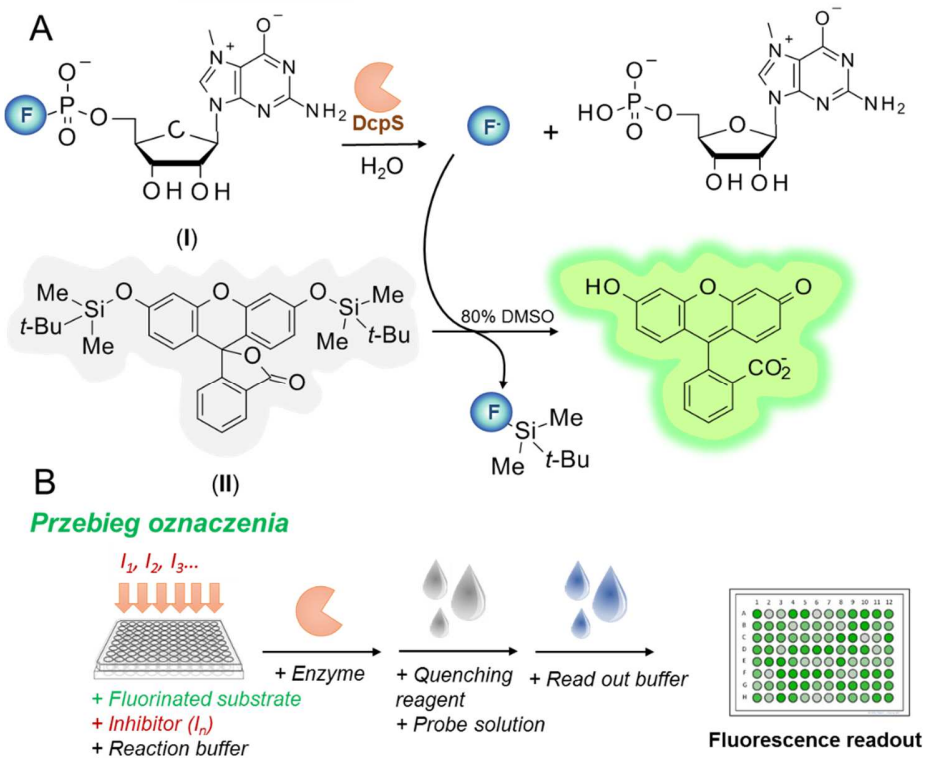


Fig. 7. General idea of the DcpS inhibitor screening assay described in the paper H6.

The developed HTS method enabled us to continue extensive studies aimed at identifying new DcpS inhibitors. The results of these studies have already been partially published in several papers (8, 21),(H6 and H9) and have contributed to the discovery of one of the strongest DcpS inhibitors so far.(21)

In the course of those studies it became apparent that fluorescence detection-based method is characterized by higher throughput and lower material consumption than the ^{19}F NMR-based method. The major advantage of fluorescence detection is higher sensitivity, which entails lower consumption of the substrate, enzyme, and the tested inhibitor. Another advantage is the adaptability to 96-well format, which enables automatized measurements by using a fluorescence reader. The fluorescence method has also some disadvantages, such as the possibility of obtaining false negative results if the compound tested as an inhibitor has fluorescent properties or false positive if it is a fluorescence quencher. However, in such cases, ^{19}F NMR method can be used for additional verification of inhibitor activity. Another limitation of the HTS method based on the detection of fluoride ions is the need to determine the concentration of fluoride ions in a solution containing at least 80% DMSO, which makes it impossible to monitor the enzymatic reaction in a continuous manner.

Therefore, we continued searching for alternative methods to test DcpS activity. We were particularly interested in the possibility of creating molecular probes that would allow us to monitor DcpS enzyme activity using spectrofluorimetric methods on a continuous basis. The first probes

with such properties designed by us were 7-methylguanine nucleotides labeled with acetylpyrene, developed in the paper H7. These probes were obtained by efficient functionalization of the terminal thiophosphate moiety in nucleotide analogs with bromoacetylpyrene. These compounds showed very interesting fluorescent properties. Attaching 7-methylguanine nucleotide to the acetylpyrene dye increased the fluorescence emission up to threefold. This phenomenon was used to develop an alternative method of DcpS enzyme inhibitors screeening and to monitor DcpS activity in real time. We also tried to explain the observed fluorescent properties of the probe, finding, inter alia, that they result from the stacking interactions between acetylpyrene moiety and 7-methylguanine in ground state.

We continued looking for new ways to increase the sensitivity and application possibilities of fluorescent probes for DcpS. We considered both the possibility of increasing the sensitivity of single-labeled probes by replacing the acetylpyrene residue with a more sensitive label (Kasprzyk et al. 2019, accepted paper) as well as modifying the probe structure to enable dual detection (ratiometric). Double-labeled nucleotide probes have been described in the literature, particularly probes labelled with a pair of fluorophores exhibiting Förster resonance energy transfer (FRET) effect have been used to monitor many enzymatic processes dependent on adenine nucleotides.(22-24) However, the synthesis of this type of nucleotides, labeled with two different fluorophores, is usually a multistage and inefficient process, which makes the optimization of this type of structures challenging. In paper H8 we proposed nucleotide fluorescence probes allowing dual fluorescence detection that are synthetically available due to the applied labeling strategy, which relies on two identical fluorophores forming homodimers with unique emission properties such as pyrene excimers. A general mechanism of action for such probes is shown in Fig. 8. In a probe double-labeled with pyrene, efficient formation of excited dimers (excimers) is observed under UV radiation, which are characterized by emission with a maximum at ~500 nm. As a result of enzymatic hydrolysis of the probe, the molecule fragments labeled with pyrene are separated, the efficiency of excimer formation decreases, so the monomeric emission of pyrene appears with a maximum of about 400 nm (Fig. 8). A such, the progress of the enzymatic reaction can be monitored either by observing a decrease in emission intensity at 500 nm, an increase in emission intensity at 400 nm or ratiometrically, i.e., by monitoring changes in the I_{500}/I_{400} intensity ratio. In order to obtain such probes, we first developed a method of synthesis of nucleotides double-functionalized with alkyne group, wherein one of the groups was introduced by a modification of terminal phosphate and the other by appropriate modification of ribose moiety at the 2'- or 3'-position. In order to verify the overall usefulness of this type of excimer probes, we obtained compounds containing different variants of nucleobases, different types of "clicked" phosphate residues and different variants of fluorescent labels with similar emission properties (Fig. 8). The obtained compounds were subjected to detailed characterization in order to assess their chemical stability, emission properties and susceptibility to enzymatic degradation. Based on these experiments, we selected the best compounds to monitor the activity and inhibition of various enzymes, including DcpS, in real time, which was also demonstrated by appropriate experiments. Importantly, we have also shown that using excimer probes, we can monitor not only the activity of

the recombinant DcpS, but also its endogenous activity in cell lysates. Due to the amphiphilic structure of the probes, we also decided to test whether they are able to penetrate trough the cell membrane into the cells. We found that some of the probes have such a property, and importantly, it is possible to detect them in a dual way inside the cells (Fig. 9). This has inspired us to continue this project and to undertake research into the possibility of monitoring DcpS activity in living cells. This topic is currently being intensively studied in our team.

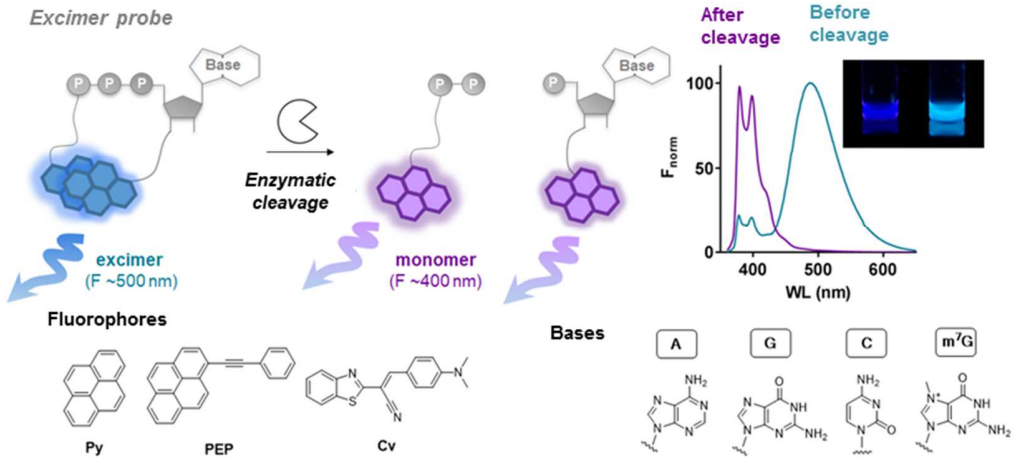


Fig. 8. The overview of structures and properties of excimer probes designed to study the activity of pyrophosphatases described in paper H8.

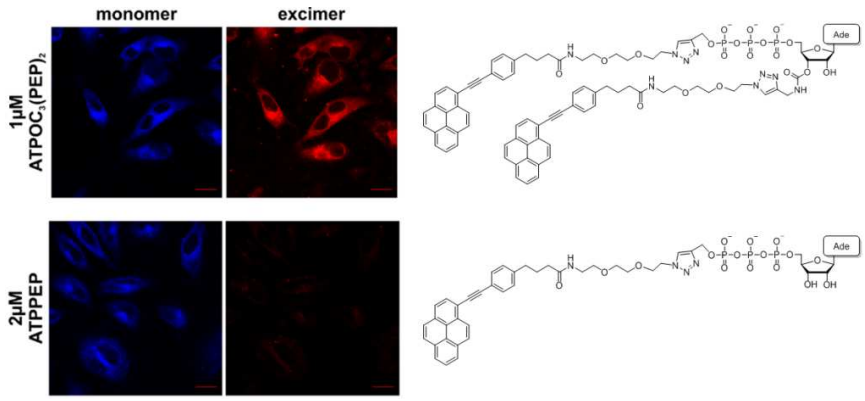


Fig. 9. Permeability of an excimer probe into HeLa cells. The presence of the excimer probe in the cells can be detected by observing both fluorescence corresponding to monomers (blue) and excimer (red) of phenylethynylpyrene (PEP). For comparison, shown is also the result of a similar experiment performed for a single-labeled probe, which also penetrates into the cells but, as expected, does not show excimer fluorescence (data published in paper H8).

4.3. Search for cNIIIB protein inhibitors

As a result of cap degradation by DcpS, 7-methylguanosine 5′-monophosphate (m^7GMP) is released as a unique nucleotide metabolite of the 5′ eukaryotic mRNAs terminus on the 3′-5′ degradation pathway. On the 5′-3′ pathway, m^7GDP is released as a result of mRNA cap degradation by the Dcp1-Dcp2 complex. It has been hypothesized that the accumulation of m^7GMP and m^7GDP in healthy cells would be toxic for them, as the compounds could inhibit important

cap-dependent processes such as eIF4E-mediated translation initiation or mRNA transport. As a logical consequence, it seems obvious that cells have evolved mechanisms for the rapid degradation of 7-methylguanine nucleotides. Nonetheless, until recently the exact mechanisms by which the degradation of m^7GMP and m^7GDP occurs have remained unexplained. Only in 2012 in human and *Drosophila Melanogaster* cells, cNIIIB enzyme encoded by NT5C3B gene, which catalyzes the reaction of m^7GMP degradation to 7-methylguanosine and inorganic phosphate was identified (Fig. 10A) (25). This enzyme belongs to the family of cytosolic 5′-nucleotidases (cN), responsible for the dephosphorylation of nucleoside 5′-monophosphates and it is the eighth 5′-nucleotidase identified in human cells. Apart from its activity towards m^7GMP, the enzyme also exhibits high activity towards CMP and, which is unusual, pyrophosphatase activity in relation to m^7GDP (Fig. 10B), while its activity in relation to nucleotides such as GMP and AMP is significantly lower. The kinetic parameters indicate that m^7GMP is the substrate best tailored to the protein binding pocket (has the lowest K_m value among all substrates). In 2014, the crystallographic structure of the cNIIIB enzyme from *Drosophila Melanogaster* has been solved, indicating that the preference for m^7GMP may result from the existence of elastic hydrophobic pocket formed mainly by the three tryptophan residues in protein structure (26). High affinity of cNIIIB to m^7GMP suggested that this enzyme may participate in the degradation of end of 5′ mRNA cap metabolites in the cell. Moreover, similar to other enzymes from the 5′-nucleotide family, it may participate in the degradation of therapeutics of nucleotide origin used, inter alia, in anticancer therapies (27, 28). However, the actual biological function (or functions) of cNIIIB has not been established so far.

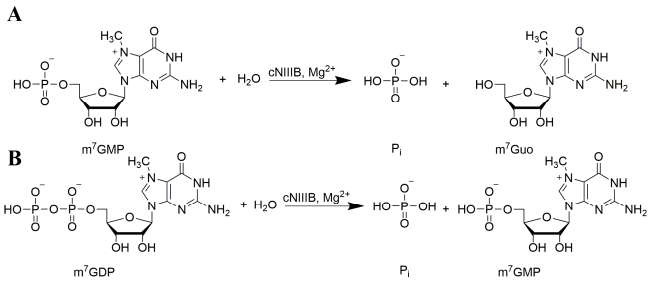


Fig. 10. Enzymatic activity of cNIIIB towards 7-methylguanine metabolites of mRNA 5' end.

In H9 work, taking the m^7GMP structure as the starting point, we designed and synthesized a group of potential inhibitors and molecular tools for the study of cNIIIB. The designed m^7GMP analogs carried a modification in 5′-position of 7-methylguanosine in the form of a 1,2,3-triazole residue functioning as a link between nucleoside and phosphate or phosphate residue (Fig. 11A). Since the cNIIIB enzyme accepts both m^7GMP and m^7GDP as substrates *in vitro*, which suggests quite high flexibility of the catalytic pocket, we assumed that the introduced modification, although sterically demanding, may enable maintaining the interaction with the enzyme while preventing the catalytic step. The compounds were obtained by an efficient copper-catalyzed azide-alkyne cycloaddition (CuAAC) between 5′-azido-5′-deoxy-7-methylguanosine and four different phosphate or

phosphate derivatives containing a terminal alkyne group, the synthesis of which has been developed in paper H5 (Fig. 11B). The ability to inhibit cNIIIB enzyme activity was initially evaluated by two independent assay: an HPLC-based assay and a colorimetric test using malachite green. These studies revealed that one of the compounds studied, namely the one containing 1,2,3-triazoylphosphonate moiety directly connected to the phosphorus atom (compound 2a in Fig. 11B), inhibited the activity of the cNIIIB enzyme at micromolar concentrations (IC_{50} ~88 μM), while other analogs did not show inhibitory activity in the studied concentration range. Interestingly, the compound containing phosphate moiety (2d) turned out to be a good unnatural substrate for cNIIIB, which indirectly confirmed our initial assumption that the triazole modification is accepted in the binding site. The inhibitor found was subjected to further studies, which led to the demonstration of its selectivity for cNIIIB in the context of other 7-methylguanine recognizing proteins, eIF4E and DcpS. The inhibitor we discovered is currently used to verify the role of cNIIIB enzyme in the degradation ofmRNA 5′ end metabolites. We are also conducting research aimed at the synthesis of second-generation compounds with increased inhibitory potential.

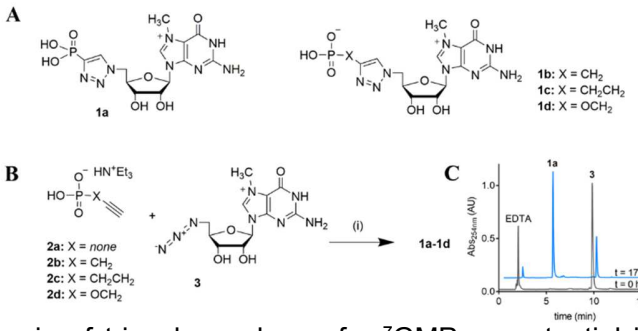


Fig. 11. Synthesis of triazole analogs of m^{7}GMP as potential inhibitors of the cNIIIB enzyme reported in paper H9.

4.4. Hydrolysis-resistant NAD-RNA analogs

Nicotinamide adenine dinucleotide (NAD) is an extremely important natural compound with numerous biological functions. The earliest known and therefore best-studied function of NAD is participation in cellular redox reactions. However, NAD is also a substrate for enzymes introducing or removing post-translational modifications of proteins (poly(ADP-ribosylation) and deacetylation, respectively), participates in signal transduction (as a precursor of signal molecule, cyclic ADP-ribose) (29), or DNA repair. Recently, it has also been discovered that NAD serves as a molecule that modifies the 5′ terminus of some RNAs. The NAD RNA molecules (Fig. 12) were first discovered in bacteria (30), and called "bacterial cap"' due to a certain analogy to 7-methylguanosine cap (m^{7}G) in Eukaryotes. (2, 31, 32) However, most recent studies revealed that NAD-terminated RNAs also occur in eukaryotic organisms (33-35) and in human cells (36). The biological functions of this modification have not yet been elucidated, but studies carried out so far indicate that they may differ depending on the organism. Nevertheless, it has been found that in both bacteria and Eukaryotes the presence of NAD at 5′ terminus affects RNA stability. Accordingly, several enzymes that are capable of removing NAD structures from the 5′ RNA terminus have also been identified (the so-called deNADing enzymes) (Fig. 12)(3).

In *E. coli*, the presence of NAD at the 5′ mRNA terminus is likely to increase RNA stability as a result of reduced susceptibility to degradation by the enzyme RppH (37), i.e., bacterial pyrophosphatase involved in the RNA dephosphorylation of 5′-triphosphates to 5′-monophosphates, thus initiating their further degradation. However, it is also known that NAD moiety in RNA can be hydrolyzed by NudC enzyme, a bacterial pyrophosphatase that specifically cleaves the pyrophosphate bond in NAD *in vitro* and *in vivo* by removing the nicotinic mononucleotide (MNM) (37, 38).

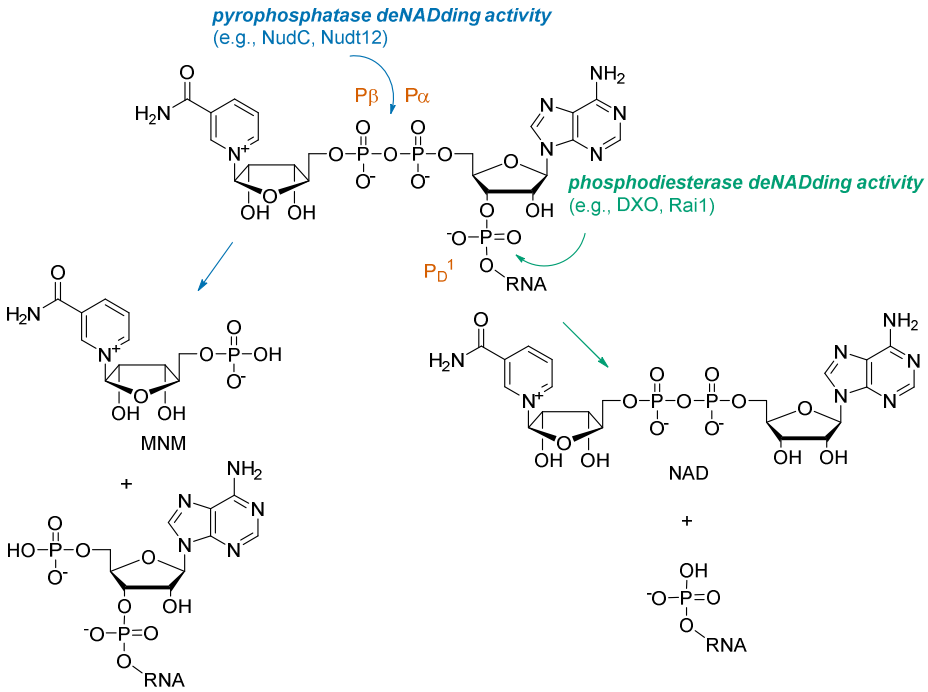


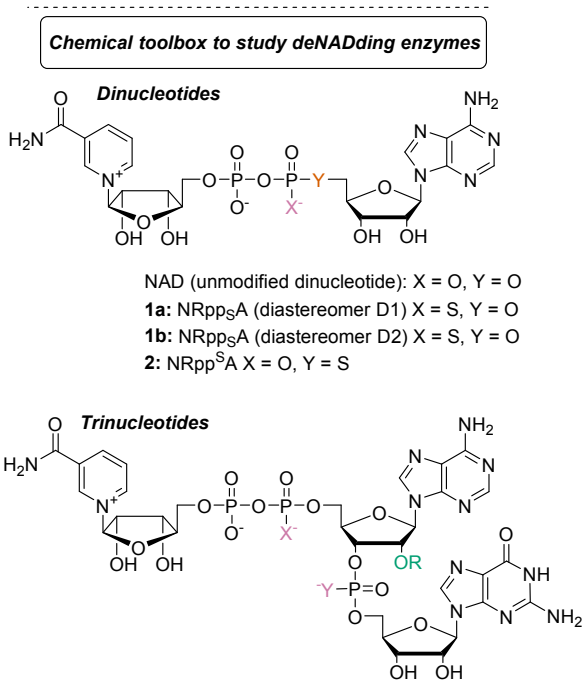
Fig. 12. The structure of NAD-RNA and its hydrolysis products by two classes of deNADing enzymes identified so far: pyrophosphatase NAD-RNAs and phosphodiesterase NAD-RNA. α, β and phosphodiester (P_D) phosphates are shown in the figure.

Unlike prokaryotic organisms, the presence of NAD at the RNA 5′ terminus in Eukaryota destabilizes RNA and directs it to rapid degradation. Degradation is initiated by the removal of the entire NAD molecule from the 5′ terminus by the DXO nuclease, which hydrolyzes the first phosphodiester bond in the RNA (33). Recently it has been discovered that the human enzyme Nudt12, which is a homolog of the NudC enzyme, has also an *in vitro* deNADing activity as well as participates in the degradation of NAD-RNA in human cells (39). Unlike DXO, Nudt12 has NAD-RNA pyrophosphatase activity.

Despite the increasing number of molecular tools and biological chemistry methods designed to investigate NAD-dependent processes (40-43), the biological significance of NAD-RNA is still largely unexplained. One of the most important questions remains whether this modification has any definite biological function in eukaryotic cells, e.g. as a response to stress conditions, or is merely the result of erroneous transcription initiation and, as an abnormal product, has to be quickly removed by the RNA quality control mechanisms.

Fig. 13. Structures of NAD analogs obtained as resistant to enzymatic degradation of molecular tools for NAD-RNA studies in paper H10.

We envisaged that research on NAD-RNAs and their specific proteins could be facilitated by the use of rationally designed molecular tools that would enable the synthesis of NAD-RNA analogs resistant to degradation by deNADing enzymes. We considered that such tools would allow access



NAD (unmodified dinucleotide): X = O, Y = O
1a: NRpp$_S$A (diastereomer D1) X = S, Y = O
1b: NRpp$_S$A (diastereomer D2) X = S, Y = O
2: NRpp^{S}A X = O, Y = S

3: NRppApG (unmodified trinucleotide); X = O, Y = O, R = H
4a: NRppAp$_S$G; X = O, Y = S, R = H (diastereomer D1)
4b: NRppAp$_S$G; X = O, Y = S, R = H (diastereomer D2)
5: NRppA$_m$pG; X = O, Y = O, R = CH_3
6a: NRpp$_S$A$_m$pG; X = S, Y = O, R = CH_3 (diastereomer D1)
6b: NRpp$_S$A$_m$pG; X = S, Y = O, R = CH_3 (diastereomer D2)

to NAD-RNAs with increased stability, which would facilitate structural studies of NAD-RNA complexes with specific enzymes, enable assessment of the contribution of individual deNADing enzymes to overall NAD-RNA degradation, facilitate experiments aimed at the identification of new NAD-RNA binding proteins in cells, and open up many other research opportunities.

Therefore, in paper H10, we received a set of NAD analogs designed to provide partial or complete resistance to enzymatic deNADding, thanks to chemical modifications introduced to them when integrated into RNA. In order to give the analogs potential resistance to enzymes with pyrophosphatase activity (NudC and Nudt12), we developed NAD analogs containing 5′-thiophosphate or 5′-phosphothioster modification within α phosphate (Fig. 13). Such modifications were successfully used earlier to stabilize eukaryotic of 5′ mRNAs termini (7-methylguanine caps) in order to give them resistance to degrading enzymes without disturbing their translational activity at the same time (21, 44, 45). In order to make the NAD analogs resistant to phosphodiesterase, such as DXO, the thiophosphate modification was introduced within the first phosphodiester bond in the

trinucleotide NAD analog (NRpppApG) (Fig. 13). In addition, we also obtained a trinucleotide with a 2'-O-methyl group within adenosine. According to the latest reports, such a modification protects against degradation RNA terminated with guanine or 7-methylguanine cap by DXO (46), so we tested if a similar effect could be achieved for NAD. Finally, in order to obtain potential resistance to both types of deNADing enzymes, we combined the α-thiophosphate modification with the 2'-O-methyl modification in one trinucleotide. We have synthesized all the compounds using methods previously developed in our team, first performing all the necessary optimizations first. The synthesis of both di-and trinucleotides required the production of nicotinic mononucleotide, activated with imidazole, as well as variously modified mono-and dinucleotide subunits, the latter obtained by synthesis on a solid support. The final stage of synthesis of all analogs was to create a pyrophosphate bond using imidazolide chemistry. NAD analogs containing α-thiophosphate moiety were synthesized in the form of two P-diastereomers, which were separated by HPLC method, which made it possible to subject them to biological tests in the diastereomerically pure form (Fig. 14A). The structures and purity of the compounds obtained were confirmed by spectroscopic methods, whereas phosphoric magnetic resonance was a particularly useful technique in this case (Fig. 14B).

The obtained set of compounds was subjected to biochemical characterization in order to determine their (i) ability to incorporate into RNA by transcription catalyzed by polymerase T7 and (ii) susceptibility to various deNADing enzymes. *In vitro* transcription experiments have confirmed that these compounds, as expected, can be the initiators of transcription catalyzed by T7 polymerase and therefore enable the formation of RNA molecules terminated with NAD at the 5' terminus. Moreover, it turned out that the use of trinucleotide analogs of NAD gives RNA of exceptionally high purity and homogeneity, so it is more advantageous than the currently used methods of NAD-RNA synthesis, which are characterized by lower efficiency of NAD incorporation (47, 48). Susceptibility of modified NAD to degradation by deNADing enzymes: NudC, Nudt12 and DXO was studied in collaboration with the group of Prof. Mike Kiledjian from Rutgers University (M. Kildjian and E. Grudzien-Nogalska). In agreement with the initial assumptions, as a result of these studies we identified analogs that are susceptible to hydrolysis by deNADing enzymes of only one type, both types, or completely resistant to hydrolysis. These results were also reported in the paper H10. The NAD analogs we have obtained are currently used for further studies on the properties and biological functions of NAD-RNA in our team in the framework of my SONATA BIS project and in cooperation with Prof. Kiledjian's group.

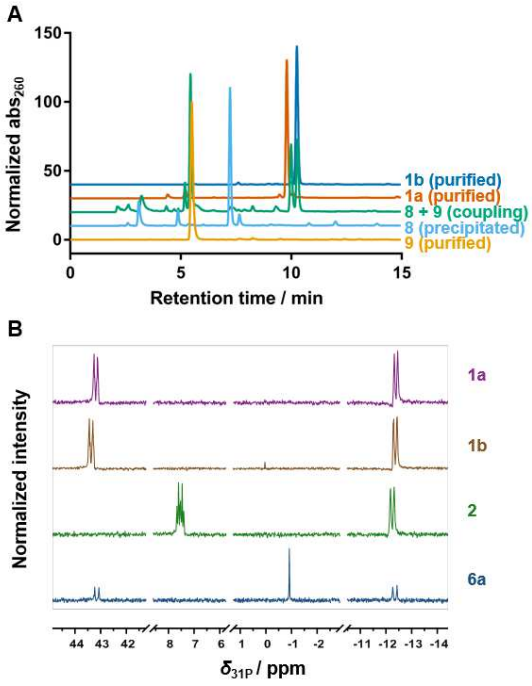


Fig. 14 A) Representative HPLC profiles showing the progress of the synthesis of NAD analog modified with thiophosphate group at the α position; B) Comparison of ^{31}P NMR spectra of selected NAD analogs. Data published in paper H10.

4.5. Analysis of synthetic nucleotide analogs by tandem mass spectrometry

Mass spectrometry is an extremely useful analytical tool in the study of nucleotides and their analogs. The literature describes examples of the use of mass spectrometry to solve specific research problems, such as the analysis of the degree of phosphorylation of selected nucleoside drugs, or quantitative determination of selected natural derivatives. Mass spectrometry using "soft" ionization techniques such as MALDI or electrospray (usually in the positive ionization mode) is usually used to solve such problems. A very important aspect is the ability to identify compounds on the basis of their fragmented spectrum. Tandem mass spectrometry allows for the fragmentation of ion with a selected mass-to-charge ratio, and the obtained fragments with specific values of *m/z* can serve as a valuable clue in predicting the chemical structure. Quantitative examinations often use selective monitoring of selected fragmentation reactions (single reaction monitoring, SRM or multiple reaction monitoring, MRM). In case of suitable selection of primary-secondary ion pairs, this method ensures high selectivity and sensitivity of the determination.

In the case of nucleotide metabolite analysis, problems often encountered in qualitative and quantitative s are structurally similar isomeric and isobaric compounds (i.e., of different composition but of the same nominal mass), which may interfere with the results of the determinations carried out (e.g. in the case of adenine ribonucleotide determinations it is necessary to distinguish them from isobaric guanine deoxyribonucleotides). In order to avoid these errors, a careful selection of chromatographic conditions and an appropriate selection of secondary ions observed by MRM, preferably those that are not commonly found in the fragmented nucleotide spectra, are necessary. Before the start of the project, we realized that there had not yet been an attempt to conduct a

comprehensive study on nucleotide fragmentation aimed at drawing more general conclusions, rules and guidelines for the identification and selection of secondary ions in quantitative studies. Moreover, most of the literature reports focused on the analysis of fragmentation ions from the nucleobases, while there were only few reports of fragmentation ions containing elements of ribose and phosphate moieties. In particular, very little has been known about the fragmentation of mono- and dinucleotides modified within the oligophosphate bridge. We also envisaged that an interesting and rather poorly understood aspect would be to compare the fragmentation of nucleotide ions generated in the positive and negative ions mode. In a project carried out together with the team within the Iuventus Plus grant, we planned to conduct a series of systematic studies on variously modified nucleotides in order to supplement and systematize the current knowledge on the fragmentation of nucleotides and their analogs in MS experiments.

These results were published in paper H11, in which we used the unique resources of our laboratory to analyze more than 150 mononucleotides, including some of those published in papers H1-H10, by tandem mass spectrometry with electrospray ionization. We analyzed primarily nucleoside 5′-mono-, 5′-di-and 5′-triphosphates, as well as their structurally similar analogs modified within the nitrogen base, phosphate residues, and ribose residues to identify characteristic fragmentation ions that may be helpful in the determination of the structure. Comparing ionization in the positive and negative modes, we noticed that in the positive ionization mode, fragmentation spectra contain mainly signals from nucleobases, while in the negative ion mode, fragments from phosphate and phosphoribose residues are also present, which makes it possible to determine certain elements of the structure such as the number of phosphate groups in the compound or the presence of modifications in the phosphates and ribose moieties. Based on these results, we proposed the structures of the most important fragmentation ions, which we additionally verified by studying isotopologues of selected compounds labeled with a heavy oxygen isotope ([^{18}O]) within the phosphate residues. We also demonstrated the possibility of using the ESI(-)/MS/MS method to distinguish structurally similar compounds through comparative analysis of isomeric and isobaric spectra of nucleotides and their synthetic analogs, as well as the potential of this method to quickly identify nucleotide products of organic synthesis from a very small amount of material. Finally, we have formulated general principles for nucleotide fragmentation and defined relationships between the fragmented spectrum and the structure, which may be helpful in the analysis of ESI(-)/MS/MS nucleotide spectra of unknown structure. In order to disseminate the results of our research as widely as possible, we have created a database of ESI(-)/MS/MS spectra in which we placed the spectra of all compounds analyzed in the study (msTide database; www.msTide-db.com).

The results of this work are currently used to solve specific research problems related to mRNA stability, e.g. to develop a method for quantifying the efficiency of incorporation of modified nucleotides into RNA transcribed *in vitro* (this project is carried out within the PRELUDIUM grant by Ph.D. student, Dominika Strzelecka). We are also developing a method of quantitative determination of 5′ mRNA cap metabolites, which will be used to determine the metabolites of cap in selected plant and animal cells and to study the activity and inhibition of cap degradation enzymes in cell lysates. Thanks to these studies it was also possible to develop a method for monitoring the degradation of enzymatic probes applied in paper H8. Currently, we also routinely using ESI(-)/MS/MS analysis for quick and unambiguous identification of nucleotides obtained by chemical synthesis or from biological material, which is particularly useful in the case of testing

significantly modified and fluorescently labeled compounds which are not available in quantities enabling NMR analysis.

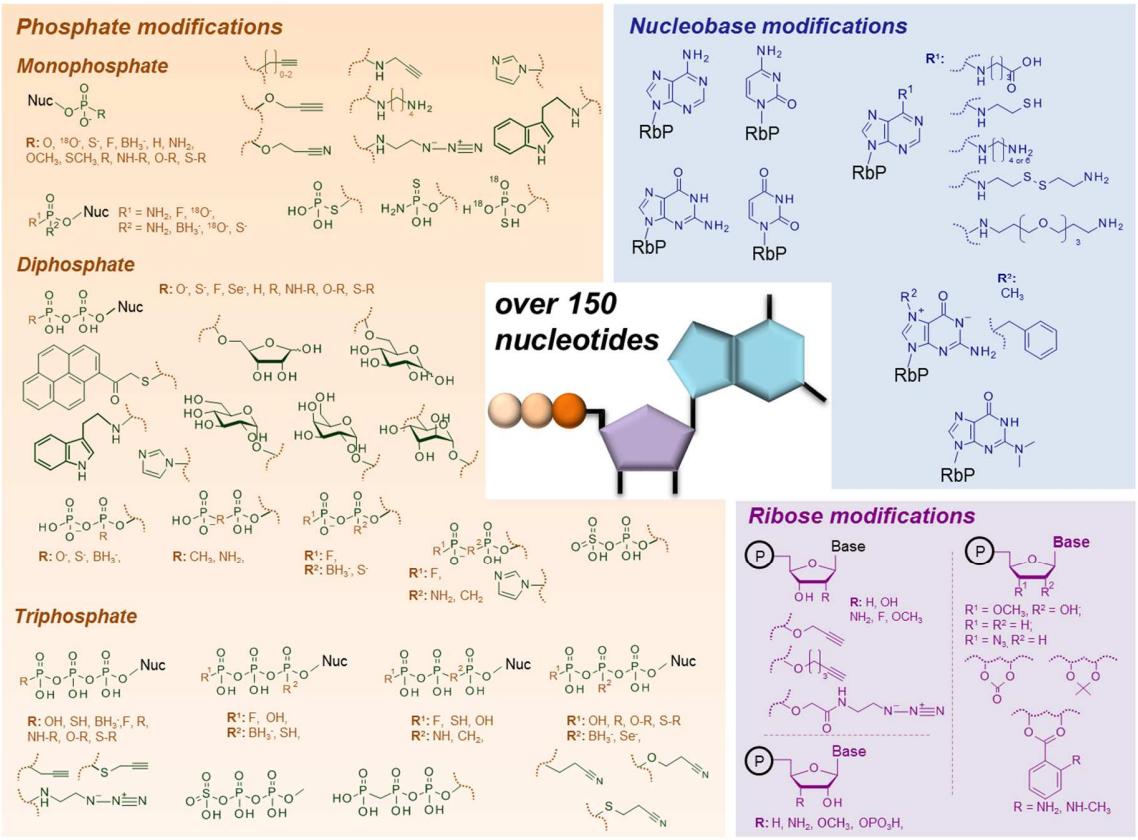


Fig. 15. An overview of the nucleotide library studied in the paper H11.

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