Nucleotide based activity probes: Synthesis and biological application of doubly dye labelled adenosine triphosphates

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Introduction

Relevance and synthesis of adenosine triphosphate (ATP)

ATP is present in all organisms from bacteria up to mammals. It is a key substance, the general currency of energy conversions in all living organisms. Due to the hydrolysis of its high-energy phosphate bonds, free energy becomes available for a variety of biochemical activities.[1] Up to current knowledge, adenosine and its nucleotides play a central role in many cellular processes such as muscle contraction, nerve excitation, active transport, post-translational modifications of proteins and many other matters of vital importance for any livings. Therefore, the number of enzymes utilizing ATP as a substrate is almost innumerable.[2,3]

In nature ATP is synthesised from adenosine diphosphate (ADP) and phosphate during both glycolysis in the cytosols and oxidative phosphorylation in mitochondria.[1] The ptoton-translocating ATP synthase, also known as proton pumping ATPase and F_1F_0-ATPase, is known to be the enzyme producing all ATP required in living organisms. ATP, after being exported to the rest of the cell, fuels its various energy-consuming processes.[4]

The discovery of ATP was first reported in 1929 by Karl Lohmann, a German chemist. Lohmann isolated ATP from muscle and liver extracts.[5] Actually, in 1847 Justus von Liebig was already able to isolate a compound he referred to as “inosinic acid” from meat extracts that later turned out to be ATP.[6] The determination of the structure was in close competition with Cyrus H. Fiske and Yellapragada Subbarow.[7] In 1931 Lohmann performed acidic hydrolysis of ATP which provided two moles of phosphoric acid, one mole of adenine and one of D-ribose-5-phosphate. The correct structure of

ATP was first proposed by Katashi Makino in 1935.[1] ATP´s central role in energy metabolism was first recognized by Fritz Lipmann and Herman Kalckar in 1941.[8] But it was only twenty years after Lohmann's findings, that the structure of ATP was finally confirmed by Alexander Todd due to its chemical synthesis (Figure 1).[1,9,10]

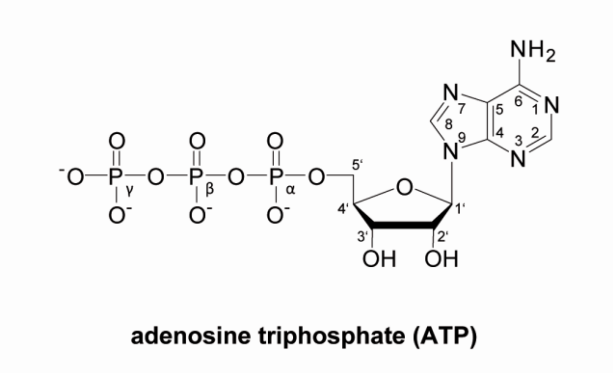


Figure 1: Structure of ATP and its nomenclature. Structure of ATP composed of a ribose, adenine as nucleobase and a triphosphate chain. Carbon atoms of the ribose building block are termed using Arabic numerals from 1’ up to 5’. Atoms building the aromatic purine structure are named using Arabic numerals from 1 up to 9. The triphosphate chain is labelled using Greek letters α, β and γ, and the phosphate connected to the ribose is called the α-phosphate by definition.

Since the beginnings of these spadeworks, nucleosides and their triphosphates opened up a huge field in scientific research. Nucleoside triphosphates (NTPs) and their developed analogues not only have important therapeutic and diagnostic applications, they are also applied to further enlighten actual issues of natural science.[11] Nevertheless, NTPs are still challanging to synthesise, isolate, characterize and store due to their ionic character and their ability to undergo hydrolysis. Consequently, the need for a general synthetic, high-yielding route towards NTPs is high. Although numerous diverse ways exist to synthesise triphosphates the need for a general applicable and high-yielding route to NTPs is an unsolved problem.[11] Usually, NTPs are chemically synthesised according to one of the concepts of either nucleophilic attack of pyrophosphate on an activated nucleoside monophosphate[12,13], syntheses involving activated phosphites or phosphoramidites derived from nucleotides[14], nucleophilic attack of nucleoside diphosphate on an activated phosphate species[15], nucleophilic attack of phosphate on an activated nucleoside pyrophosphate[16] or

syntheses involving direct displacement of 5'-*O*-leaving groups by triphosphate nucleophiles[17], respectively (Figure 2A-E).[11]

However, one of the most common ways to synthesise nucleotide triphosphates is *via* the nucleophilic attack of pyrophosphate on an activated nucleoside monophosphate. The required pyrophosphate salts are commercially available; however, the activated monophosphate must always be prepared freshly.[11]

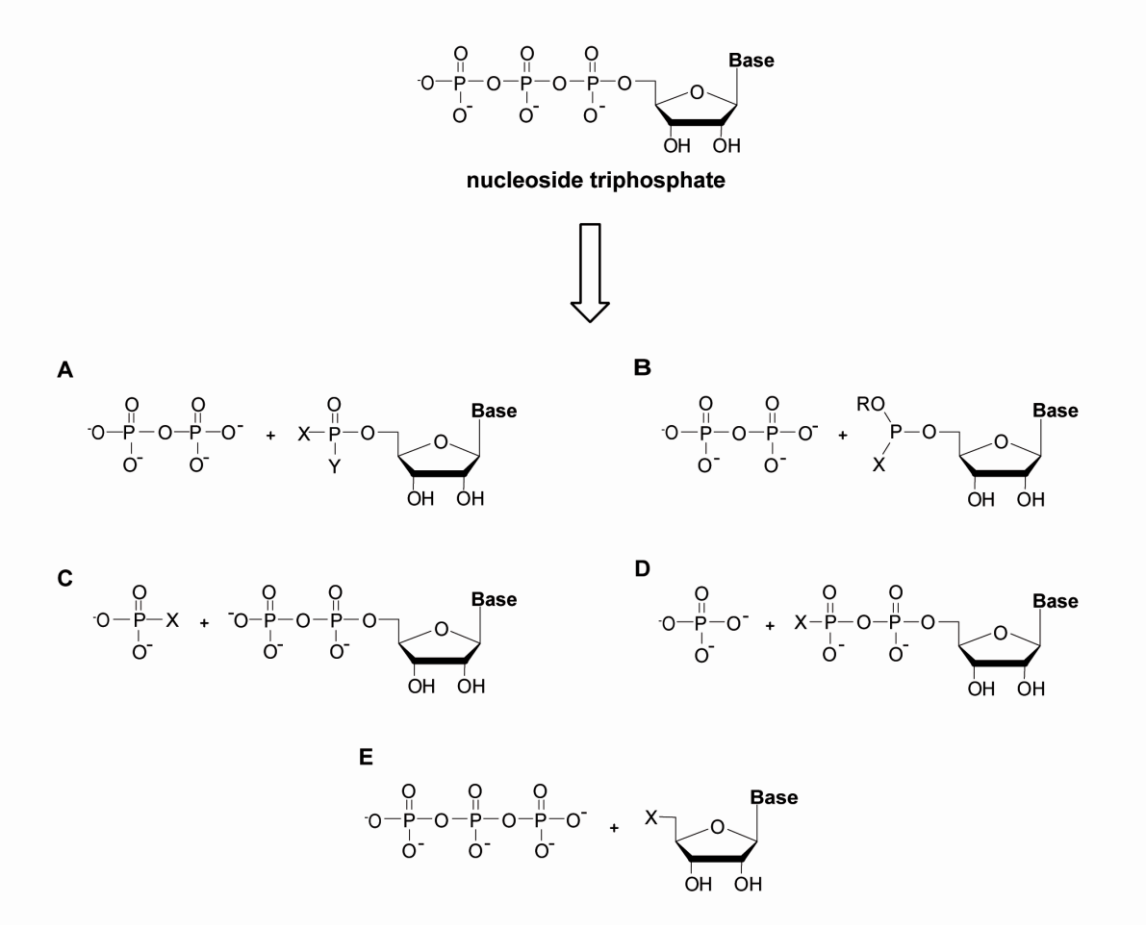


Figure 2: Overview of different chemical syntheses of nucleoside triphosphates. Five main concepts of chemical triphosphate synthesis are depicted. **A**) Nucleophilic attack of pyrophosphate on an activated nucleoside monophosphate. **B**) Syntheses involving activated phosphites or phosphoramidites derived from nucleotides. **C**) Nucleophilic attack of nucleoside diphosphate on an activated phosphate species. **D**) Nucleophilic attack of phosphate on an activated nucleoside pyrophosphate. **E**) Syntheses involving direct displacement of 5'-O-leaving groups by triphosphate nucleophiles. X; Y: leaving groups.

Early approaches to generate activated nucleoside monophosphates are based on the utilisation of phosphorous (V) oxychloride ($POCl_3$) (Figure 3). Important work and crucial improvements in respect of solvent, pH value and stereochemistry were achieved by Yoshikawa *et al.*[12], Ludwig *et al.*[13] and Kovacs and Ötvös[18]. The usage of $POCl_3$ as activating agent is even suitable to selectively activate unprotected nucleosides at the 5'-position and a valid method to synthesise nucleotide triphosphates in reasonable yields.[11] Ludwig and Eckstein alternatively used a phosphorous (III) species that was oxidised after the triphosphate reaction to activate the 5'-position of protected nucleosides (Figure 3). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4one therefore undergoes three nucleophilic displacement reactions with the nucleoside itself, pyrophosphate and an oxidative step using iodine in water to oxidise the phosphate and hydrolysis the built intermediate to give the nucleoside triphosphate.[14] It is noteworthy that in both cases, either using phosphor (V) or phosphor (III) species as activated monophosphates there is first a *meta*-triphosphate intermediate built upon the reaction with pyrophosphate. This intermediate then is quenched to the corresponding triphosphate by subsequent addition of buffer (Figure 3).[11,14] These *meta*-triphosphates also open up the scope for direct γ-modifications of triphosphates due to direct nucleophilic attack and subsequent ring opening.[19,20]

Using the herein presented phosphor species is by far not the only way to get activated nucleoside monophosphates for further triphosphate reactions. An other common way to provide these activated phosphor species is the usage of phosphoramidates for example.[11] Therefore, nucleoside monophosphates are activated with *N*,*N*'-dicyclohexylcarbodiimid (DCC)[21,22] and morpholine, to result in the formation of an activated phosphoramidate intermediate (Figure 4A).[23,24] 2,2,2-Tribromoethyl-morpholinochlorophosphate was also described as an alternative reagent to react with the 5'-hydroxyl group of the nucleoside to give direct access to a morpholine activated monophosphate species (Figure 4B).[25]

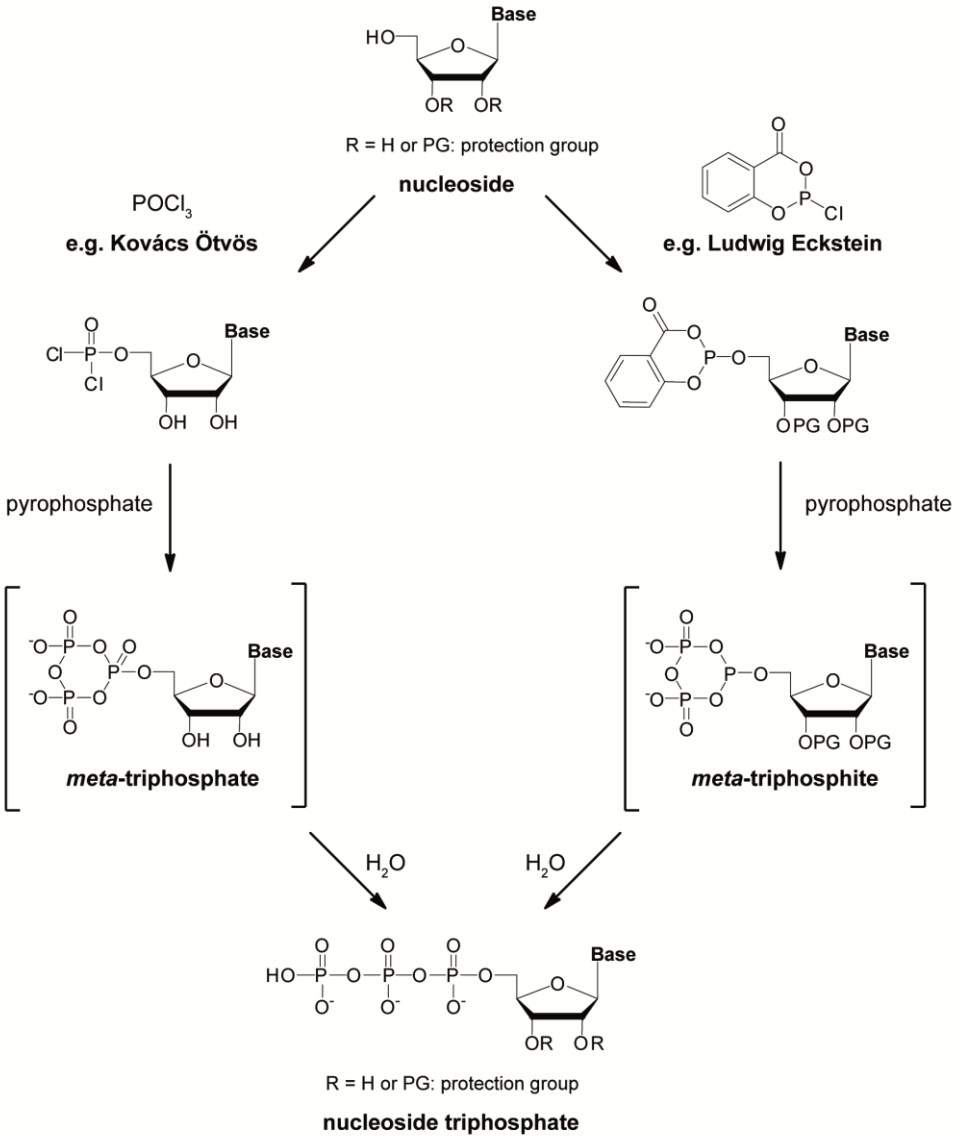


Figure 3: The Kovács Ötvös and Ludwig Eckstein procedure for the synthesis of nucleotides and their intermediates. The method of Kovács Ötvös uses phosphorous (V) oxychloride ($POCl_3$) as reagent to generate an activated nucleoside monophosphate. Alternatively, Ludwig and Eckstein used 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one and generated an activated phosphorous (III) species that is oxidised after the triphosphate reaction with pyrophosphate using iodine in general. Either way a *meta*-triphosphate or a *meta*-triphosphite intermediate is created during the reaction to give the corresponding triphosphate by quenching with water.[11]

Phosphorimidazolates resulting from activation of nucleotide monophosphates with 1,1'-carbonyldiimidazole ($(Im)_2CO$) or an pre-activated monophosphate with *N*-methylimidazole are other methods to access activated monophosphates for further triphosphate synthesis (Figure 4C-D).[26-29] Moreover, 8-quinolyl monophosphate is reported to give activated nucleoside monophosphate due to their reaction with nucleosides as well (Figure 4E).[30]

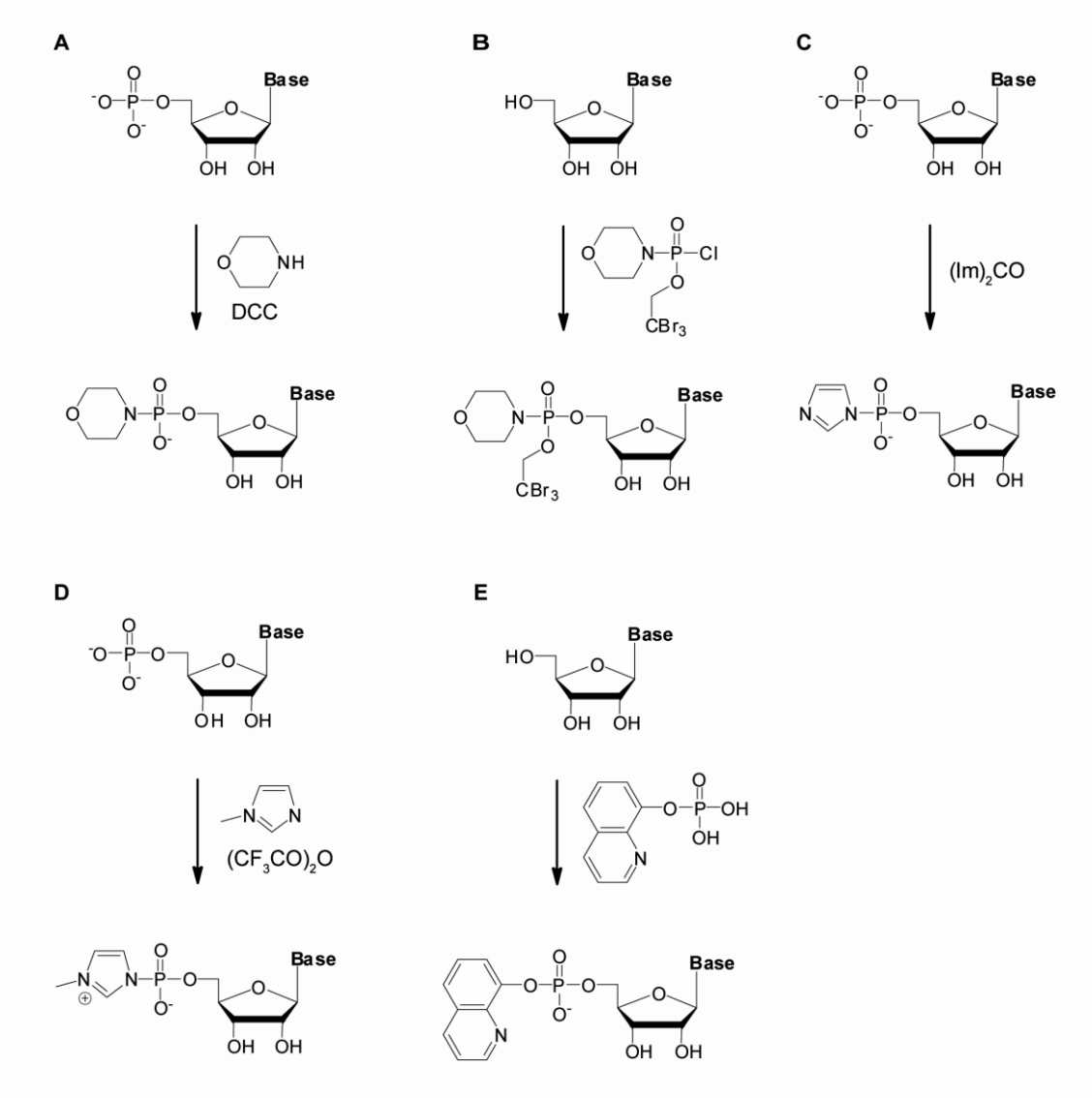


Figure 4: Overview of syntheses of activated monophosphate species. A) Morpholine activated monophosphate. **B**) 2,2,2-Tribromoethyl morpholine activated monophosphate. **C**) Imidazole activated monophosphate. **D**) *N*-Methylimidazole activated monophosphate. **E**) 8-Quinolyl activated monophosphate. All activated monophosphates are commonly used for trihosphate synthesis.

ATP and its analogues

One of the great challenges in the post-genomic era is to analyse the function and regulation of cellular molecules.[3] ATP is the universal energy currency of living organisms and plays a central role in biochemistry.[31] Hence, a vast amount of enzymes utilize the energy provided by ATP to fulfil various tasks, like to couple the chemical free energy to mechanical, electrical, or photoelectrical events or to transducer extracellular signals into intracellular processes.[32,33] Understanding the different enzymes and their way of action can largely help to understand and modify various biological pathways.[32] In the context of elucidation of cellular function and regulation, adenosine and especially its modified nucleotide analogues play a crucial role and represent powerful tools in biomedicine and molecular biology.[2,34,35] For this purpose vast amount of modified ATPs were synthesised in the last decades. Mainly these modifications can be subdivided in modifications at the γ-phosphate group[19,20,36-50], into sugar moiety modifications[2,51-62] and modifications at the nucleobase[55,63-69] (Figure 5). Many of these modifications result in changes in the essential properties of the NTPs, and most importantly their behaviour in the enzymatic reactions. Until now multiple modified ATP analogues are still unknown but highly desirable due to their ability to combine different properties and their enlarged variety of features.

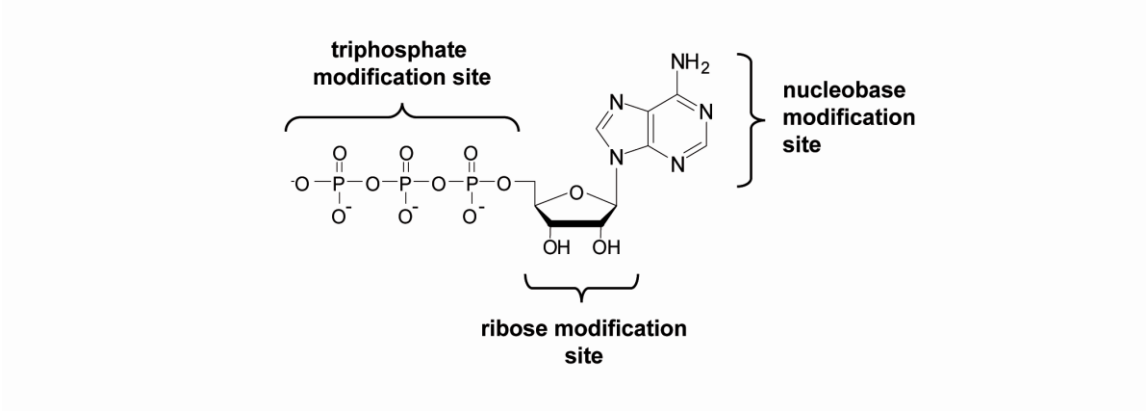


Figure 5: Overview of ATP modification sites. Modifications of ATP can be located at the phosphate group, the ribose moiety or at the nucleobase.

Phosphate modified ATP analogues

Modifications at the γ-position of the triphosphate chain of NTPs are known to be recognized and utilized by certain enzymes.[70] That allows the use of nucleotide triphosphates carrying substituents at these points as effective substrates of enzymes.[70,71] This possibility to introduce modifications at the γ-phosphate allows the creation of a variety of ATP probes. Nucleotides carrying sensor molecules at the γ-phosphate are widely used in biological research and molecular biotechnology. They represent valuable tools to understand and modify biological processes.[19,50,72,73] Usually, γ-modifications are attached by a phosphoramide-[20,36-44], phosphoresther-[19,20,37,39,45-48] or phosphonyl-[49,50] bond to the nucleoside triphosphate (Figure 6).

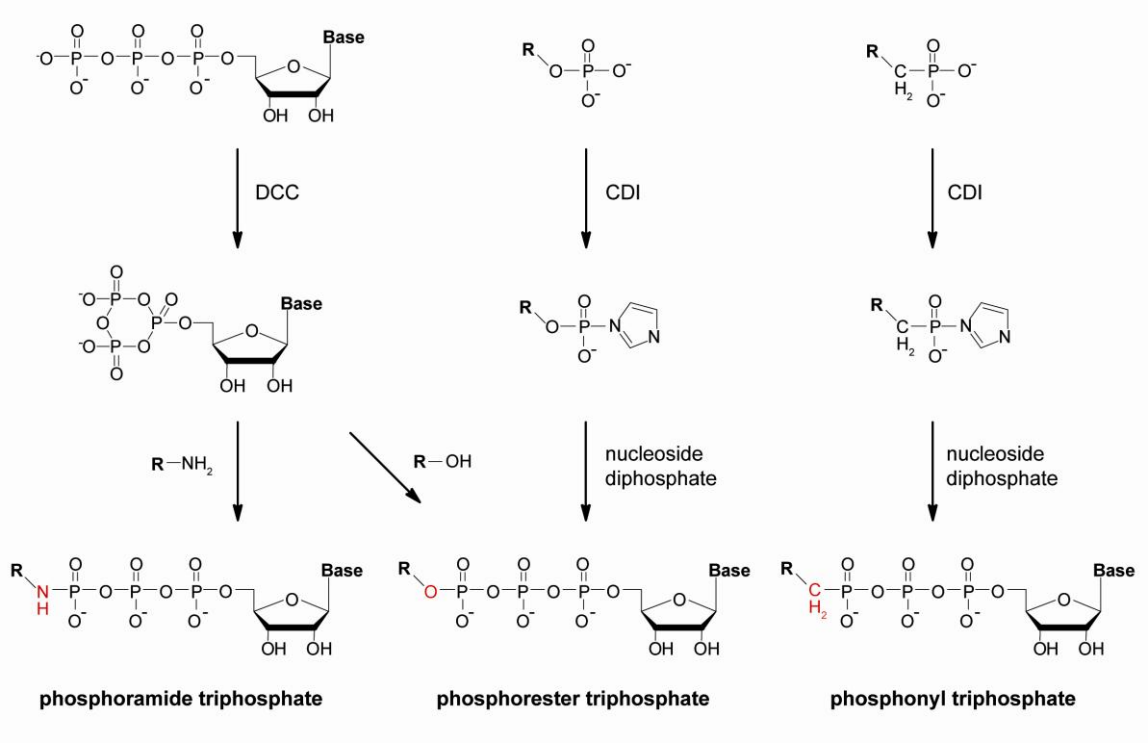


Figure 6: Different kinds of γ-modified triphosphates and their synthesis. Ring opening reaction of a corresponding *meta*-triphosphate using amines or alcohols as nucleophile result in the corresponding phosphoramide or phosphorester modified triphosphate. Activated monophosphates and monophosphites can be converted with nucleoside diphosphates to result in the corresponding phosphorester or phosphonyl modified triphosphate. R: Any residue.

The original synthesis route for triphosphate-linked nucleotides, is to employ a cyclisation step to the *meta*-triphosphate intermediate due to activation with DCC, followed by a nucleophilic attack of an amine or alcohol on the phosphate ring resulting in a newly formed phosphoramide- or phosphoresther bond.[19,20] Direct quenching of the *meta*-triphosphate intermediate, which is build during the triphosphate reaction using $POCl_3$ to generate an activated monophosphate species followed by addition of pyrophosphate, with nucleophilic amines or alcohols is possible as well to get γ-modified triphosphates. However, this reaction strongly depends on the nucleophilic strength of the amine or alcohol, respectively (Figure 6).[19] To result in γ-phosphate modified triphosphates of weak nucleophilic compounds, their corresponding phosphoric- or phosphonic acid is activated with *N*,*N*'-carbonyldiimidazole (CDI) and coupled to adenosine diphosphate to create phosphoresther- and phosphonyl triphosphates, respectively (Figure 6).[19,46,49] Applying this method, tetra- (δ) and penta- (ε) phosphate labelled nucleotides become available, too.[74] For this purpose the activated monophosphates are coupled with tri- or tetra-phosphates.[74] However, according to findings within this work it could recently be shown, that nitrogen-linked triphosphates readily decompose at slightly acidic conditions.[50] In contrast, oxygen- and carbon-linked γ-modified triphosphates turned out to be very stable over a broad range of pH values. These findings clearly limit the biological application of nitrogen-linked probes. This fact has to be kept in mind regarding the design of new γ-modified triphosphate probes.[50]

One of the applications of γ-modified triphosphates is the study of DNA polymerase catalysed DNA synthesis[19,38] and DNA- and RNA- dependent RNA polymerases[36,38,47,49,75]. Different γ-modified nucleoside triphosphate analogues were already used to monitor the activity of these enzymes in real time.[50] Therefore, these analogues are efficiently employed for continuous single molecule DNA sequencing.[76-78] Moreover, these analogues were widely used for studying protein kinases. In this context peptides and proteins can be modified with the label attached to the γ-phosphate before. This opens up a wide range of applications depending on the label of choice. Therefore, a lot of different probes bearing different labels were synthesised for such purposes. Shokat *et al.*[79,80] for example used radioactive ATP analogues, like [γ-^{32}P]ATP (Figure 7A), for specific kinase substrate labelling.

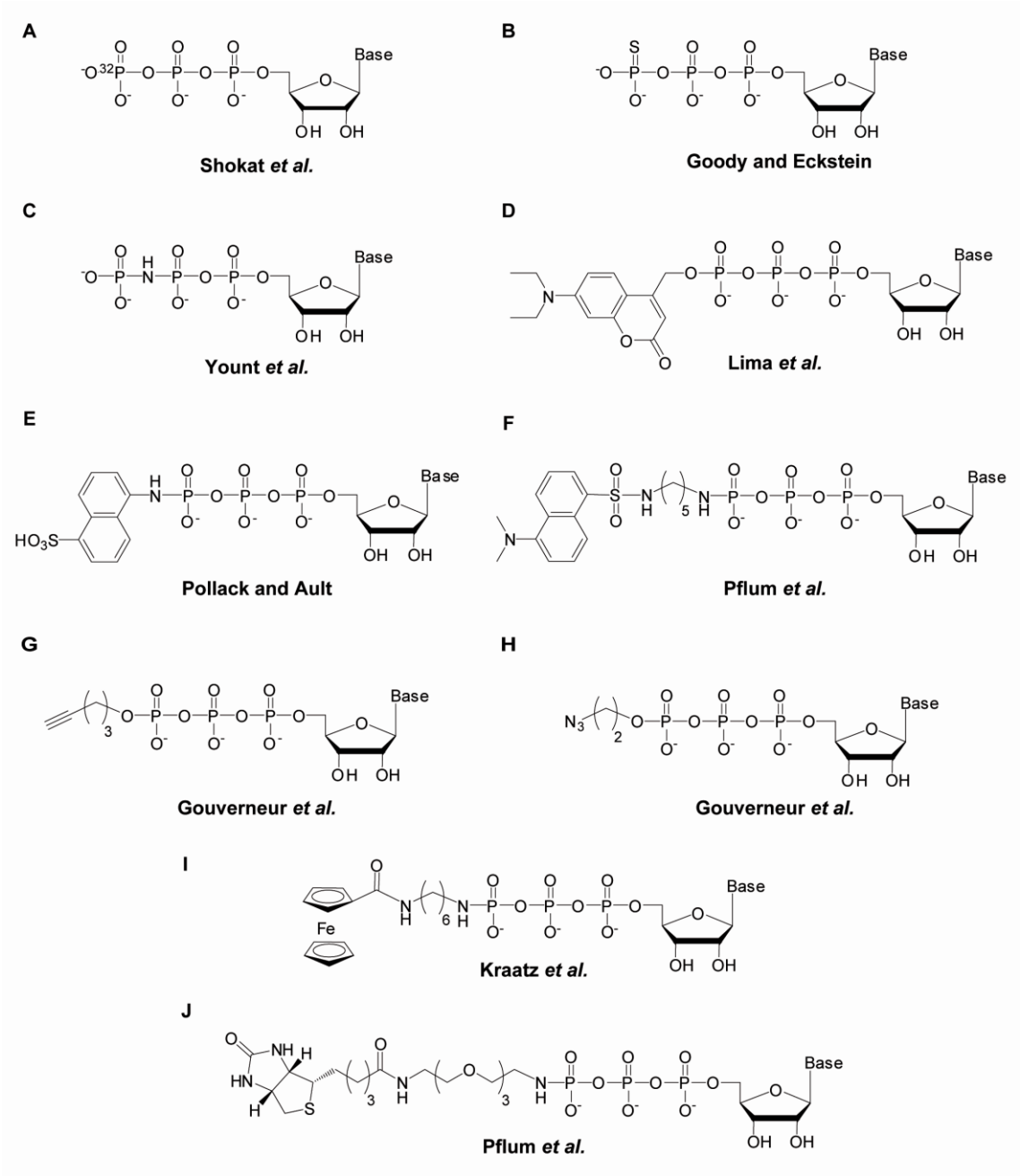


Figure 7: Overview about γ-modified ATPs used to understand and modify biological processes. A) Radioactively labelled ATP analogue [γ-^{32}P]ATP is used by Shokat *et al.* for specific kinase substrate labelling. **B**) ATPγS analogues show interesting behaviours with enzymes. **C**) Non-hydrolysable analogues like AMP-PNP are widely used to study binding energy in the transduction processes and in crystallographic studies. **D**) Caged-ATP derivatives like DEACM-ATP used by Lima *et al.* can be activated by light. **E-F**) Fluorophore labelled ATP analogues were used to study their spectral properties when cleaved by SVPD and to study kinase-catalysed protein phosphorylation. **G**-H) ATP analogues modified

with cross-linking precursors on the γ-phosphate were investigated to allow further derivatization by Huisgen chemistry or Staudinger ligation. **I**) Electrochemical sensors attached to the γ-phosphate of ATP can be assigned to surface-immobilised peptides *via* kinase-catalysed protein phosphorylation. **J**) γ-Biotin modified ATP analogues can be used by different kinases for subsequent detection or manipulation of protein substrates, for example.

This can be achieved by engineering the kinase of interest to accept bio-orthogonal ATP analogues that are not used by the remainder of the kinome. Goody and Eckstein[81] established an easy synthetic way towards ATPγS analogues (Figure 7B) that show interesting behaviours with enzymes involved. These analogues can be hydrolysed in many systems but usually show much reduced turnover rates compared to natural ATP. This fact was used in kinase and phosphatase studies; because once an amino acid side chain has been thiophosphorylated it may be resistant to rapid dephosphorylation processes. Sulphur analogues in α- and β-position generate stereo isomers that can be used to probe the specificity of binding sites.[82] In energy transducing enzyme reactions, ATP is normally hydrolysed between the β- and γ-phosphate groups. Modification of this region produces slowly hydrolysable or non-hydrolysable ATP analogues like AMP-PNP[36,83,84] (Figure 7C) synthesised by Yount *et al.* for example. These derivatives can be used to study the role of binding energy in the transduction process. Non-hydrolysable analogues are also useful in crystallographic studies, as they form stable complexes between protein and bound AMP-PNP.[82] A similar concept is applied using caged-ATP derivatives[85] like DEACM-ATP[86] (Figure 7D) used by Lima *et al.* to light activate the transcription of RNA polymerisation. Flashes of light can release ATP within milliseconds and can be used to initiate reactions *in vitro* or within cells. Different caging groups have different absorption characteristics and photolysis rates.[82] However, triphosphates can be modified with fluorophores[42-44,48,87], too. A fundamental study of Pollack and Auld[44] investigates a γ-aminonaphthalene modified triphosphate analogue (Figure 7E), its cleavage due to Snake Venomn phosphodiesterase treatment and its spectral properties. The work of Pflum *et al.*[42,87] regarding the use of γ-dansyl modified triphosphate (Figure 7F) to fluorescently label proteins *via* kinase-catalysed protein phosphorylation is basic, as well. The use of ATP analogues modified with cross-linking precursors[39,88] was investigated by Gouverneur *et al.*[39], presenting a variety of γ-modified ATPs bearing functional groups like alkynes (Figure 7G) or azides (Figure 7H)

at the γ-phosphate. These probes are able to undergo further derivatization due to highly selective chemical manipulations like Huisgen chemistry or Staudinger ligation for example.[39] Kraatz *et al.* opened up the field of electrochemical sensors attached to the γ-phosphate of ATP.[40,89,90] These sensors, mainly ferrocenes, can be assigned to surface-immobilised peptides *via* kinase-catalysed protein phosphorylation of γ-ferrocene modified ATP (Figure 7I) by different kinases. Affinity labels like biotin for example can be transferred to polypeptides and proteins as well. Pflum *et al.*[41,87,91,92] studied labelling reactions of peptides and proteins *via* kinase-catalysed protein phosphorylation using γ-biotin modified ATP (Figure 7J) and different kinases for subsequent detection or manipulation of protein substrates. Moreover, many other processes can be studied using such probes, including modification of nucleic acids by polynucleotide kinases[93], detection of GTP binding to GTPases[94] and affinity purification of nucleotide binding proteins[95].[50] The field of application of γ-modified ATP analogues is wide, nevertheless the elegance of this approach to investigate biochemical processes creates a raising desire for further investigations and applications.

Ribose modified ATP analogues

Nowadays chemistry is able to attach modifications to every desired carbon or oxygen atom of the ribose moiety of an ATP. However, considering ribose modified ATP analogues, the importance of 4'-*C*-modified nucleosides was clearly established among the scientific community over the past decade.[51] This is not only driven by findings, indicating certain entities of this class of nucleosides showing promising anti-viral activity.[96] Above, 4'-*C*-modified nucleotides were extensively explored in order to reveal insights into complex biological processes.[51,97,98] In that context it is noteworthy to mention that 4'-*C*-modifications of nucleosides always include the generation of quaternary carbon centres comprising the challenges associated with the respective chemistry.[51] Over the recent years mainly three synthetic routes for the synthesis of 4'-*C*-modified nucleosides evolved and proved to be beneficial.[51,96] Route one focuses on the synthesis of suitable 4-*C*-modified ribose derivatives bearing a desired functional group at that position, after conversion to glycosyl donors the

building blocks are used for nucleoside synthesis applying Vorbrüggen´s method (Figure 8A).[51,52,96,99,100] Route two involves the asymmetric (S)-(-)-1-amino-2-methoxy-methylpyrrolidin (SAMP)/(R)-(+)-1-amino-2-methoxy- methylpyrrolidin (RAMP)-hydrazone α-alkylation and a diastereoselective nucleophilic 1,2-addition with Grignard and organocerium reagents to open up the way of synthesis towards 4'-quarternary 2'-deoxy-3'-*epi*-β-*C*-nucleoside derivatives (Figure 8B).[53,54,96] The third route is especially designed for the direct synthesis of 4'-*C*-modified 2'-deoxynucleosides. Hereby, a 4'-*C*-branch is directly introduced to the corresponding, protected nucleoside of choice. This method possesses the benefit to skip the glycosylation step (Figure 8C).[51]

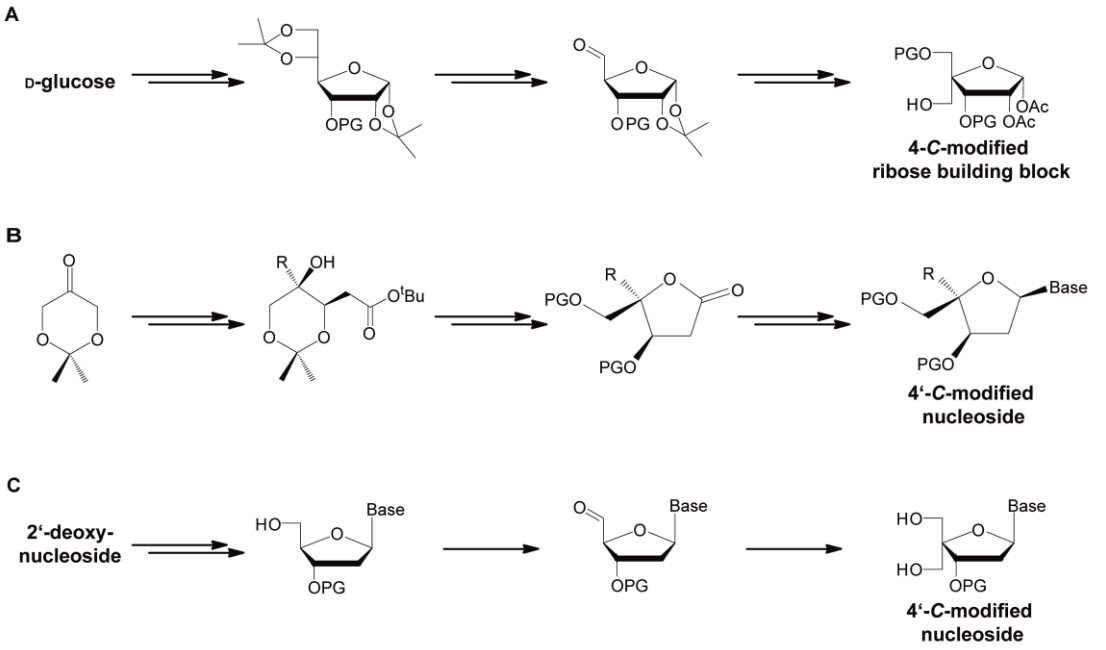


Figure 8: Three synthetic routes for the synthesis of 4'-*C*-modified nucleosides. A) Synthesis *via* suitable 4-*C*-modified ribose derivatives acting as precursors for nucleoside synthesis *via* glycosylation. **B**) Synthesis *via* asymmetric SAMP/RAMP-hydrazone α-alkylation and a diastereoselective nucleophilic 1,2-addition with Grignard and organocerium reagents. **C**) Synthesis *via* direct synthesis of 4'-*C*-modified 2'-deoxynucleosides. PG: Protection group.

However, with regard to study ATPases, mainly modifications attached *via* an ester or carbamate bond at the *O*2'- and *O*3'-position of the ribose were used until now.[2] One

of the pioneering molecule in this field is *N*-methyl-anthraniloyl-ATP (mant-ATP) that was introduced in 1983 by Hiratsuka *et al.*[56] (Figure 9A).

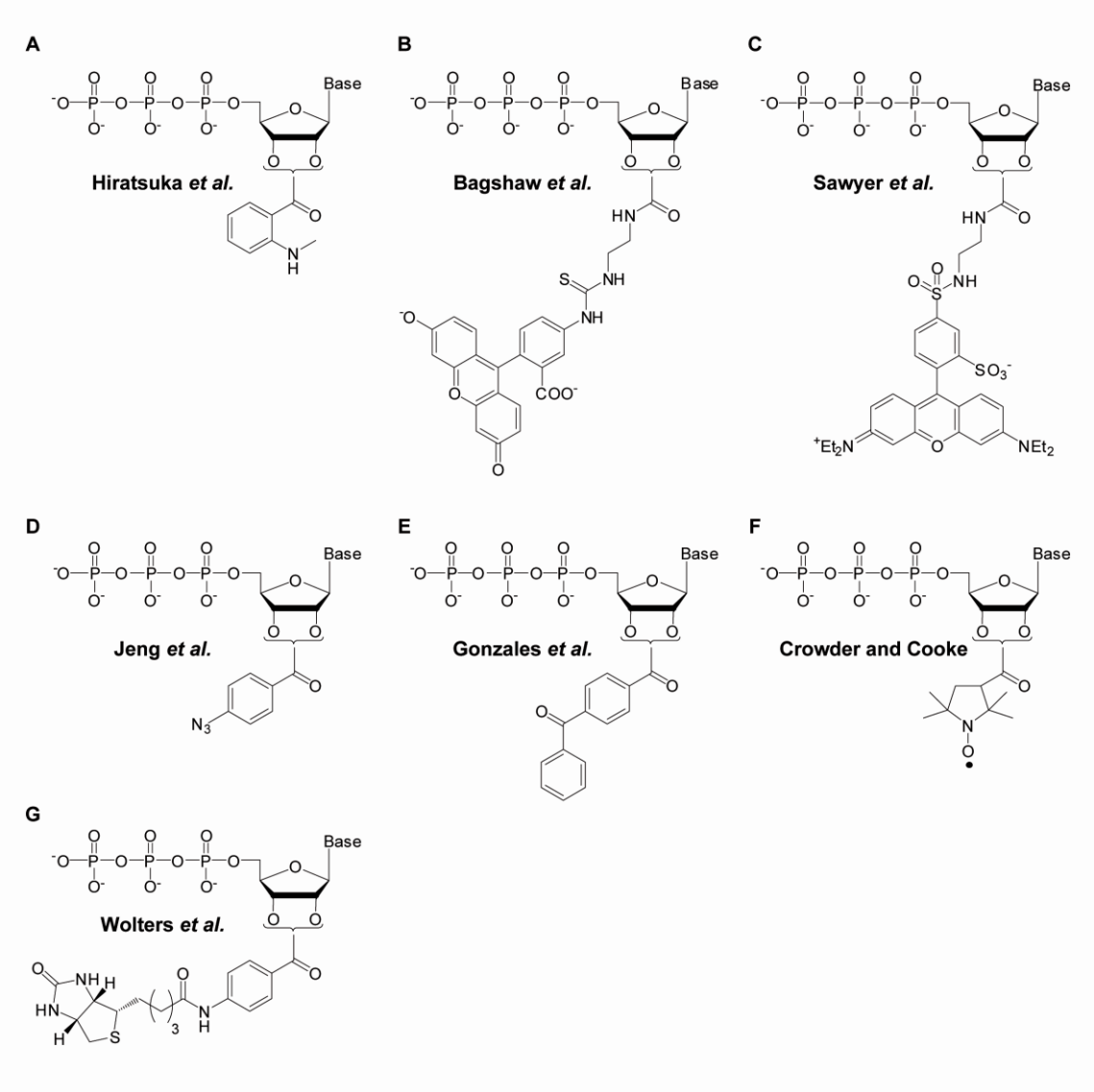


Figure 9: Overview about ribose modified ATPs used to understand and modify biological processes. **A**) mant-ATP was introduced in 1983 and extensively used to study the kinetics of binding and release of ATP. **B**) FEDA-ATP was used to investigate the kinetic pathway and mechanism of muscle contraction. **C**) Rhodamine labelled ATP analogues were exploited to monitor the distance between the ATP binding site and a fluorescent labelled DNA substrate. **D/E**) Photo-crosslinking reagents, such as aryl azides (**D**) or benzophenones (**E**) were used to affinity label the active site of ATPases. **F**) Spin labelled ATP analogues were introduced to the ribose of ATP to investigate ATPases in EPR studies. **G**) Biotin-labelled *p*-BABA-ATP is used for affinity purification of certain ATPases.

This fluorophore conjugated ATP analogue was extensively used to study the kinetics of binding and release of ATP to various enzymes using the change of fluorescence intensity as readout.[2,57-62] However, alternative fluorophore conjugated to the *O*2'- and *O*3'-position of the ribose were also used to overcome the drawbacks of the short wavelength absorption and emission of mant-ATP. 2'-(3')-*O*-(*N*-2-(3-(5-fluoresceinyl) thioureidol) ethyl carbamoyl) adenosine triphosphate (FEDA-ATP)[102] (Figure 9B) – an analogue in which a fluorescein moiety is linked *via* an ethylenediamine chain to the ribose of ATP – was used for example by Bagshaw *et al.* to investigate the kinetic pathway and mechanism of muscle contraction at the molecular level.[102-104] Moreover, these analogues were used to monitor the distance between the ATP binding site and a fluorescent labelled DNA substrate in the case of a bacterial transcription regulatory protein using the Förster resonance energy transfer (FRET) mechanism, done for example by Sawyer *et al.*[105] (Figure 9C).[2,105] Besides dyes, further modifications of ATP including photo-crosslinking reagents, such as aryl azides[106] (Figure 9D) used by Jeng *et al.* or benzophenones (Figure 9E) used by Gonzales *et al.* and Kambouris *et al.* were introduced by ester- or carbamate-attachments to the *O*2'- and *O*3'-position. These analogues were used to affinity label the active site of ATPases and to gain information on the active site architecture of the enzyme of interest.[2,106-108] Spin labels were recruited and introduced to the ribose of ATP to investigate ATPases by EPR studies.[2,109,110] Crowder and Cooke[109] used ATP analogues with a nitroxide free radical moiety attached to the *O*2'- and *O*3'-position of the ribose of the ATP (Figure 9F) to investigate myosin in glycerinated muscle fibres. Furthermore, biotin-labelled *p*-biotinyl amidobenzoic acid adenosine triphosphate (*p*-BABA-ATP) (Figure 9G) was used by Wolters *et al.*[111] for affinity purification of certain ATPases. However, all modified ATPs bearing an ester bond have the drawback that labels attached to the *O*2'- and *O*3'-position of ATP isomerise relatively fast.[112] Using the carbamate linkage this isomerisation can be slowed down, but nevertheless it is still difficult to obtain pure isomers.[2,113] Oiwa *et al.* showed that in the case of a Cy3 labelled ATP analogue the two isomers can have very different spectroscopic features.[2,114] Other attachment sites than the *C*4'-, *O*2'- and *O*3'-position of an ATP were only used in a few cases mainly requiring modifications at the nucleobase to generate nucleotide probes for investigation of ATPases.[55,64,65,115]

Nucleobase modified ATP analogues

In the last years, an increased interest in the study of modified purine nucleosides, substituted in the purine ring, arose.[63] Modified purines containing substituents in the 2-, 6- or 8-position are associated with interesting biological properties, such as antiviral-[116], anticancer-[116] and antihypertensive effects[117,118] or cytokinin activity[119,120].[121] The surge of interest in the synthesis of especially 2-*C*-modified adenosines is not only due to their potency of antiviral[122] and anticancer activity[123] but to their pharmacological activities as A_1, A_{2A} and A_3 adenosine (ADO) agonists with their peripheral vasodilator effect for the treatment of hypertension[117,124,125] and to their enzymological usefulness as biochemical probes.[122] Despite the well elaborated access to this class of compounds, up to now 2-*C*-modified adenosine probes for the investigation of bimolecular processes are still rare. Pd-catalysed coupling between halopurines and alkynes emerged to a popular reaction for C – C bond formation in the purine 2-position.[123,124,126,127] The reaction of 2-iodo adenosine with terminal alkynes in the presence of bis(triphenylphosphine)palladium dichloride and cuprous iodide – the Sonogashira reaction –opened up a synthetic way to that class of substances in excellent yields. The key intermediate therefore is represented by 2-iodo adenosine. This precursor can be prepared from guanosine in four steps according to Nair *et al.* (Figure 10).[122,126-128]

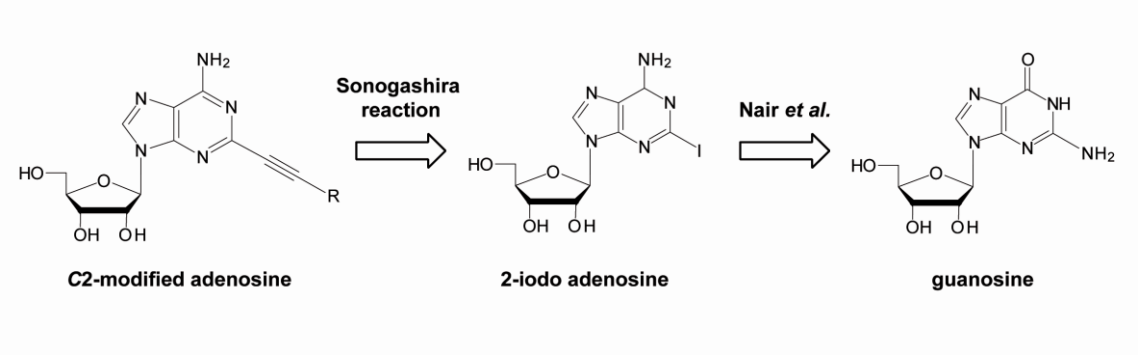


Figure 10: Retrosynthesis of *C*2-modified adenosine. Retrosynthetic scheme for *C*2-modified adenosine using 2-Iodo adenosine derived from guanosine as precursor for Sonogashira couplings.

However, attachment sites at the nucleobase were only used in a few cases to generate nucleotide probes for investigation of ATPases. Mainly modifications at the *N*6- and *N*8-position were required for those purposes.[2]

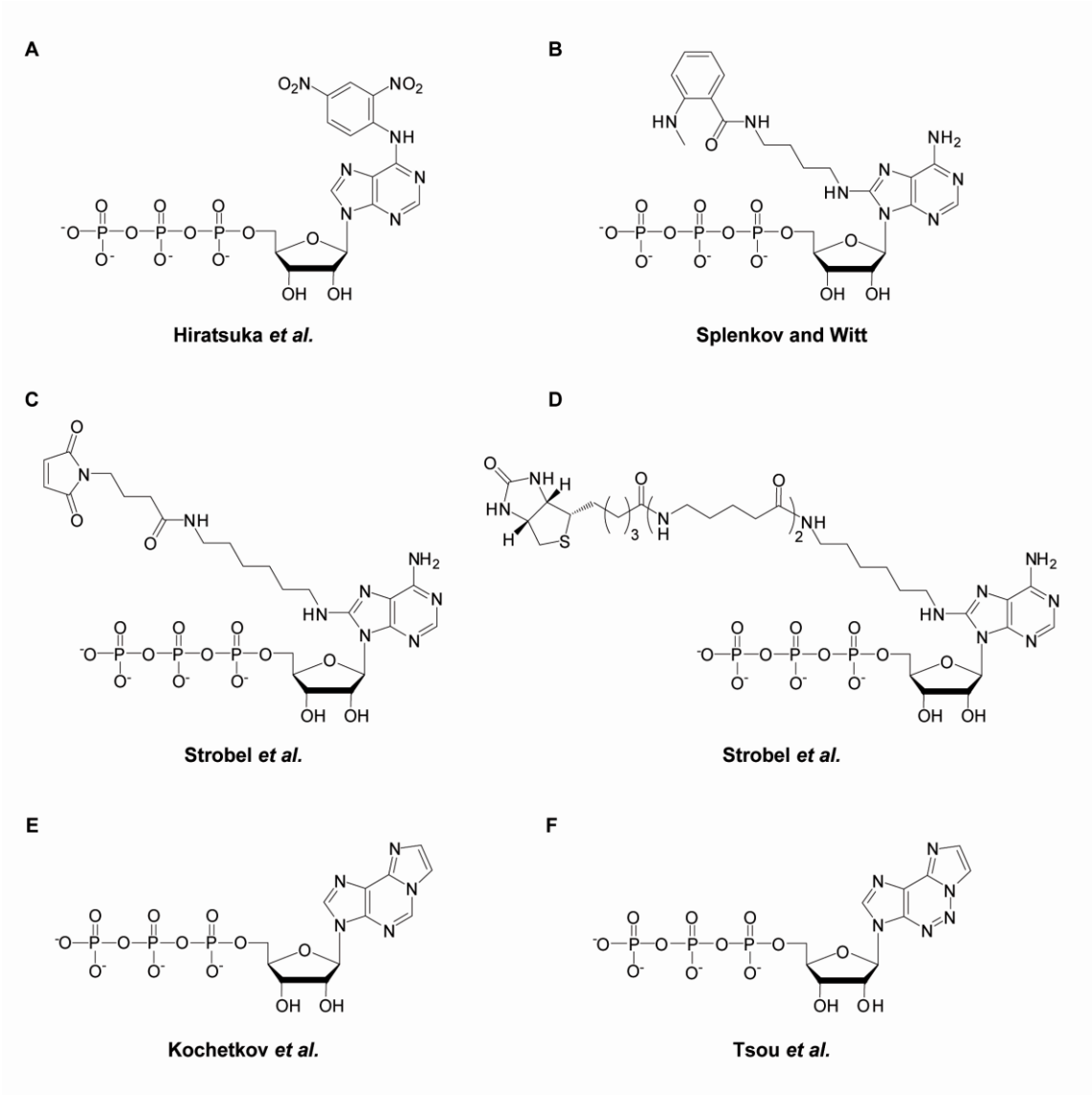


Figure 11: Overview of nucleobase modified ATPs used to understand and modify biological processes. A) DNP-ATP was used to investigate heavy meromyosin ATPase, for example. **B**) MABA-ATP was used to investigate MABA-ATP*DnaK comlexes and their dynamics due to the change rate of the fluorescent signals generated by varying peptide concentrations. **C-D**) ATP analogues modified with cross linking agencies (**C**) and affinity labels (**D**) were used to study the enzymatic activity of Bordetella pertussi adenylate. **E-F**) Autofluorescent analogues like etheno-ATP (**E**) and azaetheno-ATP (**F**) were elaborated and used to investigate kinase activities.

Hiratsuka *et al.*[55] used an analogue of ATP in which a dinitrophenyl group replaces a hydrogen of the 6-amino group of the adenosine base – *N*6-(2,4-dinitrophenyl)-adenosine 5'-triphosphate (DNP-ATP) (Figure 11A) – to investigate for example heavy

meromyosin ATPase. *N*8-(4-*N*'-Methylanthraniloylaminobutyl)-8-adenosine 5'-triphosphate (MABA-ATP) (Figure 11B) was used by Splenkov and Witt[64] to investigate MABA-ATP*DnaK complexes and their dynamics due to the change rate of the fluorescent signals generated by varying peptide concentrations. Strobel *et al.*[65] used ATP analogues that were modified at the *N*8-position with cross linking agencies (Figure 11C) and affinity labels (Figure 11D) to study the enzymatic activity of Bordetella pertussi adenylate cyclase *via* their conversion into 3'-5'-cyclic AMP analogues. A new approach was established by Kochetkov *et al.*[66] synthesising 1,*N*6-ethenoadenosine triphosphate (Figure 11E), an autofluorescent analogue of ATP. Secrist *et al.*[67] further elaborated this nucleic acid modification and used it to investigate kinase activities. Tsou *et al.*[68,69] modified 1,*N*6-ethenoadenosine and synthesised 2-aza-1,*N*6-ethenoadenosine triphosphate (Figure 11F) which is an autofluorescent derivative of ATP, as well.

However, to the best of my knowledge no ATP analogues modified at the *C*2-position have been used to study ATPases prior to this work.

Fluorescence imaging and Förster resonance energy transfer (FRET)

During the last decades FRET has emerged to one of the most powerful techniques to study conformational distribution, dynamics of biological molecules and molecular distances and interactions at the nanoscale level.[129-135] Moreover, the fact of strong dependence of transfer efficiency on probe separation makes FRET perfectly suited for on/off experiments and probes.[135] FRET, first theorized in 1948 by Theodor Förster[136], was initially used by a small number of biologists who measured distances in proteins at a scale of 10 nanometre (nm) and below.[137] In principle, FRET is a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (donor in S_1) to another fluorophore (the acceptor in S_1 state), by means of intermolecular long-range dipole-dipole coupling (Figure 12).[138]

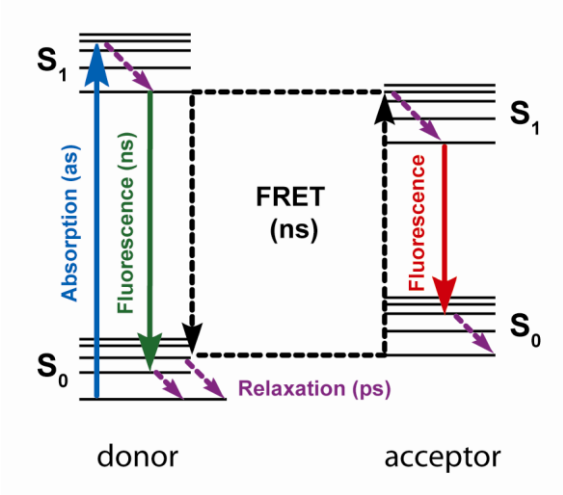


Figure 12: Jablonski diagram with FRET. FRET (black, dotted arrow) is a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (donor in S_1) to another fluorophore (the acceptor in S_1 state), by means of intermolecular long-range dipole-dipole coupling.

It is important to remember that resonance energy transfer is a process that does not involve emission and reabsorption of photons, the theory of energy transfer is based on the concept of a fluorophore as an oscillating dipole that can exchange energy with other dipoles with similar resonance frequency.[139] This process occurs whenever the emission spectrum of the donor overlaps with the absorption spectrum of an acceptor.[139] The efficiency of this transfer is inversely proportional to the sixth power of the distance between the donor and acceptor dye, making it a sensitive technique for investigating a variety of biological phenomena that produces changes in molecular distance. The efficiency of the energy transfer is given by equation (1):

$$E = \frac{1}{1+(r/R_0)^6} \qquad (1)$$

Whereas r is the distance between the donor and acceptor and R_0 is the distance at which 50% of the energy is transferred – the so called Förster radius.[134,139] R_0 is also a function of the properties of the dyes and contains a contribution from the relative orientation between the two dyes, called κ^2.[134] Technological advances in light microscopy imaging in combination with the technique of FRET has great potential and can lead to accurate measurement of molecular proximity at Ångstrom distances (10-

100 Å)[138] and therefore constitutes one of the preferred techniques to design on/off probes for spatiotemporal readout in real-time.

Protein kinases and the focal adhesion kinase (FAK)

There are approximately 500 protein kinases encoded in the human genome, which imply one of the largest protein families and together they control virtually every cellular process.[2,140] The phosphorylation of proteins represents one of the most dominant and evolutionarily conserved post-translational modifications, catalysed by this class of enzymes, for information in organisms and cells.[140-142] Protein phosphorylation mechanisms control processes as diverse as cell division, metabolism and differentiation.[143,144] Therefore it is perhaps the most universal regulatory modification used by organisms.[145] Protein kinases are enzymes capable to catalyse the transfer of the γ-phosphoryl group of ATP to serine, threonine and tyrosine residues in a wide variety of protein substrates.[146-148] This reversible modification often serves as a switch to alter for example the functionality of the substrate protein.[2] More than 30% of all eukaryotic proteins are post-translational modified by a phosphorylation step, with 99% of those modifications occurring on serine or threonine residues, respectively.[145] However, in multicellular animals the subset of proteine tyrosine kinases, which transfer the γ-phosphate to tyrosine residues of their substrate, have a key role in signal transduction, regulating cell proliferation and other cellular behaviour.[2] Accordingly, deregulation of kinase function and abnormalities in signalling pathways has been implicated in a wide variety of diseases such as cancer, diabetes, inflammatory disease, immunological disorders and arteriosclerosis.[146,147,149] Therefore, kinases have become one of the most intensively pursued classes of drug target with approximately 30 kinase targets.[150] Hence new label-free and fluorescence detection methods are required for an effective high throughput screening (HTS) of new active compounds.[151] The huge interest in kinases illustrates the urge for further investigations regarding their triggers and pathways of action. Moreover, to understand phosphorylation-mediated regulation of the proteome, it is essential to determine the substrates of each individual protein kinase and to define how signalling pathways operate.[145] The identification of such substrates of protein kinases proved

to be difficult due to the multitude of structurally related protein kinases present in cells, their apparent redundancy of function and the lack of tailor made tools to investigate their specific activity.[145]

An important representative of protein tyrosine kinases is FAK; its initial identification was done in 1992 by Schaller *et al.* and Hanks *et al.* (Figure 13).[152-154]

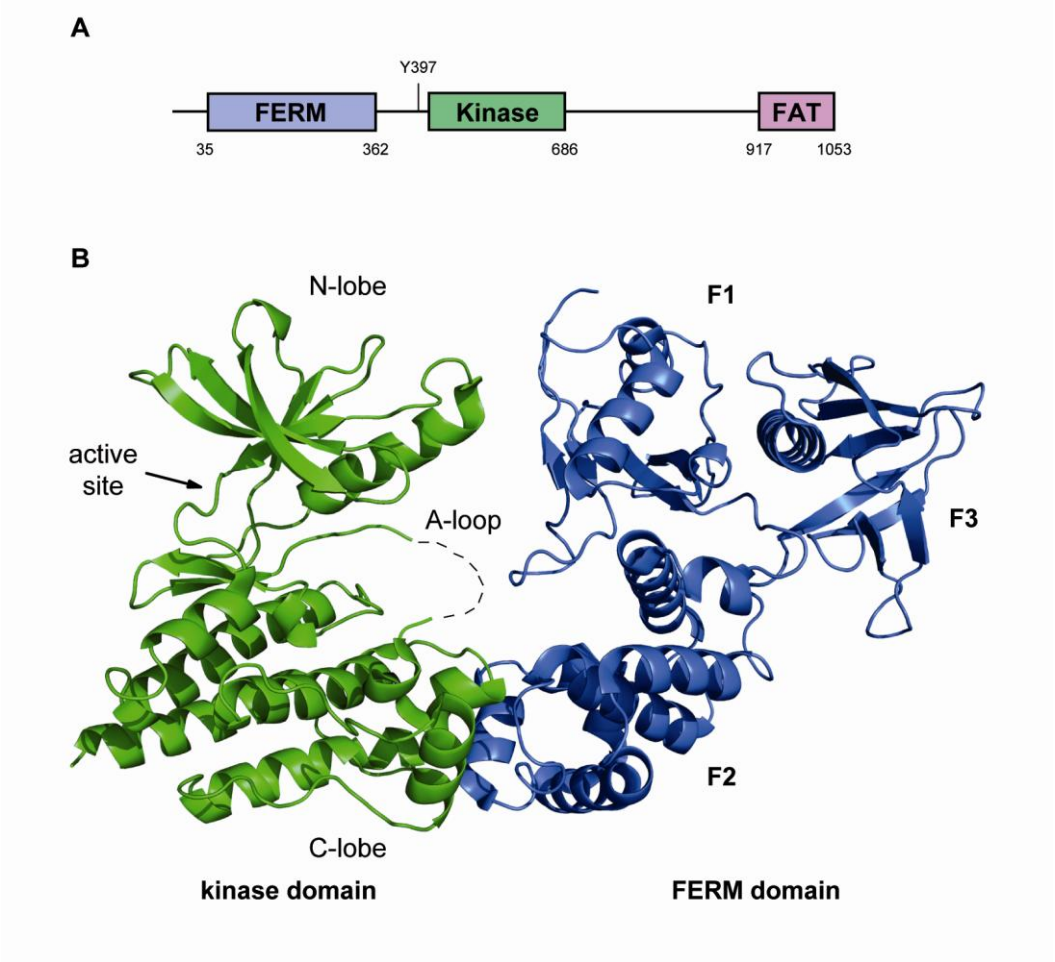


Figure 13: Structure of auto inhibited FAK. A) Domain structure of FAK. Auto phosphorylation site (tyrosine 397) is indicated. **B**) Overall structure of FAK including the FERM, linker and kinase regions. In the auto inhibited state, the FERM domain (blue ribbon) binds the kinase domain (green ribbon), primarily through an interaction between the FERM F2 lobe and the kinase C-lobe. FERM domain also blocks the access to the active site cleft and to the kinase active loop (A-loop). Disordered segments are indicated as dashed lines. (PDB: 2J0M)

Focal adhesions are integrin-mediated contact points between the surface of the cell and the extracellular matrix. Coordinated assembly and disassembly of these points of contact as well as intracellular signalling from these sites modulate cell adhesion, migration, proliferation, differentiation and survival in response to cues in the extracellular milieu.[155-157] FAK plays a central role in the signalling network emanating from focal adhesions.[152,155,158] Actually, FAK is a non-receptor tyrosine kinase that regulates signals involved in the abovementioned processes of cell proliferation, migration and survival. [152,155,158] FAK is recruited to focal adhesions *via* its *C*-terminal focal adhesion targeting (FAT) domain[159] and activated by signals from growth factor and integrin receptors[152].[84] The activation of FAK therefore is initiated by breaking an intramolecular autoinhibitory interaction between the *N*-terminal FERM (4.1, erzin, radixin, moesin homology) and kinase domains resulting in fast autophosphorylation of Tyr397 in the linker between the FERM and kinase domain (Figure 13).[84,155]

Among all duties of FAK that are essential for survival, any deregulation of the activity of FAK may cause mortal diseases. Like many kinases post translation modification, particularly phosphorylation, is a major regulatory mechanism.[160] Cell migration and invasion are fundamental components of tumour cell metastasis whereby increased FAK expression and tyrosine phosphorylation are connected with increased tumour genesis.[161] Especially, overexpression of FAK is identified in many types of cancer[162], experiments using animal models revealed the role of FAK in tumour formation and metastasis in a number of neoplasms, including breast, brain and skin cancer.[160,163-165] Moreover, aberrant migration of smooth muscle cells caused by FAK is a key feature of restenosis which describes the re-narrowing of dilated blood vessels after an operative procedure of these, occurring in approximately 10-20% of all patients.[166,167] These facts show the necessity of deeper insight to the function of the signal cascade of cell migration and adhesion, especially in the case of FAK.

Desulfococcus biacutus (*D. biacutus*) and the background of acetone degradation

D. biacutus KMRActS was isolated from anaerobic digestor sludge of a waste water treatment plant many years ago in the microbiology laboratory of Prof. Dr. B. Schink.[168] It was described as strictly anaerobic sulfate-reducing bacterium. It grows only chemoorganoheterotrophically with different substrates as electron donors and uses sulfate as electron acceptor. This bacterium belongs to the group of microorganism that are classified as complete oxidizers because of their ability to oxidize completely the growth substrate to CO_2, while coupling this oxidation to the reduction of sulfate.[168]

D. biacutus is grown with acetone as sole carbon source. Physiological experiments indicated that growth on acetone requires always the presence of CO_2 for acetone degradation.[168] Therefore, a possible carboxylation reaction was postulated at that time as the initial acetone activating reaction. Then the possibility of this type of activation by sulfate-reducing bacteria became interesting, especially by *D. biacutus*. The exciting point was to discover how sulfate-reducing bacteria that are energy-limited microorganisms can deal with such an activation reaction. Thermodynamically this reaction seems to be impossible for sulfate reducers because in the complete process they only obtain two ATP per degraded acetone. Since the activation of acetone is an endergonic reaction that requires the investment of two ATP, the cell would not have enough energy to support growth.[168]

The carboxylation of acetone to acetoacetate was first proposed with a methanogenic enrichment culture.[168] The reaction was also studied with the nitrate reducing strain Bun N under anaerobic conditions, and it was concluded that acetoacetate was formed in an ATP-dependent carboxylation of acetone.[169,170] A similar activation reaction was observed with the aerobic bacterium *Xanthobacter autotrophicus* strain Py2; a CO_2- and ATP-dependent carboxylation to form acetoacetate was again proposed.[171] The same acetone carboxylase was found with the nitrate reducer *Aromatoleum aromaticum*[172], and in *Alicycliphilus denitrificans*, *Paracoccus denitrificans* and *Paracoccus pantotrophus*.[173] Thus, it is well known that aerobic and nitrate reducing bacteria employ carboxylation in a similar reaction mechanism in the activation of

acetone. However, the degradation of acetone studied with *D. biacutus* indicated that acetoacetate in this case is not an intermediate in the metabolism of acetone because carboxylase or acetoacetate decarboxylase activities were not found in cell-free extracts.[174]

Recently, Gutiérrez and Hardt *et. al.*, showed evidence that a possible carbonylation reaction might be involved in the degradation.[175] Some evidence was provided for this type of reaction suggesting that acetone is carbonylated to form the intermediate acetoacetaldehyde in a reaction that also proved to be ATP dependent. This novel pathway has been difficult to assay, therefore in order to investigate the requirement of cofactors new nucleotide activity probes could be useful.

Concept and objective

The understanding and modification of biological processes is still a highly challenging task in natural science. However, to bring new insights to the functionality, location and synchronization of proteins with enzymatic activity, in the context of a living cell, is one of the most interesting questions in present-day research. Adenosine and its nucleotides play a major role that context, since the free energy provided by ATP hydrolysis is essential to many cellular processes, the number of enzymes utilizing ATP as a substrate is almost innumerable.[2,31] Modified nucleotides are valuable tools to understand and modify biological processes of ATP hydrolysing enzymes.[50] However, current assays do not allow direct observation of the activity of these proteins at spatiotemporal resolution. Any probes that allow monitoring protein activity in real time are therefore highly desirable. For that purpose, we decided to elaborate a conceptually novel ATPase assay allowing easy detection of enzyme activity at local and temporal resolution. At the starting point of my research a concept to visualize the cleaving process of ATP by an enzyme of interest without the introduction of mayor modifications to this enzyme was at that time not reported. Inspired by the widespread application of doubly fluorophore labelled substrates to monitor enzymatic activity of other enzyme classes, especially proteases[74,176-178], we aimed to modify an ATP with two different fluorophores that are suitable to undergo Förster resonance energy transfer (FRET). Applying our concept of a doubly labelled ATP analogue, the excitation of the fluorescent donor (D) leads to the transfer of excitation energy *via* FRET to the fluorescent acceptor (A) which emitted fluorescence can be observed. Upon the cleavage of an doubly labelled ATP analogue, FRET is not longer possible and the direct emission of the fluorescent donor can be observed. In that way, the activity of ATP hydrolysing enzymes should result in a significant change in fluorescent characteristics of the probe and can be detected in an easy way at spatiotemporal resolution (Figure 14).

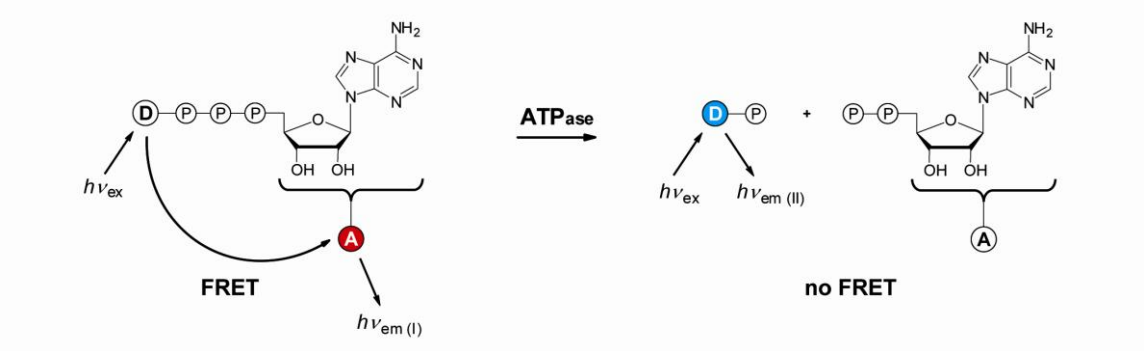


Figure 14: Concept of a doubly dye labelled ATP analogue.

Aware of the fact that two bulky fluorophores have to be attached to ATP, it was fundamental to investigate the optimal sites of attachment to substantially limit the loss of enzyme activity. Limited in the attachment of one fluorophore to the γ-phosphate of ATP, various possible attachment sites at the core body of ATP are conceivable and should be evaluated. Although ATPases have been evolved towards binding to ATP, large differences in active site architectures within different families of ATPases can be found. Therefore, we focused to optimise the attachment site of the second fluorophore towards the family of kinases with the main interest in FAK. Aware of the most promising attachment sites, a synthesis strategy that allows obtaining doubly labelled ATP analogues of this type in a straight-forward manner should be established. Special attention should be given to the stability of the probe, the flexibility of the synthesis strategy in concern of sensor attachment, and to the chemical and fluorescent characteristics of the FRET cassette. After the synthesis of conceptually novel ATP analogues their concept and spectral properties should be investigated and evaluated. Finally, the novel doubly fluorescent dye labelled ATP analogues should be assayed facing actual issues in life sciences *in vitro* and *in vivo*.

Within the framework of this research the idea of a nucleotide based activity probe towards ATPases should be conceptualised, a straight-forward strategy of synthesis in line with all requirements should be established, the novel concept should be proven and evaluated to finally address prominent issues in life sciences.

Results and Discussion

Exploring sterical constraints of ATP modifications

One of the first questions emerging while trying to realize a concept of doubly dye labelled nucleotide probes is not only how, but especially where to add the relatively large constructs of a FRET cassette to an ATP molecule without diminishing the natural recognition of ATP by the ATPase of interest. Since there already exist several ATP probes bearing a fluorescent tag attached *via* a linker to the γ-phosphate of ATP[39,40,42,44,86-90] for the evaluation of enzyme activity, the attachment of one fluorophore to the γ-phosphate of ATP is not a limiting factor. Although ATPases have been evolved towards binding to natural ATP, the active site architectures within different families of ATPases have shown large differences.[2] Between same families of ATPases there may also exist differences in the active site architecture. Having this fact in mind, we focused on the evaluation of different linker attachment sites at the core body of ATP towards FAK and other kinases.

Therefore, the first goal within this study was to systematically explore the active site constraints of FAK using a set of ATP analogues that bear modifications at different positions within the nucleotide. In this way, fingerprints of site specific demands of different kinase binding sites can be obtained. Various attachment sites at the core body of ATP are possible. However, the crystal structure of FAK shows the *C*4'-position and the *C*2-position of ATP as highly suitable for modifications, because these two positions point out of the binding pocket and promise minimal interaction of the linker with the protein (Figure 15).

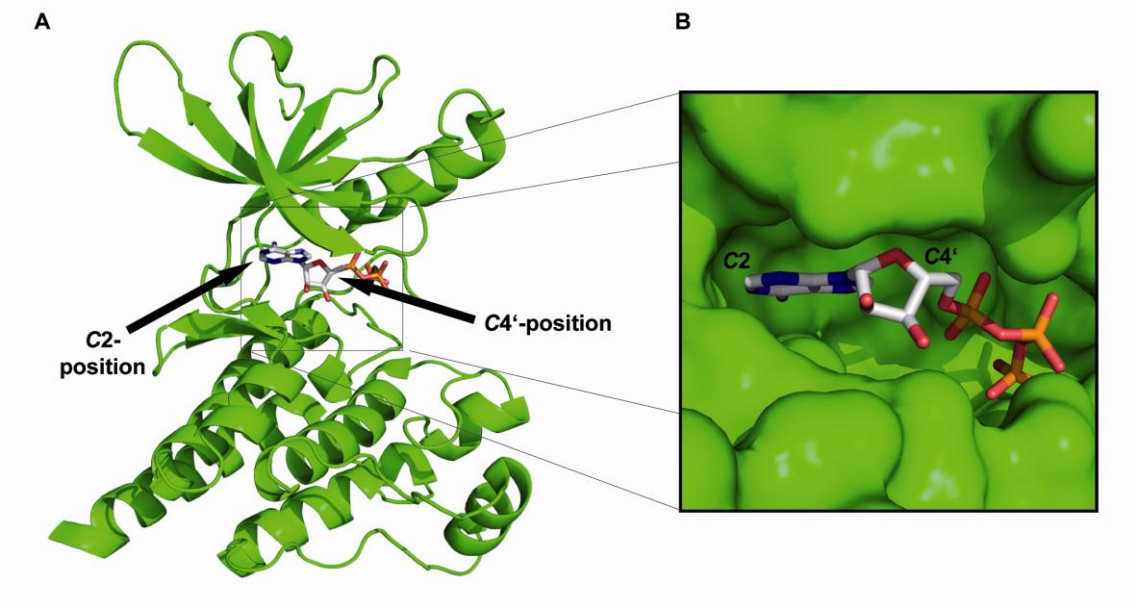


Figure 15: ATP binding to kinase domain of FAK. A) Kinase domain of FAK (green, Cartoon, PDB: 2IJM) with ATP (sticks) bound to the active site. *C*4'- and *C*2-positions are highlighted (black arrow). **B**) Close-up surface view of FAK (green, Connoly surface, PBB: 2IJM) with ATP (sticks) bound to the active site. *C*4'- and *C*2-positions are labelled.

Thus, ATP analogues with modifications at the *C*4'-position of the ribose and *C*2-position of the nucleobase were synthesised within the framework of this research. However, to systematically explore the whole space around ATP and in close cooperation with Stephan M. Hacker (S. M. Hacker, *Novel Fluorogenic Nucleotide Probes for Online Monitoring of ATP Consumption*, **2013**, Uni Konstanz) and the work of his dissertation it was possible to enlarge the scope up to six modified ATP analogues (**2**-**7**) and two autofluorescent ATP analogues (**8**,**9**) (Figure 16). Hence, the *O*2'- (**2**), *O*3'- (**3**) and *C*4'-position (**4**) at the ribose were chosen, since they were expected to interfere less with conformations of the glycosidic bond and the triphosphate in comparison to *C*1'- and *C*5'-modifications. Modifications at *C*2- (**5**), *N*6- (**6**) and *C*8-position (**7**) at the nucleobase were chosen, because the positions of the nitrogen atoms within the heterocycle are retained. The autofluorescent ATP analogues etheno-ATP (**8**) and azaetheno-ATP (**9**) represent interesting precursors concerning the concept of FRET based activity probes and were also tested towards their ability to act as co-substrate for kinases.

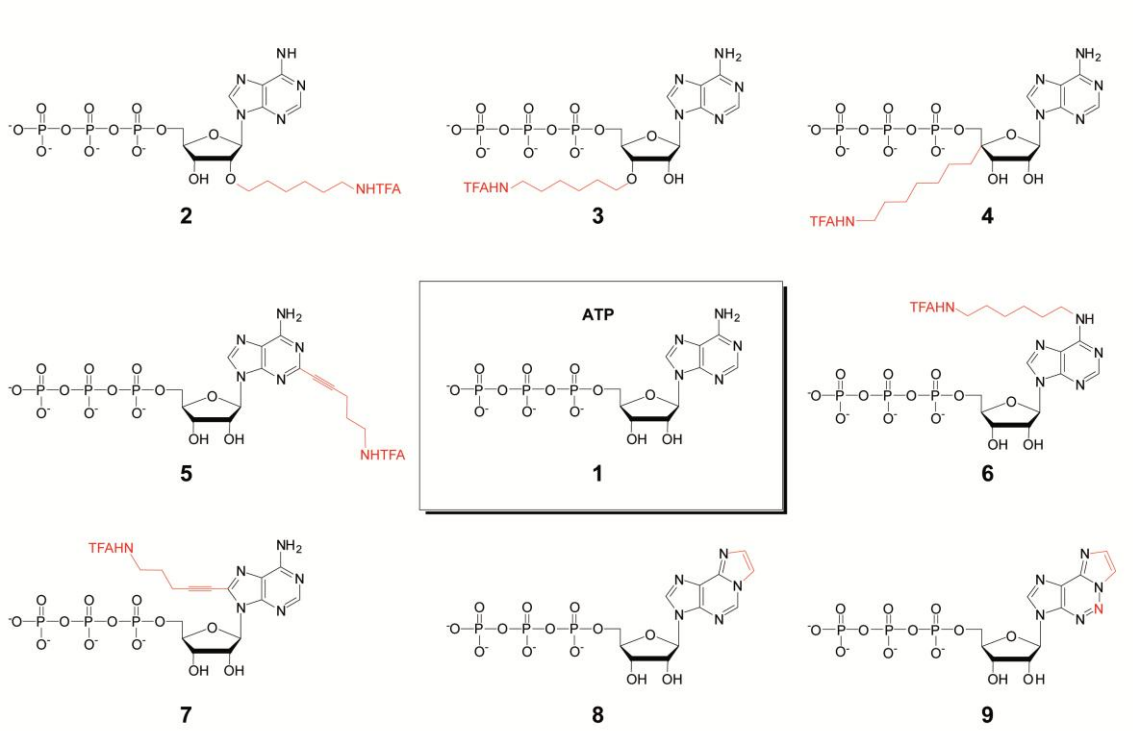


Figure 16: Natural ATP (1) and eight ATP analogues (2-9) used in this study. Analogues (**2-7**) bear a modification fused to a trifluoroacetamide group at three different positions of the nucleobase as well as at the *O*2'-, *O*3'- and *C*4'-posotion of the ribose, respectively. Analogues (**8-9**) represent the autofluorescent ATP analogues etheno-ATP (**8**) and azaetheno-ATP (**9**).

In general it was set out to synthesise analogues bearing an alkyl chain as linker with a trifluoroacetamide group at the end. This modification was used, since it acts as surrogate for any fluorescent dye, which should be attached later on. Moreover, it can be easily converted to the free amine by deprotection and afterwards used applying amine-reactive chemistry.

Synthesis of 4'-*C*-(7-(trifluoroacetamido)-heptyl)-adenosine-triphosphate (4)

A *C*4'-modified ATP analogue was obtained according to the investigated *de novo* synthesis of *C*4'-(7-(trifluoroacetamido)-heptyl)-modified ATP (**4**). The used approach to synthesise a 4-*C*-modified ribose derivative with subsequent conversion to a glycosyl

donor building block was explained earlier (See Introduction). This approach was chosen due to its suitability to synthesise non 2'-deoxy nucleoside derivatives and its securely established application in the laboratory of Prof. Dr. A. Marx. The synthesis of *C*4'-(7-(trifluoroacetamido)-heptyl)-modified ATP (**4**) starts with the *C*4-aldehyde activated building block (**10**). From a retrosynthetic point of view, this building block was synthesised according to Lomhold *et al.*[179] and Rangam *et al.*[96,180] in a six step synthesis starting from D-allofuranose. D-Allofuranose itself can be obtained from D-glucose according to Moffat *et al.*[52] in a three step synthesis (Figure 17). The aldehyde at the *C*4-position was converted to a modification containing the trifluoroacetamide moiety *via* Wittig reaction using triphenyl-(6-(trifluoroacetamido)-hexyl)-phosphonium iodide (**18**) resulting in the *C*4-position modified ribose building block (**11**) in 86% yield. Compound **11** is bearing a newly formed *cis* C=C double bond. The used Wittig salt **18** was prepared due to the reaction of *N*-trifluoroacetyl-(6-aminohexyl)-1-iodide (**17**) and triphenylphosphine refluxed in toluene in quantitative yield, whereas compound **17** could be prepared in a three step procedure according Noe *et al.* starting from 6-amino-1-hexanol.[181] Next, the previously formed C=C double bond and the benzyl protection group of **11** were removed using 10% Pd/C under H_2-atmosphere, to give compound **12** in quantitative yield. Subsequent protection group manipulations of **12** using AcOH/Ac_2O and catalytic amounts of H_2SO_4 yielded the acetyl protected glycosyl donor building block **13** in 84% yield. Glycosylation was done *via* the Vorbrüggen approach using *N*6-benzoyl protected adenine to give the protected *C*4'-modified adenosine nucleoside (**14**).[99] The protected adenosine bearing a modification at the *C*4'- position was obtained in 50% yield, whereas the main side product could be identified as *N*7-linked adenosine. By deprotection of the *O*5'-hydroxyl group of **14** using 1 M TBAF solution, compound **15** could be obtained in 63%. The partially deprotected **15** was then used for triphosphate synthesis following the approach of Kovács and Ötvös using an one-pot procedure to result in protected *C*4'-modified ATP (**16**) in 14% yield.[18]

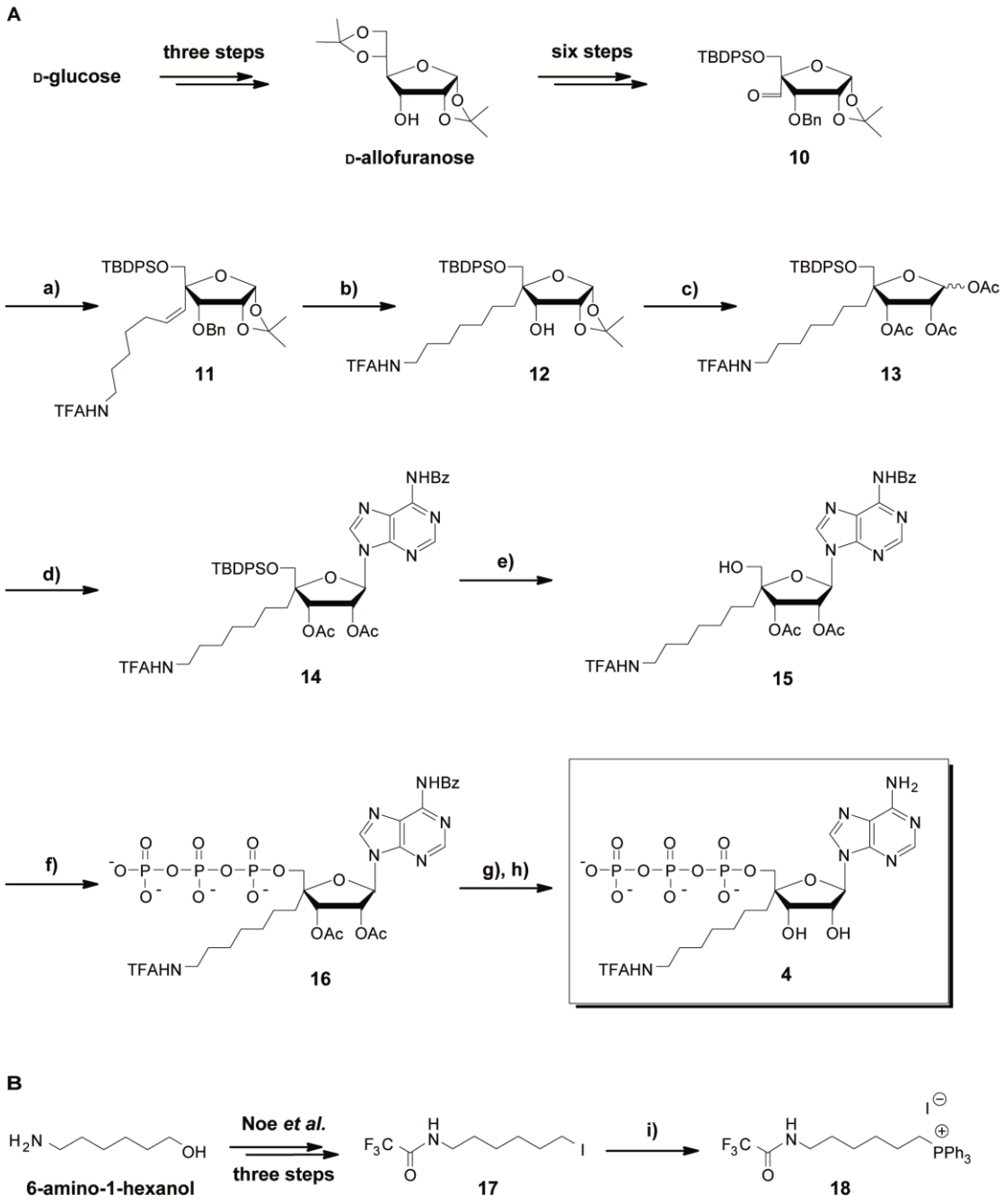
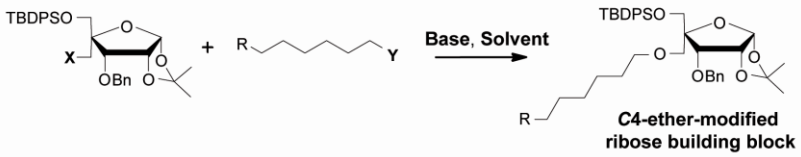


Figure 17: Synthesis of *C*4'-(7-(trifluoroacetamido)-heptyl)-modified ATP (4). A) Synthesis of ribose building block, glycosylation reaction and subsequent triphosphate synthesis. Compound **10** is prepared in a six step synthesis starting from D-allofuranose. a) Triphenyl-(6-(trifluoroacetamido)hexyl)-phosphonium iodide (**18**), *t*-BuOK, tetrahydrofuran (THF), r.t., 2 h, 86%. b) H_2/Pd, THF, r.t., 2 h, quantitative yield. c) AcOH/Ac_2O, r.t., 24 h, 84%. d) 1.) *N*6-Benzoyl-adenine, N,O-

bis(trimethylsilyl)acetamid (BSA), acetonitrile (ACN), reflux, 2 h, 0°C, 2.) Trimethylsilyl trifluoro methane sulfonate (TMSO-Tf), reflux, over night, 50%. e) Tetrabutylammonium fluoride (TBAF), AcOH, THF, r.t., 24 h, 63%. f) 1.) $POCl_3$, proton sponge, trimethyl phosphate (TMP), 0°C, 1 h, 2.) $(Bu_3NH^+)_2H_2P_2O_7^{2-}$, Bu_3N, r.t., 30 min, 3.) 0.1 M triethylammonium bicarbonate (TEAB) buffer, pH=7.5, r.t., 30 min, 14%. g) 33% NH_3 (aq.), r.t., 3.5 h. h) Ethyl trifluoroacetate, MeOH, NEt_3, r.t., 3 h, 30% over the last two steps. **B**) Synthesis of Wittig salt **18**. Compound **17** is prepared in a three step synthesis according to Noe *et al.* starting from 6-amino-1-hexanol. i) PPh_3, toluene, reflux, over night, quantitative yield.

Next, the resulting triphosphate **16** was deprotected using 33% $NH_{3(aq.)}/H_2O$ (1:2) to give *C*4'-(7-aminoheptyl)-modified adenosine triphasphate (**16i**). Intermediate **16i** was treated without further purification with ethyl trifluoroacetate to give the desired *C*4'-(7-(trifluoroacetamido)-heptyl) adenosine triphosphate (**4**) in 30% yield over the last two steps.

It is noteworthy, that the connection of the modification to the ribose building block due to the formation of a new C-C bond at the *C*4-position *via* Wittig reaction was done for the reason of stability. The formation of a C-C bond provides the advantage of a fairly stable connection of the introduced modification. A pH-dependent migration of modifications, as it is known for ester-modifications for example, is impossible. For that reason, linker attached *via* an ester bond formation was never a choice for the synthesis of a *C*4'-modified ATP analogue. In contrast, linker attached by ether bond formation was highly desired due to its similar mode of connection compared to linker-modified ATP analogue **2** and **3**. Attempts to introduce a linker to the *C*4-position *via* an ether bond, using nucleophilic substitution reactions, were not successful (Figure 18). In that context it is noteworthy to remind of the quaternary carbon centre in close proximity to the linker attachment site and the high sterical demand of neighbouring groups to the reactive centre at the ribose moiety.[51] These facts might play a major role for a possible explanation of the unlucky try to attach a linker to the *C*4-position *via* an ether bond. However, having a securely established method, fusing a linker to the *C*4-position *via* a new C-C bond formation, providing all mentioned advantages, no further investigations towards any other types of *C*4-linker connections were done.



X	R	Y	Base	Solvent	Yield [%]
OH	FmocHN	OTs	NaH	DMF	0
OH	FmocHN	OTs	KH	DMF	0
OH	FmocHN	OTs	*t*-BuOK	DMF	0
OH	FmocHN	OTs	NaH	THF	0
OH	N_3	I	NaH	THF	0
OH	N_3	Br	NaH	THF	0
OH	N_3	Br	*t*-BuOK	THF	0
OH	N_3	Br	DIPEA	THF	0
OH	N_3	Br	NEt_3	THF	0
OTf	N_3	OH	NaH	THF	0
OTf	N_3	OH	DIPEA	THF	0
OTf	N_3	OH	NEt_3	THF	0
OTf	N_3	OH	DBN	THF	0
OTf	N_3	OH	NaH	DMF	0
OTf	N_3	OH	*t*-BuOK	DMF	0
OTf	MeOH		NaH	DMF	0

Figure 18: Attempts to synthesise *C*4-ether-modified ribose building block *via* substitution reactions. Substitution reactions were performed under various conditions (see table). Variations of the ribose (X= reactive group), the linker (R= protected amine, Y= reactive group), the base and the solvent used are listed.

In conclusion, the novel *C*4'-(7-(trifluoroacetamido)-heptyl) adenosine triphosphate (**4**) could be synthesised *de novo* elaborating a 14 step synthesis with an overall yield of 0.7%. The novel *C*4'-(7-(trifluoroacetamido)-heptyl) adenosine triphosphate (**4**) was now available to further use and evaluate active sites of kinases to their sterical constraints.

Synthesis of *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate (5)

To attach a modification to the C2-position of the nucleobase, 2-iodo-adenosine was synthesised in a four step synthesis according to Engels and coworkers, starting from guanosine.[126] A Sonogashira coupling reaction was performed with 5-trifluoroacetamido-pentyne, tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) and copper iodine to give *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-adenosine (**19**) in 94% of yield. *C*2-(5-Trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate (**5**) was obtained in 40% yield, following the approach of Kovács and Ötvös (Figure 19).[18]

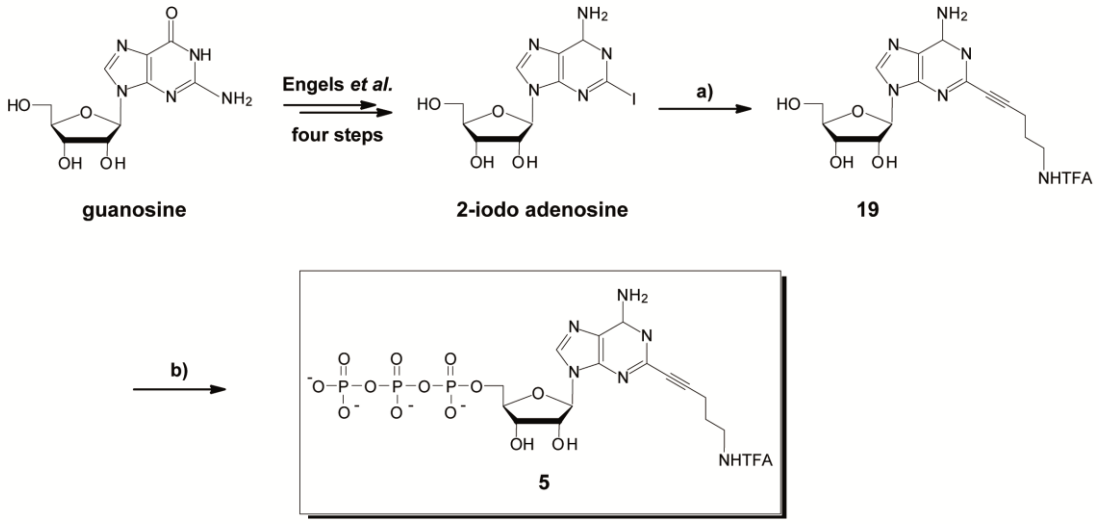


Figure 19: Synthesis of *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate (5). Synthesis of 2-iodo adenosine was done *via* Engels *et al.* in a four step synthesis. a) 5-Trifluoroacetamido-pentyne, $Pd(PPh_3)_4$, CuI, NEt_3, N,N'-dimethylformamide (DMF), r.t., 24 h, 94%. b) 1.) $POCl_3$, proton sponge, TMP, 0°C, 1 h, 2.) $(Bu_3NH^+)_2H_2P_2O_7^{2-}$, Bu_3N, r.t., 30 min. 3.) 0.1 M TEAB buffer, pH = 7.5, r.t., 30 min., 40%.

Finally, the novel *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate (**5**) could be synthesised in a six step synthesis with an overall yield of 2.0%. The novel *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate (**5**) could now be used for further investigations of the active sites of kinases to their sterical constraints.

Fingerprinting differential active site constrains of FAK and other kinases

During the course of this research, six novel trifluoroacetamide modified triphosphates (**2**-**7**) and two autofluorescent ATP analogues (**8**, **9**) were available to investigate their acceptance as co-substrates by different kinases. The approximately 500 protein kinases encoded in the human genome comprise one of the largest protein families and together control virtually every cellular process.[2,140] Protein kinases catalyze the transfer of the γ-phosphate group of ATP onto their substrate proteins, whereas this reversible modification often serves as a switch to change the functionality of the substrate protein.[2,140-142] In multicellular organisms the subset of protein tyrosine kinases (PTKs), that transfer the γ-phosphate to tyrosine residues of their substrates, hold a key role in signal transduction, regulating cell proliferation and other cellular behaviour.[2,143,144] As mentioned before, deregulated PTK activity is often causally involved in diseases, such as cancer, making this class of enzymes a prime target for therapeutics.[2]

Within the scope of this research five kinases were investigated towards their ability to use eight differently modified ATP analogues (**2**-**9**) as co-substrate in order to asure optimal positions for following modifications regarding the desing of doubly dye modified ATP probes. Since within the same family of ATPases, there exist differences within the active site architecture, all ATP analogues were evaluated towards FAK and four other kinases.[2] The additional four kinases were chosen to cover the most prominent kinase families like the tyrosine kinase family represented by FAK and Abl; Ca^{2+}/calmodulin-dependent protein kinase family represented by CaMKII; Sterile 20 serine/threonine kinase family covered by p21-activated kinase 1 (PAK1) and the cyclin-dependent kinase family with casein kinase II (CKII) as representative.

For investigation of the ability of the modified ATP analogues (**2**-**9**) to act as co-substrates, an *in vitro* enzyme-linked immunosorbent assay (ELISA) was used. The assay was investigated and established for each class of kinases based on the enzyme and a model substrate containing the unique target peptide sequence (Figure 20 A). Therefore, a surrogate kinase substrate peptide for each kinase (see experimental part) was immobilized to a 96-well plate. One of the eight modified ATP analogues (**2**-

9) or ATP (**1**) respectively was added to the substrate either with or without kinase and incubated at 37°C. Phosphorylation of the immobilized substrate was detected by a phosphotyrosine- or serine/threonine-specific antibody, in combination with a secondary antibody coupled to horseradish-peroxidase (HRP). HRP activity was measured by the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) and its specific colorimetric response to determine the kinase-mediated substrate phosphorylation.

These activity screenings investigated the ability of the kinase to utilize ATP analogues. To ensure that under these conditions the enzyme activity is still in the linear range, a time-dependent study was performed (Figure 20B). A linear increase of the target substrate phosphorylation can be seen in this experiment for a selected choice of active ATP analogues allowing quantification of the substrate properties of the different ATP analogues.

The results of the *in vitro* assays show that the evaluated kinases possess defined preferences towards the modified ATP analogues (**2**-**9**). The results also show significant differences between different kinase family members. This is an interesting fact, since the active site of kinases is considered to be highly conserved among all kinase family members (Figure 20B-H).[2] However, our results indicated that changes in the direction of the attached modification directly influence the ability of the modified ATP analogue to act as co-substrate for a specific kinase of interest.

In the case of FAK, modifications at *O*2'-position (**2**), *O*3'-position (**3**) and *C*2-position (**5**) of ATP are tolerated best (Figure 20C). While *O*2'-modified ATP (**2**) shows about 70% of the phosphorylation activity of natural ATP, the *O*3'-modified analogue (**3**) shows only about 40%. Interestingly, using the ATP analogue bearing a 5-(pent-1-yn-1-yl)-trifluoroacetamide at the *C*2-position (**5**) almost 90% of the kinase activity observed with natural ATP can be seen. In contrast, the *C*4'-modified (**4**), *N*6-modified (**6**), and *C*8-modified (**7**) analogues are not used as a co-substrate by FAK. To create a highly potential doubly dye labelled ATP analogue to act as co-substrate for FAK, either the *O*2'-position, *O*3'-position or *C*2-position respectively should be considered to attach a linker. It might also be interesting to have an inactive ATP analogue available, which can be used specifically by one single enzyme due to enzymatic engineering, for example.

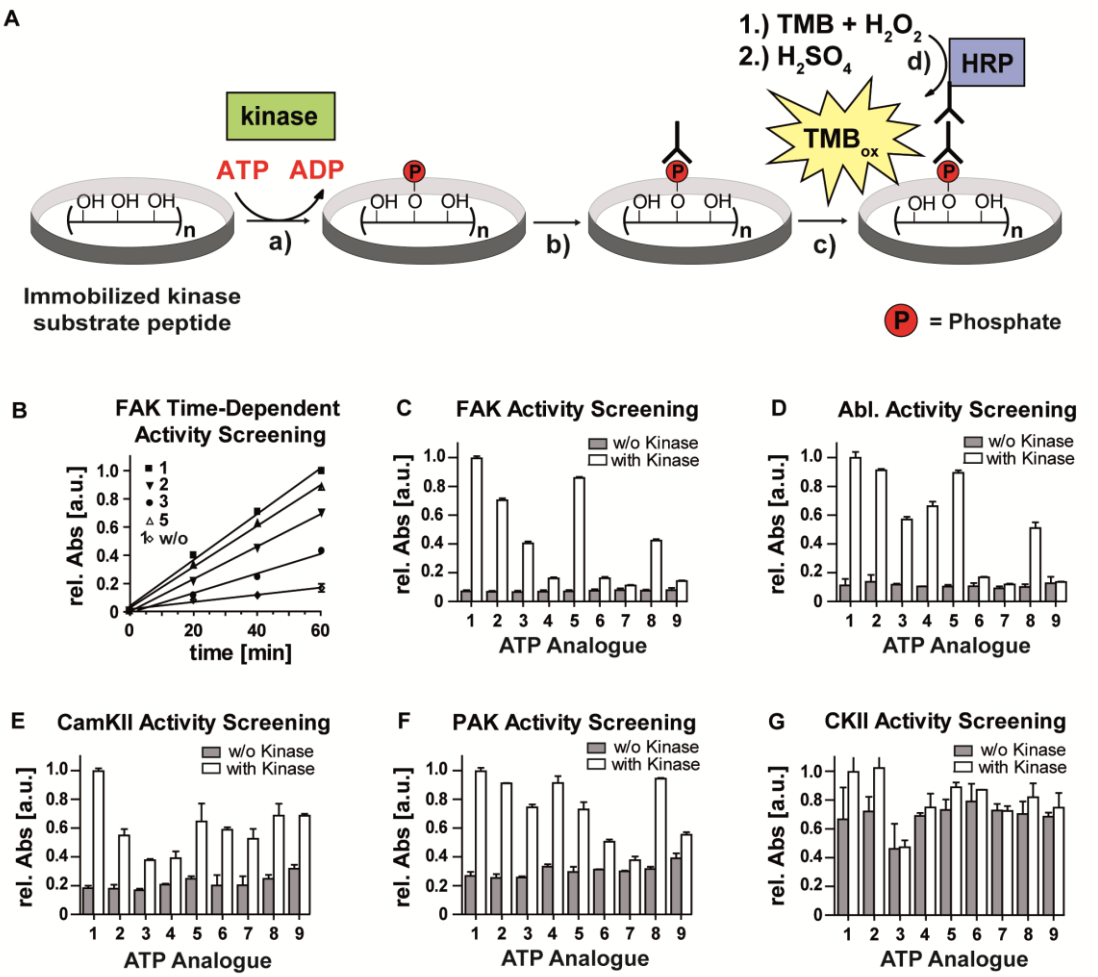


Figure 20: ELISA activity screening of natural ATP (1) and eight ATP analogues (2-9) towards a selection of kinases. A) Schematic depiction of the *in vitro* kinase assay using modified ATP analogues. a) Incubation of immobilized, peptide substrate, kinase and the ATP of interest at 37°C. b) Immobilized substrate is incubated with anti-phosphotyrosine or anit-phosphothreonine/serine antibody. c) The primary antibody is detected by a HRP-conjugated secondary antibody. d) HRP oxidizes TMB to its oxidated form TMB_{ox}. The reaction was quenched after 3 min with 2 M H_2SO_4 and the absorption of TMB_{ox} was measured at 450 nm. **B**) Absorption of TMB_{ox} in the reaction solutions of the indicated ATP analogue after incubation for indicated time intervals. The absorptions were normalized to incubation with unmodified ATP (**1**) for 1 h. Values represent mean ± SEM of three independent experiments. Negative controls were performed without ATP. The *O*2'-modified (**2**), *O*3'-modified (**3**) and *C*2-modified (**5**) analogues can clearly serve as a co-substrate for FAK. **C-H**) Absorption of TMB_{ox} in the reaction solutions of the indicated kinase and ATP analogue after 1 h of incubation. The absorptions were normalized to unmodified ATP (**1**). Negative controls were performed without addition of the kinase. Values represent mean ± SEM of three independent experiments.

In case that it is desirable to have a specific ATP analogue for a specific enzyme, the *C*4'-modified (**4**), *N*6-modified (**6**), and *C*8-modified (**7**) analogues would be interesting choices regarding directed mutagenesis in the case of FAK. Since the concept of a nucleotide based activity probe is based on FRET, auto fluorescent analogues of ATP were also investigated towards their activity. Interestingly, in the case of etheno-ATP (**8**) and azaetheno-ATP (**9**) a clear discrimination between these two analogues can be observed. Whereas azaetheno-ATP (**9**) is clearly not accepted by FAK as co-substrate, etheno-ATP (**8**) shows an activity of 42% compared to natural ATP, commending itself for further investigations. The non natural nitrogen atom at the *C*3-position (**9**) clearly influences and decreases the recognition of the probe as co-substrate by FAK.

Next, these results were compared to other kinases and kinase families. Hence the eight ATP analogues (**2**-**9**) were tested towards their activity in the case of Abl protein tyrosine kinase (Abl). Since Abl and FAK belong to the same PTK kinase family[152,155,158,182-184], Abl is perfectly suited to further investigate tyrosine phosphorylating kinases, since the assay was optimised for PTKs. Abl is a truncated form of the v-Abl PTK, a partner in the Gag-Abl fusion protein of the Abelson murine leukemia virus and contains 407 amino acids, which include the kinase catalytic domain, SH2 domain on the N-terminus and the I237M mutation.[182-184] Interestingly, Abl shows a similar pattern of activity towards the ATP analogues compared to FAK (Figure 20D). Modifications at *O*2'-position (**2**), *O*3'-position (**3**) and *C*2-position (**5**) of ATP were also accepted very well with the same preferences. However, the general acceptance of these modifications is increased compared to FAK. This result can be explained, since FAK and Abl both belong to the same kinase family of PTKs. The activity of the *O*2'-modified ATP (**2**) is clearly increased up to 90% of the activity of natural ATP (**1**) and as well accepted as the *C*2-modified ATP (**5**). The *O*2'- and *C*2-position are obviously the best attachment site for a modification in the case of Abl. Nevertheless, slight differences can be observed in the specific activity of the ATP analogues between both PTKs, the *C*4'-modified (**4**) analogue which is totally inactive in the case of FAK, is well accepted in the case of Abl. This fact indicates the main difference of both activity patterns. *N*6-modified (**6**), and *C*8-modified (**7**) analogues are neither used as a co-substrate by Abl. In the case of the two auto fluorescent

analogues, etheno-ATP (**8**) turned out to be as well accepted as co-substrate with slightly increased activity compared to FAK.

Next, the eight differently modified analogues were evaluated towards the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII belongs to the family of Ca^{2+}/calmodulin-dependent protein kinases and is a serine/threonine specific protein kinase.[185] To research on serine/threonine specific enzymes the ELISA had to be elaborated and optimised towards this class of kinases. Therefore, optimisation regarding the choice of substrate and the corresponding pair of antibodies was done. It is known that antibodies recognizing phosphoserine/threonine are less specific than phosphotyrosine antibodies which results in a general increase of the background signal. However, it was still possible to evaluate preferences towards the activity of the ATP analogues with this class of kinases. CaMKII shows a very different activity pattern and no clear discrimination of the ATP analogues (**2**-**9**) can be seen (Figure 20E). However, nucleobase modified ATPs like the *C*2-modified (**5**), *N*6-modified (**6**), *C*8-modified (**7**) ATP as well as both auto fluorescent analogues are slightly better accepted as co-substrates by CaMKII. The activity of nucleobase modified ATP analogues (**2**-**9**) varies from 60% to 70% compared to natural ATP (**1**). Ribose modified ATP analogues with modifications at *O*2'-position (**2**), *O*3'-position (**3**) and *C*4'-position (**4**) are less accepted but still act as co-substrate for CaMKII with activities from 40% to 60% compared to **1**. Altogether CaMKII shows a very wide spectrum of possible modification sites with a preference to nucleobase modifications. However, the activity was limited to 70% compared to natural ATP.

Furthermore, p21-activated kinase 1 (PAK1) was investigated. PAK1 represents a serine/threonine protein kinase belonging to the sterile 20 serine/threonine kinase family. PAK1 is a signaling kinase that interacts with GTP-bound but not GDP-bound CDC42/P21 and RAC1 to modulate cytoskeletal reorganization.[186] PAK1 shows a very interesting activity pattern with a wide spectrum of acceptance (Figure 20F). In contrast to CaMKII ribose modified ATP analogues are preferred as co-substrate. Ribose modified ATP analogues with modifications at *O*2'-position (**2**), *O*3'-position (**3**) and *C*4'-position (**4**) are very well tolerated as co-substrate for PAK1 with activities of 80% to 95% compared to ATP (**1**). *C*4'-modified ATP (**4**) and *O*2'-modified ATP (**2**) serve best as co-substrate commending their self for building blocks for activity probes

towards PAK1. *N*6-modified (**6**) and *C*8-modified (**7**) ATP show minimal activity. Etheno-ATP (**8**) is also accepted as co-substrate with activity comparable to natural ATP. In sum, PAK1 accepts almost all modifications with high activities comparable to natural ATP except of *N*6-modified (**6**) and *C*8-modified (**7**).

Last, the casein kinase II (CK2), a member of the cyclin-dependent kinase family was investigated which is a constitutively active serine/threonine protein kinase composed of two 44 kDa catalytic α-subunits and two 26 kDa regulatory β-subunits in a α_2 β_2 configuration. CK2 holoenzyme undergoes auto phosphorylation at two serine residues of its β-subunit. Recently, it has been shown that the CK2 α-subunit undergoes intermolecular tyrosine-autophosphorylation at Y182, which may represent a specific regulatory mechanism.[187,188] In the case of CK2 conclusions about activity of modified ATP analogues are not possible. Because false positive background signals occurre in all measurements, any conclusions regarding CK2 are not valid (Figure 20G).

In conclusion, six novel trifluoroacetamide modified triphosphates (**2**-**7**) and two autofluorescent ATP analogues (**8, 9**) were successfully evaluated to their potential to act as co-substrate towards four different kinases out of three different kinase families. Special effort was put into the concrete attachment site of modification in order to create a highly potential active nucleotide activity probe with two fluorophores attached. One can highlight the attachment site at the *O*2'-position (**2**) and *C*2-position (**5**) of ATP in the case of Protein Tyrosine Kinases FAK and Abl commending themselves for further investigations. In the case of CaMKII a clear preference of nucleobase modified ATP analogues can be seen. Considering the creation of an activity probe with an auto fluorescent ATP analogue, ehteno-ATP (**8**) would be the best choice for all four studied kinases.

Strategy towards the synthesis of doubly dye labelled ATP analogues

After investigation of the optimal position for the attachment of the second linker for the kinase of interest, it was fundamental to elaborate a synthesis strategy towards the most promising doubly dye labelled ATP analogues.

In general, the strategy should be applicable for the synthesis of any stable doubly labelled ATP activity probe of choice, and it should be easily performable with high yields. The strategy should be highly variable regarding the attachment of the sensor molecules, meaning that their introduction should happen on the very last steps of the synthesis. The strategy has to be performed under mild conditions to eliminate the risk of any damage to labile sensor molecules or the triphosphate itself. Moreover, the synthesis has to be performed under aqueous conditions generally required in triphosphate chemistry.

In line with all these requirements, several synthesis strategies were elaborated and evaluated. The steps of triphosphate synthesis and the attachment of the linker to the γ-phosphate were crucial to the success of the overall strategy, since they generally represent the most critical steps regarding yield. As a consequence the very first synthesis strategy tried to combine these two steps into a single one. A nucleoside triphosphate should be synthesised and the formed nucleoside *meta*-triphosphate intermediate should directly be converted to the corresponding γ-labelled triphosphate *via* a direct ring opening reaction as mentioned earlier. Afterwards, the sensor molecules had to be introduced using an orthogonal attachment strategy. Therefore, easily applicable *N*-hydroxysuccinimide (NHS)-ester chemistry was used since the second linker of the ATP analogues already bears a protected amide group. For the second sensor the Huisgen cycloaddition was used due to its elaborated application under aqueous conditions.[189]

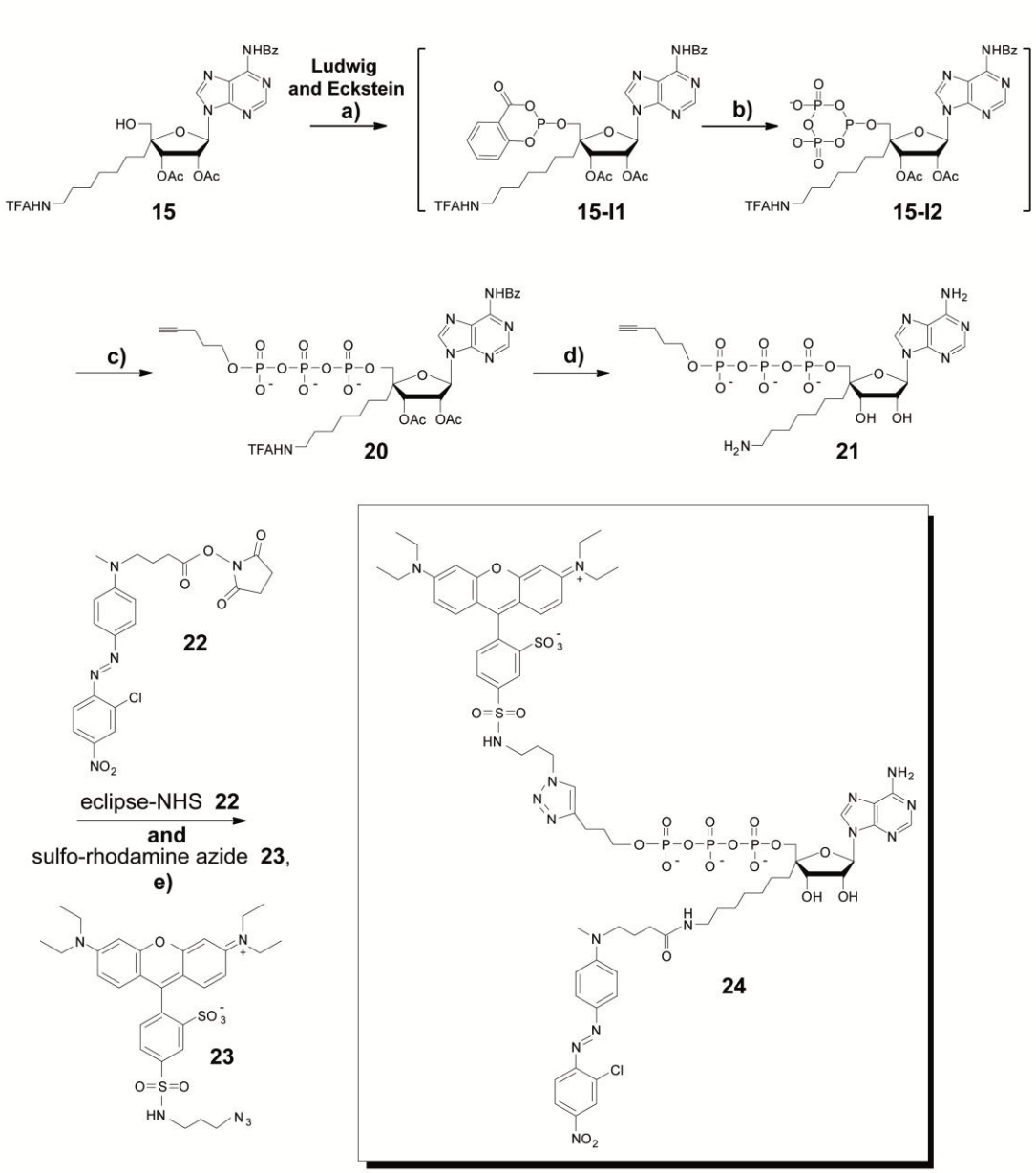


Figure 21: Synthesis of the first doubly dye labelled ATP analogue γ-(sulfo-rhodamine)-4'-*C*-(7-(eclipse)-heptyl)-adenosine-triphosphate (24). a) 2-Chloro-1,3,2-benzodioxa-phosphorin-4-one, pyridine (pyr), dioxane, 0°C, 1 h. b) $(Bu_3NH^+)_2H_2P_2O_7^{2-}$, Bu_3N, r.t., 10 min. c) 1.) 4-Pentyn-1-ol, r. t., 24 h, 2.) 2% I_2 in pyr/H_2O (1:1), r.t., 14 h, 1,5%. d) 33% NH_3 aq., 50°C, 24 h, 87%. e) Eclipse-NHS (**22**), sulfo-rhodamine azide (**23**), ACN, DMF, diisopropylethylamin (DIPEA), CuI, r.t., 24 h.

The synthesis of the first doubly dye labelled ATP analogue γ-(sulfo-rhodamine)-4'-C-(7-(eclipse)-heptyl)-adenosine-triphosphate (**24**) starts with the protected nucleoside 2'-*O*-acetyl-3'-*O*-acetyl-5'-*O*-hydroxy-4'-*C*-(7-(trifluoroacetamido)-heptyl)-*N*6-benzoyl-

adenosine (**15**). Nucleoside **15** was activated with 2-chloro-1,3,2-benzodioxaphosphorin-4-one following the Ludwig-Eckstein approach for triphosphate synthesis of protected nucleosides. The activated monophosphite (**15-I1**) was reacted *in situ* with pyrophosphate and tributylamine to the corresponding compound (**15-I2**) (Figure 21). Next, intermediate was converted in a ring opening reaction using 4-pentyn-1-ol first and subsequently treated with aqueous iodine solution to yield the γ-modified triphosphate (**20**) in 1.5% while the unmodified triphosphate (**16**) was obtained in 16% within the same reaction. These results clearly show that a ring opening reaction at **15-I2** using primary alcohols is not reacting as well as using water, resulting in bad yields even after 24 hours of reaction time. Ring opening reactions at **15-I2** were also performed with primary amines with slightly increased yields. However, in contrast to already existing studies of phosphoramide-linked triphosphates they turned out to be unstable under various pH conditions and therefore useless to the purpose of creating an activity probe. These findings were further investigated by Hacker *et al.* and could be confirmed.[50] However, next the γ-modified triphosphate **20** was completely deprotected using aqueous ammonia giving γ-modified triphosphate (**21**) in 87% yield. Doubly linker modified triphosphate **21** was then converted in a one pot reaction with the activated NHS-ester of the eclipse-quencher (**22**) and an azide functionalised sulforhodamine dye (**23**). This one pot reaction was performed in a mixture of methanol, DMF, ACN (2 µL:100 µL:100 µL) and DIPEA to give the doubly dye labelled dark quenched ATP analogue γ-(sulfo-rhodamine)-4'-*C*-(7-(eclipse)-heptyl)-adenosine-triphosphate (**24**). Using this synthesis strategy the doubly dye labelled dark quenched ATP analogue **24** could be obtained in traces only. This synthesis strategy has two major drawbacks; the synthesis of the γ-modified triphosphate *via* a direct ring-opening reaction and the one pot reaction to attach two dyes at once. Both reactions could only be performed in moderate to low yields. An alternative synthesis strategy towards doubly sensor molecule labelled ATP analogues was strongly needed.

In conclusion, the first and conceptually novel doubly dye labelled nucleotide based activity probe could be synthesised *de novo* in a total 14 step synthesis and was not only of great value for basic research but opened up novel avenues for subsequent synthesis strategies towards doubly labelled ATP analogues.

One of the main challenges that had to be solved was the low yield of a direct ring opening reaction of the *meta*-triphosphate since this reaction strongly affects the overall yield of the synthesis of doubly dye labelled ATP analogues. Therefore, the synthesis of doubly modified ATP analogues had to be optimised. For this purpose, two alternatives to γ-modify an ATP were investigated. One approach focused on the synthesis of a pre-modified pyrophosphate analogue, which should be used for triphosphate synthesis later on (Figure 22A). However, this approach turned out to be uneconomic, since the pre-modified pyrophosphate had to be used in high excess in the following triphosphate reaction. The second approach was focused on taking advantage of the nucleophilic character of the triphosphate chain itself by converting the triphosphate with an electrophile in a nuclephilic substitution reaction to the corresponding γ-modified triphosphate (Figure 22B).

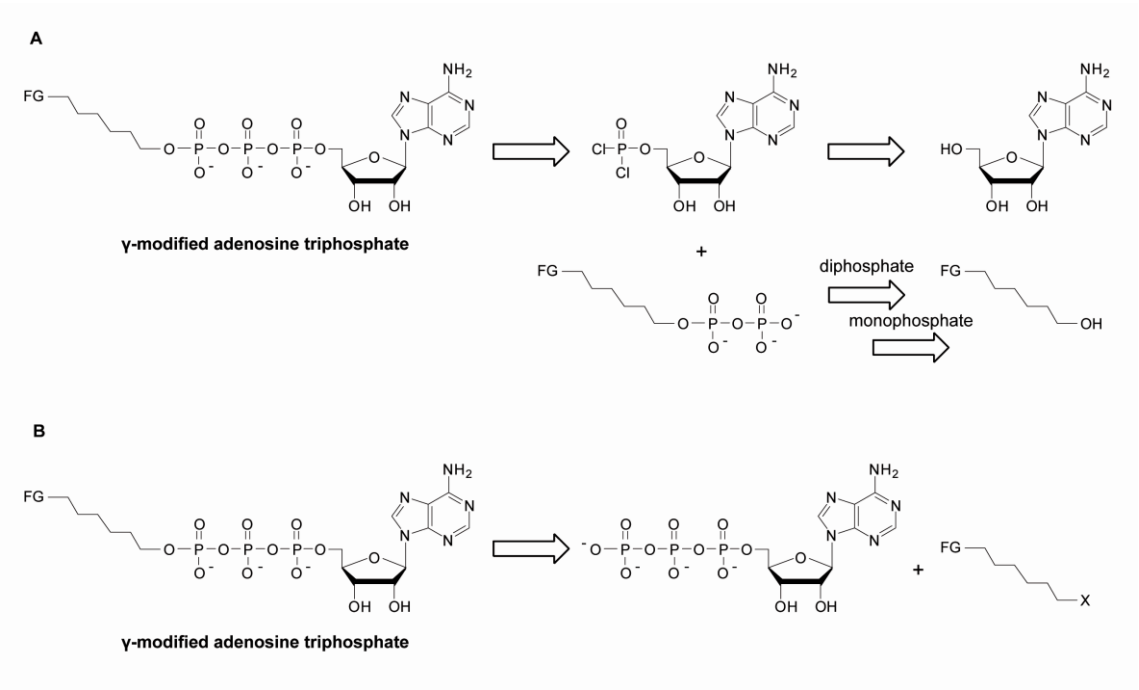


Figure 22: Retrosynthetic scheme of the two main attempts to optimise the synthesis of γ-modified triphosphates. A) A functionalised linker should be transferred to the corresponding monophosphate and diphosphate later on, to substitute pyrophosphate used in excess in a common triphosphate synthesis with an activated species of a nucleoside monophosphate. **B**) The nucleophilic γ-phosphate undergoes a nucleophilic substitution reaction with a functionalised linker molecule bearing a leaving group to form a γ-modified triphosphates. FG= functional group, X= leaving group.

This way resulted to be quite effective in turning any triphosphate into a γ-modified triphosphate and opened up an elegant approach towards doubly modified ATP analogues in good yields.[50]

Next, the attachment strategy of the sensor molecules had to be improved since the one pot reaction including NHS- and click-chemistry turned out to give bad yields. To obtain a maximum degree of freedom for choosing the best sensor molecules, it was decided to synthesise the doubly linker modified ATP scaffold with two protected amines and to introduce the sensors at the end of the synthesis sequence *via* NHS-ester chemistry, which is well performable under aqueous conditions and giving the corresponding products in good yields (Figure 23).

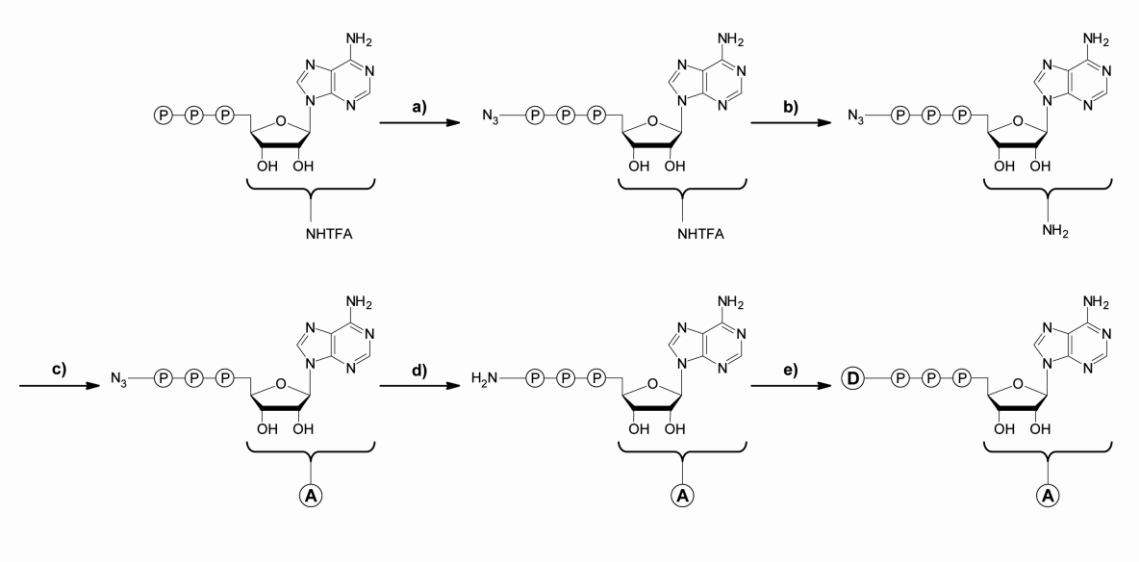


Figure 23: Concept of the optimised synthesis strategy towards doubly dye labelled ATP analogues. a) Nucleophilic substitution reaction with an azide functionalised linker to result in a doubly modified ATP analogue. b) Deprotection of the trifluoroacetamide protected amine under basic conditions. c) Attachment of the acceptor dye *via* NHS-ester chemistry. d) Reduction of the azide to the corresponding amine group under mild conditions *via* Staudinger reduction. e) Attachment of the donor dye *via* NHS-ester chemistry to result in any doubly dye labelled ATP analogue of choice. A= acceptor; D= donor.

For this purpose, two orthogonal amine protection groups are needed. One of these has to be deprotected in the presence of the first dye. Therefore, this deprotection reaction had to be as mild as possible. For that reason a trifluoroacetamide and an azide were used. The synthesis of trifluoroacetamide modified triphosphates was

already well elaborated.[2] First, the trifluoroacetamide can be deprotected under alkaline conditions and coupled with the first label. This leaves the azide completely intact. Finally, the azide can be reduced and coupled to the second label. This Staudinger reduction can be performed using very mild conditions that are tolerated by a large selection of fluorescent dyes. In this work, mainly polymethine dyes and the eclipse-quencher are used as they showed to be very well suited for FRET applications at short donor acceptor distances as used in this work.[190] This synthesis strategy can be performed under aqueous and mild conditions and therefore opens an elegant way of synthesis towards any doubly labelled ATP analogue in high yields.

Synthesis of novel doubly dye labelled ATP analogue 29a, 29b and 29c

According to the optimised synthesis strategy, three novel doubly dye labelled ATP analogues were synthesised. Thereby special interest was given to the *C*2-modified doubly dye labelled analogues because of the high acceptance of *C*2-modified ATP by FAK and other kinases.

The synthesis of these analogues is starting from the already described precursor *C*2-(5-trifluoroacetamidopentynyl)-adenosine triphosphate (**5**)[2], that was synthesised *via* a seven step synthesis starting from guanosine (Figure 23). The modification of the triphosphate at the γ-position was introduced by treatment with 1-bromo-6-azido-hexane to give the doubly linker modified triphosphate (**25**) in 70% yield. Subsequently, the trifluoroacetamide protection group was cleaved by ammonolysis to generate the free amine in compound **26** in 53% yield. This analogue already bears the first free amine ready for conjugation as well as the azide that can be used to form the second amine later on.

Next, the doubly modified ATP analogue was labelled with different combinations of dyes. First the combination of Sulfo-Cy3 and Sulfo-Cy5 was synthesised, since this FRET cassette provides good solubility in aqueous conditions and good spectral properties at close distances.[190] Therefore, compound **26** was coupled with the Sulfo-Cy5 NHS ester in a mixture of DMF and sodium carbonate buffer at pH 8.9 to give compound **27a** in

58% yield. Next, the Staudinger reduction of the azide was performed using TCEP in water, methanol and triethylamine at room temperature to give compound **28a** in 66% yield. This was followed by conjugation of the Sulfo-Cy5 NHS ester in 86% yield to give the final doubly labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29a**.

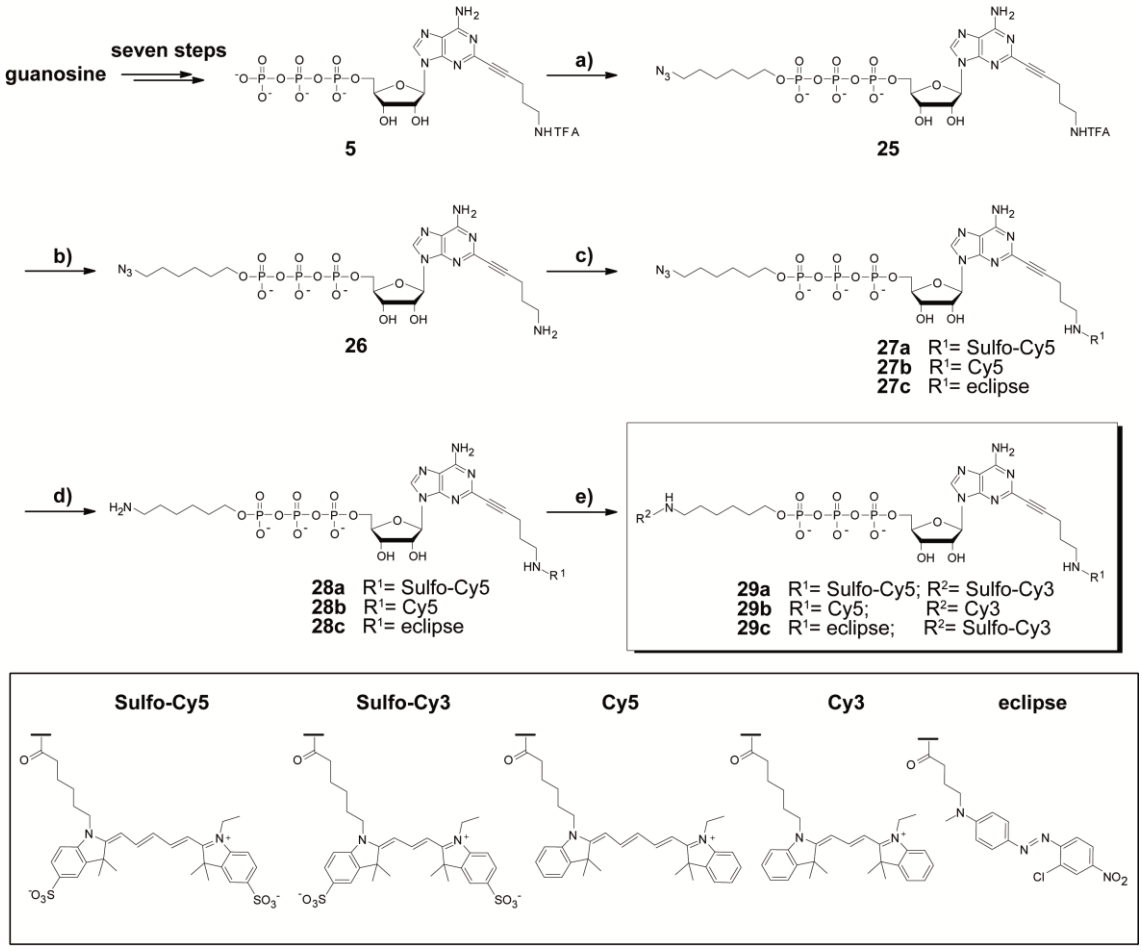


Figure 23: Synthesis of three novel doubly dye labelled ATP analogues 29a, 29b and 29c. a) 6-Azido-1-bromohexane, molecular sieves, DMF, r.t., 12 h, 70%. b) Ammonia in water, r.t., 2 h, 53%. c) Sulfo-Cy5-NHS ester/Cy5-NHS ester/eclipse-NHS ester, DMF, carbonate buffer (pH 8.9), r.t., 12 h, 58%/47%/26%. d) tris-(2-carboxyethyl)-phosphine (TCEP)-HCl, Et_3N, methanol, water, r.t., 4 h, 66%/89%/57%. e) Sulfo-Cy3-NHS ester/Cy3-NHS ester, DMF, carbonate buffer (pH 8.9), r.t., 12 h, 86%/42%/80%. Structures of attached dyes are named and depicted in the box.

Finally, the novel doubly dye labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29a** could be synthesised over eleven steps with an overall yield of 0.2%.

In the context of this work using this synthesis protocol, it was possible to attach two further combinations of dyes to compound **26**.

In this way the novel doubly dye labelled ATP analogue γ-(6-cyanine3-amindohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29b** could be synthesised in an eleven multi step synthesis with an overall yield of 0.1%.

The novel dark quenched doubly labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate **29c** could be synthesised in a synthesis sequence of eleven steps with an overall yield of 0.1%.

Hence, three different doubly labelled ATP analogues (**29a**, **29b** and **29c**) with different properties were obtained in very good yields according to triphosphate chemistry. γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29a** provides an excellent solubility in water promoting its usage in buffers for example. On the other hand γ-(6-cyanine3-amindohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29b** is very unpolar; this fact may be very interesting regarding cell permeability for further applications. A slightly different spectral concept is provided by the dark quenched doubly labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate **29c**, in an intact state of the probe no fluorescence can be seen, only after cleavage fluorescence arises at specific wavelength of 570 nm, creating interesting possibilities of applications. The three novel nucleotide activity probes were subsequently investigated on their fluorescence characteristics.

Fluorescent characteristics of doubly dye labelled ATP analogues

To proove the concept of doubly dye labelled ATP analogues, investigation of the fluorescence characteristics was crucial. Hence a model system being able to

completely cleave these ATP activity probes was needed. The snake venom phosphodiesterase from *C. adamanteus* (SVPD) is well known to cleave phosphodiester bonds in a quite efficient way and therefore presented an ideal model enzyme system to study the cleavage of the ATP based activity probes and to investigate their fluorescent characteristics during this process.[2,32,39,41,42,88] All ATP analogues were incubated with or without SVPD at 30°C for 30 minutes, until complete cleavage was observed (Figure 24A, 25A, 26A). The completion of cleavage was monitored by analytical reversed phase high pressure liquid chromatography (RP-HPLC) and is depicted in a two dimensional plot of absorbance and time (Figure 24B, 25B, 26B). Fragments of negative and positive control were identified by high resolution mass spectrometry (HRMS). Furthermore, all reaction samples were diluted to 1 µM with 1 x phosphate buffered saline (PBS) buffer and fluorescent spectra were measured (Figure 24C, 25C, 26C).[32] ATP analogue **29a** and **29b** bear a combination of fluorescent donor and fluorescent acceptor. These two FRET probes have the advantage that fluorescence intensity changes at the two specific wavelengths of the fluorophores. Therefore, analysis of the ratio of the two fluorescence intensities enables monitoring the cleavage of the ATP analogue, independently of concentrations.[32] ATP analogue **29c** containing a combination of Sulfo-Cy3 and eclipse quencher is perfectly soluble in water. Upon hydrolysis, the fluorescence intensity of Sulfo-Cy3 arises and makes this probe a very valuable tool to study ATP cleavage as well.[32]

Compound **29a** with its combination of Sulfo-Cy3 and Sulfo-Cy5 was investigated first (Figure 24). Upon cleavage of the intact probe **29a** significant shifts in retention time are observed during RP-HPLC analytics representing fragments **30a** and **31a**. All fragments were confirmed using HRMS analysis. Also a large increase of Sulfo-Cy3 emission could be detected accompanied by a strong decrease in Sulfo-Cy5 fluorescence. The ratio of the two fluorescence intensities changes fundamentally. This large change of fluorescence can be used for very robust detection of ATP cleavage making this ATP analogue a very well suited ratiometric FRET probe.[32]

The combination of the non-sulfonated fluorescent dyes Cy3 and Cy5 was investigated in ATP probe **29b** (Figure 25). The less polar dyes could be advantageous over their sulfonated analogues for cellular studies as they have the potential of passive cellular

uptake, however, the solubility is strongly decreased.[32] In this case, significant shifts in retention time are also observed during RP-HPLC analytics.

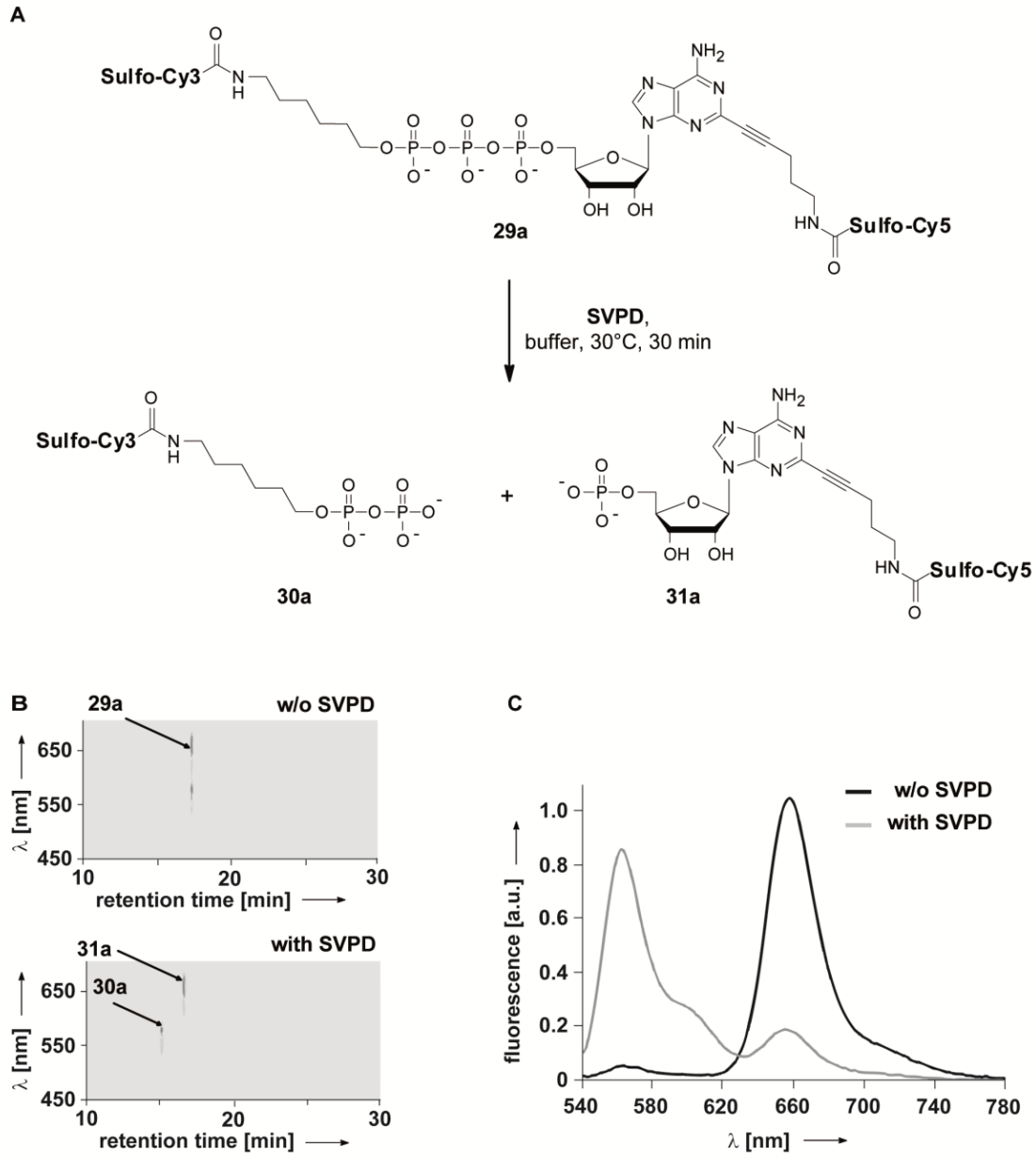


Figure 24: SVPD cleavage and analysis of γ-Sulfo-Cy3, *C*2-Sulfo-Cy3-ATP 29a. A) Fluorogenic ATP activity probe **29a** is incubated with SVPD. **B**) RP-HPLC analytics of the SVPD digested ATP probe (bottom) and the negative control (top). After 30 min of incubation quantitative conversion is detected. Fragments of negative and positive control were identified by HRMS. **C**) Fluorescence emission spectra of the ATP probe without (black) and after digestion (grey); excitation wavelength 532 nm.

Fragments **30b** and **31b** could be confirmed using HRMS analysis. An increase of Cy3 emission and decrease of Cy5 emission could be detected as well. The ratio of fluorescence intensities also changes significantly in this case.

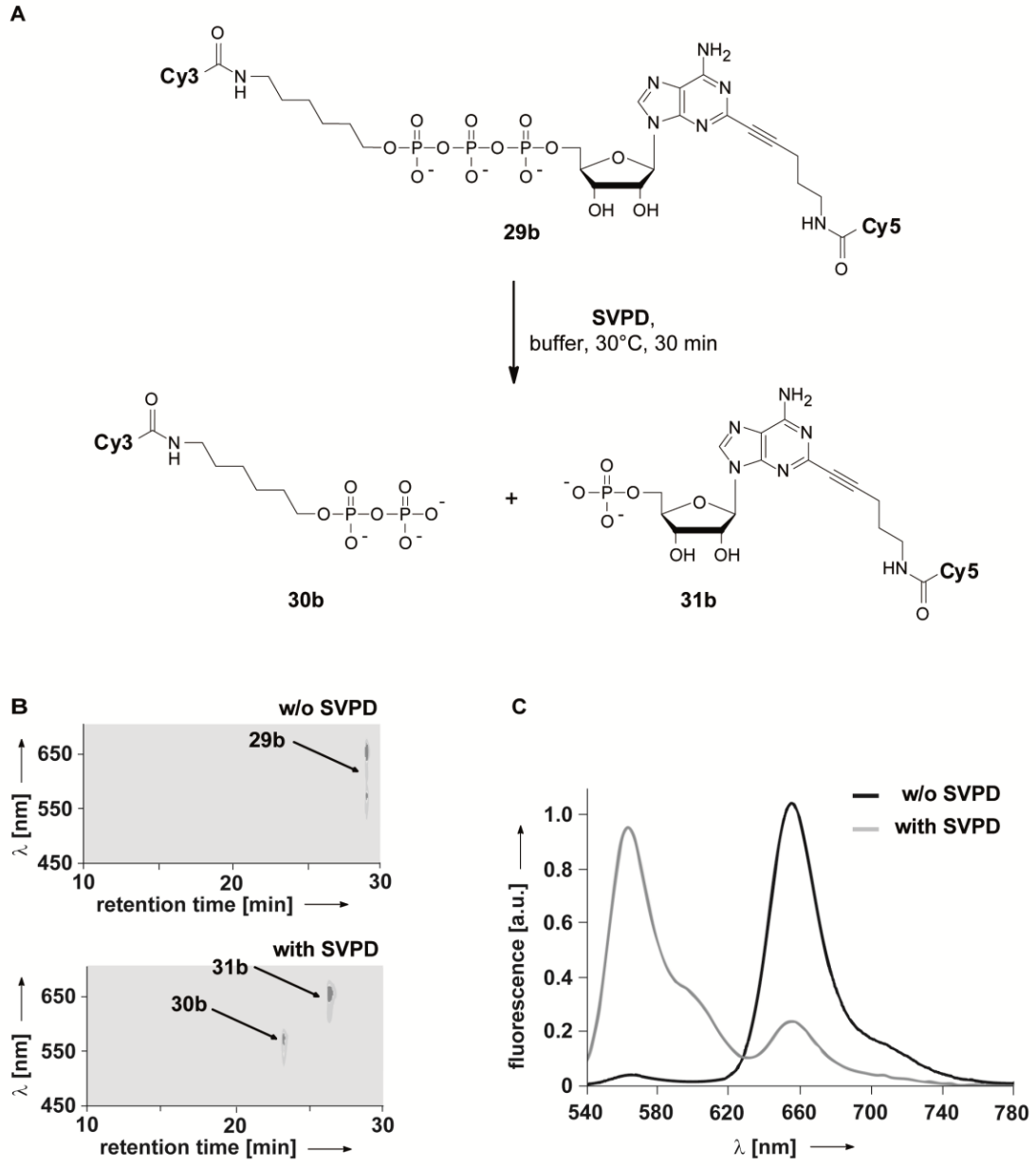


Figure 25: SVPD cleavage and analysis of γ-Cy3, *C*2-Cy5-ATP 29b. A) Fluorogenic ATP activity probe **29b** is incubated with SVPD. B) RP-HPLC analytics of the SVPD digested ATP probe (bottom) and the negative control (top). After 30 min of incubation quantitative conversion is detected. Fragments of negative and positive control were identified by HRMS. C) Fluorescence emission spectra of the ATP probe without (black) and after digestion (grey); Excitation wavelength 532 nm.

This probe is therefore very promising for the monitoring of ATPases and could have advantages over probe **29a** concerning cellular uptake. Nevertheless, concerning *in vitro* applications probe **29a** has the advantage of higher solubility in aqueous buffer.

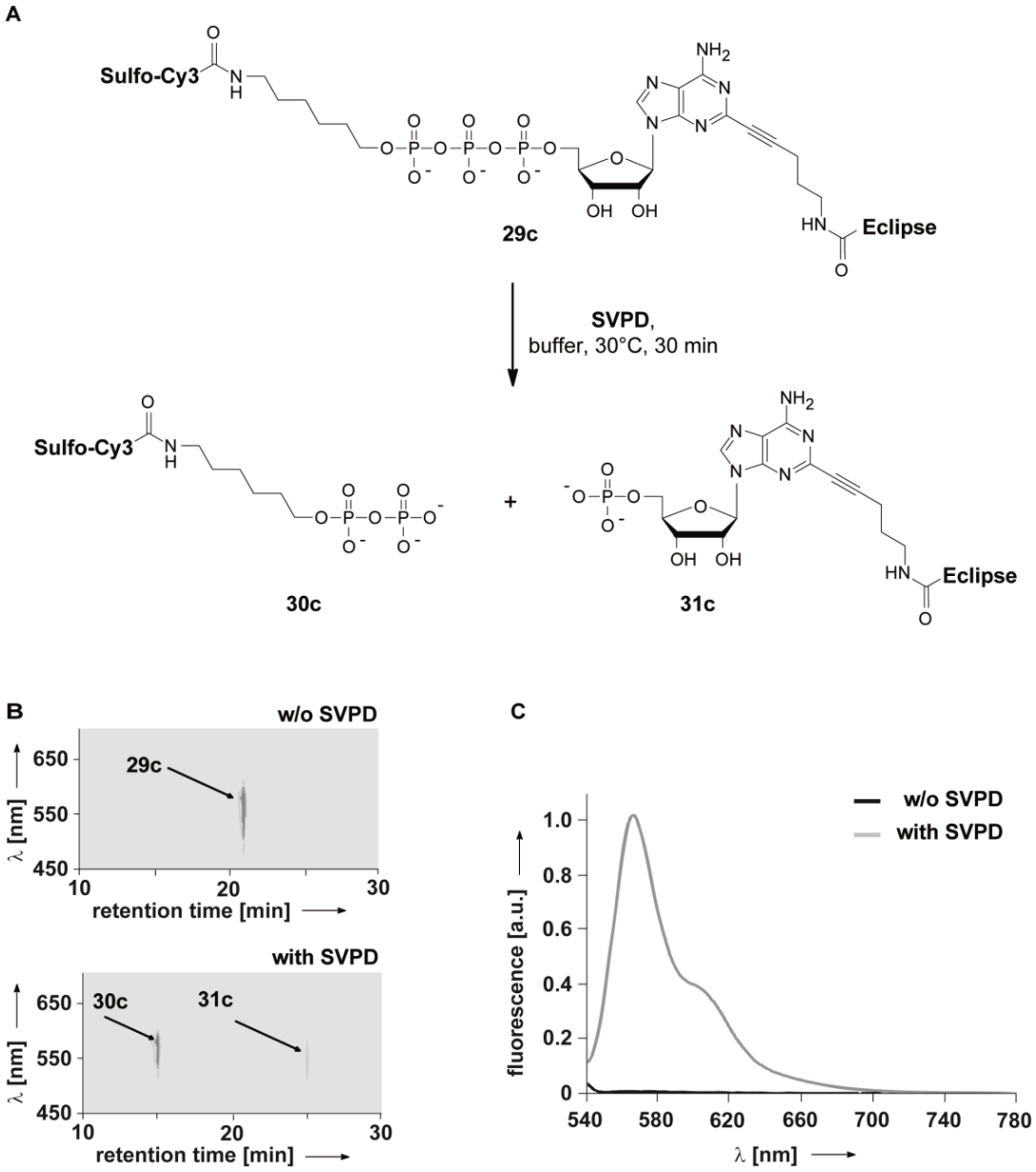


Figure 26: SVPD cleavage and analysis of γ-Sulfo-Cy3, *C*2-Eclipse-ATP 29c. A) Fluorogenic ATP activity probe **29c** is incubated with SVPD. **B**) RP-HPLC analytics of the SVPD digested ATP probe (bottom) and the negative control (top). After 30 min of incubation quantitative conversion is detected. Fragments of negative and positive control were identified by HRMS. **C**) Fluorescence emission spectra of the ATP probe without (black) and after digestion (grey); excitation wavelength 532 nm.

ATP analogue **29c** bearing a combination of Sulfo-Cy3 and eclipse quencher is soluble under aqueous conditions. Upon cleavage, significant shifts in RP-HPLC analysis are observed, fragments **30c** and **31c** were confirmed by HRMS analytics and the fluorescent intensity of Sulfo-Cy3 is initiated (Figure 26). **29c** is almost non-fluorescent in an intact state and allows monitoring of ATP cleavage by a rise of fluorescence and makes this probe a very good dark-quenched FRET probe and a very valuable tool to study ATP cleavage as well.

In conclusion, the rational design of the most promising activity probes towards the class of kinases, proved with kinase *in vitro* tests, is presented. The development of a suitable synthesis strategy for doubly labelled ATP analogues and the synthesis of three novel doubly dye labelled ATP analogues was investigated. The characterisation of spectral properties along the process of the cleavage of all three activity probes **29a**, **29b** and **29c** has been shown and the potential to act as nucleotide based activity probe towards ATPases was proved. These three novel analogues now build a repertoire of doubly dye labelled ATP probes that can be further used to study ATP cleavage.

Doubly dye labelled ATP analogues and their application towards kinases

Since the three novel ATP analogues were designed to study ATP cleavage, first *in vitro* experiments were performed with a focus on the protein family of kinases, especially FAK. Therefore, the ATP analogues should be incubated with FAK and its substrate sequence GST-4xhFAKaa378-406_myc at 37°C for one hour. Fluorescent read-out of the crude reaction mixture could be performed after incubation time to ascertain ATP cleavage and phosphorylation. The cleavage of the ATP analogue and phosphorylation of the substrate should cause significant changes in fluorescent (Figure 27).

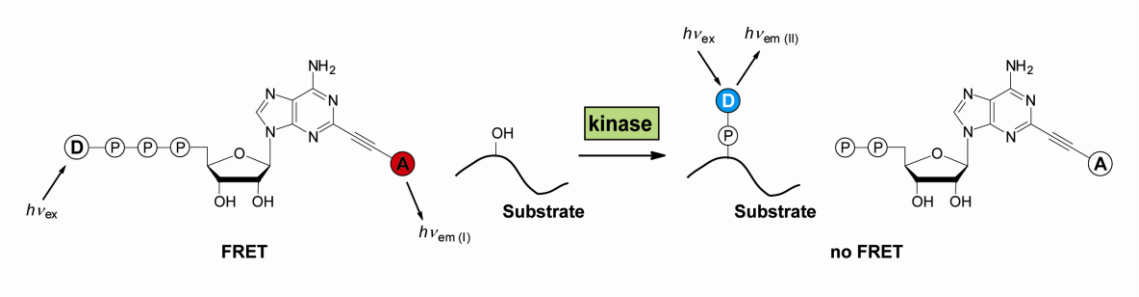


Figure 27: Concept of *in vitro* studies of kinase activities by the novel ATP analogues. ATP analogues serve the kinase of interest as co-substrate to phosphorylate and fluorescently mark a corresponding substrate. By phosphorylation the donor is separated from the acceptor and no FRET can occur resulting in a detectable shift in emitted wavelength.

ATP analogue **29a** was chosen for the in vitro studies of kinase activities since it has good optical properties and good solubility regarding aqueous conditions. Hence, 1 mM **29a** was incubated with or without 0.2 µg·µL^{-1} GST-4xhFAKaa378-406_myc in kinase buffer for one hour at 37°C. Fluorescent analysis of the crude reaction mixture with specific donor excitation wavelength showed no difference between positive and negative control. Moreover an acceptor specific fluorescent signal at 670 nm was still detected, indicating an intact non-cleaved ATP analogue **29a**. Kinase activity could not be detected, indicating that the three novel ATP probes may not be able to serve as co-substrate for FAK activity studies. This circumstance can only be explained by the two bulky rests attached to one ATP molecule. This work already showed that FAK is able to tolerate attachments at *C*2-position and moreover it is known that kinases are able to accept γ-substituents.[40-42,87-92] Therefore, it is most probable that the combination of both attachments interferes the recognition of these probes in the active site.

However, since it is not directly proven in literature that specifically FAK can process γ-substituted ATP analogues the initial activity studies of FAK *via* doubly dye labelled ATP analogues was extended to other kinases that were tested to their acceptance of *C*2-modifications within this work and are literature known to be able to process γ-substituted ATP. Therefore activity probe **29a** was incubated with the kinase of interest and their corresponding substrate at 30°C for several hours while performing fluorescent measurements every 5 minutes. Negative controls were performed without the kinase. According to this procedure five different kinases FAK, Abl, CaMKII, PAK and PKA were elaborated. However, none of the assays indicated any ATP

cleavage. These results indicate that kinases do not accept the combination of two modifications attached to natural ATP without complete loss of recognition. However, further investigations regarding visualisation of the phosphorylation step of kinases in a spatiotemporal resolution *via* nucleotide activity probes should be done since kinases constitute an area of research with absorbing interest.

Doubly dye labelled ATP analogues and their application in the novel metabolic pathway of acetone degradation in *Desulfococcus biacutus*

ATP is crucial for all living organisms and especially for organisms living under harsh conditions like sulphate-reducing bacteria because in the energetic tower they are located in a position where they cannot gain much energy. *D. biacutus* is a sulphate-reducing bacterium that was isolated with acetone as the sole organic source.[170] It has been used as a model organism to research the activation of acetone under sulphate-reducing conditions. The mechanism of activation became of interest due to thermodynamic reasons; first, acetone needs the investment of energy in order to be degraded, and second, *D. biacutus* does not get enough energy during the full reaction. This question was addressed recently by the laboratory of Prof. Dr. B. Schink (University Konstanz).[175]

Unlike aerobic and nitrate-reducing bacteria, *D. biacutus* activates acetone not by carboxylation, but uses CO in the activation reaction. The product of this carbonylation reaction is acetoacetaldehyde, as shown recently by O. B. Gutierrez Acosta and N. Hardt *et al.*.[175,191] However, the exact mechanism of this novel reaction is still under investigation. Proteomic analysis of acetone-grown cells of *D. biacutus* indicated that a thiamine pyrophosphate (TPP)-requiring enzyme may be involved in the activating reaction (data not published). The role of TPP in already known mechanisms is to help in the catalysis of C-C bond-forming and bond-breaking reactions that occur adjacent to a carbonyl group.[191,192] Consequently, a main concern was to evaluate and understand a possible involvement of TPP in the activation of acetone in cell-free extracts of *D. biacutus*. In order to further investigate the novel ATP-dependent

acetone activation reaction the concept of doubly dye labelled ATP analogues was applied. In order to use these analogues as activity probe towards *D. biacutus*, it is required to ensure their stability under cell-free extract conditions.[191] Preliminary tests were done by incubation of fluorescent dyes in cell-free extracts. The fluorescent analysis and HPLC analytics after the reaction showed that Cy5 and Sulfo-Cy5 are not stable under these conditions and therefore not suitable to serve for these investigations. Hence, the analysis of the metabolism of acetone was done using ATP probe **29c** since both dyes used for that probe turned out to be stable under cell free extract conditions.

On the basis of proteomic analysis (data not published), specific interest was given to elaborate a possible involvement of TPP as cofactor in the metabolism of acetone. The activating reaction was elaborated under strictly anaerobic conditions in cell-free extracts of acetone-grown cells in the presence of TPP. According to the concept of the novel ATP activity probe **29c**, fluorescence emission of the donor Sulfo-Cy3 can be measured after cleavage of the ATP analogue. The increase of the fluorescent signal over time of the hydrolysed ATP analogue **29c** due to the enzymatic acetone-activating reaction was measured (Figure 27A). The addition of TPP to the reaction mixture could increase the hydrolysis rate of ATP analogue **29c** up to two-fold (Figure 28B), indicating that TPP is a cofactor in the enzymatic activation of acetone in *D. biacutus*. The activity also depends on acetone, CO and TPP. The ATP analogue **29c** could also be used by other ATPases present in the cell-free extract, as indicated by the background hydrolysis activity measured in the absence of acetone. In the control assay that was performed under N_2, no significant activity could be detected and, hence, it is confirmed that CO is required for the activation of acetone (Figure 28A and B). RP-HPLC analysis of the reaction mixtures showed clear spectral properties and retention times in line with the SVPD experiments (Figure 28C). The negative control assay in the absence of CO gave a clear signal after 21 minutes retention time, representing the non-cleaved probe **29c**. The positive reaction mix containing TPP, CO, acetone, and crude cell extract (CCE) gave a signal after 16 minutes retention time, representing fragment **34** of the cleaved probe **29a** (Figure 28C). Fragments of negative control and positive reaction mix were further analysed and identified by HRMS.

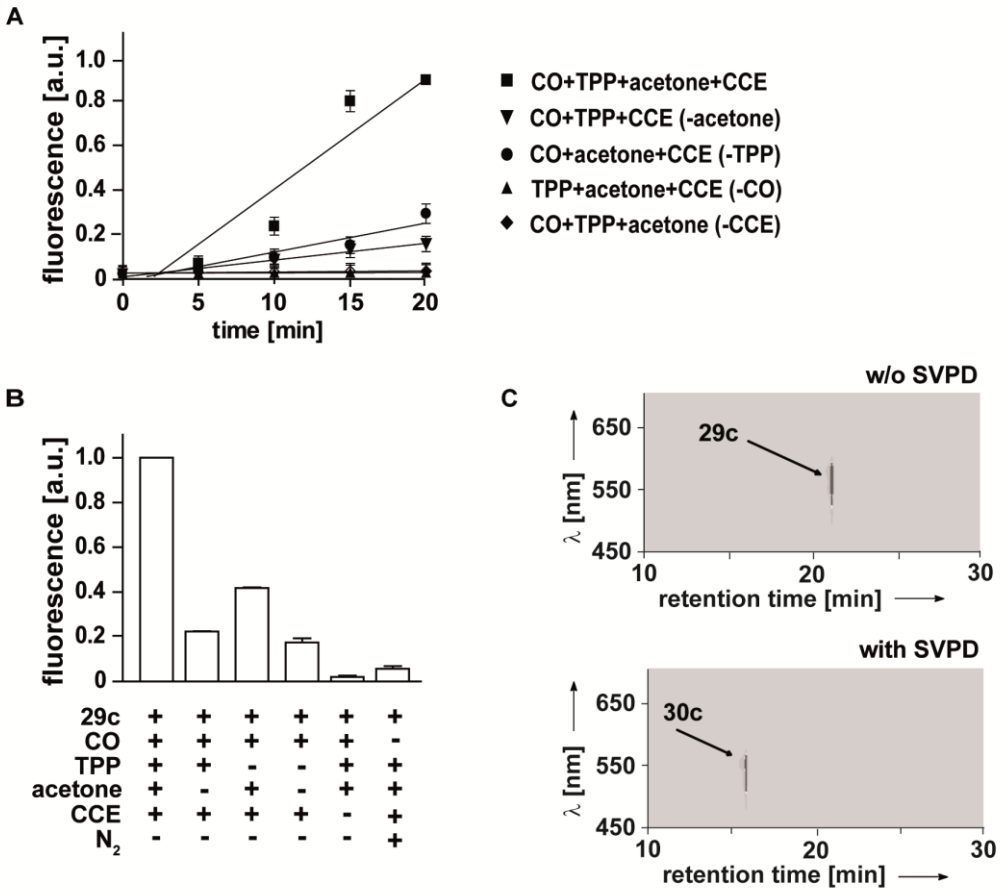


Figure 28: Reaction of 29c in cell-free extract of *D. biacutus*. All experiments were performed under strictly anoxic conditions at 30°C. Concentrations of the substrates were 1 mM acetone, 0.5 mM ATP probe **29c**, 2 mM TPP and 10% CO in the headspace. A) Time course experiments of the hydrolysis of ATP probe **29c** in the acetone activation reaction. B) Activity of hydrolysis of ATP probe **29c** after 60 min of enzymatic reaction. C) RP-HPLC analysis after 60 min of the negative control (top) gave intact ATP probe **29c** with retention time of 21 min; positive control (bottom) showed fragment **34** with retention time of 16 min, and typical spectral properties. Fragments of negative and positive control were further identified by HRMS.

The mechanism in which TPP is involved in the acetone activation reaction has still to be further investigated. However, due to the help of the novel ATP analogue **29c** and the presented findings, a hypothetical role of TPP in the novel metabolic pathway of acetone degradation in *D. biacutus* could be proposed (Figure 29). Thus, TPP attacks a carbon monoxide molecule to form the activated intermediate (**I**).

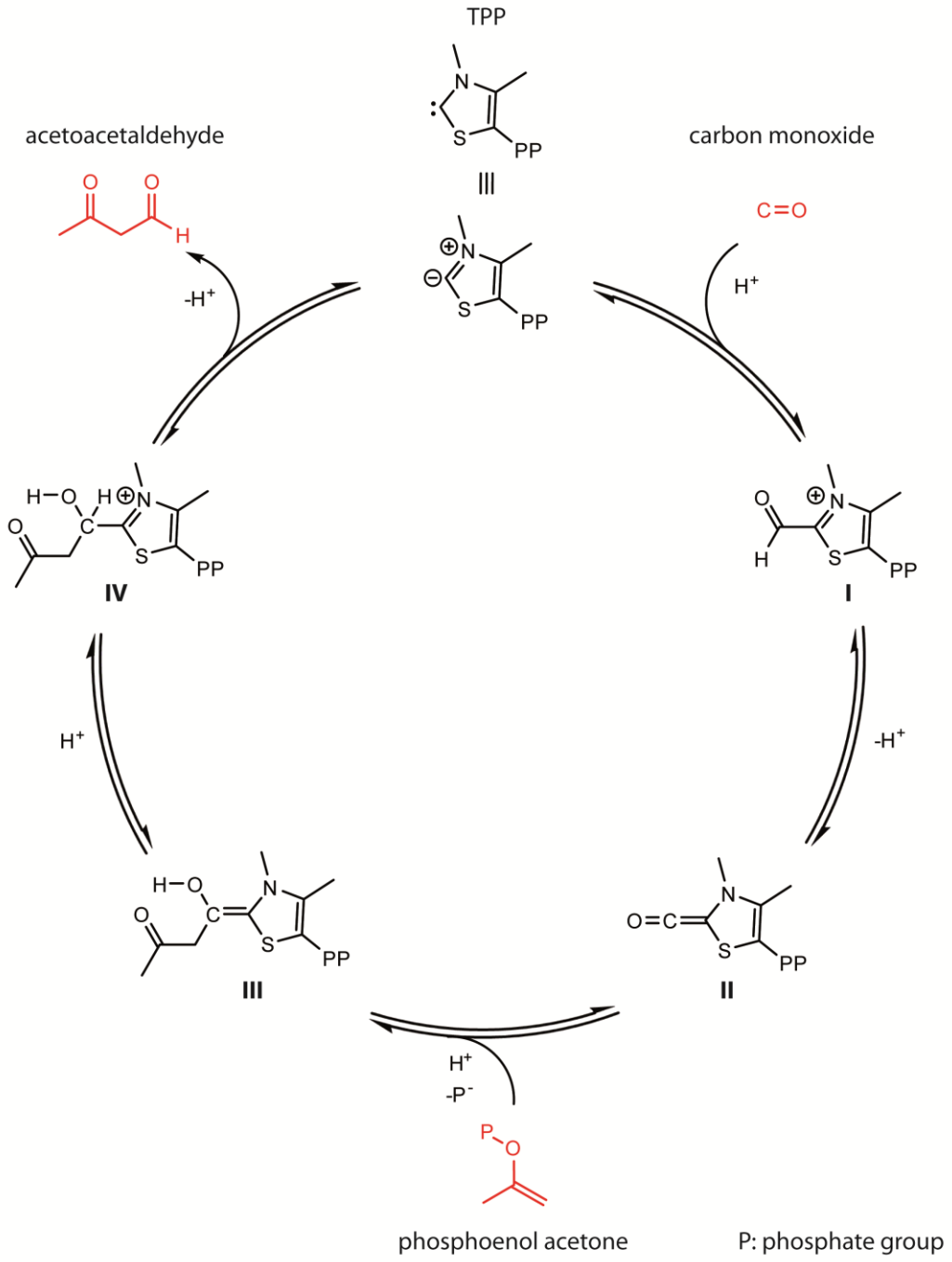


Figure 29: Hypothetical role of TPP in the novel metabolic pathway of acetone degradation in *D. biacutus*. TPP acts as catalyst for the reaction of carbon monoxide with an activated phosphoenol acetone to acetoacetaldehyde. Acetoacetaldehyde was identified as first intermediate in the novel metabolic pathway of acetone in *D. Biacutus*. Intermediates of this reaction are depictured and named using Roman numerals (**I**-**IV**).

Due to deprotonation intermediate (**II**) is formed. Subsequently, intermediate **II** can react with phosphoenol acetone, the activated form of acetone, to result in intermediate (**III**). Protonation of intermediate **III** will form intermediate (**IV**) that is able to relase, due to deprotonation, one equivalent of acetoacetaldehyde and one equivalent of TPP, that is now able to further catalyze the reaction.

Anyway, the application of one novel ATP activity probe, to further clarify this novel postulated mechanism of acetone activation reaction proposed to occur in the sulfate-reducing bacterium *D. biacutus*, could be demonstrated. Enhancement of the ATP hydrolysis rate during acetone activation was proved using the novel activity probe.

Therefore, it could be shown that the application of the conceptually novel doubly dye labelled ATP analogues are very useful tools, for further applications in complex biological processes and help to answer specific questions in life sciences.

Summary

The deep understanding of accurately timed regulatory processes in living cells is still one of the most challenging tasks in today's life sciences. Therefore, one of the most interesting questions in timely research is to bring new insights to the functionality, location and synchronization of proteins with enzymatic activity in the context of a living cell. To address these questions, conceptually novel nucleotide based activity probes were designed, elaborated, and established within the framework of this research. These Förster resonance energy transfer (FRET) based activity probes allow easy detection of enzyme activity at local and temporal resolution. It could be shown within this work, that doubly dye labelled ATP analogues represent a valuable tool to understand and modify biological processes of ATP hydrolysing enzymes. This new concept may not only be of great value for basic research but may open up novel avenues for subsequent applications in complex biological processes.

Initially, crystal structure analysis of the focal adhesion kinase (FAK) binding to ATP was performed to investigate the optimal positions for further attachment of sensor molecules to ATP without substantial loss of enzyme activity. Limited in the attachment of one fluorophore to the γ-phosphate of ATP, the work focused on exploring sterical constraints of attachment sites. The crystal structure of FAK indicated the *C*4'-position and the *C*2-position of ATP as highly potent for modifications, since these positions are exposed out of the active site and do not allow a possible linker to interact with the direct binding sites of the enzyme.

Therefore, novel ATP analogue 4'-*C*-(7-(trifluoroacetamido)-heptyl)-adenosine-triphosphate (**4**) bearing a trifluoracetamide surrogate at the *C*4'-position of the ribose was synthesised *de novo* elaborating and establishing a fourteen step synthesis with an overall yield of 0.7%. Novel ATP analogue *C*2-(5-trifluoroacetamido-pent-1-yn-1-yl)-

adenosine-triphosphate (**5**) modified at *C*2-position of the nucleobase with a trifluoracetamide surrogate was synthesised in a six step synthesis with an overall yield of 2% within this work.

An enzyme-linked immunosorbent assay (ELISA) to investigate the activity of proteine thyrosine kinases (PTKs) as well as for serine/threonine-specific kinases was optimised and established. All together six novel trifluoracetamide modified ATP analogues (**2**-**7**) and two auto fluorescent ATP analogues (**8**, **9**) were successfully used to systematically explore the active site constraints of a total of four different kinases out of three different kinase families, according to this assay. In this way, fingerprints of site specific demands of different four different kinase binding sites could be obtained. Conclusively, the *O*2'-position (**2**) and *C*2-position (**5**) of ATP were commending themselves for further investigations in the case of PTKs like FAK and Abl with regards to activity. With regards to site specific mutation studies, the *C*4'-position (**4**) resulted in the most promising candidate in the case of FAK. In the case of CaMKII a clear preference of nucleobase modified ATP analogues could be seen, as PAK ribose modified ATP analogues show most activity. Considering auto fluorescent ATP analogues, etheno-ATP (**8**) showed better activity towards all four kinases.

According to these findings the first synthesis strategy was designed. This strategy applies a direct ring-opening reaction of an intermediately formed *meta*-triphosphate followed by a one pot reaction to attach both dyes *via* Huisgen cycloaddition and NHS-ester chemistry. The first and conceptually novel doubly dye labelled nucleotide based activity probe **24** was synthesised *de novo* in a total fourteen step synthesis, according to this strategy.

However, low yields at the end of a fourteen step synthesis were the main disadvantage of the first strategy and the reasoning behind the effort of optimisation and development of the meanwhile securely established route towards the synthesis of any doubly labelled nucleotide of interest. This strategy includes an orthogonal amine group protection strategy and NHS-ester chemistry to attach any sensor of choice, both performable under mild and gentle conditions with high yields according to challenging triphosphate chemistry.

According to the new elaborated strategy three novel and differently doubly dye labelled ATP analogues **29a**, **29b** and **29c** have been synthesised. Sulfo-Cy3 and Sulfo-Cy5 dye labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29a** could be synthesised in an eleven step synthesis with an overall yield of 0.2%. Cy3 and Cy5 dye labelled ATP analogue γ-(6-cyanine3-amindohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate **29b** could also be synthesised in an eleven step synthesis with an overall yield of 0.1%. And finally the novel dark quenched doubly labelled ATP analogue γ-(6-sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate **29c** was synthesised analogous with an overall yield of 0.1%.

To prove the novel concept, the fluorescent characteristics of these three novel ATP probes were investigated, applying a model system based on the use of the snake venom phosphodiesterase from *C. adamanteus* (SVPD), which is well known to cleave phosphodiester bonds in a quite efficient way. The cleavage reaction of each activity probe was monitored by analytical RP-HPLC, the detected fragments of negative and positive control were identified by HRMS and the fluorescent characteristics of each doubly dye labelled ATP analogue were analysed. All three probes showed excellent fluorescent properties during cleavage, giving either huge shifts of wavelength of the fluorescent emission, or in the case of the dark quenched probe a significant arise of fluorescent emission at specific wavelengths. Therefore all three novel doubly dye labelled ATP analogues were qualified as nucleotide based activity probes.

Sulfo-Cy3 and Sulfo-Cy5 dye labelled ATP analogue **29a** was chosen due to its spectral properties and excellent solubility to investigate the activity of five different kinases *in vitro*. The kinases FAK, Abl, CaMKII, PAK and PKA were investigated using ATP analogue **29a** as co-substrate. Since none of these kinases was able to use probe **29a** as co-substrate further investigations have to be done on that topic, especially concerning composition of the linker and the length of the linker might have enormous impact on the acceptance of the probes.

Finally, ATP analogue **29c** was applied in cell extract studies to further clarify and confirm a novel mechanism of acetone activation reaction to occur in the sulfate-reducing bacterium *Desulfococcus biacutus* postulated by O. B. Gutierrez Acosta and

me. Enhancement of the ATP hydrolysis rate during acetone activation due to TTP was observed and proven using the novel concept of nucleotide based activity probes.

In conclusion, the novel concept of doubly dye labelled ATP analogues and its application as nucleotide based activity probes was designed, intensive investigations of attractive attachment sites for modifications was done and the first doubly dye labelled ATP analogue was synthesised. Due to these achievements, an optimised synthesis strategy towards doubly labelled ATP analogues could be established, opening therefore the way to any doubly labelled nucleotide analogue of interest. Three novel doubly dye labelled ATP analogues were synthesised and subsequently applied in complex biological processes. It could be shown that the conceptually novel doubly dye labelled ATP analogues are very useful tools and may serve for innovative applications in complex biological processes.

Zusammenfassung

Tiefgreifendes Verständnis von zeitgenauen, regulatorischen Prozessen in lebenden Zellen ist noch immer eine der anspruchsvollsten Herausforderungen in der heutigen Naturwissenschaft. Daher ist eine der interessantesten Fragestellungen der aktuellen Forschung, neue Erkenntnisse in Sachen Funktionalität, Lage und Zusammenspiel von Proteinen mit enzymatischer Aktivität im Zusammenhang einer lebenden Zelle hervorzubringen. Um diesen aktuellen Fragestellungen nachzukommen, wurden im Rahmen dieser Arbeit, konzeptionell neue Nukleotid-basierte Aktivitätssonden konzipiert, erarbeitet und etabliert. Diese FRET-basierten Aktivitätssonden ermöglichen eine einfache Erkennung von enzymatischer Aktivität in räumlicher und zeitlicher Auflösung. Im Rahmen dieser Arbeit konnte gezeigt werden, dass doppelt Farbstoff-markierte ATP-Analoga eine wertvolle Methode darstellen, um die biologischen Prozesse der ATP-Hydrolyse besser verstehen und modifizieren zu können. Dieses neue Konzept mag nicht nur für die Grundlagenforschung von großem Wert sein, sondern eröffnet möglicherweise neue Wege für nachfolgende Anwendungen in komplexen biologischen Prozessen.

Zunächst wurde die Kristallstruktur der Focal Adhesion Kinase (FAK), welche ein ATP in der Bindungstasche trägt analysiert, um die optimalen Positionen für die weitere Anbringung von Sensormolekülen am ATP zu finden, ohne größeren Verlust der enzymatischen Aktivität zu verursachen. Limitiert in der Befestigung eines Fluorophors am γ-Phosphat des ATPs, konzentriert sich diese Arbeit unter anderem auf die Erforschung sterischer Einschränkungen von Grundgerüstmodifikationen des ATP-Moleküls im Hinblick auf die enzymatische Aktivität. Die Analyse der Kristallstruktur der FAK deutete auf eine Favorisierung der *C*4'-Position und der *C*2-Position von ATP für mögliche Modifikationen hin, da diese Positionen aus der Bindungstasche des

Enzyms heraus zeigen und eine mögliche Modifikation an dieser Stelle nicht direkt mit den Bindungsstellen des Enzyms interagieren sollte.

Daher wurde das neue ATP-Analog 4'-*C*-(7-(Trifluoracetamido)-heptyl) Adenosintriphosphat (**4**), welches ein Trifluoracetamid-Surrogat an der *C*4'-Position der Ribose trägt, mittels einer neu erarbeiteten vierzehn stufigen Synthese, *de novo* mit einer Gesamtausbeute von 0,7% synthetisiert. Das neue ATP-Analog *C*2-(5-Trifluoracetamido-pent-1-in-1-yl)-Adenosintriphosphat (**5**), welches an der C2-Position der Nukleobase mit einem Trifluoracetamid-Surrogat modifiziert wurde, wurde im Rahmen dieser Arbeit mittels einer sechs stufigen Synthese mit einer Gesamtausbeute von 2% synthetisiert.

Ein Enzyme Linked Immunosorbent Assay (ELISA), um die Aktivität von Protein Thyrosin Kinasen (PTK) sowie Serin- und Throenin-spezifische Kinasen zu untersuchen, wurde optimiert und etabliert. Insgesamt wurden mit diesem Assay sechs Trifluoracetamid-modifizierte ATP-Analoga (**2**-**7**) und zwei autofluoreszierende ATP-Analoga (**8**, **9**) erfolgreich verwendet, um systematisch Einschränkungen der Bindungsstellen von insgesamt vier verschiedene Kinasen aus drei verschiedenen Kinasefamilien gegenüber der eingeführten Modifikationen zu untersuchen. Auf diese Weise konnten ortsspezifische Anforderungen der vier verschiedenen Bindungsstellen erhalten werden. Schließlich konnte die *O*2'-Position (**2**) und *C*2-Position (**5**) von ATP im Hinblick auf Aktivität für weitere Untersuchungen im Fall von FAK und Abl hervorgehoben werden. Im Hinblick auf eventuell folgende Mutationsstudien hat sich die *C*4'-Position (**4**) im Fall der FAK als sehr geeignete Modifikationsstelle hervor getan. Im Falle der CaMKII ist eine klare Präferenz der Nucleobasen-modifizierten ATP-Analoga zu erkennen, während für die PAK Ribose-modifizierte ATP-Analoga die höchste Aktivität zeigten. Im Fall der autofluoreszierenden ATP-Analoga zeigte Etheno-ATP (**8**) die bessere Aktivität gegenüber allen vier Kinasen.

Anhand dieser Erkenntnisse wurde die erste Synthesestrategie entwickelt. Diese Synthesestrategie beinhaltet eine direkte Ringöffnung des intermediär gebildeten *meta*-Triphosphats und eine Eintopfreaktion, in der beide Farbstoffe simultan über eine Huisgen-Cycloaddition und NHS-Ester Chemie eingebracht werden. Die erste und konzeptionell neue, zweifache Farbstoff markierte Nukleotid-basierende

Aktivitätssonde **24** konnte auf diesem Weg *de novo* in insgesamt vierzehn Stufen synthetisiert werden.

Allerdings waren niedrige Ausbeuten am Ende einer vierzehn-stufigen, arbeitsintensiven Synthese der Hauptnachteil der ersten Strategie und der Grund der Optimierung und Entwicklung einer mittlerweile fest etablierten Route zur Synthese von doppelt markierten Nukleotid-Analoga. Diese Strategie umfasst nun eine orthogonale Aminoschutzgruppen-Strategie und NHS-Ester-Chemie, welche erlaubt, jedes beliebige Sensormolekül unter milden Bedingungen mit hohen Ausbeuten einbringen zu können.

Nach dieser neu ausgearbeiteten Strategie konnten die drei neuartigen und unterschiedlichen, doppelt Farbstoff-markierte ATP-Analoga **29a**, **29b** und **29c** synthetisiert werden. Das Sulfo-Cy3 und Sulfo-Cy5 Farbstoff-markierte ATP-Analog γ-(6-Sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-in-1-yl)-Adenosin-triphosphat **29a** konnte dabei mittels einer elf-stufigen Synthese mit einer Gesamtausbeute von 0,2% synthetisiert werden. Das Cy3 und Cy5 Farbstoff-markierte ATP-Analog γ-(6-Cyanine3-amindohexyl)-2-(5-cyanine5-amidopent-1-in-1-yl)-Adenosin-triphosphat **29b** konnte ebenfalls mittels einer elf-stufigen Synthese mit einer Gesamtausbeute von 0,1% synthetisiert werden. Schließlich konnte das neue dunkel gequenchte doppelt-markierte ATP-Analogon γ-(6-Sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-in-1-yl)-Adenosintriphosphat **29c** analog mit einer Gesamt-ausbeute von 0,1% synthetisiert werden.

Die Fluoreszenzeigenschaften aller drei neuen ATP-Sonden wurden mittels des Modellsystems der Phosphodiesterase aus *C. adamanteus* (SVPD), welche für ihre effiziente Spaltung von Phosphodiesterbindungen bekannt ist, analysiert. Die Spaltungsreaktion der jeweiligen Aktivitätssonde wurde mittels analytischer RP-HPLC verfolgt, die erhaltenen Fragmente von Negativ- und Positivkontrollen wurden mittels HRMS identifiziert und die spektralen Eigenschaften jedes doppelt Farbstoff-markierten ATP-Analogons analysiert. Alle drei Proben zeigten ausgezeichnete spektrale Eigenschaften während ihrer Spaltung. Es konnten entweder große Verschiebungen in der Wellenlänge der Fluoreszenzemission oder im Falle der dunkel gequenchten Probe das Entstehen einer intensiven, spezifisch fluoreszierenden

Emission beobachtet werden. Es zeigten sich alle drei doppelt Farbstoff-markierten ATP-Analoga als geeignet zur Anwendung als Nukleotid-basierte Aktivitätssonden.

Das Sulfo-Cy3 und Sulfo-Cy5 Farbstoff markiert ATP-Analog **29a** wurde aufgrund seiner hervorragenden spektralen Eigenschaften und der ausgezeichneten Löslichkeit in Wasser verwendet, um die Aktivität von fünf verschiedenen Kinasen *in vitro* zu untersuchen. Die Kinasen FAK, Abl, CaMKII, PAK und PKA wurden alle mittels diesem ATP-Analogon auf Aktivität hin untersucht. Da keine dieser Kinasen das doppelt Farbstoff-modifizierte ATP **29a** als Co-Substrat akzeptierte, müssen weitere Untersuchungen auf diesem Thema unternommen werden, um die Aktivität von Kinasen mittels der neuen ATP-Analoga visualisieren zu können. Einen erheblichen Einfluss könnten vor allem die Konstellation der Linker und deren Länge auf die Akzeptanz dieser Sonden haben.

Schließlich konnte ATP-Analog **29c** in Zellextraktstudien angewandt werden, um zur weiteren Aufklärung des neu von O. B. Gutierrez Acosta und mir postulierten Mechanismus der Acetonaktivierung innerhalb des Acetonmetabolismus in dem Sulfat-reduzierenden Bakterium *Desulfococcus biacutus* beizutragen. Hierbei konnte eine Steigerung der Rate der ATP-Hydrolyse während der Acetonaktivierung durch Thiamin Pyrophosphat (TTP) mittels des neuartigen Konzepts der Nukleotid-basierten Aktivitätssonden beobachtet und bestätigt werden.

Zusammenfassend wurde das neuartige Konzept der doppelt Farbstoff-markierten ATP-Analoga und deren Anwendung als Nukleotid-basierten Aktivitätssonden entworfen, intensive Untersuchungen von attraktiven Bindungsstellen für Modifikationen unternommen und das erste doppelt Farbstoff-markierte ATP-Analogon synthetisiert. Aufgrund dieser Errungenschaften wurde eine optimierte Strategie zur Synthese beliebig doppelt-markierten ATP-Analoga entwickelt und etabliert. Nach dieser Synthese wurden drei neue zweifach Farbstoff-markierte ATP-Analoga synthetisiert, analysiert und zur Aufklärung komplexer biologischer Prozesse verwendet. Es konnte gezeigt werden, dass die konzeptionell neuartigen doppelt Farbstoff-markieren ATP-Analoga sehr nützliche Sonden darstellen mit denen neue Aufgabenfelder in komplexen biologischen Prozessen erschlossen werden können.

Experimental Part

General

Chemicals and solvents

Commercially available standard chemicals were purchased from *Acros*, *Merck*, *Fluka*, *TCI*, *MCAT*, *Sigma-Aldrich* or *Carbosynth* and were used without further purification. Dry solvents were purchased from *Sigma-Aldrich*, solvents for column chromatography were either distilled from technical grade (dichloromethane) or purchased as for chromatography grade (hexane, ethyl acetate, methanol). For chemical synthesis and experiments involving enzymes and nucleotide triphosphates water was drawn from a combined reverse osmosis / ultrapure water system (*Sartorius, arium-series*). Intermixtures of solvents are stated as percent by volume. Triethylamine used in Sonogashira reactions was distilled from calcium hydride, degassed, and stored over 4 Å molecular sieves. Trimethylphosphate was distilled and dried over 3 Å molecular sieves.

Chemical reactions

All reactions were conducted under exclusion of air and moisture in oven dried glassware (120°C) using anhydrous reagents and solvents. All temperatures quoted are uncorrected. Molecular sieves with 4 Å pore size were used for alkylation of triphosphates. The reported yields refer to the analytically pure substance and are not optimized.

Chromatography

Thin layer chromatography (TLC) was performed using precoated aluminium plates (silica gel 60 F_{254}; *Merck* or RP-18 F_{254}; *Merck*). Compounds were detected by the extinction of the fluorescence under UV light at 254 nm and stained by moistening the TLC plates with the following solutions and moderate heating afterwards:

– 20 mL *Para*-anisaldehyde, 20 mL conc. sulfuric acid, 4 mL acetic acid in 360 mL ethanol.

– 5 g Cer(IV)sulfate-tetrahydrate, 12.5 g ammonium heptamolybdate-tetrahydrate, 50 mL conc. sulfuric acid in 450 mL water.

– 3 g Potassium permanganate, 20 g potassium carbonate, 2.5 mL 10% sodium hydroxide solution in 400 mL water.

– 4.5 g Ninhydrin in 600 mL ethanol.

Preparative flash chromatography was performed using silica gel G_{60} (*Merck*) with a pressure of 0.2 - 0.4 bar and solvent mixtures or gradients as stated in the corresponding procedures. For medium pressure liquid chromatography (MPLC), a Büchi unit with a Büchi controller C-620, two pumps C-605, a UV monitor C-630 (λ = 254 nm) and fraction collector C-660 was used. For the purification of nucleosides and nucleotides, a 310-25 LiChroprep® RP-18 ready-to-use column (Merck, 40–63 mm) with a linear gradient (5 to 100%) of ACN in 50 mM aqueous triethylammonium acetate (TEAA buffer, pH 7.0) was used. Purification of triphosphates was performed on a BioLogic DuoFlow System (Bio-Rad Laboratories) with DEAE Sephadex™ A-25 (GEHealthcare Bio-SciencesAB) column using a linear gradient of TEAB (0.1 - 1.0 M, flow 2 $mL \cdot min^{-1}$, pH 7.5). For triphosphate purification a LiChroprep® RP-18 Lobar prepacked column size B (Merck), for other compounds SVF D26 - RP18 prepacked columns (25 - 40 µm, Merck Chimie SAS) or RP-18 prepacked cartridges (40 - 63 µm, Büchi) were used. For analytical or preperative HPLC a system from Shimadzu with a nucleosil-100-5(250/4)-C-18 column from Macherey-Nagel was used (flow: 1 $mL \cdot min^{-1}$). Chromatograms were recorded with a polychromatic photo diode array, usually using λ = 260 nm for chromatogram peak detection. Ion exchange chromatography was done using DEAE Sephadex A-25 (Pharmacia LKB) with a linear concentration gradient

of TEAB buffer (0.1 – 1 M, pH 7.5) in a BioLogic DuoFlow system (Bio-Rad Laboratories) at 4°C (UV monitor λ = 254 nm). RP-HPLC was performed using a Shimadzu unit. For the purification of nucleotides a EC 250/4 NUCLEODUR 100-5 C18 ec (Macherey-Nagel), VP 250/10 NUCLEODUR 100-5 C18 ec (Macherey-Nagel) or VP 250/21 NUCLEODUR C18 HTec, 5 µm (Macherey-Nagel) column and a linear gradient (5 to 100%) of acetonitrile in 50 mM TEAA buffer (pH 7.0) was used.

Instrumental and chemical analysis

Electron spray ionization mass spectrometry (ESI-MS) spectra were measured on an *Esquire 3000 plus* (Bruker) in positive or negative mode, samples were diluted to 1-15 $\mu g \cdot mL^{-1}$ with acetonitrile or acetonitrile/water (1:1) and directly injected with a flow rate of 5 $\mu L \cdot min^{-1}$. High resolution (HR) mass spectra were recorded on a *Daltronics micrOTOF-Q II ESI-Qq-TOF* (*Bruker*) in positive or negative mode, samples were diluted to 10-200 $\mu g \cdot mL^{-1}$ with acetonitrile or acetonitrile/water (1:1) and processed by HPLC (column: *Chromolith FastGradient RP-18e 50-2* (*Merck*), linear gradient acetonitrile/water 2 - 100%), before. Nucleic magnetic resonance (NMR) spectra were recorded with a Bruker Avance III 400 MHz spectrometer and Bruker AVIII 600 MHz spectrometer. ^{1}H and ^{13}C chemical shifts are reported relative to the residual solvent peak and are given in ppm (δ). Due to the high temperature dependence of the residual HDO peak spectra in D_2O were referenced according to $\delta = 5.060 - 0.0122T + 0.00211T2$ for the residual proton (T being the temperature in °C).[193] A BBFOplus probe with actively shielded z-gradient was used with its inner (BB-) coil tuned to ^{19}F or ^{31}P respectively. A sample of 85% phosphoric acid in $CDCl_3$ was used as an external standard for ^{31}P NMR spectra, setting the ^{31}P NMR resonance of phosphoric acid to 0 ppm. Assignments of chemical shifts are based on ^{1}H, ^{13}C, ^{1}H-COSY, $^{1}H/^{13}C$-HSQC, $^{1}H/^{13}C$-HMBC, ^{19}F and ^{31}P NMR spectra. Spectra were measured between 291K and 303K and processed using MestReNOVA (v6.1.1-6384). For fluorescent measurements a Perkin Elmer Luminescence Spectrometer LS50 was used.

Buffers and solutions for biochemical experiments

1 M TEAB buffer was prepared freshly before usage by saturation of a 1 M solution of triethylamine (139 mL, 1 mol) in water (filled to a total volume of 1 L) with carbon dioxide from dry ice (pH 7.5). 1 M TEAA buffer was prepared by addition of acetic acid

(57 mL, 1 mol) and triethylamine (139 mL, 1 mol) to 700 mL water. After adjusting pH to 7.0 using acetic acid the solution was filled with water to a total volume of 1 L. FAK buffer (125 mM NaCl, 48 mM $MgCl_2$, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) was freshly prepared before usage in kinase activity assays.

Quantification of nucleoside triphosphates

Quantification of nucleotides was conducted by measuring absorbance at 260 nm using a Nanodrop ND-1000 (Peqlab). In accordance to Lambert Beer's law, the concentration of the nucleotide was calculated using the specific extinction coefficients of adenosine ε_{260} = 13200 $L \cdot mol^{-1} \cdot cm^{-1}$. For quantification and concentration measurements $c = A_{(260\ nm)}\ \varepsilon\ d$ was used. c= concentration; $A_{(\lambda)}$= absorption at specific wavelength; ε_{λ}= extinction coefficient at specific wavelength; d= thickness.

Enzymes, peptides, antibodies

The kinase domain of human FAK was provided by Dr. Alexander Buntru. Therefore the kinase domain of human FAK (aa 411-689, hFAK-KD) was expressed with an amino-terminal 6xHis-tag and a carboxy-terminal PEYFK sequence (FYVE epitope-tag; tag-tools). Expression of the fusion protein was performed in Sf9 insect cells using the pVL1392 transfer vector and the BaculoGold Transfection Kit (*BD Biosciences*). Cells were lysed in Sf9 lysis buffer (0.33% Tween™20, 1 mM ethylene diamine tetra acetic acid (EDTA), 500 mM NaCl, 100 µM phenylmethylsulfonylfluorid (PMSF), 1 mM β-mercaptoethanol in 25 mM Tris-HCl, pH 7.5) for 20 min at 4°C. The lysate was cleared for 30 min at 20,000 x g at 4°C. The recombinant hFAK-KD was purified by fast protein liquid chromatography using a HisTrapFFcrude column (*GE Healthcare*). Abl and respective reaction buffer was purchased from *New England Biolabs* and used with the respective buffers from *New England Biolabs*. PKA and respective reaction buffer was purchased from *New England Biolabs* and used with the respective buffers from *New England Biolabs*. CK2 and respective reaction buffer was purchased from *New England Biolabs* and used with the respective buffers from *New England Biolabs*. CaMKII and respective reaction buffer was purchased from *Promega* and used with the respective buffers from *Promega*. PAK was purchased from *Calbiochem* and used with FAK buffer. Poly-Glu-Tyr peptide in a molar ratio of 4:1 (*Sigma*) was used as substrate for *in vitro*

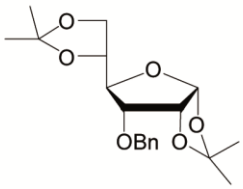
protein tyrosine kinase assay with FAK. Abl substrate peptide (EAIYAAPFAKKK) (*Jena Bioscience*) was used as substrate for *in vitro* protein tyrosine kinase assay with Abl. Myelin Basic Proteine (MBP) was used as substrate for *in vitro* protein Serine/Threonine-kinase assays with PKA, CK2, CaMKII, and PAK. Phosphorylated peptides were detected by monoclonal anti-phosphotyrosine antibody (*Upstate Biotechnology*) in the case of FAK and Abl. Phosphorylated peptides were detected by pSer/Thr Antibody (*CellSignaling*) in the case of PKA, CK2, CaMKII and PAK. SVPD was purchased from *Worthington Biochemical Corporation*.

Crystal structure models

Protein crystal structure models were prepared by the respective PDB-code using PyMOL Molecular Viewer (v0.99rc6) for visualisation.

Synthesis of Compounds

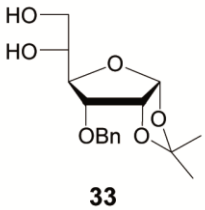
3-*O*-Benzyl-1,2;5,6-di-*O*-isopropylidene-α-D-allofuranose 32 [180]



32

To a stirred solution of NaH (6.72 g, 280 mmol, 1.4 eq.) in 30 mL anhydrous mixture of THF and DMF (5:1) at 0°C, 1,2;5,6-di-*O*-isopropylidene-α-D-allofuranose (52 g, 200 mmol, 1.0 eq.) dissolved in 100 mL anhydrous THF was slowly added and stirred for 1 h. After completion of the reaction, Benzylbromide (36.00 g, 210 mmol, 1.1 eq.) was added and the reaction mixture was stirred over night at room temperature. Reaction mixture was quenched with 100 mL water and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and crystalised from THF to give 60.27 g of compound **32.** The crude product was converted without further analysis.

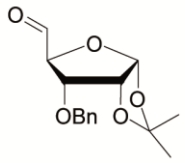
3-*O*-Benzyl-1,2-*O*-isopropylidene-α-D-allofuranose 33 [180]



33

Crude compound **32** (39.00 g, 111 mmol, 1.0 eq.) was dissolved in 250 mL acetic acid and 100 mL water at 0°C and stirred over night at room temperature. After completion of the reaction, the mixture was neutralized with saturated $NaHCO_3$ and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (1:1 hexane - ethyl acetate) to give 27.20 g of compound **33.** The crude product was converted without further analysis.

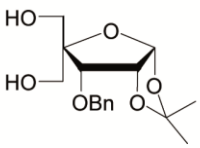
3-*O*-Benzyl-1,2-*O*-isopropylidene-α-D-*ribo*-pentodialdofuranose 34 [180]



34

Compound **33** (27.00 g, 87 mmol, 1.0 eq.) was dissolved in 200 mL THF and 200 mL water. By degrees $NaIO_4$ (22.00 g, 103 mmol, 1.18 eq.) was added to the stirred solution. The mixture was stirred over night at room temperature. After completion of the reaction, the mixture was extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (2:1 hexane - ethyl acetate) to give 23.23 g of compound **34.** The crude product was converted without further analysis.

3-*O*-Benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-*erythro*-pentofuranose 35 [180]



35

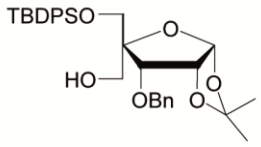
Compound **34** (23.00 g, 84 mmol, 1.0 eq.) was dissolved in 250 mL dioxan, 120 mL 37% $H_2CO_{(aq)}$ and 270 mL 4 M $NaOH_{(aq)}$ were added and the reaction was stirred over night at room temperature. After completion of the reaction, the mixture was extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (1:4 hexane - ethyl acetate) to give 25.84 g of compound **35** (83.26 mmol, 98%) as white crystals; R_f 0.30 (1:4 hexane in ethyl acetate).

^{1}H NMR (d_6-DMSO, 400 MHz): δ 7.41 – 7.24 (m, 5H, ArH), 5.71 (d, J = 3.9 Hz, 1H, H1), 4.73 (dd, *J* = 5.2, 3.9 Hz, 1H, H2), 4.68 – 4.62 (m, 2H, Bn**H**a, OH), 4.52 (d, J = 11.9 Hz, 1H, Bn**H**b), 4.16 (d, J = 5.2 Hz, 1H, H3), 4.09 (dd, J = 6.5, 5.2 Hz, 1H, OH), 3.86 – 3.77 (m, 1H, H5a), 3.56 (ddd, J = 11.7, 8.7, 5.6 Hz, 2H, H5b, H5'a), 3.40 – 3.30 (m, 1H, H5'b), 1.49 (s, 3H, CH_3), 1.27 (s, 3H, CH_3).

^{13}C NMR (d_6-DMSO, 101 MHz): δ 138.30, 128.18, 127.40, 127.23, 112.26, 103.71, 87.76, 78.85, 78.05, 71.48, 62.55, 61.61, 26.69, 26.34.

ESI-MS: found: 333.0; calculated: 333.3 ($M+Na^+$, $C_{16}H_{22}NaO_6^+$).

3-*O*-Benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-*erythro*-pentofuranose 36 [180]



40

Triethylamine (4.04 g, 40 mmol, 3.3 eq.) and *tert*-butyldiphenylsilyl chloride (10.64 g, 39 mmol, 3.3 eq.) were added at 0°C to a stirred solution of compound **35** (3.75 g, 12 mmol, 1.0 eq.) in 40 mL dichloromethane. The reaction was stirred over night at room temperature. After completion of the reaction, the mixture was washed with saturated $NaHCO_3$ and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (3:2 hexane in

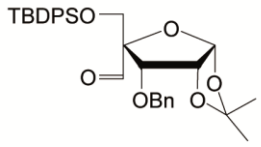
ethyl acetate) to give 3.65 g of compound **36** (6.65 mmol, 56%) as white crystals; R_f 0.70 (3:2 hexane - ethyl acetate).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.61 (ddd, J = 10.8, 7.9, 1.3 Hz, 4H, ArH), 7.47 – 7.28 (m, 11H, ArH), 5.82 (d, J = 3.7 Hz, 1H, H1), 4.82 (d, J = 11.9 Hz, 1H, Bn**H**a), 4.70 (dd, J = 5.0, 3.9 Hz, 1H, H2), 4.52 (d, J = 11.9 Hz, 1H, Bn**H**b), 4.44 (d, J = 5.2 Hz, 1H, H3), 3.90 – 3.80 (m, 2H, **H**5'a,b), 3.77 (d, J = 11.1 Hz, 1H, H5a), 3.68 (d, J = 11.1 Hz, 1H, H5b), 1.65 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 0.99 (s, 9H, 3x CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 137.36, 135.65, 135.61, 133.24, 133.02, 129.80, 129.74, 128.62, 128.14, 127.84, 127.78, 127.74, 113.84, 104.55, 87.51, 79.21, 78.03, 77.26, 72.63, 65.50, 63.29, 26.96, 26.87, 26.36, 19.27.

HR-ESI-MS: found: 571.24679; calculated: 571.24864 ($M+Na^+$, $C_{32}H_{40}NaO_6Si^+$); deviation: 3.2 ppm.

3-*O*-Benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-formyl-1,2-*O*-isopropylidene-α-D-ribofuranose 10 [96]



10

Compound **36** (12.00 g, 21.87 mmol, 1.0 eq.) was dissolved in 150 mL dichloromethane and Dess-Martin periodinane (16.70 g, 39.36 mmol, 1.8 eq.) was added. The reaction was stirred for 3 h at room temperature. After completion of the reaction, the mixture was washed with saturated $NaHCO_3$ and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (4:1 hexane - ethyl acetate) to give 11.36 g of compound **10** (20.78 mmol, 95%) as white crystals; R_f 0.60 (4:1 hexane in ethyl acetate).

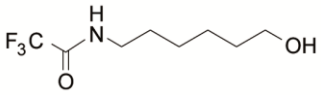
^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.85 (s, 1H, CHO), 7.57 – 7.47 (m, 4H, ArH), 7.39 – 7.19 (m, 11H, ArH), 5.81 (d, J = 3.3 Hz, 1H, H1), 4.69 (d, J = 12.2 Hz, 1H, Bn**H**a), 4.60 (dd, J = 4.2,

3.5 Hz, 1H, H2), 4.56 (d, J = 12.2 Hz, 1H, Bn**H**b), 4.48 (d, J = 4.4 Hz, 1H, H3), 3.82 (d, J = 11.5 Hz, 1H, H5a), 3.74 (d, J = 11.5 Hz, 1H, H5b), 1.56 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 0.92 (s, 9H, 3 x CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 200.28, 137.06, 135.65, 135.57, 132.88, 132.57, 129.96, 129.90, 128.60, 128.17, 127.92, 127.88, 127.85, 114.23, 104.98, 90.69, 79.12, 78.62, 72.79, 63.09, 26.83, 26.73, 26.21, 19.29.

HR-ESI-MS: found: 569.23125; calculated: 569.23299 ($M+Na^+$, $C_{32}H_{38}NaO_6Si^+$); deviation: 3.1 ppm.

N-(6-Hydroxyhexyl)trifluoroacetamide 37 [181]



37

To a solution of 6-aminohexan-1-ol (18.78 g, 160.25 mmol, 1.0 eq.) in 50 mL anhydrous dichloromethane ethyl trifluoroacetate (45.37 g, 319.34 mmol, 2.0 eq.) was added slowly at 0°C. The reaction mixture was stirred over night at room temperature. After completion of the reaction, the mixture was concentrated and purified by column chromatography (2:1 hexane in ethyl acetate) to give 26.41 g of compound **37** (123.88 mmol, 77%) as white solid; R_f 0.30 (1:1 hexane - ethyl acetate).

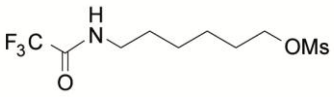
^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.63 (s, 1H, NH), 3.63 (t, J = 6.5 Hz, 2H, C**H**$_2$OH), 3.35 (dd, J = 13.5, 6.8 Hz, 2H, C**H**$_2$NH), 1.68 (s, 1H, OH), 1.64 – 1.52 (m, 4H, 2 x CH_2-linker), 1.45 – 1.30 (m, 4H, 2 x CH_2-linker).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 157.4 (q, ^{2}J = 36.7 Hz, **C**OCF_3), 116.0 (q, ^{1}J = 287.8 Hz, CF_3), 62.7, 40.0, 32.5, 29.0, 26.4, 25.3.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.9 (s, 3F).

ESI-MS: found: 214.2; calculated: 213.2 ($M+H^+$, $C_8H_{15}F_3NO_2^+$).

N-Trifluoroacetyl-(6-aminohexyl)-1-mesylate 38



38

Compound **37** (26.40 g, 123.85 mmol, 1.0 eq.) was dissolved in 100 mL anhydrous THF, triethylamine (17.50 g, 172.91 mmol, 1.4 eq.) and methanesulfonyl chloride (19.83 g, 173.13 mmol, 1.4 eq.) were added at 0°C. The reaction mixture was stirred over night at room temperature. After completion of the reaction, the mixture was washed with ice water and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (2:1 hexane - ethyl acetate) to give 34.95 g of compound **38** (120.00 mmol, 97%); R_f 0.50 (1:1 hexane - ethyl acetate).

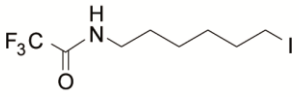
^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.43 (s, 1H, NH), 4.23 (t, J = 6.4 Hz, 2H, C**H**$_2$OMs), 3.37 (dd, J = 13.5, 6.8 Hz, 2H, C**H**$_2$NH), 3.00 (s, 3H, CH_3), 1.81 – 1.71 (m, 2H, CH_2-linker), 1.66 – 1.56 (m, 2H, CH_2-linker), 1.51 – 1.32 (m, 4H, 2 x CH_2-linker).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 151.4 (q, ^{2}J = 36.9 Hz, **C**OCF_3), 116.0 (q, ^{1}J = 287.9 Hz, CF_3), 69.9, 39.8, 37.5, 29.1, 28.9, 26.0, 25.0.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.9 (s, 3F).

ESI-MS: found: 292.0; calculated: 291.3 ($M+H^+$, $C_9H_{17}F_3NO_4S^+$).

N-Trifluoroacetyl-(6-aminohexyl)-1-iodide 17



17

Mesylate **38** (33.00 g, 113.29 mmol, 1.0 eq.) was dissolved in 200 mL acetone and lithium iodide (16.60 g, 124.02 mmol, 1.1 eq.) was added. The reaction mixture was heated to reflux over night. After completion of the reaction, was cooled to room temperature and filtered. The filtrate was washed with 0.1 M $Na_2S_2O_3$ solution and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (6:1 hexane in ethyl acetate) to give 35.03 g of compound **17** (108.42 mmol, 96%); R_f 0.90 (1:1 hexane - ethyl acetate).

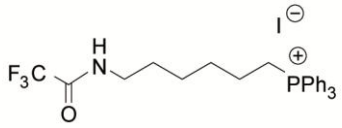
^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.41 (s, 1H, NH), 3.36 (q, J = 6.5 Hz, 2H, C**H**$_2$OMs), 3.18 (td, J = 6.9, 0.7 Hz, 2H, C**H**$_2$NH), 1.82 (p, J = 6.9 Hz, 2H, CH_2-linker), 1.70 – 1.50 (m, 2H, CH_2-linker), 1.50 – 1.27 (m, 4H, 2 x CH_2-linker).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 157.3 (q, ^{2}J = 37.2 Hz, **C**OCF_3), 116.0 (q, ^{1}J = 288.1 Hz, CF_3), 40.0, 33.3, 30.1, 29.0, 25.7, 6.8.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.9 (s, 3F).

ESI-MS: found: 346.2; calculated: 346.1 ($M+Na^+$, $C_8H_{13}F_3INNaO^+$).

Triphenyl-(6-(trifluoroacetamido)hexyl)-phosphonium iodide 18



18

To a stirred solution of compound **17** (1.00 g, 3.10 mmol, 1.0 eq.) in anhydrous toluene, triphenylphosphine (2.81 g, 10.71 mmol, 3.5 eq.) was added and the mixture was refluxed for 16 h. After completion of the reaction, the mixture was allowed to cool to room temperature. The precipitate was collected by filtration, washed with toluene and dried in vacuo to give 1.83 g of compound **18** (3.06 mmol, 99%) as a pale yellow solid; R_f 0.80 (4:1 hexane - ethyl acetate).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.12 (bs, 1H, NHTFA), 7.86 – 7.76 (m, 9H, ArH), 7.76 – 7.67 (m, 6H, ArH), 3.64 – 3.54 (m, 2H, NHTFA-C**H**$_2$), 3.43 – 3.36 (m, 2H, C**H**$_2$PPh_3), 1.65 – 1.57 (m, 6H, 3 x CH_2-linker), 1.52 – 1.40 (m, 2H, CH_2-linker).

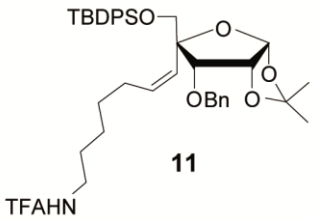
^{13}C NMR ($CDCl_3$, 101 MHz): δ 157.4 (q, ^{2}J = 37.2 Hz, **C**OCF_3), 137.8, 135.3, 135.3, 135.2, 133.6, 133.5, 130.7, 130.6, 129.0, 128.7, 128.5, 128.4, 128.2, 125.3, 118.3, 117.5, 116.0 (q, ^{1}J = 287.9 Hz, CF_3), 39.0, 29.5, 29.4, 28.0, 25.6, 23.1, 22.6, 22.3, 21.4.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.4 (s, 3F).

^{31}P NMR ($CDCl_3$, 162 MHz): δ 24.39 (s, 1P).

ESI-MS: found: 459.2; calculated: 458.5 (M^+, $C_{26}H_{28}F_3NOP^+$).

3-*O*-Benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-(*Z*)-(7-(trifluoroacetamido)-hept-6-enyl)-1,2-*O*-isopropylidene-α-D-ribofuranose 11



11

Intermediate **18** (1.61 g, 2.74 mmol, 1.1 eq.) and *t*-BuOK (0.72 g, 6.41 mmol, 3.5 eq.) were suspended in 50 mL dry THF under nitrogen atmosphere and stirred at room temperature for 1.5 h. The synthesised aldehyde **10** (1.00 g, 1.83 mmol, 1.0 eq.), dissolved in dry THF (10 mL) was added and stirring was continued for 15 h. Reaction mixture was quenched with 20 mL saturated $NaHCO_3$ solution and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (6:1 hexane - ethyl acetate) to give 1.14 g of compound **11** (1.57 mmol, 86%) as a pale yellow foam; R_f 0.55 (4:1 hexane - ethyl acetate).

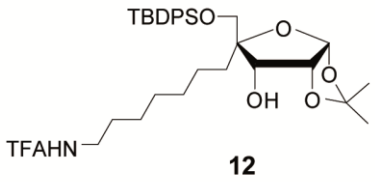
^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.77 – 7.56 (m, 4H, ArH), 7.50 – 7.29 (m, 11H, ArH), 6.52 (bs, 1H, NHTFA), 5.88 (d, J = 11.8 Hz, 1H, linker-CHb=C**H**$_a$), 5.76 (d, J = 3.9 Hz, 1H, H1), 5.41 (dt, J = 10.6, 7.5 Hz, 1H, linker-C**H**b=CHa), 4.87 (d, J = 12.3 Hz, 1H, Bn**H**a), 4.72 (d, J = 12.3 Hz, 1H, Bn**H**b), 4.66 (t, J = 4.3 Hz, 1H, H2), 4.43 (d, J = 4.7 Hz, 1H, H3), 3.68 (d, J = 11.8 Hz, 1H, H5a), 3.52 (d, J = 11.8 Hz, 1H, H5b), 3.34 (td, J = 13.7, 6.5 Hz, 1H, NHTFA-C**H**$_2$a), 3.21 (td, J = 13.4, 6.9 Hz, 1H, NHTFAC**H**$_2$b), 2.31 – 2.16 (m, 1H, C**H**$_2$b-CH=CH), 2.10 – 1.95 (m, 1H, C**H**$_2$a-CH=CH), 1.55 (s, 3H, CH_3), 1.52 – 1.40 (m, 2H, CH_2-linker), 1.40 – 1.28 (m, 2H, CH_2-linker), 1.32 (s, 3H, CH_3), 1.25 – 1.11 (m, 2H, CH_2-linker), 1.00 (s, 9H, 3 x CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 138.1, 136.1, 135.7, 134.1, 133.8, 133.2, 129.8, 129.7, 128.6, 128.1, 128.0, 127.8, 127.7, 126.4, 113.3, 103.9, 86.1, 78.2, 77.4, 72.7, 64.6, 39.9, 28.7, 28.5, 28.4, 26.9, 26.3, 26.2, 25.7, 19.4.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.83 (s, 3F).

HR-ESI-MS: found: 748.32206; calculated: 748.32517 ($M+Na^+$, $C_{40}H_{50}F_3NaNO_6Si^+$); deviation: 4.2 ppm.

5-*O*-*tert*-butyldiphenylsilyl-4-*C*-(7-(trifluoroacetamido)-heptyl)-1,2-*O*-isopropylidene-α-D-ribofuranose 12



12

Compound **11** (0.10 g, 0.14 mmol, 1.0 eq.) and one weight equivalent of 10% Pd/C were suspended in 4 mL THF and stirred under H_2-atmosphere (balloon) for 1.5 h at room temperature. After completion of the reaction, the mixture was filtered through celite on a sintered funnel and washed with acetone thoroughly. The solvent was removed under reduced pressure and the resulting pale yellow foam was purified by

column chromatography (3:1 hexane in ethyl acetate) to give compound **12** in quantitative yields as a white foam; R_f 0.4 (3:1 hexane - ethyl acetate).

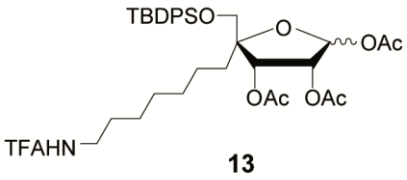
^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.74-7.58 (m, 4H, ArH), 7.51-7.32 (m, 6H, ArH), 6.23 (bs, 1H, NHTFA), 5.93 (d, J = 4.2 Hz, 1H, H1), 4.76 (dd, J = 6.3, 4.2 Hz, 1H, H2), 4.37 (dd, J = 7.5, 6.4 Hz, 1H, H3), 3.63 (d, J = 10.6 Hz, 1H, H5a), 3.53 (d, J = 10.5 Hz, 1H, H5b), 3.25 – 3.40 (m, 2H, NHTFA-C**H**$_2$), 2.64 (d, J = 7.7 Hz, 1H, 3OH), 1.82-0.78 (m, 12H, 6 x CH_2-linker), 1.54 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 1.04 (s, 9H, 3 x CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 135.8, 135.7, 130.0, 129.9, 128.0, 127.9, 113.6, 105.1, 100.1, 90.0, 81.1, 72.5, 68.2, 40.1, 31.7, 30.2, 29.0, 27.1, 27.0, 26.9, 26.7, 23.4, 19.3.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.95 (s, 3F).

HR-ESI-MS: found: 660.29255; calculated: 660.29387 ($M+Na^+$, $C_{33}H_{46}F_3NaNO_6Si^+$); deviation: 2.0 ppm.

1,2,3-Tri-*O*-acetyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-(7-(trifluoroacetamido)-heptyl)-α-D-ribofuranose 13



13

To a solution of compound **12** (1.25 g, 1.96 mmol, 1.0 eq.) in a mixture of 25.6 mL acetic acid and acetic anhydride (2.2 mL, 19.6 mmol, 10.0 eq.) was added 100 µL of concentrated H_2SO_4 and the mixture was stirred for 24 h at room temperature. After completion of the reaction, the mixture was concentrated and coevaporated with toluene. The residue was diluted with 100 mL dichloromethane and washed with 25 mL saturated $NaHCO_3$ and 25 mL water, dried over $MgSO_4$, concentrated, and purified by column chromatography (4:1 hexane - ethyl acetate) to give 1.19 g of compound **13** (1.65 mmol, 84%) as a pale yellow foam; R_f 0.4 (2:1 hexane - ethyl acetate).

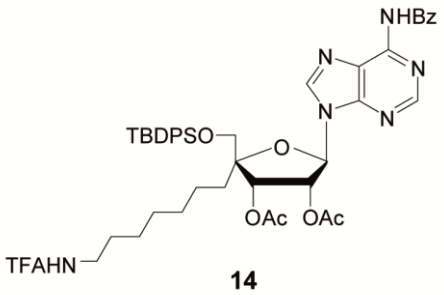
^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.72 – 7.65 (m, 4H, ArH), 7.46 – 7.38 (m, 6H, ArH), 6.44 (bs, 1H, NHTFA), 6.41 (d, J = 4.7 Hz, 1H, H1), 5.65 (d, J = 6.3 Hz, 1H, H3), 5.58 (dd, J = 6.3, 4.7 Hz, 1H, H2), 3.60 – 3.54 (m, 2H, H5a,b), 3.37 – 3.28 (m, 2H, NHTFA-CH_2) 2.15 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.74 – 1.62 (m, 2H, CH_2-linker), 1.54 (m, 4H, 2 x CH_2-linker), 1.33 – 1.16 (m, 6H, 3 x CH_2-linker), 1.07 (s, 9H, 3 x CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 170.2, 170.1, 169.3, 135.8, 135.8, 132.8, 132.5, 130.0, 130.0, 128.0, 94.5, 89.5, 71.6, 71.0, 67.3, 40.0, 32.8, 30.2, 29.4, 28.9, 26.9, 26.6, 23.4, 21.4, 20.8, 20.5, 19.2.

^{19}F NMR ($CDCl_3$, 376 MHz): δ -75.90 (s, 3F).

HR-ESI-MS: found: 746.29209; calculated: 746.29426 ($M+Na^+$, $C_{36}H_{48}F_3NaNO_9Si^+$); deviation: 2.9 ppm.

2'-*O*-Acetyl-3'-*O*-acetyl-5'-*O*-tert-butyldiphenylsilyl-4'-*C*-(7-(trifluoroacetamido)-heptyl)-*N*6-benzoyl-adenosine 14



14

Compound **13** (60 mg, 0.083 mmol, 1.0 eq.) and *N*6-benzoyl protected adenine (22 mg, 0.091 mmol, 1.1 eq.) were solved in 6 mL anhydrous acetonitrile and *N,O*-bis(trimethylsilyl)acetamide (120 µL, 0.71 mmol, 8.5 eq.) were added. The mixture was refluxed for 2 h and after cooling to 0°C, trimethylsilyltriflate (30 µL, 0.07 mmol, 0.9 eq.) was added. After refluxing over night the mixture was quenched with 10 mL saturated $NaHCO_3$ solution, evaporated and extracted with dichloromethane. The organic layer was dried over $MgSO_4$, concentrated and purified by column

chromatography (1:1 hexane - ethyl acetate) to give 67 mg of compound **14** (0.075 mmol, 90%) as a white foam; R_f 0.4 (1:2 hexane - ethyl acetate).

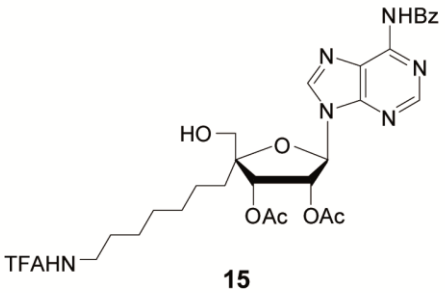
^{1}H NMR (d_6-DMSO, 400 MHz): δ 11.23 (bs, 1H, NHBz), 9.38 (t, J = 5.4 Hz, 1H, NHTFA), 8.63 (s, 1H, H8), 8.42 (s, 1H, H2), 8.07 – 7.99 (m, 2H, ArH), 7.69 – 7.32 (m, 13H, ArH), 6.42 – 6.36 (m, 1H, H2'), 6.33 (d, J = 6.8 Hz, 1H, H1'), 5.95 (d, J = 5.6 Hz, 1H, H3'), 4.09 (d, J = 10.7 Hz, 1H, H5'), 3.72 (d, J = 10.7 Hz, 1H, H5''), 3.19 – 3.11 (m, 2H, NHTFA-C**H**$_2$), 2.19 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.86 – 1.68 (m, 2H, CH_2-linker), 1.45 (d, J = 6.3 Hz, 2H, CH_2-linker), 1.22 (s, 8H, 4 x CH_2-linker), 1.02 (s, 9H, 3 x CH_3).

^{13}C NMR (d_6-DMSO, 101 MHz): δ 208.4, 172.0, 169.3, 169.2, 156.3, 156.0, 151.7, 151.5, 150.7, 144.0, 135.2, 135.1, 132.5, 132.5, 132.4, 130.0, 128.5, 128.5, 128.0, 127.8, 126.1, 117.4, 114.6, 87.0, 85.1, 71.9, 71.5, 68.5, 65.1, 55.8, 32.1, 30.6, 29.6, 29.5, 28.5, 28.2, 26.5, 26.1, 22.3, 21.1, 20.3, 20.2, 18.9.

^{19}F NMR (d_6-DMSO, 376 MHz): δ -74.38 (s, 3F).

HR-ESI-MS: found: 903.36700; calculated: 903.36731 ($M+H^+$, $C_{46}H_{54}F_3N_6O_8Si^+$); deviation: 3.4 ppm.

2'-*O*-Acetyl-3'-*O*-acetyl-5'-*O*-hydroxy-4'-*C*-(7-(trifluoroacetamido)-heptyl)-*N*6-benzoyl-adenosine 15



15

To a solution of compound **14** (750 mg, 0.83 mmol, 1.0 eq.) in 50 mL THF, 100% acetic acid (60 µl, 1.0 mmol, 1.2 eq.) and a 1 M solution of TBAF in THF (1.0 mL, 1.0 mmol, 1.2 eq.) were slowly added. The mixture was stirred at room temperature for 12 h,

concentrated and purified by column chromatography (0 - 5% methanol in dichloromethane) to give 384 mg of compound **15** (0.58 mmol, 70%) as a white foam; R_f 0.3 (5% methanol in dichloromethane).

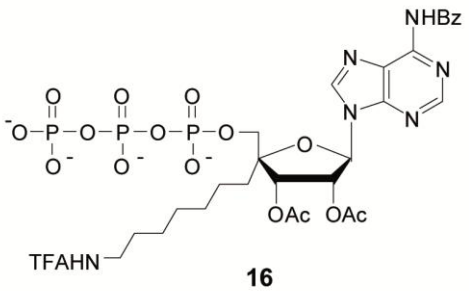
^{1}H NMR (d_6-DMSO, 400 MHz): δ 11.28 (bs, 1H, NHBz), 9.39 (bs, 1H, NHTFA), 8.79 (s, 1H, H8), 8.75 (s, 1H, H2), 8.05 (d, J = 7.2 Hz, 2H, ArH), 7.66 (t, J = 7.4 Hz, 1H, ArH), 7.56 (t, J = 7.6 Hz, 2H, ArH), 6.32 (d, J = 7.7 Hz, 1H, H1'), 6.22 (dd, J = 7.7, 5.4 Hz, 1H, H2'), 5.65 (d, J = 5.3 Hz, 1H, H3'), 5.60 (t, J = 5.4 Hz, 1H, OH), 3.71 (dd, J = 11.7, 4.8 Hz, 1H, H5'), 3.60 (dd, J = 11.7, 6.0 Hz, 1H, H5''), 3.20 – 3.12 (m, 2H, NHTFA-C**H**$_2$), 2.20 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.71 – 1.56 (m, 2H, CH_2-linker), 1.54 – 1.42 (m, 2H, CH_2-linker), 1.33 – 1.20 (m, 8H, 4 x CH_2-linker).

^{13}C NMR (d_6-DMSO, 101 MHz): δ 208.3, 169.1, 169.0, 156.7, 156.3, 155.9, 155.6, 134.5, 132.4, 129.1, 128.4, 127.5, 117.4, 114.6, 88.2, 85.9, 74.5, 72.5, 68.5, 64.3, 55.8, 32.0, 31.8, 29.6, 29.5, 28.3, 28.1, 26.5, 26.0, 22.7, 20.0.

^{19}F NMR (d_6-DMSO, 376 MHz): δ -74.41 (s, 3F).

HR-ESI-MS: found: 665.25206; calculated: 665.25412 ($M+H^+$, $C_{30}H_{36}F_3N_6O_8^+$); deviation: 3.1 ppm.

2'-*O*-Acetyl-3'-*O*-acetyl-4'-*C*-(7-(trifluoroacetamido)-heptyl)-*N*6-benzoyl-adenosine-triphosphate 16



16

The protected nucleoside **15** (100 mg, 0.15 mmol, 1.0 eq.) and proton sponge (48 mg, 22.5 mmol, 1.5 eq.) were coevaporated with acetonitrile twice and dried at high

vacuum. The solids were dissolved in 3 mL trimethylphosphate and cooled to -25°C. Phosphorous oxychloride (45 µL, 76 mg, 50 mmol, 2.0 eq.) was added dropwise. The reaction was allowed to warm up to 0°C and stirred for 1 h. Tributylamine (740 µL, 575 mg, 3.11 mmol, 16.0 eq.) and bis-(tributylammonium)-pyrophosphate (2 mL, 0.5 M in DMF, 1 mmol, 5.0 eq.) were added simultaneously, the solution was warmed to room temperature and stirred for 30 min. 5 mL 0.1 M TEAB buffer (pH 7.5) were added and the reaction stirred for 30 min. The mixture was extracted with ethyl acetate three times and the solvents were evaporated under reduced pressure. The compound was purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by RP-MPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 20.62 µmol of product **16** (14%) as white fluffs.

^{1}H NMR (MeOD-d_4, 400 MHz): δ 9.03 (s, 1H, H8), 8.73 (s, 1H, H2), 8.09 (d, J = 7.3 Hz, 2H, ArH), 7.66 (t, J = 7.4 Hz, 1H, ArH), 7.57 (t, J = 7.6 Hz, 2H, ArH), 6.41 (d, J = 7.5 Hz, 1H, H1'), 6.11 (dd, J = 7.3, 5.8 Hz, 1H, H2'), 5.82 (d, J = 5.5 Hz, 1H, H3'), 4.32 – 4.22 (m, 2H, H5',H5''), 3.27 (t, J = 7.1 Hz, 2H, NHTFA-C**H**$_2$), 2.22 (s, 3H, CH_3), 1.95 (s, 3H, CH_3), 1.85 – 1.72 (m, 2H, CH_2-linker), 1.60 – 1.49 (m, 2H, CH_2-linker), 1.42 – 1.34 (m, 8H, 4 x CH_2-linker).

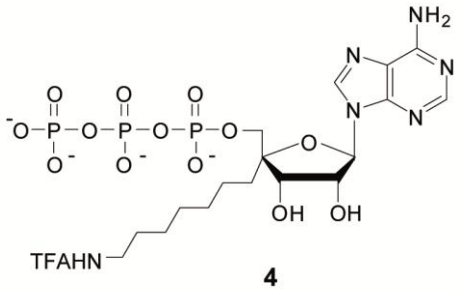
^{13}C NMR (MeOD-d_4, 101 MHz): δ 171.09, 170.64, 168.15, 153.71, 153.38, 151.15, 147.90, 144.50, 135.09, 133.89, 129.77, 129.43, 124.61, 89.07, 88.97, 87.91, 85.95, 75.70, 74.54, 40.70, 33.53, 31.22, 30.07, 29.75, 27.71, 24.34, 20.51, 20.19.

^{19}F NMR (MeOD-d_4, 376 MHz): δ -77.32 (s, 3F).

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.24 (d, J = 20.8 Hz, 1P), -11.95 (d, J = 20.0 Hz, 1P), -23.19 (t, J = 18.0 Hz, 1P).

HR-ESI-MS: found: 903.14040; calculated: 903.13750 (M-H$^+$, $C_{30}H_{37}F_3N_6O_{17}P_3^-$); deviation: 3.2 ppm.

4'-*C*-(7-(trifluoroacetamido)-heptyl)-adenosine-triphosphate 4



4

Protected nucleotide **16** (20.62 µmol, 1.0 eq.) was dissolved in 3 mL water and 6 mL $NH_4OH_{(aq)}$ (33%) was added to the solution and stirred for 3.5 h at room temperature. After completion of the reaction ammonia was removed in vacuo. The crude product was converted without further purification. The deprotected nucleotide (6.72 µmol, 33%, 1.0 eq.) was dissolved in 7 mL methanol. To the solution ethyl trifluoroacetate (8.40 µL, 70.60 µmol, 11.0 eq.) and freshly distilled triethylamine (8.40 µL, 60.6 µmol, 9.0 eq.) were added and the reaction mixture was stirred 3 h at room temperature. The reaction mixture was evaporated to dryness and purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by RP-MPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 6.22 µmol of product **4** (30% over 2 steps) as white fluffs.

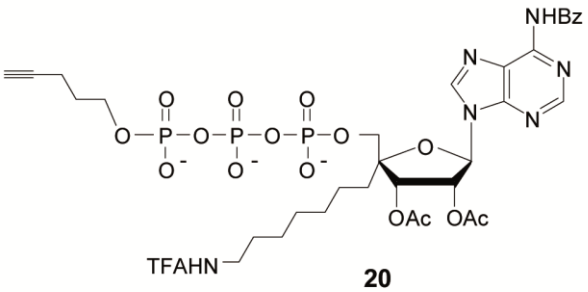
^{1}H NMR (MeOD-d_4, 400 MHz): δ 8.68 (s, 1H, H8), 8.18 (s, 1H, H2), 6.06 (d, J = 7.6 Hz, 1H, H1'), 5.06 – 5.01 (dd, J = 7.6, 5.3 Hz, 1H, H2'), 4.48 (d, J = 5.3 Hz, 1H, H3'), 4.15 (dd, J = 10.7, 5.8 Hz, 1H, H5'), 4.03 (dd, J = 10.8, 3.9 Hz, 1H, H5''), 3.25 (t, J = 7.1 Hz, 2H, NHTFA-C**H**$_2$), 1.84 – 1.70 (m, 2H, CH_2-linker), 1.58 – 1.49 (m, 2H, CH_2-linker), 1.47 – 1.38 (m, CH_2-linker), 1.37 – 1.31 (m, 6H, 3 x CH_2-linker).

^{19}F NMR (MeOD-d_4, 376 MHz): δ -77.35 (s, 3F).

^{31}P NMR (MeOD-d_4, 162 MHz): δ -9.74 (m, 1P), -11.19 (d, J = 20.6 Hz, 1P), -22.83 (m, 1P).

HR-ESI-MS: found: 715.09100; calculated: 715.09010 ($M\text{-}H^+$, $C_{19}H_{29}F_3N_6O_{14}P_3^-$); deviation: 1.3 ppm.

γ-(4-Pentynyl)-2'-*O*-Acetyl-3'-*O*-acetyl-4'-*C*-(7-(trifluoroacetamido)-heptyl)-*N*6-benzoyl-adenosine-triphosphate 20



20

The protected nucleoside **15** (120 mg, 0.18 mmol, 1.0 eq.) and 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (165 mg, 0.81 mmol, 4.5 eq.) were coevaporated with pyr twice and dried under high vacuum. The solids were dissolved in 180 µL pyr and 600 µL dioxane and cooled to 0°C. Tributylamine (500 µL, 389 mg, 2.10 mmol, 11.0 eq.) and bis-(tributylammonium)-pyrophosphate (1.5 mL, 0.5 M in DMF, 0.75 mmol, 3.8 eq.) were added simultaneously and the solution was stirred for 10 min at room temperature. 4-Pentyn-1-ol (0.6 mL, 6.45 mmol, 35.0 eq.) was added and the reaction stirred for 1 h at 0°C and 16 h at room temperature. The reaction was quenched with a 1% I_2 mixture in pyr/H_2O (98:2) and stirred at room temperature over night. Excess of I_2 was quenches with 1 M $Na_2S_2O_3$ and the solvents were evaporated under vacuo and 5 mL 0.1 M TEAB buffer (pH 7.5) were added. The mixture was extracted with ethyl acetate three times and the solvents were evaporated under reduced pressure. The compound was purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 2.70 µmol of product **20** (1.5%) as white fluffs.

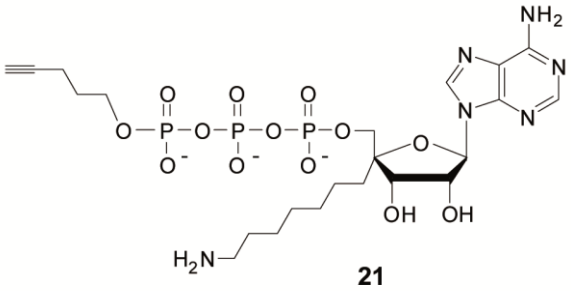
^{1}H NMR (D_2O, 400 MHz): δ 9.16 (s, 1H, H8), 8.91 (s, 1H, H2), 8.10 (d, J = 7.8 Hz, 2H, ArH), 7.75 (d, J = 7.3 Hz, 1H, ArH), 7.67 (t, J = 7.6 Hz, 2H, ArH), 5.82 – 5.73 (m, 1H, H1'), 5.69 (d, J = 5.5 Hz, H3'), 4.09 – 4.18 (m, 2H, H5', H5''), 3.27 (t, J = 6.7 Hz, 2H, NHTFA-C**H**$_2$), 3.10 – 2.98 (m, 1H, H-alkine), 1.92 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 1.71 – 1.30 (m, 10H, 5 x CH_2-linker), 1.23 – 1.06 (s, 8H, 4 x CH_2-linker).

^{31}P NMR (D_2O, 162 MHz): δ -10.30 – -10.72 (m, 1P), -11.87 (d, J = 19.5 Hz, 1P), -23.11 (t, J = 20.0 Hz, 1P).

^{19}F NMR (D_2O, 376 MHz): δ -75.78 (s, 3F).

ESI-MS: found: 969.1; calculated: 969.2 (M-H$^+$, $C_{35}H_{43}F_3N_6O_{17}P_3^-$).

γ-(4-Pentynyl)-4'-*C*-(7-aminoheptyl)-adenosine-triphosphate 21



21

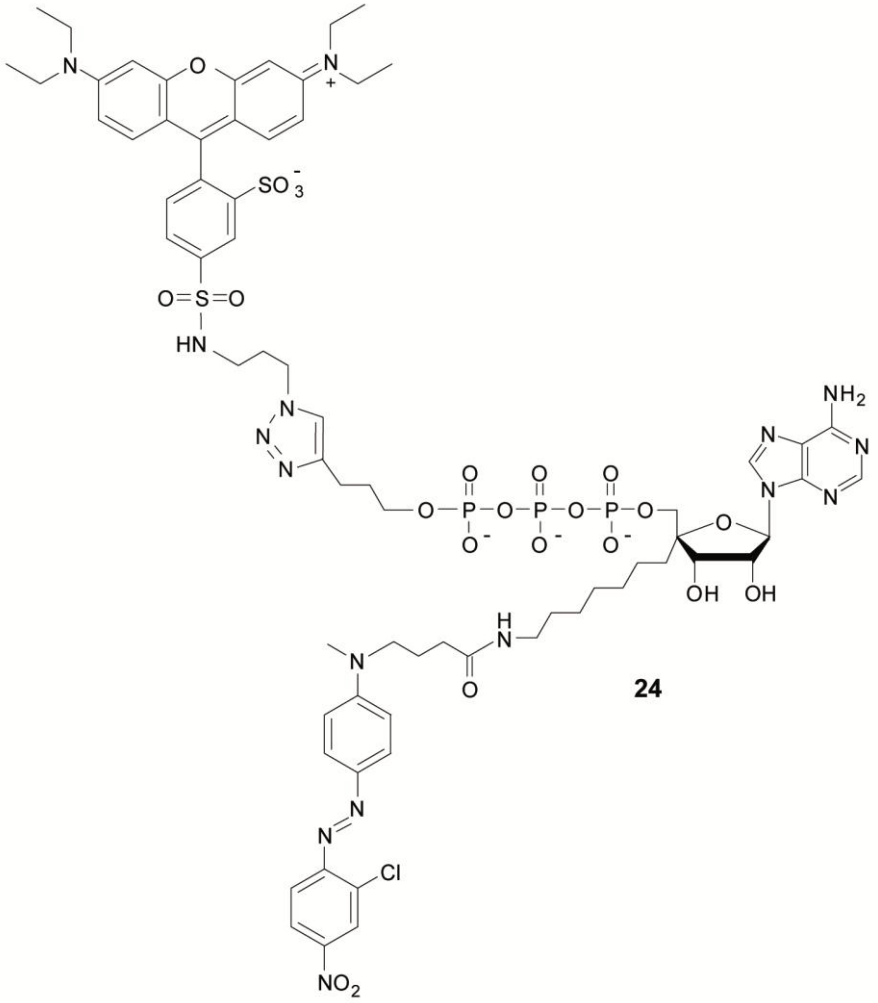
Compound **20** (2.70 µmol, 1.0 eq.) was solved in 1 mL $NH_4OH_{(aq)}$ (33%) and stirred at 50°C for 48 h. The solvent was evaporated under reduced pressure. The compound was purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 2.34 µmol of product **21** (87%) as white fluffs.

^{1}H NMR (MeOD-d_4, 400 MHz): δ 8.53 (s, 1H, H8), 8.26 (s, 1H, H2), 5.90 (d, J = 7.8 Hz, 1H, H1'), 4.51 (d, J = 6.0 Hz, 1H, H3'), 4.16 – 4.01 (m, 2H, H5', H5''), 3.97 – 3.84 (m, 2H, CH_2-linker), 3.07 – 2.97 (m, 1H, H-alkine), 2.97 – 2.88 (m, 2H, CH_2-linker), 2.18 – 2.07 (m, 2H, CH_2-linker), 1.90 – 1.57 (m, 6H, 3x CH_2-linker), 1.47 – 1.29 (m, 8H, 4x CH_2-linker).

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.00 (d, J = 19.9 Hz, 1P), -12.03 (d, J = 18.5 Hz, 1P), -23.25 (t, J = 20.0 Hz, 1P).

ESI-MS: found: 685.2; calculated: 685.1 (M-H$^+$, $C_{22}H_{36}N_6O_{13}P_3^-$).

γ-(Sulfo-rhodamine)-4'-*C*-(eclipse-7-aminoheptyl)-adenosine-triphosphate 24

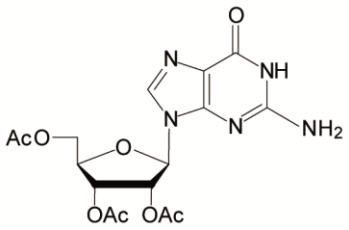


Compound **21** (1.20 µmol, 1.0 eq.) was dissolved in a mixture of ACN/DMF/MeOH (100 µL/100 µL/2 µL), NHS-activated eclipse quencher **22** (0.73 mg, 1.54 µmol, 1.3 eq.) and DIPEA (0.53 µL, 3.00 µmol, 3.5 eq.) was added to the reaction and stirred over night. Next, azidosulforodamine **23** (0.74 mg, 1.20 µmol, 1.0 eq.), CuI (0.07 mg, 0.38 µmol, 0.3 eq.) and DIPEA (0.40 µL, 2.30 µmol, 1.9 eq.) were dissolved in a mixture of ACN/H_2O (1:1) and added to the crude reaction mixture. The mixture was stirred at room temperature over night. The solvents were evaporated under reduced pressure and the compound was purified by RP-HPLC. Fractions containing the product were

evaporated. The product **24** could be identified by ESI-MS only, due to the less amount and insufficient yields.

ESI-MS: found: 1683.8; calculated: 1683.5 ($M\text{-}H^+$, $C_{69}H_{87}ClN_{16}O_{22}P_3S_2^-$).

2',3',5'-Tri-*O*-acetyl-guanosine 39 [122]



39

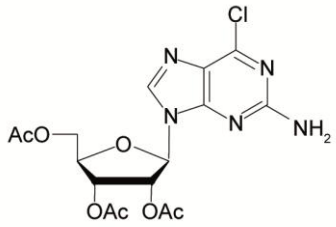
A mixture of guanosine (35.00 g, 123.57 mmol, 1.0 eq.), acetic anhydride (75.60 g, 740.52 mmol, 6.0 eq.) in 140 mL anhydrous DMF/pyr (5:2) was heated at 75°C for 4 h. The resulting solution was cooled to 4°C and the resulting crystals were filtered, washed with *iso*-propyl alcohol and dryed under vacuo to give 36.55 g of compound **39** (89.29 mmol, 72%) as white crystals; R_f 0.2 (5% methanol in dichloromethane).

^{1}H NMR (d_6-DMSO, 400 MHz): δ 10.74 (s, 1H, NH), 7.93 (s, 1H, H8), 6.54 (bs, 2H, NH_2), 5.98 (d, J = 6.1 Hz, 1H, H1'), 5.79 (t, J = 6.0 Hz, 1H, H2'), 5.49 (dd, J = 5.8, 4.2 Hz, 1H, H3'), 4.48 – 4.18 (m, 3H, H4',H5',H5''), 2.11 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.04 (s, 3H, CH_3).

^{13}C NMR (d_6-DMSO, 101 MHz): δ 170.11, 169.46, 169.29, 156.63, 153.90, 151.12, 135.64, 116.83, 84.39, 79.55, 72.05, 70.31, 63.09, 20.54, 20.39, 20.20.

ESI-MS: found: 410.3; calculated: 409.1 ($M\text{+}H^+$, $C_{16}H_{19}N_5O_8^+$).

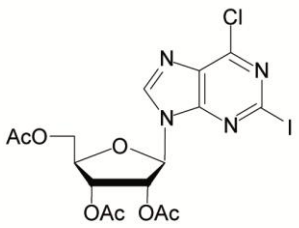
2',3',5'-Tri-*O*-acetyl-1'-deoxy-1'-(2-amino-6-chloropurine)-*β*-D-ribofuranose 40 [122]



40

To a solution of compound **39** (35.26 g, 86.19 mmol, 1.0 eq.) in 100 mL anhydrous acetonitrile, tetraethylammoniumchloride (28.55 g, 172.30 mmol, 2.0 eq.), *N*,*N*-diethylaniline (10.25 g, 68.69 mmol, 0.8 eq.) and freshly destilled phosphoryl chloride (79.25 g, 516.85 mmol, 6.0 eq.) were added in this order at room temperature. After stirring for 15 min under reflux, volatile materials were evaporated under vacuo immediately. The resulting yellowish oil was dissolved in 300 mL dichloromethane and washed with 200 mL ice water. The organic layer was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed with cold water and 5% aqueous $NaHCO_3$ solution, dried over $MgSO_4$ and concentrated under vacuo to give 33.30 g of compound **40** (77.84 mmol, 90%) as white solids; R_f 0.6 (5% methanol in dichloromethane). The crude product was used for the following step without further purification.

2',3',5'-Tri-*O*-acetyl-1'-deoxy-1'-(6-chloro-2-iodopurine)-*β*-D-ribofuranose 41 [122]



41

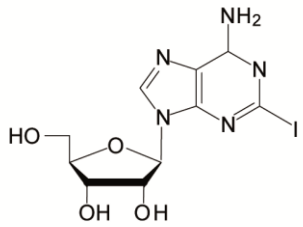
Iodine (19.35 g, 76.24 mmol, 1.0 eq.), diiodomethane (204.93 g, 765.11 mmol, 10.0 eq.), copper iodide (15.92 g, 83.59 mmol, 1.1 eq.) and isopentyl nitrite (26.60 g, 227.03 mmol, 3.0 eq.) were added to a solution of compound **40** (32.53 g, 76.17 mmol, 1.0 eq.) in 350 mL anhydrous THF. The suspension was stirred at reflux for 45 min.. After cooling to room temperature the mixture was filtered off and the solution was evaporated under vacuo and purified by column chromatography (0 - 5% methanol in dichloromethane) to give 7.86 g of compound **41** (14.60 mmol, 19%) as a white foam; R_f 0.4 (5% methanol in dichloromethane).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.20 (s, 1H, H8), 6.20 (d, J = 5.4 Hz, 1H, H1'), 5.79 (t, J = 5.5 Hz, 1H, H2'), 5.59 (dd, J = 5.5, 4.5 Hz, 1H, H3'), 4.50 – 4.47 (m, 1H, H4'), 4.42 (m, 2H, H5',H5''), 2.18 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.11 (s, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 170.28, 169.63, 169.47, 152.10, 151.18, 143.22, 132.41, 117.09, 86.82, 81.03, 73.51, 70.74, 63.05, 20.95, 20.66, 20.51.

ESI-MS: found: 539.1; calculated: 538.0 ($M+H^+$, $C_{16}H_{16}ClIN_4O_7^+$).

2-Iodo-adenosine 42 [122]



42

To 780 mL of anhydrous ethanol saturated with ammonia at -15°C, compound **41** (7.46 g, 13.85 mmol, 1.0 eq.) was added. The solution was stirred at 0°C for 1 h and over night at room temperature. The solvent was removed under vacuo and the residue was treated with 25 mM NaOMe solution for 1 h at room temperature. After completion of the reaction, the mixture was concentrated and purified by column chromatography (0 - 5% methanol in dichloromethane) to give 2.33 g of 2-iodo-

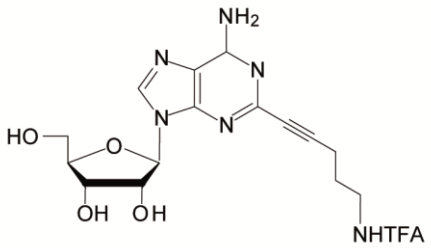
adenosine **42** (5.91 mmol, 43%) as a white foam; R_f 0.1 (5% methanol in dichloromethane).

^{1}H NMR (d_6-DMSO, 400 MHz): δ 8.30 (s, 1H, H8), 7.71 (bs, 2H, NH_2), 5.81 (d, J = 6.1 Hz, 1H, H1'), 5.44 (d, J = 6.2 Hz, 1H, 2'-OH), 5.18 (d, J = 4.9 Hz, 1H, 3'-OH), 5.02 (dd, J = 6.2, 5.2 Hz, 1H, 5'-OH), 4.57 – 4.47 (m, 1H, H2'), 4.13 (dt, J = 8.1, 4.1 Hz, 1H, H3'), 3.95 (q, J = 3.8 Hz, 1H, H4'), 3.66 (dt, J = 11.9, 4.6 Hz, 1H, H5'), 3.61 – 3.51 (m, 1H, H5'').

^{13}C NMR (d_6-DMSO, 101 MHz): δ 155.92, 149.74, 139.41, 120.79, 119.03, 87.19, 85.80, 73.57, 70.49, 61.43.

ESI-MS: found: 391.7; calculated: 392.0 ($M\text{-}H^+$, $C_{10}H_{11}IN_5O_4^-$).

*C*2-(5-Trifluoroacetamido-pent-1-yn-1-yl)-adenosine 43



43

2-Iodo-adenosine **42** (1.97 g, 4.94 mmol, 1.0 eq.) was dissolved in 25 mL dry DMF and CuI (200 mg, 1.05 mmol, 0.2 eq.) was added. The reaction mixture was degassed under vacuo before $Pd(PPh_3)_4$ (600 mg, 0.52 mmol, 0.1 eq.), trifluoro-*N*-(pent-4-ynyl)-acetamide (840 mg, 4.69 mmol, 1.0 eq.) and triethylamine (1.5 mL, 10.82 mmol, 2.2 eq.) were added. The reaction mixture was stirred for 16 h at room temperature, quenched with 25 mL saturated $NaHCO_3$ solution and extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated and purified by column chromatography (10% methanol in dichloromethane) to give 2.07 g of compound **43** (4.64 mmol, 94%) as a pale yellow foam; R_f 0.2 (5% methanol in dichloromethane).

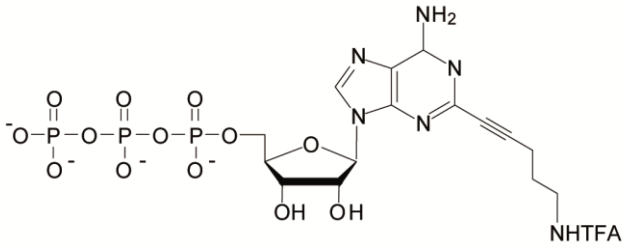
^{1}H NMR (d_6-DMSO, 400 MHz): δ 9.52 (t, J = 5.0 Hz, 1H, NHTFA), 8.40 (s, 1H, H8), 7.44 (bs, 2H, NH_2), 5.85 (d, J = 6.1 Hz, 1H, H1'), 5.46 (d, J = 6.2 Hz, 1H, 2'-OH), 5.25 (dd, J = 6.7, 4.8 Hz, 1H, 5'-OH), 5.19 (d, J = 4.8 Hz, 1H, 3'-OH), 4.55 – 4.50 (m, 1H, H2'), 4.14 – 4.10 (m, 1H, H3'), 3.95 (t, J = 3.3 Hz, 1H, H4'), 3.66 (dt, J = 12.0, 4.1 Hz, 1H, H5'), 3.60 – 3.51 (m, 1H, H5''), 3.33 – 3.27 (m, 2H, NHTFA-C**H**$_2$), 2.45 (t, J = 7.1 Hz, 2H, C**H**$_2$-CC), 1.77 (q, J = 7.1 Hz, 2H, CH_2-C**H**$_2$-CH_2).

^{13}C NMR (d_6-DMSO, 101 MHz): δ 155.8, 149.3, 145.6, 140.3, 118.6, 117.4, 114.5, 87.4, 85.8, 84.5, 81.3, 73.7, 70.5, 61.5, 38.5, 27.0, 15.8.

^{19}F NMR (d_6-DMSO, 376 MHz): δ -74.31 (s, 3F).

HR-ESI-MS: found: 445.14206; calculated: 445.14418 ($M+H^+$, $C_{17}H_{20}F_3N_6O_5^+$); deviation: 4.8 ppm.

*C*2-(5-Trifluoroacetamido-pent-1-yn-1-yl)-adenosine-triphosphate 5



5

Nucleoside **43** (50 mg, 0.11 mmol, 1.0 eq.) and proton sponge (35 mg, 0.16 mmol, 1.5 eq.) were coevaporated with acetonitrile twice and dried at high vacuum. The solids were dissolved in 2 mL trimethylphosphate and cooled to -30°C. Phosphorous oxychloride (40 µL, 67 mg, 0.55 mmol, 5.0 eq.) was added dropwise. The reaction was allowed to warm up to 0°C and stirred for 1 h. Tributylamine (300 µL, 233 mg, 1.26 mmol, 11.0 eq.) and bis-(tributylammonium)-pyrophosphate (1.2 mL, 0.5 M in DMF, 0.6 mmol, 5.0 eq.) were added simultaneously, the solution was warmed to room temperature and stirred for 30 min.. 5 mL 0.1 M TEAB buffer (pH 7.5) were added and

the reaction stirred for 30 min.. The mixture was extracted with ethyl acetate three times and the solvents were evaporated under reduced pressure. The compound was purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by RP-MPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 44.4 µmol of product **5** (40%) as white fluffs.

^{1}H NMR (MeOD-d_4, 400 MHz): δ 8.59 (s, 1H, H8), 7.19 (bs, 1H, NHTFA), 6.08 (d, J = 5.4 Hz, 1H, H1'), 4.69 (t, J = 5.1 Hz, 1H, H2'), 4.57 – 4.44 (m, 1H, H3'), 4.39 – 4.23 (m, 3H, H4', H5', H5''), 3.47 (m, 2H, NHTFA-C**H**$_2$), 2.52 (t, J = 7.1 Hz, 2H, C**H**$_2$-CC), 1.97 – 1.85 (m, 2H, CH_2-C**H**$_2$-CH_2).

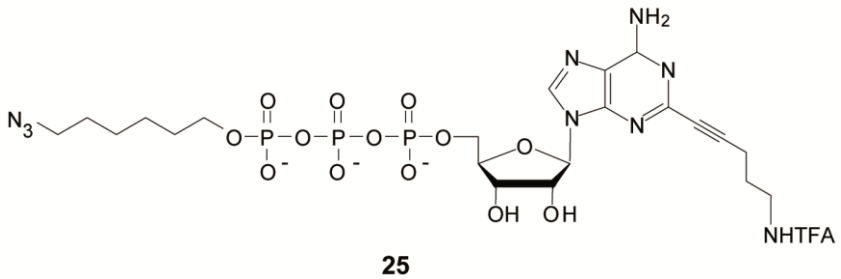
^{13}C NMR (MeOD-d_4, 101 MHz): δ 158.22 (q, J = 36.5 Hz), 148.93, 145.82, 140.14, 117.25 (d, J = 26.3 Hz), 114.28, 87.89, 86.47, 83.73, 79.06, 74.28, 70.18, 65.10, 38.74, 26.26, 15.78.

^{19}F NMR (MeOD-d_4, 376 MHz): δ -75.79 (s, 3F).

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.38 (d, J = 21.0 Hz, 1P), -11.39 (d, J = 21.2 Hz, 1P), -23.65 (t, *J* = 20.7 Hz, 1P).

HR-ESI-MS: found: 683.02920; calculated: 683.02750 ($M\text{-}H^+$, $C_{17}H_{21}F_3N_6O_{14}P_3^-$); deviation: 2.5 ppm.

γ-(6-Azidohexyl)-2-(5-trifluoroacetamidopent-1-yn-1-yl)-adenosine-triphosphate 25



25

Nucleotide **5** (89.4 µmol, 1.0 eq.) and 1-azido-6-bromohexane (55 mg, 267 µmol, 3.0 eq.) were dissolved in 4 mL DMF each and dried over molecular sieve over night. Next, the two solutions were combined and slightly stirred at room temperature over night. The solvents were evaporated under reduced pressure and the crude product was purified by MPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 62.4 µmol of product **25** (70%) as white fluffs.

^{1}H NMR (D_2O, 400 MHz): δ 8.60 (s, 1H, H8), 6.11 (d, J = 5.7 Hz, 1H, H1'), 4.77 – 4.72 (m, 1H, H2'), 4.57 (dd, J = 4.9, 3.7 Hz, 1H, H3'), 4.43 – 4.38 (m, 1H, H4'), 4.30 – 4.25 (m, 2H, H5', H5''), 3.90 – 3.80 (m, 2H, γP-O-CH_2), 3.54 (t, J = 6.8 Hz, 2H, NHTFA-C**H**$_2$), 3.20 (t, J = 7.0 Hz, 2H, N_3-C**H**$_2$), 2.58 (t, J = 6.9 Hz, 2H, CC-C**H**$_2$), 2.01 – 1.94 (m, 2H, CC-CH_2-C**H**$_2$), 1.46 – 1.34 (m, 4H, 2 x C**H**$_2$-linker), 1.16 – 1.09 (m, 4H, 2 x C**H**$_2$-linker).

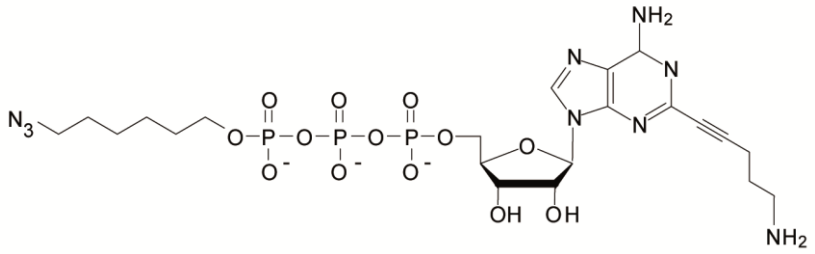
^{13}C NMR (MeOD-d_4, 101 MHz): δ 158.90 (d, J = 36.8 Hz), 157.02, 150.97, 147.67, 141.68, 119.19 (d, J = 54.2 Hz), 116.07, 88.87, 86.66, 85.47, 81.82, 76.25, 71.60, 66.90 (d, J = 6.1 Hz), 66.33, 52.33, 39.95, 31.67 (d, J = 8.1 Hz), 29.80, 28.47, 27.53, 26.44, 17.22.

^{19}F NMR (D_2O, 376 MHz): δ -75.80 (s, 3F).

^{31}P NMR (D_2O, 162 MHz): δ -11.02 (d, J = 18.4 Hz, 1P), -11.59 (d, J = 18.4 Hz, 1P), -23.38 (t, J = 19.4 Hz, 1P).

HR-ESI-MS: found: 808.1255; calculated: 808.1228 (M-H$^+$, $C_{23}H_{32}F_3N_9O_{14}P_3^-$); deviation: 3.3 ppm.

γ-(6-Azidohexyl)-2-(5-aminopent-1-yn-1-yl)-adenosine-triphosphate 26



26

Compound **25** (62.4 µmol, 1.0 eq.) was dissolved in 3 mL water and 6 mL of 33% aqueous ammonia was added. The reaction was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure and the crude product was purified by MPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 33.1 µmol of product **26** (53%) as white fluffs.

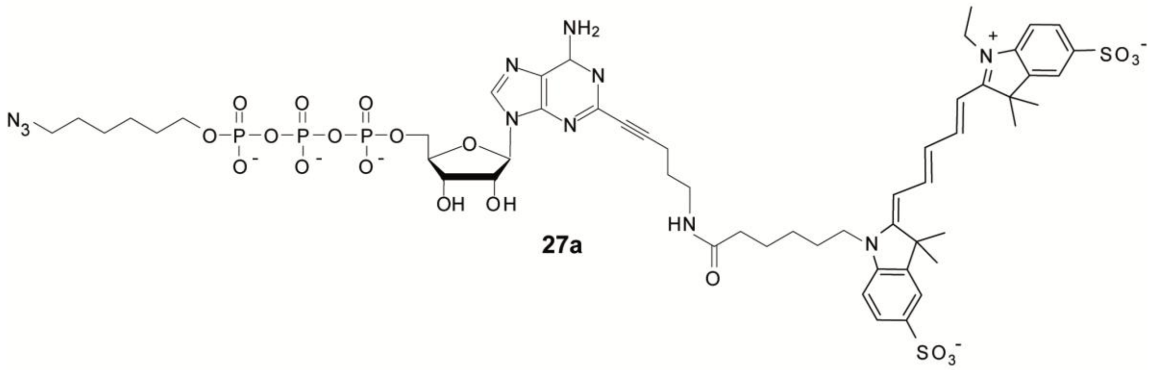
^{1}H NMR (D_2O, 400 MHz): δ 8.56 (s, 1H, H8), 6.17 (d, J = 5.9 Hz, 1H, H1'), 4.77 – 4.74 (m, 1H, H2'), 4.59 (dd, J = 5.1, 3.6 Hz, 1H, H3'), 4.44 – 4.37 (m, 1H, H4'), 4.31 – 4.22 (m, 2H, H5', H5''), 3.95 – 3.85 (m, 2H, γP-O-CH_2), 3.76 (m, 2H, NH_2-C**H**$_2$), 3.22 (t, J = 7.0 Hz, 2H, N_3-C**H**$_2$), 2.64 (t, J = 8.2 Hz, 2H, CC-C**H**$_2$), 1.97 – 1.90 (m, 2H, CC-CH_2-C**H**$_2$), 1.51 – 1.39 (m, 4H, 2 x C**H**$_2$-linker), 1.20 (m, 4H, 2 x C**H**$_2$-linker).

^{13}C NMR (D_2O, 101 MHz): δ 151.64, 113.89, 86.56, 85.67, 82.96, 67.04, 66.61, 52.42, 34.31, 31.80, 31.72, 29.90, 27.62, 26.55, 22.81.

^{31}P NMR (D_2O, 162 MHz): δ -10.99 (d, J = 21.2 Hz, 1P), -11.56 (d, J = 20.3 Hz, 1P), -23.38 (t, J = 19.5 Hz, 1P).

HR-ESI-MS: found: 713.1423; calculated: 713.1405 ($M\text{-}H^+$, $C_{21}H_{33}N_9O_{13}P_3^-$); deviation: 2.5 ppm.

γ-(6-Azidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 27a



Compound **26** (33.1 µmol, 1.0 eq.) was dissolved in 5 mL 0.1 M $NaHCO_3$ (pH 8.7) and sulfo-cyanine5-NHS (50.0 mg, 66.4 µmol, 2.0 eq.), dissolved in 1 mL dry DMF was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 19.1 µmol of product **27a** (58%).

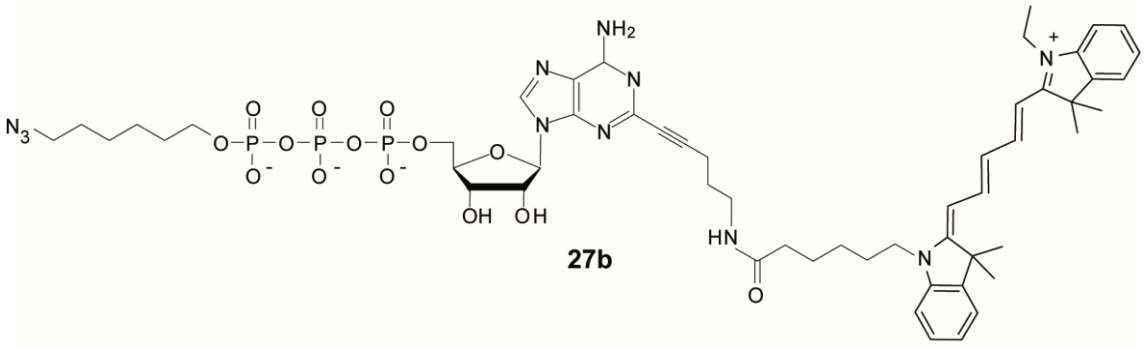
^{1}H NMR (MeOD-*d*$_4$, 400 MHz): δ 8.62 (s, 1H, H8), 8.33 (t, J = 13.0 Hz, 2H, H-β-Cy5), 7.95 – 7.82 (m, 4H, H-Ar-Cy5), 7.37 (d, J = 6.1 Hz, 1H, H-Ar-Cy5), 7.35 (d, J = 5.9 Hz, 1H, H-Ar-Cy5), 6.71 (t, J = 12.4 Hz, 1H, H-γ-Cy5), 6.37 (d, J = 13.6 Hz, 2H, H-α-Cy5), 6.08 (d, J = 5.4 Hz, 1H, H1'), 4.67 (t, J = 5.1 Hz, 1H, H2'), 4.58 – 4.51 (m, 1H, H3'), 4.38 – 4.26 (m, 1H, H4'), 4.25 – 4.07 (m, 6H, H5', H5'', 2 x C**H**$_2$-linker), 3.99 (q, J = 6.5 Hz, 2H, C**H**$_2$-linker), 3.29 – 2.24 (m, 4H, 2 x C**H**$_2$-linker), 2.48 – 2.41 (t, J = 7.1 Hz, 2H, C**H**$_2$-linker), 2.22 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 1.81 -1.75 (m, 5H, C**H**$_3$-ethyl, C**H**$_2$-linker), 1.78 – 1.72 (m, 12H, 4 x CH_3-Cy5), 1.70 – 1.64 (m, 2H, C**H**$_2$-linker), 1.64 – 1.58 (m, 2H, C**H**$_2$-linker), 1.58 – 1.50 (m, 2H, C**H**$_2$-linker), 1.49 – 1.41 (m, 2H, C**H**$_2$-linker), 1.37 – 1.34 (m, 8H, 4 x C**H**$_2$-linker).

^{13}C NMR (MeOD-*d*$_4$, 101 MHz): δ 175.79, 175.27, 174.88, 157.12, 156.32, 151.27, 147.90, 144.94, 144.47, 143.65, 143.61, 142.83, 142.70, 141.75, 128.11, 121.35, 119.57, 111.88, 111.57, 105.54, 105.20, 88.78, 87.45, 85.63, 81.98, 76.33, 71.86, 67.00 (d, *J* = 6.2 Hz), 66.55, 52.45, 50.60, 50.58, 39.77, 36.73, 31.80 (d, *J* = 8.2 Hz), 29.92, 29.21, 28.36, 28.09, 27.92, 27.64, 27.32, 26.56, 17.59, 12.73.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.22 (d, J = 18.5 Hz, 1P), -11.75 (d, J = 18.6 Hz, 1P), -23.06 (t, J = 14.9 Hz, 1P).

HR-ESI-MS: found: 1350.3569; calculated: 1350.3526 ($M\text{-}H^+$, $C_{54}H_{71}N_{11}O_{20}P_3S_2^-$); deviation: 3.2 ppm.

γ-(6-Azidohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 27b



Compound **26** (30.0 µmol, 1.0 eq.) was dissolved in 5 mL 0.1 M $NaHCO_3$ (pH 8.7) and cyanine5-NHS (50.0 mg, 84.1 µmol, 2.8 eq.), dissolved in 1 mL dry DMF was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by anion-exchange chromatography. Fractions containing the product were evaporated and further purified by MPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 14.1 µmol of product **27b** (47%).

^{1}H NMR (MeOD-d_4, 400 MHz): δ 8.66 (s, 1H, H8), 8.22 (t, J = 13.0 Hz, 2H, H-β-Cy5), 7.49 – 7.31 (m, 4H, H-Ar-Cy5), 7.27 – 7.18 (m, 4H, H-Ar-Cy5), 6.58 (t, J = 12.3 Hz, 1H, H-γ-Cy5), 6.24 (dd, J = 13.5, 5.9 Hz, 2H, H-α-Cy5), 6.07 (d, J = 5.7 Hz, 1H, H1'), 4.70 (t, J = 5.1 Hz, 1H, H2'), 4.64 – 4.56 (m, 1H, H3'), 4.37 – 4.27 (m, 1H, H4'), 4.24 – 4.14 (m, 2H, H5', H5''), 4.14 – 4.05 (m, 2H, C**H**$_2$-linker), 4.00 (q, J = 6.5 Hz, 2H, C**H**$_2$-linker), 3.61 (s, 3H, CH_3-Cy5), 3.29 – 3.21 (m, 4H, 2 x C**H**$_2$-linker), 2.43 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 2.22 (t,

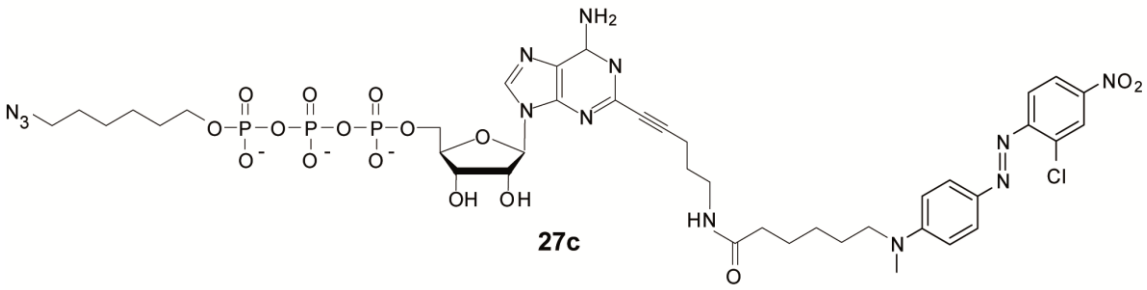
J = 6.7 Hz, 2H, C**H**$_2$-linker), 1.85 – 1.72 (m, 4H, 2 x C**H**$_2$-linker), 1.64 – 1.56 (m, 2H, C**H**$_2$-linker), 1.56 – 1.47 (m, 2H, C**H**$_2$-linker), 1.44 – 1.32 (m, 8H, 4 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 101 MHz): δ 175.90, 175.34, 174.73, 156.92, 155.39, 155.30, 151.20, 147.63, 144.17, 143.53, 142.66, 142.50, 141.96, 129.76, 129.66, 126.73, 126.19, 123.42, 123.21, 119.46, 112.06, 111.85, 104.60, 104.50, 104.41, 88.56, 88.08, 87.60, 85.94, 85.84, 81.79, 76.37, 71.96, 66.99, 52.44, 50.54, 50.48, 44.80, 39.84, 36.66, 36.49, 31.77, 31.70, 29.90, 29.02, 28.38, 28.11, 27.89, 27.62, 27.25, 26.42, 17.59.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.72 (d, J = 18.3 Hz, 1P), -11.26 (d, J = 18.9 Hz, 1P), -22.41 (t, J = 16.5 Hz, 1P).

HR-ESI-MS: found: 1176.4250; calculated: 1176.4233 ($M\text{-}H^+$, $C_{53}H_{69}N_{11}O_{14}P_3^-$); deviation: 1.5 ppm.

γ-(6-Azidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 27c



27c

Compound **26** (30.0 µmol, 1.0 eq.) was dissolved in 5 mL 0.1 M $NaHCO_3$ (pH 8.7) and eclipse-NHS (50.0 mg, 102.1 µmol, 3.4 eq.), dissolved in 2 mL dry DMF was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 7.7 µmol of product **27c** (26%).

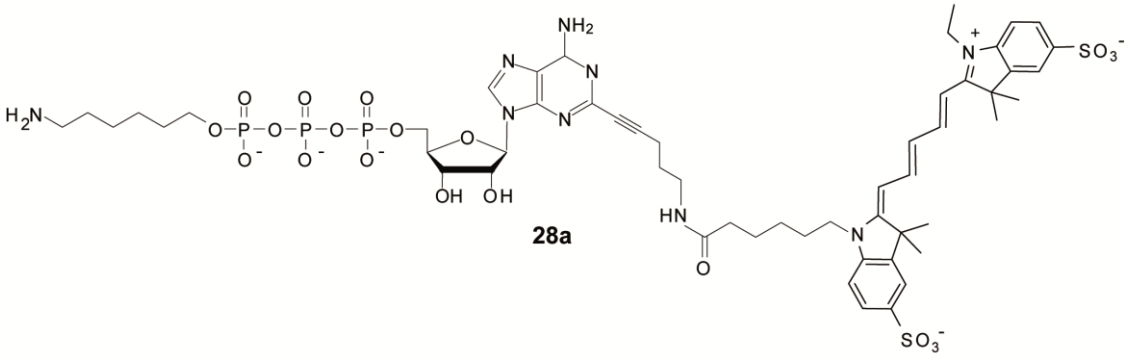
^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.65 (s, 1H, H8), 8.35 (d, J = 2.3 Hz, 1H, H-Ar_2-eclipse), 8.16 (dd, J = 8.9, 2.3 Hz, 1H, H-Ar_2-eclipse), 7.87 (d, J = 9.1 Hz, 2H, H-Ar_1-eclipse), 7.76 (d, J = 8.9 Hz, 1H, H-Ar_2-eclipse), 6.83 (d, J = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.05 (d, J = 5.2 Hz, 1H, H1'), 4.62 (t, J = 5.0 Hz, 1H, H2'), 4.54 (t, J = 4.3 Hz, 1H, H3'), 4.36 – 4.30 (m, 1H, H4'), 4.26 (dd, J = 14.4, 7.0 Hz, 2H, H5', H5''), 4.01 (q, J = 6.1 Hz, 2H, C**H**$_2$-linker), 3.56 – 3.47 (m, 2H, C**H**$_2$-linker), 3.36 (t, J = 6.6 Hz, 2H, C**H**$_2$-linker), 3.23 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.50 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 2.32 (t, J = 7.1 Hz, 2H, C**H**$_2$-linker), 2.02 – 1.91 (m, 2H, C**H**$_2$-linker), 1.84 (p, J = 6.8 Hz, 2H, C**H**$_2$-linker), 1.68 – 1.57 (m, 2H, C**H**$_2$-linker), 1.58 – 1.48 (m, 2H, C**H**$_2$-linker), 1.46 – 1.32 (m, 4H, 2 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 175.43, 154.71, 154.36, 150.74, 148.48, 145.49, 142.13, 134.66, 127.90, 126.80, 123.89, 119.07, 112.69, 89.07, 85.62, 80.78, 76.51, 71.66, 67.32, 66.46, 52.69, 52.42, 39.85, 38.79, 33.88, 31.67, 31.62, 29.88, 28.83, 27.57, 26.45, 24.09, 17.57.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.13 (d, J = 17.4 Hz, 1P), -11.45 (d, J = 19.0 Hz, 1P), -22.94 (t, J = 17.9 Hz, 1P).

HR-ESI-MS: found: 1070.2238; calculated: 1070.2238 (M-H$^+$, $C_{38}H_{48}ClN_{13}O_{16}P_3^-$); deviation: 0.1 ppm.

γ-(6-Aminohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 28a



28a

Compound **27a** (19.1 µmol, 1.0 eq.) was dissolved in 5 mL H_2O/MeOH/NEt_3 (1.25:2.5:1.25) and tris(2-carboxyethyl)phosphine (34.0 mg, 135.9 µmol, 7.1 eq.), dissolved in H_2O (1.25 mL) was added rapidly and stirred at room temperature for 3 h. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 12.6 µmol of product **28a** (66%).

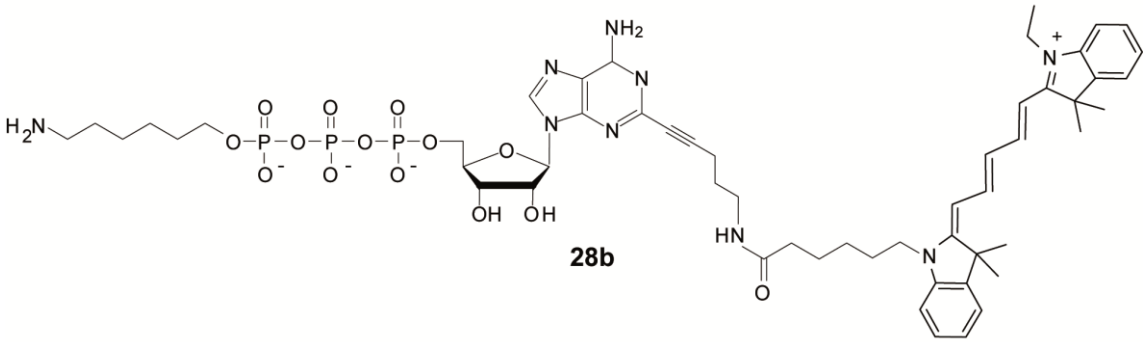
^{1}H NMR (MeOD-d_4, 400 MHz): δ 8.66 (s, 1H, H8), 8.31 (t, J = 12.9 Hz, 2H, H-β-Cy5), 7.92 – 7.84 (m, 4H, H-Ar-Cy5), 7.36 (d, J = 6.5 Hz, 1H, H-Ar-Cy5), 7.34 (d, J = 6.5 Hz, 1H, H-Ar-Cy5), 6.70 (t, J = 12.3 Hz, 1H, H-γ-Cy5), 6.36 (d, J = 13.6 Hz, 2H, H-α-Cy5), 6.08 (d, J = 4.8 Hz, 1H, H1'), 4.62 (t, J = 4.8 Hz, 1H, H2'), 4.59 – 4.51 (m, 1H, H3'), 4.34 – 4.26 (m, 1H, H4'), 4.26 – 4.08 (m, 6H, H5', H5'', 2 x C**H**$_2$-linker), 3.97 (dd, J = 12.1, 5.6 Hz, 2H, C**H**$_2$-linker), 3.28 (t, J = 6.8 Hz, 2H, C**H**$_2$-linker), 2.95 (t, J = 6.8 Hz, 2H, C**H**$_2$-linker), 2.44 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 2.22 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 1.83 – 1.76 (m, 3H, C**H**$_3$-ethyl), 1.74 – 1.71 (m, 12H, 4 x CH_3-Cy5), 1.70 – 1.65 (m, 4H, 2 x C**H**$_2$-linker), 1.63 – 1.53 (m, 2H, C**H**$_2$-linker), 1.44 (d, J = 7.4 Hz, 2H, C**H**$_2$-linker), 1.41 – 1.34 (m, 8H, 4 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 101 MHz): δ 175.81, 175.25, 174.88, 157.14, 156.33, 156.17, 151.07, 147.87, 144.95, 144.47, 143.60, 143.56, 142.83, 142.70, 141.76, 128.11, 121.34, 119.56, 111.87, 111.58, 105.51, 105.22, 89.09, 87.49, 85.45, 81.95, 76.47, 71.58, 66.64 (d, J = 6.6 Hz), 66.41, 50.60, 40.44, 39.77, 36.74, 31.19 (d, J = 7.8 Hz), 29.20, 28.39, 28.34, 28.08, 27.91, 27.33, 26.72, 26.56, 26.10, 17.58, 12.72.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.10 (d, J = 18.5 Hz, 1P), -11.75 (d, J = 19.4 Hz, 1P), -22.91 (t, J = 18.4 Hz, 1P).

HR-ESI-MS: found: 1324.3663; calculated: 1324.3621 (M-H$^+$, $C_{54}H_{73}N_9O_{20}P_3S_2^-$); deviation: 3.2 ppm.

γ-(6-Aminohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 28b



28b

Compound **27b** (14.1 µmol, 1.0 eq.) was dissolved in 4 mL H_2O/MeOH/NEt_3 (1:2:1) and tris(2-carboxyethyl)phosphine (35.0 mg, 139.9 µmol, 9.9 eq.), dissolved in water (1.0 mL) was added rapidly and stirred at room temperature for 4 h. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 12.5 µmol of product **28b** (89%).

^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.58 (s, 1H, H8), 8.08 (t, J = 13.0 Hz, 2H, H-β-Cy5), 7.27 (ddd, J = 24.5, 14.7, 7.4 Hz, 4H, H-Ar-Cy5), 7.19 – 7.03 (m, 4H, H-Ar-Cy5), 6.44 (t, J = 12.3 Hz, 1H, H-γ-Cy5), 6.11 (dd, J = 13.7, 8.0 Hz, 2H, H-α-Cy5), 5.94 (d, J = 4.2 Hz, 1H, H1'), 4.55 – 4.45 (m, 2H, H2', H3'), 4.27 – 4.17 (m, 1H, H4'), 4.17 – 4.05 (m, 2H, H5', H5''), 3.99 (t, J = 6.7 Hz, 2H, C**H**$_2$-linker), 3.85 (dd, J = 11.9, 5.7 Hz, 2H, C**H**$_2$-linker), 3.50 (s, 3H, CH_3-Cy5), 3.17 (t, J = 6.8 Hz, 2H, C**H**$_2$-linker), 2.85 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 2.33 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 2.12 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 1.73 – 1.63 (m, 4H, 2 x C**H**$_2$-linker), 1.57 – 1.53 (m, 14H, 4 x CH_3-Cy5, C**H**$_2$-linker), 1.45 – 1.38 (m, 2H, C**H**$_2$-linker), 1.34 – 1.23 (m, 8H, 4 x C**H**$_2$-linker).

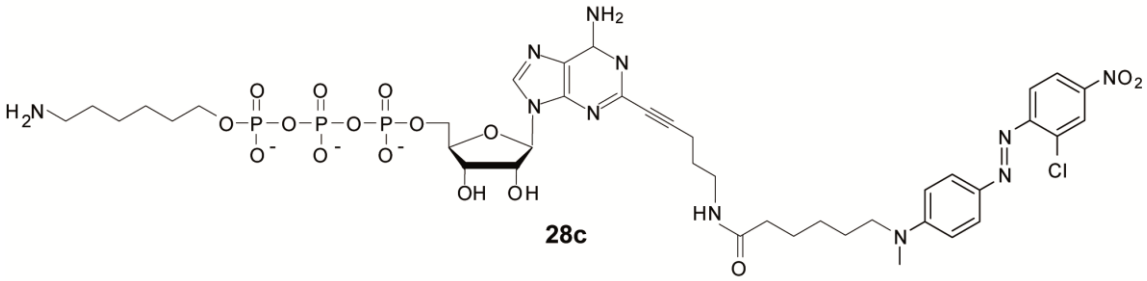
^{13}C NMR (MeOD-d_4, 151 MHz): δ 174.51, 173.89, 173.21, 155.59, 153.90, 153.80, 149.43, 146.25, 142.73, 142.10, 141.22, 141.07, 140.38, 128.34, 128.23, 125.25, 124.75, 121.98, 121.76, 118.10, 110.63, 110.44, 103.10, 102.96, 87.70, 86.14, 84.01, 83.96, 80.43, 75.21, 69.89, 65.03, 64.99, 64.63, 49.10, 49.05, 43.39, 38.82, 38.41,

35.28, 30.34, 29.53, 29.48, 27.62, 27.41, 26.94, 26.82, 26.69, 26.48, 25.86, 25.04, 24.94, 24.87, 21.87, 19.26, 19.04, 16.17, 10.14, 10.10.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.63 (d, J = 18.3 Hz, 1P), -11.40 (d, J = 18.5 Hz, 1P), -22.38 (t, J = 17.8 Hz, 1P).

HR-ESI-MS: found: 1150.4330; calculated: 1150.4328 (M-H$^+$, $C_{53}H_{71}N_9O_{14}P_3^-$); deviation: 0.2 ppm.

γ-(6-Aminohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 28c



Compound **27c** (7.7 µmol, 1.0 eq.) was dissolved in 5 mL H_2O/MeOH/NEt_3 (0.5:1.0:0.5) and tris(2-carboxyethyl)phosphine (25.0 mg, 99.9 µmol, 13.0 eq.), dissolved in water (0.5 mL) was added rapidly and stirred at room temperature for 4 h. The solvents were evaporated under reduced pressure. The compound was purified by RP-HPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 4.4 µmol of product **28c** (57%).

^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.63 (s, 1H, H8), 8.33 (d, J = 2.4 Hz, 1H, H-Ar_2-eclipse), 8.14 (dd, J = 8.9, 2.4 Hz, 1H, H-Ar_2-eclipse), 7.85 (d, J = 9.2 Hz, 2H, H-Ar_1-eclipse), 7.74 (d, J = 8.9 Hz, 1H, H-Ar_2-eclipse), 6.82 (d, J = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.03 (d, J = 4.0 Hz, 1H, H1'), 4.57 (d, J = 2.0 Hz, 2H, H2', H3'), 4.34 – 4.29 (m, 1H, H4'), 4.29 – 4.19 (m, 2H, H5', H5''), 3.98 (dd, J = 6.1, 3.7 Hz, 2H, C**H**$_2$-linker), 3.55 – 3.48 (m, 2H, C**H**$_2$-linker), 3.35 (t, J = 6.7 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.96 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 2.48 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 2.31 (t, J = 7.1 Hz, 2H, C**H**$_2$-linker), 2.00 – 1.98

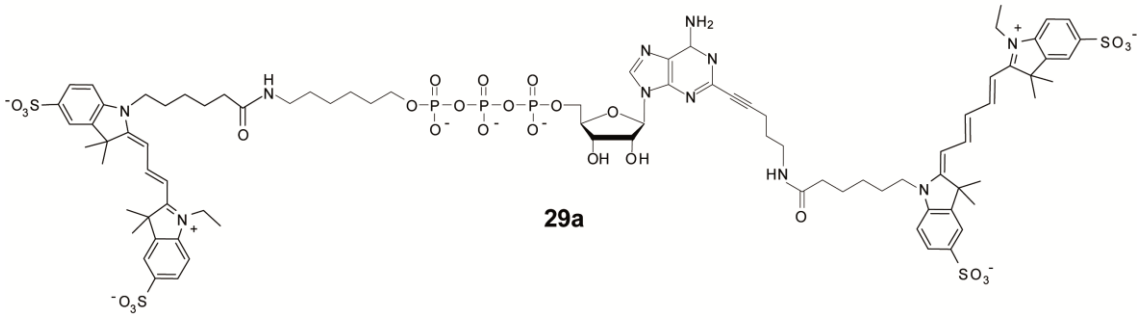
– 1.92 (m, 2H, C**H**$_2$-linker), 1.83 (p, J = 6.9 Hz, 2H, C**H**$_2$-linker), 1.72 – 1.64 (m, 2H, C**H**$_2$-linker), 1.61 – 1.54 (m, 2H, C**H**$_2$-linker), 1.46 – 1.40 (m, 4H, 2 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 173.96, 155.56, 153.27, 152.91, 149.35, 147.03, 146.28, 144.06, 140.28, 133.25, 126.49, 125.37, 122.42, 118.08, 117.62, 111.25, 87.86, 86.07, 83.92, 83.87, 80.19, 75.14, 69.82, 64.76, 51.24, 38.71, 38.42, 37.37, 32.44, 29.22, 29.17, 27.50, 26.63, 24.47, 24.17, 22.66, 22.49, 16.12.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.63 (d, J = 19.0 Hz, 1P), -11.34 (d, J = 18.9 Hz, 1P), -22.02 – -22.96 (m, 1P).

HR-ESI-MS: found: 1044.2354; calculated: 1044.2333 (M-H$^+$, $C_{38}H_{50}ClN_{11}O_{16}P_3^-$); deviation: 2.0 ppm.

γ-(6-Sulfo-cyanine3-amidohexyl)-2-(5-sulfo-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 29a



29a

Compound **28a** (12.6 µmol, 1.0 eq.) was dissolved in 3 mL 0.1 M $NaHCO_3$ (pH 8.7) and sulfo-cyanine3-NHS (21.0 mg, 28.9 µmol, 2.3 eq.), dissolved in dry DMF (0.66 mL) was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 10.9 µmol of product **29a** (86%).

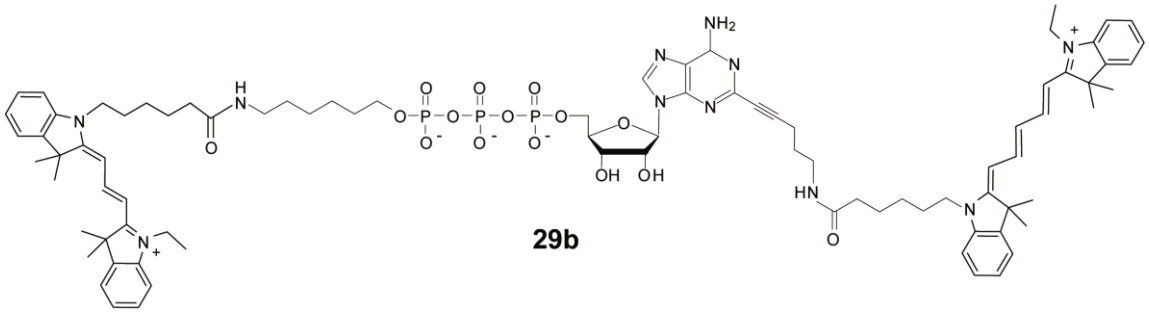
^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.64 (s, 1H, H8), 8.52 (t, J = 13.4 Hz, 1H, H-β-Cy3), 8.33 (t, J = 12.9 Hz, 2H, H-β-Cy5), 7.95 (dd, J = 3.6, 1.4 Hz, 2H, H-Ar-Cy3), 7.92 (ddd, J = 8.3, 4.4, 1.4 Hz, 2H, H-Ar-Cy3), 7.90 – 7.86 (m, 4H, H-Ar-Cy5), 7.46 (d, J = 5.1 Hz, 1H, H-Ar-Cy3), 7.44 (d, J = 5.1 Hz, 1H, H-Ar-Cy3), 7.37 (d, J = 8.8 Hz, 1H, H-Ar-Cy5), 7.35 (d, J = 8.9 Hz, 1H, H-Ar-Cy5), 6.75 (t, J = 12.3 Hz, 1H, H-γ-Cy5), 6.56 (dd, J = 16.5, 13.6 Hz, 2H, H-α-Cy3), 6.39 (d, J = 13.6 Hz, 2H, H-α-Cy5), 6.10 (d, J = 5.7 Hz, 1H, H1'), 4.71 (s, 1H, H2'), 4.58 – 4.53 (m, 1H, H3'), 4.36 – 4.29 (m, 1H, H4'), 4.28 – 4.11 (m, 10H, H5', H5'', 4 x C**H**$_2$-linker), 3.99 (dd, J = 10.9, 4.5 Hz, 2H, C**H**$_2$-linker), 3.28 – 3.22 (m, 8H, 4 x C**H**$_2$-linker), 3.12 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 2.39 (t, J = 6.9 Hz, 2H, C**H**$_2$-linker), 2.21 (td, J = 7.1, 4.2 Hz, 4H, 2 x C**H**$_2$-linker), 1.85 – 1.80 (m, 3H, CH_3-ethyl-Cy5), 1.78 – 1.75 (m, 12H, 4 x CH_3-Cy3), 1.74 – 1.70 (m, 12H, 4 x CH_3-Cy5), 1.70 – 1.63 (m, 9H, CH_3-ethyl-Cy3, 3 x C**H**$_2$-linker), 1.63 – 1.55 (m, 2H, C**H**$_2$-linker), 1.51 – 1.28 (m, 12H, 6 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 176.64, 176.37, 175.74, 175.64, 175.26, 174.84, 157.09, 152.82, 147.89, 144.91, 144.69, 144.45, 144.41, 144.36, 144.24, 143.73, 143.71, 142.81, 142.68, 142.42, 142.28, 128.36, 128.11, 121.50, 121.35, 119.57, 114.73, 112.36, 112.16, 111.89, 111.57, 105.56, 105.23, 104.90, 104.74, 88.99, 88.65, 88.43, 87.39, 85.94, 82.05, 76.24, 66.98, 59.57, 59.55, 59.52, 50.73, 50.68, 50.57, 40.38, 39.77, 36.86, 31.80, 31.72, 30.40, 29.24, 28.42, 28.36, 28.30, 28.11, 27.94, 27.79, 27.42, 27.32, 26.74, 26.63, 26.58, 24.85, 20.77, 17.59, 14.09, 12.88, 12.75.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.16 (d, J = 18.3 Hz, 1P), -11.68 (d, J = 18.1 Hz, 1P), -22.09 – -23.81 (m, 1P).

HR-ESI-MS: found: 967.7786; calculated: 967.7750 (M-2H$^+$, $C_{85}H_{108}N_{11}O_{27}P_3S_4^{2-}$); deviation: 3.7 ppm.

γ-(6-Cyanine3-amindohexyl)-2-(5-cyanine5-amidopent-1-yn-1-yl)-adenosine-triphosphate 29b



Compound **28b** (12.5 µmol, 1.0 eq.) was dissolved in 3.5 mL 0.1 M $NaHCO_3$ (pH 8.7) and cyanine3-NHS (25.0 mg, 44.0 µmol, 3.5 eq.), dissolved in dry DMF (0.7 mL) was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 5.2 µmol of product **29b** (42%).

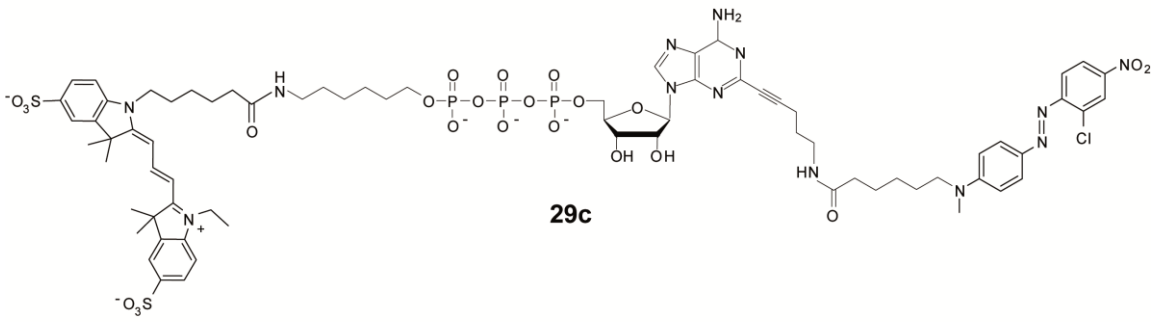
^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.70 (s, 1H, H8), 8.45 (t, J = 13.5 Hz, 1H, H-β-Cy3), 8.22 (t, J = 13.0 Hz, 2H, H-β-Cy5), 7.50 (dd, J = 6.9, 5.1 Hz, 2H, H-Ar-Cy3), 7.47 – 7.40 (m, 4H, H-Ar-Cy3), 7.35 (ddd, J = 16.3, 14.5, 8.0 Hz, 4H, H-Ar-Cy5), 7.30 – 7.18 (m, 6H, H-Ar-Cy3, H-Ar-Cy5), 6.59 (t, J = 12.4 Hz, 1H, H-γ-Cy3), 6.36 (dd, J = 13.4, 3.1 Hz, 2H, H-α-Cy3), 6.24 (dd, J = 13.3, 10.8 Hz, 2H, H-α-Cy5), 6.08 (d, J = 5.7 Hz, 1H, H1'), 4.58 (s, 1H, H2'), 4.31 – 4.24 (m, 1H, H3'), 4.19 (s, 1H, H4'), 4.13 (t, J = 7.3 Hz, 4H, H5', H5'', C**H**$_2$-linker), 4.09 (t, J = 7.3 Hz, 2H, C**H**$_2$-linker), 3.98 (dd, J = 12.7, 6.3 Hz, 2H, C**H**$_2$-linker), 3.68 (s, 3H, CH_3-Cy3), 3.60 (s, 3H, CH_3-Cy5), 3.24 (t, J = 6.8 Hz, 2H, C**H**$_2$-linker), 3.11 (t, J = 6.7 Hz, 2H, C**H**$_2$-linker), 2.34 (t, J = 7.1 Hz, 2H, C**H**$_2$-linker), 2.22 (q, J = 7.0 Hz, 4H, 2 x C**H**$_2$-linker), 1.87 – 1.78 (m, 4H, 2 x C**H**$_2$-linker), 1.74 – 1.70 (m, 12H, 4x CH_3-Cy3), 1.70 – 1.64 (m, 18H, 4 x CH_3-Cy5, 3 x C**H**$_2$-linker), 1.62 – 1.55 (m, 2H, C**H**$_2$-linker), 1.52 – 1.45 (m, 2H, C**H**$_2$-linker), 1.45 – 1.39 (m, 4H, 2 x C**H**$_2$-linker), 1.35 (q, J = 7.6 Hz, 4H, 2 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 176.51, 175.88, 175.86, 175.37, 174.71, 156.91, 155.44, 155.35, 151.90, 147.55, 144.17, 144.12, 143.54, 143.42, 142.65, 142.49, 142.20, 142.09, 130.02, 129.97, 129.75, 129.67, 126.69, 126.18, 123.49, 123.43, 123.29, 123.22, 119.47, 112.49, 112.41, 112.04, 111.85, 104.49, 104.37, 103.84, 103.70, 88.57, 87.44, 81.85, 76.43, 72.22, 68.46, 66.92, 50.59, 50.54, 50.49, 45.21, 44.77, 40.27, 39.81, 36.77, 36.66, 32.06, 31.68, 31.37, 30.10, 29.00, 28.38, 28.23, 28.09, 27.86, 27.46, 27.37, 27.26, 26.70, 26.43, 26.35, 21.13, 17.53.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -9.42 – -9.98 (m, 1P), -10.42 (d, J = 15.0 Hz, 1P), -19.86 – -20.95 (m, 1P).

HR-ESI-MS: found: 1588.6975; calculated: 1588.6999 (M-H$^+$, $C_{83}H_{105}N_{11}O_{15}P_3^-$); deviation: 1.5 ppm.

γ-(6-Sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 29c



Compound **28c** (4.4 µmol, 1.0 eq.) was dissolved in 1 mL 0.1 M $NaHCO_3$ (pH 8.7) and sulfo-cyanine3-NHS (25.0 mg, 34.4 µmol, 7.8 eq.), dissolved in dry DMF (1.0 mL) was added and stirred at room temperature over night. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze dried from water to give 3.5 µmol of product **29c** (80%).

^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.68 (s, 1H, H8), 8.46 (t, J = 13.5 Hz, 1H, H-β-Cy3), 8.36 (d, J = 2.3 Hz, 1H, H-Ar_2-eclipse), 8.16 (dd, J = 8.9, 2.4 Hz, 1H, H-Ar_2-eclipse), 7.98 – 7.89 (m, 4H, H-Ar-Cy3), 7.86 (d, J = 9.1 Hz, 2H, H-Ar_1-eclipse), 7.76 (d, J = 8.9 Hz, 1H, H-Ar_2-eclipse), 7.39 (t, J = 8.7 Hz, 2H, H-Ar-Cy3), 6.84 (d, J = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.44 (dd, J = 22.7, 13.4 Hz, 2H, H-α-Cy3), 6.08 (d, J = 6.0 Hz, 1H, H1'), 4.58 – 4.50 (m, 1H, H2'), 4.35 – 4.28 (m, 1H, H3'), 4.27 – 4.23 (m, 1H, H4'), 4.23 – 4.16 (m, 4H, H5', H5'', C**H**$_2$-linker), 4.16 – 4.08 (m, 2H, C**H**$_2$-linker), 4.04 – 3.92 (m, 2H, C**H**$_2$-linker), 3.55 – 3.48 (m, 2H, C**H**$_2$-linker), 3.13 (t, J = 6.7 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.38 (t, J = 7.0 Hz, 2H, C**H**$_2$-linker), 2.31 (t, J = 7.1 Hz, 2H, C**H**$_2$-linker), 2.22 (t, J = 7.4 Hz, 2H, C**H**$_2$-linker), 1.97 – 1.92 (m, 2H, C**H**$_2$-linker), 1.83 – 1.80 (m, 2H, C**H**$_2$-linker), 1,78 – 1.72 (m, 12H, 4 x CH_3-Cy5), 1.67 (dt, J = 14.6, 7.3 Hz, 2H, C**H**$_2$-linker), 1.62 – 1.53 (m, 2H, C**H**$_2$-linker), 1.46 (dq, J = 13.3, 6.7 Hz, 4H, 2 x C**H**$_2$-linker), 1.39 (t, J = 7.2 Hz, 3H, CH_3-ethyl), 1.36 – 1.27 (m, 6H, 3 x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 177.57, 173.91, 173.70, 173.14, 172.68, 154.28, 152.05, 151.64, 150.07, 148.53, 145.78, 145.00, 142.76, 141.98, 141.56, 141.38, 141.33, 139.72, 139.57, 131.98, 125.61, 125.30, 124.18, 121.26, 118.81, 118.76, 116.76, 116.39, 110.08, 109.55, 109.37, 101.97, 101.83, 85.85, 84.75, 78.98, 73.54, 64.32, 50.02, 48.04, 47.98, 46.91, 42.85, 38.09, 37.64, 37.11, 36.20, 34.16, 31.18, 28.83, 27.51, 26.33, 25.63, 25.60, 25.50, 24.87, 24.76, 24.03, 23.74, 21.53, 21.46, 14.82, 10.07.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.52 (d, J = 17.4 Hz, 1P), -11.03 (d, J = 17.0 Hz, 1P), -20.56 – -21.40 (m, 1P).

HR-ESI-MS: found: 827.7091; calculated: 827.7107 ($M\text{-}2H^+$, $C_{69}H_{85}ClN_{13}O_{23}P_3S_2^{2-}$); deviation: 1.9 ppm.

Proof of concept

General procedures

SVPD cleavage: 2.5 µL (1 mM) of the corresponding doubly dye labeled ATP analogue and 3.13 µL SVPD (0.2 $mg \cdot mL^{-1}$) were processed in a total volume of 25 µL of NEB buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.9) for 30 min at 30°C. Negative probes were performed in the absence of SVPD.

Fluorescence spectra: For fluorescence measurements the SVPD reactions were diluted with 3 mL 1x PBS buffer and the fluorescence spectra of the crude reaction solution was measured. Excitation of Sulfo-Cy3 and Cy3 were performed using a wavelength of 532 nm.

HPLC analysis general procedure: For HPLC analysis the SVPD reactions were diluted with 275 µL of H_2O and separated by analytical RP-HPLC (a linear gradient of acetonitrile in 50 mM TEAA buffer, pH 7.0 was used). RP-HPLC analytics were visualized using 2D-plots. Fractions containing ATP fragments were collected and further characterized by HRMS.

SVPD cleavage and analysis of γ-sulfo-Cy3, *C*2-sulfo-Cy5-ATP 29a

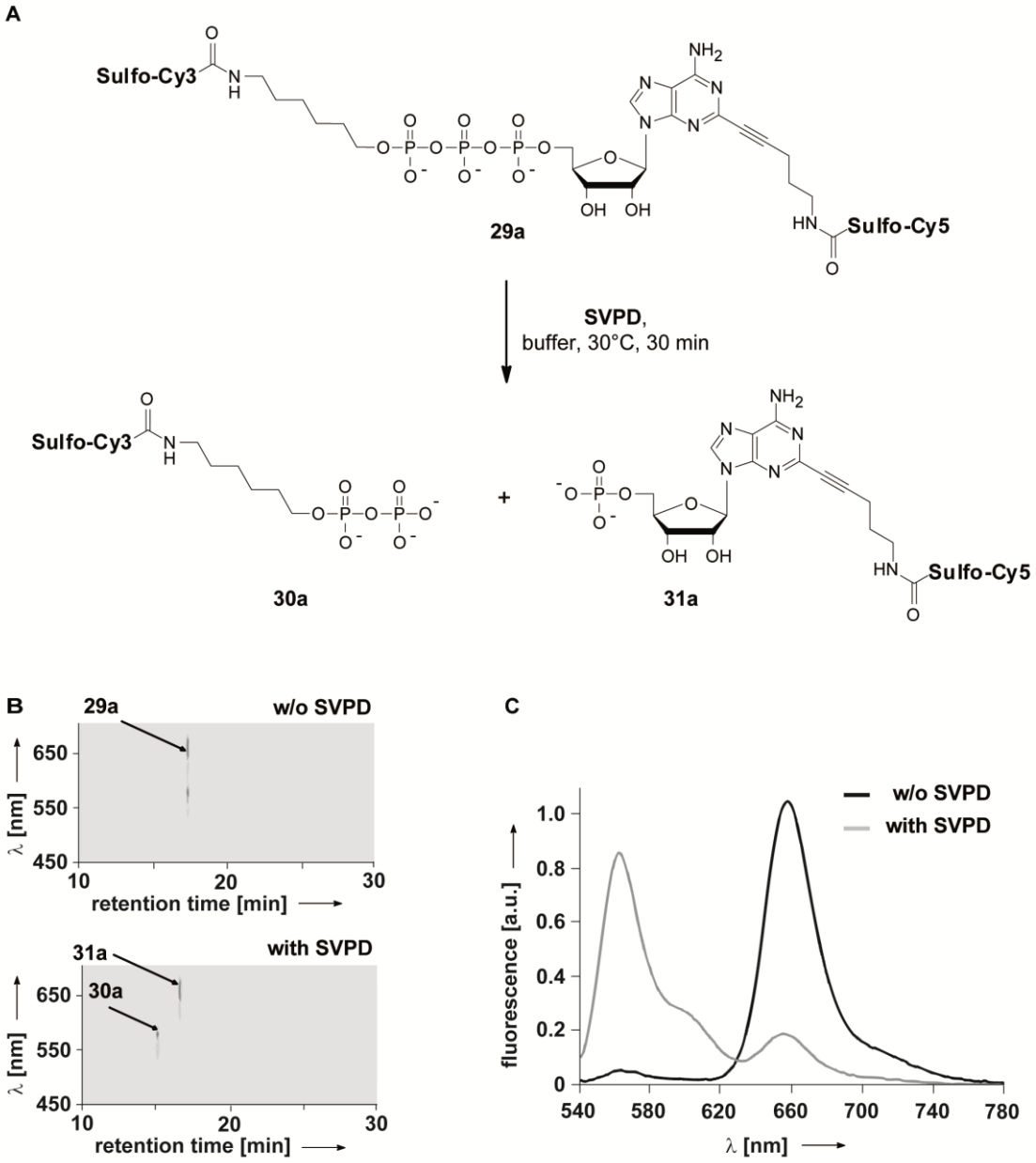


Figure S1: A) SVPD cleavage, **B**) HPLC analysis and **C**) fluorescent properties were investigated according to the general procedures using γ-sulfo-Cy3, *C*2-sulfo-Cy3-ATP **29a**. HPLC fractions of negative and positive control were analysed by HR-ESI-MS:

Negative probe:

HR-ESI-MS **29a**: found: 967.7793; calculated: 967.7750 ($M\text{-}2H^+$, $C_{85}H_{108}N_{11}O_{27}P_3S_4^{2-}$); deviation: 4.4 ppm.

Positive probe:

HR-ESI-MS **30a**: found: found: 443.6135; calculated: 443.6138 ($M\text{-}2H^+$, $C_{37}H_{52}N_3O_{14}P_2S_2^{2-}$); deviation: 0.7 ppm.

HR-ESI-MS **31a**: found: 532.1575; calculated: 532.1581 ($M\text{-}2H^+$, $C_{48}H_{38}N_8O_{14}PS_2^{2-}$); deviation: 1.2 ppm.

SVPD cleavage and analysis of γ-Cy3, *C*2-Cy5-ATP 29b

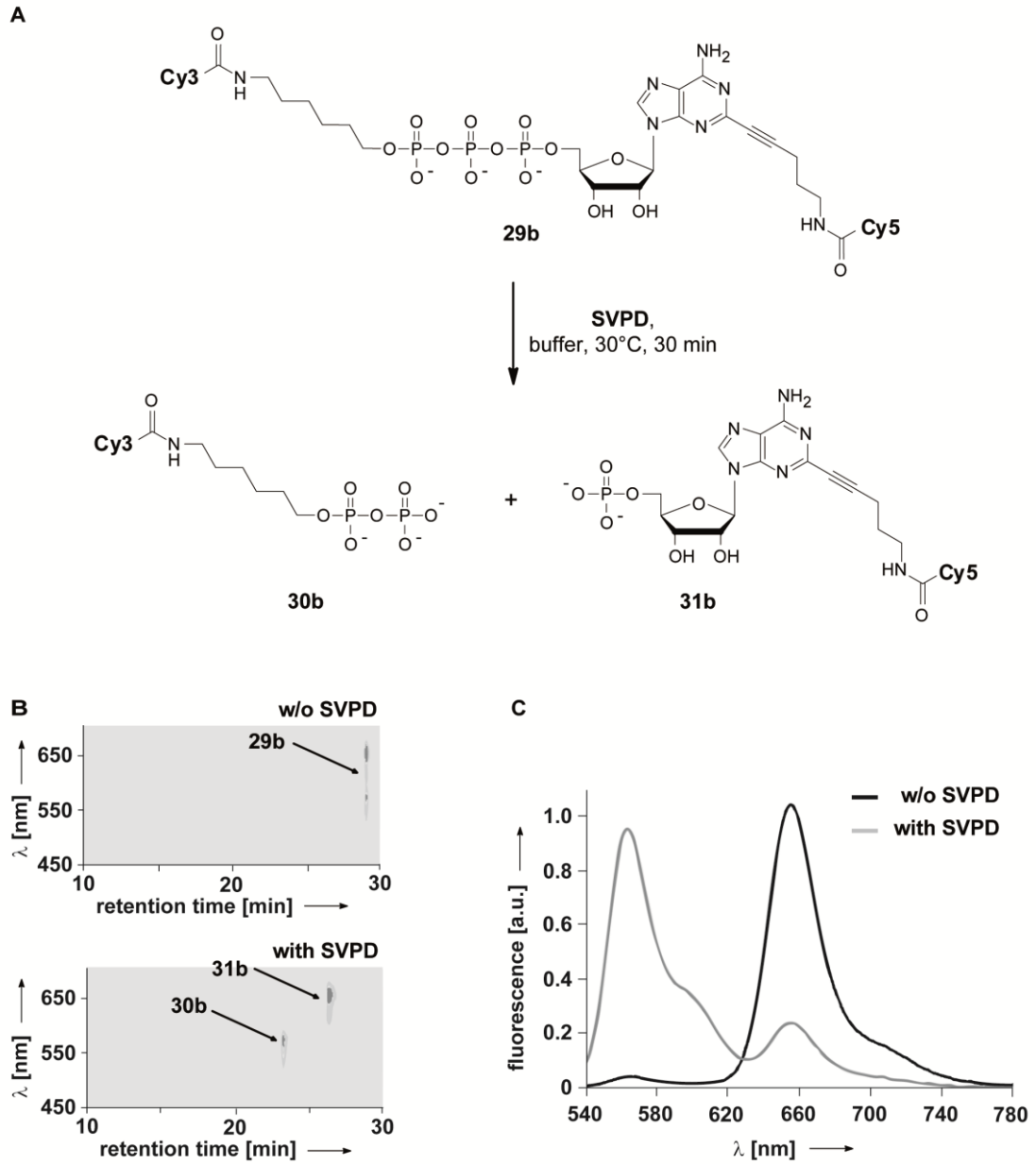


Figure S2: A) SVPD cleavage, **B**) HPLC analysis and **C**) fluorescent properties were investigated according to the general procedures using γ-Cy3, *C*2-Cy5-ATP **29b**. HPLC fractions of negative and positive control were analysed by HR-ESI-MS:

Negative probe:

HR-ESI-MS **29b**: found: 1588.6975; calculated: 1588.6999 ($M\text{-}H^+$, $C_{83}H_{105}N_{11}O_{15}P_3^-$); deviation: 1.5 ppm.

Positive probe:

HR-ESI-MS **30b**: found: 714.3058; calculated: 714.3068 ($M\text{-}H^+$, $C_{36}H_{50}N_3O_8P_2^-$); deviation: 1.4 ppm.

HR-ESI-MS **31b**: found: 891.3981; calculated: 891.3953 ($M\text{-}H^+$, $C_{47}H_{56}N_8O_8P^-$); deviation: 0.7 ppm.

SVPD cleavage and analysis of γ-sulfo-Cy3, *C*2-eclipse-ATP 29c

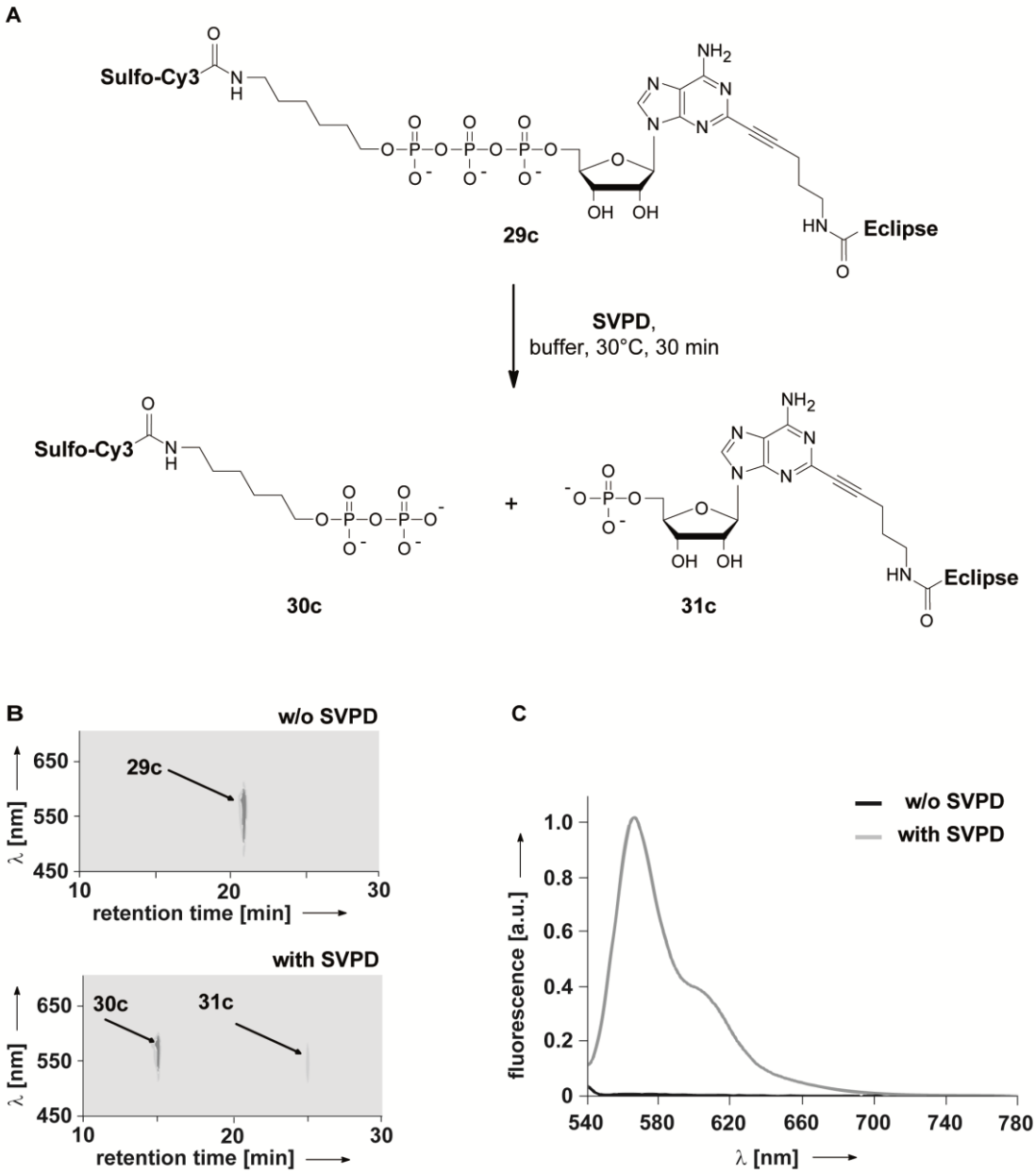


Figure S3: A) SVPD cleavage, **B**) HPLC analysis and **C**) fluorescent properties were investigated according to the general procedures using γ-sulfo-Cy3, *C*2-eclipse-ATP **29c**. HPLC fractions of negative and positive control were analysed by HR-ESI-MS:

Negative probe:

HR-ESI-MS **29c**: found: 827.7125; calculated: 827.7107 (M-2H^{+}, $C_{69}H_{85}ClN_{13}O_{23}P_3S_2^{2-}$); deviation: 2.2 ppm.

Positive probe:

HR-ESI-MS **30c**: found: 443.6127; calculated: 443.6138 (M-H^{+}, $C_{37}H_{52}N_3O_{14}P_2S_2^{-}$); deviation: 2.5 ppm.

HR-ESI-MS **31c**: found: 785.1978; calculated: 785.1958 (M-H^{+}, $C_{32}H_{35}ClN_{10}O_{10}P^{-}$); deviation: 2.5 ppm.

Enzymatic Experiments

In vitro protein tyrosine kinase assay with focal adhesion kinase (FAK)

A 96-well plate was coated with 1.0 µg per well of poly-Glu-Tyr peptide (molar ratio of 4:1; *Sigma*) in MilliQ water for 1 h at 37°C and blocked with 1% bovine serum albumin (BSA), 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without purified hFAK-KD (0.4 µg per well) in kinase buffer (125 mM NaCl, 48 mM $MgCl_2$, 50 mM HEPES, pH 7.5) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by monoclonal anti-phosphotyrosine antibody (*Upstate Biotechnology*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2 - 10 min. The absorbance was measured at 450 nm.

In vitro protein tyrosine kinase assay with Abl protein tyrosine kinase (Abl)

A 96-well plate was coated with 1.0 µg per well of Abl substrate peptide (*Jena Bioscience*) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without Abl (50 units per well) in 1x NEB buffer for protein kinases (PK) (*New England*

Bioscience) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by monoclonal anti-phosphotyrosine antibody (*Upstate Biotechnology*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2-10 min. The absorbance was measured at 450 nm.

In vitro protein serine/threonine kinase assay with cAMP-dependent proteine kinase (PKA)

A 96-well plate was coated with 1.0 µg per well of Myelin Basic Proteine (MBP) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without PKA (2,000 units per well) in 1x PKA reaction buffer (*New England Bioscience*) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by pSer/Thr Antibody (*CellSignaling*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2 - 10 min. The absorbance was measured at 450 nm.

In vitro protein serine/threonine kinase assay with casein kinase II (CK2)

A 96-well plate was coated with 1.0 µg per well of Myelin Basic Proteine (MBP) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without

CK2 (500 units per well) in 1x CK2 reaction buffer (*New England Bioscience*) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by pSer/Thr Antibody (*CellSignaling*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2 - 10 min. The absorbance was measured at 450 nm.

In vitro protein serine/threonine kinase assay with CAMKIIγ kinase enzyme system (CaMKII)

A 96-well plate was coated with 1.0 µg per well of Myelin Basic Proteine (MBP) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without CaMKII (0.4 µg per well) in 1x CaMKII reaction buffer A supplemented with 50 µM DTT and 1x Ca^{2+}/Calmodulin solution (*New England Bioscience*) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by pSer/Thr Antibody (*CellSignaling*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2 - 10 min. The absorbance was measured at 450 nm.

In vitro protein serine/threonine kinase assay with p21-activated kinase (PAK)

A 96-well plate was coated with 1.0 µg per well of Myelin Basic Proteine (MBP) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01%

Tween™ (30 min, 37°C). 100 µM ATP (or ATP analogue) were added with or without PAK (5.0 µg per well) in kinase buffer (125 mM NaCl, 48 mM $MgCl_2$, 50 mM HEPES, pH 7.5) in a total volume of 50 µL. The plate was incubated at 30°C for 1 h, then the kinase reaction was stopped by the addition of 100 µL 1 mM EDTA in PBS. The wells were washed three times with 100 µL 0.05% Triton X-100 in PBS. Phosphorylated peptide was detected by pSer/Thr Antibody (*CellSignaling*) followed by peroxidase-coupled goat anti-mouse antibody. TMB solution (0.5 mM 3,3´,5,5´-tetramethylbenzidine in 0.5% acetone, 4.5% ethanol and 1 mM H_2O_2 in 30 mM potassium citrate, pH 4.1) was added and the reaction was stopped with 2 M H_2SO_4 after 2 - 10 min. The absorbance was measured at 450 nm.

In vitro kinase assay of doubly dye labelled ATP 29a towards FAK

A 96-well plate was coated with 1.0 µg per well of GST-4xhFAKaa378-406_myc in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ (30 min, 37°C). 100 µM ATP **29a** was added with or without purified hFAK-KD (0.4 µg per well) in kinase buffer (125 mM NaCl, 48 mM $MgCl_2$, 50 mM HEPES, pH 7.5) in a total volume of 50 µL. The plate was incubated at 37°C for 1 h. After incubation fluorescent readouts were done from the crude reaction mixture at ex.530/em.670 nm; ex.530/em.570 nm; ex.630/em.670 nm wavelength.

In vitro kinase assay of doubly dye labelled ATP 29a towards FAK, Abl, PKA, CaMKII and PAK

A 96-well plate was coated with 4.0 µg per well Myelin Basic Proteine (MBP) in MilliQ water for 1 h at 37°C and blocked with 1% BSA, 0.02% NaN_3 in PBS with 0.01% Tween™ over night. 100 µM **29a**/ATP (1:99) was added with or without kinase (FAK, Abl, PKA, CaMKII or PAK) in its specific kinase buffer (FAK: 125 mM NaCl, 48 mM $MgCl_2$, 50 mM

HEPES, pH 7.5; Abl: 1x PK buffer (*NEB*); PKA: 1x PK buffer (*NEB*); CaMKII: 1x Reaction buffer A (*Promega*), 0.1 M DTT, 1x Ca^{2+}/calmoduline solution (*Pormega*); PAK: 125 mM NaCl, 48 mM $MgCl_2$, 50 mM HEPES, pH 7.5, 0.1 M DTT) in a total volume of 50 µL. The plate was incubated at 30°C and fluorescent readouts were done every 5 min at ex.530/em.670 nm; ex.530/em.570 nm; ex.630/em.670 nm wavelength over a period of 5 h.

Cell cultures of *D. biacutus*

The sulfate-reducing bacterium *D. biacutus* strain KMRActS was grown in freshwater mineral medium. The medium was reduced with 1 mM sulfide, buffered with CO_2/bicarbonate, and adjusted to a final pH of 7.2. Cells were grown in 1 L flasks with medium supplemented with 5 mM acetone or 5 mM butyrate as sole carbon source and 10 mM sulfate as the electron acceptor. Cultures were incubated under a strictly anoxic N_2/CO_2 (80/20) atmosphere at 30°C in the dark.

Preparation of *D. biacutus* cell extract

Cells of D. biacutus were harvested in the late exponential growth phase at an optical density of 0.3 (OD 600). All experiments with cell extract were done under strictly anoxic conditions. Cells were centrifuged at 6,000 x g at 4°C and the pellet was washed at least twice with 50 mM KP buffer, pH 7.2, supplemented with 3 mM DTT as reducing agent. The cell pellet was suspended in the same buffer plus 0.5 mg DNase·mL^{-1} and 1 mg·mL^{-1} of complete protease inhibitor (Complete Mini, EDTA-free protease inhibitor cocktail tablets, Roche Diagnostics GmbH). Cells were disrupted by passing them twice through a cooled French pressure cell at 100 MPa. Cell debris and un-opened cells were removed by centrifugation at 27,000 x g for 20 min at 4°C.

Reaction of γ-sulfo-Cy3, C2-eclipse-ATP 29c in cell-free extract

Cell-free extract of *D. biacutus* prepared after growth on acetone was used to test the carbonylation reaction. All enzyme assays were carried out under strictly anoxic

conditions. The buffer solution was the same as described for washing the cells pellet, and contained 1 $g \cdot L^{-1}$ NaCl, 0.6 $g \cdot L^{-1}$ $MgCl_2$ H_2O and 3 mM DTT. The enzyme reaction was tested in Eppendorf tubes sealed with a rubber stopper. Concentrations of substrates were 1 mM acetone, 0.1 mM ATP analog **29c**, 2 mM TPP and 10% CO in the headspace. The reaction was incubated for 1 h at 30°C. For the time course experiments, samples were taken at different time intervals with syringes previously flushed with N_2. All reactions were stopped with 2 mM EDTA solution. Each reaction condition was run in triplicate.

Fluorescent read out of cell extract reactions

From each sample, 10 µL aliquots were diluted and uniformly mixed with 90 µL Milli Q water and placed onto a dark 96 well plate for fluorescence measurement. The procedure was run three times for each triplicate.

HPLC analysis of cell extract reactions

Protein was precipitated by the addition of 10 µL of 2 M H_2SO_4, and removed by centrifugation at 10,000 x g. The supernatant was analysed by analytical RP-HPLC as described above. Fractions containing ATP fragments were collected and further characterized by HRMS.

HRMS characterisation of γ-sulfo-Cy3, C2-eclipse-ATP 29c and its fragments after incubation in cell-free extract

Fractions were collected during RP-HPLC separation and analysed by HRMS. Negative probe: HR-ESI-MS **29c**: found: 827.7091; calculated: 827.7107 ($M\text{-}2H^+$, $C_{69}H_{85}ClN_{13}O_{23}P_3S_{22}^-$); deviation: 1.9 ppm. Positive probe: HR-ESI-MS **30c**: found: 888.2377; calculated: 888.2360 ($M\text{-}H^+$, $C_{37}H_{53}N_3O_{14}P_2S_2^-$); deviation: 1.9 ppm.

Abbreviations

Abl	Abl protein tyrosine kinase
ACN	acetonitrile
AMP-PNP	adenylyl imidodiphosphate
ATP	adenosine triphosphate
p-BABA-ATP	*p*-biotinyl amidobenzoic acid adenosine triphosphate
br	broad
BSA	bovine serum albumin
CaMKII	Ca2+/calmodulin dependent protein kinase II
CDI	*N*,*N*'-carbonyldiimidazole
CK2	casein kinase II
CCE	crude cell extract
conc.	concentrated
d	doublet
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DEACM-ATP	(7-(diethylamino)coumarin-4-yl)methyl adenosine triphosphate
DIPEA	diisopropylethylamin (Hünig-Base)
DMF	*N*,*N*'-dimethylformamide
DNP-ATP	*N*6-(2,4-dinitrophenyl)-adenosine 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
ESI	electron spray ionization
ESI-MS	electron spray ionization mass spectrometry
FAK	focal adhesion kinase
FAT	focal adhesion targeting

FEDA-ATP	2’ (3’)-*O*-(*N*-2-(3-(5-fluoresceinyl) thioureidol) ethyl carbamoyl) adenosine triphosphate
FERM	four-point-one, ezrin, radixin, moesin
FRET	Förster resonance energy transfer
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HPLC	high pressure liquide chromatography
HR-MS	high resolution mass spectrometry
HRP	horseradish-peroxidase
HTS	high throughput screening
$(Im)_2CO$	1,1’-carbodiimidazole
m	multiplet
MABA-ATP	*N*8-(4-*N*’-methylanthraniloylaminobutyl)-8-adenosine 5’-triphosphate
mant-ATP	*N*-methyl-anthraniloyl-adenosine triphosphate
min	minutes
MPLC	medium pressure liquide chromatography
NHS	*N*-hydroxysuccinimide
nm	nanometre
NMR	nucleic magnetic resonance
PAK1	p21-activated kinase
PBS	phosphate buffered saline
PKA	cAMP-dependent protein kinase
PMSF	phenylmethylsulfonylfluorid
PTK	protein tyrosine kinase
pyr	pyridine
q	quartet
qn	quintet
RAMP	(R)-(+)-1-amino-2-methoxy- methylpyrrolidin
RP-HPLC	reversed phase high pressure liquide chromatography
r.t.	room temperature
s	singlet
SAMP	(S)-(-)-1-amino-2-methoxy- methylpyrrolidin

sat.	saturated
SVPD	snake venom phosphodiesterase
t	triplet
TBAF	tetrabutylammonium fluoride
TCEP	tris-(2-carboxyethyl)-phosphine
TEAA	triethylammonium acetate
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMB	3,3’,5,5’-tetramethylbenzidine
TMP	trimethyl phosphate
TMSO-Tf	trimethylsilyl trifluoro methane sulfonate
TPP	thiamine pyrophosphate
UV	ultraviolet
VIS	visual light

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Konstanz, im Dezember 2013

(Norman Hardt)