DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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SYNTHESIS OF ADENOSINE-PEPTIDE CONJUGATES FOR BIOLOGICAL APPLICATIONS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, referred to in text by Roman numbers I–III.

I. Enkvist, E.; Raidaru, G.; Patel, R.; Redick, C.; Boyer, J. L.; Subbi, J.; Tammiste, I.; Uri, A. Synthesis of potential purinoceptor antagonists: application of P1-tBu phosphazene base for alkylation of adenine. *Nucleosides, Nucleotides and Nucleic Acid*, **2006**, *25*, 141–157.

II. Enkvist, E.; Lavogina, D.; Raidaru, G.; Vaasa, A.; Viil, I.; Lust, M.; Viht, K.; Uri, A. Conjugation of adenosine and hexa-(D-arginine) leads to a nanomolar bisubstrate-analog inhibitor of basophilic protein kinases. *Journal of Medicinal Chemistry*. **2006**, *49*, 7150–7159.

III. Enkvist, E.; Raidaru, G.; Vaasa, A.; Pehk, T.; Lavogina, D.; Uri, A. Carbocyclic 3'-deoxyadenosine-based highly potent bisubstrate-analog inhibitor of basophilic protein kinases. *Bioorganic & Medicinal Chemistry Letters*. **2007**, *17*, 5336–5339.

Author's contribution

Paper I: The author planned and performed about a half of the syntheses, and is also responsible for writing the manuscript.

Paper II: The author contributed to the planning of the experiments (including biological tests and data analysis) and performed most of the solution syntheses, several solid-phase syntheses and all structural analyses, and is also responsible for writing of the manuscript.

Paper III: The author is responsible for planning of all experiments, data analysis and writing of the manuscript and performing of all the syntheses.

ABBREVIATIONS

Adc	1-(9-adenyl)-β-D-ribofuranuronic acid
ADP	adenosine 5’-diphosphate
Ahx	6-aminohexanoic acid
Aoc	8-aminooctanoic acid
ARC	adenosine-oligoarginine conjugate
ATP	adenosine 5’-triphosphate
ATPγS	adenosine 5'-O-(3-thiotriphosphate)
Boc	*tert*-butoxycarbonyl
CAMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3’,5’-monophoshate
cAPK	cAMP-dependent protein kinase
CDK	cyclin-dependent kinase
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	N,N'-dicyclohexylcarbodiimide
DCE	1,2-dichloroethane
DCM	dichloromethane
DIC	N,N'-diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	N,N'-dimethylformamide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FDA	Food and Drug Administration
Fmoc	9-fluorenylmethoxycarbonyl
GABA	gamma-aminobutyric acid
Hex	n-hexyl
HOBt	*N*-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IDA	iminodiacetic acid
IpAdc	1-(9-adenyl)-2’,3’-*O*-isopropylidene-β-D-ribofuranuronic acid
K_i	inhibition constant
K_m	Michaelis constant
MBHA	4-methylbenzhydrylamine
mCPBA	meta-chloroperbenzoic acid
MS	mass spectroscopy
MW	molecular weight
NMR	nuclear magnetic resonance
PKB	protein kinase B, Akt
PKI	heat-stable protein kinase inhibitor
PKC	protein kinase C
PNA	peptide nucleic acid
ROCK	Rho-associated kinase
SAM	S-adenosyl-L-methionine

SAR	structure-activity relationship
SPR	surface plasmon resonance
TAMRA	carboxytetramethylrhodamine
TBTU	*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultraviolet

INTRODUCTION

Adenosine in living systems

Adenosine moiety is a fragment of a large number of different biologically important compounds. It is a building block of nucleic acids (DNA and RNA) that carry genetic information. Several adenosine containing coenzymes play a variety of functions in living cells. The most abundant of these compounds is ATP, which is a key molecule in energy transfer and the main donor of phosphate group in phosphorylation reactions catalyzed by kinases. Energy that is released from hydrolysis of ATP is the driving force of the anabolic processes that need energy to proceed. S-adenosylmethionine is another widely used adenosine containing coenzyme that is the main donor of methyl group. Gene expression, protein functions and several other processes are regulated by methylation. This coenzyme is synthesized from ATP and methionine [1].

Most of the functions of adenosine nucleosides and nucleotides are intracellular, but there are almost 20 different extracellular membrane receptors (purinoceptors) and an unknown number of ectoenzymes, including kinases and phosphatases, which interact with adenosine-containing compounds [2]. In addition to several other functions of these compounds, the discovery of purinergic signalling also added the roles of signal molecules and neurotransmitters to adenosine and adenine nucleotides.

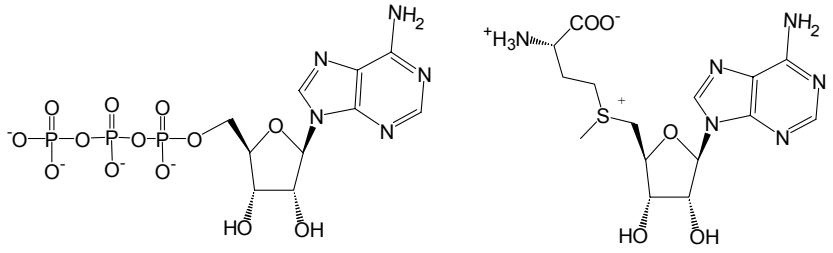

Figure 1. Natural adenosine-containing coenzymes ATP and SAM.

Nucleoside-binding proteins as potential drug targets

A large variety of functions of proteins that bind purine nucleosides and nucleotides have made their analogues attractive drug candidates. The most intensively studied area of drug research of nucleoside analogues is connected to their antiviral activity. This effect originates from the inhibition of synthesis of viral nucleic acids and thereby interruption of virus multiplication. These derivatives are mostly converted into appropriate triphosphates and incorpo-

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rated into growing chains of viral nucleic acids causing termination of the synthesis of oligonucleotide chains. Several nucleoside-based antiviral drugs are already in use in medicine and intensive continuation of research in the field promises further addition of medications [3]. The second wide area of research, associated with nucleoside analogues has been anticancer drugs. The inhibition of nucleic acid synthesis leads to reduction of proliferation and can cause cell death. Pentostatin is an anticancer drug that inhibits adenosine deaminase and therefore affects purine metabolism. Purine analogues may influence several other intracellular processes that are mediated by adenosine and guanosine derivatives by inhibiting appropriate enzymes like kinases and methyl-transferases, thus several other purine binging proteins are potential drug targets and purine analogues could serve as drug candidates [3].

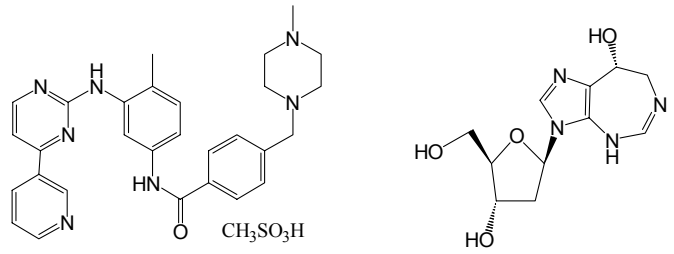


Figure 2. Anticancer drugs that act through the inhibition of nucleotide- or nucleoside-binding proteins Imatinib mesylate (Gleevec, left) and Pentostatin (right).

Last decades have provided much information about extracellural nucleoside and nucleotide receptors. Four different receptors are known to be activated by adenosine (A_1, A_{2a}, A_{2b} and A_3) and more than ten P2 receptors by adenosine nucleotides. Some of the P2 receptors are also activated by uridine nucleotides. Nucleotide receptors are divided into two families: P2Y are G-protein-coupled receptors and P2X are ligand-gated ion channels. As several pathological processes are linked to aberrant purine-mediated signalling, these receptors are important drug targets. Agonists and antagonists that influence these receptors can potentially be used as drugs. Purine derivative caffeine acts as an adenosine receptor antagonist. Most of the nucleotide receptor ligands have large negative charge that precludes their internalization to cells and eliminates the selectivity problems caused by cytosolic purine-binding proteins. This is a great advantage for the development of drugs affecting nucleoside receptors.

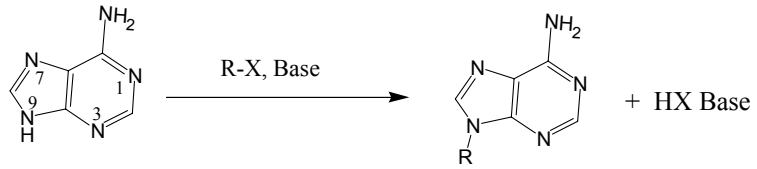
Protein kinases form one of the largest families of enzymes (518 genes in human genome) [4]. Hydrophobic ATP binding site enables development of small-molecule inhibitors. Their involvement in numerous pathological signal transduction pathways has caused the elevation of these enzymes to second largest drug target after G-protein-coupled receptors. There is one very successful drug (Gleevec) already that acts as inhibitor of protein kinase and

several other compounds have been accepted by FDA and a number of compounds are in clinical trials [5].

Chemical properties of adenine and adenosine

Adenosine has several nucleophilic functionalities in sugar and nitrogen base moieties. The most nucleophilic group for acylation reaction is 5'-hydroxyl, although base nitrogen atoms of adenine base are more basic (pK_a = 3.52) and prone to alkylation reactions [6]. 5'-Hydroxyl of adenosine can be selectively acylated, but it is also possible to acylate all sugar hydroxyl groups in one step or to perform per-acylation that involves also 6-amino group of adenine [7]. Peracylations require the presence of a strong nucleophilic catalyst like DMAP and the excess of acylating reagent. 6-Amino group of adenine has surprisingly weak nucleophilic properties due to strong resonance with aromatic ring and deactivating effect of purine nitrogens [6, 7].

Position 8 of adenine ring gives electrophilic substitution reactions easily and allows good opportunities to introduce various groups. Usually the 8th position is brominated first and then the bromine is substituted by the appropriate nucleophile. N-nucleophiles (sodium azide and R-NH_2) are used most frequently in the reaction with bromoadenosines but several O- and S-nucleophiles are also applied [8]. Substitution of the second CH hydrogen of adenine (H2) requires indirect approach for introduction of the desired fragment into 2nd position [7, 8].



Scheme 1. Base-catalysed 9-alkylation of adenine. Other possible alkylation sites (N7, N3 and N1) are shown.

Adenine has weakly acidic 9-hydrogen that can be removed by different bases, the resulting anion has remarkable nucleophilic properties and can be alkylated with a number electrophiles. In case of adenine anion, the main alkylation products are 9-substituted adenines; although the regioseletivity is not absolute (alkylation of 7- and 3- positions is often detected). Alkylation of neutral adenine gives dominantly 3-substited products. Other reactions of adenine are similar to these of the adenine ring in the structure of adenosine [9]. Effective 9-alkylation of purines is often the key in the synthesis of carbocyclic nucleosides

that are chemically and enzymatically more stable than the native adenosine [10, 11, 12]. These compounds contain a cycloalkane (usually cyclopentane) moiety instead of furan. The lack of acid-sensitive and enzymatically degradable glycoside bond makes these derivatives more stable and results in advantages of biochemical applications [10, 11].

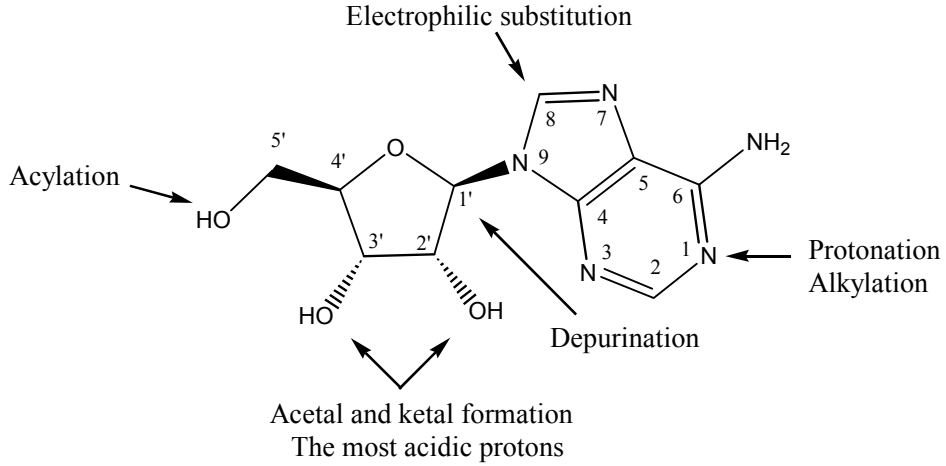


Figure 3. Chemical properties of adenosine. The most reactive positions are shown.

The introduction of alkyl groups to N6-nitrogen is performed mostly indirectly by using the reaction between a 6-chloropurine derivative and an appropriate amine. Direct alkylation of adenine causes mostly alkylation of one of the ring nitrogens instead of the exocyclic amino group. N1-alkylated adenine derivatives can be converted to monosubstituted N6-alkyl adenine derivatives by using Dimroth rearrangement [9].

Adenosine and adenine are relatively stable against reductive agents, but they can be oxidized to produce different products. 5'-Hydroxyl of the adenosine can be oxidized into aldehyde or carboxylic acid like a usual primary alcohol [8]. These reactions require prior protection of 2'- and 3'-hydroxyls to avoid side reactions [7]. Periodate oxidizes unprotected ribose into appropriate dialdehyde by cleaving of the bond between 2'- and 3'-carbons. Reactions of the obtained dialdehyde with primary amine and the following reduction with hydrides yield morfoline analogues of adenosine [13]. Peroxide fragment containing compounds (mCPBA, H_2O_2, *etc.*) can oxidize N1 nitrogen to the appropriate nitrogen oxide [7].

Syntheses starting from adenosine or adenine should take into consideration the multifunctionality of these compounds. Application of one or more protection group is often needed to achieve selective reactions. Introduction and removal of protection groups may form the majority of synthetic steps that are

used to prepare certain nucleoside analogues. Hydrophobic fragments increase solubility of nucleoside analogues in organic solvents and thereby protection groups also facilitate performance of synthetic reactions [14].

Strategies for the application of protection groups in the synthesis of adenosine analogues

The employment of protective groups is necessary if the selectivity or solubility of the reagents is not sufficient. Nucleoside analogues have commonly various functionalities with similar properties. Moreover, high polarity of these compounds makes them poorly soluble in organic solvents, which complicates the performance of transformations with the compounds. Thus, application of a suitable protection strategy is commonly needed. The optimal protection strategy depends on particular case and should be analyzed carefully during the planning of the experiments.

The most common protection group of adenosine is 2',3'-isopropylidene which is an acetal formed from acetone and diol hydroxyls of adenosine. The isopropylidene group can be removed by acid treatment without significant cleavage of glycoside bond [7, 14]. Derivatisation of 2'- and 3'-hydroxyls allows the performance of more selective acylation and oxidation reactions with 5'-CH_2OH moiety by disabling reactions with other hydroxyls and improving solubility of the compound. Other acetals and ketals are rarely used for protection but different silyl protecting groups are widely used. Silanization has often different regioselectivity than kelalization and silyl protection can be removed in mild nonacidic medium [14]. Acylation can also be used to protect adenosine functionalities. The protection of sugar hydroxyls with acyl groups usually requires nucleophilic catalysis by pyridine whereas basic hydrolysis is commonly used for deprotection [14]. Acylation of 6-amino group of adenine requires participation of stronger nucleophilic catalysts like imidazole derivatives or DMAP. Protection of N6-amine is needed only if very reactive electophiles are used or some group is activated by strong bases [15]. The introduction of an acyl or some other electron-withdrawing group to adenine ring decreases electron density and reactivity of ring nitrogens and is hence sometimes applied to avoid side reactions (like intramolecular cyclization of 5' activated adenosines) caused by N1- and N3-nitrogens [8].

Adenosine-peptide conjugates

Adenosine-peptide conjugates are compounds where adenosine or an adenosine analogue is covalently connected to a peptide moiety. The peptide nucleic acids (PNA-s) and peptide conjugates with oligonucleotides that are sometimes named analogically are not discussed here.

Combination of a nucleoside and a peptide results in a new compound carrying functionalities of both fragments and potentially endowed with unique biological and chemical properties. The adenosine analogue can be incorporated into a peptide-like structure as an unnatural amino acid or the peptide fragment may mimic a part of some natural adenosine containing coenzyme (SAM, ATP). The purpose of the production of these compounds is to influence processes where adenosine derivatives are involved. Several inhibitors of protein kinases, ATP-ases, methyltransferases, *etc.* representing this type of compounds have been developed [16, 17].

These compounds can structurally be classified according to the position of the connection between adenosine and peptide. The most common connection for these conjugates is through 5'-position of adenosine, mimicking the majority of natural adenosine derivatives. Several different chemical groups like amide, urethane, ester, phosphate ester, *etc.*, have been used for bridging of adenosine-5' and a peptide. 2'- and 3'- positions of ribose could be also used for linkage. Adenine positions 8, 2, and 6-amino might also be subjected to derivatisation and conjugation. The choice of the linker structure and position depends on the tolerance of targeted protein for adenosine modifications. For example, as derivatisations of adenine ring decrease affinity of nucleotide analogues against several protein kinases [18], the connections through adenine ring are probably not suitable for synthesis of adenosine-peptide conjugates as inhibitors of these enzymes. Peptides can be conjugated through the both termini and also by using side-chain functional groups. These different possibilities to prepare adenosine peptide conjugates allow the creation of a huge number of structurally diverse compounds with various pharmacological properties. Relatively high molecular mass and flexibility of these derivatives diminish their fit to classical rules of drug-likeliness (Lipinski's rule of five [19]). Moderate drug-likeliness has made these derivatives less attractive for big pharmaceutical companies but it does not eliminate their applicability in several areas.

Solid-phase and solution-phase synthetic strategies for preparation of adenosine-peptide conjugates

Solid-phase synthesis has become a widely used methodology for preparation of different structurally diverse compounds. This method allows to perform multi-step syntheses quickly and conveniently by virtue of easy purification of the intermediate products by filtration. The simple procedures of solid-phase synthesis have also permitted automatization resulting in further increase of the productivity. This strategy was first applied for synthesis of peptides and other oligomeric biomolecules (oligosaccharides and nucleic acids) but later has been spread to preparation of a large variety of compounds [20].

The synthesis commonly starts with the connection of the first fragment with appropriate reactive functionalities to the solid support. The following steps elongate the molecule or generate desired modifications with this immobilized fragment. The usual final step is the cleavage of protection groups and the removal of product from the solid support. Partially cross-linked polystyrene is the most widely used solid support for synthesis of organic compounds; several other organic polymers have also been used. The polymeric carrier (resin) is usually in the form of small beads. Synthesis of oligonucleotides is performed on glass beads that are rigid and do not swell like organic polymers [20].

Application of solid-phase synthetic strategy needs suitably protected reagents and usage of remarkable excesses of soluble chemicals. An additional need of functional resin increases the cost of the strategy and generally limits its applicability, although the speed of the synthesis and easy purification overcome these drawbacks in several cases.

Solution synthesis is preferred when no suitable protected reagents are available for solid phase synthesis, if large scale is needed, if heterogeneous catalysis is needed, or if yields of some steps are low and purification of intermediate products is required. Moreover, the possibilities to analyze synthetic processes on solid-phase synthesis are strongly limited compared to conventional solution phase procedures. Most of the common analytical methods (like TLC, NMR, MS, *etc.*) are not directly applicable for resin-bound intermediates. New and problematic reactions need to be verified and optimized before introduction into solid-phase synthesis [20]. Combination of the strategies takes advantages of both methodology and allows more flexible approach. Suitable reagents for solid-phase synthesis are usually prepared in solution and then used for reactions on resin.

Adenosine-peptide conjugates might be synthesized using various strategies. Peptide fragments are usually prepared on solid phase according to traditional peptide synthesis methodology. Connection of nucleoside and peptide moieties can be performed both in solution and on solid phase. The chemistry applied depends strongly on the properties of functional groups of the nucleoside

analogue used for linkage with a peptide. 5'-deoxy-5'-aminonucleoside derivatives were first precursors applied for the synthesis of adenosine-peptide conjugates [21]. Activated and properly protected amino acids were coupled to 5'-amino group of a nucleoside, protection of the N-terminal was removed and the next amino acid was added subsequently. All syntheses were carried out in solution and intermediate products were purified. Later the same group also synthesized conjugates from Adc where 5'-CH_2OH is oxidized to carboxylic acid. Carboxyl of the IpAdc was activated by DCC and reacted with free amine of an amino acid. Benzyl ester of the connected amino acid was cleaved by hydrogenation and the second amino acid was added in a similar way [22]. Products of these two approaches differ from each other mainly by connection point of the nucleoside to the peptide: either C- or N-terminus.

Several adenosine nucleotide conjugates with peptides have been synthesized with phosphate moieties serving as connection. The main intent for the synthesis of these compounds has been the development of bisubstrate analogue inhibitors for protein kinases [16]. Adenosine parts of the molecules are similar to native nucleotide where phosphates are connected to 5'-position with phosphoester bond. Peptides have been connected with phosphates by forming of ester [23], tioester [24] or amide linkage [25]. Connection can be made either upon peptide termini or by using side-chain functional groups. The lack of stability of phosphate esters and anhydrates in biological systems is a serious drawback for application of these compounds.

In the beginning of the 1990-s the research of purinergic signalling became very widespread, which caused the need for selective and biologically stable ligands for these receptors. One of the approaches for the development of the ligands of nucleotide receptors is the substitution of oligophosphate chain with a negatively charged peptide. This kind of adenosine-peptide conjugates was first synthesized by connecting IpAdc to the N-terminus of the peptide on solid phase [26]. The peptide moiety of the derivatives consisted of glutamic and aspartic acid residues. Different modifications of the structure of compounds were later done in order to increase structural diversity [27]. Urethane and urea connections led to derivatives where adenosine 5'-carbon was not oxidized and maintained its sp^3-hybridization [27, 28, 29]. Nucleoside part conformations of these derivatives are more similar to natural nucleotides if compared to analogical Adc compounds [27].

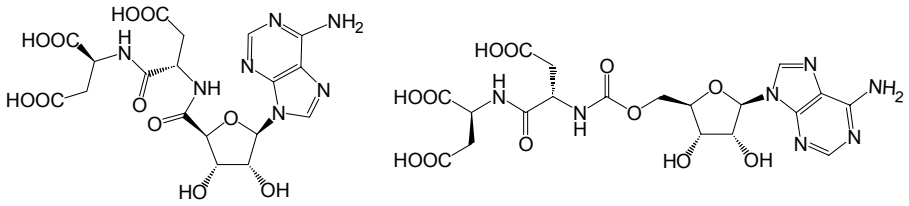
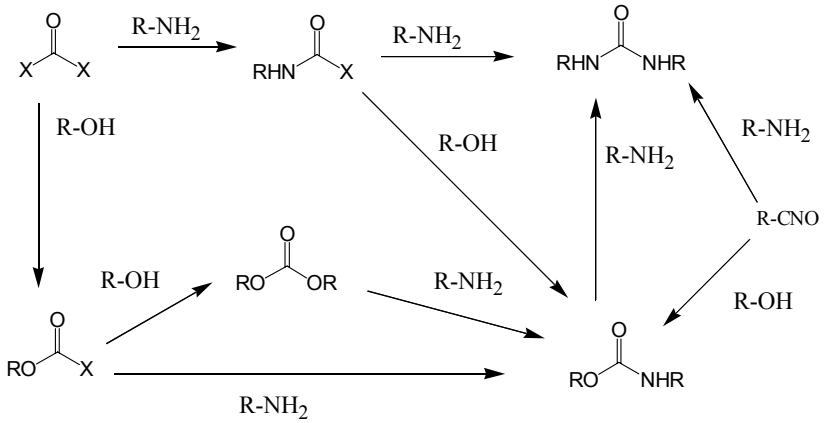


Figure 4. Examples of adenosine peptide conjugates. Possible formation of intramolecular hydrogen bond in Adc derivatives (left) that favours *syn* conformation are shown.

Amides of Adc with primary amines have tendency to form intramolecular hydrogen bonds between N1-nitrogen and 5'-amide-NH that favours *syn* conformation of the nucleoside [27, 30]. This may interrupt interactions of the compound with proteins that mainly bind nucleosides in *anti*-conformation. 5'-Urethanes have no 5'-NH-group that can give intramolecular hydrogen bond while 5'-ureas have also this possibility. The synthesis of urethanes and ureas originates usually from an activated derivative of carbonic acid where two good leaving groups are connected to carbonyl. Phosgene ($COCl_2$) is the most common starting material for synthesis of other carbonic acid derivatives. Several other activated analogues of phosgene are preferred for laboratory applications on safety reasons. 4-Nitrophenyl chloroformate has been successfully used to prepare appropriate activated carbonates or urethanes from adenosine analogues that can be used for synthesis of adenosine 5'-urethanes and ureas [27, 29]. Phosgene itself is inapplicable in these syntheses because of the reaction with adenine base leading to complicated mixture of the products [31]. Less active carbonic acid derivatives do not give these side reactions and thus can be used. Isocyanates that react with hydroxyl- and amino group producing corresponding urethanes and ureas can be used as alternatives to chloroformates.



Scheme 2. General synthetic pathways for preparation of urethane and urea linkers.

ARC-s are adenosine-peptide conjugates incorporating an oligoarginine moiety and adenosine analogue (usually Adc) that are connected by a linker (commonly Ahx). These compounds have been shown to inhibit several protein kinases [32].

Adenine-peptide conjugate can be defined as a special class of adenosine-peptide conjugates where sugar moiety of the adenosine is excluded. Structure and synthesis of these compounds is therefore different. 9-Alkyl adenines have mostly been applied for the preparation of these derivatives, but connections through 6- and 8-positions of adenine can also be used.

Peptide conjugates with functional analogues of adenosine should also be mentioned. Functional analogues of biomolecules are structurally different compounds that have similar functions in some biochemical systems. These analogues may have higher selectivity towards nucleoside-binding proteins caused by reduced similarity with diversely active adenosine. The synthesis procedure of peptide conjugates depends on the chemical character of the particular functional analogue.

P2 receptors and their ligands

P2 receptors are cell membrane receptors activated by extracellular purine and/or pyrimidine nucleotides. The receptors are divided into two families: G protein-coupled receptors, termed P2Y, and ligand-gated cation channels, termed P2X. Numerous subtypes of these receptors within both of the families with diverse range of regulational functions are known [33, 34].

The natural ligands of P2Y receptors are adenine and/or uracil nucleotides. These compounds are not selective towards different subtypes and their degradation in biological systems is fast. These two aspects are the main limitations for the application of native ligands in biochemical experiments. During the last years several selective ligands (both agonists and antagonists) have been developed for some of these receptors and the process is still going on [35, 36]. Most of these compounds are different derivatives of adenine. SAR of the ligands and the mechanism of their molecular recognition by receptors are under extensive studies [33, 34].

2-mehtylthio-substitution on adenine ring and some of the modifications in the sugar moiety increase the selectivity and potency of agonists (adenosine di- and triphosphates or their analogues) for $P2Y_1$ receptor [35]. The derivatisation of adenine moiety with N6-methyl and 2-chloro groups increases the potency of antagonists (mainly cyclic and acyclic bisphosphate derivatives of adenine) [36, 37]. Conformational locking of the sugar moiety influences remarkably agonistic and antagonistic potencies of ligands [35]. The other subtypes of P2Y receptors have more or less different SAR.

Clopidogrel is one of the first drugs acting through the P2 receptors. It is a non-nucleotide irreversible inhibitor of $P2Y_{12}$ receptor and thereby avoids

platelet aggregation and thrombosis. AZD6140 is an orally available reversible antagonist of this receptor and it has several advantages over clopidogrel: faster effect, smaller amount of side-effect and reduced response variability between patients. The clinical trials with AZD6140 are currently in progress (phase III started in 2006) [38]. The possibilities to treat other diseases through various P2 receptors are extensively studied.

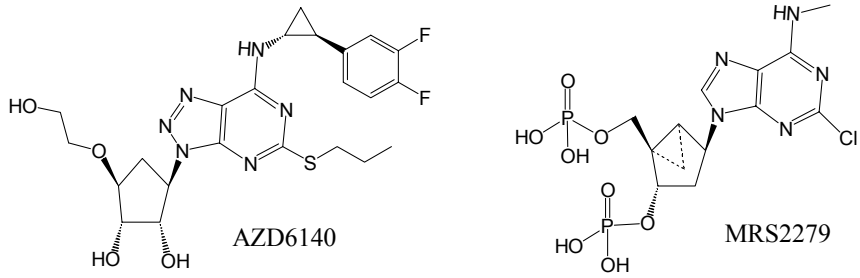


Figure 5. Antagonists of $P2Y_{12}$ and $P2Y_1$ receptors, potential antithrombosis drugs.

Conception of development of nonphosphate ligands of P2 receptors

Instability of phosphate-containing nucleotides in biological environment has caused the need for non-phosphate nucleotide analogues that have potentially longer life-times in living systems. Structure-activity studies have shown that nucleotide-selective P2X and P2Y receptors require that the ligands contain a negatively charged region (the polyphosphate chain) concentrated in close proximity to the adenine moiety. The positively charged amino acid sequence conservation in P2 nucleotide receptors supports the idea of importance of electrostatic interactions between the receptor and the negative charges of the polyphosphate moieties of nucleotides [36]. This proposes that phosphate groups can not be easily excluded from active compounds but there could be possibilities for their substitution with other negatively charged moieties. Carboxylates and sulfonates are the most common negatively charged moieties in organic compounds which be easily introduced into nucleotide analogues. Sulfonate-containing antagonists of P2 receptors have been known for a long time. These compounds are not structurally related to native nucleotides and present a family of non-nucleotide ligands of these receptors. Suramin was one of the first members in this group and several other ligands are structurally similar to it [39]. The first carboxylate-containing nucleotide analogues contained negatively charged peptide (oligoaspartate and oligoglutamate) connected to Adc through the N-terminus of a peptide. Some of these derivatives

revealed biological effects that were assumed to be mediated through P2 receptors [26]. Several modifications were introduced to the structures [27] and conjugates that incorporated carbamate connection (urethanes) between adenosine and oligoasparate were shown to be antagonists of $P2Y_1$ receptor while analogous derivatives of Adc had no activity towards this receptor [28].

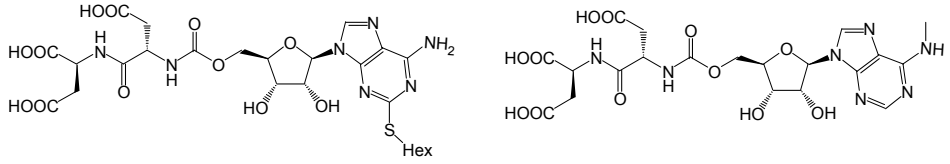


Figure 6. Examples of carboxylate-containing ligands of $P2Y_{12}$ and $P2Y_1$ receptors.

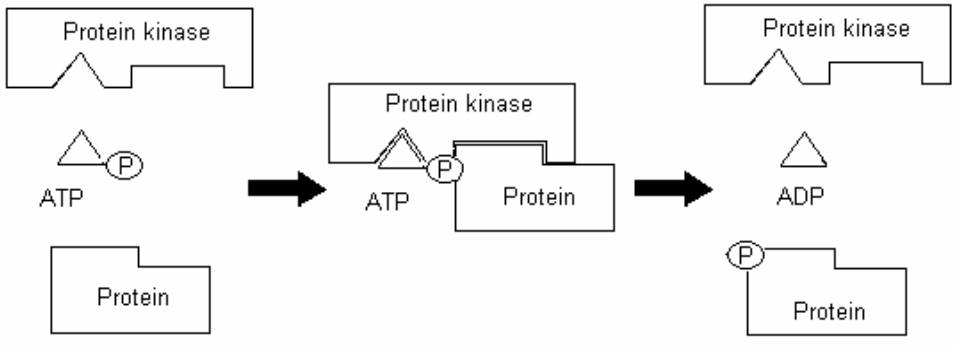
The optimal peptide incorporated two aspartates that carried three negative charges like natural agonist of this receptor ADP. The compound with hexylthio group connected to the 2-position of the adenine moiety was an antagonist of $P2Y_{12}$ receptor (IC_{50} = 20 μM) whereas a similar analogue without this group had no activity [40]. This suggests that insertion of 2-alkylthio moiety converts $P2Y_1$ receptor antagonist into $P2Y_{12}$ antagonist. Addition of methyl group to N6-position of adenine increases selectivity and affinity of $P2Y_1$ receptor antagonists [36]. Several acyclic compounds (mostly bisphosphates but also some non-phospate derivatives) have been shown to act as antagonist of P2Y receptors [37]. Some other carboxylate-containing ligands of P2 receptors have been prepared by drug companies [33, 34]. Several non-phosphate antagonist of $P2Y_{12}$ receptor (mostly without carboxyl groups) are in clinical trials carried out by AstraZeneca and Inspire Pharmaceuticals including the most promising derivative AZD6140 [38].

Carboxylate-containing nucleotide analogues have shown to be potential ligands of P2 receptors and some of them have excellent properties. Still, this approach has been successfully applied only in case of a few receptors whereas phosphate-containing analogues have usually shown higher potency.

Protein kinases

Phosphorylation of proteins is one of the most important regulatory mechanisms in living systems. It is estimated that functioning of one-third of all proteins is controlled by reversible phosphorylation catalyzed by protein kinases and phosphatases. 518 different genes have been assigned from human genome to coding protein kinases that make it one of the largest enzyme families (~2% of all the genes). This is up to 20% of druggable genome [41] and thereby the second largest drug target after G protein-coupled receptors [5]. Druggable

genome is defined as the part of the genome that codes proteins able to bind small molecular regulatory compounds and hence contains potential drug targets.



Scheme 3. Protein kinase catalyzes phosphorylation of a protein. Ternary complex of phosphoryl transfer is shown in the middle, γ-phosphate of ATP is marked with P.

Protein kinases transfer γ-phosphate from the phosphate donor ATP to the hydroxyl group of the protein. ATP and a protein substrate bind simultaneously to the kinase forming ternary complex and phosphoryl transfer follows directly without formation of covalent intermediates with the enzyme. The phosphorylated protein and ADP dissociate from the kinase and the next catalytic cycle can start. According to the phosphorylatable amino acid residue, protein kinases are divided into serine/threonine and tyrosine kinases. Some of the enzymes are able to phosphorylate both types of hydroxyls. All kinases are classified into seven main groups correspondingly to similarities of their amino acid sequences of the catalytic domains [4]. The kinases belonging to the same group show often similar substrate and inhibitor specificity profiles, which complicates studies of their function and design of selective inhibitors. Mediation of an intracellular signal-transduction is one of the major roles of protein kinases, producing cellular responses to the changes in extracellular and intracellular environment.

More than 400 human diseases have been linked to aberrant protein kinase signalling [42]. Overexpression and abnormally increased activity of protein kinases have been shown in various pathological conditions like cancers, inflammatory, autoimmune and neurological disorders, cardiovascular diseases and diabetes. This has caused a great interest towards inhibitors of protein kinases as potential therapeutic agents.

6

Inhibitors of protein kinases

Several direct and indirect strategies have been developed to inhibit protein kinases. The most successive has been the development of ATP-competitive inhibitors that bind to the well-defined hydrophobic adenine-binding cleft. This approach has apparent ease of development of high-affinity low molecular weight inhibitors that fulfil drug-likeliness criteria. On the other hand there are great challenges to solve selectivity problems because ATP-binding site is relatively similar throughout the kinome. Additionally *ca* 1500 other proteins are able to bind adenine nucleotides [43]. Finally, high concentration of competing ATP in the cellular milieu (~3 mM, while K_m values of protein kinases are usually 5 – 100 μM for ATP) results in the requirement of much higher concentrations of inhibitors to achieve desired amount of inhibition if compared to K_i values of the inhibitors.

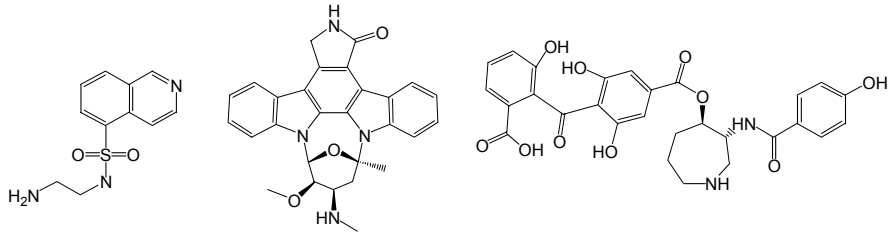


Figure 7. ATP-competitive inhibitors of protein kinases.

The simplest and first known ATP competitive inhibitors are fragments of ATP like ADP, adenosine and adenine. These compounds are not selective and have low potency (K_i > 30 μM). The first moderately potent inhibitors of protein kinases were isoquinolinesulfonamides with micromaolar activity against several AGC kinases (first published in 1984 [44]). The further development of these compounds has later yielded more active and selective derivatives [45, 46]. The first clinically applied inhibitor of protein kinase Fasudil is also isoquinolinesulfonamide derivative that has some selectivity towards Rho-kinase (ROCK) [46].

Later two different bioactive natural products staurosporine (from bacteria [47]) and balanol (from fungi [48]) were verified to act through inhibition of protein kinases. Both of the structurally different compounds inhibit several protein kinases at nanomolar level. Staurosporine is remarkably general and nonselective inhibitor. Bisindolylmaleimides (BIM-s or Bis-s) and some other compounds that have been designed by using staurosporine template show remarkable selectivity and have been widely used in biological studies [49, 50]. The complicated structure and labour-intensive synthesis of balanol and its

analogues have remarkably impeded their development and application in extensive biochemical studies [51, 52, 53]. The knowledge of binding interactions of balanol to the protein kinase [54] has facilitated the design of new type of inhibitors of AGC kinases that are easily prepared and more selective [55, 56, 57].

Tyrosine kinases and CDK-s play central role in pathogenesis of several cancers making them excellent drug targets. Massive research in this field has provided several tyrosine kinase inhibitors as new chemical entities to medicinal practice [58]. The most successful of them is Imatinib (Gleevec) that inhibits quite selectively mutated bcr-abl protein kinase. Imatinib is the current first choice drug for the treatment of chronic myelogenous leukemia [59] and is also used against other tumours like gastrointestinal stromal tumors (GISTs) [58]. It is the first member of a new class of agents that act by inhibiting particular cancer-specific enzymes instead of non-specific inhibition of rapidly dividing cells [3]. Inhibitors of CDK-s have shown promising anticancer properties during *in vitro* studies but toxic side-effects have precluded their clinical applications [60].

Aside from ATP-competitive compounds, substrate protein-competitive inhibitors of protein kinases have been developed. The recognition motifs of protein substrate-binding sites are mainly responsible for substrate specificity of protein kinases and are therefore these domains are structurally more variable than ATP-binding pockets. This allows easier design of selective inhibitors, but as longer peptidic structures are needed for achieving nanomolar potency, it leads to the problems with cellular transport and stability of the compounds [61, 62]. The easiest way to generate peptide-competitive inhibitors is the elimination of phosphorylatable hydroxyl group of substrate peptides, however the use of different peptide-mimetic structures is also possible [63]. Peptide-competitive compounds also inhibit particular protein kinases inside cells if they are delivered through the plasma membrane by using microinjection or the aid of transport peptides [64, 65, 66]. PKI, natural specific peptide inhibitor of cAPK, has been a general template for the design of other protein-competitive inhibitors [61].

Several unconventional strategies like allosteric inhibition have also been used to inhibit protein kinases [67, 68]. These approaches are not general and can not be applied for all kinases. The design of bisubstrate inhibitors seems to be a general strategy that could lead to potent and selective inhibitors [16].

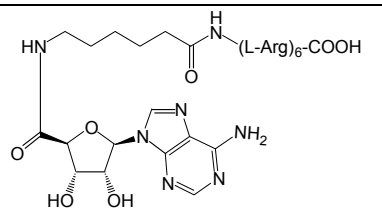
Design of bisubstrate-analogue inhibitors of protein kinases

Bisubstrate-analogue (biligand) inhibitors are compounds that are designed to associate simultaneously with both ATP and protein binding domains of protein kinase. Using of both substrate-binding pockets can result in selective and potent inhibitors of these dual substrate enzymes [16]. These compounds consist of two covalently bound fragments an ATP-mimetic part and a moiety that binds to protein/peptide substrate domain. The application of a linker that enables optimal positioning of the active fragments comprising the bisubstrate inhibitor can lead to a conjugate with kinase-binding energy substantially exceeding the sum of binding energies of the fragments [69]. In an ideal case the interactions of the moieties with the kinase are not disrupted and binding energies of the fragments summarize. Moreover, the binding of one particle to a protein is entropically less unfavourable than binding of two separate molecules. Chelate effect in the chemistry of coordination compounds has the same origin. This entropic factor may exceed 4 kcal/mol, corresponding to more than 3 orders of magnitude in potency scale [69, 70]. In the case of bisubstrate-analogue inhibitors of protein kinases this ideal case is usually not realized and the additivity of binding energies is commonly realized only partially [16].

The first successful example of the design of bisubstrate inhibitors for a kinase was the development of P1,P5-di(adenosine-5')pentaphosphate, an inhibitor with nanomolar potency towards adenylate kinase [71]. Phosphoryl transfer mechanism-based inhibitor design with connection of an adenine nucleotide *via* its phosphate groups with a substrate peptide at the phosphorylatable serine residue has been less successful. In case of these compounds, only micromolar and rarely submicromolar inhibitory potency have been achieved [23, 24, 25, 72, 73]. However, the resent developments where long and high-affinity peptides were connected with ATPγS *via* acetic linker have resulted in more potent compounds with low nanomolar activity [74, 75]. By virtue of low bioavailability and stability of substances comprising polar peptide moieties and highly charged oligophosphate fragments the pharmacological potential of these compounds has been limited. On the other hand testing of such compounds may provide valuable biochemical information about kinase functioning [73, 75].

Table 1. SAR for ARC-s variation of linker's length and number of arginines. The structure of the most active compound (right) [32]

Structure of the inhibitor	IC_{50}, μM[a]
Adc-Gly-(L-Arg)$_6$-OH	4.0 ± 0.2
Adc-β-Ala-(L-Arg)$_6$-OH	1.8 ± 0.1
Adc-GABA-(L-Arg)$_6$OH	1.3 ± 0.2
Adc-Aoc-(L-Arg)$_6$-OH	0.24 + 0.02
Adc- Ahx-(L-Arg)$_2$-OH	13.8 ± 1.0
Adc- Ahx-(L-Arg)$_4$-OH	1.2 ± 0.1
Adc-Ahx-(L-Arg)$_6$-OH	**0.12 ± 0.02**



ARC-306

[a] ATP (30 μM) and Kemptide (100 μM) were used as substrates [32].

The second large group of bisubstrate-analogue inhibitors of protein kinases is comprised of compounds with nonphosphate linker connecting adenosine and peptide moieties. These compounds do not incorporate negatively charged and biologically unstable polyphosphate chains, which could improve their utilization for cellular experiments. Different phenyl-containing linkers have been used for preparation of inhibitors for tyrosine kinases [76, 77, 78]. Usually the moieties are connected *via* an ester group between 5'-position of adenosine and the side-chain of tyrosine of the peptide mimic [16]. Using of Adc instead of adenosine enabled the application of solid-phase synthetic strategy throughout the synthesis [32]. In case of conjugates of Adc and oligoarginine (ARC-s), peptidic linkers are applied for connection of the fragments. The best inhibitors of this type showed submicromolar potency against cAPK and PKC [32]. Later it was demonstrated that ARC-s were easily immobilizable to affinity carriers [79] and could also be structurally modified to produce affinity or fluorescent ligands [80, 81]. The oligoarginine moiety renders ARC-s cell penetrating properties further increasing their potential for pharmaceutical applications [80, 81].

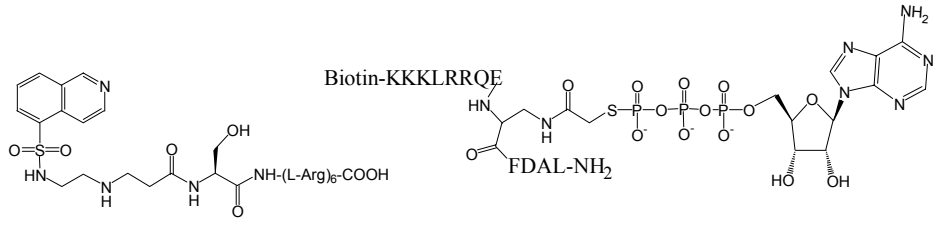


Figure 8. Examples of low nanomolar bisubtrate-analogue inhibitors of cAPK (left [82]) and CAMK-II (right [74]).

The third group of bisubstrate inhibitors are compounds where adenosine is replaced with a more active ATP-competitive inhibitor of the protein kinase. This approach has several advantages for the development of compounds with

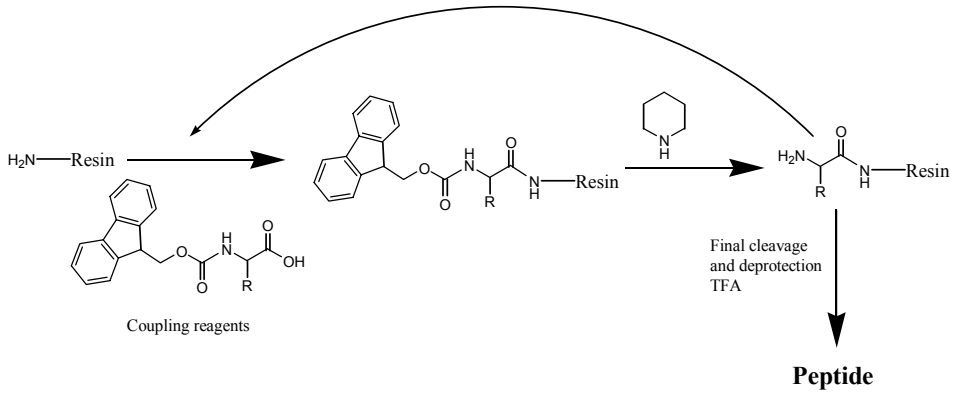
higher activity, selectivity, stability and lower molecular weight. Regardless of obvious benefits of this approach it has rarely been used. High-affinity ATP-competitive inhibitors may lose their activity during derivatisation and thus it is difficult to find a suitable connection point to the peptide. More complicated preparation of precursors for the synthesis of bisubstrate-analogues has also obstructed these developments. The first successful application of this strategy was the connection of an isoquinolinesulfonamide-based ATP-competitive inhibitor H9 (K_i = 2 μM) to the N-terminus of hexaarginine peptide *via* a linker incorporating *beta*-alanine and L-serine, which produced an inhibitor of cAPK with low nanomolar potency [82]. The further design of analogous conjugates with adenosine (ARC-s) used similar conception [32]. The isoquinolinesulfonamides have been connected with PKB-selective peptides producing compounds with up to 10 nanomolar potency, but selectivity between cAPK and PKB was not achieved [83]. Remarkably selectivity of the bisubstrate inhibitor for cAPK was achieved by connecting a staurosporine analogue k252a with PKI-containing miniature protein [84]. IC_{50} against cAPK was 3.6 nM while the same value for PKG was 680 nM and the other tested kinases (PKB, PKCα, CAMK-II) were inhibited to even less extent. High price of k252a and high molecular mass (MW > 5500) of the conjugate limits the large-scale production of this compound, but it is a good example of selective bisubstrate analogue inhibitor illustrating the general strategy.

Solid-phase peptide synthesis

Synthesis of the adenosine-peptide conjugates involves usually synthetic steps on solid phase. The preparation of the peptidic part or even entire conjugate is carried out on polymeric resin. The most widely used polymer for peptide synthesis is partially cross-linked polystyrene. The beads of this resin swell well in several organic solvents (DMF, DCM) allowing diffusion of reagents into the beads. The proper functionalisation of the polymer allows the reversible covalent connection of the molecules to the resin. Series of the synthetic steps are carried out with immobilized compound and the final cleavage from polymers produces the desired product. Easy removal of side-products and unreacted reagents by filtration is the main advantage of solid-phase synthetic strategy as it allows the usage of large excess of reagents to increase the yield [20].

The conventional peptide synthesis starts with connection of the first protected amino acid through the C-terminus to the resin, the removal of the protection group from N-terminus follows and the next amino acid can be coupled to the resin. Coupling and deprotection steps are repeated until the desired peptide sequence is prepared. Final cleavage releases the peptide from the resin and removes side chain protections. Two main strategies of peptide synthesis exist according to the N-terminal protective groups: Boc or Fmoc.

Although Boc-protected amino acids are cheaper, most of the laboratories prefer Fmoc-peptide synthesis strategy due to milder deprotection conditions of Fmoc [85]. Easy repeatable procedures have allowed automatization of solid phase synthesis and facilitated the processes even further.



Scheme 4. Solid-phase peptide synthesis with the application of Fmoc-strategy.

AIMS OF THIS STUDY

The main tasks of the Thesis were:

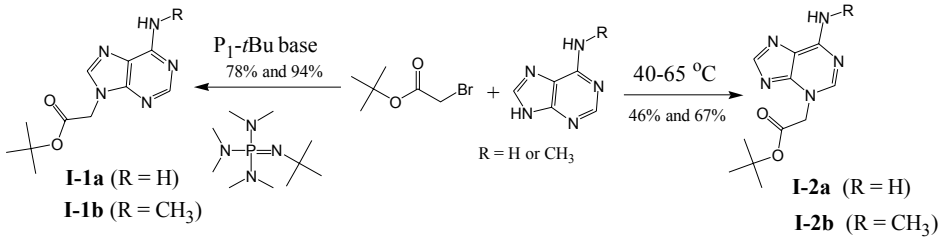
1. The development and improvement of synthetic methods for preparation of various adenosine-peptide conjugates.
2. The synthesis of appropriate adenosine- and adenine-containing precursors that could be further used in solid-phase synthesis.
3. The research of the applicability of organic bases in adenine alkylation reactions.
4. The synthesis of carboxylate-containing analogues of adenine nucleotides as potential ligands of P2 receptor.
5. The design and synthesis of various ARC-type of inhibitors for basophilic protein kinases and optimization of their structures in order to achieve high potency.
6. The synthesis of carbocyclic 3'-deoxyadenosine-containing ARC-s and evaluation of inhibition properties of the conjugates against protein kinases.

RESULTS AND DISCUSSION

Alkylation of adenine with the aid of organic and mineral bases [I]

Introduction of alkyl group to N9 position of adenine requires prior removal of 9-hydrogen to produce nucleophilic anion. Different inorganic bases like NaH and K_2CO_3 have been widely used for the activation of adenine in alkylation reactions with moderate yield producing more or less regioselectively N9-derivatives, the most common adenine-containing compounds in biological systems. Due to low solubility of adenine and corresponding salts in organic solvents, these reactions are heterogeneous and often require long times to complete. Heterogeneous mixtures can not be used in solid-phase synthesis where good swelling of the polymer resin and sufficient solubility of the reagent in the given solvent are necessary prerequisites for the organic reaction. Application of organic bases that form more hydrophobic and soluble cations could be a good alternative in case of solid-phase reactions.

DBU is one of the reagents used for the alkylation of adenine in DMF and continuous improvement of yields and regioselectivity have been reported [86]. However, the basicity of DBU is insufficient for full deprotonation of adenine in non-hydroxylic solvents as dimethyl sulfoxide, DMF and acetonitrile (pK_a = 14.2 for adenine [87] and pK_{BH+} = 13.9 for DBU in DMSO [88]). Hence, the excess of the base, higher temperatures and longer reaction times are required for quantitative alkylation of adenine. The propensity of DBU to alkylation under these conditions and incomplete solulilization of adenine limits the use of DBU as an ionizing base for the synthesis on solid phase. Schwesinger's phosphazene bases [89] possess several better characteristics for practical applications: their basicity can be varied over a wide pK_a range, they are not prone to alkylation and their cationic forms are well soluble in non-hydroxylic solvents. The commercial availability of phosphazenes with different basicity makes it possible to deprotonate the reagent selectively and quantitatively at the most acidic site in the presence of the equimolar amount of the base. P1-tBu phosphazene [tert-Butylimino-tris(dimethylamino)phosphorane] was found to be efficient ionizing base with suitable basicity characteristics (pK_{BH+} = 15.7 in DMSO [88]): only small excess of the base is sufficient for complete deprotonation of adenine.



Scheme 5. Alkylation reactions of adenine and methyladenine.

Addition of phosphazene P1-tBu to the suspension of purines in DMF resulted in complete solubilization of N6-methyladenine and partial solubilization of adenine. The following reaction with *t*-butyl bromoacetate (Scheme 5) was completed within seconds. Esters of adenine-9-acetic acids **I-1a** and **I-1b** were isolated as single N9-regioisomers with high yields (78% and 94%, respectively). The conversion of adenines into products was less effective in the presence of the same molar quantity of DBU. Alkylations with NaH were slower and resulted in lower preparative yields. Superiority of P1-tBu phosphazene base over NaH was apparent also in the reactions with other derivatives of bromoacetic acid (**I-5** in Scheme 7). Reactions under the conditions where no base was added required higher temperatures (40–65°C, 2–6 h) to proceed and gave expectedly [9] N3-substituted derivatives **I-2a** and **I-2b** as the main products.

Alkylation of adenine was also carried out on solid phase. The N-terminus of the resin-bound peptide was first bromoacetylated and the reaction with adenine anion in DMF (in the presence of P1-tBu phosphazene base) followed. The alkylation of adenine on solid support was quick and gave high yields, but the reactions produced mixtures of regioisomers (N9-, N7- and N3-alkylated products in a 4:2:1 molar ratio). The isomeric compositions were analyzed by NMR (Figure 9) after cleavage of the product from the resin with TFA. The application of DBU afforded worse solubilization of adenine and gave lower yields of alkylation mostly due to the reaction of DBU with the bromoacetylated peptide. The molar ratio of the obtained adenine regioisomers was similar in the case of both applied bases.

Chromatographic separations of the regioisomers were successful only for some derivatives. The regioisomers with smaller groups connected to adenine ring were usually more easily separatable. Structures of regioisomers were verified on the basis of NMR and UV spectra of the compounds [90].

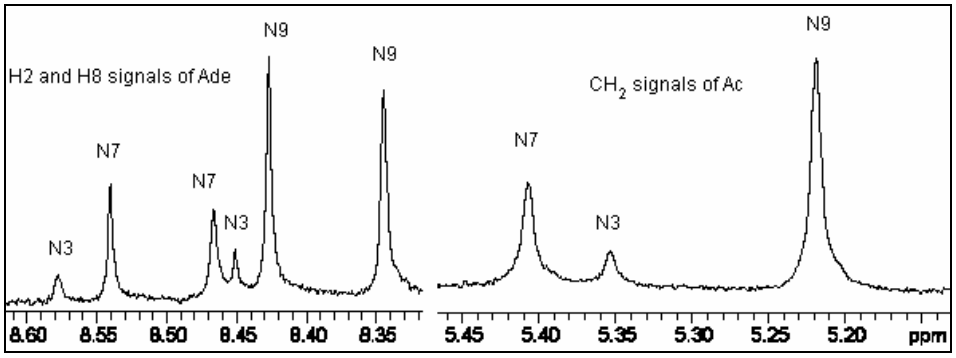
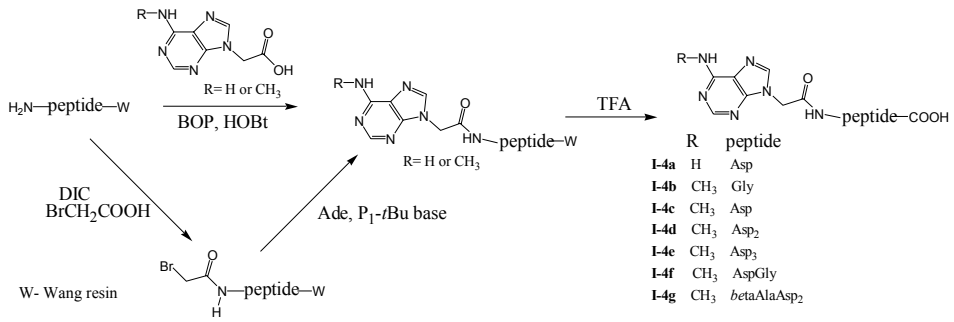


Figure 9. Fragments from ^{1}H NMR (D_2O) spectrum of the product of alkylation of adenine on solid phase (aspartic acid derivative of adenine-acetic acid **I-4a**). The signals of three regioisomers (N9-alkyl, N7-alkyl and N3-alkyl) are visible.

These results demonstrate the effectiveness of P1-tBu phosphazene base for the deprotonation and alkylation of adenine in solution. Higher yields and reactions rates may be a great benefit in several cases (*e.g.*, high prize of the electophile). However, in some cases these advantages did not realize (paper III). Most of the P1-phosphazenes are liquids, which makes their exact dosage by pipetting very facile, compared to the quantification of the suspension of NaH. The main disadvantages of using these chemicals are higher cost of the phosphazenes and slightly more complicated purification of the products. Also, an unexpected formation of mixture of regioisomers in case of reactions on polystyrene resin limits the use of this approach. The phenomenon that occurred needs further investigation in order to find out the reasons for poor regioselectivity and possible means for improvement of the yield of the desired N9-isomer.

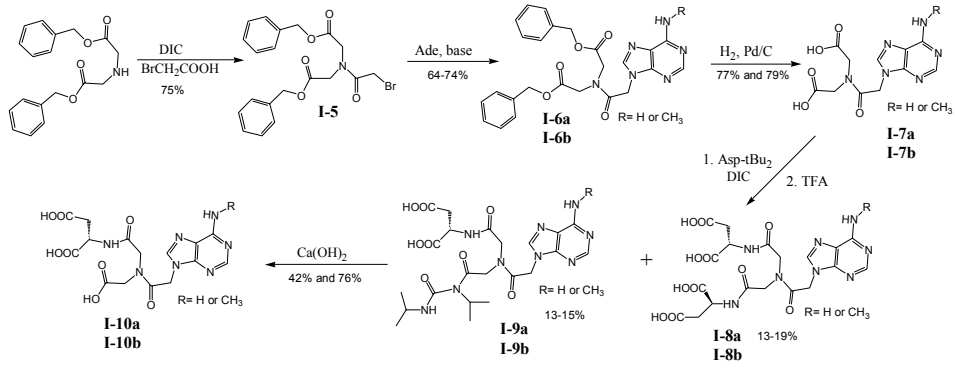
Synthesis of carboxylate-containing adenine nucleotide analogues [I]

A series of non-phosphate nucleotide analogues that does not contain ribose moiety was prepared. As acyclic bisphosphates were shown to act as antagonists of $P2Y_1$ receptor and inhibitors of ADP-induced platelet aggregation [37], we substituted carboxylates for phosphates by using negatively charged amino acids. Syntheses were carried out either on solid phase with application of conventional peptide-synthesis methodology (Scheme 6) or in solution (Scheme 7).



Scheme 6. Adenine alkylation on solid phase synthesis of carboxylate-containing nucleotide analogues.

Adenine-9-acetic acids (**I-3a** and **I-3b**) were attached to the peptides by direct coupling to polymer-bound peptides. The other possibility was preliminary bromoacetylation of the peptide and the following reaction with adenine anion in the presence of P1-tBu phosphazene base. Finally, protective groups were removed and the products were cleaved from the Wang resin with 95% TFA (Scheme 6). Purification by HPLC gave products with high preparative yield (~60%). Seven different conjugates (plus some regioisomes) were prepared by solid-phase approach. The compounds incorporate from one to four carboxylate groups that are negatively charged at physiological pH. Most of the derivatives contain methyl group at N6 position shown to increase activity and selectivity of $P2Y_1$ receptor antagonists [34, 39].



Scheme 7. Preparation of IDA-containing nucleotide analogues.

The second series of carboxylate-containing nucleotide analogues were synthesized according to the Scheme 7. These derivatives contain the IDA moiety for which no suitable reagents are available for solid-phase synthesis, hence the conventional liquid phase procedures were carried out. Bromoacetylation of

dibenzyl iminodiacetate in the presence of DIC led to the amide **I-5** which was subsequently used for alkylation of adenine and N6-methyladenine in the presence of bases (NaH, K_2CO_3 or phosphazene P1-tBu base) to produce adenine derivatives **I-6a-b**. The application of the organic base gave higher yields and the reactions were completed in a shorter time. Hydrogenation of dibenzyl esters **I-6a-b** on Pd/C gave diacids **I-7a-b** that were coupled with di-*t*-butyl ester of L-aspartic acid using DIC activation. The products were treated with TFA to remove *t*-butyl groups and the residues were purified by HPLC. Two pairs of individual compounds were separated (**I-8a-b** and **I-9a-b**). Low yields of the products were probably caused by inappropriate activation methodology (carbodiimide without auxiliary reagents). Compounds **I-9a-b** were N-acylurea derivatives formed as side-products during the activation of carboxylate groups with the carbodiimide. The removal of the N-acylurea moiety from **I-9a-b** by hydrolysis in $Ca(OH)_2$ solution yielded **I-10a-b**. Calcium ions have been shown to catalyze hydrolysis of N-acylureas and allow performance of the reaction at lower pH [91]. Six different carboxylate-containing adenine derivatives with two to four acid groups were prepared. Three of the compound contained N6-methyl group to increase activity against $P2Y_1$ receptor.

Inhibition of ADP induced platelet aggregation [I]

The new acyclic non-phosphate nucleotide analogues were evaluated for activity in human platelet aggregation assay (experiments were performed at the pharmacological company Inspire, USA). Activations of $P2Y_1$ and $P2Y_{12}$ receptors by ADP or other agonists are both required to induce platelet aggregation. Antagonists of these receptors inhibit this process and are potential antithrombosis agents in medicine. $P2Y_{12}$ receptor is more specific to platelets (found also only in brain [40]) and therefore better drug target than ubiquitous $P2Y_1$. Compounds **I-4e**, **I-7a**, **I-8a**, and **I-10b** produced a weak inhibition of ADP-induced platelet aggregation (Table 2). The mechanism of the inhibitory effect of these compounds has not yet been determined. Greater activity of N6-methyl containing compounds **I-10b** and **I-4e** suggests that the effects are mediated through $P2Y_1$ receptor. The obtained activity pointed to the possibility that acyclic non-phosphate nucleotide analogues are potential P2 receptor ligands. Lower activity of carboxylate-containing compounds if compared to the corresponding bisphosphates suggests that carboxylates mimic phosphates only partially and perfect positioning of negative charges is required for achieving detectable affinity.

Table 2. Inhibition of platelet aggregation

Compound	Inhibition (%) of platelet aggregation at 100 μM. N = 3
I-4g, **I-7b**	NE
I-4c, **I-4d**, **I-8b**, **I-10a**	NR
I-4e	28.7 ± 8.1
I-7a	11.7 ± 6.0
I-8a	9.7 ± 4.8
I-10b	53.3 ± 3.3

NE = no effect, NR = small and not reproducible effect, N – number of measurements

The synthesized compounds may have activity against several other nucleotide-binding receptors or enzymes, *e.g.,* several phosphatases that hydrolyse adenosine nucleotides are potential targets for these compounds. Inhibition of intracellular proteins by carboxylate-containing nucleotide analogues requires masking of the negative charge by their structural modification into prodrugs in order to allow their internalization into cells.

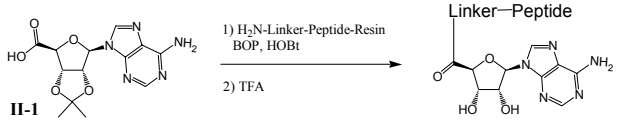
Synthesis of ARC-s with variable structure [II]

ARC-s possess several properties useful for practical applications like possibility for immobilization and derivatisation allowing preparation of affinity carriers and tagged ligands [79, 80, 81]. Most of the small-molecular ATP-competitive inhibitors do not have such properties. The oligoarginine moiety allows the use of ARC-s in cellular experiments as regulators of phoshorylation balance, but easily degradable peptide part shortens the time of their activity. Substitution of peptide that consists of natural L-amino acids with more stable structural element could therefore advance the development of these compounds. The potency of first generation ARC-s [32] extends to submicromolar range that was insufficient for several practical applications like SPR biosensors [92] or fluorescent probes for binding measurements as low nanomolar affinity of derived or immobilized ligands is required for applications that are practically valuable for these methods [93]. Moreover, the high concentration of ATP in cells demands high affinity of ATP competitive inhibitors of protein kinases in order to produce a cellular response. All these reasons pointed to requirement for significant improvement of activity and biological stability of ARC-type inhibitors.

The previous structure-activity studies had shown that the optimal length of the oligoarginine moiety was six arginine residues and the removal of two arginines from the C-terminal of the conjugate decreased the inhibitory potency by an order of magnitude (Table 1) [32]. On the other hand, the majority of basophilic serine/threonine kinases preferentially phosphorylate substrates with

Arg at P-3 position but vary greatly in additional preference for Arg at P-2 or P-5 position (P- designates amino acid residues positioned in the direction of N-terminal from the phosphorylation site) [94]. Arginines at positions P-2, P-3 and P-6 are essential for cAPK. One study reports even that arginine is the most preferred amino acid in all positions from P-2 to P-8 [95]. Hence, six sequential arginine residues in the peptide chain can enable important interactions in the peptide-binding site of target basophilic kinase. Introduction of more than 6 arginine residues into the ARC-type conjugate could lead to substantial non-specific interaction of the compound with other basophilic proteins and nucleic acids (*e.g.,* furins, RNA and DNA).

However, as the biological characterization of inhibitors with micromolar activity was easier to perform, compounds comprising four arginine residues were utilized for the optimization of structures of the linker and nucleoside moieties of the conjugates. The compounds containing six arginines were later synthesized to achieve higher inhibitory potency. For the elimination of the negative charge from the C-terminal carboxylate group, the conjugates were synthesized in the form of C-terminal amides on Rink amide MBHA resin. The intramolecular interaction of the carboxylate group with a positively charged arginine residue could reduce inhibitory potency of the conjugates towards cAPK [79, 81].



	Linker	Peptide
II-2	NH-$(CH_2)_5$CO	$(L\text{-}Arg)_4$-NH_2
II-3	NH-$(CH_2)_5$CO	$(L\text{-}Arg)_6$-NH_2
II-4	NH-$(CH_2)_5$CO	$(D\text{-}Arg)_4$-NH_2
II-5	NH-$(CH_2)_5$CO	$(D\text{-}Arg)_6$-NH_2
II-6	N(CH_3)-$(CH_2)_5$CO	$(L\text{-}Arg)_4$-NH_2
II-7	NH-CH_2-Ph-4-CO	$(L\text{-}Arg)_4$-NH_2
II-8	NH-Phe-*beta*Ala-CO	$(L\text{-}Arg)_4$-NH_2

Scheme 8. Synthesis of adenosine-5'-carboxylic acid-based ARC-s, variation of linker and peptide moiety.

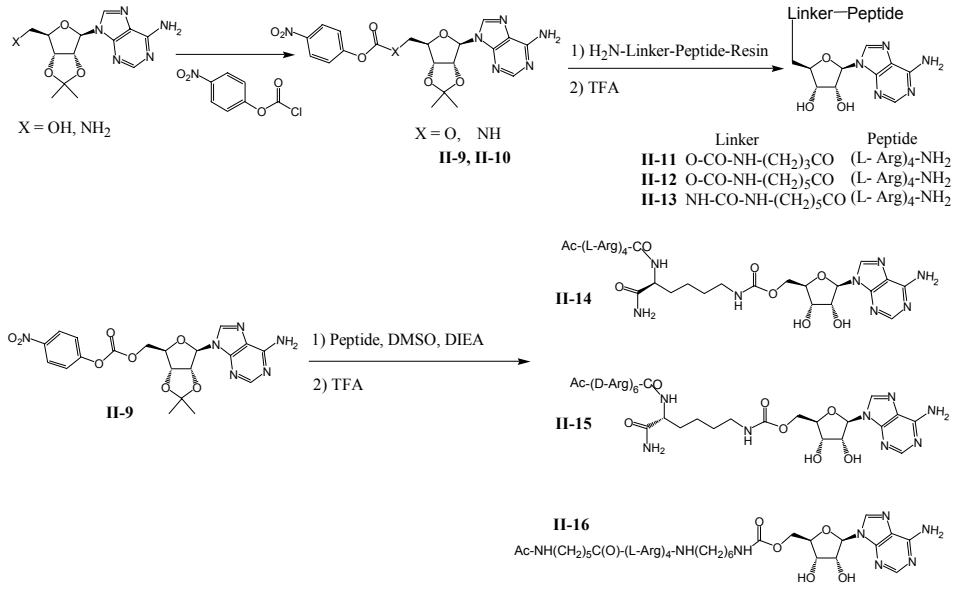
The syntheses of Adc-containing ARC-s were performed by using Rink amide resin (instead of Wang resin [32]) that yielded compounds in the form of C-terminal amides (Scheme 8). The structures of the linker and peptide components were varied, whereas the optimal length of the linker (corresponding to seven chemical bonds) between the nucleoside and peptide moieties was retained during linker variations. Methylated analogue **II-6** possessing no hydrogen bond donor characteristics was synthesized to preclude the formation of intramolecular hydrogen bond that stabilizes unflavoured *syn*-conformation of the nucleoside. The resin-bound peptide was first acylated with 6-bromohexanoic acid and then reacted with excess of methylamine. N-methylamino group of the resin-bound peptide was conjugated with IpAdc that lead to compound **II-6**. 4-(Aminomethyl)benzoic acid was used as an example of rigid aromatic linker in the compound **II-7**. Compound **II-8** with phenylalanine-(β-

alanine) dipeptide linker was prepared to investigate the effect of a bulky aromatic group in the linker region. Different lengths and the chiralities of the oligoarginine chain were used for variation of the structure of peptide part of the conjugates (Scheme 8).

The structure of 5'-carbon in adenosine 5'-urethanes is more similar to that of adenosine than to derivatives of Adc. A great difference in activity of these derivatives as antagonists of $P2Y_1$ receptors (adenosine 5'-urethanes had activity while analogous compounds of Adc were inactive [28]) points to the importance of such small changes in the structure for the biological activity.

The reaction of 4-nitrophenyl chloroformate with 2',3'-isopropylidene adenosine resulted in the activated carbonate **II-9**, which gave adenosine-5' -urethanes **II-11** and **II-12** during reactions with amines (Scheme 9). An analogous synthetic scheme was applied to prepare conjugate with carbamide linker (**II-13**). The reaction of 5'-amino-5'-deoxy-2',3'-O-isopropylidene adenosine with 4-nitrophenyl chloroformate produced activated 4-nitrophenyl urethane **II-10** that had sufficient reactivity to prepare adenosine-5'-ureas like **II-13**.

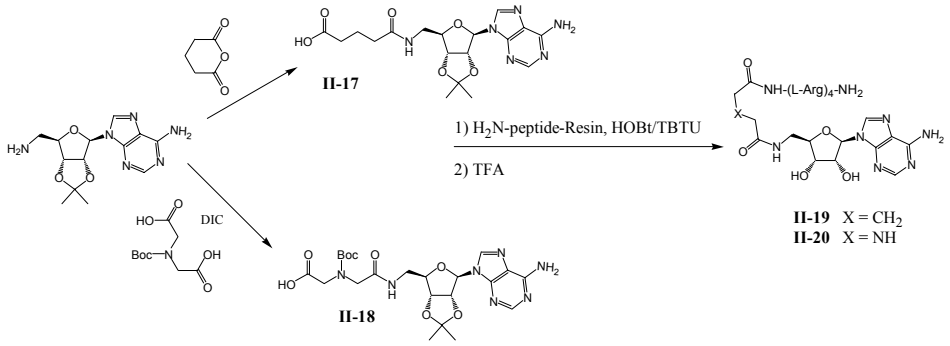
II-9 and **II-10** are good and reactive precursors for the synthesis of various adenosine derivatives. Preparation of these compounds leads to several problems and only moderate yields (30–65%) are usually achieved. Moreover, storage in cold and dry conditions in powder form is needed for these relatively unstable compounds.



Scheme 9. Synthesis of compounds with urethane and urea connections and positioning of adenosine to the C-terminus of peptides.

Variation of the structure of the conjugates also included the connection of adenosine to the C-terminus of the oligoarginine chain (compounds **II-14-16** in Scheme 9). All previous conjugates had adenosine or an adenosine mimetic connected to the N-terminal of oligoarginine [32, 82]. The important arginine residues in substrate peptides beside are situated at N-terminal part of the peptide chain from the phosphorylatable amino acid, which means that the C-terminus of arginine-rich peptide fragment points towards ATP in kinase-catalyzed phosphorylation reaction. Thus, bisubstrate inhibitors where adenosine is connected to the C-terminal of the oligoarginine should be better mimics of an activated state of the kinase reactions and therefore more potent inhibitors.

The synthesis of this kinds of compounds consisted of two parts. First, the peptides with acylated N-terminus and free amine at C-terminus were prepared on solid phase, cleaved and purified by HPLC. Secondly these peptides were reacted with **II-9** in solution and then isopropylidene protections were cleaved by TFA to produce compounds **II-14** to **II-16**. Reactive amino group was placed to C-terminal part of the peptides by using diamine linker (**II-16**) or side-chain of lysine (**II-14** and **II-15**). L- and D-arginines were both used in these series of compounds.

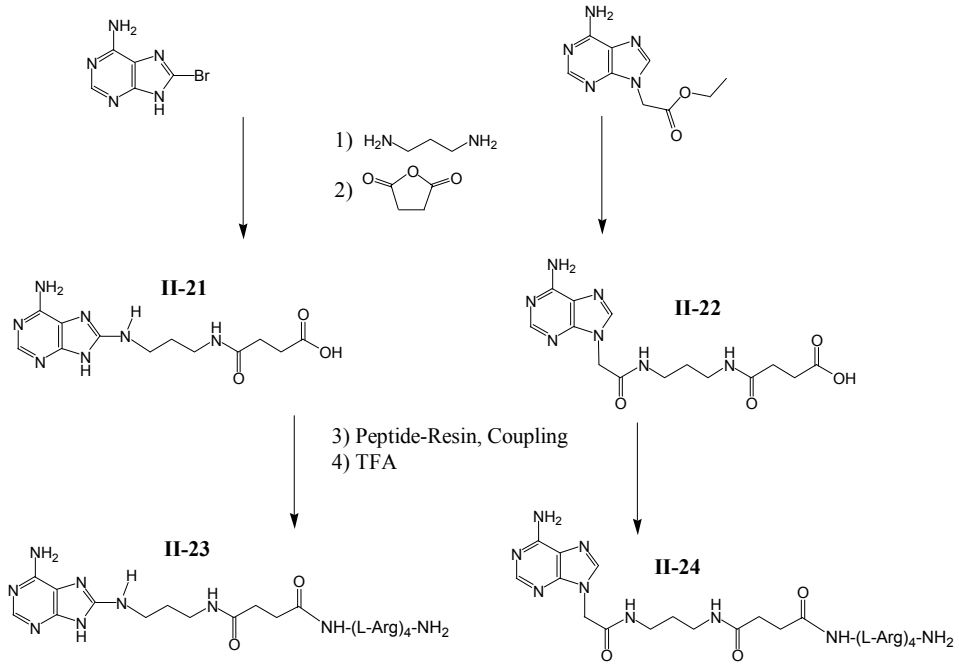


Scheme 10. Synthesis of a compound with a secondary amino group in the linker region and its CH_2-containing counterpart

Most of the highly potent inhibitors of AGC kinases contain primary or secondary amino group. This functionality binds to same position as ribose 2'- and 3'-hydroxyls (H-inhibitors, staurosporine, balanol, etc.) or to the pocket of Mg^{2+} ion [96] that is connected to triphosphate chain. The suitable positioning of an amino or imino group into adenosine part of the ARC-s may substantially increase the affinity of conjugates. One of the attempts to realize this approach is represented in Scheme 10.

The reaction of 5'-amino-5'-deoxy-2',3'-O-isopropylidene adenosine with glutaric anhydride and Boc-protected iminodiacetic acid anhydride yielded **II-17** and **II-18**, respectively. These compounds were coupled to resin-bound

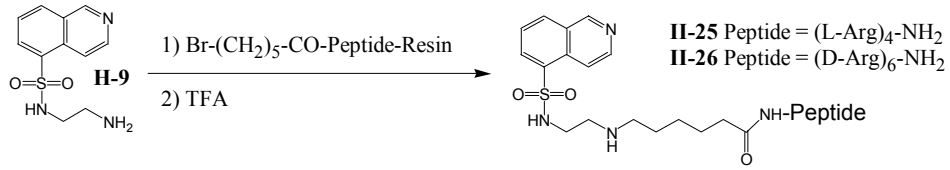
peptides with the aid of TBTU activation. Cleavage and deprotection of the conjugates with TFA gave compounds **II-19** and **II-20** that differed from each other by the presence of either CH_2 or NH group in the linker chain. These two compounds are easily comparable and the effect of imino group in the middle of the linker can be determined.



Scheme 11. Synthesis of conjugates of adenine and oligoarginine.

Comparison of inhibition potencies of adenine and adenosine with K_m of ATP indicated that the majority of binging energy of these derivatives originates from adenine moiety [97]. This leads to assumption that ribose moiety in the structure of ARC-s is not obligatory and could be removed without a loss of binding energy. Moreover, the conjugates without ribose moiety have smaller molecular weight and do not contain an unstable glycoside bond. This class of ARC-s was synthesized by connecting adenine at C8 or N9 position to a peptide *via* linker chain (Scheme 11). Precursors of these molecules containing linker with a free carboxylate group (**II-21** and **II-22**) were synthesized in solution. Coupling of **II-21** and **II-22** with the resin-bound peptides resulted in conjugates **II-23** and **II-24** after cleavage and deprotection with TFA. Synthesis of **II-21** and **II-22** contained reactions with diaminopropane that were carried out in the pure amine to increase the rate of these slow reactions. Reaction with

8-bromoadenine needed heating but aminolysis of adenine-9-acetic acid ethyl ester was surprisingly fast.



Scheme 12. Synthesis of isoquinolinesulfonamide-based conjugates.

Conjugates of isoquinolinesulfonamide and oligoarginine were the first bisubstrate-analogue inhibitors of protein kinases with low nanomolar potency [82]. Cell-penetrating peptides were not known in the beginning of 1990-s, which resulted in little interest in these compounds. Later development of ARC-s [32] used the same hexa-(L-arginine) peptide fragment. Isoquinoline-based H-series inhibitors have about 100-fold stronger inhibitory potency than adenosine and similar difference in potency was also found for their conjugates with the peptide [82, 32].

New conjugates of isoquinolinesulfonamide and oligoarginine were synthesized in order to compare their activity and selectivity to those of analogous adenosine-containing compounds. Compared to the previously synthesised isoquinolinesulfonyl peptides [82], several modifications were introduced into the structure of the conjugate and the synthesis was carried out on solid phase with the application of Fmoc-peptide and peptoid chemistry procedures. The use of Rink amide resin excluded the presence of negatively charged C-terminal carboxylate group and resulted in products in the form of C-terminal amides. The (β-Ala)-Ser linker of previous conjugates [82] was replaced with 6-aminohexanoic acid, which eliminated the potentially phosphorylatable serine residue, removed unnecessary chiral centre and simplified the overall synthetic procedure. Resin-bound oligoarginine peptides were acylated at N-terminus with 6-bromohexanoic acid and the following reaction with 5-isoquinolinesulfonyl ethylenediamine (H9) and final cleavage with TFA lead to conjugates **II-25** and **II-26** (Scheme 12).

To sum up, preparation of Adc conjugates uses cheaper reagents and gives higher yields than the synthesis of H9 derivatives. Some improvements should be introduced into the methodology if larger scale preparation of isoquinoline-peptide conjugates is needed.

Inhibition of cAPK by ARC-type inhibitors [II]

The potency of inhibitors towards cAPK Cα was measured by using the fluorometric TLC kinase activity assay [98] and the results were expressed as IC_{50} values in Table 3. TAMRA-kempide (30 μM) and ATP (0.1 mM or 1.0 mM) were used as substrates. In case of strongest inhibitors the application of higher concentration of ATP (1.0 mM) enabled to avoid tight-binding conditions of the assay. The competitiveness with ATP was also detected.

Table 3. Inhibitory potencies of compounds towards cAPK Cα [II]

compound	Code	structure	IC_{50} (μM)[a]
II-2	ARC-340	AdcAhx(L-Arg)$_4$-NH_2	2.0 ± 0.3
II-3	ARC-341	AdcAhx(L-Arg)$_6$-NH_2	0.17 ± 0.04
II-4	ARC-582	AdcAhx(D-Arg)$_4$-NH_2	0.33 ± 0.03
II-5	ARC-902	AdcAhx(D-Arg)$_6$-NH_2	0.0083 ± 0.0015 (0.109 ± 0.021)
II-6	ARC-656	AdcN(Me)Ahx(L-Arg)$_4$-NH_2	2.6 ± 0.6
II-7	ARC-351	AdcNHCH$_2$Ph-4-C(O)(L-Arg)$_4$-NH_2	23 ± 4
II-8	ARC-901	AdcPhe-betaAla(L-Arg)$_4$-NH_2	3.7 ± 0.3
II-11	ARC-348	AdoC(O)NH(CH$_2$)$_3$C(O)(L-Arg)$_4$-NH_2	3.2 ± 0.3
II-12	ARC-344	AdoC(O)Ahx(L-Arg)$_4$-NH_2	3.0 ± 0.3
II-13	ARC-346	AdnC(O)Ahx(L-Arg)$_4$-NH_2	12.5 ± 3.7
II-14	ARC-350	Ac(L-Arg)$_4$-L-Lys[AdoC(O)]-NH_2	1.4 ± 0.2
II-15	ARC-658	Ac(D-Arg)$_6$-D-Lys[AdoC(O)]-NH_2	3.4 ± 0.6
II-16	ARC-650	AcAhx(L-Arg)$_4$-NH(CH$_2$)$_6$NHC(O)Ado	0.77 ± 0.16
II-19	ARC-651	AdnC(O)(CH$_2$)$_3$C(O)(L-Arg)$_4$-NH_2	16.5 ± 4.7
II-20	ARC-652	AdnC(O)CH$_2$NHCH$_2$C(O)(L-Arg)$_4$-NH_2	13 ± 6
II-23	ARC-654	Ade-8-NH(CH$_2$)$_3$NH-Suc(L-Arg)$_4$-NH_2	75 ± 8
II-24	ARC-653	Ade-9-AcNH(CH$_2$)$_3$NH-Suc(L-Arg)$_4$-NH_2	26 ± 10
II-25	ARC-649	H9-(CH$_2$)$_5$C(O)(L-Arg)$_4$-NH_2	0.030 ± 0.007 (0.22 ± 0.03)
II-26	ARC-903	H9-(CH$_2$)$_5$C(O)(D-Arg)$_6$-NH_2	0.0053 ± 0.0007 (0.067 ± 0.019)
II-27		Ac-(D-Arg)$_6$-D-Lys-NH_2	~3000
Adenosine			350 ± 40
H89			0.10 ± 0.02 (0.85 ± 0.09)
H9			3.7 ± 0.3
Ado + II-27[b]			57 ± 19

[a] IC_{50} values for inhibition of cAPK Cα-catalysed reaction at standard substrate concentrations (ATP, 100 μM; TAMRA-kemptide, 30 μM). Values in parentheses are determined at 1.0 mM ATP concentration. [b] IC_{50} for equimolar mixture of the two compounds.

Amidation of the C-terminal carboxylate increased the activity of the conjugates by 4–6 times, leading to IC_{50} values of 2.0 and 0.17 μM for **II-2** and **II-3**, respectively. Their carboxylate counterparts showed IC_{50} values of 11.5 and 0.70 μM, respectively (values in Table 1 are measured at lower concentration of ATP). This may be caused by the lack of compensation of the positive charge of an arginine residue participating in a favourable contact with the kinase by negative charge of the carboxylate group. Amidation increases hydrophobicity of the conjugates and makes them more stable against carboxypeptidases. Similar increase of potency has been described for inhibitor peptides with C-terminal amides [99].

Several modifications in the structure of the tether between adenosine and peptide moieties of conjugates were well tolerated by cAPK (Table 3, compounds **II-2**, **II-6**, **II-8**, **II-11**, and **II-12**), while some small structural modifications lead to significant decrease of activity (**II-7**, **II-13**). The addition of N-methyl group to the 5'-position of adenosine moiety of the conjugate (**6-I** and **2-I**, respectively) left the activity unchanged, which pointed to the negligible impact of the intramolecular hydrogen bond and putative *syn*-conformation of Adc-amides to their binding to the kinase in *anti*-conformation [97]. Incorporation of phenylalanine residue into the linker chain (**II-8**) caused only minor decrease of activity, which refers to the possibility of inclusion of bulky hydrophobic moieties into this part of the inhibitor. This property may be useful for the design of tagged ligands.

The activities of adenosine-5'-urethanes **II-11** and **II-12** were well comparable to those of Adc derivatives, while the corresponding 5'-urea-connected compound **II-13** showed 4-fold lower inhibitory potency. 5'-Urethane derivatives with D-arginine-containing peptides have not been prepared yet.

The compound with 4-(aminomethyl)benzoic acid linker (**II-7**) had about 10-fold lower activity than the compound with 6-aminohexanoic acid linker (**II-2**). This effect may result from the steric hindrance of the rigid tether to the optimal positioning of interacting fragments, adenosine and tetraarginine necessary for kinase targeting. Exclusion of the ribose fragment from conjugates (**II-23** and **II-24**) lead to substantial reduction of inhibitory potency which showed the importance of interaction between the sugar-part of compounds and the kinase; still, both conjugates were better inhibitors of cAPK than their fragments, adenine and oligoarginine. These results are in contradiction to previous knowledge that adenosine and adenine have similar binding affinities [97]. More effective positioning of peptide fragment of adenosine conjugates can be one explanation. Crystal structures of complexes of purines with protein kinases have shown different binding positions for N9-unsubstituted purines [57] and adenine moiety in the structures with adenosine [97]. According to the latter description, the ribose interactions with kinases are important for binding 9-substituted derivatives of adenine like adenosine.

Variations of the peptide fragment included the connection of adenosine to the C-terminus of the oligoarginine chain (**II-14** – **II-16**) and the use of D-arginines (**II-4**, **II-5** and **II-15**). The aim of the latter approach was increase of the proteolytic stability of compounds to make them applicable in cell experiments.

The inhibitors with peptide moieties connected to the nucleoside part by C- and N-termini had similar affinities in the case of compounds containing peptides which incorporate L-amino acid residues (**II-2** and **II-14**), whereas the compound comprising the peptide with D-arginine residues connected *via* the C-terminus (**II-15**) had 500 times lower activity than the counterpart with N-terminal peptide (**II-5** = **ARC-902**). These results proved that the connection of adenosine to the C-terminus of the peptide yields active bisubstrate-analogue inhibitors. The available information concerning only three compounds is insufficient for the profound comparison of two different kinds of conjugation strategies. Easier derivatisation of N-terminus of the peptide and high potency of the corresponding D-arginine-containing compounds (**II-4**, **II-5**) are essential vantages of the first approach. The further optimization of the linker may also lead to potent inhibitors where adenosine is connected to the C-terminus of the peptide.

The compounds with adenosine connected to the N-terminus of an all-D-arginine peptide (**II-4**, **II-5**) had remarkably higher affinity than the conjugates containing L-amino acids (**II-2**, **II-3**). The compound **II-4** with four D-arginines (IC_{50} of 0.33 μM) had six-fold higher potency than its L-arginine containing counterpart **II-2**. Even greater increase of potency (20 times) was detected for compounds with six arginines (IC_{50} values of 0.17 μM and 0.0083 μM, respectively). This result was surprising because cAPK has a strong preference for L-configuration of amino acids both at the phosphorylatable serine residue as well as at the N-terminal arginine residues of the substrate [100]. On the other hand, compounds **II-2** to **II-8,** depicted in the Scheme 7, comprise oligo-arginine peptides attached to Adc N-terminally, not C-terminally, that could be the most reasonable positioning of the peptide originating from the consensus sequence of substrates of cAPK. Introduction of arginine residues into ARC in the form of their D-configuration converts the peptide motif into *retro-inverso* counterpart of the C-terminally appended oligo-(L-arginine) peptide. A *retro-inverso* peptide can be regarded as a derivative of a normal peptide in which the relative amino acid side chain topology is maintained, while the backbone termini and direction of the peptide bonds are reversed [101].

L-Ser-L-Asp-L-Val-L-Ala and D-Ala-D-Val-D-Asp-D-Ser

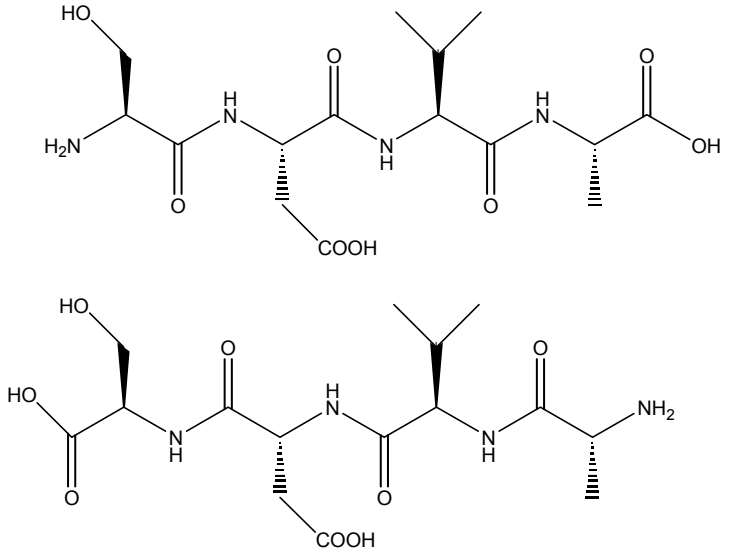


Figure 10. An example of the structure of a peptide and its *retro-inverso* analogue.

The isoquinolinesulfonylamide-based compound **II-26** incorporating six D-arginines is a highly effective inhibitor of cAPK exhibiting IC_{50} of 5.3 nM at the described assay conditions. The obtained IC_{50} value is similar to that of the most potent adenosine derivative **II-5**, whereas analogous compounds with L-arginines differ by 65-fold in activity (**II-2** and **II-25**). The reason of this different effect of D-arginines in the case of adenosine and isoquinoline-sulfonamide conjugates is unknown, hence the obtained results point to an interesting phenomenon that should be investigated more thoroughly.

Inhibition data of adenosine, H9 and H89 (Table 3) serves as good reference for the comparison ARC potencies with previously known compounds. The inhibitory potency of the equimolar mixture of adenosine and peptide **II-27** reveals the importance of covalent connection between two fragments in bisubstrate inhibitors (**II-5**).

Finally, D-arginine-containing conjugates (**II-4** and **II-5**) were stable to trypsination while their L-arginine counterparts (**II-2** and **II-3**) degraded quickly. This is first direct proof of increased proteolytic stability of these new derivatives.

Competitiveness studies of Adc-Ahx-(D-Arg$_6$)-NH$_2$ (II-5/ARC-902) [II Supporting Information]

5-II/**ARC-902** was the most active ARC of the "new generation" of compounds. Previous oligoarginine-containing bisubstrate inhibitors have declared to act as ATP competitive inhibitors but their competitiveness with peptide substrates has not been revealed [82, 32]. One study has indicated some competitiveness with the peptide substrates in addition to the competitiveness with ATP [102]. Studies of the competition mechanism of D-amino acid-containing bisubstrate-analogue inhibitors had not been performed before. Mechanism of the inhibition of cAPK by the compound **II-5** was investigated with the application of different methods: dependence of IC_{50} values on concentration of the substrates (Table 4), effect of the inhibitor for kinetic parameters of the substrates (Table 5) and Lineweaver-Burk plot analysis of these results.

Table 4. Inhibitory activity of **II-5** towards cAPK Cα

[ATP], μM	[TAMRA-kemptide], μM	IC_{50} (nM)
100	30	8.3 ± 1.5
300	30	24 ± 5
1000	30	109 ± 21
1000	10	101 ± 13
1000	100	150 ± 23
100	100	15 ± 6

IC_{50}-values of **II-5** were linearly dependent on the concentration of ATP while there was no such dependency on the concentration of the substrate peptide TAMRA-kemptide (Table 4). This supports the idea of ATP-competitive inhibition mechanism. Small effect of the concentration of peptide on IC_{50}-values was observed, which still does not confirm the conventional competitive inhibition pattern. Larger errors of measurements at 100 μM concentration of TAMRA-kemptide also complicate the interpretation of results.

Table 5. Kinetic parameters for the cAPK-catalyzed reaction between ATP and TAMRA-kemptide [a,b]

parameter	[**II-5/ARC-902**], nM			
	0	10	20	40
K_m^{App} (ATP), μM [a]	27 ± 10	139 ± 31	269 ± 126	456 ± 105
V_{max}, nM/min [a]	860 ± 46	757 ± 59	836 ± 173	840 ± 100
K_m^{App} (peptide), μM [b]	< 5	< 5	< 5	< 5
V_{max}, nM/min [b]	922 ± 85	384 ± 50	242 ± 66	140 ± 15

[a] concentration of ATP was varied from 45 to 750 μM, concentration of TAMRA-kemptide was kept at 30 μM;
[b] concentration of TAMRA-kemptide was varied from 10 to 100 μM, concentration of ATP was kept at 100 μM

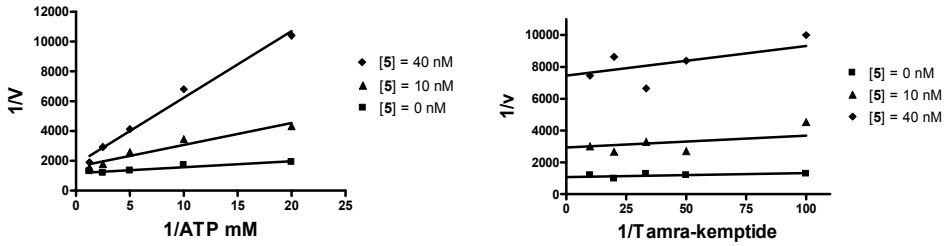


Figure 11. Lineweaver-Burk plots for the compound **II-5** with cAPK Cα.

Elevation of the concentration of the inhibitor **II-5/ARC-902** led to the increase of appeared K_m-value of ATP while the value of V_{max} was maintained at the same level (Table 5). This indicates competitive mechanism of inhibition. Other performed experiments support this interpretation (Table 4).

The obtained K_m value of ATP was slightly higher than the value that has been previously reported for this assay [98]. This may be caused by the large uncertainty of the estimation of K_m in particular conditions (the lowest ATP concentration was above its K_m-value). Lineweaver-Burk plot for ATP (Figure 11 left) also indicates the competitive inhibition.

The similar experiments with TAMRA-kemptide showed that the value of V_{max} decreased continuously when concentration of **II-5/ARC-902** was increased, which points to the non-competitive inhibition mechanism (Table 5). The applied assay method does not afford the determination of exact K_m values but these values remained below 5 μM at all inhibitor concentrations tested. The comparison with previous studies (K_m = 3 μM [98]) showed that the presence of the inhibitor **II-5/ARC-902** caused no remarkable increase of apparent K_m-values of the peptide substrate. This is a typical non-competitive inhibition pattern that appears also in Lineweaver-Burk plots (Figure 11, right).

All aforementioned kinetic results demonstrate that the inhibition of cAPK by the compound **II-5/ARC-902** is competitive with ATP and non-competitive with TAMRA-kemptide. K_i of 3.2 nM was calculated for the compound **II-5/ARC-902** from the dependence of K_m^{App} (ATP) on the concentration of the inhibitor. The value is slightly higher than the estimated inhibition constant derived from values of IC_{50}-s with the application of Cheng-Prusoff equation (K_i ~ 1-2 nM).

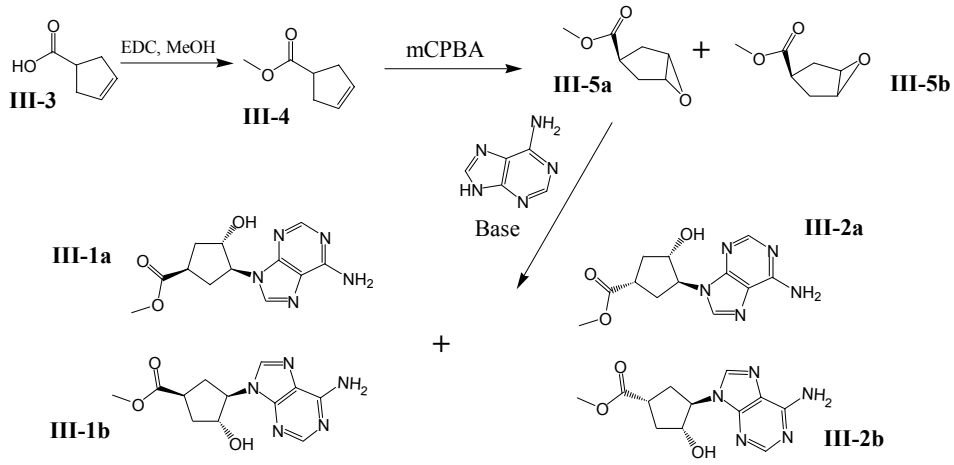
Non-competitiveness with peptide substrate raises suspicions about the bi-substrate-analogue nature of ARC-s. Generally, this kind of behaviour of biligand inhibitors has been linked to the kinetic mechanisms of the reaction (ATP first) or the mode of binding of the inhibitor (ATP-mimetic first) [103, 82] that preclude appearance of competitiveness with the substrate peptide for bisubstrate inhibitors in kinetic experiments. By using binding studies it was demonstrated later that ARC-s really behave as bisubstrate-analogue inhibitors:

12

they could be displaced from their complex with cAPK Cα by both ATP- and protein substrate-competitive inhibitors [92].

Synthesis and biological properties of carbocyclic 3'-deoxyadenosine-based ARC-s [III]

The further increase of inhibitory potency of ARC-type inhibitors could be achieved by optimization of structures of the moieties and their spatial positioning. The earlier structure-activity studies with adenosine and adenine nucleotide analogues have shown that removal of the hydroxyl group from the 3'-carbon of the ribose moiety of adenosine increases the affinity of the compounds towards several PK-s up to 5-fold while most other modifications decrease the potency [18, 104, 105]. Additionally, crystal structures of cAPK Cα complexes with adenosine derivatives reveal the absence of polar interactions between 4'-oxygen of the ribose and the kinase [97]. These facts point to the possibility to design chemically and enzymatically more stable [10] potent ARC-type inhibitors containing carbocyclic (cyclopentane) adenosine-mimics. Syntheses of carbocyclic nucleoside analogues usually involve numerous steps and arise complicated stereochemical challenges [11, 12].



Scheme 13. Synthesis of the carbocyclic nucleoside analogues.

The shortest synthetic scheme was selected for preparation of the derivatives of carbocyclic 3'-deoxyadenosine **III-1a**, **III-1b**, **III-2a** and **III-2b** that are further applicable for easy preparation of ARC-type inhibitors. An advantage that simplifies overall synthesis is the absence of 3'-hydroxyl group in the molecule of

adenosine analogue. The preparation started with conversion of cyclopentene-1-carboxylic acid (**III-3**) to its methyl ester **III-4** that was then oxidized to the appropriate epoxide (**III-5**) with mCPBA (Scheme 12). *Cis* and *trans* isomers (**III-5a** and **III-5b**, respectively) of the epoxide were separated by column chromatography (EtOAc/heptane 1/4). The products reacted with adenine in the presence of NaH or a phosphazene base. Electrophilic ring-opening of the epoxide required increased temperatures (90–110ºC) to proceed. The reaction yielded a mixture of four stereoisomers (two diastereomers as racemates – **III-1a**, **III-1b**, **III-2a** and **III-2b**), although the starting epoxide was in the form of pure single isomer. This result was apparently caused by the racemization occurring in basic medium at high temperatures (>100ºC) at the most acidic 4'-carbon adjacent to the ester group. The application of both NaH and the phosphazene base led to similar yields and similar proportion of the products. NaH appeared to be the preferred base in this case due to easier purification of the products. The adenine alkylation reaction producing mixture of **III-1a/1b** and **III-2a/2b** was the most critical step due to low overall yield (12–16%). The low yield of the synthesis was probably caused by instability of the starting epoxides (**III-5a** and **III-5b**) in the reaction medium. Using of sterically more hindered esters (like *tert*-butyl) instead of methyl may be one possible solution for the increase of the overall yields. Another solution could be the change of overall synthetic scheme and the use of enantioselective reactions [106]; however, it would also increase significantly the number of synthetic steps and the overall benefit would not be obvious.

The pairs of diastereomers (**III-1a/1b** and **III-2a/2b**) were separated by column chromatography on silica gel ($CHCl_3$/MeOH/AcOH 40/10/1). The overlapping fractions were purified repeatedly. Structural elucidation of the diastereomers was based on different NOE signals between 1'– or 2'– protons and 4'–proton of the isomers. The diastereomer **III-1a/1b** eluted faster than **III-2a/2b** in case of normal phase chromatography, which is consistent with the previous data concerning ribose counterparts of the compounds [107]. Chiral chromatography was used for the separation of enantiomers **III-1a** and **III-1b**. Derivatisation of the enantiomers (**III-1a** and **III-1b**) with Mosher chloride (R-α-methoxy-α-trifluoromethylphenylacetyl chloride) was used for the determination of absolute configuration with NMR [108]. The assigned configurations were consistent with biological data (Table 5): most active isomer is structurally similar to native adenosine.

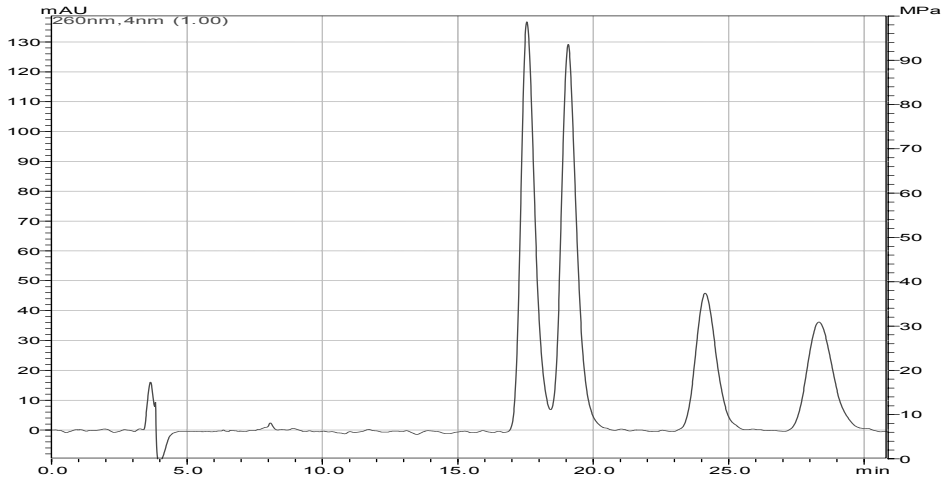
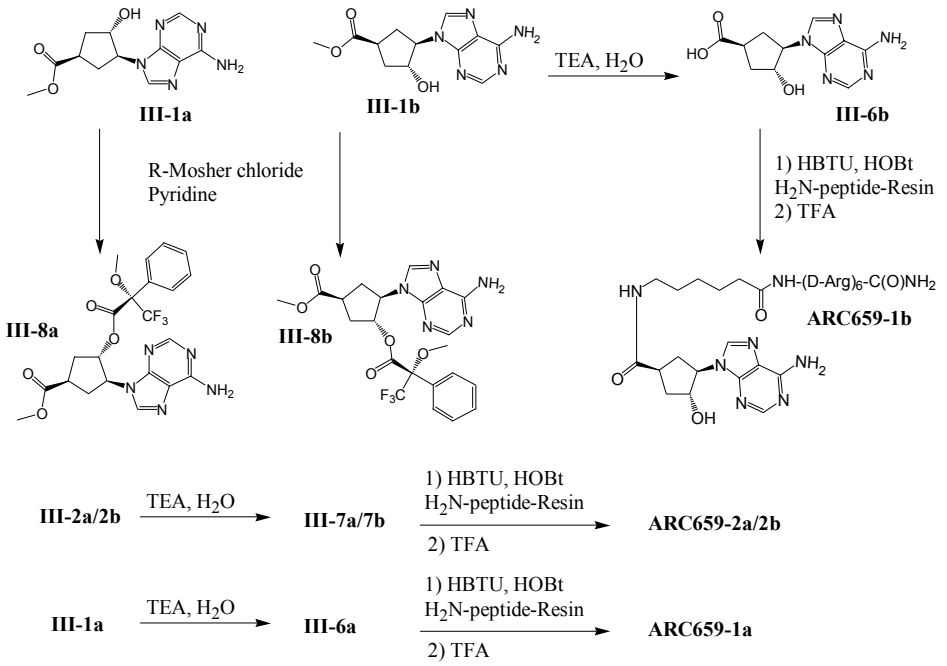


Figure 12. HPLC separation of the stereoisomers (**III-1a**, **III-1b**, **III-2a** and **III-2b**) with chiral column (Chiralpak AD-H column), isocratic elution with n-heptane/2-propanol 75/25.

Methyl esters (**III-1a**, **III-1b** and racemates **III-1a/1b** and **III-2a/2b**) were hydrolyzed and the obtained carboxylic acids (**III-6a/6b**, **III-6a**, **III-6b** and **III-7a/7b**) were coupled to the peptides (Scheme 14) by using the standard methodology. Conjugates of carbocyclic 3'-deoxyadenosine and hexa-(D-arginine) (**ARC-659**) made from different isomers are designated by adding the number of the isomer (**1a**, **1b**, etc.) to the common code of the compounds **ARC-659**.

The inhibitory potencies of the carbocyclic nucleoside derivatives were evaluated against cAPK Cα with the application of fluorometric TLC kinase activity assay [98], and the results were expressed as the IC_{50} values in Table 6. TAMRA-kempide (30 μM) and ATP (0.1 mM or 1.0 mM) were used as substrates. The application of higher concentration of ATP (1.0 mM) was essential for strongest inhibitors to avoid tight-binding conditions of the assay ($IC_{50} \sim C_{kinase}$) and make the data comparable. In the tight binding conditions the IC_{50}-values are not proportional to values of K_i and the lowest achievable value of IC_{50} is limited to half of concentration of the protein.



Scheme 14. Synthesis of the peptide conjugates and Mosher esters.

The racemic nucleoside analogues **III-1a/1b** and **III-2a/2b** were relatively weak inhibitors. The IC_{50}-values for these compounds were comparable to that of adenosine (IC_{50} = 350 μM). The diastereomer **III-2a/2b**, being structurally less similar to the native adenosine than **III-1a/1b**, showed slightly stronger inhibition. The inhibitory potency of the conjugates of these racemates with hexa-(D-arginine) differed by more than 30-fold, and **ARC-659-1a/1b** (structurally more similar to adenosine) revealed much higher potency. This difference in activity may originate from the more effective positioning of the 6-aminohexanoic acid linker and the peptide in complex with the enzyme.

The separated enantiomer **III-1b** was *ca* 10-fold more potent than **III-1a** that may point to different binding of 2'-hydroxy group to the enzyme. The conjugates with peptides (**ARC-659-1a** and **ARC-659-1b**) revealed similar one-magnitude difference in activity. The stereochemistry of the nucleoside part of most active conjugate **ARC-659-1b** is similar to that of adenosine. The estimated K_i-value (calculated according to the Cheng-Prusoff equation with K_m of 20 μM for ATP) for the compound towards cAPK was in subnanomolar range (0.2–0.5 nM). Data from Table 7 points to the similar value of K_i. **ARC-659-1b** is probably the most potent bisubstrate analogue inhibitors of protein kinase known up to date. The profound confirmation of the latter statement needs the testing of different compounds in the same assay conditions.

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ARC-659-1b has several improved properties compared to other bisubstrate-analogue inhibitors: the cell-penetrating and proteolytically more stable D-arginine-containing peptide part, the lack of enzymatically degradable glycosidic bond, slightly increased hydrophobicity (compared to **II-5**/**ARC-902**) and the highest potency. These qualities provide even greater potential to this compound compared to other bisubstrate inhibitors of protein kinases for applications in cell experiments. Low synthesic yield and time-consuming chromatographic purifications of **III-1b** are the main drawbacks of the application of **ARC-659-1b** (if compared to **ARC-902)**. Optimization of these processes may improve the preparation of this highly active compound.

Table 6. Inhibition of cAPK Cα by carbocyclic nucleoside derivatives

Compound	IC_{50} (0.1 mM ATP)[a]	IC_{50} (1 mM ATP)[a]
III-1a/1b	328 μM (3.48 ± 0.20)[b]	–
III-2a/2b	93 μM (4.03 ± 0.16)	–
III-1a	> 1000 μM	–
III-1b	168 uM (3.77 ± 0.18)	–
ARC-659-1a/1b/2a/2b[c]	3.45 nM (8.46 ± 0.22)	24.5 nM (7.61 ± 0.19)
ARC-659-1a/1b	–	16.8 nM (7.77 ± 0.23)
ARC-659-2a/2b	–	571 nM (6.24 ± 0.31)
ARC-659-1a	–	97.3 nM (7.01 ± 0.22)
ARC-659-1b	2.41 nM (8.62 ± 0.12)	12.9 nM (7.89 ± 0.25)

[a] Concentration of the substrate (ATP) in the kinetic assay. [b] Values in the parentheses express pIC_{50}-s with 95% confidence intervals. [c]**ARC-659-1a/1b/2a/2b** was synthesized from the mixture of racemic diastereomers **III-1a/1b** and **III-2a/2b**.

Selectivity studies of ARC-s [II and III]

Selectivity is one of the most important characteristics of protein kinases inhibitors in cell and *in vivo* experiments. The achievement of specificity with more than 500 human protein kinases has been a great challenge. Extensive specificity testing is very expensive and available for only some groups and companies. Usually only two to ten enzymes are used to study selectivity. Wider coverage of the kinome can be achieved by using one- or two-point inhibition experiments on selectivity panels [50]. The largest of the panels contains several hundreds of kinases. Comprehensive study of selectivity is still problematic but some attempts in the field of activity-based proteomics have been promising [109].

Selectivity of the most potent ARC-s was tested (on the commercial basis at the Division of Signal Transduction Therapy, University of Dundee) against the panel of protein kinases. To make the inhibitory potencies comparable, assays

were run at ATP concentrations which were close to the K_m-value of the kinase, essentially as it has been described previously [110, 111].

The selectivity data are presented in Table 7 and expressed as the percent of residual activities of the kinases relative to that of control incubations where the inhibitor was omitted. **II-5/ARC-902**, **II-26**, **ARC-659-1b** were tested at 1 μM concentration and **ARC-659-1b** also at 0.01 μM. The medium values of duplicate determinations are tabulated with their errors.

It should be taken into consideration that one-point inhibition data are related to significantly larger uncertainty value than measurements of IC_{50}-s that are determined by serial measurements. Activity percents over 100% and stronger inhibitions at lower concentrations (*e.g.,* PKBα, **ARC-659-1b**) of an inhibitor are good examples. Thus, the data in Table 7 could be taken as a semi-quantitative estimation of selectivity.

All tested compounds inhibited strongly several basophilic kinases and were not specific towards one particular enzyme. Kinases that have no preference for arginine-rich substrates retained their activity in the presence of ARC-s, indicating the group-selective nature of these inhibitors. Most of the inhibited kinases belong to AGC group, but some representatives of the CAMK group are also involved (Table 7). PAK5 was the only enzyme from the other groups (STE) that was significantly inhibited by **ARC-659-1b** (this kinase has not been tested with other compounds). Isoquinoline derivative **II-26** was the most general and least selective inhibitor inhibiting 13 kinases out of 52 by more than 90% at 1 μM concentration. The same values for **II-5/ARC-902** were 7/52[A] and 7/51 for **ARC-659-1b**. Additionally, **ARC-659-1b** has three kinases with inhibitory potency very close to this limit, plus PKBα which 1 μM point is apparently not correct. Stronger interaction of H-9 fragment of **II-26** with kinases and relatively smaller contribution of peptide part of the inhibitor to the binding energy may cause its more general activity.

RSK-s, SGK, PKD1, PIM2 were strongly inhibited only by **II-26.** Additionally, P70 S6K, PKCα, MST2, MNK2 were clearly more inhibited by the isoquinolinesulfonamide derivative than the adenosine analogue-containing compounds (**II-5/ARC-902** and **ARC-659-1b**).

[A] Number includes also cAPK

Table 7. Residual activities of protein kinases in the presence of inhibitors **II-5, II-26** and **ARC-659-1b**

Kinase	Kinase group	Residual activity %			
		II-5 (1 μM)	**II-26** (1 μM)	**ARC-659-1b** (0.01 μM)	**ARC-659-1b** (1 μM)
ROCK-II	AGC	0 ± 0	0 ± 0	41 ± 2	1 ± 0
MAPKAP-K1a/rsk-1	AGC	28 ± 7	0 ± 1	98 ± 3	98 ± 4
SGK	AGC	16 ± 0	1 ± 0	40 ± 4	19 ± 3
MSK1	AGC	2 ± 0	1 ± 1	37 ± 8	3 ± 0
PKBα-Δph	AGC	2 ± 0	2 ± 0	25 ± 4	32 ± 1
P70 S6K	AGC	7 ± 2	2 ± 0	81 ± 7	7 ± 0
MAPKAP-K1b/rsk-2	AGC	12 ± 0	2 ± 0	91 ± 2	28 ± 8
PRK2	AGC	2 ± 1	2 ± 0	59 ± 7	3 ± 1
PKBβ	AGC	3 ± 0	3 ± 0	74 ± 1	2 ± 0
CAMK-1	CAMK	15 ± 2	6 ± 0	62 ± 1	19 ± 1
PKA/cAPK	AGC	11 ± 7	6 ± 2	7 ±1	0 ± 0
PIM1	CAMK	–	–	92 ± 1	6 ± 1
PIM2	CAMK	38 ± 3	9 ± 0	103 ± 2	20 ± 1
PKD1	CAMK	54 ± 8	9 ± 1	81 ± 5	73 ± 15
PAK5	STE	–	–	71 ± 7	10 ± 1
MELK	CAMK	–	–	86 ± 11	10 ± 3
PKCα	AGC	31 ± 7	15 ± 5	96 ± 1	30 ± 2
CHK2	CAMK	14 ± 2	22 ± 5	90 ± 12	31 ± 3
MST2	STE	77 ± 3	24 ± 0	99 ± 5	61 ± 11
CHK1	CAMK	16 ± 4	31 ± 1	84 ± 7	27 ± 4
MNK2	CAMK	99 ± 9	31 ± 3	93 ± 2	96 ± 6
AMPK	CAMK	48 ± 6	39 ± 4	70 ± 6	12 ± 1
ERK8	CMGC	50 ± 9	52 ± 0	83 ± 15	60 ± 13
Aurora B	other	103 ± 5	53 ± 1	76 ± 6	72 ± 10
MARK3	CAMK	40 ± 3	53 ± 4	72 ± 1	65 ± 1
PDK1	AGC	62 ± 4	56 ± 1	76 ± 1	28 ± 1
NEK7	other	58 ± 1	56 ± 9	117 ± 10	117 ± 3
PBK	TK	88 ± 5	67 ± 4	–	–
MNK1	CAMK	102 ± 1	68 ± 9	95 ± 3	94 ± 8
DYRK1a	CMGC	72 ± 6	69 ± 1	83 ± 13	58 ± 0
MAPKAP-K3	CAMK	67 ± 0	69 ± 8	97 ± 4	91 ± 13
CSK	TK	48 ± 9	70 ± 1	97 ± 3	77 ± 4
CDK2/cyclin A	CMGC	88 ± 7	73 ± 3	103 ± 4	114 ± 5
JNK/SAPK1c	CMGC	70 ± 7	75 ± 4	–	–
PLK1	other	81 ± 10	76 ± 2	99 ± 10	77 ± 4
SAPK2a/p38	CMGC	79 ± 2	76 ± 7	–	–
SAPK2b/p38ß2	CMGC	85 ± 0	80 ± 2	–	–
MAPKAP-K2	CAMK	85 ± 6	80 ± 2	147 ± 3	99 ± 4
MKK1	STE	77 ± 9	84 ± 6	82 ± 10	87 ± 13
IKKβ	other	94 ± 11	87 ± 1	93 ± 0	110 ± 1
CK2	other	104 ± 9	87 ± 7	86 ± 5	88 ± 5
Src	TK	84 ± 9	88 ± 4	85 ± 9	84 ± 5
Lck	TK	70 ± 3	88 ± 3	86 ± 7	63 ± 6
smMLCK	CAMK	72 ± 0	90 ± 5	89 ± 5	108 ± 1
EF2K	atypical	92 ± 9	91 ± 8	105 ± 4	85 ± 7
JNK3	CMGC	91 ± 6	92 ± 9	103 ± 9	104 ± 10
SAPK4/p38δ	CMGC	107 ± 1	93 ± 1	94 ± 4	45 ± 5
GSK3β	CMGC	56 ± 4	94 ± 9	82 ± 0	88 ± 7
MAPK2/ERK2	CMGC	100 ± 3	95 ± 7	91 ± 3	86 ± 2
CK1	CK1	106 ± 8	97 ± 4	102 ± 6	78 ± 0
PRAK	CAMK	89 ± 4	97 ± 8	88 ± 1	96 ± 8
NEK2a	other	82 ± 2	98 ± 6	117 ± 3	123 ± 4
SAPK3/p38γ	CMGC	111 ± 4	99 ± 1	78 ± 9	92 ± 14
SRPK1	CMGC	100 ± 3	99 ± 2	80 ± 4	73 ± 1
NEK6	other	94 ± 8	100 ± 8	101 ± 11	111 ± 8

The inhibition profiles of structurally related compounds **ARC-659-1b** and **II-5/ARC-902** were similar. However, **ARC-659-1b** has remarkable cAPK selectivity while its adenosine counterpart (**II-5/ARC-902**) revealed the preference for ROCK-II. Several kinases of the CAMK group like AMPK, PIM-2 and CAMK1 were inhibited more strongly by **ARC-659-1b**, whereas **II-5/ARC-902** was more potent towards the kinases of the RSK group. PIM1, PAK5 and MELK (all known to be basophilic kinases) were also strongly inhibited by **ARC-659-1b** but they were not tested with other compounds. Distinguishably stronger inhibition (residual activity 28% at 1 μM) of PDK1 by **ARC-659-1b** should also be mentioned. The checkpoint kinases (CHK-s) were both inhibited more strongly by **II-5/ARC-902** than by other compounds.

The performance of the selectivity study of **ARC-659-1b** at two concentrations of the inhibitor allowed the discrimination of activities towards kinases that were equally strongly inhibited at 1 μM. This increased overall reliability of the data by pointing to several contradictions of one-point data.

The selectivity of L-arginine-containing compound **II-3** was tested towards 10 different protein kinases (Table 3 in paper II). The results demonstrate the similarity of the activity profile of the compound **II-3** to this of D-arginine-containing counterpart **II-5/ARC-902** (Table 7): ROCK-II was the most strongly inhibited kinase, followed by other basophilic kinases of the AGC group cAPK/PKA, MSK1 and PKCη. However, the lower potency of **II-3** compared to that of its D-arginine-containing counterparts is apparent in case of all basophilic kinases tested.

Selectivity studies with a wide panel of protein kinases (more than 50 kinases in this study) had not been performed before with bisububstrate-analogue inhibitors.

Future perspectives of ARC-type inhibitors

ARC-type inhibitors have been used previously as affinity ligands for chromatography [79] and have been labelled fluorescently for studies of cell internalization [80, 81]. Furthermore, nanomolar activity and increased proteolytic stability of the new generation of D-arginine-containing compounds enables several new applications. Derivatives of these compounds have been applied already for successful preparation of affinity surfaces for surface plasmon resonance-based biosensors [92] and for fluorescent probes for binding assays of screening and evaluation of inhibitors of protein kinases [112]. Several other types of biochemical assays can be designed arising from the high affinity of ARC-s towards protein kinases.

So far most of the biological testing of ARC-s has been performed with cAPK but the selectivity study (Table 7) shows that several other basophilic kinases could also be potential targets. Low selectivity of the ARC-s could be

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an advantage for the development of binding assays, which expands their applicability even more. Currently achieved affinities of ARC-s towards ROCK, PKB, MSK and PRK are probably sufficient for binding assays. Modification of the structures of ARC could lead to low-nanomolar affinity to other AGC kinases and some of CAMK kinases.

Optimization of the peptide part of the conjugates has the greatest potential for the tuning of properties of ARC-s. Oligo-D-arginine moiety is obviously not optimal peptide sequence for most of the kinases. The application of consensus sequences of kinases may give more selective inhibitors. High affinity of present ARC-s makes them also good lead compounds for further developments.

Several characteristics of ARC-type compounds will be optimized for their wider application, *e.g.*, selectivity, activity against several enzymes, lower molecular weight and cheaper preparation. The first obtained X-ray crystal structures of complexes of ARC-s with kinases (in collaboration with Dr Dirk Bossemeyer, unpublished data) will support these developments and make the design ARC-s more rational.

ARC-type inhibitors have various beneficial properties that make them applicable as biological tools:

a) Strong inhibition of several pharmacologically important kinases (ROCK, PKB/Akt, *etc*.) turns ARC-s into potential lead compounds for drug development.
b) Cell permeability arising from the incorporated oligoarginine transport peptide allows their use in cell experiments.
c) Increased proteolytic stability of D-amino acid-containing peptides is further advantage of second generation ARC-s if used in biological systems.
d) Introduction of carbocyclic nucleoside into the structure of ARC increases its stability toward enzymatic and acidic depurination, slight increase of hydrophobicity of the conjugates may further improve their transport into cells.
e) Various voluminous tags can be attached to the C-terminus of ARC-s without losing inhibitory properties. This characteristic of the conjugates opens way for their different applications (*e.g*., affinity adsorbents for chromatography and biosensors, fluorescent and affinity-tagged ligands).
f) Conventional peptide synthetic strategy mostly used for production of ARC-s makes the synthesis simple and the availability of large variety of building blocks supports structural modification of conjugates for structure-activity studies.

CONCLUSIONS

Application of P1-*t*Bu phosphazene base increased yields and rates of adenine alkylation reactions with alkyl bromides. Excellent regioselectivity was achieved in solution while reactions with bromoacetylated peptides on solid phase gave a mixture of regioisomes.

Series of carboxylate-containing adenosine analogues was prepared by using both solution- and solid-phase synthetic strategies. The new nucleotide analogues were tested in platelet aggregation assay and some of the compounds showed weak inhibition of ADP-promoted platelet aggregation.

Several new ARC-type inhibitors were synthesized and their potency to inhibit cAPK was tested. The novel adenosine-containing reagents suitable for conjugation with peptides on solid phase and in solution were prepared. New synthetic strategies for producing of adenosine- and isoquinoline-peptide conjugates were successfully used. Reaction of 5'-p-nitrophenyloxycarbonyl-2', 3'-*O*-isopropylidene adenosine with the amino group of the peptide was used to connect adenosine to the C-terminus of the peptide. Several structural modifications of ARC-s increased structural diversity of these inhibitors. Compounds containing oligo-D-arginine chain connected to Adc *via* the N-terminus of the peptide had low nanomolar inhibitory potency. In cAPK-catalysed phosphorylation reaction **II-5/ARC-902** was competitive with ATP and non-competitive with TAMRA-kemptide that is consistent with the results of previous studies concerning structurally similar compounds. D-Arginine-containing ARC-s were shown to be resistant to trypsinolysis. Selectivity study showed that adenosine and isoquinoline conjugates with hexa-D-arginine inhibit mainly basophilic protein kinases of AGC and CAMK groups while most of other kinases retained their activity in the presence of these inhibitors.

An analogue of carbocyclic 3'-deoxyadenosine was synthesized as a mixture of stereoisomers and probably the shortest possible synthetic scheme was chosen. The procedure started from 3-cyclopentene-1-carboxylic acid and involved four steps. The formed stereoisomers were separated by normal-phase and chiral chromatography. The following conjugation of the product with hexa-D-arginine-containing peptides yielded bisubstrate-analogue inhibitors of cAPK. The strongest inhibitor of them (**ARC-659-1b**) showed higher potency than its adenosine counterpart **II-5/ARC-902**. The selectivity study revealed remarkable cAPK selectivity, but several other basophilic protein kinases were also strongly inhibited.

SUMMARY IN ESTONIAN

Bioloogilisteks rakendusteks sobilike adenosiini ja peptiidi konjugaatide süntees

Käesoleva töö eesmärgiks oli uute sünteesimetoodikate arendamine uudsete adenosiin- ja adeniin-peptiidide konjugaatide saamiseks, nende bioloogilise aktiivsuse selgitamine P2Y retseptorite antagonistide ja proteiinkinaaside inhibiitoritena ning nende aktiivsuste suurendamine.

Töö esimeses osas sünteesiti rida adeniini derivaate negatiivselt laetud peptiididega. Orgaaniline fosfaseen *t*BuP1 osutus sobivaks aluseks adeniini deprotoneerimiseks ja tekkinud aniooni alküülimiseks N9-asendisse, omades mitmeid eeliseid anorgaaniliste aluste ees. Adeniini alküleerimisreaktsioon viidi läbi ka tahkefaasi sünteesina, mille tulemusena saadi regioisomeeride segu, mis koosnes N9-, N7- ja N3-asendi isomeeridest. Sünteesitud aineid katsetati ADP poolt indutseeritud vereliistakute agregatsiooni inhibiitoritena ning selgus, et mõned ained omavad sellele protsessile nõrka inhibeerivat toimet.

Töö teises osas valmistati ulatuslik seeria adenosiini ja oligoarginiini konjugaate (ARC-d) ja selgitati nende ainete võime inhibeerida proteiinkinaase. Disainiti ja sünteesiti rida uusi reagente adenosiini ja adeniini konjugeerimiseks peptiididega, mida õnnestus ka edukalt ARC-de sünteesiks kasutada. Uudse strateegiana kasutati adenosiini aktiveeritud derivaatide liitmist peptiidiga lahuses, mis võimaldas kasutada paindlikumaid sünteesistrateegiaid adenosiini liitmiseks peptiidi C-terminaalsesse otsa. Kõik uued ARC-d inhibeerisid cAMP-sõltuvat proteiinkinaasi (cAPK). Tugevaimateks inhibiitoriteks osutusid D-arginiine sisaldavad ained. Ühend, milles adenosiin ja heksa-D-arginiin olid ühendatud 6-aminoheksaanhappe fragmendi kaudu, omas cAPK-i suhtes K_i väärtust 3 nM. Inhibibeerimismehhanismi uurimine näitas, et **II-5/ARC-902** on ATP-konkurentne inhibiitor, samas selget konkurentsi peptiidse substraadiga kineetilistest mõõtmisest ei ilmnenud. Isokinoliinsulfoonhappe konjugaadid oligoarginiinidega, mis valmistati võrdluseks ARC-dega, olid samuti väga tugevad inhibiitorid. Samas ei täheldatud isokinoliini derivaatide juures D- ja L-arginiinidega konjugaatide aktiivsuste erinevust, mis ilmnes adenosiini ühendite korral. Isokinoliinsulfoonhappe konjugaadid peptiididega valmistati algusest lõpuni tahkel faasil, kasutades ka peptoidsünteesi elemente. D-arginiinidega ARC-d olid stabiilsed trüpsinolüüsi suhtes erinevalt analoogsetest L-aminohapetest koosnevatest ühenditest, mis tõestas oletust nende suuremast proteolüütilisest vastupidavusest.

Töö kolmandas osas sünteesiti stereoisomeeride seguna karbotsükliline adenosiini analoog, millel puudus 3'-hüdroksüülrühm. Isomeerid lahutati kromatograafiliste meetoditega ning nende konfiguratsioonid tõestati tuuma magnetresonantsspektroskoopiaga. Ühendid konjugeeriti heksa-D-arginiiniga ning

määrati saadud ARC-de võime inhibeerida cAPK-i. Kõige aktiivsem konjugaat (**ARC-659-1b**) omas subnanomolaarset K_i väärtust, olles tõenäoliselt tugevaim teadaolev bisubstraatne proteiinkinaasi inhibiitor. **ARC-659-1b** adenosiini analoogi osa on stereokeemiliselt sarnane adenosiinile. Bioloogiliselt ebastabiilse glükosiidsideme kaotamine, suurem aktiivsus ja veidi kasvanud hüdrofoobsus tõstavad **ARC-659-1b** potentsiaali kasutamisel eksperimentides elavate rakkudega.

Esmakordselt viidi läbi bisubtraatsete inhibiitorite selektiivsuse analüüs suure valiku proteiinkinaasidega (üle 50). Analüüsiks kasutati kolme kõige aktiivsemat konjugaati heksa-D-arginiiniga. Isokinoliinsulfoonhape derivaat (**II-26**) inhibeeris enim erinevaid kinaase, adenosiini derivaat (**II-5/ARC-902**) ja vastav karbotsükliline 3'-deoksüanaloog (**ARC-659-1b**) olid selektiivsemad ning inhibeerisid väiksemat hulka ensüüme. Üldiselt kuulusid kõik ARC-de poolt tugevalt inhibeeritud kinaasid basofiilsete hulka, mis tõestab kaudselt nende bisubstraatsust. **ARC-659-1b** omas märgatavat selektiivsust cAPK-i suhtes. ARC-d inhibeerisid tugevalt ka mitmeid ravimiarenduse seisukohalt olulisi kinaase, nagu ROCK ja Akt/PKB, mis võimaldab neid kasutada juht-ühenditena potentsiaalsete ravimite arendamisel.

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PUBLICATIONS

I

Enkvist, E.; Raidaru, G.; Patel, R.; Redick, C.; Boyer, J. L.; Subbi, J.; Tammiste, I.; Uri, A. Synthesis of potential purinoceptor antagonists: application of P1-tBu phosphazene base for alkylation of adenine. *Nucleosides, Nucleotides and Nucleic Acid*, **2006**, *25*, 141–157.

II

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III

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1994–1997	Hugo Treffner Grammar School (Tatru, Estonia)
1997–2001	Tartu University, Department of Chemistry, *B.Sc.*
2001–2003	Tartu University, Department of Chemistry, *M.Sc.*
2003–present	Tartu University, Department of Chemistry, *Ph.D* student

Professional employment

2003–2005	University of Tartu, Institute of Organic and Bioorganic Chemistry, researcher 0.5
2005–present	University of Tartu, Doctoral School of Material Sciences and Material Technology, researcher 0.1
2007–present	University of Tartu, Institute of Chemistry, researcher 0.9

Professional self-improvement

2001	University of Kuopio, Department of Chemistry,Kuopio, Finland, study and research, (3 months). Budapest, Hungary, Workshop "Trends and Applications of Combinatorial Chemistry and Combinatorial Technologies" (4 days).
2002	University of Kuopio, Department of Chemistry,Kuopio, Finland, study and research, (3 months).
2003	University of Tartu, Centre of Molecular and Clinical Medicine, course "Mass-spectroscopy and it's application", (6 days)

Scientific publications

1. Sak, K., Uri, A., Enkvist, E., Raidaru, G., Subbi, J., Kelve, M and Järv, J. Adenosine derived non-phosphate P2Y1 receptor antagonists. *Biochem. Biophys. Res. Commun.*, 2000, 272, 327–331.
2. Viht, K., Vaasa, A., Raidaru, G., Enkvist, E., Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. *Analytical Biochemistry*, **2005**, 340, 165–170.
3. Enkvist, E., Raidaru, G., Patel, R., Redick, C., Boyer, J. L., Subbi, J., Tammiste, I., Uri, A. Synthesis of potential purinoceptor antagonists: aplication of P1-tBu phosphazene base for alkylation of adenine. *Nucleosides, Nucleotides and Nuceic Acid*, **2006**, 25, 141–157.
4. Enkvist, E., Lavogina, D., Raidaru, G., Vaasa, A., Viil, I., Lust, M., Viht, K., Uri, A. Conjugation of Adenosine and Hexa-(D-Arginine) Leads to a Nanomolar Bisubstrate-Analog Inhibitor of Basophilic Protein Kinases. *Journal of Medicinal Chemistry*, **2006**, 49, 7150–7159.
5. Enkvist, E., Raidaru, G., Vaasa, A., Pehk, T., Lavogina, D., Uri, A. Carbocyclic 3'-deoxyadenosine-based highly potent bisubstrate-analog inhibitor of basophilic protein kinases. *Bioorganic & Medicinal Chemistry Letters*, **2007**, 17, 5336–5339.

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Haridus

1994–1997	Hugo Treffneri Gümnaasium (Tartu)
1997–2001	Tartu Ülikooli keemiaosakonna üliõpilane; B.Sc (keemia, cum laude)
2001–2003	Tartu Ülikooli keemiaosakonna magistrant, M.Sc. (keemia)
2003–2007	Tartu Ülikooli doktorand

Teenistuskäiks

2003–2005	Tartu Ülikool, Orgaanilise ja bioorgaanilise keemia istituut, erakorraline teadur 0.5k.
2005–praegu	MMTDK erokorraline teadur 0.1k.
2007–praegu	Tartu Ülikool, Keemia Istituut, erakorraline teadur 0.9k.

Erialane enesetäiendus

2001	Õpingud Kuopio Ülikooli keemiaosakonnas, Soome (kevad s. 3 kuud). Osalemine kursusel Trends and Applications of Combinatorial Chemistry and Combinatorial Technologies, Budapest, Hungari (15–18 oktoober).
2002	Õpingud Kuopio Ülikooli keemiaosakonnas, Soome (sügis s. 3 kuud).
2003	Osalemine kursusel Mass-spectroscopy and it's application, Tartu (16–21 mai)

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Teaduspublikatsioonid

1. Sak, K., Uri, A., Enkvist, E., Raidaru, G., Subbi, J., Kelve, M and Järv, J. Adenosine derived non-phosphate P2Y1 receptor antagonists. *Biochem. Biophys. Res. Commun.*, 2000, 272, 327–331.
2. Viht, K., Vaasa, A., Raidaru, G., Enkvist, E., Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. *Analytical Biochemistry*, **2005**, 340, 165–170.
3. Enkvist, E., Raidaru, G., Patel, R., Redick, C., Boyer, J. L., Subbi, J., Tammiste, I., Uri, A. Synthesis of potential purinoceptor antagonists: aplication of P1-tBu phosphazene base for alkylation of adenine. *Nucleosides, Nucleotides and Nuceic Acid*, **2006**, 25, 141–157.
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DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

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