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**Fluorometric assays for characterization of protein
kinase inhibitors**

Master thesis in bioorganic chemistry

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1. Abbreviations

ABP	activity-based probe
ABPP	activity-based protein profiling
ADP	adenosine-5'-diphosphate
AGC	A group of protein kinases including protein kinase A, G and C and related kinases
Ahx	6-aminohexanoic acid
APS	ammonium persulfate
ARC	adenosine-oligoarginine conjugate
Arg	arginine
ATP	adenosine-5'-triphosphate
BCIP	5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt
BSA	bovine serum albumine
C _{sub}	catalytic subunits
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CDK	cycline-dependent kinases
CDPK-1	Ca ²⁺ -dependent protein kinase 1
CK1	casein kinase 1
CK2	casein kinase 2
CMGC	a group of protein kinases including CDK, GSK3, MAPK and CLK-related kinases
CPP	cell-penetrating peptide
D-ARC	D-arginine-containing ARC
Delfia	delayed enhanced lanthanide fluorescence immuno assay
2-DE	two dimensional electrophoresis
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FSBA	5'-(p-(fluorosulfonyl)benzoyl) adenosine
FSBAza	5'-(p-(fluorosulfonyl)benzoyl) –8-azidoadenosine
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
GSK3	glycogen synthase kinase 3
H-7	(1-(5-isoquinolinesulfonyl)-2-methylpiperazine

H-8	N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide
H-89	N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
IRAK	interleucine-1 receptor associated kinase
IMAP	immobilized metal assay for phosphochemicals
MAPK	mitogen-activated protein kinase
MLK	mixed-lineage kinase
MS	mass spectrometry
NBT	nitro blue tetrazolium
OPK	other protein kinases
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pI	isoelectric point
PI3	phosphoinositide 3-kinase
PIKK	phosphoinositide 3-kinase related kinase
PK	protein kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PKI	protein kinase inhibitor
PLK	polo-like kinase
PVDF	polyvinylidene difluoride
RIPK	receptor-interacting protein kinase
RGC	receptor guanylate cyclase
Ser	serine
SDS	sodium dodecyl sulfate
TAMRA	carboxytetramethylrhodamine
TEMED	tetramethylethylenediamine
Thr	threonine
TKL	tyrosine kinase-like
TLC	thin layer chromatography

TTBK	tau tubulin kinase
Tyr	tyrosine
UV	ultraviolet
Vis	visible
VRK	vaccinia-related kinase

2. Introduction

Protein kinases are enzymes that catalyze the phosphorylation of protein substrates by the transfer of the phosphoryl group from ATP to Ser, Thr and Tyr residues. Phosphorylation is the most important regulatory mechanism of cellular function and signal transduction. Many human diseases have been associated with the abnormal phosphorylation of cellular proteins. Several inhibitors of protein kinases have been developed for regulating abnormal phosphorylation and have been approved for clinical trials. Designing of synthetic inhibitors for protein kinases and development of screening methods are fast developing areas in drug development.

Until recently, the most frequently used assays for evaluation of protein kinase inhibitors were radiometric methods. During recent years several non-isotopic methods have been developed. Activity-based protein profiling is a new promising approach for analysis of many proteins.

The important properties of new inhibitors for their intracellular applications are their cellular permeability and resistance to enzymatic degradation. Adenosine–oligoarginine conjugates (ARC) are cell permeable peptidic inhibitors of basophilic protein kinases. ARC-s as peptidic compounds are prone to proteolytic degradation, therefore for using them *in vivo* the enhancement of their stability is important.

The aims of the present study was to find and work out new assays for the characterization of protein kinase inhibitors, and compare the stability of the L- and D-ARC type inhibitors.

3. Survey of literature

3.1 Protein kinases

The protein kinase superfamily comprises the largest enzyme family: there are about 500 protein kinases encoded by 2 % of genes as predicted by the cataloguing of the human genome [1, 2]. Protein kinases are enzymes that catalyze the phosphorylation of proteins by the transfer of the γ -phosphoryl group from ATP to Ser, Thr, and Tyr residues. They participate in regulation of the activation, growth and differentiation of cells. Abnormal phosphorylation causes human diseases like cancer, diabetes, inflammation, immunology and cardiovascular diseases, fibrosis of liver and kidney. Therefore protein kinases have now become the second (after G-protein-coupled receptors) most important group of drug targets [1, 2, 4].

Based on the amino acid sequence of the kinase catalytic domain, protein kinases are divided into nine groups [4, 5]:

- 1) The AGC group protein kinases are mostly basophilic enzymes that phosphorylate substrates with basic amino acids Arg and Lys in close proximity to phosphorylatable Ser/Thr residues. PKA, PKG and PKC belong to this group of kinases.
- 2) Some protein kinases of the CaMK group are also basophilic enzymes, and include the kinases that are regulated by Ca^{2+} /calmodulin (CaMK1, CaMK2, CaMK4, MLCK).
- 3) The majority of enzymes of the CMGC group are proline-directed. Cycline-dependent kinases (CDK), MAP and GSK3 belong to this group.
- 4) The PTK group of kinases includes a large numbers of enzymes, which phosphorylate Tyr residues of proteins.
- 5) The STE group is named for yeast sterile-phenotype kinases, and consists of kinases involved in MAPK activation and related kinases.
- 6) The CK1 group contains CK1, TTBK and VRK families.
- 7) The TKL (tyrosine kinase-like) group is a diverse group of families that resemble both tyrosine and serine-threonine kinases. It consists of the MLK, LISK, IRAK, RIP and STRK families.
- 8) The RGC (receptor guanylate cyclase) group is also similar in domain sequence to tyrosine kinases.
- 9) Protein kinases that do not belong to the main four groups form the OPK group.

The most comprehensively studied protein kinase, cAMP-dependent protein kinase (cAPK, PKA), was discovered in 1968 (Walsh et al., 1968) [6]. It belongs to the AGC group and is as a prototype of other kinases, because it is simple, best characterized, its subunits are relatively small and they can be readily expressed in bacteria as active proteins [6, 7]. PKA regulates many physiological functions, including cell division and growth. In the absence of cAMP, PKA exists in the form of an inactive tetramer composed of two regulatory (R) and two catalytic (C_{sub}) subunits [6 –8]. The binding of cAMP to the holoenzyme causes the dissociation of the holoenzyme into a R_2 dimer and two catalytically active C_{sub} [6, 8].

Substrates and physiological inhibitors of the C_{sub} have consensus recognition site RRXS(A)I [6, 9] in which important determinants are arginines at the P-3 and P-2 positions and large hydrophobic residue (X) is preferred at the P+1 site, where P denotes the phosphorylation site [6] (Table 1). The known physiological inhibitors are R subunits and heat stable protein kinase inhibitors (PKIs) [6, 9]. Like the R subunits, the PKIs bind with high affinity ($K_D < 1$ nM) to free C_{sub} . Tight binding of PKI and RI-subunit to the C_{sub} requires the synergistic binding of ATP [6, 9].

Table 1. The high affinity binding regions of physiological inhibitors of cAPK and the sequence of a substrate Kemptide and Kemptide-derived inhibitor Ala-Kemptide

	High Affinity Site	Consensus Site	
PKI	T T Y A D F I A S G R T	G R R N A I H D	$K_i = 2.3$ nM
Kemptide		L R R A S L G	$K_m = 16$ μ M
Ala-Kemptide		L R R A A L G	$K_i = 300$ μ M
RI-Subunit	S P P P P N P V V K G R R R R G A I S A		$K_{app} = 0.2 - 0.3$ nM
RII-subunit	E E D L D V P I P G R F D R R V S V C A		$K_{app} = 0.2 - 0.3$ nM
	-6	-3-2 P +1	

3.2 Inhibitors of protein kinases

There are two specific binding domains in the active site of a protein kinase, one for the binding of ATP and the other for a protein substrate. Therefore, compounds that compete with these substrates for their binding pocket can inhibit protein kinases [1].

Most of synthetical protein-kinase inhibitors are ATP competitive [1, 2, 10, 12]. Many of them have shown high inhibitory potency (K_i in nanomolar range) [1]. A series of ATP-competitive protein kinase inhibitors, isoquinolinesulfonamide-based

H-inhibitors, was developed by H. Hidaka in 1980s [2]. These inhibitors competitively inhibit a broad range of kinases by occupying their ATP-binding sites. Examples of this class are H-7, H-8 and H-89 (Figure 1) [10, 12]. The highest selectivity and affinity has been found for H89, which has a K_i of 48 nM for cAPK, 0.48 μ M for cGPK, 31.7 μ M for PKC, etc [10].

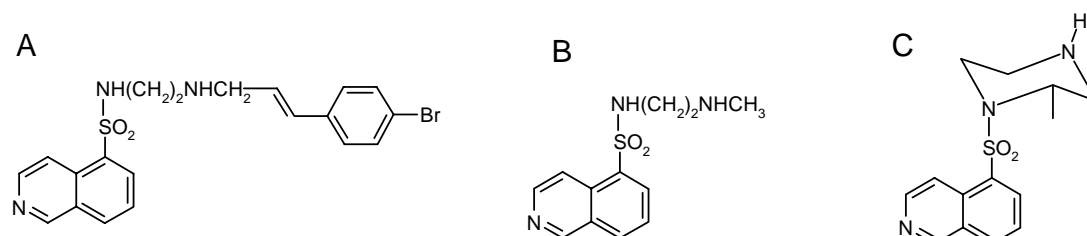


Figure 1. ATP-competitive inhibitors H-89 (A), H-8 (B) and H-7 (C).

Inhibitors competing with the protein/peptide substrate for the binding site have also been described. Shorter peptide-based inhibitors have exhibited high selectivity but relatively weak inhibition effect (K_i in micromolar range) [1, 13].

Designing of bisubstrate analog inhibitors is a promising strategy to enhance the potency and specificity of enzyme inhibition [1, 13]. Bisubstrate inhibitors of protein kinases consist of a nucleotide resembling part, often isoquinoline or adenosine derivatives, coupled with a protein mimic fragment *via* a linker (Figure 2) [1, 13].

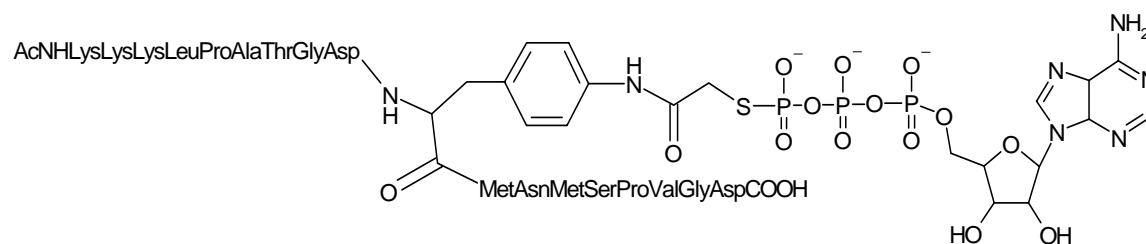


Figure 2. Bisubstrate inhibitor of insulin receptor tyrosine kinase, $K_i = 0.37 \mu$ M.

3.3 Cell Penetrating Peptides and D-analogs

One important step in the development of new inhibitors for their intracellular applications is to get them through biological barriers. Many potential drug candidates are not able to reach their target site because they cannot penetrate the cell plasma membrane. This problem can be solved by means of cell-penetrating peptides (CPP) [14-17]. These short peptides, less than 30 amino acids residues, are able to penetrate

cell membranes and translocate large cargoes (peptides, proteins, fragment of DNA, fluorescent dyes) into cells [14, 15, 17]. CPPs are capable to enter cells both *in vitro* and *in vivo*, sometimes even at low temperatures and in the presence of endocytosis inhibitors [14, 16, 17]. Several positively charged peptides, especially with multiple arginine residues, have been shown to penetrate into cell [18]. The most widely used CPPs are presented in Table 2.

Table 2. Widely used CPPs [14, 16, 17].

Name Sequence	Class
Penetratin RQIKIWFQNRRMKWKK	Protein derived CPP
Tat CGRKKRRQRRPPQC	Protein derived CPP
pVEC LLIILRRRIRKQAHASK-amide	Protein derived CPP
MAP KLALKLALKALKAALKLA-amide	Module peptide
Arg7 RRRRRRR	Module peptide
MPG GALFLGFLGAAGSTMGAWSQPKSKRKV	Designed CPP
Transportan GWTLSAGYLLGKINLKALAALAKISIL-amide	Designed CPP

One problem with the application of CPP-s as delivering vectors is their degradation by proteases before they reach the target [15]. The peptides that consist of D-amino acid residues have shown higher stability to proteolytic degradation [15]. For example, the half- life of a small peptide pVEC (from murine vascular endothelial cadherin) in phosphate buffer containing 10 units of trypsin is 10.5 min, while the corresponding all-D analog of pVEC resists enzymatic degradation in this time scale [15].

3.4 Adenosine-5'-carboxylic acid peptidyl derivatives

A class of bisubstrate inhibitors, comprising adenosine-5'-carboxylic acid and polyarginine (Figure 3), which mimic the nucleotide and peptide substrate, respectively, were designed in our research group [19-21]. These two moieties were

connected *via* various linkers (β -alanine, β -aspartate, 2,3-diaminopropanoic acid and various α,ω -aminocarboxylic acids) which structure was optimized in structure-activity studies [19]. Adenosine and peptide fragments alone had only a weak or even no inhibitory activity; however, their combination via a linker group increased the effectiveness of their interaction with the enzyme.

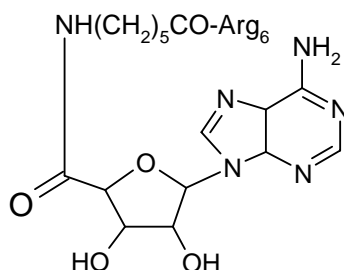


Figure 3. Structure of an ARC.

These adenosine-oligoarginine conjugates (ARC) were found to be potent inhibitors of basophilic kinases (PKA, PKC, CDPK-1), while for acidophilic protein kinases that phosphorylate Ser/Thr residues in peptides where the reaction centers are flanked with negatively charged peptide sequences (CK1 and CK2), no significant inhibition was observed [19].

It was shown that adenosine-oligoarginine conjugates, as hydrophilic compounds carrying high positive charge were able to penetrate plasma membrane [21]. This makes them potential candidates for regulation of intracellular protein kinases.

3.5 Fluorometric assays for evaluation of protein kinase inhibitors

Until recently the most frequently used protein kinase assay in research laboratories utilized ^{32}P -labeled ATP as phosphate donor and proteins or peptides as phosphate acceptors [22]. While radiometric methods have several drawbacks (short half-life of ^{32}P , personal risks, environmental pollution), the numbers of non-isotopic methods have been developed. Many of them use fluorescently labeled substrates [22].

3.5.1 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA is a technique where the peptide substrate is attached to the microtiter plate. The phosphorylation of the substrate is measured by incubating with a monoclonal anti-phospho-specific antibody, followed by secondary antibody [22, 23].

These second antibodies are coupled to the substrate-modifying enzyme, like alkaline phosphatase or horseradish peroxidase [22]. For detection, the substrate, which is converted by the enzyme to elicit a chromogenic or fluorescent signal, is added [22]. The spectrophotometer or other optical device can be used for quantification.

3.5.2 Delayed Enhanced Lanthanide Fluorescence Immuno Assay (Delfia)

Like in the case of ELISA, the peptide substrate is coated onto a microtiter plate, but phosphorylation is detected with the application of an Europium-labeled anti-phospho-specific antibody [22, 23]. After labeling, the fluorescence intensity of the bound Europium chelate is measured. This method is less time consuming than ELISA as the incubation with secondary antibody and several washing steps are eliminated [22].

Both ELISA and Delfia were first designed to measure the activity of tyrosine kinases, but are now also applicable for serine/threonine kinases [22].

3.5.3 Fluorescence Resonance Energy Transfer (FRET)

FRET is based on the distance-dependent transfer of energy from one fluorescent molecule to another [22, 23]. A fluorescence donor is excited at its specific fluorescence excitation wavelength. By a long-range dipole-dipole coupling mechanism, this excited state is then nonradiatively transferred to the second molecule, acceptor. The occurrence of FRET is characterized by a decrease in the donor fluorescence intensity, and an increased acceptor emission intensity. To detect phosphorylation by this method the two fluorophores are attached to a kinase substrate. The FRET is maintained when the peptide phosphorylation occur and FRET is lost when the peptide is not phosphorylated.

3.5.4 Fluorescence polarization (FP)

FP is based on measuring the polarization of light caused by changes in molecular size [22, 23]. When a small fluorescent molecule is excited with polarized light, the emitted light is largely depolarized, because molecules tumble rapidly in solution during its fluorescence lifetime. If this small molecule binds to a large molecule, the rotation of the fluorescent complex is slower and the emitted light will retain a proportional degree of polarization, which is seen as increase in the detected FP signal. Kinase assays based on FP use fluorescein-labeled peptide that upon

phosphorylation, are captured by binding to phospho-specific antibody [23]. Non-phosphorylated peptide have fast rotational diffusion and a low polarization value, while phosphorylated peptide bound to antibody have slower rotational diffusion and a high FP value.

3.5.5 Immobilized metal assay for phosphochemicals (IMAP)

IMAP is a FP-based kinase assay utilizing so-called IMAP nanoparticles, which are complexed with trivalent metal ions [22, 23]. These particles bind specifically to negatively charged phosphate groups of phosphopeptides. The binding of IMAP particles to fluorescence-labeled phosphopeptide results in decrease in the molecular mobility and the emission light is highly polarized.

The most important advantage of IMAP is that it does not require phosphor-specific antibodies and is possible to use for many serine/threonine kinases for which specific anti-phospho antibodies are not available [23].

3.6 Activity- Based Protein Profiling (ABPP)

The most popular method for quantitative proteome analysis combines two-dimensional gel electrophoresis with mass-spectrometry for identification of the separated proteins. This technique, 2DE-MS, is widely accepted and sensitive but several important classes of proteins, including membrane-associated and low abundance proteins, are difficult to analyze by this method [24, 25]. Additionally, 2DE-MS method measures changes in protein abundance and does not characterize protein activity [24, 25]. A new method for protein analysis has been recently developed – activity-based protein profiling (ABPP) [26, 27]. This method utilizes a tagged ligand – activity based probe (ABP) - that is capable of irreversible binding only to the target protein in its active form [26, 27] (Figure 4). The labeled proteins are separated by gel electrophoresis and detected in gel according to the reporter group, e.g., by radioactivity or fluorescence intensity. This method is able to distinguish active enzymes from their inactive zymogen or inhibitor-bound forms [26, 27].

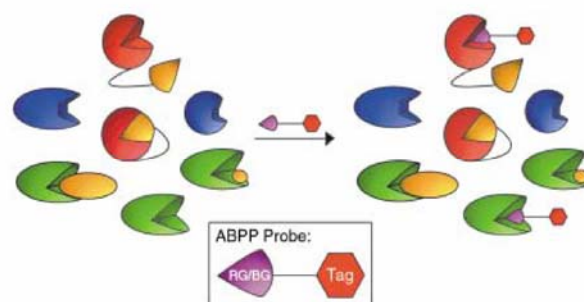


Figure 4. General strategy for activity-based protein profiling (ABPP) [26]. Proteomes are treated with chemical probes that label active enzymes, but not enzymes inhibited by intra- or intermolecular regulators (orange) or those lacking complementary binding sites (blue).

3.6.1 Activity- Based Probes (ABP)

ABP-s contain three groups: a reactive group for covalent binding to enzyme; a linker region for modulation of reactivity and specificity of the reactive group and a tag for the identification of the modified enzymes [26, 28].

Reactive group must be reactive towards the specific site of the protein and inert towards other reactive species in cell or cell extract. The reactive groups may be divided into chemically active and photoreactive groups. Examples of reactive groups that have been used for formation of a covalent bond between the inhibitor and the target protein are electrophilic ketones, acrylates; epoxides and fluorophosphonates [26, 28]. Another possibility for covalent binding is the application of photoreactive groups – e.g., an azido group [30, 31, 32] or benzoylphenone group [29] (Figure 5). Photolysis of these groups releases highly reactive radicals, which react with the target molecule.

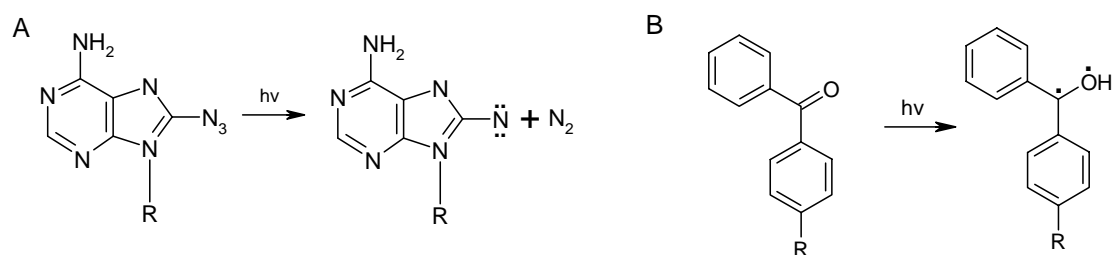


Figure 5. Formation of reactive radicals: a photoactive azido group substituted in the 8-position of adenine forms reactive nitrene (A) and double-bonded oxygen in the benzophenone group forms triplet biradical (B) upon photolysis.

When the photoreactive group is attached to competitive inhibitors, it is possible to use them as irreversible inhibitors [31, 32].

Linker is required for the connection of the reactive group to tag. Its function is to provide enough distance between these two moieties. Long-chain alkyl or polyethylene glycol (PEG) spacers can be used as linkers [28]. The alkyl linker is useful for the modulation of hydrophobicity and cell membrane permeability whereas PEG linker may enhance the solubility of hydrophobic probes in aqueous solution [28]. Peptides or peptide-like structures can also be used as linkers for the improvement of the affinity and selectivity characteristics of the probes.

Tag is for the identification and/or purification of the probe-modified proteins. The most commonly used tags are affinity, fluorescent and radioactive tags [26, 28] (Figure 6).

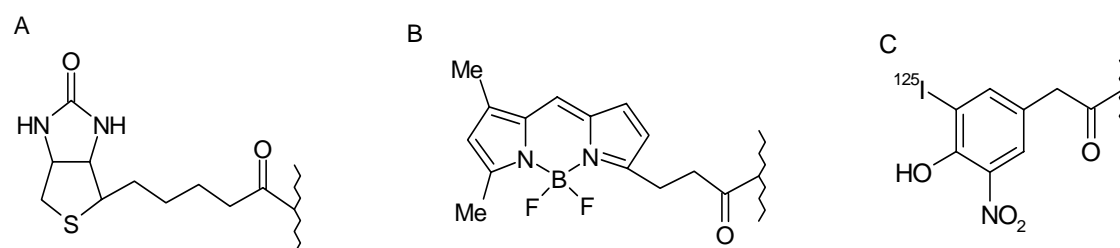


Figure 6. Structures of the tags: A, affinity tag (biotin); B, fluorescent tag (bodipy); C, radioactive tag

According to the binding mechanism four general types of ABP-s can be distinguished: mechanism-based reagents, suicide based reagents, affinity-based labeling reagents and general alkylating reagents [28]. Mechanism-based probes use the electrophilic carbon that is susceptible to attack by a nucleophile at the active-site of the enzyme. E.g., electrophilic ketones and epoxides (Figure 7) have been used for targeting the caspase and papain classes of cysteine proteases [26, 28].

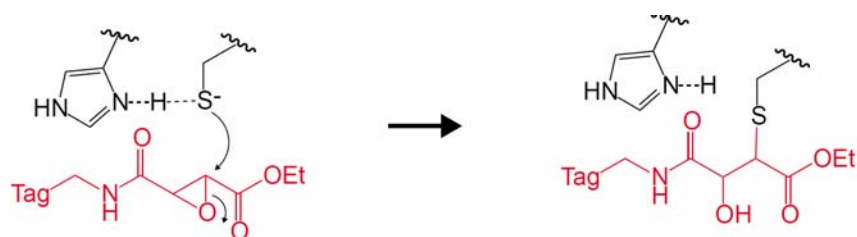


Figure 7. Targeting the papain class of cysteine protease with an epoxysuccinyl-based inhibitor.

The second type of ABPs contains an electrophile that becomes reactive after the reaction catalyzed by the target enzyme, e.g., phosphorylation [28]. This reactive electrophile is able to react with nucleophilic residues in the active site. E.g., such

probe has been used to target human prostatic acid phosphatase with 4-difluoromethylphenyl bis-(cyclohexylammonium) phosphate [28] (Figure 8).

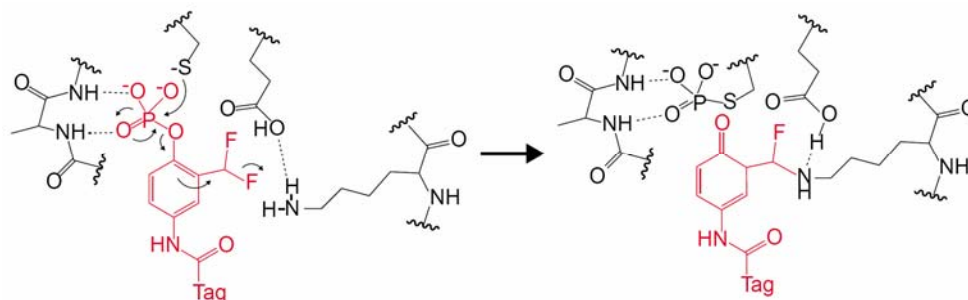


Figure 8. Targeting of human prostatic acid phosphatase with 4-difluoromethylphenyl bis-(cyclohexylammonium) phosphate.

Alkylating affinity probes require a strong nucleophile or electrophile in the active site of the enzyme and do not require the enzyme to be fully active. Usually the probes contain a reactive center or center that can be activated by chemicals or UV light. E.g., 5'-fluorosulfonylbenzoyl adenosine (5'-FSBA, Figure 9) has been used for the labeling of several nucleotide-binding proteins [28, 32].

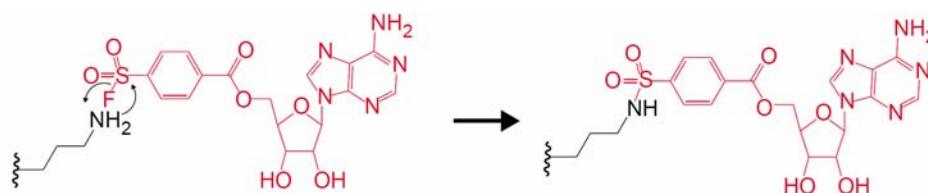


Figure 9. Targeting the nucleotide-binding enzyme with 5'-FSBA.

The final type of reactive groups used for the probe design contains non-specific alkylating groups that react with targets based only on the intrinsic reactivity of a specific amino acid residue [28] (Figure 10).

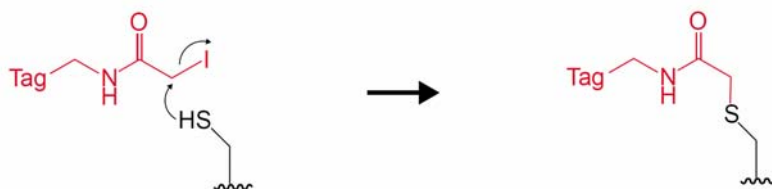


Figure 10. The iodoacetamide group of the isotope-coded affinity tag reacts with free sulfhydryls on cysteine residues.

3.6.2 Tag free ABPP

The standard ABPP method requires lysing and homogenization of cells and tissues before treatment with ABPs [33]. The obtained results are not directly comparable the situation in native environment. Applicability of ABPs with the aforementioned structure for *in vivo* analysis is limited. This limitation of ABP probes was eliminated with the introduction of “tag free” version of ABPP [25, 33], in which the tag (the major portion of probe mass) could be attached to activity-based probes after the covalent labeling of protein targets (Figure 11).

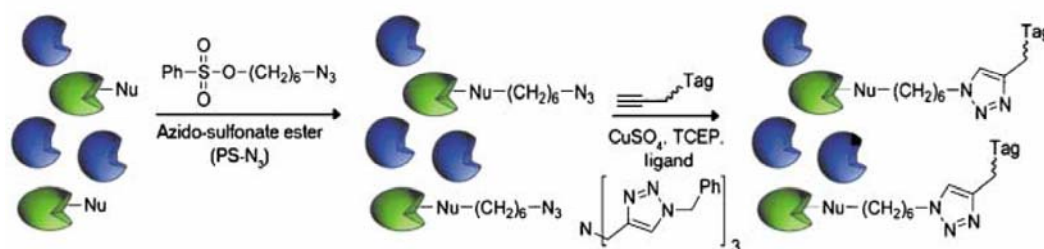


Figure 11. A “tag-free” strategy for ABPP.

This „tag free” method has several advantages relative to common ABPP: the probes are better distributed in living cells or tissues and the effect of the fluorescent tag on binding affinity of the probe is eliminated.

3.6.3 Application of ABPP

This method can be used for identification of novel drug targets [26, 34, 35] in a single experiment, without the need for the optimization of conditions for each target. Analysis of human tumors and tumor models by ABPP may identify novel enzyme activities that represent markers or targets for the diagnosis and treatment of cancer [34].

Another possibility is to use this method for screening chemical inhibitors [26, 34-36]. Initially the ABPP method was applied for identification of irreversible enzyme inhibitors. Enzymes were preincubated with inhibitor libraries and then treated with ABPP probes. Inhibitor-bound enzymes could not react with the probe and were detected by the disappearance of the fluorescence signal (Figure 12, left). With this method it is also possible to screen reversible inhibitors. ABPs compete with the inhibitors depending on the concentration and affinity of the inhibitors towards the target protein. The binding of competitive reversible inhibitors to

enzymes results in the reduction of labeling of the protein with the probe [26, 34] (Figure 12, right).

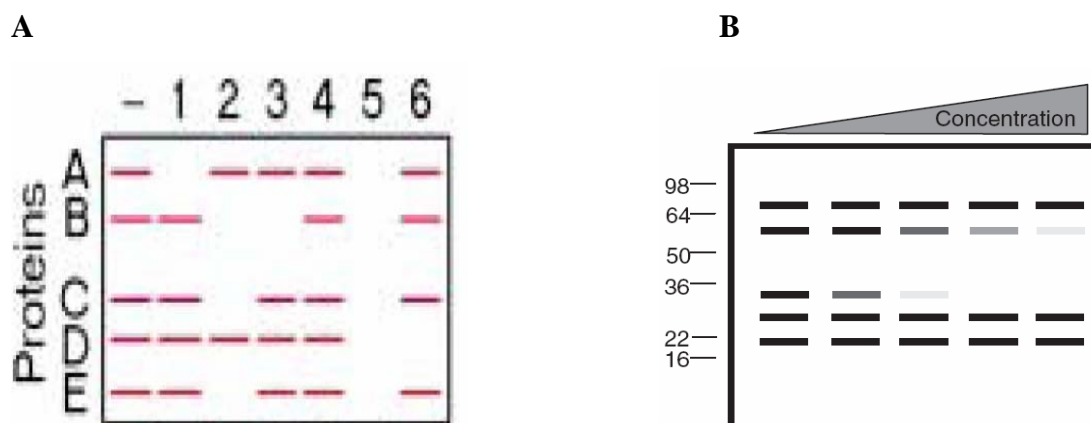


Figure 12. Screening irreversible (A) [26] and reversible (B) [34] inhibitors by ABPP. (A) Proteins (A-E) treated with different inhibitors (1-6). (B) Mixture of proteins (separated in vertical dimension) treated with increasing concentrations of inhibitor.

To date ABPP probes that target serine hydrolases, cystein proteases, oxidoreductases, protein phosphatases, metalloproteases have been developed [26]. Recent studies have shown that fluorescently labeled wortmannin, which covalently inhibits PI3, PIKK and PLK kinases, is also acting as an ABP probe [37, 38].

3.7 Gel electrophoresis [39-40]

Gel electrophoresis is a method that separates macromolecules (nucleic acids or proteins) based on their size and electric charge. Many important biological molecules such as amino acids, peptides, proteins, nucleotides, and nucleic acids are electrically charged at certain pH. Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode. Their rate of migration in the electric field depends on the strength of the field, size, shape and relative hydrophobicity of the molecules, and the ionic strength and temperature of the buffer.

Two basic types of gels are widely used: agarose and polyacrylamide. Agarose gels have very large pore size and are used to separate large molecules with molecular mass greater than 200 kDa. The pore size of polyacrylamide gel is dependent on the percentage of acrylamide in gel (used range is 3 – 30 %). This gel can be used for the separation of molecules with mass between 5 to 2.000 kDa. This is very useful range for gene sequencing, protein, polypeptide, and enzyme analysis.

The most widely used electrophoresis method is SDS-PAGE. Sodium dodecyl sulfate (SDS) is a detergent, which dissociates and denatures proteins and gives them high negative charge. Thus, in the polyacrylamide gel the SDS-protein complexes separate according to size. Separation according to charge is accomplished by isoelectric focusing (IEF). IEF separates the proteins based on their isoelectric point (pI) - pH at which the net charge of the protein is zero. At pH gradient the protein will migrate to a gradient position where its charge is zero.

Two-dimensional gel electrophoresis is the combination of isoelectric and size-based separation that are done in two different dimensions. It is the most powerful analytical method for the separation of proteins. In this way, complex mixtures consisting of thousands of different proteins can be resolved and the relative amount of each protein can be determined.

Proteins can be detected in gels by using protein stains: Coomassie blue, colloidal silver or fluorescence stains. Proteins can be transferred from gels onto a variety of membranes, including polyvinylidene difluoride (PVDF), nitrocellulose or nylon membranes. Transferred proteins can be visualized and analyzed with different stains (e.g., Amido black, Coomassie blue, colloidal gold/silver) or with antibodies (immunoblotting).

Immunoblotting is used for the identification of specific antigens that are recognized by polyclonal or monoclonal antibodies. The immunoblotted proteins can be visualized by chromogenic or luminescent products of the reactions catalyzed by specific enzymes conjugated to secondary antibodies (e.g., fluorescent substrates 4CN, TMB in the case of horseradish peroxidase based immunodetection and BCIP/NBT for alkaline phosphatase catalyzed reactions).

4. Experimental part

4.1 Materials and methods

Highly active PKA catalytic subunit (cAPK C α) recombinantly expressed in *E. coli* and purified using several chromatographic steps was purchased from Biaffin; Hepes buffer, Tris buffer, dithiothreitol, ATP, trypsin and H89 from Sigma; bovine serum albumin and pyridine were from Fluka; magnesium acetate, phosphoric acid and acetic acid from Riedel de Haën; acrylamide, bisacrylamide, TEMED, APS were from Naxo.

5'-TAMRA-kemptide and inhibitors were synthesized in our laboratory by Asko Uri and Darja Lavõgina.

- **TLC** plates (Macherey-Nagel, Polygram Sil G) were eluted with the solvent mixture of butanol:pyridine:acetic acid:water, 15:10:3:12 (by volume).
- **Fluorescence imaging** of the TLC plates and polyacrylamide gels was performed with Molecular Imager FX Pro Plus (Bio-Rad Laboratories). A diode-pumped solid-state laser at 532 nm was used for excitation and emission was measured through long pass 555 nm filter (100 μ m scanning resolution, medium detector sensitivity). Scanned images were processed by Quantity One software (Bio-Rad) and data were analyzed with GraphPad Prism version 4 software.
- **UV-Vis** spectra were recorded on Unicam UV 300 spectrometer (ThermoSpectronic). The samples were measured in quartz cells, optical path length 1.000 cm.
- **SDS-PAGE** was carried out in a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad) on 12% polyacrylamide gel (gel size 8 cm x 7.3 cm) The current intensity was 60 V for stacking gel and 120 V for separating gel.
- **RP HPLC** was performed on a C18 column Inertsil ODS with water (0.1% TFA)/acetonitrile gradient, flow rate 1 ml/min. UV-detector (260 nm) and fluorescence detector (excitation 480-520 nm, emission recorded at 570 nm) were used.
- **Cation exchange chromatography** was performed on a Mono S HR 5/5 column (Pharmacia Biotech) with the application of the gradient of buffers A (18 % isopropanol in 36 mM ammonium bicarbonate buffer) and B (18 %

isopropanol in 1.2 M ammonium bicarbonate buffer (pH = 7.8)): gradient with 0–100% of B in 8 min (flow rate 1 ml/min), and then 10 min with 100% of B. Peaks were detected at 258 nm.

- **MALDI mass** spectra were recorded on MALDI-TOF mass spectrometer Voyager-DE PRO. The used matrix was 2.5-dihydroxy benzoic acid (DHB).

Radiometric phosphocellulose paper assay was carried out by Kaido Viht and the HPLC analyses were carried out by Gerda Raidaru.

4.2 Fluorometric TLC assay for measuring protein kinase activity

The reaction was carried out in 96-well polystyrene plates at 30 °C. The reaction mixture contained 50 mM Hepes buffer (pH 7.5), 0.2 mg/ml BSA, 10 mM magnesium acetate, 1 nM cAPK C_α, 100 μM ATP and TAMRA-kemptide (various concentrations) in 40 μl reaction volume.

The reaction was initiated by the addition of ATP to the other components of the reaction mixture. At fixed time points the reaction was stopped by 20-fold dilution with 75 mM phosphoric acid. The obtained solution was analyzed by TLC.

TLC. The stopped and diluted samples (2 μl) were spotted onto a silica gel TLC plate. Then the plates were dried and developed with the solvent mixture 1-butanol:pyridine:acetic acid:water (15:10:3:12 by volume). The visualization and quantification were carried out with the fluorescence imager.

4.2.1 Evaluation of PKA inhibitors

The inhibition constant IC₅₀ was measured for three previously tested inhibitors: N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), N-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride (H9), Adc-Ahx-Arg₆ and for three new inhibitors: ARC-TAMRA (UT583), 8-N₃-ARC-TAMRA (UT908) and 8-N₃-ARC (UT907). The concentration of the inhibitors was varied from 0.5 nM to 30 μM. The reaction mixture contained 50 mM Hepes buffer (pH 7.5), 0.2 mg/ml BSA, 10 mM magnesium acetate, 1 nM cAPK C_α, 100 μM ATP, 30 μM TAMRA-kemptide and inhibitors in 40 μl volume. The analysis of the reaction mixture was performed as described in section 4.2.

4.3 Application of ABPP with ARC type inhibitors

4.3.1 Photoreaction

The reaction mixture, 1.2 μM 8-N₃-ARC-TAMRA in 50 mM Hepes buffer (pH 7.5) and 1 μM cAPK C $_{\alpha}$ (in the presence and absence of DTT), was illuminated for 30 min with a 15 W UV lamp (G15T8, wavelength 254 nm). The reaction was stopped by adding the sample buffer [0.5 M Tris-HCl, (pH 6.8); 26 % glycerol, 10% (w/v) SDS; 0.5 % (w/v) bromophenol blue].

4.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Preparation of Separating and Stacking Gels

The preparation was performed according to previously described procedure [41]. 30% acrylamide/0.8% bisacrylamide solution, 4 x TrisHCl/SDS buffer (pH 8.8 for separating gel, pH 6.8 for stacking gel) and H₂O were mixed together. After that APS (10%, w/v) and TEMED were added. Solution of separating gel was pipetted into the space between two glass plates and covered with water. The gel was allowed to polymerize for about 30 min. Then the stacking gel solution was prepared and pipetted on top of the separating gel. A comb was placed onto the stacking gel for formation of the probe wells.

Sample loading and gel electrophoresis

Samples were heated for 5 min at 85°C, and then loaded (10 μl) into the well by gel-loading tips. Low Range MW markers (BioRad) were loaded to the last well. The electrophoresis was accomplished in the electrode buffer (Tris/HCl, 0.1% SDS and glycine) with the application of voltage of 60 V and 120 V for stacking and separating gel, respectively. After gel electrophoresis the gel was dried and quantified with fluorescence scanner.

4.4 Stability studies

The stability of inhibitors was investigated by using serum and trypsin solutions. The reaction was carried out in a 0.50 ml polypropylene centrifuge tube at 30 °C. The reaction mixture (20 μl) containing 40 mM phosphate buffer (pH 7.2), 0.4 mM inhibitor solution, and trypsin at various concentrations (1, 10 and 20 U) was

prepared. At certain time points, the reaction was stopped by the injection of samples into the ion exchange HPLC column.

4.5 Reaction with reducing agents

4.5.1 Reaction with dithiothreitol

The reaction was carried out in 1.5 ml polypropylene centrifuge tubes in dark at room temperature. The reaction mixture contained 1.2 μ M of 8-N₃-ARC- TAMRA, 0.5 mM DTT and Hepes buffer in the volume of 20 μ l. The reaction was performed for 10 and 50 min and the reaction mixture was analyzed by RP HPLC.

4.5.2 Separation of 2-mercaptoethanol from kinase solution by gel-filtration

2-Mercaptoethanol from cAPK C α solution was separated with Sephadex G-25 chromatography. Sephadex G-25 swelled for 3 hours at 20 °C. 150 μ l of swollen gel was pipetted into a spin column and centrifuged at 1000 g for 2 minutes. The solution of cAPK (contains 5 mM 2-mercaptoethanol) was pipetted carefully to the center of the column, and after 2 minutes it was centrifuged at 1000 g for 4 minutes. Then the gel was eluted with 20 μ l Hepes buffer for two times.

and quantification of the fluorescence spots were carried out by fluorescence scanner. The reaction could be monitored by disappearance of the substrate and the generation of the phosphorylated product spots (Figure 14).

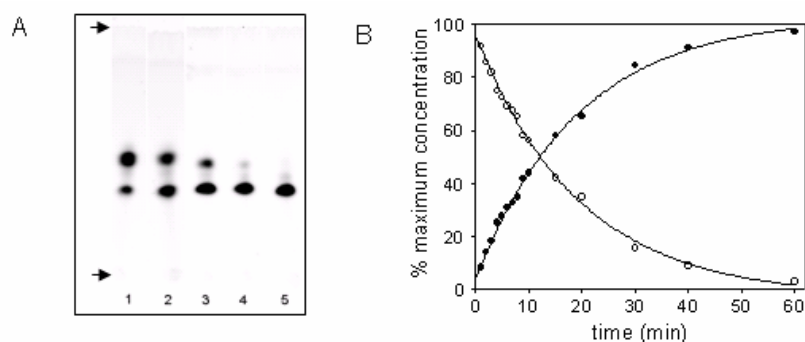


Figure 14. Time course of phosphorylation of 5-TAMRA-kemptide. A: An analysis of the reaction mixture at 2, 7, 10, 15 and 30 min by TLC. B: Kinetic curves of 5-TAMRA-kemptide (○) and 5-TAMRA-phosphokemptide (●).

To characterize 5-TAMRA-kemptide as a substrate of cAPK, values of K_m and V_{max} were determined. The kinetic parameters of kemptide and 5-TAMRA-kemptide for phosphorylation were measured in parallel by radiometric phosphocellulose paper method and by the new fluorescence TLC method (Table 3).

Table 3. Kinetic parameters of kemptide and TAMRA-kemptide

	Kemptide	5-TAMRA-kemptide	
	By radioactivity (P81)	By radioactivity (P81)	By fluorescence (TLC)
K_m^{app} (μM)	12.8 ± 2.5	2.3 ± 0.9	2.0 ± 0.5
V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	6.4 ± 0.4	6.0 ± 0.7	5.2 ± 0.4

It was shown that the attachment of a hydrophobic group (5-TAMRA) to the N-terminus of kemptide decreases the K_m value of the substrate.

Fluorometric TLC assay was used to characterize the inhibitory potency of three previously characterized and three new inhibitors of cAPK. The obtained inhibitory activities of compounds of the first group are shown in Figure 15 and Table 4.

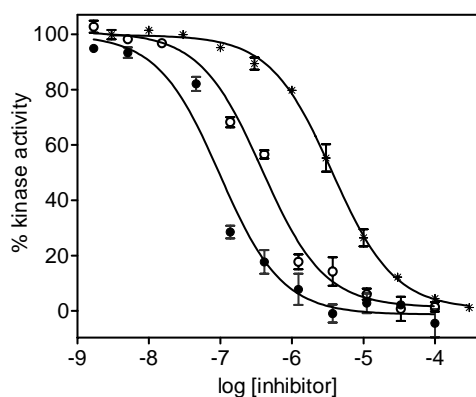


Figure 15. Inhibition of cAPK C α by H89 (●), Adc-Ahx-Arg₆ (○), and H9 (*).

Table 4. The comparison of the inhibitory potency determined by TLC and phosphocellulose paper assay

Inhibitor	Fluorometric TLC assay, pIC ₅₀ (IC ₅₀ , μ M)	Phosphocellulose paper assay (previously published data)
H89	pIC ₅₀ = 7.01 \pm 0.07 (0.10)	K _i = 0.048 \pm 0.008 μ M, IC ₅₀ = 0.135 μ M [11]
H9	pIC ₅₀ = 5.44 \pm 0.03 (3.70)	K _i 1.9 μ M [12]
Adc-Ahx-Arg ₆	pIC ₅₀ = 6.42 \pm 0.05 (0.40)	IC ₅₀ = 0.7 \pm 0.2 μ M [43]

The obtained inhibitory activities of the compounds are similar to the activities that have been determined previously proving the reliability of the new assay.

The new assay was subsequently applied for evaluation of new ARC type inhibitors (Figure 16 and in Table 5). Two of these inhibitors – ARC-TAMRA and 8-N₃-ARC-TAMRA - consisted fluorescence markers and were also visible on TLC silica plates with R_f= 0.29. The inhibitor spots disturbed little the quantification of TAMRA-phosphokemptide and increased the errors of result.

Table 5. Inhibitory potency of three new inhibitors

Inhibitor	Fluorometric TLC assay, pIC ₅₀ (IC ₅₀ nM)
ARC-TAMRA	8.54 \pm 0.10 (2.9)
8-N ₃ -ARC	8.28 \pm 0.18 (6.4)
8-N ₃ -ARC-TAMRA	8.19 \pm 0.21 (5.2)

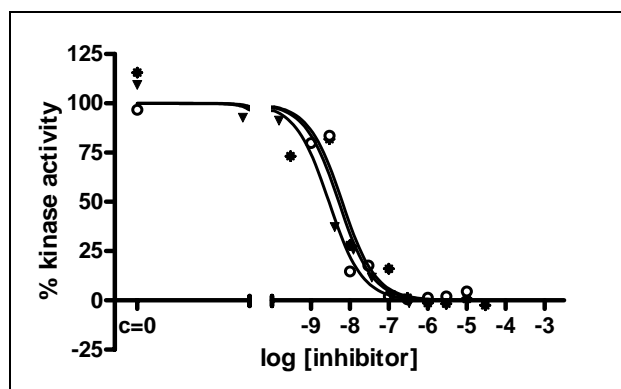


Figure 16. Inhibition of cAPK C α by ARC-TAMRA (∇), 8-N₃-ARC (*) and 8-N₃-ARC-TAMRA (\circ).

The fluorometric assay is simple to perform and relatively cheap, while many samples can be analyzed at the same time on one TLC plate, and it does not consume large amounts of chemicals. While the reaction extent is calculated from the ratio of the integrated intensity of the product spots to the sum of the integrated intensities of the product and substrate spots, the variation of the fluorescent compound concentration and pipetting errors are largely eliminated.

This method was initially worked out for cAMP-dependent protein kinase, but it should be applicable for measuring the activity of other protein kinases as well, if suitable fluorescently labeled peptide substrates are available.

5.2 The stability of ARC type inhibitors towards proteolytic degradation

The ARCs are cell permeable [21] inhibitors of basophilic protein kinases, therefore for using them *in vivo* application their resistance to proteolytic degradation is an important factor. In organisms there are many enzymes that may degrade ARC type inhibitors. Adenosine deaminases are enzymes that irreversibly deaminate adenosine, converting it to the related nucleoside inosine by the removal of an amine group. The peptide part of ARC may be prone to proteolytic degradation, specially by enzymes, which cleave peptide bond at basic amino acid residues - trypsin, carboxypeptidase A, carboxypeptidase B, etc.

The stabilities of ARC type inhibitor and D arginines containing ARC were investigated in two different media: serum and trypsin solution. Serum was used because it is a body fluid, which contains several proteins, including proteases. Trypsin was used because it specifically cleaves the peptide bond at C-terminal side of lysine and arginine residues, it is widely used and methods for measuring its

activity are well characterized. Many arginine residues of the peptide fragment of ARC make it a good substrate for trypsin degradation.

The degradation of ARC and D-ARC was monitored with cation exchange chromatography (Appendices 1- 6). The quantification of the degradation products was carried out at absorption maximum of adenosine (260 nm, $\epsilon \approx 15000 \text{ cm}^{-1} \text{ M}^{-1}$). The quantification is possible, as the absorption coefficient of adenosine does not depend on the length of the linked peptide.

It was shown that degradation of either inhibitor does not occur in serum. The trypsin solution of various concentrations was used to determine inhibitor resistance for enzymatic degradation. The determined half-life of ARC in solution containing one U of trypsin was 68 seconds, but physiological concentration of trypsin (10 U) completely degraded the ARC within 1 minute. In contrast, D-ARC remained intact for at least 20 minutes by treating with solution containing 20 U of trypsin.

Only the structures of adenosine containing fragments of degraded ARC, which can be seen in HPLC with UV detector, were verified with MALDI-TOF spectrometry. Masses of these peaks correspond to the fragments of enzymatically degraded ARC (Table 6, Appendices 7-11).

Table 6. Retention times in HPLC spectrum and molecular masses of the fragments of ARC after treatment with trypsin

Fragments of degraded inhibitor	Peak number	Retention time	MW
AdcAhxArg ₄ NH ₂	7	12.9	1018.1
AdcAhxArg ₄ OH	4	9.1	1019.1
AdcAhxArg ₃ OH	3	6.3	863.0
AdcAhxArg ₂ OH	2	3.4	706.8
AdcAhxArg ₁ OH	1	2.9	551.0

5.3 ABPP with ARC type inhibitors

The second method that was used for characterization of protein kinase inhibitors was ABPP. The probes that are capable covalently bind to the active site of enzyme, are needed for this technique. The ARC type inhibitor, which carries a photoactive azido group in the 8-position of the adenine for covalent binding of the kinase and 5-TAMRA dye for detection, was synthesized (Figure 17).

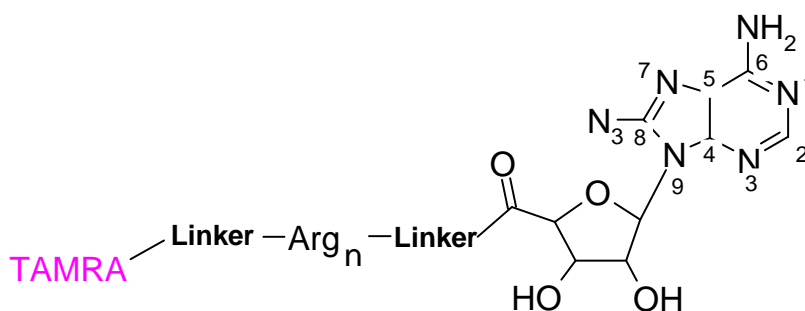


Figure 17. 8-N₃-ARC-TAMRA.

It had been determined previously that the substitution at position 8 of the purine in ATP resulted in remarkable decrease in affinity towards cAPK ($K_m=3.1 \mu\text{M}$ for ATP, $K_i=25 \mu\text{M}$ for 8-N₃-ATP). The newly designed ARC type inhibitors, ARC-TAMRA and substituted 8-N₃-ARC-TAMRA, have both high inhibitory activity ($IC_{50}=2.9 \text{ nM}$ for ARC-TAMRA and $IC_{50}=5.2 \text{ nM}$ for 8-N₃-ARC-TAMRA). The azido group in 8-position of adenine decreases the affinity of cAPK, but the effect is obviously smaller than in the case of adenine nucleotides.

To determine if ARC type inhibitor could be used as the targeting reagent, its ability to form reactive species by excitation with UV light was tested. The photolysis of the inhibitor was studied in water and found that azido reagents were reduced to amino compounds. The destruction of the azide upon photolysis was determined by HPLC. Retention times of 8-N₃-ARC-TAMRA and 8-NH₂-ARC-TAMRA in reversed phase C18 HPLC column were 20.76 min and 20.11 min, respectively (Appendix 12).

For using 8-N₃-ARC-TAMRA for enzyme targeting, its photolysis in the presence of cAPK was carried out. The irradiated mixture was analyzed by SDS-PAGE and the gel was visualized with fluorescence imager. As seen in Figure 18, no bands, which correspond to complex (~ 40 kDa) are visible, thus no labeling of cAPK occurred.

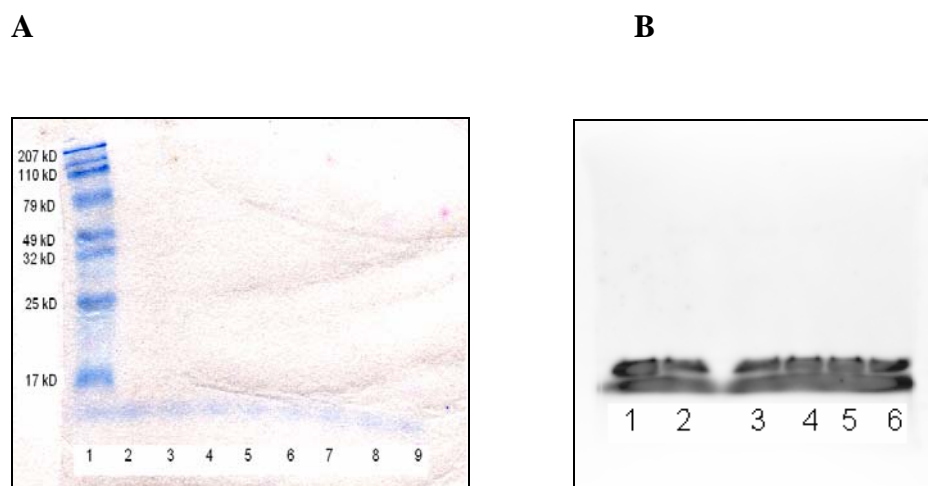


Figure 18. Analysis of complexation reaction between 8-N₃-ARC-TAMRA and cAPK C_α by SDS-PAGE. Scan images of dried SDS-PAGE gel, scanned with Epson perfection 1650 scanner (A); and scanned with fluorescence scanner (B). Numbers mark loaded samples: 1 in A is mixture of MW markers; 2-9 in A and 1-6 in B are bands of complexation samples after photoreaction.

It was assumed that the 2-mercaptoethanol as reducing agent that was present in the enzyme stock might reduce the azido groups before the photoreaction. To measure the effect of 2-mercaptoethanol on the photoreaction, the reduction reaction with another reducing agent - DTT was carried out. After 10 minutes of incubation, all 8-N₃-ARC-TAMRA was reduced to 8-NH₂-ARC-TAMRA.

2-Mercaptoethanol from cAPK solution was separated with a Sephadex G-25 column. Large cAPK C_α molecules leave the column with little retention, followed by smaller 2-mercaptoethanol molecules. Three fractions was collected. Activity of these fractions was measured and indicated that the greatest activity of the enzyme (50 %) was recovered in the second fraction. 25 % of enzyme was eluted in the first and 10 % in the third fraction. Purified enzyme was then incubated in dark with 8-N₃-ARC-TAMRA and no reducing was detected. It proves that the 2-mercaptoethanol was efficiently separated. The photolysis reaction of 8-N₃-ARC-TAMRA was performed with the purified cAPK, but no complexation was detected.

The causes why the complexation does not occur are unknown, but there may be many reasons. The complexation reaction is presumably based on the activation of the azido group which results in the formation of a highly reactive nitrene radical. The radical can react with amino acid residues of the protein. It has been shown that photoreactions of azido group do not always follow one pathway, e.g., the adenosine-

connected singlet nitrene formed after the flash can tautomerize to form a closed diazaquinodimethane.

The reaction between photoreactive affinity probe and the kinase can occur when the reactive nucleotide is positioned in the complex in suitable conformation. When the adenine nucleotides are free in solution, the orientation of the purine ring about the glycosidic bond is distributed between the *syn*- and the *anti*-conformations. If the azido group is attached to the 8-position of the adenine ring, the balance of the conformational equilibrium is shifted. While ARC type inhibitor binds to the enzyme active site with high affinity, the orientation of azido group for forming covalent bond is important. The unsuitable conformation of the substituted adenine ring may be one reason why the complexation does not occur. Inhibitors that have been used previously as ABPs, like fluorescently marked wortmannin, have shown inhibitor potency in micromolar range. 8-N₃-ARC-TAMRA is a tight-binding ligand (IC₅₀ = 5.2 nM) and its reactive azido group may be fixed in the position where it cannot find a partner for reaction in the complex with the kinase protein.

6. Summary

In the present study a new fluorometric TLC assay was worked out for determination of protein kinase activity and it was used for evaluation of protein kinase inhibitors. The assay is based on the separation of fluorescently marked substrate from its phosphorylated counterpart by TLC and their quantification by fluorescence imaging. Inhibitory activity of known inhibitors was measured and indicated that results are similar to the corresponding activities that had been determined previously.

The stability of ARC type and D-arginine-containing inhibitors was measured in serum and trypsin solution. It was shown that degradation of either inhibitor does not occur in serum. Determined half-life of ARC in solution containing 1 U of trypsin was 68 sec and the physiological concentration of trypsin (10 U) completely degraded ARC within 1 min. The D-ARC, in comparison, was not degraded under these conditions.

These adenosine-oligoarginine conjugates (ARC), especially the compounds incorporating D-arginine residues, were found to be potent inhibitors of cAPK. For using them as probes for ABPP, photoactive azido group was connected to C8 position of adenine for covalent binding to the kinase, and fluorescence dye was attached to the peptide residue for detection. It was shown that no complexation of inhibitor and cAPK occurred during the photoreaction. Thus, this inhibitor does not fit for irreversible labeling of cAPK.

7. Kokkuvõte

Käesoleva magistritöö “Fluorestsentsmeetodid proteiinkinaasi inhibiitorite iseloomustamiseks” raames töötati välja fluorestsentsdetektsiooniga planaarkromatograafilise meetod proteiinkinaasi inhibiitorite iseloomustamiseks. Meetod põhineb 5-TAMRA-kemptiidi ja 5-TAMRA-fosfokemptiidi planaarkromatograafilisel lahutamisel ning sellele järgneval reaktsioonikomponentide detekteerimisel ja kvantifitseerimisel fluorestsentskänneriga. Meetodit kasutati varem iseloomustatud inhibiitorite hindamiseks ja leiti, et saadud tulemused on võrreldavad kirjandusest saadud andmetega.

Uuriti ka ARC ja D-ARC tüüpi inhibiitorite stabiilsust seerumis ja trüpsiini lahuses. Leiti, et ARC tüüpi inhibiitor lagunes täielikult 1 minutiga füsioloogilise kontsentratsiooniga trüpsiini lahuses. Seevastu D-amiinhappeid sisaldav ühend oli vastupidav ensümaatilisele degradatsioonile. Seerumiga inkubeerimisel kummagi inhibiitori lagunemist ei toimunud.

Samuti prooviti kasutada ARC tüüpi inhibiitoreid aktiivsuspõhisel proteiinide määramisel. Analüüsiks kasutati fotoaktiivset asido rühma ja fluorestseeruvat markerit sisaldavat inhibiitorit – 8-N₃-ARC-TAMRA. Pärast fotoreaktsiooni ja geelelektroforeetilist lahutamist leiti, et kasutatud inhibiitor ei komplekseeru pöördumatult cAPK-ga ja seega ei ole kasutatav pöördumatu aktiivsuspõhise proovina. Samas on arendatud meetoodika tulevikus kasutatav proteiinkinaaside efektiivsete aktiivsuspõhiste arendamisel.

8. References

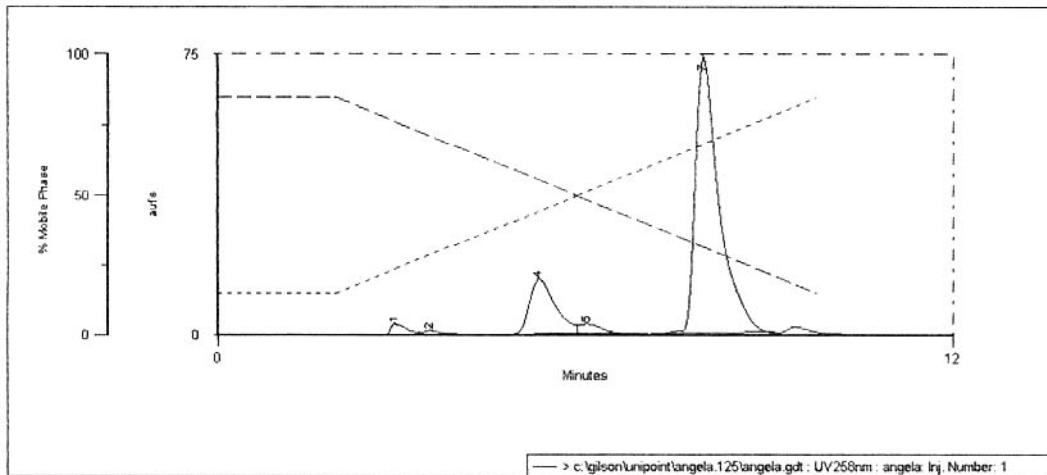
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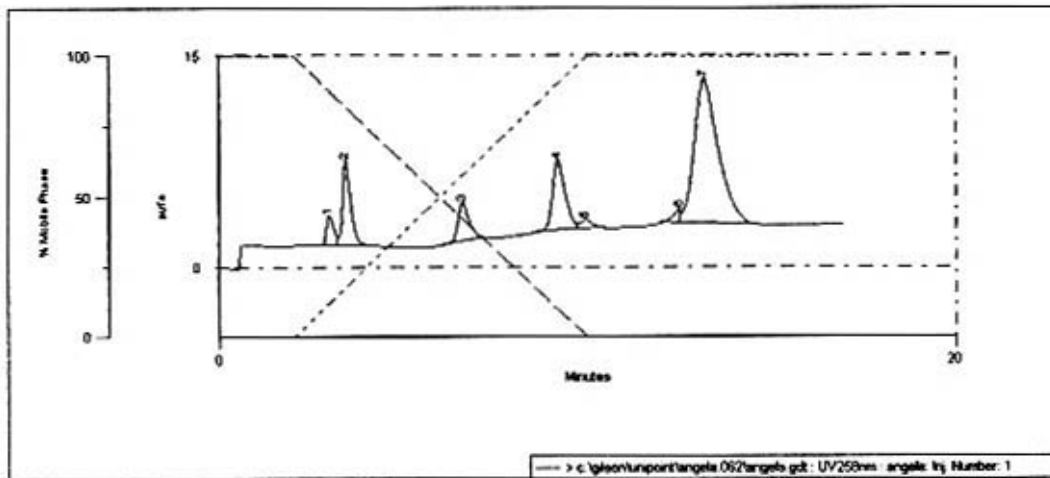
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9. Appendices



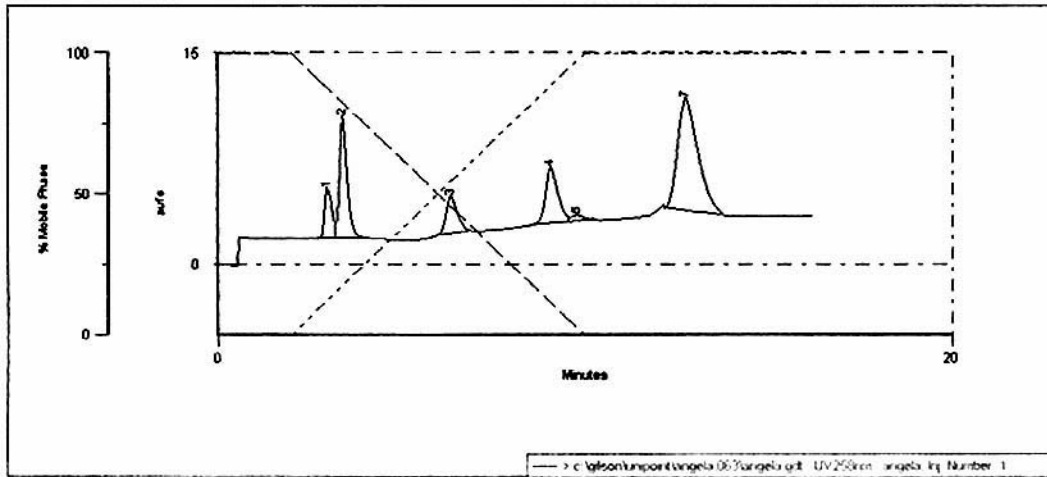
Peak Name	Area %	R. Time	Area	Sample Descrip.
1	2.16	3.01	9635687.00	angela
2	1.16	3.61	5243235.00	angela
3	17.06	5.46	77570984.00	angela
4	3.02	6.20	13742832.00	angela
5	76.61	8.25	48426208.00	angela
6				

Appendix 1. Cation exchange HPLC chromatogram of AdcAhxArg₄NH₂



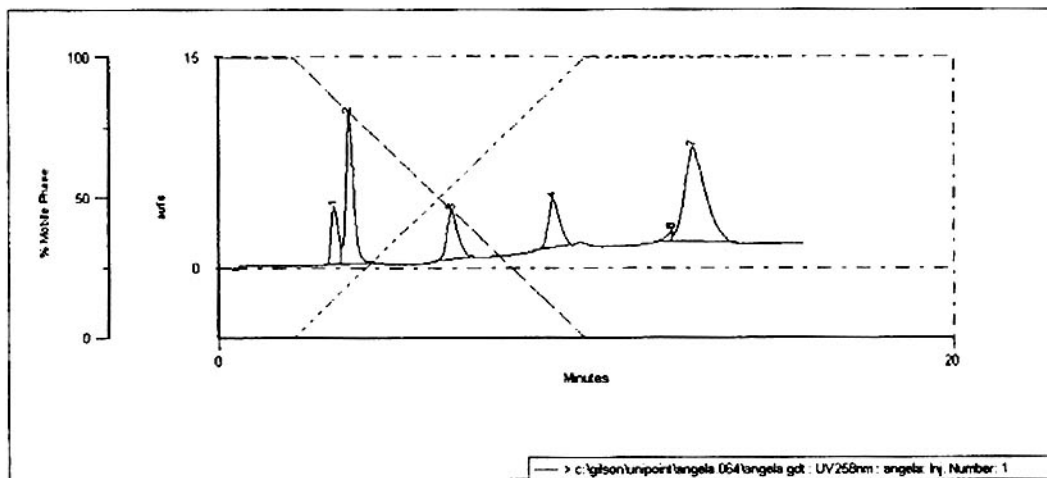
Peak Name	Area %	R. Time	Area	Sample Descrip.
1	3.80	2.97	5019133.00	angela
2	11.78	3.42	15672026.00	angela
3	7.42	6.59	9806147.00	angela
4	15.25	9.18	20166746.00	angela
5	1.99	9.94	2626690.25	angela
6	1.42	12.48	1873481.00	angela
7	58.36	13.15	77171736.00	angela
8				

Appendix 2. Cation exchange HPLC chromatogram of trypsinization mixture of AdcAhxArg₄NH₂ at 20 seconds.



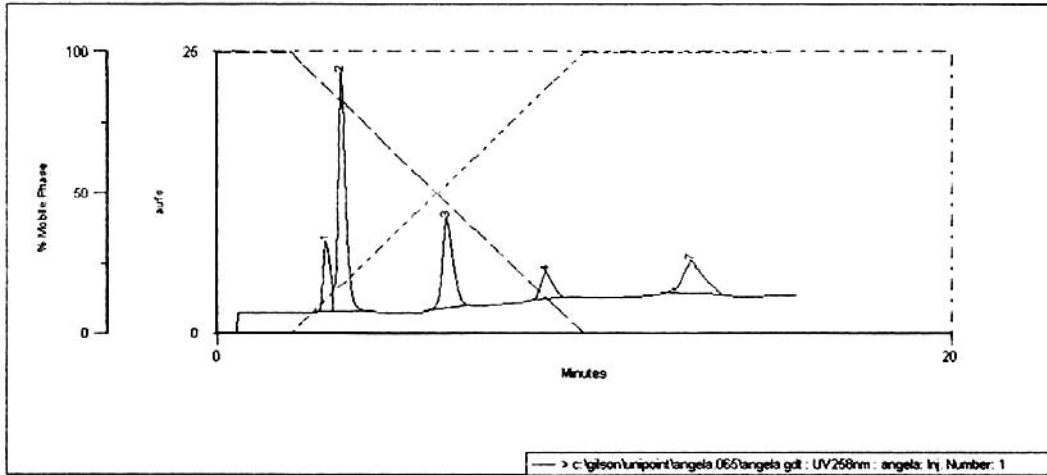
Peak Name	Area %	R. Time	Area	Sample Descrip.
1	7.53	2.96	6062963.00	angela
2	19.52	3.38	20885012.00	angela
3	9.02	6.33	9655456.00	angela
4	14.62	9.05	15642597.00	angela
5	1.74	9.76	1860121.38	angela
6	47.57	12.73	50903264.00	angela

Appendix 3. Cation exchange HPLC chromatogram of trypsinization mixture of AdcAhxArg₄NH₂ at 30 seconds.



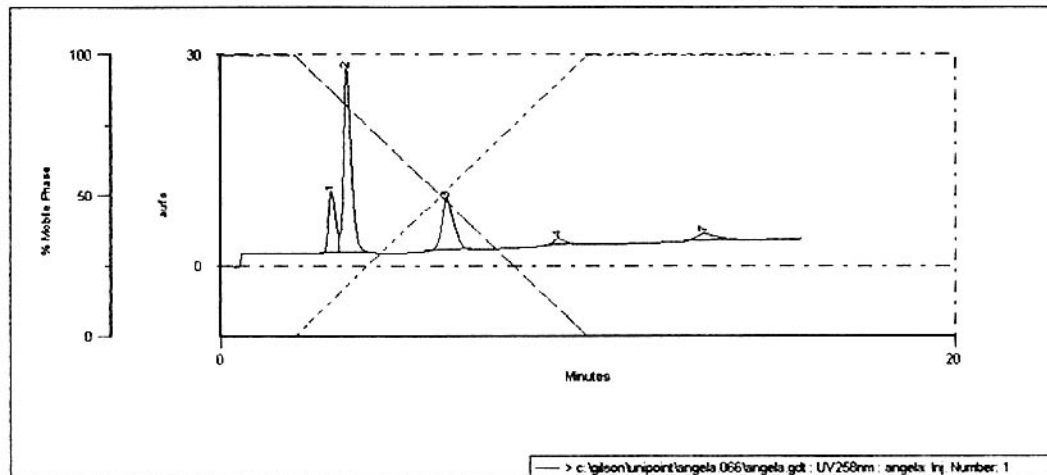
Peak Name	Area %	R. Time	Area	Sample Descrip.
1	6.70	3.13	9220016.00	angela
2	24.23	3.53	25685934.00	angela
3	11.46	6.33	12146057.00	angela
4	11.73	9.09	12438815.00	angela
5	1.39	12.33	1474210.25	angela
6	42.49	12.91	45047180.00	angela

Appendix 4. Cation exchange HPLC chromatogram of trypsinization mixture of AdcAhxArg₄NH₂ at 1 minutes.



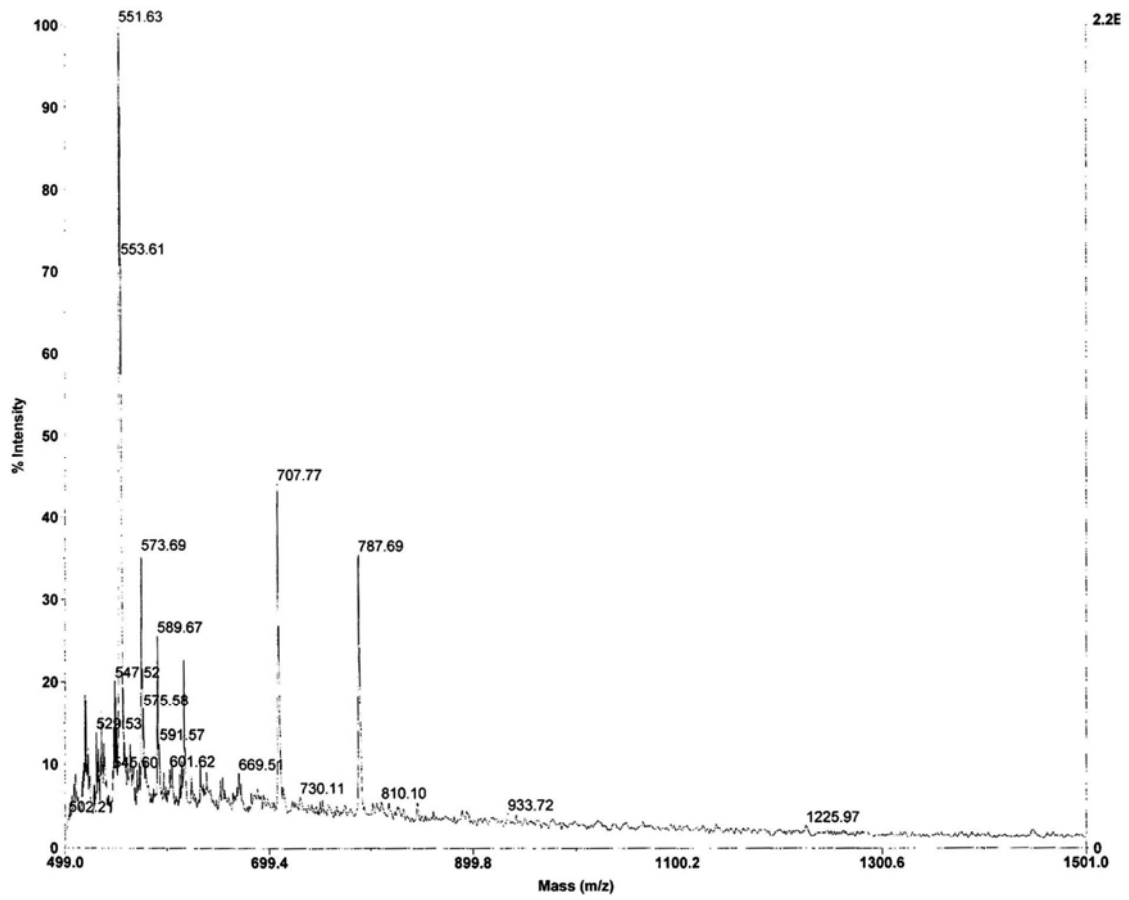
Peak Name	Area %	R. Time	Area	Sample Descrip.
1	11.60	2.96	14028425.00	angela
2	41.98	3.37	50763248.00	angela
3	22.33	6.25	27001854.00	angela
4	7.34	8.94	8870767.00	angela
5	16.75	12.91	20248718.00	angela

Appendix 5. Cation exchange HPLC chromatogram of trypsinization mixture of AdcAhxArg₄NH₂ at 2 minutes.

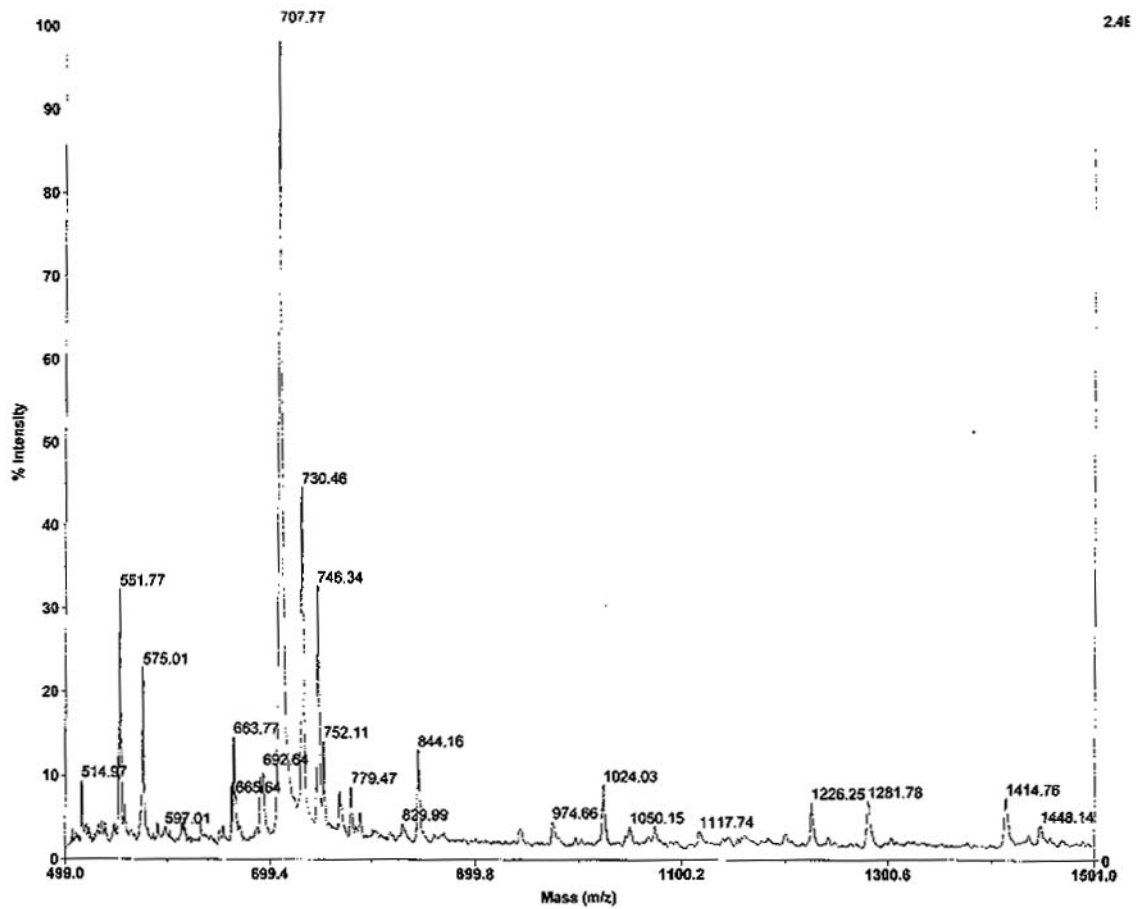


Peak Name	Area %	R. Time	Area	Sample Descrip.
1	18.11	3.00	19425270.00	angela
2	52.37	3.43	53138352.00	angela
3	23.09	6.15	27837406.00	angela
4	2.89	9.18	3479715.25	angela
5	5.55	13.16	6686435.00	angela

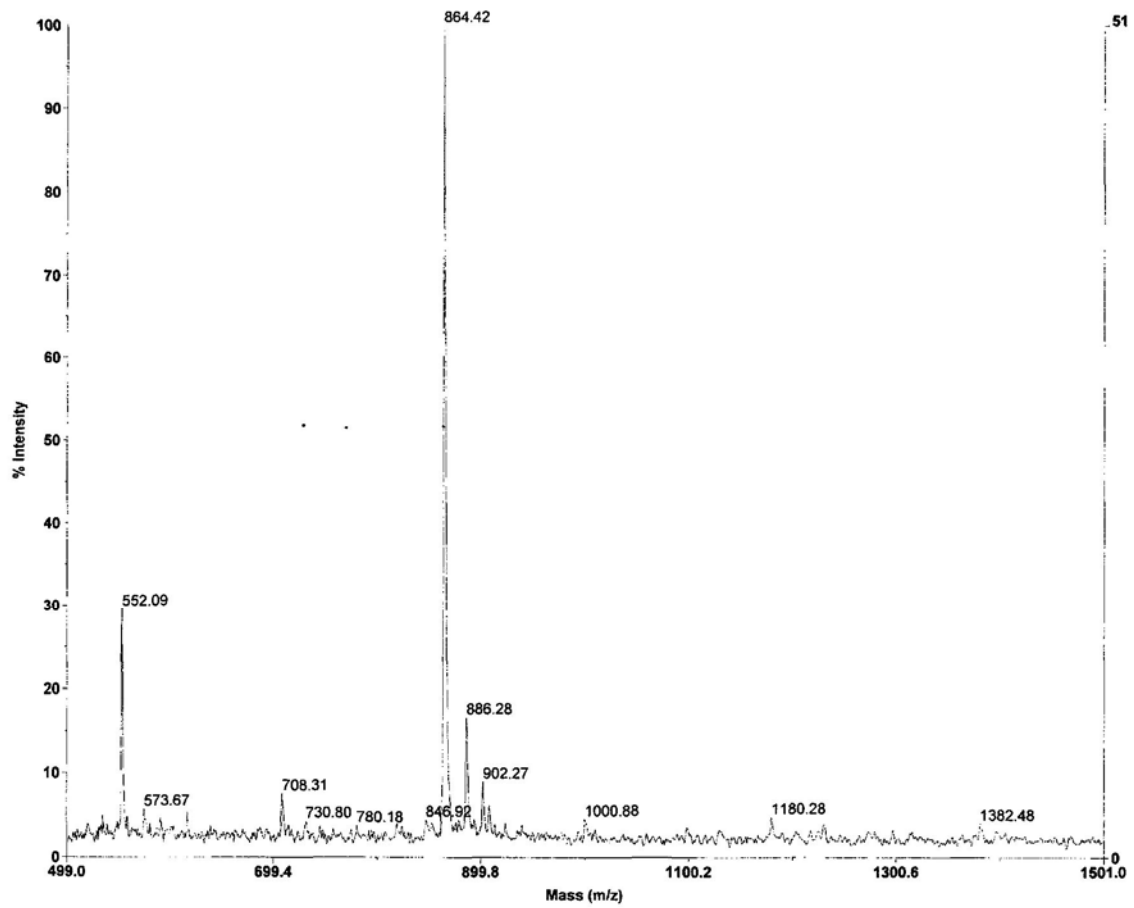
Appendix 6. Cation exchange HPLC chromatogram of trypsinization mixture of AdcAhxArg₄NH₂ at 3 minutes.



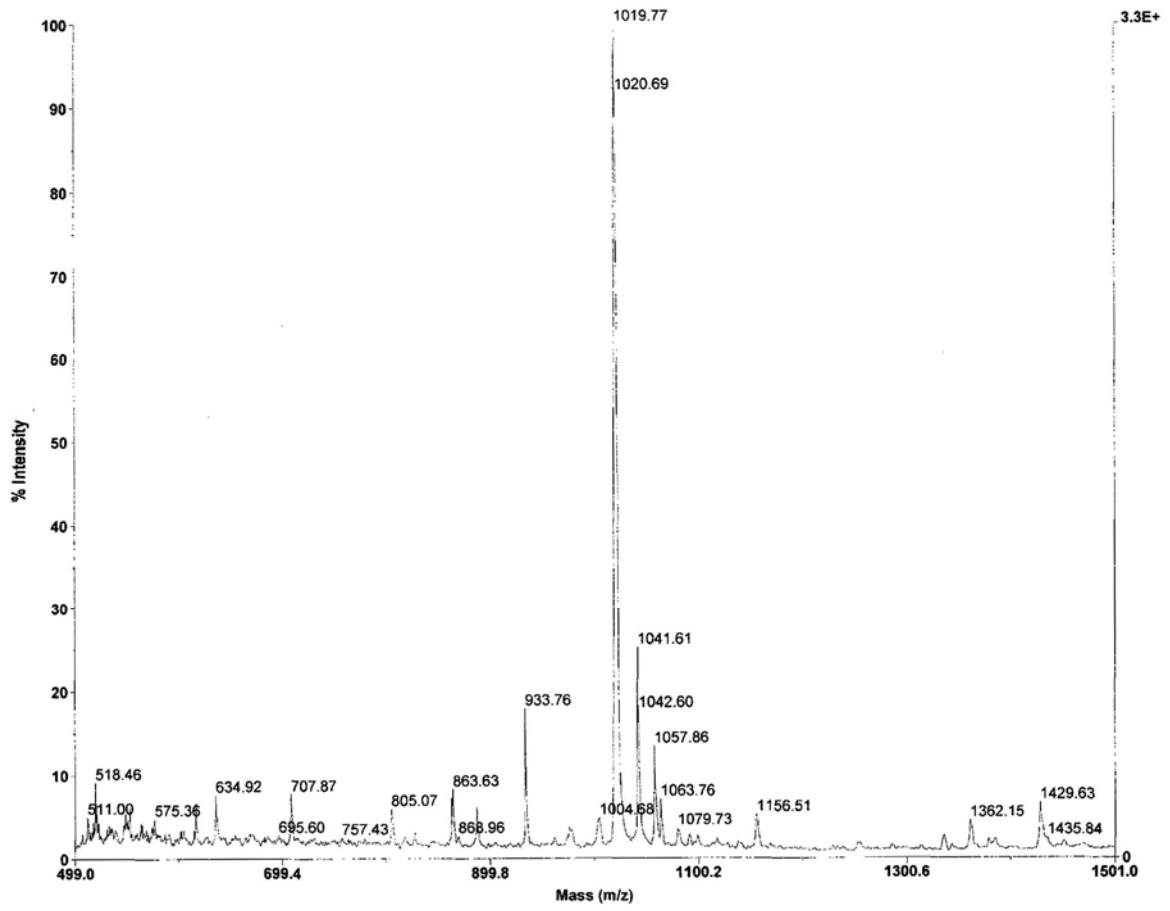
Appendix 7. MALDI-MS of a cation exchange HPLC fraction corresponding to peak 1. AdcAhxArg₁OH (M+H = 551.63), AdcAhxArg₂OH (M+H = 707.77).



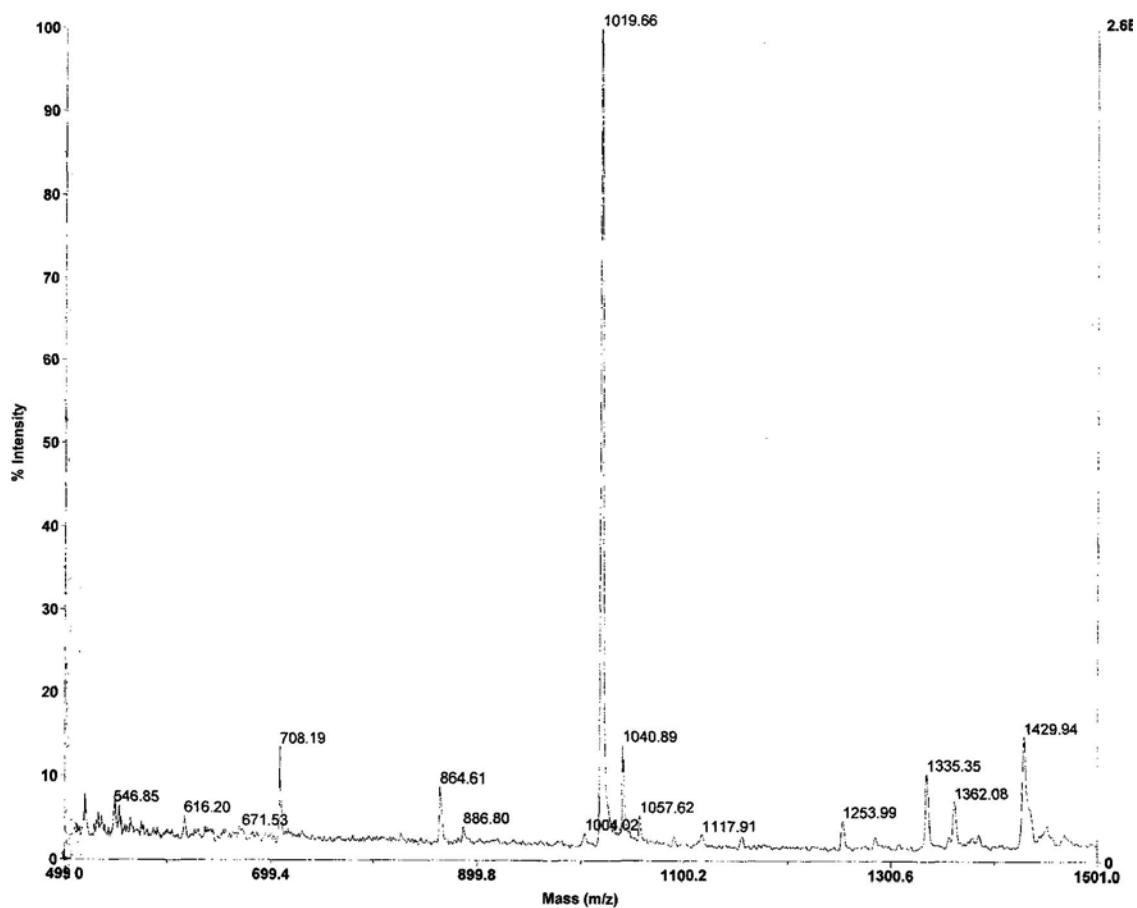
Appendix 8. MALDI-MS of a cation exchange HPLC fraction corresponding to peak 2. AdcAhxArg₂OH (M+H = 707.77).



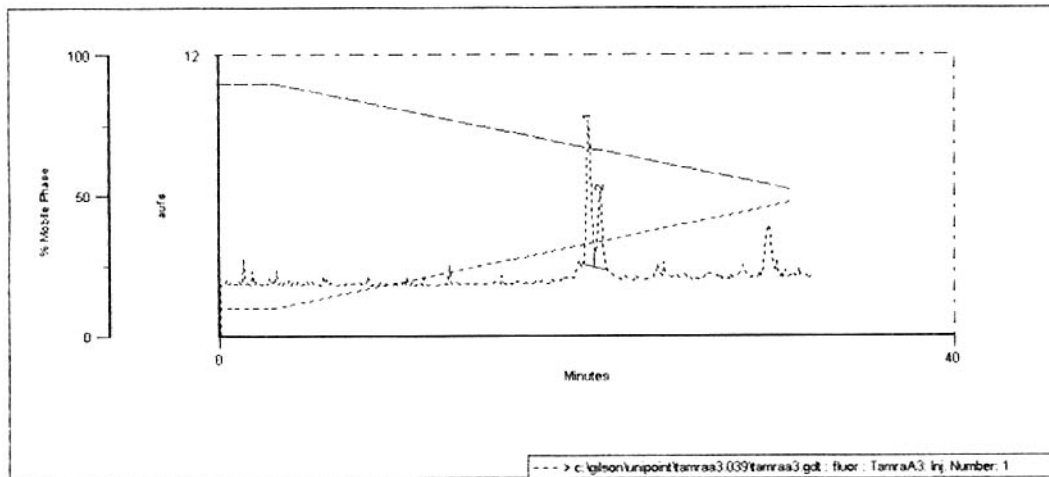
Appendix 9. MALDI-MS of a cation exchange HPLC fraction corresponding to peak 3. AdcAhxArg₃OH (M+H = 864.42).



Appendix 10. MALDI-MS of a cation exchange HPLC fraction corresponding to peak 4. AdcAhxArg₄OH (M+H = 1020.69).



Appendix 11. MALDI-MS of a cation exchange HPLC fraction corresponding to peak 7. AdcAhxArg₄NH₂ (M+H = 1019.66).



Area	Ret. Number	Peak Name	R. Time	Area	Sample Percent								
66.02	1.00	1	20.11	14670743.00	TamraA3								
33.98	1.00	2	20.76	7549386.00	TamraA3								

Appendix 12. Cation exchange HPLC chromatogram of NH₂-ARC-TAMRA (peak 1) and 8-N₃-ARC-TAMRA (peak 2).