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SYNTHESIS OF HIGH AFFINITY INHIBITORS FOR PROTEIN KINASE CK2

Master's thesis (30 EAP)

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Abbreviations

ARC	Adenosine analogue and peptide conjucates
ATP	Adenosine-5'-triphosphate
Abu	γ-aminobutyl acid
Boc	*tert*-butoxycarbonyl
DCE	1,2-dichloroethane
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
CX-4945	5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid
Fmoc	9-fluorenylmethyloxycarbonyl chloride
HBTU	O-Benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate
HOBt	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
KOtBu	Potassium tert-butoxide
MALDI-TOF	Time of flight matrix-assisted laser desorptsion/ionisation

NMM	N-methylmorpholine
SPPS	Solid phase peptide synthesis
TBBI	4,5,6,7-tetrabromobenzimidazole
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
NMR	Nuclear magnetic resonance spectroscopy
UV-VIS	Ultravioletse ja nähtava ala spektroskoopia

Contents

1. Introduction

Protein kinase CK2 is a highly conserved and acidophilic serine/threonine type of protein kinase. It is expressed in eukaryotic cells at high concentration. Like other protein kinases, CK2 catalyses the transfer of the ATP terminal phosphoryl group to a protein substrate.

Increased activity of CK2 is related to many different diseases, such as various types of cancers, Alzheimer's disease, diabetes, inflammation. That is why it is important to research CK2 inhibitors as potential drugs against diseases caused by increased activity of the enzyme.

Most of CK2 inhibitors are ATP-competitive. Since these inhibitors are binding only one active site of the enzyme, they often have low selectivity or affinity.

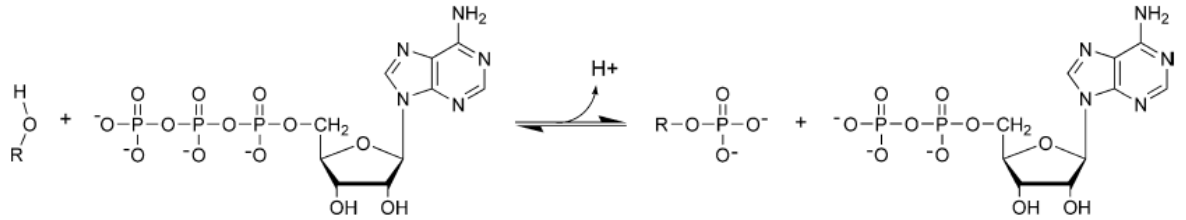
The aim of this study was to develop new, highly selective and affine bisubstrate inhibitors for CK2. Highly selective and affine inhibitors and their fluorescent probes can be useful probes for studying CK2 and its ligands. These applications include monitoring of CK2 location in cells, quantification of CK2 in samples, screening of new inhibitors, co-crystallisation. Tight-binding bisubstrate inhibitors could simplify the research of the protein binding site of CK2. Currently, the ATP binding site and its interactions are well studied, but there is lack of information about the mechanism and interactions between protein substrate and its binding site. High affinity bisubstrate inhibitors could help determine the characteristics of the binding site, which would give new information about functioning of CK2 and aids the development of new CK2 inhibitors. In addition, high affinity fluorescent probes can be used to determine the inhibition activity of new high affinity bisubstrate inhibitors.

2. Literature overview

2.1. Protein kinases

Protein kinases catalyse a simple, yet important reaction: the transfer of the terminal (also known as gamma) phosphoryl group of nucleotide (for example ATP or GTP) to the hydroxyl group of an amino acid residue (serine, threonine or tyrosine) of a protein/peptide substrate (Scheme 1). [1]

Protein kinases are scientifically interesting biological catalytic enzymes, which have a very important role in the life cycle of cells [1]. They belong to the transferase class of enzymes [2]. Because of the importance of phosphorylation, the activity of protein kinases influences most signalling pathways, affecting viability, movements, substance transport and proliferation. [1]



Scheme 1. Transfer of terminal phosphoryl group of ATP to amino acid residue hydroxyl group. [1]

As of 2011, there are 538 known protein kinases. Ninety of those kinases transfer the phosphoryl group to tyrosine residue, but the majority kinases of human genome are serine/threonine kinases. [1]

Because of protein kinases important role in cell growth, proliferation and death regulation, increased activity of protein kinases is related to different diseases, such as diabetes, Alzheimer's disease, inflammation and various types of cancer. This is the reason why many protein kinases are target molecules in drug development. [2]

2.2 Protein kinase CK2

Protein kinase CK2 is a highly conserved serine/threonine type protein kinase usually presented in cells as a tetramer, containing two catalytic subunits (α or α', expressed by different genes) and two regulatory subunits (β). CK2 is always found to be active and ubiquitously expressed. [3] The regulatory subunit does not regulate the overall activity of the catalytic subunit, but stabilises the formation of holoenzyme and affects the activity toward some peptide substrates, which are CK2β dependent. There is not complete understanding of the roll of the regulatory subunit. [4] There are more than 300 peptide substrates of CK2, and because of that it is considered to be the most pleiotropic protein kinase. [5]

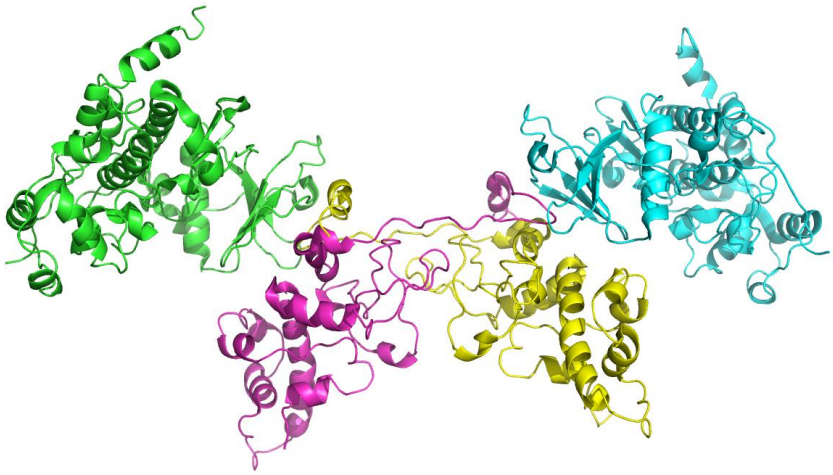


Figure 1. Crystal structure of CK2 holoenzyme. Catalytic subunits are coloured green and cyan; regulatory subunits are coloured yellow and magneta. (PDB code: 1JWH)

Such a large number of substrates indicate that CK2 is involved in many important cellular processes. [5] It is found to be a regulating enzyme for gene expression, cell growth, cell cycle and anti-apoptosis. [6]

CK2 plays an important role in tumorigenesis and is related to various types of cancers (leukaemia, neck, lung, breast, kidney and prostate cancer [7]), because CK2 is found to be over expressed and highly active in cancer cells, compared to healthy ones. It also seems that the cancer cells are relying more on CK2 than normal cells; therefore, inhibition of CK2

activity influences tumour cells more. This phenomenon is called CK2 “addiction”. Because of these reasons, CK2 is considered an important target-protein in drug development. [3]

2.3. Protein kinase inhibitors

There are four types of protein kinase inhibitors. ATP-competitive inhibitors bind to the ATP binding site of the enzyme. Peptide-substrate competitive inhibitors bind to the peptide-substrate binding site of the kinase. There are bisubstrate inhibitors, which bind both binding sites at the same time, increasing the affinity and selectivity of the inhibitor. The fourth type of inhibitors are called allosteric inhibitors, which do not bind to an active site, but some other place on the kinase, changing the conformation of the enzyme and making it inactive. Allosteric inhibitors are rare.

The majority of protein kinase inhibitors are ATP-competitive. They usually have good affinity but poor selectivity because of the high homology of ATP-binding sites. There are some highly selective peptide-substrate inhibitors. However, high affinity peptide-substrate competitive inhibitors need to be longer, which make their cellular transport and stability a problem. [2]

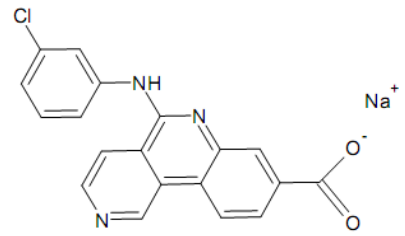
2.3.1. Inhibitors of protein kinase CK2

Among protein kinase inhibitors, most of the known CK2 inhibitors are ATP-competitive. [8]

In addition to the fact that the abnormality of CK2 activity causes serious health problems, there are two main reasons why CK2 is a good target protein. Firstly, signal pathways do not rely on CK2, but it is a modulator protein of them, hence adult organisms will cope more easily with its inhibition. Secondly, CK2 has a smaller ATP binding-site than other protein kinases, which helps to design selective and low molecular weight inhibitors. [9]

In the field of ATP-competitive inhibitors for CK2, different types of selective inhibitors have been discovered. [8] For example, many halogenated benzimidasoles have been researched as potential selective CK2 inhibitors, but their affinities toward CK2 were around micro molar range [10]. Also natural substances, for instance some flavonoids (e.g. quercetine) are found to be inhibitors for CK2. Their best inhibition activities were around sub-micromolar range. [11] In 2011, a new selective and subnanomolar affinity inhibitor was reported. It was named

CX-4945. It was also the first CK2 inhibitor to be taken into clinical trials as a potential treatment for cancer. [12]



Scheme 2. Structure of CX-4945

2.4. Bisubstrate kinase inhibitors

Bisubstrate inhibitors consist of two fragments, each targeting one of the protein kinase substrate binding-sites. The two fragments are attached to each other with a hydrophobic linker. Since both inhibitor parts are developed to inhibit the binding site of a distinct substrate, with optimal linker length, bisubstrate inhibitors are potentially highly selective and affine toward the target enzyme. [2]

There are many types of bisubstrate inhibitors for protein kinase. They vary by the ATP-competitive part (adenosine or adenosine mimic can be used) and the peptide-substrate competitive fragment (different peptide sequences can be used). The type and length of the linker between two fragments is modified to reach the optimal characteristic. [2]

2.4.1 Bisubstrate inhibitors of protein kinase CK2

Several ATP-competitive inhibitors have been developed for CK2, but other types of inhibitors have found less interest. Based on writer's bachelor thesis, an article has been published, reporting a high affinity bisubstrate inhibitor for CK2. [13] Before that, only one attempt to design biligand inhibitors that resulted with micromolar inhibitory potency has been published. [14]

The reported affinities of the novel subnanomolar compounds published in [13] reached 0.5 nM. In comparison, the affinity of CX-4945 is in the same magnitude. Since many protein

kinases are basophilic, the acidic bisubstrate inhibitors have high selectivity toward CK2 because of its acidophilic characteristics. Because of the high selectivity and affinity toward the CK2, bisubstrate inhibitors and their fluorescent probes can be used for detecting the concentration of the kinase, studying interactions of CK2 with other proteins, and they are potential drugs against cancer. [13]

Even though there is information about the interactions of the adenosine binding-site of CK2, there are still no crystal structure data of peptide substrate binding with its binding site on the CK2. High affinity bisubstrate inhibitors are potentially useful tools for that because of their tight binding to the CK2, which potentially makes the binding peptide part more stabilized, than the usual peptide substrate and thus more easily recognizable in X-ray crystallography. Enzyme interactions with the peptide substrate would help us to understand the binding mechanisms and characteristics of CK2 protein binding site. [13]

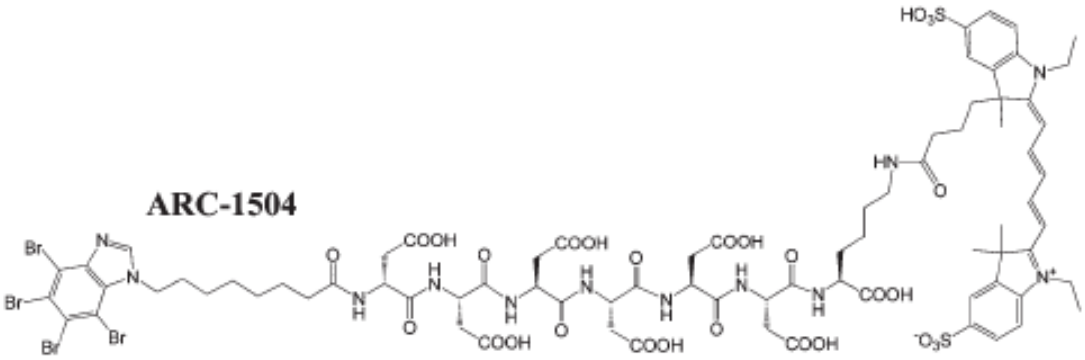


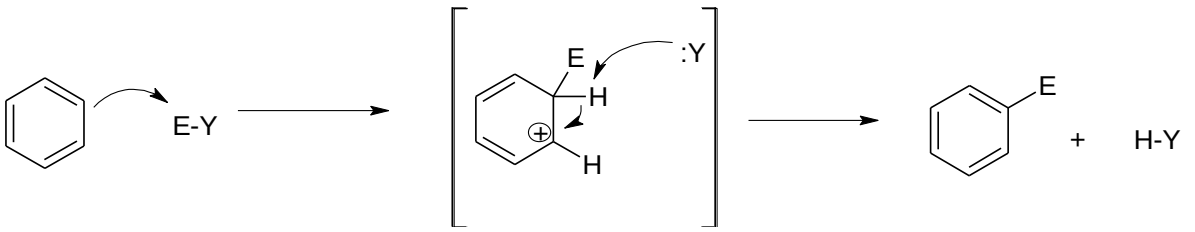
Figure 2. Structure of the fluorescent probe of CK2 bisubstrate inhibitor ARC-1502 (ARC-1504). [13]

2.5. Electrophilic substitution in aromatic ring

Electrophilic aromatic substitution reactions are very important, well-studied organic synthesis reactions. Most substitutions at an aliphatic carbon are nucleophilic, but in the case of aromatic compounds, it is electrophilic. That is because of the high electron density at the aromatic ring that leads its reactivity as Lewis base or Brønsted-Lowry base. [15]

A wide variety of electrophiles can attack the aromatic ring and effect substitution. There are compounds, which can substitute the aromatic ring without any prior activation of the ring.

On the other hand, there are substituents that can only react with an activated aromatic ring. In addition, the scale and position of substitution can be manipulated by using different substituents, activators or previously substituted aromatic compounds. [16]



Scheme 3. General mechanism of electrophilic substitution.

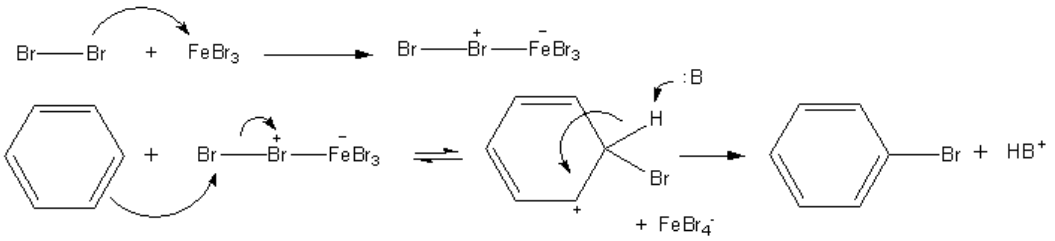
2.5.1. Halogenation

Halogenation is a very common electrophilic substitution used in organic synthesis. There are different methods and reagents used for this reaction. The reactivity of halogens grow in order $I_2<Br_2<Cl_2<F_2$, F_2 being to most reactive. [16]

Reaction with fluorine does not need any catalyst, because it reacts so rapidly, and for that synthesis special lab equipment is needed. For bromination and chlorination, usually a Lewis acid is used as a catalyst to achieve desirable rates of reaction. For multiple substitutions, protonic acid has to be used. Iodine is the least reactive halogen in aromatic substitution. In this case, an oxidizing agent has to be used for halogenation with I_2. [17, 18]

2.5.1.1. Bromination

Bromination reaction is carried out with different catalysts. As a catalyst, the most common substitute used is Lewis acid ($FeBr_3$). [19] In addition, protonic acids (HOBr, HNO3) or N-bromo amides (especially N-bromosuccimidine and tetraalkylammonium polyhalides) can be used. [15]



Scheme 4. Lewis acid catalysed bromination mechanism.

2.6. Suzuki coupling

Carbon-carbon bond formation methods are very important reactions that allow us to build molecules that are more complex from simple precursors. For a long time, there were reactions for the formation of carbon-carbon bonds between saturated sp^3 atoms but no simple method to create a bond between unsaturated groups (vinyl, aryl etc.). [20]

In this field, Suzuki (also called Suzuki-Miyaura) coupling reaction using boronic acid and halide compounds as coupling partners catalysed with palladium(0) complex, is one of the most efficient and easiest method for formation of a carbon-carbon bond between unsaturated compounds. Even though there are other coupling methods, such as Kharash coupling, Negishi coupling, Stille coupling, the Suzuki coupling has proven to be the most favoured. [21]

The Suzuki coupling offers several advantages compared to other methods. It has mild reaction conditions and is unaffected of the presence of water. The reaction also can be carried out with a broad range of functional groups; the inorganic by-products are non-toxic and easily removed, making it suitable also for industrial processes. [22]

2.7. N- and O-alkylation reaction

The N-alkylation of heterocyclic compounds bearing an acidic hydrogen atom attached to the nitrogen is usually done by the treatment of a suitable base followed by adding an alkylating agent (such as alkyl halides). [23] Usually, strong bases (KH, NaH, KOH, sodium amide etc.) are used for such kind of reactions. For cyclic N-alkylation, the use of phase transfer catalysts is reported, but there is literature available about alkylation of heterocyclic compounds with alkyl halides in basic media. [24]

Non-selective alkylation of cyclic amides is described to be carried out with anorganic bases, such as $LiCO_3$ or LiOH, using alkyl halides as alkylating reagent. With that method, the outcome is a mixture of O- and N-akylatated products. For O-selective alkylation, the carboxylic oxygen is substituted with chlorine. [25]

2.8. Solid phase peptide synthesis (SPPS)

The solid phase peptide synthesis (SPPS) method was first described in the year 1963 by R. B. Merrifield. Today, the SPPS is a widely used synthesis method. [26]

In SPPS, the synthesis is carried out on a solid support, called resin. On the resin there are functional groups, which are reaction centres for the first amino acids coupling to the resin. [26]

Peptide synthesis can be performed from N→C terminus of peptide chain or the other way around. Since carboxylic group activation on the resin is harder, synthesis from the amino terminus is more common. [26]

SPPS methods also differ for the protecting group used on the amino group. Two main protecting groups in use are Boc and Fmoc (Figure 3). The Boc group is removed with strong acids (usually trifluoroacetic acid). The Fmoc protecting group is removable with soft bases, such as piperidine, so the Fmoc method is more commonly used. In the Fmoc-method, side chains are often protected with Boc and other acid labile protecting group, which can be removed together with the final cleavage of the peptide sequence from the resin. It is usually done with trifluoroacetic acid. The fact that we can use different substances to remove protecting groups makes the protecting groups orthogonal. [27]

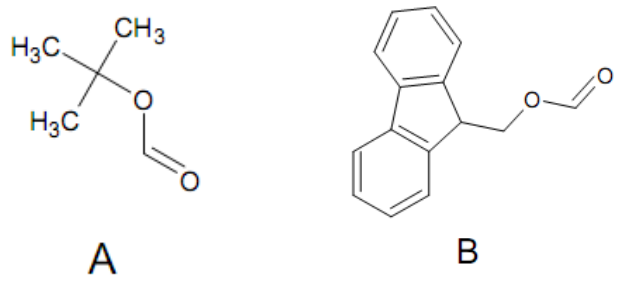


Figure 3. A – Boc protecting group, B – Fmoc protecting group

3. Experimental

3.1. Equipment and reagents

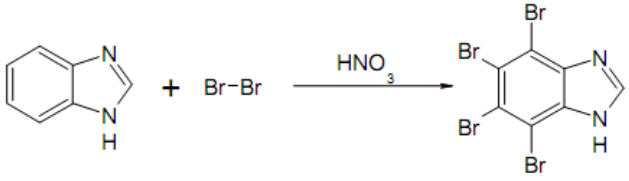
Purification of the conjugates was performed by Gerda Raidaru on a Shimadzu Prominence LC solution HPLC system with a SPD M20A diode array detector and a Phenomenex Gemini C18 5 µm column protected by a 5 µm Gemini guard column. Mass spectra were measured by Gerda Raidaru on a Shimadzu LCMS-2020 (ESI-MS) in positive ion mode and by Erki Enkvist on Applied Biosystems Voyager DE-Pro (MALDI-TOF). NMR data were recorded on a Bruker Avance III HD (16.4 T).

Bromo-esters, benzimidazole, ethyl-3-bromonicotinate and 2-amino-4-methoxycarbonyl phenylboronic acid hydrochloride were purchased from ABCR GmbH & Co. KG, dimethyl formamide was purchased from Acros Organics, TFA was purchased from Scharlau, and triisopropylsilane was purchased from Sigma-Aldrich. Fmoc protected amino acids were puschased from Advanced Chemtech, resins used in SPPS were purchased from NovaBiochem.

3.2. Methods

3.2.1. Synthesis of 4,5,6,7-tetrabromobenzimidazole (TBBI)

3 ml of Br_2 was added drop wise to 590 mg (5 mmol) of benzimidazole in 15 ml concentrated HNO_3. The solution was refluxed until the reaction completed. The reaction was monitored with a MALDI-TOF mass-spectrometer. After cooling of the solution, the precipitate was collected by filtration, washed with $Na_2S_2O_3$ and crystallized in EtOH [10]. The precipitate was dried and yielded to 1.46 g (3.36 mmol, yield 67%). Mass spectroscopy gave *m/z* M=435.05 (theoretical for TBBI=434.73).

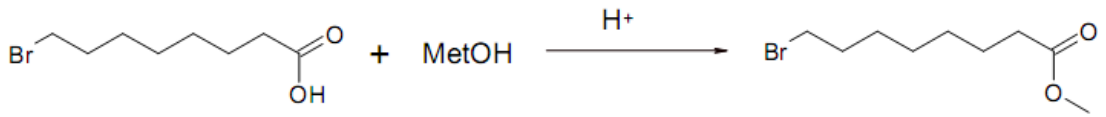


Scheme 5. Synthesis of TBBI.

3.2.3. Synthesis of methyl-8-bromooctanoate

1.5 ml MeOH was added to 539.9 mg (2.42 mmol) of 8-bromooctanoic acid. Catalytic amount of concentrated H_2SO_4 was added. The solution was stirred at 30 ºC overnight. The thin layer chromatography (TLC) (eluent 10:1 chloroform/methanol) was used to monitor the reaction. Iodine was used as developer.

Methanol was removed, and the product was partitioned in a separatory funnel; Na_2CO_3 solution and ethyl acetate were used. Ethyl acetate fraction was collected, and liquid was removed by rotatory evaporator. 466.5 mg (1.97 mmol, yield 81%) ester was collected.

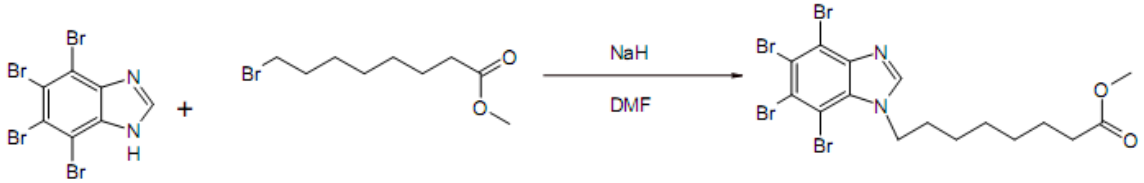


Scheme 6. Estrification of 8-bromooctanoic acid.

3.2.4. Alkylation of TBBI

TBBI (115 mg, 0.26 mmol) and sodium hydride NaH (11.3 mg, ca 0.28 mmol, 60% suspension in oil) were dissolved in 3 ml of dimehtylformamide (DMF). The solution was mixed at room temperature for 30-60 minutes. Then methyl-8-bromo-octanoate (182 mg, 0.77 mmol) was added to the reaction mixture was stirred overnight at 50 ºC. TLC (eluent 20:1 chloroform/methanol) was used for monitoring of the reaction.

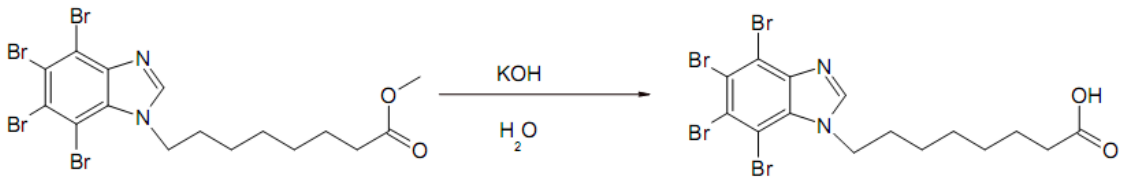
The product was partitioned in a separatory funnel using water and ethyl acetate as solvents. Ethyl acetate layer was collected, and the product was first dried in a rotatory evaporator and then freeze dried overnight. Additionally, column chromatography was used to purify the product (eluent 20:1 chloroform/methanol).



Scheme 7. Alkylation of TBBI.

3.2.5. Hydrolysis of TBBI ester.

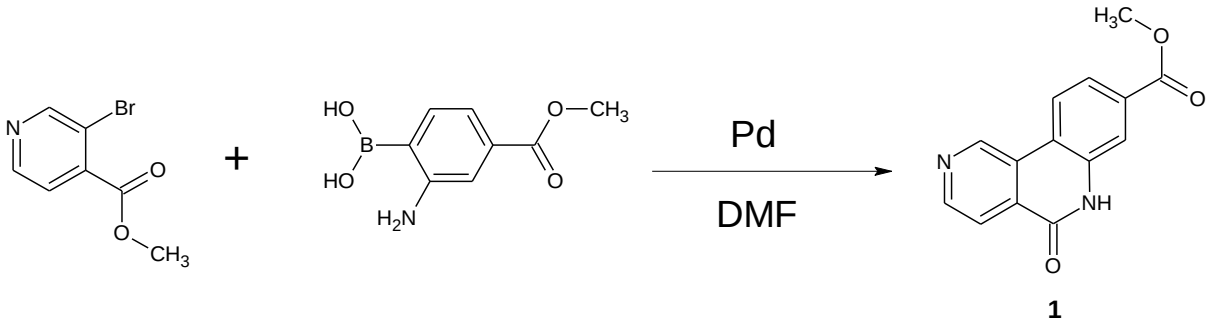
For hydrolysis, 4 ml H_2O, three pellets of KOH and 0.5 ml of EtOH was added to the purified ester. The reaction mixture was stirred until all of the ester was dissolved, and then heated for 30-60 minutes at 50 ºC. The reaction was monitored with TLC (eluent 20:1 chloroform/methanol). The residue was partitioned between ethyl acetate and $KHSO_4$ solution. Ethyl acetate was collected, and the product was dried with rotatory evaporator to yield 8-(4,5,6,7-tetrabromo-1H-benzimidazol-1-yl)octanoic acid (95 mg, 62% over two steps).



Scheme 8. Hydrolysis of alkylated TBBI ester

3.2.6. Suzuki coupling

For Suzuki coupling reaction 150 µl ethyl-3-bromonicotinate (1.1 mmol), 232 mg 2-amino-4-methoxycarbonyl-phenylboronic acid hydrochloride (1 mmol), 40.6 mg $PdCl_2$(dppf) (0.06 mmol) and 365 mg sodium acetate (4.4 mmol) were dissolved in 2 ml of DMF. Flask was filled with nitrogen. Reaction mixture was stirred for 12 hours at 125 ºC. After 12h, the reaction mixture was cooled to room temperature and 30 ml of water was added. A brown precipitate formed (compound **1**) [25]. Precipitate was washed with water twice and dried in freeze drier overnight.



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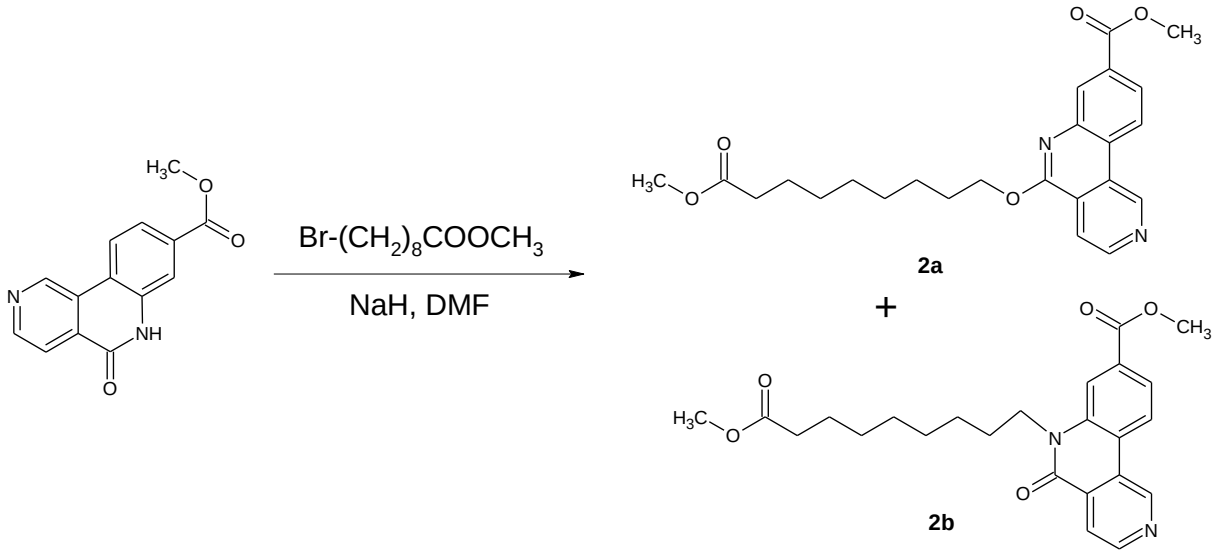
Scheme 9. Suzuki coupling

3.2.7. Alkylation of compound 1

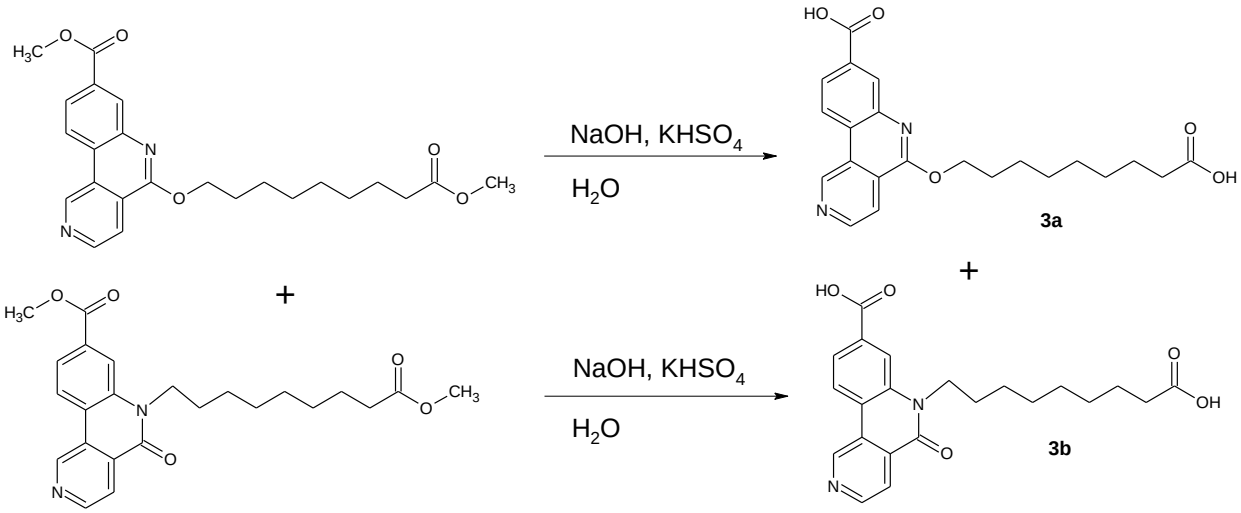
Compound **1** (32 mg, 0.13 mmol) and NaH (22.8 mg, ca 0.5 mmol) were dissolved in 1 ml of DMF. The solution was mixed at room temperature for 60 minutes and then 70 µl methyl-9-bromononanoate was added. The reaction mixture was stirred overnight at 50 ºC. Reaction was monitored with TLC (eluent 9:1 chloroform/methanol). The product was partitioned between ethyl acetate and water. Organic layer was collected and dried with rotatory evaporator. Reaction resulted with O-alkylated and N-alkylated isomers (compounds **2a** and **2b**).



Scheme 10. Alkylation of compound **1**

3.2.8. Hydrolysis of compounds 2a and 2b

For hydrolysis, 1.5 ml water and two KOH pellets were added to compounds **2a** and **2b**. Reaction mixture was stirred for 60 minutes at 50 ºC. Reaction was monitored with TLC (eluent 9:1 chloroform/methanol). After 1 hour, the mixture was cooled down to room temperature. The solution was poured to a 15 ml tube and centrifuged. The liquid was poured into a 50 ml tube, and $KHSO_4$ solution was added to the reaction mixture. Reducing pH caused precipitation (mixture of compounds **3a** and **3b**). Precipitate was centrifuged, washed with water twice and evaporated to dryness.



Scheme 11. Hydrolysis of compounds **2a** and **2b**

Since the compound has not been characterized before, then the isomers were separated and purified with HPLC and both isomers were analysed with NMR spectroscopy. Compund **2a**: ^{1}H NMR (DMSO-d6, 700MHz): δ: 1.31 (m, 4H), 1.40 (kv, J=7.0, 2H), 1.45 (m, 4H), 1.90 (kv, J=7.0, 2H), 2.19 (t, J=7.0, 2H), 4.62 (t, J=6.3, 2H), 8.09 (dd, J=8.4, J=1.4, 1H), 8.14 (dd, J=5.6, J=0.7, 1H), 8.36 (d, J=2.1,1H), 8.94 (d, J=8.4, 1H), 8.97 (d, J=4.9, 1H), 10.20 (s, 1H). ^{13}C NMR (DMSO-d6, 700MHz): δ 24.5, 25.6, 28.2, 28.5, 28.71, 28.74, 33.7, 66.7, 116.9, 123.2, 123.51, 124.2, 125.3, 127.7, 128.7, 131.8, 142.6, 147.5, 148.1, 157.9, 167.0, 171.6. Compound **2b**: ^{1}H NMR (DMSO-d6, 700MHz): δ 1.25 (m, 4H), 1.36 (kv, J=7.0, 2H), 1.42 (kv, J=7.7, 2H), 1.48 (kv, J=7.0, 2H), 1.71 (kv, J=7.7, 2H), 2.19 (t, J=8.4, 2H), 4.38 (t, J=7.7, 2H), 7.91 (dd, J=8.4, J=1.4, 1H), 8.08 (d, J=1.4, 1H), 8.19 (dd, J=4.9, J=0.7, 1H), 8.82 (d, J=8.4, 1H), 8.89 (d, J=4.9, 1H), 9.97 (s, 1H). ^{13}C NMR (DMSO-d6, 700MHz): δ 24.5, 26.3, 26.7, 28.5, 28.7 (2C), 33.7, 42.3, 116.3, 120.2, 120.4, 123.1, 124.2, 126.8, 130.5, 132.6, 136.9, 146.9, 149.0, 159.2, 166.8, 174.6.

NMR spectrums are presented in the Appendix 1.

3.2.9 Optimization of compound 1 alkylation reaction

The aim of the optimization was to increase the reaction yield and to increase the isomer ratio toward compound **2a**.

In the first step of optimisation temperature, base and solvent were varied. Every reaction was held in a different eppendorf tube. Reagents were weighed into tubes (NaH or

potassium tert-butoxide (KOtBu) was used as a base), solvent (DMF or dimethyl sulfoxide (DMSO)) was added, and the reaction mixtures were left at room temperature for 30 minutes. Then 3 µl of ethyl-9-bromononanoate and 30 µl of DMF or DMSO were added. Reactions were carried out overnight at room temperature or at 50 ºC. On the next day reactions were checked with TLC. After reactions were completed, reaction mixtures were dried in a freeze drier.

For hydrolysis, one KOH pellet was dissolved in 1 ml of deionised water. 30 µl of that solution was added to each reaction tube. In the first 2 hours the reaction was held at room temperature, and then incubated at 50 ºC for 2 hours. All tubes were centrifuged to discard the by-product of the Suzuki reaction. After centrifugation the solvent from each reaction mixture was carried to a new eppendorf tube. To each mixture 100 µl $KHSO_4$ solution was added. Precipitate was centrifuged; solvent was removed. Precipitate was washed with water twice to get rid of salts and dried with a freeze drier. All the reaction mixtures were then analysed with HPLC. Synthesis with KOtBu in DMF at room temperature showed the best result.

In the second step of optimisation cations, base and temperature were changed. The alkylation reaction was held as described in the first step, but in one reaction $LiCO_3$ was used as a base and in two reactions tetramethylammonium chloride (TMACl) or tetrabutylammonium bromide (TBABr) was used as a cation source. To see the effect of temperature, a reaction where KOtBu was used was carried out at 0 ºC. After alkylation, hydrolysis reaction was performed as described in the first step. Again, all products were analysed with HPLC.

3.2.10. Peptide fragment synthesis

For peptide fragment synthesis the traditional Fmoc solid phase peptide synthesis (Fmoc-SPPS) method was used [27]. DMF was used as a solvent. Before synthesis resin was swolen for about 45 minutes.

For the activation of an amino acid (3 equiv.) HBTU (2.85 equiv.), HOBt (2.85 equiv.) and N-methylmorpholine (NMM, 10 equiv.) were used. Amino acid and activators were dissolved, transferred to a 5 ml tube and mixed properly. Then the solution with activated amino acid was transferred to resin, and the reaction flask was put on a shaker for 60-90 minutes. After

every acylation reaction, the resin was washed 5 times with DMF and the completion of the reaction was controlled with Kaiser test.

For Kaiser test solution A (5% ninhydrin in ethanol) and B (80% phenol in ethanol) were used. Some resin beads were transferred from the reaction flask to the eppendorf tube, and 50 µl of solution A and B were added to the beads. The mixture was heated for 5-10 minutes, and if many resin beads changed their colour to blue, the reaction was repeated.

If the reaction was successful, then for adding next amino acid, the Fmoc protecting group had to be removed. The removal of Fmoc group was done with 20% piperidine solution in DMF for 3+17 minutes, and then resin was washed with DMF 5 times.

To measure the loading (amount of activated groups) of resin, Fmoc cleaving and washing solutions were collected. With UV-Vis spectrometer, the spectrum of the solution was measured, and the Fmoc concentration was calculated with Lambert-Beer law (λ=290 nm, ε=5352 l/mol*cm).

3.2.11. Conjugate synthesis

TBBI acid and the mixture of **3a** and **3b** were added to the peptide by using the SPPS method. Using resin with previously synthesised amino acid sequence, solution of TBBI or mixture of 3a and 3b (2 equiv.), HBTU (1.9 equiv.), HOBt (1.9 equiv.) and NMM (6 equiv.) were added to the resin. Synthesis was carried out for 2-3 hours and the quantity of acylation was controlled with Kaiser test.

Before cleaving, resin was washed 5 times with three different solvents: DMF, isopropyl alcohol and dichloroethane. After washing, the resin was dried with vacuum. Solution of TFA/H_2O/triisopropylsilane (ratio 90:5:5) was used for cleaving. The cleaving reaction was carried out for 2 hours. The cleaving solution was poured in to diethyl ether, where it precipitated. The precipitate was centrifuged, washed with diethyl ether twice and dried. Purification and analyses were done with reverse phase HPLC-ESI-MS.

4. Results and discussions

4.1. Selecting structural elements for inhibitors

For the ATP-competitive part of the bisubstrate inhibitor, two different fragments were chosen. TBBI is described in the literature as a selective ATP-competitive inhibitor for CK2 [10]. Compounds **3a** and **3b** were based on a high affinity ATP-competitive inhibitor CX-4945 [25], which is the first CK2 inhibitor that is in the clinical trials. Those fragments were chosen because for synthesising bisubstrate inhibitors, there has to be a functional group, to attach the fragment to the peptide part. Both fragments were suitable for adding a linker with a carboxylic group through alkylation reaction, and, for those reasons, these two inhibitors were good candidates for ATP-competitive part.

The choice of amino acids used in the peptide part of the bisubstrate inhibitor was based on the acidophilic characteristics of CK2. Aspartic acid was chosen because it was the acidic amino acid with the lowest molecular mass. To define the effect of the length and isomeric difference of the peptide part to the affinity, both characteristics were varied. To determine the importance of distinctly positioned aspartic acid in the peptide part, the sequence was altered with hydrophobic amino acid.

The length of the linker was varied to characterise its effect to the affinity of the inhibitor.

The effects of different variations are presented in the section 4.7.

4.2. Synthesis of TBBI

Synthesis of TBBI was based on the protocol described in the literature [9]. The time of the reaction was changed because in the protocol reaction mixture was heated under reflux for 24h, but in reality the reaction took place around 3-10 days. It was monitored with MALDI-TOF MS, and the reaction was stopped when the compound with four brominations was the main product. The reaction was conducted three times, and the yield varied from 63% to 67%.

4.3. Synthesis of compound 1

Synthesis of compound **1** was based on the protocol described in the literature [25]. Again, the purification of the product was not as easy as described in the publication. Reaction had a by-product, which was soluble in the same solvents as the product. That is why yield was not calculated. By-product from Suzuki coupling reaction appeared in all the following reactions.

4.4. Alkylation of TBBI and compound 1

In the first stage NaH was added to the TBBI or compound **1** solution in DMF to dehydrogenate the cyclic nitrogen and to activate the alkylation site.

In the second stage several ester of ω-bromo carboxylic acid with different lengths were added to the solution and alkylation was carried out. Alkylation of compound **1** resulted with two isomers: O- and N-alkylated derivates.

A separatory funnel was used to remove the water soluble by-products (for example, NaBr). Ethyl acetate was used as the second solvent, where the products were soluble. Ethyl acetate fraction was collected and dried with a rotatory evaporator. Since bromo-esters used in the synthesis were also hydrophobic and dissolved in ethyl acetate, in some cases column chromatography was used for additional purification. Whereas the column chromatography is time consuming and the bromo-ester did not disturb significantly the next steps of synthesis, the additional purification step was not carried out after every alkylation reaction. The isomers from the alkylation of the compound **1** were not separated after the reaction. The separation of the isomers was done with HPLC for the final conjugates.

In the article [25] the alkylation was carried out through chloride substitution of the amidic oxygen using $POCl_3$. This method was not used in this work because of unidentified reasons, the reaction did not take place as it was described in the publication.

4.5. Base catalysed hydrolysis of esters

Hydrolysis of the products of the alkylation reaction was carried out with a solution of KOH. Reaction was monitored with TLC and took place in 30-60 minutes. In that stage most of the by-product from CX-derivate synthesis was removed because it did not dissolved in aqueous

solution. The reaction was fast, and the product was easy to collect. Since base catalysis worked easily, the method was used throughout all the hydrolysis.

4.6. Optimisation of the synthesis of the compound 3a

Since the ratio of alkylation was toward compound **3b**, but measurements showed that inhibitors with compound **3a** had a higher affinity, the alkylation reaction needed to be optimized.

The next two tables show conditions used for optimisation of the alkylation reaction.

Table 1. Results of the first optimisation step

Reaction number	Compund 1 mass (mg)	Used base and it's mass (mg)	Solvent	Temperature (ºC)	Ratio between N- and O-alkylation	Reaction depth*
1.	0.22	NaH, 2.12	DMF	r.t.	O-alkylated isomer was not registered	19%
2.	0.20	NaH, 2.01	DMF	50	1.7:1	24%
3.	0.34	NaH, 2.45	DMSO	50	4.6:1	69%
4.	0.60	KOtBu, 1.16	DMF	r.t	0.8:1	75%
5.	0.71	KOtBu, 1.70	DMF	50	1.9:1	51%
6.	0.67	KOtBu, 1.20	DMSO	50	3.2:1	58%

* - calculation was based on the peak areas of HPLC spectrum

After the first stage, it showed that reaction condition nr 4 gave the best results. Reactions using NaH gave lower product yields compared to the reactions with KOtBu. There was also a difference between using DMF or DMSO as the solvent. Reactions with DMSO gave more unidentified by-products than reactions using DMF as the solvent.

Since different cation (KOtBu) gave better results than the one usually used, in the second step the cation was varied to see the effect of its size and characteristics (reactions 8-10).

Reaction at room temperature gave a better alkylation ratio toward O-alkylation than reactions carried out at 50 ºC. Therefore reaction at lower temperature (0 ºC) was carried through to see the temperature effect on the alkylation ratio (reaction 7)

Table 2. Results of the second optimisation step, TMACl – tetramethylammoniumchloride, TBABr – tetrabutylammoniumbromide

Reaction number	Compound 1 mass (mg)	Used base and it's mass (mg)	Cation source and mass (mg)	Temperature (ºC)	Ratio between N- and O-alkylation	Reaction depth*
7.	0.65	KOtBu, 0.50	-	0	3.7:1	37%
8.	0.80	Li_2CO_3, 0.45	-	r.t.	Reaction did not take place	-
9.	0.72	KOtBu 0.45	TMACl, 1.20	r.t.	6.1:1	21%
10.	0.80	KOtBu 0.48	TBABr, 2.94	r.t.	4.2:1	63%

* - calculation was based on the peak areas of HPLC spectrum

Li_2CO_3, TMACl and TBABr were used to see the effect of different cation. Lithium carbonate was probably too weak base to dehydrogenate the amide bond of compound **1**. TMACl and TBABr were used to see the steric effect of the cation. Both reactions took place, but neither the yield nor the ratio toward O-alkylation improved.

Among the 10 different alkylation reaction conditions, reaction condition nr 4 gave the best result.

4.7. Synthesised inhibitors

Altogether 25 new inhibitors were synthesised. ATP competitive part, length and composition of peptide part and linker were varied. Composition and biological activity of inhibitors are presented in Table 3. Full structures of inhibitors and their molecular weights are presented in Appendix 2.

Table 3. List of new inhibitors. All K_D values are measured and calculated by Siiri Saaver, using fluorescent anisotropy. [13]

Inhibitor code	Composition	K_D (st. dev), nM
ARC-1506	TBBI-$(CH_2)_7$-CO-L-Asp	43.4 (6.9)
ARC-1507	TBBI-$(CH_2)_7$-CO-(L-Asp)$_2$	26.8 (8.2)
ARC-1508	TBBI-$(CH_2)_7$-CO-(L-Asp)$_3$	4.64 (3.15)
ARC-1518	TBBI-$(CH_2)_7$-CO-(L-Asp)$_4$	2.21 (0.20)
ARC-1519	TBBI-$(CH_2)_7$-CO-(L-Asp)$_5$	1.72 (1.09)
ARC-1509	TBBI-$(CH_2)_7$-CO-(L-Asp)$_6$	0.55 (0.40)
ARC-1510	TBBI-$(CH_2)_9$-CO-(L-Asp)$_3$	6.99 (4.46)
ARC-1511	TBBI-$(CH_2)_9$-CO-Gly-(L-Asp)$_3$	9.58 (6.52)
ARC-1512	TBBI-$(CH_2)_9$-CO-ABut-(L-Asp)$_3$	12.0 (6.35)
ARC-1514	TBBI-$(CH_2)_9$-CO-(L-Asp)$_5$	1.73 (1.19)
ARC-1515	TBBI-$(CH_2)_9$-CO-(L-Asp)$_6$	0.72 (0.42)
ARC-1424O	CX-$(CH_2)_8$-CO-(L-Asp)$_6$-L-Lys	Undefined
ARC-1513O	CX-$(CH_2)_8$-CO-(L-Asp)$_6$-L-Lys-PF555	0.0098 (0.0018)
ARC-1520O	CX-$(CH_2)_8$-CO-(L-Asp)$_5$-L-Leu-L-Lys	0.083 (0.028)
ARC-1520N	CX-$(CH_2)_8$-CO-(L-Asp)$_5$-L-Leu-L-Lys	0.95 (0.004)

ARC-1521O	CX-$(CH_2)_8$-CO-(L-Asp)$_4$-L-Leu-L-Asp-L-Lys	0.11 (0.02)
ARC-1521N	CX-$(CH_2)_8$-CO-(L-Asp)$_4$-L-Leu-L-Asp-L-Lys	1.51 (0.11)
ARC-1522O	CX-$(CH_2)_8$-CO-(L-Asp)$_3$-L-Leu-(L-Asp)$_2$-L-Lys	0.15 (0.03)
ARC-1522N	CX-$(CH_2)_8$-CO-(L-Asp)$_3$-L-Leu-(L-Asp)$_2$-L-Lys	1.14 (0.06)
ARC-1523O	CX-$(CH_2)_8$-CO-(L-Asp)$_2$-L-Leu-(L-Asp)$_3$-L-Lys	0.18 (0.041)
ARC-1523N	CX-$(CH_2)_8$-CO-(L-Asp)$_2$-L-Leu-(L-Asp)$_3$-L-Lys	1.42 (0.13)
ARC-1524O	CX-$(CH_2)_8$-CO-L-Asp-L-Leu-(L-Asp)$_4$-L-Lys	0.37 (0.11)
ARC-1524N	CX-$(CH_2)_8$-CO-L-Asp-L-Leu-(L-Asp)$_4$-L-Lys	0.85 (0.38)
ARC-1525O	CX-$(CH_2)_8$-CO-L-Leu-(L-Asp)$_5$-L-Lys	0.24 (0.14)
ARC-1525N	CX-$(CH_2)_8$-CO-L-Leu-(L-Asp)$_5$-L-Lys	1.25 (0.07)

To find the effect of the length of the peptide part compounds **ARC-1506** to **ARC-1509**, **ARC-1518** and **ARC-1519** were prepared. The length of peptide part was varied by applying increasing the number of aspartic acids. As suspected, the number of aspartic acids does affect the affinity of the inhibitor. Inhibitors with longer peptide parts have higher affinity toward CK2. The effect shows that using smaller molecular weight bisubstrate inhibitor results in lower affinity.

In inhibitors **ARC-1510** to **ARC-1512**, **ARC-1514**, **ARC-1515** the length of the linker was varied to see the effect of different linker characteristics. For **ARC-1510**, **ARC-1514** and **ARC-1515** the length of the linker was only extended. Results showed that longer linkers do not increase the affinity of these compounds. The results are comparable with the inhibitors, which have the same number of aspartic acids in their peptide part, but different linker. For compounds **ARC-1511** and **ARC-1512** the length was changed. From those results it was concluded that the octanoic linker already has an optimal length for that type of bisubstrate inhibitors.

Compound **3a** was used as the ATP-competitive fragment for **ARC-1424**, **ARC-1513** and **ARC-1520** to **ARC-1525**. Since CX-4945 was reported as a high affinity and selectivity

inhibitor for CK2, a fragment of it was used to reach higher affinities with bisubstrate inhibitors. As the results in Table 3 shows, the prediction was right and bisubstrate inhibitors with compound **3a** gave higher affinities. The affinity of **ARC-1424O** was not measurable with probe **ARC-1504** [13] because of its too high affinity and a new fluorescent probe **ARC-1513O** was synthesised to solve this issue. The fluorescent probe reached the affinity around 10 pM. Inhibitors for CK2 with such high affinities have not been reported before. Using compound 1 as the ATP-competitive part, the conjugate synthesis resulted also with N-alkylated isomer. Compared to **1424O**, the isomer **1424N** had about 10 times lower affinity. For that reason, the N-alkylated isomers are not presented in the table.

From **ARC-1520** to **ARC-1525**, peptide part was systematically varied by using scanning with leucine to see the importance of the position of different aspartic acids in the peptide chain. Leucine was chosen because of its abundance in the positions near to the phosphorylation site (n-4 to n+10) of protein substrates of CK2. Results of the data analysis are presented in Appendix 3. Analysis was based on the publication [28].

From the results presented in the Table 3, it can be concluded that the first and second aspartic acids in the chain have greater importance, when binding to the enzyme, because the affinities of the inhibitors with those positions substituted with leucine were most affected compared to the **ARC-1513O**. Also it seems that the last three aspartic acids contribute equally to the inhibitor binding to the enzyme.

5. Summary

Phosphorylation of proteins is an important regulatory modification to the processes in cell functioning. Enzymes that catalyse this post-translational modification are called protein kinases. Increased activity of protein kinases, such as CK2, is related to different diseases, including various types of cancers. For that matter there is a need for selective and affine inhibitors for protein kinases.

Herein, 25 new bisubstrate inhibitors of CK2 were synthesised and characterised.

The aim of the study was to optimize all three parts of the bisubstrate inhibitor to result in highly selective and affine compounds for CK2. Two different ATP-competitive parts were used in the inhibitors; whereas 5-oxo-5,6-dihydrobenzo[c][2,6]naphthyridine-8-carboxylic acid was a fragment of CK2 inhibitor CX-4945 in clinical trials. Inhibitors with this fragment gave the best results, reaching affinities around 10 pM. The length of the linker was varied to find the optimal positioning of the interacting fragments. Many peptide part modifications were done, including variation of the length of the peptide part and, using leucine scanning, determination of the contribution of differently positioned amino acids. Crystallographic studies are performed on the base of the collaboration to achieve data about the peptide part interactions with CK2.

The future prospect is to improve CK2 bisubstrate inhibitors, develop and research cell-penetrating CK2 bisubstrate inhibitors and also continue to develop bisubstrate inhibitors that could help the study of the interactions between CK2 and its peptide substrates.

Kõrgafiinsete inhibiitorite süntees proteiinkinaasile CK2

Jürgen Vahter

Kokkuvõte

Fosforüülimine on oluline posttranslatoorne modifikatsioon, mille kaudu reguleeritakse paljusid protsesse raku elutsüklis. Neid reaktsioone katalüüsivaid ensüüme nimetatakse proteiinkinaasideks. Proteiinkinaaside, seal hulgas CK2, suurenenud aktiivsus seostatakse mitmete haigustega nagu näiteks erinevate vähi tüüpidega.

Töö käigus sünteesiti ja iseloomustati 25 uut CK2 bisubstraatset inhibiitorit.

Töö käigus varieeriti bisubstraatsete inhibiitorite kõiki kolme osa (ATP-konkurentset fragmenti, peptiidsubstraatset osa, neid ühendavat lüli) ning optimeeriti kasutatud sünteesi meetodeid. ATP-konkureeriva fragmendina kasutati kahte erinevat varem kirjanduses teadaolevat inhibiitorit, millest üks on hetkel kliinilistes katsetustes olev CK2 inhibiitori CX-4945 fragment. CX-4945 fragmendiga inhibiitorid andsid häid tulemusi, parima ühendi afiinsus olid 10 pM. Ühendava lüli pikkust varieeriti, et leida sellele optimaalne suurus uuritava ensüümi jaoks. Modifitseeriti ka peptiidset osa, muutes selle pikkust ning koostist, et mõista erinevates asendites olevate aminohapete olulisust. Ühendite kristallograafilised uuringud toimuvad koostöös Kölni Ülikooliga.

Tulevikus jätkatakse CK2 bisubstraatsete inhibiitorite edasiarendust kõrgema afiinsusega ühendite sünteesimiseks. Lisaks kavatsetakse kasutada modifikatsioone, mis muudaks neid aineid rakku sisenevateks. Samuti jätkub töö leidmaks inhibiitoreid, mida õnnestuks koos CK2-ga kristallida nii, et peptiidse fragmendi elektrontihedus oleks mõõdetav.

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References

1. Schwart, P. A, Murray, B. W. (2011) Protein kinase biochemistry and drug discovery, Bioorganic Chemistry, 39, 192-210
2. Lavõgina, D., Enkvist, E., Uri, A. (2010) Bisubstrate inhibitors of protein kinases: from principles to practical application, ChemMedChem, **5**, 23-34
3. Zanin, S., Borgo, C., Girardi, C., O'Brien, S. E., Miyata, Y., Pinna, L. A., Donella-Deana, A., Ruzzene, M. (2012) Effects of the CK2 inhibitors CX-4945 and CX-5011 on drug-resistant cells, PLoS ONE, **7**
4. Raaf, J., Guerra, B., Neundorf, I., Bopp, B., Issinger, O. G., Jose, J., Pietsch, M., Niefind, K. (2013) First structure of protein kinase CK2 catalytic subunit with an effective CK2β-competitive ligand, ACS Chem. Biol., **8**, 901-907
5. Lupp, S., Gumhold, C., Ampofo, E., Montenarh, M., Rother, K. (2013) CK2 kinase activity but not its binding to CK2 promoter regions implicated in the regulation of CK2α and CK2β gene expression, Mol Cell Biochem, **384**, 71-82
6. Lolli, G., Ranchio, A., Battistutta, R. (2014) Active form of the protein kinase CK2 $\alpha_2\beta_2$ holoenzyme is a strong complex with symmetric architecture, ACS Chem. Biol., **9**, 366-371
7. Olsten, M.E.K., Litchfield, D.W. (2004) Order or chaos? An evaluation of the regulation of protein kinase CK2, Biochem. Cell Biol., **82**, 681–693
8. Sarno S, Papinutto E, Franchin C, Bain J, Elliott M, Meggio, F., Kazimierczuk, Z., Orseszko, A., Zanotti, G., Battistutta, R., Pinna, L. A. (2011) ATP site directed inhibitors of protein kinase CK2: an update. Curr Top Med Chem, **11**, 1340–1351
9. Sun, H., Xu, X., Wu, X., Zhang, X., Liu, F., Jia, J., Guo, X., Huang, J., Jiang, Z., Feng, T., Chu, H., Zhou, Y., Zhang, S., Liu, Z., You, Q. (2013) Discovery and design of tricyclic scaffolds as protein kinase CK2 (CK2) inhibitors through a combination of shape-based virtual screening and structure-based molecular modification, J. Chem. Inf. Model., **53**, 2093-2102
10. Zien, P., Bretner, M., Zastapilo, K., Szyszka, R., Shugar, D. (2003) Selectivity of 4,5,6,7-tetrabromobenzimidazole as an ATP-competitive potent inhibitor of protein kinase CK2 from various sources, Biochemical and Biophysical Research Communications, **306**, 129-133

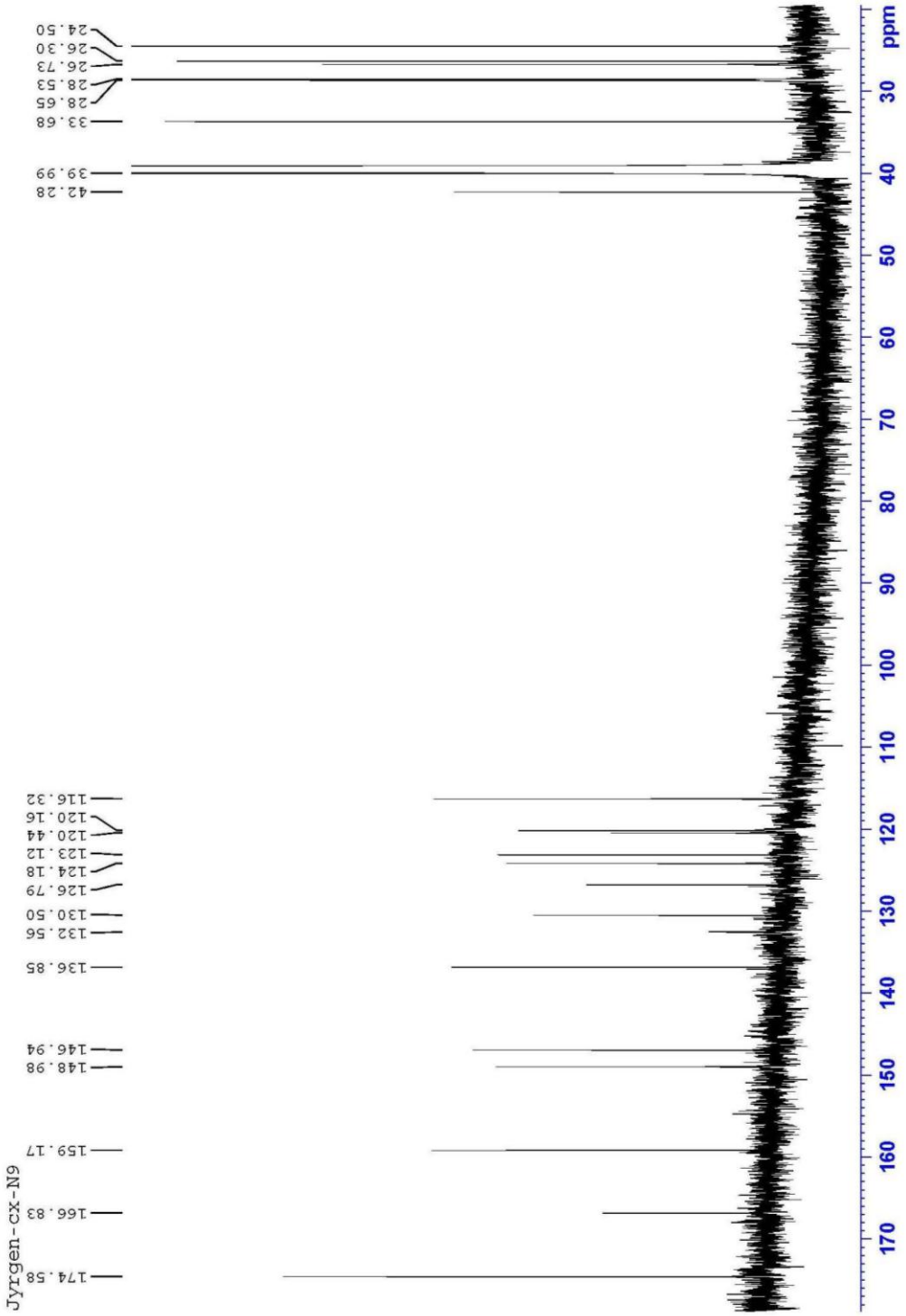
11. Lolli, G., Cozza, G., Mazzorana, M., Tibaldi, E., Cesaro, L., Donella-Deana, A., Meggio, F., Venerando, A., Franchin, C., Sarno, S., Battistutta, R., Pinna, L. A. (2012) Inhibition of protein kinase CK2 by flavonoids and tyrphostins. A structural insight, Biochemistry, **51**, 6097-6107
12. Battistutta, R., Cozza, G., Pierre, F., Papinutto, E., Lolli, G., Sarno, S., O'Brien, S.E., Siddiqui-Jain, A., Haddach, M., Anderes, K., Ryckman, D.M., Meggio, F., Pinna, L.A. (2011) Unprecedented Selectivity and Structural Determinants of a New Class of Protein Kinase CK2 Inhibitors in Clinical Trials for the Treatment of Cancer, Biochemistry, **50**, 8478−8488
13. Enkvist, E., Viht, K., Bischoff, N., Vahter, J., Saaver, S., Raidaru, G., Issinger, O.-G., Niefind, K., Uri, A. (2012) A submolar fluorescent probe for protein kinase CK2 interaction studies, Org. Biomol. Chem., **10**, 8645-8653
14. Swider, R., Maslyk, M., Martin-Santamaria, S., Ramos, A., de Pascual-Teresa, A. (2011) Multisite-directed inhibitors of protein kinase CK2: new challenges, Mol. Cell. Biochem, **356**, 117-119
15. Smith, M. B., March, J. (2007) *March's advanced organic chemistry: reactions, mechanisms and structure*, p.657, John Wiley & Sons, Inc., Hoboken, New Jersey
16. Francis A. Carey, Richard J. Sundberg (1993) *Advanced organic chemistry. Part A: Structure and Mechanisms*, 3rd ed., p.539, Springer, New York
17. Francis A. Carey, Richard J. Sundberg (1993) *Advanced organic chemistry. Part B: Reactions and Synthesis*, 3rd ed., p.572, Springer, New York
18. Graham Solomons, T.W., (1990), *Fundamentals of organic chemistry*, 3rd ed., p.483, John Wiley & Sons, USA
19. McMurry, J., (1988), O*rganic chemistry*, 2nd ed., p.523, Brooks/Cole Publishing Company, California
20. Suzuki, A. (2002) Cross-coupling reactions via organoboranes, Journal of organometallic chemistry, **653**, 83-90
21. Kotha, S., Lahiri, K., Kashinath, D. (2002) Recent applications of the Suzuki-Miyaura cross-coupling reaction in organic synthesis, Tetrahedron, **58**, 9633-9695
22. Suzuki, A. (1999) Recent advances in the cross-coupling reactions of organoboron derivatives with organic electrophiles, 1995-1998, Journal of organometallic chemistry, **576**, 147-168

23. Guida, W. C., Mathre, J. D. (1980) Phase-transfer alkylation of heterocycles in the presence of 18-crown-6 and potassium *tert*-butoxide, Journal of Organic Chemistry, **45**, 3171-3176
24. Hayat, S., Atta-ur-Rahman, Choudhary, M. I., Khan, K. M., Schumann, W., Bayer, E. (2001) N-Alkylation of anilines, carboxamides and several nitrogen heterocycles using CsF-Celite/alkyl halides/CH_3CN combination, Tedrahedron, **57**, 9951-9957
25. Pierre, F.; Chua, P. C.; O'Brien, S.; Siddiqui-Jain, A.; Bourbon, P.; Haddach, M.; Michaux, J.; Nagasawa, J.; Schwaebe, M. K.; Stefan, E.; Vialettes, A.; Whitten, J. P.; Chen, T. K.; Darjania, L.; Stansfield, R.; Anderes, K.; Bliesath, J.; Drygin, D.; Ho, C.; Omori, M.; Proffitt, C.; Streiner, N.; Trent, K.; Rice, W. G.; Ryckman, D. M. (2011) Discovery and SAR of 5-(3-Chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic Acid (CX-4945), the First Clinical Stage Inhibitor of Protein Kinase CK2 for the Treatment of Cancer, J. Med. Chem., **54**, 635-654.
26. Atherton, E., Sheppard, R. C. (1989) *Solid phase peptide synthesis: a practical approach*, IRL Press, Oxford
27. Chan W.C., White P.D. (2000) *Fmoc Solid Phase Peptide Synthesis*, Oxford University Press, Oxford
28. Meggio, F., Pinna, L. A. (2003) One thousand and one substrates of protein kinase CK2?, FASEB J., **17**, 349-368

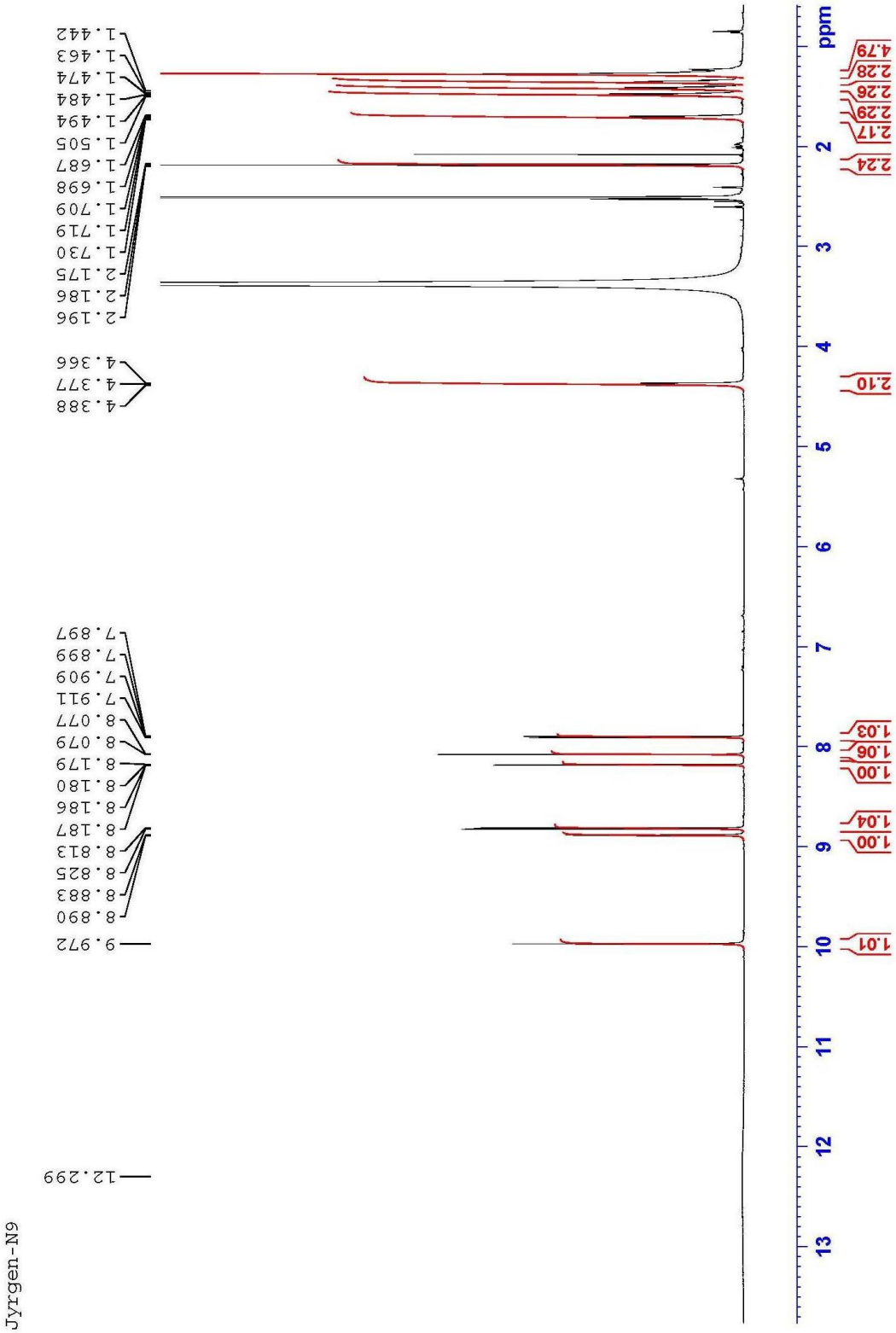
Appendices

APPENDIX 1. NMR spectrums of compounds **3a** and **3b**

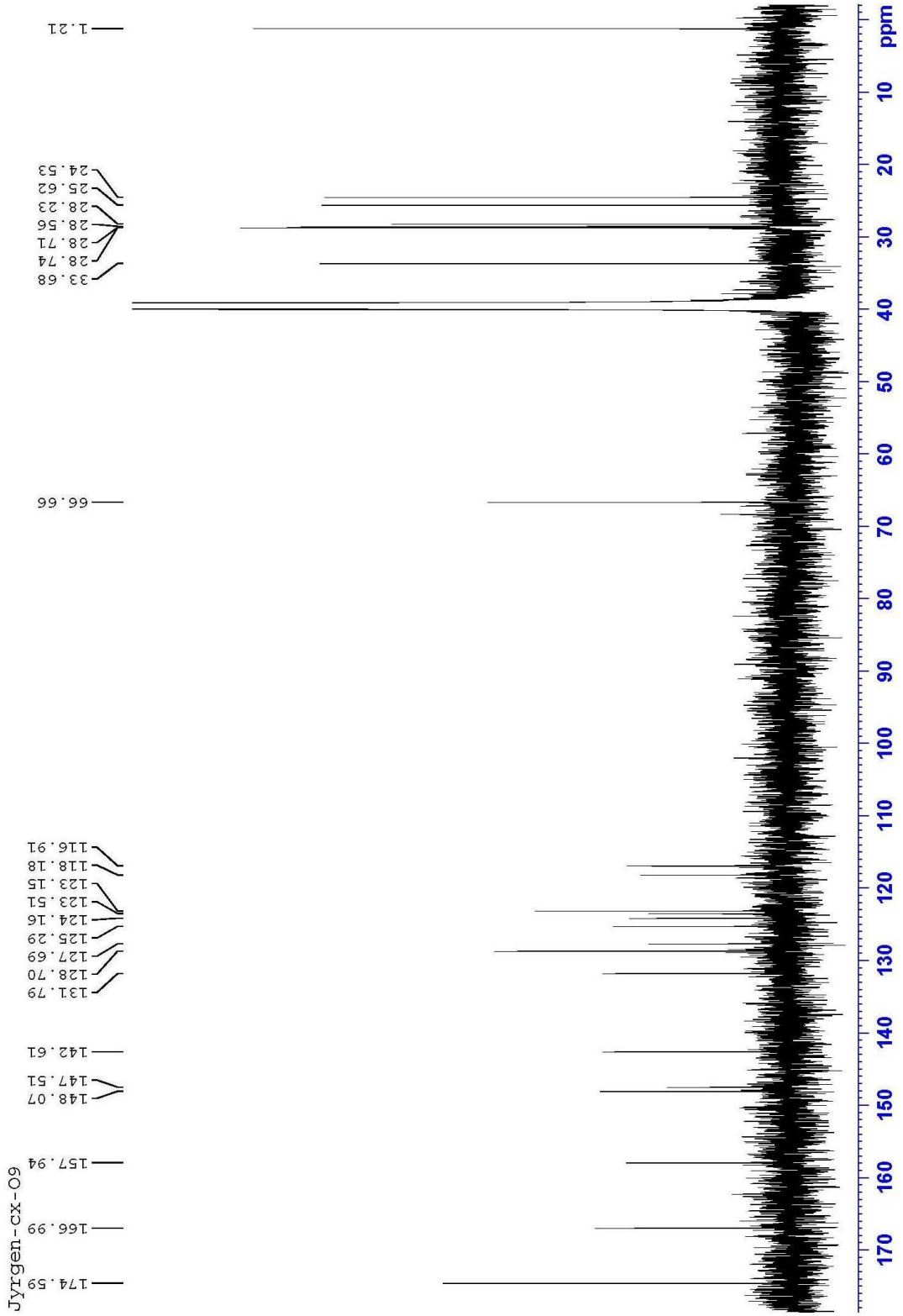
Compound **3b** ^{13}C NMR spectrum



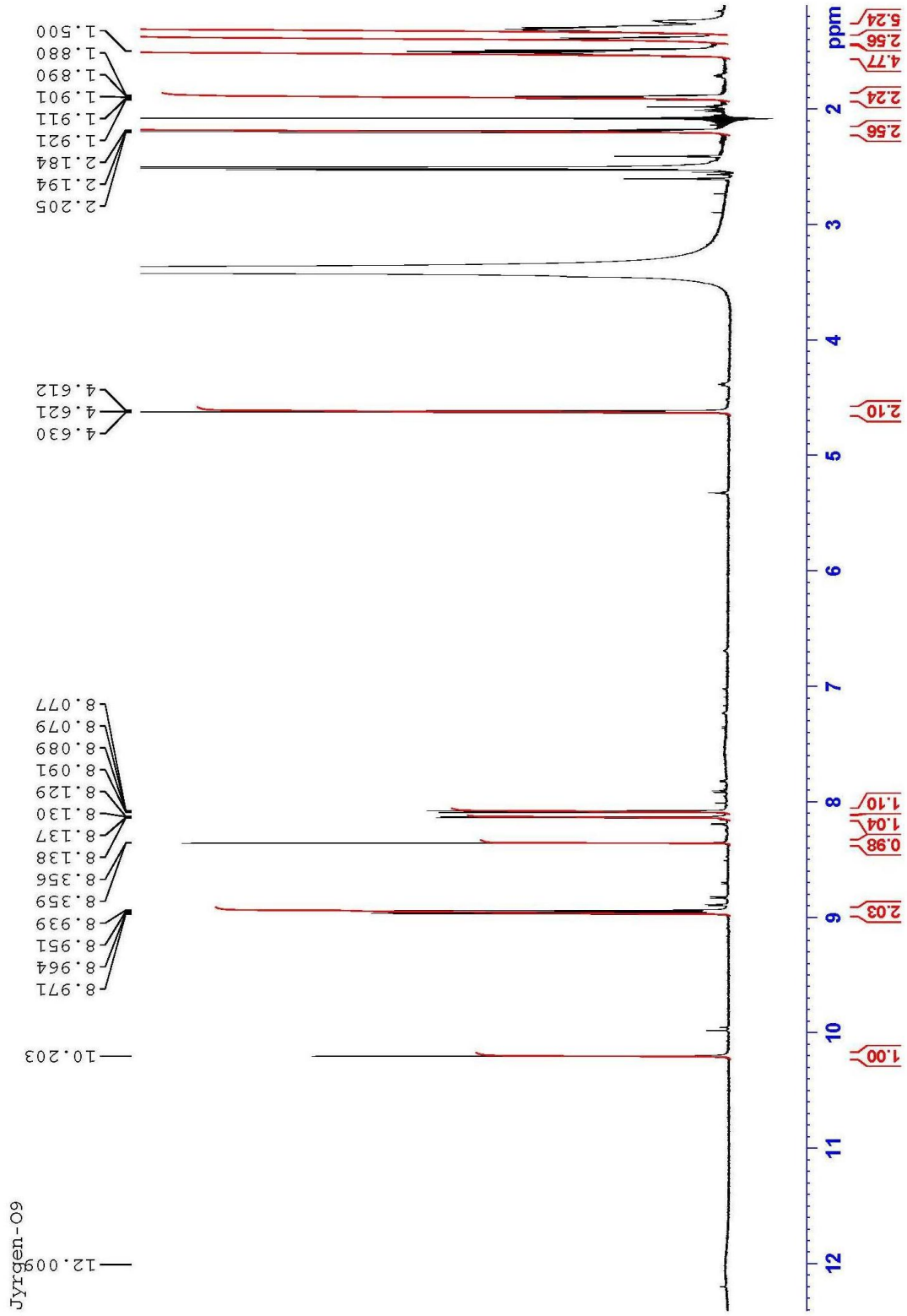
Compound **3b** ^{1}H NMR spectrum



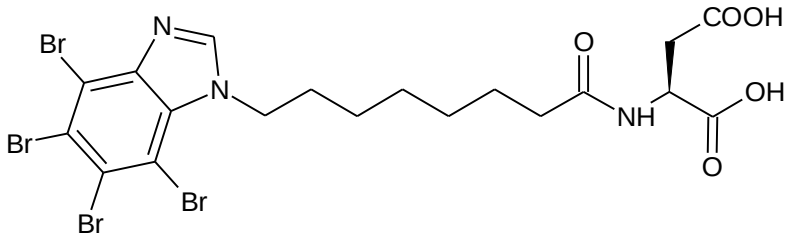
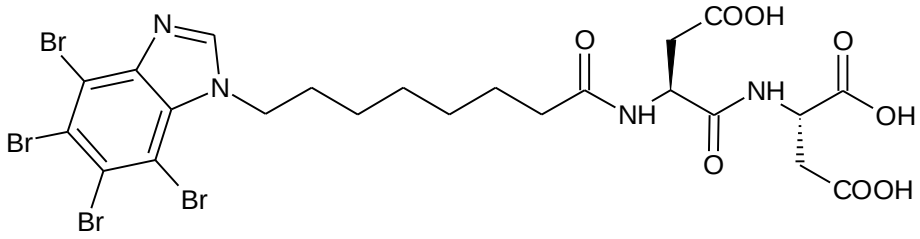
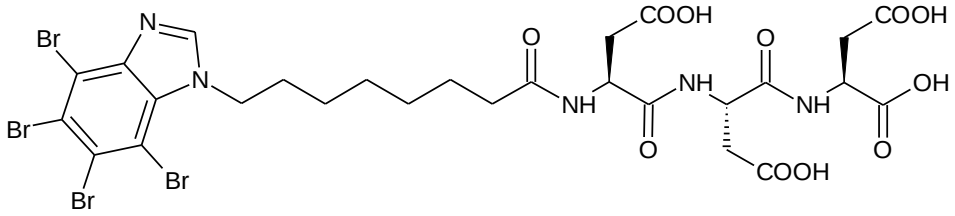
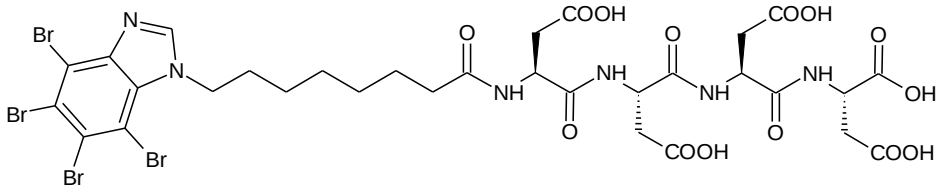
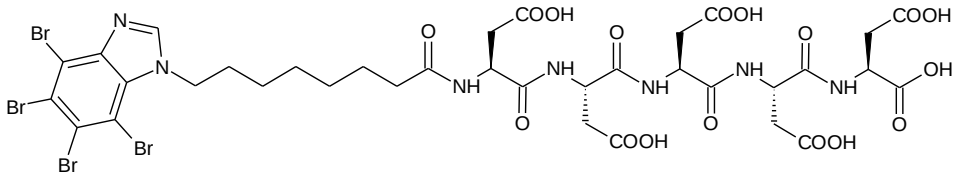
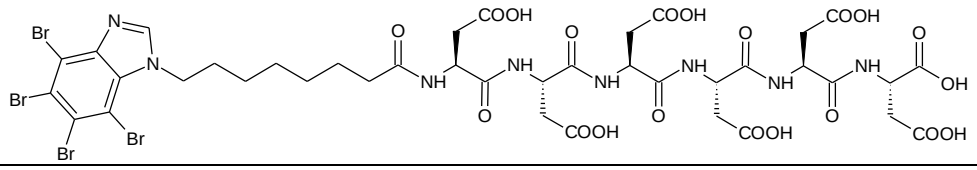
Compound **3a** ^{13}C NMR spectrum

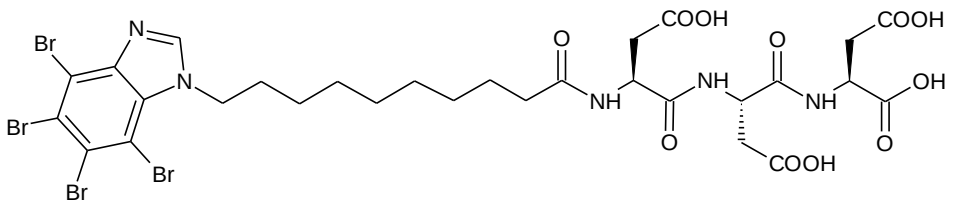
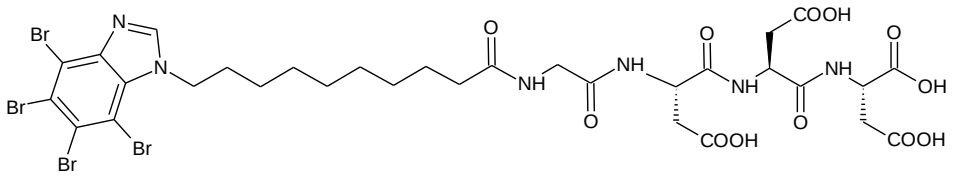
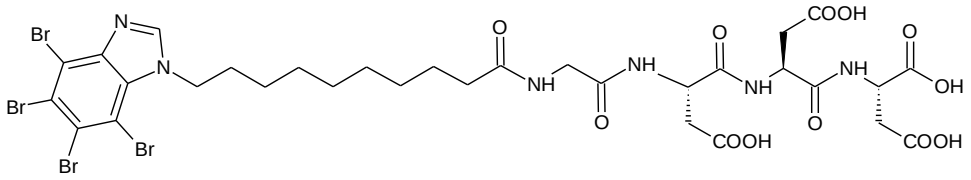
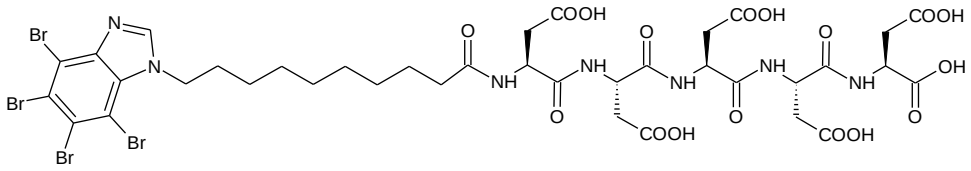
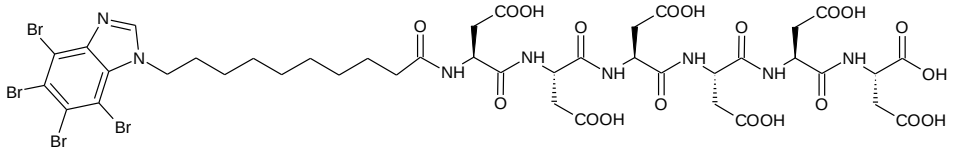
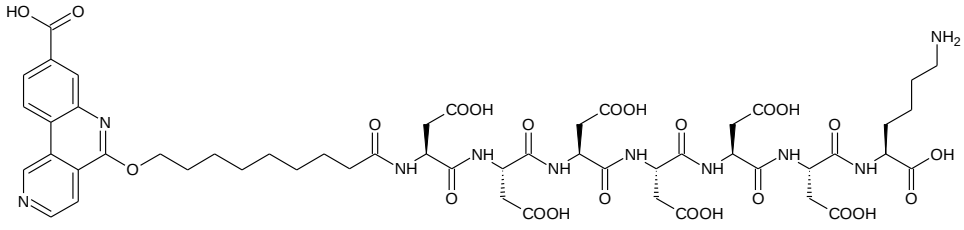


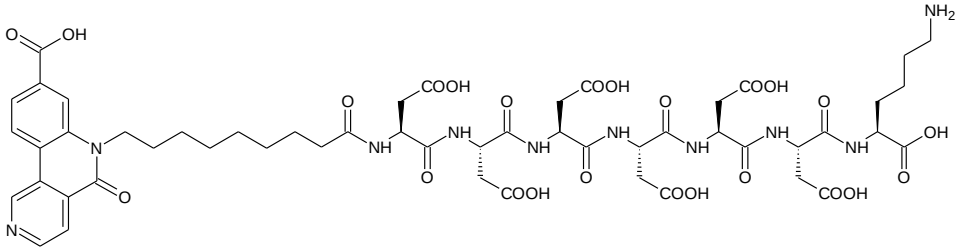
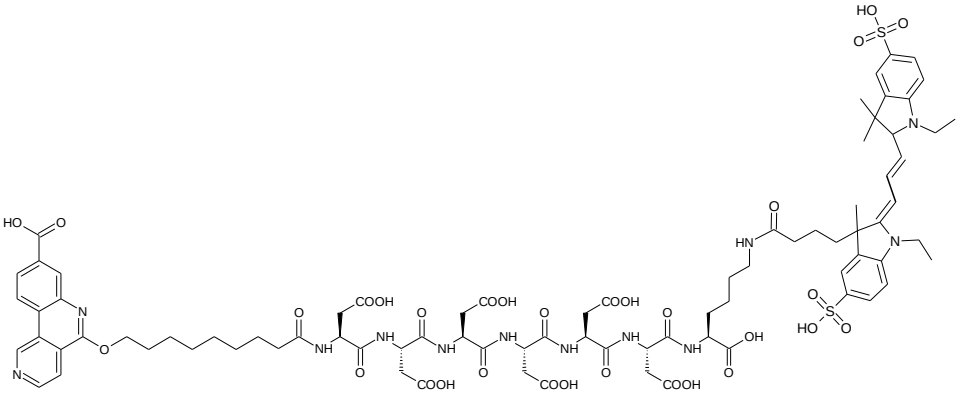
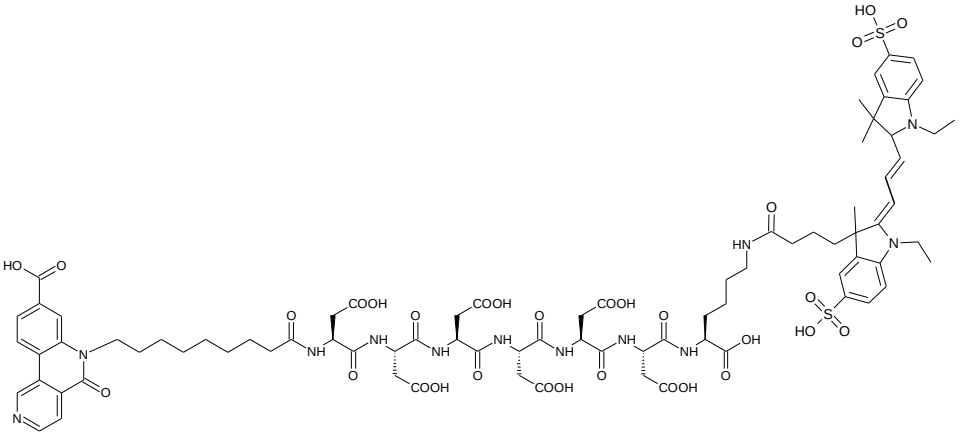
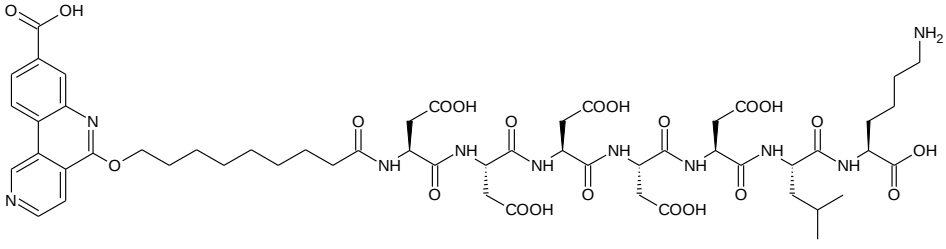
Compound **3a** ^{1}H NMR spectrum

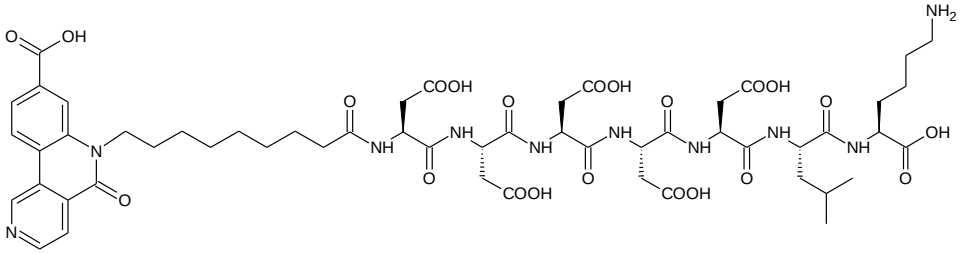
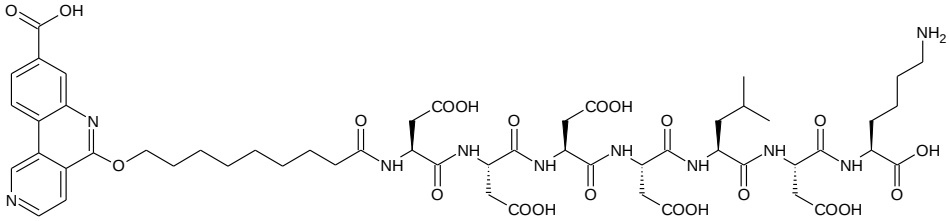
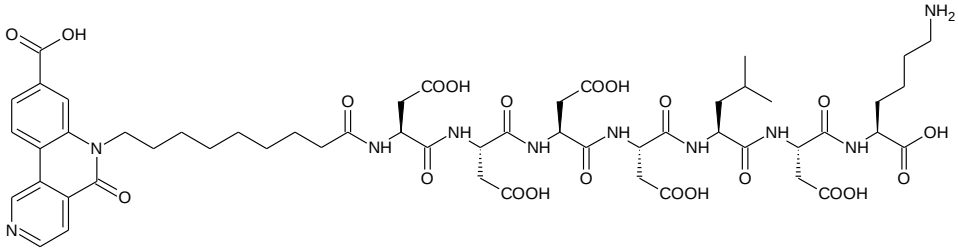
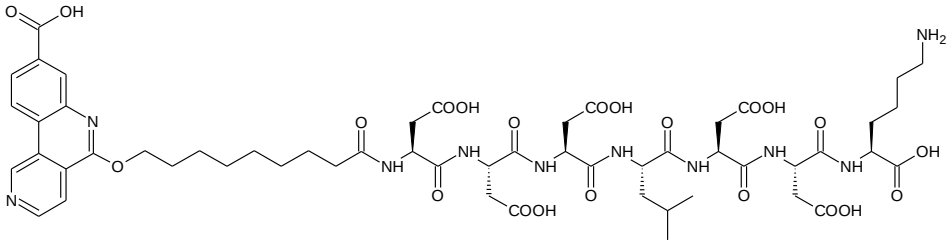
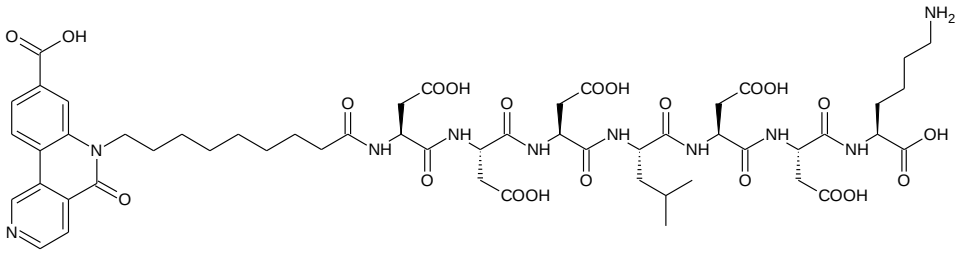


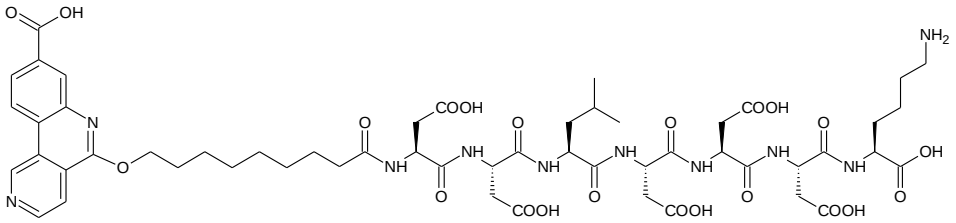
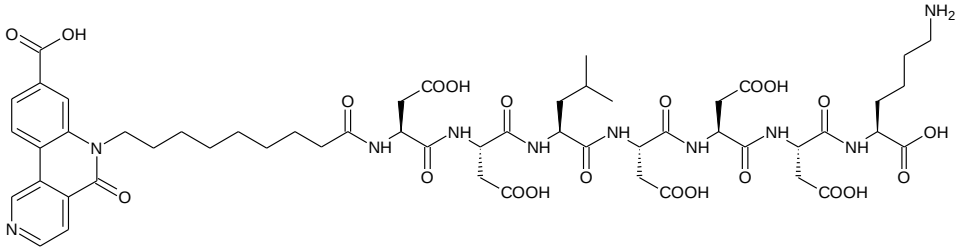
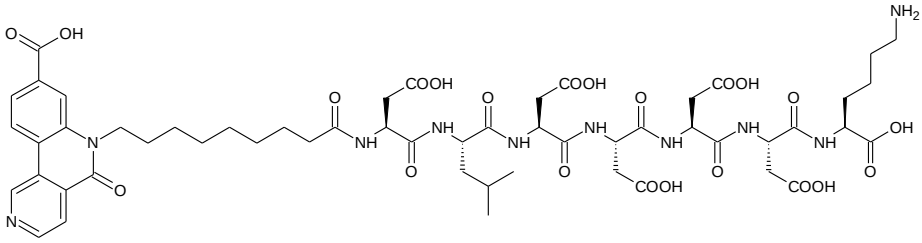
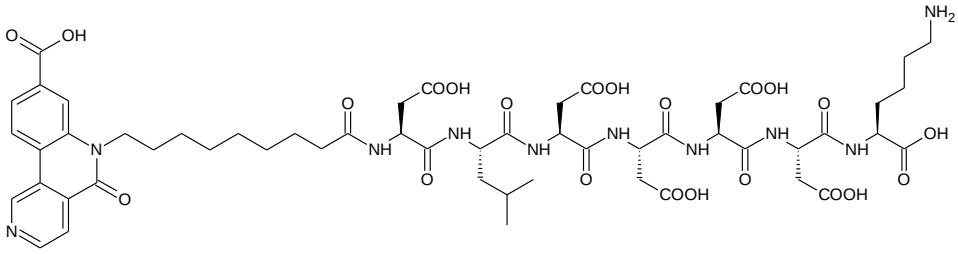
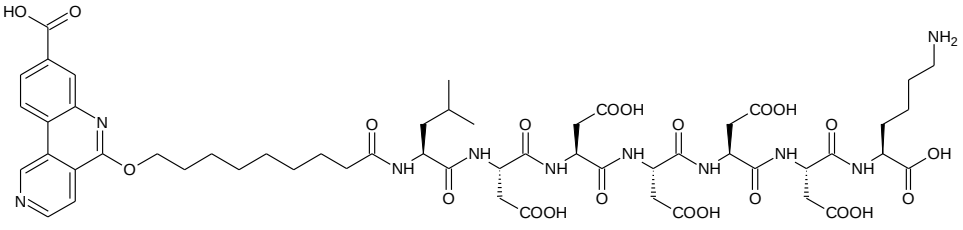
APPENDIX 2. Structures and molecular weights (m.w.) of new ARC-type inhibitors

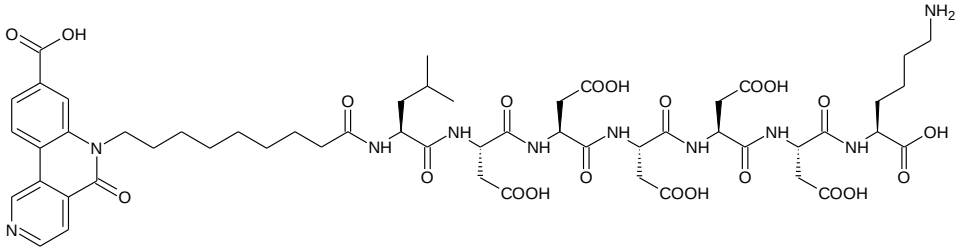
ARC code	Structure	M.w.
ARC-1506		690.0
ARC-1507		805.1
ARC-1508		920.2
ARC-1518		1035.3
ARC-1519		1150.4
ARC-1509		1265.5

ARC-1510		948.2
ARC-1511		1005.3
ARC-1512		1033.4
ARC-1514		1178.4
ARC-1515		1293.5
ARC-1424O		1215.1

ARC-1424N		1215.1
ARC-1513O		1815.9
ARC-1513N		1815.9
ARC-1520O		1213.2

ARC-1520N		1213.2
ARC-1521O		1213.2
ARC-1521N		1213.2
ARC-1522O		1213.2
ARC-1522N		1213.2

ARC-1523O		1213.2
ARC-1523N		1213.2
ARC-1524O		1213.2
ARC-1524N		1213.2
ARC-1525O		1213.2

ARC-1525N		1213.2

APPENDIX 3. Data of CK2 protein substrate sequence analysis

Number of different amino acids (AA) in different position

AA/position	n-4	n-3	n-2	n-1	n+1	n+2	n+3	n+4	n+5	n+6	n+7	n+8	n+9	n+10	SUM
A	15	16	16	18	4	13	6	19	16	17	13	23	12	12	200
C	4	1	2	2	0	2	0	3	2	1	3	3	0	3	26
D	33	32	34	64	108	75	89	59	63	43	43	30	26	29	728
E	48	51	45	52	77	70	152	67	65	54	49	40	39	32	841
F	8	6	7	7	3	1	0	4	5	5	7	6	9	4	72
G	25	27	14	24	23	17	7	18	16	13	19	12	17	1	233
H	5	10	4	3	0	2	0	4	3	4	3	4	1	4	47
I	9	5	6	7	0	5	1	13	7	9	5	11	3	6	87
K	9	6	10	6	1	3	1	4	7	19	12	5	12	10	105
L	17	18	15	22	8	10	1	14	14	13	18	12	13	4	179
M	4	5	8	6	4	2	3	4	6	2	6	1	3	4	58
N	15	10	11	8	2	10	2	7	6	4	7	7	5		94
P	14	16	25	10	2	7	1	6	13	12	14	20	21	16	177
Q	10	8	14	4	8	8	3	8	8	12	6	6	8	3	106
R	13	14	12	6	3	0	3	8	10	13	14	20	14	8	138
S	30	38	37	38	35	49	18	24	24	29	26	18	20	17	403
T	17	15	11	7	11	14	6	12	9	8	10	17	10	9	156
V	9	5	12	12	10	5	2	11	9	15	14	8	6	6	124
W	2	1	1	0	0	1	0	0	0	1	3	2	3	2	16
Y	6	10	12	6	4	5	2	7	6	9	7	6	4	4	88
No AA	12	11	9	2	3	7	9	14	17	23	27	55	80	132	401
SUM	305	305	305	304	306	306	306	306	306	306	306	306	306	306	