Chemical phosphoproteomics and development of bisubstrate based inhibitors of protein kinase C isozymes

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Chemical phosphoproteomics and development of bisubstrate based inhibitors of protein kinase C isozymes

Chemische fosfoproteomics en ontwikkeling van bisubstraat gebaseerde inhibitoren van protein kinase C isoenzymen

(met samenvatting in het Nederlands)

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Alex Johannes Poot

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Chapter 1

General introduction

1.1 Phosphoproteomics

1.1.1 Proteomics

With the unraveling of the human genome completed in 2001, it became clear that it contained between 20 000 – 25 000 genes instead of the estimated 100 000. Soon it was also concluded that not genes were really responsible for the complexity of organisms, but gene products especially proteins, which are encoded by the genome.[1-3]

The field where the proteome, that are all proteins encoded by the genome, is studied is called proteomics. Proteomics is much broader than the elucidation of all proteins encoded by the genome and it also comprises protein location, post-translational modifications, protein complexes and protein networks.[4,5] In eukaryotic organisms the majority of protein functions is regulated by post-translational modifications (Table 1). Since there are many different post-translational modifications it is a difficult task to unravel the complicated effects of the post-translational protein modifications. Therefore, various techniques have been developed aiming at a fast and reliable elucidation of the relation between the post-translational protein modifications and their function.[5]

Table 1: Examples of post-translational modifications of proteins, and the biological function of the respective modification.

Post-translational modification	Function
Phosphorylation	Enzyme activation and signaling
Acetylation	Regulation of protein-DNA interactions
Glycosylation	Cell-cell recognition
Sulfation	Protein-protein and receptor-ligand interactions
Ubiquitination	Protein destruction signalling
Methylation	Regulation of gene expression

1.1.2 Protein phosphorylation

Protein phosphorylation (Figure 1) was first observed in 1954 by Burnett *et al*. who reported the phosphorylation of casein by an enzyme present in rat liver mitochondria.[6,7] With the elucidation of the human genome it was shown that the group of protein kinases, responsible for protein phosphorylation, represented roughly 500 members.[8] It is estimated that almost half of the proteins encoded by the genome is phosphorylated at least once in their lifetime.[9] This makes it one of the most abundant post-translational modifications in eukaryotic cells, which mainly takes place at the hydroxyl functionalities of serine, threonine or tyrosine residues. It was estimated that the relative amino acid phosphorylation ratio was 90:10:0.05 for serine, threonine and tyrosine, respectively.[10] Later, Mann *et al*. published a comprehensive study towards the phosphoproteome *in vivo*, with the analysis of 2000 individual phosphorylated sites, which revealed that the relative abundance of pSer/pThr/pTyr is 86, 12 and 2%, respectively.[11]

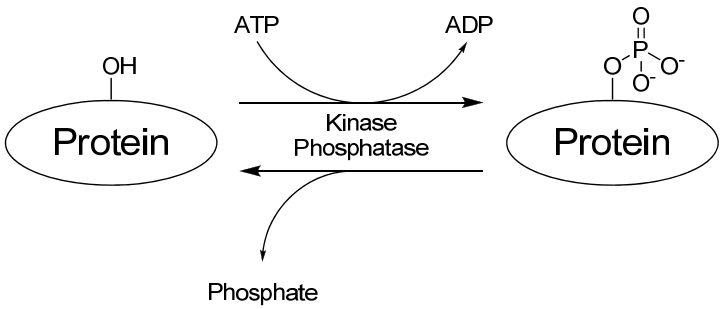


Figure 1: Schematic representation of protein phosphorylation by a kinase and the dephosphorylation by a phosphatase.

In general, phosphorylation is considered as a mechanism for either the activation or inactivation of proteins. The protein kinase substrates are involved in a large amount of cellular processes, and protein phosphorylation is essential for cell growth, proliferation and apoptosis. Uncontrolled protein phosphorylation is therefore accompanied by serious consequences, and can result in diseases like cancer, diabetes or Alzheimer's disease. Therefore, it is of major importance to elucidate the phosphoproteome and study how this post-translational protein modification is involved in different kinds of diseases.[12,13]

1.1.3 Phosphoproteome elucidation

Since protein phosphorylation plays such an important role in cellular processes, many different approaches have been used to study the phosphorylated proteins in more detail. Direct analysis of protein phosphorylation by mass spectrometry is difficult due to the low abundance of phosphorylated proteins. Also the negative charge of the phosphate moiety makes the direct measurement of these proteins by mass spectrometry not a trivial task. Therefore, different techniques have been developed for an improved analysis of the phosphoproteome. For tyrosine phosphorylation proper antibodies are available that allow selection and subsequent analysis of protein tyrosine phosphorylation from complex protein lysates.[14] For phospho-serine and -threonine containing proteins a similar tool exists, however the results are less satisfactory. Approaches for the specific investigation of serine and threonine phosphorylated proteins can generally be divided into the chemical modification approaches and the affinity-based methods.[15,16]

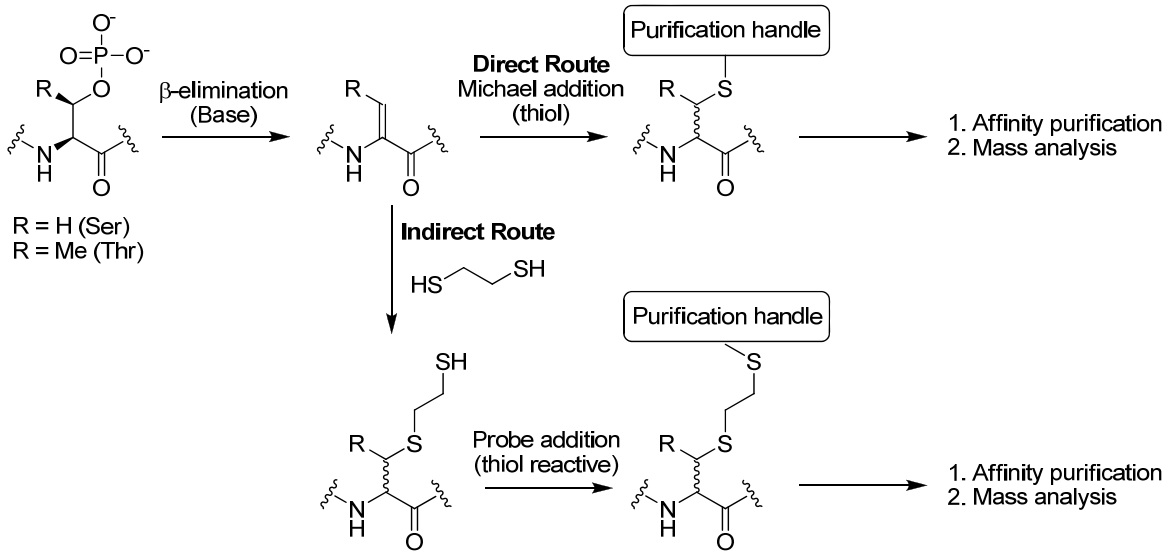


Figure 2: Chemical approach for the enrichment of phosphorylated peptides using β-elimination under basic conditions.

Chemical approaches:

These methods are mainly effective with Ser/Thr phosphorylated proteins, since these amino acid residues can undergo a β-elimination reaction.[17-19] Phosphorylated serine and threonine residues can be modified, since a phosphate group on these amino acid residues is base labile. After β-elimination a dehydro-amino acid residue is formed as a Michael

acceptor (Figure 2).[20] This β-elimination reaction has been frequently used to modify phosphorylated serine or threonine residues for enrichment purposes, and $Ba(OH)_2$ has been regarded as the most efficient base.[20] Other bases like NaOH and LiOH can be used for this reaction as well, however, the reaction rates are lower and the reaction needs more time to proceed. The resulting dehydro-amino acid is a Michael-acceptor and can be modified using sulfur nucleophiles.[21-23] Regarding the Michael addition, two different approaches have been published. In the first approach the direct addition of a thiol containing purification handle is used, since this will result in less reaction steps prior to the purification and analysis of the modified phospho-amino acid.[24] However, the use of this direct addition of purification handles often was rather disappointing, since the Michael addition is a rather slow reaction and a large excess of the thiol is required to drive the reaction to completion. Moreover, these thiol containing purification handles are relatively large molecules that cannot be easily removed, which hampers analysis by mass spectrometry.

Therefore, a second, two step approach has been used more often, in which EDT is reacted with the dehydro-derivative of the first approach.[22,23,25-27] After evaporation of the excess of EDT, a highly reactive maleimide- or iodine-functionalized purification handle can then be added to react with the thiol. This approach yielded much better results. It should be noted that in more complex mixtures all cysteine residues need to be capped with, e.g. iodoacetamide, prior to modification of the formed dehydro-amino acids with a dithiol, to prevent undesired side reactions. Later, this method has been optimized even further using solid supports and the incorporation of an acid-cleavable linker to lower the detection limit towards the picomolar range.[28,29] This method was proven to be very reliable for the detection of phosphorylated peptides, however, the analysis of phosphorylated proteins in complete cell lysates proved to be very challenging using this technique. This is due to the small amounts of phosphorylated proteins present in cell lysates, and the relative large number of chemical steps that have to be performed in quantitative yields prior to analysis by mass spectrometry. Furthermore, another challenge in optimizing this method for the enrichment of phosphorylated peptides from peptide mixtures is the issue of selectivity with respect to *O*-glycosylated peptides, which has not been addressed before. The β-elimination

reaction under alkaline conditions was not only described for phosphoproteins, but was also used for analysis and identification of *O*-glycosylated peptides.[30,31] Therefore, it would be of great value if the β-elimination of phosphorylated peptides could be performed selectively in the presence of *O*-glycosylated peptides.

Affinity-based methods:

The second method for the identification of phosphorylated peptides from complex cell lysates is the use of affinity-chromatography, which is often directly on-line connected to a mass spectrometer.[15] The use of immobilized metal affinity chromatography (IMAC) was first reported by Anderson *et al*. in 1986. In this technique metal ions like Fe^{3+}, Ga^{3+}, Al^{3+}, Zr^{3+} are immobilized onto the porous column material. Phosphorylated peptides show high affinity towards these metal ions and will be eluted later than non-phosphorylated peptides.[32,33] The best result, where IMAC is applied on phosphoproteomics, was published by White *et al*, who identified 216 phosphorylated peptides from a yeast lysate.[34] Though, IMAC was a promising method, a higher selectivity and affinity for the identification of phosphopeptides was desirable. Therefore, in 1997, TiO_2 spheres were reported as column material for the purification of phosphopeptides.[35] Phosphate-groups show a high affinity towards TiO_2 and can therefore be separated from non-phosphorylated peptides. Pinkse *et al*. demonstrated the power of this technique by reporting the use of a TiO_2-column in a LC-MS/MS setup, where phopshopeptides could by detected in a femtomolar concentrations.[36] Nevertheless, it should be noted that highly acidic peptides, containing multiple glutamic acids or aspartic acids, show affinity for this kind of column material as well. Therefore, Larsen *et al*. optimized the use of this method by the addition of 2,5-dihydroxy benzoic acid to the eluent system, which efficiently competes with the binding of acidic amino acids towards the TiO_2 spheres. The use of an appropriate concentration of 2,5-dihydroxy benzoic acid increases the performance of this method significantly.[37] Another method for the enrichment of phosphopeptides is the use of strong cation exchange (SCX) chromatography. Here the separation of phosphorylated peptides over other non-phosphorylated peptides is based on the peptide charge at a certain pH. At pH = 2.7 peptides in tryptic digests contains a charge of 2+, since all carboxylic acids in the peptide as well as the *N*-terminal amine and *C*-terminal lysine or argine are protonated. On

the other hand, a phosphate in the peptide sequence is not completely protonated and contains a charge of 1-, which brings the net charge of the peptide at 1+. This difference in charge yields the possibility to separate both peptides.[38] However, it is reported that peptides containing multiple phosphates might be lost, since these peptides have even a different charge and elution time. Therefore, SCX has been combined with previously described IMAC or TiO_2 columns, and the combination of these two analysis methods resulted in a powerful and robust system for the analysis of the phosphoproteome.[11,39-41]

1.2 The role of protein kinase C and approaches for selective inhibition

1.2.1 Protein kinase C (PKC)

The human kinome comprises over 500 members and is thereby one of the largest protein families known to date.[8] Protein kinases play important roles in a wide variety of cellular processes like signal transduction, metabolism, transcription, cell cycle progression, differentiation and apoptosis. Protein kinases phosphorylate the serine, threonine or tyrosine amino acid residues in eukaryotic cells by the consumption of ATP, and the transfer of a phosphate group to the suitable hydroxy amino acid containing peptide. The large group of protein kinases is divided into four major subclasses, namely the AGC (for protein kinase A, G, and C), the CaMK (for calmodulin-dependent protein kinases), the CMGC kinases (for cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases, and CDK-like kinases) and finally the PTK (for protein tyrosine kinases). Other kinases are assigned to three smaller kinase-families, namely PTK-like (for protein tyrosine-like kinase), CK1 (casein kinase 1), and STE (for Ste or MAP-kinases).[42] The classification of the protein kinases in subfamilies is based on the amino acid sequence homology of the enzymes. This classification of the protein kinases also reveals substrates to be phosphorylated and their substrate specificity.

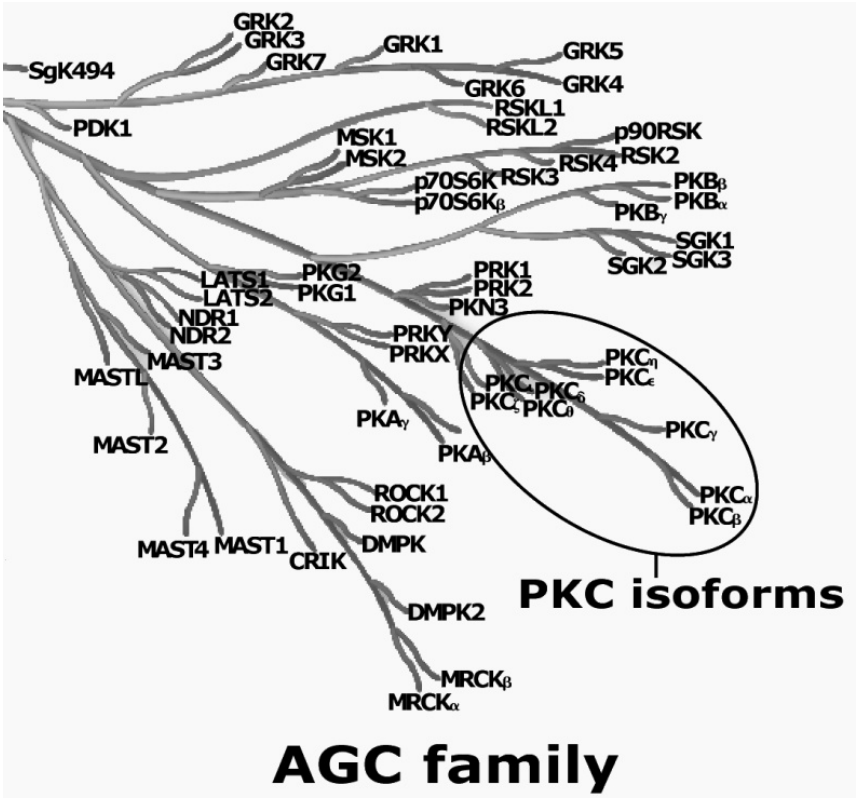


Figure 3: The kinases of the AGC family displayed in a phylogenetic tree, where highly homologous kinases are positioned in nearby braches of the tree. Reprinted with permission of AAAS.[8]

The protein kinase C subfamily is part of the AGC family (Figure 3). The AGC family name is derived from its most abundant family members, which include cyclic AMP-dependent protein kinase A, cyclic GMP-dependent protein kinase G and phospholipid-dependent protein kinase C.[43] Members of the AGC family are known to be involved in the control of critical cellular processes, such as cell growth, cell differentiation and cell survival. The kinases in this family phosphorylate serine and threonine residues.

The discovery of PKC was reported in 1977 by Inoue *et al.* They identified a novel protein serine/threonine kinase from bovine brain that was Ca^{2+} dependent.[44,45] To date, the PKC subfamily contains 11 members and these kinases show high homology at the amino acid level. The PKC subfamily is divided into three different groups depending on the co-factors required for enzyme activation (Figure 4). Firstly, PKCα, βI, βII and γ belong to the classical PKCs, and activation of these enzymes is both lipid- and Ca^{2+}-dependent. Secondly, PKCδ, ε, η and θ belong to the novel PKCs, and activation is only lipid-

dependent. Finally, PKCζ, μ and ι belong to the atypical PKCs, and the activity of these enzymes is lipid- and calcium-independent.[46-50]

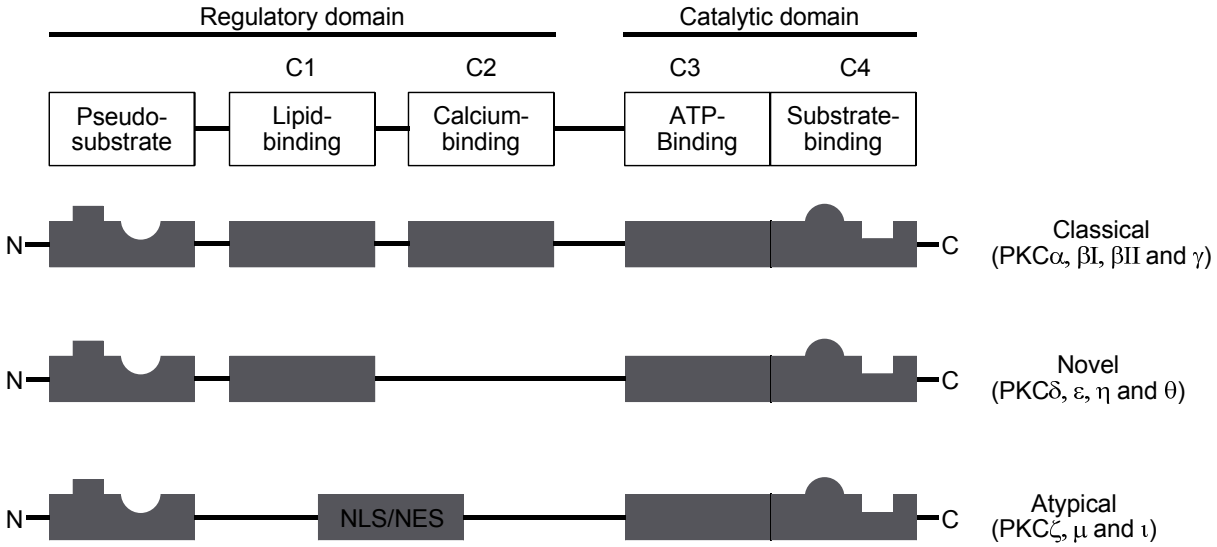


Figure 4: Schematic representation of the regulatory and catalytic domains of all PKC isoforms. Reprinted with permission of Nature Publishing Group.[46]

Activation of the PKC isoforms is the result of a series of actions that takes place in the enzyme (Figure 5). All kinases in the PKC-family consist of a regulatory and a catalytic domain. At the N-terminus a pseudosubstrate region is located, which is an amino acid sequence with multiple basic residues, which is well recognized by the substrate binding domain (Figure 4). In the inactivated form of the kinase these two regions interact with each other preventing the phosphorylation of natural PKC substrates. Classical and novel PKCs have a C1 domain, which is a lipid-binding domain that displays two zinc-finger motifs, C1a and C1b. These two zinc-fingers are highly conserved and bind diacylglycerol (DAG) to the enzyme. The binding of DAG to this region is important for the localization of PKC, which then diffuses to the lipid bilayer. As a result, it becomes anchored to the membrane before other lipids bind the C1 domain (Figure 5). C-Terminally to the C1 domain, a C2 region is located, which is only displayed by classical PKCs. The C2 domain binds Ca^{2+}, which occurs prior to activation of these enzymes (Figure 4). Since atypical PKCs lack the complete C1 and C2 domains the activation of these enzymes is independent of DAG and Ca^{2+}. Though the precise activation mechanism is not known, it is expected

that activation of atypical PKCs is regulated by nuclear localization signals (NLS) and nuclear export signals (NES) (Figure 4).[46-50]

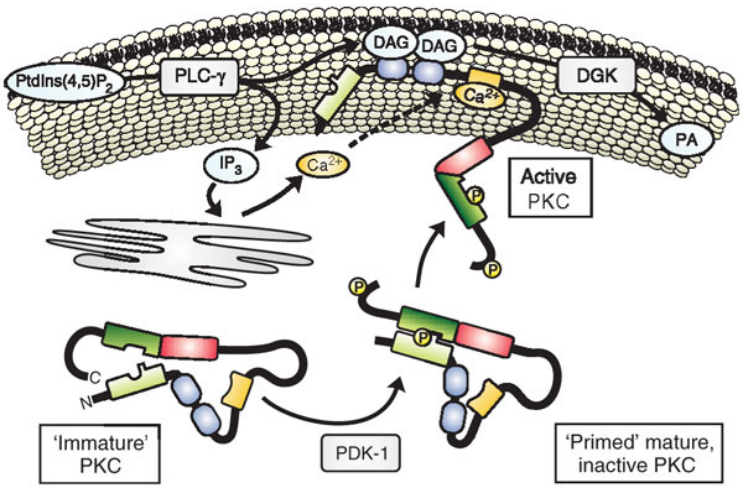


Figure 5: Activation cycle of classical- and novel-PKCs, where mature PKC is first phosphorylated. Subsequently, DAG binds to the enzyme for activation and localization at the cell membrane. In case of the classical PKCs, Ca^{2+} has to bind to the regulatory domain prior to activation. Reprinted with permission of Nature Publishing Group.[46]

PKC isozymes play important roles in the cell, and typical substrates of these enzymes are involved in processes involving growth factor receptors, ion channels and transcription factors. The amount of substrates phosphorylated by PKC is large and can be retrieved from freely accessible databases.[51,52] When sequences, which are phosphorylated by PKC, are aligned a highly basic consensus phosphorylation motif of RXXS/TXRX is found, where X can be any amino acid and R stands for arginine, while S and T represent the serine and threonine phospho-acceptor amino acid, respectively.[53] Cantley *et al.* confirmed this general consensus sequence for a majority of the PKCs experimentally[54], however when the optimal consensus sequence was determined for each individual isozyme, small differences were found, which forms the rationale for the design of PKC-selective pseudosubstrates, *vide infra*. Though this research gave a good overview of the sequence specificity of single PKC isozymes, it has to be noted that the optimal motifs are determined for 8 of the 11 isoforms.[54]

Table 2: The optimal peptide sequences that are recognized by each individual PKC isoform.[54]

PKC isozymes	Position												
	-7	-6	-5	-4	-3	-2	-1	**0**	1	2	3	4	5
PKCα	R	R	R	R	R	K	G	**S**	F	R	R	K	A
PKCβI	F	K	L	K	R	K	G	**S**	F	K	K	F	A
PKCβII	Y	K	L	K	R	K	G	**S**	F	K	K	K	A
PKCγ	R	R	R	R	R	K	G	**S**	F	K	R	K	A
PKCδ	A	R	R	K	R	K	G	**S**	F	F	Y	G	G
PKCε	Y	Y	X	K	R	K	M	**S**	F	F	E	F	F
PKCη	A	R	R	R	R	R	R	**S**	F	R	R	X	R
PKCζ	R	R	F	K	R	Q	G	**S**	F	F	Y	F	F

Since it was recognized that PKC isozymes play such a major role in vital cellular processes it became an important drug target in cancer research. Many excellent reviews have been written about PKC isozymes, which highlight their role in certain cancer cell lines or tumor types.[55-61] A striking example is the function of PKCθ, which is related to the development of human T cell leukemias.[62,63] Since the influence of PKC isozymes in the development of tumors is significant, PKC has become a major drug target and research has focused on the development of isozyme selective inhibitors.

1.2.2 General aspects of kinase inhibition

The important role of kinases in all cellular processes has led to the development of this class of enzymes to become a major drug target. However, the synthesis of selective kinase inhibitors has been proven to be difficult since these enzymes show great sequence homology, especially of their catalytic domains. For the inhibition of kinases in the catalytic domain in principle three strategies can be used, firstly, targeting the ATP-binding site (Figure 6A),[64] secondly, targeting the substrate-binding site (Figure 6B)[65] or thirdly, the design and synthesis of bisubstrate inhibitor (Figure 6C).[66]

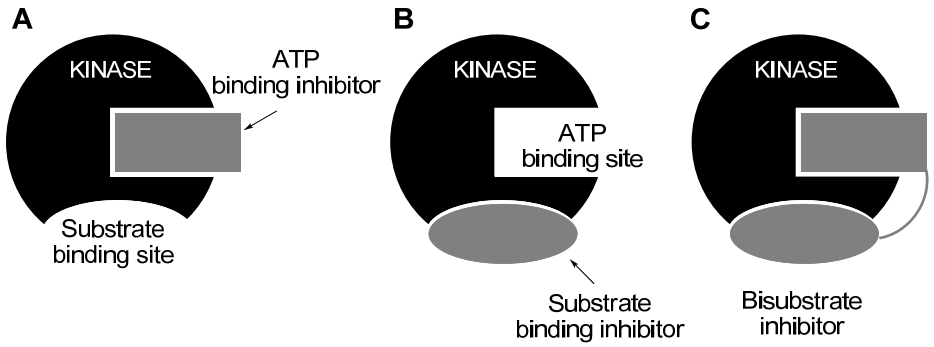


Figure 6: Schematic representation of kinase inhibitors that act on the catalytic kinase domain with **A**: an ATP competitive inhibitor, **B**: a substrate competitive blocker, **C**: a bisubstrate inhibitor binding both the ATP and substrate binding site.

ATP competitive kinase inhibition has been the most widely applied strategy for the development of highly potent and moderatly selective kinase inhibitors.[61,67,68] A major obstacle in applying this strategy is that the ATP binding site is one of the highly conserved regions within the group of kinases, and thus highly similar in each particular enzyme subclass. Therefore, despite great efforts, the design and synthesis of this kind of inhibitors that selectively interact with the ATP binding pocket has been proven to be very challenging (Figure 8).[69,70] Nevertheless several potent kinase inhibitors have reached the clinic and are FDA-approved for the treatment of cancer (Figure 7).[71,72]

Gleevec® (imatinib) was the first small molecule protein kinase inhibitor that was approved by the FDA for the treatment of chronic myelogenous leukemia, and targets the Abelson tyrosine kinase (ABL).[13] In chronic myelogenous leukemia the Abelson tyrosine kinase fuses with the breakpoint cluster region protein, resulting in a chromosomal rearrangement followed by the uncontrolled activation of the MAP kinase cascade. After the approval of Gleevec, Sprycel® (dasatinib) and Tasigna® (nilotinib) were also approved, these compounds target the Abelson tyrosine kinase as well (Figure 7).[71] Another tumor target for which Iressa® (gefitinib), Tarceva® (erlotinib) and Tykerb® (lapatinib) have been approved is the epidermal growth factor receptor (EGFR).[71] The EGFR is a growth factor receptor, and over-activation of this receptor leads to uncontrolled cell growth and proliferation. Sutent® (sunitinib) and Nexavar® (sorafenib) are two FDA-approved drugs

that inhibit the vascular endothelial growth factor receptor (VEGFR).[71] The stimulation of the VEGFR leads to angiogenesis of tumor tissue, which is required to supply the tumor with e.g. nutrients and oxygen. Initially, most kinase inhibitors that have been approved by the FDA have been were presented as selective binders to one or more target kinases. Although it should be noted that in several publications, in which a series of protein kinase inhibitors was tested against a panel of 100 to 300 kinases, it was demonstrated that not all approved kinase inhibitors were selective inhibitors.[70,73]

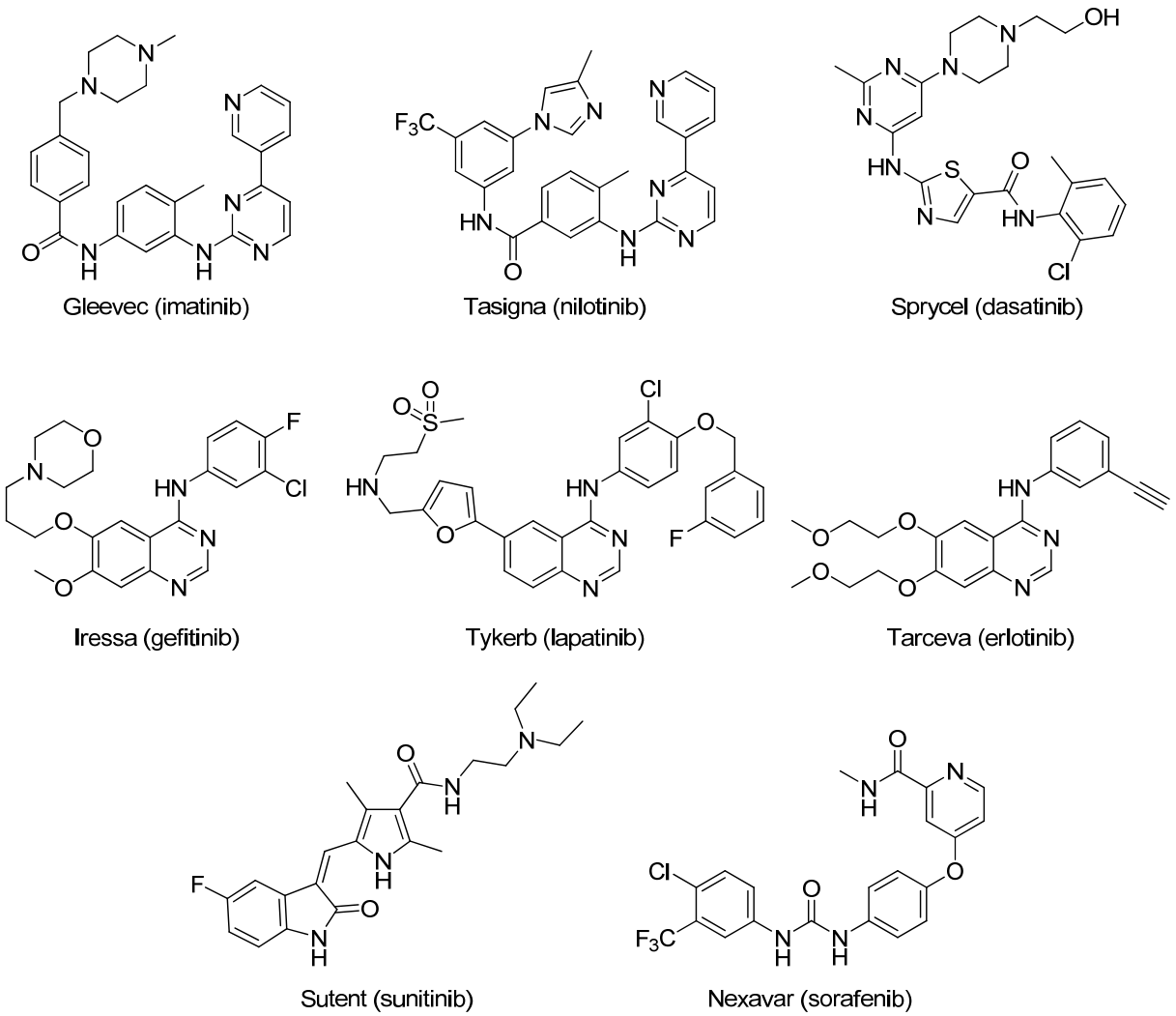


Figure 7: FDA-approved small molecule kinase inhibitors.[71]

1.2.3 Protein kinase C inhibition

In 1977, staurosporine **1** was identified as a microbial alkaloid produced by *Streptomyces staurosporeus*, and in 1986 it was discovered to be a highly potent PKC inhibitor.[74,75] Therefore, since the affinity for PKC was very high, staurosporine was used as lead structure for the development of PKC inhibitors in many studies.[64,76,77] However, inducing selectivity of staurosporine for a single protein kinase remained a huge challenge, due to the ability of staurosporine to inhibit at least half of all protein kinases known at that moment. Therefore, many analogues of staurosporine have been synthesized with varied success. Kleinschroth *et al*. published a series of staurosporine analogs lacking the glycosidic moiety e.g. **2** (Figure 8).[78] These compounds displayed equal affinity for PKC as staurosporine. Moreover, the selectivity of these compounds was improved, with **2** as the most typical example. Staurosporine analog **2** showed an increased selectivity for PKC over other protein kinases of at least 5000-fold. Though selectivity was obtained in the inhibition of PKC over other protein kinases the biggest challenge was to develop an inhibitor that was selective for a single PKC isoform. In 1996, Ishii *et al*. published a series of simplified staurosporine analogs, with **3** as the most active inhibitor, which showed comparable affinities toward PKC compared to **2**. However, **3** was found to have an even better specificity profile for PKC isoforms with a 100 fold selectivity toward PKCβ over other PKC isozymes (Figure 8 and Table 3).[79]

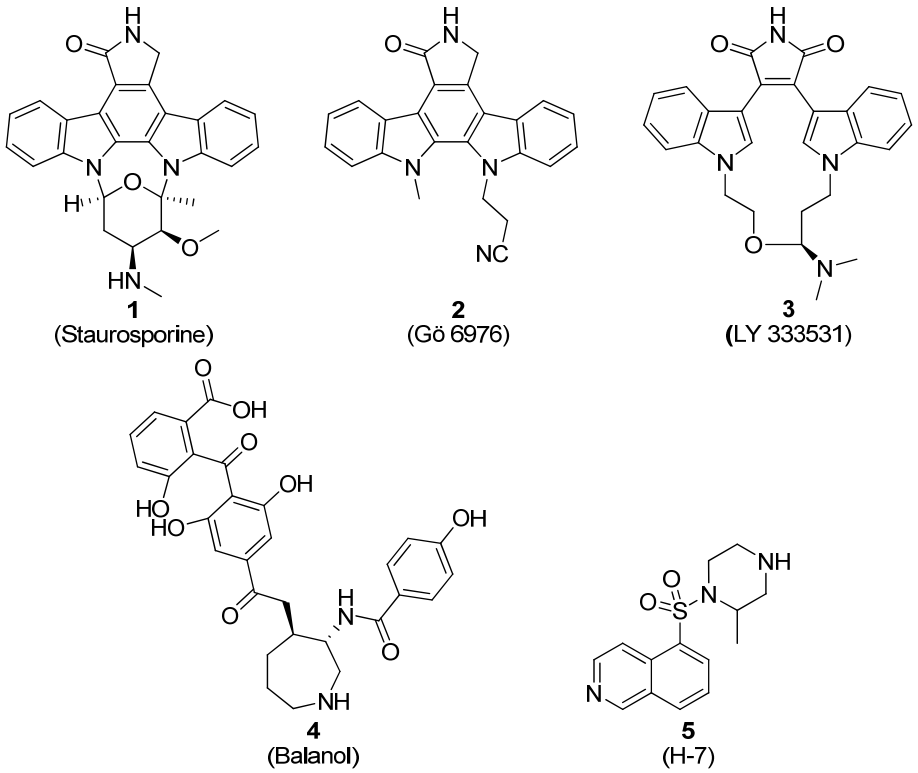


Figure 8: Potent ATP-competitive inhibitors acting against PKC.[64,78-81]

Table 3: The activity of selective PKCβ inhibitor **3** (LY333531) and broad spectrum kinase inhibitor **1** (Staurosporine).[79]

Kinase	IC_{50} (μM)	
	1 (Staurosporine)	**3** (LY 333531)
PKCα	0.0045	0.36
PKCβI	0.023	0.0047
PKCβII	0.019	0.0059
PKCγ	0.11	0.3
PKCδ	0.028	0.25
PKCε	0.018	0.6
PKCη	0.005	0.052
PKCζ	> 1.5	> 100
PKA	0.1	> 100
Ca^{2+}-calmodulin kinase	0.004	8.0
Casein kinase	14	> 100
Src tyrosine kinase	0.001	> 100

Other classes of ATP competitive inhibitors for PKC are **4** (balanol) and isoquinoline-sulfonamides derivative **5** (H-7) (Figure 8). Compound **4** is a fungal metabolite derived from *Verticillium balanoids* and shows high affinity towards PKC.[80] Though the initial affinity and selectivity of **4** toward PKC was very promising it turned out that the design of PKC isozyme selective inhibitors based on balanol as lead structure remained difficult.[82,83] Moreover, several balanol analogs published by Lampe *et al*. proved to be more selective toward PKA than PKC.[84] Since natural compounds are relatively difficult to obtain via purification or synthesis, other more easily accessible lead compounds like isoquinoline-sulfonamide **5** have been synthesized and tested as PKC inhibitors (Figure 8). Unfortunately, this class of kinase inhibitors displays a relatively low affinity towards their target, typically in the low μM range. Furthermore, the selectivity of **5** was poor, and besides PKC, other closely related kinases from the AGC family were inhibited as well with similar affinity.[81]

The substrate binding cleft is another target that can be used in the development for designing selective kinase inhibitors. Since all kinases contain a pseudosubstrate sequence in their regulatory domain, which is PKC's natural inhibitor to prevent protein phosphorylation, it is a good starting point in the development of substrate-competitive inhibitors.[85-87] Kemp *et al*. demonstrated this by determining the inhibitory constant of the PKC pseudosubstrate, Arg-Phe-Ala-Arg-Lys-Gly-*Ala*-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn, representing the sequence that is used by PKC for self-inhibition and this pseudosubstrate has an affinity of 147 ± 9 nM.[88] Though, the affinity for PKC is high, nothing is said with respect to the selectivity of this peptide sequence for single PKC isozymes. Later on, Eichholtz *et al*. reported the effective use of peptide sequences derived from the regulatory pseudosubstrate domains of PKCα and β.[89] These peptides were myristoylated at the N-terminus, which yielded a 100-fold increase in affinity compared to the unmodified peptide. The affinity observed was 7-8 μM, and it is suggested, since the peptide sequence is derived from PKCα, that there might be selectivity in inhibition between the PKC isozymes.[89] Another example where PKC pseudosubstrates were used was published by Lee and coworkers, who used the optimized pseudosubstrate **6** (shown in Figure 9) for the inhibition of PKCα, based on the peptide sequences published by Cantley

et al.[54,65] Via a combinatorial approach, four libraries were synthesized of which the most active compound was further optimized. The best peptide pseudosubstrate inhibitor **7** showed a significant increase in affinity toward PKC with K_i = 0.8 ± 0.25 nM (Figure 9). Moreover, the selectivity observed was striking, with a high preference for PKCα versus the closely related PKCβ and γ of more than 350-fold. The selectivity for PKCα versus other structurally more different PKCs was between 600-fold and 2700-fold.[65] Therefore, this pseudosubstrate approach in the development of highly potent and selective kinase inhibitors shows great potential. However, it should be noted that the use of peptides in kinase inhibitor development is still highly challenging, since peptide bonds in general are easily hydrolyzed *in vivo*, which might result in reduced half-life times of this class of inhibitors. Therefore, the development of highly stable substrate competitive inhibitors is still an emerging field of research.

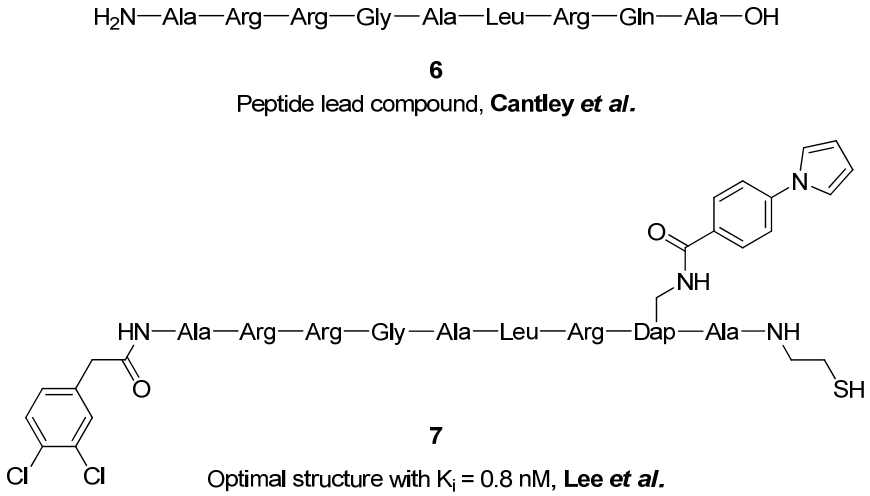
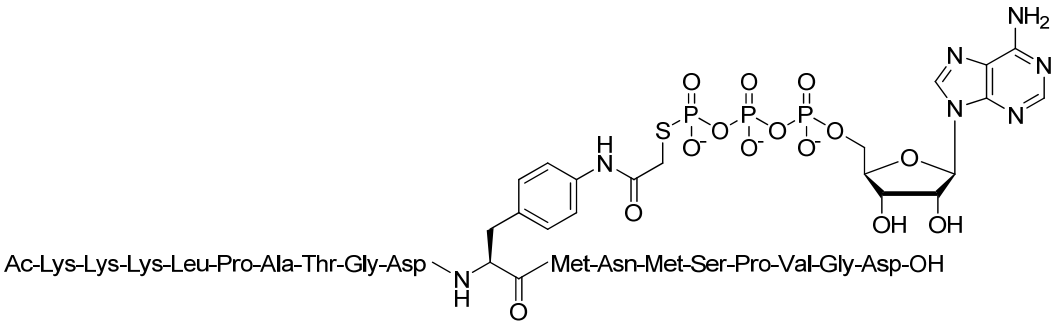


Figure 9: Substrate competitive inhibitors for PKC.[54,65]

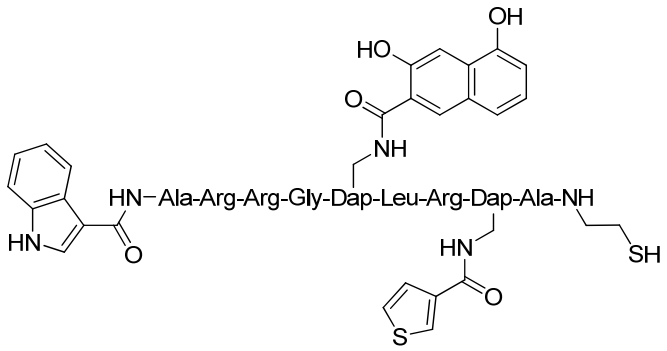
A third approach for developing selective inhibitors is the development of bisubstrate analog inhibitors targeting both the ATP and substrate binding site of the catalytic domain.[90] This method is a well known and elegant strategy to increase the potency and selectivity of enzyme inhibitors.[91] According to Parang *et al*. the ideal bisubstrate inhibitor should be designed with four characteristics.[91] Firstly, it should interact with both binding pockets. Secondly, it should be designed based on the mechanism of binding of the natural substrates of the target protein kinase. Thirdly, the molecule should be a selective inhibitor, which is far from trivial since many protein kinases display highly homologous catalytic

domains. Finally, the ideal molecule should show good bioavailability and low toxicity. Parang *et al*. demonstrated this approach of bisubstrate inhibitors by the design and synthesis of IRK inhibitor **8**, which is a protein tyrosine kinase inhibitor (Figure 10).[91-93]



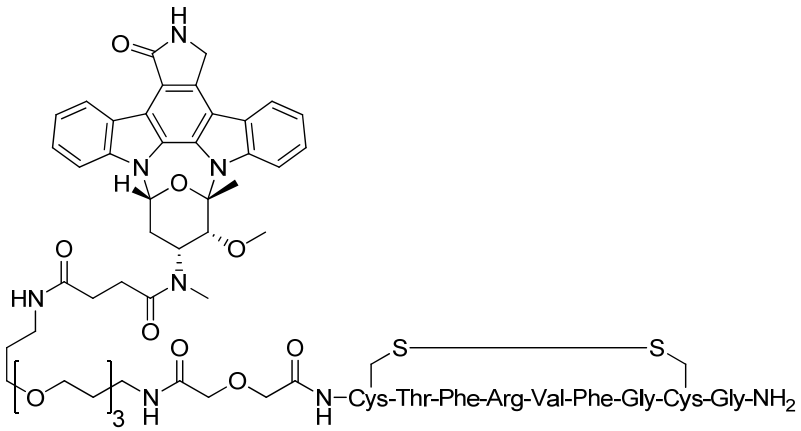
8

Target: IRK with K_i = 800 nM, **Parang *et al*.**



9

Target: PKB with K_i = 260 nM, **Lee *et al*.**



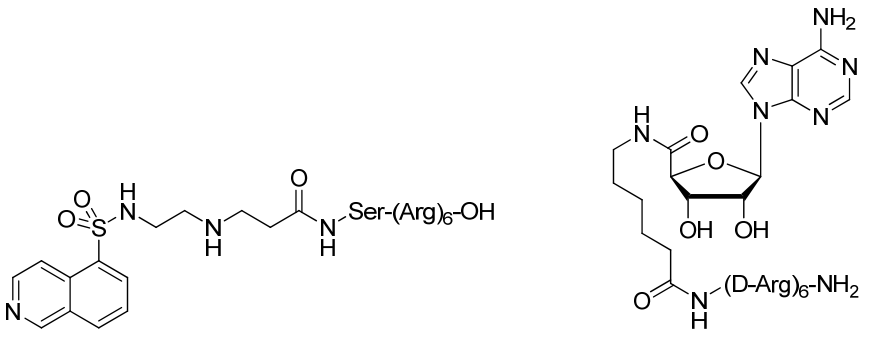
10

Target: PKA with K_i = 2.6 nM, **Meyer *et al*.**

Figure 10: Bisubstrate kinase inhibitors, acting against both the ATP and substrate binding site.[91,94,95]

The concept of bisubstrate inhibitor design was successfully used in the development of several other inhibitors for kinases from the AGC-family as well, to which PKC belongs. Several good examples of bisubstrate inhibitor design for PKA and PKB have been published to date. Lee *et al*. used a known peptide substrate for PKB with K_i = 3.2 ± 1.1 mM, and via a combinatorial approach, the peptide was modified with a moiety binding the ATP binding site, which resulted in the development of the high affinity binder **9** with K_i = 260 ± 20 nM, which acted as a bisubstrate (Figure 10).[94] For the inhibition of PKA, which is closely related to PKC, a good example is the design of staurosporine-peptide construct **10**. This bisubstrate inhibitor shows a high and selective affinity in the low nanomolar range toward PKA (Figure 10).[95,96]

This bisubstrate approach has been used for the design and synthesis of PKC selective inhibitors as well. Ricouart *et al.* reported the synthesis of kinase inhibitors by using the bisubstrate approach in which an isoquinoline sulfonamide moiety and a highly basic arginine hexapeptide were combined.[66] This resulted in an increase in affinity of the inhibitor for basophilic kinases of the AGC family. Unfortunately, however, the affinity of compound **11** was far better for PKA with a K_i = 4 nM than for PKC with a K_i = 100 nM (Figure 11). Enkvist *et al*. described bisubstrate **12** that was constructed of adenosine which was connected to a D-arginine hexapeptide as a nanomolar inhibitor for kinases from the AGC family. This compound had a high affinity for PKA with a K_i = 8.3 nM, but no significant inhibition of PKC was observed (Figure 11).[97]



11
PKC K_i = 100 nM
PKA K_i = 4 nM
Ricouart *et al.*

12
PKC no significant inhibition
PKA K_i = 8.3 nM
Enkvist *et al.*

Figure 11: Unselective bisubstrate inhibitors designed for PKC inhibition.[66,97]

Though, the bisubstrate approach has been applied successfully for the design of selective kinase inhibitors, it has not yet been successfully achieved for the inhibition of PKC. Since the bisubstrate approach has been proven to be extremely powerful in increasing the affinity as well as the selectivity, it would be a great challenge to design, synthesize and to evaluate the biological properties of novel PKC inhibitors. ATP-competitive inhibitors for PKC have been extensively described in the literature, however the peptide sequences to be phosphorylated have not been explored in great detail. Since the PKC family consists of 11 closely related members it is of great importance which peptide sequence is selectively phosphorylated by the single PKC members and to investigate which differences of peptide recognition between the PKC isozymes exist. The peptide sequence is expected to be of utmost importance for the successful design of isozyme-selective PKC inhibitors. To screen for multiple peptide substrates in a single experiment, microarray technology is an excellent tool.

1.2.4 Microarray technology

The concept of microarrays was first reported by Ekins *et al*. in the early 1990s and in their seminal articles, the microarray technique was used for DNA analysis.[98,99] Since then, peptide microarray technology has emerged to be a powerful tool to study all kind of biological interactions, like protein-protein-, enzyme-substrate- or protein-inhibitor-interactions.[100-102] Recently, the development of microarrays displaying multivalent carbohydrates to study their interactions with carbohydrate-binding proteins has been reported by our group.[103] Microarrays can display hundreds to thousands possible ligands on a surface, and a single experiment yields a comprehensive overview of interactions between the two investigated counterparts.[104-106] The material that is used for microarrays developed in time as well. The most general microarrays are 2-dimensional, which are spotted onto the surface of glass slides.[107-111] Here the substrates are covalently attached to a glass slide, and treated with an analyte, followed by the analysis. Though this approach is very general and robust, a drawback of this method is that only the end-point of an experiment can be determined, which means that it is not possible to monitor a reaction, e.g. substrate phosphorylation by a kinase, in real-time. Therefore, a novel type of flow-

through microarrays, made from porous material, has been developed by Pamgene B.V.[112,113] These microarrays are constructed from porous Al_2O_3 displaying a 3-dimensional arrangement (Figure 12A and B). This 3-dimensional arrangement has several advantages over the 2-dimensional glass slides. At first, the analyte mixture is pumped through the microarray, which provides active mixing of the reagents in the solution to give a faster reaction with the substrates on the surface. This fast interaction of the 3D-arrays is favored over the 2-dimensional arrays, where mixing relies purely on diffusion. Secondly, the flow-through microarrays have the ability to monitor a reaction between the analyte and the substrate in real-time using a CCD camera (Figure 12C). Every time when the mixture is underneath the array an image can be recorded, which is favorable compared to 2-dimensional arrays that only show end-points.

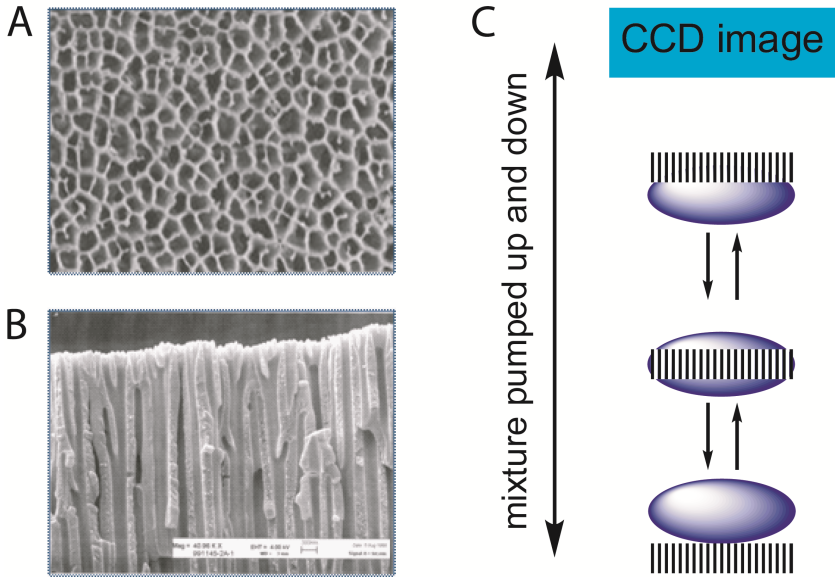


Figure 12: **A** picture of the top-view of the porous Al_2O_3 microarray magnified 3300 times. **B** picture of the side-view of the porous Al_2O_3 microarray magnified 3300 times. **C** Schematic representation of an experiment where the analyte mixture is pumped through the microarray giving good mixing properties and the ability to monitor a reaction in time.

To unravel protein networks in which kinases are involved it is of utmost importance to elucidate which substrates are recognized by a certain kinase. Furthermore, by using peptide microarrays, kinase-substrate interactions can be investigated to identify optimal peptide sequences that bind to the substrate binding site of the corresponding kinase. These

optimal sequences can then be used as lead compound in drug discovery for substrate binding site inhibitors or bisubstrates.[101]

The detection of phosphorylated peptides by a kinase on the microarray can be performed in two different approaches. Firstly, the initial reports of peptide microarrays were based on peptide phosphorylation detected by radioactivity by using radioactively labeled [γ-$^{32/33}$P]ATP (Figure 13A).[114] The use of radioactively labeled [γ-$^{32/33}$P]ATP is nowadays avoided, since operational safety, ease of handling and waste disposal became major issues.[101] Therefore, a second approach for the detection of phosphorylated amino acids can be performed by using phosphate-specific antibodies or chelators that are fluorescently labeled. Chelators are relatively small molecules, containing a positively charged metal-ion, that is designed specifically for the detection of phosphorylated amino acids.[115,116] Commercially available Pro-Q diamond or chelators described by Hamachi *et al.* have been used successfully to detect protein- or peptide phosphorylation (Figure 13B). It should be noted that the described chelating compounds do not distinguish between pSer, pThr and pTyr amino acids, and the chelators designed by Hamachi *et al*. have been used to bind highly acidic peptide sequences as well.[117]

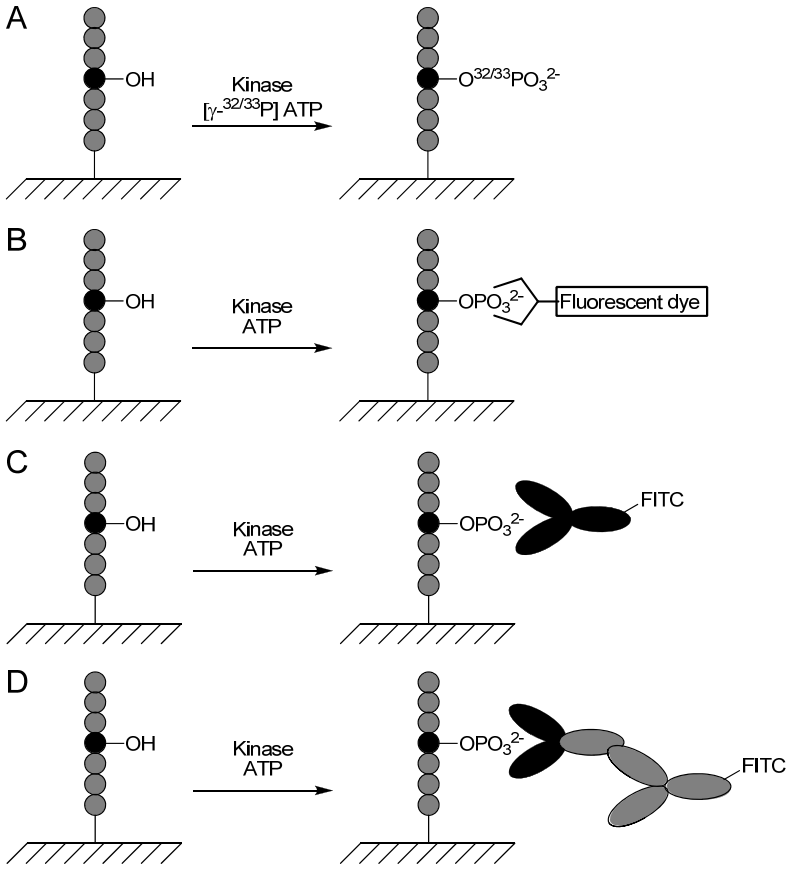


Figure 13: Possible options for the detection of kinase activity by substrate phosphorylation of the microarray surface. **A:** The use of radioactively labeled [γ-$^{32/33}$P]ATP. **B:** Using a chelator that selectively recognizes phosphorylated substrates. **C:** Fluorescently-labeled antibodies to detect tyrosine phosphorylated amino acids. **D:** A primary antibody that recognizes serine/threonine phosphorylated amino acids and on its turn is recognized by a fluorescently labeled secondary antibody.

An alternative method for the fluorescent detection of phosphorylated peptides is the use of antiphospho amino acid antibodies, which can be used in direct detection (pTyr residues) or via a two step approach with a secondary antibody that is fluorescently-labeled (pSer/pThr amino acids) (Figure 13C and D).[118] The use of antiphospho-antibodies has been proven to be a reliable method, especially for the detection of pTyr-residues. The use of antiphospho Ser/Thr antibodies is to date still very challenging, since these antibodies display a much lower selectivity between phosphorylated and non-phosphorylated peptide sequences. Therefore, it should be noted that the use of antiphospho Ser/Thr antibodies needs to be validated thoroughly, since it might yield false-positive or false-negative results.[101] In general the microarray technology is a reliable and fast method to detect protein- or

enzyme-substrate interactions for the investigation of cellular processes or the identification of peptide sequences that can serve as drug lead compounds.

1.3 Aim and outline of this thesis

Protein phosphorylation is one of the most abundant post translational modifications that plays important roles in the regulation of cellular processes. Therefore, various methods have been developed for the elucidation of the phosphoproteome. Chemical proteomics, in which a phosphorylated serine or threonine residue is chemically modified using β-elimination, is one of the methods that are used in elucidation of the phosphoproteome. Though this method has already been explored extensively, the selectivity of this method towards *O*-glycosylation is yet unknown. Therefore, **chapter 2** deals with the synthesis of pSer/pThr- and GlcSer/GlcThr-peptides, which has been be used in a model system to study the selectivity of the β-elimination reaction. Furthermore, in case of selective β-elimination the selectively modified phoshorylated peptides have been purified using affinity chromatography and characterized by MS. **Chapter 3** describes how the model system as described in chapter 2 has been translated to a natural system, where phosphorylated proteins were derivatized using the optimized β-elimination conditions. The β-eliminated product was subsequently treated with a dithiol, followed by treatment with a thiol-selective fluorescent probe. The mixture has been analyzed with gel-electrophoresis and despite the encouraging results in case of model compounds, more complex cell lysates could not be analyzed accurately by this approach.

In addition to the interest in chemical phosphoproteomics, there was a fascination for enzymes responsible for protein phosphorylation, i.e. especially PKC dates from more than two decades ago. PKC enzymes are involved in a wide range of cellular processes including gene expression and cell growth and uncontrolled activation of PKC is related to several serious diseases like cancer and diabetes. The selective inhibition of protein kinases is challenging since the catalytic kinase domain is highly conserved during evolution. However, selective inhibition may be achieved by rational design and synthesis of bisubstrate inhibitors, which targeted both the ATP- and the substrate binding-site of the

catalytic domain of the kinase. Therefore, **chapter 4** describes the application of porous flow-through microarrays, to profile the peptide substrates that are phosphorylated by the isoenzymes PKCα, ζ and θ. The flow-through microarrays allowed the real-time analysis of the peptide phosphorylation by these kinases. Peptide sequences that are rapidly phosphorylated, by their corresponding kinase, can therefore be used as lead sequences for the synthesis of selective kinase inhibitors. **Chapter 5** discusses the synthesis of ATP-competitive inhibitors. The ATP-competitive inhibitors synthesized are staurosporine analogs, isoquinoline-sulfonamides and adenosine-derivatives. All inhibitors are decorated with an acetylene functionality to couple them to the substrate binding-site inhibitors via a copper-catalyzed click-reaction. In **chapter 6** it is illustrated how the target peptides as found in chapter 4 were transformed into azide-functionalized pseudosubstrate peptide inhibitors. Furthermore, via click-chemistry, the newly designed peptide sequences were coupled to the aforementioned ATP-competitive inhibitors. The synthesized constructs as described in chapter 5 and 6 were tested for their biological activity, which is outlined in **chapter 7**. Using the same technology as described in chapter 4, the IC_{50}-values were determined of the inhibitors against PKCα, ζ and θ. One bisubstrate inhibitor showed a low nM affinity toward PKCθ in a selective manner. The selective bisubstrate inhibitor has been tested against highly homologous PKA as well, to further confirm the selectivity of this inhibitor for PKCθ. Furthermore, the mode of action of the selective bisubstrate has been studied in more detail in an ATP- and substrate-dependent experiment, which revealed that the bisubstrate indeed targeted both the ATP- and the substrate-binding pocket in a competitive manner.

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Chapter 2

Selective enrichment of Ser-/Thr-phosphorylated peptides in the presence of Ser-/Thr-glucosylated peptides

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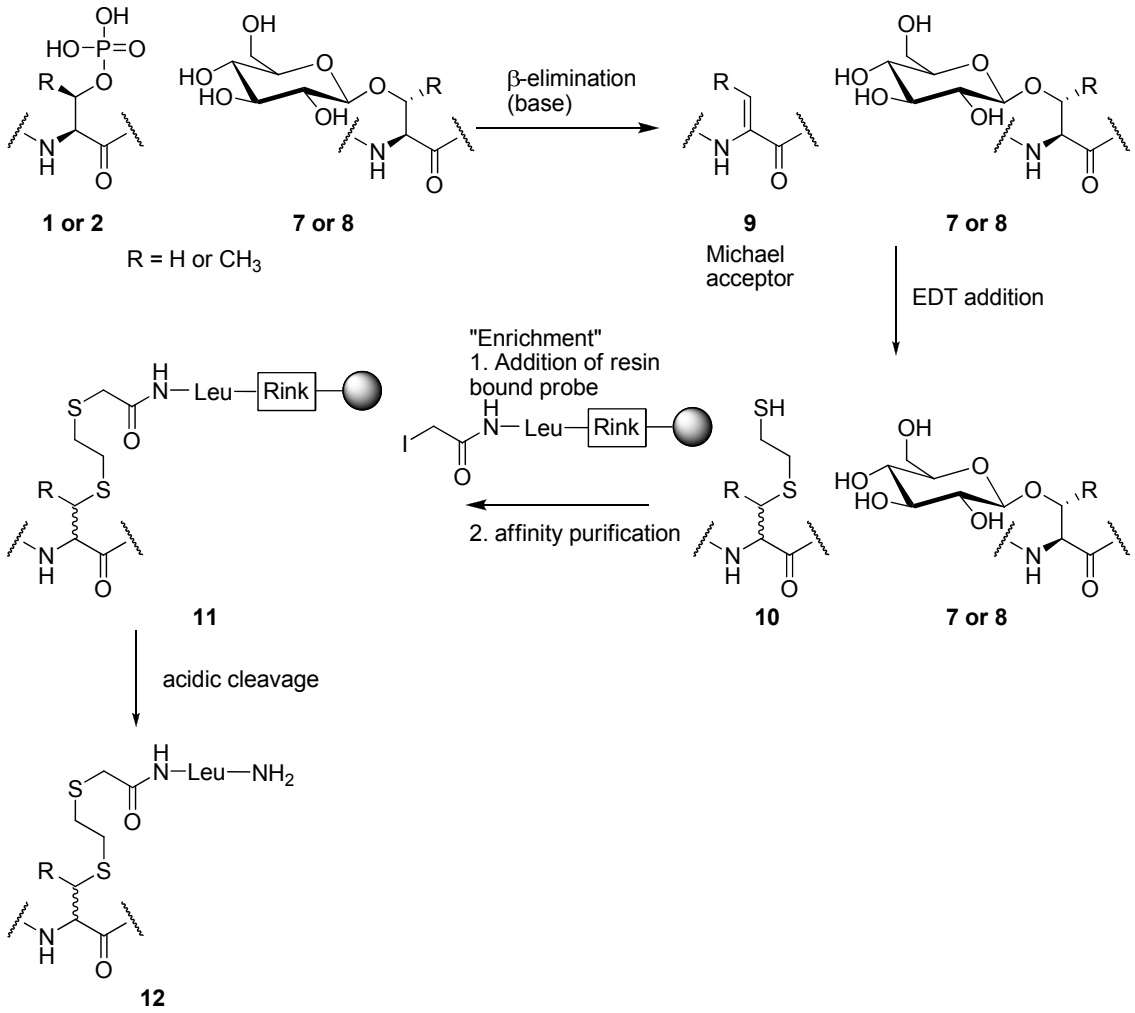
Abstract: β-Elimination of Ser-/Thr-phosphorylated peptides under basic conditions has proven to be a reliable first step in the approach for enrichment of these peptides. However, under these basic reaction conditions, Ser-/Thr-glycosylated peptides are susceptible to β-elimination as well. This chapter describes the optimization of the base-induced β-elimination that is highly selective for phosphorylated peptides *and* as an example the enrichment of phosphorylated peptides in the presence of glucosylated peptides is described.

Introduction

Protein phosphorylation is one of the most abundant post-translational modifications (PTMs), and it is estimated that half of all proteins will be phosphorylated at least once during their lifetime.[1,2] Phosphorylation is reversible, which is important for the function of proteins such as regulation of enzyme activity, signal transduction and cell division.[3-6] In contrast to the amount of phosphorylated proteins are the few methods that have been described for identification and quantification of these proteins and peptides to elucidate the phosphoproteome.[7-15] Direct MS analysis of phosphorylated peptides is difficult in positive ion mode due to the negatively charged phosphate moiety. Therefore, methods have been developed, relying on chemical modification of Ser-/Thr-phosphorylated peptides, allowing enrichment of these peptides from complex mixtures and yielding a neutral amino acid residue, making it more suitable for positive ion mode MS.[16] One of these chemical modification methods for the enrichment of Ser-/Thr-phosphorylated peptides is β-elimination under basic conditions and functionalization for affinity purification by thiol addition (Scheme 1).[5] Later, this method has been optimized by several groups including ours using solid supports and the incorporation of an acid-cleavable linker to lower the detection limit of this method to the picomolar range.[17,18] Although it has been proven that this method is reliable for the enrichment of phosphorylated peptides from peptide mixtures, the issue of selectivity with respect to *O*-glycosylated peptides has not been addressed properly before. Most articles published to date on this topic, in fact, ignore this selectivity issue. Review articles on phosphoproteomics address this lack of selectivity, but unfortunately do not present a suitable solution.[2,19] The selectivity in β-elimination under alkaline conditions is not only ignored in the field of phosphoproteomics, but also in papers dealing with the identification of *O*-glycosylated peptides.[20,21] Methods for *O*-glycopeptide identification are also based on β-elimination under alkaline conditions, and the fact that there might well be phosphorylated peptides present in the reaction mixture is also disregarded. Refusing to pay attention to this selectivity issue will most probably yield false positives when functionalization through β-elimination is applied to complex mixtures. This chapter describes an approach toward the selective enrichment of Ser-/Thr-phosphorylated peptides in the presence of Ser-/Thr-glycosylated peptides by adjusting the β-elimination conditions

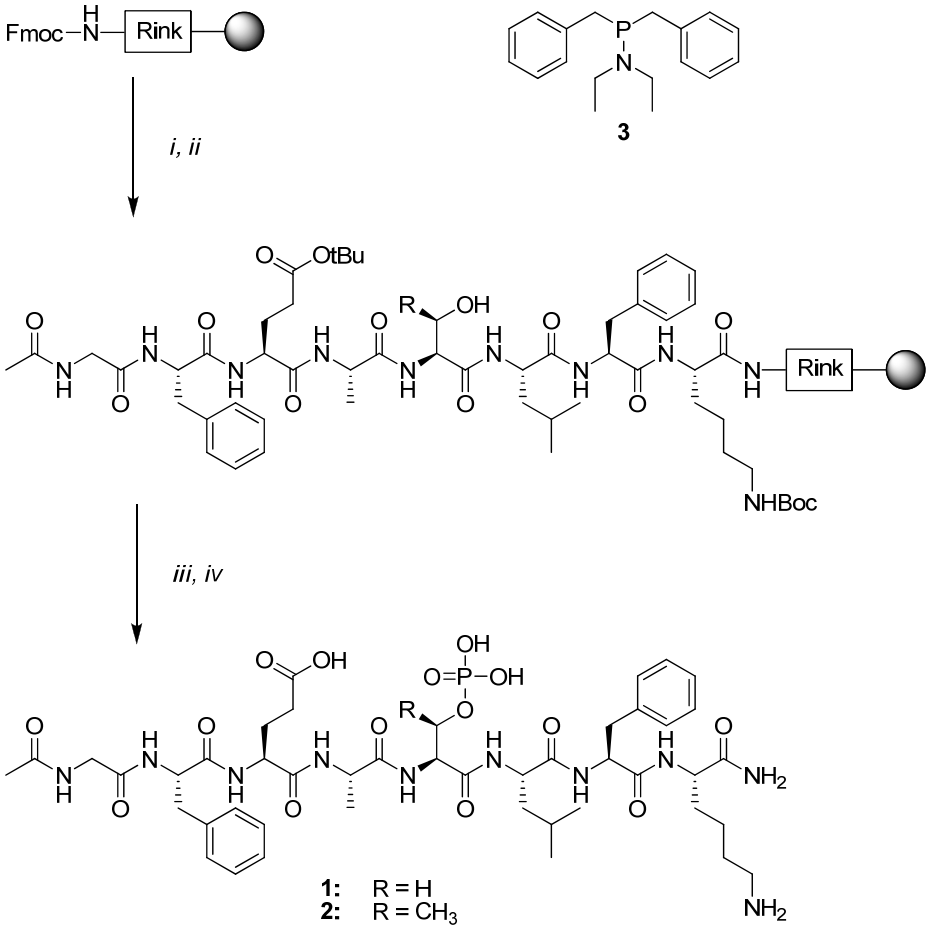
(Scheme 1). Therefore, phosphorylated and glucosylated peptides have been synthesized using SPPS and β-elimination conditions have been optimized to achieve the selective enrichment of the phosphorylated peptides. Though it is known that Ser/Thr-*O*-GlcNAc modification is more relevant in biological processes,[21,22] it was decided to investigate the β-elimination reaction in this study with Ser/Thr-*O*-Glc amino acid residues, because of their synthetic accessibility. Furthermore, when investigating the β-elimination reaction of the glucose functionality from a serine or threonine moiety, the reaction under basic conditions takes place at the α-carbon of the amino acid residue resulting in a dehydro-amino acid. The carbohydrate group does not directly participate in this β-elimination reaction, and is therefore of little influence on the reaction rate.



Scheme 1: Procedure for the enrichment of Ser-/Thr-phosphorylated peptides via chemical modification in the presence of Ser-/Thr-glucosylated peptides.

Results and Discussion

Before several β-elimination conditions could be tested, phosphorylated peptides **1** and **2** and glucosylated peptides **7** and **8** were synthesized. Phosphorylated peptides **1** and **2** were synthesized using Fmoc/tBu based SPPS starting with Fmoc-Rink-Tentagel resin (Scheme 2). After each deprotection an Fmoc-protected amino acid was coupled with HOBt and HBTU as coupling reagents and DiPEA as base. All coupling reactions and Fmoc-deprotections were monitored using a Kaiser test.[23]

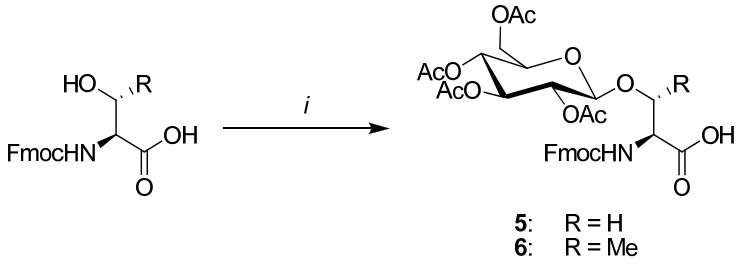


Scheme 2: Synthesis of peptides **1** and **2**. *(i)* 20% piperidine, DMF; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF; Coupled amino acids: Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Ser-OH (or Fmoc-Thr-OH), Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH and Ac-Gly-OH; *(iii)* dibenzyl *N,N*-diethylphosphoramidite **3**, 1-*H* tetrazole, dry THF, *m*-CPBA; *(iv)* TFA, TIS, H_2O (95:2.5:2.5); Overall yields: **1** (23%), **2** (16%).

In the synthesis of peptides **1** and **2**, Fmoc-Ser-OH, respectively Fmoc-Thr-OH, was coupled without *tert*-butyl as side chain protecting group since these residues were

phosphorylated on the resin at the end of the synthesis. Furthermore, the final amino acid coupled to the resin was Ac-Gly-OH to avoid acetylation of the unprotected hydroxyl functionalities prior to phosphorylation. After the coupling of the final amino acid the peptide was phosphorylated on resin using a global phosphorylation approach.[24,25] This reaction was performed in the presence of 1-*H* tetrazole and dibenzyl *N,N*-diethylphosphoramidite **3** as phosphorylating agents and the intermediate phosphite was converted into the phosphate using *m*-CPBA as the oxidizing agent.[26,27] The peptides were deprotected and cleaved from the resin by treatment with TFA and the crude peptides were purified by HPLC to give the desired phosphorylated peptides in yields of 23 and 16% for **1** and **2** respectively.

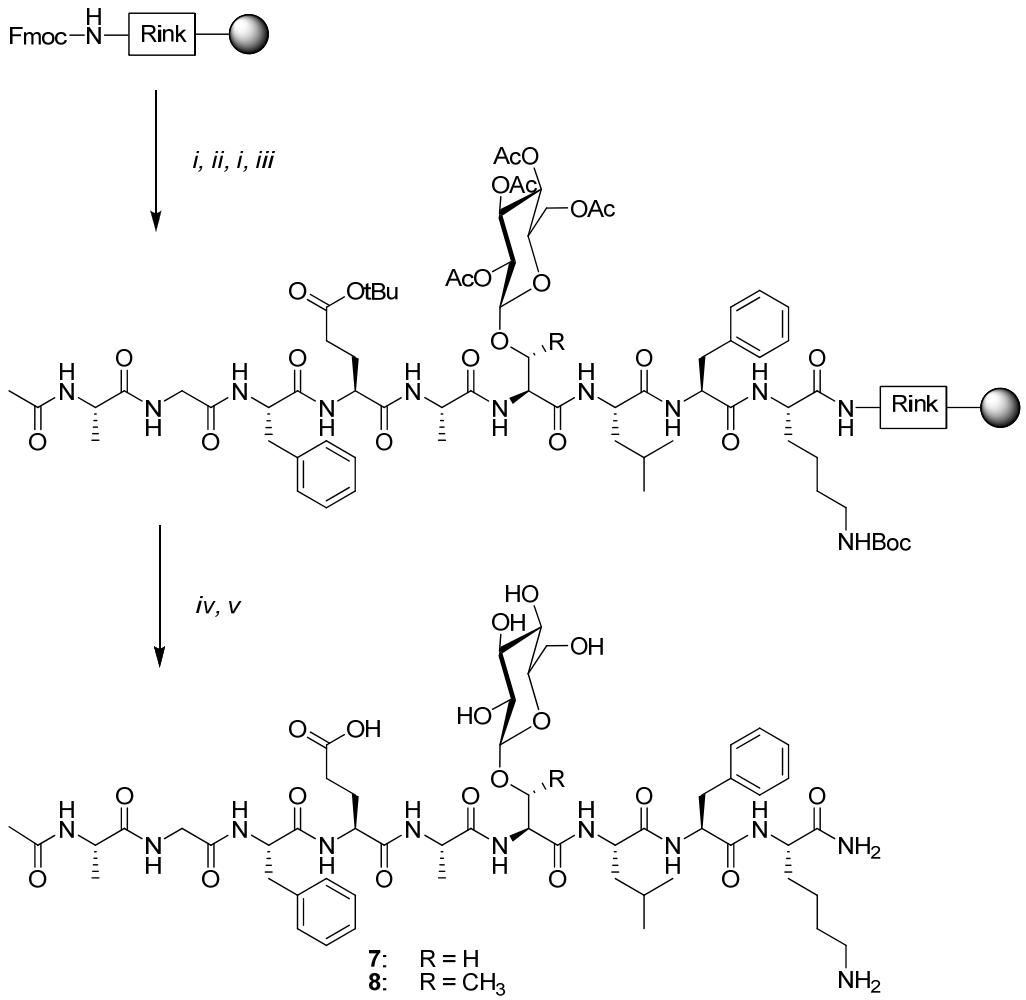
O-glucosylated amino acids **5** and **6** were synthesized as was described by Salvador *et al.*[28], in which β-D-glucose pentaacetate was activated with $BF_3{\cdot}OEt_2$ and then coupled to Fmoc-Ser-OH or Fmoc-Thr-OH, respectively. In the synthesis of peptide **7**, Fmoc-Ser[βGlc$(OAc)_4$]-OH **5**, and for peptide **8,** Fmoc-Thr[βGlc$(OAc)_4$]-OH **6** were used as building blocks without adjustments in the coupling conditions (Scheme 3).[28] Then, the glucosylated peptides **7** and **8** were synthesized as shown in Scheme 4. To distinguish the β-elimination products of both the phosphorylated and glucosylated peptides an extra *N*-terminal alanine was coupled to glucopeptides **7** and **8**. The incorporation of an alanine residue yields a mass difference of 71 *m/z* with peptide **1** and **2** for the β-eliminated products.



Scheme 3: *(i)* $BF_3{\cdot}OEt_2$, ß-D-glucose pentaacetate, CH_2Cl_2, **5**: 68%, **6**: 61%.

Each coupling cycle contained the Fmoc deprotection with 20% piperidine in DMF and the coupling of an amino acid with HOBt and HBTU as coupling reagents and DiPEA as base. After completion of the synthesis, both peptides were cleaved from the resin and all acid

labile protecting groups were removed by treatment with TFA. Subsequent deacetylation of peptides **7** and **8** was achieved by treatment with aqueous hydrazine solution (5%). The identity of the peptides was confirmed by mass spectrometry. Although, deacetylation required basic conditions, no β-elimination was observed during this treatment. A final HPLC purification gave the desired glucosylated peptides in a yield of 48 and 44% for **7** and **8**, respectively.



Scheme 4: Synthesis of peptides **7** and **8**. *(i)* 20% piperidine, DMF; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF; Coupled amino acids: Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Ser[βGlc(OAc)$_4$]-OH **5** (or Fmoc-Thr[βGlc(OAc)$_4$]-OH **6**), Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH Fmoc-Gly-OH and Fmoc-Ala-OH; *(iii)* Ac_2O, DiPEA, HOBt, DMF; *(iv)* TFA, TIS, H_2O (95:2.5:2.5); *(v)* 5% hydrazine, H_2O; Overall yields: **7** (48%) and **8** (44%).

After the successful synthesis of the phosphorylated and glucosylated peptides, initial β-elimination experiments were carried out according to the literature procedures.[5,17,29] Calculated masses of the starting materials and their β-eliminated counterparts are depicted in Table 1. Phosphopeptide **1** and glucopeptide **7** were mixed in equimolar amounts and treated with a saturated $Ba(OH)_2$ solution in aqueous MeOH. The reaction mixture was shaken and a sample was taken after 15, 30, 60, 120 and 180 min. These samples were neutralized by the addition of H_2SO_4 and centrifuged to remove the precipitated $BaSO_4$. Then, the supernatant was evaporated to dryness and the residue was desalted and concentrated using a C18 ZipTip prior to MALDI-ToF MS analysis. As was expected MS-analysis confirmed quantitative β-elimination after already 15 min for both the phosphorylated and the glucosylated peptide (Figure 1).

Table 1: Masses of peptides used in this study towards selective β-elimination of phosphorylated peptides.

Peptide	**Mass (Da)**	**Mass (dehydro-peptide) (Da)**
Ac-GFEApSLFK-NH_2 **1**	$[M+H]^+$: 1019.45	$[M+H]^+$: 921.48
Ac-AGFEA(glc)SLFK-NH_2 **7**	$[M+H]^+$: 1172.58	$[M+H]^+$: 992.51
Ac-GFEApTLFK-NH_2 **2**	$[M+H]^+$: 1033.47	$[M+H]^+$: 935.49
Ac-AGFEA(glc)TLFK-NH_2 **8**	$[M+H]^+$: 1186.59	$[M+H]^+$: 1006.53

From this result it was concluded that the use of strong alkaline conditions will result in a nonselective β-elimination. Therefore, shorter reaction times, several organic and inorganic base types and different reaction temperatures were explored for optimization to achieve a selective β-elimination.

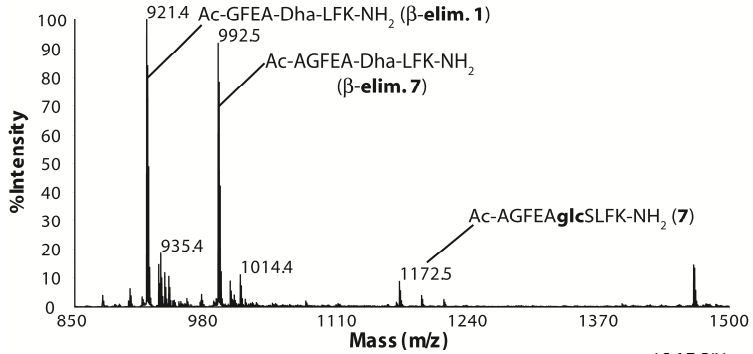
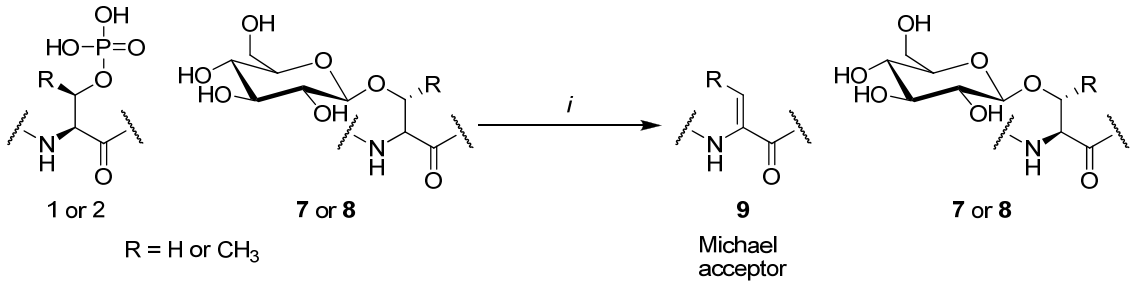


Figure 1: Complete β-elimination after 15 min with published procedures.[5,17,29]

The use of inorganic bases other than $Ba(OH)_2$ resulted in β-eliminated peptides, however, selectivity was not observed, whereas the use of organic nitrogen-based bases resulted in no or very slow β-elimination in a nonselective manner. Secondly, the reaction temperature was adjusted during the β-elimination process. Instead at 45°C, the experiments were performed at 37 and 20°C, respectively. Unfortunately, however, a selective β-elimination was not observed. The third adjustment was decreasing the base concentration during β-elimination. All experiments were carried out as in previous experiments with the use of freshly prepared $Ba(OH)_2$ solutions with concentrations of 0.15 M, 0.05 M, 0.015 M, 0.005 M or 0.0015 M and reaction times of 30 respectively 60 min. Decreasing the $Ba(OH)_2$ concentration clearly resulted in a slower β-elimination process of both the phosphorylated and glucosylated peptides. However, the combination of a lower base concentration of 0.015 M $Ba(OH)_2$ and a reaction time of 30 min, resulted in selective β-elimination of the Ser-phosphorylated peptide in the presence of the Ser-glucosylated peptide. Thus, the high $Ba(OH)_2$ concentration as used previously resulted in a fast but also nonselective β-elimination. A 10-fold reduction of the $Ba(OH)_2$ concentration indeed resulted in selective β-elimination of phosphorylated peptides.



Scheme 5: Selective β-elimination of phosphorylated peptides. *(i)*0.015 M $Ba(OH)_2$ in $MeOH/H_2O$ (9:1 v/v), 45°C, 10 min.

Based on these promising results, selectivity could be increased even further by decreasing the reaction time. Phosphorylated peptide **1** and glucosylated peptide **7** were mixed in equimolar amount and $Ba(OH)_2$ was added at a final concentration of 0.015 M. Samples were taken after 10, 20, 30, 60, 120 and 180 min and directly neutralized by the addition of H_2SO_4, to terminate the β-elimination process. The best result was obtained with a reaction time of 10 min, and a selectivity ratio of 12.7:1 in favor of phosphorylated peptide **1** was obtained. Longer reaction times led to a dramatic decrease in selectivity. When the mixture was monitored after 180 min, the ratio of β-elimination peptide **1** versus **7** had already dropped to 1.7:1 (Figure 2). Shorter reaction times than 10 min were not used since the amount of β-eliminated peptide **1** became impractically low.

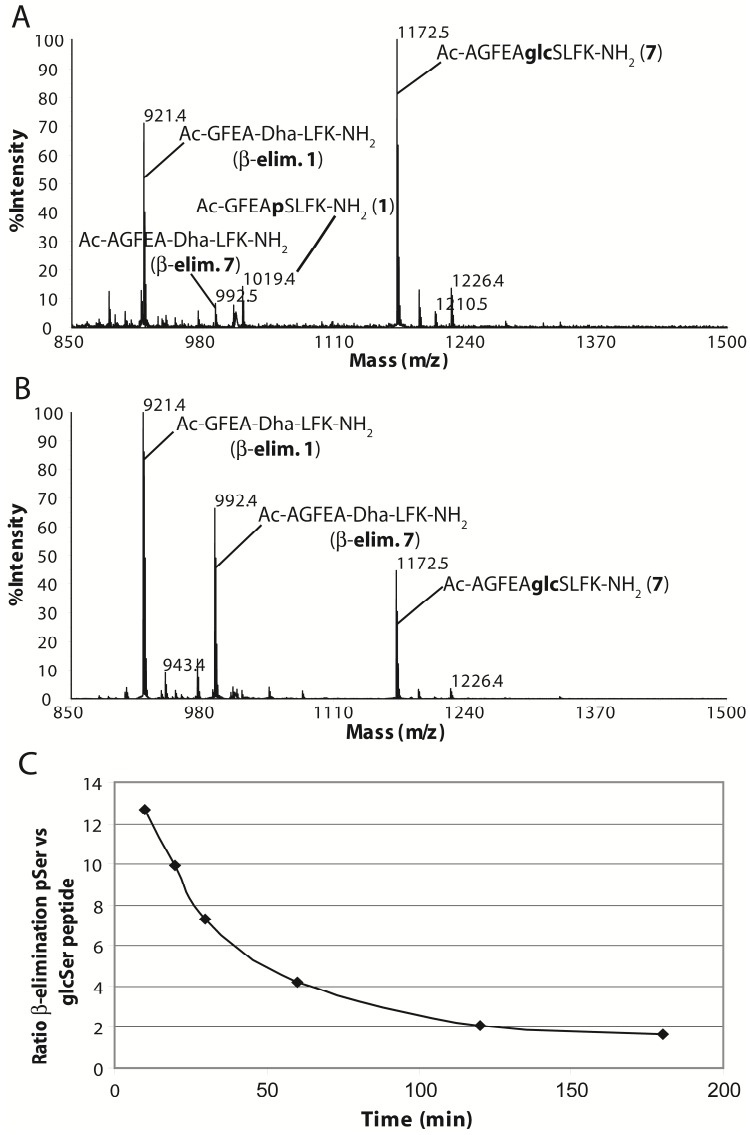


Figure 2: MALDI-ToF MS was used to monitor the β-elimination process of a 1:1 mixture of the phosphoserine and glucoserine peptides **1** and **7**. **A**: Reaction mixture after 10 min of β-elimination. **B**: Reaction mixture after 180 min of β-elimination. **C**: Selectivity ratio of β-eliminated phosphoserine and glucoserine peptides and the decreasing selectivity in time.

These optimized reaction conditions were also applied on the phosphorylated and glucosylated threonine-containing peptides **2** and **8**, respectively to see if selective β-elimination did also occur with these model peptides. Indeed, an excellent phosphate/glucose elimination ratio of 29:1 was found as judged by MALDI-ToF MS analysis, which meant that β-elimination of phosphothreonine peptides was even more selective than with phosphoserine containing peptides (Figure 3). In case of the threonine peptides similar observations were made: elongation of the reaction time resulted in a

dramatic decrease of the selectivity ratio and shortening the reaction time resulted in impractically small amounts of the dehydropeptide.

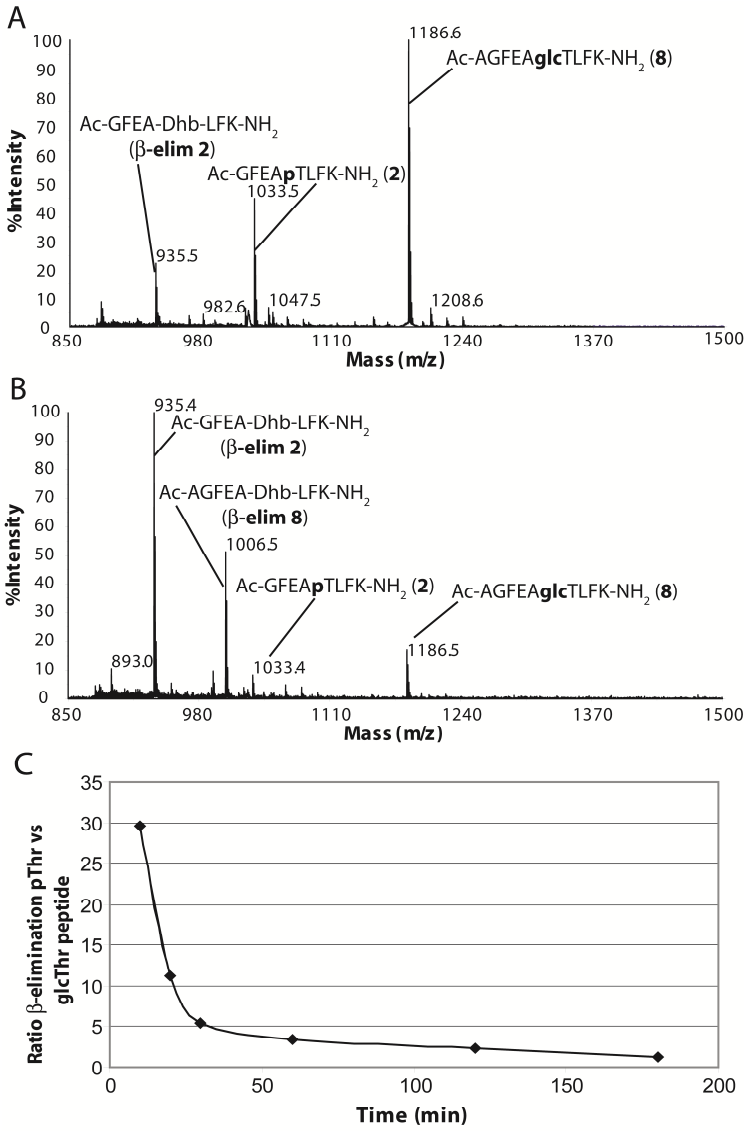
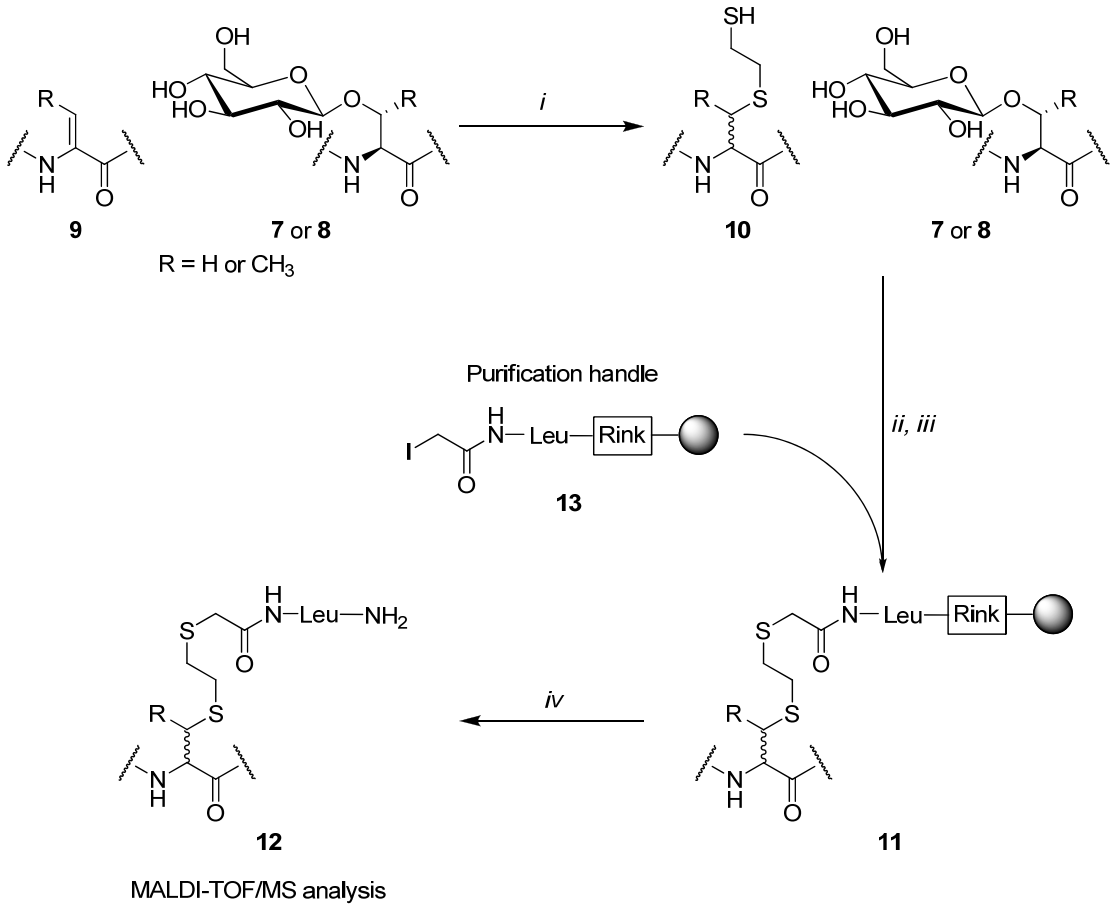


Figure 3: MALDI-ToF MS analysis to monitor the β-elimination process of a 1:1 mixture of the phosphothreonine and glucothreonine peptides **2** and **8**, respectively. **A**: Reaction mixture after 10 min of β-elimination. **B**: Reaction mixture after 180 min of β-elimination. **C**: Selectivity ratio of β-eliminated phosphothreonine and glucothreonine peptides and the decreasing selectivity in time.

Since the optimized reaction conditions for the β-elimination procedure were now known, it was tried to selectively enrich the phosphopeptide from a mixture of both peptides, as shown in Scheme 6.



Scheme 6: Affinity purification. *(i)* EDT, Et_3N, CH_3OH; *(ii)* Purification handle **13**, Et_3N, CH_3OH; *(iii)* Rinse with CH_3OH, CH_3OH /H_2O 1:1, CH_3CN, CH_3CN/H_2O 1:1, H_2O, DMF and CH_2Cl_2; *(iv)* TFA, CH_2Cl_2, TIS, H_2O (47.5: 47.5: 2.5: 2.5)

Therefore, in first instance, a mixture of peptides **1** and **7** were subjected to the optimal β-elimination conditions and after 10 min the reaction mixture was neutralized with H_2SO_4. The precipitated $BaSO_4$ was removed by centrifugation and EDT was added to the supernatant to modify the formed dehydropeptide **9**, which is a Michael acceptor, to a thiol functionalized peptide **10**. The modification with EDT required the addition of base. In this case Et_3N was used since it was shown previously that under these conditions, β-elimination of the remaining glucopeptide did not occur. EDT is a volatile reagent, and excess of this reagent can be removed by evaporation after completion of the reaction. When the dehydropeptide was fully converted, TCEP·HCl was added to prevent disulfide formation and excess EDT was evaporated. The residue was redissolved in MeOH and to this solution a resin-bound iodoacetamide probe **13** was added for affinity purification.[30] An iodoacetamide function selectively binds thiols that are present in the mixture, in this

case predominantly the thiol-modified phosphopeptide **1**. When the reaction was complete the actual purification took place by carefully rinsing the resin with various organic solvents to remove all traces of unbound material from the resin. The probe-peptide adduct was cleaved from the resin by acid treatment and the eluate, containing the probe adduct of modified phosphoserine peptide **1**, was evaporated to dryness. The analysis of the cleaved products by MALDI-ToF MS clearly showed the selective enrichment of phosphorylated peptide **1** via chemical modification in the presence of glucosylated peptide **7**. The enrichment ratio calculated from this spectrum showed a selectivity of 12.7:1 in favor of modified peptide **1**, corresponding to the initially observed β-elimination ratio (Figure 4A). This enrichment experiment was also performed under identical conditions with a mixture of threonine peptides **2** and **8** yielding a similar result after MALDI-ToF MS analysis. The spectrum clearly showed a peak from modified peptide **2**, whereas a peak from modified peptide **8** was not observed (Figure 4B).

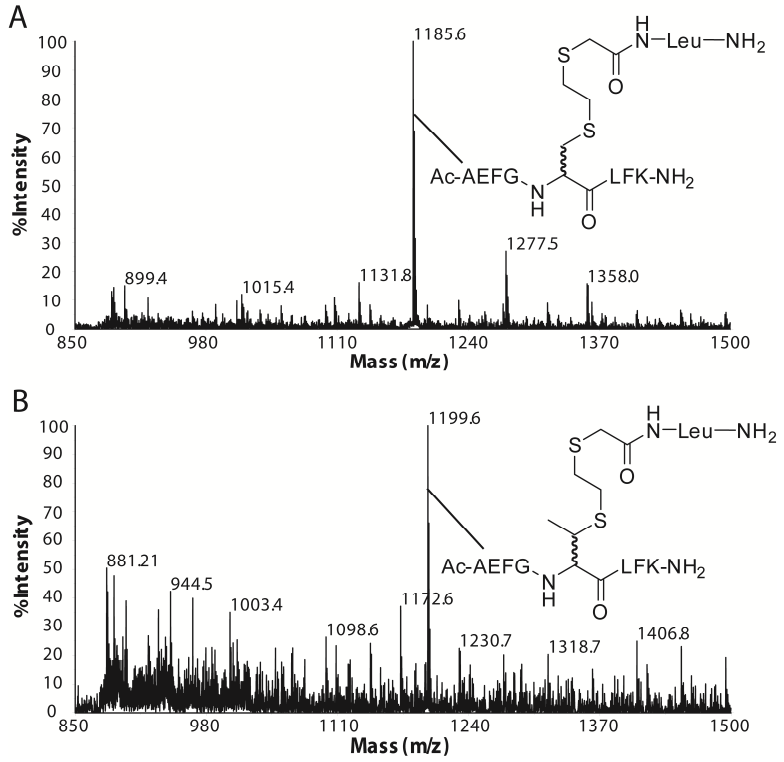


Figure 4: MALDI-ToF MS of enriched phosphorylated peptides **1** and **2**, which were selectively β-eliminated in the presence of *O*-glucosylated peptides **7** and **8**.

Conclusions

This chapter describes the robust synthesis of phosphorylated peptides **1** and **2** using SPPS followed by a global phosphorylation procedure. Furthermore, the successful synthesis of *O*-glucosylated peptides **7** and **8** is described. Regarding the β-elimination process of phosphorylated peptides, it has been shown that chemical modification is a reliable method for the enrichment of these peptides. Furthermore, it has been shown that β-elimination as reported in literature is not selective in the presence of other post-translationally modified peptides, such as *O*-glucosylated peptides. However, using a lower concentration of base and a shorter reaction time, a high selectivity toward phosphate-elimination has been achieved. Moreover, this selectivity is not lost during the ensuing reaction steps required for peptide enrichment. This study, in which model peptides were used to achieve selective β-elimination and enrichment of phosphorylated peptides, will be further exploited, as described in chapter 3. In this chapter selective β-elimination on more natural systems, using phosphorylated and glycosylated proteins, will be investigated.

Experimental section

General: All reactions were carried out at ambient temperature unless stated otherwise. All reagents were used as supplied from commercial sources unless stated otherwise. CH_2Cl_2, NMP and DiPEA were stored on molecular sieves (4Å) and THF was freshly distilled from $LiAlH_4$ prior to use. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Hanessian's stain (cerium molybdate). Column chromatography was carried out using Silicycle UltraPure silicagel (40-63 μm). Tentagel S RAM resin was purchased from Rapp Polymere (Tübingen, Germany). Fmoc amino acids were purchased from GL Biochem (Shanghai, China). All other reagents were purchased from Fluka, Sigma–Aldrich, and Acros and were used without further purification. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with a Applied Biosystems 757 absorbance detector. ESI-MS spectra were obtained in the positive ion mode on a

Shimadzu instrument. MALDI-ToF spectra were recorded in positive-ion mode on an Applied Biosystems 4700 Proteomics Analyzer with α-cyano-4-hydroxycinnamic acid as the matrix.

Ac-Gly-Phe-Glu-Ala-Ser[O-(PO_3)]-Leu-Phe-Lys-NH_2 (1): On Fmoc-Rink-Tentagel resin (Tentagel S RAM) (0.25 mmol, 1.14 g) the synthesis was carried out according to the following protocol:

1 Fmoc deprotection: The resin was treated with a 20% solution of piperidine in DMF (3×5 mL, each 8 min). The solution was removed by filtration, followed by washing with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min). Deprotection was checked with the Kaiser test.[23]

2 Coupling of Fmoc-protected amino acid: Fmoc-Xxx-OH (1.0 mmol), HOBt·H_2O (1.0 mmol, 153 mg), HBTU (1.0 mmol, 379 mg) and DiPEA (2.0 mmol, 330 µL) were added to the resin. DMF (10 mL) was added and N_2 was bubbled through the mixture for 1h. The solution was removed by filtration, followed by washing with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min). Completion of the coupling was checked with the Kaiser test. Steps 1 and 2 were repeated until the complete peptide was synthesized.

3. Coupling of Ac-Gly-OH: Ac-Gly-OH (1.0 mmol, 117 mg), HOBt·H_2O (1.0 mmol, 153 mg), HBTU (1.0 mmol, 379 mg) and DiPEA (2.0 mmol, 330 µL) were added to the resin. DMF (10 mL) was added and N_2 was bubbled through the mixture for 1 h. The solution was removed by filtration, followed by washing with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min). The coupling was checked with the Kaiser test.

4. Phosphorylation: Dry THF (10 mL) was added to the resin followed by the addition of dibenzyl *N,N*-diethylphosphoramidite **3** (2.5 mmol, 792 mg), 1*H*-tetrazole (5 mmol, 350 mg) and N_2 was bubbled through the mixture. After 3 h 70–75% *m*CPBA (5.5 mmol, 950 mg) was added followed by leading N_2 through the mixture 30 min. The solution was removed by filtration, followed by washing with THF (3×5 mL, each 2 min), DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min).

5. Cleavage: The resin was shaken in a mixture of TFA/TIS/H_2O (95:2.5:2.5) (5 mL) for 5 h. The solution was slowly poured into Et_2O (40 mL) to precipitate the cleaved peptide. The supernatant was removed and the crude product was washed twice with Et_2O (40 mL) and dried *in vacuo*. The crude product was purified by preparative HPLC (H_2O/CH_3CN/TFA 95:5:0.1 → CH_3CN/H_2O/TFA 95:5:0.1 in 60 min) and after lyophilizing the peptide **1** was obtained as a white solid 58 mg, 23%. ESI-MS: calcd for $C_{45}H_{68}N_{10}O_{15}P$: 1019.45, found: *m/z* = 1019.40 $[M+H]^+$

Ac-Gly-Phe-Glu-Ala-Thr[O-(PO_3)]-Leu-Phe-Lys-NH_2 (2): This peptide was synthesized according to the procedure described for peptide **1**. The purified peptide was lyophilized and obtaind as a white powder (41 mg, 16%). ESI-MS: calcd for $C_{46}H_{69}N_{10}O_{15}P$: 1033.47, found: *m/z* = 1033.80 $[M+H]^+$

Fmoc-Ser[βGlc(OAc)$_4$]-OH (5):[28] To a solution of Fmoc-Ser-OH (0.78 g, 2.4 mmol) and ß-D-glucose pentaacetate (0.78 g, 2 mmol) in CH_2Cl_2 (40 mL) $BF_3{\cdot}OEt_2$ (0.76 mL, 6 mmol) was added under N_2 atmosphere. The reaction mixture was stirred at ambient temperature for 24 h and diluted with CH_2Cl_2 (80 mL) and washed with 1N HCl (8 mL). The CH_2Cl_2 solution was washed with brine (1 × 40 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The crude compound was purified using column chromatography (CH_2Cl_2/CH_3OH 97:3) to give compound **5** as a white solid in 68% yield (900 mg).

R_f= 0.70 (CH_2Cl_2/CH_3OH/CH_3COOH, 89/10/1 *v/v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 7.78 – 7.27 (m, 8H, Ar*H*), 5.67 (d, *J* = 8.0 Hz, 1H, C*H*), 5.19 (t, *J* = 9.4 Hz, 1H, N*H*), 5.08 (t, *J* = 9.5 Hz, 1H, C*H*), 4.97 (m, 1H, C*H*), 4.47 (m, 3H, Fmoc CH_2C*H*), 4.22 (m, 4H, C$^{\alpha}H$, C$^{\beta}H_2$, C*H*), 3.93 (m, 1H, C*H*), 3.67 (m, 1H, C*H*), 2.07 – 2.00 (4 s, 12H, acetyl CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 172.5, 171.0, 170.3, 169.6, 169.5, 156.0 143.5, 141.2 127.7, 127.0, 124.9, 119.9 101.2, 72.5, 71.7, 71.0 69.6 68.1 67.0 53.9, 46.9 20.6, 20.5 ppm. ESI-MS: calcd for $C_{32}H_{35}NO_{14}$: 657.21, found: *m/z* = 658.50 $[M+H]^+$, 680.45 $[M+Na]^+$, 696.30 $[M+K]^+$.

Fmoc-Thr[βGlc(OAc)$_4$]-OH (6):[28] Compound **6** was synthesized according to the procedure used for Fmoc-Ser[βGlc(OH)$_4$]-OH **5**. The crude product was purified using flash chromatography (CH_2Cl_2/CH_3OH/CH_3COOH, 98/2/1 *v/v/v*) to give compound **6** as a white solid in 61% yield (820 mg).

R_f= 0.74 (CH_2Cl_2/CH_3OH/CH_3COOH, 89/10/1 *v/v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 7.76 – 7.28 (m, 8H, Ar*H*), 5.80 (d, *J* = 9.3 Hz, 1H, C*H*), 5.21 (t, *J* = 9.5 Hz, 1H, N*H*), 5.08 (t, *J* = 9.6 Hz, 1H, C*H*), 4.95 (t, *J* = 8.0 Hz, 1H, C*H*), 4.54 (d, *J* = 8.0 Hz, 2H, CH_2), 1H), 4.39 (m, 3H, Fmoc CH_2C*H*), 4.21 (m, 3H, C$^{\alpha}H$, C$^{\beta}H$, C*H*), 3.65 (d, *J* = 9.9 Hz, 1H, C*H*), 2.07 – 2.01 (4 s, 12H, acetyl CH_3), 1.23 (d, *J* = 6.3 Hz, 3H, C$^{\gamma}H_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 172.6, 171.3, 170.2, 169.3, 169.2, 156.7, 143.4, 140.9, 127.5, 126.8, 125.0, 119.7, 99.2, 75.6, 72.3, 71.3, 70.9, 68.1, 67.1, 61.5, 57.8, 46.8, 20.4, 20.3, 17.4 ppm. ESI-MS: calcd for $C_{33}H_{37}NO_{14}$: 671.22, found: *m/z* = 672.55 $[M+H]^+$, 694.45 $[M+Na]^+$.

Ac-Ala-Gly-Phe-Glu-Ala-Ser[βGlc(OH)$_4$]-Leu-Phe-Lys-NH$_2$ (7): On Fmoc-Rink-Tentagel resin (0.25 mmol, 1.14 g) the synthesis was carried out according to the following protocol:

1 Fmoc deprotection: As was described for peptide **1**.

2 Coupling of Fmoc-protected amino acid: As was described for peptide **1**.

3. Acetylation: Capping solution (10 mL) [Ac_2O (50 mmol, 4.7 mL), HOBt (1.9 mmol, 220 mg), DiPEA (12.5 mmol, 2.2 mL) in 100 mL NMP] was added to the resin and N_2 was bubbled through the

mixture for 20 min. The solution was removed by filtration and fresh capping solution (10 mL) was added for the second time. N_2 was bubbled through for 20 min followed by removal of the solution by filtration. The resin was washed with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min). Completion of the acetylation was verified with the Kaiser test.

4. Cleavage: The resin was shaken in a mixture of TFA/TIS/H_2O (95:2.5:2.5) (5 mL) for 3h. The solution was slowly poured into Et_2O (40 mL) to precipitate the cleaved peptide. Supernatant was removed and the crude precipitated product was washed twice with Et_2O (40 mL) and dried *in vacuo*.

5. Deprotection of the glucosylated amino acid residue: The crude peptide was dissolved in a mixture of 5% hydrazine in H_2O (10 mL) and stirred at room temperature for 30 min followed by lyophilization. The crude product was purified by preparative HPLC (H_2O/CH_3CN/TFA 95:5:0.1 → CH_3CN/H_2O/TFA 95:5:0.1 in 60 min) and after lyophilizing peptide **7** was obtained as a white solid (140 mg, 48%). ESI-MS: calcd for $C_{54}H_{81}N_{11}O_{18}$: 1172.58, found: *m/z* = 1172.65 $[M+H]^+$

Ac-Ala-Gly-Phe-Glu-Ala-Thr[βGlc(OH)$_4$]-Leu-Phe-Lys-NH$_2$ (8): The synthesis of this peptide was similar to the synthesis of peptide **7**. Peptide **8** was obtained as a white powder (131 mg, 44%). ESI-MS: calcd for $C_{55}H_{83}N_{11}O_{18}$: 1186.59, found: *m/z* = 1186.65 $[M+H]^+$, 1208.65 $[M+Na]^+$

Protocol for selective β-elimination and enrichment

β-elimination: A mixture of phosphopeptide (**1** or **2**) and glucopeptide (**7** or **8**) (100 pmol ≈ 0.1 μg, in 10 μL MeOH/H_2O), MeOH (70 μL) and aqueous $Ba(OH)_2$ solution (3 μL, 0.015 M, 45 nmol) in a 500 μL Eppendorf vial was shaken at 45°C for 10 min (1000 rpm) in an Eppendorf Thermomixer apparatus. Aqueous H_2SO_4 (1.5 μL, 0.03 M, 45 nmol) was added and the mixture was vortexed followed by centrifugation (5 min at 10 600 *g*) to precipitate barium salts. When the reaction product was analyzed with MALDI-ToF MS the supernatant was transferred into a new vial and dried under a continuous argon flow. The dried product was dissolved in 10 μL CH_3CN/H_2O (1:1), loaded on a C18 ZipTip and washed with an aqueous solution of 0.1 % TFA (10×10 μL). The desalted peptide mixture was then eluted with 1 μL CH_3CN/H_2O/TFA (50/50/0.1 *v*/*v*/*v*) containing 3 mg/mL α-cyano-4-hydroxycinnamic acid on a MALDI-plate. The dried mixture was then analyzed with MALDI-ToF MS.

Enrichment: The supernatant was transferred to a new 500 μL Eppendorf vial and EDT (15 μL) and Et_3N (10 μL 0.1 M in MeOH) were added followed by shaking the mixture in an Eppendorf Thermomixer apparatus at 45°C for 48 h (1000 rpm). An aqueous solution of TCEP·HCl (10 μL, 1 mM, 10 nmol) was added and the mixture was vortexed and all volatile components were evaporated by shaking briefly in an Eppendorf Thermomixer apparatus at 45°C (500 rpm) under a continuous

argon stream. The residue was redissolved in MeOH (100 μL) and transferred to a new Eppendorf vial and again evaporated in an Eppendorf Thermomixer apparatus at 45°C (500 rpm) under a continuous argon stream. This was repeated twice until there was only a very faint EDT odor left. The peptide mixture was redissolved in MeOH (90 μL) and transferred to a new vial followed by the addition of Et_3N (10 μL 0.1 M) and resin bound iodoacetamide probe **13** (2-3 mg) was added and the mixture was shaken in an Eppendorf Thermomixer apparatus at 45°C for 2 h (1000 rpm). The resin was transferred to a small filter and carefully washed with successively CH_3OH, CH_3OH/H_2O 1:1, CH_3CN, CH_3CN/H_2O 1:1, H_2O, DMF and CH_2Cl_2 (90 μL of each solvent) to remove all traces of unbound material from the resin. A mixture of TFA/ CH_2Cl_2/triisopropylsilane/H_2O (95:95:5:5) (40 μL) was added twice to the resin and then shaken for 20 min followed by washing the resin with 90 μL CH_3CN. The solution containing modified peptides was transferred to a 500 μL Eppendorf vial and evaporated to dryness under a continuous stream of argon while shaking at 20°C in an Eppendorf Thermomixer apparatus (500 rpm). The resulting peptide mixture was analyzed by MALDI-ToF MS, using the same procedure as was described for the β-eliminated product.

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Chapter 3

The application of selective β–elimination on phosphorylated proteins and subsequent fluorescent labeling

Abstract: Chemical modification through β-elimination of Ser-/Thr-phosphorylated amino acids is a reliable approach for the enrichment and detection of phosphopeptides. Translation of the optimized β-elimination conditions from chapter 2 to analyze the β-eliminated phosphorylated proteins is described next. This chapter describes these attempts, as well as a versatile synthesis of a maleimide functionalized fluorescent BODIPY probe. This probe has been used as fluorescent label for the detection of β-eliminated α-casein. This approach proved, however, to be very challenging since it was difficult to analyze the reaction products to draw reliable conclusions.

Introduction

One of the most abundant post-translational modifications in eukaryotic cells is protein phosphorylation, and it is estimated that almost half of all proteins is phosphorylated at some point in their life time.[1,2] Predominantly, serine, threonine and tyrosine residues are phosphorylated in a ratio of approximately 86, 12 and 2%, respectively.[3] Phosphorylation is reversible, which is important for the function of proteins such as regulation of enzyme activity, signal transduction and cell division.[4-7] Since protein phosphorylation is of major importance for vital cell functions, several methods have been investigated and developed for the elucidation of the phosphoproteome.[2,8] For the identification of Ser/Thr phosphorylated residues in peptide and protein sequences, chemical modification under basic conditions has been proven to be a reliable method for selective modification of these amino acids (Figure 1).[9]

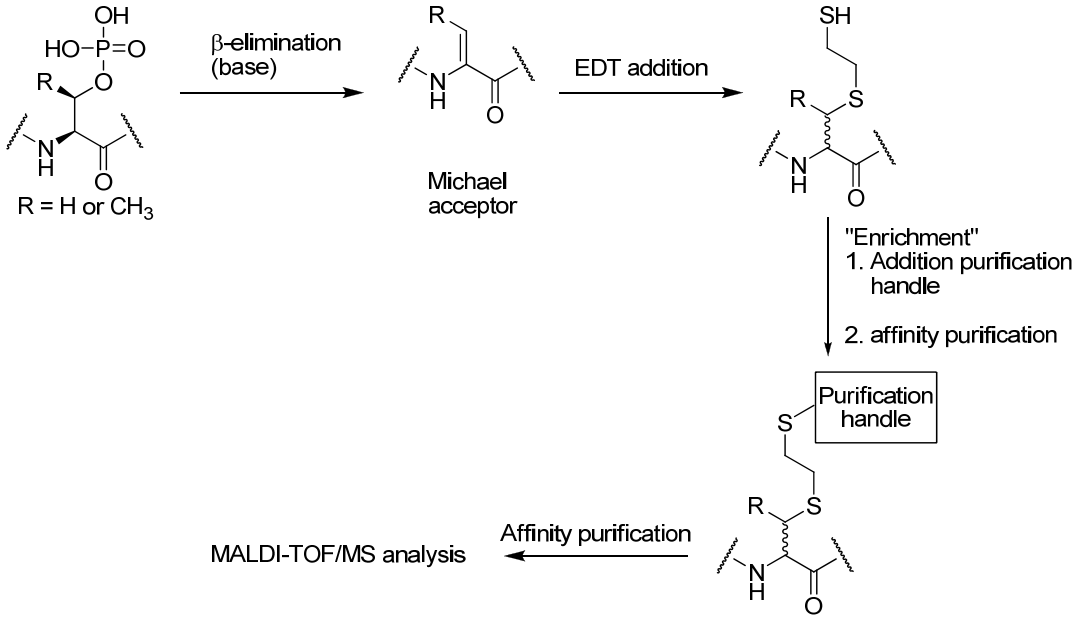


Figure 1: Procedure for the enrichment of Ser-/Thr-phosphorylated peptides via chemical modification

In chapter 2 this concept has been applied on a model system of glycosylated and phosphorylated peptides, and it has been shown that phosphorylated peptides could be selectively enriched in the presence of glycosylated peptides.[10] This base-catalyzed β-elimination reaction yields an αβ-unsaturated dehydro amino acid residue that is susceptible to a Michael-addition with a suitable nucleophile for affinity purification with

biotin/streptavidin or by means of a solid support as purification handle. Finally, the enriched peptide derivative can then be analyzed using MS or MS/MS for identification and sequencing (Figure 1).

To apply this concept for the detection of intact phosphorylated proteins, the proteins can be decorated with a fluorescent dye after β-elimination and subsequently visualized in a gel. As a fluorescent label, a BODIPY-dye can be used, which contains a maleimide functionality as the thiol-reactive group. Since BODIPY-dyes have a small emission window with a high intensity, they can be used to detect small quantities of protein.[11] This chapter describes the attempts to obtain fluorescent labelling of a phosphorylated protein, i.e. α-casein. Therefore, a BODIPY-dye is synthesized, which is functionalized with a maleimide group to be able to bind thiol functionalities. Furthermore, experiments have been performed with α-casein, a phosphorylated protein, to obtain fluorescent labelling after β-elimination.

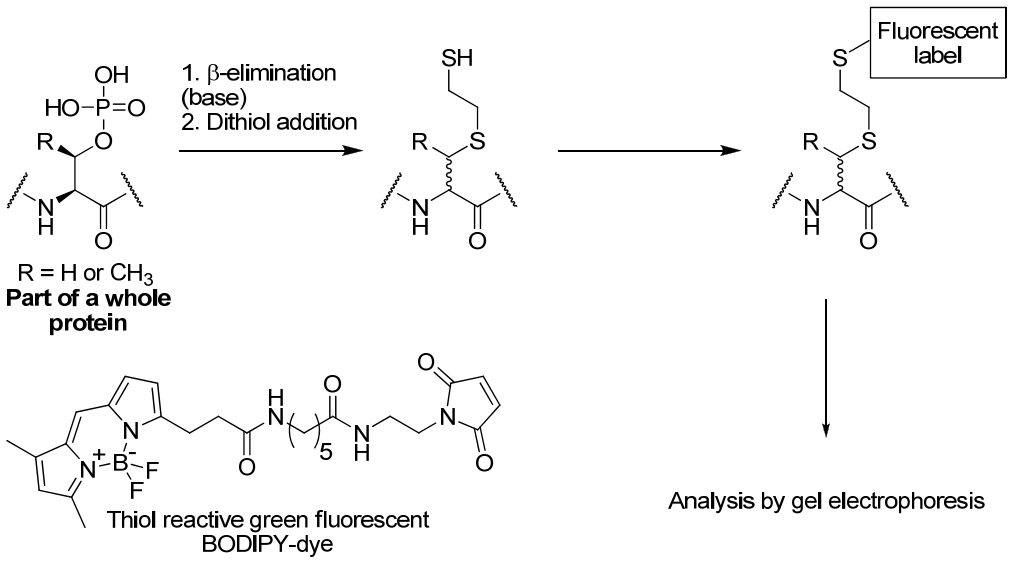
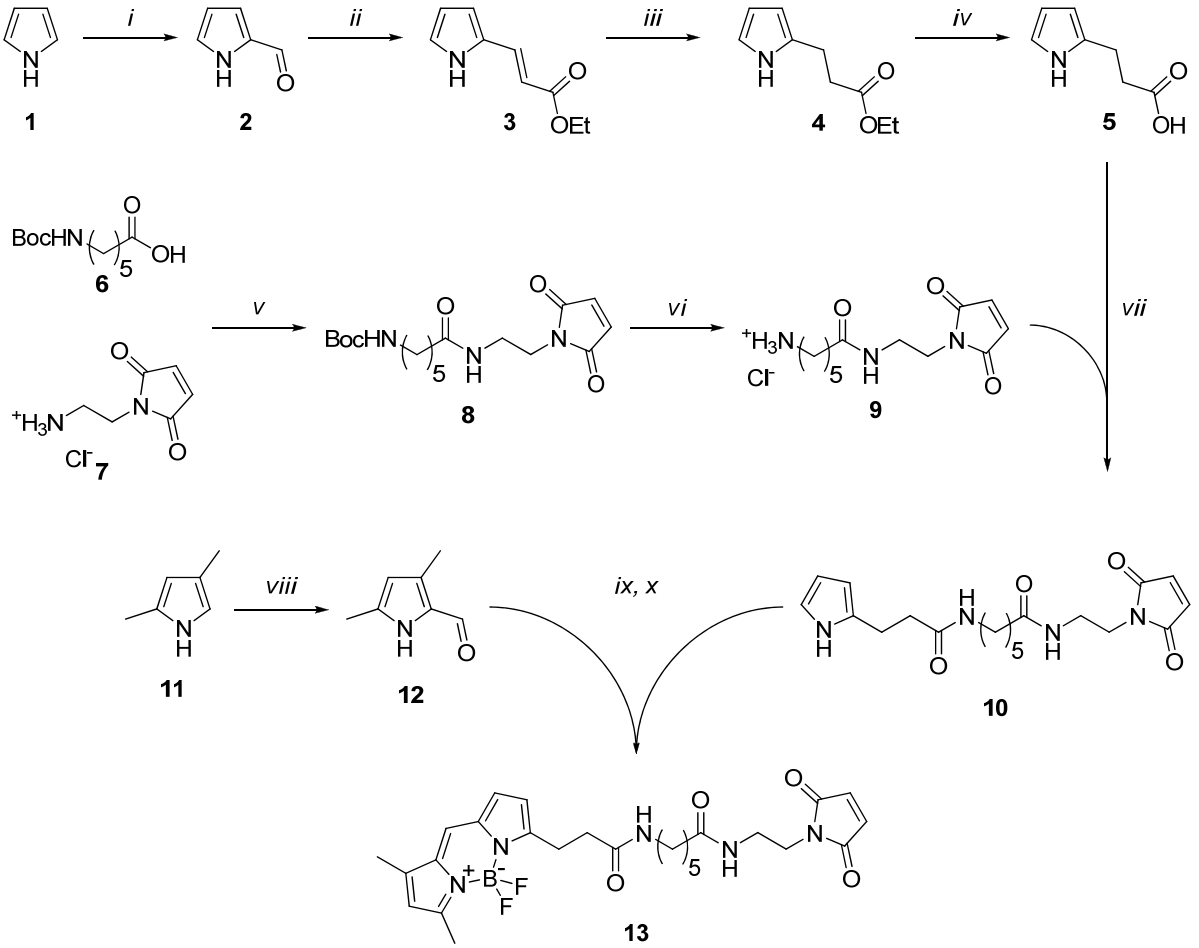


Figure 2: Procedure for the fluorescent labeling phosphorylated proteins with a thiol-reactive BODIPY-dye.

Results and Discussion

Synthesis:

The synthesis of a maleimide-functionalized BODIPY fluorescent-probe was started with the formylation of pyrrole **1** using a Vilsmeier-Haack reaction to give **2** in a yield of 82%.[12] Aldehyde **2**, in turn, was used in a Horner-Wadsworth-Emmons reaction using NaH and triethyl phosphonoacetate to give *E*-alkene **3**, which was used directly in the next reaction step.[13] The alkene double bond was reduced by treatment with $Pd/C/H_2$ and the ester was subsequently saponified to give carboxylic acid **5** in 94% overall yield based on three reaction steps. Then, Boc-aminohexanoic acid **6** was coupled to aminoethyl maleimide **7** in the presence of BOP/DiPEA as coupling reagents and the Boc-group was removed by treatment with HCl/Et_2O to give hydrochloride **9** in 57% yield. Next, carboxylic acid **5** was coupled to amine **9** using BOP/DiPEA in CH_2Cl_2 and **10** was obtained in a relatively low yield of 21%. An explanation for this low yield could be the reactivity of the maleimide moiety towards amines at elevated pH. Higher yields might be obtained when DCC is used, which reacts under neutral conditions. Then, 2,4-dimethyl-pyrrole **11** was formylated under Vilsmeier-Haack conditions and aldehyde **12** was obtained in quantitative yields. Subsequently, aldehyde **12** was reacted with pyrrole derivative **10** in the presence of $POCl_3$, and the formed bis-pyrrole was, without further purification, treated with $BF_3{\cdot}OEt_2$ under basic conditions to give the bright green fluorescent BODIPY dye **13** in a yield of 42%.

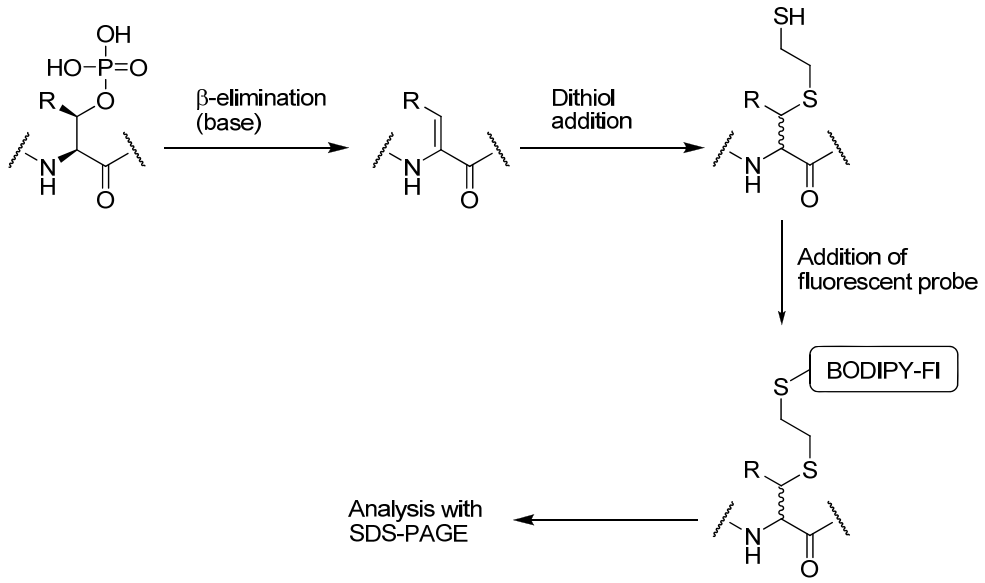


Scheme 1: Reagents and conditions. *(i)* $POCl_3$, DMF, DCE, 82%; *(ii)* NaH, triethyl phosphonoacetate, THF, quant; *(iii)* Pd/C, H_2, EtOAc/EtOH 1:1, 94%; *(iv)* 1N KOH, THF, quant; *(v)* BOP, DiPEA, CH_2Cl_2, 57%; *(vi)* HCl/Et_2O, quant; *(vii)* BOP, DiPEA, CH_2Cl_2, 21%; *(viii)* $POCl_3$, DMF, DCE, quant; *(ix)* $POCl_3$, CH_2Cl_2; *(x)* Et_3N, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 42%.

Protein labeling:

Prior to the fluorescent labeling of α-casein, the protein sample was denatured using a DTT solution, and subsequently all cysteine residues were capped with iodoacetamide to avoid undesired cysteine labeling. The initial experiments for protein labeling were done with the highly phosphorylated α-casein as model compound. α-Casein was first treated using the optimal enrichment conditions as described in chapter 2, which included treatment with 0.015 M $Ba(OH)_2$ for 10 min before neutralization with an equimolar amount of H_2SO_4 to precipitate barium salts.[10] Then, EDT was added to introduce a thiol-functionality to the protein. Finally, the protein was labeled with thiol-reactive probe **13** (Scheme 2). The

fluorescently labeled protein was then analyzed with SDS-PAGE and visualized with a fluorescent scan.



Scheme 2: Reaction circumstances required for the chemical modification and fluorescent labeling of phosphorylated proteins.

Analysis of the protein sample with gel-electrophoresis revealed a loss of protein from the sample, which was probably due to premature co-precipitation of α-casein during the reaction or during removal of precipitated barium-salts. Since these, previously described optimal β–elimination conditions from chapter did not yield the desired results, NaOH was used as a base instead. When NaOH was neutralized with H_2SO_4 to finish the β–elimination reaction no precipitates were formed, thus avoiding any premature loss of protein by co-precipitation (Figure 3).

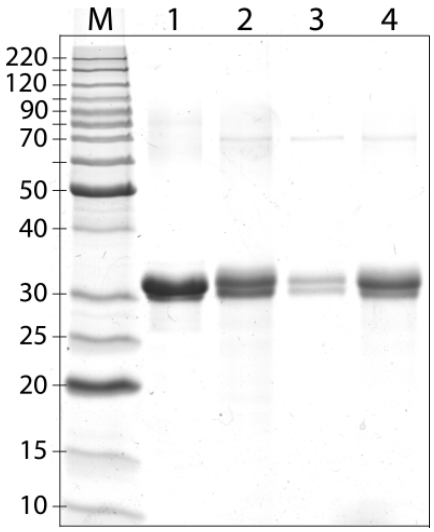


Figure 3: SDS-PAGE analysis of the β-elimination reaction visualized with coomassie blue stain. Lane **M**, molecular weight marker; lane **1**, native untreated α-casein; **2**, α-casein after denaturation with DTT and capping of cystein residues by treatment with iodoacetamide; lane **3**, α-casein sample after β-elimination with $Ba(OH)_2$; lane **4**, α-casein sample after β-elimination with NaOH.

After β-elimination with NaOH, α-casein was treated with EDT for functionalization of the β-eliminated product. Due to the insolubility of EDT in aqueous solution and its tendency to polymerize, the reaction with α-casein became less likely. Since MeOH was the solvent in the model phosphopeptide systems, which were described in chapter 2, apparently the insolubility problem did not arise earlier. Therefore, DTT was used , which is better soluble in water, for the thiol addition to the β-eliminated α-casein. This water soluble dithiol was added in huge excess (1500 equivalents) and the reaction mixture was stirred for 2 days followed by analysis of the mixture with gel-electrophoresis. The excess of DTT was removed from the mixture using microfiltration with a 5000 Da cut off (Figure 4).

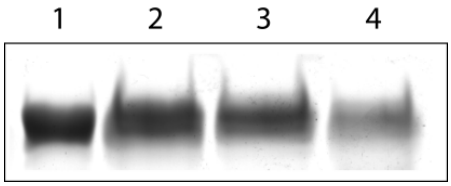


Figure 4: SDS-PAGE analysis of the α-casein with coomassie blue stain. Lane **1**, before treatment; lane **2**, after denaturation; lane **3**, after β-elimination with NaOH; lane **4**, after treatment with DTT.

Though there was some loss of protein material visible (Figure 4), the amount of protein in the sample was high enough to continue with the next reaction step, e.g. the addition of maleimide functionalized probe **13**. Under mild basic conditions, pH = 8.5, probe **13** (5-fold excess) reacted with α-casein, and after removal of the excess fluorescent material by microfiltration the sample was analyzed with gel-electrophoresis. A fluorescent protein band was visible with gel electrophoresis. However, analysis of the sample by mass spectrometry did not confirm functionalization of the protein with a fluorescent probe. Despite several attempts to adjust the protocol for fluorophore incorporation, conclusive results were not obtained.

Although the optimized β-elimination method, as described in chapter 2, was successful on small phosphorylated and glycosylated peptides, the technique could not be applied for the selective fluorescent labeling of phosphoproteins.

Conclusions

This chapter describes the synthesis of a maleimide-functionalized green fluorescent BODIPY dye. This dye was intended for the detection of phosphorylated proteins, which were thiol-functionalized through chemical modification. α-Casein was used as a phosphorylated model protein, to investigate this approach towards the fluorescent detection of phosphoproteins. Problems encountered in the chemical modification of proteins were the poor solubility and the low reactivity of the studied protein. There were some indications that α-casein was labeled with the synthesized BODIPY-probe, this presumed labelling however could not be confirmed by mass spectrometry since peaks which indicated that the protein was modified were not found.

Experimental section

General: All reactions were carried out at ambient temperature unless stated otherwise. All reagents were used as supplied from commercial sources unless stated otherwise. All solvents were stored on molecular sieves (4Å) while MeOH was stored on 3Å molecular sieves and THF was freshly distilled from $LiAlH_4$ prior to use. R_f values were determined by thin layer chromatography (TLC) on Merck

precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Hanessian's stain (cerium molybdate). Column chromatography was carried out using Silicycle UltraPure silicagel (40-63 μm). All reagents were purchased from Fluka, Sigma–Aldrich, and Acros and were used without further purification. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 absorbance detector. ESI-MS spectra were obtained in the positive ion mode on a Shimadzu instrument. MALDI-TOF spectra were recorded in positive-ion mode on an Applied Biosystems 4700 Proteomics Analyzer.

2-Formylpyrrole (2): $POCl_3$ (5.1 mL, 55 mmol) was added dropwise to ice-cold DMF (4.2 mL, 55 mmol) and the mixture was stirred for 15 min and subsequently diluted by the addition of DCE (15 mL) at room temperature. Then the mixture was cooled to 0°C and pyrrole (3.5 mL, 50 mmol) was added. After the addition was complete, the reaction mixture was refluxed for 20 min. After cooling to room temperature an aqueous solution of NaOAc (40 g in 60 mL) was added and the mixture was refluxed for 30 min. Finally, the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL) and the combined CH_2Cl_2 layers were washed with sat. aq. $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL). The CH_2Cl_2 solution was dried on $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane, 1/4 → 1/2 *v/v*) to give compound **2** as yellow crystals in 82% yield (3.9 g).

R_f = 0.43 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 10.40 (br s, 1H, N*H*), 9.51 (s, 1H, C*H*O), 7.18 (m, 1H, Ar*H*), 7.01 (m, 1H, Ar*H*), 6.53 (m, 1H, Ar*H*) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 179.4, 126.9, 121.8, 118.4, 111.3 ppm.

***(E)*-Ethyl 3-(1*H*-pyrrol-2-yl)acrylate (3):** A dispersion of 60% NaH in mineral oil (520 mg, 13 mmol) was washed with dry hexane (3 × 5 mL) and then suspended in dry THF (16 mL). At 0°C, triethyl phosphonoacetate (2.8 mL, 14 mmol) was added and the reaction mixture was stirred for 5 min before a solution of 2-formylpyrrole **2** (0.95 g, 10 mmol) in THF (10 mL) was added. The reaction mixture was stirred for 16 h at room temperature and subsequently quenched with sat. aq. NH_4Cl (30 mL). The mixture was extracted with MTBE (3 × 50 mL), the combined organic layers

were dried on $MgSO_4$ and after filtration concentrated *in vacuo*. Compound **3** was obtained in quantitative yield and was used in the next reaction steps without any further purification.
R_f = 0.50 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.26 (br s, 1H, N*H*), 7.57 (d, *J* = 15.9 Hz, 1H, C*H*=CH), 6.91 (m, 1H, Ar*H*), 6.55 (m, 1H, Ar*H*), 6.26 (m, 1H, Ar*H*), 6.07 (d, *J* = 15.7 Hz, 1H, CH=C*H*), 4.24 (q, *J* = 7.2 Hz, 2H, OCH_2), 1.31 (t, *J* = 7.2 Hz, 3H, OCH_2CH_3) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 167.9, 134.4, 128.4, 122.5, 114.2, 111.1, 110.7, 60.2, 14.3 ppm.

Ethyl 3-(1*H*-pyrrol-2-yl)propanoate (4): Crude compound **3** (10 mmol) was dissolved in a mixture of EtOAc/EtOH 1/1 (50 mL) and Pd/C (100 mg) was added. H_2 was passed through the reaction mixture for 3 h while stirring at room temperature. The mixture was filtered over Hyflo and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane, 1/3 *v.v*) to give compound **4** as an off-white solid in 94% yield (1.6 g).
R_f = 0.47 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.54 (br s, 1H, N*H*), 6.64 (m, 1H, Ar*H*), 6.08 (m, 1H, Ar*H*), 5.90 (m, 1H, Ar*H*), 4.14 (q, *J* = 7.2 Hz, 2H, OCH_2), 2.90 (t, *J* = 6.7 Hz, 2H, CH_2), 2.61 (t, *J* = 6.9 Hz, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2CH_3) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.0, 130.9, 116.7, 107.9, 105.4, 60.6, 34.5, 22.5, 14.0 ppm.

3-(1*H*-Pyrrol-2-yl)propanoic acid (5): Compound **4** (3.3 g, 20 mmol) was dissolved in THF (50 mL) and 1N KOH (50 mL) was added and the reaction mixture was stirred at room temperature for 4 h. THF was evaporated *in vacuo* and 1N HCl was added till pH 2 (indicator paper) was reached. The solution was extracted with EtOAc (3 × 100 mL), the combined organic layers were dried on $MgSO_4$ and after concentration *in vacuo* compound **5** was obtained in quantitative yield as a white solid.
R_f = 0.31 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H NMR (acetone D6, 300 MHz): δ = 10.58 (br s, 1H, COO*H*), 9.48 (br s, 1H, N*H*), 6.59 (m, 1H, Ar*H*), 5.97 (m, 1H, Ar*H*), 5.84 (m, 1H, Ar*H*), 2.87 (t, *J* = 7.4, 2H, CH_2), 2.61 (t, *J* = 7.7, 2H, CH_2) ppm. ^{13}C NMR (Acetone D6, 75.5 MHz): δ = 176.0, 131.2, 117.2, 108.4, 105.5, 34.7, 23.5 ppm.

***tert*-Butyloxycarbonyl-6-aminohexanoic acid-2-aminoethyl-maleimide (8):** To a suspension of BOP (4.4 g, 10 mmol), Boc-aminohexanoic acid (2.3 g, 10 mmol) and maleimide **7** (1.4 g, 10 mmol) in CH_2Cl_2 (100 mL), DiPEA (4.1 mL, 25 mmol) was added. The reaction mixture was stirred at room temperature for 16 h and then evaporated to dryness. The residue was dissolved in EtOAc (100 mL) and this solution was washed with 1N $KHSO_4$ (3 × 50 mL), sat. aq. $NaHCO_3$ (3 × 50 mL) and brine (50 mL). The EtOAc solution was dried on $MgSO_4$ and concentrated *in vacuo*. After purification by

column chromatography (CH_2Cl_2/MeOH, 95/5 *v/v*) compound **8** was obtained in 57% (2.0 g) yield a colourless oil.

R_f = 0.33 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.75 (s, 2H, C*H*=C*H*), 6.58 (br s, 1H, N*H*), 4.96 (br s, 1H, N*H*), 3.67 (t, *J* = 5.5 Hz, 2H, CH_2), 3.43 (q, *J* = 5.5 Hz, 2H, CH_2), 3.08 (q, *J* = 6.5 Hz, 2H, CH_2), 2.13 (t, *J* = 7.4 Hz, 2H, CH_2), 1.59 (m, 2H, CH_2), 1.43 (m, 11H, C(CH_3)$_3$, CH_2), 1.30 (m, 2H, CH_2) ppm.

6-Aminohexane-amidoethyl-maleimide hydrochloride (9): *tert*-Butyloxycarbonyl-6-aminohexanoic acid-2-aminoethyl-maleimide (2.0 g, 5.7 mmol) was suspended in HCl/diethyl ether (50 mL) and the solution was stirred at room temperature for 3 h. The reaction mixture was evaporated to dryness and coevaporated with $CHCl_3$ (3 × 10 mL) to give compound **9** in a quantitative yield (1.6 g) as a white solid.

R_f = 0.10 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H NMR (acetone D6, 300 MHz): δ = 8.44 (br s, 1H, N*H*), 6.95 (s, 2H, C*H*=C*H*), 3.54 (t, *J* = 5.6 Hz, 2H, CH_2), 3.30 (t, *J* = 5.8 Hz, 2H, CH_2), 2.86 (m, 2H, CH_2), 2.08 (m, 2H, CH_2), 1.71 (m, 2H, CH_2), 1.56 (m, 2H, CH_2), 1.36 (m, 2H, CH_2) ppm.

3-(1*H*-pyrrol-2-yl)propane-6-amidohexane-2-amidoethyl-maleimide (10): 3-(1*H*-pyrrol-2-yl)propanoic acid **5** (417 mg, 3.0 mmol), 6-aminohexane-amidoethyl-maleimide hydrochloride **9** (715 mg, 3.0 mmol), BOP (1.32 g, 3 mmol) and DiPEA (1.0 mL, 7.2 mmol) were dissolved in CH_2Cl_2 (25 mL) and the reaction mixture was stirred at room temperature for 3 h. The mixture was evaporated to dryness and the residue was redissolved in EtOAc (50 mL). This solution was washed with 1N $KHSO_4$ (3 × 25 mL), sat. aq. $NaHCO_3$ (3 × 25 mL) and brine (25 mL). The EtOAc solution was dried on $MgSO_4$ and concentrated *in vacuo*. After purification by column chromatography (CH_2Cl_2/MeOH, 95/5 *v/v*) compound **10** was obtained in 21% yield (240 mg) as a white solid.

R_f = 0.40 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.19 (br s, 1H, N*H*), 6.69 (s, 2H, C*H*=C*H*), 6.65 (m, 1H, Ar*H*), 6.06 (m, 1H, Ar*H*), 6.02 (br s, 2H, 2 × amide N*H*), 5.88 (m, 1H, Ar*H*), 3.66 (t, *J* = 5.4 Hz, 2H, CH_2), 3.43 (q, *J* = 5.5 Hz, 2H, CH_2), 3.22 (q, *J* = 6.6 Hz, 2H, CH_2), 2.91 (m, 2H, CH_2), 2.49 (t, *J* = 6.6 Hz, 2H, CH_2), 2.11 (t, *J* = 7.3 Hz, 2H, CH_2), 1.57 (m, 2H, CH_2), 1.46 (m, 2H, CH_2), 1.26 (m, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 173.4, 173.2, 170.9, 134.2, 131.5, 118.4, 116.7, 107.6, 105.1, 39.1, 38.6, 37.6, 36.5, 35.9, 28.9, 26.1, 24.6, 23.0 ppm.

3,5-Dimethyl 2-formylpyrrole (12): $POCl_3$ (2.05 mL, 22 mmol) was added dropwise to ice-cold DMF (2.1 mL, 22 mmol) and the mixture was stirred for 20 min and subsequently diluted by the addition of DCE (10 mL) at room temperature. Then the mixture was cooled to 0°C and 2,4-

dimethylpyrrole (2.06 mL, 20 mmol) was added. After the addition was complete, the reaction mixture was refluxed for 20 min. After cooling to room temperature, an aqueous solution of NaOAc (40 g in 60 mL) was added and the mixture was refluxed for 30 min. Finally, the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL) and the combined CH_2Cl_2 layers were washed with sat. aq. $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL).The CH_2Cl_2 solution was dried on $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane, 1/3 *v/v*) to give compound **12** as yellow crystals in quantitative yield (2.7 g).

R_f = 0.45 (CH_2Cl_2/MeOH, 95/5 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.95 (br s, 1H, N*H*), 9.47 (s, 1H, Ar*H*), 5.86 (s, 1H, Ar*H*), 2.32 (s, 3H, CH_3), 2.30 (s, 3H, CH_3) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 175.9, 138.3, 134.6, 128.7, 112.0, 13.1, 10.6 ppm.

BODIPY-Fl maleimide (13): 3,5-Dimethyl 2-formylpyrrole **12** (61 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (10 mL) and $POCl_3$ (46 μL, 0.5 mmol) was added. After stirring for 5 min at room temperature, 3-(1*H*-pyrrol-2-yl)propane-6-amidohexane-2-amidoethyl-maleimide **10** (180 mg, 0.5 mmol) was added. The reaction mixture was stirred for 5 h and subsequently concentrated *in vacuo* and coevaporated with $CHCl_3$ (2 × 10 mL). The residue was dissolved in a mixture of CH_2Cl_2 (100 mL) and Et_3N (0.7 mL, 5 mmol) and $BF_3{\cdot}OEt_2$ (0.6 mL, 5 mmol) was added. After 30 min of stirring, an additional amount of Et_3N (1.32 mL 10 mmol) and $BF_3{\cdot}OEt_2$ (1.2 mL, 10 mmol) was added and a bright green fluorescence appeared. Stirring was continued for another 3 h at room temperature. Then, the dark green solution was washed with H_2O (50 mL) and the organic solvent was dried with $MgSO_4$ and evaporated to dryness. After purification by column chromatography (CH_2Cl_2 → CH_2Cl_2/MeOH, 95/5 *v/v*) compound **13** was obtained as red crystals in 42% yield (110 mg).

R_f = 0.43 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.11 (s, 1H, C=C*H*), 6.89 (d, *J* = 3.85 Hz, 1H, Ar*H*), 6.69 (s, 2H, C*H*=C*H*), 6.28 (d, *J* = 4.1 Hz, 1H, Ar*H*), 6.12 (s, 1H, Ar*H*), 6.03 (m, 2H, 2 × amide N*H*), 3.66 (m, 2H, CH_2), 3.42 (m, 2H, CH_2), 3.25 (t, *J* = 7.4 Hz, 2H, CH_2), 3.18 (q, *J* = 6.9 Hz, 2H, CH_2), 2.61 (t, *J* = 7.4 Hz, 2H, CH_2), 2.55 (s, 3H, CH_3), 2.25 (s, 3H, CH_3), 2.08 (t, *J* = 7.4 Hz, 2H, CH_2), 1.55 (m, 2H, CH_2), 1.41 (m, 2H, CH_2), 1.25 (m, 4H, 2 × CH_2) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 173.3, 171.6, 170.9, 160.1, 157.5, 143.9, 134.1, 133.3, 128.3, 123.8, 120.4, 118.4, 117.4, 39.1, 38.6, 37.6, 36.2, 35.8, 29.6, 29.0, 26.2, 24.9, 14.9, 11.3 ppm. ESI-MS: calcd for $C_{26}H_{32}BF_2N_5O_4$: 527.38, found: *m/z* = 508.72 $[(M+H)-F]^+$, 528.35 $[M+H]^+$, 550.55 $[M+Na]^+$

General procedure for fluorescent protein labeling

Denaturing and thiol-capping: To a solution of α-casein (C6780, α-casein from bovine milk, Sigma-Aldrich) (10 μL, 400 pmol, 40 μM) in 50 mM NH_4HCO_3, an aqueous solution of 6 mM DTT (60 μL) was added and the reaction mixture was gently shaken at 45°C for 1 h (500 rpm) in an Eppendorf Thermomixer apparatus. To the resulting mixture an aqueous solution of 20 mM iodoacetamide (60 μL) was added and the mixture was shaken at 45°C for 1 h (500 rpm) in an Eppendorf Thermomixer apparatus. Excess of DTT and iodoacetamide was removed using microfiltration with a 5000 Da cut-off and the mixture was concentrated to ≈ 100 μL in 50 mM NH_4HCO_3. A sample of 100 pmol was used for analysis using a 12.5% SDS-PAGE gel.

β-Elimination and dithiol modification: To the denatured α-casein solution, an aqueous 0.3 M NaOH (6 μL, pH = 10) solution was added and the reaction mixture was shaken at 45°C for 3 h (1000 rpm) in an Eppendorf Thermomixer apparatus. A solution of 1.5 M DTT (5 μL) in 50 mM NH_4HCO_3 was added and the resulting mixture was shaken at 45°C for 48 h (1000 rpm). Excess of DTT was removed using microfiltration with a 5000 Da cut-off and the mixture was concentrated to ≈ 50 μL in 50 mM NH_4HCO_3. A sample of 100 pmol was used for analysis using a 12.5% SDS-PAGE gel.

Fluorescent labelling: To a solution of α-casein (400 pmol in 50 μL) in NH_4HCO_3 a solution of compound **13** (50 μL, 1 mg/mL) in ethanol was added and the mixture was shaken at 45°C for 16 h (1000 rpm) in an Eppendorf Thermomixer apparatus. Excess of compound **13** was removed using microfiltration with a 5000 Da cut-off and extensively washed with 50 mM NH_4HCO_3/EtOH (1/1, *v/v*) and the mixture was concentrated to ≈ 50 μL in 50 mM NH_4HCO_3. A sample of 100 pmol was used for analysis using a 12.5% SDS-PAGE gel.

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Chapter 4

Substrate profiling of protein kinase C isozymes α, θ and ζ using dynamic peptide microarrays

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Abstract: Protein kinases are increasingly important as drug targets. However, selective inhibition of kinases remains challenging due to the highly conserved catalytic domain of these enzymes. This may be particularly problematic for the potential drug target protein kinase C (PKC) of which 12 isoforms, with high homology, exist in humans. A promising way to design potential selective inhibitors is to target both the ATP binding site and the peptide substrate binding site of the kinase. Towards this goal the preferences of three different PKC isozymes (α, θ and ζ) for peptide substrates have been explored. An excellent way to determine kinase substrate preferences is the use of peptide microarrays. In this chapter the use of porous peptide microarrays and anti-phosphoserine/threonine antibodies allowed the identification of new isozyme specific PKC peptide substrates, that can serve as leads for the development of PKC bisubstrate based inhibitors.

Introduction

The Protein Kinase C (PKC) family is part of the AGC superfamily of serine/threonine kinases, which consists of 12 isoforms divided into three subfamilies, i.e. conventional PKC, novel PKC and atypical PKC, each of which depend on different cofactors.[1,2] The catalytic domain of all PKC isoforms shows a very high homology at the amino acid level of over 60% between all PKC subfamilies. Moreover, the homology within the subfamilies can be even higher than 80%.[3-5] PKC enzymes are involved in a wide range of cellular processes like gene expression and cell growth, and PKC dysfunctioning is related to several serious diseases like cancer and diabetes.[2,6,7] However, due to the high similarity between PKC isoforms the development of specific inhibitors for each of the PKC isoforms is very difficult. Such inhibitors would facilitate investigations of the role of PKC isoforms at cellular level. The catalytic domain of these kinases contains two binding pockets that can be targeted for kinase inhibition. These are the highly conserved ATP-binding site and the more diverse substrate binding cleft. Another promising approach for kinase inhibition is the design of bisubstrate based inhibitors, in which an ATP-binding site inhibitor is connected via a linker to a peptidic inhibitor of the substrate binding site. For the design of peptide substrate binding site inhibitors a good knowledge is required of the preferences of each PKC isozyme for a specific peptide substrate. A convenient and fast method for the screening of many substrates is the use of microarrays displaying multiple potential peptide substrates to the kinase in a single experiment. A peptide substrate that is well recognized by a single PKC isozyme can be used as a first lead compound for the design of isozyme selective PKC inhibitors. Furthermore, to date there is little knowledge about the preferences of certain kinases towards their peptide substrates. These peptide microarray experiments give insight into the cellular cascades in which these kinases are involved as well.

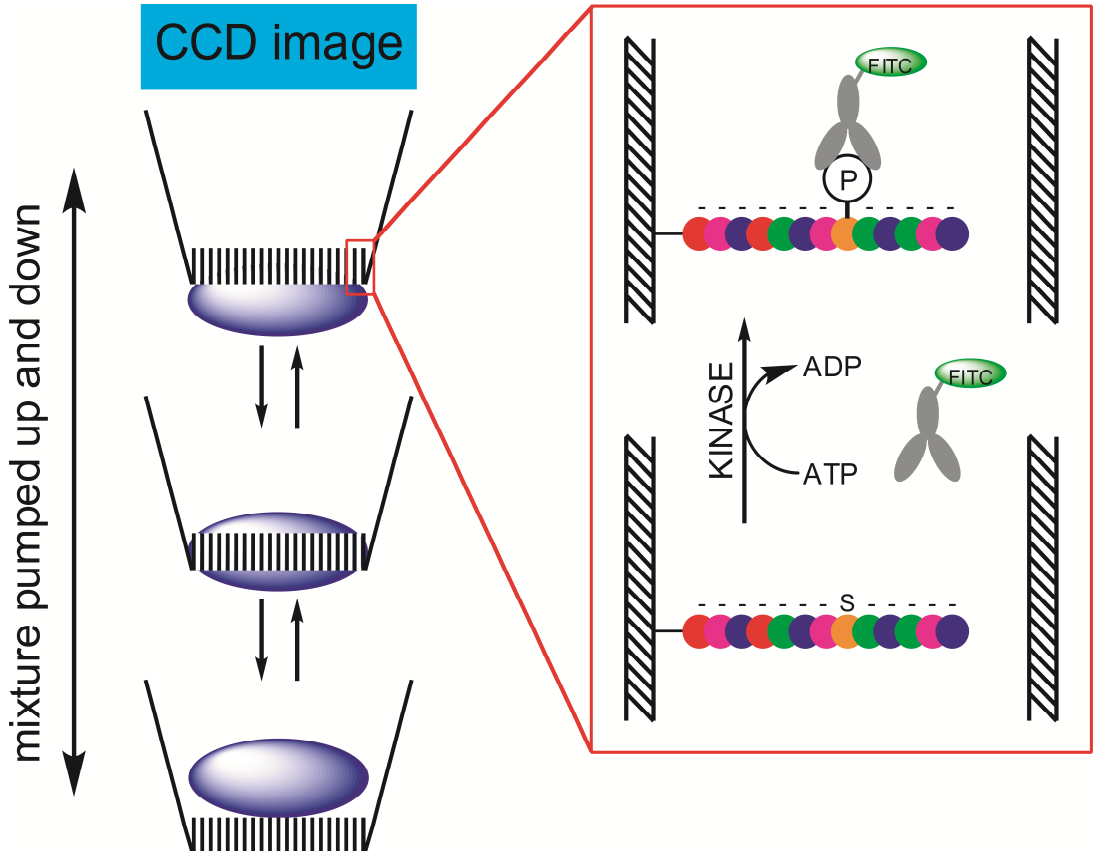

Figure 1: Schematic representation of the porous peptide microarrays used. Left: A cross-section of a dynamic microarray is shown through which an analyte mixture, containing the kinase, ATP, cofactors and detection antibodies, is pumped. Right: an enlargement of one of the pores of a dynamic microarray displaying a substrate that is phosphorylated, after phosphorylation the peptide is recognized by a fluorescently labeled antibody which can then be detected by a CCD camera.

In this chapter the development of a novel method for profiling kinase activity on porous microarrays is described. The arrays used in this study are decorated with 256 peptides per array, composed of endogenous kinase substrates containing serine/threonine phosphorylation sites derived from endogenous proteins. The use of the porous microarray material has the advantage that the analyte mixture can be pumped through the array to avoid diffusion limitation. After substrate phosphorylation, the resulting phosphopeptides are recognized by anti-phosphoserine/threonine antibodies which on their turn are detected by fluorescent secondary antibodies (Figure 1).[8] Thus far, this fluorescence based method has only been used for detecting tyrosine kinase activity using fluorescently-labeled anti-phosphotyrosine antibodies.[9,10] Serine/threonine containing peptide microarrays allowed the accurate and reproducible profiling of different PKC isozyme activities towards

multiple substrates in one single experiment. The peptide sequences found as described in this chapter served as substrates for phosphorylation and were used as starting point for further development of lead compounds towards isozyme-specific inhibitors. This process will be described in more detail in the following chapters.

Results and Discussion

Three PKC isozymes were selected for substrate profiling, one from each of the three PKC subfamilies, namely PKCα, PKCζ and PKCθ. PKCα is subject of cancer related research because of its ability to regulate phospholipase D, and plays an important role in hematology.[11,12] PKCζ was selected as a member of the atypical PKCs and is known to be involved in long-term memory processes and regulation of the immune system.[13,14] Finally, PKCθ was chosen because it recently emerged as a pharmacological target in human T cell leukemias.[6]

The peptide microarrays used, contained 256 peptide kinase substrate sequences, each consisting of 13 amino acid residues.[15] All peptide sequences were derived from known endogenous kinase substrate proteins. In order to detect the phosphorylation event, a mixture of three anti-phosphoserine/threonine antibodies was used. Since currently no antibody is available that is completely impervious to the peptide sequences flanking the phosphorylated serine or threonine residue. Next, two fluorescent secondary antibodies were used for the detection and imaging of the bound anti-phosphoserine/threonine antibodies. The porous chip material[16,17] allowed the kinase mixtures to be pumped up and down, which provided optimal reaction conditions for substrate phosphorylation. During the experiment fluorescent images of the microarray were recorded in real time and the detected fluorescence for each of the substrates was quantified. This quantification was performed simultaneously for all peptides on the microarray, and for each of the PKC isoforms, which resulted in a comprehensive picture of phosphorylation profiles and differences in substrate specificities of the used PKC isoforms. Furthermore, the ability to run multiple microarrays in a single experiment and the simultaneous incubation of four arrays reduced experimental variation.

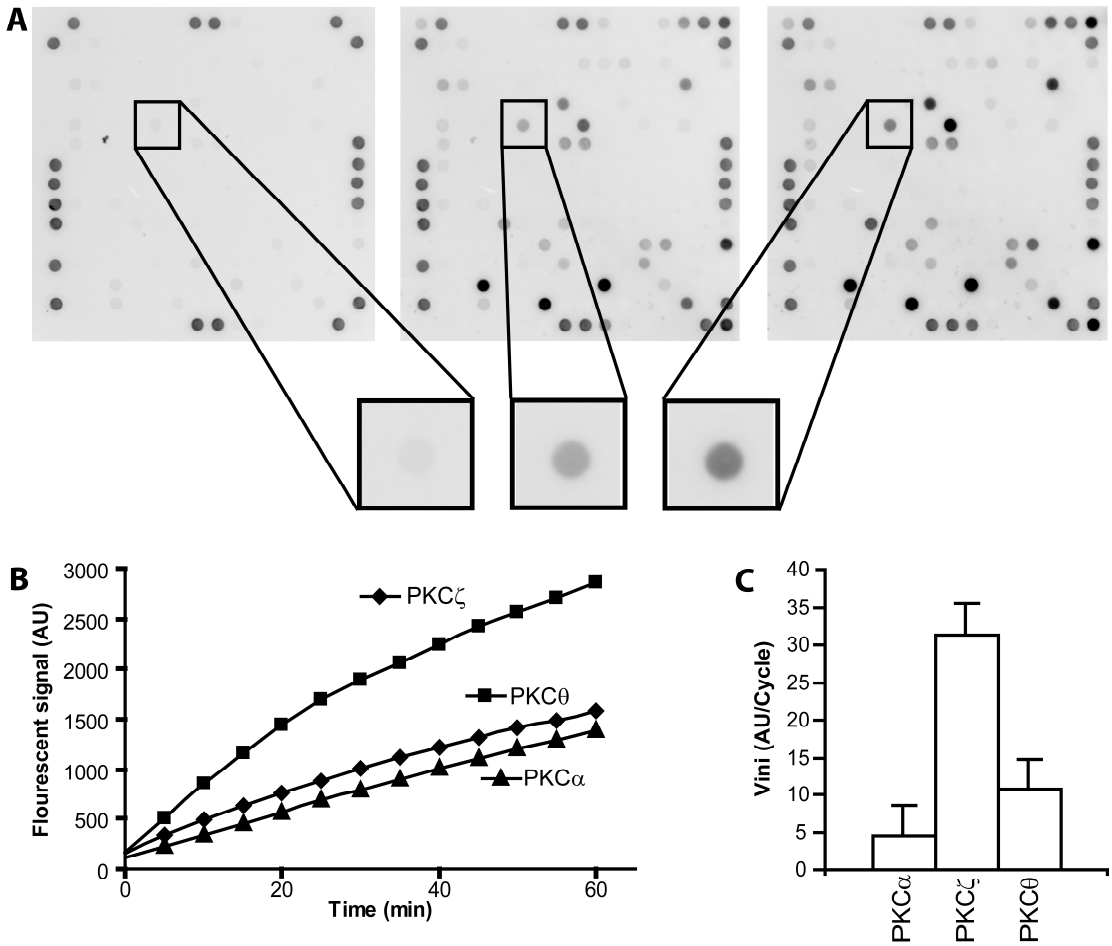

Figure 2: An example of phosphorylation of a peptide microarray by PKCθ. Each array is surrounded by 20 fluorescent reference spots for gridfinding of the CCD camera. **A:** Images of the peptide microarray after 0, 30 and 60 minutes showing increase in signal intensity. **B:** The increase in signal intensity, of the highlighted spot with peptide sequence SRLRRRASQLKIT, plotted in time versus signal intensity for every PKC isoform used. **C:** The slope of every curve (Figure 2B) showing clear differences in v_{ini} for PKCα, PKCζ and PKCθ. Data are expressed as AU/Cycle and the error bars indicate the SEM with n=3.

The analyte mixture, consisting of the protein kinase, cofactors, ATP at high concentration (100 μM) and antibodies, was incubated on the chip for 60 min and the change in fluorescence was monitored by a built-in fluorescence microscope which recorded an image every 2.5 min. Increase in fluorescence was monitored for each substrate (Figure 2A) and plotted against time (Figure 2B). Using the Bionavigator software package the initial velocity (v_{ini}) was derived by calculating the slope of the phosphorylation curve of each of the peptide substrates (Figure 2B). The substrate of which the phosphorylation is shown in Figure 2B was best recognized by PKCζ, which is an indication that this peptide substrate is

a good starting point for a substrate based inhibitor. For a quick overview, of the peptide substrates displayed on the microarray, which were effectively phosphorylated by a protein kinase, the slopes of all v_{ini}'s can be plotted in a bargraph. The assay was carried out in triplicate for all three PKC isozymes (Figure 2C).

Table 1: Substrates phosphorylated by PKC on the microarray.

Sequence	Origin[a]	v_{ini} ratio[b]		
		α	ζ	θ
KKKFRTPSFLKKS	Beta-adducin 706-718 (P35612)[18]	1	2	1
AILRRPTSPVSRE	32 kDa protein 212-224 (Q71BY8)	1	4	7
QKRREILSRRPSY	cAMP response element-binding protein 122-134 (P16220)[19]	1	7	2
EILSRRPSYRKIL	cAMP response element-binding protein 126-138 (P16220)[19]	1	6	1
RHIVRKRTLRRLL	Epidermal growth factor receptor 671-683 (P00533)[20]	1	1	1
QQKIRKYTMRRLL	Receptor tyrosine-protein kinase erbB-2 679-691 (P04626)	1	1	1
NPLMRRNSVTPLA	6-phosphofructo-2-kinase 454-466 (Q16875)	1	11	1
SRLRRRASQLKIT	GABA(A) receptor subunit beta-2 427-439 (P47870)[21]	1	7	2
FMSSRRQSVLVKS	Glutamate receptor 708-720 (Q13002)	2	1	2
PSRFNRRVSVCAE	cAMP-dependent protein kinase type II-alpha regulatory subunit 91-103 (P13861)	1	5	3
NPDEEEEDTDPRV	cAMP-dependent protein kinase type II-alpha regulatory subunit 106-118 (P13861)	1	4	2
LSAFRRTSLAGGG	Myosin-binding protein C 268-280 (Q14896)[22]	2	2	1
RSGSRRGSFDATG	Plectin-1 4635-4647 (Q15149)	3	11	1
GQKFARKSTRRSI	Pleckstrin 106-118 (P08567)[23]	2	1	1
RSAIRRASTIEMP	Cardiac phospholamban 9-21 (P26678)	1	1	1
ELNKDRTSRDSSP	Retinoblastoma-like protein 2 955-967 (Q08999)	1	1	1
IAKRRRLSSLRAS	40S ribosomal protein S6 228-240 (P62753)	2	1	2
GSRSRTPSLPTPP	Microtubule-associated protein tau 523-535 (P10636)[24]	1	1	2
RKSKRRNSEFEIF	Tryptophan 5-hydroxylase 1 51-63 (P17752)	2	1	1
NQNSRRPSRATWL	Vitronectin precursor 390-402 (P04004)	1	1	1

[a] References that describe the protein as a PKC substrate. The ranges refer to the location of the peptide in the original protein. Between brackets, the Swiss-Prot accession number is given. [b] Normalized selectivity ratio of peptide phosphorylation by three PKC isozymes.

Analysis of the phosphorylation curves for all peptide substrates on the microarray revealed that 20 peptides were phosphorylated by the PKC isozymes used in this study (Table 1). The found substrates were compared with known substrates as they are reported in several protein databases like Swissprot (http://www.expasy.org/uniprot) and the human protein reference database (http://www.hprd.org). These databases revealed that several phosphorylated peptide substrates as found in the microarray experiment, were known to be phosphorylated by all three PKC isoforms (Table 1). More interestingly, however, also peptide substrate sequences were identified that were previously unknown to be phosphorylated by one or more of the PKC isoforms (Table 1). Peptide sequences that are selectively recognized and phosphorylated by a single PKC isozyme can be used as a starting point for the development of selective inhibitors of that kinase. The initial velocity for phosphorylation of all substrates was calculated for every individual PKC isoform (Figure 3). Sequence comparison showed that most of the phosphorylated peptides derived from, both known and yet unknown PKC substrates, contain the consensus sequence R-R-X-S/T.[14] Despite the high similarity between all three PKC isoforms, a different phosphorylation pattern for all three isozymes was observed, with PKCζ as the kinase which displayed the most prominent differences in its phosphorylation pattern compared to PKCα and PKCθ (Figure 3, Table 1). For several peptides, e.g. EILSRRPSYRKIL derived from cAMP response element-binding protein and SRLRRRASQLKIT from GABA(A) receptor subunit beta-2, a significant higher relative activity of PKCζ was observed, whereas the phosphorylation by PKCα or θ was barely detectable. Though PKCα and PKCθ revealed different substrate phosphorylation patterns the differences were found to be more subtle.

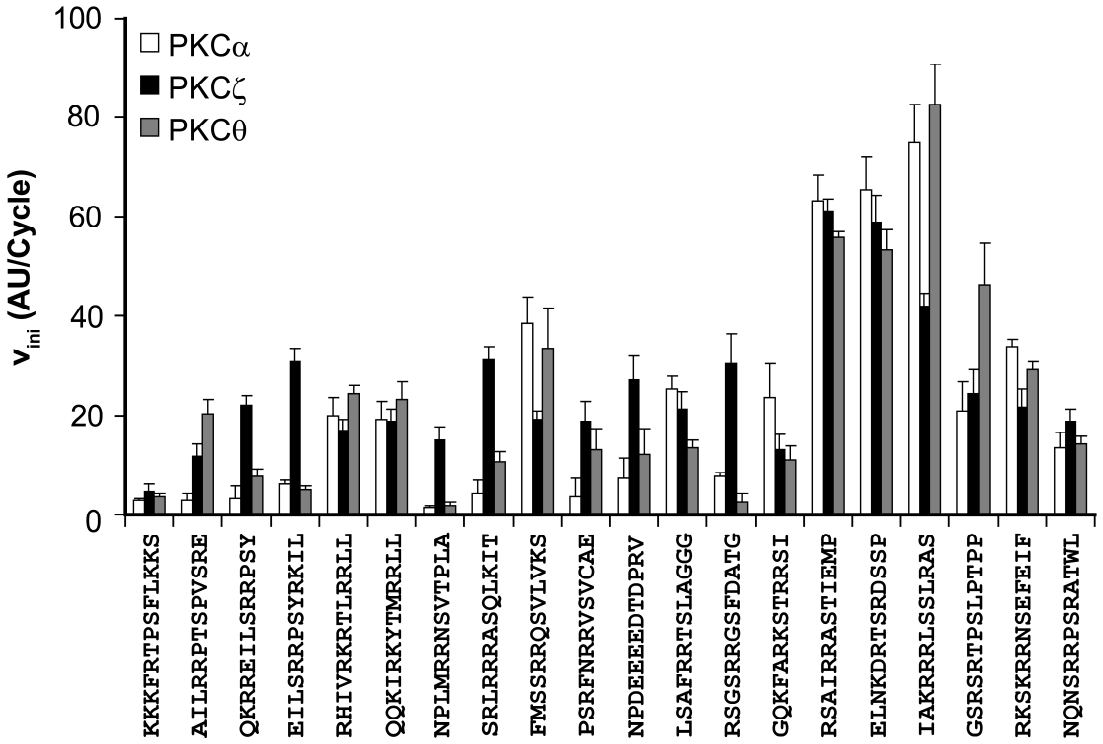

Figure 3: Calculated initial velocity (v_{ini}) of all phosphorylated substrates on the peptide microarray by three PKC isoforms. The data shown represent mean values of three v_{ini} determinations and the error bars indicate the standard error of the mean (SEM).

To confirm that the observed phosphorylation pattern shown in Figure 3 was indeed kinase-dependent, inhibition experiments were performed in the presence of staurosporine, which is a known, highly potent ATP competitive PKC inhibitor. In these experiments staurosporine was used at a concentration range from 1 nM to 1 μM, in the presence of a high ATP concentration (100 μM). The calculated v_{ini} of a phosphorylated substrate was plotted against the staurosporine concentration yielding inhibition curves from which IC_{50} values were derived (Figure 4). The IC_{50} value of inhibition of the PKC-isoforms was calculated for peptide substrate QKRREILSRRPSY, which corresponded to the amino acid residues 122-134 derived from cAMP response element-binding protein. Staurosporine showed a low inhibition towards PKCζ with an IC_{50} in the μM concentration range, whereas PKCα and PKCθ were inhibited by staurosporine with IC_{50} values in the lower nM concentrations. Since staurosporine has been extensively investigated as a protein kinase inhibitor, the obtained IC_{50} values were compared to inhibition results of PKC with staurosporine described in literature, and a good correlation was found.[25,26] These data

confirm that the observed phopshorylation pattern is indeed dependent on the presence of a protein kinase.

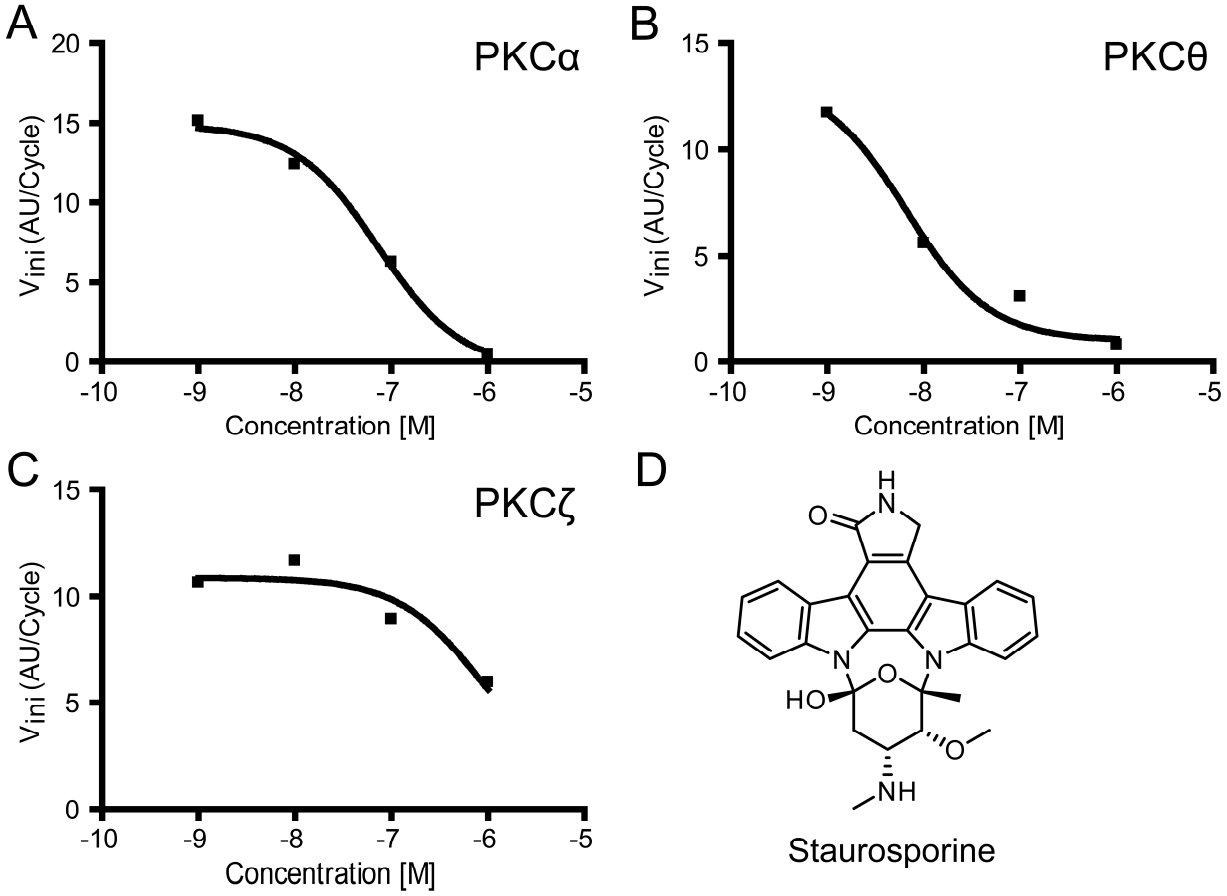

Figure 4: Phosphorylation of peptide substrate QKRREILSRRPSY with PKCα, θ and ζ and staurosporine concentrations from 1 nM up to 1 μM. **A**: IC_{50}: 73 nM of staurosporine on PKCα. **B**: IC_{50}: 6.5 nM of staurosporine on PKCθ. **C**: IC_{50}: 0.87 μM of staurosporine on PKCζ.

Conclusions

We have shown that porous peptide microarrays are a convenient tool for profiling serine/threonine kinases for peptide substrate specificities as well as for the discovery of novel kinase substrates. The porous microarray material allowed the real-time monitoring of the phosphorylation process and clearly revealed differences in phosphorylation profiles between the different PKC isoforms. Furthermore, it was shown that the application of an anti phosphoserine/threonine antibody cocktail is a useful method for the quantification of phosphorylation of a serine or threonine containing substrate. In addition to substrate

profiling of kinases, peptide microarrays were also very well suited for inhibition experiments, which has been shown by phosphorylation of the three PKC isozymes in the presence of the non-selective inhibitor staurosporine. Finally, these peptide microarrays might be used in lead finding approaches for uncovering new peptide-based inhibitors of protein kinases, and a proof of principle will be described in the next chapters.

Experimental section

General: Microarray experiments were performed using PamChip peptide arrays run on a PamStation4 instrument (PamGene, 's Hertogenbosch, the Netherlands). Four temperature-controlled peptide chips were run in parallel by pumping the sample up and down through the 3-dimensional porous chip. Data was captured by real-time imaging of the fluorescence signal by CCD imaging. The serine/threonine kinase PamChip arrays comprised 256 different peptides. Each peptide represents a 13 amino acid sequence, that is derived from a known phosphorylation site from Swissprot and Phosphobase databases (JPT Peptide Technologies GmbH). The Protein Kinase C isoforms were purchased from Invitrogen. Prior to application of the sample, the chips were blocked using a solution of 0.5% BSA (Bovine Serum Albumin, Fraction V, Calbiochem, Germany), and washed 2 times using kinase reaction buffer. During 60 min incubation at 30°C, real-time images were taken automatically every 2.5 min. Images were analysed by BioNavigator software (PamGene, 's Hertogenbosch, the Netherlands). The fluorescence intensities were expressed as arbitrary units.

Substrate profiling: A 25 μL solution was used containing 20 ng kinase, 0.125 mg/mL 3-sn-phosphatidyl-L-serine (Fluka BioChemika), Abl reaction buffer (50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij35) (New England Biolabs, Ipswich, USA) and 0.1 mg/mL BSA solution (New England Biolabs, Ipswich, USA). The primary antibodies #2351, #9611 and #9391 (Cell Signalling Technology, Danvers, USA), secondary fluorescent antibodies Goat-anti-Rabbit IgG-FITC (4 μg/mL) and Goat-anti-Mouse IgG-FITC (4 μg/mL) (Santa Cruz Biotechnology, Santa Cruz, USA) were incubated with 100 mM ATP. Samples were placed on the PS4 STK-microarray (Pamgene, The Netherlands) and during 60 min incubation at 30°C, real-time images were taken automatically every 2.5 min.

Staurosporine inhibition experiment: Staurosporine (Acros Organics, Geel, Belgium) dissolved in DMSO at a concentration range of 1 μM – 1 nM was added to the incubation solution used in the kinetic phosphorylation experiment. The final DMSO concentration was 4% in all incubations.

Samples were placed on the peptide microarray and during 60 min incubation at 30°C. Real-time images were taken automatically every 2.5 min.

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Chapter 5

Synthesis of acetylene-functionalized ATP-competitive protein kinase inhibitors

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Abstract: This chapter describes the synthesis of a series of compounds that are functionalized with an alkyne moiety. The alkyne functionality enables these compounds to be used in the Cu(I)-mediated click reaction. The newly synthesized compounds represent derivatives of known ATP-competitive kinase inhibitors as well as modified ATP-analogs.

Introduction

Protein kinases are widely recognized as important drug targets involved in many serious diseases, such as cancer and diabetes.[1-4] These phosphorylating enzymes play a pivotal role in cellular signal transduction, and many diseases are characterized by changes in the phosphorylation pattern or alterations in the expression level of a protein kinase. An example is presented by the Protein Kinase C (PKC) family, a kinase family consisting of 12 highly homologous members, which are related to various diseases.[5-8] Therefore, it is of no surprise, that kinase inhibitors are very interesting as potential drugs and indeed a sizeable number of them have reached the drug market.[4] However, most current potent kinase inhibitors only bind to the ATP binding site of a particular kinase, which obviously has a large structural similarity to the ATP binding domains of other kinases.[9-11] In order to achieve inhibition of PKC, a variety of compounds have been designed, synthesized and evaluated for their properties as potential inhibitors. In addition, all compounds have been designed in such a way that they can be turned into bisubstrate based inhibitors (Figure 1). For this purpose click chemistry will be used, and therefore all ATP-competitive inhibitors have to be decorated with an acetylene functionality (Figure 2).[12-14]

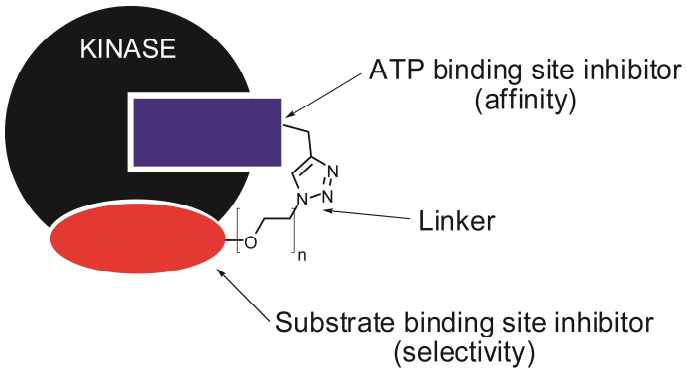


Figure 1: Schematic representation of a kinase inhibited by a bisubstrate based inhibitor interacting with the ATP-binding site and the substrate-binding site.

The first class of compounds was derived from staurosporine (Figure 2), which is a natural product isolated from *Streptomyces staurosporeus*. Staurosporine is an inhibitor with high affinity for the ATP-binding site of protein kinases, and therefore it has been used as lead compound towards the design of potent protein kinase inhibitors in many studies.[15] Despite its high affinity, the lack of selectivity of these staurosporine analogs has not

resulted in a further development as an anti-cancer drug. Removal of the pyranose moiety from staurosporine and replacing it by simpler alkyl chains, already has resulted in a higher selectivity for PKC over other kinases, while maintaining the high affinity. The carbon-carbon bond connection between both C2 and C2' indole moieties of staurosporine was not necessary for the affinity and its removal increased the selectivity for PKCs further.[16-18] Staurosporine analog **1** has been extensively described in the literature and served as a starting point for the synthesis of **2** in which one of both methyl groups has been replaced by an acetylene containing functionality (Figure 2).[18] The acetylene function is required to make the molecule suitable for bisubstrate based inhibitor formation using click-chemistry, which will be used described in chapter 6.

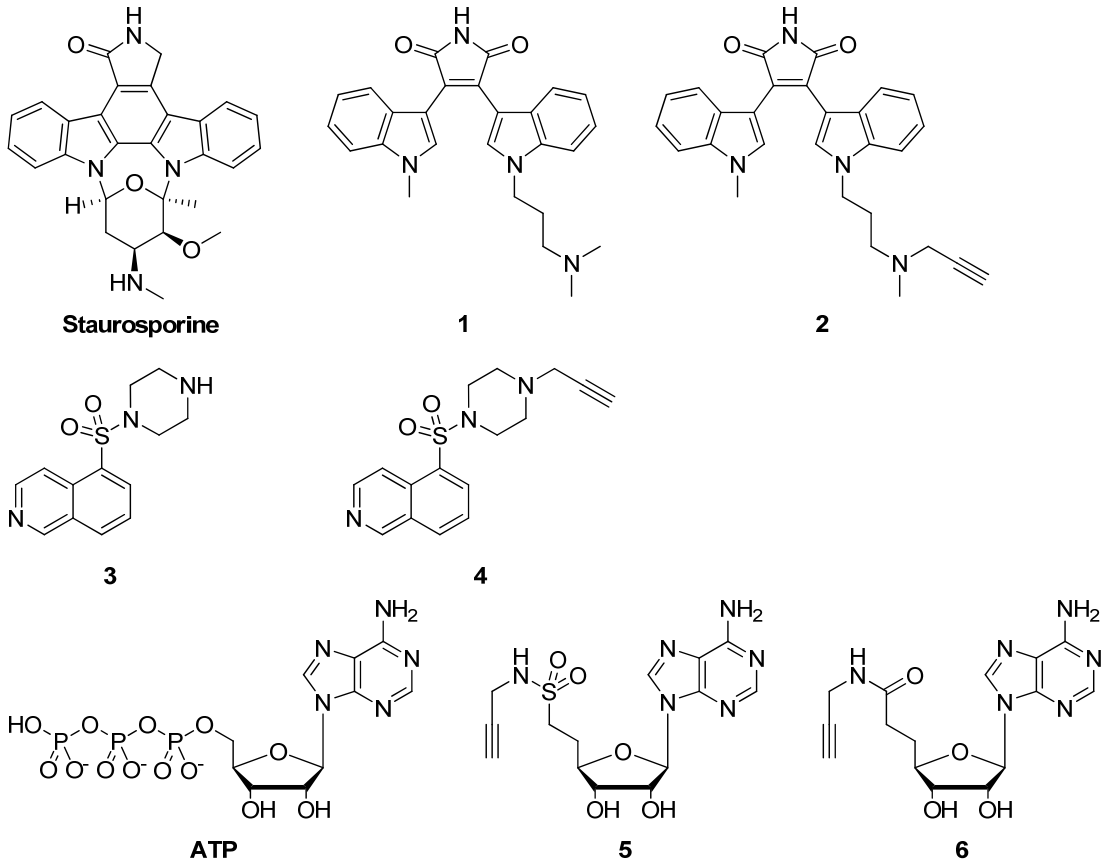


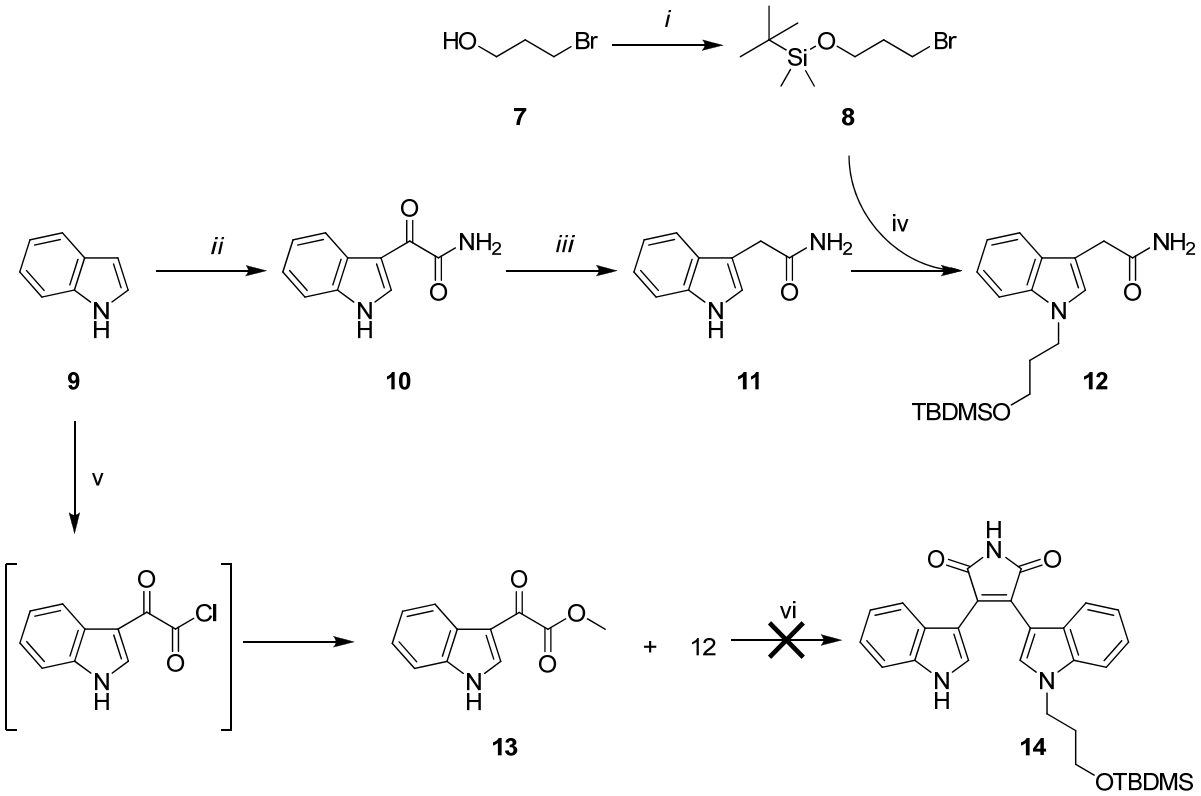
Figure 2: Broad spectrum protein kinase inhibitor staurosporine, Staurosporine mimetic **1** and acetylene functionalized staurosporine mimetic **2** suitable for bisubstrate formation. Protein kinase inhibitors isoquinoline sulfonamide **3**, and acetylene-functionalized isoquinoline sulfonamide **4**. Acetylene-functionalized analogs **5** and **6** with an amide and sulfonamide moiety to mimic a phosphate functionality of ATP.

Another class of compounds that has been studied for the inhibition properties against protein kinases consist of the isoquinoline sulfonamide **3** (Figure 2).[19] Though the reported affinities for PKC are less good than those of the staurosporine analogs the easy accessibility of the molecules justifies the synthesis of these derivatives. Compound **4** was decorated with an acetylene containing functionality for bisubstrate based inhibitor formation as will be described in chapter 6 (Figure 2). Besides ATP-competitive inhibitors known from literature, attempts have been made for the synthesis of analogs **5** and **6**. The triphosphate moiety in ATP is very labile, and therefore analogs have been made to replace a phosphate moiety by a stable amide- or sulfonamide linkage. Both ATP analogs were functionalized with alkyne functionalities for bisubstrate based inhibitor formation (Figure 2).

Results and Discussion

*Synthesis of staurosporine mimic **2***

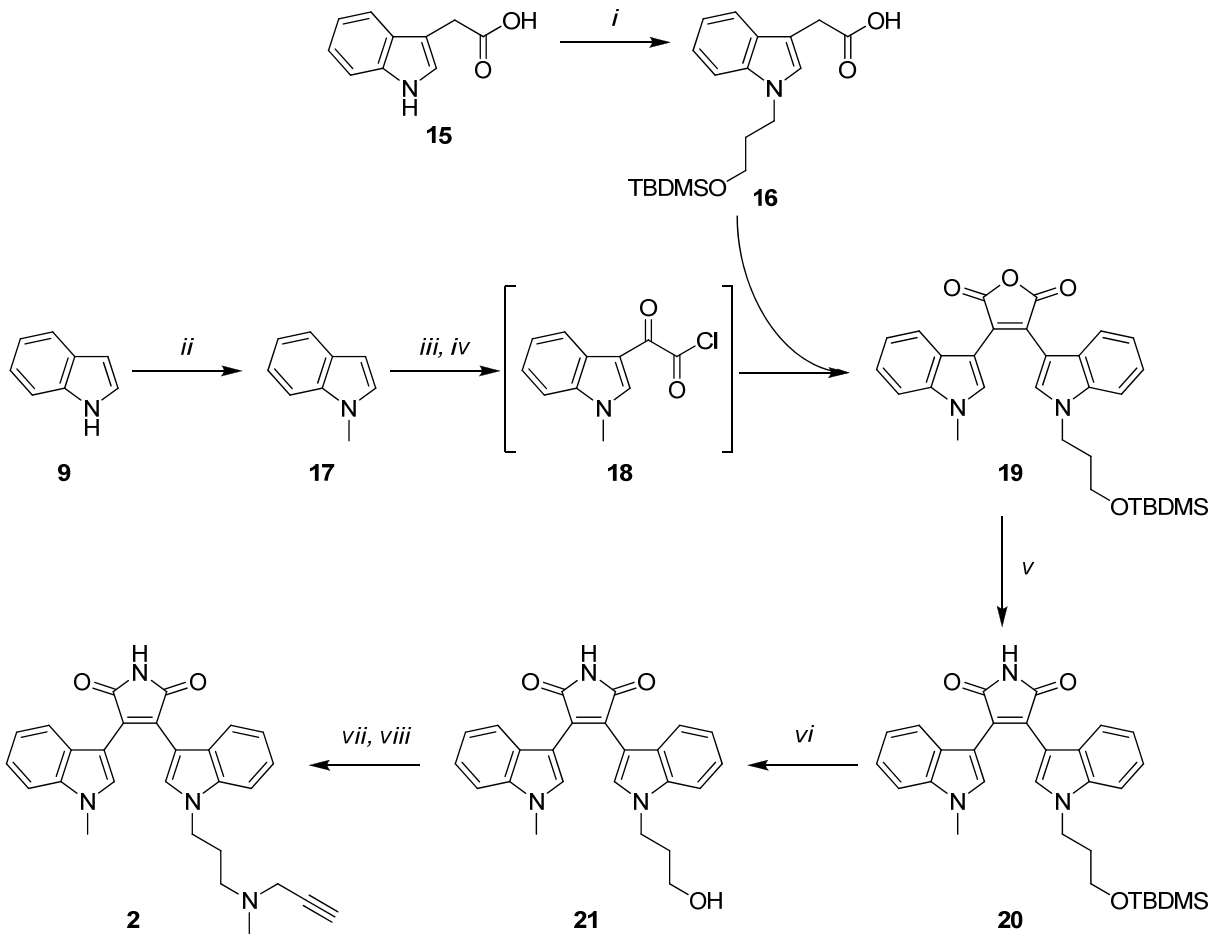
The initial attempt for the synthesis of compound **2** was based on the procedures published by Faul *et al.*[20,21], as is shown in Scheme 1. In their approach the synthesis started with the acylation of indole **9** with oxalyl chloride. Treatment of the highly reactive glyoxyl chloride intermediate with either NH_3 or MeOH resulted in the formation of glyoxylamide **10** or methyl indole-3-glyoxylate **13**, respectively. Compound **10** was reduced to its corresponding acetamide **11** by treatment with $Pd(OH)_2/NaH_2PO_2$.[20] Unfortunately however, the reduction reaction was very sluggish which resulted in a highly variable isolated yield of compound **11**. In the next step, *N*-alkylation of indole acetamide **11** was performed by treatment with NaH to deprotonate the NH-indole and subsequent reaction with bromide **8** gave compound **12** in 82% yield. Finally, carboxamide **12** and methyl indole-3-glyoxylate **13** were treated with KO^tBu as described in the literature procedure to obtain maleimide **14**.[21] However, the desired compound could not be isolated from the crude reaction product even after several attempts. Therefore, it was decided to abandon this synthetic approach in favor of the procedure as described by Davis *et al.*[22,23], as is shown in Scheme 2.



Scheme 1: Reagents and conditions. *(i)* TBDMS-Cl, pyridine, CH_2Cl_2, 88%; *(ii)* **a.** oxalyl chloride, THF, **b.** NH_4OH, quant; *(iii)* $Pd(OH)_2$, NaH_2PO_2, dioxane, reflux, 20 – 76%; *(iv)* NaH, **8**, DMF, 82%; *(v)* **a.** oxalyl chloride, THF, **b.** MeOH, 62%; *(vi)* KO^{t}Bu, THF.

This second synthetic approach started with the *N*-methylation of indole **9** in the presence of NaH/MeI to give compound **17** in quantitative yield, basically as described by Davis *et al.*[22,23] Then, commercially available indole acetic acid **15** was converted into **16** by reaction with NaH and bromide **8**. In the next step, the maleimide precursor **19** was prepared via an acylation of *N*-methyl indole **17** with oxalyl chloride. The resulting acid chloride **18** was not isolated and immediately treated with acid **16**, to afford cyclic anhydride **19** in 20% yield. This low yield could be ascribed to the loss of the TDMBS group in the acidic reaction environment of the acylation reaction. Subsequently, maleimide **20** was formed by reaction of anhydride **19** with hexamethyldisilazane (HMDS) and obtained in 91% yield. The TBDMS group of compound **20** was removed by treatment with HCl/EtOH to give alcohol **21** in quantitative yield. Finally, compound **21** was converted into its corresponding triflate with triflic anhydride/pyridine and propargyl amine **2** was

directly synthesized by reaction with commercially available *N*-methyl-*N*-propargylamine in 62% yield (Scheme 2).

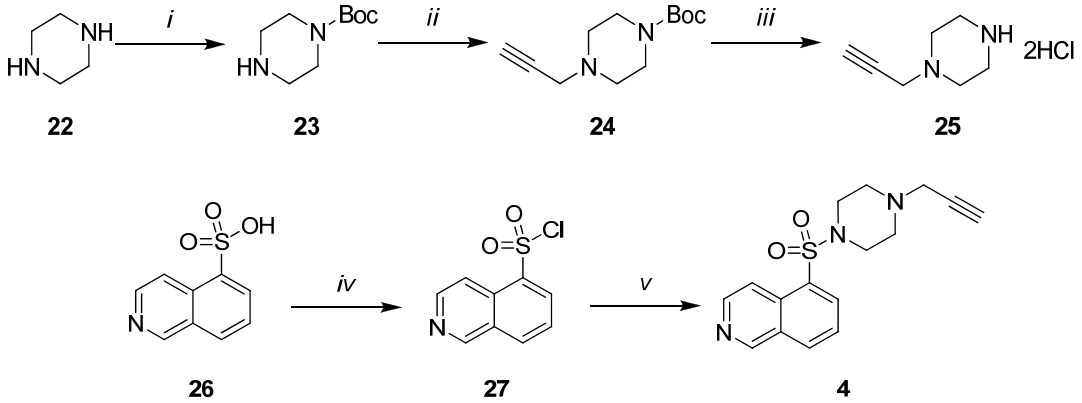


Scheme 2: Reagents and conditions. *(i)* NaH, $Br(CH_2)_3OTBDMS$, DMF, 89%; *(ii)* NaH, MeI, dry THF, quant; *(iii)* oxalyl chloride, CH_2Cl_2; *(iv)* compound **16**, Et_3N, CH_2Cl_2, 20%; *(v)* HMDS, MeOH, DMF, 91%; *(vi)* 2% HCl in EtOH, quant; *(vii)* Tf_2O, pyridine, CH_2Cl_2; *(viii)* *N*-methyl-*N*-propargyl amine, 62%.

*Synthesis of isoquinoline sulfonamide **4**:*

Not only staurosporine mimics have been described as synthetically easy accessible ATP-competitive protein kinase inhibitors, also 5-isoquinoline sulfonamide derivative **3** (Figure 2), has been reported as a protein kinase C inhibitor.[19] Despite the higher IC_{50}-values of PKC inhibition than those reported for the staurosporine derivatives, the easy synthesis of this class of PKC inhibitors makes them interesting compounds in the development of potential PKC isozyme selective bisubstrate based inhibitors. To this end, compound **4** was

synthesized starting from piperazine **22** as is shown in Scheme 3. Piperazine was treated with Boc_2O to obtain the mono-Boc protected diamine **23** which on its turn was *N*-alkylated with propargyl bromide in the presence of DIPEA to give compound **24** in a good overall yield of 70% over 2 steps.[24] The Boc functionality of alkyne **24** was removed by treatment with HCl/diethyl ether to give *N*-propargyl piperazine dihydrochloride **25** in quantitative yield. For coupling to amide **25**, 5-isoquinolinesulfonic acid **26** was converted into its corresponding sulfonylchloride **27** by treatment of the acid with thionyl chloride in nearly quantitative yield. Thus, compound **27** was coupled to amine **25** in the presence of triethylamine as a base to give alkyne **4** in a satisfactory yield of 65%.

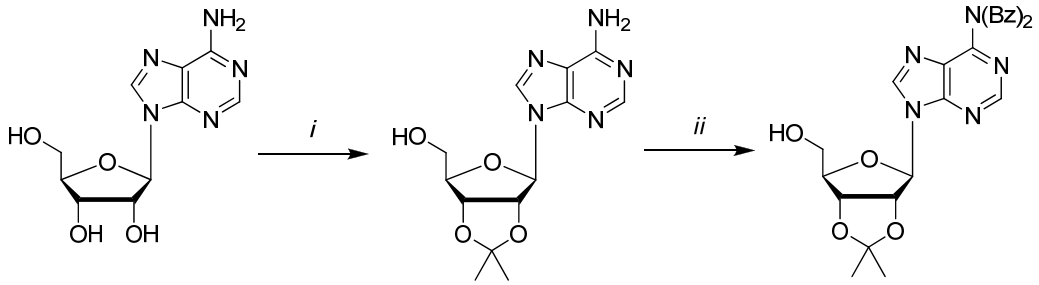


Scheme 3: Reagents and conditions. *(i)* Boc_2O, dioxane, 89%; *(ii)* propargyl bromide, DiPEA, $CHCl_3$, 79%; *(iii)* HCl/diethylether, quant. *(iv)* $SOCl_2$, DMF cat., reflux, 98%; *(v)* compound **25**, Et_3N, CH_2Cl_2, 65%.

Synthesis of ATP-based propargyl amide/-sulfonamide derivatives

As a third class of potential ATP-competitive protein kinase inhibitors, two ATP-analogs have been designed and synthesized starting from commercially available adenosine **28**. For both ATP-analogs **5** and **6** (Figure 1), protected intermediate **30** served as a general building block. Synthon **30** was prepared in two steps starting form adenosine as shown in Scheme 4. In first instance, the 2'- and 3'-hydroxyl functionalities were protected via an isopropylidene moiety by reacting adenosine with acetone in the presence of diethylorthoformate under acidic conditions to give compound **29** in quantitative yield.[25] Secondly, the exocyclic amine of **29** was converted into the bis-protected benzoyl derivative **30** in a good yield of 74%.[26] To avoid esterification with the 5'-hydroxyl

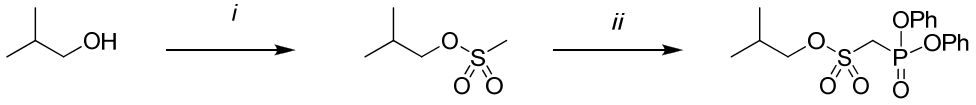
functionality, the alcohol was *in-situ* protected as a TMS-ether prior the addition of benzoyl chloride. Furthermore, due to the electron donating properties of silicon it is thought that the *in-situ* formed Si-N intermediate improves the reactivity of the exocyclic amine thereby increasing the overall yield of the bis-protection step.



28 **29** **30**

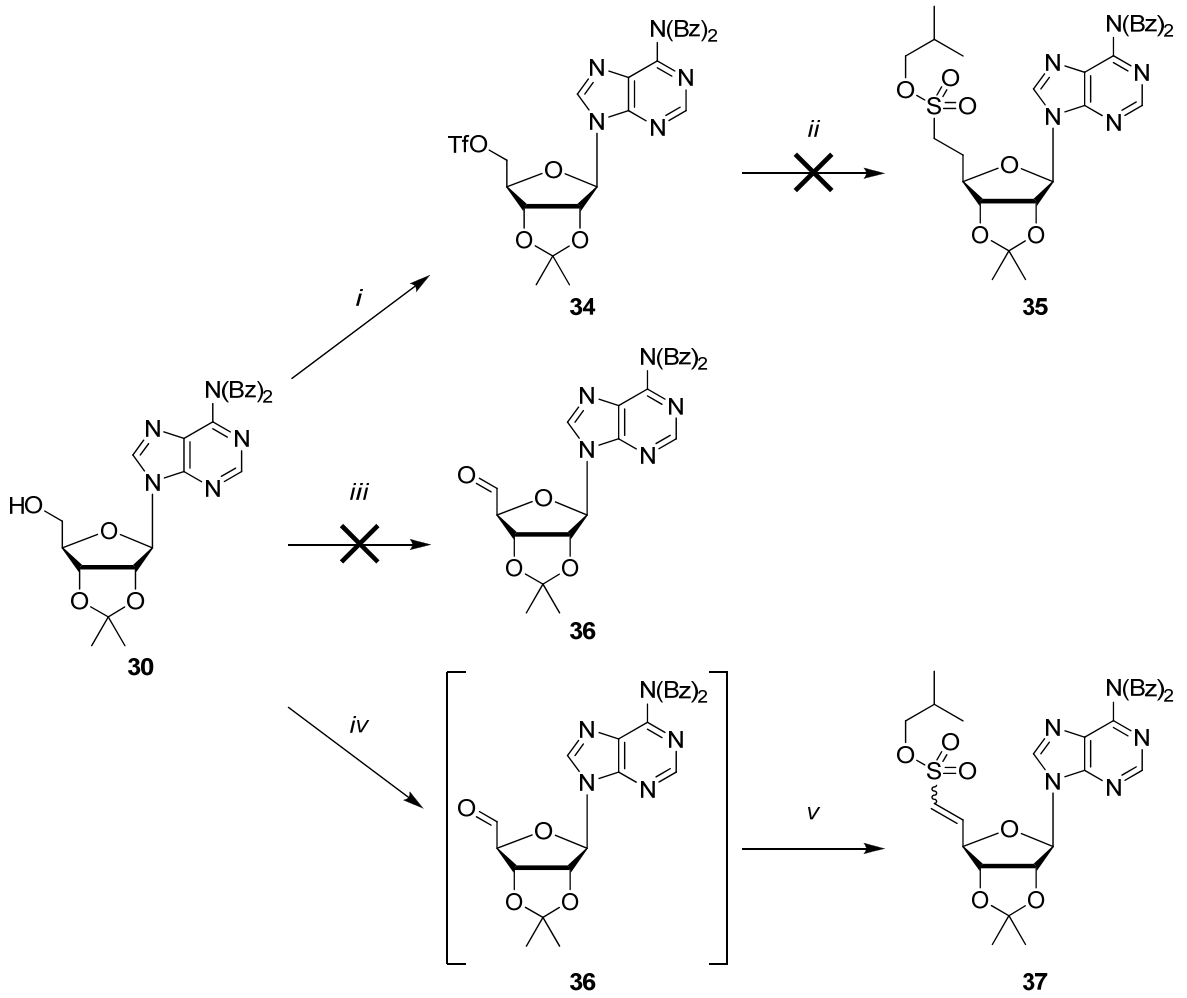
Scheme 4: Reagents and conditions. *(i)* triethyl orthoformate, *p*TsOH, acetone, quant; *(ii)* TMS-Cl, pyridine, benzoyl chloride, 74%.

For the synthesis of a sulfonamide-modified adenosine derivative, building block **33** had to be synthesized first (Scheme 5). Therefore, iso-butanol **31** was treated with methanesulfonyl chloride, and mesylate **32** was subsequently deprotonated with BuLi followed by reaction with diphenylchlorophosphate and the Horner-Wadsworth-Emmons-reagent **33** was obtained in 52% overall yield.[27,28]



31 **32** **33**

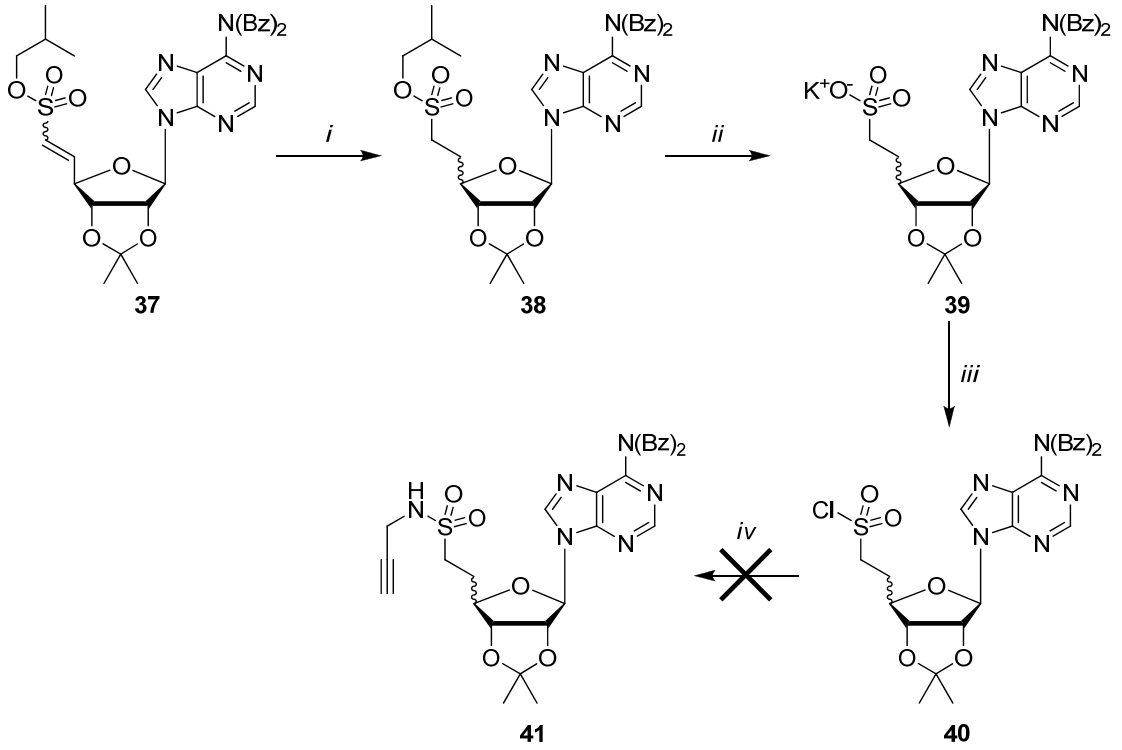
Scheme 5: Reagents and conditions. *(i)* methanesulfonyl chloride, Et_3N, CH_2Cl_2, 97%; *(ii)* nBuLi, diphenylchlorophosphate, THF, 54%.



Scheme 6: Reagents and conditions. *(i)* Tf_2O, pyridine, CH_2Cl_2, 0°C; *(ii)* BuLi, THF, HMPA, compound **33**, -78°C; *(iii)* Dess-Martin periodinane, CH_2Cl_2; *(iv)* DMSO, DCC, chloroacetic acid, oxalic acid/MeOH; *(v)* NaH, THF, compound **33**, 0°C, 68%, *E*/*Z* 2:1 (over two reaction steps).

The first attempt to incorporate a sulfonate moiety was by activation the 5'-hydroxyl functionality by treatment of alcohol **30** with triflic anhydride (Scheme 6). Then, triflate **34** was treated with a preformed sulfonate **33**/BuLi reaction mixture for obtaining the desired compound **35**. Unfortunately, sulfonate **35** could not be isolated. Therefore, in another attempt, the 5'-hydroxyl functionality of **30** was oxidized to the aldehyde via a Dess-Martin periodinane reaction. Although TLC analysis indicated full conversion of the starting material, the reaction product was an intractable material and work-up was not trivial since the material turned out to be insoluble in almost all solvents except DMSO. Gratifyingly, however, the aldehyde **36** could be obtained *in situ* via the Pfitzner-Moffat oxidation, and then directly used in a Horner-Wadsworth-Emmons reaction with building block **33** to give

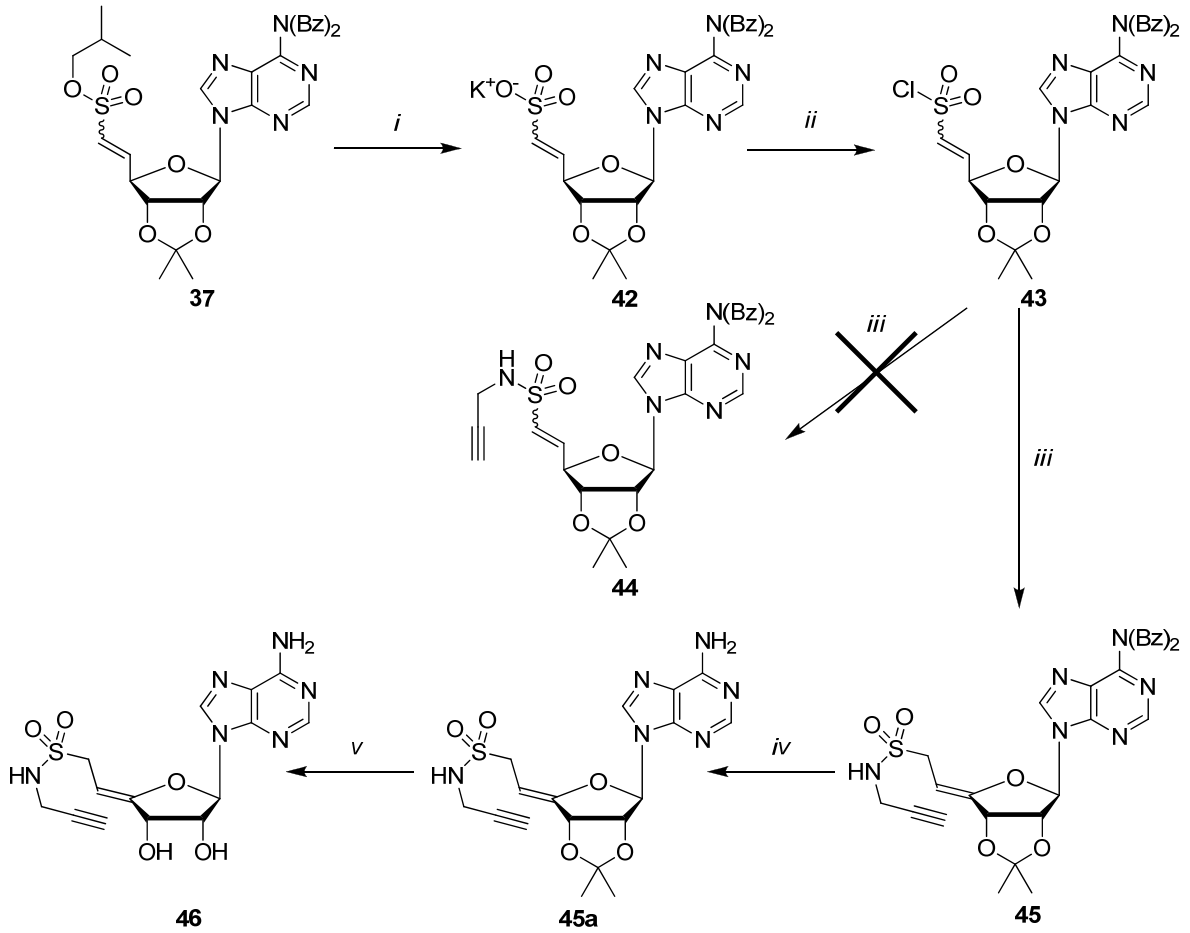
sulfonate **37** in 68% overall yield[29] (Scheme 6). After careful analysis by NMR, it turned out that compound **37** consisted of an *E*/*Z* mixture in a ratio of 2:1, which was rather unexpected since a HWE reaction is usually rather stereoselective towards the *E* enantiomer.



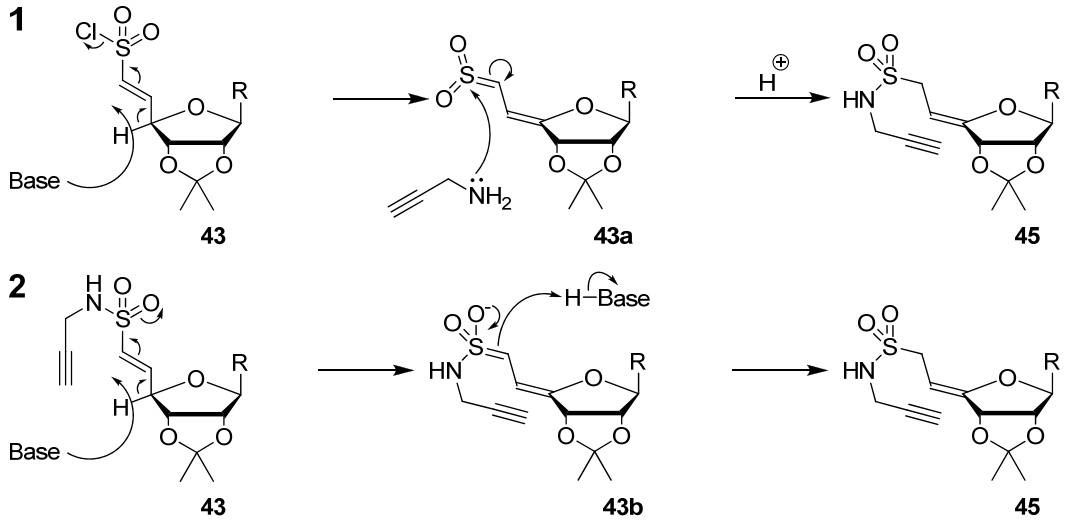
Scheme 7: Reagents and conditions. *(i)* $NaBH_4$, ethanol, 59%; *(ii)* KI, acetone; *(iii)* $COCl_2$, DMF, CH_2Cl_2, 30% (over 2 steps); *(iv)* propargylamine monohydrochloride, NMM, CH_2Cl_2.

Next (Scheme 7), the double bond in α,β-unsaturated sulfonate ester **37** was reduced followed by saponification of the sulfonate ester **38** to obtain sulfonate **39** as its potassium salt. After preparation of sulfonylchloride **40**, the final step, that consisted of the preparation of the desired sulfonamide **41**, failed for unknown reasons.

The synthesis of the more rigid ATP-analog **44**, still containing the α,β double bond, started by treatment of sulfonate ester **37** with KI in acetone and the resulting potassium salt was successfully converted into sulfonylchloride **43** by treatment with phosgene in an overall yield of 35%. Then, sulfonylchloride **43** was treated with propargylamine in the presence of NMM as a base in order to obtain sulfonamide **44** (Scheme 8).



Scheme 8: Reagents and conditions. *(i)* KI, acetone, reflux; *(ii)* $COCl_2$, DMF, CH_2Cl_2; *(iii)* propargylamine monohydrochloride, NMM, CH_2Cl_2, 24%; *(iv)* 7M NH_3, MeOH, 92%; *(v)* TFA/H_2O 5:1, 8%.



Scheme 9: Two possible reaction mechanisms of the substitution of a sulfonylchloride with propargyl amine yielding the undesired 4'-5' unsaturated adenosine derivative **45**.

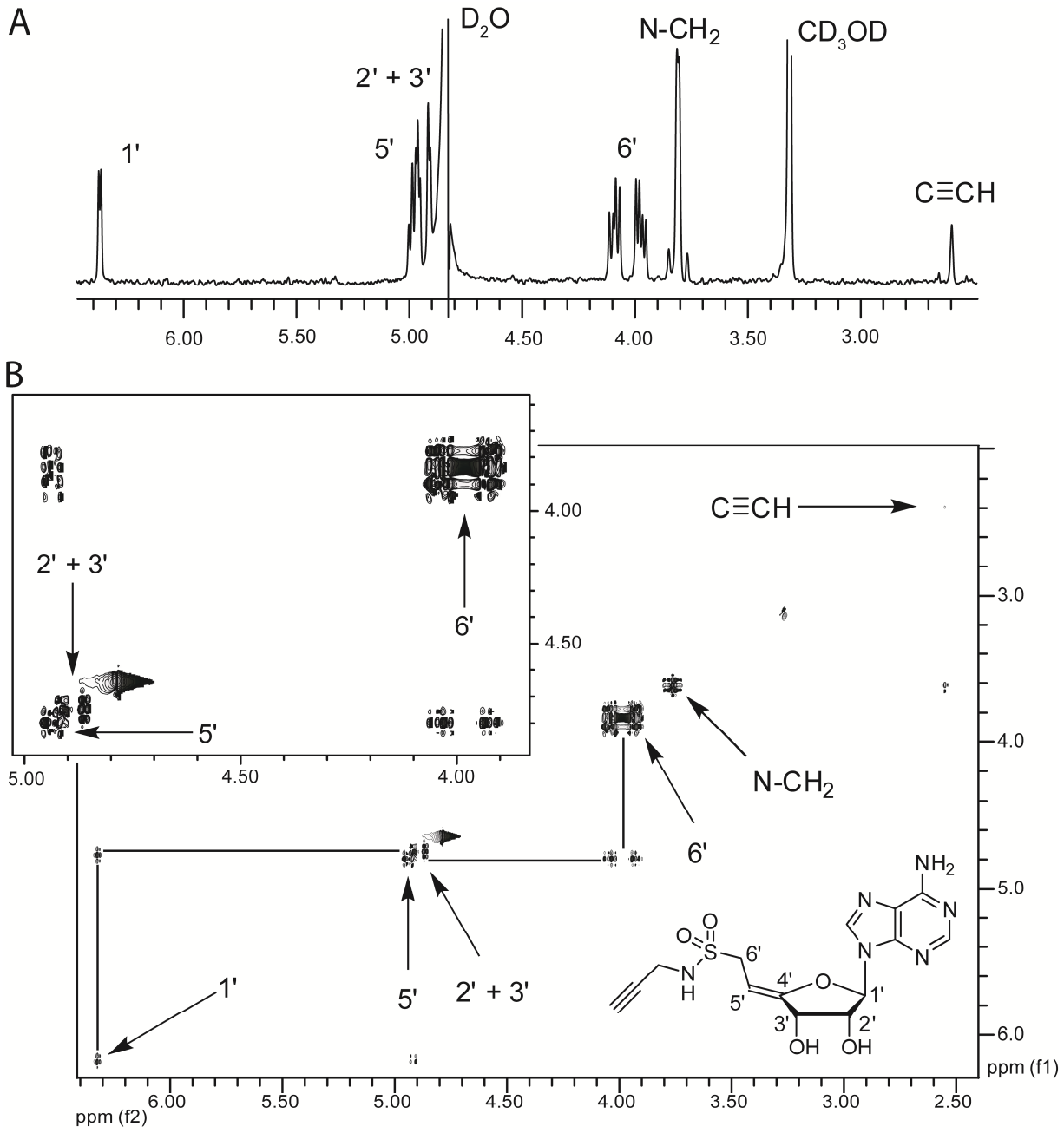


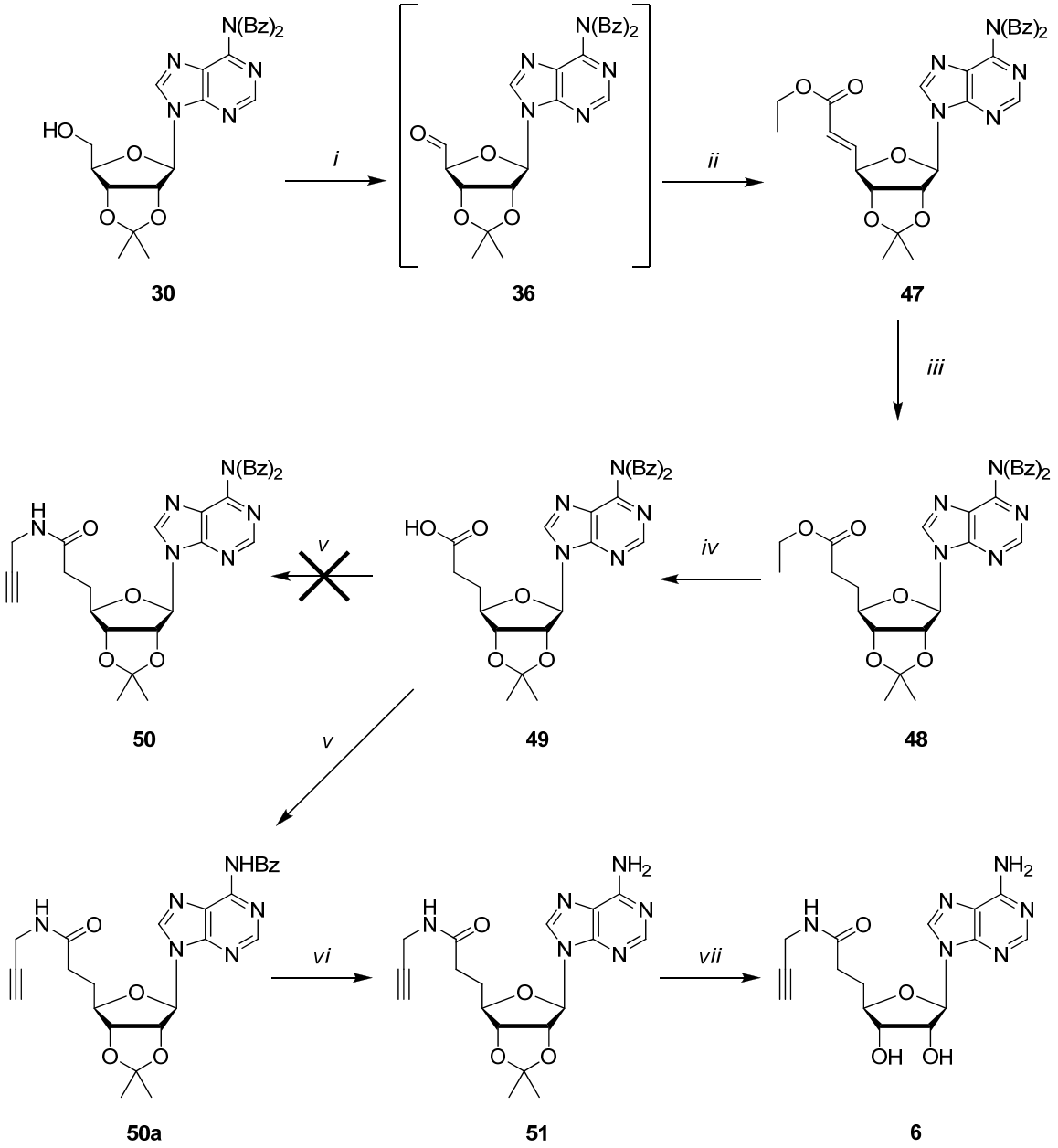
Figure 3: A: ^{1}H-NMR of **46** in D_2O/CD_3OD, 9:1 *v/v*; **B:** COSY-NMR of **46**, where a coupling is visible between the protons of 1', 2' and 3', and the protons of 5' and 6', furthermore, the 4' proton is absent.

Unexpectedly, compound **44** was not obtained and compound **45** was isolated instead in which the double had shifted from the 5'-6' to the 4'-5' position as was shown by NMR, *vide infra*. The formation of product **45** can possibly be explained by two mechanisms

(Scheme 9). As the first explanation, during the substitution reaction, the 4'-proton is abstracted by base and the free electron pair forms a double bond between the 4'- and 5'-carbon atoms thereby giving rise to the formation of sulfene **43a** by elimination of the chloride ion. Subsequently, propargylamine attacks the electron-deficient sulfene resulting in an electron shift to the 5'-position, which leads to protonation during aqueous workup yielding sulfonamide **45** (route 1, Scheme 9). The second proposed mechanism assumes a rearrangement of the desired sulfonamide **44** (route 2, Scheme 9). The 4'-proton is abstracted by base and the free electron pair forms a double bond between the 4'- and 5'-carbon atoms thereby giving rise to the formation of intermediate **43b**. After an electron shift to the 5'-position and subsequent protonation during aqueous workup, sulfonamide **45** is formed.

To proceed in the synthesis, sulfonamide **45** was first deprotected by treatment with concentrated NH_3/MeOH followed by a TFA-mediated removal of the isopropylidene moiety to furnish compound **46**. After purification by preparative HPLC, alkyne **46** was obtained in a rather low yield of 8% due to the intrinsic acid-lability of the molecule.

Since it was not possible to synthesize sulfonamide **5**, it was attempted to prepare the corresponding amide derivative **6** (Figure 2, *vide supra*). The synthesis of adenosine analog **6** started with compound **30** which was subjected to a Pfitzer-Moffat oxidation (Scheme 10). The corresponding aldehyde **36** is highly unstable and therefore immediately used in the next step featuring a Wittig-reaction in the presence of (carboethoxy methylene)triphenylphosphorane to give alkene **47** in 90% yield as the pure *trans*-isomer. After the reduction of the double bond by treatment with Pd/C/H_2 and subsequent saponification of the ethyl ester with NaOH, acid **49** was obtained in 78% overall yield. Compound **49** was coupled to propargyl amine using BOP/DiPEA as coupling reagents and the corresponding amide **50a** was obtained in a good yield of 83%. Finally, compound **50a** was subsequently treated with concentrated NH_3 in MeOH to remove the benzoyl functionality, followed by an acid-mediated removal of the isopropylidene moiety to yield the desired alkyne **6** in an acceptable overall yield of 24%.



Scheme 10: Reagents and conditions. *(i)* DCC, DMSO, dichloroacetic acid; *(ii)* (carboethoxy methylene)-triphenylphosphorane, oxalic acid monohydrate, 90%; *(iii)* Pd/C, H_2, quant; *(iv)* 1N NaOH, THF/H_2O 1:1, 78%; *(v)* propargylamine·monohydrochloride, BOP, DiPEA, 83%; *(vi)* 7M NH_3, MeOH, 96%; *(vii)* TFA/H_2O 5:1, 25%.

Conclusions

This chapter describes attempts towards the synthesis of four ATP-competitive kinase inhibitors, all of them decorated with an acetylene functionality to make them suitable for bisubstrate based inhibitor formation via Cu(I)-catalyzed click chemistry. During the synthesis of staurosporine mimetic **2** it turned out that bisindol formation was rather difficult and proceeded in low yields which might be improved by taking advance of a different protecting group strategy. The synthesis of isoquinoline sulfonamide **4** was straightforward, which was in contrast to the synthesis of considerably more complicated amide containing ATP-analogs **6** and sulfonamide containing **5**, and **46**. The synthesis of these ATP-analogs proved to be very challenging. Nevertheless, amide **6** was obtained in a good yield, while sulfonamide **5** could not be isolated at all as a result of an unprecedented side reaction. Therefore, compound **46** was prepared as a suitable alternative. All alkyne derivatives will be used in a Cu(I)-mediated click reaction with azidopeptides to obtain the newly designed bisubstrate based protein kinase inhibitors. Azidopeptide synthesis, and their application in the copper-catalyzed click reactions will be described in chapter 6. Finally, the biological evaluation of all bisubstrate based protein kinase inhibitors is extensively discussed in chapter 7.

Experimental section

General: All reactions were carried out at ambient temperature unless stated otherwise. All reagents were used as supplied from commercial sources unless stated otherwise. All solvents were dried on molecular sieves (4Å) prior to use, except for MeOH which was dried on molecular sieves (3Å). R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Hanessian's stain (cerium molybdate). Column chromatography was carried out using Silicycle UltraPure silicagel (40-63 μm). ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). For measurements in D_2O, the residual solvent peak (4.79 ppm) was used as reference. ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). For measurements in D_2O, the residual solvent peak of MeOH (49.0 ppm) was used as reference. Electrospray ionisation mass spectrometry was performed on a Shimadzu LCMS-QP8000

single quadrupole bench-top mass spectrometer in positive ionisation mode. Preparative HPLC was carried out on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Altima XL C8, 100Å, 10µm, 250×22mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95/5 *v/v*) to 100% buffer B (0.1% TFA in H_2O/CH_3CN 5/95 *v/v*) in 40 min at a flow rate of 11.5 mL/min Analytical HPLC was carried out on a Shimadzu HPLC workstation using a reverse phase C8 column (Alltech Altima XL C8, 90Å, 5 µm, 250×4.6 mm) using a linear gradient of the same buffers as above in 20 min at a flow rate of 1.0 mL/min.

1-Bromo-3-*tert*-butyldimethylsilyl-propanol (8): *tert*-Butyldimethylsilyl chloride (3.1 g, 21 mmol) was added portion-wise to a solution of 3-bromo-1-propanol (1.73 mL, 20 mmol) in CH_2Cl_2 (150 mL) in the presence of pyridine (1.79 mL, 22 mmol) and DMAP (224 mg, 2 mmol) at 0°C. The reaction mixture was stirred for 3 h at ambient temperature and subsequently quenched by the addition of H_2O (100 mL). Then, the aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL) and the combined organic layers were dried on $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified using column chromatography (EtOAc/hexane, 1/9 *v/v*) yielding compound **2** (4.2 g, 88%) as a colorless oil.

R_f = 0.66 (EtOAc/hexane, 1/5 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 3.73 (t, *J* = 5.7 Hz, 2H, OC*H*$_2$), 3.51 (t, *J* = 6.6 Hz, 2H, BrC*H*$_2$), 2.03 (m, 2H, C*H*$_2$), 0.90 (s, 9H, C(C*H*$_3$)$_3$), 0.07 (s, 6H, Si(C*H*$_3$)$_2$) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 60.4, 35.6, 30.7, 25.9, -1.4, -5.4 ppm.

2-(1-(3-*tert*-Butyldimethylsilyloxypropyl)-1*H*-indol-3-yl) acetic acid (16): 2-(1*H*-Indol-3-yl) acetic acid **15** (3.5 g, 20 mmol) was dissolved in DMF (30 mL) and at 0°C, NaH (a 60% dispersion in mineral oil, 1.76 g, 44 mmol) was added and the reaction mixture was stirred for 20 min. Then, 1-bromo-3-*tert*-butyldimethylsilyl-propanol[30] (5.57 g, 22 mmol) was added portion-wise and the reaction mixture was stirred at room temperature for 3h and subsequently evaporated to dryness. The excess of NaH was quenched by H_2O and the aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic layers were dried on $MgSO_4$, filtered and concentrated *in vacuo*. After purification by column chromatography (CH_2Cl_2/HOAc, 99/1 *v/v*) **16** was isolated as an off-white oil in 89% yield (6.9 g).

R_f = 0.29 (CH_2Cl_2/MeOH, 95/5 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 11.02 (br s, 1H, COO*H*), 7.58 (d, *J* = 7.7 Hz, 1H, C=C*H*), 7.30 (d, *J* = 8.3 Hz, 1H, Ar*H*), 7.18 (m, 1H, Ar*H*), 7.09 (m, 2H, Ar*H*), 4.16 (t, *J* = 6.7 Hz, 2H, NC*H*$_2$), 3.75 (s, 2H, C*H*$_2$COOH), 3.52 (t, *J* = 5.6 Hz, 2H, OC*H*$_2$), 1.94 (m, 2H, C*H*$_2$), 0.92 (s, 9H, C(C*H*$_3$)$_3$), 0.04 ppm (s, 6H, Si(C*H*$_3$)$_2$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ =

178.4, 136.1, 127.6, 127.1, 121.6, 119.1, 118.9, 109.5, 105.9, 59.4, 42.5, 32.9, 31.0, 25.9, 18.2, -5.5 ppm. Anal. calcd. for $C_{18}H_{27}NO_3Si$: C 64.83, H 8.16, N 4.20; found: C 64.69 H 8.10 N 4.25.

1-Methyl-1*H*-indole (17): Indole **9** (11.7 g, 100 mmol) was dissolved in freshly distilled THF and at 0°C NaH (a 60% dispersion in mineral oil, 6.0 g, 150 mmol) was added. After 15 min of stirring MeI (8.4 mL, 134 mmol) was added drop-wise and the reaction mixture was stirred for 60 min at room temperature. The reaction mixture was cooled to 0°C and sat. aq. NH_4Cl (400 mL) was added and the mixture was extracted with Et_2O (3 × 100 mL). The combined organic layers were washed with 1M $KHSO_4$ (3 × 150 mL), sat. aq. $NaHCO_3$ (3 × 150 mL) and brine (150 mL). The organic phase was dried on $MgSO_4$, filtered and evaporated to dryness. The crude product was purified using column chromatography (EtOAc/hexane, 1/4 *v/v*) yielding **17** (13 g, quant) as a clear, colorless liquid.
R_f = 0.68 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.62 (double t, *J* = 8.0, 0.8 Hz, 1H, NC*H*=CH), 7.31 (m, 1H, Ar*H*), 7.21 (m, 1H, Ar*H*), 7.10 (m, 1H, Ar*H*), 7.01 (d, *J* = 3.03 Hz, 1H, Ar*H*), 6.47 (dd, *J* = 3.0, 0.8 Hz, 1H, NCH=C*H*), 3.74 ppm (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 136.6, 128.7, 128.4, 121.4, 120.8, 119.2, 109.1, 100.8, 32.7 ppm.

2-(1-(3-*tert*-Butyldimethylsilyloxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleic anhydride (19): A solution of 1-methyl-1*H*-indole **17** (1.31 g, 10 mmol) in CH_2Cl_2 (50 mL) was added drop-wise to a solution of oxalyl chloride (3.8 mL, 40 mmol) in CH_2Cl_2 (50 mL) at 0°C and after 30 min of stirring the reaction mixture was evaporated to dryness. The residue was redissolved in dry CH_2Cl_2 (50 mL) and subsequently added to a mixture of 2-(1-(3-*tert*-butyldimethylsilyloxypropyl)-1*H*-indol-3-yl) acetic acid **16** (3.5 g, 10 mmol) and Et_3N (4.1 mL, 30 mmol) in CH_2Cl_2 (50 mL). The reaction mixture was stirred at room temperature for 3h and subsequently evaporated to dryness. The crude compound was dissolved in EtOAc (50 mL) and this solution was washed with 1M $KHSO_4$ (3 × 25 mL), sat. aq. $NaHCO_3$ (3 × 25 mL) and brine (25 mL). The organic layer was dried on $MgSO_4$, evaporated to dryness and purified using column chromatography (EtOAc/hexane, 1/4 *v/v*) yielding **17** (1.03 g, 20%) as a bright red solid.
R_f = 0.55 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.77 (s, 1H, C=C*H*), 7.70 (s, 1H, C=C*H*), 7.38 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.31 (d, *J* = 8.2, 1H, Ar*H*), 7.13 (m, 2H, Ar*H*), 7.04 (d, *J* = 8.0, 1H, Ar*H*), 6.81 (m, 2H, Ar*H*), 6.74 (m, 1H, Ar*H*), 4.28 (t, *J* = 6.9 Hz, 2H, NCH_2), 3.86 (s, 3H, NCH_3), 3.57 (t, *J* = 5.6 Hz, 2H, OCH_2), 1.99 (m, 2H, CH_2), 0.92 (s, 9H, C(CH_3)$_3$), 0.05 ppm (s, 6H, Si(CH_3)$_2$); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 166.9, 166.7, 136.9, 136.2, 133.7, 132.9, 127.3, 125.9, 125.5, 122.5, 122.3, 120.4, 109.9, 109.7, 105.1, 105.0, 59.1, 43.1, 33.3, 32.6, 25.8, 18.1, -5.5 ppm. Anal. calcd. for $C_{30}H_{34}N_2O_4Si$: C 70.01, H 6.66, N 5.44; found: C 70.20, H 6.71, N 5.29.

2-(1-(3-*tert*-Butyldimethylsilyloxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide (20): 2-(1-(3-*tert*-Butyldimethylsilyloxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleic anhydride **19** (1.0 g, 2 mmol) was dissolved in DMF (8 mL) and a mixture of hexamethyldisilazane (4.2 mL, 20 mmol) and MeOH (0.4 mL, 10 mmol) was added and the obtained reaction mixture was stirred at room temperature. After 24 h, H_2O (20 mL) was added to quench the reaction mixture and the aqueous phase was extracted with EtOAc (4 × 50 mL). The combined organic layers were dried on $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using column chromatography (EtOAc/hexane, 1/4 → 1/2 *v/v*) yielding **20** (0.92 g, 91%) as a red solid.
R_f = 0.34 (EtOAc/hexane, 1/2 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.32 (s, 1H, N*H*), 7.68 (s, 1H, C=C*H*), 7.59 (s, 1H, C=C*H*), 7.29 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.19 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.03 (m, 3H, Ar*H*), 6.71 (m, 3H, Ar*H*), 4.19 (t, *J* = 6.8 Hz, 2H, NCH_2), 3.73 (s, 3H, NCH_3), 3.51 (t, *J* = 5.5 Hz, 2H, OCH_2), 1.92 (m, 2H, CH_2), 0.89 (s, 9H, C(CH_3)$_3$), 0.01 ppm (s, 6H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 172.7, 172.5, 136.7, 136.1, 132.7, 131.8, 127.5, 127.4, 126.2, 125.9, 122.1, 122.0, 119.9, 119.8, 109.6, 109.2, 105.8, 105.5, 59.2, 42.9, 33.1, 32.8, 25.8, 11.5, -5.5 ppm. Anal. calcd. for $C_{30}H_{35}N_3O_3Si$: C 70.14, H 6.87, N 8.18; found: C 70.34, H 6.85, N 8.10.

2-(1-(3-Hydroxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide (21): 2-(1-(3-*tert*-Butyldimethylsilyloxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide **20** (382 mg, 0.75 mmol) was dissolved in 1% HCl in EtOH (20 mL) and stirred at ambient temperature for 1 h. After removing the solvent by evaporation, the residue was purified by silica gel column chromatography (EtOAc/hexane, 1/1 *v/v*) to give **21** as red crystals (300 mg) in quantitative yield.
R_f = 0.25 (EtOAc/hexane, 2/1 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 10.92 (s, 1H, N*H*), 7.86 (s, 1H, C=C*H*), 7.71 (s, 1H, C=C*H*), 7.46 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.42 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.03 (t, *J* = 7.4 Hz, 2H, Ar*H*), 6.90 (d, *J* = 8.0 Hz, 1H, Ar*H*), 6.64 (m, 3H, Ar*H*), 4.62 (t, *J* = 4.9 Hz, 2H, OCH_2), 4.27 (t, *J* = 6.7 Hz, 2H, NCH_2), 3.86 (s, 3H, NCH_3), 3.36 (m, 1H, O*H*), 1.85 ppm (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 172.9, 136.5, 135.7, 133.1, 131.9, 127.5, 126.7, 126.1, 125.5, 121.6, 121.1, 119.4, 110.1, 104.9, 104.6, 57.5, 42.7, 32.9, 32.8 ppm. Anal. calcd. for $C_{24}H_{21}N_3O_3$: C 72.16, H 5.30; N 10.52; found: C 72.01, H 5.19, N 10.39.

2-(1-(3-(*N*-Methyl-*N*-propargylamino)propyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide (2): 2-(1-(3-Hydroxypropyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide **21** (50 mg, 0.125 mmol) and pyridine (10 µL, 0.125 mmol) were mixed in CH_2Cl_2 (2 mL) and subsequently cooled to 0°C. Then, triflic anhydride (20 µL, 0.125 mmol) was added slowly and the

obtained reaction mixture was stirred for 45 min at 0°C. To this reaction mixture, *N*-methyl-*N*-propargylamine (21 µL, 0.25 mmol) was added and stirring was continued for an additional 3 h at room temperature. The reaction mixture was evaporated to dryness and the residue was redissolved in EtOAc (5 mL) and washed with 1M $KHSO_4$ (3 × 10 mL), sat. aq. $NaHCO_3$ (3 × 10 mL) and brine (10 mL). The organic layer was dried on $MgSO_4$ filtered and subsequently concentrated *in vacuo*. The residue was purified using column chromatography (CH_2Cl_2/MeOH, 98/2 *v/v*) yielding **2** (35 mg, 62 %) as a red solid.

R_f = 0.31 (CH_2Cl_2/MeOH, 95/5 *v/v*); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.72 (s, 1H, N*H*), 7.64 (s, 1H, C=C*H*), 7.60 (s, 1H, C=C*H*), 7.33 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.27 (d, *J* = 7.2 Hz, 1H, Ar*H*), 7.06 (m, 3H, Ar*H*), 6.74 (m, 3H, Ar*H*), 4.21 (t, *J* = 6.6 Hz, 2H, NCH_2), 3.84 (s, 3H, NCH_3), 3.31 (d, *J* = 2.5 Hz, 2H, CH_2C≡CH), 2.36 (t, *J* = 6.6, 2H, NCH_2), 2.21 (s, 3H, NCH_3), 2.20 (t, *J* = 2.2 Hz, 1H, C≡C*H*), 1.94 ppm (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 172.3, 172.1, 136.8, 136.1, 132.8, 131.9, 127.7, 127.5, 126.5, 126.0, 122.2, 120.0, 109.6, 109.3, 105.8, 105.6, 78.3, 73.3, 51.8, 45.6, 44.0, 33.3, 29.7, 27.5 ppm. Anal. calcd. for $C_{28}H_{26}N_4O_2$: C 74.65, H 5.82, N 12.44; found: C 74.52, H 5.90, N 12.57.

***N*^{1}-*tert*-Butyloxycarbonyl-piperazine (23):** A solution of Boc-anhydride (Boc_2O) (4.36 g, 20 mmol) in dioxane (80 mL) was added over a period of 3 h to a solution of piperazine (17.23 g, 200 mmol) in dioxane (80 mL). After the addition was complete the reaction mixture was stirred for an additional 16 h at ambient temperature and subsequently concentrated *in vacuo*. The residue was suspended in H_2O (80 mL) and the precipitate was removed by filtration and washed with H_2O. The aqueous layer was extracted with CH_2Cl_2 (3 × 80 mL) and the combined organic layers were washed with brine and dried on $MgSO_4$. The solvent was removed *in vacuo* which yielded pure compound **23** (3.32 g, 89 %) as a white solid.

R_f = 0.57 (CH_2Cl_2/CH_3OH, 9/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.39 (t, *J* = 5.1 Hz, 4H, 2 × NCH_2); 2.81 (t, *J* = 5.1 Hz, 4H, 2 × NCH_2); 1.46 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 154.8, 79.5, 45.9, 28.4 ppm.

***N*^{1}-*tert*-Butyloxycarbonyl-*N*^{4}-propargyl-piperazine (24):** DiPEA (0.86 mL, 5.25 mmol) was added to a solution of *N*^{1}-*tert*-butyloxycarbonyl-piperazine **23** (0.93 g, 5.0 mmol) in $CHCl_3$ (40 mL). This mixture was cooled on ice and propargyl bromide (0.43 mL, 5.0 mmol) was added drop-wise and the reaction mixture was stirred at room temperature for 24 h. Then, $CHCl_3$ (80 mL) was added and the obtained solution was washed with 5 % aq. $NaHCO_3$ (3 × 40 mL), dried (Na_2SO_4) and subsequently

concentrated *in vacuo*. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 97/3 *v/v*) yielding compound **24** (0.89 g, 79 %) as a colorless oil.
R_f = 0.67 (CH_2Cl_2/CH_3OH, 95:5 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.46 (t, *J* = 5.1 Hz, 4H, 2 × NC*H*$_2$); 3.31 (d, *J* = 2.5 Hz, 2H, NC*H*$_2$C≡CH); 2.51 (t, *J* = 5.1 Hz, 4H, 2 × NC*H*$_2$); 2.27 (t, *J* = 2.5 Hz, 1H, C≡C*H*); 1.46 (s, 9H, C(C*H*$_3$)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 154.6, 79.6,78.3, 73.4, 51.5, 46.9, 28.3 ppm.

***N*^{1}-Propargyl-piperazine dihydrochloride (25):** *N*^{1}-*tert*-Butyloxycarbonyl-*N*^{4}-propargyl-piperazine **24** (1.0 g, 4.5 mmol) was suspended in sat. HCl/diethyl ether (25 mL) at 0°C. The mixture was stirred for 1 h after which it was concentrated *in vacuo* to give *N*^{1}-progargyl-piperazine dihydrochloride **25** as white powder in quantitative yield (890 mg). R_f = 0.33 (CH_2Cl_2/CH_3OH, 9:1 *v/v*)

5-Isoquinoline sulfonylchloride·HCl salt (27): 5-Isoquinoline sulfonic acid (1.05 g, 5 mmol) was suspended in $SOCl_2$ (20 mL) and a catalytic amount of DMF (0.2 mL) was added. The mixture was stirred at reflux for 2.5 h, after which the starting material was completely dissolved. The reaction mixture was evaporated to dryness and the crude product was coevaporated with dry toluene (3 × 5 mL) to remove any residual $SOCl_2$ yielding to give compound **27** in nearly quantitative yield (1.1 g) as light yellow solid and this compound was used without further purification in the next reaction step.

***N*^{1}-(5-Isoquinoline sulfonamide)-*N*^{4}-propargyl-piperazine (4):** 5-Isoquinoline sulfonylchloride·HCl salt **27** (1.0 g, 4.5 mmol), *N*^{1}-propargyl-piperazine dihydrochloride **25** (0.88 g, 4.5 mmol) and Et_3N (1.8 g, 2.5 mL, 18 mmol) were dissolved in CH_2Cl_2 (50 mL) and the obtained reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed under reduced pressure and the residue redissolved in EtOAc (50 mL) and the solution was washed with sat. aq. $NaHCO_3$ (3 × 20 mL), brine (20 mL) and dried over $MgSO_4$. After filtration the EtOAc solution was evaporated to dryness and the residue was purified by flash chromatography (CH_2Cl_2/MeOH, 97:3 *v/v*) to give compound **4** in 65% yield (0.93 g) as slightly yellow powder.
R_f = 0.28 (CH_2Cl_2/CH_3OH, 95/5 *v/v*); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 9.35 (s, 1H, Ar*H*); 8.69 (d, *J* = 6.3 Hz, 1H, Ar*H*); 8.54 (d, *J* = 6.1 Hz, 1H, Ar*H*); 8.38 (d, *J* = 7.4 Hz, 1H, Ar*H*); 8.23 (d, *J* = 8.3 Hz, 1H, Ar*H*); 7.72 (t, *J* = 7.8 Hz, 1H, Ar*H*); 3.25 (d, *J* = 2.5 Hz, 2H, NC*H*$_2$C≡CH); 3.22 (t, *J* = 4.9 Hz, 4H, 2 × NC*H*$_2$); 2.59 (t, *J* = 4.9 Hz, 4H, 2 × NC*H*$_2$); 2.21 (t, *J* = 2.5 Hz, 1H, C≡C*H*) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 153.0, 144.8, 134.0 133.7,131.7, 131.5, 128.8,125.7, 117.4,77.7,73.5,50.7,

46.3, 45.3 ppm. ESI-MS: calcd for $C_{16}H_{18}N_3O_2S$: 316.10, found: *m/z* = 316.88 $[M+H]^+$. Anal. calcd. for $C_{16}H_{19}N_3O_3S$: C 57.64; H 5.74; N 12.60; found: C 57.76, H 5.31, N 12.59.

2',3'-*O*-Isopropylidene adenosine (29): To a suspension of adenosine **28** (1.0 g, 2.7 mmol) and *p*-toluenesulfonic acid (2.4 g, 12.5 mmol) in dry acetone (30 mL), triethyl orthoformate (7.5 mL, 45 mmol) was added over a period of 15 min. The obtained reaction mixture was stirred for 5 h and subsequently diluted by the addition of H_2O (23 mL) and brought to pH 8 by adding conc. NH_3. Then, all volatiles were evaporated and the residue was purified by column chromatography (EtOAc/MeOH, 4/1 *v/v*) yielding compound **29** (1.2 g, quant) as a white powder.
R_f = 0.78 ($CHCl_3$/MeOH/NH_3, 60/40/20 *v/v/v*); ^{1}H-NMR (DMSO-d6, 300 MHz): δ = 8.34 (s, 1H, Ar*H*); 8.15 (s, 1H, Ar*H*); 7.35 (s, 2H, N*H*$_2$); 6.12 (d, *J* = 3.0 Hz, 1H, H-1' C*H*); 5.34 (dd, *J* = 3.0, 6.2 Hz, 1H, H-2' C*H*); 5.24 (broad s, 1H, CH_2O*H*); 4.96 (dd, *J* = 2.5, 6.2 Hz, 1H, H-3' C*H*); 4.21 (m, 1H, H-4' C*H*); 3.53 (m, 2H, H-5' C*H*$_2$); 1.55 (s, 3H, C*H*$_3$); 1.33 (s, 3H, C*H*$_3$) ppm. ^{13}C-NMR (DMSO-d6, 75.5 MHz): δ = 156.1,152.6, 148.8, 119.1, 113.0, 89.6, 86.3, 83.2, 81.3, 61.6, 27.1, 25.2 ppm.

***N*,*N*-Dibenzoyl-2',3'-*O*-isopropylidene adenosine (30)**: Under an argon atmosphere, $(CH_3)_3$Si-Cl (504 µL, 4 mmol) was added to a solution of 2',3'-*O*-isopropylidene adenosine **29** (370 mg, 1 mmol) in dry pyridine (5 mL) which was cooled on ice. At ambient temperature the mixture was stirred for 1.5 h and subsequently cooled to 0°C before the drop-wise addition of benzoyl chloride (256 µL, 2.2 mmol). After stirring the reaction mixture for 5 h, H_2O (1 mL) was added and the solution concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (10 mL) and washed with cold 2N H_2SO_4 (3 × 10 mL), sat. aq. $NaHCO_3$ (2 × 10 mL) and H_2O (2 × 10 mL). The organic layer was dried over Na_2SO_4, filtered and subsequently evaporated to dryness. The residue was purified by column chromatography (EtOAc/hexane, 1/1 *v/v*) yielding compound **30** (380 mg, 74%) as a white foam.
R_f = 0.5 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.63 (s, 1H, Ar*H*); 8.13 (s, 1H, Ar*H*); 7.87 – 7.35 (m, 10H, 2 × Bz Ar*H*); 5.95 (d, *J* = 4.7 Hz, 1H, H-1' C*H*); 5.23 (dd, *J* = 4.4, 8.2 Hz, 1H, H-2' C*H*); 5.10 (dd, *J* = 1.2, 6.0 Hz, 1H, H-3' C*H*); 4.54 (d, *J* = 1.6 Hz, 1H, H-4' C*H*); 3.97 (d, *J* = 12.6 Hz, 1H, H-5' C*H*$_2$); 3.79 (m, 1H, H-5' C*H*$_2$); 1.65 (s, 3H, C*H*$_3$); 1.39 (s, 3H, C*H*$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 172.0, 152.2, 151.8, 133.7, 133.0, 129.3, 128.7, 128.3, 114.1, 93.5, 86.2, 83.3, 81.4, 62.9, 27.3, 25.1 ppm.

Isobutyl mesylate (32): To a mixture of iso-butyl alcohol **31** (9.3 mL, 100 mmol) and Et_3N (16.7 mL, 120 mmol) in CH_2Cl_2 (300 mL), methanesulfonyl chloride (7.8 mL, 100 mmol) was added drop-wise at 0°C. The reaction mixture was stirred for 18 h and subsequently concentrated *in vacuo*. The

residue was redissolved in CH_2Cl_2 (150 mL) and the solution was washed with 1N $KHSO_4$ (3 × 100 mL), sat. aq. $NaHCO_3$ (3 × 100 mL) and dried over Na_2SO_4. After filtration, the solution was evaporated to dryness yielding compound **32** (14.8 g, 97%) as a colorless oil.
^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.00 (d, *J* = 6.6 Hz, 2H, OC*H*$_2$); 3.00 (s, 3H, SC*H*$_3$); 2.05 (m, 1H, C*H*$(CH_3)_2$); 0.99 (d, *J* = 6.9 Hz, 6H, CH(C*H*$_3)_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 75.7, 37.2, 28.2, 18.6 ppm.

***O*-isobutyl-(diphenylphosphono)-methyl sulfonate (33):** To a solution of iso-butyl mesylate **32** (1.5 g, 10 mmol) in freshly distilled THF (25 mL), flushed with nitrogen and cooled to -78°C, a solution of BuLi (2.5 M in hexanes, 4.4 mL, 11 mmol) was added drop-wise. The obtained reaction mixture was stirred at -78°C for 30 min. Then, diphenylchlorophosphate (1.0 mL, 5 mmol) was added and this reaction mixture was stirred for 60 min at -78°C. To quench the reaction mixture, 4M NH_4Cl (2.5 mL, 10 mmol) was added at -78°C and the solution was slowly warmed to room temperature. The volatiles were evaporated *in vacuo,* the residue was diluted with H_2O (40 mL) and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified using flash chromatography (EtOAc/hexane, 1/4 *v/v*) yielding compound **33** (1.0 g, 54%) as an off-white solid.
R_f = 0.50 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.19 – 7.38 (m, 10H, Ar*H*); 4.16 (d, *J* = 6.3 Hz, 2H, SC*H*$_2$); 4.00 (d, *J* = 17.3 Hz, 2H, OC*H*$_2$); 2.04 (m, 1H, C*H*$(CH_3)_2$); 0.97 (d, *J* = 6.6 Hz, 6H, CH(C*H*$_3)_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 149.5, 129.8, 125.7, 120.4, 77.9, 48, 1 28.1, 18.3. ppm.

***N*,*N*-Dibenzoyl-5'-deoxy-5'-(sulfoxy-iso-butylvinyl)-2',3'-*O*-isopropylidene adenosine (37)**: Under an argon atmosphere, *N*,*N*-dibenzoyl-2',3'-*O*-isopropylidene adenosine **30** (515 mg, 1 mmol) and DCC (722 mg, 3.5 mmol) were suspended in dry DMSO (3 mL) at 0°C followed by the addition of dichloroacetic acid (41 μL, 0.5 mmol). After 2 h of stirring the reaction mixture was transferred via a canula to another reaction flask. In this flask, a freshly prepared ice-cold suspension of *O*-isobutyl-(diphenylphosphono)-methyl sulfonate **33** (538 mg, 1.4 mmol) and NaH (60 wt% in mineral oil, 52 mg, 1.3 mmol) in THF (4 mL) was stirred for 15 min prior to the addition of the reaction mixture containing aldehyde **36**. The resulting mixture was stirred for an additional 2.5 h at ambient temperature. Then, oxalic acid monohydrate (225 mg, 2.5 mmol) in MeOH (2 mL) was added at 0°C, and after stirring for 20 min, this finally obtained reaction mixture was concentrated *in vacuo*. The residue was taken up in cold MeOH (5 mL) and the insoluble DCU was removed by filtration. The filtrate was evaporated to dryness and redissolved in $CHCl_3$ (10 mL) and this solution was washed

with sat. aq. $NaHCO_3$ (3 × 10 mL), H_2O (3 × 10 mL) and brine (10 mL). The organic layer was dried on Na_2SO_4, filtered and subsequently concentrated *in vacuo* and the residue was purified by silicagel column chromatography (EtOAc/hexane, 1/3 → 1/1 *v/v*) to give compound **37** as an off-white foam in 68% yield (440 mg). It turned out by ^{1}H NMR and COSY NMR that the final product was obtained as a *Z*/*E* mixture with a ratio of 1:2.

R_f = 0.38 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.67, 8.61 (2s, 1H, Ar*H*); 8.15, 8.11 (2s, 1H, Ar*H*); 7.87 – 7.34 (m, 10H, 2 × Bz Ar*H*); 7.01 (dd, *J* = 15.1, 4.7 Hz, 1H, *E*, H-6' SO_2C*H*=CH); 6.44 (dd, *J* = 11.3, 8.5 Hz, 1H, *Z*, H-6' SO_2C*H*=CH); 6.29 (m, 1H, *E* + *Z*, H-5' SO_2CH=C*H*); 6.17 (d, *J* = 2.2 Hz, 1H, *E*, H-1' C*H*); 6.15 (d, *J* = 1.9 Hz, 1H, *Z*, H-1' C*H*); 5.55 (dd, *J* = 6.1, 1.9 Hz, 1H, *Z*, H-2' C*H*); 5.47 (dd, *J* = 6.3, 2.2 Hz, 1H, *E*, H-2' C*H*); 5.17 (m, 1H, *E* + *Z*, H-3' C*H*); 4.95 (m, 1H, *Z*, H-4' C*H*); 4.86 (m, 1H, *E*, H-4' C*H*); 3.89 (m, 2H, OCH_2); 1.95 (m, 1H, $CH(CH_3)_2$); 1.64 (s, 3H, CH_3); 1.40 (s, 3H, CH_3); 0.94 (m, 6H, $CH(CH_3)_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 172.2, 152.4, 152.0, 143.2, 143.0, 133.9, 133.1, 129.5, 128.8, 127.8, 126.8, 126.1, 115.5, 114.9, 91.5, 90.5, 85.5, 84.8, 84.0, 83.7, 82.0, 33.9, 28.1, 27.1, 25.3, 18.6 ppm.

***N*,*N*-Dibenzoyl-5'-deoxy-5'-(sulfonylchloride(vinyl))-2',3'-*O*-isopropylidene adenosine (43)**: To a solution of *N*,*N*-dibenzoyl-5'-deoxy-5'-(sulfoxy-iso-butylvinyl)-2',3'-*O*-isopropylidene adenosine **37** (647 mg, 1.0 mmol) in acetone (30 mL), KI (332 mg, 2 mmol) was added. The mixture was refluxed for 40 h and subsequently evaporated to dryness. The crude reaction product was suspended in CH_2Cl_2 (40 mL) and the suspension was cooled on ice before $COCl_2$ (1.25 mL 2.4 M in toluene, 3.0 mmol) and DMF (155 µL, 2 mmol) were added. After 6 h of stirring at room temperature, the reaction mixture was evaporated to dryness. The crude compound was purified using column chromatography (EtOAc/Hex, 1/1 *v/v*) yielding **45** (213 mg, 35%) as a yellow solid.

***N*,*N*-Dibenzoyl-4'-dehydro-5'-deoxy-4',5'-(*N*-propargylsulfonylamidovinyl)-2',3'-*O*-isopropylidene adenosine (45)**: *N*,*N*-Dibenzoyl-5'-deoxy-5'-(sulfonylchloride(vinyl))-2',3'-*O*-isopropylidene adenosine **43** (80 mg, 0.13 mmol) was dissolved in CH_2Cl_2 (5 mL) and propargylamine hydrochloride (13 mg, 0.14 mmol) followed by NMM (30 mg, 29 µL, 0.26 mmol) were added. After 16 h of stirring, the reaction mixture was washed with 1N $KHSO_4$ (3 × 10 mL), sat. aq. $NaHCO_3$ (3 × 10 mL) and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified using column chromatography (CH_2Cl_2/MeOH, 97/3 *v/v*) yielding **45** (20 mg, 24%) as a colorless oil.

R_f = 0.33 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.61 (s, 1H, Ar*H*); 8.17 (s, 1H, Ar*H*); 7.85 – 7.33 (m, 10H, 2 × Bz Ar*H*); 6.37 (d, *J* = 0.8 Hz, 1H, H-5' C*H*=C); 5.61 (d, *J* = 6.1 Hz,

1H, H-1' C*H*); 5.31 (dd, 1H, *J* = 0.8, 6.1 Hz, 1H, H-2' C*H*); 5.01 (t, *J* = 7.8 Hz, 1H, N*H*); 4.47 (t, *J* = 6.1 Hz, 1H, H-3' C*H*); 3.94 (dd, *J* = 8.5, 14 Hz, 1H, H-6' SO_2CH_2); 3.76 (dd, *J* = 7.2, 14.0 Hz, 1H, H-6' SO_2CH_2); 3.74 (ddd, *J* = 2.5, 6.1, 17.9 Hz, 1H, NHCH_2); 3.55 (ddd, *J* = 2.5, 6.1, 17.9 Hz, 1H, NHCH_2); 2.18 (t, *J* = 2.5 Hz, 1H, C≡C*H*); 1.57 (s, 3H, CH_3); 1.42 (s, 3H, CH_3) ppm.

4'-Dehydro-5'-deoxy-4',5'-(*N*-propargylsulfonylamidovinyl)-2',3'-*O*-isopropylidene adenosine (45a): *N*,*N*-Dibenzoyl-4',5'-deoxy-4',5'-(*N*-propargylsulfonylamidovinyl)-2',3'-*O*-isopropylidene adenosine **45** (38 mg, 60 μmol) was dissolved in 7M NH_3 in MeOH (5 mL) and stirred for 20 h at room temperature. Then, the solvent was removed by evaporation and the residue was purified by silicagel column chromatography (CH_2Cl_2/MeOH/Et_3N, 95/5/1 *v/v/v*) to give **45a** as a white solid in 98% yield (25 mg).
^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.29 (s, 1H, Ar*H*); 7.93 (s, 1H, Ar*H*); 6.33 (d, *J* = 1.1 Hz, 1H, H-5' C*H*=C); 6.22 (br s, 2H, NH_2); 5.79 (br s, 1H, N*H*); 5.58 (d, *J* = 6.0 Hz, 1H, H-1' C*H*); 5.22 (dd, *J* = 1.1, 6.0 Hz, 1H, H-2' C*H*); 5.02 (dd, *J* = 7.4, 8.5 Hz, 1H, H-3' C*H*); 3.97 (dd, *J* = 8.9, 14.2 Hz, 1H, H-6' SO_2CH_2); 3.79 (dd, *J* = 7.0, 14.0 Hz, 1H, H-6' SO_2CH_2); 3.71 (s, 2H, NHCH_2); 2.16 (t, *J* = 2.5 Hz, 1H, C≡C*H*); 1.56 (s, 3H, CH_3); 1.41 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 169.6, 160.4, 155.8, 149.0, 133.3, 132.0, 128.6, 127.3, 120.1, 114.5, 91.4, 91.2, 82.6, 79.2, 72.5, 50.2, 32.6, 26.7, 25.5. ppm

4'-Dehydro-5'-deoxy-5'-(*N*-propargylsulfonylamidovinyl) adenosine (46): 4'-Dehydro-5'-deoxy-4',5'-(*N*-propargylsulfonylamidovinyl)-2',3'-*O*-isopropylidene adenosine **45a** (25 mg, 59 μmol) was dissolved in TFA/H_2O (5/1 *v/v*, 6 mL) and the solution was stirred for 20 min after which it was concentrated *in vacuo*. The residue was purified by preparative HPLC (H_2O/CH_3CN/TFA 95/5/0.1 → CH_3CN/H_2O/TFA 95/5/0.1 in 60 min) and **46** (1.8 mg, 8%) was obtained as white fluffy solid.
R_f = 0.47 ($CHCl_3$/MeOH/NH_3, 60/40/20 *v/v/v*); Purity was confirmed by analytical HPLC (C8) and was found to be higher than 95% (R_t = 14.6 min). ^{1}H-NMR (D_2O, 300 MHz): δ = 8.50 (s, 1H, Ar*H*); 8.46 (s, 1H, Ar*H*); 6.47 (d, *J* = 3.5 Hz, H-1' C*H*); 5.05 (t, *J* = 8.3 Hz, 1H, H-5' C=C*H*); 4.98 (m, 2H, H-2' + H-3' C*H*); 4.10 (m, 2H, H-6' SO_2CH_2); 3.88 (s, 2H, NHCH_2); 2.66 (s, 1H, C≡C*H*) ppm. ^{13}C-NMR (D_2O, 75.5 MHz): δ = 150.1, 139.8, 88.9, 87.8, 72.5, 71.6, 68.8, 47.9, 31.2 ppm.

***N*,*N*-Dibenzoyl-5'-deoxy-5'-(*trans*-carbethoxyvinyl)-2',3'-*O*-isopropylidene adenosine (47)**: Under an argon atmosphere, *N*,*N*-dibenzoyl-2',3'-*O*-isopropylidene adenosine **30** (515 mg, 1.0 mmol) and DCC (722 mg, 3.5 mmol) were suspended in ice-cold DMSO (3 mL) and dichloroacetic acid (41 μL, 0.5 mmol) was added after which the reaction mixture was stirred for 2 h. Then,

(carboethoxymethylene)-triphenylphosphorane (348 mg, 1.0 mmol) was added and stirring was prolonged for 3 h. Subsequently, oxalic acid monohydrate (225 mg, 2.5 mmol) in MeOH (2 mL) was added and the reaction mixture was stirred for an additional 20 min before the mixture was concentrated *in vacuo*. The residue was dissolved in cold MeOH (5 mL) to remove DCU by filtration and the filtrate was evaporated to dryness. The residue was redissolved in $CHCl_3$ (10 mL) and the solution was washed with sat. aq. $NaHCO_3$ (3 × 10 mL), H_2O (3 × 10 mL) and brine (10 mL). The organic layer was dried on Na_2SO_4, filtered and subsequently concentrated *in vacuo.* The residue was purified by silicagel column chromatography (EtOAc/hexane, 1/3 → 1/1 *v/v*) giving compound **47** (560 mg, 98%) as an off-white foam.

R_f = 0.34 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.66 (s, 1H, Ar*H*); 8.12 (s, 1H, Ar*H*); 7.87 – 7.26 (m, 10H, 2 × Bz Ar*H*); 6.98 (dd, *J* = 5.8, 15.8 Hz, 1H, H-6' COC*H*=CH); 6.19 (d, *J* = 2.2 Hz, 1H, H-1' C*H*); 5.94 (dd, *J* = 1.6, 15.7 Hz, 1H, H-5' COCH=C*H*); 5.46 (dd, *J* = 1.9, 6.3 Hz, 1H, H-2' C*H*); 5.08 (dd, *J* = 4.1, 6.3 Hz, 1H, H-3' C*H*); 4.82 (m, 1H, H-4' C*H*); 4.15 (q, *J* = 7.2 Hz, 2H, OCH_2CH$_3$); 1.64 (s, 3H, CH_3); 1.39 (s, 3H, CH_3); 1.23 (t, *J* = 7.2 Hz, 3H, OCH$_2$CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 172.2, 165.4, 152.2, 142.6, 133.9, 130.0, 129.4, 128.7, 127.8, 123.1, 115.2, 90.4, 85.8, 83.9, 60.6, 27.1, 25.3, 14.1 ppm.

***N*,*N*-Dibenzoyl-5'-deoxy-5'-(carbethoxyethyl)-2',3'-*O*-isopropylidene adenosine (48)**: A suspension of *N*,*N*-dibenzoyl-5'-deoxy-5'-(*trans*-carbethoxyvinyl)-2',3'-*O*-isopropylidene adenosine **47** (1.65 g, 2.8 mmol) and Pd/C (165 mg, 10 wt%) in freshly distilled THF (50 mL) was stirred under a H_2 atmosphere for 48 h. The mixture was filtered through Hyflo and concentrated *in vacuo* to give compound **48** as a slightly yellow solid in quantitative yield (1.7 g).

R_f = 0.34 (EtOAc/hexane, 1/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.66 (s, 1H, Ar*H*); 8.12 (s, 1H, Ar*H*); 7.25 – 7.86 (m, 10H, 2 × Bz Ar*H*); 6.07 (d, *J* = 2.4 Hz, 1H, H-1' C*H*); 5.42 (dd, *J* = 2.4, 6.5 Hz, 1H, H-2' C*H*); 4.83 (dd, *J* = 4.0, 6.5 Hz, 1H, H-3' C*H*); 4.22 (m, 1H, H-4' C*H*); 4.09 (q, *J* = 7.2 Hz, 2H, OCH_2CH$_3$); 2.38 (t, *J* = 7.6 Hz, 2H, H-6' COCH_2); 2.04 (m, 2H, H-5' COCH$_2$CH_2); 1.60 (s, 3H, CH_3); 1.37 (s, 3H, CH_3); 1.21 (t, *J* = 7.2 Hz, 3H, OCH$_2$CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 172.5, 172.2, 152.3, 152.0, 134.0, 133.0, 129.4, 128.7, 128.0, 115.0, 90.3, 85.8, 83.9, 83.8, 60.5, 30.2, 28.4, 27.2, 25.4, 14.1 ppm.

***N*,*N*-Dibenzoyl-5'-deoxy-5'-(carbonyl)-2',3'-*O*-isopropylidene adenosine (49)**: Aqueous 1N NaOH (1 mL) was added to a solution of *N*,*N*-dibenzoyl-5'-deoxy-5'-(carbethoxyethyl)-2',3'-*O*-isopropylidene adenosine **48** (250 mg, 0.4 mmol) in THF/H_2O (5 mL, 1/1 *v/v*). The mixture was stirred for 3 h, and subsequently acidified by the addition of 1N HCl to pH 2. The acidic solution was

concentrated *in vacuo* and the residue was purified using flash chromatography (EtOAc/hexane/AcOH, 50/50/1 *v/v/v*) yielding compound **49** (151 mg, 78%) as a white foam.
R_f = 0.63 (CH_2Cl_2/MeOH/AcOH, 80/20/1 *v/v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.73 (s, 1H, Ar*H*); 8.14 (s, 1H, Ar*H*); 7.46 – 8.04 (m, 10H, 2 × Bz Ar*H*), 6.08 (d, *J* = 2.2 Hz, 1H, H-1' C*H*); 5.49 (d, *J* = 5.5 Hz, 1H, H-2' C*H*); 4.89 (dd, *J* = 3.6, 6.0 Hz, 1H, H-3' C*H*); 4.25 (m, 1H, H-4' C*H*); 2.36 (m, 2H, H-6' COCH_2); 2.04 (m, 2H, H-5' $COCH_2CH_2$); 1.60 (s, 3H, CH_3); 1.38 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 177.2, 165.6, 151.7, 150.8, 133.5, 133.0, 128.8, 128.6, 123.9, 115.0, 90.9, 86.6, 84.3, 30.5, 28.5, 27.4, 25.6 ppm.

***N*-benzoyl-5'-deoxy-5'-(propargylamidoethyl)-2',3'-*O*-isopropylidene adenosine (50a)**: *N*,*N*-Dibenzoyl-5'-deoxy-5'-(carboxyethyl)-2',3'-*O*-isopropylidene adenosine **49** (151 mg, 0.3 mmol) was dissolved in CH_2Cl_2 (3 mL), and propargylamine monohydrochloride (30 mg, 0.3 mmol) followed by BOP (153 mg, 0.4 mmol) were added. The reaction mixture was placed under an nitrogen atmosphere and cooled on ice before DiPEA (121 μL, 0.7 mmol) was added. The resulting reaction mixture was stirred for 3 h after which the volatiles were removed *in vacuo*, and the residue was redissolved in EtOAc (10 mL). This solution was washed with 1N $KHSO_4$ (3 × 10 mL), sat. aq. $NaHCO_3$ (3 × 10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. Instead of compound **50,** *N*-benzoyl-5'-deoxy-5'-(propargylamidoethyl)-2',3'-*O*-isopropylidene adenosine **50a** (134 mg, 83%) was obtained as a white foam after column chromatography (CH_2Cl_2/MeOH, 95/5 *v/v*).
R_f = 0.28 (CH_2Cl_2/MeOH, 95/5 *v/v*). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 9.07 (s, 1H, N*H*Bz); 8.79 (s, 1H, Ar*H*); 8.08 (s, 1H, Ar*H*); 7.48 – 8.02 (m, 5H, Bz Ar*H*); 6.05 (d, *J* = 2.7 Hz, 1H, H-1' C*H*); 6.01 (bs, 1H, N*H*); 5.46 (dd, *J* = 2.7, 6.3 Hz, 1H, H-2' C*H*); 4.87 (dd, *J* = 3.9, 6.3 Hz, 1H, H-3' C*H*); 4.22 (m, 1H, H-4' C*H*); 3.99 (dd, *J* = 2.5, 2.7 Hz, 2H, NHCH_2); 2.25 (m, 2H, H-6' COCH_2); 2.17 (m, 1H, C≡C*H*); 2.10 (m, 2H, H-5' $COCH_2CH_2$); 1.60 (s, 3H, CH_3); 1.37 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 171.9, 165.1, 151.5, 150.1, 133.7, 133.1, 129.0, 128.2, 124.0, 115.3, 90.6, 85.9, 84.0, 79.8, 71.8, 32.1, 29.3, 28.9, 27.4, 25.6 ppm.

5'-Deoxy-5'-(*N*-propargylamidoethyl)-2',3'-*O*-isopropylidene adenosine (51): *N*-Benzoyl-5'-deoxy-5'-(propargylamidoethyl)-2',3'-*O*-isopropylidene adenosine **50a** (164 mg, 0.3 mmol) was dissolved in 7M NH_3 in MeOH (5 mL) and stirred for 20 h. After this period of stirring the mixture was evaporated to dryness and the residue was purified by flash chromatography (CH_2Cl_2/MeOH/Et_3N, 95/5/1 *v/v/v*) to give compound **51** as white foam in 96% yield (122 mg).

R_f = 0.46 (CH_2Cl_2/MeOH/Et_3N, 90/10/1 *v/v/v*). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.33 (s, 1H, Ar*H*); 7.87 (s, 1H, Ar*H*); 6.40 (bs, 1H, N*H*); 6.02 (s, 2H, NH_2); 5.96 (d, *J* = 3.0 Hz, 1H, H-1' C*H*); 5.45 (dd, *J* = 3.0, 6.6 Hz, 1H, H-2' C*H*); 4.85 (dd, *J* = 3.8, 6.6 Hz, 1H, H-3' C*H*); 4.20 (m, 1H, H-4' C*H*); 3.98 (m, 2H, NHCH_2); 2.29 (m, 2H, H-6' COCH_2); 2.17 (t, *J* = 2.6 Hz, 1H, C≡C*H*); 2.11 (m, 2H, H-5' $COCH_2CH_2$); 1.58 (s, 3H, CH_3); 1.35 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 171.8, 155.6, 153.1, 149.3, 120.5, 115.0, 90.4, 85.2, 83.5, 79.9, 71.5, 31.7, 29.1, 28.5, 27.2, 25.4 ppm.

5'-Deoxy-5'-(*N*-propargylamidoethyl) adenosine (6): 5'-Deoxy-5'-(*N*-propargylamidoethyl)-2',3'-*O*-isopropylidene adenosine **51** (120 mg, 0.3 mmol) was dissolved in TFA/H_2O (12 mL, 5/1 *v/v*) and the solution was stirred for 2 h after which the mixture was concentrated *in vacuo*. The residue was purified by preparative HPLC (H_2O/CH_3CN/TFA 95/5/0.1 → CH_3CN/H_2O/TFA 95/5/0.1 in 60 min) and 5'-deoxy-5'-(*N*-propargylamidoethyl) adenosine **6** (26 mg, 25%) was obtained as a fluffy white solid.

R_f = 0.57 ($CHCl_3$/MeOH/NH_3, 60/40/20 *v/v/v*); Purity was confirmed by analytical HPLC (C8) and was found to be higher than 95% (R_t = 10.5 min). ^{1}H-NMR (D_2O, 300 MHz): δ = 8.33 (s, 1H, Ar*H*); 8.30 (s, 1H, Ar*H*); 5.96 (d, *J* = 5.2 Hz, 1H, H-1' C*H*); 4.71 (m, 1H, H-2' C*H*); 4.17 (m, 1H, H-3' C*H*); 4.03 (m, 1H, H-4' C*H*); 3.73 (s, 2H, NHCH_2); 2.36 (m, 1H, C≡C*H*); 2.27 (t, *J* = 7.6 Hz, 2H, H-6' COCH_2); 1.99 (m, 2H, H-5' $COCH_2CH_2$) ppm. ^{13}C-NMR (D_2O, 75.5 MHz): 145.8, 145.7, 141.3, 87.3, 87.2, 83.4, 72.8, 72.3, 70.7, 31.2, 27.9, 27.9 ppm.

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Chapter 6

Synthesis of azide-functionalized peptide-based pseudosubstrates as well as bisubstrate based potential PKC-inhibitors

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Abstract: This chapter describes the synthesis of amino acids with azide-functionalized side chains and their subsequent conversion into N^{α}-Fmoc protected building blocks for SPPS. Using these azido-functionalized pseudosubstrates, bisubstrate based inhibitors were prepared in good yields via the Cu(I)-mediated click reaction with alkyne derivatives of ATP-binding site inhibitors.

Introduction

Since the protein kinase C family has been recognized as an important drug target,[1] due to its crucial role in signal transduction and cell proliferation, many research strategies have been investigated toward kinase inhibition.[1-6] With over 500 kinases encoded by the human genome, with high homologies in the catalytic domain, the most challenging task is the design of protein kinase-selective inhibitors. To date, most kinase inhibitors have been designed for the highly conserved ATP binding pocket.[7-9]

Another promising approach for the development of selective kinase inhibitors might be targeting the substrate binding cleft. With respect to this a strategy for achieving protein kinase inhibition is the use of known protein kinase substrates and transform them into the corresponding pseudosubstrates.[10-13] The phosphorylated serine/threonine residue in the peptide sequence is then replaced by an alanine residue keeping the character of the peptide substrate intact, however the peptide cannot be phosphorylated anymore. Peptide substrates for PKC have been widely studied as lead compounds for kinase inhibition.[10] A comprehensive overview of specific PKC substrates, which were determined in a large combinatorial study, was published by Cantley *et al*.[13] This study showed an overlap in the substrate preferences of the isozymes that were used. Moreover, the pharmacologically interesting PKCθ isozyme was not included in that study.

The substrate and the ATP binding region in the catalytic domain of protein kinases are located in close proximity to each other, which gives rise to the possibility to interact with both regions simultaneously by a bisubstrate based inhibition approach.[14-23] By using this approach the advantages of both previous strategies can be combined, which is the high affinity of the ATP-competitive binder and the higher selectivity of the substrate competitive binder. For the design of selective bisubstrates, which fit well into both binding regions, a good understanding of the interactions between substrates and kinase is required. The design and synthesis of bisubstrates will be discussed in this chapter.

Results and Discussion

Design rationale:

A substrate specificity profile of PKC has been discussed in chapter 4. Based on this, a substrate peptide, which was selectively phosphorylated, was selected for transformation into a pseudosubstrate. This pseudosubstrate sequence was used for the construction of the bisubstrate based inhibitor. Peptide Ac-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-Arg-Lys-Ile-Leu-NH_2 was a selectively phosphorylated peptide by PKCζ, and was used as a starting point in the design and synthesis of a selective bisubstrate inhibitor. To this peptide two major modifications had to be introduced. Firstly, for conversion into a pseudosubstrate the serine residue that is expected to be phosphorylated was replaced by an alanine. Secondly, one amino acid residue should be replaced by an azide-functionalized amino acid residue, to make the synthesized peptides suitable for bisubstrate based inhibitor formation. An amino acid residue that is located near the phosphorylation site was replaced by an azide-functionalized amino acid (Figure 1). The replaced amino acid was an arginine residue, which is located two amino acids toward the *N*-terminus of the phosphorylated serine of peptide substrates. Since the peptide was derived from a protein (CREB) that is known to be phosphorylated, SWISSPROT and the human protein reference database were used to locate the phosphorylated serine amino acid residue to define the arginine residue that was replaced by an azide functionalized amino acid.[24,25]

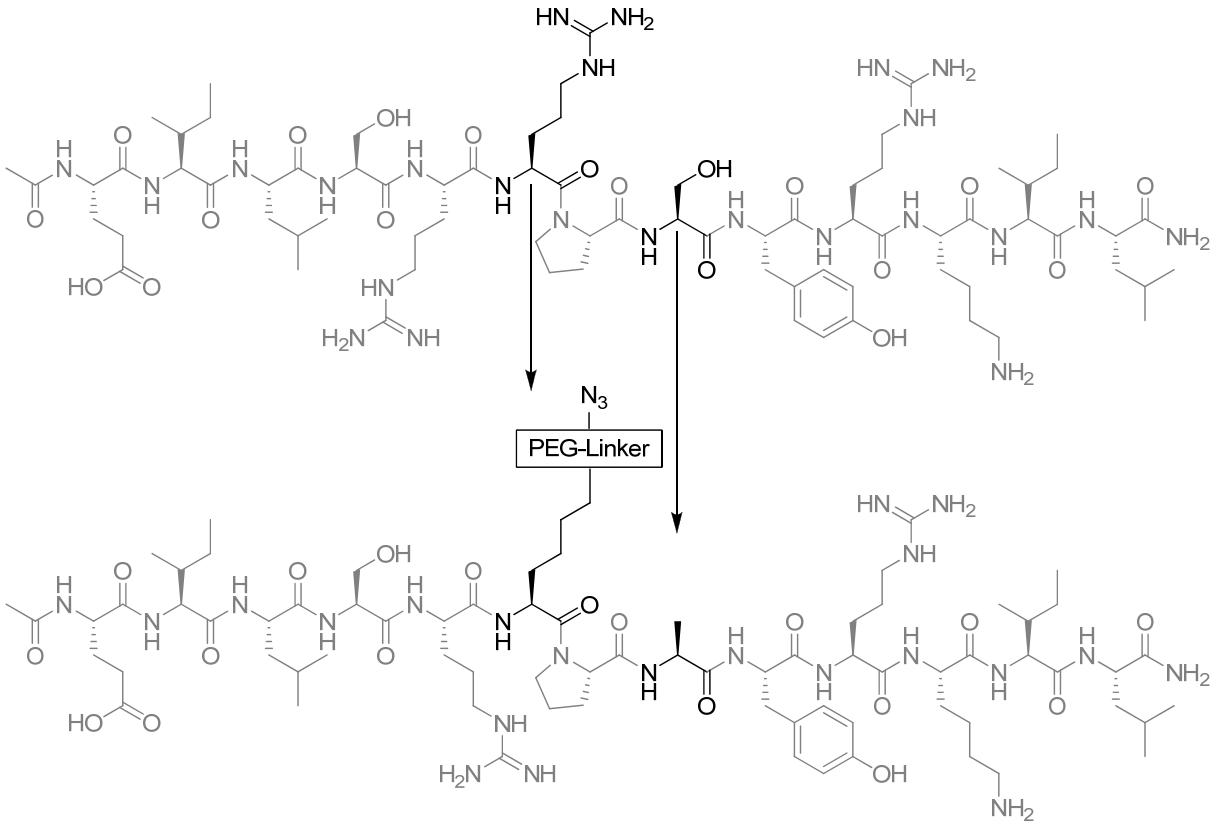


Figure 1: The top peptide is the peptide sequence derived from the peptide microarray as was described in chapter 4. In the bottom peptide the serine is replaced by an alanine residue and an azido-modified amino acid was incorporated instead of an arginine residue.

The rationale for replacement of the arginine residue, located two amino acids to the *N*-terminus from the phosphoacceptor amino acid serine, was based on a crystal structure from PKCθ (Figure 2), which was co-crystallized with staurosporine, showing the location and orientation of this ATP-competitive inhibitor in the enzyme.[26] A second Ser/Thr protein kinase crystal structure, in which a peptide substrate was co-crystallized, was overlayed in the structure of PKCθ. This gave insight into the orientation of the peptide substrate towards the ATP-binding pocket, allowing to determine the best position for modification of the peptide substrate inhibitor by incorporation of an azido-modified amino acid (Figure 2).[26]

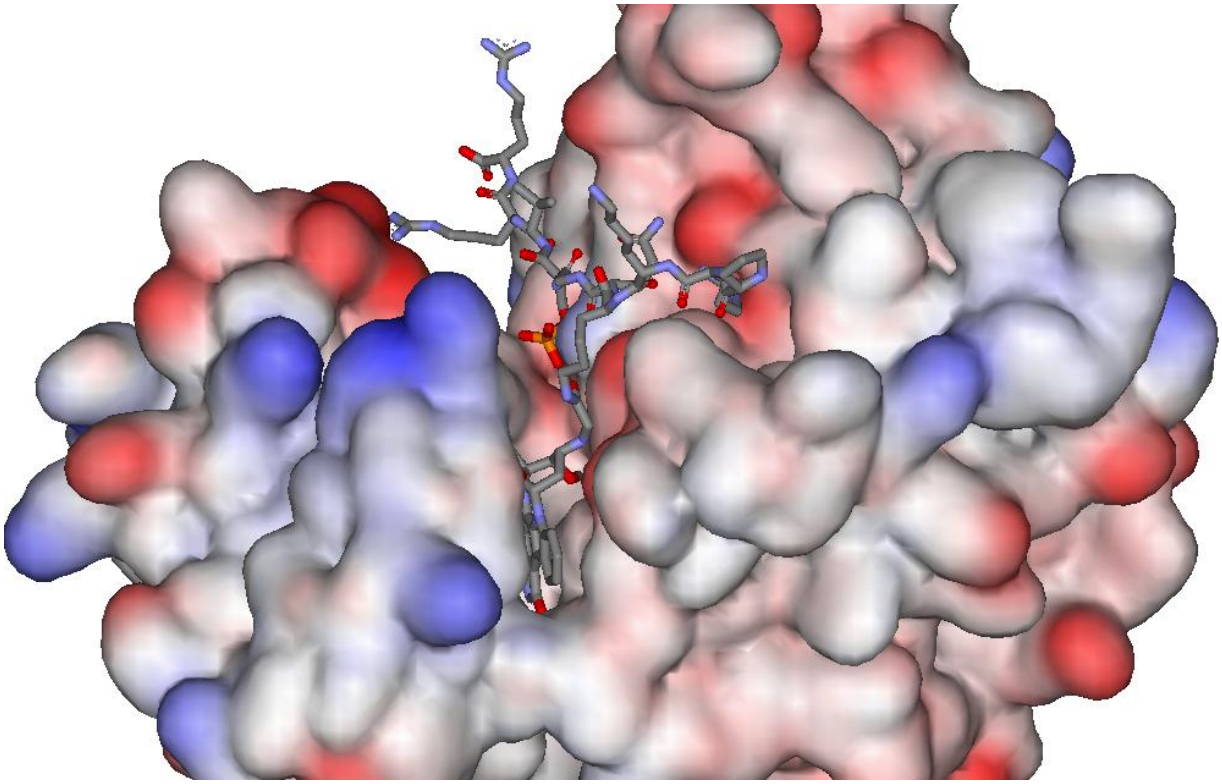
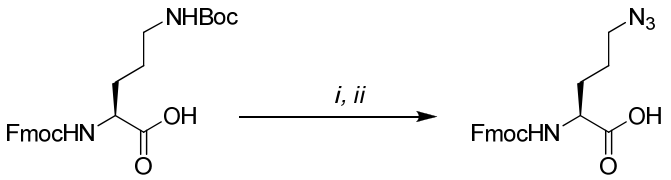


Figure 2: Bisubstrate based inhibitor, modeled in the crystal structure of PKCθ, which was co-crystallized with staurosporine. On top of this crystal structure a peptide substrate is docked.

It was expected that the synthesis of an arginine residue, functionalized with an azide moiety at the side chain, would be rather difficult. Therefore, it was decided to replace the arginine residue by an azide-functionalized ornithine or lysine residue, since the latter two are synthetically more easily accessible. Since the arginine residue is positively charged at neutral pH it has to be realized that by replacement with this azide-functionalized ornithine or lysine amino acid, a single positive charge will be removed from the peptide, which probably will influence the binding properties of the peptide towards the protein kinase. Therefore, an arginine residue decorated with an azide moiety will also be prepared to investigate if this positive charge is important in binding of the bisubstrate based inhibitor to the protein kinase.

Synthetic approach:

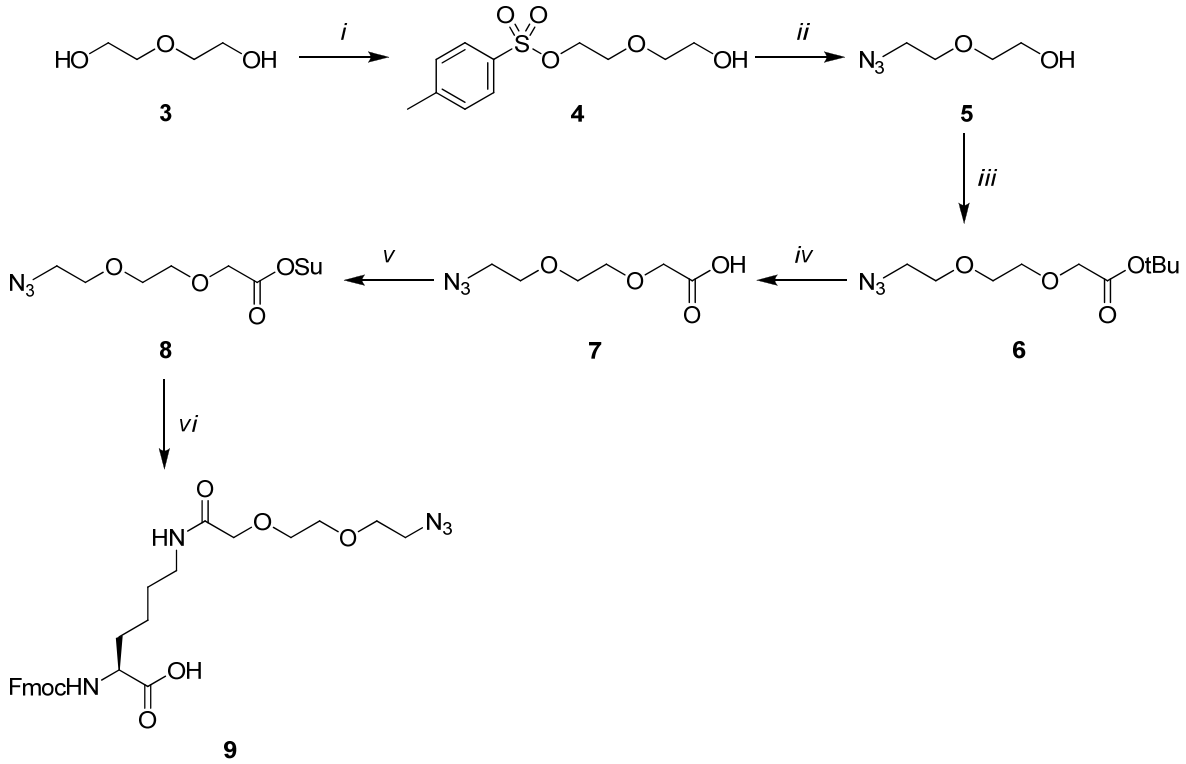
Since azide-functionalized amino acids can be incorporated in peptide sequences without adjustments to the SPPS protocol, azido amino acids **2** and **9** were synthesized prior to incorporation into pseudosubstrates **10** and **11**.



1 **2**

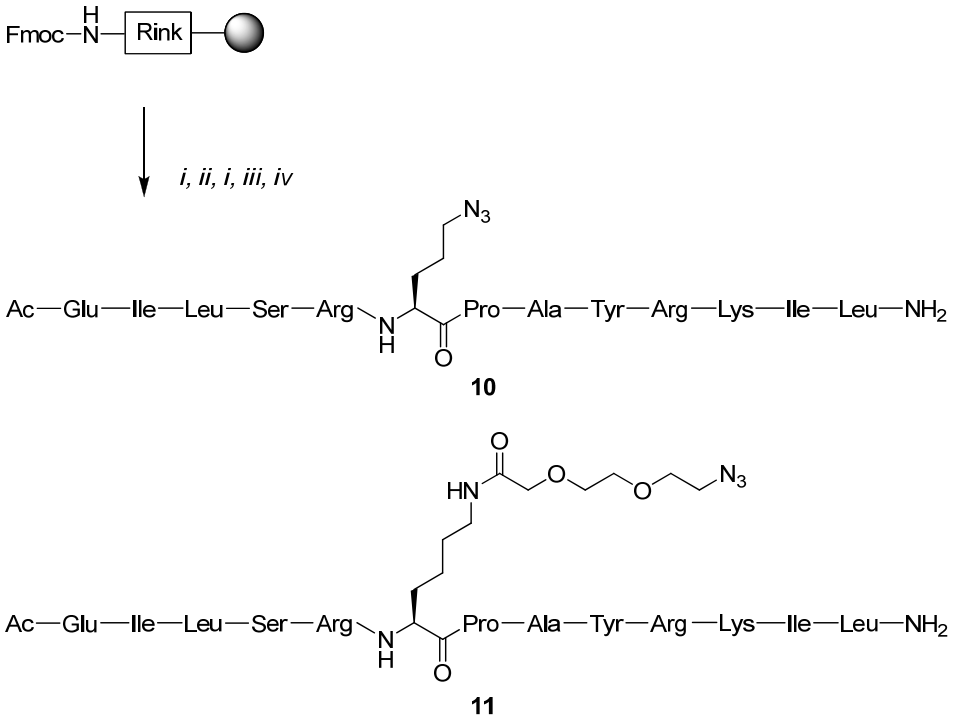
Scheme 1: *(i)* HCl/diethyl ether, CH_2Cl_2; *(ii)* triflic azide, $CuSO_4 \cdot 5H_2O$, acetate buffer pH ≈ 9, CH_2Cl_2, 92% (over 2 steps).

The conversion of amines into azides by a diazo-transfer is well described in the literature.[27,28] For the synthesis of compound **2** a procedure, as published by Meldal *et al.*[29], was used. Fmoc-Orn(Boc)-OH **1**, was treated with HCl/diethyl ether to remove the Boc-group and the crude reaction product was directly used in the next step. The diazo-transfer reaction was performed in an aqueous $AcOH/K_2CO_3$ buffer (pH 9) to avoid premature Fmoc-cleavage. Diazo-transfer was performed with freshly prepared triflic azide in the presence of $CuSO_4$ as catalyst. After workup and purification compound **2** was obtained in 92% yield (Scheme 1).



Scheme 2: Reagents and conditions. *(i)* Tosyl chloride, Et_3N, CH_2Cl_2, 26%; *(ii)* NaN_3, DMF, quant; *(iii) tert*-butyl bromoacetate, TBAI, THF, 44%, *(iv)* HCl/diethyl ether, CH_2Cl_2, quant; *(v)* DCC, HONSu, CH_2Cl_2; *(vi)* Fmoc-Lys-OH, DiPEA, CH_2Cl_2, DMF, 78% (over 2 steps).

For the preparation of **9**, diethylene glycol **3** was treated with tosyl chloride in the presence of Et_3N as a base to give tosylate **4** in a yield of 26% (based on **3**). In the next step, tosylate **4** was converted into azide **5** with NaN_3 in DMF in quantitative yield. Then, the hydroxyl functionality of **5** was deprotonated with NaH and the intermediate alkoxide was treated with *tert*-butyl bromoacetate to give ester **6** in 44% yield. Subsequent acidolysis of the *tert*-butyl ester gave quantitatively carboxylic acid **7**. The acid was converted into the corresponding *N*-hydroxysuccinimidyl ester **8** by treatment with DCC/HONSu and the active ester was directly used in the next reaction step to convert Fmoc-Lys-OH into azide **9** in 78% over two reaction steps (Scheme 2).



Scheme 3: Synthesis of peptides **10** and **11**. *(i)* 20% piperidine, DMF, 3 × 8 min; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF, 60 min; Coupled amino acids: Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, building block **2** or **9**, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH; *(iii)* Ac_2O, HOBt, DiPEA, NMP; *(iv)* TFA, TIS, H_2O (95:2.5:2.5); Overall yields: **10**, (31%), **11** (18%).

After completion of the synthesis of the azido-functionalized amino acids **2** and **9**, both building blocks were incorporated in a peptide sequence using SPPS (Scheme 3). Each coupling cycle consisted of Fmoc deprotection with 20% piperidine in DMF and the subsequent coupling of an Fmoc-amino acid with HOBt and HBTU as coupling reagents and DiPEA as base. All coupling reactions and Fmoc-deprotections were monitored by the Kaiser test.[30] After completion of the synthesis, both peptides were cleaved from the resin and all acid labile protecting groups were removed by treatment with TFA. The crude peptides were purified by preparative HPLC and characterized by mass spectrometry prior to their use in bisubstrate based inhibitor preparation via click-chemistry.

For the synthesis of peptides containing an alkyne- or azide-modified arginine residue (Figure 3), the suitably protected and functionalized building blocks **16a-e** were synthesized (Scheme 4).

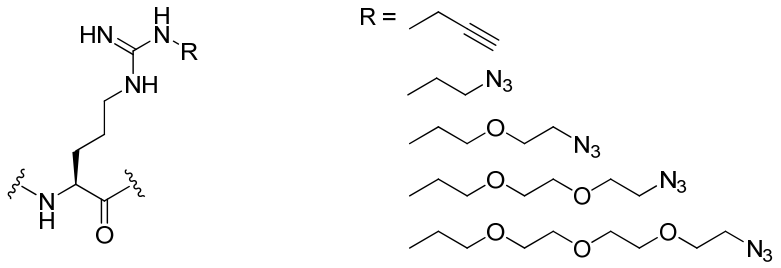
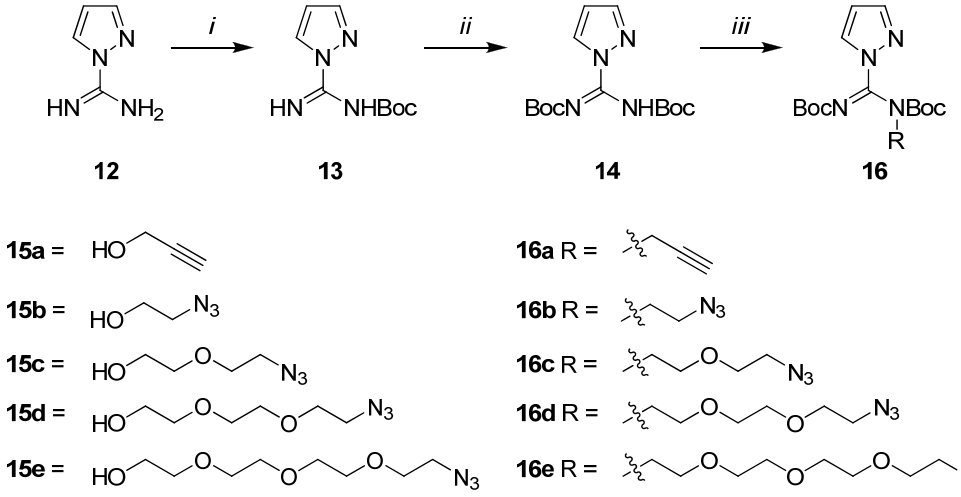


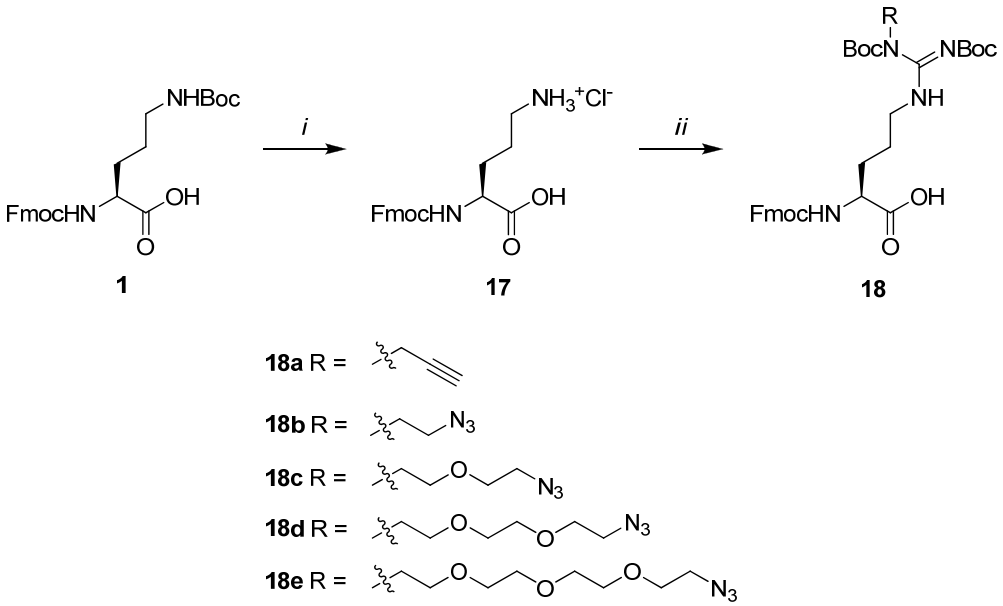
Figure 3: Structural formula of alkyne- and azide-modified residues.



Scheme 4: *(i)* Boc_2O, DiPEA, CH_2Cl_2, quant; *(ii)* Boc_2O, NaH, THF, reflux, 92%; *(iii)* DEAD, PPh_3, R-OH **15a-e**. Obtained yields: **16a** 79%, **16b** 86%, **16c** 84%, **16d** 70% and **16e** 39%.

For this purpose, guanyl pyrazole **12** was treated with Boc_2O/DIPEA to give mono Boc-protected **13** in quantitative yield. The second Boc-functionality was incorporated by treating **13** with NaH in the presence of Boc_2O in refluxing THF and bis-protected **14** was obtained in a high yield of 92%. Derivatization of **14** with a diverse set of azide containing spacers was performed, according to literature procedures, via a Mitsonubu-based *N*-alkylation in the presence of DEAD/Ph_3P and alcohol **15a-e** to give building blocks **16a-d** in good overall yields varying between 70-86%.[31] Due to the difficult purification of **16e**, this compound was obtained in only 39% yield. Gratifyingly, under these conditions, reduction of the azide functionalities into the corresponding amine via a Staudinger reaction was not observed (Scheme 4).

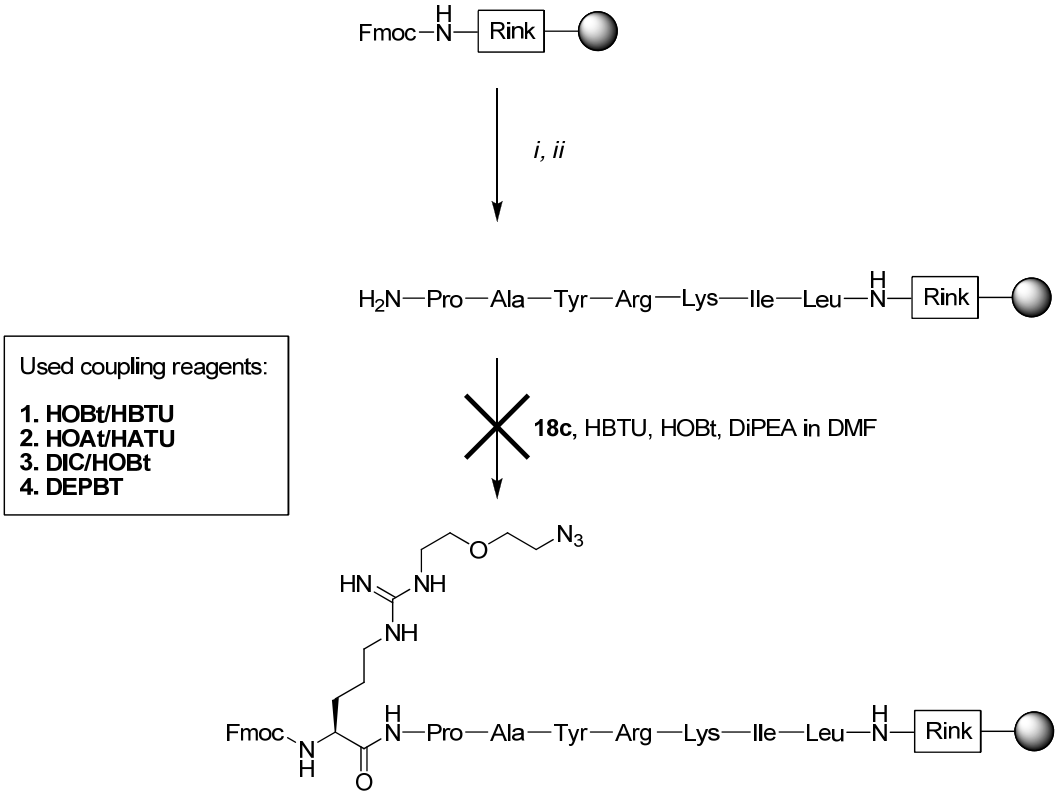
For synthesis of the desired arginine derivatives, Fmoc-Orn(Boc)-OH **1**, was treated with a saturated solution of HCl in diethyl ether, to remove the Boc-functionality. It turned out that acidolysis with HCl is preferred over TFA, since traces of the latter affects the efficiency of the guanylation reaction in the preparation of substituted arginine derivatives **18**. Hydrochloride **17** was treated with *N*,*O*-bistrimethylsilyl-acetamide/DIPEA to protect the carboxylic acid moiety *in situ* as the corresponding TMS-ester. This approach was inspired on a previously reported procedure by Goodman *et al*.[32] Since the TMS-ester is soluble in THF, guanylation proceeded smoothly and after 24h, all starting materials had disappeared. During the aqueous workup at slightly acidic conditions, the TMS-ester was hydrolyzed and the alkylated arginine derivatives **18a** – **e** were obtained in acceptable to good yields (36-82%) (Scheme 5).



Scheme 5: *(i)* HCl/diethyl ether, CH_2Cl_2; *(ii)* **16a-e**, DiPEA, *N*,*O*-bistrimethylsilyl-acetamide, dry THF. Obtained yields: **18a** 82%, **18b** 62%, **18c** 64%, **18d** 61% and **18e** 36%.

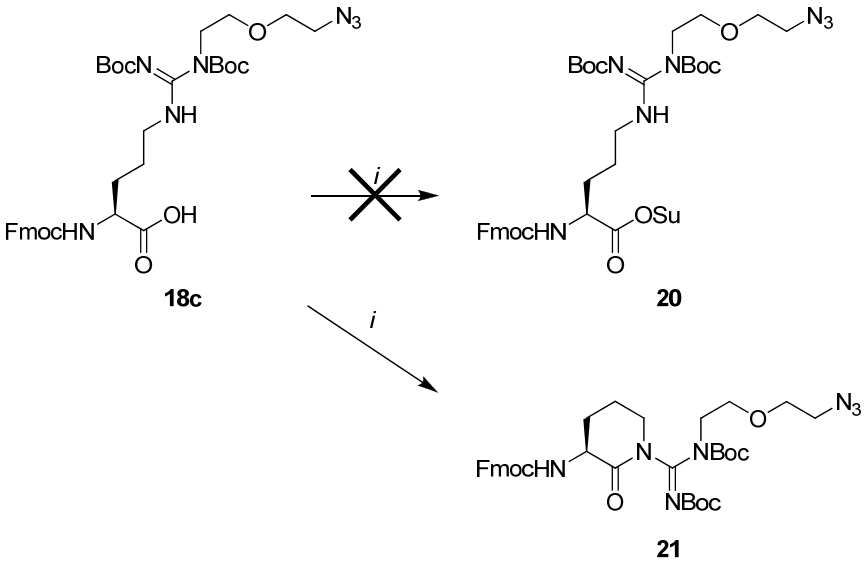
With the desired peptide building blocks now available, the synthesis of peptide **19** was attempted. Each SPPS coupling cycle consisted of an Fmoc deprotection with 20% piperidine in DMF followed by coupling of an amino acid with HOBt and HBTU as coupling reagents and DiPEA as base. The synthesis proceeded smoothly untill the coupling of Fmoc-protected arginine derivative **18c**. Analysis by mass spectrometry of the reaction product revealed that the azide-functionalized arginine derivative was not

incorporated into the peptide. Unfortunately, the coupling of **18c** in the presence of other coupling reagents, *e.g.* HATU/HOAt, DIC/HOBt, or DEPBT, also failed, despite recommendation of the latter two[33-35] for the efficient coupling of N^G-bis Boc-protected arginine derivatives (Scheme 6).



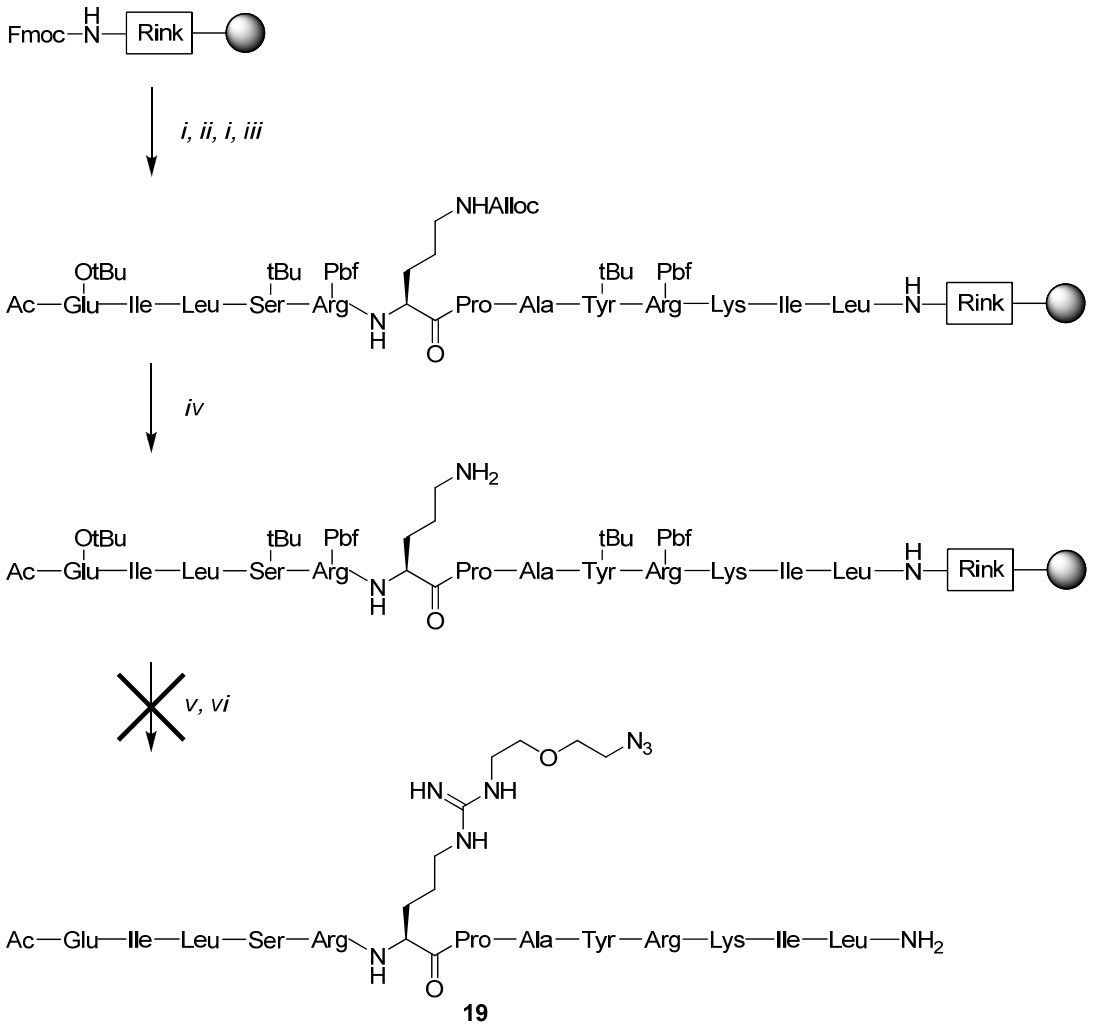
Scheme 6: Attempted synthesis of peptide **19** *(i)* 20% piperidine, DMF; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF; Coupled amino acids: Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH.

Alternatively, **18c** was treated with DCC/HONSu and a catalytic amount of DMAP (Scheme 7), and according to TLC, the reaction was complete within 2h. However, instead of the desired ONSu ester **20**, lactam **21** was isolated in 80% yield (Scheme 7). Apparently, the intramolecular cyclization is favored over the intermolecular amide formation. Since lactam **21** is inert toward amines, which might explain failure of the coupling of the arginine derivative to the solid phase bound peptide, an alternative approach toward the synthesis of peptide **19** was attempted.

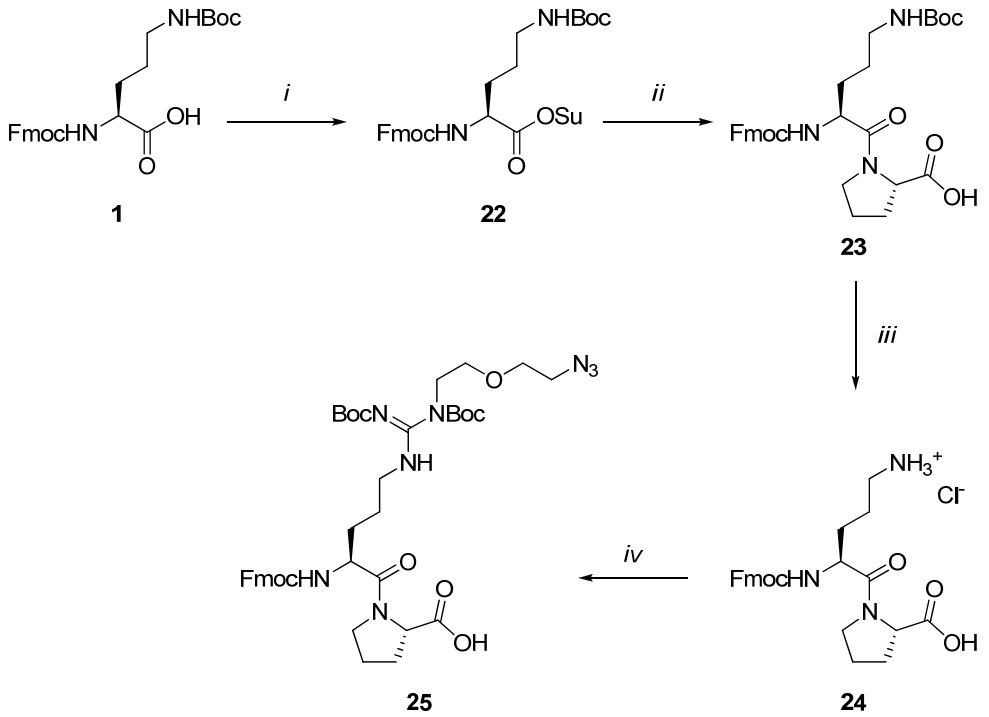


Scheme 7: HOSu, DCC, DMAP, CH_2Cl_2, only product **21** was obtained.

To prevent the formation of lactam **21** in the synthesis of peptide **19** post-assembly guanylation was tried as an alternative approach. The complete peptide sequence was synthesized with an ornithine residue, which was orthogonally protected by an Alloc functionality at the position of the arginine residue. The peptide was acetylated at the *N*-terminus and the Alloc-group was removed using $[Pd(PPh_3)_4]$/phenyl silane. A positive Kaiser test indicated the presence of free amino groups. The resin was then treated with a solution of *N*,*O*-bistrimethylsilyl-acetamide/DiPEA/guanylation reagent **16c** in DCE for 48h. *N*,*O*-Bistrimethylsilyl-acetamide was added to this reaction mixture to improve the reactivity of the free amine towards guanylating reagent **16c**. After cleavage of the peptide from the resin, it was analyzed by MS and it turned out that this post-assembly guanylation approach had failed, since a peptide with the correct mass of **19** was absent (Scheme 8).

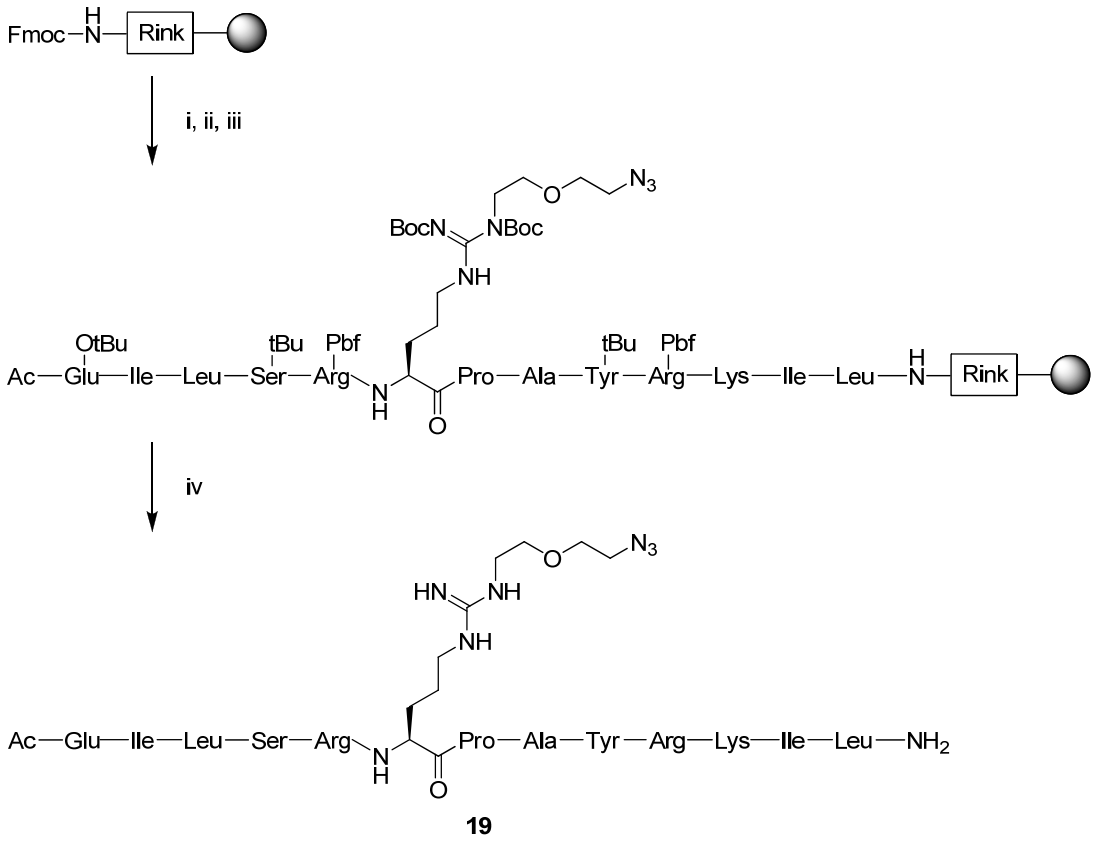


Scheme 8: Synthesis of peptide **19** by post-assembly guanylation. *(i)* 20% piperidine, DMF; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF; Coupled amino acids: Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Orn(Alloc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH; *(iii)* Ac_2O, HOBt, DiPEA, NMP; *(iv)* $[Pd(PPh_3)_4]$, $PhSiH_3$, NMP; *(v)* **16c**, DiPEA, *N*,*O*-bistrimethylsilyl-acetamide, DCE; *(vi)* TFA, TIS, H_2O (95:2.5:2.5).



Scheme 9: Reagents and conditions. *(i)* HONSu, DCC, DMAP, CH_2Cl_2; *(ii)* Et_3N, proline, CH_2Cl_2, 57%; *(iii)* HCl/diethyl ether, CH_2Cl_2; *(iv)* **16c**, DiPEA, *N*,*O*-bistrimethylsilyl-acetamide, THF, 79%.

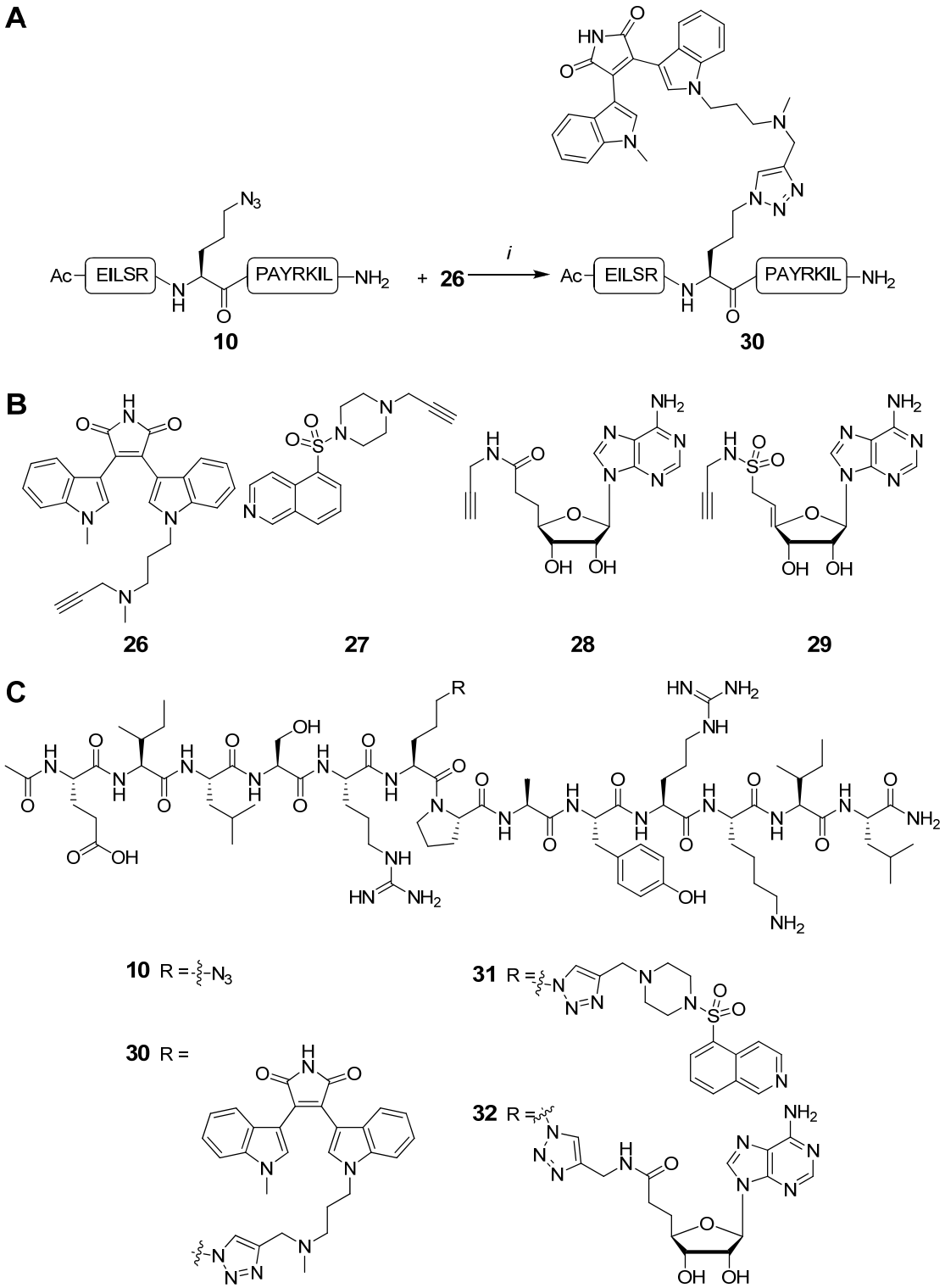
Since post-assembly guanylation did not yield the desired product, another approach for the incorporation of the substituted arginine residue was attempted by using the dipeptide Fmoc-Arg(spacer-N_3)-Pro-OH **25** (Scheme 9). Fmoc-Orn(Boc)-OH **1** was treated with DCC and *N*-hydroxysuccinimide to give active ester **22**, which was reacted without further purification with unprotected proline to afford dipeptide acid **23** in 57% yield. The Boc-group was removed by treatment with HCl/diethyl ether to give amine **24**, which was treated directly in the next step with pyrazole **16c** under basic conditions to give the desired dipeptide **25** in 79% yield. Although this approach is not the most elegant, it has at least two positive aspects since lactam formation will not occur and this well-characterized building block is already functionalized with the desired azide moiety. With this dipeptide building block, the synthesis of peptide **19** was started using SPPS. Fortunately, the synthesis proceeded smoothly and after cleavage and deprotection from the resin, the crude peptide was purified using preparative HPLC. Analysis by MS confirmed the identity of peptide **19**, which was isolated in 27% yield (Scheme 10).



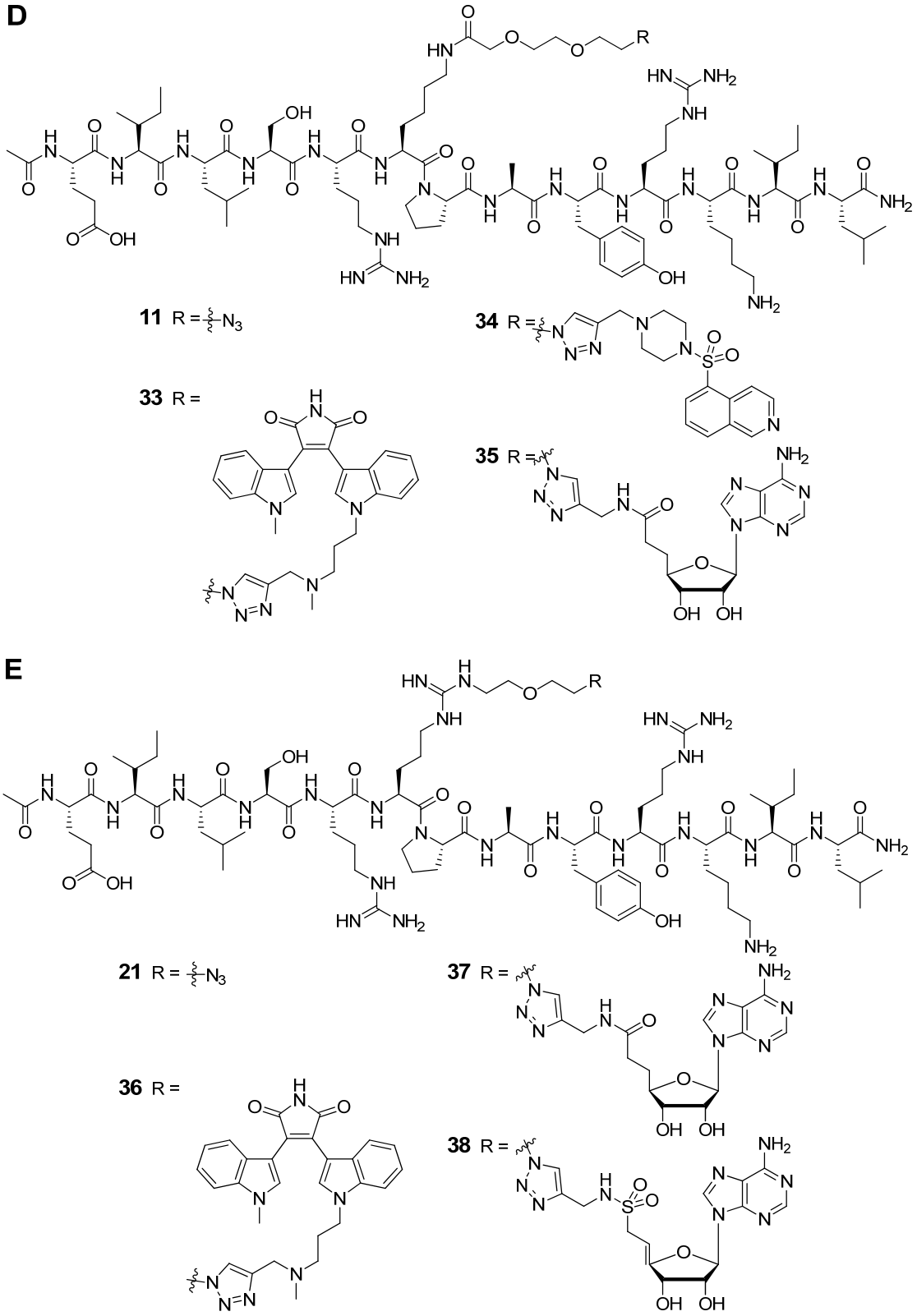
19

Scheme 10: Synthesis of peptide **19**. *(i)* 20% piperidine, DMF; *(ii)* Fmoc-Xxx-OH, HOBt, HBTU, DiPEA, DMF; Coupled amino acids: Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, dipeptide building block **25**, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH; *(iii)* Ac_2O, HOBt, DiPEA, NMP; *(iv)* TFA, TIS, H_2O (95:2.5:2.5); Overall yield of **19** (27 %).

While the SPPS of **19**, with side-chain modified arginine residues was under investigation an alternative method for the incorporation of side-chain modified arginine residues was developed by Martin *et al*.[36] In this approach the synthesis of N^G-substituted arginine derivatives was described in which the side chain is protected with a Pbf-group instead of two Boc-groups. So far the synthesis of peptides using these building blocks has not been described, although this should be in principle possible.



Scheme 11: **A**: Reagents and conditions for the click-reaction. *(i)* $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/*tert*-BuOH, 20 min, 80°C. **B**: ATP-competitive binders that are used for bisubstrate based inhibitor formation. **C**: Bisubstrate based inhibitor products from azido-pepitde **10** with ATP-binders **26**, **27**, **28**. **D**: Bisubstrate based products from azido-peptide **11** with ATP-binders **26**, **27**, **28**. **E**: Bisubstrate based products from azido-peptide **19** with ATP-binders **26**, **28**, **29**.



(Scheme 11 continued.)

Table 1: Synthesized bisubstrates and their HPLC-mass analysis with.

Product	Yield (%)	R_t/min	Mass calcd / found $[M+H]^+$
30	25	19.2	2090.17 / 2089.99[a]
31	20	17.4	1955.07 / 1955.33[a]
32	39	16.8	993.56 / 993.56[b]
33	20	19.2	2249.26 / 2249.30[a]
34	40	17.5	2114.16 / 2114.21[a]
35	17	16.9	1073.10 / 1074.51[b]
36	62	18.6	2219.26 / 2219.63[a]
37	33	16.8	2114.20 / 2114.62[a]
38	40	17.9	2148.15 / 2148.51[a]

[a] MALDI-TOF or [b] ESI-MS $[M+2H]^{2+}$.

For completion of the bisubstrate based inhibitor series **30 – 38**, azide-functionalized peptides **10**, **11** and **19** were subjected to the Huisgen 1,3-cycloaddition (Scheme 11).[37-39] The reactions were carried out at a 5 to 10 μmol scale in a *tert*-BuOH/H_2O solution in the presence of $CuSO_4$ as the catalyst and sodium ascorbate. All mixtures were heated using microwave irradiation for a fast conversion of the starting materials into the desired products. The resulting bisubstrate based inhibitors were purified using preparative HPLC and characterized by MALDI-TOF- or ESI-MS (Table 1).

Conclusions

This chapter describes the efficient synthesis of N^{α}-Fmoc protected azide-functionalized amino acids **2** and **9**, which were easily incorporated into peptides **10** and **11** by SPPS. Furthermore, a facile synthesis of orthogonally protected N^{G}-substituted arginine derivative **18a-e** was described. Unfortunately, incorporation of amino acid **18c** in peptide **19** was not successful, since carbonyl activation of **18c** resulted in the formation of an unreactive lactam **21**. Therefore, as an alternative, dipeptide building block **25** was synthesized followed by the successful incorporation in peptide **19** by means of SPPS. The acetylenic ATP-competitive inhibitors were succesfully connected to azide-functionalized peptides **10**, **11** and **19** using the Cu(I)-catalyzed click-reaction and obtained in yields ranging between

17 to 62% after purification by HPLC and characterization by MS. The then prepared bisubstrate based inhibitors **30** – **38** were purified by HPLC and characterized by mass spectrometry. All synthesized peptides and bisubstrate based inhibitors were evaluated, for their ability to selectively inhibit PKC, as described in chapter 7.

Experimental section

General: All reactions were carried out at ambient temperature unless stated otherwise. All reagents were used as supplied from commercial sources unless stated otherwise. All solvents were dried on molecular sieves (4Å) prior to use, except for MeOH which was dried on molecular sieves (3Å). R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Hanessian's stain (cerium molybdate). Column chromatography was carried out using Silicycle UltraPure silicagel (40-63 μm). ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). For measurements in D_2O, the residual solvent peak (4.79 ppm) was used as reference. ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). For measurements in D_2O, the residual solvent peak of MeOH (49.0 ppm) was used as reference. Electrospray ionisation mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer in positive ionisation mode. Preparative HPLC was carried out on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Altima XL C8, 100Å, 10μm, 250×22mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95/5 *v/v*) to 100% buffer B (0.1% TFA in H_2O/CH_3CN 5/95 *v/v*) in 40 min at a flow rate of 11.5 mL/min Analytical HPLC was carried out on a Shimadzu HPLC workstation using a reverse phase C8 column (Alltech Altima XL C8, 90Å, 5 μm, 250×4.6 mm) using a linear gradient of the same buffers as above in 20 min at a flow rate of 1.0 mL/min.

Triflic azide[27,28]: Triflic anhydride (1.32 mL, 8 mmol) was added dropwise to a vigorously stirred suspension of sodium azide (2.6 g, 40 mmol) in H_2O/CH_2Cl_2 (18 mL, 1:2 *v/v*) at 0°C and stirred for 2 h. The mixture was extracted with CH_2Cl_2 (3 × 7.5 mL) and the combined organic layers were washed with sat. aq. $NaHCO_3$ (10 mL) and dried over Na_2SO_4. The mixture was used directly in following reactions without further workup.

N^{α}-9-Fluorenylmethoxycarbonyl-δ-azido-ornithine (2)[29]: Fmoc-Orn(Boc)-OH **1** (1.8 g, 4.0 mmol) was dissolved in a mixture of CH_2Cl_2 (20 mL) and a saturated solution of HCl/diethyl ether (20 mL). After stirring for 1 h at room temperature a white precipitate was formed and the reaction mixture was evaporated to dryness. The resulting white solid was suspended in 80% aqueous AcOH (12 mL) and $CuSO_4 \cdot 5H_2O$ (20 mg, 0.09 mmol) in H_2O (1.5 mL) was added. K_2CO_3 was added until pH ≈ 9 followed by the addition of H_2O (20 mL) and MeOH (40 mL). A freshly prepared solution of triflic azide was added dropwise and the reaction mixture was stirred vigorously for 20 h at ambient temperature, maintaining the pH at 9. The CH_2Cl_2 layer was separated, washed with H_2O (2 × 40 mL) and to the combined aqueous phases 2N HCl was added till pH ≈ 2. The H_2O layer was extracted with CH_2Cl_2 (4 × 40 mL), the combined CH_2Cl_2 layers were dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by column chromatography (CH_2Cl_2/MeOH, 95/5 *v/v*) yielding compound **2** (1.4 g, 92%) as a white solid.
R_f = 0.34 (CH_2Cl_2/MeOH, 9/1 *v/v*); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 9.85 (br s, 1H, COO*H*), 7.78 – 7.31 (m, 8H, Fmoc Ar*H*), 5.33 (d, *J* = 7.9 Hz, 1H, N*H*), 4.58 (m, 1H, $C^{\alpha}H$), 4.44 (d, *J* = 6.6 Hz, 2H, Fmoc CH_2), 4.22 (t, *J* = 6.6 Hz, 1H, Fmoc C*H*), 3.32 (t, *J* = 5.8 Hz, 2H, $C^{\delta}H_2$), 2.11 – 1.45 (m, 4H, $C^{\beta}H_2$, $C^{\gamma}H_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 176.5, 156.1, 143.5, 141.2, 127.7, 127.0, 124.9, 119.9, 67.1, 53.2, 50.6, 47.0, 29.5, 24.7 ppm.

2-(2-Tosylethoxy)ethanol (4): Diethylene glycol **3** (4.8 mL, 50 mmol) and Et_3N (10.5 mL, 75 mmol) were dissolved in CH_2Cl_2 (200 mL) and at 0°C a solution of tosylchloride (9.5 g, 50 mmol) in CH_2Cl_2 (100 mL) was added. The reaction mixture was stirred at room temperature for 3 h and subsequently evaporated to dryness. The resulting solid was redissolved in EtOAc (200 mL) and the solution washed with sat. aq. $NaHCO_3$ (3 × 100 mL), 1N $KHSO_4$ (3 × 100 mL) and brine (100 mL), dried on Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (CH_2Cl_2/MeOH, 95/5 *v/v*) yielding **4** (3.5 g, 26%) as a colorless oil.
R_f = 0.25 (CH_2Cl_2/MeOH, 9/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.78 (d, *J* = 7.9 Hz, 2H, Ar*H*), 7.35 (d, *J* = 7.9 Hz, 2H, Ar*H*), 4.20 (m, 2H, SO_2OCH_2), 3.67 (m, 4H, CH_2OCH_2), 3.53 (m, 2H, CH_2OH), 2.44 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 144.8, 132.9, 129.8, 127.9, 72.4, 69.1, 68.5, 61.5, 21.5 ppm.

2-(2-Azidoethoxy)ethanol (5): To a solution of **4** (3.5 g, 13 mmol) in DMF (20 mL) NaN_3 (1.7 g, 26 mmol) was added and the mixture was stirred at room temperature for 16 h. The mixture was evaporated to dryness, suspended in H_2O (5 mL) and extracted with EtOAc (5 × 50 mL). The

combined organic layers were dried on Na_2SO_4 and concentrated *in vacuo* yielding **5** (1.7 g, quant) as a colorless oil.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.66 (m, 6H, 3 × C*H*$_2$), 3.38 (m, 2H, C*H*$_2$N$_3$), 2.41 (br s, 1H, O*H*) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 72.3, 69.9, 61.6, 50.6 ppm.

***tert*-Butyl-2-(2-(2-azidoethoxy)ethoxy)acetate (6):** At 0°C, NaH (360 mg, 15 mmol) was added to a solution of **5** (1.3 g, 10 mmol) in DMF (25 mL). After 15 min *tert*-butyl bromoacetate (2.3 mL, 16 mmol) and TBAI (740 mg, 2 mmol) were added and the mixture was stirred at room temperature for 2 h. The mixture was evaporated to dryness, and the residue was suspended in H_2O (20 mL) and the aqueous phase was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (3 × 20 mL), 1N $KHSO_4$ (3 × 20 mL) and brine (20 mL), and the organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude product was purified using column chromatography (EtOAc/hexane, 1/2 *v/v*) yielding **6** (1.09 g, 44%) as a yellow oil.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.03 (s, 2H, C*H*$_2$COOtBu), 3.71 (m, 6H, 3 × C*H*$_2$), 3.40 (t, *J* = 5.1 Hz, 2H, C*H*$_2$N$_3$), 1.48 (s, 9H, C(C*H*$_3$)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 169.5, 81.4, 70.7, 70.6, 69.9, 69.0, 50.5, 28.0 ppm.

2-(2-(2-Azidoethoxy)ethoxy)acetate (7): Compound **6** (500 mg, 2 mmol) was dissolved in HCl/diethyl ether (10 mL) and CH_2Cl_2 (10 mL) and stirred at room temperature for 2 h. The mixture was evaporated to dryness yielding **7** (300 mg, quant) as a white solid.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.59 (br s, 1H, COO*H*), 4.16 (s, 2H, C*H*$_2$COOH), 3.71 (m, 6H, 3 × C*H*$_2$), 3.42 (t, *J* = 4.4 Hz, 2H, C*H*$_2$N$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 172.5, 70.7, 70.4, 69.8, 68.3, 50.5 ppm.

2-(2-(2-Azidoethoxy)ethoxy)acetyl succinimide (8): To a solution of **7** (300 mg, 2 mmol) in CH_3CN (10 mL) was added HONSu (210 mg, 2.2 mmol) and DCC (332 mg, 3 mmol) at 0°C, and the reaction mixture was stirred for 1 h followed by stirring for 16 h at room temperature. The mixture was filtered and the filtrate was evaporated to dryness yielding **8** as a colorless oil. The product was used without further purification.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.53 (s, 2H, C*H*$_2$COOH), 3.82 (m, 2H, C*H*$_2$), 3.69 (m, 4H, 2 × C*H*$_2$), 3.40 (t, *J* = 4.8 Hz, 2H, C*H*$_2$N$_3$), 2.82 (s, 4H, succinimide 2 × C*H*$_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 168.7, 165.9, 71.4, 70.6, 70.0, 66.6, 50.6, 25.5 ppm.

N^{α}-Fluorenylmethoxycarbonyl-N^{ε}-(2-(2-(2-azidoethoxy)ethoxy)acetyl)-lysine (9): Fmoc-Lys-OH·HCl (404 mg, 1 mmol), 2-(2-(2-azidoethoxy)ethoxy)acetate) succinimide (270 mg, 1 mmol) and DiPEA (360 µL, 3 mmol) were dissolved in DMF/CH_2Cl_2 (20 mL, 9/1 *v/v*) and stirred at ambient temperature for 4 h. The mixture was evaporated to dryness, the residue was dissolved in CH_2Cl_2 (20 mL) and the solution was washed with 1N $KHSO_4$ (3 × 10 mL), sat. aq. $NaHCO_3$ (3 × 10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude compound was purified by column chromatography (CH_2Cl_2/MeOH/HOAc, 97/3/0.1 *v/v/v*) yielding **9** (420 mg, 78%) as a white oil.

R_f = 0.41 (CH_2Cl_2/MeOH, 9/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.79 (br s, 1H, COO*H*), 7.74 – 7.26 (m, 8H, Fmoc Ar*H*), 7.70 (t, *J* = 5.8 Hz, 1H, N*H*), 5.83 (d, *J* = 4.0 Hz, 1H, N*H*), 4.37 (m, 3H, Fmoc CH_2, C$^{\alpha}H$), 4.19 (t, *J* = 6.9, 1H, Fmoc C*H*), 3.99 (s, 2H, OCH_2CONH), 3.60 (m, 6H, 3 × CH_2), 3.35 (t, *J* = 5.0 Hz, 2H, CH_2N_3), 3.28 (t, *J* = 6.3 Hz, 2H, C$^{\varepsilon}H_2$), 1.91 – 1.38 (m, 6H, C$^{\beta}H_2$, C$^{\gamma}H_2$, C$^{\delta}H_2$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 174.8, 170.6, 156.1, 143.6, 141.1, 127.6, 126.9, 125.0, 119.8, 70.6, 69.9, 69.9, 66.8, 53.5, 50.4, 47.0, 38.4, 31.6, 28.7, 22.2 ppm.

General procedure for peptide synthesis: On Fmoc-Rink-Tentagel resin (Tentagel S RAM) (0.25 mmol, 1.14 g) the synthesis was carried out according to the following protocol:

1. Fmoc deprotection: The resin was treated with a 20% solution of piperidine in DMF (3×5 mL, each 8 min). The solution was removed by filtration, followed by washing of the resin with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 mL, each 2 min). Deprotection was checked with the Kaiser test.[30]

2. Coupling of Fmoc-protected amino acid: Fmoc-Xxx-OH (1.0 mmol), HOBt·H_2O (1.0 mmol, 153 mg), HBTU (1.0 mmol, 379 mg) and DiPEA (2.0 mmol, 330 µL) were added to the resin. DMF (10 mL) was added and N_2 was bubbled through the mixture for 1h. The solution was removed by filtration, followed by washing of the resin with DMF (3 × 5 mL, each 2 min) and CH_2Cl_2 (3 × 5 mL, each 2 min). Completion of the coupling was checked with the Kaiser test. Steps 1 and 2 were repeated until the complete peptide was synthesized.

3. Acetylation: Capping solution (10 mL) [Ac_2O (50 mmol, 4.7 mL), HOBt (1.9 mmol, 220 mg), DiPEA (12.5 mmol, 2.2 mL) in 100 mL NMP] was added to the resin and N_2 was bubbled through the mixture for 20 min. The solution was removed by filtration and freshly prepared capping solution (10 mL) was added a the second time. N_2 was bubbled through for 20 min followed by removal of the solution by filtration. The resin was washed with DMF (3×5 mL, each 2 min) and CH_2Cl_2 (3×5 ml, each 2 min). Completion of the acetylation was verified with the Kaiser test.

4. Cleavage: The resin was shaken in a mixture of TFA/TIS/H_2O (95/2.5/2.5) (5 mL) for 5h. The solution was slowly poured into Et_2O (40 mL) to precipitate the cleaved peptide. The supernatant was removed and the crude product was washed twice with Et_2O (40 mL) and dried *in vacuo*. The crude product was purified by preparative HPLC (H_2O/CH_3CN/TFA 95/5/0.1 → CH_3CN/H_2O/TFA 95/5/0.1 in 60 min). The pure fractions were pooled followed by lyophilization to give the pure peptide.

Ac-Glu-Ile-Leu-Ser-Arg-Orn(N_3)-Pro-Ala-Tyr-Arg-Lys-Ile-Leu-NH_2 (10): The peptide was synthesized according to the general procedure for peptide synthesis. After preparative HPLC and lyophilisation, peptide **10** (130 mg, 31%) was obtained as a white solid. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 18.3 min). MALDI-TOF-MS: calcd for $C_{74}H_{126}N_{24}O_{18}$: 1639.97, found: *m/z* = 1640.16 $[M+H]^+$. FTIR (ATR, cm^{-1}): 2102 (N_3).

Ac-Glu-Ile-Leu-Ser-Arg-Lys(Spacer-N_3)-Pro-Ala-Tyr-Arg-Lys-Ile-Leu-NH_2 (11): The peptide was synthesized according to the general procedure used for peptide **10**. After preparative HPLC and lyophilisation, peptide **11** (80 mg, 18%) was obtained as a white powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 18.2 min). MALDI-TOF-MS: calcd for 1799.06, found: *m/z* = 1800.06 $[M+H]^+$. FTIR (ATR, cm^{-1}): 2107 (N_3).

***tert*-Butoxycarbonyl guanyl pyrazole (13)**: To a solution of guanyl pyrazole·HCl **12** (1.46 g, 10 mmol,) in CH_2Cl_2 (10 mL) DiPEA (1.91 mL, 11 mmol) was added at 0°C, followed by the dropwise addition of Boc_2O (2.29 g, 5 mmol) which was dissolved in CH_2Cl_2 (7.5 mL). The reaction mixture was stirred at room temperature for 16 h and concentrated in *vacuo*. The crude compound was suspended in H_2O (30 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with 1N $KHSO_4$ (3 × 20 mL), sat. aq. $NaHCO_3$ (3 × 20 mL), brine (20 mL) and dried on $MgSO_4$. The solvent was evaporated under reduced pressure yielding **13** (2.10 g, quant) as white crystals.

R_f = 0.34 (EtOAc/hexane, 1/4 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 9.08 (br s, 1H, N*H*), 8.46 (d, *J* = 2.8 Hz, 1H, N–C*H*), 7.67 (d, *J* = 1.4 Hz, 1H, N=C*H*), 7.62 (br, s, 1H, N*H*), 6.40 (d, *J* = 2.8 Hz, 1H, CH=C*H*), 1.55 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 163.1, 1.53, 143.3, 128.8, 108.9, 80.1, 28.1 ppm.

Bis-*tert*-butoxycarbonyl guanyl pyrazole (14): A solution of **13** (1.55 g, 7.5 mmol) in dry THF (10 mL) was added dropwise to a suspension of NaH (60 wt% in mineral oil 1.05 g, 26 mmol) in dry THF (20 mL) at 0°C. The mixture was stirred at room temperature for 30 min followed by the dropwise addition of Boc_2O (3.27 g, 15 mmol) dissolved in dry THF (20 mL). The mixture was refluxed for 3 h

and cooled to 5°C followed by the careful addition of sat. aq. $NaHCO_3$ for quenching the excess NaH. THF was removed by concentration in *vacuo* and the aqueous layer was extracted with EtOAc (3 × 40 mL). The combined organic layers were washed with 1N $KHSO_4$ (3 × 40 mL), sat. aq. $NaHCO_3$ (3 × 40 mL), brine (40 mL) and dried on $MgSO_4$ and concentrated in *vacuo*. The crude product was purified using flash chromatography (EtOAc/hexane, 1/4, *v/v*) yielding **14** (2.15 g, 6.93 mmol, 92 %) as a colorless oil.

R_f = 0.26 (EtOAc/hexane, 1/4 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.93 (br s, 1H, N*H*), 8.33 (d, *J* = 3.0 Hz, 1H, N–C*H*), 7.64 (d, *J* = 1.6 Hz, 1H, N=C*H*), 6.43 (d, *J* = 2.8 Hz, 1H, CH=C*H*), 1.57 (s, 9H, C(CH_3)$_3$), 1.52 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 156.9, 149.1, 142.5, 138.8, 128.6, 109.5, 82.9, 80.9, 27.8, 27.7 ppm.

1-(*N,N*'-Bis-*tert*-butoxycarbonyl-*N*-propargyl)-guanyl pyrazole (16a) The *N,N*-bis-Boc-pyrazole **8** (620 mg, 2.0 mmol), propargyl alcohol (116 μL, 2.0 mmol) and triphenyl phosphine (786 mg, 3.0 mmol) were dissolved in freshly distilled THF (15 mL). At 0°C diethyl azodicarboxylate (471 μL, 3.0 mmol) was added dropwise over a period of 30 min. The reaction mixture was stirred at room temperature for 16 h followed by evaporation in vacuo. The crude product was dissolved in EtOAc (30 mL) and washed with 1M $KHSO_4$ (3 × 20 mL), sat. aq. $NaHCO_3$ (3 × 20 mL) and brine (20 mL). The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexane, 1/4, *v/v*) yielding **16a** (550 mg, 79%) as a white solid.

R_f = 0.80 (EtOAc/hexane, 1/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.95 (s, 1H, N–C*H*), 7.70 (s, 1H, N=C*H*), 6.41 (s, 1H, CH=C*H*), 4.48 (s, 2H, NCH_2), 2.27 (s, 1H, C≡C*H*), 1.50 (s, 9H, C(CH_3)$_3$), 1.30 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 156.9, 151.4, 143.2, 129.8, 109.0, 83.4, 82.3, 77.4, 72.8, 37.9, 27.7, 27.5 ppm. FTIR (ATR, cm^{-1}): 1737 (C=O), 1709 (C=O), 1653 (C=N). Anal. calcd. for $C_{17}H_{24}N_4O_4$: C 58.6, H 6.9, N 16.1; found: C 58.9, H 6.8, N 16.3.

1-(*N*-(2-Azidoetyl)-*N,N*'-bis-*tert*-butoxycarbonyl)-guanyl pyrazole (16b) This compound was prepared on a 2 mmol scale according to the procedure used for **16a**. The crude product was purified using column chromatography (EtOAc/hexane, 1/9 *v/v*) yielding **16b** (610 mg, 86%) as a clear, colorless oil.

R_f = 0.76 (EtOAc/hexane, 1/2 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.95 (s, 1H, N–C*H*), 7.67 (t, *J* = 0.7 Hz, 1H, N=C*H*), 6.41 (dd, *J* = 1.6, 1.1 Hz, 1H, CH=C*H*), 3.84 (t, *J* = 5.9 Hz, 2H, CH_2N), 3.63 (t, *J* = 6.3 Hz, 2H, CH_2N$_3$), 1.48 (s, 9H, C(CH_3)$_3$), 1.26 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 157.3, 152.1, 143.2, 130.0, 109.0, 83.2, 82.6, 49.6, 47.4, 27.8, 27.6 ppm. FTIR (ATR,

cm^{-1}): 2102 (N_3), 1726 (C=O), 1664 (C=N). Anal. calcd. for $C_{16}H_{25}N_7O_4$: C 50.65, H 6.6, N 25.8; found: C 50.9, H 6.5, N 25.6.

1-(*N*-(2-(2-Azidoethoxy)ethyl)-*N,N'*-bis-tert-butoxycarbonyl)-guanyl pyrazole (16c) This compound was prepared on a 2 mmol scale according to the procedure used for **16a**. The crude product was purified using column chromatography (EtOAc/hexane, 1/4, *v/v*) yielding **16c** (706 mg, 84%) as a clear, colorless oil.

R_f = 0.74 (EtOAc/hexane, 1/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.82 (s, 1H, N–C*H*), 7.52 (t, *J* = 0.82 Hz, 1H, N=C*H*), 6.26 (m, 1H, CH=C*H*), 3.77 (t, *J* = 5.0 Hz, 2H, CH_2), 3.62 (t, *J* = 5.4 Hz, 2H, CH_2), 3.42 (t, *J* = 5.0 Hz, 2H, CH_2), 3.10 (t, *J* = 4.9 Hz, 2H, CH_2), CH_2, 1.34 (s, 9H, C(CH_3)$_3$) , 1.12 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 157.2, 152.1, 142.7, 129.7, 108.5, 82.5, 81.9, 69.0, 68.4, 50.4, 47.4, 27.6, 27.4 ppm. FTIR (ATR, cm^{-1}): 2101 (N_3), 1726 (C=O), 1664 (C=N). Anal. calcd. for $C_{18}H_{29}N_7O_5$: C 51.05, H 6.9, N 23.15; found: C 51.2, H 6.7, N 23.1.

1-(*N*-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)-*N,N'*-bis-tert-butoxycarbonyl)-guanyl pyrazole (16d) This compound was prepared on a 2 mmol scale according to the procedure used for **16a**. The crude product was purified using column chromatography (EtOAc/hexane, 1/4, *v/v*) yielding **16d** (660 mg, 70%) as a clear, colorless oil.

R_f = 0.66 (EtOAc/hexane, 1/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.95 (s, 1H, N–C*H*), 7.66 (dd, *J* = 1.1, 0.5 Hz, 1H, N=C*H*), 6.38 (dd, *J* = 1.6, 1.1 Hz, 1H, CH=C*H*), 3.90 (t, *J* = 5.2 Hz, 2H, CH_2), 3.75 (t, *J* = 5.6 Hz, 2H, CH_2), 3.55 (m, 6H, 3 × CH_2), 3.32 (t, *J* = 5.1 Hz, 2H, CH_2), 1.49 (s, 9H, C(CH_3)$_3$), 1.25 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 157.7 (C=O), 152.6 (C=N), 143.2, 130.4, 108.8, 83.0, 82.4, 70.7, 70.4, 70.2, 60.0, 50.9, 48.1, 28.2, 27.9 ppm. FTIR (ATR, cm^{-1}): 2102 (N_3), 1726 (C=O), 1665 (C=N)**.** Anal. calcd. for $C_{20}H_{33}N_7O_6$: C 51.4, H 7.1, N 21.0; found: C 51.3, H 7.3, N 21.1.

1-(*N*-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-*N,N'*-bis-tert-butoxycarbonyl)-guanyl pyrazole (16e) This compound was prepared on a 2 mmol scale according to the procedure used for **16a**. The crude product was purified using column chromatography (CH_2Cl_2/MeOH, 99.5/0.5, *v/v*) yielding **16e** (400 mg, 39%) as a clear, colorless oil.

R_f = 0.66 (EtOAc/hexane, 1/1 *v/v*), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.98 (s, 1H, N–C*H*), 7.68 (d, *J* = 1.1 Hz, 1H, N=C*H*), 6.40 (dd, *J* = 1.6, 1.1 Hz, 1H, CH=C*H*), 3.91 (t, *J* = 5.2 Hz, 2H, CH_2), 3.75 (t, *J* = 5.6 Hz, 2H, CH_2), 3.59 (m, 10H, 5 × CH_2), 3.37 (t, *J* = 5.1 Hz, 2H, CH_2), 1.50 (s, 9H, C(CH_3)$_3$), 1.27 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 157.2, 152.1, 142.7, 130.0, 108.4,

82.4, 81.9, 70.3, 69.9, 69.7, 68.3, 50.4, 47.6, 27.6, 27.4 ppm. FTIR (ATR, cm^{-1}): 2102 (N_3), 1727 (C=O), 1665 (C=N). Anal. calcd. for $C_{22}H_{37}N_7O_7$; C 51.6, H 7.3, N 19.2; found: C 51.6, H 7.3, N 19.3.

N^{α}-Fluorenylmethoxycarbonyl-N^{ω},N^{ω}'-bis-*tert*-butoxycarbonyl-N^{ω}-propargyl-arginine (18a): Fmoc-Orn(Boc)-OH **1** (454 mg, 1.0 mmol) was dissolved in a mixture of CH_2Cl_2 (4.0 mL) and a saturated solution of HCl/diethyl ether (4.0 mL). After stirring at room temperature for 1 h a white precipitate was formed and the reaction mixture was evaporated to dryness. The resulting crude HCl salt was suspended in CH_2Cl_2 (5.0 mL) and DiPEA (348 µL, 2.0 mmol) was added. After addition of *N*,*O*-bistrimethylsilyl-acetamide (489 µL, 2.0 mmol) all precipitates dissolved instantly. After stirring at room temperature for 15 min, the guanylating agent **16a** (1.0 mmol) was added and the reaction mixture was stirred for 24 h at room temperature. After addition of CH_2Cl_2 (20 mL), washing with 1M $KHSO_4$ (20 mL) and brine (20 mL) and drying on Na_2SO_4, the solvents were evaporated. After column chromatography (CH_2Cl_2/MeOH, 95/5, *v/v*), **18a** (518 mg, 82%) was obtained as a white foam.

R_f = 0.45 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{22}$ +4.5 (*c* 0.27, MeOH), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 11.6 (br. s, 2H, COO*H*, N*H*), 7.60 (d, *J* = 7.4 Hz, 2H, Fmoc Ar*H*), 7.44 (m, 2H, Fmoc Ar*H*), 7.18 (m, 4H, Fmoc Ar*H*), 5.78 (br. d, *J* = 5.2 Hz, 1H, N*H*), 4.21 (m, 3H, Fmoc CH_2C*H*), 4.05 (m, 1H, C$^{\alpha}$*H*), 3.21 (br. s, 2H, CH_2C≡CH), 2.21 (br. s, 1H, C≡C*H*), 1.64 (m, 4H, C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.35 (s, 9H, C(CH_3)$_3$), 1.34 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 175.8, 156.2, 155.8, 152.9, 143.8, 143.6, 141.0, 127.4, 126.8, 124.9, 119.7, 82.8, 67.7, 66.5, 54.2, 47.0, 45.0, 41.4, 36.8, 30.9, 27.9, 25.3 ppm. Anal. calcd. for $C_{34}H_{42}N_4O_8$; C 64.3, H 6.7, N 8.8; found: C 64.5, H 6.6, N 8.7. ESI-MS calcd. for $C_{34}H_{43}N_4O_8$: 635.3; found: *m/z* = 635.5 $[M+H]^+$.

N^{α}-Fluorenylmethoxycarbonyl-N^{ω}-(2-azidoethyl)-N^{ω},N^{ω}'-bis-*tert*-butoxycarbonyl-arginine (18b): This compound was prepared on a 1 mmol scale according to the procedure used for **18a**. After column chromatography (CH_2Cl_2/MeOH, 96/4, *v/v*), **18b** (411 mg, 62%) was obtained as a white foam.

R_f = 0.35 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{22}$ +8.3 (*c* 0.28, MeOH), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 11.7 (br. s, 2H, COO*H*, N*H*), 7.73 (d, *J* = 7.1 Hz, 2H, Fmoc Ar*H*), 7.60 (m, 2H, Fmoc Ar*H*), 7.29 (m, 4H, Fmoc Ar*H*), 5.96 (br. s, 1H, N*H*), 4.33 (m, 3H, Fmoc CH_2C*H*), 4.19 (m, 1H, C$^{\alpha}$*H*), 3.69 (s, 2H, CH_2), 3.51 (s, 2H, CH_2), 3.33 (s, 2H, C$^{\delta}H_2$), 1.77 (br. m, 4H, C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.48 (s, 9H, C(CH_3)$_3$), 1.46 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 176.1, 155.9, 141.8, 143.8, 143.7, 141.0, 127.5, 126.8, 125.0, 119.7, 83.6, 66.7, 55.9, 53.3, 49.9, 47.0, 29.9, 27.8, 24.5 ppm. Anal. calcd. for

$C_{33}H_{43}N_7O_8$; C 59.5, H 6.5, N 14.7; found: C 59.7, H 6.4, N 15.0. ESI-MS calcd. for $C_{33}H_{44}N_7O_8$: 666.3; found: *m/z* = 666.2 $[M+H]^+$.

N^{α}-Fluorenylmethoxycarbonyl-N^{ω}-(2-(2-azidoethoxy)ethyl)-N^{ω},N^{ω}'-bis-*tert*-butoxycarbonyl-arginine (18c): This compound was prepared on a 1 mmol scale according to the procedure used for **18a**. After purification by column chromatography (CH_2Cl_2/MeOH, 96/4, *v/v*), **18c** (451 mg, 64%) was obtained as a white foam.

R_f = 0.31 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{22}$ +7.2 (*c* 0.20, MeOH), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 12.2 (br. s, 2H, COO*H*, N*H*), 7.73 (d, *J* = 7.3 Hz, 2H, Fmoc Ar*H*), 7.60 (m, 2H, Fmoc Ar*H*), 7.30 (m, 4H, Fmoc Ar*H*), 6.02 (br. s, 1H, N*H*), 4.34 (m, 3H, Fmoc CH_2C*H*), 4.19 (t, *J* = 7.0 Hz, 1H, C$^{\alpha}$*H*), 3.30-3.71 (br. m, 10H, C$^{\delta}H_2$, 4 × CH_2), 1.81 (br. m, 4H, C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.48 (s, 9H, C(CH_3)$_3$), 1.47 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 176.2, 155.8, 151.9, 143.9, 143.7, 141.1, 127.5, 126.9, 125.0, 119.8, 83.8, 69.4, 69.0, 66.7, 54.1, 50.4, 48.3, 47.0, 30.0, 27.9, 24.2 ppm. Anal. calcd. for $C_{35}H_{47}N_7O_9$; C 59.2, H 6.7, N 13.8; found: C 59.2, H 6.5, N 13.5. ESI-MS calcd. for $C_{35}H_{48}N_7O_9$: 710.4; found: *m/z* = 710.8 $[M+H]^+$.

N^{α}-Fluorenylmethoxycarbonyl-N^{ω}-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-N^{ω},N^{ω}'-bis-*tert*-butoxycarbonyl-arginine (18d): This compound was prepared on a 1 mmol scale according to the procedure used for **18a**. After purification by column chromatography (CH_2Cl_2/MeOH, 92/8 *v/v*), **18d** (460 mg, 61%) was obtained as a white foam.

R_f = 0.41 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{22}$ +7.3 (*c* 0.2, MeOH), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 11.0 (br. s, 2H, COO*H*, N*H*), 7.73 (d, *J* = 7.4 Hz, 2H, Fmoc Ar*H*), 7.59 (m, 2H, Fmoc Ar*H*), 7.28 (m, 4H, Fmoc Ar*H*), 6.07 (br. s, 1H, N*H*), 4.32 (br. m, 3H, Fmoc CH_2C*H*), 4.19 (m, 1H, C$^{\alpha}$*H*), 3.61 (3 x br. s, 10H, 5 × CH_2), 3.29 (br. s, 4H, C$^{\delta}$H$_2$, CH_2), 1.79 (m, 4H, C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.47 (s, 9H, C(CH_3)$_3$), 1.45 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 176.8, 155.9, 151.4, 143.9, 143.7, 141.1, 128.1, 127.5, 126.9, 125.0, 119.8, 82.9, 70.3, 70.0, 69.8, 69.1, 66.6, 54.4, 50.4, 48.3, 47.0, 30.0, 27.9, 24.4 ppm. Anal. calcd. for $C_{37}H_{51}N_7O_{10}$; C 58.9, H 6.8, N 13.0; found: C 58.8, H 6.8, N 13.1. ESI-MS calcd. for $C_{37}H_{52}N_7O_{10}$: 754.4; found: *m/z* = 753.9 $[M+H]^+$.

N^{α}-Fluorenylmethoxycarbonyl-N^{ω}-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-N^{ω},N^{ω}'-bis-*tert*-butoxycarbonyl-arginine (18e): This compound was prepared on a 1 mmol scale according to the procedure used for **18a**. After purification by column chromatography (CH_2Cl_2/MeOH, 96/4, *v/v*), **18e** (503 mg, 63%) was obtained as a white foam.

R_f = 0.51 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{22}$ +6.0 (*c* 0.20, MeOH), ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 11.6 (br. s, 2H, COO*H*, N*H*), 7.34 (d, *J* = 7.8 Hz, 2H, Fmoc Ar*H*), 7.61 (m, 2H, Fmoc Ar*H*), 7.29 (m, 4H, Fmoc Ar*H*), 6.00 (br. s, 1H, N*H*), 4.36 (m, 3H, Fmoc CH_2C*H*), 4.19 (t, *J* = 6.9 Hz, 1H, C$^{\alpha}$*H*), 3.57 (m, 14H, 7 × CH_2), 3.32 (m, 4H, C$^{\delta}H_2$, CH_2), 1.72 (br. m, 4H, C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.48 (s, 9H, C(CH_3)$_3$), 1.46 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ = 176.0, 155.8, 151.3, 143.9, 143.7, 141.1, 127.5, 126.9, 125.0, 119.8, 82.2, 70.4, 70.3, 69.9, 69.7, 68.9, 66.6, 54.1, 50.4, 48.3, 47.1, 29.9, 27.9, 24.4 ppm. Anal. calcd. for $C_{39}H_{55}N_7O_{11}$; C 58.7, H 6.9, N 12.3; found: C 58.8, H 6.8, N 12.5. ESI-MS calcd. for $C_{39}H_{56}N_7O_{11}$: 798.4; found: *m/z* = 798.9 $[M+H]^+$.

Fmoc-Orn(Boc)-Pro-OH (23): Fmoc-Orn(Boc)-OH **1** (909 mg, 2.0 mmol) and *N*-hydroxysuccinimide (230 mg, 2.0 mmol) were dissolved in dry CH_2Cl_2 (10 mL). After cooling to 0°C, EDCI (383 mg, 2.0 mmol) was added and the resulting mixture stirred for 3h at room temperature. After washing with 1M HCl (10 mL) and brine (10 mL), and drying on Na_2SO_4, the solvent was evaporated and the crude activated ester **22** was redissolved in DMF (10 mL). Proline (253 mg, 2.2 mmol) and DiPEA (383 µL, 2.2 mmol) were added and the reaction mixture was stirred overnight at room temperature. After evaporation to dryness, the residue was redissolved in EtOAc (10 mL), washed with 1M HCl (10 mL) and dried on Na_2SO_4. Column chromatography (CH_2Cl_2/MeOH, 95/5, *v/v*) gave **23** (625 mg, 57%) as a white foam.
R_f = 0.23 (CH_2Cl_2/MeOH, 9/1 *v/v*), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.80 (br. s, 1H, COO*H*), 7.73 (d, *J* = 7.4 Hz, 2H, Fmoc Ar*H*), 7.58 (m, 2H, Fmoc Ar*H*), 7.26 (m, 4H, Fmoc Ar*H*), 6.79 (br. s, 1H, N*H*), 5.06 (br. s, 1H, N*H*), 4.55 (m, 2H, Orn C$^{\alpha}$*H*, Pro C$^{\alpha}$*H*), 4.08 (m, 3H, Fmoc CH_2C*H*), 3.68 (m, 2H, Pro C$^{\delta}H_2$), 3.07 (m, 2H, Orn C$^{\delta}H_2$), 2.21-1.40 (br. m, 8H, Orn C$^{\beta}H_2$, C$^{\gamma}H_2$, Pro C$^{\beta}H_2$, C$^{\gamma}H_2$), 1.42 (s, 9H, C(CH_3)$_3$) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 174.2, 171.6, 156.4, 143.9, 143.7, 141.1, 127.5, 236.9, 125.2, 119.7, 79.3, 66.9, 59.1, 52.3, 47.1, 40.1, 37.0, 29.0, 28.7, 28.3, 25.7, 24.9 ppm. Anal. calcd. for $C_{30}H_{37}N_3O_7$; C 65.3, H 6.8, N 7.6; found: C 65.6, H 6.8, N 7.3. ESI-MS calcd. for $C_{30}H_{38}N_3O_7$: 552.3; found: *m/z* = 551.8 $[M+H]^+$.

Fmoc-Arg(2-(2-azidoethoxy)-ethyl)-Pro-OH (25): Fmoc-Orn(Boc)-Pro-OH **23** (552 mg, 1.0 mmol) was dissolved in a mixture of CH_2Cl_2 (5.0 mL) and HCl/diethyl ether (5.0 mL) and stirred for 15 min, after which the solvents were evaporated. The resulting white solid, intermediate **24**, was suspended in dry THF (10 mL) after which DiPEA (523 µL, 3.0 mmol) and *N*,*O*-bistrimethylsilyl-acetamide (734 µL, 3.0 mmol) were added. This mixture was stirred for 10 min at room temperature, at which point a clear solution was obtained. Guanylating agent **16c** (1.0 mmol) was added and the reaction mixture was stirred for 24 h at room temperature, followed by evaporation of the solvent. The residue

was suspended in EtOAc (10 mL), washed with 1M HCl (10 mL) and dried on Na_2SO_4. The resulting crude product was purified by column chromatography (CH_2Cl_2/MeOH, 92/8, *v/v*) to give **25** (637 mg, 79%) as a white foam.

R_f = 0.35 (CH_2Cl_2/MeOH, 9/1 *v/v*), $[\alpha]_D^{20}$ – 22.7 (*c* 0.10, MeOH), ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.5 (br. s, 2H, COO*H*, N*H*), 7.72 (d, *J* = 7.4 Hz, 2H, Fmoc Ar*H*), 7.59 (m, 2H, Fmoc Ar*H*), 7.23 (m, 4H, Fmoc Ar*H*), 7.14 (d, *J* = 7.1 Hz, 0.5H, N*H*), 6.98 (d, *J* = 7.9 Hz, 0.5H, N*H*), 4.56 (m, 2H, Arg C$^{\alpha}$*H*, Pro C$^{\alpha}$*H*), 4.25 (m, 3H, Fmoc C*H*$_2$C*H*), 3.80-3.23 (m, 12H, Pro C$^{\delta}$*H*$_2$, Arg C$^{\delta}$*H*$_2$, 4 × C*H*$_2$), 2.19-1.63 (m, 8H, Arg C$^{\beta}$*H*$_2$ C$^{\gamma}$*H*$_2$, Pro C$^{\beta}$*H*$_2$ C$^{\gamma}$*H*$_2$), 1.48 (s, 9H, C(C*H*$_3$)$_3$), 1.46 (s, 9H, C(C*H*$_3$)$_3$) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 174.2, 174.0, 170.9, 170.8, 162.7, 156.2, 156.0, 152.8, 151.8, 143.7, 143.5, 140.9, 128.7, 127.9, 127.3, 126.8, 125.1, 125.0, 119.5, 83.0, 82.4, 79.2, 69.0, 68.9, 66.6, 59.1, 52.1, 46.8, 40.4, 29.0, 28.6, 24.9, 24.7, 27.8 ppm. Anal. calcd. for $C_{40}H_{54}N_8O_{10}$; C 59.5, H 6.7, N 13.9; found: C 59.7, H 6.6, N 14.1. ESI-MS calcd. for $C_{40}H_{55}N_8O_{10}$: 807.4; found: *m/z* = 807.1 $[M+H]^+$.

Ac-Glu-Ile-Leu-Ser-Arg-Arg(Spacer-N_3)-Pro-Ala-Tyr-Arg-Lys-Ile-Leu-NH_2 (21): On Fmoc-Rink-Tentagel resin (Tentagel S RAM) (0.125 mmol, 1.14 g) the synthesis was carried out according to the following protocol:

1. Fmoc deprotection: According to the synthesis of peptide **10**.

2. Coupling of Fmoc-protected amino acid: As was described for peptide **10**. Dipeptide Fmoc-Arg(Spacer-N_3)-Pro-OH was coupled using the described coupling conditions.

3. Acetylation: As was described for peptide **10**.

4. Cleavage: As was described for peptide **10**. The crude product was purified by preparative HPLC (H_2O/CH_3CN/TFA 95/5/0.1 → CH_3CN/H_2O/TFA 95/5/0.1 in 60 min) and after lyophilization, peptide **20** (69 mg, 27%) was obtained as a white solid. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 17.6 min, C8). MALDI-TOF-MS: calcd for $C_{79}H_{138}N_{27}O_{19}$: 1769.06, found: *m/z* = 1769.06 $[M+H]^+$. FTIR (ATR, cm^{-1}): 2112 (N_3).

Bisubstrate based inhibitor 30: A solution of peptide **10** (13 mg, 7.2 μmol), 2-(1-(3-(*N*-methyl-*N*-propargylamino)propyl)-1*H*-indol-3-yl)-3-(1-methyl-1*H*-indol-3-yl) maleimide **26** (2.7 mg, 6 μmol), $CuSO_4 \cdot H_2O$ (1.5 mg, 6 μmol) and sodium ascorbate (1.2 mg, 6 μmol) in *tert*-BuOH/H_2O (5 mL, 1/1 *v/v*) was heated to 80°C for 20 min using microwave irradiation. The crude reaction mixture was lyophilized and purified using preparative HPLC (H_2O/CH_3CN/TFA 95/5/0.1 → CH_3CN/H_2O/TFA 95/5/0.1 in 60 min) yielding **30** (3.2 mg, 25%) as a red powder. Purity was confirmed by analytical

HPLC and was found to be higher than 95% (R_t = 19.2 min, C8). MALDI-TOF-MS: calcd for $C_{102}H_{152}N_{28}O_{20}$: 2089.17, found: *m/z* = 2089.99 $[M+H]^+$

Bisubstrate based inhibitor 31: Product was synthesized on a 12 μmol scale (**27**: 3.7 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **31** (4.8 mg, 20%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 17.4 min, C8). MALDI-TOF-MS: calcd for $C_{90}H_{143}N_{27}O_{20}S$: 1954.07, found: *m/z* = 1955.33 $[M+H]^+$

Bisubstrate based inhibitor 32: Product was synthesized on a 12 μmol scale (**28**: 4.1 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **32** (10.1 mg, 39%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 16.8 min, C8). ESI-MS: calcd for $C_{89}H_{144}N_{30}O_{22}$: 1985.11, found: *m/z* = 993.94 $[M+2H]^{2+}$

Bisubstrate based inhibitor 33: Product was synthesized on a 6 μmol scale (**26**: 2.7 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **33** (2.0 mg, 20%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 19.2 min, C8). MALDI-TOF-MS: calcd for $C_{109}H_{165}N_{29}O_{23}$: 2248.26, found: *m/z* = 2249.30 $[M+H]^+$

Bisubstrate based inhibitor 34: Product was synthesized on a 12 μmol scale (**27**: 3.7 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **34** (10.6 mg, 41%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 17.5 min, C8). MALDI-TOF-MS: calcd for $C_{97}H_{156}N_{28}O_{23}S$: 2113.16, found: *m/z* = 2114.21 $[M+H]^+$

Bisubstrate based inhibitor 35: Product was synthesized on a 6 μmol scale (**28**: 4.1 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **35** (2.2 mg, 17%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 16.9 min, C8). ESI-MS: calcd for $C_{96}H_{157}N_{31}O_{25}$: 2144.20, found: *m/z* = 1074.51 $[M+2H]^{2+}$

Bisubstrate based inhibitor 36: Product was synthesized on a 5.6 μmol scale (**26**: 2.5 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **36** (7.7 mg, 62%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 18.6 min, C8). MALDI-TOF-MS: calcd for $C_{107}H_{163}N_{31}O_{21}$: 2218.26, found: *m/z* = 2219.63 $[M+H]^+$

Bisubstrate based inhibitor 37: Product was synthesized on a 6 μmol scale (**28**: 2.1 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **37** (3.9 mg, 33%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 16.8 min, C8). MALDI-TOF-MS: calcd for $C_{94}H_{155}N_{33}O_{23}$: 2114.20, found: *m/z* = 2114.62 $[M+H]^+$

Bisubstrate based inhibitor 38: Product was synthesized on a 3.5 μmol scale (**29**: 1.3 mg) according to the procedure used for bisubstrate based inhibitor **30**. After preparative HPLC and lyophilisation, bisubstrate based inhibitor **38** (3.1 mg, 40%) was obtained as a red powder. Purity was confirmed by analytical HPLC and was found to be higher than 95% (R_t = 17.9 min, C8). MALDI-TOF-MS: calcd for $C_{93}H_{153}N_{33}O_{24}S$: 2148.15, found: *m/z* = 2148.51 $[M+H]^+$

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Chapter 7

Biological evaluation of a series of bisubstrate based inhibitors for selective inhibition of protein kinase C isozymes

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Abstract: In this chapter the determination of the biological activity of a series of bisubstrate based inhibitors against protein kinases is described. The inhibitors were tested against PKCα, ζ, θ and PKA in a competitive assay with a high ATP concentration. All experiments were performed in a 96-well microarray setup to give a complete picture of the inhibition properties of all compounds against the used kinases. This approach resulted in the uncovering of a selective inhibitor for PKCθ with IC_{50}-values in the lower nM range. Additionally, the inhibitors were tested in an ATP- and substrate-dependent experiment, which showed that they were competitive with both ATP and the peptide substrates. This confirmed that these compounds indeed acted as bisubstrate based inhibitors.

Introduction

Protein Kinase C (PKC) isozymes are recognized to play important roles in many cellular processes inducing cell growth, proliferation and signal transduction.[1-10] Abnormalities in PKC expression levels are therefore related to a variety of diseases. Therefore, PKC has emerged as a major drug target.[5] An intriguing possibility is inhibition of the protein kinase at the substrate sites in the catalytic domain, where the ATP-binding site and the peptide substrate cleft are located. These can be targeted by potential PKC inhibitors. The development of ATP competitive inhibitors has been the most widely applied strategy for inhibition of protein kinases. However, the high homology of ATP binding sites between protein kinases makes the development of selective inhibitors rather difficult and thus challenging. The peptide substrate binding cleft of the protein kinase is another site that can be targeted. By this strategy, in principle selective inhibition of the kinase should be achieved, but development of high affinity inhibitors is very challenging. Therefore, targeting both the ATP binding site and the peptide substrate binding cleft by "bisubstrate based" inhibitors might lead to high affinity compounds, which are also selective for PKC isozymes.

In this chapter it is pointed out how the protein kinase inhibitory properties of the newly synthesized compounds, which were described in chapter 6, are studied.

For biological evaluation, inhibition of the kinase activity was measured by monitoring the decrease of phosphorylation of the peptide substrates in a porous microarray. Details of this technology, which was developed by Pamgene B.V.,[11,12] are described in the general introduction (chapter 1). Thus, microarrays used in this study were decorated with known kinase peptide substrates containing serine/threonine phosphorylation sites. After phosphorylation, the amount of phosphopeptides was detected as function of time, using anti-phosphoserine/threonine antibodies which on their turn were detected by fluorescent secondary antibodies (See chapter 4, figure 1).

The affinity of the inhibitors was determined in an ATP-competitive experiment, from which IC_{50}-values were calculated. Furthermore, it was investigated whether the used inhibitors acted truly as bisubstrate based inhibitors by two independent experiments in

which the ATP substrate concentration was varied at constant peptide substrate concentration, or the peptide substrate concentration was varied at a constant ATP substrate concentration.[13-15] As a control for selectivity, the most promising PKC bisubstrate based inhibitor was tested against PKA since both enzymes have a high sequence homology.[16-18] The IC_{50}-values were determined of all described compounds and the mode of action was examined in more detail of the compound having the highest affinity and selectivity.

Results and Discussion

Staurosporine mimetic **1**, isoquinoline sulfonamide derivative **2** and ATP analogs **3** and **4** were tested as ATP competitive inhibitors, while compounds **5 – 15** are peptide substrate binding site inhibitors or bisubstrate based inhibitors (Figure 1). All these inhibitors were evaluated with respect to their inhibitory properties against several PKC isozymes.

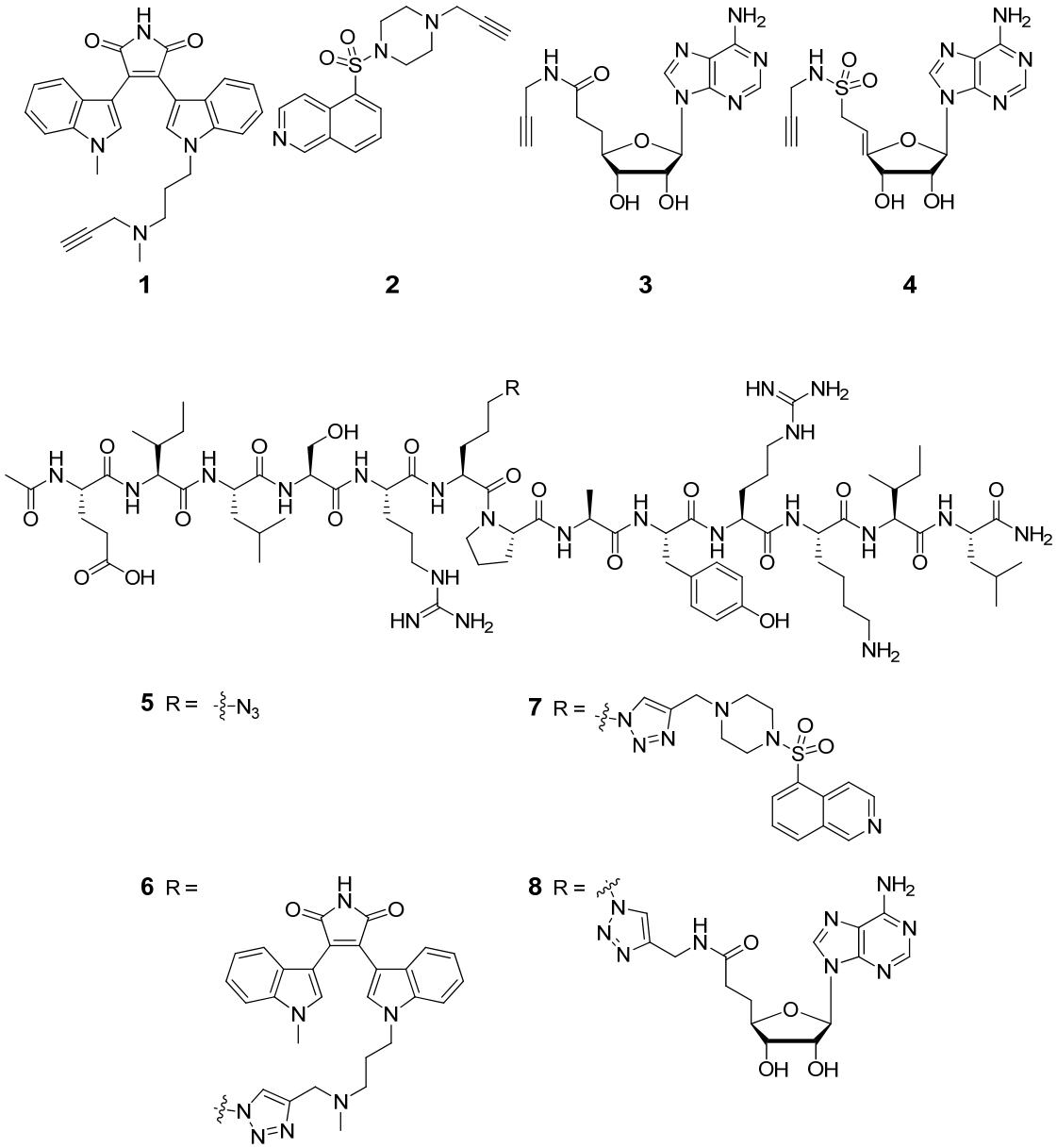
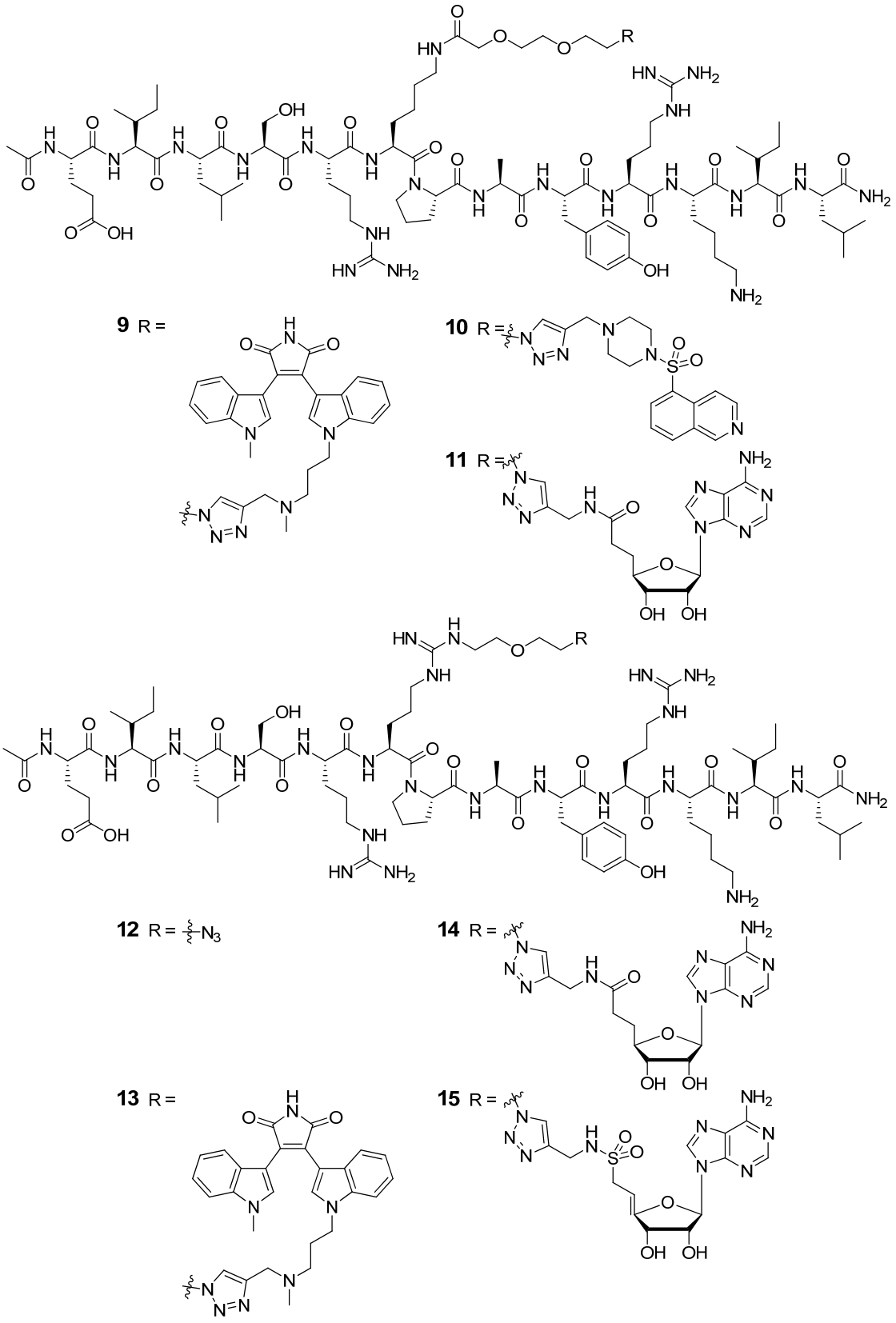


Figure 1: Overview of compounds that have been synthesized for testing of selective inhibition of PKC isozymes. Compounds **1-4** are ATP competitive inhibitors, **5**, **9** and **12** are peptide substrate competitive inhibitors and **6-8**, **10-11** and **13-15** are bisubstrate based inhibitors.



(Figure 1 continued.)

The inhibitory activity and selectivity of inhibitors **5-15** were investigated employing a 96-well microarray format to analyze four inhibitors simultaneously in various concentrations (10 nM up to 10 μM) in triplicate against one isozyme in a single experiment including positive and negative controls. A high ATP concentration of 100 μM was used in order to have a strong fluorescent read-out. This array contains only the substrates that were previously found to be phosphorylated (see chapter 4). Furthermore, the peptide sequences were immobilized on the array in 6 different concentrations (100, 300, 400, 600, 750 and 1000 μM) to enable substrate-dependent competitive inhibition. The peptide substrates used for the inhibition experiments were: CREB1 (cAMP response element binding protein 126-138, EILSRRPSYRKIL),[19] KPCB (PKC splice isoform β-II 19-31, RFARKGSLRQKNV), MARCS (myristoylated alanine-rich C-kinase substrate 152-164, KKKKKRFSKKSF)[20,21] and NCF1 (neutrophil cytosol factor 1 296-308, RGAPPRRSSIRNA).[22]

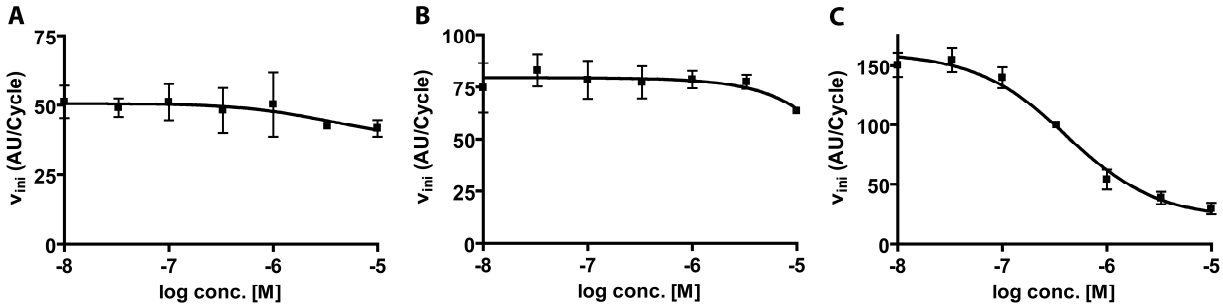


Figure 2: The phosphorylation profiles of CREB1 by PKCα (A), PKCζ (B) and PKCθ (C) in the presence of inhibitor **9**.

From these experiments the v_{ini} of each PKC isoform for a specific substrate could be calculated and plotted against the inhibitor concentration used, yielding typical inhibition curves as shown in Figure 2. Using this setup, a comprehensive insight of the affinity and selectivity of all inhibitors was obtained, and from these curves IC_{50} values were calculated. The results of the inhibition experiments are summarized Figure 3 and Table 1 and only inhibitors with reasonable affinity for one or more PKC isoforms are shown.

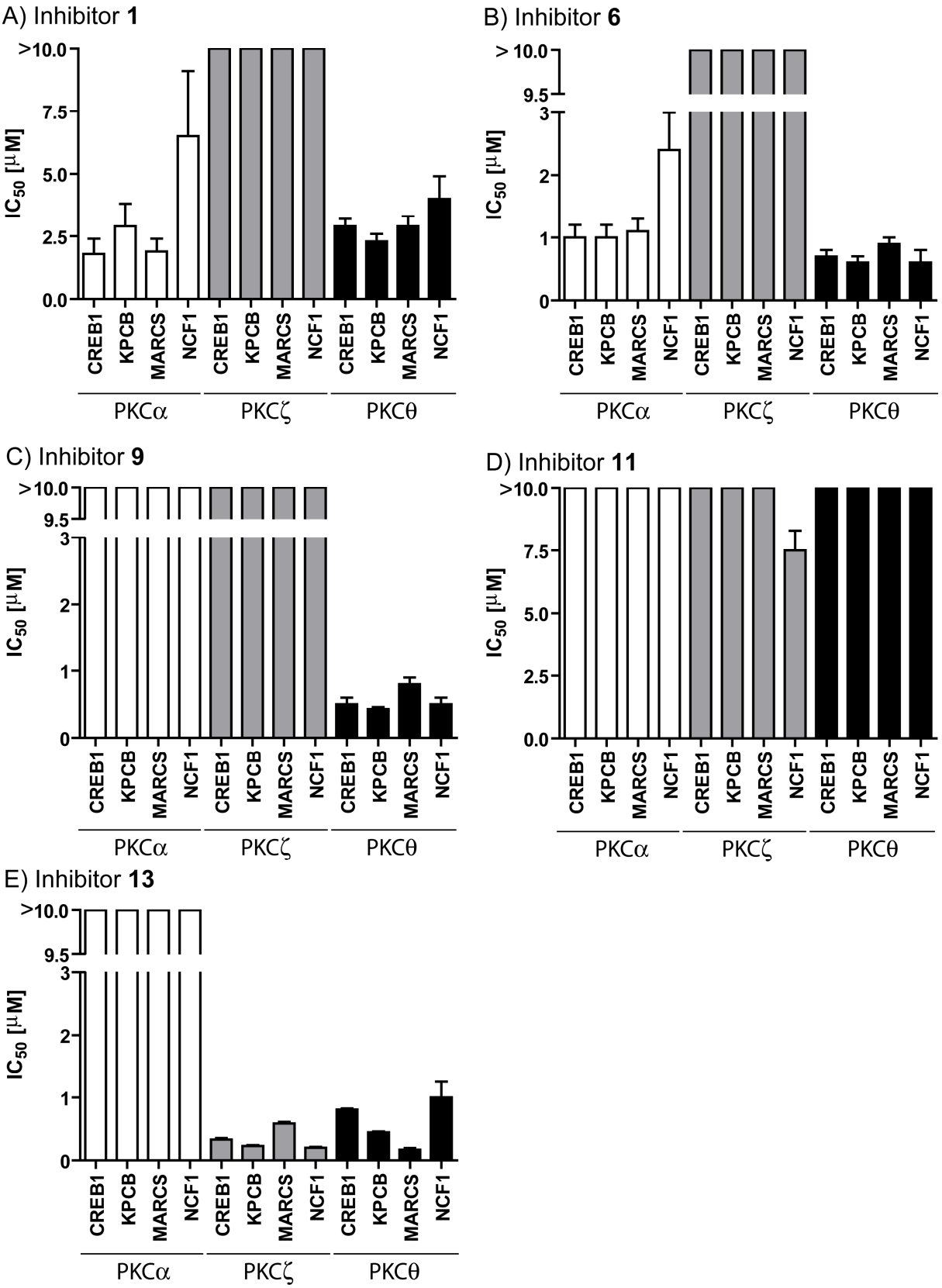


Figure 3: IC_{50}-values of inhibitor **1** (A), **6** (B), **9** (C), **11** (D) and **13** (E) of PKCα, ζ and θ using four different peptide substrates.

Table 1: Biological evaluation of bisubstrate based inhibitors against PKCα, ζ and θ.

		IC_{50} (μM)[a]				
Peptide	**Substrate[b]**	**1**	**6**	**9**	**11**	**13**
PKCα	CREB1	1.8 ± 0.6	1.0 ± 0.2	-	-	-
	KPCB	2.9 ± 0.9	1.0 ± 0.2	-	-	-
	MARCS	1.9 ± 0.5	1.1 ± 0.2	-	-	-
	NCF1	6.5 ± 2.6	2.4 ± 0.6	-	-	-
PKCζ	CREB1	-	-	-	-	0.33 ± 0.029
	KPCB	-	-	-	10.2 ± 0.7	0.23 ± 0.016
	MARCS	-	-	-	-	0.59 ± 0.026
	NCF1	-	-	-	7.5 ± 0.8	0.20 ± 0.017
PKCθ	CREB1	2.9 ± 0.3	0.7 ± 0.1	0.5 ± 0.1	-	0.81 ± 0.019
	KPCB	2.3 ± 0.3	0.6 ± 0.1	0.43 ± 0.03	-	0.45 ± 0.012
	MARCS	2.9 ± 0.4	0.9 ± 0.1	0.8 ± 0.1	-	0.17 ± 0.029
	NCF1	4.0 ± 0.9	0.6 ± 0.2	0.5 ± 0.1	-	1 ± 0.25

[a] No value is given when, IC_{50} >> 10 μM. [b] CREB1 (cAMP response element binding protein 126-138, EILSRRPSYRKIL), KPCB (PKC splice isoform β-II 19-31, RFARKGSLRQKNV), MARCS (myristoylated alanine-rich C-kinase substrate 152-164, KKKKKRFSKKSF) and NCF1 (neutrophil cytosol factor 1 296-308, RGAPPRRSSIRNA). Inhibitors **2 – 5**, **7**, **8**, **10**, **12**, **14** and **15**, did not show any inhibition of one of the three tested PKC isozymes.

Firstly, the ATP-competitive inhibitors **1** to **4** were tested. Only staurosporine mimic **1** (Figure 3A) caused a decent inhibition with IC_{50}-values of 2 – 4 μM for PKCα and PKCθ. However, **1** was found to be inactive against PKCζ. The observed inhibition pattern was comparable to the natural PKC inhibitor staurosporine since a lower affinity of staurosporine towards PKCζ was reported in the literature as well.[23,24]

Then, pseudosubstrate **5** and bisubstrate based inhibitors with a short spacer **6**-**8** were tested for their affinity towards PKC isozymes. Pseudosubstrate **5** and bisubstrate based inhibitors **7** and **8** were inactive at the concentrations tested. However, the bisubstrate based inhibitor

6 showed a similar inhibition of PKCα as the ATP binding site inhibitor **1** with IC_{50}-values of 1.0 – 1.1 μM (Figure 3B). Its potency to inhibit PKCθ mediated phosphorylation is significantly better then **1** since a 4-fold reduction in IC_{50} to 0.6 – 0.9 μM was found indicating an inhibitor in the submicromolar range. Thus, an increased selectivity towards the inhibition of PKCθ was obtained with bisubstrate based inhibitor **6**.

Next, pseudosubstrate **9** and bisubstrate based inhibitors **10** – **11** were tested for their inhibition of PKC isozymes. Interestingly, bisubstrate based inhibitor **9** was found almost inactive toward PKCα, which was in sharp contrast to bisubstrate based inhibitor **6**, demonstrating the importance of the spacer length (Figure 3C). Similar inhibitory potency (IC_{50} 0.4 – 0.8 μM) was observed of PKCθ when **9** was compared to bisubstrate **6**. Clearly, bisubstrate based inhibitor **9** has excellent isozyme selectivity for PKCθ.

Selective inhibitors for PKCθ such as **9** are particularly interesting since this kinase has been proposed as a drug target in human T cell leukemias.[2] It should be noted that this selectivity of inhibitor **9** towards isozyme PKCθ was not the same as the selectivity profile observed during selection of PKC substrates described in chapter 4. This peptide was selectively recognized by PKCζ. This may be explained by differences in the binding mode of **9** caused by the attachment of the ATP binding site inhibitor, in which a positively charged arginine residue was replaced by a neutral acylated lysine residue.

Compound **10** did not show any inhibition of the three used PKC isozymes at the tested concentrations. Inhibitor **11** showed selective inhibition of PKCζ with an IC_{50}-value between 7.5 – 10 μM. Though the affinity for PKCζ is low, the selectivity of this compound is good, which makes **11** a good lead compound for further optimization studies (Figure 3D).

Finally, pseudosubstrate **12** and bisubstrate based inhibitors **13-15** were tested, in the latter a positive charge was present by incorporation of a side chain modified arginine residue. Pseudosubstrate **12** and bisubstrate based inhibitors **14** and **15** were not capable of inhibiting PKC isozymes. However, bisubstrate based inhibitor **13**, showed a good inhibition of PKCθ with an IC_{50}-value of 0.4 – 1 μM. For PKCζ an even better inhibition, with an IC_{50}-value of 0.2 – 0.6 μM was observed, which was in agreement with the

observed selectivity profile discussed in chapter 4 (Figure 3E). Thus, the presence of a positive charge at this position of the peptide substrate sequence is of crucial importance for recognition of the inhibitor by the enzyme.

Even more, since a high ATP concentration (100 μM) was used in the inhibition experiments, the obtained IC_{50} values will be significantly higher than the inhibition constants (K_i). The Cheng-Prusoff equation may be used to calculate a K_i from the IC_{50}-value when the K_m for the original substrate, *i.e.* ATP, is known.[13]

$$K_i = \frac{IC_{50}}{(1+[S]/K_m)} \qquad \text{Cheng-Prusoff equation}$$

The K_m of ATP was determined for PKCθ using the peptide microarray by varying the ATP concentration in absence of any inhibitor. The data obtained from this experiment was fitted into a Lineweaver-Burk plot (Figure 4A and B).[15] From this plot the K_m-values were calculated, since the x-intercept represents the $-1/K_m$. These K_m-values were confirmed as derived from a Michaelis-Menten plot (Figure 4C and D),[14] and the calculated K_m-value was 4.3 ± 1.7 μM. Taking this into account, a K_i-value of 141 ± 33 nM can be derived for inhibitor **1**, while bisubstrate based inhibitor **6** had a calculated K_i of 33 ± 7 nM, and bisubstrate based inhibitor **9** was found to have a calculated K_i of 26 ± 8 nM and for bisubstrate based inhibitor **13** a calculated K_i of 28 ± 17 nM was found (Table 2).

Table 2: Calculated K_i-values of inhibitors against PKCθ using the Cheng-Prusoff equation.

	K_i (nM)			
Isozyme	**1**	**6**	**9**	**13**
PKCθ	141 ± 33	33 ± 7	26 ± 8	28 ± 17

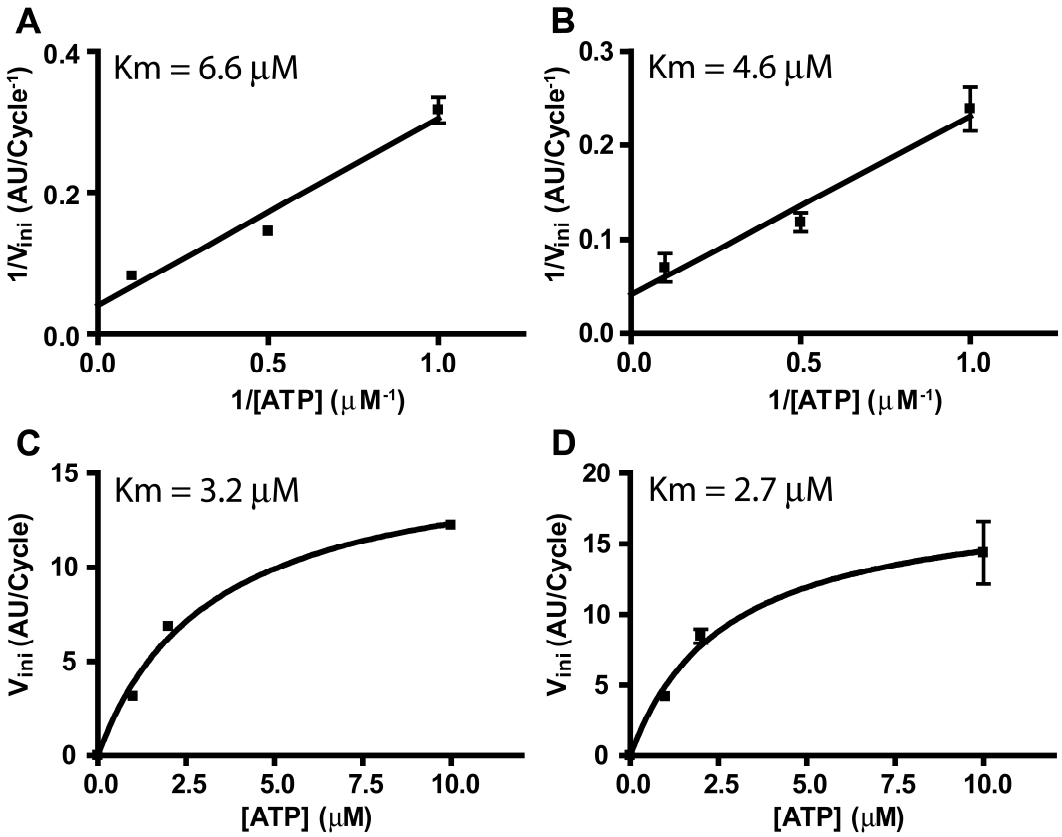


Figure 4: K_m determination of ATP for PKCθ with concentrations of 1, 2 and 10 μM tested on two substrates. **A)** Lineweaver-Burk plot on CREB1 substrate **B)** Lineweaver-Burk plot on NCF substrate **C)** Michaelis-Menten plot on CREB1 substrate **D)** Michaelis-Menten plot on NCF substrate.

An independent confirmation of the inhibition data was obtained using the FRET-based solution-phase Z'-Lyte™ assay as developed by Invitrogen.[25] In this assay, a double fluorescently labeled phosphoacceptor peptide substrate is phosphorylated by a protein kinase. After phosphorylation, this peptide is cleaved by a protease resulting in a different fluorescent emission profile than the intact peptide substrate. From this difference in fluorescent emission profile the IC_{50} values were determined by Invitrogen. Figure 5 shows the inhibition curves for bisubstrate based inhibitor **9** in the presence of PKCα (Figure 5A), PKCθ (Figure 5B) and PKCζ (Figure 5C) as assayed at high ATP concentration (100 μM). The inhibition profiles clearly showed a similar selectivity profile as was observed in the microarray experiments shown in Table 1. It should be noted that in the analysis by the Z'-Lyte™ assay an IC_{50} of 60 nM was observed for inhibitor **9** toward PKCθ. This value was 10-fold lower compared to the results obtained from the microarray assay, which might be due to less diffusion limitations for the solution phase assay as compared to the

heterogeneous microarray technology. This lower IC_{50} indicates that the described inhibitors could be even more potent, and have a K_i in the low nanomolar range.

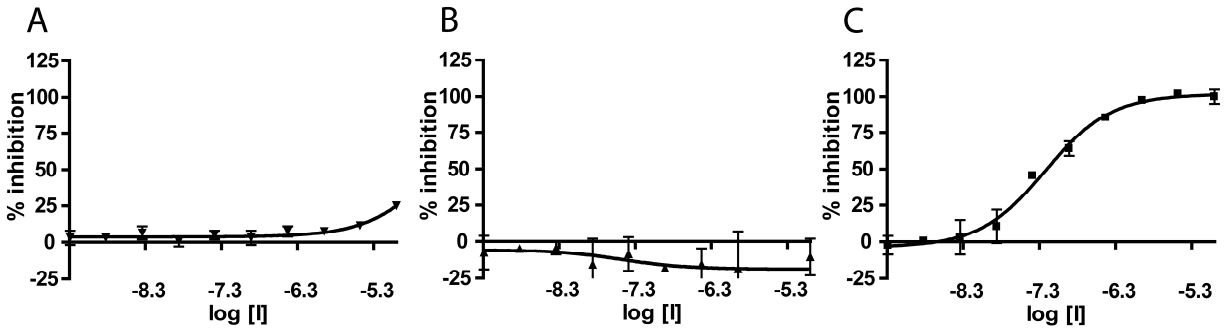


Figure 5: The inhibition curves of the FRET-based assay of inhibitor **9**. **A)** Inhibition of PKCα. **B)** Inhibition of PKCζ. **C)** Inhibition of PKCθ.

In order to determine the mechanism of inhibition of the bisubstrate based inhibitors, additional experiments were performed in which the rate of phosphorylation was monitored as a function of the inhibitor concentration by variation of either the peptide substrate- or the ATP-concentration. The dependency of the peptide substrate concentration on the phosphorylation rate could be conveniently evaluated from the excisting dataset, since each peptide on the microarray was spotted at different concentrations. This also demonstrated the potential of these microarrays for a quick and comprehensive analysis of kinase inhibitors. Thus, by plotting the reciprocal initial velocity as a function of the concentration of inhibitor **9** for different peptide substrate concentrations, a Dixon plot[26] could be constructed that showed the characteristic pattern for a competitive inhibitor (Figure 6A). From these data a K_i of 1.1 μM was calculated. A similar experiment was carried out by variation of the ATP concentration and also here a Dixon plot, characteristic of a competitive inhibitor (Figure 6B and C) with a K_i of 80 nM, was obtained.

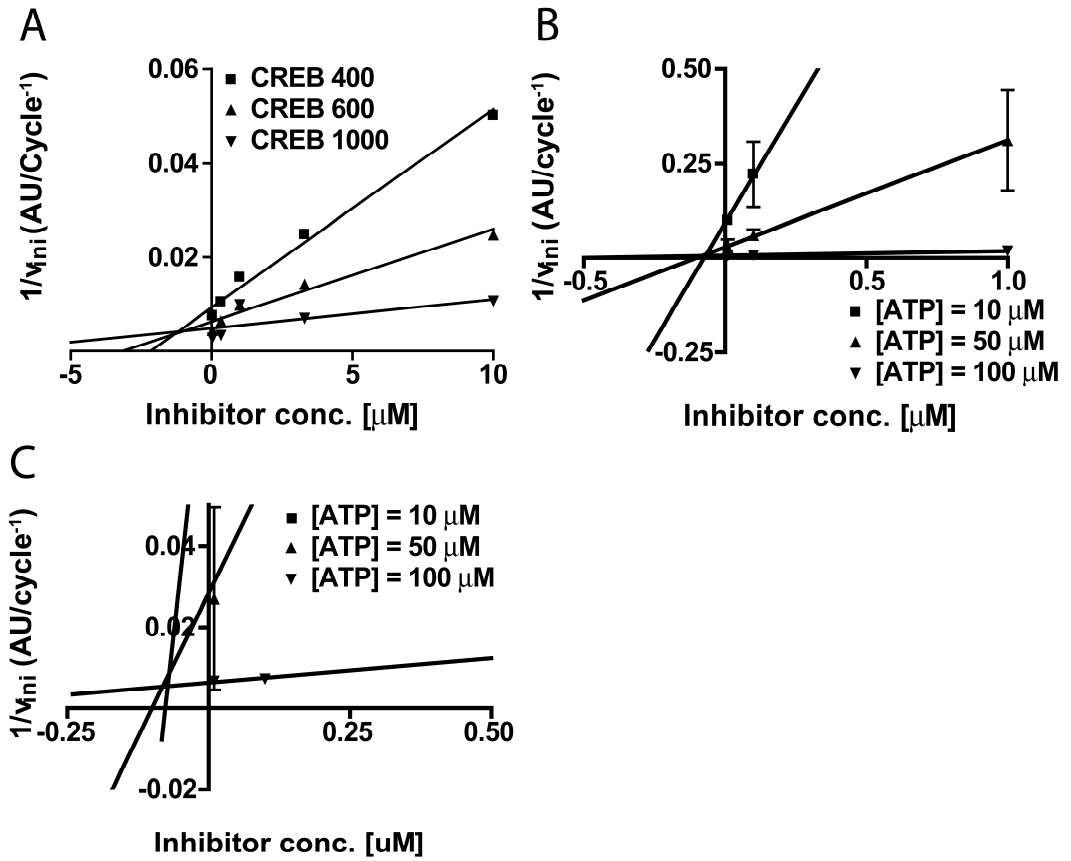


Figure 6: Kinetic binding results of bisubstrate inhibitor **9** toward PKCθ. **A)** Dixon plot of inhibitor in combination with different substrate concentrations of the CREB1 peptide on the peptide microarray of 400, 600 and 1000 μM. **B)** Dixon plot of inhibitor with different ATP concentrations of 10, 50 and 100 μM. **C)** Enlargement of **B**, clearly showing the position of the intersection.

Furthermore, the selectivity of bisubstrate inhibitor **9** was determined by testing its affinity toward PKA, which is a kinase highly homologous to the PKC isozyme family.[27] The peptide microarrays were used to study inhibition of PKA under similar conditions as the PKC inhibition assay, with inhibitor concentrations ranging from 0.33 to 10 μM. The calculated v_{ini}-values were compared with the positive controls, in which no inhibitor was present, to calculate the relative activity. No inhibition was observed at these concentrations, which is a strong indication that bisubstrate based inhibitor **9** has excellent selectivity for PKCθ (Figure 7).

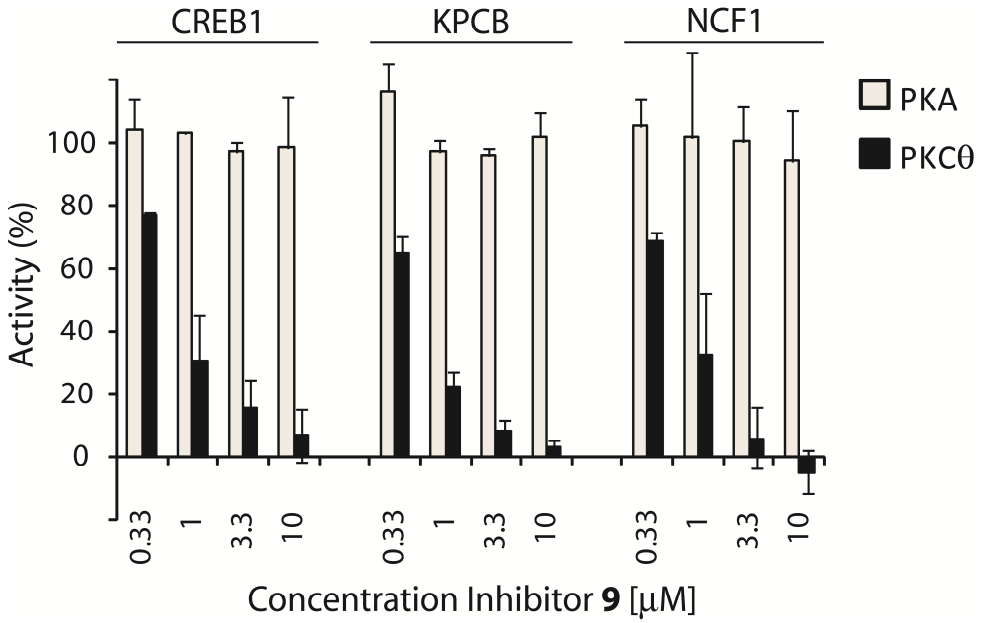


Figure 7: Relative activity of PKA and PKCθ on three peptide substrates in the presence of bisubstrate based inhibitor **9**.

Conclusions

In this chapter the biological evaluation of ATP-competitive, peptide substrate competitive and bisubstrate based PKC inhibitors, which are both ATP and peptide substrate competitive, is described. It has been shown that the dynamic peptide microarray approach is a convenient tool for the evaluation of these kinase inhibitors. Staurosporin analog **1** showed an expected high affinity towards PKCα and θ. Furthermore, the newly synthesized bisubstrate based inhibitors **6**, **9** and **13** showed a significant increase in affinity compared to the ATP analogs and the peptide pseudosubstrates giving rise to K_i-values in the low nM-range. Moreover, besides an increased affinity, selective inhibition was observed of bisubstrate based inhibitor **9** toward PKCθ. Therefore, inhibitor **9** was tested against the highly homologous protein kinase A, confirming the selectivity of this inhibitor for PKCθ, since compound **9** did not show any inhibition of PKA. To prove the robustness of the microarray setup for biological evaluation, the high affinity and selectivity of inhibitor **9** toward PKCθ was tested in an independent FRET-based assay by Invitrogen, which gave similar results. Finally, the mode of action was tested for bisubstrate based inhibitor **9**. Since this compound as well as the other compounds were designed as PKC inhibitors

capable of inhibition of both ATP and peptide substrate binding. Thus, an experiment was carried out in which the ATP- or the peptide substrate-concentration was varied and the corresponding v_{ini} was plotted in a Dixon-plot. This revealed that inhibitor **9** indeed acted as a bisubstrate based inhibitor by targeting both substrate binding sites.

Experimental section

General: Microarray experiments were performed using PamChip peptide arrays run on a PamStation4 instrument (PamGene, 's Hertogenbosch, the Netherlands). Four temperature-controlled peptide chips were run in parallel by pumping the sample up and down through the 3-dimensional porous chip. Data were collected by real-time imaging of the fluorescence signal by CCD imaging. The serine/threonine kinase PamChip arrays comprised 8 different peptides in 6 concentrations (JPT Peptide Technologies GmbH). The Protein Kinase C isozymes were purchased from Invitrogen. Prior to application of the sample, the chips were blocked using a solution of 0.5% BSA (Bovine Serum Albumin, Fraction V, Calbiochem, Germany), and washed 2 times using kinase reaction buffer. During 60 min incubation at 30°C, real-time images were taken automatically every 2.5 min). Images were analyzed by BioNavigator software (PamGene, 's Hertogenbosch, the Netherlands). The fluorescence intensities were expressed as arbitrary units.

Inhibitor evaluation on PKC: On a 96-well plate all inhibitors were tested in concentrations of 0.01, 0.033, 0.1, 0.33, 1.0, 3.3 and 10 μM on PKCα, ζ and θ in triplicate. Per array a 25 μL solution was used containing an inhibitor solution (2.5 μL, H_2O/DMSO, 4:1 *v/v*), kinase solution in Abl buffer (20 ng in 0.5 μL), H_2O (6.75 μL), 3-sn-phosphatidyl-L-serine (0.5 mg/mL, 10 μL, Fluka BioChemika), Abl buffer (2.5 μL, New England Biolabs, Ipswich, USA), a solution of BSA in H_2O (0.1 mg/mL, 0.25 μL, New England Biolabs), Phospho-(Ser) PKC Substrate Antibody #2261 in buffer (0.25 μL, Cell Signalling Technologies), fluorescein goat anti-rabbit antibody in buffer (0.1 mg/mL, 0.25 μL) and ATP in H_2O (1.25 mM, 2 μL). For positive controls the inhibitor solution was replaced by 20% aqueous DMSO (2.5 μL) and for negative controls the ATP solution was replaced by H_2O (2 μL). Samples were placed on a PamChip® 96 STK-array plate and during 60 min incubation at 30°C, real-time images were taken automatically every 2.5 min.

K_m determination of ATP for PKCθ: On a PamChip® 4 STK-array plate ATP was tested in concentrations of 1, 2 and 10 μM in duplicate for PKCθ. Per array a 25 μL solution was used containing a kinase solution in Abl buffer (20 ng in 0.5 μL), H_2O (6.75 μL), 3-sn-phosphatidyl-L-serine (0.5 mg/mL, 10 μL, Fluka BioChemika), 20% aqueous DMSO (2.5 μL), Abl buffer (2.5 μL,

New England Biolabs, Ipswich, USA), a solution of BSA in H_2O (0.1 mg/mL, 0.25 μL, New England Biolabs), Phospho-(Ser) PKC Substrate Antibody #2261 in buffer (0.25 μL, Cell Signalling Technologies), fluorescein goat anti-rabbit antibody in buffer (0.1 mg/mL, 0.25 μL) and ATP in H_2O (125, 25 and 12.5 μM, 2 μL). For negative controls the ATP solution was replaced by H_2O (2 μL). Samples were placed on a PamChip® 4 STK-array plate and during 60 min incubation at 30°C, real-time images were taken automatically every 2.5 min.

Inhibitor evaluation on PKA: The experiment was carried out as described previously for PKC on a PamChip® 4 STK-array plate. Inhibitor **9** was used in concentrations of 0.33, 1.0, 3.3 and 10 μM. Per array a 25 μL solution was used containing Protein Kinase A solution in Abl buffer (20 ng in 0.5 μL). Samples were placed on the peptide micro array and during 60 min incubation at 30°C. Real-time images were taken automatically every 2.5 min.

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Appendices

List of abbreviations

Summary

Nederlandse samenvatting

Curriculum vitae

List of publications

Dankwoord

List of abbreviations

Amino Acids

XXX	X	Amino Acid	XXX	X	Amino Acid
Ala	A	Alanine	Leu	L	Leucine
Arg	R	Arginine	Lys	K	Lysine
Asn	N	Asparagine	Met	M	Methionine
Asp	D	Aspartic Acid	Phe	F	Phenylalanine
Cys	C	Cysteine	Pro	P	Proline
Glu	E	Glutamic Acid	Ser	S	Serine
Gln	Q	Glutamine	Thr	T	Threonine
Gly	G	Glycine	Trp	W	Tryptophan
His	H	Histidine	Tyr	Y	Tyrosine
Ile	I	Isoleucine	Val	V	Valine

Å	Angstrom
Ac	Acetyl
AcOH	Acetic acid
ATP	Adenosine triphosphate
ATR	Attenuated Total Reflection
Boc	*tert*-Butyloxycarbonyl
br s	Broad singlet
BSA	Bovine serum albumin
BuLi	Butyl lithium
Bz	Benzoyl
Calcd	Calculated
CCD	Charge-coupled device
COSY	Correlation spectroscopy
d	Doublet
Da	Dalton
DAG	Diacylglycerol
DCC	*N,N*-Dicyclohexylcarbodiimide
DCE	1,2-Dichloroethane
dd	Double doublet
DEAD	Diethyl azodicarboxylate
DEPBT	3-(Diethoxy-phosphoryloxy)-3H-benzo[d][1,2,3] triazin-4-one
DIC	*N,N*-Diisopropylcarbodiimide
DiPEA	*N,N*-Diisopropylethylamine

DMAP	*N,N*-Dimethyl-4-aminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDT	1,2-Ethanedithiol
ESI-MS	Electro spray ionization mass spectrometry
EtOH	Ethanol
Fmoc	9-Fluorenylmethyloxycarbonyl
FTIR	Fourier transfrom infrared spectroscopy
FRET	Förster resonance energy transfer
Glc	Glucose
h	Hour
HATU	*N*-[(Dimethylamino)-1*H*-1,2,3-triazole[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HBTU	2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMDS	Hexamethyldisilazane
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HONSu	Hydroxy succinimide
HPLC	High pressure liquid chromatography
HWE	Horner-Wadsworth-Emmons
Hz	Hertz
IC50	Half maximal inhibitory concentration
IMAC	Immobilized metal affinity chromatography
J	Coupling constant
KOtBu	Potassium *tert*-Butoxide
LCMS	Liquid chromatography mass spectrometry
M	Molar
m	Multiplet
m/z	Mass to charge ratio
MALDI-ToF	Matrix-assisted laser desorption/ionisation time of flight
m-CPBA	*meta*-Chloroperoxybenzoic acid
MeOH	Methanol
mg	Milligram
MHz	Megahertz
min	Minutes
mL	Milliliter
μM	Micromolar
mM	Millimolar

MS	Mass Spectrometry
NES	Nuclear export signal
NLS	Nuclear localization signal
nM	Nano molar
NMM	*N*-Methyl morpholine
NMP	*N*-Methyl pyrrolidone
NMR	Nuclear magnetic resonance
Orn	Ornithine
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
Ph	Phenyl
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
ppm	Parts per milion
PTM	Post Translational Modification
q	Quartet
Rf	Retardation factor
rpm	Rotations per minute
s	Singlet
SCX	Strong cation exchange
SEM	Standard error of the mean
SPPS	Solid phase peptide synthesis
t	Triplet
TBAI	*tert*-Butylammonium iodide
TBDMS	*tert*-Butyldimethylsilyl
tBu	*tert*-Butyl
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIS	Triisopropylsilane
TLC	Thin layer chromatography
TMS	Trimethylsilane
Ts	Tosyl
UV	Ultra violet

Summary

Protein phosphorylation is one of the most abundant post-translational modifications (PTMs), and it is estimated that half of all proteins will be phosphorylated at least once during their lifetime. Phosphorylation is important for the function of the protein, for example it regulates enzyme activity, signal transduction and cell division. Protein phosphorylation is in principle a reversible process and dephosphorylation can take place by phosphatases. Because protein phosphorylation plays such a central role in virtually all crucial cellular processes, methods have been developed to determine which proteins are phosphorylated, that is to unravel the phosphoproteome. Unfortunately, direct MS analysis of phosphorylated peptides is difficult in positive ion mode due to the negatively charged phosphate moiety. Despite this, nowadays several successful methods have been developed for enrichment and determination of the phosphoproteome. However, when we started this research several analysis methods have been developed to elucidate the phosphoproteome via an indirect approach.

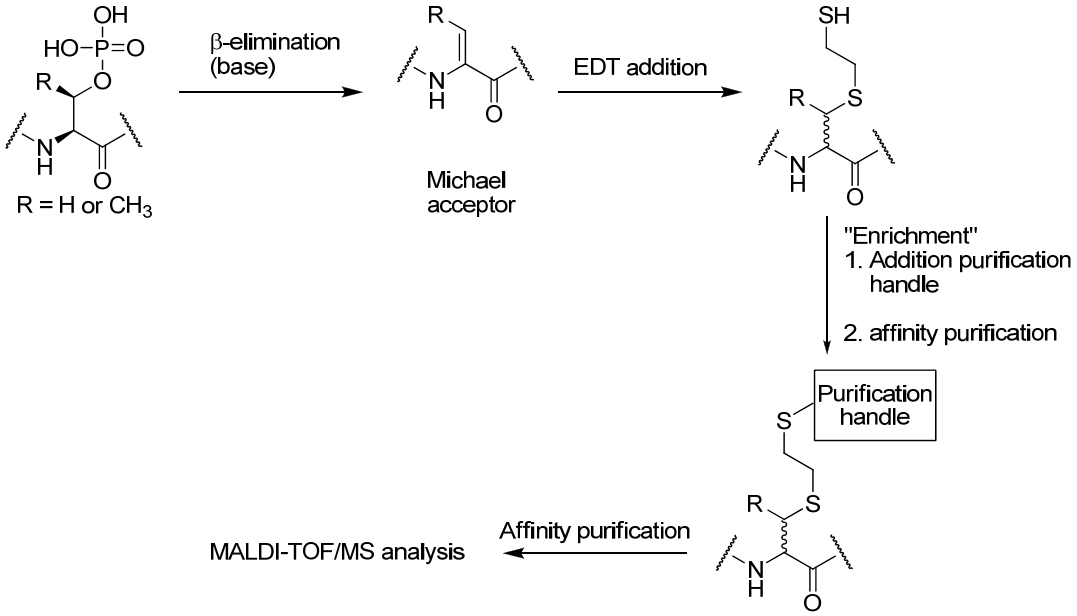


Figure 1: Procedure for the enrichment of Ser-/Thr-phosphorylated peptides via chemical modification.

One approach relies on β-elimination of Ser-/Thr-phosphorylated peptides. This chemical modification under basic conditions results in a neutral amino acid residue and allows enrichment of these peptides from complex mixtures making it more suitable for positive

ion mode MS (Figure 1). Unfortunately, this method for the identification of phosphorylated peptides is not completely selective since *O*-glycosylated peptides are also modified and thus falsely analysed using similar reaction conditions. Therefore, a reliable method for the selective analysis of phosphorylated peptides in the presence of *O*-glycosylated peptides had to be developed, which has been described in chapters 2 and 3.

The enzymes responsible for creating and inactivating the phosphoproteome are protein kinases. The protein kinases comprise over 500 members and form another subproteome, the so called kinome. As such it is one of the largest protein families known to date. A subclass within this group is the Protein Kinase C (PKC) family which consists of 12 isozymes that are highly homologous at the amino acid level. PKC enzymes are involved in a wide range of cellular processes including gene expression and cell growth. Therefore, uncontrolled activation of PKC is related to several serious diseases like cancer and diabetes. With the realization of the importance of protein kinases in cellular processes, this class of enzymes has become a major drug target. However, the synthesis of selective kinase inhibitors has been proven to be difficult since these enzymes show high sequence homology, especially with respect to their catalytic domains. For inhibition of kinases in the catalytic domain three strategies can be used. First, targeting of the ATP-binding site (A), second, targeting of the peptide substrate-binding site (B), and third, a combination of targeting both the ATP-binding site and the peptide substrate binding site (C, Figure 2). In chapter 4 to 7, the design, synthesis and evaluation of bisubstrate based inhibitors was discussed.

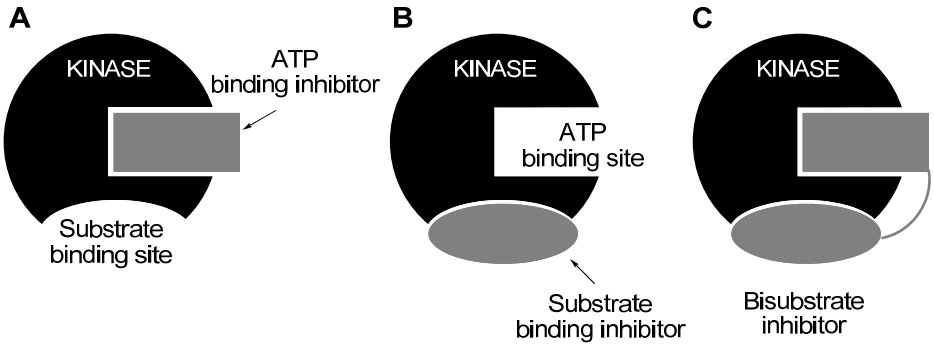


Figure 2: Schematic representation of kinase inhibitors that act on the catalytic domains.

In **Chapter 1** an overview of phosphoproteomics was provided and analytical methods for phosphorylated peptides and proteins used were reviewed. Furthermore, an introduction was given on the PKC-family and the strategies that were used for selective kinase inhibition by the bisubstrate based approach. Finally, dynamic microarrays were discussed in this chapter, since this methodology was used for the identification of peptides selectively phopshorylated by a certain kinase as well as the biological evaluation of the synthesized inhibitors for PKC.

In **Chapter 2** the synthesis of two pSer/pThr peptides as well as their corresponding GlcSer/GlcThr peptide derivatives was described. These peptides were used in a model system for the selective β-elimination and enrichment of pSer/pThr peptides in the presence of *O*-glycosylated peptides. The optimal conditions for selective β-elimination were base- and time-dependent and phosphorylated peptides could be selectively enriched from a mixture in the presence of glycosylated peptides.

In **chapter 3** the model system described in chapter two was applied on a more natural system using intact phosphorylated α-casein. After β-elimination and thiol-modification of the protein, the detection and analysis of the modified protein was performed with a thiol-reactive fluorescent dye, which was then analyzed using gel-electrophoresis. This thiol-reactive fluorescent dye, based on the BODIPY-fluorophore, was successfully synthesized. Unfortunately, despite encouraging initial results described in chapter 2, we were unable to apply this method to intact proteins or complex cell lysates.

For the design and synthesis of selective inhibitors of protein kinase C, a bisubstrate based inhibition approach was followed in which an ATP-competitive and a peptide substrate competitive inhibitor were connected via a flexible linker.

Therefore, in **chapter 4** porous flow-through microarrays were used, to profile the peptide substrates that are selectively phosphorylated by PKCα, ζ or θ. The flow-through microarrays allowed the real-time monitoring of peptide phosphorylation yielding a comprehensive overview of which peptides are selectively recognized by one of the three kinases used in this experiment. Furthermore, the kinase activity towards the peptides was confirmed in an inhibition experiment in which the broad-spectrum kinase inhibitor staurosporine was used. The peptide sequence H-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-

Arg-Lys-Ile-Leu-NH_2 was found to be selectively phosphorylated and this sequence was used in the next chapters as the peptide substrate lead-compound in the design of a bisubstrate based kinase inhibitor.

In **Chapter 5** the synthesis of various ATP-competitive PKC inhibitors suitable for bisubstrate formation was described. For this purpose, all synthesized compounds were equipped with an acetylene functionality to make them suitable for Cu(I)-mediated click-chemistry. The for this purpose synthesized ATP-competitive inhibitors were based on either staurosporine, an isoquinoline sulfonamide derivative, or two adenosine-containing ATP-mimetics.

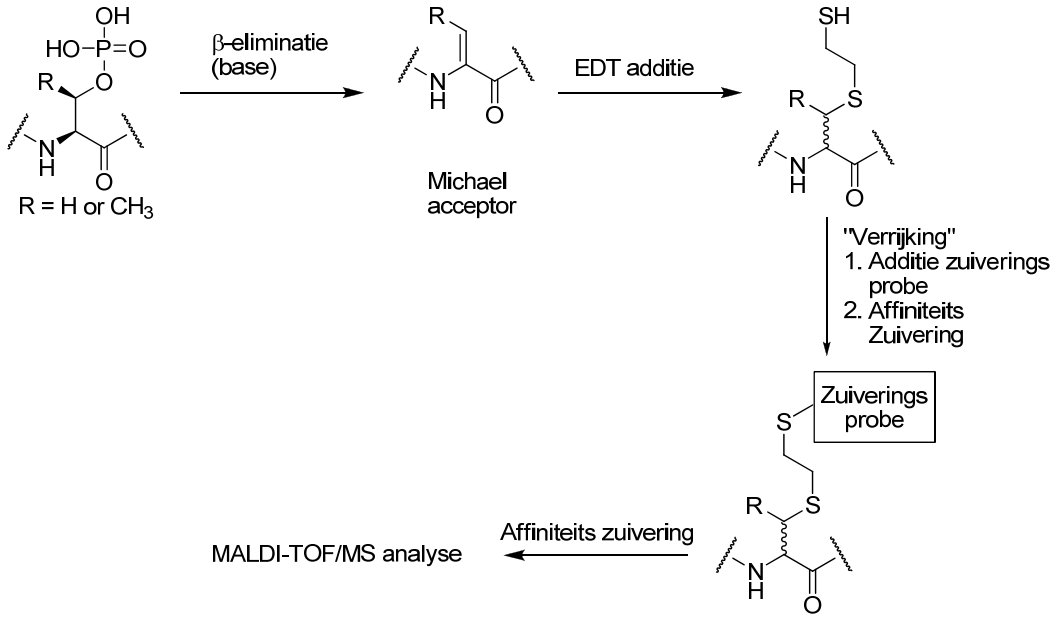
In **Chapter 6** the syntheses of azide-functionalized peptides H-Glu-Ile-Leu-Ser-Arg-Xxx(N_3)-Pro-Ala-Tyr-Arg-Lys-Ile-Leu-NH_2 were described. The required azide containing amino acid from N^{α}-Fmoc-protected ornithine, lysine and arginine residues were synthesized and incorporated into the peptide sequence via solid phase peptide synthesis. Next, the azide-functionalized peptides were coupled to acetylene-functionalized ATP-competitive inhibitors using Cu(I)-mediated click-chemistry to give in total nine bisubstrate based PKC inhibitors.

Finally, it is outlined in **Chapter 7** how all synthesized inhibitors were biologically evaluated for their activity against PKC as a selective inhibitor. The flow-through microarray, as was described in chapter 4, was used for the inhibitor evaluation as well. Bisubstrate based inhibitors containing a staurosporine analog showed an increased affinity with a K_i in the lower nM-range (0.17 – 1.0 nM) compared to the monovalent ATP or peptide substrate inhibitors. Furthermore, one bisubstrate based inhibitor showed selective inhibition of PKCθ with a low nM-affinity (0.6 – 0.9 nM). This selectivity was apparent form an inhibition experiment against the structurally similar kinase PKA, since no inhibition of the latter enzyme was observed. The most promising inhibitor was tested in an independent FRET-based inhibition experiment of Invitrogen to confirm the obtained data. Finally, the mode of action was evaluated for the most selective bisubstrate based inhibitor since this compound was designed to inhibit both the ATP- and peptide substrate binding site. These experiments showed that this compound was indeed capable to compete with both the ATP and peptide substrate.

Nederlandse samenvatting

Fosforylering van eiwitten is één van de meest voorkomende post-translationele modificaties nadat deze eiwitten door het ribosoom gesynthetiseerd zijn. Er wordt zelfs vanuit gegaan dat bijna de helft van alle eiwitten minimaal één keer in zijn bestaan wordt gefosforyleerd. Alle gefosforyleerde eiwitten tezamen vormen het fosfoproteoom. Het fosforyleren van eiwitten is een omkeerbaar proces en op deze wijze kan door middel van (de)fosforylering de functie van een eiwit aan- of uitgezet worden. Dit is belangrijk voor het regelen van processen zoals enzymactiviteit en celdeling.

Omdat fosforylering zo'n belangrijke functie vervult in veel biologische processen wordt veel onderzoek verricht om het fosfoproteoom op te helderen en in kaart te brengen. Dit gebeurt hoofdzakelijk met behulp van massaspectrometrie. Echter, door de negatieve lading van de fosfaatgroep, is de directe analyse van gefosforyleerde eiwitten en peptiden lastig en daarom zijn er verschillende methoden ontwikkeld zodat deze klasse van eiwitten beter geanalyseerd kan worden. Ondanks dit zijn er tegenwoordig enkele succesvolle methoden ontwikkeld voor het onderzoek naar het fosfoproteoom. Toen het onderzoek, zoals hier beschreven, gestart werd waren er verschillende analysemethoden in ontwikkeling voor de opheldering van het fosfoproteoom.

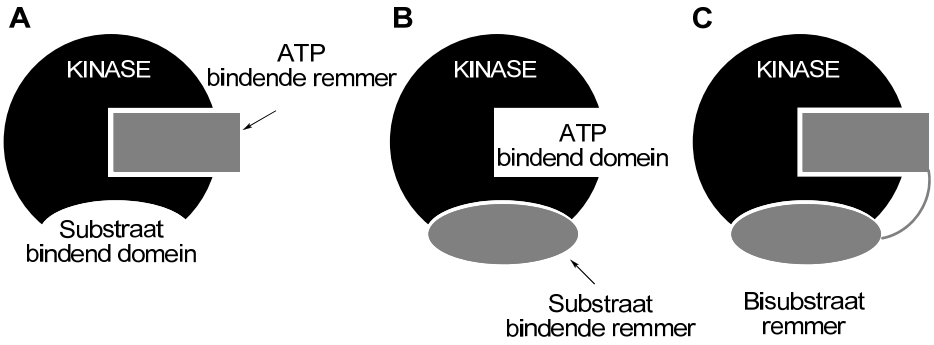


Figuur 1: Procedure voor de verrijking van gefosforyleerde peptiden door middel van chemische modificatie.

Eén methode is gebaseerd op de chemische modificatie van de fosfaatgroep gebonden aan het serine of threonine aminozuur residu (Figuur 1). Onder basische omstandigheden kan er β-eliminatie plaatsvinden en wordt er een neutrale verbinding gevormd. Deze verbinding kan selectief met een thiol reageren, waarna de gevormde sulfideverbinding verrijkt kan worden uit een mengsel van peptiden en/of eiwitten. Dit verrijkte mengsel wordt vervolgens geanalyseerd met behulp van massaspectrometrie. Naast gefosforyleerde eiwitten kunnen ook *O*-geglycosyleerde eiwitten met de hierboven beschreven methode gemodificeerd en geanalyseerd worden, wat in feite een ongewenst proces is. Om zeker te zijn welke post-translationele modificatie (fosforylering of glycosylering) bestudeerd wordt is het wenselijk een betrouwbare en selectieve methode te ontwikkelen voor analyse van alleen gefosforyleerde peptiden en eiwitten. De onwikkeling van deze β-eliminatie methode wordt beschreven in de hoofdstukken 2 en 3.

De enzymklasse die verantwoordelijk is voor de fosforylering en defosforylering van eiwitten en dus het bestaan van het fosfoproteoom zijn de proteïnkinasen en phosphatasen. Deze groep kinasen bevat ongeveer 500 verschillende enzymen, die samen het kinoom vormen. Een kleine subklasse hiervan zijn de protein kinase C (PKC) enzymen, welke 12 kinasen omvat met een zeer grote overeenkomst in de structuur en biologische activiteit. De PKC enzymen spelen een belangrijke rol in enkele processen in de cel zoals genexpressie, celgroei en celdeling. Het niet correct functioneren van deze kinasen kan ernstige consequenties hebben en wordt daarom ook in verband gebracht met het ontstaan van ernstige ziekten zoals diabetes en kanker. Sindsdien is het selectief remmen van PKC een belangrijk doel geworden in de strijd tegen deze ziekten. Het is een zeer grote uitdaging gebleken om selectief één van deze twaalf PKC enzymen te remmen vanwege de reeds eerder genoemde overeenkomsten in structuur en activiteit. Voor het selectief remmen zijn een drietal benaderingen mogelijk (Figuur 2). Ten eerste kan het kinase geremd worden door het ATP-bindend domein te blokkeren (A), zodat het substraat dat gebruikt wordt om eiwitten te fosforyleren niet meer voorhanden is. Ten tweede kan het peptide substraat-bindende domein geblokkeerd worden, zodat er geen eiwitten meer kunnen binden voor fosforylering (B). Tenslotte kan een zogenaamd bisubstraat-achtige inhibitor ontworpen worden dat zowel bindt in het ATP- als in het peptidesubstraat-bindend domein hetgeen kan

resulteren in een selectieve remming van één van de twaalf PKC enzymen. Het ontwerp en de synthese van selectieve op bisubstraat-gebaseerde PKC remmers wordt beschreven in de hoofdstukken 4 tot en met 7.



Figuur 2: Schematische weergave voor de remming van kinases.

In **hoofdstuk 1** werd een algemene inleiding gegeven op dit proefschrift. Hierin werd een overzicht gegeven van het fosfoproteoom en werden de methoden besproken waarmee dit fosfoproteoom bestudeerd kan worden. Daarnaast werd er een overzicht gegeven van PKC enzymen en de strategieën die gebruikt werden om selectief een kinase te remmen. Tenslotte werden in dit hoofdstuk ook de 'micro-arrays' besproken. Met behulp hiervan kan in één experiment veel informatie verkregen worden over de interacties tussen een kinase en de peptide/eiwit substraten die hierdoor gefosforyleerd worden.

Hoofdstuk 2 beschreef de synthese van serine/threonine gefosforyleerde- en serine/threonine geglycosyleerde peptiden. Deze peptiden werden gebruikt in een modelsysteem waar gezocht werd naar de selectieve condities voor β-eliminatie van gefosforyleerde peptiden in de aanwezigheid van *O*-geglycosyleerde peptiden. Hieruit bleek dat de optimale condities voor selectieve β-eliminatie zowel base- alsmede tijdsafhankelijk waren. Na β-eliminatie was het mogelijk om uit een mengsel van gefosforyleerde en *O*-geglycosyleerde peptiden selectief de gemodificeerde fosfopeptiden te verrijken.

In **hoofdstuk 3** werd het modelsysteem, zoals beschreven in hoofdstuk 2, toegepast op de modificatie en analyse van α-caseïne, een van nature gefosforyleerd eiwit. Na β-eliminatie en modificatie van het eiwit met een thiol-derivaat werd de detectie en analyse van het

gefosforyleerde α-caseïne uitgevoerd met een thiol-specifieke fluorescente kleurstof gevolgd door gel-electroforese. Deze thiol-specifieke fluorescente kleurstof, gebaseerd op zogenaamde BODIPY-fluoroforen, is met succes gesynthetiseerd. Ondanks eerdere bemoedigende resultaten, bleek het niet mogelijk om de detectie met succes op complexe mengsels van gefosforyleerde eiwitten toe te passen.

Tevens werd er onderzoek verricht naar de selectieve remming van PKC, een enzym dat verantwoordelijk is voor het fosforyleren van eiwitten. Om selectieve remming van dit enzym te verkrijgen zijn bisubstraat gebaseerde remmers ontworpen en gesynthetiseerd. Om bisubstraat-remmers te verkrijgen zijn ATP-competitieve en peptidesubstraat-competitieve remmers gesynthetiseerd en met een flexibele linker aan elkaar gekoppeld.
In **hoofdstuk 4** werd onderzoek beschreven naar de selectieve interactie tussen PKCα, ζ en θ en hun peptidesubstraten. Hierbij werd gebruik gemaakt van poreuze 'micro-arrays', waardoor het mogelijk was om al tijdens het experiment de interactie te bestuderen tussen een kinase en een groot aantal peptide substraten. Zodoende werd een compleet overzicht van de interactie tussen PKCα, ζ en θ en hun peptidesubstraten verkregen in één experiment. Daarnaast werd ook onderzocht in hoeverre de fosforylering van een peptide daadwerkelijk kinase-afhankelijk was, door een inhibitie-experiment uit te voeren waarin het kinase geremd werd door staurosporine. Het peptide met de aminozuursequentie H-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-Arg-Lys-Ile-Leu-NH_2 werd selectief gefosforyleerd en werd gebruikt als basisstructuur voor het ontwerp van een bisubstraat-kinase-remmer.
In **hoofdstuk 5** werd de synthese van ATP-competitieve remmers voor PKC beschreven, die ook gebruikt kunnen worden voor een bisubstraatinhibitor. Voor de vorming van bisubstraten is gekozen voor de Cu(I)-gekatalyseerde click-reactie tussen een azide en een alkyn. Daarom bevatten alle ATP-competitieve remmers een alkyneenheid. De remmers die gesynthetiseerd zijn, zijn gebaseerd op staurosporine, isoquinoline sulfonamide derivaten en twee adenosine ATP-mimetica.
In **hoofdstuk 6** werd de synthese van het peptide H-Glu-Ile-Leu-Ser-Arg-Xxx(N_3)-Pro-Ala-Tyr-Arg-Lys-Ile-Leu-NH_2, welke met een azide-eenheid gefunctionaliseerd was, beschreven. Dit peptide bevat een azide functionele groep, hiervoor zijn azide gefunctionaliseerde ornithine, lysine and arginine derivaten gesynthetiseerd, gevolgd door

de inbouw in de gewenste peptidesequentie met behulp van peptidesynthese aan de vaste drager. Vervolgens werden de azide-gefunctionaliseerde peptiden gekoppeld aan de alkyn-gefunctionaliseerde ATP-competitieve remmers. Deze koppeling werd uitgevoerd met behulp van de Cu(I)-gekatalyseerde click-reactie, waardoor er in totaal negen bisubstraten verkregen werden die getest konden worden als potentiële isoenzym-selectieve PKC remmers.

Tenslotte werden, zoals beschreven in **hoofdstuk 7**, alle gesynthetiseerde remmers getest als mogelijke selectieve PKC remmers. Hiervoor werd, net als geïllustreerd in hoofdstuk 4, gebruik gemaakt van de poreuze 'micro-arrays'. De bisubstraten die een staurosporine-mimeticum bevatten vertoonden een verhoogde inhibitie in het nanomolaire gebied (K_i = 0.17 – 1.0 nM), ten opzichte van de enkelvoudige ATP- en substraat-competitieve remmers. Daarnaast werd er een bisubstraat gevonden dat naast een verhoogde affiniteit ook nog eens selectief PKCθ (0.6 – 0.9 nM) remde. Deze selectiviteit werd verder bestudeerd door een inhibitie-experiment uit te voeren met PKA, een zeer op PKC-gelijkend kinase, en PKA werd niet geremd. In een onafhankelijk experiment, dat uitgevoerd werd door Invitrogen, werd de selectieve affiniteit van de bisubstraat inhibitor voor PKCθ bevestigd. Om te onderzoeken of de ontworpen remmer ook daadwerkelijk als bisubstraat bond aan het kinase, werden zowel ATP- als peptidesubstraat afhankelijke experimenten uitgevoerd. Hieruit is gebleken dat deze selectieve remmer inderdaad aan zowel het ATP- als aan het substraat-domein van het kinase bond.

Curriculum vitae

Alex J. Poot werd geboren op 7 augustus 1980 te Gouda. Na het behalen van het HAVO-diploma aan de CSG Willem de Zwijger te Schoonhoven in 1998, werd begonnen met de studie Chemie aan de Hogeschool van Utrecht. De afstudeeropdracht werd uitgevoerd bij Organon N.V. te Oss waar onderzoek werd gedaan naar AB-gearomatiseerde steroïden met een substitutie op de 7-positie. In juli 2002 werd het diploma behaald en in september 2002 werd begonnen met de Master Drug Innovation aan de Universiteit Utrecht. Het afstudeeronderzoek werd uitgevoerd bij de disciplinegroep Medicinal Chemistry & Chemical Biology aan de Universiteit Utrecht onder begeleiding van dr. N. Ghalit, dr. ir. D.T.S. Rijkers en prof. dr. R.M.J. Liskamp. Er werd onderzoek gedaan naar de synthese van mimetica van Nisine Z met behulp van alkyn-metathese reacties. Het masterdiploma werd behaald op 28 oktober 2004. Vanaf oktober 2004 tot en met oktober 2008 was de auteur werkzaam als assistent in opleiding bij de disciplinegroep Medicinal Chemistry & Chemical Biology onder begeleiding van dr. ir. D.T.S. Rijkers, dr. M. Slijper en prof. dr. R.M.J. Liskamp aan de Universiteit Utrecht. De behaalde onderzoeksresultaten staan beschreven in dit proefschrift en werden gepresenteerd op twee wetenschappelijke internationale congressen. Sinds februari 2009 is de auteur werkzaam als post-doc bij het radionuclidencentrum en de KNO afdeling van het VU Universitair Medisch Centrum te Amsterdam onder leiding van dr. A.D. Windhorst en prof. dr. G.A.M.S van Dongen.

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International Symposia

1st International Conference on Drug Design & Development, february 2008, Dubai UAE.
Poster Title: Design and Synthesis of Isozyme Selective Bisubstrate Based Protein Kinase C Inhibitors

7th HUPO 2008 World Congress, august 2008, Amsterdam Netherlands
Poster Title: Design and Synthesis of Isozyme Selective Bisubstrate Based Protein Kinase C Inhibitors

Dankwoord

De afgelopen jaren heb ik met veel plezier gewerkt aan dit promotieonderzoek, wat ik natuurlijk niet had kunnen voltooien zonder de hulp van enkele mensen. Ook heb ik tijdens dit promotieonderzoek de mogelijkheid gehad om met veel mensen samen te werken. Hierdoor ben ik niet beperkt gebleven tot het bekende, maar heb ik ook op het spreekwoordelijke gras van de buren rond mogen lopen.

Als eerste gaat mijn dank natuurlijk uit naar Rob Liskamp. Jouw oneindig optimisme, enorme hoeveelheid vertrouwen en de vrijheid die je mij hebt gegeven, hebben ervoor gezorgd dat ik met veel plezier aan dit onderzoek gewerkt heb. Daarnaast waren de ongedwongen werkbesprekingen op jouw kamer vaak een voedingsbodem voor nieuwe ideeën die in het lab zo snel mogelijk tot uitvoering gebracht moesten worden.

Als tweede, maar zeker zo belangrijk, voor het verschijnen van dit boekje, zijn natuurlijk mijn co-promotoren Dirk Rijkers en Monique Slijper. Dirk, al sinds mijn hoofdvak bij MedChem heb ik met je mogen samenwerken. Jouw kennis over peptiden verbaast me nog iedere keer. Ik wil je hartelijk bedanken voor de prettige samenwerking en de vele correcties die je voor me hebt gedaan voor publicaties en hoofdstukken uit dit proefschrift. Monique, jou wil ik bedanken voor je bijdrage op het gebied van massa spectrometrie en je kritische blik op de onderzoeksresultaten en geschreven publicaties.

In het lab bij MedChem heb ik mogen samenwerken met Eelco en Jeroen. In mijn ogen zijn jullie twee ‘chemische-kennis-databases’ waar ik ontzettend veel van heb geleerd. Ik heb met veel plezier met jullie samengewerkt tijdens werktijd als ook daarbuiten, waar we menig lekker biertje hebben bedacht, gemaakt en NATUURLIJK geproeft.

Eef Dirksen heeft me veel hulp geboden bij de massaspectrometrie. Ik heb de samenwerking altijd als prettig ervaren en je hebt me versteld doen staan van hetgeen er allemaal gemeten en opgehelderd kan worden met deze techniek.

Tijdens het tweede gedeelte van mijn AIO-periode heb ik mogen samenwerken met de mensen van PamGene. Rob Ruijtenbeek, Riet Hilhorst en Adriënne van de Berg hebben me

de wereld van de kinases ingeleid. Biochemie was voor mij toen nog onbekend, maar heeft me meer en meer weten te fascineren. Dit is iets wat ik niet had willen missen. De soepele "informele" manier van samenwerken heeft ervoor gezorgd dat we in korte tijd veel hebben kunnen bereiken. Het feit dat er een vervolg wordt gegeven aan dit onderzoek is daar een mooi voorbeeld van.

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Toen ik mijn AIO-periode begon, startte ik tegelijk met twee studiegenoten bij MedChem, Maarten en Joeri. Met veel plezier kijk ik terug op de tijd dat we door de verschillende stadia van het AIO-schap heen zijn gewandeld.

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Alex