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Influencing membrane-active peptides

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Influencing membrane-active peptides

Synthesis and characterization of hybrid molecules, development
towards a molecular sensor.

Hugo van Oosterhout

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Influencing membrane-active peptides

Synthesis and characterization of hybrid molecules, development
towards a molecular sensor.

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Table of Contents

List of abbreviations.....	III
Chapter 1 – Introduction	1
1.1. Antimicrobial peptides	1
1.2. Research interest.....	5
1.3. DNA in templated pore formation.	12
1.4. Goal of the thesis.....	15
1.5. References	16
Chapter 2 – Synthesis of Alamethicin and its derivatives	21
2.1. Introduction.....	21
2.2. Results and discussion	25
2.3. Conclusion	30
2.4. Supplementary Information	30
2.5. References	37
Chapter 3 – Synthesis and Characterization of Alamethicin-DNA hybrids.	39
3.1. Introduction.....	39
3.2. Results and discussion	43
3.3. Alternative approach	47
3.4. Conclusion	51
3.5. Supplementary Information	51
3.6. References	59
Chapter 4 – Evaluation of dULC-ALM–DNA hybrids in Electrophysiology	61
4.2. Black Lipid Membrane Electrophysiology	61
4.3. Goal.....	64
4.4. Results & Discussion	64
4.5. Control experiments.....	66
4.6. Summary of control experiments.....	74

4.7.	Hybrid duplex and G-quadruplex experiments	74
4.8.	Conclusion	79
4.9.	Supplementary information	80
4.10.	References	82
Chapter 5 – Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.....		83
5.1.	Acknowledgements	83
5.2.	Introduction.....	83
5.3.	Results and discussion	86
5.4.	Conclusion	93
5.5.	Supplementary information	94
5.6.	References	106
Chapter 6 – An efficient synthesis of electron-rich aminomethyl azobenzenes 109		
6.1.	Introduction.....	109
6.2.	Results and discussion	110
6.3.	Conclusion	114
6.4.	Supplementary information	114
6.5.	References	124
Chapter 7 – Overview and perspective		127
7.1.	Introduction.....	127
7.2.	Research Overview and Achievements	127
7.3.	Perspective and recommendations.....	129
Summary		131
Samenvatting.....		135
Nawoord		139

List of abbreviations

- ACN/MeCN Acetonitrile
- Aib/U α,α -dimethylglycine
- ALM Alamethicin
- BLM Black-lipid Membrane (electrophysiology), also: Planar lipid
- COMU (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate

- DCC dicyclohexylcarbodiimide
- DCM dichloromethane
- DD doubly distilled
- DIC diisopropylcarbodiimide
- DIPEA diisopropylethylamine
- DMF N,N-dimethylformamide
- DMP Dess-Martin Periodinane
- DMSO Dimethylsulfoxide
- DNA Deoxyribonucleic Acid
- DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
- DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
- DPPE 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
- dULC-ALM Alamethicin with all Aib replaced with leucine, and additionally a C-terminal cysteine

- equiv. (molar) equivalent
- EDC.HCl N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
- HATU 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate

- HBTU *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate

- HMBA 4-Hydroxymethylbenzoic acid
- HOBt 1-Hydroxybenzotriazole hydrate
- HPLC High Performance Liquid Chromatography
- HRMS High Resolution Mass Spectrometry
- IBX Iodoxybenzoic Acid
- MALDI Matrix Assisted Laser Desorption Ionisation
- MOPS 3-(*N*-Morpholino)propanesulfonic acid
- NHS *N*-Hydroxysuccinimid
- NMR Nuclear Magnetic Resonance

• PCC	Pyridiniumchlorochromate
• POPC	1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine
• SPPS	Solid Phase Peptide Synthesis
• TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
• TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
• TEA	Triethylamine
• TFA	Trifluoroacetic acid
• TFE	Trifluoroethanol
• TIPS	Triisopropylsilyl
• TIS	tetraisopropylsilane
• TLC	Thin Layer Chromatography
• TMS	Trimethylsilyl
• TOF	Time of Flight
• UPLC	Ultra Performance Liquid Chromatography

Chapter 1 – Introduction

1.1. Antimicrobial peptides

Any living organism aims to grow and thrive. With limited resources available, this results in constant competition. This competition, in turn, results in attempts at trying to outgrow or otherwise outmanoeuvre other organisms and at defending from the potentially harmful exterior pathogens to which all organisms are exposed to.

Scheme 1 Unselective defence using antimicrobial peptides (left) and selective defence using antigens (right) against prokaryotic organisms.

In order to survive, most organisms have developed complex host defence mechanisms.^[1,2] The constant production and release of antimicrobial peptides into the extracellular medium is believed to be a primitive part of this defence system.^[3] Most eukaryotic organisms have conserved this ability, ranging from fungi to insects to mammals, including humans.^[4,5] These peptides are selectively active against membranes of prokaryotes, which generally have more negatively charged membranes than eukaryotic cells.^[6,7] The selection mechanism is by no means fool proof, as some bacteria, such as *Staphylococcus Aureus*, are capable of lowering the negative charge on their outer membrane, sharply reducing the likelihood of being attacked by these antimicrobial peptides.^[8-11]

Figure 1 Overview of the different characteristics a compound may have in regards to solubility in particular kinds of solvents.

Antimicrobial peptides are typically small (<50 amino acid residues) peptides of various compositions.^[5,12] These peptides come in in a wide variety of forms of α -helices, β -sheets and loops, and they may be neutral, cationic or, less frequently, anionic.^[13,14] What the peptides generally do have in common is an amphipathic nature (i.e. an equal 'dislike' for both polar or apolar media), making these compounds favour being in or on a lipid membrane.^[15] These amphipathic characteristics are either innate to the individual peptide, or induced when multiple peptides converge on a target membrane.^[16]

Figure 2 different modes in which a pore forming peptide may disturb a (cellular) membrane. a. carpet mode; b. toroidal pores; c. barrel-stave pores.

Regarding activity, most antimicrobial peptides are known to impair the cell membrane of their target, allowing the cellular contents to leak out.^[17] For the most part they are classified as having one of three modes of action: the carpet (a), the toroidal pore (b) and the barrel-stave pore (c).^[18] It should be noted beforehand that these modes are not mutually exclusive and depending on the concentration, the same compound may exhibit one or more of these modes of action.^[19]

Figure 3 The carpet mode of membrane permeation. The actions of the pore forming peptide breaks loose micelles from the membrane until the membrane breaks up.

The carpet mode of action, as the name suggests, involves the cell-membrane being enveloped in the peptide, which has a high affinity for the head-groups of the phospholipids. As the peptide binds to the membrane, it exposes a hydrophilic flank.^[20,21] Also, as the concentration of peptide on the membrane increases, it is thought that the peptide starts to perturb the bilayer.^[22] This perturbation, combined with the now exposed hydrophilic side of the antimicrobial peptide promotes micelle formation, which creates holes through the membrane, allowing cytosol contents to spill out of the cell. As more micelles are formed and depart from the membrane, the leaking increases until finally the entire membrane has disintegrated. Notable members of this group are ovipirin and cathelicidin, both found in mammals, sheep in particular.^[23,24]

Figure 4 Toroidal pores are formed when the peptide curves the membrane inwards until a hole is formed in the bilayer. The pore is continuously lined with the polar headgroups of the lipids in the membrane

Toroidal pores are formed in a similar fashion as the micelles from the carpet method, and most antimicrobial peptides capable of forming toroidal pores will also form micelles out of the membrane at high enough concentration.^[25] The difference lies at lower concentrations, where the perturbing action of the peptides is more pronounced.^[26] At low concentrations, these peptides lie parallel with the membrane. However, due to the peptide-membrane interactions, the membrane is curved inwards (positive curvature), creating a break in the hydrophobic region of the membrane. When a threshold concentration is reached, the peptides start to self-associate, to minimize the exposure of hydrophilic groups to the apolar part of the membrane.^[27] While doing so, they switch over to an orientation perpendicular to the membrane, upon which an opening in the membrane is formed, resulting in cytosol spillage out of the cell.^[28,29] The membrane itself remains intact from a certain perspective: no material is removed from the membrane in this process and the peptide does not interact with the central lumen. Examples of this group are mellitin (also found in bee-venom) and maiganin.^[28,29]

Figure 5 Barrel stave pores are formed when the pore forming peptide penetrates through the membrane during pore formation. The centre of the pore still consists out of the apolar tails of the lipids, as opposed to the head-groups in the toroidal pores.

Lastly, barrel-stave pores are formed by mainly α -helical peptides forming a bundle straight through the membrane.^[30,31] After binding to the membrane, these peptides aggregate and undergo a conformational change.^[32,33] These changes induce local thinning of the membrane, after which the bundle inserts itself into the membrane, forming initially an ion-channel lined by the hydrophilic side groups of the peptide, while the hydrophobic groups stick into the central lumen of the membrane allowing leakage of some cytosol content out of the cell.^[34] Depending on the presence of additional peptides on the membrane, the channel may grow larger, allowing for the passage of larger molecules than simple ions (H^+ , Na^+ , Mg^{2+} etc.). The best known example of this group is alamethicin, produced by the fungus *trichoderma viride*.^[35,36]

1.2. Research interest

Over 1000 different antimicrobial peptides have been identified.^[37] While classical antibiotics are losing efficiency due to increasing resistance, most pathogenic strains still have limited to no resistance to antimicrobial peptides.^[38] This, combined with the fact that antimicrobial peptides have a predisposition to affect prokaryotic cells, has led to significant interest from the medical community. In fact, some of these peptides have been found to show significant promise as new antibiotic agents, such as mellitin, indolicidin and β -protegrin.^[39-41]

Another potential application of these compounds is found in molecular logic devices. If a pore forming peptide or cluster thereof can be treated in such a way that it will reliably give the same transmembrane pore (or pore size) to the same stimulus, the current going through can be quantified. This would form the basis for a bio-compatible sensor, or signal processing.^[42,43]

Scheme 2 Strongly simplified diagram for a temperature controller that may be used in an oven.

Let's first imagine a simplified temperature control on an oven: at start-up, the heating elements and the 'oven is warming' light are on. As the temperature rises, a calibrated temperature sensor starts to increase its reporting voltage (e.g. $+5\text{mV}/^\circ\text{C}$), until a pre-defined voltage in the comparator is reached, after which the heating elements and indicator light get switched off.^[44]

To translate the above into a molecular sensor: for analyte X, if the concentration is above a certain level, pore formation should start, allowing ions to flow through the membrane, giving a small but reproducible and quantifiable current across the membrane as output. Or in the ideal world, the pore formation and resulting transmembrane current would be a function of the concentration of analyte X. The same way the oven sensor indicates when the oven is at the desired temperature, this molecular sensor could indicate the presence of absence of a certain analyte or process in a cell.

Figure 6 By measuring the current flowing through a transmembrane haemolysing pore, a DNA sequence may be determined

Some large proteins are already adapted to function in this sense.^[45] A well-known example of this is α -haemolysin, it is a large membrane-spanning protein that normally serves, amongst others, *Staphylococcus Aureus* and *Escherichia coli* by latching on to red blood cells, perforating the membrane to release the iron content out of the cell, making it available to the bacterium.^[46,47] It also allows a single strand of DNA to pass through under the effects of a transmembrane potential. In this role, haemolysin is used as a DNA sequencing tool as the current going through the pore has discrete levels for each base going through it, i.e. the pore gets blocked to a different extent per base, which can be quantified and used to determine the sequence of the strand going through.^[48,49] However, a true sensor that may be dialled to a specific compound and concentration at will (with minimal synthetic effort) still is to be developed, and proteins in general lack a modular character that would allow for a convenient swap to another analyte or concentration level, while maintaining the same reporting moiety.

Pore forming peptides are synthetically much more accessible compared to most proteins, and material from a single synthetic campaign could be modified with a variety of functionalities without having to go through the entire procedure of expressing and then purifying a new protein.^[50] The goal of this work, ultimately, will therefore be to develop a pore forming platform capable of being tuned for various conditions, to serve as a bio-compatible sensor. However, to this end, first the platform itself will have to be developed.

In order to build even the simplest model as described above, several criteria would need to be met.

– Introduction –

- A pore forming peptide should not be detrimental to the structural integrity of the membrane.
- A pore forming peptide should tolerate modifications, without complete loss of activity
- Pores should be uniform in size
- Pores should be uniform in life time
- Pore forming activity should be switchable and controllable

Lastly, a modification to the peptide should be developed that can induce all the above in response to the presence of an analyte, however that will be beyond the scope of this work. The work presented in this manuscript will focus on developing a pore-forming peptide-based construct with control over the pore formation process.

Figure 7 Alamethicin(F30) is a peptaibol. Peptaibols are α -helical peptides with the non-proteinogenic amino acid α -aminoisobutyric acid and a C-terminal alcohol group (red highlight).

In the '80s it was already discovered that Alamethicin, of the barrel-stave method pore forming peptides, perfectly fits the description for the first criterium. First identified in 1967 Alamethicin (ALM), now identified as Alamethicin F30 (ALM F30), has been extensively studied for its pore forming behaviour and is considered the benchmark model pore forming peptides.^[51-54] It is a member of the family of peptaibols, a group ~300 of compounds deriving their name from being a peptide (**Pept**-aibols) which contain the non-proteinogenic amino acid α -aminoisobutyric acid (pept-**Aib**-ols) and have a C-terminal alcohol (peptaib-**ol**).^[55]

As mentioned before, Alamethicin (ALM) operates by forming barrel stave pores. The pores consist out of 3-11 units of ALM, with 4-8 units per pore being preferred.^[56-59] Pore formation is both concentration and voltage dependent, requiring a concentration of 1 μ M in solution and a transmembrane potential of 60 mV or more before pore formation will commence.^[60,61] Also, at these concentrations, it displays essentially no tendency to break the membrane down into micelles, making ALM a good fit for the first criterium of not disturbing the membrane.

The ALM template can also tolerate modification, both on the N- and C-terminus, as well as, at least some, modification of the ALM sequence itself.^[62]

Figure 8 Adding His-Gly to the N-terminus of Alamethicin allows for pore stabilization in the presence of metal ions, by Futaki *et al.*

For example, introducing an additional His-Gly unit on the N-terminus causes, when in the presence of metal ions (Co^{2+} , Ni^{2+} and Zn^{2+}), creates a preference towards 8-, 10- and 12-membered ALM pores over other pore sizes. It also causes a remarkable increase in pore lifetime, from a typical 20-100 ms to >4 seconds per pore. Also, if the His residue is protonated, all pore forming activity ceases.^[63]

Another modification of note is the tolerance towards extra amino acids being introduced on either terminus, the C-terminus being generally the most tolerant of the two termini.^[64,65] Of particular note is that Cysteines can be introduced as well, opening up additional modification strategies.^[66,67] Lastly, several analogues were developed where all Aib residues are replaced with Leu, variations named dUL-ALM. The resulting dUL-ALM's showed a general decrease in pore stability but remained similar enough to the original peptide to still be considered as working analogues for ALM, mostly owing to the synthetic convenience of obtaining the latter versus the original peptide.^[68]

Obtaining ALM through biosynthesis suffers from a limited ability to vary the sequence, while Solid Phase Peptide Synthesis (SPPS) generally suffered from poor yields until Skrydstrup in 2009 and later Brimble in 2017 reported more efficient methods for the synthesis of peptaibols and ALM in particular.^[69,70]

– Introduction –

With this in mind, also the second criterium of modification tolerance is met by ALM and its analogues.

The third criterium, that of producing a uniform pore size, has been met in several ways.

Figure 9 Coupling several Alamethicin units to one cyclodextrin stabilizes pores by blocking the normal migration of the monomers in and out of the pore.

A first solution was proposed by Futaki via the introduction of the aforementioned histidine residue to the N-terminus of ALM, producing selectively pores of 8, 10 or 12 ALM units.^[63] A second approach was attempted by Nishino *et al.* in 1996 and Woolley in 2007 and finally successfully implemented by Skrydstrup *et al.* where 6 and 7 ALM units were coupled on a synthetically introduced C-terminal propargylglycine residue through a copper catalysed click reaction to the same face of a cyclodextrin molecule.^[71-73] The resulting constructs would give only a single pore size per product with an extreme increase in pore lifetime.

Figure 10 when combined with a Leucine-zipper, alamethicin will only form pores in the presence of Fe^{3+} ions. Removing the ions using EDTA effectively halts pore formation.

Another method by which the number of units per pore was controlled was the introduction of a Leucine zipper to the C-terminus of ALM, again by Futaki *et al.* [74] In the presence of Fe^{3+} ions, the zippers would come together in pairs, greatly promoting pore formation, with only even numbers of ALM present per pore. Removing Fe^{3+} by adding an excess of EDTA would result in a significant reduction of the observed pore forming activity.

Criterion four, involving obtaining a uniform pore lifetime has not garnered significant attention in its own right. This may be in part due to the fact that introducing any form of switching behaviour will more than likely limit the time a pore remains open, after its formation becomes disfavoured. Furthermore, with proper statistical processing, pore longevity on its own may not need to have a significant effect on a signal under observation.

The last criterion, introducing an on/off switch in the pore forming behaviour of ALM has received more attention.

With the previous examples by Futaki, we have already discussed two instances where pore formation activity could be encouraged or strongly disfavoured, in the His-analogue and the Leucine zipper. Two more examples will be mentioned in the following, one by G.M. Whitesides *et al.*, and a last example by Futaki *et al.*

Figure 11 Sulphonamide on the C-terminus of Alamethicin allows the peptide to form pores as normal, however, introduction of Carbonic Anhydrase II effectively ceases pore formation by binding to the sulphonamide group.

In the first example, the group of Whitesides synthetically introduced a sulphonamide ligand to the C-terminus of ALM. When used in experiments, the ALM would behave normally, until the protein carbonic anhydrase II (CA II) was introduced. CA II would then bind to the sulphonamide and sterically hinder either the pore formation itself, or block the pores from letting anything through. Once 4-carboxybenzenesulphonamide was introduced into the solution, competing with the sulfo-ALM for CA II, pore formation activity would be rapidly restored. [75]

Figure 12 Alamethicin modified to contain a C-terminal Ca^{2+} -binding segment of Calmodulin will not form pores unless the binding segments are allowed to dimerize in the presence of Ca^{2+}

Similarly, when ALM (Alamethicin F50, a more hydrophobic ALM variant) was coupled on its C-terminus to the C-terminal Ca^{2+} binding segment of Calmodulin by the group of Futaki, the construct would be active, but not form any clearly identifiable pores, until Ca^{2+} ions were introduced into the mixture, allowing the binding segments to dimerize, inducing pore formation. Introducing Mg^{2+} on the other hand had no effect on the behaviour of the construct, indicating that the specific ion was required to induce activity, therefore the Calcium binding segment was still governing the pore formation as intended.^[76]

Summarizing, Alamethicin has shown great potential to serve as the basis for the switchable pore forming construct, which will be further explored in this work. However, to date no construct has been made that can reliably meet all requirements, or at least three out of the four at the same time, showing the magnitude of the challenge.

1.3. DNA in templated pore formation.

So far, mainly synthetic scaffolds, ALM analogues, synthetic ALM derivatives or modified protein fragments have been employed in attempting to better control the pore-forming process. As Futaki, Kredics and others have pointed out on several occasions, a major group of biologically active molecules, capable of changing conformation according to their chemical environment has not been investigated at all to date: DNA.^[53,62,77]

First discovered in 1871 by F. Miescher,^[78] and first fully structurally characterized by Watson and Crick in 1953,^[79,80] DNA (deoxyribonucleic acid) is known to most of the public as the primary information carrier in essentially all living organisms. In the views of the general audience, DNA is considered to be similar to the hard drive in a computer: it stores information which can be read, copies itself prior to cell division, and if too many faults or mistakes are made during copying, the whole system starts acting up and one gets cancer or another serious systemic disease.^[81,82]

Scheme 3 The four nucleobases in standard duplex binding. left: A(denosine)-T(hymine); Right: C(ytosine)-G(uanine)

DNA is built up out of four building blocks, the nucleobases Adenosine (A), Cytosine (C), Guanine (G) and Thymine (T).^[83,84] The A and T, and the G and C pairs are complementary to each other, and can dimerise through hydrogen bridging. When two single strands (ss) of DNA are fully complementary to each other, these hydrogen bridges provide enough binding energy to form the very stable and iconic double stranded (ds) DNA double helix.

DNA however can be employed in many more versatile ways. For instance, by varying the level of complementarity between two or more strands, DNA can be used to build elaborate structures, as demonstrated in DNA-origami.^[85,86] The above serves as an illustration that it is not a far stretch to use DNA as a structural directing agent or template in chemistry, and in fact has been already done on numerous occasions.^[87-92]

Figure 13 A G-quadruplex consists out of 4 Guanines coordinated around a single monovalent cation (preferably K^+)

The versatility of DNA does not stop there. Another interesting ability of DNA is to form a quadruplex of four (anti)parallel GGG structures around an ion, preferably K^+ .^[93,94] In cells,

these quadruplexes form in repeats at the end of a chromosome, forming telomeres, which help govern the DNA multiplication process, shortening with each multiplication until at a critical length, the copying process cannot occur and a cell stops dividing.^[95,96] In research, the individual quadruplexes have been studied extensively and have been found to be of remarkable stability.^[97,98] As such they may serve as a template or scaffold for other processes, either stand-alone or as part of a larger sequence.

Figure 14 Example of a DNA aptamer (thrombin binding aptamer) which will change conformation and change into a G-quadruplex in the presence of thrombin.

Another ability of DNA for the purpose of this work is aptamerism. Some sequences of ss-DNA, capable of partial self-complementarity, are known to adopt different conformations, and exposing different regions for binding of another strand, depending on their chemical environment.^[99-101] Frequently, the above-mentioned DNA GGG-quadruplex plays a role in the tertiary structure of the aptamers.^[102,103] These DNA-aptamers therefore serve as sensors, in a way similar to certain proteins.^[104,105]

Finally, DNA can be labelled, functionalized and modified, without significantly losing its innate characteristics of forming stable superstructures.^[106] Moreover, custom (short) oligomers of DNA are readily available from a large variety of commercial sources and come with a variety of linkers and functionalities to allow for derivatization.^[107]

Figure 15 the ultimate goal of this thesis is to develop a pore forming peptide which will change activity depending on the presence of a particular analyte

1.4. Goal of the thesis

Considering the above, one can at this point start to imagine how a DNA-oligomer combined with ALM or analogue may form the basis for a basic sensor. ALM would be, as discussed earlier, employed as the reporting unit by forming pores in a membrane, allowing for a small, reproducible and quantifiable current to be produced across the membrane. The attached ss-DNA, as an aptamer would be responsible for the sensing and, through its conformational changes, control pore formation, which meets the overall goal for this thesis, to develop an easily modified, switchable pore forming molecule, based on a DNA-peptide hybrid.

Before that can be exploited, a few challenges first will need to be met.

First, an effective method for conjugating a DNA oligomer with ALM needs to be established. The main challenge herein lies with combining the strong hydrophobicity of ALM with the hydrophilicity of the oligomer. Bringing the reactants together will not be the largest challenge, but a hybrid of these two extremes will have hybrid properties, particularly during workup, purification, characterization and testing making these four steps more complicated than usual, requiring adaptation of standard techniques. As significant amount of material will be consumed for this endeavour, it would also be convenient to have an in-house method to produce ALM or relevant analogue in larger amounts.

Second, it should be investigated how exactly an oligomer will affect the pore-forming behaviour of ALM: does an oligonucleotide have the ability to hinder or promote pore formation depending on which form is being used. And if so, are there benefits for pore lifetime and a more uniform size distribution?

We expect that a non-self-complementary oligomer attached to ALM will have reduced activity, as the oligomer attached will serve as steric hindrance, repelling other units and lowering the effective ALM concentration on the membrane. Furthermore, we expect that, when two complementary hybrids are mixed, they will join in a ds-DNA duplex, bringing two ALM moieties together, raising the effective local concentration, promoting pore formation. Other than this, the pairing should also ensure that only pores made out of an even number of units will be formed. Also, the effect of the DNA-quadruplex would be interesting to investigate: the oligonucleotide required would be relatively small (5'-GGGTT-3'), but it should be able to promote only single size of pore of four units of ALM and have exceptional stability, possibly even comparable to the cyclodextrin-templated pore by Skrydstrup *et al.*

The third challenge would be forming a functional sensor. Once it is determined which is the best method to influence pore formation by ALM, in terms of sequence, length and method of binding (duplex, quadruplex, a third rail system, etc.), a suitable, simple aptamer should be introduced. Only once the aptamer-based hybrid functions as intended, can the challenge of building an ALM and DNA based functionalized sensor be considered completed.

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Chapter 2 – Synthesis of Alamethicin and its derivatives

2.1. Introduction

Trans-membrane pores play a vital role in cell biology as was discussed in detail in the previous chapter. They govern the permeability of the membrane: allowing for small molecule transport across a membrane, for nourishment, cell-signalling or even offence and defence.^[1] These pores exist in various forms, ranging from complex transmembrane proteins and protein-complexes e.g. haemolysin, to relatively simple and small peptides e.g. mellitin and alamethicin.^[2-4]

Alamethicin is an amphipathic peptide i.e. the peptide has both hydrophobic and lipophilic characteristics. In this case, this manifests itself via a hydrophobic surface on one side of the folded peptide and a hydrophilic surface on the other, giving it its function. Alamethicin has been studied extensively as an ion-channel forming peptide and has been modified synthetically in order to better understand its properties.^[4-10]

Scheme 1 Alamethicin forms transmembrane pores of the barrel-stave model

As discussed in the introduction, the pores formed by Alamethicin consist of 3-11 individual peptide helices which have self-assembled in response to the local peptide concentration on the membrane, with the individual pore-sizes being related to the local concentration. Pore formation commences when alamethicin goes from its dormant form to its active form at local lipid-surface concentrations of 1 mol% alamethicin (mol alamethicin/mol lipids in membrane). Pores become stable at >3,0-3,5 mol% alamethicin when >80% of the on-surface alamethicin is active and inserting into the membrane.^[11] These bundled helices form a barrel-stave type pore which traverses the membrane, where the 'hydrophilic' face of the peptide points inward towards the centre of the pore (Scheme 1). The pores, once formed, allow for free passage through the membrane, provided its diameter is adequate. For small pores ($n = 3, 4$), this means a rapid loss of protons and a sodium ion gradient

– *Synthesis of Alamethicin and its derivatives* –

across the membrane, larger pores also may allow small molecules to pass through unhindered.

Scheme 2 Dynamic pore formation by Alamethicin. The constant association and dissociation of the individual peptides allows for new pores to be formed continually. The insertion or removal of individual peptides allows for an existing pore to grow or contract

The pores formed are dynamic as the interactions that bundle individual peptides together to form the pore are not covalent in nature. The association and dissociation of individual monomers takes place continuously (Scheme 2). Therefore, some properties of these pores, especially the number of monomers of an individual pore, the distribution of pore sizes and their life-times will change constantly. Also, N- and, particularly, C-terminal modifications have shown to give little or no disturbance towards the pore-forming behaviour of these peptides as their function is determined by their interior composition rather than their termini. These two characteristics, dynamic nature and known structure-activity relationship, are key starting points for further research aimed towards transforming these peptides into useful tools for the in-vivo investigation of biological processes. By stabilizing a particular pore size to favour its formation over other sizes, or by exclusively managing to form only one pore at a controlled given time, a highly sensitive molecular sensor could in theory be built.

However, to be able to develop any process based on these pore forming peptides, Alamethicin F30, or an analogue that can be conjugated more readily, needs to be obtained in workable quantities. Batches of approximately 100 mg or, ideally, more, need to become readily available to ensure a sufficient supply for further experimentation. To this end, a viable laboratory synthesis needs to be developed. The method preferably only employs straightforward and scalable bench-top procedures, as opposed to costly known alternatives such as design and overexpression of an Alamethicin analogue. An additional benefit of synthesis is that it is inherently more suitable to labelling and modification of the peptide. This was the first challenge we tackled during our study of these peptides. The aim was to develop a method which would allow us easy access to sufficient amounts of material to develop novel molecular sensors based on Alamethicin F30.

Figure 1 Alamethicin F30 in three representations: 3D-model, full structure and amino-acid sequence – U represents α -aminoisobutyric acid (Aib, U).

Peptaibols in general pose a significant synthetic challenge and alamethicin, being one of the largest peptaibols, is no exception to this rule.^[12] While the alcohol on the C-terminus is a unique feature that poses its own problems, it is mainly the presence of the eight α -aminoisobutyric acid (Aib, U) residues that ensure that good synthetic yields are not easy to obtain (Figure 1).

This non-proteinogenic α,α -disubstituted amino acid is the main reason for the very loose α -helix observed in peptaibols. This translates, from a synthetic point of view, into significant steric hindrance around the *gem*-dimethyl group residue, and, due to the Thorpe-Ingold effect, keeps the bond-angle of its α -carbon rigidly at a 109.5°.

– Synthesis of Alamethicin and its derivatives –

This combination causes severe difficulties when this residue needs to be incorporated into the peptide chain: both the carbonyl activating reagent and the following nucleophile are hindered in their attack angles due to the steric hindrance caused by the *gem*-dimethyl group and the sigma donation effect of the neighbouring residue lowers the electrophilicity of the carbonyl moiety.

Previous synthetic attempts indeed suffered greatly from this effect.^[12] Those using either the (asymmetric) anhydride methods or nowadays classical coupling reagents (e.g. DCC, DIC and HBTU/HOBt) gave yields as low as 70% per coupling step, which is insufficient for a workable and viable synthesis of a 19-residue peptide. Furthermore, the incorporation of the following residue also suffered from steric hindrance as the amine is more hindered than in a standard α -amino acid exalting the problem.

Scheme 3 Fluorine activated, microwave assisted synthesis of Alamethicin F30 by Skrydstrup *et al.*

Several successful syntheses of Alamethicin have been performed. Most notable among these are the works of the group of Skrydstrup and more recently, the group of Brimble (published after work on this project was completed).^[13, 14] Skrydstrup's group reported an automated, microwave assisted, method employing fluorine-activation of Aib to overcome the difficult coupling of this amino acid and a double coupling step for the residue following an Aib (Scheme 3). Using this method, overall yields of up to 24% were achieved for Alamethicin F30. However, the fluorine anions that get released during each of the coupling steps retain corrosive properties and over time form precipitates in the equipment lines, blocking or damaging them. It is also generally known that fluorine containing salts are corrosive to metals and glass, particularly when heated as these methods often require. Hence, using this synthetic approach requires the use of dedicated and costly equipment.

Scheme 4 Synthesis of Alamethicin F30 by Brimble *et al.* using a double linker strategy: Rink-AM with a second HMBA linker for SPPS followed by a final NaBH₄ reduction to cleave the HMBA linker after purification of the crude peptide product.

Brimble *et al.* employed a double linker strategy in a carefully tuned manual peptide synthesis sequence which employs optimized coupling strategies for each step, ranging from classical DIC/HOBt couplings to more specialized crosslinking agents such as COMU and HATU this combined with acid fluoride activation for the Aib steps. Their optimized approach gave the full-length peptide with a minimum of side-product formation (scheme 4). The power behind this strategy is the double linker employed, which allowed for facile clean-up of the crude peptide product. Due to this linker, the purification of the peptide by chromatography was simpler as it now displayed better visibility at 256 nm and eluted better due to a slimmer peak shape. After purification, the HMBA linker was cleaved using NaBH₄ to afford the target peptide. With their method, an overall yield of 18% could be achieved.

In this project, the goal was to develop a facile synthesis of Alamethicin, and potentially interesting analogues, using mild reaction conditions and that can be performed on a bench without the use of dedicated equipment.

2.2. Results and discussion

Initial attempts using automated solid-state peptide synthesis (SPPS) resulted in trace quantities of the desired peptide only, confirming earlier reports in literature. In order to establish a viable synthetic route, a short model peptide (Ac-VUUEQF-NH₂) was selected to test different synthetic approaches for both the Aib incorporation and the incorporation of the first amino acid after Aib.

Entry	Activator	Aib-source	Product	Des-Aib	Deprotected
1	DIC	Aib-OH	70%	30%	-
2	HOBt/HBTU	Aib-OH	80%	20%	-
3	DIC/OxymaPure	Aib-OH	75%	25%	-
4	OxymaPure/HBTU	Aib-OH	85%	15%	-
5	COMU	Aib-OH	>95%	<5%	-
6	HATU	Aib-OH	>95%	<5%	-
	-	Aib-F	90%	n.d.	10%

Table 1 Screening of activation strategies for the incorporation of Aib in the sequence Ac-UUEQF-NH₂. Scale: 0.10 mmol, 3.5 equiv. Act/3.0 equiv. Aib, reaction time: 30 min at rt.

Firstly, an optimal method to incorporate the Aib residue onto a pre-made peptide on resin (Fmoc-EQF-RinkAM) was investigated. Classical coupling reagents afforded moderate yields of up to 80% per step over 3 steps (Table 1, entries 1-3), which is in line with earlier findings of 77% per step for the Aib couplings. Alternatives, such as a combination of HBTU/Oxyma or COMU gave improved yields of up to 95% per step (entries 4 and 5). Even better results were obtained when using THF as a solvent as opposed to DMF.^[15] HATU was particularly effective (entry 6), giving rise to yields up to 98% per step.

Scheme 5 Fluorine mediated Fmoc-deprotection analogous to deprotection by TBAF as reported by Amemiya *et al.*

A test reaction with Aib(U)-fluoride to obtain a 6-residue peptide (derived from the first 6 residues in Alamethicin: Ac-VUUEQF-NH₂) gave initially only 90% yield per step, but interestingly, no Aib(U)-deletion products were found. Other than the desired compound the major side products were identified to be Ac-VUUUEQF-NH₂, Ac-UUUEQF-NH₂ and traces of Ac-UUEQF-NH₂. The lack of any products where simply one (or more) Aib residues were missing from the sequence indicated that during the coupling process the Fmoc protecting group was cleaved and the resulting amine subsequently was capped. This process was previously encountered in literature by Amemiya *et al.* when using tetrabutylammonium fluoride (scheme 5).^[16] It stands to reason that the DIPEA-HF salt can cause similar problems. To overcome this problem several potential scavengers were tested, based on the work of Jones *et al.*^[17]

Entry	Scavenger	Product	Deprot.	Other
1	-	90%	5%	5%
2	TMS-OH	85%	10%	5%
3	<i>i</i> Pr ₃ Si-H	80%	10%	10%
4	<i>i</i> PrOTMS	>95%	n.d.	n.d.
5	<i>t</i> Bu-OTMS	>95%	n.d.	n.d.
6	(<i>i</i> Pr) ₂ CHOTMS	90%	5%	5%
7	<i>n</i> BuOTBS	>95%	n.d.	n.d.
8	(<i>i</i> Pr) ₂ CHOTBS	>95%	n.d.	n.d.

Table 2 Performance of silyl-ether based scavengers in Aib-Fluoride peptide coupling

It was decided to add silyl ethers to the solution as scavengers (Table 2). This greatly improved the outcome of the reactions, with several attempts (entries 4, 5, 7 and 8) furnishing near quantitative yields per step. TMS-ethers (entries 4 and 5) gave slightly lower yields than the TBS-ethers (entries 7 and 8), most likely due to their relative lack of stability towards hydrolysis in general. More bulky silyl ethers (TIS, TBS, TBDPS) all performed similarly well, with the parent alcohol playing an apparently marginal role. Slightly fewer side-products were observed when the diisopropylcarbonyl-ether was used as opposed to isopropyl and methyl ethers.

A method for the complete synthesis of Alamethicin F30, composed of two sets of coupling conditions, was thereafter designed based on these findings. For all non-Aib couplings, the COMU/Oxyma method in DMF (single or double coupling) was employed while for Aib-F couplings, the DMF, DIPEA with TIS*o*Pr as scavenger method was used. During the actual synthesis of the complete peptide, it became apparent that the previously employed

coupling time of 30 minutes (with agitation) was insufficient and that increasing reaction time to 2 hours gave rise to too many side reactions. After some optimisation, a coupling time of 60 minutes per batch of reagents was established as being optimal.

With this procedure in hand, Alamethicin F30, and Cys-Alamethicin F30 were successfully synthesized in 4% and 3% isolated yield respectively. These yields were acceptable for initial attempts; however, more fine-tuning of the protocol would be required in due time. During the analysis of the crude peptides, it became apparent that most of the Aib-related issues were resolved and that problems now arose after both proline residues were incorporated as significant capping occurred during these steps. A similar issue existed after the Leucine and the UVU sections were incorporated, significant capping could again be observed after these steps reproducibly over several attempts. These issues could have most likely been solved by further optimizing the reaction conditions, likely by switching to the HATU coupling reagent (which was later supported by the work of Brimble *et al.*)^[14,18] and also by potentially switching to agitation with additional heating or to manual operation of an automated SPPS machine. However, because the synthesis proved to be laborious and time-consuming, a decision was made to discontinue this original approach, and switch to an alternative design.

An alternative to Alamethicin F30 was found in dULC-Alamethicin F30, an analogue where all Aib residues are replaced by Leu, a standard amino acid. This analogue has been pioneered by DeGrado *et al.* and was found to display similar pore-forming behaviour, albeit that it is less active and the pores are less stable in general.^[19,20] This behaviour was attributed to the tighter α -helix and a further increase in hydrophobicity due to the additional leucines. As the mode of action of this analogue was reported to be similar to the original peptide, it was regarded as an alternative option for Alamethicin.

ALM-F30:

Ac-UPUAUAQUVUGLPVUUEQ-Pheol

dULC-ALM-F30:

Ac-LPLALAQLVLGLLPVLLLEQFC-NH₂

The method by which dULC-ALM-F30 can be synthesized was suitable for automated SPPS although it should be noted that due to the strong apolar nature of the peptide, amino acid incorporation had to be performed as double couplings rather than single couplings.

Synthetic yields matched values reported by DeGrado and co-workers, around 20% after purification.

2.3. Conclusion

A manual SPPS method for the synthesis of ALM-F30 and derivatives was successfully developed with promising initial yields. This method uses a combination of Fluorine activation of the challenging Aib residue during its incorporation step and a tailored activating agent mixture for the remaining coupling steps.

Also, an analogue of ALM-F30, dULC-ALM-F30, where all Aib are replaced by Leu, has been successfully synthesized and characterized.

2.4. Supplementary Information

General Experimental

Unless noted otherwise, all reactions were carried out under a nitrogen atmosphere. Reactions were monitored by TLC. All solvents and reagents were obtained commercially. The solvents were used without any further purification, reagents were purified as required following procedures described in “Purification of Laboratory Chemicals (sixth edition).”

The ^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 100 MHz. The chemical shifts are reported in ppm relative to solvent residual peak.

Analytical RP-HPLC was performed on a Shimadzu LC-10AD VP machine using XTerra MS C18 Column, 125Å, 3.5 μm , 4.6 mm X 100 mm

Semi-preparative RP-HPLC purification was performed on a XTerra MS C18 Prep Column, 125Å, 5 μm , 7.8 mm X 100 mm, with a flow rate of 1.5-2.0 mL/min @ 45°C

The solvent system was: A = 0.1% TFA in MeCN, B = 0.1% TFA in H₂O (double distilled - dd)

Absorbance was monitored at 210 nm and product percentages are given by peak areas at 210 nm.

Low resolution mass spectra were recorded on a XEvo LC-Q-TOF (Waters, ESI-Pos. or Neg. mode).

High resolution mass spectra were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode).

2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-isobutyric acid (Fmoc-Aib-OH)

α -aminoisobutyric acid (5.2 g, 50 mmol, 1.0 equiv.) and Na_2CO_3 (26.4 g, 250 mmol, 5 equiv.) were dissolved in water (200 mL, 0.25 M), followed by adding 1,4 dioxane (200 mL, 0.125 M) and addition of 9-fluorenylmethoxycarbonyl chloride (15.7 g, 55 mmol, 1.1 equiv.) while stirring vigorously. After the

starting material was consumed according to TLC, water (200 mL) was added and the mixture washed with Et_2O (3 x 250 mL). Subsequently, the aqueous phase was acidified with concentrated HCl (aq., final pH = 1) and extracted with EtOAc (3 x 300 mL). The organic layer was subsequently dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The resulting white powder was used without any further purification (14.1 g, 43 mmol, 87%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ (ppm) 12.30 (s, 1H), 7.67 (d, J = 7.3 Hz, 2H), 7.56 (d, J = 7.3 Hz, 2H), 7.31 (t, J = 7.3 Hz, 2H), 7.33 (t, J = 7.3 Hz, 2H), 4.22 (d, J = 6.6 Hz, 2H), 4.14 (d, J = 6.6 Hz, 1H), 1.41 (s, 6H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ (ppm) 176.0, 155.1, 144.0, 140.8, 127.8, 127.2, 125.4, 120.2, 65.4, 55.3, 46.8, 25.3; MS (ESI-TOF): calculated for $\text{C}_{19}\text{H}_{20}\text{NO}_4$ $[\text{M}+\text{H}]^+$: m/z 326.14, found 326.14.

2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylpropanoic fluoride

In a flame-dried Schlenk flask under Argon, Fmoc-Aib-OH (1.63 g, 5 mmol, 1.0 equiv.) was suspended in CH_2Cl_2 (25 mL, 0.2 M). Pyridine (0.41 mL, 5 mmol, 1.0 equiv.) was added and the mixture was cooled to 0° C. Cyanuric Fluoride (0.19 mL, 10 mmol, 2.0 equiv.) was added dropwise and the resulting solution was stirred

overnight, while slowly warming to room temperature. CH_2Cl_2 (100 mL) was added and the mixture was washed with water (3 x 100 mL). The organic phase was dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The resulting solid was recrystallized from CHCl_3 and pentane, resulting in white needles, which were used without further purification (1.32 g, 4.0 mmol, 80%). $^1\text{H-NMR}$ (400 MHz, $\text{DMSO}-d_6$): δ (ppm) 8.17 (s, 1H), 7.89 (d, J = 7.6 Hz, 2H), 7.70 (d, J = 7.2 Hz, 2H), 7.42 (t, J = 7.2 Hz, 2H), 7.33 (t, J = 7.6 Hz, 2H), 4.38 (d, J = 6.4 Hz, 2H), 4.23 (t, J = 6.4 Hz, 1H), 1.43 (s, 6H). $^{13}\text{C-NMR}$ (100 MHz, $\text{DMSO}-d_6$): δ (ppm) 164.4, 155.4, 143.6, 140.8, 127.6, 127.0, 125.1, 120.1, 65.7, 55.0, 46.9, 24.2; MS (ESI-TOF): calculated for $\text{C}_{19}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$: m/z 328.13, found 328.13.

General procedure manual solid phase peptide synthesis (SPPS).

All resins and amino acids (excluding the acid fluorides) were purchased from Iris Biotech (Germany) and used directly. SPPS was carried out manually on a 0.10 mmol scale. Peptide grade DMF and CH_2Cl_2 were purchased from Fischer Scientific and used as provided.

– Synthesis of Alamethicin and its derivatives –

Prior to use in synthesis, the resin was loaded in a 20 mL fritted syringe, washed with DMF (3x 10 mL), and swelled in CH₂Cl₂ (10 mL) for 15 minutes.

A general coupling cycle contained the following steps (detailed below):

Single coupling:

Deprotection, wash, coupling, wash, acetylation, wash

Double coupling:

Deprotection, wash, couple, couple, wash, acetylation, wash

Fluoride coupling:

Deprotection, wash, fluoride coupling, fluorine wash, acetylation, wash

Double fluoride coupling:

Deprotection, wash, fluoride coupling, fluorine wash, acetylation, wash

Deprotection:

Added 20% piperidine in DMF (10 mL), agitated for 15 minutes, check by UV-Vis for full deprotection: 2 quartz cuvettes (1 mL) at 301 nm against a 20% piperidine in DMF as a blank; $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$ – if required, repeat the procedure.

Wash:

The resin was washed with DMF (3 x 10 mL), quickly agitated after each addition of DMF

Fluorine Wash:

The resin was washed with DMF (3 x 10 mL), CH₂Cl₂ (3x 10 mL) and again DMF (3 x 10 mL) – the resin is agitated for ~10 seconds after each solvent addition.

Coupling:

0.5 mmol of Fmoc-AA-OH was dissolved in 2 mL of a solution of COMU in DMF (0.25 M, freshly prepared), the solution was then transferred to the syringe. Subsequently 4 mL of DIPEA in DMF (0.5 M) was added to the syringe containing the resin and the resin was agitated for 60 minutes, after which the solution was discarded.

Fluorine Coupling:

0.5 mmol of Fmoc-AA-F was dissolved in 2 mL DMF, 4 mL of DIPEA in DMF (0.5 M) was added, along with 1 mmol of triisopropylsilylisopropyl ether. The solution was transferred to the resin and agitated for 60 minutes.

Acetylation:

3 mL of acetic anhydride in DMF (0.5 M) and 3 mL of DIPEA (0.5 M) were added to the resin, which was subsequently agitated for 45 minutes, after which the solution was discarded.

The previously mentioned coupling cycles were used as follows:

Standard amino acids, not after Aib	Single coupling
Standard amino acids, after Aib (and after 15 AAs)	Double coupling
Aib-fluoride, not after Aib, Ala, Val or Leu	Fluoride coupling
Aib-fluoride, after Aib, Ala, Val or Leu coupling	Double Fluoride

Kaiser test ^[21]

Solution A: 0.5 g ninhydrin dissolved in 10 mL EtOH (96%)

Solution B: 0.4 mL of 0.001 M KCN (aq) dissolved in 20 mL pyridine.

Transfer some washed and dried resin beads (~1 mg, *ad lib.*) from the resin holder to a small glass test-tube or cuvette. Add 200 µL of solutions A and B and mix well. Heat for 5 minutes in a 120 °C oven. Let cool down and inspect, a (dark) blue colour on the beads is considered “positive” and indicates an incomplete coupling reaction on the resin.

Alamethicin fragment (Ac-VUUEQF-NH₂)

Synthesized on a Rink-AM resin using the above synthetic protocol. A Kaiser test was performed after each coupling, if it was found to be positive, a second coupling step using the same residue would be initiated. After synthesis the final deprotection was carried out with 20% piperidine in DMF after which the workup was acetylation and an extensive wash cycle. The resin was subsequently cleaved using a mixture of TFA/CH₂Cl₂/TIS/H₂O (90/4/4/2 v/v) for 10 minutes, the resulting solution was concentrated to approximately 1 mL and subsequently precipitated with ice-cold Et₂O. The resulting solid underwent repeated (3 cycles) centrifugation, decantation and trituration, until the final solid was dried under reduced pressure to yield the crude peptide (143 mg, 31% purity). The crude peptide was dissolved in MeCN and purified using semi-preparative HPLC using a linear gradient of 30% to 100% B in A, which after lyophilisation gave the pure peptide (53 mg, 73%, 95% purity). Low resolution Mass spectrometry: C₃₄H₅₄N₇O₁₀ [M+H]⁺, Calculated: 720.39 found: 720.38

Alamethicin F30 (Ac-UPUAUAQUVUGLUPVUUEQPheol)

Synthesized on a 2-chlorotriyl resin loaded first with N-Fmoc-phenylalaninol, then as described, with a Kaiser test after every second coupling to monitor coupling efficiency.

Final deprotection was carried out with 20% piperidine in DMF (2 x 10 mL for 30 minutes each), followed by acetylation and an extensive wash cycle. The resin was subsequently cleaved using a mixture of TFA/CH₂Cl₂/TIS/H₂O (90/4/4/2 v/v) for 10 minutes. The resulting solution was concentrated to approximately 1 mL and subsequently precipitated with ice-cold Et₂O. The resulting solid underwent repeated (3 cycles) centrifugation (maximum setting permissible for the container, at least 10 minutes), decantation and trituration. The product was dried under reduced pressure to yield the crude peptide (92 mg, >25% purity).

The crude peptide was dissolved in MeCN:H₂O (2:1) and purified using semi-preparative HPLC using a linear gradient from 40% to 100% of A in B with a flow-rate of 2 mL/min, resulting in the pure peptide after lyophilisation (8 mg, 4%, 95% purity). MALDI-TOF, calculated (M-H)⁻: 1962.1, found: 1984.1 (M+Na-2H)⁻

Cys-AlmF30 (Ac-UPUAUAQUVUGLUPVUUEQFC-NH₂)

Synthesized on a Rink-AM resin as described, with a Kaiser test after every second coupling to monitor coupling efficiency. Final deprotection was carried out with 20% piperidine in DMF (2 x 10 mL for 30 minutes each), followed by acetylation and an extensive wash cycle. The resin was subsequently cleaved using a mixture of TFA/CH₂Cl₂/TIS/H₂O (90/4/4/2 v/v) for 10 minutes, the resulting solution was concentrated to approximately 1 mL and subsequently precipitated with ice-cold Et₂O. The resulting solid underwent repeated (3 cycles) centrifugation (maximum setting permissible for the container, at least 10 minutes), decantation and trituration. The product was dried under reduced pressure to yield the crude peptide (137 mg, >20% purity).

The crude peptide was dissolved in MeCN:H₂O (2:1) and purified using semi-preparative HPLC using a linear gradient from 40% to 100% of A in B with a flow-rate of 2 mL/min, resulting in the pure peptide after lyophilisation (7 mg, 3%, 90% purity). HRMS (ESI-Pos), Calculated (M+Na)⁺ 2102.1079, found: 2102.0995 (M+Na)⁺

General procedure Automatic SPPS

Synthesis was carried out using a CEM Liberty microwave assisted peptide synthesizer on a 0.10 mmol scale. Prior to use, the resin was swelled for 15 minutes in a mixture of DMF and CH₂Cl₂ (1:1 v/v). Only pre-installed cycles of the CEM Liberty system were employed during synthesis. Amino acids and other reagents are dissolved in DMF, unless noted otherwise. Microwave heating is only employed in the Fmoc-deprotection and μ W-heating steps.

Standard single coupling cycle (0.10 mmol scale)

Operation End temp	Reagent	Vol	Time
1) Fmoc deprotection 20 and 70 °C	20% piperidine in DMF	7.0 mL x 2	0.5 + 3 min
2) Drain and wash 20 °C	DMF	7.0 mL x 4	-
3) Add amino acid 20 °C	0.30 M Fmoc-AA-OH	3.0 mL	-
4) Add HBTU 20 °C	0.50 M HBTU	1.0 mL	-
5) Add DIPEA 20 °C	2.00 M DIPEA in DMF	0.5 mL	-
6) μ W heating 80 °C	-	-	5 min
7) Drain and wash 20 °C	DMF	7.0 mL x 3	-

Standard double coupling cycle (0.10 mmol scale)

Operation End temp	Reagent	Vol	Time
1) Fmoc deprotection 20 and 70 °C	20% piperidine in DMF	7.0 mL x 2	0.5 + 3 min
2) Drain and wash 20 °C	DMF	7.0 mL x 4	-
3) Add amino acid 20 °C	0.20 M Fmoc-AA-OH	2.5 mL	-
4) Add HBTU 20 °C	0.50 M HBTU	1.0 mL	-
5) Add DIPEA 20 °C	2.00 M DIPEA in DMF	0.5 mL	-
6) μ W heating 80 °C	-	-	5 min
7) Drain -	-	-	-
8) Repeat 3) to 7)			
9) Wash 20 °C	DMF	7.0 mL x 3	-

Standard amino acids, not after Aib

Single coupling

Aib and all amino acids after Aib

Double coupling

Attempted synthesis of Alamethicin F30 (Ac-UPUAUAQUVUGLUPVUUEQPheol)

Synthesized on a 2-chlorotrityl resin loaded first with N-Fmoc-phenylalaninol, then as described. Final deprotection was carried out with 20% piperidine in DMF (2 x 10 mL for 30 minutes each), followed by acetylation using a mixture of Ac₂O (1 mmol) and DIPEA (1 mmol) in DMF (8 mL, 0.125 M each) and a wash cycle with DMF (3 x 10 mL) and CH₂Cl₂ (3 x 10 mL). The resin was subsequently cleaved using a mixture of TFA/CH₂Cl₂/TIS/H₂O (90/4/4/2 v/v) for 10 minutes, the resulting solution was concentrated to approximately 1 mL and subsequently precipitated with ice-cold Et₂O. The resulting solid underwent repeated (3 cycles) centrifugation, decantation and trituration. The product was dried under reduced pressure. No (significant) amount of target peptide was identified.

Synthesis of dULC-Alamethicin F30 (Ac-LPLALAQLVLUGLLPVLLUEQFC-NH₂)

Synthesized on Rink-AM resin as described. Final deprotection was carried out with 20% piperidine in DMF (2 x 10 mL for 30 minutes each), followed by acetylation using a mixture of Ac₂O (1 mmol) and DIPEA (1 mmol) in DMF (8 mL, 0.125 M each) and a wash cycle with DMF (3 x 10 mL) and CH₂Cl₂ (3 x 10 mL). The resin was subsequently cleaved using a mixture of TFA/CH₂Cl₂/TIS/H₂O (90/4/4/2 v/v) for 10 minutes, the resulting solution was concentrated to approximately 1 mL and subsequently precipitated with ice-cold Et₂O. The resulting solid underwent repeated (3 cycles) centrifugation, decantation and trituration. The product was dried under reduced pressure to yield the crude peptide (157 mg, 40% purity)

The crude peptide was dissolved in MeCN:H₂O (4:1 v/v), and purified using semi-preparative HPLC using a linear gradient from 60% to 100% of A in B with a flow-rate of 1.5 mL/min, resulting in the pure peptide after lyophilisation (42 mg, 18%, 95% purity). HRMS (ESI-pos), Calculated (M+2H)²⁺: 1152.6971, found: 1152.6901.

2.5. References

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Chapter 3 – Synthesis and Characterization of Alamethicin-DNA

hybrids.

3.1. Introduction

Trans-membrane pore formation by peptides and proteins has been of research interest since the early 1950's to the present date.^[1-3] The research goals pertaining to these have evolved from trying to understand how the pores function (insertion, stabilization, (ion-)selectivity, life times, etc.) to attempting to utilize these processes, chiefly though among others, as a molecular sensors.^[2] In the world of proteins, the most well-known application of these pore forming peptides is the use of Haemolysin as a RNA/DNA-sequencing tool.^[3] When a membrane that contains Haemolysin is exposed to a transmembrane potential, these pores will allow a strand of single-stranded DNA (ss-DNA) to pass through the membrane. The variations in the transmembrane current caused by the passing of the DNA (which temporarily blocks the pore to a varying degree) can be measured. These variations can then be used to determine the nucleotide sequence of this strand.^[4] Other known applications are the detection of ions, small molecules and peptides.^[5-10]

Figure 1 Schematic overview of a triggered pore formation of a dormant modified pore-forming peptide by a target analyte.

Pore forming peptides, and Alamethicin in particular, provide more of a challenge to work with as one cannot benefit from a (large) pre-made pore generated by a single protein inserting in the membrane which can be readily accessed. Pore forming peptides typically

need to gather on a membrane and will only insert upon reaching a certain local concentration. Furthermore, in contrast to the single, steady pore offered by a large protein, the pores formed by these peptides may vary in size depending on the number of peptides participating in the pore, which depends on again the local concentration, transmembrane potential and susceptibility of the membrane for the particular peptide. However, as discussed in the previous two chapters, these peptides are far more readily available in large (mg-g) quantities at a fraction of the cost of a similar quantity of large pore forming protein. Lastly, the pore forming peptides can be subjected to significant modification as a function of the total mass of the peptide, without fear of disturbing the relatively delicate tertiary or quaternary structure of a protein. Particularly the C-termini of these peptides may be heavily modified. In all, the flexibility and relatively high accessibility of pore forming peptides counterbalances the aforementioned downsides, making them a suitable platform with which further applications may be developed. ^[11-16]

With regards to insertion and or diffusion of these peptides (acting as monomers prior to pore formation) and the resulting pore sizes and lifetimes of these pores, the pore forming peptides may be employed in potentially more complex situations. As pore formation is highly dynamic there is no need for the peptide to be present on the membrane from the start of the experiment, and similarly it may be possible to drive the peptides from the membrane prior to terminating a measurement. Potentially, these characteristics could be combined to form a functional molecular sensor which could be added to a complex sample and which would give reliable and quantifiable transmembrane activity when triggered by a target analyte, which is the ultimate goal of this project.

In order to arrive at this goal, several key issues need to be addressed before a functionalized pore-forming peptide, based on Alamethicin, can be obtained, as outlined in the introduction. First, the resulting pore stability needs to be improved. Once formed, a pore should remain stable for a certain, known, period of time. Initially, therefore, our goal will be to increase the life-time of the formed pores which should be as long as possible. Later, with specific applications in mind, the lifetime of such a pore should be tailorable to suit a particular need and also produce pores that are constant in size. Its targeted lifetime and size should also allow for reliable and facile quantification of a transmembrane signal.

Second, especially when considering that barrel-stave type pores will often consist of a variable number of monomers, such as in the case of Alamethicin, the resulting pore size needs to be more carefully controlled. Indeed, as various pore-sizes give rise to different levels of conductance, a non-controlled pore size formation will result in a high variance of readout which is contrary to the needs of a molecular sensor which requires a significant, quantifiable and reproducible output. This reliability can best be achieved by ensuring only one pore size is assembled in a membrane. This in turn can only be done by ensuring that a

fixed number of monomers are incorporated when the pore is formed, or at least that this number is strongly favoured over others in a distribution.

Last, for the development of a molecular sensor based on pore-forming peptides, the pore-forming behaviour itself should become controllable meaning that formation and dissociation should be controllable by an exterior factor.

Overall, if the first two elements could be combined with the latter ability to activate or deactivate pore-formation altogether at will one would have an efficient and tuneable molecular probe in hand. This (de-)activation of pore formation would ideally be controlled by a target analyte, and would result in a quantifiable and reproducible result, depending on the concentration of that target analyte.

Figure 2 Pore stabilization of the Alamethicin pores by means of Cyclodextrin linking (Skrydstrup) and Calmodulin binding segments (Futaki).

Two striking examples have been offered by the groups of Skrydstrup and Futaki (See Chapter 1 for a more detailed review). Skrydstrup *et al.* successfully addressed the first two challenges by tethering six monomers together, linking the Alamethicin C-termini to a cyclodextrin ring, forming a pre-fabricated pore with an unprecedented lifetime and a single pore selection. [17,18] Futaki *et al.* have managed to address all three challenges separately. They managed to stabilize the Alamethicin pore by introducing zinc-finger proteins on the C-terminus, also

introducing the ability to switch pore-formation ‘on,’ provided the initial mixture did not contain any zinc ions.^[13,19,20] Later, they improved this concept by introducing the Calcium binding domain of calmodulin to mediate pore formation, which had an even greater impact on pore formation and stability. Switching the pore forming activity ‘off’ from an earlier ‘on’ state still remains to be achieved however.

Figure 3 Modes of DNA-hybridization to stabilize a dULC-ALM-F30 pore. Left: duplex; centre: single strand (non-hybridized); right: G-Quadruplex.

DNA, however, has, to the best of our knowledge, not yet been investigated as a C-terminal additive to pore forming peptides with the aim of controlling or influencing pore formation. Yet it would be a highly interesting modification as, with its unique properties compared to small molecules and peptides or proteins, it has been successfully employed in a plethora of different applications, giving structure, stability or a design template to hybrid materials.^[21-24] As such, it could be the ideal candidate for developing a switchable artificial pore-forming peptide. DNA can stabilize a pore through duplex or G-quadruplex formation, forming either a partially pre-formed pore in solution or in the latter case, an entire premade pore.^[25-27]

The advantages of DNA do not end there. Indeed, single-strand DNA (ss-DNA) will in general not dimerize with itself. This behaviour arises due to a general lack of self-complementarity in most (but not all) sequences. This could be used to greatly reduce the activity of an attached Alamethicin unit in view of pore size control. Using the duplex to stabilize a pore could result in an increase in pore lifetime as well as a reduction in the amount of conductance states (related to the monomers per pore), as it limits the options of pore sizes to even numbers of monomers. DNA-quadruplexes are even more interesting in this regard as they should limit pore sizes to only one preferred size and greatly increase the lifetime, comparably to the work of Skrydstrup *et al.*, but with the benefit that the G-quadruplex can be disassembled.

Scheme 1 schematic representation of a thrombin binding aptamer employing a G-quadruplex

Another reason why DNA is an attractive candidate for modifying the behaviour of Alamethicin is the fact that some DNA sequences can also be used as a sensor.^[28-32] These DNA aptamers are sequences that can bind to particular target analytes (e.g. thrombin, see scheme 1) and, in doing so, undergo conformational changes. An aptamer undergoing these changes can expose previously hidden domains of its chain as a single (accessible) strand across its surface, resulting in alteration of how it interacts with its environment and what complementary strands may bind to it. Combining the ability of an aptamer to respond to its environment with an inactive ss-DNA-Alamethicin complex (complementary to the activated aptamer) would result in the complex being capable of reversibly activating pore formation only when a certain trigger is encountered.

The goal of this chapter is to prepare and characterize ss-DNA-Alamethicin conjugates. The overall objective is to generate conjugates which will allow for the evaluation of the intended deactivation of the pore-forming activity of Alamethicin by lone ss-DNA, the stabilization of pores by ds-DNA and the pre-made pores of a G-quadruplex hybrid.

3.2. Results and discussion

Initially it was envisioned to follow a conjugation strategy similar to what was reported by Whitesides *et al.*^[15] This route (Scheme 2), which employed commercially available Alamethicin F30 (eliminating any need to synthesize additional materials), began with the oxidation of the C-terminal alcohol of Alamethicin F30 to the corresponding aldehyde using Dess-Martin periodinane.^[33] The desired oxime was then formed by condensing the newly formed aldehyde with the appropriate O-hydroxylamine.

Alternatively, it was shown that the aldehyde could be used in a reductive amination, resulting in the amine analogue of the oxime proposed in Scheme 2. After a proof of principle

– *Synthesis and Characterization of Alamethicin-DNA hybrids.* –

had been established for introducing an oligonucleotide via the oxime pathway, the alternative of using reductive amination was proposed to be of particular interest for further explorations, as most commercial oligonucleotides are offered with an amine functionality, whereas hydroxyl amines are not standard on offer (or at a significantly increased price). This Alamethicin F30 Aldehyde provides a flexible platform to make several ALM-F30-Oligo hybrids, with options to readily vary the sequence, linker length and characteristics.

This flexibility greatly appealed to us as it would give us the tool we required to approach our final goal, which was to obtain a series of hybrids with different stabilisation techniques: two pairs of duplex formers (short, 11-mer and long, 24-mer), a quadruplex-former, and several reference compounds to help distinguish behaviour introduced by the oligomer from the behaviour of the native peptide (table 1).

Scheme 2 Modification of Alamethicin F30 with oligonucleotides based on the procedure by Whitesides *et al.*

Nr.	Oligonucleotide Sequence	Role
1	-	Blank
2	5'-TGA TT-3'	DNA-Blank
3	5'-GGG TT-3'	Quadruplex stabilization
4	5' ACC ATG CTT AA-3'	Duplex stabilization (with 5)
5	5'-TTA AGC ATG GT-3'	Duplex stabilization (with 4)

Table 1 Designated oligonucleotides for modification of Alamethicin, with predicted function.

We began by attempting to reproduce this model reaction on our substrate to later be better able to adapt it to our purpose. To this end, a set of three readily synthesized O-hydroxylamines was proposed for use in a series of test-reactions. The goal of these tests was first to determine if this reaction would reliably convert the terminal alcohol of our peptide into the aldehyde and subsequently to the oxime, with minimal to no formation of potentially hard to remove side products, in a one pot manner. Second, we wanted to establish if this approach would be suitable for use with our oligonucleotides and their associated purification procedures.

Initial attempts resulted in the decomposition of the starting peptide. Although Dess-Martin periodinane and IBX are generally very mild oxidants, it is possible that either a trace contaminant in these or a by-product of the reaction caused either the loss, or the total signal suppression of the starting peptide and/or product(s) as was evidenced by both MALDI-TOF and HPLC analysis of the reaction media.

#	Oxidation method	Time (h)	Conv. (%)	Prod. (%)
1	DMP	3	Full	Trace.
2	IBX	18	n.d.	n.d.
3	Parikh-Doehring	1	Full	Trace.
4	Swern	1	60%	Trace.
5	PCC	18	n.d.	n.d.

Table 2 Screened oxidants for oxidation-modification process: 40 equiv. oxidant in CH₂Cl₂, 23 equiv. benzylhydroxylamine.

Alternative oxidants were explored as well in an attempt to optimise the Whitesides protocol. With the majority of oxidants tested (Dess-Martin Periodinane – DMP, Table 2, entry #1; Iodoxybenzoic acid – IBX, #2; Pyridinium chlorochromate – PCC, #5), no product was observed. Either the starting materials were recovered or only decomposition of the (starting) product could be observed. In later attempts, we decided to use wet dichloromethane, obtained by shaking dichloromethane with deionized-water, as solvent. The Dess-Martin periodinane (DMP) oxidation strategy gave trace amounts of product under

these conditions indicating that with sufficient optimisation, the protocol could work. The more surprising results were obtained from the Swern and related Parikh-Doehring oxidations (entries #3 and #4) which gave full conversion of the starting material to the intermediate with relatively limited degradation. Trace amounts of the target product could also be observed in the reaction media^[34,35]. What drew our interest was that most of the material appeared to smoothly convert to the aldehyde but the latter did not further react with the hydroxylamine. Decomposition of the material over time seemed to only occur when the reaction was performed with excess hydroxylamine in an attempt to drive the reaction to completion. Therefore, with apparent good and reproducible conversion of the starting alcohol to the aldehyde, the Swern oxidation was selected for further testing and optimisation of the condensation.

#	Equiv. Oxidant	R	Time (h)	Prod. (%)
1	10	Benzyl	1	n.d.
2	40	Benzyl	1	Trace.*
3	200	Benzyl	1	<5%**
4	2000	Benzyl	1	Decomp.
5	200	Benzyl	0.5	Trace.*
6	200	Benzyl	5	<5%**
7	200	3,4-dimethoxybenzyl	1	n.d.
8	200	<i>n</i> -octyl	1	n.d.

Table 3 Screening of reaction conditions and various hydroxylamines for the functionalization of ALM-F30 using an initial Swern oxidation (1 equiv. oxidant equals 1 equiv. COCl_2 and 1.2 equiv. DMSO). Reaction time relates to the oxidation step only. *target peak observed in LCMS/MALDI, no LC-peak area determined. **no isolated yield, yield estimated on LC-peak area.

In order to confirm the effectiveness of the oxidation method further, the Swern conditions were varied and more electron rich O-hydroxylamines were used in an attempt to drive the reaction towards completion. However, despite repeated attempts, no significant amounts of product were observed (table 3).

As sadly no clear single reason could be identified for the lack of conversion to the oxime of the aldehyde, which was apparently formed cleanly, we had to reconsider our approach. In our new strategy, we hypothesised that an Alamethicin analogue that required no post-synthetic modification other than the coupling-step with the target oligonucleotide itself could be employed.

Scheme 3 Modification of dULC-ALM-F30 with an oligonucleotide as described by L. Gjonaj, $n=3, 6$.

3.3. Alternative approach

In her thesis, L. Gjonaj described an alternative method to arrive at the desired peptide-oligonucleotide complexes based on a Cysteine - maleimide/O-succinimide - amine linking strategy.^[36] In this approach, rather than using naturally occurring Alamethicin F30, a C-terminal Cysteine is added to the sequence to provide an anchor point for the linker and attached Oligonucleotide. For reasons of convenience the used peptide was switched for dULC-Alamethicin F30 (dULC-ALM-F30), the preparation of which was described in Chapter 2.^[37]

In this approach, the appropriate amine-functionalized oligonucleotide was first reacted with a large excess of linker agent in a buffer-DMF system, followed by purification over a size-exclusion column and lyophilisation, typically giving rise to quantitative yields of conjugate. Second, the conjugate is reacted with an excess of peptide, followed by semi-preparative

HPLC and a second lyophilisation to afford the final product. The main benefit of this procedure is that no additional steps are required on the peptide and only one reaction under mild conditions has to be performed on the oligonucleotide before it can be reacted to give the final product.

Scheme 4 attempted synthesis of maleimide functionalized oligonucleotide TGATT with 6 carbons between oligo and the amine and a 3-carbon linker to the maleimide.

Several oligonucleotides were tested, initially with a short, 3-carbon, linker (scheme 4), then with a longer, 6-carbon, linker between the amide and maleimide functionality. The synthesis of the shortest non self-complementary oligonucleotide, TGATT-linker-dULC-ALM, was approached first to test the strategy. Out of the two evaluated linkers, only the C₆-chain appeared to give the desired dULC-ALM-F30 – DNA hybrids (Scheme 5 and Table 4), whereas the 3-carbon linker failed to form the first intermediate (Scheme 4).

Scheme 5 Synthesis of dULC-ALM-F30 – Oligonucleotide hybrids in two steps. 1) 5-hexylamine functionalized oligonucleotide in phosphate buffer(300µl, pH 7) is reacted with 6-maleimidohehexanoic acid N-hydroxysuccinimide (100 µl, 20 mg/ml). 2) the maleimido modified oligonucleotide was reacted with dULC-ALM-F30 in DMF (500 µl, 6 mg/ml).

Nr.	Oligonucleotide Sequence	Isolated Yield
1	Linker only (maleimidohehexanoic acid N- hydroxylsuccinimide)	80%
2	5'-TGA TT-3'	11%
3	5'-GGG TT-3'	17%
4	5' ACC ATG CTT AA-3'	25%
5	5'-TTA AGC ATG GT-3'	27%
6	ALM-5' TAT AGT GCG CTA ATC GGA GAC GAA-3'	14%
7	5'-TTC GTC TCC GAT TAG CGC ACT ATA-3'-ALM	12%

Table 4 synthesized conjugates and final yield - established by UV-Vis absorption @270 nm

Using the C₆-linker, all intended products were obtained in medium yield, but in more than sufficient quantities for all remaining experiments and therefore no further optimisation was performed. Although not explored in this thesis, entries #6 and #7 (table 4) with the longer oligonucleotides indicate that this particular method may need further optimization if even longer oligonucleotides are pursued.

Figure 4 HPLC and MALDI traces of dULC-ALM-F30-TGATT hybrid using optimized synthetic and analytical approaches.

The conjugates displayed a strong tendency to aggregate, a tendency which became particularly apparent in HPLC and MS experiments as well as when (re)-dissolving the materials. In general, a solution would not remain stable for more than a few hours at room temperature after which a white deposit would form. It was noticed however that adding 1-3% of DMF to the solution improved the stability of the solution. The best results were obtained in this manner by first dissolving the conjugates in DMF, then diluting the solution with water to ~3% (v/v) DMF concentration

However, adding DMF to the HPLC eluents proved to be detrimental for the separation, even when it was only present in the sample as the only solvent. Omitting DMF entirely from the sample resulted in a loss of the products on the column, most likely as a result of precipitation. The optimal result was obtained by injecting into the HPLC the crude mixture after filtration, including DMF in moderate amounts (100 μ L), and using a relatively long gradient at high flowrates. The reason for this peculiar behaviour is most likely due to the

chimeric character of these conjugates which present on one side the extremely hydrophobic (amphipathic) Alamethicin and on the other side the strongly hydrophilic oligonucleotide.

Only linear (very) low-resolution MALDI-TOF in negative mode using CHCA/Citrate matrix gave a reliable signal, typically within 20 m/z units of the expected product, which was within the margin of error for this particular technique and the related equipment used. HRMS of the conjugates were obtained using DMF as a solvent at nanomolar concentrations with both Atmospheric Pressure Photoionization (APPI) and Electrospray Ionisation (ESI) being equally effective ionization methods.

Surprisingly, indications for the presence of intact quadruplexes with potassium cations was observed when measuring conjugate **3** (5'-GGGT-3'), indicating the high stability of this particular complex.

3.4. Conclusion

In order to arrive at the ALM-DNA hybrids for analysis of their pore-forming behaviour, their synthesis had to be explored and optimized. It was initially attempted to prepare ALM-DNA hybrids using a combination of oxidising the C-terminal alcohol of ALM and subsequently introducing the oligonucleotide via either reductive amination or oxime-formation. This strategy met with little success and was discontinued as generating the final products from the aldehyde in one step proved to be unreliable and would require more optimization than was considered acceptable for this investigation. An acceptable solution was found by using an adapted procedure of L. Gjonaj which ultimately resulted in the successful synthesis of a small library of dULC-ALM – Oligonucleotide hybrids and relevant control-compounds. Also, for the first time, these compounds were fully characterized.

3.5. Supplementary Information

General Information

Unless noted otherwise, all reactions were carried out under nitrogen atmosphere. Reactions were monitored by TLC Silica 60 (Merck Millipore), examined under UV (365 nm and 254 nm), and stained by KMnO₄, ninhydrin, vanillin or H₂SO₄ in MeOH (1%). Chromatography was performed using the flash-protocols by Still *et al.* on Silica gel 60 (0.040-0.063 mm) from Merck Millipore. ^[38]

All solvents and reagents were obtained commercially. Oligonucleotides were obtained from Biotez GmbH (Berlin, Germany). ALM-F30 (also: commercially from Sigma Aldrich) and dULC-ALM-F30 were obtained synthetically, as described in chapter 2. The solvents were used

without any further purification, reagents were purified as required following procedures described in "Purification of Laboratory Chemicals (sixth edition)."^[39]

The ^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 100 MHz. The chemical shifts are reported in ppm relative to solvent residual peak.

Analytical RP-HPLC was performed on a Shimadzu LC-10AD VP machine using XTerra MS C18 Column, 125Å, 3.5 μm , 4.6 mm X 100 mm

Semi-preparative RP-HPLC purification was performed on a XTerra MS C18 Prep Column, 125Å, 5 μm , 7.8 mm X 100 mm, with a flow rate of 1.5-2.0 mL/min @ 45°C

The solvent system was: A = 0.1% TFA in MeCN, B = 0.1% TFA in H_2O (dd)

Absorbance was monitored at 210 nm and product percentages are given by peak areas at 210 nm for non-fluorescent compounds (e.g. alamethicin F30), or at 270 nm (for oligonucleotide containing compounds). For reasons of convenience, yields were based on the molar absorptivity coefficient of the parent oligonucleotide, not compensating for the presence of the peptide (assumed to not absorb significantly in that region).

Low-resolution mass spectra were recorded on an Applied Biosystems MALDI-TOF 4700 in linear negative mode, using a CHCA-matrix with monosodium citrate in Ethanol/water (1:1), using pre-mixing of the sample.

High-resolution mass spectra were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode).

Experimental procedures

3-maleimido propanoic acid

β -alanine (4.45 g, 50 mmol, 1 equiv.) was dissolved in glacial acetic acid (200 mL, 0.25M). Maleic anhydride (4.9 g, 50 mmol, 1 equiv.) was added and the resulting mixture was heated under reflux for 17 h. The acetic acid was removed *in vacuo*. The residue was taken up in chloroform (100 mL) and washed with 1M HCl (2 x 100 mL) and brine (100 mL). The organic phase was dried over Na_2SO_4 filtered and concentrated *in vacuo* to yield the crude product as a white powder. Flash Chromatography (Silica, 60% EtOAc in Heptane to 80% in two steps) afforded the pure products as a white powder (7.7 g, 45.7 mmol, 91%). ^1H -NMR (400 MHz, CDCl_3): δ (ppm) 6.70 (s, 2H), 3.82 (t, $J = 7.1$ Hz, 2H), 2.70 (t, $J = 7.1$ Hz, 2H); ^{13}C -NMR (100 MHz, CDCl_3): δ (ppm) 176.6, 170.4, 134.3, 33.3, 32.6. Mass (LC-ESI+TOF): calculated for $\text{C}_7\text{H}_8\text{NO}_4$ $[\text{M}+\text{H}]^+$: m/z 170.04, found m/z 170.04. mp. 103-106 °C.

3-maleimido propanoic acid N-hydroxysuccinimide ester

3-maleimido propanoic acid (1.7 g, 10 mmol, 1 equiv.) was dissolved in HPLC-grade, dry MeCN (100 mL). N-hydroxysuccinimide (1.2 g, 10.5 mmol, 1.05 equiv.) was added and the solution cooled to 0°C. Diisopropylcarbodiimide (1.9 mL, 12 mmol, 1.2 equiv.) was added dropwise over 10 minutes after which the reaction mixture was stirred for 17 hours while letting it return to room temperature. The mixture was then filtered

and concentrated *in vacuo*. Flash Chromatography (silica, 50% EtOAc in heptane to 70% EtOAc in heptane over 3 steps), followed by a recrystallization from hot EtOAc/pentane afforded the product as a fine white powder (825 mg, 3.1 mmol, 31%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 6.72 (s, 2H), 3.94 (t, J = 7.0 Hz, 2H), 3.02 (t, J = 7.0 Hz, 2H), 2.82 (s, 4H); ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 170.0, 168.7, 166.0, 134.4, 32.9, 29.8, 25.5. Mass (LC-TOF): calculated for C₁₁H₁₁N₂O₆ [M+H]⁺: m/z 267.06, found m/z 267.06. Mp 167-171 °C

6-maleimido hexanoic acid

6-aminohexanoic acid (6.5 g, 50 mmol, 1 equiv.) was dissolved in glacial acetic acid (200 mL, 0.25M). Maleic anhydride (4.9 g, 50 mmol, 1 equiv.) was added and the resulting mixture was heated under reflux for 4 h, until monitoring by TLC indicated the full consumption of one of

the starting materials. The acetic acid was removed *in vacuo*. The residue was taken up in chloroform (100 mL) and was washed with 1M HCl (2 x 100 mL) and brine (100 mL). The organic phase was dried over Na₂SO₄ filtered and concentrated *in vacuo* to yield the crude product as a white powder. Flash Chromatography (Silica, 60% EtOAc in Heptane to 80% in two steps) afforded the pure products as a white powder (9.1 g, 43.1 mmol, 87%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 6.67 (s, 2H), 3.50 (t, J = 7.6 Hz, 2H), 2.30 (t, J = 7.5 Hz, 2H), 1.70-1.55 (m, 4H), 1.30 (quin, J = 7.5 Hz, 2H); ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 179.5, 170.9, 134.1, 37.5, 33.9, 28.2, 26.2, 24.1. Mp 87-92 °C

6-Maleimido hexanoic acid N-hydroxysuccinimide ester

6-Maleimido hexanoic acid (1055 mg, 5 mmol, 1 equiv.) was dissolved in freshly dried and distilled DMF (35 mL). N-hydroxysuccinimide (590 mg, 5.2 mmol, 1.05 equiv.) was added and the solution cooled to 0°C. Dicyclohexylcarbodiimide (1240 mg, 6 mmol,

1.2 equiv.) in DMF (15 mL) was added dropwise over 10 minutes after which the reaction mixture was stirred for 14 hours while letting it return to room temperature. The mixture

was then filtered, and concentrated to about 10 mL *in vacuo*. The residue was taken up in EtOAc (150 mL), filtered, washed with 3 M LiCl (2 x 100 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*, to yield the crude product as a light brown semi-transparent oil. Flash Chromatography (silica, 50% EtOAc in heptane to 70% EtOAc in heptane over 3 steps), followed by a recrystallization from chloroform/pentane afforded the product as a fine white powder (710 mg, 2.3 mmol, 46%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 6.67 (s, 2H), 3.50 (t, J = 7.2 Hz, 2H), 2.81 (s, 4H), 2.57 (t, J = 7.6 Hz, 2H), 1.78 (tt, J = 7.6 Hz, 2H), 1.64 (tt, J = 8.2 Hz, J = 7.6 Hz, 2H) Hz 1.41 (tt, J = 8.2 Hz, J = 7.2 Hz, 2H); ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 170.9, 169.0, 168.3, 134.0, 37.5, 30.8, 28.0, 25.9, 25.6, 24.1. Low-resolution mass (ESI-POS), calculated for C₁₄H₁₇N₂O₆ [M+H]⁺: 309.11, found: 309.10. Mp 70-73 °C.

2-iodoxybenzoic acid (IBX)^[33]

Procedure from literature, ref. 33. In a 2L round bottom flash equipped with a mechanical stirrer and internal thermometer, 2-iodobenzoic acid (50 g, 0.2 mol, 1 equiv.) was added to a solution of Oxone (200 g, 0.30 mol, 1.5 equiv.) in dd-H₂O (600 mL, 0.5 M). The solution was heated rapidly (<10 minutes, also using a heat-gun) to 70°C, ensuring the temperature does not exceed 73°C, under rapid stirring. After 4h, the solution was cooled to approximately 0°C using a bucket with an ice-salt mixture and left to stand for 2 h, stirring slowly. The resulting suspension was filtered (20 cm diameter glass filter), and washed repeatedly with water (10 x 100 mL) and acetone (2 x 100 mL). The resulting white powder was allowed to dry overnight under vacuum (40.7 g, 145 mmol, 73%) ¹H-NMR (DMSO-d₆): δ (ppm) 8.15 (dd, J = 7.8 Hz, J = 1.4 Hz, 1H), 8.01 (dd, J = 7.5 Hz, J = 1.4 Hz, 1H), 7.98 (ddd, J = 7.8 Hz, J = 7.5 Hz, J = 1.4 Hz, 1H), 7.84 (ddd, J = 7.7, J = 7.5 Hz, J = 1.4 Hz, 1H). ¹³C-NMR (DMSO-d₆) δ 167.5, 146.6, 133.4, 133.0, 131.4, 130.1, 125.0 ppm. Mp 233 °C (decomposes).

General procedure for the synthesis of hydroxylamines

The starting alcohol (5 mmol, 1 equiv.) and N-hydroxyphthalimide (820 mg, 5 mmol, 1 equiv.) were dissolved in CH₂Cl₂ (40 mL, 0.125 M) and cooled to 0°C after which tributylphosphine (1.85 mL, 7.5 mmol, 1.5 equiv.) was added. Diethylazodicarboxylate (40% wt, 3.5 mL, 7.5 mmol, 1.5 equiv.) in toluene was added dropwise and the resulting solution stirred overnight at room temperature. The mixture was concentrated *in vacuo* and subsequently recrystallized from refluxing EtOH (approx. 70 mL) and filtered off. The filtrate was then dissolved in EtOH (50 mL) at reflux, after which monomethylhydrazine (0.3 mL, 5.5 mmol, 1.1 equiv.) was added over 10 minutes by syringe pump. After 1h at reflux, the mixture was concentrated *in vacuo* after which Et₂O (50 mL) was added. After 30 minutes, the mixture was filtered, after which the liquor was left to stand and filtered again (3 cycles total). The resulting solution was concentrated *in vacuo* to afford the product.

Benzylhydroxylamine

Benzylalcohol (0.52 mL, 5 mmol, 1 equiv.) was treated as described in the above procedure. After concentration *in vacuo*, the product was obtained as a clear colourless oil that quickly yellows upon contact with air (472 mg, 3.8 mmol, 77%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 7.32-7.29 (m, 5H), 5.25 (br. s, 2H), 4.61 (s, 2H); ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 137.8, 128.4, 127.9, 77.9.

3,4 dimethoxybenzylhydroxylamine

3,4-dimethoxybenzylalcohol (840 mg, 5 mmol, 1 equiv.) was reacted as described in the above procedure. After the solution was concentrated *in vacuo*, it afforded the product as a colourless oil that turns brown over several days at -20°C (use immediately after synthesis) - (692 mg, 3.8 mmol, 76%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 6.91-6.89 (m, 2H), 6.85-6.81 (m, 1H), 4.63 (s, 2H), 3.88 (s, 3H), 3.85 (s, 3H). ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 149.2, 148.9, 129.8, 121.0, 111.5, 110.0, 77.8, 55.8 (2).

Attempted Dess-Martin & IBX oxidation of Alamethicin F30

Dess-Martin Periodinane (15 mg, 35 μmol, 14 equiv.) was added to a solution of Alamethicin F30 (5 mg, 2.5 μmol, 1 equiv.) in CH₂Cl₂ (1 mL) in a HPLC vial equipped with a magnetic stirrer and stirred for 2 h. The mixture was filtered, concentrated *in vacuo* and checked with MALDI-TOF – only degradation of the peptide was observed. The crude mixture was taken up in MeCN (0.5 mL) and analysed using semi-preparative HPLC (40 % A in B to 80% A in B over 40 minutes), no significant product or starting material was observed. Using IBX (15 mg, 54 μmol, 21 equiv.) afforded the same result.

General procedure for the synthesis of Alamethicin-oximes using the Swern oxidation

DMSO (10 μL, 140 μmol, 56 equiv.) was dissolved in dry CH₂Cl₂ (1 mL) in a 1.5 mL HPLC-vial equipped with a stirring bar and cooled to -78°C using a dry-ice/acetone bath (using metal wire to hold up the vial). Oxalyl chloride (9 μL, 104 μmol, 42 equiv.) was added and the resulting mixture was stirred for 15 minutes at -78°C. Alamethicin F30 (5 mg, 2.5 μmol, 1 equiv.) was dissolved in dry CH₂Cl₂ (1 mL) in a 4 mL capped vial equipped with a magnetic stirring bean and cooled to -78°C. The Oxalyl/DMSO mixture was transferred to the 4 mL vial using cannula techniques and additional CH₂Cl₂ (0.5 mL) was added and the mixture was stirred for an additional hour at -78°C. Freshly distilled dry triethylamine (28 μL, 200 μmol, 80 equiv.) was added and the solution stirred for 1 hour, letting the temperature slowly return to room temperature. CH₂Cl₂ (7.5 mL) was added, after which the solution was washed with a saturated ammonium chloride solution (10 mL), saturated NaHCO₃ (10 mL) and brine (10 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. MALDI-TOF was used to check both the organic and aqueous phase for decomposition. The residue

– Synthesis and Characterization of Alamethicin-DNA hybrids. –

was dissolved in MeOH (1 mL), the appropriate hydroxylamine was added (50 μ mol, 20 equiv.) and stirred at rt. MALDI-TOF was used to monitor the reaction (typically 24+ h).

The mixture was concentrated *in vacuo*, taken up in acetonitrile and purification was performed *via* semi-preparative HPLC (40% A in B to 100% A over 30 minutes).

Attempted synthesis of Alamethicin F30 benzyl oxime

Benzylhydroxylamine (7 mg, 58 μ mol, 23 equiv.) in MeOH (1 mL) was added to the Alamethicin solution (prepared as described). After 17 and 24 h, no reaction was observed by MALDI-TOF, after 40h, significant degradation was observed and the experiment terminated, trace amounts of product were observed, but insufficient for purification.

Attempted synthesis of Alamethicin F30 3,4-dimethoxybenzyl oxime

3,4-dimethoxybenzylhydroxylamine (10 mg, 55 μ mol, 22 equiv.) in MeOH (1 mL) was added to the Alamethicin solution (prepared as described above). After 17 and 24 h, no reaction was observed by MALDI-TOF. After 40h significant degradation was observed and the experiment terminated, no product was observed.

Attempted synthesis of Alamethicin F30 n-octyl oxime

n-octylhydroxylamine HCL-salt (9 mg, 55 μ mol, 22 equiv.) in MeOH (1 mL) was added to the Alamethicin solution (prepared as described above). After 17 and 24 h, no reaction was observed by MALDI-TOF. After 40h, significant degradation was observed and the experiment stopped. No (desired) product was identified.

General procedure for the synthesis of dULC-ALM-F30 – DNA hybrids

The appropriate amine-modified oligonucleotide was dissolved in Phosphate-buffer (300 μ L, pH ~7). 6-Maleimidohexanoic acid N-hydroxysuccinimide ester in DMF (100 μ L, 20 mg/ml, 65mM) was added and the mixture gently agitated for 1 hour, after which the progress of the reaction was checked using MALDI-TOF. If any starting oligonucleotide remained, an additional aliquot of the 6-maleimidohexanoic acid N-hydroxysuccinimide ester in DMF was added and agitated for an additional hour (repeat again if needed). The mixture was then purified over a Sephadex G10 size exclusion column using dd H₂O as per instructions on the package. MALDI-TOF was used to determine which fractions contained the modified oligonucleotide, which were subsequently freeze-dried (the fractions that contained impurities and oligo as well were combined and purified one more round using the same purification protocol).

The modified Oligonucleotide was taken up in dd H₂O (200 μ L), after which a solution of dULC-ALM-F30 in DMF (500 μ L, 6 mg/mL) was added. The resulting mixture was agitated for 2h at room temperature, after which the reaction was checked using MALDI-TOF. If after 6h

starting material was still observed, an additional aliquot of the alamethicin solution was added (200 μ L) and the mixture agitated overnight. Upon completion of the reaction, the mixture was passed through a syringe filter (pore size: 10 μ m) and subsequently purified using semi-preparative HPLC (40% A in B to 80% A in B over 40 minutes, then 100% A in 10 minutes, staying at 100% for 10 minutes, followed by a 10-minute flush-back). Lyophilisation afforded the final product.

Attempted synthesis of Maleimide-C₃-5'-TGATT-3'

The 5'-hexylamine modified oligonucleotide 5'-TGATT-3' was treated according to the above described protocol, with the exception of using 3-maleimido propanoic acid N-hydroxysuccinimide ester in DMF (100 μ L, 20 mg/mL, 75 mM). After purification, the product was obtained as a clear film on the vessel wall. MALDI-TOF (Pos), Calculated [M-H]⁺: 1805 m/z Found: 1811 m/z Retention time (HPLC, method A): 38 min.

Attempted synthesis of dULC-ALM-F30

The maleimide modified oligonucleotide was then treated following the second half of the above procedure. No significant product formation was observed after 72h.

Maleimide-C₆-5'-TGATT-3'

The 5'-hexylamine modified oligonucleotide 5'-TGATT-3' was treated according to the above described protocol. After purification, the product was obtained as a light white fibrous solid. MALDI-TOF (Neg), Calculated [M-H]⁻: 1863 m/z Found:

1866 m/z Retention time (HPLC, method A): 37 min. No yield was determined.

dULC-ALM-F30-C₆-5'-TGATT-3' hybrid

Maleimide-C₆-5'-TGATT-3' was subjected to the second half of the above procedure. After purification, the product was recovered as an off-white fine powder. MALDI-TOF (Neg), Calculated [M-H]⁻: 4170 m/z Found: 4181 m/z, HRMS (ESI-Neg): calculated [M-H]⁻, found: Retention time (HPLC, method B): 31 min. Yield over 2 steps: 11%.

Maleimide-C₆-5'-GGGTT-3'

5'-hexylamine modified oligonucleotide 5'-GGGTT-3' was reacted using a modified version of the general procedure: two consecutive size exclusion purifications were required in all attempts. The product was obtained as a white sticky solid that

was difficult to dissolve in dd H₂O without sonication. MALDI-TOF (Neg), Calculated [M-H]⁻: 1904 m/z Found: 1945 m/z [M-2H+K]⁺ Retention time (HPLC): 28 min. No yield was determined.

dULC-ALM-F30-C₆-5'-GGGTT-3' hybrid

Maleimide-C₆-5'-GGGTT-3' was subjected to the second half of the above procedure. After purification, the product was recovered as an off-white fine powder. MALDI-TOF (Neg), Calculated [M-H]⁻: 4207 Found: 4248 [M-2H+K]⁺, HRMS (ESI-Neg): calculated [M-H]⁻, found: Retention time (HPLC): 48 min. Yield over 2 steps: 17%.

Maleimide-C₆-5'-TTAAGCATGGT-3'

The 5'-hexylamine modified oligonucleotide 5'-TTAAGCATGG-3' was treated according to the above described protocol. After purification, the product was

obtained as a light white fibrous solid. MALDI-TOF (Neg), Calculated [M-H]⁻: 3742 m/z Found: 3748 m/z Retention time (HPLC): 35 min. No yield was determined for this step.

dULC-ALM-F30-C₆-5'-TTAAGCATGGT-3' hybrid

Maleimide-C₆-5'-TTAAGCATGGT-3' was subjected to the second half of the above procedure. After purification, the product was recovered as an off-white fine powder. MALDI-TOF (Neg), Calculated [M-H]⁻: 6045 m/z Found: 6051 m/z, HRMS (ESI-Neg): calculated [M-H]⁻, found: Retention time (HPLC): 44 min. Yield over 2 steps: 27%.

5'-ACCATGCTTAA-3'-C₆-Maleimide

The 3'-hexylamine modified oligonucleotide 5'-ACCATGCTTAA-3' was treated according to the above described protocol. After purification, the

product was obtained as a light white fibrous solid. MALDI-TOF (Neg), Calculated [M-H]⁺: 3671 m/z Found: 3680 m/z Retention time (HPLC): 39 min. No yield was determined for this step.

5'-ACCATGCTTAA-3'-C₆-dULC-ALM-F30 hybrid

5'-ACCATGCTTAA-3'-C₆-Maleimide was subjected to the second half of the above procedure. After purification, the product was recovered as an off-white fine powder. MALDI-TOF (Neg), Calculated [M-H]⁺: 5976 m/z Found: 5981 m/z, HRMS (ESI-Neg): calculated [M-H]⁺, found: Retention time (HPLC): 43 min. Yield over 2 steps: 25%.

3.6. References

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Chapter 4 – Evaluation of dULC-ALM–DNA hybrids in

Electrophysiology

4.1. Acknowledgements

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4.2. Black Lipid Membrane Electrophysiology

In the previous chapter, the synthesis and purification of several DNA-dULC-Alamethicin constructs was reported. This chapter will further investigate the pore forming properties of these compounds.

To measure and quantify the activity of a pore-forming compound, such as the pore-forming peptide-DNA hybrids created in Chapter 3, electrophysiology is commonly employed as the primary analysis tool.^[1,2] In these measurements, a lipid bilayer membrane serves as model for a cell membrane, on which the pore forming compound (optionally after being activated by a selected analyte) is to act. If the membrane is successfully perforated by the peptide complex, the pores will allow ions to cross the membrane. When a transmembrane potential is applied at the same time, this results in a small, reproducible and quantifiable current. This current is both a function of the applied potential and the pore diameter (in the case of pore-forming compounds). By dividing the observed current by the potential applied ($I/U = S(\text{Siemens})$), the conductance of the pore forming system can be determined, which is, for pore forming peptides, believed to be solely dependent on the pore diameter, i.e. the number of monomers per pore.

This model system is highly simplified, generally not taking into account certain factors such as the complex physiological environment cells usually grow in, the crowded nature of most cell surfaces or intercellular interactions, however, it remains a good primary evaluation tool for our molecules ^[3]

Scheme 1. Schematic representation of a Planar lipid bilayer (BLM) setup.

This technique comes in two main varieties: a ‘patch-clamp’ set-up and a ‘black lipid membrane’ one (BLM).^[4,5] In the former, a part of a cellular (or vesicular) membrane is deposited on a special pipette-tip and in the latter, as used in this investigation, a lipid mixture dissolved in *n*-hexadecane is deposited around a pinhole (180 μm) in a Teflon or Delrin™ cup and briefly allowed to dry. The cup is inserted into a holder and thereby two compartments of equal volume are created which are both filled with electrolyte. One compartment is considered henceforth as the *cis*-membrane compartment, the other one as the *trans*. Both compartments are then equipped with an Ag/AgCl electrode *via* a salt-bridge. Subsequently, the pore forming compound is typically introduced on the *cis* side in the solution, after which its behaviour in the membrane, in terms of conductance across it, can be studied by applying a transmembrane potential (V). If an ion-channel (pore) is formed, it will allow ions to pass through the membrane. The current generated by the ions’ passage is then recorded by the workstation as a function of time.

In a typical BLM experiment, this current (A_x) is then measured at various potentials (V_x) and resolved into conductance ($S_x = A_x/V_x$) allowing for a quantitative picture of pore formation to be established, typically in the form of a bar graph representing the populations per conductance range over a period of time or fixed number of events. Other relevant information includes pore lifetime (per conductance range), and standard deviation from the expected conductance level for that pore-size.

Measurements are typically performed in two manners. The first is manual stimulation, i.e. longer recordings with long dwell times per voltage (typically tens of seconds to minutes), following the behaviour of the formed pore over a longer period of time. The second is by episodic stimulation: a script in the software cycles the voltage every few seconds. This has as a consequence the membrane potential across the membrane gets inverted, which is intended to drive all pore-forming compounds from the membrane’s *cis* side back into solution, effectively returning the experiment to the starting conditions. A notable exception is the persistence of any stable pores that may have formed, which can resist this effect until the potential is raised to the point the bilayer collapses, as demonstrated in the experiments performed later in this chapter.

Also, any pore forming compound that has managed to fully traverse the membrane will be unaffected, as inverting the potential will now allow the pore-forming compound on the *trans*-side to engage in pore forming behaviour. This last event is highly undesirable and is reason to stop the ongoing experiment.

The main observable difference between ‘stable pores’ and membrane traversal caused pores is that a stable pore does not suffer any loss of activity, while for membrane traversal pores, a small lag is observed between the potential inversion and continuation of pore formation. The latter is typically accompanied by different conductance levels being preferred due to the different concentration of pore-forming compound on the *trans*-side of the membrane.

As stated above, once a pore has formed in the membrane, a current is generated and recorded by the system. The exact amount of conductance is a function of the pore size. ^[6] A random rift or defect in the membrane will have a randomly defined conductance, and therefore pore-size, whereas a pore generated by a pore-forming compound are defined by the characteristics of that compound and therefore will display only particular levels of conductance. When a trace is analysed, all observed currents across the membrane are identified and catalogued as events. Upon processing, random events will disappear to the background due to their uniform distribution, whereas events generated by the analyte will mainly concentrate in a few bands or levels of conductance and become clearly visible and identifiable.

Figure 1. Example trace of a BLM measurement with three conductance levels (by statistical analysis) and obscured events/noise (illustration based on own results from an active pore forming peptide).

When a sufficient length of trace and/or a sufficient number of events have been collected in BLM, the data can be processed. First, noise is filtered out if necessary (e.g. noise from the

membrane, AC interference at 50 Hz). Then conductance levels are defined by visual inspection (e.g. levels 0 and 2 in Fig 1), followed by a refinement upon statistical analysis (e.g. levels 1 and 3 in Fig 1).

It is possible to let the software detect and define all levels, however, in the set-up used for this chapter it proved to be more time-consuming than manually defining initial levels. After defining the relevant levels, relevant events are identified by analysing the stable sections of the trace (i.e. sections where no software or human interference may have affected the stability of the membrane). Of these events the following characteristics are of particular interest: their current amplitude (in pA) and dwell time (in ms). The amplitude can easily be transformed into conductance (nS: pA/mV) and is a measure for relative pore size. The dwell time can be used without further transformation and is an indication of the life-time of the pore and by extension, the stability of the system.

4.3. Goal

The goal of this investigation was two-fold. The first objective was to determine whether an Oligo-dULC-Alm hybrid still displays pore-forming activity, and if so, to what extent does the activity decrease or improve compared to the unmodified peptide. The second objective was to determine the effect that DNA-duplex or quadruplex formation of these hybrids have on the overall formation behaviour of the pore construct and to establish whether or not these techniques can be employed for the development of a (selectively) switchable pore-forming peptide.

4.4. Results & Discussion

Entry	Acronym	Hybrid makeup	Function
1	dULC-ALM	-	Control
2	HR	ALM*-5'-TGA TT-3'	Control
3	HQ	ALM*-5'-GGG TT-3'	G-quadruplex
4	HSA	ALM*-5' ACC ATG CTT AA-3'	Duplex HSB
5	HSB	5'-TTA AGC ATG GT-3'-ALM*	Duplex HSA
6	HLA	ALM-5' TAT AGT GCG CTA ATC GGA GAC GAA-3'	Duplex HLB
7	HLB	5'-TTC GTC TCC GAT TAG CGC ACT ATA-3'-ALM	Duplex HLA

Table 1. Assignment of the various DNA-dULC-ALM hybrid compounds with their assigned roles

For the evaluation of pore forming behaviour in BLM-analysis, the hybrids synthesized in Chapter 3 were studied (table 1). The single stranded compounds (entries 2, 4 and 5) were measured separately first. When using the independent strands, no duplex-formation/DNA-hybridization is expected to take place. This way, the influence of the DNA-strand can be compared with the parent dULC-ALM (entry 1). The expectation was to still observe pore-forming activity, albeit reduced when compared to the unmodified parent peptide. Subsequently, the two hybridized duplex pairs (respectively the two 11-oligomers **4,5** and the two 24-oligomers **6,7**) were evaluated, with the expectation to observe either more prolific pore-formation with higher stability, or higher selectivity towards a particular pore size compared to the single stranded compounds, or ideally, even the parent peptide.

Lastly, the G-quadruplex forming conjugate was considered (entry 2) with the expectation that it would behave more as a pre-formed pore with tendencies that may match the work previously described by Skydstrup and co-workers.^[7,8] This would include an increase in lifetime and specific selectivity towards a particular conductance level. All measurements were done both in manual and episodic stimulation to obtain optimal information both on the onset of pore-formation and the long-term behaviour of the systems.

4.5. Control experiments

dULC-ALM

The reference peptide (**dULC-ALM**) displayed well defined and spaced conductance levels (standard deviations in parentheses) at 0.10 nS (σ 0.013 nS), 0.25 nS (σ 0.018 nS), 0.40 (σ 0.038 nS) and 0.61 nS (σ 0.071 nS), (Figure 2) with indications that higher conductance states can exist, albeit that they are poorly populated and rarely reached in this under these conditions. The typical lifetime of an event was 12-15 ms, with pores typically rapidly opening and closing in an uncontrolled, unpredictable fashion, as is expected for this particular peptide, as previously reported on by DeGrado.^[9]

Figure 2. Event histogram displaying the distribution of conductance levels (nS) of native dULC-ALM (3:7 POPC:DOPE; 7.5 nM dULC Alm, 1M KCl + 10 mM MOPS, pH 7.2)

Trace 1 Representative trace for dULC-ALM at 180 mV.

Single strand hybrid HR

Figure 3. Event histogram displaying the distribution of conductance levels (nS) of **HR** (3:7 POPC : DOPE; 7.5 nM **HR** in 1M KCl + 10 mM MOPS, pH 7.2)

Trace 2. Representative trace for **HR** at 180 mV.

HR displayed a strong preference for the formation of pores with 0.05-0.06 nS (σ 0.004 nS) conductance (Figure 3), with additional smaller populations around 0.11 nS (σ 0.005 nS); 0.2

nS (σ 0.007 nS); 0.34 nS (σ 0.008 nS) and 0.54 nS (σ 0.029 nS). Overall, these pores had an average event-life time of under 10 ms. The observations that HR was not particularly active in terms of events per second and exhibited long lag-times between event-rich periods, typically in the order of minutes, combined with the fact that the pores still displayed activity, allows for the conclusion that its behaviour is similar to the parent-peptide of des-Aib-Alamethicin, albeit with lower conductance levels and a smaller difference in conductance between each level. This demonstrates that the addition of a DNA-oligonucleotide to the peptide does not obstruct the pore-forming capacity of dULC-ALM, in which case only normally distributed noise would be observed, but merely hinders pore formation.

Single strand hybrid HSA

HSA displayed a more unpredictable behaviour. Rather than slowly building up (or losing) activity on the membrane, it mainly gave short, sharply defined bursts, of pore formation. During these so-named bursts, the membrane would frequently break, bringing the experiment to an unscheduled, early halt: 5 out of the 8 attempts ended early. It displayed poorly resolved levels at 0.07 nS (σ 0.012 nS); 0.12 nS (σ 0.013 nS); 0.22 nS (σ 0.017 nS); 0.40 nS (σ 0.020 nS) and 0.75 nS (σ 0.033 nS), with average pore lifetimes of 20 ms. Its behaviour in terms of stability is more like the native peptide **dULC-ALM** than **HR**, implying a certain stabilization of the pores due to the added DNA-strand.

The main difference between **HSA** and either **HR** or **dULC-ALM** is its near total lack of population in the higher conductance-levels. This may be explained by different interactions between the individual units composing the pore, where the one oligomer sequence (in this case, **HSA**) has more affinity for itself than the other and thereby benefits from more self-stabilizing effects, resulting in slightly more stable pores. The presence of an oligomer on top of the pore forming ALM-component may hinder the flow of ions, explaining the lower conductance levels of the hybrid compared to **HR** or **dULC-ALM**.

This line of reasoning might explain the visually and statistically observed differences between the activity of the observed compounds. Compared to **HR**, **HSA** might be more prone to form aggregates in solution, which upon contact with the membrane, could explain the more pronounced differences in activity: going from completely inactive with a low local concentration of **HSA** to a ‘burst’ when an aggregate makes contact, causing a high local concentration of **HSA** (increasing the risk of a failure of the membrane as well) and back again to near inactivity upon aggregate dissipation in the electrolyte or aggregate departure from the membrane, interspersed with the occasional period of “normal” activity due to non-aggregated material.

Figure 4. Event histogram displaying the distribution of the conductance levels (nS) of **HAS** (3:7 POPC : DOPE; 7.5 nM **HSA** in 1M KCl + 10 mM MOPS, pH 7.2)

Trace 3. representative trace for **HSA** at 150 mV

Single strand hybrid HSB

Figure 5. Event histogram displaying the distribution of the conductance levels of **HSB**, insert: slightly better resolved conductance levels by using the episodic stimulation program (3:7 POPC : DOPE; 7.5 nM **HSB** in 1M KCl + 10 mM MOPS, pH 7.2).

Trace 4. representative trace for **HSB** at 200 mV

Pore formation activity in this hybrid did not commence as straight-forward as with the two previously tested materials. Pore formation would only commence on, lacking a better term, membranes capable of holding a larger electrical charge, i.e. display higher than normal capacitance. The required membrane capacitance for **HSB** to display activity would lie around 180 pF. This is a step up from the typical membrane capacitance of 130-140 pF, observed in the successful experiments for the other tested hybrids in this chapter. As membrane capacitance could not be reliably 'dialled in,' the role of membrane capacitance was not further investigated as a variable in these experiments.

The compound was significantly more active in pore formation than the other non-duplex forming compounds evaluated (**HR** and **HSA**). The lowest levels were the most populated, first 0.07 nS (σ 0.006 nS), followed by 0.12 nS (σ 0.013 nS); 0.24 nS (σ 0.022 nS) and 0.40 nS (σ 0.038 nS). A last well-defined conductance level was found at 0.58 nS (σ 0.043 nS), with broader and less populated conductance levels in the higher regions, for which no (meaningful) standard deviation could be calculated. These higher populations lie at 0.81; 0.95; 1.3; 1.75 and 2.3 nS. The spike at 0.81 could be regarded as an artefact due to its sharp definition and absence in the episodic stimulation trace (figure 5, inset), however, it proved to be too consistent in the manual stimulation traces to be simply ignored. The average lifetime per pore increased significantly compared to **HR**, increasing from 6 to 24 ms. This still indicates mostly short-lived events and noisy pores, but a certain (additional) stabilization becomes apparent compared to the previously tested compound. This is especially the case when considering the presence of several higher-level conductance states. This behaviour, unexpected but not necessarily undesirable, when compared to **HR** and **HSA** again underlines that the individual oligomer sequence may play a stronger than anticipated role in altering the pore-forming abilities of the dULC-ALM peptide.

Single strand hybrid HLA

Trace 5. best activity trace for **HLA** (observed for 3 seconds) on total 30 minutes. (3:7 POPC : DOPE; 15 nM **HLA** in 1M KCl + 10 mM MOPS, pH 7.2) at 210 mV

This particular hybrid displayed barely any activity when used in either manual or episodic stimulation. This behaviour was coherent over several independent attempts, indicating a near total loss of pore forming activity. Over 6 hours of measurements, it produced a total of 5 pore events above the noise threshold, 4 at 0.4 nS, one at 0.74 nS, each lasting about 2 seconds. Whether these are artefacts or genuine pores generated by the hybrid cannot be determined beyond reasonable doubt. The total loss of pore-forming activity is highly desirable in its own right, as potentially the duplex formation can restore it, forming a truly switchable system.

Single strand hybrid HLB

HLB displayed slightly better activity than **HLA** though these events are better described as short-lived noisy attacks/perturbations on the membrane than actual stable pore formation. Also, in this instance, insufficient data was obtained for detailed analysis due to a lack of general activity of this complex. Indications were found for the existence of 2 conductance levels at 0.08 and 0.18 nS respectively, with an average lifetime of 60-80 ms. However, with only 3000 observed events, the basis for this analysis is limited. Again, the loss of pore formation in its own right is, in principle, a good thing as it puts the goal of achieving a truly switchable system in reach.

Figure 6. Event histogram displaying the distribution of the conductance levels of **HLB** for illustration purposes (episodic stimulation data only). (3:7 POPC : DOPE; 15 nM **HLB** in 1M KCl + 10 mM MOPS, pH 7.2).

Trace 6. representative trace for **HLB** at 150 mV

4.6. Summary of control experiments

The control compounds all display similar trends and conductance levels, with some variance in average dwell-times. The main difference compared to des-Aib-ALM is that the conductance-levels have shifted closer together, becoming less resolved and that populations have shifted to favour the lowest states possible. This indicates that the presence of single-strand DNA (non-duplex forming) in general destabilizes pore formation and reduces activity. The length and sequence of the oligonucleotide used both do appear to play a significant role in the exact extent of these effects, likely due to the oligomers ability to form a complex with itself or a second unit, resulting in varying levels of pore stabilization. Overall, the behaviour of certain of these hybrids show promise by demonstrating the sought after lost tendency to form pores which will allow any positive interactions with their complementary strands to be more obvious and would make the creation of a switchable system easier.

4.7. Hybrid duplex and G-quadruplex experiments

Duplex HS(A+B)

The duplex **HS(A+B)** formed by combining **HSA** and **HSB** (1:1 proportion) displayed extraordinary characteristics compared to its individual constituent compounds, being generally more active and less disrupting to the membrane. It also displayed activity in a far more extended range in terms of conductance levels (up to 3.2 and 4.1 nS) with a poorly resolved section between 1 and 2 nS in manual stimulation. Episodic stimulation here shows conductance levels of 1.10, 1.36 and 1.50 nS. The average dwell time was increased to 120 ms overall, going up to 183 ms in one particular level at 0.88 nS, with individual events lasting up to 10s being a relatively common occurrence.

The observed conductance levels are 0.11 nS (σ 0.033 nS), 0.30 nS (σ 0.028 nS), 0.51 nS (σ 0.045 nS), 0.60 nS (σ 0.034 nS), 0.85 nS (σ 0.038 nS), 1.66 nS (σ 0.146 nS), 2.59 nS (σ 0.143 nS), 3.21 nS (σ 0.148 nS), 4.08 nS (σ 0.170 nS) 5.25 nS (σ 0.213 nS) and 6.40 nS (σ 0.232 nS).

Figure 7. Event histogram displaying the conductance levels (nS) of duplex **HS** in manual stimulation (30-minute hold times) Insert: **HS** in episodic stimulation, showing the earliest stages of pore formation (hold time: 2s); bilayer: 3:7 POPC : DOPE; 7.5 nM **HSA** + 7.5 nM **HSB** in 1M KCl + 10 mM MOPS, pH 7.2)

Trace 7. Representative trace for duplex **HS** at 150 mV

Combining these findings, it is likely that conductance-level 0,88nS (strongly observed in both techniques), which also displayed the 183 ms lifetime average, is now highly preferred, in contrast with the control traces, due to the additional stability inferred by the DNA-duplex. The lower levels are still filled significantly compared to the control compounds, with some of them having a shorter lifetime than others, ranging from 80-183 ms averages with a seemingly random distribution (it is not that a larger pore is more stable or vice versa). This may be attributed to the stepwise manner in which these pores are formed: the various states all benefit from the duplex-stabilization, with the conductance level pore size 0,88 nS being the most stabilized over time. The higher conductance states that were observed could either stem from the further increase in pore-size or might be attributed to multiple events occurring at the same time. Especially for the conductance-levels in the high region (3+ nS), it stands to reason that at least a second pore is present, which could also explain the broadening of each level to at least a certain degree.

This experiment appears to deliver the proof of principle that DNA can be used to stabilize a pore forming system and is able to introduce newly preferred conductance levels with significant occurrence not observed in the parent molecules. This indicates that a DNA-based switchable pore forming peptide can in fact be achieved through this method.

Duplex HL(A+B)

The longer 24-mer duplex **HL(A+B)**, generated from **HLA** and **HLB**, generally displayed stabilization as well, although from the experiments it became apparent that the general pore-forming activity was reduced when compared to the 11-mer duplex. The complex displayed populations at conductance levels 0.06 nS (σ 0.018 nS), 0.14 nS (σ 0.029 nS), 0.20 nS (σ 0.019 nS), 0.31 nS (σ 0.020 nS), 0.48 nS (σ 0.021 nS) and one more densely populated at 0.68 nS (σ 0.030 nS). Indications of larger pore sizes were also present. Remarkably, the average lifetime of a pore improved to 207 ms.

Overall, it can be surmised that the benefit gained from more stabilization by the oligomer is offset by the higher “activation” barrier the construct has to overcome before a pore is formed or expanded, resulting in more stable, but generally, smaller pores. The overall activity of the system was slightly reduced in terms of events/minute compared to **HS**, but **HL**, in its own right, is still a highly efficient pore-forming system. As this could indicate that preferred pore sizes can (also) be tuned *via* oligomer length, this could become useful additional tool in generating a more controlled, switchable pore-forming complex.

Figure 8. Event histogram displaying the distribution of conductance levels (nS) of the duplex **HL** (Bilayer 3:7 POPC : DOPE; 7.5 nM **HLA** + 7.5 nM **HLB** in 1M KCl + 10 mM MOPS, pH 7.2)

Trace 8. Representative trace for duplex **HL** at 180 mV

Quadruplex HQ

Figure 9. Event histogram displaying the distribution of the conductance levels (nS) of quadruplex **HQ** (Bilayer: 3:7 POPC : DOPE; 7.5 nM **HQ** in 1M KCl + 10 mM MOPS, pH 7.2);

Trace 9. Representative trace for quadruplex forming construct **HQ** at 150 mV

The G-quadruplex stabilized **HQ** was by far the most active compound during the tests in addition to generally not breaking the membrane. One highly interesting observation was that it was highly resistant to cleaning efforts. After the initial run and rigorous cleaning procedures, the Delrin sample cups used (on the *Cis*-side), generally would still be contaminated with **HQ**, in sufficient quantities that pore-forming activity would persist in freshly made membranes for 2-3 more cleaning cycles. It is possible that these quadruplexes may be working effectively and reliably at far lower concentrations than the concentration used in these experiments.

After the first conductance state observed at 0.05 nS (σ 0.028 nS), the conductance states at 0.21 nS (σ 0.022 nS), 0.30 nS (σ 0.021 nS), 0.40 nS (σ 0.026 nS), 0.52 nS (σ 0.032 nS), 0.73 nS (σ 0.033 nS) and 0.95 nS (σ 0.056 nS), were occupied evenly, with additional, but only marginally active, states up to 6 nS. The average lifetimes per event were around 130 ms, going up to 150-200 ms for the higher conductance levels with individual events lasting up to 2000 ms.

These findings are a strong indication that, as expected, the G-quadruplex is an excellent structure for stabilizing a pore. The construct prefers higher conductance states which equates to the existence of stable larger pores. While the gain in pore life-time was not particularly better than for the duplex experiments, the simplicity of this system (requiring a much shorter sequence and simpler purification) might be the way to explore in order to further improve on these characteristics. These observations, who are in accord with earlier studies by L. Gjonaj,^[10] indicate a more pronounced effect by the G-quadruplex, giving good hope that with further optimization, even better results can be obtained.

4.8. Conclusion

The goal of this chapter was to determine the effect of introducing a DNA-oligomer that can give rise to a DNA-duplex or Quadruplex structure on the pore-forming activity of dULC-ALM. It was clearly established by BLM-analysis that overall, these peptide derivatives, when unable to undergo duplex or quadruplex formation, retain their ability to form transmembrane pores, albeit at (severely) reduced activity and lower pore-lifetimes. This indicates that pore formation is discouraged by the presence of unhybridized ss-DNA.

For the duplex hybridized DNA-peptide constructs, **HS** and **HL**, a clear improvement was observed both in terms of higher conductance levels reached and the lifetime of the pores. This stabilization appears to increase with the length of the oligomer involved and therefore its increased heterodimer stabilization. It should be noted that the price for more stability is that it takes on average longer to form a pore with the longer oligonucleotide (**HL**) than with the shorter (**HS**). The latter appearing to be generally more active in terms of pore formation than both the longer oligonucleotide and the individual compounds measured in the control experiments.

The best results were, as hoped for, obtained with the G-quadruplex forming structure **HQ**, which displayed a significant increase in activity, conductance levels and a good pore-stability. As quadruplexes are part of many aptameric systems, it is expected that with further investigation and proper optimization, the quadruplex hybrids can be employed in a switchable pore-forming system without any concern for activity-loss or destabilization.

It can be concluded that the goals of this chapter were met. It was not only established that DNA-dULC-Alm hybrid compounds still display pore forming activity on a membrane but also that the attached DNA strand can have different effects on the pore forming process depending on its hybridization. A proof of principle has been obtained in that the created DNA oligomers can be used to modify and control the behaviour of a pore forming peptide, alamethicin in this case.

However, further investigation and optimization is required before a fully functional switchable pore-forming peptide can be developed. The main challenges lie in determining concentration dependence of the pore forming activity: i.e. establishing a minimum, maximum and optimal concentration for pore forming to take place in the presence of these compounds. Also determining the exact influence of the oligonucleotide length and sequence would require follow-up. Particularly the study of the influence of the length of the oligonucleotide is of the essence, as the introduction of an aptamer invariably will lengthen the oligomer-side of the construct, the pore-stabilization has to be sufficient to counteract any potential negative effects if and when they occur.

4.9. Supplementary information

General experimental

Planar lipid bilayer experiments were carried out on a Warner Instruments 1440A Bilayer Clamp Workstation using a Sun-Stir base in combination with an 8-pole low-pass Bessel Filter LPF 8, using Clampex 10.2 acquisition software for digitization. Sampling was done at a frequency of 10 kHz. All data was treated using a 99-point boxcar filter prior to further processing.

For the first two rounds of experimentation, 4 mL Delrin cups were used for bilayer support, aperture size 250 μm . Insulated 1/8 mm Ag-wire (10 cm) with 10 mm gold-plated electrode was used for the electrodes, the last 3 cm being exposed and coated in AgCl by treating the bare wire with a household grade sodium hypochlorite solution for 10 minutes. Agar salt-bridges (2% agarose, 3 M KCl, 46x1 mm) were used to complete the circuit.

The third evaluation was performed on a 1 mL setup with a Teflon membrane clamped between two chambers, aperture and remaining setup being identical to the above. No significant differences were observed between the datasets obtained with either set of cups.

The bilayers were formed by drying 1,5 μ L of a 3/7 mixture of palmitoyl oleoyl phosphatidyl choline (POPC) and di-oleoyl phosphatidyl ethanolamine (DOPE) (10 mg/ml in n-decane) for 10 minutes on the flattened face of the Delrin cup, taking care to clear the pinhole by gently brushing a Kimtech precision wipe against the inside of the pinhole until a puckering can clearly be seen in the deposited droplet of lipid-mixture.

Subsequently, the cup was placed inside the holder and both holder (Cis side) and cup (trans side) were filled with 4 mL of electrolyte solution (1M KCl with 10 mM MOPS buffer at pH 7.2) and a 2 mm magnetic stir-bar was added to the cis (holder) side after placing the assembly inside the measuring chamber. Electrodes were connected to either side, after which it was verified whether the pin-hole was open by measuring the total current going through the set-up (>10.000 pA). Next, the instrument offset was determined, with any offset between ± 2 mV and ± 1 pA being accepted.

The headstage was set to “capacitance” to observe the formation of the membrane. The membrane itself was formed by gently bubbling air from a lipid-coated pipette tip against the pinhole. If the bilayer formed within 15 minutes and reached a stable capacitance of >130 pF for over 1 minute, the headstage would be switched back to “current,” magnetic stirring engaged and the analyte (10 μ L; 3mM stock solution) added by Hamilton syringe to the Cis-side and the measurement was started. After 3 minutes, stirring was switched off, and 150 mV of transmembrane potential was applied. If within 5 minutes a signal was detected, recording on the measurement would begin. Otherwise, after checking the membrane capacitance (>130 pF), the transmembrane potential would be raised to 180 mV for 5 minutes. If that gave no result, a second dose of analyte was added, followed by 3 minutes of stirring while applying 180 mV transmembrane potential followed by another 5 minutes without stirring. If after that time no signal was observed, the potential was increased to 210 mV, before the experiment was discontinued.

At the start of the measurement, the set-up would remain at its initial voltage for at least 5 minutes, unless the membrane showed signs of excessive stimulation, in which case voltages were lowered in 30 mV increments (down to 90 mV) or the potential would be switched to negative (-10 mV) for a short period of time before being returned to a positive value.

After the initial 5-minute wait, the voltage was set to the desired value for measurements (180, 150 and 1000 mV) and left to run, while trying to keep interference to a minimum. The runs would either entail manual stimulation of the membrane (long periods of the same transmembrane voltage, runs 1 and 2), or episodic stimulation.

Episodic stimulation had the following format: 2s, +150 mV, 0.1s +0 mV, 2s -150 mV, 0.5s 0 mV, to be repeated for 200-2000 cycles, depending on the activity of the system – The target was to observe at least 20k events, taking into account the relatively high levels of noise generated by these particular pore-forming compounds.

Traces were filtered using a boxcar filter over 99 points, followed by single channel search over 8 pre-determined levels (0, 10, 20, 40, 70, 100, 250, and 500 pA). The amplitude histogram this search produces (in software) was then used to redefine the lower levels to the middle of each of the observed populations (rounded up or down to the nearest integer) and the single channel search was subsequently repeated.

The data from three measurements for each individual sample, each using a fresh membrane, were combined and copied to Excel, in particular the following event parameters: Level, Event Start, Event Stop, Amplitude, Amp. Sd., and Dwell time. All level 0 and level 8 events were discarded as being the baseline and noise/outliers (e.g. electrical switch, disturbing the table) respectively. Amplitude (in pA) was then divided by the potential applied (in mV) in the specific experiment to yield Conductance (in nS). The conductance data was subsequently combined with data from other experiments for comparison and final merger into one histogram. Each local maximum in the histogram was subsequently reported, rounded to two decimals. As in most, if not all analyses, the peaks could not be deconvoluted and levels were assigned manually, a meaningful bias could not be always be obtained and is therefore omitted for certain samples.

Dwell data was taken as is (in ms) and plotted as a logarithmic histogram for each population separately and the average value was reported if only one maximum was found in the plot, otherwise the local maxima were both given.

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Chapter 5 – Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.

5.1. Acknowledgements

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5.2. Introduction

WALP is a 19-residue α -helical model membrane peptide of sequence: GWWLALALALALALWWA, which is widely studied and modified in localization experiments in lipid bilayers.^[1,2] Computational studies indicate that WALP localization is affected by the ganglioside G_{M1}: a ceramide containing one sialic acid moiety (Figure 1). According to these reported simulations, this lipid clusters around the WALP peptide and shuttles it to the liquid ordered (L_o) phase, in phase separating membranes: a liquid crystal phase of the membrane, related to lipid rafts observed in (cellular) plasma-membranes.

Figure 10. Ganglioside G_{M1}, sialic acid moiety on the left, ceramide on the right.

In order to observe this shuttling behaviour two separate modification steps are needed on the peptide, necessitating a double workup. First, the peptide needs to be palmitoylated *in vitro*, and second, its localization studied in giant unilamellar vesicles (GUVs) by confocal microscopy. To be able to localize it, the peptide needs to be visualised by means of a

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

covalently attached dye. In order to accomplish both these steps, without disturbing the native activity of the WALP peptide, an analogue has to be used which possesses a single tethering point at its hydrophilic end to which both dye and palmitoyl moieties can be attached. Ideally, this tether point should be a scaffold that allows for the facile attachment of various dyes and lipids so that these can be screened without having to perform a long multistep synthesis for each combination, potentially losing significant amounts of peptide in the process. This scaffold would also allow for the screening of various (WALP) analogues or another peptide candidate, should the situation require it.^[3]

The goal of this research was to develop a scaffold that could be employed to quickly attach three different large functionalities together, to facilitate studies such as that of WALP. In this case, for example, these would be the peptide, the phospholipid and a dye.

In the ideal case, the construction of the complex containing the developed scaffold and three components from the individual building blocks should require minimal synthetic work, so that if needed, the work could be carried out in facilities not equipped for specialized chemical synthesis.

Figure 11. Examples of basic (non-orthogonal) trifunctional linkers used in biochemistry and labelling studies.

Trifunctional molecules are ubiquitous in chemistry: any molecule that has three functional groups can be considered trifunctional. If these groups can be readily employed to undergo bond formation with another functional group, it has linking-capabilities and therefore could be considered a candidate for being a trifunctional linker or scaffold. Even the simplest of examples can already achieve a great variety of potential outcomes, based on stochastics, or clever (sub stoichiometric) introduction of the groups that need to be incorporated (Figure 2).^[4-6] Tri(-orthogonal)-functional linkers are already more of a challenge but still several varieties exist,^[7-9] two of which are shown below (Figure 3).

Figure 12. Two tri-orthogonally functionalized linkers, **4** by Knall and co-workers,^[10] **7** by Jiracek *et al.*^[11]

With the goal in mind, of developing the versatile and easy to use scaffold, we began by considering several known trifunctional linker structures that could be used as starting points during the design phase. For example, we first considered the elegant option that is offered by a tri-orthogonal “click” scaffold which combines an inverse Electron Demand Diels-Alder (iEDDA) with a Copper-catalysed Azide-Alkyne Coupling (Cu-AAC) and a thio-Michael reaction (between an α,β -unsaturated ketone and a thiol or similar structure) as promoted Knall *et al.*^[10] Second, we considered the approach of Jiracek *et al.* which was to combine two CuAAC click reactions with an aldehyde and/or activated ester couplings.^[11] However, both the above mentioned strategies were considered to be too elaborate for application in a non-synthetic chemistry lab where robustness and convenience of the approach are key elements for success.

Scheme 2. Idealized functionalization of p-azidophenylalanine with three different functional groups (the amine being transformed to a maleimide first)

Alternatively, amino acids are also considered to be a convenient starting point to create linker scaffolds as several of them (the non-apolar amino-acids) offer three potentially interesting functional groups, two of which are normally already orthogonal to each other, can be easily protected and can already be transformed readily by non-specialists. The R-

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

group in schematic amino-acid **8** can be readily varied, either by modification of a ‘standard’ amino acid, or by synthesizing a new one, and can easily be made to play the role of third, orthogonal, functional group.

Based on this, *p*-Azidophenylalanine **9** was first selected as target scaffold for this project. In this approach, target molecule **10** would be synthesized by introducing the phospholipid through amide formation on the free acid (R1), Tamra (R2) could be introduced by a photo-mediated alkyne-azide coupling reaction, and finally the incorporation of the WALP moiety (R3) would be done via a thio-Michael reaction on a maleimide, previously formed from the amine.^[12-13] However, concerns about the stability of the dye under the proposed conditions (UV-light) and the need (from a cost-perspective) to maintain a strict order of introduction: lipid first, peptide last, meant this proposal needed to be adapted further.

Scheme

3. Use of propargylglycine **11** as a trifunctional scaffold as by J.Oelerich (**12**) and the proposed construct for this chapter (**13**)

The solution was inspired by previous work by J. Oelerich, who used propargylglycine **11** in a project in collaboration with the group of Cordes *et al.*^[14] He demonstrated the versatility of this non-proteinogenic amino acid and showed that this would be the candidate of choice to serve as basis for the scaffold.

5.3. Results and discussion

With this design, a maleimide is introduced on the amine of propargylglycine via substitution, a phospholipid on the acid via coupling (dipalmitoyl-sn-glycero-phosphatidyl-ethanolamine, DPPE), a dye (5-Carboxytetramethylrhodamine - TAMRA) via the alkyne and click chemistry. The WALP peptide is introduced last by reacting WALP-SH with the maleimide. Propargylglycine is commercially available and can alternatively be made in a short amount of time, in gram-quantities, using known procedures.^[15]

Scheme 4. Synthesis of L-propargylglycine. a) AcCl , NEt_3 , CH_2Cl_2 b) EtONa , Propargylbromide, EtOH c) LiOH , H_2O . d) Enzymatic resolution by Acylase I, H_2O

Propargylglycine **11** was therefore prepared using the procedure by Granja *et al.* (scheme 3).^[15] Starting from 2-aminodiethyl malonate **14**, acetamido malonate **15** was first formed in quantitative yield via acetylation. Introduction of the propargyl moiety via substitution onto **15** smoothly afforded **16**. After quantitative hydrolysis of both ethyl esters, free diacid **17** was subjected to mono-decarboxylation to afford racemic N-acetyl propargylglycine **18**. Enzymatic resolution using Acylase I in water then resulted in the desired L-propargylglycine (**L-11**) and R-N-acetyl propargylglycine (**R-18**).

Scheme 5. Attempted introduction of a maleimide functionality using direct functionalisation and a three-carbon linker, with the putative reaction product.

Initially, it was attempted to directly functionalize the α -amine with maleic anhydride (Scheme 4). These attempts failed, resulting either in a ring-opened product or in a complex mixture when harsher conditions were explored. Similarly, when attempting to introduce the maleimide via a short three-carbon linker, rearrangement of the desired product was the primary outcome of the reaction (**21**). Results by NMR suggest that 1,5-intramolecular addition onto one maleimide ester, followed by ring-opening was the main cause of the different outcome.

– Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids. –

Experiments with a longer, in this case six-carbon, linker gave the intended product **22** in good yield (50%), using a similar protocol to one used by Jens Oelerich (Scheme 5). In the next step, dipalmitoyl-sn-glycero-phosphatidyl-ethanolamine (DPPE) was to be coupled to the carboxylic acid to obtain **23**. The preferred solvent system to solubilise DPPE (chloroform, methanol and water, 6/3.5/0.5 v/v)^[16] proved incompatible with coupling reagents, forcing us to explore alternatives. The two-step, one-pot coupling system using EDC.HCl gave unsatisfactory results in this particular instance.^[17] However, a two-step procedure could be developed, which was composed of first generating an activated ester, followed by rapid removal of the insoluble urea side-products and then addition of the activated ester to the solution containing DPPE. This was then stirred for an extended period of time, 10 hours, which resulted in us obtaining phospholipid modified scaffold **23** in acceptable yield (41%). During optimisation, switching the DPPE solvent system for a combination of chloroform and 2,6-lutidine greatly improved the yield and scalability of the reaction, allowing for amounts of 40-70 mg to be prepared during each attempt.

Scheme 6. Synthesis of DPPE and dye functionalized peptides WALP and dULC-ALM. a) 6-maleimidohexanoic-O-succinimide, K₂CO₃ b) i)NHS, DCC; ii) DPPE, 2,6 lutidine; c) Dye-Azide, TBTA, CuSO₃, Sodium ascorbate; d)WALP or dULC-ALM in TFE.

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

The functionalization of DPPE-modified linker **23** with TAMRA or Cyanine-3 (Cy3) dyes to respectively give the penultimate products **24** or **25** was fairly straightforward and needed little optimization with approximately 30% isolated yield for both compounds.

Functionalization with the peptide gave a clear single (brightly coloured and fluorescent) product on TLC, however, the desired final products **26** and **28** could not be identified. The remaining starting scaffold after completion of the reaction could be removed through trituration. However, due to the hybrid properties of the final product, it being poorly soluble in any solvent but trifluoroethanol, DMSO and DMF, it proved challenging to purify further despite multiple attempts via chromatography (HPLC/UPLC/Flash/(Prep-)TLC), crystallization or otherwise, meaning further studies are still needed for this product in order to obtain it in high purity and verify the correct structures were obtained.^[18]

An attempt was made with an alternative peptide, a cysteine-containing synthetic analogue of Alamethicin, dULC-ALM, another membrane-active short peptide. Reacting dULC-ALM with both scaffolds also resulted in the formation of a single new product by TLC. So far, no purification was established and the final constructs were ultimately not observed in High Resolution Mass Spectrometry (HRMS). However, the complete lack of starting material and the observed characteristics strongly imply the successful formation of the conjugates in a clean manner, albeit that additional investigations remain necessary.

Scheme 7. Synthesis of a tyrosine derived scaffold until the introduction of DPPE. a) propargyl bromide, K_2CO_3 ; b) HCl in MeOH (anhydrous) c) 6-maleimidoheptanoic acid, Et_3N , DIC; d) Me_3SnOH , DCE, reflux; e) i) NHS, DCC; ii) DPPE, 2,6-lutidine.

Simultaneously, a slightly shorter synthetic route, as when compared to the propargylglycine one, was attempted based on doubly protected Boc-tyrosine methyl ester following the same general strategy (Scheme 6). This amino acid is commercially available at acceptable prices. This route would save 3 steps and avoids an enzymatic resolution which is costly in terms of time and product losses).

Starting from protected Boc-tyrosine methyl ester **30**, it was first propargylated to **31** in good yield followed by a quantitative Boc-deprotection to give the corresponding free amine. Subsequently, 6-maleimido hexanoic acid was introduced using DIC as coupling agent, resulting in maleimido hexanoic amide **32** in moderate yields. After several attempts to hydrolyse the methyl ester, it turned out that most conventional methods were too aggressive and caused the decomposition of the material rather than hydrolysis. However, when using the Nicolaou ester hydrolysis reaction, which employs trimethyltin hydroxide, the free acid was obtained in good yields.^[19] DPPE was then introduced using the same protocol as in the previous section giving product **33** in moderate yields.

During the final steps (Scheme 7), Tamra and Cy3 were introduced, without any difficulties to give the penultimate products (**34** and **35**) in similar yields (30%) as those observed with the propargylamine strategy. Lastly, the final products were formed by reacting the respective scaffolds with either dULC-ALM or WALP-SH. Here again, the formation of a single new product with bright colouring and fluorescence was observed, assumed to be the desired product (**36-39**). However, as with the previous section, the product could not be identified using HRMS. The unreacted starting scaffolds could be removed by trituration, but a positive identification of the product, along with a chromatographic purification were left to be achieved later by De Vries and Syga.^[24]

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

Scheme 8. Synthesis of the final constructs based on Tyrosine as a scaffold. a) dye-azide, CuSO_4 , TBTA, sodium ascorbate; b) WALP or dULC-ALM, TFE.

In their work, De Vries and Syga managed to tackle the final challenge by adjusting the final part of the synthetic approach, by using a Sulfo-Cy3-containing linker **38**, obtained from intermediate **33** (Scheme 8). In their approach, the Sulfo-Cy3 azide was coupled to the palmitoylated linker **33** again using CuAAC, now with the better water-soluble Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA). With Sulfo-Cy3-labeled linker **38** in hand, the final conjugation with WALP was performed in TFE overnight, using DIPEA as an activating base. The resulting mixture could be filtered and subsequently be purified by HPLC, to finally obtain the trifunctional hybrid **39**, and thereby demonstrating the feasibility of this synthetic approach. Later in the article, they describe the favourable properties the hybrid displayed for localization in biological membranes, proving a trifunctionalized scaffold is a viable tool for these localization studies.

Scheme 9. Synthesis of final WALP and Sulfo-Cy3 conjugated scaffold **39** from scaffold **33** by De Vries and Syga.^[24]
a) Sulfo-Cy3 azide, CuSO₄, THPTA, TFE, H₂O, *t*-BuOH; b) WALP-SH, DIPEA, TFE.

5.4. Conclusion

In order to obtain a scaffold that can readily host three different functionalities with a minimum of synthetic work involved, two routes were explored. Using either

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

propargylglycine or tyrosine, and, in theory, straight forward and facile tri-orthogonal functionalization was attempted. While final purification and a full characterization of these four products is still to be achieved, the method shows good promise. These difficulties in purification are attributed to the challenging properties of the peptides introduced in the construct, rather than to the label itself: both peptides are known to be challenging to handle and purify even in their unmodified states. With the success of De Vries and Syga in synthesizing, characterizing and biologically testing a very similar construct **39**, based on palmitoylated intermediate **33**, the proof of principle has been provided that this method can be employed to assemble constructs varying in, dyes, peptides and lipids, with the key steps being straight-forward to perform, likely even outside of a synthetic lab, as was our objective.

5.5. Supplementary information

General Information

Unless noted otherwise, all reactions were carried out under nitrogen atmosphere. Reactions were monitored by TLC Silica 60 (Merck Millipore), examined under UV (365 nm and 254 nm), and stained by KMnO₄, ninhydrin, vanillin or H₂SO₄ in MeOH (1%). Chromatography was performed using the flash-protocols by Still *et al.* on Silica gel 60 (0.040-0.063 mm) from Merck Millipore.^[23]

Unless specified otherwise, all solvents and reagents were obtained commercially (Sigma Aldrich, Acros Chemicals and TCI Europe). dULC-ALM-F30 was obtained synthetically, as described in chapter 2. WALP-SH was obtained from Bachem. The solvents were used without any further purification, reagents were purified if required, following procedures described in "Purification of Laboratory Chemicals (sixth edition)."^[25]

The ¹H NMR spectra were recorded at 400 MHz and ¹³C NMR spectra were recorded at 100 MHz. The chemical shifts are reported in ppm relative to the residual solvent peak.

Analytical RP-HPLC was performed on a Shimadzu LC-10AD VP machine using XTerra MS C18 Column, 125Å, 3.5 µm, 4.6 mm X 100 mm

Semi-preparative RP-HPLC purification was performed on a Sunfire ODB C8 Prep Column, 100Å, 5 µm, 10 mm X 150 mm, with a flow rate of 2.0 mL/min @ 60°C

The solvent system was: A = 20% MeOH in iPrOH with 0.1% FA, B = 0.1% FA in H₂O (dd)

Absorbance was monitored at 210 nm, 254 nm and the absorption maximum of the respective dye (550 nm range) product percentages are given by peak areas at 210 nm for non-fluorescent compounds (e.g. Alamethicin F30), or at the absorption maximum (550 nm range) for dye containing compounds. For reasons of convenience, yields were based on the

molar absorptivity coefficient of the parent dye, not compensating for the presence of lipid, linker or the peptide (assumed to not absorb in that region).

Low-resolution mass spectra were recorded on a Waters XEvo LC-QTOF (ESI, pos/neg) using direct injection of the sample (bypass of the LC system).

High-resolution mass spectra were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode).

Experimental Procedures

Synthesis of 2-acetamido-diethylmalonate (**15**)^[15]

In a 500 mL three-neck flask equipped with a magnetic stirrer, 2-aminodiethylmalonate (10.18 g, 50 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (250 mL). Triethylamine (20.8 mL, 150 mmol, 3 equiv.) was added and the mixture cooled to 0°C . Acetyl chloride (3.5 mL, 50 mmol, 1 equiv.) was added using a syringe pump over 30 minutes. The resulting solution was stirred for an additional hour, after which it was washed with dilute HCl (2 x 200 mL, 1M), water (200 mL), saturated NaHCO_3 (200 mL) and brine (200 mL). The organic phase was then dried using Na_2SO_4 , filtered and concentrated *in vacuo*. The product was obtained as a white solid and used without further purification (9.65 g 44.5 mmol, 89%). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6): δ (ppm) 8.75 (d, $J = 7.4$ Hz, 1 H), 5.05 (d, $J = 7.5$ Hz, 1 H), 4.15 (m, 4H), 1.90 (s, 3H), 1.18 (t, $J = 7.0$ Hz, 6 H). $^{13}\text{C-NMR}$ (100 MHz, DMSO-d_6): δ (ppm) 169.9, 166.5, 62.8, 56.6, 24.0, 14.0.

Synthesis of 2-acetamido-2-propargyl-diethylmalonate (**16**)^[15]

2-acetamido-diethylmalonate **15** (5.4 g, 25 mmol, 1 equiv.) and EtONa (2.2 g, 32 mmol, 1.25 equiv.) were dissolved in freshly dried, distilled EtOH (200 mL) in a 500 mL three-neck flask under a nitrogen atmosphere and stirred for 30 minutes. Freshly distilled propargyl bromide (3.2 mL, 32 mmol, 1.25 equiv.) was added in 10 minutes using a syringe pump, after which the solution was brought to reflux for 4 hours, until TLC indicated complete consumption of the starting material. The solution was concentrated *in vacuo* and subsequently dissolved in Et_2O (150 mL), washed with saturated ammonium chloride (100 mL), NaHCO_3 (100 mL), water (100 mL) and brine (100 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*, to obtain the crude product as a white powder. Recrystallization using Et_2O /pentane over three crops afforded the product as fluffy white needles (5.01 g, 19.64 mmol, 79%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ (ppm) 6.94 (s, 1H), 4.25 (m, 4H), 3.26 (d, $J = 2.7$ Hz, 2H), 2.05 (s, 3H), 1.96 (t, $J = 2.7$ Hz, 1H), 1.25 (t, $J = 7.2$ Hz, 6H); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ (ppm) 169.5, 166.4, 78.3, 71.7, 66.0, 63.4, 24.1, 23.2, 14.3.

Synthesis of 2-acetamido-2-propargylmalonic acid (**17**)^[15]

2-acetamido-2-propargyl-diethylmalonate **16** (2.5 g, 10 mmol, 1 equiv.) was added to a solution of LiOH (1 g, 40 mmol, 4 equiv.) in water (100 mL, 0.4 M) and heated under reflux for 17 h. The resulting clear solution was acidified to pH ≤ 2 using 1M HCl (aq), and subsequently extracted with EtOAc (3 x 150 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The product was obtained as a

brittle, hard and white solid and was used without further purification (1.9 g, 9.5 mmol, 95%). ¹H-NMR (400 MHz, DMSO-d₆): δ (ppm) 8.10 (s, 1H), 3.01 (d, J = 2.7 Hz, 2H), 2.84 (t, J = 2.7 Hz, 1H), 1.91 (s, 3H). ¹³C-NMR (100 MHz, DMSO-d₆): δ (ppm) 173.2, 173.1, 79.9, 71.9, 53.0, 22.5, 22.4.

Synthesis of *rac*-N-acetyl propargylglycine (**18**)^[15]

2-acetamido-2-propargylmalonic acid **17** (1 g, 5 mmol, 1 equiv.) was dissolved in water (25 mL) and heated under reflux for 3 h. The solution was acidified to pH ≤ 1 using 1M HCl (aq) and extracted with EtOAc (5 x 50 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. Recrystallisation of the crude product from

CHCl₃/pentane afforded the final product as white thin needles (640 mg, 4.1 mmol, 82%). ¹H-NMR (400 MHz, MeOD): δ (ppm) 8.05 (br.s, 1H), 4.53 (t, J = 6.2 Hz, 1H), 2.69 (dd, J = 6.2 Hz, J = 2.7 Hz, 2H), 2.35 (t, J = 2.7 Hz, 1H), 1.99 (s, 3H); ¹³C-NMR (100 MHz, MeOD): δ (ppm) 173.3, 169.0, 82.9, 71.5, 50.7, 30.2, 22.3.

Synthesis of L-propargylglycine (**L-11**)

Racemic *N*-acetyl propargylglycine **18** (1 g, 6.45 mmol, 1 equiv.) was dissolved in dd H₂O (70 mL) and neutralized with aqueous LiOH (1 M) to pH 7.5-8.0, by digital pH-meter. Acylase I (5.2 mg; 8.3 U/mmol of acetyl L-amino acid – follow instructions on the package) was added and the resulting mixture stirred at 37°C for 24 h in the oven (water bath works

too). The mixture was then adjusted to pH = 5.0 by dropwise addition of 1M HCl (aq) and filtered over fresh activated charcoal. The filtrate was acidified to pH ≤ 2.0 and extracted with EtOAc (6 x 30 mL). The resulting aqueous layer was concentrated *in vacuo* to 1-2 mL and applied to a Dowex-50 (H⁺) column. The column was rinsed with dd H₂O until the outflow was of neutral pH, after which the column was eluted with 10% NH₄OH (aq). The resulting solution was concentrated *in vacuo* and subsequently crashed with acetone to yield the product as a hygroscopic white solid (310 mg, 2.7 mmol, 42%). ¹H-NMR (400 MHz, MeOD): δ (ppm) 3.49 (dd, J = 7.4 Hz, J = 4.3 Hz, 1H), 2.75 (ddd, J = 17.2, J = 4.3, J = 2.7, 1H), 2.62 (ddd, J = 17.2, Hz, J = 7.4 Hz, J = 2.7 Hz, 1H), 2.43 (t, J = 2.7 Hz, 1H); ¹³C-NMR (100 MHz, MeOD): δ (ppm) 169.8, 77.4, 75.6, 52.1, 22.1; LCMS (ESI-POS): C₅H₈NO₂ [M+H]⁺: 114.05 m/z, found: 114.05 m/z.

Synthesis of L-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)pent-4-ynoic acid (22)

Propargylglycine **L-11** (260 mg, 2 mmol, 1 equiv.) and K_2CO_3 (500 mg, 4 mmol, 2 equiv.) were dissolved in dd H_2O (4 mL, 0.5M) under a nitrogen atmosphere in a 15 mL Schlenk flask equipped with a magnetic stirrer. 6-maleimido hexanoic acid

N-hydroxysuccinimide ester (680 mg, 2.4 mmol, 1.2 equiv.) in 1,4 dioxane (2 mL) was added and the resulting mixture stirred for 2 hours. The mixture was then acidified to pH = 1 using 1M HCl (aq) and extracted with EtOAc (5 x 20 mL). The organic phase was dried over Na_2SO_4 , filtered and concentrated *in vacuo*. Flash chromatography of the crude (silica, 5% EtOH in CH_2Cl_2 to 15% in 2 steps) afforded the product as a clear oil that later solidified to form white brittle crystals (310 mg, 1 mmol, 50%). 1H -NMR (400 MHz, $CDCl_3$): δ (ppm) 6.67 (s, 2H), 6.43 (d, $J=7.42$ Hz, 1H) 4.70 (dt, $J=7.62$, 4.78 Hz, 1H), 3.49 (t, $J=7.22$ Hz, 2H), 2.77 - 2.82 (m, 2H), 2.26 (t, $J=7.62$ Hz, 2H), 2.04 - 2.08 (m, 1H), 1.53 - 1.72 (m, 4H) 1.31 (quin, $J=7.71$ Hz, 2H); ^{13}C -NMR (100 MHz, $CDCl_3$): δ (ppm) 173.5, 173.2, 170.9, 134.1, 113.1, 78.29, 71.9, 37.6, 36.1, 28.2, 26.1, 24.9, 22.0. HRMS (ESI pos), calculated for $C_{15}H_{19}N_2O_5$ $[M+H]^+$: 307,12885, found: 307.12896.

Synthesis of (2S)-3-(((2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)pent-4-ynamido)ethoxy)(hydroxy)phosphoryl)oxy)propane-1,2-diyl dipalmitate (23)

2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)pent-4-ynoic acid **22** (92 mg, 0.3 mmol, 3 equiv.) was dissolved in dry THF (3 mL) in a 10 mL Schlenk flask under an argon atmosphere. N-hydroxysuccinimide (41 mg, 0.4 mmol, 4 equiv.) and dicyclohexylcarbodiimide (75 mg, 0.36 mmol, 3.6 equiv.) were added and the mixture was stirred for 4h until the starting material was fully consumed as judged by TLC. The mixture was then filtered over celite to remove the white precipitate and concentrated to about 1 mL in volume. A mixture of DPPE (70 mg, 0.1 mmol, 1 equiv.) in chloroform (4 mL) was made using a few drops of 2,6-lutidine to aid the dissolving process (repeated heating/sonication cycles were required, even with the added base – typically the process will take half an hour before a clear and stable solution was obtained). After cooling down the DPPE-solution to

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

room temperature, it was placed in a sonicating bath before the concentrated solution containing the O-succinimide was added drop by drop, making sure the solution did not precipitate. If the new solution remained clear after addition, it was left to stir for 12-15 h. The mixture was then diluted to 10 mL using additional chloroform and washed with 0.1 M HCl (10 mL), water (10 mL) and brine (10 mL). The organic layer was then dried over Na₂SO₄, decanted and dried *in vacuo*, to obtain the crude as a yellow oil. Flash chromatography (silica, MeOH/CH₃Cl/NH₃ 5/95/0.1 to 20/80/0.1 in 3 steps) afforded the product as a clear oil that later dried into a glassy film (41 mg, 0.042 mmol, 41%). ¹H-NMR (400 MHz, CDCl₃): δ (ppm) 7.62 (br. s., 1H), 6.91 (d, J=7.0 Hz, 1H), 6.68 (s, 2H), 5.22 (br. s., 1H), 4.61 (d, J=5.5 Hz, 1H), 4.34 (dd, J=11.9, 3.3 Hz, 1H), 4.00 - 4.22 (m, 4H), 3.60 (br. s., 1H), 3.49 (t, J=7.2 Hz, 2H), 2.62 - 2.90 (m, 2 H), 2.20 - 2.40 (m, 6H), 2.08 - 2.15 (m, 1H), 1.49 - 1.74 (m, 8H), 1.02 - 1.43 (m, 52H), 0.79 - 0.94 (m, 6H); ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 173.4, 173.0, 134.1, 61.9, 34.2, 34.0, 37.9, 29.7, 29.5 (mult.), 29.4 (mult.), 29.3 (mult.), 29.2, 28.2, 26.1, 24.9, 22.7, 14.1; HRMS (ESI POS) Calculated C₅₂H₉₁N₃O₁₂P [M+H]⁺: 980.63349, found: 980.62959. Retention time (HPLC): 43-45 min.

NB. the exact number of C in the palmitate groups could not be determined using ¹³C-NMR – the peaks labelled ‘mult.’ indicate higher than normal intensities.

Synthesis of *N*-Boc-*O*-propargyl-L-Tyrosine methyl ester (30)

Boc-L-tyrosine methyl ester (6 g, 20 mmol, 1 equiv.) was dissolved in dry DMF (100 mL, 0.2 M), K₂CO₃ (11 g, 80 mmol, 4 equiv.) was added and the suspension was stirred vigorously. Propargylbromide (80% in toluene, 5.2 mL, 40 mmol, 2 equiv.) was added and the resulting mixture stirred for 6h. The solution was concentrated to approx. 15 mL *in vacuo* and diluted with EtOAc (100 mL). The organic phase was then washed with saturated ammonia (100 mL), water (100 mL), 3M LiCl (3 x 100 mL) and brine (100 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*.

Flash chromatography of the crude product (silica, 10% EtOAc in heptane) afforded the product as a white solid (5.84 g, 17.5 mmol, 88%). ¹H-NMR (300 MHz, CDCl₃) δ (ppm) 7.05 (d, J=8.7 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H), 4.94 (br. s., 1H), 4.66 (d, J=2.1 Hz, 2H), 4.54 (br. s., 1H), 3.71 (s, 3H), 2.89 - 3.12 (m, 2H), 2.51 (t, J=2.3 Hz, 1H), 1.41 (s, 9H); ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 173.0, 157.0, 155.5, 130.7, 129.3, 115.1, 80.0, 78.6, 75.5, 55.8, 54.5, 52.1, 37.4, 28.1.

Synthesis of *O*-propargyl-L-Tyrosine methyl ester HCl salt (**30B**)

N-Boc-*O*-propargyl-L-Tyrosine methyl ester **30** (5 g, 15 mmol, 1 equiv.) was dissolved in anhydrous MeOH (50 mL) under a nitrogen atmosphere. Anhydrous HCl in MeOH was generated by slowly adding Acetyl chloride (2.5 mL, 40 mmol, 2.4 equiv.) to dry methanol (20 mL) cooled to 0°C. Upon completion of the acetyl chloride addition, the HCl solution was added via cannula to the Boc-tyrosine solution and left to stir for 30 min. The mixture was then concentrated *in vacuo*, and triturated with CHCl₃ (3 x 50 mL), to afford the pure product as a white fluffy powder (4 g, 15 mmol, quant.). ¹H-NMR (300 MHz, DMSO-*d*₆) δ (ppm) 7.10 (d, *J*=8.7 Hz, 2H), 6.93 (d, *J* = 8.7 Hz, 2H), 4.94 (br. s., 1H), 4.66 (d, *J*=2.1 Hz, 2H), 4.54 (br. s., 1H), 3.75 (s, 3H), 2.95 - 3.18 (m, 2H), 2.49 (t, *J*=2.3 Hz, 1H).

Synthesis of methyl (S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoate (**31**)

O-propargyl-L-tyrosine methyl ester HCl salt **30B** (1 g, 4 mmol, 1 equiv.) and 6-maleimidohexanoic acid (1.3 g, 6 mmol, 1.5 equiv.) were dissolved in CH₂Cl₂ (50 mL) at 0 °C, diisopropylethylamine (1.7 mL, 10 mmol, 2.5 equiv.) was added, followed by diisopropylcarbodiimide (0.95 mL, 6 mmol, 1.5 equiv.) and a single crystal of DMAP. After 30 min, the reaction was allowed to warm to

room temperature, after which the reaction was stirred for an additional 3 h, until full consumption of the starting material was observed by TLC. The mixture was filtered over a sintered glass filter (P4) and subsequently diluted with CH₂Cl₂ (50 mL) washed with 1M HCl (100 mL), saturated NaHCO₃ (100 mL) and brine (100 mL). The organic phase was then dried over Na₂SO₄, filtered and concentrated *in vacuo*. Flash Chromatography (silica, 50% Et₂O in heptane) afforded the product as a clear oil (860 mg, 2 mmol, 50%). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 7.01 (d, *J*=8.6 Hz, 2H), 6.90 (d, *J*=8.6 Hz, 2H), 6.68 (s, 2H), 5.85 (d, *J*=7.4 Hz, 1H), 4.81 - 4.88 (m, 1H), 4.66 (d, *J*=2.3 Hz, 2H), 3.72 (s, 3H), 3.49 (t, *J*=7.2 Hz, 2H), 3.06 (ddd, *J*=25.0, 13.7, 5.9 Hz, 2H), .52 (t, *J*=2.3 Hz, 1 H) 2.16 (t, *J*=7.4 Hz, 2 H) 1.60 (mult., 4H), 1.22 - 1.32 (m, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 172.1, 170.8, 156.7, 134.04, 130.3, 128.8, 115.0, 78.5, 75.5, 55.8, 53.0, 52.3, 37.6, 37.0, 36.2, 28.2, 26.23, 24.9. HRMS (ESI-POS): calculated for C₂₃H₂₆N₂O₆ [2M+Na]⁺: 875.5728, found: 875.5788 [2M+Na]⁺

Synthesis of (S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoic acid (32**)**

Methyl (R)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoate (430 mg, 1 mmol, 1 equiv.) **31** was dissolved in DCE (5 mL, 0.1 M) in a sealed tube. Me₃SnOH (360 mg, 2 mmol, 2 equiv.) was added and the solution was heated under reflux (80°C) for 2-3 h, until complete consumption of the starting material based on TLC. The solution was then

concentrated *in vacuo* and flash chromatography (silica, 8% EtOH in CH₂Cl₂) afforded the product as a clear oil that solidifies to a white solid over the course of several days (353 mg, 0.86 mmol, 86%). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 7.09 (d, J=8.7 Hz, 2H), 6.91 (d, J=8.7 Hz, 2H) 6.68 (s, 2H) 6.12 (d, J=7.2 Hz, 1H) 4.84 (dd, J=11.8, 5.6 Hz, 1H) 4.66 (d, J=2.1 Hz, 2H) 3.48 (t, J=7.2 Hz, 2H) 3.12 (ddd, J=33.8, 14.4, 5.1 Hz, 2H) 2.53 (s, 1H) 2.19 (t, J=7.4 Hz, 2H) 1.50 - 1.67 (m, 4H) 1.20 - 1.33 (m, 3H); ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 174.3, 173.5, 170.9, 156.7, 134.1, 130.4, 128.7, 115.0, 105.0, 78.6, 75.6, 55.8, 53.2, 37.5, 36.1, 28.1, 26.1, 24.9;

Synthesis of (2S)-3-(((2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanamido)ethoxy)(hydroxy)phosphoryl)oxy)propane-1,2-diyl dipalmitate (33**)**

(R)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoic acid **32** (100 mg, 0.25 mmol, 2 equiv.) was dissolved in freshly distilled THF (3 mL) in a 10

mL Schlenk flask under an argon atmosphere. N-hydroxysuccinimide (40 mg, 0.35 mmol, 2.6 equiv.) and dicycolhexylcarbodiimide (65 mg, 0.3 mmol, 2.4 equiv.) were added and the mixture was stirred for 2h until the starting material was completely consumed as judged by TLC. The mixture was then filtered over celite to remove the white precipitation and condensed to about 1 mL in volume. While stirring the first solution, a second mixture of

DPPE (50 mg, 0.08 mmol, 1 equiv.) in chloroform (4 mL) was made using a few drops of 2,6-lutidine to aid the dissolving process (repeated heating/sonication cycles were required). After cooling down the DPPE-solution to room temperature, it was placed in a sonicating bath before the concentrated solution containing the O-succinimide was added dropwise, making sure the solution did not precipitate. If the new solution remained clear after addition, it was left to stir for 20 h. The mixture is then diluted to 10 mL using additional chloroform and washed with 0.1 M HCl (10 mL), water (10 mL) and brine (10 mL). The organic layer was then dried over Na₂SO₄, decanted and dried *in vacuo*, to obtain the crude as a yellow oil. Flash chromatography (silica, MeOH/CH₃Cl/NH₃ 5/95/0.1 to 20/80/0.1 in 3 steps) afforded the product as a clear oil that later dried into a glassy film (43 mg, 0.04 mmol, 53%). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 7.14 (d, J=8.2 Hz, 2H) 6.84 (d, J=8.2 Hz, 2H) 6.69 (s, 2H) 5.17 (br. s., 1H) 4.83 (br. s., 1H) 4.64 (s, 2H) 4.33 (d, J=10.8 Hz, 1H) 3.66 - 4.12 (m, 5H) 3.47 (s, 2H) 3.39 (t, J=6.9 Hz, 2H) 3.01 - 3.12 (m, 1H) 2.82 (br. s., 1H) 2.53 (s, 1H) 1.98 - 2.38 (m, 9H) 1.17 - 1.59 (m, 59H) 1.06 (br. s., 2H) 0.87 (t, J=6.4 Hz, 6H) ¹³C-NMR (75 MHz, CDCl₃) (indicative peaks): δ (ppm) 134.1, 130.25, 58.9, 55.7, 34.0, 31.9, 29.7, 29.4, 29.2, 24.8, 22.7, 14.11. HRMS (ESI-pos): calculated for C₅₉H₉₇N₃O₁₃P [M+H]⁺: 1086.6754, found: 1086.6739. Retention time (HPLC): 42 min.

General procedure for the incorporation of the photoactive dye label using a copper-catalysed Alkyne-Azide click-reaction (example: TAMRA)

The appropriate alkyne (5 μmol, 1 equiv.) and Tamra-azide (5 mg 10 μmol, 2 equiv.) were added to a stained (brown) HPLC-vial (1.5 mL) equipped with a screw-cap and solubilized using a mixture of THF/tBuOH (0.5 mL, 9:1 v/v). A mixture of CuSO₄ (9 mg, 0.06 mmol) and TBTA (53 mg, 0.1 mmol) in dd H₂O/tBuOH (20 mL 1:19 v/v) was prepared simultaneously and a small quantity (approx. 0.2 mL) of the 'click mix' was added to the reaction vial. Finally, sodium ascorbate (3 mg, 15 μmol, 3 equiv.) was added, and the mixture agitated for 24 h. The mixture was then passed through a 10 μm filter and purified using semi-preparative HPLC (60% A in B to 90% A over 20 minutes, then 100% A in 1 minute, for 10 minutes). The product was obtained as a purple film after lyophilisation.

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

Synthesis of Propargylglycine-based linker with DPPE and Tamra (24)

Maleimido-hexyl (dipalmitoyl-glycerol-phosphatidyl)-ethanolamide modified propargylglycine **23** (5 mg, 5 μ mol, 1 equiv.) was treated according to the above procedure, resulting in a brittle purple film (2.4 mg, 1.6 μ mol, 32%). HRMS: HPLC retention time: 38-40 min (broad). HMRS (ESI-Pos) Calculated for $C_{80}H_{119}N_9O_{16}P$ $[M+H]^+$: 1492.8507, found 1492.8442.

Synthesis of Tyrosine-based linker with DPPE and Tamra (34-a)

Maleimido-hexyl (dipalmitoyl-glycerol-phosphatidyl)-ethanolamide modified O-propargyloxytyrosine **33** (5 mg, 4 μ mol, 1 equiv.) was treated according to the above procedure, resulting in a brittle purple film (3.1 mg, 1.9 μ mol, 38%). HRMS: HPLC retention

time: 40-43 min (broad). HMRS (ESI-Pos) Calculated for $C_{87}H_{125}N_9O_{17}P$ $[M+H]^+$: 1598.8926 m/z , found: 1598.9074 m/z .

Synthesis of Propargyl-based linker with DPPE and Cy3 (25)

Maleimidoethyl (dipalmitoyl-glyceryl-phosphatidyl)-ethanolamide modified propargylglycine (1.2 mg, 1 μ mol 1 equiv.) **23** was treated according to the above procedure, now using Cy3 azide (approx. 1 mg, 2 μ mol, 2 equiv.) dissolved in tBuOH/DMSO (0.2 mL, 3:1 v/v). After purification, the product was recovered as a lilac/purple film in trace quantities (100-200 μ g, 0.1 μ mol, 10%). HPLC retention time: 29-32 min (broad. HMRS: Chemical Formula: $C_{86}H_{134}N_8O_{13}P^+$ $[M+H]$, Calculated: 1517.9803 m/z , observed 1539.9796 $[M+Na]$.

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

Synthesis of Tyrosine-based linker with DPPE and Cy3 (35a)

Maleimidohexyl (dipalmitoyl-glyceryl-phosphatidyl)-ethanolamide modified propargylglycine (1.2 mg, 1 μ mol 1 equiv.) **33** was treated according with the above procedure, now using Cy3 azide (approx. 1 mg, 2 μ mol, 2 equiv.) dissolved in tBuOH/DMSO (0.2 mL, 3:1 v/v). After purification, the product was recovered as a lilac/purple film in trace quantities (400-600 μ g, 0.3 μ mol, 30%). HPLC retention time: 31-35 min. HMRS: Chemical Formula: $C_{92}H_{139}N_9O_{14}P^+$ [M+H], calculated: 1625.01736 m/z, observed 1647.03018 [M+Na].

General procedure for peptide-dye-phospholipid hybrids on a single scaffold.

The appropriate scaffold was dissolved in degassed trifluoroethanol (so that the concentration was approximately 100 μ M) under inert conditions, protected from light. The peptide (100 nmol, 2 equiv.) was dissolved in trifluoroethanol and degassed as well – the scaffold (500 μ L, 50 nmol, 1 equiv.) was then added to the peptide and the mixture was gently agitated under inert conditions and shielded from light for the next 48 h. The mixture was subsequently precipitated using freshly distilled THF. The residue was then taken up again in a minimum of trifluoroethanol and the cycle was repeated up to three times in order to remove all unreacted linker from the mixture and obtain the product as a mixture with unreacted peptide – HPLC-purification proved to be impossible to date.

WALP derivatives:

WALP-Propargyl-TAMRA-DPPE: 26

A bright purple and fluorescent spot developed at $\sim$ rF 0.05 (silica, 100% trifluoroethanol) – the unreacted fluorescent scaffold would move to rF 0.80

HRMS (ESI-Pos): calculated for $C_{212}H_{316}N_{38}O_{42}PS^+$ $[M+H]^+$: 4129,32122, not observed.

WALP-Propargyl-Cy3-DPPE: 28

A lilac (green/yellow fluorescing) spot developed at the baseline in TLC (silica, 100% TFE). The unreacted fluorescent scaffold would move to r_F 0.65.

HRMS (ESI-Pos): calculated for $C_{218}H_{331}N_{37}O_{39}PS^+$ $[M+H]^+$: 4154,45078, no resolved target peak observed. Some above-baseline activity was observed in the range of 1385-1410 m/z , but nothing that could be considered a true peak (with at least $s/n = 3$).

WALP-tyrosine-TAMRA-DPPE: 34

A bright purple and fluorescent spot developed at $\sim r_F$ 0.05 (silica, 100% trifluoroethanol).

HRMS (ESI-Pos): calculated for $C_{219}H_{322}N_{38}O_{43}PS^+$ $[M+H]^+$: 4235,36364, not observed.

WALP-tyrosine-Cy3-DPPE: 36

A lilac (green/yellow fluorescing) spot developed at the baseline in TLC (silica, 100% TFE).

HRMS (ESI-Pos): calculated for $C_{224}H_{336}N_{38}O_{40}PS^+$ $[M+H]^+$: 4261,48789, no resolved target peak observed.

dULC-ALM-F30 derivatives:

dULC-ALM-F30 -Propargyl-TAMRA-DPPE 27

A bright purple and fluorescent spot developed at $\sim r_F$ 0.10 (silica, 100% trifluoroethanol).

HRMS (ESI-Pos): calculated for $C_{191}H_{305}N_{33}O_{42}PS^+$ $[M+H]^+$: 3796,21977, no resolved target peak observed; a “bump” in the region of 1275-1290 was observed, which would coincide with the $M+3Na$ or $M+H+2Na$ $3+$ ion.

dULC-ALM-F30 -Propargyl-Cy3-DPPE 29

A lilac (green/yellow fluorescing) spot developed at the baseline in TLC (silica, 100% TFE).

HRMS (ESI-Pos): calculated for $C_{197}H_{319}N_{32}O_{39}PS^+$ $[M+H]^+$: 3820,33443, no resolved target peak observed.

dULC-ALM-F30 -tyrosine-TAMRA-DPPE 35

A bright purple and fluorescent spot developed at $\sim r_F$ 0.10 (silica, 100% trifluoroethanol).

HRMS (ESI-Pos): calculated for $C_{198}H_{311}N_{33}O_{43}PS^+$ $[M+H]^+$: 3902,26164, no resolved target peak observed. A broad peak was found in the 1310-1330 m/z region, indicating potential presence of a $3+$ ion of the desired mass.

– *Synthesis and evaluation of a trifunctional scaffold for peptide-dye-phospholipid hybrids.* –

dULC-ALM-F30 -tyrosine-Cy3-DPPE 37

A lilac (green/yellow fluorescing) spot developed at the baseline in TLC (silica, 100% TFE).

HRMS (ESI-Pos): calculated for $C_{203}H_{325}N_{33}O_{40}PS^+$ $[M+H]^+$: 3928,386453, no resolved target peak observed.

5.6. References

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Chapter 6 – An efficient synthesis of electron-rich aminomethyl azobenzenes

6.1. Introduction

Molecular switches are molecules that can reversibly interconvert between two or more stable conformations upon the action of an external stimulus. This switching behaviour has been of interest in diverse fields of research for decades, especially when it appears, or is introduced into, small organic molecules and (bio-)polymers. The ability to change the physico-chemical properties of a molecule in-situ, instead of through the application of extensive chemical transformation, by only using ‘simple’ or non-invasive modifiers such as light, heat or a messenger molecule, opens up a range of potential applications from engineering to medicine.^[1-3]

The azobenzene moiety is an interesting candidate to explore in this respect and has been extensively studied as such. The molecule itself, for example, already serves in industry as a stabilizer in plastics and as a component of ionic liquids (liquid crystals) and displays excellent switching capabilities under mild conditions (i.e. visible light switching).^[4-7]

In her 2011 review on the topic of synthesizing azobenzenes, Merino describes in great detail the most common synthetic methods currently in use, none of which appear to have been evaluated towards electron-rich azobenzenes. As electron rich azobenzenes allow for different synthetic strategies than the corresponding electron poor ones by which they could be integrated into larger constructs, it would be interesting to investigate these options further.

Of particular interest are the aminomethyl-functionalized azobenzenes, due to the many available methods in which a primary amine can be utilized in functionalization strategies.^[8] The choice of aminomethyl-functionalization over a simple aniline group is that an aromatic amine will be less nucleophilic in comparison and would probably be insufficient to be effective in labelling studies.

– An efficient synthesis of electron-rich aminomethyl azobenzenes –

Scheme 10. Attempted synthesis of aminomethylazobenzene based on patent literature.^[11]

Of the few known examples in literature, the symmetric 4,4'-aminomethyl-azobenzene that was reported by Liu *et al.*^[9] and an azobenzene-derived amino acid was reported by Sewald *et al.*^[10] However, they didn't share a common synthetic route applicable to a wider range of variants. 4-aminomethyl-azobenzene itself exists in patent literature which described the synthesis of this particular compound.^[11] However, reproduction of their results proved to be problematic as the reduction of amide **3** to the target amine **4** invariably resulted in the loss of the azo-bond and other side-reactions.^[11,12] We therefore decided to investigate a novel route to these compounds with the goal of applying it in further bioriented studies, for example those investigated in the first chapters of this manuscript.

6.2. Results and discussion

A route was proposed which would go from commercial 4-nitrobenzylalcohol **5** to the corresponding nitroso compound (**6**, Scheme 2), which would make good branching point for generating a library of various azobenzenes.^[13] Subsequently 4-nitrosobenzyl alcohol **6** was to be converted to azide **9** in three steps. The azide, which on its own could be employed in various different reactions,^[14,15] is then to be reduced to the methylamino-functionalized azobenzene **4**.^[16,17] Although it can be argued that the intermediate alcohols discussed in this chapter could be synthesized more effectively than by using these methods, the synthesis of the initial compounds was chosen based on convenience and proof of principle, rather than efficiency of the reaction itself and will not be discussed in further detail.^[8]

Scheme 11. Synthesis of azidomethyl azobenzene **9** in 5 steps from 4-nitrobenzylalcohol **5**. a) i) Zn(0), NH₄Cl, EtOH/H₂O ii) Fe(III)Cl₃, H₂O; b) aniline, AcOH, EtOH; c) MsCl, Et₃N, DMF; d) NaN₃, DMF.

#	Phosphine	Solvent	Additive	Time (h)	Yield (%)
1	PPh ₃	Toluene	-	18 ¹	SM recovered
2	PPh ₃	THF	-	18 ¹	SM recovered
3	PPh ₃	THF/H ₂ O	-	18 ¹	SM recovered
4	PBu ₃	THF	-	0.5	Decomp.
5	PBu ₃	THF/H ₂ O	-	0.5	Decomp.
6	TCEP	THF/ H ₂ O	-	2	40%
7	PBu ₃	THF/ H ₂ O	-	2	70%
8	PBu ₃	THF/ H ₂ O	HCl	2	85%

Table 2. screening and optimization of the Staudinger reduction of azidomethyl azobenzene **9** to aminomethylazobenzene **4**. THF/H₂O (95:5 v/v) ¹⁾ reaction performed at reflux

4-nitrosobenzylalcohol **6** was readily synthesized in accordance with literature,^[13] although it should be noted that its quality varies between batches as the compound appears to be sensitive to temperature. Hydroxymethyl azobenzene **7** was subsequently obtained in good yield. The sensitivity of azobenzene **7** towards residual acetic acid in the crude mixture was remarkable and could result in decomposition before chromatography purification could be attempted if the aqueous treatment of the reaction was not rigorous enough to remove all traces of acid.

The transformation of alcohol **7** to the corresponding azide **9** in two steps occurred in (near) quantitative yields. However, the subsequent Staudinger reduction using standard conditions afforded the product only in trace quantities. It became apparent that triphenylphosphine was poorly reactive towards these products (table 1, entry 1-3), whereas tributylphosphine gave mainly decomposition at various temperatures (table 1, entry 4-5). When TCEP was used as reducing agent, the reaction afforded moderate yields of the desired product (40%). This singular behaviour of TCEP was an interesting case which drew our attention, as its behaviour in a Staudinger reduction should be similar to tributylphosphine as the terminal carboxyl groups in principle only serve to solubilize TCEP in water and are not meant to strongly influence the central phosphorous atom. This supposed similarity in behaviour is also touched upon by Nepomniaschij and Arndt in their respective solutions to a similar problem.^[18,19]

After further investigations, it was hypothesised that TCEP could form a salt with the newly formed amine, causing it to precipitate from the reaction media and thereby avoiding degradation during the treatment of the reaction. This hypothesis was confirmed when the reductions with tributylphosphine were repeated but this time in the presence of HCl added

– An efficient synthesis of electron-rich aminomethyl azobenzenes –

either during workup (entry 7) or during the reaction itself (entry 8), the latter producing the best results. After further optimization, aminomethylazobenzene **4** could be obtained reliably in good yields as the HCl-salt.

Encouraged by these promising results, we turned our attention to the scope of the reaction as we hoped to be able to obtain a wide variety of derivatives, hence obtaining one of the first truly general and straightforward methods for the synthesis of aminomethyl azobenzenes without the need for extensive protection/deprotection steps. We were also interested in the obtained intermediates as we believed they could be of use as well.

#	R	Isolated yield (%)
10	-Me	47%
11	-OMe	38%
12	NH ₂	27%
13	-CH ₂ OH	10%
14	-OH	58%
15	-CH ₂ OTBS	27%
16	-COMe	25%
17	-SMe	19%
18	-CH ₂ COOMe	27%

Table 3. synthesis of a library of azobenzenes from 4-nitrosobenzylalcohol. 1) i) Zn(0), NH₄Cl; ii) Fe(III)Cl₃; 2) Aniline-derivative, AcOH.

To this end, various functional groups were added to the 4-nitrosobenzylalcohol core and these aniline derivatives were submitted to the reaction conditions (Table 2). We chose to limit our investigations to this ring due to time constraints and due to our reasoning that this position would be the one where modifications would be the most interesting for further use. Overall, the products were obtained in moderate yields, although no attempts were made to optimize these reactions. Alkyl and alkyl ether substituents on the aniline gave the best yields (38-47%) while free amines, silyl ethers, esters, thioethers and ketones gave moderate yields of about 19-27%. Diol **13** was obtained as an inseparable mixture of E/Z isomers in the ratio 3:1 (by NMR). 4-aminobenzyl alcohol gave a poor yield. The reaction with thioaniline was attempted as well, but did not give the desired product. This behaviour can most likely be attributed to its redox potential in relation to the nitrosobenzylalcohol. In general, again the sensitivity of the desired azobenzenes towards trace acetic acid after the initial workup was remarkable, to the point where we decided to modify our protocol so that

the pH of the solution after treatment of the reaction mixture was slightly basic instead of neutral (pH = 8-10). This had no significant effect on the initial reaction yield but would allow for more time to pass between the end of the reaction and purification, while also making for an easier purification due to fewer side-products.

#	-R	Isolated yield (%)
19	-Me	98%
20	-OMe	96%
21	-NH ₂	n.d.
22	-CH ₂ OH/-CH ₂ N ₃	n.d.
23	-CH ₂ OTBS	94%
24	-COMe	n.d.
25	-SMe	96%
26	-CH ₂ COOMe	81%

Table 4. Synthesis of azidomethyl azobenzene derivatives in 2 steps with modified protocol. 1) MsCl, Et₃N; 2) NaN₃

The conversion of the alcohols to their corresponding azides occurred in (near) quantitative yields in most cases.^[Note 1] Indeed, for the derivatives bearing alkyl, ether or ester substituents, the method gave 80-98% yields. Only amino-azidomethyl azobenzene **21**, acetyl functionalized azidomethyl azobenzene **24** and diazide **22** were not obtained due to degradation of the product. For the former a protecting group on the nitrogen may be required for the reaction to work as the free amine appeared highly unstable. Sadly, due to time constraints this could not be investigated further, however we hypothesise that a Boc protecting group would be the preferred initial candidate, as it could be removed during the reduction step in the presence of HCl to immediately block the amine from decomposing without further synthetic effort, making this a three-step procedure, installing it prior to the mesylation.

Scheme 12 Synthesis of two aminomethylazobenzene derivatives - PBu₃, HCl, THF;

The final conversion to the amine was attempted on two compounds: methyl-functionalized azidomethyl azobenzene **19** and methoxy-functionalized azidomethyl azobenzene **20** and appeared to work accordingly in both instances, with moderate yields for both methyl functionalized aminomethylazobenzene **27** and methoxy functionalized aminomethylazobenzene **28**. However, further investigation into these reactions is in order before a definitive conclusion regarding the scope and the application of this approach can be reached.

6.3. Conclusion

In conclusion, a new promising synthesis towards electron rich aminomethyl azobenzenes was developed for the future use in polymers and bio-constructs such as pore forming peptides. The synthetic route offers much versatility and allowed us to obtain several previously undocumented azobenzenes from azobenzylalcohol, in good overall yields. From these could be obtained versatile azide bearing intermediates which can potentially undergo several transformations of interest for both biological and synthetic applications. This included obtaining, in a straightforward manner, the corresponding amines as was demonstrated by the synthesis of compounds **27** and **28**. Also, this route may potentially be employed in an Aza-Wittig reaction, although this line was not investigated so far. Both these functional groups could in principle be used to attach these switches to the constructs described in the previous chapters, to be able to study the effect of structural changes on their activity for example, which might bring interesting insight on their mode of action.

Although this method has not yet been tested fully and more research would be in order, the results so far are promising as to both the scope and overall yield of the process.

Notes

- 1) Experiments from table 3 and beyond are single experiment only, as time had run out. This affects azide functionalization and reduction in the scope section of this chapter (compound **19** and onward).

6.4. Supplementary information

General information

Unless noted otherwise, all reactions were carried out under nitrogen atmosphere. Reactions were monitored by TLC Silica 60 (Merck Millipore), examined under UV (365 nm and 254 nm), and stained by KMnO₅, ninhydrin, vanillin or H₂SO₄ in MeOH (1%). Chromatography was performed using the flash-protocols by Still *et al.* on Silica gel 60 (0.040-0.063 mm) from Merck Millipore. ^[16]

Unless specified otherwise, all solvents and reagents were obtained commercially (Sigma Aldrich, Acros Chemicals and TCI Europe). The solvents were used without any further purification, reagents were purified if required, following procedures described in "Purification of Laboratory Chemicals (sixth edition)."^[17]

The ¹H NMR spectra were recorded at 400 MHz and ¹³C NMR spectra were recorded at 100 MHz. The chemical shifts are reported in ppm relative to the residual solvent peak.

High-resolution mass spectra were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode).

Experimental procedures

4- Nitrosobenzylalcohol (6)

p-nitrosobenzylalcohol (3.2 g, 21 mmol, 1 equiv.) and ammonium chloride (1.6 g, 29 mmol, 1.4 equiv.) were dissolved in 160 mL of an ethanol-water mixing solvent (5:1) under argon, and then zinc powder (4.1 g, 63 mmol, 3 equiv.) was added at room temperature. After 2 h stirring, the suspension was filtered, followed by pouring into 500 mL aqueous solution of FeCl₃·6H₂O (6.8 g, 25 mmol, 1.2 equiv.) in an ice bath, after which the solution was stirred for an additional 2 h. The resultant aqueous solution was extracted by CH₂Cl₂ (100 mL x3). After evaporation of the organic phase *in vacuo*, (4-nitrosophenyl)methanol was obtained as a mixture with p-nitrosobenzylalcohol (2.78 g total, calculated 1.37 g, 10 mmol, 48% (4-nitrosophenyl)methanol estimated from NMR analysis), which was used without further purification. ¹H-NMR of the mixture (400 MHz, CDCl₃): δ (ppm) 8.23 (d, J=8.6 Hz, 2H), 7.91 (d, J=8.4, 2H), 7.61 (d, J=8.6 Hz, 2H), 7.54 (d, J=9.0 Hz, 2H), 4.84 (d, 2H), 1.89 (s, br, 1H).

4-hydroxymethylaniline

Palladium on charcoal (10% w/w, 350 mg, 14mg/mmol) was charged in a three-neck flask (250 mL) and placed under nitrogen (3 cycles) and wetted with freshly distilled toluene (10 mL). 4-nitrosobenzylalcohol (3.83 g, 25 mmol, 1 equiv.) was dissolved in methanol (50 mL) and added to the flask using a syringe, after which additional methanol was added (50 mL). The flask was cooled to 0 °C after which hydrazine-hydrate (6.25 mL, 125 mmol, 5 equiv.) in methanol (14 mL), was added to the mixture dropwise over 30 minutes. The mixture was stirred at room temperature until all the starting 4-nitrosobenzylalcohol was consumed on TLC. The reaction mixture was filtered over celite and activated charcoal and concentrated *in vacuo* to about 8-10 mL. The crude was purified directly with flash-chromatography (silica, heptane:EtOAc +1% Et₃N: 1:1 to 1:4), resulting in 4-hydroxymethylaniline as white (quickly yellowing to air/light) crystals (24.5 mmol, 97%). ¹H-

– An efficient synthesis of electron-rich aminomethyl azobenzenes –

NMR (400 MHz, CDCl_3): δ (ppm) 7.10 (d, $J=8.2$ Hz, 2H), 6.60 (d, $J=8.2$ Hz, 2H), 4.48 (s, 2H), 3.40 (br.s, 2H). ^{13}C -NMR (100 MHz, $\text{DMSO}-d_6$): δ (ppm) 145.9, 131.2, 128.9, 115.1, 64.9.

***tert*-butyldimethyl((4-nitrobenzyl)oxy)silane**

To a solution of 4-nitrobenzylalcohol (3.2 g, 21 mmol, 1 equiv.) in THF (50 mL, 0.2 M) was added *tert*-butyldimethylsilyl chloride (4.8 g, 32 mmol, 1.5 equiv.), triethylamine (8.5 mL, 63 mmol, 3 equiv.) and a few crystals of imidazole. The reaction mixture was then stirred for 3h at room temperature, until TLC indicated complete consumption of the starting material. The solution was then concentrated *in vacuo*. The residue was taken up in EtOAc (50 mL), washed with saturated ammonium chloride (50 mL), NaHCO_3 (50 mL) and brine (50 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was obtained as a clear oil that solidified upon standing. Flash chromatography (silica, 5% EtOAc in heptane) afforded the pure product as a clear, viscous oil that solidified upon contact with a pipette. Yield was determined for the clear and colourless oil (5.6 g, 21 mmol, 100%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 8.20 (d, $J = 8.2$ Hz, 2H), 7.49 (d, $J = 8.2$ Hz, 2H), 4.82 (s, 2H), 0.96 (s, 9H), 0.13 (s, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 149.0; 146.9; 126.4; 123.3; 63.8; 25.8; 18.2; -5.4.

4-(((*tert*-butyldimethylsilyl)oxy)methyl)aniline

Palladium on charcoal (10% w/w, 350 mg, 14mg/mmol) was charged in a three-neck flask (250 mL) and placed under nitrogen (3 cycles), after which freshly distilled toluene (10 mL) was added to form a slurry. *Tert*-butyldimethyl((4-nitrobenzyl)oxy)silane (5.6 g, 21 mmol, 1 equiv.) was dissolved in methanol (50 mL) and added to the flask using a syringe, after which additional methanol was added (50 mL). The flask was cooled to 0 °C after which hydrazine-hydrate (6.25 mL, 125 mmol, 6 equiv.) in methanol (14 mL), was added to the mixture dropwise over 30 minutes. The mixture was subsequently stirred at room temperature until all the starting 4-nitrobenzylalcohol was consumed, indicated by TLC. The reaction mixture was filtered over celite and activated charcoal and concentrated *in vacuo* to about 8-10 mL. The crude product was directly purified with flash-chromatography (silica, 30% EtOAc in Heptanes to 60% in 2 steps), resulting in the product as a colourless oil (4.5 g, 19.8 mmol, 92%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.11 (d, $J = 8.2$ Hz, 2H); 6.68 (d, $J = 8.2$ Hz, 2H); 4.62 (s, 2H); 3.59 (v.br. s, 2H); 0.92 (s, 9H); 0.07 (s, 6H); ^{13}C NMR (100 MHz, CDCl_3): δ 145.4; 131.7; 127.5; 114.9; 65.0; 25.9; 18.3; -5.2.

(E/Z)-(4-(phenyldiazenyl)phenyl)methanol (7)

To a solution of (4-nitrosophenyl)methanol (275 mg, 2 mmol, 1 equiv.) and aniline (200 μ L, 2.2 mmol, 1.1 equiv.) in ethanol (20 mL, 0.1M) was added glacial acetic acid (2 mL). The reaction was stirred at rt until full conversion of the nitroso-compound (typically 5-8 h). To the mixture was added dd H₂O (40-100 mL) which resulted in the precipitation of a bright orange crystalline solid. The solid was dissolved in EtOAc (50 mL), washed with saturated NaHCO₃ (50 mL), dd H₂O (50 mL) and brine (50 mL), after which the organic phase was dried over Na₂SO₄ and concentrated *in vacuo*. Chromatography (silica, heptanes:EtOAc 2:1) yield the product as a bright orange powder (needles) in 40-80% yield. ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 7.95–8.89 (m, 4H), 7.62–7.51 (m, 5H), 4.76 (d, *J* = 5.8 Hz, 2H), 4.39 (br s, 1H, OH); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 153.5, 152.5, 147.1, 132.0, 130.1, 128.0, 127.7, 123.5, 64.2;

(E/Z)-1-(4-(azidomethyl)phenyl)-2-phenyldiazene (9)

To (4-(phenyldiazenyl)phenyl)methanol (1.9 g, 15 mmol, 1.0 equiv.) in dry DMF (75 mL, 0.2 M) was added freshly distilled triethylamine (4.2 mL, 30 mmol, 2 equiv.) and mesyl chloride (1.8 mL, 22.5 mmol, 1.5 equiv.), which resulted mildly exothermic reaction upon which the mixture turned bright red and opaque. After consumption of all starting material (typically within 5 min), NaN₃ (2.05 g, 31.5 mmol, 2.1 equiv.) was added and the mixture was stirred overnight at rt, during which the colour of the mixture returned to orange. The reaction mixture was concentrated *in vacuo* to approx. 5 mL, after which EtOAc (100 mL) was added and the solution washed with 1M HCl (100 mL), saturated NaHCO₃ (100 mL), and brine (100 mL). The organic phase was dried over Na₂SO₄ and dried *in vacuo*. Flash chromatography (silica, heptanes:EtOAc 8:1 for R_f = 0.25) afforded the product as a very light, brittle, pale orange compound in 95% yield. ¹H-NMR (CDCl₃, 300 MHz): δ (ppm) 8.00-7.90 (m, 4H); 7.58-7.46 (m, 5H); 4.45 (s, 2H). ¹³C-NMR (CDCl₃, 75 MHz): δ (ppm) 152.4, 152.1, 137.5, 131.2, 129.3, 127.8, 123.3, 122.2, 53.9. HRMS: calculated for C₁₃H₁₂N₅ (M+H⁺): *m/z* 238.10872, found: *m/z* 238.10905.

(E/Z)-(4-(phenyldiazenyl)phenyl)methanamine (4)

To a solution of (E/Z)-1-(4-(azidomethyl)phenyl)-2-phenyldiazene (475 mg, 2 mmol, 1 equiv.) in THF:1M HCl (95:5, 10 mL, 0.2 M) at -20°C was added PBu₃ (760 μ L, 3 mmol, 1.5 equiv.) dropwise over 30 minutes, after which the mixture was allowed to stir at rt for 2h, during which N₂ gas slowly evolved, to form a slurry of golden flakes in a red solution. To this solution were

added drops concentrated HCl until the gas-evolution subsided. After cooling to -20°C , the mixture was filtered. The residue was washed with additional ice-cold THF, resulting in the target product as HCl salt, orange crystals (190 mg, 0.9 mmol, 45%). The remaining liquid was concentrated *in vacuo*, resulting in a black oil, which, upon contact with chromatographic silica immediately turns orange. Flash Chromatography (1:1 heptanes:EtOAc $\rightarrow$ MeOH + 1% NH_3) afforded an additional 40% yield (as orange powder), resulting in a total yield of 85% (360 mg, 1.7 mmol). ^1H -NMR (MeOD, 300 MHz): δ (ppm) 7.98 (d, $J = 8.5$ Hz, 2H); 7.93 (dd, $J = 7.7, 2.1$ Hz, 2H); 7.66 (d, $J = 8.5$ Hz, 2H); 7.60–7.50 (m, 3H); ^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 152.4, 152.1, 137.4, 130.8, 129.3, 123.2, 122.4, 105.7, 42.6. HRMS (ES $^{+}$): calculated for $\text{C}_{13}\text{H}_{14}\text{N}_3$ ($\text{M}+\text{H}^{+}$): m/z 212.11822, found: m/z 212.11849.

General procedure for the synthesis of hydroxymethylazobenzenes

To a solution of freshly prepared (4-nitrosophenyl)methanol (48% w/v, 1.39 g, 5 mmol, 1 equiv.) in dry ethanol (20 mL, 0.25 M) under nitrogen was added the appropriate *p*-aminophenyl compound (6 mmol, 1.2 equiv.), followed by glacial acetic acid (2 mL). The resulting mixture was allowed to stir at room temperature for at least 12 h, or until one of the reagents was fully consumed by TLC. To the mixture was added dd H_2O (40–100 mL) until the crude product precipitated. The precipitate was filtered off and taken up in EtOAc (50 mL), and washed with saturated NaHCO_3 (50 mL), H_2O (50 mL) and brine (50 mL). The organic phase was then dried over Na_2SO_4 , filtered and concentrated *in vacuo*.

Alternatively, after the initial addition of H_2O , the solution was extracted with EtOAc (3 x 100 mL). the organic phase was then concentrated to about 100 mL in volume and processed as described before.

The pure compound was obtained by flash chromatography or by recrystallization from hot EtOAc.

(*E/Z*)-(4-(*p*-tolyl diazenyl)phenyl)methanol (10)

As described, starting from *p*-toluidine (650 mg, 6 mmol, 1.2 equiv.). Recrystallization from refluxing EtOAc with pentane afforded the product as dark orange crystals (530 mg, 2.3 mmol, 47%). ^1H -NMR (300 MHz, DMSO- d_6): δ (ppm) 7.80 (dd, $J=16.4, 8.2$ Hz, 4H) 7.50 (d, $J=8.7$ Hz, 2H) 7.36 (d, $J=8.2$ Hz, 2H) 5.35 (br. s., 1H) 4.58 (s, 2H) 2.37 (s, 3H); ^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 152.5, 150.7, 141.8, 137.9, 129.8, 128.8, 123.3 (2), 65.1, 21.5

(E/Z)-(4-((4-methoxyphenyl)diazenyl)phenyl)methanol (11)

As described, starting from *p*-anisidine (760 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 50% EtOAc in heptanes) afforded the product as brittle dark orange crystals (460 mg, 1.9 mmol, 38%). ¹H-NMR (300 MHz, DMSO-*d*₆): δ (ppm) 7.80 - 7.92 (m, 4H) 7.50 (d, *J*=8.7 Hz, 2H) 7.06 (d, *J*=9.2 Hz, 2H) 4.68 (s, 2H) 3.88 (s, 3H) ¹³C-NMR (DMSO-*D*₆, 75 Hz): δ (ppm) 162.1, 152.2, 147.0, 143.1, 127.4, 124.8, 122.8, 114.22, 64.9, 55.6.

(E/Z)-(4-((4-aminophenyl)diazenyl)phenyl)methanol (12)

As described, starting from *p*-phenylenediamine (650 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 70% EtOAc in heptanes) afforded the product as brittle bright orange crystals (300 mg, 1.3 mmol, 27%). ¹H-NMR (300 MHz, DMSO-*d*₆): δ (ppm) 7.66 (dd, *J*=18.2, 8.5 Hz, 4H) 7.42 (d, *J*=8.2 Hz, 2H) 6.64 (d, *J*=8.7 Hz, 2H) 6.04 (s, 2H) 5.26 (t, *J*=5.9 Hz, 1H) 4.54 (d, *J*=5.6 Hz, 2H); ¹³C-NMR (DMSO-*D*₆, 75 Hz): δ (ppm) 153.1, 151.8, 144.5, 143.2, 127.5, 125.5, 122.0, 113.8, 63.0.

(E/Z)-(diazene-1,2-diylbis(4,1-phenylene))dimethanol (13)

As described, starting from *p*-aminobenzylalcohol (740 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 70% EtOAc in heptanes) afforded the product as bright orange powder containing an inseparable mixture of *E/Z*-isomers (ratio 1:3) (120 mg, 0.5 mmol, 10%). ¹H-NMR (300 MHz, DMSO-*d*₆): δ (ppm) 7.85 (d, *J*=8.2 Hz, 4H); 7.44 - 7.57 (d, *J* = 8.2 Hz, 4H); 5.43 (s, 2H) 4.62 (s, 4H); ¹³C-NMR (DMSO-*D*₆, 75 Hz): δ (ppm) 145.9, 131.2, 127.7, 115.2, 65.0.

(E)-4-((4-(hydroxymethyl)phenyl)diazenyl)phenol (14)

As described, starting from *p*-aminophenol (650 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 70% EtOAc in heptanes) afforded the product as bright orange powder (800 mg, 3.5 mmol, 58%). ¹H-NMR (300 MHz, DMSO-*d*₆): δ (ppm) 10.24 (s, 1 H) 7.73 - 7.80 (m, 4H) 7.47 (d, *J*=8.7 Hz, 2H) 6.92 (d, *J*=8.7 Hz, 2H) 5.30 (t, *J*=5.9 Hz, 1H) 4.56 (d, *J*=5.6 Hz, 2H); ¹³C-NMR (DMSO-*D*₆, 75 Hz): δ (ppm) 151.9 149.7, 144.1, 143.0, 127.4, 125.9, 123.2, 129.9, 64.9.

(E/Z)-4-((4-(((tert-butyldimethylsilyl)oxy)methyl)phenyl)diazenyl)phenyl)methanol (15)

As described, starting from 4-(((tert-butyldimethylsilyl)oxy)methyl)aniline (1.4 g, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 40% EtOAc in heptanes) afforded the product as a brilliant red solid (420 mg, 1.3 mmol, 27%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.84 (dd, J=8.2, 3.6 Hz, 4H) 7.50 (dd, J=8.2, 5.1 Hz, 4H) 5.35 (t, J=5.6 Hz, 1H) 4.79 (s, 2H) 4.58 (d, J=5.6 Hz, 2H) 0.90 (s, 9H) 0.08 (s, 6H); ¹³C-NMR (DMSO-D₆, 75 Hz): δ (ppm) 151.5, 151.3, 146.8, 145.2, 127.5, 127.2, 122.9, 122.8, 64.3, 62.9, 26.3, 18.5, -4.8.

(E/Z)-1-(4-((4-(hydroxymethyl)phenyl)diazenyl)phenyl)ethan-1-one (16)

As described, starting from 4-aminoacetophenon (810 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 40% EtOAc in heptanes) afforded the product as a pale-orange solid (500 mg, 1.3 mmol, 25%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 8.04 - 8.23 (m, 2H) 7.84 - 8.01 (m, 4H) 7.54 (d, J=7.2 Hz, 2H) 5.38 (t, J=5.4 Hz, 1H) 4.60 (d, J=5.1 Hz, 2H) 2.62 (s., 3H); ¹³C-NMR (DMSO-D₆, 75 Hz): δ (ppm) 197.1, 154.4, 151.3, 147.8, 146.8, 130.1, 127.6, 123.3, 123.0, 65.6, 20.9

(E/Z)-4-((4-(methylthio)phenyl)diazenyl)phenyl)methanol (17)

As described, starting from 4-(methylthio)aniline (835 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 50% EtOAc in heptanes) afforded the product as a red sticky powder (250 mg, 1 mmol, 19%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.88 (t, J=8.2 Hz, 4H) 7.49 (d, J=8.2 Hz, 2H) 7.34 (d, J=8.7 Hz, 2H), 5.32 (s, 1H), 4.77 (s, 2H) 2.55 (s, 3H); ¹³C-NMR (DMSO-D₆, 75 Hz): δ (ppm) 152.1, 149.9, 143.6, 142.9, 127.4, 125.9, 123.4, 123.0, 64.9, 15.3.

methyl (E/Z)-2-(4-((4-(hydroxymethyl)phenyl)diazenyl)phenyl)acetate (18)

As described, starting from methyl 4-(aminophenyl)acetate (990 mg, 6 mmol, 1.2 equiv.). Flash chromatography (silica, 33% EtOAc in heptanes) afforded the product as brown brittle powder (580 mg, 1.3 mmol, 27%). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 7.90 (dd, J=8.2, 7.2 Hz, 4H) 7.38 - 7.57 (m, 4H) 4.79 (s, 2H) 4.73 - 4.77 (m, 2H)

3.70 - 3.75 (br. s, 3H); ^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 171.8 152.0 (2), 139.5, 137.1, 130.0, 129.3, 123.8, 123.5, 70.9, 65.2, 42.0.

General procedure for the synthesis of azidomethyl azobenzenes

The appropriate 4-hydroxymethylazobenzene (1 mmol, 1 equiv.) was dissolved in dry DMF (10 mL, 0.1 M) under a nitrogen atmosphere. Triethylamine (420 μL , 3 mmol, 3 equiv.) was added, followed by MsCl (120 μL , 1.5 mmol, 1.5 equiv.), resulting in an opaque solution. After 30 minutes, sodium azide (200 mg, 3 mmol, 3 equiv.) was added and the suspension was stirred vigorously for 14h. EtOAc (50 mL) was added and the mixture was then washed with 1M HCl (50 mL), saturated NaHCO_3 (50 mL), 3M LiCl (2 x 50 mL) and brine (100 mL). The organic phase was dried over Na_2SO_4 and dried *in vacuo*. Flash Chromatography (silica, 10-15% EtOAc in Heptanes) afforded the products as orange to red powders.

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(p-tolyl)diazene (19)

(E/Z)-4-(p-tolyldiazenyl)phenyl)methanol **10** (220 mg, 1 mmol, 1 equiv.) was treated following the above protocol. Flash chromatography (silica, 10% EtOAc in heptanes) afforded the pure compound as a fine orange powder (246 mg, 1 mmol, 98%). ^1H -NMR (300 MHz, DMSO- d_6): δ (ppm) 7.92 (d, $J=8.2$ Hz, 2H) 7.84 (d, $J=8.2$ Hz, 2H) 7.46 (d, $J=8.2$ Hz, 2H) 7.32 (d, $J=7.8$ Hz, 2H) 4.43 (s, 2H) 2.45 (s, 3H); ^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 154.7, 141.8, 138.0, 129.8, 128.8, 125.9, 123.2, 122.9, 54.4, 21.5.

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(4-methoxyphenyl)diazene (20)

(E/Z)-4-((4-methoxyphenyl)diazenyl)phenyl)methanol **11** (230 mg, 1 mmol, 1 equiv.) was processed using the described method. Flash chromatography (silica, 15% EtOAc in heptanes) afforded the pure compound as a red solid. (261 mg, 1 mmol, 96%). ^1H -NMR (300 MHz, DMSO- d_6): δ (ppm) 7.87 - 7.96 (m, 4H) 7.45 (d, $J=8.6$ Hz, 2H) 6.99 - 7.06 (m, 2H) 4.42 (s, 2H) 3.90 (s, 3H); ^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 162.2, 152.4, 147.0, 143.1, 128.8, 124.9, 123.0, 114.3, 63.9, 55.6.

Attempted synthesis of (E/Z)-4-((4-(azidomethyl)phenyl)diazenyl)aniline (21)

(E/Z)-4-((4-(aminophenyl)diazenyl)phenyl)methanol **12** (220 mg, 1 mmol, 1 equiv.) was treated as described above. No product was observed, also the in hindsight expected mesylamide was not recovered in significant quantities. The starting material was not recovered and was assumed to have degraded.

Putative synthesis of (E/Z)-4-((4-(azidomethyl)phenyl)diazenyl)aniline (22)

(E/Z)-4-((4-(aminophenyl)diazenyl)phenyl)methanol **14** (220 mg, 1 mmol, 1 equiv.) was treated as described above. A single product was isolated in minimal quantities. ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.83 - 7.90 (m, 2H) 7.52 (d, *J*=8.2 Hz, 2H) 7.45 (d, *J*=8.2 Hz, 2H) 6.89 - 6.96 (m, 2H) 5.65 (br. s., 1H) 4.65 (s, 2H); ¹³C-NMR (DMSO-D₆, 75 Hz): δ (ppm) 158.5, 152.4, 147.1, 139.6, 129.4, 125.1, 122.9, 115.9, 45.8.

Attempted synthesis of (E/Z)-1,2-bis(4-(azidomethyl)phenyl)diazene (23)

(E/Z)-(diazene-1,2-diylbis(4,1-phenylene))dimethanol **13** (240 mg, 1 mmol, 1 equiv.) was treated per the above method, with the exception that 3 mmol of MsCl, 6 mmol of triethylamine and 6 mmol sodium azide were used in the same volume of solvent. No diazide was recovered, nor a significant amount of monoazide. Most material appears to have undergone polymerisation, based on TLC.

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(4-(((tert-butyldimethylsilyl)oxy)methyl)phenyl)diazene (24)

(E/Z)-4-((4-(((tert-butyldimethylsilyl)oxy)methyl)phenyl)diazenyl)phenyl)methanol **15** (350 mg, 1 mmol, 1 equiv.) was treated per the above method. Flash Chromatography (silica, 5% EtOAc in heptanes) afforded the pure product as a scarlet powder (362 mg, 0.95 mmol, 94%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.92 (dd, *J*=10.7, 8.4 Hz, 4H) 7.47 (dd, *J*=8.4, 2.9 Hz, 4H) 4.83 (s, 2H) 4.43 (s, 2H) 0.97 (s, 9H) 0.13 (s, 6H); ¹³C-NMR (DMSO-D₆, 75 Hz): δ (ppm) 151.9, 151.8, 147.3, 145.2, 128.8, 126.5, 123.2, 122.9, 64.6, 54.4, 26.3, 18.5, -4.8.

(E/Z)-1-(4-((4-(azidomethyl)phenyl)diazenyl)phenyl)ethan-1-one (25)

^{13}C -NMR was not recorded.

(E/Z)-1-(4-((4-(hydroxymethyl)phenyl)diazenyl)phenyl)ethan-1-one **16** (250 mg, 1 mmol, 1 equiv.) was treated as described. Product: 120 mg, ^1H -NMR (300 MHz, DMSO-d_6): δ (ppm) 8.09 - 8.13 (m, 2H) 7.97 (dd, $J=8.2$, 5.9 Hz, 4H) 7.54 (d, $J=8.2$ Hz, 2H) 4.81 (s, 2H) 2.67 (s, 3H);

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(4-(methylthio)phenyl)diazene (26)

^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 151.8, 149.3, 144.5, 138.6, 128.9, 125.9, 123.4, 123.2, 55.1, 15.0.

(E/Z)-4-((4-(methylthio)phenyl)diazenyl)phenyl)methanol **17** (260 mg, 1 mmol, 1 equiv.) was treated as per described procedure. Flash chromatography (silica, 10% EtOAc in heptanes) afforded the product as a red solid (273 mg, 0.96 mmol, 96%). ^1H -NMR (300 MHz, DMSO-d_6): δ (ppm) 7.90 (dd, $J=16.2$, 8.4 Hz, 4H) 7.46 (d, $J=8.2$ Hz, 2H) 7.31 - 7.39 (m, 2H) 4.43 (s, 2H) 2.56 (s, 3H);

methyl (E/Z)-2-(4-((4-(azidomethyl)phenyl)diazenyl)phenyl)acetate (27)

^{13}C -NMR (DMSO- D_6 , 75 Hz): δ (ppm) 172.5 151.67, 138.2, 137.2, 130.1, 128.8, 123.3, 123.1, 72.1, 54.4, 41.4.

Methyl (E/Z)-2-(4-((4-(hydroxymethyl) phenyl) diazenyl)-phenyl)acetate **18** (280 mg, 1 mmol, 1 equiv.) was treated as described above. The azide was recovered after flash chromatography (silica, 10% EtOAc in heptanes) (251 mg, 0.81 mmol, 81%). ^1H -NMR (300 MHz, DMSO-d_6): δ (ppm) 7.91 (dd, $J=14.3$, 8.4 Hz, 4H) 7.46 (dd, $J=11.9$, 8.4 Hz, 4H) 4.43

General procedure for the synthesis of aminomethyl azobenzenes

To the appropriate 4-azidomethylazobenzene (0.5 mmol, 1 equiv.) in THF (5 mL) at -20°C was added tributylphosphine (250 μL , 1 mmol, 1 equiv.) dropwise over 30 minutes, followed by 5M HCl (0.5-1 mL). If a precipitate was formed, the mixture was filtered and the resulting solid washed with ice-cold THF and Et_2O and subsequently recovered. The remaining organic phase was treated as if no precipitation had taken place.

If no precipitate was observed, the mixture was concentrated *in vacuo*, and directly purified using Flash-Chromatography – using the acidity of the silica to break-up the intermediates (silica, double length column, 5% MeOH in CH₂Cl₂ + 0.5% NH₃). After collecting the product fractions, they were concentrated *in vacuo*, redissolved in Et₂O, filtered and finally precipitated with a drop of 5M HCl (aq), concentrated again at own discretion (the extremely fine powder gets stuck in P4 glass filters, clogging them).

(E/Z)-4-(p-tolyldiazenyl)phenyl)methanamine HCl salt (single experiment) (28)

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(p-tolyl)diazene **19** (130 mg, 0.5 mmol, 1 equiv.) was treated to the above procedure. The product was obtained after Flash Chromatography and trituration using pentane/THF (1:1 v/v) to remove the last traces of

tributylphosphinoxide. The product was recovered as a dull orange powder (240 mg, 0.4 mmol, 78%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.95 (d, J = 8.5 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 7.64 (d, J = 8.5 Hz, 2H), 7.38 (d, J = 8.5 Hz, 2H), 4.21 (s, 2H), 2.44, (s, 3H). ¹³C-NMR was not recorded.

(E/Z)-4-((4-methoxyphenyl)diazenyl)phenyl)methanamine (single experiment) (29)

(E/Z)-1-(4-(azidomethyl)phenyl)-2-(4-methoxyphenyl)diazene **20** (130 mg, 0.5 mmol, 1 equiv.) was processed according to the above procedure. The product was purified using both Flash Chromatography, followed by additional trituration (THF) after the formation of the HCl-salt. The product was recovered as a reddish-orange finely divided solid

(101 mg, 0.36 mmol, 73%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 7.92 (m, 4H), 7.62, (d, J = 8.3 Hz, 2H), 7.10 (d, J = 9.0 Hz, 2H), 4.20 (s, 2H), 3.90 (s, 3H). ¹³C-NMR was not recorded.

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Chapter 7 – Overview and perspective

7.1. Introduction

The previous chapters of this manuscript were dedicated to describing the experimental work performed and discussing the emanating results in detail. This chapter will focus on evaluating these results and accomplishments in a broader sense. In the past five chapters, a few key central concepts were put forward which will be discussed further:

- DNA as a means to influence behaviour of a pore forming peptide;
- hybrid molecules embody the best and worst of both worlds;
- the use of scaffolds for labelling peptides;
- The need for simple and robust (organic) chemistry.

7.2. Research Overview and Achievements

Chapters 2-4 are all focussed on employing DNA to modify the pore-forming behaviour of pore-forming peptide Alamethicin. Chapter 2 described the successful development of a manual SPPS technique for the synthesis of alamethicin and derivatives. The method gave the desired peptide in sufficient quantities required for the later experiments, benefitting from powerful Fluorine-mediated couplings with a scavenger, resulting in near-quantitative yields per step. However, this method to obtain the peptide was still time-consuming owing to its lack of automation, while each synthesis would yield enough peptide for only a few experiments. This pushed us to develop the alternative, more straightforward and time-efficient solution, of employing dULC-ALM: an Alamethicin analogue lacking the Aib residues in favour of Leu, which can be made in larger batch sizes in overnight automated SPPS.

In the original design, it was envisioned to directly attach amine-modified oligomers onto the C-terminus of Alamethicin, and later dULC-ALM, using an elegant two-step oxidation-conjugation technique developed by the group of Whitesides. In chapter 3, it was demonstrated that elegance does unfortunately not always indicate simplicity or robustness: a string of poor outcomes prompted us to reconsider the synthetic plan, and, ultimately, revert to an earlier peptide-oligonucleotide conjugation strategy based on the thiol-maleimide coupling, as was originally performed by L. Gjonaj.

As a result, six different DNA-Alamethicin hybrids were created, by which the influence of DNA on the activity of the peptide could be established (single stranded and hybridized vs duplex or quadruplex). Synthesizing the diverse molecules was relatively easily accomplished. However, by combining an extremely hydrophobic peptide to a very polar and hydrophilic

DNA-oligomer, we created compounds whose original characteristics, in terms of hydrophilicity and hydrophobicity, did not blend into one average, but instead stacked, creating hybrids very peculiar and unique in their behaviour. This resulted in significant challenges when attempting to purify and characterise these, not previously described, constructs. However, sufficient amounts of the pure hybrids were obtained for further use.

The true lesson of chapter 3 should therefore read: ‘in making hybrid molecules of two distinctly different species, you do not (only) combine the best characteristics, but definitely the challenges of both worlds,’ – a quote kindly offered by prof. dr. J.G. Roelfes.

In chapter 4 was demonstrated how a DNA-oligomer affects the pore forming ability of dULC-ALM. A single strand oligomer managed to destabilize and slightly inhibit pore-formation. When hybridized to double-stranded DNA with a peptide-DNA construct with a complementary sequence, it greatly improved pore stability, both in size and lifetime. The same improvements were seen when using a G-quadruplex to bring multiple peptides together. The shorter oligomer strands also displayed an increase in overall activity compared to the unmodified peptide, whereas the longer sequence appeared to still decrease the overall activity. These findings clearly provide a proof of principle that DNA-hybridization can be used as a means to develop a DNA-aptamer based switchable pore-forming system that can act as a molecular sensor.

Chapter 5 revolved around the development of connecting 3 large biologically relevant moieties on the same core element. Two readily accessible amino acids were selected and modified to host a hydrophobic peptide (WALP or dULC-ALM), one of two dyes (Cy3 or TAMRA) and a phospholipid (DPPE) with a minimum of synthetic operations required. Also, in this case, hybrid molecules embody the best and worst of both worlds, particularly during purification.

Again, the simplicity and robustness of the synthetic method behind these templates was taken as a central value, to ensure ease of use of these new tool compounds, even outside of a regular chemistry lab (e.g. the biochemical laboratory where the constructs were evaluated for their effectiveness). The method was tested in a bid to synthesize a small library of hybrids. However, the final products could not be identified in time. Despite the dissatisfying outcome, the scaffolds themselves proved to be a versatile and reliable method to combine distinctly different molecules together in close proximity, as was later shown in follow-up work by Syga and De Vries in their 2020 ChemBioChem publication.

The final synthetic chapter took place not on the interface of synthesis with biomolecular/chemical biology as the others had, but rather on that of polymer science and organic synthesis. In this chapter, the development an electron rich aminomethyl azobenzene was discussed, which intended to be incorporated in a larger construct to make it potentially a photosensitive/-switchable construct. After the target molecule was obtained in excellent yield, the route taken toward the target proved versatile: a library of previously

unexplored/inaccessible electron-rich methylazide/amine modified azobenzenes was envisioned. Unfortunately, a lack of time left part of this project unfinished and open to further experimentation.

7.3. Perspective and recommendations

The overall goal of this thesis was to develop a molecular sensor, based on an artificial, switchable pore forming peptide, using a DNA-aptamer as a recognition/switching moiety. In this thesis, a proof of principle has been clearly demonstrated that DNA can be used to control pore-formation, increase pore stability and tailor pore sizes.

However, it fell short of its ambitious ultimate goal: a functional sensor is still out of reach. Such a goal, it has become apparent, requires far more time and concerted effort than a single thesis can encompass. Future studies are needed to shed more light on critical aspects such as the role of the length of the DNA-oligomer and the impact of the sequences used. In parallel, studies can commence on using a Thrombin-aptamer in combination with either G-quadruplex stabilisation (best pore forming results), or duplex stabilisation (more easily achieved with a complementary strand) to develop a rudimentary switch.

For convenience, both in terms of handling and pore-analysis, a different peptide should be employed. dULC-ALM fulfilled a particular need when the project started, but in hindsight may have caused more issues in purification and pore-analysis than it solved in terms of availability and ease of synthesis. One can think of regular Alamethicin, which should be more readily accessible since the last publication by Brimble and co-workers. In their work, they describe a safe, reliable and surprisingly expeditious manual synthesis of Alamethicin and analogues (Chapter 2). Alternately, GALA peptides or even simpler LLS-type peptides could be considered – ultimately, any barrel-stave type of peptide will suffice, provided it has better handling/purification characteristics.

Chapter 5 had as a goal to develop a trifunctional scaffold to help visually monitor the behaviour of WALP and Alamethicin (dULC) while anchored to a lipid membrane via an attached phospholipid. This synthetic approach was met with mixed success. Up until the final incorporation of the peptide, the method proved to be robust and give the target compounds in moderate to good yield. However, presumably, upon introduction of the peptides, the: “hybrid compounds have hybrid properties,” again came into play, and with the conditions chosen for the experiments, the final products could not be identified or recovered. Syga en De Vries later managed to complete the challenge of optimizing and identifying the final products. In doing so they also managed to demonstrate the viability of the method as we had envisioned it, providing another tool in the toolbox for making (complex) hybrid compounds.

In Chapter 6, a versatile and reliable route was developed to produce a methylamino azobenzene monomer. As this route potentially gave access to electron rich azobenzenes in the azide (and later amine) form, a library was synthesized for evaluation. Preliminary results appear to indicate that this library can be finished with medium to good yields, arriving at several not previously described compounds. Furthermore, as the method employs a synthetically interesting azide intermediate, in hindsight, the azide may be as interesting to explore for conjugation purposes as the amine based azobenzenes explored in this chapter. In all, this method has not yet been tested for its full potential and should receive further attention, both in terms of the library, its potential applications and further optimisation of the reaction conditions.

Summary

In this thesis, the use of DNA to modify the behaviour of a transmembrane pore-forming peptide is described. The work presented here serves as a proof of principle towards developing a DNA-peptide hybrid molecular sensor.

Pore-forming peptides (PFP's) operate by (reversibly) opening pores in lipid bilayers (e.g. cellular membranes), these pores consist out of multiple peptides, although the exact number of peptides, per pore, may vary. When these pores are formed, ions that were previously blocked by the membrane may now freely move from either side of the membrane to the other through the pore. In a controlled environment, such as with Black-Lipid Membrane electrophysiology, the current that passes through the membrane in this manner may be quantified. The current associated with a particular pore size is quantifiable and reproducible. Furthermore, pores have discrete sizes due to number of peptides required for a certain pore size. Therefore, these peptides may serve as a basis for a sensor, for which a reproducible, quantifiable and preferably discrete output is required.

When a PFP is modified on a terminus, it typically will retain its ability to form a transmembrane pore, provided the pore-forming section of the peptide is left intact and available to the membrane. However, the terminal modification of the PFP will affect the performance of the peptide: it may reduce or even completely destroy its pore-forming activity by favouring dissociation from the membrane, or by preventing the recruitment of the additional peptides required to form a pore. Alternatively, these modifications may promote pore formation/stabilize existing pores. Also, with modifications, it is possible to selectively promote particular pore sizes. With the development of a sensor in mind, it would be favourable to find a modification that, under the appropriate environmental conditions promotes a particular pore to form (with its associated current as signal output), while blocking pore formation when the conditions are not met. In the work described in thesis, Alamethicin F30 and its dULC-analogue, both well-known pore-forming peptides that allow for extensive C-terminal modifications without completely losing activity were used.

Single-strand DNA oligomers, as explored in this thesis, are interesting as terminal modifications, as they typically would hinder pore formation due to their great hydrophilicity. However, when hybridized to form duplexes, quadruplexes or other configurations, DNA may be employed to bring together several PFP's in the same location, which should greatly promote pore formation with an even number of peptides in the pore (4, 6, etc...). Furthermore, certain sequences of (single strand) DNA, aptamers, are known to selectively respond to the presence of a target molecule, changing their conformation in the process. This combination of DNA and PFP is therefore highly interesting to pursue as a potential molecular sensor.

Several challenges arise when trying to make a hybrid out of a highly amphipathic peptide and hydrophilic oligonucleotides. The first challenge was to obtain a viable amount of the peptide in a relatively short amount of time, (over)expression being not an option.

Chapter 2 described the successful development of a manual SPPS technique for the synthesis of alamethicin and derivatives. The method gave the desired peptide in sufficient quantities required for the later experiments, benefitting from powerful Fluorine-mediated couplings with a scavenger, resulting in near-quantitative yields per step. Due to the length of the synthesis, the yields remained in the single-digit range. In conclusion, despite the promise of the method, dULC-ALM was preferred over actual ALM as it was available via automated SPPS in shorter time and higher yield.

Chapter 3 describes how the challenge of forming a hybrid peptide-DNA molecule, with hybrid properties was overcome. Initially the hybrid synthesis was attempted by using an elegant two-step oxidation-conjugation technique developed by the group of Whitesides. After initial failure, a peptide-oligonucleotide conjugation strategy based on the thiol-maleimide coupling, originally performed by L. Gjonaj in her work, yielded the desired outcome. Six different, new DNA-dULC-ALM F₃₀ hybrids were synthesised and subsequently successfully isolated and characterized for the first time.

Chapter 4 describes the BLM-electrophysiological experiments on the six hybrids obtained in chapter 3. Here it was demonstrated how the presence of various C-terminal DNA-oligomers affect the pore forming ability of dULC-ALM. Single strand oligomers destabilize pores, resulting in shorter lifetimes, and slightly inhibit pore-formation. When hybridized to double-stranded DNA with a peptide-DNA construct with a complementary sequence, it greatly improved pore stability, both in size and lifetime. The same improvements were seen when using a G-quadruplex to bring multiple peptides together. The sequence lengths also appeared to affect pore forming behaviour: shorter oligomer duplex strands displayed a higher activity compared to the longer duplex and the unmodified reference peptide. The longer oligomer duplex was less active than the reference peptide. These findings clearly provide a proof of principle that DNA-hybridization can be used as a means to develop a DNA-aptamer based switchable pore-forming system that can act as a molecular sensor.

Chapter 5 was a different approach to influencing a membrane active peptide. The goal here was to introduce two larger moieties to a hydrophobic peptide (WALP) while not affecting its interaction with the membrane. To meet the challenge, both moieties were to be introduced on the same scaffold, after which the scaffold was attached to the peptide. Two viable cores were identified and successfully conjugated to one of two dyes (Cy3 or TAMRA) and a phospholipid (DPPE) with a minimum of synthetic operations required. The final constructs with WALP unfortunately could not be isolated. This promising lead was later followed up on and completed in work by Syga en De Vries in their 2020 ChemBioChem publication.

In chapter 6 there is a change of pace, as this work took place not on the interface of synthesis with biomolecular/chemical biology as the others had, but rather on that of polymer science and organic synthesis. A new, electron rich aminomethyl azobenzene was synthesized and characterized, which intended to be incorporated in a larger polymer, to make the latter into a photosensitive/-switchable material. After the target molecule was obtained in excellent yield, the route taken toward the target proved versatile: a library of previously unexplored/inaccessible electron-rich methylazide/amine modified azobenzenes was envisioned. Time constraints left several questions unanswered.

The final chapter puts each of the previous chapters in the perspective of the bigger picture: which questions have been answered, which ones are left open. And more importantly, which new questions have appeared, and what might be done to continue the project to a success.

Samenvatting

In dit proefschrift wordt het gebruik beschreven van DNA om het gedrag van transmembraan porievormende peptiden te beïnvloeden. Het hier beschreven werk dient als bewijs voor het principe dat een DNA-peptide hybride verbinding kan dienen als moleculaire sensor.

Porievormende peptiden (PVP's) werken door (reversibel) poriën in een lipide membraan te vormen (zoals bijvoorbeeld een celmembraan). Deze poriën worden uit meerdere PVP's gevormd, het exacte aantal in een porie kan variëren. Als een porie is gevormd in een membraan, kunnen o.a. ionen die eerder door het membraan werden geblokkeerd nu vrij van de ene kant van het membraan naar het andere bewegen via de porie. Als er een spannings-gradiënt over het membraan staat, zal de stroom van ionen specifiek een kant op bewegen. In een gecontroleerd experiment, zoals bij "Black-Lipid Membrane"-elektrofysiologie, kan een dergelijke stroom reproduceerbaar worden gemeten en gekwantificeerd. Hieruit is gebleken dat PVP's discrete niveaus aan stroomsterkte door een membraan kunnen opwekken. Deze discrete niveaus ontstaan doordat de poriegrootte afhangt van het aantal peptiden (altijd een heel getal) dat deelneemt aan de porie. De stroomsterkte door een porie kan daarmee gebruikt worden als output voor een sensor en de PVP als systeem om een dergelijk signaal mee te genereren.

Als een PVP wordt gemodificeerd aan een van zijn uiteindes, behoudt deze doorgaans zijn porievormende eigenschappen, mits het porievormend domein beschikbaar blijft voor het membraan en niet anderszins is aangetast. Een modificatie aan het uiteinde kan het gedrag van het peptide zelf wel beïnvloeden: het porievormend gedrag kan geremd worden, of zelfs volledig stilgelegd door de affiniteit voor het membraan te verlagen, of door te verhinderen dat een tweede peptide bij het aangepaste peptide in de buurt komt, wat wel vereist is voor het vormen van een porie. Anderzijds kan het porievormend gedrag, of de levensduur van een individuele porie ook verbeterd worden. Daarnaast is het ook mogelijk om de formatie van poriën met een specifiek aantal peptiden te selectief stimuleren. Met de ontwikkeling van een sensor in het achterhoofd zou het gunstig zijn om een modificatie te ontwikkelen die bij de gewenste omstandigheden het PVP aanzet tot het vormen van een specifieke porie (met bijbehorende stroomsterkte) en bij afwezigheid van die omstandigheden alle porievorming verhindert. In dit proefschrift zijn als PVP Alamethicine F₃₀ en het dULC analoog ervan gebruikt. Beiden zijn uitvoerig gebruikt in onderzoek naar PVP's en de modificaties ervan en zijn daarbij uitermate geschikt bevonden als platformen die zelfs bij relatief grote modificaties niet noodzakelijkerwijs hun activiteit verliezen.

Enkelstrengs-DNA, zoals ook bestudeerd in dit proefschrift, is een interessante modificatie voor aan het C-terminale uiteinde van een PVP. Ongehybridiseerd zou het hydrofiele

karakter van het oligomeer de interacties van het PVP met een membraan moeten verminderen en daarmee porievorming afremmen. Gehybridiseerd tot een duplex, quadruplex of andere vorm, brengt het DNA meerdere PVP's bij elkaar en verhoogt daarmee de lokale concentratie. Op het membraan zou dit tot meer porievormende activiteit en/of stabielere poriën kunnen leiden. Daarnaast zou hiermee een specifiek aantal PVP's in een porie gestimuleerd kunnen worden. Tot slot kunnen sommige, specifieke DNA-sequenties (aptameren), binden aan een bepaalde doelverbinding en daarmee hun conformatie aanpassen. Deze combinatie van eigenschappen maakt dat een hybride van DNA en een PVP een interessante kandidaat is voor de ontwikkeling van een moleculaire sensor.

Bij het maken van een DNA-PVP hybride, waarbij het DNA zeer hydrofiel is en het PVP amphipatisch, komen een aantal uitdagingen bovendien.

Hoofdstuk 2 beschrijft de succesvolle ontwikkeling van een handmatige SPPS-methode voor het synthetiseren van alamethicine en afgeleiden daarvan. De methode leverde het gewenste peptide op, met voldoende opbrengst voor vervolg-experimenten. De kracht van de methode zat in de Fluoride-activering van de inkomende aminozuren tijdens de koppeling, gecombineerd met een silyl ether 'scavenger,' waardoor de opbrengst per individuele stap bijna volledig was. Door de lengte van de synthese bleef de opbrengst van de gehele route beperkt tot enkele procenten na opzuivering. Als gevolg daarvan, ondanks het succes met de methode, is gekozen om toch het dULC-ALM analoog in te zetten in plaat van het originele peptide.

Hoofdstuk 3 beschrijft de hoe de uitdaging van de conjugatie van het PVP met DNA oligomeren is aangegaan en hoe deze materialen zijn gezuiverd en gekarakteriseerd. In eerste instantie werd geprobeerd om met een elegante oxidatie-conjugatie in twee stappen, origineel door de groep van Whitesides ontwikkeld, de gewenste hybriden te verkrijgen. Na tegenslag in de vertaling van de methode, is met succes teruggevallen op een thiol-maleïmide koppeling, eerder verkend door L. Gjonaj in haar proefschrift. Zes nieuwe, verschillende DNA-dULC-ALM F₃₀ hybriden zijn op deze manier verkregen en vervolgens voor het eerst succesvol gezuiverd en gekarakteriseerd.

Hoofdstuk 4 behandelt de BLM-elektrofysiologische experimenten die vervolgens met de zes DNA-ALM hybriden uit hoofdstuk 3 zijn uitgevoerd. Hierin werd aangetoond hoe de aanwezigheid van verschillende DNA-oligomeren op de C-terminus van dULC-ALM het porievormende karakter van het PVP beïnvloedt. Enkelstrengs oligomeren remmen, zoals verwacht, de mate waarin poriën worden gevormd in het membraan en verkorten de levensduur van de poriën wanneer ze gevormd zijn, tegenover een ongemodificeerd peptide als referentie. Wanneer de enkelstrengs verbindingen worden gehybridiseerd tot dubbelstrengs DNA door het toevoegen van een verbinding met een complementaire streng, verbetert de levensduur en grootte van de poriën sterk. Eenzelfde verbetering

wordt waargenomen als een G-quadruplex wordt gebruikt om meerdere peptiden bijeen te brengen. De lengte van de oligomeer lijkt ook effect te hebben op de activiteit. De hybrides met een kortere streng vertoonden als duplex een hogere activiteit t.o.v. de ongemodificeerde referentie, terwijl de langere oligomeren een lagere activiteit vertoonden. Op basis van deze verbindingen wordt duidelijk dat DNA geschikt is om het gedrag van een PVP te beïnvloeden en het in principe mogelijk moet zijn om een DNA-PVP gebaseerde sensor te ontwikkelen.

Hoofdstuk 5 omschrijft een andere aanvliegroute naar het beïnvloeden van een membraanactief peptide. Het doel was hier om twee grote functionele groepen te introduceren op het uiteinde van een peptide (WALP), zonder hierbij de werking van het peptide op een lipide membraan significant te verstoren. Hier werd de uitdaging aangegaan door beide functionele groepen eerst te koppelen aan een centraal platform, om vervolgens het platform aan het peptide te verbinden. Twee kandidaat-platformen werden geselecteerd en succesvol geconjugerd met een kleurstof (Cy3 of TAMRA) en een fosfolipide (DPPE), met slechts een minimum aan synthetische handelingen. De verbindingen werden daarna met WALP geconjugerd, maar het gewenste eindproduct kon op dat moment niet worden geïsoleerd. De belofte van dit platform werd in een vervolgonderzoek door Syga en De Vries wel waargemaakt, zoals ook staat beschreven in hun ChemBioChem publicatie.

Hoofdstuk 6 heeft een andere focus, aangezien het werk zich niet afspeelt op het grensvlak tussen synthese en biomoleculaire chemie/chemische biologie, maar meer op het vlak van polymeren en synthese. In dit hoofdstuk wordt de synthese en karakterisatie van een nieuw, elektronrijk aminomethyl azobenzeen beschreven, met het doel om deze in een polymeer in te bouwen en zo doende het polymeer fotosensitief/schakelbaar te maken. Nadat het gewenste molecuul was verkregen met een hoge opbrengst, bleek de methode geschikt om verschillende, niet eerder zo eenvoudig verkregen elektronrijke methylazide/amine azobenzenen te maken. Helaas was er te weinig tijd meer beschikbaar om alle openstaande vragen voor dit hoofdstuk te beantwoorden.

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Nawoord

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