Double-CLIPS technology for the mimicry of structurally complex antibody binding sites on proteins
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SYNTHESIS OF FUNCTIONALIZED SCAFFOLDS FOR CLIPS-CYCLIZATION AND LIGATION OF PEPTIDES

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2.1 GENERAL INTRODUCTION

In this chapter, the design and synthesis of a novel set of scaffolds is described that can be used to i) cyclize side-chain unprotected linear peptides containing two cysteine thiol groups [C(AA)₉C], and ii) chemically connect two such constrained peptides to form a double-CLIPS peptide construct.¹ ² In contrast to the divergent strategies published by others, involving the step-wise addition of peptides onto a single central scaffold molecule (see first example in Figure 2.1), the convergent strategy that we describe here is based on bio-orthogonal ligation reactions between two functionalized peptides, forming a central scaffold upon reaction with each other (second example in Figure 2.1). Therefore, our method does not require the protection of sensitive side-chain functionalities in the peptide, or pre-activation of the individual functionalities at the central scaffolds prior to ligation.

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**Figure 2.1:** Schematic representation of divergent and convergent strategies towards the synthesis of two cyclic peptides attached onto a single central scaffold.

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Theoretically, the simplest scaffold that can be used for cyclization and subsequent ligation of two dissimilar peptides using CLIPS chemistry is 1,2,4,5-tetramermodulene, also referred to as ‘T₄’ (for structure, see Figure 2.2). However, using the symmetrical molecule T₄ typically introduces characteristic problems associated with scaffolding peptides in this manner, i.e. the fact that reaction of two different peptides (X and Y) with T₄ will produce three different products: two symmetric products XX and YY, and an additional asymmetric product XY (Figure 2.2). Due to the equal reactivity of the four bromomethyl groups on T₄, each product can additionally exist in three regioisomeric forms (ortho, meta or para, shown for XX in Figure 2.2), whereby each of these three regioisomers again can exist in either the syn-syn or the syn-anti orientation (shown for ortho XX in Figure 2.2), resulting in a total of 18 different compounds (6 different isomers of in total three different products).
Synthesis of Functionalized Scaffolds for CLIPS-Cyclization and Ligation of Peptides

Figure 2.2: Illustration of the formation of three different products (XX, YY and XY) when attaching two dissimilar peptides (X and Y) onto T4. Each product gives three different regioisomers (ortho, meta and para shown for XX) whereby each regioisomer can be formed as two different isomers (syn-syn and syn-anti shown for ortho-XX).

The formation of 18 different compounds from a reaction between two peptides and one scaffold is obviously unacceptable and also creates huge problems when the most active compound in the mixture needs to be isolated and purified. Introducing some degree of flexibility into the scaffold, e.g. allowing free rotation of the individually looped peptides, could for example exclude the formation of the syn-anti and syn-syn isomers (Figure 2.3), but this does not fully solve the formation of different isomers, because the ortho/meta/para products are still formed as the result of equal reactivity of all four bromomethyl groups of the T4 scaffold.

Figure 2.3: Ligation strategies using three types of scaffolds: Application of two functionalized scaffolds for separate peptide cyclization and ligation is the only route that gives one single product, excluding the formation of isomers.
Chapter 2

Clearly, the best solution to prevent the formation of any type of isomeric product mixtures is the use of two differently functionalized CLIPS-scaffolds, both equipped with a complementary reactive group (represented with A and B in Figure 2.3), to which the two dissimilar peptides are first connected, followed by a subsequent ligation reaction to fuse the two CLIPSed peptides together. The synthesis of such functionalized scaffolds is described in detail in this chapter, starting with the 1st-generation scaffolds based on the non water-soluble \textbf{mT2} scaffold. The solubility problem encountered during cyclization and ligation of various C(AA)$_n$C peptides when using this type of scaffolds was the major reason that we developed the design, synthesis and application of a 2nd-generation of water-soluble CLIPS scaffolds (o\textbf{S2}) as described in § 2.3.
2.2 SYNTHESIS OF 1ST-GENERATION CLIPS SCAFFOLDS

The first attempted synthesis towards functionalized CLIPS-scaffold was based on functionalization of the mT2 (chapter 1) scaffold. Recently, Hartman et al. introduced a general method towards functionalized CLIPS-scaffolds via a simple mono-carboxylation reaction on 1,3,5-tribromomesitylene, resulting in a mT2 CLIPS-scaffold carrying an ester functionality at the 5-position (strategy A in Figure 2.4).

Inspired by this strategy, we first investigated the synthesis of two azide and one alkyne functionalized CLIPS-scaffolds (strategy B in Figure 2.4), accordingly named mT2-CH₂N₃, mT2-N₃, and mT2- (Figure 2.5), that were suitable for a follow-up ligation to a double-CLIPS construct via the Cu(I) catalyzed [3+2] azide-alkyne cycloaddition (CuAAC).

First, a simple route to the 5-(azidomethyl)-1,3-bis(bromomethyl)benzene scaffold mT2-CH₂N₃ was developed. According to literature, reaction of excess 1,3,5-tribromomesitylene (T3) (4.0 equiv) with NaN₃ was expected to yield mainly the mono-substituted product mT2-CH₂N₃. Indeed, we found that this compound was formed as the major product (~70%) in the above reaction, together with the di- (~20%) and tri-substituted product (5%) and starting material. Unfortunately, the mono-substituted product could not easily be separated from the starting material or its poly-substituted equivalents, neither by normal-phase silica gel nor reversed-phase HPLC due to extensive hydrolysis of the reactive bromides in the presence of TFA. We therefore turned our attention to the synthesis of scaffold mT2-N₃ that starts from 5-amino-isophthalic acid (Scheme 2.1). 5-Amino-isophthalic acid was esterified in 83% yield by refluxing in ethanol after slow addition of H₂SO₄ (2 equiv). The resulting ethyl ester 2 was reduced using LiAlH₄, to give the diol 3 in 97% isolated yield.
Scheme 2.1: Multistep synthesis of scaffold mT2-N₃ starting from 5-amino-isophthalic acid.

Transformation of the amine functionality of 3 into the corresponding azide 4 was achieved via diazotization,⁶ by treating the amine with nitrous acid to form a diazonium salt that subsequently reacts with NaN₃ to form the azide. Product formation was confirmed by IR-spectroscopy (peak observed at 2108 cm⁻¹ of the azide). The conversion of the remaining alcohol groups into bromides, however, turned out to be more difficult than expected. Reaction under typical Sₐ1 conditions using HBr only gave the mono-substituted product. Reaction under Sₙ2 conditions using CBr₃/PPh₃⁷ resulted in the formation of a large number of side-products. Fortunately, reaction with PBr₅ in DCM delivered mT2-N₃ as a white powder in 68% yield. ¹H NMR spectroscopy and mass spectrometry measurements confirmed the substitution, showing a shift from 4.60 to 4.45 ppm for both benzylic CH₂ protons and a mass of 302.92 (M⁺ calculated = 302.90). The compound was sufficiently stable at room temperature and could be used for prolonged times when stored at -18 °C.

Next, the mT2-■ scaffold was synthesized in three steps starting from 5-hydroxy-isophthalic acid (Scheme 2.2).

Scheme 2.2: Synthesis towards mT2-■ from 5-hydroxy-isophthalic acid.

Base-mediated reaction of 5-hydroxy-isophthalic acid with propargyl bromide (3.0 equiv) followed by reduction of the resulting diester 5 using LiAlH₄ gave 5-propargyloxy-1,3-bis(hydroxymethyl)benzene (6) that was subsequently converted to the corresponding dibromide using the same protocol as used for mT2-N₃. The characteristic shift in the ¹H-NMR spectrum for the benzylic protons from 4.60 to 4.46 ppm indicated a successful conversion of the hydroxyl groups into bromides. Moreover, mT2-■ gave a clear mass signal at 315.90 (M⁺), which perfectly matches with the calculated mass of 315.92 (M⁺). Scaffold mT2-■ showed similar stability as mT2-N₃ at room temperature and was also stable for extended periods of time when stored at -18 °C.

Despite their optimized syntheses, the 1st-generation scaffolds frequently showed to be highly hydrophobic, disturbing the water-solubility of the CLIPS-cyclized peptides when used in the CuAAC reaction. We therefore envisioned the use of an alternative water-soluble CLIPS-scaffold that was mainly developed in order to overcome the encountered solubility problems.
2.3 SYNTHESIS OF 2nd-GENERATION CLIPS SCAFFOLDS

A straightforward and generally applicable method for the synthesis of water-soluble functionalized CLIPS scaffolds named ‘oS2’ (“o” for the “ortho” bromide positions and “S” for “soluble”) was developed. Reaction of T4 with a variety of secondary amines gave the quaternary ammonium salts that were easily separable from the water insoluble starting materials (Figure 2.6). The use of symmetrical secondary amines, like for example 1,4-piperazine, prevents the formation of diastereoisomers that would otherwise have formed (R, R2NH) due to the introduced quaternary center. Moreover, the remaining amine of 1,4-piperazine is now available for further functionalization via robust amide bond formation using a wide variety of functionalized carboxylic acids. Piperazine functions as the ideal ‘connector’ unit between the functionalities on the scaffold and the dibromoxyylene unit (Figure 2.6).\(^1\)

![Formation of quaternary ammonium salt](image)

**Figure 2.6:** Strategy towards water-soluble 2nd-generation scaffolds introducing a Functional Group (FG) and a quaternary ammonium salt on a Piperazine connector.

For convenience, the synthesis of this type of scaffolds was conducted by prior coupling of a functionalized carboxylic acid to a mono-N-protected 1,4-piperazine using standard amide coupling conditions (HBTU, DIEA), followed by reaction with an excess of T4. The alternative approach, i.e. coupling of the mono-N-protected 1,4-piperazine to T4 prior to amide coupling, was not an option due to the instability of the benzyl bromide functionalities under the sometimes harsh conditions for protective group removal. For the final reaction in which the quaternary ammonium salt is formed, it was found that the use of relatively low amine concentrations (typically 10 mM) and excess of T4 (3 equiv) were required to completely suppress further reaction of the product with a second equivalent of amine. Moreover, the choice of solvent and base in this step strongly influenced the reaction and was optimized in test reactions of T4 with diethanolamine using DIEA as the base in dry MeCN (see Table 2.1 in § 2.3.1). The final workup was done by washing with cold Et2O, removing the excess of T4 and obtaining the final product by precipitation and filtration. Little contamination of the DIEA salt was sometimes observed by NMR, but was found to be harmless in the follow up CLIPS reactions.
2.3.1 Scaffolds for an ‘Azide-alkyne Cycloaddition’ Ligation Reaction

The optimized approach towards the 2nd-generation of highly water-soluble scaffolds resulted in the design of three new azide and alkyne functionalized scaffolds called oS2-N3, oS2- and oS2-cyclooctyne and is shown in Figure 2.7.

![Figure 2.7: 2nd-generation scaffolds for the azide-alkyne cycloaddition.](image)

For the synthesis of the azide-scaffold oS2-N3 (Scheme 2.3), Boc-protected piperazine was used as the starting material because the strongly acidic conditions used for Boc-removal were not expected to harm the functional azide group at the scaffold. Coupling of azido-glycine to Boc-piperazine was achieved in quantitative yields and removal of the Boc-group of 8 with 50% TFA in DCM was complete within one hour.

![Scheme 2.3: Synthesis towards oS2-N3 from Boc-piperazine.](image)

Immediate reaction of the resulting TFA salt with T4 after Boc-removal failed, due to Br-TFA exchange on the final product. Therefore, Boc-deprotection was directly followed by purification on a silica column flushed with 2% ammonia, to liberate the amine of 9 from the formed TFA salt. Formation of the quaternary ammonium salt was additionally investigated by testing several solvents and bases in order to optimize this reaction (Table 2.1). Some of the bases tested slowed down the reaction considerably (K2CO3, 2,6-lutidine), while others (triethylamine, pyridine) interfered in the process by reacting with the benzylic bromides. DIEA was the only base capable of driving the reaction cleanly towards completion in ~30 minutes. In addition, solvent influences were also investigated. Product formation was not at all observed in toluene and only at a low rate in CHCl3, THF and DCM, while formation of numerous side products was observed in DMSO and DMF. MeCN turned out to be the best solvent. This resulted in using DIEA in MeCN (entry 5), because those conditions were found to result in 100% conversion after 30 minutes. Treatment of the purified amine 9 with 3 equivalents of T4 under the optimized conditions of entry 5 was indeed successful and resulted in the azide-functionalized scaffold oS2-N3 in 93% yield.
### Table 2.1: Influence of solvent and base for the reaction of diethanolamine and T4.

<table>
<thead>
<tr>
<th></th>
<th>Solvent</th>
<th>Base</th>
<th>Conv 30 min (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conv 24 h (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MeCN</td>
<td>2,6-lutidine</td>
<td>&lt;5</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2</td>
<td>MeCN</td>
<td>K&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>&lt;5</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>MeCN</td>
<td>TEA</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>MeCN</td>
<td>Pyridine</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5</td>
<td>MeCN</td>
<td>DIEA</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>DIEA</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>THF</td>
<td>DIEA</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
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<td>DIEA</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
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<td>DIEA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>DIEA</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>DIEA</td>
<td>&lt;5</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Conversion was determined by peak integration of UPLC-MS.

Product formation was confirmed by exact mass experiments (M<sup>+</sup> calculated for oS2-N<sub>3</sub> is 458.0015 and was found 458.0021) and IR spectrometry (the peak at 2104 cm<sup>-1</sup> confirmed the presence of an azide). Product purity was finally determined by <sup>1</sup>H and <sup>13</sup>C NMR (Figure 2.8), obtained in a mixture of 10% MeCN in water to enhance the solubility of the final scaffold. The two aromatic protons (2xCH) at 7.73 ppm and the two singlets of the eight benzylic protons at 5.18 and 4.96 ppm (2xCH<sub>2</sub>Br and 2xCH<sub>2</sub>N<sup>+</sup>), together with the three multiplets at 4.43, 4.17 and 4.04 ppm of the piperazine protons (4xCH<sub>2</sub>) were found characteristic for the ammonium based scaffold salts. Moreover, it was observed from the <sup>1</sup>H NMR spectrum that oS2-N<sub>3</sub> was obtained as a mixture of scaffold salt and DIEA salt (1:0.37). This latter observation was, however, considered to be irrelevant for the CLIPS reaction under basic conditions and the scaffold therefore was used as such in the follow up CLIPS reactions.

Synthesis of the alkyne-scaffold was also performed starting from Boc-piperazine (Scheme 2.4). Coupling Boc-piperazine to Fmoc-propargyl glycine was followed by Fmoc-removal and acetylation to replace the bulky and hydrophobic Fmoc group (95% over two steps). Next, Boc-removal from 11 and amine liberation to give compound 12 was achieved in 77% yield. Reaction of 12 with T4 resulted in the final alkyne-functionalized scaffold oS2- in 79% yield, that was like oS2-N<sub>3</sub> partially obtained as a DIEA salt (Scaffold:DIEA = 4:3).

![Scheme 2.4: Synthesis towards oS2- from Boc-piperazine.](image)
Figure 2.8: $^1$H NMR (top) and $^{13}$C NMR (bottom) of oS2-N$_3$ after reaction of 9 with T4. Scaffold oS2-N$_3$ was obtained together with some DIEA salt and used without further purification.
In addition, the synthesis of a cyclooctyne-functionalized scaffold was established, allowing a strain-promoted cycloaddition reaction to avoid the use of copper. It was expected that the cyclooctyne would not be stable under acidic conditions and therefore the cyclooctyne carboxylic acid 16 (see experimental section for the synthesis) was first coupled to Fmoc-piperazine. However, this turned out to be a difficult coupling reaction giving low yields (<20%) and poor reproducibility, despite testing several different coupling reagents. Therefore, we returned our attention to the previously used Boc-piperazine, and fortunately we observed that coupling of the cyclooctyne-carboxylic acid 16 with Boc-piperazine using HATU as coupling reagent gave the amide 17 in 65% yield (Scheme 2.5).

![Scheme 2.5: Synthesis towards CLIPS-scaffold oS2-cyclooctyne starting from Boc-piperazine.](image)

In view of the possible acid sensitivity of the alkyne bond, Boc-removal was performed in 50% TFA in DCM for 30 seconds, immediately followed by dilution in toluene and direct evaporation of the solvents. NMR analysis revealed that the cyclooctyne remained intact and the Boc-group was removed in quantitative yield. Final reaction of amine 18 with T4 after the TFA salt liberation gave oS2-cyclooctyne in 67% yield, though product precipitation in this example was slow and relatively troublesome due to the hydrophobicity of the cyclooctyne moiety. Product formation was confirmed by exact mass measurements, revealing the correct mass of 527.0535 (calculated mass M+ = 527.0533) and by 1H and 13C NMR spectrometry, which illustrated the characteristic multiplets of the piperazine protons (4xCH2) at 4.53, 4.33 and 4.02 ppm, as well as the three singlets, one from the two aromatic protons (2xCH) at 7.88 ppm and two from the eight benzylic protons at 5.32 ppm (2xCH2Br) and 5.12 (2xCH2N+). 19F NMR additionally elucidated a single peak at 75.3 ppm for the fluorine substituent. The scaffold/DIEA salt ratio was finally determined from 1H NMR, being 1:0.12.

### 2.3.2 Scaffolds for a ‘Thiol-ene’ Ligation Reaction

In order to use the thiol-ene reaction as a ligation route, the straightforward synthetic route towards oS2-type scaffolds was also applied for the synthesis of alkene (oS2-=) and thiol (oS2-S(Trt)) functionalized scaffolds (Figure 2.9). In contrast to the azide and alkyne functionalities, the reactive thiol group could only be successfully applied when properly protected at first, to prevent side-reactions during the CLIPS reaction.
The synthesis of the alkene-functionalized scaffold oS2= started from commercially available allyl chloroformate (Scheme 2.6). Coupling of allyl chloroformate to Boc-piperazine using TEA (1.5 equiv) as the base in DCM gave 19 in 93% isolated yield. Quantitative Boc-removal and TFA salt liberation resulted in 20, which was reacted with T4 to afford oS2= as a mixture of scaffold salt and DIEA salt (1:0.07) as determined from the 1H NMR spectrum. The final step proceeded in 68% yield and the formation of oS2= was also confirmed by exact mass determination and found 459.0107 (M+ calculated for oS2= is 459.0107).

In contrast to the alkene functionality, the thiol group required for the thiol-ene ligation could not be introduced on a scaffold without prior protection, as it would interfere with the CLIPS reaction. Therefore, S-trityl-protected mercaptoacetic acid 21 was prepared from bromoacetic acid and triphenylmethanethiol and coupled to Fmoc-piperazine (Scheme 2.7). The choice of switching to Fmoc-protected piperazine was based on the presence of the acid-labile trityl group that obviously prevents the use of Boc-protected piperazine. The alternative Cbz-piperazine was also excluded as an option due to possible poisoning of the catalyst for Cbz removal by the thiols. Coupling between Fmoc-piperazine and S-trityl-protected mercaptoacetic acid proceeded in quantitative yield using HBTU as the coupling reagent. Fmoc deprotection of 22 using diethylamine was followed by reaction of the amine 23 with T4, resulting in oS2-S(Trt) as a mixture of scaffold and DIEA salt (1:0.77) and in 63% over the last two steps. Exact mass measurements confirmed the calculated mass of oS2-S(Trt) at 691.0891 (M+ calculated for oS2-S(Trt) is 691.0922).
2.3.3 Scaffolds for ‘Oxime’ Formation

Lastly, in order to study an oxime formation reaction as a final alternative ligation of two dissimilar peptides, the aldehyde- and hydroxylamine-functionalized scaffolds os2-CHO and os2-ONH₂ were synthesized (Figure 2.10).

Since aldehydes and hydroxylamine groups were expected to be highly reactive under the conditions of the CLIPS reaction, the aldehyde functionality was protected as a diethyl acetal, enhancing its stability under the slightly basic CLIPS conditions. The hydroxylamine functionality was introduced in its N-Boc-protected form. Both protective groups are acid labile and therefore can easily be removed by acidification of the mixture directly after the CLIPS reaction. In both cases, Cbz-piperazine thus was used as the connector.

For the diacetal protected aldehyde scaffold os2-CHO, coupling of sodium 4-hydroxybutyrate (24) to Cbz-piperazine was followed by oxidation of alcohol 25 to the corresponding aldehyde 26 using Dess-Martin periodinane in 98% yield (Scheme 2.8). Next, refluxing 26 in ethanol in the presence of a catalytic amount of p-toluenesulfonic acid gave the corresponding diethyl acetal 27. Final Cbz removal by hydrogenation of 27 was achieved using Pd on charcoal to give the free amine 28 in 94% yield. os2-CHO was obtained as scaffold/DIEA salt (1:0.30, determined from ¹H NMR) in 71% yield after the reaction of 28 with T4, confirmed by exact mass experiments (M⁺ calculated for os2-CHO is 533.0839 and was found 533.0840). The ¹H NMR again illustrated the central core of this os2-scaffold by the indicative singlets of the benzylic and aromatic protons (i.e. at 7.71, 5.15 and 4.94 ppm, respectively) and the multiplets of the piperazine protons at 4.12 and at 3.95 – 3.45 ppm.
Scheme 2.8: Synthesis towards oS2-CHO from Cbz-piperazine.

Finally, synthesis of the Boc-protected hydroxylamine scaffold oS2-ONH$_2$ was performed by coupling of commercially available (Boc-aminoxy)acetic acid with Cbz-piperazine, providing amide 29 in quantitative yield (Scheme 2.9).

Scheme 2.9: Synthesis towards oS2-ONH$_2$ from Cbz-piperazine.

Cbz removal resulted in 63% yield of the desired mono-functionalized piperazine 30. This relatively low yield is probably due to partial hydrogenolytic reduction of the N-O bond of 29. Final reaction with an excess of T4 (3 equiv) gave the desired oS2-ONH$_2$ scaffold in 97% yield as a scaffold and DIEA salt (1:0.17, determined from $^1$H NMR) and exact mass experiments confirmed the calculated M$^+$ mass of 394.1978 by detection of 394.1976.

In conclusion, eight distinct 2nd-generation CLIPS-scaffolds were developed for application in the field of peptide cyclization by CLIPS reaction and follow up peptide ligation by azide-alkyne cycloaddition (oS2-N$_3$, oS2- and oS2-cycloocycne), thiol-ene reaction (oS2= and oS2-S(Trt)) or oxime formation (oS2-CHO and oS2-ONH$_2$). The straightforward and successful syntheses of these water-soluble scaffolds resulted in overall yields of 43-89% and product analyses were achieved by exact mass experiments in combination with IR and $^1$H/$^1$C/$^1$F NMR. Product purity was additionally determined by $^1$H NMR that often revealed a mixture of the scaffold salt (major) and the DIEA salt (minor) of the product, which was considered irrelevant for follow up CLIPS reactions.
2.4 SUMMARY

This chapter describes the synthesis of a series of functionalized CLIPS-scaffolds that were
designed for the cyclization of C(AA),C-type peptides and subsequent covalent ligation of
two such peptides to form double-CLIPS peptides (described in chapter 3). The first strategy
was largely based on exploring the use of an mT2-type scaffold, bearing an additional
functional group. These 1st-generation scaffolds were synthesized from two different
isophthalic acids, resulting in mT2-N3 and mT2-N with an overall yield of 53% for both
scaffolds. However, the use of these scaffolds led to major solubility problems on the level of
the double-CLIPS peptides (chapter 3), which led us to develop an alternative type of
scaffolds.

Therefore, a set of 2nd-generation scaffolds was synthesized with largely improved
solubility in aqueous solutions and giving access to a much more simplified introduction of
eventually any type of functionality desired. These scaffolds all contain a quaternary
ammonium functionality as a central element (explaining the water-soluble character), and
they also share a piperazine-based central core. Functional groups for azide-alkyne
cycloadditions, thiol-ene ligations or oxime formations were introduced via robust amide
bond forming reactions and the only restriction in this respect was the fact that functional
group reactivity was required to be chemically orthogonal to the removal of Boc, Cbz or
Fmoc protective groups. In this way it was shown that the procedures were straightforward
(three steps in general), reproducible and high yielding (the overall yields of scaffold
synthesis was 43-89%). Application of the scaffolds described in this chapter for peptide
cyclization and ligation will be further discussed in chapter 3.
2.5 EXPERIMENTAL SECTION

General
All reagents were used as received. Flash chromatography was performed employing Sigma-Aldrich 230-400 Mesh, 60 Å. Thin-layer chromatography (TLC) was performed using silica gel coated aluminum plates with F-254 indicator (250 μm, 20x20 cm, Whatman). Visualization was accomplished by fluorescence quenching (256 nm), in combination with coloring using ninhydrine/Li/KMnO₄. Nuclear magnetic resonance spectra (¹H, ¹³C and ¹⁹F) were recorded with Bruker DRX-400 spectrometer at 25 °C using tetramethylsilane as an internal standard. IR spectra were recorded on a Bruker Alpha-P FT-IR spectrometer. UPLC analysis was performed on a Waters Acquity Ultra Performance LC System, equipped with a Waters Acquity UPLC BEH130 C18 1.7 μm column. A linear gradient of 5-55% MeCN (0.05% TFA) in H₂O (0.05% TFA) was used. Preparative HPLC purification was done on a Waters Prep LC System equipped with a Waters Delta-Pak C18 column (15 μm, 25x10 mm). Depending on the UPLC retention time, a gradient over 25 min of H₂O (+ 0.05% TFA) and MeCN (+ 0.05% TFA) was used, starting at 100:0 and going to a maximum of 70:30 at 40 ml/min with simultaneous UV detection at 215 nm. Photo induced reactions were carried out in a glass vial (diameter: 2 cm; wall thickness: 0.55 mm), located 10 cm away from the UV lamp (irradiation on sample: 365 nm). Mass spectra and accurate mass determinations were performed on a JEOL JMX SX/SX102A, coupled to a JEOL MSMP7000 data system.

Procedures for Synthesis of the 1'-generation Scaffolds

5-Azidomethyl-1,3-bis(bromomethyl)-benzene (1)

Route 1: To a solution of 1,3,5-tribromomesitylene (T3) (0.6 mmol, 214 mg, 4 equiv) in MeCN (4 ml), NaN₃ (0.15 mmol, 65 mg, 1 equiv) was added slowly. The solution was stirred for 4 h at room temperature. The solvent was evaporated in vacuo, re-dissolved in PE and the salts were filtrated of. The remaining solution was purified by column chromatography (PE:EtOAc 30:1) only resulting in a 1:1 mixture of product and T3 (65 mg). Reversed phase HPLC purification resulted in complete hydrolysis.

Route 2: T3 (0.6 mmol, 214 mg, 4 equiv) was dissolved in MeCN (4 ml). NaN₃ (0.15 mmol, 65 mg, 1 equiv) was added slowly and the reaction was stirred for 4 h at room temperature. This step was repeated until all starting material was gone. The solvent was evaporated in vacuo, re-dissolved in PE and the salts were filtered off. Column chromatography (PE:EtOAc 30:1) was not successful for purification of 1.

5-Amino-1,3-benzenediethylester (2)

5-Aminoisophthalic acid (28.0 mmol, 5.0 g; 1 equiv) was dissolved in 30 ml ethanol and cooled on ice. H₂SO₄ (56.0 mmol, 3 ml, 2 equiv) was added dropwise to the solution and the mixture was refluxed (95 °C) overnight. Next, 50 ml of H₂O was added to dilute the mixture, followed by the addition of a 4M NaOH solution (+/- 15 ml) until pH 10. The mixture was cooled on ice for 1 h and the product precipitated from the solution. The mixture was filtered and washed with cold (0 °C) H₂O (3x 50 ml). The white powder was dried overnight on high vacuum, resulting in 5.4 g (83%) of the product 2.
Synthesis of Functionalized Scaffolds for CLIPS-Cyclization and Ligation of Peptides

$^{1}$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.07 (s, 1H), 7.53 (s, 2H), 4.38 (q, $J = 7.1$ Hz, 4H), 1.41 (t, $J = 7.1$ Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 165.9 (2 C$_{a}$), 146.4 (C$_{a}$), 131.6 (2 C$_{a}$), 120.5 (CH), 119.5 (2 CH), 61.0 (2 CH$_{2}$), 14.1 (CH$_{3}$). IR (NaCl) v 3456; 3362; 1689; 1626; 1607; 1442; 1397; 1375; 1336; 1242 cm$^{-1}$. HRMS (EI) m/e (rel. intensity) data calculated for C$_{12}$H$_{16}$NO$_{4}$ (MH$^+$) 238.10, found 238.12.

5-Amino-1,3-benzenedimethanol (3)

To a solution of LiAlH$_4$ (50.4 mmol, 1.9 g, 3 equiv) in 60 ml dry THF was added dropwise a solution of 2 (16.8 mmol, 4.0 g, 1 equiv) in 40 ml dry THF under N$_2$. The mixture was refluxed (90 °C) overnight. Then, the reaction was quenched with 2 ml of a NaOH solution (15% w/w) was added, followed by 8 ml H$_2$O. The precipitated salts were filtered and the solvent was evaporated in vacuo, giving compound 3 as a white powder (97%, 2.5 g).

$^{1}$H NMR (400 MHz, MeOD): $\delta$ 6.71 (s, 1H), 6.66 (s, 2H), 4.51 (s, 4H); $^{13}$C NMR (100 MHz, MeOD): $\delta$ 147.2 (C$_{a}$), 142.1 (2 C$_{a}$), 115.0 (CH), 112.6 (2 CH), 63.8 (2 CH$_{2}$). IR (NaCl) v 3396; 1567; 1417 cm$^{-1}$. HRMS (EI) m/e (rel. intensity) data calculated for C$_{8}$H$_{12}$NO$_{2}$ (MH$^+$) 154.08, found 154.10.

5-Azido-1,3-benzenedimethanol (4)

Compound 3 (10.1 mmol, 1.6 g, 1 equiv) was suspended in 7.5 ml H$_2$O. After the dropwise addition of concentrated H$_2$SO$_4$ (38.5 mmol, 2.0 ml, 3.8 equiv), the mixture was stirred for 30 min while cooling on ice. A solution of NaN$_3$ (12.1 mmol, 0.84 g, 1.2 equiv) in 4.5 ml H$_2$O was added dropwise, followed by the addition of 15 ml of hexane. Next, a solution of NaN$_3$ (15.2 mmol; 0.9 g, 1.5 equiv) in 4.5 ml H$_2$O was added dropwise. The mixture was stirred on ice for 3 h. In order to change the pH to basic, 7.5 ml of a NaOH solution (20% w/v) was added to the mixture. The resulting solution was extracted 3x with 10 ml EtOAc. Solvent evaporation in vacuo resulted in 1.8 g (97%) of 4, isolated as a brown powder.

$^{1}$H NMR (400 MHz, MeOD): $\delta$ 7.13 (s, 1H), 6.98 (s, 2H), 4.60 (s, 4H); $^{13}$C NMR (100 MHz, MeOD): $\delta$ 143.6 (2 C$_{a}$), 140.0 (C$_{a}$), 121.1 (CH), 112.6 (2 CH), 63.8 (2 CH$_{2}$). IR (NaCl) v 3288; 2108; 1595; 1451 cm$^{-1}$. HRMS (EI) m/e (rel. intensity) data calculated for C$_{8}$H$_{10}$N$_3$O$_2$ (MH$^+$) 180.07, found 180.09.

5-Azido-1,3-bis(bromomethyl)-benzene (mT2-N$_3$)

To a solution of 4 (6.6 mmol, 1.2 g, 1 equiv) in 200 ml CH$_2$Cl$_2$, PBr$_3$ (13.2 mmol, 1.24 ml, 2 equiv) was added. The mixture was stirred overnight. H$_2$O (150 ml) was added and the organic layer was washed 3x with 50 ml H$_2$O. The solvent was evaporated in vacuo, and the product was purified by flash chromatography (100:1 PE:EtOAc) resulting in 1.6 g (55%) of mT2-N$_3$ obtained as a yellow oil.

$^{1}$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.21 (s, 1H), 7.00 (s, 2H), 4.45 (s, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 140.9 (C$_{a}$), 140.0 (2 C$_{a}$), 125.8 (CH), 119.4 (2 CH), 31.7 (2 CH$_{2}$). IR (NaCl) v 2924: 2853; 2111; 1725; 1595; 1456 cm$^{-1}$. HRMS (EI) m/e (rel. intensity) data calculated for C$_{8}$H$_{2}$Br$_3$N$_3$ (M$^+$) 302.90, found 302.92.

55
1,3,5-Tris(prop-2-yn-1-yloxy)benzene (5)

5-hydroxyisophthalic acid (27.5 mmol, 5.0 g, 1 equiv), K₂CO₃ (82.4 mmol, 11.4 g, 3 equiv) and propargylbromide (93 mmol, 11.0 g, 10.3ml) were dissolved in 50 ml DMF. The mixture was stirred overnight at room temperature. Next, the mixture was poured onto 150 ml of ice cold H₂O in order to precipitate the product. The product was filtered and washed with hexane (3x 100 ml), resulting in 6.0 gram (74%) of 5 as a white powder after drying under high vacuum.

¹H NMR (400 MHz, CDCl₃): δ 8.37 (s, 1H), 7.87 (s, 2H), 4.96 (d, J = 2.4 Hz, 4H), 4.80 (d, J = 2.4 Hz, 2H), 2.56 (t, J = 2.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 164.4 (C₉), 157.4 (C₈), 131.0 (C₇), 124.0 (CH), 120.7 (CH), 77.3 (CH), 77.2 (CH), 76.3 (C₆), 75.2 (C₅), 56.1 (CH₂), 52.7 (CH₂). IR (NaCl) ν 3287; 2362; 1725; 1307; 1226 cm⁻¹. HRMS (EI) m/e (rel. intensity) data calculated for C₉H₇O₃ (M⁺) 296.07, found 296.09.

5-(2-Propyn-1-yloxy)-1,3-benzenedimethanol (6)

To a solution of LiAlH₄ (61.0 mmol, 2.3 g, 3 equiv) in 60 ml dry THF, was dropwise added a solution of 5 (20.3 mmol, 6.0 g, 1 equiv) in 40 ml dry THF under N₂. The mixture was refluxed (90 °C) overnight. To quench the reaction, 2.5 ml of a NaOH solution (15% w/v) was added, followed by 10 ml H₂O. The salts that formed were filtered and the solvent was evaporated in vacuo, resulting in 3.8 g of a yellow powder 6 (96%).

¹H NMR (400 MHz, MeOD): δ 6.98 (s, 1H), 6.91 (s, 2H), 4.74 (d, J = 2.4 Hz, 2H), 4.60 (s, 4H), 2.94 (t, J = 2.4 Hz, 1H); ¹³C NMR (100 MHz, MeOD): δ 158.0 (C₉), 143.0 (C₈), 117.8 (CH), 111.7 (CH), 78.4 (CH), 75.2 (C₇), 63.5 (CH₃), 55.1 (CH₃). IR (NaCl) ν 3290; 1568; 1417; 1293; 1155; 1020 cm⁻¹. HRMS (EI) m/e (rel. intensity) data calculated for C₉H₁₃O₃ (MH⁺) 193.08, found 193.11.

5-(2-Propyn-1-yloxy)-1,3-bis(bromomethyl)-benzene (mT2■)

To a solution of 6 (17 mmol, 3.3 g, 1 equiv) in 300 ml CH₂Cl₂, PBr₃ (34.1 mmol, 3.2 ml, 2 equiv) was added. The mixture was stirred overnight. H₂O (150 ml) was added and the organic layer was washed 3x with 100 ml H₂O. The solvent was evaporated in vacuo, and the product was purified by flash chromatography (20:1 PE:EtOAc) resulting in 3.8 g (71%) of mT2■ as a white powder.

¹H NMR (400 MHz, CDCl₃): δ 7.07 (s, 1H), 6.96 (s, 2H), 4.73 (d, J = 2.4 Hz, 2H), 4.46 (s, 4H), 2.57 (t, J = 2.4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ 157.7 (C₉), 139.5 (C₈), 122.5 (CH), 115.4 (CH), 75.9 (C₇), 55.8 (CH₃), 32.5 (CH₂). IR (CDCl₃) ν 3291; 1720; 1597; 1459; 1298; 1051; 913 cm⁻¹. HRMS (EI) m/e (rel. intensity) data calculated for C₁₁H₁₆Br₂O (M⁺) 315.90, found 315.92.

Procedures for Synthesis of the 2nd-generation Scaffolds

oS2-OH

To a solution of 1,2,4,5-tetrakis(bromomethyl)-benzene T4 (0.55 mmol, 250 mg, 3 equiv) and DIEA (0.4 mmol, 61 µl, 2 equiv) in dry MeCN (40 ml), diethanolamine (0.2 mmol, 20.0 mg, 1 equiv) was added drop wise as a solution in 2 ml dry MeCN. The mixture was
stirred for 1 h at room temperature. The solvent was evaporated in vacuo. The mixture was re-dissolved in 10 ml MeCN and Et$_2$O was added until a white powder precipitated from the solution. The solvent was removed and this was repeated 3x. This resulted in 86 mg of product 4, being a mixture of scaffold salt and DIEA salt (1:0.14). Yield 85%. The scaffold was used as such.

$^1$H NMR (400 MHz, D$_2$O/CD$_3$CN 9:1) δ 7.71 (s, 2H), 5.23 (s, 4H), 4.97 (s, 4H), 4.18 (t, $J=5.2$ Hz, 4H), 3.95 (q, $J=4.0$ Hz, 4H). $^{13}$C NMR (100 MHz, D$_2$O/CD$_3$CN 9:1): δ 137.3 (C$_6$), 133.4 (C$_6$), 125.0 (CH), 67.8 (CH$_2$), 62.6 (CH$_2$) 55.0 (CH$_2$), 29.0 (CH$_2$). IR ν 3301; 2941; 1443; 1355; 1018; 541 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{14}$H$_{30}$Br$_2$NO$_2$ (M$^+$) 393.9841, found 393.9846.

2-Azidoacetic acid (7)$^9$

Bromoacetic acid (20 mmol, 2.8 g, 1 equiv) and NaN$_3$ (40 mmol, 2.6 g, 2 equiv) were dissolved in 25 ml DMSO and stirred for 1 h. The reaction was allowed to come to room temperature and stirred overnight. The solution was diluted with water (20 ml) and acidified using concentrated hydrochloric acid until pH 4. The aqueous solution was extracted 3x with EtOAc and dried on MgSO$_4$. Evaporation in vacuo yielded 1.45 g (73%) of 7 as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.90 (s, 1H), 3.96 (s, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 173.5 (C$_6$), 50.2 (CH$_2$). IR ν 2111; 1726; 1220 cm$^{-1}$.

$N$-Boc-$N'-(2$-azidoacetyl)$-1$-4-piperazine (8)$^{10}$

To a solution of 7 (4.0 mmol, 406 mg, 1.5 equiv), HBTU (4.0 mmol, 1.5 g, 1.5 equiv) and DIEA (6.7 mmol, 1.1 ml, 2.5 equiv) in 30 ml THF was added 1-Boc-piperazine (2.68 mmol, 0.5 g, 1 equiv). The suspension was stirred for 2 h after which the suspension became a clear solution. The solvent was evaporated in vacuo. The residue was re-dissolved in EtOAc (30 ml) and extracted with 1M KHSO$_4$ (30 ml), a saturated solution of NaHCO$_3$ (30 ml) and brine (30 ml). Drying on MgSO$_4$ and evaporation in vacuo was followed by flash chromatography (1% MeOH), which resulted in 728 mg of 8 (>99%) obtained as a yellow oil.

$^1$H NMR (400 MHz, CDCl$_3$): δ 3.92 (s, 2H), 3.57 (m, 2H), 3.40 (m, 4H), 3.33 (m, 2H), 1.43 (s, 9H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 165.9 (C$_6$), 154.5 (C$_6$), 80.6 (C$_6$), 50.8 (CH$_2$), 45.0 (CH$_2$), 41.9 (CH$_2$), 28.4 (CH$_2$). IR ν 2103; 1657; 1417; 1235; 1166 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{11}$H$_{29}$N$_3$O$_3$ (M$^+$) 270.1566, found 270.1561.

$N$-(2-Azidoacetyl)-1,4-piperazine (9)

Compound 8 (2.7 mmol, 728 mg, 1 equiv) was dissolved in 40 ml of a 1:1 mixture of TFA and DCM. The mixture was stirred for 1 h, followed by evaporation of the solvents in vacuo. The residue was purified by flash chromatography (8% MeOH and 2% NH$_3$ in DCM), resulting in 439 mg (97%) of 9 as a yellow oil.

$^1$H NMR (400 MHz, MeOD): δ 4.01 (s, 2H), 3.53 (m, 2H), 3.38 (m, 2H), 2.85 (m, 4H). $^{13}$C NMR (100 MHz, MeOD): δ 168.3 (C$_6$), 51.5 (CH$_2$), 45.6 (CH$_2$), 45.4 (CH$_2$), 45.3 (CH$_2$), 42.3 (CH$_2$). IR ν 2107; 1652; 1456; 1236; 1200 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{10}$H$_{12}$N$_3$O (M$^+$) 170.1042, found 170.1040.

oS2-N$_3$

To a solution of 1,2,4,5-tetraiodobenzene T4 (1.2 mmol, 540 mg, 3 equiv) and DIEA (0.8 mmol, 132 µl, 2 equiv) in dry MeCN (40 ml), 9 (0.4 mmol, 67.5 mg, 1 equiv) was added
dropwise as a solution in 2 ml dry MeCN. The mixture was stirred for 1 h at room temperature. The solvent was evaporated in vacuo. The mixture was re-dissolved in 10 ml MeCN and Et$_3$O was added until a white powder precipitated from the solution. The solvent was removed and this was repeated 3x. This resulted in 228 mg (93%) of the product oS2-N$_3$, being a mixture of scaffold and DIEA salt (1:0.37). The scaffold was used without further purification.

$^1$H NMR (400 MHz, D$_2$O/CD$_3$CN 9:1) $\delta$ 7.73 (s, 2H), 5.18 (s, 4H), 4.96 (s, 4H), 4.43 (s, 2H), 4.17 (m, 2H), 4.04 (m, 2H), 3.87 (m, 4H). $^{13}$C NMR (100 MHz, D$_2$O/CD$_3$CN 9:1): $\delta$ 167.6 (C$_2$), 137.6 (C$_3$), 132.4 (C$_4$), 125.5 (CH), 66.2 (CH$_2$), 58.4 (CH$_3$), 58.2 (CH$_2$) 49.6 (CH$_3$), 41.8 (CH$_2$), 39.2 (CH$_3$) 36.4 (CH$_2$), 28.8 (CH$_3$). IR v 2978; 2104; 1643; 1424; 1182; 1133; 932; 552 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{16}$H$_{20}$Br$_2$N$_2$O(M$^+$) 458.0015, found 458.0021.

1-Boc-4-(2-Fmoc-aminopent-4-ynoyl)piperazine (10)

To a solution of Fmoc-propargylglycine (1.7 mmol, 557 mg, 1.5 equiv), HBTU (1.7 mmol, 630 mg, 1.5 equiv) and DIEA (2.8 mmol, 0.5 ml, 2.5 equiv) in 15 ml THF was added 1-Boc-piperazine (1.1 mmol, 205 mg, 1 equiv). The suspension was stirred over night after which it turned into a clear solution. The solvent was evaporated in vacuo and extracted with 1M KHSO$_4$ (15 ml), a saturated solution of NaHCO$_3$ (15 ml) and brine (15 ml). Drying on MgSO$_4$ and evaporation in vacuo was followed by flash chromatography (4:1 PE:EtOAc - 1:1 PE:EtOAc), which resulted in 787 mg (>99%) of a yellow oil 10.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.72 (d, $J = 7.4$ Hz, 2H), 7.57 (d, $J = 7.4$ Hz, 2H), 7.36 (t, $J = 7.4$ Hz, 2H), 7.27 (t, $J = 7.4$ Hz, 2H), 6.10 (d, $J = 7.5$ Hz, 1H), 4.86 (dd, $J = 16.7$, 7.5 Hz, 1H), 4.43 – 4.28 (m, 2H), 4.18 (t, $J = 7.1$ Hz, 1H), 3.56 (m, 4H), 3.45 (m, 4H). 2.68 (dd, $J = 16.7$, 7.5, 2.5 Hz, 1H), 2.60 (dd, $J = 16.7$, 7.5, 2.5 Hz, 1H), 2.05 (t, $J = 2.5$ Hz, 1H), 1.55 (s, 9H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 172.6 (C$_2$), 157.2 (C$_4$), 155.9 (C$_3$), 145.2 (C$_1$), 142.8 (C$_2$), 129.2 (CH), 128.6 (CH), 126.6 (CH), 121.5 (CH), 81.9 (C$_4$), 80.5 (C$_3$), 72.9 (CH), 68.6 (CH$_2$) 50.6 (CH$_2$), 48.6 (CH), 47.3 (CH$_3$), 43.7 (CH$_3$), 29.8 (CH$_3$), 24.9 (CH$_2$). HRMS (FAB+) $m/z$ calculated for C$_{26}$H$_{34}$N$_3$O$_2$(MH$^+$), 504.2420, found 504.2425.

1-Boc-4-(2-acetimidopent-4-ynoyl)piperazine (11)

Compound 10 (1.1 mmol, 0.5 g, 1 equiv) was dissolved in 50 ml of a 1:1 mixture of Et$_3$NH and THF. The mixture was stirred for 2 h, after which the solvents were evaporated in vacuo. The residue was dissolved in 50 ml THF, followed by the addition of acetic anhydride (2.7 mmol, 253 $\mu$l, 2.5 equiv) and TEA (1.7 mmol, 236 $\mu$l, 1.5 equiv). The mixture was stirred overnight. The solvents were evaporated in vacuo and purification using flash chromatography (2% MeOH in DCM) resulted in 336 mg (95%) of a colorless oil 11.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.63 (s, 1H), 5.12 (td, $J = 7.6$, 5.3 Hz, 1H), 3.63 (m, 4H), 3.45 (m, 4H), 2.69 (ddd, $J = 16.7$, 7.5, 2.7 Hz, 1H), 2.59 (ddd, $J = 16.7$, 7.5, 2.7 Hz, 1H), 2.05 (t, $J = 2.7$ Hz, 1H), 1.48 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 171.2 (C$_3$), 170.6 (C$_4$), 155.9 (C$_1$), 82.0 (C$_3$), 80.2 (C$_4$), 72.8 (CH), 48.6 (CH), 47.3 (CH$_2$), 43.7 (CH$_3$), 29.9 (CH$_3$), 24.7 (CH$_2$), 24.6 (CH$_3$). HRMS (FAB+) $m/z$ calculated for C$_{16}$H$_{26}$N$_3$O$_4$(MH$^+$), 324.1845, found 324.1850.
**4-(2-Acetamidopent-4-ynoyl)piperazine (12)**

Compound 11 (1.0 mmol, 336 mg, 1 equiv) was dissolved in 20 ml of a
1:1 mixture of TFA and DCM. The mixture was stirred for 1 h,
followed by evaporation of the solvents *in vacuo*. The residue was
purified by flash chromatography (8% MeOH and 2% NH₃ in 90%
DCM), resulting in 180 mg (81%) of a white powder 12.

¹H NMR (400 MHz, MeOD) δ 5.00 (t, J = 7.3 Hz, 1H), 3.90 (m, 4H), 3.28 (m, 4H), 2.66
(ddd, J = 17.0, 7.6, 2.6 Hz, 1H), 2.60 (ddd, J = 17.0, 7.6, 2.6 Hz, 1H), 2.43 (t, J = 2.6 Hz,
1H), 1.99 (s, 3H). ¹³C NMR (100 MHz, MeOD): δ 173.6 (C₆), 171.2 (C₅), 80.9 (C₄), 73.0
(CH), 48.8 (CH), 47.7 (CH₂), 44.2 (CH₃), 25.1 (CH₃), 24.9 (CH₃). HRMS (FAB+) m/z
calculated for C₁₁H₁₈N₃O₂ (MH⁺), 224.1321, found 224.1320.

**oS2-**

To a solution of 1,2,4,5-tetrakis(bromomethyl)-benzene
(2.4 mmol, 1.1 g, 3 equiv) and DIEA (1.6 mmol, 0.27 ml,
2 equiv) in dry MeCN (70 ml), 12 (0.8 mmol, 180 mg, 1
equiv) was added dropwise as a solution in 10 ml dry
MeCN. The mixture was stirred for 1 h at room
temperature. The solvent was evaporated *in vacuo*. The mixture was re-dissolved in 10 ml
MeCN and Et₂O was added until a white powder precipitated from the solution. This
resulted in 538 mg of oS2-, being a mixture of the dimer, scaffold salt and DIEA salt
(1:8:6). Yield 79%. The scaffold was used as such.

¹H NMR (400 MHz, D₂O/CD₃CN 9:1) δ 7.67 (s, 2H), 5.13 (s, 4H), 5.10 (t, J = 6.8 Hz, 1H),
4.21 (m, 4H), 4.07 (m, 2H), 3.84 (m, 2H), 2.78 (m, 2H), 2.60 (t, J = 2.4 Hz, 1H), 2.15 (s,
3H). ¹³C NMR (100 MHz, D₂O/CD₃CN 9:1): δ 172.8 (C₆), 167.6 (C₅), 143.3 (C₄), 138.4
(C₃), 126.5 (CH), 79.9 (C₂), 72.6 (CH), 66.4 (CH₂), 60.2 (CH₃), 49.3 (CH), 41.8 (CH₂), 39.0
(CH₃) 36.2 (CH₂), 25.4 (CH₂), 24.0 (CH₃). HRMS (FAB+) m/z calculated for C₁₂H₂₆Br₂N₃O₂
(M⁺), 510.0386, found 510.0398.

**Methyl-2-oxocyclooctane-1-carboxylate (13)¹¹**

NaH (61.6 mmol, 2.4 g, 2.8 equiv) was suspended in 30 ml toluene. Diethyl
carbonate (44 mmol, 5.2 g, 2 equiv) was added and the mixture was heated up
to reflux temperature (120 °C). Cyclooctanone (22 mmol, 2.8 g, 1 equiv)
was dissolved in 20 ml toluene and this solution was added dropwise over 1
h. After stirring for 1 h, the mixture was cooled down to room temperature.
To quench the reaction, 6-10 ml glacial acetic acid was added carefully, followed by 20-30
ml ice cold H₂O. The acidic water layer was extracted 3x with toluene (50 ml) and the
combined toluene layers were washed with ice cold H₂O (100 ml). The solvent was
evaporated *in vacuo* and the product was purified by flash chromatography (20:1
PE:EtOAc). This resulted in 4.2 g (96%) of a colorless oil 13, which existed as a mixture of
ketone and enol in a ratio of 0.5:1.

¹H NMR (400 MHz, CDCl₃) δ 12.55 (s, 1H) 4.16 (q, J = 7.1 Hz, 2H), 4.09 (q, J = 7.1 Hz,
1H), 3.52 (dd, J = 10.7, 4.5 Hz, 0.5H), 2.57 (ddd, J = 13.9, 11.0, 4.5 Hz, 0.5H), 2.44 (ddd, J = 13.9, 5.9, 4.5 Hz, 0.5H), 2.35 (m, 2H) 2.30 (m, 2H), 2.04 (m, 1H), 1.86 (m, 1H), 1.67 (m,
3H), 1.54-1.32 (m, 8H), 1.25 (t, J = 7.1 Hz, 3H), 1.19 (t, J = 7.1 Hz, 1.5H). ¹³C NMR (100
MHz, CDCl₃) δ 212.2 (C₆), 176.0 (C₅), 172.9 (C₄), 170.1 (C₃), 99.2 (C₂), 61.1 (CH₃), 60.1
(CH₃), 57.1 (CH), 41.7 (CH₂), 32.3 (CH₃), 29.9 (CH₃), 29.0 (CH₃), 28.7 (CH₂), 27.0 (CH₃),
26.6 (CH₃), 26.1 (CH₃), 25.5 (CH₂), 25.3 (CH₂), 24.6 (CH₃), 23.9 (CH₃), 14.3 (CH₃), 14.1
(CH₃). IR ν 2923; 1641; 1224; 850 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₁H₁₉O₃ (MH⁺) 199.1334, found 199.1330.

2-(Ethoxycarbonyl)-2-fluoro-octanone (14)¹²

To a stirred solution of 13 (19.4 mmol, 3.8 g, 1 equiv) in 100 ml dry MeCN cooled to 0 °C was added Selectfluor (23.2 mmol, 8.2 g, 1.2 equiv). The resulting mixture was then heated in a 55 °C oil bath and stirred overnight. After cooling to room temperature, the reaction was quenched with water (50 ml) and extracted 4x with EtOAc (4x 50 ml). The combined organic layer was dried on Na₂SO₄, filtered and concentrated in vacuo. This resulted in 14, obtaining 4.2 gram (98%) of a white solid.

¹H NMR (400 MHz, CDCl₃) δ 4.20 (q, J = 7.2 Hz, 2H), 2.70 - 2.66 (m, 1H), 2.60 - 2.55 (m, 2H), 2.21 - 2.17 (m, 1H), 1.91 - 1.83 (m, 2H), 1.68 - 1.54 (m, 3H), 1.49-1.32 (m, 3H) 1.35 (t, J = 7.2 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 208.42 (d, J = 21.4 Hz, C₆), 166.8 (d, J = 24.7 Hz, C₇), 98.8 (d, J = 198.8 Hz, C₈), 62.3 (CH₃), 38.6 (CH₂), 32.9 (d, J = 22.0 Hz, CH₃), 27.4 (CH₂), 26.2 (CH₂), 24.1 (CH₃), 21.1 (d, J = 2.5 Hz, CH₂), 13.7 (CH₃). IR ν 2933; 1720; 1259; 1222; 1056; 1022 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₁H₁₉FΟ₂ (MH⁺) 217.1240, found 217.1237.

1-Fluorocyclooct-2-yne-1-carboxylic acid ethyl ester (15)

A solution of KHMDS (0.5 M in toluene, 43.5 mmol, 87.1 ml, 2.25 equiv) was added dropwise to a stirred solution of 14 (19.3 mmol, 4.2 g, 1 equiv) in THF (250 ml) at -78 °C. After the addition was complete the reaction mixture was maintained for 30 min and then Tf₂NPh (21.2 mmol, 7.6 g, 1.1 equiv) in THF (50 ml) was added slowly. After stirring for 1 h, the reaction mixture was allowed to warm to room temperature and stirred overnight. Ethanol (100 ml) was then added and the reaction mixture was concentrated in vacuo. The crude residue was purified by flash column chromatography (50:1 PE:EtOAc), which yielded 3.3 g (88%) of 15 as a yellow oil.

¹H NMR (400 MHz, CDCl₃) δ 4.25 (q, J = 7.1, 1.4 Hz, 2H), 2.40 - 2.18 (m, 4H), 2.06 - 1.80 (m, 4H), 1.75 - 1.63 (m, 1H), 1.47 - 1.35 (m, 1H), 1.31 (t, J = 7.1 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 168.4 (d, J = 28.4 Hz, C₆), 108.5 (d, J = 10.2 Hz, C₇), 91.9 (d, J = 186.2 Hz, C₈), 87.1 (d, J = 31.8 Hz, C₉), 62.4 (CH₃), 46.3 (d, J = 24.9 Hz, CH₂), 33.9 (CH₂), 29.1 (CH₂), 25.5 (d, J = 1.4 Hz, CH₃), 20.6 (d, J = 2.6 Hz, CH₂), 14.1 (CH₂). IR ν 2935; 1748; 1209; 1145; 1086; 1026 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₁H₁₉FΟ₂ (MH⁺) 199.1056, not found.

1-Fluorocyclooct-2-yne-1-carboxylic acid (16)

Compounds 15 (3.73 mmol, 743 mg, 1 equiv) and LiOH (7.46 mmol, 180 mg, 2 equiv) were combined in 50% aqueous MeOH (20 ml). This mixture was heated in a 50 °C oil bath for 10 min. The reaction was allowed to cool to room temperature and stirred an additional 2 h. Next, the mixture was cooled to 0 °C, diluted with H₂O (10 ml) and acidified to pH ~2 with dilute aq. HCl solution. The mixture was extracted 3x with EtOAc (3x 50 ml) and the combined organic layer was dried over Na₂SO₄, filtered and concentrated in vacuo. The crude product was purified by flash chromatography (1:1 PE:EtOAc) to afford 16 as a yellow oil (264 mg, 91%).

¹H NMR (400 MHz, CDCl₃) δ 10.12 (bs, 1H), 2.70 - 2.61 (m, 1H), 2.41 - 2.23 (m, 3H), 2.10 - 1.80 (m, 3H), 1.80 - 1.58 (m, 2H), 1.57 - 1.41 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 173.7 (d, J = 28.9 Hz, C₆), 109.1 (d, J = 9.9 Hz, C₇), 91.2 (d, J = 185.6 Hz, C₈), 86.0 (d, J
= 31.7 Hz, C₆), 46.0 (d, j = 24.7, CH₂), 33.6 (CH₂), 28.8 (CH₂), 25.2 (d, j = 1.2 Hz, CH₂), 20.3 (CH₂). IR v 2931; 2360; 1731; 1205; 1143 cm⁻¹. HRMS (FAB+) m/z calculated for C₉H₁₂FO₂ (MH⁺) 171.0821, found 171.0818.

**N-[^Boc]-N’-((1-fluorocyclooct-2-ynyl)carbonyl)-1,4-piperazine (17)**

To a solution of 16 (1.47 mmol, 250 mg, 1 equiv), HATU (1.76 mmol, 669 mg, 1.2 equiv), HOAt (1.76 mmol, 239 mg, 1.2 equiv) and DIEA in THF (30 ml) was added Boc-piperazine (1.76 mmol, 328 mg, 1.2 equiv) and the mixture was stirred overnight. The solvent was evaporated in vacuo and the residue was purified by flash chromatography (10:1 PE:EtoAc), resulting in 325 mg of yellow oil 17 (65%).

¹H NMR (400 MHz, CDCl₃) δ 3.70 (m, 2H), 3.60-3.42 (m, 6H), 2.71-2.58 (m, 1H), 2.38 – 2.15 (m, 3H), 1.93 (m, 2H), 1.84 – 1.73 (m, 3H), 1.68 – 1.61 (m, 1H), 1.45 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 154.7 (C₆), 108.8 (d, J = 28.7 Hz, C₆), 91.7 (d, J = 182.5 Hz, C₆), 87.9 (d, J = 25.4 Hz, C₆) 80.3 (C₆), 46.3 (CH₂), 45.4 (d, J = 24.1 Hz, CH₂) 42.8 (CH₂) 34.0 (CH₂), 29.4 (CH₂), 28.5 (CH₂) 26.2 (d, J = 1.2 Hz, CH₂), 20.7 (CH₂).IR v 2931; 1699; 1664; 1417; 1243; 1170 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₈H₂₃FN₂O₃ (MH⁺) 339.2084, found 339.2089.

**N-((1-fluorocyclooct-2-ynyl)carbonyl)-1,4-piperazine (18)**

Compound 17 (0.89 mmol, 300 mg, 1 equiv) was dissolved in TFA/DCM (10 ml, 1:1) for 30 seconds. The mixture was diluted with toluene (50 ml) and evaporated in vacuo. Flash column chromatography (1-10% MeOH in DCM) yielded 321 mg of a white powder 18 (>99%).

Mp 40-42 °C. ¹H NMR (400 MHz, CDCl₃) δ 9.73 (bs, 1H), 4.05 (m, 4H), 3.19 (m, 4H), 2.66 (m, 1H), 2.39 – 2.18 (m, 3H), 1.95 (bs, 2H), 1.85 – 1.56 (m, 4H). ¹³C NMR (100 MHz, CDCl₃) δ 165.3 (d, J = 26.7 Hz, C₆), 110.2 (d, J = 10.4 Hz, C₆) 91.6 (d, J = 181.9 Hz, C₆) 86.9 (d, J = 30.6 Hz, C₆) 45.1 (d, J = 23.9 Hz, CH₂), 43.5 (CH₂), 39.7 (CH₂) 34.0 (CH₂), 29.3 (CH₂), 26.2 (CH₂), 20.7 (CH₂).IR v 2934; 2359; 1667; 1438 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₈H₂₃FN₂O (MH⁺) 239.1560, found 239.1555.

**oS2-cyclooctyne**

To a solution of 1,2,4,5-tetrabromodurene T₄ (1.11 mmol, 500 mg, 3 equiv) and DIEA (0.74 mmol, 122 μl, 2 equiv) in dry MeCN (70 ml), 17 (0.37 mmol, 88.3 mg, 1 equiv) was added dropwise as a solution in dry MeCN (10 ml). The mixture was stirred for 1 h at room temperature. The solvent was evaporated in vacuo until 1 ml of MeCN was remaining. Et₂O was added until a white powder precipitated from the solution. The solvent was removed and this was repeated 3x. This resulted in 156 mg (67%) of the product **oS2-cyclooctyne**, being a mixture of scaffold and DIEA salt (1:0.12). The scaffold was used without further purification.

¹H NMR (400 MHz, D₂O/CD₃CN 9:1) δ 7.88 (s, 2H), 5.32 (s, 4H), 5.12 (s, 4H), 4.53 (s, 2H), 4.33 (m, 2H), 4.02 (m, 4H), 2.87 (m, 1H), 2.66 (m, 3H), 2.27 (m, 2H), 2.11-1.98 (m, 3H), 1.88-1.80 (m, 1H). ¹³C NMR (100 MHz, D₂O/CD₃CN 9:1) δ 165.3 (C₆), 137.7 (C₆), 132.6 (C₆), 125.7 (CH), 111.1 (d, J = 10.6 Hz, C₆), 91.9 (d, J = 180.6 Hz, C₆), 85.3 (d, J = 31.7 Hz, C₆), 66.5 (CH₂), 59.6 (CH₂), 58.7 (CH₂) 44.3 (d, J = 24 Hz, CH₂), 41.0 (CH₂) 37.6 (CH₂), 32.9 (CH₂), 28.9 (CH₂), 28.1 (CH₂), 25.0 (CH₂), 19.4 (CH₂). ¹⁹F NMR (282 MHz,
D$_2$O/CD$_3$CN 9:1) $\delta$ 75.3. IR v 2930; 1649; 1440; 1219; 950; 606 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{23}$H$_{25}$Br$_3$F$_3$N$_2$O (M$^+$) 527.0533, found 527.0535.

N-$\text{Boc-N'-(allyloxycarbonyl)}$-1,4-piperazine (19)

Boc-piperazine (0.82 mmol, 153 mg, 1 equiv), allyl chloroformate (1.23 mmol, 0.13 ml, 1.5 equiv) and TEA (1.23 mmol, 0.17 ml, 1.5 equiv) were dissolved in DCM (20 ml) and stirred overnight at room temperature. The solvent was evaporated in vacuo and the crude mixture was dissolved in EtOAc (20 ml), washed with 1 M KHSO$_4$ (20 ml), a saturated solution of NaHCO$_3$ (20 ml) and brine (20 ml). The organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. This afforded 206 mg of a yellow oil (93 %).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 5.91 – 5.85 (m, 1H), 5.24 (ddd, $J = 17.2, 3.1, 1.5$ Hz, 1H), 5.16 (ddd, $J = 10.5, 2.6, 1.2$ Hz, 1H), 4.55 (dt, $J = 5.6, 1.4$ Hz, 2H), 3.41 – 3.35 (m, 8H), 1.41 (s, 9H). $^{13}$C-NMR (100MHz, CDCl$_3$): 154.9 (C$_4$), 154.5 (C$_1$), 132.8 (CH), 117.5 (CH$_2$), 80.0 (C$_{8}$), 66.1 (CH$_3$) 43.5 (CH$_2$), 28.3 (CH$_3$). IR v 1698; 1416; 1231 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{13}$H$_{23}$N$_2$O$_4$ (MH$^+$) 271.1655, found 271.1658.

N-(allyloxy carbonyl)-1,4-piperazine (20)

Compound 19 (0.76 mmol, 206 mg, 1 equiv) was dissolved in DCM/TFA (20 ml, 1:1). The mixture was stirred for 1 h. The solvent was evaporated in vacuo. Product 20 (254 mg, >99 %) was found as a brown oil.

$^1$H-NMR (400 MHz, MeOD): $\delta$ 9.51 – 5.84 (m, 1H), 5.24 (ddd, $J = 17.2, 1.4$ Hz, 1H), 5.20 (dd, $J = 10.5, 1.1$ Hz, 1H), 4.57 (d, $J = 5.6$ Hz, 2H), 3.74 (s, 4H), 3.15 (s, 4H). $^{13}$C-NMR (100 MHz, MeOD): 154.6 (C$_4$), 132.2 (CH), 118.5 (C$_1$), 67.0 (CH$_2$), 43.4 (CH$_3$), 40.6 (CH$_3$). IR v 1672; 1438; 1257; 1171; cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_9$H$_{12}$N$_2$O$_2$ (MH$^+$) 171.1136, found 171.1134.

oS2$^-$

To a solution of 1,2,4,5-tetraminobromourene T4 (1.11 mmol, 500 mg, 3 equiv) and DIEA (0.74 mmol, 122 $\mu$l, 2 equiv) in dry MeCN (70 ml), 20 (0.37 mmol, 63 mg, 1 equiv) was added dropwise as a solution in dry MeCN (10 ml). The mixture was stirred for 1 h at room temperature. The solvent was evaporated in vacuo. The mixture was re-dissolved in MeCN (10 ml) and Et$_3$O was added until a white powder precipitated from the solution. The solvent was removed and this was repeated 3x. This resulted in 140 mg (68%) of the product oS2$^-$, being a mixture of scaffold and DIEA salt (1:0.07). The scaffold was used without further purification.

$^1$H NMR (400 MHz, D$_2$O/CD$_3$CN 9:1) $\delta$ 7.61 (s, 2H), 6.07 (m, 1H), 5.44 (dd, $J = 10.4, 1.2$ Hz, 1H), 5.34 (dd, $J = 10.4, 1.2$ Hz, 1H), 5.03 (s, 4H). $^{13}$C NMR (100 MHz, D$_2$O/CD$_3$CN 9:1) $\delta$ 155.2 (C$_4$), 139.0 (C$_3$), 134.3 (C$_9$), 133.7 (CH), 127.1 (CH), 117.8 (CH$_2$), 67.7 (CH$_2$), 67.1 (CH$_3$), 60.2 (CH$_3$), 39.7 (CH$_3$), 30.2 (CH$_3$). IR v 2977; 2250; 1678; 1445; 1248; 1149; 933; 733; 616 cm$^{-1}$. HRMS (FAB+) $m/z$ calculated for C$_{15}$H$_{23}$Br$_3$N$_2$O$_4$ (M$^+$) 459.0107, found 459.0107.

2-(Tritylmercapto)acetic acid (21)

Bromoacetic acid (7.48 mmol, 1.0 g, 1 equiv) and triphenylmethanethiol (8.22 mmol, 2.3 g, 1.1 equiv) were dissolved in DMF (8 ml). DIEA (9.27 mmol, 1.61 ml, 1.24 equiv) was added. The mixture was stirred at room temperature for 4 h. The solvents were evaporated in vacuo. The residue was re-dissolved in DCM and purified by flash chromatography (9:1
PE:EtOAc – 1:1 PE:EtOAc). Product 21 1.3 g (>99%) was obtained as a light yellow solid.

$^1$H-NMR (400 MHz, MeOD): δ 7.38 (m, 6H), 7.27 (m, 6H), 7.22 (m, 3H), 2.91 (s, 2H). $^{13}$C-NMR (100 MHz, MeOD): 173.1 (C), 145.6 (C), 130.7 (CH), 129.0 (CH), 128.0 (CH), 35.7 (CH$_3$). IR ν: 3055, 1708, 743, 700 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{21}$H$_{19}$O$_2$S (MH$^+$) 335.1108, found 335.1106.

$\textbf{N-Fmoc-N'}-(2-(tritylmercapto)acetyl)-1,4-piperazine (22)$

Fmoc-piperazine (0.40 mmol, 156 mg, 1 equiv), 21 (0.48 mmol, 161 mg, 1.2 equiv), HBTU (0.6 mmol, 228 mg, 1.5 equiv) and DIEA (1.0 mmol, 174 µl, 2.5 equiv) were dissolved in THF (20 ml) and stirred overnight at room temperature. The solvent was evaporated in vacuo and the crude mixture was dissolved in EtOAc (20 ml), washed with 1M KHSO$_4$ (20 ml), a saturated solution of NaHCO$_3$ (20 ml) and brine (20 ml). The organic layer was dried over Na$_2$SO$_4$, filtrated and concentrated in vacuo. This afforded 270 mg of 22 as an orange oil (>99%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.78 (d, J = 7.5 Hz, 2H), 7.57 (t, J = 6.7 Hz, 2H), 7.49 (t, J = 8.5 Hz, 6H), 7.43 (t, J = 7.4 Hz, 2H), 7.39 – 7.23 (m, 11H), 4.52 (s, 2H), 4.24 (t, J = 6.3 Hz, 1H), 3.59 – 3.06 (m, 8H), 2.96 (s, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 167.0 (C$_{\beta}$), 154.9 (C$_{\alpha}$), 143.8 (C$_{\alpha}$), 143.7 (C$_{\alpha}$), 141.3 (C$_{\alpha}$), 129.4 (CH), 128.0 (CH), 127.7 (CH), 127.0 (CH), 126.9 (CH), 124.7 (CH), 119.9 (CH), 67.2 (C$_{\beta}$), 60.3 (CH$_2$), 47.3 (CH$_3$), 45.5 (CH$_2$), 43.8 (CH$_3$), 43.3 (CH$_2$), 41.4 (CH$_3$), 34.4 (CH$_2$). IR ν: 1736, 1372, 1233, 1043 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{40}$H$_{37}$N$_5$O$_2$S (MH$^+$) 625.2523, found 625.2525.

$\textbf{N-(2-(tritylmercapto)acetyl)-1,4-piperazine (23)}$

Compound 22 (0.40 mmol, 250 mg, 1 equiv) was dissolved in THF/DEA (30 ml, 1:1). The solution was stirred for one h. The solvent was evaporated in vacuo. The product was purified by flash chromatography (5% MeOH and 2% NH$_3$ in 93% DCM), yielding 124 mg (77%) of a brown oil 23.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.46 (d, J = 7.2 Hz, 6H), 7.29 (t, J = 7.2 Hz, 6H), 7.21 (t, J = 8.0 Hz, 3H), 3.51 (t, J = 4.8 Hz, 2H), 3.01 (t, J = 4.8 Hz, 2H), 2.93 (s, 2H), 2.77 (t, J = 4.8 Hz, 2H), 2.70 (t, J = 4.8 Hz, 2H), 2.59 (bs, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 167.0 (C$_{\beta}$), 144.0 (C$_{\alpha}$), 129.5 (CH), 128.1 (CH), 126.9 (CH), 67.2 (C$_{\beta}$), 47.0 (CH$_2$), 46.3 (CH$_3$), 45.6 (CH$_2$), 42.7 (CH$_3$), 34.7 (CH$_2$). IR ν: 1637, 1444, 841, 701 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{23}$H$_{27}$N$_5$O$_2$S (MH$^+$) 403.1843, found 403.1844.

$\textbf{oS2-S(Trt)}$

To a solution of 1,2,4,5-tetraphenylmethylene T4 (0.52 mmol, 235 mg, 3 equiv) and DIEA (0.34 mmol, 56 µl, 2 equiv) in dry MeCN (35 ml), 23 (0.17 mmol, 70 mg, 1 equiv) was added dropwise as a solution in dry MeCN (5 ml). The mixture was stirred for 1 h at room temperature. The solvent was evaporated in vacuo. The mixture was re-dissolved in MeCN (5 ml) and Et$_2$O was added until a white powder precipitated from the solution. The solvent was removed and this was repeated 3x. This resulted in 146 mg (92%) of the product oS2-S(Trt), being a mixture of scaffold and DIEA salt (1:0.77). The scaffold was used without further purification.

$^1$H NMR (400 MHz, D$_2$O/CD$_3$CN 9:1) δ 7.75 (s, 2H), 7.71-7.69 (m, 6H), 7.63 – 7.54 (m, 9H), 5.12 (s, 4H), 5.01 (s, 4H), 4.04 (br s, 2H), 3.80 (br s, 4H), 3.64 (s, 4H). $^{13}$C NMR (100 MHz D$_2$O/CD$_3$CN 9:1) δ 168.1 (C$_{\beta}$), 143.0 (C$_{\alpha}$), 137.7 (C$_{\alpha}$), 132.4 (C$_{\alpha}$), 128.6 (CH), 127.7
(CH), 126.7 (CH), 125.6 (CH), 66.2 (C₆), 58.6 (CH₃), 58.3 (CH₂), 41.8 (CH₃), 40.3 (CH₂), 36.4 (CH₃), 32.9 (CH₂), 28.8 (CH₃). IR: 1629; 1441, 744, 700 cm⁻¹. HRMS (FAB+) m/z calculated for C₃₃H₃₅Br₂N₂O₇S (M⁺) 691.0891, found 691.0822.

Sodium 4-hydroxybutyrate (24)⁴

\[
\text{NaO} \quad \text{O} \quad \text{OH}
\]

To a solution of butyrolactone (11.6 mmol, 1 g, 1 equiv) in EtOH (3 ml) a solution of NaOH (11.6 mmol, 464 mg, 1 equiv) in water (2 ml) was added. The mixture was refluxed for 5 h and the solvents were evaporated in vacuo. Yielding 24 as a white solid, 1.2 g (81%).

¹H NMR (400 MHz, MeOD) δ 3.57 (t, J = 6.8, 2H), 2.24 (t, J = 7.2, 2H), 1.81 (p, J = 6.8, 2H). ¹³C NMR (100 MHz, MeOD): δ 182.7 (C₆), 63.2 (CH₂), 35.9 (CH₃), 30.4 (CH₂). IR ν 3333; 1549; 1403; 1054 cm⁻¹.

\[\text{N-(benzyloxy carbonyl)-N'-(4-hydroxybutanoyl)-1,4-piperazine (25)}\]

Cbz-piperazine (3.8 mmol, 732 μl, 1 equiv), 24 (4.6 mmol, 580 mg, 1.2 equiv), HBTU (5.7 mmol, 2.1 g, 1.5 equiv) and DIEA (9.5 mmol, 1.5 ml, 2.5 equiv) were dissolved in THF (50 ml) and stirred overnight at room temperature. The solvent was evaporated in vacuo and the crude mixture was dissolved in EtOAc (50 ml), washed with 1 M KHSO₄ (50 ml), a saturated solution of NaHCO₃ (50 ml) and brine (50 ml). The organic layer was dried over Na₂SO₄ and concentrated in vacuo. Column chromatography (2% MeOH in DCM) afforded 0.9 g of 25 as a colorless oil (74%).

¹H NMR (400 MHz, CDCl₃) δ 7.31 (m, 5H), 5.10 (s, 2H), 3.60 (t, J = 5.9 Hz, 2H), 3.54 (s, 2H), 3.45 (m, 6H), 2.43 (t, J = 7.1 Hz, 1H), 1.82 (p, J = 6.7 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 172.2 (C₆), 155.1 (C₆), 136.2 (C₆), 128.5 (CH), 128.1 (CH), 127.9 (CH), 67.4 (CH₂), 61.7 (CH₂), 45.2 (CH₂), 41.3 (CH₂), 30.1 (CH₃), 27.6 (CH₃). IR ν 3439; 1700; 1627; 1428; 1231 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₈H₂₈N₂O₄ (MH⁺) 307.1656, found 307.1658.

\[\text{N-(benzyloxy carbonyl)-N'-(4-oxobutanoyl)-1,4-piperazine (26)}\]

Dess-Martin periodinane (2.24 mmol, 950 mg, 1.2 equiv) was dissolved in dry DCM (60 ml) and the solution was cooled to 0 °C. 25 (1.86 mmol, 570 mg, 1 equiv) was dissolved in dry DCM (10 ml) and added dropwise to the solution. The mixture was stirred for 2 h at 0 °C. The reaction was quenched by adding Et₃O (60 ml), followed by a saturated 1:1 Na₂S₂O₃/NaHCO₃ solution (60 ml). The mixture was stirred for 1 h. The layers were separated and the organic layer was dried on Na₂SO₄. After filtration and evaporation of the solvent, product 26 was obtained as a yellow solid (53 mg, 98%).

¹H NMR (400 MHz, CDCl₃) δ 9.82 (s, 1H), 7.33 (m, 5H), 5.13 (s, 2H), 3.53 - 3.47 (m, 8H), 2.81 (t, J = 6.3 Hz, 2H), 2.62 (t, J = 6.3 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 200.9 (CH), 169.7 (C₆), 155.1 (C₆), 136.4 (C₆), 128.6 (CH), 128.2 (CH), 128.0 (CH), 67.5 (CH₂), 45.1 (CH₂), 43.7 (CH₂), 41.5 (CH₂), 38.5 (CH₂), 25.7 (CH₃). IR ν 1700; 1645; 1426; 1230 cm⁻¹. HRMS (FAB+) m/z calculated for C₁₈H₁₅N₂O₄ (MH⁺) 305.1659, found 305.1659.

\[\text{N-(benzyloxy carbonyl)-N'-(4,4-diethoxybutanoyl)-1,4-piperazine (27)}\]

Compound 26 (1.82 mmol, 553 mg, 1 equiv) was dissolved in EtOH (150 ml). After addition of pTSA (0.182 mmol, 34.6 mg, 0.1 equiv), the mixture was refluxed (90 °C) for 1.5 h. The reaction was quenched with TEA (0.91 mmol, 126 μl, 0.5 equiv) and the solvents
were evaporated in vacuo. The product 27 was obtained as yellow oil (658 mg, 96%).

\[ \text{H NMR (400 MHz, CDCl$_3$)} \delta 7.33 (m, 5H), 5.12 (s, 2H), 4.52 (t, J = 5.2 Hz, 1H), 3.63 (m, 4H), 3.47 (m, 8H), 2.39 (t, J = 7.5 Hz, 2H), 1.93 (td, J = 7.5, 5.2 Hz, 2H), 1.17 (t, J = 7.5 Hz, 6H). \]

\[ \text{C NMR (100 MHz, CDCl$_3$)} \delta 171.2 (C$_6$), 155.0 (C$_5$), 136.3 (C$_4$), 128.4 (CH), 128.1 (CH), 127.9 (CH), 102.0 (CH), 67.3 (CH$_2$), 61.7 (CH$_3$), 45.1 (CH$_2$), 41.5 (CH$_3$), 41.2 (CH$_2$), 29.0 (CH$_3$), 27.7 (CH$_2$), 15.2 (CH$_3$). \]

IR ν 1701; 1646; 1426; 1230 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{20}$H$_{31}$N$_2$O$_5$ (MH$^+$) 379.2233, found 379.2227.

\(N\)-(4,4-dioctoxybutanoyl)-1,4-piperazine (28)\)

Compound 27 (2.25 mmol, 850 mg, 1 equiv) was dissolved in EtOAc and i-PrOH (100 ml, 1:1). Pd/C (50% w/w, 400 mg) was added and the mixture was flushed several times with H$_2$ gas and stirred overnight under H$_2$. After filtration over celite and washing with EtOAc (3x 100 ml), the solvents were evaporated in vacuo and the product was purified by flash chromatography (5% MeOH and 1% TEA in DCM). This resulted in 516 mg 28 (94%) as a yellow oil.

\[ \text{H NMR (400 MHz, MeOD)} \delta 4.54 (t, J = 5.2 Hz, 1H), 3.66 (m, 4H), 3.50 (m, 4H), 2.92 (m, 4H), 2.40 (t, J = 7.4 Hz, 2H), 1.95 (m, 2H), 1.20 (t, J = 7.0 Hz, 1H). \]

\[ \text{C NMR (100 MHz, MeOD)} \delta 170.8 (C$_6$), 101.8 (CH), 61.5 (CH$_2$), 45.0 (CH$_3$), 44.9 (CH$_2$), 44.7 (CH$_3$), 40.9 (CH$_2$), 28.9 (CH$_2$), 27.4 (CH$_2$), 15.0 (CH$_3$). \]

IR ν 2973; 1640; 1440; 1123; 1033 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{12}$H$_{25}$N$_2$O$_5$ (MH$^+$) 245.1865, found 245.1865.

oS2-CHO

To a solution of 1,2,4,5-tetra-bromodurene T4 (2.20 mmol, 1.0 g, 3 equiv) and DIEA (1.48 mmol, 244 µl, 2 equiv) in dry MeCN (140 ml), 27 (0.74 mmol, 192 mg, 1 equiv) was added dropwise as a solution in dry MeCN (10 ml). The resulting solution was stirred for 1 h at room temperature. The solvent was evaporated in vacuo until 1 ml of MeCN was remaining. Et$_3$O was added until a white powder precipitated from the solution. The Et$_3$O was removed and this procedure was repeated 3x, resulting in 354 mg (71%) of the product oS2-CHO, being a mixture of scaffold and DIEA salt (1:0.30). The scaffold was used without further purification.

\[ \text{H NMR (400 MHz, CDCl$_3$)} \delta 7.71 (s, 2H), 5.15 (s, 4H), 4.94 (s, 4H), 4.12 (m, 4H), 3.95 – 3.45 (m, 10H), 2.69 (t, J = 7.4 Hz, 2H), 2.05 (q, J = 7.4 Hz, 2H), 1.33 (t, J = 7.8Hz, 6H). \]

\[ \text{C NMR (100 MHz, D$_2$O/CD$_3$CN 9:1)} \delta 172.7 (C$_6$), 137.6 (C$_5$), 132.4 (C$_4$), 125.5 (CH), 101.4 (CH), 66.1 (CH$_2$), 61.9 (CH$_2$), 58.6 (CH$_3$), 39.9 (CH$_2$), 36.2 (CH$_2$), 28.9 (CH$_2$), 27.9 (CH$_2$), 26.6 (CH$_2$). \]

IR ν 2976; 1652; 1443; 1366; 1249; 1161; 1110; 843 cm$^{-1}$. HRMS (FAB+) m/z calculated for C$_{22}$H$_{33}$Br$_2$N$_2$O$_5$ (MH$^+$) 533.0839, found 533.0840.

\(N\)-(benzyloxycarbonyl)-N'-(2-(N-Boc-aminoxy)acetyl)-1,4-piperazine (29)\)

To a solution of (Boc-aminoxy)acetic acid (2.1 mmol, 400 mg, 1.2 equiv), HBTU (2.1 mmol, 797 mg, 1.2 equiv) and DIEA (4.4 mmol, 0.7 ml, 2.5 equiv) in THF (30 ml) was added 1-Cbz-piperazine (1.7 mmol, 327 µl, 1 equiv). The suspension was stirred overnight, after which the suspension became a clear solution. The solvent was evaporated in vacuo. The residue was re-dissolved in EtOAc (30 ml) and extracted with 1M KH$_2$SO$_4$ (30 ml), a saturated solution of NaHCO$_3$ (30 ml) and brine (30 ml). Drying on MgSO$_4$, filtration and evaporation in vacuo resulted in 751 mg (>99%) of 29 as a yellow oil.

\[ \text{H NMR (400 MHz, CDCl$_3$)} \delta 8.19 (s, 1H), 7.33 (m, 5H), 5.13 (s, 2H), 4.52 (s, 2H), 3.57 (s, 2H), 3.51 (s, 4H), 3.38 (s, 2H), 1.44 (s, 9H). \]

\[ \text{C NMR (100 MHz, CDCl$_3$)} \delta 166.9 (C$_6$), \]

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156.2 (C\textsubscript{9}), 155.1 (C\textsubscript{9}), 136.3 (C\textsubscript{9}), 128.6 (CH), 128.3 (CH), 128.1 (CH), 73.4 (CH\textsubscript{2}), 67.6 (CH\textsubscript{2}), 44.4 (CH\textsubscript{2}), 43.5 (CH\textsubscript{2}), 41.5 (CH\textsubscript{2}), 28.2 (CH\textsubscript{3}). IR \textnu \textperiodcentered 3175; 2938; 1690; 1629; 1427; 1228; cm\textsuperscript{-1}. HRMS (FAB+) \textit{m/z} calculated for C\textsubscript{19}H\textsubscript{28}N\textsubscript{3}O\textsubscript{6} (MH\textsuperscript{+}) 394.1978, found 394.1976.

\textit{N-}(2-(N-\textit{Boc}-aminoxy)acetyl)-1,4-piperazine (30)  

\[\text{HN} - \text{O} \quad \text{O} \quad \text{NH}_{\text{Boc}}\]

Compound 29 (1.7 mmol, 668 mg, 1 equiv) was dissolved in EtOAc and i-PrOH (250 ml, 1:1). Pd/C (50\% w/w, 300 mg) was added and the mixture was flushed several times with H\textsubscript{2} gas and stirred overnight under H\textsubscript{2}. After filtration over celite and washing with EtOAc (3x 150 ml), the solvents were evaporated \textit{in vacuo} and the product was purified by flash chromatography (8\% MeOH and 2\% 7M ammonia in MeOH in DCM). This resulted in 275 mg (63\%) of a colorless oil 30.

\textsuperscript{1}H NMR (400 MHz, MeOD) \textdelta 4.85 (bs, 2H), 4.49 (s, 2H), 3.51 (m, 4H), 2.80 (m, 4H), 1.43 (s, 9H). \textsuperscript{13}C NMR (100 MHz, MeOD) \textdelta 168.5 (C\textsubscript{9}), 158.6 (C\textsubscript{9}), 82.4 (CH\textsubscript{2}), 75.0 (2x CH\textsubscript{2}), 46.8 (CH\textsubscript{2}), 46.4 (CH\textsubscript{2}), 46.0 (CH\textsubscript{2}), 43.3 (CH\textsubscript{2}), 28.5 (CH\textsubscript{3}). IR \textnu 3369; 2928; 1718; 1633; 1447; 1249; 1163; 837 cm\textsuperscript{-1}. HRMS (FAB+) \textit{m/z} calculated for C\textsubscript{19}H\textsubscript{28}N\textsubscript{3}O\textsubscript{6} (MH\textsuperscript{+}) 260.1610, found 260.1610.

\textit{oS2-ONH\textsubscript{2}}

To a solution of 1,2,4,5-tetramethylurea T4 (3.3 mmol, 1.48 g, 3 equiv) and DIEA (2.2 mmol, 363 \textmu l, 2 equiv) in dry MeCN (170 ml), 30 (1.1 mmol, 273 mg, 1 equiv) was added dropwise as a solution in dry MeCN (30 ml). The mixture was stirred for 1 h at room temperature. The solvent was evaporated \textit{in vacuo}. The mixture was re-dissolved in MeCN (10 ml) and Et\textsubscript{2}O was added until a white powder precipitated from the solution. The Et\textsubscript{2}O was removed and this procedure was repeated 3x, resulting in 710 mg (97\%) of the product \textit{oS2-ONH\textsubscript{2}}, being a mixture of scaffold and DIEA salt (1:0.17). The scaffold was used without further purification.

\textsuperscript{1}H NMR (400 MHz, D\textsubscript{2}O/CD\textsubscript{3}CN 9:1) \textdelta 7.81 (s, 2H), 5.25 (s, 4H), 5.05 (s, 4H), 4.90 (s, 2H), 4.17 – 4.23 (m, 4H), 3.96 (m, 4H), 1.73 (s, 9H). \textsuperscript{13}C NMR (100 MHz, D\textsubscript{2}O/CD\textsubscript{3}CN 9:1) \textdelta 167.7 (C\textsubscript{9}), 157.2 (C\textsubscript{9}), 137.6 (C\textsubscript{9}), 132.5 (C\textsubscript{9}), 125.6 (CH), 82.5 (CH\textsubscript{2}), 72.6 (CH\textsubscript{2}), 66.3 (CH\textsubscript{2}), 65.1 (CH\textsubscript{2}), 59.5 (CH\textsubscript{2}) 58.4 (CH\textsubscript{2}) 39.3 (CH\textsubscript{2}) 36.3 (CH\textsubscript{2}), 28.9 (CH\textsubscript{2}), 26.7 (CH\textsubscript{2}). IR \textnu 2971; 1633; 1452; 1132; 1059; 999; 937; 615 cm\textsuperscript{-1}. HRMS (FAB+) \textit{m/z} calculated for C\textsubscript{19}H\textsubscript{30}Br\textsubscript{2}N\textsubscript{3}O\textsubscript{4} (M\textsuperscript{+}) 394.1978, found 394.1976.
2.6 REFERENCES