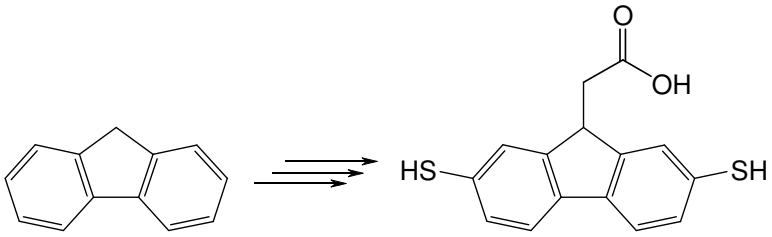
Peptide self-replication driven by self-assembly

Synthesis of 2-(2,7-dimercapto-9H-fluoren-9-yl)acetic acid



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List of abbreviations

m-CPBA	-	*meta*-Chloroperbenzoic acid
DABCO	-	1,4-diazabicyclo[2.2.2]octane
DCM	-	Dichloromethane
DMF	-	Dimethylformamide
DMSO	-	Dimethyl sulfoxide
MeOH	-	Methanol
DCC	-	Dynamic combinatorial chemistry
DCL	-	Dynamic combinatorial libraries
~GLKLK	-	Peptide chain with ~Gly-Leu-Lys-Leu-Lys

Abstract

Self-replication of molecules is probably an essential process in the origin of life. Self-replicating molecules can be observed with dynamic combinatorial chemistry and already a small numbers of self-replicators are found with this technique. Peptide-derived self-replicating behaviour is achieved with small dynamic combinatorial libraries of a self-binding building block. Fibres are formed from the macrocycles of the building block, because of the stabilisation by the β-sheets between the peptides of the building blocks. To understand better which factors provide the self-assembly and self-replication of molecules, varying the building block structure is a useful tool. Here the first steps of the synthesis of 2-(2,7-dimercapto-9H-fluoren-9-yl)acetic acid out of fluorene is described.

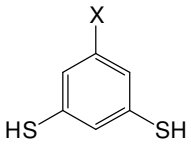
Introduction

Self-replicating systems very likely played a central role in the organisation of the very first organisms and the formation and assembly of prebiotic systems [1]. These processes are still far from understood. By studying the self replication of small molecules in an aqueous medium, researchers try to understand which factors stabilise the far-from-equilibrium distribution characterizing even simple organisms. The group of S. Otto reported self-replicating peptide-derived macrocycles that emerge from a small dynamic combinatorial library [2], thereby providing information about the possible role of self-assembly and self-replication in the origin of life.

Dynamic combinatorial chemistry

Dynamic combinatorial chemistry (DCC) is a useful tool for the discovery of self-replicating systems. DCC uses large sets of molecules, which are called dynamic combinatorial libraries (DCLs). In a DCL, building blocks are added to the same environment where they can interact with each other. The building blocks are linked by non-covalent or reversible covalent bonds, forming components of the DLC. These components are in equilibrium and can interconvert by a reversible process. The composition is determined by the thermodynamic stability of the components; the more stable a component, the larger its concentration in the DCL [3]. One

main product can emerge form the library, which is not the most thermodynamic stable product, but a kinetic product formed by self-replication (See fibril formation).



X = Peptide chain ~GLKLK

*Figure 1. Building block **1***

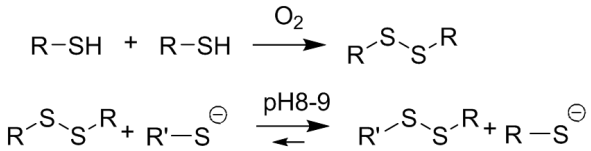


Figure 2. Thiol oxidation and dynamic disulfide exchange

Carnall et al. studied the DCLs of an aromatic building block (building block **1**) containing two thiol groups and a peptide chain (figure 1). The thiols can form disulfide bonds in the presence of oxygen by thiol oxidation (figure 2). In an aqueous media with a pH of 8-9, the disulfide bonds can exchange, so one of the building blocks can be replaced by another [6]. Building block **1** is a useful tool for setting up DCLs because the thiols in this molecule can exchange, forming a dynamic system. During thiol oxidation, the building blocks bind each other covalently, producing a mixture of macrocycles of different sizes, which are all in equilibrium (figure 3). [2]

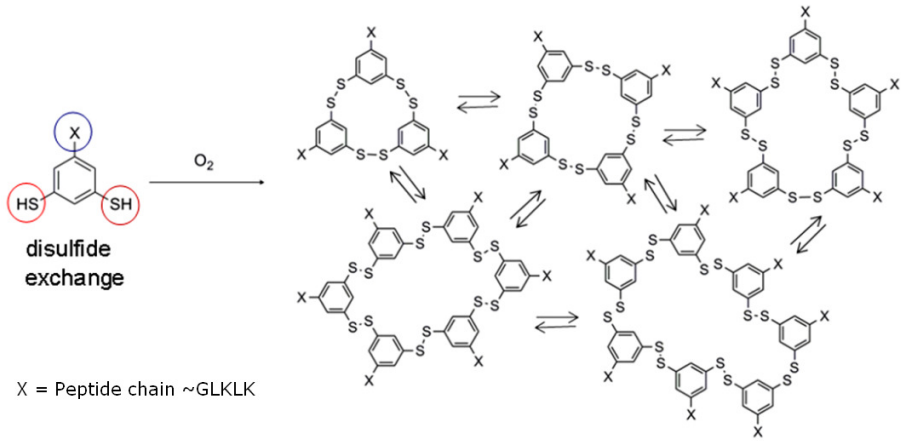


*Figure 3. Schematic illustration of the DCL of building block **1***

Fibril formation

DCC is used to make a self-assembling system of building block **1**, where macrocycles are formed [2]. At the beginning of the reaction, only monomers of the building block are present, but soon thereafter trimers and tetramers are formed. After about 15 days the trimer and tetramers disappear and a larger macrocycle is formed, the hexamer or heptamer (figure 4).

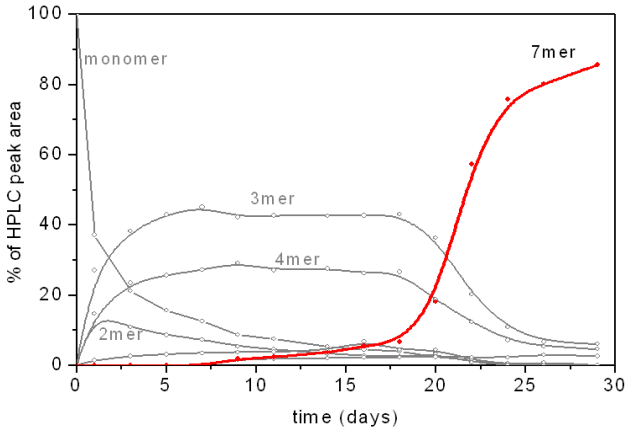


Figure 4. Evolution of product distribution in time during the formation of the heptamer fibers. The evolution of the macrocycles during the reaction is followed with high performance liquid chromatography (HPLC). The components corresponding to the peaks of the HPLC analyses are determined with mass-spectroscopy.

Building block **1** has a peptide chain. The peptide chain used by Carnall et al. contains the amino acids glycine, leucine and lysine. The arrangement of alternating hydrophobic (leucine) and hydrophilic (lysine) amino acid residues of the peptide chain is known to be favorable for β-sheet formation [4]. The macrocycles are able to stack on top of each other because of this β-sheet formation. Only for the large macrocycles, the hexamer and the heptamer, the non-covalent interactions are strong enough to form fibers (figure 5). The equilibrium of all components (figure 3) will shift to this macrocycle [2].

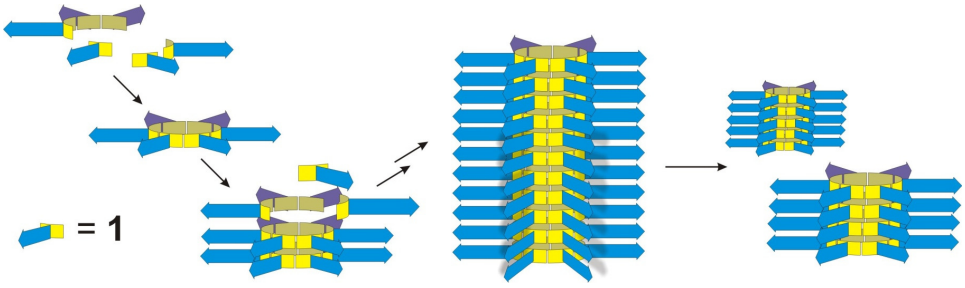


*Figure 5. Illustration of the self assembly of building block **1** into fibers; The monomer is oxidized forming macrocycles, which then stack into fibers, stabilized by the β-sheet between the peptide chains. Shear stress, a mechanical force, can break the stabilised fibre.*

When shear stress is applied to the library, the fibers can break (see figure 5, last step). More fibre-ends are formed, which is the location where fibre formation takes place, which results in more fibres. The fibre formation starts once the macrocycle is formed which can be stabilised into fibres, resulting in "trapping" the compounds of the library into the fibers of the stabilised macrocycle (figure 4)

So the reaction starts with thermodynamically controlled self-assembly of the building block and ends in a kinetic product: the fibres of the stabilised macrocycle. This kinetic product is only formed if the non-covalent interactions between the peptides are strong or numerous enough to stabilise the fibers. J. Carnall concludes that "such a transition from thermodynamically controlled self-assembly to kinetic control must have been an important step in the origin of life, as life is far from equilibrium" [2].

Building block design

The current study aims to synthesize a new building block (building block **2**) (figure 6). This building block consists of fluorene with two thiol-groups and a carboxyl acid. A peptide chain can be connected to this carboxyl acid. Future studies may investigate whether building block **2**, with a peptide chain, is able to form macrocycles and fibres, in similar manner to building block **1**.

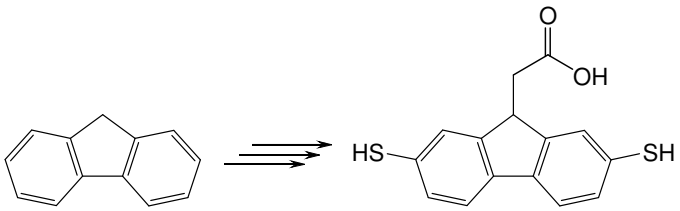


*Figure 6. Synthesis of Building block **2***

Building block **2** has a larger conjugated π-system than building block **1** and therefore can potentially form stronger pi-pi interactions. Flat aromatic rings such as building block **2** can arrange themselves on top of each other, especially because of the strong Van der Waals bonding between the surfaces of these rings [5]. This phenomenon is called aromatic stacking. Future studies will show if the pi-pi interactions have an influence on the fibre formation.

Another difference between the building blocks is the angle between the peptide chain and the thiols. This angle is smaller in building block **2** than in building block **1**. Moreover, the aromatic ring system of molecule **2** is larger. Because aromatic rings can not bend, it might be more difficult to form small rings of building block **2** (figure 7). It is possible that the strain in a small ring of building block **2** will be larger then in the same macrocycle of building block **1**. Therefore, the macrocycles of building block **2** might be larger.

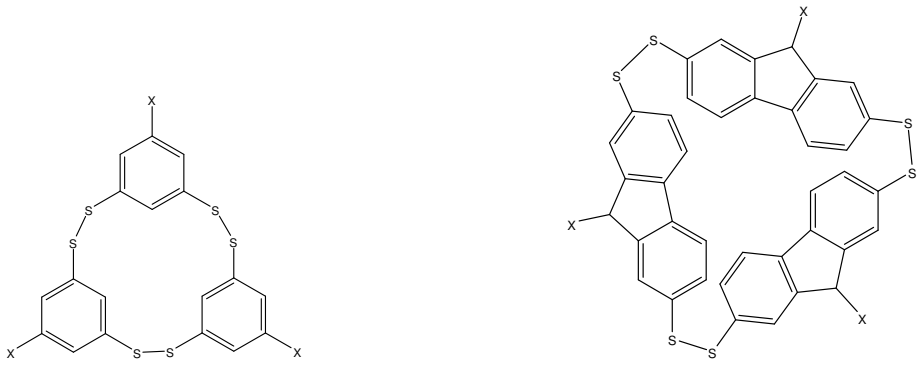
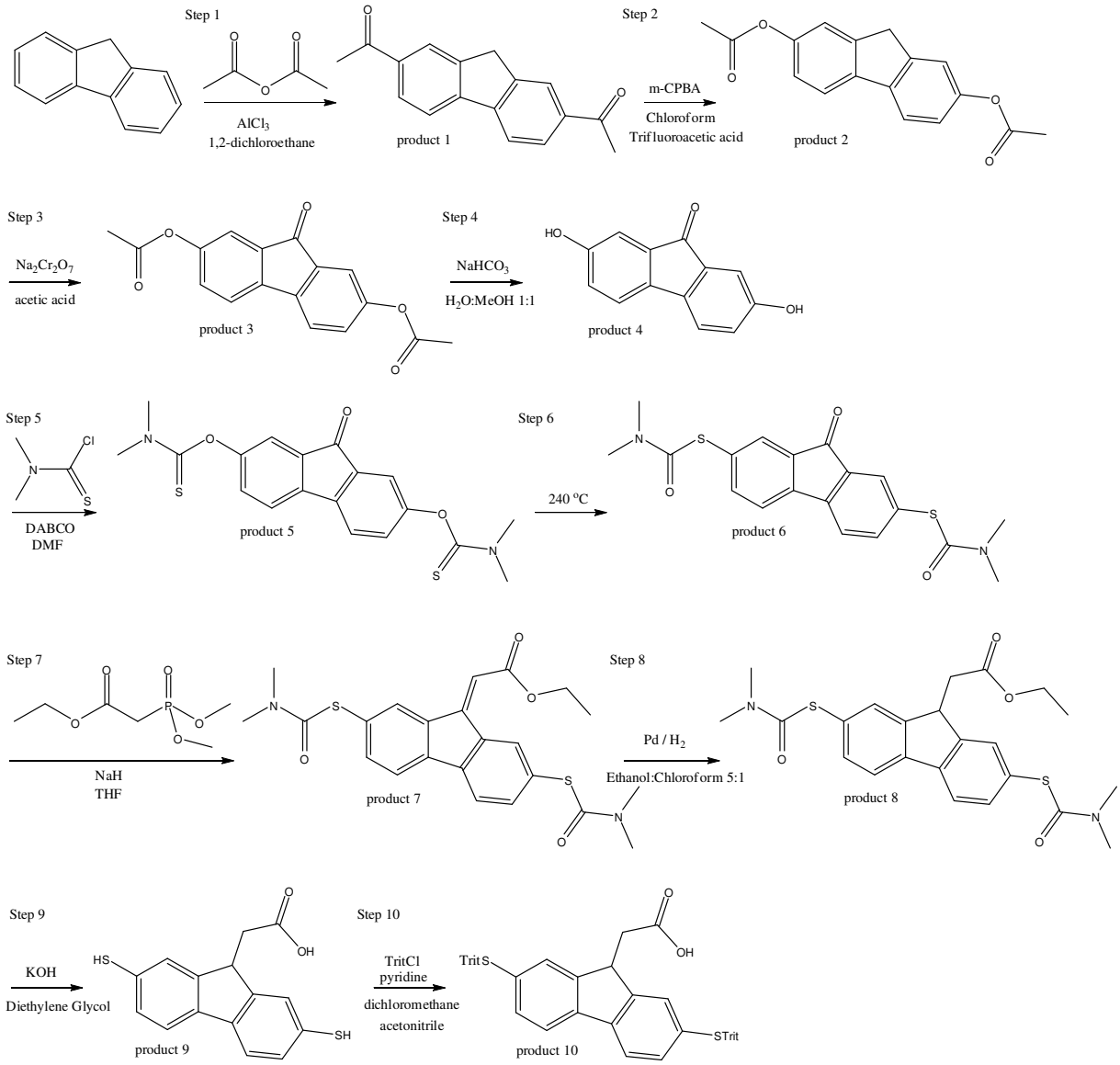


*Figure 7. Trimer of building block **1** (right) and building block **2** (left)*

Future studies will analyze which macrocycles of building block **2** are formed, and when the non-covalent interactions are large enough to stabilise the fiber formation. However, before this study can be carried out, the building block should be synthesised. Therefore we aim to synthesise building block **2** using a ten-step synthesis starting with fluorene.

Results and Discussion

We aimed to synthesise building block **2** using a ten-step synthesis starting with fluorene (Scheme 1). Because of the lack of time, only the first six steps are done. First fluorene reacted with acetic anhydride and aluminium chloride to 2,7-diacetylfluorene. This product was oxidized with m-CPBA to 2,7-fluorenediol diacetate. The esters at 2 and 7 position (figure 8) were hydrolysed to give to the alcochol, after the fluorene was oxidized at the 9 position with sodium chromate, forming 2,7-dihydroxy-9-fluorenone. Then dimethylthiocarbamoyl chloride was reacted with the product, and after a rearrangement 2.7-Bis(dimethylcarbamoylthio)-9H-fluoren-9-one was formed.



*Scheme 1. Formation of building block **2***

Synthesis of 2,7-fluorenediol diacetate [7,8]

In the first step acetyl chloride was formed by the reaction of acetic anhydride with aluminium chloride in 1,2-dichloroethane. This solution was dropwise added to fluorene and twice a Friedel-Crafts acylering occurred forming 2,7-diacetylfluorene (product **1**).[7] After the reaction was quenched with ice-HCL, the aqueous layer was decanted and the residue was dissolved in hot acetone, according to the route described by Sulzberg et. al.[7] Product was still present in the decanted aqueous layer, which was collected by filtration. It turned out to be easier to filter the mixture after it was quenched with HCl, and wash the residue with hot acetone, which was done the second time. The acetone mixture was cooled, and filtered again to improve the yield.

Once a separation of two layers was found after the reaction was quenched. The product was found when the solvent of the organic layer was evaporated. After collection of the product by filtration of the cooled acetone layer, the solid was dried under vacuum. The product was obtained in yields between 68 and 73%.

The next step was a Baeyer-Villiger rearrangement between product **1** and m-CPBA, a peroxy acid which was used as strong oxidizing agent as described in Synth. Commun[8]. Because m-CPBA is sensitive to light, aluminium foil was put around the flask. A catalytic amount of trifluoroacetic acid was added to make the reaction mixture more acetic and the ketone groups more reactive. After stirring the mixture for 3 days product **2** (2,7-fluorenediol diacetate) was formed. After recrystallization the product was obtained in yields between 44 and 50 %. Probably product was lost in the purification steps, when the mixture was washed with water and during the recrystallization

The product was recrystallized from chloroform and methanol. The impurity is m-CPBA, which was added in excess, and probably the reacted form of m-CPBA. It is difficult to separate these two compounds through recrystallization; it helps to add a little more solvent then is necessary to dissolve the solid.

Synthesis of 2,7-dihydroxy-9-fluorenone [8,9,10]

The 9 position of fluorene was oxidized with sodium dichromate dihydrate in acetic acid and 2,7-fluoren-9-onediol diacetate was formed, as described by Burke et.al.[8] The reaction time was difficult to predict, so the reaction was followed with TLC. With full conversion, yields up to 90% can be obtained.

Once, after quenching the reaction with water, no solid percipitated. A part of the mixture was neutralized, but still no solid precipitated. Evaporation of the water give a dark green oil. Direct recrystallization of this oil with ethanol gave no product. Dissolving this material in water and extracting it with DCM gave a small amount of solid which was impure. After the solid was twice recrystallized from ethanol, the product was still impure. Why the outcome of this reaction is different, is still unknown.

Burke et al [8] proposed a recrystallization from ethanol, but then a lot of solvent is necessary. So the residue was dissolved in DCM and then ethanol was added. The product crystallized and a lot less solvent was needed. If there was no full conversion, further recrystallization of the residue gave the starting material back.

The fourth step was the deprotection of the diols forming 2,7-dihydroxy-9-fluorenone. Product **3** reacted with sodium bicarbonate in water and methanol. The starting material did not solve in the mixture, but the product of this reaction did. After the reaction was quenched with hydrochloric acid, the mixture was filtered. A portion of the solid was still in the filtrate, which was collected after a part of the solvent had been evaporated. The solid was washed extensively with water to remove the salts. After drying the solid under vacuum, a yield of 90 % was obtained.

Synthesis of 2.7-Bis(dimethylcarbamoylthio)-9H-fluoren-9-one [10,11]

First product **4** and DABCO, a weak base, were dissolved in DMF, before dimethylthiocarbamoyl chloride dissolved in DMF was added. The reaction was finished after 16 hr stirring, and the solid was collected after filtration. This reaction was proposed by Vial et.al.[10] When water was added to the DMF-mixture, more product percipitated. The product, 2,7-Bis(2-dimethylcarbamoylthio)-9H-fluoren-9-one, was dried under vacuum, and obtained in a yield of 49 %. Expected is that all solids were precipitated when water was added to the DMF layer. Because no starting material was found in the product by ^{1}H-NMR, full conversion is expected. Probably product was lost in the purification step, when the solid was washed with water.

It is important the solid is dry, because the presence of water in the next step can lead to side reactions. A ^{1}H-NMR of the product in DMSO is taken, and DMSO always contains water. So it is difficult to decide if the product is dry enough. Experience show that drying the solid 24 hr under vacuum gives a good result.

The next reaction was a Newman-Kwart rearrangement [12]. When product **5** was heated to 240 ºC for 45 minutes, the solid changed to the thermodynamic more stable product, where the sulfur and oxygen atoms were exchanged. The used temperature was high, because the melting point of product **5** was ~235 ºC.[11] After the mixture was cooled, the solid was dissolved in DMF and precipitated again when water was added. Yields between 70 and 95 percent are found. Probably product is lost, if the solid was dissolved in too much DMF.

Conclusion + Future plans

It was aimed to synthesize building block **2** out of fluorene. The synthesis is not finished yet. The synthesis of 2.7-bis(dimethylcarbamoylthio)-9H-fluoren-9-one, with fluorene as starting material, is finished successfully with an overall yield of 14 %.

In the future it will be tried to complete the synthesis. First product **6** will be reacted with triethyl phosphonoacetate and the strong base sodium hydride in THF, forming the alkene. Then the double bond is reduced with palladium on carbon in ethanol and chloroform (5:1). With potassium hydroxide the thiocarbamoyl groups and the ester are deprotected to the thiols and the carboxylic acid, forming the product 2-(2,7-dimercapto-9H-fluoren-9-yl)acetic acid. The last step is the protection of the thiols with triphenylmethyl chloride, which is a standard procedure before the building block is used in the peptide synthesizer. [2, 13, 14]

When the synthesis of building block **2** is succesfully finished, a peptide chain will be connected to the carboxyl group with a microwave peptide synthesizer. The self-replicating behaviour of building block **1** with the peptide chain ~Gly-Leu-Lys-Leu-Lys was studied. If it is possible, the same sequence of amino acids will be connected to building block 2. Next, libraries of the molecule will be made, and the self-assembling and self-replicating behavior will be investigated.

Probably the sequence of the peptide chain has an influence on which macrocycles are stabilized, because if the sequence is changed the strength of the non-covalent interactions changes. In the future it will be interesting to study the self-replicating behavior of building block **1** and **2** with different peptide chains. The results of building block **1** and **2** with the original peptide chain can be compared, but also the results of building block 2 with different peptide chains.

Experimental Section

*2,7-Diacetylfluorene (Product **1**)* [7]

A mixture of aluminium chloride (26.3 g, 197 mmol, 6.8 eq) in 34 ml freshly distilled anhydrous 1,2-dichloroethane was cooled on ice and acetic anhydride (11.0 ml, 111 mmol, 3.8 eq) was added dropwise in 20 min. This gray-yellow mixture was added dropwise to 4.82 g of fluorene (29.0 mmol, 1.0 eq) in 33 ml anhydrous 1,2-dichloroethane and the mixture was refluxed for 4 hr while 30 ml of 1,2-dichloroethane was distilled off. The dark-brown reaction mixture was poured onto ice-HCl and the aqueous phase was discanted. The residue was dissolved in hot acetone, cooled in the cold room and the solid was collected. This solid was dried under vacuum, giving 5.23 g of 2,7-diacetylfluorene (20.9 mmol, 73 % yield).
The reaction was followed by TLC (chloroform/acetonitrile 99:1) ($Rf_{Fluorene}$ = 0.86, $Rf_{product\ 1}$ = 0.71).

^{1}H-NMR (400 MHz, $CDCl_3$): δ/ppm = 2.65 (s, 6H, CH_3), 4.00 (s, 2H, CH_2), 7.89 (d, 2H, J = 4Hz, C*H*CHCCO), 8.02 (d, 2H, J = 4Hz, CHC*H*CCO), 8.16 (s, 2H, C*H*CCO)

*2,7-Fluorenediol diacetate (Product **2**)* [8]

2,7-Diacetylfluorene (11.31 g, 45.2 mmol, 1.0 eq) was dissolved in 480 ml chloroform and 0.36 ml trifluoroacetic acid (0.25 g, 2.22 mmol) and the mixture was cooled on ice. m-CPBA (27.96 g, 162.0 mmol, 3.6 eq) was added and the mixture was protected from light. The chloroform solution was allowed to warm to room temperature and was stirred for four days. The reaction mixture was washed with a saturated sodium bicarbonate solution and with a saturated sodium chloride solution and then dried over magnesium sulphate. The mixture was filtered and the solvent was evaporated under reduced pressure. The residue was recrystallized from chloroform and methanol 1:1, giving 6.38 g of pale yellow crystals (22.6 mmol, 50 %).
The reaction was followed with TLC (chloroform/acetonitrile 99:1) ($Rf_{m\text{-}CPBA}$ = 0.33, $Rf_{product\ 1}$ = 0.71, $Rf_{product\ 2}$ = 0.79)

^{1}H-NMR (400 MHz, $CDCl_3$): δ/ppm = 2.31 (s, 6H, CH_3), 3.88 (s, 2H, CH_2), 7.89 (dd, 2H, J = 8Hz, J = 4Hz, CHC*H*CO), 7.25 (s, 2H, C*H*CO), 7.70 (d, 2H, J = 8Hz, C*H*CHCO)

*2,7-Fluoren-9-onediol diacetate (Product **3**)* [9]

A mixture of product **2** (3.30 g, 11.68 mmol, 1.00 eq), sodium dichromate dehydrate (9.90 g, 33.22 mmol, 2.85 eq) and 33 ml acetic acid was heated until reflux for 45 min, and the colour of the mixture turned from orange to dark green. After the reaction mixture was cooled with ice, 50 ml water was added and a solid precipitated. This green mixture was filtered and the solid was recrystallized from dichloromethane and ethanol, yielding bright yellow 2,7-fluoren-9-onediol diacetate (2.92 g, 9.86 mmol, 85 %).
The reaction was followed with TLC (chloroform/acetonitrile 99:1) ($Rf_{product\ 2}$ = 0.79, $Rf_{product\ 3}$= 0.45)

1H-NMR (400 MHz, $CDCl_3$): δ/ppm = 2.30 (s, 6H, CH_3), 7.19 (dd, 2H, J = 8Hz, J = 4Hz, CHC*H*CO), 7.37 (s, 2H, C*H*CO), 7.47 (d, 2H, J = 8Hz, CHC*H*CO)

*2,7-Dihydroxy-9-fluorenone (Product **4**)* [9] [10]

3.645 g 2,7-Fluoren-9-onediol diacetate (12.3 mmol, 1.0 eq) was added to 260 ml 0.8 M $NaHCO_3$ in H_2O:MeOH 1:1 (208.3 mmol, 16.9 eq) and refluxed for 2 hr. The dark purple mixture was cooled in ice and 60 ml concentrated HCl was added. The dark solid was filtered and washed extensively with water. The filtrate was concentrated to yield another part of solid, which was washed again with water. The dark red solids were combined, yielding 2.402 g of product (11.3 mmol, 92 %)
The reaction was followed with TLC (chloroform/acetonitrile 99:1) ($Rf_{product\ 3}$= 0.64, $Rf_{half\ deprotected}$ = 0.15 $Rf_{product\ 4}$ = 0.0)

^{1}H-NMR (400 MHz, DMSO): δ/ppm = 6.80-6.84 (m, C*H*CO, CHC*H*CO), 7.33 (d, J = 8 Hz, C*H*CHCO), 9.79 (s, O*H*)

*2,7-Bis(2-dimethylthiocarbamoyl)-9H-fluoren-9-one (Product **5**)* [10]

Product **4** (7.30 g, 34.24 mmol, 1.0 eq) was added to a mixture of 2.17 g DABCO (19.3 mmol, 4.11 eq) in 12.0 ml dry DMF under a nitrogen atmosphere. The mixture was cooled with an ice bath and stirred until product **4** dissolved. 2.3 g Dimethylthiocarbamoyl chloride (18.6 mmol, 4.00 eq) was dissolved in 6.0 ml DMF and added dropwise to the reaction mixture. After stirring for 40 hrs the mixture was filtered and the residue was washed extensively with water and dried under vacuum to give 6.44 g of yellow solid (16.66 mmol, 49 %).

^{1}H-NMR (400 MHz, DMSO): δ/ppm = 3.33 (s, -CH_3), 3.37 (s, -CH_3), 7.31 (s, *H*CCO), 7.31-7.33 (m, C*H*CHCO), 7.82 (d, CHC*H*CO, J = 8.8 Hz)

*2,7-Bis(dimethylcarbamoylthio)-9H-fluoren-9-one (Product **6**)* [11]

1.00 g Of product **5** (2.59 mmol) was heated using a metal bath to 240 ºC for 50 min under a nitrogen atmosphere. After the mixture was cooled, it was dissolved in DMF and precipitated again by addition of water. Filtration leaves product 6 (0.79 g, 2.05 mmol, 79 %) as a brown colored solid.

^{1}H-NMR (400 MHz, DMSO): δ/ppm = 2.89 (s, -CH_3), 3.06 (s, -CH_3), 7.64 (s, HCCO), 7.08 (d, C*H*CHCO, J = 7.6 Hz), 7.10 (d, CHC*H*CO, J = 7.2 Hz)

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