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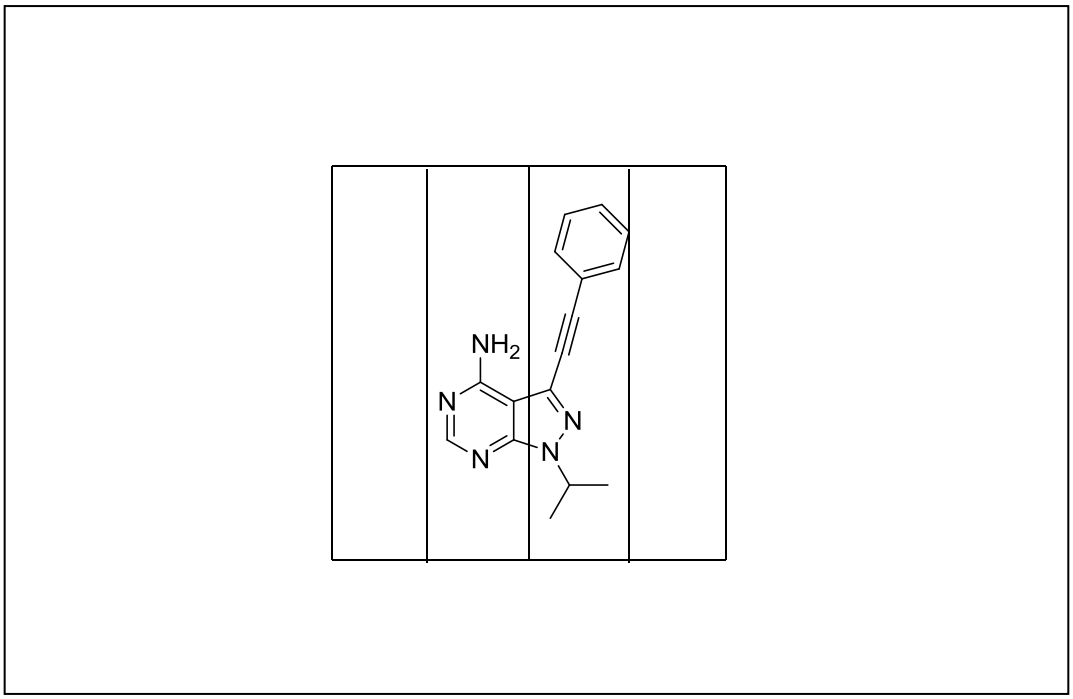

Synthesis and Evaluation of a Caged Hog1-Inhibitor

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Abstract

Caged compounds consists of a biologically active compound that has lost its activity when it is protected by a photolabile protecting group. These compounds are useful tools for molecular biologists since they give an increased control of location of the biological activity and the start of said activity. A caged compound based on a Hog1 inhibitor was synthesized. Three strategies for the synthesis were attempted and two of these yielded the target product. Neither of the strategies yielded the product in sufficiently large quantities for further testing. One strategy produced a usable byproduct in sufficient quantities. The byproduct was tested for suitable wavelengths for release.

Key words: HOG1, inhibitor, caged compound, photolabile group, synthesis.

Sammanfattning

Caged compounds består av en biologiskt aktiv molekyl som har förlorat sin aktivitet när den är skyddad av en fotolabil skyddsgrupp. Dessa molekyler är användbara verktyg för molekylär biologer eftersom de ger ökad kontroll av plats och start av biologisk aktivitet. En caged compound baserad på en Hog1 inhibitor synthetiserades. Tre strategier för syntesen prövades och två av dessa producerade målmolekylen. Ingen av strategierna gav dock produkten is stora nog mängder för fortsatta tester. En av strategierna producerade en sidoprodukt i tillräcklig mängd. Sidoprodukten testades för passande frigörande våglängder.

Nyckelord: HOG1, inhibitor, caged compound, photolabil grupp, syntes.

Abbreviations used

ATP-Adenosine-5’-triphosphate

AcOH-Acetic Acid

ASKA-analog sensitive kinase allele

DCM-Dichloromethane

DIPEA-DiIsoPropanylEthylAmine

DMAP- Dimethyl amine pyridine

DMF-Dimethylformamide

DMNB-4,5-Dimethoxy-2-nitro-benzylol.

DMSO-Dimethylsulfoxide

EtoAc-Ethyl Acetate

eq.-equivalents

FT-NMR-Fourier Transform NMR.

Hep-Heptane

HOG-High Osmolarity Glycerol

HPLC-High Performance Liquid Chromatography

LC/MS–Liquid Chromatography Mass Spectrometer

MAP-mitogen-activated protein

MeOH-Methanol

n-BuLi–n-Butyllithium

NEt_3-Triethylamine

NMR-Nuclear Magnetic Resonance Spectrometry

ONB-O-Nitro-Benzyl

PL-Photolabile group

rt-room temperature

STP-Signaling Transduction Pathway

THF-Tetrahydrofuran

TMS-Tetramethylsilane

TLC-Thin Layer Chromatography

Acknowledgements

I would here like to take the time to thank Morten Grøtli and to David Bliman for giving me the opportunity to work with this project and for all the help, advice and optimism during the last semester.

Special thanks also extend to Mariell Pettersson, Christopher Lawson, Petra Båth, Kristina Luthman and Itedal Namro Redwan for help, advice and a generally cheerful attitude.

Special thanks goes to Jesper Nilsson at the division of physical chemistry at Chalmers University of Technology for his photochemical lab work and help with analysis of said work.

The rest of the people at floor 8 and 9 also receive a special mentioning. No one mentioned and no one forgotten.

Best wishes to all of you.

/Jakob Wåhlander

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1. Introduction

Spatial and temporal control is an issue in Molecular biology. A biologist wants to control where a process starts and when it starts. As an example the study of nerve cells might demand both the stimulation of several different neurons simultaneously in some cases and the stimulation of a single synapse in other cases. Another problem is that control over a molecule is completely lost as soon as the molecule enters the cell. [1][2] Chemists can provide the biologists with adequate tools for addressing these problems. One method that can be used is that of caged compounds.[1][2] These compounds consist of a molecule with some kind of activity that is prevented by a photolabile protecting group. Since the photolabile group is easy to remove in a non-invasive manner and in principle also possible to remove within a controlled area the desired control is gained with the help of these molecules. [1][3][2][4][5][6]

In this project the goal is to create such a caged compound from a known kinase inhibitor through organic synthesis. The compound can then be evaluated at other laboratories. The main focus of the project will be to create a compound in large enough quantities for further testing to be possible. No attempts to optimize the reactions beyond production of useful amounts of substance will be done.

2. Theory

The theory section is divided into three sections: A background section, a chemistry section and a methodology section. Further description of each section can be found at the beginning of each section.

2.1 Background

The background section will cover the areas necessary for placing the project in a context. It includes subjects necessary for understanding the lab work done, but also subjects that are not necessary for understanding the project itself but without which the utility of the project will be lost.

2.1.1 Signaling transduction pathways, kinases and Hog 1 kinase

Cells in larger organisms have a need to communicate with each other. This can be in order to control everything from cell death to migration in an orderly fashion. There are several ways in which the cells can communicate with each other. One way is to release small signaling substances that can be picked up by other cells and in that way send a message between cells. This is what is known as a Signaling transduction pathway (STP).[7]

Most STPs starts with a receptor on the cell membrane that binds to a signaling substance that has been released by another cell. The binding of the signaling substance will in some way lead to the transferal of the signal across the membrane. There are multiple ways in which the receptor protein can transfer the signal into the cell. For example the receptor could attract one or more “mates” when binding to a signal substance to form a di- or multi- receptor complex. These complexes can either activate each other’s active sites or form the basis of larger protein complexes on the inside of the cell. Another example is where the binding to the receptor makes the receptor activate another factor which in its turn transfers the signal across the membrane. The later is the method used by a large group of proteins known as G-protein coupled receptors.[7] See Figure 1 for a schematic description. It is also possible for the binding of a ligand to directly change the structure of the receptor so that it for example allows water or certain ions to pass through the cell membrane.[8] These receptors are however of no importance of the project.

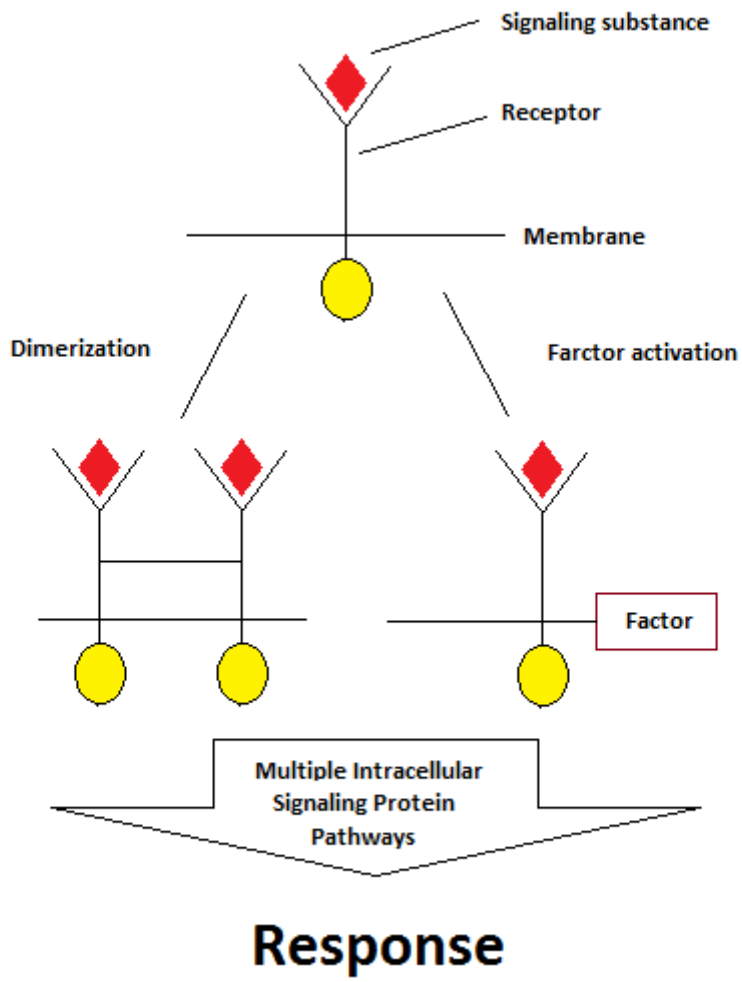


Figure 1: Signaling Transduction pathways. A signaling substance will bind to a receptor on the cell membrane which will transfer the signal into the cell. Multiple intracellular signaling protein pathways will then direct, amplify and stabilize the signal. The intracellular pathways consists mainly of long chains of kinases activating and deactivating other kinases with one final kinase activating the response.

After a signal has been received and transferred across the membrane a long chain of intracellular signaling protein activation and deactivation is initiated. The initial step of the chain is activated by the receptors and each step then activate and inactivate new proteins until a response is reached such as activation of a gene or a specific protein.[8] There are several of these intercellular pathways and they do not just exist in parallel but rather several pathways can share certain steps or crosstalk with each other.[7] A large part of these chains of protein activation and deactivation is performed by different kinases known as MAP-kinases. The chain of kinases will amplify the signal and stabilize it within the cell, this since the initial binding of the signaling molecule at the receptor only activates that molecule for a few minutes.[8]

Kinases are enzymes that catalyze the transfer of a phosphate group from an ATP molecule onto a specific molecule. The subgroup of these called protein kinases transfer a phosphate group onto a serine, tyrosine or threonine side chain in another protein (see Figure 2). The act of phosphorylation of a protein change the structure of the protein and this might lead to the activation and deactivation depending on the protein in question. A single kinase can often activate large numbers of different proteins even if specific kinases also exist.[8]

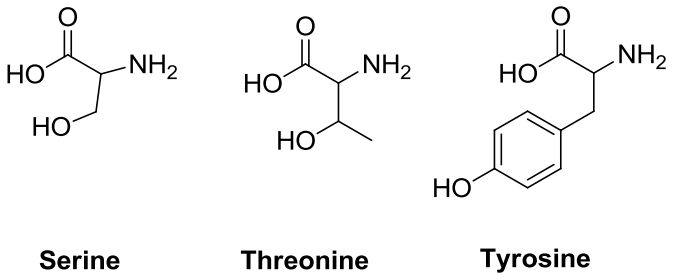


Figure 2: The structure of Serine, Threonine and Tyrosine.[9]

One particular MAP kinase is Hog1, Hog being short for High Osmolarity Glycerol, which can be found in *Saccharomyces Cerevisiae*. It is a homolog to the mammalian MAP-kinase p38 and respond to extracellular changes in osmolarity. During high osmolarity conditions the kinase is essential for cell survival. [10]

2.1.2 Caged compounds

A caged compound is in essence a molecule protected by a photolabile protecting group.[2][3][4][5] A functional group is allowed to react with a protecting group. The protecting group then prevents both electronic and steric interaction of the functional group to such a degree that the molecule can be seen as "turned off". However when exposed to light at the right wavelength the protecting group will fall off and the molecule regains its function. An example of this specifically using an enzyme inhibitor is described in Figure 3.

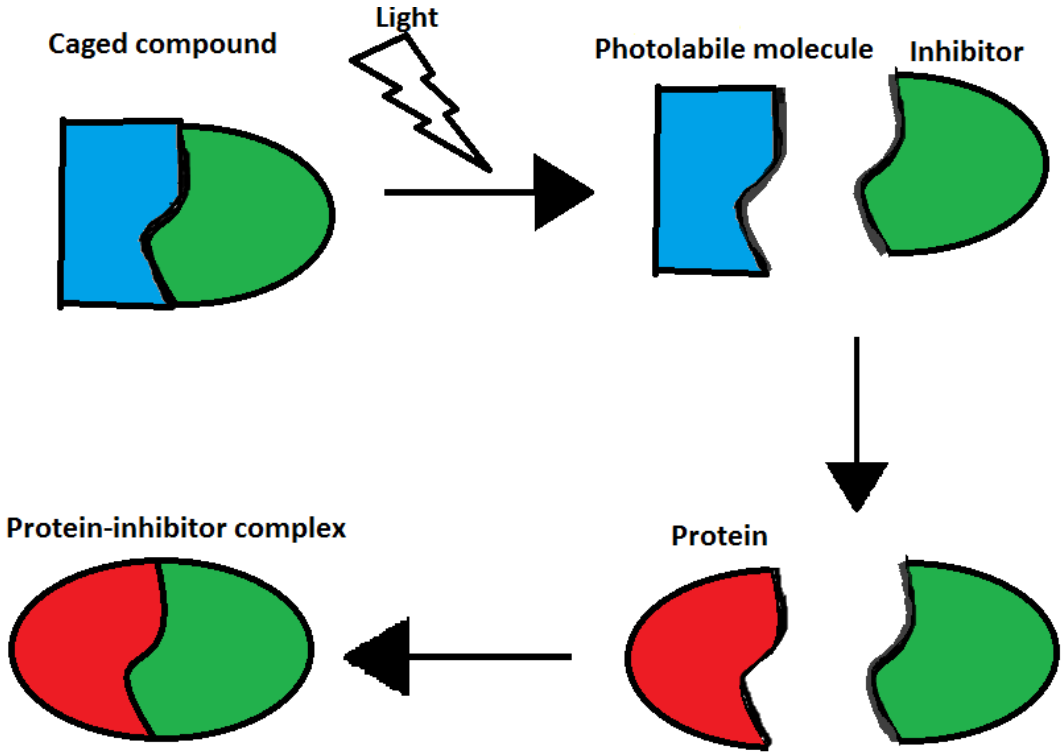


Figure 3: Example of a caged protein inhibitor. A caged compound is exposed to light which cleaves of the photolabile protecting group and therefore leaving a reactivated inhibitor. The inhibitor in its turn interacts with the protein and thus giving rise to protein inhibition.

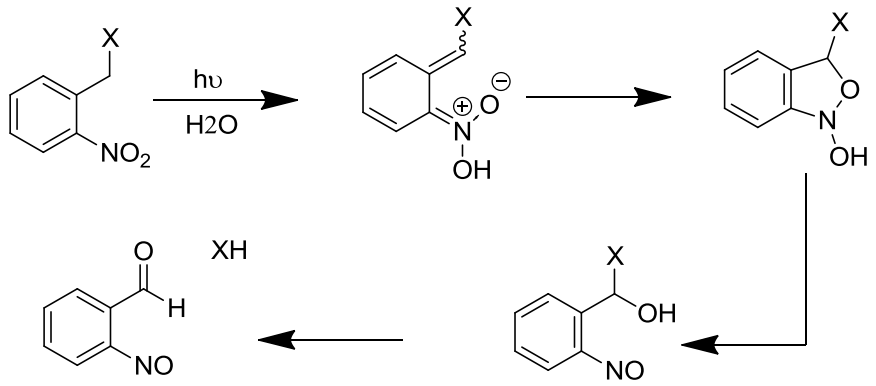
With a caged compound some obvious new possibilities open up. It will be possible to control the exact start of molecular activity since exposure of light of the correct wavelength, and therefore cleavage of the PL, is possible to control. The compounds also give a great gain in spatial control since the area that is exposed to light of a specific wavelength can be controlled. This would for example allow single cell experiments.[2][3][4][5]

Some examples of how caged compounds have been used are: caging a gene activator and thus getting a photocontroled genetic regulation [4], bacterial lithography [4], control of ion channels [3] and photocontroled Taq polymerases [5]. The reader is referred to the references 2-5 for further information.

2.1.3 Photolabile groups

A photolabile group (PL) is a protecting group that can be cleaved of by light. A good PL should as a rule have a clean cleavage and occur with a high quantum yield. It should also have a high absorption coefficient at wavelengths above 300 nm and the products of the release should not interfere with the photoreaction. [11]

There are a myriad of PLs available like for example coumarins[6][11], p-hydroxyphenacyl-groups[6][11], 1-Acyl-7-nitroindolines[6][11] and the benzoin group[11]. The largest family is the so called ONB group (Figure 4A). [6][11] It is possible to tune ONBs by adding substitution groups onto the phenyl ring. The structure DMNB (**1)** seen in Figure 4B is an example of this.[6] The mechanism for the light activated deprotection can be seen below in scheme 1.



Scheme 1: The mechanism for ONB deprotection.[6][11]

The chosen PL **1** belongs to the ONB group as can be seen in Figure 4 and was chosen for its general availability. It is also tuned for being able to release at wavelengths as long as 365 nm.[6]

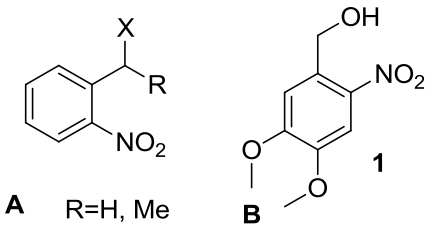


Figure 4: A, the general structure of an ONB PL group. [6] B, the structure of 4,5-dimethoxy-2-nitro-benzyl (DMNB) alcohol (**1**).[6][11]

A release at longer wavelengths is, as noted above, desirable. This since exposure to short wavelength, high energy, UV-radiation is harmful to the cells. The UV-radiation damages DNA, and therefore low energy, long wavelength, light is desirable.[8]

2.1.4 The inhibitor

The inhibitor 1-Isopropyl-3-(phenylethynyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine **(2)** that was used in the project can be seen in Figure 5 I below. It was developed by the Grøtli group at The University of Gothenburg and is synthesized in a three step reaction from 4-amino-pyrazolopyrimidine. [12]

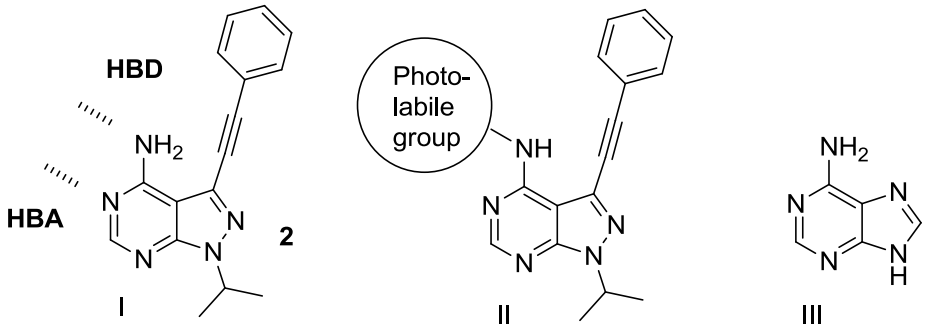


Figure 5: I) The inhibitor **2**. The inhibitor binds to a mutated Hog1 protein by the Hydrogen Bond Donator (HBD) and the Hydrogen Bond Acceptor (HBA). II) A Photolabile group is attached to the inhibitor and blocks both the Hydrogen Bonding interactions.[12] III) The structure of Adenine, the base of ATP. Adenine is the part of ATP that binds to kinases. [9]

The inhibitor **2** has been designed as an orthogonal inhibitor.[12] An orthogonal inhibitor is designed from a known inhibitor of a protein in such a way that it loses its binding affinity to the native protein. The native protein is simultaneously mutated in such a way that it binds to the orthogonal inhibitor without losing its function when not binding to the inhibitor.[13][14] The ASKA-technology is based on this strategy. The technology uses the homology of the catalytic domain of kinases to its advantage. There is a bulky amino acid residue covering a hydrophobic pocket on all kinases. This "gate keeper" group is easy to locate using amino acid sequence alignment, thus eliminating the need of 3d-structure information, and can be mutated into a glycine or alanine amino acid without loss of kinase function. With this method a rather small number of analogue inhibitors can be made to inhibit with specificity each and every kinase in the kinase superfamily.[13] The specific inhibitor **2** inhibits a mutated Hog1-kinase.[12]

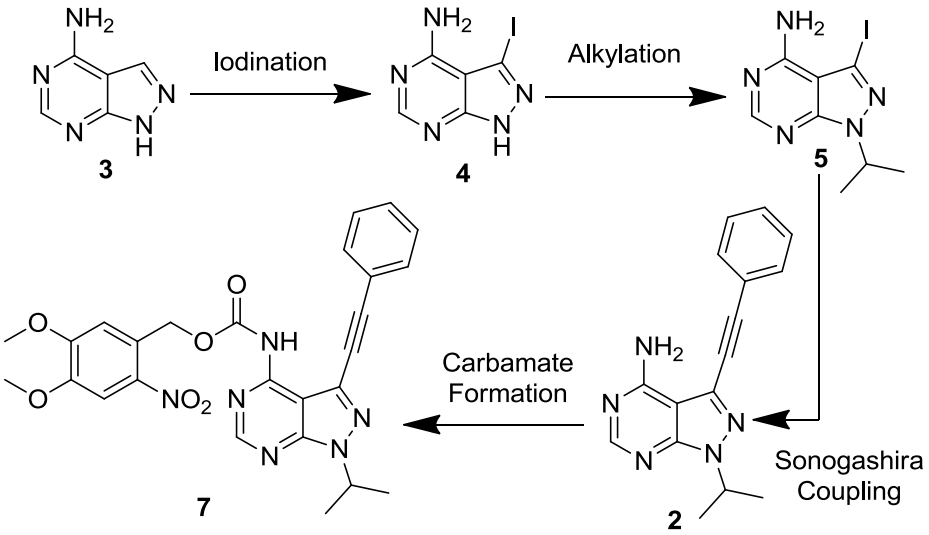
As can be seen in Figure 5 I the inhibitor **2** binds to the modified Hog1 using a "hinge area" that is very similar to the interaction that the kinase would have with adenine, see Figure 5 III for a comparison of the structures.[12] A suitable position for the PL in order to create a caged version of **2** would therefore be on the exocyclic amine as can be demonstrated in Figure 5 II.

2.2 Chemistry

The Chemistry part will present the general plan for the lab work, some of the reactions used in that plan and some useful concepts that will be used in the discussion of the results.

2.2.1 General synthesis plan

The initial plan was to start with 4-amino-pyrazolopyrimidine and synthesizes the caged compound in four steps (Scheme 2). The first three steps were based on previous work while the main focus of the project was the development of the last "carbamate formation"-step.



Scheme 2. The General synthetic plan for the caged compound. Compound **3** was first iodinated and then alkylated. The iodine was then exchanged for a phenylacetylen group by a Sonogashira coupling. Compound **1** was then coupled to the inhibitor in the last step.

2.2.2 **The synthesis of the inhibitor**

The initial three steps of the general synthesis plan were for the synthesis of the inhibitor **2**. The steps are an iodination, an alkylation and a Sonogashira coupling (Scheme 2) . A mechanism for each step is described below. The starting point was 4-amino-pyrazolopyrimidine which can be seen in Figure 6.

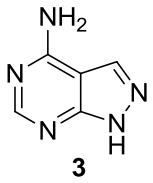


Figure 6: 4-amino-pyrazolopyrimidine, the starting material used for synthesis of the inhibitor **2**.

Iodination using N-iodosuccimide

N-iodosuccinimide, which can be seen in scheme 7A, is used in step 1 of the synthesis of the inhibitor. It is used as an iodine source. The molecule falls apart to form Iodine which in its turn can attack the double bond in **3** to form **4**. An example of a mechanism can be seen in Figure 7A and B.[15]

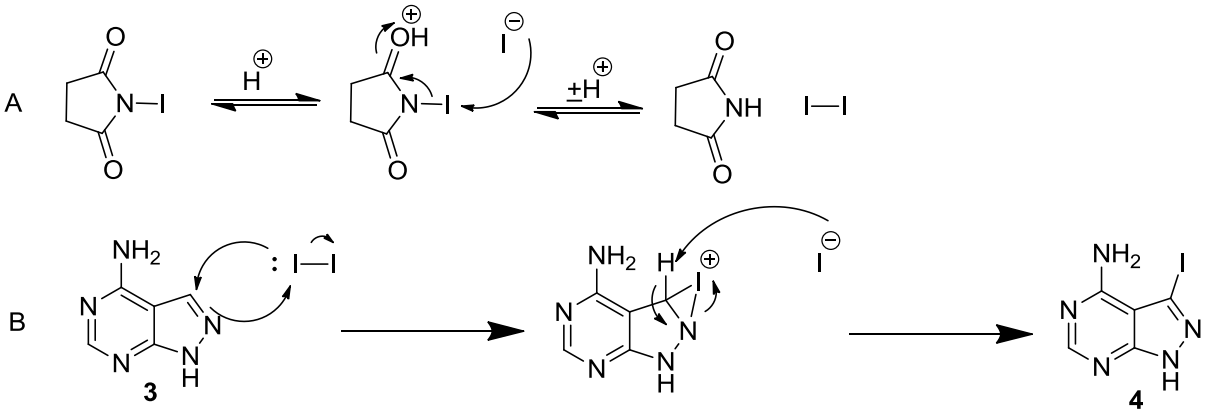


Figure 7A: A scheme of how N-iodosuccinimide provides I_2. The original iodine ions could originate from breakage of N-iodosuccinimide due to light or heat. 7B: A possible mechanism presented by the author with the aid of a textbook.[15]

Alkylation

The alkylation reaction was a S_N2 reaction. A possible mechanism is as described in Figure 8. First **4** acted as a nucleophile onto the 2-chloropropane. The chlorine ion left and the positively charged intermediate is formed. After the intermediate was formed the carbonate base deprotonated the positively charged nitrogen on the intermediate to give **5**. [15]

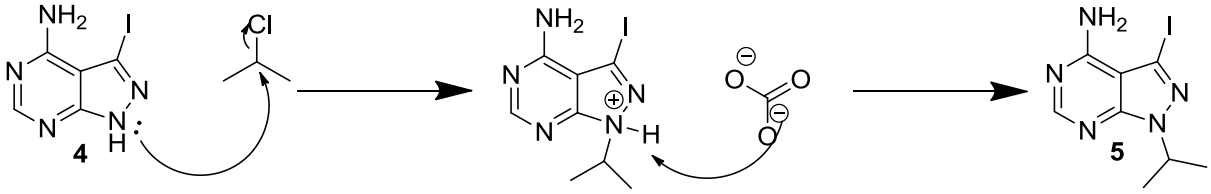
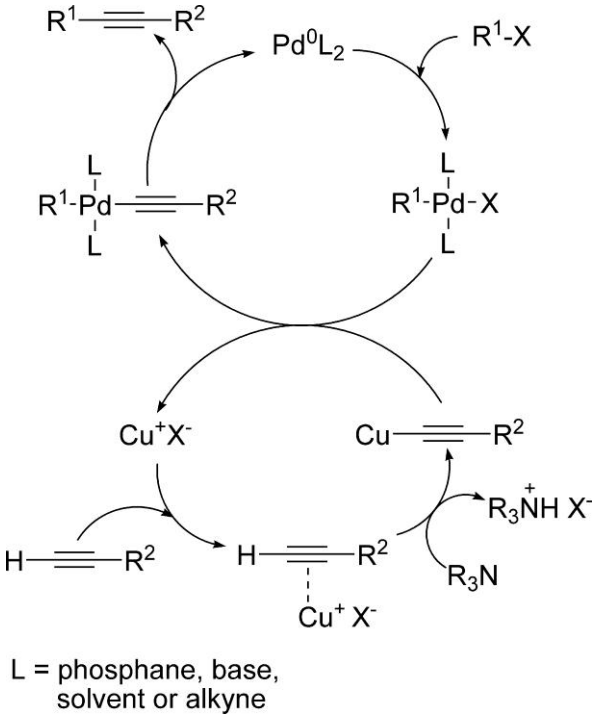


Figure 8: The S_N2 reaction used for the alkylation of **4**. The base K_2CO_3 removes one hydrogen and the negative ion of **4** then attacks the 2-chloropropane. [15]

Sonogashira coupling

The Sonogashira coupling is a reaction used in the synthesis of the inhibitor. It couples an aryl-, hetaryl- or vinyl-halide to an aryl-, hetaryl-, alkenyl-, alkyl- or silylalkyne. The mechanism is rather extensive and the exact steps are unknown. It is however believed to consist of a double cycle system where one cycle takes place on palladium and the other on copper (Scheme 3).[16]



Scheme 3: A proposed mechanism for the Sonogashira coupling. R_1 is an aryl, hetaryl or vinyl group and R_2 is an aryl,hetaryl, alkenyl, alkyl or silyl group. X is a halogen or a trifluorosulfonate-group. The picture is directly borrowed from Chinchilla and Nàjera[16]

The first step in the Sonogashira reaction is that the halide, R_1-X, is incorporated with the palladium atom using an oxidative addition. A transmetalation of the alkyne from the copper ion to the palladium atom, in exchange of the halogen ion, follows after that. The desired product, a molecule in which the previous halide is bound to the alkyne, is then finally released from the palladium by a reductive elimination. A second cycle using the copper ions is also present but less understood. It is believed that the alkyne group is coordinated to the copper ion using π-bonding. This would make the acetylenic proton acidic enough for the amine base to remove it. The amine then leaves together with the halide anion and thus giving rise to a copper-acetylide. After that the cyles interact with each other in the transmetalation step. The copper ion leaves with the halogen ion and can coordinate a new alkyne.[16]

In the project the palladium complex used is $Pd(PPh_3)_2Cl_2$ where palladium initially has got the oxidation number II. The palladium is then reduced by either the PPh_3 or the amine in a before the catalytic cycles can commence.[16] For more information concerning the Sonogashira reaction the reader is refered to reference 16.

2.2.2 **Carbonyl substitution**

The PL and the inhibitor are supposed to be connected with a carbamate group (Figure 9). This structure can be formed by carbonyl substitution.

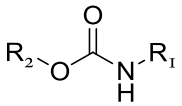
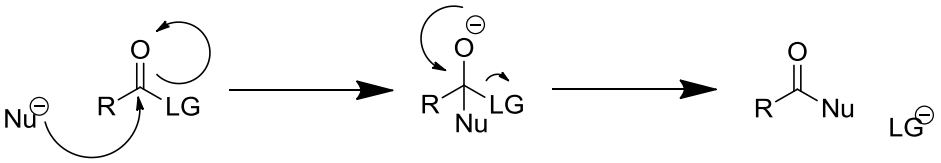


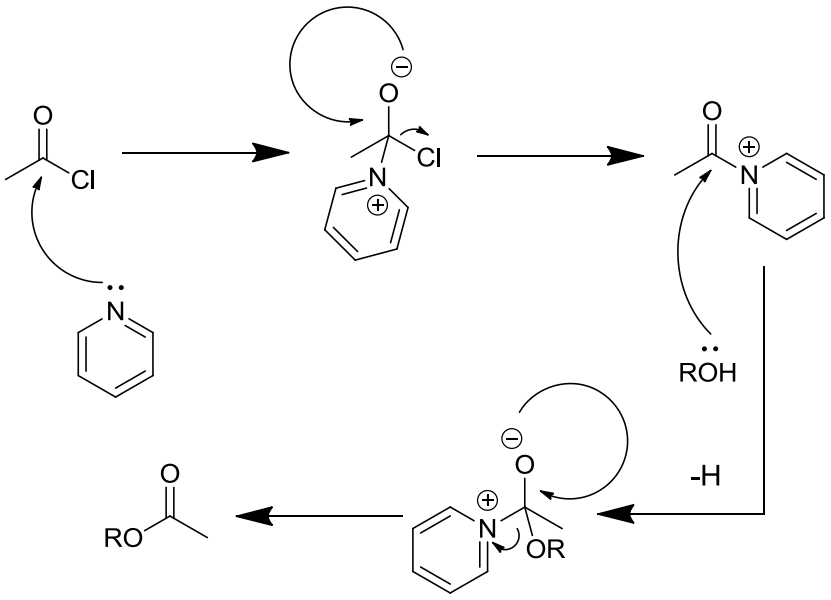
Figure 9: A carbamate group. R_1 and R_2 can be anything with a single bond to the nitrogen and oxygen respectively. When coupling the inhibitor **2** to the PL R_1 will be the inhibitor and R_2 the PL.

Carbonyl substitution takes place when a molecule with a free electron pair, called a nucleophile, attacks a carbonyl group, a carbon double bonded to an oxygen atom, and another group, the leaving group, leaves the carbonyl group. See scheme 4 below for a schematic example of the mechanism.[15]



Scheme 4: Mechanism for a carbonyl substitution. The Nucleophile (Nu) attacks the carbonyl group and forms a tetrahedral intermediate. Electron rearrangements in the intermediate then leads to the removal of the Leaving group (LG).[15]

Any molecule with a free electron pair will be able to attack the carbonyl group. However it might not necessarily be able to have the group already placed at the carbonyl group removed. Which group that leaves from the tetrahedral intermediate depend on how good a leaving group both groups are. From Scheme 4 it can be seen that the leaving group gains a charge when it leaves the carbonyl group. How well the leaving group stabilizes this charge is what will determine how good a leaving group it is. As a rule a good leaving group is usually a weak base, and a good nucleophile is thus often a strong base, but other characteristics play a role as well. For example a group that is strongly electrophilic, for example strongly positively charged, will be a good leaving group since it will gain electrons (see scheme 4) when it leaves. An example of this is shown in scheme 5.[15]



Scheme 5: Mechanisms for using pyridine as a nucleophilic catalyst.[15]

In scheme 5 it can be seen how pyridine, which is actually a better nucleophile than the alcohol, becomes a good leaving group due to the charge on the nitrogen that arise when it binds to the carbonyl group. Thus the reaction is actually helped by the addition of a catalytical amount of pyridine.[15]

It should also be noted just as reactivity can be increased by making a good leaving group it can also be increased by making a good nucleophile. Since good nucleophiles are strong bases one strategy is to increase the nucleophilicity by increasing the basicity. One example of this is that a deprotonated alcohol will be a better nucleophile than the alcohol itself.[15]

2.3 Methods

The Methods section describes the main methods used in the labwork. The methods are divided into three sections: Purification, Analysis and Microwave heating.

2.3.1 Purification

The purification methods are, with the exception of TLC, used for separating the product from remaining starting material and possible side products. All purification methods used are some kind of chromatography.

2.3.1.1 Chromatography

All types of chromatography consist of a stationary phase and a mobile phase. The stationary phase is normally a porous solid, usually of SiO_2 or Al_2O_3 if the regular absorption chromatography is used, that will bind both to the mobile phase and the crude product that should be purified. The mobile phase normally consists of a liquid or gas that will be allowed to pass through the solid carrying the crude product. Since different molecules in the crude product bind with different ease to the stationary phase they will move through the phase at different speed and thus be separated when they have passed through the phase completely. Different mobile phases will also bind the crude products with different ease so such parameters as elution speed and separation can be controlled by varying the liquids used for the mobile phase. It is also possible to use mixed solvent systems for the mobile phase or even, though it might be difficult without some mechanical aid, gradients going from one composition of a mixed solvent system to another over time. In the end the mobile phase is collected in fractions after passing through the solid phase and the separated product can be retrieved from the appropriate fractions.

The principles above apply to all forms of chromatography. There are however a number of different variations in chromatography techniques. The ones used in this project are the regular absorption chromatography which uses a hydrophilic stationary phase and reversed phase chromatography which uses a hydrophobic stationary phase. There is however others such as ion-exchange chromatography or different Liquid-Gas and Liquid-Liquid systems which uses stationary and mobile phases with different physical states.[17][18]

2.3.1.2 Flash Chromatography

Flash chromatography is a type of column chromatography, the stationary phase is loaded into a column and the mobile phase is poured through, where high pressure is used. In practice the technique works as described above but by laying a gas pressure onto the mobile phase it can be forced through the stationary phase quickly. The method requires small uniform stationary phase particles, 40-63 µm in diameter, which allows better packing and finer separation. It also needs strengthened glassware compared to a column were the mobile phase is poured through by gravity alone but apart from that only a valve to control the gas pressure. If a UV-detector is connected to the outflow of the columns it is possible to quickly identify the fractions containing UV-absorbing products. [17] The interested reader can refer to the original paper where Flash chromatography was introduced.[19]

2.3.1.3 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is another type of column chromatography which works on the same principles as described in Flash chromatography. It does however take the principles to even further extremes. Extremely small stationary phase particles are used, 5-10 µm in diameter, and this gives a very fine separation. The small particles pack so tightly that a very high

pressure is needed for the mobile phase to pass through the column. This means that the method demands a high pressure pump and strengthened columns. It also demands more in the way of degassed solvents. An injection loop where the product is injected into a loop that is then connected in between the pump and the column is also used. Different detectors, for example a UV-detector, can also be connected to the line going out from the pump in order to detect fractions containing product.[17][18]

2.3.1.4 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a method used for analysis rather than purification but since it uses the same principles as all other chromatography it is listed among the purification methods. In this type of chromatography the stationary phase is spread out on thin glass or aluminum plates. The sample is spotted directly onto the plate which is then placed with one end in the mobile phase. The mobile phase will then climb the plate using capillary forces. When the front of the mobile phase reaches the top of the plate it is removed and visualized using appropriate stains. The stationary phase on the plates is usually mixed with a fluorescent substance which helps illuminating UV-absorbing substances when the plates are viewed under a UV-light. The plates are mainly used for analysis of reactions and of testing mobile phase mixtures for future column chromatography systems.[17]

2.3.2 Analysis

The analysis methods are used for identification of products. The most important of these is NMR since this method gives structural information of the molecule. The LC/MS is mostly used for quickly detecting the presence of a specific molecule in a solution.

2.3.2.1 Nuclear Magnetic Resonance

Spin is an intricate property of atomic nuclei with an odd mass. It appears as if the nuclei is spinning in one of a number of states decided by a physical constant called the quantum spin constant. Since the nucleus is a charged particle its movement will generate a magnetic field. This leads to different spin states having different energy states when placed in a uniform magnetic field. The energy states will be affected of the environment that the nuclei are placed in, for example a hydrogen nucleus on an aromatic ring is in a different environment compared to a hydrogen nucleus on a methyl group. Consequently different amounts of energy will be needed to be absorbed in order for nuclei in different environments to change energy state. It should be noted that nuclei only absorb exactly the energy necessary for going from one state to another.[20]

A sample of a pure molecule in a uniform magnetic field can therefore be scanned with radio waves at different frequencies giving rise to a spectrum with peaks at wavelengths where energy is absorbed. Since these peaks are dependent on the environment of the nuclei specific types of environments will give rise to characteristic peaks which can be recognized with experience and large databases. The strength of the magnetic field can also be tuned so that only a specific type of nuclei, the most common being ^{1}H and ^{13}C, can be studied. Other important parameters are the integral of the peaks, which gives information concerning the proportions of different nucleus environments in a sample, and the "splitting of the peaks", that is the splitting of one distinct peak into several closely placed peaks due to interaction with adjacent nuclei. The spectra are usually presented as the part per million shift in frequency compared to TMS. The direct scanning method described above is used by old NMR machines, nowadays FT-NMR is used. FT-NMR build on the same principles but scan in a lot more efficient since a simple pulse is sent in and the out signal is separated using Fourier transforms. Using the information in the spectra most simple molecular structures can be determined. For more

complicated structures more advanced NMR methods such as 2D-NMR or DEPT experiments can be used.[20] Description of these are however beyond the scope of this text.

2.3.2.2 Liquid Chromatography/Mass Spectrometry

The HPLC technique can be coupled with a Mass Spectrometer giving the very sensitive Liquid Chromatography-Mass Spectrometry (LC/MS) instrument. A mass spectrometer (MS) is in practice an instrument that scans through a sample for different weights giving rise to a spectrum containing the different masses found within the sample. This is usually done by ionizing the molecules within the sample and then accelerating with an electric field to an analyzer and detector system.[17] A MS-system contains a high vacuum pump, a sample inlet, an ion source, an analyzer system and a detector. The pumps are used to ensure that the ions are travelling through a vacuum and thus do not collide with other molecules. The sample inlet contains the different samples that should be analyzed. The ion source will be responsible for creating ions that can be accelerated by the electric field. There are multiple ways in which this can be performed such as electron impact ionization, chemical ionization, fast atom bombardment and electrospray ionization. These techniques differ in which types and how small fragments are formed. Some molecules might not survive some techniques. In practice it should also be pointed out that the mass spectrometer does not record mass as such but rather mass per charge[18] The analyzer system differentiate the ions so that only one weight to charge ratio enter into the detector at any time. Also here there are several different versions such as the quadrupole analyzer, the ion trap analyzer, and the magnetic sector analyzer[18] The detector detects ions that enter into it and thus give the relative difference in occurrence between different ions.[18] When the MS-system is connected to a HPLC system the result is a system that separates the different molecules within the sample and then weight each molecule individually.

2.3.3 Microwave assisted heating

Reactions that use microwaves for heating have increased drastically during the 1980s and -90s.[21][22][23] When using microwave heating two different mechanisms give rise to the heat and both are due to the oscillating electric field of the microwaves, electromagnetic waves consisting of oscillating electric and magnetic fields.

The first mechanism consists of dipoles who, due to their dipole moment, will try to arrange themselves in the direction of the electric field. Since the electric field is oscillating the dipoles will increase rotation. The microwaves being at just the right frequency to be too quick for the molecules to actually align with the field and yet to slow for the molecules to freeze in position due to the field being back at the initial direction again.[21][22][23] To illustrate this the reader can imagine having two persons (A and B) being assigned spots on the floor, when a third person (C) shouts “Now” A and B will change places. If C shouts very rarely A and B will change places and then wait at their new places for a while before changing places again at the next “Now”. If C is shouting to often A and B will not have time to change places and will be freezed at their spots. At a particular frequency of “Now”-s A and B will be constantly changing places and this frequency is equivalent to the microwaves described above. When the molecules rotate with increased frequency, and therefore increased energy from the electric field, some of the energy will be lost due to molecular friction and given off as heat.[21][22][23]

The other mechanism is the similar behavior of ions. The ions will move through the liquid, as charged particles do in electric fields, and constantly changing direction due to the changes in

electromagnetic fields. These movements will once again give rise to molecular friction and from that heat is generated.[21][22]

It should be clear to the reader that a solution containing only nonpolar molecules will not heat up in microwaves. Microwave heating have two main advantages to regular heating. First of all microwave heating gives rise to heat profiles that are a lot more uniform than the ones of regular heating. Since regular heating consist of letting heat conduct through the container into the solution a gradient within the solution is formed where the region close to the container is warmer than the region in the middle of the solution. Microwaves on the other hand get through the usually microwave transparent containers and heat the solvent directly by itself giving rise to a much more uniform heating. Since the microwaves pass through the containers directly to the sample microwave heating heats up the sample a lot more quickly as well.[21][22][23]

Microwave assisted heating is a usually performed in dedicated instruments, even if the use of regular microwave ovens was common early in the development of microwave heated reactions. These instruments can usually contain methods for measuring the temperature such as IR-sensors and fiber optic sensors.[23]

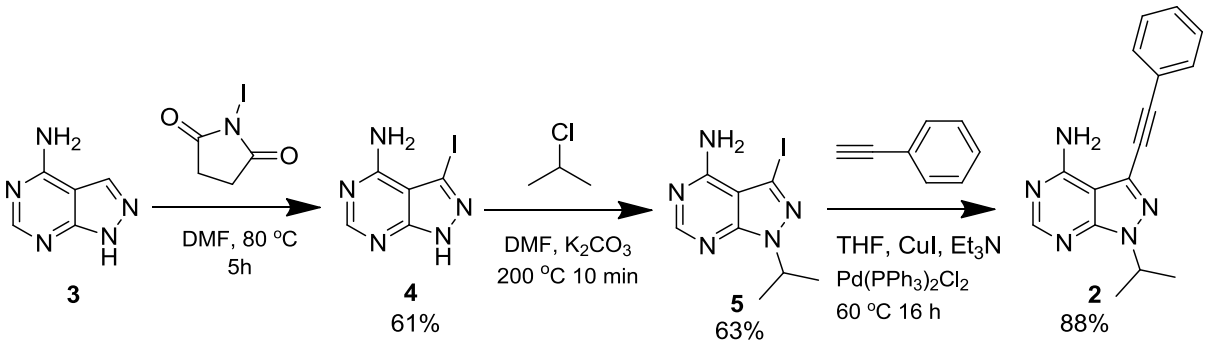
3. Results and Discussion

The project can be divided into three different parts; the synthesis of the inhibitor, the coupling of the PL to the inhibitor and the analysis of the function of the finished inhibitor. The first two parts consist of synthetic lab work while the last part consists mainly of photo spectrometric work.

The NMR-spectra for produced molecules can be found in appendix 1.

3.1 Synthesis of the inhibitor

The synthesis of the inhibitor mainly followed the literature of Klein et al [12] and can be seen in scheme 6 below. The starting material was 4-Aminopyrazolo[3,4-d]pyrimidine and the inhibitor was synthesized by three steps as can be seen in scheme 6 below.



Scheme 6: General synthesis of the inhibitor **2**. The first step is an iodination, the second step is an alkylation and the third step is a sonogashira coupling.

Step 1: iodination

The first reaction was an iodination of **3** with the goal of creating a handle for the Sonogashira coupling used in step 3 (Scheme 6). The protocol used was an inhouse protocol by Peter Dinér and not from Klein et al.[12] The used protocol differs from Dinér's only in in scale of the reaction. The reactions performed were with 2 grams of **3** grams was used rather than 6 used by Dinér. The reported yield was 77% while a yield of 61% was found in the project.

Compound **3** was mixed with a slight excess of N-iodosuccinimide in DMF. Heated at 80 ºC for five hours and then left to cool. The solvent was removed and the remaining solid was washed and rinsed with ethanol.

Step 2: alkylation

In the second reaction **4** is alkylated by 2-chloropropane (Scheme 6). The reaction is performed according to the literature with some deviations described below.[12]

The general procedure was that under dry conditions **4** was dissolved in dry DMF and mixed with dry K_2CO_3. Excess 2-chloropropane was added to the mixture which was then placed in a microwave for 10 minutes at 200ºC. This differs from the literature where a small excess is added first, followed by 5 minutes at 200 ºC, then a slight addition of 2-chloropropane followed by 5 more minutes at 200 ºC. The change was made for the sake of practicality; the literature procedure made it impossible for other researchers to queue samples in the microwave. The solution was filtered, diluted with more DMF and the solvent was then removed. It was then purified using Flash chromatography.[12] The protocol was performed on a 2 g scale while the reaction never was performed on a larger scale than 0.5 g. The reported yield was 97% compared to 63% found in the project.

Step 3: Sonogashira

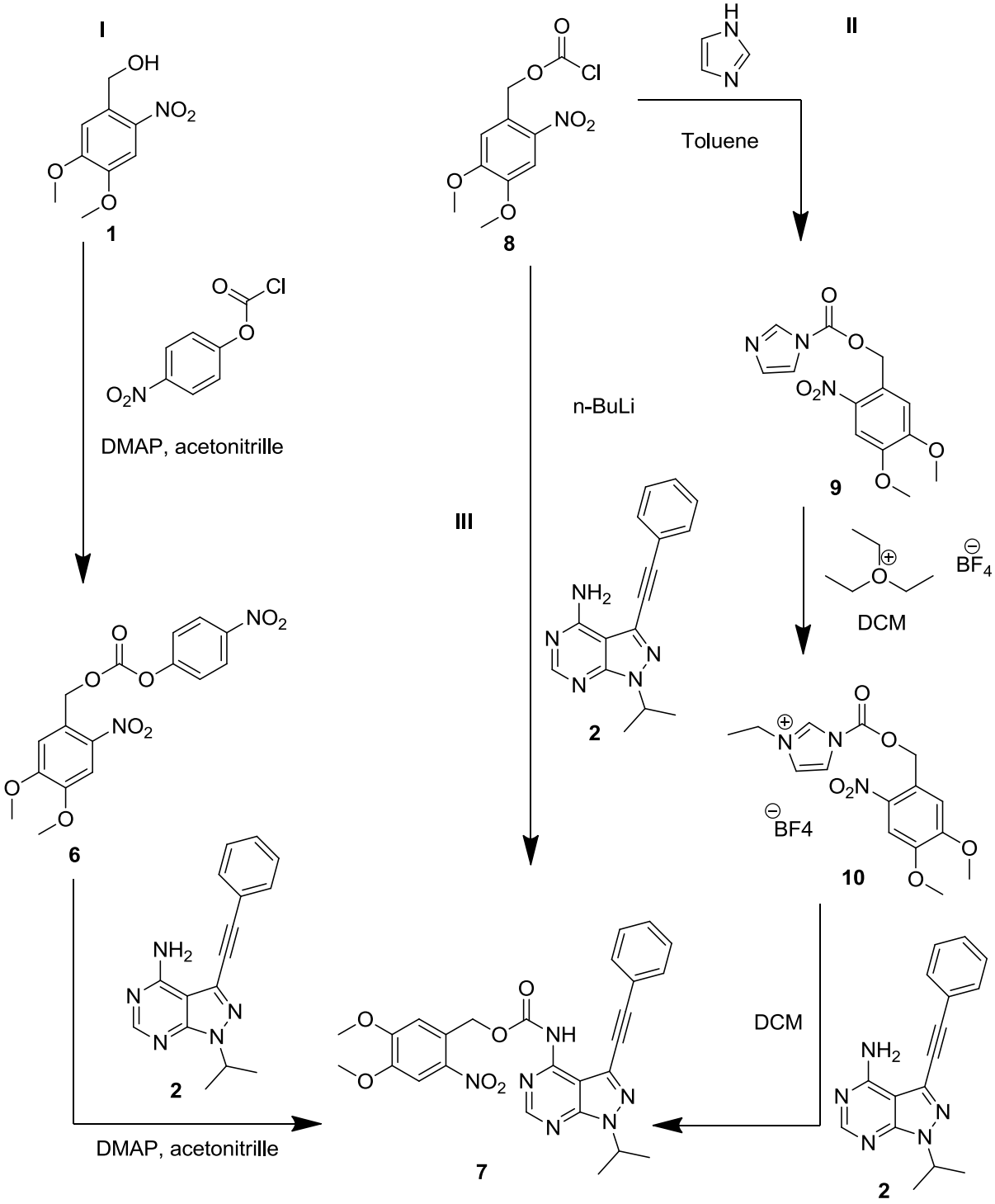
The last step is a Sonogashira coupling reaction. In this step phenylacetylene is coupled to **5** on the site of the iodine to form the inhibitor **2** (Scheme 6). The reaction was performed according to literature and the reported yield was 81% compared to 88% found in the project.[12]

The general procedure was that **5**, CuI and bis(triphenylphospine)palladium(II)dichlorine ($Pd(PPh_3)_2Cl_2$) were mixed in dry THF in a dry container under nitrogen. Phenylacetylene and NEt_3 were added to the solution which then was heated to 60 ºC for 16 hours.

The synthesis yielded the inhibitor in useful amounts even if yields in step 1 and 2 were lower than reported in the literature [12] and by Peter Dinér. Step 3 had yields similar but better results. The difference in yield for both step 1 and 2 might be due to difference in scale. This is most apparent in step 2

3.2 Coupling of the photolabile group onto the inhibitor

Three main strategies were attempted in order to couple the PL to the inhibitor **2**: Using a p-nitrophenyl chloroformate, using Rapaport's reagent and directly using a PL chloroformate and a strong base (Scheme 7).



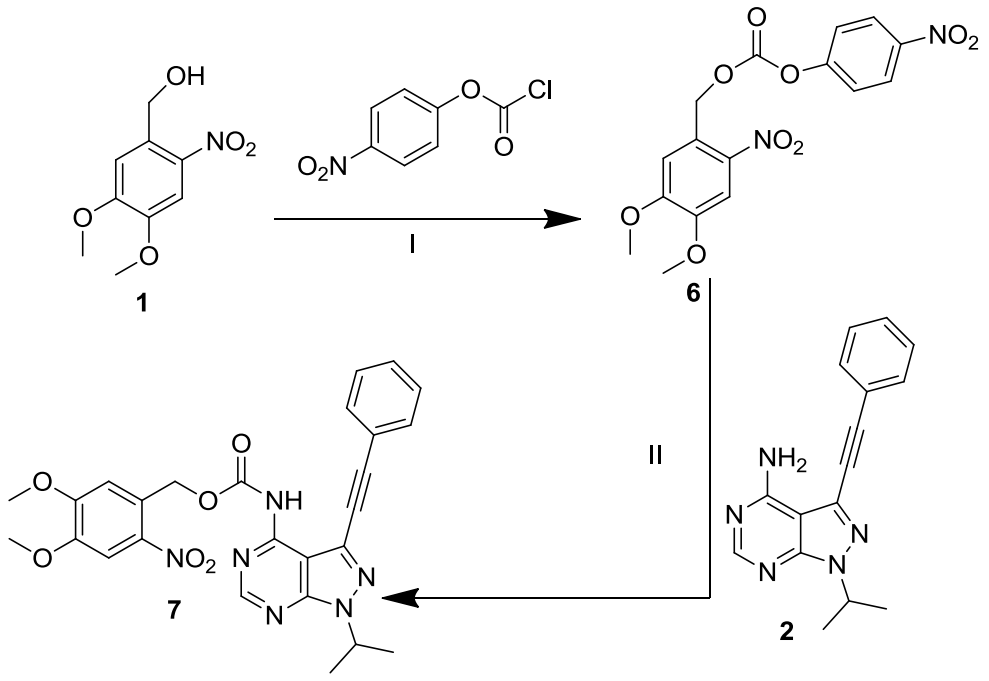
Scheme 7: Overview of the synthetic strateigies tried within the project. I is the initial strategy chosen was a p-nitropehnyl chloroformate is used for the coupling of the PL to **2**. In II an attempt was made using "Rapoport's reagent". In III a direct attempt using the chloroformate of the PL and activating **2** with n-BuLi was made.

3.2.1 Using a p-nitrophenyl chloroformate

The first strategy was to use p-nitrophenol chloroformate which is a carbonyl group with two good leaving groups on (path I scheme 7). The alcohol **1** would attack the chloroformate first forming the intermediate **6** by a carbonyl substitution aided by DMAP. In the same pot the inhibitor **2** would then

be added so that the carbonyl group in **6** could be attacked by the exocyclic amine on the inhibitor **2** to result in the final product **7**.

The pathway was based on the article by Subramaniam et al where similar couplings between coumarins and amino acids had given between 50-60% yield.[24] Several different variations of the conditions of this reaction were attempted with negative results. The general outline for this pathway, which is a one pot reaction, can be seen in Scheme 8 and table 1.

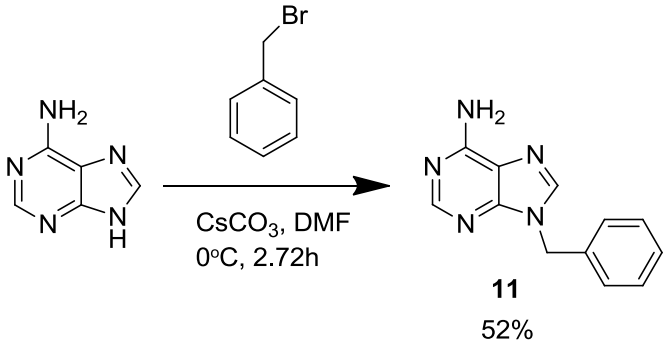


Scheme 8. I) 1.2 eq. p-nitrophenol chloroformate, 1.2 eq. DMAP, 1 eq. **1** in acetonitrile at X1ºC for T1 h II) 1eq. **9**, 2.4 eq. DMAP at X2ºC for T2 h. See Table 1 for variation in parameters.

Condition set	T1 (h)	T2 (h)	X1 (ºC)	X2 (ºC)	Result
1	24	Interrupted	rt	-	-
2[a]	3.5	18.25	rt	Rt	-
3[b]	1.5	19.5	60	60	-
4[c]	3.5	19.25	rt	60	-
5[d]	1.33	21.75	60	60	-
6[e]	0.62	1.5	60	60	-
7[f,g,h]	0.73	1.5	60	60	**6** isolated
8	0.8	21.75	60	60	-
9[i]	0.5	24.75	60	60	-

Table 1: Different conditions for the p-nitro pathway. a, Used Benzyl amine instead of **2**/**11**. b, 1.4 eq. p-nitrophenol chloroformate. c, 1 eq. p-nitrophenol chloroformate, 0.82 eq. PL, 3 eq. DMAP. d, , 1.1 eq. p-nitrophenol chloroformate, 0.9 eq. PL, 3.54 eq. DMAP. e, 4 eq. DMAP. f, Used Benzyl amine. g, 1.3 eq. p-nitrophenol chloroformate, 3.7 eq. DMAP. h, Purified using Flash chromatography first isocratic 70:30 EtoAc:Hep and then on the resulting crude from the first purification a gradient system going from 25:75 to 50:50 EtoAc:Hep. i, **11** was activated by letting it stir for 26 minutes in 12.87 l/mol dry acetonitrile together with 1.1 eq. DIPEA.

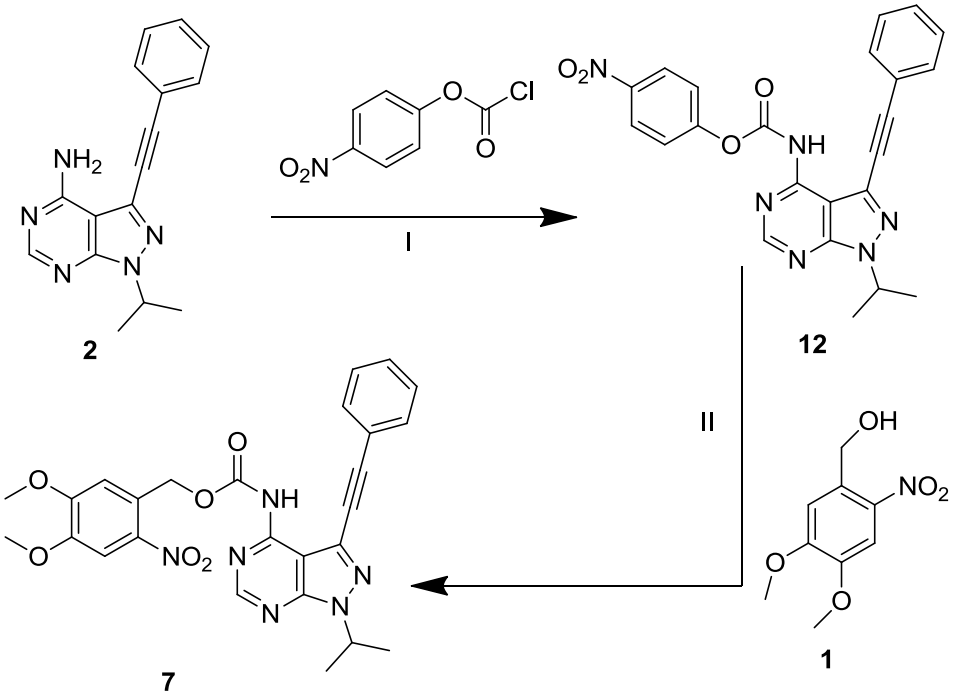
In order to avoid wasting hard-won the inhibitor **2** a dummy molecule, **11**, was synthesized and used in place of **2** for testing the reactions (Scheme 9). The reaction used an in-house protocol by Christopher Lawson.



Scheme 9. Synthesis of 9-Benzyl adenine using nucleophilic substitution. 1 eq. benzyl bromide, 1.2 eq. $CsCO_3$, DMF, 0 ºC to rt for 2.72 minutes.

A number of different conditions were tried in both step I and II (scheme 8) of the reaction which can be seen in table 1. The changes mostly concerned reaction time and temperature but in some cases the dummy molecule **11** was exchanged for benzylamine mainly to test if the reaction worked at all. At one point it was tried to activate the dummy molecule **11** using DIPEA aided by a different protocol.[25] The choice of DIPEA might surprise the reader since it is a fairly weak base and n- BuLi was used later in the project. DIPEA was chosen due to it being easy to handle and the rather good results at deprotonating aniline in the literature.[25] Some improvements in reactivity might be possible with the base. None of the attempts, with or without base, yielded any product. The intermediate **6** was isolated once together with a large amount of p-nitrophenol or possibly p-nitrophenyl chloroformate in the same fraction.

The existence of the intermediate **6** (in Scheme 8) proves that the linking of the alcohol **1** was possible. Since **7** was never found the problem therefore lies either with the dummy molecule **11** not being a good enough nucleophile or with the p-nitrophenol not being a good enough leaving group. It was therefore tried to change the order of addition of the inhibitor **2** (**11**) and the alcohol **1**. The purpose of this was to expose the dummy molecule **11** to the better leaving group of the chlorine ion. The protocol used for these tests can be seen in Scheme 10 and table 2 below.



Scheme 10. I) 1.2 eq. p-nitrophenol chloroformate, 1.2 eq. DMAP, 1 eq. **11** in acetonitrile at X1ºC for T1 h. In cases of base activation 1 eq. **11**, 1.05 eq. DIPEA. 1.2 eq. p-nitrochlorformate. T hours at rt in acetonitrile. II) 1eq. **1**, 2.4 eq. DMAP at X2ºC for T2 h. No second step ever attempted with base activation. See table 2 for variation of parameters.

Condition set	T1 (h)	T2 (h)	X1 (ºC)	X2 (ºC)	T (h)	Results
1[a]	12 days	Interrupted	Rt	-	-	-
2[b,c,d]	29+57	18.25	40+rt	-	-	-
3[e]	45.6h	26h	60	60	-	Some **13**
4[f]	-	-	-	-	96	-
5[f,g]	-	-	-	-	23	Some **13**

Table 2: Different conditions for the reversed p-nitro pathway. a,1.3 eq. P-nitro chloroformate, 3.7 eq. DMAP. b, Underwent the workup used in "General procedure for P-nitro pathway". c, First left at 40 ºC for 29 hours and then rt for 57 hours. d, 1.3 eq. p-nitrophenol chloroformate. e, , 1.1 eq. p-nitrophenol chloroformate, 0.9 eq. PL, 3.54 eq. DMAP. e, 4 eq. DMAP. f, base activated. g. Used dichloromethane instead of acetonitrile.

The attempts in scheme 10 and table 2 failed to give rise to the intermediate **12**, which was never found, but **13** was found though only identified on LC/MS. Some attempts were made with using DIPEA in this pathway, also with negative results. Thus attempts with this pathway were discontinued. After these attempts it seemed as if **11,** and the equivalent was assumed for **2,** did indeed give rise to the intermediate **12**. However since only the byproduct could be found it seemed as if **12** were too reactive for further work to be practical.

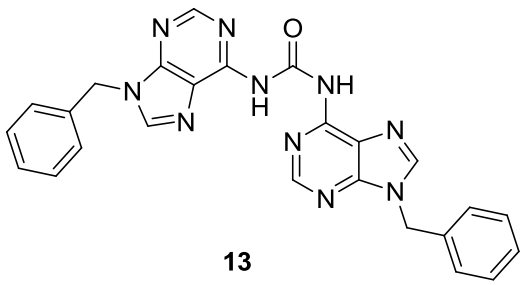
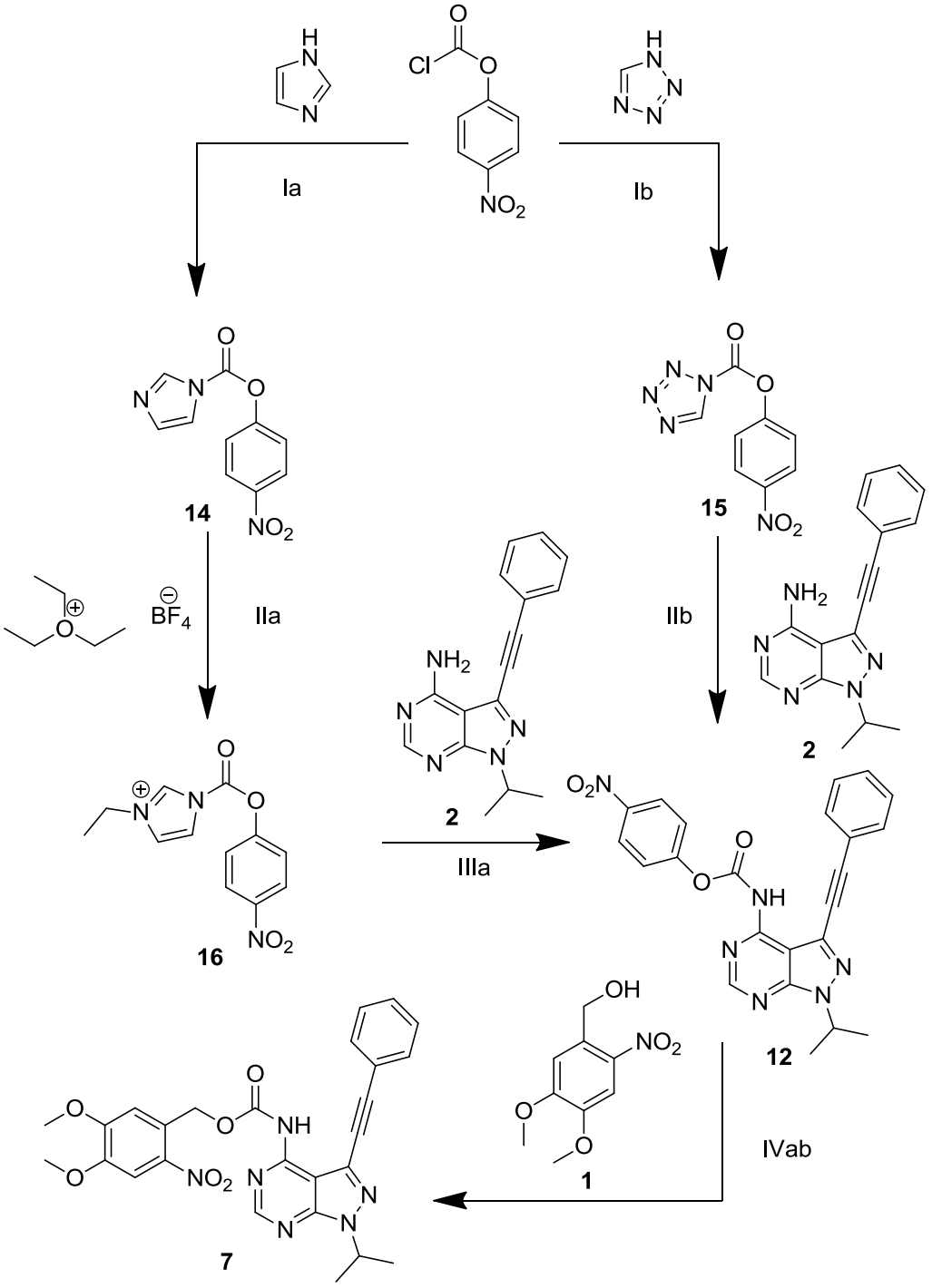


Figure 10: A double side product found while attempting the reverse order reaction seen in Scheme 14.

3.2.2 Using Rapoport's reagent

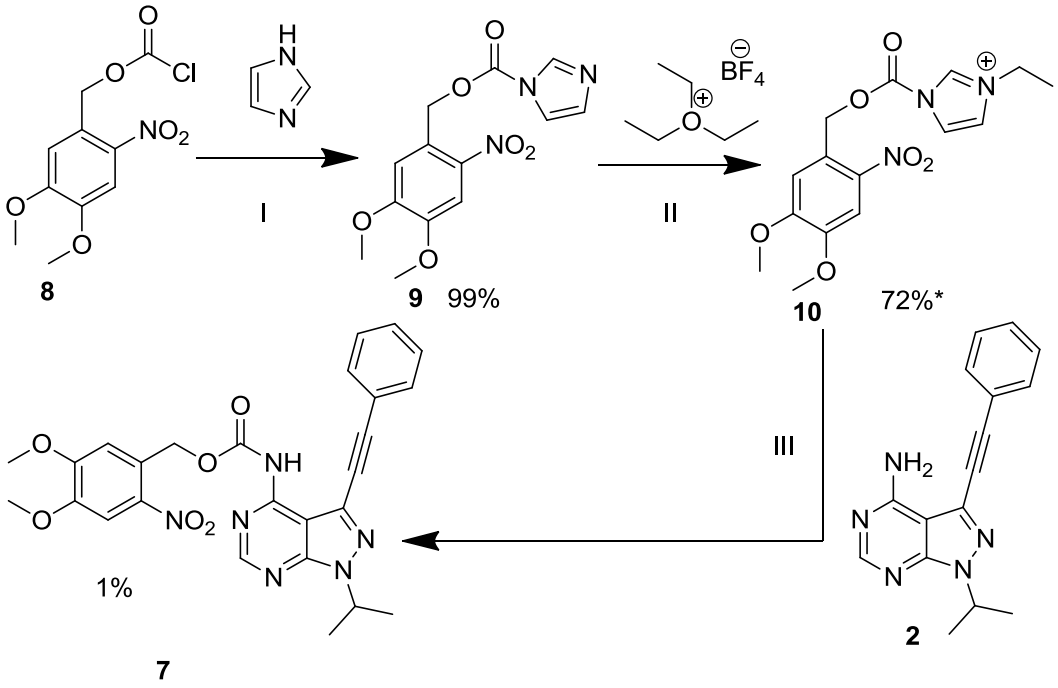
In order to enhance the reaction some attempts to increase the ability of the leaving groups were made. An attempt was made by creating a leaving group that was strongly electrophilic. Inspiration for this came mainly from the work of Rapoport et al[26][27] and the strategy followed path a in scheme 11 below. Another, similar, strategy based on the work of Baraldi et al[28] was also tried and can be seen in the b-path in scheme 11.



Scheme 11: Overview of attempts to create better leaving groups for p-nitrophenol chloroformate. Ia: 1 eq. p-nitrophenol chloroformate, 2.01 eq. imidazole in toluene over night at rt. IIa: 1 eq. **14,** 1.13 eq. Triethylamine Tetrafluoroborate in DCM at rt for 6h. IIIa not attempted. Ib: 1 eq.p-nitrophenol chloroformate, 1 eq. 1H-tetrazole, 1.05 eq. triethyl amine in acetonitrile at 0ºC for 33 min . IIb:2.83 eq. **15** 1 eq. **11** in THF for 6 h at 39 ºC. IVab: Never attempted.

The a-path produced **14** (only confirmed by LC/MS) in the first step, Ia in scheme 11, but was difficult to recrystallize for purification purposes. The second step, IIa, did however not yield any product **16**, it is possible that it was too instable, and only p-nitrophenol or possibly starting material were found in NMR studies of the crude product. Why the imidazole or the ethyl-groups are not present remains uncertain. The b-path did indeed yield **15**, not pure but with p-nitrophenol or possibly starting material, but when tried on the dummy molecule **11** no product could be found. In the end both strategies were abandoned when the strategies seen in scheme 7 (path II and III) were made available through the purchase of the newly commercially available PL chloroformate **8**.

The strategy first tried with **8** is based on the works of Rapoport et al [26][27] and **10** is a so called Rapoport's reagent. Once again multiple reaction conditions were tested. The general outline of the strategy can be seen in Scheme 12.



Scheme 12. I) 2eq. Imidazole 1 eq. **8** in toluene over night at rt. II) 1 eq. **9**, 1.05 eq. Triethyloxonium Tetrafluoroborate in DCM at rt over night. III) 1 eq. **2**, 4 eq. **10**, DMF 40ºC 72 h then rt 69 h. The third step was also tested with: 8 eq. **10** at rt for 101 h, 4 eq. **10** at 40 ºC for 101 h, 8 eq. **10** at 40 ºC for 163 h. The last three conditions were not isolated but followed on analytical HPLC and LC/MS. *Calculated using the theoretical value of **10**.

The reaction I (scheme 12) worked excellently giving **9** in 99% yields. Reaction II did yield a product that precipitated and was generally non-soluble in anything less polar than DMF. NMR did however indicate that the product in question was not **10** but something different. The product also seemed to be moving very readily on a TLC-plate (Solvent used was 9:1 $CHCl_3$:MeOH) which was unexpected for a charged compound such as **10**. Even if the identity of the intermediate compound was not settled it was decided to use the compound in the next step of the synthesis. Reaction III was performed under a few different conditions, changes in heat and equivalences, and did manage to produce **7**. Compound **7** was possible to purify on a HPLC system. The yields were however not large enough to be useful for further testing and the reactions took several days. It should be mentioned that the non-test 40 mg reaction that produced **7** was chosen for effectiveness of reagent and not of yield and that some errors in estimating the yields were made. The reaction had a tendency to precipitate when introduced to the

1:1 H_2O: acetonitrile solution necessary for LC/MS measurements. It was therefore decided to use the analytical HPLC instead. Unfortunately the wrong peak in the chromatogram was monitored, it turned out that it was a decomposed reagent peak and not the product peak, and rather optimistic estimates of the yield were made based on those data.

The low yield could be explained with two different mechanisms. The first mechanism is the fact that the inhibitor **2** (scheme 12) is a poor nucleophile, which has been noted earlier in the report, and therefore won't attack the carbonyl group. The fact that DNA-base and their analogs are unimpressive as bases was one of the reasons for the development of the first Rapoport's reagent which was designed to benzylate original DNA bases. [27] The low reactivity of the desired amine on this type of structure can be seen by the fact that even the original protocols use 4 equivalents of reagent.[26] Compared to the base used in the original protocol **2** have got a large phenylacetylenenyl group which adds some steric hindrance. Add to that the fact that the chloroformate used for the reagent also contain some bulky side chains not present in the original reagent, which was based on benzyloxy chloroformate, and a lowered reactivity can be expected. It is however very low considering the fact that the yields for similar reactions in the literature where close to or over 90% contrasted with the 1% obtained in these attempts.[26][27] Steric hindrance is therefore probably not the single reason for the lowering of the yield.

A second, and more likely, reason for the lack of reactivity is that the molecule produced to be used as a reagent, **10**, (Scheme 12) is not as a matter of fact **10**. The NMR shows no peaks corresponding to the peaks associated with the newly alkylated ethyl group in the literature and instead 2 new peaks, which are coupling to each other, show up in the aromatic region. The peaks at 2.5(m) (solvent), 3.89 (3H, s), 3.94 (3H, s), 5.74 (2H, s), 7.13 (1H, s), 7.36 (1H, s), 7.66 (1H, s), 7.75 (1H, s) and 8.39 (1H, s) in the spectra (Figure 11) are all accounted for. However the peaks 7.69 (2H, d) and 9.10 (1H, d) are new (see Figure 11 or Appendix 1). These have the internal proportion 2 to 1 but they don't always seem to match the other peaks with reference to size.

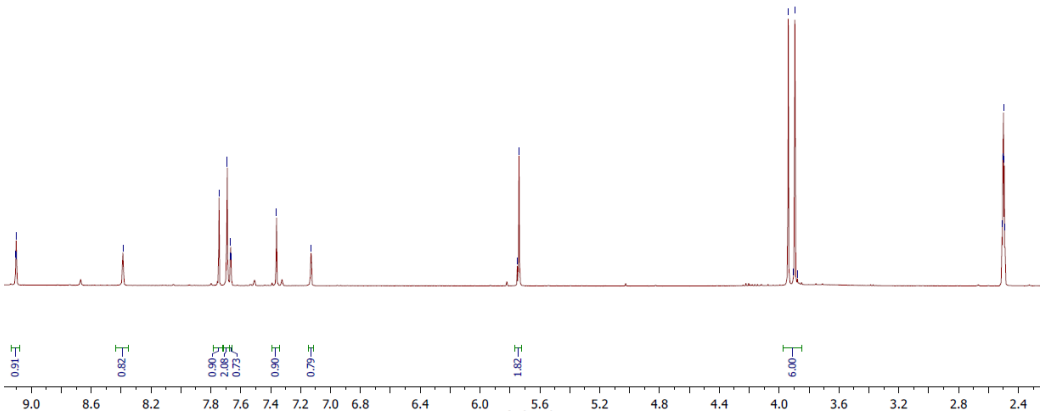


Figure 11: The NMR of the intermediate synthetized according to scheme 16. The solvent used is DMSO.

Exactly what it is remains uncertain but the NMRs seem to be indicating a structure similar to the one seen in Figure 12 A below. A vinyl group on an aromatic ring will give rise to signals in the aromatic region although only in the lower part. However the hydrogens might be deshielded even further by the presence of the charged nitrogen. The splitting of the peaks also seems to suggest this structure. It is however hard to see why this molecule should exist at all in the solution.

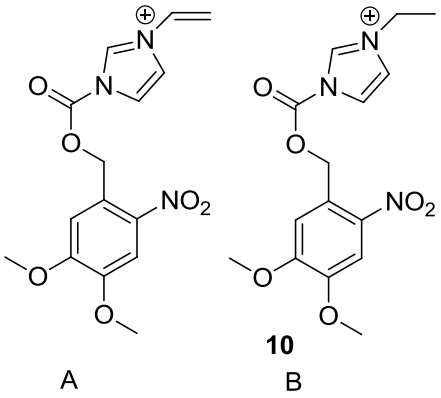


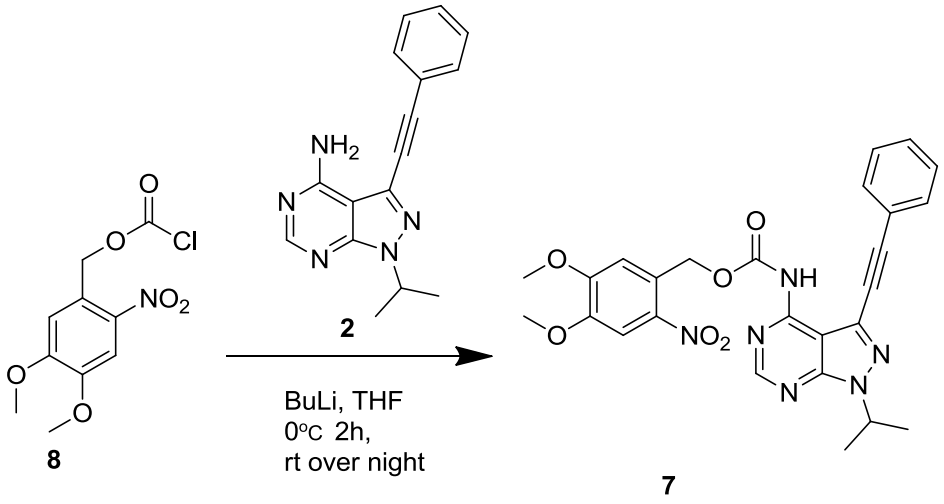
Figure 12:A: A possible structure of the reagent used instead of **10**. B: **10**.

The reason for moving on with this unsatisfactory product was that test reactions actually indicated that the final product **7** was formed. The end result did also yield some useful knowledge such as the fact that the product can be produced and that it survives HPLC-treatment.

As have been mentioned before the protocol chosen for the large scale reaction wasn't the one that showed the most promise before the reaction was set up but rather the one that seemed to demand least reagent to use of the tested conditions. The optimal conditions would demand having half as much starting material as the used synthesis. The probable gain in yield was not deemed large enough to compensate for the loss in absolute mass. This follows the same logic as the fact that 30% of 100 grams is more than 50% of 50 grams. Some improvements might therefore be possible.

3.2.3 Using the chloroformate of the photolabile group directly

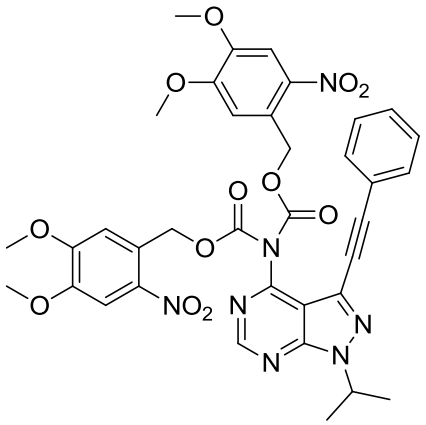
After managing to produce the molecule using Rapoport's reagent we decided to focus the last few weeks of laboratory work on a different, simpler, strategy with hope of getting better yields. The idea this time was to make the inhibitor **2** into a better nucleophile by removing one of the hydrogens on the exocyclic amine. This final strategy is based on the work of Pintér et al.[29] The general outline of the pathway can be seen in scheme 13.



Scheme 13:Carbonyl substitution on **8** performed with n-BuLi-activated **2**. The proportions tried were 1.1 eq. n-BuLi + 1 eq. benzyl chloroformate**,** 3 eq. n-BuLi+ 2 eq. **8**, 1.5 eq. n-BuLi+1.2 eq. **8** and 1.5 eq. n-BuLi+1.2 eq. **8** using **11** not **2**.

Different ratios of n-BuLi, the PL chloroformate **8** and the inhibitor **2** were attempted. This pathway does also seem to produce **7** in small amounts. Side products are present but their amount seems to vary with the amount of n-BuLi put in. When having large excess of n-BuLi, 3 equivalents, an unidentified side product dominated the output on the LC/MS and no product could be found. Admittedly this might also be due to the large excess of chloroformate. However, the smaller amounts of used n-BuLi were tested with a small variation of the amount of chloroformate giving rise to the product in lower amounts. When using smaller amounts of n-BuLi, 1.5 equivalents, **17** (Figure 13) was isolated instead and some product could be observed on LC/MS. The reason for the favored di-alkylation seen in **17** is probably due to the increased resonance stability gained when the amine becomes a carbamide and thus making the nitrogen easier to deprotonate.

Another discovery during this part of the project was that **7** were more resistant to 254 nm UV-light, used by both LC/MS and in Flash chromatography, than previously expected. This made the use of regular Flash purifications using the Biotage SP4 Flash purification system possible, previously these separations had been attempted by manual flash chromatography. However time constrains unfortunately stopped the finding of a sufficient flash chromatography system. The byproduct **17** was however isolated in a 14% yield amounts large enough for further testing to be performed.



17

Figure 13: The byproduct of the alkylation which contains two PLs.

The byproduct **17** is actually also a caged compound and its photochemical properties were therefore tested. The author does however feel that its utility in biological studies might be less useful than the original target **7**. This since the double PLs with necessity will lead to the need of twice as much exposure to light as **7**. This could be potentially harmful for the cells in question. Since it could however give a clue concerning the suitable wavelengths for uncaging of **7** studies on **17** could still yield useful results.

3.3 Evaluation of the molecule

The compound **17** (Figure 13) was sent to the division of physical chemistry at the Department of Chemistry and Bioengineering at Chalmers University of Technology for testing. The absorpotion spectra of the inhibitor **2** and the byproduct **17** (Scheme 13 and Figure 17) were recorded and **17** was exposed to 365 nm UV-light for 30 minutes. The results can be seen in Figure 14 below.

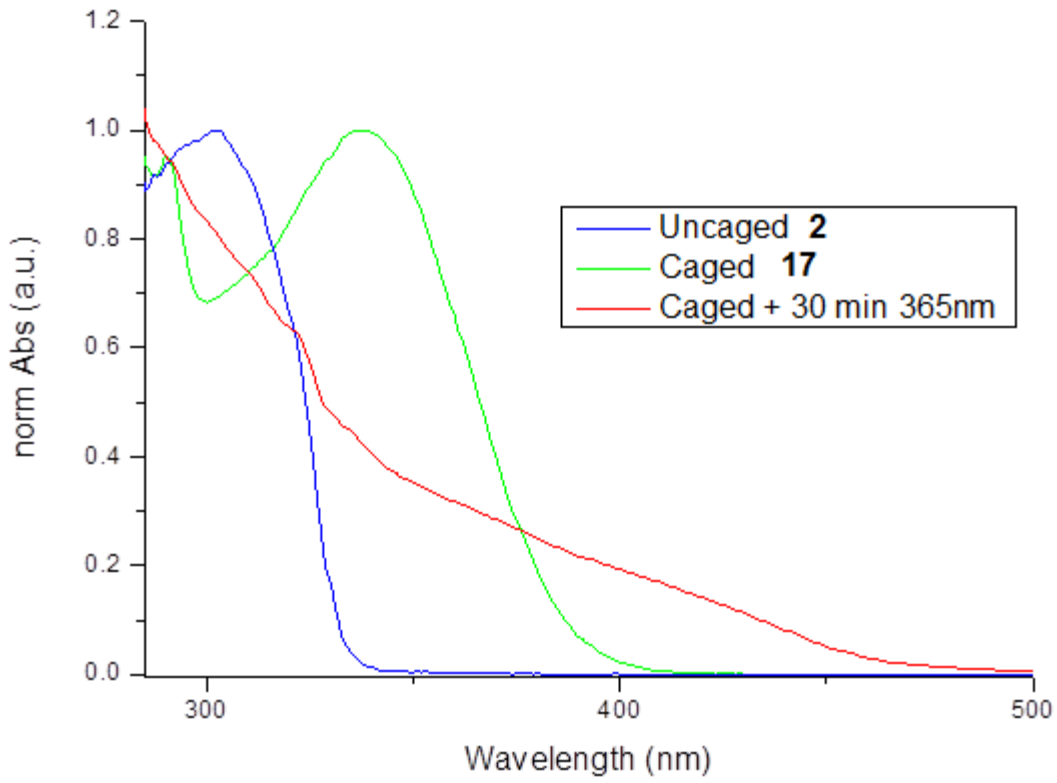


Figure 14: Absorption spectra for the uncaged molecule (**2),** the caged molecule (**17**) and the caged molecule after 30 minutes of irradiation by a 365 nm lamp. Compound **2** is represented by the blue line, compound **17** is represented by the green line and compound **17** after 30 of 365 nm irradiation. All absorption measurements were performed in toluene. Picture by Jesper Nilsson.

As can be seen there are a distinct difference in absorption between the inhibitor **2** and the byproduct **17**. After 30 minutes of irradiation the spectra changes. This could indicate that **17** is uncaged to form **2**. However the fact that there is absorption above 400 nm which is not present in either of the molecules indicates that something other than **2** and **17** is present and therefore that something more than cleavage of the PL might be taking place.

Figure 15 shows the change of the spectra the byproduct **17** as a function of time when the sample is exposed to light. The two circle areas indicate the points where the absorption never changes. This means that all the species in the sample that absorb at the plotted spectra have exactly the same molar absorption coefficient at these points. This clearly favors the idea that there are two species present since the odds at having more than two species with the same molar absorption coefficient are small.

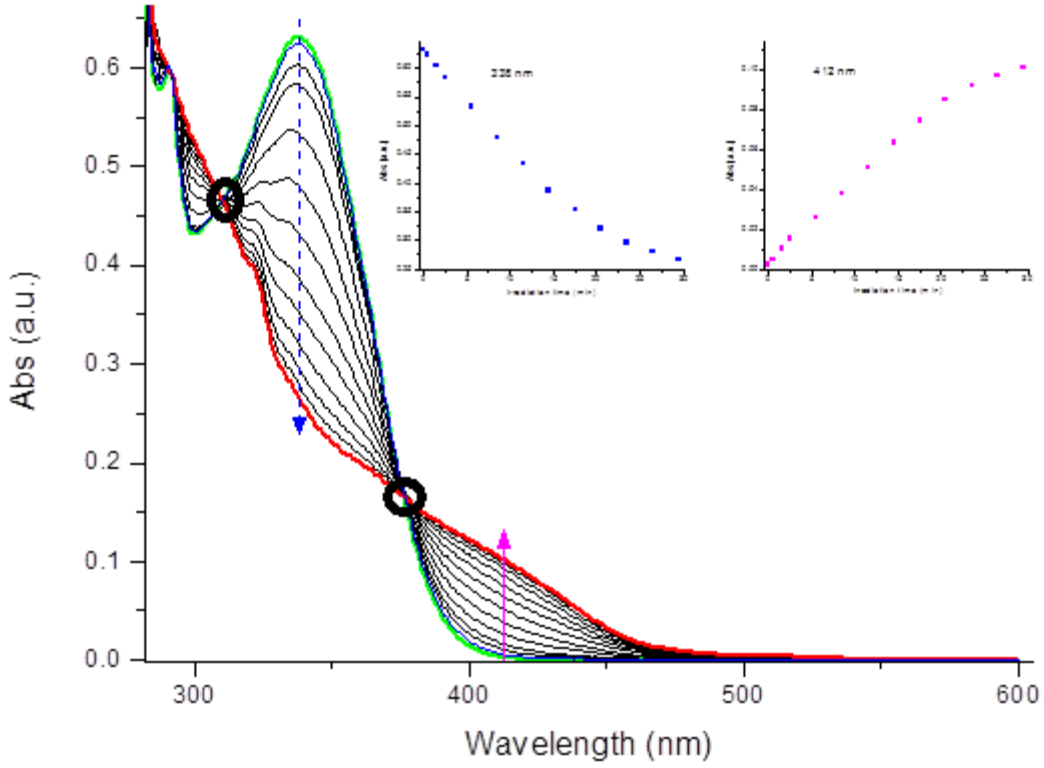


Figure 15: Spectral changes of the caged compound upon 365 nm irradiation. The insets show absorbance changes as a function of irradiation time at λ = 338 nm and λ = 412 nm. Larger copies of these graphs can be found in appendix 2. Picture by Jesper Nilsson with black circles added by the author.

Since there seem to be a presence of two different molecules and one of them must be the byproduct **17** and the other does not seem to be the inhibitor **2**, due to the presence of absorption above 400 nm, it does seem unlikely that the PL is cleaved of from **17** and resulting in **2**. Further NMR studies will hopefully answer the question of what happens when **17** is exposed to UV-light.

4. Conclusion

In conclusion the project has managed to produce two molecules, **7** and **17**, in large enough amounts for identification to be possible. Two possible synthetic pathways for **7** have also been identified, although with different success. One of the two pathways can also be used to synthesize **17**. Of the two molecules **17** have been produced in such amounts that further analysis was possible to perform. The UV-absorption analysis indicated that the photo cleavage did not run smoothly in **17**. Therefore continued efforts in producing **7** are encouraged.

Further work should be performed mainly on the last, direct, pathway (scheme 13) since this one is both least explored, quickest and requires the least amount of steps to perform. It also uses the same starting material as the Rapoport's reagent pathway so the initial costs are similar.

The Rapoport's reagent pathway (Scheme 12) does however have some uses. These mainly become clear when the present PL is exchanged for another one. The direct pathway requires a chloroformate. Chloroformates of PLs are however not very common in the catalogues of major chemical producers and can therefore be difficult to get a hold on. It is however common to find them as alcohols. The synthesis of a chloroformate from an alcohol normally requires the use of phosgene or other similar

substances which are rather unpleasant to use. It should however be possible to circumvent this with the help of a carbonyl diimidazole (see Figure 16) to get to the first step of the Rapoport synthesis directly. Since this will include the use of wastly different compounds compared to the one used now there is no telling concerning how well the chemistry might work.

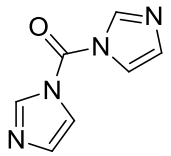


Figure 16: Carbonyl diimidazole.

5. Experimental

For the synthesis a normal organic laboratory equipped with a Biotage Initiator Microwave Syntheziser with a 60 position sample bed robot, a Biotage SP4 Flash purification system and a HPLC system used during the project used a reversed phase column with a mobile phase consisting of different mixtures of water and acetonitrile. Manual silica columns were also performed. For analysis of produced substances a Varian 400Hz NMR Spectrometer was used. A LC/MS-system of with a reversed phase column system was also used for analysis. The solvent of the loaded samples was 1:1 H_2O: acetonitrile, the ionization method was electro spray ionization and the analysis method was quadropole mass. The databases Reaxys and SciFinder were used to find protocols.

5.1 Synthesis of the inhibitor

Iodination of 4-Aminopyrazolo[3,4-d]pyrimidine (3): 4-Aminopyrazolo[3,4-d]pyrimidine (2.25 g , 14.7 mmol) and N-Iodosuccinimide (4.11 g , 16.2 mmol) were dissolved in 40 ml dry DMF. The solution was heated to 80 ºC and held there for 5 hours. The solution was then cooled to rt and then co-distilled four times with toluene. The solid product was washed with ethanol and then filtered and washed with ethanol again. The precipitate was washed rigorously with ethanol and then placed on an oil pump overnight. Yield: 61.3%. ^{1}H-NMR (d-DMSO): δ 8.15 (1H, 2), 13.77 (1H, s). ^{13}C-NMR (d-DMSO): δ 89.67, 102.48, 155.01, 156.02, 157.56 .

Alkylation of 4-amino-3-iodopyrazolopyrimidine (4): Compound **3** (503.5 mg , 1.93 mmol) and K_2CO_3 (530.3 mg , 3.83 mmol) were dissolved in 4.5 ml of dry DMF under nitrogen. 2-chloropropane (0.28 ml , 3.06 mmol) was added to the solution. The solution was placed in a microwave at 200 ºC for 10 min with 20 seconds pre-stirring and fixed hold time. An LC/MS sample was taken which showed mostly product. The solution was filtered and diluted with more DMF. The filtrate was co-distilled with toluene until dry and then left on a pump overnight. The dry product was then chromatographed on silica using a 1 to 5% MeOH in $CHCl_3$ gradient. This gave product with solvent remaining and a weight of 367.4 mg. Yield: 62.8% ^{1}H-NMR($CDCCl_3$): δ 1.55 (6H, d), 5.08 (1H, m), 8.45 (1H,s). ^{13}C-NMR ($CDCl_3$): δ 22.03, 49.51, 88.47, 110.11, 153.41, 155.06, 156.71.

Sonogashira coupling of phenylacetylene onto 3-iodo-1-isopropyl-1H-pyrazolo[3,4-d] pyrimidine-4-amine (5): Compound **4** (405.7 mg , 1.34 mmol), CuI (17.2 mg , 0.09 mmol) and $Pd(PPh_3)_2Cl_2$ (23.7 mg, 0.03 mmol) were added to a dry microwave vial flushed with nitrogen. Dry THF, 20 ml, was added to the container and nitrogen gas was bubbled through the liquid. Phenylacetylene (0.19ml , 1.73 mmol) and triethylamine (0.38ml , 2.72 mmol) were added to the closed container. The container was placed in an oil bath at 60 ºC for 16 hours and 13 minutes. The solution was cooled to rt and then filtered. TLC analysis of the filtrate indicated that most of the starting material has been used. The filtrate was purified using Flash Chromatography with a 99:1 to a 97:3 $CHCl_3$:MeOH gradient. The solvent was evaporated and placed on a pump overnight to give a light brown solid with a weight of 327 mg. Yield: 88%. ^{1}H-NMR ($CDCl_3$): δ 1.57 (6H, d), 5.13 (1H, m), 6.45 (2H, s broad), 7.36 (3H, m), 7.57 (2H, m), 8.33 (1H, s). ^{13}C-NMR ($CDCl_3$): δ 21.97 (2C), 49.43, 81, 93.84, 121.6, 125.92, 128.55, 129.36, 131.75, 152.46, 156.05, 157.98.

5.2 Coupling of the photolabile group onto the inhibitor

The protocols are divided into the respective strategy. For those cases were a number of different conditions have been used a table is present that lists all the tested conditions.

5.2.1 Using a p-nitrophenyl chloroformate

Synthesis of 9-Benzyl adenine (11): Adenine (504 mg, 3.73 mmol) and Cs_2CO_3 (1458.3 mg , 4.48 mmol) was added to a dry vial while flushed with nitrogen. 5 ml of dry DMF was added to the vial and the solution was stirred while cooled to 0 ºC using an ice bath. Dropwise addition of (0.5ml, 4.21 mmol) of Benzyl bromide while stirring with great vigour for 2 hours and 43 minutes and followed on LC/MS. The solution was filtered and the filtrate was dried for a while and then placed on a pump. Flash chromatography was performed using a gradient from 99:1 CHCl3:EtOH to 97:3 $CHCl_3$:EtOH/MeOH. Received 481.4 mg prod. Yield: 52% ^{1}H-NMR (DMSO): δ 5.37 (2H, s), 7.31 (7H, m), 8.16 (1H, s), 8.25 (1H, s). ^{13}C-NMR (DMSO): δ 46.59, 119.12, 127.94 (2C), 128.13, 129.09 (2C), 137.58, 141.23, 149.92, 153.08, 156.45.

General procedure for P-nitro pathway: P-nitrophenyl chloroformate (1.2 eq.), DMAP (1.2 eq.) and **1** (1 eq.) were added to a dry container under nitrogen. 108.7 ml/g **1** of dry acetonitrile was added and the mixture was wrapped in alumina foil and left under nitrogen at X1 ºC for T1 hours. Reaction mixture is a clear yellow. **9** (1 eq) and DMAP (2.4 eq) were then added. Left at X2 ºC for T2 hours. The solution was quenched with 15% AcOH, extracted with DCM and washed with brine. The DCM fraction was dried with large amounts of sodium sulfate and the solvent was evaporated.

Table 3 on the next page shows different conditions used. Condition set 7 is the only one which was purified.

Condition set	T1 (h)	T2 (h)	X1 (ºC)	X2 (ºC)
1	24	Interrupted	rt	-
2[a]	3.5	18.25	rt	Rt
3[b]	1.5	19.5	60	60
4[c]	3.5	19.25	rt	60
5[d]	1.33	21.75	60	60
6[e]	0.62	1.5	60	60
7[f,g,h]	0.73	1.5	60	60
8	0.8	21.75	60	60
9[i]	0.5	24.75	60	60

Table 3: Different conditions for the p-nitro pathway. a, Used Benzyl amine. b, 1.4 eq. p-nitrophenol chloroformate. c, 1 eq. p-nitrophenol chloroformate, 0.82 eq. PL, 3 eq. DMAP. d, , 1.1 eq. p-nitrophenol chloroformate, 0.9 eq. PL, 3.54 eq. DMAP. e, 4 eq. DMAP. f, Used Benzyl amine. g, 1.3 eq. p-nitrophenol chloroformate, 3.7 eq. DMAP. h, Purified using Flash chromatography first isocratic 70:30 EtoAc:Hep and then on the resulting crude from the first purification a gradient system going from 25:75 to 50:50 EtoAc:Hep. i, **11** was activated by letting it stir for 26 minutes in 12.87 l/mol dry acetonitrile together with 1.1 eq. DIPEA.

General procedure for reversed P-nitro pathway: P-nitrophenyl chloroformate **(**1.2 eq.), DMAP (1.2 eq.) and **9** (1 eq.) were added to a dry container under nitrogen. 95.3 ml/g **9** of dry acetonitrile was added and the mixture was wrapped in alumina foil and left at X1 ºC for T1 min. Monitor using TLC and LC/MS. Compound **1** (1 eq.) was dissolved in 50.3 ml/g **1** of dry acetonitrile and injected into the vessel. Left the reaction at X2 ºC for T2 hours.

Table 4 below shows different conditions used.

Condition set	T1 (h)	T2 (h)	X1 (ºC)	X2 (ºC)
1[a]	12 days	Interrupted	Rt	-
2[b,c,d]	29+57	18.25	40+rt	-
3[e]	45.6h	26h	60	60

Table 4: Different conditions for the reversed p-nitro pathway. a,1.3 eq. P-nitro chloroformate, 3.7 eq. DMAP. b, Underwent the workup used in "General procedure for P-nitro pathway". c, First left at 40 ºC for 29 hours and then rt for 57 hours. d, 1.3 eq. p-nitrophenol chloroformate. e, , 1.1 eq. p-nitrophenol chloroformate, 0.9 eq. PL, 3.54 eq. DMAP. e, 4 eq. DMAP.

General procedure for base activated reversed p-nitro pathway: Dissolve **11 (**1 eq.) into 40 ml/g **11** of dry acetonitrile in a dry vial under nitrogen. DIPEA (1.05 eq.) is added to the container which is placed in a small water bath at rt. Mixed 0.24M p-nitrophenyl chloroformate solution in acetonitrile and added 1.2-1.3 eq. dropwise. Left for T hours. The reaction was monitored by both TLC and LC/MS but not necessarily simultaniously. Since no intermediate was ever found no second step was attempted.

Table 5 below shows different conditions used.

Condition set	T (h)
1	4 days
2[a]	23h

Table 5: Different conditions for the base activated reversed p-nitro pathway. a, used DCM as a solvent instead.

5.2.2 The Rapoport pathway

Synthesis of 14: P-nitrophenyl chloroformate (317.8 mg, 1.58 mmol) and imidazole (216.1mg, 3.17 mmol) were weighted into a round bottle flask. 30 ml of toluene was added and left to stir under a nitrogen atmosphere over night. Precipitate was formed and filtered away the next morning. The filtrate was evaluated using LC/MS. The solvent was evaporated giving white crystals. Attempts were made to try and recrystallize white crystals in Et_2O without success and the product was left solved in Et_2O in the refrigerator overnight. After some inspection and concentration of solvent the next day the crystals were left in the refrigerator again over the weekend. On Monday the solvent was evaporated and the end product was weighted 151.7 mg. This crude was impure but still used in the next step.

Synthesis of 16: The complete crude from the previous reaction was dissolved in 3 ml of DCM. The solution was cooled to 0 ºC while under nitrogen. Weighted up Triethyloxonium tetrafluoroborate (95% in Et_2O) (139.4 mg, 0.7 mmol). Dissolved it in 1 ml of DCM and added it slowly to the cold container under nitrogen. The container was left to stir for 6 hours. Evaporated the solvent and performed NMR on crude product. No product found.

Synthesis of 15: 3wt% 1H-tetrazole in acetonitrile **(**3.4 ml, 1mmol) was added to a container placed on ice and stirred together with Et_3N (0.21 ml, 2.9 mmol). 1 ml of acetonitrile was used to dissolve p-nitrophenyl chloroformate (288.9mg, 1.43mmol) and the solution was then added unevenly over 20 minutes to the cold solution which went from clear to yellow and left to stir on ice for 33 minutes. The solvent was evaporated in rt giving yellow white crystals. The solution was diluted with 2.7 ml EtoAc and then filtered through a silica plug using 3% Et_3N in EtoAc for eluent. The solvent of the filtrate was evaporated giving a yellow/white solid with a weight of 302.8mg product. This mixture also containing Et_3N and p-nitrophenol chloroformate was used in the next step. ^{1}H-NMR ($CDCl_3$): δ 7.50 (2H, m), 8.33 (2H, m), 8.64 (1H, s)

Linking 15 to 11: From an NMR-sample of the product in the previous step 147.8 mg (0.58 mmol) of **15** were estimated to exist. So the total amount from the previous step was weighted up and added to dry container containing **11** (20mg, 0.088mmol). Dissolved both of these in 0.6 ml THF and placed in an oil bath starting at 32 ºC and quickly rising to 39 ºC for 6 hours. The reaction was tracked with LC/MS. The solvent was evaporated and the crude product was left over the weekend in the dry state. No product found on LC/MS.

Synthesis of 9: Imidazole (2 eq) was added to 12 ml/mg imidazole of toluene in a closed dry container and placed in an ice bath with a stirrer for 12 min. Compound **8 (**1 eq.) was added and the container was resealed and left on ice for another five minutes then placed at rt wrapped in alumina foil overnight. Interrupted the reaction the next morning and filtered the solution. Collected precipitate from filter and place on pump over night. Yield: 99% ^{1}H-NMR ($CDCl_3$): δ 3.98 (6H, s), 5.81 (2H,s), 7.02 (1H, s), 7.09 (1H, d), 7.45 (1H, d), 7.76 (1H, s), 8.16 (1H, t). ^{13}C-NMR ($CDCl_3$): δ 56.49, 56.52, 66.63, 108.52, 111.37, 117.08, 124.30, 130.95, 137.04, 149.08, 153.53.

Synthesis of 10: Compound **9** (1 eq.) was dissolved in 9.1ml/g DCM and then placed under nitrogen in a sealed container and put on ice for 5 minutes. Added Triethyloxonium tetrafluoroborate (1. eq) (1 M in DCM, bringing total liquid volume to 12.5ml/g) and placed the vial at rt over night. Precipitate formed almost instantaneously. The solvent was evaporated the next morning leaving large amounts of crystals. Stored under nitrogen at rt. Yield: 71.6%. ^{1}H-NMR (d-DMSO): δ 3.89 (3H, S), 3.94 (3H, s), 5.74 (2H, s), 7.13 (1H, s), 7.36 (1H, s), 7.67 (1H, s), 7.69 (2H, d), 7.75 (1H, s), 9.10 (1H, t). ^{13}C-NMR

(d-DMSO): δ 56.62, 56.82, 66.78, 108.75, 109.01, 112.80, 118.22, 119.67, 124.66, 130.17, 134.69, 137.82, 140.45, 148.87, 153.65.

General procedure for synthesis of 7:A dry container was filled with **2** (1 eq.) and **10 (**4 eq.). Dry DMF (62 ml/g **2**) was then added to the mixture and the solution was stirred for hours at 40 ºC for 72h, then left at rt for another 69h. After interruption the solution was codistilled with toluene 4 times thus removing the solvent. The crude product was purified using HPLC, 100% water to 100% acetonitrile. Yield 1.3%. ^{1}H-NMR (d-MeCN): δ 1.55 (6H, s) 3.80 (3H, s), 3.90 (3H, s), 5.20 (1H, s), 5.57 (2H, s), 7.21 (1H, s), 7.32 (2H, m), 7.42 (1H, m), 7.57 (2H, m), 7.71 (1H, s), 8.63 (1H, s).

The reaction was attempted at a few other conditions, using the same general procedure in other respects, on an analysis only basis (did not purify crude product). The conditions can be seen in table 6.

Condition set	Equivalents of 18	Temperature (ºC)	Time (h)
1	8	Rt	101
2	4	40	101
3	8	40 for 117h, then rt	163

Table 6: Conditions tested using the Rapoport reagent for alkylation of **2** by PL in non-purified reactions.

5.2.3 The direct usage of a chloroformate

General procedure: Compound **2 (**1 eq.) was weighted up and added to a dry microwave vial under a nitrogen atmosphere. Dry THF (14.8ml/g **2**) was added to the vial which was then left to stirr at 0 ºC for 1 hour. Added n-BuLi (1.5 eq.) to the solution and let it stir under nitrogen for 1 hour. Compound **8** (1.2 eq.) was dissolved, with great difficulty, in dry THF (30 ml/g **8**) and then added to the solution. Wrapped the vial in alumina foil and left it for 2 more hours at 0 ºC. After 2 hours the ice had melted away. The reaction was left over night and tracked with LC/MS the next morning. The reaction was interrupted by adding dest. H_2O until n-BuLi stopped precipitate. The product was extracted the product using Diethyl ether (3x 5ml). Filtered solution and removed solvent. Purification was attempted using flash chromatography (100:0 till 0:100 heptane:EtoAc). Did not manage to separate **7** from **2** but did isolate 9.5 mg (theoretically 13.8% yield) of **17. [1]**H-NMR: δ 1.66 (6H, d), 3.82 (6H, s), 3.91 (6H, s), 5.31 (1H, m), 5.60 (2H,s), 7.06 (2H,s), 7.20 (2H, m), 7.29 (1H, m), 7.45 (2H, m), 7.62 (2H, s), 8.96 (1H, s). ^{13}C-NMR ($CDCl_3$): δ 21.94, 50.59, 56.47, 66.29, 94.90, 108.01, 110.61, 120.98, 125.72, 128.32, 129.34, 131.76, 139.42, 148.29, 150.47, 152.33, 153.59, 155.36.

The reaction was attempted at a few other conditions on an analysis only basis (did not purify crude product). The conditions can be seen in table 7.

Condition set	Equivalents of n-BuLi	Equivalents of **8**
1[a]	1.1	1
2	3	2
3[b]	1.5	1.2

Table 7: Analytical conditions tested on the direct coupling attempts. a, Used benzyl chloroformate, showed product. b, Used dummy molecule **11**.

5.3 Photochemical testing

Testing of photochemical properties of **17** was performed by Jesper Nilsson at the division of Physical Chemistry at the Department of Chemistry and Bioengineering at Chalmers University of Technology.

The absorption measurements were performed in toluene at rt on a Cary Bio 50 UV/Vis spectrometer. The 365 nm UV-light was generated by a UVP hand-held UV-lamp model UVGL-25 with a power density of 760 μW/cm^2 at the sample. Total sample volume was *ca.* 2.5 mL. When applying UV-light, the whole sample volume was exposed to light at any given time.

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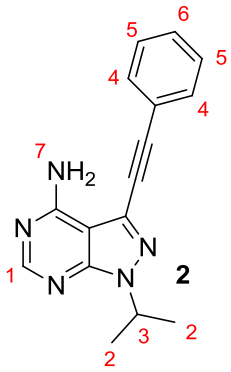
[28] P.G. Baraldi, A.R. Moorman, P.A. Borea, "WO 2005/028489 A2", International Patent,PCT/US2004/030514 pg. 61-62

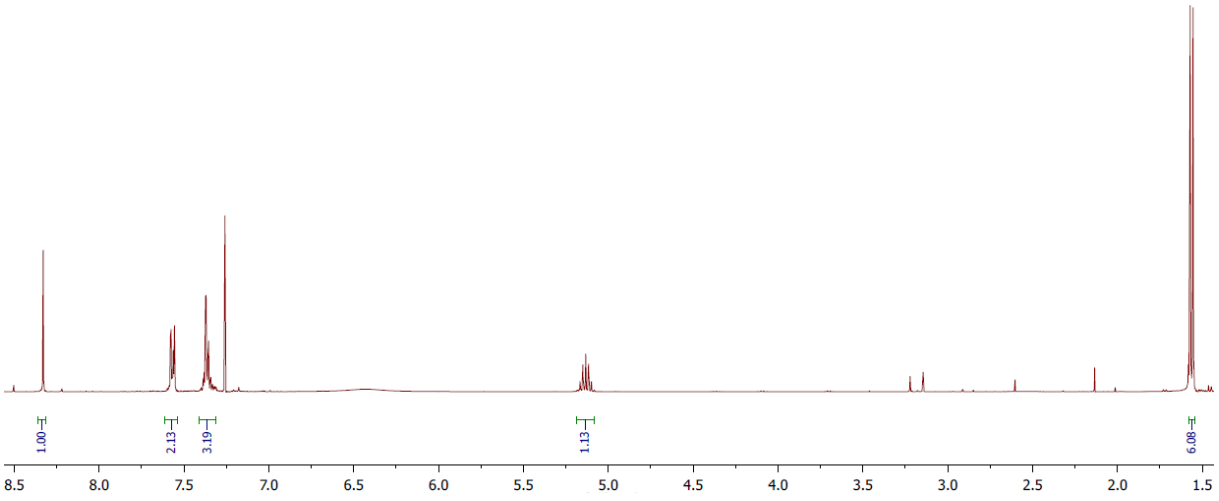
[29] Pintér Á., Sub A., Sureshkumar D., Klussmann M., "Autoxidative Carbon–Carbon Bond Formation from Carbon–Hydrogen Bonds", Angewandt Chemie International edition, 2010, 49, 29, 5004-5007,DOI: 10.1002/anie.201000711

Appendix 1 - Structures and NMR-spectra

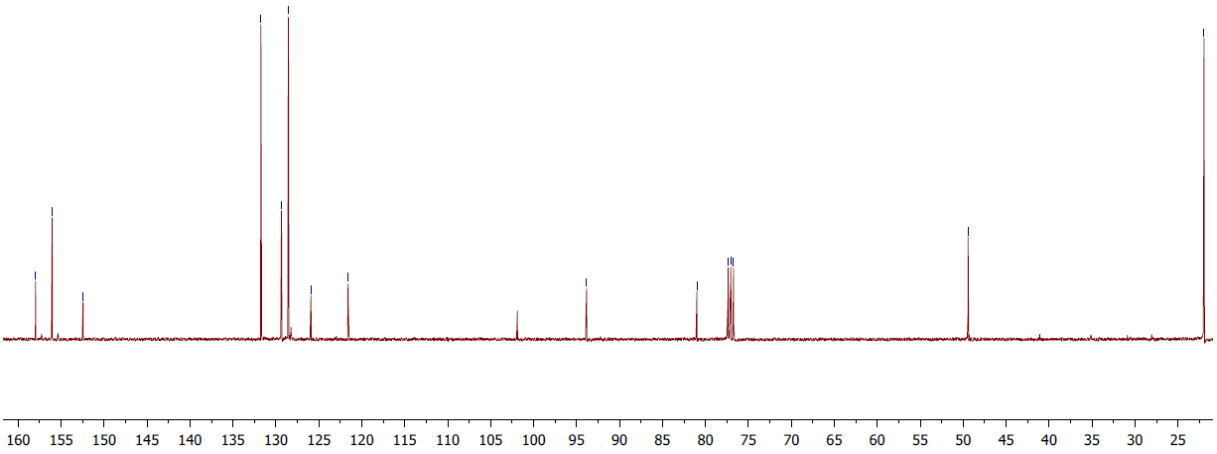


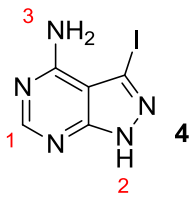
Molecule 2

^{1}H-NMR: The solvent was $CDCl_3$. The peak at 8.33 ppm corresponds to hydrogen 1. The six hydrogen atoms at 2 give rise to the doublet at 1.57 ppm while the single hydrogen 3 gives rise to the multiplette at 5.13 ppm. The multiplette at 7.36 ppm is caused by the hydrogens 5 and 6 while the multiplette at 7.58 ppm is caused by the the hydrogen atoms 4. The hydrogens 7 gives rise to a barely visible broad peak at 6.5 ppm. The 7.26 ppm peak is the solvent shift.



^{13}C-NMR ($CDCl_3$):21.97 (2C), 49.43, 81, 93.84, 121.6, 125.92, 128.55, 129.36, 131.75, 152.46, 156.05, 157.98.

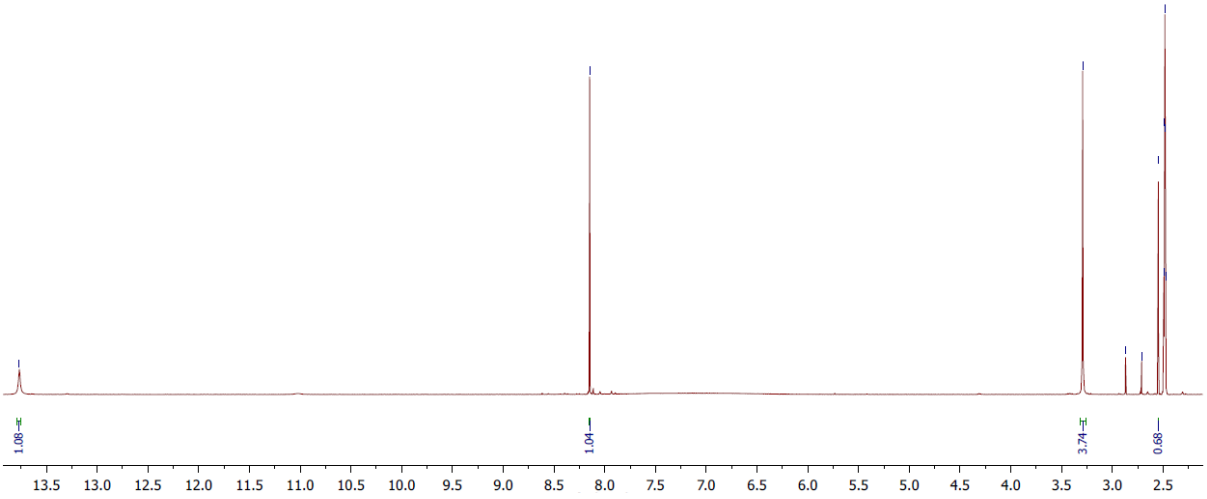




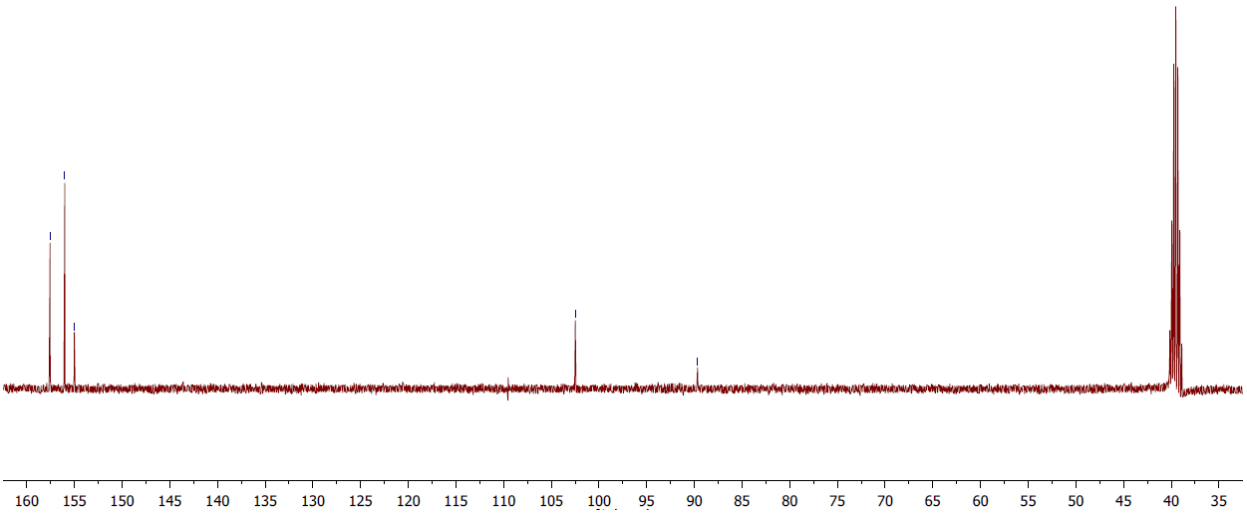
4

Molecule 4

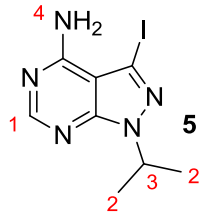
[1]H-NMR: The solvent used was d-DMSO. The peak at 3.29 ppm is H_2O and the one at 2.55 ppm is regular DMSO. The 8.15 ppm peak corresponds to 1. The peak at 13.77 ppm is likely 2. The amine hydrogens 3 are not visible. 2.5 ppm corresponds to the DMSO.



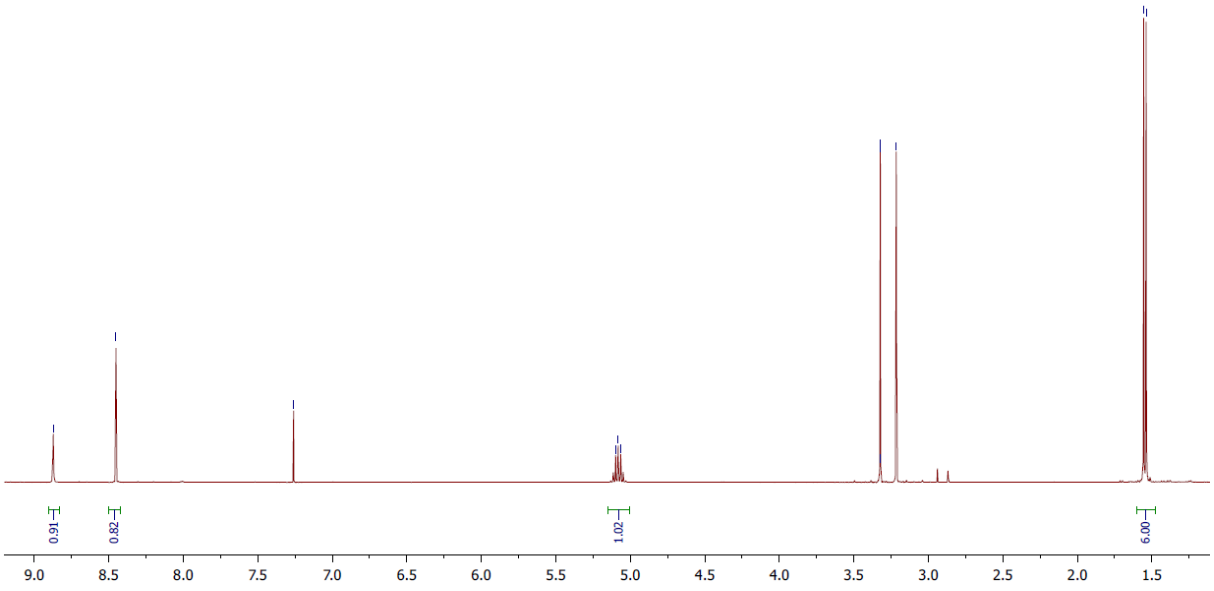
[13]C-NMR (d-DMSO): 89.67, 102.48, 155.01, 156.02, 157.56



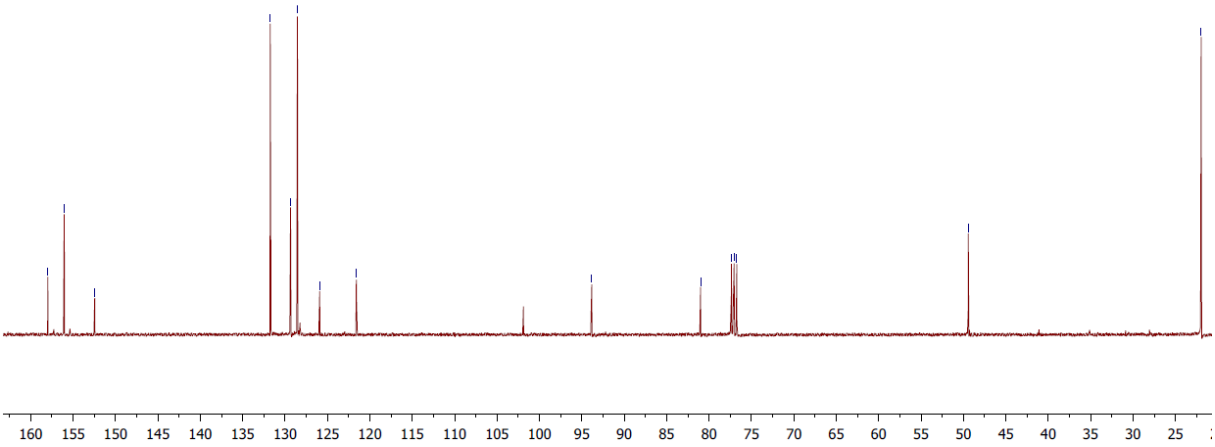
Molecule 5

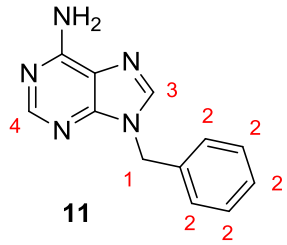


^{1}H-NMR: The solvent used was $CDCl_3$. The peak at roughly 8.45 ppm is caused by 1. The large doublet at 1.54 ppm is caused by the hydrogens at 2. The multiplette at 5.08 ppm is caused by the single hydrogen at 3. The amine hydrogens at 4 are not seen. The 7.26 ppm peak is caused by the solvent. While peaks at 3.22 and 3.32 ppm are a bit high they are probably caused by the methyl groups of DMF and the 8.87 ppm peak is caused by the aldehyde hydrogen in the very same molecule.



^{13}C-NMR ($CDCl_3$): Contains DMF (35.41, 41.17, 161.86). 22.03, 49.51, 88.47, 110.11, 153.41, 155.06, 156.71.

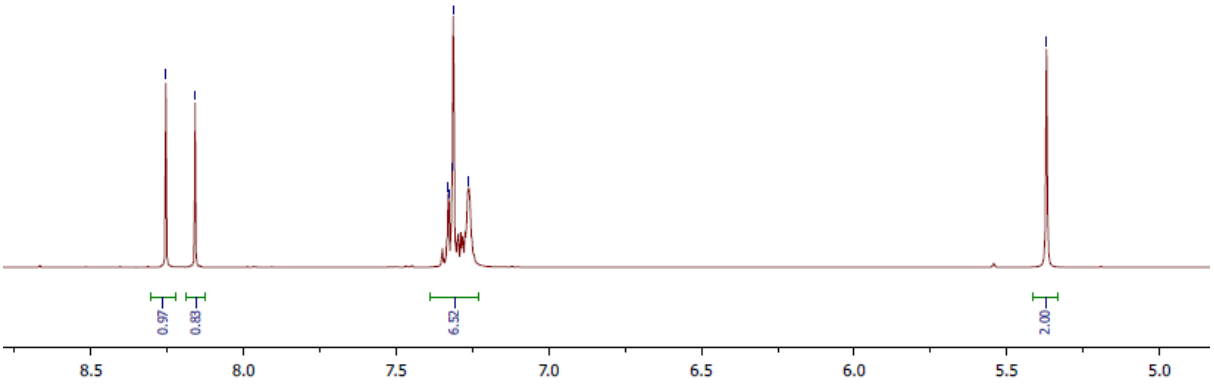




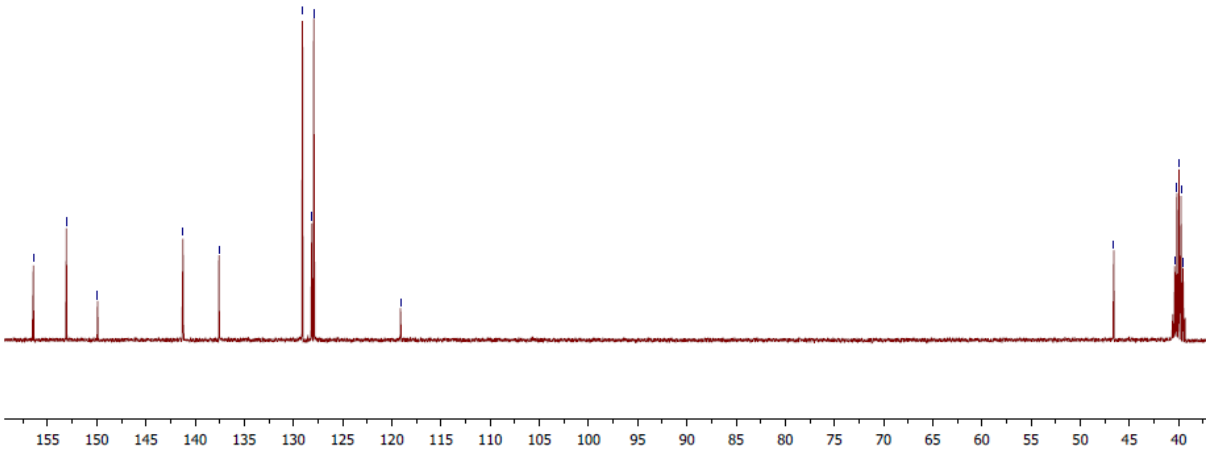
11

Molecule 11

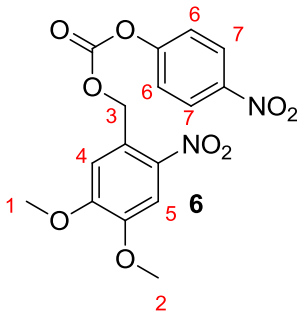
^{1}H-NMR: The solvent was d-DMSO. The peak at 5.37 ppm is caused by 1. The multiplette at 7.31 ppm is most likely caused by the aromatic hydrogen atoms marked 2 even if the integral is too large in size. The peak at 8.16 ppm is caused by 3 and hydrogen 4 gives rise to the peak at 8.25 ppm.



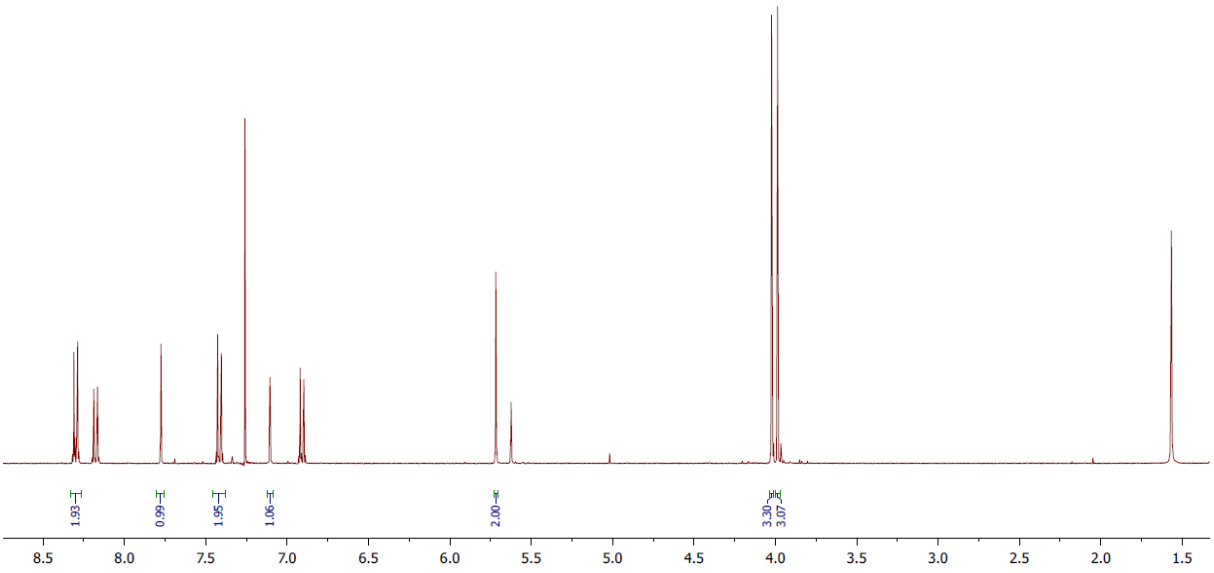
^{13}C-NMR (d-DMSO): δ 46.59, 119.12, 127.94 (2C), 128.13, 129.09 (2C), 137.58, 141.23, 149.92, 153.08, 156.45.



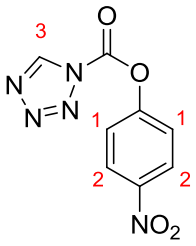
Molecule 6



^{1}H-NMR: The solvent used was $CDCl_3$. The two peaks at 4.02 ppm and 3.99 ppm corresponds to the hydrogens at 1 and 2. The peak at 5.72 ppm is caused by the methane group at 3. The hydrogen atoms 4 and 5 give rise to the peaks at 7.11 ppm and 7.78 ppm respectivly. The hydrogen atoms 6 give rise to doublet at 7.42 ppm and the hydrogen atoms at 7 give rise to the doublet 8.30 ppm. The smaller doublets at 8.18 ppm and 6.91 ppm are caused by free para-nitro-phenol or para-nitro-phenol chloroformate. The peak at 7.26 ppm is due to chloroform and the one at 1.57 ppm is due to water.

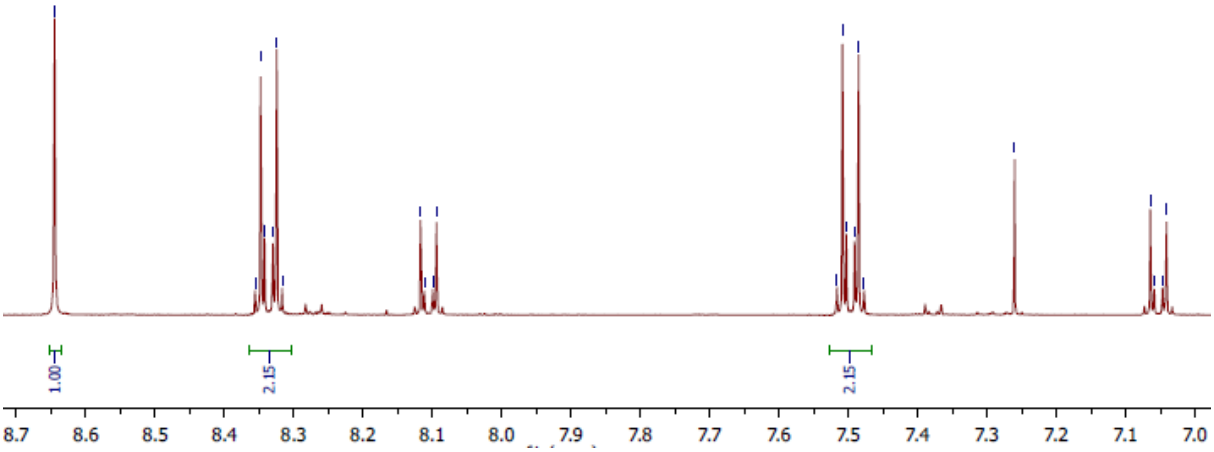


^{13}C-NMR- Not obtained.

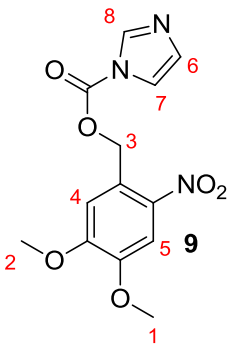


Molecule 15

^{1}H-NMR: The solvent was $CDCl_3$. The doublet at 7.50 ppm is caused by the two hydrogen atoms 1. The atoms 2 give rise to the doublet at 8.33 ppm. Finally the hydrogen 3 gives rise to the singlet at 8.64 ppm. The doublets at 7.05 ppm and 8.11 ppm are caused by the starting material p-nitro-phenol chloroformate. The singlet peak at 7.26 ppm is caused by the solven.

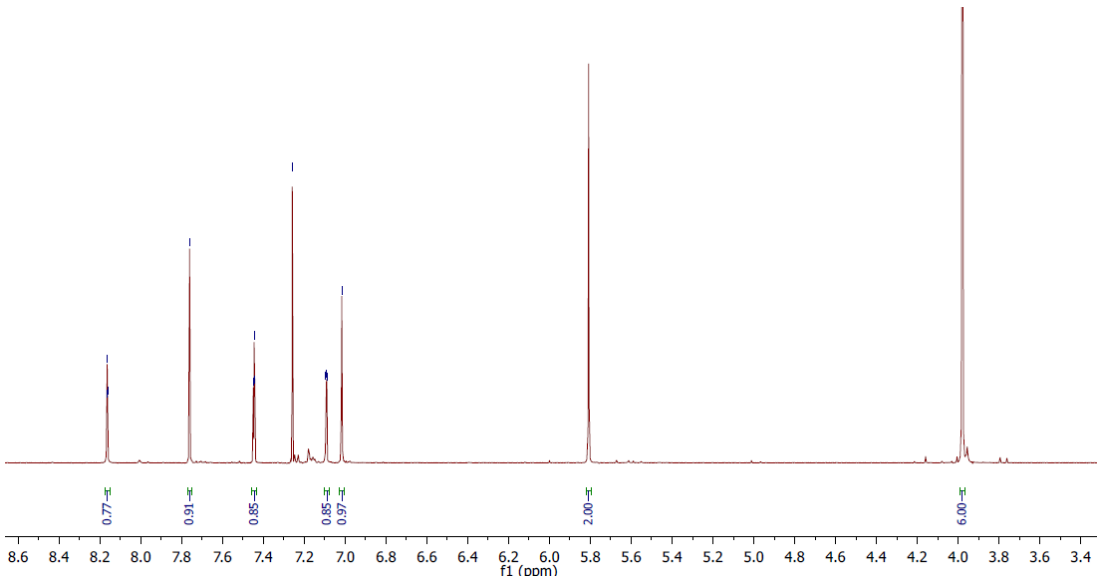


^{13}C-NMR was not obtained.

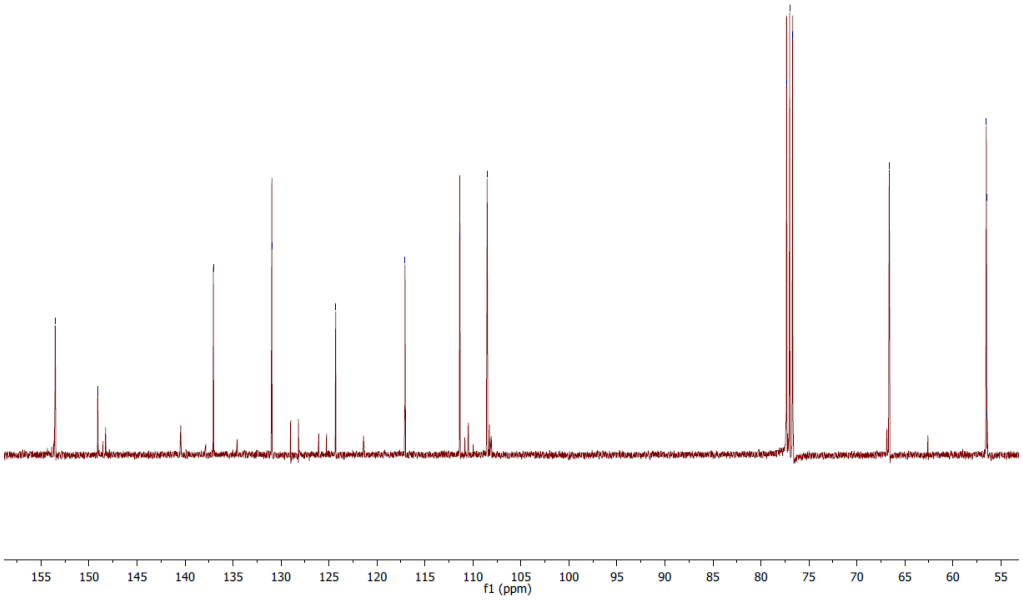


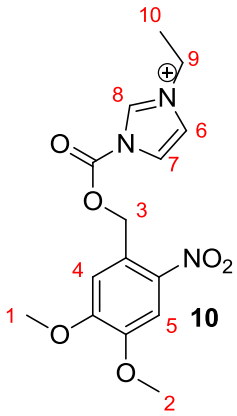
Molecule 9

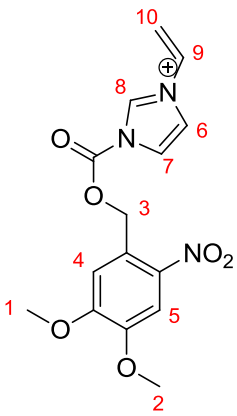
^{1}H-NMR: The solvent used was $CDCl_3$. The large single peak at 3.98 ppm is caused by the hydrogen atoms at 1 and 2. The 5.81 ppm peak is caused by the hydrogen 3. The hydrogen atoms at 4 and 5 give rise to the peaks at 7.02 ppm and 7.76 ppm respectively. The peaks at 7.09 ppm and 7.45 ppm are caused by 6 and 7. The final peak at 8.16 ppm is caused by 8.



^{13}C-NMR ($CDCl_3$): 56.49, 56.52, 66.63, 108.52, 111.37, 117.08, 124.30, 130.95, 137.04, 149.08, 153.53 (one peak for either the carbonyl carbon or the carbon bound to the nitro group is missing)

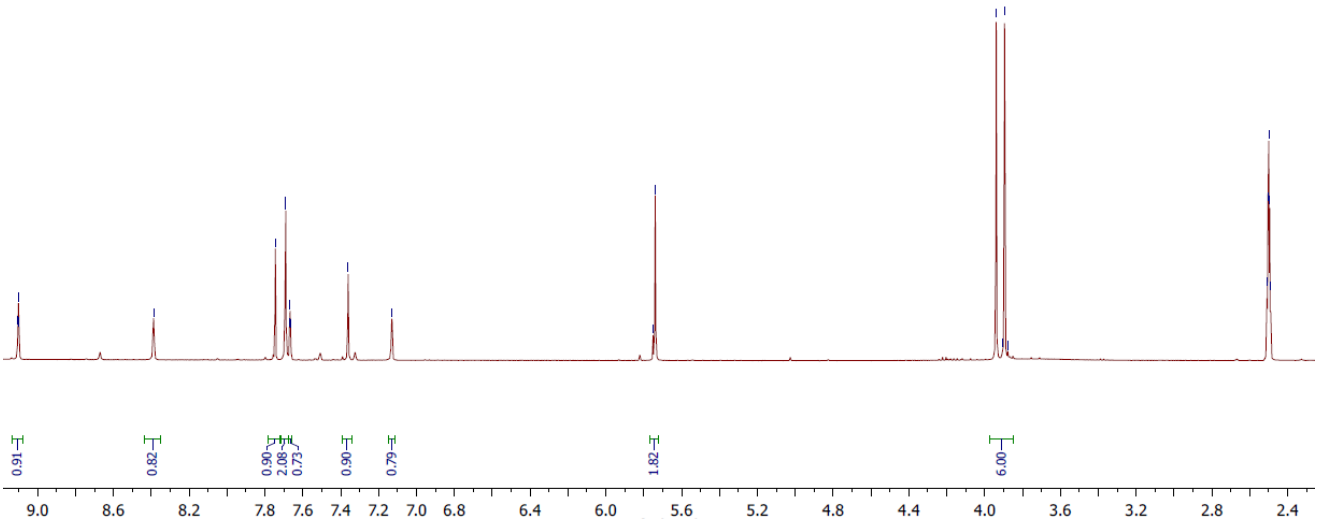




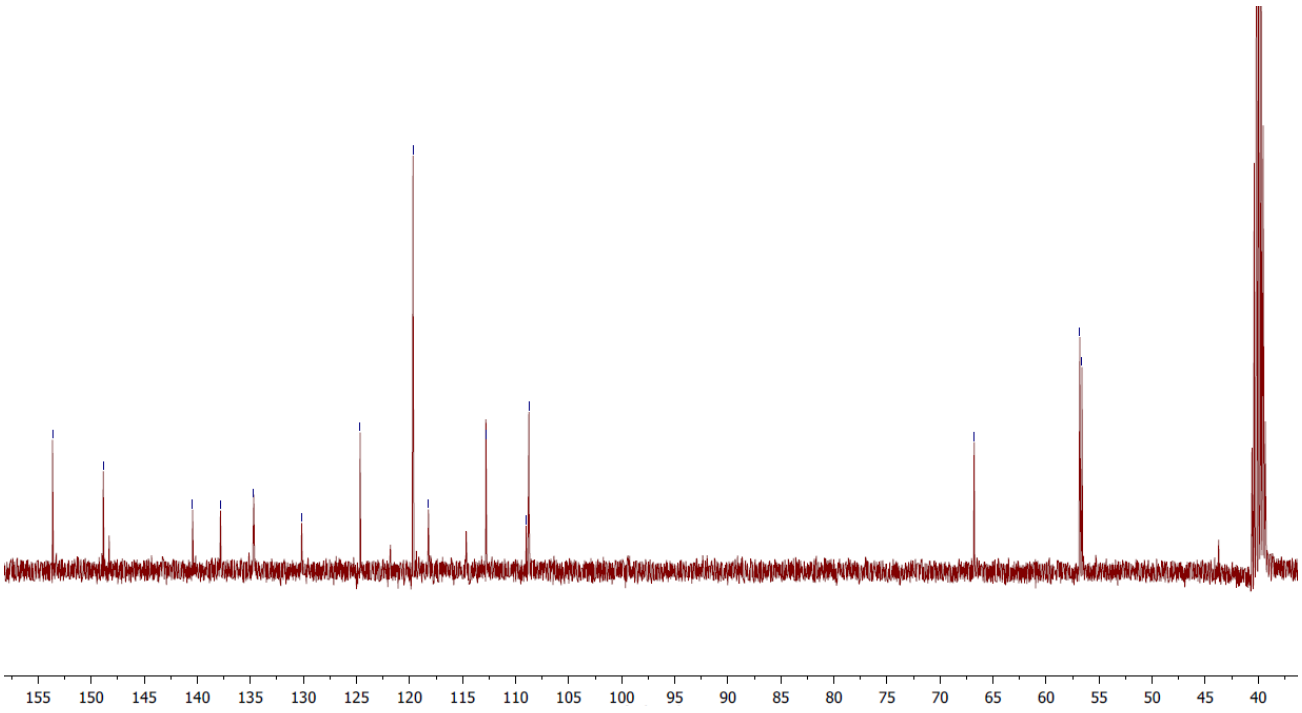


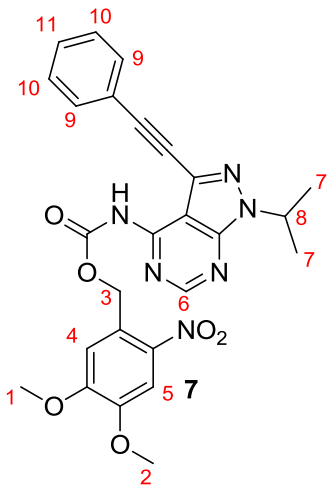
Molecule 10

^{1}H-NMR: The picture above is not obviously **10** it could be something else, like for example the picture next to 10. It does however remain unclear why anything other than the described **10** would form. The solvent used was d-DMSO and the peak can be seen at 2.50 ppm. The two peaks at just below 4 ppm, at 3.89 ppm and 3.94 ppm, are caused by the hydrogen atoms at 1 and 2. The 5.74 ppm peak is caused by the hydrogen 3. The hydrogen atoms at 4 and 5 give rise to the peaks at 7.36 ppm and 7.75 ppm respectively. The peaks at 7.13 ppm, 7.67 ppm and 8.39 ppm are caused by 6, 7 and 8. This remains true for both alternatives. What remains are two peaks at 9.10 (size 1H, tripplet) and 7.69 ppm (size 2H, doublet). These peaks are obviously too small for being 9 and 10 in **10** and also they behave like aromatic peaks. If they are placed in a conjugated system with a vinyl group they are however not impossible. They are further downfield than expected which might be explained with the bond to the charged nitrogen. Also the split confirms this. What is harder to explain is why the two hydrogens at the 10 position would be equivalent. All in all it is not obvious what kind of structure we have.



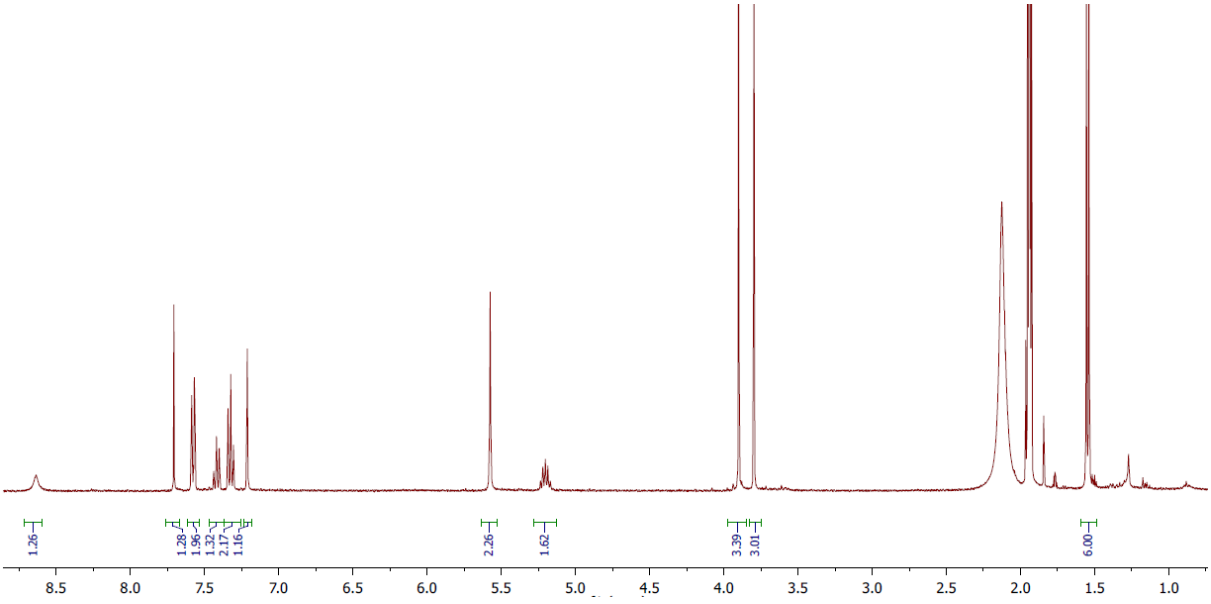
^{13}C-NMR (d-DMSO): 56.62, 56.82, 66.78, 108.75, 109.01, 112.80, 118.22, 119.67, 124.66, 130.17, 134.69, 137.82, 140.45, 148.87, 153.65





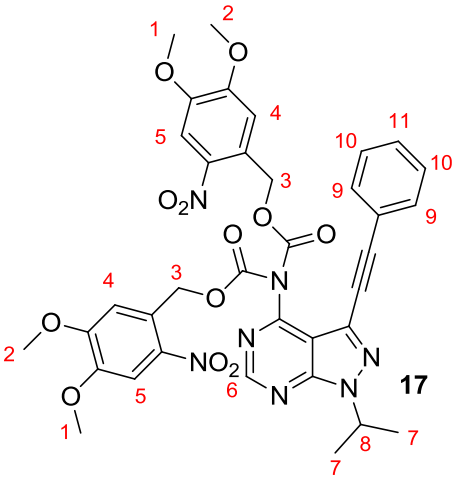
Molecule 7

^{1}H-NMR: The solvent used is d- acetonitrile. The hydrogen atoms from 1 and 2 can be found at 3.90 ppm and 3.80 ppm. 3 causes the peak at 5.57 ppm. At 7.21 ppm the peak caused by 4 is found and at the one at 7.71 ppm is caused by 5. The small broad peak at 8.63 ppm is caused by 6. At 1.53 ppm the doublet caused by the hydrogen atoms at 7 can be found. The multiplet found at 5.20 ppm is caused by the single hydrogen at 8. 9, 10 and 11 give rise to the multiplets at 7.3-7.6 ppm. The hydrogen on the amid is not visible. The small broad peak at 2.13 is caused by water and the peak at 1.90 is caused by the d-acetonitrile.

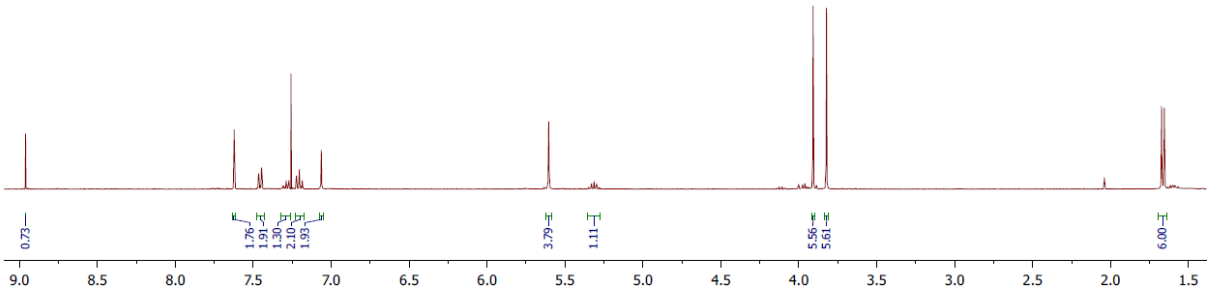


^{13}C-NMR: Not obtained

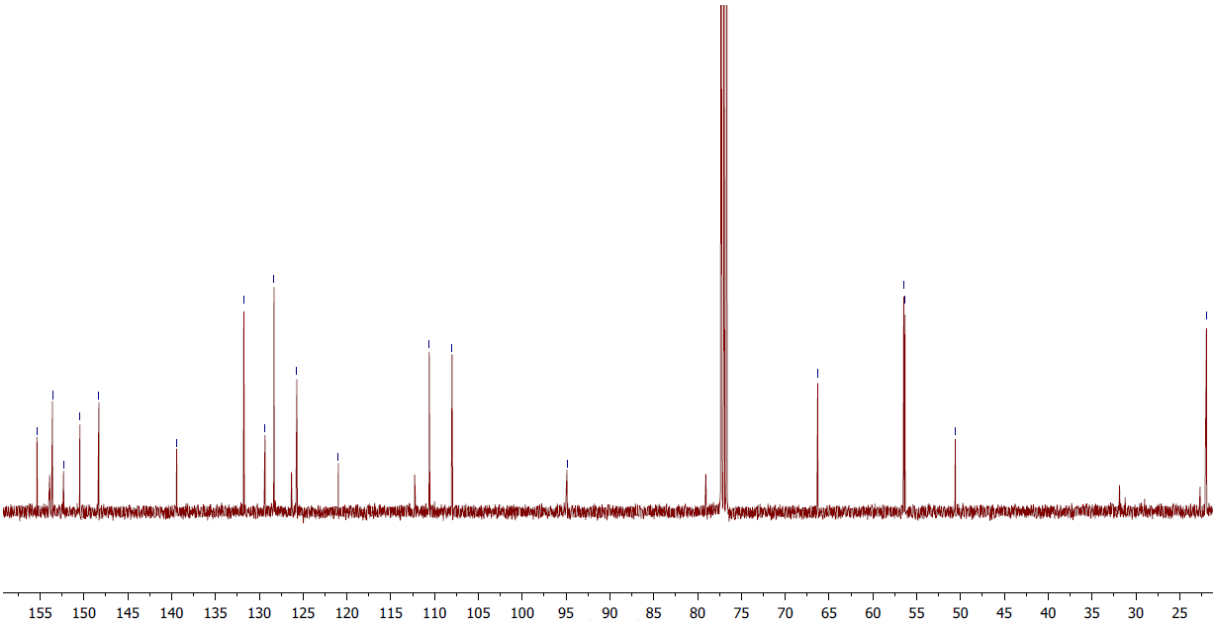
Molecule 17



^{1}H-NMR: The solvent used is $CDCl_3$. The peaks are similar to the ones found with **7**. 1 and 2 can once again be found just below 4.0 ppm, 3.91 and 3.82 ppm, but are twice as large compared to the peaks caused by 6-11 before. All the peaks caused by 1-5 have roughly twice as large integrals compared to peaks caused by 6-11 when compared to **7** since there are twice as many hydrogen atoms in the environments 1-5. The peak caused by 3 is found at 5.60 ppm. The peaks caused by 4 and 5can be found at 7.06 ppm and 7.62 ppm. The peak for 6 can be found at 8.96 ppm. The peak at 1.7 is caused by the atoms at 7. The multiplet at 5.31 ppm is caused by the hydrogen atom 8. Once again the atoms 9-11 cause the multiplets between 7.20 ppm to 7.47 ppm. The singlet peak at 7.26 ppm is the solvent peak.



^{13}C-NMR ($CDCl_3$): 21.94, 50.59, 56.47, 66.29, 94.90, 108.01, 110.61, 120.98, 125.72, 128.32, 129.34, 131.76, 139.42, 148.29, 150.47, 152.33, 153.59, 155.36.



Appendix 2 – Changes in absorption at 338nm and 412 nm over time

338 nm

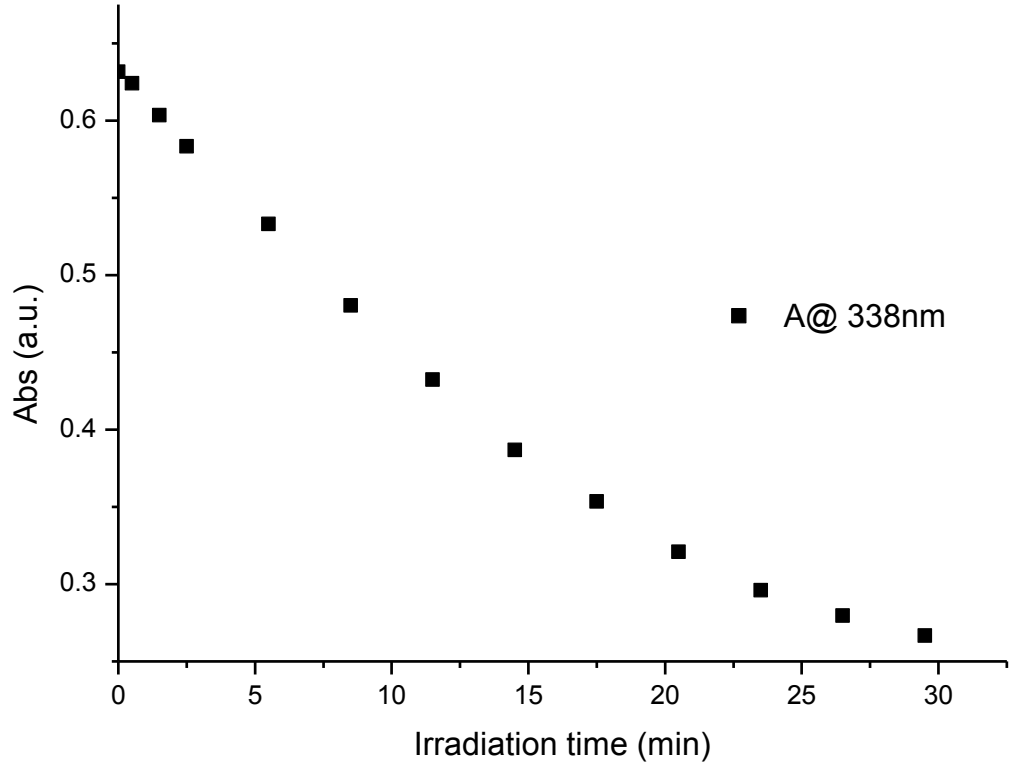


Figure 17: Change in absorption at 338 nm over time.

412 nm

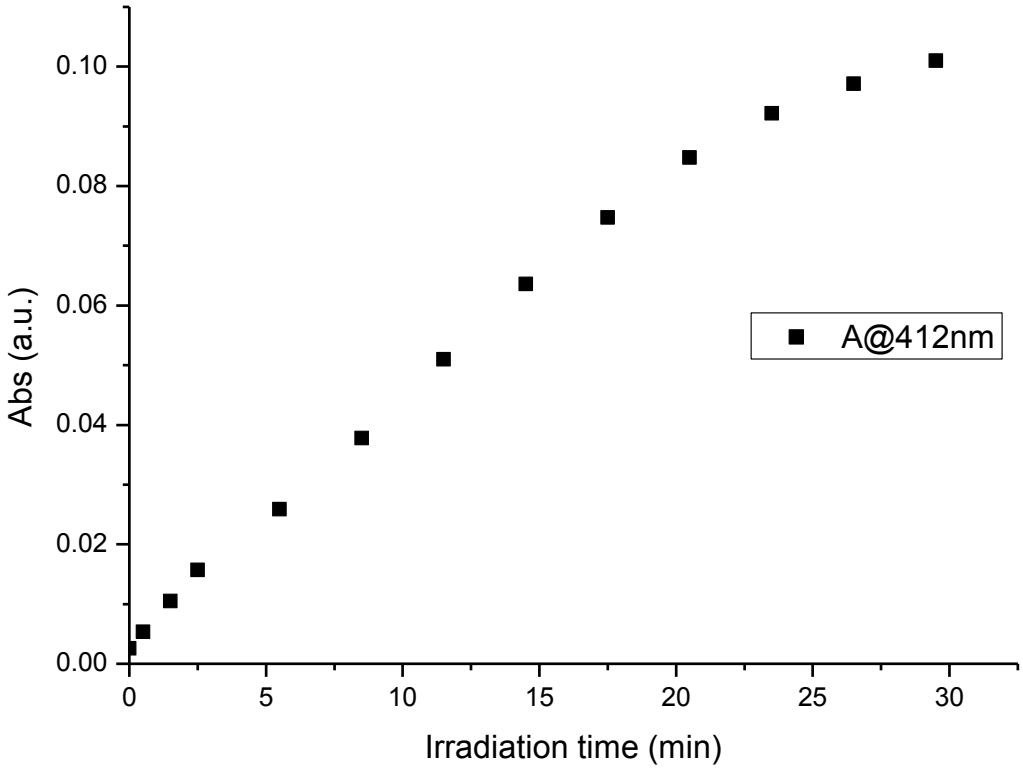


Figure 17: Change in absorption at 412 nm over time.