University of Groningen

Photoresponsive antibiotics and cytotoxic agents

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Chapter 5

Photoprotection of a model antibiotic and anticancer agent

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General Overview of the Two Sections

In this chapter we present our attempts towards applying the previously obtained BODIPY photoprotecting groups as solutions to counter two of modern day society's most pressing healthcare problems: side effects of chemotherapy in treating cancer and the buildup of bacterial resistance towards antibiotics.

In part 1 of this chapter, a photoprotected derivative of Mitomycin C – a drug commonly used for chemotherapy is described. In part 2 of this chapter, a photoprotected derivative of Neomycin – a commonly used antibiotic for treating infections caused by Gram-negative bacteria is described.

Even though both of the aforementioned drugs find application in treating their target diseases, their use is limited by their, often severe, adverse effects. The goal of our research was to lessen these adverse effects by enhancing the selectivity of the drugs with the use of photoprotecting groups.

Photocleavable Mitomycin C: towards limiting the side effects of HIPEC therapy

Introduction

Cancer is a major concern for today's society. In the US, only in 2017, 1,688,780 new cancer cases and 600,920 cancer death cases were projected for 2018.[1] Pancreatic cancer is one of most abundant and lethal tumors, with 10 - 20% of diagnosed patients able to undergo potential curative surgery that shows 15% 5-year-survival rate.[2] Only in the USA, pancreatic cancer killed over 35600 of people in 2011.[3]

To fight cancer, clinicians usually turn to surgery, but sometimes this kind of treatment is not enough and alternative methods need to be explored. Other commonly used cancer treatments are radiation therapy, chemotherapy, immunotherapy, hormone therapy and stem cell transplants.[4] In some cases though, one type of cancer treatment is not enough to help the patient recover and a combination of treatments has to be used.[5]

The decision of employing a second type of treatment can be made during or after the surgery. After the surgeon has removed the tumor along with some additional tissue around it, this excess tissue, called the cancer margin, is examined thoroughly by a pathologist. If no trace of cancer cells is found in it, the margin is described as negative (Figure 24, a). This result suggests that the entire tumor has been successfully removed and no additional treatment is needed. On the other hand, if any cancer cells are present in the margin, it is described as positive and shows that some of the tumor cells are still left in the patient's body (Figure 24, b). This result either means that the surgery has to continue or a different therapy is needed.
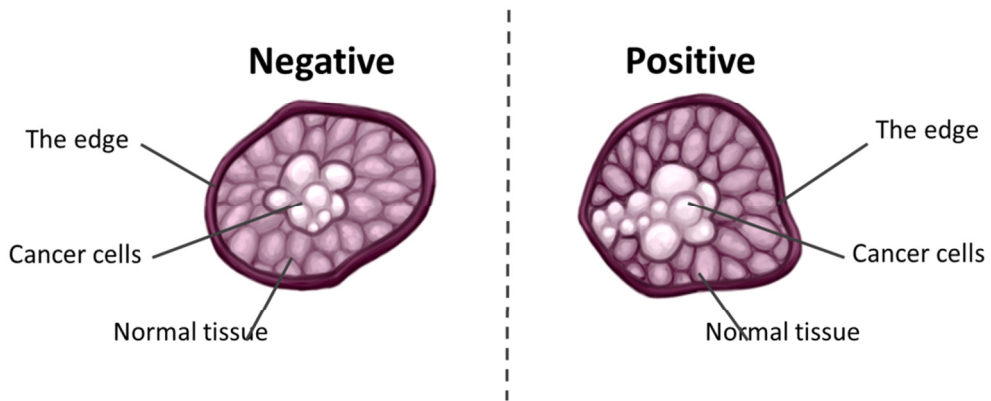
Figure 24. Schematic interpretation of when a tumour is easy to eliminate by surgery (negative) or needs additional treatments (positive)

In the treatment of pancreatic cancer[6] but also colon cancers,[7] a therapy called HIPEC (Hyperthermic Interperitoneal Chemotherapy) can be successfully applied during the surgery,[8] after the ablative part. The main idea of HIPEC treatment is to chemically kill the cancer cells left after the initial ablation. The standard procedure consists of pumping in and circulating a heated chemotherapy drug solution through the abdominal cavity through the same cut that was made during the tumor removal surgery.[9] This method of administration is favored over IV injections because of the existence of a barrier to diffusion between the plasma and the peritoneum. If the chemotherapeutic is injected to the bloodstream, it spreads evenly through the body and only a small amount of it is able to pass through the peritoneum to the targeted area. If, however, the drug is administrated via a direct injection to the peritoneum, the same barrier does not allow for its passage to the bloodstream, resulting in a much higher concentration of the drug in the peritoneum, compared to the IV injection method.[10] This modification, combined with the applied heat allows for better penetration of the cancer cells by the chemotherapeutic which can lead to an increase of its activity and, in some cases, to overcoming the cell resistance to the used drug.[11]

As a result, the survival rates and the quality of life of the patients are greatly improved, making HIPEC therapy superior to standard chemotherapy. Its drawbacks, however, remain severe and certain adverse effects can be observed in a majority of patients (up to 50% and may be the cause of death for 0-12%).[12]

These drawbacks include: leukopenia, heart and kidney disorders, small bowel fistulas, fatigue, weakness and cell necrosis when the temperature of the drug solution is too high (>42 °C). Because of these adverse effects, determining the patients which would benefit from HIPEC therapy and the optimal chemotherapeutics to use is an ongoing challenge faced everyday by the oncologists.[13]

As HIPEC therapy is relatively young[14], it has not been yet standardized: the preferably used chemotherapeutics and their doses vary from center to center[14]. What has been agreed upon is, however, how an optimal candidate for this kind of drugs should behave.[15] First of all, the drug should lack severe toxicity after the intraperitoneal administration and have a well-established activity against the malignancy it is meant to treat. Second, the drug has to be active immediately; those that need to be activated metabolically in the liver are not usable in the treatment. Third, the drug should be globally effective, even on the more resistant tumor cell lines of the targeted cancer. And finally, the chemotherapeutics have to be stable at elevated temperatures.

The pool of commonly used drugs for HIPEC is therefore rather narrow and consists of: cisplatin, carboplatin, oxaplatin, melphalan, doxorubicin, docetaxel, paclitaxel, 5-fluorouracil, irinocetan, and Mitomycin C. The last compound of this list is the drug of choice in most centers where patients with pancreatic cancer of colorectal and appendiceal origin are treated.[16]
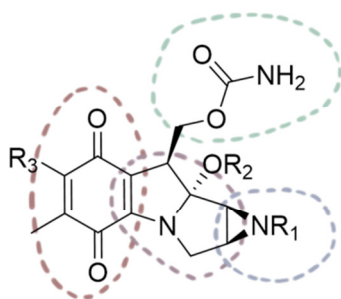
Name	R_1	R_2	R_3
Mitomycin A	H	CH_3	OCH_3
Mitomycin B	CH_3	H	OCH_3
Mitomycin C	H	CH_3	NH_2
Mitomycin D	H	OH	NH_2
Mitomycin E	CH_3	OH	NH_3

Scheme 36. Structures of mitomycins A-E

Mitomycins are a group of biologically active (both antibacterial and antitumor) compounds derived from mitosane. Originally isolated from *Streptomyces*

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caespitosus by Hata and all in 1956,[17] they are closely related in their structures and activity (Scheme 36).

These compounds contain four reactive moieties (Scheme 36). These are: an aziridine ring (circled in blue), a carbamate side chain (green), a methoxy or amino group-bearing benzoquinone (red) and a pyrrolizidine fragment (purple). Substituents on the benzoquinone ring and the aziridine N atom differ in many natural mitomycins and their role on the biological activity of the compounds is only partially understood.

Even though mitomycins are commonly used as chemotherapeutics, their mode of action was originally evaluated on bacteria.[18] The toxic effects of mitomycins are associated with their ability to covalently cross-link complimentary DNA strands. By forming two covalent bonds in 5'CpG3' sequences they stop DNA replication leading to cell death. Szybalski and coworkers[19] demonstrated that DNA isolated from cells (*Escherichia coli* strain B, *Sarcina lutea* strain ARCC-272 and various *Bacillus subtilis* mutant lines) exposed to mitomycins for 1 minute already show signs of being affected by it. The experiment consisted of treating the bacterial DNA with high temperature denaturation followed by rapid cooling (Figure 25).

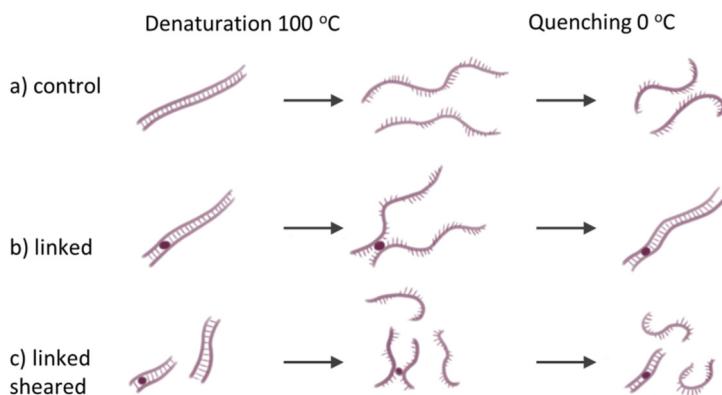Figure 25. A schematic representation of the possible events happening in the experiment of Szybalski group: A) control sample of noncross-linked DNA, B) Cross-linked DNA, C) Cross-linked DNA with additional breaking of the initial double strand before denaturation

For a control sample of DNA, the process of denaturation was irreversible and the separated strands did not renaturate (Figure 25. A). For the samples with

Mitomycin-crosslinked DNA, however, the situation was different. The partially separated strands were still covalently connected and close together enough to bind (Figure 25. B). Also partial renaturization has been observed, probably as a result of an earlier double strand break (Figure 25. C). They also observed that mitomycins did not react with isolated DNA unless a cell extract was added, which led them to the conclusion that the antibiotic must be activated by the cell before it can act.

Later on, Szybalski,[20] along with others,[21] has worked on deciphering the mechanism of action of mitomycins. Initially, based on the observations by Schwarz and coworkers[22] that mitomycin C can be reduced and metabolically inactivated in liver, it was hypothesized that reducing steps are responsible for mitomycins metabolic activation. It was later proven to be true [23] through various attempts of chemical reduction of mitomycin C and subsequent cross-linking of isolated bacterial DNA.

It turned out, however, that the active form of the drug is fairly unstable, as, for the cross-linking reaction to take place, the DNA needed to be added within seconds after the reduction. Reoxidizing mitomycin C also did not give the original compound back: the product had no more cross-linking properties (when submitted to the same reaction sequence again) and its spectrum was similar to the one of the product of acidic degradation of mitomycin C (Scheme 37).[24]

It was in 1972 that the idea of using bioreductive alkylation as a method of treating cancer has emerged.[25] It was reasoned that the oxygen-deficient cells of solid neoplasms with increased tissue concentrations of NADH and NADPH may provide a great target for mitomycins. The group of Sartorelli, inspired by this idea, performed a comprehensive study on the effect of mitomycins on hypoxic tumor cells.[26] It has been proven that Sarcoma 180 and EMT6 mammary carcinoma cells not only are able to uptake mitomycin C under the proposed conditions, but also that the drug indeed exhibited significant toxicity towards them. Surprisingly, one of the tested compounds, BMY-25282, a mitomycin C derivative modified on the amine group on the quinolone ring, showed greater toxicity towards the used cells, even if oxygen was present in the system.
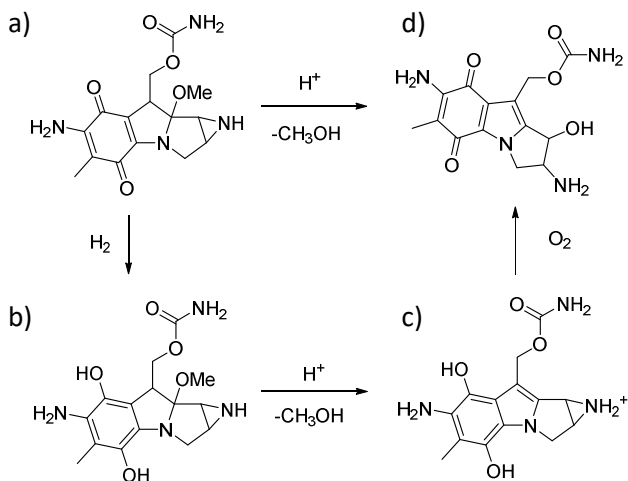
Scheme 37. Structures of Mitomycin C in the studied reactions: a) Mitomycin C before the reactions, b) Product of reduction, c) A postulated structure after a rearangement taking place after reduction, d) Product of acidic degradation

This kind of differences in biological activity of derivatives of mitomycins was throughfully studied by the groups of Uzu, Oboshi and Miyamura.[27] The researchers synthesized a series of mitomycin derivatives varying the substituents believed to be responsible for their respective activity.

It turned out that changing the tertiary methoxy group to a hydroxyl did not significantly alter the cross-linking abilities of the compound but resulted in a reduced bactericidal and antineoplastic activity. This group was already suspected of not being responsible for binding to DNA, as it was not present in the active intermediate formed after the catalytic hydrogenation of mitomycin. Its reoxidation product also lacks this group (compound c and d on Scheme 37).

Changing the amine group on the quinone ring to a hydroxy, did, however, make a difference on the compounds DNA cross-linking ability (a difference which varied with the bacterial strain used for testing). It was observed that, in most cases, the compounds bearing the OH moieties were less active than these with N-pyridine, tertiary amines, methoxy, aziridino and amino groups in the same position. The activity of these derivates increased in the same respective order. It was therefore postulated that the electronic character of the substitution on this position changes the redox potential of mitomycins, resulting in an altered activity.

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Lastly, they proved that, although the methylation of the aziridine nitrogen atom did not alter by much the activity of the studied compound, acylation or sulphonylation did. This finding was rationalized by considering the fact that the aziridine nitrogen atom has to be protonated before being able to react further (Scheme 37, c), which is much easier done for amines that amides or suplhonamides.

Their study has shown that changing the structures of mitomycins by modifying either their aziridine groups or the groups connected to the quinolone ring can greatly affect the activity of the drugs towards their targets. These groups were later proven to play a major role in the mode of action of mitomycins, which is of key importance for the molecular design presented in this chapter.

Even though these findings shone some light on the initial mechanism of cross-linking DNA by mitomycin and multiple research groups have devoted tremendorous effort to describe the full mechanism of action of the drug (Scheme 38),[28] our understanding of its mode of action is still not complete, as new findings arise.[29] However, a resonable picture of its mode of action has been drawn from the aforementioned experiments (Scheme 38).

The commonly accepted mechanism of action of Mitomycin C starts with the reduction and protonation of of the quinone moiety. This reaction can proceed via either one- or double-electron reduction followed by aromatization **2** and be catalyzed by a variety of enzymes,[30] thiols (and even more efficiently dithiols)[31] as well as simple reducing agents.[23] After this step, the methoxy group is eliminated and a fully substituted iminium **3** is formed which then tautomerises to the corresponding enamine **4** via elimination of the proton on the tertiary carbon at the foot of the methyl urea side chain. This step is followed by protonation of the aziridine and an intramolecular rearrangement which leads to opening of the now protonated aziridine ring to give **5**. The stage has now been set for a 1,6-attack of a guanidine from a DNA lone strand onto α,β,γ,δ-unsaturated ketone **5** to give intermediate **6**. This indole intermediate can further rearange to eliminate the carbamate and form α,β-unsaturated iminium **7** which can be trapped by another guanidine from a second DNA lone strand resulting in a stable polysubstituded indole **8** bearing both DNA strands.
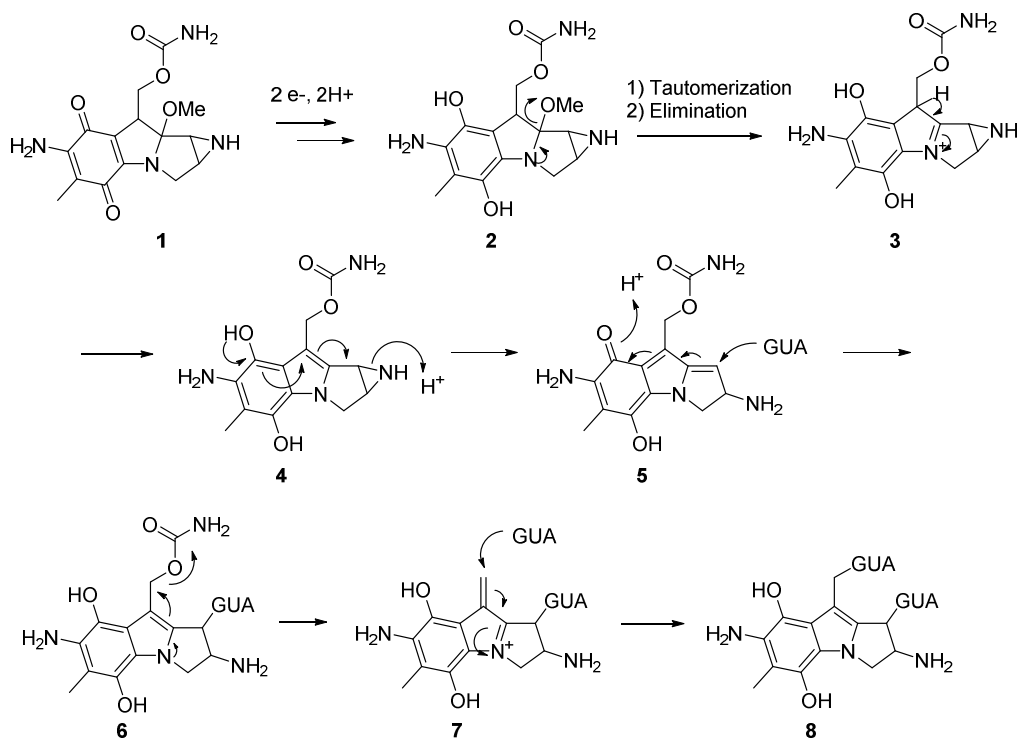
Scheme 38. Mechanism of DNA cross-linking by Mitomycin C

The whole reaction takes less than a minute to complete and is proven to be deadly for a variety of bacteria,[32] viruses[33] and bigger organisms.[34]

Because of its effective mode of action, Mitomycin C is used as a chemotherapeutic [35] and an antibiotic for persistent bacteria.[36] This same mechanism is, however, responsible for some of the side effects caused during the therapy.[37] These include severe nausea and vomiting, temporary hair loss, bruising and bleeding, breathlessness, pathological changes in organs, skin rash, constipation, etc. In some rare cases, because of its lack of selectivity, secondary cancers can occur. This problem is crucial to address to ensure a more effective treatment when using this drug in chemotherapy, especially during the HIPEC procedure, where the concentration of the drug is high and the surface of its administration vast. Because

of all of the aforementioned side effects, a modification of the drug is needed to render it more effective and safer to use in long term.

Results and discussion

In our approach, we wanted to design a photolabile protected derivative of mitomycin C, which would not show biological activity unless activated (deprotected) with light. This kind of modification would allow us to obtain more control on the region affected by the drug by activating it only at the site of action with the high spatio-temporal precision which is characteristic for light based methods. Once protected, the compound would not exhibit its cytotoxicity, which hopefully would stop the bacteria from gaining resistance to its active form.

To prepare compounds which would work in this desired manner, we designed mitomycin C derivatives **5** and **9** (Scheme 39). Both of these compounds consist of mitomycin C connected with a photoprotecting group (coumarin for compound **5** and BODIPY for compound **9**) via the nitrogen of the aziridine ring. We hoped that this modification would prevent the opening of the ring as described in the mechanism of action of the drug therefore deactivating the drug till its release.[38]
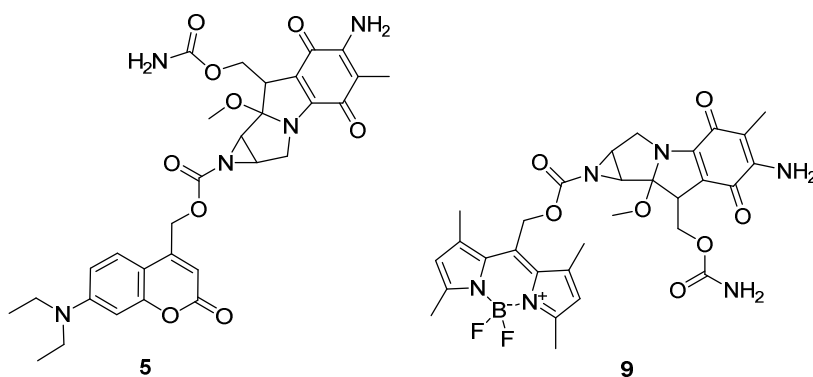
Scheme 39. Structures of proposed derivatives of mitomycin C

The main difference between these compounds is the wavelength of their deprotection. Unsubstituted coumarin PPGs usually absorb light at wavelengths shorter than 450 nm[39], which is effectively being absorbed also by the tissues of the body[40], while the BODIPY PPGs absorb the light of above 520 nm[41], making them more clinically applicable.

To obtain the desired compounds, we used the synthetic routes shown below (Scheme 40 for compound **5** and Scheme 41 for compound **9**).
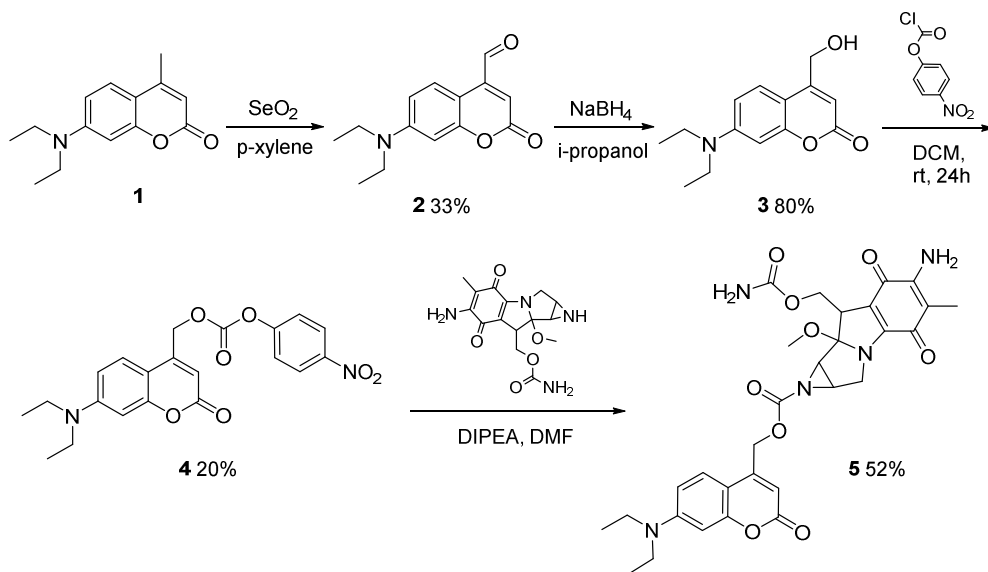
Scheme 40. Synthetic route for obtaining mitomycin derivative **5**

The synthesis of compound **5** started with oxidation of commercially available compound **1** to compound **2** using SeO$_2$,[42] yielding the desired aldehyde in 33% yield. Next, the compound was reduced with NaBH$_4$ leading to alcohol **3** in 80% yield.[43] Subsequently, a reactive carbonate **4** was obtained by reaction of **3** with *p*-nitrophenyl chloroformate.[44] During this reaction, it is crucial for the chloroformate to be added slowly, as otherwise a side reaction occurs yielding the corresponding alkyl chloride instead of the carbonate. It was also observed that any basic aqueous treatment of the reaction mixture caused degradation of the desired product. Due to these stability issues, carbonate **4** was obtained in a poor yield of 20% (unoptimized). It was then coupled with mitomycin C to afford the desired carbamate **5** in 52% yield.

Alternative compound **9** was obtained as follows (Scheme 41).
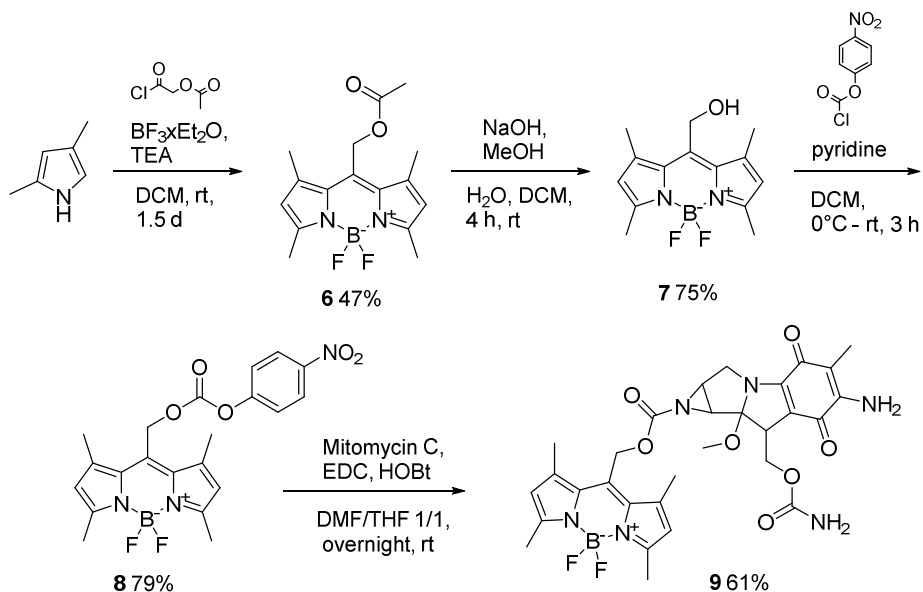
Scheme 41. Synthetic route for obtaining mitomycin derivative **9**

Compound **8** was obtained as described in detail in Chapter 2. With this compound in hand, we tried to apply the same reaction conditions as employed for the model fluorobenzylamines for the coupling with mitomycin C. However, no product was obtained. We attempted to optimize the conditions of the reaction by exploring various coupling reagents and solvent conditions (Scheme 42, Table 9). Adding mitomycin C to a THF solution of compound **8** in the presence of mixtures of DIPEA, DMAP, DCC or EDCI did not give any traces of the desired compound. However, after the addition of HOBt, the reactions with DCC or EDCI, formation of the mitomycin carbamate was observed. In the end, using this method, the desired compound was obtained in 61% yield.
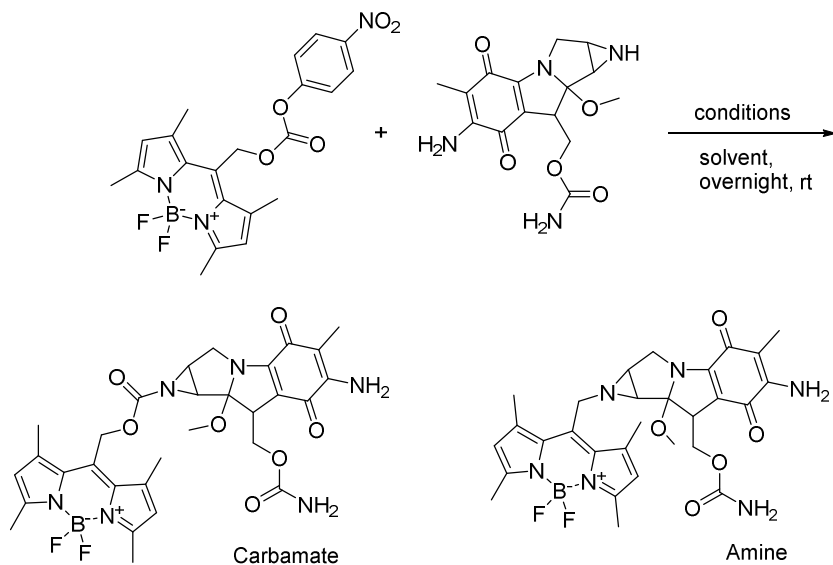
Scheme 42. Coupling of mitomycin C and carbonate **8**

Table 9. Conditions of the coupling reaction

Entry	Additive	Additive [equiv.]	Solvent	Outcome
1	-	-	DMF	no product
2	DIPEA	2	DMF	no product
3	DCC	1	DMF	no product
4	EDCI	1	DMF	no product
5	DMAP	1	DMF	no product
6	DCC+HOBt	1+1	DMF	Amine and carbamate
7	EDCI+HOBt	1+1	DMF	Amine and carbamate
8	EDCI+HOBt	1+1	DMF+THF	Mostly carbamate

With compounds **5** and **9** in hand, we started the deprotection studies. First, a 0.125 mM solution of compound **5** in 20% DMSO in phosphate buffer pH = 7.5 was irradiated with UV light (λ = 365 nm) for 1 hour. Although the compound seemed to fully react under these conditions, no traces of mitomycin C were found (data not shown). Instead, a variety of small signals was visible. As we were suspecting that Mitomycin C might be sensitive to UV light,[45] we did not conduct any additional studies on compound **5** but moved our attention to compound **9**.

To check the photostability of Mitomycin C under irradiation with light of longer wavelengths, we prepared samples of pure mitomycin C in 20% DMSO in phosphate buffer pH = 7.5 and irradiated them with light of λ = 530 nm and 650 nm for 10 min, taking UV-Vis spectra every minute. During this measurements no significant loss of absorbance was observed, leading us to the assumption, that the compound was not light-sensitive to red or green light (Figure 26).
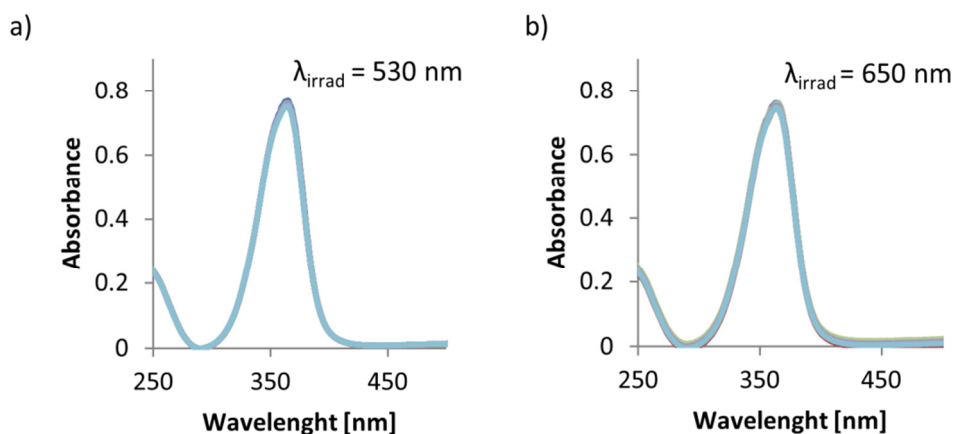
Figure 26. UV-Vis spectra of 20 µmol solutions of mitomycin C in 20% DMSO in phosphate buffer pH = 7.5: a) irradiation with green light (λ_{max} = 530 nm); b) irradiation with red light (λ_{max} = 650 nm)

Next, to check if compound **9** is photosensitive, we prepared its analogous solution and irradiated it with green light (λ_{max} = 530 nm). This time, to our delight, the absorbance was decreasing quickly (Figure 27).
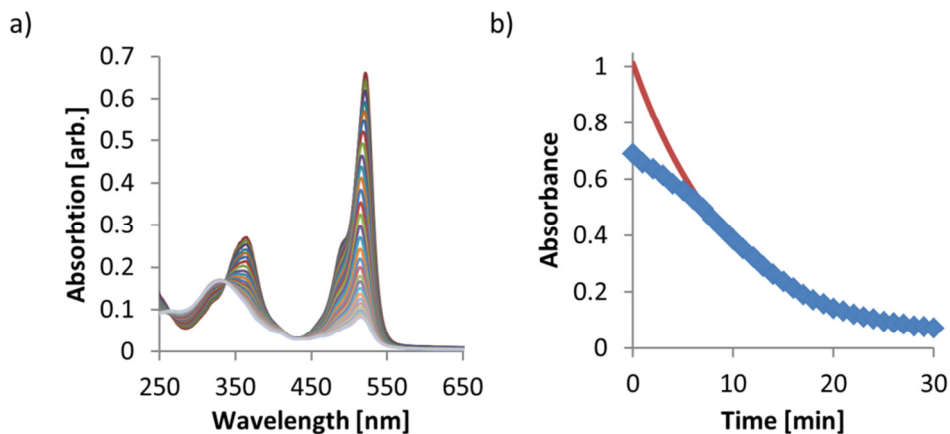
Figure 27. Irradiation of compound **5** (in 20% DMSO in phosphate buffer pH = 7.5) with green light: a) UV-Vis spectra taken every min; b) decrease of absorbance at λ_{max}

We noticed, however, that the sample was behaving a bit differently than those described in Chapters 2 and 3. Firstly, we observed an additional shift in the λ_{max} value of the compound during the deprotection (Figure 27, a). This led to the appearance of a lag time when we plotted absorbance at the λ_{max} of our compound versus time, suggesting that more than one process was taking place during the irradiation (Figure 27, b). We hypothesized that it could be caused either by the formation of a BODIPY photocleavage product as described by Winter and co-workers,[46] or that another reaction might have been taking place. Secondly, the λ_{max} of the absorption band attributed to mitomycin C moiety was also shifting to a value not corresponding to the one of the free drug (363 vs. 326 nm). Trying to rule out solvent effects in the process, we also attempted the photocleavage in 20% acetonitrile in phosphate buffer pH = 7.5. However, this change did affect the results (Figure 28.).
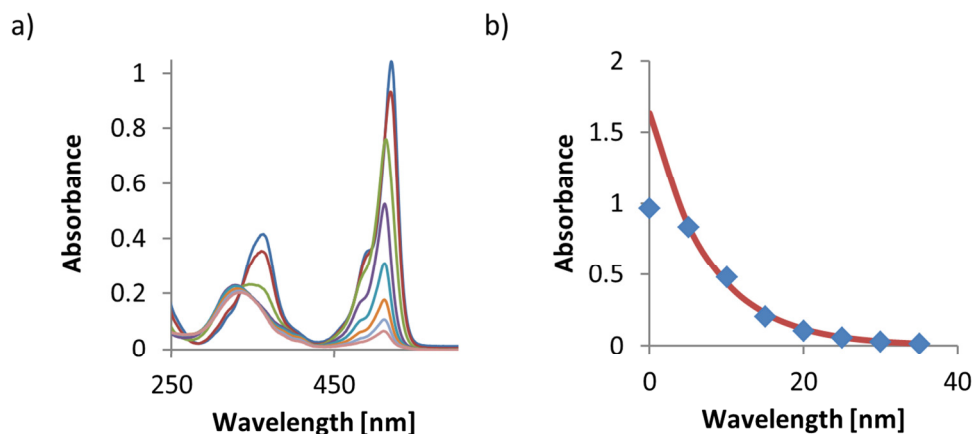
Figure 28. Irradiation of compound **9** (in 20% acetonitrile in phosphate buffer pH = 7.5) with green light: a) UV-Vis spectra taken every min; b) decrease of absorbance at λ_{max}

Next, using LCMS we attempted to determine the products of the photocleavage and establish the stability of compound **9**. Therefore we prepared two 0.125 mM solutions of compound **9** in 20% DMSO in phosphate buffer pH = 7.5. After measuring LCMS traces of both of them, one was irradiated with green light (λ_{irrad} = 530 nm) for 40 min and the other one was kept at room temperature in the dark.

To our delight, compound **9** was stable in the used media if stored in the dark at room temperature for 24 hours (Figure 29, a-c). As expected, it also reacted fully under the irradiation with green light (Figure 29, d-e). Analogously to the results for compound **5**, a variety of deprotection products was formed (Figure 29), of which none could be obviously attributed to the desired compounds or their close derivatives. No traces of free mitomycin were found in the resulting sample according to MS measurements.

With these results in hand we hypothesized that either mitomycin is released under the irradiation with green light but undergoes an additional reaction, or that the deprotection follows a different mechanism than we anticipated and the aziridine ring is opened during the experiments, what allows for the molecule to react further. With no conditions found for Mitomycin C being released, we did not conduct any additional studies on the obtained compounds and terminated the project.
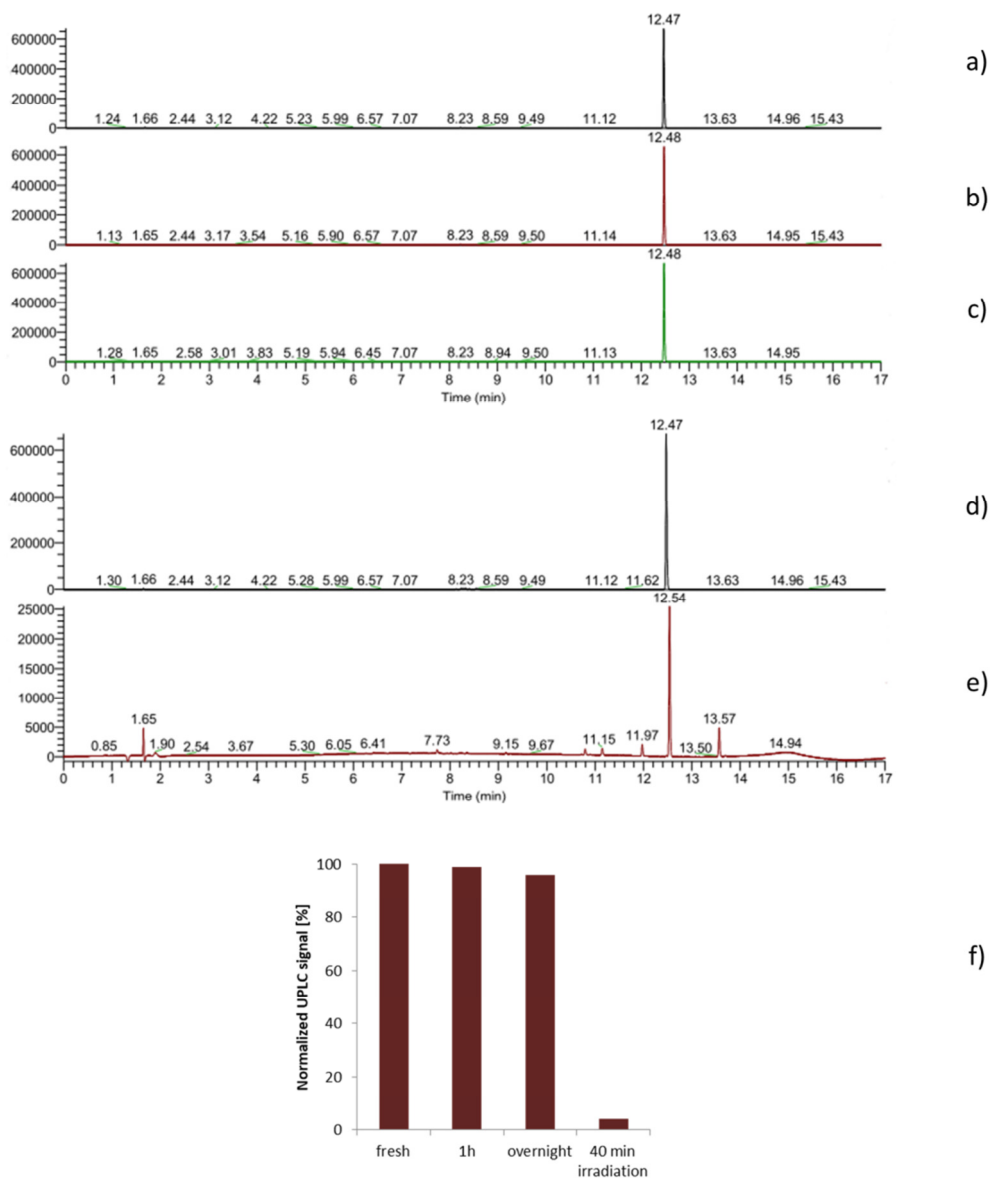
Figure 29. UPLC trace for compound **9**, 0.125 mM in 25% DMSO / 5 mM phosphate buffer, pH=7.5. λ_{obs}= 520 nm. a-c: stability study; a) freshly prepared sample, b) sample after 3h at rt, c) sample after one day at rt, d-e: photodeprotection study; d) freshly prepared sample, e) sample after irradiation with λ_{irr}=530 nm for 1 h, f: comparison of the normalized UPLC signals

Conclusions

We prepared two carbamate derivatives of Mitomycin C protected with coumarin (**5**) and BODIPY (**9**) photoprotecting groups. Neither of these compounds, however, furnished the free drug upon irradiation with light despite reacting fully. While in the case of compound **5** it could have been caused by the possible instability of Mitomycin C under UV irradiation, it could not have been the problem of compound **9** in view of the wavelength used (λ = 523 or 650 nm) as Mitomycin C turned out to be stable under these irradiation conditions. We hypothesized that either the deprotection proceed via a two-step mechanism in which the aziridine ring of the Mitomycin C opens before the PPG is cleaved or one of the photocleavage products reacts further with Mitomycin C. However, to prove any of these pathways additional studies are needed.

Experimental procedures

General Information

Starting materials, reagents and solvents were purchased from Sigma–Aldrich, Acros and Combi-Blocks and were used without any additional purification. Solvents for the reactions were purified by passage through solvent purification columns (MBraun SPS-800). 4-nitrophenol chloroformate was obtained from Combi-Blocks. Unless stated otherwise, all reactions were carried using standard Schlenk techniques and were run under nitrogen atmosphere in the dark. The reaction progress was monitored by TLC. Thin Layer Chromatography analyses were performed on commercial Kieselgel 60, F254 silica gel plates with fluorescence-indicator UV254 (Merck, TLC silica gel 60 F254). For detection of components, UV light at λ = 254 nm or λ = 365 nm was used. Column chromatography was performed on commercial Kieselgel 60, 0.04-0.063 mm, Macherey-Nagel.

UPLC traces were measured on Thermo Fisher Scientific LC/MS: UPLC model Vanquish, MS model LTQ with an iontrap and HESI (Heated ESI) ionisation source with positive and negative mode. UV-Vis absorption spectra were recorded on an Agilent 8453 UV/vis absorption Spectrophotometer. Irradiation at 532 nm was performed using Sahlmann Photochemical Solutions LEDs, type LXMLPM01, opt. power 810 mV. UV/vis spectra were baseline corrected. Nuclear Magnetic Resonance spectra were measured with an Agilent Technologies 400-MR (400/54 Premium Shielded) spectrometer (400 MHz) at room temperature (25°C). Chemical shifts for the specific NMR spectra were reported relative to the residual solvent peak in ppm; $CDCl_3$: δ_H = 7.26; $CDCl_3$: δC = 77.16; d_6-DMSO: δ_H = 2.50; d_6-DMSO: δ_C = 39.52. The multiplicities of the signals are denoted by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). All ^{13}C-NMR spectra are [1]H-broadband decoupled. High-resolution mass spectrometric measurements were performed using a Thermo scientific LTQ OrbitrapXL (ion trap) spectrometer with ESI ionization. The molecule-ion M+, [M + H]+ and [M–X]+, respectively are given in m/z-units. Melting points were recorded using a Stuart analogue capillary melting point SMP11 apparatus.

Compound Characterisation

7-(diethylamino)-4-formylcoumarin (2) (According to a literature procedure)
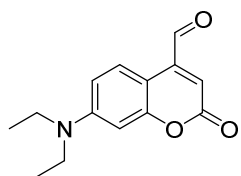
Selenium dioxide (1.4 g 13 mmol, 1.5 equiv.) was added to a solution of 7-(diethylamino)-4-methylcoumarin (2.0 g, 8.7 mmol) in *p*-xylene (60 mL) and the mixture was heated for 2 days at 130°C. The solution was then cooled down and filtrated. The filtrate was washed 1M aq. HCl solution (6 x 20 mL). The aqueous layers were basified with aq. Na$_2$CO$_3$ and extracted with DCM (3 x 20 mL). The combined organic layers were dried with MgSO$_4$ and concentrated *in vacuo* to yield a brown oil, which was subjected to column chromatography (pentane/acetone 13:2 -> 10:5, v/v) to afford desired product **2** as red crystals (630 mg, 30% yield).

Rf. = 0.75 (pentane/acetone 13:2), M.p. = 58-61 °C, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 1.21 (t, *J* = 7.1 Hz, 6H, **CH$_3$**), 3.42 (q, *J* = 7.2 Hz, 4H, **CH$_2$**) , 6.44 (s, 1H, OCHC**CH**), 6.51 (d, *J* = 2.6 Hz, 1H, NC**CH**CHCCO), 6.62 (dd, *J* = 9.2, 2.6 Hz, 1H, NCCH**CH**CCO) , 8.29 (d, *J* = 9.2 Hz, 1H, OCOC**CH**C), 10.02 (s, 1H, CO**H**). The NMR data was in agreement with the literature data[47].

7-(diethylamino)-4-(hydroxymethyl)coumarin (3) (According to a literature procedure)
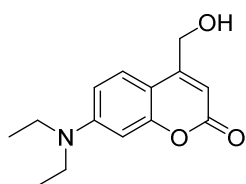
Sodium borohydride (80 mg, 2.2 mmol, 1.5 equiv.) was added to a solution of compound **2** (350 mg, 1.4 mmol) in *iso*-propanol (50 mL). After stirring the mixture for 2 hours, a 1N aq. HCl solution was added until no gas formation was detected. The excess of the acid was neutralized by addition of aq. Na$_2$CO$_3$ and the obtained solution was extracted with DCM (3 x 20 mL). Combined organic layers were dried with MgSO$_4$ and concentrated *in vacuo* to yield a brown oil, which was subjected to column chromatography (pentane/acetone 13:15 to 1:1 v/v) to afford desired product **3** as yellow crystals (150 mg, 42% yield).

Rf. = 0.3 (pentane/acetone 13:2), M.p. = 127-128 °C, ^{1}H NMR (400 MHz, Chloroform-*d*): δ 1.20 (t, *J* = 7.1 Hz, 6H, **CH$_3$**), 3.41 (q, *J* = 7.1 Hz, 4H, **CH$_2$**), 4.83 (d, *J* =

5.3 Hz, 2H, CC**CH₂**OH), 6.25 (s, 1H, HOCH₂C**CH**), 6.52 (d, *J* = 2.6 Hz, 1H, NCC**CH**CHCCO), 6.56 (dd, *J* = 9.0, 2.6 Hz, 1H, NCC**CH**CHCCO), 7.32 (d, *J* = 9.0 Hz, 1H, OCOC**CH**C). The NMR spectrum was in agreement with the literature data[48].

(7-(diethylamino)-coumarin-4-yl)methyl-(4-nitrophenyl)-carbonate (4)
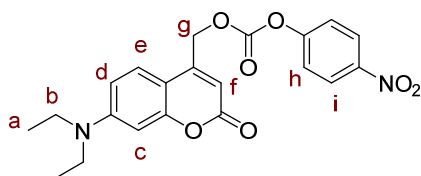

4-nitrophenyl-chloroformate (150 mg, 0.73 mmol, 1.2 equiv.) was added to a solution of compound 2 (0.607 mmol, 150 mg) and DMAP (0.910 mmol, 111 mg) in dry DCM (20 mL). The mixture was stirred under argon at rt for 24h and afterwards it was washed 6 times with a saturated aq. NaHCO₃ solution. The organic layer was dried with MgSO₄ and concentrated *in vacuo* to yield yellow solid, which was subjected to column chromatography (DCM) to afford product **4** as orange crystals (50 mg, 20% yield).

Rf. = 0.6 (DCM), M.p. = 150-153 °C, ¹H NMR (400 MHz, Chloroform-*d*) δ 1.21 (t, *J* = 7.1 Hz, 6H, a), 3.42 (q, *J* = 7.1 Hz, 4H, b), 5.40 (d, *J* = 1.0 Hz, 2H, g), 6.21 (s, 1H, f), 6.53 (d, *J* = 2.5 Hz, 1H, c), 6.60 (dd, *J* = 9.0, 2.6 Hz, 1H, e), 7.31 (d, *J* = 9.0 Hz, 1H, d), 7.42 (d, *J* = 9.1 Hz, 2H, h), 8.29 (d, *J* = 9.1 Hz, 2H, i). ¹³C NMR (101 MHz, Chloroform-*d*) δ 12.4, 44.8, 65.6, 76.7, 77.0, 77.3, 97.9, 105.5, 106.9, 108.8, 121.7, 124.3, 125.4, 145.6, 147.7, 150.9, 152.1, 155.2, 156.5, 161.6.

(7-(diethylamino)-2-oxo-2H-chromen-4-yl)methyl 6-amino-8-((carbamoyloxy)methyl)-8a-methoxy-5-methyl-4,7-dioxo-1a,4,7,8,8a,8b-hexahydroazirino[2',3':3,4]pyrrolo[1,2-a]indole-1(2H)-carboxylate (5)
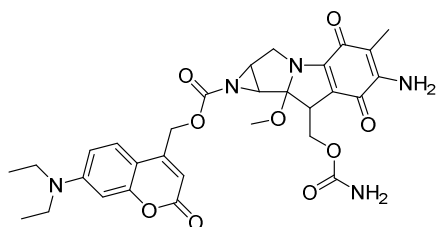
Mitomycin (25 mg, 75 µmol, 1.3 eq.) was added to a solution of compound **3** (25 mg, 59 µmol) and DIPEA (20 µL, 120 µmol, 2 eq.) in DMF (1mL). After 24 h of stirring in RT, AcOEt (50 mL) was added and the mixture was washed subsequently with brine (2 x 20 mL), aq. NaHCO₃ (2 x 20 mL) and again brine (2 x 20 mL). The organic solution was dried over MgSO₄ and concentrated. The product was purified by column chromatography (DCM/MeOH

98:2, v/v). Product **5** was obtained as brown solid (19 mg, 52% yield). Rf = 0.95 (DCM/MeOH 95:5), M.p. = 121-125 $^\circ$C, ^{1}H NMR (400 MHz, Chloroform-*d*) δ = 1.20 (t, *J* = 7.1, 6H), 1.77 (s, 3H), 3.21 (s, 3H), 3.41 (q, *J* = 6.9, 5H), 3.52 (dd, *J* = 13.4, 1.8, 1H), 3.58 (d, *J* = 4.6, 1H), 3.88 (dd, *J* = 11.2, 4.5, 1H), 4.30 (t, *J* = 11.2, 1H), 4.51 (d, *J* = 13.4, 1H), 5.00 – 5.09 (m, 3H), 5.21 (s, 2H), 5.48 (d, *J* = 14.5, 1H), 6.22 (s, 1H), 6.50 (d, *J* = 2.5, 1H), 6.56 (dd, *J* = 9.0, 2.5, 1H), 7.22 (d, *J* = 9.0, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ = 7.9, 12.4, 40.2, 41.7, 44.1, 44.7, 48.5, 49.7, 62.1, 63.0, 77.2, 97.8, 105.3, 105.5, 105.56, 108.8, 110.7, 124.1, 146.9, 149.4, 150.8, 156.6, 160.3, 162.3.

(5,5-difluoro-1,3,7,9-tetramethyl-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)methyl 6-amino-8-((carbamoyloxy)methyl)-8a-methoxy-5-methyl-4,7-dioxo-1a,4,7,8,8a,8b-hexahydroazirino[2',3':3,4]pyrrolo[1,2-a]indole-1(2H)-carboxylate (9)
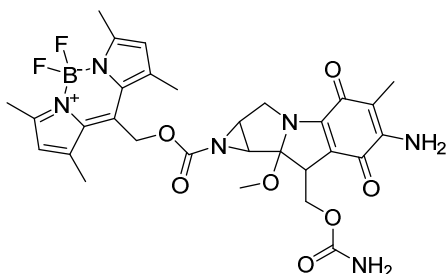

Mitomycin C (21.0 mg, 62.8 μmol) was dissolved in dry DMF (0.3 mL) under nitrogen. The resulting solution was added to a dry THF (0.3 mL) solution of carbonate **3** shown in Chapter 2 (33.4 mg, 75.4 μmol, 1.2 equiv.) with EDCI (9.75 mg, 62.6 μmol, 1 equiv.) and HOBt (8.52 mg, 63.5 μmol, 1 equiv.). After stirring the reaction mixture overnight, EtOAc (10 mL) was added and it was washed with aq. NaHCO$_3$ (3 x 10 mL), 1M aq. HCl (3 x 10 mL) and brine (2 x 10 mL). The organic phase was then dried with MgSO$_4$ and concentrated *in vacuo*. The crude mixture was then purified by flash chromatography using a mixture of DCM and methanol as the eluent (0.1-0.3% MeOH in DCM). The product was obtained as dark purple precipitate (31 mg, 77% yield).

M.p. = 167-171°C, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 1.76 (s, 3H), 2.30 (d, s, 6H), 2.52 (s, 6H), 3.15 (s, 3H), 3.31 (d, *J* = 4.5 Hz, 1H), 3.37 (d, *J* = 0.9 Hz, 1H), 3.45 (d, *J* = 4.1 Hz, 1H), 3.48 – 3.56 (m, 1H), 3.63 (dd, *J* = 11.2, 4.4 Hz, 1H), 4.01 (t, *J* = 11.1 Hz, 1H), 4.44 (d, *J* = 14.1 Hz, 3H), 4.82 (dd, *J* = 10.9, 4.4 Hz, 1H), 5.18 – 5.40 (m, 5H), 6.08 (s, 2H), ^{19}F NMR (376 MHz, Chloroform-*d*) δ -145.77 (dd, *J* = 65.5, 32.2 Hz), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 30.9, 41.0, 42.2, 44.0, 48.4, 49.7, 59.9, 61.4, 105.0, 105.1, 110.3, 122.5, 131.8, 132.7, 141.4, 147.0, 153.8, 155.8, 156.9, 160.6, 175.7,

178.3, 207.0. HRMS (ESI+) calc. for $[M+H]^+$ ($C_{30}H_{34}BF_2N_6O_7$): 639.2592, found 639.2547.

Photocleavable Neomycin: preventing the buildup of bacterial resistance against aminoglycoside antibiotics

Introduction

Antimicrobial resistance is one of the most dangerous threats to modern society.[49] Every year, around 23 000 people die of infections caused by multi drug resistant bacteria.[50] In 2005, it was already known that around 70% of hospital acquired neonatal infections could not be treated by employing the World Health Organization (WHO) regimes and each year this number has been increasing.[51] It is predicted that in 2050 resistant bacteria are going to take more lives than cancer.[52]

The existence of antibiotic resistance was postulated already by in 1942 by Renè Dubos[53] and addressed directly at the Nobel Prize ceremony in 1945[54] by Howard Florey and Alexander Fleming, who is also acknowledged as the one who discovered the first antibiotic to be later used as a drug – penicillin.[55] Even though nowadays WHO and other health-oriented organizations are trying to limit the damage done by antibiotic resistance by forcing laws on the use of antibiotics, the problem is still far from being solved and it's a matter of time when a new epidemic of resistant bacteria caused disease is going to arise.

Misuse of antibiotics, both in animals (including humans) and plants, is considered as the main cause of the rise of antibacterial resistance.[56] A commonly accepted idea on how bacteria can gain resistance when an antibiotic is used is deciphered below (Scheme 43).[50]
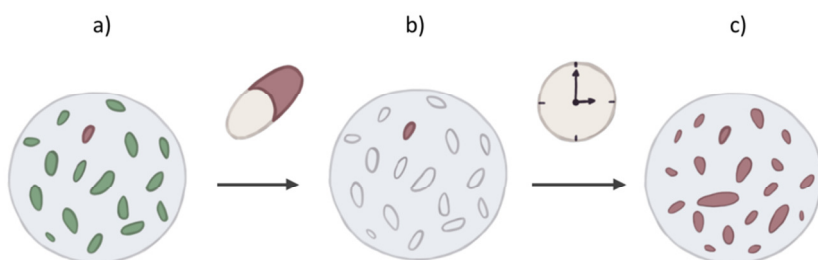

Scheme 43. Idea of the build-up of bacterial resistance: a) bacterial colony before the treatment, b) after antibiotic misuse, c) after a resistant colony is formed

In a bacterial colony, there is a high chance that some of its members will naturally possess genes responsible for resistance to an antibiotic. As these bacteria represent a small percentage of the whole population, and multiply and die at the same rate, this specific resistance will not spread (a). Therefore, in case of an infection by this bacterial colony, it can be treated with antibiotics and generally be cured as this small percentage of bacteria surviving after the antibiotic will be destroyed by defending organism and represent a too small percentage to be a threat.

However if an antibiotic is misused, either taken under the prescribed effective amount or wrongly prescribed, most of the non-resistant bacteria, in addition to ones protecting the organism from the infection,[57] will be killed leaving the resistant ones alive in conditions where they will not be necessarily dealt with by the defending organism (b). With much better conditions to grow, the surviving, resistant, bacteria will multiply and a fully resistant colony is now formed. This new infection is no longer curable by the initially used drug (c) and can even begin to spread further. Poor hygiene and sanitation problems, as well as poor infection control in hospitals and clinics, additionally help to spread the bacteria further.[50]

Incorporating photosensitive moieties in the structures of known drugs has already proven to show potential in slowing the bacterial resistance buildup.[58] Using molecular switches can lead to pharmaceuticals which activity can be turned on and off when needed.[59] Modifying the drug structure with photoprotecting groups would make the new compounds light activable prodrugs, which could be activated only once.[39] These methodologies would allow for slowing the bacterial resistance by limiting the exposure of the active drug to the outside environment (storage in its non-active form) and not infected parts of the organisms (selective activation on the site of action).[60] For more details on the matter, see Chapter 1.

Some of the studies on the topic of modifying drug structures with photosensitive moieties for pioneering light-initiated control of their activity were conducted in our group (Scheme 44).

a)

b)
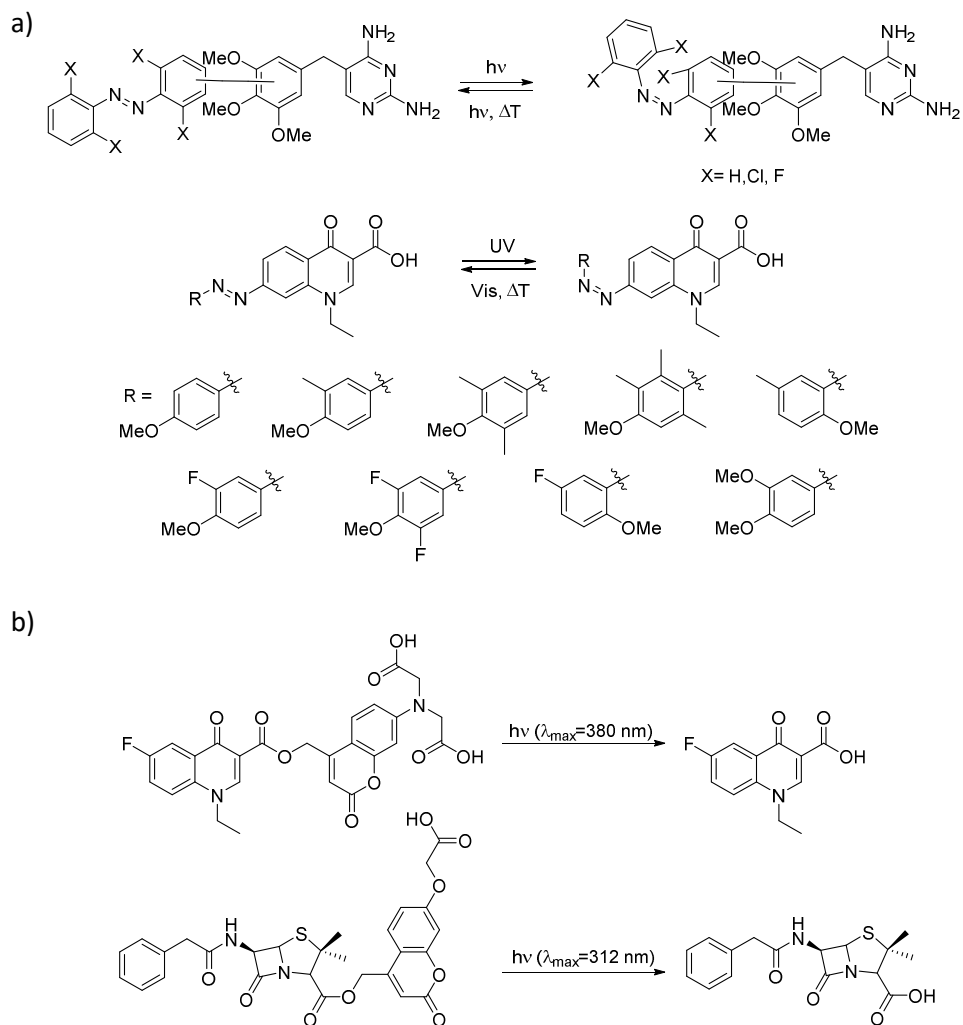
Scheme 44. Structures of light sensitive antimicrobial agents

Our group incorporated azobenzenes, widely used molecular switches, into the structures of a quinolone antibiotic and into trimethoprim (Scheme 44, a). This modification allowed for on and off switching of their activity by irradiating these compounds with light.[58, 61] In the case of trimethoprim, the system was designed in a way for it to be responsive to NIR light rather than UV thus solving the main issue of the methodology which is scattering and absorption of the used light by living tissues.[62]

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In another study, fluoroquinolone and benzylpenicillin were protected with coumarines sensitive to light of different wavelengths (Scheme 44, b).[63] This modification allowed for the selective activation of one of the drugs in a sample where both were present, leaving the other one dormant. Both of these methods might be used for the prevention of bacterial resistance by limiting the contact of bacteria with the active forms of antibiotics.

A somewhat different approach for slowing bacterial resistances was explored by the group of Mobashery.[64] In their work, the researchers presented a photoprotected β-lactam antibiotic, which would deactivate when submitted to UV light (Scheme 45).
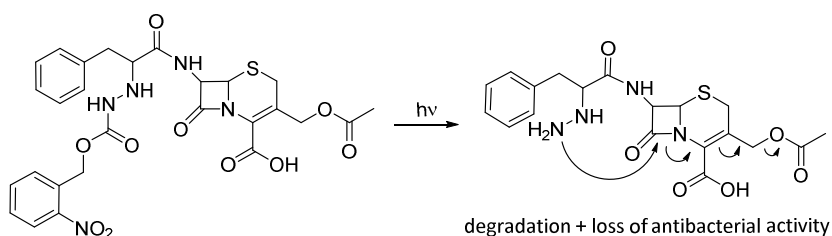
Scheme 45. Reaction with light of a photoprotected β-lactam antibiotic

Irradiating the compound with light would deprotect its hydrazine moiety, which would then lead to an intramolecular rearrangement resulting in the opening of its four member ring and deactivating the drug. In this way, if the compound were ever to be released into the environment, it will deactivate itself before being in contact with bacteria.

Despite all the attempts of preventing or slowing down antibiotic resistance, many antibiotics are becoming less and less active against certain bacteria, forcing medical personnel to prescribe stronger drugs with higher risks of adverse effects.[49] A class of antibiotics whose use had nearly disappeared but which is currently getting more attention is aminoglycosides. These compounds, consisting of glycosides with some of their hydroxyl substituents replaced by amine groups, form a family of narrow range antibiotics sharing similar structures and activities (Scheme 46).[65]
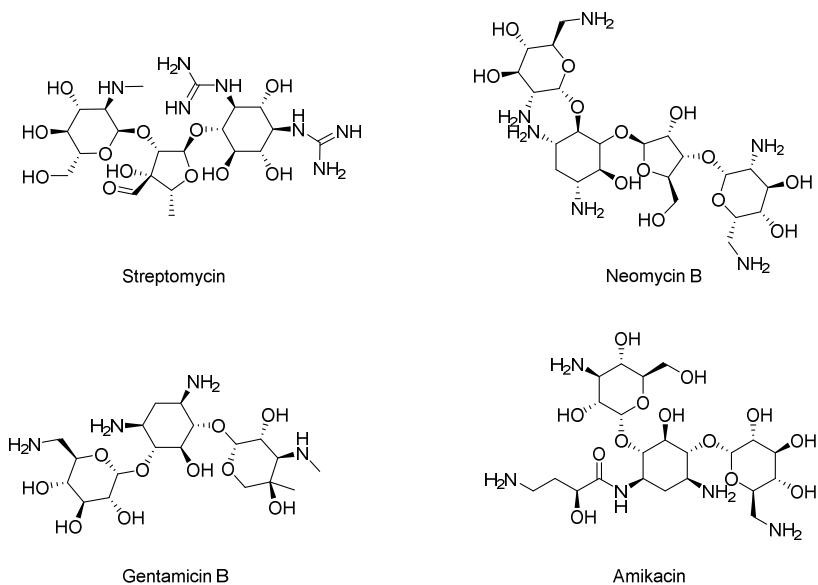
Scheme 46. Structures of chosen aminoglycoside antibiotics

The first known aminoglycoside antibiotic, Streptomycin, was discovered in 1943 by Albert Schatz in Selman Waksman's lab.[66] The drug has found its use against tuberculosis and plague, saving countless lives at the times of epidemics.[67] Other members of the aminoglycoside class of antibiotics, such as Neomycin, Gentamycin and Amikacin, share a similar mode and targets of action. The compounds are rarely used orally, due to their low absorption in the gastrointestinal tract.[68] Most aminoglycoside antibiotics are prescribed as ointments, creams, or injections.[69]

Aminoglycoside antibiotics are mostly used for treating infections caused by gram-negative aerobic bacteria[70] and function by interfering with translation and protein synthesis processes within the bacteria. To do so, they bind irreversibly to ribosomal 16S rRNA proteins and S12 RNA binding proteins which represent the starting points for protein synthesis from RNA. This binding leads to a shape change of the ribosome which means mRNA will no longer be read correctly and that protein synthesis either stops or produces structurally flawed proteins which render the cell nonfunctional.[71]

There have been already many reports on bacterial resistance towards aminoglycosides over the past years.[72] Most commonly, these antibiotics are

either chemically inactivated by enzymes,[73] or their targets, 16S rRNA proteins, mutate, so that the binding affinity of the drugs is decreased.[74] Resistance to one of the compounds in the aminoglycosides group can, but does not imply, resistance to the whole group.[75]

In developed countries, aminoglycosides are rarely the initially prescribed antibiotics against bacterial infections,[70] potentially due to the possible severe adverse effects of these compounds. It has been reported that aminoglycosides tend to accumulate in the inner ear and kidneys causing damage to these organs in a significant amount of patients (above 10%).[76] In large doses it has been observed that Amikacin can be neurotoxic leading to paralysis and breathing problems.[77] Allergy caused by the use of these antibiotics affect around 10 % of patients in the US. Aminoglycosides are therefore the most prone to causing side effects in patients among the diverse antibiotic families.[78]

While aminoglycoside antibiotics have proven to be a powerful tool to fight bacteria despite the high price in terms of adverse effects they come with, they are also suffering from bacterial resistance buildup like other families and would require reinvestigation in the view of storing/eliminating them in a non-active form to prevent further resistance buildup and to minimize their side effects.

To address this problem, we designed and attempted to prepare a derivative of a commonly used aminoglycoside antibiotic, which would be activated by light (Scheme 47).

We chose Neomycin as our model antibiotic as it constitutes an ideal target to evaluate a decrease in adverse effects in living organisms due to photocontrol of its release.
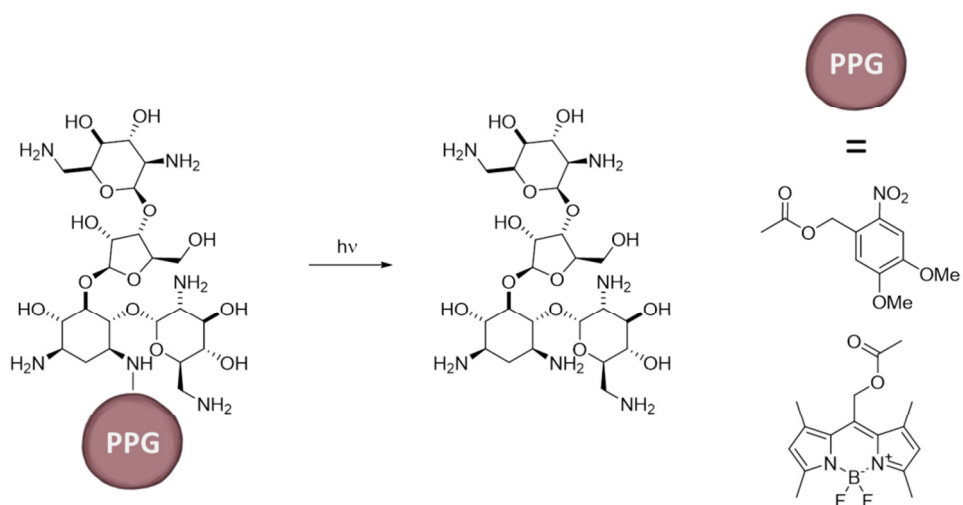
Scheme 47. Idea of the project

We prepared derivatives of this drug covalently bound to *o*-nitrobenzyl and BODIPY groups as PPG's. The chosen photoprotective groups would be responsible for different λ_{max} and speeds of the activation.

Results and discussion

The synthesis of the desired compounds started with the preparation of photoprotecting groups **1** and **2** (Scheme 48). Compound **1** was obtained following a literature procedure.[79] The synthesis of BODIPY carbamate **2** is described in detail in Chapter 2.
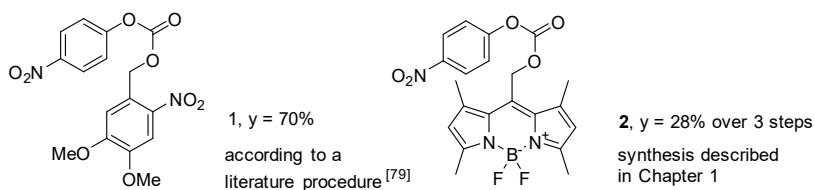
Scheme 48. Synthesis route to obtain compound **1** and **2**

Next, with these compounds in hand, we attempted their coupling to a Boc-protected derivative of Neomycin B using different reaction conditions (Scheme

49). For a more detailed description of the route used to obtain this derivate; see the thesis manuscript of Eliza Warszawik in collaboration with which this work was done.[80]
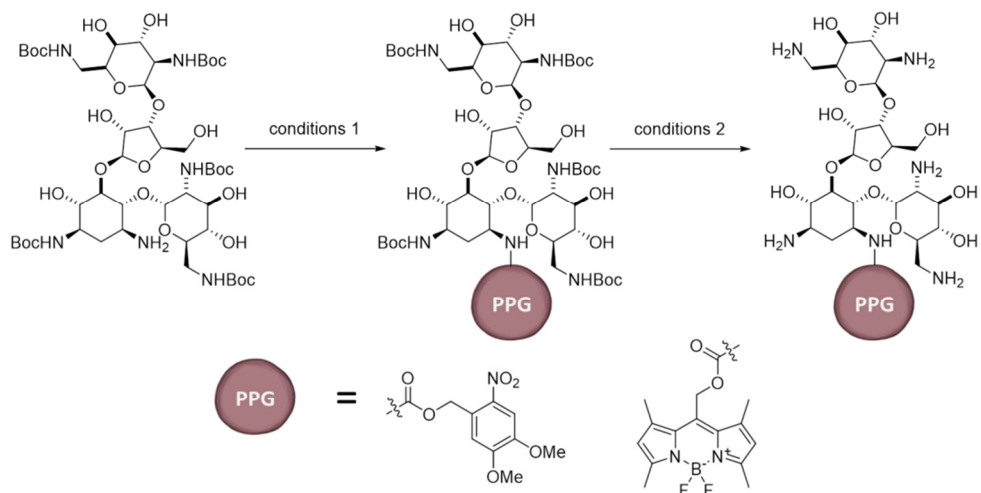
Scheme 49. Coupling of compounds **1** and **2** to a Boc-protected derivative of Neomycin B (**3**)

The reaction of Neomycin B with compound **1** in the presence of DIPEA in DMF and the subsequent deprotection of Boc groups gave the desired compound in 16% yield over two steps (Scheme 50).
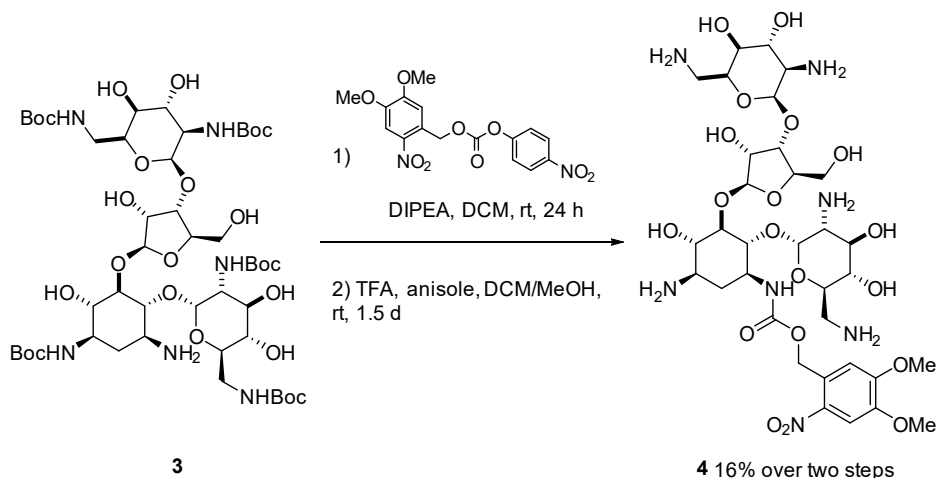
Scheme 50. Synthetic road to obtain compound **4**[81]

The same conditions did not, however, furnish the corresponding derivative when starting from BODIPY **2.** We therefore decided to explore different coupling methods (Scheme 51, Table 10). Unfortunately, the screening of various conditions did not give the expected result. Most of the performed reactions gave compound **5**, an amine side product of the coupling (for more details see Chapter 2) as the main obtained compound. A moderate conversion to the desired carbamate **6** was observed (TLC and LCMS-TOF measurements) when the reaction was performed in DMF with pyridine as the base. This compound was then partially purified and used for the next step without thorough characterization.[82]
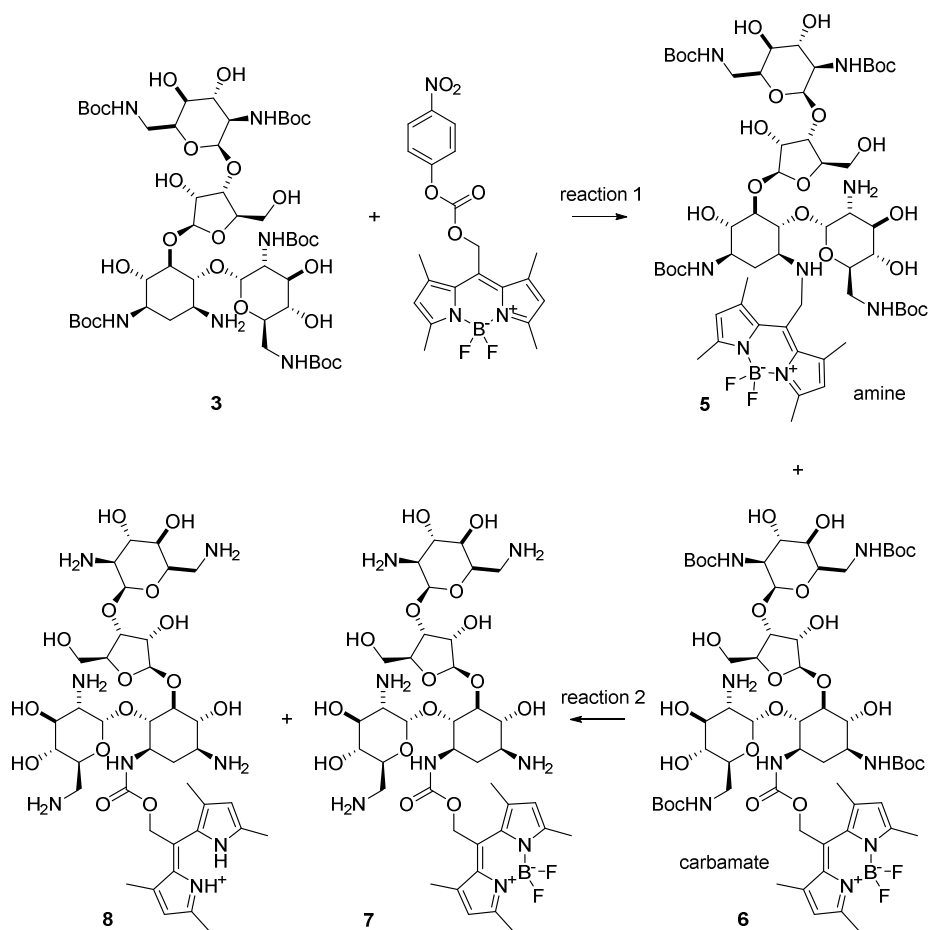
Scheme 51. Synthetic route to prepare compound **8**

Our attempts at this step were, however, unsuccessful, as even if compound **7** was obtained, we were not able to purify it. In view of this, we decided to postpone the preparation of compound **7** until we had preliminary data for compound **4** and we knew it was worth pursuing further.

Table 10. Conditions of the coupling of compound **2** to a Boc-protected derivative of Neomycin B **3** and subsequent Boc deprotection

Entry	Reaction no.	Starting Material	PPG (equiv.)	Reactants (equiv.)	Conditions	Product
1	1	**3** (1 eq)	**2** (1.2 eq)	DIPEA (0.1)	DMF, rt, 24h, Ar	Mostly **5**
2	1	**3** (1 eq)	**2** (1.2 eq)	DIPEA (1)	DMF, rt, 24h, Ar	Mostly **5**
3	1	**3** (1 eq)	**2** (1 eq)	DIPEA (1), HATU (1)	DMF, rt, 3h, Ar	Mostly **5**
4	1	**3** (1 eq)	**2** (1 eq)	Pyridine (1)	DMF, rt, 24h, N_2	**6*** + **5**
5	1	**3** (1 eq)	**2** (1 eq)	Pyridine (1)	THF, rt, 24h, N_2	Mostly **5**
6	2	**7** (1 eq)	-	TFA (10), Anisole (cat)	DCM, rt, 24h, N_2	**8**
7	2	**7** (1 eq)	-	$SnCl_4$ (10)	EtOAc, rt, 24h, N_2	**7** + **8** hard to separate

*25% conversion estimated from LCMS-TOF

Thus, we proceeded to study the deprotection of **4** with light (λ_{max} = 365 nm). To do so, we prepared 80 μM solutions of compound **4** in water and irradiated them with UV light for 90 min taking UV-Vis spectra every minute (Scheme 52).
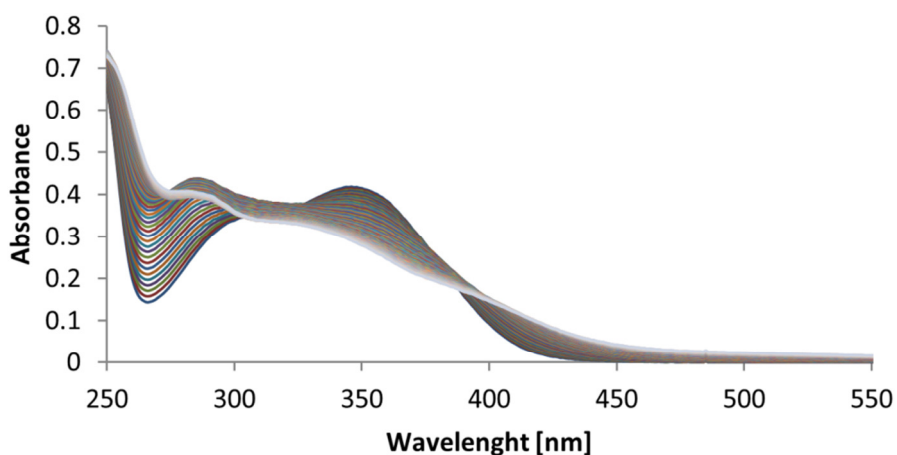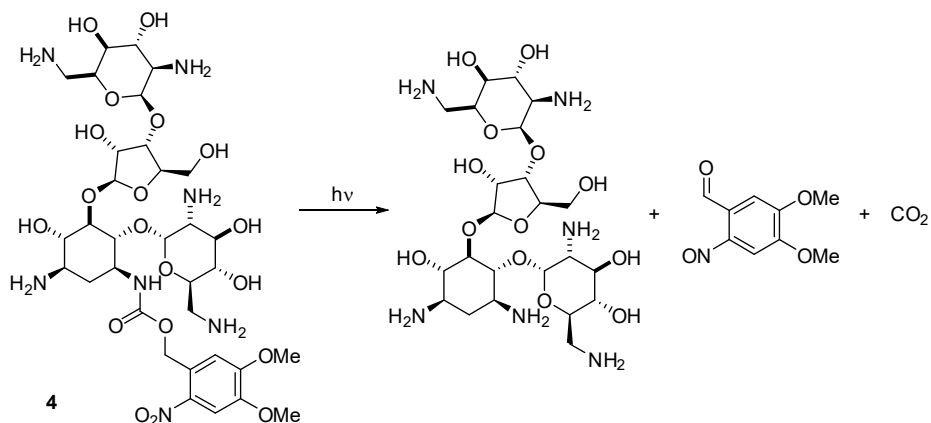
Scheme 52. a) Photocleavage of compound **4**; b) UV-Vis spectra of a sample of compound **4** (80 µM in water) during irradiation with UV light (λ_{max} = 365 nm); spectra taken every min.

To our delight, we were able to observe a decrease of absorbance at 350 nm and an increase at around 400 nm which could be attributed to the appearance of 4,5-dimethoxy-2-nitrosobenzaldehyde released during the deprotection. The presence of the deprotection product was further confirmed by UPLC-TOF measurements.

Next, to determine if the deprotected Neomycin remained active against bacteria after deprotection, we compared the bacteria growth curves of *E. coli* ATTC29522 incubated with or without the antibiotic (Figure 30).
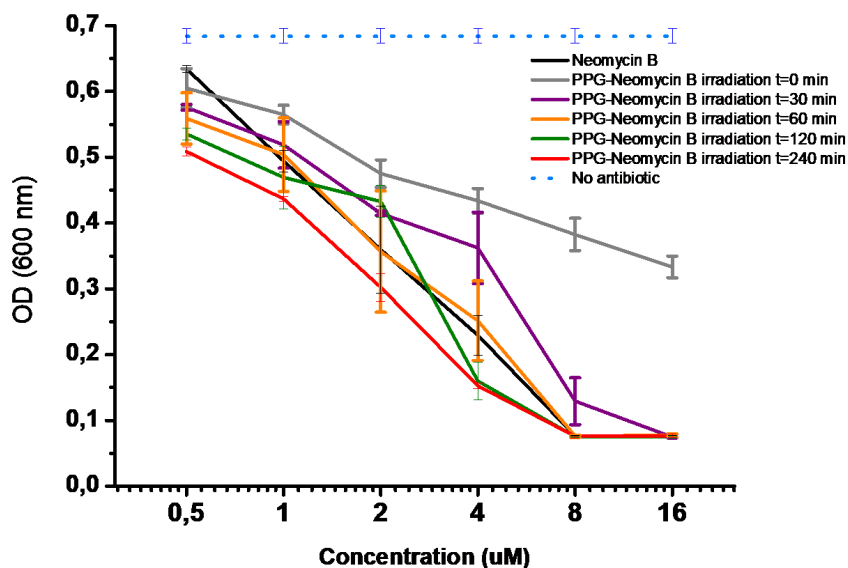
Figure 30. Bacterial growth of E. coli ATTC29522 at different concentrations pure Neomycin B and samples of compound **4** taken after irradiation with light at λ= 365 nm at 37°C for 0, 30, 60, 120 and 240 min.

To do so, we first repeated the irradiation experiment using a 250 µM sample of compound **4** and took aliquots of 25 µL of solution after 30, 60, 120 and 240 min of irradiation. Then, the chosen bacteria strain was incubated in the dark with, in parallel, either the pure Neomycin, non-irradiated compound **4** or each of the before mentioned aliquots.

To our pleasant surprise, it turned out that indeed, the products of the irradiation of compound **4** were active towards *E. coli*. When comparing the reference bacterial culture containing unmodified Neomycin B, showing about 50% growth inhibition at a concentration of 2 – 4 µM, to the ones containing our aliquots, we could observe that a similar result was obtained for the solution of **4** that had undergone 60 min of irradiation.

Unfortunately, some activity could be also observed for the non-irradiated samples of compound **4** which were kept in the dark (IC50 = 16 µM). This lead us to the assumption that protecting neomycin with the *o*-nitrobenzyl PPG was not enough to render it fully inactive. This could be either due to a wrong choice in PPG for this

experiment or due to an underestimation the role of the other groups of the drug in its activity.

Sadly this only partial loss of overall activity makes compound **4** only moderately interesting for further use as the difficulty and high costs of its modification make it too expensive to be produced in a bigger scale in view of the limited potential gains.

Conclusions

We prepared two types of PPGs (*o*-nitrobenzyl and BODIPY derivatives) and attempted coupling them with Neomycin B. After successfully obtaining compound **4**, we performed the photodeprotection reaction using UV light and described the activity of the obtained products against *E. coli*. Even though the deprotected neomycin showed similar activity compared to the pure drug, residual activity was also observed for non-irradiated compound **4**. This observation led us to the conclusion that either blocking a single site of neomycin does not render the drug fully inactive or that a different PPG would be needed for this task. Even though a possibility that using a BODIPY (or another) PPG would solve this problem exists, more studies are needed to prove this hypothesis.

Experimental procedures

General Information

Starting materials, reagents and solvents were purchased from Sigma–Aldrich, Acros and Combi-Blocks and were used without any additional purification. Solvents for the reactions were purified by passage through solvent purification columns (MBraun SPS-800). 4-nitrophenol chloroformate was obtained from Combi-Blocks. Unless stated otherwise, all reactions were carried using standard Schlenk techniques and were run under nitrogen atmosphere in the dark. The reaction progress was monitored by TLC. Thin Layer Chromatography analyses were performed on commercial Kieselgel 60, F254 silica gel plates with fluorescence-indicator UV254 (Merck, TLC silica gel 60 F254). For detection of components, UV light at λ = 254 nm or λ = 365 nm was used. Column chromatography was performed on commercial Kieselgel 60, 0.04-0.063 mm, Macherey-Nagel.

UPLC traces were measured on Thermo Fisher Scientific LC/MS: UPLC model Vanquish, MS model LTQ with an iontrap and HESI (Heated ESI) ionisation source with positive and negative mode. UV-Vis absorption spectra were recorded on an Agilent 8453 UV/vis absorption Spectrophotometer. Irradiation at 532 nm was performed using Sahlmann Photochemical Solutions LEDs, type LXMLPM01, opt. power 810 mV. UV/vis spectra were baseline corrected. Nuclear Magnetic Resonance spectra were measured with an Agilent Technologies 400-MR (400/54 Premium Shielded) spectrometer (400 MHz) at room temperature (25°C). Chemical shifts for the specific NMR spectra were reported relative to the residual solvent peak in ppm; CDCl$_3$: δ_H = 7.26; CDCl$_3$: δC = 77.16; d_6-DMSO: δ_H = 2.50; d_6-DMSO: δ_C = 39.52. The multiplicities of the signals are denoted by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). All ^{13}C-NMR spectra are [1]H-broadband decoupled. High-resolution mass spectrometric measurements were performed using a Thermo scientific LTQ OrbitrapXL (ion trap) spectrometer with ESI ionization. The molecule-ion M+, [M+H]+ and [M–X]+, respectively, are given in m/z-units. Melting points were recorded using a Stuart analogue capillary melting point SMP11 apparatus.

Compound Characterisation

3-N-(4,5-dimetoxy2-nitrobenzylcarbamate)neomycin B (4).
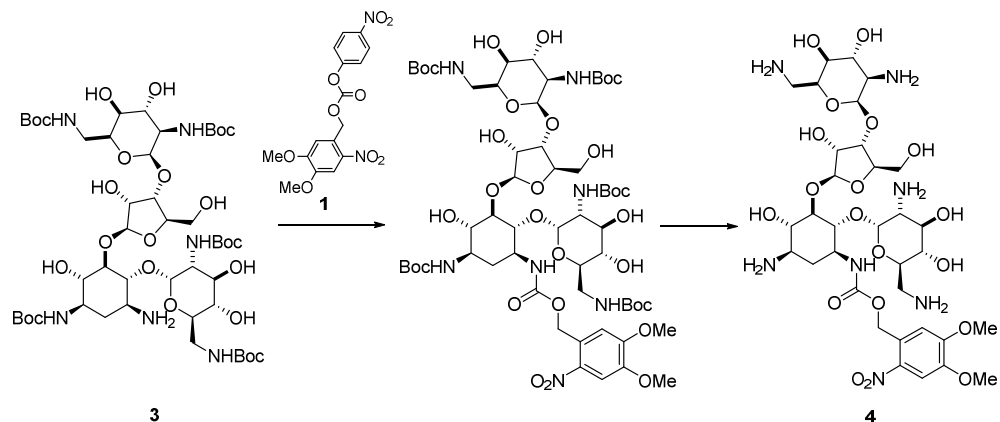
To a solution of compound **3** (98 mg, 88 μmol) in DMF (5 mL), DIPEA (9 μL, 0.1 equiv.) was added. Then a solution of compound **1** (40 mg, 0.1 mmol, 1.2 equiv.) in DMF (7 mL) was added and the reaction mixture was stirred under argon atmosphere at rt for 24 h. After the reaction was completed (TLC), the mixture was diluted with water (140 mL), extracted with EtOAc (3 x 150 mL) and dried with $MgSO_4$. After the solvents were removed in *in vacuo,* the crude was redissolved in a mixture of DCM/MeOH 1/1 (v/v) to which TFA (111 equiv.) and anisole (2.5 equiv.) were added. The resulting mixture was then stirred at RT for 1.5 days. After completion the solvents were removed *in vacuo* and the crude mixture was dissolved in water and purified by HPLC, using a a mixture of acetonitrile, water and TFA as the eluent (95% H_2O, 5 % AcCN, containing 100 mM TFA -> 95% H_2O, 5 % AcCN, containing 100 mM TFA). After removing acetonitrile *in vacuo*, the residual mixture was liophilized, yielding the product as a TFA salt. (20 mg, 16% yield over two steps).

^{1}H NMR (500 MHz, D$_2$O) δ 1.68 (q, J = 12.4 Hz, 1H), 2.28 (dt, J = 11.9, 3.8 Hz, 1H), 3.06 (dd, J = 13.8, 4.6 Hz, 1H), 3.22 (dd, J = 13.5, 3.5 Hz, 1H), 3.27 − 3.48 (m, 5H,), 3.56 − 3.64 (m, 2H), 3.70 − 3.88 (m, 7H), 3.91 (d, J = 3.0 Hz,1H), 3.94 (s, 3H), 3.97 (s, 3H), 4.19 − 4.27 (m, 2H), 4.32 (t, J = 4.8 Hz, 1H), 4.37 − 4.43 (m, 1H), 4.46 − 4.49 (m, 1H), 5.30 (s, 1H), 5.36 (m, 2H), 5.51 (d, J = 13.5 Hz, 1H), 5.72 (d, J = 3.6 Hz, 1H), 7.16 (s, 1H), 7.81 (s, 1H). ^{13}C NMR (151 MHz, D$_2$O) δ 33.3, 41.8, 43.2, 52.0, 52.9, 53.6, 56.5, 59.0, 59.1, 63.3, 67.1, 70.1, 70.3, 71.1, 71.8, 72.3, 72.8, 75.3, 76.3, 78.4, 80.6, 84.2, 88.6, 98.2, 111.5, 113.2, 114.9, 118.1, 120.0, 129.1, 143.1, 150.7, 155.9, 160.1. HRMS (ESI+) (m/z) calculated for (C$_{33}$H$_{55}$N$_7$O$_{19}$Na) [M+Na]$^+$: 876.3445 , found 876.3469.

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[81]   Full experimental details and characterisation can be found in the thesis of Eliza Warszawik

[82]   For details see the thesis of Eliza Warszawik.