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„Novel Equatorially Substituted Platinum(IV) Complexes and their Properties“

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Abstract

Since the discovery of the cytotoxic effect of cisplatin in 1965 and the subsequent development of carboplatin and oxaliplatin, Pt(II) drugs have become indispensable in anticancer therapy. Due to their high effectiveness against various cancer types, they are used in approximately 50% of all cancer treatments. To mitigate side effects and increase tumor selectivity, platinum(IV) prodrugs have become the primary focus of research for platinum-based drugs. These compelling alternatives to platinum(II) complexes have the advantage of additional axial ligands that can be utilized for bioactive moieties or targeting purposes, i.e. maleimides for tumor targeting via binding to the most abundant blood protein, albumin. Contrary to established theories, our recent research has revealed that the equatorial ligands of intact platinum(IV) drugs can undergo hydrolysis under physiological conditions, resulting in two equatorial hydroxido groups. This phenomenon was observed for chlorido ligands and the oxalato moiety of an oxaliplatin(IV) complex. Further studies have shown that one or both hydroxido groups can be substituted by the bioactive ligand biotin, forming a stable complex.

This thesis aimed to synthesize targeted, equatorially substituted platinum(IV) complexes with different types of bonds, specifically ester, carbamate or amine, starting from [(DACH)Pt(OH_{eq})$_2$(OAc_{ax})$_2$]. To target albumin, maleimide ligands were successfully coupled equatorially with an ester or a carbamate bond to the platinum(IV). The generated complexes showed high stability and slow reduction kinetics and a high binding affinity to albumin. *In vivo* studies revealed a significantly prolonged plasma half-life and markedly enhanced anticancer activity compared to oxaliplatin. This new type of platinum(IV) prodrugs will enable the attachment of two different (axial) bioactive ligands, thereby allowing the development of the first targeted triple-action platinum(IV) complexes.

Kurzfassung

Seit der Entdeckung der zytotoxischen Wirkung von Cisplatin im Jahr 1965 und der anschließenden Entwicklung von Carboplatin und Oxaliplatin sind Platin(II)-Wirkstoffe in der Krebstherapie unverzichtbar geworden. Aufgrund ihrer hohen Wirksamkeit gegen eine Vielzahl von Krebsarten werden sie bei rund 50 % aller Krebsbehandlungen eingesetzt. Mit dem Ziel Nebenwirkungen zu verringern und die Tumorselektivität zu erhöhen, sind Platin(IV)-Prodrugs in den Mittelpunkt der Forschung für platinbasierte Medikamente gerückt. Diese Alternativen haben den Vorteil vom zusätzlichen axialen Liganden, welche mit bioaktiven Molekülen oder für Targeting-Zwecke funktionalisiert werden können, z. B. Maleimide für die gezielte Behandlung von Tumoren durch Bindung an das am häufigsten vorkommende Blutprotein, Albumin. Im Gegensatz zur gängigen Theorie, haben unsere neuesten Forschungsergebnisse gezeigt, dass die äquatorialen Liganden intakter Platin(IV)-Komplexe bei physiologischen Bedingungen hydrolysieren können, wodurch zwei neue äquatoriale Hydroxidogruppen entstehen. Dieses Phänomen wurde für Chloridoliganden und dem Oxalatoliganden eines Oxaliplatin(IV)-Komplexes beobachtet. Weitere Versuche haben gezeigt, dass eine oder beide Hydroxidogruppen von bioaktiven Liganden, zum Beispiel Biotin, ersetzt werden können. Im Falle des Biotins wurde ein stabiler Komplex gebildet.

Ziel dieser Arbeit war die Synthese äquatorial substituierter Platin(IV)-Komplexe unter Verwendung einer Ester-, Carbamat- oder Aminbindungen, ausgehend von dem Komplex [(DACH)Pt(OH_{eq})$_2$(OAc_{ax})$_2$]. Um gezielt Albumin zu binden, wurde ein Maleimidligand erfolgreich mittels einem Ester oder einem Carbamat äquatorial an Platin(IV) gekoppelt. Die synthetisierten Komplexe wiesen eine hohe Stabilität und langsame Reduktion auf sowie eine hohe Bindungsaffinität zu Albumin. Anschließend durchgeführte *in vivo* Studien ergaben eine stark verlängerte Plasmahalbwertszeit und eine im Vergleich zu Oxaliplatin deutlich verbesserte antitumorale Wirkung. Diese neuartigen Platin(IV)-Prodrugs ermöglichen die Koordination von zwei verschiedenen bioaktiven (axialen) Liganden und damit die Entwicklung der Ersten zielgerichteten Platin(IV)-Prodrugs mit Dreifachwirkung.

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1. Introduction

1.1. Cancer

Cancer is the collective term for diseases in which abnormal cell growth occurs. This happens due to damaged DNA, which leads to dysfunctional gene expression. Because of that, every cell in the human body can be affected, resulting in a wide variety of different types of cancer. Mutated cells can grow abnormally, leading to uncontrolled cell division and multiplication in an uncontrolled way. These rapidly growing cells can accumulate and form tumors. Depending on multiple factors, cancerous tumors (also referred to as malignant tumors) can spread into nearby tissues to form metastases. Left untreated, this can be fatal.[1] In 2020, roughly 19.3 million new cancer cases were diagnosed (Figure 1). The most common types for women are breast, colorectum, lung and cervical uteri cancer. For men, it is lung, prostate, colorectum and gastric cancer. Therefore, cancer resulted in nearly ten million deaths worldwide and was one of the leading causes of death in 2020 (Figure 1).[2]

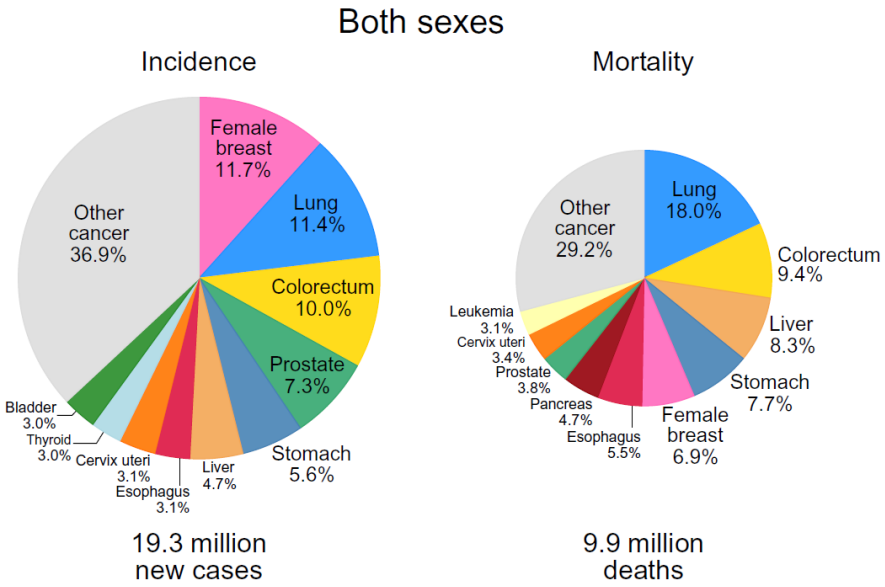


Figure 1: Total cancer incidence and mortality for both sexes in 2020. taken from[3]

In general, there are three main cancer-causing categories of carcinogens: chemical, physical and biological. Chemical carcinogens are a wide variety of chemical molecules like heavy metals, organic structures, or gases, which can damage the DNA. Physical carcinogens are ionizing radiation, like ultraviolet sunlight or radioactive decay, which can break DNA strands. Biological carcinogens consist of bacteria and viruses, like the

human papillomavirus, Kaposi's sarcoma-associated herpesvirus or hepatitis B and C that can cause cancer after infection.[4] Apart from these carcinogens, a manifold amount of risk factors and exposure variables influence the probability of developing a malignant tumor. These factors include the diet and level of physical activity, consumption of addictive substances, or different environmental risks. Low fruit and vegetable intake, obesity, smoking, alcohol consumption, unsafe sex or urban air pollution lead to an increased likelihood of cancer.[5]

1.2. Cancer Therapies

According to antique scripts from ancient Egypt, Rome, and the Arabic region, malignant diseases were already known at that time. It can also be inferred that these illnesses were already treated by performing mastectomy or removing neoplasms. So, the first form of treatment was surgery.[6] Nowadays, surgery is also one of the primary procedures to remove major parts of tumor tissue from the body. One example is a mastectomy for breast cancer. However, such operations can trigger the formation of metastases or a micrometastatic disease. This occurs because of local and systemic inflammation.[7] Therefore, surgery is always combined with at least one other treatment method.

Until the 1960s, radiotherapy was the second standard for treating solid tumors. Based on the discovery of X-rays by Henri Becquerel and Wilhelm Röntgen, it was possible to potentially cure a solid tumor exclusively by radiation in 1898. Electromagnetic radiation can ionize the molecules of the DNA or surrounding water. This leads to double-strand breaks, which subsequently cause cell death. However, the possible damage to the surrounding tissue is critical. To minimize this risk various methods, such as three-dimensional X-ray therapy or intensity-modulated radiation therapy (IMRT), have been developed. The use of radiotherapy on its own or as a post-surgical treatment led to improved cure rates but was no complete solution for the growth of uncontrolled micrometastases.[8] To tackle this problem, a large number of different types of anticancer drugs have been developed in the last decades. These agents can be categorized into the following groups: chemotherapy, hormone therapy, targeted therapy, and immunotherapy.

1.2.1. Chemotherapy

The class of chemotherapeutics includes all agents with a cytotoxic effect. They can be further classified, but in general, they interfere with the DNA and DNA-associated processes, which ultimately leads to apoptosis. The first cytotoxic agent was nitrogen mustard back in 1942. At that time two pharmacologists, Louis Goodman, and Alfred Gilman, proposed its cytotoxic effect based on evidence they gathered from sulfur mustard gas victims from the First World War, who had serious lymphoid hypoplasia and myelosuppression. By treating with nitrogen mustard they were able to achieve a temporary tumor volume reduction in patients affected with lymphoid tumors. This drug belongs to the group of alkylating agents[9]. As the name suggests these compounds work by transferring an alkyl group to the nucleophilic side of the DNA bases. The preferred binding site is the N7 position of guanine, but also the N1 and N3 of adenine, N3 of cytosine and O6 of guanine can be alkylated. Drugs like melphalan, carmustine or altretamine are representatives of this group (Figure 2).[10] Moreover, within the class of chemotherapeutics, there are further subdivisions in antimetabolites, antitumor antibiotics, topoisomerase inhibitors, plant alkaloids and platinum-based anticancer drugs.[11] The latter will be discussed in more detail in Chapter 1.3.

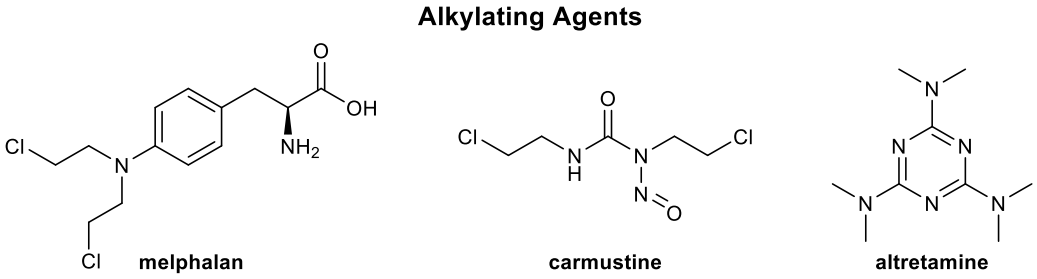


Figure 2: Structure of the alkylating agents melphalan, carmustine and altretamine.

An antimetabolite is a structural analog of an essential biomolecule that naturally occurs in the human body and has a metabolic relevance. By replacing the original metabolite with a slightly modified molecule, it is possible to interfere with or even inhibit DNA-associated processes, like DNA synthesis. Established approaches use antimetabolites of urea, folic acid or DNA bases. Drugs like cytarabine, methotrexate, or fluorouracil have an exchanged or additional functional group compared to their natural analog (Figure 3).[12]

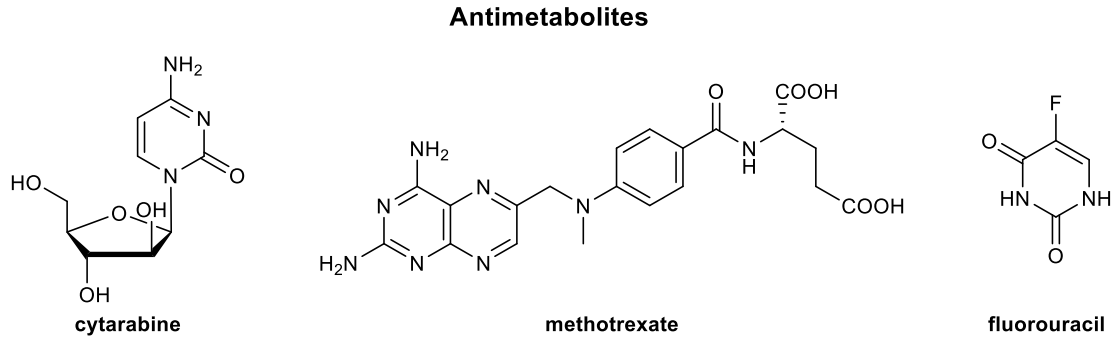


Figure 3: Structure of the antimetabolites cytarabine, methotrexate and fluorouracil.

Antitumor antibiotics are microbial products that interfere with DNA synthesis or can intercalate the DNA. The majority of the approved drugs are isolated from different strains of *Streptomyces*. Two of the most commonly used representatives in the clinic are daunorubicin and doxorubicin (Figure 4). Doxorubicin can be used to treat a variety of solid tumors, such as small-cell lung cancer, breast cancer or ovarian cancer. In addition to their DNA binding ability, daunorubicin and doxorubicin can intercalate the DNA, due to their anthracycline structure. Moreover, both drugs can impede the function of topoisomerase II, resulting in DNA double-strand breaks.[13]

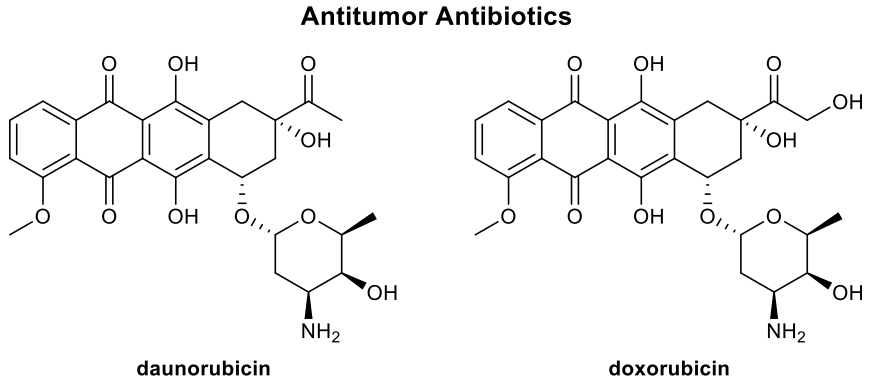


Figure 4: Structure of the antitumor antibiotics daunorubicin and doxorubicin.

The enzymes topoisomerase I and II catalyze the unwinding of the DNA strands prior to DNA replication. Topoisomerase I can cut and uncoil one DNA strand at a time and does not require energy for the process. The two approved drugs, irinotecan and topotecan, can bind to the enzyme after the DNA is broken and stabilize this intermediate state, resulting in an inhibited recombination of the broken strand (Figure 5). Topoisomerase II, on the other hand, can break both strands simultaneously but requires adenosine triphosphate (ATP) for this process. Inhibitors of this enzyme can impede the activity by binding directly to the protein or by inserting a planar part of the molecule in between two adjoining base pairs of double-stranded DNA. This prevents the rejoining of the double-

strand broken DNA. An inhibiting effect has been found for different antitumor antibiotics and plant alkaloids. For example, the already mentioned doxorubicin or amsacrine can intercalate the DNA and insert a moiety in between base pairs. Etoposide, a plant alkaloid, is an example of a non-intercalating topoisomerase II inhibitor (Figure 5).[14]

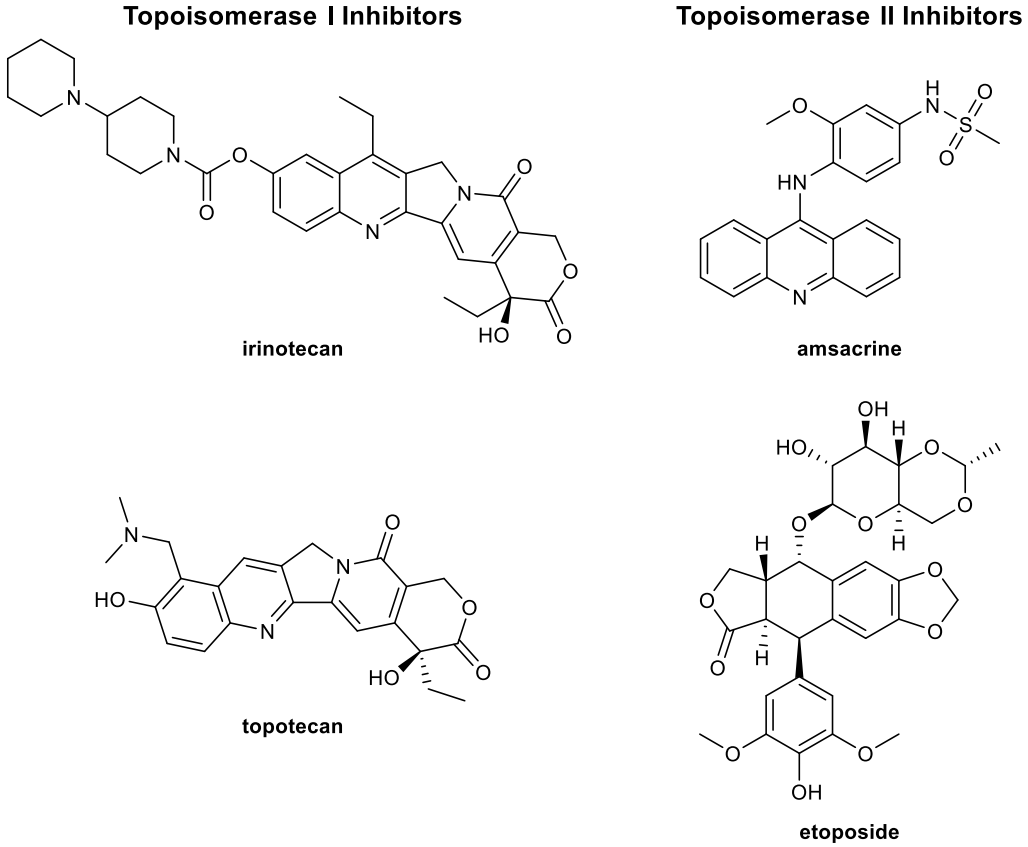


Figure 5: Structure of the mentioned topoisomerase I and II inhibitors.

Plant alkaloids are secondary plant metabolites with a physiological effect on the human body. Their potential anticancer effect was discovered in the 1960s and since then, about nine different compounds have been approved by the FDA. Two of the best-known representatives are paclitaxel and vinorelbine (Figure 6).[15]

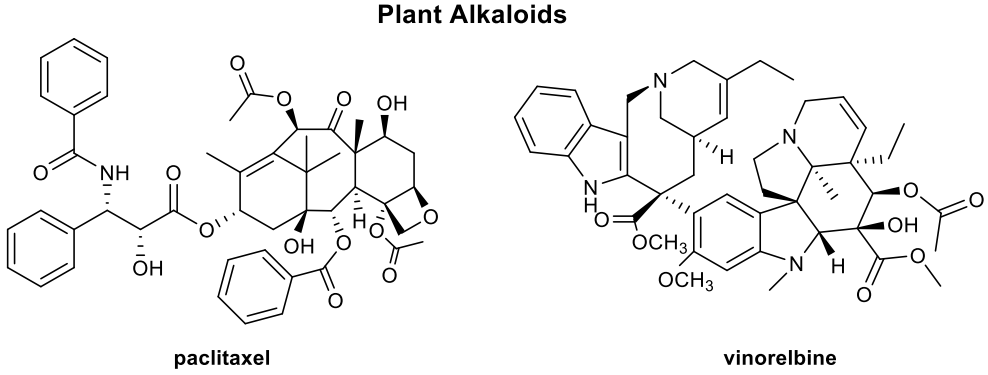


Figure 6: Structure of the plant alkaloids paclitaxel and vinorelbine.

1.2.2. Hormonal Therapy

Hormonal therapy is an effective method mainly used to treat breast and prostate cancer. In both types, the sex hormones estrogen, androgen and progestogen can play crucial roles as growth factors. By blocking different receptors, it is possible to inhibit further growth. For hormone receptor-positive breast cancer, the drug target is the estrogen receptor, aiming to prevent estradiol from binding. The inhibition of this enzyme results in reduced estrogen production. Therefore, drugs like tamoxifen, anastrozole, or letrozole block the estrogen receptor (Figure 7).[16] For prostate cancer, the main target is the androgen receptor on the surface of androgen receptor-positive cancer cells. By blocking this receptor, it is possible to downregulate the production of testosterone and halt the proliferation of these cells.[17]

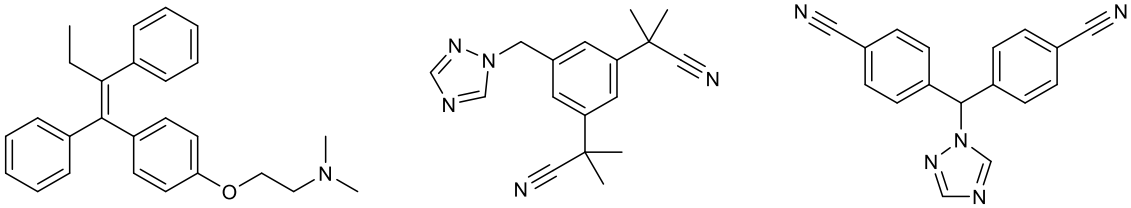


Figure 7: Structure of tamoxifen, anastrozole and letrozole used in hormonal therapy.

1.2.3. Targeted Therapy

In targeted therapy, small molecular drugs or antibodies interfere with tumor cell-specific expressed markers that induce tumor proliferation. Most frequently, receptor tyrosine kinases are targeted and tried to be disabled. Kinases are proteins with a major influence on the signaling pathways and thus also on the growth and development of tumors. By blocking the binding site of ATP, the tyrosine kinase can be inactivated. Imatinib is an example of such an adenosine triphosphate site binder. It was approved in 2001 and was the first competitive tyrosine kinase inhibitor. Other targets are the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor (VEGF).[18] EGFR is a transmembrane protein to which primarily epidermal growth factors bind. Overexpressed in cancer cells, the receptor promotes uncontrolled cell growth. Drugs like osimertinib, erlotinib or cetuximab can target and bind to this receptor, thereby inhibiting its activity (Figure 8).[19] VEGF is the signaling protein involved in the formation of new blood vessels. By impeding the activity of the growth factor, no new blood vessels are formed around the tumor and the lack of oxygen and nutrients should lead to death of the cancer cells. Bevacizumab and sorafenib are approved VEGF-targeted drugs (Figure 8).[20]

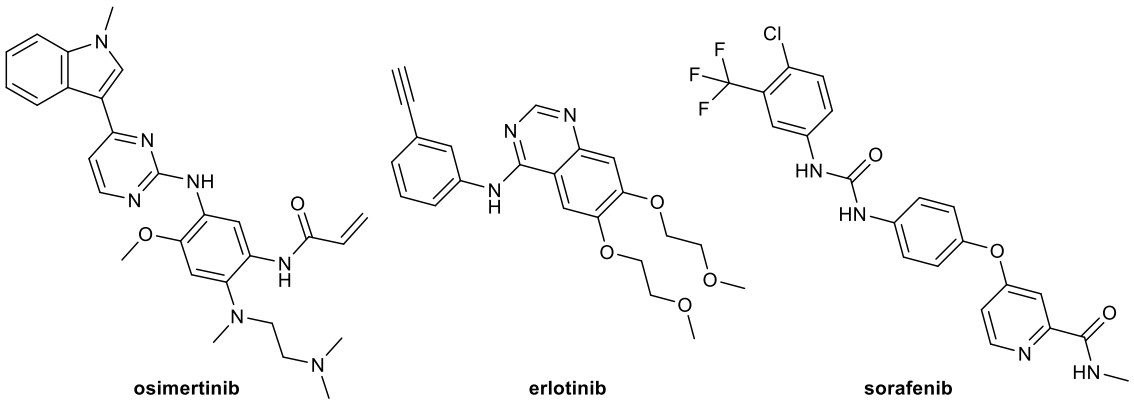


Figure 8: Structure of the EGFR inhibitors osimertinib and erlotinib and the VEGF inhibitor sorafenib.

1.2.4. Immunotherapy

Usually, the human immune system can detect defective cells and eliminate them. However, cancer cells can circumvent the immune response by creating multiple resistance mechanisms and interfering with the functionality of immune cells, like T-cells. In immunotherapy, the goal is to overcome these resistances so that the immune system can attack the cancer.[21] In general, the field can be divided into two concepts, which, however, can merge: immune modulation and antigen-specific vaccines. Immune modulation uses immune checkpoint inhibitors (ICIs) and adoptive cell transfer (ACT) to upregulate the T-lymphocyte activity. Cancer cells can downregulate the activity of T-cells using the programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) receptors on their surface. Immune checkpoint inhibitors are antibodies that can bind to these receptors and thereby increase the anticancer immune response. The first ever approved drug that targets this receptor was ipilimumab. It is a CTLA-4 antagonist and can downregulate its activity.[22] The approach for adoptive cell transfer is slightly different. The idea here is to use modified T-cells. These lymphocytes can be genetically modified or originate from resected tumors. Such T-cells are called tumor-infiltrating lymphocytes.[23] The idea of antigen-specific vaccines is to train the immune system with injected antigens. In this way, an immune response to cancer cells can be triggered. The body recognizes the antigens produced by the tumors and can attack them. Unfortunately, this technique is currently very limited in its applications. However, vaccination can be used very successfully against the two cancer-causing viruses hepatitis B and human papillomavirus.[21]

1.3. Platinum(II)-based Anticancer Chemotherapy

Since the coincidental observation of the cytostatic effect of platinum complexes by Barnett Rosenberg in 1965, Pt(II) anticancer drugs have established themselves as one of the most commonly used chemotherapeutics.[24] After cisplatin, the first clinically approved platinum-based anticancer drug, two other representatives, carboplatin and oxaliplatin, made it through the clinical phases (Figure 9).[25] Only these three highly effective platinum complexes are approved worldwide and they are used in approximately 50% of all anticancer treatments.[24]

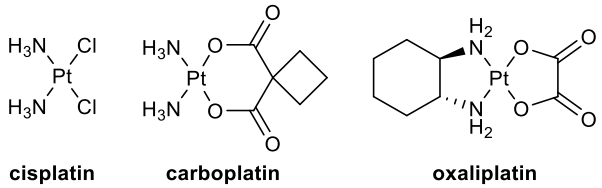


Figure 9: Structure of the three worldwide approved platinum(II) anticancer drugs.

Apart from the three drugs mentioned, there are three additional complexes: nedaplatin, iobaplatin, and heptaplatin (Figure 10). However, these are only approved in several Asian countries.[26]

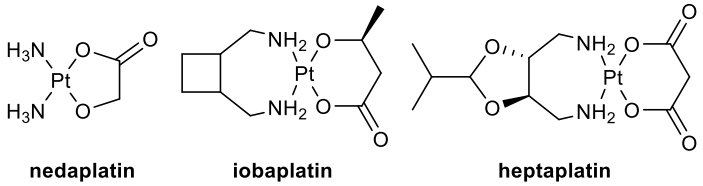


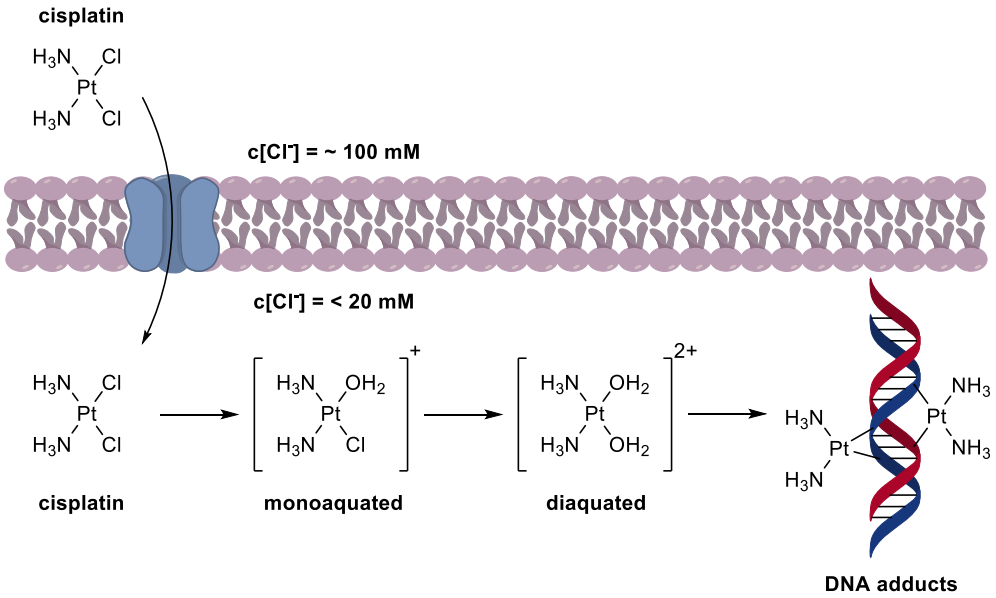
Figure 10: Structure of nedaplatin, iobaplatin and heptaplatin.

1.3.1. Cisplatin – The First Generation

Cisplatin was approved as the first platinum-containing anticancer drug in 1978. The first application was to treat testicular cancer. Besides this application, nowadays, it is used to treat e.g. head and neck squamous cell carcinoma, (non)-small cell lung, ovarian, and brain cancer.[24]

Cis-diamminedichloridoplatinum(II), commonly known as cisplatin, is a square planar complex with a platinum central atom in the oxidation state +2. It has two ammine and two chlorido moieties in cis configuration. The two chlorido ligands are the so-called leaving group and play an important role in the mechanism of action.[24] Usually, cisplatin is administered intravenously with a dose of 10 – 100 mg/m^2.[27] After administration, the

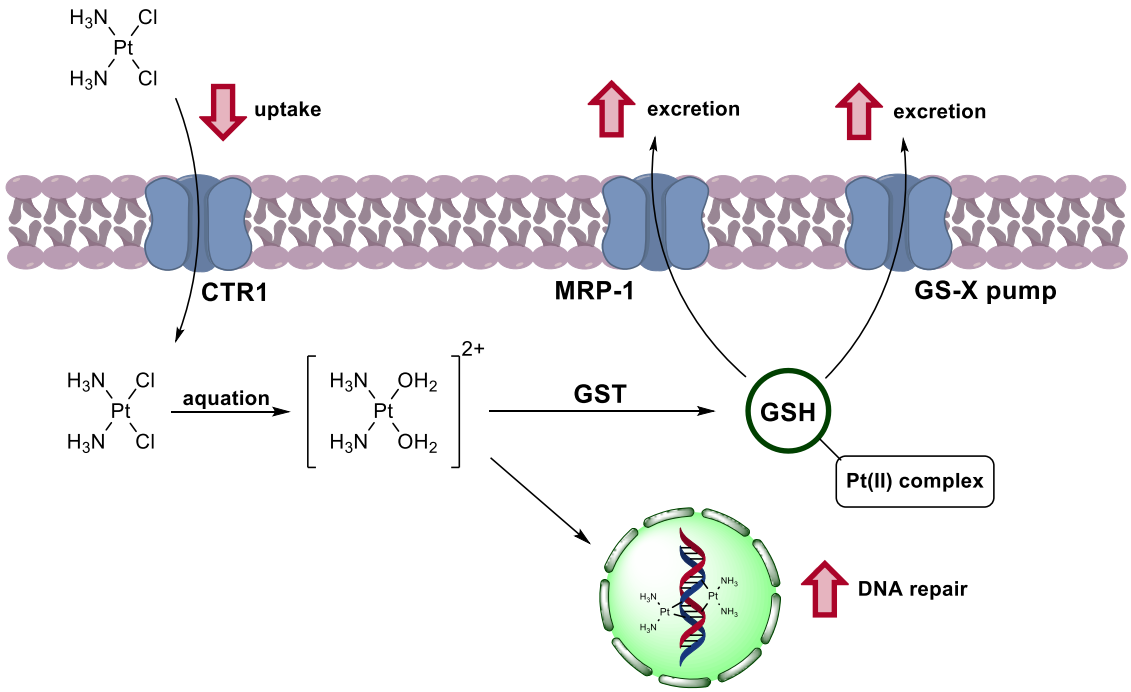
drug enters the human cells *via* passive diffusion or with the help of an organic anion transporter.[28] Additionally, it is assumed that the drug uptake is mediated by the copper transporter CTR1. Due to the significantly lower concentration of chloride ions in the intracellular space, the drug is hydrolyzed.[27] This means that one or both labile chlorido ligands get displaced with water molecules, forming single or double positively charged cationic hydrates (Scheme 1). The activated drug is a highly reactive electrophile. It can bind to a variety of nucleophiles, like different nitrogen or thiol groups of proteins or nucleic acids.[29] Regarding the latter, the main target is the nucleophilic N7 atom of purine bases, with a preference for the N7 of guanine. This reaction results in different DNA adducts (Scheme 1). To a small extent, mono adducts between the complex and the DNA are formed. But in most cases, cisplatin forms 1,2-intrastrand adducts with adjacent guanines or adenines. The other possibility is 1,3-interstrand DNA adducts, where the bases are located on different DNA strands. In all cases, the result is damaged DNA.[23,24] This causes a disrupted DNA replication and, thus a dysfunctional cell cycle, leading to the initiation of apoptosis. Due to the labile nature of the chlorido ligands and the direct correlation between the cytotoxicity and the rate of hydrolysis of the leaving group, cisplatin is the most toxic complex out of the three approved platinum-based drugs. In addition to the DNA adducts, cisplatin can induce oxidative stress, resulting in elevated levels of reactive oxygen species (ROS) with lethal consequences for cells.[24]



Scheme 1: Activation of cisplatin and schematic illustration of the formed DNA adducts.

Unfortunately, during cisplatin therapy, cancer cells develop different defense mechanisms and consequently become resistant to the drug. Until today this remains a major problem even during individual therapy cycles. Cells can reduce the drug uptake,

bind free cisplatin with glutathione or metalloproteins and find ways to remove bound platinum from the DNA or tolerate it. By diminishing the expression of CTR1 the uptake of the drug into the cells gets downregulated. On the other hand, the multidrug resistance protein 1 (MRP-1) and the ATP-dependent glutathione S-conjugate export pump (GS-X pump) are overexpressed, leading to an increased excretion of glutathione-bound platinum from cells. Both of these effects result in a lower drug concentration in the cell (Scheme 2). The majority of already damaged DNA is repaired by nucleotide excision repair (NER). This general DNA repair mechanism can locate the DNA adduct, unwind the DNA strand and excise the lesion from the strand. In the end, the missing DNA bases are inserted with the help of a DNA polymerase. All these different mechanisms lead to a decreased apoptosis rate and reduced effectiveness of cisplatin.[29,30]



Scheme 2: Developed defense mechanisms of cancer cells against cisplatin.

Due to its low selectivity, cisplatin accumulates in healthy cells in the same way as in cancer cells, leading to severe side effects. The most common are nephrotoxicity, ototoxicity and neurotoxicity. Cardiotoxicity, gastrointestinal toxicity, hepatoxicity, and hematological toxicities can also occur. Nephrotoxicity, which includes over a dozen different side effects, is the dose-limiting toxicity (DLT) for cisplatin[31]. Therefore, the dose has to be lowered or the treatment adapted if patients already have pre-existing renal disease or show reduced functionality during the treatment. The mechanism behind kidney toxicity is not fully understood, but a few of the most critical factors are acute renal failure and hypomagnesemia. Acute renal failure, otherwise known as acute kidney

injury, is the accumulation of waste products in the blood due to decreased functionality of the filtration organ. In the case of hypomagnesemia, the concentration of magnesium ions in serum is decreased. Ototoxicity is another major side effect, ranging in severity from simple ear pain, tinnitus, and affected balance to irreversible hearing loss. The reason for this toxicity is also not fully clarified. It is suspected that it is related to the accumulation of cisplatin in the inner ear and the resulting increase in ROS. The problem is compounded by the lack of medication to mitigate this side effect. In addition, cisplatin can damage the nervous system (neurotoxicity). However, this is typically more associated with oxaliplatin (see Chapter 1.3.3).[24,32]

1.3.2. Carboplatin – The Second Generation

Carboplatin is the result of intense research after the anticancer properties of cisplatin have been established. It has the same two ammines but differs in the leaving group. Instead of the two chlorido moieties, it has a bidentate dicarboxylate, specifically a 1,1-cyclobutene dicarboxylic acid moiety.[24] This ligand drastically changes the properties of the complex compared to cisplatin. The hydrolysis rate of the “leaving group” is significantly slower.[27] The decreased reactivity results in a slower DNA binding rate, lower toxicity and also a reduced potency. Additionally, a major fraction of the given carboplatin is excreted in the urine.[24] Therefore, the administered dose has to be higher, typically 300 – 450 mg/m^2.[32] However, the mode of action and the formed DNA adducts do not differ from cisplatin. Thus, cancer cells develop the same resistance against carboplatin as they do to cisplatin. There is one significant difference when it comes to the excretion of the hydrolyzed platinum from the cancer cells. The lower hydrolysis rate leads to a decreased formation of glutathione adducts, resulting in a lower amount of protein-bound platinum being transported out of the cell.[27]

Generally, carboplatin is used to treat ovarian, testicular, lung, bladder and cervical cancer, which are similar types of cancer as those treated with cisplatin. As for cisplatin, carboplatin is usually administered in combination with other drugs e.g. paclitaxel.[25] Of special note is that modern immunotherapeutics like pembrolizumab targeting PD-1, are frequently combined with carboplatin.[33] Overall, the side effects of carboplatin are similar to those of cisplatin, but in general less severe and carboplatin is significantly less nephrotoxic. The DLT for carboplatin is myelosuppression, a condition in which there is a severe decrease in the number of white and red blood cells and platelets. This results from reduced blood production due to impaired bone marrow. Usually, 17 to 21 days

after single-dose administration, the lowest platelet levels are reached, followed by recovery in the following days.[32]

1.3.3. Oxaliplatin – The Third Generation

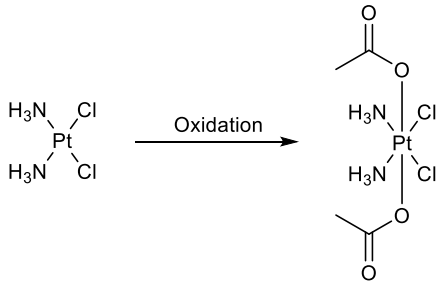
The latest and third worldwide approved platinum anticancer drug is oxaliplatin, which is different in structure compared to the other two, resulting in changed properties. Not only the leaving group but also the inert group has been replaced. The two ammino ligands have been exchanged to a much bulkier and bidentate (1*R*,2*R*)-cyclohexane-1,2-diamine, abbreviated as *R*,*R*-DACH.[26] Among the three different stereoisomers of 1,2-diamino cyclohexane, research has shown that the complex with *R*,*R*-DACH results in the most potent drug. The leaving group is a bidentate oxalate. Despite the significantly different structure, the general mode of action is the same as for cisplatin and carboplatin. To get activated, the oxalato ligand has to be hydrolyzed. This step can proceed *via* the direct replacement of the oxalato with two aquo moieties or through an intermediate with two chlorido ligands, which subsequently get exchanged in the intracellular space forming the monoaquo and diaquo complex.[34] Then, the platinum can bind to the DNA. The mostly generated GpG intrastrain adducts of the DNA are bulkier compared to the ones of cisplatin and carboplatin. Additionally, the cyclohexane can position itself in the major groove of the DNA and form non-covalent bonds with the strands. As a result, the DNA repair mechanism, more specifically the mismatch repair (MMR) mechanism, can no longer recognize the damaged DNA.[34] The most significant difference between oxaliplatin and the two other platinum(II)-based drugs is that oxaliplatin needs a functional immune system to be effective because it can induce immunogenic cell death (ICD). This form of cell death triggers an immune response and the release of molecules like ATP, calreticulin or high mobility group box 1 (HMGB1), which belong to the group of damage-associated molecular patterns (DAMPs). These released biomarkers lead to a “find me” and “eat me” signal for antigen-presenting cells (APCs), like dendritic cells. The APCs can then present the released antigens to T cells, initiating an adaptive immune response against the cancer cells. Cell studies have shown that in immune-suppressed cell lines, oxaliplatin loses its anticancer activity.[25] These might be the reasons why oxaliplatin shows antitumor activity against cisplatin-resistant cell lines and cancer types.[34,35] Also, oxaliplatin does not develop a cross-resistance with cisplatin and carboplatin.[27]

Compared to the other two drugs, oxaliplatin is less toxic than cisplatin and more toxic than carboplatin. A typical dose is 85 – 130 mg/m^2. Oxaliplatin is always formulated in a

5% glucose solution without sodium chloride. Otherwise, the ligand exchange of the oxalato with the chlorides would occur, forming [$PtCl_2$(*R*,*R*-DACH)].[31] Oxaliplatin is usually used to treat colorectal and pancreatic cancer.[25] The DLT for this platinum anticancer drug is neurotoxicity. The most common symptom patients have to deal with is peripheral sensory neuropathy (PSN). This condition is reversible but can be severe. It appears with drug accumulation in the body over the treatment period. Contrary to what may be assumed, the side effect is caused by free oxalate ions and not by the activated platinum complex. The free oxalate has a high binding affinity to calcium and magnesium ions. The chelation of these ions in high quantity results in affected voltage-gated Na^+ and K^+ channels. There are no specific treatments to control or attenuate this toxicity.[31]

1.4. Platinum(IV) Prodrugs

In the last two to three decades, the main research focus for platinum-based anticancer drugs has shifted to platinum(IV) anticancer prodrugs.[36] These octahedral, low-spin d^6 complexes are usually based on the three common platinum(II) drugs mentioned (see Chapter 1.3).[37] By oxidation, for example, with hydrogen peroxide, it is possible to generate a platinum core in the oxidation state +4.[38] During this reaction, two new axial moieties are inserted into the complex, which can be functionalized (Scheme 3). In general, platinum(IV) complexes are much more inert than the respective platinum(II) analogs (see Chapter 1.4.1).[37]



Scheme 3: Schematic illustration of the oxidation of cisplatin to obtain a Pt(IV) prodrug with two axial acetato ligands.

The general established idea is that Pt(IV) complexes need to be activated which is achieved by reduction inside cancer cells. The intracellular concentration of reducing agents is significantly higher and can be divided into two fractions: high and low molecular weight. The most prominent representatives for the low molecular weight fraction are glutathione and ascorbic acid. It was suspected that these two molecules are mainly responsible for the reduction for a long time. However, Wexelbach and Gibson

showed in 2012 that this is not the case. They were able to show that the platinum complexes were mainly reduced by the high molecular weight reducing agents.[37] Due, to the reduction, the axial ligands get released inside the cell and the platinum(II) complex is formed, subsequently acting according to the mode of action described in Chapter 1.3.1. Because of this activation step, these compounds are considered as prodrugs.[39]

Until now, four Pt(IV) anticancer prodrugs have made it into clinical trials: tetraplatin, iproplatin, satraplatin, and LA-12 (Figure 11). However, none of them have been approved by the FDA so far. Satraplatin and iproplatin have reached phase III of clinical testing. The phase III trial for iproplatin showed no significant improvement compared to cisplatin or carboplatin therapy.[38] Satraplatin was tested in phase III against refractory cancer in 950 patients in 2013. The result was that the overall survival rate had not increased significantly.[40] Interestingly, satraplatin has currently still one ongoing clinical trial (NCT03258320).[41]

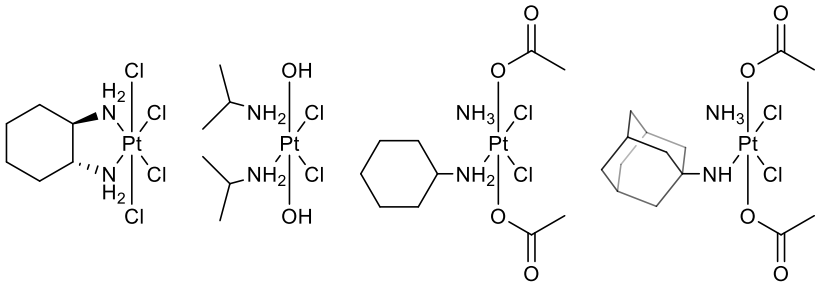


Figure 11: Structure of the four platinum prodrugs that were investigated in clinical trials.

These four drugs share the common characteristic that their axial ligands are not bioactive. However, using one or even two different bioactive moieties for creating double or even triple-action platinum(IV) prodrugs holds a lot of potential. The possibilities for suitable ligands are almost limitless. During the oxidation process, it is synthetically possible to add two axial hydroxido groups.[38] These can then be functionalized and bound *via* an ester, a carboxylate, or a carbamate different cytotoxic moieties, biomolecules or even other drugs can be found as ligands in the literature.[39] Some examples would be dichloroacetic acid, which is a dehydrogenase kinase inhibitor, or ethacrynic acid as a glutathione-S-transferases inhibitor (Figure 12 A). Furthermore, the attachment of well-known drugs like aspirin (Figure 12 B), indomethacin, or ibuprofen (Figure 12 C) has demonstrated promising results.[38,42] Another possibility for which an axial moiety can be used is drug targeting (see Chapter 1.5).

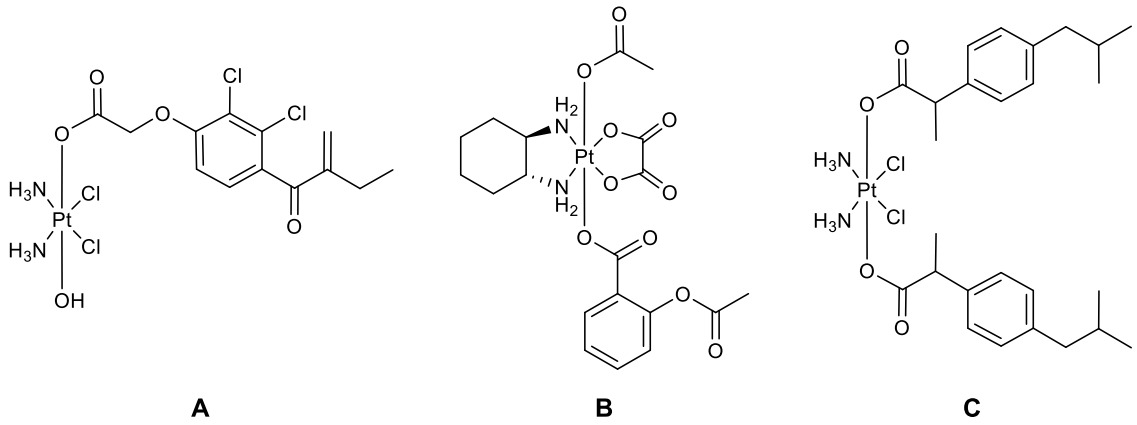
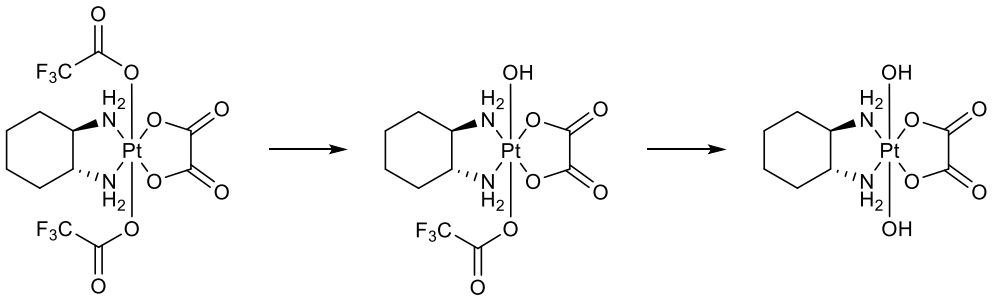


Figure 12: Structure of platinum(IV) complexes with different bioactive axial ligands.

1.4.1. Hydrolytic Stability of Platinum(IV) Complexes

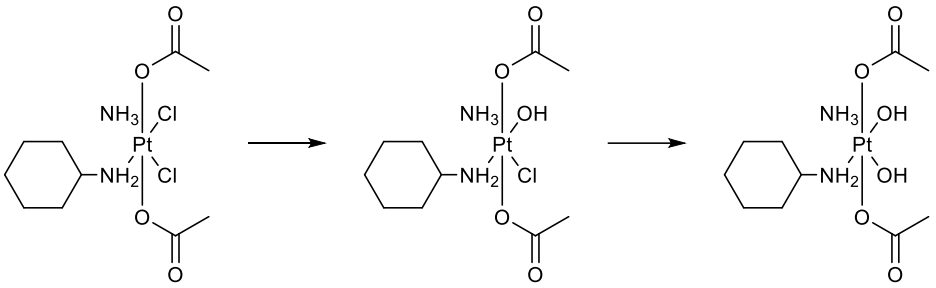
For a long time, it was generally assumed that platinum(IV) prodrugs are inert, meaning that the axial ligands are only released by reduction and that no hydrolysis of the leaving group of the Pt(IV) core can occur under physiological conditions. Both of these assumptions have been disproved. In 2013 Wexselblatt et all showed that for Pt(IV) complexes with axial haloacetato moieties, these ligands are released under physiological conditions without the need for reduction. This indicates that at pH 7 and 37°C in human plasma, one or even both haloacetates are cleaved and substituted by hydroxido ligand(s) (Scheme 4).[43]



Scheme 4: Reaction scheme of the hydrolyzation of axial haloacetato ligands.

The first compound for which the hydrolysis of the equatorial leaving group of an intact Pt(IV) prodrug was reported is satraplatin (Scheme 5). It was possible to isolate the mono- and dihydroxido species from the human plasma of patients, indicating that the chlorido ligands must have been exchanged without reduction.[44] Subsequent studies show that the chlorido moieties of Pt(IV) complexes with axial acetato ligands rapidly hydrolyze in HEPES buffer under physiological conditions. It is also proposed that the

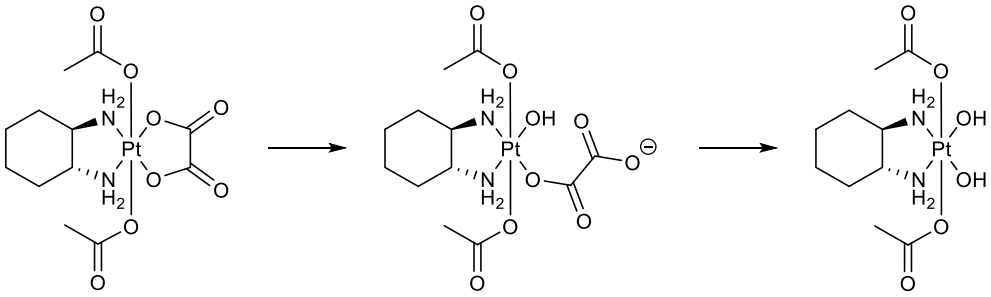
inert am(m)ine ligand(s) with a strong σ-donor ability can accelerate the hydrolysis rate.[45]



Scheme 5: Equatorial hydrolysis of satraplatin.

Kastner et all demonstrated that this phenomenon not only occurs for chlorido ligands but also for the more stable oxalato-leaving group. Under physiological pH in phosphate buffer at 37°C the compound [Pt(DACH)$(OAc)_2$oxalato] forms 41% monohydroxido and 13% dihydroxido complex in 24 h (Scheme 6). The conversion rate for [Pt(DACH)$(OAc)_2Cl_2$] was even faster, resulting in nearly no unhydrolyzed complex left after 24 h. Additionally, the notable influence of the stable ligand was further illustrated. Exchanging the *R,R*-DACH ligand with NH_3 groups resulted in a complete absence of hydrolysis. This impact proved to be even more significant than the exchange of the leaving groups. By increasing the pH to 8 or even 9, it is possible to fully hydrolyze the oxalato moiety quantitatively. The reaction rate can be further accelerated by elevating the temperature. For the most stable platinum core with the structure of carboplatin, no hydrolysis can be observed.[46]

This leads to new opportunities for the functionalization of platinum(IV) complexes at these two equatorial positions e.g. using a targeting moiety or a bioactive ligand.



Scheme 6: Equatorial hydrolysis of [Pt(DACH)$(OAc)_2$oxalato].

1.5. Targeted Platinum Drugs

One commonly employed strategy in medicinal chemistry to enhance the desired therapeutic effect of a drug is the use of targeting or selective delivery.[47] For platinum-based chemotherapeutics, a variety of different drug targeting and delivery (DTD) systems have been designed, offering multiple advantages. Firstly, they demonstrate high selectivity, allowing for a reduction of damage to healthy tissues and thereby minimizing side effects. Moreover, these methods can help to overcome drug resistance. Generally, two different strategies for DTD exist active targeting and passive targeting. In active targeting, the aim is to bind the drug *via* a bioactive ligand to a cancer cell-specific side, like an overexpressed receptor. The other strategy, passive targeting, relies on the so-called enhanced permeability and retention (EPR) effect in tumor tissue.[48,49]

1.5.1. Active Targeting

For active targeting a ligand that can bind to a tumor-specifically overexpressed cell receptor is necessary. The most common examples are:

1) The sodium-dependent multivitamin transporter (SMVT), which e.g. transports the vitamin biotin into cells. Thus, by using biotin as axial ligands, it is possible to actively target cancer cells and increase the cytotoxic effects of the drugs.[50]

2) On breast and ovarian cancer cells the estrogen receptors on the cell surface are overexpressed. By using estrogen as an axial ligand for platinum(IV) complexes, these estrogen receptors can be targeted.[51]

3) Due to the elevated energy consumption of cancer cells the expression of carbohydrate transporters is upregulated. It is possible to use monosaccharides, oligosaccharides, and polysaccharides to target them.[49]

1.5.2. Passive Targeting

Passive targeting is based on the enhanced permeability and retention effect. Rapidly growing tumor cells have an increased demand for oxygen and nutrition compared to healthy tissues. As a result, vascular density is higher. However, the newly formed blood vessels differ significantly from healthy ones. They are irregular in size and shape and the endothelial cells lining the inside of the vessels have gaps. This leads to large fenestrations, resulting in enhanced permeation and passive accumulation of

macromolecules (30 – 200 nm and > 40 kDa), lipidic particles and nanosystems into the tissue. Additionally, this leakage is facilitated by the underdeveloped lymphatic drainage in tumors, enabling these leaked molecules to remain in the tissue for a longer period. A benefit of passive targeting over active targeting is that it is not limited to overexpressed receptors, allowing the targeting of cells without specific surface properties.[48,52] To leverage the EPR effect for DTD, two possible strategies exist. One approach involves the use of nanoparticles, liposomes, or micelles as carriers. These macromolecules can encapsulate the platinum drug. Their size, circulation time, and surface charge are important factors for their cellular uptake.[48,49] The other strategy would be the use of an *in situ* carrier molecule. Here, the drug is equipped with a functional group to bind to a macromolecule in the bloodstream. An example of such a molecule is human serum albumin.[53]

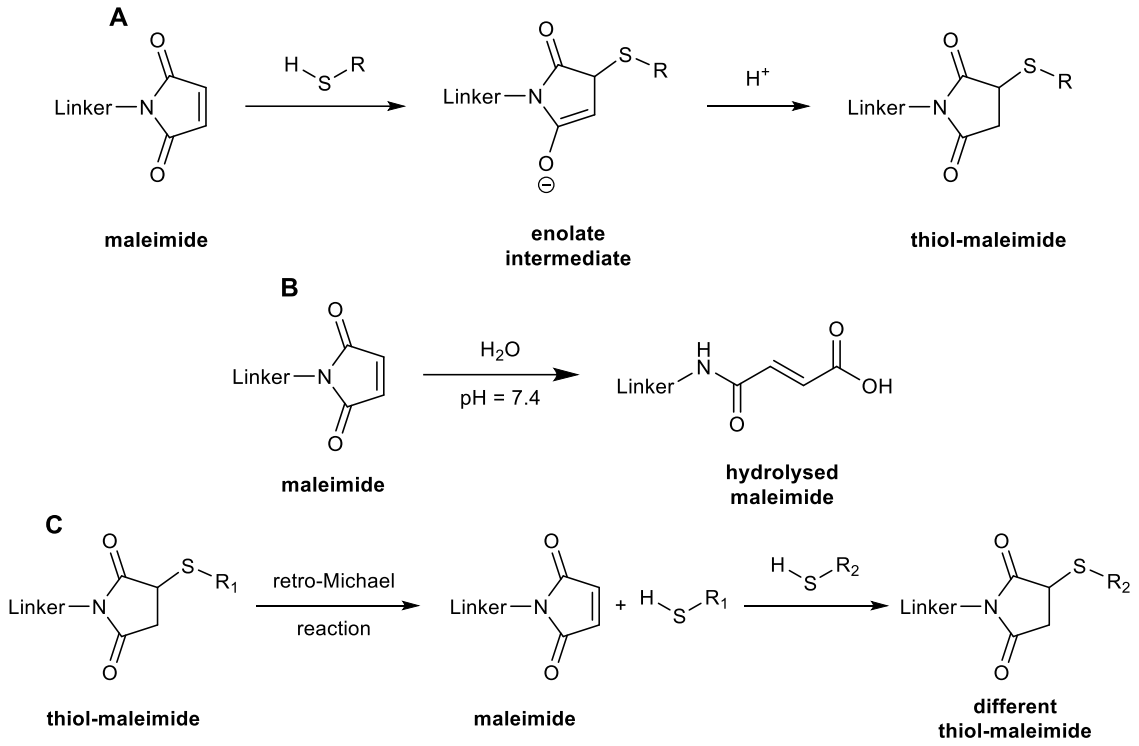
1.5.2.1. Albumin and Maleimide

Albumin is the most abundant human blood protein (35 – 50 g/L) with a molecular weight of around 67 kDa. This high concentration is possible due to its long half-life of 19 days and the daily production of 13 – 14 g in the liver.[54] From the vascular space, extravasated albumin can be brought back into the bloodstream through the lymphatic system. As mentioned, the lymphatic system in cancer cells is underdeveloped, so accumulated albumin is removed less effectively.[49] Additionally, the protein can bind and subsequently transport a variety of different endogenous and exogenous molecules. All these properties make albumin a perfectly suitable macromolecule to exploit the EPR effect. There are two strategies for *in situ* albumin binding: covalent and noncovalent binding. For noncovalent binding, fatty acids, small molecule binders or nanobodies can be used. They can be employed because albumin has different noncovalent binding regions, like fatty acid binding sites. For covalent *in situ* binding the free thiol of the amino acid cysteine-34 is primarily used. One way to link a drug to this sulfur atom would be another thiol group, resulting in a disulfide bond. In the cancer cell, the bond is then simply broken by reduction leading to the cleavage of the compound. Another option are maleimides.[53]

Maleimides have established themselves as the most prominent endogenous albumin-targeting moiety. Due to the release of ring strain and the withdrawing effects of the two activating carbonyls, the unsaturated imide has a selective binding affinity to thiols via a Michael-addition reaction. Albumin bears only one free thiol group at the cysteine-34, where maleimides can bind highly efficiently (Scheme 7 A).[55] However, the use of

maleimides poses two major problems. 1) At physiological pH, hydrolysis of the double bond can occur, resulting in a carboxylic acid. Through this hydrolysis, the molecule loses its ability to bind to the thiol group (Scheme 7 B). 2) It is possible that a retro-Michael reaction occurs, thereby breaking the bond between the maleimide and the thiol. Reconjugation can take place, but in biological systems, the abundance of excess thiols leads to multiple different thiol-maleimide species (Scheme 7 C).[56]

Especially for Pt(IV) prodrugs, where the two axial positions bear the opportunity for additional moieties, the use of maleimides to target albumin is of major interest. In combination with other bioactive moieties, it is even possible to generate triple-action prodrugs (Figure 13).[57,58]



Scheme 7: A) Michael-addition reaction of a maleimide to a thiol. ***B)*** Maleimide hydrolysis in water at physiological pH. ***C)*** Retro-Michael reaction of a thiol-maleimide conjugate and a subsequent conjugation to a different thiol.

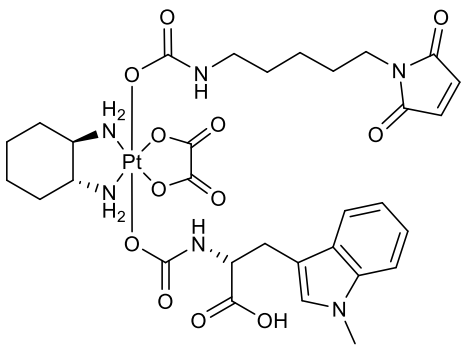
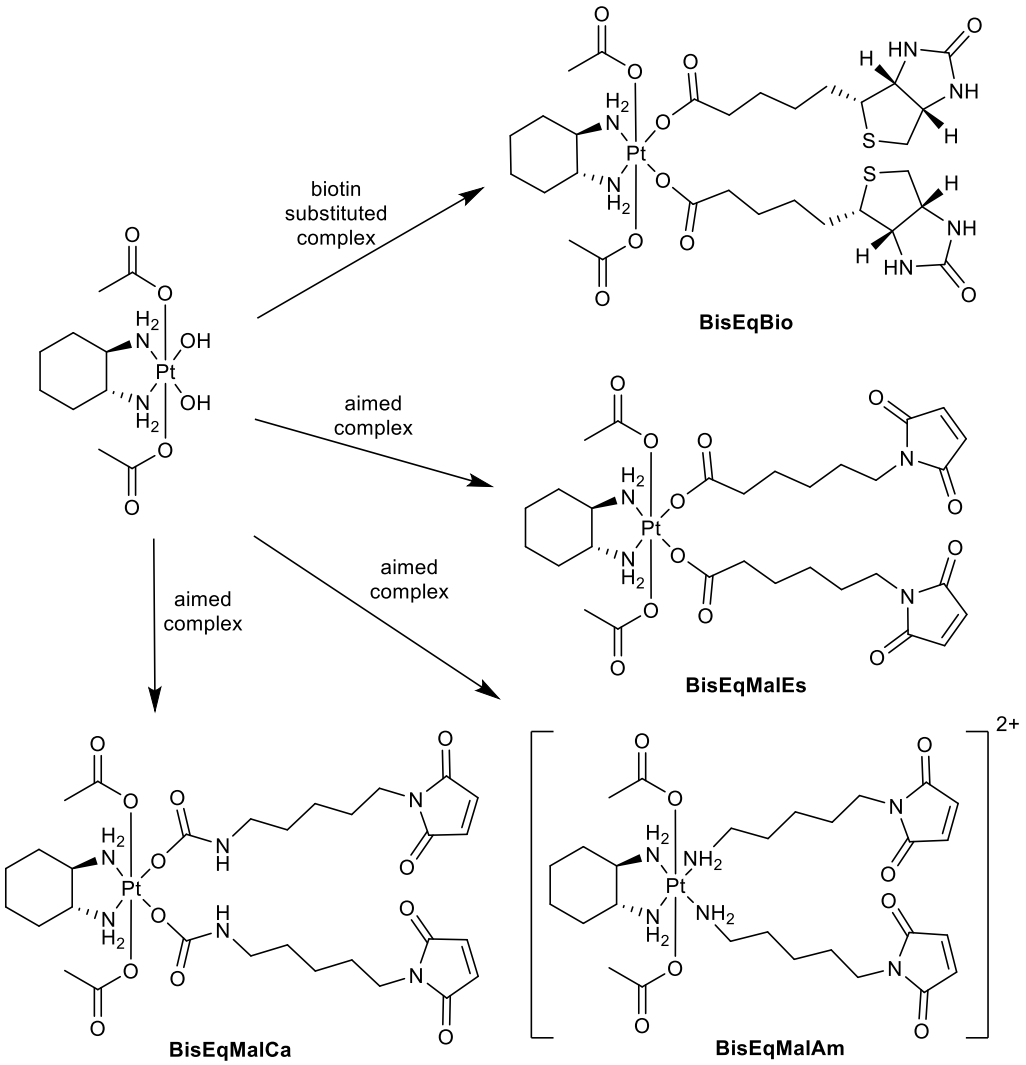


Figure 13: Targeted platinum(IV) prodrug with an oxaliplatin core, one axial maleimide and one axial indoximod moiety.

2. Aim of the Thesis

Recently, Kastner et all reported on the equatorial substitution of an intact platinum(IV) complex after oxalato hydrolysis. The study investigated the ability of different solvents to bind to the two new hydroxido groups and the resulting complexes were studied to get a better understanding of their properties. Subsequently, biotin was equatorially bound to the complex *via* an ester and the overall stability, reduction behavior and biological activity were examined (Scheme 8 **BisEqBio**).[59]

The aim of this thesis was to synthesize and investigate the properties of different types of bonds in the equatorial position. Therefore, maleimide moieties were bound through an ester (**BisEqMalEs**), a carbamate (**BisEqMalCa**) or an amine (**BisEqMalAm**) to the hydroxido groups of a hydrolyzed oxaliplatin(IV) with two axial acetato ligands (Scheme 8).



Scheme 8: Structure of the equatorially substituted biotin platinum complex (**BisEqBio**) and the three aimed products with maleimide ligands (**BisEqMalEs, BisEqMalCa and BisEqMalAm**).

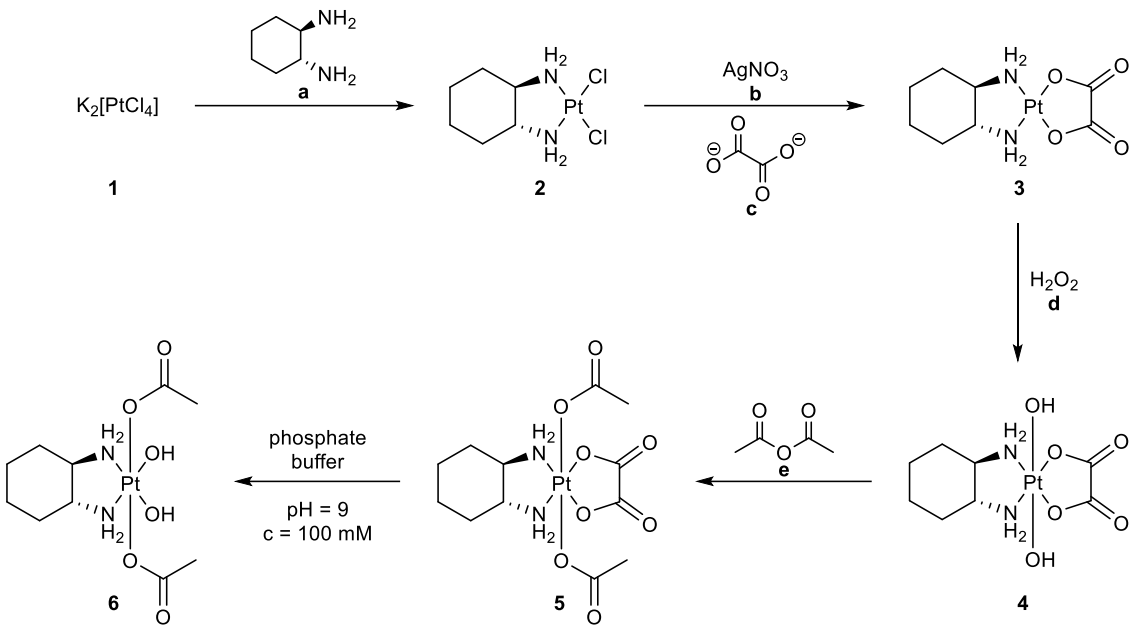
3. Results and Discussion

3.1. Synthesis

The synthesis can be roughly divided into three parts: first, the synthesis of the hydrolyzed oxaliplatin(IV), then the preparation of the ligands and lastly, the equatorial coordination of the moieties to the complex.

3.1.1. Synthesis of the Hydrolyzed Oxaliplatin(IV)

The synthesis of the hydrolyzed oxaliplatin(IV) (**6**) was a five-step procedure consisting of the following parts: Potassium tetrachloridoplatinate(II) ($K_2[PtCl_4]$) (**1**) was dissolved in water and (R,R)-1,2-diaminocyclohexane (**a**) was added. The reaction was stirred at room temperature, under light exclusion. After 24 h, the resulting complex **2** was filtered off. To obtain oxaliplatin (**3**), the chlorido ligands of **2** were precipitated with silver nitrate (**b**) in an aqueous solution and after removing the silver chloride, potassium oxalate (**c**) was added to the filtrate. Subsequently, oxaliplatin (**3**) was oxidized using hydrogen peroxide (**d**). By stirring **4** in acetic anhydride (**e**), the diacetato complex **5** was formed. The last step involved the hydrolysis of the oxalato ligand. Therefore, **5** was dissolved in 100 mL phosphate buffer (pH 9, c = 100 mM) heated to 50°C and stirred for 24 h, forming **6** (Scheme 9). The buffer capacity needed to be high enough to prevent a decrease in pH caused by the released oxalate ions. Compound **6** was purified by preparative RP-HPLC in acid-free conditions. This was necessary to avoid the substitution of the two equatorial hydroxido groups by formic acid (FA) or trifluoroacetic acid (TFA).



Scheme 9: Synthesis scheme of the equatorially hydrolyzed oxaliplatin(IV) **(6)**.

3.1.2. Ligand Synthesis

As mentioned in Chapter 2, this thesis aimed to establish different kinds of bonds in the equatorial position of an intact platinum(IV) complex. In addition, a maleimide moiety should be introduced into the platinum(IV) complexes to enable tumor-targeting via albumin binding. Kastner et all demonstrated that carboxylic acids can bind via an ester bond to the platinum core.[59] Thus, the commercially available maleimide with a C5 linker and a carboxylic acid was chosen (**7,** 6-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoic acid; Figure 14).

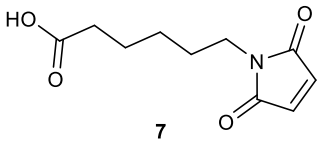
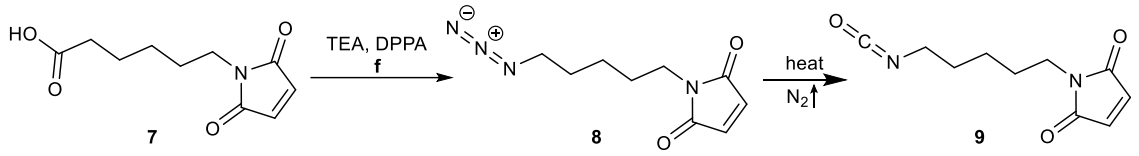


Figure 14: Structure of the purchased ligand **7**.

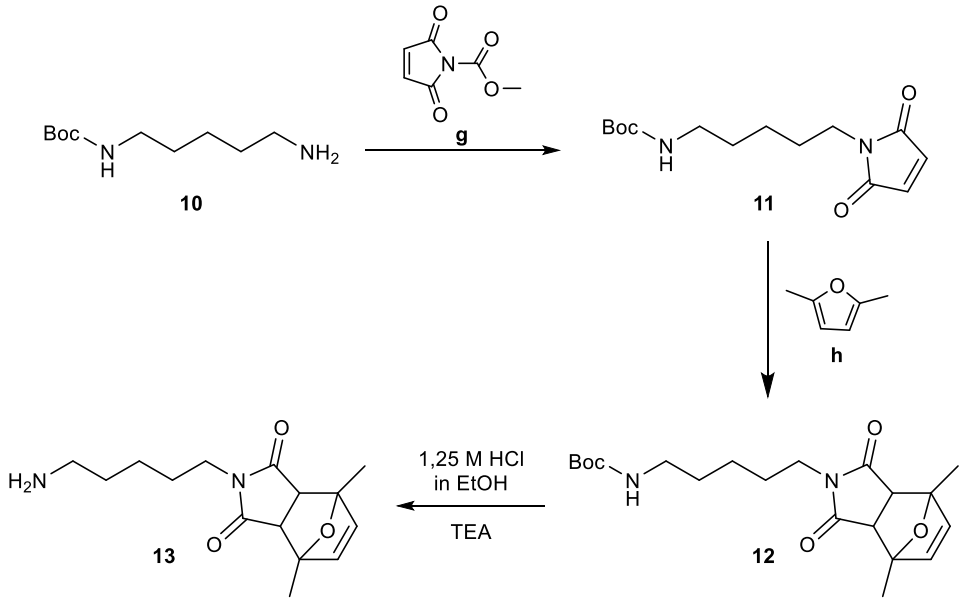
To synthesize the carbamate, the carboxylic acid of ligand (**7**) had to be converted to an isocyanate (**9**). This was done according to literature.[58] Using triethylamine (TEA) and diphenyl phosphorazidate (DPPA) (**f**) the maleimide **7** was converted into an acyl azide (**8**). Upon exposure to heat, the intermediate underwent a Curtius rearrangement, leading to its decomposition into the desired product, 1-(5-isocyanatopentyl)-1H-pyrrole-2,5-dione (**9**), along with the release of nitrogen gas (Scheme 10).[60]



Scheme 10: Synthesis scheme of the isocyanate moiety **9**.

Critical was the shelf life of compound **9**. Despite storage in the freezer, dimerization of the product was observed. This common reaction for isocyanates led to a urea derivative, which could no longer be coupled to the platinum.

For the moiety with the amine functionalization, a ligand with a protected maleimide had to be synthesized. Therefore, N-methoxycarbonyl-maleimide (**g**) was coupled to N-Boc-1,5-pentanediamine (**10**) in a saturated sodium hydrogen carbonate solution, forming **11**. Keller and Rudinger first described this reaction in 1975 and proposed that it proceeds *via* a ring opening of the maleimide, forming the imide-amide derivative followed by subsequent ring closure with the cleavage of the methyl carbamate.[61] Since maleimides react vigorously with nucleophiles like primary amines or thiols, they must be protected before the Boc-protection group can be cleaved from the amine. Through a Diels-Adler reaction, 2,5-dimethylfuran (**h**) reacted with **11**, forming the protected maleimide species **12**. The protection of maleimides with furans is a common method. By using 2,5-dimethylfuran instead of furan as a protection group, it is possible to lower the deprotection temperature from 120°C to 80°C.[62] For the Boc-deprotection 1,25 M HCl in ethanol was used. Because the amine was protonated and present as a chloride salt, it was neutralized with 5 equivalents of TEA, forming the final ligand **13** (Scheme 11).



Scheme 11: Synthesis scheme of the protected maleimide moiety **13**.

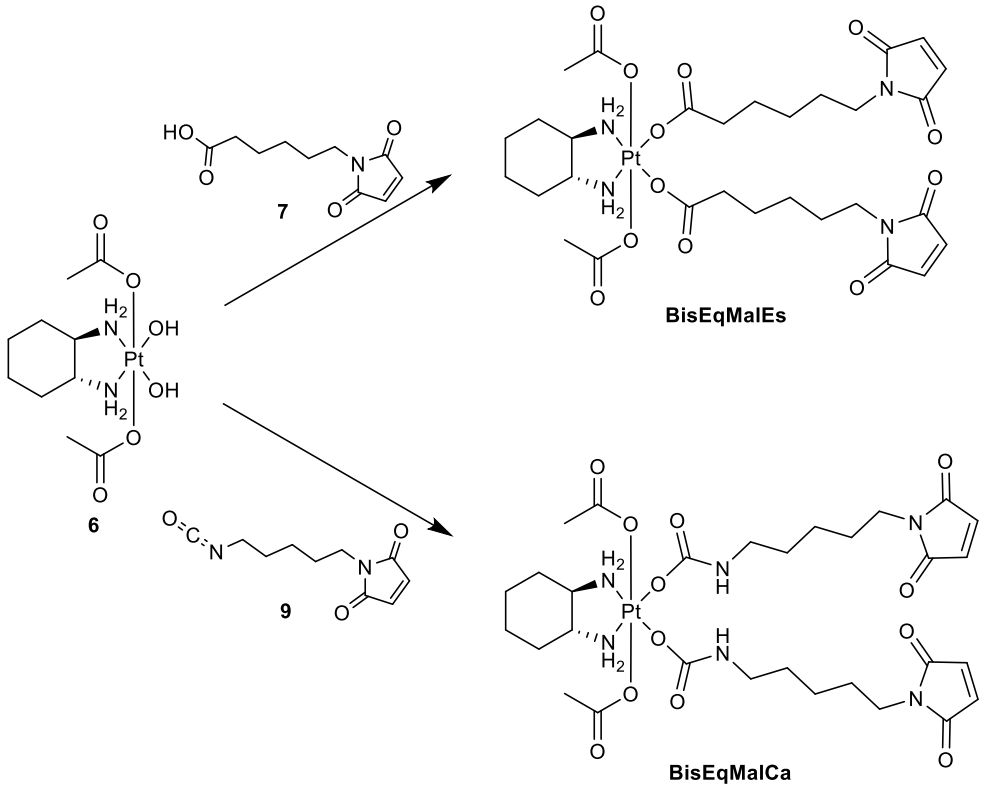
3.1.3. Synthesis of the Equatorially Substituted Platinum(IV) Complexes

The coordination of a carboxylic acid in equatorial position was reported by Kastner et all. The general procedure for the ligand coupling to the equatorial position involved suspending the complex in a non-coordinating solvent and adding the ligand in excess. Dimethylformamide (DMF) was used as the solvent. A. Kastner showed that first the mono-substituted and afterward the bis-substituted complex is formed. Depending on the reaction conditions and the equivalents the equilibrium between the mono- and bis-substituted product can be influenced and shifted to the desired side.[59] To form the "ester" type complex, 6-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoic acid (**7**) was used (see Chapter 3.1.2). The dihydroxido complex **6,** along with 10 equivalents of maleimide **7** was suspended in DMF and stirred at room temperature for 24 h. These conditions led to the formation of the mono- and bis-substituted products. However, to prioritize the synthesis of the bis-substituted complex the reaction temperature was increased to 50°C. Due to the high binding affinity of **7** at 50°C, the excess of the ligand was reduced to 6 equivalents. Initially, the crude reaction was purified by preparative RP-HPLC with the addition of 0.1% TFA. The addition of an acid resulted in a minor fraction of cleaved ligand. Thus, for subsequent purifications acid-free eluents were used with isocratic 32% ACN. These optimized conditions resulted mainly in the bis-substituted product **BisEqMalEs** with a yield of 40% (Scheme 12). The purity of the compound was verified by ^{1}H-NMR, mass spectroscopy, HPLC and elemental analysis.

The **BisEqMalEs** complex had to be stored under an inert argon atmosphere due to its extremely high hygroscopicity. Of note, short exposure to air did not lead to any visible changes but drastically decreased the solubility of the compound, rendering it practically insoluble in Milli-Q water or 5% glucose solution with 20% propylene glycol. This insolubility could be temporarily eliminated by dissolving the complex in methanol, diluting it with water and subsequent lyophilization. This procedure was repeated several times, and no structural change of the complex could be determined by ^{1}H-NMR.

For the synthesis of equatorial carbamates, an approach following literature-known strategies for axial carbamates was used. The most common methods to form this kind of bond in the axial position are the use of the coupling agent N,N-disuccinimidyl carbonate (DSC) or an isocyanate of the carboxylic acid ligand. First, the activation of the hydroxido ligands with DSC was attempted. In theory, DSC forms a stable active ester, which can be isolated. Through the addition of a primary amine, this intermediate reacts to a carbamate. In pre-tests, neither the DSC-activated complex nor the final product could be found. For the second approach, the carboxylic acid was converted into

an isocyanate as already described in Chapter 3.1.2 (Scheme 10). This activated ligand (**9**) was able to form the equatorial carbamate with the oxaliplatin(IV) complex (**6**). Test reactions indicated that the coupling needed to be carried out at room temperature, owing to the higher reactivity of isocyanate **9** compared to the carboxylic acid **6**. A higher reaction temperature resulted in the formation of side products and a lower yield. Furthermore, only 4 equivalents of the moiety were required and the reaction was completed after 4 h. This yielded the bis-substituted product **BisEqMalCa** (yield: 40%; Scheme 12). The complex was purified by preparative RP-HPLC (see below). The purity of the compound was verified by ^{1}H-NMR, mass spectroscopy, HPLC and elemental analysis. The hygroscopicity of this compound was significantly lower compared to the "ester" type complex **BisEqMalEs** and also no solubility problems occurred.



Scheme 12: Synthesis scheme of **BisEqMalEs** and **BisEqMalCa**.

BisEqMalCa had to be purified by preparative RP-HPLC in acid-free conditions. The addition of an acid, like TFA, led to the cleavage of the carbamate(s), resulting in the mono-substituted and TFA-substituted complex (Figure 15). As expected the released carbamate ligand decomposed into the primary amine.

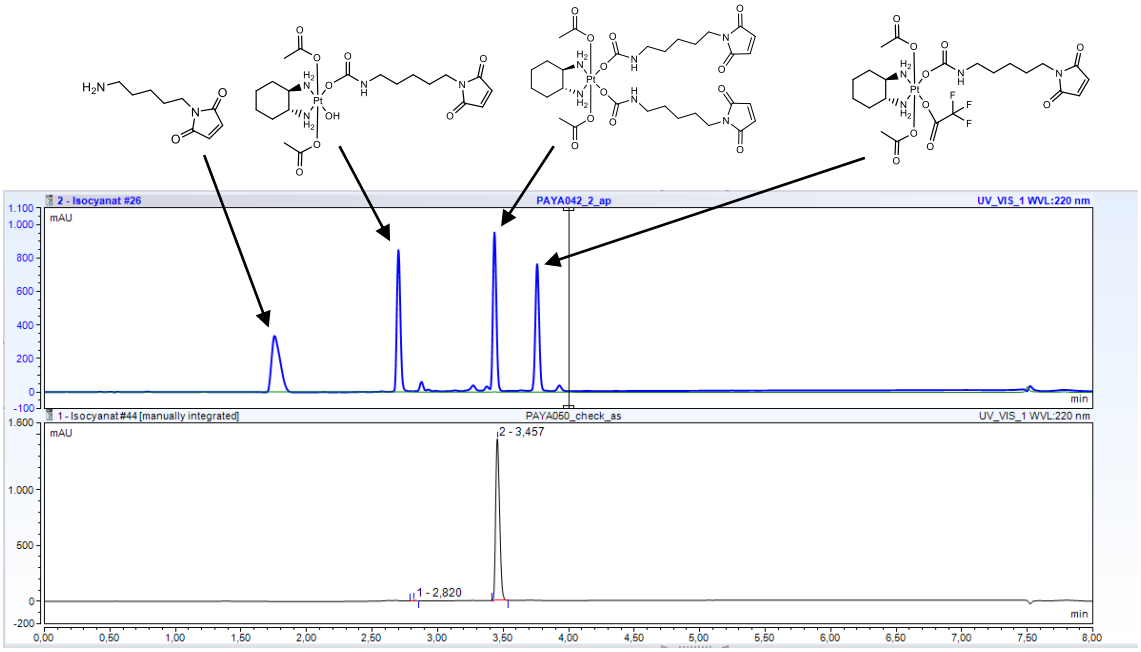


Figure 15: Chromatogram of **BisEqMalCa** purified under acidic conditions (0.1% TFA) (blue). With LC-MS, the peaks were assigned to the corresponding structures drawn above. The reference chromatogram of **BisEqMalCa** purified under acid-free conditions is shown below (black).

Because the attachment of amines in equatorial position is not documented in the literature and there are hardly any comparable axial reactions that could serve as a reference, preliminary tests had to be conducted. To determine if amines can coordinate to the equatorial positions of a hydrolyzed oxaliplatin(IV), propyl-1-amine was used as a test ligand. To compare the previously used conditions the reaction was carried out as described above: The starting material **6** was suspended in DMF and 10 equivalents of propyl-1-amine were added (Scheme 13). The reaction was stirred at 40°C for 24 h. However, with HPLC it could be observed, that the formed product peak rapidly decreased and side products occurred over time. Consequently, the reaction temperature was lowered. After 24 h at 30°C, mainly **BisEqProAm** was formed, but after purification by preparative RP-HPLC with 0.1% TFA the isolated compound showed additional NMR peaks (Figure 16).

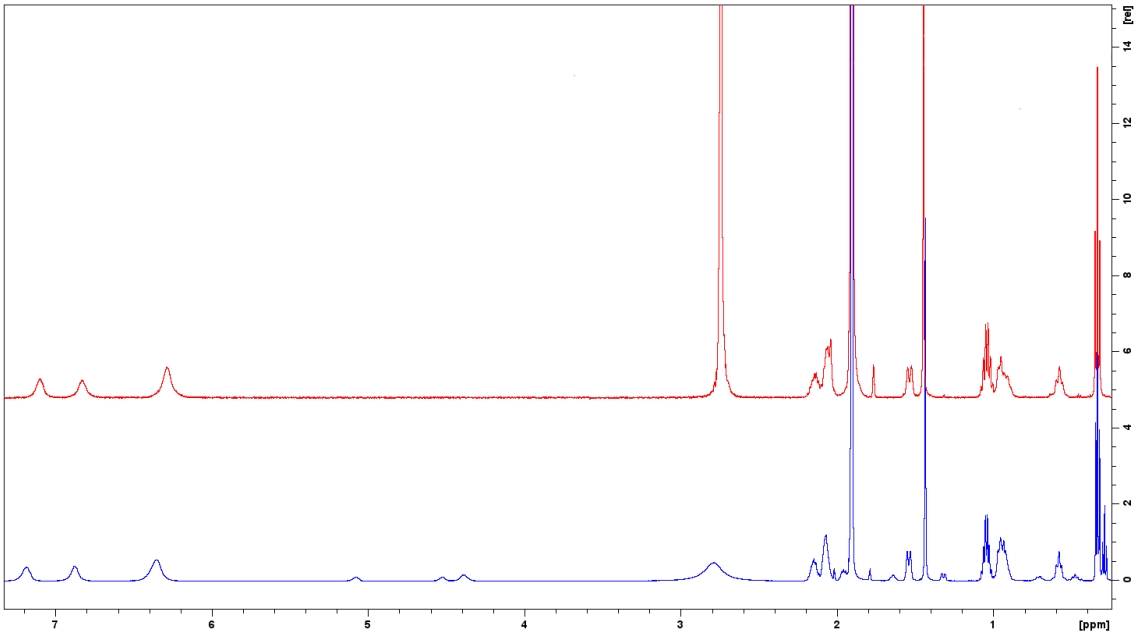
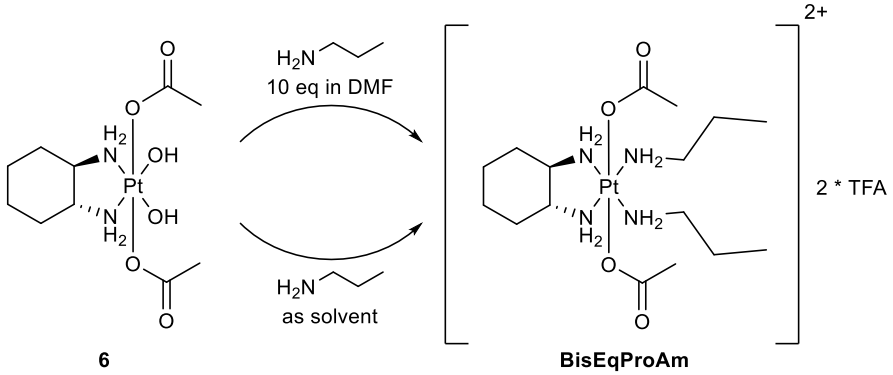


Figure 16: ^{1}H-NMR of **BisEqProAm** with unidentified impurities (blue). As a reference ^{1}H-NMR of pure **BisEqProAm** (red).

In order to minimize side-products, it was attempted to use the liquid ligand propyl-1-amine also as the solvent. Therefore, compound **6** was dispersed in the liquid amine and stirred. Initially, the reaction was carried out at 40°C for 24 h. However, no **BisEqProAm** was detected using HPLC. Subsequently, monitoring of the reaction *via* HPLC revealed that the majority of the product was already formed after 1 h at 40°C. However, again several side-products were identified and thus the reaction temperature was decreased. After stirring for 1 h at 30°C, the excess ligand was removed and the crude reaction was purified by preparative RP-HPLC with 0.1% TFA. This led to complex with two trifluoroacetates as counter ions compensating the twofold negative charge. The yield of the bis-substituted product **BisEqProAm** was 29% (Scheme 13). The purity of the compound was verified by ^{1}H-NMR, mass spectroscopy, HPLC and elemental analysis.



Scheme 13: Both synthesis strategies for **BisEqProAm**.

To investigate if the synthesis of the maleimide analogue **BisEqMalAm** is possible, a test reaction was set up and monitored by LC-MS. Therefore, hydrolyzed oxaliplatin(IV) (**6**) was suspended in 5 mL DMF, the protected maleimide moiety **13** was added and the reaction was stirred at 30°C for 24 h. *Via* LC-MS the formation of the bis-substituted product **BisEqMalAm** could be confirmed. However, the reaction was not further purified.

3.2. Characterization Studies

3.2.1. Hydrolytic Stability

For the three isolated complexes **BisEqMalEs**, **BisEqMalCa** and **BisEqProAm**, the hydrolytic stability at physiological pH was studied. Therefore, 1 mM of the compound was incubated in phosphate buffer (150 mM, pH 7.4) at 20°C and the reaction was monitored with HPLC for 24 h. Acid-free eluents were used for **BisEqMalEs** and **BisEqMalCa**, but for **BisEqProAm,** the addition of 0.1% TFA to the eluents was necessary to improve the peak resolution. A gradient of 5–95% acetonitrile over 6.5 min was used for all measurements.

As discussed in Chapter 1.5.2.1, maleimides tend to hydrolyze at physiological pH. For **BisEqMalEs** and **BisEqMalCa,** exactly this phenomenon was observed during the stability measurements. The hydrolyzed maleimide moiety led to the formation of a new peak with a shorter retention time compared to the parental complex, which could also be confirmed by LC-MS measurements. Besides that, no other newly formed peaks were observed, leading to the conclusion that the platinum core is stable and no axial or equatorial hydrolysis occurred (Figure 17).

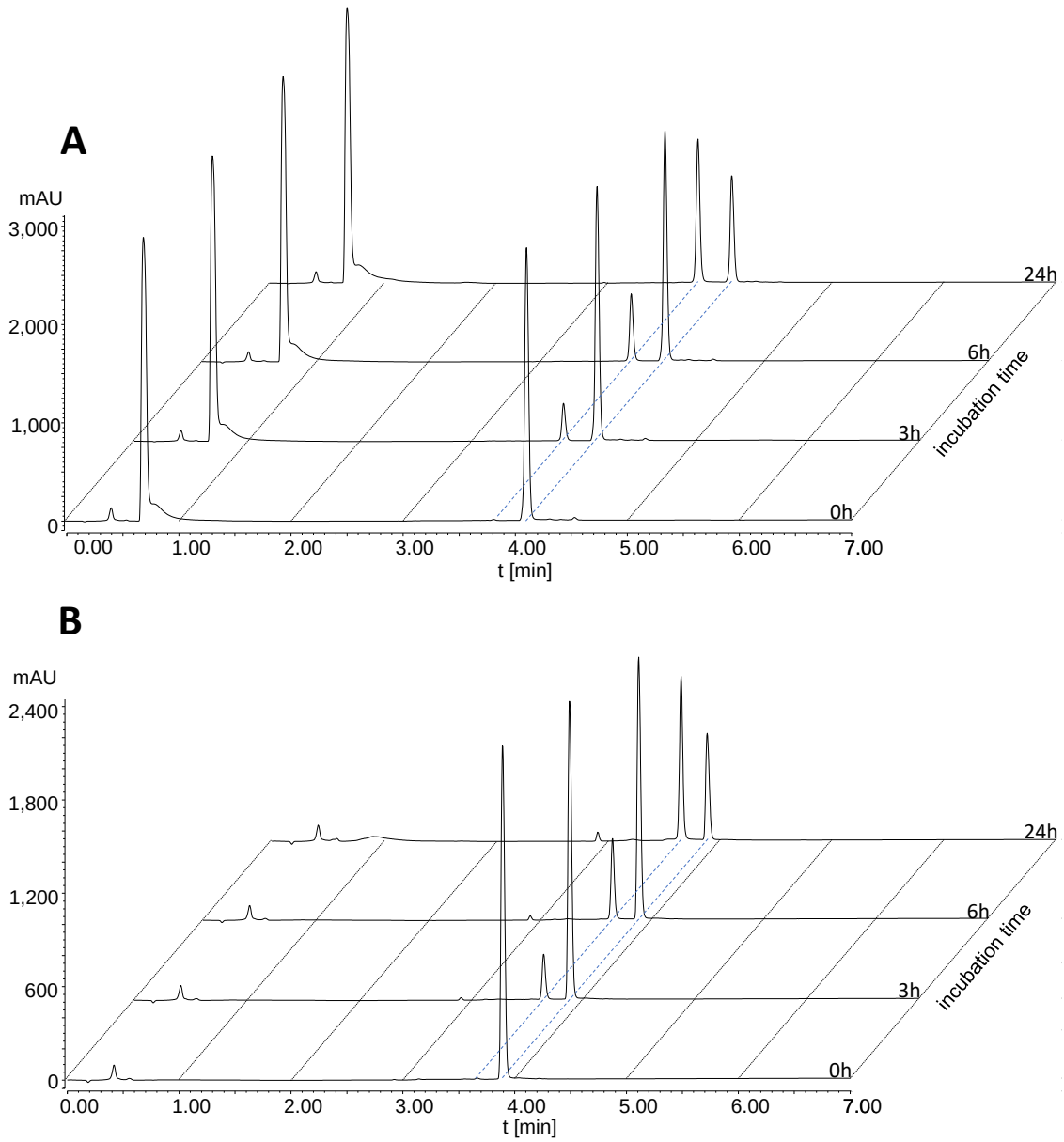


Figure 17: Hydrolytic stability of **BisEqMalEs** (A, 4.1 min) and **BisEqMalCa** (B, 3.9 min) at 0 h, 3 h, 6 h and 24 h. The hydrolysis of the maleimide led to the formation of a new peak (A, 3.8 min; B, 3.7 min), but no hydrolyzed platinum species occurred.

The measurements for **BisEqProAm** showed very high stability and no newly formed peaks (Figure 18). Therefore, it can be assumed that under physiological conditions in phosphate buffer, no axial or equatorial hydrolysis occurs within 24 h.

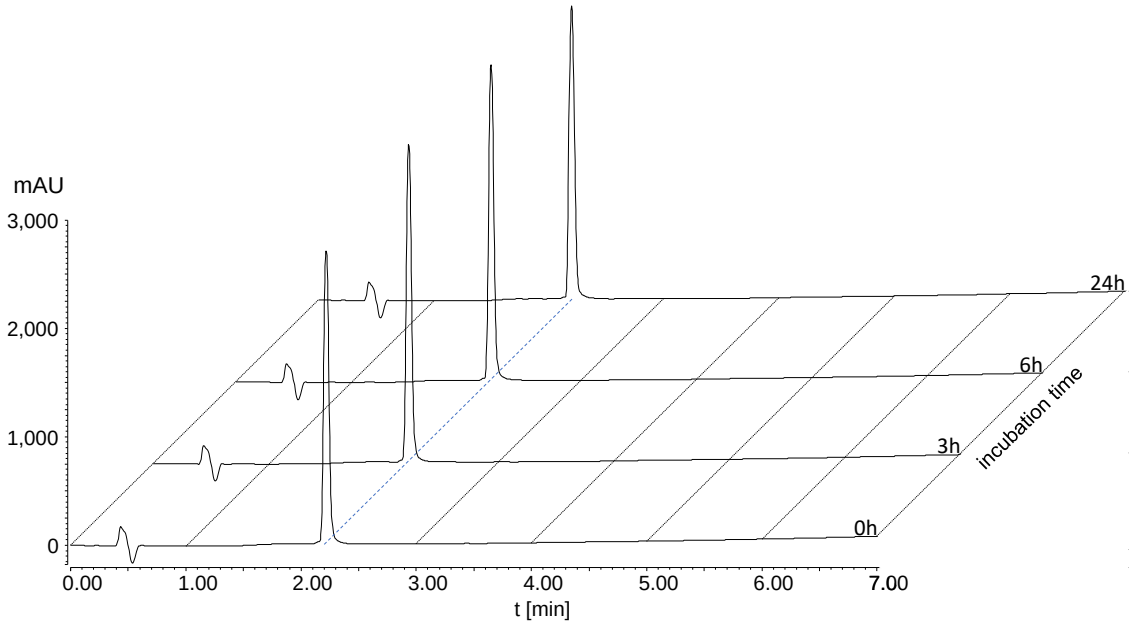


Figure 18: Hydrolytic stability of **BisEqProAm** (2.4 min) at 0 h, 3 h, 6 h, 24 h.

3.2.2. Reduction Kinetics

For the three isolated complexes **BisEqMalEs**, **BisEqMalCa** and **BisEqProAm**, in addition the reduction kinetics at physiological pH was studied. Therefore, 1 mM of the compounds was incubated in phosphate buffer (150 mM, pH 7.4) with 10 equivalents of L-ascorbic acid at 20°C and the reaction was monitored with HPLC for 24 h. Acid-free eluents were used for **BisEqMalEs** and **BisEqMalCa**, but for **BisEqProAm,** the addition of 0.1% TFA to the eluents was necessary for improved peak resolution. A gradient of 5–95% acetonitrile over 6.5 min was used for all measurements.

During the reduction kinetics of **BisEqMalEs** and **BisEqMalCa**, also the hydrolysis of the maleimide ligand occurred. In order to calculate the reduction rate, this decrease in the educts had to be considered. For this, the previously measured hydrolyses data, shown in Figure 17 were used. This resulted in a calculated 21% reduction for both **BisEqMalEs** and **BisEqMalCa** after 6 h. To illustrate the difference between the hydrolysis and the reduction the chromatograms of the measurements were overlaid for the two compounds at the given time points (Figure 19).

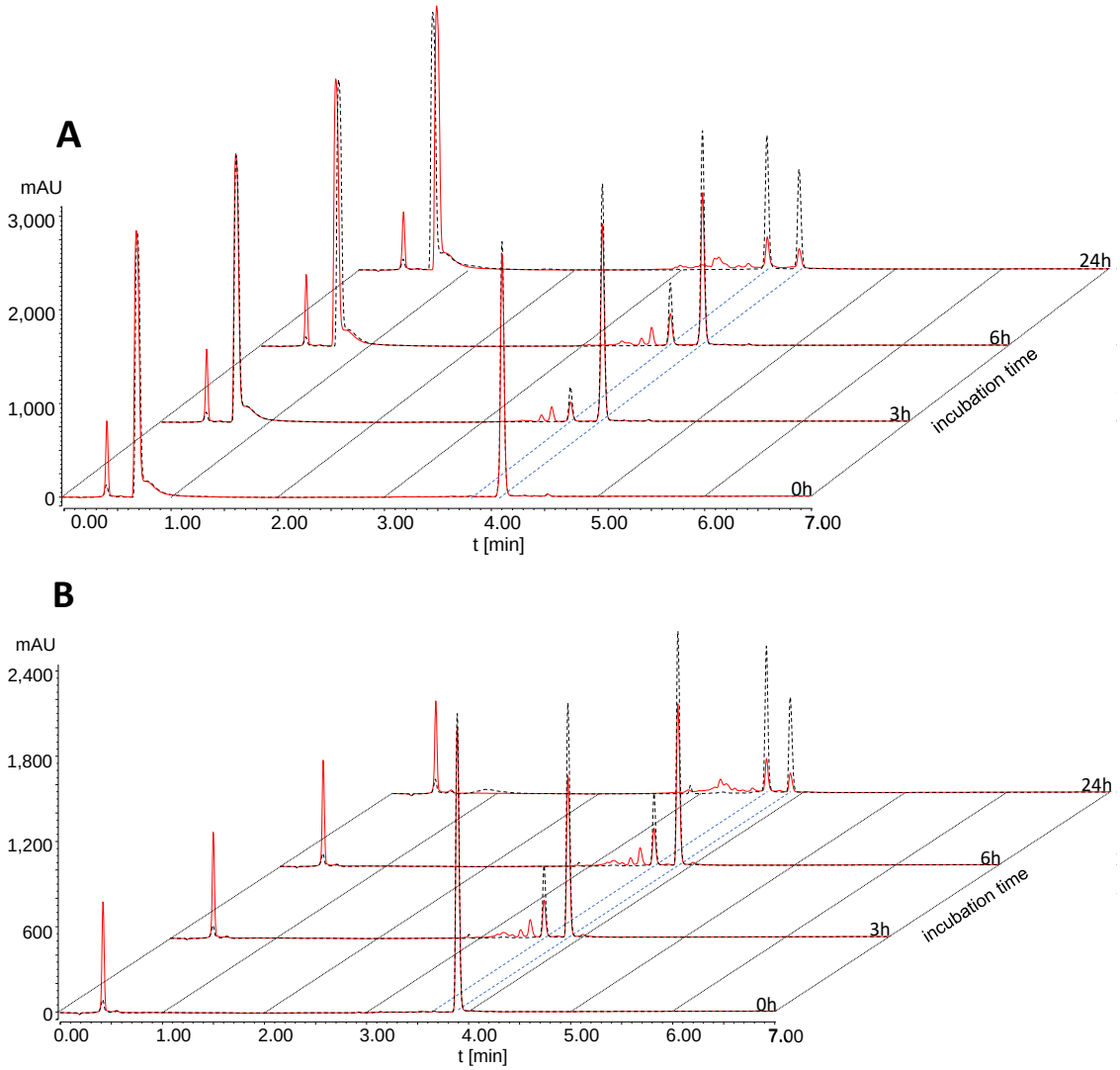


Figure 19: Reduction kinetics of **BisEqMalEs** (A, 4.1 min) and **BisEqMalCa** (B, 3.9 min) at 0 h, 3 h, 6 h, 24 h (red line). For comparison, overlaid with the respective hydrolytic stability chromatograms shown in ***Figure 17*** (black dotted lines). The reduction of the platinum(IV) can be determined by analyzing the difference in the signal intensities of the two spectra.

Because the hydrolytic stability for **BisEqProAm** showed no formation of additional products, it could be assumed that the reduction rate corresponded directly to the decrease of the educt peak. The data revealed that 28% of **BisEqProAm** were reduced after 6 h (Figure 20).

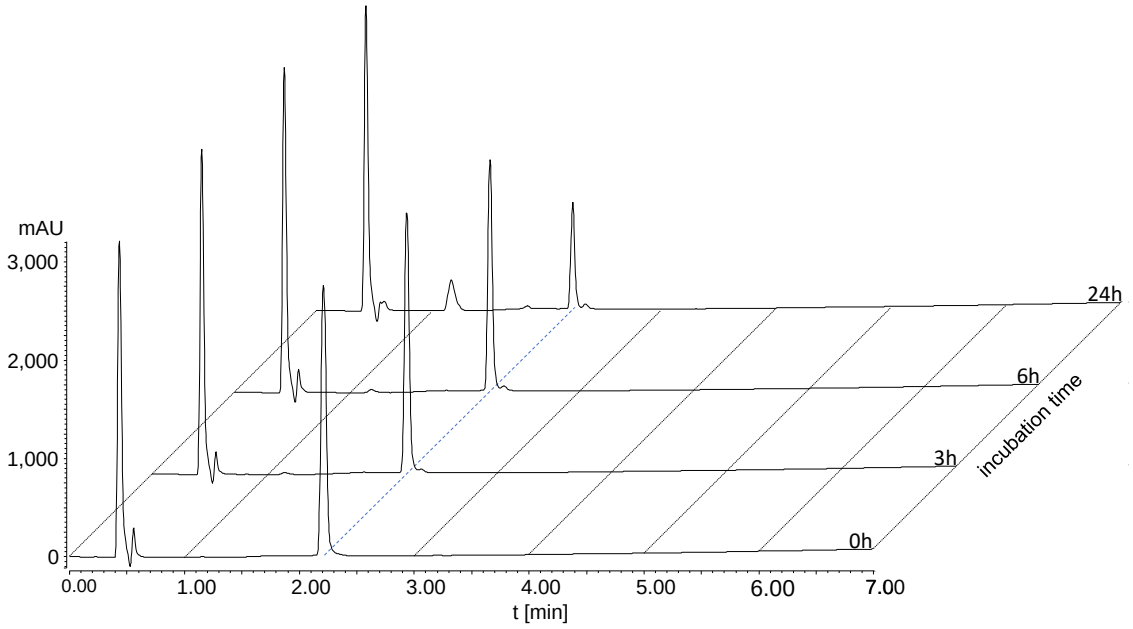


Figure 20: Reduction kinetics of **BisEqProAm** (2.4 min) at 0 h, 3 h, 6 h, 24 h.

3.2.3. Albumin Binding Study

For **BisEqMalEs** and **BisEqMalCa** albumin binding studies were conducted in fetal calf serum. The compounds (5 mM) were dissolved in phosphate buffer (150 mM, pH 7.4) and diluted with the buffered serum to a final concentration of 100 µM. The samples were incubated at 37°C and measured every hour for 5 h using size exclusion chromatography-inductively coupled plasma mass spectrometry (SEC-ICP-MS). To determine the retention time of albumin in fetal calf serum first the sulfur trace of pure albumin and fetal calf serum was measured, revealing a retention time of 3.9 min for albumin. Also, the albumin dimer peak could be observed at around 3.3 min (Figure 21).

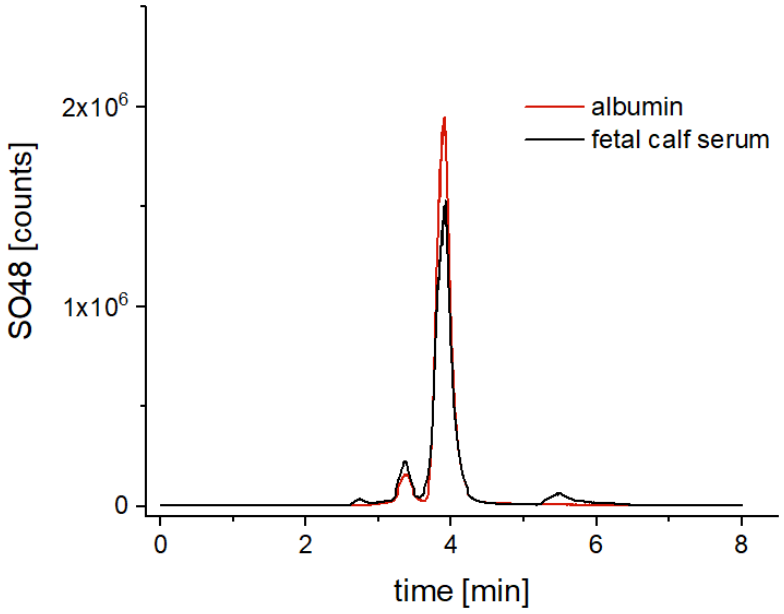


Figure 21: Sulfur trace of albumin (red, 3.9 min) and fetal calf serum (black) in phosphate buffer (pH 7.4) measured with SEC-ICP-MS. The albumin dimer can be seen at ~3.3 min.

Figure 22 shows the results of the ^{195}Pt SEC-ICP-MS measurements of fetal calf serum incubated with **BisEqMalEs** and **BisEqMalCa**. It can be seen that for both complexes already at the first time point (30 s after incubation) the majority (**BisEqMalEs**: 83% and **BisEqMalCa**: 73%) of the platinum was bound to albumin at ~3.9 min (Figure 22). For both compounds, the maximum binding was reached after 1 h of incubation. At this point, 93% of **BisEqMalEs** and 98% of **BisEqMalCa** were bound to albumin. As discussed in Chapter 1.5.2.1, the maleimide albumin binding may undergo a retro-Michael reaction, which can be observed in a decrease of platinum levels at 3.9 min. After 24 h, 81% of **BisEqMalEs** and 82% of **BisEqMalCa** were still bound to the blood protein. Over the incubation time of 24 h, the peak of the unbound compounds shifted from >6 min to shorter retention times (<6 min) most probably due to maleimide hydrolysis or binding to low-molecular weight sulfur-containing compounds (cysteine, glutathione etc.).

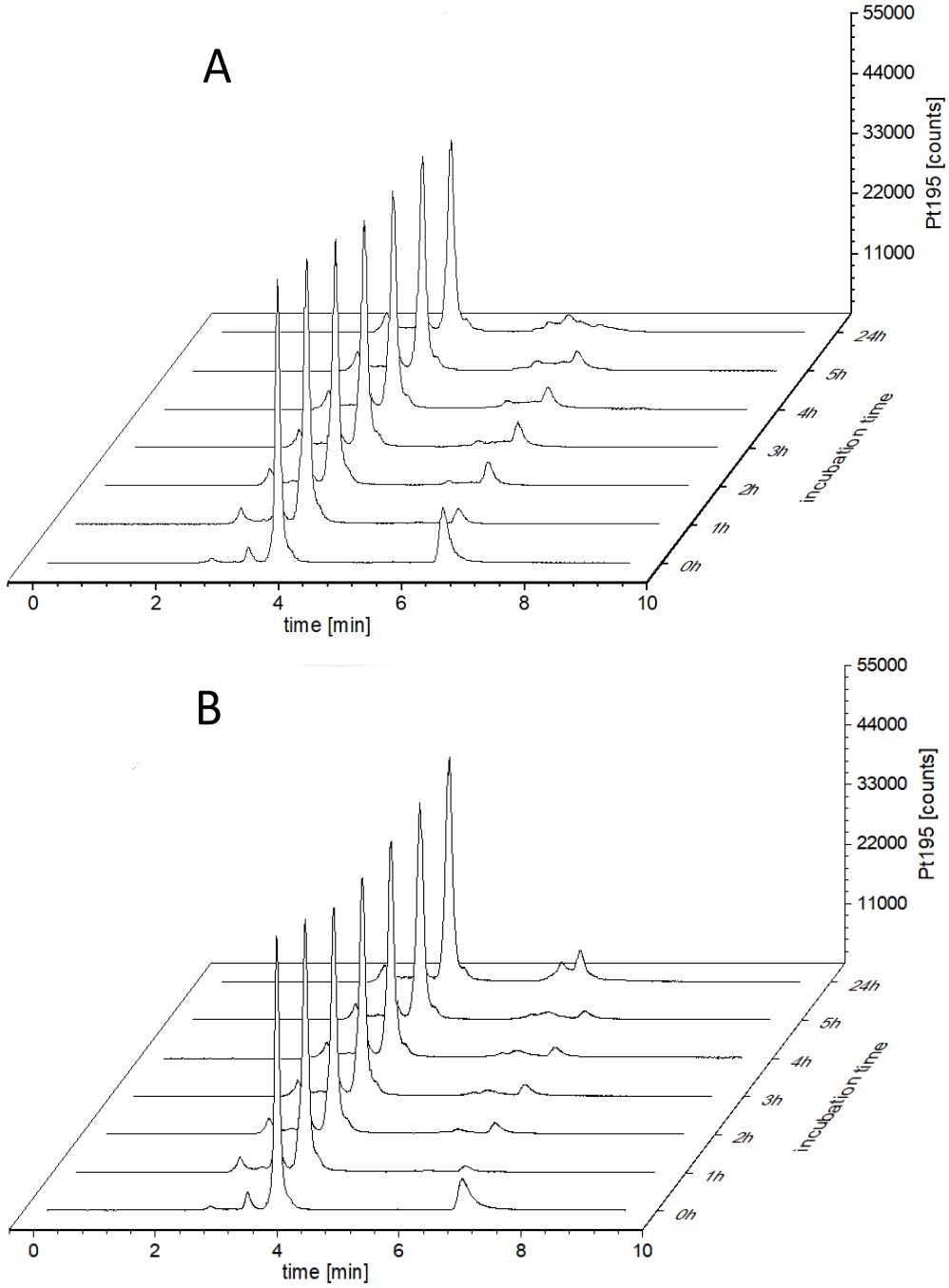


Figure 22: Albumin binding study for **BisEqMalEs** (A) and **BisEqMalCa** (B) measured with SEC-ICP-MS.

The synthesis and characterization of **BisEqMalEs** and **BisEqMalCa** together with first *in vivo* data performed at the Center of Cancer Research of the Medical University of Vienna have already been published in Angewandte Chemie International Edition 2023, 62, e202311468. The main manuscript can be found below.

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Bioinorganic Chemistry

Insertion of (Bioactive) Equatorial Ligands into Platinum(IV) Complexes

Alexander Kastner[+], Hemma Schuefffl[+], Patrick A. Yassemipour, Bernhard K. Keppler, Petra Heffeter, and Christian R. Kowol**

Abstract: Platinum(IV) prodrugs are highly interesting alternatives to platinum(II) anticancer therapeutics due to their increased tumor selectivity and reduced side effects. In contrast to the established theory, we recently observed that the equatorial ligand(s) of e.g. oxaliplatin(IV) complexes can be hydrolyzed with formation of $[(DACH)Pt(OH_{eq})_2(OAc_{ax})_2]$. In the work presented here, we investigated the reactivity and synthetic usability of this complex to be exploited as a precursor for the development of novel platinum(IV) complexes, not able to be synthesized by conventional protocols. Indeed, we could substitute the equatorial hydroxido ligand(s) e.g. by one or two monodentate biotin ligands (which would be oxidized under standard methods). The formed complexes turned out to be very stable with slow ligand release after reduction, ideal for long-circulating tumor-targeting strategies. Therefore, two platinum(IV) complexes with equatorial maleimides, capable of exploiting serum albumin as a natural nanocarrier, were synthesized as well. The complexes showed massively prolonged plasma half-life and distinctly improved anticancer activity in vivo compared to oxaliplatin. Taken together, the newly developed synthetic platform allows the simple and specific insertion of equatorial ligands into platinum(IV) complexes. This will enable the attachment of three different (bioactive) moieties generating targeted triple-action platinum(IV) prodrugs within one single platinum complex.

Introduction

Chemotherapy is still a very important part of cancer treatment. Especially platinum complexes are frequently used, also in combination with modern immunotherapeutics.[1] So far, only three platinum complexes have been clinically approved for worldwide use (cisplatin, carboplatin and oxaliplatin), while five more are applied in Asia exclusively.[2] The mode of action involves hydrolysis of the leaving ligands inside the cell and subsequent binding to guanine and adenine of the DNA, thus inducing apoptosis.[3] However, these processes can also occur in healthy tissues resulting in a variety of dose-limiting side effects.[4] One possibility to reduce this problem is the use of platinum(IV) complexes, as these oxidized counterparts are kinetically more stable, preventing many side reactions. Furthermore, the two introduced axial ligands can be utilized for targeting, attachment of bioactive ligands or the fine-tuning of physico-chemical properties.[5]

Different prerequisites exist for axial and equatorial ligands: While the axial ligands are often used to implement additional functionalities into the platinum(IV) complexes and should be stable against hydrolysis, the equatorial "leaving group" ligands should hydrolyze once the complex is reduced to platinum(II) inside the cancer cell. Nevertheless, some research groups synthesized and compared complexes with e.g. dichloroacetate,[6] acetate,[7] or ethacrynic acid,[8] coordinated as axial ligands to platinum(IV) and as equatorial ligands to platinum(II), respectively. These data suggest that the equatorial plane can also be utilized for direct attachment of bioactive ligands.

Recently, we reported on the unexpected hydrolysis of the equatorial "leaving group" of certain platinum(IV) complexes under physiological conditions. We could show that oxali- and satraplatin(IV) complexes hydrolyze rapidly, while the respective carboplatin(IV) complex was completely stable and the cisplatin(IV) analogue was in between. This also enabled the synthesis of a novel oxaliplatin(IV) complex bearing two equatorial hydroxido ligands: $[(DACH)Pt(OH_{eq})_2(OAc_{ax})_2]$ (DACH = (1*R*,2*R*)-1,2-diaminocyclohexane; Scheme 1).[9] The question arose, if this complex could be used as synthetic precursor, enabling the direct and selective introduction of novel equatorial ligands

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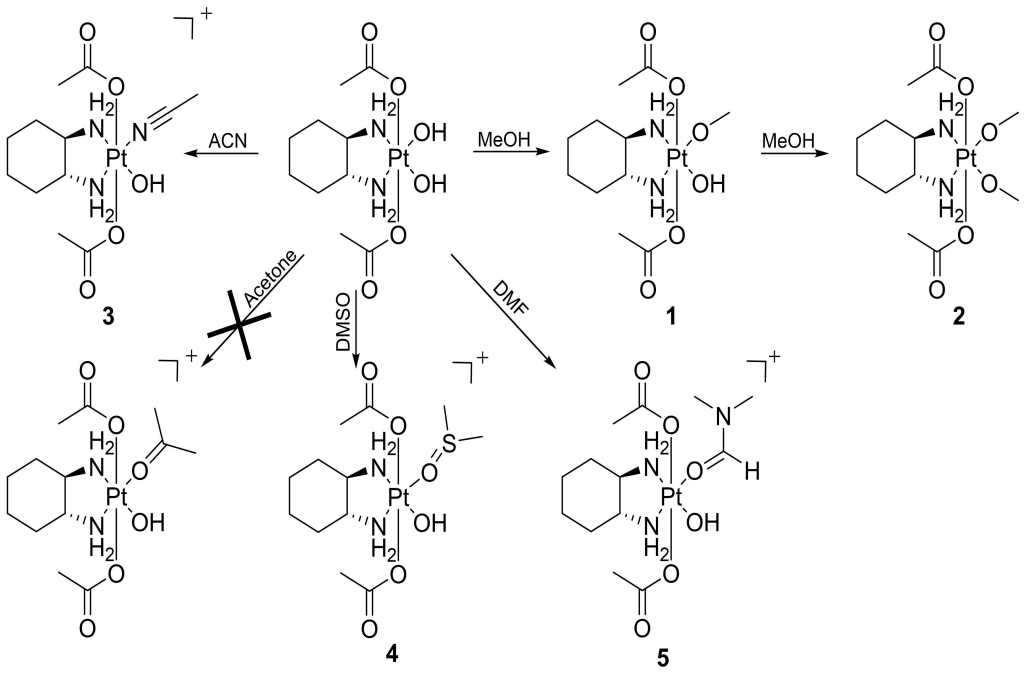


Scheme 1. Overview of the reactions of $[(DACH)Pt(OH)_2(OAc)_2]$ with different solvents.

into platinum(IV) complexes. In literature only very few publications with tetra-hydroxidoplatinum(IV) complexes exist, where such substitutions have been reported.[10] However, the presence of four hydroxido groups, two axial and two equatorial, does not allow for specific reactions in the equatorial plane, resulting in isomeric mixtures,[10c] or the same ligand in all four positions.[10b]

Conventionally, the development of platinum(IV) complexes starts with the synthesis of the respective platinum(II) core, which is then oxidized e.g. with hydrogen peroxide to introduce two axial hydroxido groups, which can subsequently be functionalized to esters, carbonates or carbamates.[11] In addition, few examples of axial halido ligand exchange reactions have been recently reported to introduce e.g. axial N-heterocycles.[12] Selective substitution of the equatorial "leaving group" in platinum(IV) complexes would be an elegant new synthetic pathway. Moreover, the oxidation step is not suitable for every ligand, as some of them might be prone to oxidation themselves. One example is biotin, for which the thioether can be oxidized into a sulfoxide or even a sulfone.[13] Furthermore, such sulfur atoms also have a high affinity for direct coordination to the "soft" platinum(II) center; which is prevented when introducing the ligand in the "hard" platinum(IV) oxidation state. Biotin is an important cofactor in a variety of enzymes related to gluconeogenesis or fatty acid synthesis.[14] It is taken up into the cell by the sodium-dependent multivitamin transporter (SMVT), which is overexpressed in many types of cancer, and has therefore been suggested as a targeting moiety.[15] Biotin was already investigated as an axial ligand for cisplatin(IV) complexes, showing promising results.[16]

Herein, we chose biotin for the establishment of the equatorial substitution reaction using the specific properties of $[(DACH)Pt(OH_{eq})_2(OAc_{ax})_2]$, generating complexes that are not accessible by conventional synthetic routes. Subsequently, their hydrolytic and reductive behavior was compared with analogues bearing biotin in axial position. Furthermore, the biological activity against cancer cells was analyzed, indicating that equatorial substitution of platinum(IV) drugs results in reduced cytotoxicity. This renders such complexes ideal tools for prodrug concepts as shown by the synthesis of the first equatorial maleimide-bearing platinum(IV) complexes (also not possible to be obtained by standard synthetic methods) for albumin-mediated drug delivery.

Results and Discussion

In the first step, we wanted to understand if the two equatorial hydroxido groups of $[(DACH)Pt(OH_{eq})_2(OAc_{ax})_2]$ are suitable for substitution reactions and can be used for new synthetic strategies. Therefore, $[(DACH)PtOx(OAc)_2]$ (Ox = oxalate) was stirred in aqueous solution at pH 9 for 24 h leading to the release of the oxalate ligand and formation of $[(DACH)Pt(OH_{eq})_2(OAc_{ax})_2]$.[9] Subsequently, the isolated complex was incubated for 24 h at room temperature (RT) with different solvents (acetone, acetonitrile (ACN), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol (MeOH)) (Scheme 1). The reactions were monitored via high-performance liquid chromatography coupled to a mass spectrometer (HPLC-MS).

In MeOH, $[(DACH)Pt(OH)_2(OAc)_2]$ formed the mono- and bis-methanolato complexes **1** and **2**. This is in agreement with the X-ray crystal structure of $[(DACH)Pt(OH)_2$-

$(OAc)_2$] from MeOH/diethyl ether, where also both hydroxido ligands were exchanged by MeOH.[9] In ACN, exclusively the mono-ACN complex **3** was formed. In contrast, acetone did not coordinate to platinum, instead again the mono-ACN complex **3** could be observed due to the ACN in the HPLC solvent (also direct injection of the acetone solution into the MS did not show coordination to platinum). In case of DMSO and DMF, also the respective mono-solvent complexes **4** and **5** were formed. Comparing all tested solvents, MeOH coordinated most readily and was the only solvent resulting in the formation of a bis-species.

The stability of the formed complexes in aqueous solution at physiological pH was investigated after incubation of 100 μM [(DACH)Pt$(OH)_2(OAc)_2$] in the different solvents for 24 h and drying of the samples under reduced pressure. The remaining solids were taken up in phosphate buffer at pH 7.4 and tested for their stability against hydrolysis with HPLC-MS. The mono-ACN and -DMF complexes **3** and **5** hydrolyzed quickly, again forming [(DACH)Pt$(OH)_2(OAc)_2$] after 1 h. The MeOH complexes **1** and **2** showed slower hydrolysis of their methoxido ligands (Figure S2), with a rate comparable to the oxalate release from [(DACH)PtOx$(OAc)_2$].[9] The sample dissolved in DMSO turned black over the course of 24 h. As a result, it could not be tested for hydrolytic stability. However, further examination of the HPLC-MS runs revealed the formation of the tetra-acetato complex, which could also be isolated via preparative HPLC (data not shown). This suggests that axial acetate ligands, which are released during decomposition/reduction, can coordinate to still intact molecules of [(DACH)Pt$(OH)_2(OAc)_2$] (or displace a DMSO ligand). Consequently, already quite low levels of carboxylic acids (e.g. acetate) seem to be sufficient to replace hydroxido ligand(s) in [(DACH)Pt$(OH)_2(OAc)_2$]. Therefore, we investigated the reaction of [(DACH)Pt$(OH)_2(OAc)_2$] dissolved in acetone (where the complex is stable) and 2–250 eq. of acetic acid for 24 h at RT. Even with only 10 eq. full conversion to the tetra-acetato complex in an analytical scale was observed. Therefore, we scaled up and optimized this reaction (Table 1).

All reactions were carried out with 5 mg of [(DACH)Pt$(OH)_2(OAc)_2$], 10 eq. of the respective ligand and a reaction time of 24 h. First, the reaction with acetic acid in different solvents was tested: in water the reaction did not take place at all, which is also in agreement with the high stability of [(DACH)Pt$(OH)_2(OAc)_2$] in medium and serum.[9] In contrast, in both acetone and DMF approx. 60 % of the dihydroxido complex reacted to the mono- or bis-acetato species. Hence, further experiments were conducted in DMF, being the more versatile solvent. Of note, using [(DACH)Pt$Cl_2(OAc)_2$] as the precursor under the same conditions did not result in any conversion. Next, it was investigated if [(DACH)Pt$(OH)_2(OAc)_2$] could be transformed back to its original oxaliplatin core. Addition of both sodium oxalate and oxalic acid resulted in the desired complex (20 % and 30 %, respectively). This comparison furthermore revealed that acids might be preferred in this type of reaction. Finally, the biologically active targeting ligand biotin was used. While under the "standard" conditions only the mono-complex was formed, the bis-complex could be obtained in good yields (60 %) by 4-fold higher concentrated solution and 50 °C reaction temperature. Under these new conditions the mono- and bis-complexes formed even with only one (10 % mono; 5 % bis) or two eq. of biotin (5 % mono; 15 % bis). Of note, when using 2.5 eq. biotin and 2.5 eq. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) as coupling agent exclusively the bis-complex was formed in 30 % yield. Consequently, when only limited amounts of the carboxylate ligand are available, this could be an interesting alternative strategy.

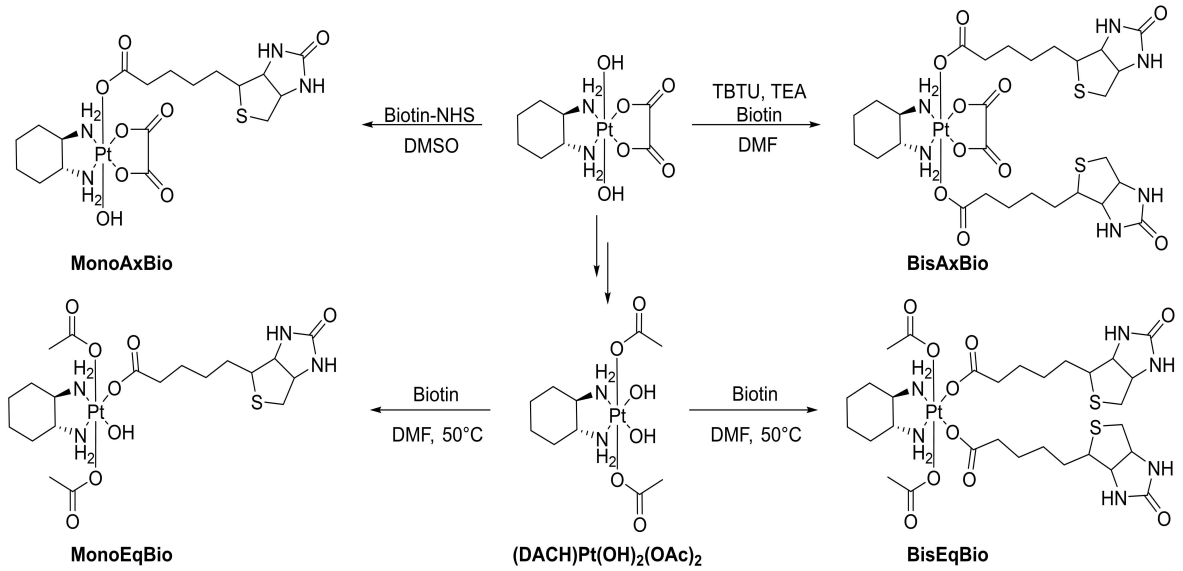
Table 1: Overview on the reaction conditions and yields of [(DACH)Pt$(OH)_2(OAc)_2$] and different ligands. 10 eq. of ligand were added in all cases.

Ligand	Temp	Solvent	[(DACH)Pt$(OH)_2(OAc)_2$] [mmol/l]	Yield[b]
Acetic acid	RT[a]	Acetone	7.2	40 % Mono + 20 % Bis
Acetic acid	RT	Water	7.2	no reaction
Acetic acid	RT	DMF	7.2	40 % Mono + 20 % Bis
Sodium oxalate	RT	DMF	7.2	10 % Mono + 20 % Bis
Oxalic acid	RT	DMF	7.2	10 % Mono + 30 % Bis
Biotin	RT	DMF	7.2	30 % Mono + 0 % Bis
Biotin	RT	DMF	27	20 % Mono + 20 % Bis
Biotin	50 °C	DMF	27	30 % Mono + 60 % Bis

[a] RT = room temperature; [b] yield determined via HPLC-MS after 24 h reaction time.

As proof of principle, we investigated if equatorial ligand exchange is also possible for a cisplatin core, which is generally much more resistant against equatorial hydrolysis.[9] Therefore, we further increased the pH value and temperature, and incubated [$(NH_3)_2PtCl_2(OAc)_2$] in aqueous phosphate buffered solution at pH 10 at 50 °C, resulting in a mixture of 80 % [$(NH_3)_2Pt(OH_{eq})_2(OAc_{ax})_2$] and 20 % [$(NH_3)_2PtOH_{eq}Cl_{eq}(OAc_{ax})_2$] after 24 h. The obtained $(OH_{eq})_2$ species was then incubated with 10 eq. biotin at 50 °C for 24 h, resulting in exclusive formation of the biotin/OH complex (even after 48 h reaction time). Notably, when incubating the equatorially mixed OH/Cl species [$(NH_3)_2PtOH_{eq}Cl_{eq}(OAc_{ax})_2$], the OH was exchanged at the same rate as for the $(OH_{eq})_2$ compound, resulting in the biotin/Cl complex. Consequently, also cisplatin(IV) species can be used for equatorial ligand substitution reactions.

However, our focus was to compare the properties of equatorially substituted complexes with the respective axial counterparts. Therefore, the following oxaliplatin(IV) panel was synthesized (Scheme 2): the equatorial mono-biotin (**MonoEqBio**) and bis-biotin complex (**BisEqBio**), and as axial references the oxaliplatin(IV) complexes with one (**MonoAxBio**) or two biotin ligands (**BisAxBio**). For



Scheme 2. Structures of synthesized biotin-platinum(IV) complexes.

purification of **MonoEqBio** on the preparative HPLC, pure solvents had to be used, as the usually added 0.1 % formic acid or trifluoroacetic acid (TFA) could replace the OH at the platinum core. The purity of all complexes was confirmed via NMR, MS and elemental analysis. Efforts to synthesize the respective biotin-platinum(II) reference complexes were not successful. Most probably, the soft platinum(II) readily coordinates the thioether of biotin resulting in various different species.

In order to compare the properties of the different oxaliplatin(IV)-like biotin complexes (Scheme 2), first the hydrolytic stability of the equatorial ligands was evaluated. The complexes (1 mM) were incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C (Figure 1A). While **MonoAxBio** was quite stable against equatorial oxalate hydrolysis (8 % mono-OH species after 24 h), **BisAxBio** showed substantial parts of hydrolyzed species after 24 h of incubation (24 % mono-hydroxido; 4 % di-hydroxido). The high stability of the **MonoAxBio** derivative compared to the **BisAxBio** complex could be confirmed by the reference complexes [(DACH)PtOxOH$_{ax}$OAc$_{ax}$] and [(DACH)PtOx-(OAc)$_2$], showing 2 % and ≈50 % equatorial hydrolysis at the platinum(IV) center after 24 h, respectively (Figure S3). This trend was reversed for the complexes with equatorially bound biotin: **BisEqBio** showed only minimal hydrolysis (4 % after 24 h), while 22 % of **MonoEqBio** were hydrolyzed to the di-hydroxido species after 24 h, which also allowed the detection of released biotin (Figure S4). This data shows that regarding equatorial hydrolysis of platinum(IV) complexes the two directly adjacent carboxylic acid groups in

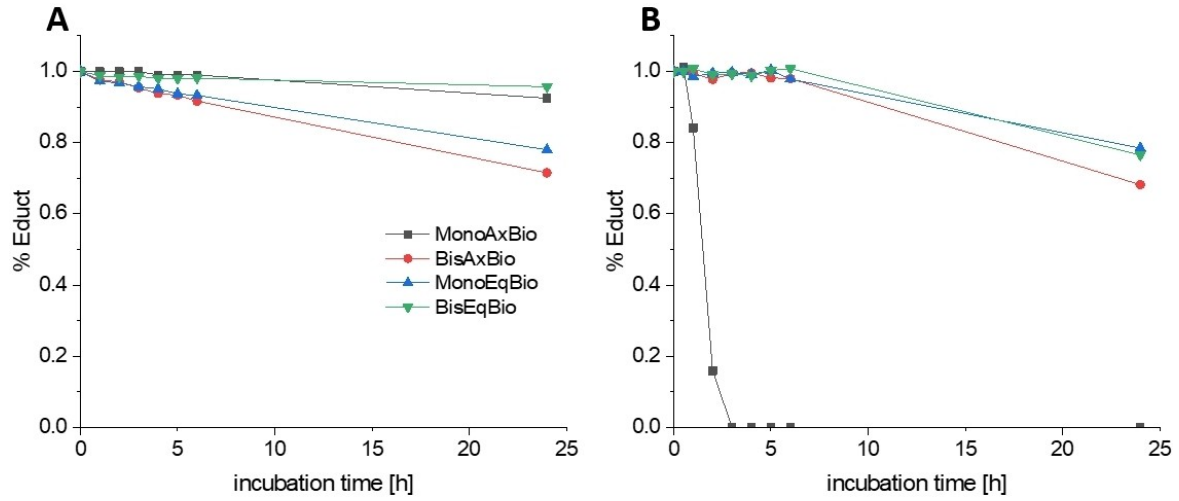


Figure 1. A) Equatorial hydrolysis of the investigated platinum complexes at 37 °C (1 mM complex in 100 mM phosphate buffer pH 7.4). B) Reduction kinetics of the investigated platinum complexes at 20 °C (1 mM complex with 10 eq. of ascorbic acid in 150 mM phosphate buffer pH 7.4).

the oxalate ligand of **BisAxBio** have a distinctly lower coordination strength, when compared to the two monodentate carboxylate ligands in **BisEqBio**. In line, the carboplatin analogue [(DACH)Pt(CBDCA)$(OAc)_2$] (CBDCA = 1,1-cyclobutanedicarboxylic acid) with one carbon atom between the bidentate carboxylates was very stable (2 % hydrolysis after 24 h; data not shown) indicating that **BisEqBio** behaves more like a carboplatin than an oxaliplatin derivative.

The reduction kinetics of the complexes in the presence of 10 eq. ascorbic acid at 20 °C (Figure 1B) revealed that **MonoAxBio** was completely reduced after 3 h, in accordance to literature on other platinum(IV) complexes with one axial OH group.[17] The other three complexes were much more stable, with **MonoEqBio** and **BisEqBio** at ≈80 % intact after 24 h and **BisAxBio** at ≈70 % (again, for the axial biotin complexes **MonoAxBio** and **BisAxBio** the release of biotin and oxaliplatin could be confirmed; Figure S5). Interestingly, the free OH moiety in equatorial position (**MonoEqBio**) did not accelerate the reduction, as was observed for the axial analogue **MonoAxBio**.

To test the impact of the equatorial/axial ligand coordination on the cellular drug uptake, two cancer cell models with different SMVT expression, indicative for their biotin uptake ability, were employed (human colorectal cancer HCT116 and human breast cancer MCF-7 cells).[18] The superior and active biotin uptake of MCF-7 cells (compared to HCT116 cells) was first confirmed by flow cytometry after incubation with FITC-labeled biotin (Figure 2A). Subsequently, inductively coupled plasma mass spectrometry (ICP-MS) was used to detect the intracellular platinum levels after treatment with the new biotin-targeted platinum(IV) derivatives. [(DACH)PtOx$(OAc)_2$] was used as a reference. As shown in Figure 2B, especially in case of the two bis-biotin drugs, the SMVT-high MCF-7 cells had significantly higher platinum uptake than SMVT-low HCT116 cells. A similar pattern was observed for the monobiotin drugs (although it did not reach statistical significance). In contrast, the drug uptake of [(DACH)PtOx$(OAc)_2$] was not influenced by the SMVT expression levels.

To test the anticancer activity of the complexes, we first compared the impact of free oxaliplatin and [(DACH)PtOx$(OAc)_2$] in MCF-7 and HCT116 cells after 72 h via MTT-based assays (Figure S6). Noteworthy, oxaliplatin was distinctly more active against HCT116 (IC_{50} of 1.88 μM) than MCF-7 cells ($IC_{50} > 10$ μM). This is in good agreement with oxaliplatin being the standard therapy against advanced colorectal cancer.[19] Also in case of [(DACH)PtOx$(OAc)_2$], the anticancer activity was more pronounced against HCT116 than MCF-7 cells (16.8 μM vs 34.4 μM, respectively), but in general distinctly less active due to the prodrug nature of the platinum(IV) complex.[20] The four biotin-conjugated platinum(IV) compounds (Scheme 2) revealed strong differences under the same conditions (Figure 2C). The two equatorially biotin-conjugated substances showed no activity up to the highest concentration of 100 μM, **BisAxBio** was slightly cytotoxic and **MonoAxBio** was the most active derivative. Overall, the general pattern and activity of the tested drugs was similar in both cell models (especially interesting considering the reduced sensitivity of MCF-7 to oxaliplatin) indicating that the differences in anticancer activity cannot originate from the different biotin uptake efficiency. The higher activity of **MonoAxBio** can be explained by the very fast reduction compared to the other derivatives (Figure 1B). Accordingly, viability experiments with the reference complex [(DACH)PtOxOHOAc] revealed similar anticancer activity (Figure 2D). However, that **MonoEqBio** and **BisEqBio**, bearing monodentate biotin ligand(s), were even less active than **BisAxBio** with a bidentate oxalate ligand was unexpected. As the reduction properties of these three complexes are quite similar (Figure 1B), we hypothesized that the underlying reason has to be the hydrolysis rate of the formed platinum(II) complexes. Indeed, subsequently performed viability experiments with the carboplatin reference [(DACH)Pt(CBDCA)$(OAc)_2$] showed activity comparable to the equatorial-biotin complexes (Figure 2D). This suggests, that the specific reduction and hydrolysis characteristics of the individual derivative is in fact crucial for their anticancer activities.

Noteworthy, prolonged drug incubation (10 days clonogenicity tests) not only enhanced the general activities of the drugs (due to longer time for prodrug and platinum(II) activation), but also inverted the response pattern of the equatorial drugs (Figure S7). Thus, the drugs with equatorial biotin were now more active against the oxaliplatin-resistant SMVT-high MCF-7 than HCT116 cells. In contrast, the axial-conjugated biotin drugs showed an activity pattern comparable to the 72 h experiments. This indicates that the exact position of the ligands can distinctly influence the activity as well as the (platinum) resistance pattern of the complexes.

Taken together the data clearly show that **MonoEqBio** and **BisEqBio** are highly stable platinum(IV) complexes, which after reduction generate platinum(II) derivatives with slow ligand release more comparable to carboplatin than oxaliplatin. Consequently, we hypothesized that these properties of equatorial monodentate carboxylate ligands are ideal for the use of long-circulating drug delivery systems, where slow release/activation is desired. Miriplatin emulsions[21] (approved in Japan against hepatocellular carcinoma) or liposomal aroplatin[22] (studied up to clinical phase II) are representatives of such type of platinum(II) complexes with two monodentate carboxylate ligands. One elegant drug delivery strategy is the use of albumin as a drug carrier via maleimide chemistry. We recently developed platinum(IV) complexes with axial maleimide ligand(s).[20b,23] However, introduction of the albumin-targeting maleimide at the equatorial position(s) would allow the attachment of two (different) synergistic drugs at the free axial positions, generating targeted triple-action prodrugs within one platinum complex.

We therefore synthesized two different equatorial maleimide-platinum(IV) complexes (Figure 3) following the procedure described above. **BisEqMalEs** was synthesized by incubation of 6-maleimidehexanoic acid and [(DACH)Pt$(OH)_2(OAc)_2$], whereas for the carbamate analogue (**BisEqMalCa**) the maleimide isocyanate was used. Of note, such

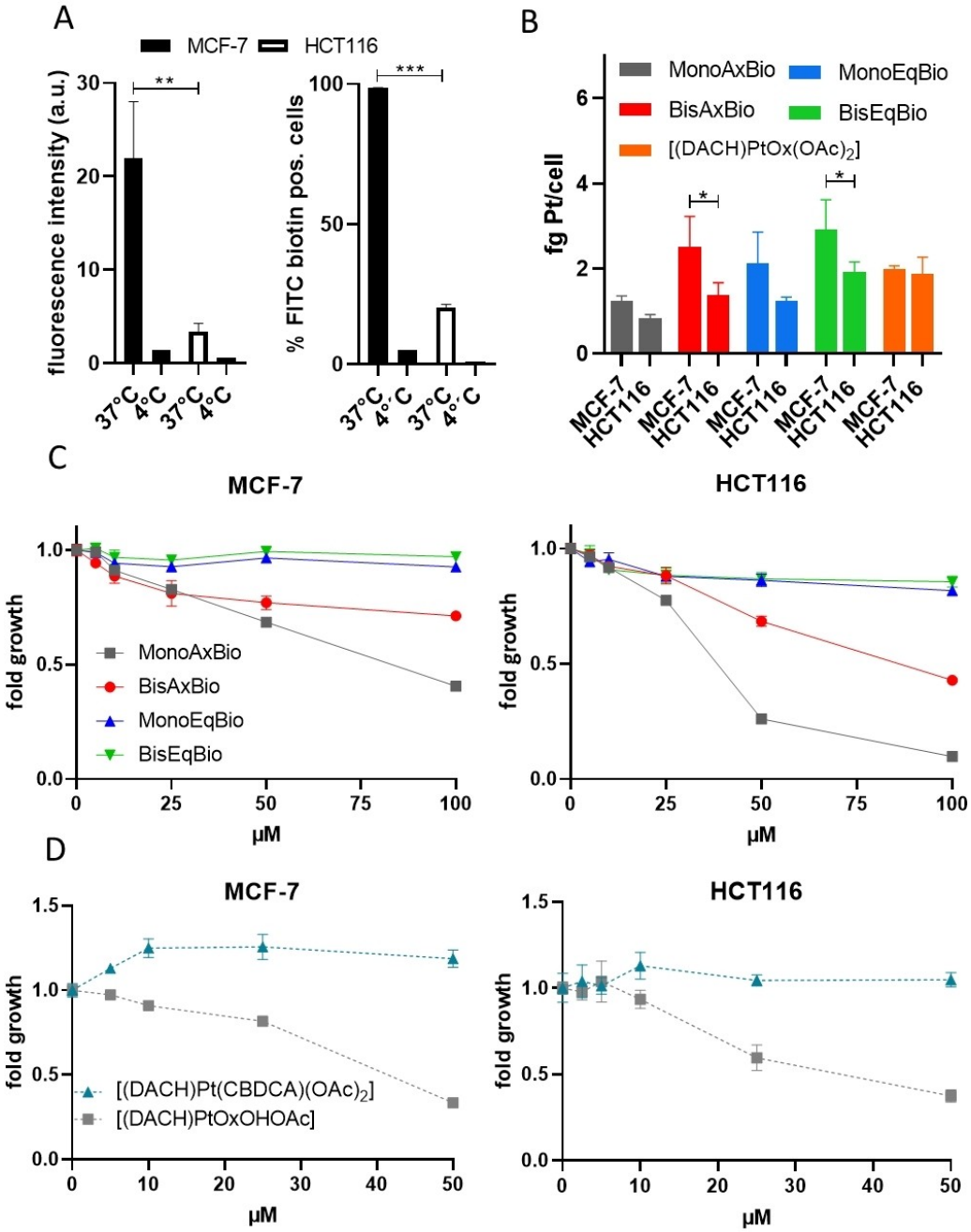


Figure 2. Characterization of the cellular biotin and drug uptake and anticancer activity of the platinum(IV) complexes in MCF-7 (SMVT high) and HCT116 cells (SMVT low). A) Cells were incubated with FITC-labeled biotin (20 μM) at 37 °C or 4 °C for 5 h. Mean FITC fluorescence intensity (left) FITC-positive cells (right) were determined by flow cytometry (ex/em: 488/530 nm, fluorescence intensity was normalized to auto fluorescence control). B) Cellular uptake of indicated drugs (10 μM) was determined by ICP-MS after 3 h incubation. Values in (A) and (B) are presented as means ± SD of three independent experiments. Statistical significance was tested by one-way ANOVA and Dunnett's multiple comparison test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). (C) Anticancer activity of biotin-containing platinum compounds and platinum reference compounds after 72 h was determined via MTT-based assays. In case of (C) and (D), values refer to means ± SD of one representative experiment performed in triplicates.

maleimide-bearing complexes could not be synthesized from the respective platinum(II) precursors by conventional synthetic methods, as already the oxidation with H_2O_2 leads to maleimide hydrolysis. To the best of our knowledge **BisEqMalCa** is also the first platinum(IV) complex with equatorial carbamate leaving groups. Incubation of the complexes at pH 7.4 revealed high stability (Figure S8), except for the well-known maleimide hydrolysis at physiological pH (Figure S9).[24] This phenomenon also hampered a quantitative evaluation of the reduction rate as both processes are overlaying; nevertheless similar reduction rates could be observed for **BisEqMalEs** and **BisEqMalCa**

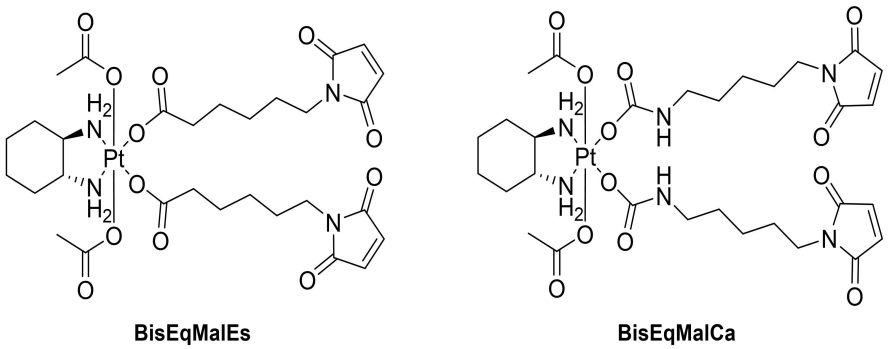


Figure 3. Structures of synthesized maleimide-bearing platinum(IV) complexes.

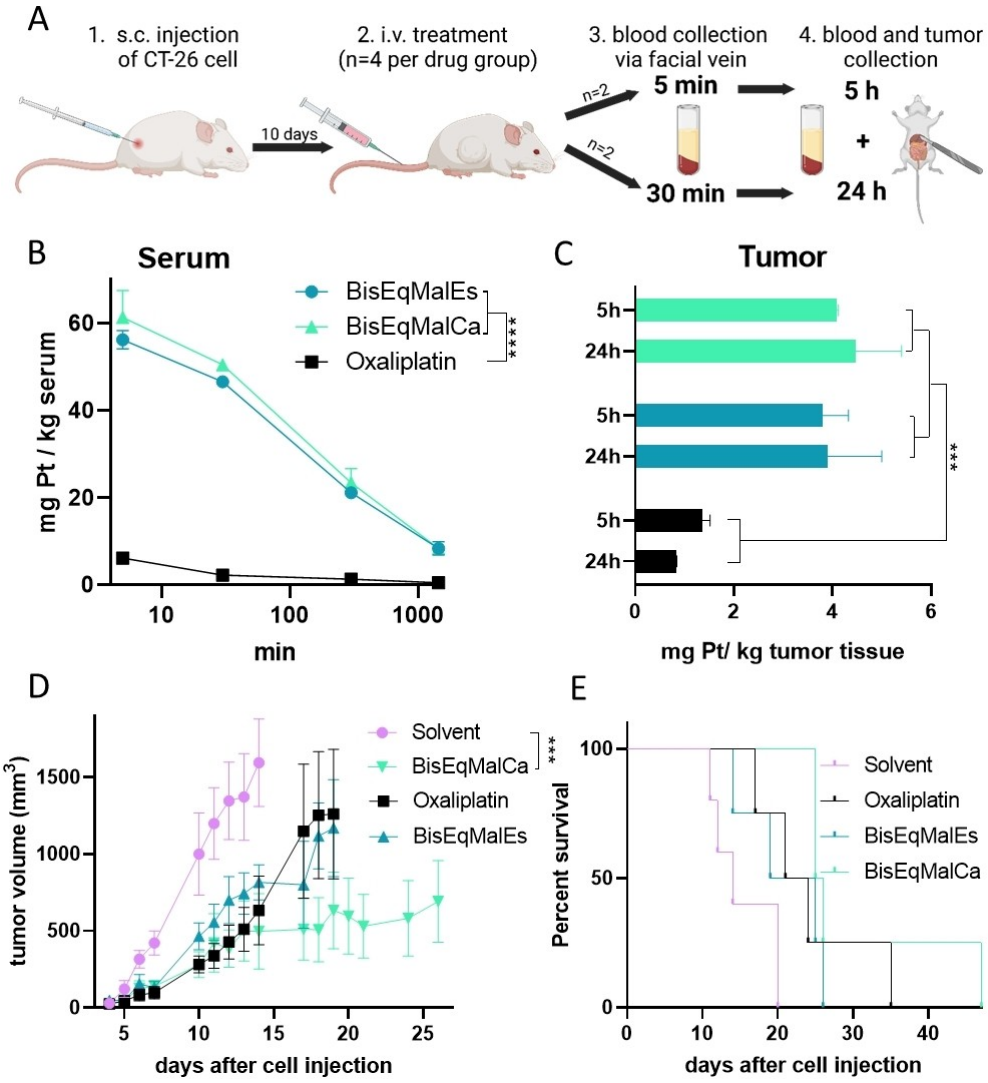


Figure 4. Pharmacological evaluation and anticancer activity of the platinum complexes in CT-26-bearing Balb/c mice. For the pharmacological studies, animals were treated once i.v. with concentrations equimolar to 9 mg/kg oxaliplatin. A) treatment and sample collection scheme for pharmacological studies. B) serum samples were collected after 5 min, 30 min, 300 min and 1440 min; C) tumor samples were collected after 5 h and 24 h. Platinum levels of all samples were measured via ICP-MS. In case of the evaluation for the anticancer activity, CT-26-bearing Balb/c mice were treated twice a week for two weeks i.v. with concentrations equimolar to 9 mg/kg oxaliplatin. (D) Impact on tumor growth; data are presented as means ± SEM. (E) The overall survival is depicted via a Kaplan–Meier curve. Statistical significance for (B) and (D) was tested by two-way ANOVA (*** $p < 0.001$). The oxaliplatin data for the pharmacological analysis were used from Schueffl et al.[20b]

(Figure S10). In addition, albumin-binding studies via size exclusion chromatography (SEC) coupled to ICP-MS, showed fast binding rates for both complexes in fetal calf serum (FCS, buffered with 150 mM phosphate buffer to ensure a stable pH) at 37 °C (Figure S11).

Evaluation of maleimide complexes in cell culture is usually difficult, due to the reaction with the artificially high content of amino acids in the culture medium. Consequently, the two new albumin-binding compounds were directly tested for their pharmacokinetic properties in vivo using CT-26 colon cancer-bearing Balb/c mice (Figure 4A). Indeed, the maleimide-functionalization not only resulted in a distinctly prolonged plasma half-life time, but also in enhanced drug accumulation in the tumor tissue compared to free oxaliplatin (Figure 4B&C), in good agreement with axial maleimide-containing platinum(IV) derivatives.[20b,25] This also resulted in superior anticancer activity of **BisEqMalCa** compared to oxaliplatin (Figure 4D). Of note, this was not the case for **BisEqMalEs**. Interestingly, a distinctly higher activity of the "carbamate-linked" maleimide compared to the "ester-linked" analog was recently also observed for axial albumin-targeting platinum(IV) drugs,[25] although the underlying reasons are so far unknown.

Conclusion

Taken together, this work presents a new strategy for the synthesis of platinum(IV) complexes, with introduction of the equatorial ligands as the final step. This especially enables the insertion of sensitive ligands, which do not survive the platinum(II) oxidation process using H_2O_2. The equatorial biotin ligands of the newly synthesized complexes **MonoEqBio** and **BisEqBio** showed very slow release kinetics (comparable to carboplatin), ideal for long-circulating drug delivery strategies. Indeed, two complexes with equatorially inserted maleimide ligands (**BisEqMalEs** and **BisEqMalCa**) for endogenous albumin-binding revealed greatly improved pharmacokinetic properties and tumor accumulation in mice. **BisEqMalCa** also showed distinctly higher anticancer activity compared to the approved reference oxaliplatin. Shifting the drug-targeting moiety from the typical axial position into the equatorial plane generates two free axial positions for attachment of (different) synergistic drugs, which will enable the development of new types of triple-action platinum(IV) prodrugs.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Antitumor Agents · Bioinorganic Chemistry · Platinum · Synthesis Design

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4. Conclusion and Outlook

In conclusion, the equatorial hydroxido groups of an oxaliplatin(IV) complex could be reacted/substituted with new ligands forming an ester, a carbamate or an amine type connectivity. For targeting purposes an albumin-binding maleimide moiety was coupled in the equatorial position via ester or carbamate (**BisEqMalEs**, **BisEqMalCa**). Of note, **BisEqMalCa** is the first equatorial carbamate. The complexes showed no axial or equatorial ligand hydrolysis at the platinum core at physiological pH and revealed high binding affinity to albumin. In cooperation with the Center for Cancer Research of the Medical University of Vienna the pharmacokinetic and anticancer properties of these two targeted drugs were evaluated *in vivo*. Both drugs showed a substantially longer plasma half-life and enhanced drug accumulation in tumor tissue compared to the approved reference platinum(II) drug oxaliplatin. In addition, **BisEqMalCa** showed a significantly higher anticancer activity than oxaliplatin.

By moving the tumor-targeting maleimide ligand from the axial into the equatorial plane, one or even two additional free axial positions are created. In the next step they will be utilized for the attachment of bioactive ligands, allowing the development of the first targeted triple-action platinum(IV) complexes.

For the complexes with four equatorial am(m)ine ligands (**BisEqProAm** and **BisEqMalAm**), the stability of the “leaving group” will be of high interest. We expect an inert platinum core without hydrolysis of the “leaving” ligands even after reduction to the platinum(II) species, thus no cytotoxic activity from the platinum core. However, the reduction data already show that we still have an intermediate reduction rate perfectly suitable for tumor-specific release of axial ligands. Thus, it will be possible to use these complexes as transporters for bioactive axial ligands. With **BisEqMalAm,** even a target platinum complex transporter would become feasible.

5. Experimental Section

5.1. Materials and Instrumentation

Potassium tetrachloridoplatinate ($K_2[PtCl_4]$) was purchased from Johnson Matthey (Switzerland) and 6-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoic acid (**7**) from Alfa Aesar (USA). Water for synthesis was taken from a reverse osmosis system. For HPLC measurements Milli-Q water (18.2 MΩ·cm, Merck Milli-Q Advantage, Darmstadt, Germany) was used. Other chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Merck, and Fisher Scientific). Electrospray ionization (ESI) mass spectra were recorded on a Bruker amaZon SL ion trap mass spectrometer in positive and/or negative mode by direct infusion. High-resolution mass spectra were measured on a Bruker maXis™ UHR ESI time of flight mass spectrometer. One- and two-dimensional ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance III 500 or AV III 600 spectrometer at 298 K. For NMR spectra the solvent residual peak was taken as an internal reference. Elemental analysis measurements were performed on a Perkin Elmer 2400 CHN Elemental Analyzer at the Microanalytical Laboratory of the University of Vienna. Compounds were purified by preparative RP-HPLC using a Waters XBridge C18 column on an Agilent 1200 Series system. Milli-Q water and acetonitrile were used as eluents and a flow rate of 17 ml/min.

Hydrolytic Stability and Reduction Studies

1 mM platinum compound was incubated in phosphate buffer (150 mM, pH 7.4). For both studies, the samples were incubated at 20°C for 24 h. For the reduction kinetics 10 eq. L-ascorbic acid was added. The reaction was monitored on a Thermo Scientific Dionex UltiMate 3000 UHPLC-system using a Waters Acquity UPLC BEH C18 1.7 µm 3.0x50 mm column. Milli-Q water and acetonitrile with or without TFA were used as eluents. A gradient of 5-95% over 6.5 minutes was used.

SEC-ICP-MS Measurements

Fetal calf serum (FCS) was purchased from Sigma-Aldrich and buffered with 150 mM phosphate pH 7.4 in order to guarantee a stable pH over the duration of the experiment. The platinum(IV) complexes (5 mM) were dissolved in 150 mM phosphate buffer (pH 7.4)

and diluted 1:50 in the buffered serum to obtain a final concentration of 100 µM. The samples were then incubated in the autosampler at 37°C and analyzed every 1 h for 5 h as well as after 24 h. Between each sample a pure water blank was measured. For SEC-ICP-MS measurements an Agilent 1260 Infinity system coupled to an Agilent 7800 ICP-MS equipped with a dynamic reaction cell was used. Oxygen (purity 5.5, Messer Austria GmbH, Gumpoldskirchen, Austria) was used as reaction gas.

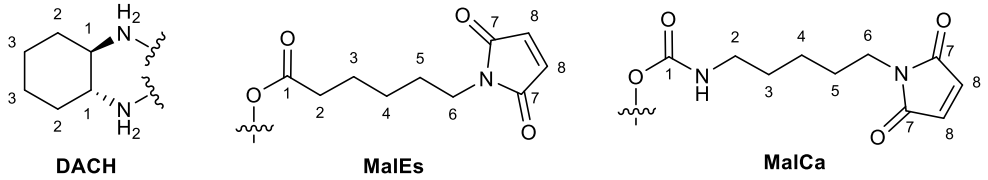
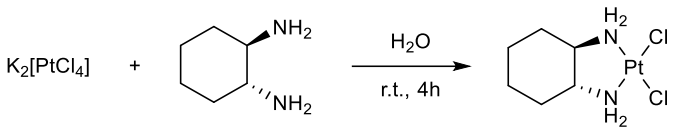


Figure 23: NMR numbering for the ligands **DACH**, **MalEs** and **MalCa**.

5.2. Synthesis

(SP-4-2)-Dichlorido[(1R,2R)-cyclohexane-1,2-diamine]platinum(II) (2)



K_2PtCl_4 (**1**, 3.00 g, 7.23 mmol) was dissolved in 30 mL Milli-Q water and (R,R)-1,2-diamino cyclohexane (0.835 mg, 7.23 mmol, 1 eq.) was added. The reaction was stirred at room temperature and light exclusion for 4 h. The resulting precipitate was filtered off and washed three times with 3 mL of water. The product was dried over phosphorus pentoxide. The filtrate was stirred under the same conditions overnight. The precipitate was filtered off, washed, and dried separately over phosphorus pentoxide. Yield: 1. fraction 2.29 g (83%) as a yellow powder; 2. fraction 0.369 g (13%) yellow solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 5.58-5.57 (d, *J* = 8.08 Hz, 2H, N*H*$_2$), 5.05 (m, 2H, N*H*$_2$), 2.08 (b, 2H, DACH-1), 1.85-1.83 (d, *J* = 12.48 Hz, 2H, DACH-2), 1.44-1.43 (b, 2H, DACH-3), 1.22-1.20 (b, 2H, DACH-2), 0.97-0.93 (m, 2H, DACH-3) ppm.

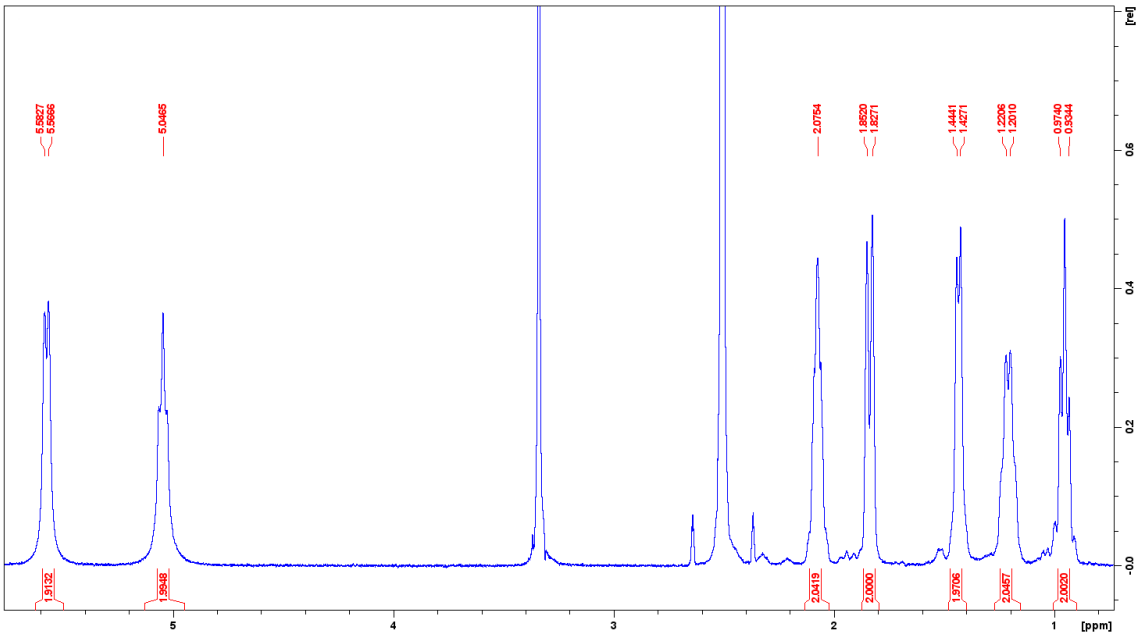
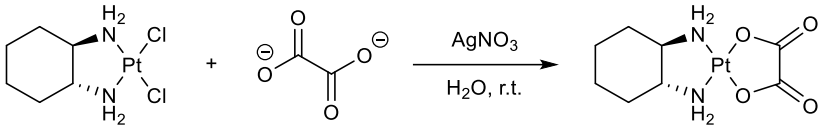


Figure 24: ^{1}H-NMR of **2**.

(SP-4-2)-[(1R,2R)-Cyclohexane-1,2-diamine]oxalatoplatinum(II) (3)



(SP-4-2)-Dichlorido[(1R,2R)-cyclohexane-1,2-diamine]platinum(II) (**2**, 2.66 g, 7.00 mmol) was suspended in 35 mL Milli-Q water and $AgNO_3$ (2.31, 13.6 mmol, 1.95 eq.) was added. The reaction was stirred at room temperature under light exclusion for 6 h. The white precipitate was filtered off and to the filtrate potassium oxalate monohydrate (1.25 g, 6.81 mmol, 0.973 eq) was added. The mixer was stirred at room temperature under light exclusion for 18 h. The reaction was placed in the fridge for 3 h and the resulting precipitate was filtered off. The product was dried over phosphorus pentoxide. Yield: 2.11 g (76%) white solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 6.12-6.11 (d, *J* = 8.47 Hz, 2H, N*H*$_2$), 5.39-5.36 (m, 2H, N*H*$_2$), 2.03-2.00 (b, 2H, DACH-1), 1.84-1.82 (d, *J* = 12.42 Hz, 2H, DACH-2), 1.47-1.45 (b, 2H, DACH-3), 1.22-1.20 (b, 2H, DACH-2), 1.04-1.00 (m, 2H, DACH-3) ppm.

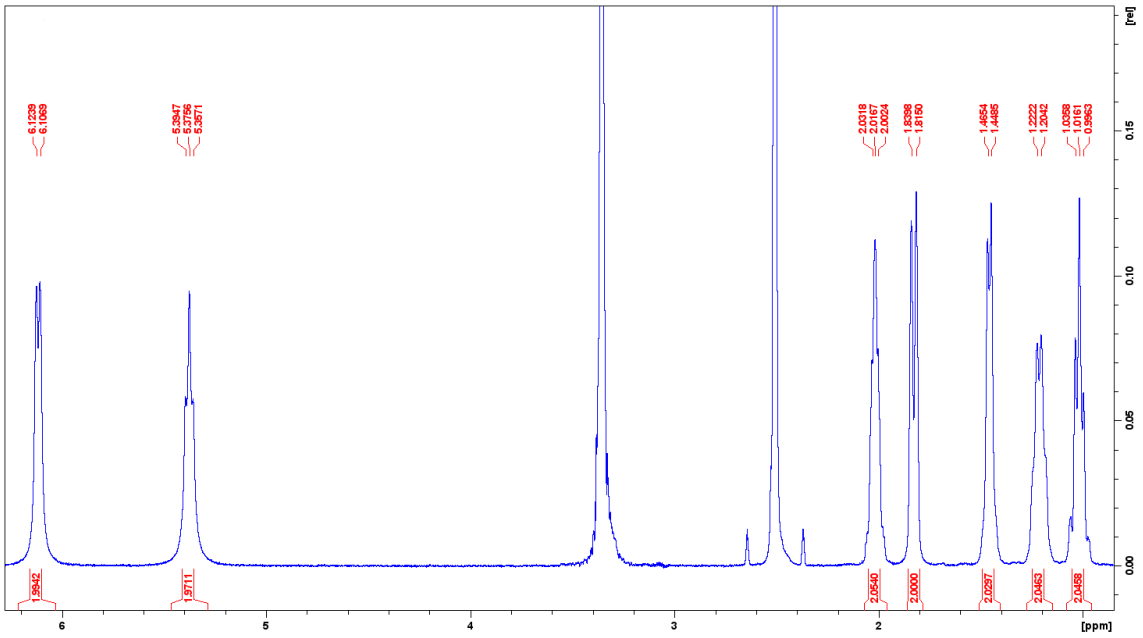
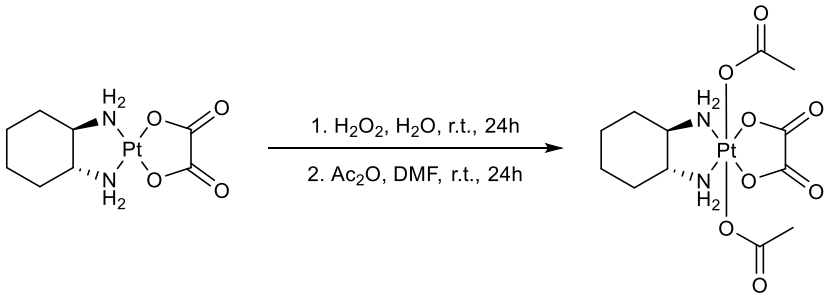


Figure 25: ^{1}H-NMR of **3**.

(OC-6-33)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]oxalatoplatinum(IV) (5)



(SP-4-2)-[(1R,2R)-Cyclohexane-1,2-diamine]oxalatoplatinum(II) (**3**, 2.11 g, 5.32 mmol) was suspended in 35 mL Milli-Q water and 3.5 mL hydrogen peroxide (50%) was added. The mixer was stirred at room temperature under light exclusion for 24 h. The solvent was removed and the residue was dissolved in methanol. The intermediate product (OC-6-33)-[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidooxalatoplatinum(IV) was precipitated with diethyl ether, filtered off, washed three times with 5 mL of diethyl ether, and dried in vacuo. Yield: 1.96 g (86%) white solid.

(OC-6-33)-[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidooxalatoplatinum(IV) (**4**, 1.96 g, 4.54 mmol) was dissolved in 14 mL of dimethylformamide and 14 mL acetic anhydride was added. The mixer was stirred at room temperature under light exclusion for 24 h. The solvent was removed and the residue was dissolved in methanol. The product was precipitated with diethyl ether, filtered off, washed three times with 5 mL of diethyl ether, and dried in vacuo. Yield: 1.70 g (73%) white solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 8.38-8.29 (b, 4H, NH_2), 2.56 (b, 2H, DACH-1), 2.12-2.10 (m, 2H, DACH-2), 1.96 (s, 6H, Acetate), 1.51-1.50 (b, 2H, DACH-3), 1.43-1.41 (b, 2H, DACH-2), 1.18-1.14 (m, 2H, DACH-3) ppm.

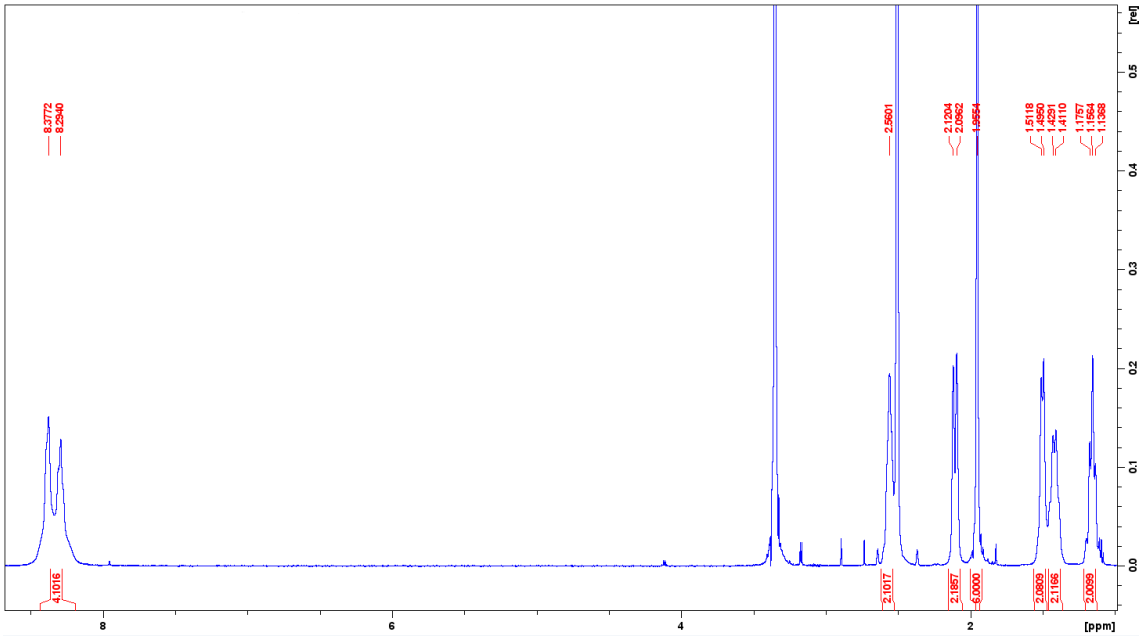
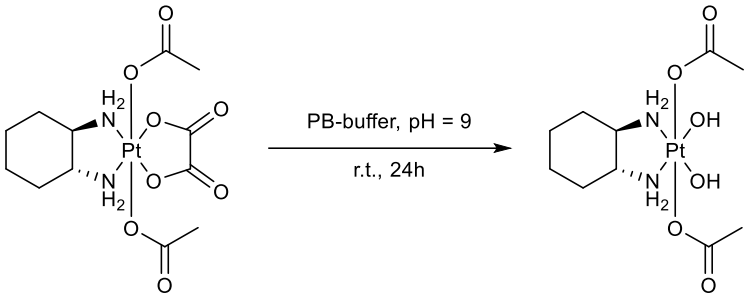


Figure 26: ^{1}H-NMR of **5.**

(OC-6-13)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidoplatinum(IV) (6)



(OC-6-33)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]oxalatoplatinum(IV) (**5**, 250 mg, 0,097 mmol) was suspended in 100 ml phosphate buffer (100 mM, pH 9) and stirred at 50°C for 24 h. Afterward, the solution was lyophilized. The crude product was dissolved in 20 mL H_2O and purified by preparative RP-HPLC (Atlantis T3). 5 mL of product solution was purified per run using H_2O/ACN and a gradient of 1–15% ACN in 15 min at a flow rate of 17 mL/min. The product eluted after approximately 11 minutes. All product fractions were combined and lyophilized. Yield: 111 mg (50%) yellow solid.

^{1}H-NMR (500 MHz, D_2O): δ = 2.62-2.61 (b, 2H, DACH-1), 2.10-2.08 (m, 2H, DACH-2), 1.96 (s, 6H, Acetate), 1.52-1.51 (b, 2H, DACH-3), 1.35-1.34 (b, 2H, DACH-2), 1.12-1.04 (m, 2H, DACH-3) ppm.

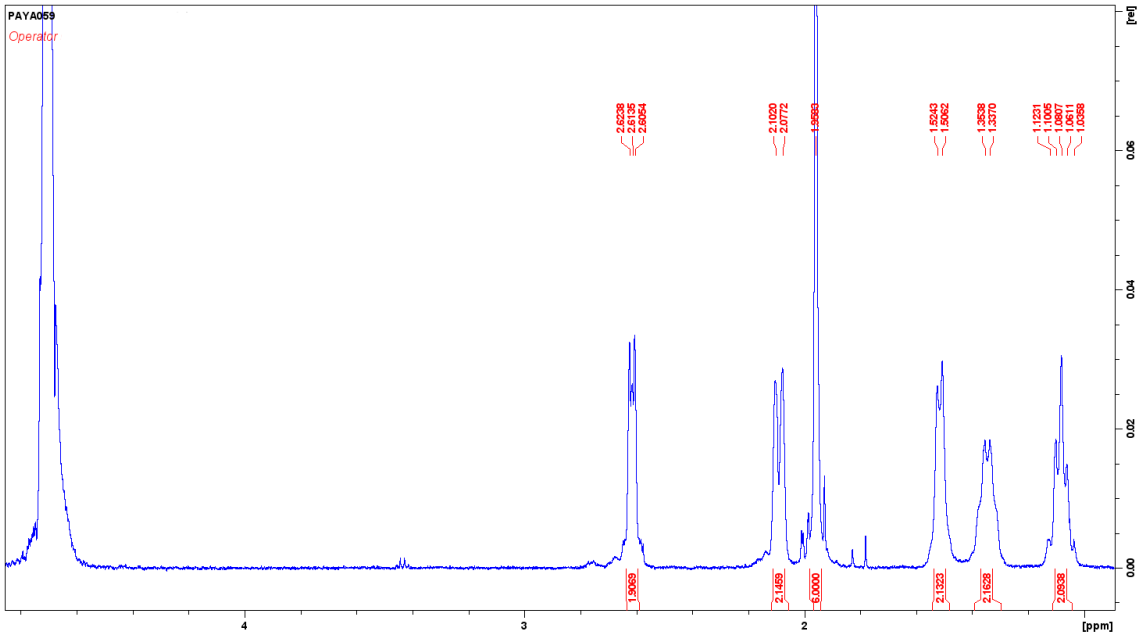
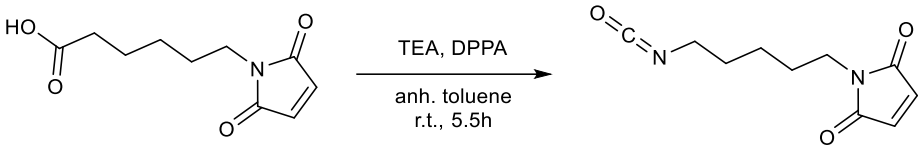


Figure 27: ^{1}H-NMR of **6**.

1-(5-Isocyanatopentyl)-1H-pyrrole-2,5-dione (9)



6-(2,5-Dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoic acid (**7**, 500 mg, 2.37 mmol) was dissolved in 14 mL anhydrous toluene and triethylamine (396 µL, 2.84 mmol, 1.20 eq) was added. To the mixer diphenyl phosphorazidate (DPPA) (509 µL, 2.37 mmol, 1.00 eq) was added drop-wise, and the reaction was stirred at room temperature under an inert atmosphere for 6 h. To quench the reaction 14 mL saturated $NaHCO_3$ solution was added. The organic phase was washed three times with 20 mL brine and dried over Na_2SO_4. The mixer was refluxed under an inert atmosphere for 17 h. The solvent was removed in vacuo. Yield: 450 mg (91%) yellow oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 6.63 (s, 2H, C*H*$_{Maleimide}$), 3.48-3.45 (t, 2H, C*H*$_2$, *J* = 7.16 Hz), 3.25-3.22 (t, 2H, C*H*$_2$, *J* = 6.63), 1.60-1.53 (m, 4H, C*H*$_2$), 1.34-1.31 (m, 2H, C*H*$_2$) ppm.

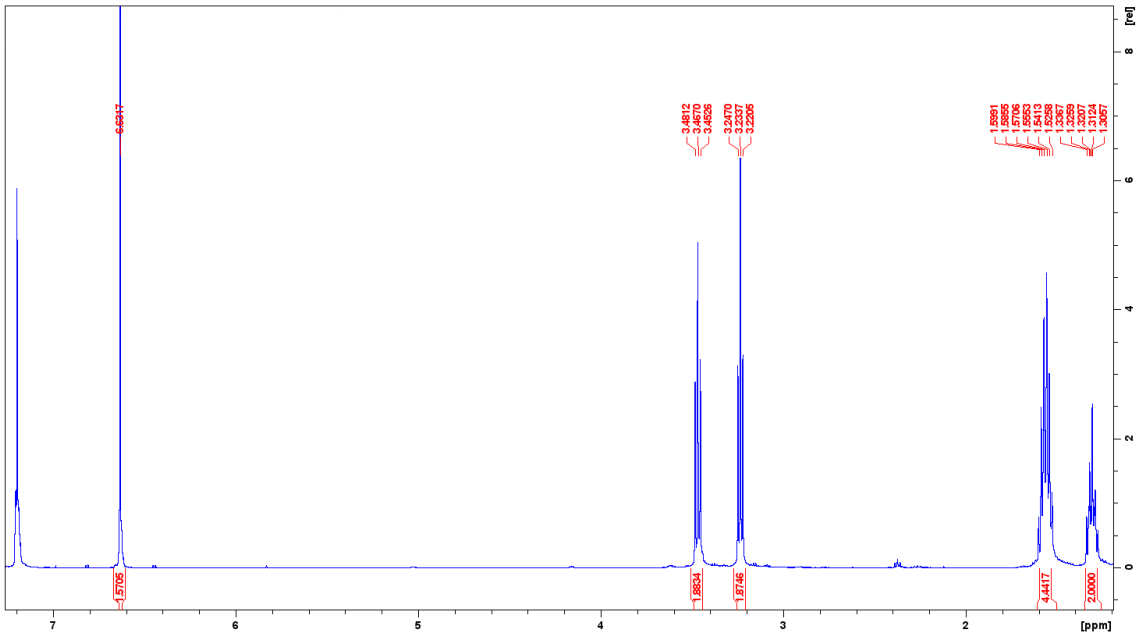
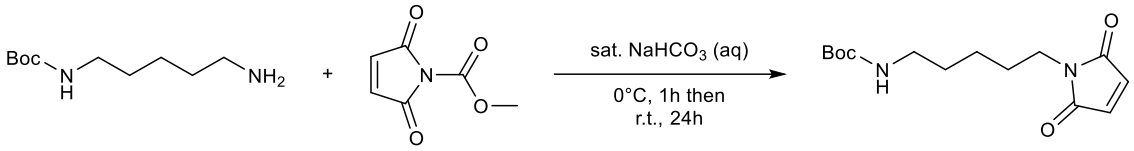


Figure 28: ^{1}H-NMR of **9**.

tert-Butyl (5-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)pentyl)carbamate (11)



tert-Butyl (5-aminopentyl)carbamate (**10**, 500 mg, 2.47 mmol) was dissolved in 18 mL saturated $NaHCO_3$, cooled to 0°C and methyl 2,5-dioxo-2,5-dihydro-1H-pyrrole-1-carboxylate (651 mg, 4.20 mmol, 1.70 eq) was added. The reaction was stirred at 0°C for 1 h, then warmed up to room temperature and stirred for another 24 h. Afterwards, 20 mL of water was added and the product was extracted three times with 40 mL ethyl acetate. The organic phases were combined, washed with brine, dried over $MgSO_4$ and the solvent was removed in vacuo. Yield: 554 mg (79%) yellow oil.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.01 (s, 2H, C*H*$_{Maleimide}$), 3.39-3.37 (m, 2H, C*H*$_2$), 2.89-2.85 (dd, 2H, C*H*$_2$, *J* = 6.72), 1.50-1.44 (m, 2H, C*H*$_2$), 1.37 (s, 9H, C*H*$_3$), 1.34-1.33 (m, 2H, C*H*$_2$), 1.20-1.18 (m, 2H, C*H*$_2$) ppm.

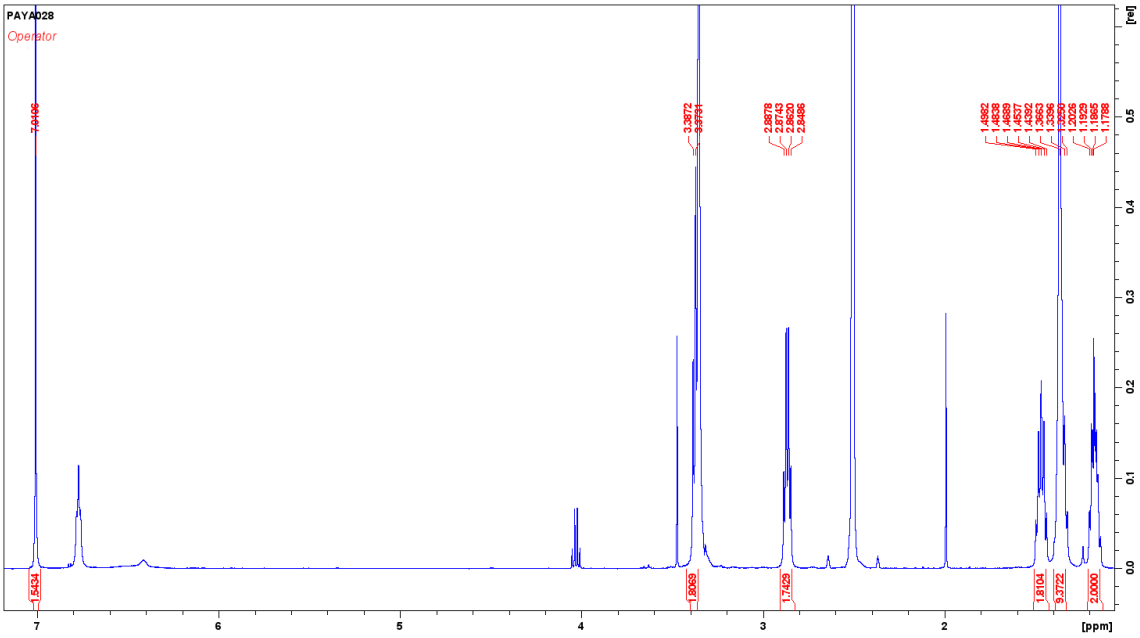
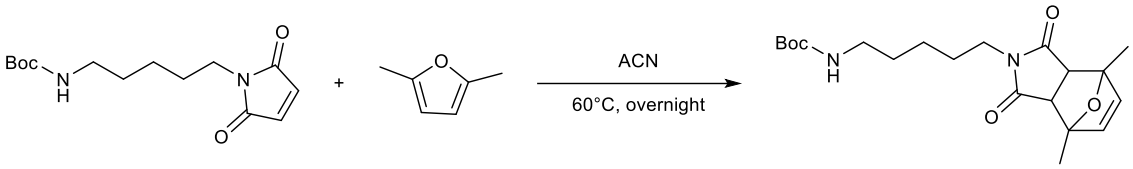


Figure 29: ^{1}H-NMR of **11**.

tert-Butyl (5-(4,7-dimethyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-epoxy-isoindol-2-yl)pentyl)carbamate (12)



tert-Butyl (5-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)pentyl)carbamate (**11**, 250 mg, 0.885 mmol) was dissolved in 10 mL acetonitrile and 2,5-dimethylfuran (1.70 g, 17.7 mmol, 20.0 eq.) was added. The reaction was heated to 60°C and stirred overnight. The solvent was removed in vacuo. The crude product was purified by flash column chromatography (SiO_2, DCM:EtOAc 90:10). Yield: 285 mg (85%) yellow oil.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 6.37 (s, 1.75H, C$H_{\text{double bond}}$), 6.26 (s, 0.25H, C$H_{\text{double bond}}$), 3.33-3.31 (m, 2H, CH_2), 2.86-2.83 (m, 2H, CH_2), 1.63 (s, 2H, CH), 1.53 (s, 6H, CH_3), 1.45-1.40 (m, 2H, CH_2), 1.37 (s, 9H, CH_3), 1.35-1.33 (m, 2H, CH_2), 1.19-1.15 (m, 2H, CH_2) ppm.

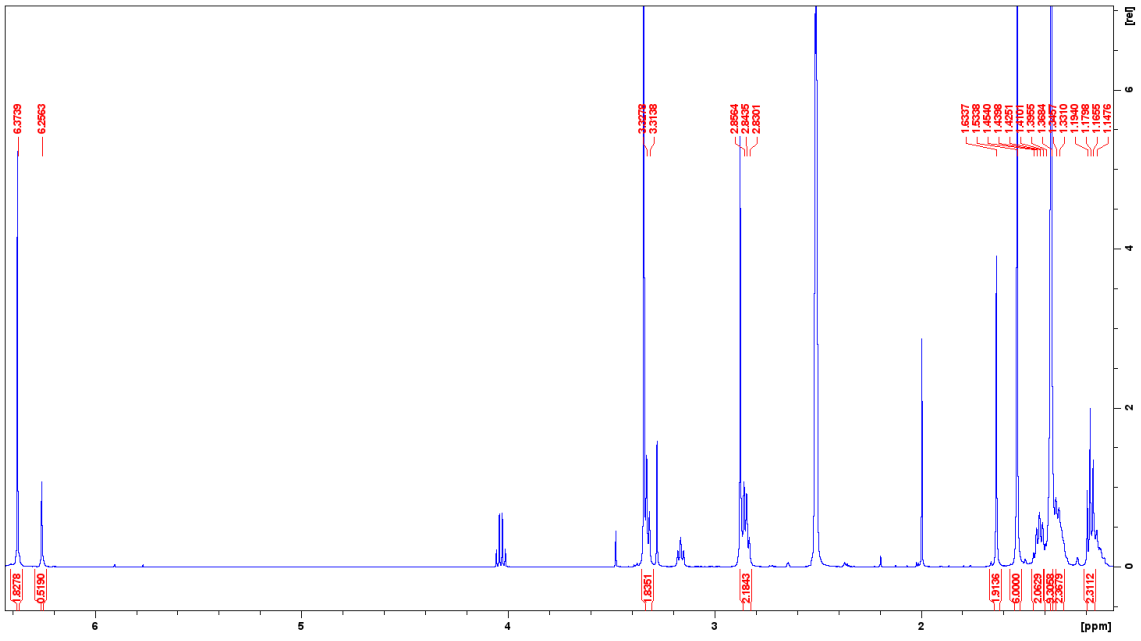
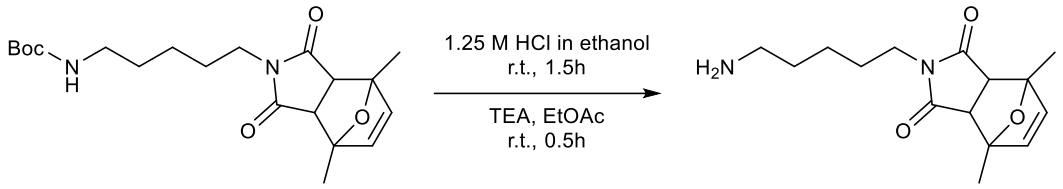


Figure 30: ^{1}H-NMR of **12**.

2-(5-Aminopentyl)-4,7-dimethyl-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (13)



tert-Butyl (5-(4,7-dimethyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-epoxyisoindol-2-yl)pentyl)carbamate (**12**, 260 mg, 0.687 mmol) was dissolved in 5 mL 1,25 M HCl in ethanol and stirred at room temperature for 1.5 h. The solvent was removed in vacuo. The residue was taken up in methanol, dried over $MgSO_4$ and the solvent was again removed in vacuo yielding the chloride salt. The intermediate was dissolved in EtOAc and 5 eq. of TEA was added. The reaction was stirred for 30 minutes, the precipitate was filtered off and subsequently the solvent was removed in vacuo. Yield: 95 mg (50%) yellow oil.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 6.38 (s, 1.75H, C*H*$_{\text{double bond}}$), 6.27 (s, 0.25H, C*H*$_{\text{double bond}}$), 1.64 (s, 2H, C*H*), 1.54 (s, 6H, CH_3), 1.47-1.43 (b. 4H, CH_2), 1.34 (b, 2H, CH_2), 1.24 (b, 2H, CH_2) ppm.

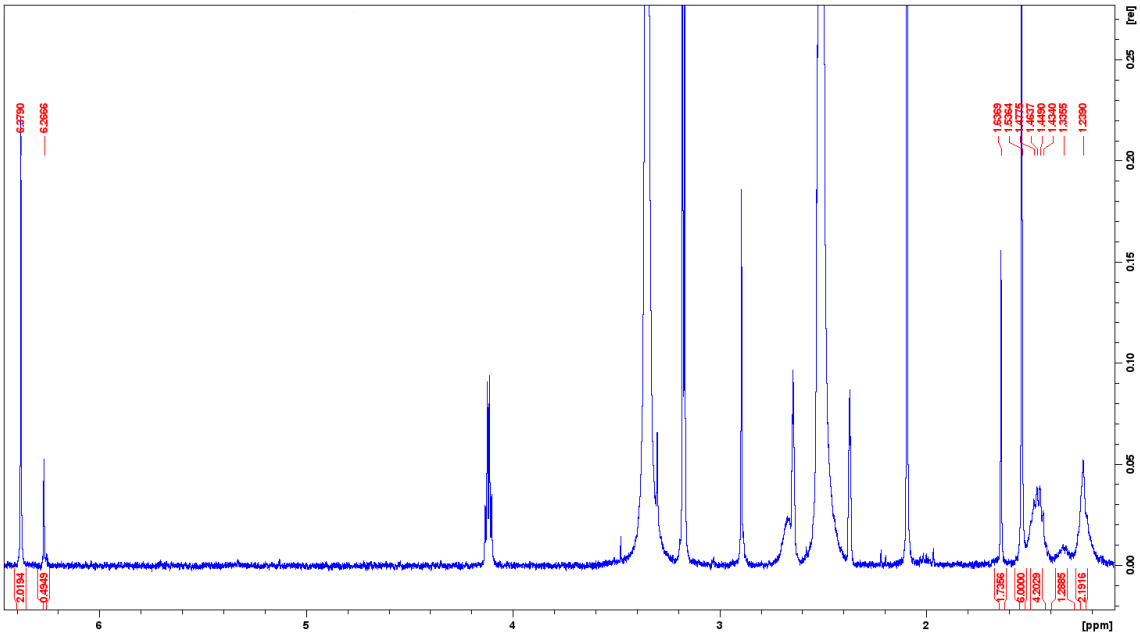
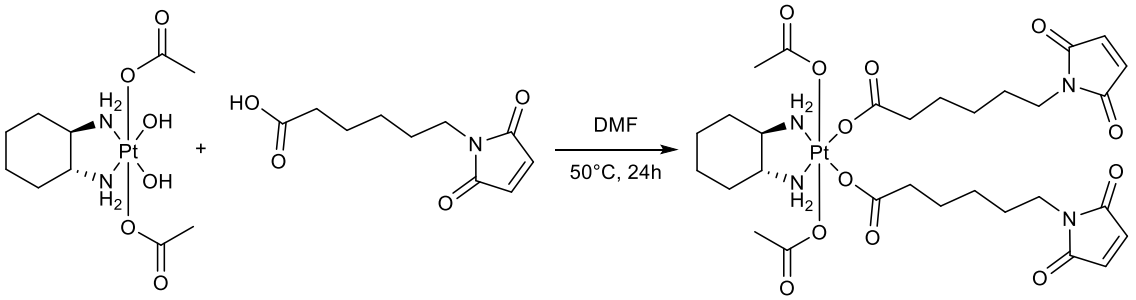


Figure 31: ^{1}H-NMR of **13**.

(OC-6-33)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]di[6-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoato]platinum(IV) (BisEqMalEs)



Purified (OC-6-13)-diacetato[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidoplatinum(IV) (**6**, 100 mg, 0.217 mmol) was suspended in 4 ml DMF and 6-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)hexanoic acid (**7**, 275 mg, 1.30 mmol; 6.00 eq.) was added. The reaction was stirred at 50°C for 24 h. The solvent was removed in vacuo. The crude product was dissolved in 40 mL H_2O/ACN (68:32) and purified by preparative RP-HPLC (XBridge Prep C18). 10 mL of product solution were purified per run using H_2O/CAN (+0.1% TFA) and an isocratic method with 32 % ACN over 15 min at a flow rate of 17 mL/min. The product eluted after approximately 10 min. All product fractions were combined and lyophilized. Due to impurities the product was purified a second time using acid free eluents (H_2O/ACN). Yield: 75 mg (41%) white solid.

^{1}H-NMR (700 MHz, DMSO-d_6): δ = 8.87 (b, 2H, NH_2), 8.15 (b, 2H, NH_2), 6.99 (s, 4H, MalEs-8), 3.36 (t, 4H, MalEs-6, *J* = 7.19 Hz), 2.58 (b, 2H, DACH-1), 2.23 (m, 6H, DACH-2,MalEs-2), 1.90 (s, 6H, Acetate), 1.50-1.44 (m, 10H, DACH-3,MalEs-3,MalEs-5), 1.34 (b, 2H, DACH-2), 1.23 (m, 4H, MalEs-4, *J* = 7.60 Hz), 1.09 (t, 2H, DACH-3, *J* = 9.59 Hz) ppm. ^{13}C-NMR (175 MHz, DMSO-d_6): δ = 179.47 (Acetate), 178.15 (MalEs-7), 171.05 (MalEs-1), 134.43 (MalEs-8), 61.37 (DACH-1), 37.01 (MalEs-6), 34.99 (MalEs-2), 30.57 (DACH-2), 27.83 (MalEs-5), 25.63 (MalEs-4), 25.28 (MalEs-3), 23.65 (DACH-3), 22.92 (Acetate) ppm.

MS: calculated. for [$C_{30}H_{44}N_4O_{12}$Pt-Na^+]$^+$: 870.2496, found: 870.2485.

Elemental analysis calculated for $C_{30}H_{44}N_4O_{12}Pt \cdot H_2O$: C: 41.62, H: 5.36, N: 6.47, found: C: 41.58, H: 5.10, N: 6.86.

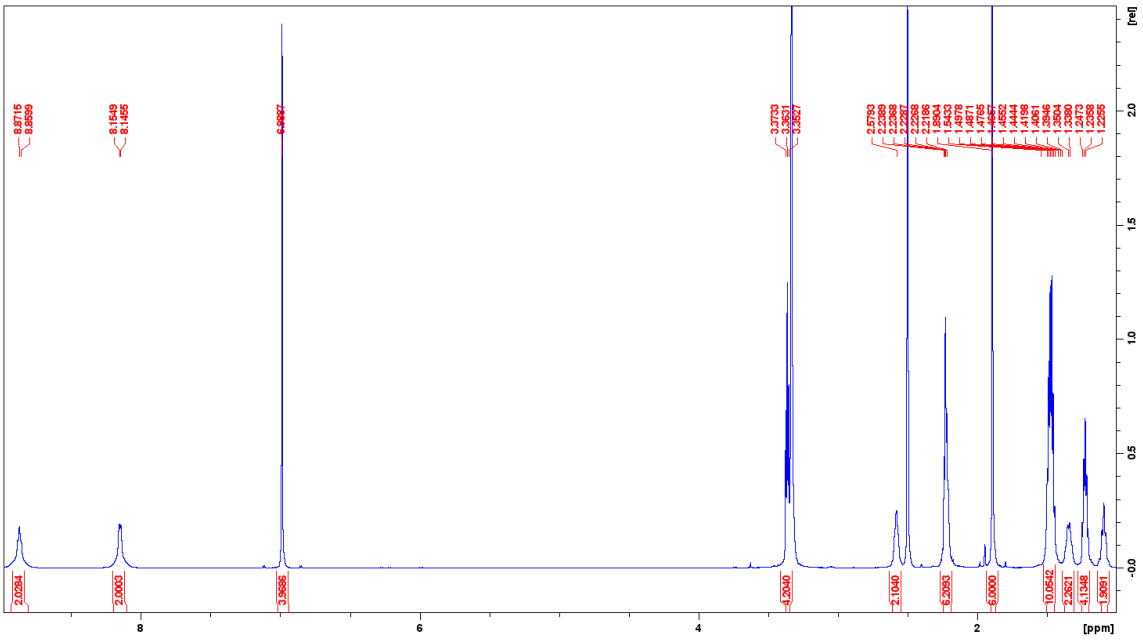


Figure 32: ^{1}H-NMR of **BisEqMalEs**.

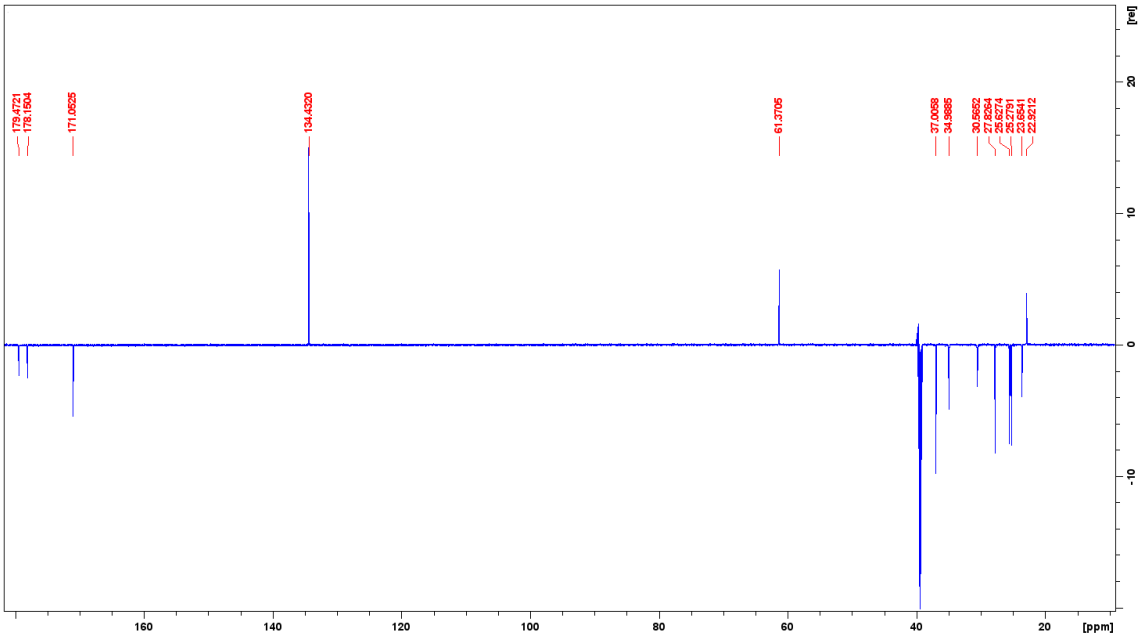
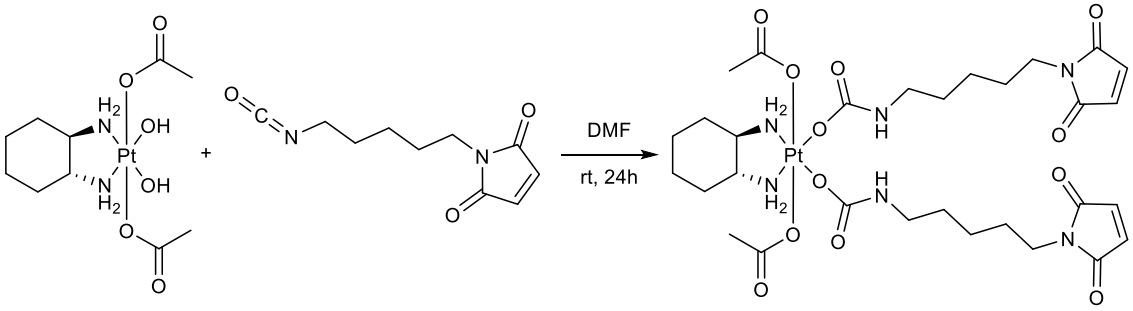


Figure 33: ^{13}C-NMR of **BisEqMalEs**.

(OC-6-33)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]di[5-(2,5-dioxo-2,5-dihydro-1H-pyrrole-1-yl)pentylcarbamato]platinum(IV) (BisEqMalCa)



Purified (OC-6-13)-diacetato[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidoplatinum(IV) (**6**, 100 mg, 0.217 mmol) was suspended in 4 mL DMF and 1-(5-isocyanatopentyl)-1H-pyrrole-2,5-dione (**9**, 181 mg, 0,868 mmol, 4.00 eq.) was added. The reaction was stirred at room temperature for 24 h. The solvent was removed in vacuo. The crude product was dissolved in 50 mL H_2O/ACN (74:26) and purified by preparative RP-HPLC (XBridge Prep C18). 10 mL of product solution were purified per run using H_2O/ACN without TFA and an isocratic method with 26% ACN over 20 min at a flow rate of 17 mL/min. The product eluted after approximately 12 minutes. All product fractions were combined and lyophilized. Yield: 77 mg (40%) white solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 9.00 (b, 2H, N*H*$_2$), 8.28 (b, 2H, N*H*$_2$), 7.00 (s, 4H, MalCa-8), 6.20-5.70 (m, 2H, MalCa-N*H*), 3.40-3.35 (m, 4H, MalCa-6, *J* = 4,78 Hz), 2.89 (b, 4H, MalCa-2), 2.66 (b, 2H, DACH-1), 2.22-2.20 (dd, 2H, DACH-2, *J* = 11.50 Hz), 1.88 (s, 6H, Acetate), 1.51-1.43 (m, 6H, DACH-3,MalCa-5), 1.37-1.32 (m, 6H, DACH-2,MalCa-3), 1.22-1.17 (m, 4H, MalCa-4), 1.09 (t, 2H, DACH-3) ppm. ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 179.93 (Acetate), 178.15 (MalCa-7), 134.42 (MalCa-8), 61.40 (DACH-1), 40.91 (MalCa-2), 37.06 (MalCa-6), 30.64 (DACH-2), 27.74 (MalCa-5), 23.67 (DACH-3), 25.63 (MalCa-4), 23.59 (MalCa-3).

MS: calculated for $[C_{30}H_{44}N_4O_{12}Pt\text{-}Na^+]^+$: 900.2714, found: 900.2684.

Elemental analysis calculated for $C_{30}H_{46}N_6O_{12}Pt$: C: 41.05, H: 5.28, N: 9.57, found: C: 40.67, H: 5.27, N: 9.84.

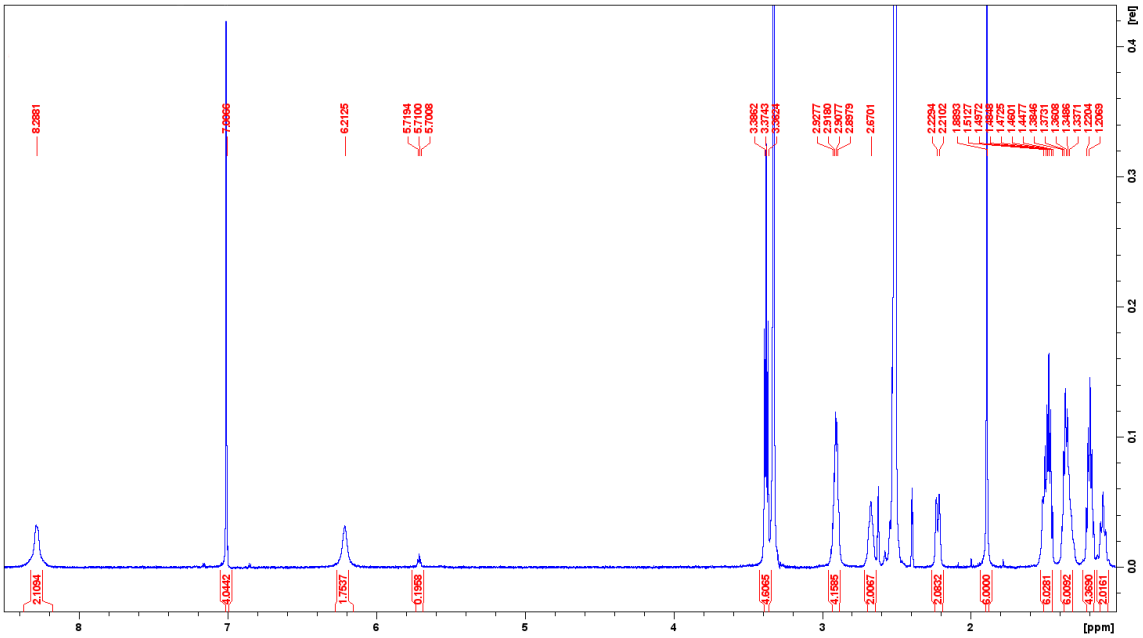


Figure 34: ^{1}H-NMR of **BisEqMalCa**.

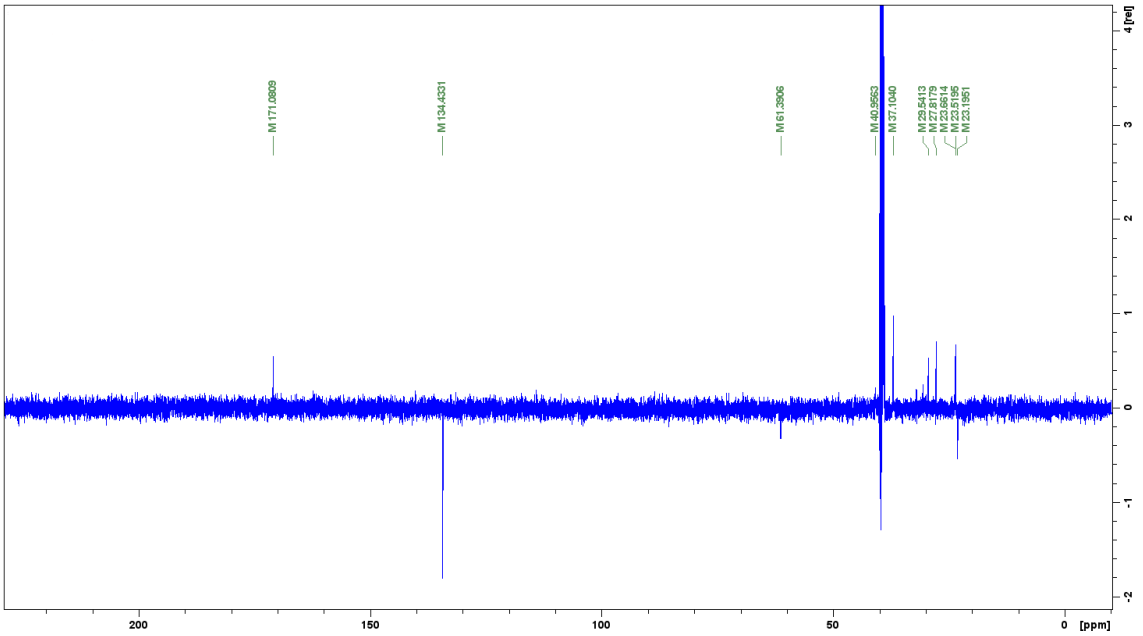
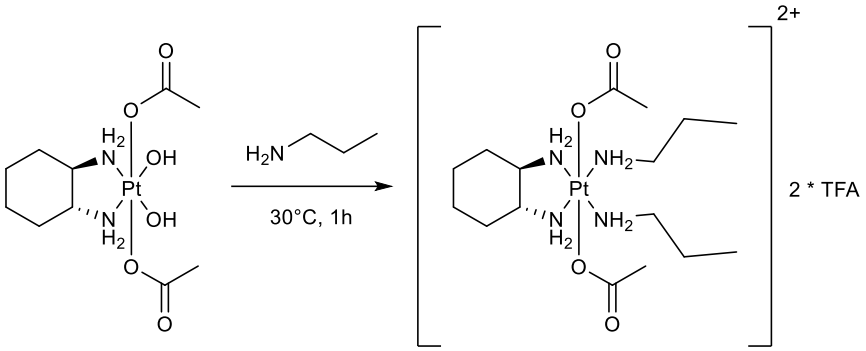


Figure 35: ^{13}C-NMR of **BisEqMalCa**.

(OC-6-13)-Diacetato[(1R,2R)-cyclohexane-1,2-diamine]dipropane-1-amineplatinum(IV) (BisEqProAm)



Purified (OC-6-13)-diacetato[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidoplatinum(IV) (**6**, 100 mg, 0.217 mmol) was suspended in 2 mL propane-1-amine and the reaction was stirred at 30°C for 1 h. The solvent was removed in vacuo. The crude product was dissolved in 10 mL H_2O/ACN (+0.1% TFA, ratio 92:8) and purified by preparative RP-HPLC (XBridge Prep C18). 3 mL of product solution were purified per run using H_2O/ACN (+0.1% TFA) and an isocratic method with 8% ACN over 15 min at a flow rate of 17 mL/min. The product eluted after approximately 10 min. All product fractions were combined and lyophilized. Yield: 49 mg (29%) white solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.70 (b, 2H, NH_2), 7.43 (b, 2H, NH_2), 6.89 (b, 4H, NH_2), 2.76-2.72 (m, 2H, CH_2), 2.66-2.64 (b, 4H, CH_2), 2.15-2.13 (d, 2H, CH_2, *J* = 11.80 Hz), 2.05 (s, 6H, Acetate), 1.68-1.61 (m, 4H, CH_2), 1.57-1.52 (b, 4H, CH_2), 1.20-1.16 (b, 2H, CH_2), 0.95-0.92 (t, 3H, CH_3, *J* = 7.42 Hz) ppm.

MS: calculated for [$C_{16}H_{38}N_4O_4Pt$]: 272.6265, found: 272,6265.

Elemental analysis calculated for $C_{16}H_{38}N_4O_4Pt \cdot 2\ CF_3COO \cdot H_2O$: C: 30.42, H: 5.11, N: 7.10, found: C: 30.57, H: 4.84, N: 7.03.

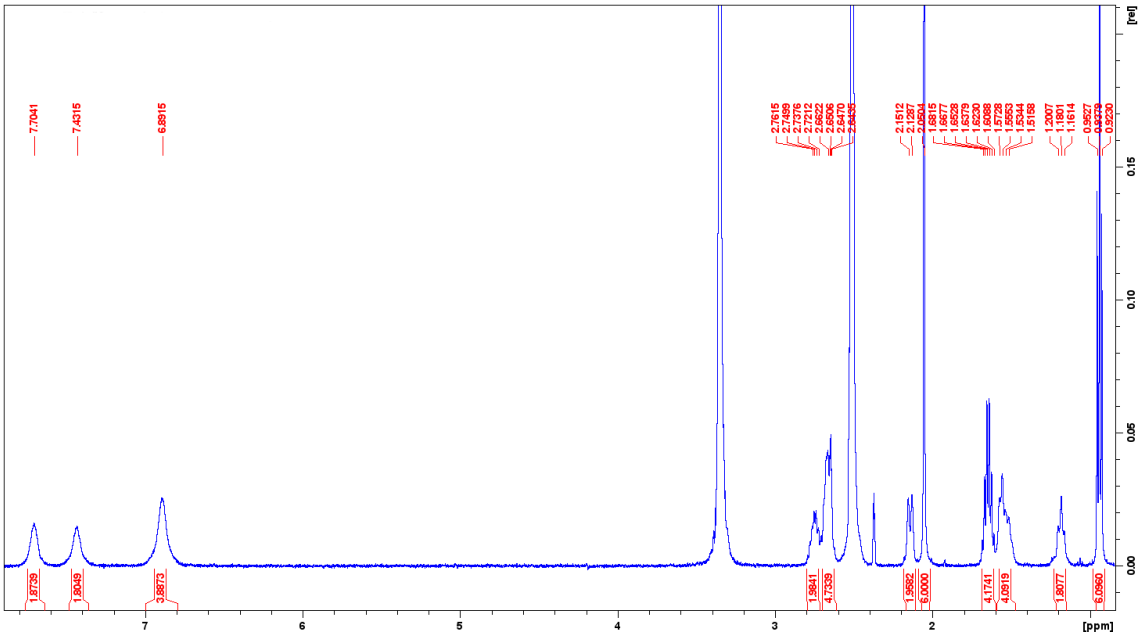
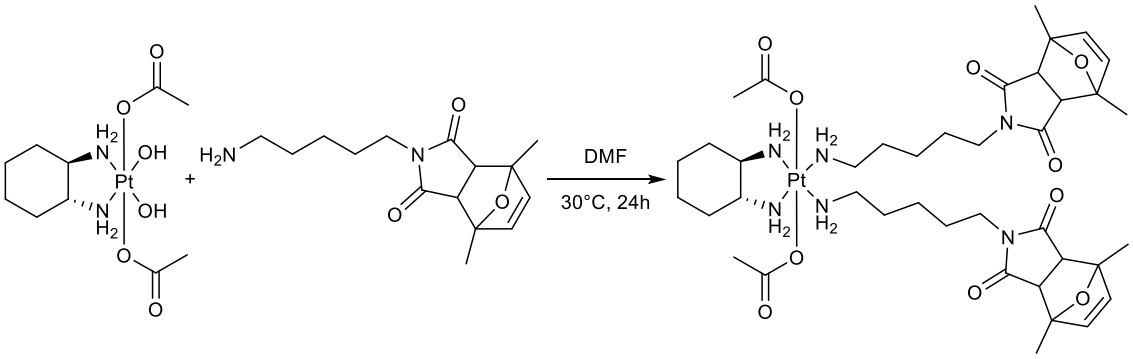


Figure 36: ^{1}H-NMR of **BisEqProAm**.

(OC-6-13)-Diacetatodi[2-(5-aminopentyl)-4,7-dimethyl-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione][(1R,2R)-cyclohexane-1,2-diamine]platinum(IV) (BisEqMalAm)



(OC-6-13)-diacetato[(1R,2R)-cyclohexane-1,2-diamine]dihydroxidoplatinum(IV) (**6**, 25 mg, 0.054 mmol) was suspended in 5 mL DMF and 2-(5-aminopentyl)-4,7-dimethyl-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (**13**, 30.2 mg, 0.109 mmol, 2.00 eq.) was added and the reaction was stirred at 30°C for 24 h. The reaction was monitored by LC-MS. The solvent was removed in vacuo and the crude product analyzed by LC-MS.

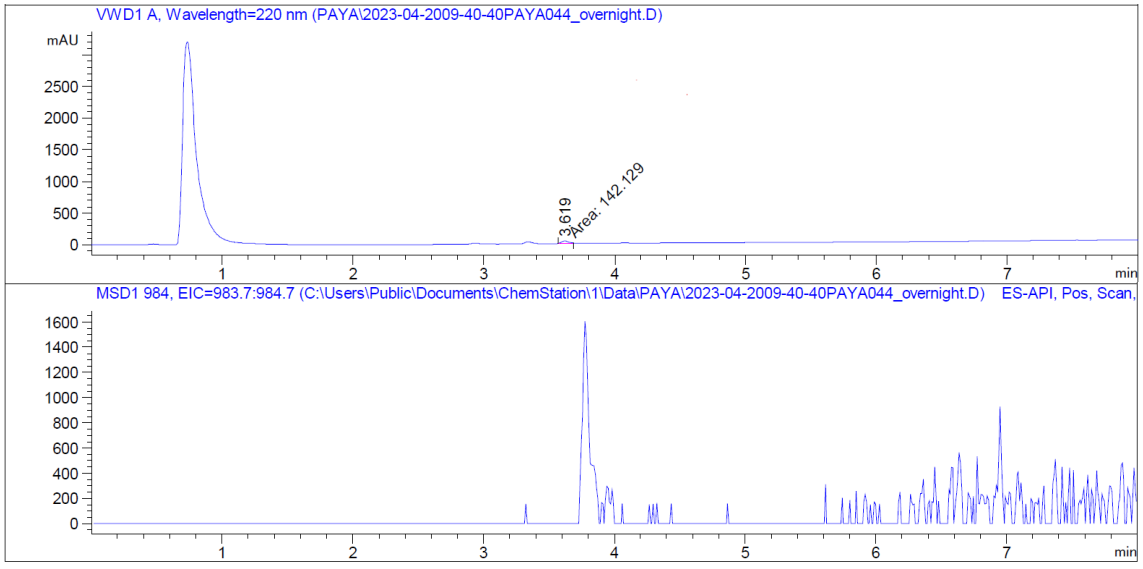


Figure 37: LC-MS run of **BisEqMalAm** (above) and the corresponding extracted ion count for 984 m/z (below).

Abbreviations

ACN	acetonitrile
ACT	adoptive cell transfer
APCs	antigen-presenting cells
ATP	adenosine 5’-(tetrahydrogen triphosphate)
Boc	di-tert-butyl dicarbonate
CTLA-4	cytotoxic T lymphocyte antigen 4
CTR1	copper transporter
DAMPs	damage-associated molecular patterns
DLT	dose-limiting toxicity
DMF	*N*,*N*-dimethylformamide
DMSO-d_6	deuterated dimethyl sulfoxide
DPPA	diphenyl phosphorazidat
DSC	*N,N*-disuccinimidyl carbonate
DTD	drug targeting and delivery
EGFR	epidermal growth factor receptor
EPR effect	enhanced permeability and retention effect
eq	equivalent
FA	formic acid
FDA	Food and Drug Administration
GS-X pump	glutathione S-conjugate export pump
HCl	hydrogen chloride

HMGB1	high mobility group box 1
(RP)-HPLC	(reversed-phase) high-performance liquid chromatography
ICD	induce immunogenic cell death
ICI	immune checkpoint inhibitor
IMRT	intensity-modulated radiation therapy
LC-MS	liquid chromatography-mass spectroscopy
MMR	mismatch repair
MRP-1	multidrug resistance protein 1
NER	nucleotide excision repair
NMR	nuclear magnetic resonance
PD-1	Programmed Cell Death 1
PSN	peripheral sensory neuropathy
ROS	reactive oxygen species
RP-HPLC	reversed-phase high-performance liquid chromatography
SEC-ICP-MS	size-exclusion chromatography-inductively coupled plasma mass spectrometry
SMVT	sodium-dependent multivitamin transporter
TEA	*N,N*-diethylethanamine
TFA	trifluoroacetic acid
VEGF	vascular endothelial growth factor

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