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„Synthesis and characterisation of a cell-penetrating
homing peptide (CPHP) conjugated to a cytotoxic
metal-based paullone“

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To my family

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Abstract

As cancer represents one of the leading death causes, its treatment based on among others chemotherapy is of huge significance. Concerning this approach, great success can be ascribed to platinum-based antineoplastic agents. Nevertheless their application is accompanied by severe limitations, including side-effects due to lacking selectivity for cancerous cells and intrinsic/acquired resistances leading to activity against a limited number of tumours. These aspects prompted research on development of other metal-based therapeutics. Among various metal-containing developing anticancer agents, the compounds based on ruthenium represent the best investigated and most promising drug candidates, in particular, NAMI-A and KP1019, finished phase I and preliminary phase II clinical trials. Moreover, a synergistic effect can be achieved by coordination of antiproliferative ligands to ruthenium. Paullones, a class of heterocyclic compounds with high antiproliferative activity, were discovered as potential cyclin dependent kinase (CDK) inhibitors involved in regulation of cell cycle. In addition, metal binding sites could be created at the periphery of paullone molecule backbone able to accommodate metal ions. Further exploration of a strategy based on selective drug delivery is supposed to minimise off-target effects. Among such compounds cell-penetrating homing peptides (CPHPs) are suitable representatives, as they are able to be conjugated to cargos, to home to specific cell-surface receptors and to internalise into the cell through endocytosis. *Svensen, N. et al.* have identified the sequence of LKKP as a CPHP in respect to K562 myeloid leukemia cells.

In the course of this thesis, two ruthenium-based complexes coordinated to a paullone and a paullone-peptide-conjugate, respectively, were synthesised. These isolated compounds were characterised by 1D/2D-NMR, X-ray diffraction, RP-HPLC, HR-ESI-MS, IR- and UV-vis spectroscopy. Furthermore stability of the complexes was investigated in aqueous media by optical spectroscopy. Cytotoxic activity of the peptide, the paullone-ligands and the ruthenium-based compounds was assayed in the K562 myeloid leukemia cancer cell line.

Zusammenfassung

Da Krebserkrankungen zu den führenden Todesursachen zählen, ist ihre Behandlung unter anderem durch Chemotherapie von großer Bedeutung. Unter den zugehörigen Therapeutika wird besonders auf Platin basierenden, antineoplastischen Wirkstoffen ein großer Erfolg zugeschrieben. Nichtsdestotrotz ist deren Anwendung mit schwerwiegenden Nachteilen verbunden, die Nebenwirkungen aufgrund von mangelnder Selektivität gegenüber kanzerogenen Zellen beinhalten, sowie intrinsische/erworbene Resistenzen, die eine Wirksamkeit nur gegenüber einer begrenzten Tumoranzahl zur Folge haben. Ausgelöst durch diese Aspekte begann die Forschung mit der Entwicklung anderer metallbasierender, tumorinhibierender Verbindungen. Unter den verschiedensten, metallhaltigen Chemotherapeutika in Entwicklung stellen die rutheniumenthaltenden die am besten untersuchten und vielversprechendsten Kandidaten dar, besonders da NAMI-A und KP1019 die klinischen Phase I und einleitende Versuche der Phase II absolviert hatten. Zusätzlich kann ein synergistischer Effekt durch Koordination antiproliferativ wirkender Liganden an Ruthenium erreicht werden. Paullone, eine Klasse heterozyklischer Verbindungen mit hoher antiproliferativer Aktivität, wurden als potentielle CDK(*Cyclin abhängige Kinase*)-Inhibitoren, die in die Regulation des Zellzyklus involviert sind, entdeckt. Des Weiteren konnten Metallbindungsstellen an der Peripherie des Paullongrundgerüsts generiert werden, um so Metalle zu koordinieren. Durch weitere Erforschung einer auf selektivem Transport von Zytostatika basierenden Strategie sollen auftretende Nebenwirkungen reduziert werden. Unter den Verbindungen, die dazu in der Lage sind, stellen *Cell-penetrating homing Peptide* (CPHPs) geeignete Vertreter dar. Sie können durch Konjugation als Träger diverser Verbindungen dienen, mit diesen in Kombination spezifische, sich an Zelloberflächen befindliche Rezeptoren anvisieren und sich in deren Zellen durch Endozytose einschleusen. Svensen, N. et al. gelang es die Sequenz LKKP als CPHP mit Selektivität gegenüber K562 myeloischen Leukemie Zellen zu identifizieren.

Im Rahmen dieser Arbeit konnten zwei Ruthenium-Komplexe mit einem Paullon bzw. dessen Peptid-Konjugat synthetisiert werden. Die isolierten Verbindungen wurden mittels 1D/2D-NMR, Röntgendiffraktometrie, RP-HPLC, HR-ESI-MS, IR- und UV-vis Spektroskopie charakterisiert. Außerdem erfolgte eine Untersuchung der Stabilität der Komplexe in wässrigem Medium mit optischer Spektroskopie. Die zytotoxische Wirkung des Peptids, der Paullonliganden und der rutheniumbasierten Verbindungen wurde in der K562 myeloischen Leukemie Zelllinie getestet.

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1. Introduction

1.1. Global and Austrian death causes

Cancer is among the leading death causes worldwide, accounting for 7.9 million deaths in 2011.¹ With a percentage of 14%, it follows cardiovascular and infectious diseases, causing 30% and 18%, respectively, of all deaths. Nevertheless the WHO projects an increase of deaths resulting from malign neoplasms to estimated 13.1 million in 2030. This rise can be ascribed to the ageing of the population.

However, there are differences in the mortality of high- and low-income countries.² On the one hand in the latter people have a low life expectancy – nearly four in every ten deaths are among children under 15 years – and almost one third dies of infectious diseases. On the other hand industrial countries, like Austria, show dominance of diseases of the cardiovascular system and cancer at high age (see Figure 1). In 2012 these caused together seven of ten of 79.436 Austrians' deaths.³ Moreover, as it can be seen in Figure 1, 10.713 men and 9.553 women died because of malignant neoplasms, accounting for 28.6% and 22.6%, respectively, of all deaths, making cancer the second leading cause of death in Austria.

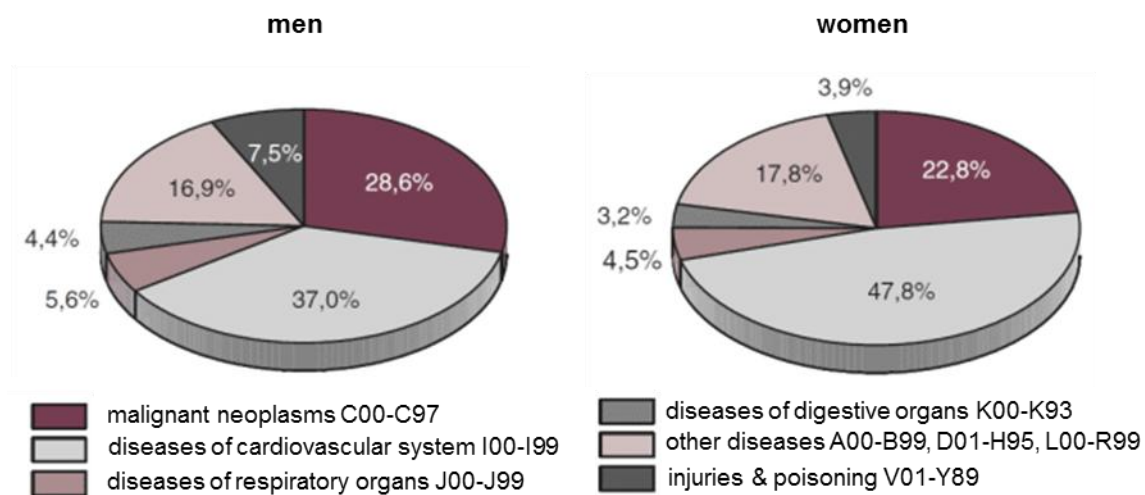


Figure 1. Percental distribution of death causes with corresponding ICD-codes concerning Austrian men & women in 2012. Data taken from STATISTIK AUSTRIA.³

Figure 2 shows that worldwide a significant difference in the appearance between the genders exists for the various types of cancer. Predominantly malign neoplasms in breast, prostate, lung and colorectum occur. In 2011 these most frequent types were assigned to 25% prostate, 30% breast, 13/11% colorectum and 13/9% lung cancer of overall tumour incidence among Austrian men/women.⁴

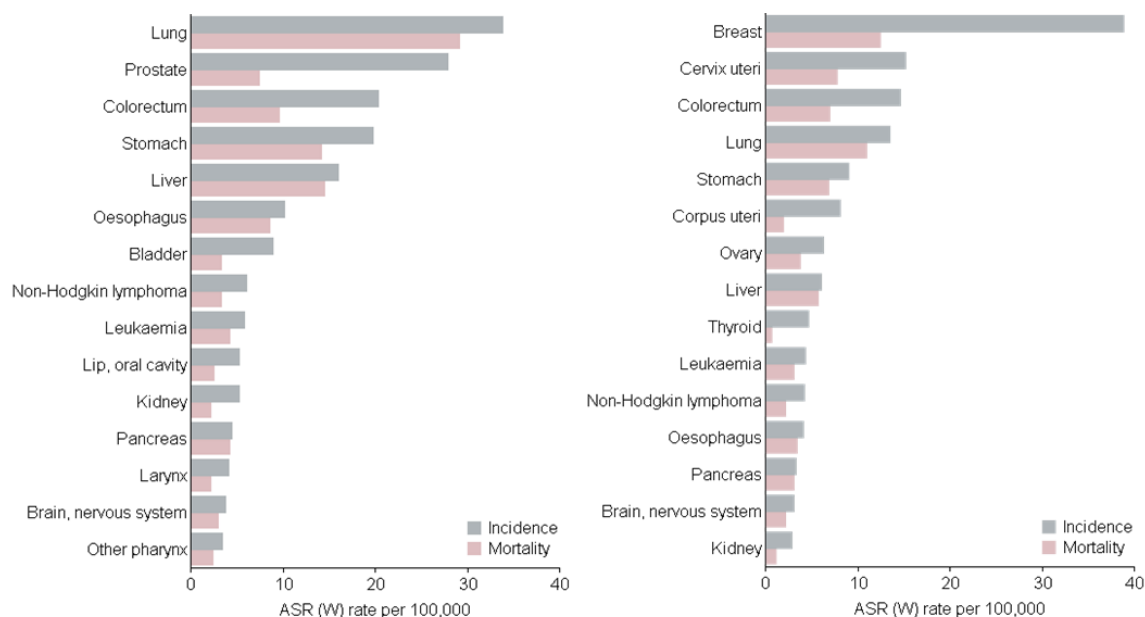


Figure 2. Most frequent cancers worldwide in men (right) & women (left) in 2008. Data taken from GLOBOCAN.²

By comparing worldwide incidence and mortality of the single tumour types, high healing chances can be attributed to prostate and breast cancer. In Austria between 1983 and 2011 tumour incidence increased from 29.000 to 37.000 affected people, whereas mortality stagnated at around 19.000.^{5,6} Decisive for this success are early diagnoses and new, improved therapy approaches.

Consideration of all mentioned facts above leads to the conclusion that the fight against cancer represents a significant task for research in medicine and biochemistry.

1.2. Carcinogenesis⁷

The emergence of cancer is a complex process characterised by a long incubation time. It can be divided in three steps, starting with the phase of initiation. Hereby DNA damage occurs due to endogenic or exogenic factors. The first implies genetic inheritance and the latter comprises biological – e.g. human papilloma virus, hepatitis C –, physical – e.g. UV and ionising radiation – and chemical influences – e.g. tobacco smoke, aflatoxin, asbestos, arsenic, alcohol, unhealthy diet, physical inactivity. If repair mechanisms fail and the mutation in the cell is stored, irreversible initiation is taking place, followed by promotion. This phase includes a series of genetic mutations. Attaching to that, progression as a last step occurs. Thereby benign turn into malign tumours, further leading to angiogenesis and metastasis.

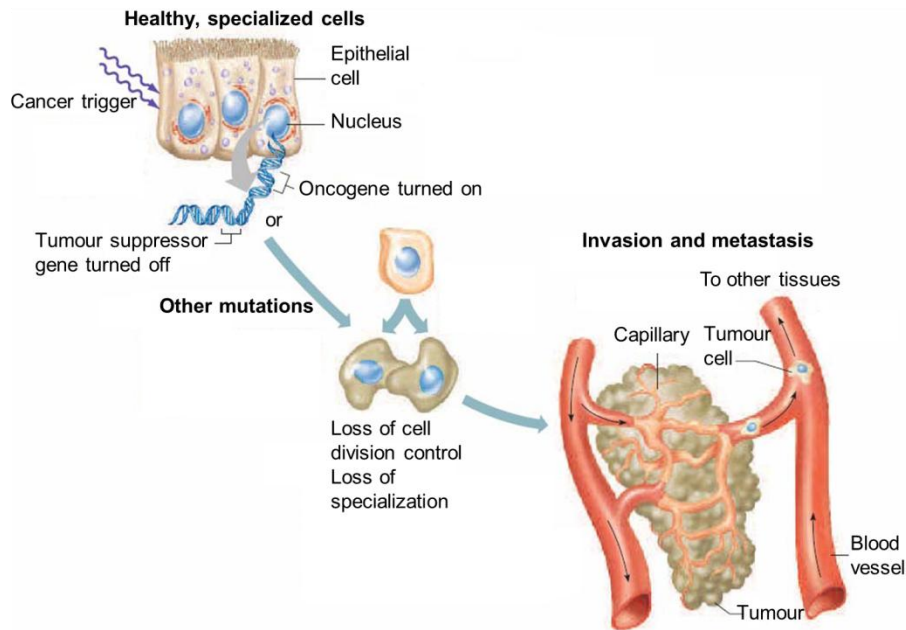


Figure 3. Carcinogenesis.⁸

1.3. Cancer therapy

Nowadays there are three possibilities used separately and combined, respectively, for cancer treatment in orthodox medicine, namely surgery, radiation therapy and chemotherapy.

Although surgery represents the oldest method, it is still a first-line therapy due to the progress in diagnostic techniques. However, it is only applicable to solid tumours, excluding disseminating cancer types.

Radiation therapy uses high energetic γ -, radioactive and X-rays in the affected areas. As this description implies, again, a localised, non-disseminating malignancy is required. For the application it is furthermore advantageous in the case of the tumour's position being close to the skin. However, ionising radiation itself is mutagenic and can result in the formation of secondary neoplasms. In respect to this therapy approach, today's research focuses on the development of sensitisers that are selectively enriched in tumour tissues and activated *via* irradiation.

Chemotherapy is understood as the treatment of cancer with cytotoxic antineoplastic drugs. Thereby a systematic approach on molecular level is applied, enabling attack on solid tumours and, additionally, metastases. In the case of leukemia chemotherapy represents the only method of treatment. Chemotherapy is mainly based on the fact that tumour cells are attacked at a larger extent compared to healthy cells, since cancer cells are characterised by higher proliferation rates. Nevertheless, selectivity of the drugs presents a problem. Side-effects like hair loss, myelosuppression and mucositis can be

ascribed to attack of healthy, high-proliferating cells. Therefore among the aims of today's research is the improvement of selectivity through usage of cancer cell specific targets, e.g. homing peptides (HPs).

According to the ATC classification system, antineoplastic agents (L01) are divided after their target location, therapeutic, pharmacological and chemical properties as follows:⁹

- alkylating agents (L01A; e.g. chlorambucil)
- antimetabolites (L01B; e.g. fluorouracil)
- plant alkaloids and other natural products (L01C; e.g. paclitaxel)
- cytotoxic antibiotics and related substances (L01D; e.g. doxorubicin)
- other antineoplastic agents (L01X; e.g. cisplatin)

The last group of antineoplastic agents contains among others the subgroups platinum compounds (L01XA) and protein kinase inhibitors (L01XE).

As this master thesis focuses on the synthesis and characterisation of metal-based kinase inhibiting agents conjugated to targeting peptides, the following sections discuss in more detail the corresponding compound classes.

1.4. Metal-based therapeutics

Research in the field of metal-based cytotoxic agents originated from investigations of various platinum compounds and further extended to platinum group metals (PGMs)-, i.e., ruthenium-,¹⁰ osmium-, rhodium-¹¹ and iridium-containing¹² substances.

1.4.1. Platinum-based drugs (L01X)

The breakthrough in the development of platinum-based drugs was achieved with the discovery of cisplatin (*cis*-diamminedichloridoplatinum(II), L01XA01) in 1969.¹³ While investigating the effect of an electric field on mitosis of *E. coli* bacteria under usage of platinum electrodes, Barnett Rosenberg observed inhibition of cell division.¹⁴ This outcome was ascribed to oxidised platinum, namely *cis*-diamminetetracloridoplatinum(IV), and its intracellularly reduced cisplatin. As a result of these investigations cisplatin was approved for clinical use by the FDA as the first metal-based antineoplastic agent in 1978,¹⁵ after having been already synthesised in 1844 by Michele Peyron.¹⁶

Although the mode of action of cisplatin is still not completely clarified, the mono- and diaqua-compounds, namely, $[\text{Pt}(\text{NH}_3)_2(\text{OH}_2)\text{Cl}]^+$ and $[\text{Pt}(\text{NH}_3)_2(\text{OH}_2)_2]^{2+}$, respectively, were proved to be the biologically active species.¹⁷ Their formation occurs in the cells as hydrolysis is facilitated there due to the lower chloride concentration of 4 mM compared to 100 mM in the blood plasma. $[\text{Pt}(\text{NH}_3)_2]^{2+}$ fragment interacts with DNA and binds to N⁷ of

guanine base. The resulting 1,2-d(GpG) intrastrand adducts further lead to apoptosis of the affected cells.

Today, as one of the world's bestselling anticancer drugs, cisplatin is utilised in almost 50% of all chemotherapies. In particular, it is successfully used in the treatment of advanced and metastatic testicular germ-cell cancer, which had become in 90% a curable disease.¹⁸ In addition, it is applied in the case of ovarian, lung, cervical, bladder and esophageal cancer. Despite cisplatin's frequent application, severe side-effects represent a strong limitation. These include the dose-limiting nephrotoxicity, neurotoxicity, nausea and vomiting. Further drawbacks are intrinsic/acquired resistances, activity against a limited number of tumours and low selectivity for cancerous cells – $\leq 1\%$ of the intravenously administered drug reaches the tumour tissue, whereas the main part reacts with sulphur-containing biomolecules. Thus, motivated by the aim to circumvent these limitations, search for more effective metal-based anticancer drugs was extended.

As a result the second generation of platinum-complexes, carboplatin (*cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II), L01XA02) achieved worldwide approval in clinical use, as well. The higher stability of the 1,1-cyclobutanedicarboxylato platinum chelate compared to cisplatin led to slower hydrolysis and less cytotoxicity. However, the DNA adduct formation rate is only one tenth of that for cisplatin and 20–40 times higher doses have to be administered.¹⁹ A different profile of side-effects emerged. In particular nephrotoxicity disappeared, neurotoxicity became less pronounced, but myelosuppression, especially thrombocytopenia, was dose-limiting.¹⁸ Concerning application, carboplatin replaced cisplatin in ovarian and small cell lung cancer. Nevertheless, an identical spectrum of activity due to the same DNA binding moiety $[\text{Pt}(\text{NH}_3)_2]^{2+}$, carboplatin's effect is analogously limited in certain tumours. This inevitably led to a third generation of platinum-based drugs.

Since 2004 oxaliplatin (1*R*, 2*R*)-diaminocyclohexaneoxalatoplatinum(II), L01XA03) is in clinical use worldwide.²⁰ In comparison to cisplatin it offers a different way of accumulation in tumour cells. It is, therefore, used in the case of metastatic colorectal cancer, priorly untreatable with platinum drugs. As a dose-limiting factor neurotoxicity should be noted.

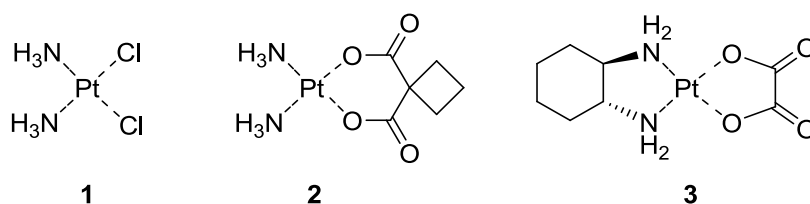


Figure 4. **1** – cisplatin, *cis*-diamminedichloridoplatinum(II); **2** – carboplatin, *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II); **3** – oxaliplatin, (1*R*, 2*R*)-diaminocyclohexaneoxalatoplatinum(II).

Although a large number of platinum complexes entered clinical trials, only three other achieved regional approval, namely nedaplatin in Japan, heptaplatin in South Korea and lobaplatin in China.¹⁸

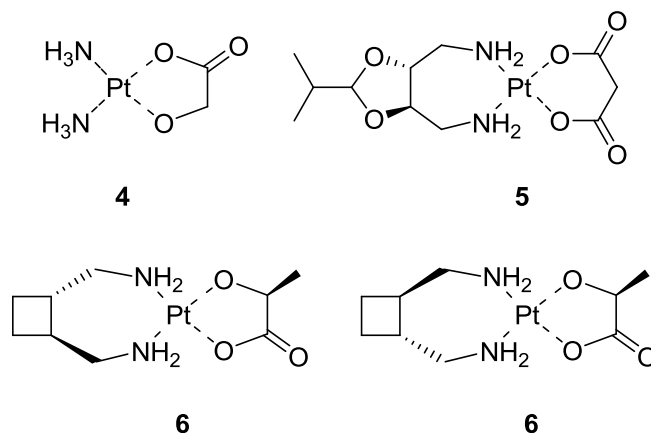


Figure 5. 4 – nedaplatin, *cis*-diammineglycolatoplatinum(II); 5 – heptaplatin, [*cis*-malonato [(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]platinum(II)]; 6 – lobaplatin, (*R*, *R*/*S*,*S*)-[(1,2-cyclobutanedimethanamine) [(2*S*)-2-hydroxypropanoato²⁻-O1,O2]platinum(II)].

Concerning the current state of research on platinum anticancer agents, the approach towards platinum(IV) complexes seems to be promising.²¹ These compounds due to their kinetic inertness and the opportunity to be used as prodrugs are expected to enter a reduced number of side reactions *in vivo*. In addition, they offer the possibility for oral administration and increased structural diversity resulting from the octahedral configuration, contrary to the square-planar, intravenously administered platinum(II) compounds. One such drug, satraplatin, had failed in the SPARC (phase III) study to show benefit in overall survival, and the manufacturing company has withdrawn the application for admission.²²

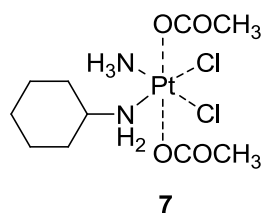


Figure 6. 7 – satraplatin, (OC-6-43)-bis(acetatato)amminedichloridocyclohexylamineplatinum(IV).

Because of the known drawbacks of platinum complexes, investigations on novel metal-based antiproliferative agents have also emerged.

1.4.2. Ruthenium-based cytostatics

Today after cisplatin and related compounds the ruthenium-based coordination and organometallic compounds are considered as the most promising potential anticancer drugs.

Among them, the most prominent drug candidates are the octahedral complexes NAMI-A (imidazolium *trans*-[tetrachlorido(1H-imidazole)(DMSO)ruthenate(III)], KP1019 (indazolium *trans*-[tetrachloridobis(1H-indazole)ruthenate(III)]) and the corresponding sodium salt KP1339. This is justified since these compounds finished phase I and preliminary phase II clinical trials with minor side-effects and the patients showed marked tolerance towards the administration dose. It should be noted that NAMI-A inhibits metastasis formation,²³ whereas KP1019 and KP1339 show activity in solid tumours.^{24,25}

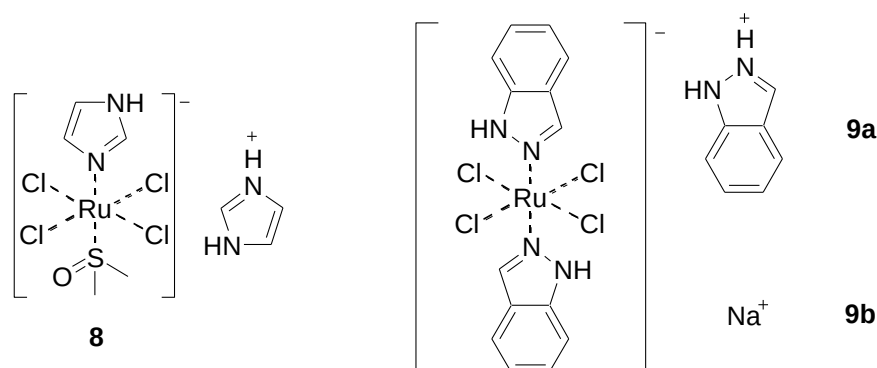


Figure 7. **8** – NAMI-A, $\text{Him}[\text{trans-RuCl}_4(\text{DMSO})\text{Im}]$ with Im = imidazole, imidazolium *trans*-[tetrachlorido(1H-imidazole)(DMSO)ruthenate(III)]; **9a** – KP1019, $\text{Hind}[\text{trans-RuCl}_4(\text{Ind})_2]$ with Ind = 1H-indazole, indazolium- or **9b** – KP1339, $\text{Na}[\text{trans-RuCl}_4(\text{Ind})_2]$, sodium- *trans*-[tetrachloridobis(1H-indazole)ruthenate(III)].

Ruthenium itself is a noble, second row transition metal with atomic number 44 and an average mass of 101.07 g/mol. Although it belongs to the same group as iron, it exhibits different physical and chemical properties. Ruthenium is available in the oxidation states from +VIII to -II, with +II and +III being the most common. The metal is considered as chemically inert element. Among its seven naturally occurring isotopes, ⁹⁹Ru and ¹⁰¹Ru are suitable for NMR measurements.²⁶

In addition to these general features, there are aspects highlighting application of ruthenium as a potential antineoplastic agent.²⁷

- A convenient access to various ruthenium complexes enabled due to established coordination chemistry and synthesis pathways.
- Ligand exchange rates comparable to those of platinum(II) and tunable through appropriate ligands.
- The octahedral coordination geometry provides reactivity and mode of action different from that of square-planar platinum(II) compounds.

- In addition to the accessibility of the oxidation states +II, +III, +IV under physiological conditions, the electron transfer rates and redox potentials can also be tuned. This is especially significant for the "activation by reduction" mechanism, which offers redox-based cancer-selectivity. Due to the presence of reducing agents in tumour tissues, e.g. glutathione, hypoxic and acidic conditions, reduction of ruthenium(III) to ruthenium(II) is more readily to occur than in healthy cells. This characteristic environment is a consequence of the rapid proliferation in combination with chaotic angiogenesis and increased lactate formation from anaerobic glycolysis as energy providing process.²⁸ The resulting ruthenium(II) compounds show a higher activity due to filled t_{2g} orbitals, accompanied by a weakening of metal-ligand bond and enhancement of hydrolysis of the ligand.²⁹
- Iron mimicking properties in respect to the binding affinity towards biomolecules are presumably responsible for a decrease in the toxicity compared to platinum(II) complexes. In line with the HSAB principle, ruthenium(III) shows affinity towards nitrogen and oxygen, but concerning the latter to a lesser extent, whereas bonds with sulfur are kinetically labile. Thus, imines of imidazoles originating from purines or histidine appear as frequent binding sites of biomolecules, such as human serum albumin and transferrin. Furthermore, through close relation to iron utilisation of the same delivery ways into the cell *via* transferrin receptor-mediated endocytosis seems likely. This is significant for the selectivity and reduced toxicity for healthy cells of ruthenium-based anticancer agents, as malignancies generate a high number of transferrin receptors due to increased demand for iron. Hereby, a pharmacological study during the clinical phase I trials in the case of intravenously administered KP1019 (see Figure 7) is noteworthy. It revealed that in the blood stream the ruthenium complex is nearly completely bound to proteins, namely to HSA (80–90%)³⁰ and transferrin (1%).³¹ After several infusion cycles molar ratios of KP1019 bound to HSA and transferrin, respectively, were determined as 1.6 to 0.3. The low cargo efficiency of transferrin is relevant as *in vitro* assays indicated inhibition of the cellular drug uptake in the case of too high ruthenium loading.
- The latter aspect is based on increased knowledge of biological effects induced by ruthenium compounds, representing another advantageous issue. As possible modes of action have been considered: DNA binding,³² topoisomerase inhibition^{33,34} and direct interaction with mitochondria. Influence on the formation of ROS and inhibition of metastasis are also under investigation.³⁵

As ruthenium(II) complexes are assumed to show higher activity than their ruthenium(III) congeners, synthesis of Ru(II)-based compounds was a natural extension of research in the field of development of non-platinum anti-cancer drugs. In particular, organoruthenium(II) complexes bearing η^6 -arene ligands have been increasingly studied. They adopt a "three leg piano-stool geometry", with the arene ligand representing the seat and the other three monodentate ligands the corresponding legs.

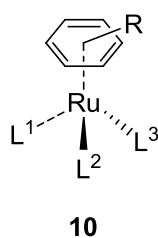


Figure 8. "Three leg piano-stool geometry" of Ru-arene complex.

The arene ligand modulates the chemical properties of the compound and affects the cytotoxicity to a great extent. As can be seen in Table 1 below, IC_{50} values of the complex $[RuCl(arene)(en)]PF_6$ increase with substitution of selected aromatic moieties in the following order: tetrahydroanthracene < biphenyl < *p*-cymene < benzene < ethylbenzoate < methylbenzoate, showing a 100-fold IC_{50} value variation in A2780 human ovarian cancer cells. Nevertheless, although complexes with tetrahydroanthracene show the lowest IC_{50} values, *p*-cymene is most frequently used as it exhibits the best compromise between lipophilicity that correlates with cytotoxicity and solubility in aqueous media, which is required for the application and testing of the antiproliferative substances.³⁶

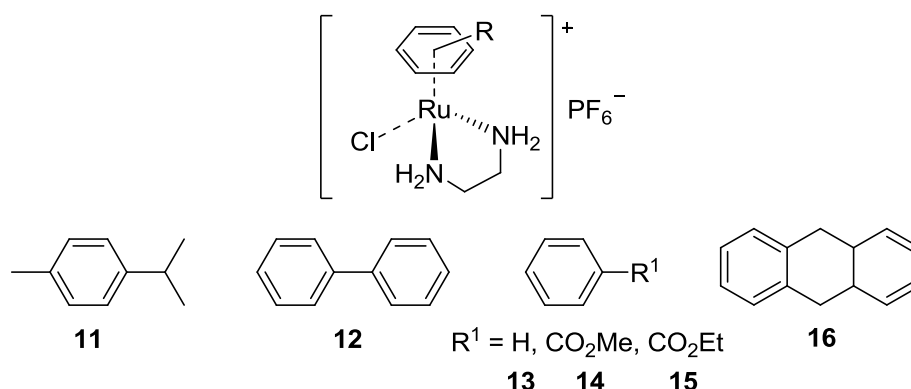


Figure 9. **11** – *p*-cymene, **12** – biphenyl, **13** – benzene, **14** – methylbenzoate, **15** – ethylbenzoate, **16** – tetrahydroanthracene as arene ligands in $[RuCl(arene)(en)]PF_6$ complexes.

Table 1. IC₅₀ values for [RuCl(arene)(en)]PF₆ complexes, evaluated in A2780 human ovarian cancer cells.³⁷

η^6 -arene ligand	IC ₅₀ [μ M]
<i>p</i> -cymene	10 $\pm$ 1
biphenyl	5.0 $\pm$ 0.4
benzene	17 $\pm$ 8
methylbenzoate	56 $\pm$ 1
ethylbenzoate	52 $\pm$ 2
tetrahydroanthracene	0.5 $\pm$ 0.1

However, not only variation of the arene enables the tuning of the redox potential, kinetic lability and reactivity of the metal complex. Different leaving groups and co-ligands constitute important parameters determining the biological activity. Therefore, synthetic approaches are based on derivatisations of mono- and bidentate *N,N*³⁸-, *N,O*³⁹-, *P,N*⁴⁰-, *S,O*⁴¹- and *O,O*⁴¹-coordinated ligands.

Among complexes with monodentate ligands, the class of RAPTA compounds, shown in Figure 10, is very well studied.⁴² In general RAPTA compounds offer various possibilities for derivatisation. PTA confers aqueous solubility to these compounds. Exhibition of low, but selective cytotoxicity towards TS/A murine adenocarcinoma cells was also observed.

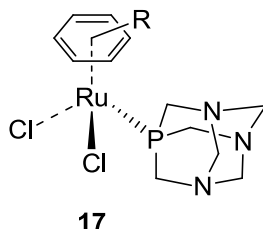


Figure 10. Example of RAPTA compounds.

As *N,N*-bidentate ligand, en is frequently used (see Figure 9). Coordination to sp²-hybridised nitrogen-containing ligands was also realised.³⁶

1.4.3. Cytotoxic osmium-based compounds

Osmium belongs to the noble, third row transition metals with atomic number 76, an average mass of 190.23 g/mol and the highest density of all elements with ~22 g/cm³. It is available in the oxidation states from +VIII to -II, with +III and +IV being the most common. Like ruthenium, osmium differs in its physical and chemical properties from iron and is also relatively inert in its compounds. Among its seven naturally occurring isotopes, ¹⁸⁷Os and ¹⁸⁹Os are suitable for NMR measurements.²⁶

Although osmium resembles ruthenium in its features, there are also differences in their ligand exchange kinetics, rooted in the stronger spin orbit coupling and π -back donation

from lower oxidation states of osmium. In addition, the preference for higher oxidation states deserves to be mentioned.

The effect of different ligand exchange kinetics can be illustrated by different pKa-values of the aqua species of the complexes. In the case of $[M(\text{maltolato})(\text{H}_2\text{O})]^+$ ($M = \text{Ru}, \text{Os}$) the pKa of the coordinated H_2O in ruthenium compound is 9.23 and with 7.6 far lower in the osmium congener. As a result the osmium compound exists at physiological pH to a huger extent in its deprotonated, less reactive form $[\text{Os}(\text{maltolato})(\text{OH})]$, what affects the biological activity.⁴³ The extent of hydrolysis for the osmium-based compound is only one half compared to that of the ruthenium analogue within 72 h. Additionally, the aqua ligand of the first was subsequently deprotonated, in contrast to the ruthenium one, where no further reaction was observed.⁴⁴

1.4.4. Paullones as potential cyclin dependent kinase inhibitors

Due to the crucial role of CDKs in cell proliferation and cancer, research focused on their investigation as possible targets for cancer chemotherapy. Flavopiridol was the first CDK inhibitor that entered clinical trials.⁴⁵ The search for further potential CDK inhibitors started thereafter. In 1999 paullones were identified as potential CDKs in a comparative algorithm search at the NCI.⁴⁶ Kenpaullone as lead compound was first described in 1992 by Kunick, C.⁴⁷

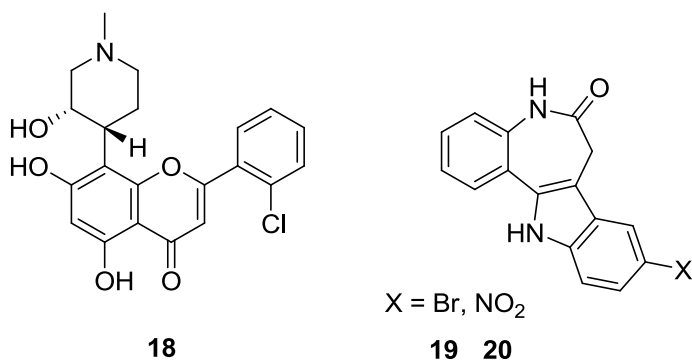


Figure 11. **18** – flavopiridol; **19** – kenpaullone, 9-bromo-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6-one; **20** – alsterpaullone, 9-nitro-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6-one.

In vitro tests of the 9-bromo-substituted paullone showed an activity profile similar to that of flavopiridol. Investigation of numerous paullones for their pharmacological properties revealed that electron-withdrawing substituents in position 9 were favourable for CDK1 inhibitory potency, e.g. alsterpaullone.⁴⁸ Structure–activity studies indicated that kinase inhibition efficiency did not necessarily correlates with antiproliferative activity. Hence, other intracellular targets have been suggested.⁴⁹ Inhibition of glycogen synthase kinase-3, mitochondrial malate dehydrogenase and sirtuins were additionally found. Modifications of

the lactam moiety led to an increase of the inhibitory potency of sirtuins compared to unsubstituted paullones, which is reversed in the case of CDK-inhibition.⁴⁶

However, most paullones lack sufficient aqueous solubility and bioavailability for potential medical application. In order to overcome these limitations and achieve a synergistic antiproliferative effect, metal coordination was explored. However, since paullone-backbones do not provide any coordination sites, modifications by introducing chelating sites were required.

Cytotoxicity of one of the first metal-based paullones was, depending on the cell line, 1.5–18 times higher, compared to its free ligand.⁵⁰ However, both showed promising IC₅₀ values in the low micro- and submicromolar concentration range, respectively.

Paullones were coordinated to ruthenium, osmium,⁵¹ gallium,⁵⁰ copper(II),⁵² etc.

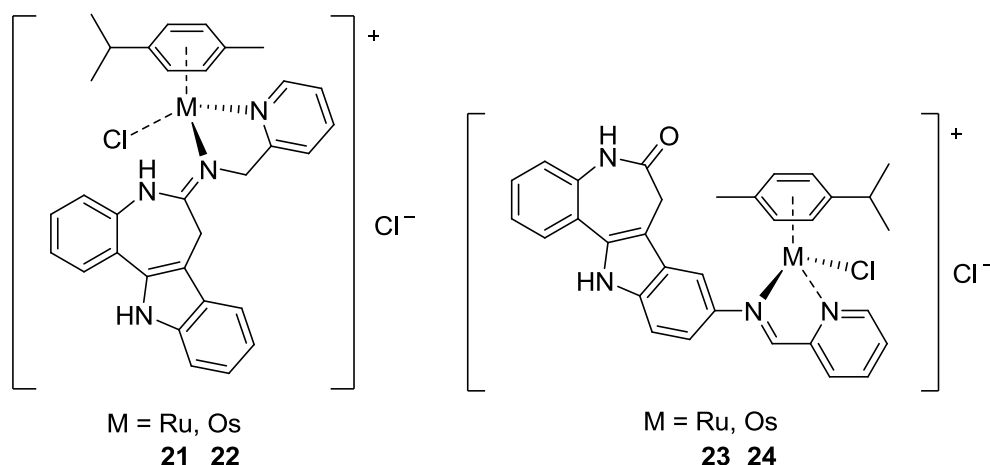


Figure 12. paullone complexes with ruthenium and osmium as central atoms, and coordination based on modifications in position 9 (**23, 24**) and the lactam unit (**21, 22**), respectively.

The structure–activity relationship showed, that cytotoxicity of paullones bound to metals in position 9 was lower than that of the corresponding derivative with lactam-modified binding site. This fact indicated the evidence that CDKs are not the main targets of paullones, as unsubstituted lactam units are presumably required for efficient CKD1 inhibition.

1.4.5. Cell-penetrating homing peptides

In order to minimise off-target effects, strategies for selective drug delivery based on expression of tissue-specific markers, comprising receptors and proteins, are emerging. By identification and usage of selectively binding peptides, their further conjugation to anticancer agents, reduction of the toxicity risk for healthy tissue could be achieved.⁵³

Generally for specific peptide-mediated delivery, there are three possibilities: a) HPs that deliver their cargos only to specific cell-surface receptors; b) CPPs, which are linked to HPs and have the ability of cargo internalisation through endocytosis or pore formation; and c) CPHPs, that are able to get into intracellular matrix without requiring external agents. Methods for identification of these particular peptides represent the *in vivo* phage-display, SPLs and direct targeting.

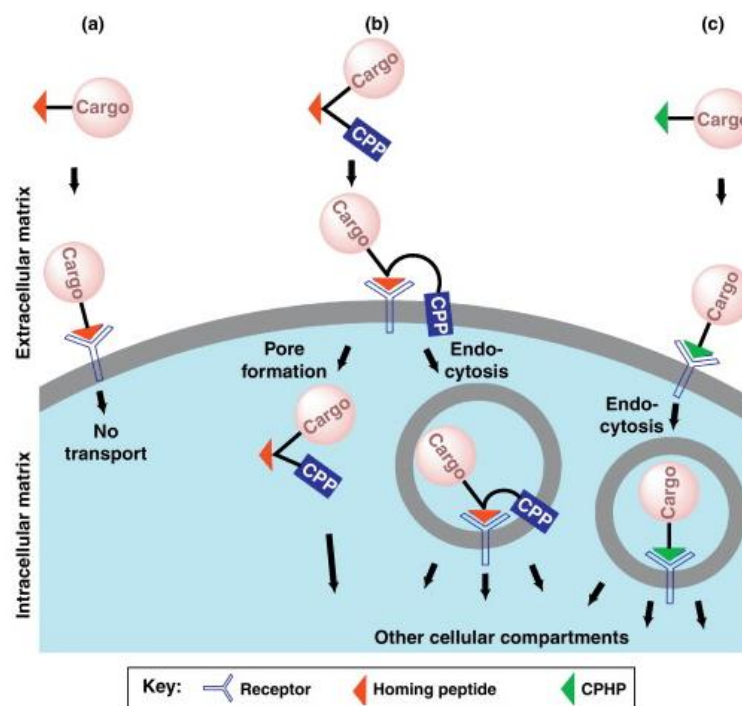


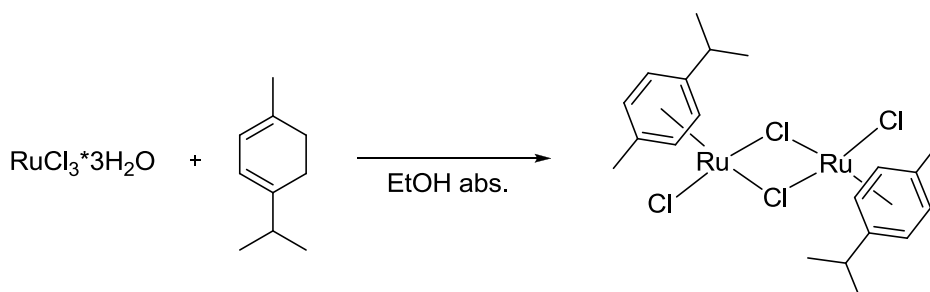
Figure 13. Selective targeting and delivery possibilities of peptides: strategy of a) HPs, b) HP-CPPs and c) CPHPs. Illustration taken from *Svensen, N. et al.*⁵³

By application of the third and the first techniques two CPHPs have been discovered and investigated in clinical phase III and II trials, respectively.⁵⁴ The first is cilengitide, a salt of the cyclised RGD-based pentapeptide cRGDf(NMeV), which binds selectively to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins that are highly expressed in tumour cells and involved in angiogenesis.⁵⁵ Another peptide in clinical trials is NGR. It shows affinity towards the aminopeptidase N/CD13 that is overexpressed in many cancers.

Recently, four CPHPs have been identified in a chemical library screen by *Svensen, N. et al.*⁵⁶ One of them consists of the short, polar sequence LKKP. It homes and internalises in K562 myeloid leukemia cells. The uptake mechanism is based on endocytosis, but with no receptor being reported. Nevertheless, as the peptide offers small size and cell permeability, its conjugation to antineoplastic agents might allow benefiting from the resulting selective delivery system.

Bis[chloride- μ -chlorido(η^6 -*p*-cymene)ruthenium(II)]

Reaction



Reagents

Ruthenium(III) chloride trihydrate (261.47 g/mol): 2.02 g (7.7 mmol, 1.0 eq)

α -Terpinene ($\text{C}_{10}\text{H}_{16}$, 136.23 g/mol, 0.837 g/mL, 90%): 13 mL (71.9 mmol, 9.3 eq)

Ethanol abs.: 60 mL

Preparation

Ruthenium(III) chloride trihydrate and α -terpinene in ethanol abs. under Ar-atmosphere were refluxed 4 h. The solution was subsequently concentrated under reduced pressure to 2/3 of its original volume and allowed to stand overnight at $-20\text{ }^\circ\text{C}$. The precipitate formed was filtered off, washed with cold acetone ($-20\text{ }^\circ\text{C}$), diethyl ether and dried *in vacuo*.

Yield ($\text{C}_{20}\text{H}_{28}\text{Cl}_4\text{Ru}_2$; 612.39 g/mol)

4.36 g (92%) red crystals

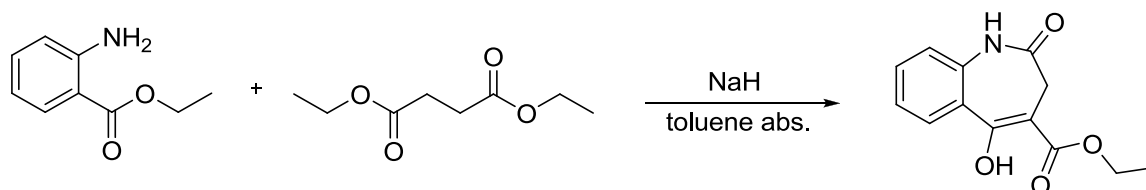
Analytical data

^1H NMR (500 MHz, $\text{DMSO}-d_6$)¹: 5.82 (d, 4H, $^3J = 6.3\text{ Hz}$, $4\times\text{H}^{\text{ar}}$), 5.77 (d, 4H, $^3J = 6.3\text{ Hz}$, $4\times\text{H}^{\text{ar}}$), 2.88–2.79 (m, 2H, $2\times\text{H}^{\text{ipr}}$), 2.09 (s, 6H, $2\times 3\text{H}^{\text{Me}}$), 1.19 (d, 12H, $^3J = 7.0\text{ Hz}$, $2\times 6\text{H}^{\text{ipr}}$) ppm. Data is in accordance with the literature.⁵⁷

¹ ipr = isopropyl, Me = methyl

2,3-Dihydro-5-hydroxy-2-oxo-1H-1-benzazepine-4-carboxylic acid ethyl ester

Reaction



Reagents

Ethyl anthranilate ($C_9H_{11}NO_2$; 615.19 g/mol): 57.6 g (348.7 mmol, 1.0 eq)

Diethyl succinate ($C_8H_{14}O_4$; 174.19 g/mol): 81.2 g (466.2 mmol, 1.3 eq)

Sodium hydride (24.00 g/mol; 60% dispersion in paraffin oil): 20.5 g (512.5 mmol, 1.5 eq)

Toluene abs.: 800 mL

Preparation

Ethyl anthranilate, diethyl succinate and sodium hydride were refluxed under Ar-atmosphere in toluene abs. for 4 h. After cooling to room temperature hydrochloric acid (10%, 800 mL) was added. The suspension was subsequently mixed thoroughly and petroleum ether (500 mL; bp 30-40 °C) was added. Then the precipitate formed was filtered off, washed with petroleum ether, H_2O dist., 3% $NaHCO_3$ aq., repeatedly with H_2O dist., petroleum ether and finally dried *in vacuo* at 60 °C.

Yield ($C_{13}H_{13}NO_4$; 247.25 g/mol)

41.47 g (48%) white solid

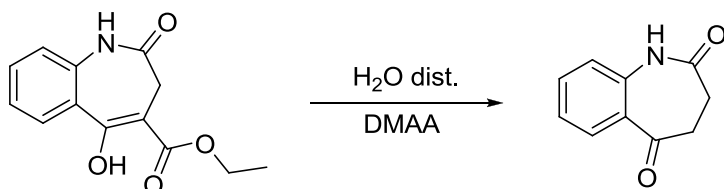
Analytical data

1H NMR (500 MHz, $DMSO-d_6$)²: 12.56 (s, 1H, OH), 10.33 (s, 1H, NH), 7.79 (dd, 1H, $^3J = 8.0$ Hz, $^4J = 1.5$ Hz, H^{ar}), 7.56–7.51 (m, 1H, H^{ar}), 7.28–7.24 (m, 1H, H^{ar}), 7.20–7.17 (m, 1H, H^{ar}), 4.30 (q, 2H, $^3J = 7.1$ Hz, $2H^{Et}$), 2.94 (s, 2H), 1.31 (t, 3H, $^3J = 7.1$ Hz, $3H^{Et}$) ppm. Data is in accordance with the literature.⁵⁸

² ar = aromatic, Et = ethyl

3,4-Dihydro-1H-1-benzazepine-2,5-dione

Reaction



Reagents

2,3-Dihydro-5-hydroxy-2-oxo-1H-1-benzazepine-4-carboxylic acid ethyl ester (C₁₃H₁₃NO₄; 247.25 g/mol): 27.730 g (112.2 mmol, 1.0 eq)
H₂O dist. (18.02 g/mol): 5 mL (277.5 mmol, 2.5 eq)
DMAA: 300 mL

Preparation

To a solution of enol ester in DMAA under stirring, H₂O dist. was added. The reaction mixture was heated under reflux for 4 h. Then the solvent was removed under reduced pressure. The resulting precipitate was suspended in acetone under sonication, filtered off and dried *in vacuo* at 60 °C.

Yield (C₁₀H₉NO₂; 175.18 g/mol)

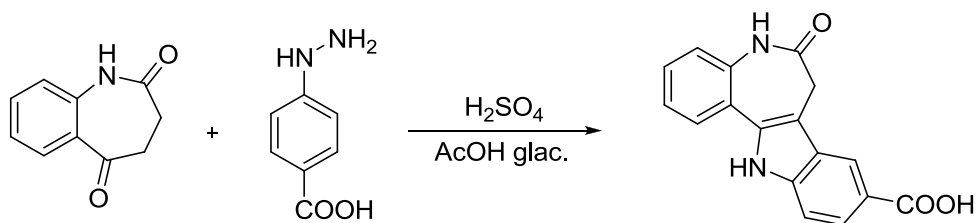
9.48 g (48%) fawn solid

Analytical determination

¹H NMR (500 MHz, DMSO-*d*₆)²: 10.08 (s, 1H, NH), 7.82 (dd, 1H, ³*J* = 7.8 Hz, ⁴*J* = 1.7 Hz, H^{ar}), 7.57–7.52 (m, 1H, H^{ar}), 7.20–7.14 (m, 2H, 2×H^{ar}), 2.93–2.88 (m, 2H), 2.69–2.64 (m, 2H) ppm. Data is in accordance with literature.⁵⁸

6-Oxo-5,6,7,12-tetrahydroindolo[3,2-d][1]benzazepine-9-carboxylic acid

Reaction



Reagents

3,4-Dihydro-1H-1-benzazepine-2,5-dione (C₁₀H₉NO₂; 175.18 g/mol): 9.48 g (54.1 mmol, 1.0 eq)

4-Hydrazinobenzoic acid (C₇H₈N₂O₂, 152.15 g/mol): 9.63 g (63.3 mmol, 1.2 eq)

Sulfuric acid (98.08 g/mol, 1.84 g/mL, 96%): 4 mL (72.0 mmol, 1.3 eq)

Acetic acid glac.: 90 mL

Preparation

Acetic acid glac. was added to benzazepinedione and hydrazinobenzoic acid under Ar-atmosphere and the reaction mixture was stirred at 75 °C for 1 h. Then sulfuric acid was added and heating continued for 3 h. The mixture was allowed to stand at room temperature overnight. The precipitate formed was filtered off, washed with H₂O dist., diethyl ether and dried *in vacuo* at 60 °C.

Yield (C₁₇H₁₂N₂O₃; 292.29 g/mol)

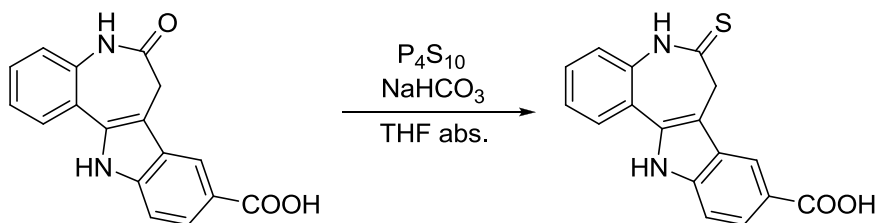
12.17 g (77%) white solid

Analytical determination

¹H NMR (500 MHz, DMSO-*d*₆)²: 12.43 (brs, 1H, OH), 11.97 (s, 1H, NH), 10.14 (s, 1H, NH), 8.31 (s, 1H, H^{ar}), 7.82–7.74 (m, 2H, 2×H^{ar}), 7.50 (d, 1H, ³J = 8.6 Hz, H^{ar}), 7.43–7.38 (m, 1H, H^{ar}), 7.32–7.25 (m, 2H, 2×H^{ar}), 3.54 (s, 2H) ppm. Data is in accordance with the literature.⁵⁹

5,6,7,12-Tetrahydro-6-thioxo-indolo[3,2-d][1]benzazepine-9-carboxylic acid

Reaction



Reagents

6-Oxo-5,6,7,12-tetrahydroindolo[3,2-d][1]benzazepine-9-carboxylic acid (C₁₇H₁₂N₂O₃; 292.29 g/mol): 7.68 g (26.3 mmol, 1.0 eq)

Phosphorus pentasulfide: (444.56 g/mol): 12.29 g (27.6 mmol, 1.1 eq)

Sodium hydrogen carbonate (84.01 g/mol): 18.43 g (219.3 mmol, 8.4 eq)

THF abs.: 550 mL

Triethylamine (101.19 g/mol, 0.728 g/mL): 14.5 mL (104.3 mmol, 4.0 eq)

Acetic acid (60.05 g/mol, 1.048 g/mL): 6 mL (104.7 mmol, 4.0 eq)

Preparation

The lactam-containing-paullone and sodium hydrogen carbonate were dissolved in THF abs under Ar-atmosphere and phosphorus pentasulfide was added. The solution was refluxed for 4 h. Then it was poured on ice (2 L) and allowed to stand at room temperature overnight. The precipitate formed was filtered off, dissolved with THF in a round-bottom flask and the solvent was removed under reduced pressure. The solid was dissolved in triethylamine and methanol (300 mL) and the remains were filtered off. The filtrate was neutralised with acetic acid and concentrated under reduced pressure until first precipitation could be observed. After addition of H₂O dist. (10 mL), the solid was filtered off, washed with H₂O dist., acetone and dried *in vacuo* at 80 °C.

Yield (C₁₇H₁₂N₂O₂S; 308.36 g/mol)

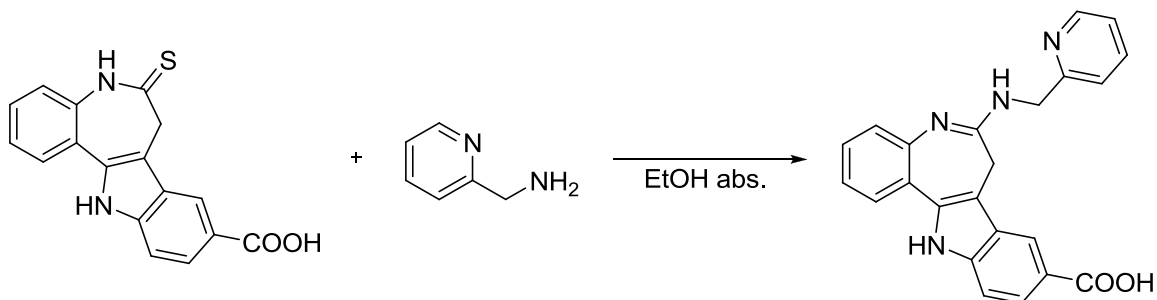
2.99 g (37%) yellow solid

Analytical data

^1H NMR (500 MHz, $\text{DMSO-}d_6$)²: 12.13–12.07 (m, 2H, 2×NH), 8.30 (s, 1H, H^{ar}), 7.84–7.79 (m, 2H, 2× H^{ar}), 7.52 (d, 1H, $^3J = 8.6$ Hz, H^{ar}), 7.47–7.40 (m, 3H, 3× H^{ar}), 3.95 (s, 2H) ppm. ^1H signal of carboxylic group could not be observed. Data is in accordance with the literature.⁶⁰

7,12-Dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine-9-carboxylic acid (L**¹)**

Reaction



Reagents

5,6,7,12-Tetrahydro-6-thion-indolo[3,2-*d*][1]benzazepine-9-carboxylic acid (C₁₇H₁₂N₂O₂S; 308.36 g/mol): 0.50 g (1.6 mmol, 1.0 eq)

2-(Aminomethyl)pyridine (C₆H₈N₂, 108.14 g/mol, 1.049 g/mL): 0.5 mL (4.9 mmol, 3.0 eq)

Ethanol abs.: 14 mL

Preparation

To a suspension of thionated paullone in ethanol abs. 2-(aminomethyl)pyridine was added. The mixture was refluxed for 24 h. Then the precipitate formed was filtered off and washed with ethanol. Recrystallisation in THF abs. (25 mL) afforded the product, which was washed with acetone and finally dried *in vacuo* at 80 °C.

Yield (C₂₃H₁₈N₄O₂; 382.41 g/mol)

0.5 g (80%) white solid

Analytical data

HR-ESI-MS (methanol, positive): *m/z* 383.1513 [**L**¹+H]⁺.

IR (ATR, selected bands, ν_{max}): 1649, 1608, 1527, 1430, 1361, 1321, 1255, 901, 753, 676, 644, 615 cm⁻¹.

¹H NMR (500 MHz, DMSO-*d*₆): 12.33 (brs, 1H, H^{9a}), 11.78 (s, 1H, H¹²), 8.49 (d, 1H, *J* = 4.1 Hz, H¹⁹), 8.43 (s, 1H, H⁸), 7.98–7.88 (m, 1H, H¹³), 7.76 (d, 1H, ³*J* = 8.5 Hz, H¹⁰),

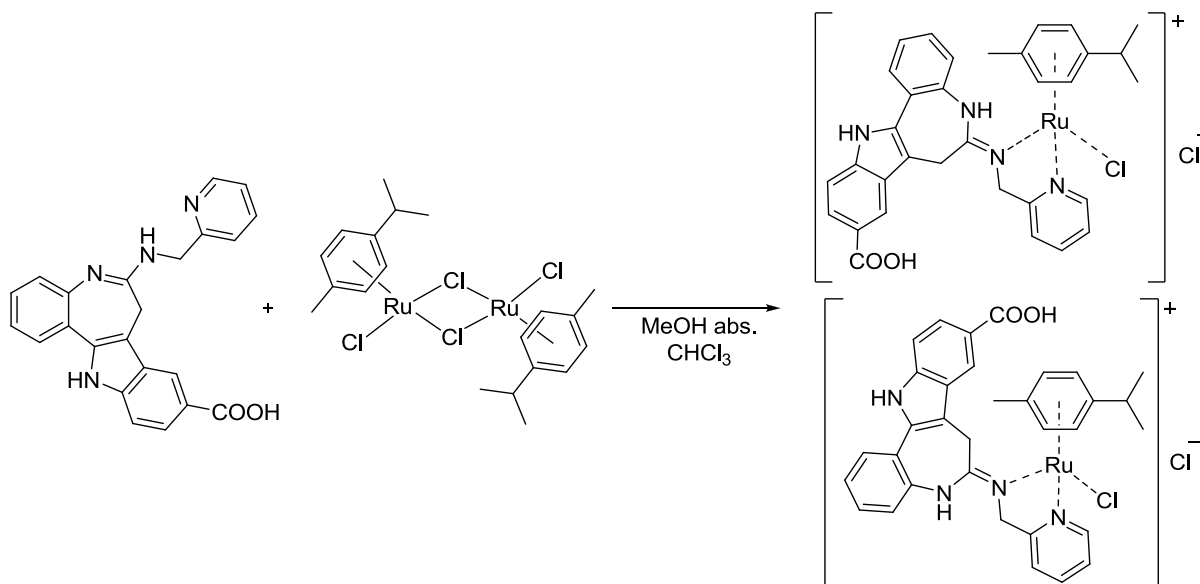
7.73–7.62 (m, 2H, $H^1 + H^{17}$), 7.45 (d, 1H, $^3J = 8.5$ Hz, H^{11}), 7.32–7.18 (m, 3H, $H^{16} + H^3 + H^{18}$), 7.13–7.00 (m, 2H, $H^4 + H^2$), 4.5 (d, 2H, $^3J = 3.9$ Hz, H^{14}), 3.47 (s, 2H, H^7) ppm.

^{13}C NMR (125 MHz, $\text{DMSO-}d_6$): 168.93 (Cq, C^{9a}), 159.11 (Cq, C^{15}), 155.00 (Cq, C^6), 149.27 (CH, C^{19}), 146.52 (Cq, C^{4a}), 140.19 (Cq, C^{11a}), 137.00 (CH, C^{17}), 135.70 (Cq, C^{12a}), 127.96 (CH, C^4), 127.80 (CH, C^3), 126.96 (CH, C^1), 126.53 (Cq, C^{7b}), 123.24 (CH, C^{10}), 122.49 (CH, C^{18}), 122.35 (Cq, C^{12b}), 121.97 (CH, C^{16}), 121.22 (CH, C^8), 120.95 (CH, C^2), 111.29 (CH, C^{11}), 110.31 (Cq, C^{7a}), 47.10 (CH_2 , C^{14}), 28.23 (CH_2 , C^7) ppm. C^9 signal could not be observed.

Data is in accordance with the literature, besides the not detected C^9 signal.⁶⁰

Chlorido(η^6 -*p*-cymene){7,12-dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine-9-carboxylic acid}ruthenium(II) chloride – [RuCl(cymene)L¹]Cl (1)

Reaction



Reagents

7,12-Dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine-9-carboxylic acid (**L**¹) (C₂₃H₁₈N₄O₂; 382.41 g/mol): 0.11 g (0.3 mmol, 2 eq)

Bis[chlorido-μ-chlorido(η^6 -*p*-cymene)ruthenium(II)] (C₂₀H₂₈Cl₄Ru₂; 612.39 g/mol): 0.09 g (0.1 mmol, 1 eq)

Methanol abs.: 4 mL

Chloroform: 0.5 mL

Preparation

Under Ar-atmosphere a solution of ruthenium-dimer in chloroform was added dropwise to a solution of imine-modified paullone in methanol abs.. Afterwards the solution was stirred at room temperature for 24 h and filtered. By slowly adding the filtrate to diethyl ether (dried over Na₂SO₄) yellow solid precipitated. This was filtered off and dried *in vacuo* at 50 °C.

Crystals in *E*- and *Z*-configuration could be obtained from a solution of methanol and diethyl ether, stored 4 days at 4 °C, and a solution of ethanol and hexane, stored 3 days at 4 °C, respectively.

Yield ($C_{33}H_{32}Cl_2N_4O_2Ru$; 688.61 g/mol)

0.17 g (89%) yellow solid

Analytical data

HR-ESI-MS (methanol, positive): m/z 653.1270 [$1-Cl$]⁺.

IR (ATR, selected bands, ν_{max}): 3059, 2968, 1689, 1622, 1570, 1381, 1217, 1038, 875, 763, 645, 601 cm^{-1} .

UV-vis (H_2O), λ_{max} (ϵ , $M^{-1}cm^{-1}$): 256 (46744) nm.

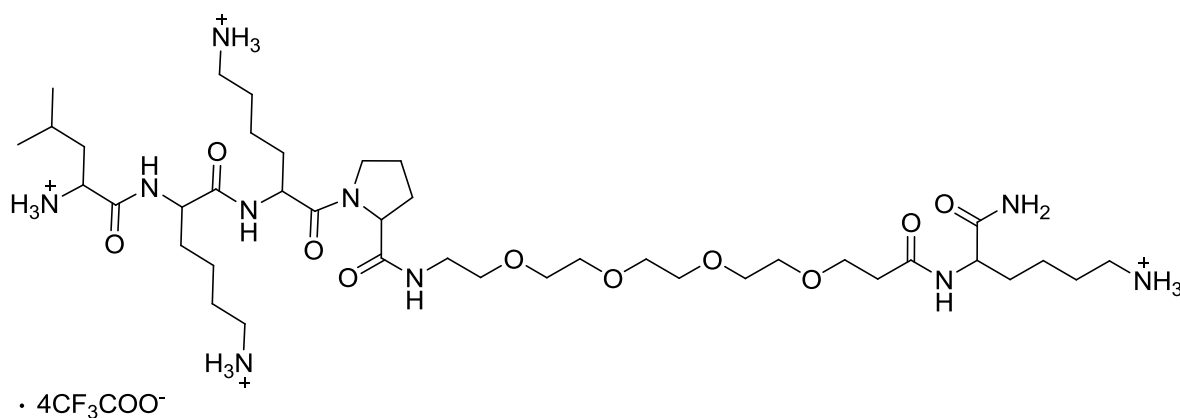
1H NMR (500 MHz, $DMSO-d_6$)^c: 12.28 (brs, 2H, $H^{9a(E)+(Z)}$), 12.23 (s, 1H, $H^{12(E)}$), 11.97 (s, 1H, $H^{12(Z)}$), 9.62 (s, 1H, $H^{5(Z)}$), 9.28 (s, 1H, $H^{5(E)}$), 9.02–8.96 (m, 2H, $2 \times H^{19(E)+(Z)}$), 8.81 (s, 1H, $H^{8(Z)}$), 8.62 (s, 1H, $H^{8(E)}$), 8.08 (dt, 1H, $^3J = 7.8$ Hz, $^4J = 1.3$ Hz, $H^{17(E)}$), 7.96 (dt, 1H, $^3J = 7.8$ Hz, $^4J = 1.4$ Hz, $H^{17(Z)}$), 7.89–7.85 (m, 2H, $H^{1(E)} + H^{10(E)}$), 7.81 (d, 1H, $^3J = 7.8$ Hz, $^4J = 1.2$ Hz, $H^{1(Z)}$), 7.77–7.72 (m, 2H, $H^{4(Z)} + H^{10(Z)}$), 7.68 (d, 1H, $^3J = 7.8$ Hz, $H^{16(E)}$), 7.62 (t, 1H, $^3J = 7.8$ Hz, $H^{18(E)}$), 7.58 (d, 1H, $^3J = 8.6$ Hz, $H^{11(E)}$), 7.55–7.46 (m, 3H, $H^{18(Z)} + 2 \times H^{3(E)+(Z)}$), 7.45–7.35 (m, 4H, $H^{11(Z)} + H^{16(Z)} + 2 \times H^{2(E)+(Z)}$), 7.18 (d, 1H, $^3J = 8.1$ Hz, $H^{4(E)}$), 6.05–5.98 (m, 2H, $H^{22(Z)} + H^{21(Z)}$), 5.96–5.89 (m, 2H, $H^{20(Z)} + H^{14(E)}$), 5.86 (d, 1H, $^3J = 5.9$ Hz, $H^{23(Z)}$), 5.82 (d, 1H, $^3J = 6.1$ Hz, $H^{22(E)}$), 5.73–5.70 (m, 1H, $H^{21(E)}$), 5.61–5.57 (m, 2H, $H^{20(E)} + H^{23(E)}$), 5.19–4.95 (m, 5H, $2 \times H^{14(Z)} + H^{14(E)} + 2 \times H^{7(E)+(Z)}$), 3.80 (d, 1H, $^2J = 14.2$ Hz, $H^{7(Z)}$), 3.47–3.40 (m, 1H, $H^{25(E)}$), 2.87–2.79 (m, 1H, $H^{25(Z)}$), 2.33 (s, 3H, $H^{24(Z)}$), 1.79 (s, 3H, $H^{24(E)}$), 1.24 (d, 3H, $^3J = 6.9$ Hz, $H^{27(Z)}$), 1.18 (d, 3H, $^3J = 6.9$ Hz, $H^{26(Z)}$), 0.99 (d, 3H, $^3J = 6.9$ Hz, $H^{27(E)}$), 0.91 (d, 3H, $^3J = 6.9$ Hz, $H^{26(E)}$) ppm. $H^{7(E)}$ signal under DMSO-solvent peak (2.53–2.48 ppm).

^{13}C NMR (125 MHz, $DMSO-d_6$): 168.86 (Cq, $C^{9a(Z)}$), 168.78 (Cq, $C^{9a(E)}$), 167.08 (Cq, $C^{6(E)}$), 165.35 (Cq, $C^{6(Z)}$), 161.51 (Cq, $C^{15(E)}$), 160.36 (Cq, $C^{15(Z)}$), 155.13 (CH, $C^{19(Z)}$), 154.85 (CH, $C^{19(E)}$), 140.37 (Cq, $C^{11a(E)}$), 140.18 (Cq, $C^{11a(Z)}$), 139.89 (CH, $C^{17(E)}$), 139.58 (CH, $C^{17(Z)}$), 136.11 (Cq, $C^{4a(Z)}$), 135.72 (Cq, $C^{4a(E)}$), 135.48 (Cq, $C^{12a(E)}$), 133.74 (Cq, $C^{12a(Z)}$), 129.52 (CH, $C^{3(E)}$), 128.42 (CH, $C^{3(Z)}$), 127.83 (CH, $C^{1(E)}$), 127.49 (CH, $C^{1(Z)}$), 126.70 (Cq, $C^{7b(Z)}$),

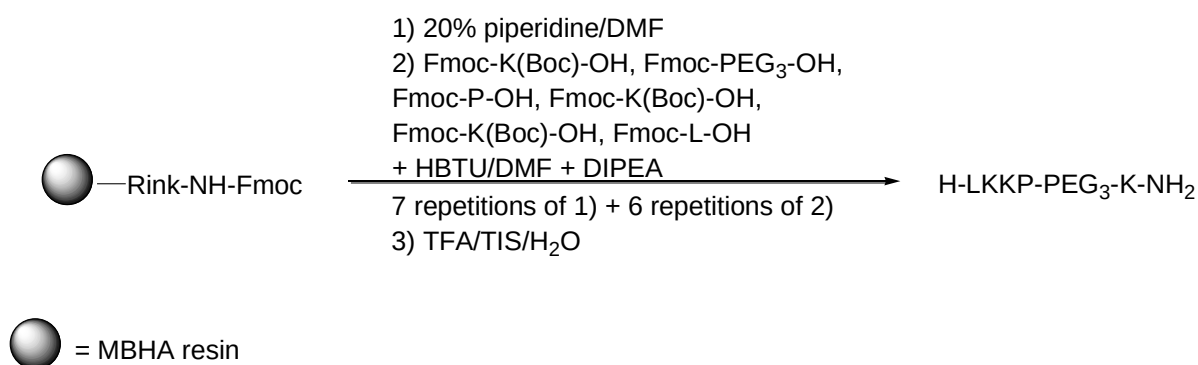
^c Actual ratio of *E/Z*-isomers is not considered in this data, as it is dependent on various parameters, including time, pH, etc. and was not determined at the equilibrium.

126.50 (Cq, C^{7b(E)}), 125.57 (CH, C^{18(E)}), 125.23 (CH, C^{18(Z)}), 124.82 (CH, C^{2(Z)}), 124.53 (CH, C^{2(E)}), 124.17 (CH, C^{10(E)}), 123.86 (CH, C^{8(Z)}), 123.82 (CH, C^{10(Z)}), 123.44 (CH, C^{4(Z)}), 122.82 (Cq, C^{9(E)}), 122.60 (Cq, C^{12b(Z)}), 121.85 (Cq, C^{12b(E)}), 121.71 (Cq, C^{9(Z)}), 121.66 (CH, C^{4(E)}), 121.48 (CH, C^{16(E)}), 121.37 (CH, C^{8(E)}), 121.07 (CH, C^{16(Z)}), 111.99 (CH, C^{11(E)}), 111.18 (CH, C^{11(Z)}), 111.07 (Cq, C^{7a(Z)}), 108.91 (Cq, C^{7a(E)}), 105.42 (Cq, C^{21a(Z)}), 105.19 (Cq, C^{21a(E)}), 100.43 (Cq, C^{23a(Z)}), 99.76 (Cq, C^{23a(E)}), 85.27 (CH, C^{20(E)}/C^{23(E)}), 85.19 (CH, C^{20(Z)}), 84.97 (CH, C^{22(E)}), 84.67 (CH, C^{21(Z)}), 84.32 (CH, C^{21(E)}), 84.10 (CH, C^{23(Z)}), 83.64 (CH, C^{23(E)}/C^{20(E)}), 82.76 (CH, C^{22(Z)}), 61.66 (CH₂, C^{14(Z)}), 61.56 (CH₂, C^{14(E)}), 33.00 (CH₂, C^{7(Z)}), 31.00 (CH, C^{25(Z)}), 30.63 (CH, C^{25(E)}), 24.39 (CH₂, C^{7(Z)}), 22.81 (CH₃, C^{26(Z)}), 22.34 (CH₃, C^{27(E)}), 22.14 (CH₃, C^{27(Z)}), 22.00 (CH₃, C^{26(E)}), 18.57 (CH₃, C^{24(Z)}), 17.64 (CH₃, C^{24(E)}) ppm.

L-Valinyl-di(L-lysanyl)-L-prolinyl-(15-amino-4,7,10,13-tetraoxapentadecanoic)-L-lysine – H-LKKP-PEG₃-K-NH₂ (P)



Reaction



Reagents^d

Rink MBHA resin (loading: 0.62 mmol/g; 100–200 mesh): 323 mg (0.2 mmol, 1 eq)

Fmoc-K(Boc)-OH (468.54 g/mol): 1.: 469 mg (1 mmol, 5 eq)

2.+3., each: 234 mg (0.5 mmol, 2.5 eq)

Fmoc-P-OH·H₂O (355.37 g/mol): 178 mg (0.5 mmol, 2.5 eq)

Fmoc-L-OH (353.40 g/mol): 177 mg (0.5 mmol, 2.5 eq)

Fmoc-PEG₃-OH (487.55 g/mol): 244 mg (0.5 mmol, 2.5 eq)

HBTU (379.25 g/mol, used as 0.5 M HBTU/DMF):

1.aa: 1.920 mL (0.96 mmol, 4.8 eq)

^d All used aa are L-configured.

2.–6.aa: 0.960 mL (0.48 mmol, 2.4 eq)
DIPEA (129.24 g/mol; 0.755 g/mL): 1.aa: 0.342 mL (2 mmol, 10 eq)
2.–6.aa: 0.171 mL (1 mmol, 5 eq)
20% piperidine/DMF: 2×20 mL (7×)
DMF: ~20 mL for swelling and in each washing cycle
CH₂Cl₂
TFA/TIS/H₂O dist. (92.5/5/2.5%)^e: 5 mL
Diethyl ether
ACN/H₂O

Preparation

Synthesis of H-LKKP-PEG₃-K-NH₂ was carried out in a 50 mL plastic fritted syringe in combination with a rotating device on a Rink MBHA resin *via* SPPS using Fmoc-strategy, starting with lysine at the C-terminus. After 1 h swelling of the resin in DMF, Fmoc was removed *via* 20% piperidine/DMF (3 min incubation, 1×1 min washing with DMF, 7 min incubation) and the first aa, dissolved in HBTU/DIPEA/DMF, was coupled to the free amino group for 40 min, forming an amide bond. Then 5×1 min washing with DMF was performed. Repetition of the chronological steps Fmoc-removal (6×), washing (6×), coupling (5×) and again washing (5×) finally led to the Boc-protected, resin-linked peptide, H-LK(Boc)K(Boc)P-PEG₃-K(Boc)-NH-resin, which was subsequently dried by incubation in dichloromethane (5×1 min) and storage in the desiccator for at least 30 min. Completeness of the coupling steps was confirmed through the Kaiser test and quantitative, photometric determination of the Fmoc-cleaved chromophore at 301 nm. In order to achieve deprotection and cleavage, a mixture of TFA/TIS/H₂O dist. was added to the dried resin and allowed to react 2 h. Thereafter, the solution was collected in a falcon tube and combined with 300 µL TFA from the resin washing. Precipitation was performed by addition of 20 mL diethyl ether (cooled at 4 °C) and 15 min incubation in an ice bath. After centrifugation (4 °C, 5 min, 5500 rpm/5411 g) the supernatant was removed. Hereafter, the precipitate was twice mixed with diethyl ether, 10 min incubated, centrifuged and the supernatant solutions decanted. The crude product was dried under Ar-stream, lyophilised in ACN/H₂O (1/1) and stored at –20 °C.

Purification was carried out with RP-HPLC on a preparative scale with acetonitrile and MilliQ water (18.2 MΩ, Millipore Advantage A10, 185 UV Ultrapure Water System,

^e Amount depends on weight of dried Boc-protected, resin-linked peptide (generally: 10–20 mL are required for 1 g).

Molsheim, France), both supplemented with 0.05% TFA, following a linear gradient (15→35% acetonitrile with a slope of 1.3% per min) with 15 mL/min.

Yield ($C_{40}H_{78}N_{10}O_{10} \cdot 4CF_3COOH$, 1315.20 g/mol)

0.169 g (64%) white lyophilisate

Analytical data

HR-ESI-MS (methanol, positive): m/z 859.5958 $[P+H]^+$, 430.3029 $[P+2H]^{2+}$, 287.2047 $[P+3H]^{3+}$.

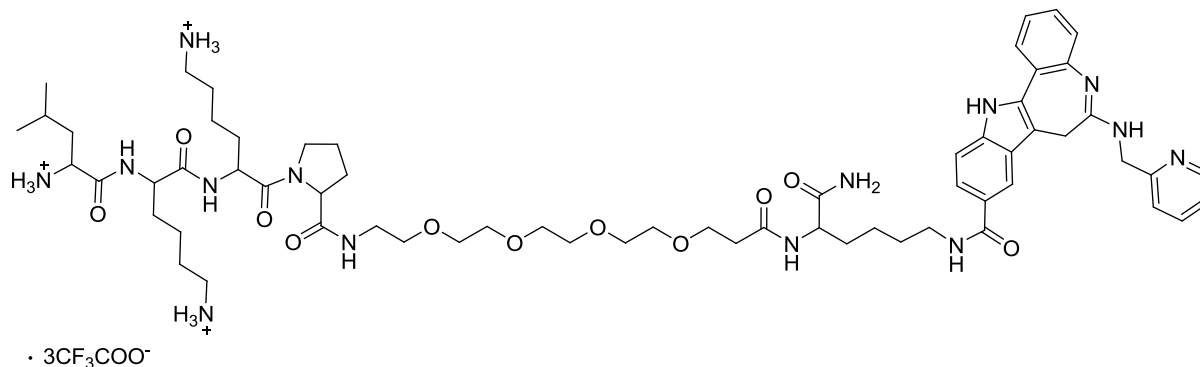
UV-vis (H_2O/ACN , 1/1), λ_{max} : 210 nm.

Analytical HPLC (solvent: acetonitrile(0.05%TFA)/MilliQ water, linear gradient: 5→45% acetonitrile with slope of 1% per min, 1 mL/min): t_R = 15.244 min (214 nm: h = 1246.906 mAU, A = 324.075 mAU*min (99%)).

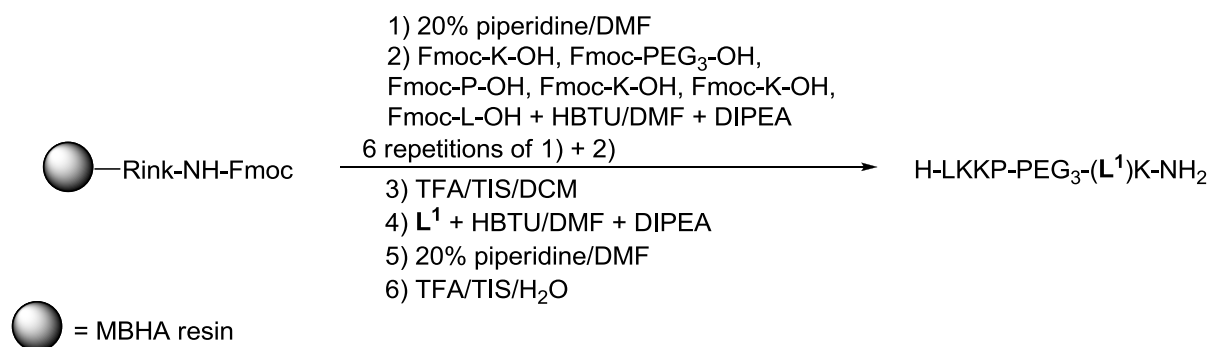
1H NMR (500 MHz, $DMSO-d_6$): 8.61 (d, 1H, 3J = 8.1 ppm, H^4), 8.29–7.63 (m, 15H, $H^7 + H^{27} + 3H^1 + H^{15} + 3H^{5e} + 3H^{8e} + 3H^{28e}$), 7.26 (s, 1H, H^{30}), 7.00 (s, 1H, H^{30}), 4.47–4.40 (m, 1H, H^8), 4.36–4.28 (m, 1H, H^5), 4.28–4.23 (m, 1H, H^{13}), 4.18–4.11 (m, 1H, H^{28}), 3.84–3.77 (m, 1H, H^2), 3.67–3.53 (m, 4H, $2 \times H^{10} + 2H^{24}$), 3.52–3.46 (m, 12H, $2H^{18} + 2H^{19} + 2H^{20} + 2H^{21} + 2H^{22} + 2H^{23}$), 3.28–3.22 (m, 1H, H^{16}), 3.15–3.07 (m, 1H, H^{16}), 2.81–2.66 (m, 6H, $2H^{5d} + 2H^{8d} + 2H^{28d}$), 2.44–2.32 (m, 2H, $2 \times H^{25}$), 2.07–1.98 (m, 1H, H^{12}), 1.96–1.88 (m, 1H, H^{11}), 1.86–1.79 (m, 1H, H^{11}), 1.78–1.71 (m, 1H, H^{12}), 1.70–1.59 (m, 4H, $H^{2b} + H^{5a} + H^{8a} + H^{28a}$), 1.58–1.43 (m, 11H, $2 \times H^{2a} + H^{5a} + H^{8a} + H^{28a} + 2H^{5c} + 2H^{8c} + 2H^{28c}$), 1.39–1.21 (m, 6H, $2H^{5b} + 2H^{8b} + 2H^{28b}$), 0.91–0.84 (m, 6H, $3H^{2c} + 3H^{2d}$) ppm. $2H^{17}$ signals under H_2O -solvent peak (3.43–3.31 ppm).

^{13}C NMR (125 MHz, $DMSO-d_6$): 174.15 (Cq, C^{29}), 172.12 (Cq, C^{14}), 171.25 (Cq, C^6), 170.65 (Cq, C^{26}), 170.20 (Cq, C^9), 169.28 (Cq, C^3), 70.23 (CH_2 , $C^{19/20/21/22}$), 70.20 (CH_2 , $C^{19/20/21/22}$), 70.16 (CH_2 , $C^{19/20/21/22}$), 70.13 (CH_2 , $C^{19/20/21/22}$), 70.03 (CH_2 , C^{18}), 69.95 (CH_2 , C^{23}), 69.43 (CH_2 , C^{17}), 67.39 (CH_2 , C^{24}), 59.91 (CH, C^{13}), 52.68 (CH, C^5), 52.52 (CH, C^{28}), 51.18 (CH, C^2), 50.76 (CH, C^8), 47.30 (CH_2 , C^{10}), 40.72 (CH_2 , C^{2a}), 39.15 (CH_2 , $C^{5d/8d}$), 39.12 ($2CH_2$, $C^{28d} + 5d/8d$), 38.94 (CH_2 , C^{16}), 36.42 (CH_2 , C^{25}), 31.93 (CH_2 , C^{5a}), 31.75 (CH_2 , C^{28a}), 30.79 (CH_2 , C^{8a}), 29.89 (CH_2 , C^{12}), 27.12 ($2CH_2$, $C^{5c/8c/28c}$), 27.08 (CH_2 , $C^{5c/8c/28c}$), 24.93 (CH_2 , C^{11}), 23.94 (CH, C^{2b}), 23.15 (CH_3 , C^{2d}), 22.82 ($2CH_2$, $C^{28b} + 5b$), 22.35 (CH_3 , C^{2c}) ppm. Both shifts of 22.61(CH_2) and 22.25(CH_2) ppm might be correlated with C^{8b} , an exact determination is not possible.

***L*-Valinyl-di(*L*-lysinyl)-*L*-prolinyl-(15-amino-4,7,10,13-tetraoxapentadecanoic)-(ε-(7,12-dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine)-9-amide)-*L*-lysine – H-LKKP-PEG₃-(*L*¹)K-NH₂ (*L*²)**



Reaction



Reagents^d

Rink MBHA resin (loading: 0.62 mmol/g; 100–200 mesh): 323 mg (0.2 mmol, 1 eq)

Fmoc-K(Mtt)-OH (624.79 g/mol): 625 mg (1 mmol, 5 eq)

Fmoc-K(Boc)-OH (468.54 g/mol): 2.+3., each: 234 mg (0.5 mmol, 2.5 eq)

Fmoc-P-OH·H₂O (355.37 g/mol): 178 mg (0.5 mmol, 2.5 eq)

Fmoc-L-OH (353.40 g/mol): 177 mg (0.5 mmol, 2.5 eq)

Fmoc-PEG₃-OH (487.55 g/mol): 244 mg (0.5 mmol, 2.5 eq)

7,12-Dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine-9-carboxylic acid (*L*¹) (C₂₃H₁₈N₄O₂; 382.41 g/mol): 115 mg (0.3 mmol, 1.5 eq)

HBTU (379.25 g/mol, used as 0.5 M HBTU/DMF):

combined with 300 μ L TFA from the resin washing. Precipitation was performed by addition of 20 mL diethyl ether (cooled at 4 °C) and 15 min incubation in an ice bath. After centrifugation (4 °C, 5 min, 5500 rpm/5411 g) the supernatant was removed. Hereafter, the precipitate was twice mixed with diethyl ether, 10 min incubated, centrifuged and the supernatant solutions decanted. The crude product was dried under Ar-stream, lyophilised in ACN/H₂O (1/1) and stored at –20 °C.

Purification was carried out with RP-HPLC on a preparative scale with acetonitrile and MilliQ water (18.2 M Ω , Millipore Advantage A10, 185 UV Ultrapure Water System, Molsheim, France), both supplemented with 0.05% TFA, following a linear gradient (15→28% acetonitrile with a slope of 1% per min) with 15 mL/min.

Yield (C₆₃H₉₄N₁₄O₁₁·3CF₃COOH, 1565.58 g/mol)

0.185 g (59%) white lyophilisate

Analytical data

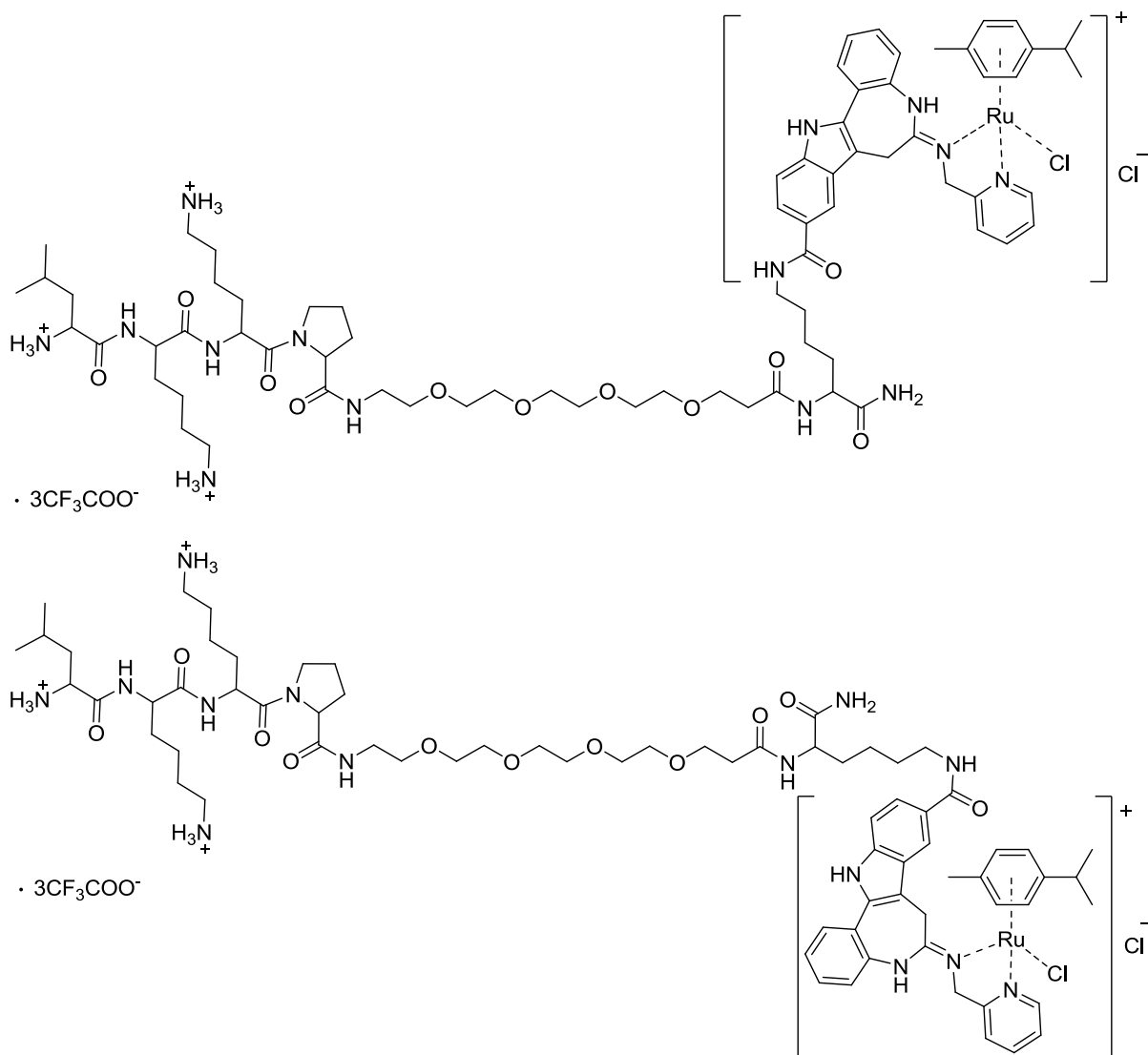
HR-ESI-MS (methanol, positive): *m/z* 1223.7288 [L²+H]⁺, 612.3694 [L²+2H]²⁺, 408.5829 [L²+3H]³⁺.

UV-vis (H₂O(+0.05% TFA)/ACN(+0.05% TFA), 72/28), λ_{max} : 249 nm.

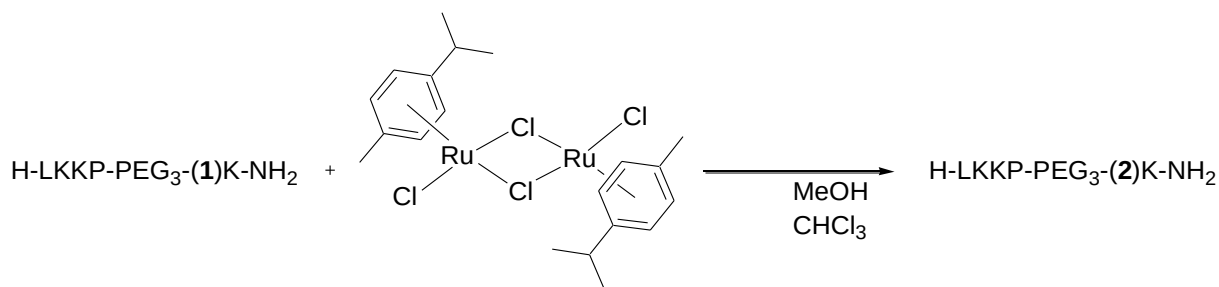
Analytical HPLC (solvent: acetonitrile(0.05%TFA)/MilliQ water, linear gradient: 5→65% acetonitrile with slope of 2% per min, 1 mL/min): *t_R* = 14.204 min (250 nm: *h* = 527.702 mAU, *A* = 59.780 mAU*min (98%)).

¹H NMR (500 MHz, DMSO-*d*₆): 12.16 (s, 1H, H¹²), 8.62 (d, 1H, ³*J* = 4.6 Hz, H¹⁹), 8.58 (d, 1H, ³*J* = 7.9 Hz, H⁴), 8.36 (t, 1H, ³*J* = 5.5 Hz, H^{28e}), 8.33 (s, 1H, H⁸), 8.18–8.10 (m, 4H, 3H¹ + H⁷), 7.95 (d, 1H, ³*J* = 8.2 Hz, H²⁷), 7.91–7.87 (m, 2H, H¹⁵ + H¹), 7.85 (t, 1H, ³*J* = 7.7 Hz, H¹⁷), 7.82–7.71 (m, 7H, 3H^{5e} + 3H^{8e} + H¹⁰), 7.63–7.52 (m, 3H, H² + H³ + H⁴), 7.50 (d, 1H, ³*J* = 8.6 Hz, H¹¹), 7.45 (d, 1H, ³*J* = 7.7 Hz, H¹⁶), 7.42–7.38 (m, 1H, H¹⁸), 7.24 (s, 1H, H³⁰), 6.96 (s, 1H, H³⁰), 4.83 (2H, ³*J* = 3.6 Hz, H¹⁴), 4.47–4.41(m, 1H, H⁸), 4.36–4.30 (m, 1H, H⁵), 4.28–4.23 (m, 1H, H¹³), 4.19–4.13 (m, 1H, H²⁸), 4.02 (s, 2H, H⁷), 3.84–3.77 (m, 1H, H²), 3.68–3.52 (m, 4H, 2×H¹⁰ + 2H²⁴), 3.52–3.44 (m, 12H, 2H¹⁸ + 2H¹⁹ + 2H²⁰ + 2H²¹ + 2H²² + 2H²³), 3.29–3.23 (m, 3H, H¹⁶ + 2H^{28d}), 3.15–3.07 (m, 1H, H¹⁶), 2.79–2.70 (m, 4H, 2H^{5d} + 2H^{8d}), 2.43–2.34 (m, 2H, 2×H²⁵), 2.07–1.98 (m, 1H, H¹²), 1.96–1.87 (m, 1H, H¹¹), 1.86–1.79 (m, 1H, H¹¹), 1.78–1.59 (m, 5H, H¹² + H^{2b} + H^{5a} + H^{8a} + H^{28a}), 1.58–1.45 (m, 11H, 2×H^{2a} + H^{5a} + H^{8a} + H^{28a} + 2H^{5c} + 2H^{8c} + 2H^{28c}), 1.40–1.21 (m, 6H, 2H^{5b} + 2H^{8b} + 2H^{28b}), 0.92–0.84 (m, 6H, 3H^{2c} + 3H^{2d}) ppm. Shift of H¹³ could not be allocated. 2H¹⁷ signals under H₂O-solvent peak (3.43–3.31 ppm).

***L*-Valinyl-di(*L*-lysiny)-*L*-prolinyl-(15-amino-4,7,10,13-tetraoxapentadecanoic)-(ϵ -(chlorido(η^6 -*p*-cymene)){7,12-dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-*d*][1]benzazepine)-9-amide ruthenium(II) chloride)-*L*-lysine – H-LKKP-PEG₃-(L²)K-NH₂ (2)**



Reaction



Reagents

L-Valinyl-di(L-lysiny)-L-prolinyl-(15-amino-4,7,10,13-tetraoxapentadecanoic)-(ε-(7,12-dihydro-6-[(2-pyridinylmethyl)amino]-indolo[3,2-d][1]benzazepine)-9-amide)-L-lysine – H-LKKP-PEG₃-(L¹)K-NH₂ (L²) (C₆₃H₉₄N₁₄O₁₁·3CF₃COOH, 1565.58 g/mol): 73.4 mg (0.047 mmol, 2 eq)

Bis[chloride-μ-chlorido(η⁶-p-cymene)ruthenium(II)] (C₂₀H₂₈Cl₄Ru₂; 612.39 g/mol): 14.2 mg (0.023 mmol, 1 eq)

Methanol abs.: 2 mL

Chloroform: 0.5 mL

Preparation

Ruthenium-dimer dissolved in chloroform was added dropwise to the paullone-peptide conjugate L² in methanol abs. under Ar-atmosphere. The solution was stirred at room temperature for 3 h, filtered and subsequently concentrated under reduced pressure to 1/5 (bath-T: 30 °). To the residual methanolic solution acetonitrile (4 mL) was added and the mixture was lyophilised.

Yield (C₇₃H₁₀₈Cl₂N₁₄O₁₁Ru·3CF₃COOH, 1868.75 g/mol)

79.84 mg (91%) yellow solid

Analytical data

HR-ESI-MS (methanol, positive): *m/z* 747.3558 [2-Cl+H]²⁺, 498.5744 [2-Cl+2H]³⁺, 374.1824 [2-Cl+3H]⁴⁺, 366.2551 [L¹(-OH)+H]⁺, 365.1872 [2-2Cl+2H]⁴⁺.

UV-vis (H₂O), λ_{max} (ε, M⁻¹cm⁻¹): 259 (42634) nm.

Analytical HPLC (solvent: methanol(0.1% formic acid)/MilliQ water, linear gradient: 5→95% methanol with slope of 4.5% per min, 1 mL/min):

- isomeric peaks, non-baseline-separated: *t_R* = 12.122 min (250 nm: A = 247.122 mAU*min (96%)).
- isomeric peaks, separated at minimum in between: *t_R* = 11.862 min (250 nm: h = 612.254 mAU, A = 161.929 mAU*min (63%)); *t_R* = 12.282 min (250 nm: h = 281.224 mAU, A = 85.202 mAU*min (33%)).

¹H NMR (500 MHz, DMSO-*d*₆)^c: 12.12 (s, 1H, H^{12(E)}), 11.88 (s, 1H, H^{12(Z)}), 9.68 (s, 1H, H^{5(Z)}), 9.26 (s, 1H, H^{5(E)}), 9.02 (t, 1H, ³*J* = 6.2 Hz, H^{19(Z)}), 8.98 (d, 1H, ³*J* = 5.6 Hz, H^{19(E)}), 8.66–8.60 (d, 2H, ³*J* = 8.0 Hz, H^{4(E)+(Z)}), 8.58–8.53 (m, 2H, H^{8(E)+(Z)}), 8.47–8.41 (m, 1H, H^{28e(E)}), 8.24–8.14 (m, 8H, 3H^{1(E)+(Z)} + H^{7(E)+(Z)}), 8.08 (t, 1H, ³*J* = 7.7 Hz, H^{17(E)}), 8.03–7.68

(m, 23H, $3H^{8e(E)+(Z)} + 3H^{28e(E)+(Z)} + H^{27(E)+(Z)} + H^{15(E)+(Z)} + H^{28e(Z)} + H^{1(E)+(Z)} + H^{17(Z)} + H^{10(E)} + H^{4(Z)} + H^{16(E)}$), 7.64–7.58 (m, 2H, $H^{18(E)} + H^{10(Z)}$), 7.54–7.47 (m, 4H, $H^{11(E)} + H^{18(Z)} + H^{3(E)+(Z)}$), 7.42–7.35 (m, 4H, $H^{11(Z)} + H^{16(Z)} + H^{2(E)+(Z)}$), 7.29–7.23 (m, 2H, $H^{30(E)+(Z)}$), 7.19 (d, 1H, $^3J = 8.0$ Hz, $H^{4(E)}$), 7.02–6.95 (m, 2H, $H^{30(E)+(Z)}$), 6.12–6.06 (m, 2H, $H^{22(Z)} + H^{21(Z)}$), 5.98 (d, 1H, $^3J = 6.2$ Hz, $H^{20(Z)}$), 5.94–5.85 (m, 2H, $H^{23(Z)} + H^{14(E)}$), 5.81 (d, 1H, $^3J = 6.1$ Hz, $H^{22(E)}$), 5.70 (d, 1H, $^3J = 6.2$ Hz, $H^{21(E)}$), 5.62–5.58 (m, 2H, $H^{20(E)} + H^{23(E)}$), 5.15–5.06 (m, 2H, $H^{14(E)} + H^{14(Z)}$), 5.00–4.88 (m, 3H, $H^{14(Z)} + 2 \times H^{7(E)+(Z)}$), 4.47–4.40 (m, 2H, $H^{8(E)+(Z)}$), 4.37–4.29 (m, 2H, $H^{5(E)+(Z)}$), 4.28–4.14 (m, 4H, $H^{13(E)+(Z)} + H^{28(E)+(Z)}$), 3.52–3.46 (m, 24H, $2H^{18(E)+(Z)} + 2H^{19(E)+(Z)} + 2H^{20(E)+(Z)} + 2H^{21(E)+(Z)} + 2H^{22(E)+(Z)} + 2H^{23(E)+(Z)}$), 3.41–3.07 (m, 14H, $2H^{28d(E)+(Z)} + H^{25(E)} + 2 \times H^{10(E)+(Z)} + 2 \times H^{16(E)+(Z)} + 2H^{17(E)+(Z)}$), 2.93–2.86 (m, 1H, $H^{25(Z)}$), 2.80–2.69 (m, 8H, $2H^{5d(E)+(Z)} + 2H^{8d(E)+(Z)}$), 2.43–2.34 (m, 4H, $2 \times H^{25(E)+(Z)}$), 2.33 (s, 3H, $H^{24(Z)}$), 2.06–1.98 (m, 2H, $H^{12(E)+(Z)}$), 1.98–1.88 (m, 2H, $H^{11(E)+(Z)}$), 1.87–1.78 (m, 5H, $H^{11(E)+(Z)}$, $H^{24(E)}$), 1.79–1.47 (m, 32H, $H^{12(E)+(Z)} + 2 \times H^{2a(E)+(Z)} + H^{2b(E)+(Z)} + 2 \times H^{5a(E)+(Z)} + 2H^{5c(E)+(Z)} + 2 \times H^{8a(E)+(Z)} + 2H^{8c(E)+(Z)} + 2 \times H^{28a(E)+(Z)} + 2H^{28c(E)+(Z)}$), 1.44–1.29 (m, 14H, $2H^{5b(E)+(Z)} + 2H^{8b(E)+(Z)} + 2H^{28b(E)+(Z)} + H^{27(Z)}$), 1.21 (d, 3H, $^3J = 6.9$ Hz, $H^{26(Z)}$), 0.99 (d, 3H, $^3J = 6.9$ Hz, $H^{27(E)}$), 0.94–0.86 (m, 15H, $H^{26(E)} + 3H^{2c(E)+(Z)} + 3H^{2d(E)+(Z)}$) ppm. $H^{7(Z)}$, $H^{2(E)+(Z)}$, $2H^{24(E)+(Z)}$ under solvent peak at 4.00–3.53 ppm and $H^{7(E)}$ at 2.53–2.48 ppm.

2.2. Physical measurements

^1H , ^{13}C , ^{19}F NMR and two-dimensional ^1H – ^1H COSY, ^1H – ^1H TOCSY, ^1H – ^1H NOESY, ^1H – ^1H ROESY, ^1H – ^{13}C HSQC, ^1H – ^{13}C HMBC, ^1H – ^{15}N HSQC NMR spectra were recorded with two Bruker Avance III spectrometers at 500.32 or 500.10 (^1H), 50.70 or 50.68 (^{15}N), 125.81 or 125.75 (^{13}C) and 470.53 (^{19}F) MHz, respectively, by using as a solvent DMSO- d_6 at room temperature and standard pulse programs. ^1H and ^{13}C shifts are quoted relative to the solvent residual signals.

IR spectra were measured on a Bruker Vertex 70 FT-IR-spectrometer using ATR technique. UV-vis spectra were recorded with a Perkin Elmer Lambda 650 spectrophotometer.

HR-ESI mass spectra were measured on a Bruker maXIS ESI-Qq-oaRTOF spectrometer.

Purification of the crude peptide and its conjugate, respectively, was carried out on an Agilent 1200 series HPLC instrument under usage of a Waters XbridgeTM Prep C18 10 μm OBDTM (19 $\times$ 250 mm) column. HPLC analysis was performed on a Dionex Summit system in combination with Chromeleon 6.60 software and a Waters Xbridge C18 5 μm (4.6 $\times$ 150 mm) column. Both systems work with UV-vis detection, and runs were followed at 214, 250, 275 and 300 nm.

X-ray diffraction measurements were performed on a Bruker X8 APEXII CCD diffractometer.

2.3. Cytotoxicity assays

K562 cells were grown as cultures in 75 cm^2 culture flasks in RPMI 1640 medium, supplemented with 10% FCS and 4 mM *L*-glutamine at 37 °C under a moist atmosphere containing 5% CO_2 and 95% air. Cytotoxicity was determined by means of a colorimetric MTT assay. Therefore cells were harvested by trypsination and seeded into 96-well microculture plates. After 24 h preincubation, cells were exposed to solutions of the test compounds (200 μL) for 72 h. To the medium, EZ4U solution (20 μL per well) was added, and plates were incubated for 2 h. Formazan was formed by viable cells. Optical densities at 450 nm (reference wavelength: 620 nm) of the plates were measured with a microplate reader. The quantity of vital cells was expressed as percentage of untreated controls, and IC_{50} values were calculated from concentration–effect curves by interpolation. Evaluation is based on at least four independent experiments.

3. Results and discussion

3.1. Synthesis

The paullone synthesis started from ethyl anthranilate and diethyl succinate undergoing a Dieckmann condensation, that resulted in the formation of an enol ester with 48% yield. Then ester hydrolysis and decarboxylation at boiling temperature in dimethyl acetamide, followed by addition of stoichiometric amount of H₂O, led to benzazepinedione. The latter by Fischer indole reaction in acetic acid glac. with catalytic amounts of sulfuric acid was converted into the paullone backbone in one step with 77% yield. Activation of the lactam moiety through thionation with phosphorus pentasulfide and sodium hydrogen carbonate in THF abs. allowed further imine formation with 2-aminomethylpyridine, providing finally a binding site for ruthenium. Complexation of both paullone-containing ligands could be performed at room temperature in dry methanol as solvent.

The peptide-containing compounds were obtained *via* SPPS following Fmoc-strategy carried out on a Rink MBHA resin, starting with lysine at the C-terminus. Formation of **P** and **L**² occurred under similar conditions, by using lysine with protected ε-amino group. The paullone was conjugated under formation of an amide bond at this group. In the case of **L**² Mtt was used at the C-terminal aa, allowing selective deprotection. After RP-HPLC lyophilisates with purities of 99% (214 nm, **P**) and 98% (250 nm, **L**²) were obtained.

3.2. Crystal structures

Results of X-ray diffraction studies of the structures of the two ruthenium-based isomeric species **E-1** and **Z-1** are shown in Figures 15 and 16, respectively. In the solid state both complexes have the characteristic "three leg piano-stool" geometry of ruthenium(II) arene compounds, with *p*-cymene as the seat, the bidentate paullone moiety and the chlorido ligand as the legs of the stool.

The cymene ligand is oriented with the isopropyl group above C¹⁴ of the paullone chelate ring.

In the crystal structure of **E-1** N⁵ and C¹⁴ are in *trans*-positions with respect to C⁶=N¹³ bond. The methylene azepine group points away from the *p*-cymene ligand. Intramolecular hydrogen bonding between N⁵ and Cl¹ [N⁵–H 0.880 Å, H...Cl¹ 2.460 Å, N⁵...Cl¹ 3.210 Å, N⁵–H...Cl¹ 143 °] stabilises the ligand in *E* configuration with the exocyclic double C⁶=N¹³ bond (1.291(4) Å). Another hydrogen bond of note exists between N¹² and Cl² [N¹²–H 0.880 Å, H...Cl² 2.316 Å, N¹²...Cl² 3.182 Å, N¹²–H...Cl² 168 °].

In Figure 15 the chirality of the shown *E*-**1** species can be described with $R\Theta_-$ because of the planar chirality of the paullone ligand characterised by the torsion angle $\Theta_{\text{N12-C12A-C12B-C1}}$ of

$-33.3(4)^\circ$ and the *R/S*-configuration at the metal centre. As the chelate ring is planar, no δ/λ chirality is induced. Next to this complex with $R\Theta_-$ the corresponding enantiomeric species $S\Theta_+$ exists in equal amounts, which results in a racemate and is in accordance to the NMR spectrum showing one signal for *E*-**1**.

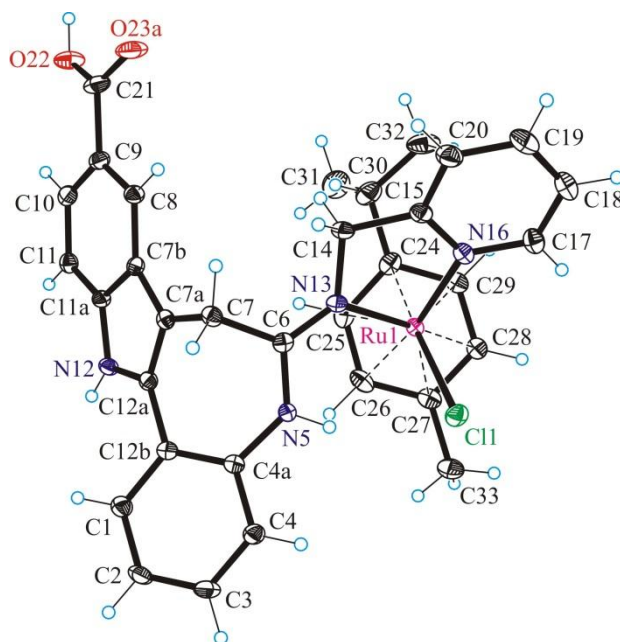


Figure 15. ORTEP view of the structure of the cation of *E*-[RuCl(cymene) L^1] Cl (*E*-**1**) with atom numbering scheme. Thermal ellipsoids are drawn at the 50% probability level. Selected bond distances (Å) and angles ($^\circ$): N13–Ru1 2.088(2), N16–Ru1 2.078(2), Cl1–Ru1 2.4103(7), Ru1–C(24–29)_{av} 2.195(18), C14–C15 1.498(4), C14–N13 1.467(3), C6–N13 1.291(4), C6–N5 1.364(3), C15–N16 1.346(4), C17–N16 1.351(4), N16–Ru1–N13 76.98(9), N13–Ru1–Cl1 87.02(6), N(16)–Ru1–Cl1 86.66(7), $\Theta_{\text{N5-C6-N13-C14}}$ $-167.4(2)$, $\Theta_{\text{N13-C14-C15-N16}}$ $22.2(3)$, $\Theta_{\text{N12-C12A-C12B-C1}}$ $-33.3(4)$, $\Theta_{\text{C7-C6-N13-C14}}$ $17.4(4)$.

Table 2. Crystal data and details of data collection for *E*-1.

compound	[RuCl(cymene)(HL ¹)]Cl + [RuCl(cymene)L ¹] ^f
empirical formula	C _{33.15} H ₃₄ Cl _{1.5} N ₄ O _{2.85} Ru
fw	688.29
space group	<i>Pnna</i>
<i>a</i> [Å]	14.6501(7)
<i>b</i> [Å]	35.5449(18)
<i>c</i> [Å]	11.5040(6)
<i>V</i> [Å ³]	5990.6(5)
<i>Z</i>	8
<i>λ</i> [Å]	0.71073
ρ_{calcd} [g cm ⁻³]	1.526
crystal size [mm ³]	0.25 × 0.07 × 0.04
μ [cm ⁻¹]	0.70
<i>T</i> [K]	100(2)
<i>R</i> ₁ ^g	0.0377
<i>wR</i> ₂ ^h	0.0972
GOF ⁱ	1.094

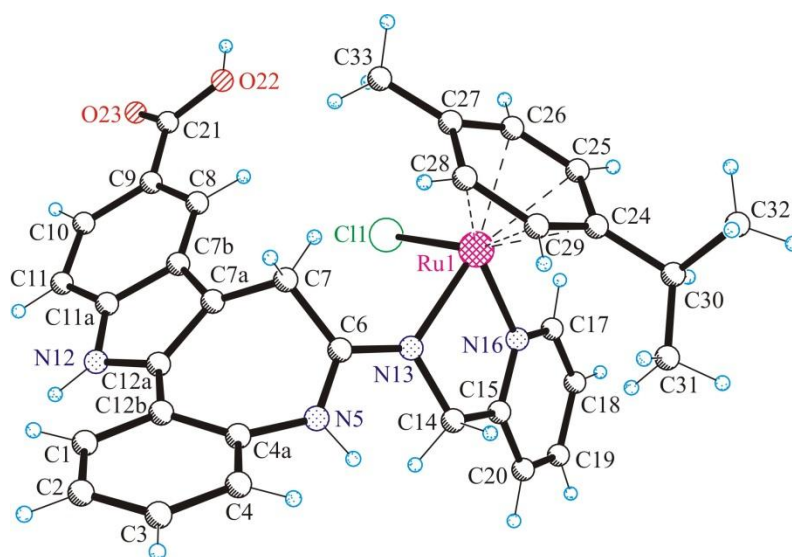


Figure 16. ORTEP view of the structure of the cation of *Z*-[RuCl(cymene)L¹]^fCl (*Z*-1) with atom numbering scheme. Thermal ellipsoids are drawn at the 50% probability level.

^f L¹ is deprotonated at O²² and HL¹ represents the corresponding protonated acid.

^g $R_1 = \sum ||F_0| - |F_c|| / \sum |F_0|$.

^h $wR_2 = \{ \sum [w(F_0^2 - F_c^2)^2] / \sum [w(F_0^2)^2] \}^{1/2}$.

ⁱ $GOF = \{ \sum [w(F_0^2 - F_c^2)^2] / (n - p) \}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

In the crystal structure of **Z-1** atoms N⁵ and C¹⁴ are in *cis*-positions with respect to C⁶=N¹³ bond. The sp³ hybridised carbon atom of the seven-membered azepine ring points towards the *p*-cymene ligand.

In Figure 16 **Z-1** complex with S Θ ₊ chirality is shown. Here, as well as with **E-1**, a racemate of S Θ ₊/R Θ ₋ exists, which agrees with one NMR signal in solution.

3.3. NMR

¹H NMR spectra of **L**¹ and **L**² in DMSO-*d*₆ indicate that the ligands adopt a configuration with an endocyclic double bond C⁶=N⁵ in solution, as can be evidenced by a triplet^j of the amidine nitrogen proton H¹³ at 7.98–7.88 ppm and a doublet of the methylene protons H¹⁴ of the 2-aminomethyl pyridine moiety at 4.5 ppm and 4.83 ppm, respectively.

Upon coordination to ruthenium the signal of the amidine nitrogen proton H¹³ in **1** and **2** is shifted downfield by 1.3 (*E*) or 1.6 ppm (*Z*) and appears as a singlet, which indicates an exocyclic double bond C⁶=N⁵ with N⁵ being protonated instead of N¹³. Additionally the methylene (H¹⁴) and azepine (H⁷) protons show diastereomeric splitting.

Evidence for the assignment of the signals to the *E*- and *Z*-isomers was provided by ¹H–¹H ROESY NMR experiments. As expected, crosspeaks between the nitrogen proton H⁵ and the methylene protons H¹⁴ were observed in **Z-1**, whereas missing in **E-1**. In the crystal structures of **Z-1** and **E-1** the methylene group of the azepine ring points towards and away, respectively, from the *p*-cymene ligand. As the crosspeaks of the azepine (H⁷) to the cymene protons are stronger in the *Z*- than in the *E*-form, retention of this structural feature can be concluded. For both isomers crosspeaks between H¹⁹ and all four aromatic cymene protons were observed. NOEs of H²¹ and H²² show similar intensities to H⁷ and H¹⁹, which indicates an equal population of the cymene orientations, in which one has the isopropyl group above the chelate ring and the other differs by 180 ° rotation of the cymene. Similar behaviour of related compounds was described in literature.⁵¹

In *d*₆-DMSO *E/Z* ratio of **2** was observed to be 1:0.28 after dissolution, but changed to 1:1 over a time period of 19 h 30 min. **1** showed similar ratios of 1:0.25 and 1:1, but also of 0.33:1.

3.4. UV-vis

UV-vis measurements of the two paullone-based complexes **1** and **2** in aqueous media show absorption maxima at 256 and 259 nm, respectively. Investigation of the stability

^j As the signal is not properly resolved, it is listed as multiplett for **L**¹. In the case of **L**², the shift of H¹³ could not be allocated.

revealed their kinetic inertness towards ligand exchange. The spectra showed only a negligible decrease in absorption over 24 h. No isosbestic points were observed, indicating that the coordination sphere remained intact during the measurements.

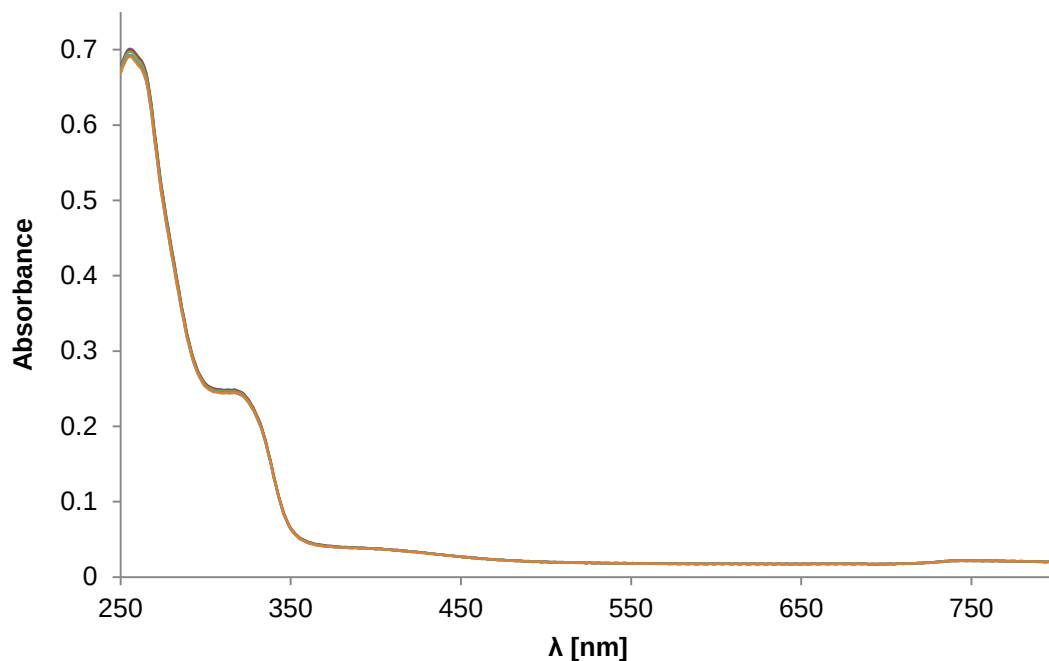


Figure 17. UV-vis spectra of $[\text{RuCl}(\text{cymene})\text{L}^1]\text{Cl}$ (**1**) (15 μM) in H_2O . Spectra were recorded at time points after dissolution – 0 h, blue; 5 h, red; 10 h, green; 15 h, violet; 20 h, light blue; 24 h, orange.

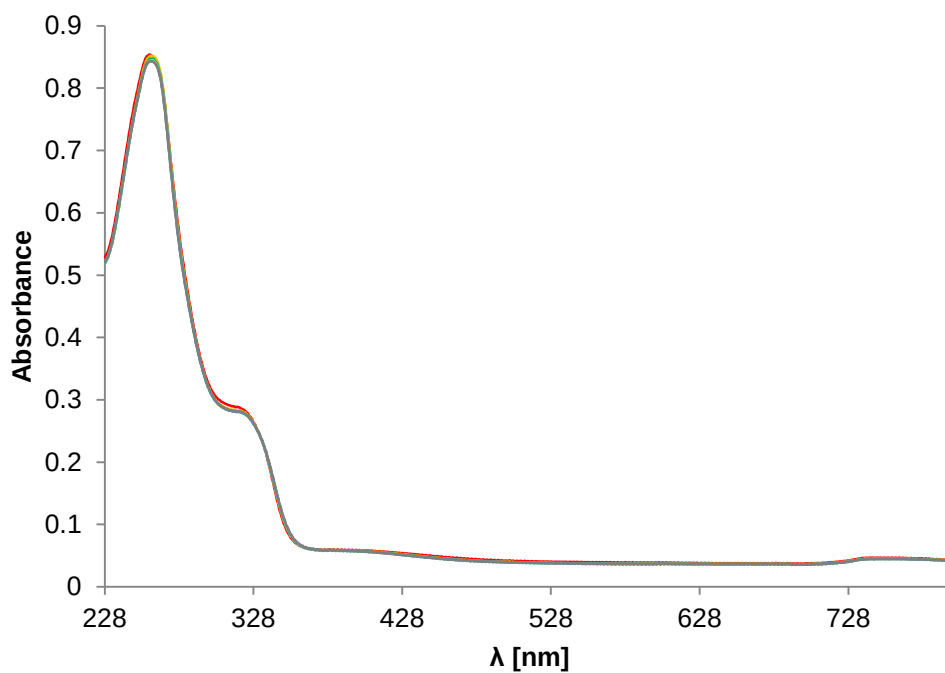


Figure 18. UV-vis spectra of $[\text{RuCl}(\text{cymene})\text{L}^2]\text{Cl}$ (**2**) (20 μM) in H_2O . Spectra were recorded at time points after dissolution – 0 h, red; 5 h, orange; 10 h, green; 15 h, light green; 20 h, blue; 24 h, grey.

3.5. Cytotoxicity assays

As the sequence LKKP was found to home and internalise in K562 myeloid leukemia cells, MTT assays on this cell line were performed.⁵⁶ To estimate the influence of the cell line on the toxicity of ruthenium complexes, a published, related paullone-based compound **1a** with known IC₅₀ values in A549, CH1 and SW480 cells from Schmid, *et al.*,⁵¹ differing only in position 9 from **1**, was also tested and compared. In A549, CH1 and SW480 cell lines incubation time was 96 h, whereas in K562 cell line, it was 72 h. Complex **1a** showed in K562 cells a twice as high IC₅₀ value compared to that in A549 cells and 10 times lesser cytotoxicity than in CH1 and SW480 cells. From the tested compounds the IC₅₀ value of **L**¹ could be determined. The other compounds showed no pronounced toxicity in the evaluated concentration range. In the case of **L**² and the corresponding complex **2** a trend towards the IC₅₀ value could be perceived at approximately 10 µM and 30 µM, respectively. Antiproliferative activity of **P** and **1** could not be observed.

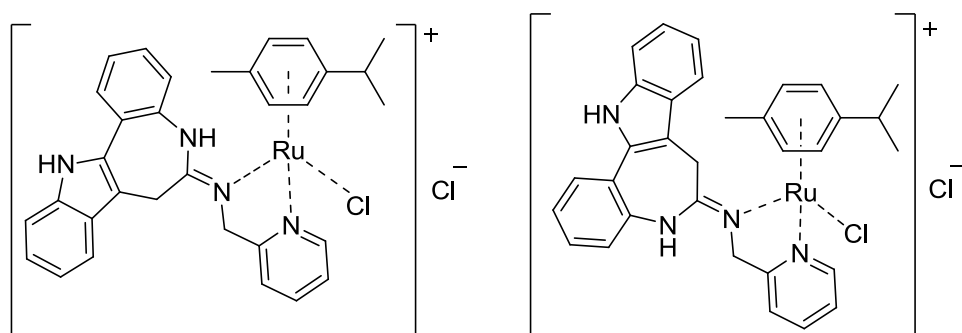


Figure 19. ruthenium complex **1a** from Schmid, *et al.*⁵¹

Table 3. cytotoxicity of **1a** and **L**¹ in human cancer cell lines.

	K562	A549 ⁵¹	CH1 ⁵¹	SW480 ⁵¹
compound	IC ₅₀ [µM]			
1a	13.9 ± 1.7	5.2 ± 0.9 ^k	1.6 ± 0.5 ^k	1.6 ± 0.3 ^k
L ¹	92.7 ± 4.0	–	–	–

^k IC₅₀ values are means ± standard deviations, obtained from at least three independent experiments, after exposure for 96 h in the MTT assay.

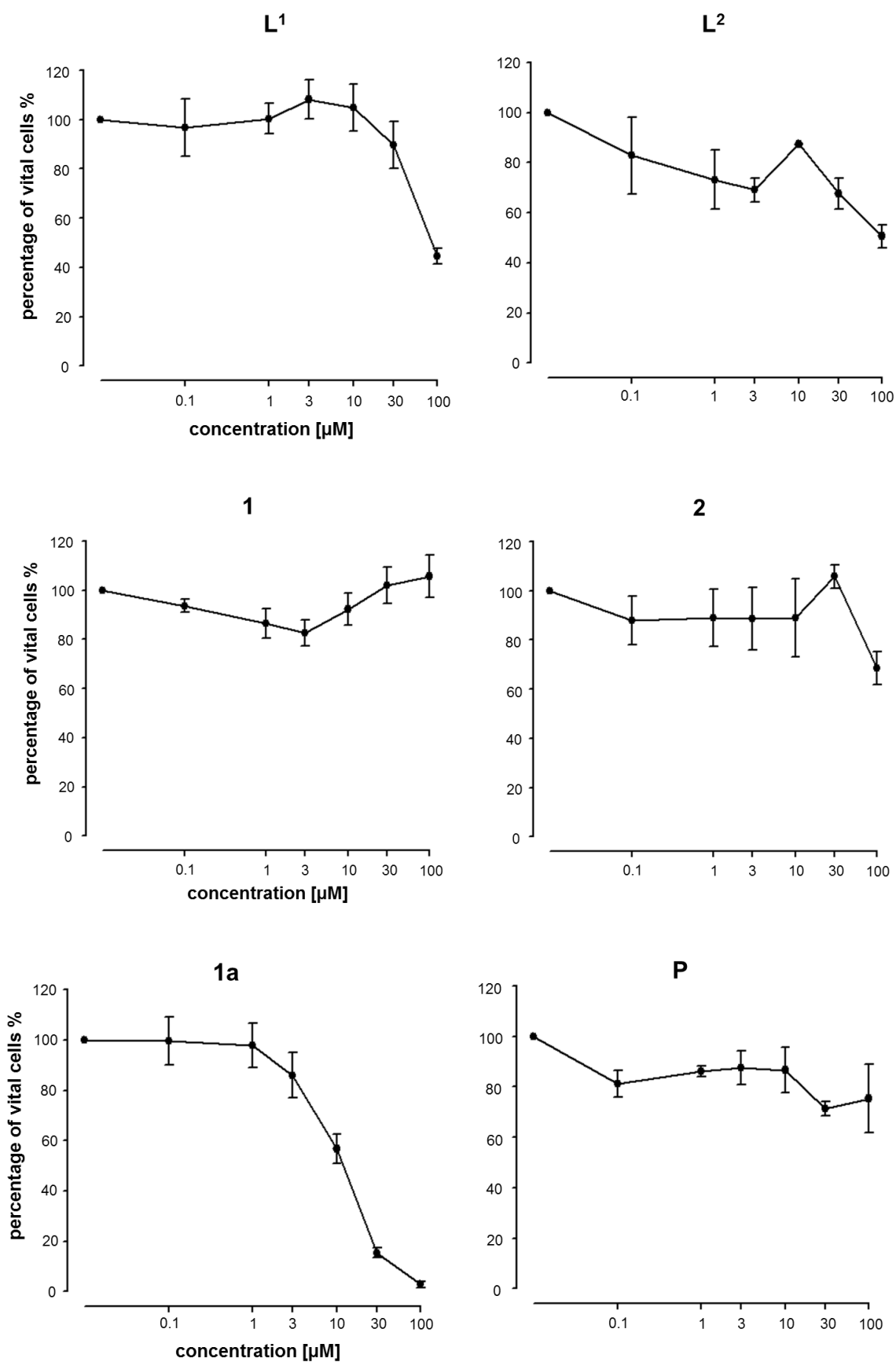


Figure 20. concentration–effect curves of the peptide **P**, the paullone-based ligands **L¹**, **L²** and the ruthenium complexes **1a**, **1**, **2** in K562 cells, obtained by MTT assay.

4. Supplementary

4.1. HR-ESI mass spectra

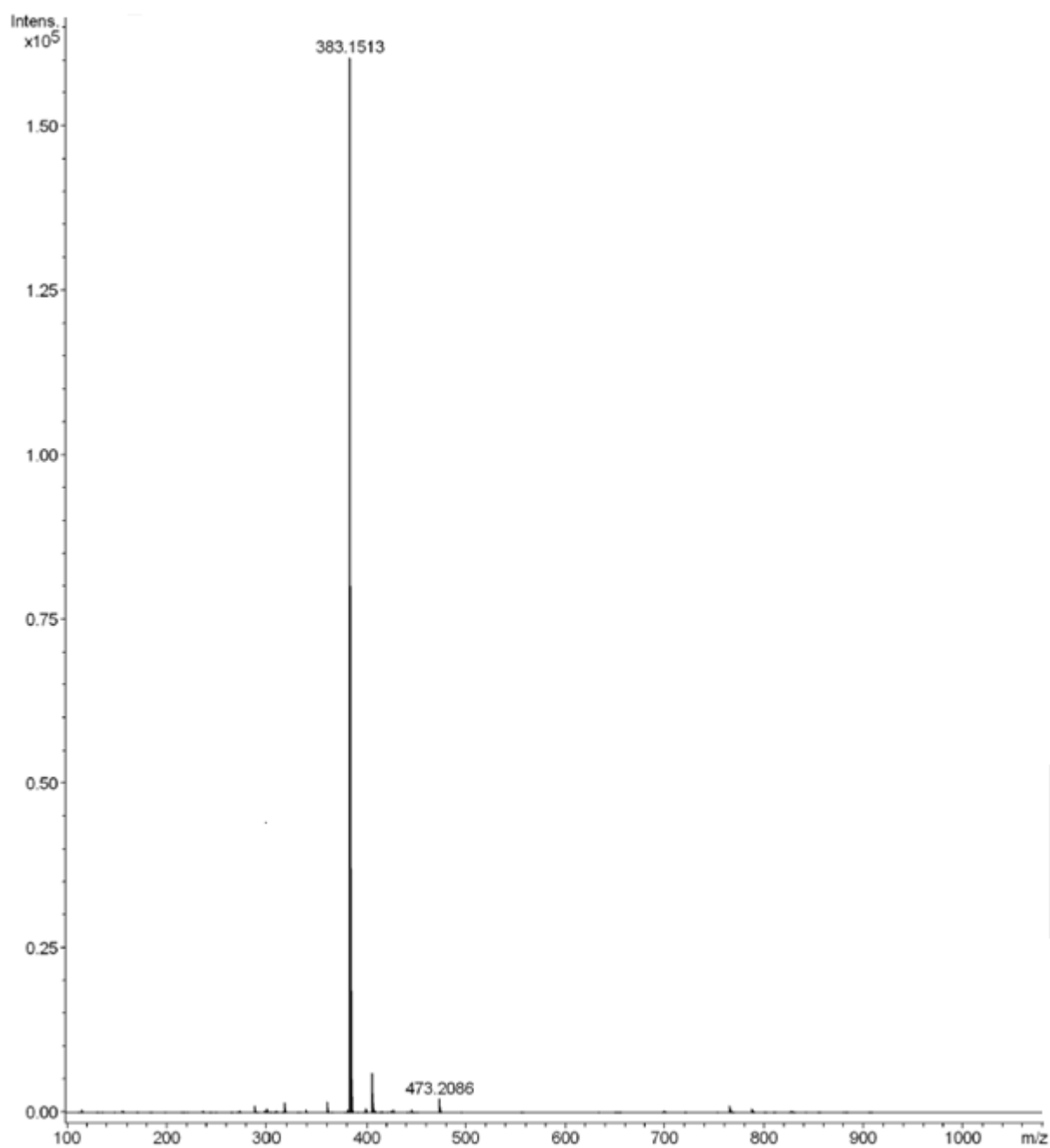


Figure 21. HR-ESI-MS spectrum (full, mass range: m/z 100–1080) of L^1 .

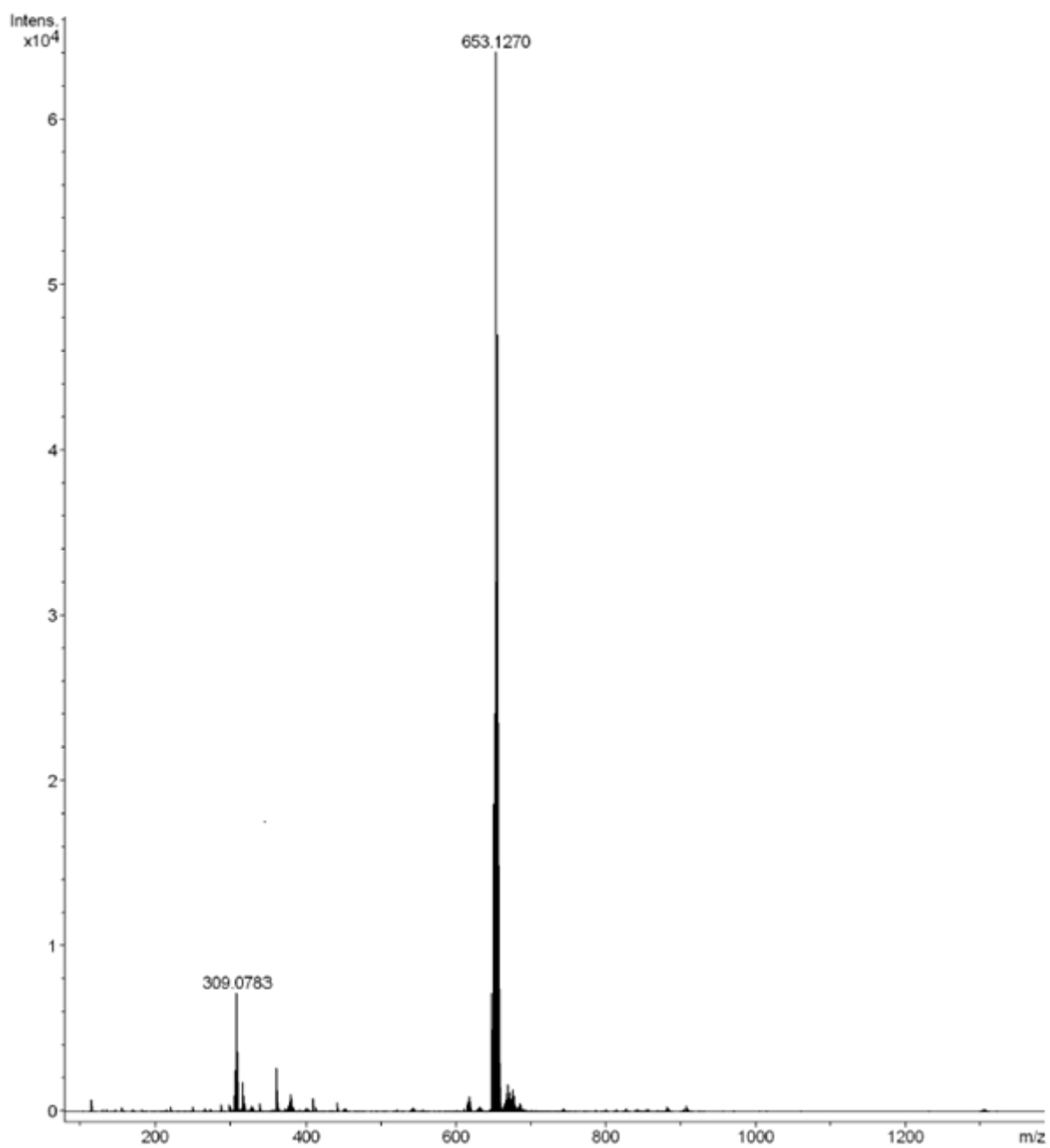


Figure 22. HR-ESI-MS spectrum (full, mass range: m/z 100–1300) of $[\text{RuCl}(\text{cymene})\text{L}^1]\text{Cl}$ **1**.

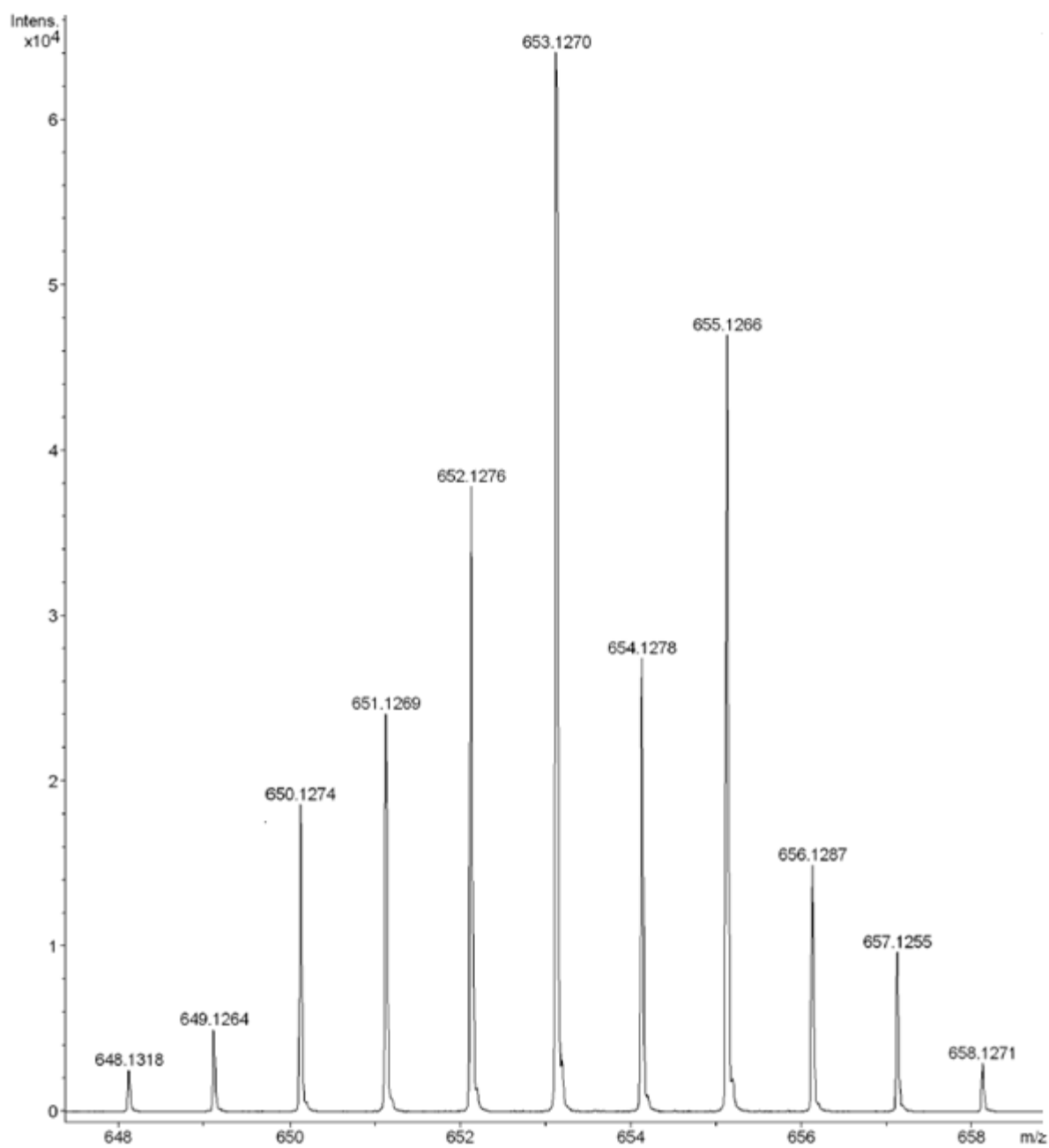


Figure 23. Isotopic distribution of 653.1270 $[1-Cl]^+$.

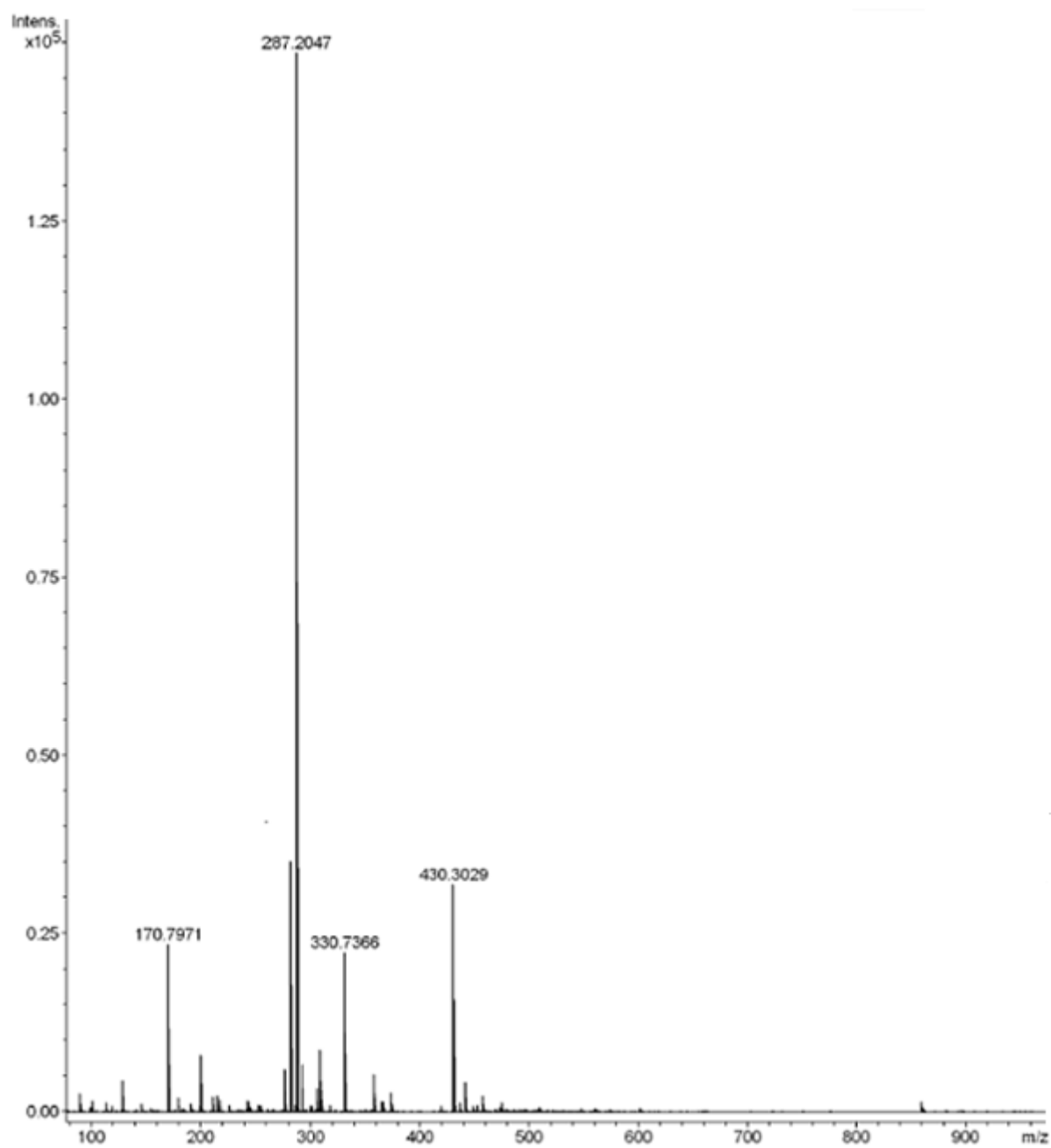


Figure 24. HR-ESI-MS spectrum (full, mass range: m/z 100–900) of H-LKKP-PEG₃-K-NH₂ (**P**).

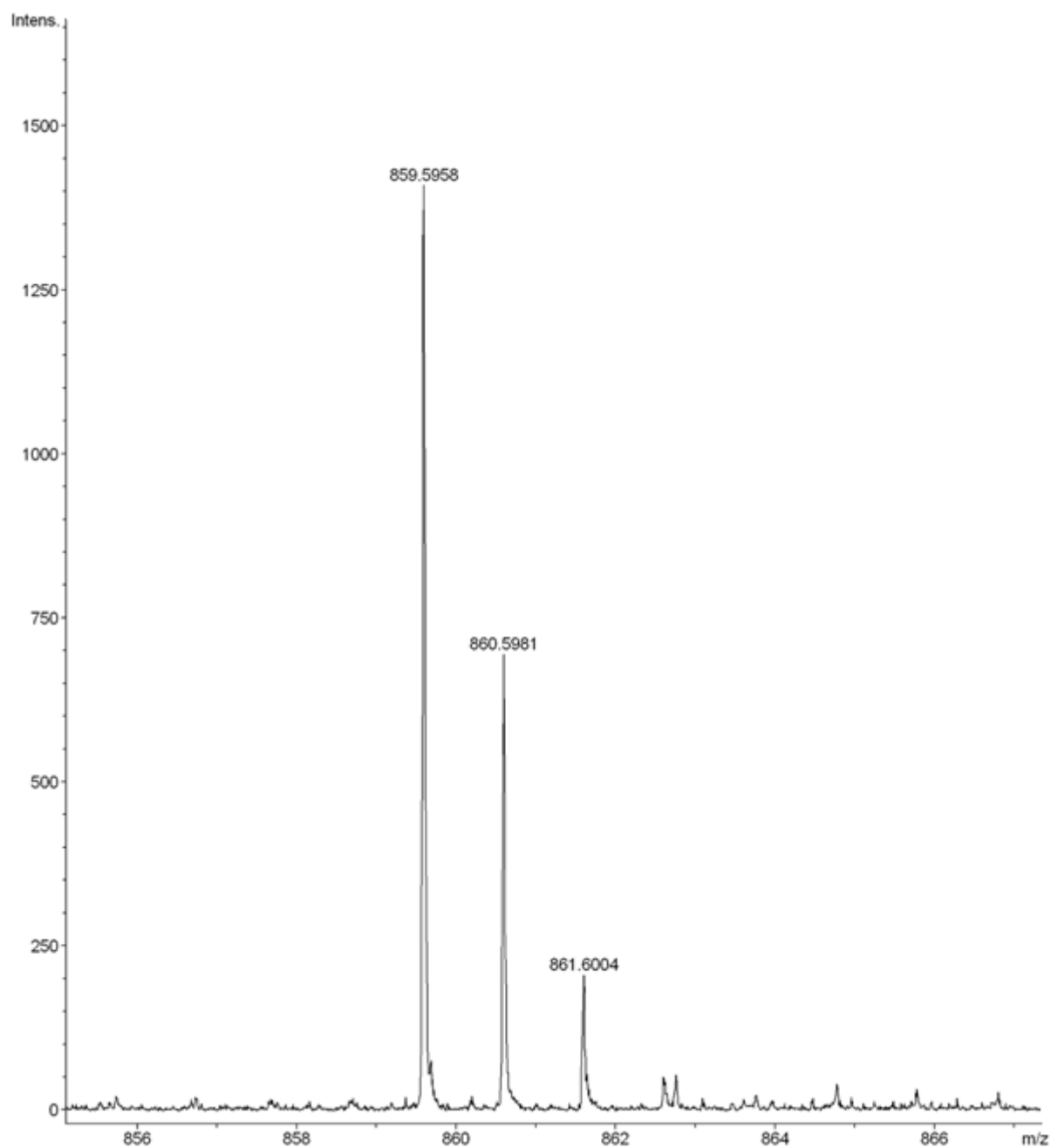


Figure 25. Isotopic distribution of 859.5958 $[P+H]^+$.

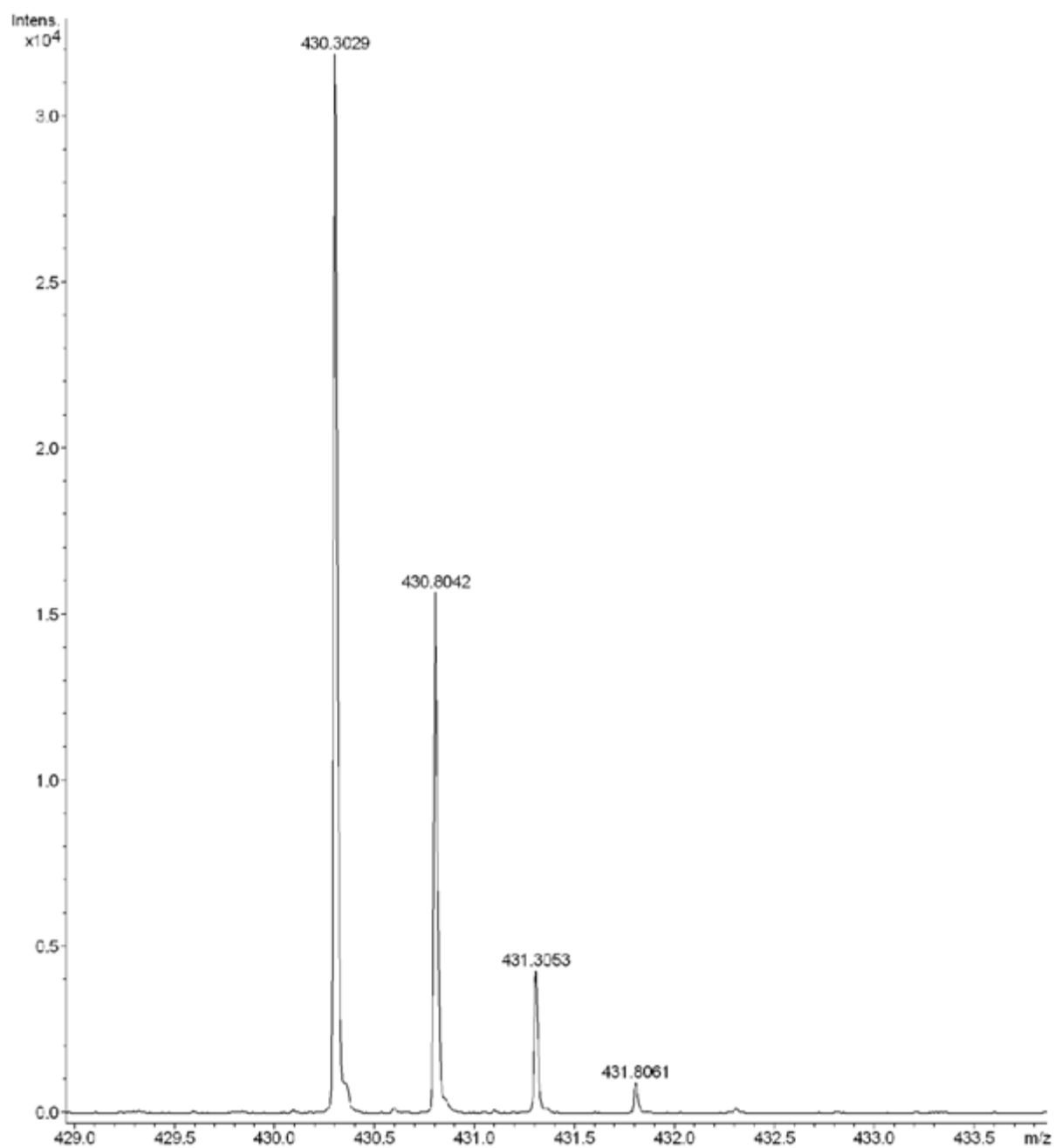


Figure 26. Isotopic distribution of 430.3029 $[P+2H]^{2+}$.

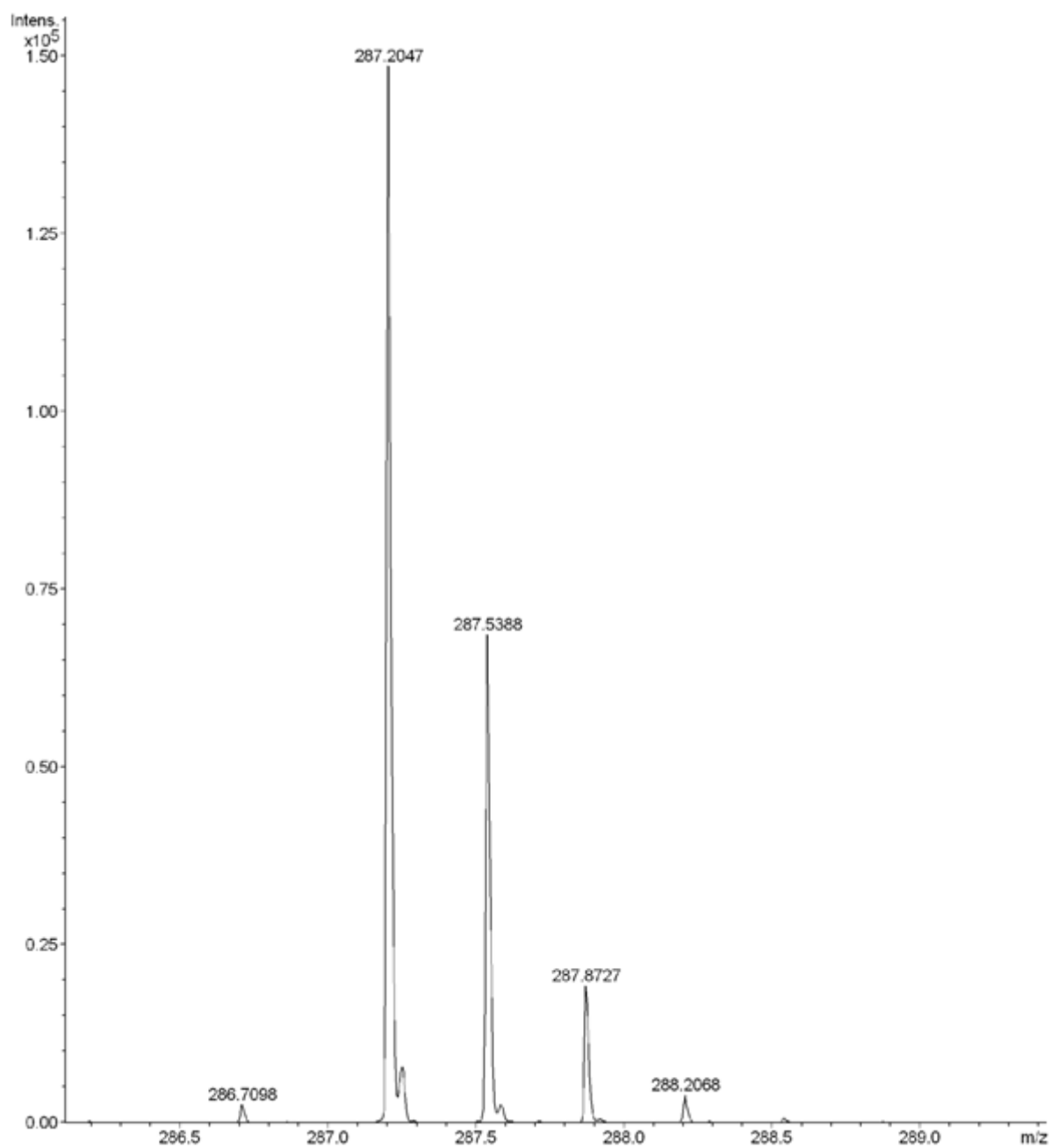


Figure 27. Isotopic distribution of 287.2047 $[P+3H]^{3+}$.

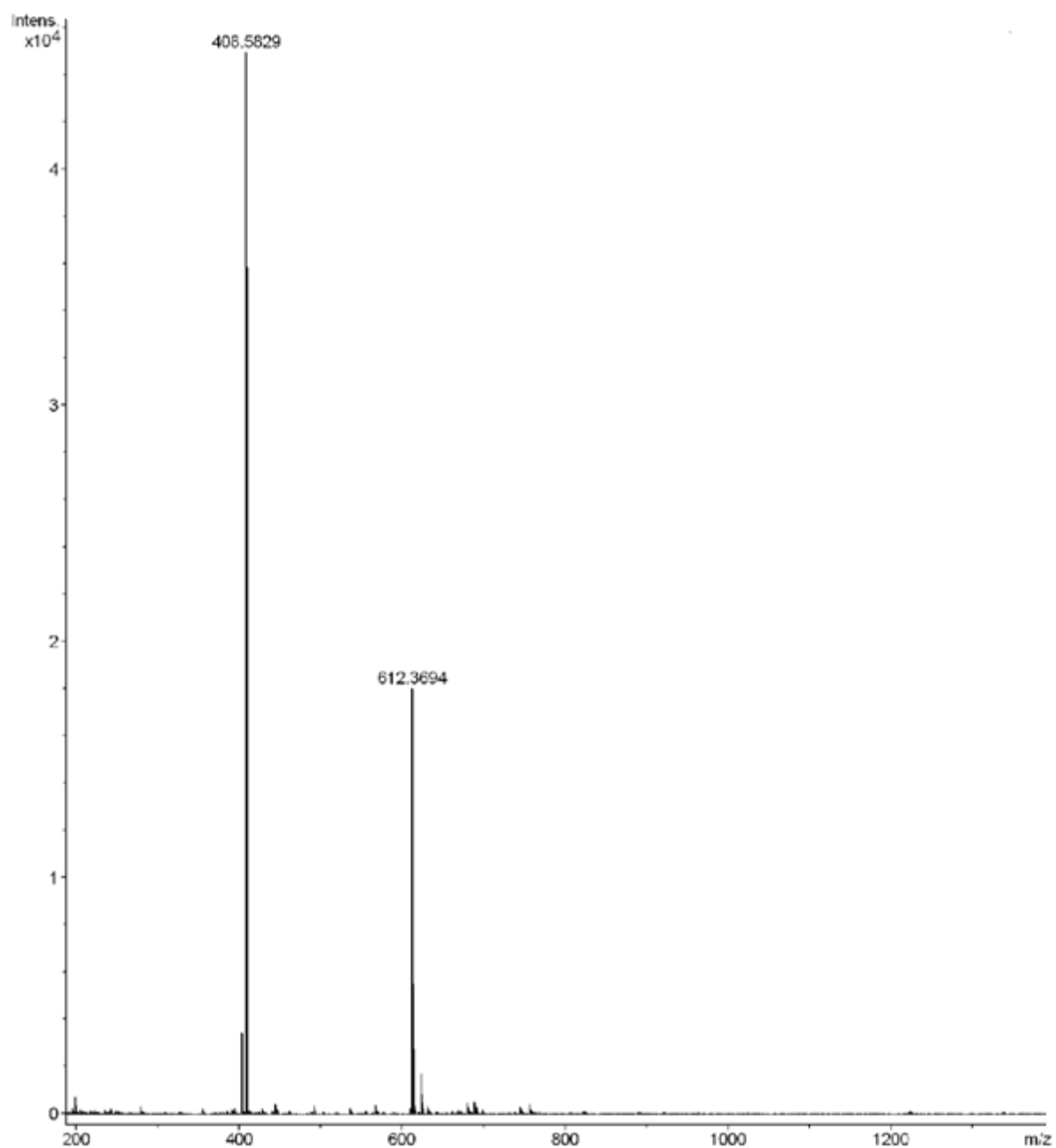


Figure 28. HR-ESI-MS spectrum (full, mass range: m/z 200–1400) of H-LKKP-PEG₃-(L¹)K-NH₂ (L²).

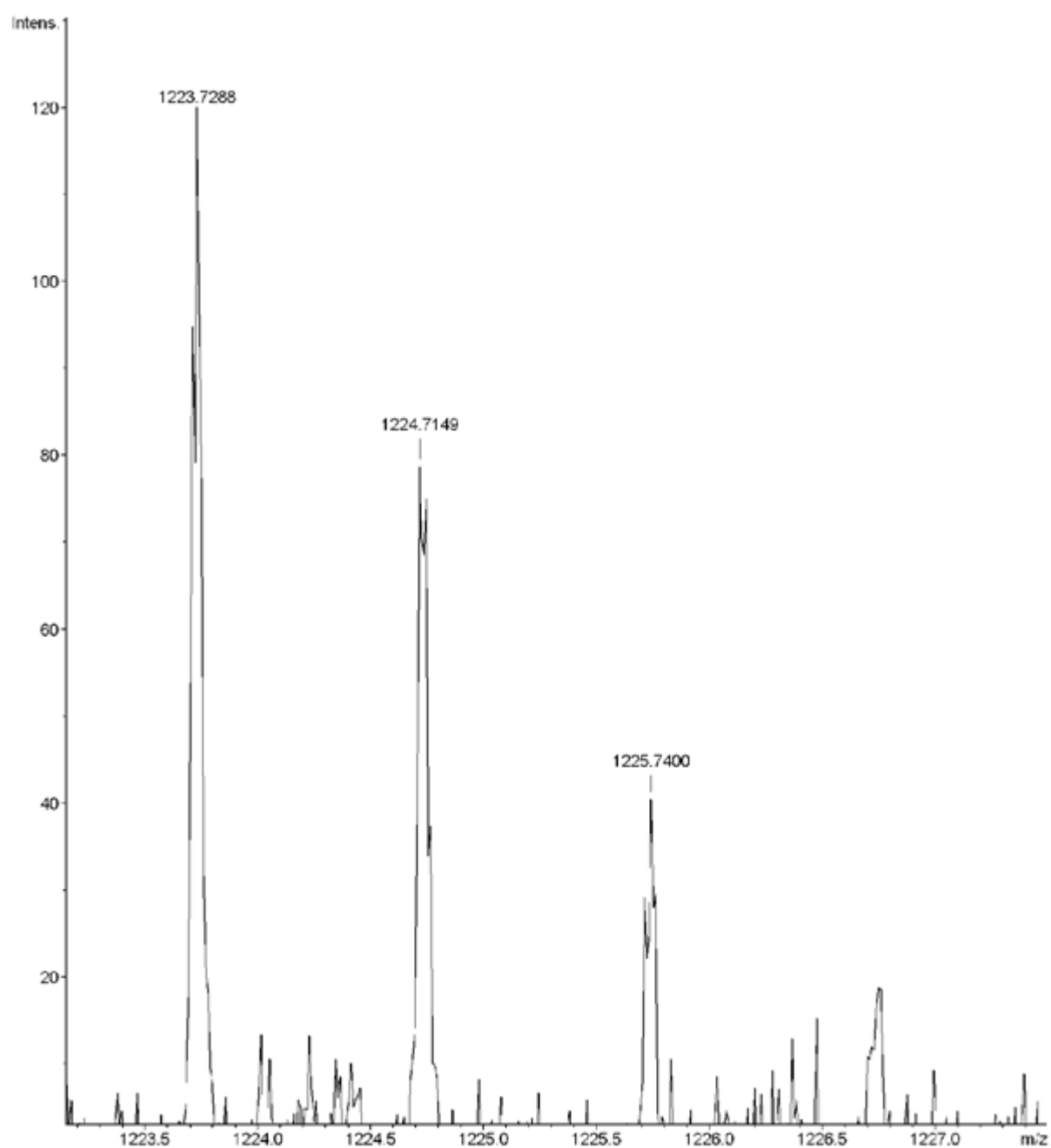


Figure 29. Isotopic distribution of 1223.7288 [L^2+H] $^+$.

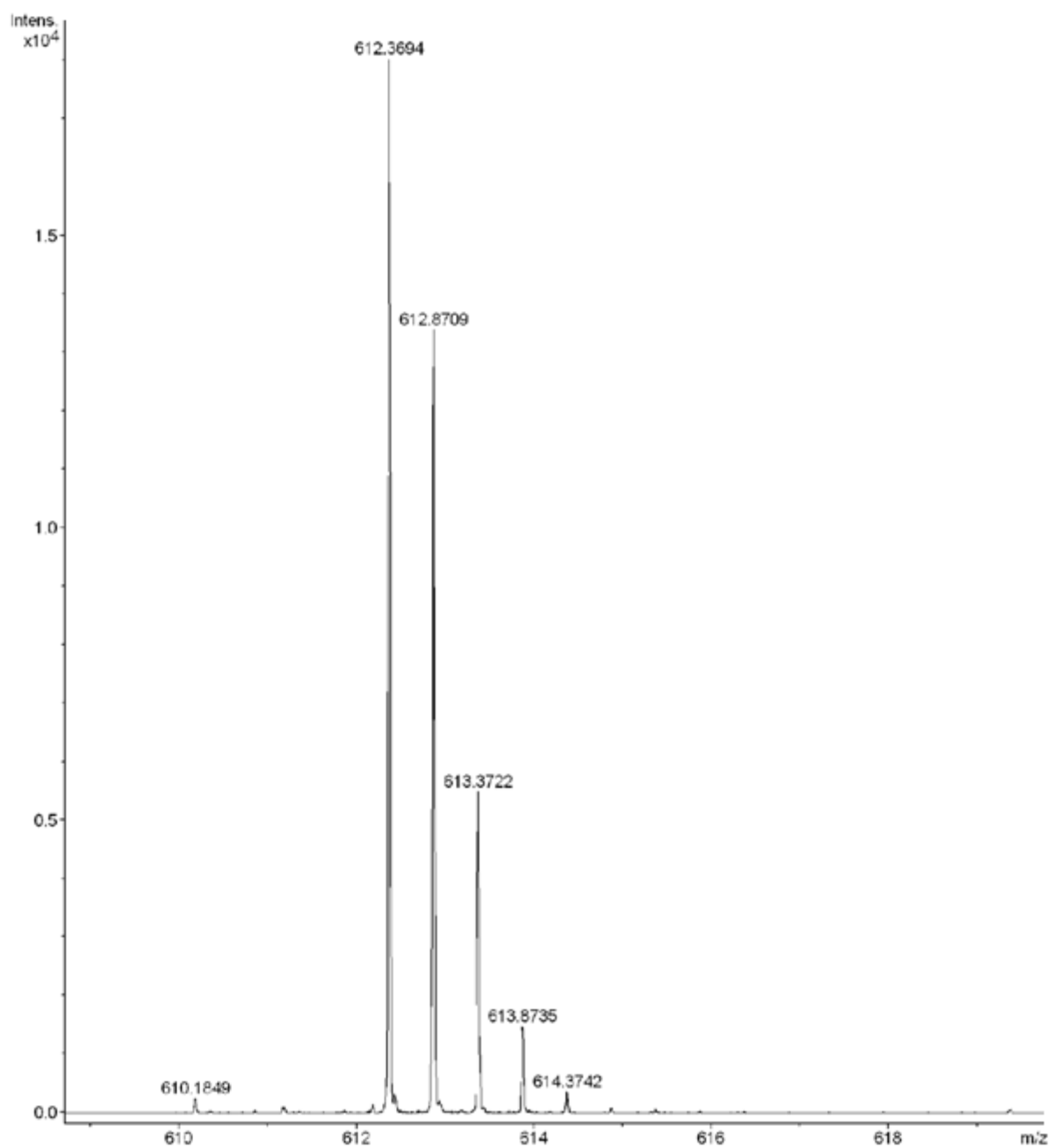


Figure 30. Isotopic distribution of 612.3694 $[L^2+2H]^{2+}$.

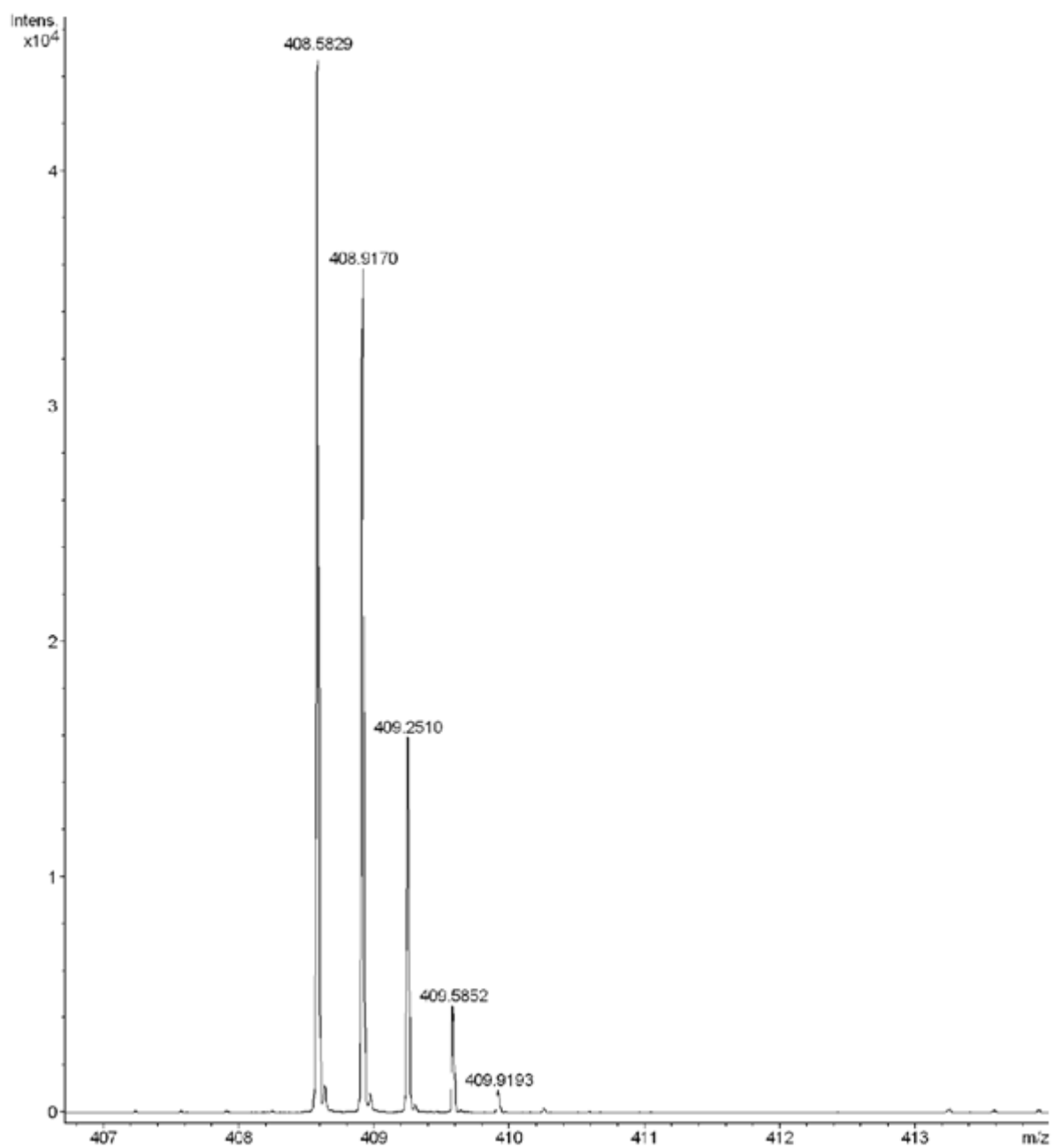


Figure 31. Isotopic distribution of 408.5829 $[L^2+3H]^{3+}$.

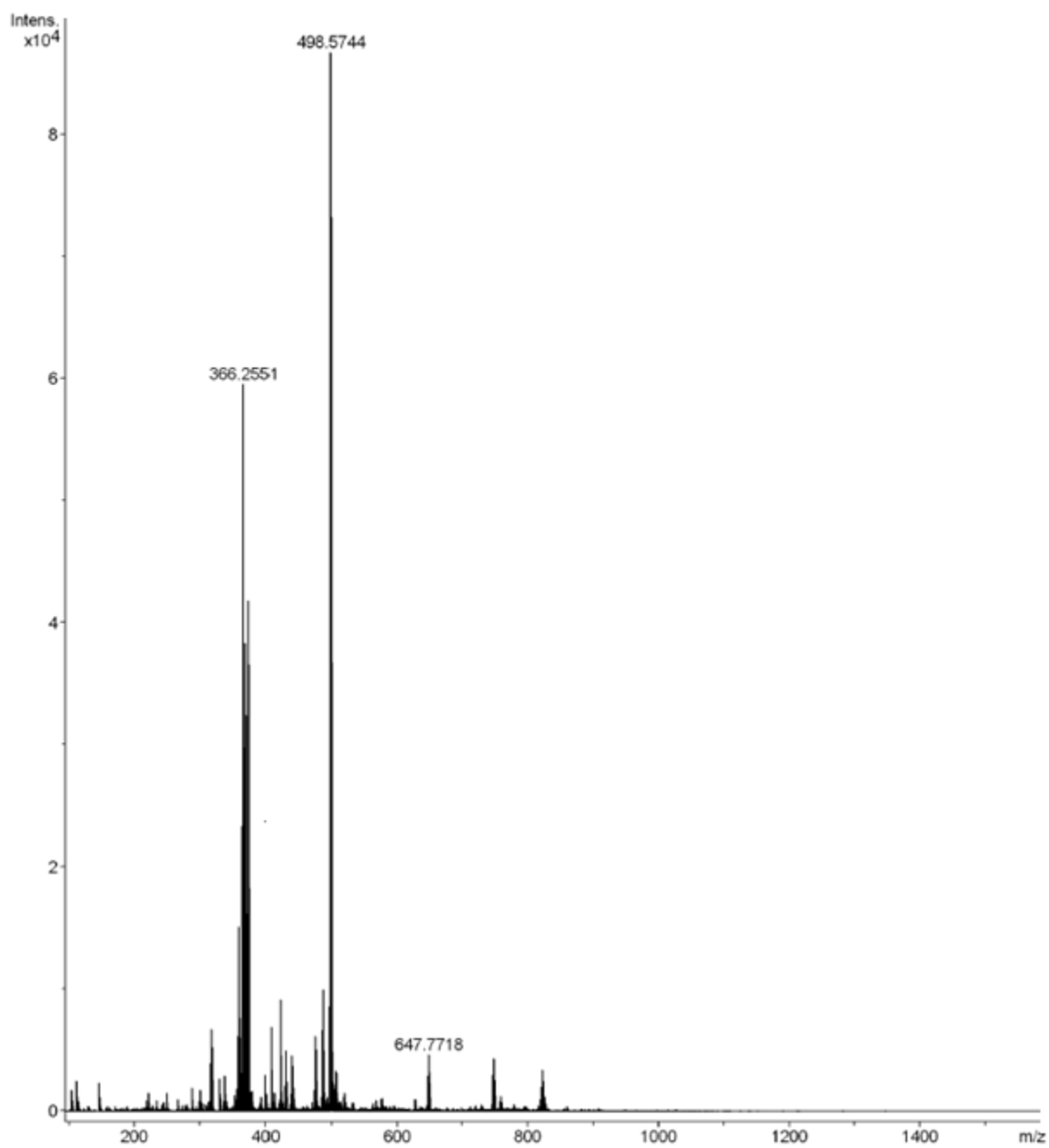


Figure 32. HR-ESI-MS spectrum (full, mass range: m/z 100–1500) of $[\text{RuCl}(\text{cymene})\text{L}^2]\text{Cl}$ **2**.

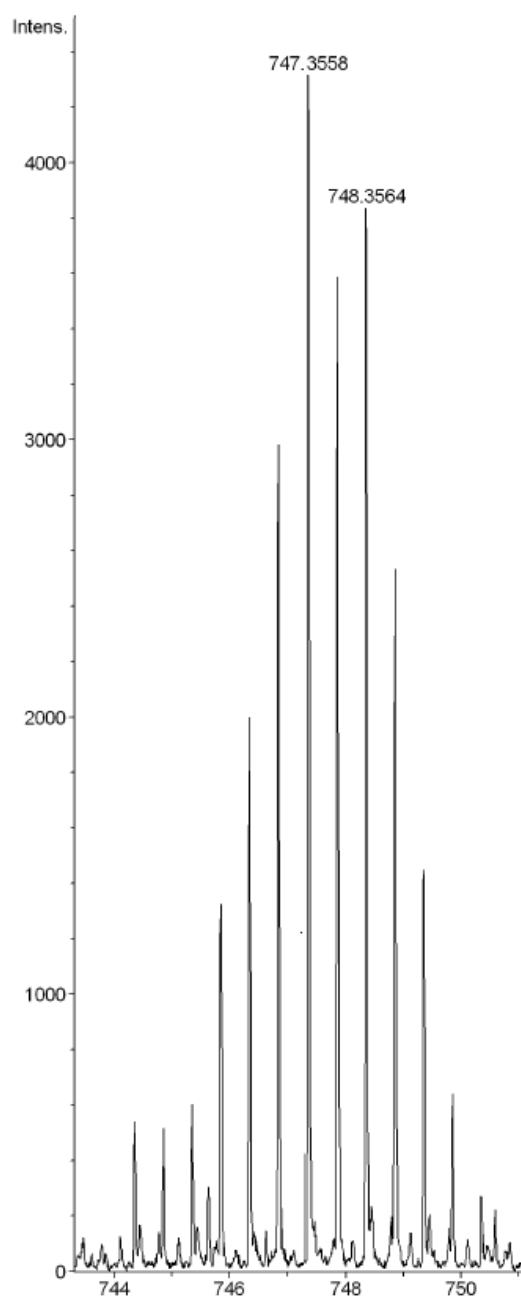


Figure 33. Isotopic distribution of 747.3558 $[2\text{-Cl+H}]^{2+}$.

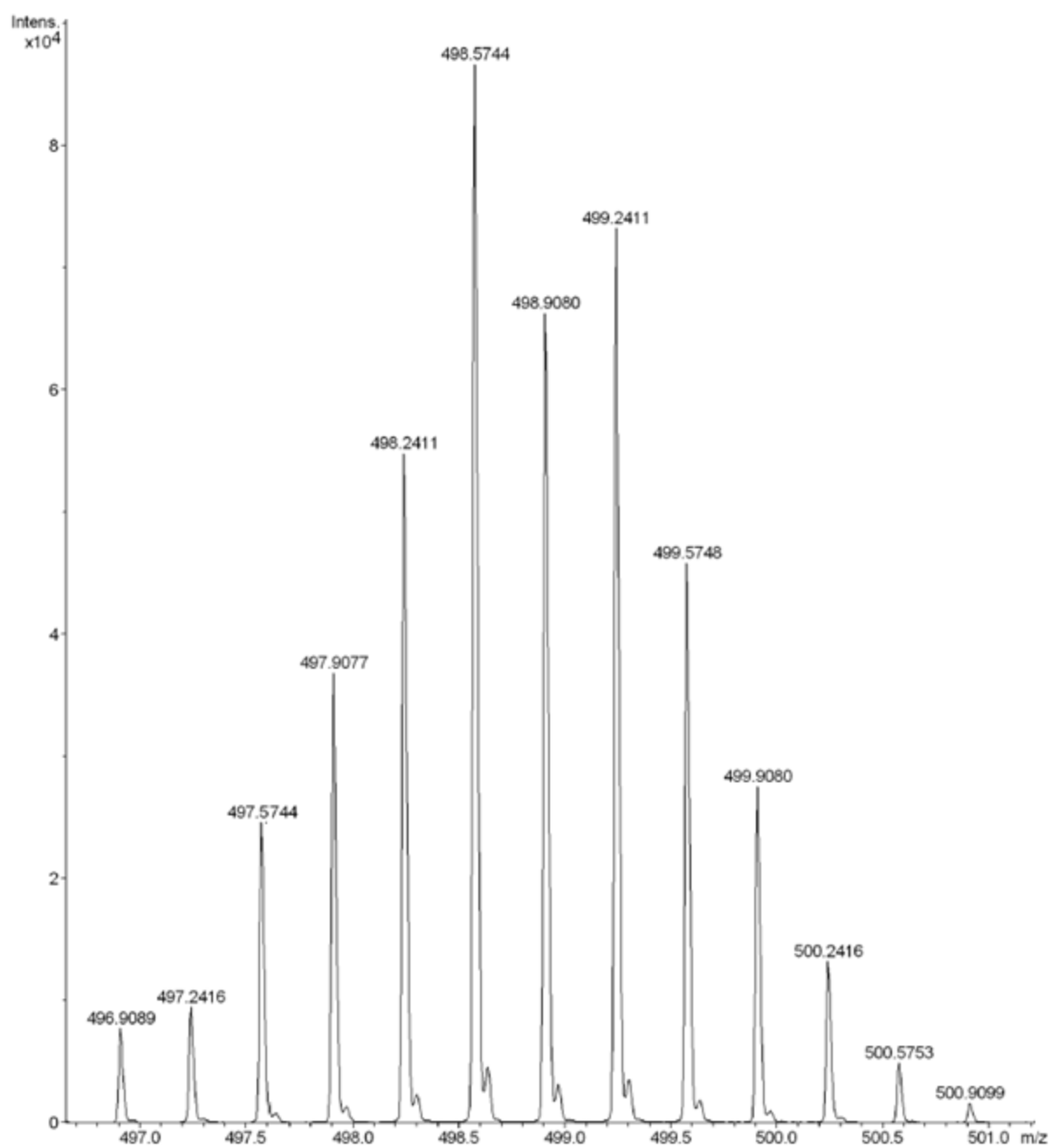


Figure 34. Isotopic distribution of 498.5744 $[2\text{-Cl}+2\text{H}]^{3+}$.

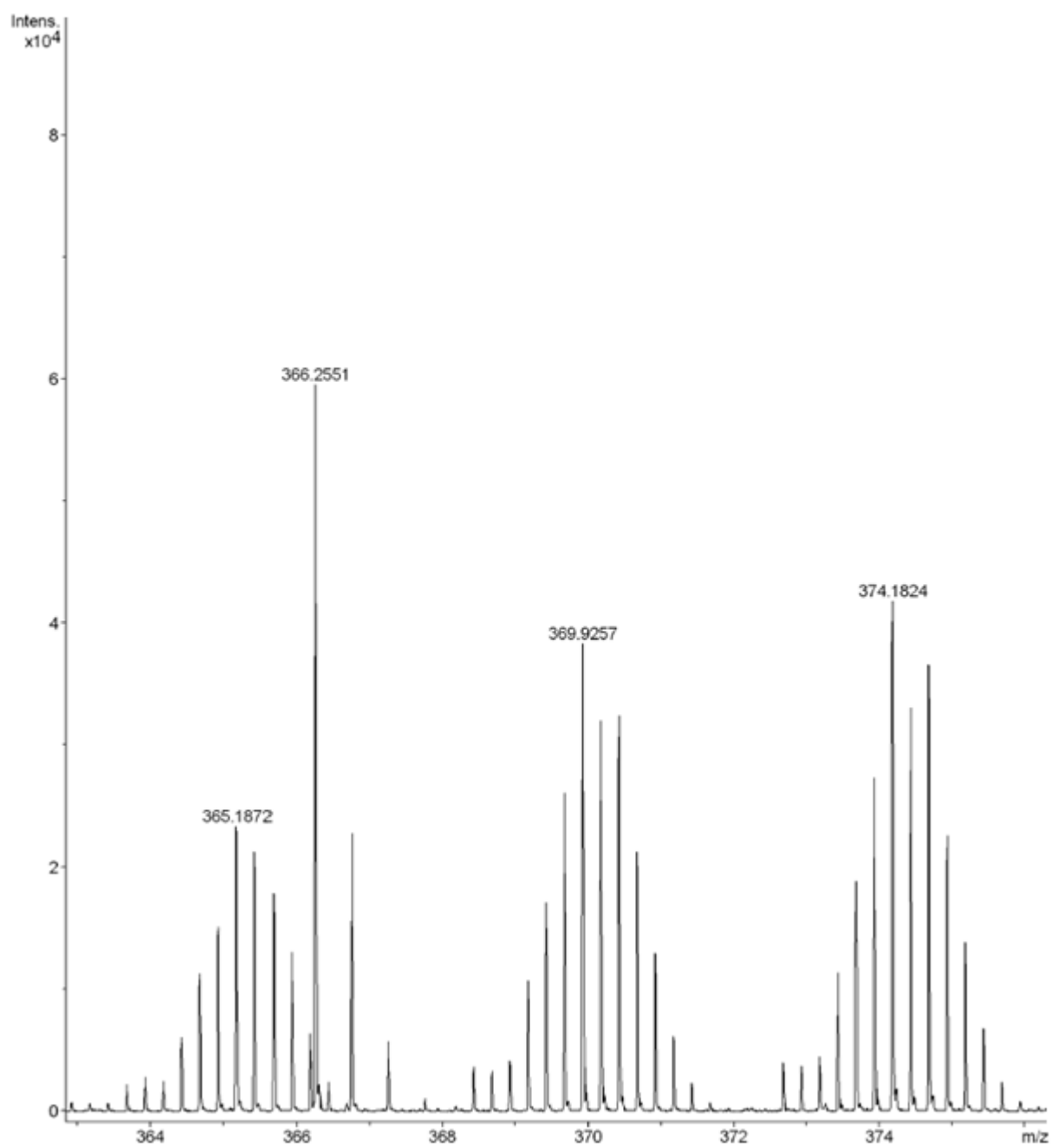


Figure 35. Isotopic distributions of 365.1872 [2-2Cl+2H]⁴⁺, 366.2551 [L¹-OH+H]⁺ and 374.1824 [2-Cl+3H]⁴⁺.

4.2. Chromatograms

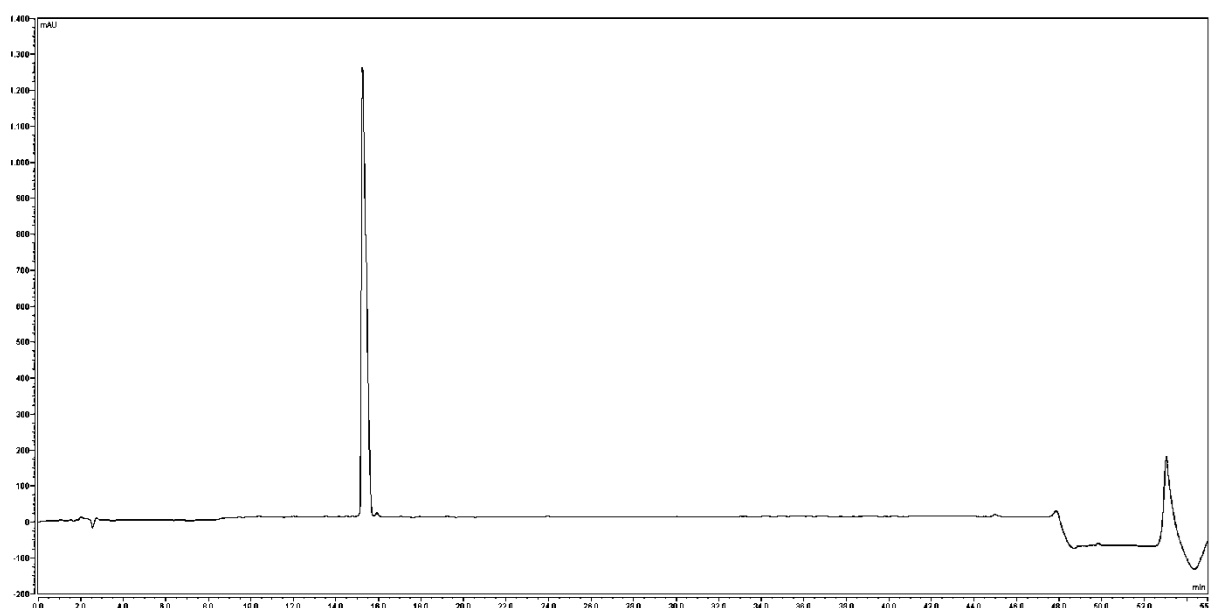


Figure 36. chromatogram of **P**. Analytical HPLC (solvent: acetonitrile(0.05%TFA)/MilliQ water, linear gradient: 5→45% acetonitrile with slope of 1% per min, 1 mL/min): $t_R = 15.244$ min (214 nm: $h = 1246.906$ mAU, $A = 324.075$ mAU*min (99%)).

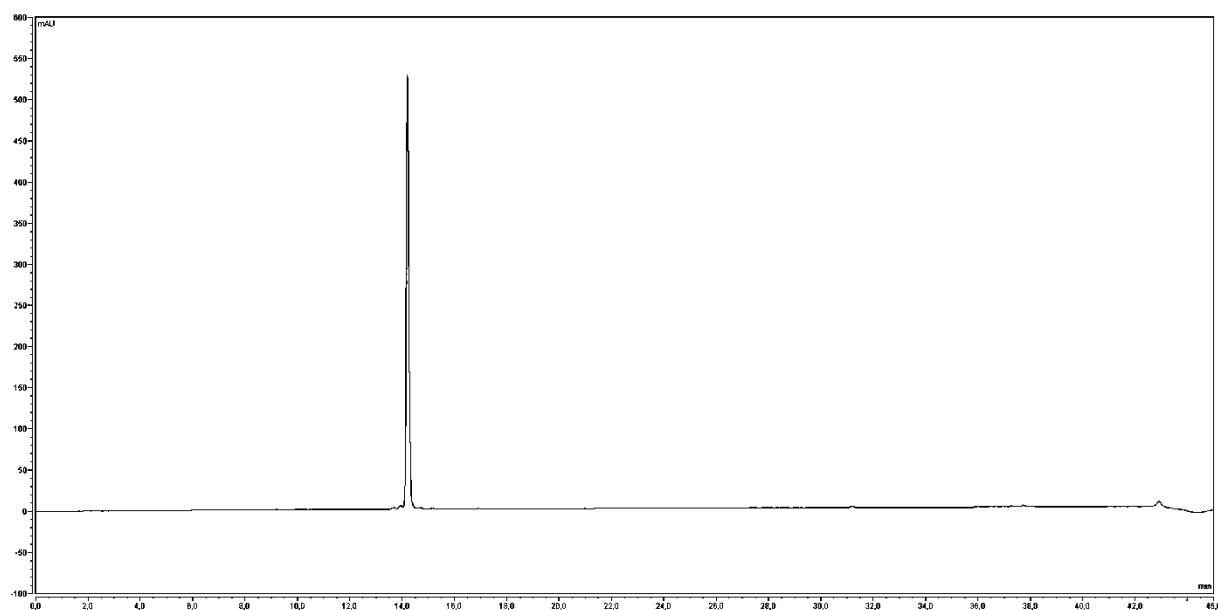


Figure 37. chromatogram of **L²**. Analytical HPLC (solvent: acetonitrile(0.05%TFA)/MilliQ water, linear gradient: 5→65% acetonitrile with slope of 2% per min, 1 mL/min): $t_R = 14.204$ min (250 nm: $h = 527.702$ mAU, $A = 59.780$ mAU*min (98%)).

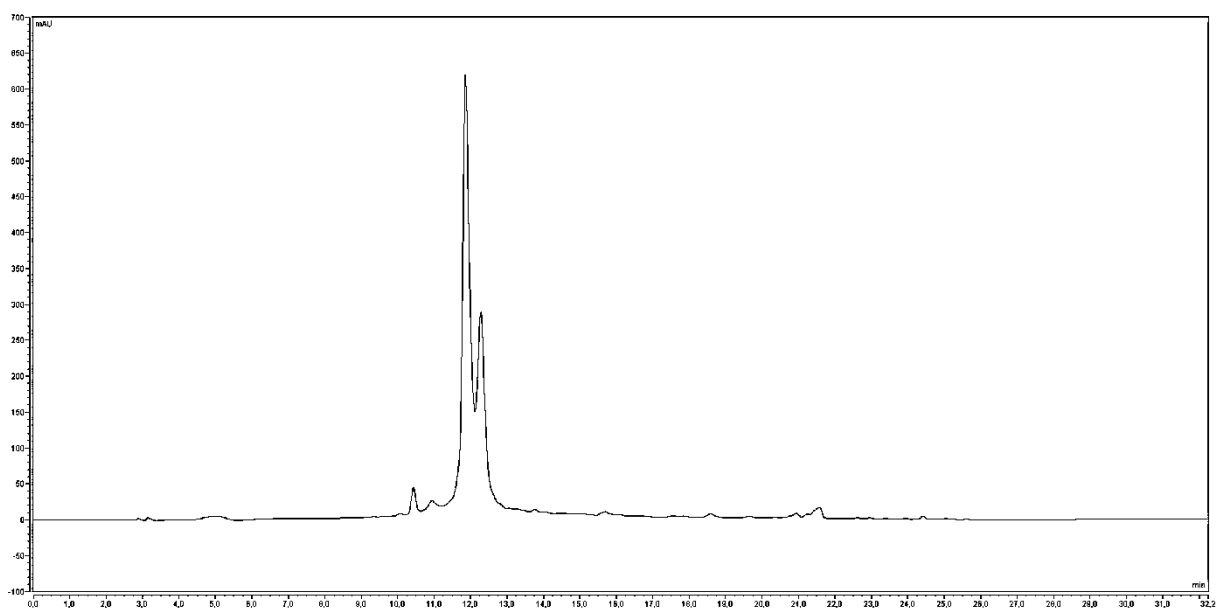


Figure 38. chromatogramm of **2**. Analytical HPLC (solvent: methanol(0.1% formic acid)/MilliQ water, linear gradient: 5→95% methanol with slope of 4.5% per min, 1 mL/min): isomeric peaks, non-baseline-separated: $t_R = 12.122$ min (250 nm: $A = 247.122$ mAU*min (96%)); isomeric peaks, separated at minimum in between: $t_R = 11.862$ min (250 nm: $h = 612.254$ mAU, $A = 161.929$ mAU*min (63%)) and $t_R = 12.282$ min (250 nm: $h = 281.224$ mAU, $A = 85.202$ mAU*min (33%)).

4.3. NMR

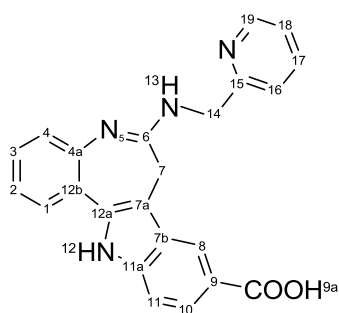


Figure 39. NMR-numbering scheme of paullone-ligand (L^1).

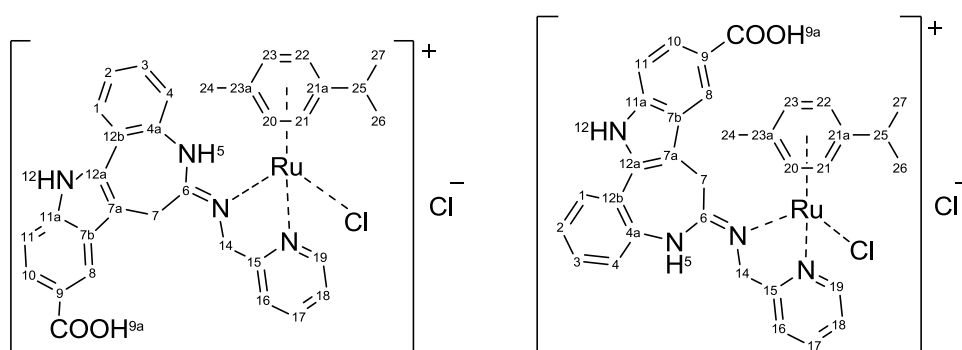


Figure 40. NMR-numbering scheme of ruthenium-*p*-cymol-based paullone *E*(left)- & *Z*(right)-configured complexes **1**.

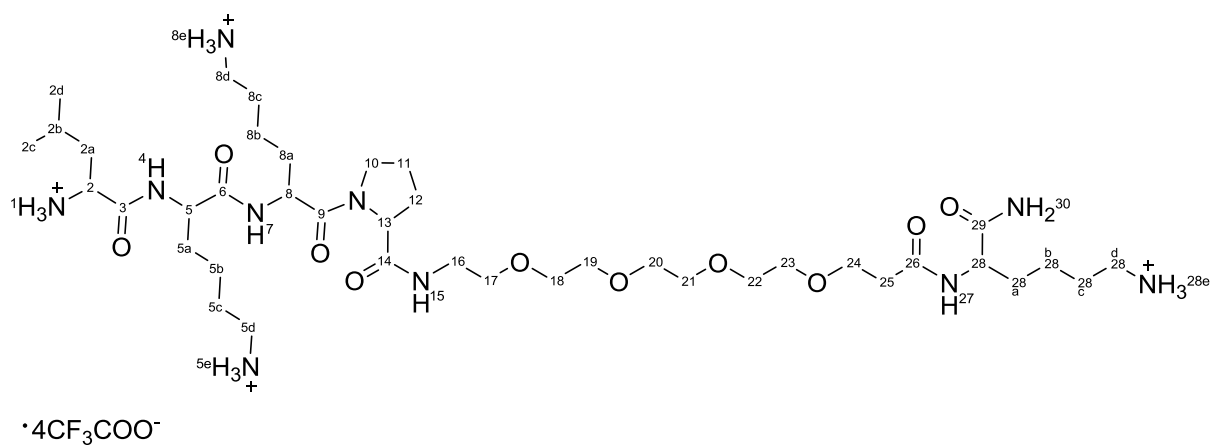


Figure 41. NMR-numbering scheme of peptide H-LKKP-PEG₃-K-NH₂ (**P**).

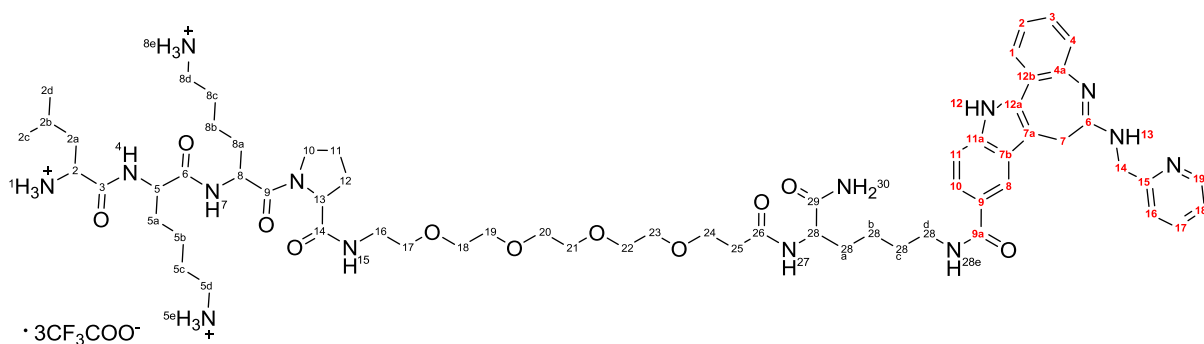


Figure 42. NMR-numbering scheme of paullone-peptide conjugate H-LKKP-PEG₃-(Pa)K-NH₂ (**L**²) (black numbering: peptide, **red bold numbering**: paullone).

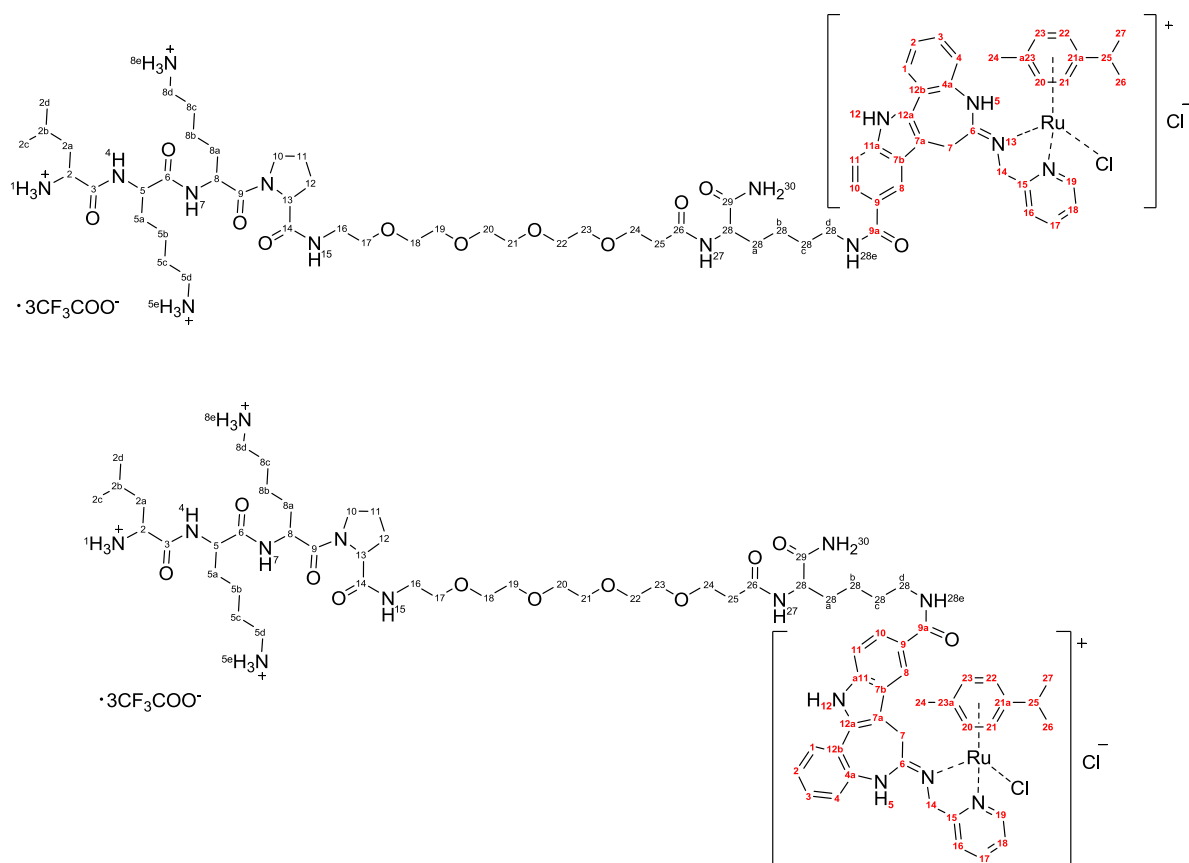


Figure 43. NMR-numbering scheme of ruthenium-*p*-cymol-based peptide-conjugated paullone *E*(above)- & *Z*(below)-configured complexes **2** (black numbering: peptide, **red bold numbering**: ruthenium based paullone complex).

Abbreviations

aa	amino acid
ATC	anatomical therapeutic chemical
ATR	attenuated total reflectance
Boc	<i>tert</i> -butoxycarbonyl
CDK	cyclin dependent kinase
COSY	correlation spectroscopy
CPHP	cell-penetrating homing peptide
CPP	cell-penetrating peptide
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAA	<i>N,N</i> -dimethylacetamide
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
en	ethylenediamine
FCS	fetal calf serum
FDA	US food and drug administration
Fmoc	fluorenylmethoxycarbonyl
FT	fourier transformation
fw	formula weight
FWF	Fonds zur Förderung der wissenschaftlichen Forschung
glac.	glacial
GOF	goodness of fit
GSK-3	glycogen synthase kinase
HBTU	<i>O</i> -benzotriazole- <i>N,N,N',N'</i> -tetramethyluronium-hexafluorophosphate
HMBC	heteronuclear multiple bond correlation
HP	homing peptide
HR-ESI-MS	high resolution electrospray ionisation mass spectrometry
HSA	human serum albumin
HSAB principle	"hard and soft acids and bases" principle
HSQC	heteronuclear single quantum coherence
IC ₅₀	(half maximal = 50%) inhibitory concentration
ICD	international statistical classification of diseases and related health
IR	infrared spectroscopy
K	lysine (one letter code)
L	leucine (one letter code)
MBHA	4-methylbenzhydrylamine
mMDH	mitochondrial malate dehydrogenase
MTT	3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
NCI	national cancer institute
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser enhancement spectroscopy
P	proline (one letter code)
PEG ₃ ¹	polyethylene glycol
PGMs	platinum group metals
pta	7-phospha-1,3,5-triaza-tricyclo[3.3.1.1]decane
Rink	4-[(<i>R,S</i>)-(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid
ROESY	rotating-frame overhauser effect spectroscopy
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography
SIRT	sirtuin

¹ three: number of ethylene glycols

SPARC	satraplatin and prednisone against refractory cancer
SPL	synthetic peptide library
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TOCSY	total correlation spectroscopy
UV-vis	ultraviolet-visible spectroscopy
WHO	world health organisation
WHOCC	world health organisation collaboration centre

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